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Research Article

Determination of 8 Endogenous Alkaloid Components in *Boletus* Using Ultrahigh-Performance Liquid Chromatography Combined with Quadrupole-Time of Flight Mass Spectrometry

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A ultrahigh performance liquid chromatography coupled with quadrupole-time of flight mass spectrometry (UPLC-Q-TOF/MS) method was developed for simultaneous determination of 8 endogenous alkaloid compounds in *Boletus*. *Boletus* samples were extracted by 50% (V/V) methanol-water solution, then separated by CORTECS UPLC HILIC column using a binary solvent system by gradient elution. The analytes were determined by Q-TOF/MS in TOF MS and information dependent acquisition (IDA)-MS/MS mode. The results showed that mass accuracy error of the 8 endogenous alkaloids were lower than 5.0×10^{-6}, good linear relationship was got in range of 0.2–500 μg/L, and correlation coefficient was higher than 0.9990. The limit of detection was in the range of 0.002–0.100 mg/kg and the limit of quantification was in the range of 0.004–0.200 mg/kg. Recovery of the method was in range of 80.1%–101.5% with spike levels of 0.004–2.00 mg/kg, relative standard deviations were lower than 10%. The method was simple, specific, and reliable. It could be used for the rapid screening and quantitative analysis of 8 endogenous alkaloids in *Boletus*.

1. Introduction

The *Boletus* is an edible fungus belonging to the *Fungi*, *Basidiomycotina*, *Hymenomycetes*, *Agaricates* [1]. It is mainly distributed in Yunnan Province of China. Due to a wide variety of secondary metabolites from fungus and their biological activities, extensive attention has been paid to the researches of fungus both in China and abroad [2]. *Boletus* is popular because of its delicious taste, appreciated nutrition value [3], and a variety of medicinal effects [4]. The chemical composition of the *Boletus* was mainly terpenes, flavonoid [5], phenols [6], and alkaloids, which possessed comprehensive biological activities, such as antioxidant, antifatigue, antitumor, and anti-inflammatory [7, 8]. Generally, the species for which a particular alkaloid structure is characteristic are closely related and can be comprised within the next higher taxon, genus, or family.

Alkaloids have been reported as a group of basic organic substances of *Boletus*, containing at least one nitrogen atom in a ring structure in the molecule [9]. A number of alkaloids were discovered and reported with biological activities in *Boletus*. Nicotine is an important and most well-known alkaloid, and it is the most important alkaloid in tobacco. In addition, nicotine is also widely present in *Boletus* and other plants. Some countries restricted the nicotine content in some foods. For example, the European Commission stipulated that the maximum limit of nicotine in edible fungus fresh and dry products is 0.036 mg/kg and 1.17 mg/kg, while the maximum limit in *Boletus* dried product is 2.3 mg/kg. Besides nicotine, *Boletus* contains some important and

pharmacologically secondary alkaloids. Choline is an indispensable substance for normal metabolism, which has the functions of promoting brain development and improving memory. *Boletus* is rich in amino acids. Arginine and ornithine produce putrescine under the decarboxylation of bacterial amino acid decarboxylase. Tryptamine is a monoamine alkaloid, based around the indole ring structure. It is regarded as the backbone for tryptamines, a group of compounds that include many pharmacologically active compounds, including serotonin (neurotransmitters), melatonin (hormone), and psilocybin (psychedelic drugs) [10]. Du et al. [11] isolated nicotinamide from the *Boletinus pictus*. Zhang et al. [12] found that anabasine, cotinine, and muscarine exist in *Boletus*. According to references, nicotine, anabasine, choline, tryptamine, putrescine, nicotinamide, muscarine, and cotinine can be detected in *Boletus*. However, due to a lack of research on the detection of alkaloids in *Boletus*, the inconsistency of the species, and the maturity of the tested samples, there are certain differences in the data.

At present, there are few studies on the detection of endogenous alkaloids in *Boletus*. The analytical methods of these alkaloids mainly include gas chromatography [13–15], high-performance liquid chromatography [16–21], and liquid chromatography-tandem mass spectrometry [22–28]. However, gas chromatography requires derivation and determination, and the process is relatively cumbersome; high-performance liquid chromatography has poor anti-interference ability [29]. Liquid chromatography-tandem mass spectrometry has a limited ability to identify isomers, and the ion dwell time is limited due to the scan rate. UPLC-Q-TOF/MS has the characteristics of high resolution, high sensitivity, high accuracy, and wide scanning range [30]. It has been widely used in quantitative analysis of pesticide residues, veterinary drug residues, and other research fields [31, 32]. In this paper, UPLC-Q-TOF/MS was first used for fast and accurate determination of endogenous alkaloids in the *Boletus*. It can obtain accurate and stable test results and provide reliable technical support for enterprises and regulatory authorities. This study lays the foundation for the development and establishment of quality standards in *Boletus* resources, provide a reference for comprehensive quality control and evaluation of *Boletus* and its products, and promote sustainable development of the industry.

