A data-independent acquisition workflow for qualitative screening of new psychoactive substances in biological samples

A data-independent acquisition workflow for qualitative screening of new psychoactive substances in biological samples

Juliet Kinyua[1], Noelia Negreira[1], María Ibáñez[2], Lubertus Bijlsma[2], Félix Hernández[2], Adrian Covaci[1*], Alexander L.N. van Nuijs[1]

[1] Toxicological Center, Department of Pharmaceutical Sciences, Campus Drie Eiken, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium

[2] Research Institute for Pesticides and Water, University Jaume I, Avenida Sos Baynat s/n 12071, Castellón de la Plana, Spain

Corresponding author: Prof. Dr. Adrian Covaci

E-mail: adrian.covaci@uantwerpen.be; Fax: +32-3-265-2722

Abstract

Identification of new psychoactive substances (NPS) is challenging. Developing targeted methods for their analysis can be difficult and costly due to their impermanence on the drug scene. Accurate mass-mass spectrometry (AMMS) using a quadrupole time-of-flight (QTOF) analyzer can be useful for wide-scope screening since it provides sensitive, full-spectrum MS data.

Our article presents a qualitative screening workflow based on data-independent acquisition mode (All-ions MS/MS) on liquid chromatography (LC) coupled to QTOFMS for the detection and identification of NPS in biological matrices. The workflow combines and structures fundamentals of target and suspect screening data processing techniques in a structured algorithm. This allows the detection and tentative identification of NPS and their metabolites.

We have applied the workflow to two actual case studies involving drug intoxications where we detected and confirmed the parent compounds ketamine, 25B-NBOME, 25C-NBOMe and several predicted Phase I and II metabolites not previously reported in urine and serum samples. The screening workflow demonstrates the added value for the detection and identification of NPS in biological matrices.

Keywords: designer drugs, All-Ions MS/MS, LC-QTOFMS, 25B-NBOMe, suspect screening, qualitative screening

Introduction

Liquid chromatography (LC) coupled to accurate-mass mass spectrometry (AMMS) based on a quadrupole time-of-flight (QTOF) provides sensitive full-spectrum MS data for the identification of known and previously unknown compounds [1-3]. Several applications using LC-QTOFMS have been reported in the literature for the screening of many different families of compounds in biological samples [4-7].

While AMMS in full-acquisition mode provides meaningful information for the characterization of unknowns in complex samples (molecular formula, isotopic patterns, double bond equivalents (DBE)), MS/MS fragment ions are normally required for a tentative structural elucidation. In this regard, several approaches can be applied to conduct MS/MS experiments. The data-dependent acquisition mode presents an advantage as it allows getting accurate mass information on both the precursor ion and its product ions (MS and MS/MS) in a single injection. However, MS/MS information for the less abundant ions is very often lost, since acquisition of MS/MS is triggered by the detection of ions above a certain threshold. In addition, the maximum number of precursors selected per cycle and active exclusion for compounds overlapping or with close retention time can affect acquisition of MS/MS spectra. Therefore, additional injections in targeted MS/MS would be required. Novel acquisition modes are available in more recent high-resolution/accurate-mass (HRAM) instruments, which aim to increase the throughput of unknown identification.

In data-independent acquisition mode, all ions are fragmented without a specific isolation of a precursor ion in the first mass analyzer [8-10]. This mode is also known as MS^E, All Ions MS/MS or all-ion fragmentation (depending on the manufacturer). In a single injection, different collision energies can be applied, providing accurate fragmentation spectra for each 'precursor ion' [2,11]. This acquisition mode has been proven ideal for qualitative purposes and allows for retrospective analysis using the accurate mass full-acquisition and 'MS/MS'

information even years after data are acquired [12]. In addition, there exist a variety of sophisticated data processing approaches that utilize advanced software programmes, which can be applied depending on the goals of the research and tools available [8].

One data processing approach for the identification of compounds is 'Suspect screening' and has been described by some researchers [3,13-15]. It involves extraction of the exact masses (calculated based on molecular formula) of expected ions $[M+H]^+$ or $[M-H]^-$ from the acquired data [3]. It relies on the information of the molecular formula and structure for the tentative identification of compounds present in a sample and can be useful when no reference standards are available to confirm mass spectra and retention time information. However, in 'Target screening' - another data processing approach, a reference standard is required to match measured retention time and MS/MS spectrum [3,16].

The different advantages of these data processing approaches makes it very attractive to apply them for the detection and tentative identification of new psychoactive substances (NPS). NPS are an interesting group of compounds that mimic effects of illicit drugs like cocaine, cannabis and amphetamines but evade law enforcement by introducing slight modifications to chemical structures of controlled substances. NPS are easily acquired legally through online vendors and smart shops where they are sold under false labels with misleading information about their effects and safety. They are considered a growing problem in many communities and are responsible for numerous fatal intoxications [17,18]. There are currently around 450 NPS being monitored in the market [18], but not much is known about their actual use. The detection of NPS is challenging due to their rapid transience on the drug scene creating a scenario with constantly moving analytical targets. Furthermore, there is limited experimental data on their pharmacokinetics and biotransformation pathways [19]. It is therefore difficult to determine target NPS biomarkers for further analysis. In addition, the reference standards of NPS and their metabolites are

often costly and not always available. Taking this into account a suspect screening based on AMMS data would be a good approach to determine their occurrence prior to purchasing expensive reference standards for experimental analysis.

Several studies have used AMMS techniques to characterize NPS in various samples [13,20-22]. However, most studies have focused on target screening of NPS, with available reference standards. Furthermore, only one study at this point [13] has applied a suspect screening approach and detailed their sophisticated data processing techniques, showing their contribution to compound identification of NPS.

In this work, we combine and structure fundamentals of suspect and targeted screening data processing techniques. The aims were to i) develop a robust workflow for the analysis of NPS in biological samples using AMMS data, acquired through data-independent acquisition mode; ii) provide structured detail into how to process and handle the data acquired; iii) demonstrate the advantages and application of this workflow in the identification of co-eluting and isomeric compounds; and iv) discuss challenges and complications related to the workflow by demonstrating the feasibility of its application.

Materials and Methods

Chemical and reagents

Chemicals standards for cocaine (COC), benzoylecgonine (BE), ecgonine methyl esther (EME), amphetamine (AMP), methamphetamine (METH), 3,4-methylenedioxy-methamfetamine (MDMA), methadone (MTD), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 6-monoacetylmorphine (6-MAM), ketamine (KET), norketamine (NK), dehydronorketamine (DHNK), mephedrone, methylenedioxypyrovalerone (MDPV), methoxetamine (MXE), butylone, ethylone, methylone, methiopropamine (MPA),

4-methoxy-methamphetamine (PMMA), and 4-methoxyamphetamine (PMA) were obtained from LGC Standards SARL (Molsheim, France) and Cerilliant (Round Rock, Texas, USA) at the concentration of 1 mg/mL or 100 µg/mL in methanol or acetonitrile.

LC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by purifying demineralized water in an Elga LabWater Purelab Flex system (Veolia Water Solutions & Technologies Belgium, Tienen, Belgium). Formic acid (eluent additive for LC-MS, 98%) was obtained from Sigma-Aldrich (Steinheim, Germany). The internal reference standards ranitidine-D_6 and fluoxetine-D_5 used (with purity > 98%) were purchased from Cerilliant (Round Rock TX, USA) at concentrations of 1 mg/mL in methanol. Working solutions were prepared for concentrations ranging between 0.005 - 100 ng/µL in methanol.

Liquid Chromatography

The LC system consisted of an Agilent Infinity 1290 SL binary pump with an integrated two-channel solvent degasser, a thermostated Agilent 1290 HiP-ALS autosampler system (20 µL injection loop) and a 1290 Agilent TCC SL column compartment (Agilent Technologies, Santa Clara, USA). Chromatographic separation was achieved with a Phenomenex Biphenyl (100 mm x 2.1 mm, 2.6 µm) column fitted to a SecurityGuard ULTRA Holder for UHPLC columns (2.1 - 4.6 mm) and maintained at 32 ºC. Mobile phase composition consisted of water (A) and of 80:20 acetonitrile:water (B) both with 0.04% of formic acid, with the following gradient: 0 min, 2% B; 2 min, 2% B; 18 min, 40% B; 25 min, 90% B; 29 min, 90% B; 29.5 min, 2% B; 33 min, 2% B. The total run time including column equilibration was 33 min. The injection volume was optimized based on peak shape and set to 2 µL and the flow rate was 0.4 mL/min.

