Trends in Analytical Chemistry 162 (2023) 117023

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Forensic drug screening by liquid chromatography hyphenated with high-resolution mass spectrometry (LC-HRMS)

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ARTICLE INFO

Article history:
Received 13 November 2022
Received in revised form
13 March 2023
Accepted 14 March 2023
Available online 15 March 2023

Keywords:
Forensic toxicology
Drug screening
LC-HRMS
Broad-scope screening
Wide-scope screening
Retrospective data analysis
New psychoactive substances

ABSTRACT

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) has been widely used for screening small organic molecules in complex samples. Its selectivity and sensitivity allow for broad-scope screening of thousands of analytes. However, the complexity of the acquired data has complicated its implementation in high-throughput laboratories that analyze hundreds of samples per week and require that multiple users be able to analyze the data. Forensic laboratories have managed to harvest the merits of LC-HRMS technology using robust and often leveled data analysis(/acquisition) workflows, without spending a disproportionate amount of time evaluating inconclusive or false positive identifications. This critical review describes the full analytical process of LC-HRMS-based forensic drug screening, from sample preparation to data analysis and beyond. Interesting solutions are highlighted, and two emerging trends will be discussed: *i*) the use of free online tools to improve forensic drug screening, and *ii*) re-use of data to improve forensic services.

1. Introduction

The increase in the number of drugs and toxins that a forensic laboratory must be able to detect in biological samples has intensified the need for flexible broad-scope drug screening [1–15]. Targeted methods that focus on a limited number of compounds are suitable for frequently encountered analytes or when analysis of specific analytes are requested [16,17], but they are inadequate for general screening of unknown compounds, such as emerging or infrequently detected drugs and toxins. Notable examples that have raised great concern among forensic analysts are the new psychoactive substances (NPS). The NPS are characterized by their large numbers and their typically low frequency of use when compared with the drugs covered by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances [18,19]. Currently, over 1150 NPS have been reported to the United Nations Office on Drugs and Crime [20]. These compounds typically have increased potencies and, as such, can be more toxic and potentially fatal.

The large number of analytical targets analyzed, in combination with the variable frequency of use, increases the need for selective analytical methods and flexible target lists that can be easily updated when a new drug emerges. Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) offers this selectivity and flexibility, as well as higher sensitivity for important drug classes compared with previous immunoassay and Gas Chromatography - Mass Spectrometry screening methods [3,4,9]. On the other hand, an advantage of Gas Chromatography - Electrospray Ionization - Mass Spectrometry is spectral reproducibility and access to online databases such as the SWGDRUG Mass Spectral Library [21]. LC-HRMS is widely used to screen small organic molecules, including in the neighboring disciplines of environmental and food monitoring [22,23]. However, these merits are accompanied by great complexity, which may not be problematic in research projects where special focus is put on a few samples, but it quickly becomes a problem when data analysis workflows must be scaled up for a large volume of samples and for users with different levels of expertise who will analyze the data. Forensic science is not the area of research to push the scientific

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https://doi.org/10.1016/j.trac.2023.117023

frontiers on the ultimate capabilities of LC-HRMS instrumentation, but it is our impression that forensic toxicologists have been pioneers in establishing the functionality of broad-scope LC-HRMS screening workflows in high-throughput laboratories. LC-HRMS drug screening papers present innovative solutions on how to tailor LC-HRMS drug screening workflows to best harvest the technology's benefits for broad-scope screening while maintaining a reasonable data analysis time.

This article provides a critical review of the full analytical process of LC-HRMS-based forensic drug screening, from sample preparation to data analysis workflows, as outlined in Fig. 1. Fifteen papers on LC-HRMS drug screening methods used in forensic toxicology and written in English were selected from peer-reviewed scientific journals. Targeted acquisition or data analysis methods covering only selected groups of compounds and methods not developed for forensic toxicology were excluded. The review also provides perspectives on new trends in forensic drug screening: *i*) how to improve drug screening using free online and/or *in silico* tools; and *ii*) how to re-use historic screening data to improve forensic services. Previous reviews have covered in detail the guidelines for data interpretation, quality assurance, and MS data acquisition in forensic drug screening [24] and recent advancements in HRMS for the detection of NPS [25]. However, no review has comprehensively covered the forensic drug-screening process from sample preparation to data analysis workflows for the past ten years. Our aim was to provide a review of LC-HRMS drug screening that can help forensic researchers interested in implementing LC-HRMS in their laboratories. Furthermore, to update general analytical chemists on developments in LC-HRMS drug screening with a focus on how the field has assured scalability for this type of data.

2. Choice of sample and sample preparation

Analytes covered in forensic drug screening span a broad range of physiochemical properties. Sample preparation should consequently be unspecific to avoid losing analytes while still removing matrix components. Most forensic samples contain analytes of interest at relatively high concentrations, so large concentration increases are not required during sample preparation. Notable exceptions include potent NPS such as fentanyl analogues or NBOMes. By contrast, robustness is imperative for sample preparation, as postmortem matrixes will have different textures and variable matrix effects, depending on the nature of the sample. Forensic drug screening is well suited for automated liquid handlers, since many samples must be prepared the same way every week, and time is available to accumulate samples [13,26].

The biological samples used for drug screening in the reviewed methods are whole blood [1,5–9,11,13], urine [2,3,5,7,12–15], and/or others [4,5,7,10]. Drugs can be metabolized and subsequently eliminated in urine as metabolites; therefore, each drug may be present as multiple analytical targets in urine, sometimes even without the drug target [27]. Reference materials of drugs are more readily available than metabolites [7], and the active drugs are relatively more abundant in whole blood. Conversely, the concentration levels can be higher for the drug targets in urine and have a longer detection window. Urine samples can be hydrolyzed with β-glucuronidase, possibly in combination with arylsulfatase, thereby increasing the concentration of phase I metabolites [3,12,14,15]. This reduces the number of targets and increases the relative concentration of otherwise conjugated metabolites. A deconjugation step is advantageous if the screening library applied later does not contain phase II metabolites. The addition of deuterated morphine-glucuronide prior to the deconjugation step can serve as a quality control for glucuronidase performance [12]. The choice of biological sample naturally influences the hydrophilicity of the target chemical space and should therefore also influence downstream analytical decisions.

Forensic drug screening sample preparation is mostly protein precipitation [1,2,5,7–9], (assisted) liquid-liquid extraction [4–7], or solid-phase extraction [11,13,15]. Using less selective sample preparation methods can broaden the chemical range of analytes extracted, but at the expense of dirtier extracts increasing the matrix effect [1,6,14]. Threshold accurate calibration can be used to correct for inter-case differences in ion responses for analytes in a screening setup [11] to improve the confidence of an identification being above a set decision point.

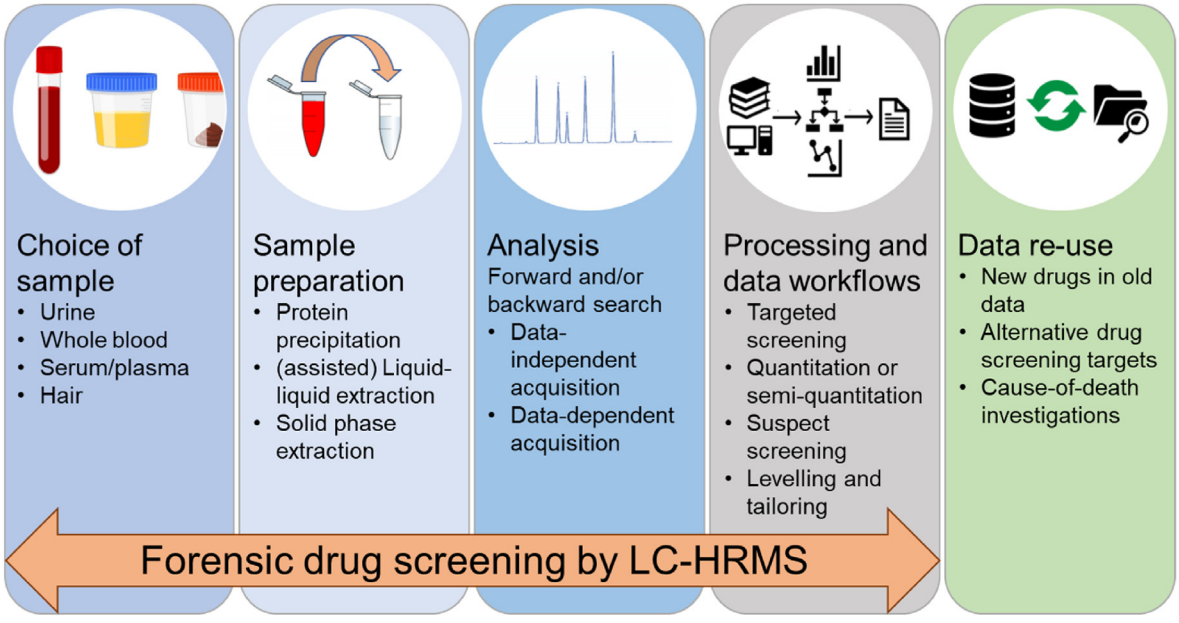

Fig. 1. Schematic representation of main protocol variables in Liquid Chromatography - High Resolution Mass Spectrometry (LC-HRMS) forensic drug screening.

