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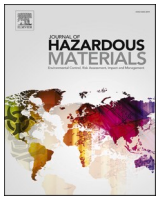

Workflow to facilitate the detection of new psychoactive substances and drugs of abuse in influent urban wastewater

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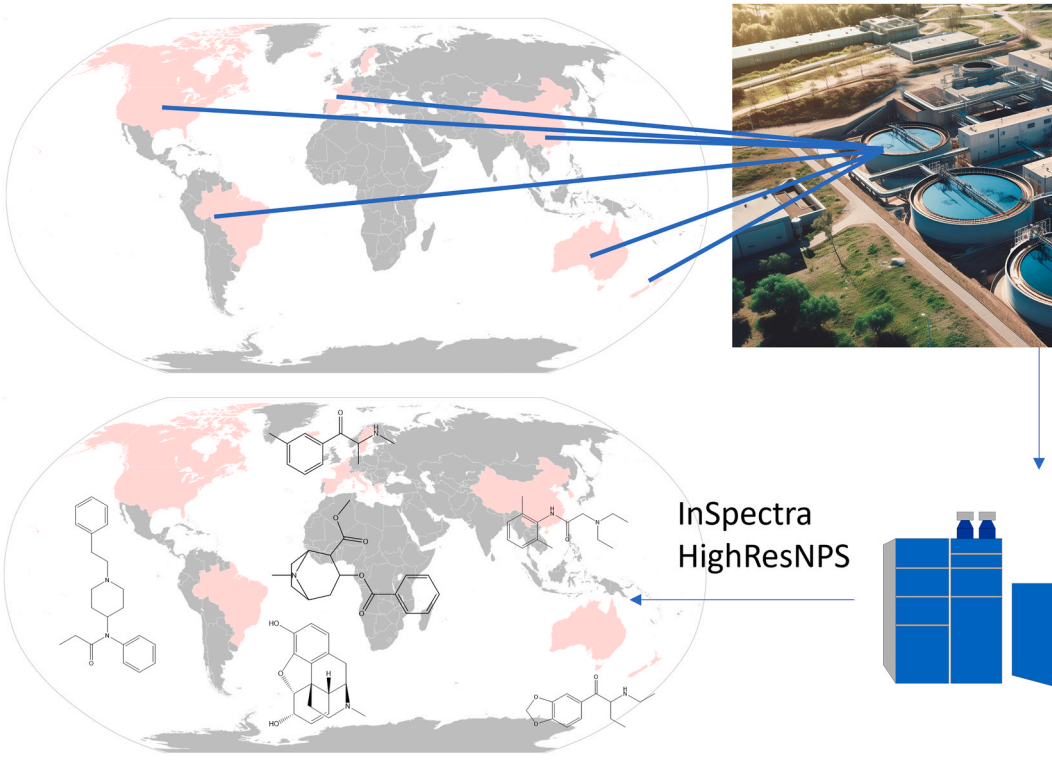
HIGHLIGHTS

- New open-access workflow to facilitate the analysis of emerging drugs in wastewater.
- A total of 50 compounds detected in wastewater samples from 16 countries.
- Trimethoxyamphetamine, 25-I NBOH and phenibut detected for the first time in wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

The complexity around the dynamic markets for new psychoactive substances (NPS) forces researchers to develop and apply innovative analytical strategies to detect and identify them in influent urban wastewater. In this work a comprehensive suspect screening workflow following liquid chromatography – high resolution mass spectrometry analysis was established utilising the open-source *InSpectra* data processing platform and the HighResNPS library. In total, 278 urban influent wastewater samples from 47 sites in 16 countries were collected to investigate the presence of NPS and other drugs of abuse. A total of 50 compounds were detected in samples from at least one site. Most compounds found were prescription drugs such as gabapentin (detection frequency 79%), codeine (40%) and pregabalin (15%). However, cocaine was the most found illicit drug (83%), in all countries where samples were collected apart from the Republic of Korea and China. Eight NPS were also identified with this protocol: 3-methylmethcathinone 11%), eutylone (6%), etizolam (2%), 3-chloromethcathinone (4%), mitragynine (6%), phenibut (2%), 25I-NBOH (2%) and trimethoxyamphetamine (2%). The latter three have not previously been reported in municipal wastewater samples. The workflow employed allowed the prioritisation of features to be further investigated, reducing processing time and gaining in confidence in their identification.

1. Introduction

Wastewater has been widely investigated as a surveillance tool for a multitude of analytes including lifestyle markers (i.e. drugs, tobacco, alcohol), exposure markers (i.e. pesticides, mycotoxins, plasticisers), population size markers (i.e. food additives and neurotransmitters) and health markers (i.e. pharmaceuticals, viruses) [1,2]. The focus of these studies has primarily been on predefined analytes through target analysis using liquid chromatography – tandem mass spectrometry (LC-MS/MS), hence requiring analytical reference standards. However, there are some classes of compounds where predetermined lists are not necessarily appropriate. For example, new psychoactive substances (NPS) are a particularly difficult class of drugs, for which to develop appropriate target methods, in part due to number of reported substances, their short life cycles and high rates of turnover. NPS are compounds which skirt the border of legality and mimic the effects of known licit and illicit drugs such as benzodiazepines, cannabis, opioids and stimulants [3]. With blanket legislation not enforced in all jurisdictions, slight structural modifications can inevitably lead to 'new' NPS. As such, the combination of a dynamic market and lack of analytical reference standards has made it very difficult for laboratories to develop and validate methods, while ensuring 'new' NPS are included and monitored.

