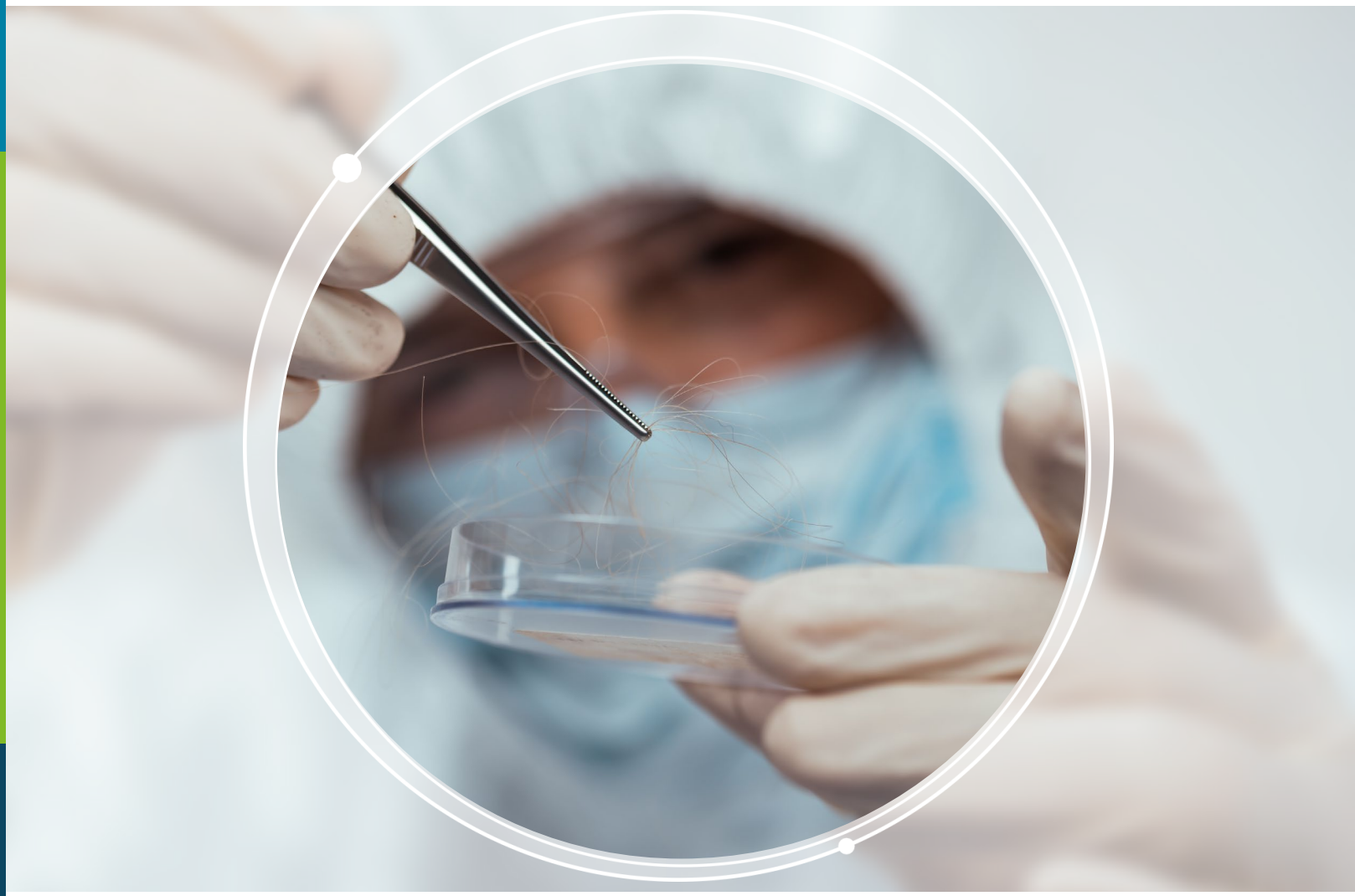
2024 法醫毒理應用文集

第一期 -質譜技術應用於法醫相驗鑑識



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目錄

Highly sensitive MS/MS detection for confident identification of potent novel synthetic opioids and their metabolites

HRMS analysis of discarded authentic postmortem case samples using the SCIEX ZenoTOF 7600 system, powered by SCIEX OS software

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The introduction of highly potent novel synthetic opioids (NSO) to the illicit drug market has been a major driver for the recent rise in the number of accidental drug overdoses. NSO are a class of novel psychoactive substances (NPS) that are commonly used as adulterants in heroin and counterfeit preparations to mimic the effects of controlled opioids. These substances vary greatly in potency and purity and thus often require only a small amount to cause acute intoxications. Their increasing occurrence in combined opioid drug toxicity cases, resulting in accidental and fatal drug overdoses, continues to create a major challenge for public health officials.

Traditionally, screening for ultra-potent substances was performed using targeted workflows, such as multiple reaction monitoring (MRM) using triple quadrupole mass spectrometers, because of the higher selectivity and sensitivity performance. However, the continuous emergence of NPS on the recreational drug market is creating an additional challenge for drug tracking agencies and laboratories to meet. High-resolution mass spectrometry has provided forensic toxicology laboratories with a unique tool for the untargeted detection and identification of these new emerging substances, with little or no method optimization necessary. In addition, accurate mass instruments are affording additional levels of certainty by reliably obtaining comprehensive MS/MS spectral fragment information that can be used for identification, confirmation, and/or library matching.

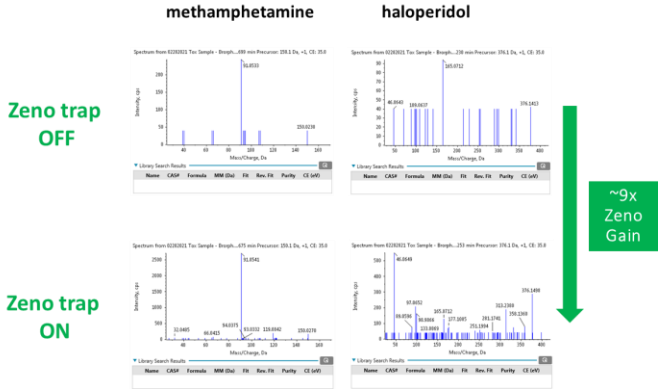


Figure 1: TOF MS/MS sensitivity gains using Zeno IDA for representative analytes. An average of ~9X gain in TOF MS/MS sensitivity was observed across all analytes identified in this study.

In this technical note, a highly sensitive method for the detection and identification of potent NSO in human whole blood is described. The technological enhancements of the ZenoTOF 7600 system[1] provide a high degree of sensitivity, selectivity and confidence for MS/MS experiments. They enable accurate and reliable detection of potent substances in poly-drug, authentic, case samples at trace levels that were not previously achievable.

Key features of Zeno IDA for untargeted detection of low level NSO in blood samples

- Zeno trap provides ≥90% duty cycle across the entire mass range for MS/MS acquisition
- Improved duty cycle leads to an MS/MS sensitivity increase, resulting in higher numbers of detections, improved spectral library matching and increased confidence in identification
- MS/MS sensitivity improvements of ~9X, on average, across all MS/MS fragments for the positively identified substances
- Increased MS/MS sensitivity leads to confident detection of low level NPS, metabolites and other potent drugs in discarded authentic postmortem case samples, providing the necessary evidence to support medicolegal death investigations

Experimental details

Target analytes: An NSO panel including 3 newly emerging non-fentanyl opioids (brorphine, isotonitazene, metonitazene), one metabolite (4'-hydroxy nitazene) and two halogenated fentanyl analogs (*para*-fluorofentanyl and *para*-chlorofentanyl) was selected for method development. A 1 µg/mL standard mixture containing the 6 target analytes and a 1 ng/mL fentanyl-D5 internal standard solution were prepared in water.

Calibrator preparation: The 1 µg/mL standard mixture containing the 6 target analytes was used to fortify 500 µL of human whole blood. This freshly spiked whole blood mixture was used to prepare a series of 9 calibrator solutions covering concentrations ranging from 10 pg/mL to 100 ng/mL.

Sample preparation: NSO were extracted from human whole blood using a liquid-liquid extraction (LLE) procedure summarized in Figure 2.

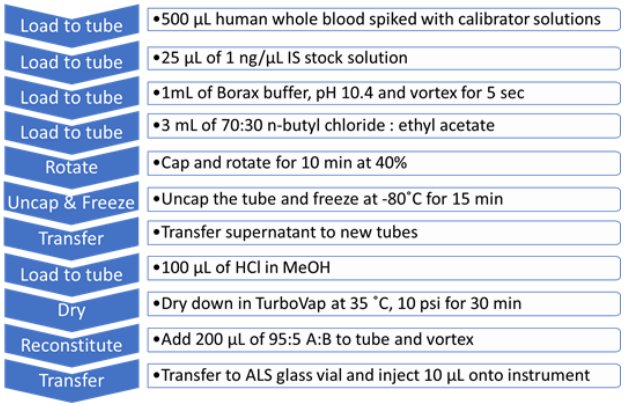


Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used for selectively extracting drugs from human whole blood samples for analysis with the ZenoTOF 7600 system.

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 with a total LC runtime of 15.5 minutes. The injection volume was 10 µL.

Mass spectrometry: MS and MS/MS data were collected for each sample using Zeno IDA for optimal sensitivity 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 TOF MS/MS full scan ranging from 25 to 700 Da to ensure all fragments were captured for identification using a maximum of 16 candidate ions. Data was acquired using SCIEX OS software 2.0.1.

Data analysis: Data was processed using SCIEX OS software 2.0.1. Detection and integration of the peaks from the background was accomplished using the MQ4 algorithm in the Analytics module of the software where quantitative and qualitative analyses were performed. Positive analyte identification was accomplished based on confidence criteria as previously described.[2] The four main confidence criteria used include mass error (M), retention time (R), isotope ratio difference (I), and library score (L). An in-house library was used to perform spectral library matching and identification of the drugs present in the discarded authentic postmortem case samples.

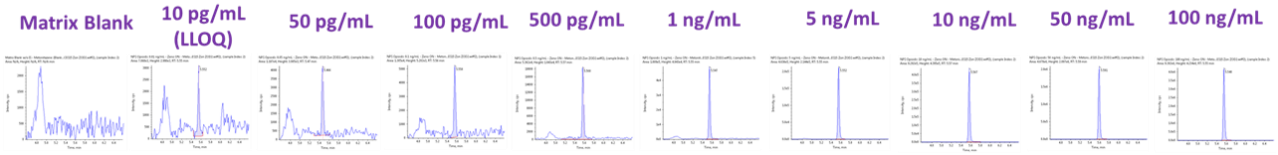
Optimized IDA method leads to accurate and reliable drug quantification

Information dependent acquisition (IDA) is a non-targeted data dependent acquisition technique that provides high confidence in compound identification by generating high-resolution, accurate mass spectra in both MS and MS/MS modes for spectral library matching or for structural elucidation purposes. Accurate quantification can also be performed simultaneously using the accurate mass of precursor ions from the TOF MS experiment.

A series of 9 calibrator solutions were prepared by spiking control human whole blood samples with the 6 targeted analytes at final concentrations ranging from 10 pg/mL to 100 ng/mL. The series of calibrator solutions were injected to evaluate the quantitative performance of the system and its ability to accurately measure low level analytes with a high level of precision and accuracy in TOF MS mode. Each calibrator was injected in triplicate.

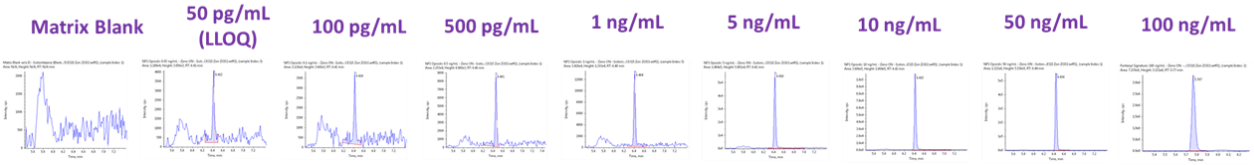
Figure 3 shows representative extracted ion chromatograms (XICs) for A) metonitazene and B) isotonitazene, two highly potent NSO that have been linked to accidental drug overdoses at low concentrations. The series of XIC displays shows the resulting signal for a blank injection (left) and for concentrations ranging from 10 pg/mL (LLOQ) to 100 ng/mL for metonitazene and from 50 pg/mL (LLOQ) to 100 ng/mL for isotonitazene, respectively. Figure 3 also displays the statistical results from the peak area integration of A) metonitazene and B) isotonitazene. Excellent precision and accuracy were observed across the series of calibrators, proving the robustness of the assay. Full quantification, including detection and integration of the peaks and area, concentration and quantitative performance value calculations (precision and accuracy) was automatically performed in Analytics in SCIEX OS software. The software is designed for quick, intuitive and streamlined data processing with accurate and reliable results.

A Metonitazene



Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
1	Metonitazene	0.01	3 of 3	9.875e-3	1.477e-3	14.95	98.75	9.474e-3	8.641e-3	1.151e-2
2	Metonitazene	0.05	3 of 3	5.166e-2	1.685e-3	3.26	103.33	5.079e-2	5.059e-2	5.361e-2
3	Metonitazene	0.10	3 of 3	9.053e-2	4.580e-3	5.06	90.53	8.525e-2	9.292e-2	9.342e-2
4	Metonitazene	0.50	3 of 3	5.183e-1	2.291e-2	4.42	103.66	5.432e-1	4.982e-1	5.134e-1
5	Metonitazene	1.00	3 of 3	1.023e0	1.640e-2	1.60	102.34	1.039e0	1.026e0	1.006e0
6	Metonitazene	5.00	3 of 3	5.091e0	5.074e-2	1.00	101.82	5.086e0	5.043e0	5.144e0
7	Metonitazene	10.00	3 of 3	9.814e0	3.087e-1	3.15	98.14	1.011e1	9.492e0	9.842e0
8	Metonitazene	50.00	3 of 3	5.137e1	8.919e-1	1.74	102.73	5.219e1	5.042e1	5.149e1
9	Metonitazene	100.00	3 of 3	9.870e1	2.265e0	2.29	98.70	9.996e1	1.000e2	9.608e1

B Isotonitazene



Row	Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
1	Isotonitazene	0.01	0 of 3	N/A	N/A	N/A	N/A	~~1.706e-1~~	~~9.531e-1~~	N/A
2	Isotonitazene	0.05	3 of 3	5.563e-2	5.110e-3	9.19	111.27	5.854e-2	4.973e-2	5.863e-2
3	Isotonitazene	0.10	3 of 3	9.682e-2	1.776e-3	1.83	96.82	9.478e-2	9.801e-2	9.767e-2
4	Isotonitazene	0.50	3 of 3	5.135e-1	1.879e-2	3.66	102.70	4.994e-1	5.062e-1	5.348e-1
5	Isotonitazene	1.00	3 of 3	9.619e-1	4.868e-2	5.06	96.19	1.011e0	9.601e-1	9.141e-1
6	Isotonitazene	5.00	3 of 3	4.974e0	1.625e-1	3.27	99.47	5.091e0	5.042e0	4.788e0
7	Isotonitazene	10.00	3 of 3	9.240e0	2.412e-1	2.61	92.40	9.289e0	8.977e0	9.452e0
8	Isotonitazene	50.00	3 of 3	5.035e1	5.170e-1	1.03	100.70	5.091e1	5.025e1	4.989e1
9	Isotonitazene	100.00	3 of 3	1.005e2	7.097e-1	0.71	100.46	1.013e2	1.002e2	9.992e1

Figure 3. Extracted ion chromatogram (XIC) traces and statistical results for A) metonitazene and B) isotonitazene, two potent NSO targeted in this study. XIC traces and resulting statistics panes from 10 pg/mL (LLOQ) to 100 ng/mL for: A) metonitazene and from 50 pg/mL (LLOQ) to 100 ng/mL for B) isotonitazene, respectively. Both NSO showed excellent accuracy and precision across the calibration levels, proving the overall robustness of the assay.

XIC area values resulting from the TOF MS experiment were used to generate regression plots for each of the 6 targeted analytes. Figure 4 shows the resulting calibration curves which demonstrate excellent linearity across the concentration ranges analyzed. They were calculated with R^2 values observed to be greater than 0.99 for all 6 targeted NSO.

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 6 target analytes used in this panel. These values demonstrate the quantitative performance of the ZenoTOF 7600 system in TOF MS mode.

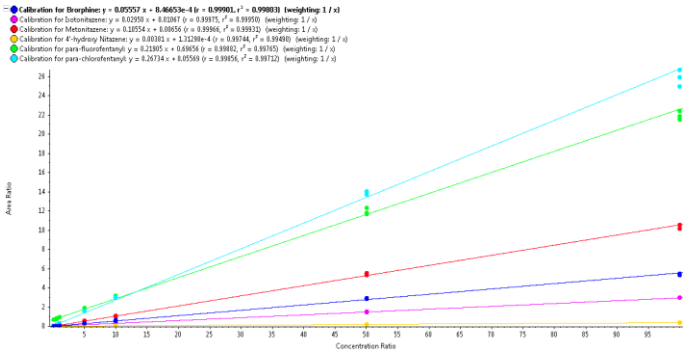


Figure 4. Excellent linearity for the 6 targeted NSO. Calibration curves resulting from the series of 9 calibrators extracted from human whole blood at concentrations ranging from 10 pg/mL to 100 ng/mL. R^2 values greater than 0.99 were observed for the 6 targeted analytes.

Table 1. Statistical results for the 6 targeted drugs. 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 6 targeted drugs.

Compound	Calibration Range (ng/mL)	Linear Correlation (R2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
Brorphine	*0.05 - 100*	*0.99803*	*0.05*	*88.95*	*8.47*
Isotonitazene	*0.05 - 100*	*0.99950*	*0.05*	*111.27*	*9.19*
Metonitazene	*0.01 - 100*	*0.99931*	*0.01*	*98.75*	*14.95*
4-Hydroxy Nitazene	*0.1 - 100*	*0.99490*	*0.1*	*103.58*	*7.52*
para-Fluorofentanyl	*0.5 - 100*	*0.99765*	*0.5*	*85.75*	*2.23*
para-Chlorofentanyl	*0.01 - 100*	*0.99712*	*0.01*	*88.24*	*8.17*

Zeno trap technology leads to MS/MS sensitivity gains

QTOF mass spectrometers commonly make use of an orthogonal TOF geometry which has been shown to maximize MS and MS/MS resolution and mass accuracy for an entire spectrum, but results in a significant loss of ions through this region of the MS (only 5-20% duty cycle).[1] To overcome this limitation, a Zeno trap was added at the end of the collision cell on the ZenoTOF 7600 system, which increases the duty cycle in the orthogonal injection region of the MS to ≥90% across the entire mass range. Therefore, the technological enhancements on the ZenoTOF 7600 system significantly increase MS/MS sensitivity which results in improved MS/MS spectral quality at low analyte concentration. This improvement ultimately yields improved MS/MS spectral library matching which provides greater confidence in analyte identification.

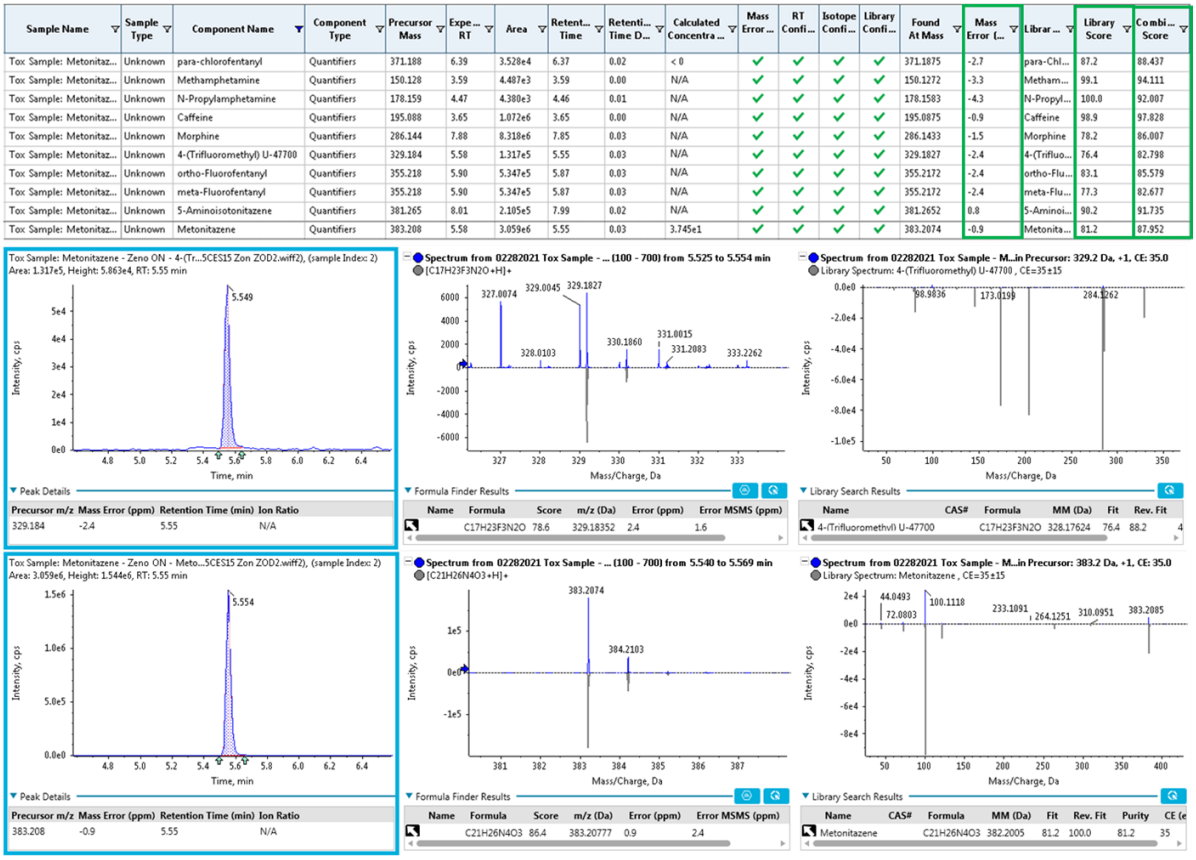
Zeno MS/MS increases confident identifications of low drug levels in authentic postmortem case samples

The MS/MS sensitivity improvements resulting from the use of the Zeno trap on the ZenoTOF 7600 system was investigated by analyzing discarded authentic postmortem case samples from subjects suspected of NSO ingestion resulting in accidental overdoses. These biological specimens were prepared using the aforementioned LLE procedure. Data were acquired on the ZenoTOF 7600 system with both the Zeno trap on and off for each sample and the results were compared to assess the impact of the MS/MS sensitivity gains. The concentrations of the targeted NSO detected in the discarded authentic postmortem case samples were calculated automatically in SCIEX OS software using the calibration curves generated for each of the 6 target analytes. Each case sample was run in triplicate.

Case study 1

Figure 5 (top) shows the results table from the analysis of discarded authentic postmortem case sample #1, using Zeno IDA, where 10 analytes were successfully identified. Figure 5 (bottom) also displays the XIC, TOF MS and TOF MS/MS spectra of two representative drugs positively identified in the sample: methamphetamine and 4-(Trifluoromethyl) U-47700, a potent synthetic opioid that has been reported to cause opioid-like effects similar to heroin and fentanyl. The results table shows the successful detection of two of the targeted NSO: *para*-chlorofentanyl and metonitazene, as well as other non-targeted NPS such as 4-(Trifluoromethyl) U-47700 and fluorofentanyl (the para and meta isomers were not resolved chromatographically). The presence of fentanyl analogs (*para*-chlorofentanyl and *para-/meta*- fluorofentanyl) and the potent synthetic opioid 4-(Trifluoromethyl) U-47700 suggest that the subject ingested a preparation originating from the illicit drug market. The presence of multiple potent NSO could support the case of combined opioid drug toxicity leading to death. Positive identification determination was accomplished using the four confidence criteria and sorted out using the traffic light system. The mass errors (ranging from -4.3 to 0.8 ppm), the mass spectra library scores (ranging from 76 to 100%) and the combined scores (ranging from 82.677 and 97.828%) provided excellent measures of the confident identification of the ten compounds in the discarded postmortem sample #1.

