Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MS^{E} accurate-mass spectra

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ABSTRACT

A study of the fragmentation pathways of several classes of drugs of abuse (cannabinoids, ketamine, amphetamine and amphetamine-type stimulants, cocaine and opiates) and their related substances has been made. The knowledge of the fragmentation is highly useful for specific fragment selection or for recognition of related compounds when developing MS-based analytical methods for the trace level determination of these compounds in complex matrices. In this work, accurate-mass spectra of selected compounds were obtained using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, performing both MS/MS and MS^E experiments. As regards fragmentation behavior, the mass spectra of both approaches were quite similar, and were useful to study the fragmentation of the drugs investigated.

Accurate-mass spectra of 37 drugs of abuse and related compounds, including metabolites and deuterated analogues, were studied in this work and structures of fragment ions were proposed. The accurate-mass data obtained allowed to confirm structures and fragmentation pathways previously proposed based on nominal mass measurements, although new insights and structure proposals were achieved in some particular cases, especially for amphetamine and amphetamine-type stimulants, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) and opiates.

KEYWORDS

Illicit drugs of abuse, fragmentation pathways, metabolites, accurate mass, liquid chromatography, time-of-flight mass spectrometry

1. INTRODUCTION

In the recent years, there has been an increasing concern on the occurrence of drugs in the environment. In one of the first papers, Ternes *et al.* [1] reported significant concentrations of different classes of drugs in sewage and surface waters. Since then, a notable number of studies have been reported, and the number of articles published in this area is still increasing. [2] A group of drugs of particular interest are (illicit) drugs of abuse, which are frequently found in the aquatic environment, particularly in urban wastewater. Methods based on liquid chromatography-tandem mass spectrometry (LC–MS/MS) using triple quadrupole (QqQ) analyzers are important for determination of these compounds and their metabolites. This allows the rapid and efficient simultaneous quantification and confirmation at low analyte concentrations, e.g. ng/L levels, in samples such as surface water and urban wastewater with little sample manipulation. [3, 4]

Wide-scope screening of organic contaminants in environmental samples is gaining popularity thanks to the hyphenation of LC to high-resolution mass spectrometry (HR MS), e.g. orbitrap and time-of-flight (TOF) instruments. HR MS has strong potential for detection and identification purposes as a consequence of the full-spectrum acquisition with satisfactory sensitivity - allowing accurate-mass measurements of the analyte molecule and/or its main fragments -, the ability of performing retrospective analysis without the need of additional sample injections, and the feasibility of investigating a large number of contaminants after MS acquisition using a post-target approach. [5] LC–HR MS has been successfully applied for accurate-mass screening of (polar) target compounds and/or their metabolites, and to discover non-target contaminants. [6-10] Some papers report on the use of LC–HR MS for identification of illicit drugs and metabolites in surface water and wastewater. [11, 12] Accurate-mass measurements of protonated molecules and their fragments allowed the reliable identification of the target compounds.

Data obtained on the levels of drugs of abuse in urban wastewater have been used to estimate illicit drugs consumption of a certain community and to appreciate their potential environmental impact. [13-15] To fully understand the real impact of these compounds in the environment, not only parent drugs should be taken into account but also their metabolites. Some of them are well known to be excreted by human beings and might be observed in the aquatic environment. In addition, other potentially

hazardous transformation products (TPs) might be formed in the aquatic environment or in wastewater treatment plants (WWTPs) as well. From an analytical point of view, knowledge on the fragmentation of different classes of illicit drugs is of great interest, as the recognition of specific product ions and the application of basic fragmentation rules may help to elucidate "unknown" related compounds. The selection of more specific fragment ions would allow minimizing potential interferences and would be of great help to confirm the identity of the compounds detected in complex matrices.

When using a QTOF instrument, application of the MS^E mode is feasible, i.e. performing simultaneous acquisition of MS spectra at low (LE) and high (HE) collision energy. [12] In addition, conventional MS/MS experiments can be made to obtain accurate-mass product ion spectra, which is highly useful for confirmative purposes. Both approaches are powerful for investigation of fragmentation of drugs of abuse and have been applied in this paper.

Several papers report on fragmentation of drugs of abuse, making use of GC–MS [16, 17] and/or LC–MS with different analyzers. Only LC–MS was taken into account in this paper, as fragmentation of molecular ions generated by electron ionization, typically applied in GC–MS, notably differs from fragmentation of protonated molecules generated by atmospheric pressure ionization typically employed in LC–MS. LC–MS is nowadays the technique of choice for the wide majority of drugs of abuse and metabolites.

Mass analyzers, acquiring in nominal or accurate mass, have been used to the study of fragmentation of drugs of abuse. Recently, Castiglioni *et al.* [3] reviewed mass spectra and fragmentation patterns of several classes of illicit drugs. In most cases, low-resolution MS analyzers have been applied to elucidate the most abundant fragments. [18-22] However, for more detailed fragmentation and in cases of ambiguity, HR MS or a combination of techniques (including the use of isotope labeled analogues) needs to be applied. [23-27] In this paper, we report a detailed study on the fragmentation of several classes of illicit drugs and their related substances (metabolites/degradation products) using accurate mass measurements provided by LC–QTOF MS. Whereas some information already exists in the literature, often data are yet incomplete or, in some cases, the interpretation of mass spectra might be questionable. Information provided in this work will be of help for future method development and data interpretation in LC–MS based analytical methodology.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Illicit drugs and metabolites studied were the following: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), R(+)-methcathinone, 1R,2S(-)ephedrine, cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, norcocaine, ecgonine, ketamine, heroin, codeine, norcodeine, morphine, 6-monoacetylmorphine (6-MAM), normorphine and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH). These compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA), Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol (MeOH), acetonitrile or as salt. Standard stock solutions of each compound were prepared at 100 mg/L in MeOH or acetonitrile.

Deuterium labeled compounds were all obtained from Cerilliant as solutions in MeOH or acetonitrile at a concentration of 100 mg/L: amphetamine-d_6, methamphetamine-d_5 MDA-d_5, MDMA-d_5, MDEA-d_5, 1S,2R(+)ephedrine-d_3, cocaine-d_3, cocaethylene-d_8, benzoylecgonine-d_3, ecgonine-d_3, ketamine-d_4, heroin-d_9, codeine-d_6, morphine-d_3, 6-MAM-d_6 and THC-COOH-d_3.

Intermediate solutions (10 mg/L) were prepared by diluting the stock solutions with MeOH. All standard solutions were stored in amber glass bottles at −20 ºC. Working solutions of individual standards were prepared at a concentration of 100 µg/L in MeOH:water (5:95, v/v) just before MS^E and MS/MS experiments.

Chemical structure, exact mass of the protonated molecule, and CAS number of the selected illicit drugs and their deuterated analogues are listed in Table SI 1 of Supporting Information (SI).

2.2. Instrumentation

A Waters Acquity ultra-high-performance liquid chromatography (UHPLC) system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QTOF Premier, Waters Micromass, Manchester, UK) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operated in positive-ion mode.

Two types of acquisition, MS/MS and MS^E, were performed. For MS/MS experiments, cone voltage and collision energy ramp were optimized for each compound individually (Table SI 2). For MS^E experiments, two acquisition functions with different collision energies were created: the low energy function (LE), and the high energy (HE) function, with the same compound-dependent optimized cone voltage and collision energy ramp as for MS/MS.

Further details on instrument operating conditions both chromatographic and spectrometric can be found elsewhere. [12]

3. RESULTS AND DISCUSSION

3.1. General aspects

Several papers report on MS fragmentation of drugs of abuse. Recently, Castiglioni *et al.* [3] published an excellent review on fragmentation of several drugs of abuse and metabolites frequently found in wastewater and surface water, based on nominal mass measurements by LC–MS/MS (QqQ). A more detailed investigation of the fragmentation should be based on accurate mass data. This would allow the elucidation of the chemical formulae of fragments with higher confidence. The information obtained would permit a more rational selection of structural specific fragments, which is of interest for LC–MS analytical methods, e.g. using TOF or QqQ mass analyzers, giving a more reliable identification of analytes and minimizing the possibility of reporting false positives or false negatives. A reduction of interferences and an increase in the signal-to-noise ratio could also be obtained by performing extracted ion chromatograms (XIC) from full-spectrum accurate-mass data, selecting narrow *m/z* windows (e.g., ± 0.01 Da). [28, 29] In analyses involving selected reaction monitoring (SRM) in a QqQ instrument, the same effect could be expected by selection of more specific ions.

For the present paper, data were acquired in both MS/MS and MS^E mode using a QTOF instrument in positive-ion ESI. The MS^E acquisition strategy was recently introduced for QTOF instruments [12, 30-32]: a continuous scan-wise switching is made between low collision energy, to detect ions from intact molecules, and high collision energy, to acquire fragmentation data. This technique enables acquisition of ions from parent molecules, their isotopic patterns, and their fragment ions in a single injection. It has become a powerful tool for wide-scope screening of a large number of compounds with strong identification capabilities.

Although product ion and MS^E mass spectra were highly comparable, the different characteristics of each mode were useful for the elucidation of certain fragments. When applied to samples, e.g. urban wastewater, product ion mass spectra are generally cleaner and the sensitivity is higher, as specific precursor ions are selected in the quadrupole.

