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Overview of systematic toxicological analysis strategies and their coverage of substances in forensic toxicology

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Abstract

Systematic toxicological analysis (STA) is the process of using an adequate analytical methodology to detect and identify as many potentially toxicologically relevant compounds as possible in biological samples. STA is an important part of everyday routine work within forensic toxicology, and several methods for STA have frequently been published and reviewed independently. However, the many drugs and other substances involved, as well as the constant emergence of new ones, may pose a major challenge in STA, which often demands a strategy involving multiple analytical methods in parallel. Such strategies have been published and evaluated less frequently despite their relevance in forensic toxicology. This mini-review briefly summarizes commonly applied methods for STA in forensic toxicology, including gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–MS (LC–MS) methods, and highlights some of their potential pitfalls. Second, it provides an overview of previously reported strategies to conduct STA, including a presentation of the STA strategy applied in the authors' laboratory. This involves broad drug screening by LC–high-resolution MS, supported by targeted screening and quantification using LC–tandem MS, headspace (HS)-GC–MS, HS-GC–flame ionization detector and other complementary methods. The STA strategy aims to cover as many potentially relevant drugs as possible and seeks to reduce potential pitfalls arising in forensic casework. The review underlines that not every substance can be identified in all circumstances even with a comprehensive STA strategy.

KEYWORDS
drug screening, forensic toxicology, high-resolution, mass spectrometry, STA

Abbreviations: BHB, beta-hydroxybutyric acid; DFSA, drug-facilitated sexual assault; DOA, drugs of abuse; EMCDDA, European Monitoring Centre for Drugs and Drug Addiction; ESI, electrospray ionization; GC, Gas chromatography; GHB, gamma-hydroxybutyric acid; HbA1c, hemoglobin A1c; HRMS, high-resolution MS; HS, headspace; ICP, inductively coupled plasma; LC, liquid chromatography; MS, mass spectrometry; NPSs, New psychoactive substances; PPT, protein precipitation; SPE, solid phase extraction; STA, systematic toxicological analysis; TOF, Time of flight.

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1 | INTRODUCTION

In forensic toxicology postmortem cases, drug-facilitated sexual assault cases, and other poisoning cases, the analysis of a wide variety of substances with diverse physicochemical properties is an essential task to identify potential drug consumption, intoxications, and metabolic diseases. This is also a process that often requires comprehensive analytical methods because any substance may be involved, and depending on the case type a high sensitivity is needed when very potent compounds might be involved.[1–3] Frequently implicated substances include medical drugs (i.e. small molecules), drugs of abuse and their metabolites, ethanol, as well as rarer substances such as natural toxins, cyanide, doping agents, gasses, nitrite, metals, and other substances. In some regions, natural plant toxins as well as pesticides are of importance. The detection and identification of as many potential toxicologically relevant substances as possible and their metabolites in biological and related samples by employing an adequate analytical methodology is referred to as systematic toxicological analysis (STA).[4,5] Biological samples commonly analyzed to verify recent consumption include blood, plasma, serum, urine, and tissues.[5,6] STA is an important part of the everyday routine work in forensic toxicology cases, and various methods for STA have been described and evaluated.[2,5,7] In the past decades, immunoassays, gas chromatography (GC), liquid chromatography (LC), and other chromatographic methods using various detectors, such as UV-diode array detection, have commonly been used in STA.[3,8] More recently, there has been an increase in the application of hyphenated chromatographic and spectrometric methods, such as LC–tandem mass spectrometry (LC–MS/MS), GC–MS/MS, and highly selective and sensitive screening procedures using LC–high-resolution MS (LC–HRMS).[9,10] Despite the comprehensive description and review of the numerous methods employed for STA, the overall STA strategy comprising an overview of the method setups used for STA in the individual laboratories has been less commonly reported. The constant emergence of new drugs, differences in capabilities, and factors influencing the interpretation of toxicological findings may pose a major challenge in STA, thereby demanding the deployment of an STA strategy that comprises comprehensive analytical methods that are used in parallel and continuously refined and in which potential pitfalls are considered.[6] Strategies that employ continuous adjustments and improvements in sample preparation and instrumentation may increase toxicology testing efficiency by allowing for better sensitivities, improved specificity, and reduced resource utilization.[11]

This mini-review briefly summarizes the individual methods for STA commonly applied in forensic toxicology cases and highlights some of their potential pitfalls. Second, it provides an overview of published STA strategies and of the STA strategy and method setups in the authors' laboratory, including sample preparation, broad drug screening by LC–HRMS supported by screening and quantification of relevant drugs using additional chromatographic methods, and other analytical methods. The compound coverage and potential pitfalls are described.