QTOFMS

The MS system consisted of an Agilent 6530 Accurate-Mass QTOF instrument (Agilent Technologies, Santa Clara, USA) operated with jet stream electrospray ion source (Dual AJS ESI source). The source parameters were as follows: gas temperature, 325 °C; gas flow, 8 L/min; nebulizer gas, 40 psi; sheath gas temperature, 325 °C; sheath gas flow, 11 L/min; capillary voltage, 3,500 V and the nozzle voltage, 0 V. The data-independent acquisition (All-ions MS/MS) was set-up to acquire three scan segments in MS mode alternating the collision energies: 0 eV, 15 eV, and 35 eV, respectively. With this acquisition mode, in only one injection data are acquired in scan segment one to display the 'precursor ion', and scan segment two and three to provide the product ions. The mass accuracy (within ± 2 ppm) of the QTOFMS was calibrated before each analysis using a reference solution for scanning up to *m/z* 1700. The scan range was set to acquire between *m/z* 50-1000 at a rate of 2.5 spectra/s for each scan segment, with a minimum of 12 data points per peak. For measurements, the MS was operated in 4 GHz High Resolution mode with a typical resolution of 9000-20000 full width at half maximum (FWHM) for the mass range *m/z* 118.0862-622.0289.

Analyses were performed in positive and negative ESI modes. Mass calibration of the QTOFMS system was controlled by constant infusion of a reference mass solution (provided by Agilent Technologies) into the source of the QTOFMS system during the analysis. The ions selected for recalibrating the mass axis, ensuring the accuracy of mass assignations throughout the chromatographic run were the protonated reference ions ($[M+H]^+$ =121.0509) and $[M+H]^+$ =922.0098) for the positive mode and the deprotonated reference ions ($[M-H]^-$ = 112.9856 and $[M-H]^-$ = 980.0164) for negative mode.

MassHunter software qualitative analysis (Version B.06.00) and the personal compound database and library manager (PCDL, Version Rev. B.04.01, Agilent Technologies, Santa Clara, USA) were used to develop the data processing workflow. The workflow combines

suspect screening [16] and target screening [13,16] techniques for verification and tentative identification of compounds.

Samples

To test the applicability of the workflow, a mix of 20 reference standards in methanol with concentrations ranging between 0.12 and 150 ng/mL (Table 1) was prepared and injected into the LC-QTOFMS system. We tested anonymized urine and serum samples from two different subjects collected at a first aid station during a Belgian dance festival in 2013. This study was approved by the Ethical Committee of the Ghent University Hospital (no. 2013-931) and the participants provided a written informed consent. Details on sample preparation are described in the supplementary information.

Data processing workflow

A- Pre-processing tips

An exclusion mass list was generated from the acetonitrile blank sample data file and used to subtract background noise from the chromatograms. In addition, we monitored consistency of our reference masses during a sample run by extracting ion chromatograms (EICs) for the reference masses in each chromatogram.

B- In-house library

PCDL software was used to build an in-house library comprised of molecular formulae, retention time (t_R) and MS/MS centroid spectra (if reference standards were available). The in-house target list was made up of NPS and classical drugs for which analytical reference standards were available. In addition, data of extracts containing metabolites derived from laboratory experimental sources (*in vitro* and *in vivo* metabolites of some NPS) were acquired on the LC-QTOFMS instrument and included as MS/MS centroid spectra.

The suspect list comprised of NPS from the following groups: cannabinoids, cathinones, phenylethylamines, piperazines, tryptamines, opioids, benzodiazepines, plant extracts and others (medicinal products, intermediates, precursors and common product ions of known compounds). Information was primarily obtained from existing literature on NPS (*in vitro* and *in vivo* studies), from organizations such as European Early Warning System (EWS), European Monitoring Center for Drugs and Drug Addiction (EMCDDA), United Nations Office on Drugs and Crime (UNODC) and TICTAC Communications Limited (London) and from some few *in silico* predictions of Phase I and Phase II metabolites. The *in silico* predictions were performed using Meteor Nexus program (Lhasa 145 limited, UK). In summary, the library consists of more than 1500 entries.

C- Structuring of identification parameters

A variety of data processing techniques can be used to extract information from accurate mass data. In this work, the identification and scoring system is divided into two parts, one for the precursor and another one for the product ion.

'Precursor ion' identification

The molecular formulae in the library are searched against the acquired data using a narrow formula matching window of ± 10 ppm. Additionally, it allowed a maximum of 10 possible matches per formula as well as the possibility to detect sodium, potassium and ammonium adducts in positive mode, plus formic acid adducts in negative mode. The overall match score for each candidate species was calculated by the software based on the *m/z*, molecular formula, and isotopic pattern match of > 75% (weights to be specified by the operator: mass-100%; isotope abundance-60%; isotope spacing-50%; t_R-100%).

Product ion identification

The inclusion of product ion confirmation is useful in both cases of target and suspect screening. As shown previously [13], the elution profile of precursor and product ions is significant in the identification of compounds. In this work, the co-elution profile of each candidate accounted for peak shape (symmetry), and the t_R difference between precursor ion and product ions.

The product ion identification criteria and specified product ion EIC's parameters were defined as: ± 20 ppm extraction window; S/N > 3; t_R window ± 0.1 min of precursor ion; overall co-elution score (precursor and product ion correlation) > 80%. To reduce the number of qualified product ions, the option to automatically generate formulae for product ions of the proposed candidate was included. Qualified product ions for which a possible molecular formulae could not be proposed were discarded.

For the generation of qualified product ions for target compounds (with MS/MS spectra and t_R in the library), the ten most abundant product ions in the library for the candidate compound are extracted as EICs in the acquired data file and overlaid with EIC of the precursor ion. Similarly, for suspect compounds (with only molecular formulae in the library) 20 EICs of the most abundant product ions from average spectra (15 eV and 35 eV) of the acquired data file are extracted and overlaid with EIC of the precursor ion.

In the present work, we used the five levels of identification and confirmation described by Schymanski et al. (2014) [16] to communicate the confidence of identification. Confirmation by injection of a reference standard for t_R, MS and MS/MS spectra were designated as level one while with level two a probable structure was proposed based on matching existing (library or literature) spectrum data or using non-reported diagnostic MS/MS product ion evidence. In the case of level three, a tentative candidate was proposed with a possible structure, however, the exact structure remained assumed. With a level four, identification of a molecular formula was assigned based on the spectral information however, there was

insufficient evidence to propose possible structure. Lastly, level five was designated to a specific measured accurate mass (*m/z*) of interest when there was insufficient information to assign a formula.

D- Qualified product ion elucidation

Structures of qualified product ions were elucidated manually using basic fragmentation rules and the software ChemDraw Ultra 14.0. At the end of this workflow a list of confirmed candidates at different confidence levels is shown.

Results and Discussion

Compound identification

The reference standard mixture was useful for confirming the identity of some targeted compounds contained in the library (Table 1). It was also useful in assessing the capabilities such as the resolving power of the LC-QTOFMS instrument. This aided in setting thresholds for algorithm parameters by monitoring % scores for identification of candidate compound, and the co-elution profiles of candidate compounds and their product ions. The ideal workflow algorithm scores were set to > 75% for parent compound and > 80% for product ion identification.

The proposed workflow (Fig 1) successfully identified and confirmed 90% of the compounds in the reference standard mixture (Table 1) within a ± 10 ppm mass tolerance and their qualified product ions within ± 20 ppm mass tolerance. For instance, methylone in the reference standard mixture had an overall workflow score of 89% which accounted for isotopic pattern score of 98% (data not shown), mass error 7.7 ppm, t_R of 6.7 min, and qualified product ions with co-elution scores > 90% (Table 1). Since an MS/MS spectrum of the candidate compound methylone was included in the library (target compound), the most

abundant product ions in the library spectra were extracted (EICs) in the data file and overlaid with the EIC of the precursor ion (Fig 2). The co-elution score for all four qualified product ions was assigned based on predefined criteria for product ions.

When the candidate compound had no MS/MS spectrum in the library (suspect compound), the 20 most abundant product ions (with S/N >3; within the specified t_R) in the combined spectra (collision energies of 15 eV and 35 eV) were extracted as EICs. They were overlaid with that of the precursor ion and evaluated based on abundance, peak shape (symmetry), peak width and t_R (Fig SI-1). A co-elution plot was generated for suspect compounds by plotting the normalized ratio of the product to precursor ion abundance over acquisition time (Fig SI-1). Following this structural elucidation of the qualified product ions was done to deliver a tentative identification and confirmation (Fig SI-1).

*Application to sample*s

Serum and urine samples from two different subjects were used to assess the applicability of the screening workflow. In these two cases, the screening workflow was applied leading to the identification and confirmation of ketamine (KET) and 25*X*-NBOMe and the tentative identification of several *in silico* predicted metabolites (Table 2). Ketamine was detected in the serum sample while 25B-NBOMe and 25C-NBOMe were detected in the urine sample.