Optimum MS/MS and (HE) MS^E conditions for each compound were established and are listed in Table SI 2 of Supporting Information (SI). Data

interpretation was based on accurate *m/z* values observed for the fragment ions, often both of the drugs and of a deuterated analogue, the molecular formulae derived from the measured accurate *m/z*, and the general concept that fragmentation should involve logical neutral losses and no major rearrangements in the structure of the drug. Based on these concepts, structures for the fragment ions have been proposed. No attempts were made to prove the proposed structures by further experiments or theoretical calculations. Isotope information from the MS^E was also of help. Accurate-mass information allowed us to confirm the fragment ion identity previously proposed by other authors, e.g. based on nominal mass measurements. In addition, accurate-mass data were essential to understand the fragmentation and/or to discard structures suggested in the literature.

3.2. Cannabinoids

The most psychoactive cannabinoid is Δ^9-tetrahydrocannabinol (THC). The amount of THC is used as a measure of "cannabis potency". But as marker compounds of cannabis consumption, the THC metabolite 11-nor-9-carboxy-Δ^9-THC (THC-COOH) and its glucuronic-acid conjugate (THC-COOH-glucuronide) is generally analyzed by LC–MS/MS in human plasma [18] and in urine samples. [33, 34] In wastewater, where glucuronide conjugates are easily hydrolyzed to the free acid by beta-glucuronidase of fecal bacteria [35], THC-COOH is the analytical target of choice. THC-COOH has been determined by LC–MS/MS both in negative-ion [36, 37] and positive-ion mode. [38, 39] The most abundant fragment ions in the product ion mass spectra of the deprotonated and protonated molecule of THC-COOH have been discussed before. [3, 18] A structure of a less abundant, but more specific fragment ion, with *m/z* 193, to be (2,6-dihydroxy-4-pentylphenyl)methylium was proposed by Maralikova *et al.* based on triple quadrupole data. [18]

The proposed fragments of the protonated molecule could be confirmed by our accurate-mass data. Additionally, with the help of the product ion mass spectra (Figure 1B and C) of the protonated THC-COOH ion and THC-COOH-d_3 deuterated analogue, several less abundant fragment ions, not reported previously, could be identified (Figure 1A). As the D_3C group is at the end of the pentyl chain, any +3 shift between the *m/z* values in the product ion mass spectra of the $[M+H]^+$ ion of THC-COOH and its deuterated analogue indicates the pentyl chain is unaffected by the fragmentation. This is true for the *m/z* 193 fragment ion, but also for *m/z* 257, corresponding to the loss of

propene (C_3H_6) from the fragment ion *m/z* 299 $[M+H–HCOOH]^+$, which involves ring opening of the saturated six-member ring. On the other hand, the fragment ions with *m/z* 229 and 187 do not show the +3 shift, indicating that these fragments originate from the loss of pentene (C_5H_{10}) and the combined loss of pentene and propene, respectively, from the fragment ion with *m/z* 299.

In the case of THC-COOH, accurate-mass information served as a confirmation tool of fragments previously suggested based on nominal mass measurements. Comparison of mass spectra of this metabolite and of its deuterated analogue was essential for the identification of less abundant fragments and understanding the fragmentation route.

3.3. Ketamine

In the aqueous environment, detection of ketamine in wastewater has been reported a few times. [40, 41] Ketamine is a licit pharmaceutical, and as such, has some abuse potential and is connected to the "club drug" scene. [42]

As shown in prior work by the authors, the MS^E spectrum of the $[M+H]^+$ ion and fragment ions of ketamine showed useful isotopic information related to the presence of one chlorine atom. [40] This information together with the accurate mass of both precursor and fragment ions confirmed the fragmentation proposed by Wang *et al.* [19] with high confidence. The chlorine atom is still present in all fragments, with the exception of those with *m/z* 115 and 116. Most of them can be explained being due to the sequence of losses of water and methylamine (in either order) to the ion *m/z* 189, with intermediate ions *m/z* 220 and 207, respectively, and further fragmentation to ions *m/z* 179 (CO loss from the ion with *m/z* 207) and *m/z* 125. Most likely, the (2-chlorophenyl)methylium ion with *m/z* 125 is more readily formed from the ion *m/z* 179, due to an inductive cleavage involving the loss of C_4H_6, because the alternative route from *m/z* 189 requires the cleavage of two C-C bonds, which seems less likely (Figure SI 1).

The ions with *m/z* 115 and 116 ($C_9H_7^+$ and $C_9H_8^{+\bullet}$, respectively) involve the loss of chlorine, either as HCl or as a radical. From the ketamine-d_4 spectrum (Figure SI 1B), one may conclude that this may be a Cl-radical, HCl or DCl. However, the pathway involved in the loss of chlorine is certainly not evident, because for instance an ion with *m/z* 151/153 is not clearly observed.

The fragmentation of ketamine derivatized heptafluorobutyric anhydride has been studied by Pieri *et al.* [17] using GC–MS and electron ionization. Obviously, a fragmentation pathway of the derivatized ketamine under these conditions significantly differs from that reported here.

3.4. Amphetamine and amphetamine-type stimulants

Since Jones-Lepp *et al.* reported methamphetamine and MDMA in effluent wastewater in the USA [43], amphetamine and amphetamine-type stimulants (ATS) have frequently been detected at ng/L level in influent wastewater and sometimes in effluent samples. [38, 41, 44 - 46] ATS are a group of synthetic stimulants, including predominantly amphetamine, methamphetamine, MDA, MDMA (ecstasy) and MDEA. Amphetamine analogues, such as methcathinone, may also be included in this group. Furthermore, the most commonly used ATS chemical precursors such as ephedrine fall under international control, and their seizure can provide some limited indications about manufacturer trends. [42]

The MS/MS fragmentation patterns of the $[M+H]^+$ ion of amphetamine (Figure 2A) and methamphetamine are straightforward with the fragment ion *m/z* 119, resulting from the loss of ammonia or methylamine, respectively, the tropylium ion ($C_7H_7^+$ with *m/z* 91) due to a β-C-C cleavage, and the secondary fragment *m/z* 65 ($C_5H_5^+$) due to the loss of acetylene from the ion *m/z* 91, as common fragment ions. The product ion mass spectrum of amphetamine-d_6 leads to some interesting novel observations (Figure 2B). Given the deuterium positions in the amphetamine-d_6 ($C_6H_5–CD_2–CD(–NH_2)–CD_3$), a straightforward β-C-C cleavage would lead to a tropylium ion with the formula $C_7H_5D_2^+$ (*m/z* 93). However, next to the ion with *m/z* 93, two other ions were observed consistent with *m/z* 94 ($C_7H_4D_3^+$) and *m/z* 95 ($C_7H_3D_4^+$), which indicate scrambling of the labels during fragmentation (Figure 2C). NMR spectroscopy was applied to confirm the specified positions of the D-labels. In turn, the ions *m/z* 93, 94 and 95 provide secondary fragmentation involving the loss of either HCCH, HCCD, or DCCD, leading to fragment ions with *m/z* 65 ($C_5H_5^+$), 66 ($C_5H_4D^+$), 67 ($C_5H_3D_2^+$), 68 ($C_5H_2D_3^+$) and 69 ($C_5HD_4^+$) (Figure 2D). Essentially the same results were obtained by collision-cell fragmentation of the precursor ions *m/z* 93, 94 and 95, generated by in-source fragmentation of amphetamine-d_6. In our opinion, these observations are most readily explained if the fragment ion *m/z* 91 has a tropylium ion and not a benzyl cation

structure. Moreover, it puts some light on the complexity of the fragmentation mechanism of a protonated molecule, as the β-C-C cleavage apparently involves scrambling of ring and chain hydrogen atoms. Further complexity is added by the product ion mass spectrum of methamphetamine-d_5, where next to the most abundant ion *m/z* 92 ($C_7H_6D^+$), consistent with a straightforward β-C-C cleavage, the ions *m/z* 91 (one D-label lost, $C_7H_7^+$) and *m/z* 93 (one D-label gained, $C_7H_5D_2^+$) are also observed. Similar secondary fragmentation was obtained, involving losses of HCCH, HCCD, and DCCD.

Compared to other amphetamine analogues, methcathinone and ephedrine have been scarcely studied. To the best of our knowledge, a detailed study on their fragmentation pathway has not been reported. The structures of these compounds are comparable to methamphetamine, yet with either a ketone- or hydroxyl-group at the C^1 position, respectively. This significantly affects the fragmentation. Methcathinone ($[M+H]^+$ with *m/z* 164) (Figure 3A) and ephedrine ($[M+H]^+$ with *m/z* 166) (Figure 3B) both show the loss of water with remarkable ease from the keto- or hydroxyl-function, respectively, and subsequently the loss of $CH_3^{\bullet}$, resulting in the radical cations *m/z* 131 and *m/z* 133, respectively. To this end, there are two methyl-groups that could be lost: N-CH_3 and C^2-CH_3. From the ephedrine-d_3 spectrum (Figure 3C), one can deduce, that both losses actually occur, with the loss from C^2-CH_3 resulting in a more abundant fragment (*m/z* 136) than the loss from N-CH_3. The loss of CH_4 is also observed, leading to fragments with *m/z* 130 and 132 of the unlabeled compounds, respectively. Structures for these fragment ions are proposed in Figure 3. At low *m/z*, the spectra differ considerably (Figure 3A and B). Methcathinone shows a fragment with *m/z* 105.0699. Accurate-mass data (Figure 3A) prove that this ion corresponds to $C_6H_5–C^+H–CH_3$ (calculated *m/z* 105.0704, mass error 0.5 mDa) rather than the expected $C_6H_5–C{\equiv}O^+$ (calculated *m/z* 105.0340, mass error 35.9 mDa). Ephedrine, on the other hand, shows a fragment with *m/z* 117, owing to subsequent losses of water and methylamine, as well as a fragment with *m/z* 115, which can be written as a stable ring structure with a resonance-delocalized charge, after an apparently easy loss of H_2 from the ion with *m/z* 117.

