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DOI
10.1016/j.chemosphere.2017.06.101

Publication date
2017

Document Version
Final published version

Published in
Chemosphere

Chemosphere 184 (2017) 1186–1193

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

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HIGHLIGHTS

- Wastewater collected during city-wide festival to track recreational substances use.
- Combination of independent and dependent data acquisition with LC-HRMS.
- Qualitative screening and reporting hits of NPS is more useful.
- Results suggest prevalence of classical drugs and low NPS use.

ARTICLE INFO

Article history:
Received 26 April 2017
Received in revised form
20 June 2017
Accepted 23 June 2017
Available online 26 June 2017

Handling Editor: Klaus Kümmerer

Keywords:
Wastewater-based epidemiology
Drugs abuse
Designer drugs
High-resolution mass spectrometry
Suspect screening

ABSTRACT

The inclusion of new psychoactive substances (NPS) in the wastewater-based epidemiology approach presents challenges, such as the reduced number of users that translates into low concentrations of residues and the limited pharmacokinetics information available, which renders the choice of target biomarker difficult. The sampling during special social settings, the analysis with improved analytical techniques, and data processing with specific workflow to narrow the search, are required approaches for a successful monitoring. This work presents the application of a qualitative screening technique to wastewater samples collected during a city festival, where likely users of recreational substances gather and consequently higher residual concentrations of used NPS are expected. The analysis was performed using liquid chromatography coupled to high-resolution mass spectrometry. Data were processed using an algorithm that involves the extraction of accurate masses (calculated based on molecular formula) of expected *m/z* from an in-house database containing about 2,000 entries, including NPS and transformation products. We positively identified eight NPS belonging to the classes of synthetic cathinones, phenethylamines and opioids. In addition, the presence of benzodiazepine analogues, classical drugs and other licit substances with potential for abuse was confirmed. The screening workflow based on a database search was useful in the identification of NPS biomarkers in wastewater. The findings highlight the specific classical drugs and low NPS use in the Netherlands. Additionally, *meta*-chlorophenylpiperazine (mCPP), 2,5-dimethoxy-4-bromophenethylamine (2C-B), and 4-fluoroamphetamine (FA) were identified in wastewater for the first time.

1. Introduction

A current trend in analytical and environmental chemistry is the chemical analysis of raw wastewater in order to identify specific biomarkers that could inform on the health and lifestyle of the population living in the catchment area under study (Daughton,

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http://dx.doi.org/10.1016/j.chemosphere.2017.06.101
0045-6535/

2001). This approach, named wastewater-based epidemiology (WBE), has been successfully applied in revealing the use of illicit substances (Ort et al., 2014; Thomas et al., 2012) and other licit substances, such as pharmaceuticals (Baz-Lomba et al., 2016; Causanilles et al., 2016), alcohol (Ryu et al., 2016), tobacco (Castiglioni et al., 2014), stress biomarkers (Ryu et al., 2015), and more recently new psychoactive substances (NPS) (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016; Kinyua et al., 2015a; Senta et al., 2015).

NPS are psychotropic drugs that produce similar effects to those produced by illicit substances, and are not directly controlled by international conventions (Reid and Thomas, 2016b). They may pose a public health threat, because there is no scientific evidence of their pharmacokinetics, recommended dose, effects or safety. Furthermore, they are easily acquired through the internet and smart shops where they are sold under various product labels with often misleading information. To date, more than 560 NPS have been reported (EMCDDA, 2016). Their monitoring and control is a challenging task because the NPS market is very dynamic with analogues constantly emerging to satisfy consumers' demands and avoid criminalization.

Typically, WBE studies focus on target analysis of well-known biomarkers that are present at a sufficient concentration to be detected and quantified. In the case of NPS, several challenges may arise: the choice of target biomarker is difficult; analytical standards are high-priced and in some cases not available, particularly for metabolites. These recreational substances are mostly consumed to a lesser extent compared to popular substances (cocaine, ecstasy), which will be translated into very low ng L^{-1} residue concentrations in wastewater. As such, recent WBE studies that focused on target screening of NPS with available reference standards using low and high resolution MS have shown that extremely low levels are detected or none at all (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016; Kinyua et al., 2015a; Senta et al., 2015). In contrast, studies that applied qualitative screening techniques based on high-resolution mass-spectrometry (HRMS) to biological matrices from individuals, namely blood and urine, (Andreasen et al., 2015; Kinyua et al., 2015b; Negreira et al., 2016b; Sundström et al., 2015), or collective pooled urine from festivals (Archer et al., 2013, 2014; Kinyua et al., 2016) have detected and identified several NPS and their metabolites. The results from the previously cited works stress the importance of different sample matrices, sampling locations and the role of improved analytical techniques to track NPS use. Pooled urine from recreational areas has the advantage that levels of drug biomarkers are higher and easier to detect compared to wastewater (Kinyua et al., 2016; Mardal et al., 2017). However, the collection of pooled urine may likely miss samples from female users (Kinyua et al., 2016). Wastewater influent samples collected at a wastewater treatment plant (WWTP) are representative for populations connected to the sewer system and would provide useful information about an entire community's use of NPS within a catchment area if advanced analytical techniques are applied. However, consumption by the population, and hence concentrations of NPS in wastewater, are much lower than those of the more traditional illicit drugs and the daily wastewater levels may thus not be sufficiently high to allow detection. A modification aimed at gathering qualitative information on NPS use by targeting sampling at some social settings (festivals or events) where substance use is elevated would increase the likelihood of successfully identifying and detecting NPS (Reid and Thomas, 2016a). Another powerful improvement which can be applied when no reference standards are available to confirm mass spectra and retention time information is the use of HRMS using the "suspect screening" approach, which involves extraction of the exact masses of expected ions $[M+H]^+$ or $[M-H]^-$ from the acquired data (Hernández et al., 2016; Kinyua et al., 2015b; Krauss et al., 2010). Fundamentally, we can cast a wider net for screening of NPS by creating a suspect list that includes all potential biomarkers of interest.

