Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A Tutorial

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Abstract

New psychoactive substances (NPS), often designed as (legal) substitutes to conventional illicit drugs, are constantly emerging in the drug market and being commercialized in different ways and forms. Their use continues to cause public health problems and is therefore of major concern in many countries. Monitoring NPS use, however, is arduous and different sources of information are required to get more insight of the prevalence and diffusion of NPS use. The determination of NPS in pooled urine and wastewater has shown great potential, adding a different and complementary light on this issue. However, it also presents analytical challenges and limitations that must be taken into account such as the complexity of the matrices, the high sensitivity and selectivity required in the analytical methods as a consequence of the low analyte concentrations as well as the rapid transience of NPS on the drug market creating a scenario with constantly moving analytical targets. Analytical investigation of NPS in pooled urine and wastewater is based on liquid chromatography hyphenated to mass spectrometry and can follow different strategies: target, suspect and non-target analysis. This work aims to discuss the advantages and disadvantages of the different data acquisition workflows and data exploration approaches in mass spectrometry, but also pays attention to new developments such as ion mobility and the use of *in-silico* prediction tools to improve the identification capabilities in high-complex samples. This tutorial gives an insight into this emerging topic of current concern, and describes the experience gathered within different collaborations and projects supported by key research articles and illustrative practical examples.

Keywords

New psychoactive substances; biological samples; wastewater-based epidemiology; monitoring strategies; mass spectrometry; ion mobility separation

1. Introduction

New psychoactive substances (NPS) are continually evolving and introduced in different ways in the drug market. The NPS retail market is characterized by its dynamic nature and the large number of substances covering a broad range of drug categories [1,2]. Whereas most NPS disappear after a short time, others seem to establish a niche market [2,3]. They are often introduced as legal substitutes for known controlled drugs, but also explored for their novel effect. Some substances have been known for years and are now misused for recreational purposes, but most NPS are newly synthesized with little or no safety data regarding their short or long-term toxicity. Furthermore, purity and composition of products containing NPS are often not known, which places users at an even higher risk compared to well-known conventional illicit drugs [1,2]. The NPS market is extremely diverse and differs between countries. Governments have responded in different ways to the NPS market, but have not been able to act upon all the NPS which have emerged in an effective way in terms of penalizing its supply and use [4]. Hence, NPS continue to cause public health problems [5,6] and challenge healthcare professionals, toxicologists and policymakers in terms of identification, prevention, treatment and control.

The Early Warning Systems (EWS) established by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), Europol and the United Nations Office of Drugs and Crime (UNODC) play a key role in collecting data on new NPS appearing on the market. This information together with indications of the health and social risks associated with these substances is pivotal to respond to the emergence of NPS [7]. Analytical chemistry has a prominent role in gathering more thorough data which allows to better understand the situation of NPS use in the population. To complement the existing sources of information and improve our knowledge about the categories and characteristics of NPS present on the market, the application of appropriate analytical strategies is of utmost importance.

The discovery and characterization of new substances in commercially available products and drug seizures is an important source of information for EWS. Since reference standards for unambiguous confirmation of the identity are often not available, a combination of several techniques, such as nuclear magnetic resonance (NMR), liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS), gas chromatography mass spectrometry (GC-MS) and X-ray crystallography, is normally applied [8–11]. Although there is a correlation, the identification of new substances in seized products mainly gives information on the NPS available on the market rather than information on the prevalence of use. Therefore, the analysis of biological samples is needed, but this implies a different analytical strategy to deal with the complexity of the matrix and the low analyte concentrations normally present in the samples [12–14].

The analysis of biological samples can be considered a frontline in the detection of consumed NPS. Samples of individuals can be collected from, for example, hospital emergency rooms, drug testing campaigns or post-mortem examinations, where concentrations of some NPS in acute intoxications may be relatively high. This may facilitate the identification of hitherto unknown intoxicants by means of the abovementioned analytical techniques [3]. However, it does not give a full picture of NPS use within a community, rather individuals, and the analyses of many samples required to have a wider picture is time consuming and expensive. In contrast, pooled urine and urban wastewater can anonymously provide information of many people in one single aggregated sample. Although the dilution factor can be rather high in these matrices, for example dilution of the sample with urine of non-consumers or water used in households and industry, it has demonstrated its utility for community-wide monitoring of illicit drug use and showed possibilities for getting complementary insight into the consumption and diffusion of NPS use [15–20].

Liquid chromatography hyphenated to tandem mass spectrometry instruments (LC-MS/MS) with triple quadrupole mass analyzers (QqQ) or hybrid HRMS/MS systems are the preferred analytical techniques that have the required high sensitivity and selectivity to deal with the challenges related to the screening of NPS in pooled urine and wastewater. Furthermore, the polar characteristics of most NPS and their metabolites, as well as the sample matrix, make them compatible with these techniques. This article aims to discuss the advantages and disadvantages of relevant mass spectrometry (MS) data acquisition workflows and data exploration approaches to confront the low analyte concentrations and ever-changing NPS market and will be supported using key research articles and illustrative practical examples. This tutorial is not intended to be an extensive review of the existing literature, but to give an insight into this timely topic and describes the experience gathered within different collaborations and projects. It also pays attention to new developments such as ion mobility separation (IMS) and the use of *in-silico* prediction tools to improve the identification capabilities.

2. Sample collection and sample treatment

Well-designed protocols for sample collection and storage, and versatile sample treatment of pooled urine and wastewater are essential for getting data that provide meaningful information on NPS use. The collection of anonymous pooled urine samples from portable street urinals has recently demonstrated its utility to detect the use of recreational drugs, including NPS [18,19]. Generally, multiple samples are taken from various urine reservoirs, over a 12-hour period, and then mixed to form pooled urine samples. This sampling method can be applied in cities where stand-alone urinals are routinely used at weekends [19], but can also be used for monitoring specific night time settings or recreational events such as music festivals [20–24]. Sampling urine aliquots from urinals ensures the collection of anonymous and representative samples and results may reflect the direct use of NPS. Yet, some limitations are related to the fact that urinals are designed for male use only and normally have no 'flushing' mechanism [25]. Thus, the number of contributors to the samples is unknown and, although quantitative analysis is possible, the comparison of concentrations gives little additional insight rather than a qualitative overview of the actual use of a certain drug compared to the other substances quantified in that specific sample.

Wastewater analysis may circumvent these limitations by providing anonymous population-normalized information of an entire community and has recently been explored to gather information on NPS use [3,15,17,26,27]. The successful application of wastewater-based epidemiology for assessing spatial differences and temporal changes in illicit drug use has been demonstrated [28,29] where population-normalized data can be calculated taking into account the measured concentration, the daily flow rate of sewage and the number of people connected to the wastewater treatment plant (WWTP) [28,30]. Specific sampling protocols have been developed to obtain representative 24-hour composite wastewater samples collected at the inlet of a WWTP [28]. In addition, a standardized questionnaire facilitates the collection of relevant meta-data such as the daily flow rate of sewage and the number of people connected to the WWTP [31]. This meta-data allows quantitative population-normalized information for a limited number of target NPS to be explored. The information provided by wastewater analysis can be integrated with existing epidemiological data because of the unique ability to provide objective, updated and nearly real-time information on drug use [16,32].

One sampling technique not yet fully explored but with potential for monitoring NPS in wastewater is passive sampling [33], which ensures the concentration of analytes from longer periods (days or weeks) and increases the possibility to detect substances with low prevalence of use. The main advantage is that passive samplers, consisting of polymeric-based sorbent material, deployed for longer periods, can accumulate trace analytes on the sorbent during this period. Moreover, as some NPS might be consumed sporadically (and thus might not always be present in wastewater), one does

not need to collect multiple wastewater samples, which all eventually need to be processed *i.e.* increasing labor costs. Hence, this technology offers practical and economic advantages for gathering long-term data. But it has also some challenges related to calibration and quantification, since they require knowledge about uptake and diffusion of the different substances and are subject to the variability associated with NPS stability and environmental factors (e.g., flow rates, biofouling) [33,34]. The uptake of target analytes on sorbent materials needs, therefore, to be determined prior to deployment in the sampling site.

Stability of NPS is an important aspect of sample collection for both pooled urine and wastewater analysis. While specific stability studies in pooled urine samples are lacking, they have been carried out on urine samples for forensic toxicology purposes. Metabolites of synthetic cannabinoids have been shown to be stable up to 14 days when refrigerated [35]. Many synthetic cathinones, benzodiazepines and amphetamine-type derivatives are very stable under freezing (-20 °C) storage conditions for months-years. However, when stored at room temperature or even refrigerated, degradation of these compounds can occur within days [36–38]. Therefore, it is recommended to freeze pooled urine samples immediately upon collection to avoid degradation. Regarding wastewater, it has been shown that acidification to pH 2 improves the stability in both filtered and unfiltered wastewater for up to 14 days for a wide variety of NPS such as cathinones, phenethylamines, opioid-derivatives and amphetamine-like stimulants [39]. If samples cannot be acidified, it is recommended that they are kept either refrigerated (4 °C) or frozen (-20 °C) for no longer than one week prior to sample processing [39–42]. Several synthetic cannabinoids have been shown to be unstable at pH 2 and in raw wastewater *i.e.* the hydroxypentyl metabolites of JWH 122, AM 2201, RCS-4 and JWH 073, while JWH 018 n-pentanoic acid, JWH 073 N-butanoic acid and JWH018 N-5-hydroxypentyl were stable at room temperature for up to 24 hours [42]. Moreover, the use of sodium metabisulfite as a preservative has been recommended to improve the stability of synthetic cannabinoids [43].

