Application Note

High Throughput Determination of Emerging Synthetic Amphetamine Drugs in Untreated Wastewater Using SPE-LC-MS/MS

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Abstract

Sewage-based epidemiology (SBE) is a pioneering approach utilizing sewage samples to detect and quantify illicit drugs usage trends within a community. While SBE has been traditionally applied to estimate the prevalence of conventional illicit drugs since 2005, there has only been limited exploration on the detection of new psychoactive substances (NPS) in untreated wastewater samples. These substances mimic the effects of illicit drugs with the introduction of subtle modifications to the chemical structures of controlled illicit drugs. Identifying and determining these NPS poses challenges for government control bodies due to the evolving nature of their production, while presenting a pressing concern.

In this study, we present a new analytical method employing liquid chromatography coupled to positive electrospray ionization tandem quadrupole mass spectrometry (LC-ESI-MS/MS) for the determination of 18 emerging amphetamine NPS in sewage wastewater samples. These substances include methoxetamines (MXE, MBDB, MDA, MDEA, MDMA, MDPV), Butylone, methylone, 4-methoxyamphetamine (PMA), 1,3-Benzodioxolyl butamine (BDB), 4-Bromo-2,5-Dimethoxy phenethylamine (2C-B), and 2,5-Dimethoxy-4-iodo phenethylamine (2C-I), among others.

Sample preparation involved solid-phase extraction (SPE) utilizing an Oasis™ MCX LP 96 Well Plate. The LC separation was achieved using an ACQUITY™ Premier BEH™ C_{18} Column (1.7 µm, 2.1 x 100 mm), ensuring optimal resolution of analytes within a total runtime of 12 minutes. The limits of quantification (LOQ) ranged between 0.033–300.0 µl/L for all compounds on the Xevo™ TQ-XS Tandem Quadrupole Mass Spectrometer. Method validation encompassed sensitivity, specificity, linearity, accuracy, precision, recoveries, and matrix effects.

Benefits

- Lower untreated wastewater sample processing volumes and simplified analytical workflows resulted in quicker analysis times and the potential for high sample throughput
- In-house method validation demonstrated the performance of the method and its ease of implementation
- Method is suitable for high throughput analysis of 18 different amphetamine drugs in untreated wastewater to check compliance against regulatory limits

Introduction

The rapid emergence of numerous new psychoactive substances (NPS) known as legal highs, designer drugs, or bath salts synthacaines in the illegal drug market poses significant risk to public health and challenges drug policy creation.[1,2,3] While traditional illicit drugs (***e.g.,*** cannabis, cocaine, heroin, ecstasy) continue to dominate the market, the significant rise in the variety of NPS with largely unknown toxicological effects and social harm warrants increased vigilance. Continuous monitoring, information sharing, early warning and risk awareness are essential to respond to the emerging NPS. A large portion of these NPS comprise various amphetamine-like substances, including synthetic cathinones and sympathomimetic amines which are designed to replicate the pharmacological effects of the original drugs. Demand is increasing for the analysis and identification of a large number of chemically diverse substances present in drug markets in different sample matrices.[4,5,6]

The quantification of drugs of abuse (DOA) in aquatic habitats has grown in popularity in recent years due to advantages such as their sample collection being a non-invasive technique of larger sample volumes with no legal concerns. Quantitative examination of untreated wastewater provides significant data for estimating drug consumption at local and national levels, as well as providing insights through comparison against international data. Routine monitoring can establish an early warning system for the government bodies. This approach is a viable path for epidemiological research on illegal drug use and addiction, as it provides real-time, evidence-based insights that supplement survey-based studies.

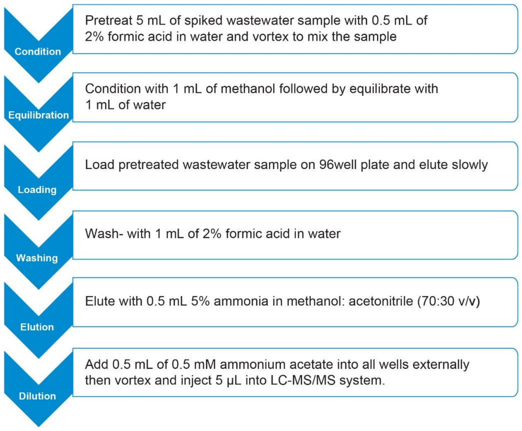
Accurate and reliable methods with the lowest possible LOQ are essential for monitoring untreated wastewater samples to understand the prevalence of these compounds and assess their temporal variability.[1] This study aims to develop and validate a high-throughput, multi-residue chromatographic method for the detection of 18 synthetic amphetamine designer drugs at the possible LOQ in untreated wastewater samples. The method focuses on synthetic cathinones and other substituted phenylalkylamines, providing a tool for early detection of these compounds in urban communities as part of drug abuse mitigation efforts, as well as studying their occurrence and fate in aquatic environments.[1]

Experimental

Calibration Standards and Sample Preparation

An analytical standard mix of 18 amphetamine analog drugs was used to prepare an intermediate working solution. This was then used to create 8 calibration standards and three QC standards at different levels independent of the calibration concentrations in untreated wastewater matrix. The calibration standards were prepared over the range of 0.033 to 300 µg/L. An internal standard mix was prepared and added before sample extraction.

