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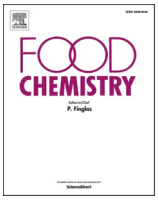

Paper spray and direct mushroom spray mass spectrometry methods for the analysis of psycho-neurological disorder toxins

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ABSTRACT

Fast screening approaches based on paper spray ionization were developed to identify psycho-neurological disorders mushrooms. Directly spraying from mushroom tissue already allowed direct identification of muscarine, psilocin, and ibotenic acid within 2–3 min. For quantitative analysis of the more challenging ibotenic acid, a new anion-exchange modified paper spray tandem mass spectrometry (AEPS-MS/MS) method was established to increase selectivity and sensitivity. The developed method was benchmarked against HPLC-MS/MS (with detection limits of 0.009 μg mL^{-1}) and could reach detection limits of 1.3 μg mL^{-1} for ibotenic acid spiked in mushroom extract, with acceptable accuracy (−14.0 % to +5.1 %) and precision (10.8 % to 18.0 %). In addition, the developed method was compared with solid-phase extraction coupled with PS-MS/MS (with detection limits of 2.8 μg mL^{-1}). Finally, the AEPS-MS/MS was applied for the quantitative analysis of ibotenic acid in *Amanita griseopantherina*, matching results from HPLC-MS/MS.

1. Introduction

Mushroom poisoning is a seasonal phenomenon that occurs relatively frequently in areas where people pick wild mushrooms for consumption (Gonmori, Fujita, Yokoyama, Watanabe, & Suzuki, 2011). Wild edible mushrooms are popular, as they are considered delicious in taste, rich in nutrients or even beneficial to health and beauty (Xu et al., 2017). Unfortunately, toxic mushroom ingestion can easily occur due to confusion between edible and poisonous species (Persson, 2016). According to the report on mushroom poisoning outbreaks from the Chinese Center for Disease Control and Prevention, there were 2266 incidents of mushroom poisoning from 2019 to 2023, with 6046 patients and 111 deaths (Li et al., 2020; Li et al., 2021; Li et al., 2022; Li et al., 2023; Li et al., 2024). It is thus very important to handle acute poisoning emergencies in a timely manner to minimize morbidity and mortality. To do this, it is imperative for the emergency physicians to know the species of mushroom ingested and whether different mushroom species have been co-ingested. However, it is obviously difficult, and prone to error, to distinguish mushrooms by morphology alone.

Seven types of mushroom poisoning symptoms have been identified for clinical diagnosis and treatment (Chen, Zhang, & Zhang, 2014). Each type of symptoms requires a different approach. To better understand the type of toxic mushroom ingested, chemical analysis can be applied, including thin layer chromatography (TLC), (ultra) high-performance liquid chromatography ((U)HPLC), gas chromatography (GC) or (U)HPLC coupled with (tandem) mass spectrometry (MS(/MS)) (Goff et al., 2024; Hasegawa et al., 2013; Morel et al., 2016; Stříbrný, Sokol, Merová, & Ondra, 2011; Zhao et al., 2022). For example, ibotenic acid – a psycho-neurotic disorder-inducing toxin, produced mainly by the genus *Amanita* Pers. (Kalichman, Kirk, & Matheny, 2020; Su et al., 2023) – and muscimol have been determined by UHPLC-MS/MS (Hasegawa et al., 2013). In addition, a method for determination of psilocybin and

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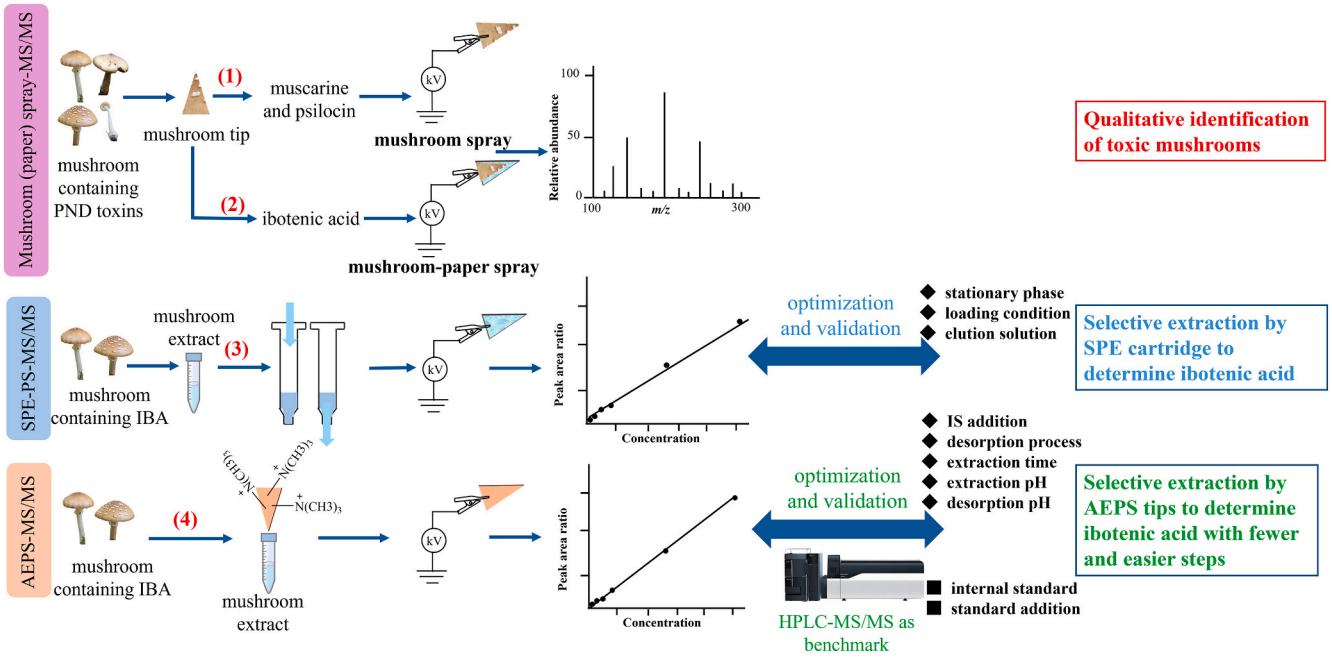

Fig. 1. Overview of the experimental workflow. (1) Direct spray from mushroom tissue (mushroom spray) for muscarine and psilocin. (2) Combination of mushroom tissue on a paper spray tip (mushroom-paper spray) for ibotenic acid. (3) SPE-PS-MS/MS for ibotenic acid. (4) AEPS- MS/MS for ibotenic acid.

psilocin was developed using HPLC-MS/MS (Goff et al., 2024). However, these methods rely on sample pretreatment or column-based separation (Aigumov et al., 2025; Aigumov, Vishnevskii, Novikov, & Savchuk, 2025), which are labor- and time-consuming. In emergency poisoning cases, it is crucial to provide information on the ingested toxins as soon as possible.

Ambient ionization mass spectrometry (AIMS) techniques have the potential to greatly reduce the analytical time to mere minutes (with little or no sample pretreatment). Such techniques have already been demonstrated in the area of food analysis (Bartella et al., 2022; Birse et al., 2022; Klampfl & Himmelsbach, 2015; Lara-Ortega et al., 2018), and for emergency toxicology (Lee et al., 2016; Lee, Su, & Shiea, 2022; Su, Huang, Shiea, & Lee, 2023; Wen et al., 2018). For example, electrospray laser desorption ionization MS was used for herb analysis and rapid morphological recognition of herbs (Wen et al., 2018). Moreover, direct electrospray probe MS, combined with multivariate statistical analysis, was recently reported for rapid toxin identification in mushrooms (Su et al., 2024). This method could detect six mushroom toxins and distinguish toxic vs. edible species by principal component analysis and clustering. Despite this significant progress, to our knowledge, no ambient MS method has been published for selective detection of ibotenic acid.

