FLEXIBLE HIGH RESOLUTION-MASS SPECTROMETRY APPROACH FOR SCREENING NEW PSYCHOACTIVE SUBSTANCES IN URBAN WASTEWATER

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ABSTRACT

The number of new psychoactive substances (NPS) on the recreational drug market has increased rapidly in the last years, creating serious challenges for public health agencies and law enforcement authorities. Epidemiological surveys and forensic analyses to monitor the consumption of these substances face some limitations for investigating their use on a large scale in a shifting market. The aim of this work was to develop a comprehensive and flexible screening approach for assessing the presence of NPS in urban wastewater by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Almost 200 substances were selected as "priority NPS" among those most frequently and recently reported by the Early Warning Systems (EWS) of different agencies and were included in the screening. Wastewater samples were collected from several cities all over Europe in 2016 and 2017, extracted using different solid-phase cartridges and analysed by LC-HRMS. The screening workflow comprised two successive analytical steps and compounds were identified and confirmed following specific criteria from the current guidelines. Thirteen NPS were identified at different confidence levels by using analytical standards or information from libraries and literature, and about half of them were phenethylamines. As far as we know, this is the first time that four of them (i.e. 3,4-dimethoxy-*α*-pyrrolidinovalerophenone, *para*-methoxyamphetamine, 2-phenethylamine and α – methyltryptamine) have been found in urban wastewater. The proposed screening approach was successfully applied in the largest NPS European wastewater monitoring, providing an innovative and easily adapted procedure for investigating NPS. In the light of current challenges and specific future research issues, this approach may complement epidemiological information and help in establishing measures for public health protection.

Keywords: *New drugs; monitoring; LC-HRMS; qualitative analysis; phenethylamines.*

INTRODUCTION

New psychoactive substances (NPS) are drugs that have similar structures and effects to common illicit drugs, but are often not under any legislation (UNODC, 1971). Many NPS appearing on the recreational drug market are synthesized by adding or changing a functional group in the chemical structure of conventional illicit drugs (e.g. amphetamine, cannabis, heroin and 3,4-methylendioxymethamphetamine (MDMA)), or previously marketed NPS (EMCDDA and Europol, 2013).

The global market is characterized by the emergence of a large number of new substances belonging to different categories, reported to amount to 803 different NPS according to the most recent report by the United Nations Office on Drugs and Crime (UNODC) (UNODC, 2018). In Europe, more than 670 NPS were reported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) until 2017 (EMCDDA and Europol, 2017), and the number of new substances identified per year considerably rose from 13 in 2005 to 101 in 2014, while in the last few years these numbers have fallen (51 in 2017) (EMCDDA and Europol, 2017). Synthetic cannabinoids are the largest NPS category (179 substances), followed by synthetic cathinones (127) and phenetylamines (77). Synthetic opioids have recently become the fourth largest category, with thirteen new substances identified in 2017 and a total number of 38 substances. In view of the rapid transience of new substances on the drug scene, UNODC and EMCDDA established an Early Warning System (EWS) for promptly monitoring their appearance on the market and identifying risks for human health (EMCDDA, 2018; UNODC, 2017).

Estimating the prevalence of NPS use is problematic and challenging, as indirect information obtained from drug seizures, forensic analyses, and medical reports is limited in extension and time and might not be representative of the constantly shifting market (EMCDDA and Europol, 2013; Reid and Thomas, 2016). Furthermore, population surveys can be biased by users' limited knowledge of the substance they are consuming. In this context, the analysis of specific metabolic residues in urban

wastewater (WW) can give some information on the real use of NPS and serve as a complementary tool to other epidemiological indicators. This approach, called *Wastewater-based epidemiology* (WBE) (Castiglioni et al., 2014; Zuccato et al., 2008), has been successfully applied to monitor the consumption of illicit drugs in several countries (Banta-Green et al., 2009; Castiglioni and Vandam, 2016; Lai et al., 2016; Ort et al., 2014; van Nuijs et al., 2011), the intake of caffeine (Gracia-Lor et al., 2017), nicotine (Castiglioni et al., 2015), alcohol (Lai et al., 2018; Ryu et al., 2016) and pharmaceutical compounds (Jose Antonio Baz-Lomba et al., 2016; van Nuijs et al., 2015) and to evaluate human exposure to pesticides (Rousis et al., 2017) and phthalates (González-Mariño et al., 2017).

In the case of NPS, the analysis of WW presents several challenges (Reid et al., 2014a): low concentrations are expected because of the large number of different substances on the market; limited information is available on pharmacokinetics and human metabolism, and there are few studies related to NPS stability in this matrix. These factors make it hard to select suitable biomarkers for WW monitoring. Despite these limitations, some attempts to detect NPS in urban WW have been made using quantitative target analysis (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016b; Kinyua et al., 2015a; Reid et al., 2014b; Senta et al., 2015). However, this strategy reduces the investigation to a few substances for which reference standards are available, and prevent the possibility to investigate the much larger number of NPS available on the market. A qualitative screening approach using high-resolution mass spectrometry (HRMS) is therefore a promising tool to monitor the broad spectrum of NPS even without reference standards. Moreover, HRMS allows retrospective analyses, i.e. reprocessing data already acquired to search for the appearance of new substances.

Some screening methodologies has been applied to investigate biological samples (blood and urine (Concheiro et al., 2015; Kinyua et al., 2015b) and collective pooled urine from festivals (Kinyua et al., 2016), but there are very few applications for investigating NPS in WW (Bade et al., 2019b,

2019a; J.A. Baz-Lomba et al., 2016, p.; Causanilles et al., 2017; González-Mariño et al., 2016a). González-Mariño et al. (González-Mariño et al., 2016a) proposed a screening method for the identification of 52 NPS in Italy, focusing on synthetic cathinones and synthetic cannabinoids. Baz-Lomba et al. included some NPS and metabolites in the target screening of 51 psychoactive substances in Norway (J.A. Baz-Lomba et al., 2016). Causanilles et al. (Causanilles et al., 2017) monitored NPS and their metabolites during Amsterdam street festivals and recent studies by Bade et al. investigated the use of NPS in Australia, both on a national scale (Bade et al., 2019b) and on a community level in combination with forensic analyses (Bade et al., 2019a).

In the present work a comprehensive and flexible screening approach was developed using HRMS for monitoring NPS in urban WW. Almost 200 NPS were investigated according to a "priority list" built in the framework of the research project *NPS Euronet*, in which the most frequently and recently reported NPS (2015-2017) were included (NPS-Euronet, 2018). WW samples were extracted by solid phase extraction (SPE) and analysed following a screening workflow. Potential "suspect compounds" were (tentatively) identified at different confidence levels by using analytical standards or information from libraries and literature. This screening approach was successfully applied in the largest NPS monitoring, analysing urban WW from different European countries over a two-year period. To the best of our knowledge, this is the first time that a HRMS-screening approach, including a considerable number of NPS, has been applied in an extensive WW monitoring in Europe, demonstrating its relevance and significance for investigating the NPS phenomenon.