To circumvent this limitation, liquid chromatography-high resolution mass spectrometry (LC-HRMS), is an analytical methodology that has emerged as the necessary instrumental approach to meet the analytical challenge. LC-HRMS takes advantage of the acquisition of high resolution, full-spectrum data, which can be screened against

databases to confirm the presence of predefined drugs or find 'suspect' compounds [4,5]. These databases contain information pertaining to the accurate mass of the compound, fragment ions, and experimental (or predicted) retention time, thus facilitating identification. Criteria have been developed to better understand identification confidence, thereby allowing detections to be communicated even without the availability of analytical standards [6]. These identification levels range from unequivocal identification through the exact mass of precursor and fragment ions and confirmation with analytical reference standards (i.e. Level 1) through to exact mass of precursor ion only (i.e. Level 5) [6]. Further identification confidence can be given to analytes for which a reference standard is unavailable through the use of predicted retention time [7–10].

Such LC-HRMS workflows have previously been used for the determination of NPS in wastewater, primarily in Europe and Australia [11–16]. Most of these studies utilized in-house databases, with compounds therein based on national intelligence, while some studies also incorporated the HighResNPS database [15,17]. HighResNPS is a comprehensive crowd-sourced database, containing compounds of toxicological relevance – primarily NPS but also illicit drugs, pharmaceuticals of abuse potential and related metabolites [18]. Vendor-specific software is typically used to conduct these studies, but there is a move to open-source processing software to allow more flexibility around data processing, while also allowing data from different vendors to be processed on the same software [19,20].

In this work, we developed and applied a new workflow for suspect screening. To facilitate the data processing, the newly developed open-source platform *InSpectra* [21] was used. The primary aim of this work was to develop a comprehensive workflow using an open-source, free platform for the identification of NPS and related psychoactive drugs. In this work, we analysed influent 278 wastewater samples from 47 sites in 16 countries over the 2021–2022 New Year period. We have previously analysed these samples using a targeted LC-MS/MS method for 32 NPS, and identified nine across all sites [22]. As such, a secondary aim was to evaluate whether a LC-HRMS suspect screening analysis could complement targeted analysis and whether additional spatial trends can be ascertained.

2. Materials and methods

2.1. Chemicals and reagents

All standards used in this work were either purchased from Cerilliant (Round Rock,Texas, USA), Dr Ehrenstorfer (Augsburg, Germany), Cayman Chemical (Ann Arbor, Michigan, USA) or kindly donated by Forensic Science Queensland. LC-MS grade methanol and hydrochloric acid (HCl) were obtained from Merck Pty Ltd (Victoria, Australia) and formic acid was obtained from Sigma-Aldrich Pty Ltd. (Castle Hill, Australia). LC-MS grade acetonitrile and formic acid were obtained from Thermo Fisher Scientific (Scoresby, VIC, Australia), as was ammonia (28%). Ammonium formate (≥99.9995% trace metal basis) was purchased from Sigma-Aldrich Pty Ltd. (Castle Hill, Australia). Ultrapure water was produced using a Milli-Q system (Millipore, Bedford, USA).

2.2. Sampling and sample treatment

Influent wastewater samples (200–500 mL) were collected for at least three days between 23 December 2021 and 5 January 2022 from 47 sites in 16 countries (Australia (4 sites), Belgium (1), Brazil (3), Canada (1), China (1), Cyprus (2), France (1), Greece (1), Iceland (1), Italy (1), New Zealand (4), Republic of Korea (1), Slovenia (4), Spain (3), Sweden (2) and the United States (17)). All collaborators were instructed to collect 24-hour composite samples using flow or time proportional autosamplers and to acidify the samples (pH 2, using HCl) upon collection. At the conclusion of the sampling period, the samples were transported to the laboratory in the country of collection and stored at −20 °C until sample treatment. Further information on the specific site data has previously been published [22,23]. Samples (100 mL) were initially spiked with internal standards (illicit drug reference standards, spiked between 20 ng/L and 1000 ng/L, Table S1) and then loaded onto the SPE cartridges (UCT XtracT DAU, 500 mg/6 mL; UCT Inc., Bristol, PA, USA). Following loading and drying, the cartridges were frozen at −20 °C before being shipped to The University of Queensland, Australia for the elution and analysis. Previous studies have investigated the stability of illicit drugs on dried SPE cartridges for both storage at −20 °C [24] and international shipment [25], as well as for the preservatives used in this work [26]. The samples were reconstituted with 0.1% formic acid in methanol (20 μL) and 0.1% formic acid in ultrapure water (80 μL) giving a final volume of 100 μL.

2.3. Instrumentation

All wastewater samples and reference standards were analysed at The University of Queensland, Australia with a SCIEX Triple TOF 5600 + LC-HRMS instrument. A subset of wastewater samples was analysed using a Waters Corporation Xevo G2-XS LC-HRMS instrument to gain more confidence in some tentatively identified compounds. Finally, a second SCIEX Triple TOF 5600 + and a SCIEX X500R LC-HRMS instrument at The Center for Forensic Science Research and Education in the United States (CFSRE; Willow Grove, PA, USA) was used to analyse standards of trimethoxyamphetamine that were not available at the time of the study in the Australian laboratories, using an analogous instrument and similar method to that at The University of Queensland. All LC-HRMS instruments used during this study were liquid chromatographs coupled to quadrupole time-of-flight mass spectrometers (LC-QTOF-MS).

