2022 法醫毒理應用文集

第二期 --- 新興影響精神物質

(New psychoactive substances, NPS)

SCIEX

The Power of Precision

目錄

2022 精彩會議內容回顧：

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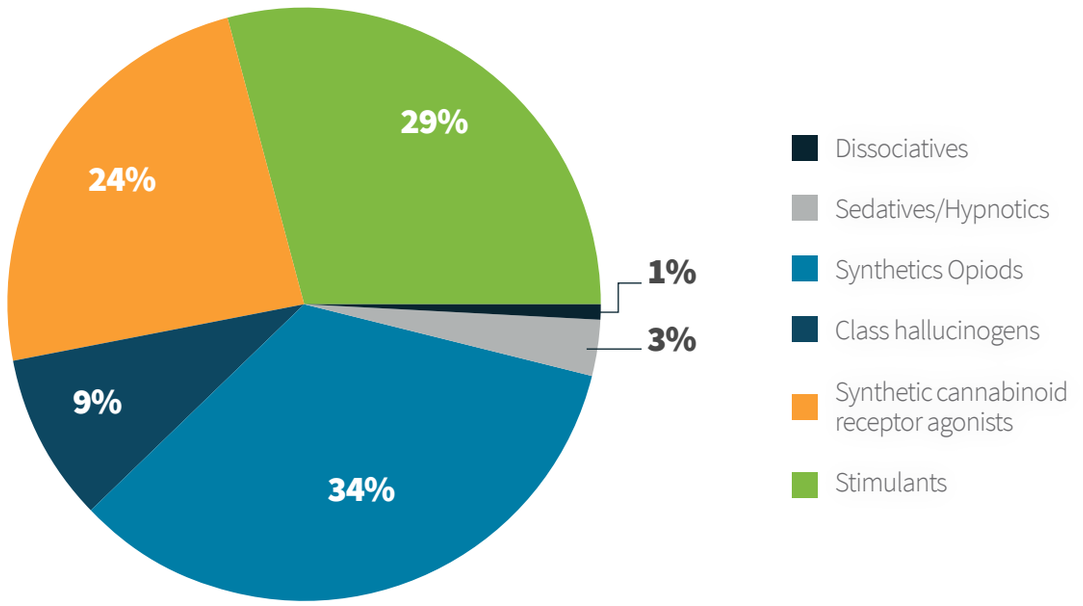
The growing problem of NPS

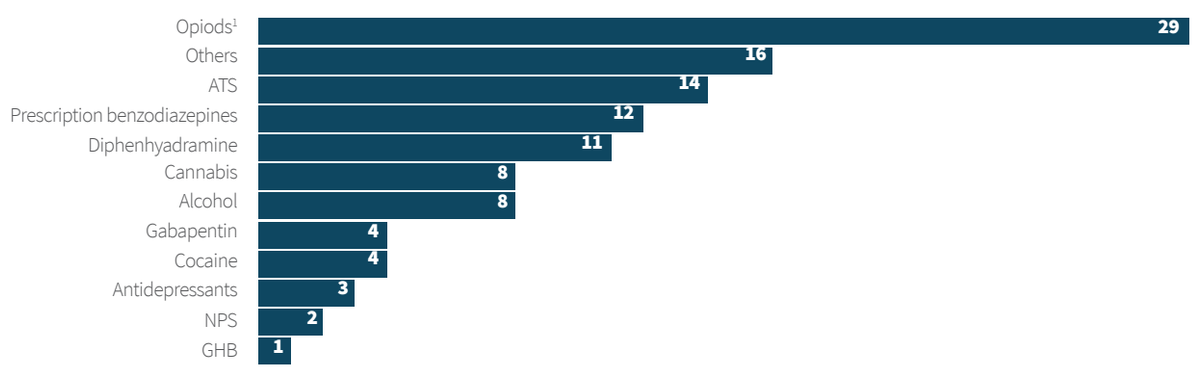
The United Nations Office on Drugs and Crime (UNODC) Early Warning Advisory (EWA) monitors, analyzes and reports on NPS trends to help provide effective evidence-based policy responses and improve the understanding of NPS distribution patterns and use worldwide.

As of January 2020, 120 countries and territories reported the cumulative emergence of 950 individual NPS.[1]
The distribution of new cases reported to the UNODC EWA since the beginning of 2018 is shown below:



Between 2016 and 2018, just over half of all NPS toxicology cases reported to the Tox-Portal involved opioids or synthetic cannabinoids. However, the most recent information from 2019 indicates that benzodiazepine-type NPS now account for most cases, demonstrating the dynamic nature of NPS trends.[1,2]

Additionally, poly-drug use is very common; in 2019, a high proportion of reported NPS fatalities involved kratom and in all these cases, additional substances were detected. This presents a significant challenge when trying to assess the significance and contribution of a particular drug in a person's death.[1,2]



**Kratom is the colloquial name of the plant Mitragyna speciose which has some opioid and stimulant properties, containing pharmacologically active alkaloids especially mitragynine and 7-hydroxymitragynine.*

1. United Nations Office on Drugs and Crime. 2019. Current NPS Threats Volume II. [online] Available at: https://www.unodc.org/documents/scientific/Current_NPS_Threats_Volume_II_Web.pdf [Accessed 3 February 2020].
2. United Nations Office on Drugs and Crime. 2019. Current NPS Threats Volume I. [online] Available at: https://www.unodc.org/pdf/opioids-crisis/Current_NPS_Threats_-_Volume_I.pdf

聲波激發耦合質譜儀（AEMS）超快速檢測污水中17種毒品的含量

使用Echo™ MS 系統，以每秒鐘一個樣品的分析速度突破定量質譜分析通量的瓶頸

AEMS ultra-fast detection of the concentration of 17 drugs in sewage

Using the SCIEX Echo™ MS system to break bottlenecks in quantitative mass spectrometry throughput at the rate of one sample per second

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Key Words：Echo™ MS, AEMS, ultra-fast, quantitative, sewage, drug

儀器簡介

Echo™ MS系統（見圖1），即聲波激發耦合質譜儀（Acoustic Ejection Mass Spectrometry, AEMS）是一款由聲波液滴噴射技術（Acoustic droplet ejection technology, ADE），開放埠探針採樣介面（Open-port probe sampling interface, OPI）以及強大定量能力的SCIEX Triple Quad™ 6500+ 系統（配備電噴霧離子源，ESI電離模式）三位一體耦合在一起的開創性新產品。

Echo™ MS系統集成了ADE技術和OPI技術；ADE的作用是通過優化設計， 用聲波能量將樣品從樣品板中極小的樣品量（2.5 nL）激發出來，激發出來的小液滴傳輸到一個固定倒置的OPI中，樣品小液滴在OPI中與傳輸流體相遇並稀釋，通過OPI將樣品輸送到常壓下ESI電離模式的質譜中進行分析檢測。基本工作原理如圖2：

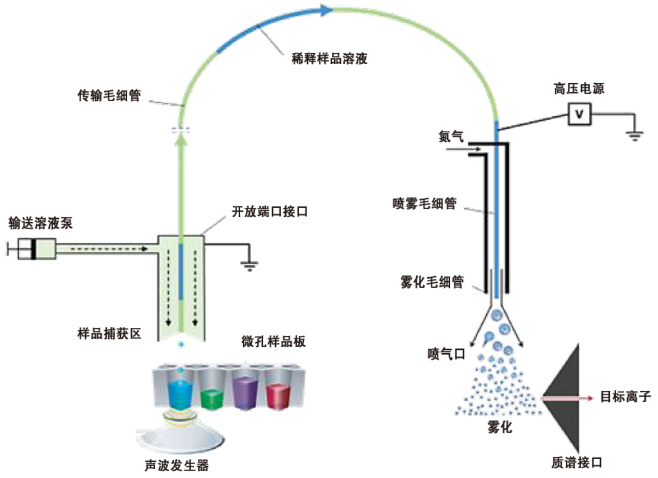

圖**2.** Echo™ MS系統基本工作原理示意圖

圖**1.** Echo™ MS 系統

污水驗毒背景

污水毒品檢測技術是通過提取生活污水中存在的毒品及其代謝物的含量，通過高靈敏的液質聯用檢測技術，對特定區域的生活污水進行抽樣檢測，結合污水水質參數和污水排放人口數量，推算出該區域吸毒人群規模、毒品消耗量等。在禁毒工作中，生活污水毒品檢測技術對開展制毒窩點緝查、毒品犯罪打擊和新精神活性物質預警等工作有著重大的參考價值和指導意義，污水驗毒堪稱禁毒的又一大“黑科技”。污水驗毒與傳統毒情調查方法完全不同，該方法具有更加客觀、準確、快速等優點。

污水驗毒行業難點

1. 傳統的液質聯用通常會採用梯度洗脫，通常一個樣品的分析時間可能超過10 min，耗時較長。污水驗毒實際檢測的樣品量很大，對於成百上千份實際樣品的檢測，連續進樣也需要數天到數周的資料獲取時間，該方法的實用性目前存在較大問題。
2. 傳統的液質聯用方法需要消耗預柱、色譜柱（幾千針後需要更換）等常規液相色譜耗材，此外，對流動相配置重現性要求較高。
3. 通常毒品及其代謝物進入到生活污水後被稀釋上千甚至上萬倍，其在污水中的含量通常只有納克甚至皮克級別。行業內通常採用固相萃取法進行樣品前處理，該方法耗費時間較長，通常一个样品的前处理时间一般会超过2小时，且固相萃取柱耗材成本较高。

高通量定量的Echo™ MS 系統污水驗毒特點

1. Echo™ MS 系統通過優化流速，以每秒鐘1個樣品的檢測速度進行快速取樣分析檢測，每個樣品的分析速度比傳統液質聯用方法的分析速度（每個樣品10 min）快幾百倍以上，對於成百上千份實際樣品的檢測，僅需要幾小時即可完成，Echo™ MS 系統具有遠超傳統液質聯用檢測的分析速度。
2. Echo™ MS 系統配備標準的384孔或1536孔進樣板，適合快速大批量樣品檢測。
3. Echo™ MS 系統無需使用色譜柱、預柱等常規液相色譜耗材。
4. 該方法採用磁珠吸附法對污水樣品進行前處理，耗時相對較短，且操作簡單。

實驗方法

Echo™ MS 條件：

耦合流體：純水

流動相：甲醇+0.1%甲酸

流速：360 µL/min

進樣模式：SP模式（即樣品粘度小於水）

進樣體積：2.5 nL

質譜條件：

離子源參數：

Curtain gas (psi)：20

CAD gas：9

Ionspray voltage (V)：5500

Temperature(℃)：300

Ion source gas1 (psi)：90

Ion source gas2 (psi)：45

表**1.** MRM離子對資訊表（共17種毒品，包含17種氘代內標）

編號	毒品名稱	Q1	Q3	DP	CE
1	苯丙胺	136.1	119.1	20	13
		136.1	91.1	20	23
	苯丙胺-D5	141.1	124.1	20	13
2	甲基苯丙胺	150.1	119.1	25	16
		150.1	91.1	25	27
	甲基苯丙胺-D5	155.2	121.1	25	16
3	O6-單乙醯嗎啡	328.2	211.1	120	34
		328.2	165.1	120	50
	O6-单乙酰吗啡-D3	331.2	211.1	120	34
4	吗啡	286.1	201.1	110	36
		286.1	165.1	110	57
	吗啡-D3	289.2	201.1	110	36
5	氯胺酮	238.1	207.1	35	19
		238.1	125	35	35
	氯胺酮-D4	242.1	211.1	35	19
6	去甲氯胺酮	224.1	207.1	30	18
		224.1	125	30	35
	去甲氯胺酮-D4	228.1	211.1	30	18
7	可卡因	304.2	182.1	80	27
		304.2	150.1	80	32
	可卡因-D3	307.2	185.1	80	27
8	苯甲醯愛康寧	290.1	168.1	70	26
		290.1	105	70	36
	苯甲酰爱康宁-D3	293.1	171.1	70	26
9	3,4-亚甲二氧基苯丙胺	180.1	133.1	15	25
		180.1	105.1	15	30
	3,4-亚甲二氧基苯丙胺-D4	184	167	15	16
10	3,4-亚甲二氧基甲基苯丙胺	194.1	163.1	30	16
		194.1	105.1	30	32
	3,4-亚甲二氧基甲基苯丙胺-D4	198.1	167.1	30	16
11	卡西酮	150.4	117.2	30	31
		150.4	132.2	30	17
	卡西酮-D5	155.3	122	40	31
12	甲卡西酮	164.1	105.1	70	31
		164.1	131.1	70	26
	甲卡西酮-D5	169.1	136.1	70	31
13	枸橼酸芬太尼	337.2	188.3	90	31
		337.2	105.2	90	45
	芬太尼-D5	342.2	105	90	45
14	地西泮	285.1	193	125	44
		285.1	154	125	35
	地西泮-D5	290	198	125	44
15	艾司唑仑	295.2	267.3	130	32
		295.1	205.2	130	54
	艾司唑仑-D5	300	272	130	34
16	美沙酮	310.2	265.2	40	21
		310.2	105.1	40	34
	美沙酮盐酸盐-D10	320	275	40	21
17	5F-MDMB-PICA	377	232	110	20
		377	144	110	54
	5F-MDMB-PICA-D4	381	236	110	20

污水樣品前處理過程

取50 mL污水樣品，磁性顆粒分散吸附20-30 min，磁性顆粒和污水分離後，加入3 mL有機溶劑洗脫磁性顆粒15 min後，移除磁珠，氮氣吹幹後，200 µL甲醇水（甲醇: 水 = 2 : 8，v/v）溶液複溶，濾膜過濾後，進樣分析。

實驗結果

標準曲線及定量下限考察

表**2.** 17種毒品標準曲線的線性範圍及定量下限表（以污水中含量計）

編號	毒品名稱	線性範圍（ng/mL）	定量限（ng/mL）
1	苯丙胺	0.008-2	0.008
2	甲基苯丙胺	0.004-1	0.004
3	O6-單乙醯嗎啡	0.008-2	0.008
4	嗎啡	0.008-2	0.008
5	氯胺酮	0.004-2	0.004
6	去甲氯胺酮	0.008-2	0.008
7	可卡因	0.004-2	0.004
8	苯甲醯愛康寧	0.004-2	0.004
9	MDA	0.008-2	0.008
10	MDMA	0.008-2	0.008
11	凱西酮	0.004-2	0.004
12	甲凱西酮	0.004-2	0.004
13	枸櫞酸芬太尼	0.004-2	0.004
14	地西泮	0.004-2	0.004
15	艾司唑侖	0.004-2	0.004
16	美沙酮	0.004-2	0.004
17	5F-MDMB-PICA	0.004-2	0.004

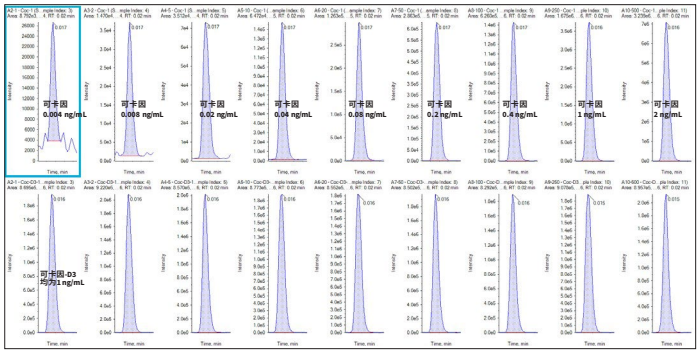

圖**3.** 標準曲線色譜圖舉例（可卡因及可卡因-D3）

基質樣品重現性考察

實驗考察了污水空白樣品添加17種毒品（相當於污水中添加濃度1 ng/mL）後經過前處理樣品，3平行樣本考察（即，sample-5、sample-6、sample-7），每個樣品分別連續進樣6針，資料表明：17種毒品峰面積的RSD值均小於5%，詳細資料清單如下表3。

表**3.** 17種毒品重現性考察資料統計表

編號	化合物名稱	化合物峰面積重複性RSD %（6針進樣）		
		sample-5	sample-6	sample-7
1	苯丙胺	3.4	2.3	2.5
2	甲基苯丙胺	3.4	3.8	4.5
3	O6-单乙酰吗啡	2.7	3.4	3.4
4	吗啡	1.6	4.9	4.7
5	氯胺酮	2.6	2.7	3.7
6	去甲氯胺酮	4.0	0.8	4.8
7	可卡因	4.8	4.8	3.6
8	苯甲酰爱康宁	1.4	2.5	2.7
9	MDA	2.3	3.4	4.1
10	MDMA	4.4	3.4	3.6
11	卡西酮	1.9	4.3	3.7
12	甲卡西酮	2.5	4.1	2.9
13	枸橼酸芬太尼	4.2	4.6	4.9
14	地西泮	3.3	4.4	4.5
15	艾司唑仑	2.1	3.3	1.9
16	美沙酮	5.0	4.9	2.3
17	5F-MDMB-PICA	1.1	3.7	2.0

基質效應和提取回收率考察

實驗考察了污水空白樣品經過前處理後添加17種毒品（相當於污水中添加濃度1 ng/mL），雙平行樣本考察（即，sample-3、sample-4），每個樣品在標準曲線下計算其濃度後除以250（理論添加濃度），計算可得該方法的基質效應（以 % 計），資料表明，該方法基質效應均大於70 %，即基質干擾小於30 %，詳細資料清單如下表4。

此外，實驗考察了污水空白樣品添加17種毒品（相當於污水中添加濃度1 ng/mL）後經過前處理樣品，3平行樣本考察（即，sample-5、sample-6、sample-7），每個樣品在標準曲線下計算其濃度後除以250（理論添加濃度），計算可得該方法的提取回收率（以 % 計），資料表明，該方法提取回收率均在80 %以上（除去甲氯胺酮和凱西酮外），詳細資料清單如下表4。

表**4.** 17種毒品基質效應和提取回收率考察資料統計表

編號	化合物名稱	基質效應 %		回收率 %		
		sample3	sample4	sample-5	sample-6	sample-7
1	苯丙胺	81	95	88	100	96
2	甲基苯丙胺	90	100	93	91	91
3	O6-单乙酰吗啡	97	102	105	95	97
4	吗啡	96	91	92	101	97
5	氯胺酮	89	92	101	93	96
6	去甲氯胺酮	67	70	68	70	67
7	可卡因	93	98	94	93	92
8	苯甲酰爱康宁	84	98	94	92	99
9	MDA	89	94	97	96	97
10	MDMA	93	89	82	95	88
11	卡西酮	71	30	46	43	44
12	甲卡西酮	111	102	95	98	93
13	枸橼酸芬太尼	86	82	93	88	90
14	地西泮	97	104	97	94	95
15	艾司唑仑	87	90	95	88	89
16	美沙酮	92	82	96	87	92
17	5F-MDMB-PICA	86	80	99	93	96

總結

1. 本文通過Echo™ MS 系統（即聲波激發耦合質譜儀，AEMS）開發了高通量分析污水中的17種毒品檢測方法，該方法前處理採用磁珠吸附法，前處理操作簡單，耗時更短，適合大批量樣品的檢測。

2. Echo™ MS 系統配備的ADE技術，用聲波能量將樣品從樣品板中極小的樣品量（2.5 nL）激發出來，樣品小液滴通過OPI技術將樣品輸送到質譜系統中進行分析檢測，配備OptiFlow™ Turbo V離子源穩定高效的電噴霧電離技術的SCIEX Triple Quad™ 6500+ 質譜系統可以實現了廣泛的化合物覆蓋度，整個方案無需使用色譜柱、預柱等常規液相色譜耗材。

3. Echo™ MS 系統擁有超快的進樣速度，以每秒鐘1個樣品的檢測速度進行快速取樣分析檢測，配備標準384孔或1536孔進樣板，適合快速大批量樣品檢測。目前該實驗共檢測了127個樣品，以1個毒品的檢測為例，127個樣品可以在6.3 min完成全部檢測，與傳統污水驗毒液質聯用檢測方法相比（11 min每個樣品），127個樣品共需要耗時至少1397 min（約23.3 h），Echo™ MS 系統的樣品分析速度比傳統的液質聯用方法至少提升200倍以上。

4. 配備SCIEX Triple Quad™ 6500+質譜系統的Echo™ MS 系統同樣擁有強大的定量能力，資料顯示17種毒品的定量限可以達到0.004-0.008 ng/mL（以污水中含量計）；其重現性考察實驗中Echo™ MS 系統可以做到基質樣品RSD < 5%；此外，該方法的基質效應考察資料顯示17種毒品均可以做到70 %以上，即基質干擾小於30 %，表明該方法抗基質干擾能力強；提取回收率考察實驗中17種毒品均可達到80 %以上（除去甲氯胺酮和凱西酮外）。各項方法學資料顯示，通過Echo™ MS 系統開發的污水中的毒品檢測的方法適用性良好。

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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; [3]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.

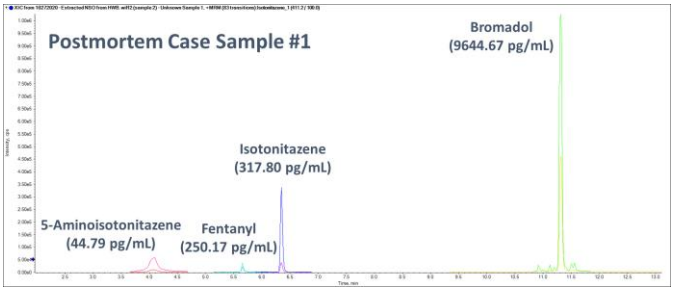


Figure 1. Trace level quantification of potent NSO in postmortem case sample #1. Extracted ion chromatogram (XIC) showing the successful detection of two potent NSO, a metabolite, and fentanyl at low concentrations in a case sample from a subject suspected of accidental overdose from combined drug toxicity.

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.

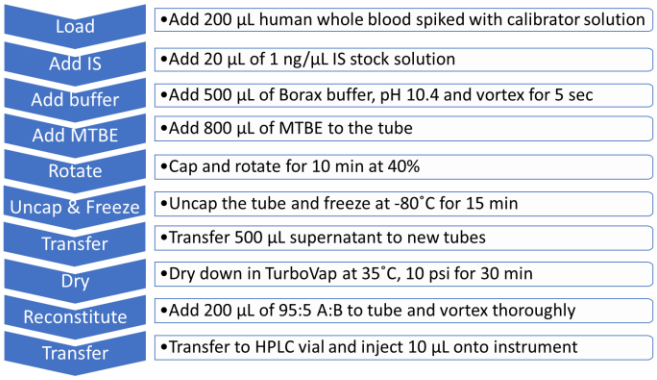


Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used for selectively extracting NSO from human whole blood samples for analysis with the SCIEX 7500 System.