3. LC-HRMS methods

The combination of efficient separation with Ultra-high performance LC (UHPLC) and the selectivity of the HRMS detector makes it a good analytical strategy for the screening of drugs in complex matrixes. As presented in Table 1, Reversed-phase LC in gradient mode is the preferred mode of separation for LC-HRMS drug screening. The analytical column stationary phase is typically C18- or phenylhexyl. The preferred organic mobile phase constituent(s) are acetonitrile [1,4–6,8,11,12,14], methanol [3,7,13,15], or 50:50 mixtures of the two [2,9,10], possibly including added formic acid or buffer. Aqueous mobile phase additives mostly consist of ammonium formate buffer [2–5,8–14] or formic acid [1,4,6], under acidic conditions. Three methods [4,8,11], are largely based on a vendor-developed chromatography with a more selective gradient ranging from 13 to 50% mobile phase B in 9.5 min, followed by a 0.75 min increase to 95%. This gives an enhanced focus on the chromatographic hotspot for drugs, but at the expense of retention and separation of very hydrophilic or hydrophobic targets. Another reported method has a wider range for the main gradient, from 0% to 80% B, resulting in retention of hydrophilic molecules like Gamma-hydroxybutyric acid [1].

The ionization mode is mostly positive electrospray ionization [1,3,4,6–15]. Fewer toxicologically relevant analytes are preferentially ionized with negative than with positive electrospray ionization, as exemplified by Fels et al., where the panel analyzed was more than 15 times larger in positive mode than in negative mode [3]. Intelligence on known caveats and insufficient sensitivity in the screening are important in forensic services, since some analytes may need to be covered by alternative methods [1,6].

The choice of MS acquisition modes and parameters will be influenced by the fact that vendor-specific designs and configurations have certain optimum scan settings and software screening compatibilities. All methods have a full MS acquisition, either in combination with fragmentation mode or with fragmentation mode used only for confirmation. Fragmentation information is collected by either data-dependent acquisition (DDA) [2,3,6,7,9], possibly with inclusion lists for priority analytes, or data-independent acquisition (DIA) [1,4,8,11,12,14,15]. Inclusion lists give the option for customized collision energies for analytes that do not fragment well under standard conditions [6], as well as providing lower identification levels [2,7].

Unit-resolution precursor-ion-selected MS/MS spectra are easy to interpret, but DDA also comes with the inherent risk of missing fragment ion information if an MS/MS spectrum is not generated [2,6,28]. Identification confidence and sensitivity based on all-ion fragmentation DIA data are more multifaceted; therefore, a functional data analysis workflow may be more challenging to set up, and many decisions must be made. Whether spectrum-based screening based on DDA or fragment ion screening based on DIA is better for LC-HRMS-based forensic drug screening is an ongoing discussion. Sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH) can be considered a compromise between the two. In our opinion, DDA-based screenings are well suited for laboratories new to LC-HRMS drug screening, as these need a general-unknown screening with results that are easy to interpret, possibly with a purchased library and a curated inclusion list. If a laboratory knows in detail what purpose the drug screening should fulfill, and if it has the capacity to develop a customized workflow, then DIA-based screening can be more flexible and can give more conclusive results in retrospective data analyses.

4. Data processing and workflows

The quantity and complexity of LC-HRMS forensic drug screening data are immense. A robust and manageable workflow is imperative for broad-scope screening to work in a routine setting where toxicologists who are not methods experts will also perform data analyses. Priorities must be established, and compromises made to allow functional, high-throughput analyses of hundreds of cases per week. This involves finding a balance between sensitivity and identification confidence and a compromise between acceptable false positives and false negatives. The drug screening role is discussed in the papers reviewed here, as one mentions that the presented method is a confirmation-level screening where confirmation only is required in few cases [15], and another indicates that false positive identifications are not critical since positive identifications are confirmed by another specific method [1]. Brilliant and functional solutions are presented in the reviewed papers for improvement of the interpretability or confidence in the data analysis workflows. We will highlight some of these solutions to inspire toxicologists who are in the process of developing or interested in improving a screening method. The application domains of the reviewed methods cover forensic toxicology broadly, as they include hair drug testing, driving-under-the-influence(-of-drugs) (DUID), and emergency or forensic toxicology applications. Data processing and workflow parameters are summarized in Table 2. The reviewed methods are discussed in this review as fully functional forensic drug screening methods. However, an important point to emphasize is that this is not always the intended focus of the articles. For this reason, key parameters such as total analysis time including data analysis is not given. Validation is performed for all or a subset of analytes, as summarized in Table 2. Evidently, variation occurs in the parameters that are included, as also recently pointed out by Wille et al. [24], who discuss guidelines and provide suggestions for adequate identification and validation, and recommend finding a consensus on how to report tentative results if the data do not allow for unambiguous identification. A focus of method validation and quality assurance should be the establishment of reproducible identification around decision points.

Not all drugs analyzed are equally important in forensic toxicology, and not all types of cases warrant the same amount of attention. Careful curation and traceability of screening library entries are preferable. An increased number of screening targets, particularly when not all analytical parameters are available, also increases false positive identifications that need manual evaluation by a toxicologist [1,6,15] at the expense of data analysis time. Consequently, the presented methods may be evaluated differently based on the type of sample, the type of analyte, and/or the number of analytical identification parameters available as strategies to balance acceptable false positives and false negatives. Generally, a special effort is focused on a set of analytes deemed particularly important for the laboratory. In methods operating with DDA, inclusion lists of prioritized or tentatively identified analytes are used for improved sensitivity and to ensure the generation of MS/MS spectra [2,3,6,9,10]. Telving et al. used a higher intensity threshold for selected drugs in DUID cases than in postmortem and clinical samples. The addition of calibrators in the analytical run can facilitate quantitative evaluations of selected analytes [3,6], which then extend beyond quantitation to also serve to improve identification confidence for these analytes. Extended screening libraries, often with fewer analytical diagnostic variables are mentioned for use in special cases or when requested [1,3,6,8].

The quality control (QC) samples with assigned test parameters and their role in drug screening workflows are not always mentioned in detail in the reviewed methods. Monitoring of deuterated internal standard signals in each sample can verify the successful extraction and injection of each sample, but this is not always included (Table 1). The first steps in the workflows are checking the QC samples and/or measurement uncertainties using

Table 1
Summary of sample preparation and analytical methods from a selection of LC-HRMS-based forensic drug screening methods.

Ref	Matrices	Sample preparation Method	Internal standard	Reconstitution of vaporized sample	Mobile phase	Analytical column and injection volume	Flow rate (run time)	MS system	Ionization mode	Acquisition mode	Acquisition parameters
[1]	Whole blood	Protein precipitation using AcN	Four deuterated internal standards	15% aqueous MeOH with 0.1% FA	A: 0.1% aqueous FA B: AcN	ACQUITY UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm), 10 μL	0.6 mL/min (13.5 min)	maXis Impact QTOF (Bruker Daltonics)	ESI^+	DIA: bbCID	Scan range: *m/z* 50-1000 Scan rate (Spectra scan): 10 Hz
[2]	Urine	Protein precipitation using AcN	–	A mixture of mobile phase A and B	A: 2 mM aqueous NH_4HCO_2 (pH 3) B: 2 mM NH_4HCO_2 in AcN:MeOH (50:50) with 0.1% FA	TF Accucore PhenylHexyl (100 mm × 2.1 mm, 2.6 μm), 10 μL	0.5 −0.8 mL/min (13.5 min)	Q-Exactive system (Thermo Fisher Scientific)	Switching between ESI^+ and ESI^-	General screening: DDA with loop count = 5. Targeted screening: As above, also inclusion list	Scan range: *m/z* 130-1000 Resolution 35,000
[8]	Whole blood	Protein precipitation using AcN	Five deuterated internal standards	74:25:1 v/v/v of water, MeOH, and FA.	A: 5 mM aqueous NH_4HCO_2 (pH 3) B: AcN with 0.1% FA	ACQUITY UPLC HSS C18 (150 mm × 2.1 mm, 1.8 μm), 3 μL	0.4 mL/min (15 min)	Xevo G2-S qTOF (Waters Corporation)	ESI^+	DIA: MS^E, Low (6 eV) and ramped (10 −40 eV) collision energies	Scan rate: 5 Hz, Resolution FWHM: 32,500
[9]a	Whole blood	Protein precipitation using 75:25 (AcN:Acetone)	–	20% aqueous MeOH	A: ~15 nM aqueous NH_4HCO_2 with 0.1% FA B: ~15 nM NH_4HCO_2 in MeOH:AcN (50:50) with 0.1% FA	Agilent 120 EC-C18 (2.1 × 100 mm, 2.7 μm) with guard column, 5 μL	0.6 mL/min (6 min)	Q-Exactive Focus™ system (Thermo Fisher Scientific)	ESI^+	DDA with inclusion list (N = 183) triggered within 0.2 min of expected RT	
[9]b	Whole blood	Protein precipitation using 75:25 (AcN: acetone) followed by liquid-liquid extraction	–	75% aqueous MeOH	A: ~15 nM aqueous NH_4HCO_2 with 0.1% FA B: ~15 nM NH_4HCO_2 in MeOH:AcN (50:50) with 0.1% FA	Agilent 120 EC-C18 (2.1 × 100 mm, 2.7 μm) with guard column, 20 μL	0.2 mL/min (10 min)	Q-Exactive Focus™ system (Orbitrap)	Switching between ESI^+ and ESI^-	DDA with inclusion list (N = 10) triggered within 0.2 min of expected RT	
[10]	Hair	Washed with water and acetone. Then dried and cut followed by sonication in MeOH	15 deuterated internal standards	–	A: 5 mM aqueous NH_4HCO_2 with 0.1% FA B: MeOH:AcN (50:50) with 0.1% FA	Thermo Acclaim RSLC 120C18 (2.1 × 100 mm, 2.2 μm), 5 μL	0.4 mL/min (14.5 min)	Single-stage Orbitrap Exactive MS (Thermo Fisher Scientific)	ESI^+	Screening: Full scan mode. Confirmation: in-source collision-induced dissociation with voltage set at 40 V	Screening: Scan range: *m/z* 110-800 Resolution: 100,000 Confirmation: Scan range: *m/z* 50−500, resolution: 50,000
[11]	Whole blood	SPE and sonication	Methapyrilene as internal standard	87% mobile phase A and 13% mobile phase B	A: 5 mM aqueous NH_4HCO_2 (pH 3.0) B: 0.1% v/v FA in AcN	ACQUITY UPLC HSS C18 (2.1 × 150 mm, 1.8 μm), 5 μL	0.4 mL/min (15 min)	Xevo G2 QTOF (Waters Corporation)	ESI^+	DIA: MS^E, Low (6 eV) and ramped (10 −40 eV) collision energies	Scan range: *m/z* 50-1000 Resolution FWHM: 20,000 Scan rate: 10 Hz
[12]	Conjugate-cleaved urine	Dilute-and-shoot	–	–	A: 5 mM aqueous NH_4HCO_2 (pH 3.0) B: 0.1% FA in AcN	ACQUITY UPLC HSS C18 (2.1 × 150 mm, 1.7 μm)	-(15 min)	G2-XS QTOF (Waters Corporation)	ESI^+	DIA: MS^E, Low (6 eV) and ramped (10 −40 eV) collision energies	