2.4. SCIEX Triple TOF 5600 + (The University of Queensland, Australia)

Samples were analysed using a Shimadzu UHPLC system (Nexera X2) coupled to a SCIEX Triple TOF 5600 + mass spectrometer. Chromatographic separation was achieved using a Phenomenex Kinetex Biphenyl (50 × 2.1 mm × 2.6 μm) column fitted with a Security Guard ULTRA Cartridges UHPLC Biphenyl 2.1 mm ID columns, at a flow rate of 0.3 mL/min with an injection volume of 10 μL and a column oven temperature of 40 °C. The mobile phase was MilliQ water: methanol (95:5); 0.1% formic acid (mobile phase A) and methanol: MilliQ water (95:5); 0.1% formic acid (mobile phase B). The initial percentage of B was 5%, which was kept steady for the first 1.5 min. The concentration of B was linearly increased to 95% over 12.5 min and held for 3 min before being brought back to the starting conditions over 0.1 min and kept steady for the final 2.9 min to equilibrate the system. The total run time was 20 min. MS data were collected over a m/z range of 50–650 to cover all the compounds in the database. Data were acquired in Sequential Window Acquisition of all THeoretical fragment-ion spectra (SWATH) mode, utilising one full scan MS (collision energy of 10 V) and 8 subsequent experiments, each of which had a collision energy of 25 V with a collision energy spread of 15 V.

All data were acquired in positive ionisation mode using SCIEX Analyst (version 1.7) and processed using *InSpectra* and SCIEX OS (version 3.1.6.44) software.

Instrumental details for the instruments (i.e. Waters Xevo G2-XS (Royal Brisbane and Women's Hospital, Australia) and SCIEX X500R and TripleTOF®5600 + (CFSRE, United States)) used for confirmation experiments are in the supporting information (Section S1).

2.5. Quality assurance and quality control

All samples were spiked with between 1 and 30 internal standards (Table S1) to monitor instrumental performance in terms of sensitivity, sample-to-sample variation, and to cater for retention time drift. For the analysis of wastewater samples, a mixture of NPS and illicit drug

standards were injected at the beginning of each batch run and at regular intervals to monitor chromatographic and mass spectrometric performance. Instrumental blanks (Milli-Q) water was analysed alongside the samples. Mass calibration was performed prior to each batch run using the SCIEX ESI Positive Calibration Solution and after every 15 samples to ensure mass accuracy.

Quality control for the Waters Corporation Xevo G2-XS LC-QTOF-MS analysis consisted of two levels of urine QC (MEDICHEM) containing 59 analytes for mass, retention time and fragmentation to confirm appropriate system performance and a drug free urine control (Biorad).

2.6. Suspect screening database

The HighResNPS consensus [27] database as at April 2022 was downloaded from https://highresnps.com. This consensus database contained 2186 unique compounds distilled from 5647 entries, with 1480 of these unique compounds containing at least one fragment ion. Most of the compounds were NPS, but the database also included pharmaceuticals of abuse potential or toxicological relevance (e.g. gabapentin, amantadine, oxycodone, fentanyl) and illicit drugs (e.g. cocaine and its metabolite benzoylecgonine, 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine). Predicted retention times using a validated model as outlined below were also included in the database.

2.7. Retention time prediction

The retention time prediction was performed according to the study by Pasin *et al.*[7]. Briefly, a sub-library of the HighResNPS database was generated by taking retention time data from twenty laboratories, who had provided information to the HighResNPS database. The developed retention time prediction model allows for the inclusion of retention times from methods with different chromatography and thus a standardised chromatography is not required. Four molecular descriptors (variables) including the log of the distribution coefficient between octanol and water (logD), log of the partition coefficient (log P), number of carbon atoms and number of oxygen atoms were calculated from entries simplified molecular-input line-entry system (SMILES) string using JChem for Excel 20.11.0.644 (ChemAxon, https://www.chemaxon.com). Furthermore, categorical data such as the laboratory and drug class names were one-hot encoded. This process takes *n* labels in a column of categorical data and transforms it to *n*-1 columns where each column contains a 0 or 1 to indicate the absence or presence of that category, respectively. This encoding produced an additional 19 and 12 variables, respectively, where the first label in each category was removed. Therefore, a total number of 35 variables were used for modelling). The retention times were then split into a training, optimization, validation, and test set at a proportion of 55:15:15:15, corresponding to 2090:571:571:571. The retention time prediction model was a multi-layer perceptron, a type of artificial neural network, of which the optimal architecture was determined by training many different architectures in replicate ($n = 5$) with different numbers of hidden layers (up to 2) and many hidden neurons (50−200). The optimal architecture was the one which gave the lowest average mean absolute error on the validation set predictions. The model was then used to predict retention times for 2186 compounds included in the HighResNPS database. Further information relating to the prediction accuracy is in the supporting information (Section S2 and Table S2).

2.8. Data processing with InSpectra

Initially, the raw data files from the samples analysed at The University of Queensland were converted to mzXML with Proteowizard MsConvert, using 32-bit precision and an absolute intensity threshold of 100 counts [28]. The *InSpectra* platform was used to perform suspect screening using specific parameters Table 1:

Table 1
Parameters within InSpectra for suspect screening.

Parameter	Value	Units	Definition
mode	Positive		Mode of the Mass Spectrometer
mz_thresh	100 to 650	Da	Minimum and Maximum *m/z* values to consider
Int_thresh	1000	Count	Minimum MS1 Intensity needed for consideration
mass_tol	0.05	Da	Predicted mass accuracy of the analyser of the MS/MS
iso_depth	5		The number of peaks in the isotopic distribution.

The suspect screening algorithm that is part of *InSpectra* can also be found in the ULSA repository (https://bitbucket.org/SSamanipour/ulsa.jl/src/master/src/) in case local processing of data is preferred. After executing suspect screening, a csv file containing all possible suspect detections present in the sample was obtained. Subsequently, outside of *InSpectra*, the list of suspects was further refined by including thresholds around MS1 intensity (min = 5000), number of fragment ions (min = 1), match factor (the dot product between the database and unknown spectral vectors, representing their similarity level [29–31]; min = 0.5) and retention time tolerance between predicted and actual (max = 3 min).

