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Qualitative screening of new psychoactive substances in pooled urine samples from Belgium and United Kingdom

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HIGHLIGHTS

- Pooled urine samples from Belgium and UK were collected to detect NPS use.
- Data-independent acquisition with LC-QTOFMS was applied.
- Cathinones and phenylethylamines were the most detected substances.
- Results suggest higher NPS use in UK than in Belgium.

GRAPHICAL ABSTRACT

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ABSTRACT

Concerns about new psychoactive substances (NPS) are increasing due to the rising frequency of serious intoxications. Analysis of biological fluids (urine) is necessary to get reliable information about the use of these substances. However, it is a challenging task due to the lack of analytical standards and the dynamic character of the NPS market. In the present work, a qualitative screening of NPS was carried out in 23 pooled urine samples collected from a city center in the UK and festivals in the UK and Belgium. The analytical method was based on data-independent acquisition mode using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. An in-house library was used with >1500 entries corresponding to NPS, classical drugs and metabolites. All samples contained 53 and 28 compounds of interest from the UK and Belgium respectively. Of the different compounds detected, about 70% were confirmed using retention time and product ions while the remaining compounds were identified using elucidated fragmentation pathways. The highest numbers of NPS identified in both countries were from the cathinone and phenylethylamine families, with a higher number being detected in samples from the festival in the UK. Moreover, several cathinone metabolites in human urine

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were detected and identified. The screening method proved useful to detect a large number of compounds and determine the use of NPS.

1. Introduction

Since 2005, the drug scene has changed dramatically with an increasing number of new psychoactive substances (NPS) entering the market through various modes of distribution, including the internet and the so-called "smart shops" (Prosser and Nelson, 2012). These new substances are mostly synthetic and have psychoactive properties that are similar to traditional drugs of abuse (DOA) such as cannabis, amphetamine-like stimulants or cocaine; often simply by adding or changing a functional group of an already controlled drug molecule. In addition, some of these substances were invented years ago for their possible therapeutic effects, but never made it into the pharmaceutical market (Brandt et al., 2014; King and Kicman, 2011; Shulgin and Pikhal, 1991). Several classes of NPS exist, of which the synthetic cannabinoids, cathinones, phenylethylamines and tryptamine analogues are the most important (Favretto et al., 2013). They are often sold as legal alternatives to controlled drugs ("legal highs") and given camouflaging names, such as "bath salts", "spice", "plant food" and "research chemicals", with claims to contain only legal substances and marked "not for human consumption" (Helander et al., 2013; Prosser and Nelson, 2012). This misleading information on content often comes without identification of the true active ingredients or their purity increasing the risk of overdose and adverse health effects. Consequently, users are often unaware of which compounds they have consumed. The attempt to control them has led to the synthesis of newer analogues to meet market demands and evade regulations. In 2015, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported 98 new NPS found in Europe, bringing the number of monitored NPS to >560 (EMCDDA, 2016; UNODC, 2016). These compounds are attracting increasing attention, due to numerous cases of acute and fatal intoxications associated with NPS intake (Andreasen et al., 2015; Blanckaert et al., 2013; Rojek et al., 2012).

The European Union's Early Warning System (EWS) compiles highly relevant information on NPS in Europe, but due to the new and highly dynamic character of these substances, reliable information regarding the extent of their actual use is difficult to obtain. The development and application of alternative approaches for the identification of NPS on the market and the evaluation of their use is urgently needed. Pooled urine analysis (PUA) is the analysis of the content of stand-alone male urinals placed in locations with a high number of nightclubs, festivals, etc. (Archer et al., 2014a). Analysis of pooled urine provides information on substance use, such as drugs and NPS, in an anonymous way through measurements of excreted parent compounds and metabolites. Previous studies conducted by Archer et al. (2013, 2014a, 2014b) demonstrated that PUA of samples collected from the city centre of London and from a nightclub could be used to detect and monitor trends in NPS use.

The analytical methods typically used for the detection of DOA and NPS in biological matrices are mostly based on targeted analysis developed from available reference standards (Strano-Rossi et al., 2014; Strano Rossi et al., 2014). It is very difficult to know which biomarkers (parent or metabolites) are present in biological samples (urine or serum) to subsequently determine which NPS are used. Consequently, these targeted methods miss very likely information on NPS present in samples. In addition, the cost and availability of reference standards for target NPS biomarkers is a major challenge for analytical chemists. In order to overcome this and to get a wider assessment of the usage of NPS, applied suspect and non-targeted screening techniques (Gago-Ferrero et al., 2015; Hernández et al., 2011; Kinyua et al.,

2015b) are desirable. The separation of analytes based on liquid chromatography (LC) coupled to detection with accurate-mass quadrupole time-of-flight mass spectrometry (QTOFMS) or high-resolution linear trap quadrupole (LTQ)-Orbitrap mass spectrometry enables the detection of a large number of compounds. The accurate mass of a precursor ion obtained from MS scan enables the molecular formula generation and MS/MS spectra provide confirmatory information after structural elucidation of the precursor and product ions (Hogenboom et al., 2009). In addition, target and non-target data handling techniques (Bletsou et al., 2015; Ibáñez et al., 2014; Kinyua et al., 2015b; Schymanski et al., 2014a) can be applied to comprehensively process the acquired data from a sample. The PUA studies conducted by Archer et al. (2013, 2014b) were based on a LTQ-Orbitrap instrument for screening of DOA and NPS, however information about data processing technique, accurate masses and MS/MS spectra were not provided (Archer et al., 2013, 2014b). This information could be useful for inclusion into screening databases for routine analysis in forensic laboratories.

