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Quantitation of novel psychoactive substances in wastewater by direct injection analysis

Using the SCIEX 7500 system powered by SCIEX OS software

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Introduction

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

Wastewater surveillance of illicit drugs has been routinely used to study recreational drug consumption trends in many countries.[2] There has been an emerging interest in monitoring NPS at hotspots, such as music festivals at which drug usage might be increased.[1,3] Direct injection analysis minimizes contamination and irreproducible results but is challenged by low concentrations of NPS due to their infrequent consumption and their dilution in wastewater. Solid phase extraction (SPE) and liquid-liquid extraction (LLE) are typically required for the quantitation of low-level NPS, but these methods are laborious and time-consuming.[4,5] In addition, extraction conditions must be optimized to cover the wide range of physicochemical properties of different classes of NPS, such as synthetic cannabinoids, synthetic

cathinones, synthetic opioids, benzodiazepines and phenylethylamines. In this work, the sensitivity of the SCIEX 7500 system allowed direct injection analysis of low-level NPS present in wastewater while enabling matrix dilution of interferences that would have otherwise been concentrated during SPE and LLE. High-throughput direct injection analysis can rapidly provide wastewater surveillance data to inform public health alerts and proactive drug education programs.

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

- High sensitivity of the SCIEX 7500 system achieved low- to mid-ppt (0.5–195.3 ng/L) LOQs, resulting in the detection of eutylone, etizolam and clonazolam in Australian wastewater for the first time by direct injection

- A rapid direct injection LC-MS/MS method for the analysis of 32 NPS in wastewater was developed and validated

- Reduced matrix interferences from direct injection enabled the use of solvent-based calibration for quantitation

- Custom calculations and flagging in SCIEX OS software expedited the review of method validation acceptance criteria
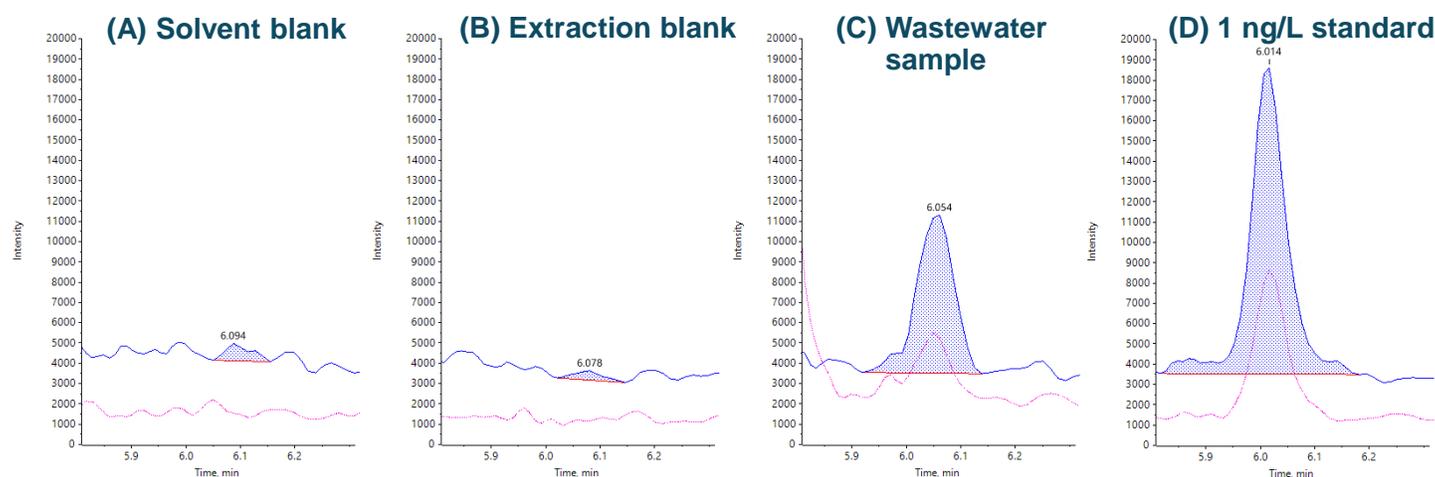
Figure 1. Detection of eutylone in wastewater compared to quality control samples. A) MilliQ water solvent blank. B) Extraction blank comprised of acidified MilliQ water prepared in the same manner as the wastewater samples. C) Wastewater sample. D) 1 ng/L standard. The blue trace represents the quantifier transition (m/z 236.0 > 188.1) and the pink trace represents the qualifier transition (m/z 236.0 >174.0).