2.9. Data processing with SCIEX OS

An equal weighting was applied to each of the four criteria (i.e. MS1 intensity, number of fragment ions, match factor and difference between predicted and experimental retention time) to ensure that one did not skew the results. All compounds with a weighted score of 1.8 from the *InSpectra* analysis were included in a database for data processing using SCIEX OS. This included predicted retention time, protonated molecule, and fragment ion exact masses. A minimum peak height of 300 was set and flagging rules were employed for precursor and fragment ion mass error (less than 5 ppm), retention time error (less than 10%) and percentage difference in isotope ratio (less than 20%). A less strict 10 ppm 'marginal acceptance' threshold was included for the precursor and fragment ion mass errors to ensure that no compounds would be missed if they had a mass error slightly greater than 5 ppm. Each sample was compared with a standard, where available, to enable confirmation. If a standard was unavailable, but fragment ions were present and mass error and predicted retention time within the flagging limits, the compound was deemed tentatively identified (i.e. Level 2 in the Schymanski Scale) [6,32].

3. Results and discussion

3.1. Workflow

This work took advantage of the in-house open-source automated platform, *InSpectra*, to facilitate data processing. It has been applied to assess environmental pollution, exposure to chemicals and public health [21,33]. While we have previously investigated these samples for NPS using targeted analysis [22], the dynamic NPS market makes it very difficult to have a targeted method encompassing all potential substances. As such, this suspect screening analysis took advantage of the comprehensive HighResNPS database as it can readily be applied and combines data from not only NPS but also other psychoactive drugs of abuse already detected around the world.

After data processing was completed with *InSpectra* (Step 1), the final output was manually examined to establish a ranking of compounds potentially present in the samples. A visual summary of the methodology is shown in Fig. 1, together with the number of features filtered at each step. The specific number of features filtered per site is in the Supporting Information (Table S3). A normalised weighting was applied

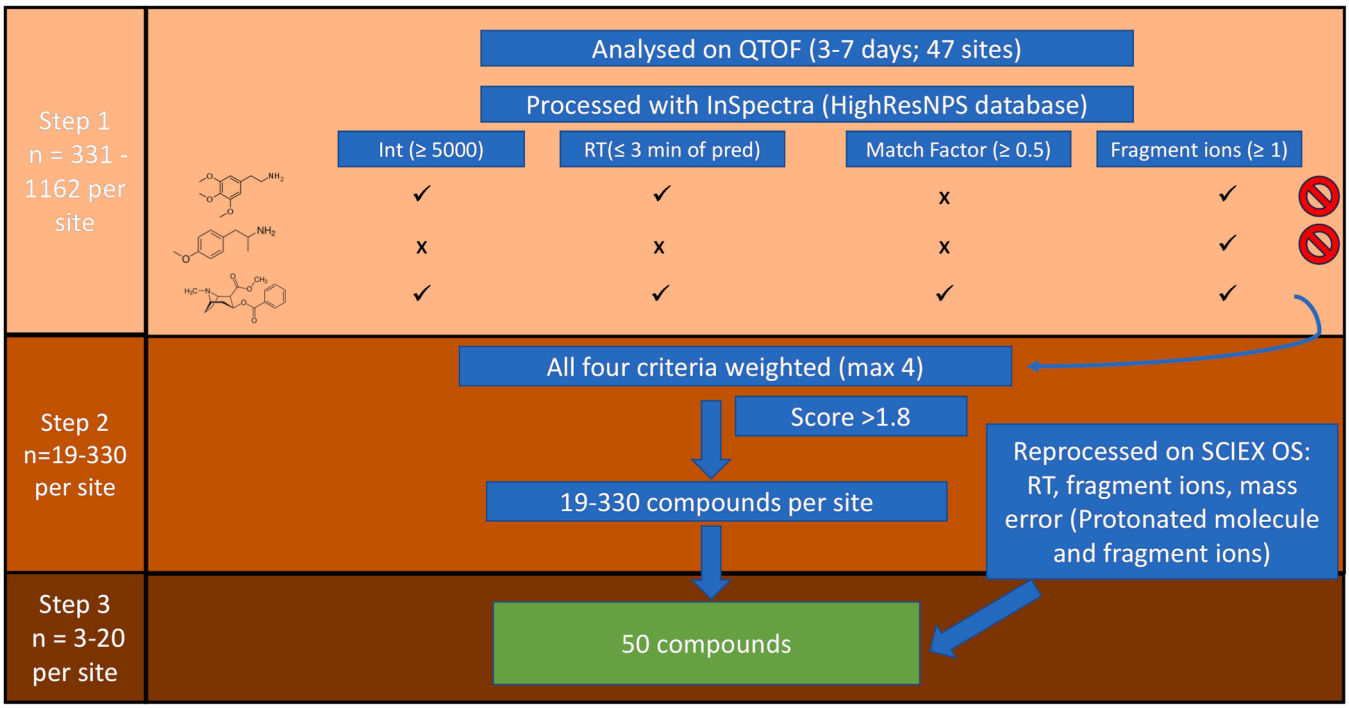

Fig. 1. Suspect screening workflow. The three colour bands show how the samples and sites are combined: from initially all samples/sites (light orange), all samples combined per site (orange) and finally all sites combined (brown).

to MS1 intensity, number of fragment ions, match factor and retention time tolerance between predicted and actual to ensure that one did not skew the results. The normalisation was based on the maximum and minimum values for each sample. For example, for the match factor criterion, the minimum was 0.5, and the maximum was 1. If a compound within a sample had a match factor of 0.8, the normalised score would be 0.53 (0.8/ (0.5 +1)). This was calculated for each of the four criteria for each suspect compound. As such, a highly intense compound with a large difference between predicted and experimental retention time and poor match factor (e.g. <0.5) would not rank highly. A combined score of 1.8 across the four criteria, which was optimised based on the quality of match the time spent reviewing the data, was deemed the minimum for further analysis (Step 2) and final investigation by SCIEX OS (Step 3) to add confidence in their detection. As an example, in one sample, a suspected finding of mescaline had a high MS1 intensity, but a poor match factor (< 0.5) and predicted retention time error (> 3 min). As such, it did not reach the threshold for further analysis.