MDA, MDMA and MDEA, containing a dioxole ring, all give similar fragmentation patterns, with the exception of some minor fragments at the lower end of the spectra and, obviously, the protonated molecule. As an example, MDA ($[M+H]^+$ with *m/z* 180) (Figure 4A) shows a loss of NH_3 to a fragment ion with *m/z* 163 and a β-

C-C cleavage leading to a 1,3-benzodioxol-5-ylmethylium ion with *m/z* 135 due to the loss of ethylamine. Structure of these and some other fragment ions are proposed in Figure 4. This pattern is similar to what is observed for amphetamine and related compounds, as described above. With MDMA and MDEA, the same β-C-C cleavage also leads to minor immonium ions with *m/z* 58 and 72, consistent with the loss of 5-methyl-1,3-benzodioxole from $[M+H]^+$. The fragment ion with *m/z* 133 can be considered as a secondary fragment of the ion with *m/z* 163 (after loss of ammonia) and corresponds to a loss of $H_2C=O$ due to cleavage of the methylenedioxy ring. An interesting fragment is the ion with *m/z* 105, since this ion has been interpreted as being due to the loss of $H_2C=O$ from the ion with *m/z* 135 (thus with formula $C_7H_5O^+$, calculated *m/z* 105.0340) [3], whereas our accurate-mass data (*m/z* 105.0703) suggests that the correct formula is $C_8H_9^+$ (calculated *m/z* 105.0704, mass error 0.1 mDa). Structures of both $C_7H_5O^+$ and $C_8H_9^+$ are shown in Figure 4. In addition, data of its labeled analogue (Figure 4B) proofs this interpretation of *m/z* 105.0704 to be correct, as it would be the only way to account for five labels in this fragment.

Study of the labeled analogues of MDA, MDMA and MDEA was consistent with our expectation. Some H/D scrambling was observed, similar to the observations of the labeled analogues of (meth)amphetamine. No H/D scrambling was observed in the spectra of MDEA-d_5, where D-labeling is at the N-ethyl group.

3.5. Cocaine

Cocaine and its metabolites are frequently reported to be found in the aquatic environment. Concentrations are relatively high (sometimes up to µg/L level), especially for its major metabolite benzoylecgonine, compared to other illicit drugs. [41, 44, 45]

By using product ion mass spectra of labeled and unlabeled compounds, Wang *et al.* [20] comprehensively described fragmentation pathways for cocaine, its metabolites and pyrolytic degradation products. Later, Castiglioni *et al.* [3] also reported fragmentation data on this group of compounds, supporting them with the corresponding mass spectra. The fragmentation mechanisms proposed by these authors are confirmed by our accurate-mass spectra (data not shown). The most abundant fragment of cocaine and most of its metabolites can be assigned to the neutral loss of benzoic acid (122 Da). The remaining fragment ions can be considered as subsequent

fragmentation of the resulting ion $[M+H - 122]^+$, involving a further loss of methanol or water.

3.6. Opiates

As they are frequently used as narcotic to relief pain, opiates can enter the environment not only by way of drug abuse but also due to clinical usage. The opiates most widely used as abusive drug are heroin, morphine and codeine. These compounds have been found (few ng/L) in wastewater, together with their related metabolites, 6-monoacetylmorphine (6-MAM) and norcodeine. [36, 37, 39] Glucuronide conjugates such as morphine-3-β-glucuronide or morphine-6-β-glucuronide have also been found in sewage water. [36] Fragmentation of these conjugates shows an intense fragment ion corresponding to the characteristic loss of the dehydroglucuronic acid group (loss of 176 Da). [3] Here, only the fragmentation of unconjugated opiates was studied.

The structures of the opiates and their deuterated analogues studied in this work are illustrated in the supporting information (Table SI 1). And, in addition to the mentioned references, visual assistance is available to the reader (Figure SI 2) to get more insight in the proposed fragmentation route of opiates. Fragmentation pathways at the high end of the spectra have been studied by other authors [23-25] and our accurate-mass data confirm the earlier interpretation. However, fragment ions at the lower end of the spectra have generally been ignored.

The loss of the ketene $H_2C=C=O$ and acetic acid (from heroin), acetic acid (from 6-MAM) and water (from morphine) in all three cases results in a fragment ion at *m/z* 268 ($C_{17}H_{18}NO_2^+$) (see Figure SI 2). Subsequent fragmentation involves cleavages in the morphinan backbone. This results in complex fragmentation patterns, in which heroin, 6-MAM and morphine, but also nor-morphine, codeine and nor-codeine, show many similar features.

Taking heroin as an example (Figure 5A), an initial cleavage of the piperidine ring occurs. The most abundant fragment results from the loss of $H_3C–CH_2=N–CH_3$ (57 Da) to fragment ion with *m/z* 211 ($C_{14}H_{11}O_2^+$), although fragment ions due to the loss of $H_2C=N–CH_3$ (43 Da) to *m/z* 225 ($C_{15}H_{13}O_2^+$) and $H_2N–CH_3$ (31 Da) to *m/z* 237 ($C_{16}H_{13}O_2^+$) are also observed. The structure proposed by Zhang *et al.* [25] for the ion with *m/z* 225, involving a seven-member ring, seems unlikely, as it would require significant rearrangement of the structure; an alternative proposal is given in Figure 5 (top).

Next to the ion with *m/z* 211, an ion with *m/z* 209 ($C_{14}H_9O_2^+$) is observed, which would involve an additional double bond in the remaining structure with four fused rings. Similar behavior is observed for the fragment ions with *m/z* 193 ($C_{14}H_9O^+$) and 183 ($C_{13}H_{11}O^+$), which are due to losses of water or CO, respectively, from the ion with *m/z* 211; thus ions with *m/z* 191 ($C_{14}H_7O^+$) and 181 ($C_{13}H_9O^+$) are observed as well (see Figure SI 2). The lower *m/z* satellite peaks (*m/z* 191 and 181) show greater aromaticity, which may be the driving force in their formation.

Careful comparison of the spectra of heroin and heroin-d_9 reveals that the loss of water to give *m/z* 193 from ion with *m/z* 211 (Figure 5A) involves either the oxygen from 4,5-epoxide or the 3-hydroxy group, whereas the loss of CO mainly appears to involve the oxygen from 4,5-epoxide. It should be pointed out that in the heroin-d_9 spectrum the loss of water from *m/z* 212 would lead to both fragment ions with *m/z* 193 and *m/z* 194 (Figure 5B). This is only possible if the loss of water involves the oxygen of either of the two groups, with the loss of the hydroxy-O (as HDO) leading to *m/z* 193 and the loss of the epoxy-O (as H_2O) to *m/z* 194. In heroin-d_9, the three methyl groups of heroin are CD_3 groups. In the fragment ion with *m/z* 212, two CD_3 groups have been lost with the loss of acetic acid and the N–CD_3 substituent; one D-label is still present, resulting from D-rearrangement upon the loss of the ketene $D_2C{=}C{=}O$.

Regarding the CO loss, only the fragment ion *m/z* 184 is observed in the heroin-d_9 spectrum, but not the *m/z* 183 (Figure 5B), suggesting that only 4,5-epoxide is involved in this loss, maintaining the 3-hydroxy group. Moreover, an abundant fragment (m/z 182) was also observed, due to H_2 loss. This demonstrates that D-labeling of heroin and related compounds was important in elucidation of the fragmentation pathway. From our point of view, the structure suggested by others for the fragment ion *m/z* 181 of heroin and related compounds, with a 4-hydroxy group, is unlikely. [24, 25] We think that the 3-hydroxy group is more appropriate (Figure 5 (top)).

An abundant fragment in the spectra of opiates is the ion *m/z* 165. It is frequently selected in SRM analysis for quantification or confirmation of opiates in wastewater. [37, 39] The ion *m/z* 165/166 can be derived from the ion *m/z* 193/194 due to the loss of CO or from the ion *m/z* 183/184 due to the loss of water.

It is noteworthy that the fragment ions involving additional loss of H_2 and thus the formation of a double bond (*m/z* 191, 181 and also 153, see below) as well as the most abundant ion in the quadrupole spectra (*m/z* 165) were not observed in ion-trap MS^2 spectra, but only as minor ions in subsequent MS^3 and MS^4 spectra. [23, 25]

Fragment ions at the lower end of the spectra have been generally ignored. The formation of ions such as *m/z* 157, 141, and 115 is complex and involves multiple neutral losses and/or losses of larger neutral fragments (Figure 5A). Structure elucidation is complicated and unambiguous structures cannot always be proposed. At this point, it was helpful to perform additional experiments using a triple-quadrupole MS/MS instrument. By performing precursor-ion scans of the more abundant low-*m/z* fragment ions, more insight was obtained in their fragmentation pathways.

The precursor ions of the morphine product ion with *m/z* 157 ($C_{11}H_9O^+$) are the *m/z* 185, 201 and 229 ions, involving neutral losses of CO, H_3C-CH=O and CO + H_3C-CH=O, respectively. We compared these data with the precursor-ion scan data of the related product ion of codeine, *i.e.*, *m/z* 171. Codeine contains a methoxy- instead of a hydroxy-group on the C^3 position resulting in a mass shift of +14 in the *m/z* value of various product ions relative to morphine. Furthermore, the deuterated analogue of codeine (codeine-d_6) contains three additional D-labels on this methoxy-group compared to morphine-d_3. Thus, the corresponding codeine fragment ion with *m/z* 171 results from precursors *m/z* 199, 215 and 243; the mass shift of 14 Da indicates an identical fragmentation pathway in which the methoxy-group is kept.