[13]	Blood and urine	Dilution followed by SPE	SKF-525	10% aqueous MeOH	A: 5 mM aqueous NH_4HCO_2 B: MeOH	Agilent Eclipse Plus C18 (3.0 × 100 mm, 1.8 μm), 4 μL	0.6 mL/min (13 min)	6230 TOF-MS (Agilent Technologies)	ESI^+	Full scan mode	Scan range: *m/z* 100-1000
[14]	Conjugate-cleaved urine	Dilute-hydrolyze-shoot	Five deuterated internal standards	–	A: 5 mM aqueous NH_4HCO_2 (pH3) B: 0.1% FA in AcN	UPLC BEH C18 (2.1 × 150 mm, 1.7 μm) with guard column	0.4 mL/min (11.05 min)	Xevo G2 TOF (Waters Corporation)	ESI^+	DIA: MS^E, Low (6 eV) and ramped (10 −50 eV) collision energies	Scan range: *m/z* 50-650 Resolution FWHM: 20,000 (at *m/z* 400)
[15]	Conjugate-cleaved urine	SPE	Four deuterated internal standards	45% aqueous MeOH with 0.1% FA	A: 2 mM aqueous NH_4Ac with 0.1% FA B: MeOH	Waters HSS T3 (2.1 × 150 mm, 1.8 μm) with guard column, 1 μL	0.3 mL/min (22 min)	maXis Impact qTOF (Bruker Daltonics)	ESI^+	DIA: bbCID	Mass range: *m/z* 50−700, Resolution FWHM: 24,900
[3]a	Conjugate-cleaved urine	Dilute-and-shoot	22 deuterated internal standards	–	A: 5 mM aqueous NH_4HCO_2 with 0.1% FA B: 5 mM NH_4HCO_2 in MeOH with 0.01% FA	Zorbax Eclipse XDB-C8 (4.6 × 150 mm, 5 μm) with precolumn, 10 μL	0.85 mL/min (15 min)	TripleTOF 5600 system (ABSciex)	ESI^+	DDA: fullMS with 10 information-dependent MS/MS scans	fullMS: Mass range: *m/z* 100 −1000 Resolution: 30,000 MS/MS: mass range: *m/z* 50 −600, Resolution: 15,000
[3]b	Conjugate-cleaved urine	Dilute-and-shoot	22 deuterated internal standards	–	A: 5 mM aqueous NH_4HCO_2 with 0.1% FA B: 5 mM NH_4HCO_2 in MeOH with 0.01% FA	Zorbax Eclipse XDB-C8 (4.6 × 150 mm, 5 μm) with precolumn, 10 μL	0.85 mL/min (15 min)	TripleTOF 5600 system (ABSciex)	ESI^-	DDA: fullMS with 10 information-dependent MS/MS scans	fullMS: Mass range: *m/z* 100 −1000 Resolution: 30,000 MS/MS: mass range: *m/z* 50 −600, Resolution:15,000
[4]a	Serum	Liquid-liquid extraction	Two deuterated internal standards	50% mobile phase A and 50% mobile phase B	A: 5 mM aqueous NH_4HCO_2 (pH 3) B: AcN with 0.1% FA	HSS C18 (2.1 × 150 mm, 1.8 μm), 1 μL	0.4 mL/min (15 min)	Xevo G2-S QTof (Waters Corporation)	ESI^+	DIA: MS^E, Low (6 eV) and ramped (10 −40 eV) collision energies	Mass range: *m/z* 50-1000
[4]b	Serum	Liquid-liquid extraction	Two deuterated internal standards	50% mobile phase A and 50% mobile phase B	A: 0.001% aqueous FA B: AcN with 0.001% FA	HSS C18 (2.1 × 150 mm, 1.8 μm) (Waters Corporation), 1 μL	0.4 mL/min (7.5 min)	Xevo G2-S QTof (Waters Corporation)	ESI^-	DIA: MS^E, Low (6 eV) and ramped (10 −40 eV) collision energies	Mass range: *m/z* 50-1000
[5]	Whole blood, serum, and urine	Various protein precipitation and online SPE	Five deuterated internal standards	–	A: 10 mM aqueous NH_4HCO_2 with 0.1% FA B1: ACN with 0.1% FA B2: solvent mixture	Betasil® phenyl/hexyl (3 × 100 mm, 3 μm), 20 μL	0.3 mL/min (33.58 min)	Thermo Exactive benchtop Orbitrap (Thermo Fisher Scientific)	ESI^+ and ESI^-	Full scan mode and higher energy collisional dissociation	Mass range: *m/z* 100−1300, resolution: 50,000 FWHM
[6]	Whole blood	Alkaline liquid-liquid extraction	A mixed internal standard solution	Ethanol	A: 0.1% aqueous FA B: AcN	BEH C18 (3.0 × 50 mm, 1.7 μm), 0.3 μL	0.35 −0.40 mL/min (14 min)	6545 QTOF (Agilent Technologies)	ESI^+	DDA: Auto MS/MS mode, with inclusion list (Ñ450 masses) triggered within 0.5 min of expected RT, then on precursor ion abundance.	FullMS mass range: *m/z* 100 −1000, MS/MS mass range: *m/z* 40-700
[7]	Whole blood, serum, plasma and urine	Liquid-liquid extraction, protein precipitation, or dilute-and-shoot	–	35% AcN in 0.1% aqueous FA	A: 10 mM aqueous NH_4Ac B: MeOH	Poroshell 120 EC-C18 (2.1 × 100 mm, 2. μm), 5 μL	0.4 mL/min (27 min)	6530 Q-TOF LC/MS (Agilent Technologies)	ESI^+	DDA, loop count = 3, Precursor mass-dependent collision-induced dissociation (offset 4 eV, slope 6 eV/100 *m/z*)	Scan range full MS: *m/z* 100 −1000, MS/MS: *m/z* 50−600, Mass resolutions: 5000−10,000. Scan rate: 4 Hz

AcN: acetonitrile, bbCID: broadband collision induced dissociation, DDA: data dependent acquisition, DIA: Data-independent acquisition, ESI: electron spray ionization, FA: formic acid, FWHM: full width at half maximum, MeOH: methanol, MS^E: elevated collision energy, NH_4Ac: Ammonium acetate, NH_4HCO_2: Ammonium formate, QTOF: quadrupole time-of-flight, SPE: solid-phase extraction.

Table 2
Summary of data analysis workflow and test parameters used in forensic drug screening methods together with their application domain(s).

