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Nota applicativa

Combating New Psychoactive Substances (NPS) Abuse: Adapting a Forensic Toxicology Screening Solution for Environmental Wastewater Surveillance with ACQUITY UPLC H-Class PLUS Coupled with Xevo G2-XS QTof Instrument

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For forensic toxicology use only.

Abstract

This application note demonstrates how the advantages of the Forensic Toxicology Screening Application Solution with UNIFI™ can be used and adopted to provide comprehensive screening of new psychoactive substances (NPS) at low levels of concentration in wastewater. The approach incorporates tailored sample preparation protocols designed to overcome the analytical challenges posed by complex wastewater matrices and limitations of conventional detection methodologies.

Benefits

- Single sample preparation protocol using Oasis™ MCX 6 cc/150 mg
- Established limit of detection (LOD) parameters reduced significant false positive determinations
- Screening and identification of more than 1,975 analytes are possible within a single injection
- Fully automated workflows with Forensic Toxicology Screening Application Solution with UNIFI, enabling the identification and detection of NPS and other drugs in wastewater

Introduction

NPS have emerged as a significant global issue; however, the understanding of their adverse health effects and social consequences remains limited, complicating efforts for effective prevention and treatment. The increasing prevalence of NPS poses a substantial threat to public health and further complicates the formulation and implementation of drug policies. Identifying and analyzing these chemically diverse substances present on the drug market is a complex task; therefore, effective monitoring and early warning systems are crucial to addressing the issue.

The detection and characterization of emerging anthropogenic substances in wastewater is an indirect but efficient approach for assessing environmental and public health risks. Traditional monitoring methods like ultraviolet spectroscopy, immunoassay, and thin layer chromatography (TLC) have often been inadequate for tracking the continuously evolving landscape of substances, due to limitations in selectivity and specificity of the techniques employed and the quantification at lower sensitivity levels.[1] A more effective technology recently applied for wastewater surveillance is liquid chromatography coupled to mass spectrometry (LC-MS), providing high throughput capabilities for identifying a broad spectrum of organic contaminants. Here, the high-resolution mass spectrometry (HRMS) technique is described for the detection, identification, and monitoring of the

presence of illicit substances using a broad screening approach.

This work is based on adapting environmental screening methodology to facilitate the use of the Forensic Toxicology Screening Application Solution in waters_connect™ Software with UNIFI.[2,3] In this study, the monitoring and identification of controlled substances in wastewater samples is demonstrated by developing an in-house solid phase extraction (SPE) protocol and a cutting edge UPLC™-based chromatographic method by using the UNIFI workflow. To evaluate the method performance, the forensic toxicology standards kit from Waters™ was used and the method performance and instrument parameters were evaluated using the system suitability mix (SSM) supplied in the kit.

Experimental

Sample Preparation

A total of 100 wastewater samples were collected from different geographical locations. The samples were collected in 500 mL polypropylene bottles and transported to the RASID Environmental Laboratory at Abu Dhabi. An SPE protocol was developed using SPE Oasis MCX Cartridges (6 cc/150 mg, p/n: 186000256 < https://www.waters.com/nextgen/global/shop/sample-preparation--filtration/186000256-oasis-mcx-6-cc-vac-cartridge-150-mg-sorbent-per-cartridge-30--m-.html>). The cartridges were conditioned with 1 mL methanol and equilibrated with 1 mL Milli-Q® water. A volume of 50 mL untreated wastewater sample was initially centrifuged before being loaded onto the cartridges. The cartridges were washed with 1 mL 2% (v/v) formic acid in water, and analytes were eluted with 1 mL methanol: acetonitrile (70:30, v/v) with 5% ammonia.

ACQUITY UPLC Conditions

UPLC system:	ACQUITY™ UPLC H-Class PLUS System (FTN)
Column:	ACQUITY UPLC HSS™ C_{18}, 100Å, 1.8 µm, 2.1 mm x 150 mm, (p/n: 186003534)
Vials:	Screw Neck Vials, Max Recovery, 12 x 32 mm, screw neck (p/n: 186000327c)

Column temperature:	50 °C
Sample temperature:	10 °C
Injection volume:	5 µL
Flow rate:	0.4 mL/min
Mobile phase A:	5 mM ammonium formate pH 3.0 in water with 0.15% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile
Gradient:	87% A for 0.5 min then to 50% A at 10 min and 5% A at 10.75 min hold for 2 min before switching to 87% A
Run time:	15 min

MS Conditions

MS system:	Xevo™ G2-XS QTof Mass Spectrometer
Ionization mode:	ESI Positive
Source temperature:	150 °C
Desolvation temperature:	400 °C
Desolvation gas:	800 L/h
Reference mass:	Leucine enkephalin $[M-H]^+$ m/z = 556.2766