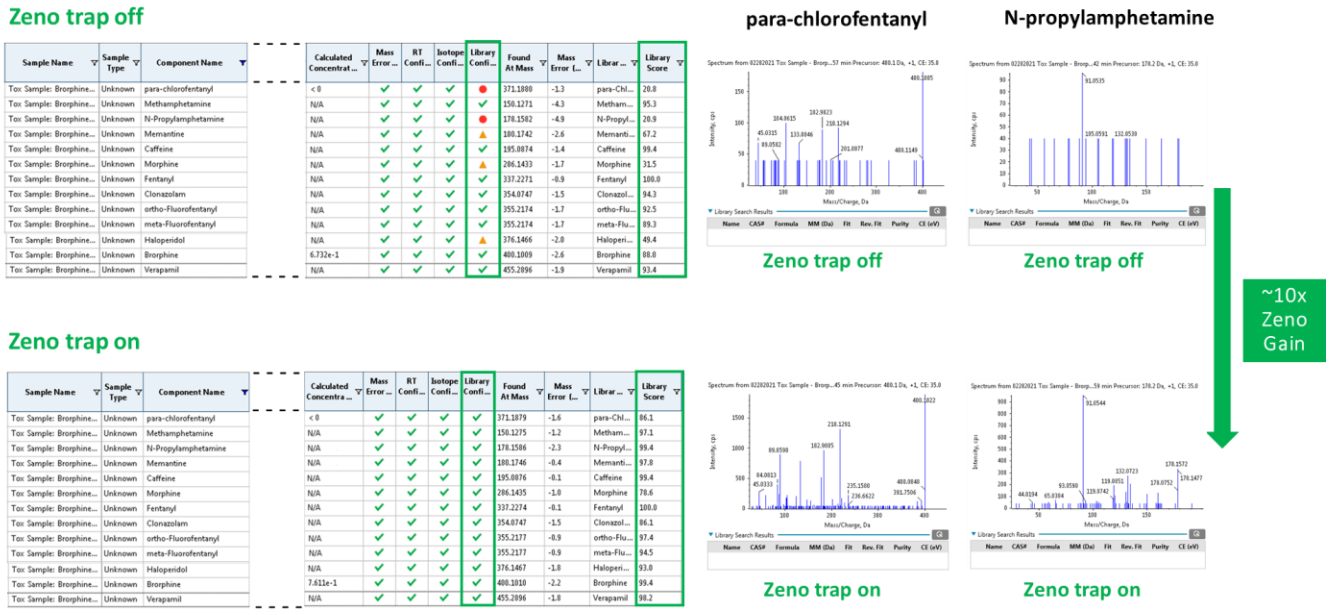
Discarded authentic postmortem case sample # 1



Sample Name	Sample Type	Component Name	Component Type	Precursor Mass	Expe... RT	Area	Retent... Time	Retenti... Time D...	Calculated Concentra ...	Mass Error ...	RT Confi...	Isotope Confi...	Library Confi...	Found At Mass	Mass Error (...	Librar ...	Library Score	Combi... Score
Tox Sample: Metonitaz...	Unknown	para-chlorofentanyl	Quantifiers	371.188	6.39	3.528e4	6.37	0.02	< 0	✓	✓	✓	✓	371.1875	-2.7	para-Chl...	87.2	88.437
Tox Sample: Metonitaz...	Unknown	Methamphetamine	Quantifiers	150.128	3.59	4.487e3	3.59	0.00	N/A	✓	✓	✓	✓	150.1272	-3.3	Metham...	99.1	94.111
Tox Sample: Metonitaz...	Unknown	N-Propylamphetamine	Quantifiers	178.159	4.47	4.380e3	4.46	0.01	N/A	✓	✓	✓	✓	178.1583	-4.3	N-Propyl...	100.0	92.007
Tox Sample: Metonitaz...	Unknown	Caffeine	Quantifiers	195.088	3.65	1.072e6	3.65	0.00	N/A	✓	✓	✓	✓	195.0875	-0.9	Caffeine	98.9	97.828
Tox Sample: Metonitaz...	Unknown	Morphine	Quantifiers	286.144	7.88	8.318e6	7.85	0.03	N/A	✓	✓	✓	✓	286.1433	-1.5	Morphine	78.2	86.007
Tox Sample: Metonitaz...	Unknown	4-(Trifluoromethyl) U-47700	Quantifiers	329.184	5.58	1.317e5	5.55	0.03	N/A	✓	✓	✓	✓	329.1827	-2.4	4-(Triflu...	76.4	82.798
Tox Sample: Metonitaz...	Unknown	ortho-Fluorofentanyl	Quantifiers	355.218	5.90	5.347e5	5.87	0.03	N/A	✓	✓	✓	✓	355.2172	-2.4	ortho-Flu...	83.1	85.579
Tox Sample: Metonitaz...	Unknown	meta-Fluorofentanyl	Quantifiers	355.218	5.90	5.347e5	5.87	0.03	N/A	✓	✓	✓	✓	355.2172	-2.4	meta-Flu...	77.3	82.677
Tox Sample: Metonitaz...	Unknown	5-Aminoisotonitazene	Quantifiers	381.265	8.01	2.105e5	7.99	0.02	N/A	✓	✓	✓	✓	381.2652	0.8	5-Aminoi...	90.2	91.735
Tox Sample: Metonitaz...	Unknown	Metonitazene	Quantifiers	383.208	5.58	3.059e6	5.55	0.03	3.745e1	✓	✓	✓	✓	383.2074	-0.9	Metonita...	81.2	87.952

Figure 5. Results from authentic postmortem case study #1. (Top) Results table in SCIEX OS software showing the analytes positively identified in postmortem case sample #1 along with mass error, library score and combined score using the confidence criteria. (Bottom) XICs, TOF MS and TOF MS/MS spectra collected provide detailed and confident identification of two of the positively identified analytes: methamphetamine and 4-(Trifluoromethyl) U-47700.

Discarded authentic postmortem case sample # 2



Sample Name	Sample Type	Component Name	Calculated Concentra ...	Mass Error ...	RT Confi...	Isotope Confi...	Library Confi...	Found At Mass	Mass Error (...	Librar ...	Library Score
Tox Sample: Morphine...	Unknown	para-chlorofentanyl	< 0	✓	✓	✓	●	371.1880	-1.3	para-Chl...	20.8
Tox Sample: Morphine...	Unknown	Methamphetamine	N/A	✓	✓	✓	✓	150.1271	-4.3	Metham...	95.3
Tox Sample: Morphine...	Unknown	N-Propylamphetamine	N/A	✓	✓	✓	●	178.1582	-4.9	N-Propyl...	20.9
Tox Sample: Morphine...	Unknown	Memantine	N/A	✓	✓	✓	▲	180.1742	-2.6	Memanti...	67.2
Tox Sample: Morphine...	Unknown	Caffeine	N/A	✓	✓	✓	✓	195.0874	-1.4	Caffeine	99.4
Tox Sample: Morphine...	Unknown	Morphine	N/A	✓	✓	✓	▲	286.1433	-1.7	Morphine	31.5
Tox Sample: Morphine...	Unknown	Fentanyl	N/A	✓	✓	✓	✓	337.2271	-0.9	Fentanyl	100.0
Tox Sample: Morphine...	Unknown	Clonazolam	N/A	✓	✓	✓	✓	354.0747	-1.5	Clonazol...	94.3
Tox Sample: Morphine...	Unknown	ortho-Fluorofentanyl	N/A	✓	✓	✓	✓	355.2174	-1.7	ortho-Flu...	92.5
Tox Sample: Morphine...	Unknown	meta-Fluorofentanyl	N/A	✓	✓	✓	✓	355.2174	-1.7	meta-Flu...	89.3
Tox Sample: Morphine...	Unknown	Haloperidol	N/A	✓	✓	✓	▲	376.1466	-2.0	Haloperi...	49.4
Tox Sample: Morphine...	Unknown	Bromphine	6.732e-1	✓	✓	✓	✓	400.1009	-2.6	Bromphine	88.0
Tox Sample: Morphine...	Unknown	Verapamil	N/A	✓	✓	✓	✓	455.2896	-1.9	Verapamil	93.4

Sample Name	Sample Type	Component Name	Calculated Concentra ...	Mass Error ...	RT Confi...	Isotope Confi...	Library Confi...	Found At Mass	Mass Error (...	Librar ...	Library Score
Tox Sample: Morphine...	Unknown	para-chlorofentanyl	< 0	✓	✓	✓	✓	371.1879	-1.6	para-Chl...	86.1
Tox Sample: Morphine...	Unknown	Methamphetamine	N/A	✓	✓	✓	✓	150.1275	-1.2	Metham...	97.1
Tox Sample: Morphine...	Unknown	N-Propylamphetamine	N/A	✓	✓	✓	✓	178.1586	-2.3	N-Propyl...	99.4
Tox Sample: Morphine...	Unknown	Memantine	N/A	✓	✓	✓	✓	180.1746	-0.4	Memanti...	97.8
Tox Sample: Morphine...	Unknown	Caffeine	N/A	✓	✓	✓	✓	195.0876	-0.1	Caffeine	99.4
Tox Sample: Morphine...	Unknown	Morphine	N/A	✓	✓	✓	✓	286.1435	-1.0	Morphine	78.6
Tox Sample: Morphine...	Unknown	Fentanyl	N/A	✓	✓	✓	✓	337.2274	-0.1	Fentanyl	100.0
Tox Sample: Morphine...	Unknown	Clonazolam	N/A	✓	✓	✓	✓	354.0747	-1.5	Clonazol...	86.1
Tox Sample: Morphine...	Unknown	ortho-Fluorofentanyl	N/A	✓	✓	✓	✓	355.2177	-0.9	ortho-Flu...	97.4
Tox Sample: Morphine...	Unknown	meta-Fluorofentanyl	N/A	✓	✓	✓	✓	355.2177	-0.9	meta-Flu...	94.5
Tox Sample: Morphine...	Unknown	Haloperidol	N/A	✓	✓	✓	✓	376.1467	-1.8	Haloperi...	93.0
Tox Sample: Morphine...	Unknown	Bromphine	7.611e-1	✓	✓	✓	✓	400.1010	-2.2	Bromphine	99.4
Tox Sample: Morphine...	Unknown	Verapamil	N/A	✓	✓	✓	✓	455.2896	-1.8	Verapamil	98.2

Figure 6. Results comparison between Zeno trap on and off for authentic postmortem case study #2. (Top) Results table and representative TOF MS/MS spectra with (top) and without (bottom) the Zeno trap enabled. The use of the Zeno trap resulted in a 10x improvement, on average, in sensitivity, which resulted in greater confidence in analyte identification confirmation through MS/MS spectral library matching.

Case study 2

The use of the Zeno trap for this qualitative workflow should provide substantial improvements in the observed TOF MS/MS spectral quality which should ultimately result in greater confidence in spectral library matching confirmation. Figure 6 (left) shows the results table from the analysis of discarded authentic postmortem case sample # 2 without (top) and with (bottom) activation of the Zeno trap. The analysis of this sample with the Zeno trap on resulted in greater library confidence for the majority of the positively identified compounds, as evidenced by comparing the green icons (bottom table) with the Zeno trap on to the red and yellow icons (top table) with the Zeno trap off.

For example, the library score for the identification of I-chlorofentanyl and N-propylamphetamine (ISTD) increased from 20.8% to 86.1% and from 20.9% to 99.4%, respectively, when the Zeno trap was activated. This drastic improvement in library score is the consequence of the MS/MS sensitivity enhancements afforded by the Zeno trap, which resulted in improved TOF MS/MS spectral quality. The sensitivity gains are shown in the TOF MS/MS spectra comparison for *para*-chlorofentanyl and N-propylamphetamine in Figure 6 (right). Overall, the average library score for the positively identified analytes in this sample increased from 74.5% to 94.4% when the Zeno trap was activated. It also resulted in a 10x improvement, on average, in sensitivity, which resulted in greater confidence in analyte identification confirmation through MS/MS spectral library matching.

Case study 3

Figure 7 shows the results tables and representative TOF MS/MS spectra comparison from the analysis of discarded authentic postmortem case sample #3. Similar observations can be drawn from the observations made for the analysis of the first case sample. First, the use of the Zeno trap resulted in the confident detection of all ten compounds with high confidence as evidenced by the high library scores ranging from 77.3 to 99.3%. Without the Zeno trap activated, analysis of this case sample resulted in two poorly matched analytes (*para*-chlorofentanyl and morphine returned a yellow and red library match icon, respectively) and three unmatched analytes (N-propylamphetamine, memantine and 5-aminoisonitazene returned a red library match icon) because of the poor quality of the triggered TOF MS/MS spectra. This is evidenced by comparing the TOF MS/MS spectra for two of these analytes, memantine and 5-aminoisonitazene (right). Without the Zeno trap activated, the generated TOF MS/MS spectra did not contain unique fragment ions to yield a library match. Overall, a 9x improvement, on average, in sensitivity was observed across the TOF MS/MS spectra positively identified in the three authentic postmortem case samples analyzed when the Zeno trap was activated (Figure 1). In addition, the use of the Zeno trap enabled acquisition of a much richer MS/MS spectrum that was used for confident compound identification, which resulted in an average library score increase from 56.3% to 88.6%.

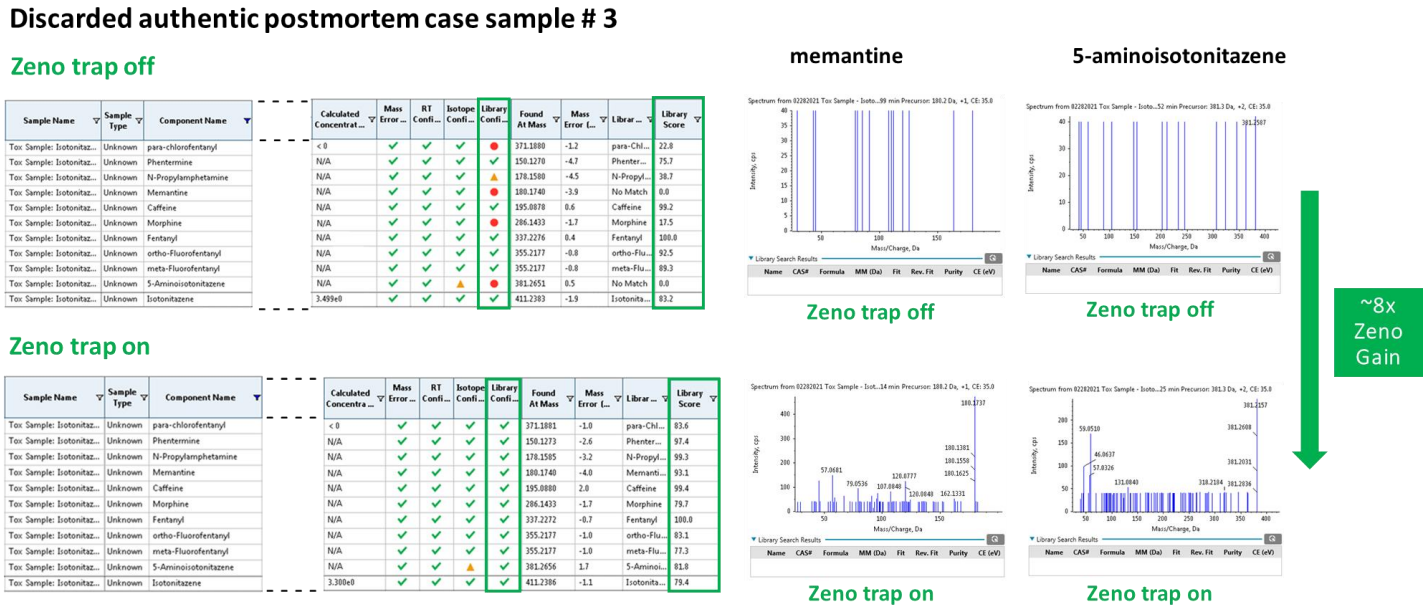


Figure 7. Results comparison between Zeno trap on and off for authentic postmortem case study #3. (Top) Results table and representative TOF MS/MS spectra with (top) and without (bottom) the Zeno trap enabled. The use of the Zeno trap enabled acquisition of a much richer MS/MS spectra that contained unique fragment ions that were used for confident compound identification.

A few observations can be drawn from the results highlighted in Figure 7. First, the added MS/MS sensitivity afforded by use of the Zeno trap enabled the accurate identification of 5-aminoisonitazene, one of metabolites of the potent NSO isotonitazene, with a library score of 81.8%. Second, the detection of fentanyl and other fentanyl analogs (*para*-chlorofentanyl and *para*-/*meta*-fluorofentanyl) suggest that the drug ingested by the subject might have originated from the illicit market. Although the presence of fentanyl might have been a contributing factor to the accidental overdose, the presence of the potent NSO isotonitazene and its metabolite could support the case of combined opioid drug toxicity leading to death.

Conclusions

A comprehensive and highly sensitive method for the screening and identification of potent NSO in human whole blood is described. The significant gains in MS/MS sensitivity on the ZenoTOF 7600 system yielded an improvement in confident identifications of low-level analytes through spectral library matching. The observed sensitivity gains afforded by the use of the Zeno trap resulted in a 9x improvement, on average, in TOF MS/MS sensitivity across the drugs positively identified in the authentic case samples analyzed. This improvement enabled confident identification of key drugs and metabolites at trace levels that were not previously achievable.

The MS/MS sensitivity levels afforded by ZenoTOF 7600 system provide a means to monitor low levels of ultra-potent NSO in poly-drug intake scenarios. This advancement could support the case of combined opioid drug toxicity leading to death, which offers a valuable insight into the causation of accidental overdoses.

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.

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Streamlined Unknown Screening for Postmortem Analysis

Using the SCIEX X500R QTOF System and SWATH® Acquisition in a Forensic Toxicology Laboratory

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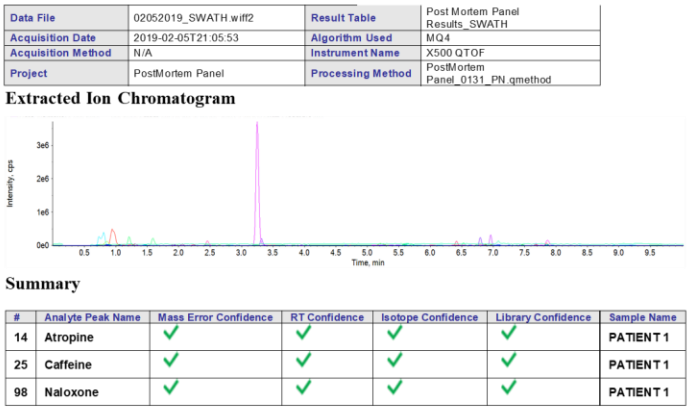
Gathering evidence to determine the cause of death is paramount in the service of the public interest and the judicial process. To this end, accurate identification of drugs present in postmortem samples is crucial for forensic toxicologists to successfully conduct case examinations as the findings of these examinations often raise important questions and provide immediate answers about cause of death and other related antemortem events.

The rapid emergence of novel psychoactive substances (NPS), designer drugs, and the abuse of prescribed drugs require fast and comprehensive drug screening approaches. Traditionally, postmortem drug screens are either performed by immunoassay or GC-MS. However, immunoassay techniques are often not conclusive enough (false positives) and lack sensitivity. GC-MS requires sample derivatization and lengthy chromatographic runs to accurately identify NPS and other drugs present in a biological postmortem sample. As a result, there is a need for rapid and robust screening methods that allow positive identification of NPS and other drugs with a high level of sensitivity and selectivity.



Data File	02052019_SWATH.wiff2	Result Table	Post Mortem Panel Results_SWATH
Acquisition Date	2019-02-05T21:05:53	Algorithm Used	MQ4
Acquisition Method	N/A	Instrument Name	X500 QTOF
Project	PostMortem Panel	Processing Method	PostMortem Panel_0131_PN.qmethod

Extracted Ion Chromatogram

Summary

#	Analyte Peak Name	Mass Error Confidence	RT Confidence	Isotope Confidence	Library Confidence	Sample Name
14	Atropine	✓	✓	✓	✓	PATIENT 1
25	Caffeine	✓	✓	✓	✓	PATIENT 1
98	Naloxone	✓	✓	✓	✓	PATIENT 1

Figure 1. Confidently Identify all Analytes Present Within an Unknown Postmortem Blood Sample. Obtain accurate mass data of all novel psychoactive substances and other drugs of interest present in a postmortem blood sample using SWATH® acquisition. Chromatogram and results table showing all positive target compounds identified in a blood sample based on the different acceptance criteria are easily generated using SCIEX OS Software.

High resolution mass spectrometers (HRMS) in the forensic laboratory allows toxicologists to rapidly obtain complete chemical profiles from biological samples. The acquisition of accurate mass, analyte specific MS/MS spectra often provides increased confidence in compound identification at low analyte concentrations.

In this technical note, a comprehensive drug screening workflow for the analysis of postmortem blood samples is described. The workflow was streamlined using a simplified sample preparation approach in combination with SWATH® Acquisition on the SCIEX X500R QTOF System.

Key Features of Postmortem Method

- Postmortem panel consisted of 151 drugs with limits of detection (LOD) down to the sub-ng/mL range
- Sample preparation was significantly simplified, using a protein precipitation with methanol and acetonitrile, followed by reconstitution with mobile phase
- Robust and reliable chromatographic separation was achieved using the vMethod™ Application for 664 forensic compounds[1] using the ExionLC™ AC HPLC system
- Analytes were monitored in positive ionization mode using SWATH® Acquisition on the SCIEX X500R QTOF with SCIEX OS Software
- The method allowed identification and quantification of nanogram (ng) detection limits of these drugs in a complex biological matrix

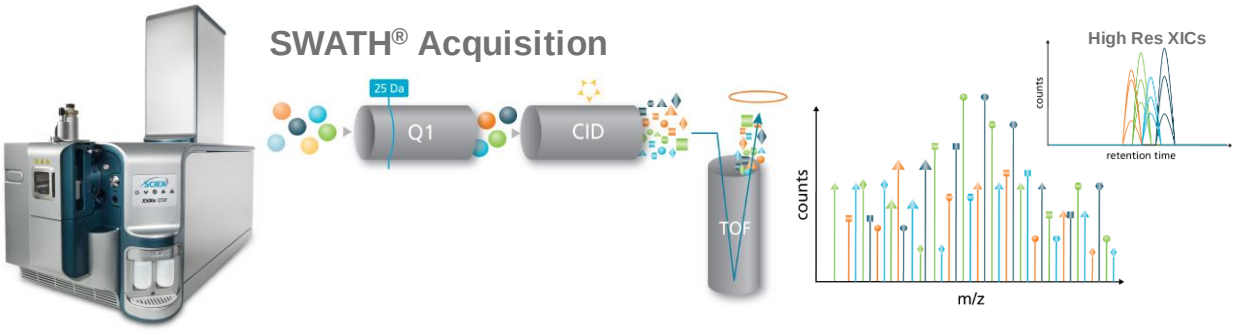


Figure 2. Avoid Missing Important Forensic Compounds with SCIEX X500R QTOF System and SWATH® Acquisition. In this workflow, instead of the quadrupole (Q1) transmitting a narrow mass range through to the collision cell, a wider window containing more analytes is passed. This produces a richer MS/MS spectrum which is a composite of all the analytes within that Q1 m/z window. This MS/MS is performed across the full m/z range of target compounds, ensuring MS/MS is collected on every detectable compound. Because the fragment ions are generated using high resolution acquisition, detected compounds can be accurately identified through extraction of the specific accurate mass fragment ions.

Experimental Details

Sample Preparation: Stock standard mixtures in neat solutions were prepared by diluting with methanol: water (20:80, v/v) to appropriate concentrations. These diluted standard mixtures were used to determine the retention times of the 151 targeted compounds. The full list of the forensic compounds used this method, including accurate mass information and limits of detection (LOD) is detailed in Table 1.

10 µL of the stock standard solution mixture containing the 151 different drugs were spiked into 90 µL of whole blood matrix for initial method development. Forensic case postmortem blood samples were extracted by using a protein precipitation procedure. In short, 900 µL of Methanol: MeCN (50:50, v/v) were added into the above mixture and vortexed for 1 min then followed by 3 min sonication and another 1 min vortex mixing. Then the samples were centrifuged for 5 min at 8,000 rpm. The supernatant was transferred out and completely dried down under nitrogen gas. The residues were reconstituted with 500 µL methanol: water (20:80, v/v). The protein precipitation procedure is shown in Figure 3.

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	•1 min
Sonicate	•3 min
Vortex	•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 MeOH: water (20:80, v/v)

Figure 3. Protein Precipitation Procedure for Whole Blood Samples. A 9-step protein precipitation protocol was used for selectively extracting drugs from whole blood samples for analysis with the X500R QTOF System.