In the Netherlands, NPS appear to be used at a lower rate compared to other countries, more in particular the UK, because of the high quality, low price and availability of classical drugs such as cocaine and 3,4-methylenedioxymethamphetamine (MDMA) (Hondebrink et al., 2015). It is noteworthy to mention that amphetamine-type stimulants are easy to acquire because The Netherlands and Belgium are the most important European production areas (EMCDDA and Europol, 2016). Thus far, NPS have been found as adulterants or as a replacement of classical drugs without users' awareness. However, their use is increasing progressively in recent years as drug of choice, as data from the Poisons Information Centre show (Hondebrink et al., 2015).

The aims of the present study were to: i) analyse wastewater collected during a special social setting with a suspect screening approach; ii) elucidate the identity of NPS and provide a qualitative snapshot of recreational substances used during a festival in the Netherlands; iii) discuss the challenges in applying a suspect screening approach to wastewater samples. For this purpose, we used liquid chromatography coupled to HRMS for the screening of eight 24-h composite raw wastewater samples collected at the WWTP serving the catchment area of Amsterdam in 2012 and 2014, during festivals that brought approximately 300,000 visitors to the city.

2. Materials and methods

2.1. Sample collection

Eight 24-h flow-dependent influent composite samples were collected after the sand trap at the main WWTP serving the city of Amsterdam, representing 769,000 inhabitants (according to census data). The sampling campaign was performed in the summer of 2012 and 2014 just prior to and during a festival that attracted ~300,000 visitors to the city. Such festivals are key sites for drug use among young people (Dilkes-Frayne, 2016). Four samples corresponding to 24-h composite samples from Thursday to Sunday were collected in both years. WWTP characteristics and sample details can be found in Table SI-1 (in Supplementary Information).

2.2. Sample treatment

Samples were stored in HDPE containers and frozen immediately after collection at −20 °C until analysis. Samples were thawed at 4 °C for 24 h, and after homogenization, a 50 mL aliquot was filtered on glass microfiber filters GF/A (1.6 μm). Solid-phase extraction (SPE) was performed with Oasis HLB cartridges (150 mg, 6 cc) pre-conditioned with 8 mL of methanol and 8 mL of ultra-pure water. After sample loading, the SPE cartridge was washed with 4 mL of ultra-pure water and vacuum dried for 30 min. After elution with 8 mL methanol, the eluate was evaporated to dryness with a gentle nitrogen stream in a water bath at 35 °C. The final extract was reconstituted to 250 μL water/methanol 90:10, v/v.

2.3. Instrumental analysis

Extracts were analysed twice by liquid chromatography coupled to high-resolution mass spectrometry: (i) with an Agilent LC-QTOFMS at the Toxicological Centre at the University of Antwerp, Belgium; and (ii) with a Thermo LC-LTQ-Orbitrap at the KWR Watercycle Research Institute in Nieuwegein, The Netherlands.

Chromatographic separation was achieved for both systems with a Phenomenex Biphenyl (100 mm × 2.1 mm, 2.6 μm) column fitted to a SecurityGuard ULTRA Holder for UHPLC columns (2.1–4.6 mm). The mobile phase was ultrapure water (A) and 80:20 acetonitrile:water (B) both with 0.04% of formic acid, and with the following gradient: 0 min: 2% B; 2 min: 2% B; 18 min: 40% B; 25 min: 90% B; 29 min: 90% B; 29.5 min: 2% B; 33 min: 2% B. The total run time including column equilibration was 33 min. The injection volume was set to 2 μL and the flow rate was 0.4 mL min^{-1}.

