The key role of mass spectrometry in comprehensive research on new psychoactive substances

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Abstract

New psychoactive substances (NPS) are a wide group of compounds that try to mimic the effects produced by the "classical" illicit drugs, including cannabis (synthetic cannabinoids), cocaine and amphetamines (synthetic cathinones), or heroin (synthetic opioids), and which health effects are still unknown for most of them. Nowadays, more than 700 compounds are being monitored by official organisms, some of which have been recently identified in seizures and/or intoxication cases. Toxicological analysis plays a pivotal role in NPS research. A comprehensive investigation on NPS, from the first identification of a novel substance until its detection in drug users to help in diagnostics and medical treatment, requires the use of a wide variety of instruments and analytical strategies.

This paper illustrates the key role of mass spectrometry (MS) along a comprehensive investigation on NPS. . The synthetic cannabinoid XLR-11 and the synthetic cathinone 5-PPDi have been chosen as representative substances of the most consumed NPS families. Moreover, both compounds have been investigated at our laboratory in different stages of the three-step strategy considered in this article. The initial identification and characterisation of the compound in consumption products, the first reported metabolic pathway, and the development of analytical methodologies for its determination (and/or their metabolites) in different toxicological samples are described. The analytical strategies and MS instruments are briefly discussed to show the reader the possibilities that MS instrumentation offer to analytical scientists. This publication aims to be a starting point for those interested on the NPS research field from an analytical chemistry point of view.

Keywords: New psychoactive substances; high-resolution mass spectrometry; tandem mass spectrometry; compound elucidation; toxicological analysis.

A brief introduction on mass spectrometry in NPS research

Mass spectrometry (MS) is, undoubtedly, one of the most powerful analytical techniques at present, and it is used in many research fields, from public health to development of new materials. This work will be focused on the application of MS in toxicological and forensic analysis [1]. This analytical technique is pivotal in these fields, due to the complexity of the samples to be analysed, but also to the wide number of compounds that can be subject of study from a toxicological point of view.

The psychoactive "market" has notably changed in the last decade due to the increment in the use and availability of the so-called new psychoactive substances (NPS). Although the "classical" illicit drugs (such as cannabis or cocaine) are the most consumed [2], the NPS use has increased since 2010, as illustrated by the number of novel compounds reported every year and the number of NPS seizures, according to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [3]. By the end of 2018, more than 730 NPS were monitored in Europe, the most important ones being (in terms of number of reported compounds and seizures) synthetic cannabinoids, synthetic cathinones, recently reported amphetamines and synthetic opioids [3]. Similarly to the drugs of abuse, the analytical determination of NPS in biological samples, seizures, surfaces or environmental compartments is mostly based on the use of MS for a reliable compound identification [4].

The selection of the MS instrumentation in toxicological analysis mainly depends on the aim of the study and the complexity of the sample. However, in many cases the major limitation is the equipment available in the laboratory. By one side, single low-resolution MS and low-resolution tandem MS (MS/MS) are usually applied for the targeted determination of a limited number of compounds mainly for quantitative

purposes [5–9]. By other side, high-resolution MS (HRMS) is more appropriate for the screening of a large number of compounds [4,10,11], identification and characterisation of novel NPS [12–15], and elucidation of their metabolites [16–19].

Although almost all the toxicological analyses use chromatographic separations coupled to MS, the recent use of ambient ionisation sources, which allow the direct analysis of NPS in different matrices, has proved to be an interesting approach. Thus, analytical strategies based on direct analysis in real time (DART) [20–22], desorption electrospray (DESI) [23], atmospheric solids analysis probe (ASAP) [24,25], or other techniques recently developed such as swab-touch spray [26] , can be found in the literature. These techniques are specially indicated for the analysis of seized drugs as they are relatively pure in comparison with biological specimens; hence, the reduced need of a chromatographic separation [25,27].

Nowadays, different MS techniques and analytical strategies (including ionisation sources, mass analysers and data processing) are available to face the different steps included in a comprehensive research on NPS. In order to illustrate the key role of MS in this field, two compounds representative of the most consumed NPS families have been selected: the synthetic cannabinoid XLR-11 and the synthetic cathinone 5-PPDi (**Figure 1**). The authors of this feature article have experience on NPS research, particularly on these families of compounds. We aim to take profit of such knowledge to better illustrate the challenges and achievements related to the application of MS in this exciting field of research. The role of MS is focused on the three main stages associated to the monitoring of NPS consumption (**Figure 2**): firstly, detection and analytical characterisation of NPS in seizures; secondly, metabolic and pharmacokinetics studies to select the most suitable consumption biomarkers; and thirdly, detection/identification/quantification of these biomarkers in biological fluids

from potential human consumers. The instrumentation and analytical strategies applied along these stages are discussed as well.

Facing the unknown: identification and characterisation of novel compounds

When a novel or unknown NPS is suspected to be present in a seizure or sample delivered to a drug testing service, the first step is the structural elucidation and full-analytical characterisation of this unknown compound. The drug testing laboratories are commonly equipped with gas chromatography-MS using electron ionisation (GC-MS) and Fourier-transformed infrared (FTIR) spectroscopy instruments. These instruments present ease of use and the availability of spectral libraries, such as the SWGDRUG MS Library (swgdrug.org/ms.htm, examined by the NIST Mass Spectrometry Data Center), the Cayman Chemical GC-MS spectral library [28] or the RESPONSE FTIR database [29]. Nevertheless, these techniques do not usually allow the complete structural elucidation of the putative NPS, and additional analytical techniques are required [30,31]. The complementary use of HRMS and nuclear magnetic resonance (NMR) has proven to be a powerful tandem for the unequivocal identification and characterisation of organic compounds [32], including NPS [33–35]. In this step, technical expertise plays an essential role, at least, to categorize an unknown compound based on its HRMS spectrum.

In the case of the NPS selected in this paper, the synthetic cannabinoid XLR-11 was found in a "legal" smoking mixture available in a kiosk in Russia in 2011 [36], while the synthetic cathinone 5-PPDi was detected in 2018 in a drug sample provided by an anonymous user to a drug analysis service [37]. In both cases, the combination of MS-

based techniques and NMR was mandatory for the unequivocal identification of compound structures.