A non-selective and versatile sample preparation protocol for the enrichment and clean-up of samples capable of retaining a wide range of NPS with broad physicochemical properties is preferred and applied by the vast majority of reported studies. Pooled urine samples are usually treated by performing a hydrolysis step to cleave drug-glucuronide conjugates with β-glucuronidase and arylsulfatase prior to solid-phase extraction (SPE), liquid-liquid extraction and/or dilute and shoot techniques [21,44,45], while wastewater samples do generally not require this hydrolysis step due to in-sewer deconjugation [46–50] and are normally filtered and solid-phase extracted [17], although a less labor-intensive and quicker preparation procedure following the QuECHeRS principle has also been applied [51]. In order to cover the broadest range of substances possible, multiple SPE cartridges

or cartridges consisting of several layers with different stationary phase chemistries can be used [27,52]. The use of more cartridges implies several separate extractions, yet these can be optimized to specific NPS categories of interest such as cathinones or synthetic cannabinoids [15,21]. Typically, cartridges containing polymeric-based SPE sorbents with reversed phase (RP) properties built of generic hydrophilic and lipophilic balanced monomers or strong cation-exchange mixed mode sorbents incorporating RP copolymers are used. For the latter, samples should be acidified to pH 2-3 to ensure that the analytes are positively charged during extraction [53]. This especially aids the recovery of cathinones, amphetamine-like stimulants, opioid derivatives and phenethylamines [21,39,45,54,55]. Online SPE has also been utilized for a limited number of NPS using a RP cartridge, with satisfactory recovery (*i.e.* 70-120%) [56]. LLE has been shown to aid in the detection of synthetic cannabinoids in pooled urine [57] and wastewater [43,58]. For wastewater studies, it is important to note that the removal of the solid fraction through filtration can greatly affect the overall recovery of synthetic cannabinoids due to their lipophilicity. Therefore, when performing wastewater analysis, both the aqueous and particulate fraction should be extracted together for optimal recovery of cannabinoids.

Although both pooled urine and wastewater analyses incorporate SPE, there is a much lower pre-concentration factor needed for pooled urine, with initial volumes of 1-2 mL, due to the generally higher concentrations found [23,45,57]. Furthermore, lower pre-concentration results in less matrix effects and potentially an improved chromatographic performance. Higher pre-concentration factors in wastewater are commonly applied to deal with the very low concentrations of NPS expected in these samples. However, this can also result in strong matrix effects due to the pre-concentration of unremoved components present in the sample extract. Matrix effects are alterations of the MS signal (enhancement or suppression), which have been linked to co-eluting interferences such as proteins, lipids, sugars or salts, that affect the ionization process [59]. Frequently, isotopically labelled internal standards (ILIS) are used as surrogates and added to samples prior to processing (i.e., SPE) or analysis (in the case of dilute and shoot approaches applied in pooled urine analysis), to account for potential matrix effects, but also to correct for potential errors due to sample preparation. Ideally ILIS of the corresponding NPS are used as they are supposed to be affected in a similar manner as their non-labelled counterparts. However, ILIS are often expensive and not always commercially available, especially in the case of NPS. Therefore, ILIS are regularly used to correct for several compounds [15,40]. Nevertheless, the performance of each ILIS for correcting matrix effects need to be carefully evaluated. When appropriate ILIS are unavailable, matrix effects may be minimized by applying an additional clean-up step, but also lower pre-concentration factors may occasionally be desired for some substances in order to reduce ionization suppression and increase their detection limit [27,60].

In general, even when ILIS are available, a reduction of matrix effects is recommended for better precision, sensitivity and robustness in complex matrix samples [60].

3. Chromatographic separation

Good chromatographic separation is important to reach the required levels of selectivity, sensitivity and identification power to monitor NPS through wastewater and pooled urine analysis. GC-MS has been applied for the determination of NPS in urine. However, because of the high levels of selectivity and sensitivity provided by this technique, it requires the derivatization of the analytes which results in a more time-consuming and less generic sample treatment [61,62]. Alternatively, LC-MS allows the determination of compounds with a broad range of polarity, low volatility and thermolability with the application of more generic sample treatment strategies. In addition, the aqueous nature of the matrices makes LC-MS fully compatible with the determination of NPS in wastewater and pooled urine samples [63].

Reverse-phase LC (RPLC) separates compounds within the range of low-polarity to non-polarity. Therefore, it seems to be the most suitable chromatographic technique to achieve generic and good chromatographic separation especially for wide-scope monitoring of NPS. Consequently, the vast majority of studies dealing with multi-residue methods in wastewater and/or pooled urine samples applied RPLC as the separation technique [13,23,25,64,65]. However, more polar (or ionic) substances such as amphetamine-like stimulants or synthetic cathinones and their metabolites, might require more specific methodologies. Recent developments in column chemistries and improvement in robustness of existing stationary phases allowed the analysis of more particular scenarios. Hydrophilic interaction LC (HILIC) is an alternative approach to effectively separate small and highly polar NPS. For example, Kinyua et al. [55] successfully developed a multi-residue methodology for the determination of 7 synthetic cathinones and amphetamine-like stimulants by means of HILIC separation.

Additionally, enantiomeric analysis has also been explored for the determination of NPS [66–68]. Chiral NPS are usually consumed as racemic mixtures of different forms (*i.e.* with an enantiomeric fraction (EF) between the two forms of approximately 0.5), even though both forms might differ quantitatively and qualitatively in the pharmacological activity [69]. Therefore, enrichment of the R (or S) form, depending on the stereoselective metabolism in humans, is expected in biological samples [66]. Consequently, an EF found in wastewater or pooled urine samples deviated from the original EF value could help in distinguishing between human consumption and direct disposal of unused substances [66]. Other chromatographic techniques such as capillary chromatography and supercritical fluid chromatography (SFC) are promising strategies for the monitoring of NPS. The improvement in sensitivity provided by capillary chromatography, especially for the small amphetamine-like structures, revealed a technique to explore for this purpose [26]. Also, recent developments in commercially available instruments has seen an increase in applications of ultra-high performance (UHP) SFC - MS/MS, in particular using (sub)supercritical carbon dioxide (CO_2) with

241 various organic additives as mobile phase [58,70]. One of the main advantages of UHPSFC compared
242 to conventional UHPLC is its increased chromatographic efficiency and resolution [71] also permitting
243 the separation of several NPS isomers with good results [72].

4. Quantitative target monitoring

As discussed above, the determination of NPS can be challenging due to the large number of potentially relevant compounds and the low concentrations expected in samples, in particular when considering wastewater and pooled urine. In fact, due to the often low prevalence of use of individual compounds, concentrations of these substances are often orders of magnitudes lower compared to conventional illicit drugs (< 10 ng L^{-1}) [55]. For this reason, targeted methods, specifically using LC-MS/MS with QqQ or ion-trap mass analyzers, have been implemented for the reliable identification and quantification of selected NPS in urine and wastewater samples [15,17,73,74]. The development of such quantitative target methods, however, requires access to reference standards for precursor-product ion transition selection in the Selected Reaction Monitoring (SRM) mode and MS parameters optimization. Identification and confirmation is achieved through the acquisition of at least two SRM transitions and matching of the retention time (RT) and ion-intensity ratios between the sample and reference standard [75,76]. The most sensitive SRM transition is commonly selected for the quantification at low concentration levels, whereas the second transition allows confident confirmation [26,40,46]. However, since NPS often retain high structural similarity, the risk of selecting common transitions is present and therefore the acquisition of more transitions (if feasible) is recommended to gain more confidence to the confirmation process. Hence, it is also important to understand fragmentation of each NPS as it allows the selection of specific product ions and avoid non-specific transitions such as a neutral loss of water or CO_2[77]. The latter is especially relevant to minimize potential matrix interferences when analyzing NPS at low concentrations in highly complex matrices such as pooled urine and raw wastewater samples. Although quantitative target monitoring can be performed using LC-HRMS instruments, their application in the field is limited due to the generally lower sensitivity compared to low resolution MS/MS instruments [17]. Hence, the advantage of low-resolution instruments for quantitative analysis lies in the robustness, selectivity and sensitivity which can be achieved by monitoring these specific precursor-product ion transitions. Combined with their high scanning speed, these instruments can monitor many transitions almost simultaneously, and consequently high-throughput, multi-residue methods that include many targeted NPS biomarkers, can relatively easily be developed.