Experimental methods

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

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

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

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

Table 1. Chromatographic gradient.

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

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

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

Table 2. Source, gas and temperature conditions.

Parameter	Value
Curtain gas (CUR)	40 psi
Collision gas (CAD)	10 psi
IonSpray voltage (ISV)	2600 V
Temperature (TEM)	450°C
Nebulizer gas (GS1)	60 psi
Heater gas (GS2)	60 psi
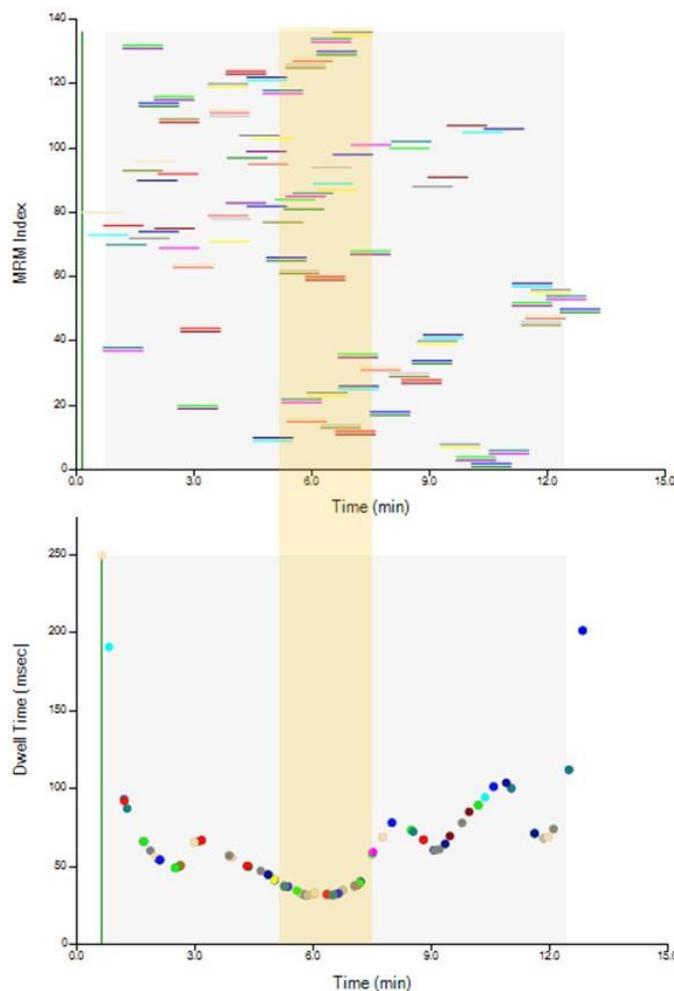
Figure 2. sMRM summary plots of the RT distribution of analytes (top) and their dwell times (bottom). The yellow bar indicates regions of high MRM concurrency, as shown by the lower dwell times for those transitions.