Based on these results, possible fragmentation pathways for the low-*m/z* fragments are discussed in more detail starting from the product ions with *m/z* 243 and 229 of codeine and morphine, respectively, formed by the complete loss of the piperidine ring (see Figure SI 2).

The formation and structure of fragment ions *m/z* 141 ($C_{11}H_9^+$) and *m/z* 115 ($C_9H_7^+$) are less evident. Results from precursor-ion scans for *m/z* 141, showed the presence of *m/z* 201 and 229 for morphine, and with *m/z* 215 and 243 for codeine, pointing out an identical fragmentation route, retaining the oxygen-group at the C^3 position. A key-role can be attributed to the precursor ions *m/z* 201 (morphine) and *m/z* 215 (codeine), which can be formed by the loss of CO from the *m/z* 229 and *m/z* 243 ions, respectively. However, the loss of CO can be associated with the oxygen either from the 4,5-epoxide or the 6-hydroxy group. In Figure 6 (top), three possible structures for fragment ion *m/z* 201 (and *m/z* 215) are proposed: A and B involving the loss of 6-hydroxy oxygen, whereas C involves the loss of 4,5-epoxide group. Structures A and B are convincing to explain the formation of the ion *m/z* 141 from the precursor ion *m/z* 201 (or *m/z* 215). It appears to correspond to a one-step loss of HC(=O)-CH_2OH for

morphine and of HC(=O)-CH_2OCH_3 for codeine. Structure C is unlikely to be involved in the formation of m/z 141.

Regarding fragment ion m/z 115, the precursor ion with *m/z* 201 (and *m/z* 215 for codeine) possibly also plays an important role in the formation of this fragment, although in this case an intermediate ion, *m/z* 173 (and *m/z* 187 for codeine) is also participating in the fragmentation pathway based on precursor ion scan data. According to the high-resolution accurate-mass data (Figure 6 (bottom)), two isobaric fragment ions with *m/z* 173 ($C_{11}H_9O_2^+$ or $C_{12}H_{13}O^+$) are formed. Zhang *et al.* proposed two different structures for this fragment ion, involving a one-step or two-step fragmentation from m/z 229. Our TOF MS data clearly shows the presence of both these fragment ions, *m/z* 173.0609 and *m/z* 173.0981, demonstrating that both pathways are surely involved. However, we believe that fragment ions are formed only from the precursor ion *m/z* 201, involving a one-step loss of either C_2H_4 from structure 6A (supported by the presence of *m/z* 173.0609) or a loss of CO from structure 6 C (supported by *m/z* 173.0981).

In both cases, a further one-step fragmentation to *m/z* 115 is complicated, implying less logic rearrangements and fragmentation of aromatic rings. Identical complicated one-step fragmentation is involved in the formation of *m/z* 115 from the codeine intermediate ions *m/z* 187 ($C_{13}H_{15}O^+$ or $C_{12}H_{11}O_2^+$). Therefore, unequivocal structure for the fragment ion with *m/z* 115 could not be suggested.

3.7. Applied issues

LC–QTOF MS is a powerful technique for identification and confirmation purposes, very attractive for wide-scope screening of drugs of abuse in the environment. To take full profit of its qualitative and elucidative potential, a detailed knowledge of the fragmentation pathways is required. The possibility of searching for drug-related compounds is one of the strong points of TOF MS. Assuming that most of these compounds share similar fragmentation pathway with the parent drug, the knowledge of fragment ions of the main drugs of abuse is essential to search for metabolites, transformation and/or degradation products. Different approaches can be applied based on accurate-mass measurements of common fragment ions. Nowadays, manufacturers provide automated software algorithms to detect expected and unexpected metabolites. Most of these algorithms are designed to compare and contrast chromatograms of a presumptive positive sample with a control or blank sample. However, in environmental

analysis, a representative blank sample is practically impossible to obtain, and this approach seems less useful. An alternative way is to obtain narrow-mass window extracted ion chromatograms (e.g., ± 0.01 Da) for specific fragment ions from the HE and/or LE functions of MS^E data. When these chromatograms show additional chromatographic peaks at retention times different to that of the parent drug, the presence of parent-chemically related compounds can reasonably be expected in the sample. This approach, based on assuming similar fragmentation pathways for parent analyte and their metabolites has successfully been applied for investigation of pesticide metabolites. [47] Another example illustrating the relevance of knowing the fragmentation in detail is the possibility to differentiate isomers from their specific fragments, as although sharing the same empirical formula and exact mass they can suffer different fragmentation. [12, 40]

The selection of specific fragment ions is not only relevant when searching for metabolites or transformation products using accurate-mass data, but also for the selection of appropriate (selective) SRM transitions in quantitative analyses using QqQ instruments. As pointed out by Pozo *et al.* [48], non-specific transitions such as those involving the loss of water or CO may result in reporting false positives or false negatives, as the probability of finding interferences sharing the same transition increases. This is more likely to occur when dealing with analytes at sub-ppb levels in complex matrices, e.g., the determination of drugs of abuse in urban wastewater. In addition, the most sensitive transitions (typically selected in quantitative methods) not necessarily are the most selective ones. Therefore, the selection of fragment ions taking into account not only their abundance but also their specificity is of great relevance for trace-level analysis. The European Union established the acquisition of at least two SRM transitions in low-resolution MS instruments, together with the measurement of their intensity ratio for confirmation of contaminants in samples of animal origin.[49] This criterion is also frequently used in the determination of organic contaminants in environmental samples. However, no requirements are given on the selectivity/ specificity of the selected ions. In our opinion, more attention needs to be paid to the specificity of the (fragment) ions in order to minimize the possibility of reporting false positives, especially when legal or societal implications are involved. An interference sharing one of the transitions may lead to report the sample as negative because of the non-compliance of the Q/q ratio, even in cases where the analyte is present. In any case, the maximum tolerances established for Q/q ratio deviations are subject of controversy,

especially in complex-matrix samples. [48, 50] Unfortunately, information on Q/q ratio values in samples and reference standards is not always given in the literature. A detailed knowledge of fragmentation pathways and the expertise of the researcher on MS are among other aspects of great importance to obtain reliable data in LC/MS based methods.

4. CONCLUSIONS

Accurate-mass spectral data for some classes of illicit drugs and their related substances (metabolites, degradation products), that are frequently detected in environmental and wastewaters, have been obtained using QTOF MS in both MS/MS and MS^E mode. Based on these data, fragmentation pathways have been carefully elucidated. Although nominal-mass measurement can already be useful for elucidation purposes in many cases, accurate-mass information is imperative for confirmation of the identity of such fragment ions as well as to allow elucidation of structures of several important key ions. In some cases, accurate-mass data of deuterated analogues also played a key role in the elucidation of the fragmentation. All information provided by QTOF MS helped us to elucidate structures with high reliability and to complete fragmentation pathways.

The information in this paper can help the reader to interpret related spectra and to select specific fragments for the safe identification of these emerging contaminants in environmental, food or biological sample matrices.

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SUPPORTING INFORMATION

In this section, a table with useful information for 37 drugs of abuse, their metabolites and deuterated analogues investigated is shown (Table SI 1). Chemical structures, exact masses of the protonated molecules and CAS numbers are given. In addition, a table with optimum MS/MS and MS^E conditions used for the interpretation of these compounds are also given (Table SI 2). Furthermore two figures, one reporting fragmentation pathway of ketamine and MS^E (HE) spectra of the $[M+H]^+$ ion of both ketamine and its deuterated analogue (Figure SI 1), and another including a fragmentation route of opiates via morphinan backbone (Figure SI 2), are added to have supportive visual information on the written text.

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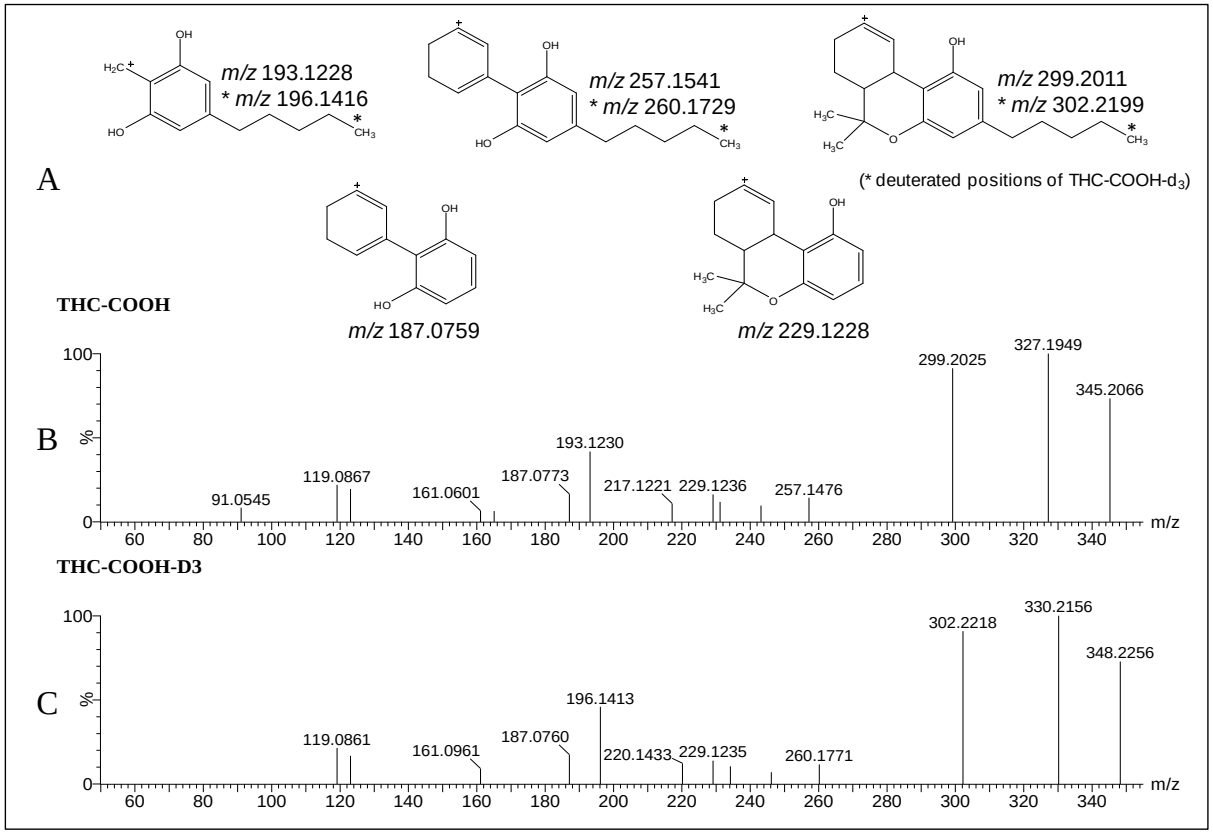
FIGURE CAPTIONS