In an earlier method (Kinyua et al., 2015b), a workflow based on data-independent acquisition (DIA) mode on LC-QTOFMS was optimized and applied for the screening of NPS and their metabolites in biological matrices. A major advantage of the DIA mode is that all ions are fragmented without the need for a specific isolation of a precursor ion in the first mass analyser (Bern et al., 2009; Roemmelt et al., 2014; Wrona et al., 2005) and, therefore, MS and "MS/MS" information are obtained in one single injection without losing useful information. It has been ideal for qualitative purposes allowing retrospective analysis even years after data are acquired particularly since the acquisition of product ion data is not dependent on a threshold trigger of the 'precursor' ion, and thus no information is lost (Hernández et al., 2015).

In the present work, we applied this DIA workflow to detect the use of different classes of NPS through the analysis of 23 pooled urine samples collected from Belgian and UK festivals and from a UK city centre. The aims of this work were i) to detect the presence of NPS and their metabolites in pooled urine samples from two countries using a data independent acquisition method and workflow; ii) demonstrate actual usage of NPS in the areas studied iii) demonstrate the identification of NPS and their metabolites without the use of reference standards and iv) demonstrate the usefulness of PUA in the detection of the actual use of NPS.

2. Materials and methods

2.1. Chemicals and reagents

LC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by purifying demineralized water in an Elga LabWater Purelab Flex system (Veolia Water Solutions & Technologies Belgium, Tienen, Belgium). Formic acid (eluent additive for LC-MS, 98%) was obtained from Sigma-Aldrich (Steinheim, Germany). The reference standards ranitidine-D_6 and fluoxetine-D_5 (with purity > 98%) were purchased from Cerilliant (Round Rock TX, USA) at concentrations of 1000 mg/L in methanol. Working solutions were prepared for concentrations ranging between 0.005 and 100 mg/L in methanol.

2.2. Liquid chromatography

The LC system consisted of an Agilent Infinity 1290 SL binary pump with an integrated two-channel solvent degasser, a thermostated

Agilent 1290 HiP-ALS autosampler system (20 μL injection loop) and a 1290 Agilent TCC SL column compartment (Agilent Technologies, Santa Clara, USA). Chromatographic separation was achieved 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) and maintained at 32 °C. The mobile phase was 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 optimized based on peak shape and set to 2 μL and the flow rate was 0.4 mL/min.

2.3. 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; nebulizer gas, 40 psi; sheath gas temperature, 325 °C; sheath gas flow, 11 L/min; capillary voltage, 3500 V and the nozzle voltage, 0 V. The data-independent 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. With this acquisition mode, in only one injection, data are acquired in scan segment one to display the 'precursor ion', and scan segment two and three to provide the product ions. The mass accuracy of the QTOFMS was calibrated before each analysis using a reference solution for scanning up to m/z 1700. The scan range was set to acquire between m/z 50–1000 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 9000–20,000 full width at half maximum (FWHM) for the mass range m/z 118.0862–622.0289.

Analyses were performed in positive and negative ESI modes in two separate runs. 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 assignments throughout the chromatographic run were the protonated reference ions ($[M + H]^+ = 121.0509$ and $[M + H]^+ = 922.0098$) for the positive mode and the deprotonated reference ions ($[M - H]^- = 112.9856$ and $[M - H]^- = 980.0164$) for negative mode.

MassHunter qualitative analysis software (Version B.06.00) and the personal compound database and library manager (PCDL, Version Rev. B.04.01, Agilent Technologies, Santa Clara, USA) were used for data processing using a workflow described in a previous study (Kinyua et al., 2015b).

Briefly, the workflow combines and structures fundamentals of target and suspect screening data processing techniques, since the data are acquired utilizing all-ions MS/MS mode we utilized a structured algorithm. The algorithm called 'Find by Formula (FbF)' searches (matching window of ± 10 ppm) the acquired data file (scan segment one - 0 eV) for the exact masses of expected ions $[M + H]^+$ or $[M - H]^-$ (calculated based on molecular formula) of compounds contained in an in-house built library comprising of > 1500 compounds. The in-house library contains 130 DOA, 1200 NPS (parent, their derivatives and metabolites), and 200 pharmaceutical compounds. The overall match score for each candidate compound was calculated based on the mass accuracy, and isotopic pattern match. The product ion confirmation for target compounds (with MS/MS spectra and t_R in the library) involves the extraction as EICs of the ten most abundant product ions in the library for the candidate compound in the acquired data file within t_R window ± 0.1 min of precursor ion and overlaid with EIC of the precursor ion. Similarly, for suspect compounds (with only molecular formulae in the library) 20 EICs within t_R window ± 0.1 min of precursor ion of the most abundant product ions from average spectra (15 eV and 35 eV) of the acquired data file are extracted and overlaid with EIC of

the precursor ion. To reduce the number of qualified product ions, the option to automatically generate formulae for product ions of the proposed candidate was included. Finally, structures of qualified product ions were elucidated manually using basic fragmentation rules and the software ChemDraw Ultra 14.0. which led to a list of confirmed candidates at different confidence levels. To communicate confidence of the identifications we used three of the five levels described by Schymanski et al. (2014b). Confirmation by injection of a reference standard for determination of retention time, MS and MS/MS spectra were designated as level one, while for level two, a probable structure was proposed based on matching existing (library or literature) spectrum data or using non-reported diagnostic MS/MS product ions evidence. For level three, a tentative candidate was proposed with a possible structure, however, the exact structure remained unconfirmed. Further details on the workflow can be found in Kinyua et al. (2015b).