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

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

Smaller injection load to reduce matrix effects

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

Sample Name	Sample ID	Sample Type	Inj... Vo...	Compo... Name	Ret... Time	Area	Ion Ratio	Ion Ratio...	RT Co...	*Solve...	*ME Low	*ME Med	*ME High	*RSD Low	*RSD Med	*RSD High
5 ppt	Solvent	Standard	10.00	Eutylone 1	6.08	2.543e5	0.4173	✔	✔							
50 ppt	Solvent	Standard	10.00	Eutylone 1	6.07	1.735e6	0.4147	✔	✔							
500 ppt	Solvent	Standard	10.00	Eutylone 1	6.07	2.409e7	0.3868	✔	✔							
WW 5 ppt replicate 1	Low	Quality Control	10.00	Eutylone 1	6.06	2.177e5	0.3882	✔	✔	2.543e5	86			2.0		
WW 5 ppt replicate 2	Low	Quality Control	10.00	Eutylone 1	6.08	2.179e5	0.4435	✔	✔	2.543e5	86			2.0		
WW 5 ppt replicate 3	Low	Quality Control	10.00	Eutylone 1	6.07	2.171e5	0.4525	✔	✔	2.543e5	85			2.0		
WW 5 ppt replicate 4	Low	Quality Control	10.00	Eutylone 1	6.06	2.206e5	0.4493	✔	✔	2.543e5	87			2.0		
WW 5 ppt replicate 5	Low	Quality Control	10.00	Eutylone 1	6.06	2.275e5	0.4362	✔	✔	2.543e5	89			2.0		
WW 50 ppt replicate 1	Med	Quality Control	10.00	Eutylone 1	6.07	2.182e6	0.4075	✔	✔	1.735e6		126			3.3	
WW 50 ppt replicate 2	Med	Quality Control	10.00	Eutylone 1	6.06	2.355e6	0.4035	✔	✔	1.735e6		136			3.3	
WW 50 ppt replicate 3	Med	Quality Control	10.00	Eutylone 1	6.07	2.255e6	0.3952	✔	✔	1.735e6		130			3.3	
