

Article

Simultaneous Determination of Multi-Class Mushroom Toxins in Mushroom and Biological Liquid Samples Using LC-MS/MS

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Abstract: A comprehensive analytical method based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) was developed for the simultaneous detection of 12 mushroom toxins (ibotenic acid, muscimol, muscarine, β -amanitin, α -amanitin, desoxoviroidin, γ -amanitin, phallisin, illudin S, phalloidin, phalloidin and illudin M) in mushrooms, serum, urine and simulated gastric fluid. The samples were extracted with water or acetonitrile solution, and the serum sample was further purified with PSA sorbent. Chromatographic separation was performed on an ACQUITY UPLC HSS T3 column with gradient elution using methanol and water containing 1 mM ammonia fluoride as a mobile phase. Mass spectrometric acquisition was performed in electrospray positive ionization mode. Good linearities ($R^2 > 0.994$) were obtained for 12 toxins over the range of 0.05–200 $\mu\text{g/L}$. Matrix-matched calibration curves were used for quantification. The method limits of quantification were 0.01–0.2 mg/kg for mushrooms and 0.15–2.0 $\mu\text{g/L}$ for three biological liquid samples. The mean recoveries of 12 target toxins (spiked at three concentration levels) ranged from 73.0% to 110.3%, with relative standard deviations not exceeding 19.4%, which meets the requirements for the determination of trace compounds in a biological matrix. This method was applied to the analysis of mushroom samples from Yunnan Province. As a result, 11 toxins, not including illudin M, were detected with a concentration range of 0.61–2143 mg/kg.



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Keywords: mushroom toxins; amanitin; ibotenic acid; muscimol; muscarine; illudin S; serum; urine; LC-MS/MS

1. Introduction

Wild mushrooms are appreciated by many people around the world for their delicious taste and unique flavor. Picking wild mushrooms in some rural areas not only satisfies the taste buds of diners, but also brings economic benefits to local people [1–3]. However, some wild mushrooms are poisonous due to the presence of natural toxins [4]. According to The Situation of Food Poisoning Incidents Nationwide published by the China National Health Commission, more than half of all poisoning deaths are attributed to the ingestion of poisonous mushrooms or poisonous animals and plants [5–9]. For example, in Yunnan, a province on the southwestern border of China, where more than half of the world's edible mushroom production originates, a significant proportion of which is for domestic consumption, there are consistently several or dozens of deaths from mushroom poisoning each year [10,11].

The identification and detection of mushroom toxins is essential for the prevention and control of food poisoning, especially in spring and summer when mushroom poisoning is common. Tests can accurately identify mushrooms containing toxins, thus preventing poisoning incidents caused by accidental ingestion. Toxin analysis in human biosamples can aid clinical decision-making in patient management and pharmacotherapy. Therefore,

analytical methods have been developed to determine different types of mushroom toxins in mushrooms, serum or urine samples. Laboratory tests based on immunoassays [12,13] or HPLC [14] as well as LC-MS/MS [15,16] techniques are commonly used. LC-MS/MS, which offers high sensitivity and selectivity in the identification of toxin molecules based on their specific mass-to-charge ratio and retention times, has become the most widely used technology for the detection of mushroom toxins.

In terms of LC-MS/MS techniques, most publications focused on the analysis of amanitin, as it was recognized as one of the most toxic compounds in the mushroom realm, including α -amanitin, β -amanitin, γ -amanitin, phalloidin and phalloidin [17,18]. Muscarine, muscimol and ibotenic acid are other frequently implicated mushroom toxins [19]. In recent decades, Japanese researchers have identified illudin S and illudin M mainly from the genus *Omphalotus* (such as *Omphalotus olearius*, *Omphalotus nidiformis* and *Omphalotus guepiniformis*), which can cause gastroenteritis poisoning [20–22]. At present, most LC-MS/MS analytical methods for the detection of mushroom toxins only follow one class of compounds or no more than six target compounds. However, in real circumstances, there are many types of wild poisonous mushrooms, and edible mushrooms tend to be similar in morphology and difficult to distinguish, resulting in the coexistence of multiple classes of toxins [23]. Therefore, the simultaneous analysis of multiple toxins by rapid LC-MS/MS qualitative and quantitative methods is of great importance. The aim of this study was to develop an efficient sample preparation procedure and an accurate analytical technique for the detection of a total of 12 toxins in mushroom, urine, serum and gastric fluid samples.

2. Materials and Methods

2.1. Reagents and Chemicals

The mushroom toxins α -amanitin, β -amanitin, γ -amanitin, phalloidin, phalloidin, muscarine, muscimol and ibotenic acid (purity $\geq 96.0\%$) were purchased from Sigma-Aldrich (Alexander, MI, USA). Illudin S and illudin M (purity 98%) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Phallisacin and desoxoviroidin (purity 98%) were purchased from First Standard (Tianjin, China).

Fisher Scientific (Fair Lawn, NJ, USA) provided LC-MS-grade methanol and acetonitrile. J&K Chemicals (Shanghai, China) provided HPLC-grade formic acid, ammonium formate, ammonium acetate, ammonium carbonate and ammonium fluoride. Simulated gastric fluid (SGF) was purchased from HongFeng Corp. (Dongwan, China). Solid-phase extraction (SPE) sorbents HLB, MCX and PSA used for the purification of serum extracts were purchased from Agela Technologies (Tianjin, China). Ultrapure water (18.2 M Ω /cm) was obtained from a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Preparation of Standard Solutions

The standard stock solution with a concentration of 100 mg/L was prepared by accurately weighing 1.0 mg toxins and dissolving them individually in 10.0 mL methanol. To prepare calibration standards of different concentrations, aliquots of different stock solutions were combined and then diluted with water.

2.3. LC-MS/MS Analysis

LC-MS/MS analysis was performed using a Waters ACQUITY UPLC system coupled to a Waters Xevo TQ-XS mass spectrometer (Waters Corp., Milford, MA, USA). An ACQUITY (Waters Corp., Milford, MA, USA) UPLC[®] HSS T3 column (100 mm \times 2.1 mm, 1.7 μ m) was used for the LC separation. The mobile phase consisted of water containing 1 mM ammonium fluoride and methanol, designated A and B, respectively. The flow rate was kept at 0.3 mL/min. The initial composition of the mobile phase started at 0% B and was held for 0.5 min. This was gradually increased to 65% B over 3 min, held for 2 min and washed with 95% B for 1.5 min. The column was equilibrated with 0% B for 2 min until the next injection. A column oven temperature of 40 °C was set, and a 2 μ L injection volume

was used. Mass spectrometry was performed using the parameters listed in Table 1. The specific parameters for each compound are given in Section 3.1.

Table 1. Mass tuning parameters of 12 mushroom toxins.