The NPS compound information for the suspect library was obtained from various existing literature on NPS (in vitro and in vivo studies) listed in Table SI-1, reports from organizations such as EMCDDA (EMCDDA, 2002, 2003, 2013, 2014a, 2014b, 2014c, 2015, 2016), United Nations Office on Drugs and Crime (UNODC) (UNODC, 2014, 2016), and verbal communications from TICTAC Communications Limited (London).

2.4. Sample collection

2.4.1. United Kingdom (UK)
Mobile male urinals were placed in a city centre at a railway station in the evening (between 5:00 and 7:00 PM) in the UK. Pooled urine samples (25 mL) were collected on one weekend from Thursday to Sunday in September and December 2013 between 6:30 and 8:30 PM using a plastic tube connected to a 15 mL disposable syringe and transported at room temperature to the laboratory within 1 h, where they were stored at − 20 °C.

Sampling at a music festival in the UK (number of attendees: 35,000) was performed during the summer of 2014 at the same five locations on three consecutive days. The collection was done from reservoir tanks, connected to male urinals. Samples were collected (using the same procedure for the city samples above) in the afternoon of each day, kept on ice and frozen to − 20 °C within 12 h. Overall, 20 samples were analysed (Table SI-2).

2.4.2. Belgium
Sampling at a Belgian music festival (number of attendees ~ 80,000) was conducted during the summer of 2015. Twenty milliliter of pooled urine samples (n = 7) were collected from a reservoir tank that was linked to male urinals located around the mainstage of the festival (Table SI-2). A plastic tube connected to a 10 mL pipette was used to draw the sample from the tank. In total seven samples were collected at two different time points over three consecutive days with an extra time point on one of the three days (Table SI-2). The samples were immediately placed on ice before transporting to the laboratory where they were stored at − 20 °C within 12 h. For analysis, samples were pooled per day of collection resulting in three samples.

2.5. Sample preparation

To 200 μL of pooled urine 400 μL of acetonitrile was added and this was vortexed for 30 s and centrifuged for 10 min at 8000 rpm. The supernatant was then evaporated to dryness under a gentle nitrogen stream at 40 °C and reconstituted in 60 μL of a mixture water: acetonitrile (98:2, v/v) and vortexed for 30 s after addition of 10 μL of a standard mix of fluoxetine-D₅ and ranitidine-D₆ at 250 μg/L. The purpose of this standard mix was to monitor instrument performance by confirming their presence, abundance and retention time in each sample.

3. Results and discussion

3.1. Compound identification

In this study, we report compounds identified at levels 1 and 2a and consider them to be definite. We also chose to add the level 3 identifications to the supplementary information. For the level 1 confirmation, we injected commercial reference standards to the LC-QTOFMS to confirm the tR and product ions. The level 2a identification as described by Schymanski et al. (2014b) was based on evidence of accurate mass of parent compound and their product ions, tR, matching literature and/or library spectrum data. The library spectra data were available from previous experiments and from intoxication cases received at our forensic toxicology laboratory (including *in vivo* samples from individual users) (Erratico et al., 2015; Kinyua et al., 2015b; Lai et al., 2015; Negreira et al., 2015; Negreira et al., 2016a; Negreira et al., 2016b). The level 2a identification is definite, but it lacks commercial reference standard to warrant its level 1 identification. On the other hand, the level 3 identification is speculative and has been previously described as a "grey zone" (Schymanski et al., 2014b) where evidence exists for possible structure(s), but inadequate information to confirm one definite structure. We consider it worth reporting since the data were obtained from urine samples and thus the compounds present are more

likely from consumption by individuals, furthermore, the samples were collected from recreational areas and events which are considered 'hotspots' for drug use.

3.2. Overall number of compounds detected

In total, we detected 53 and 28 compounds (DOA, NPS, some pharmaceuticals and their metabolites) from the samples from UK and Belgium, respectively with confirmation levels one to three. Eight NPS (methiopropamine (MPA), methylone, ethylone, methedrone, mephedrone, 5-(2-aminopropyl)-benzofuran (5-APB), ketamine and methoxetamine (MXE)), and five DOA (amphetamine, methamphetamine 3,4-methylenedioxymethamphetamine (MDMA), cocaine and tetrahydrocannabinol (THC)) were identified and confirmed using reference standards in the samples from UK (Table 1). In the samples from Belgium, three NPS (4-fluoroamphetamine (4-FA), α-pyrrolidinopentiophenone (α-PVP) and ketamine), and four DOA (amphetamine, 3, 4-methylenedioxyamphetamine (MDA), MDMA and cocaine) were identified and confirmed using reference standards (Table 2). The other detected compounds (NPS and metabolites) in both UK and Belgium samples were identified based on their accurate mass, structural elucidated product ions and classified using the identification levels proposed by Schymanski et al. (2014b) described above. For

Table 1
Compounds detected in 20 pooled urine samples from a festival in UK in 2014 and urinals set up in London city in 2013.