2. Materials and Methods

2.1. Materials and Reagents. Methanol, acetonitrile, ethanol, and formic acid were HPLC grade and were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q-System (Millipore, Guyancourt, France). Ammonium acetate (NH_4OAc) was purchased from Beijing Chemical Reagent Factory (Beijing, China). Formic acid was purchased from Duksan Pure Chemicals (Ansan, Korea). Eight alkaloids reference materials with purity ≥95% were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

2.2. Instruments and Equipment. The high-speed refrigerated centrifuge (CR22N, HITACHI, Germany), the vortex mixer (Vortex Genius 3, IKA, Germany), and the ultrasonic cleaner (Elmasonic P300H, Elma, Germany) were used in the procedure of extraction. The separation of compounds was carried out on a LC-30AD UPLC system equipped with a binary solvent manager, sample manager, and column manager (Shimadzu, Japan). Quantitative analysis of target compounds was conducted on a TripleTOFTM 5600^+ quadrupole/time of flight mass spectrometry (AB Sciex, USA).

2.3. Sample Preparation. The sample was pulverized uniformly on a small pulverizer, and one gram of pulverized sample was weighed and transferred to a 50 mL centrifuge tube. Following spiking 20 mL of 50% methanol-water solution, vortexing for 1 min, and sonicating for 20 min, the sample was centrifuged at 8000 r/min for 5 min. After centrifugation, the supernatant was filtered through a 0.22 μm nylon membrane before UPLC-Q-TOF/MS detection.

2.4. Chromatographic Conditions. The chromatographic separation was performed on a CORTECS UPLC HILIC column (100 mm × 2.1 mm, 1.6 μm; Waters, USA). The column temperature was 40°C. The injection volume was 5.0 μL. The flow rate was 300 μL/min. 20 mmol/L NH_4OAc solution containing 0.1% (v/v) formic acid (phase A) and acetonitrile containing 0.1% (v/v) formic acid (phase B) were used as mobile phase. The consecutive program was as follows: 0–3.00 min, 85% B; 3.0–6.0 min, 85% to 60% B; 6.0–11.0 min, 60% B; 11.0–12.0 min, 60% to 85% B; 12.0–15.0 min, 85% B.

2.5. Mass Spectrometry Conditions. The MS analysis was performed using an electrospray ion source (ESI) in positive ionization mode. The optimized parameters of ion source were as follows: the ionization voltage was 5.5 kV, the source temperature was 400°C, the pressure of curtain gas was 35 psi, the pressure of nebulizer gas was 50 psi, and the pressure of auxiliary gas was 55 psi; TOF MS conditions were as follows: scan range was 50~500 m/z, duration time was 15 min, and accumulation time was 0.15 s. IDA-MS/MS conditions were as follows: accumulation time was 0.05 s, high sensitivity mode was set, exclude isotopes were within 4 Da. Declustering potential was 80 V, collision energy was 35 V, and the dynamic energy of collision was 15 eV. Before each experiment, the instrument performed mass accuracy calibration through the CDS system. During the experiment, every five samples were run, and the mass accuracy calibration was automatically performed. The mass spectrum information of 8 endogenous alkaloids was shown in Table 1.

2.6. Optimization of Sample Extraction. To find out an optimum extraction method for samples, this experiment investigated the extraction effect when acetonitrile, methanol, ethanol, water, 50% acetonitrile-water solution, 50% methanol-water solution, and 50% ethanol-water solution were used as the extraction solvent, and the extraction time of 10, 15, 20, and 30 min was compared.

Table 1: Mass parameters for the 8 endogenous alkaloids.