Synthetic cathinones, phenethylamines, tryptamines and piperazine-derivatives have been quantitatively determined in pooled urine samples collected during weekends at specific night settings [25] or at music festivals [23]. Although data obtained from quantitative determination of NPS in pooled urine samples only gives an indication on the extent of use for an NPS compared to other substances found in a specific sample [23], these findings are still very valuable, as the application of these selective and sensitive target quantitative methods give high confidence and allows

confirmation of the NPS identified at low concentration levels. Synthetic cathinones are by far the most studied group of NPS in wastewater, followed by synthetic cannabinoids and phenethylamines. Studies using LC-MS/MS to monitor these substances have been carried out in Europe, Asia and Australia [15,17,26,39,40,78,79] and have shown spatial and temporal trends using population-normalized data. Although LC-MS/MS methods are highly sensitive and multi-residue methods can be developed, they have a major drawback, namely reference standard materials need to be available for method development as previously highlighted. Given the high number of NPS that have been detected in the market and their transient nature, reference standards are mostly available for only a limited number of compounds. Moreover, by the time reference standards become available, these compounds might have already disappeared from the market as they may have been less popular or added to the lists of regulated substances and can thus not be sold legally anymore. Further exacerbating the determination of these substances is the extent of their metabolism. There have been studies carried out on the metabolism of NPS using human liver microsome incubations to better understand the metabolism of certain NPS [80–84]. In addition, recent advances in computing power have permitted the development of comprehensive knowledge based software to predict the metabolic fate [85,86]. However, reference standards of most of the metabolites proposed are not commercially available and therefore unsuitable for quantitative target monitoring. Thus, quantitative target LC-MS/MS methods, although indispensable to achieve the highest sensitivity needed for certain types of substances (e.g., fentanyl and its derivatives), need to be complemented by other analytical approaches which allow a quick and broader monitoring, without the necessity for reference standards. Although low-resolution mass spectrometry (LRMS), especially tandem MS instruments, are highly appreciated in quantitative analysis, its application to qualitative analysis and capabilities in detecting unknowns is, limited due to the relative low resolving power (approximately 1 Da) and low sensitivity in full scan mode [77]. The use of HRMS offers new possibilities in the determination of NPS as well as circumventing some of the limitations of LRMS.

5. Qualitative screening approaches

HRMS presents strong potential for monitoring a large number of substances, due to its acquisition of accurate-mass full spectrum data at good sensitivity [63,77,87]. In order to facilitate the reading of this tutorial, terms that will be used in this section are defined below:

Target screening based on HRMS allows the qualitative screening of NPS after data acquisition based on large databases, thus evading the pre-selection of analytes for method development and the need of reference standards. However, the information included in the database is limited by the availability of reference standards. When reference standards are available, information such as accurate masses of fragment ions, adduct formation and RT can be included, whereas only the elemental composition, exact mass and theoretical isotopic pattern can be included when no reference standard is available. Although the acquisition of data is performed in an untargeted way, the approach is considered targeted and generally known as *suspect screening* [77,87], since the search is based on a list of target compounds that can be expected to be found in the samples. An advantage of this approach is that retrospective analysis can also be performed at any time from the acquired data to search for substances initially not considered and included in the database, such as novel NPS or newly discovered metabolites [88,89]. It should, however, be noted that the detection of some substances might be restricted by the sample treatment, the chromatographic conditions or the ionization efficiency [90], since usually a generic analysis is performed and no optimization has been executed for the NPS included in the database.

Non-targeted screening, without any selection of analytes, allows the investigation of any other NPS biomarker not included in the database. However, it implies an examination of each chromatographic peak and extensive investigation of its accurate mass spectrum. This process is challenging and time consuming and probably does not outweigh the rate of success in identifying of unknown NPS. Alternatively, the screening can be directed to discover related compounds of known NPS using characteristic mass spectral information and applying mass-defect filtering or common fragmentation pathways.

As a starting point for researchers interested in undertaking qualitative screening of NPS by HRMS, the review article written by Hernandez et al. [63] describing different mass spectrometric strategies for the investigation of illicit drug biomarkers in wastewater is recommended. Although similar strategies and identification criteria can be applied for the investigation of NPS in pooled urine and wastewater, the challenges are different due to the rapid turnover in the NPS drug market creating a scenario with constantly moving analytical targets and the often lower prevalence of use compared to conventional illicit drugs. Moreover, the structural similarities of NPS and their metabolites often requires increased identification confidence in order to minimize reporting false positives. In the text

337 below, practical examples are given to discuss different data acquisition workflows and data
338 exploration approaches to illustrate how HRMS can help in the confident identification of NPS in high-
339 complex pooled urine and wastewater samples.
340
341 ***5.1. Acquisition modes for hybrid high resolution mass spectrometric systems***
342 The most commonly used HRMS analyzers are time-of-flight (TOF) and Orbitrap, which can be coupled
343 with LC and possess high mass resolving power (> 20,000 Full Width at Half Maximum (FWHM)) and
344 mass accuracy (< 5 ppm) for wide scope screening of NPS in pooled urine and wastewater [17,75,76].
345 However, hybrid configurations, such as quadrupole-TOF (QTOF) or quadrupole-Orbitrap (Q-Orbitrap),
346 are nowadays more the standard than the exception as they considerably increase the potential of
347 HRMS for screening NPS [20,21,27,44,52,91]. When working in MS/MS mode, it is possible to record
348 accurate mass product-ion spectra of previously detected candidates and obtain relevant structural
349 information to allow suspected NPS to be confidently identified or disregarded as false positives.
350 However, the simultaneous accurate-mass acquisition of both full-spectrum and product-ion spectra
351 data is preferable and collects accurate mass data of both the (de)protonated molecules and its
352 fragment ions in a single acquisition and without the selection of precursor ions.
353 In data-dependent acquisition (DDA) mode, the instrument first performs a “survey scan” from which
354 the analyst chooses (or not) certain ions that fit specific criteria based on, for example, intensity
355 thresholds. Ions for which these conditions are met, are then selected to be included in a list of
356 preselected masses and fragmented to provide information-rich product ion scans. Unlike intensity
357 thresholds, an inclusion (or exclusion) list allows large matrix interferences to be ignored, thereby
358 facilitating the identification process and saving effort and time [27,52,63,92]. However, the size of
359 the inclusion list (i.e., suspects to be fragmented) can adversely affect the cycle time of the instrument.
360 Therefore, a decrease in the number of scans (or data points) across a chromatographic peak will
361 occur, reducing its detectability. Moreover, any compound not included in the initial inclusion list
362 cannot later be retrospectively analyzed, so the sample would have to be re-extracted and re-
363 analyzed. Yet, there is a way around this limitation, utilizing complementary targeted and untargeted
364 DDA. This technique initially conducts an MS scan followed by targeted MS/MS using an inclusion list
365 and then untargeted MS/MS on *n*-selected precursors. For example, analysts can look at MS/MS of
366 the *n* most abundant precursor ions, which would be of great utility for samples with high levels of
367 NPS such as seizure samples [14,93]. However, the generally low concentration of NPS found in pooled
368 urine and wastewater might mask the detection of low abundant peaks, and therefore, many NPS may
369 remain undetected [94].

Data independent acquisition (DIA) allows the acquisition of accurate-mass full-scan spectra under different collision induced dissociation conditions within a single injection. This acquisition mode is known under different names depending on the manufacturer (e.g. All-ion-fragmentation (AIF), all-ion MS/MS, MS^E and broadband collision-induced dissociation (bbCID)), where all ions generated in the ion source are sent to the collision cell for fragmentation without precursor ion selection or any predefined selection criteria. This alternation between full-scan and untargeted MS/MS events at low collision energy (LE) and high collision energy (HE), respectively, allows one to obtain information relating to the accurate masses of the (de)protonated molecule as well as their fragment ions. Furthermore, it conserves highly valuable information on adducts and isotopes since the quadrupole works as an ion guide [63,77]. The main limitation of DIA is that spectra are non-selective and contain product ions for all ions formed in the ion source. Hence, the interpretation can be challenging, since co-eluting compounds or matrix interferences may "contaminate" the spectra, and makes it difficult to associate product ions with the correct (de)protonated molecule [14,95,96].

Slightly different modes compared to the other DIA modes mentioned above in terms of specificity have been developed by manufacturers with the objective to have HE spectra approaching to MS/MS quality data. As an example, in Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH) mode, a TOF MS full scan at LE is acquired, alternated by SWATH experiments at HE obtaining MS/MS data by fragmenting only the (de)protonated molecules present in a much narrower window (e.g. 15 -25 *m/z*). In this way, SWATH can distinguish co-eluting compounds of different masses by having specific experimental mass fragmentation windows which filter out all masses not included in the specified mass range. This results in cleaner spectra, which facilitates identification [96,97]. This is a particular important point in the determination of NPS, which are notorious for the analytical challenges associated with common fragments. **Figure 1** shows the utility of SWATH in differentiating two co-eluting NPS, butyryl fentanyl with *m/z* 351.2431 and furanylfentanyl with *m/z* 375.2067 in a spiked wastewater sample. In the full scan acquisition at LE, it can be observed from the individual extraction ion chromatograms (XICs) that the two NPS seemingly elute at 12.50 min (**Figure 1A, top**), with the mass spectra at this RT showing both masses (**Figure 1A, bottom**). However, when applying SWATH, the HE experiments carried out at different mass windows (*m/z* 340.2 – 357.4; **Figure 1B** and *m/z* 372.6 – 389.8; **Figure 1C**) allowed them to be distinguished by extracting the mass of each of these fentanyl derivatives in their corresponding acquisition window. With the mass of butyryl fentanyl and furanylfentanyl falling within separate experiments, they can be individually extracted and identified using cleaner spectra. This exemplifies the power of this acquisition mode in the elucidation of NPS.

