Suitability of high-resolution mass spectrometry in analytical toxicology: focus on drugs of abuse

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Abstract

High-resolution mass spectrometry (HRMS) is now the method of choice in several toxicology contexts. This paper reviews HRMS approaches for research and application in various toxicology fields, focusing on drugs of abuse in clinical and forensic toxicology. Papers concerning HRMS applications in screening, quantification and metabolism of drugs of abuse in biological and non-biological samples were included. Specific applications for new psychoactive substances in contexts such as online libraries, bioinformatic tools (molecular networking) and methods combinations were also included.

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1. Introduction

Drugs of abuse (DoA) are psychoactive substances that affect mood or behavior and are consumed for non-medical purposes. Their use is likely to induce addiction and psychic or somatic disturbances. For these reasons, DoA are defined and legally regulated at the international level.

In France, "the illicit transport, possession, supply, transfer, acquisition or use of narcotics" is punishable by ten years' imprisonment and a fine of 7,500,000 euros according to Article 222-37 of the Penal Code (2002) (1). It is also forbidden to drive under the influence of narcotics according to the French highway code, a rule that differs from one country to another (2). In 2019, data from the French Observatory of Drugs and Drug Addiction (OFDT) reported different consumption levels depending on the substance used: cannabis (45% of experimenters, 11% of users in the year) and cocaine (1.6% of users in the year) are widely consumed in France. However, other illicit drugs are marginally used by adults (5% of experimenters use hallucinogenic mushrooms or MDMA/ecstasy) (3). Classified at the international level by the Single Convention of 1961, DoA were transposed into French law by the decree of 22 February 1990. This classification includes cannabis (THC and its metabolites), cocaine (and its metabolite benzoylecgonine), opiates (opium, morphine, etc.), amphetamines (e.g. MDMA), and some of the new psychoactive substances (NPS) such as mephedrone and benzylpiperazine (4).

The detection and quantitation of DoA is of major importance from a forensic and public health point of view and represents an analytical challenge due to the wide variety of molecules used, particularly with the arrival of NPS. Since the 2000s, more and more NPS, also called research chemicals (RC), "legal highs", "designer drugs" and "synthetic drugs", have appeared on the drug market with the advent of the Dark Web. In general, only one function or chemical moiety differs from the original molecule, thus modifying its pharmacological properties. This makes the use of a molecule legal, simply because the derivative is not yet registered on the list of narcotic products (5). These molecules are of great interest for toxicologists, even if the constant renewal of substances and the difficulty of obtaining reference materials make it difficult to analyze and detect them. Above all, by investigation their consumption, toxicologists can report information on these emerging molecules to national and international authorities.

Mass spectrometry has a long history in toxicology laboratories. Since its development in the 1990s, liquid chromatography (LC) coupled to single-stage mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has evolved as a complementary technique to the gold standard of gas chromatography coupled to mass spectrometry (GC-MS). Originally used mainly for targeted qualitative and quantitative analysis of a limited number of compounds, the broad spectrum of scan types of modern LC-MS(/MS) have opened new possibilities for non-targeted toxicological screening. This allows the detection and quantification of a large number of molecules in a sample of unknown composition, particularly in clinical and forensic toxicology (6). While LC-MS/MS is able to determine the characteristics of a given drug, its lack of mass accuracy sometimes does not allow it to distinguish two molecules of the same mass and with the same fragments, so a more accurate technology is needed.

More recently, high-resolution MS (HRMS) has proved superior to other methods in terms of specificity and flexibility, allowing the exact mass of the molecule to be determined with an accuracy close to 1 ppm mass resolution (7). This resolution enables peaks of compounds with close m/z to be separated. It is also possible to assign an exact molecular formula, thus limiting the number of candidate compounds. The sensitivity of HRMS is also much higher than low resolution MS, which is particularly useful in forensic or roadside expertise cases where the notion of threshold is important. Furthermore, improvements in the specificity and chromatographic separation performance of HRMS have made it possible to differentiate isomers or molecules with very similar accurate masses, which was previously tricky (8).

In 2012, researchers showed that the chromatographic system influenced the mass accuracy of untargeted HRMS. In particular, they fund that high chromatographic separation power is essential to obtain high-quality HRMS data, as the mass accuracy and thus the number of detected compounds are strongly influenced by the co-eluting compounds (9). This also demonstrated the need to optimize HRMS-based detection methods, especially when the results are for use in clinical or forensic settings. In this context, new powerful bioinformatics tools are also emerging such as molecular networks or metabolomics, since the optimization steps include the reprocessing of the acquired data (10,11). Despite their great diversity, more and more databases used in HRMS for toxicological screening, listing NPS in particular, are emerging and allow faster detection, efficient medical management and retrospective data processing.