2 | A BRIEF OVERVIEW OF COMMONLY APPLIED STA METHODS IN FORENSIC TOXICOLOGY

2.1 | Immunoassay methods

Immunoassays are commonly used in forensic contexts and are often applied together with other analytical methods.[12] Immunoassays provide rapid and simple screening findings for specific drugs or drug classes and may thus cover some of the most frequently occurring drugs. However, these assays are only available for a selected subset of new psychoactive substances (NPS), thereby limiting the scope of the screening.[13,14] Other drawbacks of immunoassays include false positive and negative results, and the requirement for a separate independent approach to confirm the exact drug finding.[3] False positive results may be caused by cross-selectivity when an antibody binds potential new substances other than the target analyte and by interferences from other drugs or biomolecules. False-negative results can be caused by adulterations, such as the denaturation of antibodies and enzymes by acids, bases, or salts or by measurement disturbances.[14] Nevertheless, pre-screening of conventional drugs of abuse by immunoassays is often performed and further confirmed by GC–MS or LC–MS.[14]

2.2 | GC–MS methods

GC–MS in scan mode is an established screening procedure encompassing a wide range of compounds that are related to comprehensive libraries (e.g. NIST[15] and the Maurer/Pfleger/ Weber mass spectral library[16]). GC–MS methods combine the high separation power of GC with the high selectivity of electron ionization MS.[2] However, the use of GC–MS to analyze compounds that are polar, large, or thermally unstable may cause a failure. In some cases, these challenges can be circumvented by sample preparation with derivatization or cleavage of conjugates.[14] Moreover, artefacts can be formed by heating in the injection port, for example, the formation of methamphetamine from ephedrine.[17] Therefore, the choice of sample preparation prior to analysis by GC–MS is especially crucial. To avoid such issues, especially in cases with low drug concentrations, the use of LC–MS methods may be preferable.[12] Nevertheless, GC–MS can be used as a complementary tool to detect compounds which are not detected by LC–MS such as volatile compounds.

2.3 | LC–MS methods

LC–MS and LC–MS/MS-based methods for STA in forensic toxicology are regularly published and reviewed.[2,3,6,18] Both low-resolution and HR instruments are frequently employed for untargeted or targeted screening and/or quantification in STA.[2,6,18] Some of the most recent publications have been reviewed and outlined by Peters et al.[2] and Remane et al.[18] These reviews underlined the importance of employing

proper sample preparation to include a wide variety of toxicologically relevant substances. Techniques such as liquid–liquid extraction, protein precipitation (PPT) and solid-phase extraction (SPE) were often applied.[2,18] The reviews reported common analysis of aqueous samples using chromatography with reversed-phase stationary phases and instruments with MS or MS/MS capabilities operated mostly with positive electrospray ionization (ESI) as an ionization technique.[6,18] By applying LC-MS/MS screening, several hundred substances can be included for simultaneous analysis as described in previous publications by Rosano et al.[19] and Di Rago et al.[20] or in application notes from major instrument manufacturers. In contrast, with greater mass resolution and accuracy, HRMS-based screening approaches can cover thousands of drugs in a single analytical run, which is advantageous in STA.[10,14,21] Most common HRMS instruments are of the time-of-flight (TOF) or orbitrap types with compound libraries used for data interpretation.[10] One usually distinguishes between targeted screening based on reference substances with confirmed fragmentation patterns and retention times and suspect screening based on reported fragmentation patterns and no verified retention time. However, if chromatography corresponding to a published method is used, the presumed retention time will be close to the published one, and a tentative window can be specified. Both data-independent and data-dependent acquisition have been employed in STA.[18] The former is more comprehensive but may be subject to interference from endogenous substances, whereas the latter is more selective. Exact mass, retention time, isotopic pattern match and isotope match intensity are commonly used criteria for the identification of drugs.[1,18] Grapp et al.[1] and Broecker et al.[4] reported a higher number of positive identifications using LC–QTOF-MS compared to traditional screening by GC–MS. However, it has also been found that some compounds are not detectable by the LC–QTOF-MS methods, implying that GC–MS may still be the recommended choice for the detection of some compounds such as pregabaline,[1] as well as propofol, and other volatile compounds such as chloral hydrate and its metabolite. In addition, Broecker et al.[4] reported that some compounds (i.e. furosemide, ibuprofen, barbiturates and salicylic acid) are not detectable by their method that was operated only with positive ESI and stated that negative ESI is necessary for the detection of these compounds. This challenge may apply to many HRMS approaches used for STA, as only positive ESI is frequently applied since it covers most of the typical targets.[6]