Untreated wastewater samples were screened and stored at 4 °C. For analysis, aliquots (5 mL) were transferred to 15 mL centrifuge tubes, spiked with the internal standard mix and acidified. Before loading the samples onto the SPE 96 well plate, they were vortexed. The detailed extraction protocol is shown in Figure 1.



Pretreat 5 mL of spiked wastewater sample with 0.5 mL of 2% formic acid in water and vortex to mix the sample

Condition with 1 mL of methanol followed by equilibrate with 1 mL of water

Load pretreated wastewater sample on 96well plate and elute slowly

Wash- with 1 mL of 2% formic acid in water

Elute with 0.5 mL 5% ammonia in methanol: acetonitrile (70:30 v/v)

Add 0.5 mL of 0.5 mM ammonium acetate into all wells externally then vortex and inject 5 µL into LC-MS/MS system.

Figure 1. SPE workflow to extract Amphetamines from untreated wastewater.

LC Conditions

LC system:	ACQUITY UPLC I-Class Plus System with BSM and FTN SM
96-well collection plate:	Polypropylene 2 mL Square Collection Plate (p/n: 186002482)
Column:	ACQUITY Premier BEH C_{18} (1.7 µm, 2.1 x 100 mm) (p/n: 186009453)
Column temperature:	55 °C

Sample temperature:	10 °C
Injection volume:	5 µL
Mobile phase A:	0.5 mM ammonium acetate with 0.025% acetic acid in water
Mobile phase B:	methanol:acetonitrile (70:30 v/v) with 0.025% acetic acid

Gradient Table

Time (min)	Flow (mL/min)	%A	%B
0	0.35	98.0	2.0
0.50	0.35	98.0	2.0
0.60	0.35	85.0	15.0
8.0	0.35	45.0	55.0
9.50	0.35	2.0	98.0
10.50	0.35	2.0	98.0
10.60	0.35	98.0	2.0
12.00	0.35	98.0	2.0

MS Conditions

MS system:	Xevo TQ-XS
Ionization mode:	Electrospray (positive ion mode)
Capillary voltage:	1.2 kV
Source temperature:	150 °C

Disolvation temperature:	600 °C
Disolvation gas flow:	1000 L/hr
Cone gas flow:	150 L/hr

Data Management

MS acquisition software:	MassLynx™ 4.2 Software
Quantitation software:	TargetLynx™ 4.2 Software

Method Validation

Single laboratory validation was performed by replicate analysis of spiked, screened blank, untreated wastewater samples. The following performance factors were assessed: specificity, sensitivity, calibration graph characteristics, trueness (% recovery), repeatability (RSD_r), and within-laboratory reproducibility (RSD_{ip}), also called intermediate precision. Trueness and RSD_{ip} were determined from replicate analysis (n=18) of spiked samples prepared at three QC levels HQC, MQC, and LQC in screened, untreated wastewater matrix and analyzed on three different days.

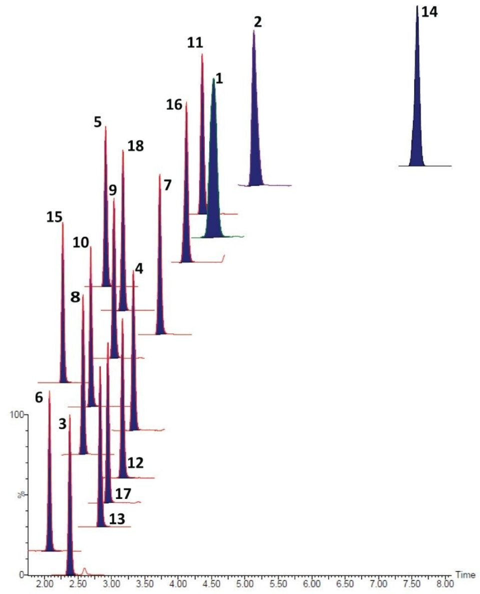
Results and Discussion

Chromatography and Sensitivity

The method provided excellent chromatographic retention and peak shape for all the amphetamine drugs. Average retention time and %RSD in untreated wastewater (n=42) are summarized in Table 1 with excellent retention time reproducibility observed and all %RSDs <0.21. Figure 2 shows typical total ion chromatograms (TIC) for all 18 amphetamine analogs, from the analysis of untreated wastewater samples spiked at the LOQ level. The observable peak shapes and signal-to-noise ratios demonstrate that the high throughput method can quantify these analytes in a complex untreated wastewater matrix at low concentrations. Typical TICs of the isotope labelled internal standards in untreated wastewater are shown in Figure 3.