Ketamine and metabolites

KET is closely related to phencyclidine and has been used for its therapeutic value in veterinary and human medicine as an anesthetic and analgesic [23, 24]. However, it has also been used as a new psychoactive substance since the 1960s [25,26] and several intoxication cases have been reported [25,27]. Some research groups have studied the *in vitro* metabolism

290 of KET [28,27] and identified two major metabolites: norketamine (NK) and
291 dehydronorketamine (DHNK). Additionally, these studies [28,27] found several isomers of
292 hydroxyketamine (HK) and hydroxynorketamine (HNK). However, the confirmation of these
293 metabolites was not done by AMMS. At this point, reference standards for the isomers of
294 HNK were not commercially available; however by including chemical formulae of
295 metabolites from the literature into our library we were able to tentatively identify six isomers
296 of HNK (Table 2) in one of the serum samples. The tentative identification of the three
297 detected isomers HNK 1, 2 and 3 (Fig SI-1 and Fig SI-2) was facilitated by the elucidation of
298 fragmentation pathways of the qualified product ions, by the possible isomeric structures
299 sourced from the literature [28,29] and by *in silico* predictions from Nexus software.
300 Additionally, HNK 4, 5 and 6 were detected based on their accurate mass. However, at very
301 low abundances and therefore their isotopic patterns and product ions could not be
302 determined, which resulted in a low overall score (Fig 3 and Table 2).
303 The product ions at *m/z* 125.0145 (Δm = -6.4 ppm) and 125.0157 (Δm = 3.2 ppm) were
304 labeled as qualified ions for KET and NK, respectively (Table 2). This fragment
305 corresponded to the methylbenzene with the chlorine atom ($[C_7H_6Cl]^+$). The presence of a
306 chlorine atom was confirmed by the existence of the characteristic chlorine isotopic pattern:
307 the abundance of the $[M+H+2]^+$ ion was about $1/3^{rd}$ of the $[M+H]^+$ ion. For DHNK (*m/z*
308 222.0676, Δm = -1.8 ppm), three product ions were qualified (Table 2; Fig SI-2). F1 at *m/z*
309 205.0413 (Δm = -1.0 ppm) corresponded to the loss of the amine group from the parent
310 compound. Subsequent loss of carbon monoxide moiety lead to F2 at *m/z* 177.0460 (Δm = -
311 3.4 ppm) and finally, the loss of a propenal group from the parent compound to F3 at *m/z*
312 170.0717 (Δm = -8.2 ppm) was observed.
313 Seven product ions (HNK 1, F1 to F7) confirmed the presence of HNK 1 (t_R 4.75 min) at *m/z*
314 240.0804 (Δm = 7.5 ppm) (Fig SI-1 and Table 2). F1 at *m/z* 195.0577 (Δm = 3.1 ppm)

corresponded to the loss of amine and carbon monoxide groups, leading to the formation of a double bond or to the formation of a ring. A subsequent loss of a water molecule resulted in F2 at *m/z* 177.0470 (Δm = 2.3 ppm) indicating that the hydroxyl group could not be positioned in the aromatic ring. Additional losses of an ethylene moiety and the chlorine atom yielded F3 (*m/z* 151.0306, Δm = -2.0 ppm) and F4 (*m/z* 142.0768, Δm = -6.3 ppm), respectively. F5 corresponded to the common fragment ion at *m/z* 125.0148 (Δm = -4.0 ppm) observed also for KET and NK. Loss of an ethylene group from F4 lead to F6 at *m/z* 116.0611 (Δm = -8.6 ppm) and successive cyclization to F7 at *m/z* 115.0536 (Δm = -5.2 ppm). Though the position of the hydroxyl group could not be elucidated, it could be in the cyclohexanone ring since the loss of water was observed in F2, discarding its possible position in the aromatic ring.

In the case of isomer 2 (HNK 2, t_R 5.83 min) at *m/z* 240.0793 (Δm = 2.9 ppm), the presence of F1 at *m/z* 107.0494 corresponding to the molecular formula $[C_7H_7O]^+$ (Δm = -1.9 ppm) suggests that the hydroxyl group is positioned in the aromatic ring (see Fig. SI.2).

HNK 3 (t_R 6.12 min) at *m/z* 240.0786 (Δm = 0.0 ppm) was qualified by three product ions (Table 2). F1 at *m/z* 223.0527 (Δm = 3.1 ppm) corresponded to the loss of ammonia. An additional loss of water results in F2 at *m/z* 205.0418 (Δm = 1.5 ppm). F3 at *m/z* 142.0769 (Δm = -5.6 ppm), also observed for HNK 1, resulted from F1 after losing water, the chlorine atom and a carbon monoxide moiety (SI-Fig 2). The position of the hydroxyl group might be in the cyclohexanone ring since the loss of water was observed in F1, discarding its possible position in the aromatic ring.

25X-NBOMe and metabolites

In the NPS market, phenethylamine derivatives account for about 23% of the total number of reported NPS between 2009 and 2012 [24]. The 25X-NBOMe series of NPS are classified under the '2C-substitutes' of phenylethylamines. This recently emerging group of compounds has been detected in several countries [30-32]. Fatal intoxications have been already attributed to the parent compounds [33,34]. However, no metabolite biomarkers have been identified through *in vitro* and *in vivo* studies, and no actual information exists on the best biomarkers in urine or serum. Furthermore, there are no commercially available reference standards for their metabolites.

Our method was able to confirm presence of 25B-NBOMe (*m/z* 380.0876, Δm = 5.3 ppm) and 25C-NBOMe (*m/z* 336.1360, Δm = -0.3 ppm) in the sample (Table 2). For 25B-NBOMe ($C_{18}H_{22}BrNO_3$), two product ions were qualified at *m/z* 121.0651 (F1) and 91.0545 (F2) corresponding to the methoxymethylbenzene ($[C_8H_9O]^+$, Δm = 2.5 ppm) and methylbenzene, the tropylium ion ($[C_7H_7]^+$, Δm = 3.3 ppm), respectively (Fig 4), yet both product ions are not very specific to 25B-NBOMe. The characteristic bromine isotopic pattern with the $[M+H+2]^+$ ion at the same abundance as the $[M+H]^+$ ion confirmed the existence of the parent compound in the sample. Regarding 25C-NBOMe, it presented the characteristic isotope cluster of chlorine and the product ion F1 at *m/z* 121.0635 (Δm = -10.7 ppm). In the end, both compounds were confirmed through the injection of reference standards. 25H-NBOMe at m/z 302.1778 (Δm = 8.9 ppm), structurally related to 25C-NBOMe and 25B-NBOMe but without any halogen substituent, could not be confirmed with any product ion, so its tentative identification was only based on its accurate mass, isotopic pattern and molecular formula in the spectrum of the precursor ion.

The *in silico* predictions added to our library were useful in the tentative identification of some 25B-NBOMe and 25C-NBOMe metabolites in a non-hydrolyzed urine sample (Table 2,

Fig SI. 3-4). For 25B-NBOMe, Phase I oxidative *O*-demethylated metabolite ($C_{17}H_{20}BrNO_3$, CYP 2b), Phase II *N/O*-glucuronidated metabolite ($C_{23}H_{29}BrNO_9$, GLU 2a) and *O*-sulphated metabolite ($C_{17}H_{20}BrNO_6S$, SUL 1) along with their product ions were identified (Table 2 and Fig 4). 25B-NBOMe (CYP 2b) ($C_{17}H_{20}BrNO_3$) at *m/z* 366.0710 (Δm = 3.0 ppm) differed only of 25B-NBOME by 14.018 u due to a methyl group. F1 at *m/z* 121.0648 (Δm = 0.0 ppm) confirmed its presence. The fragments at *m/z* 121.0709 (Δm = -19.8 ppm) and 121.0656 (Δm = 6.6 ppm) were also labelled as qualified product ions of the glucuronidated and sulphated conjugate, respectively.

Additionally, four extra metabolites were identified solely based on the *m/z*, molecular formulae and isotopic pattern thereby confirmed under level four [16] due to lack of qualified product ions (Table 2). These metabolites corresponded to another *O*-demethylation (CYP 2a), demethylation of both methoxy groups (CYP 1) and two extra glucuronidated conjugates (GLU 1 and GLU 3). The errors ranged from 0.4 to 8.1 ppm. Proposed structures for these predicted metabolites are shown in Fig SI.3.

For 25C-NBOMe, the *in silico* sulphated conjugate was identified at *m/z* 402.0809 (Δm = 9.0 ppm). In addition, the fragment at *m/z* 121.0651 (Δm = -1.7 ppm) was present. The *O*-demethylated metabolite ($C_{17}H_{20}ClNO_3$, CYP 1) at *m/z* 322.1234 (Δm = 9.3 ppm) was also identified but no fragments were qualified. Structures of the predicted metabolites are shown in Fig SI.4.

Common fragments' method

Since NPS are often produced by only small modifications of the chemical structures of controlled substances, many of them share structural moieties [35]. Thus, the search for common fragment ions can be performed on the data acquired to identify significant peaks. For instance the 25B-NBOMe and its metabolites (Fig 4) have two common fragments (*m/z*

121.0648 ($[C_8H_9O]^+$ and *m/z* 91.0542 ($[C_7H_7]^+$)). EICs generated for 121.0648 from the scan segments with collision energies at 15 eV and 35 eV (Fig SI.5) show additional peaks from those previously identified that were not included in our suspect list. The additional peaks could be significant and can be further analyzed using non-target strategies described by Schymanski et al. 2013 [36].