Reference	Screening software	Screening library	Data filters	Validated for a subset of analytes	Test parameters	Application
[1]	Data Analysis 4.1 (Bruker Daltonics)	In-house library with 467 compounds and suspect screening libraries from Bruker (Bruker ToxScreener™ and Bruker PesticideScreener™.)	Mass error (15 ppm), Retention time (0.18 min), isotopic pattern	Most toxicologically relevant drugs and metabolites in forensic cases. (N = 232–325)	Sensitivity, matrix effects, recovery, robustness (retention time deviation, area intensity deviation, mass error and resolving power)	DUID, post-mortem toxicology
[2]	TF Xcalibur Qual Browser software version 3.0.63, and TF TraceFinder Clinical Research 3.2 for target screening (Thermo Fisher Scientific)		Identification: Mass error (5 ppm) and comparison with the full HR-MS/MS library spectra Detection: Mass error (5 ppm)	Cardiovascular drugs (N = 63)	Selectivity, recovery, matrix effects, process efficiency, LOD, LOI	Routine toxicological analysis, emergency toxicology and medication adherence monitoring
[8]	UNIFI 1.8.1 (Waters Corporation)	In-house expansion of the Waters Forensic Toxicology Screening Application Solution library of 1457 compounds	General: Mass error (3 mDa), intensity count (200), Retention time (0.5 min), one fragment ion Analytes in QC mixtures (N = 285) analyzed in each run: retention time (0.02 min)	Designer benzodiazepine analogues (N = 12)	LOI	DUID, post-mortem toxicology The study presents a routine targeted drug screening workflow together with a non-targeted workflow
[9]	TraceFinder software (Thermo Fisher Scientific)	In-house library of 200 analytes	Mass error (5–10 ppm), Retention time (0.5 min), library score (>50)	Drugs relevant for toxicological analysis in forensic cases: positive mode (N = 183) and negative mode (N = 10)	LOI, matrix effects, recovery, interferences, selectivity, parallel run(s)	Post-mortem and antemortem toxicology
[10]	–	In-house library of 177 analytes	Mass error (2 ppm), isotopic pattern (2 ppm), and retention time (0.2 min)	Drugs relevant for toxicological analysis in forensic cases (N = 177)	LOD, selectivity, matrix effects, and carry-over	DUI, post-mortem toxicology, workplace drug testing, drug-facilitated crime, and judicial cases.
[11]	ChromaLynx software (Waters Corporation)	Library from Waters with 950 toxicologically relevant drugs and metabolites and an in-house library	Mass error (5 ppm), retention time (0.3 min), and qualifier fragment ion (5 ppm) Additional variables used to evaluate screening specificity: two or more qualifier fragment ions (5 ppm) and isotope ratio (10%)	Drugs relevant for toxicological analysis in forensic cases (N = 81)	LOD, Sensitivity, Column recovery, matrix effects, overall process efficiency, and parallel run(s)	Post-mortem toxicology
[12]	UNIFI (Waters Corporation)	Targeted screening library: 64	Mass error (5 ppm), retention time, at least one fragment ion, TAC ratio ≥ the working calibrator TAC ratio, and acceptable quality control and injection recovery performance.Software custom calculation for TAC ratio	Drugs relevant for toxicological analysis in forensic cases (N = 64)	Method accuracy and precision, matrix effects, LOD, and parallel run(s)	Probation, drug court, social services, chemical dependency, pain management and addiction medicine casework
[13]	Agilent MassHunter (Agilent Technologies)	Accurate-mass retention-time Personal Compound Database and Library: >100	"Find by Formula": Mass error for main adduct (15 ppm), retention time (0.15 min). Compound score ≥50 out of 100	A selection of stimulants, benzodiazepines, opiates, muscle relaxants, hypnotics, antihistamines, antidepressants, synthetic cannabinoids and cathinones (N = 96)	Extraction recovery and matrix effects, isobaric interferences, and parallel run(s)	Post-mortem cases, DUI and drug facilitated sexual assault toxicology casework
[14]	UNIFI version 1.6.1 (Waters Corporation)	In-house library of 61 analytes	Mass error (5 ppm), Retention time (0.2 min), ≥ one fragment ion (5 ppm)	Drugs relevant for toxicological analysis in forensic cases (N = 61)	Matrix effect, specificity, and parallel run(s)	Post-mortem and antemortem toxicology
[15]	OTOFcontrol 3.2 and DataAnalysis 4.2 (version 376) were used for data acquisition and Target Analysis 1.3 (Bruker Daltonics) for post-run mass calibration, processing of data, and	In-house library of 526 compounds (280 which was included based on a reference compound)	Mass error (2.5 mDa), Retention time (0.2 min) SigmaFit isotopic ratio (<400), and minimum area count for precursor peaks: 20,000, and qualifier ion peaks (2,200). Specific area criteria applied for: THC-	Opiates, THC-COOH, buprenorphine, benzodiazepines and amphetamines (N = 8)	Sensitivity, specificity, LOQ, and parallel run(s)	Post-mortem urine

Table 2 (*continued*)

Reference	Screening software	Screening library	Data filters	Validated for a subset of analytes	Test parameters	Application
	creating automated reports		COOH, internal standards, clonazepam, bupropion, buprenorphine, and synthetic cannabinoids.			
[3]	PeakView 2.2 software with the integrated MasterView 1.1 software (ABSiex)	Two in-house libraries consisting of 480 compounds where retention time were available for 365. Extended (suspect) screening with the Weinmann ESI-MS/MS library. Additional commercially available library spectra used for peak identification	Global intensity threshold (500 counts). Three levelled identification filters used, with scope for interpreting the classifications made: Narrowest tolerance range for identification: Mass error (5 ppm), retention time (2%), isotope ratio difference (10%), library hit score (>70)	Drugs relevant for toxicological analysis in forensic cases (N = 34)	LOD, LOQ, and parallel run(s)	Post-mortem urine
[4]	UNIFI 1.8.2 (Waters Corporation)	Positive ionization mode: Waters library containing 1279 target compounds complemented with further compounds of interest. Negative ionization mode: In-house library of 74 target compounds	Fixed: Mass error $[M+H]^+$ (5 ppm), $[M-H]^-$ (10 ppm), retention time (0.35 min), isotopic pattern match (<10 ppm), and relative abundance of the isotopic peaks (<20% RMS) Additionally, identification of at least two or more fragment ions (mass error (5 ppm)). *In silico* fragmentation used for further verification of positive hit	Drugs relevant for toxicological analysis in forensic cases (N = 31)	Recovery, Matrix effects, LOD, and parallel run(s)	Post-mortem cases and forensic toxicology
[5]	Xcalibur® Quan Browser for quantification and Exactfinder® version 2.0 for qualitative analysis (Thermo Fisher Scientific)	In-house library of 654 compounds (544 in positive mode and 72 in negative mode)	Automated blank subtraction. Automated target report with retention time, area count, mass tolerance, isotopic pattern match (score >75%) and at least one higher energy collisional dissociation fragment match	Drugs relevant for toxicological analysis in forensic cases (N = 14)	Serum and whole blood: Selectivity, accuracy, precision, recovery, LLOQ and ULOQ, and matrix effects Urine: within-day precision and accuracy for LLOQ and theoretical LOD	Toxicological screening
[6]	MassHunter Qualitative Analysis version B07 and Quantitative Analysis version B07 (Agilent Technologies)	In-house library of 470 compounds	"Find by Formula". Qualitative screening settings: Mass error (10 mDa), retention time (0.5 min), area (5000 counts). Confirmation settings: Mass error (2 mDa), retention time (2%), full scan MS/MS spectrum consistent with a contemporaneously analyzed reference standard	Drugs relevant for toxicological analysis in forensic cases (N = 320)	Recovery, LOD, matrix effects, selectivity, extract stability, and instrument carry-over.	Toxicological screening
[7]	MassHunter Acquisition version B.02.01 with service pack 3, and MassHunter Qualitative Analysis version B.03.01 with service pack 3 (Agilent Technologies)	In-house library of theoretical masses of 7500 suspect toxic compounds, from which 2500 MS/MS spectra were acquired at three collision energies	Automated background removal Mass error (3–5 ppm), isotopic pattern scoring, matching with library MS/MS spectra recorded with all collision energies	–	Parallel run(s)	Toxicological screening. The focus of the study is the development of a spectral database of toxicologically relevant substances, rather than presentation of a drug screening workflow ready for implementation in routine forensic analysis

LOD: limit of detection, LOI: limit of identification, DUI(D): Driving under influence (of drugs), LOQ: limit of quantification, LLOQ: lower limit of quantification, QC: quality control, TAC: Threshold accurate calibration, ULOQ: upper limit of quantification.

internal standards [1,5]. QC mixtures with hundreds of common analytes can serve to improve identification confidence for analytes by reducing the retention time window [1,8]. This within-run retention time benchmarking is particularly relevant for analytes with common drug or matrix interferences. One method using Bruker software generates a report that shows the detection of these QC mixture analytes in each run [1]. This shows compound-specific analytical run performance that improves the confidence in negative results. If isomer pairs are assigned to different QC mixtures, the method can also be used to differentiate between closely eluting isomers with otherwise identical fragment ions, such as quinine/quinidine or morphine/hydromorphone [8]. Buprenorphine, which is not easily fragmented with collision-induced dissociation, will result in a high lower limit of identification if at least one fragment ion is required for a library hit. Inclusion of buprenorphine in QC mixtures for retention time benchmarking can improve confidence in a drug screening hit of buprenorphine even in the absence of fragment ions [1].