Parameter	Value
Polarity	ES+
Scan type	MRM
Capillary voltage (kV)	2.0
Offset multiplier voltage (V)	30
Source temperature (°C)	150
Desolvation temperature (°C)	500
Desolvation gas flow (L/h)	900
Cone gas flow (L/h)	150
Collision gas	Ar
Collision gas flow (mL/min)	0.14

2.4. Sample Pretreatment

The edible mushroom *Lentinus edodes*, collected from a local supermarket, was used for the development and validation of the analytical method. Homogenized samples were stained using an Alpha 1-2 LD plus freeze-dyeing machine (Christ/Sigma, Osterode am Harz, Germany). Aliquots of 100 mg dried samples were sonicated with 5 mL of pure water for 30 min and centrifuged at 10,000 rpm for 5 min at 4 °C. Then, 0.5 mL of the supernatant was transferred to a vial for LC-MS/MS analysis.

Serum and urine samples for method development were kindly provided by healthy volunteers in our laboratory. The study was approved by the Medical Ethical Review Committee of the National Institute for Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention [No. NIOHP202323] on 19 June 2023. One-half-milliliter urine samples were mixed with 0.5 mL of water and then centrifuged at 10,000 × g rpm for 5 min at 4 °C. For the serum, 0.4 mL samples were placed in 2 mL polypropylene tubes, and then 0.6 mL of acetonitrile was added for protein precipitation. The mixture was sonicated for 15 min and then centrifuged at 10,000 × g rpm for 5 min at 4 °C. Then, 0.8 mL of the supernatant was pipetted into a clean Eppendorf tube containing 20 mg of PSA SPE sorbent. The tubes were subjected to vortex-induced vibration at 2000 rpm for 1 min and then centrifuged at 10,000 × g rpm for 5 min at 4 °C. The top layer was collected for LC-MS/MS analysis.

SGF samples were diluted with an equal volume of acetonitrile, and the next step was the same as for the urine sample.

2.5. Method Validation

The validation of this method included the assessment of the linear range and linearity of the calibration curves, evaluation of the matrix effect (ME), determination of the method limit of detection (MLOD) and method limit of quantitation (MLOQ), and examination of the accuracy and precision.

3. Results and Discussion

3.1. Optimization of Instrumental Method

3.1.1. MS/MS Parameters

Twelve mushroom toxins, namely ibotenic acid, muscimol, muscarine, β-amanitin, α-amanitin, desoxoviroidin, γ-amanitin, phallisin, illudin S, phallacidin, phalloidin and illudin M, were selected in this study. The physico-chemical properties and toxicity of these 12 mushroom toxins are given in Table 2.

Table 2. Physico-chemical properties and toxicity of mushroom toxins in this study.

Name	CAS No.	Formula	ACD/LogP ^a	Toxicity	Source
Ibotenic acid	2552-55-8	C ₅ H ₆ N ₂ O ₄	−1.07	Impairment of central γ -aminobutyric acid system. LD ₅₀ is 15 mg/kg	<i>Amanita citrina</i> ; <i>Amanita farinosa</i> ; <i>Amanita muscaria</i> ; <i>Amanita pantherina</i> ; <i>Amanita vaginata</i> ; <i>Amanita pseudosynchopyramis</i> ; <i>Amanita subjunquillea</i>
Muscimol	2763-96-4	C ₄ H ₆ N ₂ O ₂	−0.93	Impairment of central γ -aminobutyric acid (GABA) system. LD ₅₀ is 8.1 mg/kg	<i>Amanita citrina</i> ; <i>Amanita muscaria</i> ; <i>Amanita pantherina</i> ; <i>Amanita pseudosynchopyramis</i>
Muscarine	2303-35-7	C ₉ H ₂₀ NO ₂	−2.79	The inhibition of acetylcholinesterase. LD ₅₀ is 93–166 U/kg	<i>Amanita citrina</i> ; <i>Amanita melleiceps</i> ; <i>Amanita muscaria</i> ; <i>Amanita pantherina</i> ; <i>Amanita phalloides</i> ; <i>Boletus luridus</i> ; <i>Clitocybe dealbata</i> ; <i>Clitocybe phyllophila</i> ; <i>Hebeloma crustuliniforme</i> ; <i>Hebeloma fastibile</i> ; <i>Inocybe asterospora</i> ; <i>Inocybe erubescens</i> ; <i>Inocybe flavobrunnea</i> ; <i>Inocybe geophylla</i> ; <i>Inocybe rimosa</i> ; <i>Lactarius torminosus</i> ; <i>Mycena pura</i> ; <i>Omphalotus olearius</i> ; <i>Panaeolus antillarum</i> ; <i>Panaeolus subbalteatus</i> ; <i>Russula emetica</i> ; <i>Amanita pseudosynchopyramis</i>
β -Amanitin [24]	21150-22-1	C ₃₉ H ₅₃ N ₉ O ₁₅ S	−7.98	Necrotic damage of liver and kidney, lesion of intestinal mucosa. LD ₅₀ is 0.5 mg/kg	<i>Amanita phalloides</i> ; <i>Agaricus pequinii</i> ; <i>Amanita bisporigera</i> ; <i>Amanita citrina</i> ; <i>Amanita exitialis</i> ; <i>Amanita fritillaria</i> ; <i>Amanita fuliginea</i> ; <i>Amanita gymnopus</i> ; <i>Amanita japonica</i> ; <i>Amanita kohiraensis</i> ; <i>Amanita melleiceps</i> ; <i>Amanita muscaria</i> ; <i>Amanita neo-ovoidea</i> ; <i>Amanita oberwinklerana</i> ; <i>Amanita pallidorozea</i> ; <i>Amanita pantherina</i> ; <i>Amanita sepiacea</i> ; <i>Amanita subjunquillea</i> ; <i>Amanita sychnopyramis</i> ; <i>Amanita tenuifolia</i> ; <i>Amanita vaginata</i> ; <i>Amanita verna</i> ; <i>Amanita virgineoides</i> ; <i>Amanita virosa</i> ; <i>Galerina fasciculata</i> ; <i>Galerina marginata</i> ; <i>Galerina subpectinata</i> ; <i>Galerina sulciiceps</i> ; <i>Galerina venenata</i> ; <i>Lepiota brunneo-incarnata</i> ; <i>Lepiota castanea</i> ; <i>Lepiota helveola</i> ; <i>Mycena pura</i> ; <i>Russula subnigricans</i>
α -Amanitin	23109-05-9	C ₃₉ H ₅₄ N ₁₀ O ₁₄ S	−9.14	Necrotic damage of liver and kidney, lesion of intestinal mucosa. LD ₅₀ is 0.25–0.3 mg/kg [25,26]	Same with β -Amanitin
Desoxoviroidin	74125-14-7	C ₃₈ H ₅₆ N ₈ O ₁₄ S	−8.52	Hemorrhagic necrosis of hepatocytes. LD ₅₀ is 2.5 mg/kg	<i>Amanita phalloides</i> ; <i>Amanita verna</i> ; <i>Amanita virosa</i> ; <i>Galerina marginata</i> ; <i>Galerina sulciiceps</i> ; <i>Galerina venenata</i> ; <i>Lepiota helveola</i> ; <i>Amanita pallidorozea</i> ; <i>Amanita exitialis</i> ; <i>Amanita subjunquillea</i>
γ -Amanitin	21150-23-2	C ₃₉ H ₅₄ N ₁₀ O ₁₃ S	−8.19	Necrotic damage of liver and kidney, lesion of intestinal mucosa. LD ₅₀ is 0.2 mg/kg	Same with β -Amanitin
Phallisacin	58286-46-7	C ₃₇ H ₅₀ N ₈ O ₁₃ S	−5.42	Hemorrhagic necrosis of hepatocytes. LD ₅₀ is 2.5 mg/kg	<i>Amanita exitialis</i> ; <i>Amanita phalloides</i> ; <i>Amanita pallidorozea</i> ; <i>Amanita subjunquillea</i>
Illudin S	1149-99-1	C ₁₅ H ₂₀ O ₄	−0.29	Lesion of intestinal mucosa. LD ₅₀ is 50 mg/kg	<i>Omphalotus guepiniformis</i> ; <i>Omphalotus olearius</i> [27,28]; <i>Omphalotus nidiformis</i> [29]
Phallacidin	26645-35-2	C ₃₇ H ₅₀ N ₈ O ₁₃ S	−4.78	Hemorrhagic necrosis of hepatocytes. LD ₅₀ is 1.5 mg/kg	<i>Amanita exitialis</i> ; <i>Amanita phalloides</i> ; <i>Lepiota brunneo-incarnata</i> ; <i>Amanita pallidorozea</i> ; <i>Amanita subjunquillea</i>
Phalloidin	17466-45-4	C ₃₅ H ₄₈ N ₈ O ₁₁ S	−5.75	Hemorrhagic necrosis of hepatocytes. LD ₅₀ is 1–2 mg/kg	<i>Agaricus pequinii</i> ; <i>Amanita citrina</i> ; <i>Amanita exitialis</i> ; <i>Amanita fritillaria</i> ; <i>Amanita fuliginea</i> ; <i>Amanita griseofarinosa</i> ; <i>Amanita griseoverrucosa</i> ; <i>Amanita oberwinklerana</i> ; <i>Amanita pallidorozea</i> ; <i>Amanita pantherina</i> ; <i>Amanita phalloides</i> ; <i>Amanita sepiacea</i> ; <i>Amanita subjunquillea</i> ; <i>Amanita sychnopyramis</i> ; <i>Amanita verna</i> ; <i>Amanita virosa</i> ; <i>Lepiota brunneo-incarnata</i> ; <i>Lepiota helveola</i> ; <i>Russula subnigricans</i> ; <i>Lepiota brunneo-incarnata</i>
Illudin M	1146-04-9	C ₁₅ H ₂₀ O ₃	0.99	Lesion of intestinal mucosa	<i>Omphalotus olearius</i> ; <i>Omphalotus nidiformis</i>