MATERIALS AND METHODS

Selection of NPS

Since over 450 NPS were on the market at the beginning of the project in 2015, a list of "priority NPS" was created by reviewing the EWS reports from UNODC (UNODC, 2017),

EMCDDA (EMCDDA, 2018) and the National EWS in Italy (Presidenza del Consiglio dei Ministri, Dipartimento Politiche Antidroga, 2013). Priority NPS were selected among those most recently reported and most frequently recorded on the market or during seizures (NPS-Euronet, 2018). The resulting list included 197 substances belonging to ten categories: 70 synthetic cannabinoids, 53 synthetic cathinones, 38 phenethylamines, 9 synthetic opioids, 7 tryptamines, 6 piperidines, 3 aminorex derivatives, 4 natural NPS, 4 benzodiazepines and 3 ketamine analogues. A database was built, collecting information on the chemical properties, the first alert of intoxication (date and place), the fragmentation pathways (including analytical techniques and mass analysers used) and human metabolism data when available.

Chemicals and materials

A total of 40 analytical standards were used for the identification and/or confirmation of NPS (Table 1). Thirty-nine were supplied by LGC (Teddington, UK), Cerilliant (Round Rock, TX, USA) or Cayman Chemicals (Ann Arbor, MI, USA) as solutions of 0.1, 0.4 or 1 mg mL^{-1} in acetonitrile (ACN) or methanol (MeOH). The remaining one, α-methyltryptamine (AMT), was synthetized and characterised using NMR and UHPLC-HRMS (Bijlsma et al., 2017). Working solutions were prepared at concentrations of 1 and 0.1 µg mL^{-1} in MeOH before each analytical run, and were stored at -20°C in the dark. An additional standard solution containing 19 deuterated analogues of illicit drugs was prepared in MeOH (0.1 µg mL^{-1}) and injected before and after each analytical batch of samples to check the instrumental sensitivity and selectivity. Details on this mixed solution are reported in the Supplementary Material (SM).

MeOH for pesticide residue analysis, ammonium hydroxide solution (25%) and ACN for LC-MS were acquired from Fluka (Buchs, Switzerland), formic acid (FA, 98%) from Tokyo Chemical Industry UK Ltd. (Oxford, United Kingdom), and hydrochloric acid (HCl, 37%) from Carlo Erba (Italy). HPLC grade Milli-Q water was obtained directly from a MILLI-RO PLUS 90 apparatus (Millipore, Molsheim, France). Glass micro fibre filters 1.6 µm GF/A (Whatman, Kent, UK) and 0.45

μm nitrocellulose filters (Millipore, Bedford, MA, USA) were used to filtrate the samples. Cartridges for SPE were 6 mL disposable Oasis® MCX (150 mg) and 3 mL disposable Oasis® HLB (60 mg), both from Waters Corporation (Milford, MA, USA).

Sample collection

Raw wastewater samples were collected in 2016 and 2017 from the inlet of several urban wastewater treatment plants (WWTPs) in different European countries. Automatic devices working in volume or time-proportional mode and collecting aliquots at high frequency in order to obtain representative samples (Castiglioni et al., 2013) were used to take 24h composite samples. In 2016, the sampling campaign included 26 cities from 15 countries, and in 2017, 11 cities from 7 countries (Table 2). On arrival in the laboratory, WW samples were vacuum-filtered and stored at −80 °C to inhibit microbial activity until analysis.

Sample preparation

WW samples collected daily from Friday to Monday were pooled for analyses, and a "weekend" composite sample was created, as NPS are expected to be more used recreationally over the weekend. Samples were extracted using two different SPE cartridges in order to cover the wide range of physical-chemical properties of the selected NPS. The volume of sample loaded on the cartridges was 50 mL. The first SPE cartridges were Oasis® HLB, which were conditioned with 6 mL MeOH and 3 mL Milli-Q water, vacuum-dried for 10 min after sample percolation, and eluted with 4 mL of MeOH. Sample pH was checked before extraction and kept around 7. SPE was performed using an automated system GX-274 ASPEC (Gilson, Middleton, WI, USA) at a flow rate of 5 mL min^{-1}. The second cartridges were Oasis® MCX, conditioned with 10 mL MeOH, 5 mL Milli-Q water, and 5 mL water acidified to pH 2, vacuum-dried for 10 min after percolation, and eluted with 2 mL of MeOH and 2 mL of a 2% ammonia solution in MeOH. Samples for extraction on Oasis® MCX cartridges were acidified to pH~2 with 37 % HCl and SPE was performed manually at a flow rate of 5 mL min^{-1}. Both HLB and MCX eluates were dried under a gentle nitrogen stream, reconstituted in

200 µL of a mixture of Milli-Q water:MeOH (90:10), centrifuged for 2 min at 2500 rpm, and transferred into glass vials for instrumental analysis.

Instrumental analysis

WW samples were analysed by LC coupled to HRMS using a Q-Exactive™ Hybrid Quadrupole-Orbitrap™ (Thermo Scientific, Bremen, Germany). Chromatographic separation was done using an Agilent 1200 series HPLC including a membrane degasser, a binary high-pressure gradient pump, and an autosampler. The chromatographic column was an XBridge® C_{18} (2.1x100 mm, 3.5 µm) from Waters Corporation (Milford, MA, USA). A dual eluent system consisting of (A) 0.1 % FA in MilliQ water and (B) ACN was employed at a constant flow rate of 200 µL min^{-1}. The gradient was: 0 min (10% B), 20 min (60% B), 25 min (99% B), 30 min (99% B) and 31 min (10% B), the initial conditions were finally kept for 6 min in order to re-equilibrate the column (total run time 38 min). The column temperature was set at 30°C. The volume of injection was 8 µL. The mass spectrometer was equipped with a heated electrospray ionization (HESI) source. Analyses were carried out in positive mode, under the following working conditions: sheath gas pressure 45 bar, auxiliary gas pressure 5 bar, auxiliary gas temperature 160°C, ion spray voltage 3.5 kV, heated capillary temperature 320°C, S-lens RF 60. MS^2 experiments were done in the collision-induced dissociation (CID) mode by applying normal collision energy (NCE) values of 35 and 50 V and with a precursor ion isolation window of ± 3.0 m/z that ensures a good sensitivity avoiding interferences. Data were acquired with Full-scan mode at 70,000 resolution and with MS^2 mode at 35,000 resolution, using Thermo Xcalibur™ 4.0 software (Thermo Scientific, Bremen, Germany). More details about the parameters of MS and MS^2 experiments are reported in SM.

Identification criteria and confidence levels for qualitative analysis

Setting screening approaches using HRMS calls for particular attention to the confidence of identification of a substance and the confirmation of its identity. Schymanski et al. (Schymanski et al., 2014) proposed a scheme for identifying chemicals consisting of five different confidence levels

198 (1 to 5), where level 1 has the highest confidence indicating a chemical structure confirmed by a
199 reference standard, and levels 4 and 5 have the lowest confidence including cases when only the exact
200 mass or the molecular formula can be identified. We used the same scheme for identifying NPS, but
201 only confidence levels 1-3 have been reported, according to the specific information available case
202 by case. Level 1 was assigned when confirmation was obtained with a reference standard, considering
203 retention time (RT), MS and MS^2 spectra matching. Level 2 was assigned when no reference standard
204 was available, but the chemical structure could be elucidated from diagnostic evidence or with
205 matching spectra data (library or literature). Level 3 was assigned to "tentative candidates" when the
206 information was not enough to confirm the exact structure of the analyte, but the exact mass was
207 matching and some fragments were identified with a potential chemical structure.