Liquid Chromatography: HPLC separation was performed on a Phenomenex Kinetex Phenyl-Hexyl column (50 × 2.1 mm, 2.6µm, 00B-4495-E0) on the SCIEX ExionLC™ AC system. Mobile phases used were water and methanol with appropriate additives. The injection volume was 5 µL and the total LC runtime was 8.5 minutes.

Mass Spectrometry: MS and MS/MS data were collected using SWATH® Acquisition on the SCIEX X500R QTOF System with SCIEX OS Software, each SWATH® Acquisition scan beginning with a TOF MS experiment.

Data Analysis: Targeted data processing was performed using SCIEX OS Software for positive analyte identification based on previously determined criteria. Four main confidence criteria were used including mass error (M), retention time (R), isotope ratio difference (I), and library score (L). Subsequently, a combined score (C) was computed based on these four confidence categories (MRIL) with custom weightings, as shown in Figure 4.

Qualitative Rule	✓ Acceptable Difference		▲ Marginal Difference		● Unacceptable Difference		Combined Score Weight (%)
Mass Error (ppm)	<	5	<	10	>=	10	15
Fragment Mass Error (ppm)	<	5	<	10	>=	10	0
Error in Retention Time	<	5	<	10	>=	10	30
% Difference Isotope Ratio	<	20	<	40	>=	40	5
Library Hit Score	>	60	>	30	<=	30	50
Formula Finder Score	>	50	>	20	<=	20	20

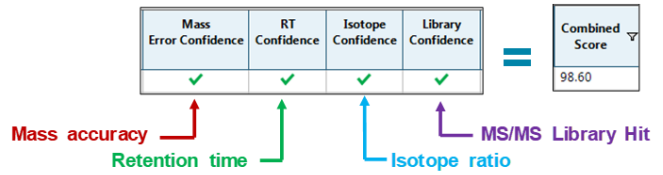


Figure 4. Confidence Criteria Used for Data Processing Using SCIEX OS Software. Mass Error (15%), Retention Time (30%), Isotope Ratio Difference (5%) and Library Score (50%) were used to generate a combined score.

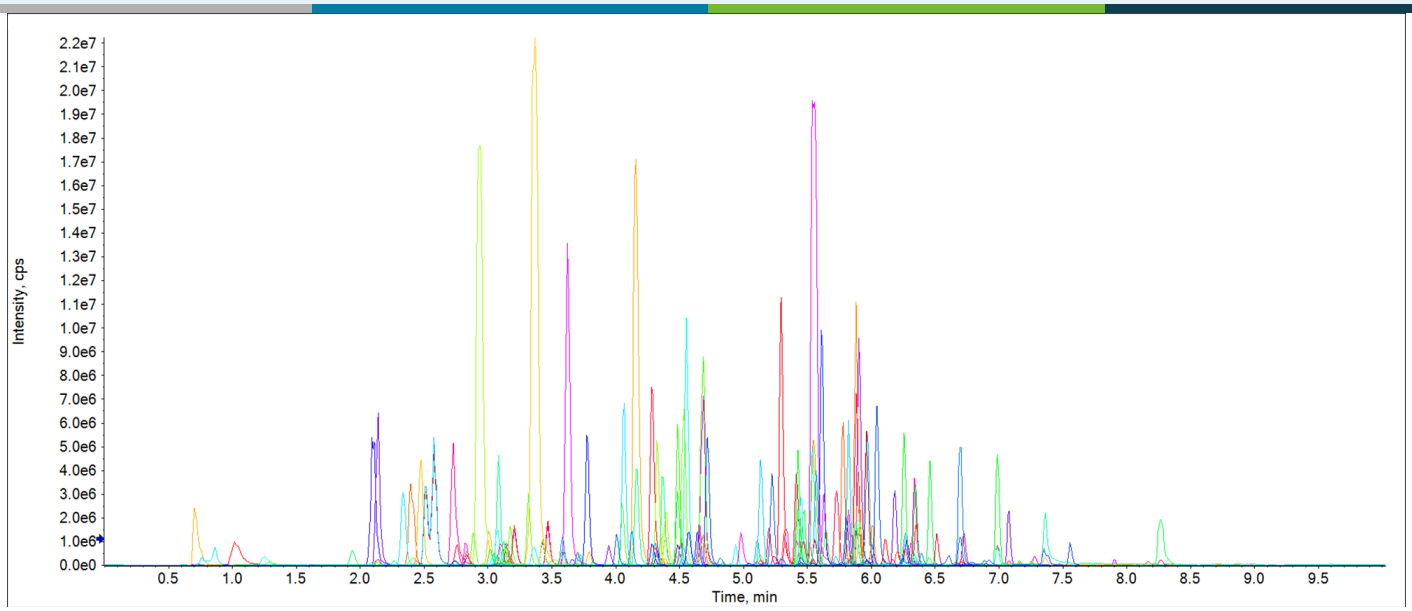


Figure 5. Obtain Fast and Confident Identification of Hundreds of NPS and Other Drugs of Interest in Biological Matrices. Extracted Ion Chromatogram (XIC) shows a rapid LC separation (8.5 min) and identification of 151 forensic compounds of interest spiked in whole blood using SWATH® acquisition.

vMethod Application for Comprehensive Screening of Postmortem Samples

Control whole blood samples spiked with all 151 forensic compounds of interest were prepared at various concentrations ranging from 2-12000 ng/mL. These standard solutions were extracted and injected to build a data analysis processing method.

The separation conditions for the vMethod Application for 664 forensic compounds[1] were initially used and further optimized to a final 8.5 min LC run time. Figure 5 shows the extracted ion chromatogram (XIC) for all 151 forensic compounds in a control whole blood sample using the optimized LC conditions.

Information-dependent acquisition (IDA) was initially applied to acquire and store MS/MS spectra for each target compound of interest. However, SWATH® Acquisition was chosen as the preferred data acquisition method as this strategy provides comprehensive fragment ion spectra generation over the whole run, minimizing the risk of missing potential forensic compounds present in postmortem blood samples in comparison to IDA[2,3].

Through the method development process, it was important to obtain the limit of detections (LOD) of all 151 compounds. Figure 6 shows the XIC, TOF-MS and MS/MS spectra of 3 representative forensic compounds spiked in whole blood matrix at different LOD concentrations. SWATH® Acquisition generated comprehensive and high-quality MS/MS spectra, enabling reliable compound fragmentation for spectral library database searching for the analytes.

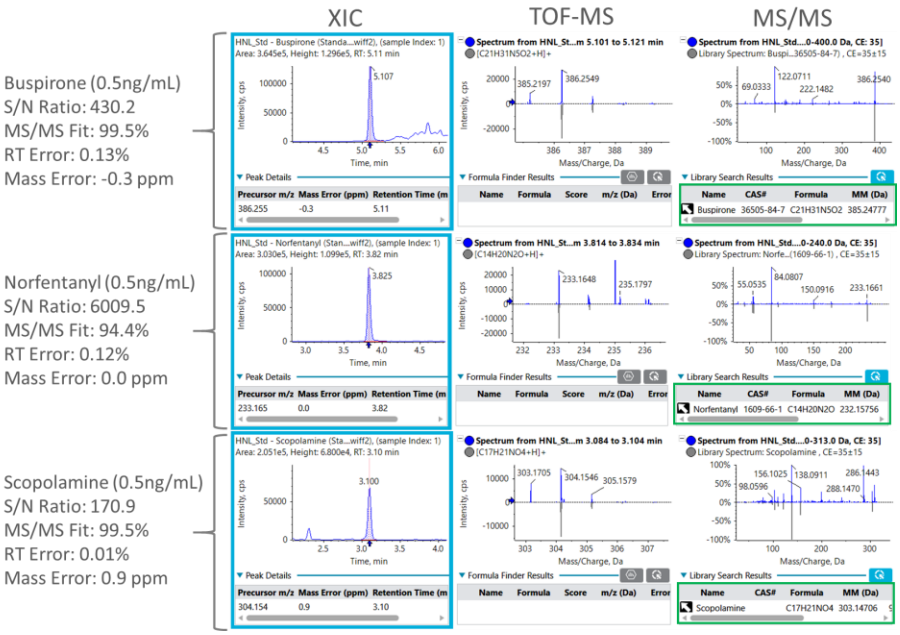


Figure 6. SWATH Acquisition Leads to Increased Compound Identification. XICs, TOF-MS and MS/MS spectra obtained showing confident and detailed identification of buspirone (top), norfentanyl (middle) and scopolamine (bottom) spiked in whole blood at low ng/mL concentrations.

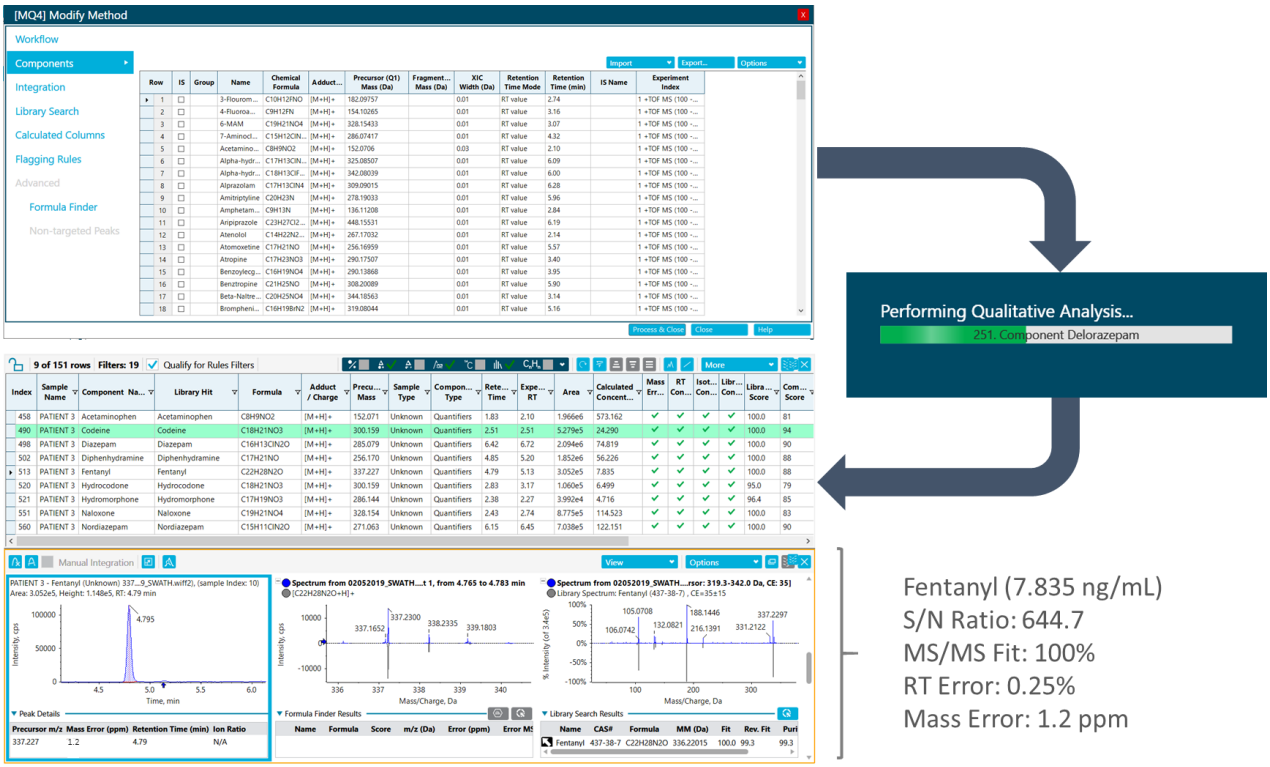


Figure 7. Re-Interrogate the Data Obtained from the Analysis of Postmortem Samples Using SCIEX OS Software. Data acquired from a postmortem blood sample, suspected of containing an NPS, was reprocessed after modifying the processing method window (top). The novel opioid Fentanyl (bottom) was retrospectively identified from the SWATH® Acquisition data.

Discovering Novel Psychoactive Substances Present in Postmortem Samples Through Retrospective Analysis

As a data independent acquisition strategy, SWATH® Acquisition allows the forensic toxicologists to collect MS and MS/MS information on every detectable peak within a sample, essentially creating a digital record of the sample. This allows the option to re-interrogate the sample data should new questions arise in the future.

Figure 7 displays a postmortem blood sample screened using SWATH® Acquisition, where 10 forensic compounds targeted in the data processing method were successfully identified and quantified above the LOQ in the first round of data processing. Next, the same sample was re-interrogated for the presence of a potential known NPS (i.e., Fentanyl), by extracting the compound's molecular formula ($C_{21}H_{26}N_2O$). Based on the confidence criteria set in SCIEX OS Software Fentanyl was detected with good confidence in the interrogated sample. This highlights the ability for users to retrospectively analyze previously acquired SWATH data sets and screen for new compounds without having to re-inject samples, when newly identified forensic targets are discovered.

Streamlined Sample Reporting for Efficient Forensic Case Turnaround Time

The data analysis component of SCIEX OS Software is designed to provide a centralized results grid for streamlined review and efficient sample report processing. Retention time, mass, isotope ratio error, and mass spectral library search score are calculated automatically and visualized using "traffic lights". Compounds identified with high confidence are indicated using green check symbols.

The results table can then be sorted by the 'traffic light' columns and/or filtered by 'identification criteria' for review and reporting of the positively identified compounds.

Figure 8 shows a customized report generated by SCIEX OS Software, after the processing of a postmortem blood sample, where 10 forensic compounds of interest were confidently identified based on the acceptance criteria. The sample report included XICs, TOF-MS and MS/MS spectra as well as the library matches. Table 2 summarizes the results of the postmortem blood sample and includes the list of compounds detected along with their concentration, library score and combined score.

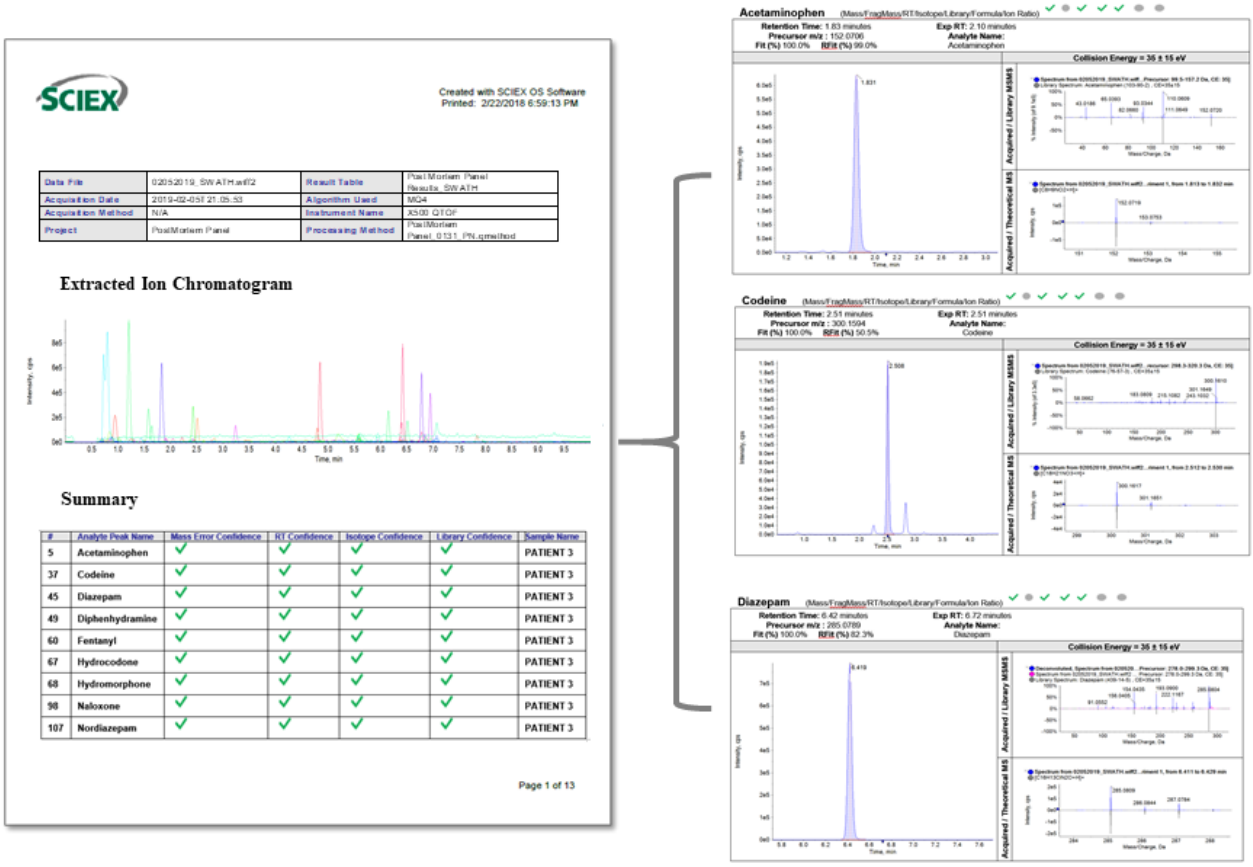


Figure 8. Streamlined Data Processing Through Customized Sample Reports Using SCIEX OS Software. A postmortem blood sample report was generated identifying all of the forensic compounds identified (left**)** and representative XICs, TOF-MS and MS/MS spectra (right).

Table 2. Summary Table for Postmortem Blood Sample for Case Sample #3. Inter-day average (n=3) for the detection of 10 compounds screened in a postmortem blood sample.

Drug Detected	Concentration (ng/mL)	Library Score (%)	Combined Score (%)
Acetaminophen	*773.162 ± 7.4*	*100*	*81.3 ± 0.2*
Codeine	*24.290 ± 2.1*	*95.7*	*89.5 ± 0.1*
Diazepam	*74.819 ± 2.4*	*98.3*	*94.6 ± 0.6*
Diphenhydramine	*56.226 ± 1.2*	*100*	*94.9 ± 1.1*
Fentanyl	*7.835 ± 0.3*	*100*	*90.3 ± 0.8*
Hydrocodone	*6.499 ± 0.4*	*95*	*79.3 ± 0.5*
Hydromorphone	*4.716 ± 0.1*	*96.4*	*85.4 ± 1.2*
Morphine	*29.732 ± 1.8*	*89.8*	*73.2 ± 0.5*
Naloxone	*114.523 ± 3.4*	*100*	*82.8 ± 1.2*
Nordiazepam	*122.151 ± 1.4*	*100*	*90.1 ± 0.4*

Conclusions

A comprehensive drug screening workflow for the analysis of postmortem blood samples was successfully developed using the SCIEX X500R QTOF System.

- The adaptation of the vMethod Application LC-MS conditions enabled the rapid implementation and optimization of the screening workflow for 151 forensic compounds of interest.
- SWATH® Acquisition generated comprehensive and high-quality MS/MS spectra, which enabled reliable compound fragmentation comparison to library spectra for confident drug identification. The data independent nature of SWATH® Acquisition allows for retrospective analysis to avoid missing potential NPS present in postmortem samples.
- The data analysis component of SCIEX OS Software provided a simplified interface for streamlined data review based a rigorous scoring system, a “traffic light” display, and an efficient sample report generation process.

Table 1. List of Compounds Used in the Postmortem Panel Along with Chemical Formula, Precursor Mass and Cutoff Concentration (LOD).

Component Name	Retention Time	Chemical Formula	Precursor (Q1) Mass (Da)	Adduct & Charge	LOD (ng/mL)
3-Fluoromethcathinone HCl	*2.74*	$C_{10}H_{12}FNO$	*182.09757*	*[M+H]+*	*5*
4-Fluoroamphetamine	*3.16*	$C_9H_{12}FN$	*154.10265*	*[M+H]+*	*5*
6MAM	*3.07*	$C_{19}H_{21}NO_4$	*328.15433*	*[M+H]+*	*2.5*
7-Aminoclonazepam	*4.32*	$C_{15}H_{12}ClN_3O$	*286.07417*	*[M+H]+*	*5*
Acetaminophen	*2.1*	$C_8H_9NO_2$	*152.0706*	*[M+H]+*	*250*
Alphahydroxyalprazolam	*6.09*	$C_{17}H_{13}ClN_4O$	*325.08507*	*[M+H]+*	*5*
Alphahydroxymidazolam	*6*	$C_{18}H_{13}ClFN_3O$	*342.08039*	*[M+H]+*	*5*
Alprazolam	*6.28*	$C_{17}H_{13}ClN_4$	*309.09015*	*[M+H]+*	*5*
Amitriptyline HCl	*5.96*	$C_{20}H_{23}N$	*278.19033*	*[M+H]+*	*12.5*
Amphetamine	*2.84*	$C_9H_{13}N$	*136.11208*	*[M+H]+*	*10*
Aripiprazole	*6.19*	$C_{23}H_{27}Cl_2N_3O_2$	*448.15531*	*[M+H]+*	*25*
Atenolol	*2.14*	$C_{14}H_{22}N_2O_3$	*267.17032*	*[M+H]+*	*50*
Atomoxatine HCL	*5.57*	$C_{17}H_{21}NO$	*256.16959*	*[M+H]+*	*5*
Atropine	*3.4*	$C_{17}H_{23}NO_3$	*290.17507*	*[M+H]+*	*1*
Benzoylecgonine	*3.95*	$C_{16}H_{19}NO_4$	*290.13868*	*[M+H]+*	*2.5*
Benztropine	*5.9*	$C_{21}H_{25}NO$	*308.20089*	*[M+H]+*	*25*
Beta-Naltrexol	*3.14*	$C_{20}H_{25}NO_4$	*344.18563*	*[M+H]+*	*5*
Brompheniramine maleate	*5.16*	$C_{16}H_{19}BrN2$	*319.08044*	*[M+H]+*	*1*
Buphedrone HCl	*3.43*	$C_{11}H_{15}NO$	*178.12264*	*[M+H]+*	*5*
Buprenorphine	*5.26*	$C_{29}H_{41}NO_4$	*468.31084*	*[M+H]+*	*0.5*
Bupropion HCl	*4.51*	$C_{13}H_{18}ClNO$	*240.11497*	*[M+H]+*	*12.5*
Buspirone	*5.1*	$C_{21}H_{31}N_5O_2$	*386.25505*	*[M+H]+*	*0.5*
Butorphanol tartrate	*4.66*	$C_{21}H_{29}NO_2$	*328.22711*	*[M+H]+*	*5*
Butylone HCl	*3.47*	$C_{12}H_{15}NO_3$	*222.11247*	*[M+H]+*	*0.5*
Caffeine	*3.25*	$C_8H_{10}N_4O_2$	*195.08765*	*[M+H]+*	*250*
Carbamazepine	*5.54*	$C_{15}H_{12}N_2O$	*237.10224*	*[M+H]+*	*2500*
Carboxyzolpidem	*3.59*	$C_{19}H_{19}N_3O_3$	*338.14992*	*[M+H]+*	*2.5*
Carisoprodol	*5.63*	$C_{12}H_{24}N2O_4$	*261.18088*	*[M+H]+*	*25*
Chlorpheniramine maleate	*4.98*	$C_{16}H_{19}ClN_2$	*275.13095*	*[M+H]+*	*5*
Chlorpromazine HCl	*6.31*	$C_{17}H_{19}ClN_2S$	*319.10302*	*[M+H]+*	*5*
Citalopram HBr	*5.33*	$C_{20}H_{21}FN_2O$	*325.17107*	*[M+H]+*	*5*
Clomipramine	*6.34*	$C_{19}H23ClN_2$	*315.16225*	*[M+H]+*	*12.5*

