Novel Approaches in Clinical Toxicology Using High-Resolution MS/MS and Dual LC for Analyzing Biological Samples

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VORWORT

Die nachfolgende Arbeit entstand unter der Anleitung von Herrn Univ.-Prof. Dr. rer. nat. Markus R. Meyer in der Abteilung für Experimentelle und Klinische Toxikologie der Fachrichtung 2.4. Experimentelle und Klinische Pharmakologie und Toxikologie der Universität des Saarlandes in Homburg in der Zeit von Februar 2021 bis Januar 2025.

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- Rapid analysis of amatoxins in human urine by means of affinity column chromatography and liquid chromatography-high-resolution tandem mass spectrometry, Vollmer et al., Sci Rep, 2024. (DOI: 10.1038/s41598-024-72463-3)
- Polyclonal antibodies towards abrin and ricin – Design and potential application for mass spectrometry-based analysis of human biosamples, Vollmer et al., ATOX-S-25-01100, submitted, 05/2025. (DOI not yet provided)
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“All our dreams can come true,

if we have the courage to pursue them”

Walt Disney

ZUSAMMENFASSUNG

Analytische Methoden basierend auf hochauflösender Tandem-Massenspektrometrie (HRMS/MS) gekoppelt an Flüssigchromatographie (LC) ermöglichen die Detektion und Quantifizierung von schädlichen Verbindungen und Arzneistoffen in verschiedenen biologischen Proben. Diese Dissertation zielte darauf ab, die Möglichkeiten eines modernen Orbitrap-basierten Massenspektrometers in Verbindung mit einem dualen LC System zu untersuchen. Der Schwerpunkt lag auf der Entwicklung von LC-HRMS/MS Methoden, um eine zeitsparende Probenvorbereitung, flexible und nachhaltige Analysestrategien sowie eine ausreichende Empfindlichkeit für den Nachweis von Analyten mit unterschiedlichen physikalisch-chemischen Eigenschaften zu ermöglichen. Vier verschiedene Methoden wurden entwickelt und validiert, welche die Analyse von Verbindungen mit niedrigem und hohem Molekulargewicht in Plasma, die Anwendung von Extraktionstechniken auf Antikörperbasis zum Nachweis toxischer Peptide in Plasma und Urin sowie eine vergleichende Studie zur Mikrofluss LC und analytischen LC zur Identifizierung ausgewählter missbräuchlich verwendeter Drogen in Speichel umfassten. Methodenentwicklung und Validierung zeigten bestimmte Limitationen auf, die resultierenden Methoden erwiesen sich aber als wirksam bei der Beantwortung kritischer toxikologischer Fragestellungen. Diese Arbeit verdeutlicht die Flexibilität und Vielseitigkeit aktueller LC-HRMS/MS-Instrumente und Analysen im Bereich der analytischen Toxikologie.

SUMMARY

Analytical methods utilizing high-resolution tandem mass spectrometry (HRMS/MS) coupled to liquid chromatography (LC) allow the reliable detection and quantification of harmful compounds and drugs across various biological samples. This dissertation aimed to explore the capabilities of a state-of-the-art Orbitrap-based mass spectrometer paired with a dual LC system. The focus was on developing LC-HRMS/MS methods that prioritize time-efficient sample preparation, flexible and sustainable analytical strategies, and sufficient sensitivity to enable detection of analytes with diverse physico-chemical properties. Four distinct methods were developed and validated, encompassing the analysis of both low and high molecular weight compounds in plasma, the implementation of antibody-based extraction techniques for detecting toxic peptides in plasma and urine, and a comparative study of microflow LC versus analytical flow LC for identifying selected abused drugs in oral fluid. While certain limitations were observed during method development and validation, the resulting methods proved effective in addressing critical toxicological questions. This work highlighted the flexibility and versatility of current LC-HRMS/MS instruments and analysis in the field of analytical toxicology.

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1. GENERAL PART

1.1 MASS SPECTROMETRY-BASED ANALYSIS

Mass spectrometry (MS)-based analysis has become the technique of choice in analytical toxicology for comprehensive screening and quantification of drugs, foreign compounds, and their metabolites across different biological specimens [4]. Therefore, MS is widely applied for drug metabolism studies, the investigation of pharmacokinetics and toxicokinetics, metabolomics and proteomics studies, but also the development of qualitative and/or quantitative analytical procedures [5-7]. MS instruments basically consist of an ion source responsible for generating ions that enter a mass analyzer to be separated based on their mass-to-charge (*m/z*) ratio, followed by the conversion of the ions into electrical signals that are measured by a mass detector [8, 9]. Depending on the structure, molecular weight, polarity, and volatility of an analyte, different ionization techniques are used in clinical and forensic practice e.g., electron impact ionization (EI) or (heated) electrospray ionization ((H)ESI) [10-14]. EI is preferred for volatile, low molecular weight, and thermally stable compounds, produces singly charged ions, and is commonly used in gas chromatography (GC)-MS analysis, which is applied in the clinical and forensic setting to perform drug screening in plasma and urine or for the quantification of drugs within the context of emergency toxicology [11, 15-17]. However, limitations associated with this technique are the detection of low-dosed compounds and the need to derivatize e.g., thermolabile and very polar compounds prior to their analysis [18, 19]. (H)ESI proved to be suitable for non-volatile, thermally unstable, and higher molecular weight compounds including proteins or peptides as multiple charged ions can be generated and analyzed [20]. To be considered, this ionization type is reported to be susceptible to matrix effects (ME) [21]. The centerpiece of MS instruments, which significantly affects sensitivity, is the

mass analyzer (quadrupole (Q), ion trap, time-of-flight (TOF), Fourier transform ion cyclotron, or Orbitrap mass analyzer) [22]. Focusing on high-resolution tandem MS (HRMS/MS), Orbitrap mass analyzers or even hybrid instruments equipped with two mass analyzers (e.g., Q-Orbitrap) are commonly used [13]. Different acquisition modes such as full scan (FS), targeted selected ion monitoring, product ion scan amongst others can be chosen for analysis. And although targeted analysis can improve sensitivity and selectivity, scan modes need to be thoroughly tested and evaluated for each procedure to maximize the potential of HRMS/MS analysis [23]. Furthermore, Orbitrap analyzers of state-of-the-art instruments can provide high mass resolution up to 480,000 at *m/z* 200 and accurate mass measurements (at the order of sub parts per million) [24, 25]. Thus, HRMS/MS analysis provides high selectivity and allows reliable identification of precursor ions of isobaric compounds in the MS^1 spectrum and their fragments in the MS^2 spectrum [26, 27]. In contrast, MS experiments with unit resolution pose a challenge for the discrimination of isobaric compounds without different fragmentation patterns or sufficient chromatographic separation prior to MS analysis [26]. Nevertheless, systems with unit resolution are still preferred for targeted quantitative analysis [28-30]. With the advent of more versatile mass analyzers, the power of Orbitrap-based HRMS/MS instruments is growing [31]. Thus, the evaluation of the performance of Orbitrap-based HRMS/MS analysis for qualitative but also quantitative purposes needs therefore to be an ongoing process to extend the current range of applications in analytical toxicology.