The structure of XLR-11 was tentatively identified based on the GC-MS data. Its fragmentation was similar to another related synthetic cannabinoid (later known as UR-144) also present in the smoking mixture [36], which, in turn, had been previously found based on the common fragmentation with the reported synthetic cannabinoid JWH-210 [36]. The common fragmentation pathway strategy for the identification of related compounds has been successfully applied in metabolism experiments [16,17,38], and has been found very helpful for the identification of unknown NPS, such as the opioid U-49900 [14]. **Figure 3** shows the GC-MS spectra obtained for the synthetic cannabinoids JWH-210, UR-144 and XLR-11 (also known as 5-fluoro UR-144). On the one hand, and as highlighted in this figure, ion at *m/z* 144 is shared by the three compounds, indicating that this part of the molecule is not altered. On the other hand, ion *m/z* 214 is observed for JWH-210 and UR-144, while this fragment presents a mass shift of +18 Da (*m/z* 232) for XLR-11, indicating that the compound includes a fluorine atom. Due to the lack of analytical reference standard for XLR-11, the structure confirmation was finally performed by NMR and HRMS [36]. In this case, HRMS was used for determining the elemental composition of the synthetic cannabinoid, which in combination with ^{1}H and ^{13}C NMR experiments, provided enough evidences for the unequivocal identification of XLR-11. In early 2013, this compound was also found in smoking mixtures in Japan, being characterised by GC-MS, LC-MS, DART-HRMS and NMR [39].

In the case of the cathinone 5-PPDi, preliminary analysis by GC-MS and FTIR did not allow the identification of the compound, as its spectra were not available in scientific libraries [37]. Moreover, the compound presented high fragmentation in GC-MS and the molecular ion was not present in the spectrum [37]. On the contrary, the analysis by liquid

chromatography (LC)-HRMS (QTOF instrument) allowed the tentative identification based on the accurate mass data obtained for product ions and neutral losses, as carefully detailed in the corresponding publication [37]. **Figure 4** shows the schematically elucidation of 5-PPDi based on the accurate-mass fragmentation observed. A plausible fragmentation pathway for this compound was proposed based on the product ions and neutral losses observed [37]. The availability of the fragmentation pathway facilitates the subsequent identification of related compounds that may be present in seizures as well as the structural elucidation of its metabolites, as discussed in the next section. Similarly to XLR-11, the structure of 5-PPDi was unequivocally confirmed by different NMR experiments, as the analytical reference standard was not available when its characterisation was published [37]. Additionally, GC-MS and FTIR data were also acquired to provide other laboratories information that might be useful for future suspect identifications of 5-PPDi[37].

Undoubtedly, the availability of GC-MS spectral libraries makes easier the identification of novel compounds when related molecules have been previously analysed. However, not all NPS are GC-MS amenable (for example, the thermolabile 25X-NBOH, where X can be Cl, Br or I) [40,41], and therefore, require a previous derivatization. Additionally, when analytical data are not available for similar/related compounds, HRMS instruments result crucial for a structure elucidation from scratch. Ideally, the suspicious compound should be confirmed by the use of analytical reference standards, which unfortunately uses to be commercially unavailable for new NPS. Under these circumstances, the compound might be synthetized and used as analytical reference. In any case, the confirmation by the use of reference standards implies a delay until the (commercial or synthetized) standard is available. Additional analytical techniques providing structural information, especially NMR, are much useful if an

unequivocal compound identification is required. Thus, most of the published studies dealing with the identification/characterisation of unknown compounds use the combination HRMS and NMR [12–15,34,35,42,43].

The selection of the instrumentation to solve a specific problem is sometimes limited to the MS equipment available in the laboratory. While, GC-MS(/MS) and LC-MS/MS equipment are common in toxicological laboratories and some drug checking services due to their easy use and suitability for quantification purposes, HRMS instrumentation requires an important financial investment, and highly trained technicians due to the complexity of the technique and data generated. When facing to unknowns, a deep knowledge on mass spectrometry and on fragmentation rules is required to reach an initial tentative identification. In the special case of drug checking services, where rapid turnaround time is crucial, the selection of an appropriate MS must be performed taking into account the need of a fast response. For that, GC-MS can be found in different drug checking services, such as those included in the Trans-European Drug Information project (TEDI) [44]. Moreover, the recently developed direct-MS techniques coupled to single-MS or MS/MS would also be much useful for these services, as they provide fast identification of major compounds present in samples [20,21,25].

Although, HRMS is highly appropriate for identification/elucidation of compounds, some problems can also be faced with low-resolution instruments, although an in-depth knowledge of MS is required. As an example, unknown compounds may be elucidated by MS/MS instruments, performing a full scan acquisition for detection of the precursor ion (usually $[M+H]^+$) and a product scan acquisition for compound elucidation based on fragmentation data. Recent studies have demonstrated the applicability of MS/MS instruments (e.g. triple quadruple, QqQ) for tentative identification of NPS making use of fragmentation data and spectral libraries in consumption products [25], and for tentative

identification of synthetic cathinones using common fragmentation strategies in combination with precursor ion scan and neutral loss scan acquisition methods [45].

Another issue to be addressed is the difference between a seized research chemical (pure powder or crystal) and a classical legal high (for example, herbal blends or pills). On one hand, the high purity of the research chemicals facilitates the analysis by HRMS and NMR, since only one organic compound is usually found[14,15,31]. On the other hand, legal highs present more complexity due to the presence of additional compounds, such as those coming from the herbs (in the case of herbal blends) or additives (in pills). In these cases, NMR cannot be directly used and the compound must be identified based on HRMS data [11,46].

Understanding NPS metabolism based on MS data

Once a new compound is detected, characterised and reported, the next step in MS-based NPS research would be the identification of its metabolites and the study of its pharmacokinetics. These studies have two main objectives: the proposal of consumption biomarkers in biological samples from consumers, and the identification of potential metabolites that could present certain toxicity [38].

The metabolic pathway of xenobiotics can be assessed by *in vitro* experiments. This approach presents high versatility as different models can be used, such as single enzymes, pooled human liver microsomes (pHLM), pooled human hepatocytes (pHH), or even an isolated whole organ [47]. In addition, *in vivo* approaches use living animals, and allow performing ADME (Absorption, Distribution, Metabolism and Excretion) studies [47]. *In vitro* approaches are most used to obtain metabolite profiling as they can be performed easier and faster than *in vivo* experiments. Although both approaches are

useful, the metabolites selected as biomarkers might sometimes differ from the human ones. So, the most accurate (but also more problematic due to sample availability and ethical issues) is to perform NPS metabolism studies using biological samples obtained from NPS users [47].