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.

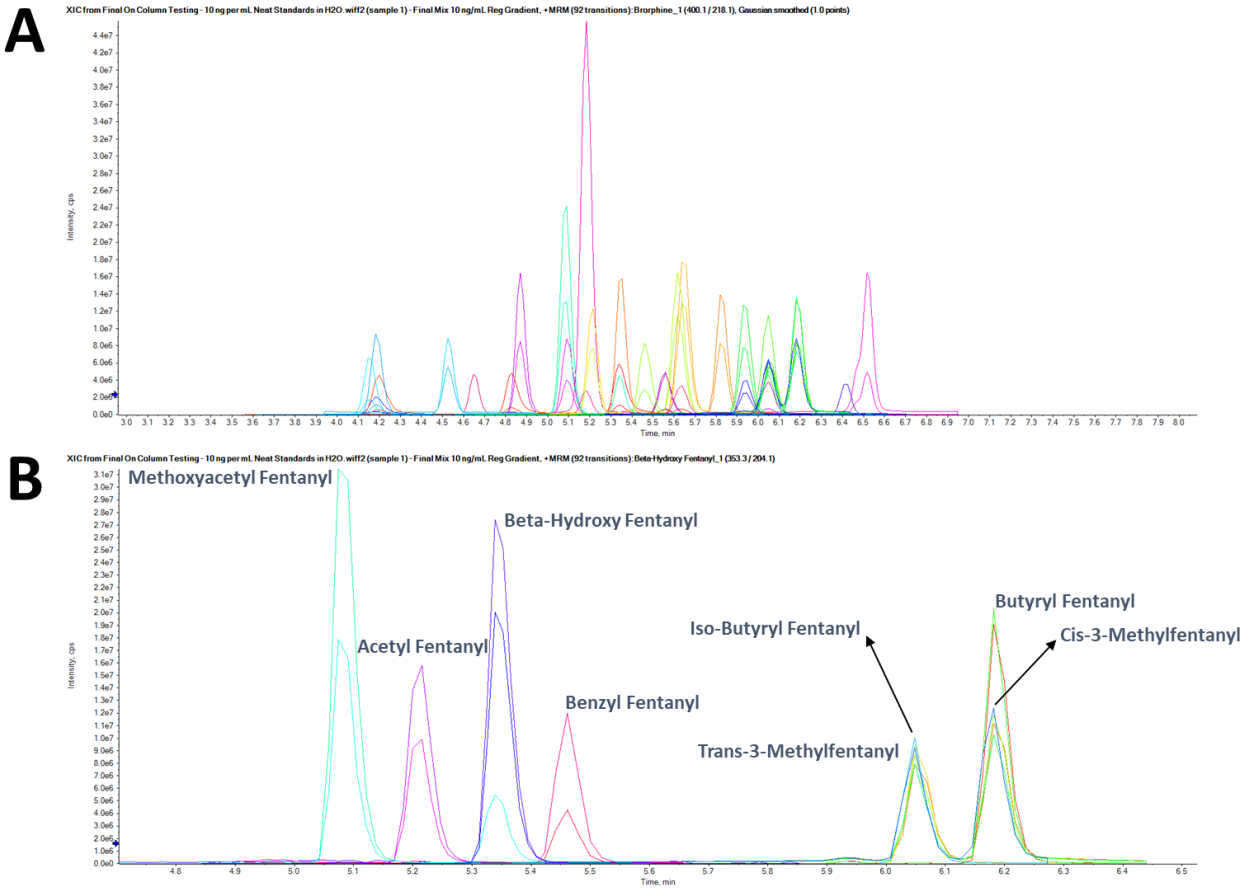


Figure 3. Chromatographic profile of the 32 NSO targeted in this study. A) Extracted ion chromatogram (XIC) resulting from the optimized LC conditions and data acquisition method using a 10 ng/mL neat standard mixture containing the 32 NSO included in the panel. B) Representative extracted ion chromatogram (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:

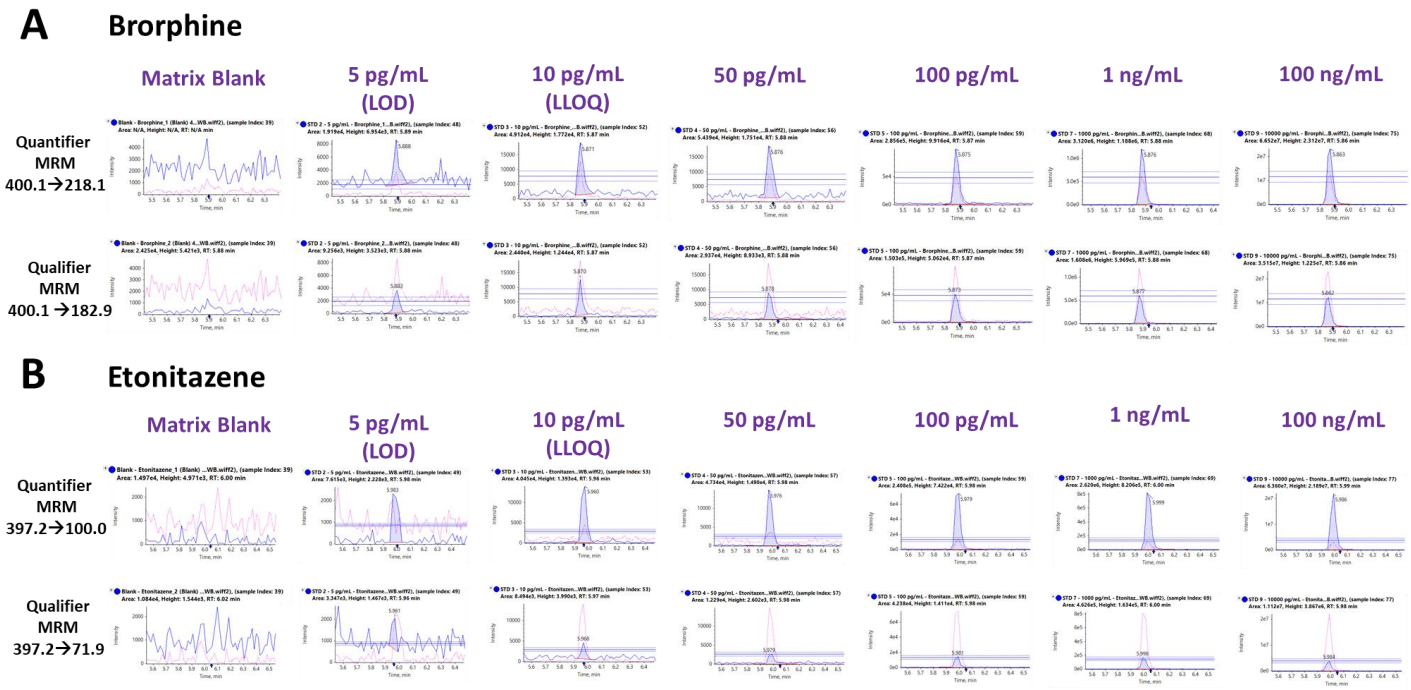


Figure 4 Accurate quantification of two potent NSO extracted from blood samples using the SCIEX 7500 System. Extracted ion chromatograms (XICs) for A) brorphine and B) etonitazene, two potent NSO recently found in accidental overdose case samples. The series of XICs display overlays of both the quantifier 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.

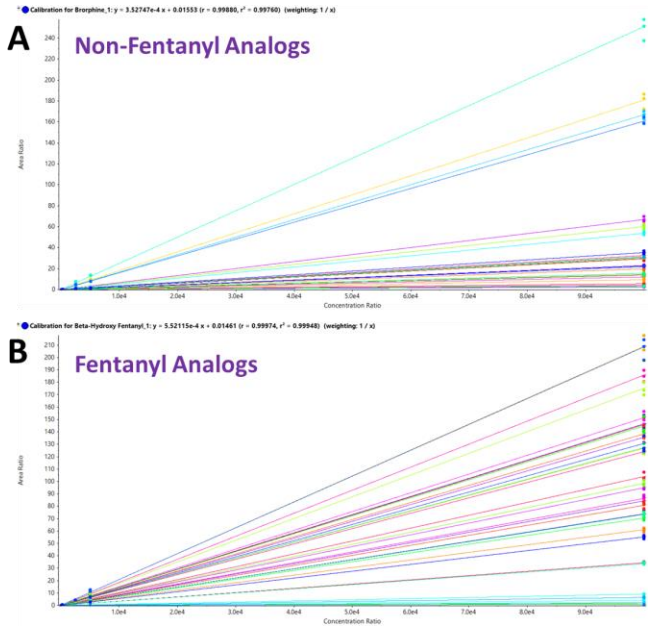


Figure 5. Excellent linearity for the 32 NSO. Linear regression curves resulting from the calibration series from 10 to 100000 pg/mL for A) non-fentanyl analogs and B) fentanyl analogs extracted from human whole blood samples. R^2 values greater than 0.99 were observed for all the NSO in the panel.

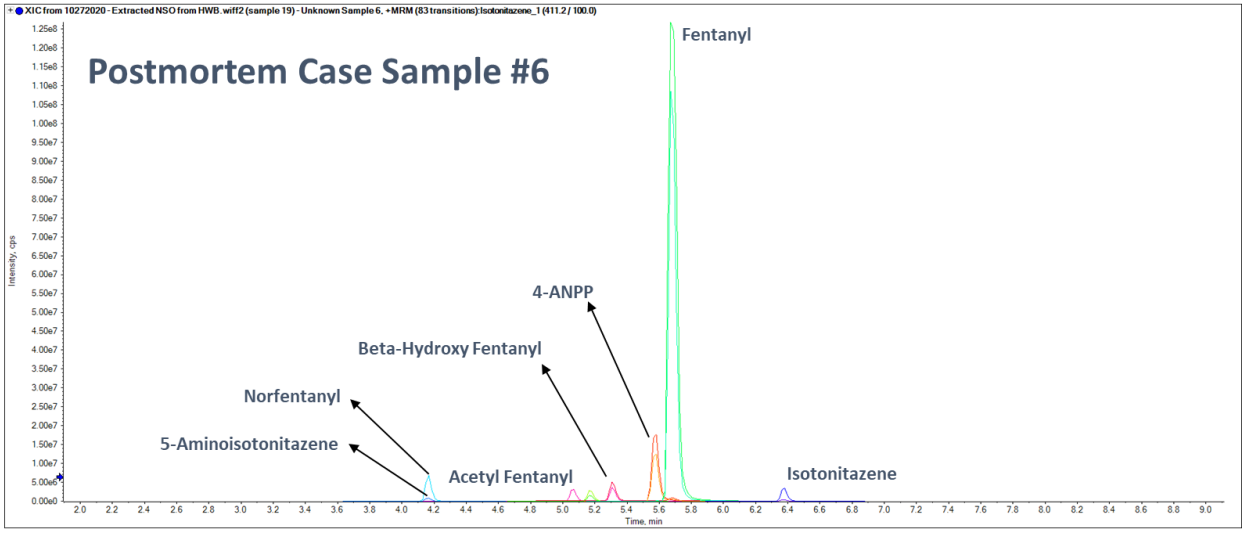
Enhanced sensitivity leads to low level detection of potent NSO in case samples

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

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

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

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

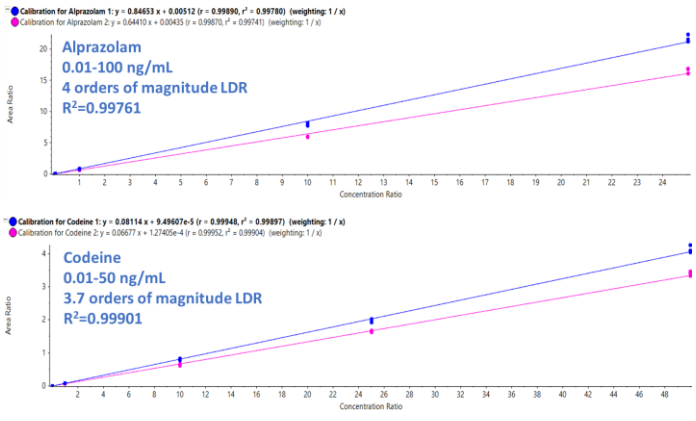


Figure 1. High linearity and linear dynamic range (LDR) demonstrated for chemically diverse panel of drugs. Calibration curves resulting from the calibration series of alprazolam (0.01-100 ng/mL) and codeine (0.01-50 ng/mL) showing excellent linear response even at pg/mL concentrations.

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

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

Experimental details

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

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

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

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

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

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

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

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

Method development and optimization

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

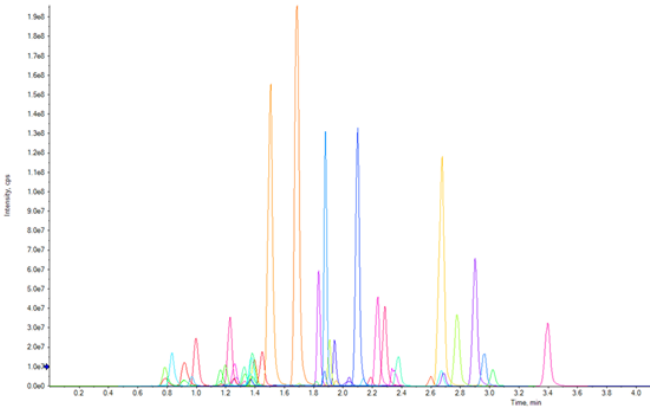


Figure 3. Chromatographic profile of the 49 drugs targeted in this study. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition using a neat standard mixture. Method optimization using the Scheduled MRM™ Algorithm Pro in SCIEX OS Software enabled collection of optimal data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

Optimized detection method enables accurate and reliable drug quantification

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

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

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

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

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

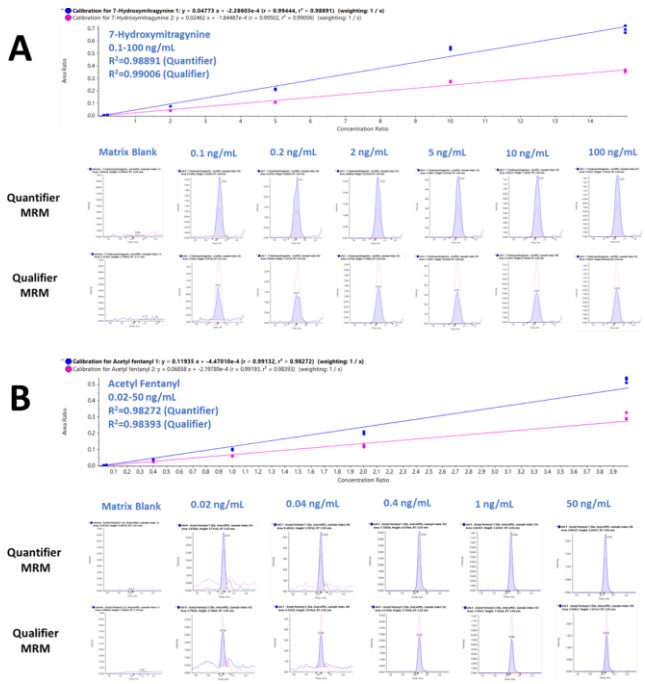


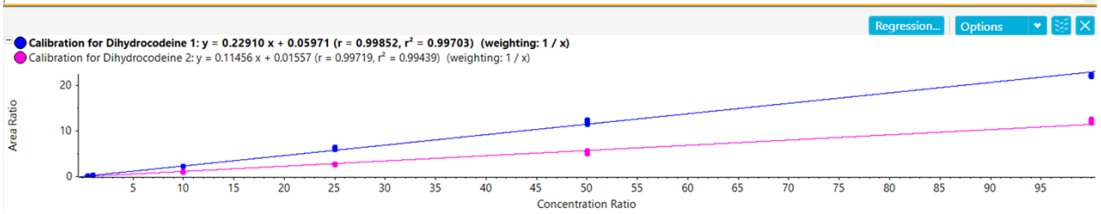
Figure 4: High sensitivity and linearity for selected drugs in the forensic panel. A) Calibration curves and XIC traces resulting from the calibration series for the two transitions of 7-hydroxymitragynine from 0.1 to 100 ng/mL. B) Calibration curves and XIC traces resulting from the calibration series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

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

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

A

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



B

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

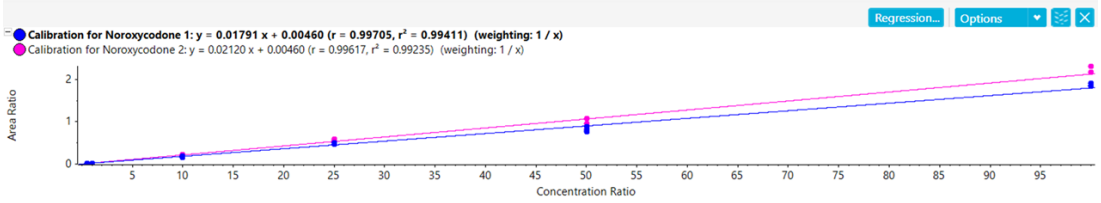


Figure 5. Statistical results and calibration curves for selected drugs in the forensic panel. Statistics pane and calibration curves for dihydrocodeine (A) and noroxycodone (B) from 0.5 to 100 ng/mL. Both analytes showed excellent linearity, reproducibility, accuracy and precision across the six calibration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

Conclusions

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

References

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

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

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

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

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

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High sensitivity drug analysis using dried blood spots

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

Pierre Negri[1], Marta Massano[2], Enrico Gerace[3], and Alberto Salomone[2,3]
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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.

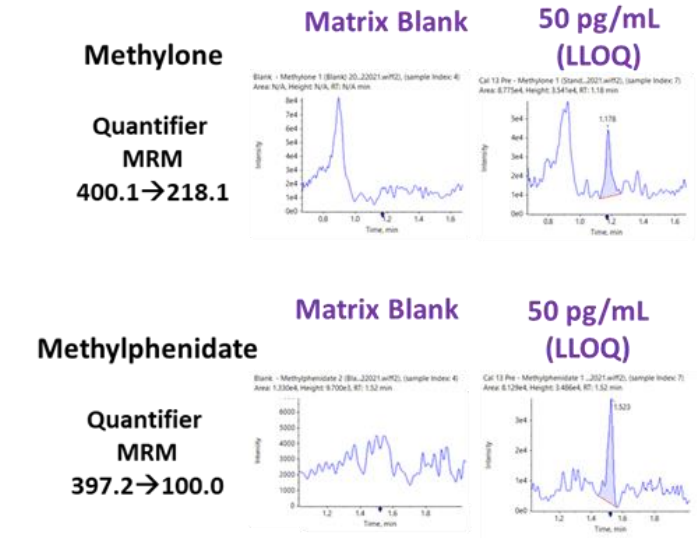

Figure 1. High sensitivity detection of drugs and metabolites extracted from DBS. Extracted ion chromatogram (XIC) traces for methylone (top) and methylphenidate (bottom) showing the quantifier ion traces for the matrix blank (left) and at the LLOQ at 50 pg/mL (right).

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.

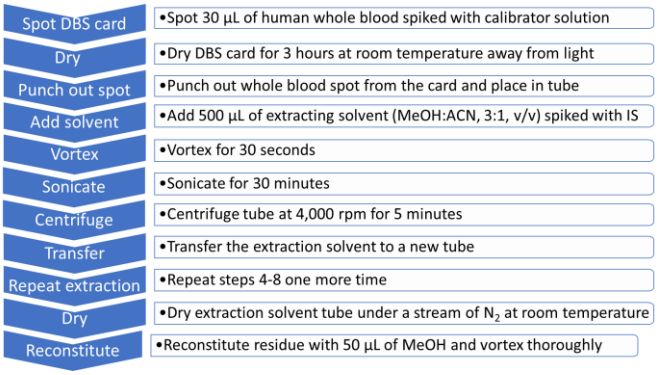

Figure 2. Analyte extraction workflow from DBS cards. An 11-step sample extraction protocol was optimized to selectively extract the 24 analytes from DBS cards for analysis using the SCIEX 7500 system.

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.

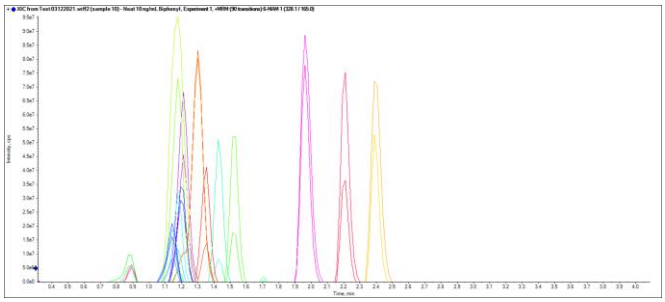

Figure 3. Chromatographic profile of the 24 analytes included in the panel. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition method, obtained from the 10 ng/mL neat standard mixture containing the 24 targeted analytes. Method was built using the Scheduled MRM algorithm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

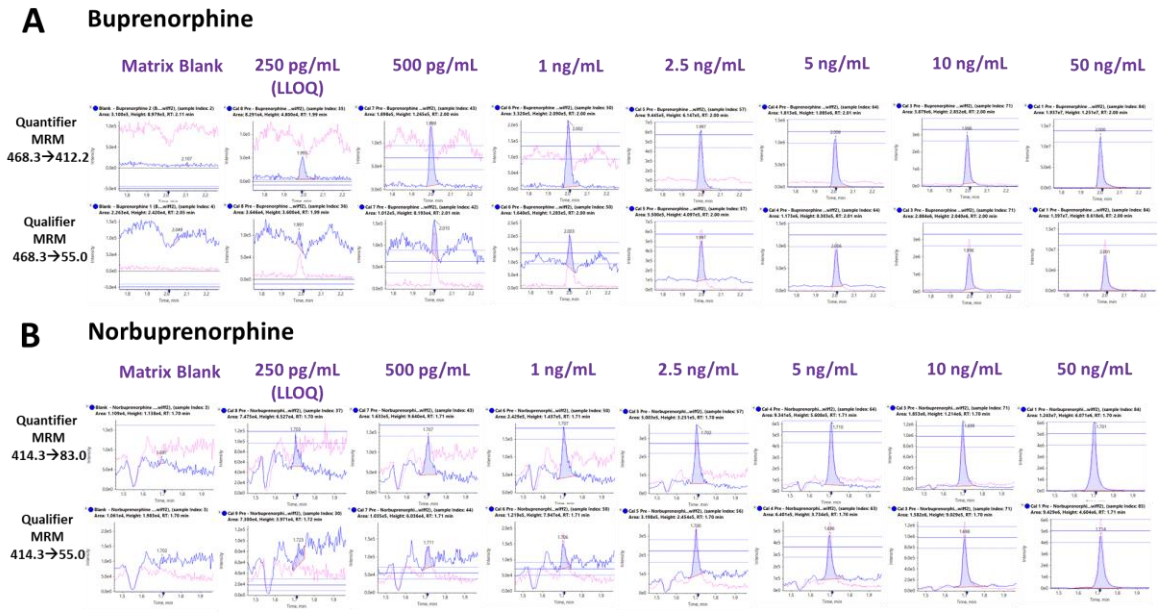

Figure 4. Representative extracted ion chromatograms (XICs) for selected drugs in the forensic panel. XICs for buprenorphine (A) and norbuprenorphine (B) from 0.25 to 50 ng/mL, including the blank injection. Both the quantifier and qualifier traces are shown. Ion ratios were also monitored across the 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).

