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RESEARCH ARTICLE

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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

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Abstract

Recently, we presented a strategy for analysis of eight biomarkers in human urine to verify toxic mushroom or *Ricinus communis* ingestions. However, screening for the full panel is not always necessary. Thus, we aimed to develop a strategy to reduce analysis time and by focusing on two sets of analytes. One set (A) for biomarkers of late-onset syndromes, such as phalloides syndrome or the syndrome after castor bean intake. Another set (B) for biomarkers of early-onset syndromes, such as pantherine–muscaria syndrome and muscarine syndrome. Both analyses should be based on hydrophilic-interaction liquid chromatography coupled with high-resolution mass spectrometry (MS)/MS (HILIC-HRMS/MS). For A, urine samples were prepared by liquid–liquid extraction using dichloromethane and subsequent solid-phase extraction of the aqueous supernatant. For B urine was precipitated using acetonitrile. Method A was validated for ricinine and α- and β-amanitin and method B for muscarine, muscimol, and ibotenic acid according to the specifications for qualitative analytical methods. In addition, robustness of recovery and normalized matrix factors to matrix variability measured by urinary creatinine was tested. Moreover, applicability was tested using 10 urine samples from patients after suspected mushroom intoxication. The analytes α- and β-amanitin, muscarine, muscimol, and ibotenic acid could be successfully identified. Finally, psilocin-*O*-glucuronide could be identified in two samples and unambiguously distinguished from bufotenine-*O*-glucuronide via their MS^2 patterns. In summary, the current workflow offers several advantages towards the previous method, particularly being more labor-, time-, and cost-efficient, more robust, and more sensitive.

KEYWORDS
HILIC-HRMS/MS, matrix variability, mushroom intoxication, mushroom toxins, urinary biomarker

1 | INTRODUCTION

Exposure to toxic fungi or plants is an important issue in areas such as forensics, food safety, and intensive care.[1–5] An overview on relevant syndromes after toxic mushroom intake was provided by White et al.[6] Intoxications by fungi or plants often occur by accident, when wild-picked toxic mushrooms are consumed after a mix-up or after an ingestion by toddlers.[3,7,8] Furthermore, mushrooms like *Amanita phalloides* or castor beans, the seeds of *Ricinus communis*, can be used by intention to attempt suicide or murder due to their high toxicity.[1,9–11] Other mushrooms like *Psilocybe* species and *Amanita muscaria* or *Amanita pantherina* contain psychoactive ingredients, such as psilocybin and psilocin or muscimol and ibotenic acid, respectively.[12] Due to their potential of being used as a psychoactive drug, they are also relevant in driving under influence of drugs (DUID) events.[13] In particular, *Psilocybe* species and their active ingredients are currently gaining attention due to their promising therapeutic potential in mental disorders and may be used as pharmaceuticals in the future.[14,15] Today, they are still controlled by legislature in many countries but recent acts of decriminalizing could increase their availability and popularity.[16,17] This demonstrates the need for suitable analytical methods for specific demands in clinical and forensic toxicology, which can be applied to detect mushroom toxins or specific biomarkers in complex human or animal samples.

In a recently published study, we presented a validated analytical strategy based on hydrophilic-interaction liquid chromatography (LC) coupled with high-resolution mass spectrometry (MS)/MS (HILIC-HRMS/MS) analysis (termed as "Method R" throughout the following parts of the manuscript) to identify ricinine, biomarker of castor bean intoxication, and seven mushroom toxins in urine.[18] In detail, the method included α- and β-amanitin, related to phalloides syndrome, muscimol and ibotenic acid, related to pantherine–muscaria syndrome, muscarine, related to muscarine syndrome, psilocin, related to psilocybin mushroom intoxication, and bufotenine, related to bufotenine mushroom or Bufo toad intoxication.[5,19,20] Besides, bufotenine is discussed as a potential human metabolite of serotonin, and it should therefore be discriminated from its structural isomer psilocin.[21–23] In the context of intoxication after suspected consumption of wild fungi, the presence of amatoxins should either be confirmed or excluded by analysis in order to start a proper treatment as soon as possible. Even if ingestions of wild mushrooms lead to neuropsychiatric or cholinergic symptoms, a potential co-ingestion of other toxic wild-growing fungi, leading to dangerous late-onset symptoms, should be evaluated. Such a co-ingestion of *A. muscaria* and *A. phalloides* was described in a case report by Garcia et al.[24] However, in many cases of suspected mushroom intoxication, there is no necessity to cover all above-mentioned analytes in one analytical run. In case of *Ricinus* seed or amatoxin intoxication, first symptoms are observed after 4–10 h and 6–12 h of ingestion, respectively.[5,25,26] Therefore, the absence of early-onset (typical up to 2 [–3] h after ingestion) neuropsychiatric or neurologic/vegetative symptoms of the other mentioned toxidromes would make the analysis of their corresponding toxins/biomarkers not necessary.[5] Moreover, *Psilocybe* fungi brought on the drug market are usually cultivated and not collected in the forests. Thus, a mix-up or co-ingestion of different toxic mushrooms of other genera would be unlikely.[27,28] Therefore, in intoxication cases after (suspected) abuse of such mushrooms or in forensic DUID cases, analyses of the hallucinogens or corresponding biomarkers might be sufficient.