Compound	t_R[a]	Ion formula $[M + H]^+$	Calculated m/z $[M + H]^+$	Δm (average ppm)[b]	Qualified product ions	Level[c]	Hits
Hydroxyamphetamine	2.21	$[C9H14NO]^+$	152.1070	1.24	135.0806, 107.0496	2a	19
Hordenine	2.90	$[C10H16NO]^+$	166.1226	2.33	121.0667, 103.0551, 91.0542, 77.04	1	18
Cotinine	3.13	$[C10H13N2O]^+$	177.1022	4.25	80.0495	1	5
Hydroxymethamphetamine	3.40	$[C10H16NO]+$	166.1226	4.67	121.0671, 77.0396	2a	1
Hydroxynorketamine (HNK)	4.10	$[C12H15ClNO2]^+$	240.0786	−6.69	223.0520, 205.0415, 142.0777	2a	2
4-Hydroxy-3-methoxymethamphetamine (HMMA)-sulphate	4.47	$[C11H18NO5S]^+$	276.0900	−0.68	196.1336	1	15
HMMA	4.67	$[C11H18NO2]^+$	196.1332	−2.63	165.0924, 137.0607, 105.0704	1	12
Hydroxycodeine	4.96	$[C18H22NO4]^+$	316.1543	−0.80	151.0390, 86.0964	2a	2
Amphetamine	5.50	$[C9H14N]^+$	136.1121	1.68	91.0548, 58.0662	1	18
Methiopropamine	5.78	$[C8H14NS]^+$	156.0841	1.72	97.0106	1	2
Ranitidine-D_6	6.70	$[C13H17D6N4O3S]+$	321.1862	−0.31	265.1282, 130.0496	1	20
Methamphetamine	6.80	$[C10H16N]+$	150.1274	2.16	91.055, 65.0394	1	5
Methylone	6.90	$[C11H14NO3]^+$	208.0968	3.84	132.0808, 120.0808, 117.0573	1	3
Dihydromephedrone	7.30	$[C11H18NO]^+$	180.1383	1.93	162.127, 147.1058	1	6
Codeine-6-glucuronide	7.16	$[C24H30NO9]^+$	476.1915	0.21	300.1594	1	1
Normephedrone	7.40	$[C10H14NO]^+$	164.1070	−3.05	130.0643, 115.0534, 91.0537	1	4
Codeine	7.52	$[C18H22NO3]^+$	300.1594	3.63	58.0651	1	1
3,4-Methylenedioxy-methamphetamine (MDMA)	7.55	$[C11H16NO2]^+$	194.1166	−5.15	163.0754, 135.0441, 133.0648, 105.0699	1	2
Dihydromephedrone 2	7.65	$[C11H18NO]^+$	180.1383	−3.45	162.127, 147.1058	2a	5
Ethylone	7.72	$[C12H16NO3]^+$	222.1125	4.58	204.1041, 174.0927	1	1
Methedrone (4-methoxymethcathinone)	7.82	$[C11H16NO2]^+$	194.1176	5.47	146.0581, 103.0534, 91.0528	1	1
Dehydronorketamine	7.94	$[C12H13ClNO]^+$	222.0680	−2.59	205.0415, 177.0466, 170.0731	1	2
Mephedrone	8.10	$[C11H16NO]^+$	178.1226	1.53	160.1117, 145.0884	1	12
Ritalinic acid	8.41	$[C13H18NO2]+$	220.1332	2.75	174.1277, 84.0808	1	2
Norketamine	8.75	$[C12H15ClNO]^+$	224.0837	6.69	125.0153	1	2
5-(2-aminopropyl)benzofuran, (5-APB)	8.86	$[C11H14NO]^+$	176.1070	1.70	131.0479, 91.0543, 77.0393	1	1
Norbenzoylecgonine	9.09	$[C15H18NO4]^+$	276.1230	4.71	154.0863, 136.0757	2a	1
Benzoylecgonine	9.38	$[C16H20NO4]^+$	290.1387	1.45	168.1019, 105.0335, 77.0386	1	10
Ketamine	9.80	$[C13H17ClNO]^+$	238.0993	3.78	125.01525	1	8
Methoxetamine	10.90	$[C15H22NO2]^+$	248.1666	3.65	203.1073, 121.066	1	3
Norcocaine	11.43	$[C16H20NO4]^+$	290.1387	−2.71	105.0352	1	2
Cocaine	12.87	$[C17H22NO4]^+$	304.1543	2.07	109.1012, 105.0335, 91.0542, 86.0964	1	2
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	18.24	$[C20H24N]^+$	278.1903	3.78	234.1277, 158.0964	1	1
Fluoxetine-D_5	18.60	$[C17D5H14F3NO]+$	315.1727	0.63	124.0868, 107.0508	1	
Methadone	19.47	$[C21H28NO]^+$	310.2165	1.93	265.1587, 91.0542, 57.0335	1	1
THC	21.33	$[C21H31O2]^+$	315.2319	6.02	161.1325	1	1

Hits = number of times detected in 20 samples.
[a] Retention time (min).
[b] m/z accurate mass measurement error.
[c] Identification level according to Schymanski et al. (2014b).