WW 50 ppt replicate 4	Med	Quality Control	10.00	Eutylone 1	6.07	2.321e6	0.4138	✔	✔	1.735e6		134			3.3	
WW 50 ppt replicate 5	Med	Quality Control	10.00	Eutylone 1	6.07	2.364e6	0.4013	✔	✔	1.735e6		136			3.3	
WW 500 ppt replicate 1	High	Quality Control	10.00	Eutylone 1	6.06	2.600e7	0.3984	✔	✔	2.409e7			108			3.8
WW 500 ppt replicate 2	High	Quality Control	10.00	Eutylone 1	6.05	2.679e7	0.3949	✔	✔	2.409e7			111			3.8
WW 500 ppt replicate 3	High	Quality Control	10.00	Eutylone 1	6.06	2.660e7	0.3927	✔	✔	2.409e7			110			3.8
WW 500 ppt replicate 4	High	Quality Control	10.00	Eutylone 1	6.07	2.499e7	0.4018	✔	✔	2.409e7			104			3.8
WW 500 ppt replicate 5	High	Quality Control	10.00	Eutylone 1	6.08	2.456e7	0.4055	✔	✔	2.409e7			102			3.8
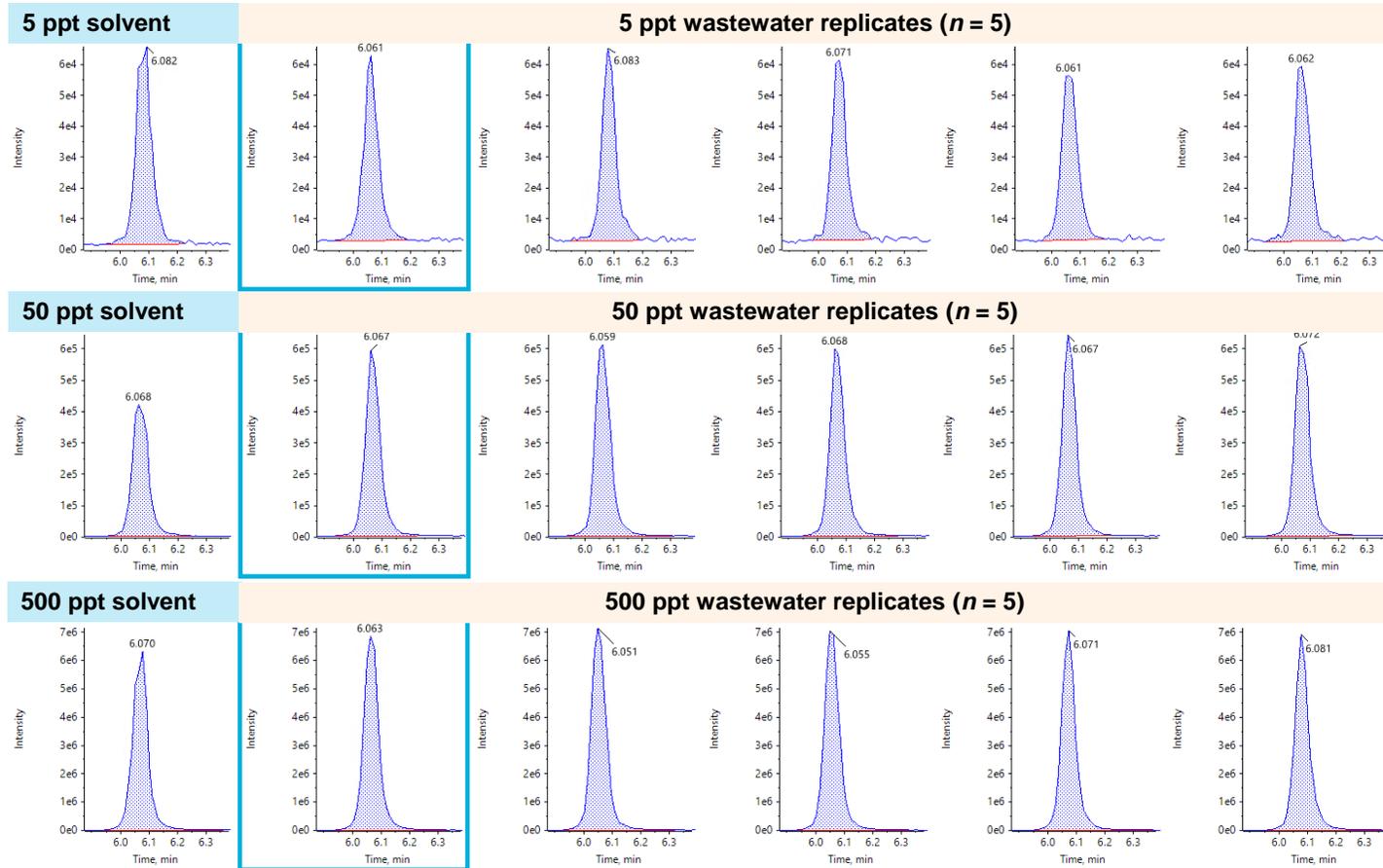
Figure 3. Matrix effects (%) for the quantifier transition of eutylone. Top) Results table showing parameters calculated for solvent spikes (highlighted in blue) and wastewater spikes (highlighted in brown). Bottom) Extracted ion chromatograms (XICs) demonstrating the peak intensity of eutylone in the spiked samples. The matrix effects were determined by calculating the percent quotient of the peak areas between wastewater (brown) and solvent (blue) spikes at low, medium and high concentration levels.
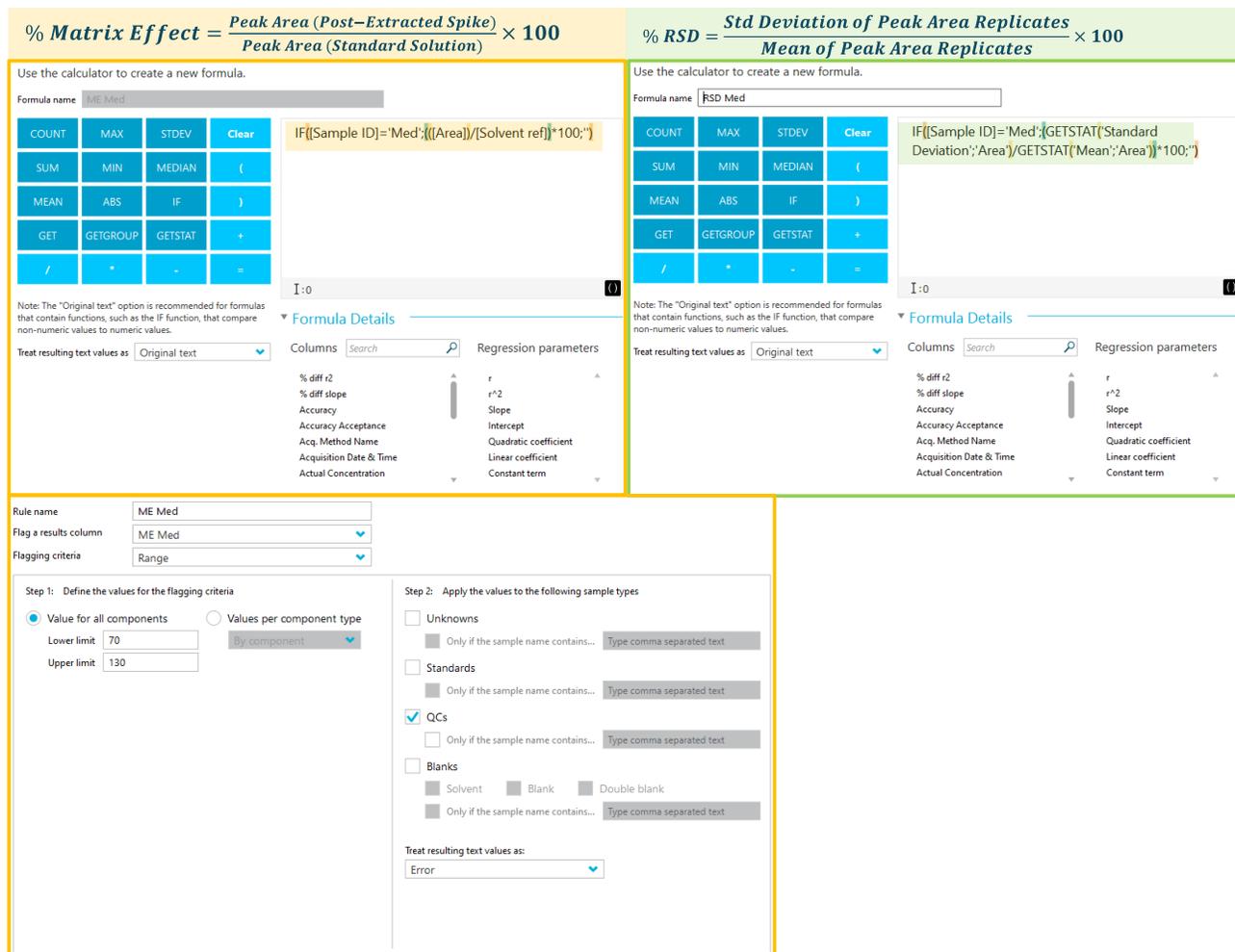
$$\% \, Matrix \, Effect = \frac{Peak \, Area \, (Post-Extracted \, Spike)}{Peak \, Area \, (Standard \, Solution)} \times 100$$