1.2 CHROMATOGRAPHIC SEPARATION

Chromatographic separation using liquid chromatography (LC) allows analytes to be separated due to interactions with a mobile phase and a stationary phase [32]. Hereby, reversed-phase LC (RPLC) and hydrophilic interaction LC (HILIC) are common

separation modes applied [33]. Choosing the appropriate stationary phase is crucial and depends on the physico-chemical properties of the analytes [34]. HILIC is preferred for the separation of polar and ionic compounds (e.g., sugars, amino acids, peptides, proteins) as they indicate better retention on polar stationary phases [35]. RPLC, mainly using alkyl bonded stationary phases (C_{18} columns) or phenyl-hexyl columns demonstrated to be suitable in metabolism studies, multi-analyte procedures for the separation of antipsychotic drugs, cardiovascular drugs, sedatives, stimulants amongst others but also for toxic plant and mushroom ingredients such as colchicine, yew constituents, or amatoxins [28, 36-42].

Moreover, different LC configurations can be used (nanoflow LC, capillary flow LC, microflow LC, analytical flow LC) which differ in terms of the inner column diameter (ID) and flow rate. Nanoflow LC characterized by column ID < 100 µm and flow rates below 1 µL/min as well as capillary flow LC defined by column ID between 150-300 µm and flow rates between 1-10 µL/min are preferred in proteomics and metabolomics workflows [43]. To be considered, the conduction of e.g., comprehensive proteome analysis necessitates gradient elution that is time-consuming and requires one or more hours [44]. Microflow LC using column ID between 0.5-1.0 mm and flow rates between 10-200 µL/min can be applied for large and small molecule analysis and is expected to reduce the injection volume, solvent consumption, column back pressure, and ME, but also to increase analytical sensitivity [43, 45-48]. Laboratories focusing on e.g., emergency toxicology usually use analytical flow LC with pumps operating columns with ID of 2.1 mm (or higher) and flow rates > 200 µL/min [43].

Currently, LC-based methods are usually carried out using single-channel LC systems [12, 41, 49-51]. However, the capabilities of LC analysis can be extended by the use of dual LC systems, which are expected to increase sample throughput, ensure greater analytical flexibility, enable better regeneration of LC columns due to an extended re-

equilibration phase, and avoid the need to change LC columns for each application [52]. Dual LC systems can operate two identical columns or two different columns (e.g., RP and HILIC) in parallel [53, 54]. Hence, dual LC systems are a worthwhile investigation for smooth analytical workflows.

1.3 BIOLOGICAL MATRICES

Different biological matrices can be used to develop analytical approaches for LC-HRMS/MS analysis such as blood (plasma, serum), urine, and oral fluid (OF) [37, 50, 51, 55-57]. In blood, analytes may be detected qualitatively, however, drugs are primarily quantified, as their concentrations offer valuable insights into their pharmacological or toxic effects [58]. However, venous blood sampling is invasive, must be performed by healthcare professionals, and carries higher infection risks [59].

Therefore, OF, which has emerged as an alternative matrix to blood and urine, provides simple and non-invasive sample collection, which can be performed in an open environment decreasing the risk of adulteration [59, 60]. Drug transfer from the blood into OF via passive diffusion is influenced by multiple factors including physico-chemical properties (drug pK_a, extent of protein binding, lipophilicity, molecular weight), its composition, the salivary flow, and the pH [60]. Basic substances (e.g., amphetamines) are expected to be present in higher concentrations, as they are ionized in the acidic environment (pH 6.2-7.4) and thus remain trapped in OF [59]. Altered concentrations in OF are also influenced by e.g., cigarette smoking or the intake of drugs with anticholinergic activity, which can lead to the ´dry mouth syndrome´ and to a reduced salivary flow [59]. OF is used to monitor drug consumption in clinical toxicology, doping control as well as workplace and roadside drug testing [61]. For the

latter, the detection of abused drugs (e.g., benzodiazepines, opioids, or stimulants) is at the center of interest [62].

Urine is still recognized as the preferred matrix for drug screening purposes [59]. In contrast to venous blood sampling, larger volumes can easily be collected without interfering with the patient's privacy. However, if urine collection is not supervised, drug screening results may be affected due to possible adulteration, dilution, drug spiking, or sample substitution [59, 63]. After compounds have been metabolized, they are eliminated mainly via the renal system into the urine, where they accumulate and can be detected in higher concentrations than in blood up to several weeks [63]. To be considered, the drug concentration can be influenced by the urinary flow, the urinary pH, the frequency of bladder emptying, or the intake of fluids, leading to urine dilution [59, 64].

To be considered, the selection of the appropriate sample matrix is crucial and depends on the availability, the pharmacokinetics of compounds, and the objective of the study [59].

1.4 SAMPLE PREPARATION TECHNIQUES

For the development of sensitive and selective qualitative and/or quantitative LC-HRMS/MS-based procedures, appropriate sample preparation is required e.g., to achieve reproducible recovery (RE) or reduce the occurrence of ion enhancement or suppression effects [58, 59]. Protein precipitation (PP), liquid-liquid extraction (LLE), solid-phase extraction (SPE), or combinations of these methods are well-established and routinely used in analytical toxicology [15, 37, 65, 66]. For PP, organic solvents or zinc sulfate are used to remove proteins or phospholipids from plasma or urine [36,

65]. LLE is based on the transfer of analytes between two immiscible phases (aqueous phase and organic phase) due to their different solubility and is applied for the extraction of a variety of toxicologically relevant compound classes e.g., antidiabetics, antidepressants, neuroleptics, and benzodiazepines [15, 29, 67, 68]. Although PP and LLE are simple to perform, both extraction techniques are often not sufficient to remove interfering matrix components (fatty acids, lipids, phospholipids) responsible for ion suppression or enhancement effects and thus affecting analytical sensitivity [69]. This limitation can be addressed by SPE which consists of preconditioning the SPE column, sample application, washing steps, and finally compound elution. The target analytes are retained by the stationary phase while interfering components are washed away [32]. Different SPE-based procedures with subsequent LC-HRMS/MS or GC-MS analysis are already established for the detection or quantification of amatoxins, stimulants, designer drugs, and peptides [20, 70, 71]. Besides, for the extraction of proteins or peptides, antibody-based enrichment using magnetic beads can be performed prior to LC-HRMS/MS analysis. Proteins or peptides (antigens) are isolated from complex matrices using antigen-specific monoclonal or polyclonal antibodies (mAB or pAB) that are covalently or non-covalently attached to a solid support [72]. Protein A and G magnetic beads proved to be suitable as they can capture immunoglobulins from different mammalian species (e.g., human, rabbit, mouse), prevent non-specific binding and thus reduce the risk of co-elution of interferences during LC-HRMS/MS analysis [73, 74].

To state, conventional sample preparation procedures form an essential part within LC-HRMS/MS-based workflows. Hence, continuous research is required to expand the landscape of current sample preparation techniques.