In untargeted drug screening, data preprocessing will involve componentization, where *m/z*-RT features are grouped with (depending on the software used) possible isotopes, adducts, multicharged ions, and/or fragmentation data. The result is a list of analytes that can be matched with the library based on set match criteria. Common filter variables used to match the library and sample data include the mass of the preferred precursor ion, retention time, and possibly the presence of a fragment ion (Table 2). The screening workflows that involve library matching will have fixed parameters with minimum requirements for a library hit. Other parameters, such as the isotopic pattern or *in silico* fragmentation, can help to improve confidence in an identification without being mandatory for a positive hit [3,4,8,12]. Two methods report that the isotopic pattern is not a good fixed parameter, as its use may lead to false negatives of low-abundance peaks [2,14]. A major concern is false negative hits caused by peak overloading or detector saturation by a very high concentration of an analyte. Of course, a forensic drug screening cannot allow a false negative hit caused by very high concentration levels. The accepted error in the mass and retention time domains is thus set higher than necessary to reduce this risk [1,6,8]. The risk of detector saturation in orbitrap-systems should be reduced using automated gain control. The origin of the measurement reference determines the accepted error for a library hit. For example, Mollerup et al. reported the accepted error between the measurement of an analyte in an unknown sample and within-run QC sample as ±0.02 min, whereas the error for identification from a library hit was ±0.5 min [8]. Using the same method, these authors elsewhere suggested ±2 min for predicted retention time values [29].

A simulated LC-HRMS forensic drug screening is presented in Fig. 2 from sample preparation to compound identification check, where QC samples are used to support identification. The simulated example includes representative problem analytes from routine drug screening. A tramadol identification is accepted in the absence of diagnostic fragment ions, because of absence in blank and retention time within ±0.02 min from a QC sample spiked with tramadol. Quinine and quinidine are enantiomers that can be chromatographically separated. Quinidine is an antiarrhythmic agent and quinine, although also registered to treat malaria, is frequently detected in blood samples being a constituent of the soft drink tonic water. In Fig. 2, a measured feature is identified as both quinine and quinidine including diagnostic fragment ions; by having quinine and quinidine in two different spiked QC mixtures, the quinidine identification can be set to false positive by comparing within-run retention times.

Uniform instrument and workflow settings are convenient in broad-scope screening designed for the best detection of as many relevant analytes as possible. However, some analytes are better detected with special settings, while still others need special attention to prevent disruption of the data analysis workflows. LC-HRMS drug screening workflows may therefore be improved by some tailoring with compound-specific settings. Some authors highlight the importance of the careful choice of qualifier ions to improve accuracy and selectivity [2,5,6,15]. Compound-specific filters can remove recurring false positives by increasing area criteria [15] or can enhance sensitivity by using in-source fragment ions as targets [4,5,30]. Metabolites can also be used as markers for analytes that are not easily identified in positive ionization and at toxicologically relevant levels; these include buprenorphine, propofol, and THC [1]. Endogenous molecules, artifacts, or contaminants that are of no forensic relevance can be added to compound libraries and then either added to exclusion lists to avoid wasting MS/MS scans [2,6] or assigned and filtered out in data analysis workflows to avoid false positive hits [8]. The detection of multiple analytical targets or metabolites can increase the confidence in the toxicological evaluation [2,3,7,12,15], as this is particularly relevant in emergency toxicology where identification may not be confirmed by complementary methods.

5. Online tools and computational methods to improve forensic drug screening

A trend in the reviewed methods is to have a panel of analytes with special focus, while having additional strategies for suspect screening involving special cases or analytes. Analysis of NPS does not warrant the same focus as prescription opioids or benzodiazepines, but a strategy is needed for the detection of NPS. Their increasing numbers and rate of development present ongoing challenges for forensic and clinical laboratories. Finding a proportional allocation of time and resources to analyze NPS is not a simple task, considering the other continuous improvements and analyses that need to be undertaken in modern laboratories. Consequently, a large variation exists between laboratories from country to country, and even within a country, regarding how much time and effort are spent on developing and curating analytical NPS methods. Independent of how a laboratory chooses to deal with the issue, several excellent and free online tools are available to support their analytical strategies.

Notably, while NPS receive substantial attention in the forensic community and at forensic toxicology conferences, other groups of infrequently detected but toxicologically relevant analytes can be detected with the same strategies. Based on the choice of screening strategy, we now discuss some of the online tools and computational methods available to support forensic drug screening. Further information on the developments in HRMS analyses of NPS is available in a recent review by Klingenberg et al. [25].

5.1. Non-targeted screening

Non-targeted screening is a bottom-up approach in which a reduced number of features that may allude to an NPS are filtered from the HRMS raw data [8] and online tools are then used to aid in compound identification. The features can then be compared with online MS libraries, as reviewed elsewhere [31], or used for *de novo* structural elucidation. Ranking of hits can be supported with the *in silico* fragmentation also used in the LC-HRMS drug screening [3,4,8,12] and/or by predicted analytical parameters, such as retention time and collision cross section values [29,32]. Since only a few features are selected from each sample in non-targeted screening, the concern of false positive identifications from routine drug screening does not apply here. The online tool DarkNPS can be used for automatic elucidation of structures of

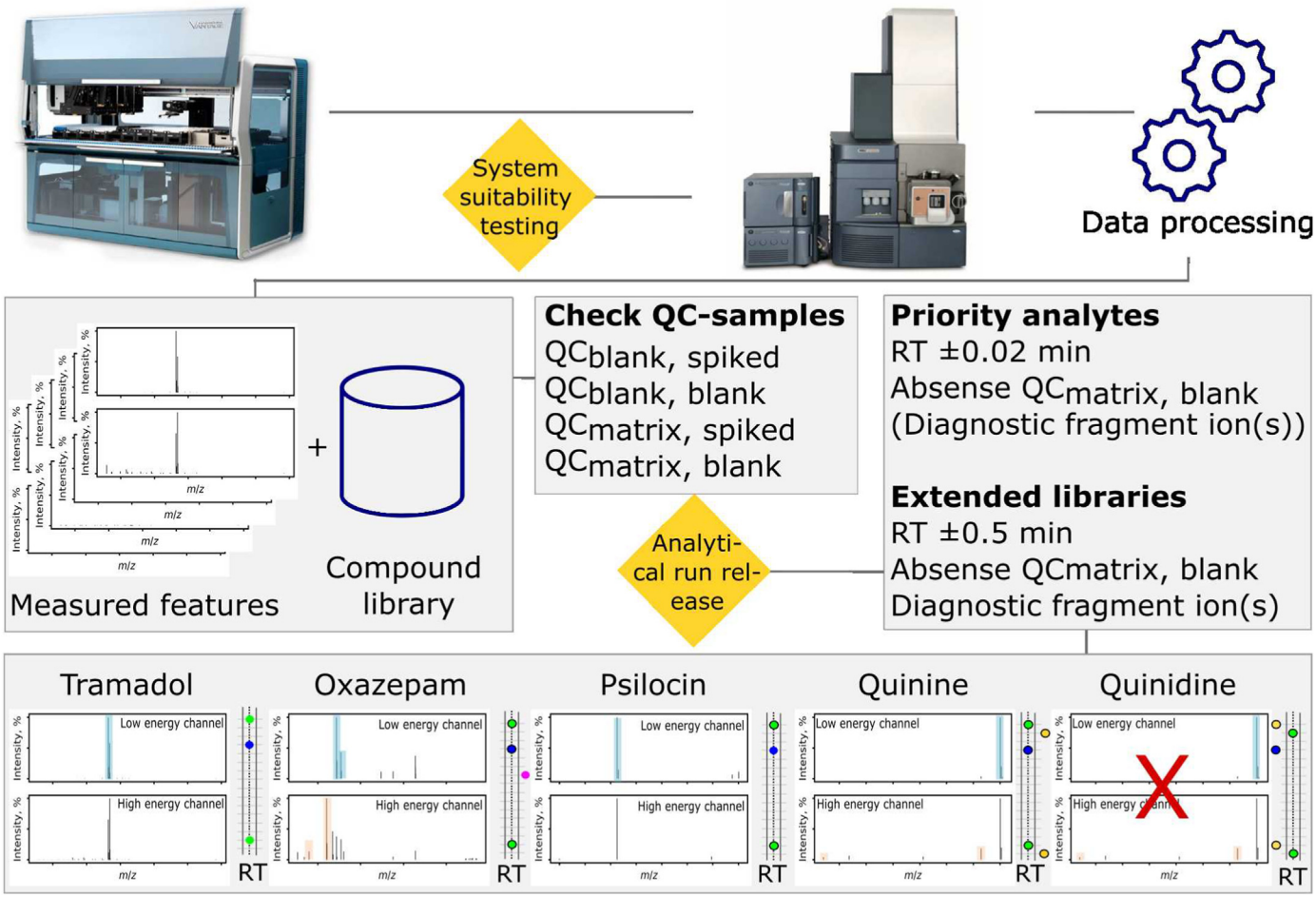

Fig. 2. Simulated example of an LC-HRMS forensic drug screening workflow. After data pre-processing, measured features are matched with a compound library. QC samples possibly spiked with reference material in matrix or solvent, are monitored to evaluate analytical run performance. If the analytical run is accepted, a levelled data analysis workflow with set criteria for priority analytes and others for extended library analytes is applied. Five common identifications are given as examples with low-energy and high-energy channel spectra where a blue box indicates a precursor ion match, and an orange box indicates diagnostic fragment ion match. To the right of spectra are paired retention time (RT) plots, where horizontal lines represent an injection, a circle indicates an identification of the precursor ion and a black line around the circle further indicates identification of at least one diagnostic fragment ion. Dotted, black, vertical line: within-run analyte RT, grey vertical line: ±0.02 min from dotted, black line. Blue circle: identification evaluated with spectra, green circle: with-in run QC spiked with the evaluated analyte (true positive), yellow circle: within-run QC spiked with known isomer (false positive), purple circle: false positive identification.

unidentified NPS from mass spectrometric data through a deep learning-enabled approach [33]. Non-targeted screening is the only way to detect unknown toxicological substances, but it is labor intensive and therefore best suited for special cases rather than for routine analysis of biological samples [8]. A real-time non-targeted screening step that could filter out only drug-like features that had never been encountered previously would be an ideal supplement for a routine LC-HRMS drug screening workflow, but this may not be achievable with current vendor screening software limitations.