$$\% \, RSD = \frac{Std \, Deviation \, of \, Peak \, Area \, Replicates}{Mean \, of \, Peak \, Area \, Replicates} \times 100$$

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

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

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

Out-of-bound validation results were identified using flagging rules to highlight values that did not meet user-specified tolerance thresholds. For example, the calculated matrix effects for some of the 50 ppt wastewater spikes were flagged (red) in

Figure 3 because they exceeded the upper limit of 130%, as defined in Figure 4.

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

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

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

Compound	MRM transition (Q1 > Q3$_{quant}$, Q3$_{conf}$)	Internal standard	LOD (ng/L)	LOQ (ng/L)	Range (ng/L) (r^2)	Matrix Effects (%, %RSD)			Precision (%RSD)		
						Low	Med	High	Low	Med	High
25C-NBOMe	337.1 > 121.0, 91.0	EDDP-d3	8.7	28.9	10–500 (0.9980)		155 (4)	116 (2)		8.9	6.9
2F-Deschloroketamine	222.1 > 109.0, 163.1	Norketamine-d4	7.3	24.5	10–1000 (0.9979)		139 (3)	96 (2)		4.3	1.9
2-Methyl AP-237	287.2 > 117.1, 91.1	Fentanyl-d5	1.8	6.0	1.5–300 (0.9960)	84 (16)	104 (14)	90 (17)		4.7	1.9
2-Oxo-PCE	218.2 > 173.1, 145.1	Ketamine-d4	4.0	13.3	5–1000 (0.9985)	62 (3)	114 (1)	96 (1)	2.7	5.0	4.0
3-MMC	178.1 > 144.8, 160.0	Mephedrone-d3	3.7	12.2	5–500 (0.9989)	95 (3)	178 (2)	131 (1)	2.7	5.8	4.1
4-Fluoroamphetamine	154.1 > 109.0, 137.0	Oxycodone-d3	27.3	90.9	10–500 (0.9924)	N/A	135 (4)	122 (5)		5.6	5.9
5F-EMB-PICA	377.2 > 232.1, 144.0	Diazepam-d5	0.2	0.6	0.3–300 (0.9993)	59 (3)	117 (4)	97 (2)	2.4	4.1	2.8
5F-MDMB-PICA	377.2 > 232.1, 144.0	Diazepam-d5	0.2	0.7	0.5–1000 (0.9995)	63 (4)	124 (4)	105 (3)	2.8	5.2	2.1
5F-MDMB-PINACA	378.0 > 233.0, 318.0	Diazepam-d5	0.5	1.7	1–1000 (0.9990)	61 (3)	118 (2)	92 (3)	4.8	3.0	1.9
7-Hydroxymitragynine	415.3 > 190.0, 238.1	Cocaine-d3	4.5	15.2	5–1000 (0.9993)	90 (2)	152 (3)	125 (4)	2.2	3.7	5.4
AMB FUBINCA	384.2 > 109.0, 253.0	Diazepam-d5	5.5	18.2	5–1000 (0.9992)	60 (14)	134 2)	102 (1)		4.5	5.0
AP-238	287.2 > 117.1, 169.1	Oxycodone-d3	5.2	17.2	5–212 (0.9968)	107 (21)	83 (7)	69 (4)	9.7	4.2	2.5
Brorphine	400.1 > 218.1, 104.0	Fentanyl-d5	4.1	13.7	1–200 (0.9985)	N/A	N/A	118 (3)			9.5
Butylone	222.1 > 174.0, 131.0	Benzoylecgonine-d3	0.2	0.8	0.5–1000 (0.9987)	74 (3)	145 (4)	115 (4)	3.8	2.9	5.2