This paper reviews the applications and innovations of HRMS in human toxicology (advantages and limitations), especially in clinical and forensic toxicology focusing on DoA including NPS.

2. General applications for drugs of abuse including new psychoactive substances

The advent of LC-MS/MS has revolutionized DoA detection and HRMS goes one step furrther. In addition, HRMS has led to much more efficient screening methods both in terms of sensitivity and specificity, especially for forensic screening to identify drugs without a clinical context. NPS are also of great interest to toxicologists since the reference standards are still very expensive or not available and most of the metabolites are unknown. More sophisticated techniques are therefore required to solve these problems. In this section, we present the advantages of HRMS for the identifying and quantifying DoA including NPS.

2.1. Screening for drugs of abuse

Toxicological screening is a specific approach to analytical toxicology that allows the detection and simultaneous identification of a large number of compounds (12). DoA screening is suitable for both forensic and clinical applications.

In the emergency hospital setting, the rapid delivery of results is an important prerequisite as an early diagnosis of intoxication may improve patient prognosis. Sine urine is the major matrix for drug screening, immunoassay techniques predominate in most hospital laboratories because of their rapidity and ease of execution. However, only a few relevant molecules can be covered by currently available immunoassay techniques: classical DoA (THC, cocaine, opiates, MDMA and amphetamines are usually detected) while some drugs require specific chromatography coupled to mass spectrometry such as NPS due to their lack of commercial alternatives. Kronstrand et al. compared the performance of an immunoassay (IA) for synthetic cannabinoids with a recently developed confirmatory method using quadrupole-coupled time-of-flight mass spectrometry (QTOF-MS). The MS procedure proved superior, as new compounds and their metabolites can be quickly included and thus identified (13). The advantage of HRMS over the still widely used IA techniques is its high flexibility, which allows toxicologists to react to the dynamic and ever-changing NPS market.

In forensic toxicology, screening should allow the identification of drugs without reference to any clinical context. The same biological matrices are used as in clinical toxicology, but the difference is that the data are used for judicial purposes. In addition, the application of analytical methods on *post-mortem* samples involves dealing with sometimes putrefied samples and variable matrices (14). In this context, it is important to have comprehensive and specific screening methods such as LC-HRMS to identify new or rare drugs, or metabolites that can shed light on the investigation.

HRMS screening procedures are mainly qualitative and can be applied to most matrices, e.g. urine, serum, hair, or whole blood samples. Most chromatographic systems are coupled to an LC-QTOF or an Orbitrap. The main difference between most HRMS and MS/MS methods is that HRMS is flexible and cater for non-targeted screening. Previously, HRMS was considered best suited for multi-component targeting and the screening of unknowns. Nowadays, large-scale screening by HRMS is one of the most powerful and efficient analytical tools in the NPS field. HRMS accurate establishes the mass of analytes and their fragments, thereby allowing elucidation of their molecular formulas. The list of possible candidates can be narrowed down to one or a limited number of compounds, which can be further investigated in the available databases. In other words, the analysis can be performed initially without reference standards, because the overall information provided by HRMS (full-spectrum acquisition, precise mass of the (de)protonated molecule and relevant fragment ions, and isotopic profile) is relevant and allows the putative identification of the detected compounds (15). The ability to screen without having standards a priori is very useful, as laboratories do not need to purchase all the reference standards prior to analysis, with the attendant problems of cost and expiration dates. Reference standards are needed for final confirmation, but only as a last step, when there is strong, well-founded evidence that the compound has been identified in the sample. The addition of new NPS into existing qualitative workflows is straightforward and only requires the availability of reference compounds. An advantage of HRMS accurate-mass full-spectrum acquisition is the possibility of performing retrospective analysis. Thus, while initial data inspection may involve a targeted approach, designer analogues that are unreported or unknown at the time of analysis can be targeted subsequently in post-acquisition data processing without the need for additional analysis. This is mainly the case for NPS, which are often absent from them. (16).