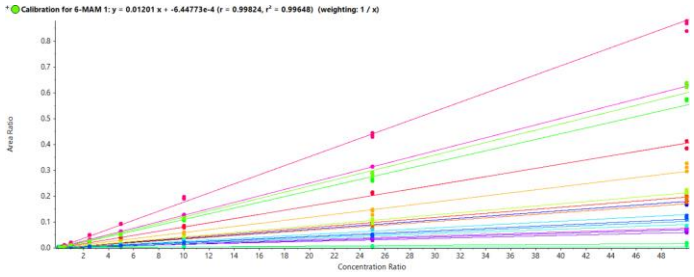

Figure 5. Excellent linearity for the 24 analytes extracted from DBS. Calibration curves generated using the two MRM transitions monitored for each of the 24 analytes targeted in this study. The assay showed excellent linearity with R^2 values greater than 0.99 for all the analytes.

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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Enhancing NPS characterization using electron-activated dissociation (EAD)

Using the ZenoTOF 7600 system 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

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

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

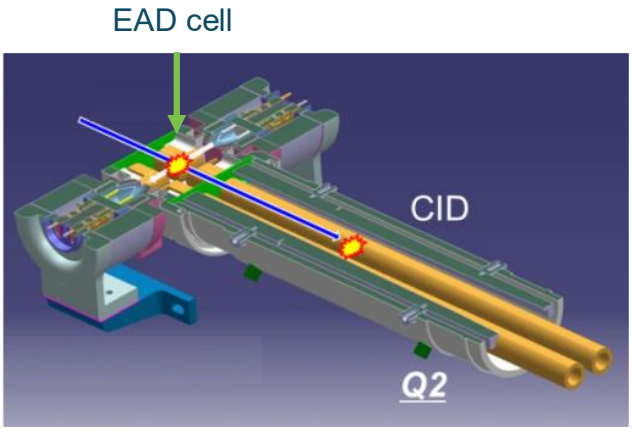


Figure 1. Schematic of the EAD cell on the ZenoTOF 7600 system. EAD provides reproducible and unique fragment ions that enhance the characterization of NPS.

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

Advantages of EAD on the ZenoTOF 7600 system for NPS characterization

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

Experimental details

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

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

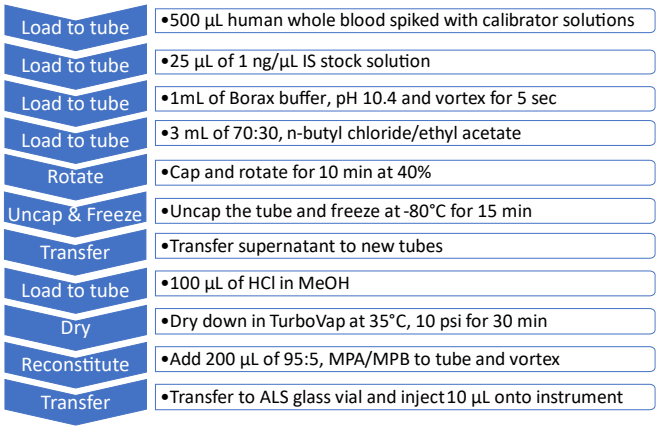


Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used to selectively extract drugs from human whole blood samples for analysis with the ZenoTOF 7600 system.

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

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

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

Optimized EAD conditions for reproducible and comprehensive fragment information

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

Zeno MS/MS for improved sensitivity

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

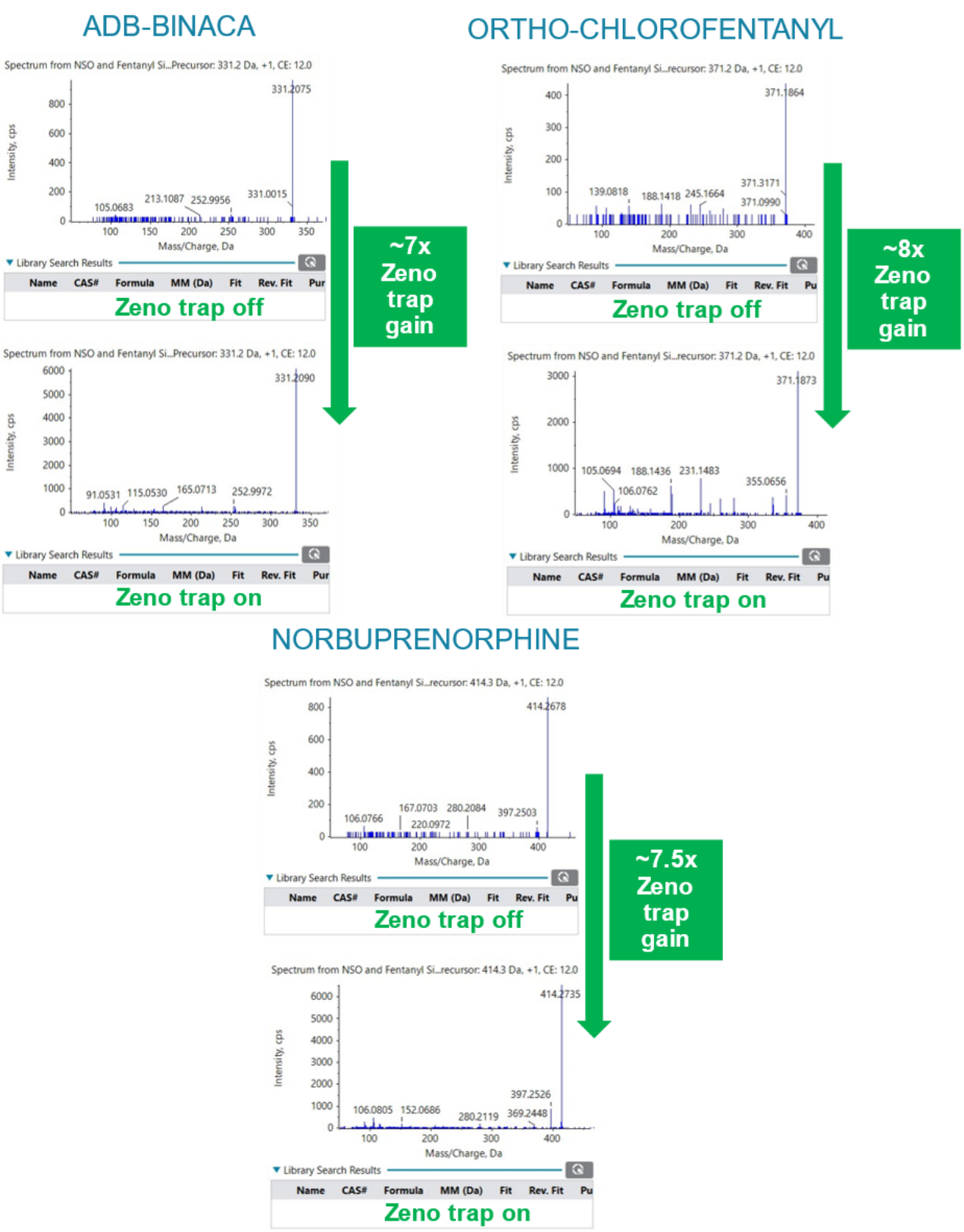


Figure 3. TOF MS/MS sensitivity gains when combining Zeno DDA with EAD for representative NPS. An average of ~8x gain in TOF MS/MS sensitivity was observed across all the analytes positively identified in the discarded postmortem case samples when the Zeno trap was used in combination with EAD.

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

Comprehensive characterization of synthetic opioids

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

Differentiation of AP-series NSO

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

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

In-depth characterization of synthetic cannabinoids

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

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

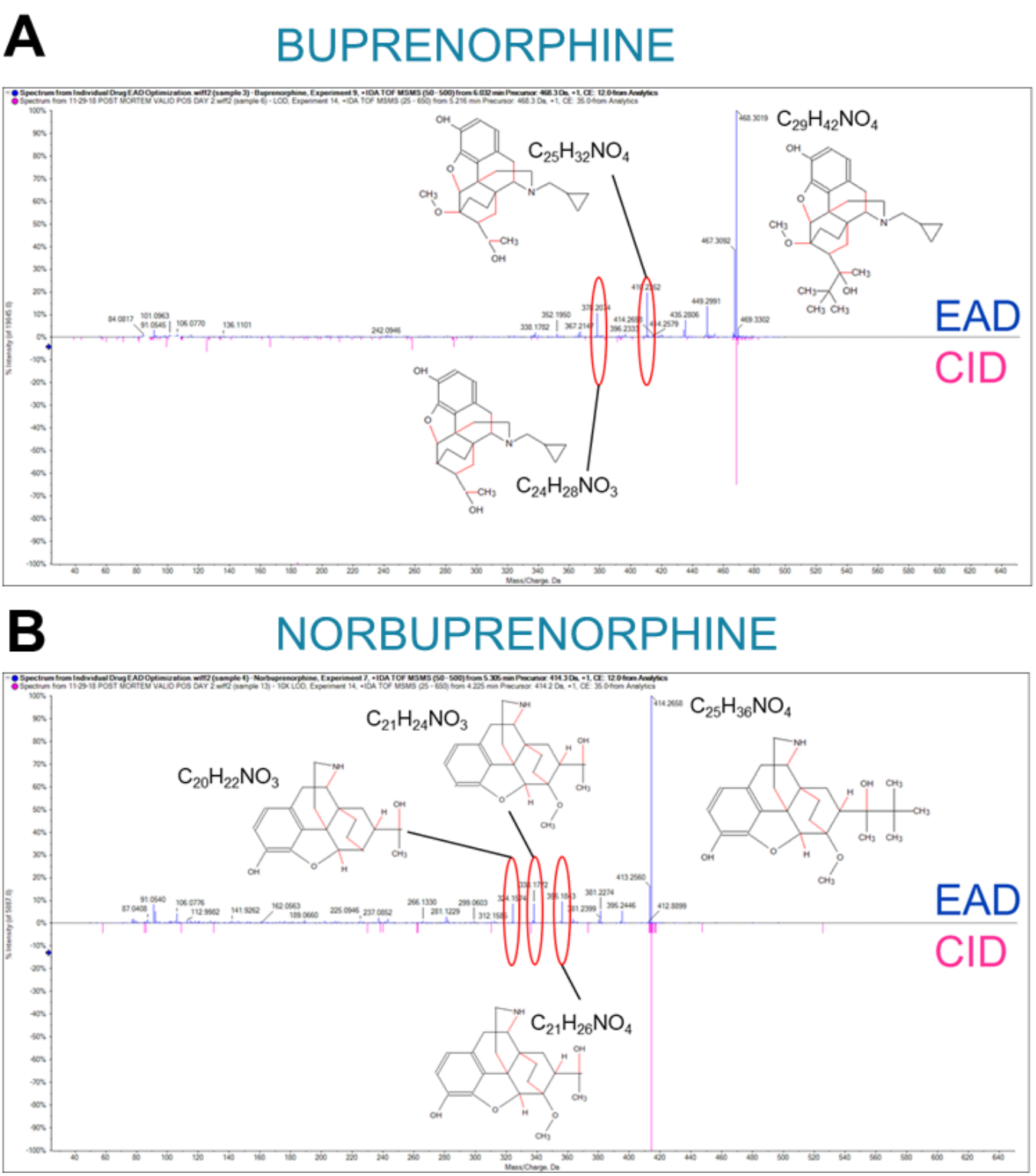


Figure 4. EAD enables in-depth characterization of challenging synthetic opioids. Zeno EAD MS/MS (top) and CID MS/MS (bottom) spectra for A) buprenorphine and B) its main active metabolite norbuprenorphine. The Zeno EAD MS/MS show unique diagnostic fragment ions that enable in-depth characterization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

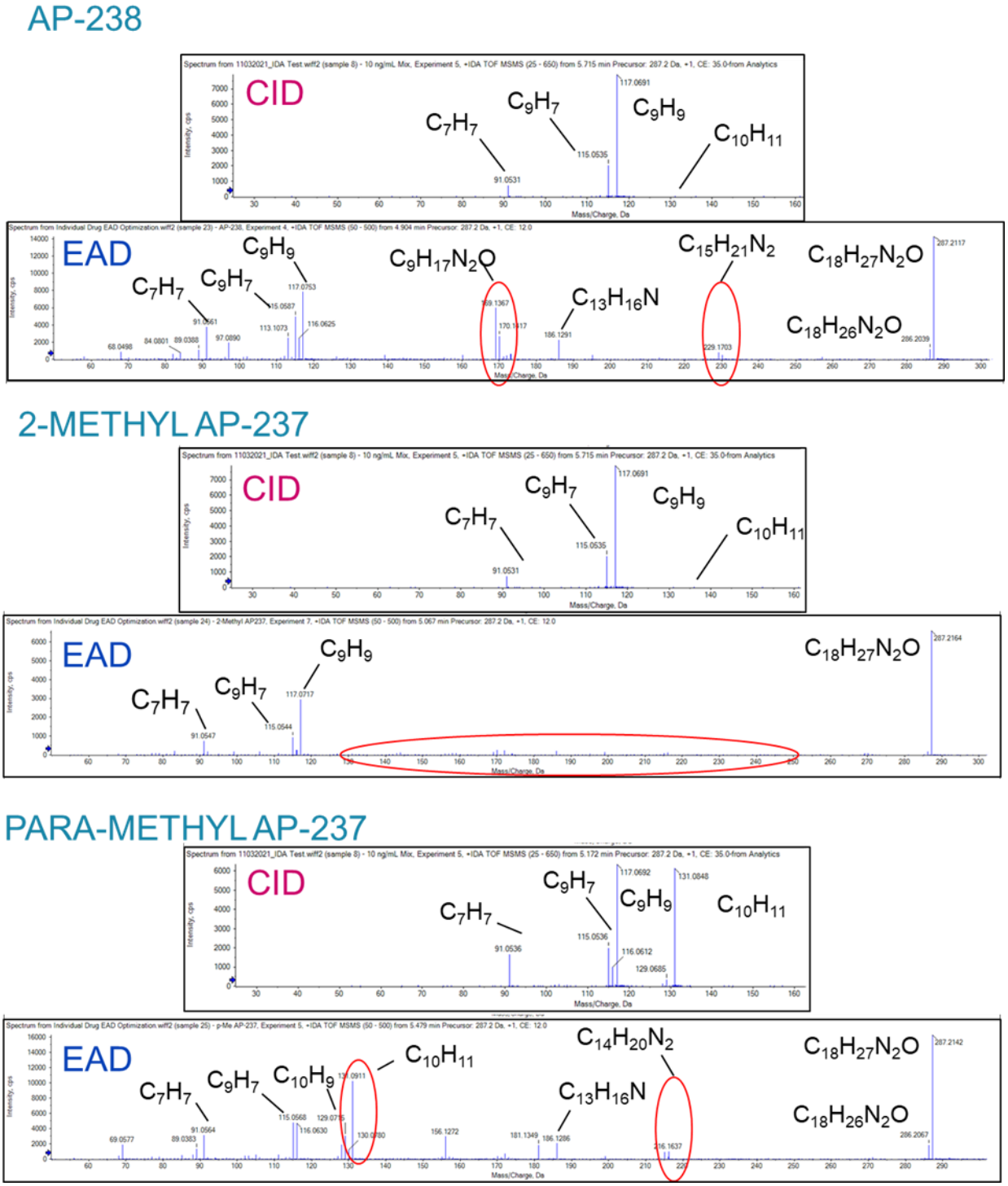
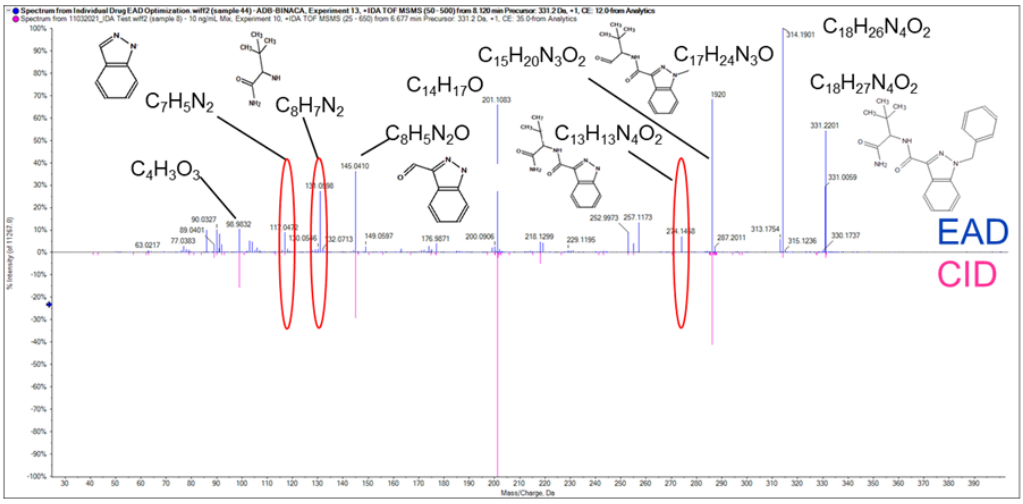
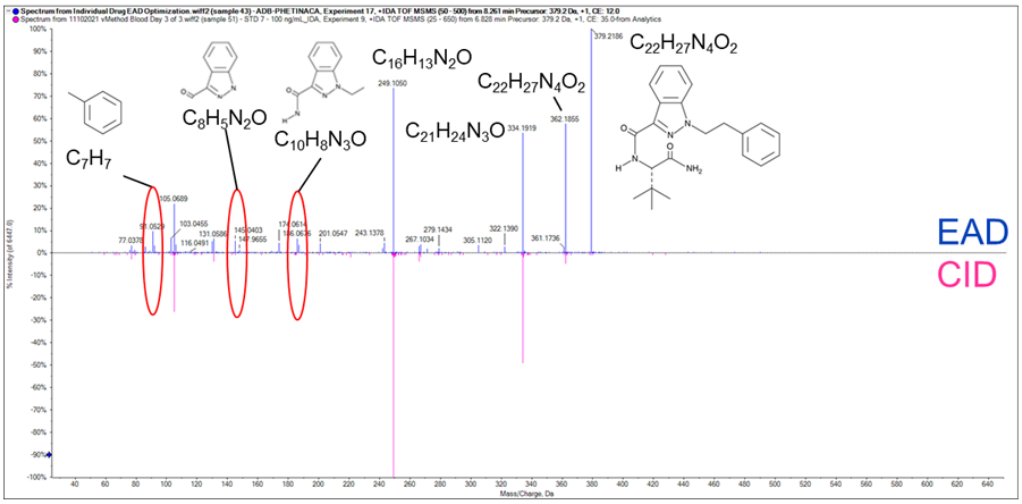


Figure 5. EAD provides rich MS/MS spectral features to enable the differentiation of the isobaric synthetic opioids from the AP series. CID and EAD MS/MS spectra for 3 synthetic opioids: AP-238 (top), 2-methyl AP-237 (middle) and para-methyl AP-237 (bottom). The CID MS/MS spectra are indistinguishable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

A. ADB-BINACA



B. ADB-PHETINACA



C. 4F-MDMB-BINACA

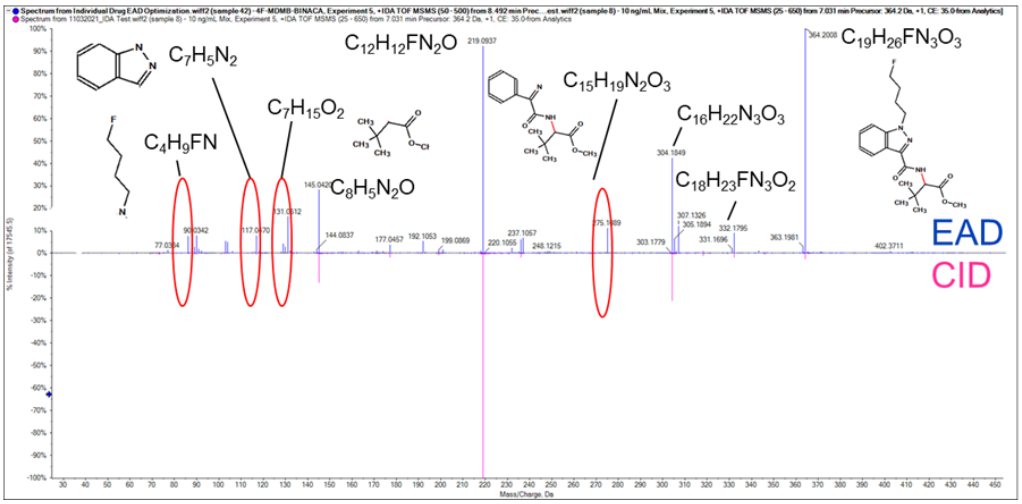


Figure 6. EAD enables in-depth characterization of challenging synthetic cannabinoids. Spectral comparisons between Zeno EAD MS/MS (top) and Zeno CID MS/MS (bottom) for 3 synthetic cannabinoids: A) ADB-BINACA, B) ADB-PHENITACA and C) 4F-MDMB-BINACA. EAD provides richer fragmentation in the form of unique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