Clonazolam	354.0 > 308.0, 326.0	Diazepam-d5	2.7	8.9	5–500 (0.9978)	94 (3)	75 (3)		3.8	3.3	2.0
Cumyl pegaclone	373.2 > 255.1, 185.1	Diazepam-d5	2.4	8.1	1–1000 (0.9974)	45 (2)	109 (6)	100 (5)	3.7	6.0	5.9
Cumyl-5F-pegaclone	391.2 > 273.1, 119.1	Diazepam-d5	2.1	7.1	5–1000 (0.9984)	45 (5)	96 (5)	66 (5)	3.5	3.1	4.2
Dibutylone	236.1 > 161.0, 86.0	Benzoylecgonine-d3	1.5	5.2	5–1000 (0.9981)	101 (3)	144 (2)	104 (4)	6.5	5.2	4.2
Etizolam	343.0 > 314.0, 289.1	Diazepam-d5	0.9	2.9	1–1000 (0.9957)	64 (4)	96 (4)	81 (1)	6.8	1.9	5.4
Eutylone	236.0 > 188.1, 174.0	Benzoylecgonine-d3	0.2	0.6	0.5–1000 (0.9996)	87 (2)	132 (4)	107 (4)	2.9	2.5	3.8
Flualprazolam	327.2 > 292.2, 223.0	Temazepam-d5	0.1	0.5	0.5–1000 (0.9976)	50 (3)	96 (2)	80 (3)	1.9	6.0	4.9
Flubromazolam	371.0 > 223.0, 292.0	Temazepam-d5	15.8	52.6	10–1000 (0.9942)		101 (3)	76 (2)		5.5	3.6
Isotonitazene	411.2 > 100.0, 106.9	EDDP-d3	22.1	73.5	1–1000 (0.9983)	101 (4)	215 (4)	155 (3)	9.4	3.5	3.6
MDMB-4en-PINACA	358.2 > 298.2, 213.1	Diazepam-d5	0.2	0.5	0.5–1000 (0.9988)	53 (3)	107 (3)	89 (3)	4.7	3.3	3.7
Methcathinone	164.3 > 130.2, 146.2	Amphetamine-d6	3.2	10.6	10–500 (0.9965)	119 (6)	215 (2)	161 (1)		2.9	4.1
Metonitazene	383.2 > 100.0, 121.0	Fentanyl-d5	7.5	24.9	1–1000 (0.9984)		220 (1)	144 (2)	2.6	4.6	3.6
Mitragynine	399.2 > 174.1, 159.0	EDDP-d3	44.8	149.3	50–1000 (0.9804)		187 (4)	125 (3)		5.5	3.9
N-ethylheptedrone	234.1 > 146.1, 91.1	Cocaine-d3	5.4	18.0	5–1000 (0.9993)		130 (4)	101 (1)		2.0	3.9
N-ethylhexedrone	220.2 > 130.0, 91.1	MDMA-d5	58.6	195.3	50–1000 (0.9983)			103 (5)			4.7
N-ethylpentylone	250.2 > 202.0, 175.3	MDMA-d5	4.5	15.2	5–1000 (0.9969)	82 (4)	134 (3)	103 (5)	5.6	3.3	3.3
Pentylone	236.0 > 188.0, 205.0	MDMA-d5	0.2	0.8	0.5–1000 (0.9996)	65 (5)	127 (5)	112 (3)	5.6	4.6	2.4
Protonitazene	411.2 > 100.0, 72.1	EDDP-d3	0.5	1.7	1–1000 (0.9969)	104 (4)	229 (3)	159 (2)	3.7	1.3	3.0

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

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

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

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

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

Calibration for Eutylone 1: y = 4.47620e4 x + 2.94261e5 (r = 0.99652, r² = 0.99304) (weighting: None)

Calibration for Eutylone 1: y = 4.72216e4 x + -1.20783e5 (r = 0.99980, r² = 0.99959) (weighting: None)