1.5 LIQUID CHROMATOGRAPHY-HIGH-RESOLUTION MASS SPECTROMETRY FOR SMALL MOLECULE AND PEPTIDE ANALYSIS

1.5.1 ABUSED DRUGS

While cannabis is the most abused (illicit) drug in Europe, other substances such as cocaine, synthetic stimulants (amphetamine, methamphetamine, synthetic cathinones), 3,4-methylenedioxymethamphetamine, heroin, opioids, new psychoactive substances, ketamine, or lysergic acid diethylamide also contribute to the growing issue of drug abuse [75]. Drugs of abuse testing can be carried out by means of antibody-based procedures (immunoassays, IA), which are commonly used in clinical practice as they provide rapidity and simplicity. Drug classes typically covered include benzodiazepines, cannabinoids, hallucinogens, opiates, or stimulants. However, IA are limited by their sensitivity and are susceptible to cross-reactions, leading to false-positive or false-negative results [76]. A commercially available opiate-based enzyme-linked immunosorbent assay for morphine detection is reported to show cross-reactivities towards 6-monoacetylmorphine, codeine, dihydrocodeine, morphine-*β*-3-glucuronide, oxycodone, hydromorphone, and oxymorphone. Thus, increased sensitivity and specificity as well as the possibility to perform comprehensive screening support MS-based testing as analytical tool to confirm (illicit) drug abuse [77]. As the problem of drug use continues to rise, it is becoming increasingly important to develop LC-HRMS/MS-based methods using advanced analytical techniques to reliably and sensitively detect abused drugs.

1.5.2 ANTIDIABETIC DRUGS

Globally, more than 500 million people aged between 20-79 years suffer from type I or type II diabetes mellitus (T1DM or T2DM), and the number is expected to rise in the coming years [78]. T1DM is characterized by the destruction of insulin-producing beta

cells in the pancreatic islets, resulting in an absolute insulin deficiency requiring insulin therapy, while T2DM is characterized by a progressive decline in the beta cell function leading to insulin resistance [79]. The intake of antidiabetic drugs can be associated with the occurrence of life-threatening hypoglycemia, which underlines the need for rapid, sensitive, and reliable quantitative procedures [67]. Human insulin is an endogenous peptide hormone (5807 Da, amino acids 51) consisting of two chains (A- and B-chain) connected via two disulfide bridges and plays an essential role in the regulation of blood glucose levels and the energy and anabolic metabolism [20, 80]. Synthetic insulin analogues have been further developed by amino acid substitutions (short-acting, insulin lispro, insulin aspart, insulin glulisine; long-acting, insulin detemir, insulin glargine, insulin degludec) [81]. Insulins or insulin mimetics can be misused either for suicide or homicide, but also to enhance athletic performance, which is why they are classified as prohibited substances by the World Anti-Doping Agency [80, 82]. Thomas et al. focused on the development of analytical methods to detect and quantify human insulin, its synthetic analogues, and the C-peptide using LC-HRMS/MS or LC-ion mobility-MS after SPE or immunoaffinity purification [20, 83, 84]. Particularly, LC-HRMS/MS by means of HESI is preferred as these peptide drugs are detected multiple charged with *m/z* 800-1,500 [81]. With respect to T2DM, oral antidiabetic drugs (OAD) such as the biguanide metformin, dipeptidyl peptidase-4 inhibitors (e.g., saxagliptin, sitagliptin, vildagliptin), sodium-glucose transport protein 2 inhibitors (e.g., dapagliflozine, empagliflozine), glinides (e.g., repaglinide), and sulfonyl urea (e.g., glibenclamide, glimepiride) can be used, but also the subcutaneously administered glucagon-like peptide-1 (GLP-1) analogues (e.g., exenatide, semaglutide) [85, 86]. LC-MS-based methods for the detection and/or quantification of OAD or GLP-1 analogues are reported in plasma or urine after LLE or SPE [87-89].

To date, the development of LC-MS-based methods has focused exclusively on either OAD or insulins, thus creating a significant gap of knowledge in the analysis of antidiabetic drugs which can be addressed by the development of a combined workflow.

1.5.3 MUSHROOM AND PLANT TOXINS

Amatoxin-containing mushrooms (e.g., *amanita phalloides*, death cap), which mainly contain the bicyclic octapeptides α-, β-, and γ-amanitin, play an important role in the forensic and clinical setting as they are reported to be the main cause of severe to fatal mushroom poisonings [90]. Amatoxins enter the hepatocytes via the organic anion transporting protein 1B3 and inhibit the transcription process in eukaryotic cells by binding to the RNA polymerase II leading to apoptosis [90, 91]. After amatoxin ingestion, patients are asymptomatic for the first 6-12 h followed by gastrointestinal disorders, which might appear within the next 12-24 h. Thereafter, the kidneys and the liver are damaged, accompanied by an increase in liver enzyme activities. Finally, multi-organ failure and death may occur a few days after consumption [92, 93]. In the case of amatoxin intoxication, therapy is primarily based on stabilizing the patient's condition, electrolyte balancing, and treatment with silibinin as antidote, which is recommended within 48 h following toxin ingestion [92]. LC-HRMS/MS is the most commonly applied analytical technique for the detection and also quantification of amatoxins in different biological matrices given the fact that high separation efficiency is combined with high sensitivity, specificity, and mass accuracy [93]. After SPE-based extraction, Bambauer et al. used HILIC-HRMS/MS for qualitative α- and β-amanitin detection in urine down to 1 ng/mL and RPLC-HRMS/MS for their quantification in plasma down to 0.2 ng/mL [70, 90, 94]. The study reported by Maurer et al. describes the enrichment of α- and β-amanitin from urine using immunoaffinity extraction [95].

Abrin and ricin are highly toxic plant proteins contained in the seeds of *Abrus precatorius* or *Ricinus communis*. They can be used to commit suicide or as biological weapon, which is why they are mentioned on the US Select Agents and Toxins list [96-98]. Both proteins (60-64 kDa) consist of two polypeptide chains (A- and B-chain) linked by a disulfide bridge [99]. On the cellular level, they are type-II ribosome inactivating proteins, interfering the process of eucaryotic protein synthesis causing cell death. After intravenous injection, both toxins exhibit similar lethal doses, 50 % (LD_{50}) at 2.7 and 0.7 µg/kg body weight, respectively. Patients suffer from unspecific gastrointestinal symptoms and in contrast to the amatoxins, no antidote is yet available [96, 97]. Immunoaffinity or carbohydrate affinity enrichment including enzymatic digestion using trypsin in combination with MS-based analysis (LC-ESI-MS/MS, LC-ESI-HRMS/MS, or matrix assisted laser desorption ionization TOF MS) is reported to detect abrin or ricin with sufficient sensitivity in environmental samples and food matrices with reduced matrix background [97, 99-101].

However, clinical toxicologists typically receive plasma and urine samples to be analyzed for the presence of abrin or ricin [102]. Therefore, strategies to confirm the presence of abrin, ricin, or also amatoxins in human biosamples remains crucial in analytical toxicology.

2. AIMS AND SCOPES

The detection and/or quantification of harmful compounds or drugs are important tasks to be performed in analytical toxicology. This thesis aimed to address these analytical challenges by evaluating the performance of HRMS/MS analysis provided by a state-of-the-art instrument in combination with a dual LC system.

Therefore, the following steps had to be conducted:

- Development of LC-HRMS/MS-based methods for the detection and/or quantification of selected small molecule drugs and peptides
- Evaluation of dual LC for smooth analytical workflows
- Investigation of different biological matrices and sample preparation techniques
- Investigation of microflow LC and comparison with analytical flow LC
- Validation of the established approaches in accordance with international guidelines and recommendations including proof-of-concept studies

3. PUBLICATIONS OF THE RESULTS

The results of the studies were presented in the following publications:

3.1 Simultaneous analysis of antihyperglycemic small molecule drugs and peptide drugs by means of dual liquid chromatography high-resolution mass spectrometry [1]

(DOI: 10.1515/cclm-2022-1316)

Author contribution

Aline C. Vollmer (conceptualization, methodology, validation, formal analysis, writing original draft); Armin A. Weber (resources, writing review & editing); Lea Wagmann (conceptualization, methodology, writing review & editing); Markus R. Meyer (conceptualization, methodology, resources, writing review & editing, supervision).