Common fragments' method can be particularly useful when data is acquired using data-independent acquisition mode since no information (MS and MS/MS) is lost. However, for phenylethylamine compounds the common fragment approach is challenging since there are many possible modifications to the basic amphetamine/ phenylethylamine structure [37] and thus fragments are not so specific and may generate many peaks.

Advantages and challenges of the data-independent acquisition workflow

Data-dependent acquisition generates MS/MS spectra for pre-selected compounds and may miss potential compounds of interest, particularly co-eluting compounds [10]. Since with a data-independent method no compounds are pre-selected, all compounds are subject to collision induced dissociation including co-eluting compounds. Furthermore, the workflow searches the entire library (suspect and target compounds) against the acquired data, which allows identification of co-eluting compounds like amphetamine and methiopropamine (Table 1). This also indicated that the QTOFMS instrument set to High-Resolution mode (4 GHz, 10000-15000 resolving power for lower masses) was sufficient to distinguish co-eluting analyte ions. Additionally, allowing for multiple matches per formula is useful in the case of identification of isomeric compounds at different t_R like in the case of ethylone and butylone (Table 1). Furthermore, it is possible to get the information on the co-elution of analytes without additional injections, which is a very difficult task in data-dependent acquisition.

One of the challenges associated with working in data-independent acquisition mode is the difficult association of product ions to a specific precursor ion. Since that precursor ion is not previously selected, other ions, even with very different *m/z*, co-elute and produce a mixed spectra. Also, the likelihood of false positives is increased mostly depending on isobaric interferences in the matrix. Another potential limitation would be the possibility of isomeric compounds co-eluting which would not be easily distinguishable. Lastly, one has to regularly update new compounds into the library to keep the suspect list current and avoid missing compounds.

Future perspectives

Detection of NPS is a challenge due to the high number of potential compounds to be investigated and their rapid transience in the drug scene. Furthermore, reference standards are often not available which makes target analysis impossible. Pooled urine analysis [38,39] and analysis of samples from hospital emergency intoxication cases would be useful for detecting the occurrence of NPS in order to prioritize the essential NPS for the purchase of reference standards. Detection of NPS and their metabolites particularly with a workflow such as the one showed in this work would contribute to the identification of possible biomarkers that can be applied in the monitoring of community health. Since some substances [40] are extensively metabolized, targeting the parent compound may be redundant. In such cases, and considering that not much experimental data exist on the biotransformation of NPS, this workflow would be useful since it includes screening for not only the parent compounds but also potential biomarkers like predicted metabolites. Furthermore, other fields might benefit from having biomarker information- such as sewage-based epidemiology [41-44].

Conclusions

In this article we demonstrate the application of data-independent acquisition mode in qualitative screening for NPS. The proposed workflow combines two approaches of data processing proposed in the literature. Furthermore, we detail the handling of the acquired data stressing the importance of precursor and product ion correlation in the tentative identification of a suspect compound. Furthermore, the applicability of the workflow in distinguishing co-eluting compounds and isomers is demonstrated. The potential use of 'common fragments' approach' is outlined and the difficulties in identification of amphetamine-like compounds are emphasized. The application and significance of *in silico* predicted metabolites is shown, which can be especially useful for NPS detection particularly when *in vitro* and *in vivo* studies have not been performed.

Acknowledgments

We would like to thank the staff at Toxicological Center (UA), Philanthropic Educational Organization (PEO) and American Association of University Women (AAUW) for their support. We would like to thank Dr. Kristof Maudens for providing the samples used in the application of the workflow. Financial support: EU International Training Network SEWPROF (Marie Curie- Grant number 317205). Dr. Alexander van Nuijs and Dr. Noelia Negreira acknowledge the Flanders Foundation for Research (FWO) and University of Antwerp for their post-doctoral fellowships. The University Jaume I acknowledge the financial support from Generalitat Valenciana (Group of Excellence Prometeo II 2014/023); ISIC 2012/16 and from the Spanish Ministry of Economy and Competitiveness (Project Ref CTQ2012-36189).

Conflict of Interest

The authors have no conflicts of interest to declare.

Supplementary information is available

The supplementary information contains five figures.

References

1. Roman M, Ström L, Tell H, Josefsson M (2013) Liquid chromatography/time-of-flight mass spectrometry analysis of postmortem blood samples for targeted toxicological screening. Analytical and Bioanalytical Chemistry 405 (12):4107-4125
2. Hernández F, Bijlsma L, Sancho JV, Díaz R, Ibáñez M (2011) Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography–quadrupole-time-of-flight-mass spectrometry. Analytica Chimica Acta 684 (1–2):96-106
3. Krauss M, Singer H, Hollender J (2010) LC–high resolution MS in environmental analysis: from target screening to the identification of unknowns. Analytical and Bioanalytical Chemistry 397 (3):943-951
4. Gergov M, Boucher B, Ojanperä I, Vuori E (2001) Toxicological screening of urine for drugs by liquid chromatography/time-of-flight mass spectrometry with automated target library search based on elemental formulas. Rapid Communications in Mass Spectrometry 15 (8):521-526
5. Pelander A, Ojanperä I, Laks S, Rasanen I, Vuori E (2003) Toxicological screening with formula-based metabolite identification by liquid chromatography/time-of-flight mass spectrometry. Analytical chemistry 75 (21):5710-5718
6. Georgakopoulos CG, Vonaparti A, Stamou M, Kiousi P, Lyris E, Angelis YS, Tsoupras G, Wuest B, Nielen MWF, Panderi I (2007) Preventive doping control analysis: liquid and gas chromatography time-of-flight mass spectrometry for detection of designer steroids. Rapid communications in mass spectrometry 21 (15):2439-2446
7. Diaz R, Ibanez M, Sancho JV, Hernandez F (2012) Target and non-target screening strategies for organic contaminants, residues and illicit substances in food, environmental and human biological samples by UHPLC-QTOF-MS. Analytical Methods 4 (1):196-209
8. Roemmelt AT, Steuer AE, Poetzsch M, Kraemer T (2014) Liquid Chromatography, in Combination with a Quadrupole Time-of-Flight Instrument (LC QTOF), with Sequential Window Acquisition of All Theoretical Fragment-Ion Spectra (SWATH) Acquisition: Systematic Studies on Its Use for Screenings in Clinical and Forensic Toxicology and Comparison with Information-Dependent Acquisition (IDA). Analytical Chemistry 86 (23):11742-11749

9. Bern M, Finney G, Hoopmann MR, Merrihew G, Toth MJ, MacCoss MJ (2009) Deconvolution of Mixture Spectra from Ion-Trap Data-Independent-Acquisition Tandem Mass Spectrometry. Analytical Chemistry 82 (3):833-841
10. Wrona M, Mauriala T, Bateman KP, Mortishire-Smith RJ, O'Connor D (2005) 'All-in-One' analysis for metabolite identification using liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry with collision energy switching. Rapid Communications in Mass Spectrometry 19 (18):2597-2602
11. Geromanos SJ, Vissers JPC, Silva JC, Dorschel CA, Li G-Z, Gorenstein MV, Bateman RH, Langridge JI (2009) The detection, correlation, and comparison of peptide precursor and product ions from data independent LC-MS with data dependant LC-MS/MS. PROTEOMICS 9 (6):1683-1695
12. Hernández F, Ibáñez M, Portolés T, Cervera MI, Sancho JV, López FJ (2015) Advancing towards universal screening for organic pollutants in waters. Journal of Hazardous Materials 282 (0):86-95
13. Ibáñez M, Sancho JV, Bijlsma L, van Nuijs ALN, Covaci A, Hernández F (2014) Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. TrAC Trends in Analytical Chemistry 57 (0):107-117
14. Bletsou AA, Jeon J, Hollender J, Archontaki E, Thomaidis NS (2015) Targeted and non-targeted liquid chromatography-mass spectrometric workflows for identification of transformation products of emerging pollutants in the aquatic environment. TrAC Trends in Analytical Chemistry 66:32-44
15. Schymanski EL, Singer HP, Longrée P, Loos M, Ruff M, Stravs MA, Ripollés Vidal C, Hollender J (2013) Strategies to Characterize Polar Organic Contamination in Wastewater: Exploring the Capability of High Resolution Mass Spectrometry. Environmental Science & Technology 48 (3):1811-1818
16. Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, Hollender J (2014) Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology 48 (4):2097-2098
17. Walterscheid JP, Phillips GT, Lopez AE, Gonsoulin ML, Chen H-H, Sanchez LA (2014) Pathological Findings in 2 Cases of Fatal 25I-NBOMe Toxicity. The American Journal of Forensic Medicine and Pathology 35 (1):20-25
18. EMCDDA (2015) New psychoactive substances in Europe. An update from the EU Early Warning System (March 2015). http://www.emcdda.europa.eu/publications/2015/new-psychoactive-substances. Accessed 12 April 2015
19. Reid MJ, Baz-Lomba JA, Ryu Y, Thomas KV (2014) Using biomarkers in wastewater to monitor community drug use: A conceptual approach for dealing with new psychoactive substances. Science of The Total Environment 487 (0):651-658
20. Bijlsma L, Sancho JV, Hernández F, Niessen WMA (2011) Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MSE accurate-mass spectra. Journal of Mass Spectrometry 46 (9):865-875