Wastewater-based calibration

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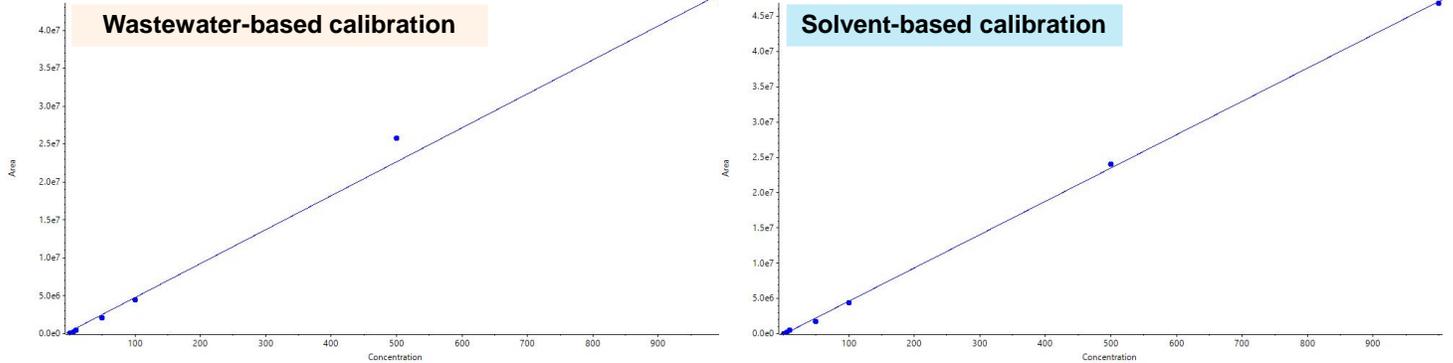
Figure 5. Comparison of regression parameters derived from wastewater- and solvent-based calibration curves. Top) Results table showing parameters calculated for wastewater- (highlighted in brown) and solvent-based (highlighted in blue) calibration samples. Custom formulas were used to calculate the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