The first metabolic profiling of the synthetic cannabinoid XLR-11 was reported in 2013, using pHH in combination with LC-HRMS [48]. The QTOF instrument was operated in data-dependent acquisition (DDA) [48], which provides useful information on both accurate-mass full-range MS spectrum and accurate-mass product ions, in a single run [49]. DDA mode has been applied for the elucidation of NPS metabolites [17,19,50–52] as the low presence of interferents in pHH incubation media reduces the risk of non-detecting potential metabolites due to automatic MS/MS triggering collapse. The strategy used for phase I and phase II XLR-11 metabolite detection was the establishment of a set of expected biotransformations, selected on the basis of the structure of this NPS, which were searched for in raw data (suspect screening). When a suspect metabolite was detected, its structure was elucidated based on the observed DDA MS/MS product ions [48]. As expected, most of the metabolites shared product ions with the parent compound. This information was used to stablish the position where the biotransformation (hydroxylation, oxidation, dealkylation, defluorination, conjugation, etc) [48] occurred. In this way, up to 30 metabolites were detected for XLR-11 using pHH and LC-HRMS DDA acquisition, some of them common to its analogue NPS UR-144 [48].

Later, in 2016, 18 urinary XLR-11 metabolites were reported after analysis by LC-HRMS (QTOF) from an intoxication case, following a similar analytical strategy than the previously explained [53], and quantified by LC-MS/MS.

Figure 5 shows the MS/MS spectra of XLR-11 and two metabolites selected as suitable consumption biomarkers for this compound [48,53]: 5-hydroxypentyl UR-144 and the UR-

144 N-pentanoic acid metabolite [48]. As it can be seen, the three compounds share up to three product ions (*m/z* 97, 125 and 144), corresponding to unaltered moieties. The fourth highlighted product ion at *m/z* 232 (XLR-11), *m/z* 230 (hydroxylated metabolite) and *m/z* 244 (carboxylated metabolite) indicates the biotransformation site, corresponding to a hydroxylation+defluorination for the first metabolite, and an oxidation to carboxylic acid for the second one.

The same strategy -pHH incubation and subsequent analysis by LC-HRMS (Q-Orbitrap instrument in this case) using DDA mode- was used for the first reported metabolic pathway of the synthetic cathinone 5-PPDi [54]. In addition to the metabolites searching based on expected biotransformations, the compounds detected in the MS scan of 5-PPDi pHH incubations were automatically compared with blank pHH incubations, using an appropriate processing software [54]. This approach, also known as binary comparison, is typically used in metabolism experiments when a control sample is available [38]. The main objective is to detect unexpected metabolites, not considered in the expected biotransformation list, and to discard possible false positives from the incubation media that could present the same exact mass than a putative metabolite. In this way, only those compounds present in 5-PPDi pHH incubations but not in blank pHH incubations are considered as potential metabolites, performing subsequent automated MS/MS fragmentation to complement the information obtained [54]. When the detected compound presented MS/MS fragmentation compatible with a metabolic biotransformation, the MS/MS spectra were again acquired at three different collision energies, in order to obtain the maximum information possible [54]. The observed metabolite fragmentation was finally compared with 5-PPDi MS/MS fragmentation, in order to locate the position of the biotransformation [54]. Up to 12 phase I metabolites were identified and justified proposing their fragmentation pathways [54] . As illustrative

example, **Figure 6** shows the proposed fragmentation pathway for 5-PPDi and for the indanyl-carboxylated metabolite, as well as the MS/MS spectrum at 50 eV collision energy for the metabolite: the common fragments shared with 5-PPDi are highlighted in green whereas those fragments with a known mass shift are marked in red. It can be observed that all product ions were successfully justified based on metabolite structure and typical fragmentation routes [54] . Oppositely to most synthetic cannabinoids, after 180 min of pHH incubation, the 5-PPDi signal was around 67% respect to its initial response, indicating a relative high hepatic stability of this type of NPS [54]. The stability of the parent compound was also observed for other cathinones, which were detected in authentic urine samples [16,38]. Finally, the most suitable consumption biomarkers for this compound were proposed based on hepatic stability and analytical signal observed [54]. Other strategies that may be useful for the detection of metabolites, in addition to the previously commented based on binary comparison and search of expected biotransformations, would be the mass defect filtering, common fragment and neutral loss search, and *in silico* prediction.

Although the first metabolic study of XLR-11 and 5-PPDi was based on HRMS DDA data, the data-independent acquisition (DIA) mode has also been used for metabolite identification and elucidation [16,38,55]. Both acquisition approaches have advantages and limitations. On one hand, DIA is a standardized acquisition mode, whose related parameters are usually well established for HRMS analysis using a specific instrument. In this mode, all the information on the ionisable compounds in the sample is acquired at both low and high collision energy, i.e. information on the (de)protonated molecules (and/or adducts) and on their fragment ions is acquired in a single injection. Instead, DDA triggering must be carefully optimised in order to promote the MS/MS acquisition of the compounds of interest. On the other hand, the use of DDA allows the acquisition

of a “pure” MS/MS spectrum, while the DIA spectrum can contain fragment ions coming from different co-eluting precursor ions, hampering the fragment assignment and spectrum interpretation.

In the light of the scientific literature, most NPS metabolite profiling studies are carried out using HRMS. As commented above, low resolution instruments, such as QqQ, can also be used for the detection of compound-related compounds in biological matrices [56]. Taking profit of the common fragmentation between metabolites and parent compound, QqQ has been used for the investigation of prednisolone [57], betamethasone [58] or corticosteroid [59] metabolites in human urine, as well as for NPS metabolite profiling (e.g. of the synthetic tryptamines *N,N*-diallyltryptamine and 5-methoxy-DALT [52], and the piperazine methoxypiperamide [60]). In subsequent steps, the product ion scan of the putative metabolite can be acquired in order to assess its fragmentation. A detailed comparison of the metabolite spectrum with that of the parent compound may be feasible to establish the position where the biotransformations occurred. Up to our knowledge, this strategy for the use of QqQ instruments has not been used for NPS metabolite detection yet, but it might be successfully applied when HRMS instrumentation is not available in the laboratory.