^a Available on <https://www.chemspider.com/> accessed on 10 March 2024.

Optimization of the quadrupole mass spectrometry parameters was performed in ESI positive mode using the single standard (0.1 μ g/mL) via a syringe pump at 10 μ L/min with direct injection. The calibration of each toxin was initially performed automatically using Waters Intelli Start and then checked manually to ensure accuracy. For the majority of the analytes, [M + H]⁺ is the most abundant ion, except for illudin S and illudin M. For these two compounds, the dominant ion observed was [M + H − H₂O]⁺, with m/z 247 showing

the highest intensity for illudin S, which was produced by in-source collision-induced dissociation with the loss of a water molecule. These ions were selected as precursor ions, and their product ions were subsequently identified by optimization. The underlined transition marked in Table 3 is chosen for quantitation, while the second, less sensitive one is used for confirmation.

Table 3. LC-MS/MS acquisition parameters for 12 mushroom toxins.

Toxin Name	RT (min)	MRM Trace	Cone (V)	Collision Energy (eV)
Ibotenic acid	0.80	<u>159 > 113</u> ^a	20	11
		159 > 70		11
Muscimol	0.84	<u>115 > 98</u>	20	11
		115 > 86		11
Muscarine	2.62	<u>174 > 115</u>	25	20
		174 > 59.9		20
β-Amanitin	3.33	<u>920.5 > 85.9</u>	55	95
		920.5 > 259		60
α-Amanitin	3.51	<u>919.5 > 85.9</u>	55	90
		919.5 > 259		60
Desoxoviroidin	3.56	<u>849.5 > 85.9</u>	45	90
		849.5 > 157		80
γ-Amanitin	3.63	<u>903.5 > 85.9</u>	65	90
		903.5 > 243		90
Phallisacin	3.67	<u>863.4 > 86</u>	60	90
		863.4 > 157		82
Illudin S	3.77	<u>247 > 201</u>	20	12
		247 > 229		12
Phallacidin	3.84	<u>847.4 > 85.9</u>	60	90
		847.4 > 157		82
Phalloidin	3.97	<u>789.4 > 157</u>	60	80
		789.4 > 330.1		50
Illudin M	4.67	<u>231 > 213</u>	20	12
		231 > 203		12

^a The underline transition was used for quantitation.

3.1.2. Optimization of LC Columns

The complex variations in chemical composition and physical properties of the 12 toxins present a challenge for their separation and analysis by chromatography. A series of tests were carried out on five different 2.1 mm × 100 mm columns to evaluate the efficacy and specificity of all 12 target compounds. The brand names, particle size, technical characteristics and manufacturers of the columns are summarized in Table 4. The specific details of the mobile phase conditions are given in Tables S1–S5 in Supplementary Materials.

As can be seen from Figure 1, the performance of the HSS T3 column shows favorable sensitivity and efficiency in the analysis of interest, while the retention of ibotenic acid and muscimol on this column is slightly insufficient. The CSH PFP column, with a weak positive charge on the stationary particles, has shown exceptional peak shapes and homogeneous distribution of analyte peaks in various bioanalytical applications and metabolomics [30]. However, in this study, the performance of this column shows deficiencies in either peak shape or response. The Trinity P1 column is designed with a modified inner and outer

pore area with an organic layer that provides both reversed-phase and anion-exchange properties and cation-exchange functionality, respectively. Thus, trifunctional retention mechanisms including reversed phase, anion exchange and cation exchange were combined in one column. Although this gave good retention for the highly polar compounds ibotenic acid (RT 2.79 min) and muscimol (RT 1.56 min), the signal intensity of all compounds except muscarine was relatively low, resulting in a decrease in peak intensity, possibly due to the inclusion of 20 mM ammonia formate. A total of twelve toxins were tested on the C18 amide column. This alternative mixed retention phase column showed a comparable elution sequence to that observed on the HSS T3 column, indicating that the predominant retention mechanism is likely to be reversed-phase one. The tailing peak observed for muscarine and the relatively weak response further suggest that the C18 amide column may not be the most appropriate choice (see Figure 1d). In addition, the BEH amide column was also not selected due to suboptimal sensitivity for most compounds and inadequate retention for illudin S and illudin M.

Table 4. Summary of the chromatographic columns evaluated.