2.3.1. Quadrupole-time-of-flight mass spectrometry

The MS system consisted of an Agilent 6530 Accurate-Mass QTOF instrument (Agilent Technologies, Santa Clara, USA) operated with a jet stream electrospray ionisation source (Dual AJS ESI source). The source parameters were as follows: gas temperature, 325 °C; gas flow, 8 L min-1; nebulizer gas, 40 psi; sheath gas temperature, 325 °C; sheath gas flow, 11 L min^{-1}; capillary voltage, 3500 V and the nozzle voltage, 0 V. The data-indpendent acquisition (All-ions MS/MS) was set-up to acquire three scan segments in MS mode alternating the collision energies to 0 eV, 15 eV, and 35 eV, in only one injection. With this acquisition mode, all ions are fragmented without a specific isolation of a precursor ion in the first mass analyser. The mass accuracy (within ±2 ppm) of the QTOFMS was calibrated before each analysis using a reference solution for scanning up to *m/z* 1,700. The scan range was set to acquire between *m/z* 50–1,000 at a rate of 2.5 spectra/s for each scan segment. For measurements, the MS was operated in 4 GHz High Resolution mode with a typical resolution of 9,000–20,000 full width at half maximum (FWHM) for the mass range *m/z* 118.0862–622.0289. Analyses were performed in positive ESI mode. Mass calibration of the QTOFMS system was controlled by constant infusion of a reference mass solution (acquired from Agilent Technologies) into the source of the QTOFMS system during the analysis. The ions selected for recalibrating the mass axis, ensuring the accuracy of mass assignations throughout the chromatographic run were the protonated reference ions ($[M+H]^+$ 121.0509 and 922.0098).

2.3.2. Linear Trap Quadrupole Orbitrap mass spectrometry

The MS system consisted of an LTQ FT Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a Heated Ion Max Electrospray Ionization (HESI) probe and operated in the positive ion mode. The conditions were: source voltage 3000 V, heated capillary temperature 300 °C, vaporizer temperature 350 °C, capillary voltage 24 V and tube lens 70 V. The mass spectrometer operated under data-dependent-acquisition (DDA) mode during the complete chromatographic run, in which both MS and MS^n spectra were acquired simultaneously. The instrument was initially set to operate in full-scan mode with accurate mass measurements. Full-scan accurate mass spectra (mass range from 50 to 1,000 Da) were obtained at a mass resolution of 60,000 FWHM (*m/z* 400). When an ion exceeded a pre-set threshold or corresponded to the inclusion mass list specified by the user, the instrument switched to product-ion scan mode (MS^n) in the ion trap with nominal mass measurements. This inclusion list was built with the masses previously tentatively identified by the QTOFMS (see Tables SI-2 and SI-3 in Supporting Information) with a retention time window of ±2 min and collision energy of 35%. In this way, relevant information for identification and confirmation, e.g., retention time, molecular weight and fragmentation, was obtained in a single analysis. The total cycle time at the selected resolution corresponded to 0.15 s. Mass calibration was performed before each batch run by using flow injection of a Polytyrosine-1,3,6 solution ($[M+H]^+$ 182.01170, 508.20783 and 997.39781) at a flow rate of 10 μL min−1.

2.4. Data analysis workflow for suspect screening

MassHunter qualitative analysis software Version B.06.00 with the personal compound database and library manager (PCDL, Version Rev. B.04.01, Agilent Technologies, Santa Clara, USA), ACD/MS Workbook Suite 2015 (MS Fragment), and Xcalibur software Version 2.1 (Thermo Fisher Scientific, Breda, The Netherlands) were used for data processing using a modification of the workflow described elsewhere (Kinyua et al., 2015b).

An updated version of our in-house spectral library was used, comprised of (il)licit drugs and pharmaceuticals of potential abuse, NPS, and their metabolites and transformation products of the different chemical families. Since wastewater can be considered a diluted urine sample, and metabolites/transformation products are expected to be more abundant (Meyer and Maurer, 2016), an effort was made to include the latter on the suspect list. To this end, the database was built upon the currently available knowledge on pharmacokinetics of NPS. This information was primarily obtained from existing literature (*in vitro* and *in vivo* studies) and the few Phase I and Phase II metabolites listed, from organizations such as European Early Warning System (EWS), European Monitoring Center for Drugs and Drug Addiction (EMCDDA), United Nations Office on Drugs and Crime (UNODC) and TICTAC Communications Limited (London), and from the section Forensic Science Products in caymanchem.com. The library currently consists of almost 2,000 entries.

The workflow is based on a 'Find by Formula (FbF)' algorithm (Agilent Technologies, Santa Clara, USA) that involves the extraction of accurate masses (calculated based on molecular formula) of expected ions $[M+H]^+$ in combination with specific parameters from the data acquired. The criteria for the proposal of a candidate list according to these parameters is defined as follows: error in the accurate mass lower than 10 ppm for the precursor ion and 20 ppm for the product ions, retention time deviation of maximum 0.1 min, signal to noise ratio lower than 3, and isotope abundance and spacing of minimum 50%. Eventually a list of candidates with their proposed fragments is generated. See Fig. 1 for the schematic of the followed workflow, and Fig. 2 for the schematic workflow used to eliminate false positives and prioritize compounds for purchase.