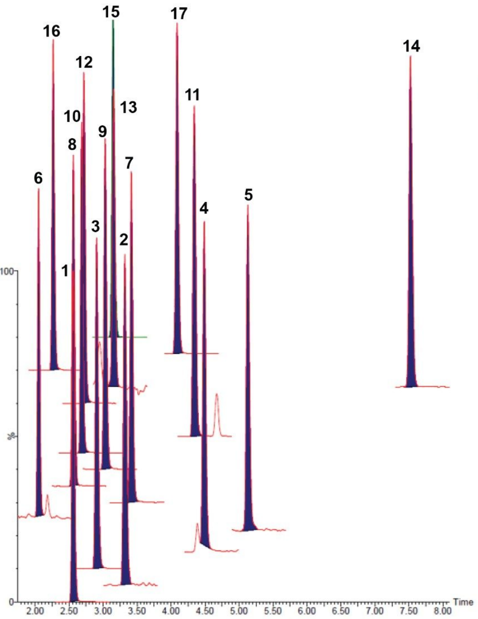
Analyte	Precursor Ion (*m/z*)	Quantifier Ion/ qualifier ion (*m/z*)	Cone voltage (V)	Collision energy (CV)	Average RT in min (n=26).	RT %RSD (n=26)
2C B	260.0	228.0	25	16	3.96	0.13
		244.0		14		
2C I	309.0	277.0	20	13	4.50	0.00
		292.0		10		
Amphetamine	136.0	91.0	25	19	2.27	0.09
		119.0		15		
BDB	194.0	147.0	15	21	2.90	0.17
		177.0		21		
Butylone	221.8	173.8	15	15	2.57	0.18
		203.7		12		
Cathinone	150.0	105.0	12	22	1.87	0.20
		117.0		22		
MBDB	209.0	136.0	20	22	2.99	0.07
		178.0		25		
MDA	180.0	133.0	15	20	2.28	0.21
		164.0		20		
MDEA	209.0	106.0	11	22	2.67	0.18
		164.0		20		
MDMA	194.1	105.0	12	11	2.37	0.20
		163.0		18		
MDPV	276.1	126.0	17	23	3.82	0.05
		134.9		25		
Mephedrone	178.1	144.9	24	19	2.70	0.00
		160.0		15		
Methamphetamine	150.0	90.9	23	15	2.41	0.17
		118.9		9		
Methaqualone	251.0	91.0	5	41	7.38	0.05
		131.9		27		
Methylone	208.1	132.1	25	29	2.03	0.00
		160.1		20		
Methylphenidate	234.1	55.9	39	39	3.63	0.09
		83.9		19		
PMA	166.0	121.0	18	25	2.46	0.16
		149.0		25		
Ritalinic acid	220.0	55.9	41	35	3.05	0.09
		83.9		17		

Table 1. Retention performance of the amphetamines with optimized MRM transitions on the XEVO TQ-XS.



L. no	Internal standards
1	2C B
2	2C I
3	Amphetamine
4	BDB
5	Butylone
6	Cathinone
7	MBDB
8	MDA
9	MDEA
10	MDMA
11	MDPV
12	Mephedrone
13	Methamphetamine
14	Methaqualone
15	Methylone
16	Methylphenidate
17	PMA
18	Ritalinic acid

Figure 2. Typical total ion chromatograms of all 18 amphetamine analogs from the analysis of untreated wastewater samples spiked at LOQ level.