21. Zuba D (2012) Identification of cathinones and other active components of ‘legal highs’ by mass spectrometric methods. TrAC Trends in Analytical Chemistry 32 (0):15-30.
22. Fornal E (2014) Study of collision-induced dissociation of electrospray-generated protonated cathinones. Drug Testing and Analysis 6 (7-8):705-715
23. EMCDDA (2002) Report on the risk assessment of ketamine in the framework of the joint action on new synthetic drugs. European Monitoring Center for Drugs and Drug Addiction, Lisbon, Portugal. http://www.emcdda.europa.eu/html.cfm/index33341EN.html. Accessed 12 April 2015
24. UNODC (2014) Global Synthetic Drugs Assessment. (United Nations publication, Sales No. E.14.XI.6). https://www.unodc.org/documents/scientific/2014_Global_Synthetic_Drugs_Assessment_web.pdf. Accessed 12 April 2015
25. Siegel RK (1978) Phencyclidine and Ketamine Intoxication: A Study of Four Populations of Recreational Users. In. NIDA Research Monograph, vol 21. pp 119-147
26. Morgan CJA, Curran HV, the Independent Scientific Committee on D (2012) Ketamine use: a review. Addiction 107 (1):27-38
27. Weiner AL, Vieira L, McKay Jr CA, Bayer MJ (2000) Ketamine abusers presenting to the Emergency Department: A case series. The Journal of Emergency Medicine 18 (4):447-451.
28. Turfus SC, Parkin MC, Cowan DA, Halket JM, Smith NW, Braithwaite RA, Elliot SP, Steventon GB, Kicman AT (2009) Use of Human Microsomes and Deuterated Substrates: An Alternative Approach for the Identification of Novel Metabolites of Ketamine by Mass Spectrometry. Drug Metabolism and Disposition 37 (8):1769-1778
29. Adams JD, Baillie TA, Trevor AJ, Castagnoli N (1981) Studies on the biotransformation of ketamine 1-Identification of metabolites produced in vitro from rat liver microsomal preparations. Biological Mass Spectrometry 8 (11):527-538
30. Papoutsis I, Nikolaou P, Stefanidou M, Spiliopoulou C, Athanaselis S (2015) 25B-NBOMe and its precursor 2C-B: Modern trends and hidden dangers. Forensic Toxicol 33 (1):1-11
31. Zuba D, Sekuła K, Buczek A (2013) 25C-NBOMe – New potent hallucinogenic substance identified on the drug market. Forensic Science International 227 (1–3):7-14
32. Stellpflug S, Kealey S, Hegarty C, Janis G (2014) 2-(4-Iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe): Clinical Case with Unique Confirmatory Testing. J Med Toxicol 10 (1):45-50
33. Poklis JL, Nanco CR, Troendle MM, Wolf CE, Poklis A (2014) Determination of 4-bromo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25B-NBOMe) in serum and urine by high performance liquid chromatography with tandem mass spectrometry in a case of severe intoxication. Drug Testing and Analysis 6 (7-8):764-769

585 34. Tang MHY, Ching CK, Tsui MSH, Chu FKC, Mak TWL (2014) Two cases of severe
586 intoxication associated with analytically confirmed use of the novel psychoactive
587 substances 25B-NBOMe and 25C-NBOMe. Clinical Toxicology 52 (5):561-565
588 35. Grabenauer M, Krol WL, Wiley JL, Thomas BF (2012) Analysis of Synthetic
589 Cannabinoids Using High-Resolution Mass Spectrometry and Mass Defect Filtering:
590 Implications for Nontargeted Screening of Designer Drugs. Analytical Chemistry 84
591 (13):5574-5581
592 36. Schymanski EL, Gerlich M, Ruttkies C, Neumann S (2014) Solving CASMI 2013
593 with MetFrag, MetFusion and MOLGEN-MS/MS. Mass Spectrometry 3
594 (Special_Issue_2):S0036-S0036
595 37. Logan BK (2001) Amphetamines: An Update on Forensic Issues. Journal of
596 Analytical Toxicology 25 (5):400-404
597 38. Archer JRH, Dargan PI, Hudson S, Wood DM (2013) Analysis of anonymous pooled
598 urine from portable urinals in central London confirms the significant use of novel
599 psychoactive substances. QJM 106 (2):147-152
600 39. Archer JRH, Dargan PI, Hudson S, Davies S, Puchnarewicz M, Kicman AT, Ramsey
601 J, Measham F, Wood M, Johnston A, Wood DM (2013) Taking the Pissoir – a novel
602 and reliable way of knowing what drugs are being used in nightclubs. Journal of
603 Substance Use:1-5
604 40. Erratico C, Negreira N, Norouzizadeh H, Covaci A, Neels H, Maudens K, van Nuijs
605 ALN (2015) In vitro and in vivo human metabolism of the synthetic cannabinoid AB-
606 CHMINACA. Drug Testing and Analysis. Doi:10.1002/dta.1796
607 41. Reid MJ, Derry L, Thomas KV (2014) Analysis of new classes of recreational drugs
608 in sewage: Synthetic cannabinoids and amphetamine-like substances. Drug Testing
609 and Analysis 6 (1-2):72-79
610 42. van Nuijs ALN, Gheorghe A, Jorens PG, Maudens K, Neels H, Covaci A (2014)
611 Optimization, validation, and the application of liquid chromatography-tandem mass
612 spectrometry for the analysis of new drugs of abuse in wastewater. Drug Testing and
613 Analysis 6 (7-8):861-867
614 43. Kankaanpää A, Ariniemi K, Heinonen M, Kuoppasalmi K, Gunnar T (2014) Use of
615 illicit stimulant drugs in Finland: A wastewater study in ten major cities. Science of
616 The Total Environment 487 (0):696-702
617 44. Kinyua J, Covaci A, Maho W, McCall A-K, Neels H, van Nuijs A (2015) Sewage-
618 based epidemiology in monitoring the use of new psychoactive substances: Validation
619 and application of an analytical method using LC-MS/MS. Drug testing and analysis.
620 Doi:10.1002/dta.1777

621

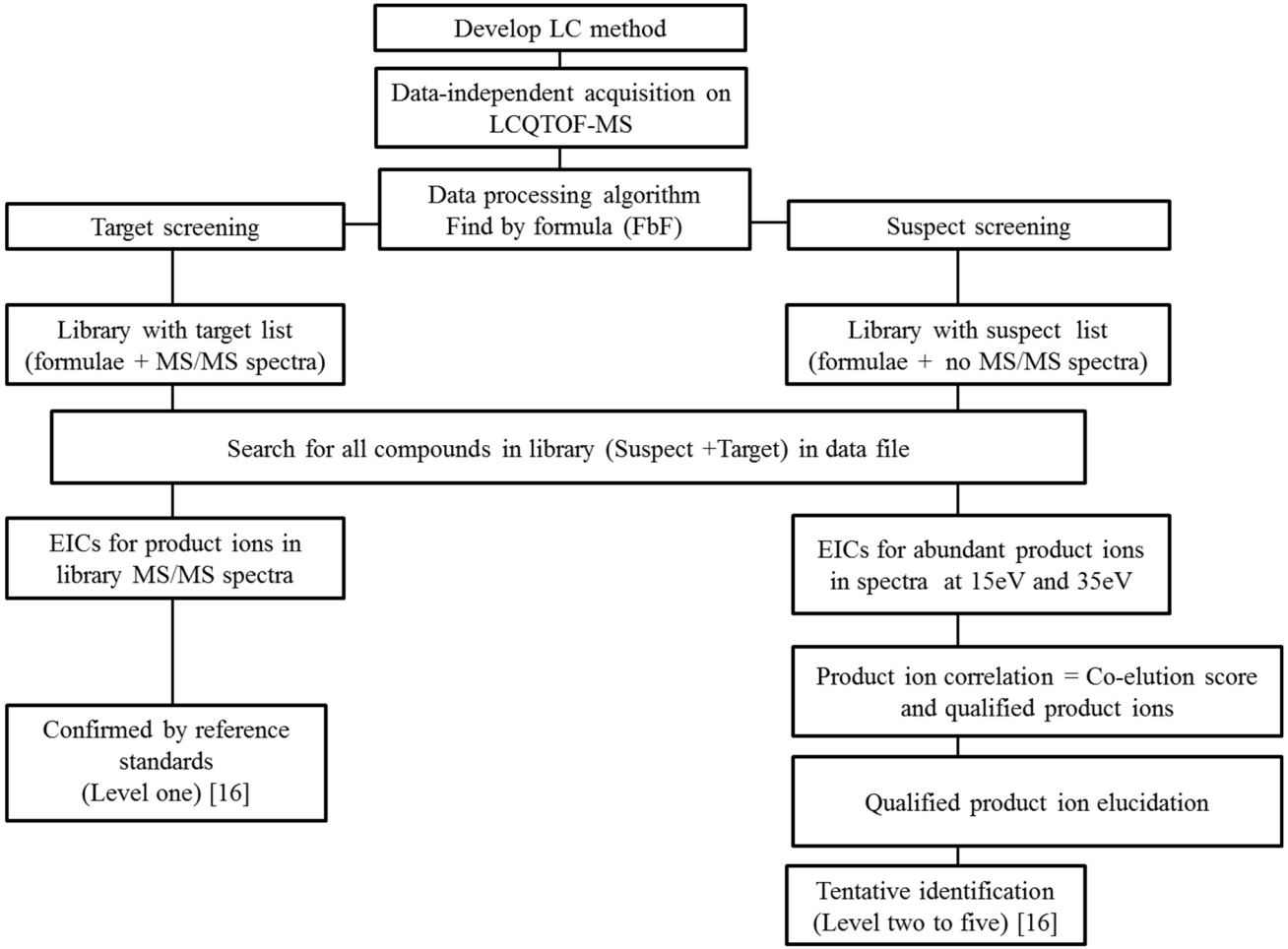

Fig. 1 Illustration of workflow components. FbF- Find By molecular Feature.