We note that paper spray mass spectrometry (PS-MS) has become a popular direct ionization method as it is straightforward and cost-effective, requiring only a paper tip for generation of electrospray (Wang, Liu, Cooks, & Ouyang, 2010). A variation on this method, plant spray MS (also known as leaf spray MS), was introduced by spraying from pieces of ginseng tissue or spray from leaves in 2011 (Liu, Wang, Cooks, & Ouyang, 2011). Leaf spray is a method derived from paper spray, which uses the leaf directly as substrate instead of paper. As the method is simple, and does not require any add-on, it is a promising tool for simple and rapid identification of botanical species (Freund, Martin, Cohen, & Hegeman, 2017; Liu, Gu, Yao, Zhang, & Chen, 2016).

Despite their merits, paper spray and leaf spray directly introduce a complex sample into the MS, which often leads to matrix effects and might cause ion suppression. As a result, the selective and targeted determination and quantification of trace level analytes is typically compromised. To overcome such issues, in previous research, different types of modified papers as substrate for PS-MS/MS have been developed to improve sensitivity and selectivity for targeted detection of analytes in different matrixes, including urine, plasma, and olive oil (Luo et al., 2024; Luo, van Beek, Chen, Zuilhof, & Salentijn, 2022; Luo, van Beek, Chen, Zuilhof, & Salentijn, 2023). In these studies modified paper spray MS was applied to the screening for markers of disease or food safety hazards.

Considering the application of leaf spray, it was hypothesized that a similar approach could be used for the analysis of psycho-neurological disorders (PND) mushroom toxins, as a convenient, fast, cheap and environmentally friendly screening tool. Moreover, as became clear from previous reports, some toxins, such as ibotenic acid are challenging to detect directly. Considering the zwitterionic properties of ibotenic acid, we hypothesized that a selective extraction using modified paper could be a feasible route for enhancing its detectability. Anion-exchange modified paper spray (AEPS) tips were thus developed and evaluated for ibotenic acid enrichment to thus enhance the selectivity of detection of this target compound over other matrix constituents.

2. Experimental

2.1. Experiment design

The aim of this work was to first develop a fast-screening method for three PND toxins (muscarine, psilocin and ibotenic acid) in mushrooms, and a rapid and easy method for ibotenic acid quantification in mushrooms. The experimental design, which consisted of four phases, is presented in Fig. 1. (1) Direct spray from mushroom tissue (mushroom spray) was first developed for fast screening of muscarine and psilocin. (2) To improve method robustness, a combination of mushroom tissue and a paper spray tip was also explored as mushroom-paper spray. (3) After that, SPE cartridges were used before the PS-MS/MS method to selectively enrich ibotenic acid. (4) Based on the best-performing SPE stationary phase and protocol, AEPS tips were prepared to replace the SPE cartridge, and AEPS-MS/MS was established for ibotenic acid analysis. SPE-PS-MS/MS and AEPS-MS/MS methods were then validated against HPLC-MS/MS as benchmark.

Fig. 2. Fresh basidiomata of toxic mushrooms. A: *Inocybe squarrosolutea* (MHHNU31427, containing muscarine); B: *Psilocybe cubensis* (MHHNU31391, containing psilocin); *C. Amanita griseopantherina* (MHHNU 34547, containing ibotenic acid); D. *Amanita griseopantherina* (MHHNU 34548, containing ibotenic acid). Bar = 2 cm.

2.2. Chemicals and materials

Ibotenic acid was purchased from Sigma Aldrich (Shanghai, China). Sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), epichlorohydrin (EPI), and trimethylamine (43 %–49 % in water) were purchased from Sigma Aldrich Trading Co., Ltd. (USA). 1-Butylimidazole, methanol (MeOH, HPLC-grade) and acetonitrile (ACN, HPLC-grade) were purchased from Innochem Scientific Co., Ltd. (Beijing, China). Formic acid (wt%, ≥ 88.0 %), ammonium hydroxide (wt%, 25.0–28.0 %), ethanol (EtOH), and acetone were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chromatography paper was purchased from J&K Scientific Co., Ltd. (Beijing, China). Filters (13 mm * 0.22 μm, Nylon6) were purchased from BKMAM Biotechnology Co., Ltd. (Changde, China). Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). Solid phase extraction columns with different types of stationary phase (details are described in the Supporting Information, SI, Fig. S1, mixed cation-exchange column (MCX), hydrophilic-lipophilic balance column (HLB), mixed anion-exchange columns (MAX), weak anion-exchange columns (WAX), strong anion-exchange columns (SAX), mix of C_8 and strong anion-exchange columns (C_8/SAX)) were purchased from Hangzhou Micron Pai Technology Co., Ltd. (Hangzhou, China). Four specimens of toxic mushroom (Fig. 2, details in SI, Table S1) were collected from China; the fresh basidiomata were dehydrated using an electric dryer at 45 °C for 8–10 h; specimens were stored in the Mycological Herbarium of Hunan Normal University (MHHNU), Changsha, China. Dried edible mushrooms including *Boletus edulis*, *Coprinus comatus*, *Pholiota nameko*, *Morchella esculenta* and *Lyophyllum decastes* were bought from local supermarkets, Changsha, China.

2.3. Solutions

A 1:1 (*v*/v) mixture of MeOH and water was made, hereafter referred to as MeOH/H_2O. 0.5 % ammonium hydroxide was added to MeOH/H_2O and this is hereafter referred to as alkaline MeOH/H_2O. 0.5 % ammonium hydroxide was added to pure MeOH and this is hereafter referred to as alkaline MeOH. 10 % formic acid was added to MeOH and is hereafter referred to as acidic MeOH. Internal standard (IS) was dissolved in acidic MeOH (0.50 μg mL^{-1}) and used as spray solvent.

2.4. Preparation of blank paper spray tips

The preparation of blank paper spray tips has been described in previous papers (Luo et al., 2022; Luo et al., 2023; Luo et al., 2024). In short, a CUTOK DC craft cutting plotter (Hefei CNC Equipment Co. Hefei, China) was used to cut the paper triangles (base width = 9 mm, height = 13.2 mm). All cut tips were washed with methanol twice, dried overnight in a fume hood, and stored in a clean plastic bottle for the next steps.

2.5. Preparation of anion-exchange modified paper spray (AEPS) tips

AEPS tips were prepared using trimethylamine (detailed protocol in SI, Fig. S2) in a similar fashion as reported in a previous paper (Luo et al., 2023). Briefly, blank paper tips were added to a beaker with EPI and DMSO under alkaline conditions for 4 h at room temperature. After washing and drying, treated papers were added to trimethylamine and water (1,1, *v*/v) for reaction during 10 h at room temperature. Afterwards, papers were washed until the washing solution remained colorless. Then, the obtained AEPS tips were placed on a clean glass plate, dried overnight in a fume hood and stored in a clean plastic bottle until use.

2.6. Instrumental setups

2.6.1. Paper spray-tandem mass spectrometry (PS-MS/MS)

PS-MS/MS experiments were carried out on a Shimadzu LCMS-8040 triple quadrupole MS, using a custom-made paper spray ionization source (SI, Fig. S3) as described previously (Luo et al., 2022; Luo et al., 2023; Luo et al., 2024). For blank paper spray, the sample extract was pipetted onto the paper tip and voltage (4.0 kV) was applied after adding spray solvent. For AEPS tips (extraction mode), the tips were immersed in the sample solution to allow extraction; after a 5 min extraction and subsequent washing/drying, spray solvent was added and voltage