Acquisition range:	*m/z* 50–1200
Scan time:	0.1 s
Capillary voltage:	0.8 KV
Cone voltage:	25 V
MS^E conditions:	Collision energy function 1: 6 eV Collision energy function 2: ramped 10 to 45 eV
Software:	waters_connect Software with UNIFI application

Results and Discussion

This study highlights the precise mass screening capabilities of a novel in-house extraction method and a modified chromatographic approach by utilizing the ACQUITY UPLC H-Class PLUS System coupled with the Xevo G2-XS QTof Mass Spectrometer. The screening solution incorporates the readily available forensic toxicology installation standards kit to evaluate system parameters and performance. This method offers user-friendly workflows, a comprehensive readily available database of more than 1975 toxicologically relevant compounds, and data-independent acquisition (DIA) of time-aligned precursor and fragment ions in a single injection (MS^E) for effectively analyzing very complex wastewater matrix samples.

The acceptance criteria for a positive identification were as follows: retention time to be within ±0.35 minutes of reference method, the observed precursor mass to be within 5 ppm, minimum of one diagnostic fragment ion should be present within 5 ppm of mass error, and the precursor response should be ≥10,000 intensity.

To apply the primary identification criteria, adjustments to the UPLC method had to be made, as the forensic toxicology screening solution was originally developed on the ACQUITY UPLC I-Class System, and the system used in this study was the ACQUITY UPLC H-Class PLUS System. The dwell volume of the ACQUITY UPLC H-

Class PLUS System plays a crucial role in matching the retention times of ACQUITY UPLC I-Class System. It can be adjusted with the gradient start time relative to the injection, which can either advance or delay the peak elution. Starting the gradient “before injection” shifts peaks to earlier retention times, while starting “after injection” delays them. For this study, the gradient starts when the volume reaches 400 µL with the gradient start set “before injection” as shown in Figure 1.

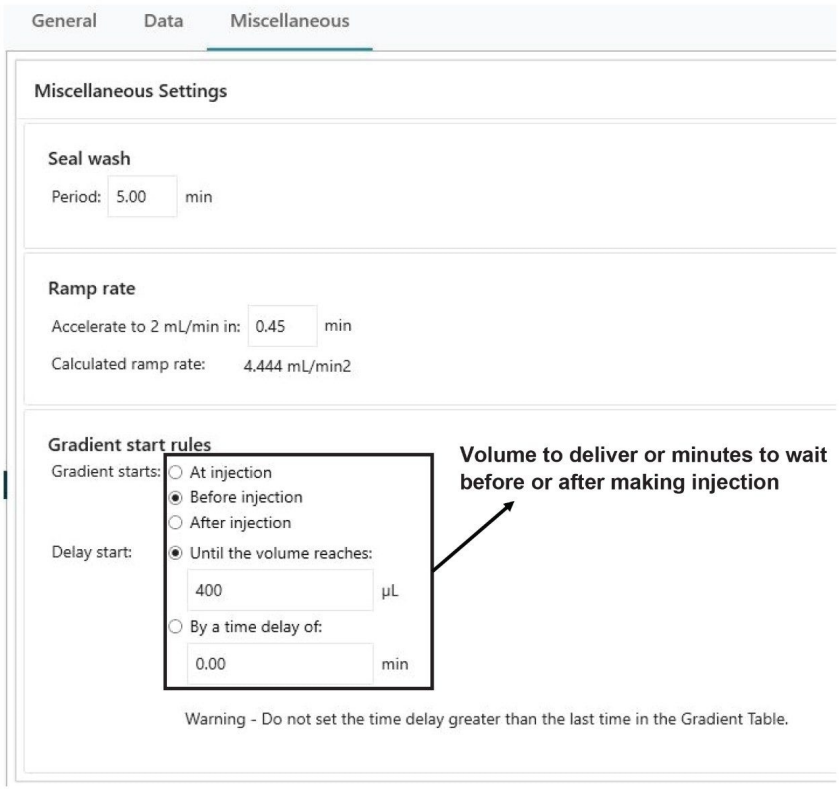


Figure 1. Tools for gradient adjustments between ACQUITY UPLC I-Class System and ACQUITY UPLC H-Class PLUS System method development.

System performance was evaluated using a SSM contained in the forensic tox installation standards kit (p/n: 186007361 <https://www.waters.com/nextgen/global/shop/standards--reagents/186007361-forensic-tox-installation-standards-kit.html>) for positive ionization mode analysis. All 10 analytes in the SSM were accurately identified, confirming the method’s suitability as shown in Table 1.