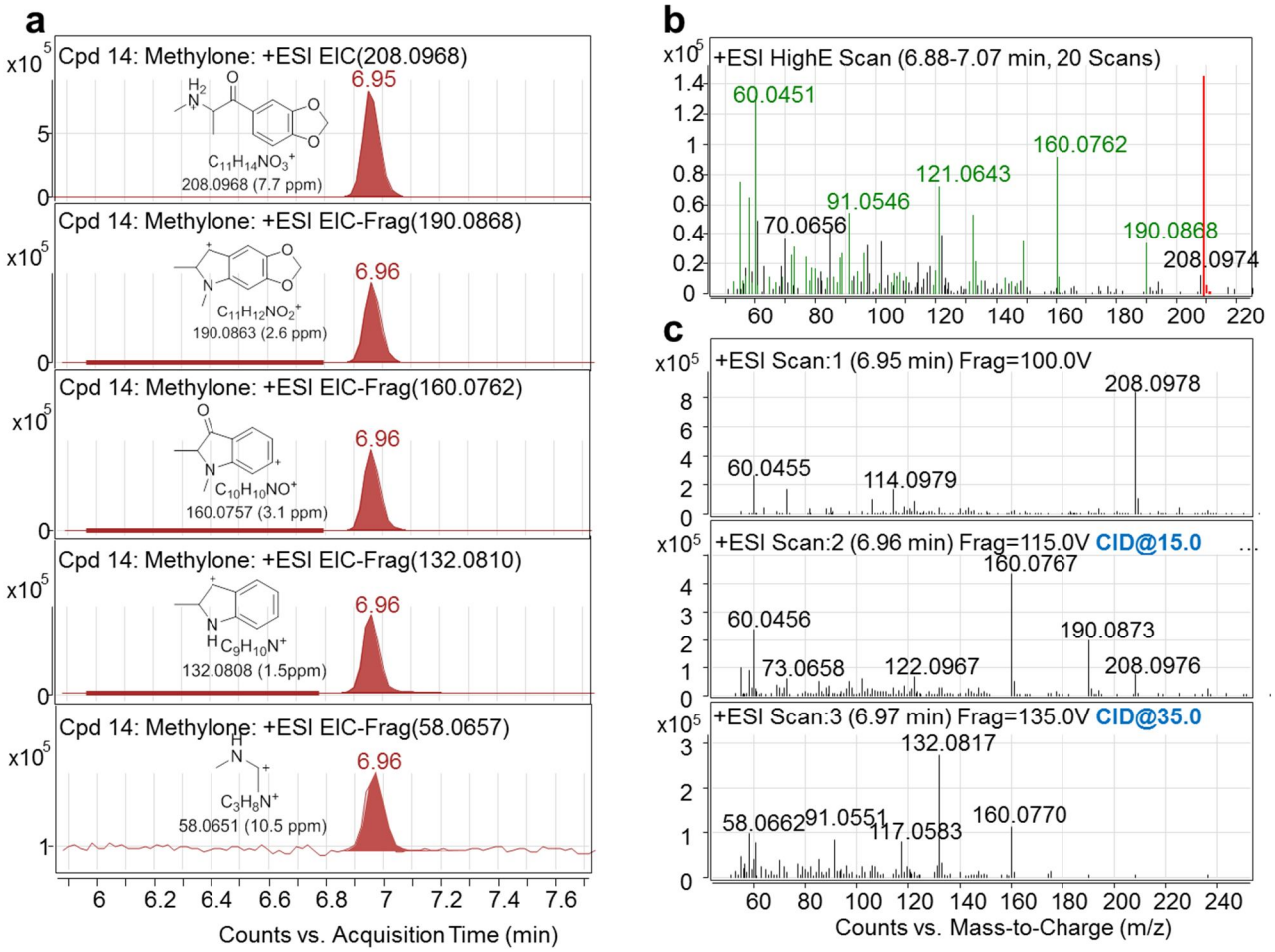

Fig. 2 Identification of methylone in the reference standard mixture. A) EICs for product ions (*m/z* 190, 160, 132 and 58) in the library spectrum of methylone are extracted from acquired data and overlaid with candidate precursor ion EIC. B) Combined spectra for three scan segments (0, 15 and 35 eV) at t_R 6.88-7.07 min. C) Spectra of the three scan segments at t_R 6.97 min.

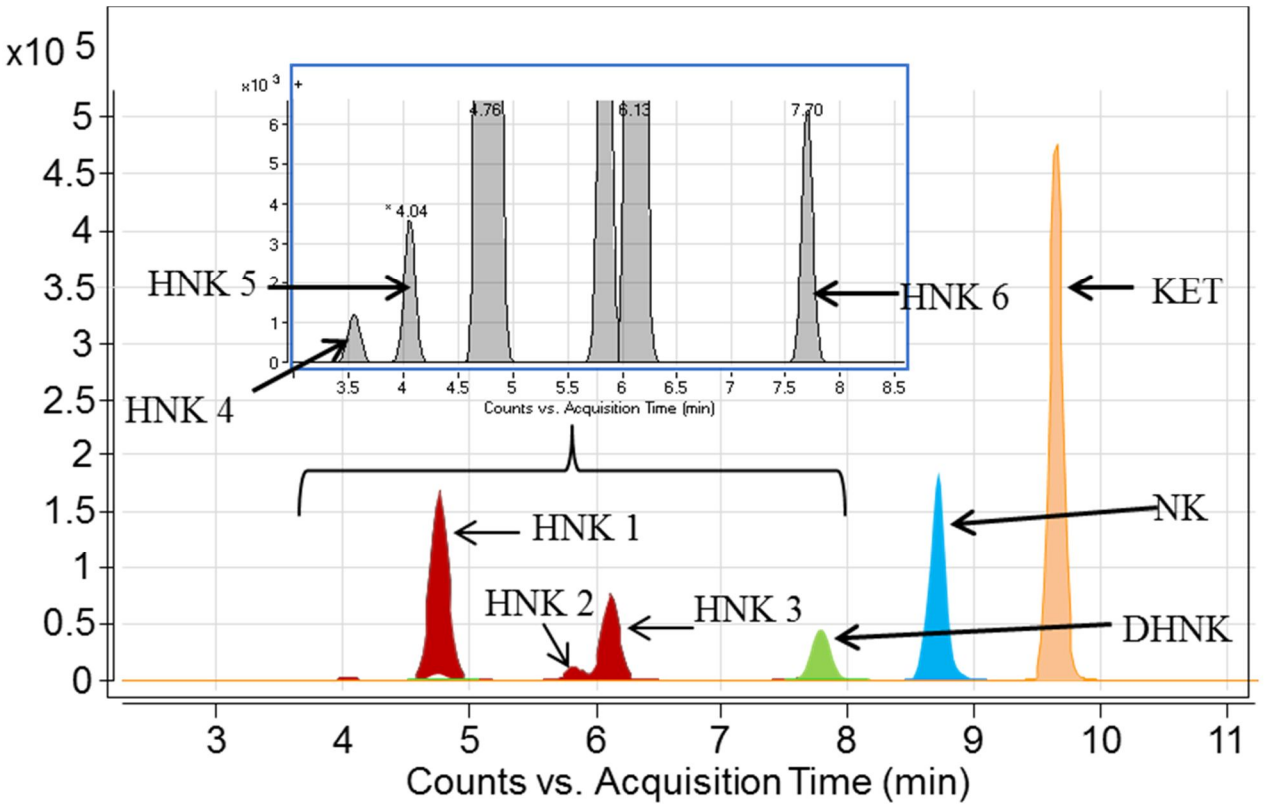

Fig. 3 Chromatogram showing ketamine and its metabolites detected in serum. Six isomers of HNK were detected of which HNK 4, 5 and 6 had very low abundance and were identified solely based on their accurate mass.