The aim of the current study was therefore to simplify and further develop the qualitative analysis of eight biomarkers (α-amanitin, β-amanitin, bufotenine-*O*-glucuronide, ibotenic acid, muscarine, muscimol, psilocin-*O*-glucuronide, and ricinine; structures shown in Figure 1) in human urine compared with the previous method.[18] Psilocin and bufotenine glucuronide should be included as psilocin-*O*-glucuronide showed a higher stability in biosamples amongst others and as it can be distinguished from bufotenin-*O*-glucuronide via their MS^2 patterns.[18,29,30]

A strategy should be developed and validated offering an easier of workflow and less turnaround time. Applicability should be tested by analyzing human urine samples obtained from suspected mushroom intoxication cases. Finally, robustness towards matrix variability (high-level creatinine) should be evaluated.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Ricinine was obtained from Latoxan (Portes-lès-Valence, France), ibotenic acid from Hello Bio (Bristol, UK), muscimol from Tocris (Wiesbaden, Germany), α-, β-, and γ-amanitin, and (+)-muscarine chloride from Sigma-Aldrich (Taufkirchen, Germany), and L-tryptophan-d5 from Alsachim (Illkirch-Graffenstaden, France), each as pure substances. Water was purified using a Milli-Q water purification system (Merck KGaA, Darmstadt, Germany) to reduce its resistivity to 18.2 MΩ •cm. Methanol (MeOH), ACN, dichloromethane (DCM), formic acid, and other chemicals were obtained from VWR (Darmstadt, Germany).

2.2 | Preparation of stock and working solutions

Stock solutions of α-amanitin, β-amanitin, γ-amanitin, (+)-muscarine, ricinine, and L-tryptophan-d5 were prepared at a concentration of 1000 mg/L in MeOH. Ibotenic acid and muscimol were dissolved in purified water at 1500 mg/L and 5000 mg/L, respectively. These stock solutions and all working solutions mentioned hereinafter were stored at −20°C.

2.2.1 | Method A

Working Solution A1 was aqueous and contained α- and β-amanitin, each 0.5 mg/L. Working Solution A2 consisted of 4.5 mg/L ricinine in

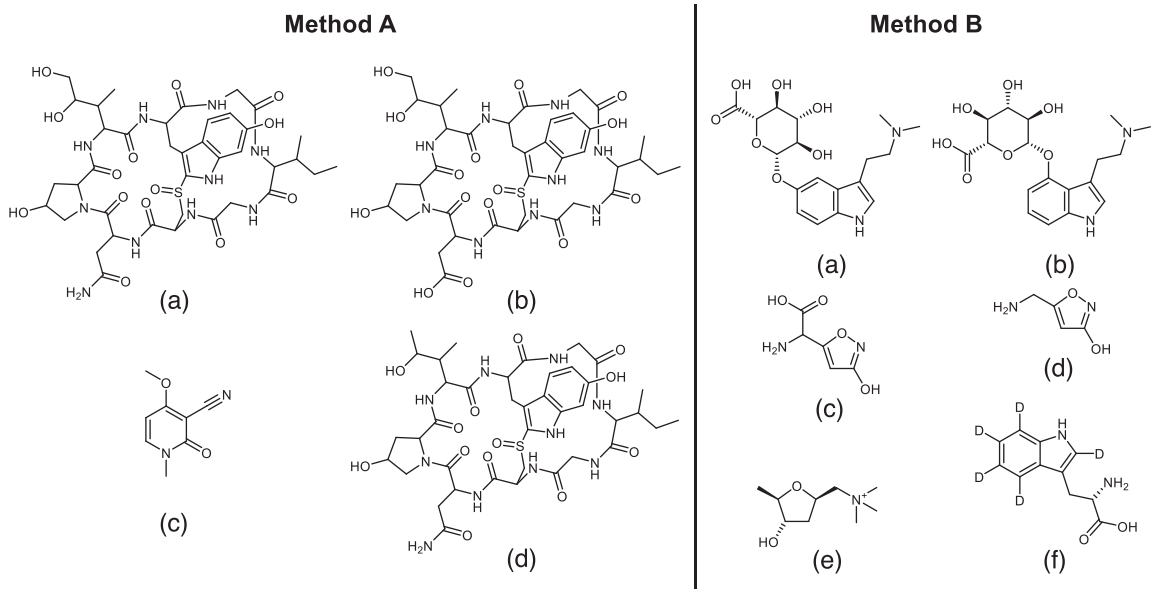

FIGURE 1 Method A structures of analytes and the internal standard (IS): (a) α-amanitin, (b) β-amanitin, (c) ricinine, (d) γ-amanitin (IS). Method B structures of analytes and the IS: (a) bufotenine-*O*-glucuronide, (b) psilocin-*O*-glucuronide, (c) ibotenic acid, (d) muscimol, (e) muscarine, (f) L-tryptophan-d5 (IS)

acetonitrile. The internal standard (IS) working Solution A contained 3.0 mg/L γ-amanitin in MeOH.

2.2.2 | Method B

Analyte working Solution B consisted of ibotenic acid (420 mg/L), muscimol (200 mg/L), and muscarine (1.2 mg/L) and was prepared with water. IS working Solution B was prepared by dilution of L-tryptophan-d5 stock solution to a concentration of 20 mg/L using MeOH.