Compound name	Formula	Adduct/charge	Theoretical mass (m/z)	Experimental mass (m/z)	Mass error ($\times 10^{-6}$)	Retention time (min)
Nicotine	$C_{10}H_{14}N_2$	$[M+H]^+$	163.123	163.1231	0.9	4.69
Anabasine	$C_{10}H_{12}N_2$	$[M+H]^+$	161.107	161.1069	−2.5	2.97
Choline	$C_5H_{14}NO^+$	$[M+H]^+$	104.107	104.1071	1.5	4.30
Tryptamine	$C_{10}H_{12}N_2$	$[M+H]^+$	161.107	161.1075	0.9	1.80
Putrescine	$C_4H_{12}N_2$	$[M+H]^+$	89.107	89.1073	−0.1	6.55
Nicotinamide	$C_6H_6N_2O$	$[M+H]^+$	123.055	123.0554	0.8	1.04
Muscarine	$C_9H_{20}NO_2^+$	$[M+H]^+$	174.149	174.1491	0.6	3.79
Cotinine	$C_{10}H_{12}N_2O$	$[M+H]^+$	177.102	177.1025	1.4	1.34

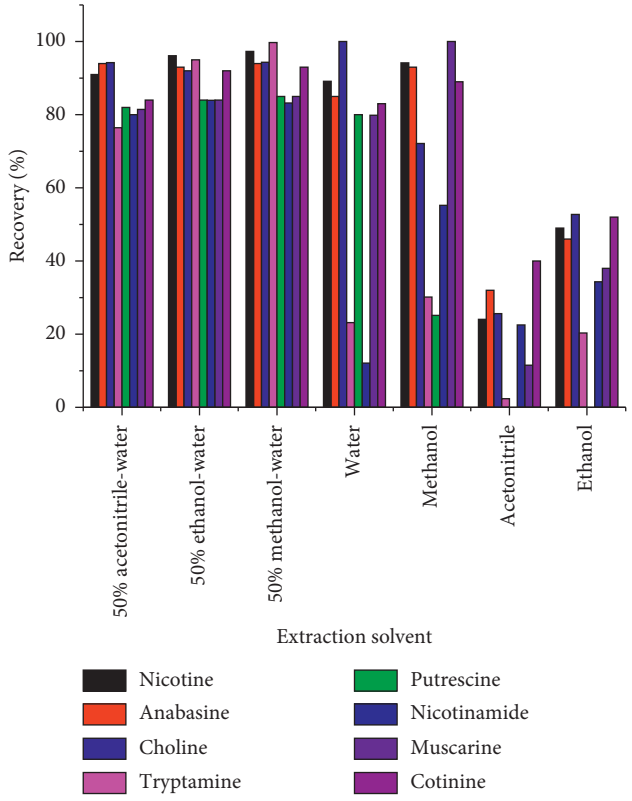


Figure 1: Effect of different solvent on the recoveries of 8 endogenous alkaloids.

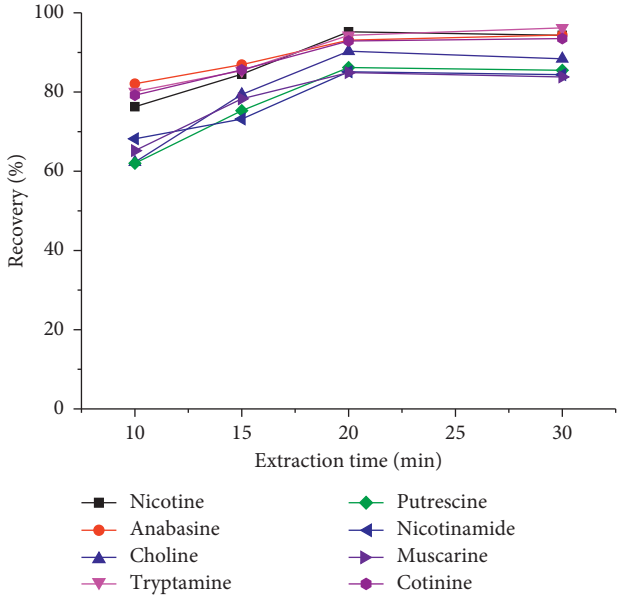
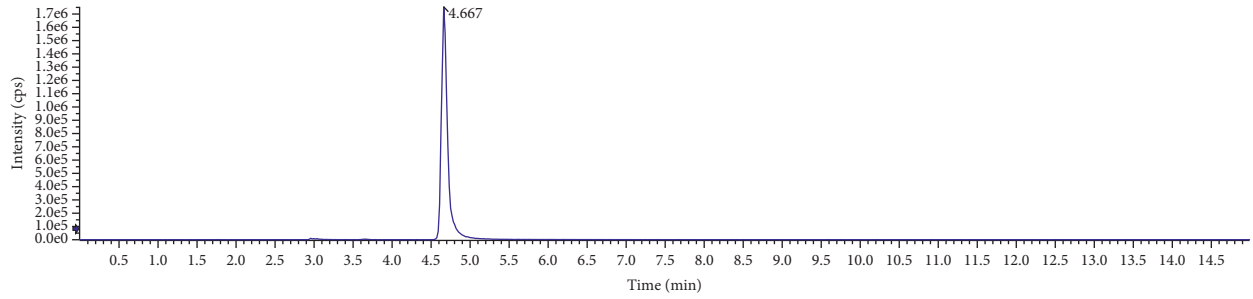