Compound name	Neutral mass	Observed neutral mass	Observed *m/z*	Mass error (ppm)	Expected RT (min)	Observed RT (min)	Detector counts	Response	Adducts
Buflomedil	307.1784	307.1785	308.1858	0.4	4.30	4.24	184250	151848	+H
Clozapine	326.1298	326.1298	327.1371	-0.1	6.20	5.96	38598	31259	+H
Milnacipran	246.1732	246.1733	247.1806	0.4	4.99	4.99	70657	58744	+H
Nicotine	162.1157	162.1156	163.1229	-0.3	1.03	0.99	18526	16449	+H
Perphenazine	403.1485	403.1476	404.1549	-2.2	8.80	8.80	6043	4810	+H
Scopolamine	303.1471	303.1471	304.1544	0.1	2.19	2.17	77259	63331	+H
Tianeptine	436.1224	436.1221	437.1294	-0.6	6.90	6.95	106877	84000	+H
Tiapride	328.1457	328.1460	329.1533	1.0	1.69	1.75	156621	130179	+H
Trazodone	371.1513	371.1515	372.1588	0.6	5.60	5.38	173845	139179	+H
Triprolidine	278.1783	278.1779	279.1852	-1.5	5.80	5.86	50682	40647	+H

Table 1. List of SSM compound summary with mass error, retention time, observed m/z information.

Additionally, an internal method validation was performed by including over 100 CRMs to determine their LOD, retention times, and instrument parameters. All CRMs were individually procured from ISO-certified labs and combined in an analyte mix in aqueous solution with concentration levels from 1 µg/L to 100 µg/L. To determine LOD levels for each analyte, the minimum precursor intensity was set at 10,000 counts. The determined LOD levels helped to reduce the number of false positive identifications significantly (Figure 2).

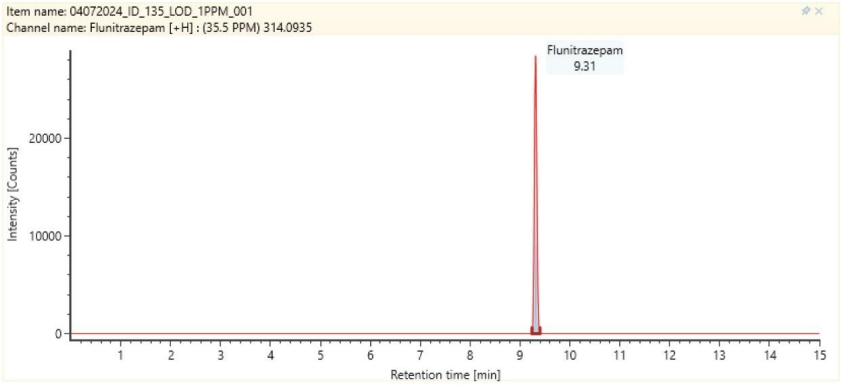


Figure 2. Representative chromatography of detection of flunitrazepam 1 µg/L concentration for limit of detection establishment.

The forensic toxicology screening solution database provided by Waters Corporation includes a wide range of compound classes, such as amphetamines, analgesics, antidepressants, antipsychotics, benzodiazepines,

cannabinoids, cathinones, designer drugs, fentanyls, pesticides, and more. This database has more than 1,975 relevant compounds in the library. The methodology employed for this qualitative assessment was applied to test wastewater samples, setting the aforementioned identification criteria in the screening workflow.

The identification of accurate mass fragments of more than 100 potential target analytes served as conclusive proof of the presence of controlled substances and significantly minimizes false positives. It was possible to identify 42 controlled substances and other classes of compounds in the wastewater with high confidence as presented in Table 2. Detailed fragment ion information from high-energy data (MS^E approach) reveals specific fragment ion characteristics.[9] Fragment ions that match those in the scientific library are automatically annotated with their mass error. Fragment ions identified via the fragment match tool are automatically annotated with suggested structures derived from theoretically calculated masses as depicted in Figure 3. The final confirmation of a match is evident in the chromatogram window where the extracted ion chromatogram is displayed for both precursor ions and matched fragment ions.