Component Name	Retention Time	Chemical Formula	Precursor (Q1) Mass (Da)	Adduct & Charge	LOD (ng/mL)
Clonidine	2.82	$C_9H_9Cl_2N_3$	230.02463	[M+H]+	5
Clozapine	5.42	$C_{18}H_{19}ClN_4$	327.1371	[M+H]+	5
Cocaethylene	4.72	$C_{18}H_{23}NO_4$	318.16998	[M+H]+	2.5
Cocaine	4.31	$C_{17}H_{21}NO_4$	304.15433	[M+H]+	2.5
Codeine	2.51	$C_{18}H_{21}NO_3$	300.15942	[M+H]+	2.5
Cotinine	2.73	$C_{10}H_{12}N_2O$	177.10224	[M+H]+	250
Cyclobenzaprine HCl	5.82	$C_{20}H_{21}N$	276.17468	[M+H]+	5
Delorazepam	6.29	$C_{15}H_{10}Cl_2N_2O$	305.02429	[M+H]+	0.5
Desalkylflurazepam	6.17	$C_{15}H_{10}ClFN_2O$	289.05385	[M+H]+	5
Desipramine HCl	5.88	$C_{18}H_{22}N_2$	267.18558	[M+H]+	25
Desomorphine	3.09	$C_{17}H_{21}NO_2$	272.16451	[M+H]+	5
Dextromethorphan	5.23	$C_{18}H_{25}NO$	272.20089	[M+H]+	1
Diazepam	6.72	$C_{16}H_{13}ClN_2O$	285.07892	[M+H]+	5
Diazepam D5	6.7	$C_{16}H_8[2H]_5ClN_2O$	290.1103	[M+H]+	5
Dihydrocodeine HCL	2.76	$C_{18}H_{23}NO_3$	302.17507	[M+H]+	5
Diltiazem HCL	5.73	$C_{22}H_{26}N_2O_4S$	415.1686	[M+H]+	12.5
Diphenhydramine HCl	5.2	$C_{17}H_{21}NO$	256.16959	[M+H]+	5
Diphenoxylate	6.51	$C_{30}H_{32}N_2O_2$	453.25365	[M+H]+	5
Doxepin HCl	5.42	$C_{19}H_{21}NO$	280.16959	[M+H]+	12.5
Doxylamine succinate	4.28	$C_{17}H_{22}N_2O$	271.18049	[M+H]+	25
Duloxetine HCL	5.84	$C_{18}H_{19}NOS$	298.12601	[M+H]+	12.5
EDDP perchlorate	5.29	$C_{20}H_{23}N$	278.19033	[M+H]+	25
Ephedrine HCl	2.52	$C_{10}H_{15}NO$	166.12264	[M+H]+	25
Estazolam	6.1	$C_{16}H_{11}ClN_4$	295.0745	[M+H]+	5
Ethylone HCl	3.47	$C_{12}H_{15}NO_3$	222.11247	[M+H]+	5
Etizolam	6.36	$C_{17}H_{15}ClN_4S$	343.07787	[M+H]+	5
Etomidate	6.27	$C_{14}H_{16}N_2O_2$	245.12845	[M+H]+	5
Fentanyl	5.13	$C_{22}H_{28}N_2O$	337.22744	[M+H]+	0.5
Flunitrazepam	6.21	$C_{16}H_{12}FN_3O_3$	314.09355	[M+H]+	5
Fluoxetine HCl	5.9	$C_{17}H18F_3NO$	310.14133	[M+H]+	12.5
Flurazepam	5.32	$C_{21}H_{23}ClFN_3O$	388.15864	[M+H]+	5
Fluvoxamine maleate	5.78	$C_{15}H_{21}F_3N_2O_2$	319.16279	[M+H]+	25

Component Name	Retention Time	Chemical Formula	Precursor (Q1) Mass (Da)	Adduct & Charge	LOD (ng/mL)
Gabapentin	2.39	$C_9H_{17}NO_2$	172.13321	[M+H]+	125
Haloperidol	5.54	$C_{21}H_{23}ClFNO_2$	376.14741	[M+H]+	1
Hydrocodone	3.17	$C_{18}H_{21}NO_3$	300.15942	[M+H]+	2.5
Hydromorphone	2.27	$C_{17}H_{19}NO_3$	286.14377	[M+H]+	2.5
Hydroxybupropion	4.39	$C_{13}H_{18}ClNO_2$	256.10988	[M+H]+	5
Hydroxyzine di-HCl	6.01	$C_{21}H_{27}ClN_2O_2$	375.18338	[M+H]+	5
Imipramine	5.88	$C_{19}H_{24}N_2$	281.20123	[M+H]+	25
Ketamine HCl	3.79	$C_{13}H_{16}ClNO$	238.09932	[M+H]+	2.5
Lamotrigine	4.16	$C_9H_7Cl_2N_5$	256.01513	[M+H]+	250
Levamisole	3	$C_{11}H_{12}N_2S$	205.0794	[M+H]+	5
Levetiracetam	2.48	$C_8H_{14}N_2O_2$	171.1128	[M+H]+	250
Lidocaine	3.37	$C_{14}H_{22}N_2O$	235.18049	[M+H]+	250
Loperamide	6.37	$C_{29}H_{33}ClN_2O_2$	477.23033	[M+H]+	1
Lorazepam	6	$C_{15}H_{10}Cl_2N_2O_2$	321.01921	[M+H]+	5
mCPP [1(3Chlorophenyl)piperazine HCL	4.38	$C_{10}H_{13}ClN_2$	197.084	[M+H]+	12.5
MDMA (methylenedioxymethamphetamine)	3.32	$C_{11}H_{15}NO_2$	194.11756	[M+H]+	10
MDPV HCL (3,4methylenedioxypyrovalerone)	4.39	$C_{16}H_{21}NO_3$	276.15942	[M+H]+	5
MEGX (monoethylglycycinexylidide)	2.94	$C_{12}H_{18}N_2O$	207.14919	[M+H]+	250
Meperidine	4.32	$C_{15}H_{21}NO_2$	248.16451	[M+H]+	12.5
Mephedrone HCL	3.43	$C_{11}H_{15}NO$	178.12264	[M+H]+	5
Meprobamate	4.53	$C_9H_{18}N_2O_4$	219.13393	[M+H]+	250
Methadone	5.91	$C_{21}H_{27}NO$	310.21654	[M+H]+	2.5
Methamphetamine	3.07	$C_{10}H_{15}N$	150.12773	[M+H]+	10
Methedrone HCl	3.18	$C_{11}H_{15}NO_2$	194.11756	[M+H]+	5
Methylone HCl	2.88	$C_{11}H_{13}NO_3$	208.09682	[M+H]+	5
Methylphenidate HCl	4.16	$C_{14}H_{19}NO_2$	234.14886	[M+H]+	12.5
Metoprolol TARTRATE	4.05	$C_{15}H_{25}NO_3$	268.19072	[M+H]+	5
MHC ((±)-10,11-Dihydro-10-Hydroxycarbamazepine)	4.69	$C_{15}H_{14}N_2O_2$	255.1128	[M+H]+	250
Midazolam	5.65	$C_{18}H_{13}ClFN_3$	326.08548	[M+H]+	5
Mirtazapine	4.48	$C_{17}H_{19}N_3$	266.16517	[M+H]+	12.5
Mitragynine	5.43	$C_{23}H_{30}N_2O_4$	399.22783	[M+H]+	1.25
Morphine	1.94	$C_{17}H_{19}NO_3$	286.14377	[M+H]+	5

Component Name	Retention Time	Chemical Formula	Precursor (Q1) Mass (Da)	Adduct & Charge	LOD (ng/mL)
Morphine-3-beta-glucuronide	*1.05*	$C_{23}H_{27}NO_9$	*462.17586*	*[M+H]+*	*24.7*
Naloxone	*2.74*	$C_{19}H_{21}NO_4$	*328.15433*	*[M+H]+*	*2.5*
Naltrexone	*3.02*	$C_{20}H_{23}NO_4$	*342.16998*	*[M+H]+*	*5*
Naphyrone HCl	*5.48*	$C_{19}H_{23}NO$	*282.18524*	*[M+H]+*	*5*
Naproxen	*6.26*	$C_{14}H_{14}O_3$	*231.10157*	*[M+H]+*	*250*
N-desmethylclomipramine HCL	*6.35*	$C_{18}H_{21}ClN_2$	*301.1466*	*[M+H]+*	*12.5*
N-Desmethyldoxepin	*5.43*	$C_{18}H_{19}NO$	*266.15394*	*[M+H]+*	*12.5*
Nefazodone HCl	*6.46*	$C_{25}H_{32}ClN5O_2$	*470.23173*	*[M+H]+*	*12.5*
Nitrazepam	*6.1*	$C_{15}H_{11}N_3O_3$	*282.08732*	*[M+H]+*	*50*
Norbuprenorphine	*4.84*	$C_{25}H_{35}NO_4$	*414.26389*	*[M+H]+*	*1.25*
Nordiazepam	*6.45*	$C_{15}H_{11}ClN_2O$	*271.06327*	*[M+H]+*	*5*
Norfentanyl oxalate	*3.82*	$C_{14}H_{20}N_2O$	*233.16484*	*[M+H]+*	*0.5*
Norhydrocodone HCL	*3.12*	$C_{17}H_{19}NO_3$	*286.14377*	*[M+H]+*	*12.5*
Norketamine HCl	*3.72*	$C_{12}H_{14}ClNO$	*224.08367*	*[M+H]+*	*2.5*
Normeperidine	*4.37*	$C_{14}H_{19}NO_2$	*234.14886*	*[M+H]+*	*12.5*
Noroxycodone HCl	*3.04*	$C_{17}H_{19}NO_4$	*302.13868*	*[M+H]+*	*5*
Nortriptyline HCl	*5.97*	$C_{19}H_{21}N$	*264.17468*	*[M+H]+*	*12.5*
O-Desmethyl tramadol HCL	*3.08*	$C_{15}H_{23}NO_2$	*250.18016*	*[M+H]+*	*12.5*
O-desmethyl venlafaxine	*3.7*	$C_{16}H_{25}NO_2$	*264.19581*	*[M+H]+*	*12.5*
Olanzapine	*3.5*	$C_{17}H_{20}N_4S$	*313.14814*	*[M+H]+*	*1*
Orphenadrine HCl	*5.54*	$C_{18}H_{23}NO$	*270.18524*	*[M+H]+*	*25*
Oxazepam	*6.13*	$C_{15}H_{11}ClN_2O_2$	*287.05818*	*[M+H]+*	*5*
Oxycodone	*3.07*	$C_{18}H_{21}NO_4$	*316.15433*	*[M+H]+*	*2.5*
Oxymorphone	*2.09*	$C_{17}H_{19}NO_4$	*302.13868*	*[M+H]+*	*2.5*
Paroxetine Maleate	*5.88*	$C_{19}H_{20}FNO_3$	*330.15*	*[M+H]+*	*12.5*
Pentazocine HCL	*4.55*	$C_{19}H_{27}NO$	*286.21654*	*[M+H]+*	*25*
Phenazepam	*6.39*	$C_{15}H_{10}N_2OBrCl$	*348.97378*	*[M+H]+*	*5*
Phencyclidine	*4.94*	$C_{17}H_{25}N$	*244.20598*	*[M+H]+*	*2.5*
Phentermine	*3.36*	$C_{10}H_{15}N$	*150.12773*	*[M+H]+*	*50*
Phenytoin	*5.45*	$C_{15}H_{12}N_2O_2$	*253.09715*	*[M+H]+*	*250*
Pregabalin	*2.33*	$C_8H_{17}NO_2$	*160.13321*	*[M+H]+*	*125*
Primidone	*4.07*	$C_{12}H_{14}N_2O_2$	*219.1128*	*[M+H]+*	*250*

Component Name	Retention Time	Chemical Formula	Precursor (Q1) Mass (Da)	Adduct & Charge	LOD (ng/mL)
Promethazine HCl	*5.72*	$C_{17}H_{20}N_2S$	*285.142*	*[M+H]+*	*1*
Propranolol HCL	*5.13*	$C_{16}H_{21}NO_2$	*260.16451*	*[M+H]+*	*10*
Protriptyline HCl	*5.82*	$C_{19}H_{21}N$	*264.17468*	*[M+H]+*	*12.5*
Pseudoephedrine	*2.58*	$C_{10}H_{15}NO$	*166.12264*	*[M+H]+*	*500*
Quetiapine fumarate	*5.57*	$C_{21}H_{25}N_3O_2S$	*384.17402*	*[M+H]+*	*12.5*
Risperidone	*5.11*	$C_{23}H_{27}FN_4O_2$	*411.21908*	*[M+H]+*	*2.5*
Ritalinic Acid	*3.58*	$C_{13}H_{17}NO_2$	*220.13321*	*[M+H]+*	*12.5*
Scopolamine HBr	*3.1*	$C_{17}H_{21}NO_4$	*304.15433*	*[M+H]+*	*0.5*
Sertraline HCl	*6.27*	$C_{17}H_{17}Cl_2N$	*306.08108*	*[M+H]+*	*12.5*
Tapentadol HCL	*4.13*	$C_{14}H_{23}NO$	*222.18524*	*[M+H]+*	*2.5*
Temazepam	*6.39*	$C_{16}H_{13}ClN_2O_2$	*301.07383*	*[M+H]+*	*5*
Thioridazine	*6.7*	$C_{21}H_{26}N_2S_2$	*371.16102*	*[M+H]+*	*25*
Topiramate	*4.82*	$C_{12}H_{21}NO_8S$	*340.10606*	*[M+H]+*	*250*
Tramadol HCl	*4.01*	$C_{16}H_{25}NO_2$	*264.19581*	*[M+H]+*	*2.5*
Trazodone HCl	*5.22*	$C_{19}H_{22}ClN_5O$	*372.15856*	*[M+H]+*	*12.5*
Trimipramine	*6.04*	$C_{20}H_{26}N_2$	*295.21688*	*[M+H]+*	*12.5*
Venlafaxine HCl	*4.72*	$C_{17}H_{27}NO_2$	*278.21146*	*[M+H]+*	*12.5*
Verapamil HCl	*5.61*	$C_{27}H_{38}N_2O_4$	*455.29043*	*[M+H]+*	*25*
Zaleplon	*5.8*	$C_{17}H_{15}N_5O$	*306.13494*	*[M+H]+*	*10*
Ziprasidone	*5.45*	$C_{21}H_{21}ClN_4OS$	*413.11974*	*[M+H]+*	*5*
Zolpidem	*4.63*	$C_{19}H_{21}N_3O$	*308.17574*	*[M+H]+*	*2.5*
Zonisamide	*3.77*	$C_8H_8N_2O_3S$	*213.03284*	*[M+H]+*	*25*
Zopiclone	*4.57*	$C_{17}H_{17}ClN_6O_3$	*389.11234*	*[M+H]+*	*25*

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Ultra-Sensitive Forensic Analysis of Cocaine and its Metabolites in Hair Samples

Using the QTRAP® 6500+ LC-MS/MS System

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Cocaine is one of the most commonly abused recreational drugs, with an estimated 16.5 million people or 0.35% of the worldwide population abusing the substance.[1] Detection of its use can be performed in several biological matrices such as blood, urine, oral fluid and hair. While urine and oral fluid are very useful for determining cocaine use in short term, hair samples are becoming extremely valuable in testing the long-term use. Additional benefits of hair testing include but are not limited to (1) ease and non-invasive nature of sample extraction, (2) absence of storage requirement and (3) long term stability and little risk of sample degradation overtime.

Presence of cocaine and its metabolites in hair indicate active drug use. However, there are two major analytical challenges associated with detecting cocaine and its metabolites in hair sample: (1) low concentration of these compounds and (2) high abundance of matrix interferences associated with hair samples.

Herein, a sensitive and reliable analytical workflow is presented which combines the use of QTRAP 6500+ LC-MS/MS system with solid phase extraction (SPE) for picogram per mg of hair detection of cocaine and its metabolites. This method for quantification of cocaine and its metabolites was demonstrated to provide unique advantages in the ability to maximize selectivity when confirming and quantifying low level metabolites in hair.

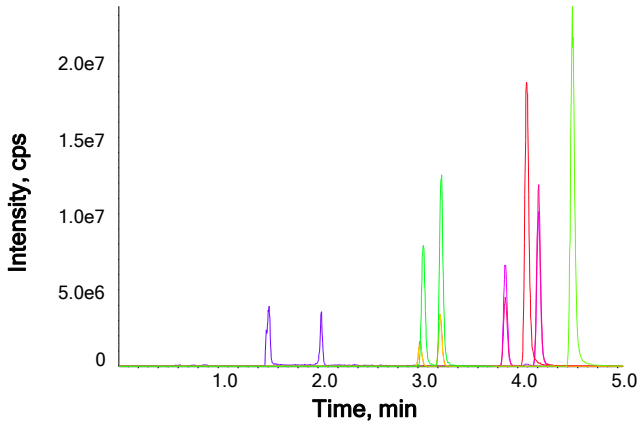

Figure 1: Sensitive Detection of Cocaine and its 10 Metabolites in Hair Using the QTRAP® 6500+ LC-MS/MS System. Detection of cocaine and its 10 metabolites in hair at 0.05 ng/mg of hair level.

Key Features of the QTRAP® 6500+ LC-MS/MS System with Optimized SPE

- IonDrive™ Technology on the QTRAP® 6500+ system provides improvements in ionization efficiency, ion sampling efficiency and detection dynamic range for very high sensitivity of detection (high fg/mL to low pg/mL).
- Simultaneous identification and confirmation of illicit drugs and their metabolites through the acquisition of full scan MS/MS data (Enhanced Product Ion (EPI) and automated MS/MS library searching).
- Improved sample preparation with SPE provides a robust and easily implemented method for selective analysis of trace levels of cocaine and its metabolites in hair.
- Optimized chromatography allows separation of cocaine and its metabolites in less than 5 minutes with a high level of selectivity.
- Automated generation of optimized linear curves using SCIEX OS Software 1.4 with less manual intervention and quick flagging of outliers.

Methods

Hair Sample Preparation and Digestion: Hair samples were washed according to accepted laboratory procedure, dried and cut into segments of ~ 2 mm lengths. Approximately 20 mg of each hair sample was transferred into a suitable and sealable container with cap and 1 mL of 0.1 N HCl was added into each container. The containers were incubated overnight at 45ºC for complete digestion of the hair samples. The next day, the containers were removed from the incubator and allowed to cool down to room temperature. 10 µL of each internal standard (IS) were added to the 800 µL of hair extract solution and the containers were thoroughly mixed. The resulting solutions underwent solid phase extraction using the Phenomenex Strata®-X-C, 30 mg/3mL (Part No. 8B-S029-TBJ) according to the procedures shown in Figure 2.

HPLC Conditions: HPLC separation was performed on a Phenomenex Kinetex® Biphenyl column (100x3mm, 2.6µm, 00A-4723-AN) on the SCIEX ExionLC™ AC system. Mobile phase A (MPA) and mobile phase B (MPB) were 0.1% formic acid in water and methanol, respectively. The HPLC flow rate was 600 µL/min, column temperature was held at 30˚C, and the total HPLC runtime was less than 5 minutes.

MS/MS Conditions: A SCIEX QTRAP® 6500+ system with IonDrive™ Turbo V source and Electrospray Ionization (ESI) probe was used. Cocaine and its 10 metabolites were detected using two MRM transitions per compound to allow quantification and identification based on the ratio of quantifier to qualifier MRM transitions (Table 3).

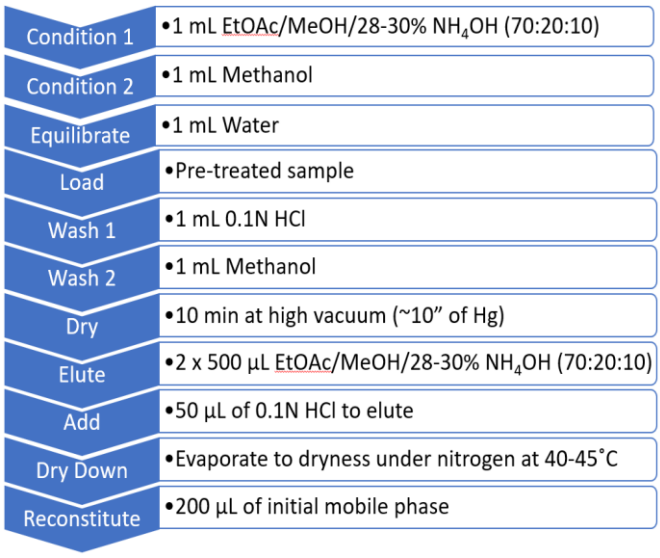

Figure 2: Strong Anion Exchange (SAX) Solid Phase Extraction (SPE) Workflow Using Phenomenex Strata®-X-C, 30 mg/3mL cartridges. An 11-step extraction protocol was developed and optimized for selectively extracting cocaine and its metabolites from hair samples for analysis with the QTRAP® 6500+ LC-MS/MS System.

The source parameters are provided in Table 1. The compound-dependent voltages of Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Collision Cell Exit Potential (CXP) were optimized for each transition and are represented in Table 3.

Table 1. Source Conditions.

Parameter	Value
Curtain gas (CUR)	*30*
Collision gas (CAD)	*8*
IonSpray Voltage (IS)	*4500 V*
Temperature (TEM)	*600 ℃*
Ion Source Gas 1 (GS1)	*60*
Ion Source Gas 2 (GS2)	*20*

Separation of Isomers

Upon ingestion, cocaine is rapidly adsorbed and broken down in the body into several metabolites. Detection and identification of those metabolites is paramount to ensure correct quantification of cocaine. The existence of isomeric analogues within the panel of cocaine metabolites adds an additional level of complexity to the assay as these analogues have no unique fragments that can be used for detection. Therefore, chromatographic separation of these analogues from their isomers is critical for confident identification and quantitation and therefore was the focus of this study.

Optimal chromatographic separation was accomplished by using a Phenomenex Kinetex® Biphenyl column (100x3mm, 2.6µm, 00A-4723-AN) which allowed for better retention and selectivity of the more polar analytes throughout the gradient. The column was held at 30˚C during the course of the experiment. The column in conjunction with an optimized mobile phase composition produced the separation that was needed to correctly distinguish all isomers. This chromatographic separation was optimized for this MRM assay but can also be used on the SCIEX X500R QTOF System, for additional screening or confirmation techniques. Figure 3 shows the separation of three sets of isomeric metabolites of cocaine.

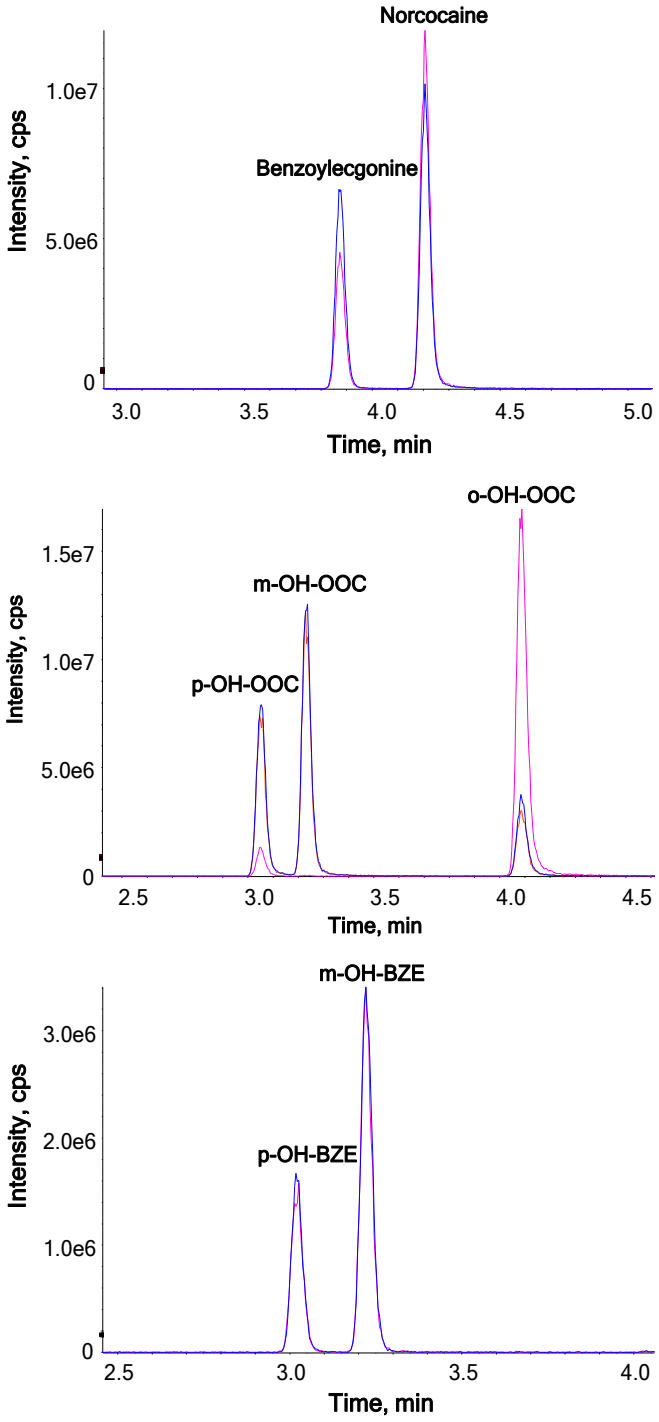

Figure 3: Examples of Separation of Specific Cocaine Metabolites Isomers. (Top) Separation of the isomers Benzoylecgonine and Norcocaine. (Middle) Separation of the isomers p-OH-OOC, m-OH-OOC and o-OH-OOC. (Bottom) Separation of the isomers p-OH-BZE and m-OH-BZE. These individual chromatograms showing isomeric separation were extracted from the full chromatogram shown in Figure 1 that includes the full panel of analytes used in this study.