To communicate confidence of the identifications, we used the levels described by Schymanski and colleagues (Schymanski et al., 2014). Level one was assigned after confirmation by injection of a reference standard for determination of retention time (t_R), MS and MS/MS spectra. For Level two (2a), a probable structure was proposed based on matching existing library and literature spectra data or using non-reported diagnostic MS/MS product ions evidence. The Level 2a confirmations were based on the in-house library spectra data available from previous experiments, and intoxication cases received at our forensic toxicology laboratory (including *in vivo* samples from individual users) (Kinyua et al., 2015b; Lai et al., 2015; Mardal et al., 2017; Negreira et al., 2015, 2016a, 2016b). In addition, we used literature spectra from *in vitro* and *in vivo* studies on NPS. The Level 2a identification is definite, but lacks a commercial reference standard to warrant the Level 1 identification. For Level 3, a tentative candidate was proposed where the evidence existed for possible structure(s), however the exact structure remained unconfirmed.

Using MetFrag 2.2 developed by Ruttkies et al., (2016), available at (http://msbi.ipb-halle.de/MetFragBeta/), we input the neutral molecular formula of the precursor, and input the qualified measured product ion accurate masses and their abundances. Then MetFrag retrieves candidate structures from selected databases (ChemSpider, PubChem, KEGG and STOFF-IDENT). The candidates were *in silico* fragmented (two fragmentation steps) and the generated fragments were compared with the product ions in the acquired measured mass spectrum.

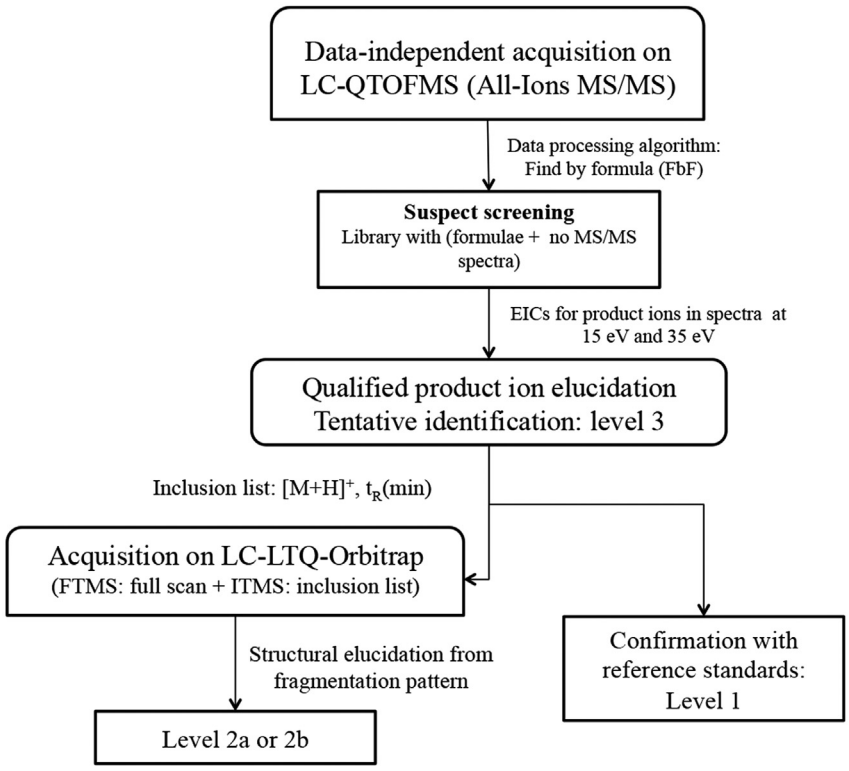

Fig. 1. Schematic of suspect screening workflow.

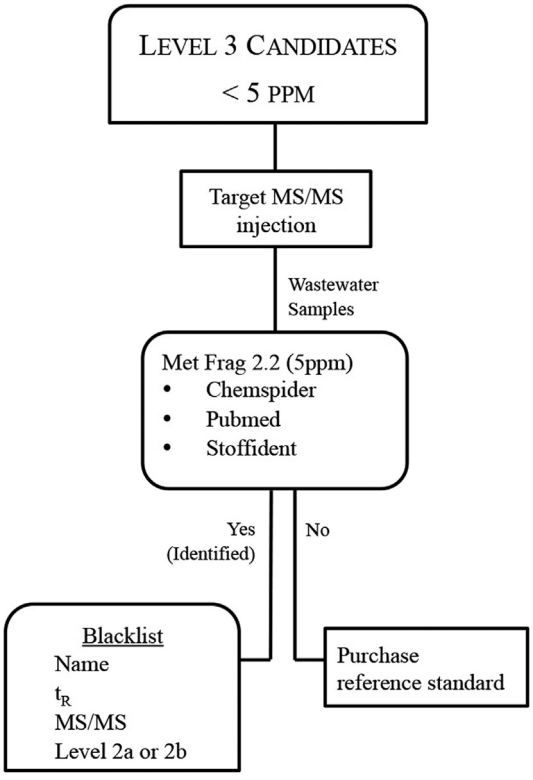

Fig. 2. Schematic of workflow used to eliminate false positives and prioritize compounds for purchase.