HRMS has not changed the role that separative methods plays upstream of detection. Chromatography, whether liquid or gas, has pride of place in the final detection of compounds (16,17). The way in which the molecules are derived, the solvents used and the preparation of

the samples play an important role in the final detection, especially for isobaric substances. For example, the NPS beta-keto-2C-B ((2-amino-1-(4-bromo-2,5-dimethoxyphenyl)ethenone; exact mass: 274.0079 Da) and DOB (2,5-dimethoxy-4-bromamphetamine; exact mass: 274.0364 Da) can be differentiated by selecting the protonated masses of the precursors and by comparing the different elemental compositions of certain fragment ions (18,19).

Incorporating metabolites into urine screening procedures is more difficult (20). Reference NPS metabolite standards are usually not immediately available and may have already been withdrawn from the market. Kinyua et al. presented a qualitative screening method also based on all-ion MS to allow the detection and tentative identification of NPS and their metabolites (21). An HRMS method was also published for the simultaneous screening and confirmation of selected synthetic cannabinoids, cathinones, amphetamines, natural cannabinoids, opioids, cocaine, and other important DoAs, as well as their major metabolites in urine samples (22). Solid-phase extraction (SPE) was used as sample preparation prior to ultra-high performance liquid chromatography and TOF-MS using fragmentation without precursor selection (all-ion MS/MS) to avoid loss of information. Application data were included for 50 authentic urine samples. However, particularly extensive sample preparation, such as SPE and liquid-liquid extraction (LLE), could be replaced by simple dilution or precipitation using these highly sensitive systems (23,24). This approach is also contributive in judicial seizures, allowing the rapid identification of products and subsequent legal sanctions. Furthermore, the detection of impurities and cutting products is easier, sometimes revealing a ‘fingerprint’ that allows the location of the manufacturers to be traced.

These techniques also have limitations such as the cost of instruments, the complexity of data processing software and the requirement of skilled analysts (25). Other disadvantages of HRMS are the enormous size of the data, which can be a problem in terms of data storage. In addition, the examination of unknown HR data is laborious and time-consuming, and the analyst must be familiar with MS. Moreover, HRMS does not always provide additional knowledge. This is the case with already characterized molecules, for which detection and separation methods are robust. For example, roadside narcotics as defined by French legislation comprise only four families of well-known molecules: amphetamines, cocaine, cannabis, opiates, so HRMS is of little interest in this setting. HRMS systems will become more attractive not only as their cost decreases over time but also with the advent of more

robust software that is easier to use for identifying new compounds. However, HRMS may hold promise in situations where a combination of screening and quantification is required. Overall, HRMS appears to be the gold standard for non-targeted screening procedures because of its high flexibility, sensitivity, and selectivity.

2.2. Quantification of drugs of abuse

After DoA detection, their quantification allows the prediction of potential damage from intoxication, especially in blood. DoA should be quantified in most clinical and forensic cases of overdose. This is a prerequisite for evidence-based case interpretation, which can have a significant impact on clinical or forensic consequences and outcomes (26). This is particularly the case in clinical and forensic toxicology, where blood concentrations can be compared to reference values in order to evaluate the degree of intoxication. Quantification also allows the kinetics of elimination to be assessed, a necessary step in the follow-up of acute intoxication or withdrawal. Currently, LC-MS/MS is the most widely used technique for (multi-target) quantification (27–29). However, thanks to the high sensitivity it achieves by excluding all compounds with different elemental composition, LC-HRMS has become a powerful alternative for DoA quantification. Several recent studies have quantified DoA and/or their metabolites in biological samples using LC-HRMS with TOF or Orbitrap mass analyzers (30). These are summarized in Table 1. Multi-analyte LC-HRMS approaches to DoA quantification allow for much easier method development without the need for extensive MS optimization for each analyte and a limitation on the number of compounds investigated. This is also the case for “pharmaco-toxics” such as buprenorphine and ketamine, where dosing techniques are now well established (31,32). The Q-TOF detection and quantification of cathinones, hallucinogenic phenethylamines, and piperazines after salting-out assisted LLE with acetonitrile in whole blood was presented by Pasin et al. (33). Identification was based on the precise masses of the molecular ion, the two ions produced, and the retention time and quantification performed over the range of 0.05-2 mg/L. Concheiro et al. described the simultaneous quantification of 28 synthetic cathinones and metabolites (34) as well as 40 novel psychoactive stimulants in urine by LC-HRMS (35). A particularly interesting application for simultaneous identification and quantification of NPS in blood without the need for authentic reference standards was published by Ojanpera et al. (36). They combined gas chromatography coupled with nitrogen chemiluminescence detection (NCD) and atmospheric pressure chemical ionization Q-TOF MS. The GC stream was split between NCD

for single-calibration quantification, using the equimolar response of the detector to nitrogen, and Q-TOF MS for identification. This configuration showed promise for simultaneous qualitative and quantitative drug analysis in the absence of authentic reference standards.