Recovery and Matrix Effects

Hair is a very complex matrix, which may represent a problem when detecting analytes at low concentration levels. Robust and reliable extraction procedures are critical in achieving the desired reproducibility, good linear response and limits of quantitation. To assess the recoveries of the analytes used in this experiment, recovery (RE) and the matrix effect (ME) were calculated using 0.005 ng/mg of each internal standard. If one depicts the peak areas obtained in neat solution standards as A, the corresponding peak areas for internal standard spiked after extraction into hair extracts as B, and peak areas for internal standards spiked before extraction as C, the RE and ME values can be calculated as follows:

$$RE\ (\%) = C/B \times 100 \quad (1)$$

$$ME\ (\%) = B/A \times 100 \quad (2)$$

The extraction procedures demonstrated excellent recoveries of the analytes of interest, as shown in Figure 4.

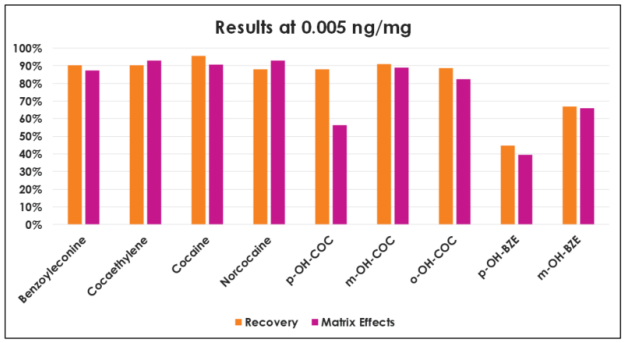

Figure 4: Recovery (RE) and Matrix Effects (ME) Calculated for Cocaine and Its Metabolites using 0.005 ng/mg of Each Internal Standard. (Top) Separation of the isomers Benzoylecgonine and Norcocaine. (Middle) Separation of the isomers p-CH-OOC, m-CH-OOC and o-CH-OOC. (Bottom) Separation of the isomers p-OH-BZE and m-OH-BZE.

Analytical Performance of the SCIEX QTRAP 6500+ LC-MS/MS System

Following the SPE procedure, 10 µL of the reconstituted solution were injected for each compound. Calibration curves were generated for each of the compounds to determine limits of quantitation (LOQ). The results demonstrated excellent linearity of the generated regression curves covering linear dynamic range from 3 to 4 orders of magnitude; coefficients of variations (Cs) within 10% and good accuracies. Signal-to-noise ratios (S/N) at LLOQ were found to vary from 10 to 50. Table 2 summarized the lower limits of quantitation (LLOQ) for cocaine and metabolites panel. Figure 5 shows a few representative calibrations curves and statistics of quantitation generated using this comprehensive method.

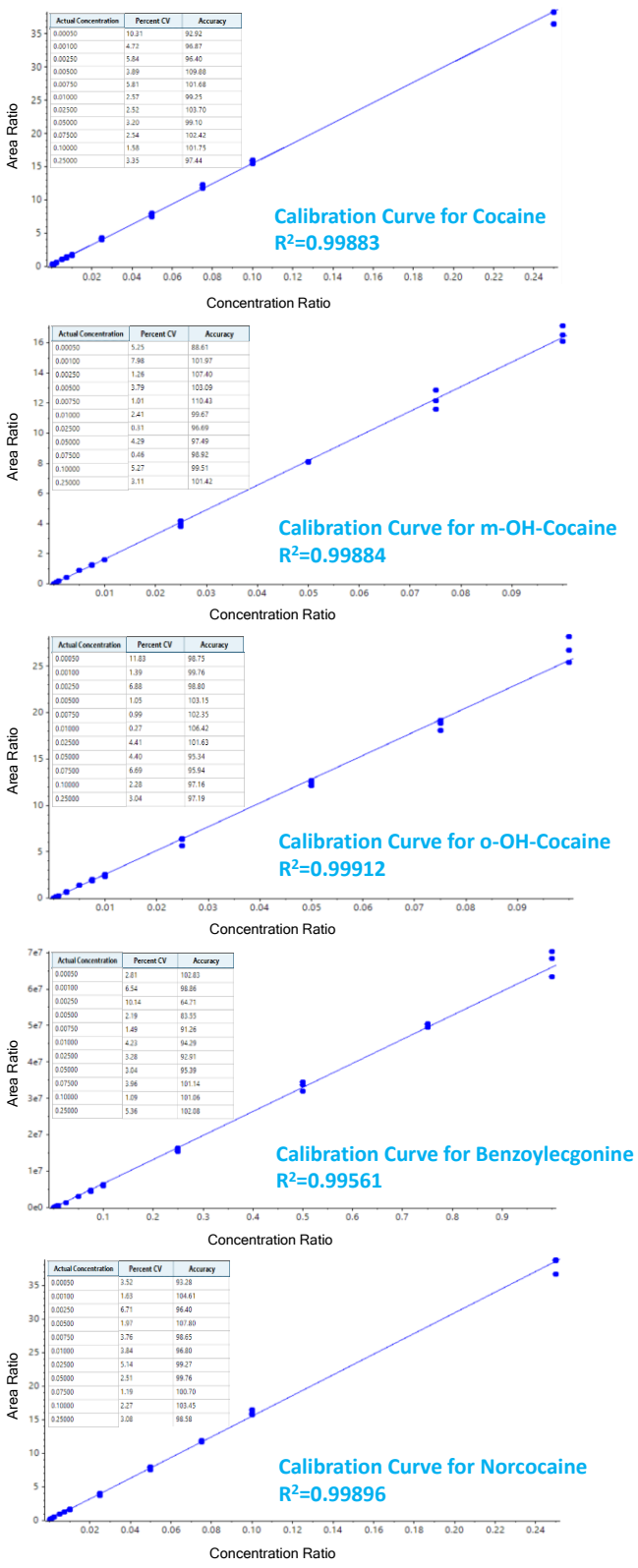

Figure 5: Excellent Linearity Was Achieved for Cocaine and Its Metabolites. Calibration curves and statistic information are shown for Cocaine, m-OH-Cocaine, o-OH-Cocaine, Benzoylecgonine and Norcocaine. R^2 values were equal to or higher than 0.9990 for all the analytes used in the comprehensive method.

Table 2. Lower Limits of Quantitation (LLOQ) for Cocaine and Metabolites Panel.

Analyte	LLOQ (ng/mg)
Ecgonine	*0.05*
Ecgonine Methyl Ester	*0.0025*
Benzoylecgonine	*0.001*
Norcocaine	*0.0005*
Cocaine	*0.0005*
p-OH-Benzoylecgonine	*0.01*
m-OH-Benzoylecgonine	*0.01*
Cocaethylene	*0.0001*
m-OH-Cocaine	*0.00005*
o-OH-Cocaine	*0.00005*
p-OH-Cocaine	*0.001*

Full Scan MS/MS for Confirmation

The QTRAP 6500+ System is a hybrid triple quadrupole linear ion trap mass spectrometer which allows to easily switch between quantitative MRM scans and qualitative trap scans. For this assay, the method uses an MRM survey scan (Table 3) followed by two EPI scans (full scan MS/MS) which are triggered when signal is detected for each specific MRM transition. The acquired full scan MS/MS spectra contain the complete molecular fingerprint of cocaine and its metabolites and can be searched against relevant spectral libraries for confirmation of detection. This approach provides both high sensitivity quantitation with compound confirmation which significantly reduces the risk of false positives in the unknown samples.

Using this comprehensive method, cocaine and its metabolites were identified, and confirmation was achieved using MS/MS library searching. Figure 6 illustrates typical results of MS/MS library searching.

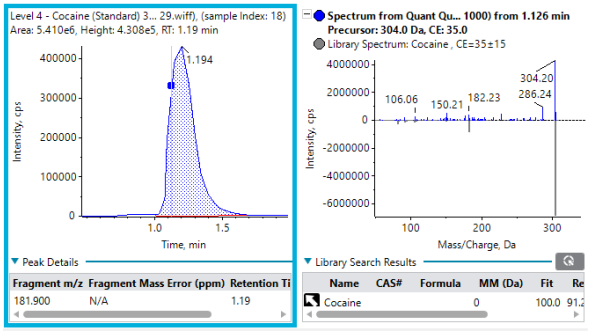

Figure 6: Typical Results of MS/MS Library Searching Using SCIEX OS Software 1.4. MS/MS library searching for Cocaine in a standard solution prepared by spiking in blank hair extract.

Conclusions

The combination of a solid phase extraction (SPE) procedure and optimized chromatography with the highly sensitive QTRAP 6500+ System allowed the efficient and sensitive detection of trace levels of cocaine and its metabolites (0.05 pg/mg) in hair samples, making the workflow easily adaptable into a forensic toxicology laboratory.

- An 11-step extraction protocol using SAX SPE can be rapidly implemented and optimized for selective analysis of cocaine and it metabolites.
- Optimized chromatographic separation was achieved with a high level of selectivity for cocaine and its metabolites using Phenomenex's Kinetex Biphenyl column for a total HPLC runtime of less than 5 minutes.
- Analyte extraction recoveries were demonstrated to be greater than 80% enabling the analytical workflow to obtain sub pg/mg lower limits of quantification (LLOQ) in hair matrix for the two hydroxycocaine isomers.
- Successful quantitation of cocaine and its metabolites was performed using SCIEX OS Software 1.4 allowing streamlined and accurate data processing of trace level concentrations (0.05 pg/mg) in hair samples.
- The workflow showed excellent accuracy (>95%) and precision (<15%), with excellent linearity resulting in R^2 values of 0.9990 for all analytes.
- In addition to quantitation, the QTRAP 6500+ System enabled simultaneous identification and confirmation of cocaine and its metabolites by acquiring full MS/MS data and using automated MS/MS library searching.

References

1. Peacock A, Leung J, Larney S, Colledge S, Hickman M, Rehm J, et al. Global statistics on alcohol, tobacco and illicit drug use: 2017 status report. Addiction. 2018. DOI: 10.1111/add.14234.

Table 3. MRM Transitions for Cocaine and its Metabolites, Including Optimized Compound Dependent Parameters.

Analyte	Q1	Q3	DP	EP	CE	CXP
BZE_1	*290.2*	*168.1*	*65*	*10*	*25*	*10*
BZE_2	*290.2*	*105*	*65*	*10*	*36*	*10*
Norcocaine_1	*290.2*	*168*	*50*	*10*	*21*	*10*
Norcocaine_2	*290.2*	*136.1*	*50*	*10*	*30*	*10*
Cocaine_1	*304.2*	*182.2*	*70*	*10*	*26*	*10*
Cocaine_2	*304.2*	*82.1*	*70*	*10*	*35*	*10*
Cocaine_3	*304.2*	*105*	*70*	*10*	*37*	*10*
Ecgonine_1	*186.2*	*168.1*	*60*	*10*	*23*	*10*
Ecgonine_2	*186.2*	*100.3*	*60*	*10*	*31*	*10*
EME_1	*200.201*	*182.1*	*45*	*10*	*23*	*10*
EME_2	*200.201*	*82.1*	*45*	*10*	*32*	*10*
p-OH-BZE_1	*306.1*	*168.1*	*70*	*10*	*26*	*10*
p-OH-BZE_2	*306.1*	*186.1*	*70*	*10*	*27*	*10*
m-OH-BZE_1	*306.102*	*168.1*	*80*	*10*	*27*	*10*
m-OH-BZE_2	*306.102*	*121.1*	*80*	*10*	*35*	*10*
Cocaethylene_1	*318.2*	*196.1*	*50*	*10*	*26*	*10*
Cocaethylene_2	*318.2*	*82.1*	*50*	*10*	*37*	*10*
m-OH-COC_1	*320.1*	*182.1*	*70*	*10*	*27*	*10*
m-OH-COC_2	*320.1*	*82.1*	*70*	*10*	*42*	*10*
o_OH-COC_1	*320.101*	*200.1*	*50*	*10*	*27*	*10*
o_OH-COC_2	*320.101*	*182.1*	*50*	*10*	*37*	*10*
p_OH-COC_1	*320.102*	*182.2*	*80*	*10*	*26*	*10*
p_OH-COC_1	*320.102*	*82.1*	*80*	*10*	*44*	*10*

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High sensitivity drug analysis using dried blood spots

Using the SCIEX Triple Quad 7500 system, powered by SCIEX OS software

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The ability to accurately measure low levels of drugs and their metabolites is critical for a wide variety of toxicology applications, including roadside testing (driving under the influence of drugs, or DUID cases), postmortem investigations, drug-facilitated sexual assault cases, follow-up of drug and alcohol addicts. As some drugs are rapidly metabolized in the body, comprehensive drug analysis approaches are critically needed to confirm the presence of these substances and provide the necessary drug concentration evidence to support their toxicity level with a high level of sensitivity and specificity.

Drug monitoring is typically performed using serum or plasma obtained by venous blood sampling. However, there is a growing interest in dried blood spots (DBS) as an alternative sampling strategy. Compared to traditional venous blood sampling, DBS have many advantages including (1) minimally invasive sample collection procedure, (2) small sample volume requirement, (3) increased analyte stability, and (4) convenient sample storage and transport with minimal chance of sample adulteration. Given the small amount of sample available for testing (usually in the 5-50 µL range), accurate quantification of low levels of drugs and their metabolites requires the use of a sensitive analysis technique.

In this technical note, an optimized sample extraction procedure was used in combination with the SCIEX 7500 system for picogram/mL detection of a panel of 24 drugs and metabolites extracted from DBS. This robust and comprehensive drug monitoring workflow is shown to provide the required sensitivity levels for accurate quantification of low levels of analytes with a wide range of physical and chemical properties.

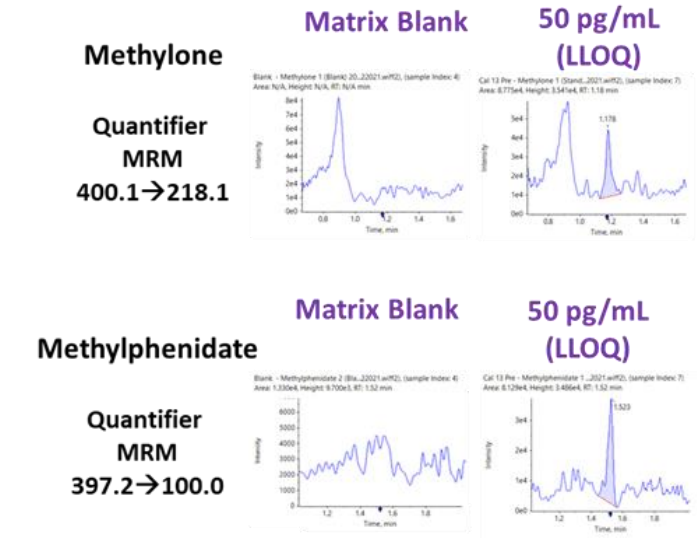

Figure 1. High sensitivity detection of drugs and metabolites extracted from DBS. Extracted ion chromatogram (XIC) traces for methylone (top) and methylphenidate (bottom) showing the quantifier ion traces for the matrix blank (left) and at the LLOQ at 50 pg/mL (right).

Key advantages of drug monitoring method for DBS analysis on the SCIEX 7500 system

- Optimized sample extraction procedure in combination with a robust detection method using the Scheduled MRM algorithm in SCIEX OS software enables pg/mL detection levels for a wide diversity of drug classes
- Ion ratio difference was <20% for the quantifier and qualifier ions of the targeted analytes, showing the quantitative robustness of the developed workflow
- Overall performance of the system resulted in excellent correlation (R^2 >0.98) with optimal precision (below 20%) and accuracy (with bias ±15%) across the calibration range
- Combination of low pg/mL LLOQs with acceptable analyte recoveries provides a sensitive and robust method fit for rapid implementation of DBS analysis for routine drug monitoring

Experimental details

Target analytes and solutions: A total of 24 drugs and 10 deuterated internal standards were purchased from Cerilliant Corporation (Round Rock, TX). Two solutions were prepared in water: a standard mixture containing the 24 target analytes and an internal standard mixture containing the 10 deuterated internal standards. Table 1 lists the name, the calibration range, linear correlation value (R^2), LLOQ, accuracy and precision reported at the LLOQ, as well as the recovery values calculated at two concentrations levels (1 and 5 ng/mL) for each of the 24 target analytes targeted in this panel.

Calibrator preparation: Thirteen levels of calibrators were prepared by spiking the standard mixture containing the 24 target analytes in human whole blood to final concentrations ranging from 1 pg/mL to 50 ng/mL. A 10 ng/mL IS standard stock solution containing the 10 deuterated internal standards was prepared in methanol/acetonitrile (3:1, v/v) and used as the extracting solvent to extract the analytes from the DBS cards.

Sample preparation and DBS sample extraction procedures: Protein saver cards (also known as DBS cards) were purchased from Whatman (Piscataway, NJ). Human whole blood calibrator samples spiked with various concentrations of the 24 analytes were spotted onto the DBS cards and the analytes were extracted using the sample extraction procedure summarized in Figure 2.

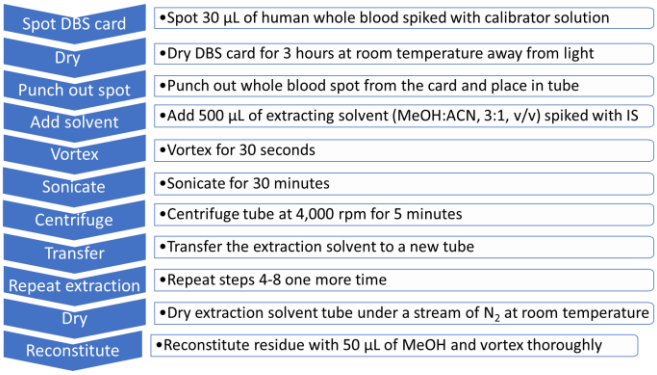

Figure 2. Analyte extraction workflow from DBS cards. An 11-step sample extraction protocol was optimized to selectively extract the 24 analytes from DBS cards for analysis using the SCIEX 7500 system.

Liquid chromatography: HPLC separation was performed on an ExionLC system using a Phenomenex Kinetex Phenyl-Hexyl column (50 × 2.1 mm, 2.6 µm, 00B-4495-AN). 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 10 µL and the LC runtime was 6.5 min.

Mass spectrometry: A SCIEX 7500 system was equipped with an OptiFlow Pro ion source using an electrospray ionization (ESI) analytical probe and E Lens probe and was operated in positive mode. A single acquisition method consisting of 68 MRM transitions (48 for the drugs and 20 for the internal standards) was created using the Scheduled MRM algorithm in SCIEX OS software 2.0. Two MRM transitions were monitored for each of the targeted analytes and each sample was injected in triplicate to build a data analysis processing method.

Data analysis: Data processing was performed using SCIEX OS software. Rapid and automated quantitative data analysis was performed using the MQ4 algorithm in the Analytics module to streamline data processing. Peak area values, calibration curves, concentration calculations, assay precision and accuracy statistics were automatically generated in the Analytics module of the software.

Method development and optimization using the Scheduled MRM algorithm

A diluted, 10 ng/mL neat standard mixture containing the 24 analytes was used for initial method development. The Scheduled MRM Algorithm was used to automatically compute an optimized acquisition method based on user supplied analyte retention times based on MRM concurrency.[3] As a result, data sampling was optimal across each peak, maintaining good dwell times and desired cycles times. Most MRM transitions had 15 or more data points across each of the LC peaks, with 10 being the minimum number of data points observed on a peak for the 24 target analytes in this panel. Figure 3 shows the chromatographic profile of the 24 targeted analytes resulting from the optimized data acquisition method using the 10 ng/mL neat standard mixture.

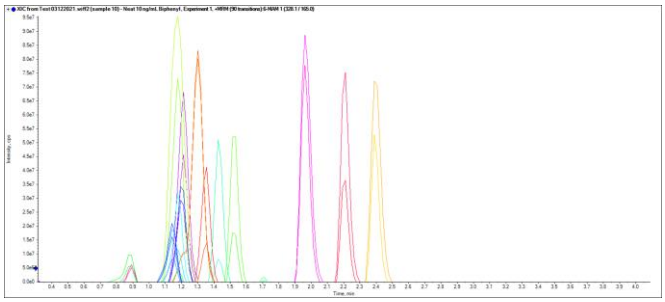

Figure 3. Chromatographic profile of the 24 analytes included in the panel. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition method, obtained from the 10 ng/mL neat standard mixture containing the 24 targeted analytes. Method was built using the Scheduled MRM algorithm Pro in SCIEX OS software.

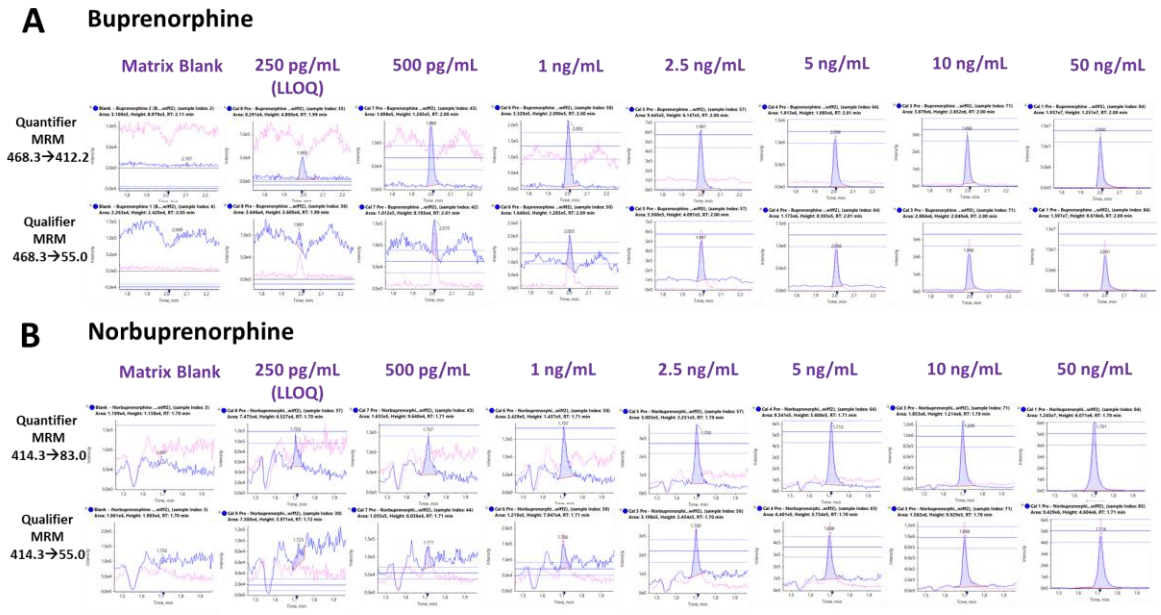

Figure 4. Representative extracted ion chromatograms (XICs) for selected drugs in the forensic panel. XICs for buprenorphine (A) and norbuprenorphine (B) from 0.25 to 50 ng/mL, including the blank injection. Both the quantifier and qualifier traces are shown. Ion ratios were also monitored across the dataset and tolerance lines are shown. The ion ratio difference was <20% for the quantifier and qualifier ions of each of the targeted analytes across the calibration range.

Robust detection method leads to accurate analyte quantification

Reliable measurements of drug concentrations are key to the successful implementation of drug monitoring workflows in testing laboratories. To that extent, reproducible and accurate quantification of drugs and metabolites extracted relies on the use of a robust detection method. Human whole blood calibrator samples spiked with concentrations ranging from 1 pg/mL to 50 ng/mL were injected to evaluate the quantitative performance of the system and its ability to accurately measure various levels of drugs and metabolites extracted from DBS with a high level of precision and accuracy.