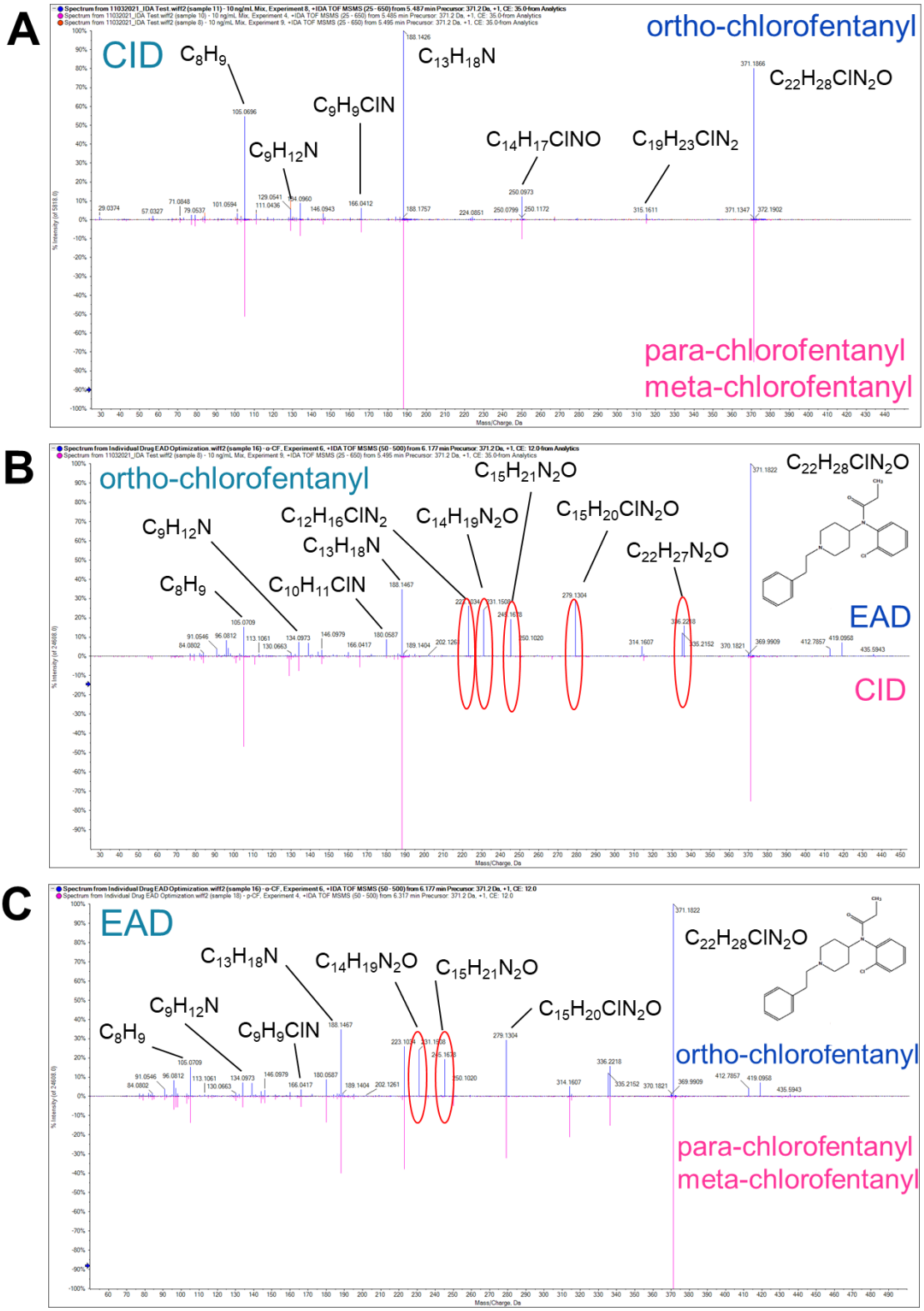


Figure 7. EAD generates unique fragment ions that enable differentiation of isobaric species. A) Zeno CID MS/MS spectra for isobaric NSO ortho-chlorofentanyl (top) and para-chlorofentanyl and meta-chlorofentanyl (bottom) showing no spectral differences, B) spectral comparison between Zeno EAD MS/MS (top)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

Differentiation of halogenated fentanyl analogs

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

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

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

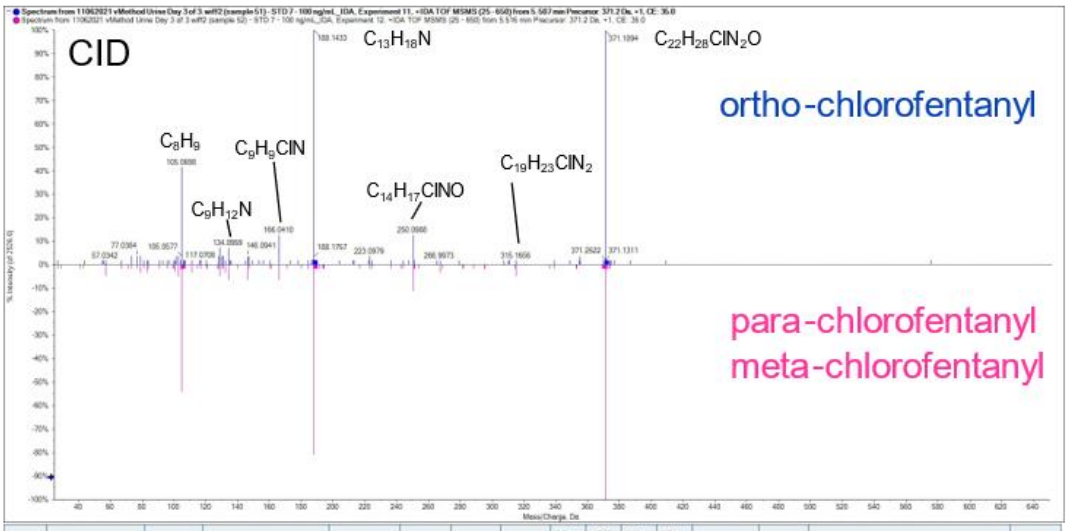
Conclusions

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

References

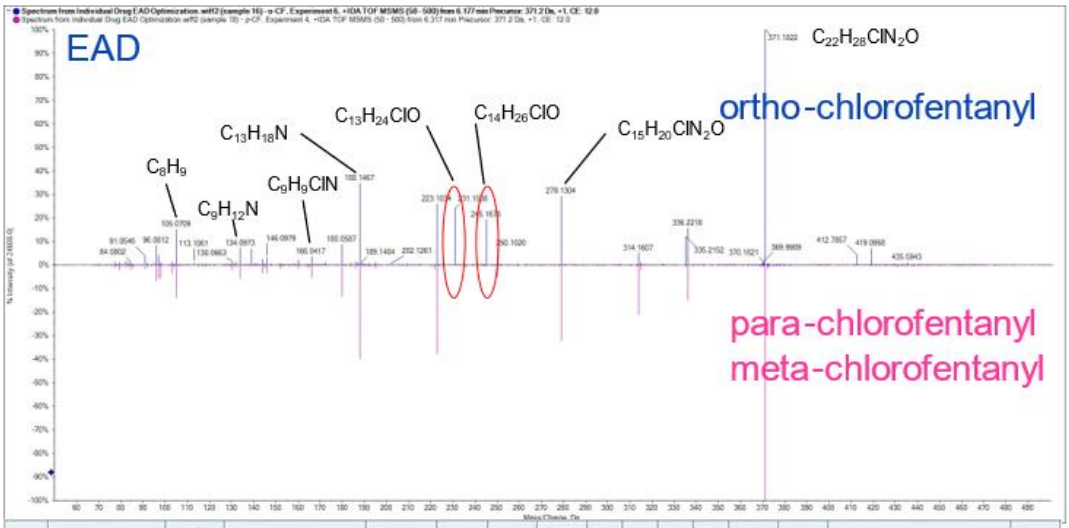
1. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.
2. vMethod Application – Single-Injection Screening of 664 Forensic Toxicology Compounds on a SCIEX X500R QTOF System.
3. Highly sensitive MS/MS detection for confident identification of potent novel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

A



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

B



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

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

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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

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 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.

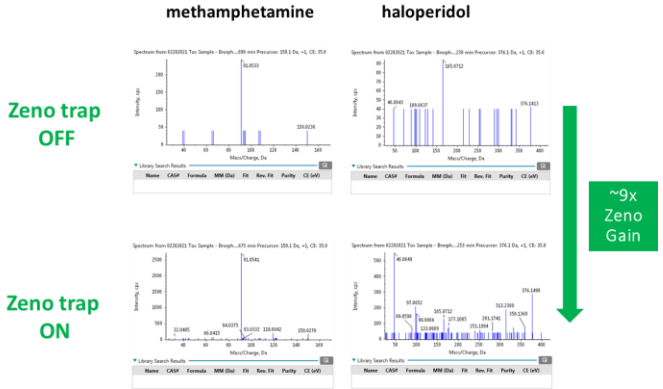


Figure 1: TOF MS/MS sensitivity gains using Zeno IDA for representative analytes. An average of ~9X gain in TOF MS/MS sensitivity was observed across all analytes identified in this study.

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.

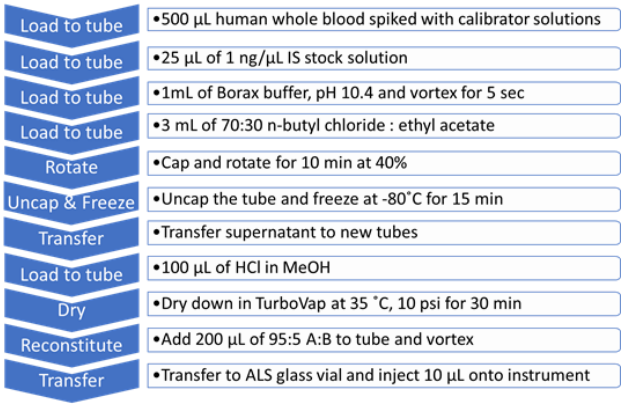


Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used for selectively extracting drugs from human whole blood samples for analysis with the ZenoTOF 7600 system.

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.

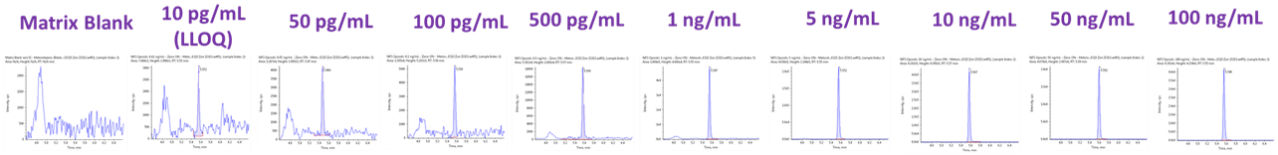
Optimized IDA method leads to accurate and reliable drug quantification

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.

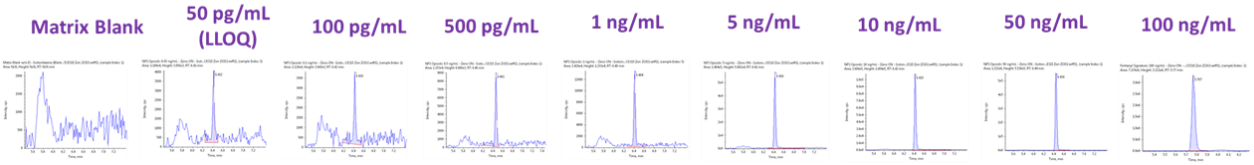
Figure 3 shows representative extracted ion chromatograms (XICs) for A) metonitazene and B) isotonitazene, two highly potent NSO that have been linked to accidental drug overdoses at low concentrations. The series of XIC displays shows the resulting signal for a blank injection (left) and for concentrations 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.

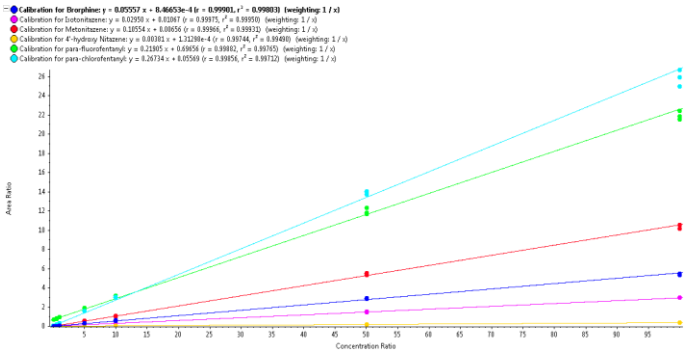


Figure 4. Excellent linearity for the 6 targeted NSO. Calibration curves resulting from the series of 9 calibrators extracted from human whole blood at concentrations ranging from 10 pg/mL to 100 ng/mL. R^2 values greater than 0.99 were observed for the 6 targeted analytes.

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.

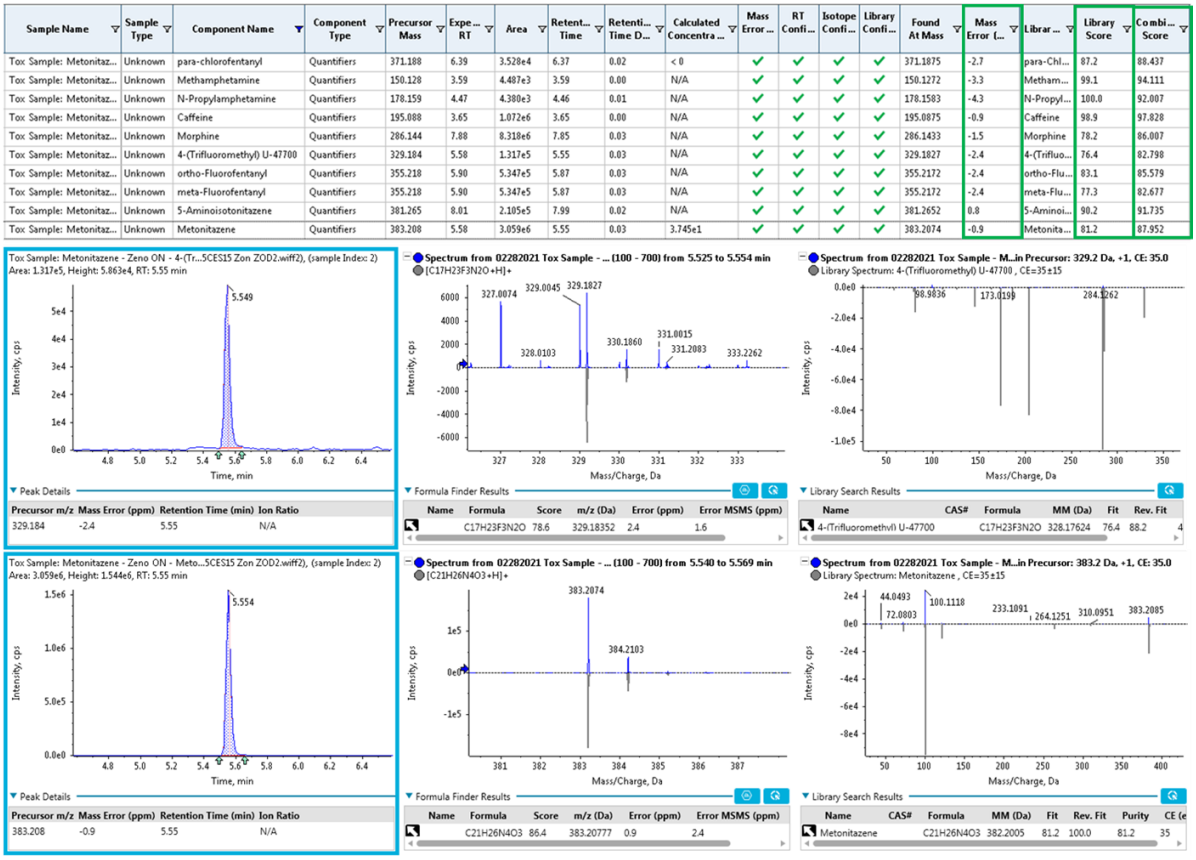
Zeno MS/MS increases confident identifications of low drug levels in authentic postmortem case samples

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.

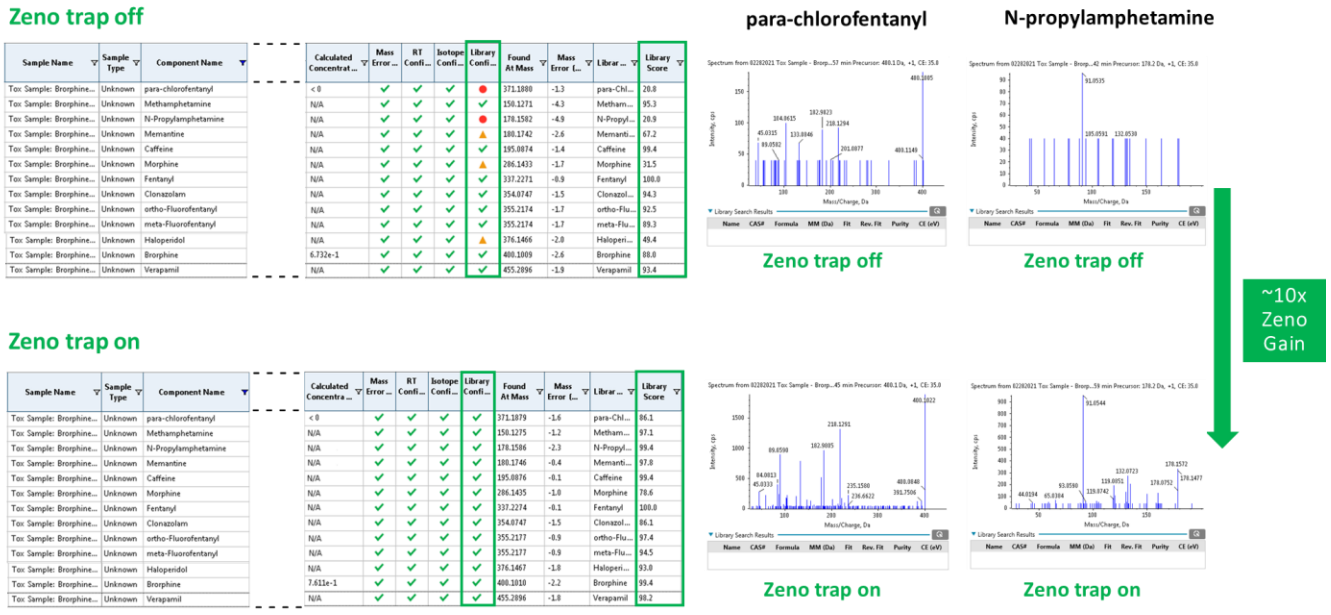
Discarded authentic postmortem case sample # 1



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%.

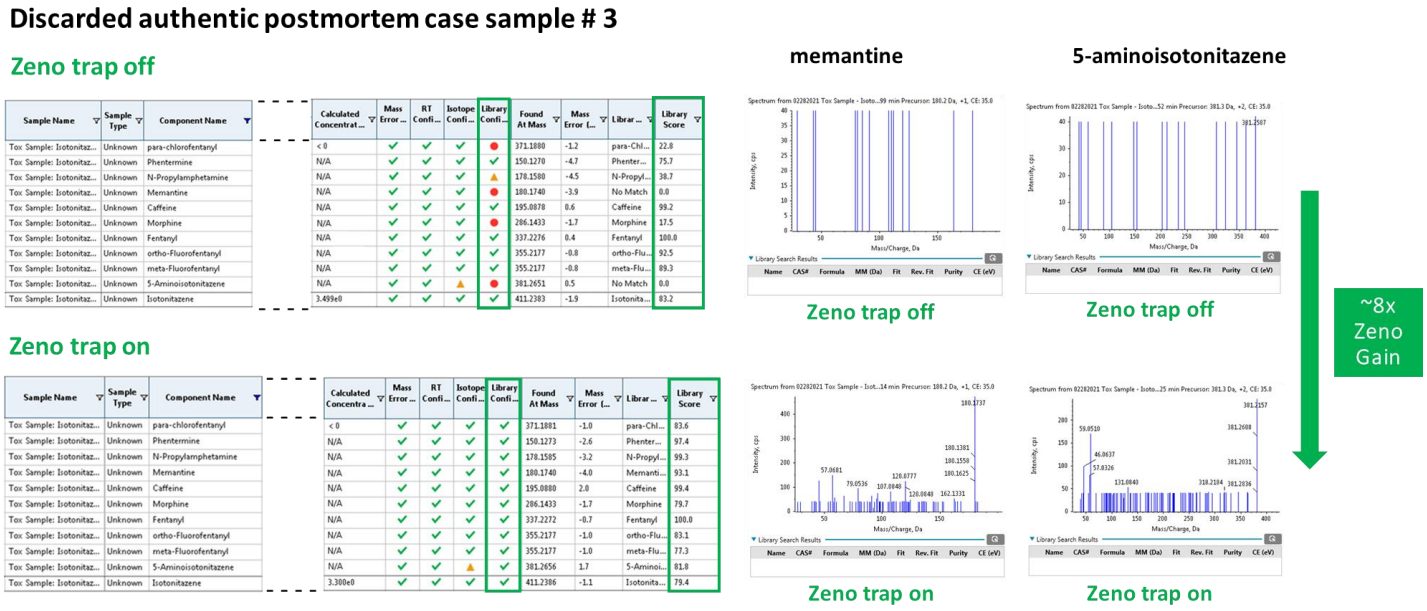


Figure 7. Results comparison between Zeno trap on and off for authentic postmortem case study #3. (Top) Results table and representative TOF MS/MS spectra with (top) and without (bottom) the Zeno trap enabled. The use of the Zeno trap enabled acquisition of a much richer MS/MS spectra that contained 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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Expanding NPS screening capabilities in the forensic toxicology laboratory

Screen for over 900 compounds using the vMethod application on the SCIEX X500R QTOF system

Pierre Negri
SCIEX, USA

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

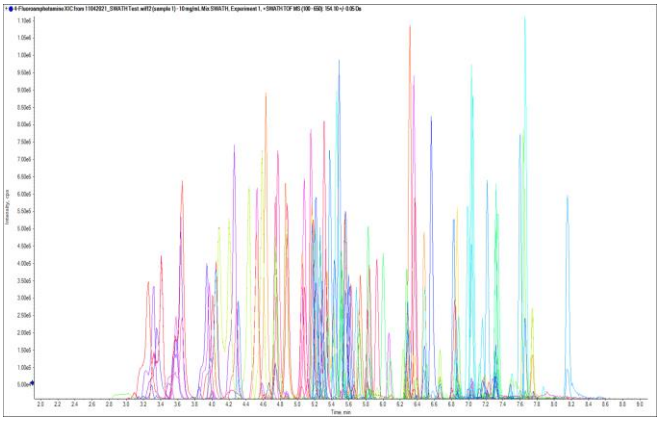


Figure 1. Chromatographic profile of the 130 NPS targeted in this study using the LC conditions of the SCIEX vMethod™ application for forensic toxicology screening on the X500R QTOF system.
Extracted ion chromatogram (XIC) resulting from the near baseline separation of the 130 NPS in a 9.5-minute gradient.