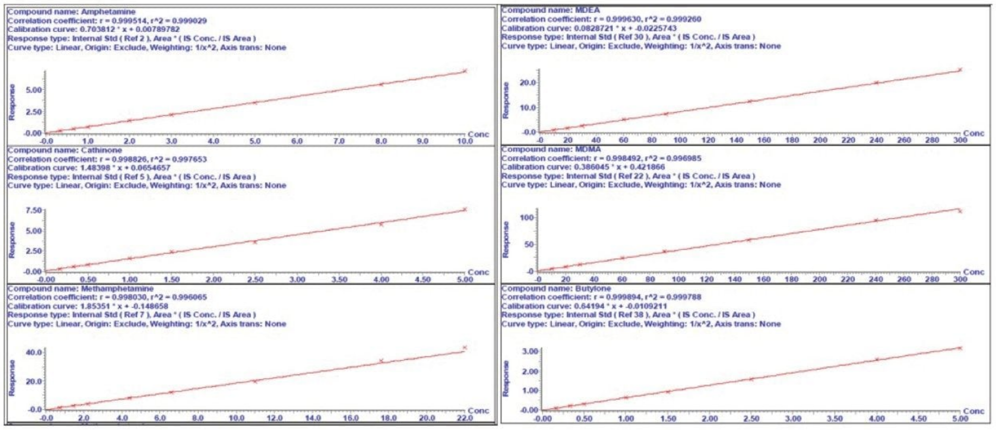


L. No	Internal standards
1	Amphetamine-d6
2	BDB-d2
3	Butylone-d2
4	2C-B-d5
5	2C-I-d5
6	Cathinone-d5
7	MBDB-d4
8	MDA-d5
9	MDEA-d4
10	MDMA-d2
11	MDPV-d7
12	Mephedrone-d2
13	Methamphetamine-d4
14	Methaqualone-d6
15	Methylone-d2
16	Methylphenidate-d9
17	Ritalinic acid-d3

Figure 3. Typical total ion chromatograms of all 17 Internal standards, from the analysis of raw wastewater samples. Note: MDA-d5 is used as ISTD for PMA.

Specificity, Identification, and Calibration Criteria

Untreated wastewater was screened and used as matrix blank to check the specificity of the method. No signal was detected in the blanks that could potentially lead to false reporting of non-compliant samples or impact the sensitivity of the method. The ion ratios and the respective retention times of each analyte were within the tolerances given in the ISO 21253–1:2019 standard (part 1).[7] 8-point calibration curves were acquired on each day after SPE sample extraction. Linear fit with $1/X^2$ weighing was applied and all correlation of determination (R^2) values were >0.99, with deviation from back calculated concentrations below 15%. Examples of typical calibration curves are given in Figure 4.



Compound name: Amphetamine
Correlation coefficient: r = 0.999514, r^2 = 0.999029
Calibration curve: 0.703812 * x + 0.00789782
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Compound name: MDEA
Correlation coefficient: r = 0.999630, r^2 = 0.999260
Calibration curve: 0.0828721 * x + -0.0225743
Response type: Internal Std (Ref 30), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Compound name: Cathinone
Correlation coefficient: r = 0.998826, r^2 = 0.997653
Calibration curve: 1.48398 * x + 0.0654657
Response type: Internal Std (Ref 5), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Compound name: MDMA
Correlation coefficient: r = 0.998492, r^2 = 0.996985
Calibration curve: 0.386045 * x + 0.421866
Response type: Internal Std (Ref 22), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Compound name: Methamphetamine
Correlation coefficient: r = 0.998030, r^2 = 0.996065
Calibration curve: 1.85351 * x + -0.148658
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Compound name: Butylone
Correlation coefficient: r = 0.999894, r^2 = 0.999788
Calibration curve: 0.64194 * x + -0.0109211
Response type: Internal Std (Ref 38), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None

Figure 4. Calibration graphs of exemplary amphetamines in untreated wastewater.

Laboratory Validation Results

Validation is crucial in analytical chemistry to evaluate the performance characteristics of an analytical method. Within-laboratory validation ensures that a method is fit for its intended purpose. The validation results demonstrated good accuracy for the quantification of all 18 compounds in untreated wastewater (Table 2). The accuracy was determined from the measured values of concentration in the spiked samples, after adjustment using the appropriate internal standard, Trueness, determined as measured mean %recovery (RSD_r) between 93.3 to 111.7% and within laboratory precision (RSD_{wr}) between 1.97 and 11.8%. These values are within the acceptance criteria when using internal standards: "yield" (measured recovery) between 70 and 120% and intermediate precision (RSD_{ip}) ≤20%. Figure 5 shows a summary of the mean measured recovery values, with associated repeatability.