Figure 4 shows representative extracted ion chromatograms (XICs) for the two MRM transitions monitored for buprenorphine and norbuprenorphine, two of the drugs targeted in this study. The XIC traces display overlays of both the quantifier and qualifier ion transitions monitored for each drug, for a blank injection (left) and for concentrations ranging from 250 pg/mL to 50 ng/mL. The confirmatory ion ratio lines between the two transitions are also displayed showing the tolerance limit. The lower limits of quantification (LLOQ) for the drugs and metabolites targeted in this workflow ranged from 50 to 250 pg/mL (Table 1). Figure 1 shows the XIC traces for methylone and methylphenidate, two drugs with LLOQs of 50 pg/mL. Overall, the quantifiable concentration ranges showcased in this workflow are well within the range of concentrations relevant for drug monitoring.

The ability to accurately quantify low levels of drugs and metabolites extracted from DBS is important, but the ability to consistently deliver high levels of data quality with high precision and accuracy is critical. The quantification performance of the SCIEX 7500 system was demonstrated with calculated precision compliance reported as CV% (values below 20%) and accuracy reported as bias% (values in the interval ±15%) across the calibration range for all 24 targeted analytes across the calibration range (Table 1).

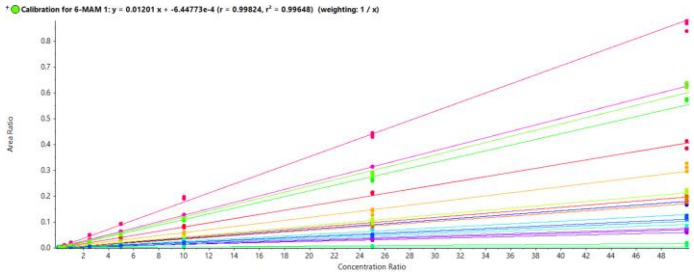

Figure 5. Excellent linearity for the 24 analytes extracted from DBS. Calibration curves generated using the two MRM transitions monitored for each of the 24 analytes targeted in this study. The assay showed excellent linearity with R^2 values greater than 0.99 for all the analytes.

Calibration curves were quickly generated using the two MRM transitions monitored for each analyte. Figure 5 shows the resulting regression lines plotted across the calibrator levels. The calibration curves demonstrated excellent linearity with R^2 values greater than 0.98 for all the drugs and metabolites in the panel (Table 1).

Optimized extraction procedure leads to acceptable levels of analyte recovery

One of the critical aspects of DBS analysis is the efficiency of the extraction method for analytes with a wide range of physical and chemical properties such as those included in this panel. An inefficient extraction method typically yields low analyte recovery, which can result in poor linear response, limits of quantification (LOQ) and assay reproducibility. The efficiency of the DBS extraction procedure used in this workflow was investigated by calculating the analyte recovery at two concentration levels (1 and 5 ng/mL). The recovery values were calculated by expressing ratio of the average (n=3) peak areas of each analyte spiked before and after the extraction procedure as a percentage. The recovery values at the two concentration levels ranged between 21% and 56% for the drugs and metabolites targeted in the panel. The range of recovery values can be explained in part by the generic sample preparation procedure used for the 24 analytes and the wide chemical diversity of the drugs and metabolites making up the panel. Overall, the recovery values were acceptable given the high reproducibility of the assay and the range of LLOQ values achieved for the analytes. In this scenario, the use of deuterated internal standards is recommended to compensate the analytes loss. The recovery values at the two concentration levels for each analyte are summarized in Table 1.

Conclusions

A highly sensitive workflow for the detection of 24 drugs and metabolites in DBS has been described using the SCIEX 7500 system. The broad applicability of the optimized sample extraction procedure in combination with the sensitivity of the system enabled accurate quantification at low levels of a panel of 24 chemically diverse drugs and metabolites.

- An 11-step optimized sample extraction procedure was developed to efficiently extract a broad panel of 24 drugs and metabolites from DBS
- Analyte extraction recovery values were found to be between 21% and 56% for the panel of 24 analytes
- The use of the Scheduled MRM Algorithm optimized data acquisition and ensured high data quality for all analytes in the fast 6.5-minute runtime
- The high sensitivity of the SCIEX 7500 system enabled accurate quantification with low levels of drugs and metabolites, with LLOQ values ranging from 50 to 250 pg/mL
- Excellent precision (CV%< 20%), accuracy (bias ±15%) and correlation (R^2>0.98) were observed across the calibration range, proving the robustness of the workflow and the quantification performance of the SCIEX 7500 system
- The method can be easily implemented by testing laboratories in routine drug analysis for low-level detection of drugs and their metabolites extracted from DBS

References

1. Enabling new levels of quantification — using the SCIEX Triple Quad 7500 system, 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.
3. Using Scheduled MRM algorithm in SCIEX OS software. SCIEX community post, RUO-MKT-18-11941-A.

Table 1. Statistical results for the 24 analytes targeted in this workflow. The table includes calibration range, linear correlation coefficient (R^2 Value), and the LLOQ, as well as the accuracy and precision measured at the LLOQ. The analyte recovery values at 1 and 5 ng/mL are also reported.

Compound	Calibration range (ng/mL)	Linear correlation (R^2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)	Recovery at 1 ng/mL (%)	Recovery at 5 ng/mL (%)
6-MAM	*0.1-50*	*0.99648*	*0.1*	*111.91*	*1.85*	*20*	*18*
Acetyl fentanyl	*0.1-50*	*0.99101*	*0.1*	*106.23*	*6.77*	*45*	*36*
Buphedrone	*0.25-50*	*0.99561*	*0.25*	*93.85*	*11.04*	*29*	*27*
Buprenorphine	*0.25-50*	*0.99075*	*0.1*	*89.69*	*5.20*	*26*	*28*
EDPP	*0.01-50*	*0.99168*	*0.01*	*105.62*	*4.77*	*37*	*23*
Fentanyl	*0.05-50*	*0.98625*	*0.05*	*91.35*	*8.94*	*47*	*56*
Hydromorphone	*0.25-50*	*0.98176*	*0.25*	*112.41*	*1.92*	*24*	*21*
Imipramine	*0.05-50*	*0.99323*	*0.05*	*87.28*	*7.23*	*49*	*45*
MDEA	*0.1-50*	*0.99210*	*0.1*	*93.50*	*8.97*	*39*	*42*
MDPV	*0.05-50*	*0.99817*	*0.05*	*92.51*	*6.45*	*30*	*21*
Mephedrone	*0.05-50*	*0.99224*	*0.05*	*95.24*	*0.89*	*31*	*31*
Methadone	*0.05-50*	*0.99299*	*0.05*	*96.87*	*8.33*	*44*	*40*
Methamphetamine	*0.1-50*	*0.99619*	*0.1*	*91.42*	*8.17*	*45*	*56*
Methedrone	*0.05-50*	*0.99346*	*0.05*	*89.73*	*13.13*	*45*	*38*
Methylone	*0.05-50*	*0.99687*	*0.05*	*94.97*	*13.09*	*37*	*38*
Methylphenidate	*0.05-50*	*0.99515*	*0.05*	*85.84*	*17.27*	*44*	*46*
Morphine	*0.1-50*	*0.99563*	*0.1*	*105.94*	*3.05*	*37*	*34*
Norbuprenorphine	*0.25-50*	*0.98456*	*0.5*	*104.60*	*9.76*	*43*	*36*
Norfentanyl	*0.05-50*	*0.99842*	*0.05*	*106.44*	*9.12*	*47*	*42*
Norhydrocodone	*0.1-50*	*0.99796*	*0.1*	*95.98*	*3.29*	*38*	*37*
Noroxycodone	*0.1-50*	*0.99426*	*0.1*	*97.35*	*10.08*	*46*	*39*
Oxycodone	*0.05-50*	*0.99230*	*0.05*	*94.33*	*12.94*	*51*	*58*
Oxymorphone	*0.1-50*	*0.99765*	*0.1*	*103.91*	*9.18*	*28*	*24*
Sufentanil	*0.01-50*	*0.99772*	*0.01*	*112.79*	*12.58*	*42*	*43*

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Reaching new sensitivity levels for the detection of fentanyl analogs and highly potent novel synthetic opioids in blood

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

Pierre Negri[1] and Alex J. Krotulski[2]

[1]SCIEX, USA; [2]Center for Forensic Science Research and Education at the Fredric Rieders Family Foundation, USA

The continuous emergence of novel synthetic opioids (NSO) on the recreational drug market has been a major contributor to the ongoing opioid crisis. NSO are a class of novel psychoactive substances (NPS) that includes analogs of fentanyl and newly emerging non-fentanyl compounds. These illicitly manufactured substances are designed to mimic the effects of conventionally controlled opioids but greatly vary in potency and purity. The continuous introduction of these new opioid substances on the drug market, in combination with the rapidly changing trends in drug consumption, has created a significant challenge for law enforcement agencies and health professionals.

NSO can be consumed as stand-alone products but have more commonly been used as adulterants in heroin or counterfeit prescription preparations. The frequent occurrence of these substances in counterfeit pills is presenting an additional health and safety threat that requires vigilance and monitoring from drug tracking agencies and laboratories. NSO have been responsible for an increasing number of acute intoxications that often result in accidental and fatal combined drug overdoses. As newer and more potent synthetic opioids are synthesized and introduced to the recreational drug market, timely and comprehensive analytical drug screening approaches focused on rapid identification of these novel substances in biological matrices are critically needed. However, prior mass spectrometry-based detection platforms are lacking the sensitivity requirements for trace level detection of potent NSO linked to increasing intoxications, adverse events, and death.

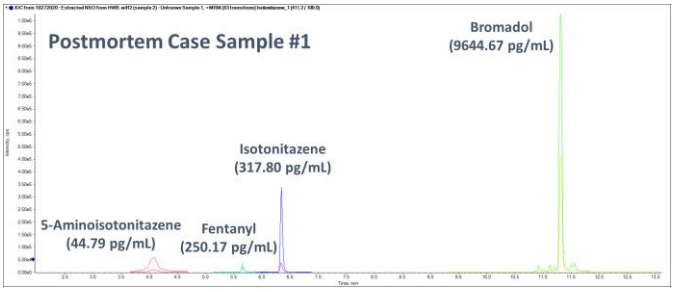


Figure 1. Trace level quantification of potent NSO in postmortem case sample #1. Extracted ion chromatogram (XIC) showing the successful detection of two potent NSO, a metabolite, and fentanyl at low concentrations in a case sample from a subject suspected of accidental overdose from combined drug toxicity.

In this technical note, the sensitivity of the SCIEX 7500 System[1] was investigated for the detection of 32 NSO, including fentanyl analog isomers, in human whole blood. The unparalleled quantification performance of the system enabled accurate detection of potent substances in poly-drug, authentic case samples at trace levels that were not previously achievable. This advancement enables toxicologists to develop a better picture of the overdose causation.

Key features of sensitive detection method for low levels of NSO in blood samples

- Optimized LC conditions in combination with a robust detection method using the Scheduled MRM™ Algorithm in SCIEX OS Software enabled sensitive detection of 32 NSO extracted from authentic forensic blood samples
- Method demonstrated excellent linearity, accuracy and precision for NSO concentrations ranging from 10 to 100000 pg/mL, even at the low end of the calibration curve
- The remarkable quantification performance of the SCIEX 7500 System enabled robust quantification of NSO down to 10 pg/mL, with limits of the detection below 5 pg/mL for the majority of the NSO in the panel
- The SCIEX 7500 System provided the ability to detect low levels of NSO in postmortem case samples that would normally go undetected, providing a clearer picture for help in determining the cause of death

Experimental details

Target analytes and solutions: A total of 32 NSO including 17 fentanyl analogs and 15 newly emerging non-fentanyl opioids were selected for this panel. Two solutions were prepared in water: a 1 µg/mL standard mixture containing the 32 target analytes and a 1 ng/mL internal standard mixture containing two deuterated internal standards (fentanyl-D5 and carfentanil-D5). Table 1 lists the 32 target analytes used in this method.

Calibrator preparation: The 1 µg/mL standard mixture containing the 32 target analytes was used to fortify 200 µL of human whole blood. This fresh spiked whole blood mixture was used to prepare a series of 9 calibrator solutions covering concentrations ranging from 1 pg/mL to 100 ng/mL.

Sample preparation: NSO were extracted from the 200 µL spiked whole blood mixtures using a liquid-liquid extraction (LLE) procedure summarized in Figure 2.

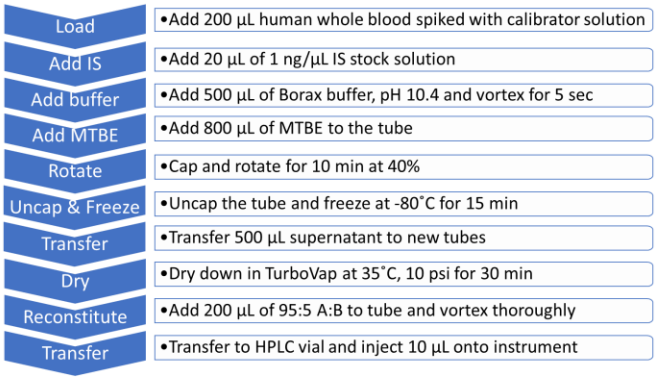


Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used for selectively extracting NSO from human whole blood samples for analysis with the SCIEX 7500 System.

Liquid chromatography: HPLC separation was performed on an ExionLC™ system using a Phenomenex Kinetex C18 column (50 × 3.0 mm, 2.6µm, 00B-4462-Y0). Mobile phase A (MPA) and mobile phase B (MPB) were ammonium formate with formic acid and formic acid in methanol and acetonitrile, respectively. The LC gradient and runtime were optimized to enable baseline separation of all the analytes in the panel, including isomeric species. The injection volume was 10 µL and the total LC runtime was 15.5 minutes.

Mass spectrometry: A SCIEX 7500 System equipped with an OptiFlow® Pro Ion Source with an analytical probe and E Lens™ Technology was used. The ionization source was operated with electrospray ionization (ESI) in positive mode. A single acquisition method consisting of 68 MRM transitions (64 for the NSO and 4 for the internal standards) was created using the Scheduled MRM Algorithm in SCIEX OS Software 2.0. Two MRM transitions were monitored for each of the targeted NSO 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 accomplished within the viewing window using the MQ4 algorithm. Quantitative analysis was performed in the Analytics module of the software. Here calibration curves, concentration calculations, assay precision and accuracy statistics were automatically generated.

Optimized LC conditions lead to separation of isomeric species

A diluted, 10 ng/mL neat standard mixture containing the 32 NSO was used for initial method development. Figure 3A shows the chromatographic profile of the NSO panel resulting from the optimized data acquisition method. Baseline separation of the 32 analytes, including fentanyl and non-fentanyl isomeric species, was accomplished by using a combination of appropriate gradient, adequate mobile phase composition and ideal column choice (Phenomenex Kinetex C18). Together, this enabled better retention of the more polar NSO throughout the course of the 15.5 minute long gradient.

A few fentanyl analogs in the panel are isomeric with other analogs and have no unique fragments that can be used for analyte differentiation. That is the case with *trans*-3-methyl fentanyl and *cis*-3-methylfentanyl (sharing fragment ions of 202.1 and 105.0 Da), as well as iso-butyryl fentanyl and butyryl fentanyl (sharing fragment ions of 188.1 and 105.0 Da). Figure 3B displays representative extracted ion chromatograms (XICs) for four sets of isomeric fentanyl analogs (trans-3-methylfentanyl and cis-3-methylfentanyl, iso-butyryl fentanyl and butyryl fentanyl, acetyl fentanyl and benzyl fentanyl, and β-hydroxy fentanyl and methoxyacetyl fentanyl). As seen in Figure 3, the optimized LC conditions used in this workflow produced the level of separation needed to correctly distinguish the four sets of isomeric species, including the two sets that share the same fragment ions. Chromatographic separation of these four pairs of analogs from their isomers was critical for accurate identification and quantification.

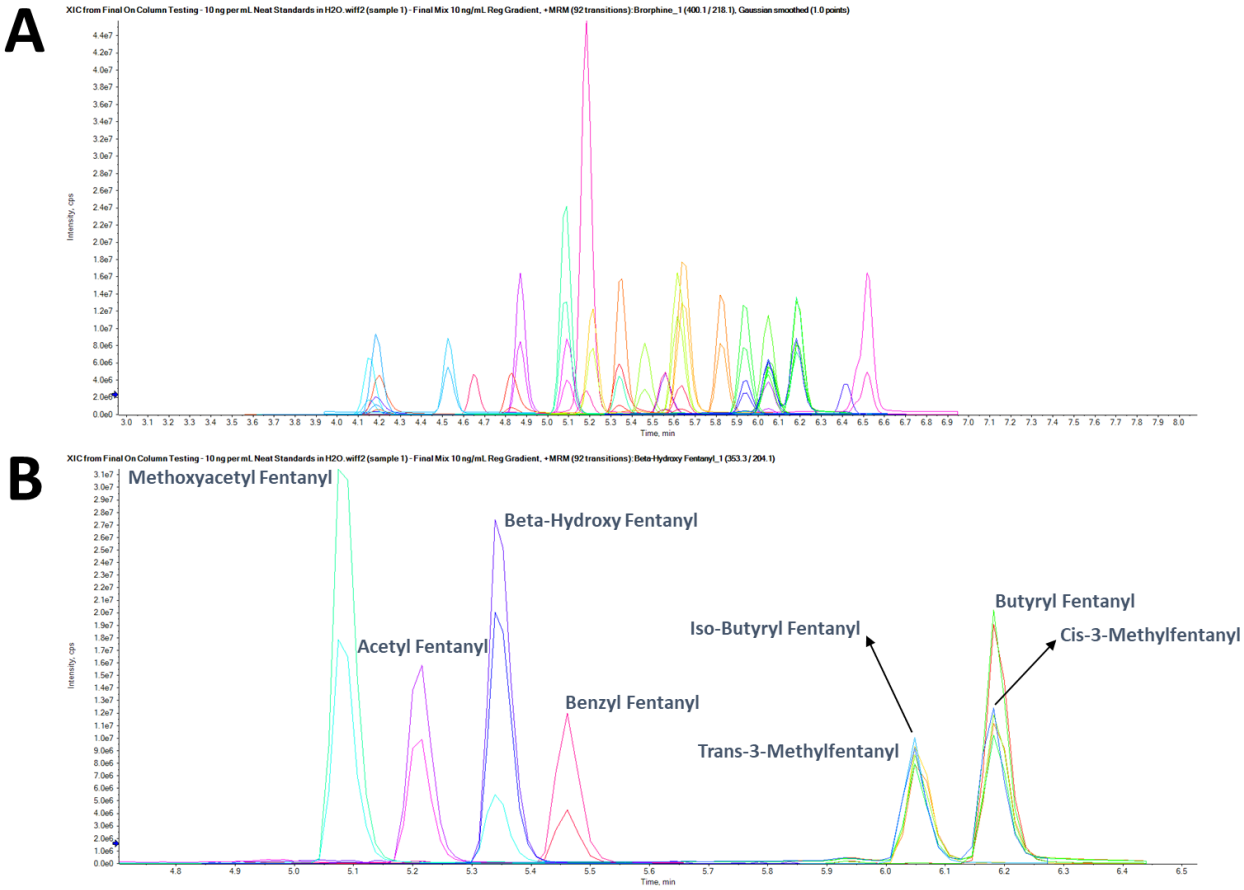


Figure 3. Chromatographic profile of the 32 NSO targeted in this study. A) Extracted ion chromatogram (XIC) resulting from the optimized LC conditions and data acquisition method using a 10 ng/mL neat standard mixture containing the 32 NSO included in the panel. B) Representative extracted ion chromatogram (XIC) of four sets of isomeric fentanyl analogs (*trans*-3-methylfentanyl and *cis*-3-methylfentanyl, iso-butyryl fentanyl and butyryl fentanyl, acetyl fentanyl and benzyl fentanyl, and β-hydroxy fentanyl and methoxyacetyl fentanyl). The optimized LC conditions enabled the level of separation required to separate the fentanyl isomeric species in this NSO panel.

Optimized data collection using the Scheduled MRM Algorithm in SCIEX OS Software enables robust drug quantification

Control human whole blood samples spiked with the 32 target analytes were prepared at concentrations ranging from 1 pg/mL to 100 ng/mL. These standard human whole blood mixtures were spiked with the internal standard mixture, extracted using the aforementioned liquid-liquid extraction procedure and injected to build a data processing method. The Scheduled MRM Algorithm in SCIEX OS Software was used to optimize the dwell time of each MRM transition, ensuring sufficient data sampling across each peak and providing reliable peak integration, quantification and confirmation for each of the NSO in the panel. Detection and integration of the peaks was performed automatically using the MQ4 Algorithm in the Analytics module of SCIEX OS Software. Analyte concentration and ion ratios were calculated automatically in the software.

The ability to accurately detect trace levels of NPS extracted from human whole blood is critical for a toxicologist's interpretation of drug testing results and their help in determining the cause of death. The series of calibrator solutions were injected to evaluate the quantification performance of the system and its ability to accurately measure low levels of drugs and their metabolites with a high level of precision and accuracy. Figure 4 shows representative extracted ion chromatograms (XICs) for the two MRM transitions monitored for A) brorphine and B) etonitazene, two highly potent NSO that have been linked to accidental drug overdoses at low concentration. The series of XIC display overlays both the quantifier and qualifier ions for a blank injection and for concentrations ranging from 5 pg/mL (LOD) to 100 ng/mL. Also displayed in Figure 4 is the tolerance in the form of the ion ratio line overlay which helps visualize the confidence levels. The signal shown for 5 pg/mL is well above the blank signal. The signal for 10 pg/mL is the lower limit of quantification (LLOQ). The LLOQ is the lowest concentration level meeting the following standard performance requirements:

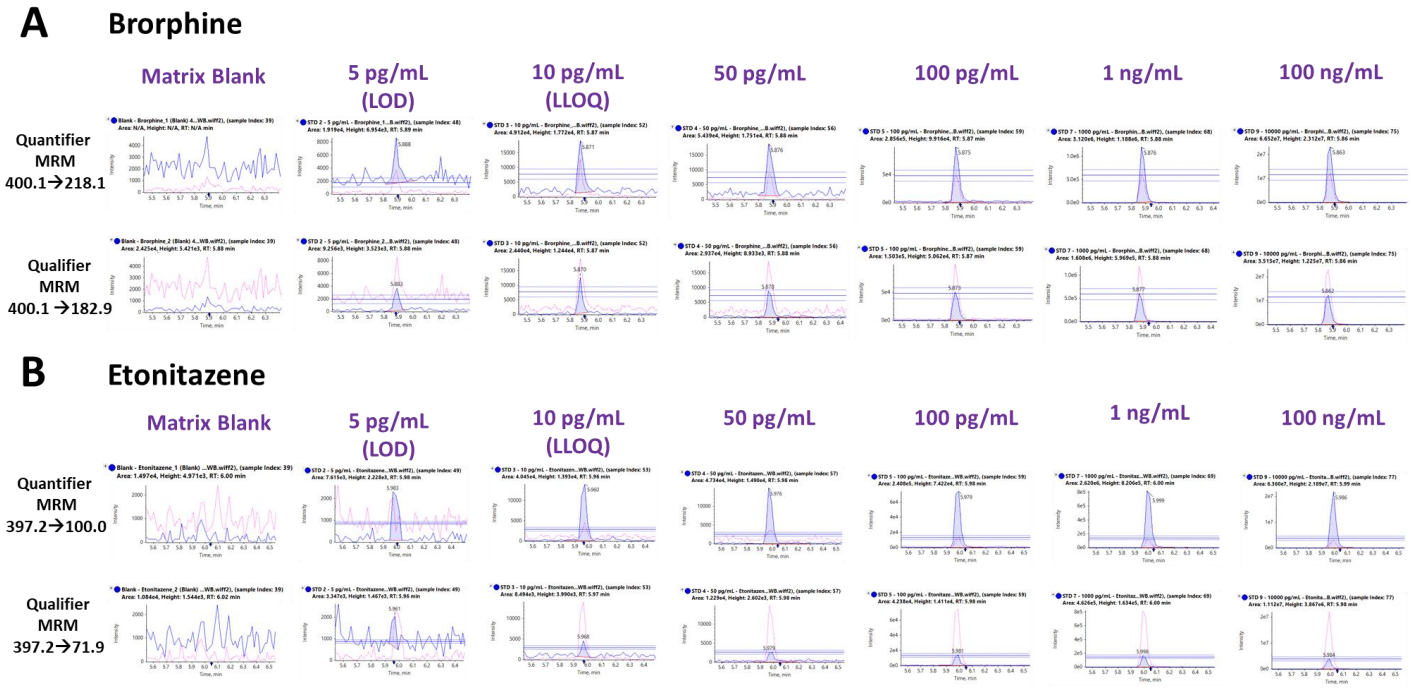


Figure 4 Accurate quantification of two potent NSO extracted from blood samples using the SCIEX 7500 System. Extracted ion chromatograms (XICs) for A) brorphine and B) etonitazene, two potent NSO recently found in accidental overdose case samples. The series of XICs display overlays of both the quantifier and qualifier ions from 5 to 100000 pg/mL. Also shown is the ion ratio line tolerance overlay to visualize the ion ratio confidence levels. The sensitivity of the SCIEX 7500 System enabled robust quantification of NSO down to 10 pg/mL, with limits of detection down to 5 pg/mL for brorphine and etonitazene. Similar quantification performance was observed for the rest of the NSO in the panel.

signal-to-noise ratio (S/N) > 10, calculated concentration accuracy (%bias) within 20%, precision less than 20%, ion ratio acceptance criteria below 20% and calibrators falling on a linear regression curve with an R^2 value of at least 0.99. Overall, the developed method provided robust and accurate quantification of the drugs in the panel without any sacrifice or compromise in data quality.