Figure 1. Structure proposals for some THC-COOH fragment ions (A), product ion (QTOF) mass spectra of $[M+H]^+$ of THC-COOH (B) and THC-COOH-d_3 (C).



740

Figure 2. Product ion (QTOF) mass spectra of $[M+H]^+$ of amphetamine (A) and amphetamine-d_6 (B) (range *m/z* 60-70, 82x magnified), H/D scrambling of the tropylium ion (C) and the secondary fragments of the tropylium ion (D) of amphetamine-d_6.

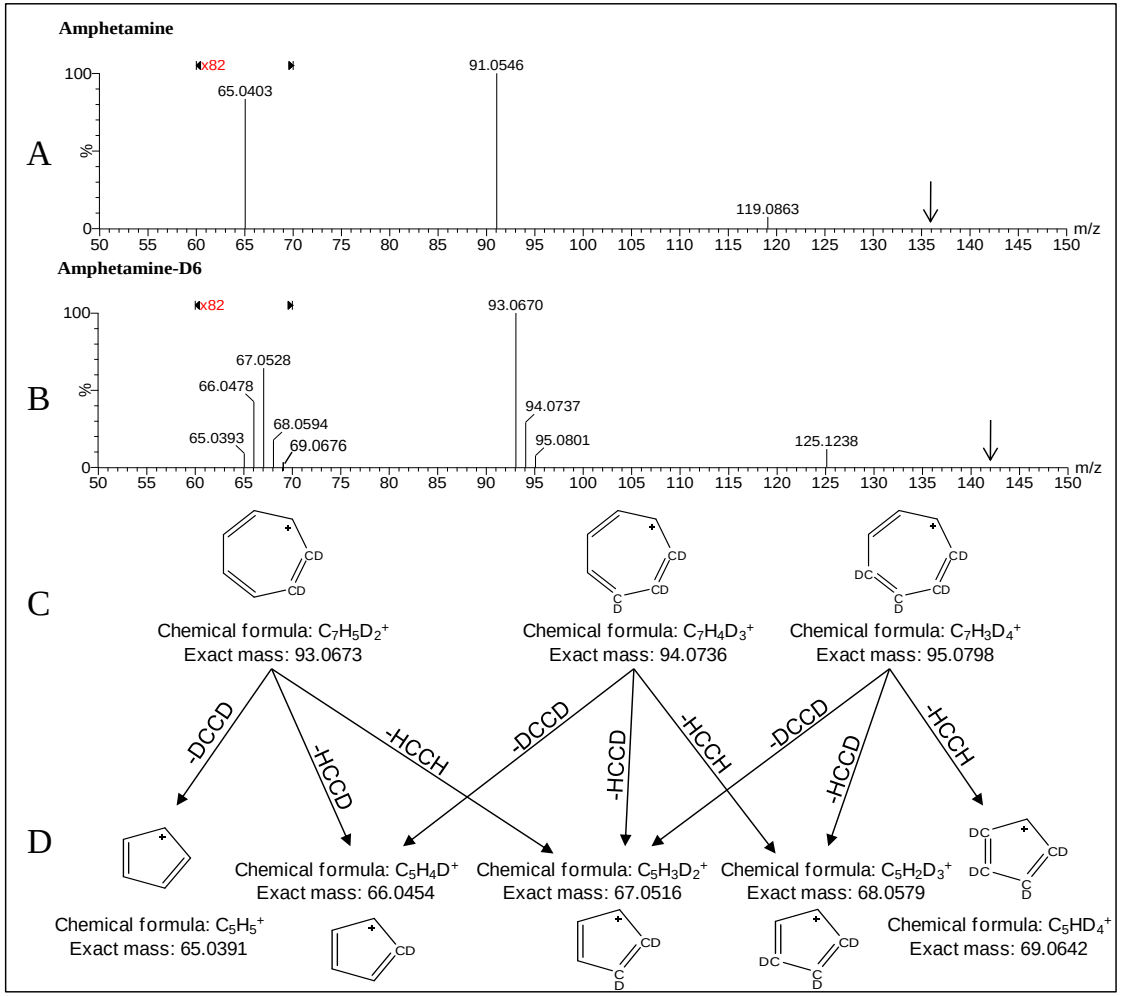


Figure 3. Structure proposals for some of the fragment ions in the product ion mass spectra of $[M+H]^+$ of methcathinone (left) and ephedrine (right). MS^E (HE) spectra (middle) of methcathinone (A), ephedrine (B) and ephedrine-d_3 (C).

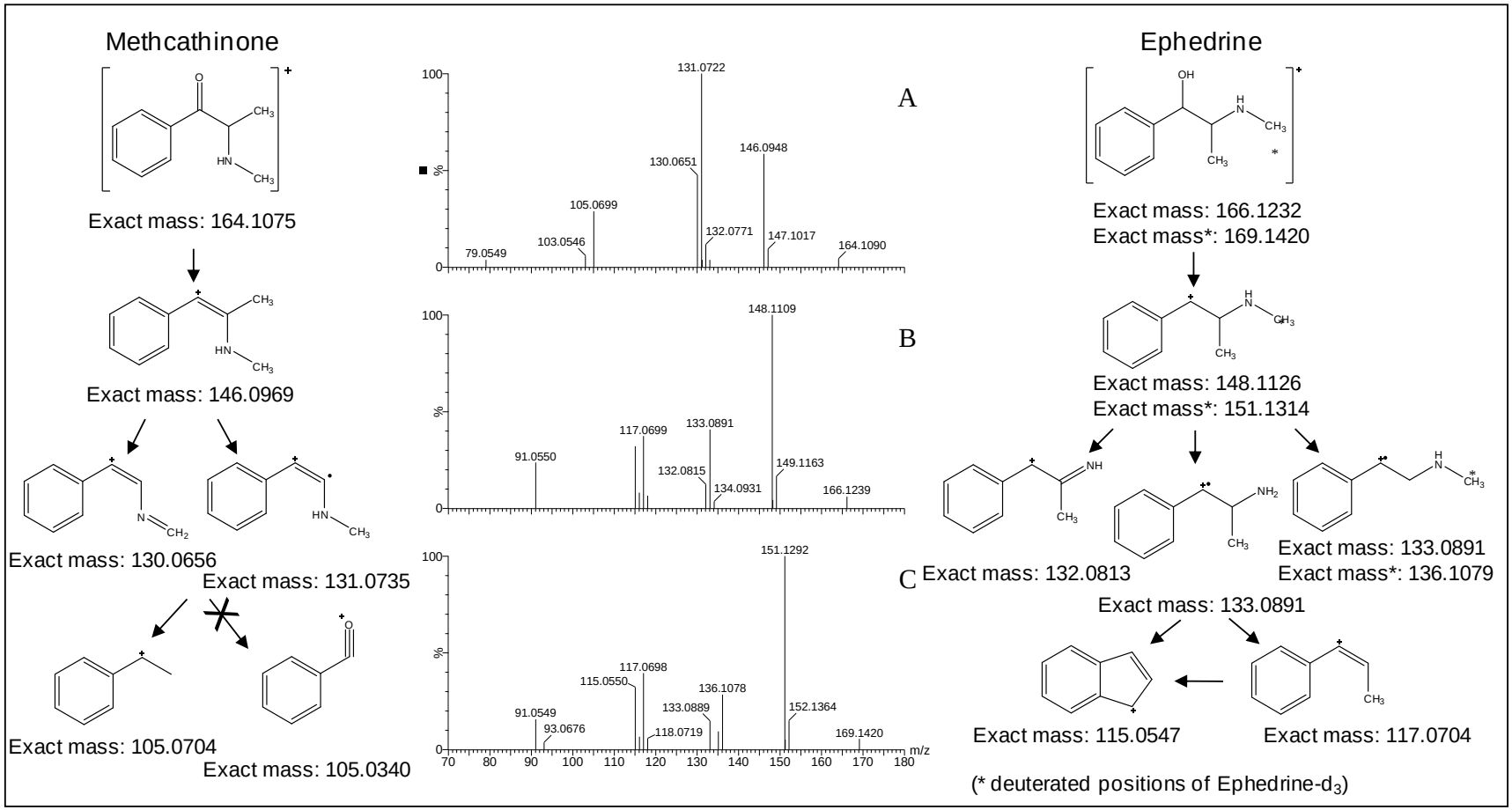


Figure 4. Structure proposals for some of the fragment ions of $[M+H]^+$ of MDA. MS^E (HE) spectra (right) of MDA (A), MDA-d_5 (B): Insert, structure of MDA deuterated fragment *m/z* 110.