5.2. Targeted screening

A common analytical approach for emerging drugs in forensic sciences is to curate a list relevant for the region and include the analytes in a targeted data analysis with library entries from reference material. Since the NPS market is dynamic, this strategy requires periodic reevaluation, as well as purchase and analysis of reference material. However, data analysis in terms of compound identification is easier and fits well into normal routine forensic drug screening. This strategy requires quality intelligence about the NPS drug market, ideally with geographic and temporal resolution. In the United States, the Center for Forensic Science Research & Education collects data on NPS monitoring and shares well-informed quarterly trend reports on their home page [34]. Government reports, including early warning systems [20], scientific literature, or Reddit [35] discussions, are other relevant sources of information available for compilation of targeted lists. The European Union has a large workforce mobility; consequently, monitoring the European Medicines Agency article 57 database is relevant for collecting intelligence on registered psychoactive drugs in neighboring countries [36].

5.3. Suspect screening

Suspect screening here refers to the matching of measured analytical features with a library of external LC-HRMS information, such as online databases, suspect lists, or *in silico* data. Examples of the reviewed methods are vendor or commercial libraries [1,3] or suspect lists without retention times [8,15]. Another approach is to use HighResNPS, which is a free crowd-sourced LC-HRMS data repository for NPS mass spectrometric data [37]. The HighResNPS database holds diagnostic fragment ions rather than spectra, and

these can be exported with consensus fragment ions [38]. The database can be downloaded as a screening database compatible with most vendor formats and can be directly added as a step in leveled forensic drug screening [30]. If a laboratory uploads sufficient analytical data with retention times to HighResNPS, a retention time model will be trained on the supplied data, and all database analytes can be provided with predicted retention times [32]. Note, however, that the accuracy of predicted retention times does not allow distinguishing of positional isomers, although it will help filter false positive hits [29].

6. Re-use of forensic drug screening data

Forensic drug screening data are suitable for re-use because of their traceability and the quality assurance under which they were first collected. For this reason, forensic data is being applied more frequently to improve and verify quality of the drug screening procedure, but also for other purposes such as establishing cause of death. Retrospective data analysis is sometimes referred to as retrospective screening. Here, we exclusively discuss studies where data are acquired as part of a routine LC-HRMS-based forensic drug screening and then re-used for other purposes.

6.1. New drugs in old data

The reprocessing of screening data files when new drugs are included in MS libraries or databases has become a new and attractive option in forensic toxicology for approaching unsolved drug-related cases or for testing the performance of screening library coverage [8,39]. Screening data reprocessed using updated libraries with novel drugs also form part of the surveillance and monitoring strategies used by forensic labs and law enforcement agencies. The fact that previously acquired LC-HRMS drug screening can be re-used for retrospective data analyses has been pointed out in several screening method papers [1,3–5,8–10,13,15].

Partridge et al. used retrospective data analysis with an updated screening library in a coronial case 8 months after the original analysis. The detection of NPS in this case was confirmed by reanalysis [40]. Axelsson et al. reprocessed LC-HRMS drug screening data collected over one year in 14,367 clinical oral fluid samples from mainly psychiatric and addiction clinics to search for NPS. Their analysis revealed 34 NPS that had not been identified in the samples by the original sample testing. The size of the data set allowed its use in epidemiological evaluation, and the authors found that 1.87% of the patients tested positive for NPS drug use [41]. A retrospective data analysis of 2339 forensic cases from 2016 by Noble et al. used reprocessed data files with a targeted data analysis method for 50 fentanyl analogues [39]. The study relied on variable identification parameters from full analytical data to theoretical fragments, supported by online spectral databases, as mentioned in section 5 [39]. Gundersen et al. also performed a retrospective data analysis of 1314 postmortem cases analyzed with DDA. The authors built a new in-house library ($n = 374$) based on HighResNPS data. Four new substances were identified in the data files that had MS/MS scans available. The authors discussed challenges in the interpretation of library hits where MS/MS scans were not generated [28].

Most retrospective data analysis studies are made using the vendor software also used for drug screening [28,39–41], but this can be laborious for a large number of samples. A more scalable approach involves parsing LC-HRMS drug screening raw data to a SQL archive, making it available for querying and analysis with the general-purpose programming language Python [42]. This SQL archive was applied for a retrospective data analysis, tailored to benzodiazepines from paired LC-HRMS drug screening files using quantitative results from a complementary method as true condition [43]. The benzodiazepine-tailored workflow was then applied for retrospective data analysis of designer benzodiazepines in drug screening data files. The workflow could screen 47 new drugs across 13,514 data files in 1 min and generated only 9 false positive identifications; however, it required retention time as an analytical parameter [43].

6.2. Alternative drug markers

The reviewed drug screening methods used a range of solutions to improve the detection confidence of problem analytes. This included using known metabolites as markers or compound-customized identification parameters. The identification of alternative screening targets for drugs that are otherwise not detectable at relevant levels can help with the inclusion of these drugs into a drug screening method. Differential analysis of features in the historic data in LC-HRMS drug screening data files from cases with paired quantitative results used as a true condition can reveal alternative targets and subsequently rank them regarding selectivity and specificity. Alternative targets have been identified with this method for several drugs, such as valproic acid [44], barbiturates [45], and salicylic acid and ibuprofen [46], that all ionize poorly with positive electrospray ionization. Drug screening data can also reveal markers of exposure to drugs with metabolomic strategies. Nielsen et al. mined two years of LC-HRMS screening data with known MDMA exposure and identified known MDMA metabolites, thereby providing a proof-of-principle of the developed untargeted metabolomic strategy. The authors also observed changes in the endogenous production of serotonin and tryptophan, likely induced by MDMA [47]. Similarly, another study has shown the benefits of using metabolomics to elucidate new markers and metabolic changes associated with Gamma-hydroxybutyric acid use [48]. This drug is otherwise hard to detect in forensic case work because of its short biological detection window.

6.3. Identification of markers to establish cause of death

Toxicologic evaluation of opioid blood concentration levels is complicated by the large overlap in therapeutic and lethal ranges. Elmsjö et al. sought to identify potential biomarkers of lethal oxycodone overdoses from 934 postmortem cases using a metabolomics-type approach by correlating potential biomarkers with hypoxia and toxicological effects. These models showed that acylcarnitines might be potential biomarkers of the hypoxia and ischemia observed in oxycodone-involved intoxications [49]. The same group used a similar setup to identify post-mortem biomarkers of pneumonia compared with control cases [50]. Other studies have explored the use of metabolomics in a forensic setting based on new rather than re-used data. Metabolomics in clinical and forensic toxicology is reviewed elsewhere [51].

Alternative screening targets that are not identified through the analysis of reference material or controlled studies always require confirmation of the presence of the main target using a complementary analytical method. Although further studies are needed on metabolomics to corroborate its use as a reliable tool in forensic analysis, it is a highly promising approach for evaluating drug intake and its effects on humans and for identifying the cause of death.

7. Conclusion and future perspectives

The introduction of LC-HRMS has been a paradigm shift in forensic drug screening, although not implemented yet in all

laboratories. The methods reviewed here show that LC-HRMS is flexible, robust, and sensitive and therefor suitable for modern high-throughput laboratories. It is our ambition and hope with this review to inspire more toxicologists to implement the technology in forensic labs.

The ability of forensic toxicologists to reliably determine when drugs and other toxins are involved in criminal cases is important for a sense of justice and security in society. Forensic drug screening by LC-HRMS can ensure that less frequently consumed drugs, such as NPS, do not fly under the radar while maintaining the compound-resolved detection. However, the implementation of a functional data analysis workflow that is scalable to high-throughput laboratories remains challenging and requires many resources.

The reviewed analytical methods do not vary substantially in terms of sample preparation and chromatography. However, the data analysis workflows and application domains vary widely, and a number of savvy steps that can ease the data analysis burden are highlighted in this review. One trend noted is to use a panel of analytes with special focus and easier interpretation, while also having suspect screening libraries used in special cases. This involves a leveling of compound-specific acquisition or tailoring of workflows, thereby enhancing the focus on toxicologically relevant analytes and diminishing disturbances from recurring matrix interferences, while collecting information on anything in between. This can be achieved by the active use of QC samples to improve identification confidence or by mining historic data to identify troublesome or alternatively targeted analytes. In the future, further leveling of data analysis workflows may be necessary to adhere to data protection legislation.