3. Results and discussion

3.1. Snapshot of recreational substances in use

The application of the suspect screening workflow was helpful in the search for NPS. The database allowed narrowing the search from a list of almost 2,000 entries, including 560 parent NPS in addition to their known metabolites and transformation products. Table 1 shows the final list of 8 detected NPS that were identified at least at level 3. The identity of two NPS could not be confirmed at Level 1 due to the lack of reference standards. m-Chlorophenylpiperazine (mCPP), 2,5-dimethoxy-4-bromophenethylamine (2C-B), and 4-fluoroamphetamine (FA) were identified for the first time ever in wastewater samples.

Synthetic cathinones and phenethylamines were in general terms the most frequently detected families, considering the full list of tentative hits (levels 4–5, results not shown). They are commonly used as stimulants to substitute or complement MDMA, amphetamine and cocaine. 3-methoxy-4-methylamphetamine (MMA) was detected in 5 out of the 8 samples analysed. The detection was classified with identification level 2a thanks to the library spectrum match from its previous identification by Kinyua et al., (2016) in pooled urine samples from London. 2C-B was confirmed as Level 1 in one sample in the present study. 2C-phenethylamines became list I substances of the Opium Act in the Netherlands in the late 90s despite no health incidents occurring. FA was confirmed at Level 1, in agreement with being widely reported in the Netherlands starting sometime between 2007 and 2009 until nowadays, and also elsewhere in Europe (Al-Saffar et al., 2013; Röhrich et al., 2012). In the form of 4-FA, it is found as an

Table 1
NPS identified and confirmed (Level 1–3) in wastewater samples collected during Amsterdam street festivals in 2012 and 2014.

Compound	t_R[a] (min)	Ion formula $[M+H]^+$	Measured *m/z* $[M+H]^+$	Average Δm (ppm)[b]	Qualified product ions[c]	Level[d]	Hits[e] 2012	Hits[e] 2014
3-Methoxy-4-methylamphetamine (MMA)	3.6	$[C_{11}H_{18}NO]^+$	180.1380	−1.7	77.0406, 91.0567, 121.0595	2a	4	1
Methylhexanamine	5.5	$[C_7H_{18}N]^+$	116.1433	−0.9	57.0706	1	2	
4-fluoroamphetamine (4-FA)	6.2	$[C_9H_{13}FN]^+$	154.1030	1.9	83.0297, 109.0449, 137.0761	1	1	
3,4-Methylenedioxy-*N*-ethylamphetamine (MDEA)	8.8	$[C_{12}H_{18}NO_2]^+$	208.1341	4.3	105.0697, 135.0439, 163.0755	1	1	
meta-Chlorophenylpiperazine (mCPP)	10.4	$[C_{10}H_{14}ClN_2]^+$	197.0840	−2.5	77.0385, 91.0547,118.0649, 154.0424	1	1	
2,5-dimethoxy-4-bromophenethylamine (2C-B)	11.1	$[C_{10}H_{15}BrNO_2]^+$	260.0274	−2.7	91.0573	1	1	
Fentanyl	15.5	$[C_{22}H_{29}N_2O]^+$	337.2274	0.0	79.0531, 105.0686, 188.1418	1	1	
L-759,633	19.2	$[C_{26}H_{41}O_2]^+$	385.3101	0.0	253.1219, 367.2564	3		4

[a] Retention time in minutes, measured on the QTOF.
[b] *m/z* accurate mass measurement error as the deviation from the theoretical protonated ion.
[c] DDA.
[d] Identification level according to (Schymanski et al., 2014).
[e] Hits = number of times detected in 4 samples.

adulterant in ecstasy and speed, but also as a drug of choice due to its subjective effects ranged between those of amphetamine and MDMA (Linsen et al., 2015). Its presence in urine and blood samples has been reported occasionally (Al-Saffar et al., 2013; Röhrich et al., 2012).

Piperazines are mainly used as tranquilizers or antidepressants, although some also have a recreational use as stimulants. mCPP was detected once, and confirmed at Level 1 (Fig. 3). Recently reported by the Drugs Information and Monitoring System in the Netherlands, mCPP was found to be one of the contents in a new liquid designer drug that appeared on the Dutch drug market called 'Explosion' (Bossong et al., 2005). From the opioid family, fentanyl was detected in one sample, and we confirmed its presence using a reference standard. Fentanyl is a potent, synthetic opioid analgesic known to have a rapid onset and short duration of action (Clotz and Nahata, 1991). Fentanyl is estimated to have hundreds of times the potency of pure, pharmacy-grade heroin and about 80 times the potency of morphine (Poklis, 1995). In the Netherlands it is a