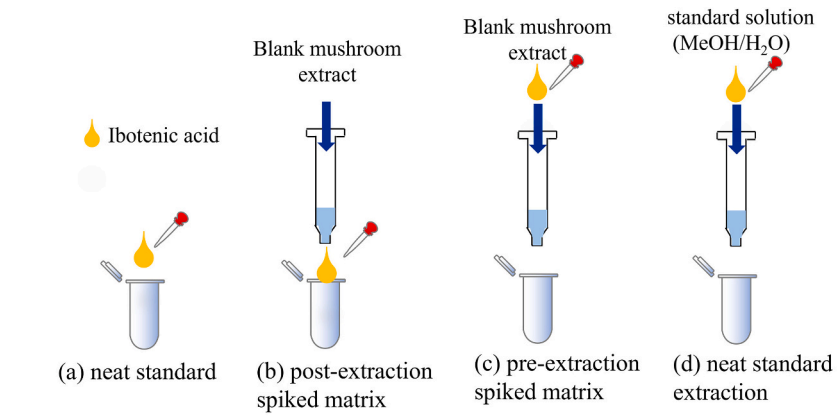

Fig. 3. Representation of samples required for matrix effect evaluation and calculation. Ibotenic acid spiked in MeOH is referred to neat standard; blank mushroom extract is pretreated by SPE cartridge, then ibotenic acid spiked in the eluate is referred to post-extraction spiked matrix; ibotenic acid spiked in blank mushroom extract and pretreated by SPE cartridge is referred to pre-extraction spiked matrix; ibotenic acid spiked in MeOH/H_2O and pretreated by SPE cartridge is referred to neat standard extraction.

applied. Full scan mass spectra were acquired (*m/z* 50–300) under the following conditions: 250 °C desolvation temperature; 300 °C heating block temperature; positive mode. Multiple reaction monitoring (MRM) was used for the analysis of the three kinds of PND toxins and IS (see SI, Table S2 and Fig. S4 for ibotenic acid, muscarine, psilocin and 1-butylimidazole MRM settings and fragmentation).

2.6.2. *Mushroom spray-tandem mass spectrometry*

Mushroom spray mass spectrometry analyses were performed with a custom-made paper spray setup; MS settings were the same as for PS-MS/MS. Two modes were used for mushroom spray (SI, Fig. S3). (i) A dried mushroom was cut into triangular pieces of ca. 9 × 13 mm, and clamped with an alligator clip. After that, 20 μL of spray solvent was deposited on the center of the mushroom, and spray voltage was applied to generate spray from the tip of mushroom. (ii) The second operational mode was mushroom-paper spray mass spectrometry, in which the triangular mushroom piece was positioned on top of a paper tip and clamped together with the alligator clip. Then, spray solvent was deposited on the center of the mushroom and solvent flowed through the mushroom tissue onto the paper. Then the spray voltage was applied and a spray was generated from the paper tip.

2.6.3. *HPLC-MS/MS*

HPLC-MS/MS analyses were performed using an LCMS 8050 triple quadrupole mass spectrometer (Shimadzu Corp., Japan). The MS/MS conditions were same as PS-MS/MS mentioned before, except for: nebulizing gas 3.0 L min^{-1}, drying gas and heating gas both 10.0 L min^{-1}, and interface temperature 250 °C. An amide column (5 μm, 250 × 4.6 mm, GL Sciences Inc., Japan) was employed for the determination of ibotenic acid (Su, Liu, et al., 2023). The mobile phase consisted of (A) 0.5 % formic acid in water and (B) acetonitrile. The gradient elution conditions (with a constant flow of 0.5 mL min^{-1} with high pressure gradient) were as follows: linear decrease from 80 % to 50 % B in 5 min; 50 % B until 10 min, then linear increase of B to 80 % in 1 min; 80 % B until 15 min.

2.7. *Extraction procedure and efficiency*

A 10 mg portion of mushroom was added to 1000 μL of MeOH/H_2O and homogenized by vortexing at 1500 rpm for 2 min at room temperature. The mixture was treated in an ultrasonic bath for 15 min, then centrifuged at 10,000 rpm for 5 min, and all supernatant was collected. The supernatant from an edible mushrooms (*Boletus edulis)* was used as blank mushroom extract for evaluation of matrix effects and pretreatment optimization.

Assessment of the extraction efficiency: A 10 mg portion of toxic mushroom (*Amanita griseopantherina*) containing ibotenic acid was added to 1000 μL of MeOH/H_2O, then homogenized, ultrasonicated, and centrifuged as above, all supernatant was collected. After that, 1000 μL of fresh MeOH/H_2O was added and extraction was repeated. The extraction was repeated up to 5 times. Supernatant of each extraction was collected separately and analyzed by HPLC-MS/MS.

2.8. *Optimization of solid-phase extraction (SPE) procedure*

To assess the matrix effects, 20 μg mL^{-1} ibotenic acid in MeOH/H_2O and toxic mushroom extract (*Amanita griseopantherina*) containing ibotenic acid were analyzed separately by HPLC-MS/MS. For comparison, the above two solutions were analyzed by MS/MS without HPLC column. Next, 100 μg mL^{-1} ibotenic acid dissolved in MeOH/H_2O and toxic mushroom extract (*Amanita griseopantherina*) were separately analyzed by a traditional PS-MS/MS method (using unmodified paper). Besides, 100 μg mL^{-1} ibotenic acid dissolved in five different edible mushroom extracts (*Boletus edulis*, *Coprinus comatus*, *Pholiota nameko*, *Morchella esculenta*, *Lyophyllum decastes*) were separately analyzed by such traditional PS-MS/MS method.

Evaluation of extraction procedure In order to evaluate the influence of the matrix on the MS analysis of ibotenic acid and assess the use of SPE procedures to reduce this, several experiments were conducted with four solutions: (i) 50 μg mL^{-1} ibotenic acid spiked in standard solution was prepared as *neat standard*; (ii) 50 μg mL^{-1} ibotenic acid spiked in post-extraction matrix (matrix after cleanup) was prepared as *post-extraction spiked matrix*; (iii) 50 μg mL^{-1} ibotenic acid spiked in pre-extraction matrix (matrix before cleanup) was pretreated with SPE steps and prepared as *pre-extraction spiked matrix*; (iv) 50 μg mL^{-1} ibotenic acid standard solution was pretreated with SPE steps as *neat standard post-extraction*. These four solutions were used for all SPE procedure optimization experiments, unless otherwise specified. Matrix effects (change in signal due to matrix presence), SPE recovery for standard solution (percentage of analyte recovered after SPE with standard solutions), SPE recovery for matrix extract (percentage of analyte recovered after SPE with matrix extract), and the process efficiency (analyte recovered after

SPE of matrix extract compared to neat standard solution) can be calculated after the analysis of the above four solutions (Marchi et al., 2010) (Fig. 3).

SPE cartridge MAX, MCX and HLB cartridges were used separately to compare different kinds of SPE cartridges (SI, Fig. S1). There are four steps for SPE pretreatment, namely preconditioning, loading, washing and eluting; detailed information about each step for different cartridges can be found in SI, Table S3. Neat standard and post-extraction spiked matrix with different kinds of SPE cartridge were analyzed by PS-MS/MS; the average signal of ibotenic acid was used for assessing matrix effects.

MAX, WAX, SAX, and C_8/SAX cartridge were applied separately for the comparison of different kinds of anion-exchange cartridges; detailed procedures of SPE pretreatments can be found in the SI, Table S3. Neat standard, post-extraction spiked matrix, pre-extraction spiked matrix, neat standard extraction with different kinds of cartridge were analyzed by PS-MS/MS; average signal of ibotenic acid was used for matrix effect and SPE recovery assessment.

SPE loading The pH of the sample solution was optimized for the MAX cartridge. Sample solutions (1000 μL) with different ammonium hydroxide concentrations (0 %, 0.5 %, 2 %, 5 % and 10 %) were loaded on MAX cartridges. The preconditioning solutions were changed to match each ammonium hydroxide concentration. Neat standard and pre-extraction spiked matrix were analyzed by PS-MS/MS; average signal of ibotenic acid was used for process efficiency evaluation.

SPE elution solution The elution step was optimized by assessing the effects of pH on the purified extract for the MAX cartridge. After washing with ACN/H_2O (9,1, *v*/v), 6.0 mL 0 %, 0.5 %, 2 %, 5 % and 10 % formic acid in MeOH was used for elution. Neat standard and pre-extraction spiked matrix were analyzed by PS-MS/MS; average signal of ibotenic acid was used for process efficiency evaluation. In addition, the elution volume was optimized; elution solution was collected per 1000 μL and analyzed by PS-MS/MS.