3.2 Rapid analysis of amatoxins in human urine by means of affinity column chromatography and liquid chromatography-high-resolution tandem mass spectrometry [2]

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Author contribution

Aline C. Vollmer (Data curation, formal analysis, investigation, methodology, software, validation, visualization, writing original draft, review & editing); Claudia Fecher-Trost (conceptualization, methodology, writing review & editing); Lea Wagmann (writing review & editing); Candace S. Bever (writing review & editing); Christina C. Tam (writing review & editing); Markus R. Meyer (data curation, conceptualization, funding acquisition, investigation, methodology, resources, supervision, writing review & editing).

3.3 Polyclonal antibodies towards abrin and ricin – Design and potential application for mass spectrometry-based analysis of human biosamples

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Aline C. Vollmer (conceptualization, data curation, methodology, validation, formal analysis, visualization, investigation, writing original draft); Claudia Fecher-Trost (investigation, writing review & editing); Martin Jung (investigation, writing review & editing); Marnie Cole (methodology, writing review & editing); Tilman F. Arnst (methodology, conceptualization, writing review & editing); Veit Flockerzi (resources, writing review & editing); Lea Wagmann (investigation, writing review & editing); Markus R. Meyer (conceptualization, methodology, resources, writing review & editing, supervision).

3.4 Advancing drug testing in oral fluid: Comparison of microflow and analytical flow LC-Orbitrap analysis [3]

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4. DISCUSSION

A state-of-the-art Orbitrap-based instrument coupled to a dual LC system for chromatographic separation, was evaluated using the four presented approaches. These studies clearly expanded the current spectrum of analytical methods for the selected compound classes through the application of advanced and sustainable techniques.

The first study focused on the simultaneous analysis of ten OAD and nine antihyperglycemic peptides and their quantification in plasma. Methods for the detection and/or quantification of OAD or human insulin, its synthetic analogues, and GLP-1 agonists are reported [20, 67, 83, 84]. If glucose levels fluctuate e.g., due to uncontrolled T2DM, the patient's treatment is based on the intake of different OAD and/or insulin therapy [103]. This increased the necessity to develop an approach suitable for analyzing antihyperglycemic small molecule drugs and peptide drugs in a smooth workflow. First, a PP of plasma samples was carried out. Then, the supernatant was split into two aliquots. One aliquot was analyzed immediately for the OAD, while the other underwent a subsequent SPE for enrichment of the antihyperglycemic peptides before analysis. In LC analysis, it is important to adequately adjust the column to the initial gradient conditions after the elution of the last compound in the chromatogram [104]. This facilitates reproducible chromatographic results [105]. Additionally, carry-over can occur, as compounds remain in the LC system and can therefore be detected in the subsequent analysis. Although matrix blanks indicated to be more efficient in reducing column carry-over, flushing the column with mobile phase is a supportive option [106, 107]. Based on a dual LC system, two similar RPLC columns were operated in parallel. Total runtime was set to 22 min while chromatographic separation of the OAD or the peptide drugs was already completed

after 12 min. This allowed the sample analysis on column 2 to be performed at the same time as the equilibration of column 1. The main limitation of this approach lies in accurately quantifying the peptide drugs at lower concentration levels. The peptide drugs were analyzed in the product ion scan mode to achieve sufficient sensitivity but also reproducible peak areas, which are required for quantitative analysis. However, due to missing scan points at low concentrations, the lower limit of quantification of human insulin and its synthetic analogues had to be set to 5 ng/mL, although physiological insulin plasma levels may vary between 0.2-3.0 ng/mL [81, 108]. Insulin glargine, exenatide, and semaglutide were only considered further for qualitative detection as reproducible quantification could not be guaranteed.

However, the analysis of patient samples submitted for toxicological analysis demonstrated the application of the established method in case of insulin overdoses, which particularly contribute to severe hypoglycemia. Furthermore, from an analytical perspective, this approach offers significant advantages. Small molecule drugs and peptide drugs can be extracted from plasma by means of one sample preparation procedure. The use of dual LC has been demonstrated to enhance time efficiency, as it enables the analysis of compounds and column equilibration in parallel.

The second study focused on the detection of α-, β-, and γ-amanitin in human urine after antibody-based enrichment using magnetic beads followed by LC-HRMS/MS analysis. Methods established for amatoxin detection and/or quantification rely mainly on SPE [70, 90, 94, 109]. However, SPE require organic solvents and, if performed manually, the process is time-consuming. To avoid time-demanding sample clean-up while achieving sufficient sensitivity for the determination of suspected amatoxin poisoning, the mAB AMA9G3, which was affinity purified from mouse ascites fluid and non-covalently attached to the surface of protein A and G magnetic beads, allowed the

detection of α-, β-, and γ-amanitin to as low as 1 ng/mL in human urine, which was in line with previous reported SPE-based procedures [40, 70, 94, 109]. After antibody binding, amatoxins were extracted using affinity column chromatography. As shown during method development and validation, the prepared antibody-bead columns can be stored in buffer solution (pH 7.4) at 4 °C for at least two months.

Thus, with this method, suspected amatoxin ingestion can be determined in less than 90 min, as it only requires affinity column chromatography to extract the target compounds, followed by LC-HRMS/MS analysis. The proof-of-concept study, conducted on suspected cases of amatoxin intoxication, compared this method to previously developed SPE-based procedures and showed agreement in amatoxin detection results [40, 94].

The strategy of antibody-based enrichment was pursued further in the third study, which aimed to design pAB (pABAbrin and pABRicin) towards an abrin-A-peptide and ricin-peptide by repeated immunization of rabbits and to investigate their potential application in MS-based analysis. Since antibody specificity was demonstrated by antibody epitope mapping, two approaches (A and B) were investigated for the detection of abrin, ricin, or related peptides in human biosamples. In approach A, blood and urine samples from two suspected ricin intoxications were analyzed after affinity column chromatography, followed by overnight trypsin digestion and preparation for nanoLC-MS analysis using a 48 min LC gradient. While ricin intake was confirmed in one of the two cases, the time-consuming nature of this workflow limited its utility in situations requiring rapid diagnosis. In contrast, approach B only involved affinity column chromatography, followed by LC-Orbitrap analysis based on a 6 min gradient, allowing the detection of an abrin-A-peptide and ricin-peptide in plasma to as low as 5 ng/mL. Due to the greater importance of feces for excretion, this approach was only

based on plasma [110]. Within the validation of approach B, peptide degradation occurred under different storage conditions (short-term, 4 °C; benchtop, 22 °C; freeze/thaw, -20 °C; storage period 7 h and 24 h in blank plasma), although the peptides were handled in Protein Lo Bind tubes to avoid adsorption and therefore a loss of analyte. Stability issues were addressed by the use of tubes spray coated with different proteases inhibitors, which are reported to be present in blood plasma and responsible for proteolytic degradation [111]. Even though the stability of the abrin-A-peptide and ricin-peptide was improved, the availability of these coated tubes in hospitals need to be considered. One important limitation for future consideration is the use of enzymatic digestion prior to MS analysis of the peptides.