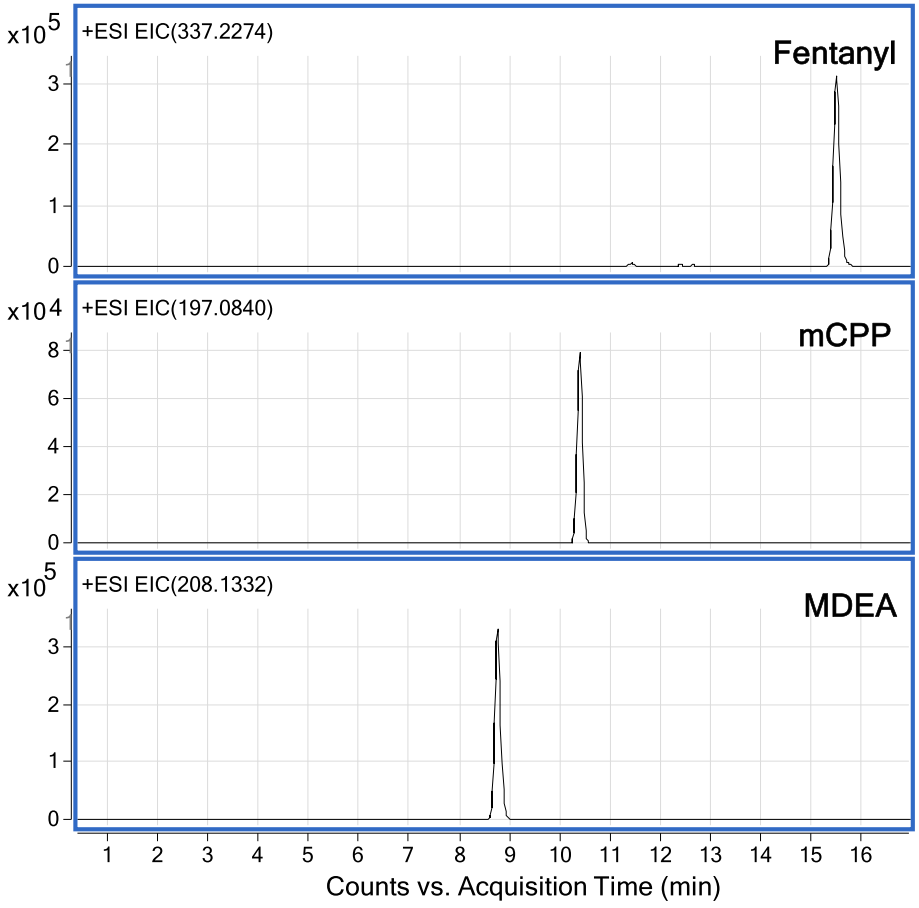

Fig. 3. NPS detected in a wastewater sample collected in 2012 during a festival in Amsterdam (Data from LC-QTOFMS).

Table 2
List of pharmaceuticals with potential of abuse and other licit substances identified and confirmed (Level 1) in wastewater samples collected during Amsterdam street festivals in 2012 and 2014.

Compound	t_R[a] (min)	Ion formula $[M+H]^+$	Measured *m/z* $[M+H]^+$	Average Δm (ppm)[b]	Qualified product ions[c]	Level[d]	Hits[e] 2012	Hits[e] 2014
Cotinine	3.1	$[C_{10}H_{13}N_2O]^+$	177.1022	−0.22	70.0658	1	4	4
Hordenine	3.1	$[C_{10}H_{16}NO]^+$	166.1226	1.56	77.0387, 121.0642	1	4	3
Paraxanthine	6.3	$[C_7H_9N_4O_2]^+$	181.0720	1.31	130.1598	1	3	2
Caffeine	8.0	$[C_8H_{11}N_4O_2]^+$	195.0877	0.42	138.0666	1	2	1
Ritalinic acid	8.4	$[C_{13}H_{18}NO_2]^+$	220.1332	1.26	82.0648	1	2	4
Methylphenidate	8.7	$[C_{14}H_{20}NO_2]^+$	234.1489	−0.24	84.1078	1	1	
Venlafaxine	12.2	$[C_{17}H_{28}NO_2]^+$	278.2115	1.19	58.0656	1	4	4
Clozapine	12.9	$[C_{18}H_{20}ClN_4]^+$	327.1371	−1.43	221.1377	1	3	
Nordiazepam	17.3	$[C_{15}H_{12}ClN_2O]^+$	271.0633	0.12	243.0174	1	1	
Oxazepam	17.3	$[C_{15}H_{12}ClN_2O_2]^+$	287.0582	−2.72	269.0834	1	3	4
Temazepam	19.4	$[C_{16}H_{14}ClN_2O_2]^+$	301.0738	−0.94	255.0675	1	4	
Diazepam	20.1	$[C_{16}H_{14}ClN_2O]^+$	285.0789	0.29	257.1232	1	1	

[a] Retention time in minutes, measured on the QTOF.
[b] *m/z* accurate mass measurement error as the deviation from the theoretical protonated ion.
[c] DIA.
[d] Identification level according to (Schymanski et al., 2014).
[e] Hits = number of times detected in 4 samples.

prescribed pharmaceutical, therefore its presence could be related to such approved use. Synthetic cannabinoids are the largest group of substances monitored by the EMCDDA in Europe. In the Netherlands, the ready availability of cannabis for adults through the coffee shop system conflicts with the interest in synthetic cannabinoids. Nevertheless, one of the substances from this class was detected. L-759,633 was detected (Level 3) in four samples (Figure SI-1).