Detection of NPS biomarkers in toxicological samples: from quantitative MS/MS analysis to HRMS screening

The last step in this comprehensive work would be the detection of the selected NPS biomarkers in forensic samples collected from potential consumers or intoxication cases. To this aim, a HRMS screening strategy directed towards detection and identification, and/or a target quantitative MS/MS analysis when analytical standards

are available, can be applied. Different identification criteria have been used for MS/MS analysis and/or HRMS screening, such as the recommendations of the Food and Drug Administration for animal drug residues [61], or the identification confidence levels in HRMS for small molecule identification proposed by Schymanski and co-authors [62]. Although quantitative analysis can be performed by LC-HRMS, the most used instrumentation for that purpose is (low resolution) LC-MS/MS. So, the next paragraphs will not consider HRMS quantification methodologies.

The first detection of XLR-11 in a consumer was published in 2014 [63]. The parent compound was detected in blood sample collected from a 22-year-old male driver in San Francisco by using LC-MS/MS (QqQ). The identity of the compound was confirmed by the acquisition of two selected reaction monitoring (SRM) transitions (**Figure 7**). The concentration of XLR-11 in blood was found to be 1.34 ng/mL. Prior to LC-MS/MS analysis, the blood sample was analysed by ELISA (enzyme-linked immunosorbent assay) and GC-MS as screening techniques, retrieving negative results for over one hundred of different drugs and metabolites. The publication remarks the need of specialized equipment and well-trained scientists for detecting these novel (by the time of publishing the study) substances in toxicological matrices [63]. XLR-11 was also related to two deaths (29-year-old and 32-year-old females, USA). Similarly to the case described above, the parent compound was found in blood by LC-MS/MS (QqQ), but it was not detected by ELISA methods [64].

The mentioned studies were focused on the detection of the parent compound (XLR-11) in blood. However, as XLR-11 is highly metabolised [48,65,66], it is important to monitor its major metabolites as well, especially when analysis are made on urine samples. Thus, the metabolites 5-hydroxypentyl UR-144, UR-144 N-pentanoic acid (both also common to UR-144) and XLR-11 N-4-hydroxypentylwere selected as analytical targets for XLR-

11 in the simultaneous quantification of 37 synthetic cannabinoids metabolites in urine by LC-MS/MS (QqQ) [67]. The methodology applied did not include parent compounds as the high hepatic metabolism showed by synthetic cannabinoids made quite improbable their detection in urine samples [67]. In the case of authentic hair samples, the parent compound XLR-11 was the major compound found by LC-MS/MS (QqQ), while its major metabolites were determined at lower concentrations [68].

LC-HRMS has also been used for the screening of synthetic cannabinoids metabolites in urine from NPS users. Several metabolites of XLR-11 were detected in urine by QTOF MS, obtaining the urinary metabolite profiling. Additional quantification was performed by LC-MS/MS (QTRAP instrument) [53]. In the case of HRMS screening, DIA and DDA strategies can be used for sample analysis; regarding data processing, different approaches can be applied (target, suspect, or non-target screening) depending on the aim of the analysis and the information available for the compounds investigated in the samples [69].

A recent work illustrates the convenience of performing complementary analyses of urine from potential synthetic cannabinoids consumers with the analysis of the herbal blends available in the geographical area of the users [46]. The aim was to identify the synthetic cannabinoids potentially consumed, and perform subsequent LC-HRMS (QTOF, DIA) suspect screening searching for the major reported metabolites for the compounds found in herbs [46]. In this study, XLR-11 and UR-144 were found in herbal blends available in a local smartshop. The XLR-11 major metabolite UR-144 N-pentanoic acid metabolite [53], was tentatively identified in some urine samples (**Figure 8**) collected from teenagers in juvenile offenders' centres [46]. Following this strategy, more evidences exist on potential NPS consumption, an issue that is particularly troublesome in the case of synthetic cannabinoids due to their extensive and rapid metabolism.

Additionally, this study remarked the relationship between screening the local supply market and the urine of the local population. Drugs are often localized, so it helps to look for what compounds have high local prevalence.

The metabolic behaviour of synthetic cannabinoids (the great majority excreted in urine as metabolites mixture but not as parent compound) is rather different to that observed for synthetic cathinones. A study published in early 2020 describes the detection of parent 5-PPDi, as well as the identification of phase I metabolites, in urine. Up to 4 metabolites were identified by LC-HRMS, and their structures confirmed after synthetizing the corresponding analytical reference standards [70]. Quantitative analysis resulted in 972 ng/mL for the major metabolite (indanyl-hydroxylated metabolite) and 335 ng/mL for unaltered 5-PPDi, the remaining metabolites being present at levels below 200 ng/mL [70]. In the case of the metabolic fate observed for 5-PPDi using pHH [54], the parent compound was the major one found after 180 min of incubation, illustrating that some differences can be observed when comparing *in vivo* and *in vitro* data. For other synthetic cathinones, such as MDPV and mephedrone, the parent compound was also detected in urine [16,38]. Up to our knowledge, there are no additional research articles dealing with 5-PPDi, probably because it has been recently reported. The identification of one or more specific metabolites of a certain drug in authentic human samples reinforces the evidence of its consumption, and is a clear evidence that the drug was actually consumed by the user and not a consequence of a contamination (e.g. during sample collection or manipulation)

An interesting area of research is known as wastewater-based epidemiology (WBE), a recent discipline able to provide data in near-real time about the consumption of illicit drugs, and other life-style substances, such as tobacco, coffee and alcohol, in a defined population by analysing the appropriate biomarkers in urban wastewater [71]. Although

less explored and with some limitations, WBE can be also applied to obtain information about the use of NPS [10]. To this aim, it is necessary to know the metabolic fate of NPS in order to direct the analysis towards appropriate biomarkers of consumption (i.e. parent compound and/or metabolites excreted in urine with enough stability in wastewater). Apart from the limited metabolic information for many NPS, their low concentration levels in wastewater, due to the low prevalence of NPS consumption compared with classical illicit drugs, and the matrix complexity, are another handicaps that complicate the reliable identification and accurate quantification (mainly due to important signal suppression produced by matrix effect). Some examples are the determination of synthetic cathinones by LC-MS/MS (acquiring in SRM mode) and/or LC-HRMS (DIA and DDA) in wastewater samples collected in urban populations and during music festivals [9,72,73]. The role of MS in this recent discipline is essential, as the reliable determination of most biomarkers in wastewater requires the use and deep knowledge of hyphenated chromatography-MS, particularly in research related with illicit drugs and NPS [74].