Significantly, the presence of abrin or ricin in human matrix can be verified using both approaches while avoiding direct exposure to the toxic proteins in the laboratory. Approaches A and B have also demonstrated that pABAbrin and pABRicin can be flexibly integrated in different MS-based workflows, signifying their potential value in analytical toxicology.

The fourth study focused on the development and validation of a microflow LC-Orbitrap-based method for the detection of 29 abused drugs in OF and its comparison with analytical flow LC-Orbitrap analysis. Reducing the flow rate (analytical flow LC, 250 to 500 µL/min; microflow LC,100 µL) and the column ID (analytical flow LC, ID, 2.1 mm; microflow LC, ID 1 mm) allowed in particular for more narrow peaks, reduced injection volume (analytical flow LC, 5 µL; microflow LC, 1 µL) and solvent consumption. Since the injection volumes were different for both LC setups, the absolute amount of analyte applied on the microflow column was five times lower than on the analytical column, which resulted in increased sensitivity for most compounds with limits of detection (LOD) between 0.2-25 ng/mL. It has been demonstrated that

lower flow rates result in smaller droplets following ionization with (H)ESI. These droplets have a larger surface area, leading to a higher ionization efficiency [112]. Increased sensitivity was finally demonstrated by comparing microflow LC-HRMS/MS with analytical flow LC-HRMS/MS using pooled OF samples, as microflow LC-HRMS/MS achieved to detect a higher number of analytes. Specific challenges relate to Δ9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol. Despite the enrichment of the cannabinoids with SPE, THC was detectable only as low as 25 ng/mL, which did not correspond to the reported cut-off concentration of 2.0 ng/mL by the Substance Abuse and Mental Health Services Administration and the European Workplace Drug Testing Society, that establish guidelines to allow countries to conduct legally compliant OF drug testing [113, 114]. The lack of sensitivity was attributed to the low RE and the presence of ME. Moreover, methods targeting cannabinoids are typically based on LC-triple quadrupole MS as its offers both selectivity and sensitivity with LOD as low as 1 ng/mL [115].

Nonetheless, this study expanded the applicability of microflow LC for small molecule analysis while contributing to enhanced sustainability.

The conducted studies are well suited to address analytical challenges within the field of analytical toxicology and merit application particularly in clinical practice. As each approach covered different compound classes, the need to employ distinct acquisition modes in HRMS/MS workflows to achieve sufficient sensitivity became important. Dual LC was evaluated and demonstrated to be valuable for LC-HRMS/MS-based procedures. The analysis of study samples successfully determined the presence or absence of suspected intoxications. Limitations encountered in each study were identified and discussed, with considerations for future research.

5. CONCLUSION

The discussed projects demonstrated that LC-HRMS/MS analysis is an essential technique in analytical toxicology that offers both selectivity and sensitivity. This makes it crucial for applications involving the qualitative and quantitative determination of a diverse range of compounds. Using advanced LC separation techniques and sample preparation methods has led to the development of time-efficient, reliable, sensitive, and more sustainable approaches. The four established and validated methods demonstrated the suitability, flexibility, and versatility of LC-HRMS/MS analysis for detecting and quantifying selected small molecule drugs and peptide-based compounds across various biological matrices.

6. REFERENCES

1. Vollmer, A.C., et al., Simultaneous analysis of antihyperglycemic small molecule drugs and peptide drugs by means of dual liquid chromatography high-resolution mass spectrometry. Clinical Chemistry and Laboratory Medicine (CCLM), 2023. **61**(7): p. 1300-1308.
2. Vollmer, A.C., et al., Rapid analysis of amatoxins in human urine by means of affinity column chromatography and liquid chromatography-high-resolution tandem mass spectrometry. Sci Rep, 2024. **14**(1): p. 21397.
3. Vollmer, A.C., et al., Advancing drug testing in oral fluid: Comparison of microflow and analytical flow LC-Orbitrap analysis. Microchemical Journal, 2025. **212**: p. 113508.
4. Maurer, H.H., Analytical toxicology. Anal Bioanal Chem, 2007. **388**(7): p. 1311.
5. Maurer, H.H. and M.R. Meyer, High-resolution mass spectrometry in toxicology: current status and future perspectives. Arch Toxicol, 2016. **90**(9): p. 2161-2172.
6. Manier, S.K., et al., Untargeted metabolomics by high resolution mass spectrometry coupled to normal and reversed phase liquid chromatography as a tool to study the in vitro biotransformation of new psychoactive substances. Sci Rep, 2019. **9**(1): p. 2741.
7. Deschamps, E., et al., Advances in Ultra-High-Resolution Mass Spectrometry for Pharmaceutical Analysis. Molecules, 2023. **28**(5).
8. Murayama, C., Y. Kimura, and M. Setou, Imaging mass spectrometry: principle and application. Biophys Rev, 2009. **1**(3): p. 131.
9. de Hoffmann, E. and V. Strobant, Mass Spectrometry Principles and Applications. 2007: Wiley & Sons Ltd.
10. Meyer, M.R., A.A. Weber, and H.H. Maurer, A validated GC-MS procedure for fast, simple, and cost-effective quantification of glycols and GHB in human plasma and their identification in urine and plasma developed for emergency toxicology. Anal Bioanal Chem, 2011. **400**(2): p. 411-4.
11. Meyer, M.R., et al., Development, validation, and application of a fast and simple GC-MS method for determination of some therapeutic drugs relevant in emergency toxicology. Ther Drug Monit, 2011. **33**(5): p. 649-53.
12. Jacobs, C.M., L. Wagmann, and M.R. Meyer, Development, validation, and application of a quantitative volumetric absorptive microsampling-based method in finger prick blood by means of LC-HRMS/MS applicable for adherence monitoring of antipsychotics. Anal Bioanal Chem, 2021. **413**(6): p. 1729-1737.
13. Maurer, H.H., Hyphenated high-resolution mass spectrometry-the "all-in-one" device in analytical toxicology? Anal Bioanal Chem, 2021. **413**(9): p. 2303-2309.
14. Couchman, L. and P.E. Morgan, LC-MS in analytical toxicology: some practical considerations. Biomed Chromatogr, 2011. **25**(1-2): p. 100-23.
15. Maurer, H.H., et al., GC-MS Library of Drugs, Poisons, and Their Metabolites. 6. Auflage ed. Mass Spectral Library of Drugs, Poisons, Pesticides, Pollutants, and Their Metabolites 5th Edition Upgrade. 2023, Weinheim: Wiley-VCH. DVD.
16. Meyer, M.R., F.T. Peters, and H.H. Maurer, Automated mass spectral deconvolution and identification system for GC-MS screening for drugs, poisons, and metabolites in urine. Clin Chem, 2010. **56**(4): p. 575-84.