Free online and computational tools are used *via* different strategies to expand and otherwise improve the forensic drug screening. However, more efforts are necessary to allow true broad-scope screening that is not restricted to special cases. One of the next major transformations in forensic drug screening will be data-driven by means of machine learning. Models based on structured, historic data could help filter out recurring false positives or predict whether an emerging drug can be detected at relevant concentration levels. Automation and digitalization are already being deployed in forensic laboratories. In combination with the continuity guaranteed by rigorous quality assurance schemes, this may result in the structured connectivity necessary to fully attain data-driven forensic services. Retrospective data analyses aimed to detect new drugs in old data or determine biological markers to establish cause of death, are examples discussed where forensic drug screening data already is being used to improve forensic services prospectively. The direction has been set, but there are obstacles on the way. Vendor software is unfortunately a limiting factor in data analysis workflow development and does not easily support integration of machine learning code. Software limitations are mentioned as a limitation in several forensic drug screening articles, which cause unnecessary data analysis obstructions or restrictions. Ideally, the data or toxicologist skill level and not software should define the limits of what can be achieved.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors would like to thank Juliet Wawira Kinyua for valuable input. PJH acknowledges Antidoping Denmark; MM acknowledges the Research Council of Norway (#312267).

References

[1] R. Telving, J.B. Hasselstrøm, M.F. Andreasen, Targeted toxicological screening for acidic, neutral and basic substances in postmortem and antemortem whole blood using simple protein precipitation and UPLC-HR-TOF-MS, Forensic Sci. Int. 266 (2016) 453–461. https://doi.org/10.1016/j.forsciint.2016.07.004.

[2] A.G. Helfer, J.A. Michely, A.A. Weber, M.R. Meyer, H.H. Maurer, Orbitrap technology for comprehensive metabolite-based liquid chromatographic-high resolution-tandem mass spectrometric urine drug screening - exemplified for cardiovascular drugs, Anal. Chim. Acta 891 (2015) 221–233. https://doi.org/10.1016/j.aca.2015.08.018.

[3] H. Fels, T. Dame, H. Sachs, F. Musshoff, Liquid chromatography-quadrupole-time-of-flight mass spectrometry screening procedure for urine samples in forensic casework compared to gas chromatography-mass spectrometry, Drug Test. Anal. 9 (2017) 824–830. https://doi.org/10.1002/DTA.2039.

[4] M. Grapp, C. Kaufmann, F. Streit, L. Binder, Systematic forensic toxicological analysis by liquid-chromatography-quadrupole-time-of-flight mass spectrometry in serum and comparison to gas chromatography-mass spectrometry, Forensic Sci. Int. 287 (2018) 63–73. https://doi.org/10.1016/j.forsciint.2018.03.039.

[5] L. Roche, J. Pinguet, P. Herviou, F. Libert, C. Chenaf, A. Eschalier, N. Authier, D. Richard, Fully automated semi-quantitative toxicological screening in three biological matrices using turbulent flow chromatography/high resolution mass spectrometry, Clin. Chim. Acta 455 (2016) 46–54. https://doi.org/10.1016/J.CCA.2016.01.017.

[6] E. Partridge, S. Trobbiani, P. Stockham, T. Scott, C. Kostakis, A validated method for the screening of 320 forensically significant compounds in blood by LC/QTOF, with simultaneous quantification of selected compounds, J. Anal. Toxicol. 42 (2018) 220–231. https://doi.org/10.1093/jat/bkx108.

[7] S. Broecker, S. Herre, B. Wüst, J. Zweigenbaum, F. Pragst, Development and practical application of a library of CID accurate mass spectra of more than 2,500 toxic compounds for systematic toxicological analysis by LC-QTOF-MS with data-dependent acquisition, Anal. Bioanal. Chem. 400 (2011) 101–117. https://doi.org/10.1007/S00216-010-4450-9.

[8] C.B. Mollerup, P.W. Dalsgaard, M. Mardal, K. Linnet, Targeted and non-targeted drug screening in whole blood by UHPLC-TOF-MS with data-independent acquisition, Drug Test. Anal. 9 (2017) 1052–1061. https://doi.org/10.1002/dta.2120.

[9] J.B. Stephenson, M.L. Flater, J. Austin, L.T. Bain, L.A. Holt, J.M. Mehan, Comprehensive drug screening of whole blood by LC–HRMS–MS in a forensic laboratory, J. Anal. Toxicol. 45 (2021) 243–251. https://doi.org/10.1093/JAT/BKAA073.

[10] S. Odoardi, V. Valentini, N. De Giovanni, V.L. Pascali, S. Strano-Rossi, High-throughput screening for drugs of abuse and pharmaceutical drugs in hair by liquid-chromatography-high resolution mass spectrometry (LC-HRMS), Microchem. J. 133 (2017) 302–310. https://doi.org/10.1016/J.MICROC.2017.03.050.

[11] T.G. Rosano, M. Wood, K. Ihenetu, T.A. Swift, Drug screening in medical examiner casework by high-resolution mass spectrometry (UPLC–MSE-TOF), J. Anal. Toxicol. 37 (2013) 580–593. https://doi.org/10.1093/JAT/BKT071.

[12] T.G. Rosano, P.Y. Ohouo, M. Wood, Application of high-resolution UPLC–MSE/TOF confirmation in forensic urine drug screening by UPLC–MS/MS, J. Anal. Toxicol. 43 (2019) 353–363. https://doi.org/10.1093/JAT/BKY106.

[13] F. Guale, S. Shahreza, J.P. Walterscheid, H.H. Chen, C. Arndt, A.T. Kelly, A. Mozayani, Validation of LC–TOF-MS screening for drugs, metabolites, and collateral compounds in forensic toxicology specimens, J. Anal. Toxicol. 37 (2013) 17–24. https://doi.org/10.1093/JAT/BKS084.

[14] N.S. Chindarkar, M.R. Wakefield, J.A. Stone, R.L. Fitzgerald, Liquid chromatography high-resolution TOF analysis: investigation of MSE for broad-spectrum drug screening, Clin. Chem. 60 (2014) 1115–1125. https://doi.org/10.1373/CLINCHEM.2014.222976.

[15] M. Sundström, A. Pelander, I. Ojanperä, Comparison between drug screening by immunoassay and ultra-high performance liquid chromatography/high-resolution time-of-flight mass spectrometry in post-mortem urine, Drug Test. Anal. 7 (2015) 420–427. https://doi.org/10.1002/DTA.1683.

[16] A.G. Helfer, M.R. Meyer, J.A. Michely, H.H. Maurer, Direct analysis of the mushroom poisons α- and β-amanitin in human urine using a novel on-line turbulent flow chromatography mode coupled to liquid chromatography-high resolution-mass spectrometry/mass spectrometry, J. Chromatogr., A 1325 (2014) 92–98. https://doi.org/10.1016/j.chroma.2013.11.054.

[17] M.K.K. Nielsen, M. Nedahl, S.S. Johansen, K. Linnet, Validation of a fully automated solid-phase extraction and ultra-high-performance liquid chromatography–tandem mass spectrometry method for quantification of 30 pharmaceuticals and metabolites in post-mortem blood and brain samples, Drug Test. Anal. 10 (2018) 1147–1157. https://doi.org/10.1002/dta.2359.

[18] UN General Assembly, 1971 Convention on Psychotropic Substances, 1975.