[Insert **Figure 1** here: Identification of two co-eluting NPS, butyryl fentanyl (*m/z* 351.2431) and furanylfentanyl (m/z 375.2067) in a spiked wastewater sample using Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH). (A) overlapping extraction ion chromatograms (XICs) of the two NPS with chromatographic peaks eluting at 12.50 min (top); full scan acquisition mass spectra with low collision energy (LE) (10 V) at retention time 12.50 ± 0.10 min (bottom). (B) SWATH mass window *m/z* 340.2-357.4, XIC at *m/z* 351.24 (middle) and high collision energy (HE) mass spectra (bottom); (C) SWATH mass window m/z 372.6-389.8, XIC at *m/z* 375.21 (middle) and HE mass spectra (bottom)]

5.2. Suspect screening

Suspect screening approaches usually take advantage of home-made databases. However, the information included therein is limited by the availability of reference standards, as previously explained. When no reference standard is available, the minimum suggested requirements for a tentative identification is the accurate mass of the (de)protonated molecule and, at least, one significant fragment ion together with the corresponding isotopic pattern. This is in the line with proposed quality procedures recommended in other research fields [76,98]. The observed fragments need to be in accordance with the chemical structure and, preferably, in agreement with previously reported data in scientific literature or online spectral databases [27,52,99,100]. Ideally, reference standards are available, and information such as accurate masses of fragment ions, adduct formation and RT can be included, which allow unequivocal identification. However, this entails high costs due to the high number of compounds and, therefore, huge efforts have been devoted, in the recent years, to develop community-made or online mass spectral databases for NPS. The best known databases are NPS Data Hub [101] and HighResNPS [102,103] with more than 2800 and 3350 entries, respectively (date accessed: 26 June 2020). The HighResNPS library currently has active users from more than 10 laboratories around the world with the intention to ensure up-to-date analytical information from the moment a specific NPS becomes available to a given participating laboratory [102]. These libraries are available to help and facilitate the screening of NPS and their metabolites [101,104–106].

In most laboratories, a suspect screening based on large home-made databases is often the first step for monitoring samples. Due to the high number of NPS and metabolites, the rapid transience of these compounds on the market, high costs and limited availability of reference standards, home-made databases are normally built of merely accurate masses of the (de)protonated NPS and fragment ions. Yet, the low concentration levels of NPS present in combination with strong matrix interferences makes the tentative identification of NPS challenging and remark often the necessity to perform some additional research or experiments to increase the confidence in the tentative identification. As an

example, **Figure 2** shows the tentative identification of 4-chloro-α-pyrrolidinopropiophenone (4-chloro-α-PPP) in a pooled urine sample. Its protonated molecule, the isotopic information related to the presence of one chlorine atom and at least one fragment ion was observed at accurate mass (**Figure 2A**). However, a known and abundant fragment of 4-chloro-α-PPP at *m/z* 167.0258 [107] showed an undue high mass error (+143 ppm) under the initial screening conditions, which made the tentative identification of this NPS questionable. By increasing the mass resolution of the Orbitrap MS from 20.000 to 35.000 FWHM and zooming in the *m/z* range of the fragment, it was possible to distinguish three peaks at *m/z* 167, one corresponding to the fragment ion *m/z* 167.0258 (+5.3 ppm) of 4-chloro-α-PPP (**Figure 2B, bottom**). This allowed more confidence to be gained in the identification. Subsequently, the feature could be identified as 4-chloro-α-PPP by means of a reference standard. The latter is pivotal for the confirmation of the identity of the NPS. However, by using this approach, laboratories do not need to purchase all reference standards *a priori* to the analysis [108] and could prioritize those NPS for which more reliable evidence is obtained.

[Insert **Figure 2** here: Tentative identification of 4'-chloro-α-pyrrolidinopropiophenone (4-chloro-α-PPP) in a pooled urine sample. (A) Extracted ion chromatogram of 4-chloro-α-PPP and ^{37}Cl isotope (top); Product ion mass spectra of $[M+H]^+$ at *m/z* 238.10 (bottom). (B) Structure of 4-chloro-α-PPP (top); Zoom in the range of fragment ion with *m/z* 167 at resolution (R) of 35.000 Full Width at Half Maximum (FWHM) (bottom)]

Positional isomers or homologues are frequently the first choice to substitute banned NPS [109]. Hence, NPS often have only minor modifications to a backbone structure and the structural similarities of NPS and their metabolites are often reflected by their common fragmentation pathways, this poses one of the principal challenges in suspect screening strategies. As an example, the analysis of a raw wastewater sample showed a chromatographic peak at 4.51 min giving a positive hit for the isomers α-methyltryptamine (AMT) and 5-(2-aminopropyl)indole (5-IT) based on the accurate mass of their protonated molecule and their fragment ions (**Figure 3A**). These two isomers share the same chemical backbone with the only difference being the position of the substituent (**Figure 3B and 3C, top**). The following MS fragment ions were found: *m/z* 158.0954, *m/z* 143.0724, *m/z* 132.0799, *m/z* 117.0577 and *m/z* 115.0541, with the most abundant fragment at *m/z* 143.0724 (**Figure 3A, bottom**). The only difference, described in the literature, between the spectra of AMT and 5-IT resides in the relative intensities of the fragment ions [110]. The most intense fragment ion of 5-IT has an *m/z* of 130, whereas the most abundant fragment ion for AMT corresponds to *m/z* 143. This slight difference in the fragmentation pattern (i.e. intensities) gave more confidence in the tentative identification of AMT

instead of 5-IT in this sample. Therefore, AMT was synthesized and a reference standard of 5-IT was donated by a collaborating laboratory. When comparing that empirical data to AMT and 5-IT reference standard MS fragment ions (**Figure 3b and 3c, bottom**), it can be observed that both substances share the same fragment ions (in nominal mass; *m/z* 143, *m/z* 130, *m/z* 117 and *m/z* 115) coinciding with the fragment ions observed in the sample, but that AMT indeed show a more abundant fragment ion with *m/z* 143. This gave more confidence in the positive identification of this NPS and together with its RT, AMT could finally be confirmed.

[Insert **Figure 3** here: Identification of α-methyltryptamine in a raw wastewater sample using QTOF MS. (A) feature detection of *m/z* 175.1235 at 4.51 min (top, insert) together with the low collision energy (LE) spectra (top) and high collision energy (HE) spectra with emphasis on *m/z* 130-145 (grey areas) (bottom); (B) Structure, fragment ions, LE and HE spectra of α-methyltryptamine; (C) Structure, fragment ions, LE and HE spectra of 5-(2-aminopropyl)indole]

5.3. In-silico approaches

In some cases, the instrument-specific parameters (i.e. accurate mass ions and isotopic patterns) do not suffice to tentatively propose a chemical structure, and, therefore, additional studies are required. For that purpose, predictive models have been used to filter out false positives and increase the confidence of compound identification when reference standards are unavailable or no information is within reach in previously reported data [27,111,112]. Aalizadeh et al. developed a RT prediction model using Quantitative Structure-Retention Relationships (QSSR) and Support Vector Machines (SVM) to model the RT data for both HILIC and RPLC with high accuracy [111]. A different approach was proposed by Bade et al. considering the application of Artificial Neural Networks (ANNs) for the development of a RT predictor for gradient-RPLC using a dataset of more than 500 compounds with an predictor accuracy of ±2 min [112]. Such RT predictive tools are highly valuable for the determination of NPS in complex matrices as demonstrated by Diamanti et al. [27]. Since the availability of reference standards is limited, the suspect screening of NPS usually results in many candidate structures because of the structural similarity of many NPS, as for example, in the case of the two isomeric phenethylamines 3,4-methylenedioxy-N-hydroxyethylamphetamine (MDHOET) and N-hydroxy-*N*-methyl-3,4-ethylenedioxyamphetamine (EFLEA). The predicted RT using a QSSR predictor model matched the one for MDHOET and discarded the one for EFLEA, thereby reducing the number of candidates and increasing the confidence in the tentative identification of MDHOET in influent wastewater from Athens [27]. *In-silico* fragmentation tools, such as the MetFrag software, are pivotal in a suspect screening workflow. This software generates a predicted fragmentation of

505 molecules based on their structure and compare it to the empirical data gathered proposing a list of
506 fitting candidates together with a scoring parameter [113,114]. However, it is common that many
507 structurally related substances can be assigned to the empirical data with a similar score value [113],
508 which is a drawback particularly for the investigation of NPS because of the similarity of several
509 substances.