17. Vargas Medina, D.A., et al., The overshadowed role of electron ionization–mass spectrometry in analytical biotechnology. Current Opinion in Biotechnology, 2023. **82**: p. 102965.
18. Maurer, H.H., What is the future of (ultra) high performance liquid chromatography coupled to low and high resolution mass spectrometry for toxicological drug screening? J Chromatogr A, 2013. **1292**: p. 19-24.
19. Sasse, M. and M. Rainer, Mass Spectrometric Methods for Non-Targeted Screening of Metabolites: A Future Perspective for the Identification of Unknown Compounds in Plant Extracts. Separations, 2022. **9**(12): p. 415.
20. Thomas, A., et al., Simplified quantification of insulin, its synthetic analogs and C-peptide in human plasma by means of LC-HRMS. Drug Test Anal, 2020. **12**(3): p. 382-390.
21. Dams, R., et al., Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. J Am Soc Mass Spectrom, 2003. **14**(11): p. 1290-4.
22. Li, C., et al., Towards Higher Sensitivity of Mass Spectrometry: A Perspective From the Mass Analyzers. Front Chem, 2021. **9**: p. 813359.
23. Kaufmann, A., Analytical performance of the various acquisition modes in Orbitrap MS and MS/MS. J Mass Spectrom, 2018. **53**(8): p. 725-738.
24. Meyer, M.R. and H.H. Maurer, Current applications of high-resolution mass spectrometry in drug metabolism studies. Anal Bioanal Chem, 2012. **403**(5): p. 1221-31.
25. Denisov, E., E. Damoc, and A. Makarov, Exploring frontiers of orbitrap performance for long transients. International Journal of Mass Spectrometry, 2021. **466**: p. 116607.
26. Habler, K., et al., Understanding isotopes, isomers, and isobars in mass spectrometry. J Mass Spectrom Adv Clin Lab, 2024. **33**: p. 49-54.
27. Zarrouk, E., et al., High-resolution mass spectrometry: Theoretical and technological aspects. Toxicologie Analytique et Clinique, 2022. **34**(1, Supplement): p. 3-18.
28. Michely, J.A. and H.H. Maurer, A multi-analyte approach to help in assessing the severity of acute poisonings - Development and validation of a fast LC-MS/MS quantification approach for 45 drugs and their relevant metabolites with one-point calibration. Drug Test Anal, 2018. **10**(1): p. 164-176.
29. Remane, D., et al., Fast and simple procedure for liquid-liquid extraction of 136 analytes from different drug classes for development of a liquid chromatographic-tandem mass spectrometric quantification method in human blood plasma. Anal Bioanal Chem, 2010. **397**(6): p. 2303-14.
30. Caspar, A.T., M.R. Meyer, and H.H. Maurer, Blood plasma level determination using an automated LC-MS(n) screening system and electronically stored calibrations exemplified for 22 drugs and two active metabolites often requested in emergency toxicology. Drug Test Anal, 2019. **11**(1): p. 102-111.
31. Hecht, E.S., et al., Fundamentals and Advances of Orbitrap Mass Spectrometry, in Encyclopedia of Analytical Chemistry. p. 1-40.
32. Thomas, S.N., et al., Liquid chromatography-tandem mass spectrometry for clinical diagnostics. Nat Rev Methods Primers, 2022. **2**(1): p. 96.
33. Harrieder, E.M., et al., Current state-of-the-art of separation methods used in LC-MS based metabolomics and lipidomics. J Chromatogr B Analyt Technol Biomed Life Sci, 2022. **1188**: p. 123069.
34. Hemmer, S., et al., Comparison of reversed-phase, hydrophilic interaction, and porous graphitic carbon chromatography columns for an untargeted

toxicometabolomics study in pooled human liver microsomes, rat urine, and rat plasma. Metabolomics, 2024. **20**(3).
35. Buszewski, B. and S. Noga, Hydrophilic interaction liquid chromatography (HILIC)--a powerful separation technique. Anal Bioanal Chem, 2012. **402**(1): p. 231-47.
36. Helfer, A.G., et al., Liquid chromatography-high resolution-tandem mass spectrometry using Orbitrap technology for comprehensive screening to detect drugs and their metabolites in blood plasma. Anal Chim Acta, 2017. **965**: p. 83-95.
37. Helfer, A.G., et al., Orbitrap technology for comprehensive metabolite-based liquid chromatographic-high resolution-tandem mass spectrometric urine drug screening - exemplified for cardiovascular drugs. Anal Chim Acta, 2015. **891**: p. 221-33.
38. Jacobs, C.M., L. Wagmann, and M.R. Meyer, Analytical toxicology of yew constituents in human blood and urine by liquid chromatography-high-resolution tandem mass spectrometry. Drug Test Anal, 2022.
39. Vollmer, A.C., L. Wagmann, and M.R. Meyer, Toxic plants-Detection of colchicine in a fast systematic clinical toxicology screening using liquid chromatography-mass spectrometry. Drug Test Anal, 2021.
40. Helfer, A.G., et al., Direct analysis of the mushroom poisons alpha- and beta-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, 2014. **1325**: p. 92-8.
41. Hemmer, S., et al., Comprehensive Qualitative Drug Screening in Emergency Toxicology Using an Automated LC–MSn System:Simultaneous Quantification of Relevant Drugs and Metabolites in Blood Plasma. Drug Testing and Analysis, 2025.
42. Wagmann, L., et al., Going deeper into the toxicokinetics of synthetic cannabinoids: in vitro contribution of human carboxylesterases. Arch Toxicol, 2022. **96**(10): p. 2755-2766.
43. Bian, Y., C. Gao, and B. Kuster, On the potential of micro-flow LC-MS/MS in proteomics. Expert Rev Proteomics, 2022. **19**(3): p. 153-164.
44. Fecher-Trost, C., et al., The in vivo TRPV6 protein starts at a non-AUG triplet, decoded as methionine, upstream of canonical initiation at AUG. J Biol Chem, 2013. **288**(23): p. 16629-44.
45. Steuer, A.E., et al., Comparison of conventional liquid chromatography-tandem mass spectrometry versus microflow liquid chromatography-tandem mass spectrometry within the framework of full method validation for simultaneous quantification of 40 antidepressants and neuroleptics in whole blood. J Chromatogr A, 2015. **1381**: p. 87-100.
46. Wang, H. and P. Bennett, Performance assessment of microflow LC combined with high-resolution MS in bioanalysis. Bioanalysis, 2013. **5**(10): p. 1249-67.
47. Geller, S., et al., Comparison of Microflow and Analytical Flow Liquid Chromatography Coupled to Mass Spectrometry Global Metabolomics Methods Using a Urea Cycle Disorder Mouse Model. J Proteome Res, 2022. **21**(1): p. 151-163.
48. Bian, Y., et al., Robust, reproducible and quantitative analysis of thousands of proteomes by micro-flow LC-MS/MS. Nat Commun, 2020. **11**(1): p. 157.
49. Richter, L.H.J., et al., Development and application of a LC-HRMS/MS method for analyzing antihypertensive drugs in oral fluid for monitoring drug adherence. Anal Chim Acta, 2019. **1070**: p. 69-79.