The SCIEX vMethod application for forensic toxicology screening on the X500R QTOF system provides a comprehensive workflow for sample preparation and LC-MS/MS detection of 664 forensic compounds, including many NPS, in biological matrices in a single injection method.[1] The use of high-resolution instrumentation in the forensic toxicology laboratory, such as the X500R QTOF system, has provided a unique tool for screening unknown compounds such as NPS in complex biological samples with little or no method optimization required. The ability to acquire accurate mass information on all precursor ions, followed by the acquisition of multiple, dependent and analyte-specific MS/MS spectra provides confident identification, confirmation and/or library matching of known or newly emerged substances in a routine testing laboratory environment.

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

Advantages of the vMethod for NPS screening and identification

- The vMethod application for forensic toxicology screening on the X500R QTOF system provides:
  - A comprehensive solution for screening NPS with sample preparation procedures for both human whole blood and urine, detailed LC conditions and MS/MS detection methods
  - A robust data processing method to confidently monitor and identify new NPS
- A panel of 130 relevant NPS was analyzed using the vMethod conditions to determine retention times and generate structural information in the form of fragment-rich TOF MS/MS spectra, enabling the confident identification of structurally related NPS isomers
- A components table including the compound name, formula, precursor mass and retention time for the 130 NPS was generated
  - The list can be downloaded and directly imported into the data processing method in SCIEX OS software and used for both targeted and untargeted data processing to identify NPS in authentic case samples
  - The updated list can be used for retrospective analysis of previously acquired MS and MS/MS datasets to screen for the presence of NPS without having to re-inject samples
- The presented workflow provides a significant update that now allows X500R QTOF system users to screen for more than 900 compounds in biological matrices in a single injection method

Experimental details

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

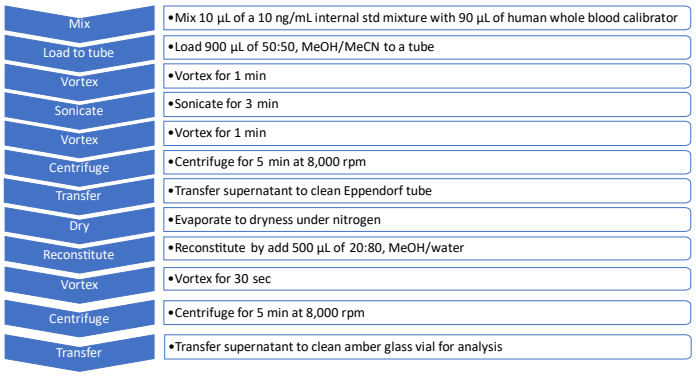


Figure 2. Protein precipitation procedure for human whole blood samples. A 12-step protein precipitation procedure was used for extracting the 130 NPS from human whole blood samples for analysis with the X500R QTOF system.

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

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

Human whole blood samples: 10 µL of the 10 ng/mL internal standard mixture was added to 90 µL of each of the human whole blood calibrator solutions. Then 900 µL of a 50:50, methanol/acetonitrile solution was added to deproteinize the human whole blood samples. The resulting solution was vortexed for 1 minute and then sonicated for 3 minutes. The solution was then vortexed for 1 minute and centrifuged for 5 minutes at 8000 rpm. The supernatant was transferred to a clean Eppendorf tube and completely dried down under nitrogen gas. The residues were reconstituted with 500 µL of 20:80, methanol/water. The reconstituted solution was mixed for 30 seconds then centrifuged for 5 minutes at 8000 rpm. The supernatant was transferred to a clean amber glass vial for analysis. The protein precipitation procedure for human whole blood samples is summarized in Figure 2.

Human urine samples: 10 µL of the 10 ng/mL internal standard mixture was added to 90 µL of each of the human urine calibrator solutions. Then 700 µL of water and 200 µL of methanol were added and the resulting solution was vortexed for 30 seconds and centrifuged for 5 minutes at 8000 rpm. The supernatant was transferred to a clean amber glass vial for analysis. The dilute-and-shoot preparation procedure for human urine is summarized in Figure 3.

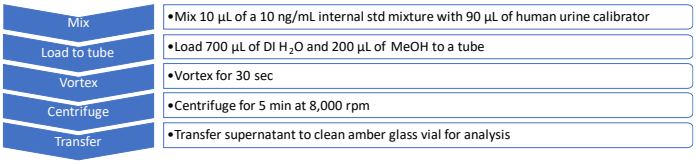
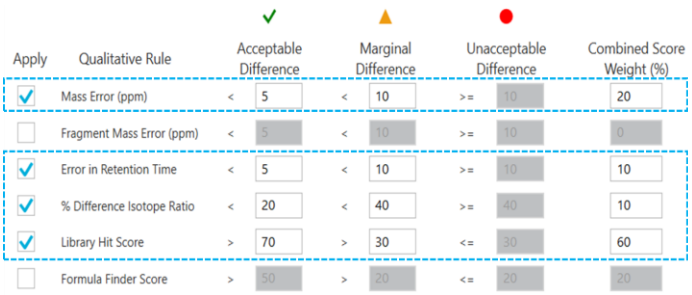


Figure 3. Dilute-and-shoot preparation procedure for human urine samples. A 5-step dilute and shoot precipitation procedure was used for extracting the 130 NPS from human urine samples for analysis with the X500R QTOF system.

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

Mass spectrometry: Two non-targeted data acquisition methods were used and compared. Both data dependent acquisition (DDA) and SWATH data independent acquisition (DIA) generated data that could be analyzed retrospectively. Both experiments started with a TOF MS scan to collect accurate mass precursor ions from 100 to 650 Da. For DDA, a TOF MS/MS full scan ranging from 25 to 650 Da was acquired to ensure all fragments were captured for identification using a maximum of 14 candidate ions. For SWATH DIA, 14 variable Q1 windows ranging from 25 to 650 Da were acquired. Both acquisition methods generated comprehensive and high-quality MS/MS spectra, enabling reliable compound fragmentation to search against spectral library databases to identify analytes. Data acquisition was performed using SCIEX OS software, version 2.0. Samples were injected in triplicate over the course of 3 consecutive days to build a data processing method.

Data analysis: Data processing was performed using SCIEX OS software, version 2.0 for positive analyte identification based on confidence criteria, as previously described.[2,3] The 4 main confidence criteria used are outlined in Figure 4 and included mass error (M), retention time (R), isotope ratio difference (I) and library score (L). A new processing method was created in the Analytics workspace of SCIEX OS software. The components tab was populated by entering the name, molecular formula, precursor mass and retention time of the 130 NPS that were determined following the neat standard mixture injection. The components table for the 130 NPS included in this panel is available for download in the Supporting Information. Spectral library database searching was accomplished by matching the high-quality TOF MS/MS spectra acquired from the matrix calibrator samples to those of a custom-built library using the TOF MS/MS spectra generated using the neat standard mixture. Rapid and automated quantitative data analysis was performed



Apply	Qualitative Rule		✓ Acceptable Difference		▲ Marginal Difference		● Unacceptable Difference	Combined Score Weight (%)
☑	Mass Error (ppm)	<	5	<	10	>=	10	20
☐	Fragment Mass Error (ppm)	<	5	<	10	>=	10	0
☑	Error in Retention Time	<	5	<	10	>=	10	10
☑	% Difference Isotope Ratio	<	20	<	40	>=	40	10
☑	Library Hit Score	>	70	>	30	<=	30	60
☐	Formula Finder Score	>	50	>	20	<=	20	20

Figure 4. Confidence criteria used for data processing in SCIEX OS software. Qualitative rules including mass error (20%), retention time (10%), isotope ratio difference (10%) and library score (60%) used to assess positive analyte identification using the traffic light system.

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.

Optimized LC conditions to separate isomeric species

The separation conditions of the vMethod application for forensic toxicology screening on the X500R QTOF system were used for these experiments. A diluted 10 ng/mL neat standard mixture containing the 130 NPS was used to determine the retention times of the analytes. Figure 1 shows the chromatographic profile of the 130 NPS. Baseline separation was achieved for most of the analytes, except for a few isomeric species that shared the same precursor mass and chemical formula and therefore had similar structures and MS/MS fragmentation patterns. Although the 9.5-minute LC method gradient and conditions enabled baseline separation of most analytes, the introduction of challenging isobaric substances in this panel might prevent chromatographic resolution and thus identification of those species based only on retention time.

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

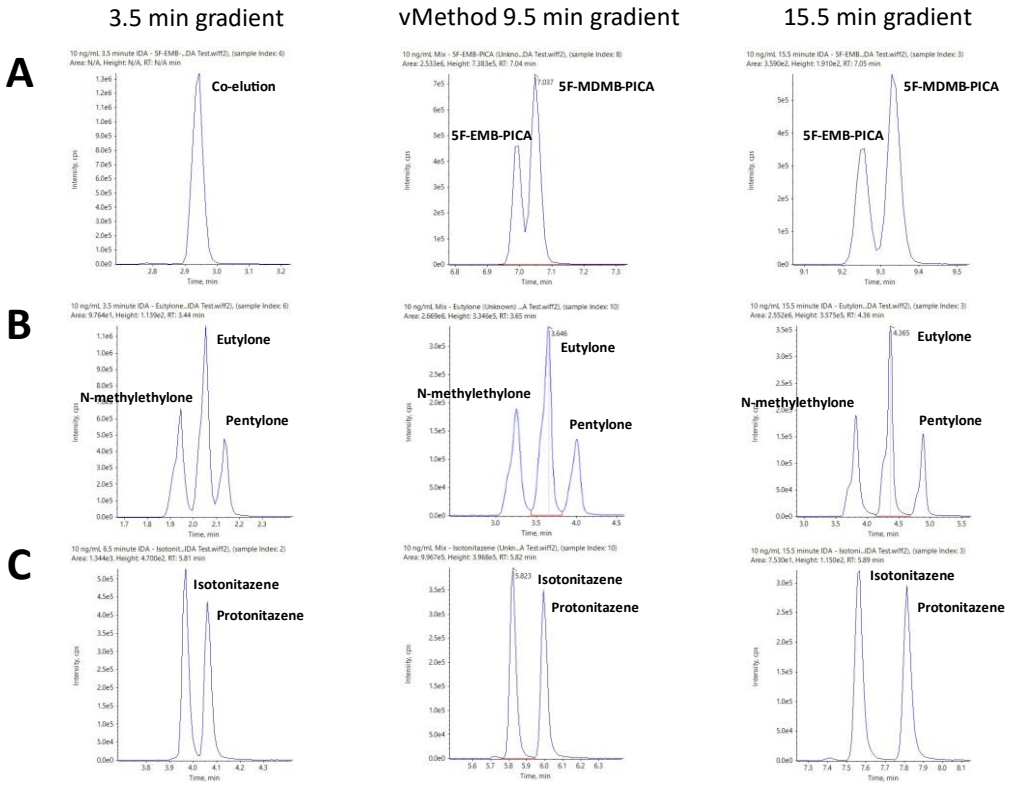


Figure 5. Effect of LC gradient runtime on the separation of isobaric species. XIC traces for A) 5-MDMB-PICA and 5F-EMB-PICA, B) eutylone, N-methylone and pentylone and C) isotonitazene and protonitazene using 3.5-, 9.5- (vMethod conditions) and 15.5-minute LC gradient runtimes. The results show that the LC separation conditions are important for separating challenging isobaric species.

However, near-baseline resolution of these isobaric species was achieved using the 3.5-minute gradient (Figure 4B). Full baseline separation of 2 potent novel synthetic opioids, isotonitazene and protonitazene, was achieved using the 3.5-minute gradient (Figure 4C). The results shown in Figure 4 demonstrate that the LC separation conditions of the vMethod provide a good generic method to separate challenging isobaric species. LC conditions such as the runtime and gradient can be modified based on the nature of the analytes screened using this workflow. Good judgement and caution are recommended when making these changes to ensure the method is fit for purpose and tailored based on the screening requirements.

Criteria for confident NPS identification

Although chromatographic separation is key to resolve and distinguish analytes in a mixture, retention time information alone is often not sufficient to confidently identify compounds with similar structures and MS/MS fragmentation patterns, such as isobaric species. One of the benefits of the use of high-resolution mass spectrometry (HRMS) and data independent acquisition, such as SWATH DIA, is that it enables the generation of a comprehensive digital archive of the sample at both the precursor and fragment levels on all detectable components in the sample. The acquisition of accurate mass data enables accurate identification of drugs through a combination of precursor mass accuracy, by incorporation of the chemical formula into criteria for positive identification (TOF MS), and comprehensive, high-quality MS/MS spectra, through spectral library database searching (TOF MS/MS). As a result, acceptance criteria such as mass error, isotope difference/score and library matching score are typically used in addition to retention time error to confidently assess compound identification. Figure 4 shows the confidence criteria used with corresponding performance levels, used as qualitative rules, for positive analyte identification in SCIEX OS software. The integration of these confidence criteria to the Analytics module of SCIEX OS software provides the ability to filter the positively identified compounds using the “traffic lights” system, which provides a visual assessment of the match quality based on the customized weightings assigned for each of the confidence criteria.

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
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
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
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
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
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
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-(Trifluo...	76.4
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
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
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
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

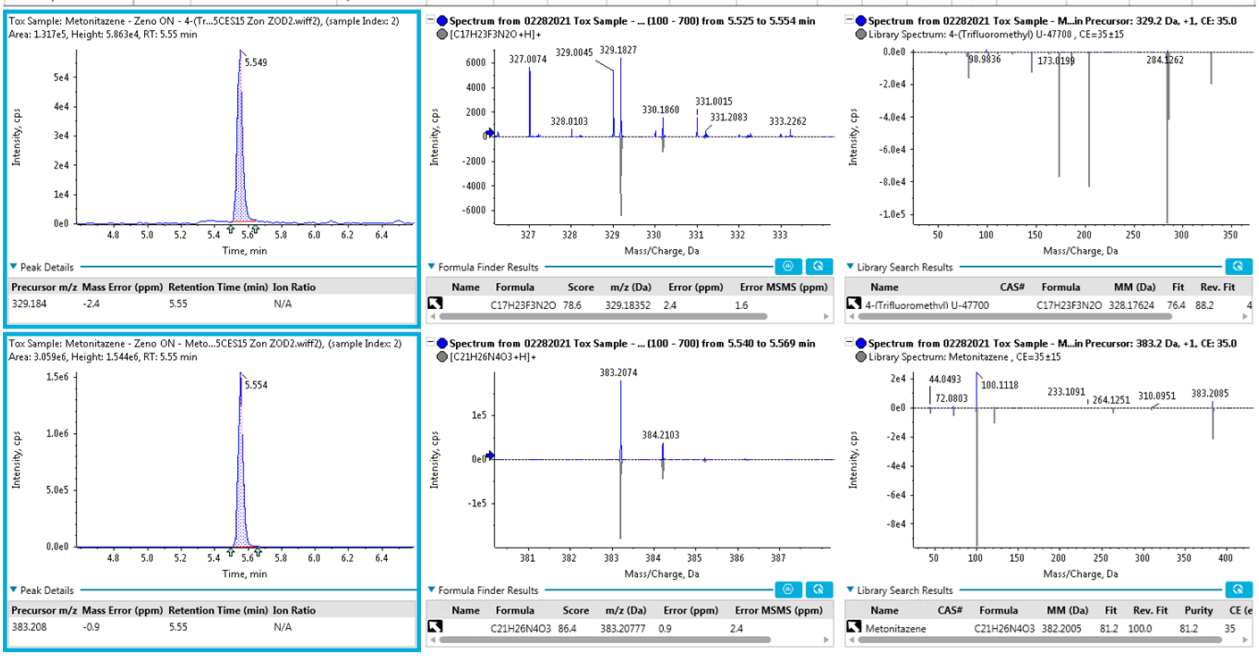


Figure 6. Streamlined and confident NPS identification using SCIEX OS software. (Top) Results table in SCIEX OS software showing the analytes positively identified in an authentic case sample. Mass, library match and library score were assessed using the confidence criteria. (Bottom) XICs, TOF MS and TOF MS/MS spectra collected provided detailed and confident identification of 2 of the positively identified analytes, 4-(trifluoromethyl) U-47700 and metonitazene, 2 synthetic opioids included in the NPS panel.

Streamlined analyte identification

The data analysis component of SCIEX OS software provides an integrated data processing and management platform that allows streamlined data review based on the scoring and reporting criteria set by the user. This step is systematically performed as part of the data review process to ensure correct drug identification while minimizing false positives and/or false negatives. Identification of the analytes was performed by displaying the XIC and TOF MS and MS/MS spectra of the sample with a library search match for each of the NPS included in the screening workflow. Figure 6 (bottom) shows examples of XICs and TOF MS and TOF MS/MS spectra with library matches for 4-Trifluoromethyl-U-47700 and metonitazene, 2 synthetic opioids included in the NPS panel. The retention time error, mass error, isotope ratio difference and MS/MS library score were automatically calculated in SCIEX OS software. Figure 6 (top) shows the parameters and metrics associated with the identification of the analytes. SCIEX OS software provides a simplified interface for streamlined data review based on a robust and reliable scoring system, enabling confident identification of the NPS.

Improved analyte identification capabilities

The use of high-resolution mass spectrometry enables collection of untargeted, accurate mass TOF MS scans of precursor ions followed by acquisition of comprehensive, high-quality TOF MS/MS spectra of the precursor ion fragments. As a result, the non-targeted nature of the DDA and SWATH DIA data acquisition methods used in this workflow allows for expanded drug detection capabilities. Identification of these new drugs in previously acquired datasets can be performed retrospectively using the updated list of NPS provided in this technical note, which includes the name, molecular formula and retention time of each compound. This allows datasets to be re-processed when newly identified forensic targets are discovered without the need to re-inject samples. Figure 7 shows the results of the retrospective analysis of a postmortem case sample that was initially analyzed in 2019. The results table generated in SCIEX

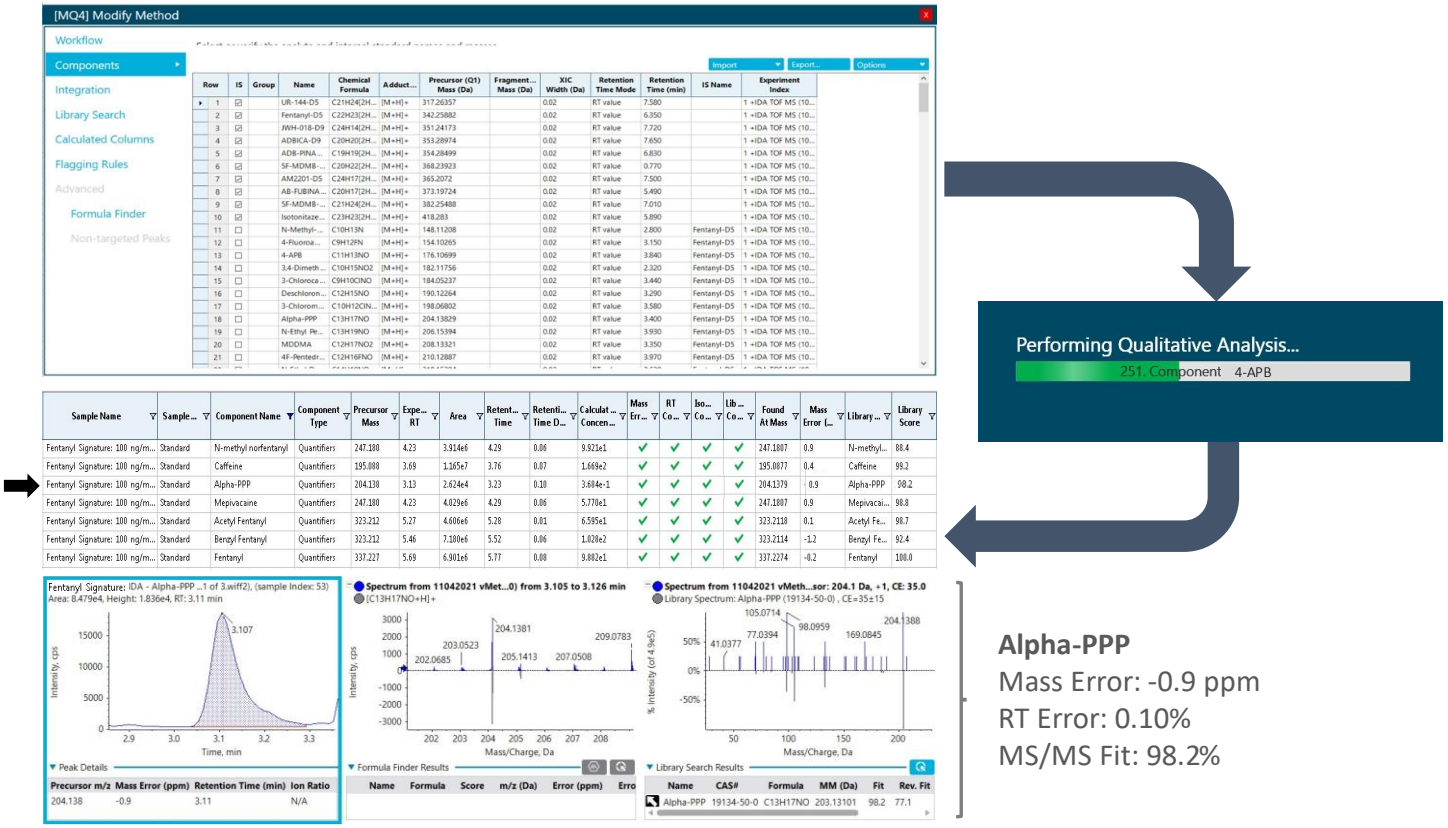


Figure 7. Retrospective analysis performed on a previously acquired dataset using SCIEX OS software. Data acquired using an authentic case sample was reprocessed after modifying the processing method window by adding the 130 NPS included in this panel (top). The stimulant Alpha-PPP was retrospectively identified in the authentic case sample from the SWATH DIA data. A precursor mass error of -0.9 ppm, retention time error of 0.10% and an MS/MS fit score of 98.2% provided excellent measures for the confident identification of Alpha-PPP in the postmortem case sample.