Table 1. Examples of HRMS methods for drugs of abuse

Analytes	Matrix	Sample preparation	HRMS technology	Qual/quant	Ref.
THC	Urine	LLE	Orbitrap	Quant	(8)
Multidrug including Cocaine, amphetamines, and opiates	Blood	PP	MS^E-TOF	Quant	(37)
Methadone, Buprenorphine	Blood	LLE	Orbitrap	Quant	(38)
Ketamine	Hair	DPE	Orbitrap	Quant	(39)
>2,500 toxic compounds	Blood, urine	PP	QTOF	Qual	(40)
39 NPS	Urine	SALLE	QTOF	Quant	(41)
47 synthetic cannabinoid metabolites	Urine	SLE	QTOF	Quant	(42)
30 acidic and neutral pharmaceuticals	Blood	SPE	MS^E-TOF	Qual	(43)
30 common DoA	DBS	SLE	Orbitrap	Qual	(44)
Fentanyl and analogs	Oral fluid	SPE	QTOF	Quant	(45)
MDMA and phase II metabolites	Urine	SPE	Orbitrap	Quant	(46)
28 cathinones and metabolites	Urine	SPE	Orbitrap	Quant	(47)
Hallucinogens and opioids	Blood	LLE	Orbitrap (ThermoFisher)	Quant	(48)
LSD, GHB	No analytical method found in the literature				

LLE: liquid/liquid extraction; PP: protein precipitation; DPE: decontamination; pulverization, extraction; SALLE: Salting-out Liquid-Liquid Extraction; SLE: Solid-Liquid extraction; DBS: Dried blood spot; Qual: qualitative; Quant: quantitative

3. Innovative applications for new psychoactive substances

3.1. HRMS online libraries

An analytical reference standard is essential in forensic and clinical screening procedures for unequivocal identification. Even if they are available, however, the cost of purchasing samples of each NPS is prohibitive, given their volume and diversity. Moreover, delays in the manufacture and/or delivery of these products are unavoidable, so newly emerging drugs may be overlooked. A full-scan screening workflow may be needed to keep the laboratory up to speed for evaluating new cases where new NPS drugs may be present in the sample(s) being analyzed. This consists of screening large lists of compounds of interest in complex samples using their molecular formula (and the resulting calculated exact mass) if no reference standards are available (49). Regardless of how a laboratory chooses to handle the issue, there are several excellent free online tools to support NPS analysis, some of which are crowd- and open-sourced. One of the key ideas of crowd-sourced science is to alleviate the burden of a single lab and improve collective know-how by sharing user data and using the summed data of a group of peers. Hopefully, the implementation of crowd-sourcing science in NPS screening and intelligence will help to improve the quality and coverage of NPS analysis in forensic toxicology. Indeed, the use of external data sources can help laboratories around the world to detect emerging NPS by qualitative screening.

Query online compound databases and/or perform structure elucidation

In untargeted screening for NPS analysis, peak selection and filtering are performed on the full HRMS data to select a smaller number of peaks that may provide a clue about an NPS. In the untargeted approach, peak searching involves extracting all detectable peaks in a single analysis using a peak selection algorithm to generate a list of candidate masses that can be reliably used for identification. This shortlist of candidate masses can then be compared to existing MS libraries and/or used for mass spectral structure elucidation. The peak selection algorithm uses raw full-scan MS data (without prior knowledge of specific ions) to search for peaks that have a common elution profile. The masses are then grouped into compound masses, resulting in a list of candidate masses. The algorithm can apply user-defined compound filters, including blank subtraction, peak intensity and ion number to facilitate peak prioritization. This approach significantly reduces false positives and limits features that do not contain enough information to result in an identification attempt. The list of accurate candidate masses can then be subjected to molecular formula assignment and/or compared to a list of suspects, and the results can be confirmed using online MS data.