Line number	Component name	Expected mass (Da)	*Observed m/z*	Mass error (ppm)	Expected RT (min)	Observed RT (min)	Expected fragments count	Expected fragments found	Response	Adducts	Formula
1	19-Norandrosterone	276.2089	277.2171	3.3	11.8	11.7	2	1	12658	+H	$C_{18}H_{28}O_2$
2	Alpha-solanine	867.4980	868.5059	0.7	6.2	6.0	6	2	16390	+H	$C_{45}H_{73}NO_{15}$
3	Azithromycin	748.5085	749.5168	1.4	4.9	4.9	4	4	26137	+H	$C_{38}H_{72}N_2O_{12}$
4	Betaxolol	307.2147	308.2225	1.4	6.5	6.6	4	1	11300	+H	$C_{18}H_{29}NO_3$
5	Bisoprolol	325.2253	326.2329	1.0	5.5	5.4	3	2	24822	+H	$C_{18}H_{31}NO_4$
6	Caffeine	194.0804	195.0880	1.9	2.1	2.0	3	3	187036	+H	$C_8H_{10}N_4O_2$
7	Capsaicin	305.1991	306.2070	2.0	11.2	11.4	3	3	25534	+H	$C_{18}H_{27}NO_3$
8	Carbamazepine	236.0950	237.1028	2.3	7.3	7.1	3	3	18299	+H	$C_{15}H_{12}N_2O$
9	Citalopram/Escitalopram	324.1638	325.1722	3.4	6.7	6.5	4	4	13253	+H	$C_{20}H_{21}FN_2O$
10	Clarithromycin	747.4769	748.4858	2.1	9.0	8.9	2	2	71472	+H	$C_{38}H_{69}NO_{13}$
11	Cocaine	303.1471	304.1542	-0.5	4.5	4.7	4	1	11958	+H	$C_{17}H_{21}NO_4$
12	Cotinine	176.0950	177.1028	3.1	1.1	1.1	4	4	55508	+H	$C_{10}H_{12}N_2O$
13	DEET	191.1310	192.1388	2.9	9.1	8.8	3	2	15716	+H	$C_{12}H_{17}NO$
14	Dimethylone-M (methylated)	223.1208	224.1284	1.2	1.9	1.7	4	3	17714	+H	$C_{12}H_{17}NO_3$
15	Eslicarbazepine	254.1055	255.1138	3.9	4.8	4.6	3	3	11940	+H	$C_{15}H_{14}N_2O_2$
16	Fexofenadine	501.2879	502.2953	0.3	8.2	8.1	3	2	479085	+H	$C_{32}H_{39}NO_4$
17	Irbesartan	428.2325	429.2403	1.3	9.5	9.3	2	2	137247	+H	$C_{25}H_{28}N_6O$
18	Itopride	358.1893	359.1968	0.6	3.6	3.3	4	3	12053	+H	$C_{20}H_{26}N_2O_4$
19	Ketoconazole	530.1488	531.1565	0.9	7.9	8.1	1	1	33674	+H	$C_{26}H_{28}Cl_2N_4O_4$
20	Ketoprofen	254.0943	255.1023	3.0	9.5	9.3	3	3	45147	+H	$C_{16}H_{14}O_3$
21	Lidocaine	234.1732	235.1807	0.9	3.2	3.1	1	1	39267	+H	$C_{14}H_{22}N_2O$
22	Losartan	422.1622	423.1696	0.3	8.9	8.6	4	3	19538	+H	$C_{22}H_{23}ClN_6O$
23	Mebeverine	429.2515	430.2593	1.2	8.0	7.9	2	2	50190	+H	$C_{25}H_{35}NO_5$
24	Methocarbamol	241.0950	242.1032	3.9	4.2	4.0	4	4	12179	+H	$C_{11}H_{15}NO_5$
25	Metipranolol	309.1940	310.2021	2.5	5.8	6.1	4	3	21276	+H	$C_{17}H_{27}NO_4$
26	Metronidazole	171.0644	172.0721	2.7	1.8	1.7	3	3	17773	+H	$C_6H_9N_3O_3$
27	Minoxidil	209.1277	210.1352	1.1	3.0	2.8	2	2	11137	+H	$C_9H_{15}N_5O$
28	Nicotine	162.1157	163.1234	2.4	1.0	0.8	4	4	21472	+H	$C_{10}H_{14}N_2$
29	NM-2AI (N-Methyl-2-Aminoindane)	147.1048	148.1126	3.4	2.0	1.8	3	3	89269	+H	$C_{10}H_{13}N$
30	Oxcarbazepine	252.0899	253.0974	1.2	6.0	5.8	4	3	14310	+H	$C_{15}H_{12}N_2O_2$
31	Paracetamol	151.0633	152.0710	2.8	1.5	1.5	2	2	284507	+H	$C_8H_9NO_2$
32	Pregabalin	159.1259	160.1339	4.0	1.8	1.8	1	1	10397	+H	$C_8H_{17}NO_2$
33	Rosuvastatin	481.1683	482.1759	0.7	9.1	8.9	4	2	33651	+H	$C_{22}H_{28}FN_3O_6S$
34	Sitaglipin	407.1181	408.1258	1.0	4.7	4.4	4	4	47143	+H	$C_{16}H_{15}F_6N_5O$
35	Telmisartan	514.2369	515.2447	1.1	9.4	9.3	2	2	294135	+H	$C_{33}H_{30}N_4O_2$
36	Theobromine	180.0647	181.0726	3.5	1.2	1.2	3	2	20779	+H	$C_7H_8N_4O_2$
37	Theophyline/aminophylline	180.0647	181.0726	3.1	1.5	1.4	1	1	50694	+H	$C_7H_8N_4O_2$
38	Thiamethoxam	291.0193	292.0268	0.9	2.7	3.0	1	1	22383	+H	$C_8H_{10}ClN_5O_3S$
39	Trimethoprim	290.1379	291.1457	2.0	2.6	2.4	3	3	20953	+H	$C_{14}H_{18}N_4O_3$
40	Valsartan	435.2270	436.2349	1.5	10.6	10.4	3	3	133438	+H	$C_{24}H_{29}N_5O_3$
41	Venlafaxine-M (O-demethyl metabolite)	263.1885	264.1962	1.7	3.2	3.1	4	3	11725	+H	$C_{16}H_{25}NO_2$
42	Vildagliptin	303.1947	304.2020	0.0	1.6	1.6	4	4	20654	+H	$C_{17}H_{25}N_3O_2$