208 The key parameters for the identification of NPS were defined following specific criteria from
209 the current guidelines (WADA, 2010), which include accurate mass for the protonated molecule with
210 delta mass lower than 5 ppm, the isotope pattern, and at least one fragment ion identified with a delta
211 mass lower than 5 ppm. When reference standards were available, the RT was also considered for
212 identification with an acceptable variability of ± 2% min.

213

214 **RESULTS AND DISCUSSION**

215 **Workflow for screening analysis**

216 The developed screening workflow comprised two successive analytical steps reported in Fig.
217 1. The first was a full-scan analysis at 70,000 resolution, looking for molecules with a mass-to-charge
218 ratio (m/z) between 100 and 600 to specifically include NPS. The software Trace Finder™ 3.1
219 (Thermo Scientific, Bremen, Germany) was used to screen suspects from HRMS chromatograms.
220 This software allows quick identification of suspects based on the following parameters: m/z value,
221 RT, fragment ions, isotopic pattern, and library search (González-Mariño et al., 2016a). "Preliminary

222 suspect compounds" were identified considering the full "priority list" of NPS and using the m/z
223 values as main parameter; the RTs were also recorded, while the fragmentation spectra were typically
224 missing at this stage.

225 The second step consisted in confirmation of the "preliminary suspect compounds". First, a
226 data-dependent analysis (FullMS-ddMS^2) mode was used, in which the first data event was a full-
227 scan MS (scan range 100–500 m/z), and the next *n* events (e.g., five events for a ddMS^2 TOP-5) were
228 MS^2 scans of the *n* most intense m/z recorded in the first event among the specific list of "preliminary
229 suspect compounds". If fragmentation was not obtained with this method, the full-scan MS (scan
230 range 100–500 m/z) was followed by a data-independent analysis (DIA), in which the MS^2 scans did
231 not depend on the first event as before, but were acquired by a specific list of m/z ("suspects to be
232 confirmed") indicated as targets in a retention time window (±1 min).

233 In the third step, "suspect compounds" were finally identified at confidence levels 1-3, by
234 comparison with standards, libraries or literature data, following the already mentioned criteria.

235 **NPS spectral database**

236 An *in-house* HRMS spectra database was built analysing the reference standards of 40 NPS
237 (Table 1). The fragmentation spectra (MS^2) were acquired using a full-scan MS (scan range 100–500
238 m/z) followed by a data independent analysis (DIA), and applying normal collision energy (NCE)
239 values of 35 and 50 V. Dataset is reported in Table S1 (SM), including for each NPS the chemical
240 formula and theoretical accurate m/z values of the parent substance, its RT, and the most abundant
241 fragments (three when available) with the calculated chemical formulae. The MS^2 spectra are
242 available on request.

243 Since information on NPS fragmentation in the literature is very limited (Bade et al., 2019b;
244 González-Mariño et al., 2016a; Seither et al., 2018; Urbas et al., 2018), and depends on the HRMS
245 mass analyzers used, that are often equipped with a different collision cell or devise for fragmentation,

this NPS database can be particularly helpful for integrating the existing information and improving the confidence level in the NPS identification.

Identification of NPS in wastewater samples

The proposed workflow (Fig.1) was applied for NPS identification in WW samples, resulting in a flexible and comprehensive analytical tool. The initial screening step included 197 NPS and the first analysis (full-MS acquisition) identified around 20-30 compounds as "preliminary suspect compounds". The next steps then aimed for the collection of MS/MS spectra with product ions and reduced the number of "suspect compounds" to about 10. Finally, "suspect compounds" NPS were positively identified at the three different confidence levels as previously described.

Level 1 – NPS confirmed

NPS were identified and confirmed at level 1 by comparison with the corresponding reference standard in terms of RT, MS and MS^2 spectra. Four NPS were confirmed at this level: one synthetic cathinone (3,4-DMeO-alpha-PVP), two phenethylamines (PMA and 2-PEA) and one tryptamine (AMT) (Table 3). The variability of RTs was within the acceptable range (± 2% min). The precursor ion (Q) and the two most abundant product ions were identified with a delta mass lower than 5 ppm. As an example, AMT chromatograms and MS^2 spectra for a reference standard and a WW sample are reported in Fig. 2. Results for the other NPS confirmed at level 1 are shown in Fig. S1-S3 (SM).

Level 2 – NPS identified (exact/possible structures)

Level 2 was assigned when no reference standards for suspects were available, but comparison was possible with analytical standards of related compounds (for which a common fragmentation pattern is expected) or information reported in the literature or in MS^2 spectra libraries. In any case, subsequent confirmation with analytical standards would be required to achieve the level 1.

Three phenethylamines (25E-NBOMe, 25H-NBOMe and 2-methoxyamphetamine (2-MA)) were identified at this level (Table 4). The first two are N-methoxybenzyl derivatives, thus the

analytical standards of four analogues were used for identification (i.e. 25B-NBOMe, 25C-NBOMe, 25I-NBOMe and 25iP-NBOMe). All had a common fragmentation pattern, based on two main product ions m/z 121.0650 ($C_8H_9O^+$) and m/z 91.0548 ($C_7H_7^+$). The first corresponds to cleavage of the N-C bond, and the second, less abundant one, corresponds to the loss of CH_2O (-30.0112u) leading to a tropylium ion. The same fragments and the corresponding exact masses were found for 25E-NBOMe and 25H-NBOMe with delta mass lower than 5 ppm. The RTs for the suspects (~14.5 min) were also similar to those of the analogues, ranging from 14.0 min (25C-NBOMe) to 17.2 min (25iP-NBOMe). This was considered sufficient to identify the "suspect compounds" at level 2. Chromatograms of 25E-NBOMe and 25H-NBOMe and their common MS^2 spectra are shown in Fig. 3.

For 2-MA (also called *ortho*-methoxyamphetamine), the structural isomer PMA was used as standard for identification at confidence level 2. As isomers, they have the same chemical formula (exact mass) and the same fragmentation pattern, being the two most abundant ions 149.0234 ($C_{10}H_{13}O^+$) and 121.0653 m/z ($C_8H_9O^+$). Thus, the RT is essential for identifying both compounds. With the proposed method, the RT of 2-MA was 4.8-4.9 min, only slightly different from PMA (5.2 min). This can be explained considering that *ortho*- isomers are generally more polar than *para*-isomers, and are therefore less retained in a reversed-phase chromatographic column. Although this information was sufficient to consider a confidence level 2, the reference standard of 2-MA would be required to confirm the presence of this NPS. LC-HRMS-data related with 2-MA are shown in SM (Fig. S4).