Figure 2: Comparison of extraction efficiency at extraction times.

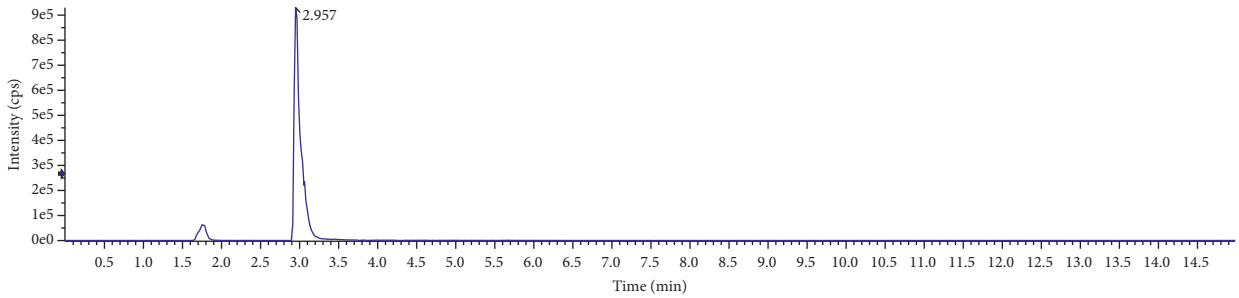
2.7. Optimization of Liquid Chromatography and Mass Spectrometry Conditions. Three analytical columns were tested prior to the selection of any additional chromatographic parameters, the Acquity BEH C18 column (100 mm × 2.1 mm, 2.5 μm), the Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm), and the Cortecs UPLC HILIC column (100 mm × 2.1 mm, 1.6 μm) were used. The mobile phase was evaluated. First, methanol and acetonitrile were tested as organic solvents in the mobile phase. In relation to the aqueous phase, an aqueous solution with various concentrations of NH_4OAc (0, 5 mmol/L, 10 mmol/L, 15 mmol/L, 20 mmol/L) were tested.

Syringe injection was used to inject 8 endogenous alkaloids directly into the mass spectrometer. The experiment investigated the response of target compounds under positive and negative ionization modes. For this method, TOF MS and IDA MS/MS modes were adopted for the detection of a target analyte. Under TOF MS mode, the experiment investigated the response of target compounds under different declustering potentials (50–300 V) and found the declustering potentials with the highest response.

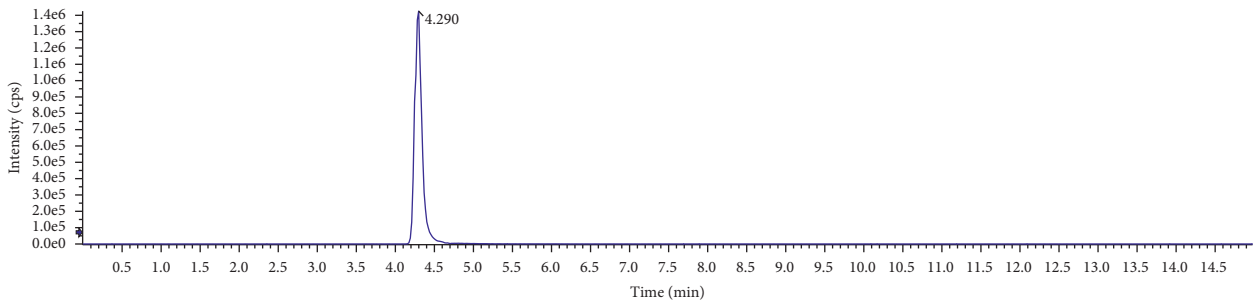
2.8. Method Validation. Validation of the whole analytical method was performed with linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, accuracy, and recovery. The linearity of the method was evaluated by constructing calibration curves with different concentrations of 8 alkaloids. The LOD and LOQ under the present chromatographic conditions were calculated on the basis of the response and slope of each regression equation at signal-to-noise ratios (S/N) of 3 : 1 and 10 : 1, respectively. The recoveries of analytes were evaluated by adding the standard solutions with three different concentration levels (1*x* LOQ, 3*x* LOQ, 10*x* LOQ) to the known amounts of fungus samples, and each level was repeated six times. The precision of the method was expressed by a relative standard deviation (RSD).