Table 1 summarizes the statistical results obtained for the 32 NSO and includes the LOD and LLOQ for each of the two MRM transitions monitored for each analyte. Also included in the table is the calibration range and linear correlation coefficient (R^2), as well as the accuracy and precision at the LLOQ. Reported LLOQ values ranged between 10 to 50 pg/mL for the 32 analytes in the panel. The accuracy and precision of measurements ranged from 80.50-116.64% of target and 0.42-17.80%, respectively. The excellent accuracy and precision were observed over the entire concentration range, including at the LLOQ. Overall, the developed method showed excellent reproducibility and linearity, proving the robustness of the developed method and quantitative performance of the SCIEX 7500 System even at low concentration levels for each of the targeted NSO in this study.

Figure 5 shows the resulting calibration curves for the fentanyl analogs (A) and non-fentanyl analogs (B) in the panel. Excellent linearity was observed across the concentration ranges analyzed with R^2 values greater than 0.99 for all of the NPS in the panel.

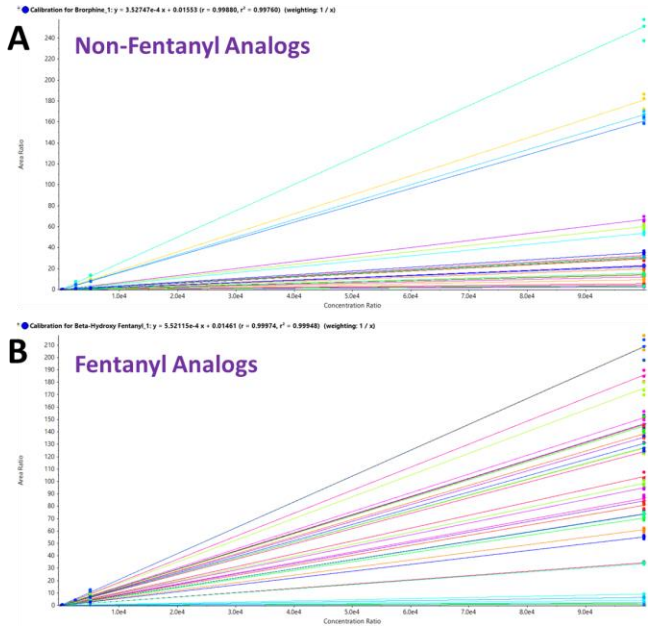


Figure 5. Excellent linearity for the 32 NSO. Linear regression curves resulting from the calibration series from 10 to 100000 pg/mL for A) non-fentanyl analogs and B) fentanyl analogs extracted from human whole blood samples. R^2 values greater than 0.99 were observed for all the NSO in the panel.

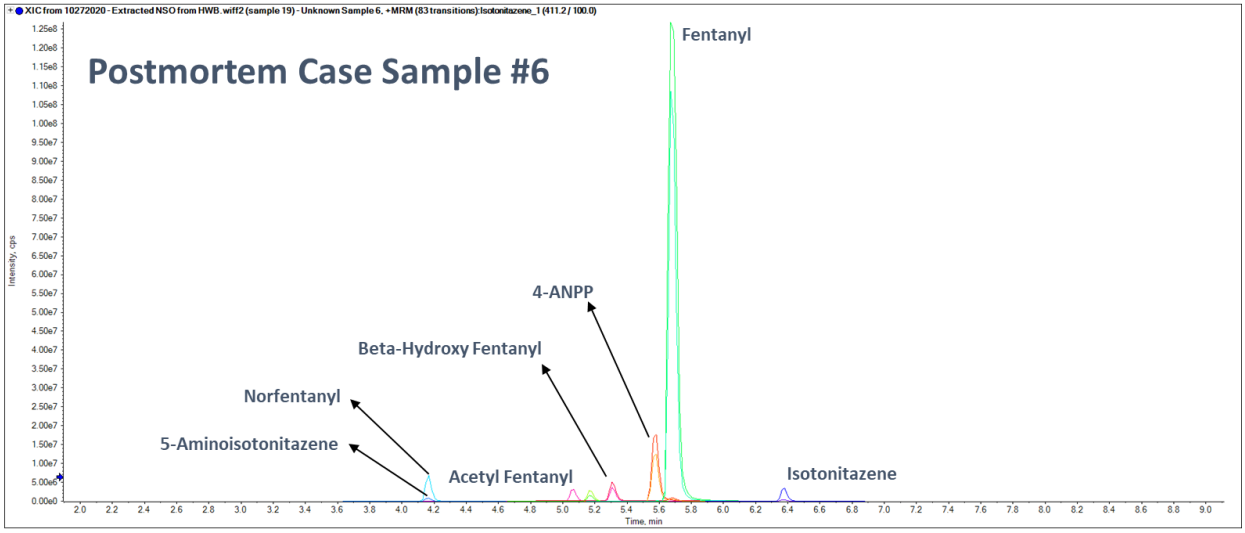
Enhanced sensitivity leads to low level detection of potent NSO in case samples

The robustness of the method and the quantitative performance of the SCIEX 7500 System were further investigated by analyzing ten discarded authentic postmortem case samples from subjects suspected of NPS ingestion resulting in accidental overdoses. These biological specimens were prepared using the aforementioned liquid-liquid sample extraction method and analyzed using the developed acquisition method. The concentration of the positively detected NSO in the postmortem samples were calculated automatically in SCIEX OS Software using the calibration curves generated for each of the 32 NPS. Each case sample was run in triplicate.

Figure 6 shows the results of the successful detection of one NSO and its metabolite: isotonitazene and 5-aminoisotonitazene, as well as fentanyl and four of its analogs/metabolites: beta-hydroxy-fentanyl, norfentanyl, 4-ANPP and acetyl fentanyl. These results are from postmortem case sample #6 at concentrations of 1434.33, 7.93, 599.10, 9756.67, 147.69 and 1465.00 pg/mL, respectively. The displayed XIC in Figure 6A shows the seven analytes positively identified in the postmortem sample. The results summary table shown in Figure 6B lists the analyte peak name, retention time, area and calculated concentration of each of the positively identified analytes. It also shows ion ratio, precursor mass, and the accuracy and concentration acceptance criteria.

A few observations can be drawn from the results highlighted in the summary table. First, detection of the potent NSO isotonitazene is supported by the presence of one of its metabolites, 5-aminoisotonitazene, at low (<10 pg/mL) concentration. Second, detection of fentanyl is confirmed by the presence of its metabolites (*beta*-hydroxy-fentanyl, norfentanyl) as well as synthesis precursors (e.g. 4-ANPP) and by-products (e.g. acetyl fentanyl). This data might suggest that the drug ingested by the subject originated from the illicit market. Although the presence of fentanyl might have been a contributing factor to the accidental overdose, the presence of the potent NSO isotonitazene could support the case of combined opioid drug toxicity leading to death.

A



B

Analyte Peak Name	Retention Time	Area	Calculated Concentration	Accuracy	Ion Ratio	Precursor Mass	Accuracy Acceptance	Concentration Acceptance
Isotonitazene_1	6.36	8.972e+06	1.417e+03	N/A	0.1134	411.239	Pass	Pass
Isotonitazene_2	6.36	1.018e+06	1.440e+03	N/A	0.1134	411.239	Pass	Pass
5-Aminoisotonitazene_1	4.12	2.242e+04	7.596e+00	N/A	0.1454	411.239	Pass	Pass
5-Aminoisotonitazene_2	4.12	2.326 e+04	7.687e+00	N/A	0.1454	381.265	Pass	Pass
Beta-Hydroxy Fentanyl_1	5.30	9.492e+06	6.077e+02	N/A	1.3138	353.300	Pass	Pass
Beta-Hydroxy Fentanyl_2	5.30	1.247e+07	5.029e+02	N/A	1.3138	353.300	Pass	Pass
4-ANPP_1	5.57	5.581e+07	1.484e+03	N/A	0.7001	281.200	Pass	Pass
4-ANPP_2	5.57	3.907e+07	1.467e+03	N/A	0.7001	281.200	Pass	Pass
Acetyl Fentanyl_1	5.16	7.986e+06	1.548e+02	N/A	0.5167	323.200	Pass	Pass
Acetyl Fentanyl_2	5.16	4.126e+06	1.391e+02	N/A	0.5167	323.200	Pass	Pass
Fentanyl_1	5.67	4.699e+08	1.126e+06	N/A	0.3129	337.200	Pass	Pass
Fentanyl_2	5.67	4.350e+08	1.063e+06	N/A	0.3129	337.200	Pass	Pass
Norfentanyl_1	4.15	1.747e+07	9.950e+03	N/A	0.1201	233.200	Pass	Pass
Norfentanyl_2	4.15	2.098e+06	9.931e+03	N/A	0.1201	233.200	Pass	Pass

Figure 6. Accurate and sensitive quantification of low levels of potent NSO in postmortem case sample #6. A) Extracted ion chromatogram (XIC) and B) results summary table showing the analytical and quantitative details of the successful detection of seven potent NSO in a postmortem sample at low concentration. The robustness and sensitivity performance of the SCIEX 7500 System enabled accurate quantification of these analytes at trace levels.

The potency of some non-fentanyl analog NSO showcased in this workflow is a true testament of their ability to cause fatal outcomes. Figure 1 shows the detection of four analytes that were detected in postmortem case sample #1 which was analyzed using the described method. The XIC shows the successful detection of fentanyl at 250.17 pg/mL and two NSOs: isotonitazene (317.80 pg/mL) and its metabolite 5-aminoisotonitazene (44.79 pg/mL), and bromadol, (9644.67 pg/mL). The presence of fentanyl alone at 250 pg/mL might not be sufficient to explain the overdose. However, the two potent non-fentanyl analog NSOs, isotonitazene and bromadol, can contribute to additive opioid effects leading to a combined drug overdose scenario.

The results from the analysis of the postmortem case samples demonstrated the robustness of the developed method and showed that the Scheduled MRM Algorithm in SCIEX OS Software 2.0 on the SCIEX 7500 System enabled sensitive detection and accurate quantification of trace levels of potent NSO. The information that can be interpreted from the results offers valuable insight into the causation of accidental death. As seen with the case samples presented, the sensitivity of the SCIEX 7500 System can support the necessary evidence in postmortem cases where combined intake of high potency drugs at low concentration is responsible for or contributes to an unintentional drug overdose.

Conclusions

A robust and sensitive drug screening workflow for the analysis of 32 potent NSO was successfully developed using the SCIEX 7500 System. The combination of optimized LC conditions with the use of the Scheduled MRM Algorithm in SCIEX OS Software enabled robust and sensitive quantification and identification of isomeric species with a high level of precision and accuracy, even at trace level concentrations The applicability of the developed workflow to accurately detect low concentrations of NSO in authentic forensic samples was further evaluated for the analysis of postmortem case samples from a subject suspected of accidental drug overdose. The results indicate the high quantification performance of the method and its ability to detect low levels of NSO, providing the necessary evidence for toxicologists and medical examiners to determine the cause of death. Overall, the remarkable quantification performance of the SCIEX 7500 System enabled accurate detection of potent NSO at concentrations that were not previously achievable, providing a means to monitor ultra-potent NSO in overdose scenarios. The adaptation of this robust method to postmortem case samples from a subject suspected of combined NSO intake, using the SCIEX 7500 System, offers a valuable insight into the causation of accidental overdoses.

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.

Table 1. Statistical results for the 32 NPS targeted in this workflow. The table includes calibration range, linear correlation coefficient (R^2 Value), LOD and LLOQ, as well as the accuracy and precision at the LLOQ for each of the two MRM transitions monitored for each of the targeted NSO.

Compound	Calibration Range (pg/mL)	Linear Correlation (R^2)	LOD (pg/mL)	LLOQ (pg/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
Brorphine 1	*10-100000*	*0.99760*	*5*	*10*	*84.25*	*1.25*
Brorphine 2	*10-100000*	*0.99889*	*5*	*10*	*89.32*	*15.11*
Isotonitazene 1	*10-100000*	*0.99881*	*5*	*10*	*87.80*	*4.72*
Isotonitazene 2	*50-100000*	*0.99936*	*10*	*50*	*85.00*	*4.26*
5-Aminoisotonitazene 1	*10-100000*	*0.99858*	*5*	*10*	*100.57*	*0.83*
5-Aminoisotonitazene 2	*50-100000*	*0.99937*	*10*	*50*	*87.75*	*4.83*
Metonitazene 1	*50-100000*	*0.99797*	*10*	*50*	*82.48*	*2.74*
Metonitazene 2	*50-100000*	*0.99718*	*10*	*50*	*83.09*	*2.49*
Etonitazene 1	*10-100000*	*0.99726*	*5*	*10*	*88.35*	*12.31*
Etonitazene 2	*10-100000*	*0.99758*	*5*	*10*	*86.84*	*5.17*
AP-237 1	*10-100000*	*0.99752*	*5*	*10*	*99.15*	*16.41*
AP-237 2	*50-100000*	*0.99741*	*10*	*50*	*95.17*	*12.00*
2-methyl AP-237 1	*50-100000*	*0.99608*	*10*	*50*	*94.51*	*17.39*
2-methyl AP-237 2	*50-100000*	*0.99096*	*10*	*50*	*81.50*	*10.97*
2F-Viminol 1	*10-100000*	*0.0.99812*	*5*	*10*	*104.64*	*4.33*
2F-Viminol 2	*10-100000*	*0.99779*	*5*	*10*	*98.53*	*4.35*
Butorphanol 1	*10-100000*	*0.99413*	*5*	*10*	*103.86*	*14.11*
Butorphanol 2	*10-100000*	*0.99178*	*5*	*10*	*107.15*	*9.02*
N-Desethyl Isotonitazene 1	*50-100000*	*0.99750*	*10*	*50*	*80.50*	*0.42*
N-Desethyl Isotonitazene 2	*50-100000*	*0.99828*	*10*	*50*	*96.91*	*7.61*
4'-Hydroxy Nitazene 1	*10-100000*	*0.99807*	*5*	*10*	*98.80*	*14.71*
4'-Hydroxy Nitazene 2	*10-100000*	*0.99904*	*5*	*10*	*93.47*	*17.80*
Flunitazene 1	*50-100000*	*0.99850*	*10*	*50*	*87.94*	*14.34*
Flunitazene 2	*50-100000*	*0.99857*	*10*	*50*	*98.22*	*0.51*
Isotodesnitazene 1	*10-100000*	*0.99789*	*5*	*10*	*81.64*	*6.99*
Isotodesnitazene 2	*50-100000*	*0.99857*	*10*	*50*	*83.65*	*3.64*
Etodesnitazene 1	*10-100000*	*0.99896*	*5*	*10*	*84.74*	*1.47*
Etodesnitazene 2	*50-100000*	*0.99889*	*10*	*50*	*85.79*	*1.76*
Metodesnitazene 1	*10-100000*	*0.99879*	*5*	*10*	*87.73*	*13.55*
Metodesnitazene 2	*10-100000*	*0.99882*	*5*	*10*	*96.67*	*10.94*
Beta-Hydroxy Fentanyl 1	*50-100000*	*0.99948*	*10*	*50*	*88.20*	*4.34*
Beta-Hydroxy Fentanyl 2	*100-100000*	*0.98615*	*50*	*100*	*88.44*	*7.60*
2-Furanyl Fentanyl 1	*10-100000*	*0.99888*	*5*	*10*	*88.78*	*1.84*
2-Furanyl Fentanyl 2	*10-100000*	*0.99919*	*5*	*10*	*97.75*	*2.25*

Compound	Calibration Range (pg/mL)	Linear Correlation (R^2)	LOD (pg/mL)	LLOQ (pg/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
4-ANPP 1	*10-100000*	*0.99911*	*5*	*10*	*88.22*	*5.83*
4-ANPP 2	*10-100000*	*0.99945*	*5*	*10*	*110.72*	*7.71*
Acetyl Fentanyl 1	*10-100000*	*0.99884*	*5*	*10*	*87.10*	*1.96*
Acetyl Fentanyl 2	*10-100000*	*0.99944*	*5*	*10*	*104.51*	*4.96*
Acrylfentanyl 1	*10-100000*	*0.99925*	*5*	*10*	*89.53*	*10.24*
Acrylfentanyl 2	*10-100000*	*0.99971*	*5*	*10*	*88.55*	*16.63*
Benzyl Fentanyl 1	*10-100000*	*0.99943*	*5*	*10*	*108.57*	*8.84*
Benzyl Fentanyl 2	*50-1000000*	*0.99876*	*10*	*50*	*94.42*	*1.60*
Butyryl Fentanyl 1	*10-100000*	*0.99909*	*5*	*10*	*85.64*	*0.53*
Butyryl Fentanyl 2	*10-100000*	*0.99934*	*5*	*10*	*98.81*	*11.39*
Carfentanil 1	*10-100000*	*0.99608*	*5*	*10*	*94.51*	*17.39*
Carfentanil 2	*10-100000*	*0.99811*	*5*	*10*	*82.24*	*0.87*
Cis-3-Methylfentanyl 1	*10-100000*	*0.99689*	*5*	*10*	*99.76*	*14.18*
Cis-3-Methylfentanyl 2	*10-100000*	*0.99920*	*5*	*10*	*83.95*	*2.36*
Cyclopropyl Fentanyl 1	*10-100000*	*0.99895*	*5*	*10*	*109.59*	*7.98*
Cyclopropyl Fentanyl 2	*10-100000*	*0.99956*	*5*	*10*	*91.83*	*9.10*
Fentanyl 1	*10-100000*	*0.99378*	*10*	*50*	*85.68*	*5.94*
Fentanyl 2	*10-100000*	*0.99138*	*10*	*50*	*95.66*	*5.98*
Iso-Butyryl Fentanyl 1	*10-100000*	*0.99846*	*5*	*10*	*80.66*	*5.47*
Iso-Butyryl Fentanyl 2	*10-100000*	*0.99909*	*5*	*10*	*105.30*	*7.05*
Methoxyacetyl Fentanyl 1	*10-100000*	*0.99944*	*5*	*10*	*86.35*	*6.59*
Methoxyacetyl Fentanyl 2	*10-100000*	*0.99945*	*5*	*10*	*108.48*	*3.87*
N-Methyl Norfentanyl 1	*10-100000*	*0.99932*	*5*	*10*	*104.74*	*3.51*
N-Methyl Norfentanyl 2	*10-100000*	*0.99944*	*5*	*10*	*103.20*	*2.15*
Norcarfentanil 1	*50-100000*	*0.99675*	*10*	*50*	*115.85*	*12.50*
Norcarfentanil 2	*50-100000*	*0.99825*	*10*	*50*	*108.52*	*6.85*
Norfentanyl 1	*10-100000*	*0.99808*	*5*	*10*	*116.64*	*2.98*
Norfentanyl 2	*50-100000*	*0.99852*	*10*	*50*	*105.62*	*12.85*
Trans-3-Methylfentanyl 1	*10-100000*	*0.99762*	*5*	*10*	*107.72*	*4.64*
Trans-3-Methylfentanyl 2	*50-100000*	*0.99546*	*10*	*50*	*83.32*	*5.88*

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Sensitivity improvement for the detection of steroid hormones and endocannabinoids in keratinized matrices

Using the SCIEX 7500 system, powered by SCIEX OS software

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Steroid hormones and endocannabinoids (ECs) are endogenous substances that play a key role in the regulation of the human stress response. The concentrations of these regulators greatly fluctuate throughout the day, making it challenging to correlate their relationship to the range of stress-related conditions experienced by humans. Analysis of these substances is analytically challenging due to their low levels in biological matrices and chemical similarity to one another. As a result, sensitive analytical techniques are required to provide accurate quantification of these stress markers in biological matrices.

Detection of these endogenous markers can be performed in many biological matrices including urine, blood, saliva and keratinized matrices, such as hair and nails. Among these matrices, hair and nails are becoming extremely valuable for assessing the long-term and retrospective determination of these endogenous stress markers, which are known to steadily accumulate in the keratinized matrix over time. Other benefits include the non-invasive nature of sample collection, minimal storage requirements and long-term stability with low risk of sample degradation over time.

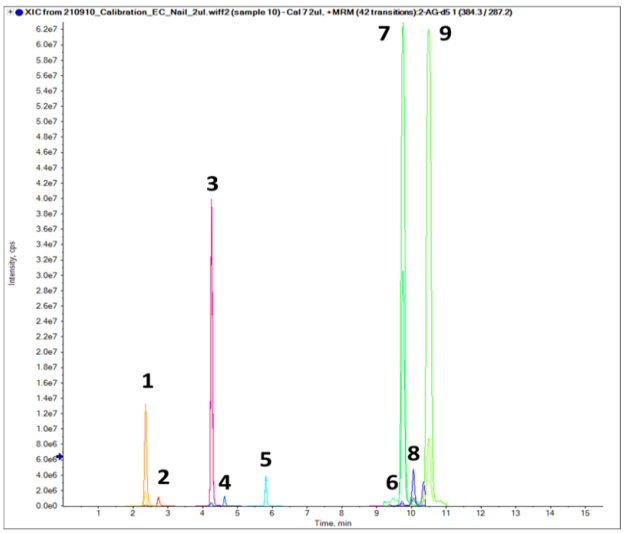

Figure 1. Chromatographic profile of the 5 steroid hormones and 4 endocannabinoids targeted in this study. Extracted ion chromatogram (XIC) resulting from the baseline separation of the 9 compounds in the panel. The numbered peaks are assigned as follows: 1) cortisone $^{13}C_3$, 2) cortisol $^{13}C_3$, 3) androstenedione $^{13}C_3$, 4) testosterone $^{13}C_3$, 5) progesterone $^{13}C_3$, 6) AEA D_4, 7) PEA D_4, 8) 2-AG D_5 and 9) N-OEA D_4. The total LC runtime was 15.5 min.

One of the challenges associated with the detection and accurate quantification of endogenous compounds in biological matrices is that the matrices themselves already contain varying levels of the endogenous analytes of interest. Therefore, no blank, analyte-free matrices are available that can be used for calibration and validation. An approach commonly used to overcome this challenge is the use of surrogate analytes, such as $^{13}C_3$-labeled or deuterated analogs that enable calibration in the biological matrix of interest. This approach was successfully used previously for the accurate quantification of endogenous steroids and endocannabinoids in keratinized matrices[1].

Here, an analytical workflow that combines the use of a supported liquid extraction (SLE)-based sample preparation and the SCIEX 7500 system for sub-pg/mg calibration of 5 steroid hormones and 4 ECs is described. Calibration results from the SCIEX 7500 system were compared with results from the QTRAP 6500+ system, an established platform that is routinely used for low-level quantification of endogenous species. Significant gains in peak area and signal-to-noise ratio (S/N) were observed on the SCIEX 7500 system, demonstrating the benefits of this system for sensitive quantification of low-level stress markers in keratinized matrices.

Advantages of the SCIEX 7500 system for high-sensitivity stress marker analysis in keratinized matrices

- Analyte extraction followed by a SLE-based sample preparation in combination with a robust detection method using the scheduled MRM algorithm in SCIEX OS software enabled calibration in the sub-pg/mg range using surrogate endogenous steroid hormones and ECs in keratinized matrices
- Sensitivity gains observed on the SCIEX 7500 system improved quantification of low-level stress markers in keratinized matrices

Experimental details

Target analytes: The following surrogate analytes were used for quantification: cortisone $^{13}C_3$, cortisol $^{13}C_3$, androstenedione $^{13}C_3$, testosterone $^{13}C_3$, progesterone $^{13}C_3$, AEA D_4, PEA D_4, 2-AG D_5 and N-OEA D_4. The internal standard included cortisone D_7, progesterone D_9 and AEA D_{11}.