Table 2
Compounds detected in pooled urine samples from a festival in Belgium in 2015.

Compound	t_R[a]	Ion formula [M + H]+	Calculated m/z [M + H]+	Δm (ppm)[b]	Qualified product ions	Level[c]	Hits
Hydroxyamphetamine	2.21	[C9H14NO]$^+$	152.1070	−5.16	135.0806, 107.0496	2a	1
Hordenine	2.90	[C10H16NO]$^+$	166.1226	2.09	121.0653, 103.0551, 77.0400	1	3
HMMA-sulphate	4.47	[C11H18NO5S]+	276.0900	−0.68	196.1336	2a	3
HMMA	4.67	[C11H18NO2]+	196.1332	4.23	165.0924, 137.0607, 105.071	1	3
Amphetamine	5.50	[C9H14N]+	136.1121	1.00	91.0546	1	3
4-Fluoroamphetamine	6.23	[C9H13FN]+	154.1027	1.79	109.0451	1	3
Paraxanthine	6.30	[C7H9N4O2]+	181.0720	2.32	130.0498, 105.0337	1	3
Ranitidine-D$_6$	6.70	[C13H17D6N4O3S]+	321.1862	−0.62	265.1282, 130.0496	1	3
MDA	6.80	[C10H14NO2]+	180.1019	0.48	163.0750	1	3
MDMA	7.88	[C11H16NO2]+	194.1176	1.90	163.0754, 135.0441, 133.0648, 105.0699	1	3
Caffeine	8.04	[C8H11N4O2]+	195.0877	0.48	138.0656, 117.0332	1	3
Benzoylecgonine	9.45	[C16H20NO4]+	290.1387	0.95	168.1015, 105.0333	1	3
Ketamine	9.62	[C13H17ClNO]+	238.0993	1.97	125.0153	1	2
M-264 (α-PVP metabolite)[d]	10.40	[C15H22NO3]+	264.1594	−0.26	246.149, 91.0546, 118.0644, 105.0339,	2a	2
α-Pyrrolidinopentiophenone (α-PVP)	10.77	[C15H22NO]+	232.1696	0.08	105.0340, 91.0548, 77.0398	1	1
M-234 (α-PVP metabolite)[d]	11.54	[C15H24NO]+	234.1852	−0.92	216.1751, 173.1187, 104.0623	2a	1
Cocaine	12.19	[C17H22NO4]+	304.1543	−0.38	182.1178, 105.0335	1	3
Venlafaxine	12.25	[C17H28NO2]+	278.2115	−0.01	118.0865, 58.0659	1	1
Cocaethylene	13.86	[C18H24NO4]+	318.1700	−0.15	196.1324, 278.1743	1	3
Fluoxetine-D$_5$	18.60	[C17D5H14F3NO]+	315.1727	−0.63	124.0868, 107.0508	1	3

Hits = number of times detected in 3 samples.
[a] Retention time (min).
[b] m/z measurement error.
[c] Identification level according to Schymanski et al. (2014b).
[d] α-PVP metabolites identified *in vitro* by Negreira et al. (2015).

several compounds (Table SI-3 and 4), no reference standards were available to confirm their identification at level 1. Overall, the detection of DOA in both sample locations was a clear indication of drug use in the two locations. Ketamine, an older generation NPS, remains popular as evidenced by the detection of parent compound and metabolites (hydroxynorketamine (HNK), norketamine (NK), and dehydronorketamine (DHNK)) in both UK and Belgium (Tables 1 and 2).

3.2.1. Metabolites (levels 1 and 2a)

With pooled urine analysis it is difficult to determine at which point a drug was consumed by users and which compound (parent or metabolite) to target for screening. The inclusion of metabolites to the in-house database and subsequent product ion elucidation of proposed candidates proved to be useful in detecting DOA and NPS since some compounds are extensively metabolized and only the corresponding metabolites are detectable in urine, this was also the case in a previous study by Mardal et al. (2016). The detection of hydroxy-methoxymethamphetamine (HMMA), HMMA-sulphate (HMMA-S), *p*-hydroxyamphetamine (AMP-OH), *p*-hydroxymethamphetamine (METH-OH), and (HNK) at a higher frequency than their parent compounds (Tables 1, 2) proves that one can likely miss the parent compounds if they are (bio)transformed or at concentrations below the limit of detection at the time of sampling. However, it is important to note that AMP-OH and METH-OH are not specific since they are also metabolites of para-methoxyamphetamine (PMA) and PMMA respectively (Baselt, 2014; Lai et al., 2015).

The compounds identified as isomers of 4-methylephedrine (dihydromephedrone) m/z 180.1383 at 7.3 and 7.6 min are metabolites of mephedrone. Only the isomer at 7.3 min could be confirmed using a reference standard (Fig. SI-1). The isomer with t_R 7.6 min was distinguished from tentative identified compound m/z 180.1383 at 3.5 min (level 3) using qualified product ion m/z 162.1270 (Fig. 1) which is consistent with *in vivo* detected dihydro-mephedrone (Pedersen et al., 2013). Additionally, by including metabolites from previous *in vitro* experiments (Negreira et al., 2015; Shima et al., 2014; Tyrkkö et al., 2013) we were able to detect and identify (level 2a) two α-PVP metabolites (m/z 264.1599; 234.1858) in pooled urine samples from Belgium (Table 2; Fig. 2). The identification of these metabolites indicates that the inclusion of metabolites might improve the detection of NPS.