2.3 | LC-HRMS/MS apparatus

The instrument was the same as for Method R.[18] A Dionex UltiMate 3000 RS ultra-high-performance LC (UHPLC) system (Thermo Fisher Scientific, Dreieich, Germany) equipped with a quaternary UltiMate 3000 RS pump and an UltiMate 3000 RS autosampler was used, all controlled by Aria MX 2.2 software (Thermo Fisher Scientific). A Nucleodur HILIC column (Macherey-Nagel, Düren, Germany), 100 × 2 mm, 1.8 μm, was coupled to a TF Q-Exactive Focus, equipped with a heated electrospray ionization II source (HESI-II). The adopted HESI-II conditions were as follows[31]: sheath gas, 60 arbitrary units (AU); auxiliary gas, 10 AU; spray voltage, 4.00 kV; heater temperature, 320°C; ion transfer capillary temperature, 320°C; and S-lens RF level, 60.0. External mass calibration was conducted as recommended by the manufacturer.

2.4 | LC settings

2.4.1 | Method A

Eluent A consisted of 40-mM ammonium formate, solved in a mixture of 98.5% (*v*/*v*) MeOH, 1% (*v*/*v*) purified water, and 0.5% (*v*/*v*) formic acid. Eluent B was 100% ACN and eluent C purified water. The multistep gradient consisted of six time windows (TWs) as follows: TW 1, step: from 0:00 to 0:20 min, A: kept at 4%, B: kept at 96%; TW 2, ramp: from 0:20 to 8:20 min, A: to 12%, B: to 88%; TW 3, ramp: from 8:20 to 9:20 min, A: to 28%, B: to 72%; TW 4, ramp: from 9:20 to 12:50 min, A: to 40%, B: to 60%, C: to 0%; TW 5, step: from 12:50 to 15:20 min, A: kept at 25%, B: kept at 25%, C: kept at 50%; TW 6, step: from 15:20 min to 16:50 min, A: kept at 4%; B: kept at 96%, C kept at 0%; the flow rate was set to 0.80 ml/min in TW 1, 0.75 ml/min in TW 2, 0.70 ml/min in TW 3, 0.60 ml/min in TW 4 and 5, and 0.50 ml/min in TW 6. The injection volume was 5 μl. The elution profile is shown in Figure S1A.

2.4.2 | Method B

Eluent A was MeOH acidified with 2% (*v*/*v*) formic acid, Eluent B was ACN, and eluent C purified water. The gradient consisted of four TWs: TW 1, step: from 0:00 to 0:20 min, A: kept at 1%, B: kept at 99%; TW 2, ramp: from 0:20 to 8:50 min, A: to 55%; B: to 45%, C: to 0%; TW 3, step: from 8:50 to 11:50 min, A: kept at 25%, B: kept at 25%, C: kept at 50%; TW 4, step: from 11:50 to 13:20 min, A: kept at 1%; B: kept at 99%, C kept at 0%; the flow rate was set to 0.70 ml/min in TW 1 and

2, 0.50 ml/min in TW 3, and 0.60 ml/min in TW 4. The injection volume was 5 µl. The elution profile is shown in Figure S1B.

2.5 | MS settings

2.5.1 | Method A

Parameters of full scan experiments: polarity, positive; resolution, 70,000; scan range 1, 110–390 *m/z*; scan range 2, 890–932 *m/z*; automatic gain control (AGC) target, 2×10^5; maximum injection time (IT), 120 ms; microscans, 1; spectrum data type, profile. In the following, data-dependent MS/MS (dd-MS^2) experiments were performed in discovery mode. An inclusion list containing the exact masses of the following positively charged analytes was used: α-amanitin (*m/z* 919.3614), β-amanitin (*m/z* 920.3455), γ-amanitin (*m/z* 903.3665), muscarine (*m/z* 174.1489), muscimol (*m/z* 115.0502), ibotenic acid (*m/z* 159.0400), psilocin/bufotenine (*m/z* 205.1335), ricinine (*m/z* 165.0659), L-tryptophan-d5 (*m/z* 210.1285), psilocin-d10 (*m/z* 215.1963), psilocin-*O*-glucuronide/bufotenine-*O*-glucuronide (*m/z* 381.1656). The customized tolerance of mass deviations of this inclusion list was set to 5 ppm. The settings for dd-MS^2 mode were as follows: resolution, 70,000; isolation window, 3.0 *m/z*; normalized collision energy (NCE) in high collision dissociation (HCD) cell, 28%; default charge state, 1; AGC target, 5×10^4; maximum IT, 120 ms; loop count, 1; minimum AGC target, 5×10^1; dynamic exclusion, 2.0 s; charge exclusion, ≥2; exclude isotopes, on; spectrum data type, profile.

2.5.2 | Method B

Parameters of full scan experiments: polarity, positive; resolution, 35,000; scan range, 105–390 *m/z*; AGC target, 1×10^6; maximum IT, 120 ms; microscans, 1; spectrum data type, profile. In the following, dd-MS^2 experiments were performed in discovery mode. An inclusion list containing the exact masses of the following positively charged analytes was used: muscarine (*m/z* 174.1489), muscimol (*m/z* 115.0502), ibotenic acid (*m/z* 159.0400) psilocin/bufotenine (*m/z* 205.1335), L-tryptophan-d5 (*m/z* 210.1285), psilocin-d10 (*m/z* 215.1963), psilocin-*O*-glucuronide/bufotenine-*O*-glucuronide (*m/z* 381.1656). The customized tolerance of mass deviations of this inclusion list was set to 10 ppm. The settings for the dd-MS^2 mode were as follows: resolution, 35,000; isolation window, 1.0 *m/z*; NCE, 28%; default charge state, 1; AGC target, 1×10^5; maximum IT, 160 ms; loop count, 1; minimum AGC target, 5×10^1; dynamic exclusion, 2.0 s; charge exclusion, ≥2; exclude isotopes, on; spectrum data type, profile.