2.4 | Other methods

Supplementary methods frequently required to cover additional substances include GC–flame ionization detector (GC–FID) for analyzing ethanol as well as other volatiles.[22] Other methods may be required in special cases and other rarer cases, including the LC–UV methods for analyzing inorganic ions such as nitrite/nitrate[23] and inductively coupled plasma MS (ICP–MS), a method used for the elemental analysis of metals, metalloids and minerals, and may include the evaluation of arsenic, thallium, lead, mercury, lithium and others.[24,25] Goullé et al.[25] previously evaluated the current role of ICP–MS in forensic toxicology and reported that deaths attributed to metals are infrequent and often unexpected and therefore suggested that a whole blood multielemental analysis should be routinely carried out in all unexplained deaths.[25]

TABLE 1 Examples of published STA strategies in forensic toxicology.

Analytical method	Approach	Case type	Reference
LC–HRMS	Screening	DFSA cases	Wille et al.[9]
UHPLC–MS/MS, GC–FID, GC–MS	Quantification		
Immunoassay, GC–MS, HPLC–DAD, HS–GC–FID	Screening	Postmortem cases	Lefrancois et al.[26]
GC–MS, HPLC–DAD, HS–GC–FID, LC–MS/MS	Quantification		
GC–MS	Screening	Casework samples	Pitterl et al.[28]
LC–MS/MS	Untargeted screening		
GC–MS, immunochemical tests, LC–MS/MS, SPME–GC/MS	Screening	Postmortem case	Gottzein et al.[27]
GC–MS, HPLC–DAD, LC–MS/MS	Quantification		

Abbreviations: DAD, diode-array detector; DFSA, drug-facilitated sexual assault; FID, flame ionization detector; GC, gas chromatography; HPLC, high-performance liquid chromatography; HS, head-space; LC, liquid chromatography; MS, mass spectrometry; SPME, solid-phase microextraction.

3 | OUTLINE OF STA STRATEGIES IN FORENSIC TOXICOLOGY

Despite the abundance of papers on screening methodologies, relatively few published studies present an overview of all the methods used in parallel for STA and do not elaborate further on the method setups. To sufficiently cover the analysis of toxicologically relevant compounds, an STA strategy customized for different case types and comprising several analytical methods may be necessary. Table 1 lists examples of recently published STA strategies. These usually involve various stages of sample preparation followed by a variety of drug screening and quantification methods.[9,26–28]

Wille et al.[9] recently described the application of an STA strategy that contained multiple methods for drug analysis in urine and blood samples from victims of drug-facilitated sexual assault (DFSA). They applied drug screening using LC–HRMS with a QTOF instrument followed by confirmation and quantification using several targeted methods. Illicit drugs such as amphetamines, cocaine, opioids, and relevant analogues and metabolites as well as antidepressants, benzodiazepines, and neuroleptics were quantified using LC–MS/MS in the multiple-reaction-monitoring (MRM) mode, whereas ethanol was

quantified via headspace (HS)-GC–FID and gamma-hydroxybutyric acid (GHB) via GC–MS (See Table 1). They emphasized the need to combine a wide screening technique, such as LC–HRMS, with sensitive multi-target quantitative methods to ensure up-to-date screening of all potential compounds with appropriate sensitivity, which is especially crucial for DFSA cases.[9] Lefrancois et al.[26] reported an STA strategy performed on postmortem cases that includes several methods for screening and quantification of therapeutic drugs, drugs of abuse, volatiles, cyanide, and pesticides. Analysis of carboxyhemoglobin formed in cases of carbon monoxide intoxication and GHB in cases with suspicion of exposure were performed in special cases.[26] Pitterl et al.[28] described their primarily established method for STA on human body fluids, which is a GC-MS method, and in order to increase the range of detectable compounds, they added an LC-MS/MS method. Samples were analyzed using both methods by splitting the eluate after SPE. Gottzein et al.[27] used an STA strategy involving multiple screening and quantification methods covering the analysis of multiple drug classes in a variety of body fluids and organs retained from the autopsy. They demonstrated that an STA involving various methods is an important part of the postmortem investigation and that it also needs to be adapted to the facts presented in each case to produce satisfying and relevant results.[27]