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

Flexible quantification

Another advantage of the use of untargeted data acquisition for NPS screening is that it provides flexibility for quantification of the analytes. Since both TOF MS and TOF MS/MS scans are acquired, users can perform quantification using either precursor or fragment ion information. The use of precursor ion information is usually sufficient but the use of fragment ion information is helpful for complex sample matrices. Figure 8 shows representative XICs for 4F-MDMB-BINACA 3,3-dimethylbutanoic acid and its quantification using the precursor ion (206.1534 ±0.005 m/z; Figure 8A) and the fragment ion (206.1534 →130.0629 m/z; Figure 8B). The series of XICs show the resulting signal for a blank injection (left) and at concentrations ranging from 0.5 to 100 ng/mL. The use of the fragment ion information significantly reduced the background signal. A few other analytes, including most synthetic cannabinoids, benefited from this approach.

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

Robust and precise quantification

The ability to deliver reproducible and accurate results for every injection of every batch is important for a forensic toxicology laboratory to achieve reliable quantification in an NPS screening workflow. The robustness of the vMethod application for forensic toxicology screening on the X500R QTOF system was investigated by analyzing extracted human urine and whole

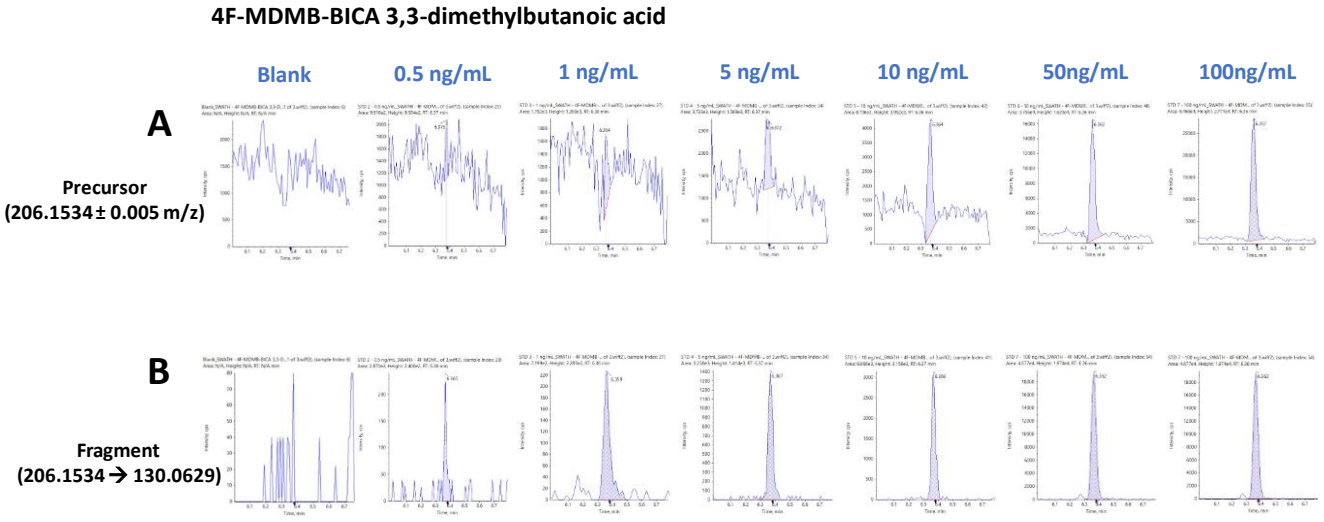


Figure 8. SWATH DIA enables flexible and reliable quantification using precursor (top) and fragment (bottom) ion information. Quantification of 4F-MDMB-BICA 3,3-dimethylbutanoic acid at concentrations ranging from 0.5 to 100 ng/mL using A) precursor ion information (206.1534 ±0.01 m/z) and B) fragment ion information (206.1534 → 130.0629 ±0.01 m/z). The use of the fragment ion significantly reduced the background signal.

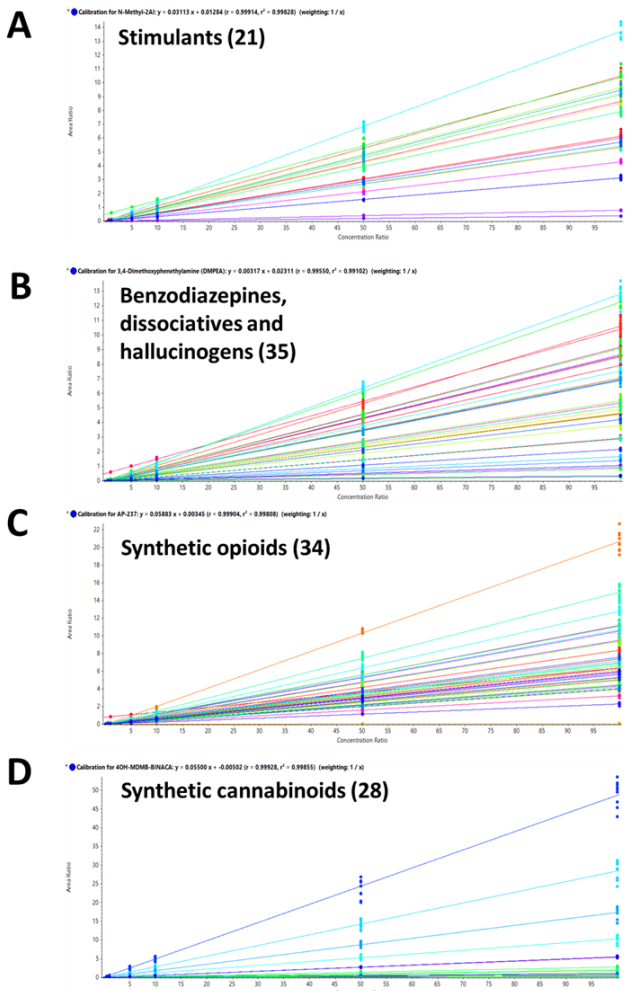


Figure 9. Excellent linearity for the 130 NPS included in the panel. Calibration curves resulting from the calibration series for A) 22 stimulants; B) 35 benzodiazepines, dissociatives and hallucinogens; C) 34 synthetic opioids and D) 28 synthetic cannabinoids. Excellent linear response and sensitivity were observed with R^2 values greater than 0.99 for all the molecules included in this panel.

blood samples at concentrations ranging from 0.5 to 100 ng/mL. Detection and integration of peaks from the background were performed automatically within the viewing window using the MQ4 algorithm in the Analytics module of the software to generate peak area and concentration values for each of the 130 NPS included in this workflow. The use of SCIEX OS software enabled efficient and streamlined analysis of replicate injection averages (n=9, 3x for 3 days) which were used to verify the calibration range, linearity and inter- and intra-day precision at 10 ng/mL.

Calibration curves were generated for each of the 130 NPS included in the panel. Figure 9 shows the resulting regression lines for A) 22 stimulants; B) 35 benzodiazepines, dissociatives and hallucinogens; C) 34 synthetic opioids and D) 28 synthetic cannabinoids, including the 2 cannabinoids, delta-8 THC and delta-8 carboxy THC. Each of the calibrator solutions was injected in triplicate on 3 consecutive days to yield 9 total injections. These calibration curves demonstrated excellent correlation of the generated regression curves, with R^2 values >0.99 for all NPS targeted in the panel, regardless of the acquisition method used.

The performance of the SWATH DIA and DDA workflows was compared for each of the 2 sets of biological samples. The series of calibrator solutions was injected in triplicate over the course of 3 consecutive days to accurately measure the precision of measurements. Overall, the assay showed great reproducibility over the course of 3 consecutive days with intra- and inter-day precision %CV values below 10% for all the calibrator solutions. These results demonstrate the quantitative robustness of the 2 untargeted acquisition methods in both human urine and whole blood samples.

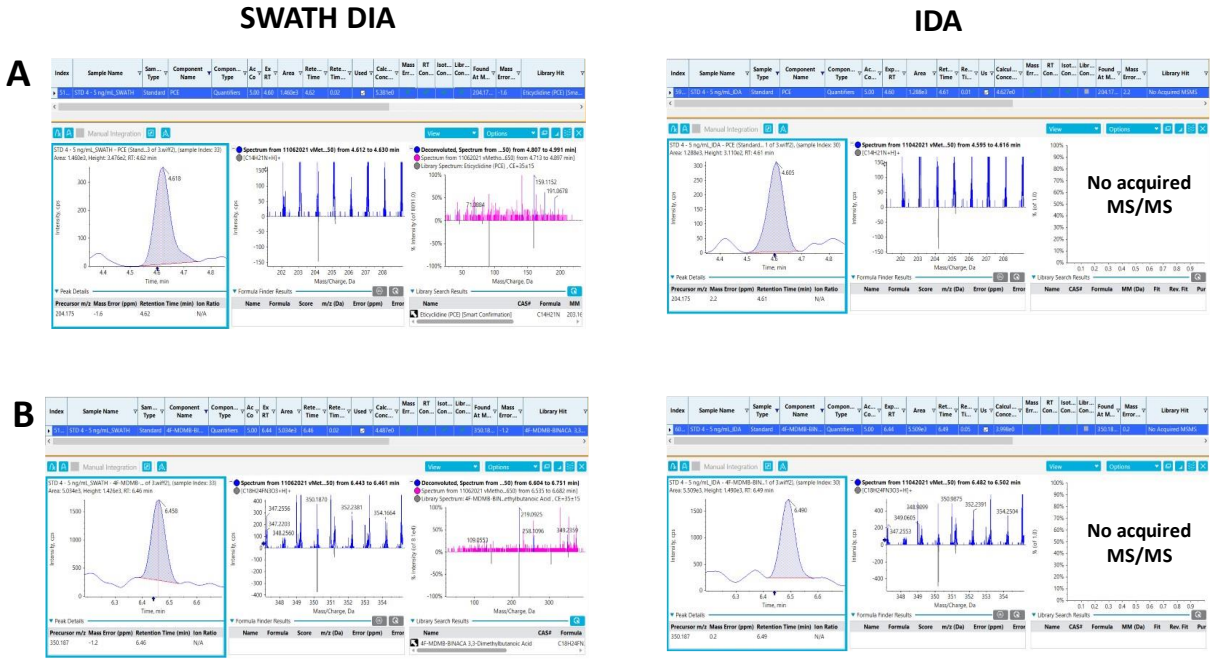


Figure 10. Comparison between SWATH DIA and DDA for TOF MS/MS spectral acquisition. XICs, TOF MS and TOF MS/MS spectra acquired using SWATH DIA (left) and DDA (right) for A) PCE and B) 4F-MDMB-BINACA 3,3-dimethylbutanoic acid. The comparisons demonstrate that no TOF MS/MS spectra were acquired when using DDA at the same concentration levels due to the acquisition method prioritizing the most abundant ions in each data cycle to trigger the TOF MS/MS experiments and missing the low-abundant species present in the biological samples. SWATH DIA, however, generated TOF MS/MS spectra with high (>90%) MS/MS library scores at the same concentration levels.

Tables 1 and 2 summarize the statistical results obtained for the 130 NPS and includes the calibration range, linear correlation coefficient (R^2) and the intra- and inter-day precision at 10 ng/mL for both the SWATH DIA and DDA workflows in human urine and whole blood, respectively. In addition to providing accurate identification of the NPS using TOF MS/MS spectral library matching, the vMethod application for forensic toxicology screening on the X500R QTOF system provided robust and accurate quantification of the drugs in the panel without any compromise to data quality.

DDA vs SWATH DIA: Performance of the data acquisition methods

DDA and SWATH DIA are 2 untargeted data acquisition methods that provide TOF MS and TOF MS/MS information in the form of high-resolution precursor mass and comprehensive MS/MS spectral fragment information that can be used for identification, confirmation and/or library matching. Both data acquisition methods start with a TOF MS experiment covering a mass range determined by the user. However, DDA differs from SWATH DIA in the way the MS/MS information is triggered and acquired. In SWATH DIA, MS/MS information is collected for all detectable compounds in the sample, whereas DDA relies on the user to define the maximum number of candidate ions and therefore the maximum number of dependent TOF MS/MS scans triggered from the TOF MS survey scan in each data cycle. As a result, the MS/MS information for the least abundant species present in the biological matrix might be missed in DDA data, as the more intense ions are prioritized within each data cycle.

MS/MS spectral library matching is the most integral part of the screening workflow, as an MS/MS spectrum provides the fingerprint-like structural details of a compound that are used to perform MS/MS spectral library matching for confident compound identification. The ability to acquire TOF MS/MS information on low-abundant species present in the complex biological samples analyzed was assessed by comparing the TOF MS/MS positivity rates for the 2 data acquisition methods used in this workflow. Figure 10 shows 2 instances in which TOF MS/MS spectra were acquired with SWATH DIA and DDA, but only SWATH DIA data were able to confirm the presence of low concentration drugs. Figures 10A and 10B show the detection and accurate identification of PCE and 4F-MDMB-BINACA 3,3-dimethylbutanoic acid, respectively, using SWATH DIA (left) and DDA (right) at a concentration of 5 ng/mL. The use of SWATH DIA generated TOF MS/MS spectra that passed the library match criteria for accurate identification of the NPS. However, the MS/MS information was not triggered at the same concentration level when DDA was used as the acquisition method because the most abundant ions in each data cycle were prioritized to trigger the TOF MS/MS experiments, causing the

low-abundant species present not to be detected. As a result, some NPS included in this panel did not meet all the confidence criteria at the lower calibrator levels. This is primarily attributed to unacquired MS/MS spectra or poor MS/MS spectral library matching.

In terms of overall performance, however, DDA and SWATH DIA yielded comparable results. The statistical results summarized in Tables 1 and 2 show that the 2 acquisition methods demonstrated excellent performance in terms calibration range, linearity and inter and intra-day assay precision and accuracy. For both acquisition methods, inter-and intra-day concentration accuracies were more than 90% and inter-and intra-day precisions (% bias) were below 10% at the 10 ng/mL calibration level.

Last, it should be noted that SWATH DIA enables users to select fixed or variable Q1 isolation windows for the TOF MS/MS experiment. This feature enables isolation of the compounds with similar mass, such as isobaric species, into different SWATH DIA windows to minimize the amount of convolution, or multiple precursor ions generating common fragment ions at the same time, in each TOF MS/MS experiment. The results demonstrate that SWATH DIA generates more analyte-specific MS/MS information, significantly improving MS/MS library matching and hence providing more confidence in analyte identification. This can be leveraged to differentiate structurally related compounds and generate a more specific digital record of the sample analyzed, which is an advantage of SWATH DIA over DDA that is highly valuable for retrospective data analysis.

Conclusions

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

The use of SCIEX OS software provided a simplified interface for streamlined data review based on a robust and reliable scoring system. This integrated software platform provides the ability to simultaneously perform compound quantification and library matching within the same workspace. Compound identification was confirmed using MS/MS spectral library matching by leveraging the fragment-rich TOF MS/MS spectra generated.

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

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2. Single-Injection Screening of 664 Forensic Toxicology Compounds on a SCIEX X500R QTOF System. SCIEX technical note, RUO-MKT-08-6395-A.
3. Intelligently designed SWATH Acquisition for novel psychoactive substances (NPS) detection in whole blood. SCIEX technical note, RUO-MKT-02-9780-A.

Acknowledgements

The author would like to thank Alex Krotulski and Alberto Salomone for curating the list of relevant NPS and providing some of the standards used in these experiments.

Table 1. Statistical results for the 130 NPS extracted from human urine samples using DDA and SWATH DIA. The table includes the name of the compound, calibration range (ng/mL), linear correlation (R^2 value), and the inter- and intra-day accuracy (%bias) and precision (%CV) for each of the 2 acquisition methods investigated.

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
2Br-Deschloroketamine	*1-100*	*0.9981*	*2.88*	*4.72*	*93.10*	*98.30*	*1-100*	*0.9985*	*2.10*	*4.52*	*95.18*	*100.56*
2-Bromo-4,5-Dimethoxyphenethylamine (2Br-DMPEA)	*1-100*	*0.9966*	*4.00*	*8.07*	*88.87*	*97.83*	*1-100*	*0.9961*	*3.71*	*9.25*	*91.47*	*98.27*
2F-Deschloroketamine	*1-100*	*0.9984*	*5.37*	*5.51*	*92.72*	*96.05*	*1-100*	*0.9989*	*2.81*	*5.52*	*91.22*	*97.09*
2-Methyl AP-237	*1-100*	*0.9990*	*6.16*	*5.39*	*84.33*	*83.84*	*1-100*	*0.9990*	*3.61*	*4.55*	*94.51*	*98.29*
2-Naphthyl U-47700	*5-100*	*0.9918*	*7.73*	*3.69*	*90.46*	*98.72*	*5-100*	*0.9900*	*3.82*	*5.75*	*97.24*	*102.77*
3,4-Difluoro U-47700	*1-100*	*0.9985*	*3.68*	*3.92*	*94.77*	*95.49*	*1-100*	*0.9986*	*1.49*	*3.16*	*94.99*	*96.81*
3,4-Dimethoxyphenethylamine (DMPEA)	*5-100*	*0.9910*	*4.43*	*9.32*	*96.49*	*97.39*	*5-100*	*0.9941*	*5.18*	*9.92*	*94.80*	*101.02*
3-Chlorocathinone	*5-100*	*0.9980*	*5.50*	*5.19*	*97.26*	*98.09*	*5-100*	*0.9984*	*3.39*	*5.97*	*103.39*	*96.87*
3-Chloromethcathinone (3-CMC)	*1-100*	*0.9986*	*4.67*	*6.94*	*96.33*	*95.98*	*1-100*	*0.9987*	*2.87*	*2.65*	*100.99*	*100.43*
3-Cl-PCP	*5-100*	*0.9975*	*4.11*	*5.76*	*93.85*	*92.01*	*5-100*	*0.9978*	*6.09*	*7.47*	*105.49*	*104.17*
3-fluoro-N-Ethylbuphedrone	*5-100*	*0.9984*	*2.17*	*9.12*	*109.23*	*104.45*	*5-100*	*0.9989*	*1.81*	*3.36*	*94.40*	*97.55*
3F-PCP	*1-100*	*0.9984*	*2.97*	*3.72*	*91.94*	*94.80*	*1-100*	*0.9992*	*3.31*	*3.71*	*93.68*	*96.44*
3-HO-PCE	*1-100*	*0.9986*	*8.34*	*9.27*	*93.05*	*99.24*	*1-100*	*0.9988*	*3.28*	*4.68*	*92.77*	*97.51*
3-HO-PCP	*1-100*	*0.9988*	*3.23*	*3.52*	*97.13*	*97.27*	*1-100*	*0.9984*	*2.19*	*3.04*	*98.20*	*101.39*
3-MeO-PCE	*1-100*	*0.9988*	*2.14*	*2.95*	*94.68*	*97.44*	*1-100*	*0.9990*	*3.08*	*4.99*	*95.69*	*100.64*
3-MeO-PCP	*1-100*	*0.9981*	*2.17*	*2.97*	*94.24*	*97.01*	*1-100*	*0.9980*	*2.18*	*2.53*	*91.08*	*90.77*
3-Methyl PCP	*1-100*	*0.9981*	*4.05*	*5.25*	*92.48*	*90.76*	*1-100*	*0.9988*	*4.38*	*4.78*	*93.20*	*93.61*
4-AcO-EPT	*1-100*	*0.9987*	*3.89*	*4.55*	*92.90*	*96.37*	*1-100*	*0.9989*	*2.14*	*3.55*	*96.76*	*99.71*
4-APB	*5-100*	*0.9961*	*3.19*	*9.43*	*91.48*	*93.53*	*5-100*	*0.9969*	*2.96*	*9.72*	*105.42*	*99.60*
4Br-Alpha-PVP	*1-100*	*0.9985*	*4.47*	*5.56*	*91.23*	*95.84*	*1-100*	*0.9991*	*2.84*	*4.81*	*92.83*	*97.98*
4Cl-Alpha-PVP	*1-100*	*0.9984*	*4.22*	*7.05*	*92.10*	*99.18*	*1-100*	*0.9990*	*6.34*	*6.98*	*95.10*	*100.37*
4Cl-Pentedrone	*1-100*	*0.9978*	*7.21*	*8.89*	*90.40*	*97.99*	*1-100*	*0.9982*	*6.09*	*9.68*	*92.87*	*98.23*
4CN-CUMYL-BINACA	*1-100*	*0.9977*	*5.46*	*8.30*	*107.16*	*100.63*	*1-100*	*0.9911*	*5.45*	*8.63*	*94.31*	*98.99*
4F-ABINACA	*1-100*	*0.9930*	*6.90*	*6.87*	*104.12*	*105.26*	*1-100*	*0.9920*	*1.42*	*5.72*	*100.21*	*102.48*
4F-ABINACA N-Butanoic Acid	*1-100*	*0.9925*	*6.17*	*5.65*	*102.93*	*104.94*	*1-100*	*0.9951*	*6.14*	*7.73*	*98.89*	*99.93*
4F-Alpha-PHP	*1-100*	*0.9985*	*4.06*	*4.86*	*91.92*	*95.20*	*1-100*	*0.9988*	*2.11*	*4.11*	*92.45*	*97.07*
4-Fluoroamphetamine	*5-100*	*0.9965*	*7.79*	*7.28*	*93.72*	*95.98*	*5-100*	*0.9959*	*8.21*	*9.26*	*100.08*	*99.64*
4F-MDMB-BICA	*1-100*	*0.9975*	*4.63*	*6.73*	*101.10*	*95.32*	*1-100*	*0.9949*	*7.21*	*6.44*	*98.01*	*97.48*
4F-MDMB-BICA 3,3-Dimethylbutanoic Acid	*5-100*	*0.9912*	*5.78*	*7.90*	*102.95*	*100.51*	*5-100*	*0.9901*	*9.32*	*9.48*	*106.77*	*103.21*
4F-MDMB-BINACA	*1-100*	*0.9984*	*3.32*	*4.84*	*101.73*	*98.55*	*1-100*	*0.9928*	*7.82*	*7.31*	*106.90*	*103.71*