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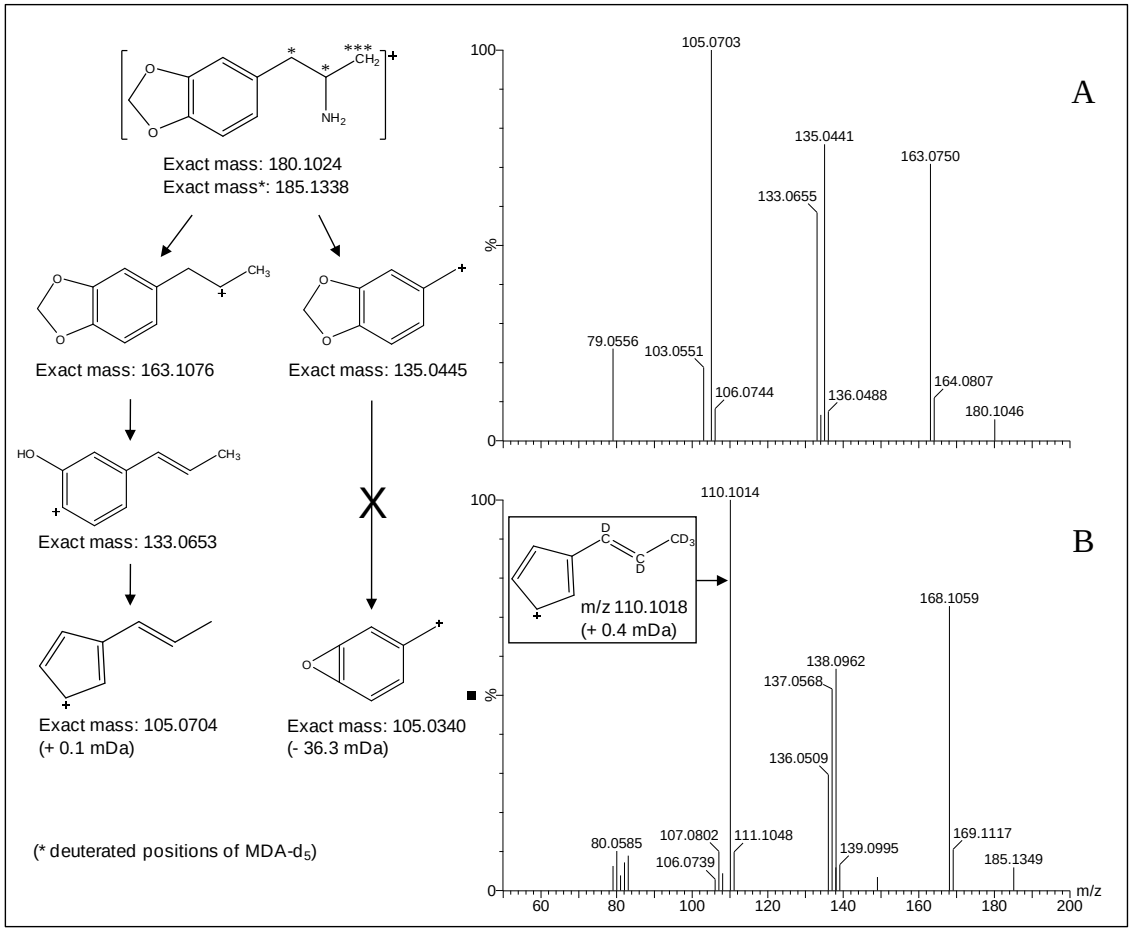

Figure 5. Product ion mass spectra of $[M+H]^+$ of heroin (A) and heroin-d_9 (B) (bottom) with zoom in the range *m/z* 175-215. Proposed structures for product ions *m/z* 181 ($C_{13}H_9O^+$) and *m/z* 225 ($C_{15}H_{13}O_2^+$), involving cleavage in the morphinan backbone (top).

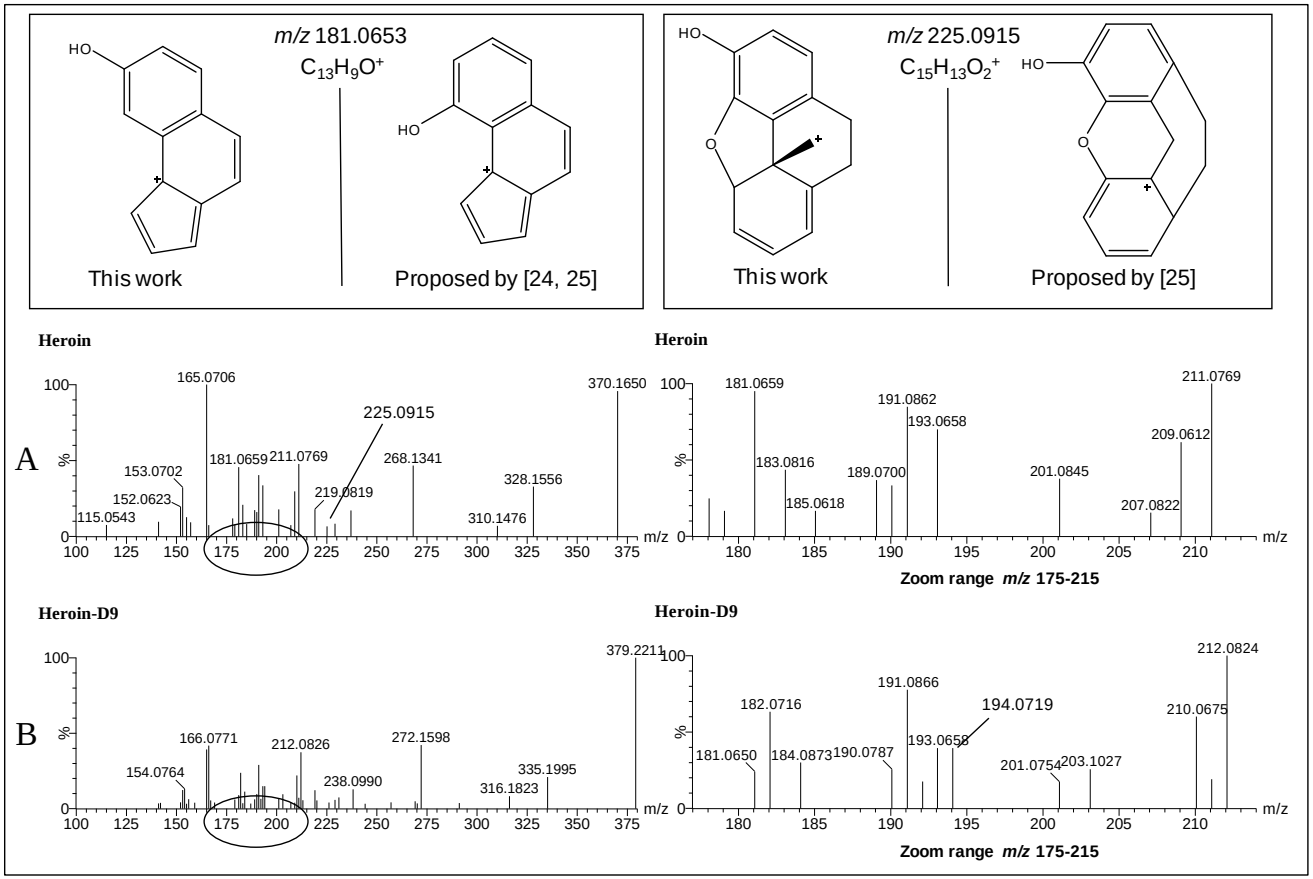

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Figure 6. Product ion mass spectrum of $[M+H]^+$ of morphine and three possible chemical structures (A, B, C) suggested for morphine fragment *m/z* 201.0915 (R=H, $C_{13}H_{13}O_2^+$) and codeine fragment *m/z* 215.0915 (R=CH_3, $C_{14}H_{15}O_2^+$). The loss of HC(=O)-CH_2OR to *m/z* 141.0704 is illustrated as a dotted line (top).