Type	Column	Particle (μm)	Ligand	Manufacturer
RP	HSS T3	1.8	C18, low density	Waters (Milford MA, USA)
RP	CSH PFP	1.7	PFP, CSH ^a	Waters (Milford MA, USA)
HILIC	BEH Amide	1.7	Trifunctional amide	Waters (Milford MA, USA)
MMC	Trinity P1	3	NSH ^b mixed mode	Thermo Scientific (Waltham, MA, USA)
MMC	C18-Amide	2	C18 and amide	ACE (Aberdeen, Scotland, UK)

^a Charged surface hybrid, weak positive charge on the surface of the particles. ^b Nano-polymer silica hybrid technology. The inner pore region is modified with an organic layer that provides both reversed-phase and anion-exchange properties, while the outer pore region is modified with cation-exchange functionality.

3.1.3. Optimization of Mobile Phase

Based on the above information, the HSS T3 column was selected for this research. The optimization of the mobile phase then took into account the presence of an ammonia buffer along with the organic solvent.

In the literature, ammonia acetate in the concentration range of 1–100 mM is commonly observed as an additive in LC-MS analysis, including in the analysis of mushroom toxins [31]. Other different ammonia buffers have also been reported. Here, we compared ammonium formate, ammonium acetate, ammonium carbonate and ammonium fluoride solution at 1 mM with methanol as a mobile phase component on the effect of LC-MS/MS analysis. Figure 2 shows that ammonium fluoride, ammonium formate and ammonium acetate as modifiers induced similar TIC chromatograms. However, the former gave the best sensitivity for most of the targets. When 1 mM ammonium carbonate was used, strong broadening and tailing of the chromatographic peaks of muscimol and muscarine were observed.

The composition of the organic solvent and the addition of formic acid were then further optimized. The peak response was investigated using acetonitrile–1 mM ammonium fluoride, acetonitrile–1 mM ammonium fluoride and 0.1% formic acid, methanol–1 mM ammonium fluoride, and methanol–1 mM ammonium fluoride and 0.1% formic acid as mobile phases. The results are shown in Figure 3. The addition of 0.1% formic acid results in a significant decrease in most target compounds in both acetonitrile and methanol systems. The maximum sensitivity was achieved using methanol–1 mM ammonium fluoride.

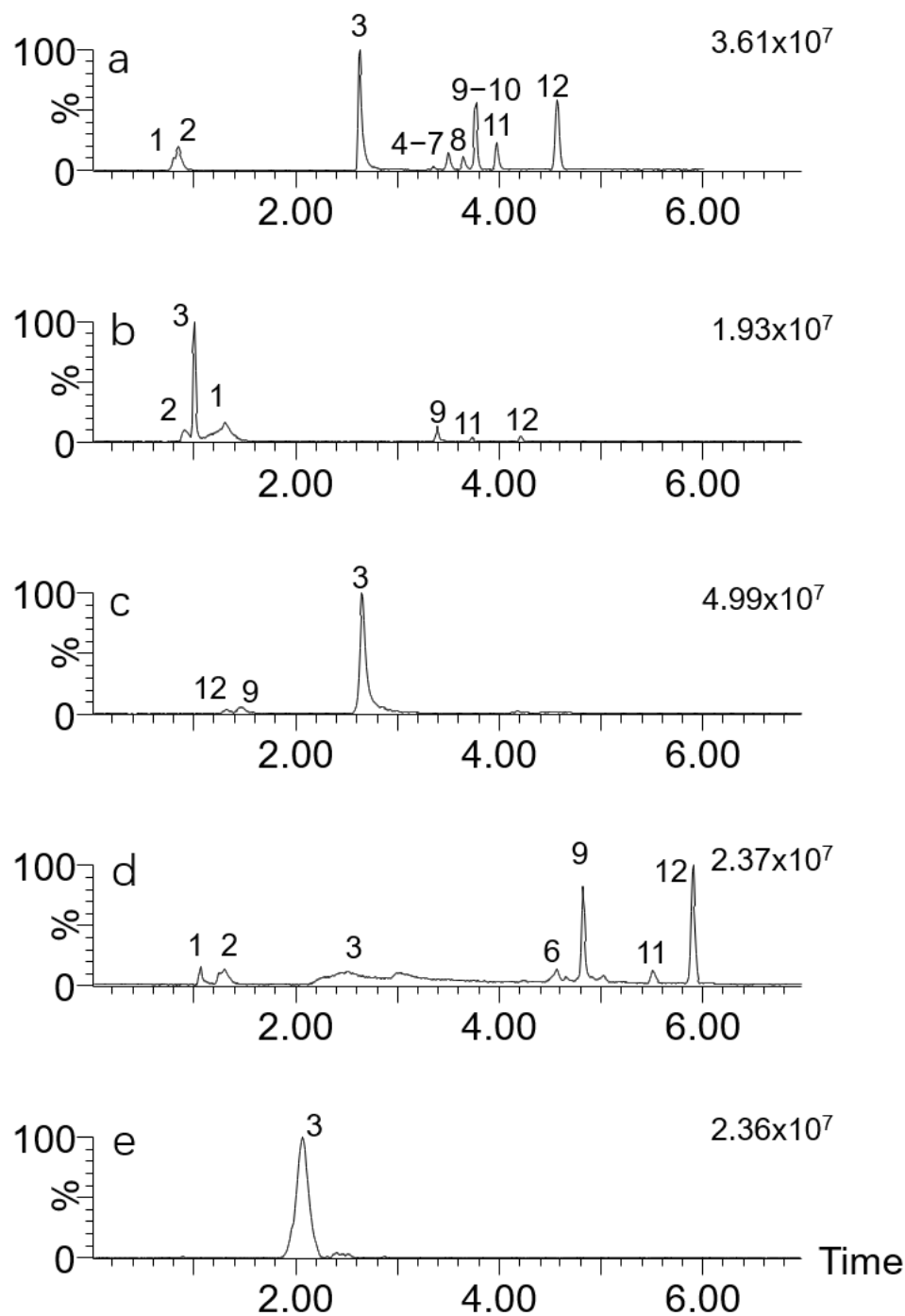


Figure 1. TIC chromatograms of 12 mushroom toxins on different columns. Toxins: 1. ibotenic acid; 2. muscimol; 3. muscarine; 4. β -amanitin; 5. α -amanitin; 6. desoxoviroidin; 7. γ -amanitin; 8. phallisacin; 9. illudin S; 10. phallacidin; 11. phalloidin; 12. illudin M. Columns: (a) HSS T3; (b) CSH PFP; (c) Trinity P1; (d) C18 amide; (e) BEH amide.