50. Wagmann, L., et al., Assessing Adherence to Antihypertensive Medication by Means of Dose-Dependent Reference Plasma Concentration Ranges and Ultra-High Performance Liquid Chromatography-Ion Trap Mass Spectrometry Analysis. Molecules, 2021. **26**(5).
51. Maalouli Schaar, J., et al., Studying drug excretion into exhaled breath aerosol – A workflow based on an impaction sampling device and LC-HRMS/MS analysis. Analytica Chimica Acta, 2024. **1323**.
52. Diaz-Galiano, F.J., et al., Cutting-edge approach using dual-channel chromatography to overcome the sensitivity issues associated with polarity switching in pesticide residues analysis. Anal Chim Acta, 2021. **1180**: p. 338875.
53. Zijp, T.R., et al., Ultra-high throughput dual channel liquid chromatography with tandem mass spectrometry for quantification of four immunosuppressants in whole blood for therapeutic drug monitoring. J Chromatogr A, 2023. **1702**: p. 464086.
54. Rajski, L., et al., Dual-channel chromatography a smart way to improve the analysis efficiency in liquid chromatography coupled to mass spectrometry. J Chromatogr A, 2020. **1633**: p. 461614.
55. Wagmann, L., et al., Method development for quantitative determination of seven statins including four active metabolites by means of high-resolution tandem mass spectrometry applicable for adherence testing and therapeutic drug monitoring. Clin Chem Lab Med, 2020. **58**(5): p. 664-672.
56. Jacobs, C.M., et al., Evaluation and analytical applicability of a novel volumetric absorptive microsampling strategy for adherence monitoring of antihypertensive drugs by means of LC-HRMS/MS. Anal Chim Acta, 2021. **1187**: p. 339137.
57. Jacobs, C.M., et al., Towards clinical adherence monitoring of oral endocrine breast cancer therapies by LC-HRMS-method development, validation, comparison of four sample matrices, and proof of concept. Anal Bioanal Chem, 2024. **416**(12): p. 2969-2981.
58. Maurer, H.H., Advances in analytical toxicology: the current role of liquid chromatography-mass spectrometry in drug quantification in blood and oral fluid. Anal Bioanal Chem, 2005. **381**(1): p. 110-8.
59. Jacobs, C.M., L. Wagmann, and M.R. Meyer, Sample Matrices for Mass Spectrometry-Based Adherence Monitoring: A Systematic Critical Review. Ther Drug Monit, 2024. **46**(1): p. 6-15.
60. Desrosiers, N.A. and M.A. Huestis, Oral Fluid Drug Testing: Analytical Approaches, Issues and Interpretation of Results. J Anal Toxicol, 2019. **43**(6): p. 415-443.
61. Anizan, S. and M.A. Huestis, The Potential Role of Oral Fluid in Antidoping Testing. Clin Chem, 2013.
62. Drummer, O.H., Drug testing in oral fluid. Clin Biochem Rev, 2006. **27**(3): p. 147-59.
63. Allen, K.R., Screening for drugs of abuse: which matrix, oral fluid or urine? Ann Clin Biochem, 2011. **48**(Pt 6): p. 531-41.
64. Cone, E.J., et al., Normalization of urinary drug concentrations with specific gravity and creatinine. J Anal Toxicol, 2009. **33**(1): p. 1-7.
65. Wissenbach, D.K., et al., Drugs of abuse screening in urine as part of a metabolite-based LC-MSn screening concept. Anal Bioanal Chem, 2011. **400**(10): p. 3481-9.

66. Beyer, J., et al., Detection and validated quantification of toxic alkaloids in human blood plasma--comparison of LC-APCI-MS with LC-ESI-MS/MS. J Mass Spectrom, 2007. **42**(5): p. 621-33.
67. Hess, C., et al., Simultaneous determination and validated quantification of human insulin and its synthetic analogues in human blood serum by immunoaffinity purification and liquid chromatography-mass spectrometry. Anal Bioanal Chem, 2012. **404**(6-7): p. 1813-22.
68. Mazzola, P.G., et al., Liquid–liquid extraction of biomolecules: an overview and update of the main techniques. Journal of Chemical Technology & Biotechnology, 2008. **83**(2): p. 143-157.
69. Cortese, M., et al., Compensate for or Minimize Matrix Effects? Strategies for Overcoming Matrix Effects in Liquid Chromatography-Mass Spectrometry Technique: A Tutorial Review. Molecules, 2020. **25**(13).
70. Bambauer, T.P., et al., Development and application of a strategy for analyzing eight biomarkers in human urine to verify toxic mushroom or ricinus communis ingestions by means of hydrophilic interaction LC coupled to HRMS/MS. Talanta, 2020. **213**: p. 120847.
71. Peters, F.T., et al., Screening for and validated quantification of amphetamines and of amphetamine- and piperazine-derived designer drugs in human blood plasma by gas chromatography/mass spectrometry. J Mass Spectrom, 2003. **38**(6): p. 659-76.
72. Stewart, A. and R.A. Fisher, Chapter 2 - Co-Immunoprecipitation: Isolation of Protein Signaling Complexes from Native Tissues, in Methods in Cell Biology, P.M. Conn, Editor. 2012, Academic Press. p. 33-54.
73. Whiteaker, J.R., et al., Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. Anal Biochem, 2007. **362**(1): p. 44-54.
74. Safarik, I. and M. Safarikova, Magnetic techniques for the isolation and purification of proteins and peptides. Biomagn Res Technol, 2004. **2**(1): p. 7.
75. EUDA. European Drug Report 2024. 2024; 2024:[Available from: https://www.euda.europa.eu/publications/european-drug-report/2024_en#pdf.
76. Tamama, K., Advances in drugs of abuse testing. Clin Chim Acta, 2021. **514**: p. 40-47.
77. Allen, K.R., et al., Replacement of immunoassay by LC tandem mass spectrometry for the routine measurement of drugs of abuse in oral fluid. Ann Clin Biochem, 2005. **42**(Pt 4): p. 277-84.
78. Lehner, C.T., et al., Incidence trend of type 2 diabetes from 2012 to 2021 in Germany: an analysis of health claims data of 11 million statutorily insured people. Diabetologia, 2024. **67**(6): p. 1040-1050.
79. WADA. TUE Physician Guidelines – Diabetes Mellitus 2023; 2023:[Available from: https://www.wada-ama.org/sites/default/files/2023-12/tue_physician_guidelines_diabetes_mellitus_-_version_5.1_-_october_2023.pdf.
80. Bian, C., et al., Biochemical Toxicological Study of Insulin Overdose in Rats: A Forensic Perspective. Toxics, 2023. **12**(1).
81. Thomas, A. and M. Thevis, Recent advances in the determination of insulins from biological fluids. Adv Clin Chem, 2019. **93**: p. 115-167.
82. WADA. Prohibited List. 2025; 2025:[Available from: https://www.wada-ama.org/sites/default/files/2024-09/2025list_en_final_clean_12_september_2024.pdf.