Level 3 – Tentative NPS

Level 3 was assigned when no reference standards were available and no information on the pattern of fragmentation was found in the literature. Databases of MS^2 spectra of NPS are still very limited and depend on the instrument and the acquisition mode used for analyses; consequently, only a tentative identification based on interpretation of the accurate mass spectra is possible in these cases.

295 Six NPS were identified at level 3 (Table 5) and were: two phenethylamines (N-methyl-2AI, DOIP),
296 three piperidines (isopropylphenidate, HDMP-28, diphenidine) and one synthetic cannabinoids
297 (AMB-FUBINACA). For these compounds, an exact mass (Q) with a delta mass lower than 5 ppm
298 and two product ions related to their possible structures were identified on the basis of the molecular
299 formula for the specific fragments. Fig. 4 shows the chromatogram, the MS^2 spectra and the proposed
300 fragmentation pattern for the phenethylamine N-methyl-2-AI. Results for the other tentative NPS are
301 reported in the SM (Fig. S5-S9). These NPS could only be tentatively identified because of the scant
302 information available for confirmation, being the confidence level much lower than in the previous
303 cases. Reference standards are therefore mandatory for confirming the identity of the substance at
304 higher confidence level.

305 **Overview of NPS use in Europe**

306 A total of 13 NPS were confirmed or tentatively identified in WW samples: four at confidence
307 level 1, three at level 2 and six at level 3. Generally, the most frequent category identified was
308 phenethylamines, potentially used as substitute for amphetamines, MDMA or cocaine. Other less
309 common NPS categories such as tryptamines and piperidines were also identified. Synthetic
310 cannabinoids and synthetic cathinones, the largest groups of NPS on the European market (EMCDDA
311 and Europol, 2017; UNODC, 2018), were identified to a lesser extent in this study. Synthetic
312 cannabinoids are rapidly metabolized in human liver and therefore, very low concentrations of the
313 parent compounds are expected in WW, making their determination difficult. This may explain the
314 present findings and previous investigations (González-Mariño et al., 2016a; Reid et al., 2014b). In
315 contrast, some synthetic cathinones, which are metabolised less in the human body, were found as
316 parent substances in several studies where quantitative analysis of WW using low-resolution mass
317 spectrometry was performed (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016b;
318 Kinyua et al., 2015a; Senta et al., 2015).

The number of NPS identified in the present study was 1 in 2016 but 12 in 2017. The sole NPS found in 2016 was PMA, which was confirmed in four countries (five cities) located in Eastern Europe (Table 3). Although PMA was not detected in 2017, its isomer 2-MA was identified at confidence level 2 in three countries (four cities). Among the other confirmed NPS, 3,4-DMeO-α-PVP and 2-PEA were found in one and two samples respectively, in 2017, and AMT was the NPS found most frequently in six countries (seven cities) from Western and Central Europe. About half the NPS identified in 2017 were detected in at least two countries (2-PEA, 25E-NBOMe, 25H-NBOMe, DOIP), but considering the large number of samples and NPS investigated, these results indicate the low use of these substances in the general population.

It is hard to compare our results with previous ones, considering the number and transience of NPS on the market, their variable use over time and the different characteristics of the analytical methods employed in each study. González-Mariño et al. identified three synthetic cathinones in WW from Italy applying a screening method for the identification of 52 NPS (González-Mariño et al., 2016a), and Baz-Lomba et al. found two NPS in Norway (J.A. Baz-Lomba et al., 2016). Causanilles et al. identified eight NPS in Amsterdam street festivals, phenethylamines and synthetic cathinones being the most frequently detected (Causanilles et al., 2017). Bade et al. found 22 NPS in Australia, where the most detected compounds were synthetic cathinones (two were found in all the regions studied), but also five synthetic cannabinoids were identified, despite their extensive metabolism in the human body (Bade et al., 2019b). In another study, Bade et al. found six different cathinones in Adelaide (South Australia) in 2012 - 2017 and tentatively identified 25H-NBOMe (Bade et al., 2019a), as in the present study.

To the best of our knowledge, in the present study 3,4-DMeO-alpha-PVP, PMA, 2-PEA and AMT were found for the first time in WW samples. These results evidence the changing use of NPS in Europe and the importance of adopting tools for continuous monitoring over time. They also

343 demonstrate the applicability of the proposed screening approach for NPS identification, even on a
344 large scale.

345 **Challenges of the HRMS screening approach for monitoring NPS in wastewater**

346 HRMS techniques for WW analysis offer a powerful tool to screen the presence of NPS in
347 urban wastewater, which may indicate their use in the population. Wide-scope HRMS screening
348 strategies allow the identification of a wide panel of NPS and can be easily adapted to identify the
349 new substances that are continuously appearing on the drug market, performing also retrospective
350 analyses. Despite the first promising applications of this approach, several limitations must be
351 considered to improve future studies.

352 One of the main challenges is the confirmation of "suspect compounds". This is normally
353 done by comparison with the corresponding reference standard. However, the high costs, the
354 complicated bureaucracy and limited availability of NPS reference standards, mean this option is not
355 always feasible, decreasing the confidence level in the identification of "suspect compounds". In
356 these cases, the strong potential of HRMS allows well-founded tentative identifications thanks to the
357 value of accurate-mass obtained from MS and MS^2 data. Thus, it is possible to compare experimental
358 MS^2 spectra with those in the literature and/or spectra libraries. Even so, current information about
359 fragmentation patterns is scarce and heterogeneous, as it depends on the mass analysers and the
360 fragmentation mode used. Although recent works have presented databases and/or HRMS spectral
361 libraries (Bade et al., 2019b; González-Mariño et al., 2016a; Seither et al., 2018; Urbas et al., 2018),
362 they commonly focused on one specific mass analyzer and a limited number of substances and
363 therefore, more effort is needed to supply new information about fragmentation patterns. The present
364 study provides information for 40 NPS, including analytical conditions and MS^2 fragmentation
365 (available in the SM) to help further research. A valuable alternative for identifying MS^2
366 fragmentation is the application of *in silico* fragmentation tools, such as MassFrontier (HighChem)

or MSFragmenter (ACD/Labs)) software. Different algorithms are available for elucidating the fragmentation pattern of "suspect compounds" allowing a time-effective data handling.

It is hard to identify NPS in WW due to the low levels expected, mostly because of the limited use of these substances and the large numbers of interchangeable drugs on the market. Screening approaches can be affected by the lower sensitivity of HRMS instruments compared with low-resolution tandem mass spectrometers (e.g. QqQ analyzers). Thus target analysis using QqQ analysers can be used to confirm and quantify "suspect compounds", when possible, but require additional analytical effort and costs. It is therefore suggested that future studies optimize their experimental design and choose the most appropriate instrumentation considering the specific need for qualitative or quantitative profiles of NPS, the matrix to be investigated, and the analytical results of previous investigations.