As a final note, recently developed ionisation sources that allow direct-MS analysis of psychoactive substances open a new scenario for NPS biomarker detection in toxicological samples. Some examples are the determination of amphetamine in urine by ASAP-MS/MS (QqQ) [24], or different psychoactive substances in saliva by swab-touch spray-MS/MS (QqQ) [75]. This approach will facilitate the development of fast NPS detection in toxicological analysis, and could be useful for the *in situ* analysis of biological fluids or seizures. It is expected to see more publications in the near future dealing with this issue, in which MS will be again the key of the success in the applications.

Conclusions

In this feature article, the role of mass spectrometry in the comprehensive research of NPS has been briefly discussed and illustrated with key examples. The synthetic cannabinoid XLR-11 and the synthetic cathinone 5-PPDi have been selected as model compounds to give more detailed information and better understand the power of MS in this research area. The use of different MS instruments and the analytical strategies applied have been discussed for both compounds along the different steps, comprising from the first identification of a novel NPS until its detection during toxicological analysis in authentic samples from drug users.

From the point of view of analytical chemistry, the first step consists on the identification and analytical characterisation of these two NPS. The compound XLR-11 was identified by GC-MS after comparing its fragmentation spectrum with related synthetic cannabinoids previously reported. Nevertheless, 5-PPDi was elucidated from scratch based on HRMS accurate-mass fragmentation and NMR. In a second step, the metabolic pathway for both compounds is studied, using pHH incubation and LC-HRMS instruments. Additional metabolite identification strategies based on the use of low-resolution MS/MS instruments are also discussed in this paper, in order to show different possibilities of MS-based analysis. Finally, the third step of a comprehensive research is the development of analytical methodologies (target LC-MS/MS and suspect screening LC-HRMS) for the detection of these compounds, as well as their major metabolites, in biological samples such as hair, blood and urine from drug consumers.

As briefly shown in this paper, MS-based forensic analysis is pivotal in NPS research. The essential role of MS not only applies to NPS but to many other analytes that are relevant from the toxicological point of view. An overview to the most recent scientific

literature allows concluding that forensic and toxicological analysis cannot be conceived without this powerful technique, a fact that can be extended to other areas or research where analytical chemistry is essential, such as environmental pollution or food safety, among others.

Acknowledgements

Authors acknowledge financial support from the University Jaume I (UJI-B2018-19) and from Generalitat Valenciana (Group of Excellence PROMETEO/2019/040). D. Fabregat-Safont acknowledges Ministerio de Educación, Cultura y Deporte in Spain for his predoctoral grant (FPU15/02033).

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Figures

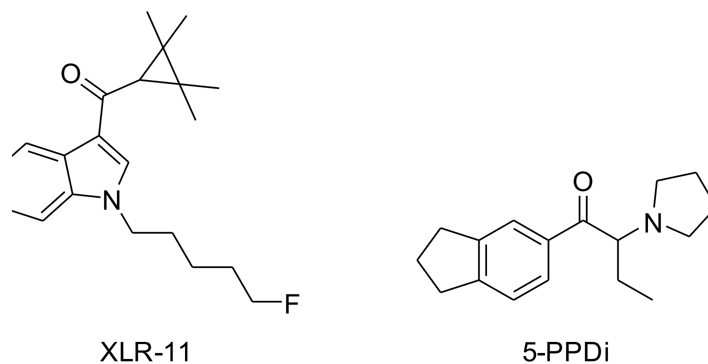


Figure 1. Chemical structures of the synthetic cannabinoid XLR-11 and the synthetic cathinone 5-PPDi.

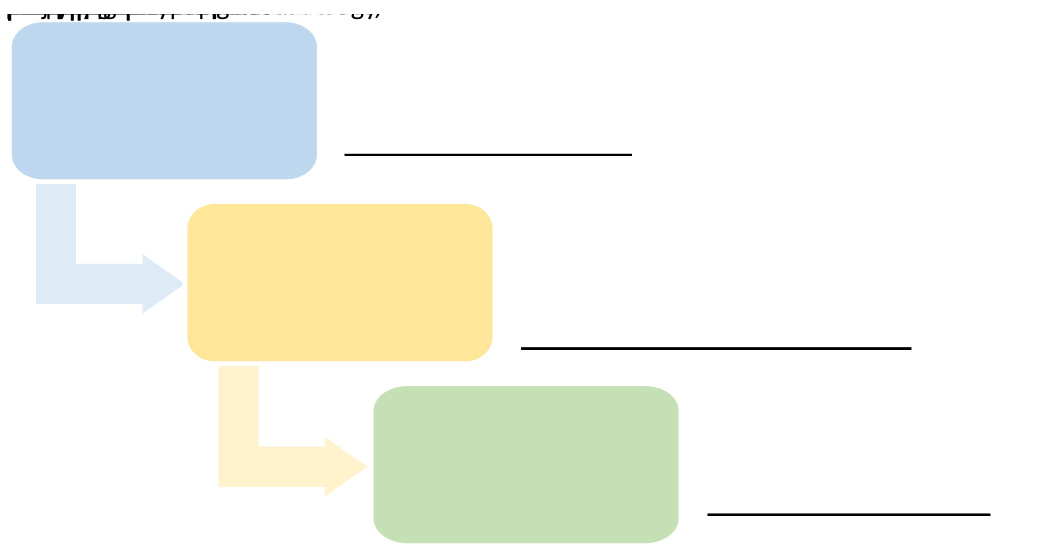


Figure 2. Diagram of the three-stage strategy for a comprehensive NPS investigation based on MS techniques. The main objective of each step, which will be the basis of the next step, is underlined.