Acquire/purchase curated spectral databases for NPS

Purchasing a mass spectra database for NPS screening ensures that the screening data are accurate, collected systematically, and often accompanied by recommended LC and MS acquisition parameters and workflows for use in the intended contexts. This screening strategy does not rely on free online tools, but it is a very feasible alternative. HRMS screening libraries can be purchased, such as the "LC-HRMS/MS Library of Drugs, Poisons, and Their Metabolites" (50), the "Mass Spectra of Designer Drugs" (51), or the "Wiley Registry of Tandem Mass Spectral Data, MS for ID" (52). Waters®, Thermo Fisher®, Agilent® and SCIEX® also provide spectral libraries tailored to their respective instruments.

Download crowd-sourced screening library

MS data from one instrument can be used as reference data on another MS device with comparable parameters for screening purposes (53). For this reason, several users can share and use screening data. These data sources include data repositories such as the NPS Data Center (54), the RESPONSE database (55) or HRMS databases such as Massbank (56), MzCloud (57) or HighResNPS (58) (Table 2). The HighResNPS.com homepage was designed as a repository of HRMS data for NPS detection that can easily be converted into a screening library. The primary molecular identifier on HighResNPS is the InChIKey, which provides an unambiguous identifier, as opposed to a given name, SMILES string, or IUPAC name. Each data entry must include the protonated molecular ion and optionally one to three diagnostic fragment ions. Each user adds information about the analytical setup they use to generate their results. If some users have identical analytical setups, they can also use retention times with the MS data, which increases confidence in detection. HRMS identification of unknowns using HighResNPS does not distinguish between positional isomers, which could account for most of the incorrectly assigned NPS. In addition, HighResNPS.com has been used successfully to identify NPS in seizures (59), environmental samples (60), and for the retrospective analysis of *post-mortem* biological samples (61). In the current setup, data quality in HighResNPS increases when multiple users add data on the same compound. Unlike the NPS datahub, user-entered data are not manually verified and classified. Diagnostic fragment ions are added to the database with the molecular formula and exact mass, reducing the risk of rounding errors or mistakenly adding exact masses. Two limitations of HighResNPS.com are the manual data curation and the lack of peer reviewing. Continuous curation is an advantage of the commercial databases, which have designated funds and labor

for this. When a given NPS has multiple analytical entries in the database, the data self-validate, since different users with different standards are unlikely to report the same faulty data. Thus, if a positive hit is obtained after screening with HighResNPS, the user can further support identification by retrieving full HRMS spectra on MzCloud and/or additional information on NPS Data Hub, and ultimately obtain an unambiguous identification with an analytical reference standard. Note that when identification is based on HighResNPS screening data, it must always be confirmed using additional identification techniques, multiple analytical targets and/or analytical reference material.

Table 2. Examples of crowed-sourced online libraries available for HRMS

Online library	**Type**	**References**
General libraries		
Massbank	HRMS database public repository for small compounds (<3000 Da)	(56)
MzCloud	HRMS database	(57)
NPS libraries		
NPS data hub	Web-based community driven analytical data repository	(54)
RESPONSE database		(55)
HighResNPS	HRMS database	(58)

3.2 Combination with other structural identification methods

The confirmation of NPS identification requires the development of a specific, high-quality, comprehensive method. Toxicology laboratories are therefore limited and must adapt to the material they can purchase and try to set up legally acceptable exchanges between colleagues. In addition to the difficulties associated with molecule characterization such as identification, quantification and purity, analytical methods used routinely are not always sufficient to firmly identify NPS, especially in cases of powders or e-liquids. It is therefore sometimes necessary to combine several analytical methods. A selection of studies dealing with combination methods is summarized in Table 3.

For example, the analysis of seized drugs depends on the available instrumental facilities. Traditionally, this includes a combination of analytical techniques such as GC-MS, LC-MS,