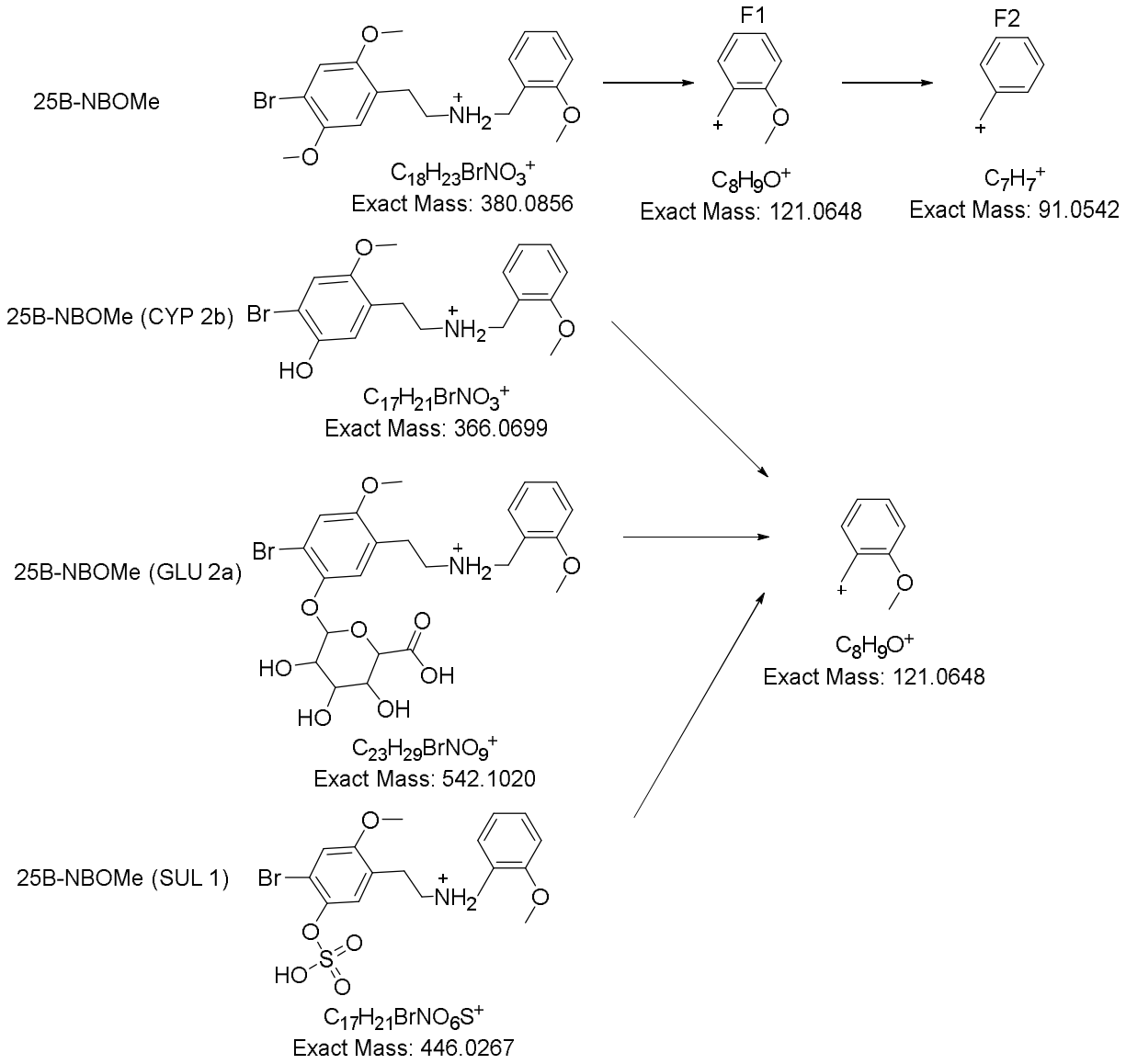

Fig. 4 Proposed fragmentation pathway for 25B-NBOMe and its metabolites detected in a patient's urine sample.

Table 1 Reference standard mix used to set parameter thresholds for the FbF (Find By molecular Feature) algorithm. The workflow scores range from 70%- 99% for precursor and product ions. [a] Retention time (min); [b] *m/z* measurement error; [c] collision energy

Compound	[a] t_R	Ion formula	Score	Measured *m/z* $[M+H]^+$	[b] Δm (ppm)	[c] CE
EME	1.13	$[C_{10}H_{18}NO_3]^+$	99.4	200.1281	0.0	
EME F1	1.13	$[C_{10}H_{16}NO_2]^+$	98.8	182.1187	6.0	15
EME F2	1.13	$[C_5H_8N]^+$	96.8	82.0653	2.4	35
AMP	5.57	$[C_9H_{14}N]^+$	93.6	136.1125	2.9	
AMP F1	5.58	$[C_7H_7]^+$	99.6	91.0546	4.4	35
AMP F2	5.58	$[C_3H_8N]^+$	95.9	58.0657	10.3	35
MPA	5.57	$[C_8H_{14}NS]^+$	97.1	156.0847	3.8	
MPA F1	5.55	$[C_5H_5S]^+$	99.5	97.0112	6.2	35
MPA F2	5.58	$[C_7H_7]^+$	94.6	91.0547	5.5	35
MPA F3	5.58	$[C_3H_8N]^+$	97.3	58.0662	18.9	35
MPA F4	5.55	$[C_4H_5]^+$	98.3	53.0395	17.0	35
METH	6.81	$[C_{10}H_{16}N]^+$	95.9	150.1274	-2.0	
METH F1	6.82	$[C_7H_7]^+$	99.7	91.0550	8.8	35
METH F2	6.80	$[C_5H_5]^+$	97.4	65.0394	12.3	35
Methylone	6.95	$[C_{11}H_{14}NO_3]^+$	89.4	208.0984	7.7	
Methylone F1	6.96	$[C_{11}H_{12}NO_2]^+$	98.2	190.0868	2.6	15
Methylone F2	6.96	$[C_{10}H_{10}NO]^+$	99.1	160.0762	3.1	15
Methylone F3	6.96	$[C_9H_{10}N]^+$	99.2	132.0810	1.5	35
Methylone F4	6.96	$[C_3H_8N]^+$	98.4	58.0657	10.3	35
PMA	7.03	$[C_{10}H_{16}NO]^+$	79.4	166.1219	-4.2	
PMA F1	7.04	$[C_{10}H_{13}O]^+$	98.4	149.0965	2.7	15
PMA F2	7.04	$[C_8H_9O]^+$	98.3	121.0654	5.0	15
PMA F3	7.02	$[C_7H_7]^+$	72.8	91.0548	6.6	35
DHNK	7.79	$[C_{12}H_{13}ClNO]^+$	97.9	222.0676	-1.8	
DHNK F1	7.78	$[C_{12}H_{10}ClO]^+$	98.9	205.0413	-1.0	15
DHNK F2	7.78	$[C_{11}H_{10}Cl]^+$	96.4	177.0460	-3.4	15
DHNK F3	7.78	$[C_9H_{13}ClN]^+$	98.4	170.0717	-8.2	35
6-MAM	7.70	$[C_{19}H_{22}NO_4]^+$	76.4	328.156	5.2	