3.2. Compound identification

As shown in Fig. 1, for each site (n = 3–7), between 19 and 330 compounds were identified at Level 1–3, following initial analysis by *InSpectra* and normalised weighting of the four criteria of predicted retention time compared to experimental, match factor, intensity, and number of fragment ions. Following final reprocessing, a total of 50 compounds were found across in at least one site (Table 2, visualisation in Supporting Information html file). Almost half (26) of these were at level 1 confidence, with a reference standard available to confirm their identity. These compounds included illicit drugs, pharmaceuticals (e.g., anticonvulsants, pain killers, stimulants, and antidepressants), NPS and human metabolites. Another 22 were identified at level 2 confidence, based on library matching with fragmentation reported to HighResNPS and actual retention time within the set threshold of 3 min, while two were at level 3 confidence due to the possibility of a number of isomers. It is worth noting that due to the structural similarity of many NPS, isomers and shared fragment ions are common [34]. For the final identification, we included all fragment ions within the HighResNPS database to ensure identification confidence, recognising that having an increased number to match against would lead to high accuracy matches. Compounds identified with known isomers (and identical fragmentation), are noted in Table 2.

3.3. Detection of established illicit drugs

Cocaine was the most detected compound, appearing in samples from 39 sites, with its metabolite, benzoylecgonine detected in 35 sites. Although benzoylecgonine is excreted at a higher quantity than cocaine [35], previous work has shown increased matrix effects for benzoylecgonine compared to cocaine, which could reflect this detection frequency discrepancy [36]. Only in samples collected from sites in China and the Republic of Korea were neither of those compounds detected, which reflects previous findings [37–39]. Methamphetamine was the next most common illicit drug (n = 29). With cocaine and amphetamine-type stimulants (such as methamphetamine) being the fastest-growing, in terms of trafficking and seizures, it is potentially unsurprising that these were the most common illicit drugs in this study [40]. Methamphetamine was found in samples from sites in Australia, New Zealand, the United States and parts of eastern Europe, similar to previous wastewater-based epidemiology studies [16,37,41–43]. MDMA and ketamine are party drugs with known increased use during festivals [13,44–46] and holiday periods [47,48]. Ketamine was found in samples from several sites across Europe. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has reported that the levels of ketamine seized across the continent has plateaued at relatively high levels, suggesting that it has become entrenched in the community as a recreational drug [49]. Ketamine was also found in samples from sites in Brazil. Previous work has shown that ketamine is the main NPS – in approximately 75% of all oral fluid samples collected from 13 music festivals across the country [50]. MDMA was found in samples from sites in Australia, New Zealand, United States, Canada, Brazil, as well as most of the European sites. Interestingly, MDMA was detected at all of the sites in this study where an increase in MDMA levels in the most recent

Table 2
List of compounds found across all sites, ordered by detection frequency.

Compound	Percentage detection	Number of sites found	Countries found	Confirmation level according to Schymanski et al.[6]	Type
Cocaine	83	39	AU, BE, BR, CA, CY, ES, FR, GR, IS, IT, NZ, SE, SI, US	1	Illicit Drug
Gabapentin	79	37	AU, BE, BR, CA, CY, ES, FR, IS, IT, KR, SE, SI, US	1	Anticonvulsant
Benzoylecgonine	77	36	AU, BE, BR, CY, ES, FR, GR, IS, IT, NZ, SE, SI, US	1	Metabolite of cocaine
Hydroxybupropion	62	29	AU, BE, BR, CY, ES, IS, IT, SE, US	2	Metabolite of bupropion
MDMA	62	29	AU, BE, BR, CA, CY, ES, FR, IS, NZ, SE, SI, US	1	Illicit Drug
Methamphetamine	62	29	AU, BE, CY, ES, NZ, SE, US	1	Illicit Drug
Morphine	57	27	CA, CY, IS, ES, GR, SE, SI, US	1	Pain killer
Levorphanol	55	26	AU, BE, CA, CY, ES, IT, US	2	Pain killer
Tramadol	51	24	BE, CN, ES, FR, GR, IS, SE, US	1	Pain killer
Pregabalin	45	21	AU, BR, FR, GR, IS, SE, US	2	Anticonvulsant
Bupropion	43	20	BR, CA, SI, SE, US	2	Antidepressant
O-Desmethylvenlafaxine	43	20	BE, BR, GR, IS, US	1	Metabolite of venlafaxine
Codeine	40	19	AU, BE, BR, CY, IS, NZ, SE, SI, US	1	Pain killer
Ketamine	38	18	AU, BE, BR, CA, CY, ES, FR, GR, IT, NZ, SE, SI, US	1	Anaesthetic/illicit drug
Lidocaine	38	18	AU, BR, CN, KR, IS, IT, SE, SI	2	Anaesthetic/cocaine adulterant
Ephedrine	36	17	AU, BE, BR, CA, CY, ES, IS, KR, US	1	Stimulant
Tapentadol	36	17	AU, BE, CY, ES, IT, KR, SE, SI, US	1	Pain killer
Levamisole	34	16	BE, BR, ES, IT, SE, SI, US	2	Anthelmintic/cocaine adulterant
Amphetamine	32	15	BE, BR, CA,CY, ES, KR, US	1	Illicit Drug
Amantadine	23	11	AU, BR, CN, ES, KR	2	M2 ion channel inhibitor
Diazepam	15	7	AU, BR, CY, ES	1	Benzodiazepine
Oxycodone	15	7	US	1	Pain killer
Pregabalin methyl ester	15	7	AU, BR, FR, SE	2	Metabolite of pregabalin
Fentanyl	13	6	BR, US	1	Pain killer
Nordazepam	13	6	AU, ES, SE	2	Benzodiazepine
Noroxycodone	13	6	US	1	Metabolite of oxycodone
Oxazepam	13	6	AU, BE, FR, SE	1	Benzodiazepine
Venlafaxine	13	6	AU, CA, ES, GR	1	Antidepressant
3-methylmethcathinone[a]	11	5	BE, ES, SI	1	NPS – Stimulant
Orphenadrine	9	4	AU, BR	2	Anticholinergic
4-methylaminoantipyrine	6	3	BR, ES	2	Metabolite of aminopyrine
Alpha-hydroxymidazolam	6	3	BR	2	Metabolite of Midazolam
Clozapine	6	3	CY, NZ, SI	2	Antipsychotic
Eutylone[a]	6	3	NZ	1	NPS – Stimulant
Mitragynine	6	3	US	1	NPS – Plant-based
Norketamine	6	3	BR, IT	1	Metabolite of ketamine
Oxymorphone	6	3	AU	2	Pain killer
3-chloromethcathinone[a]	4	2	ES, SE	3 *	NPS – Stimulant
Ritalinic Acid	4	2	ES, IS	2	Metabolite of methylphenidate
Sertraline	4	2	CA, CY	2	Antidepressant
trans-Cinnamoylcocaine	4	2	BR	2	Coca alkaloid
25I-NBOH[a]	2	1	BR	2	NPS - Hallucinogen
Dehydronorketamine	2	1	BE	2	Metabolite of ketamine
Etizolam[a]	2	1	IS	1	NPS benzodiazepine
Methylephedrine	2	1	KR	2	Metabolite of ephedrine
Norephedrine	2	1	CY	2	Metabolite of amphetamine
Phenibut	2	1	CY	1	NPS - stimulant
Temazepam	2	1	AU	1	Benzodiazepine
Trimethoxyamphetamine[b]	2	1	US	3 *	NPS – Stimulant