https://www.unodc.org/pdf/convention_1971_en.pdf.
[19] UN General Assembly, 1961 Single Convention on Narcotic Drugs, 1961. https://www.unodc.org/pdf/convention_1961_en.pdf.
[20] UNODC, Current NPS Threats - Volume V, 2022. https://www.unodc.org/documents/scientific/Current_NPS_Threats_V.pdf.
[21] Scientific Working Group for the Analysis of Seized Drugs, SWGDRUG Mass Spectral Library, 2023. https://www.swgdrug.org/ms.htm.
[22] M. Paszkiewicz, K. Godlewska, H. Lis, M. Caban, A. Białk-Bielińska, P. Stepnowski, Advances in suspect screening and non-target analysis of polar emerging contaminants in the environmental monitoring, TrAC, Trends Anal. Chem. 154 (2022), 116671. https://doi.org/10.1016/J.TRAC.2022.116671.
[23] Y. Fu, C. Zhao, X. Lu, G. Xu, Nontargeted screening of chemical contaminants and illegal additives in food based on liquid chromatography–high resolution mass spectrometry, TrAC, Trends Anal. Chem. 96 (2017) 89–98. https://doi.org/10.1016/J.TRAC.2017.07.014.
[24] S.M.R. Wille, B. Desharnais, S. Pichini, A. Di Trana, F.P. Busardò, D.K. Wissenbach, F.T. Peters, Liquid chromatography high-resolution mass spectrometry in forensic toxicology: what are the specifics of method development, validation and quality assurance for comprehensive screening approaches? Curr. Pharmaceut. Des. 28 (2022) 1230–1244. https://doi.org/10.2174/1381612828666220526152259.
[25] J. Klingberg, B. Keen, A. Cawley, D. Pasin, S. Fu, Developments in high-resolution mass spectrometric analyses of new psychoactive substances, Arch. Toxicol. 964 (96) (2022) 949–967. https://doi.org/10.1007/S00204-022-03224-2, 2022.
[26] D. Andersen, B. Rasmussen, K. Linnet, Validation of a fully automated robotic setup for preparation of whole blood samples for LC–MS toxicology analysis, J. Anal. Toxicol. 36 (2012) 280–287. https://doi.org/10.1093/JAT/BKS014.
[27] T. Sobolevsky, I. Prasolov, G. Rodchenkov, Detection of JWH-018 metabolites in smoking mixture post-administration urine, Forensic Sci. Int. 200 (2010) 141–147. https://doi.org/10.1016/j.forsciint.2010.04.003.
[28] P.O.M. Gundersen, S. Broecker, L. Slørdal, O. Spigset, M. Josefsson, Retrospective screening of synthetic cannabinoids, synthetic opioids and designer benzodiazepines in data files from forensic post mortem samples analysed by UHPLC-QTOF-MS from 2014 to 2018, Forensic Sci. Int. 311 (2020), 110274. https://doi.org/10.1016/j.forsciint.2020.110274.
[29] C.B. Mollerup, M. Mardal, P.W. Dalsgaard, K. Linnet, L.P. Barron, Prediction of collision cross section and retention time for broad scope screening in gradient reversed-phase liquid chromatography-ion mobility-high resolution accurate mass spectrometry, J. Chromatogr., A 1542 (2018) 82–88. https://doi.org/10.1016/j.chroma.2018.02.025.
[30] M. Mardal, O.-M. Fuskevåg, P.W. Dalsgaard, Comprehensive UHPLC-HR-MS^E screening workflow optimized for use in routine laboratory medicine: four workflows in one analytical method, J. Pharm. Biomed. Anal. 196 (2021). https://doi.org/10.1016/j.jpba.2021.113936.
[31] M. Vinaixa, E.L. Schymanski, S. Neumann, M. Navarro, R.M. Salek, O. Yanes, Mass spectral databases for LC/MS- and GC/MS-based metabolomics: state of the field and future prospects, TrAC, Trends Anal. Chem. 78 (2016) 23–35. https://doi.org/10.1016/J.TRAC.2015.09.005.
[32] D. Pasin, C.B. Mollerup, B.S. Rasmussen, K. Linnet, P.W. Dalsgaard, Development of a single retention time prediction model integrating multiple liquid chromatography systems: application to new psychoactive substances, Anal. Chim. Acta 1184 (2021), 339035. https://doi.org/10.1016/J.ACA.2021.339035.
[33] M.A. Skinnider, F. Wang, D. Pasin, R. Greiner, L.J. Foster, P.W. Dalsgaard, D.S. Wishart, A deep generative model enables automated structure elucidation of novel psychoactive substances, Nat. Mach. Intell. 311 (3) (2021) 973–984. https://doi.org/10.1038/s42256-021-00407-x, 2021.
[34] A.J. Krotulski, D.M. Papsun, S. Walton, M. Fogarty, B.K. Logan, NPS Discovery: Year in Review 2021, 2021.
[35] E. Barenholtz, A.J. Krotulski, P. Morris, N.D. Fitzgerald, A. Le, D.M. Papsun, B.K. Logan, W.E. Hahn, B.A. Goldberger, L.B. Cottler, J.J. Palamar, Online surveillance of novel psychoactive substances (NPS): monitoring Reddit discussions as a predictor of increased NPS-related exposures, Int. J. Drug Pol. 98 (2021), 103393. https://doi.org/10.1016/J.DRUGPO.2021.103393.
[36] Public data from Article 57 database | European Medicines Agency, (n.d.). https://www.ema.europa.eu/en/human-regulatory/post-authorisation/data-medicines-iso-idmp-standards/public-data-article-57-database (accessed November 7, 2022).
[37] M. Mardal, M.F. Andreasen, C.B. Mollerup, P. Stockham, R. Telving, N.S. Thomaidis, K.S. Diamanti, K. Linnet, P.W. Dalsgaard, HighResNPS.com: an online crowd-sourced HR-MS database for suspect and non-targeted screening of new psychoactive substances, J. Anal. Toxicol. 43 (2019) 520–527. https://doi.org/10.1093/jat/bkz030.
[38] A. Davidsen, M. Mardal, K. Linnet, P.W. Dalsgaard, How to perform spectrum-based LC-HR-MS screening for more than 1,000 NPS with HighResNPS consensus fragment ions, PLoS One (2020). https://doi.org/10.1371/journal.pone.0242224.
[39] C. Noble, P. Weihe Dalsgaard, S. Stybe Johansen, K. Linnet, Application of a screening method for fentanyl and its analogues using UHPLC-QTOF-MS with data-independent acquisition (DIA) in MSE mode and retrospective analysis of authentic forensic blood samples, Drug Test. Anal. 10 (2018) 651–662. https://doi.org/10.1002/dta.2263.
[40] E. Partridge, S. Trobbiani, P. Stockham, C. Charlwood, C. Kostakis, A case study involving U-47700, diclazepam and flubromazepam—application of retrospective analysis of HRMS data, J. Anal. Toxicol. 42 (2018) 655–660. https://doi.org/10.1093/jat/bky039.
[41] M.A.B. Axelsson, H. Lövgren, R. Kronstrand, H. Green, M.A. Bergström, Retrospective identification of new psychoactive substances in patient samples submitted for clinical drug analysis, Basic Clin. Pharmacol. Toxicol. 131 (2022) 420–434. https://doi.org/10.1111/BCPT.13786.
[42] M. Mardal, P.W. Dalsgaard, B.S. Rasmussen, K. Linnet, C.B. Mollerup, Scalable analysis of untargeted LC-HRMS data by means of SQL database archiving, Anal. Chem. 95 (2023) 4592–4596. https://doi.org/10.1021/acs.analchem.2c03769, 10.
[43] M. Pan, B.S. Rasmussen, P.W. Dalsgaard, C.B. Mollerup, M.K.K. Nielsen, M. Nedahl, K. Linnet, M. Mardal, A new strategy for efficient retrospective data analyses for designer benzodiazepines in large LC-HRMS datasets, Front. Chem. 10 (2022) 1–11. https://doi.org/10.3389/fchem.2022.868532.
[44] C.B. Mollerup, B.S. Rasmussen, S.S. Johansen, M. Mardal, K. Linnet, P.W. Dalsgaard, Retrospective analysis for valproate screening targets with liquid chromatography–high resolution mass spectrometry with positive electrospray ionization: an omics-based approach, Drug Test. Anal. 11 (2019) 730–738. https://doi.org/10.1002/dta.2543.
[45] L.J. Høj, C.B. Mollerup, B.S. Rasmussen, S.S. Johansen, K. Linnet, P.W. Dalsgaard, Identification of phenobarbital and other barbiturates in forensic drug screening using positive electrospray ionization liquid chromatography–high resolution mass spectrometry, Drug Test. Anal. 11 (2019) 1258–1263. https://doi.org/10.1002/dta.2603.
[46] D. Pasin, M. Pan, B.S. Rasmussen, K. Linnet, P.W. Dalsgaard, C.B. Mollerup, Metabolomics-driven determination of targets for salicylic acid and ibuprofen in positive electrospray ionization using LC-HRMS, Drug Test. Anal. 14 (2022) 747–756. https://doi.org/10.1002/dta.3215.
[47] K.L. Nielsen, R. Telving, M.F. Andreasen, J.B. Hasselstrøm, M. Johannsen, A metabolomics study of retrospective forensic data from whole blood samples of humans exposed to 3,4-methylenedioxymethamphetamine: a new approach for identifying drug metabolites and changes in metabolism related to drug consumption, J. Proteome Res. 15 (2016) 619–627. https://doi.org/10.1021/acs.jproteome.5b01023.
[48] T. Wang, K.L. Nielsen, K. Frisch, J.K. Lassen, C.B. Nielsen, C.U. Andersen, P. Villesen, M.F. Andreasen, J.B. Hasselstrøm, M. Johannsen, A retrospective metabolomics analysis of gamma-hydroxybutyrate in humans: new potential markers and changes in metabolism related to GHB consumption, Front. Pharmacol. 13 (2022) 1–16. https://doi.org/10.3389/fphar.2022.816376.
[49] A. Elmsjö, C. Söderberg, G. Jakobsson, H. Green, R. Kronstrand, Postmortem metabolomics reveal acylcarnitines as potential biomarkers for fatal oxycodone-related intoxication, Metabolites 12 (2022) 109–112. https://doi.org/10.3390/METABO12020109.
[50] A. Elmsjö, S. Vikingsson, C. Söderberg, F.C. Kugelberg, H. Green, Post-mortem metabolomics: a novel approach in clinical biomarker discovery and a potential tool in death investigations, Chem. Res. Toxicol. 34 (2021) 59. https://doi.org/10.1021/acs.chemrestox.0c00448.
[51] A.E. Steuer, L. Brockbals, T. Kraemer, Metabolomic strategies in biomarker research-new approach for indirect identification of drug consumption and sample manipulation in clinical and forensic toxicology? Front. Chem. 7 (2019). https://doi.org/10.3389/fchem.2019.00319.