(a)



(b)



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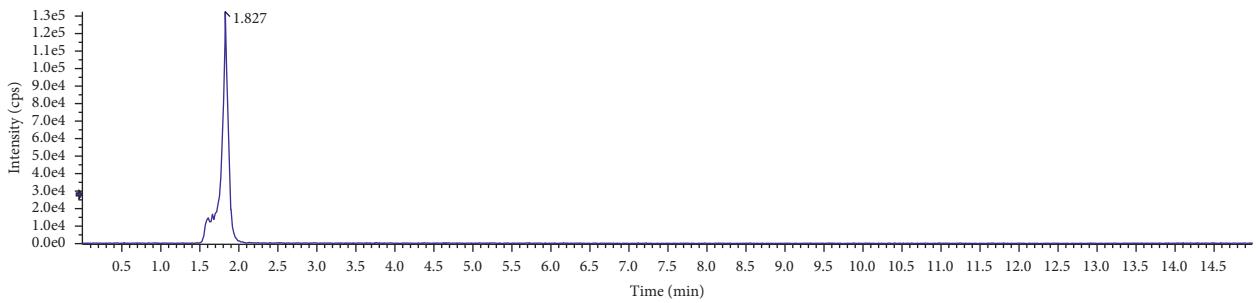

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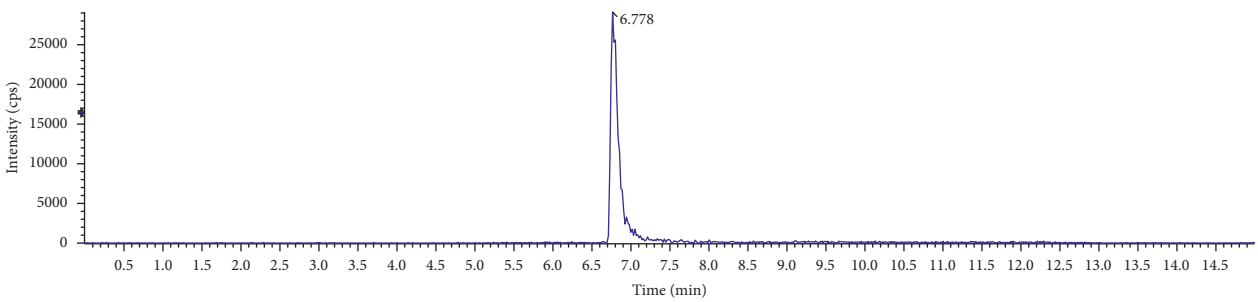

(e)

Figure 3: Continued.