AU (Australia); BE (Belgium); BR (Brazil); CA (Canada); CN (China); CY (Cyprus); ES (Spain); FR (France); GR (Greece); IS (Iceland); IT (Italy); KR (Republic of Korea); NZ (New Zealand); SE (Sweden); SI (Slovenia); US (United States)
NPS: New Psychoactive Substance
* : This compound has isomers, with identical fragmentation.
[a]: compounds also confirmed with subsequent analysis with Waters QTOF instrument
[b]: Level 2 identification achieved through comparison of standards analysed using an analogous instrument in the United States

EMCDDA report was observed [49].

3.4. Detection of pharmaceuticals

Many of the more frequently found compounds were legal pharmaceuticals such as gabapentin, codeine, bupropion, pregabalin and amantadine, so their detection was unsurprising. Tapentadol is a relatively new opioid analgesic, only receiving FDA approval in 2008 and was approved for use in other countries in the early 2010 s. Despite its infancy, it has become a popular analgesic and our study found it in 17 sites across nine countries. It has previously been found across Australia through wastewater analysis [51], while a study from Greece showed that its use declined during the COVID-19 pandemic [52] – which could be the reason we did not detected it in samples collected

from Greece.

Morphine and codeine were the most found opioid analgesics, while oxycodone and its metabolite noroxycodone as well as fentanyl were only found in samples from sites in the United States and Brazil. The ongoing 'opioid epidemic' and high prescribing rates of opioids in the United States could be the reason behind oxycodone and fentanyl being found in sites there. In Brazil, there has been an approximate 500% increase in the pharmacy sales of opioids, which this has been driven mostly by codeine [53]. However, there have been reports of the rising use of fentanyl in Brazil, following the COVID-19 pandemic, and a large seizure was reported in the southeast of the country in early 2023 [53, 54]. These fentanyl findings were especially interesting considering its low dose size.

3.5. Detections of new psychoactive substances

With quite strict thresholds in place for identification in terms of intensity and presence of fragment ions, paired with general lower use compared to traditional illicit drugs and pharmaceuticals, NPS were not expected to be found in high numbers. Nevertheless, eight were found: 3-methylmethcathinone, 3-chloromethcathinone eutylone, mitragynine, etizolam, 25I-NBOH, phenibut and trimethoxyamphetamine. The former five have previously been found using a targeted method [16, 22], while the latter three had not yet been found in wastewater. 3-Methylmethcathinone and 3-chloromethcathinone were previously found in highest levels in sites in Spain, Slovenia and Sweden, at mass loads up to 120 mg/day/1000 people, while etizolam was found in a site in Iceland at approximately 20 mg/day/1000 people. Eutylone was previously found in high levels in New Zealand (>50 mg/day/1000 people) and mitragynine in sites in the United States (1000–5000 mg/day/1000 people) [22]. Perhaps unsurprisingly, the NPS found at lower levels (< 10 ng/L) using the targeted method were not detected using the current workflow, as the concentrations present were likely below the instrumental limit of detection.

To increase the confidence around their identification, some wastewater extracts were reanalysed at a second independent laboratory, using a different instrument (Table 2). This reacquisition also enabled the samples to be processed using the Waters Forensic Toxicology Screening Application Solution with UNIFI and associated scientific libraries, vendor-specific software, which were incompatible with the initial analysis. To make the findings as similar as possible to the initial analysis, positive mode acquisition data was also reprocessed using the HighResNPS spectral library. In the end, this confirmatory analysis also identified 25I-NBOH, thereby increasing confidence in the initial Level 2 identification.