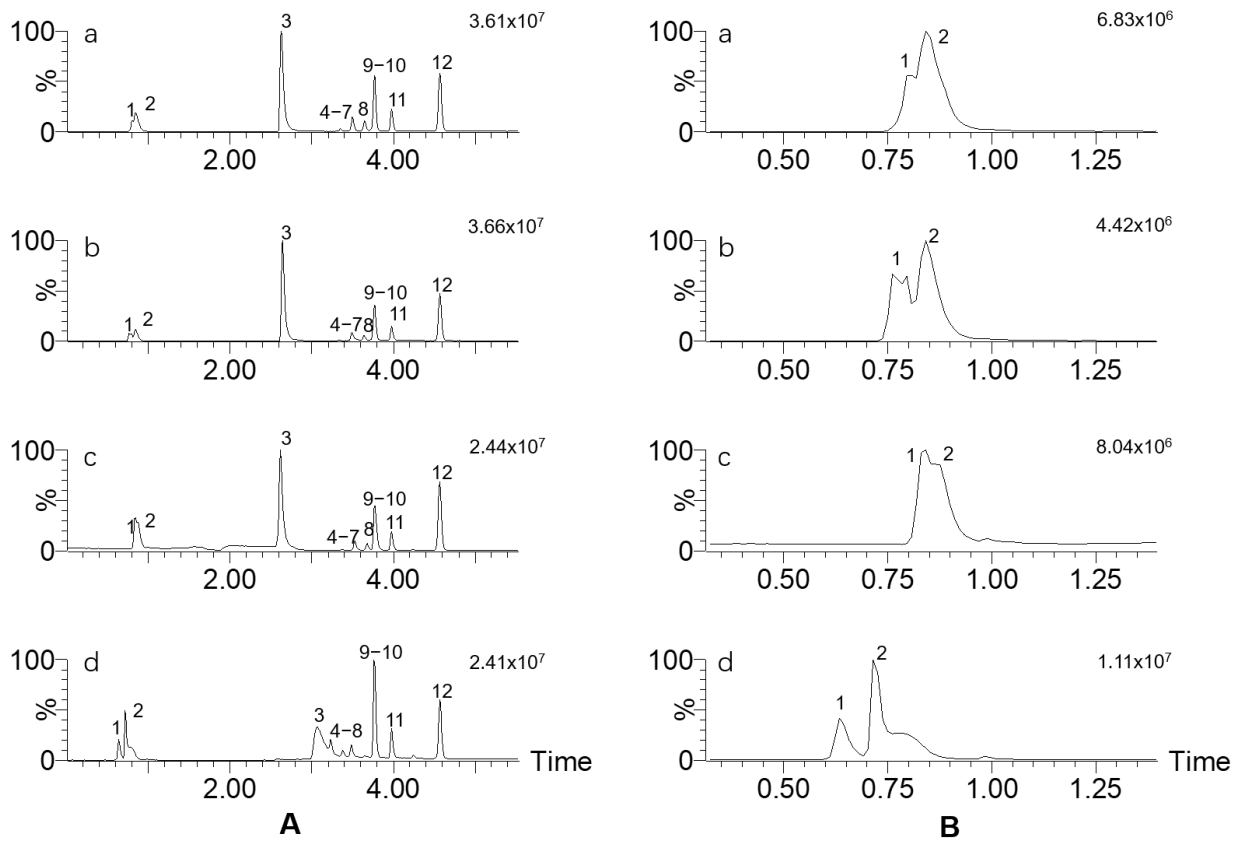


Figure 2. (A) TIC chromatograms of 12 mushroom toxins using different ammonia buffers and methanol as mobile phase; (B) partial enlargement at 0.25–1.4 min from (A). Ammonia buffer: a, ammonium fluoride; b, ammonium formate; c, ammonium acetate; d, ammonium carbonate. The toxin numbers are the same as in Figure 1.

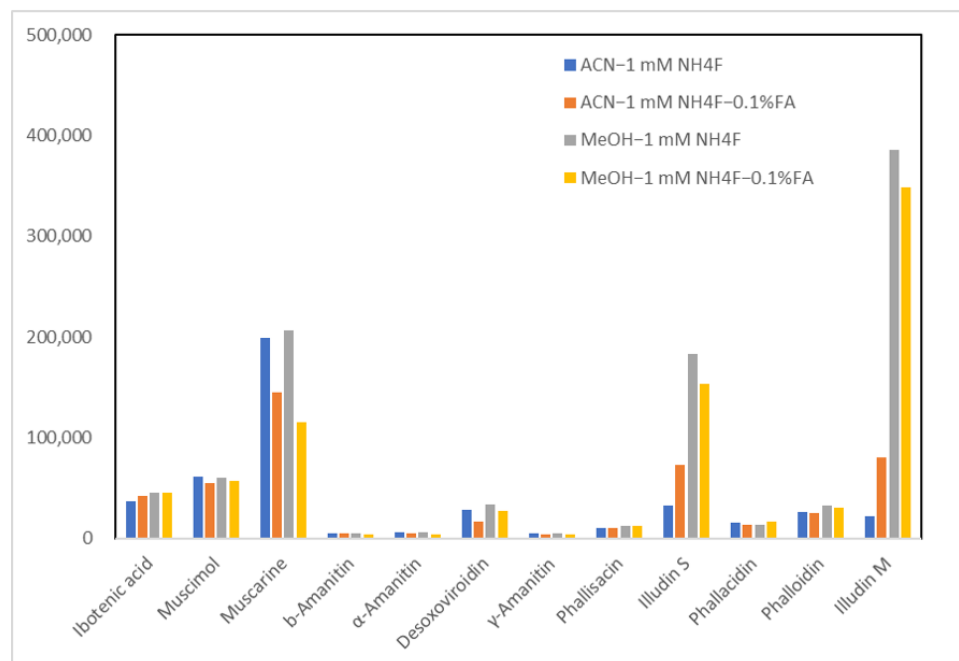


Figure 3. Peak response of 12 mushroom toxins using different mobile phase components. Abbreviations in the legend: ACN, acetonitrile; MeOH, methanol; NH4F, ammonium fluoride; FA, formic acid.

3.2. Optimization of Sample Pretreatment

As the amount of toxins in poisonous mushrooms is usually high, generally in the mg/kg or even g/kg range, the analysis of mushroom samples is relatively simple compared to other biological matrices. Using an ultrasonic apparatus, 100 mg samples of dried mushrooms were extracted with 5 mL of water, and the resulting extracts were subjected to LC-MS/MS analysis. Urine and SGF samples were diluted with one volume of water and acetonitrile, respectively, followed by centrifugation and transfer of the supernatant for analysis. As for the serum matrix, it contains a high abundance of proteins and phospholipids, which could suppress the spectrometric response during the assay. Acetonitrile extracts of serum were further purified using three types of SPE sorbents: MCX, HLB and PSA. MCX is a mixed-mode exchanger that combines both strong ion exchange and reversed phase in one sorbent, specifically designed for improved cation retention. HLB contains only the reversed-phase component in the sorbent. PSA, the abbreviation for primary secondary silica, has proven to be the most effective sorbent for the removal of various matrices, significantly reducing the matrix effect and playing a vital role in the removal of fatty acids and polar interferences. Purification performance was evaluated in terms of recovery and ME, which is represented by the ratio of the peak area of matrix-matched standards to the pure standard solution at the same concentration level (50 µg/L) and multiplied by 100 for expression in percent. Values of $100 \pm 15\%$ indicate that no matrix effect was observed, whereas values above 115% or below 85% indicate the presence of ionization enhancement or suppression, respectively.

The two least retained toxins, ibotenic acid and muscimol, were completely suppressed in serum and urine samples. This result can be explained by the inhibition of ionization caused by the different interferences co-eluting in serum and urine. Therefore, SPE purification of serum samples only gives results for the other 10 toxins as shown in Figure 4. When MCX powder was used, muscarine was tightly bound to the sorbent, and recovery was zero. HLB and PSA showed recoveries between 79 and 112%, while the former achieved ME 41~72%, significantly less than that for PSA (52~84%). It was shown that PSA has a better ability to purify the serum sample compared to MCX sorbent. Therefore, 20 mg of PSA was selected for purifying 0.8 mL of serum extracts.