6. Ion mobility separation coupled to high resolution mass spectrometry

The recent development of the hyphenation of IMS with LC-QTOF MS instruments (LC-IMS-QTOF MS) represents an innovative tool for their application in target and non-targeted screening strategies. IMS separates ions depending on their size, shape and charge in a gas phase, (usually nitrogen or helium), and in the presence of an electric field [115]. Ion separation occurs in the millisecond time scale, making it compatible with fast TOF MS acquisitions [116]. The time an ion takes to travel through the mobility cell i.e. the drift time (DT), adds an extra dimension to the obtained chromatographic RT and accurate mass, which results in increased selectivity and improved identification, particularly in DIA modes [116,117]. The increased selectivity is translated into much cleaner and higher-quality spectra than conventional HRMS DIA spectra, since (de)protonated molecules and fragment ions of interest with the same DT can be aligned and separated from co-eluting matrix components. Although data sets inherently become more complex and more comprehensive, the utilization of IMS-HRMS instruments does not overcomplicate the data revision process thanks to the four-dimensional automatic feature detection. This allows the software to both deconvolute peaks based on chromatographic and MS data and align ions with the same RT and DT into unique features. Thus, LE and HE spectra are DT filtered for the deconvoluted ions (*i.e*. for each ion detected in the LE spectra its DT is used to correlate it with the fragment ions obtained in the HE spectra). Cleaner spectra can also be obtained by improving the chromatographic separation. Although improvements in the quality of the spectra often relies on spectral discrimination of the compounds, a good chromatographic separation is recommended especially when analyzing complex matrices such as pooled urine and wastewater that contain many co-eluting interferences. Yet, IMS provides an extra dimension of separation which fits between chromatography and MS and results in cleaner spectra, but without increasing the chromatographic run time or mass resolving power.

A further advantage of IMS is that Collision Cross Section (CCS) values can be derived from the DT and represent the surface of the sphere created by the ion when moving in the gas phase. Unlike DT, CCS is an instrument independent value, provided that the same drift gas and ion mobility calibration standards are used [116,118,119]. The importance of CCS values relies on the fact that they are robust across multiple platforms (*i.e.* deviation up to 2%), independent of the chromatographic conditions used and not affected by matrix composition [118–120]. CCS values depend on the calibration procedure applied, and the deviation between instruments is caused by the slight experimental variations in room temperature, gas pressures and other hardware settings. Hence, CCS is a parameter that can give support to MS-based compound identification in addition to RT, *m/z*, isotopic pattern and fragment ions. Finally, IMS enables, in theory, the separation of isomeric compounds not previously resolved using LC, since they are expected to have a different mobility in the drift cell, and

therefore different CCS values [121,122]. Although there is a relationship between the *m/z* and CCS, Bijlsma et al. [123] showed that a range of 35 $Å^2$ could be observed for molecules of approximately 300 Da, therefore, demonstrating that no direct correlation between *m/z* and CCS could be established and that thus IMS may separate isomers.

Figure 4 illustrates the benefits of IMS in terms of higher-quality spectra in DIA MS/MS events. In this example, a positive finding of ketamine in a wastewater sample is shown using an ion mobility separation QTOF MS (Vion from Waters). When searching for ketamine (with *m/z* 238.0993 ≤ 5ppm) a chromatographic peak at a RT of 3.33 min was observed (**Figure 4A, top** (yellow arrow)). The corresponding conventional DIA MS^E spectra (LE and HE) show many ions when no DT alignment is applied (**Figure 4B, top**) resulting in a base peak with *m/z* 263.1386, which does not correspond to ketamine (i.e. *m/z* 238.0993, highlighted in green). However, when applying the IMS MS^E acquisition mode ($HDMS^E$, High-Definition MS^E), several co-eluting ions at 3.33 min are separated in the mobility cell, illustrated as red or black dots in **Figure 4A, bottom**. The DT of the ion with *m/z* 238.0993 was 4.89 ± 0.20 ms and the corresponding fragment ions in this range, the blue highlighted areas, can be aligned. All other ions outside this area are filtered out, which results in much cleaner and easier to interpret spectra (**Figure 4B, bottom**). Despite the presence of some co-eluting interferences with similar DT, the resulting spectra contains fragment ions which could be primarily assigned to ketamine [124].

[Insert **Figure 4** here: Identification of ketamine in a wastewater sample using IMS QTOF MS. (A) feature detection of *m/z* 238.0993 at 3.33 min and drift time (DT) 4.89 ms, yellow arrow (top); co-eluting ions at 3.33 min illustrated as red or black dots and separated by DT. Blue highlighted areas are the DT ranges of 4.89 ± 0.20 ms at *m/z* 238.0993 at low collision energy (LE) and high collision energy (HE) (bottom). (B) LE and HE mass spectra without IMS DT alignment (top); LE and HE mass spectra with IMS DT alignment (bottom)]

The additional cleaning of spectra provided by IMS is of particular relevance for the determination of NPS in challenging matrices such as wastewater or pooled urine where thousands of naturally occurring compounds can hamper the identification of these substances at the low concentration levels expected. Moreover, since the CCS value of a certain molecule is not affected by matrix composition, their utilization as an additional identification point in the determination of NPS pushes IMS-HRMS as a promising technique in the monitoring of these substances [125,126]. Therefore, the development of home-made or collaborative on-line databases including ion mobility data will enhance the efficiency of target NPS screening. However, as has been discussed earlier, due to the

lack of analytical standards for most of the NPS and metabolites and the still sparse accessibility to IMS-HRMS instruments in research centers, the availability of CCS values for these substances is still very limited. Hence, *in*-silico predictive tools similar to those for RT and MS fragmentation may help to increase the confidence in the identification of tentative candidates. Several data-driven CCS predictor systems have been developed for the prediction of CCS values for small molecules [123], pharmaceuticals and drugs of abuse [127] and metabolites [128]. As an example, the predictor reported by Bijlsma and Bade et al. [123] was developed using 205 CCS values for small molecules including pharmaceuticals, pesticides and drugs of abuse with ANNs for modelling the ion mobility data. Although the empirical variability of CCS measurements across instruments for a certain molecule is known to be up to 2%, with the developed CCS predictive model, the maximum deviation at the 95% confidence interval was only 6%. Mollerup et al. [127] were able to reduce the deviation in the predicted CCS to a 4%, consequently increasing the accuracy of the model. In the case of the predictor model developed by Zhou et al. [128], support vector regression was applied to the development of predictive models for different molecular adducts with median relative errors of approximately 3%. Regardless of the predictive model applied for the prediction of CCS, the utilization of these strategies facilitates the tentative identification of NPS in suspect screening strategies [125], especially when combined with RT and MS fragmentation predictive tools.

7. Future perspectives

The determination of NPS in pooled urine and urban wastewater has shown several challenges due to distinct factors as discussed in this manuscript. Current analytical instrumentation based on LC combined with LRMS and HRMS and the application of complementary data acquisition workflows and data exploration approaches helps to circumvent or confront certain barriers. However, more research related to NPS biomarkers is required and several trends in analytical chemistry, which is under continuous development, can be highlighted:

i. ***NPS biomarker selection***. The high number of existing NPS and the constant introduction of new compounds on the drug market creates a dynamic scenario of moving target biomarkers. Hence, monitoring of all NPS is complex and efforts could therefore be initially focused on NPS which are relatively high-dosed or frequently consumed and excreted (partly) unchanged such as amphetamine-like substances and cathinones. Especially since scant information on NPS pharmacokinetics is currently available, which complicates the choice of suitable biomarkers (parent substance or urinary metabolites) [129,130]. This is particularly relevant for synthetic cannabinoids and compounds like NBOMes that are highly metabolized in the human body [42,131,132] and for synthetic opioids that are consumed at very low doses [39], leading in both cases to very low concentration levels of the corresponding biomarkers in urine and, consequently, in wastewater. However, there are some published works on the metabolism of NPS [80–84] and different computational tools exist that predicts the metabolic fate of chemicals [86,87]. Although the proposed metabolites therein are generally not commercially available for quantitative target monitoring, these compounds should be included within screening databases as well as aiding in retrospective data analysis to ensure that the most appropriate analytical targets are investigated.

ii. ***Sample collection, storage and treatment*** of pooled urine and wastewater is pivotal for getting meaningful information on NPS use. Pooled urine analysis of samples collected from portable toilets and urinals give an informative snapshot of the NPS used, but is often limited to men only and it is difficult to extrapolate results to the total number of toilet users. All-gender toilets with an improved design, complying specific technical requirements like a flushing mechanism and a visitor counter could circumvent these limitations in future studies. Currently, daily composite wastewater samples are more representative and analysis provides population-normalized quantitative information on NPS. A best practice protocol to collect representative wastewater samples of an entire community is available [32] to ensure the comparability of results from different countries. However, wastewater is more diluted compared to pooled urine resulting in

lower concentrations, which may complicate the detection of some NPS. Passive sampling increases the possibility to detect substances with low prevalence of use, because of the sampling and concentration of analytes over a longer period of time. Yet, passive sampling also merely gives a snapshot and has several limitations that need to be overcome or optimized as previously described. Recent developments, using diffusive gradients in thin films which, in contrast to conventional samplers, consist of a diffusive and binding gel and are exposed to the medium, are less dependent to hydrodynamic condition (e.g. flow rates) and can hence overcome some of the limitations encountered with conventional passive samplers [133,134].