However, in most of the cases, confirmed level 2a and 3 compounds (particularly metabolites) are not available as commercial reference standards, and thus a comprehensive confirmation (level 1) cannot be performed.

3.2.2. Level 3 identification

The most detected tentatively identified (level 3) compound (m/z 180.1383 at 3.5 min) present in 13 samples from the UK (Table SI-4) was proposed to be one of two possibilities: 2- or 3-methoxymethamphetamine (MMA) (Fig. SI-2) with respect to our library. 2-MMA (methoxyphenamine) and 3-MMA are positional isomers of *para*-methoxymethamphetamine (PMMA) having the methoxy group in the *ortho*- or *meta*-position respectively. The tentative identification of 2- or 3-MMA involved elucidation of product ions m/z 121.0648, 91.0542, 77.0386 and 60.0808 (Fig. SI-3). Firstly, the cleavage between the α-carbon to the nitrogen atom and the aromatic ring led to the ions at m/z 121.0648 and 60.0808. Secondly, the loss of the methoxy group from 121.0648 led to the ions at m/z 91.0542 and 77.0386. Though these fragments belong clearly to 2- or 3-MMA, they are not so specific since they are common to other phenylethylamines, such as 4-MMA (PMMA) which can be confirmed with retention time at 7.8 min from a previous study utilizing the same workflow (Kinyua et al., 2015b). 2-MMA is available as a bronchodilator (Dal Cason, 2001) and not much has been reported on 3-MMA (Dal Cason, 2001).

The second most detected tentatively identified (level 3) compounds in the UK samples were 5-methoxy-*N*-methyl-*N*-isopropyltryptamine (5-MeO-MiPT) and *O*-acetylpsilocin (4-ACO-DMT) (Table SI-3). 5-MeO-MiPT (m/z 247.1805) is a tryptamine which is structurally similar to the amino acid tryptophan. Many tryptamines are found in nature, such as psilocybin (*O*-phosphoryl-4-hydroxy-*N,N*-dimethyltryptamine) and psilocin (4-hydroxy-*N,N*-dimethyl tryptamine), which are obtained from certain mushrooms indigenous to tropical and sub-tropical regions of South America, Mexico, and the USA (Martins et al., 2010; Zuba, 2012). Tentative identification of 5-MeO-MiPT (Fig. SI-3) indicated that the fragmentation pathway started with the loss of the isopropyl moiety leading to the ion at m/z 205.1335. Additional losses of the *N*-ethyl-methylamine chain or *N,N′*-dimethylamine and methoxy group resulted in the fragments at m/z 146.0600 and 130.0651, respectively. From the ion at m/z 130.0651 to the ion at m/z 120.0808, loss of a methyl group and opening of the
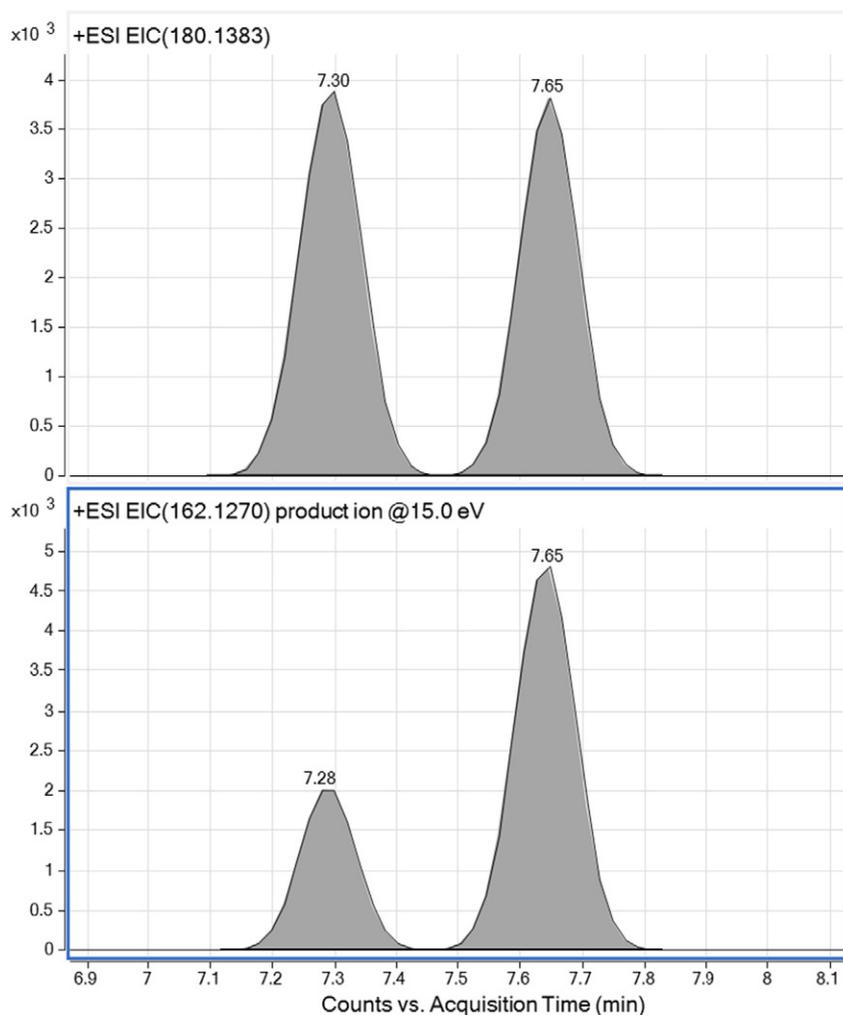
Fig. 1. Chromatogram showing isomers of dihydro-mephedrone (m/z 180.1383) with co-eluting fragment ion (m/z 162.1270) detected in 13 pooled urine samples from UK.