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

Sample Name	Component Name	Area	*WW Prespk	*WW Matrix Rec
WW post spk 5 ppt	Eutylone 1	2.481e5	2.940e5	118.50
WW post spk 50 ppt	Eutylone 1	2.181e6	2.066e6	94.72
WW post spk 500 ppt	Eutylone 1	1.863e7	2.090e7	112.16
WW post spk 5 ppt	Etizolam 1	4.158e4	4.779e4	114.94
WW post spk 50 ppt	Etizolam 1	3.298e5	3.411e5	103.44
WW post spk 500 ppt	Etizolam 1	3.253e6	3.425e6	105.30
WW post spk 5 ppt	Clonazolam 1	5.237e4	5.132e4	98.00
WW post spk 50 ppt	Clonazolam 1	2.904e5	2.854e5	98.27
WW post spk 500 ppt	Clonazolam 1	2.867e6	2.832e6	98.76
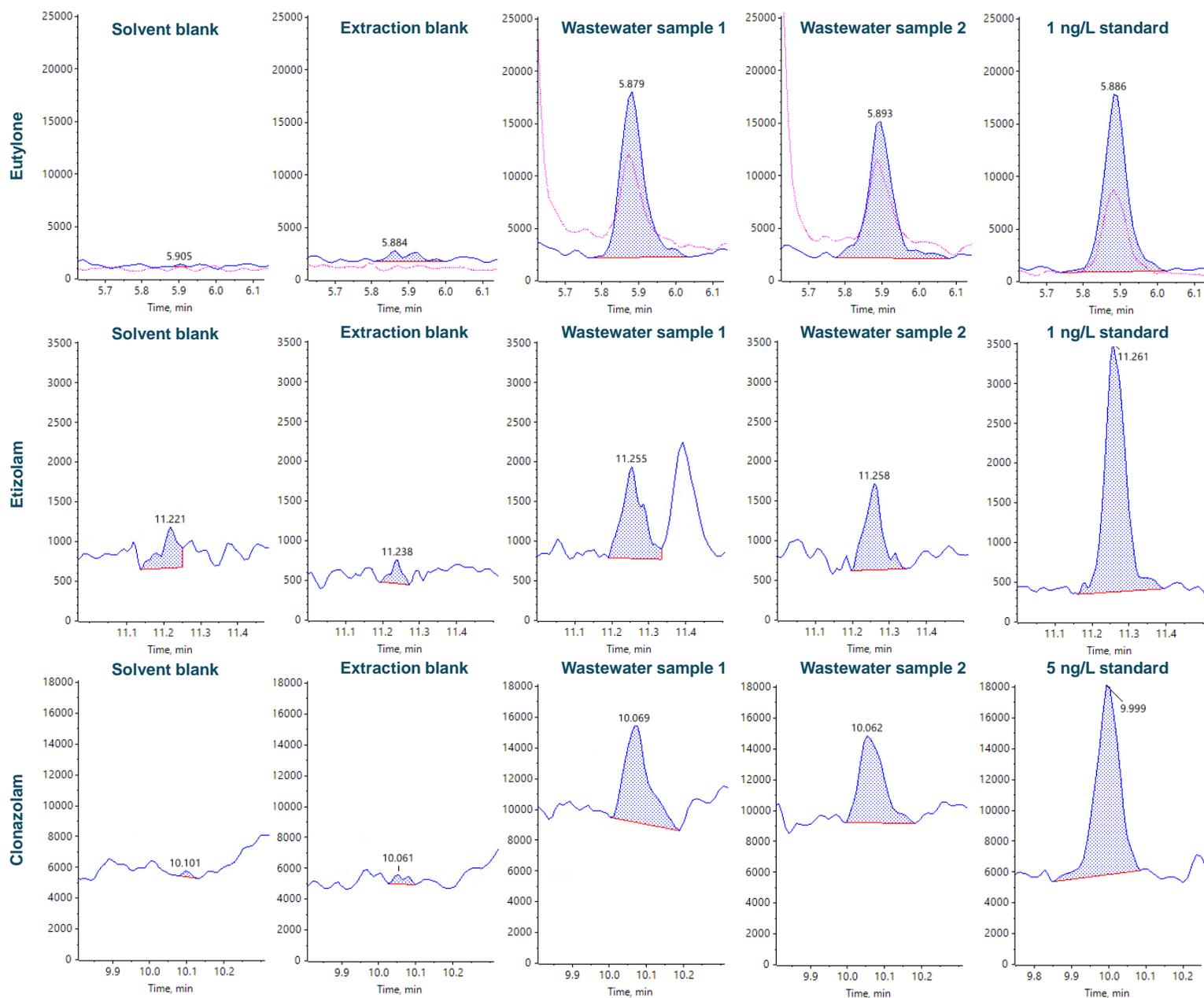
Figure 7. Detection of eutylone, etizolam and clonazolam in 2 wastewater samples compared to quality control samples. Top) Results for eutylone are shown. The blue trace represents the quantifier transition (*m/z* 236.0 > 188.1) and the pink trace represents the confirmation transition (*m/z* 236.0 > 174.0). Middle, Bottom) For both etizolam (middle) and clonazolam (bottom), only the quantifier transitions (*m/z* 343.0 > 314.0, etizolam and *m/z* 354.0 > 308.0, clonazolam) demonstrated peaks with matching RTs compared to the calibration standards. Neither of the confirmation transitions (*m/z* 343.0 > 289.1, etizolam and *m/z* 354.0 > 326.0, clonazolam) demonstrated any observable peaks. The solvent blank was MilliQ water and the extraction blank was acidified MilliQ water prepared in the same manner as the wastewater samples. The calibration standard was prepared at 1 ng/L for eutylone, 1 ng/L for etizolam and 5 ng/L for clonazolam.

Conclusions

- A rapid and sensitive direct injection method was developed for the quantitation of 32 NPS in wastewater with LOQs ranging from 0.5 to 195.3 ng/L

- Application of the method to Australian wastewater samples collected during the holiday period of 2021–2022 revealed the detection of eutylone, etizolam and clonazolam for the first time by a direct injection LC-MS/MS approach

- The high sensitivity of the SCIEX 7500 system enabled the use of a small injection volume (10 µL), which reduced matrix interferences without the need for SPE, LLE or any other pre-concentration steps

- Linearity performance was comparable between wastewater-based calibration and solvent-based calibration, which enabled the simpler solvent-based approach to be used for quantitation

- Acceptable method validation performance was achieved for a diverse range of NPS classes, allowing for easy expansion to include new and emerging substances in future analysis

- The speed and simplicity of the direct injection approach allowed for high-throughput and rapid turnaround time of wastewater surveillance of recreational drug use

- Visualization software tools, such as sMRM summary plots, provide real-time updates of large and complex acquisition methods while editing parameters that propagate across many MRM transitions

- Custom calculations enable the user to perform direct calculations in SCIEX OS software without the need to export the data elsewhere. Flagging rules expedite the review of outlier data in SCIEX OS software.

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