Another limitation is the lack of information about the human metabolism of the NPS, which restricts the choice of target metabolic residues as biomarkers of consumption. In fact, the analysis of a metabolic residue (called *biomarker*) in WW may directly indicate the use of the parent substance, provided that the percentage of excretion in humans is known. Different studies have been designed to assess NPS metabolism over recent years (Erratico et al., 2015; Franz et al., 2017; Lai et al., 2015; Mardal et al., 2016; Richter et al., 2017), but they are still few in relation to the number of NPS. Consequently, the most common strategy to date is to directly investigate parent substances as markers of use (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016b, 2016a; Kinyua et al., 2015a; Senta et al., 2015) and a few studies also include some NPS metabolites (J.A. Baz-Lomba et al., 2016; Causanilles et al., 2017; Reid et al., 2014b). An adequate biomarker of consumption should also be stable in WW, but the little information available for NPS is limited to a few substances (Bade et al., 2017; González-Mariño et al., 2016a; Senta et al., 2015). Further research therefore needs to address also in this field in order to identify specific and suitable biomarkers for monitoring NPS in WW.

CONCLUSIONS

This study proposed a comprehensive and flexible HRMS approach successfully applied for screening almost 200 NPS in urban WW collected in the largest NPS European monitoring. Thirteen substances were identified at different confidence levels according to the current guidelines, demonstrating the low prevalence of use of NPS in the general population already reported by epidemiological surveys. The proposed screening approach was confirmed as a useful tool for assessing NPS use on an international scale by WW analysis and therefore, it could complement current epidemiological information and help in establishing appropriate measures for public health protection.

Several challenges have been highlighted and discussed and some suggestions for future research were identified, such as sharing analytical standards and HRMS data obtained with different mass analyzers, or increasing information on NPS metabolism and on stability in WW. Despite the mentioned limitations, a wide-scope screening by LC-HRMS seems the best option nowadays to face the detection/identification of a large number of NPS in WW. To have better options to detect these compounds, it would be also recommended to focus the research on samples collected during events when higher consumption of NPS is expected, e.g. music festivals, festivities, weekends.

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Table 1. Analytical standards used for the identification and confirmation of new psychoactive substances (ordered by class)

Synthetic cathinones		**Phenethylamines**	
Methcathinone	METC	2C-B-NBOMe	25B-NBOMe
Ethcathinone	ETHC	2C-C-NBOMe	25C-NBOMe
3,4-dimethylmethcathinone	3,4-DMMC	2C-I-NBOMe	25I-NBOMe
3-methylmethcathinone	3-MMC	2C-iP- NBOMe	25iP- NBOMe
3-methoxymethcathinone	3-MeOMC	2-chloro-4,5-methylendioxy-methylamphetamine	2-Cl-4,5-MDMA
4-fluoromethcathinone	4-FMC	*para*-methoxyamphetamine	PMA
4-methylethcathinone	4-MEC	*para*-methoxy-N-methylamphetamine	PMMA
Methylone	METL	N-ethyl-1,2-diphenylethylamine	NEDPA
Butylone	BUTL	2-phenethylamine	2-PEA
Pentylone	PENTL	5-(2-Aminopropyl)indole	5-IT
Dipentylone	bk-DMBDP	6-(2-Aminopropyl)-benzofuran	6-APB
Methedrone	METH	**Synthetic opioid**	
Mephedrone	MEPH	1-cyclohexyl-4-(1,2-diphenylethyl)piperazine	MT-45
Buphedrone	BUPH	**Tryptamines**	
Pentedrone	PENT	5-Methoxy-N-isopropyl-N-methyltryptamine	5-MeO-MiPT
Methylenedioxypyrovalerone	MDPV	5-Methoxy-N,N-diallyltryptamine	5-MeO-DALT
α-pyrrolidinovalerophenone	α-PVP	α -methyltryptamine	AMT
α-pyrrolidinopentiothiophenone	α -PVT	**Aminorex derivates**	
3,4-dimethoxy-α-pyrrolidinovalerophenone	3,4-DMeO-α-PVP	4-methylaminorex	4-MAR
4'-chloro-α-Pyrrolidinopropiophenone	4-Cl- α -PPP	4,4-dimethylaminorex	4,4-DMAR
Synthetic cannabinoids		**Ketamine analogue**	
5-fluoropentyl-3-pyridinoylindole	5Fpentyl-3-pyr	Methoxetamine	MXE
MDMB-CHMICA	MDMB-CHMICA		

Table 2. Countries and number of cities included in the two sampling campaigns (2016 and 2017)

2016		**2017**	
Countries	Number of cities	Countries	Number of cities
Bosnia and Herzegovina	1	Germany	2
Bulgaria	1	Italy	3
Italy	1	Poland	1
Ireland	1	Portugal	2
Poland	1	Slovakia	1
Portugal	2	Slovenia	1
Romania	3	Spain	1
Serbia	2		
Slovakia	4		
Slovenia	1		
Spain	4		
Switzerland	1		
the Netherlands	1		
Ukraine	2		
United Kingdom	1		
15 countries	**26 cities**	**7 countries**	**11 cities**

Table 3. NPS identified at confidence level 1 in WW samples collected in Europe in 2016 and 2017.

NPS	Category	Year	Country	LC-HRMS Characteristics				
				Retention time ± Δt (min)	Precursor ion (Q)		Product ions (q)	
					Formula $[M+H]^+$	Measured m/z (Δm, ppm)	Formula	Measured m/z (Δm, ppm)
3,4-DMeO-α-PVP	Synthetic cathinones	2017	Portugal	8.2 ± 0.2	$C_{17}H_{26}NO_3$	292.1908 (+0.34)	$C_9H_{11}O_2$ $C_8H_{16}N$	151.0754 (0) 126.1279 (0)
PMA	Phenethylamines	2016	Romania	5.1 ± 0.1	$C_{10}H_{16}NO$	166.1228 (+1.2)	$C_{10}H_{13}O$ C_8H_9O	149.0964 (+1.3) 121.0653 (+1.6)
		2016	Serbia	5.1 ± 0.1		166.1228 (+1.2)	$C_{10}H_{13}O$ C_8H_9O	149.0963 (+0.67) 121.0652 (+0.83)
		2016	Serbia	5.2 ± 0		166.1227 (+0.6)	$C_{10}H_{13}O$ C_8H_9O	149.0964 (+1.3) 121.0653 (+1.6)
		2016	Slovakia	5.1 ± 0.1		166.1229 (+1.8)	$C_{10}H_{13}O$ C_8H_9O	149.0964 (+1.3) 121.0653 (+1.6)
		2016	Ukraine	5.1 ± 0.1		166.1227 (+0.6)	$C_{10}H_{13}O$ C_8H_9O	149.0964 (+1.3) 121.0652 (+0.83)
2-PEA	Phenethylamines	2017	Poland	3.2 ± 0	$C_8H_{11}N$	122.0965 (+0.82)	C_8H_9 C_6H_7	105.0702 (-0.95) 79.0548 (-1.3)
		2017	Slovenia	3.2 ± 0		122.0966 (+1.6)	C_8H_9 C_6H_7	105.0702 (-0.95) 79.0548 (-1.3)
AMT	Tryptamine	2017	Germany	5.8 ± 0.1	$C_{11}H_{15}N_2$	175.1228 (-0.57)	$C_{11}H_{12}N$ C_9H_8N	158.0963 (0) 130.0653 (+0.76)
		2017	Germany	5.8 ± 0.1		175.1228 (-0.57)	$C_{11}H_{12}N$ C_9H_8N	158.0963 (0) 130.0651 (-0.76)
		2017	Italy	5.8 ± 0.1		175.1229 (0)	$C_{11}H_{12}N$ C_9H_8N	158.0963 (0) 130.0652 (0)
		2017	Portugal	5.8 ± 0.1		175.1228 (-0.57)	$C_{11}H_{12}N$ C_9H_8N	158.0963 (0) 130.0651 (-0.76)
		2017	Slovenia	5.8 ± 0.1		175.1229 (0)	$C_{11}H_{12}N$ C_9H_8N	158.0963 (0) 130.0651 (-0.76)
		2017	Slovakia	5.8 ± 0.1		175.1229 (0)	$C_{11}H_{12}N$ C_9H_8N	158.0963 (0) 130.0652 (0)
		2017	Spain	5.8 ± 0.1		175.1227 (-1.1)	$C_{11}H_{12}N$ C_9H_8N	158.0962 (-0.63) 130.0651 (-0.76)