2.6 | Data handling

Data were processed by using Xcalibur Qual Browser 2.2 software (Thermo Fisher Scientific) was used. The mass tolerance for integration of peaks was set to 10 ppm.

2.7 | Sample preparation

2.7.1 | Method A

A volume of 1470 µl urine was fortified with 15 µl of working Solution A1, 10 µl of working Solution A2, and 5 µl of IS Solution A. Patient samples and blank urine were fortified with 25 µl of a mixture of ACN and MeOH (40/60, *v/v*) for volume adjustment to replace working solutions. Afterwards, the mixture was centrifuged at −10°C for 10 min at 21,130 × *g*. Then, 1300 µl of the supernatant (Supernatant A) was added to 600 µl of DCM and a hydrophilic-phase liquid–liquid extraction (HP-LLE) was performed during 2 min of shaking and afterwards centrifuged for 10 min at 18,407 × *g*. A volume of 1100 µl of the aqueous upper phase (Supernatant B) was diluted with 4400 µl of 0.1% formic acid before solid-phase extraction (SPE) was performed. Strata X-Drug B 33 µm Polymeric Strong Cation cartridges (Phenomenex, Aschaffenburg, Germany), containing 60 mg of sorbent mass and 3-ml capacity were preconditioned and equilibrated with 2 × 1 ml of MeOH and 1 ml of 0.1% formic acid, respectively. Then, the mixture was loaded, followed by two washing steps, using 1 ml of 1% formic acid and 2 × 1 ml of 0.1% formic acid, respectively. In the next step, analytes were eluted by loading 0.6 ml of MeOH in duplicate. This eluate was gently evaporated at 70°C under a stream of nitrogen and reconstituted by 100 µl of Eluent A of Method A (see Section 2.4.1).

2.7.2 | Method B

A volume of 190-µl urine was fortified with 5 µl of working Solution B and 5 µl of IS Solution B. Patient samples and blank urine were fortified with 5 µl of purified water for volume adjustment to replace working Solution B. Afterwards, the mixture was centrifuged at −10°C for 10 min at 21,130 × *g*. Then, 100 µl of this supernatant were added to 500 µl of ACN, followed by 2 min of shaking and 2 min of centrifugation using 18,407 × *g*. A 500 µl of the resulting ACN-containing supernatant was evaporated at 60°C under a stream of nitrogen and reconstituted by 100 µl of Eluent A of Method B (see Section 2.4.2).

2.8 | Method validation

Methods A and B were validated according to the recommendations for validation of qualitative methods, covering selectivity, carry-over, matrix effects, and limits of identification (LOIs).[31–34] For better comparison, all analyzed urine samples in the current validation experiments were the same samples as those for validation of Method R.[18] Selectivity was tested by analyzing 10 blank urine samples from different human donors. Analyte carry-over was evaluated by injecting zero samples after a quality control (QC) sample, spiked with toxins in high concentrations (ibotenic acid and muscimol, 100 mg/L, each, muscarine, psilocin, bufotenine, and ricinine, 5 mg/L, each; α- and β-amanitin, 1 mg/L, each).

Recovery (RE) and matrix effects (MEs) experiments were conducted by using blank urine samples from six different human donors, processed in triplicate. RE was determined by comparing peak areas of the analytes (MH^+) spiked into urine samples before extraction with areas of the extracts of blank samples spiked with the analytes afterwards. MEs were calculated by comparing peak areas of spiked extracts with those in neat standards. Coefficients of variation (CV) of RE and ME were calculated on the means of the replicates ($n = 3$) of each individual sample. IS-normalized matrix factors (MF_{IS}) were calculated as specified in European Medicines Agency (EMA) guidelines.[33] The MF of the IS γ-amanitin was used to normalize MFs of α- and β-amanitin and ricinine (Method A), and the MF of the IS L-tryptophan-d5 was used for muscarine, muscimol, and ibotenic acid (Method B). Analyte concentrations spiked into the samples, and extracts are listed in Table 1. All calculations were performed in Excel 2016 (Microsoft, Redmond, USA).

LOIs were determined by dilution of QC samples prepared in triplicate using pooled blank urine of 10 donors. Those samples were analyzed in two runs on two consecutive days. The LOI was defined as the lowest concentration at which the identification of the analytes was still possible in each analysis according to the identification criteria.[31,35] Both, the accurate mass of the precursor ion must be detected and the corresponding high-resolution MS^2 spectrum or selected fragment ions must match the reference data and retention window. Reference spectra can be found in the supporting information of Method R.[18] The criteria for each analyte are given in Table 1.