Fourier-transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy for confirmation and structural elucidation (62). NMR is a physical phenomenon in which spin (most commonly 1H,13C,19F,31P) placed in a strong magnetic field absorbs and re-emits electromagnetic radiation. This method not only makes it possible to identify a compound unambiguously: it also confirms the absence of other organic compounds and determines the purity of a product (63–66). FTIR is gaining ground as the gold standard for fast non-destructive analysis and the detection of different compounds in complex matrices. When used alone, however, all these methods have limitations for the unequivocal identification of an unknown molecule (62). For example, LC which is the most widely used separative method, may not discriminate positional isomers (such as chloro-N,N-dimethylcathinone (CDC) and chloroethcathinone (CEC) or 3-MMC and mephedrone (4-MMC)) (59,67). In LC-MS/MS, their retention time may be identical as well as their fragmentation pattern. It is therefore necessary to use another method, such as nuclear magnetic resonance (NMR) spectroscopy, GC-MS, infrared spectroscopy or supercritical fluid chromatography (SFC) (68) to discriminate the structures. The availability of GC/MS libraries helps greatly in the identification of new drugs. For relatively pure samples, a GC–MS analysis together with an FTIR-spectrum can lead to the full identification of the substance if both spectra give a match with existing data. Unfortunately, in most cases involving NPS, commercial GC-MS libraries do not contain novel analogues. In this case, HRMS is often used to perform MS/MS to evaluate collision-induced dissociation (CID) pathways for putative structural elucidation, followed by confirmation using NMR and/or IR spectroscopy (Figure 1) (69). The combination of results from HRMS and NMR with data obtained by MS, MS/MS and IR can help to elucidate the structures of unknown substances prior to the syntheses of ad hoc standards. Systematic use of the multi-methods approach allows the structural definition and successful confirmation of unknown samples thanks to powerful complementary analytical techniques, despite the lack of a reference standard (Table 4).

Table 3. Studies of HRMS methods combinations for NPS identification

Context	Matrix	Analytes	HR-MS technique	Combined methods	Ref.
Seizure	Powders; crystals	NPSs	LC-Orbitrap	GC-MS; NMR	(62)
Seizure	Tablet powder	bk-2C-B	LC-Orbitrap	NMR	(19)
Seizure	Powder	deschloroketamine	LC-Orbitrap	GC-MS; NMR	(19)
Seizure	Powders	3-MeO-PCP; 3-MMC	LC-Orbitrap	sd-GC-FTIR	(70)
Seizure	41 powders	NPSs	GC-QTOF	NMR	(64)
Users	5 powders	SC	LC-QTOF	NMR; IR; GC-MS	(63)

NPS: new psychoactive substances; SC: synthetic cannabinoids

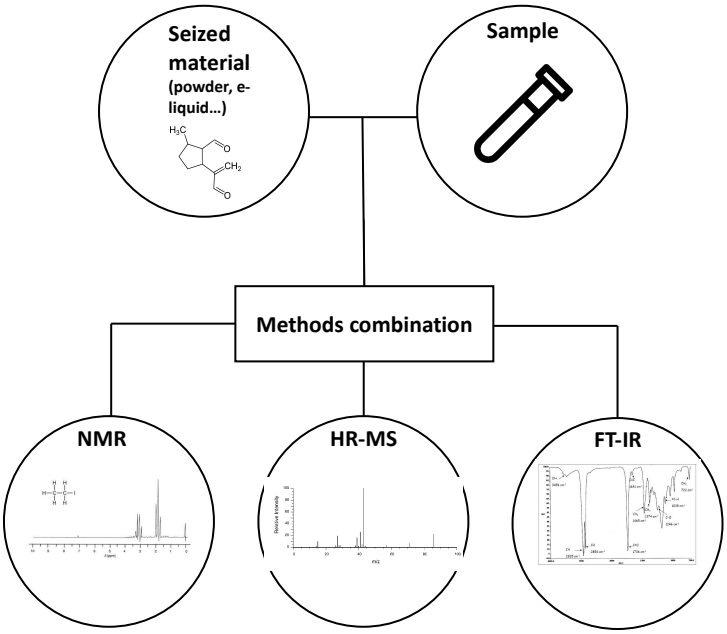

Figure 1. Methods combination with spectra

Table 4. Methods for identifying unknown powders

Step	Objective	Methods
1	Study of **mass spectrometric** behavior using electron impact ionization.	GC-MS
2	Study of **accurate molecular mass, raw formulas and isotopic clusters** obtained without fragmentation	LC-HRMS (ESI+)
3	Applying different fragmentation voltages to study **accurate fragment masses** obtained to determine their structures	LC-HRMS (ESI+)
4	**Molecular structure evaluation** by molecular weight determination and significant fragment interpretation using these two methods	GC-MS/LC-HRMS (ESI+)
5	**Isomer identification** in absence of certified standard	NMR/FTIR

3.3. Contribution of HRMS in metabolism studies

New varieties of NPS are constantly emerging on the market yet data on their pharmacodynamics or pharmacokinetics are lacking. This is especially an issue for compounds that are extensively metabolized such as synthetic cannabinoids (71) and NBOMe (72), where the parent compound itself is not a reliable target. To increase the chances of detecting the consumption of these NPS to increase the detection window in biological matrices, the identification of NPS metabolites appears unavoidable so metabolism studies need to be done. This is the case for both clinical and forensic cases, especially in matrices known to accumulate metabolites such as bile and urine.