2.9. Characterization of anion-exchange modified paper

X-ray photoelectron spectroscopy (XPS) XPS was performed with a K-Alpha instrument (Thermo Fisher Scientific, USA). Blank papers and AEPS papers were cut to 5 × 5 mm. The instrument conditions were as follows: Al Kα ray ($h\nu = 1486.6$ eV, excitation source); 400 μm beam spot; 12 kV voltage; 6 mA heater current.

PS-MS/MS Blank papers and AEPS papers were immersed separately in 5.0 mL of fresh 5.0 μg mL^{-1} ibotenic acid in alkaline MeOH/H_2O. After washing with alkaline MeOH/H_2O, alkaline MeOH, and drying, the papers were analyzed by PS-MS/MS directly, and acidic MeOH was used as spray solvent. Also, AEPS papers were immersed in blank alkaline MeOH/H_2O solution (0 μg mL^{-1} ibotenic acid) as comparison.

2.10. Optimization of AEPS tips

The AEPS-MS/MS method was optimized with respect to the addition of IS, desorption process, duration and pH of extraction and desorption pH. The final protocol used is listed here: AEPS tips were immersed in 1000 μL of neat standard solution, after 5 min of extraction by slight manual stirring of paper in solution, the treated paper was washed with alkaline MeOH/H_2O (2×) and alkaline MeOH (1×), and then the tip was dried. With the double-tip method described in a previous paper (Luo et al., 2023), 40 μL of acidic MeOH without IS was added once without applying voltage, and then 40 μL of acidic MeOH with IS (0.50 μg mL^{-1}) was used as spray solvent and the voltage was applied. This protocol has been used for all experiments, unless otherwise specified.

2.11. Internal standard addition

Quantitative analysis was performed by IS (1-butylimidazole) addition with the developed AEPS-MS/MS method. Acidic MeOH with IS was used as spray solvent during analysis. To confirm the IS does not bind to AEPS paper under acidic conditions, it was dissolved in acidic MeOH as spray solvent at 5.0 μg mL^{-1} and sprayed directly with blank paper and AEPS paper. In addition, eight standard solutions (0, 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10 μg mL^{-1} ibotenic acid in alkaline MeOH/H_2O) were prepared to construct a calibration curve with 1-butylimidazole in acidic MeOH (spray solvent) as IS.

2.12. Desorption process

The desorption process was optimized. AEPS paper tips were first immersed in 5.0 mL of 5.0 μg mL^{-1} ibotenic acid in alkaline MeOH/H_2O for 20 min. After washing and drying, the treated papers were analyzed by PS-MS/MS; 40 μL of acidic MeOH with IS (0.50 μg mL^{-1}) was applied as spray solvent on a single paper 1, 2, 3, 4 or 5 times. Next, AEPS paper tips were first immersed in 5.0 mL of 5.0 μg mL^{-1} ibotenic acid in alkaline MeOH/H_2O for 20 min. After washing and drying, the treated papers were analyzed by PS-MS/MS; acidic MeOH (40 μL) was added to a single paper 1, 2, 3, 4, or 5 times without voltage, and only with the last addition voltage was applied.

2.13. Extraction time

AEPS tips were immersed in 5.0 mL of 5.0 μg mL^{-1} ibotenic acid in alkaline MeOH/H_2O for 1, 3, 5, 10, or 20 min. After washing and drying, PS-MS/MS was performed as described above.

2.14. Extraction pH

AEPS paper tips were immersed in 5.0 mL of 5.0 μg mL^{-1} ibotenic acid solution in MeOH/H_2O with different ammonium hydroxide concentrations (0 %, 0.5 %, 1 %, 2 %, 5 %, 10 %) for 20 min. After extraction, washing with alkaline MeOH/H_2O and alkaline MeOH (same ammonium hydroxide concentration as for the preparation of ibotenic acid solution) and drying, the treated papers were analyzed by the AEPS-MS/MS method as described above.

2.15. Desorption pH

AEPS tips were immersed in 5.0 mL of 5.0 μg mL^{-1} ibotenic acid solution in alkaline MeOH/H_2O with 0.5 % ammonium hydroxide for 20 min. After washing and drying, the treated papers were analyzed by the AEPS-MS/MS method. MeOH with different formic acid concentrations (0 %, 0.5 %, 1 %, 2 %, 5 %, 10 %) and IS (0.50 μg mL^{-1}) was used as spray solvent.

2.16. Analytical performance of AEPS-MS/MS

2.16.1. Method validation

Calibration curves were constructed for ibotenic acid in blank mushroom extract, with different spiked ibotenic acid concentrations (0, 0.50, 1.0, 2.0, 5.0, 10, 20, 40 μg mL^{-1}); every sample was measured in triplicate. The AEPS tips were immersed in the above samples for 5 min, and after washing with alkaline MeOH/H_2O and alkaline MeOH, and drying, they were analyzed by the AEPS-MS/MS method with the double-tip technique. The ratio between ibotenic acid and IS signals was used to construct a calibration curve.

Limit of detection (LOD) and quantification (LOQ) were estimated from the standard deviation of 10 replicates at the lowest spiking concentration (Sa) and the slope of the calibration curve (b): $LOD = 3Sa/b$ and $LOQ = 10Sa/b$.

2.16.2. Method comparison

Experimental details for the AEPS-MS/MS and HPLC-MS/MS method validation are shown in SI, Table S4. In short: three different types of