2.9 | Estimation of matrix variability robustness

As for validation experiments, the same urine samples were processed using the methods A, B, and R. The same set of urine samples used for recovery and matrix effect experiments was extended by three

TABLE 1 Analytes and internal standards (IS) of Methods A and B and their expected retention window (RW), mass spectrometry (MS) identification criteria, and concentrations in QC samples used for evaluation of recovery (RE) and matrix effects (ME)

Method	Compound	RW (min)	MS-identification criterium	QC samples (ng/ml)
Method A	α-Amanitin m/z (MH^+) = 919.3614	11.9–12.4	dd MS^2 fragment ions: m/z = 259.1275 m/z = 86.0600	10
	β-Amanitin m/z (MH^+) = 920.3455	12.9–14.0	dd MS^2 fragment ions: m/z = 259.1275 m/z = 86.0600	10
	γ-Amanitin (IS) m/z (MH^+) = 903.3665	10.2–10.9	dd MS^2 fragment ions: m/z = 243.1339 m/z = 86.0600	10
	Ricinine m/z (MH^+) = 165.0659	0.45–0.75	Full dd MS^2 spectrum	100
Method B	Muscarine m/z (M^+) = 174.1489	0.60–2.2	Full dd MS^2 spectrum	100
	Muscimol m/z (MH^+) = 115.0502	4.8–6.5	dd MS^2 fragment ions: m/z = 115.0502 m/z = 98.0236	5,000
	Ibotenic acid m/z (MH^+) = 159.0400	7.0–8.7	dd MS^2 fragment ions: m/z = 114.0185 m/z = 113.0345	15,000
	L-tryptophan-d5 (IS) m/z (MH^+) = 210.1285	5.4–7.4	m/z = 192.0957	500
	Psilocin-*O*-glucuronide[a] m/z (MH^+) = 381.1656	6.0–6.7	Full dd MS^2 spectrum Incl. m/z = 336.1078 Rel. abundance = 0.5%	
	Bufotenine-*O*-glucuronide[a] m/z (MH^+) = 381.1656	5.2–6.2[b]	Full dd MS^2 spectrum Incl. m/z = 336.1078 Rel. abundance = 15%	

Abbreviations: QC, quality control; dd, data dependent.

[a]The *O*-glucuronides of psilocin and bufotenine were not included in validation experiments.

[b]RT determined for incubate of pooled human liver microsomes.[18]

selected samples with high urinary creatinine >300 mg/dl. In order to evaluate RE, ME, and MF_{IS} of the extended sample set ($n = 9$), those additional samples were processed like the other six as outlined above for Methods A and B or as described previously.[18] Then, CVs of RE and MF_{IS} were calculated as described for the extended set and compared against the original set ($n = 6$). The changes of CVs after extending the sample set were finally used to compare Methods A and B against Method R concerning robustness towards matrix variability. Urinary creatinine was quantified using the P.I.A.2 device (Protzek, Lörrach, Germany).

2.10 | Applicability

Applicability was tested by analyzing urine samples from subjects after suspected consumption of toxic mushrooms. These samples had been sent to the authors' laboratory for regular toxicological analysis. Urinary creatinine of these samples was determined as mentioned in Section 2.8.

3 | RESULTS AND DISCUSSION

In clinical toxicology, time-saving comprehensive methods are favorable, because they allow fast decision making and a rapid start of a specific therapy. So far, several analytical methods have been published that allow detection of biomarkers of one mushroom intoxication syndrome or *Ricinus* seed intoxication in human urine or plasma.[29,36–44] Tomková et al. developed a method for a simultaneous determination of α- and β-amanitin and muscarine in urine by LC-high-resolution (HR)-time-of-flight (TOF) MS.[45] Recently, we presented a comprehensive and validated analytical solution to identify a total of eight biomarkers of castor bean intoxication, phalloides syndrome, pantherine–muscaria syndrome, muscarine syndrome, psilocybin syndrome, and bufotenine intoxication in urine within one single run.[18] The samples were prepared by SPE and urine precipitation performed in parallel and the use of a four-eluent system for normal-phase chromatography were necessary to realize the aims of selectivity and earmarked sensitivity for all analytes. However, as there is not always the need to screen for all biomarkers using such an extensive method, the method should be simplified by saving cost and turnaround time while enhancing flexibility in toxicological analysis by development of two separate methods for biomarker identification of late- and early-onset syndromes, respectively.

Psilocin is known to be prone to oxidative decomposition due to effects of light. Furthermore, discrimination against the isobaric bufotenine via MS^2 spectrum alone is not possible. Thus, psilocin-*O*-glucuronide was preferred in this study over psilocin as screening target as it shows higher amounts of excretion (>80% of total psilocybin), a higher stability, and can be distinguished from bufotenine-*O*-glucuronide via their MS^2 spectra (Table 1).[18,29,30,46,47] Kamata et al. demonstrated that unbound psilocin was excreted more rapidly from the body than the glucuronide, indicating an extended detection window for the latter—in their study up to 52 h for plasma analysis.[47] However, psilocin-*O*-glucuronide and bufotenine-*O*-glucuronide were not available as pure standards, and thus, full method validation could not be performed.

3.1 | Extraction

The sample preparation protocol of Method R was further optimized and finally separated into SPE (Method A) and precipitation (Method B).[18] The addition of ascorbic acid before and after several extraction steps to prevent decomposition of psilocin was not necessary anymore. The intermediate step of HP-LLE after 10 min of cooled centrifugation of urine was kept only for Method A to reduce matrix effects. For Method B, the benefit of LLE before precipitation was negligible. Furthermore, the SPE protocol of Method A was shortened, compared with Method R, as the second elution step with a mixture of aqueous ammonia/isopropanol/DCM (2/18/80, *v*/*v*/*v*) to recover psilocin and bufotenine was removed. The thermal stability of the remaining analytes allowed a faster nitrogen evaporation of the methanolic eluate of Method A and the ACN-containing supernatant of Method B at 70°C and 60°C, respectively, instead of 45°C in Method R.