A relevant requirement for an NPS biomarker is its stability in pooled urine and wastewater in order to avoid any loss that can prevent detecting its use. Further work need to be addressed to test biomarkers stability and potential degradation or transformation in raw wastewater and urine [39–41,94]. Until more information is available, it is recommendable to store samples in the dark at -20 °C directly after sample collection in order to minimize possible degradation.

Sample treatment is very important to improve detection. However, a versatile sample treatment to retaining a wide range of NPS is not always feasible and specific treatments for certain NPS classes such as synthetic cannabinoids and synthetic opioids (i.e. high potency NPS such as fentanyl) need to be developed.

iii. Good ***chromatographic separation*** might seem less important when coupled to highly sensitive and selective mass spectrometers, although it can be essential in the detection and identification of NPS. Taking into account the many isomers or structurally related compounds and the often strong matrix effects, more effort could be put into chromatographic separation in future work. HILIC and enantiomeric analysis have demonstrated a strong potential to move a step forward into a more comprehensive determination of NPS in wastewater and pooled urine. Capillary chromatography and UHPSFC-MS/MS have also been explored. Yet, some concerns have also been raised related to the robustness of the technique to routinely analyze complex matrices. Future developments in terms of more robust column chemistries will open a new scenario for the monitoring of NPS. Additionally, UHPSFC has the potential to combine the advantages of LC and GC, thus improving analytical capabilities of laboratories dealing with the determination of NPS.

iv. Highly sensitive ***targeted methodologies*** based on LRMS will continue to play an important role in monitoring NPS use, particularly for those compounds which have established a niche market and/or are highly potent and require low detection limits. In addition, complementary ***suspect screening approaches*** based on large home-made databases, including many substances for which reference standards are not available, will remain the common practice for the foreseeable

future. Furthermore, the improved sensitivity and quantitative capabilities of HRMS instruments combined to multi-stage off-line or on-line solid-phase extraction allow achieving targeted quantitative and qualitative screening analyses in a single run, thus overcoming the need of having two distinct instruments/methods [27]. Similarly, machine learning algorithms used to relate peak area of features recorded in HRMS analyses, chromatographic and mass spectrometric conditions to concentrations, might overcome the need for reference standards to obtain an (indicative) information about analyte concentrations in measured samples [135]. Qualitative information about the presence or absence of given NPS in wastewater is informative and studies have shown some spatial and temporal trends [23,27,136], but only quantitative data can provide absolute comparisons by showing changes in community prevalence through concentrations or mass loads.

v. ***Non-target screening*** remains predominantly unexplored for the identification of NPS in pooled urine or wastewater. A genuine non-target screening without any selection of analytes to be searched is a very challenging and time consuming process and a more successful strategy would be the application of non-target screening directed towards the discovery of compounds structurally related to known NPS. In this case, the higher concentrations generally present in pooled urine makes this matrix most interesting for this approach. The expected improvements for the forthcoming years in the mass-resolving power of HRMS instruments in combination with higher scan-speed will allow the acquisition at higher mass resolution with more efficient chromatography. This development in instrumentation will improve sensitivity and can also be very useful to differentiate between isobaric compounds (i.e. compounds with the same nominal mass but different chemical formula and thus different exact mass). Moreover, improved mass resolving power does not only improve the separation of parent compounds, but can also help finding characteristic fragment ions and gain confidence in the obtain identification. Furthermore, improvements in software tools for peak picking and data deconvolution (*i.e.* the capability to find chromatographic peaks of compounds and to obtain high quality spectra) will aid to a successful identification of NPS, but the knowledge of basic rules in mass fragmentation and thus the expertise of the mass spectrometrists should not be overlooked in both suspect and non-target screening.

vi. The rapid transience of NPS in the drug market as well as the limited availability of reference standards for both NPS and known metabolites poses an analytical challenge for the full confirmation of substances detected. Therefore, the development and continuous updating of ***collaborative and public NPS mass spectral databases*** will smooth the identification process since contributors and users to those databases will have access to empirical information without the need of having the reference standards in their own laboratories. Hence, the number of false

positive identification (based on suspect and non-target screening) will be reduced since tentative identifications will be supported by empirical data from other researchers.

vii. As is the case with online databases, ***prediction tools*** ease the tentative identification of NPS. The development of metabolic, RT and CCS predictive models represent a turning point in the investigation of NPS. The continual development of more accurate and refined predictive models will make prediction tools even more powerful for the application of NPS consumption – particularly the complexity associated with structural similarities among NPS families. The small differences in the chemical backbone for most NPS classes and consequently similar physicochemical properties often make the current predictive tools less than ideal due to the analogous outcome obtained from the prediction.

viii. ***Retrospective analysis*** will continue to play an important role in uncovering trends in NPS consumption. HRMS analyses allow analysts to continually explore samples, without the time expense associate with re-extracting and re-analyzing samples. Reprocessing samples should be performed periodically, which can be a laborious task. Nevertheless, it is an interesting tool, as 'new' NPS and metabolites are found, standards become more available and predictive techniques become more commonplace, retrospective analyses can be performed to better reveal community use of NPS.

ix. ***Ion mobility separation*** coupled to HRMS has arisen as a useful technique and it is expected that it will gain in popularity. The cleaner and higher-quality mass spectra as well as the increased sensitivity of the instruments facilitates the identification process of NPS at low concentration levels and in complex wastewater or pooled urine samples. Future improvements will be related to the resolution of IMS instrument to enhance the separation of isobaric or isomeric substances that cannot be previously resolved by chromatography.

8. Conclusions

Comprehensive analytical strategies can be applied to investigate NPS in pooled urine and wastewater, from quantification of target biomarkers to the detection and (tentative) identification of new substances and metabolites. The investigation of NPS in pooled urine and wastewater is a subject of current interest because, integrated with additional epidemiological information, it can be a useful tool for a comprehensive assessment of NPS use. In this context, data triangulation with traditional indicators, such as public surveys, online forums, data of drug testing services, police seizures and forensic analyses, is pivotal to gauge community consumption. Thus, the analysis of pooled urine and wastewater can complement other data and provide a more complete picture of community consumption.

Acknowledgements

The authors acknowledge EuSeME (project number 861602) funded by the European Union's Justice Programme - Drugs Policy Initiatives, and NPS-Euronet (HOME/2014/JDRUG/AG/DRUG/7086) funded with support from the European Commission. This communication reflects the views only of the authors, and the European Commission cannot be held responsible for any use that may be made of the information contained therein. Alberto Celma acknowledges the Spanish Ministry for Economy and Competitiveness for his pre-doctoral grant (BES-2016-076914). Richard Bade acknowledges the financial support of the Thyne Reid Foundation. The authors also acknowledge Juan Vicente Sancho of the Research Institute for Pesticides and Water for his help and fruitful discussions, Florenci Vicent González Adelantado and Lledo Bou-Iserte of the Department of Inorganic and Organic Chemistry, University Jaume I (Spain) for the synthesis of α-methyltryptamine.

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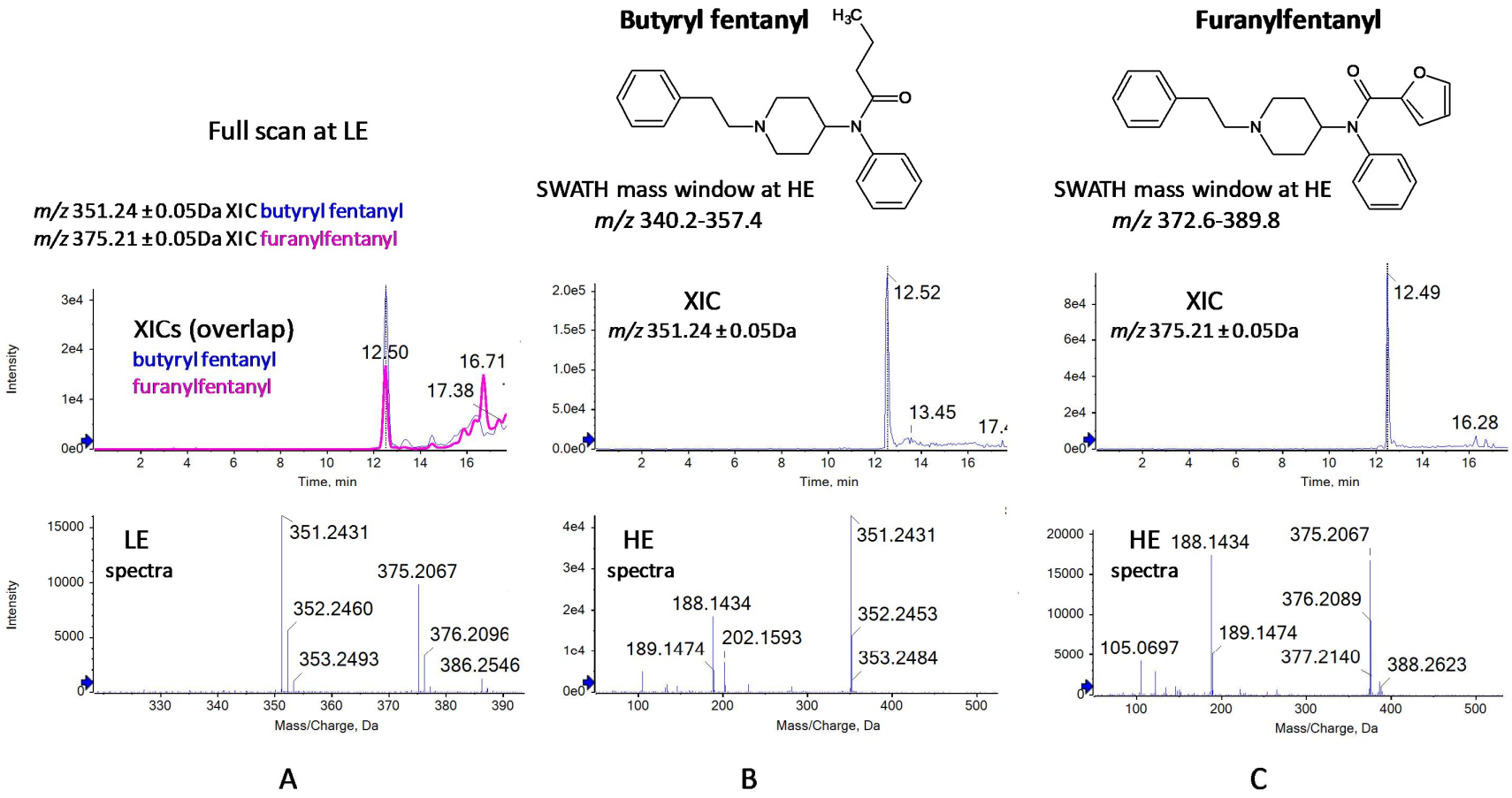
Figure captions