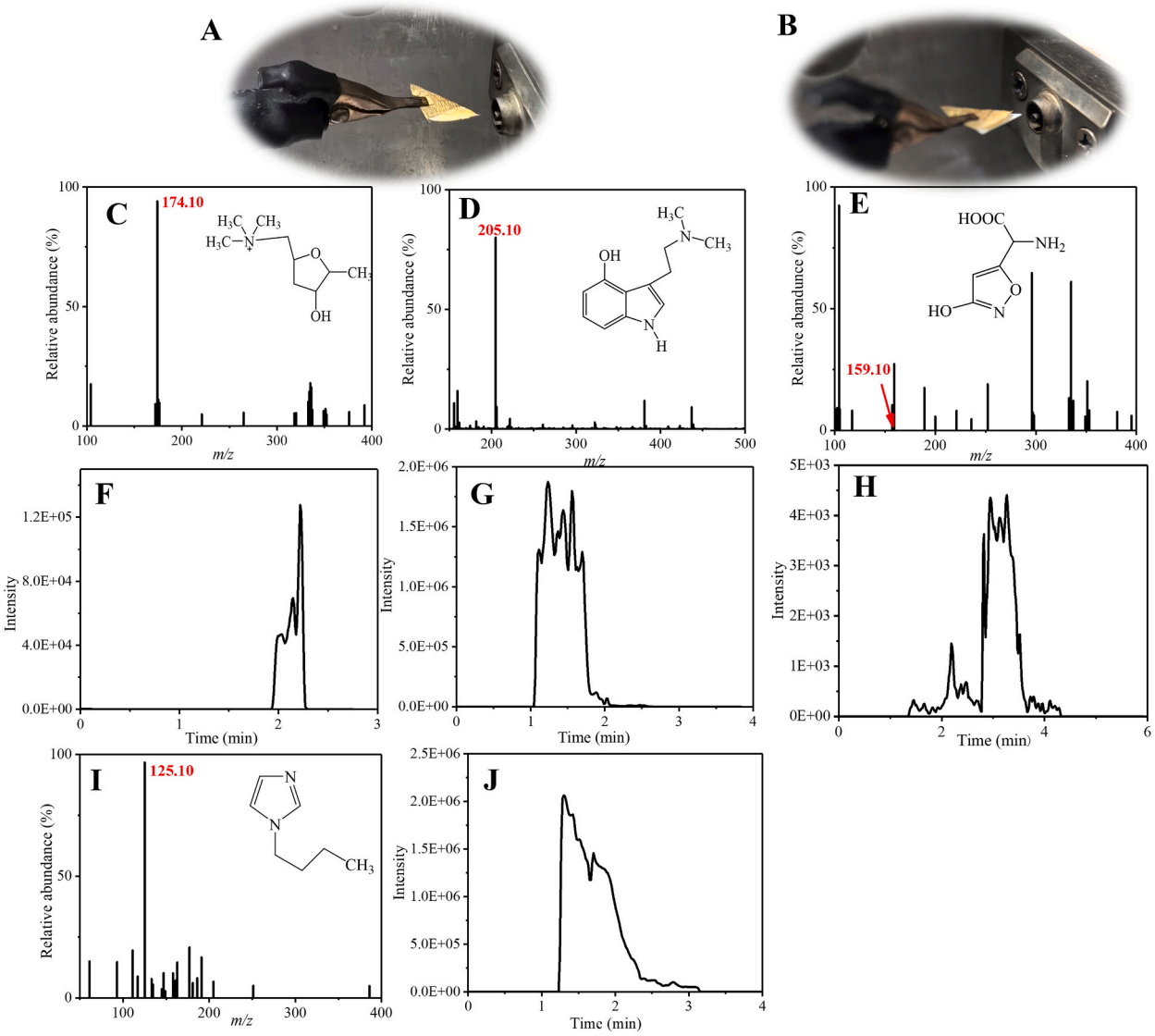

Fig. 4. (A) Mushroom spray and (B) mushroom-paper spray mass spectra of three kinds of toxic mushroom, and paper spray mass spectra of internal standard. Signal of (C, $[M]^+$, *m/z*: 174.10) muscarine and (D, $[M + H]^+$, *m/z*: 205.10) psilocin that can be detected by the direct mushroom spray method; MRM was used to confirm identity (F, *m/z*: 174.10 → 115.10; G, *m/z*: 205.10 → 58.10). Signal of ibotenic acid is limited (E, $[M + H]^+$, *m/z*: 159.10) and extracted ion signal over time in MRM mode (*m/z*: 159.10 → 113.10) can be observed by mushroom-paper spray method. Paper spray mass spectrum of internal standard is shown in I ($[M + H]^+$, *m/z*: 125.10); MRM was used for quantitative analysis (J, *m/z*: 125.10 → 69.10).

edible mushrooms (*Boletus edulis*, *Coprinus comatus*, *Pholiota nameko*) were used for blank mushroom extract comparison; different concentrations in these three blank mushroom extracts were spiked and compared after analysis; selected samples were analyzed on three different days. Each sample condition was replicated six times.

Repeatability and intermediate precision were calculated from three samples with the same spiked concentrations in the same blank mushroom extract, measured on three different days. One-way ANOVA (SPSS Statistics software) was used for statistical evaluation. Repeatability represents the within-group variance; intermediate precision is calculated by combining the within- and between-group variance components (Magnusson & Ornemark, 2014).

Robustness was evaluated with three samples with the same spiked concentration in different blank extracts, measured on three different days, and statistically evaluated by one-way ANOVA (SPSS Statistics software).

Accuracy was calculated as the relative deviation (%) of the calculated mean value from the actual concentration. Precision was expressed as the relative standard deviation (RSD %).

The SPE-PS-MS/MS method was used as a comparison. Mushroom samples were pretreated by a MAX cartridge and analyzed by the PS-MS/MS method.

HPLC-MS/MS was used as benchmark. Mushrooms were pretreated according to extraction procedure (2.6) and filtered (13 mm * 0.22 μm, Nylon 6 filter). 1000 μL filtered supernatant was taken for ibotenic acid analysis.

2.17. Mushroom analysis by AEPS-MS/MS

Two toxic mushrooms suspected of containing ibotenic acid were analyzed by the developed AEPS-MS/MS method. HPLC-MS/MS was used as benchmark. Standard addition calibration curves by AEPS-MS/MS and HPLC-MS/MS method were also established (0, 0.20, 0.50, 1.0, 2.0, 5.0, 10, 20 μg mL^{-1}) for two toxic mushrooms as comparison.

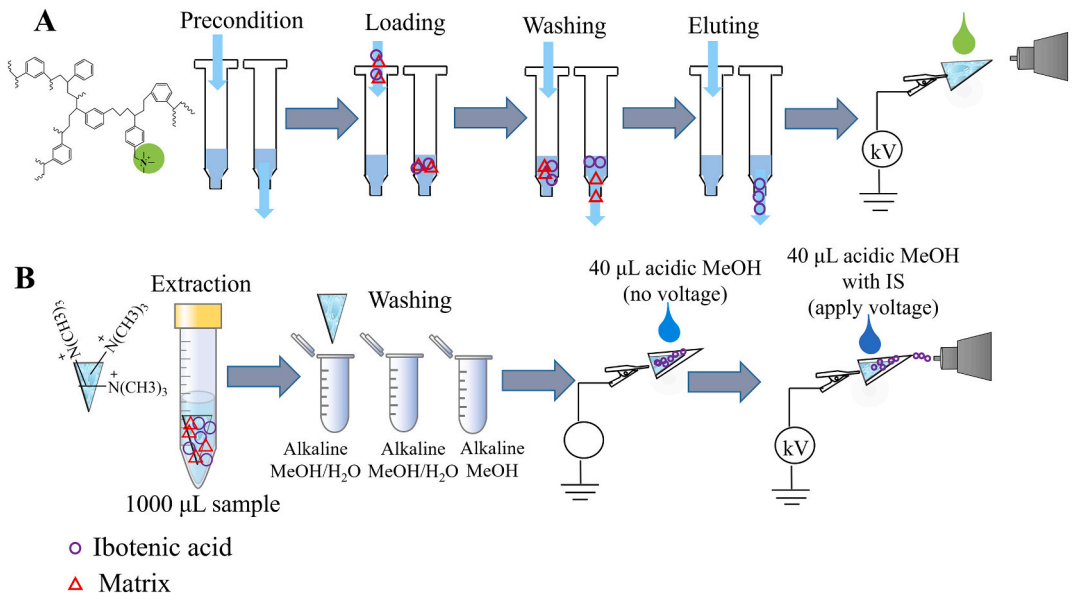

Fig. 5. Schematic diagram of the (A) SPE-PS-MS/MS and (B) AEPS-MS/MS procedures. For SPE-PS-MS/MS method, MAX cartridge is preconditioned by 2.0 mL MeOH and 2.0 mL 0.5 % ammonium hydroxide in water, after which 1000 μL mushroom extract is loaded. After that, 2.0 mL ACN/H_2O (9,1, *v*/v) is used as washing solution, and 3.0 mL 5 % formic acid in MeOH is used for elution and analyzed with IS by PS-MS/MS. For AEPS-MS/MS method, AEPS tips were immersed in 1000 μL of extract, and after 5 min of extraction, the treated paper was washed with alkaline MeOH/H_2O (2×) and alkaline MeOH (1×), and then the tip was dried. With the double-tip method, 40 μL of acidic MeOH without IS was added once without applying voltage, and then 40 μL of acidic MeOH with IS was used as spray solvent and the voltage was applied.

3. Results and discussion

3.1. Distinction of psycho-neurological disorder (PND) toxic mushroom by mushroom spray MS/MS

Muscarine, ibotenic acid and psilocin are three representative PND toxins. Even though these toxins result in similar symptoms when ingested, they actually originate from different species. For example, muscarine can come from *Inocybe* or *Clitocybe,* psilocin from *Psilocybe,* and ibotenic acid from *Amanita species.* To evaluate whether a direct mushroom spray mass spectrometry method is feasible for distinguishing PND mushroom toxins, four toxic mushrooms (Fig. 2) from different species were investigated. As evident from Fig. 4, muscarine (*m/z:* 174.10) and psilocin (*m/z:* 205.10) are easily detected by the direct mushroom spray method, since the targeted compounds are easily or naturally charged and present in high concentration. Detected ion signals for these toxins are corresponding to reported literature by direct electrospray probe MS method or HPLC-MS/MS (Goff et al., 2024; Su et al., 2024). However, ibotenic acid cannot be detected by such direct mushroom spray, as no discernible peak at *m/z* 159.10 corresponding to ibotenic acid was observed in the spectra. Considering that the tip of a cut mushroom triangle is dried, fragile and not very sharp, it results in unstable spray current and poor spray quality. Thus, we introduced the mushroom-paper spray mode (placing the tissue on a paper tip) to improve spray stability and sensitivity for compounds like ibotenic acid. As a result, the signal of the target compound could be observed in MRM mode, but unfortunately remained weak (Fig. 4), which we hypothesize is due to persistent matrix effects.