Calibrator preparation: Seven levels of calibrators were prepared by spiking the surrogate analyte mixture and internal standard in hair and nail samples across various concentrations. The concentrations tested included: 5 to 200 pg/mg for 2-AG D_5; 0.1 to 10 pg/mg for AEA D_4 and testosterone $^{13}C3$; 1 to 500 pg/mg for androstenedione $^{13}C3$, cortisone $^{13}C3$ and progesterone $^{13}C3$; 500 to 10,000 pg/mg for N-OEA D_4 and PEA D_4 and 0.5 to 50 pg/mg for cortisol $^{13}C3$.

Hair and nail sample washing: Hair and nail samples were first washed for 3 minutes with deionized water, followed by a 2-minute wash with acetone. The tubes containing the hair and nail samples were thoroughly shaken by hand during the washing steps and the washing solutions were decanted and discarded after each washing step. The washed hair and nail samples were dried overnight at room temperate.

Hair and nail sample preparation: Nail clippings (20 mg) were weighed into an Eppendorf tube and 3 milling balls (stainless steel, 5 mm diameter) were added. Nail clippings were pulverized for 10 minutes at 30 Hz. Hair segments were manually cut into snippets and 20 mg of hair was weighed into an Eppendorf tube. Then, 1 mL of methanol and 50 uL of the internal standard were added to each tube containing either the hair or pulverized nail samples. The tubes were briefly shaken and placed in a sonicated bath (35 kHz, 600 W) for 4 hours (hair) or 1 hour (nail) at 55°C for extraction. The tubes were centrifuged at 9000 g for 5 minutes and the methanolic extracts were transferred to a column rack for SLE.

SLE procedure: A supported liquid extraction (SLE) was performed using an automated Biotage Extrahera system (Biotage, Sweden). Sample extracts were automatically loaded onto Isolute SLE+ columns and allowed to absorb for 5 minutes. Analytes were then eluted 2 times with 2.5 mL ethyl acetate with a wait time of 5 minutes between the 2 elution steps. The extracts were dried in a Turbovap solvent evaporator system (Biotage, Sweden) and resuspended in 60 µL of methanol and 140 µL of a reconstitution solution consisting of 0.2mM ammonium formate in 97:3, water/methanol.

Liquid chromatography: HPLC separation was achieved using a Phenomenex Kinetex XB-C_{18} column (50 x 2.10 mm, 2.6 µm, 00B-4496-AN). The column was held at 40°C on a Prominence UFLC system on the QTRAP 6500+ system or on a Nexera 40 Series UHPLC system on the SCIEX 7500 system. Mobile phases A and B consisted of 0.2 mM ammonium formate in 97:3, water/methanol and 3:97, water/methanol, respectively. The LC flow rate was 0.4 mL/min and the total runtime was 20 min. The injection volume was 2 µL.

Mass spectrometry: A SCIEX 7500 system equipped with an OptiFlow Pro ion source with an electrospray ionization (ESI) analytical probe and E Lens probe was used. For comparison purposes, a QTRAP 6500+ system equipped with an IonDrive Turbo V ion source was also used. Both instruments were optimized for maximum sensitivity and operated in positive ESI mode. Source parameters and compound-dependent parameters for all compounds and their corresponding internal standards were also optimized on each system, including the Q0D dissociation on the SCIEX 7500 system.

Data acquisition and processing: Data were acquired using SCIEX OS software on the SCIEX 7500 system. For the QTRAP 6500+ system, data were acquired using Analyst software, version 1.7. All data were processed using SCIEX OS software, in which detection and integration of the peaks from the background were accomplished within the viewing window using the MQ4 algorithm. The peak-to-peak algorithm was used for S/N calculations in the Analytics module of SCIEX OS software.

Analytical methodology for robust and accurate quantification of stress markers

For the initial method development, a diluted, 10 ng/mL neat standard mixture containing the 9 target analytes was used. Figure 1 shows the chromatographic profile of the 5 steroid hormones and 4 endocannabinoids targeted in this study. The combination of an appropriate gradient, adequate mobile phase compositions and ideal column choice (Phenomenex Kinetex XB-C_{18}) enabled baseline separation of the 9 analytes in a 15-minute total runtime.

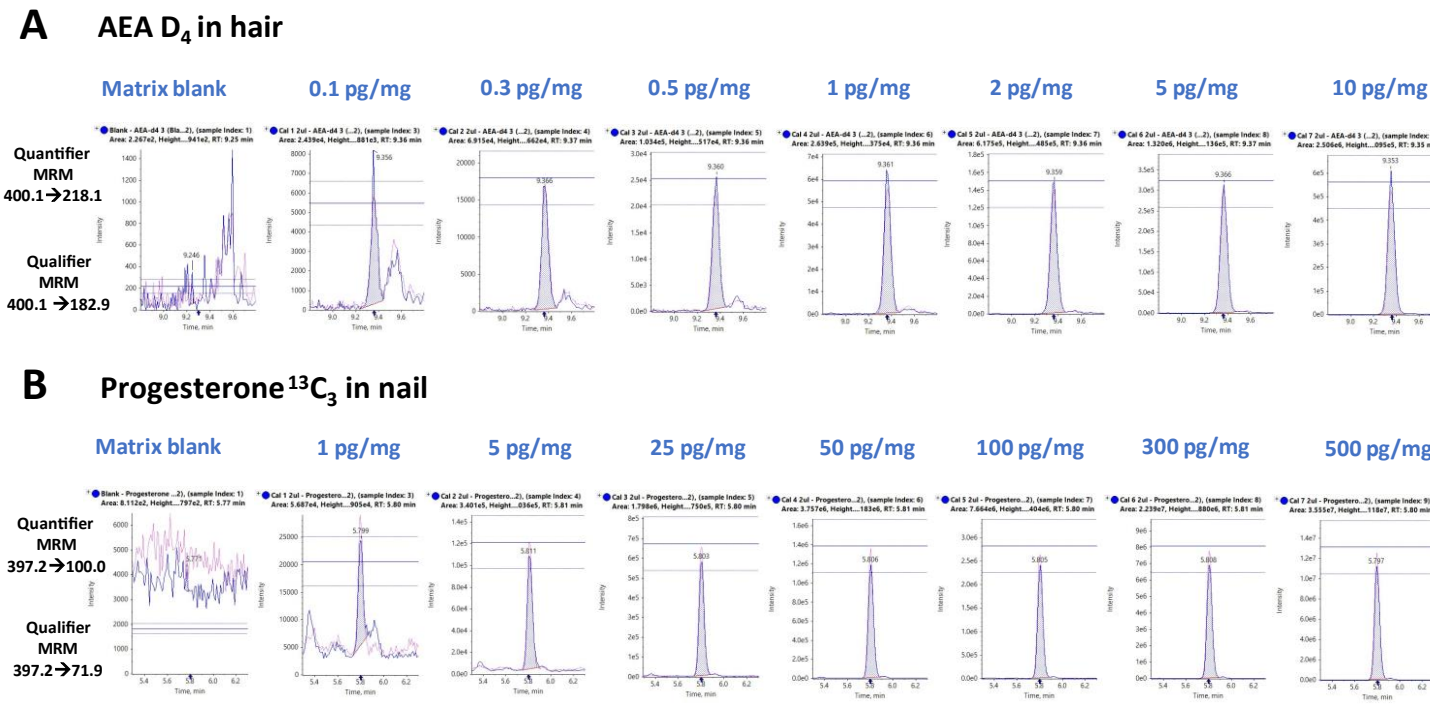

Figure 2. Representative XICs acquired on the SCIEX 7500 system for an endocannabinoid and steroid hormone extracted from hair or nail samples. XICs for A) the deuterated EC analog, AEA D_4, extracted from hair samples at concentrations ranging from 0.1 to 10 pg/mg and B) the $^{13}C_3$ labeled steroid hormone, progesterone $^{13}C_3$, extracted from nail samples at concentrations ranging from 1 to 500 pg/mg. Both the quantifier and qualifier traces are shown. Ion ratios were monitored across the dataset, indicated by the tolerance lines.

Authentic human hair and nail samples spiked with 5 $^{13}C_3$-labeled steroid hormones and 4 deuterated ECs were prepared at concentrations ranging from 0.1 to 10,000 pg/mg, extracted using the SLE procedure and injected to the Nexera 40 Series UHPLC system on the SCIEX 7500 system to build a data processing method. The series of calibrator solutions was injected to evaluate the quantification performance of the system and its ability to accurately measure low levels of endogenous biomarkers with high levels of precision and accuracy.

Figure 2 shows representative extracted ion chromatograms (XICs) acquired on the SCIEX 7500 system for 2 MRM transitions monitored for AEA D_4 extracted from hair samples (Figure 2A) and progesterone $^{13}C_3$ extracted from nail samples (Figure 2B). The series of XICs displayed includes overlays of both the quantifier and qualifier ions for a blank injection (left) and for concentrations ranging from 0.1 to 10 pg/mg for AEA D_4 and from 1 to 500 pg/mg for progesterone $^{13}C_3$. Also displayed in Figure 2 is the tolerance set for these analyses, indicated by the ion ratio line overlay that helps visualize the confidence levels. The ion ratio difference was <20% for the quantifier and qualifier ions of each of the targeted analytes across the calibration range. Overall, the developed method enabled robust and accurate quantification of endogenous steroids and endocannabinoids in keratinized matrices.

Calibration curves were generated using the quantifier MRM transition for each of the analytes targeted in this method. Figure 3 shows the resulting regression lines plotted across the calibrator levels for androstenedione $^{13}C_3$, cortisone $^{13}C_3$ and progesterone $^{13}C_3$ from to 500 pg/mg (Figure 3A), 2-AG D_5 from 5 to 200 pg/mg (Figure 3B), N-OEA D_4 and PEA D_4 from 500 to 10,000 pg/mg (Figure 3C) and AEA D_4 and testosterone $^{13}C_3$ from 0.1 to 10 pg/mg (Figure 3D) extracted from hair samples. The resulting calibration curves showed excellent linearity across the calibration ranges with R^2 values greater than 0.99 for all the steroid hormones and endocannabinoids targeted in this study. Excellent linearity was also observed for the analytes extracted from nail samples (data not shown).

Leveraging sensitivity improvement for low-level detection of endogenous steroids and endocannabinoids in hair and nail samples

The use of sensitive mass spectrometry instrumentation is critical to accurately quantify low levels of these endogenous species in complex biological matrices. In this study, the sensitivity of the SCIEX 7500 system was compared to that of a previous generation instrument, the QTRAP 6500+ system using both the peak area and signal-to-noise ratio (S/N). Figure 4 shows the XIC series, respective peak area and S/N values for

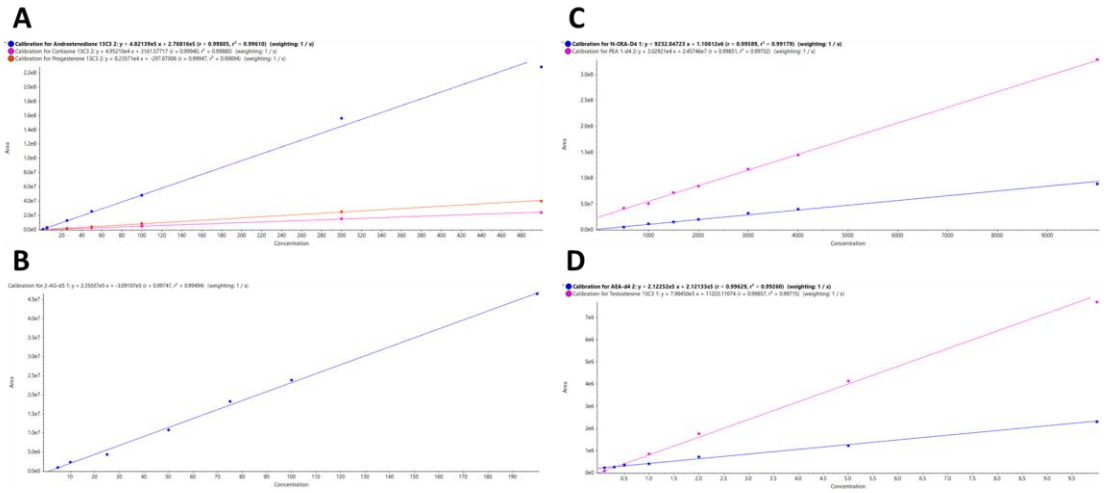

Figure 3. Excellent linearity for 8 steroids and endocannabinoids targeted in this panel. Calibration curves were generated using the quantifier MRM transition for A) androstenedione $^{13}C3$, cortisone $^{13}C3$ and progesterone $^{13}C3$ from 1 to 500 pg/mg, B) 2-AG D_5 from 5 to 200 pg/mg, C) N-OEA D_4 and PEA D_4 from 500 to 10,000 pg/mg and D) AEA D_4 and testosterone $^{13}C3$ from 0.1 to 10 pg/mg extracted from hair samples and acquired on the SCIEX 7500 system. The assay showed excellent linearity with R^2 values greater than 0.99 for all the analytes.

androstenedione $^{13}C_3$ in hair samples acquired on the SCIEX 7500 system (top) and QTRAP 6500+ system (bottom) for 7 concentration levels, ranging from 1 to 500 pg/mg. The average peak area and S/N gains across the 7 concentration levels for androstenedione $^{13}C_3$ were 9.9 ±1.2 and 4.4 ±2.2, respectively. Similar observations were made for the other 7 analytes included in the panel, with peak area gains ranging from 9.9x for androstenedione $^{13}C_3$ to 71.2x for PEA D_4 and S/N gains ranging from 0.6x for progesterone $^{13}C_3$ to 13.8x for PEA D_4 in hair samples. Overall, the SCIEX 7500 system provided a significant increase in both peak areas and S/N ratios across all the analytes extracted from hair samples, with average peak area gains of 30.0x and average S/N gains of 5.6x across all the analytes extracted from hair samples.

Figure 5 shows the XIC series for 2-AG D_5 in nail samples acquired on the SCIEX 7500 system (top) and QTRAP 6500+ system (bottom) for 7 concentration levels ranging from 5 to 200 pg/mg. Similarly, the average peak area and S/N gains across the 7 concentration levels for 2-AG D_5 were 35.5 ±4.8 and 4.5 ±4.0, respectively. As observed with analytes from hair samples, significant gains in the peak area and S/N were observed for the analytes extracted from nail samples. Peak area gains ranged from 3.7x for progesterone $^{13}C_3$ to 426x for PEA D_4 and S/N gains ranged from 0.7x for progesterone $^{13}C_3$ to 19.0x for PEA D_4 in nail samples. Overall, the peak area gains and S/N gains averaged 15.4x and 5.6x, respectively, across all the analytes extracted from nail samples. Table 1 summarizes the peak area gains and S/N gains observed for the 9 analytes targeted in this study in the 2 keratinized matrices investigated.

Conclusions

- The technology improvements of the SCIEX 7500 system were leveraged to improve the sensitivity for accurate detection of endogenous markers in keratinized matrices
- The ion ratio difference was <20% for the quantifier and qualifier ions of the targeted analytes, showing the quantitative robustness of the developed workflow
- The overall performance of the system resulted in excellent linearity with R^2 values >0.99 for all the steroids and endocannabinoids targeted in this study
- The impact of these sensitivity gains was investigated by comparing the signals observed on the SCIEX 7500 system and a previous generation instrument, the QTRAP 6500+ system
- Improvements were observed in both peak area and S/N gains for the 9 analytes targeted in this study in the 2 keratinized matrices investigated
- Average peak area gains were attained that ranged from 9.85x to 71.21x and 3.69x to 42.55x for compounds extracted from hair and nails, respectively
- Average S/N ratio gains were achieved that ranged from 0.62x to 13.82x and 0.68x to 19.04x for compounds extracted from hair and nails, respectively
- The use of the SCIEX 7500 system demonstrated the ability to routinely and robustly detect ultra-low levels of analytes extracted from challenging biological matrices
- The presented workflow provided the sensitivity levels required for the long-term retrospective measurement of endogenous biomarkers in keratinized matrix

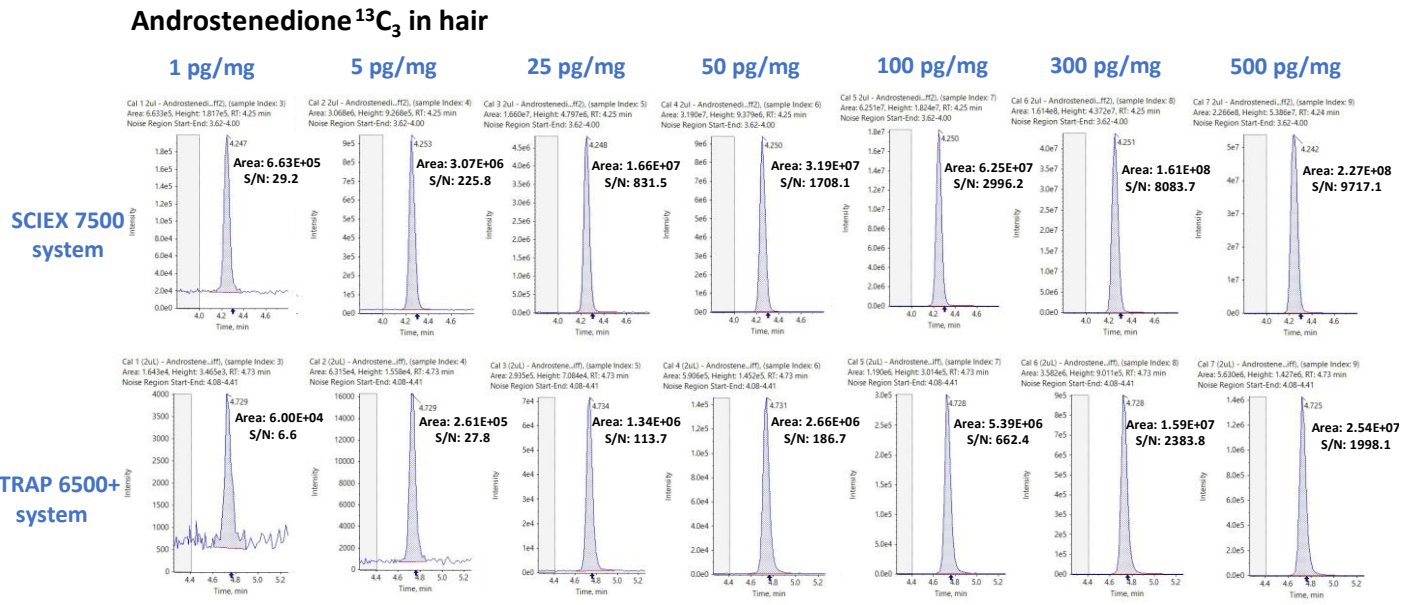

Figure 4. Sensitivity improvement for the detection of steroid hormones extracted from hair samples. XIC comparisons between the SCIEX 7500 system (top) and the QTRAP 6500+ system (bottom) for the 7 levels androstenedione $^{13}C_3$ calibrators extracted from hair samples at concentrations ranging from 1 to 500 pg/mg. The SCIEX 7500 system showed significant improvements in both peak area and S/N gains over the QTRAP 6500+ system.

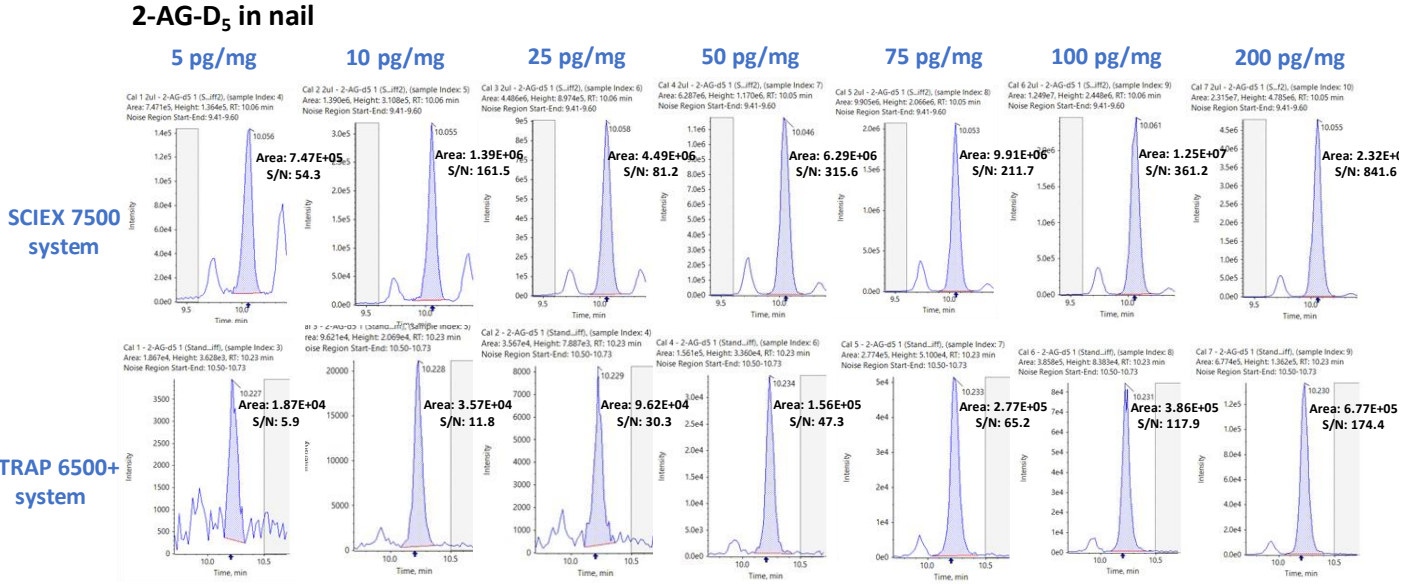

Figure 5. Increased sensitivity for the detection of endocannabinoids extracted from nail samples. XIC comparisons between the SCIEX 7500 system (top) and the QTRAP 6500+ system (bottom) for the 7 levels of 2-AG D_5 calibrator extracted from nail samples at concentrations ranging from 5 to 200 pg/mg. The SCIEX 7500 system showed significant improvements in both peak area and S/N gains over the QTRAP 6500+ system.

References

1. Voegel CD, Baumgartner MR, Kraemer T, Wüst S, Binx TM (2021) "Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS - Insights into EC baseline values and correlation to steroid concentrations", *Talanta*, **222**: 121499.

Table 1. Mean peak area and S/N ratios of the 7 levels of calibrators for each of the 9 analytes spiked in hair and nail samples. Seven levels of calibrators were prepared and injected to the SCIEX 7500 system and QTRAP 6500+ system. The ratios of the peak areas and S/N were determined at each calibrator level and averaged for each of the 5 steroid hormones and 4 endocannabinoids for each of the 2 keratinized matrices analyzed (hair and nail).

Compound	Hair matrix		Nail matrix	
	Mean peak area ratio	Mean S/N ratio	Mean peak area ratio	Mean S/N ratio
2-AG D_5	*63.0 ± 11.0*	*11.7 ± 4.2*	*35.5 ± 4.8*	*4.5 ± 4.0*
AEA D_4	*57.6 ± 10.2*	*1.2 ± 0.5*	*21.4 ± 121.0*	*3.9 ± 7.4*
Androstenedione $^{13}C3$	*9.9 ± 1.2*	*4.4 ± 2.2*	*6.5 ± 0.3*	*1.1 ± 0.2*
Cortisol $^{13}C3$	*15.2 ± 0.5*	*3.4 ± 1.0*	*6.7 ± 0.7*	*1.8 ± 0.4*
Cortisone $^{13}C3$	*13.9 ± 0.9*	*3.5 ± 1.4*	*6.1 ± 0.5*	*2.7 ± 0.8*
N-OEA D_4	*13.7 ± 5.6*	*9.2 ± 60.9*	*8.1 ± 2.2*	*14.4 ± 65.5*
PEA D_4	*71.2 ± 21.0*	*13.8 ± 11.8*	*42.6 ± 9.0*	*19.0 ± 12.2*
Progesterone $^{13}C3$	*10.8 ± 1.8*	*0.6 ± 0.1*	*3.7 ± 0.6*	*0.7 ± 0.3*
Testosterone $^{13}C3$	*14.4 ± 1.7*	*2.6 ± 1.0*	*8.2 ± 1.1*	*2.0 ± 0.3*

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