Trimethoxyamphetamine was found at one site in the United States. This compound has at least six isomers, the reference standard of none of which were available in the laboratory in Australia. Tentative identification (Level 3) was achieved through the comparison of multiple fragment ions with that on the HighResNPS database as well as retention time prediction, while the exact conformation of the trimethoxyamphetamine could not be ascertained. There is no literature data of exact mass fragmentation, so to increase the confidence and the possible configuration of the compound, additional information was sourced. The Center for Forensic Science Research and Education (CSFRE) operates NPS Discovery – an initiative to track emerging drug trends through the re-analysis of authentic forensic casework samples. As they have a comprehensive suite of NPS reference standards, a collaboration was initiated to confirm the presence of trimethoxyamphetamine, with or without exact isomer configuration. Due to insufficient sample volume, it was not possible to send the wastewater extract to be analysed at the CSFRE. However, they had recently obtained the reference standards for six trimethoxyamphetamine isomers, as part of their work. The standards were analysed using an analogous instrument and analytical setup to the initial analysis performed in Australia (Fig. 2; Figs. S1-S6). The comparison with the wastewater extract shows near exact fragmentation at a similar collision energy. The primary fragmentation seen was the loss of the amine group (-NH_2) and one methoxy group (-CH_3O) to form the major fragment ions at *m/z* 194.0930 and 181.0868. In total, up to 20 fragment ions were analogous between the reference standard and the wastewater extract, with the most intense being: *m/z* 209.1159, 194.0927, 181.0868, 178.0965, 151.0752, 123.0433, 121.0658 and 91.0533. While there are other isomers of trimethoxyamphetamine that have the same fragmentation, it is not possible to distinguish the exact positions of the methoxy groups using this approach. However, the fragmentation between all isomers and the sample were compared and based on the specific fragment ions found and their associated intensities, it is hypothesised that the wastewater sample contained either 2,3,6- or 3,4,5-trimethoxyampehtamine (Fig. 2; all isomers in supporting information). As a reference standard was not available at The University of Queensland to directly compare retention times, Level 3 identification confidence was maintained, albeit with now only two rather than six potential isomers.

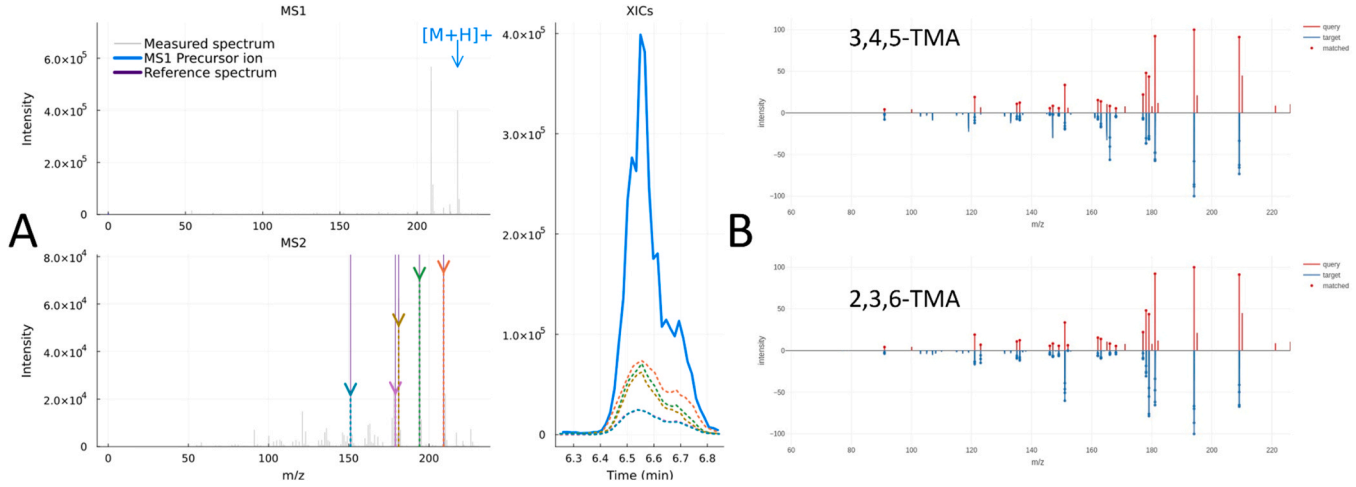

Fig. 2. Identification of trimethoxyamphetamine. A: The top panel shows the MS1 spectrum at the apex of the precursor ion, and the bottom panel the componentised fragments. The matched fragments are coloured for easier reference. The right panel shows the extracted ion chromatogram (XIC) for the precursor ion (solid blue line), matched Suspect Screening fragments as dashed lines. B: The comparison of the sample (red) to the reference standards (blue) for 3,4,5-trimethoxyamphetamine (TOP) and 2,3,6-trimethoxyamphetamine (BOTTOM).

4. Spatial trends

This workflow allowed spatial trends to be ascertained. Illicit drugs and pharmaceuticals did not appear to have distinct spatial disparity, being found across all sites, in line with previous WBE studies [37]. However, trans-cinnamoylcocaine was only found in sites in Brazil. Previous studies have shown that the South American coca plant contains this alkaloid, and therefore this finding is unsurprising [55,56]. The primary spatial trends were observed for NPS. For example, eutylone and mitragynine were only found in sites in New Zealand and the United States, respectively, and 3-methylmethcathinone was only found in sites in Europe, similar to previous studies [22,57]. 25I-NBOH was found in one site in Brazil. It is not yet under the control of any of the United Nations Conventions but has been scheduled in Brazil since 2016. However, it has previously been found in blotter paper seizures in three states of Brazil [58]. Trimethoxyamphetamine (either 2,3,6- or 3,4,5-) was found in one site in the United States. Interestingly, 3,4,5-trimethoxyamphetamine was found through NPS Discovery in late 2022, at a similar time to the sampling carried out in this work, providing further confidence to this detection. Ongoing surveillance of international wastewater samples could help unveil further hidden spatial patterns in the illicit drug market.