3.2. Optimization of solid-phase extraction

A preliminary experiment to evaluate matrix effects of mushroom extract on ibotenic acid was conducted. Mushroom extraction efficiency was assessed. One 10 mg toxic mushroom sample was extracted 5 times by the extraction procedure. When the extraction procedure was applied a second time, only 4 %–11 % of ibotenic acid was extracted compared to the first time. The amount of the first extraction was 88 %–94 % compared to the total amount obtained after five subsequent extractions; thus nearly all ibotenic acid is recovered in the first extraction (SI, Fig. S5). Thus, a single extraction step was applied in all further experiments. Ibotenic acid in MeOH/H_2O and toxic mushroom extract containing ibotenic acid were analyzed by MS/MS with or without prior HPLC separation. Signal of targeted compound was suppressed and no detectable ibotenic acid signal was observed in the direct MS analysis of the extract, whereas it was readily detected with HPLC-MS/MS (SI, Fig. S6). Next, 100 μg mL^{-1} ibotenic acid was dissolved in toxic mushroom extract and the signal was also suppressed (SI, Fig. S7). In addition, ibotenic acid was separately dissolved in five different edible mushroom extracts, and analyzed by PS-MS/MS. The content of these – as far as available – is compared in SI, Table S5. In all cases, the signal was seriously suppressed and no signal was obtained (SI, Fig. S7). Therefore, in order to improve the performance, sample preparation must be applied for ibotenic acid analysis, and direct mushroom spray is not feasible for ibotenic acid.

SPE is a widely used sample-preparation technique for isolation, concentration, clean-up and medium exchange. Six kinds of SPE cartridges were evaluated in this study. As shown in the SI, Fig. S8, compared to polarity-based (HLB) and cation exchange (MCX) SPE, the matrix effect on ibotenic acid in solution pretreated by anion exchange (MAX) SPE was substantially reduced, as signals of 85 %–123 % were obtained compared to the corresponding concentrations in standard solution. Then, different types of anion-exchange cartridges (MAX, WAX, SAX, SAX/C_8) were compared (SI, Fig. S9), and matrix effect, SPE recovery for standard solution, and SPE recovery for matrix extract were calculated. Ibotenic acid has an amino acid structure with carboxyl acid and amino group, and there is an additional amino group in the 5-membered ring, resulting in three pKa values for ibotenic acid (2, 5.1, 8, obtained from PubChem (National Center for Biotechnology Information, 2025). Ibotenic acid can thus have a negative or positive charge depending on the pH (SI, Fig. S10). The quaternary ammonium group on the anion-exchange cartridge can capture the ibotenic acid under alkaline conditions and it can be eluted under sufficiently acidic conditions. WAX with a tertiary amine shows worse performance, as did the SAX/C_8 with an additional hydrophobic carbon chain. SAX has better

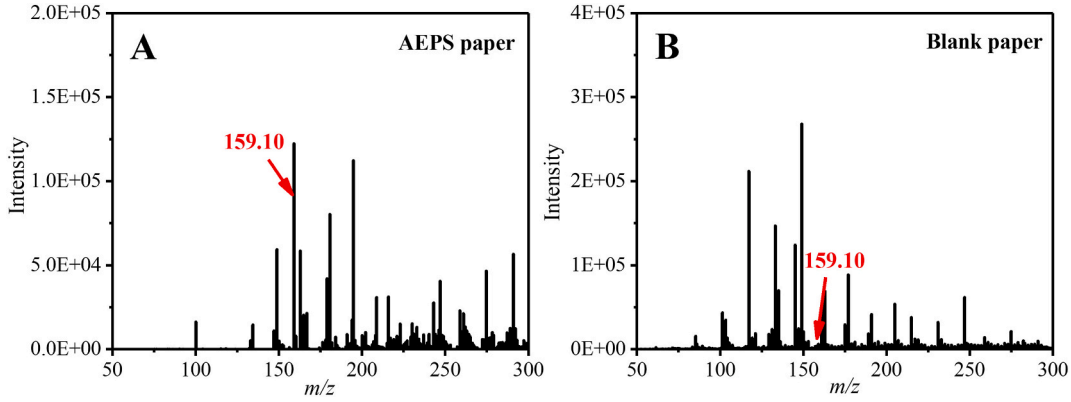

Fig. 6. Comparison between AEPS paper and blank paper. Mass spectrum of ibotenic acid ($[M + H]^+$, *m/z*: 159.10) released by acidic MeOH as spray solvent from (A) AEPS paper and (B) blank paper, after immersing in 5.0 μg mL^{-1} ibotenic acid in alkaline MeOH/H_2O, washing and drying. MRM was used to confirm identity.

performance on SPE recovery from standard solution but worse from matrix extract. Thus, the MAX cartridge was chosen.

Next, the influence of pH during loading and eluting was assessed. Different concentrations of ammonia in the aqueous loading solution do not have an obvious influence on total process efficiency (SI, Fig. S11) since ibotenic acid is always fully negatively charged when pH is above 10. At the same time, higher concentrations of formic acid up to 5 % during elution can significantly improve the process efficiency (SI, Fig. S12) since the carboxylic acid group has a quite acidic pKa of 2.

Finally, the elution volume was studied (SI, Fig. S13). A small elution volume is not enough for ibotenic acid elution, while an excess volume results in a longer procedure and a decreased final concentration. Based on the optimized steps for SPE, the final SPE-PS-MS/MS protocol is shown in Fig. 5A. MAX cartridge is preconditioned by 2.0 mL MeOH and 2.0 mL 0.5 % ammonia in water; then, 1000 μL mushroom sample is loaded. After that, 2.0 mL ACN/H_2O (9,1, *v*/v) is used as washing solution, and finally 3.0 mL 5 % formic acid in MeOH is used for elution, collected, and 1000 μL is taken and analyzed with 1-butylimidazole as IS by PS-MS/MS.

3.3. Optimization of anion-exchange modified paper

Pretreatment with SPE can effectively decrease the matrix effect caused by mushroom extract and increase the sensitivity of ibotenic acid analysis. However, integration of SPE into the workflow also increases the complexity of the analytical process. As mentioned before, the functional group in SPE is a quaternary ammonium, which can capture and release ibotenic acid under different pH conditions. Thus, it was hypothesized that paper tips themselves might replace the SPE column (Fig. 5B), which is both cheaper and more convenient, and also tips do not require preconditioning. Paper modification to obtain AEPS paper was thus conducted (Experimental 2.5 for details).

Based on our previous research (SI, Fig. S2) (Luo et al., 2023), paper spray tips were functionalized, resulting in a quaternary ammonium group. This allows their use for the extraction of ibotenic acid from complex mixtures, which is similar to the anion-exchange cartridge. Based on the modification procedures, nitrogen should be present on the surface of the paper fibers, because of the reagent trimethylamine. The presence of nitrogen on the paper was confirmed by XPS (SI, Fig. S14).

In order to verify that AEPS tips work for ibotenic acid under different conditions, blank papers and AEPS papers were immersed in ibotenic acid solution under alkaline conditions. After washing and drying, the AEPS tip was eluted with acidic MeOH, producing a clear ibotenic acid signal (Fig. 6A). In contrast, a blank paper tip treated identically yielded no detectable signal (Fig. 6B). Also, AEPS papers were immersed in fresh 0 μg mL^{-1} ibotenic acid solution as a control to check for carryover or contamination, which yielded no ibotenic acid signal. In addition, AEPS papers immersed in 5.0 μg mL^{-1} ibotenic acid solution and desorbed with pure MeOH (without acidification), also did not show signal, as the ibotenic acid is not released with pure MeOH (SI, Fig. S15).