3.3.1. Study models

Before the analytical phase involving mass spectrometry, care must be taken in choosing the right model for characterizing metabolites, since this conditions the relevance of the subsequent steps. For ethical reasons, pharmacokinetic studies in humans are not usually carried out as soon as an NPS emerges, and animal experiments should be conducted

parsimoniously. Consequently, several *in vitro* models for identifying the metabolism of NPS have been designed to anticipate the behavior of these xenobiotics *in vivo* (73).

Primary human hepatocytes (PHH) are considered the gold standard in *in vitro* metabolism studies, as they express relevant human enzyme clusters, co-substrates, and drug transporters (74,75). It is now possible to explore the metabolism of numerous NPS such as cannabinoids (71,76), cathinones (77) NBOMe (78) or benzodiazepines (79). However, their limited availability, high cost, variability in the expression of metabolic enzymes and the loss of their phenotype over time may limit their applicability.

Metabolism studies should be conducted in a fast, simple, cost-efficient and reliable way. Consequently, alternative stable cell sources have emerged, allowing NPS metabolism studies in pooled human liver microsomes (pHLM) (80–83), pooled human S9 fraction (pS9) (73,84) and differentiated HepaRG cells (83,85). Interestingly, these three models have shown comparable results to PHH for identifying both major phase I and phase II metabolites (86). The HepG2 human hepatoma cell line, another model capable of expressing metabolic enzymes, has also been used, although it produces a lower number and lower abundance of metabolites compared to HepaRG (87). Data of *in vitro* model systems were also compared to human urinary metabolites (73). Surprisingly, no 3D model seems to have been used to study NPS metabolism so far.

In recent years, zebrafish (*Danto reto*) larvae (embryos) have increasingly been used as a new *in vivo* model to study metabolism (73,85), and a recent study revealed the NPS metabolism of new cannabinoids by using zebrafish (88). NPS metabolism *in vivo* investigations are frequently performed using authentic clinical or forensic cases (89,90). They provide data that reflect the behavior of xenobiotics in the body and take into account the absorption, distribution, and elimination of the substance, which also underlines the relevance of urinary consumption markers. The importance of this work has even led some researchers to swallow NPS to study their pharmacokinetic parameters (91).

3.3.2. Bioinformatic tools

HRMS coupled with bioinformatics methods is very effective in identifying metabolites. Table 5 shows some examples of bioinformatics tools allowing (i) the prediction of

metabolism sites and metabolite structures, (ii) the processing and analysis of mass spectrometry data, and (iii) the visualization of mass spectrometry data. Recently, molecular networks (MN) have led to the organization and representation of tandem mass spectrometry (MS/MS) data in a graphical form (92). MN visually represent chemical similiarity among analytes in an untargeted MS/MS analysis. Each node represents an ion and its associated fragmentation spectrum, and the links between the nodes indicate similarities between spectra (Figure 2). Since some mass differences correspond to well known biotransformation reactions, it is possible to determine the structure of potential metabolites by information propagation. In addition, the MN approach offers valuable insights into drug metabolism by facilitating sample-to-sample comparison, and increases the number of metabolites compared to HRMS analysis without data reprocessing (93).

Table 5. Bioinformatics tools used in xenobiotic metabolic exploration

SOFTWARE	PURPOSE	REFERENCES
Metabolism prediction		
FAME 2	Prediction of sites of metabolism	(94)
SMARTCyp		(95)
SOMP		(96)
Xenosite		(97)
CYPReact		(98)
BioTransformer	Prediction of sites of metabolism and metabolite structures	(99)
MetaSite		(100)
META Ultra		(101)
GLORY		(102)
MetaTox	Web application for predicting structure and toxicity of xenobiotic metabolites	(103)
Tandem mass-spectrometry data processing and analysis		
MZMINE	Open-source software for mass-spectrometry data processing, visualizing and analysis	(104)
OpenMS		(105)
Tandem mass-spectrometry data visualization		
SIRIUS 4	Turn tandem mass spectra into metabolite structure information	(106)
GNPS	Open-source software for visualizing tandem mass spectrometry data and facilitating identification and discovery	(92)
METGEM		(107)
CYTOSCAPE	Open-source software for visualization and analysis of complex networks	(108)

The Multimatrix approach provides a semi-quantitative visualization of molecule distribution in different samples. It has already shown its value in the analysis of drug metabolism, *in vivo* (90,109,110), *in vitro* (111), and in samples of suspected substances consumed, even when metabolites are poorly expressed (112). Interestingly, MN combining interpretation of NPS metabolism data obtained *in vivo* and by different *in vitro* models would therefore be of particular interest in the future.