5. Future perspectives and limitations

This work presents an open-source workflow to facilitate the rapid identification of NPS and other psychoactive drugs of abuse in influent wastewater samples. It must be noted that the SPE procedure utilised in this study was not optimised for all compounds present in the HighResNPS database, so some compounds could have been present in the samples but not retained on the cartridge, such as synthetic cannabinoid receptor agonists [59]. Although we provided a standardised protocol for all participants to follow, it is important to acknowledge that sample handling and processing can influence the final analysis. For example, temperature of the autosampler during collection, use of a preservative, and temperature during transport, storage and sample processing are all known to influence the final results [26] and the stability of many of these compounds remains unknown.

One known limitation of HRMS analyses is the insensitivity compared to targeted LC-MS/MS methods. We have previously shown instrumental detection limits for an analogous instrument to that utilised in this work is up to 100 times greater than an LC-MS/MS instrument [23,60]. However, it is impractical to have a targeted method including the thousands of compounds that are included in the current suspect screening method. For example, although some of the NPS, illicit drugs and pharmaceuticals that were found in this method are commonplace in various targeted methods [23,36], several such as trimethoxyamphetamine, phenibut and trans-cinnamoylcocaine have not previously been found in wastewater analyses.

This work allowed a comparison to our previous targeted work, where nine NPS were found [22]. While the compounds included in that study were selected based on findings from around the world [61], it was not possible to include all in a single targeted method. In the current study, several of these compounds were also found (e.g. eutylone, etizolam, mitragynine and 3-methylmethcathinone). However, the qualitative screening approach allowed additional NPS and illicit drugs to be identified – such as trimethoxyamphetamine and 25I-NBOH. As NPS continually evolve and as new (and more potent) compounds emerge, it is important to understand their impact on the community. With the database included in the current workflow easily able to be expanded when 'new' NPS emerge, a qualitative screening may be able to detect such compounds more easily. Moreover, with HRMS data able to be retrospectively analysed, additional temporal and spatial trends can be ascertained without the need to re-analyse the samples.

With most of the workflow automatised, it considerably reduced the data processing, which has previously been a bottleneck for HRMS screening analyses. However, the intensity thresholds set could have resulted in potential false negatives (i.e. compounds were present but did not fit within our thresholds set for intensity, predicted retention time or number of fragment ions). Nevertheless, with the generous thresholds set, there is high confidence in the identity (at either Level 1 or 2) for the compounds found in this work. The code for this workflow is publicly available through the ULSA repository (https://bitbucket.org/SSamanipour/ulsa.jl/src/master/src/Screening_alignment.jl), so any laboratories undertaking similar research can adapt our workflow to better suit their needs.

Statement of environmental implication

Little is known about the hazardous nature of new psychoactive substances due to the limited information relating to their global presence. However, with their known human bioactivity, it is likely that they will also have environmental activity. To circumvent this issue, we have developed an open-access workflow to facilitate their detection following analysis by high resolution mass spectrometry. This workflow considerably reduces the time necessary to process data files and with the code available for other researchers to use, it can be utilized for a variety of compounds to better understand the hazardous materials present in our environment.

CRediT authorship contribution statement

Richard Bade: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. **Denice van Herwerden:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Nikolaos Rousis:** Formal analysis, Investigation, Writing – review & editing. **Sangeet Adhikari:** Writing – review & editing, Resources. **Darren Allen:** Formal analysis, Investigation, Writing – review & editing. **Christine Baduel:** Writing – review & editing, Resources. **Lubertus Bijlsma:** Supervision, Writing – review & editing, Resources. **Tim Boogaerts:** Writing – review & editing, Resources. **Dan Burgard:** Writing – review & editing, Resources. **Andrew Chappell:** Writing – review & editing, Resources. **Erin M. Driver:** Writing – review & editing, Resources. **Fernando Fabriz Sodre:** Resources, Writing – review & editing. **Despo Fatta-Kassinos:** Resources, Writing – review & editing. **Emma Gracia-Lor:** Resources, Writing – review & editing. **Elisa Gracia-Marín:** Resources, Writing – review & editing. **Rolf U. Halden:** Resources, Writing – review & editing. **Ester Heath:** Resources, Writing – review & editing. **Emma Jaunay:** Resources, Writing – review & editing. **Alex Krotulski:** Investigation, Resources, Writing – review & editing. **Foon Yin Lai:** Resources, Writing – review & editing. **Arndís Sue Ching Löve:** Resources, Writing – review & editing. **Jake W. O'Brien:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Jeong-Eun Oh:** Resources, Writing – review & editing. **Daniel Pasin:** Resources, Visualization, Writing – review & editing. **Marco Pineda Castro:** Resources, Writing – review & editing. **Magda Psichoudaki:** Resources, Writing – review & editing. **Noelia Salgueiro-Gonzalez:** Resources, Writing – review & editing. **Cezar Silvino Gomes:** Resources, Writing – review & editing. **Bikram Subedi:** Resources, Writing – review & editing. **Kevin V. Thomas:** Funding acquisition, Resources, Writing – review & editing. **Nikolaos Thomaidis:** Resources, Writing – review & editing. **Degao Wang:** Resources, Writing – review & editing. **Viviane Yargeau:** Resources, Writing – review & editing. **Saer Samanipour:** Resources, Supervision, Writing – review & editing. **Jochen Mueller:** Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133955.

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