Our findings partially match the information available from different information points in the Netherlands: the Drugs Information and Monitoring System from Trimbos Institute and the Dutch Poisons Information Centre from the University Medical Centre in Utrecht. According to their last Drug annual report (Trimbos Instituut, 2015), the most popular NPS are the phenethylamines: 4-FA, 2C-B and 5/6-APB; and ketamine and methoxetamine. The general conclusion provided by the aforementioned information shows that NPS use is not yet widespread in the Netherlands, although it is recently increasing. In the authors' opinion, the snapshot of recreational substances in use during the specific social setting sampled in this work highlights the wastewater chemical analysis as a complementary source of information in the quest for knowledge on the use of NPS. Furthermore, it supports the information from the report reflecting low use of NPS in the country, and the rising concern after the positive identification of the phenethylamines 4-FA and 2C-B.

In addition, the application of the workflow led to the positive detection and identification of 12 pharmaceuticals and other licit substances associated with the potential of abuse, and 14 classical drugs and metabolites (Table 2 and 3). These findings might not be directly linked to festival attendees themselves, but rather to the city population in general, as they have been previously reported in WBE studies including the city of Amsterdam (Bijlsma et al., 2012; Ort et al., 2014).

The most frequently detected compounds were the antidepressant venlafaxine and the anxiolytics from the benzodiazepine family oxazepam, temazepam, diazepam and nordiazepam. The use of Ritalin was observed by the presence of its main active ingredient methylphenidate and its metabolite ritalinic acid. This pharmaceutical is used to treat attention deficit disorder (ADD), attention deficit hyperactivity disorder (ADHD), and narcolepsy. But its use is prevalent in some regions as a central nervous system stimulant (Clemow and Walker, 2014). In the Netherlands, methylphenidate prescriptions have quadrupled in recent years (Health Council of the Netherlands, 2014), and this is the first time that it has been reported in wastewater. The antipsychotic clozapine, used for the treatment of schizophrenia, was also detected. Finally, biomarkers of beer (hordenine), coffee (caffeine and paraxanthine) and tobacco (cotinine) were positively identified. The presence of hordenine, which is a controlled substance in the UK (Archer et al., 2013), has not been reported before in the Netherlands.

In the case of the classical illicit drugs and metabolites the most frequently detected one was THC-COOH, the main cannabis biomarker. This is expected in the Netherlands, since cannabis use is tolerated in this country. Cocaine use was detected by the presence of the parent compound as well as two human metabolites, benzoylecgonine and ecgonine methyl ester. Additionally, the transformation product cocaethylene, which is formed when cocaine and alcohol are consumed together, was also detected. In the case of amphetamine type stimulants, amphetamine and MDMA were widely detected, while the metabolites MDA and 4-hydroxy-3-methoxymethamphetamine (HMMA) were also present. Other recreational substance positively identified was ketamine, together with 6-MAM and EDDP, the metabolites of the opioids heroin and methadone, respectively. Finally, LSD was initially tentatively identified (Level 3). This identification was made definite (Level 1) after the injection of a reference standard. The chromatograms corresponding to the accurate mass of the LSD protonated molecule in both the analytical standard and the sample can be consulted in Figure SI-2.

3.2. Filtering of false positives

Wastewater is a very complex matrix containing numerous compounds like pharmaceuticals and personal care products (PPCPs), pesticides, and emerging contaminants from many sources-residential, agricultural and industrial (Kosma et al., 2014; Ort et al., 2010). These compounds are likely interferences when analysing influent wastewater, the reason why a Level 3 identification (the exact structure is unconfirmed) remains speculative and could likely be a false positive from isobaric compounds. Considering we have a matching accurate mass (<5 ppm), isotopic pattern information and qualified product ions for the tentative candidates, searching its corresponding structural formula in databases like Chemspider can still generate > 500 possibilities. Consequently, we tried to improve the identification from Level 3 to Level 2 (before purchasing standards), and considered it worthwhile to explore their likely matches in various databases.

Since LTQ Orbitrap has a superior selectivity compared to QTOF,

Table 3
List of classical illicit drugs and/or metabolites thereof identified and confirmed (Level 1) in wastewater samples collected during Amsterdam street festivals in 2012 and 2014.