Table 4. Phenethylamines identified at confidence level 2 in WW samples collected in Europe in 2016 and 2017.

NPS	Analytical standard used as reference			Year	Country	LC-HRMS Characteristics				
						RT (min)	Precursor ion (Q)		Product ions (q)	
	Compound (Formula)	RT (min)	MS^2 information				Formula $[M+H]^+$	Measured m/z (Δm, ppm)	Formula	Measured m/z (Δm, ppm)
25E-NBoMe	25C-NBoMe ($C_{18}H_{23}ClNO_3$) 25iP-NBoMe ($C_{21}H_{30}NO_3$)	14-17	121.0650 (C_8H_9O) 91.0548 (C_7H_7)	2017	Germany	14.2	$C_{20}H_{28}NO_3$	330.2065 (+0.30)	C_8H_9O C_7H_7	121.0649 (-0.82) 91.0547 (-1.1)
					Slovenia	14.2		330.2065 (+0.30)	C_8H_9O C_7H_7	121.0649 (-0.82) 91.0547 (-1.1)
25H-NBoMe	25C-NBoMe ($C_{18}H_{23}ClNO_3$) 25iP-NBoMe ($C_{21}H_{30}NO_3$)	14-17	121.0650 (C_8H_9O) 91.0548 (C_7H_7)	2017	Portugal	14.1	$C_{18}H_{24}NO_3$	302.1751 (0)	C_8H_9O C_7H_7	121.0649 (-0.82) 91.0547 (-1.1)
					Spain	14.1		302.1751 (0)	C_8H_9O C_7H_7	121.0649 (-0.82) 91.0547 (-1.1)
2-MA	PMA ($C_{10}H_{16}NO$)	5.2	149.0962 ($C_{10}H_{13}O$) 121.0650 (C_8H_9O)	2017	Italy	4.9	$C_{10}H_{16}NO$	166.1226 (0)	$C_{10}H_{13}O$ C_8H_9O	149.0960 (-1.3) 121.0649 (-0.82)
					Italy	5.0		166.1227 (+0.60)	$C_{10}H_{13}O$ C_8H_9O	149.0963 (+0.67) 121.0649 (-0.82)
					Portugal	4.9		166.1227 (+0.60)	$C_{10}H_{13}O$ C_8H_9O	149.0962 (0) 121.0649 (-0.82)
					Spain	4.9		166.1226 (0)	$C_{10}H_{13}O$ C_8H_9O	149.0960 (-1.3) 121.0650 (0)

Table 5. NPS tentatively identified at confidence level 3 in WW samples collected in Europe in 2016 and 2017.

NPS	Category	Year	Country	LC-HRMS Characteristics						
				RT (min)	Precursor ion (Q)		Product ions (q)			
					Measured m/z (Δm, ppm)	Formula $[M+H]^+$	Measured m/z	Formula	Measured m/z	Formula
N-methyl-2AI	Phenethylamine	2017	Poland	3.5	148.1120 (-0.68)	$C_{10}H_{14}N$	117.0701	C_9H_9	133.0887	$C_9H_{11}N$
DOIP	Phenethylamine	2017	Italy	15.7	238.1800 (-0.83)	$C_{14}H_{24}NO_2$	219.1379	$C_{14}H_{19}O_2$	159.1167	$C_{12}H_{15}$
			Italy	15.7	238.1800 (-0.83)	$C_{14}H_{24}NO_2$	219.1378	$C_{14}H_{19}O_2$	159.1167	$C_{12}H_{15}$
			Poland	15.7	238.1800 (-0.83)	$C_{14}H_{24}NO_2$	219.1379	$C_{14}H_{19}O_2$	159.1167	$C_{12}H_{15}$
			Portugal	15.7	238.1800 (-0.83)	$C_{14}H_{24}NO_2$	219.1379	$C_{14}H_{19}O_2$	159.1167	$C_{12}H_{15}$
HDMP-28	Piperidine	2017	Germany	13.5	284.1644 (-0.35)	$C_{18}H_{22}NO_2$	252.1383	$C_{17}H_{18}NO$	224.1435	$C_{16}H_{18}N$
Isopropyl phenidate	Piperidine	2017	Germany	15.1	262.1801 (-0.38)	$C_{16}H_{24}NO_2$	86.0969	$C_5H_{12}N$	175.0753	$C_{11}H_{11}O_2$
Diphenidine	Piperidine	2017	Italy	15.9	266.1903 (0)	$C_{19}H_{24}N$	86.0969	$C_5H_{12}N$	181.1008	$C_{14}H_{13}$
AMB-FUBINACA	Synthetic cannabinoids	2017	Germany	11.9	384.1731 (+3.3)	$C_{21}H_{23}FN_3O_3$	253.0789	$C_{15}H_{10}FN_2O$	221.1071	$C_{15}H_{13}N_2$

FIGURE CAPTIONS

Figure 1. Scheme of suspect screening workflow and confidence levels of identification (1-3) using a Q-Exactive mass analyzer.

Figure 2. Chromatograms and MS^2 spectra for α-methyltryptamine (AMT) in A) wastewater and B) analytical standard. Identification at confidence level 1.

Figure 3. Chromatograms and MS^2 spectra for (A) 25E-NBOMe and 25H-NBOMe in wastewater, and (B) analytical standards of NBOMe derivatives. Identification at confidence level 2.

Figure 4. Chromatogram and MS^2 spectra for phenethylamine N-methyl-2AI in wastewater. Identification at confidence level 3.