4 | PRESENTATION OF AN EXAMPLE OF AN STA STRATEGY

The STA strategy applied in the authors' laboratory includes an LC–HRMS screening method for several thousand compounds using positive ESI supported by a set of LC–MS/MS and GC methods to supplement the detection of compounds not covered by the LC–HRMS method and for the quantitation/confirmation of positive screening results. The STA strategy was developed to perform a quick drug analysis by running multiple methods simultaneously and to cover as many relevant compounds as possible. The most common samples to be examined routinely are blood, brain, and urine; however, other samples such as muscle and hair may also be examined in some cases.[29–31]

4.1 | Analytical methods used in the STA strategy

An example of the methods included in the STA strategy for postmortem cases is outlined in Figure 1. The STA strategy is performed for all postmortem cases but can be expanded depending on the case circumstances. Validation has been performed in accordance with international standards, and the majority of the methods have been independently published in peer-reviewed scientific journals.[21,29–39] A number of the methods have undergone additional modifications to meet the current requirements.[21] The sample preparation carried out in many of the methods was based on fully automated robotic handling, including fully automated SPE and/or PPT.[21,29–33,35,37–39]

4.1.1 | Screening by LC–HRMS

The STA strategy performed for postmortem cases comprises targeted drug screening supplemented by a suspect-modified screening (semi-targeted workflow) using LC–TOF-MS (Waters Xevo-G2-S QTOF) and the UNIFI software as well as a screening library comprising more than 5000 compounds (*Method I*, Figure 1).[21] Protein precipitation, used for sample preparation, facilitates the detection of a wide range of compounds. The targeted screening is based on compound identification by matching the measured data with the molecular formula, structure, retention time, and mass-to-charge ratio for the precursor and product ions obtained from reference standards. The targeted screening library consists of approximately 2000 compounds representing pharmaceutical drugs, drugs of abuse, and their metabolites.[21] Data acquisition is limited to positive ESI, but by identifying adducts of substances that normally require negative ESI modes for detection, such as salicylic acid, ibuprofen, barbiturates, and valproate, they can be identified in the LC–HRMS screening, thereby overcoming some of the potential pitfalls of this screening method.[40,41] Because the reference standards for some metabolites and compounds, such as NPSs, are not always accessible, the targeted screening is supplemented by a suspect screening involving the identification of approximately 3000 compounds based on predicted reference data.[21] Untargeted screening is rarely performed and only if specifically requested. Untargeted screening consists of various steps, such as filtering, elucidation of the molecular formula, comparison and matching with other libraries like the Human Metabolome Database, DrugBank and ChemIDplus, and tentative identification. Reference standards are needed for final confirmation.[21]

4.1.2 | Chromatographic methods used in parallel

The LC–HRMS screening is accompanied by a variety of other chromatographic methods including LC–MS/MS, HS-GC–MS and HS-GC–FID methods (*Method II–V, VIII* and *IX*, Figure 1), to quantify the relevant LC–HRMS screening findings as well as identify and quantify common substances, that is, GHB, THC, ethanol and other volatiles, not covered by LC–HRMS.[21] The methods are applied in parallel to reduce waiting time for scanning results and due to the relatively frequent number of positive findings in these methods. LC–MS/MS in the MRM mode is applied to quantify drugs of abuse and benzodiazepines (*Method II*),[29] pharmaceuticals (i.e. antidepressants, antipsychotics and analgesics) separated based on their acidic, neutral and basic properties by splitting the eluate after SPE (*Method III*),[31] and GHB, beta-hydroxybutyric acid (BHB) and cannabinoids separated for analysis by PPT followed by SPE (*Method IV*).[35,36] BHB is used as a marker for diabetic/alcoholic ketoacidosis, and in cases of high blood BHB levels (>1000 µmol/L), the measurement of vitreous/urine glucose and haemoglobin A1c (HbA1c) is subsequently included to evaluate potential metabolic diseases (See section 4.1.3 and *Method VII* Figure 1). The quantification of ethanol, acetone, isopropanol, and methanol and the screening of lighter gas