Table 1. Statistical results for the 130 NPS extracted from human urine samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
4F-MDMB-BINACA 3,3-Dimethylbutanoic Acid	1-100	0.9968	6.78	6.48	103.08	102.03	1-100	0.9977	6.52	6.57	98.56	96.22
4F-Pentedrone	1-100	0.9985	2.97	3.70	92.39	94.83	1-100	0.9989	1.81	3.36	94.40	97.56
4'-Hydroxy Nitazene	1-100	0.9942	2.61	9.21	106.33	95.45	1-100	0.9958	3.08	2.91	104.00	103.85
4OH-5F-MDMB-PICA	5-100	0.9934	7.18	8.45	94.67	99.22	5-100	0.9936	8.66	9.23	100.74	101.15
4OH-ADB-BINACA	5-100	0.9908	5.51	8.15	97.39	104.41	5-100	0.9906	6.89	7.39	94.68	96.96
4OH-MDMB-BICA	1-100	0.9942	4.76	5.16	103.11	103.54	1-100	0.9947	7.56	9.83	97.12	96.51
4OH-MDMB-BINACA	1-100	0.9986	2.92	3.33	97.10	98.75	1-100	0.9973	5.56	6.77	93.21	97.49
4-Trifluoromethyl-U-47700	1-100	0.9967	2.82	6.41	97.43	105.47	1-100	0.9942	3.31	9.64	90.03	102.47
5-Amino Isotonitazene	1-100	0.9960	3.98	4.25	91.06	93.61	1-100	0.9955	8.41	9.10	92.14	92.00
5Cl-MDMB-PICA	1-100	0.9978	4.38	6.91	105.88	102.65	1-100	0.9938	8.69	9.05	108.60	103.06
5F-BZO-POXIZID (5F-MDA-19)	1-100	0.9936	5.35	6.90	102.03	106.41	1-100	0.9914	3.60	2.29	103.21	100.21
5F-EDMB-PICA	1-100	0.9976	3.72	5.35	104.85	101.52	1-100	0.9933	5.87	9.18	92.79	95.39
5F-EMB-PICA	1-100	0.9980	3.22	5.78	103.54	98.82	1-100	0.9943	5.29	6.04	108.10	103.04
5F-MDMB-PICA	1-100	0.9980	3.20	5.76	103.55	98.84	1-100	0.9930	6.05	9.22	99.74	100.47
5-MeO-MiPT	1-100	0.9988	3.24	3.63	95.43	97.97	1-100	0.9988	2.10	3.61	95.42	99.52
5OH-MDMB-PICA	1-100	0.9978	4.57	6.48	101.26	96.96	1-100	0.9955	6.35	8.62	97.30	99.80
8-Aminoclonazolam	1-100	0.9980	3.87	4.97	92.79	96.52	1-100	0.9985	2.72	7.22	91.65	98.37
ADB-4en-PINACA	1-100	0.9978	4.63	3.72	103.02	95.79	1-100	0.9963	2.76	2.14	97.58	96.44
ADB-BINACA	1-100	0.9954	9.75	8.07	96.08	102.96	1-100	0.9944	8.76	8.68	95.93	92.02
ADB-BINACA N-Butanoic Acid	1-100	0.9946	4.16	7.41	109.27	103.25	1-100	0.9931	8.70	7.11	98.11	96.85
ADB-HEXINACA	5-100	0.9951	5.86	6.99	96.11	91.02	5-100	0.9947	2.24	2.82	97.51	98.16
ADB-PHETINACA	1-100	0.9977	2.42	3.60	96.92	95.26	1-100	0.9933	5.50	10.60	91.54	94.11
Adinazolam	1-100	0.9980	1.93	3.99	93.19	97.65	1-100	0.9983	2.51	5.73	92.21	99.21
Alpha-PPP	1-100	0.9981	3.34	4.84	92.95	97.74	1-100	0.9989	2.07	4.59	91.78	96.32
AMP-4en-PINACA	5-100	0.9943	6.01	11.29	100.50	95.38	5-100	0.9927	5.13	5.50	98.07	97.03
AP-237	1-100	0.9980	5.45	6.22	102.13	99.45	1-100	0.9978	3.51	6.32	100.15	98.15
AP-238	1-100	0.9990	7.20	7.47	98.13	101.76	1-100	0.9926	3.60	4.54	94.63	98.41
Bentazepam	1-100	0.9957	4.08	4.48	94.58	97.96	1-100	0.9985	2.75	5.63	92.78	99.33
Bipiperidinyl 4-ANPP	1-100	0.9909	9.88	9.92	91.38	93.89	1-100	0.9944	6.52	7.52	102.50	99.79
Bromazepam	5-100	0.9967	6.65	6.02	92.59	91.62	5-100	0.9966	5.73	7.52	90.70	90.57
Bromazolam	1-100	0.9984	2.01	4.21	91.35	95.77	1-100	0.9988	2.06	3.60	95.28	99.01
Brorphine	1-100	0.9966	6.97	7.61	97.31	99.93	1-100	0.9969	5.83	6.32	99.61	95.36

Table 1. Statistical results for the 130 NPS extracted from human urine samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
Butonitazene	1-100	0.9974	3.06	4.59	94.60	98.76	1-100	0.9975	2.27	2.62	102.50	100.96
BZO-POXIZID (MDA-19 pentyl analogue)	1-100	0.9984	4.04	4.35	100.38	101.73	1-100	0.9980	2.07	2.27	104.40	105.50
Carfentanil	1-100	0.9981	3.35	3.60	94.26	94.82	1-100	0.9986	2.73	2.72	98.35	99.00
Clonazolam	1-100	0.9980	3.19	4.39	96.00	98.52	1-100	0.9992	2.51	2.97	97.51	99.67
Clonitazene	5-100	0.9937	6.69	9.12	103.25	94.14	5-100	0.9958	8.90	9.05	92.64	90.97
CUMYL-NBMICA	1-100	0.9908	6.77	6.45	106.05	106.41	1-100	0.9926	5.06	5.47	101.97	101.90
Cyclopropyl U-47700	1-100	0.9982	4.11	5.35	98.94	101.87	1-100	0.9987	3.45	6.28	90.63	96.73
Delta-8 Carboxy THC	5-100	0.9929	8.71	9.04	99.75	98.28	5-100	0.9941	2.89	3.40	90.75	93.59
Delta-8 THC	5-100	0.9914	4.69	7.94	102.95	105.95	5-100	0.9937	0.72	0.84	91.67	93.93
Deoxymethoxetamine	1-100	0.9990	3.42	3.19	96.90	98.40	1-100	0.9985	1.40	3.11	96.19	99.31
Desalkylflurazepam	1-100	0.9982	3.10	4.00	95.49	96.30	1-100	0.9988	2.89	3.41	96.92	98.89
Deschloroetizolam	1-100	0.9987	2.40	2.49	94.11	95.39	1-100	0.9989	2.66	3.58	96.10	99.09
Deschloroketamine	1-100	0.9981	3.32	4.82	93.00	97.77	1-100	0.9989	2.08	4.60	91.58	96.12
Deschloronorketamine	1-100	0.9987	4.26	4.96	102.17	100.29	1-100	0.9982	3.81	5.64	92.31	96.47
Despropionyl Carfentanil	1-100	0.9990	3.82	4.17	94.16	96.81	1-100	0.9991	3.61	4.56	94.59	98.38
Despropionyl ortho-Fluorofentanyl	1-100	0.9987	5.20	5.55	99.45	103.02	1-100	0.9989	2.14	3.54	96.95	99.91
Despropionyl para-Chlorofentanyl	1-100	0.9978	6.76	8.37	90.43	95.62	1-100	0.9988	4.68	6.14	95.98	97.73
Diclazepam	1-100	0.9986	2.71	3.46	95.39	98.50	1-100	0.9988	2.17	5.32	93.68	99.79
Dimethylpentylone	1-100	0.9983	3.84	3.86	94.31	96.02	1-100	0.9985	2.88	3.16	97.00	99.13
Etodesnitazene	1-100	0.9971	5.06	4.90	95.14	93.48	1-100	0.9974	2.85	4.88	97.18	95.55
Etonitazene	1-100	0.9970	3.59	3.90	98.21	99.06	1-100	0.9968	3.45	3.58	98.95	96.72
Eutylone	1-100	0.9987	2.43	3.61	95.44	98.23	1-100	0.9986	2.29	3.43	98.33	101.73
Flualprazolam	5-100	0.9968	7.20	9.12	92.38	99.00	5-100	0.9972	9.47	9.04	95.08	98.08
Flunitazene	5-100	0.9973	2.88	7.40	99.13	92.07	5-100	0.9952	2.27	2.62	102.50	100.96
Flunitrazolam	1-100	0.9982	3.96	3.73	98.82	99.59	1-100	0.9984	2.20	2.83	94.96	96.72
Furanyl UF-17	1-100	0.9984	3.56	4.00	95.09	97.54	1-100	0.9989	4.19	4.66	95.43	98.98
Isotodesnitazene	1-100	0.9966	2.50	3.39	96.20	94.88	1-100	0.9980	1.75	3.55	101.48	97.60
Isotonitazene	1-100	0.9978	7.23	6.56	102.64	101.67	1-100	0.9930	3.09	2.80	98.66	98.71
MDDMA	1-100	0.9981	4.47	4.45	93.12	95.61	1-100	0.9988	5.01	4.81	94.28	96.60
MDMB-4en-PINACA	1-100	0.9976	5.08	6.34	102.79	101.14	1-100	0.9952	7.69	7.31	106.77	103.13
MDMB-4en-PINACA 3,3-Dimethylbutanoic Acid	1-100	0.9965	8.35	9.99	98.54	96.33	1-100	0.9957	8.55	9.79	98.82	96.44

Table 1. Statistical results for the 130 NPS extracted from human urine samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
Meclonazepam	1-100	0.9983	4.93	5.38	90.91	94.79	1-100	0.9984	5.83	5.94	94.33	97.20
Methoxy U-47700	1-100	0.9983	6.31	6.24	96.41	97.87	1-100	0.9908	3.38	4.59	96.47	99.68
Methylenedioxy-PV8	1-100	0.9979	2.46	4.53	95.84	97.00	1-100	0.9928	3.48	4.45	101.09	97.04
Metodesnitazene	1-100	0.9973	4.72	4.97	100.09	96.98	1-100	0.9982	2.29	2.76	97.50	97.58
Metonitazene	1-100	0.9967	3.52	5.96	102.33	98.76	1-100	0.9966	3.15	6.16	102.50	97.12
MXPr	1-100	0.9983	3.82	4.15	93.31	96.11	1-100	0.9986	5.83	6.32	99.61	95.36
N-Butyl Hexedrone	5-100	0.9965	5.29	5.92	102.24	102.67	5-100	0.9922	3.00	11.17	91.99	102.63
N-Desethyl Etonitazene	1-100	0.9945	9.50	8.30	91.92	86.88	1-100	0.9958	3.08	2.91	104.07	103.93
N-Desethyl Isotonitazene	1-100	0.9971	3.52	3.89	97.98	97.68	1-100	0.9967	3.14	6.14	102.90	97.51
N-Desmethyl U-47700	1-100	0.9984	4.46	4.39	95.90	97.25	1-100	0.9985	3.47	4.91	92.55	96.70
N-Ethyl Deschloroketamine (O-PCE)	5-100	0.9987	1.80	3.42	94.35	98.22	5-100	0.9989	1.88	4.07	94.36	99.07
N-Ethyl Heptedrone	1-100	0.9987	2.14	2.95	94.77	97.53	1-100	0.9989	3.07	4.98	95.77	100.72
N-Ethyl Pentedrone	1-100	0.9985	2.16	2.56	94.27	96.25	1-100	0.9990	2.08	2.80	96.77	99.44
N-Ethyl Pentylone	1-100	0.9983	6.02	6.79	97.41	101.96	1-100	0.9984	2.88	3.16	96.97	99.09
N-Ethylhexedrone	1-100	0.9986	4.47	4.53	91.68	94.54	1-100	0.9987	3.26	4.64	93.38	98.10
Nitrazolam	1-100	0.9979	4.51	6.31	91.51	97.46	1-100	0.9989	4.18	4.46	98.51	100.22
N-Methyl-2AI	1-100	0.9983	4.14	5.68	92.85	96.82	1-100	0.9985	4.20	5.53	95.73	101.19
N-Methylethylone	1-100	0.9986	1.67	2.57	95.59	97.71	1-100	0.9986	2.28	3.43	98.40	101.80
N-Methyl-Hexylone	1-100	0.9984	6.92	7.12	94.37	99.40	1-100	0.9985	2.92	3.20	96.05	98.19
N-Piperidinyl Etonitazene	1-100	0.9972	4.15	5.71	102.33	99.25	1-100	0.9981	2.30	2.76	96.84	96.91
N-Pyrrolidino Etonitazene (Etonitazepyne)	1-100	0.9964	4.87	4.76	99.09	98.77	1-100	0.9967	4.21	7.41	105.74	100.09
para-bromo Fentanyl	1-100	0.9982	5.75	5.54	98.69	97.73	1-100	0.9978	4.64	5.77	93.97	98.36
para-Chlorofentanyl	1-100	0.9983	2.33	3.11	93.37	95.44	1-100	0.9988	2.59	3.30	97.04	99.20
para-Fluoro Phenethyl 4-ANPP	1-100	0.9978	4.10	4.17	98.02	98.68	1-100	0.9986	3.62	5.31	96.62	101.14
para-Fluorofentanyl	1-100	0.9987	3.49	4.80	99.10	103.68	1-100	0.9989	2.14	3.54	96.87	99.82
para-methyl AP-237	1-100	0.9973	5.42	12.02	90.82	91.35	1-100	0.9979	1.91	9.76	92.29	97.37
para-Methyltetrahydrofuranylfentanyl	1-100	0.9985	4.72	5.03	92.29	93.68	1-100	0.9984	1.87	2.89	92.23	94.01
PCE	5-100	0.9948	9.23	8.27	105.46	101.94	5-100	0.9958	7.93	7.75	93.06	92.47
Pentylone	1-100	0.9986	3.07	4.27	91.91	95.11	1-100	0.9986	2.26	3.42	98.55	101.95
Phenazolam	1-100	0.9955	3.89	4.80	93.27	96.45	1-100	0.9970	3.09	4.42	97.87	100.77
Phenethyl 4-ANPP	1-100	0.9987	3.53	3.59	93.51	94.83	1-100	0.9989	2.14	3.55	96.91	99.86

Table 1. Statistical results for the 130 NPS extracted from human urine samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
Piperidylthiambutene	*1-100*	*0.9914*	*9.16*	*8.67*	*106.47*	*103.62*	*1-100*	*0.9963*	*9.24*	*8.90*	*101.40*	*102.44*
Protonitazene	*1-100*	*0.9985*	*3.90*	*5.17*	*95.79*	*99.47*	*1-100*	*0.9988*	*2.18*	*3.62*	*94.59*	*98.16*
Tenocyclidine	*5-100*	*0.9958*	*5.87*	*9.58*	*95.90*	*98.19*	*5-100*	*0.9973*	*4.28*	*5.58*	*103.78*	*102.61*
U-48800	*1-100*	*0.9981*	*5.95*	*5.77*	*93.01*	*94.66*	*1-100*	*0.9986*	*5.32*	*7.01*	*90.96*	*92.90*
U-49900	*1-100*	*0.9987*	*3.89*	*3.94*	*97.00*	*98.05*	*1-100*	*0.9981*	*2.52*	*3.08*	*95.88*	*98.65*
UF-17	*1-100*	*0.9974*	*6.53*	*6.94*	*98.33*	*96.77*	*1-100*	*0.9982*	*4.71*	*5.43*	*93.81*	*97.22*

Table 2. Statistical results for the 130 NPS extracted from human whole blood samples using DDA and SWATH DIA. The table includes the name of the compound, calibration range (ng/mL), linear correlation (R^2 value) and the inter- and intra-day accuracy (%bias) and precision (%CV) for each of the 2 acquisition methods investigated.

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
2Br-Deschloroketamine	1-100	0.9955	4.48	4.76	92.76	96.01	1-100	0.9976	3.41	5.75	104.30	99.49
2-Bromo-4,5-Dimethoxyphenethylamine (2Br-DMPEA)	1-100	0.9950	6.17	6.00	99.85	97.91	1-100	0.9974	2.62	5.13	107.07	101.43
2F-Deschloroketamine	1-100	0.9917	2.67	3.29	91.14	93.40	1-100	0.9972	2.44	4.59	101.40	96.73
2-Methyl AP-237	1-100	0.9948	3.88	4.84	99.82	97.82	1-100	0.9955	3.97	9.15	101.77	91.84
2-Naphthyl U-47700	5-100	0.9949	5.59	5.90	97.06	99.71	5-100	0.9974	2.96	4.99	107.77	102.86
3,4-Difluoro U-47700	1-100	0.9957	3.54	3.43	95.07	94.04	1-100	0.9982	2.83	6.25	108.30	100.74
3,4-Dimethoxyphenethylamine (DMPEA)	5-100	0.9949	8.99	8.70	94.15	99.23	5-100	0.9940	3.95	8.11	108.87	102.00
3-Chlorocathinone	5-100	0.9919	4.82	5.09	91.80	93.40	5-100	0.9909	5.94	7.70	101.45	94.82
3-Chloromethcathinone (3-CMC)	1-100	0.9919	5.03	5.18	91.86	90.45	1-100	0.9923	4.00	7.58	107.00	99.05
3-Cl-PCP	5-100	0.9938	7.50	8.57	100.19	95.37	5-100	0.9964	7.10	6.57	97.27	98.05
3-fluoro-N-Ethylbuphedrone	5-100	0.9950	3.56	3.43	92.10	91.34	5-100	0.9918	4.87	9.57	100.67	90.84
3F-PCP	1-100	0.9911	7.25	9.28	107.83	100.99	1-100	0.9956	3.62	4.10	95.48	92.47
3-HO-PCE	1-100	0.9930	2.62	2.66	91.71	90.13	1-100	0.9903	3.78	7.05	101.48	94.52
3-HO-PCP	1-100	0.9964	3.54	4.45	96.27	97.73	1-100	0.9984	2.77	5.24	109.23	103.52
3-MeO-PCE	1-100	0.9944	3.03	3.19	92.04	90.41	1-100	0.9930	3.49	6.62	104.00	97.49
3-MeO-PCP	1-100	0.9945	3.51	3.81	95.53	93.42	1-100	0.9979	2.55	7.79	104.40	95.83
3-Methyl PCP	1-100	0.9904	1.83	2.63	90.66	91.82	1-100	0.9955	3.09	6.74	98.63	91.69
4-AcO-EPT	1-100	0.9953	6.30	7.91	92.40	97.48	1-100	0.9946	3.68	2.37	100.46	103.67
4-APB	5-100	0.9951	2.92	3.55	92.57	94.92	5-100	0.9973	3.02	6.79	106.90	98.82
4Br-Alpha-PVP	1-100	0.9919	3.58	3.22	94.27	93.74	1-100	0.9936	3.34	13.31	107.90	92.91
4Cl-Alpha-PVP	1-100	0.9942	5.02	4.94	90.50	90.33	1-100	0.9938	1.82	7.22	106.27	97.53
4Cl-Pentedrone	1-100	0.9945	4.06	8.14	90.89	98.67	1-100	0.9952	5.18	6.82	104.48	97.84
4CN-CUMYL-BINACA	1-100	0.9952	5.86	7.08	90.53	95.62	1-100	0.9926	5.47	5.27	94.68	96.10
4F-ABINACA	1-100	0.9922	2.16	2.72	82.60	83.16	1-100	0.9926	2.13	4.59	94.55	91.94
4F-ABINACA N-Butanoic Acid	1-100	0.9975	8.46	8.00	97.18	97.24	1-100	0.9925	7.19	7.58	100.55	95.51
4F-Alpha-PHP	1-100	0.9934	6.04	5.83	92.01	94.79	1-100	0.9927	3.26	9.20	100.81	90.52
4-Fluoroamphetamine	5-100	0.9916	8.74	8.43	97.52	100.64	5-100	0.9948	7.75	9.51	106.77	99.30
4F-MDMB-BICA	1-100	0.9931	6.44	9.81	91.26	90.44	1-100	0.9911	7.64	8.05	105.53	100.51
4F-MDMB-BICA 3,3-Dimethylbutanoic Acid	5-100	0.9903	9.57	9.43	108.63	99.57	5-100	0.9926	5.65	5.52	95.90	94.85
4F-MDMB-BINACA	1-100	0.9975	6.73	7.34	92.31	96.71	1-100	0.9945	7.34	6.72	95.46	94.32