indole ring were observed. Successive losses of an amine group and a CH_2 moiety yielded the ions at m/z 103.0542 and m/z 91.0542, respectively. O-acetylpsilocin (also known as psilacetin, 4-acetoxy-

dimethyltryptamine, or 4-AcO-dimethyltryptamine) presented the characteristic fragments at m/z 188.0706, 118.0651 and 60.0808 corresponding to the 2-methyl-4-acetoxy-indole, indole moiety, and
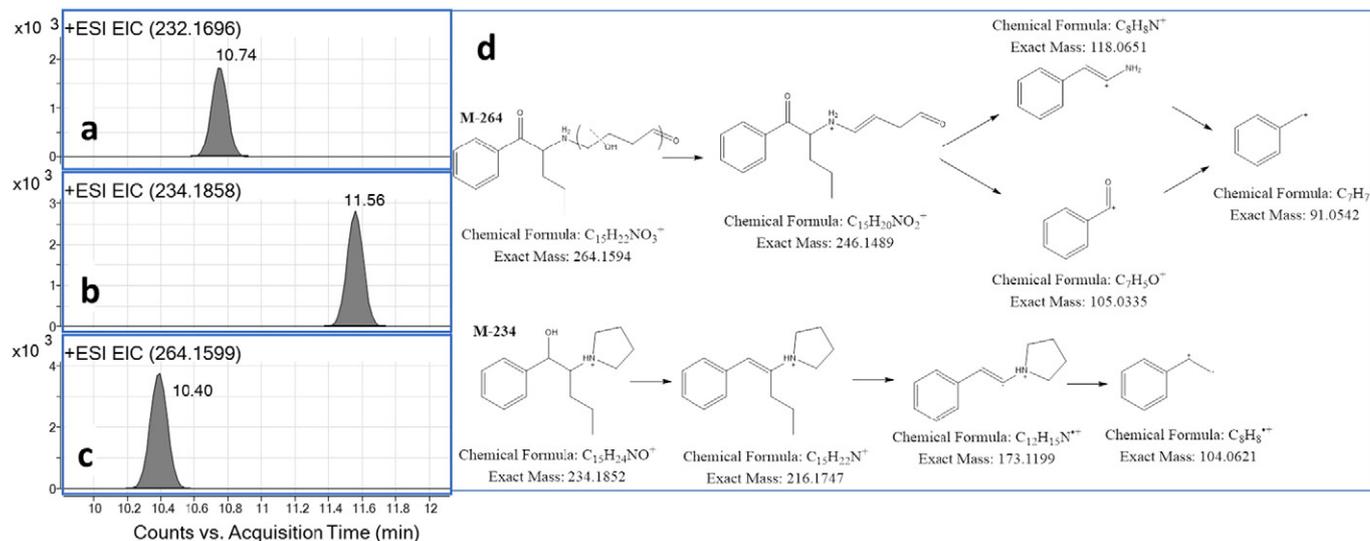

Fig. 2. Identification of α-PVP and its metabolites in pooled urine samples from Belgium. a. Chromatogram showing α-PVP m/z 232.1696 at t_R 10.74 min. b. Chromatogram showing α-PVP metabolite m/z 234.1858 at t_R 11.56 min. c. Chromatogram showing α-PVP metabolite m/z 264.1599 at t_R 10.40 min. d. Identification of two α-PVP metabolites (m/z 264.1594; m/z 234.1852) (previously identified by Negreira et al. (2015)) detected in pooled urine from a Belgian festival.

trimethylamine respectively (Fig. SI-3). Formation of the amine moiety (m/z 60.0808) is very typical in other tryptamine compounds (Martins et al., 2010; Zuba, 2012). In some cases, a fragment at m/z 146.0600 was also qualified, corresponding to 2-methyl-4-hydroxy-indole.

3.2.3. Challenge of suspect screening identifications for NPS

Suspect screening has opened up the opportunity to tentatively identify compounds when no reference standards are available. However, in the case of level 3 identifications it may be difficult to make a definite identification where one might have many possible structures that match the acquired mass and can be justified by the qualified product ions. In such a case, it would be difficult to determine which suspects' reference standards to purchase. Furthermore, in many cases the reference standards are costly, unavailable commercially and may require long wait time for purchase permits.

3.3. NPS in UK samples

A larger number of samples were collected over a longer period in the UK (Table SI-2) which explains the high number and diversity of NPS detected. Considering this study and the study by Archer et al. (2014b), it is notable that MPA, mephedrone and 5-APB are consistently detected NPS in the UK.