Then, several steps in the analytical procedure for the ibotenic acid extraction and desorption were optimized. Below, the optimization procedures, including IS addition, desorption process, extraction time and pH, and desorption pH are described.

As IS, 1-butylimidazole (structure and mass spectrum are shown in Fig. 4) was chosen for the quantitative analysis of ibotenic acid, as it does not have interaction with the AEPS paper, and exhibits a good response in mass spectrometry. IS in acidic MeOH is used as spray solvent during analysis, and thus a solution of IS in acidic MeOH was separately analyzed on blank paper and AEPS paper by PS-MS. The results show that for both papers, there is a clear signal for the IS (SI, Fig. S16). Also, there is a good linearity over the range of 0.10–10 μg mL^{-1} ibotenic acid in MeOH (SI, Fig. S17) after IS addition to the spray solvent, with a correlation coefficient (*r*) of 0.992.

AEPS modification is based on previous work (Luo et al., 2023), and there are several steps involved in the paper modification process, during which modified papers need to be immersed in different solutions, at different pH values. The resulting papers are more frail than the original paper, and thus a double-tip spray approach is chosen for AEPS method, in which the modified paper is placed on top of a regular piece of paper for stability and spray generation. First, 40 μL of acidic MeOH with IS as spray solvent was added and voltage was applied. Then, a fresh aliquot was added to the same paper, and this was repeated up to 5 times (SI, Fig. S18A). This experiment indicates that ibotenic acid was released each time when fresh spray solvent was added. In addition, one paper with different desorption steps was tested: 40 μL of acidic MeOH (without IS) was added onto the paper and dried (without application of voltage, i.e. no spray was generated) for several times; then 40 μL of acidic MeOH with IS was applied and spray was generated (SI, Fig. S18B). The results show that multiple additions of solvent can increase the amount of ibotenic acid released and sprayed. This is likely due to the fact that more time is available to keep the paper wet for the release of ibotenic acid and for ibotenic acid to be transported to the tip. Finally, two desorption steps (no spray was generated during the first addition) were employed for all ibotenic acid analyses.

Next, extraction time was optimized. Ibotenic acid in alkaline MeOH/H_2O was extracted with AEPS paper tips. The extraction of ibotenic acid did not improve beyond an extraction time of 5 min (SI, Fig. S19), which was thus selected for the final protocol.

Then, the influence of the pH during extraction and desorption was assessed. Ibotenic acid can only be combined with quaternary ammonium under certain conditions because of its different charge states (SI, Fig. S10), and thus different concentrations of ammonium hydroxide in

Table 1
Matrix-matched calibration curve of ibotenic acid analysis.

	Linear equation	Correlation coefficients (r)	Linear range (μg mL^{-1})	LOD/LOQ in extract (μg mL^{-1})	LOD/LOQ in dried mushroom* (g kg^{-1})
AEPS-MS/MS for the determination of IBA in dried edible mushroom extract	$y = 0.030x + 0.020$	0.994	5–40	1.3/4.3	0.13/0.43
SPE-PS-MS/MS for the determination of IBA in dried edible mushroom extract	$y = 0.015x + 0.036$	0.993	10–40	2.8/9.4	0.28/0.94
HPLC-MS/MS for the determination of IBA in dried edible mushroom extract	$y = 2.7E^6x + 1.1E^4$	0.999	0.5–10	0.009/0.03	0.0009/0.003

* This is estimated based on extraction volume (1000 μL), weight (10 mg) of dried mushroom sample, and an assumed extraction recovery of 100 %.

Table 2
Precision and accuracy of different mushroom extraction samples by AEPS-MS/MS and HPLC-MS/MS methods.

Analyzing day	Edible mushroom	Analyte	Spiked Concentration (μg mL^{-1})	AEPS-MS/MS		HPLC-MS/MS	
				Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)	Accuracy (%)
First day	Edible mushroom 1	IBA	6	14.5	+5.1	1.1	−5.9
Second day	Edible mushroom 2	IBA	6	14.6	−20.9	0.8	+5.5
Third day	Edible mushroom 3	IBA	6	18.6	−24.3	0.5	−1.6
First day	Edible mushroom 1	IBA	25	18.0	−10.2	1.2	+5.4
First day	Edible mushroom 1	IBA	35	12.2	−13.4	0.7	+8.1
Second day	Edible mushroom 1	IBA	35	10.8	−14.0	0.9	+6.8
Third day	Edible mushroom 1	IBA	35	12.9	−14.0	1.2	+7.8
Repeatability (RSD, 100 %)		IBA	/	3.4		0.9	
Intermediate precision (RSD, 100 %)		IBA	/	5.7		1.1	
p value for different edible mushroom matrix		IBA	/	0.009		0.057	

MeOH/H_2O were tested. No substantial differences were observed since there is no effect on ionization when the pH is above 10 (SI, Fig. S20). This trend is similar to that observed with the MAX cartridge. In addition, extraction under acidic conditions was applied as control, which – as expected – did not lead to extraction and did not show any signal for ibotenic acid. 0.5 % ammonium hydroxide is therefore used in the final protocol. In addition, experiments with different concentrations of formic acid in MeOH as spray (desorption) solvent showed that higher amounts of acid can increase the signal in line with earlier results with the SPE cartridges (SI, Fig. S21), and thus 10 % formic acid was chosen for the final procedure. The final protocol is shown in Fig. 5B.

3.4. Analytical performance of AEPS-MS/MS

3.4.1. Method validation

As described above, there is good linearity for the calibration curves of an ibotenic acid standard solution in MeOH/H_2O by AEPS-MS/MS. First, matrix-matched calibration curves were constructed for ibotenic acid in blank mushroom extract ($r > 0.99$). Also, calibration curves using the SPE-PS-MS/MS and HPLC-MS/MS methods were obtained for comparison (see SI, Fig. S22 for an example chromatogram). The results of the AEPS-MS/MS method show that the presence of matrix somewhat worsened the LOD (to 1.3 μg mL^{-1} in extract, from 0.06 μg mL^{-1} in pure solvent), but this LOD is still quite low (see Table 1). The reported concentration of ibotenic acid in 24 species of *Amanita* ranges from 0.6 to 32 g kg^{-1} (Su, Liu, et al., 2023), so the concentration of ibotenic acid could be 6 to 320 μg mL^{-1} according to the extraction procedure, assuming 100 % extraction recovery (§2.7). Therefore, the developed AEPS-MS/MS method is appropriate for fast screening of toxic mushrooms. Also, precision, accuracy, repeatability, intermediate precision and robustness were evaluated by the analysis of five samples with different spiked concentrations, on different days, and in three different edible mushroom matrixes (Table 2). The accuracy ranged from −14.0 % to +5.1 % for three different levels of spiked concentration in the same matrix; the repeatability (3.4 %) and intermediate precision (5.7 %) are <10 % for the same sample type across three different days according to a within-group and between-group comparison. To assess robustness, three different sample types were measured across 3 different days with the same spiked concentrations, and a significant difference was found ($p < 0.05$). This may be attributed to the fact that the matrix-matched calibration curve was developed with edible mushroom 1 – and thus does not fully correct for matrix effects from different mushroom types. Still, while the accuracy for edible mushroom 2 and edible mushroom 3 are −20.9 % and − 24.3 %, and the precision <20 % (14.6 % and 18.6 %), these would give an indication of the amount of mushroom toxins present. While spiking of edible mushroom extracts is the appropriate approach to obtain validation samples in absence of reference materials, it evidently suffers from drawbacks as well. Therefore, a standard addition approach was applied as well for two toxic mushrooms (Section 3.5).

3.4.2. Method comparison

SPE-PS-MS/MS and HPLC-MS/MS methods were used as comparison. The results (Table 1, SI, Figs. S23 & S24, Tables S6 & S7) show that

Table 3
Comparison of AEPS-MS/MS, SPE-PS-MS/MS and HPLC-MS/MS method for different parameters.