3.2 | Analysis

The analytes of Method A and B were separated by using two different optimized gradients on the same HILIC column (Figures 2 and S1A,B). The expected retention windows are listed in Table 1. The LC gradient could be reduced to a duration of 16:50 min for Method A and to 13:20 min for Method B, compared with 20:50 min for Method R.[18] To enhance sensitivity of muscimol and ibotenic acid in Method B, the ionization efficiency of their MH^+ ions was improved by avoiding the use of eluent buffers. While four eluents had to be used in Method R, a third aqueous eluent was still needed in gradients of Methods A and B, to keep the column clean from impurities and for reconstitution of the stationary phase (Figure S1A,B). However, it might be even possible to work only with the two organic Eluents A and B by adding an injection for maintenance with an aqueous eluent after a couple of injections, but this was not thoroughly tested. For higher MS sensitivity, especially for the amatoxins, the MS^2 isolation window was set to 3.0 *m/z*. The NCE of 28% used in Method R was kept for Methods A and B; thus, MS^2 spectra are the same as published previously.[18]

3.3 | Method validation

Interfering signals that may lead to false identification according to criteria given in Table 1 were not observed. Analyte recoveries of Method A (>86%) were higher than of Method B (46%–62%, see Table 2). Ricinine showed a much higher recovery (106%) than in

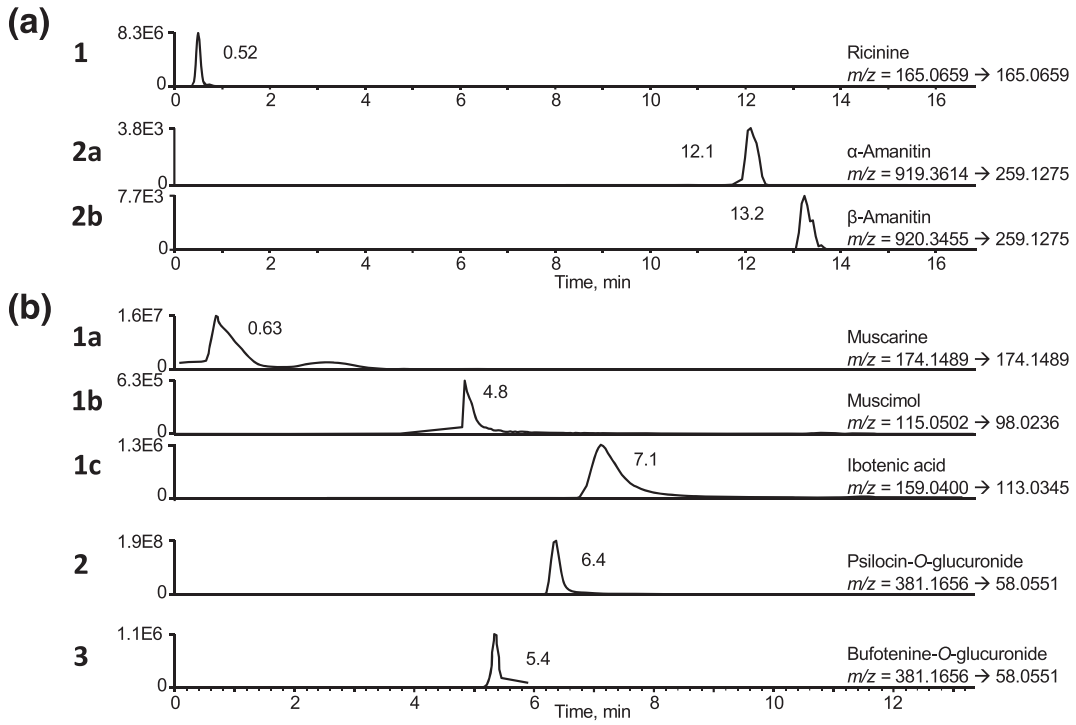

FIGURE 2 Extracted fragment ion chromatograms after data dependent MS^2 of analytes (MH^+) in human urine samples, if available ((a)2a–b; (b)1a–c, (b)2), matrix-matched quality control (QC) sample (A1) and incubate of human liver microsomes (HLM) ((b)3). Analytes were labeled with retention times. Mass deviation was set to 10 ppm, NCE = 28; A1, QC blank urine sample, spiked with ricinine; (a)2a–b, *Amanita phalloides* intake; (b)1a–c, *Amanita muscaria* intake; (b)2, *Psilocybe* sp. intake; (b)3, HLM incubate of bufotenine

TABLE 2 Methods A and B: limits of identification (LOIs), analyte recoveries (REs) and internal standard-normalized matrix factors (MF_{IS}) (6 individuals; $n = 3$)

Compound	LOI (ng/ml)	RE, % (CV, %)	MF_{IS} (CV, %)
α-Amanitin	1	86 (3.9)	1.0[a] (6.9)
β-Amanitin	1	87 (6.5)	1.2[a] (11)
γ-Amanitin (IS)		97 (5.8)	0.56[b] (17)
Ricinine	5	106 (12)	0.19[a] (25)
Muscarine	5	55 (11)	4.0[c] (31)
Muscimol	250	46 (19)	0.51[c] (13)
Ibotenic acid	750	62 (13)	3.4[c] (7.2)
L-tryptophan-d5 (IS)		38 (8.7)	0.13[b] (37)

Abbreviations: IS, internal standard; CV, coefficient of variation.
[a]Normalized by γ-amanitin peak area (method A).
[b]MFs of IS are not normalized.
[c]Normalized by L-tryptophan-d5 peak area (method B).