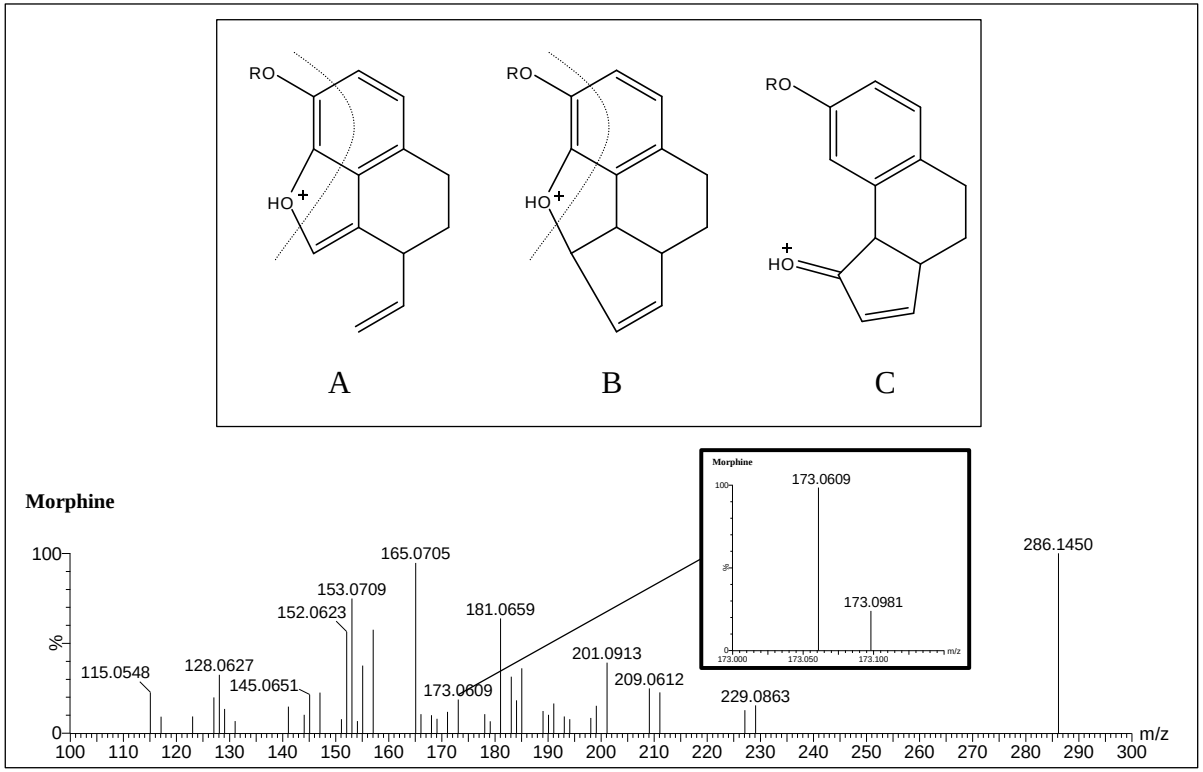

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Table SI 1: Chemical structure, exact mass of the protonated molecule, and CAS number of the selected illicit drugs and their deuterated analogues.

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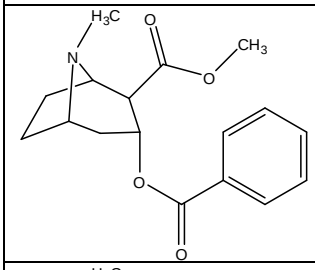
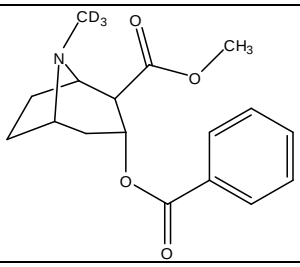
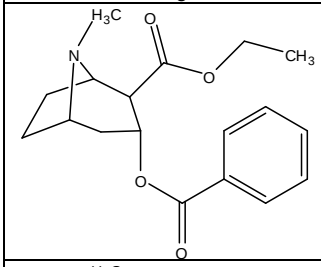
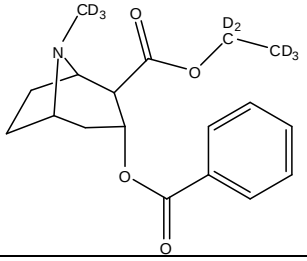
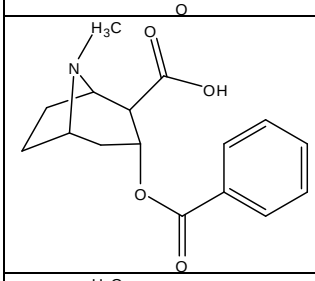
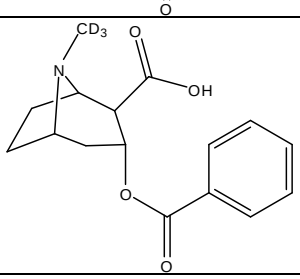
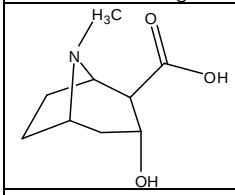
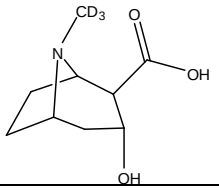
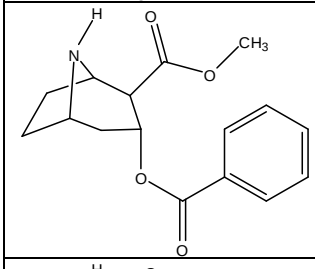
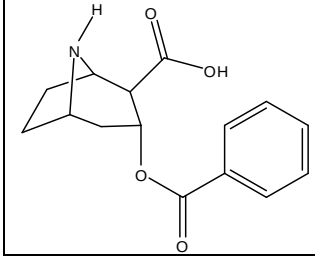
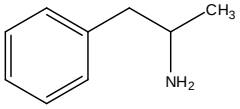
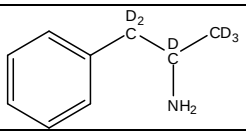
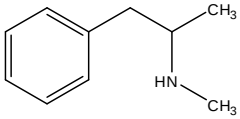
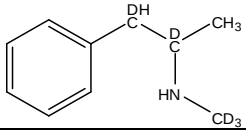
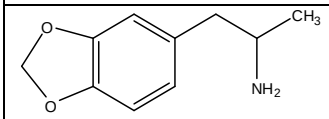
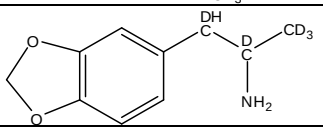
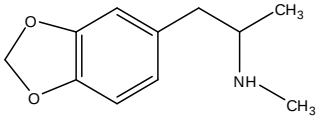
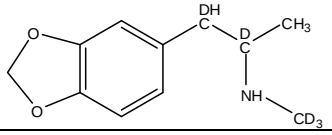
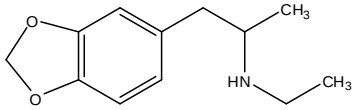
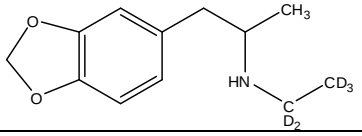
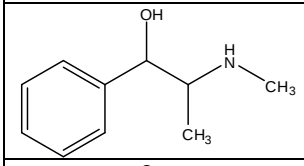
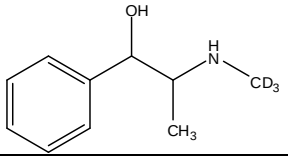
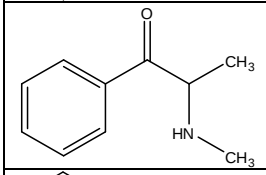
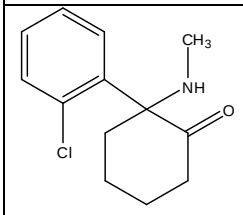
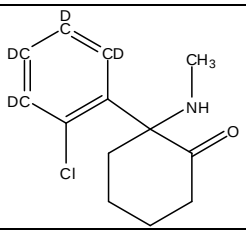
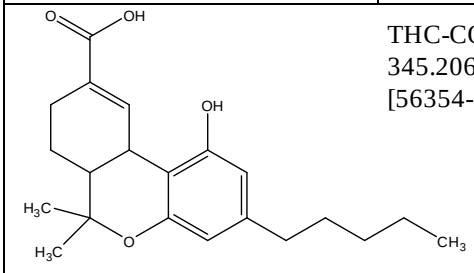
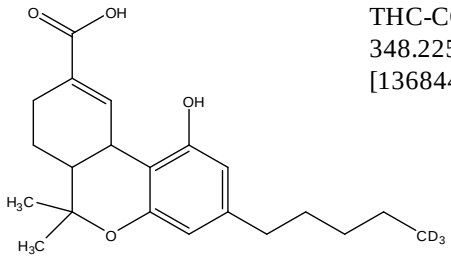
Structure	**Compound** **Exact mass $[M+H]^+$** **CAS number**	**Structure**	**Compound** **Exact mass $[M+H]^+$** **CAS number**
	Cocaine 304.1549 [50-36-2]		Cocaine-d_3 307.1737 [65266-73-1]
	Cocaethylene 318.1705 [529-38-4]		Cocaethylene-d_8 326.2207 [152521-09-0]
	Benzoylecgonine 290.1392 [519-09-5]		Benzoylecgonine-d_3 293.1580 [115732-68-8]
	Ecgonine 186.1130 [5796-31-6]		Ecgonine-d_3 189.1318 [N/A]
	Norcocaine 290.1392 [61585-22-6]		
	Norbenzoylecgonine 276.1236 [60426-41-7]		

Structure	Compound Exact mass $[M+H]^+$ CAS number	Structure	Compound Exact mass $[M+H]^+$ CAS number
	Amphetamine 136.1126 [300-62-9]		Amphetamine-d_6 142.1503 [N/A]
	Methamphetamine 150.1282 [537-46-2]		Methamphetamine-d_5 155.1596 [60124-88-1]
	MDA 180.1024 [4764-17-4]		MDA-d_5 185.1338 [136765-42-9]
	MDMA 194.1181 [42542-10-9]		MDMA-d_5 199.1495 [13675-43-0]
	MDEA 208.1337 [82801-81-8]		MDEA-d_5 213.1651 [160227-43-0]
	Ephedrine 166.1232 [50-98-6]		Ephedrine-d_3 169.1420 [N/A]
	Methcathinone 164.1075 [152610-69-0]		
	Ketamine 238.0998 [1867-66-9]		Ketamine-d_4 242.1249 [N/A]
	THC-COOH 345.2066 [56354-06-4]		THC-COOH-d_3 348.2254 [136844-96-7]