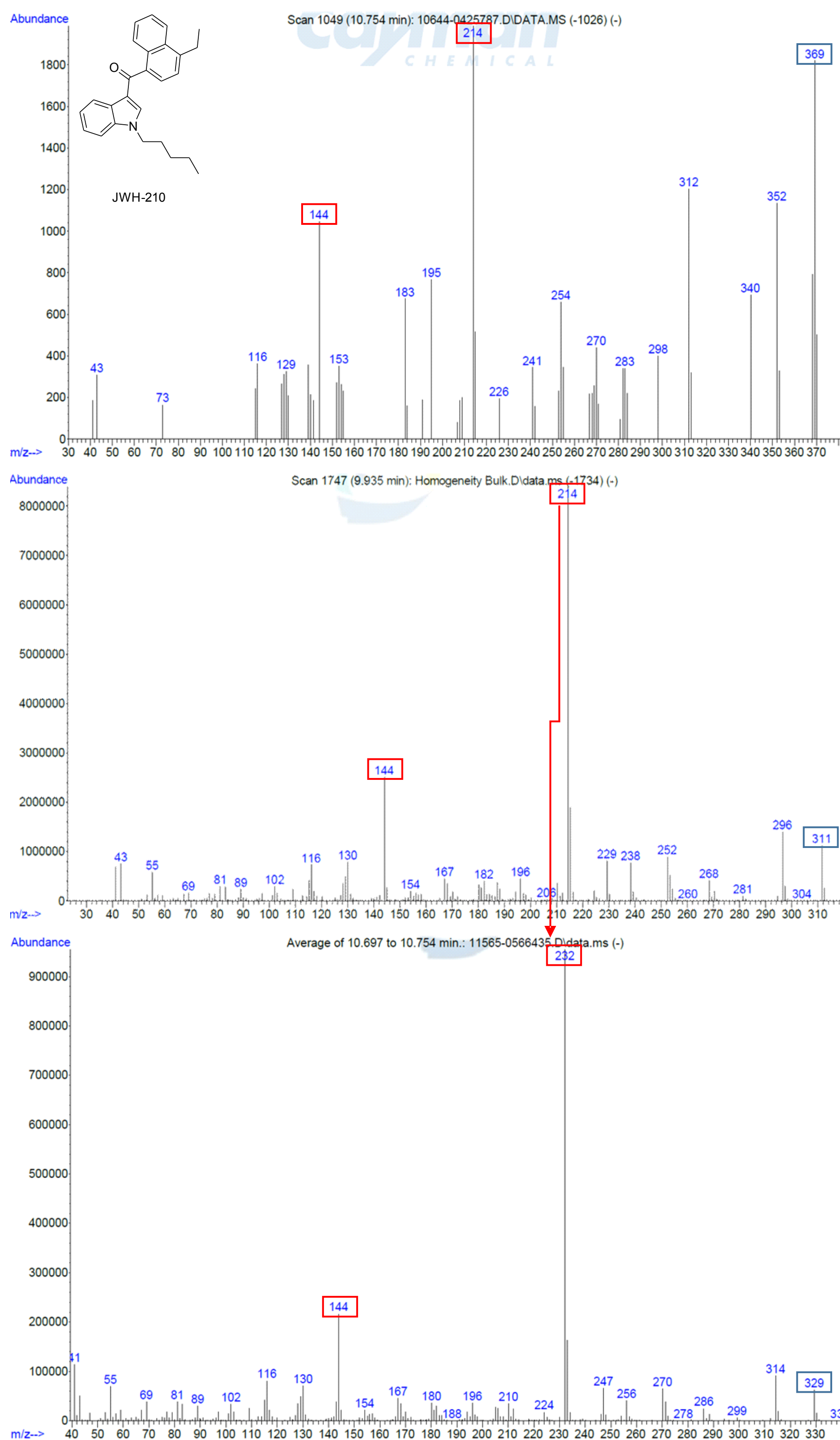


Figure 3. GC-MS spectra for JWH-210, UR-144 and XLR-11, obtained from Cayman Chemical webpage (https://www.caymanchem.com/). Common fragments, as well as fragments with a known mass shift are highlighted.

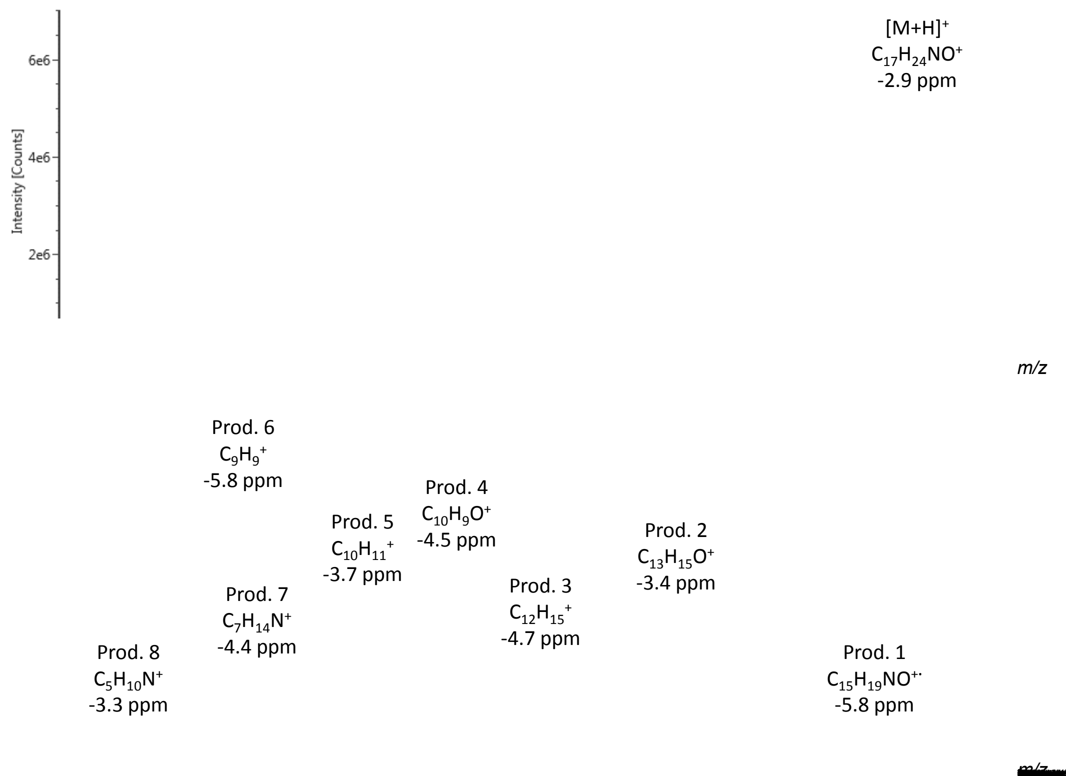


Figure 4. Spectra obtained for 5-PPDi by HRMS QTOF instrument using DIA acquisition. **A** Spectrum obtained without fragmentation, observing the protonated molecule. **B** Fragmentation spectrum obtained at a 15-40 eV collision energy ramp. Elemental composition and mass error are indicated for each ion. The interpretation of the fragmentation spectrum is also included in the figure. Adapted with permission from the corresponding publication [37].