Table 2. Statistical results for the 130 NPS extracted from human whole blood samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
4F-MDMB-BINACA 3,3-Dimethylbutanoic Acid	1-100	0.9930	3.55	3.42	91.79	91.03	1-100	0.9916	6.96	7.35	103.18	98.05
4F-Pentedrone	1-100	0.9949	2.60	4.71	100.43	95.66	1-100	0.9901	4.90	9.63	102.23	92.19
4'-Hydroxy Nitazene	5-100	0.9929	3.63	4.62	92.40	94.71	1-100	0.9958	3.11	3.93	100.19	103.88
4OH-5F-MDMB-PICA	5-100	0.9958	4.79	5.32	98.29	97.44	5-100	0.9928	8.01	8.91	96.81	101.85
4OH-ADB-BINACA	1-100	0.9976	5.68	8.46	95.54	103.79	5-100	0.9930	8.96	8.08	102.58	104.02
4OH-MDMB-BICA	1-100	0.9962	5.51	5.27	95.55	94.70	1-100	0.9929	6.08	5.67	95.25	94.26
4OH-MDMB-BINACA	1-100	0.9955	4.65	6.26	110.33	106.29	1-100	0.9931	8.12	8.08	92.19	93.32
4-Trifluoromethyl-U-47700	1-100	0.9917	4.48	3.18	91.87	95.12	1-100	0.9928	6.57	8.73	109.07	101.57
5-Amino Isotonitazene	1-100	0.9956	7.16	7.34	105.97	102.28	1-100	0.9903	4.96	3.66	106.06	93.56
5Cl-MDMB-PICA	1-100	0.9967	5.22	5.90	102.35	105.95	1-100	0.9917	4.03	7.34	97.47	90.06
5F-BZO-POXIZID (5F-MDA-19)	1-100	0.9967	4.85	3.59	92.40	95.66	1-100	0.9946	3.52	6.68	97.46	95.53
5F-EDMB-PICA	1-100	0.9919	3.69	3.59	105.10	105.91	1-100	0.9926	5.89	7.85	101.49	94.18
5F-EMB-PICA	1-100	0.9924	0.83	0.89	96.69	96.44	1-100	0.9961	8.37	8.16	92.92	97.56
5F-MDMB-PICA	1-100	0.9959	4.10	4.22	97.13	98.88	1-100	0.9916	3.72	5.35	104.85	101.52
5-MeO-MiPT	1-100	0.9913	6.16	6.44	105.78	105.69	1-100	0.9976	4.24	5.54	108.61	104.30
5OH-MDMB-PICA	1-100	0.9955	4.35	4.40	97.10	97.32	1-100	0.9948	4.97	4.81	105.47	106.02
8-Aminoclonazolam	1-100	0.9944	7.36	5.79	102.64	105.64	1-100	0.9973	3.98	5.31	104.74	102.35
ADB-4en-PINACA	1-100	0.9960	5.70	8.87	90.62	93.82	1-100	0.9944	4.60	3.76	103.69	102.46
ADB-BINACA	1-100	0.9908	6.28	5.98	91.84	92.04	1-100	0.9911	5.33	6.79	100.30	95.70
ADB-BINACA N-Butanoic Acid	5-100	0.9946	4.28	6.44	105.10	103.28	1-100	0.9941	5.67	6.37	98.80	95.61
ADB-HEXINACA	1-100	0.9951	2.63	1.84	90.96	92.69	5-100	0.9974	8.45	8.01	102.46	102.95
ADB-PHETINACA	1-100	0.9950	4.76	5.10	90.69	92.14	1-100	0.9961	7.59	9.21	96.21	101.22
Adinazolam	1-100	0.9924	2.84	3.54	90.40	92.58	1-100	0.9971	2.57	6.88	107.50	99.44
Alpha-PPP	5-100	0.9961	6.31	7.09	100.50	95.84	1-100	0.9966	3.38	7.46	105.40	97.21
AMP-4en-PINACA	1-100	0.9936	3.95	3.83	97.72	96.16	5-100	0.9922	5.21	5.95	97.47	95.10
AP-237	1-100	0.9945	2.71	3.51	94.07	94.03	1-100	0.9968	2.42	7.64	101.61	92.88
AP-238	1-100	0.9930	4.35	3.91	91.56	90.96	1-100	0.9967	7.20	7.47	98.13	101.76
Bentazepam	1-100	0.9944	2.60	4.59	92.40	92.41	1-100	0.9955	2.60	4.93	94.21	92.58
Bipiperidinyl 4-ANPP	5-100	0.9951	3.87	4.45	96.44	97.28	1-100	0.9932	7.65	5.04	102.86	101.94
Bromazepam	1-100	0.9947	4.59	5.32	90.96	92.41	5-100	0.9978	2.50	4.10	107.33	103.03
Bromazolam	1-100	0.9953	1.15	1.57	99.79	98.59	1-100	0.9970	3.42	8.45	108.47	98.34
Brorphine	1-100	0.9930	3.55	3.42	91.79	91.03	1-100	0.9948	1.79	6.77	100.93	93.33

Table 2. Statistical results for the 130 NPS extracted from human whole blood samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
Butonitazene	3.48	4.09	99.95	103.32	3.48	4.09	1-100	0.9954	2.47	9.15	103.55	90.64
BZO-POXIZID (MDA-19 pentyl analogue)	4.29	4.74	96.48	94.56	4.29	4.74	1-100	0.9917	1.77	4.66	104.33	98.67
Carfentanil	7.43	9.24	96.40	99.65	7.43	9.24	1-100	0.9963	1.51	5.05	104.13	98.70
Clonazolam	4.85	4.59	96.90	95.12	4.85	4.59	1-100	0.9971	3.97	6.20	103.86	98.74
Clonitazene	7.53	7.91	92.18	92.13	7.53	7.91	5-100	0.9917	2.46	8.67	103.35	90.90
CUMYL-NBMICA	8.01	8.16	98.07	93.25	8.01	8.16	1-100	0.9924	9.95	9.41	95.27	93.94
Cyclopropyl U-47700	3.99	4.11	93.07	93.39	3.99	4.11	1-100	0.9966	4.54	8.84	102.11	92.97
Delta-8 Carboxy THC	8.17	9.20	98.57	98.19	8.17	9.20	5-100	0.9911	4.13	4.84	90.64	99.71
Delta-8 THC	4.58	4.11	93.28	94.03	4.58	4.11	5-100	0.9914	3.34	3.20	104.74	102.86
Deoxymethoxetamine	3.94	4.17	90.17	91.69	3.94	4.17	1-100	0.9968	2.32	8.13	106.90	96.85
Desalkylflurazepam	5.78	5.94	93.19	94.50	5.78	5.94	1-100	0.9972	4.33	7.32	107.73	99.68
Deschloroetizolam	3.02	3.55	91.59	92.40	3.02	3.55	1-100	0.9970	1.96	7.83	107.10	97.68
Deschloroketamine	2.00	2.71	90.01	92.07	2.00	2.71	1-100	0.9967	3.37	7.46	105.33	97.13
Deschloronorketamine	2.72	4.88	95.93	96.83	2.72	4.88	1-100	0.9939	8.77	9.98	94.36	95.21
Despropionyl Carfentanil	3.60	3.42	95.24	94.05	3.60	3.42	1-100	0.9954	3.82	4.59	92.15	90.69
Despropionyl ortho-Fluorofentanyl	3.87	3.87	100.19	103.28	3.87	3.87	1-100	0.9969	3.64	4.65	96.56	95.10
Despropionyl para-Chlorofentanyl	3.87	8.70	103.13	96.99	3.87	8.70	1-100	0.9958	3.45	3.61	96.62	98.70
Diclazepam	2.18	2.18	102.14	103.28	2.18	2.18	1-100	0.9956	3.53	4.20	93.52	91.13
Dimethylpentylone	4.00	3.80	93.07	93.95	4.00	3.80	1-100	0.9963	3.13	6.96	104.51	98.09
Etodesnitazene	2.27	6.16	92.84	97.84	2.27	6.16	1-100	0.9961	3.20	7.82	103.64	94.59
Etonitazene	1.44	1.73	93.79	94.14	1.44	1.73	1-100	0.9938	3.54	6.77	94.32	91.96
Eutylone	5.07	5.18	93.35	95.66	5.07	5.18	1-100	0.9962	2.04	5.85	108.40	101.97
Flualprazolam	9.23	8.46	95.59	94.74	9.23	8.46	5-100	0.9979	5.42	7.47	102.23	97.95
Flunitazene	2.63	2.60	93.08	92.69	2.63	2.60	5-100	0.9948	2.47	9.16	103.75	90.80
Flunitrazolam	3.60	3.63	96.28	96.90	3.60	3.63	1-100	0.9979	2.23	3.13	104.27	101.71
Furanyl UF-17	3.31	3.22	94.56	94.70	3.31	3.22	1-100	0.9976	3.92	6.65	104.73	98.77
Isotodesnitazene	2.55	2.92	97.92	97.04	2.55	2.92	1-100	0.9955	3.59	9.85	104.11	92.83
Isotonitazene	2.00	1.84	91.87	91.55	2.00	1.84	1-100	0.9914	2.96	7.28	97.68	91.34
MDDMA	3.70	3.68	91.56	92.60	3.70	3.68	1-100	0.9966	1.75	8.57	106.17	95.51
MDMB-4en-PINACA	2.24	2.53	90.60	90.62	2.24	2.53	1-100	0.9934	2.70	3.82	96.24	94.01
MDMB-4en-PINACA 3,3-Dimethylbutanoic Acid	5.23	5.63	98.65	102.56	5.23	5.63	1-100	0.9916	3.89	5.76	91.19	94.66

Table 2. Statistical results for the 130 NPS extracted from human whole blood samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
Meclonazepam	3.94	4.28	91.97	91.15	3.94	4.28	1-100	0.9966	4.38	8.12	107.43	99.08
Methoxy U-47700	4.05	3.69	92.69	93.43	4.05	3.69	1-100	0.9942	4.03	8.01	99.71	91.42
Methylenedioxy-PV8	1.98	2.21	90.84	91.41	1.98	2.21	1-100	0.9961	2.16	8.05	102.03	92.73
Metodesnitazene	2.69	3.21	101.67	103.43	2.69	3.21	1-100	0.9970	1.60	8.11	109.07	98.78
Metonitazene	7.18	7.38	100.82	100.13	7.18	7.38	1-100	0.9966	5.05	6.09	97.98	93.56
MXPr	4.53	5.14	90.93	93.28	4.53	5.14	1-100	0.9979	3.43	6.95	107.37	99.27
N-Butyl Hexedrone	7.41	7.77	96.58	101.01	7.41	7.77	5-100	0.9916	2.54	7.25	93.36	102.64
N-Desethyl Etonitazene	3.76	5.02	97.05	94.63	3.76	5.02	1-100	0.9954	4.27	3.85	104.22	104.76
N-Desethyl Isotonitazene	1.47	2.83	102.53	99.87	1.47	2.83	1-100	0.9967	2.57	10.22	107.67	95.49
N-Desmethyl U-47700	4.71	4.60	95.30	96.43	4.71	4.60	1-100	0.9969	3.79	6.27	108.57	101.92
N-Ethyl Deschloroketamine (O-PCE)	3.12	4.64	90.24	93.52	3.12	4.64	5-100	0.9954	2.97	7.60	106.47	97.37
N-Ethyl Heptedrone	3.05	3.20	92.97	91.31	3.05	3.20	1-100	0.9905	3.71	8.05	103.03	93.90
N-Ethyl Pentedrone	2.16	1.91	90.78	90.69	2.16	1.91	1-100	0.9924	3.83	9.48	102.29	92.01
N-Ethyl Pentylone	4.00	3.80	93.09	93.97	4.00	3.80	1-100	0.9964	3.47	7.95	106.57	98.72
N-Ethylhexedrone	1.80	2.63	90.06	90.87	1.80	2.63	1-100	0.9913	4.96	7.95	101.57	93.87
Nitrazolam	5.16	4.58	98.89	97.72	5.16	4.58	1-100	0.9974	4.37	7.41	109.93	101.88
N-Methyl-2AI	3.93	3.76	99.93	99.19	3.93	3.76	1-100	0.9961	3.76	6.73	105.63	98.69
N-Methylethylone	5.08	5.19	93.07	95.37	5.08	5.19	1-100	0.9962	2.17	5.38	106.97	101.48
N-Methyl-Hexylone	3.99	3.80	93.58	94.47	3.99	3.80	1-100	0.9963	2.27	8.22	108.77	99.48
N-Piperidinyl Etonitazene	2.44	2.81	102.81	103.71	2.44	2.81	1-100	0.9971	1.60	8.14	109.17	98.84
N-Pyrrolidino Etonitazene (Etonitazepyne)	7.29	7.19	94.41	96.66	7.29	7.19	1-100	0.9968	2.43	7.65	101.60	92.87
para-bromo Fentanyl	3.96	4.81	99.05	97.42	3.96	4.81	1-100	0.9951	2.26	3.71	93.93	90.49
para-Chlorofentanyl	4.77	9.49	92.91	99.70	4.77	9.49	1-100	0.9951	4.17	9.13	105.90	95.78
para-Fluoro Phenethyl 4-ANPP	5.76	7.48	97.99	103.42	5.76	7.48	1-100	0.9938	5.49	6.09	100.79	98.89
para-Fluorofentanyl	7.29	3.43	90.93	93.42	7.29	3.43	1-100	0.9967	3.43	6.69	100.81	102.64
para-methyl AP-237	5.50	5.54	97.96	97.82	5.50	5.54	1-100	0.9960	4.90	8.82	100.59	97.21
para-Methyltetrahydrofuranylfentanyl	3.41	3.88	92.60	92.22	3.41	3.88	1-100	0.9971	1.64	8.64	109.73	98.68
PCE	7.34	8.58	97.66	98.14	7.34	8.58	5-100	0.9946	8.61	8.43	96.98	99.10
Pentylone	5.08	5.19	93.07	95.37	5.08	5.19	1-100	0.9962	2.98	4.98	104.26	100.71
Phenazolam	5.44	6.30	96.28	91.72	5.44	6.30	1-100	0.9975	4.50	7.36	103.40	95.61
Phenethyl 4-ANPP	7.16	5.32	92.40	92.84	7.16	5.32	1-100	0.9946	8.70	7.35	102.46	103.86

Table 2. Statistical results for the 130 NPS extracted from human whole blood samples using DDA and SWATH DIA. Continued

Compound	DDA						SWATH DIA					
	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)	Cal range (ng/mL)	R^2 value	Intra-day accuracy at 10 ng/mL (%bias)	Inter-day accuracy at 10 ng/mL (%bias)	Intra-day precision at 10 ng/mL (%CV)	Inter- day precision at 10 ng/mL (%CV)
Piperidylthiambutene	*6.44*	*5.88*	*98.82*	*99.95*	*6.44*	*5.88*	*1-100*	*0.9970*	*5.17*	*6.84*	*92.45*	*90.51*
Protonitazene	*4.95*	*5.07*	*90.66*	*93.46*	*4.95*	*5.07*	*1-100*	*0.9966*	*7.40*	*9.32*	*93.33*	*96.31*
Tenocyclidine	*3.59*	*5.22*	*92.13*	*97.03*	*3.59*	*5.22*	*5-100*	*0.9953*	*4.33*	*6.35*	*103.73*	*99.33*
U-48800	*2.93*	*3.87*	*90.23*	*90.27*	*2.93*	*3.87*	*1-100*	*0.9974*	*3.02*	*3.27*	*96.56*	*94.59*
U-49900	*4.03*	*3.98*	*91.50*	*91.14*	*4.03*	*3.98*	*1-100*	*0.9968*	*3.36*	*9.14*	*107.00*	*96.30*
UF-17	*4.19*	*4.76*	*91.88*	*93.56*	*4.19*	*4.76*	*1-100*	*0.9976*	*3.57*	*7.35*	*108.47*	*99.93*

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Wide Compound Coverage for Confident Analyte Identification Using SWATH® Acquisition on the SCIEX TripleTOF® and X-Series QTOF Systems

Using the Wiley MMHW LC-MS/MS Spectral Library

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

The ability to develop comprehensive screening procedures for the detection of toxic compounds, drugs of abuse and their metabolites is critical for forensic laboratories to accurately identify and quantify substances with a high level of confidence. SWATH® Acquisition on the SCIEX TripleTOF® and X-Series QTOF Systems provides a fast, comprehensive analysis of forensics samples and allow detection and identification of drugs, metabolites and other emerging compounds present in complex biological matrices.

The Wiley MMHW LC-HR-MS/MS spectral library enables accurate detection and identification of novel psychoactive substances (NPS), toxic compounds and drugs of abuse through library spectral matching. The flexibility of SCIEX accurate mass instruments and supporting software allows the acquisition of high resolution MS/MS spectra, enabling library searching using the Wiley MMHW LC-HR-MS/MS library for increased confidence in compound identification.

In the example below, a processing method was built in SCIEX OS Software by adding the precursor and fragment ions of various drugs of abuse in the components table. The “Library Search” function was enabled and the Wiley MMHW LC-MS/MS Spectral Library was selected. As shown in Figure 1, pentylone, fentanyl, 24I-NBOMe and ADB-FUBINACA were detected from a urine sample spiked with multiple compounds of interest. Multiple points of high-quality data such as analyte retention time and exact fragment masses are used to detect and identify these drugs of abuse. The exact fragment masses (m/z 236.128, 337.227, 428.072, and 383.188 Da) were extracted and the experiment fragment mass errors were found to be 1.9, 0.3, 1.7 and -0.5 ppm for pentylone, fentanyl, 24I-NBOMe and ADB-FUBINACA, respectively. These mass errors indicate excellent mass accuracy across the compounds detected. Further, the MS/MS spectra match those of the library with a fit score above 99% for all the analytes detected.

Key Advantages of SWATH Acquisition with the Wiley MMHW LC-MS/MS Library

- High speed acquisition of the TripleTOF® and X-Series QTOF Systems enables the use of SWATH Acquisition, even at fastest gradient speeds
- Comprehensive MS and MS/MS data collected on every sample for wide compound coverage
- Compatible with the Wiley MMHW LC-HR-MS/MS spectral library which includes over 5,000 drugs and metabolites, for drugs commonly tested in forensic samples, including blood and urine
- Library is compatible with SWATH® Acquisition workflows on the TripleTOF®, X-Series QTOF and QTRAP® Systems with SCIEX OS Software
- Accurate screening of drug metabolites increases selectivity, allows confirmation of the body passage and minimizes the risk of false negative results, hence enhancing the screening accuracy of illicit substances and other drugs of abuse

Analyte: Pentylone
RT: 4.89 min
Precursor m/z: 236.128 Da
Mass error: 1.9 ppm
MS/MS Fit: 100%

Analyte: Fentanyl
RT: 6.10 min
Precursor m/z: 337.227 Da
Mass error: 0.3 ppm
MS/MS Fit: 99.2%

Analyte: 25I-NBOMe
RT: 7.36 min
Precursor m/z: 428.072 Da
Mass error: 1.7 ppm
MS/MS Fit: 100%

Analyte: ADB-FUBINACA
RT: 8.49 min
Precursor m/z: 383.188 Da
Mass error: -0.5 ppm
MS/MS Fit: 100%

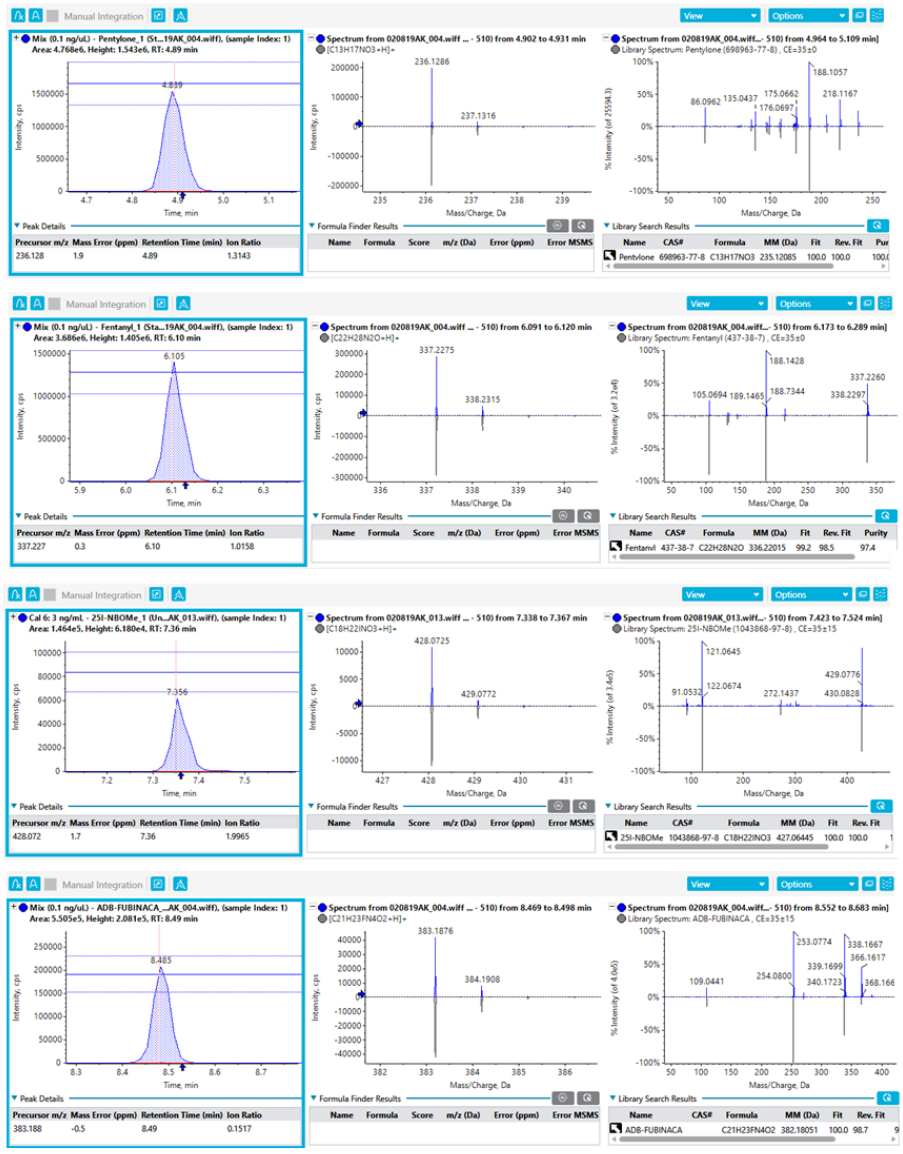

Figure 1: Gain Comprehensive Analyte Coverage and Increase Confidence in Analyte Identification Using the Wiley MMHW LC-HR-MS/MS Library. XIC, TOF MS and MS/MS spectra obtained showing confident and accurate identification of pentylone, fentanyl, 24I-NBOMe and ADB-FUBINACA from a urine sample spiked with several forensic compounds of interest. The sample was analyzed using SWATH® Acquisition on the SCIEX X500R QTOF System and the acquired data was searched against the Wiley MMHW LC-HR-MS/MS Library.

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