Mephedrone and its metabolite normephedrone were present in 12 and 6 samples, respectively, with >60% of these positives being detected in festival samples (Table 1 and SI-5). MPA, ethylone, 5-APB and MXE were detected only in festival samples whereas methedrone (4-methoxymethcathinone) was only detected in a city sample. Methylone and ketamine were detected in both festival and city samples, furthermore, ketamine and its metabolites appear to be consistently used in both environments (Table SI-5).

Few synthetic cannabinoids were detected in this study, despite being the largest class of NPS (EMCDDA, 2015). This could indicate their low availability in the city and at the festival in the UK. However, since they are frequently found in herbal mixtures in unknown amounts (Seely et al., 2012), and are known to be extensively metabolized (Erratico et al., 2015; Wintermeyer et al., 2010) with one study revealing that the parent compound could not be detected within 12 h of consumption (Sobolevsky et al., 2010). It is also likely the levels of parent and/or metabolite in the urine samples were below detection limit.

Overall, a higher number of compounds were detected in festival samples compared to samples from the city. In addition, specific NPS families (phenylethylamines, amphetamine type substances (ATS), cathinones, classic DOA, psychedelic drugs, and pyrolidinophenones) were detected more often in festival samples than in city samples (Table SI-5).

3.4. NPS in Belgian samples

In general, fewer NPS and metabolites were detected in the festival samples from Belgium compared to the samples from the UK festival (Table 2). Two possible explanations can be found for this observation: i) the different nature of the festivals and the fact that the samples from the Belgian festival covered only the mainstage; and ii) a lower use of NPS in Belgium compared to the UK. The main NPS detected and confirmed in these samples were 4-FA, α-PVP and ketamine (Table 2). There have been a high number of notifications to EMCDDA since 2008 regarding 4-FA (King, 2014), and this compound was detected in all samples collected over the three day festival suggesting popularity of this NPS (Table 2). The use of α-PVP at the festival was obvious from the detection of the parent (one sample) and its identified metabolites at m/z 264.1599 (two samples) and at m/z 234.1858 (one sample) (Fig. 2; Table 2). Ketamine remains popular as evidenced by the detection of the parent compound in samples from two days of the festival. The tentatively identified NPS (4-fluoromethamphetamine, 2-methoxyketamine and 5-fluoropentyl-3-pyridinoylindole) were only detected once over the three day festival (Table SI-4). The DOA (amphetamine, MDA, MDMA, and cocaine) and their metabolites (AMP-OH, HMMA, HMMA-S, and benzoylecgonine) were consistently confirmed in all samples collected over the three-day event, which suggests a significant use of these substances at this event.

3.5. Benefits and limitations of pooled urine analysis

It is difficult to determine which NPS are currently used in communities However, it is known that in some social settings drug and alcohol use is elevated. PUA proposes an approach to target these social settings to gather qualitative information on the presence of NPS. On the contrary, monitoring consumption of NPS using wastewater-based epidemiology (WBE) has been explored in some studies (Borova et al., 2015; González-Mariño et al., 2016; Kinyua et al., 2015a; Thai et al., 2016). In these studies, target methods using commercially available reference standards were applied that upon deployment detected very low levels of NPS or none at all. This could be due to several factors; firstly, popularity of the NPS influenced by unknown shifts in the dynamic NPS market. Secondly, the substantial dilution in sewer systems affects the detection levels of the NPS. Thirdly, they could be targeting unsuitable biomarkers (parent or metabolite) since not much is known about the stability of most of the NPS in wastewater. Furthermore, most metabolites are not available as commercial standards so to be included in targeted methods.

PUA offers several advantages; firstly, the levels of biomarkers in pooled urine are expected to be higher since there is less dilution in the reservoir tanks of the urinals. Secondly, one can apply suspect screening techniques after analysis with LC-HRMS instruments, allowing us to confirm NPS present even without reference standards. Thirdly, PUA is ideal for the detection of many metabolites, revealing potential target biomarkers and showing which NPS are actually used. Fourthly, PUA provides a snapshot of NPS use, which can in-turn be used to develop ideas for potential quantitative methods.

However, PUA data are purely qualitative. Consequently, further studies on stability would be required to determine the best target biomarker for NPS. In addition, it is important to note that since samples are collected from urinals, one major limitation of PUA is that the data acquired is only representative of use by male populations. Furthermore, since the goal is to capture a snapshot of NPS usage, population fluxes within these events cannot be predicted; thus, in our study, several samples were collected from each day of the events. Lastly, there is a chance of missing unstable targets during analysis if sample collection and storage (freezing) are delayed. It is recommended to sample frequently and, if possible, actively freezing samples as they are collected; in order to reduce the possibility of loss of compound through degradation.

4. Conclusions

NPS availability and supply is highly complex because new substances are continually introduced on the market under different brands and mixtures. Therefore, it is important to adapt existing analytical methodologies or to develop new ones that enable determination of the new compounds. The presented results demonstrate the potential of PUA in combination with a screening method to detect a greater number and diversity of substances. Furthermore, this provides an alternative method to get fast information about which NPS are the most frequently detected and, therefore, more consumed. Further studies are still necessary to quantify these NPS in order to get reliable data on community use and NPS occurrence however, for this, reference standards are required. Nevertheless, the provided information on accurate masses can be useful for inclusion in other screening methods, especially the new metabolites detected in authentic human urine for the first time in this study.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.08.124.

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