Compound	t_R[a] (min)	Ion formula $[M+H]^+$	Measured *m/z* $[M+H]^+$	Δm (average ppm)[b]	Qualified product ions[c]	Level[d]	Hits[e] 2012	Hits[e] 2014
Ecgonine Methyl Ester	1.0	$[C_{10}H_{18}NO_3]^+$	200.1281	0.90	82.0658	1	4	1
HMMA	4.6	$[C_{11}H_{18}NO_2]^+$	196.1332	−1.77	165.1389	1	1	4
Amphetamine	5.3	$[C_9H_{14}N]^+$	136.1121	0.91	91.0542	1	4	3
MDA	6.8	$[C_{10}H_{14}NO_2]^+$	180.1019	4.97	163.2555	1	1	
MDMA	7.8	$[C_{11}H_{16}NO_2]^+$	194.1176	3.91	163.0781	1	4	3
6-MAM	8.1	$[C_{19}H_{22}NO_4]^+$	328.1543	3.25	211.0827, 268.1868	1	1	
Benzoylecgonine	9.4	$[C_{16}H_{20}NO_4]^+$	290.1387	2.47	105.0338, 168.1023	1	3	3
Ketamine	9.6	$[C_{13}H_{17}ClNO]^+$	238.0993	1.04	220.1161, 207.0640	1	3	
Cocaine	12.2	$[C_{17}H_{22}NO_4]^+$	304.1543	−0.44	182.0812	1	3	
LSD	13.3	$[C_{20}H_{26}N_3O]^+$	324.2070	−2.28	223.1910, 197.1519	1	1	
Cocaethylene	13.7	$[C_{18}H_{24}NO_4]^+$	318.1700	0.15	196.1324, 278.1743	1		3
EDDP	17.9	$[C_{20}H_{24}N]^+$	278.1903	−1.95	234.1253	1	4	2
Methadone	19.1	$[C_{21}H_{28}NO]^+$	310.2165	−2.17	265.1570, 57.0348	1	4	
11-nor-9-carboxy-THC	22.6	$[C_{21}H_{29}O_4]^+$	345.2060	0.06	327.1843, 299.3178	1	4	4

[a] Retention time in minutes, measured on the QTOF.
[b] *m/z* accurate mass measurement error as the deviation from the theoretical protonated ion.
[c] DIA.
[d] Identification level according to (Schymanski et al. 2014).
[e] Hits = number of times detected in 4 samples.

by using the Orbitrap we could likely achieve resolution >50,000 for most compounds with which the tentative candidates could be distinguished from interferences of the same nominal *m/z*. Furthermore, we could apply MetFrag 2.2, an *in silico* fragmenter, which allows one to combine compound searches from databases with fragmentation prediction in order to compare to measured data. The databases linked could be large with millions (ChemSpider and PubChem) or smaller with several thousands (KEGG, HMDB, STOFF-IDENT) of structures and ensured an exhaustive search. Additionally, MS Fragment (ACD Labs) also provides *in silico* fragmentation generating possible product ions and their fragmentation pathway. On completion of this search one can either generate a 'blacklist' backed by literature (Level 2a) or diagnostic evidence from MetFrag (Level 2b); and a justified reason to purchase reference standards to confirm t_R and spectra. The blacklist remains an excellent way to quickly identify false positives in future analysis.

The piperazines para-methoxyphenylpiperazine (MeOPP) and 3-trifluoromethyl-phenylpiperazine (TFMPP) were initially tentatively identified (Level 3) but finally rejected after MetFrag analysis and standard injection because of the mismatch of the retention time. This shows the challenge associated with reporting Level 3 hits in wastewater samples.

3.3. Future of suspect screening in monitoring NPS in WBE

In the case of NPS, the number of substances with recreational use reported for the first time to the EWS keeps steadily increasing. It seems important to adapt a common "one-step-ahead" strategy where epidemiologists, analytical chemists, policy makers, law enforcement and forensic practitioners work together. Analytical toxicologists have brought forward the suspect screening of NPS in wastewater samples collected in specific social settings as a complimentary source of information. The findings of this work have shown how certain tools available in HRMS can be applied to the data processing workflow in order to reduce false positives, which seems to be the main disadvantage thus far. The future in this application of WBE seems to be directed towards the reporting of frequency of use as opposed to quantifying. This is so because of the little pharmacokinetic information, which renders the choice of target biomarkers difficult, and the uncertainties related to estimating the number of people contributing to the sample during such festivals or events. Besides, the use of information from external sources to build stronger databases for the "suspect" screening is essential for its successful application. This relates to findings from forensic laboratories on intoxication cases and biotransformation studies. Altogether, the efforts to curb NPS use would streamline since the information will be available at a faster pace.

4. Conclusions

This work has shown that sampling during specific social settings, in combination with the analysis using high resolution mass spectrometry and a specific "suspect" screening processing workflow based in a database search can be very useful to narrow the quest for NPS in wastewater analysis. As a result, the snapshot of recreational substances in use during city festivals has been presented. NPS from several groups were detected within the synthetic cathinone, phenethylamine, and synthetic cannabinoid families. The screening method helped to identify the presence of mCPP, 2C-B and 4-FA in wastewater for the first time. In addition, the use of *in silico* fragmentation tools has proven to be useful in the rejection of false positives when analytical standards are not available.

Acknowledgements

This work was supported by EU-International Training Network SEWPROF (Marie Curie-PEOPLE Grant no 317205) and COST Action "SCORE – Sewage biomarker analysis for community health assessment" [ES1307]. Dr. Alexander van Nuijs acknowledges the Flanders Foundation for Research (FWO) for his postdoctoral fellowship. Alex Veltman and Peter Theijssen from Waternet are acknowledged for providing the samples. We also thank Mr. Jorrit van den Berg from the Netherlands Forensic Institute and Mr. Trevor Shine from TICTAC Communications Ltd for sharing their knowledge about NPS with us.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.06.101.

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