83. Thomas, A. and M. Thevis, Analysis of insulin and insulin analogues from dried blood spots by means of LC-HRMS. Drug Test Anal, 2018.
84. Thomas, A., W. Schanzer, and M. Thevis, Determination of human insulin and its analogues in human blood using liquid chromatography coupled to ion mobility mass spectrometry (LC-IM-MS). Drug Test Anal, 2014. **6**(11-12): p. 1125-32.
85. Schwabe, U. and W.D. Ludwig, Arzneiverordnungs-Report 2020, ed. S. U. and L. W.D. 2020, Berlin, Heidelberg: Springer.
86. Fachi, M.M., et al., A systematic and critical review on bioanalytical method validation using the example of simultaneous quantitation of antidiabetic agents in blood. J Chromatogr B Analyt Technol Biomed Life Sci, 2017. **1055-1056**: p. 61-71.
87. Lam, Y.H., et al., Simultaneous detection of 24 oral antidiabetic drugs and their metabolites in urine by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci, 2020. **1141**: p. 122020.
88. Hess, C., F. Musshoff, and B. Madea, Simultaneous identification and validated quantification of 11 oral hypoglycaemic drugs in plasma by electrospray ionisation liquid chromatography-mass spectrometry. Anal Bioanal Chem, 2011. **400**(1): p. 33-41.
89. Esposito, S., et al., A liquid chromatography high-resolution mass spectrometry in vitro assay to assess metabolism at the injection site of subcutaneously administered therapeutic peptides. J Pharm Biomed Anal, 2018. **159**: p. 449-458.
90. Bambauer, T.P., et al., Analysis of alpha- and beta-amanitin in Human Plasma at Subnanogram per Milliliter Levels by Reversed Phase Ultra-High Performance Liquid Chromatography Coupled to Orbitrap Mass Spectrometry. Toxins (Basel), 2020. **12**(11).
91. Bever, C.S., et al. Rapid, Sensitive, and Accurate Point-of-Care Detection of Lethal Amatoxins in Urine. Toxins, 2020. **12**, DOI: 10.3390/toxins12020123.
92. Vetter, J., Amanitins: The Most Poisonous Molecules of the Fungal World. Molecules, 2023. **28**(15).
93. Barbosa, I., et al., Analytical methods for amatoxins: A comprehensive review. J Pharm Biomed Anal, 2023. **232**: p. 115421.
94. Bambauer, T.P., et al., Further development of a liquid chromatography-high-resolution mass spectrometry/mass spectrometry-based strategy for analyzing eight biomarkers in human urine indicating toxic mushroom or Ricinus communis ingestions. Drug Test Anal, 2021. **13**(9): p. 1603-1613.
95. Maurer, H.H., et al., Validated electrospray liquid chromatographic-mass spectrometric assay for the determination of the mushroom toxins alpha- and beta-amanitin in urine after immunoaffinity extraction. J Chromatogr B Biomed Sci Appl, 2000. **748**(1): p. 125-35.
96. Feldberg, L., et al., Rapid, Sensitive and Reliable Ricin Identification in Serum Samples Using LC-MS/MS. Toxins (Basel), 2021. **13**(2).
97. Hansbauer, E.M., et al., Rapid Detection of Abrin Toxin and Its Isoforms in Complex Matrices by Immuno-Extraction and Quantitative High Resolution Targeted Mass Spectrometry. Anal Chem, 2017. **89**(21): p. 11719-11727.
98. CDC, HHS and USDA Select Agents and Toxins. 2025.
99. Livet, S., et al., Development and Evaluation of an Immuno-MALDI-TOF Mass Spectrometry Approach for Quantification of the Abrin Toxin in Complex Food Matrices. Toxins (Basel), 2021. **13**(1).

100. Fredriksson, S.A., et al., Identification of RIP-II toxins by affinity enrichment, enzymatic digestion and LC-MS. Anal Chem, 2015. **87**(2): p. 967-74.
101. Drinkard, K.K., J.R. Barr, and S.R. Kalb, Mass Spectrometric Detection and Differentiation of Enzymatically Active Abrin and Ricin Combined with a Novel Affinity Enrichment Technique. Chem Res Toxicol, 2024. **37**(7): p. 1218-1228.
102. Hallbach, J., et al., Bedeutung der Analytik in der Klinischen Toxikologie: Auswirkungen auf die Diagnostik und Behandlung vergifteter Patienten / Analytical role in clinical toxicology: impact on the diagnosis and treatment of poisoned patients. LaboratoriumsMedizin, 2009. **33**(2): p. 71-87.
103. Hollander, P., et al., Combination therapy with insulin glargine plus metformin but not insulin glargine plus sulfonylurea provides similar glycemic control to triple oral combination therapy in patients with type 2 diabetes uncontrolled with dual oral agent therapy. J Diabetes Complications, 2015. **29**(8): p. 1266-71.
104. Cole, L.A. and J.G. Dorsey, Reduction of reequilibration time following gradient elution reversed-phase liquid chromatography. Anal Chem, 1990. **62**(1): p. 16-21.
105. Schellinger, A.P., D.R. Stoll, and P.W. Carr, High-speed gradient elution reversed-phase liquid chromatography of bases in buffered eluents: Part I. Retention repeatability and column re-equilibration. Journal of Chromatography A, 2008. **1192**(1): p. 41-53.
106. Yamagaki, T. and T. Yamazaki, Troubleshooting Carry-Over in the LC-MS Analysis of Biomolecules: The Case of Neuropeptide Y. Mass Spectrom (Tokyo), 2019. **8**(2): p. S0083.
107. Jogpethe, A., et al., Critical strategies to pinpoint carryover problems in liquid chromatography-mass spectrometry: A systematic direction for their origin identification and mitigation. Microchemical Journal, 2022. **179**: p. 107464.
108. Cai, J. and Z. Yan, Re-Examining the Impact of Minimal Scans in Liquid Chromatography-Mass Spectrometry Analysis. J Am Soc Mass Spectrom, 2021. **32**(8): p. 2110-2122.
109. Abbott, N.L., et al., Detection of alpha-, beta-, and gamma-amanitin in urine by LC-MS/MS using (15)N(10)-alpha-amanitin as the internal standard. Toxicon, 2018. **152**: p. 71-77.
110. Moshiri, M., F. Hamid, and L. Etemad, Ricin Toxicity: Clinical and Molecular Aspects. Rep Biochem Mol Biol, 2016. **4**(2): p. 60-5.
111. Bottger, R., R. Hoffmann, and D. Knappe, Differential stability of therapeutic peptides with different proteolytic cleavage sites in blood, plasma and serum. PLoS One, 2017. **12**(6): p. e0178943.
112. Jiang, D. and L. Yuan, Microflow LC-MS/MS to improve sensitivity for antisense oligonucleotides bioanalysis: critical role of sample cleanness. Bioanalysis, 2022. **14**(21): p. 1365-1376.
113. SAMHSA, Mandatory Guidelines for Federal

Workplace Drug Testing Programs—

Oral/Fluid. 2019.
114. EWDTS, European Guidelines for Workplace Drug Testing in Oral Fluid. 2022.
115. da Cunha, K.F., et al., Screening of 104 New Psychoactive Substances (NPS) and Other Drugs of Abuse in Oral Fluid by LC-MS-MS. J Anal Toxicol, 2020. **44**(7): p. 697-707.

7. ABBREVIATIONS

MS	Mass spectrometry
m/z	Mass-to-charge
EI	Electron impact ionization
(H)ESI	(Heated) electrospray ionization
GC-MS	Gas chromatography-mass spectrometry
ME	Matrix effect
Q	Quadrupole
TOF	Time-of-flight
HRMS/MS	High-resolution tandem mass spectrometry
FS	Full scan
LC	Liquid chromatography
RP	Reversed-phase
HILIC	Hydrophilic interaction chromatography
ID	Inner column diameter
OF	Oral fluid
RE	Recovery
PP	Protein precipitation
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
mAB	Monoclonal antibody
pAB	Polyclonal antibody
IA	Immunoassay
T1DM	Type I diabetes mellitus
T2DM	Type II diabetes mellitus
OAD	Oral antidiabetic drug
GLP-1	Glucagon like peptide-1
LD	Lethal dose
LOD	Limit of detection
Δ9-THC	Δ9-Tetrahydrocannabinol