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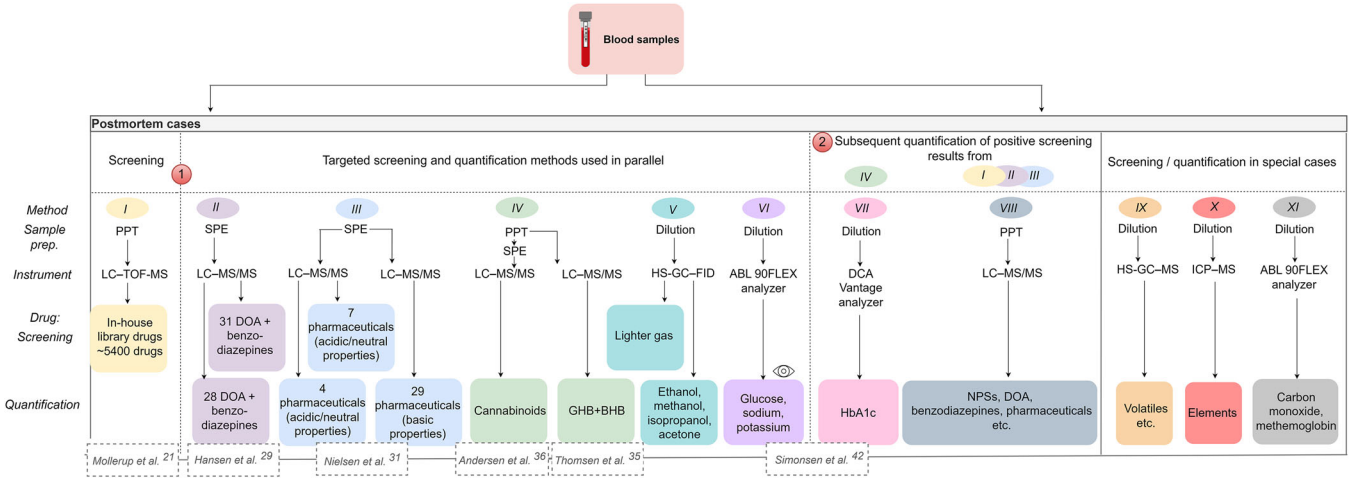

FIGURE 1 Overview of the STA strategy and method setups applied for postmortem cases in the authors' laboratory. Screening and simultaneous targeted screening and quantification are initially performed, indicated by (1), and quantification of positive screening results is then performed, indicated by (2). The methods are indicated by I–XI. Method VI is performed in vitreous humour/urine. For further explanations see section 4.1. Abbreviations: BHB: beta-hydroxybutyric acid, DOA: drugs of abuse, GC: Gas chromatography, GHB: gamma-hydroxybutyric acid, HbA1c: hemoglobin A1c, LC: liquid chromatography, MS: mass spectrometry, NPSs: New psychoactive substances, PPT: protein precipitation, SPE: solid phase extraction, TOF: Time of flight.

is performed via GC–FID-MS (*Method V*). For substances not covered by broad LC–HR–MS screening, targeted screenings for the concerned substances are performed in parallel on LC–MS/MS (*Method II, III and V*). The positive findings from the LC–HRMS and LC–MS/MS screenings are subsequently quantified using LC–MS/MS (*Method VIII*).

4.1.3 | Other complementary methods and methods used in special cases

Other complementary methods covering additional substances are also included for simultaneous analysis in STA for postmortem cases. This includes measurements of glucose, sodium and potassium in vitreous humour/urine using an ABL 90FLEX blood gas analyzer instrument (Radiometer) to evaluate possible electrolyte imbalances (*Method VI*, Figure 1).[42]

In addition to the methods described above, other methods may be used in special cases, depending on the case history and if requested. Measurement of HbA1c in the blood is only included in cases of high blood BHB levels (>1000 µmol/L) in order to differentiate between the different kinds of ketoacidosis, by a combination of immunology and spectrometry methods using a DCA Vantage Analyzer (Siemens) (*Method VII*, Figure 1). Carbon monoxide is analyzed in victims of fire and methemoglobin in cases where nitrite poisoning is suspected using an ABL 90FLEX instrument (*Method XI*, Figure 1). HS-GC–MS is used for the analysis of other volatile compounds including laughing gas (nitrous oxide) and lighter gas, as well as other toxic compounds such as cyanide and 2,2,2-trichlorethanol (a metabolite of chloral hydrate) (*Method IX*, Figure 1). ICP–MS may be included in the analysis of elements such as lithium, toxic metals and arsenic compounds (*Method X*, Figure 1).