Figure 1: Identification of two co-eluting NPS, butyryl fentanyl (*m/z* 351.2431) and furanylfentanyl (*m/z* 375.2067) in a spiked wastewater sample using Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH). (A) overlapping extraction ion chromatograms (XICs) of the two NPS with chromatographic peaks eluting at 12.50 min (top); full scan acquisition mass spectra with low collision energy (LE) (10 V) at retention time 12.50 ± 0.10 min (bottom). (B) SWATH mass window *m/z* 340.2-357.4, XIC at *m/z* 351.24 (middle) and high collision energy (HE) mass spectra (bottom); (C) SWATH mass window *m/z* 372.6-389.8, XIC at *m/z* 375.21 (middle) and HE mass spectra (bottom).

Figure 2: Tentative identification of 4'-chloro-α-pyrrolidinopropiophenone (4-chloro-α-PPP) in a pooled urine sample. (A) Extracted ion chromatogram of 4-chloro-α-PPP and ^{37}Cl isotope (top); Product ion mass spectra of $[M+H]^+$ at *m/z* 238.10 (bottom). (B) Structure of 4-chloro-α-PPP (top); Zoom in the range of fragment ion with *m/z* 167 at resolution (R) of 35.000 Full Width at Half Maximum (FWHM) (bottom).

Figure 3: Identification of α-methyltryptamine in a raw wastewater sample using QTOF MS. (A) feature detection of *m/z* 175.1235 at 4.51 min (top, insert) together with the low collision energy (LE) spectra (top) and high collision energy (HE) spectra with emphasis on *m/z* 130-145 (grey areas) (bottom); (B) Structure, fragment ions, LE and HE spectra of α-methyltryptamine; (C) Structure, fragment ions, LE and HE spectra of 5-(2-aminopropyl)indole.

Figure 4: Identification of ketamine in a raw wastewater sample using IMS QTOF MS. (A) feature detection of *m/z* 238.0993 at 3.33 min and drift time (DT) 4.89 ms, yellow arrow (top) (*70 µs/scan); co-eluting ions at 3.33 min illustrated as red or black dots and separated by DT. Blue highlighted areas are the DT ranges of 4.89 ± 0.20 ms at *m/z* 238.0993 at low collision energy (LE) and high collision energy (HE) (bottom). (B) LE and HE mass spectra without IMS DT alignment (top); LE and HE mass spectra with IMS DT alignment (bottom).



1268

1269 **Figure 1**

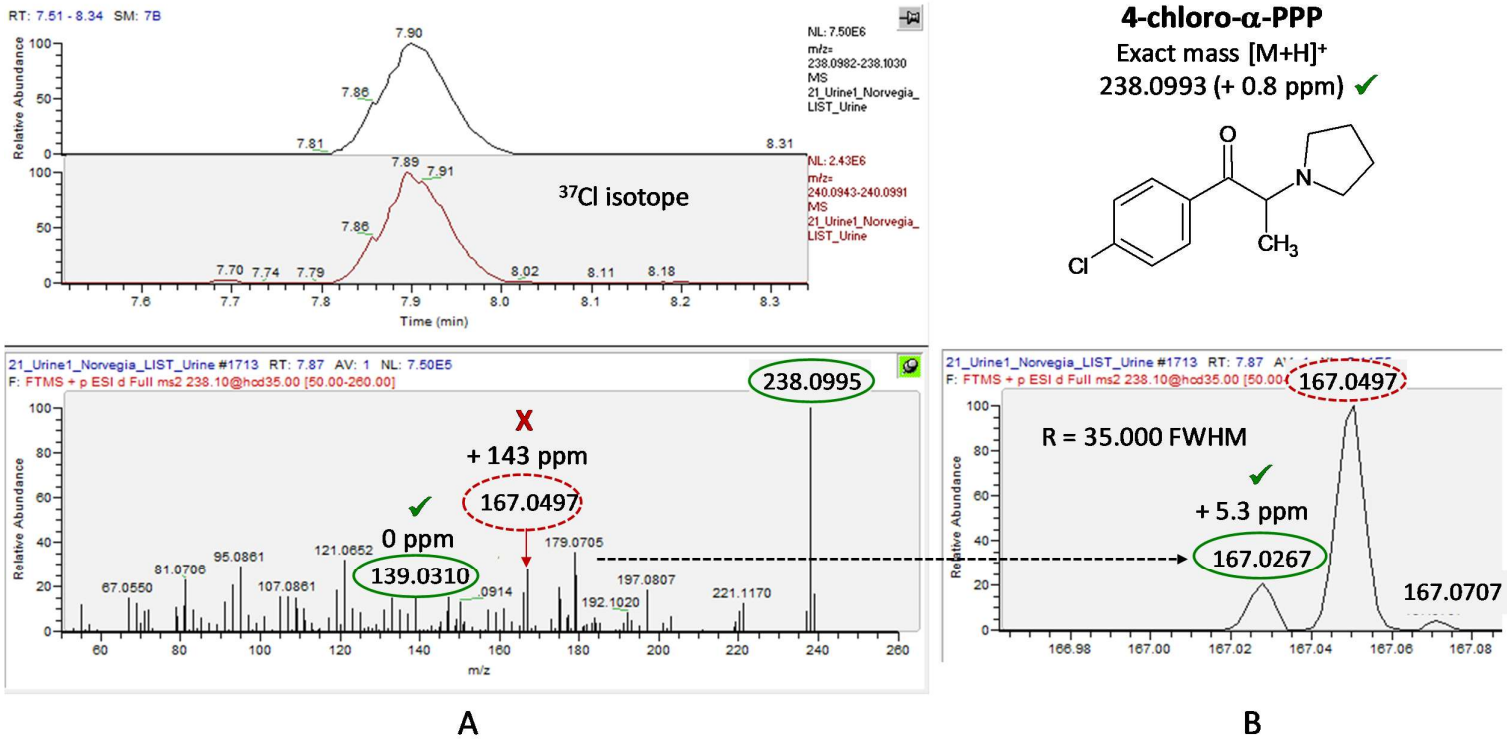
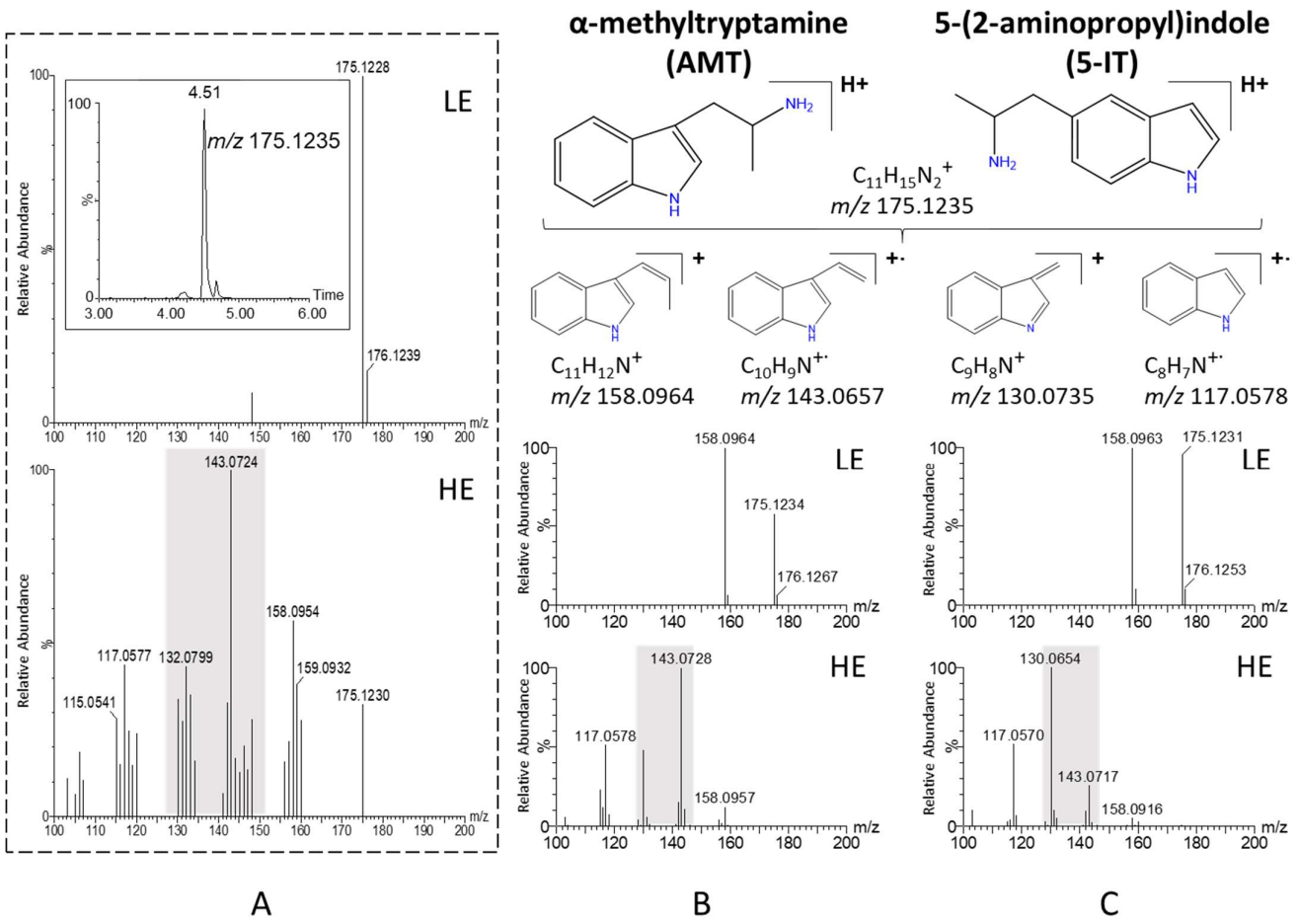