3.3. Method Validation

The calibration curve was initially constructed using a clean standard series from 0.02 µg/L to 200 µg/L. Satisfactory linearity was observed over the range of all 12 target toxins from 0.05 to 200 µg/L with correlation coefficients of $R^2 > 0.994$ (Table 5). The isotope dilution method is an ideal way to compensate for the depletion of specific compounds during sample preparation and the interferences of mass spectrometric analysis. However, the lack of isotopic standards for the target toxins in this study poses a significant challenge. Therefore, quantification of real samples was performed using matrix-matched calibration. As shown in Table 5, the ME was obtained by the ratio of the slope of the matrix-matched calibration plot to the slope of the solvent-dissolved standard calibration plot and multiplied by 100 for expression in percent. As a result, 33~91% ME was observed for 12 toxins in mushroom, urine, serum and SGF, indicating varying degrees of response suppression.

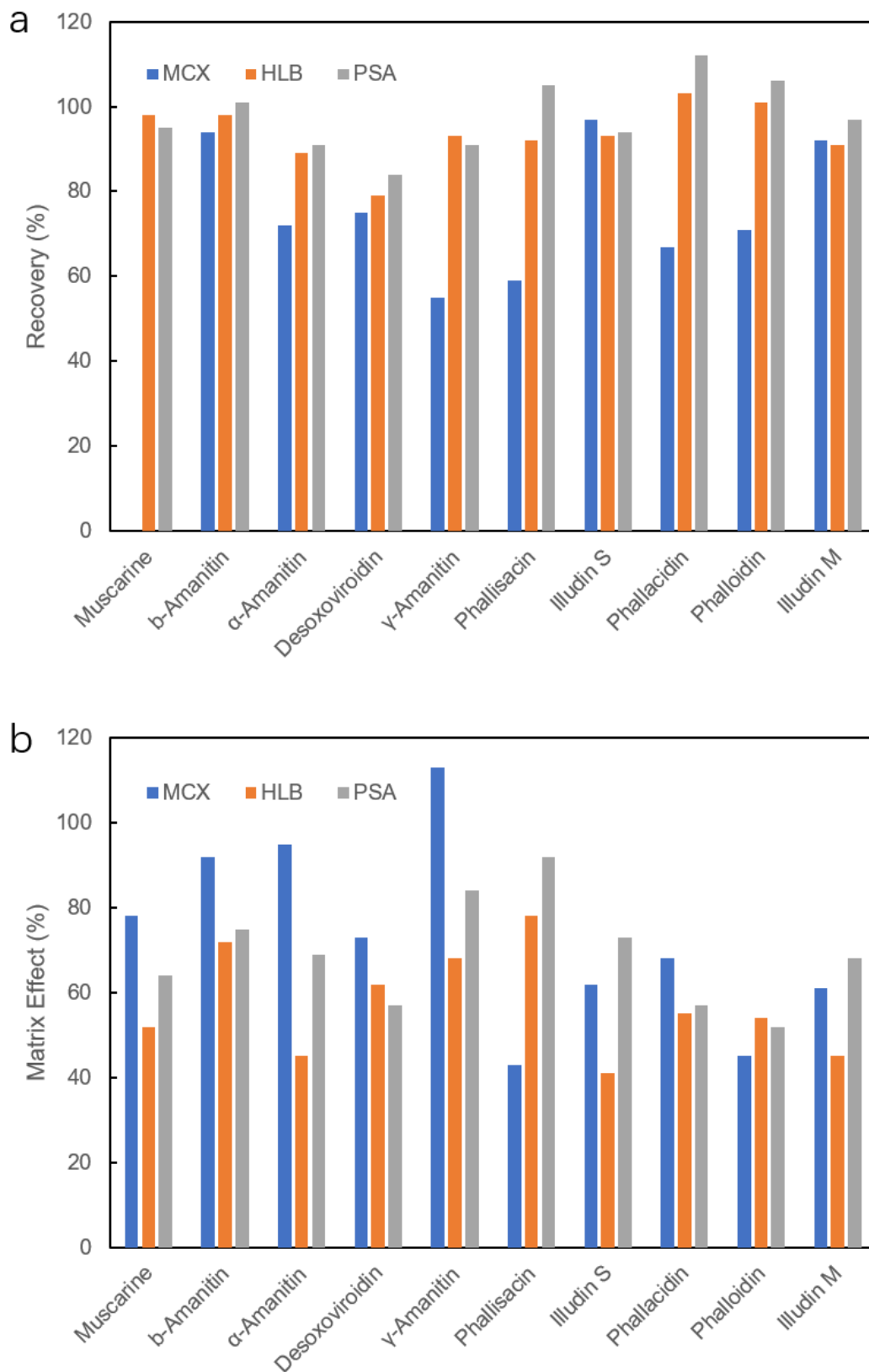


Figure 4. Recovery (a) and matrix effect (b) of 10 mushroom toxins in serum extracts after different SPE purifications.

Table 5. Linearity and matrix effects of 12 mushroom toxins.

Toxin Name	Linear Range (µg/L)	Calibration Curve	R ²	Mushroom	Matrix Effects (%)		SGF
					Serum	Urine	
Ibotenic acid	1~200	Y = 1327.29x + 339	0.9954	54	0	0	45
Muscimol	2~200	Y = 3424x + 215	0.9985	40	0	0	73
Muscarine	0.05~100	Y = 34137x + 83	0.9975	66	61	58	58
β-Amanitin	0.1~200	Y = 1354x - 75	0.9966	38	74	71	45
α-Amanitin	1~200	Y = 2139x - 95	0.9981	56	67	63	72
Desoxoviroidin	1~200	Y = 1466x + 218	0.9980	33	53	59	75
γ-Amanitin	0.5~200	Y = 1942x + 153	0.9982	42	79	72	91
Phallisacin	1~200	Y = 955x - 48	0.9954	41	89	55	54
Illudin S	0.2~100	Y = 3842x + 143	0.9985	53	67	62	70
Phallacidin	0.5~200	Y = 1250x + 132	0.9962	61	57	60	56
Phalloidin	0.5~200	Y = 2638x - 216	0.9983	52	47	84	53
Illudin M	0.2~100	Y = 16074x + 2528	0.9947	55	69	51	63

The MLODs (identified as the concentration giving a signal-to-noise ratio (S/N) of 3) and MLOQs (identified as the concentration giving an S/N of 10) are summarized in Table 6. The MLODs of 12 toxins in mushroom samples were 0.005~0.1 mg/kg, and the MLOQs were 0.01~0.2 mg/kg. Muscarine shows more sensitive results compared to the other toxins in all four matrices. The MLOQs of the 12 toxins in SGF were 0.20~2.0 µg/L. The values for ibotenic acid and muscimol in serum and urine are not available, and the other 10 toxins showed MLOQs of 0.15~2.0 µg/L. These results were in line with or even lower than the majority of previous studies on mushroom toxins [19,32–34]. A preliminary summary of the literature on the detection of mushroom toxins by LC-MS/MS is given in Table S6 in Supplementary Materials. Most of these reports focused on mushroom analysis targeting 1~11 toxins, with MDLs ranging from 0.00098 mg/kg to 10 mg/kg. Urine and blood samples were extracted with acidic methanol aqueous solution or other reagents followed by SPE purification, resulting in MDLs of 1~2000 µg/mL. Compared to this literature, this method is very simple, has high sensitivity and has a high coverage of the fungal toxins as well as the sample matrix.