4.2 | Addressing potential pitfalls

The mini-review presents an example of an STA strategy and method setups implemented for postmortem samples. The STA involves the use of multiple comprehensive methods in parallel with an in-house library comprising several thousand compounds, which have been established to cover as many relevant compounds as possible. Despite this, potential pitfalls may emerge in situations involving substances that are not included in the screening library and are not suspected.

The LC–HRMS screening performed as a part of the STA strategy addresses small organic molecules, including medical drugs and illicit drugs of abuse, and is primarily operated with positive ESI to cover most of the typical target compounds. Some target compounds included in the LC-HRMS screening may be associated with sensitivity problems, for example, potent NPSs like nitazenes and NBOMes. To circumvent this issue, the LC–MS/MS targeted methods are structured to cover the majority of such potent drugs. Substances that are exclusively detectable with negative ESI are typically not included in libraries and may therefore potentially go undetected if there is no suspicion of consumption. This applies to newer antidiabetic drugs such as the A10BK SGLT-2 inhibitors empagliflozin, canagliflozin, and ertugliflozin among others, which are only detectable with negative ESI. The libraries are continuously updated with new substances according to their appearance in cases or in line with new trends worldwide and early warning systems, for example, from European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). More recently, attention has been directed toward fatal methemoglobinemia, as a new trend in intentional ingestion of sodium nitrite has been observed as a method of suicide.[43] An analogous trend of consuming nitrite as a suicide method, which has been communicated on the inter-

net, includes ingestion of plant toxins such as Taxus Baccata.[44] This underlines the importance of continuously searching the internet for new trends and adapting accordingly.

This mini-review presents an STA strategy that covers a large number of substances with global relevance and includes the majority of cases without expending excessive resources. The STA can be modified to fit the local environment and avoid pitfalls. Nevertheless, these pitfalls may differ from other forensic toxicology laboratories in different regions and nations, depending on the surrounding environment and circumstances. This may especially apply to the analysis of obsolete substances, which are not present worldwide, and some substances are obscure in some countries but not in others, as is the case for some rodenticides and pesticides. The STA procedure does not cover compounds such as biologics and other proteins, and peptides such as insulin and snake venoms. These substances are emerging with an increased prevalence and may constitute future challenges when performing STA.

4.3 | Keeping up to date

To keep up with trends in the expanding drug market and to overcome some of the potential pitfalls associated with an STA procedure, the in-house LC–HRMS library should be continuously updated with new targets from analyzed cases and retrospective data analysis for recently reported NPS. Furthermore, systematically reviewing the internet for relevant home pages is important, for instance, those of the EMCDDA, United Nations Office on Drugs and Crime and The Center of Forensic Science Research and Education. Recent scientific publications or handbooks on new trends in use and misuse patterns as well as the occurrence of new NPS are also important. Libraries can conveniently be expanded by participating in a database sharing with other forensic laboratories, as in the case of the crowd-sourced HighResNPS.com database.[45] Moreover, application notes from the major vendors may be helpful.[45,46]

Recently, an efficient and scalable retrospective data analysis workflow was developed and established for use in the author's laboratory for the retrospective data analysis of previously analyzed samples to identify new targets from analytes missed in the first data analysis in order to improve the STA.[47] More extensive in-house libraries may improve the STA in cases with unknown compounds and ultimately lead to more solved cases. However, it must be considered that more extensive libraries can also lead to a greater workload of identifying the relevant compounds in the standard routine cases and lead to potentially more false-positive screening results. Partridge et al.[48] previously described the usefulness of applying an LC-HRMS screening method comprising 320 fully validated compounds, which is capable of screening for many more compounds using an expanded spectral database. The method proved to be simple and robust for the screening and identification of several hundred forensic significant compounds consistent with normal use, and it provides the flexibility to identify non-targeted compounds, thus minimizing the possibility of false positive and negative results.[48] Alternatively, machine learning used in combination with the available methods has been proposed as another approach for better and smarter structure elucidation.[49]

5 | CONCLUSION

This mini-review briefly summarized the commonly applied methods for STA in forensic toxicology, including GC–MS and LC–MS methods, and some of their potential pitfalls. The summary implies that to overcome some of the potential pitfalls in forensic toxicology casework, it is generally necessary to apply a strategy in STA involving a variety of analytical methods to cover a wider range of toxicologically relevant compounds. Previously reported strategies in STA were outlined, and the STA strategy used in the authors' laboratory was presented with its drug coverage and potential pitfalls. However, the mini-review underlines that not every substance can be identified under all circumstances, even with a comprehensive STA strategy.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable - no new data generated, or the article describes entirely theoretical research.

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