	AEPS-MS/MS	SPE-PS-MS/MS	HPLC-MS/MS
Pretreatment time	~ 10 min	~ 10 min	~ 2 min
Steps for pretreatment	2	4	1
Equipment or materials for analysis	plastic tube, paper	SPE cartridge, paper	vial, filter, column
Equipment analytical time	~ 2 min	~ 30 s	15 min
Time for one sample (including pretreatment)	~ 15 min	~ 10 min	~ 20 min
Time for fifty samples (including pretreatment)	~ 4 h	~ 8.5 h	~ 17 h

Pretreatment in table is for sample solutions. Before pretreatment, mushrooms are extracted by MeOH/H_2O and homogenized. The mixture was treated in an ultrasonic bath and then centrifuged, and all supernatant was collected (details are shown in section 2.7).

AEPS-MS/MS reaches a better LOD than SPE-PS-MS/MS, which indicates that AEPS tips can indeed be used instead of the SPE cartridge. Moreover, our new approach is cheaper, more easily operated and environmentally friendly. As expected, HPLC-MS/MS (SI, Table 1, Fig. S25, Table S8) has superior performance, yet needs a column-based separation and re-equilibration of 15 min after each analysis – as well as has a much higher solvent consumption. A detailed comparison between these three methods with different parameters is shown in Table 3. AEPS-MS/MS does not have obvious advantages with regard to the pretreatment time, but the pretreatment steps are easier compared to SPE-PS-MS/MS, and do not require column-based separation compared to HPLC-MS/MS. Moreover, the analysis time of AEPS-MS/MS for one sample, including pretreatment, is comparable to the other two methods, yet has large advantages when the number of samples increases. SPE pretreatment includes several steps and cannot be easily operated in parallel; in comparison, the pretreatment process of AEPS-MS/MS is easy and multiple samples can be analyzed at the same time. This is beneficial for toxic effect assessment during emergencies. Also, AEPS-MS/MS is less expensive and easier applied in this field since it does not need an HPLC system and could in the future be combined with portable MS/MS to further increase mobility (Szalwinski, Hu, Morato, Cooks, & Salentijn, 2021).

3.5. Mushroom analysis by AEPS-MS/MS method

The validated quantitative method was then applied to determine the concentrations of ibotenic acid in different real toxic mushrooms to evaluate its applicability. The extracted ion signal over time of ibotenic acid in MRM mode for edible mushroom and toxic mushroom is shown in Fig. 7, and the calculated concentration is shown in SI, Table S9. Ibotenic acid can be detected by the newly established method for two mushrooms giving concentrations of 12 ± 2 and 6.3 ± 0.8 g kg^{-1}. These values are somewhat lower than the results obtained by HPLC-MS/MS (15 ± 1 and 7.4 ± 0.1 g kg^{-1}).

Standard addition was then applied for ibotenic acid detection by AEPS-MS/MS (SI, Fig. S26) and HPLC-MS/MS (SI, Fig. S27) as a control to determine to what extent the specific mushroom may have influenced the analysis. The ibotenic acid concentrations in the two mushrooms were 12 and 6.3 g kg^{-1}, which is comparable to the results obtained by HPLC-MS/MS (12 and 6.9 g kg^{-1}); see SI, Table S9. Still, as a larger portion of mushroom sample and ibotenic acid standard are needed when the standard addition method was applied, and substantially more measurements need to be conducted, this method is not suitable when time or the amount of toxic mushroom sample is limited. In future applications, one should choose the quantitation approach based on sample availability: matrix-matched calibration (using a closely related matrix) or standard addition can be employed for different mushroom samples as appropriate to the situation.

4. Conclusions

Four different kinds of MS methods were developed for the purpose of PND toxin mushroom analysis. Firstly, mushroom spray (1) and mushroom-paper spray (2) were applied for qualitative analysis. Then, an SPE-MS/MS method (3) was established for quantitative analysis of ibotenic acid. Finally, a new anion-exchange modified paper spray tandem mass spectrometry (AEPS-MS/MS) method (4) was established to replace an SPE-MS/MS method, with an LOD of 1.3 μg mL^{-1} in mushroom extract. The AEPS-MS/MS method is easy to use, fast, and does not need complicated sample pretreatment. It is also quite convenient and faster compared to HPLC when analyzing larger sample numbers. The method has potential for the application to the analysis and identification of different kinds of toxins for food safety. Also, the methodology might be translatable to the analysis of other toxic compounds with specific functional groups, if appropriate interaction chemistry can be developed.

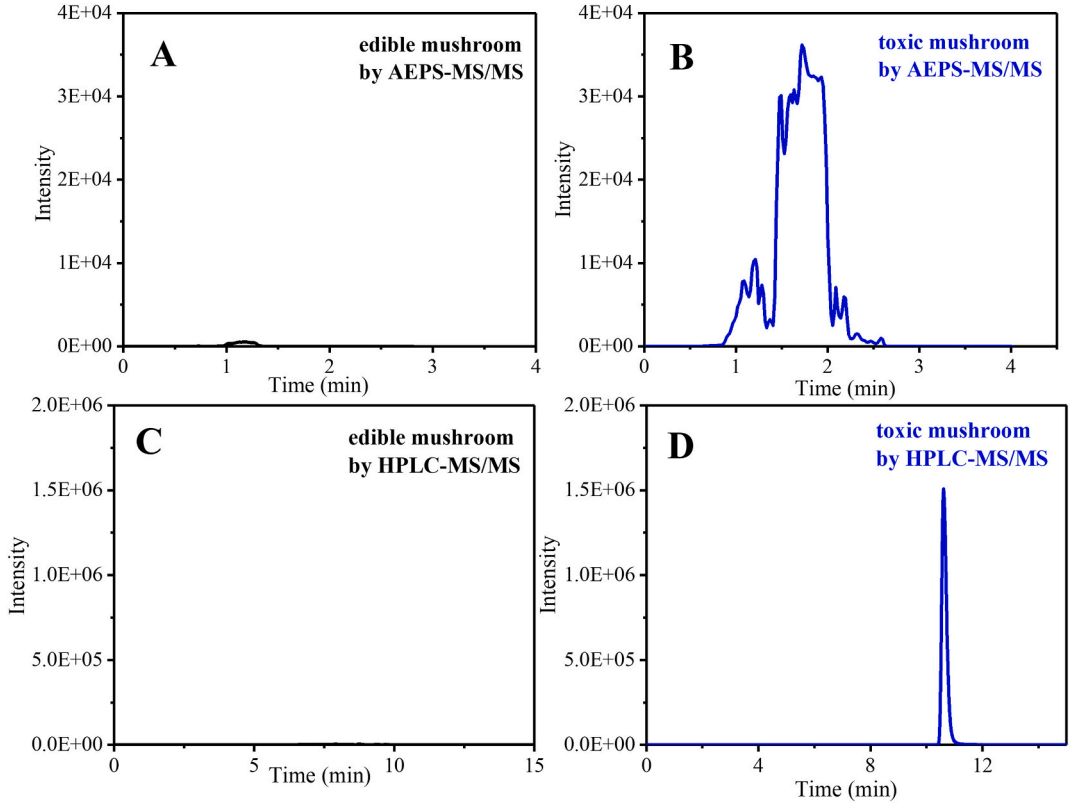

Fig. 7. Extracted ion signal over time of ibotenic acid under (+) mode in MRM mode (*m/z*: 159.10 → 113.10) by (A, B) AEPS-MS/MS and (C, D) HPLC-MS/MS for (A, C) edible and (B, D) toxic mushrooms.

CRediT authorship contribution statement

Wei Luo: Writing – original draft, Methodology, Investigation. **Qiaolian Dai:** Methodology. **Teris A. van Beek:** Writing – review & editing. **Han Zuilhof:** Writing – review & editing, Supervision, Funding acquisition. **Bo Chen:** Writing – review & editing, Supervision, Funding acquisition. **Zuohong Chen:** Writing – review & editing, Supervision. **Gert IJ. Salentijn:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A

Supplementary data includes photo's of instruments, calibration curves, optimization data, chromatograms, and supporting data. Supplementary data to this article can be found online at [https://doi.org/10.1016/j.foodchem.2025.145369].

Data availability

Data will be made available on request.

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