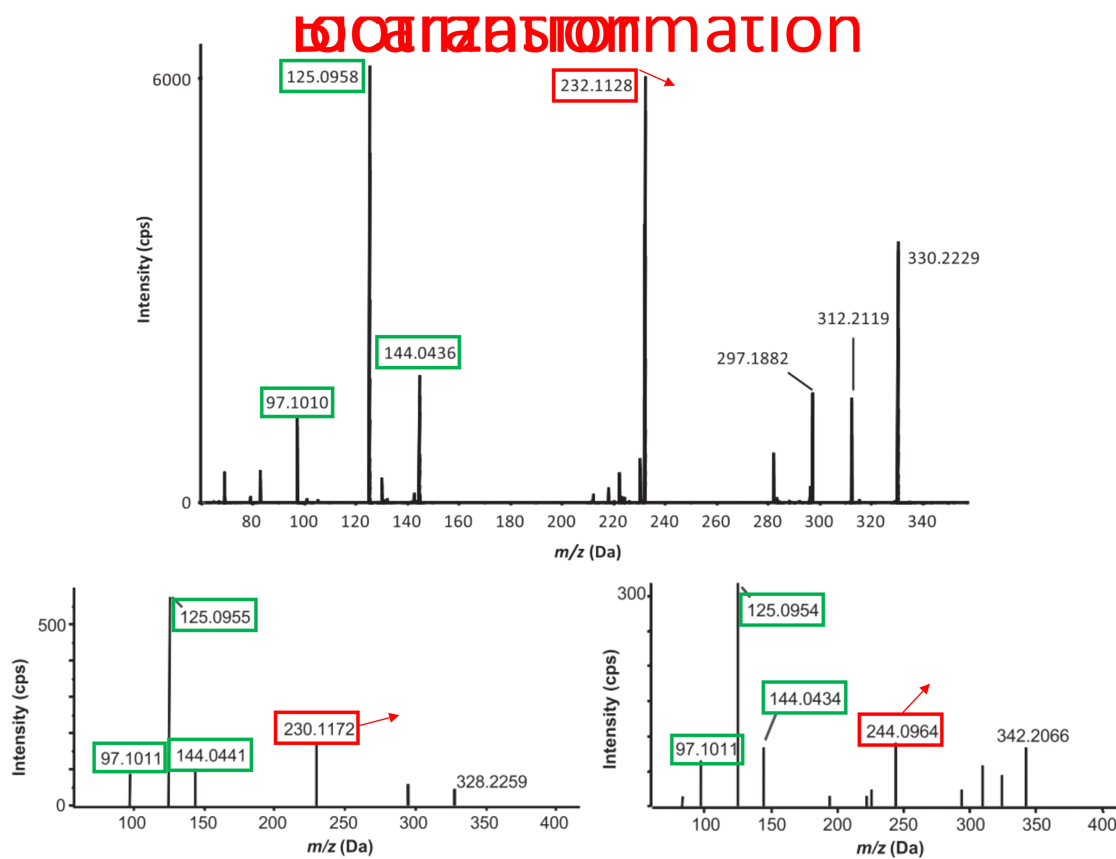
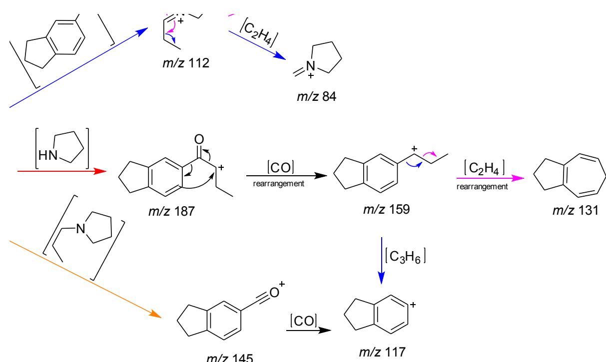


Figure 5. MS/MS spectra for XLR-11, 5-hydroxypentyl UR-144 and UR-144 N-pentanoic acid metabolite. Product ions corresponding to unaltered moieties, and product ions with a mass shift in concordance to the biotransformation that has occurred are highlighted. Adapted with permission from the corresponding publication [48].

boxylated indole metabolit



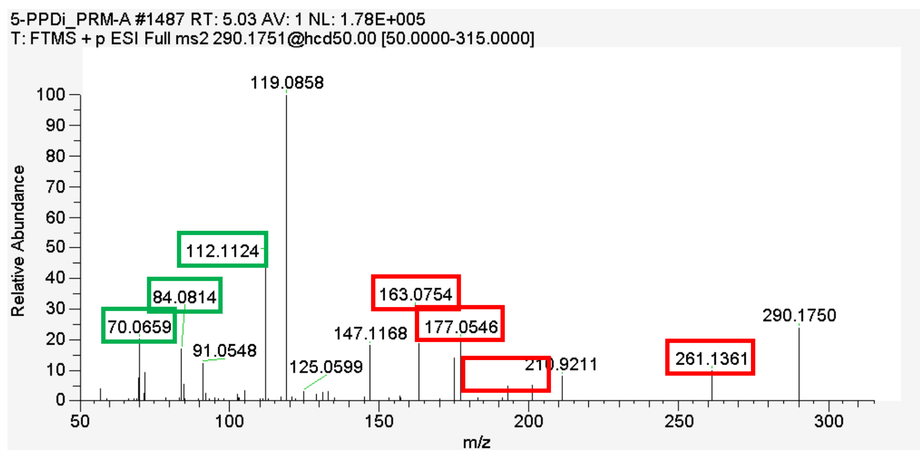
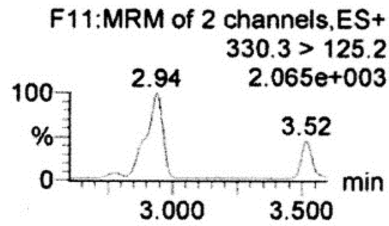
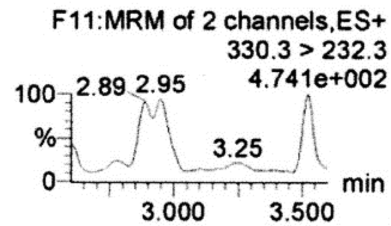


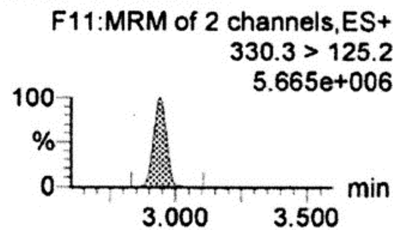
Figure 6. Proposed fragmentation pathway for 5-PPDi and the indanyl-carboxylated-metabolite. MS/MS spectrum at 50 eV for the indanyl-carboxylated-metabolite is included, highlighting product ions related to 5-PPDi. Adapted with permission from the corresponding publication [54].