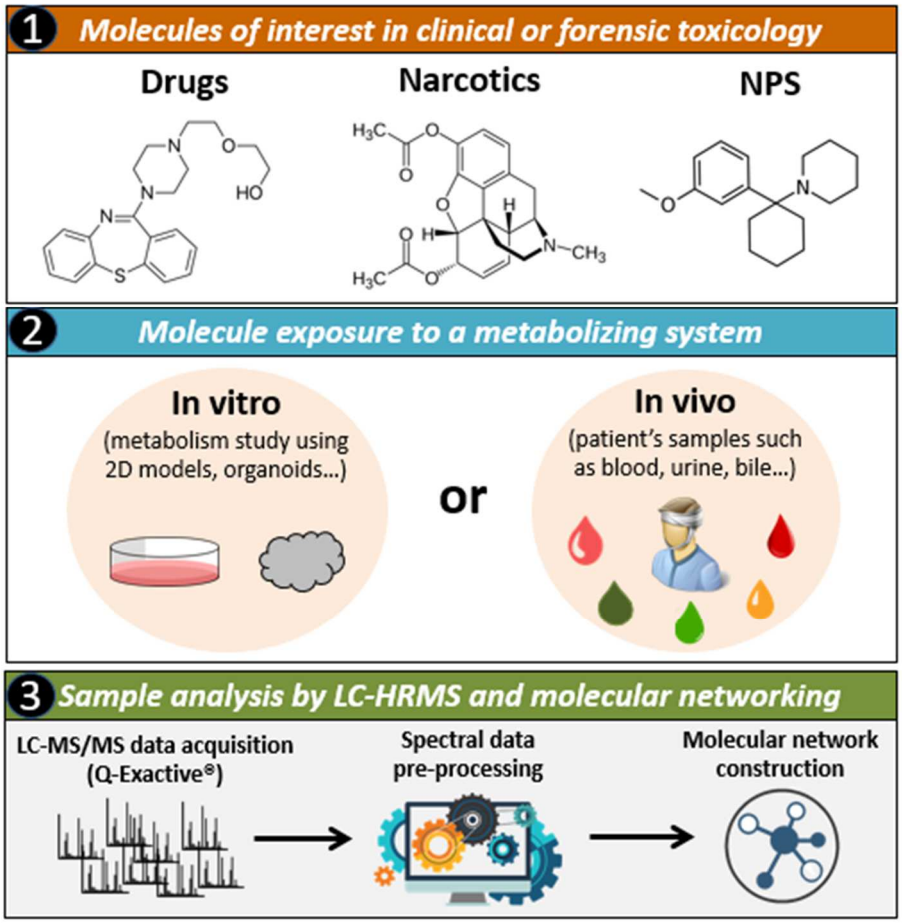

Figure 2: Principles of metabolism study using LC-HRMS and molecular networking

To go further, combined *in silico* analysis by creating MN of high-precision spectral data ($[M+H]^+$ and fragments) from different sources (*in vitro* and *in vivo / in cadaver*) (i) provides a solution for identifying unknown metabolites of NPS, and (ii) represents a visual model for interpreting and understanding toxicological results in the context of a death involving NPS.

4. Conclusion

HRMS in forensic drug chemistry and analytical toxicology has gained ground in recent years, providing a highly versatile analytical platform for the targeted and non-targeted screening of suspected metabolites. It is highly sensitive and specific for DoA identification and allows the development of qualitative and quantitative methods. It is clearly the gold standard for analyzing samples suspected of containing NPS thanks to its ability to acquire full-scan MS data that can then be assessed retrospectively. The advent of bioinformatics methods in recent years has taken LC-HRMS data one step further, allowing for the in-depth analysis of xenobiotic metabolism, both in vivo and in vitro, and leading to the discovery of new markers of consumption of these substances.

Disclosure statement

No potential conflict of interest is reported by the author(s).

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