6-MAM F1	7.70	$[C_{11}H_{12}NO]^+$	83.4	174.1234	-2.9	15
MDMA	7.89	$[C_{11}H_{16}NO_2]^+$	81.5	194.1193	8.8	
MDMA F1	7.90	$[C_{10}H_{11}O_2]^+$	98.9	163.0760	3.7	15
Ethylone	7.83	$[C_{12}H_{16}NO_3]^+$	87.8	222.1165	18.0	
Ethylone F1	7.82	$[C_{12}H_{14}NO_2]^+$	98.3	204.1041	10.8	15
Ethylone F2	7.82	$[C_{11}H_{12}NO]^+$	99.2	174.0927	8.0	15
PMMA	8.05	$[C_{11}H_{18}NO]^+$	84.8	180.1383	0.0	
PMMA F1	8.06	$[C_{10}H_{13}O]^+$	99.1	149.0971	6.7	15
PMMA F2	8.06	$[C_8H_9O]^+$	99.5	121.0657	7.4	15
PMMA F3	8.04	$[C_7H_7]^+$	92.8	91.0550	8.8	35
PMMA F4	8.04	$[C_6H_6]^+$	97.0	78.0475	14.1	35
PMMA F5	8.04	$[C_6H_5]^+$	95.1	77.0395	11.7	35
Mephedrone	8.26	$C_{11}H_{15}NO$	77.7	178.1241	8.4	
Mephedrone F1	8.27	$C_{11}H_{14}N$	99.0	160.1122	0.6	15
Mephedrone F2	8.27	$C_{10}H_{11}N$	99.8	145.0879	-4.8	15
Butylone	8.48	$[C_{12}H_{16}NO_3]^+$	97.5	222.1128	1.4	
Butylone F1	8.49	$[C_{11}H_{12}NO]^+$	99.3	174.0918	2.9	15
Butylone F2	8.47	$[C_7H_{14}O_3]^+$	98.9	146.0937	0.0	35
Butylone F3	8.49	$[C_6H_{13}O_3]^+$	95.5	133.0864	3.8	15
Butylone F4	8.49	$[C_4H_{10}N]^+$	99.7	72.0815	9.7	15
NK	8.84	$[C_{12}H_{15}ClNO]^+$	95.6	224.0839	0.9	
NK F1	8.83	$[C_7H_6Cl]^+$	98.9	125.0169	12.8	40
Benzoylecgonine	9.54	$[C_{16}H_{20}NO_4]^+$	81.2	290.1414	9.3	
Benzoylecgonine F1	9.53	$[C_9H_{14}NO_2]^+$	99.7	168.1024	3.0	15
Benzoylecgonine F2	9.53	$[C_7H_5O]^+$	99.2	105.0340	4.8	35
Benzoylecgonine F3	9.53	$[C_5H_8N]^+$	97.4	82.0671	24.4	35
Benzoylecgonine F4	9.53	$[C_6H_5]^+$	98.7	77.0401	19.5	35
KET	9.85	$[C_{13}H_{17}ClNO]^+$	85.0	238.1001	3.4	
KET F1	9.84	$[C_7H_6Cl]^+$	99.2	125.0155	1.6	35
MXE	10.97	$[C_{15}H_{22}NO_2]^+$	86.3	248.1666	8.5	
MXE F1	10.98	$[C_{13}H_{15}O_2]^+$	98.8	203.1073	3.0	15
MXE F2	10.98	$[C_8H_9O]^+$	98.5	121.066	9.9	35

MXE F3	10.98	$[C_7H_7]^+$	99.2	91.0552	11.0	35
MXE F4	10.98	$[C_7H_7]^+$	85.9	91.0530	-13.2	35
MDPV	12.34	$[C_{16}H_{22}NO_3]^+$	95.7	276.1605	4.0	
COC	12.69	$[C_{17}H_{22}NO_4]^+$	85.1	304.1584	13.5	
COC F1	12.70	$[C_{10}H_{16}NO_2]^+$	70.7	182.1178	1.1	15
COC F3	12.70	$[C_7H_5O]^+$	72.9	105.0340	4.8	35
COC F2	12.70	$[C_5H_8N]^+$	70.6	82.0661	12.2	35
EDDP	19.05	$[C_{20}H_{24}N]^+$	93.7	278.1916	4.7	
EDDP F1	19.04	$[C_{17}H_{16}N]^+$	98.8	234.1288	4.7	35
MTD	19.95	$[C_{21}H_{28}NO]^+$	84.8	310.2188	7.4	
MTD F1	19.96	$[C_{19}H_{21}O]^+$	98.2	265.1616	10.9	15
MTD F2	19.96	$[C_7H_5O]^+$	98.7	105.0359	22.8	35

Table 2 Compounds detected in serum and urine samples. [a] Retention time (min); [b] *m/z* measurement error; [c] collision energy; [d] Identification level according to Schymanski et. al (2014)

Compound	tR [a]	Ion formula	Score	Measured *m/z*	[b] Δm (ppm)	[c] CE	Confirmation Level [d] (1-5)
Serum sample (patient one)							
KET	9.63	$[C_{13}H_{17}ClNO]^+$	93.9	238.0998	2.1		1
KET F1	9.64	$[C_7H_6Cl]^+$	98.9	125.0145	-6.4	35	1
NK	8.67	$[C_{12}H_{15}ClNO]^+$	79.7	224.0832	-2.2		1
NK F1	8.66	$[C_7H_6Cl]^+$	99.6	125.0157	3.2	35	1
DHNK	7.79	$[C_{12}H_{13}ClNO]^+$	97.9	222.0676	-1.8		1
DHNK F1	7.78	$[C_{12}H_{10}ClO]^+$	98.9	205.0413	-1.0	15	1
DHNK F2	7.78	$[C_{11}H_{10}Cl]^+$	96.4	177.0460	-3.4	15	1
DHNK F3	7.78	$[C_9H_{13}ClN]^+$	98.4	170.0717	-8.2	35	1
HNK 1	4.75	$[C_{12}H_{15}ClNO_2]^+$	86.9	240.0804	7.5		3
HNK 1 F1	4.76	$[C_{11}H_{12}ClO]^+$	97.8	195.0577	3.1	15	3
HNK 1 F2	4.76	$[C_{11}H_{10}Cl]^+$	97.7	177.0470	2.3	15	3
HNK 1 F3	4.76	$[C_9H_8Cl]^+$	99.1	151.0306	-2.0	35	3
HNK 1 F4	4.74	$[C_{11}H_{10}]^+$	97.6	142.0768	-6.3	35	3
HNK 1 F5	4.74	$[C_7H_6Cl]^+$	98.8	125.0148	-4.0	35	3
HNK 1 F6	4.74	$[C_9H_8]^+$	97.8	116.0611	-8.6	35	3
HNK 1 F7	4.74	$[C_9H_7]^+$	97.3	115.0536	-5.2	35	3
HNK 2	5.83	$[C_{12}H_{15}ClNO_2]^+$	87.4	240.0793	2.9		3
HNK 2 F1	5.76	$[C_7H_7O]^+$	81.3	107.0494	-1.9	35	3
HNK 3	6.12	$[C_{12}H_{15}ClNO_2]^+$	96.3	240.0786	0.0		3
HNK 3 F1	6.11	$[C_{12}H_{12}ClO_2]^+$	97.2	223.0527	3.1	15	3
HNK 3 F2	6.11	$[C_{12}H_{10}ClO]^+$	98.0	205.0418	1.5	15	3
HNK 3 F3	6.11	$[C_{11}H_{10}]^+$	96.1	142.0769	-5.6	35	3
HNK 4	4.04	$[C_{12}H_{15}ClNO_2]^+$	47.0	240.0792	2.5		5
HNK 5	3.53	$[C_{12}H_{15}ClNO_2]^+$	47.2	240.0772	-5.8		5
HNK 6	7.67	$[C_{12}H_{15}ClNO_2]^+$	45.6	240.0799	5.4		5

Urine sample (patient two)							
25B-NBOMe	19.40	$[C_{18}H_{23}BrNO_3]^+$	83.6	380.0876	5.3		1
25B-NBOMe F1	19.41	$[C_8H_9O]^+$	99.3	121.0651	2.5	15	1
25B-NBOMe F2	19.41	$[C_7H_7]^+$	92.3	91.0545	3.3	35	1
25B-NBOMe (GLU 1)	12.37	$[C_{23}H_{29}BrNO_{10}]^+$	81.9	558.0977	1.4		4
25B-NBOMe (GLU 2a)	13.69	$[C_{23}H_{29}BrNO_9]^+$	88.2	542.1024	0.7		3
25B-NBOMe (GLU 2a) F1	13.68	$[C_4H_{11}NO_3]^+$	73.0	121.0709	-19.8		3
25B-NBOMe (GLU 2b)	13.88	$[C_{23}H_{29}BrNO_9]^+$	96.9	542.1022	0.4		4
25B-NBOMe (CYP 1)	13.98	$[C_{16}H_{19}BrNO_3]^+$	74.8	352.0572	8.2		4
25B-NBOMe (GLU 3)	14.53	$[C_{24}H_{31}BrNO_{10}]^+$	90.3	572.1134	1.4		4
25B-NBOMe (SUL 1)	15.61	$[C_{17}H_{21}BrNO_6S]^+$	93.1	446.0281	3.1		3
25B-NBOMe (SUL 1) F1	15.60	$[C_8H_9O]^+$	90.0	121.0656	6.6	35	3
25B-NBOMe (CYP 2a)	16.22	$[C_{17}H_{21}BrNO_3]^+$	81.8	366.0710	3.0		4
25B-NBOMe (CYP 2b)	16.56	$[C_{17}H_{21}BrNO_3]^+$	98.8	366.0698	-0.3		3
25B-NBOMe (CYP 2b) F1	16.55	$[C_8H_9O]^+$	97.3	121.0648	0.0	35	3
25C-NBOMe	18.77	$[C_{18}H_{23}ClNO_3]^+$	84.7	336.1360	-0.3		1
25C-NBOMe F1	18.78	$[C_8H_9O]^+$	92.4	121.0635	-10.7	15	1
25C-NBOMe (SUL 1)	15.18	$[C_{17}H_{21}ClNO_6S]^+$	70.1	402.0809	9.0		3
25C-NBOMe (SUL 1) F1	15.17	$[C_8H_9O]^+$	99.3	121.0651	-1.7	35	3
25C-NBOMe (CYP 1)	15.84	$[C_{17}H_{21}ClNO_3]^+$	81.3	322.1234	9.3		4
25H-NBOMe	5.13	$[C_{18}H_{24}NO_3]^+$	77.0	302.1778	8.9		4