Fig.1

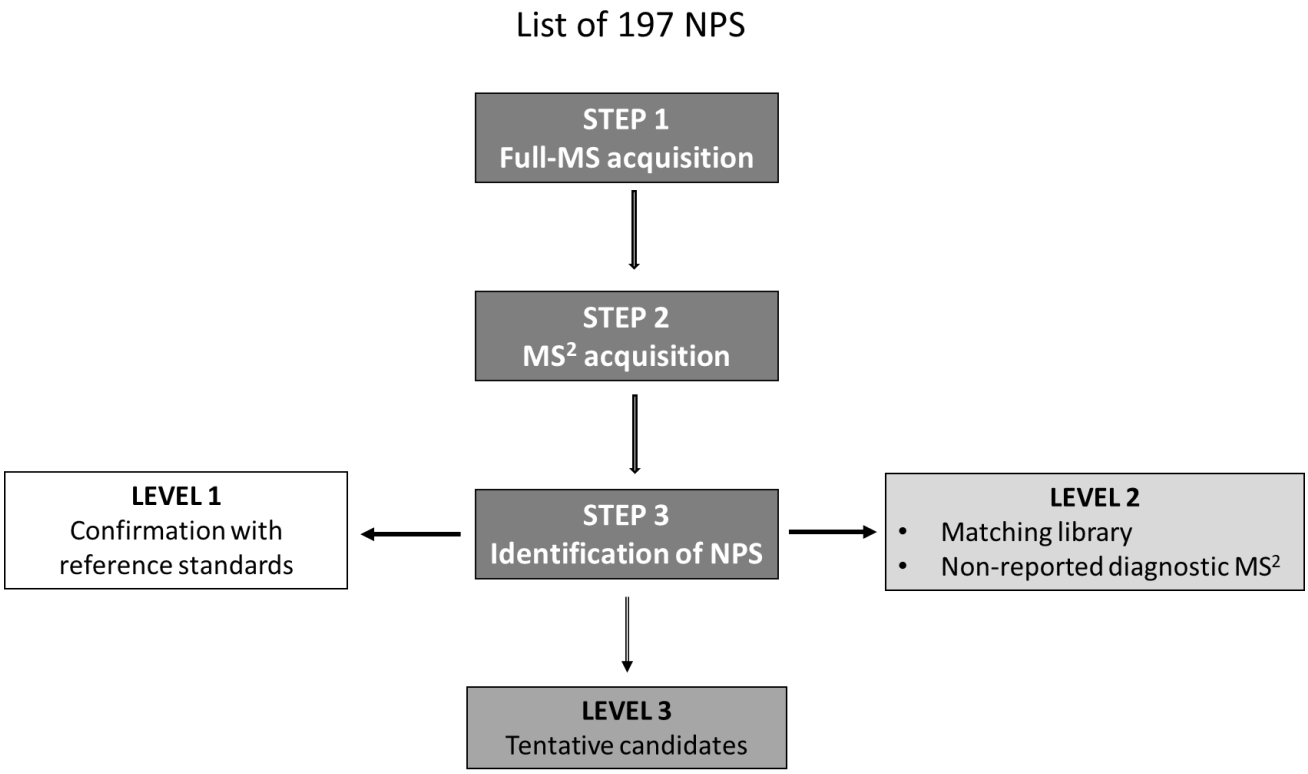

List of 197 NPS
STEP 1
Full-MS acquisition
STEP 2
MS2 acquisition
STEP 3
Identification of NPS
LEVEL 1
Confirmation with reference standards
LEVEL 2
• Matching library
• Non-reported diagnostic MS2
LEVEL 3
Tentative candidates

Fig.2

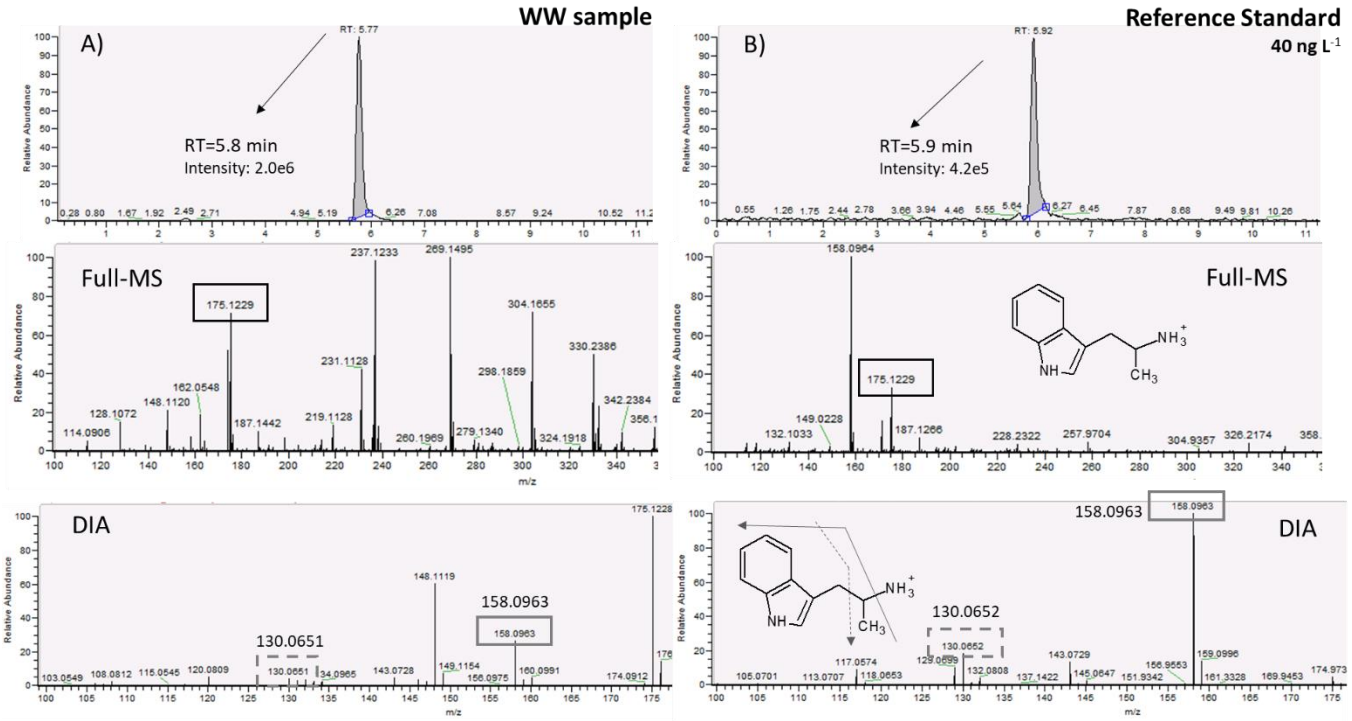

WW sample
Reference Standard
40 ng L-1
A)
RT=5.8 min
Intensity: 2.0e6
B)
RT=5.9 min
Intensity: 4.2e5
Full-MS
175.1229
Full-MS
158.0964
175.1229
DIA
158.0963
130.0651
DIA
158.0963
130.0652
Relative Abundance
m/z