Table 6. MLODs and MLOQs of 12 mushroom toxins in different matrices.

Toxin Name	Mushroom (mg/kg)		Serum (µg/L)		Urine (µg/L)		SGF (µg/L)	
	MLOD	MLOQ	MLOD	MLOQ	MLOD	MLOQ	MLOD	MLOQ
Ibotenic acid	0.05	0.10	NA ^a	NA	NA	NA	0.70	2.0
Muscimol	0.10	0.30	NA	NA	NA	NA	0.70	2.0
Muscarine	0.005	0.01	0.06	0.20	0.10	0.15	0.07	0.20
β-Amanitin	0.08	0.20	0.70	2.0	0.50	1.5	0.70	2.0
α-Amanitin	0.08	0.20	0.80	2.0	0.50	1.5	0.70	2.0
Desoxoviroidin	0.10	0.30	0.70	2.0	0.50	1.5	0.70	2.0
γ-Amanitin	0.10	0.30	0.30	1.0	0.30	1.0	0.30	1.0
Phallisacin	0.03	0.10	0.70	2.0	0.70	2.0	0.70	2.0
Illudin S	0.02	0.05	0.20	0.5	0.15	0.5	0.15	0.5
Phallacidin	0.08	0.20	0.30	1.0	0.30	1.0	0.30	1.0
Phalloidin	0.07	0.20	0.30	1.0	0.30	1.0	0.30	1.0
Illudin M	0.02	0.05	0.20	0.6	0.20	0.5	0.15	0.5

^a NA is stand for Not Applicable.

The accuracy of the method was validated by analyzing the average recovery from spiked blank at three different concentration levels, each performed in six replicates. Four different matrix samples were tested. As shown in Table 7, the accuracy of the method ranged from 73.0% to 110.3%. The precision of this method was represented by the percentage RSD at each spiked level for each toxin, and these values are also summarized in Table 7, with values not exceeding 19.4%.

Table 7. Recoveries and RSDs of 12 toxins in mushroom, serum, urine and SGF (*n* = 6).

Toxin Name	Mushroom (%)			Spiked (µg/L)	Serum (%)		Urine (%)		SGF (%)	
	Spiked (mg/kg)	Recovery	RSD		Recovery	RSD	Recovery	RSD	Recovery	RSD
Ibotenic acid	0.30	80.1	5.2	2.0	NA ^a	NA	NA	NA	93.7	16.5
	0.50	91.2	10.7	10	NA	NA	NA	NA	98.5	13.2
	1.00	85.5	8.3	100	NA	NA	NA	NA	91.2	9.7
Muscimol	0.30	88.1	8.2	2.0	NA	NA	NA	NA	95.3	7.0
	0.50	85.7	12.7	10	NA	NA	NA	NA	89.7	13.7
	1.00	86.5	9.4	100	NA	NA	NA	NA	85.6	11.6
Muscarine	0.03	94.3	11.2	2.0	79.4	11.1	87.9	4.9	105.7	13.5
	0.05	91.4	5.6	10	76.0	14.8	92.7	7.0	85.1	8.0
	0.10	89.2	8.5	100	85.3	7.2	98.7	11.4	96.6	6.4
β-Amanitin	0.30	95.7	8.2	2.0	89.8	13.5	99.0	8.0	98.0	4.7
	0.50	87.2	10.5	10	82.4	14.2	84.8	10.2	110.3	7.5
	1.00	98.7	4.3	100	91.5	10.6	91.7	2.6	92.8	8.8
α-Amanitin	0.30	89.2	9.7	2.0	84.8	15.7	81.2	7.9	94.6	15.7
	0.50	95.9	10.6	10	87.1	11.5	98.0	14.5	100.9	5.5
	1.00	90.1	13.9	100	88.1	13.2	88.5	2.9	85.1	7.1
Desoxoviroidin	0.30	85.3	16.1	2.0	83.0	18.9	83.5	14.6	90.3	19.2
	0.50	90.5	13.5	10	88.5	11.3	86.6	5.8	94.8	5.3
	1.00	95.6	8.7	100	87.0	8.9	96.9	6.8	99.3	13.9
γ-Amanitin	0.30	92.8	7.5	2.0	84.8	17.1	84.8	14.6	95.0	6.8
	0.50	83.5	6.8	10	87.1	14.9	97.8	8.7	88.5	10.0
	1.00	80.2	12.4	100	89.2	13.5	99.6	13.6	101.5	10.7
Phallisacin	0.30	89.5	8.4	2.0	77.7	17.5	106.2	6.8	73.0	5.5
	0.50	91.7	13.8	10	81.3	16.3	92.2	12.6	76.4	17.5
	1.00	94.2	12.6	100	79.3	14.2	95.9	9.7	97.7	14.7
Illudin S	0.10	93.9	7.9	2.0	84.4	13.4	92.2	8.1	83.7	5.6
	0.50	95.3	10.5	10	81.4	15.9	97.8	8.7	83.3	12.5
	1.00	97.4	9.1	100	83.1	11	91.3	8.7	78.2	6.9
Phallacidin	0.30	78.4	15.2	2.0	86.5	13.2	93.1	6.8	87.2	7.1
	0.50	105.2	7.9	10	84.8	7.8	83.8	4.9	105.6	13.3
	1.00	107.9	5.7	100	87.0	12.1	92.2	8.7	90.9	5.1
Phalloidin	0.30	97.1	7.6	2.0	77.4	8.5	91.3	2.9	101.0	10.2
	0.50	95.2	13.2	10	83.4	7.3	101.5	4.9	102.8	8.2
	1.00	102.5	8.4	100	85.4	12.4	99.6	3.9	91.8	11.2
Illudin M	0.10	107.4	6.3	2.0	96.6	15.6	92.0	8.7	99.4	19.4
	0.50	98.2	10.6	10	87.3	11.4	93.2	4.9	88.1	11.2
	1.00	100.5	8.9	100	85.0	7.8	94.9	7.8	82.6	14.3

^a NA: Not Applicable.

3.4. Method Application

This novel analytical method was used to analyze 34 samples of wild mushrooms from Yunnan Province. Figure 5 shows LC-MS/MS chromatograms of 12 toxins in standard and real mushroom samples. All toxins except illudin M were detected in these real samples, among which illudin S was found in 15 *Omphalotus olearius* samples in the concentration range of 4.37~141 mg/kg (Table 8). Ibotenic acid, muscimol and muscarine were detected in five *Amanita pseudosychnopyramis* samples at 23.6~489 mg/kg. *Amanita pallidorosea*, *Amanita exitialis* and *Amanita subjunquillea* samples contained α-amanitin, β-amanitin, γ-amanitin, phallisacin, phallacidin and phalloidin at 0.61~2143 mg/kg. No toxin was detected in the *Chlorophyllum molybdites* sample.