Table 2. Component summary.

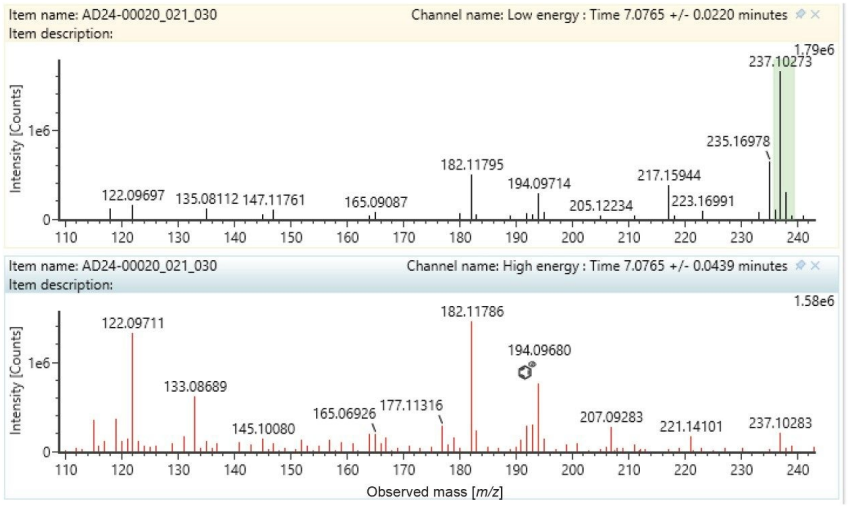


Figure 3. Low energy and high energy data with fragmentation identification for carbamazepine.

Conclusion

This study presents an internally developed analytical workflow specifically designed for detecting controlled substances in complex wastewater matrices. Utilizing advanced analytical techniques, the study employed the Xevo G2-XS QTof Mass Spectrometer in conjunction with the ACQUITY UPLC H-Class PLUS System. The focus was on developing an environmental toxicology screening method, particularly a suspect screening approach for identifying controlled substances in untreated wastewater against a library. The method incorporates a unique in-house SPE protocol using Oasis MCX to capture compounds of interest from wastewater samples. Unlike standard approaches such as toxicology untargeted screening in blood, urine, hair, and saliva, this methodology was custom-designed within the RASID ADQCC lab to address the limitations of conventional detection techniques, especially for emerging psychoactive substances in wastewater. Data processing was conducted using waters_connect Software through the UNIFI application. The innovative integration of suspect screening with high-resolution MS^E acquisition and the UNIFI application forms the core of this internally developed system.

This integrated approach demonstrates the effectiveness of combining sophisticated instrumentation with

powerful software tools for comprehensive substance detection and identification in environmental samples. The combination of information-rich MS^E acquisition and an integrated scientific information system facilitates routine laboratory screening for compounds of interest, their adducts, and potential metabolites. Having retention times and accurate mass fragment ions in Waters toxicology libraries allows for identifications based on more than just the accurate mass of precursor ions. This is crucial for reducing false detection rates and expediting data review in screening experiments. Leveraging UNIFI's identification capabilities, 42 compounds were identified in a wastewater sample.

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