Method R (33%), whereas recoveries of muscarine and muscimol appeared to be lower than in the referenced method (110% and 68%, respectively).[18] The latter could be the result of muscarine and muscimol recovery in the SPE fraction of Method R, not existing in Method B.

According to the EMA guidelines, acceptance criteria of CV (MF_{IS}) are <15%.[33] All analytes (see Table 2) but not ricinine (25%, Method A) and muscarine (31%, Method B) fell below this threshold value, showing general appropriateness for IS γ-amanitin and L-tryptophan-d5, to indicate ME (Table 2). As this EMA acceptance limit is given for quantitative bioanalytical methods, higher variations of ME might be accepted, as long the sensitivity aimed for the purpose is reached.

LOIs based on previously defined identification criteria are given in Table 2. They are comparable with other reported LC–MS/MS methods and sufficient for application in clinical toxicology.[38,39,41,45,48,49] Compared with Method R, LOIs were the same except of muscimol and ibotenic acid, which could be reduced from 2000 to 250 ng/ml and from 1500 to 750 ng/ml, respectively.

Usually, stability experiments are mandatory tests in validation of bioanalytical methods.[33] For all analytes and IS, bench-top and autosampler stability tests were already executed in a previous study.[18] As the same laboratory, autosampler, and stock solutions were used, these tests were not considered necessary anymore. In the former study, the only analyte that showed low bench-top stability was psilocin, which neither was included in Method A nor in Method B. Although slightly different solvents for reconstitution were used in Methods A and B, we do not recommend exceeding 5 days of storage in the cooled (10°C) autosampler.[18]

3.4 | Matrix variability robustness and comparison with Method R

There is a high interindividual and intraindividual variability of concentrations of excreted substances in spontaneous urine samples.[50] In

order to overcome this variance due to changes of urinary filtration rates, urinary creatinine concentration is commonly used as reference for interpretation of urinary biomarker concentrations in different fields, such as occupational health, medicinal research, and forensics.[50-53] Despite the amount of urinary creatinine that does not always reflect urinary excretion, especially when glomerular filtration rate is reduced, normalization by creatinine is still often used as an approximation.[54] The width of published reference ranges for urinary creatinine, 28–217 mg/dl for women and 39–259 mg/dl for men, indicates the extent of the variability.[55] In LC–MS-based analytical methods, the quantity of compounds that can interfere with the analyte's signal and, thus, the quantity of potential matrix effects (e.g., ion suppression) would be affected as well. In addition, a high variability could lead to deviations in analyte recovery due to influences in stages of sample preparation. Therefore, CVs of both RE and MF_{IS} of the validation sample set (6 urines, $n = 3$) and an extended sample set (9 urines, $n = 3$) with a wider range of urinary creatinine were compared. Creatinine values of the validation samples were in the range of 41–154 mg/dl (determined after the validation was completed), and those of the additional three were 301, 304, and >500 mg/dl. For Methods A, B, and R, the REs, MF_{IS}, and corresponding CVs of the extended set are summarized in Table S1. The absolute and relative effects of the higher variability of urinary creatinine by extension of sample set on CVs are specified in Table 3.

CVs of RE for β-amanitin and ricinine showed a moderate absolute enhancement in the range of 10%–20% for Methods A and R, apart from other analytes, which showed only minor deviations (Table 3). The higher variations of REs for β-amanitin could be a reason of increasing pH differences of the diluted urine extract prior to loading on the SPE columns, because a sufficiently low pH is required for a high recovery of this analyte. In summary, matrix variability robustness of Method A or B and Method R based on CVs of RE was comparable.

In Methods A and B, with exception of ibotenic acid, absolute changes of CV of MF_{IS} were below 11% under circumstances of a higher variability of urinary creatinine regarding the extended sample set (Table 3). In contrast, concerning Method R, absolute changes of CV of MF_{IS} surpassed 24% for four of the six shared analytes, α-amanitin, β-amanitin, muscarine, and ibotenic acid. As a result, based on CVs of RE, matrix variability robustness appears to be higher for Method A and B compared with those for Method R. This could be explained in general by a larger amount of matrix compounds in the extract of Method R that was injected into the LC system and potentially lead to interferences, disturbing the ionization process of analytes in the following. Because ibotenic acid showed high absolute and relative changes of CVs of MF_{IS} in both Methods B and R, L-tryptophan-d5 is probably not the best choice for normalization, when this analyte is exposed to high matrix effects.