RT: 7.51 - 8.34 SM: 7B
Relative Abundance
Time (min)
7.90
7.86
7.81
8.31
NL: 7.50E6
m/z= 238.0982-238.1030 MS 21_Urine1_Norvegia_LIST_Urine
7.89
7.91
7.86
37Cl isotope
7.70 7.74 7.79
8.02
8.11
8.18
NL: 2.43E6
m/z= 240.0943-240.0991 MS 21_Urine1_Norvegia_LIST_Urine
4-chloro-α-PPP
Exact mass [M+H]+
238.0993 (+ 0.8 ppm)
O
N
Cl
CH3
21_Urine1_Norvegia_LIST_Urine #1713 RT: 7.87 AV: 1 NL: 7.50E5
F: FTMS + p ESI d Full ms2 238.10@hcd35.00 [50.00-260.00]
238.0995
X
+ 143 ppm
167.0497
0 ppm
139.0310
179.0705
121.0652
95.0861
81.0706
67.0550
107.0861
197.0807
192.1020
221.1170
m/z
21_Urine1_Norvegia_LIST_Urine #1713 RT: 7.87
F: FTMS + p ESI d Full ms2 238.10@hcd35.00 [50.00
167.0497
R = 35.000 FWHM
+ 5.3 ppm
167.0267
167.0707
A
B

Figure 2


α-methyltryptamine
(AMT)
5-(2-aminopropyl)indole
(5-IT)
H+
NH2
C11H15N2+
m/z 175.1235
C11H12N+
m/z 158.0964
C10H9N+·
m/z 143.0657
C9H8N+
m/z 130.0735
C8H7N+·
m/z 117.0578
LE
HE
Relative Abundance
m/z
4.51
m/z 175.1235
Time
175.1228
176.1239
143.0724
158.0954
117.0577
132.0799
159.0932
115.0541
175.1230
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175.1234
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158.0963
175.1231
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117.0570
143.0717
158.0916
A
B
C

Figure 3

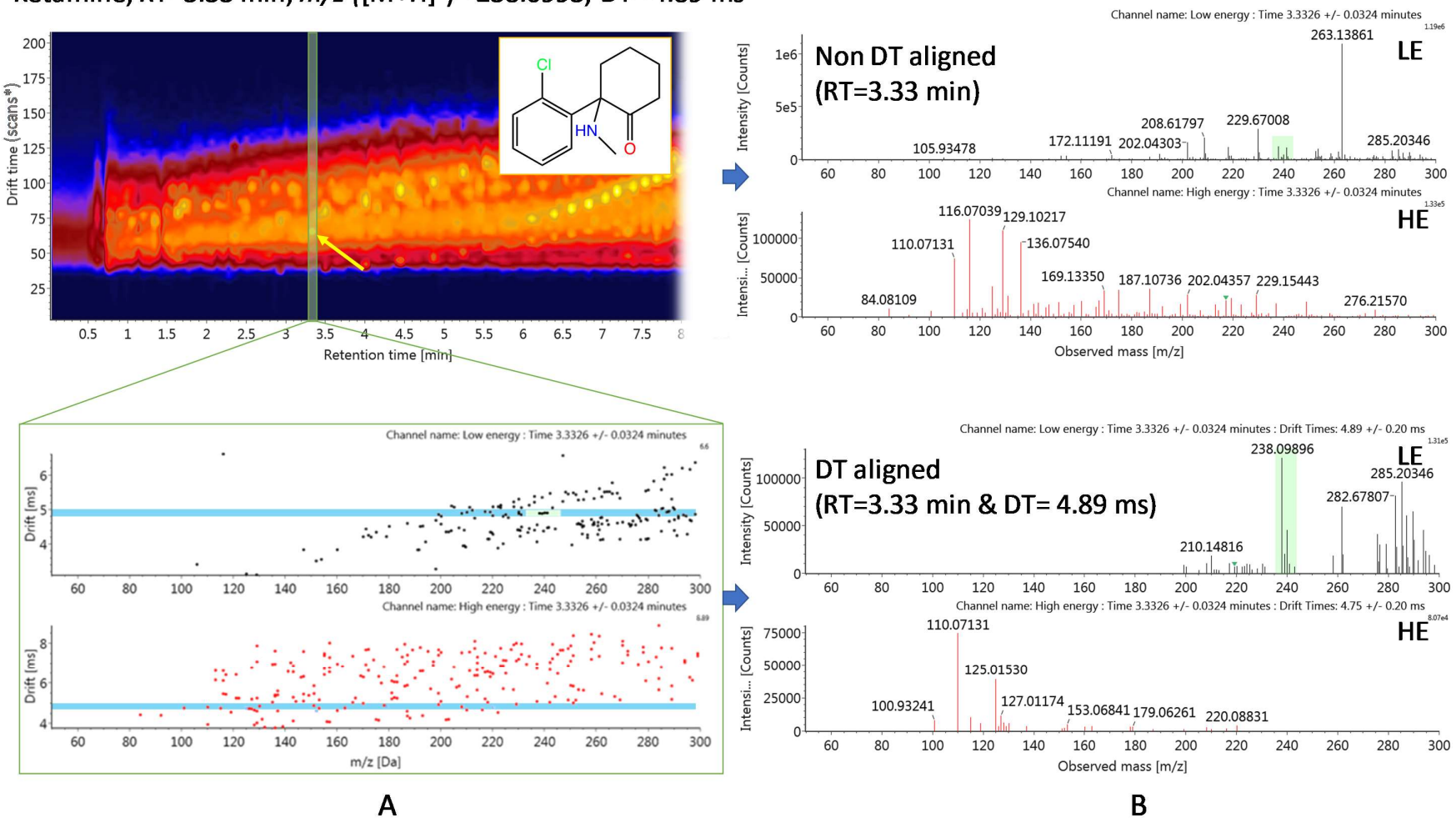
Ketamine, RT=3.33 min, m/z ([M+H]+)= 238.0993, DT= 4.89 ms
Drift time (scans*)
Retention time [min]
Cl
HN
O
Channel name: Low energy : Time 3.3326 +/- 0.0324 minutes
Channel name: High energy : Time 3.3326 +/- 0.0324 minutes
Drift [ms]
m/z [Da]
Non DT aligned
(RT=3.33 min)
LE
HE
Intensity [Counts]
Observed mass [m/z]
263.13861
208.61797
229.67008
105.93478
172.11191
202.04303
285.20346
116.07039
129.10217
110.07131
136.07540
169.13350
187.10736
202.04357
229.15443
84.08109
276.21570
Channel name: Low energy : Time 3.3326 +/- 0.0324 minutes : Drift Times: 4.89 +/- 0.20 ms
Channel name: High energy : Time 3.3326 +/- 0.0324 minutes : Drift Times: 4.75 +/- 0.20 ms
DT aligned
(RT=3.33 min & DT= 4.89 ms)
238.09896
285.20346
282.67807
210.14816
110.07131
125.01530
100.93241
127.01174
153.06841
179.06261
220.08831
A
B

Figure 4