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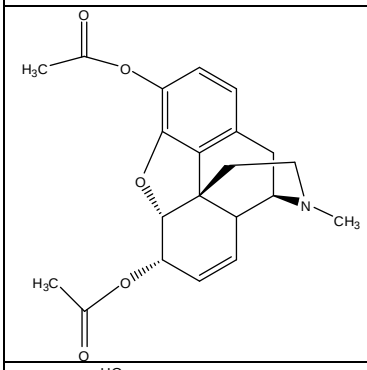
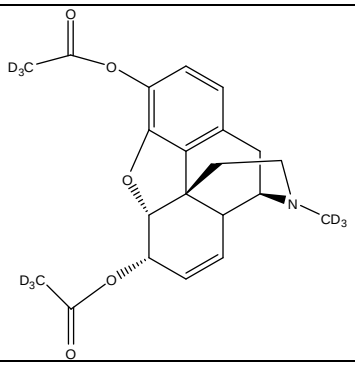
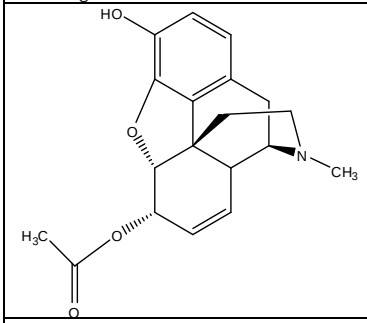
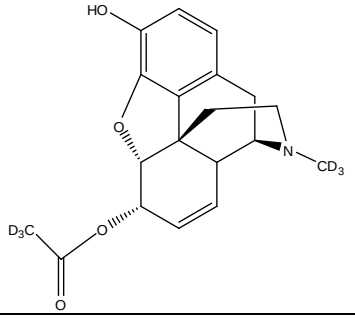
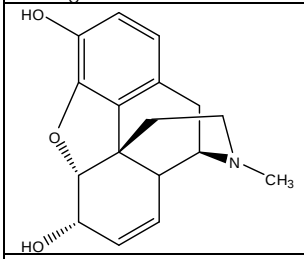
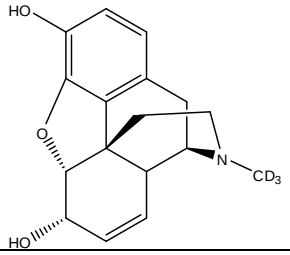
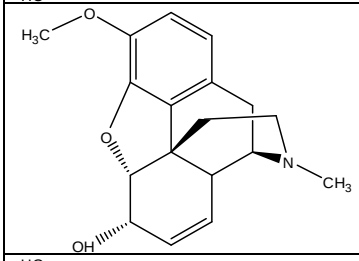
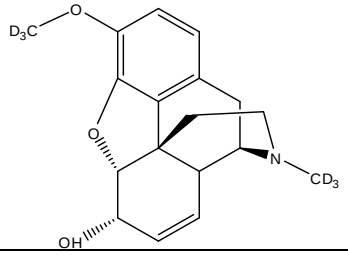
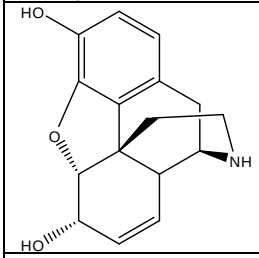
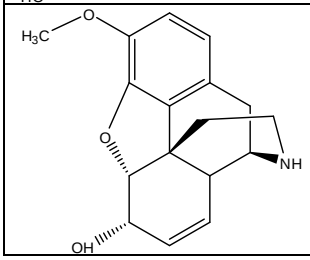
Structure	Compound Exact mass $[M+H]^+$ CAS number	Structure	Compound Exact mass $[M+H]^+$ CAS number
	Heroin 370.1654 [561-27-3]		Heroin-d_9 379.2219 [N/A]
	6-MAM 328.1549 [2784-73-8]		6-MAM-d_6 334.1925 [152477-90-2]
	Morphine 286.1443 [57-27-2]		Morphine-d_3 289.1631 [67293-88-3]
	Codeine 300.1599 [76-57-3]		Codeine-d_6 306.1976 [N/A]
	Normorphine 272.1286 [466-97-7]		
	Norcodeine 286.1443 [467-15-2]		

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Table SI 2: Optimum MS/MS [(a)] and MS^E [(b)] conditions used for the interpretation of spectra of illicit drugs and their metabolites. (ESI operated in positive ionization mode)

Compound	Retention time (min)	Cone voltage (V)	High Collision Energy (eV)
THC-COOH	8.77	25	10-45
THC-COOH-d_3	8.77	25	10-40
Ketamine	3.76	15	10-40
Ketamine-d_4	3.74	10	10-40
Amphetamine	3.05	10	10-50
Amphetamine-d_6	3.03	10	10-40
MDA	3.08	10	10-45
MDA-d_5	3.03	10	10-40
MDEA	3.43	15	10-40
MDEA-d_5	3.42	15	10-45
MDMA	3.12	10	10-40
MDMA-d_5	3.11	10	10-45
Methamphetamine	3.15	15	10-40
Methamphetamine-d_5	3.12	15	10-45
Benzoylecgonine	3.78	15	10-40
Benzoylecgonine-d_3	3.78	15	10-40
Cocaethylene	4.67	20	10-40
Cocaethylene-d_8	4.66	20	10-40
Cocaine	4.10	15	10-40
Cocaine-d_3	4.10	20	10-40
Ecgonine	1.00	20	10-50
Ecgonine-d_3	0.99	20	10-45
Norbenzoylecgonine	3.92	15	10-45
Norcocaine	4.37	15	10-40
6-Acetylmorphine	2.83	25	20-60
6-Acetylmorphine-d_6	2.79	25	20-50
Codeine	2.45	20	20-60
Codeine-d_6	2.46	25	20-50
Ephedrine	2.66	10	10-45
Ephedrine-d_3	2.64	10	10-40
Heroin	3.97	30	20-60
Heroin-d_9	3.95	35	20-50
Methcathinone	2.64	15	10-40
Morphine	1.64	30	20-50
Morphine-d_3	1.62	35	20-50
Norcodeine	2.55	25	20-50
Normorphine	1.54	25	20-50

a) MS/MS conditions, selecting the protonated molecule $[M+H]^+$ as precursor ion.

785 b) Conditions used for MS^E in the HE mode. The collision energy applied in the LE mode was in all cases 4eV.

FIGURE CAPTIONS

Figure SI 1. Proposed ketamine fragmentation pathway (left). MS^E (HE) spectra (right) of $[M+H]^+$ of ketamine (A), ketamine-d_4 (B).

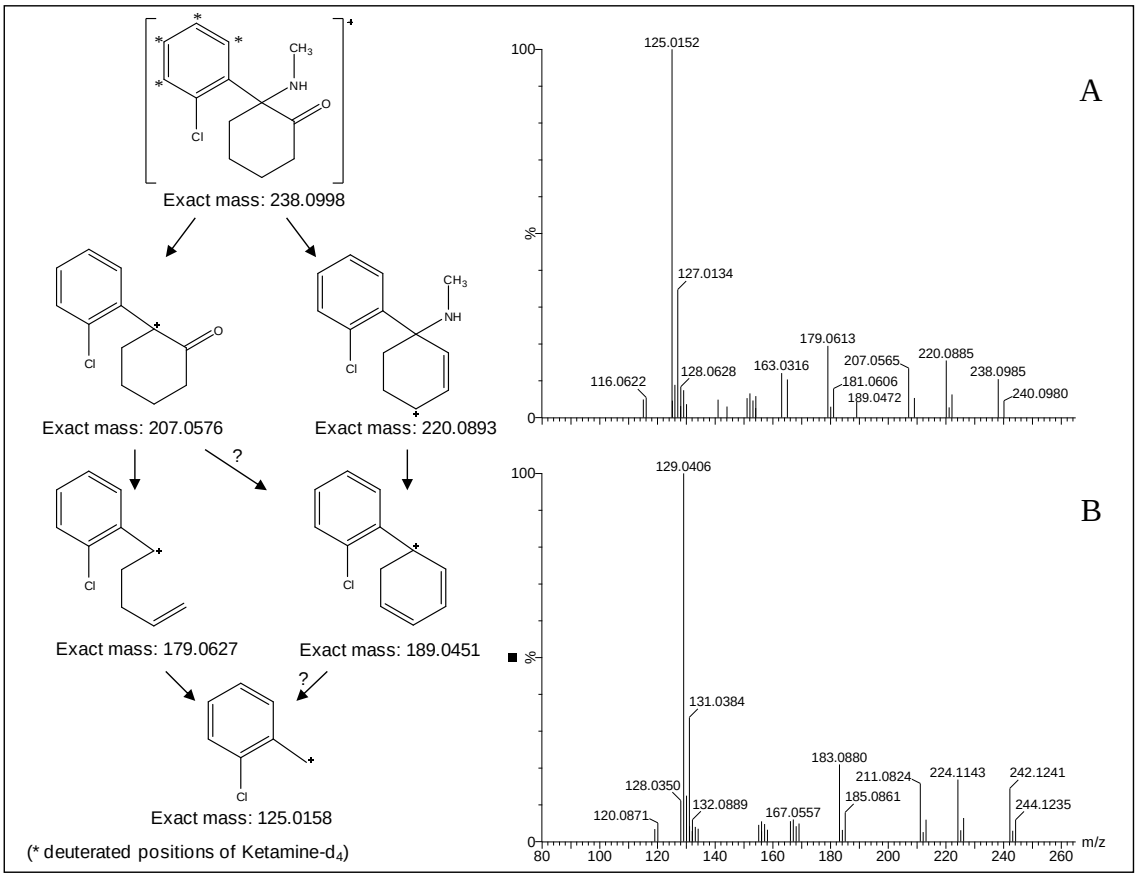

Figure SI 2. Proposed fragmentation route of opiates via morphinan backbone.