3.5 | Applicability

Methods A and B were used to analyze 10 urine samples of patients showing intoxication symptoms after suspected consumption of toxic fungi (Table 4). Patients 1–4 suffered from phalloides syndrome, and Patients 6–8 suffered from pantherine–muscaria syndrome after accidentally mixing up wild mushrooms. Patient 5 ingested *A. muscaria* and Patients 9 and 10 ingested *Psilocybe* sp. for recreational purposes. Analysis results of these urine samples by use of Method R were already shown with exception of samples of Patients 7 and 8.[18] By using Methods A and B, the same analytes could again be identified in all cases, except of psilocin and bufotenine. In addition, ibotenic acid could be identified by Method B in Sample 6, most likely due to higher

TABLE 3 Robustness to matrix variability of Methods A and B compared with a previously published reference method (Method R),[18] represented by relative changes of CV values of internal standard-normalized matrix factors (MF_{IS}) and those of analyte recovery, when spiked QC urine samples from three individuals with elevated creatinine levels (>300 mg/dl) were additionally included to the original setup (six individuals; $n = 3$) of matrix effect evaluation. The same urine samples were processed in each method's setup

	Absolute (and relative) changes of analyte recovery CVs (%)		Absolute (and relative) changes of MF_{IS} CVs (%)	
Compound	Methods A and B	Method R	Methods A and B	Method R
α-Amanitin	+4.8 (+120)	−0.9 (−5.9)	+7.0 (+100)	+30 (+330)
β-Amanitin	+16 (+240)	+19 (+230)	−0.2 (−1.4)	+24 (+240)
γ-Amanitin (IS)[a]	+0.1 (+2.2)	−1.5 (−14)	+5.4 (+33)	+7.0 (+84)
Ricinine	+20 (+170)	+11 (+55)	+11 (+44)	−5.4 (−17)
Muscarine	+2.7 (+26)	+2.6 (+30)	−0.8 (−2.7)	+29 (+130)
Muscimol	+0.9 (+5.0)	−2.1 (−7.4)	−1.6 (−13)	+15 (+46)
Ibotenic acid	−3.9 (−31)	−3.9 (−10)	+22 (+300)	+50 (+750)
L-tryptophan-d5 (IS)[a]	−0.4 (−5.0)	−1.4 (−11)	+3.6 (+9.7)	+32 (+550)

Note: MFs normalized by IS γ-amanitin: α-amanitin, β-amanitin, ricinine. MFs normalized by IS L-tryptophan-d5: muscarine, muscimol, ibotenic acid. MFIS and analyte recoveries based on six different urine samples of Methods A and B are given in Table 2, and those based on different urine samples of all methods are given in Table S1

Abbreviations: CV, coefficient of variation; IS, internal standard; n.d., not determined; QC, quality control.

[a]Not normalized MFs: γ-amanitin, L-tryptophan-d5.

TABLE 4 Results of analyzing authentic urine samples of intoxicated patients

	Patient									
	1	2	3	4	5	6	7	8	9	10
Suspected intake	?	*Amanita phalloides*	*Amanita phalloides*	*Amanita phalloides*	*Amanita muscaria*	*Amanita pantherina*	*Amanita pantherina*	*Amanita pantherina*	*Psilocybe* sp.	*Psilocybe* sp.
Sampling time after ingestion (ca.)	3 days	2–3 days	2–3 days	?	5–8 hours	19 hours	5 hours	3 hours	?	?
Identified compound										
Creatinine (mg/dl)	<20	223	70	175	91	<20	46	49	354	22
α-Amanitin	X	X	X	X						
β-Amanitin	X			X						
Ricinine										
Muscarine					X					
Muscimol					X		X	X		
Ibotenic acid					X	X	X	X		
Psilocin-*O*-glucuronide									X	X
Bufotenine-*O*-glucuronide										

Note: "X" marks the identification of the analyte in the urine sample.

sensitivity compared with Method R. In the course of this study, urine samples of Patients 7 and 8 have also been analyzed by using Method R, and again, all analytes shown in Table 4 could be found. In Cases 9 and 10, MS^2 spectra of the precursor ion at *m/z* 381.1656 (retention time = 6.4 min) could be identified as psilocin-*O*-glucuronide (Table 1 and Figure 2). Bufotenine-*O*-glucuronide is expected to elute 0.1–0.2 min later (see Figure 2) and can be distinguished from psilocin-*O*-glucuronide via its MS^2 pattern (15% abundance of MS^2 fragment ion at 336.1078 in contrast to 0.5% abundance in the MS^2 spectrum of psilocin-*O*-glucuronide). Bufotenine-*O*-glucuronide was generated by pooled human liver microsomes as described previously.[18] However, in cases where intake of α- and β-amanitin need to additionally be excluded, the more comprehensive Method R should be used.

4 | CONCLUSIONS

Two time- and cost-saving analytical procedures were developed for analyzing biomarkers of late-onset and early-onset syndromes in urine after suspected mushroom or castor bean intoxication. They might particularly be used in the context of clinical and forensic toxicology, for example, to support therapeutic decision making. Compared with the previous method, the total turnaround time and the number of used eluents could be reduced and the analysis of psilocin- and bufotenine-*O*-glucuronides instead of the unstable parent molecules was introduced. Furthermore, the optimized workflow offers much easier handling and a higher sensitivity for ibotenic acid and muscimol as well as being more robust by compensating for high variances of urinary excretion. The strategy was successfully applied to identify α- and β-amanitin, muscarine, muscimol, ibotenic acid, and psilocin-*O*-glucuronide in human urine samples after suspected ingestion of the respective mushrooms.

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CONFLICT OF INTEREST

The authors have no competing interests to declare.

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