Compound	Linearity range in µg/L	Regression value r²	QC trueness (% Mean recovery) RSDr % (n=18)			Within laboratory precision (%CV) RSDwr% (n=18)		
			LQC (µg/L)	MQC (µg/L)	HQC (µg/L)	LQC	MQC	HQC
2C B	1,078 - 34,5	0.997	101.8	99.1	102.6	5.21	4.90	5.83
2C I	0,775 - 24,8	0.998	97.2	112.1	98.39	9.12	11.81	4.92
Amphetamine	0,330 - 10,0	0.999	98.7	99.3	98.8	5.90	5.41	2.84
BDB	0,863 - 27,6	0.991	98.1	101.9	103.4	7.67	4.82	3.24
Butylone	0,165 - 5,00	1.000	104.9	99.3	100.9	8.00	6.06	3.36
Cathinone	0,165 - 5,0	0.998	95.7	101.1	100.3	6.94	4.24	2.75
MBDB	0,943 - 30,2	0.997	103.1	95.9	102.5	3.91	2,63	1,97
MDA	9,90 - 300,0	0.998	97.3	103.9	101.83	5.50	4.22	2.05
MDEA	9,90 - 300,0	0.999	98.6	98.8	98.2	5.63	5.39	2.80
MDMA	9,90 - 300,0	0.997	93.3	104.1	99.7	6.34	5.06	2.89
MDPV	0,033 - 1,00	0.998	104.7	102.0	102.56	8.32	5.81	4.91
Mephedrone	0,033 - 1,00	0.999	101.6	100.5	101.1	4.82	4.03	2.90
Methamphetamine	0,726 - 22,0	0.996	100.5	99.1	99.3	5.68	5.20	4.27
Methaqualone	0,033 - 1,00	0.998	100.4	99.6	100.7	2.72	4.30	2.47
Methylone	0,165 - 5,00	0.995	99.8	96.1	99.5	4.49	4.52	3.47
Methylphenidate	0,033 - 1,00	0.998	100.2	97.1	99.9	5.34	3.81	4.73
PMA	9,90 - 300,0	0.945	97.2	111.7	101.7	11.2	6.59	9.25
Ritalinic acid	0,033 - 1,00	0.994	109.1	105.5	109.2	9.72	6.22	5.93

Table 2. Summary of the results from the laboratory validation study.

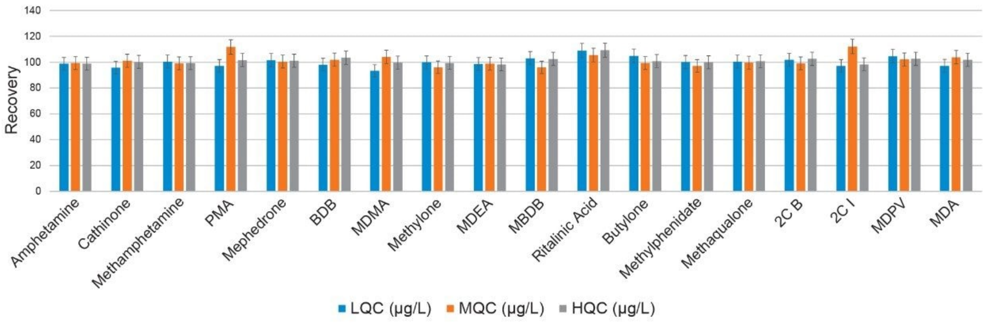


Figure 5. Overview of the mean measured recovery values at LQC, MQC, and HQC spiking levels (according to calibration range given in Table 2) in wastewater sample, with associated repeatability displayed in error bars.

Conclusion

Advancements in public health surveillance increasingly rely on robust, real-time data from unconventional sources like untreated wastewater. A newly developed inhouse analytical method now enables fast, sensitive, and high-throughput detection of 18 synthetic amphetamine analogs, marking a major step forward in wastewater-based epidemiology.

This innovative workflow involves injecting SPE-extracted untreated wastewater samples analysed on a Xevo TQ-XS tandem quadrupole mass spectrometer operated in positive ESI mode coupled to an ACQUITY Premier UPLC I-Class Plus System equipped with a ACQUITY BEH C_{18} Column. By automation we simplified the sample treatment process and reduced labour-intensive preparation. This method transforms routine testing into a faster and more scalable operation when compare with traditional methods.

Validated according to ISO 17025 and EPA guidelines, the method reliably detects compounds at concentrations as low as 0.033 µg/L using just 5 mL of sample volume. Its design enables high-throughput analysis of up to 100 samples per day, making it highly suitable for real-time public health monitoring and regulatory compliance.

The novelty of this method lies not only in its sensitivity and minimal sample volume but also in its operational efficiency. By integrating automated streamlined sample preparation with tandem mass spectrometry, it significantly enhances lab productivity while maintaining analytical integrity. This makes it particularly valuable for large-scale studies on human drug exposure and temporal trend analysis across urban populations.

This approach demonstrates how environmental monitoring can be both efficient and insightful — delivering near-real-time data that supports public health responses, urban drug policy, and environmental safety with unprecedented speed and accuracy.

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