Fig.3

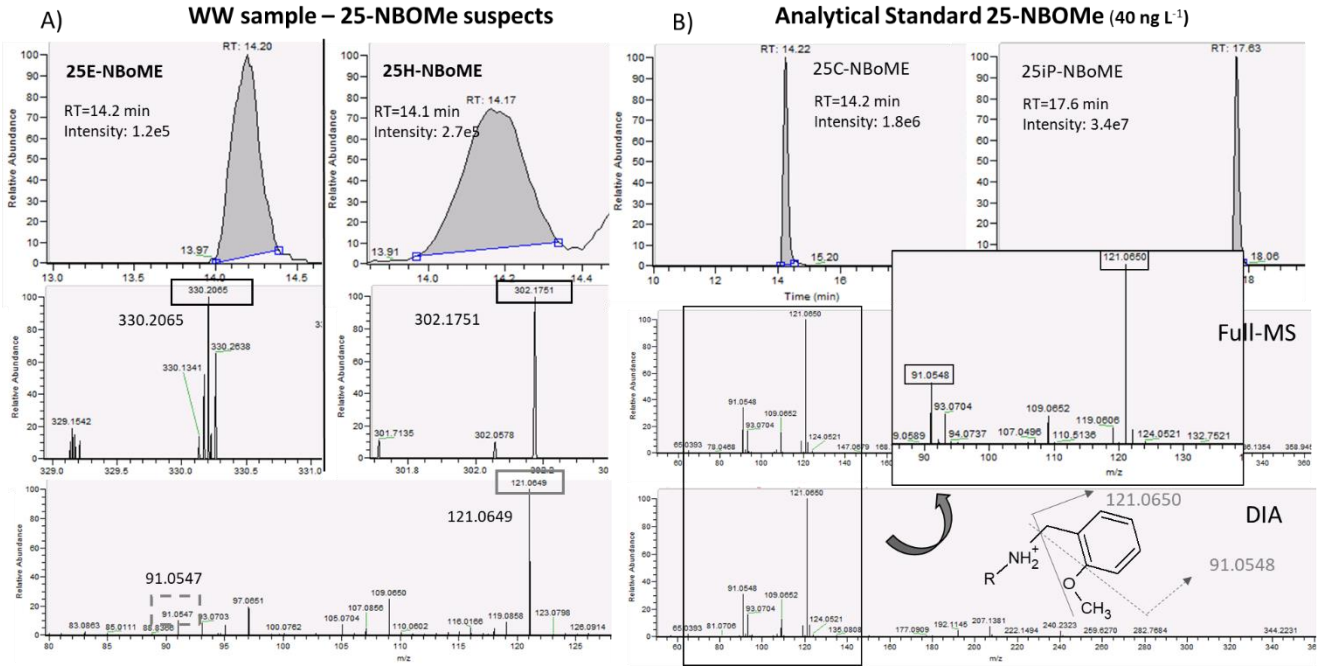
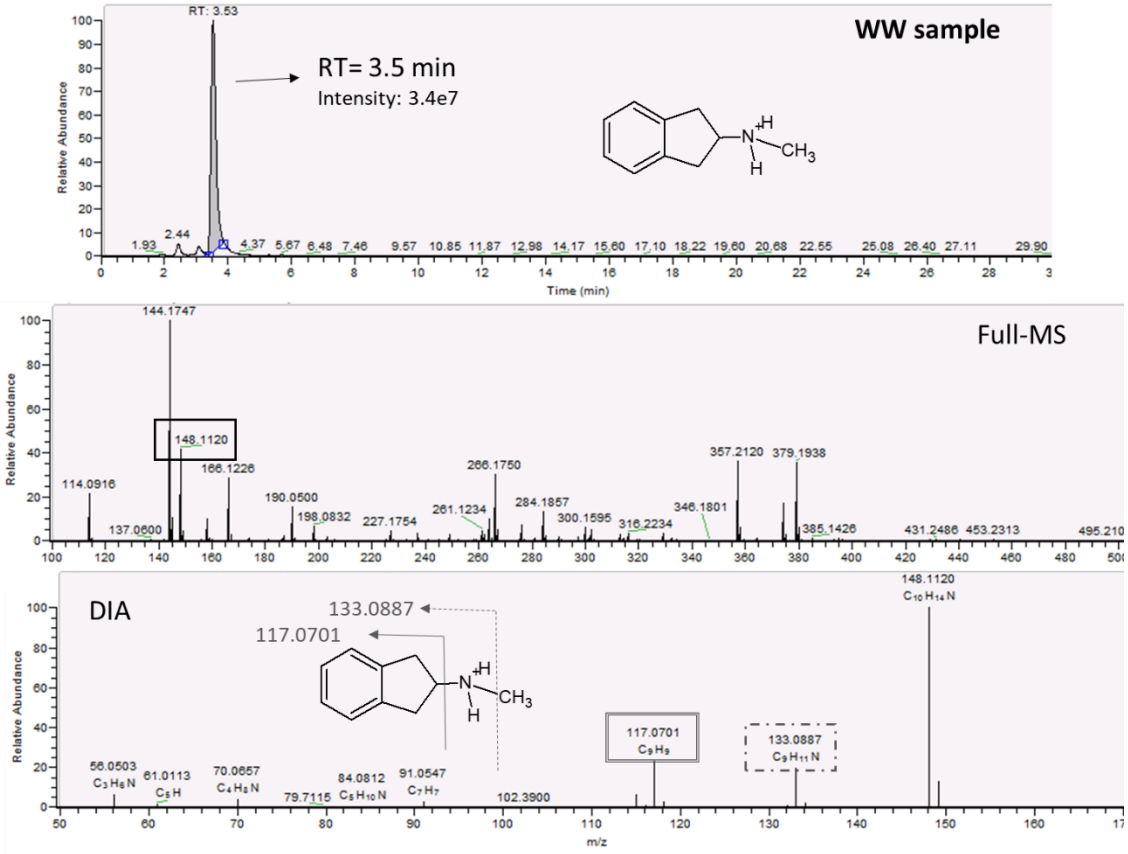

A)
WW sample – 25-NBOMe suspects
25E-NBoME
RT=14.2 min
Intensity: 1.2e5
25H-NBoME
RT=14.1 min
Intensity: 2.7e5
330.2065
302.1751
121.0649
91.0547
B)
Analytical Standard 25-NBOMe (40 ng L-1)
25C-NBoME
RT=14.2 min
Intensity: 1.8e6
25iP-NBoME
RT=17.6 min
Intensity: 3.4e7
Time (min)
Full-MS
DIA
121.0650
91.0548
m/z
Relative Abundance

Fig.4


WW sample
RT: 3.53
RT= 3.5 min
Intensity: 3.4e7
Relative Abundance
Time (min)
Full-MS
144.1747
148.1120
166.1226
114.0916
137.0600
190.0500
198.0832
227.1754
261.1234
266.1750
284.1857
300.1595
316.2234
346.1801
357.2120
379.1938
385.1426
431.2486
453.2313
DIA
133.0887
117.0701
148.1120
C10H14N
117.0701
C9H9
133.0887
C9H11N
56.0503
C3H6N
61.0113
C5H
70.0657
C4H8N
79.7115
84.0812
C6H10N
91.0547
C7H7
102.3900
m/z