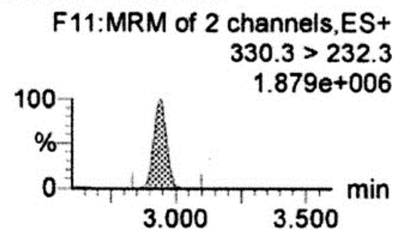
(A) BLANK



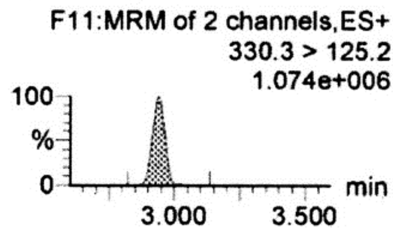


(B) POSITIVE QUALITY CONTROL





(C) DRIVER S1 WHOLE BLOOD SPECIMEN



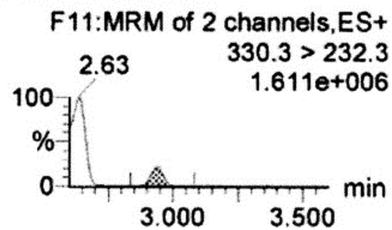
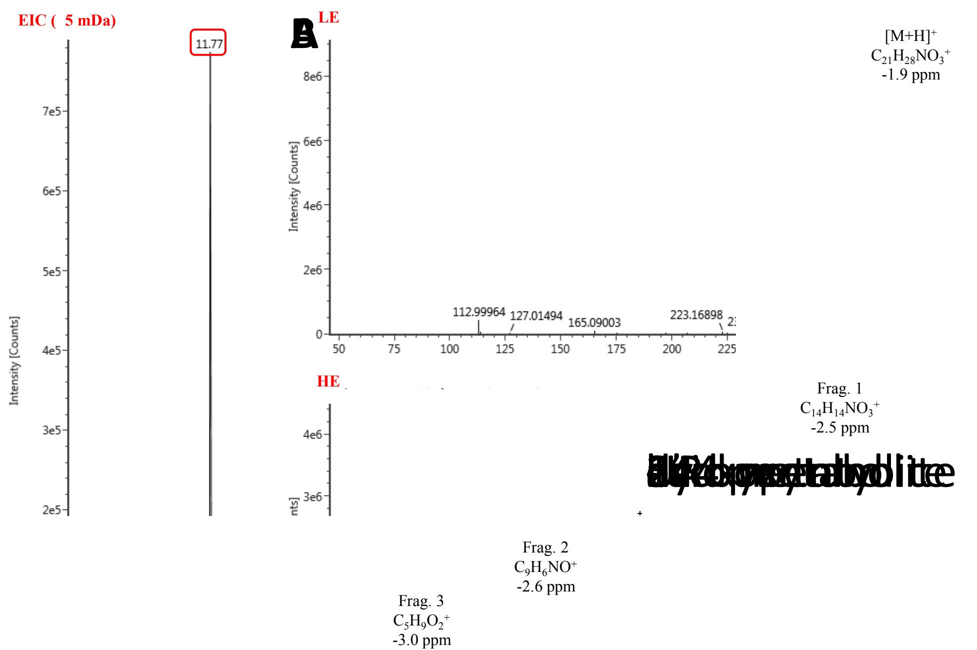


Figure 7. SRM XLR-11 chromatograms of (**A**) blank, (**B**) positive quality control, and (**C**) driver's whole blood specimen. Adapted with permission from the corresponding publication [63].



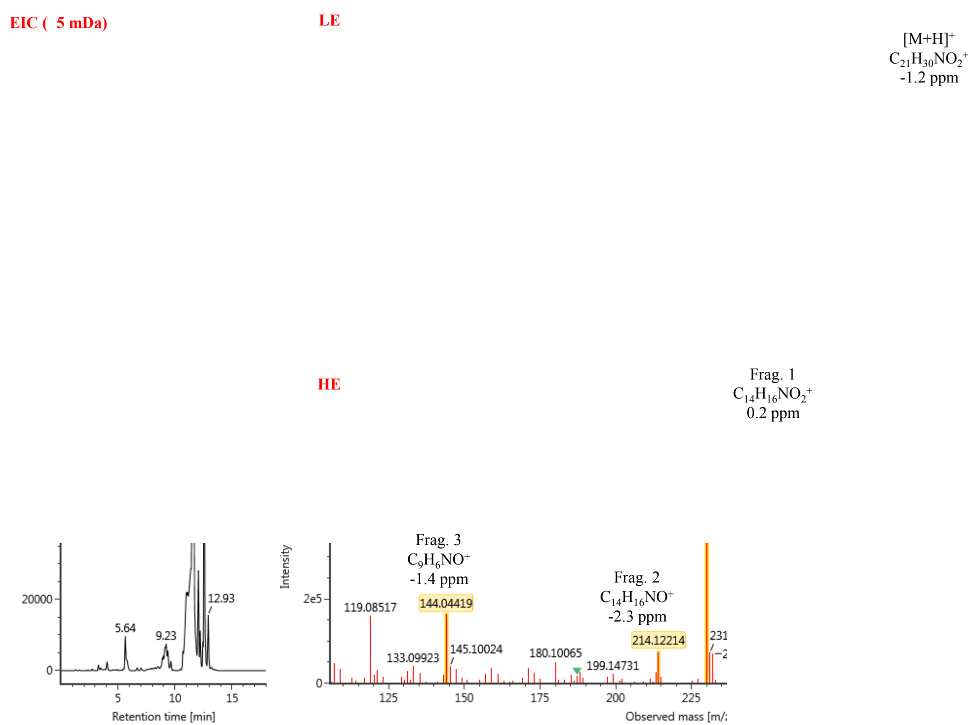


Figure 8. Tentative identification of the two major XLR-11 metabolites found in a urine sample from a teenager. Extracted ion chromatograms and accurate-mass spectra for UR-144 N-pentanoic acid metabolite (**A**) and 5-hydroxypentyl UR-144 (**B**) metabolites. Adapted with permission from the corresponding publication [46].