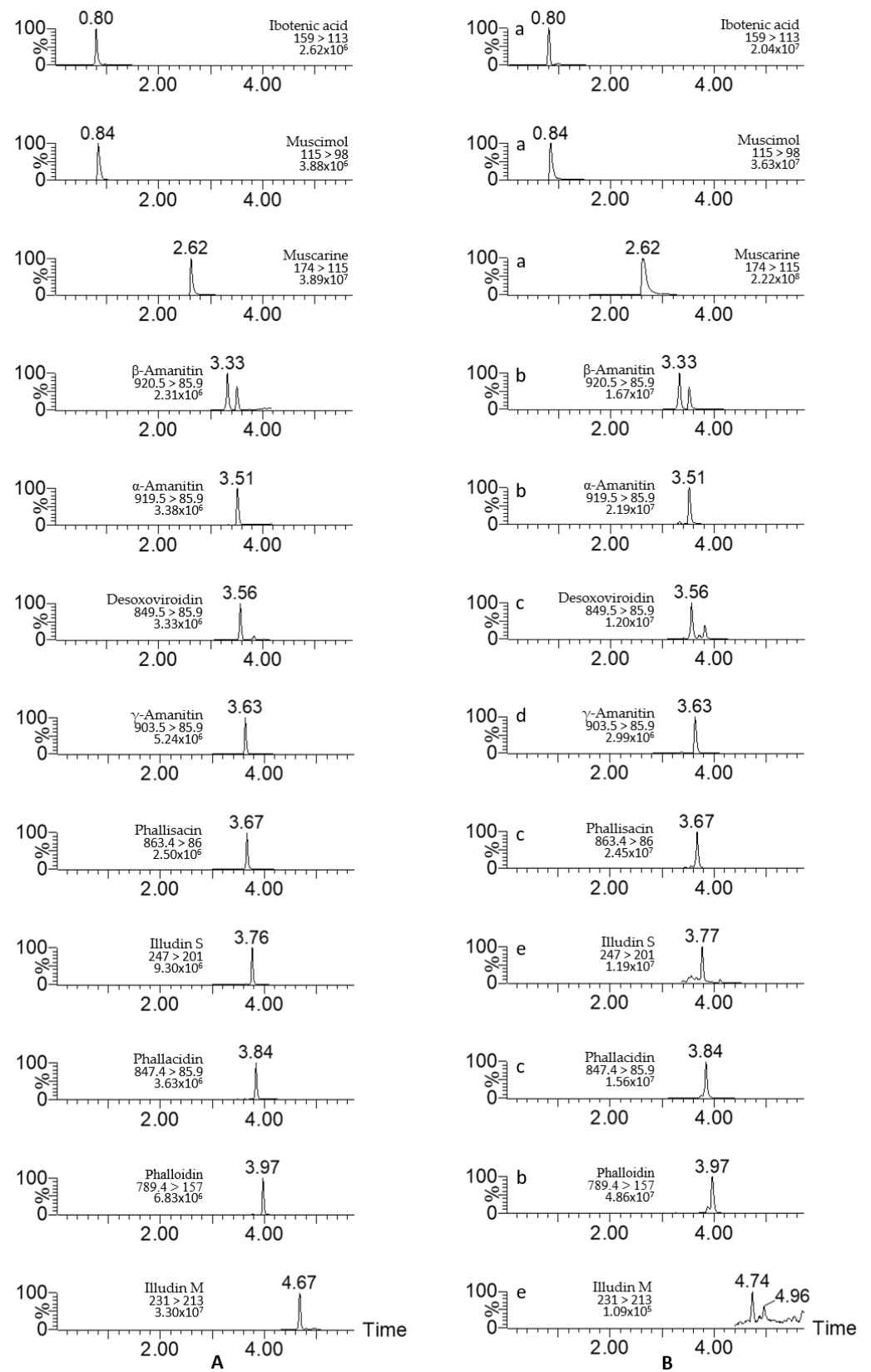


Figure 5. LC-MS/MS chromatograms of 12 mushroom toxins in standard solution (A) and wild mushroom samples (B). Mushroom species: a, *Amanita pseudosyncnopyramis*; b, *Amanita pallidorozea*; c, *Amanita exitialis*; d, *Amanita subjunquillea*; e, *Omphalotus olearius*.

Table 8. Occurrence of 12 toxins in wild mushrooms.

Toxin Name	Mushroom (mg/kg, n = 34)					
	<i>Amanita Pseudoschnopyramis</i> (n = 5)	<i>Amanita Pallidorosea</i> (n = 6)	<i>Amanita Exitialis</i> (n = 4)	<i>Amanita Subjunquillea</i> (n = 3)	<i>Chlorophyllum Molybdites</i> (n = 1)	<i>Omphalotus Olearius</i> (n = 15)
Ibotenic acid	42.8–252	<MLOD	<MLOD	<MLOD–4.92	<MLOD	<MLOD
Muscimol	23.6–195	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
Muscarine	154–489	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD
β-Amanitin	<MLOD ^a	4.52–281	172–1938	10.4–51.7	<MLOD	<MLOD
α-Amanitin	<MLOD	6.98–475	76.1–2143	39.8–245.6	<MLOD	<MLOD
Desoxoviroidin	<MLOD	<MLOD–9.87	<MLOD–73.4	<MLOD–3.76	<MLOD	<MLOD
γ-Amanitin	<MLOD	<MLOD–3.07	0.61–4.52	1.01–10.4	<MLOD	<MLOD
Phallisin	<MLOD	5.83–26.2	2.62–215	2.13–18.5	<MLOD	<MLOD
Illudin S	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	4.37–141
Phallacidin	<MLOD	4.12–78.8	4.37–87.8	9.24–82.7	<MLOD	<MLOD
Phalloidin	<MLOD	2.34–252	<MLOD	<MLOD–96.3	<MLOD	<MLOD
Illudin M	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD	<MLOD

^a MLOD: Determination of the Method Limit of Detection.

4. Conclusions

A comprehensive LC-MS/MS method was developed for the simultaneous detection of 12 mushroom toxins from different species and sources in mushroom and biological samples. The entire method consisted of a simple extraction followed by dilution injection or SPE clean-up using PSA sorbent for serum samples. The method was well validated. The main advantages of the method presented in this paper are its high coverage of mushroom toxins, simplicity, relatively high selectivity and high sensitivity. It can be used in the laboratory for real sample analysis.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations11060183/s1>, Table S1: Gradient mobile phase conditions of HSS T3 column; Table S2: Gradient mobile phase conditions of CSH PFP column; Table S3: Gradient mobile phase conditions of Trinity P1 column; Table S4: Gradient mobile phase conditions of ACE C18-Amide column; Table S5: Gradient mobile phase conditions of BEH Amide column; Table S6: Literature summary of mushroom toxins detection using LC-MS/MS [14–16,19,35–37].

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Conflicts of Interest: The authors declare no conflicts of interest.

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