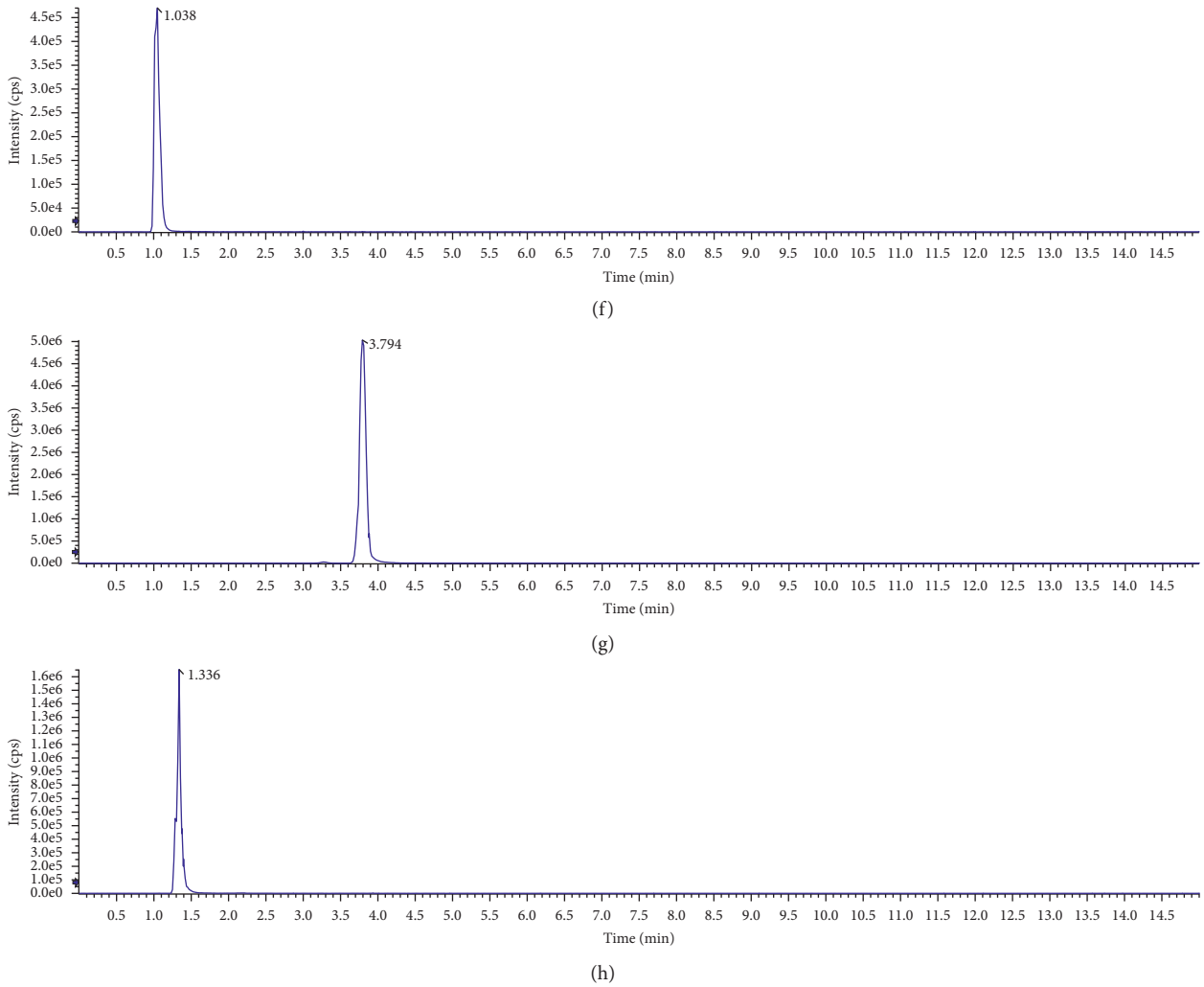

Figure 3: Extracted ion chromatograms of the 8 endogenous alkaloids. (a) Nicotine. (b) Anabasine. (c) Choline. (d) Tryptamine. (e) Putrescine. (f) Nicotinamide. (g) Muscarine. (h) Cotinine.

3. Results and Discussion

3.1. Optimization of Sample Extraction

3.1.1. Optimization of Extraction Solvents. As Figure 1 shows, the extraction efficiency of extraction solvents for various endogenous alkaloids was different. 50% methanol-water solution yielded the best reproducibility and recovery of the 8 alkaloids. Therefore, a 50% methanol-water solution was selected as the extraction solvent.

3.1.2. Optimization of Extraction Times. As Figure 2 shows, when the ultrasonic extraction time was increased from 10 min to 20 min, the extraction efficiency of alkaloids was greatly improved. After 20 min, the growth trend decelerates, and the extraction efficiency of alkaloids changes little. Considering the extraction efficiency of the experiment and time cost, 20 min was chosen as extraction time.

3.2. Optimization of Liquid Chromatography and Mass Spectrometry Conditions

3.2.1. Optimization of Liquid Chromatography Conditions. The results showed that the nicotine, anabasine, choline, tryptamine, putrescine, and muscarine were not retained on the Acquity BEH C_{18} column and the Acquity UPLC HSS T3 column. They can be retained on the CORTECS UPLC HILIC column, and the separation effect was good. Because they are strong polar hydrophilic compounds, which were weakly retained on the C_{18} and T3 columns, so it is necessary to replace hydrophilic columns for separation. Therefore, the CORTECS UPLC HILIC column (100 mm × 2.1 mm, 1.6 μm) was selected as the analytical columns.

The mobile phase was evaluated. First, methanol and acetonitrile were tested as organic solvents in the mobile phase, observing that methanol and acetonitrile as mobile phases have little effect on the signal intensity, but when methanol was used as the mobile phase for gradient elution, column pressure varies widely, and equilibration takes

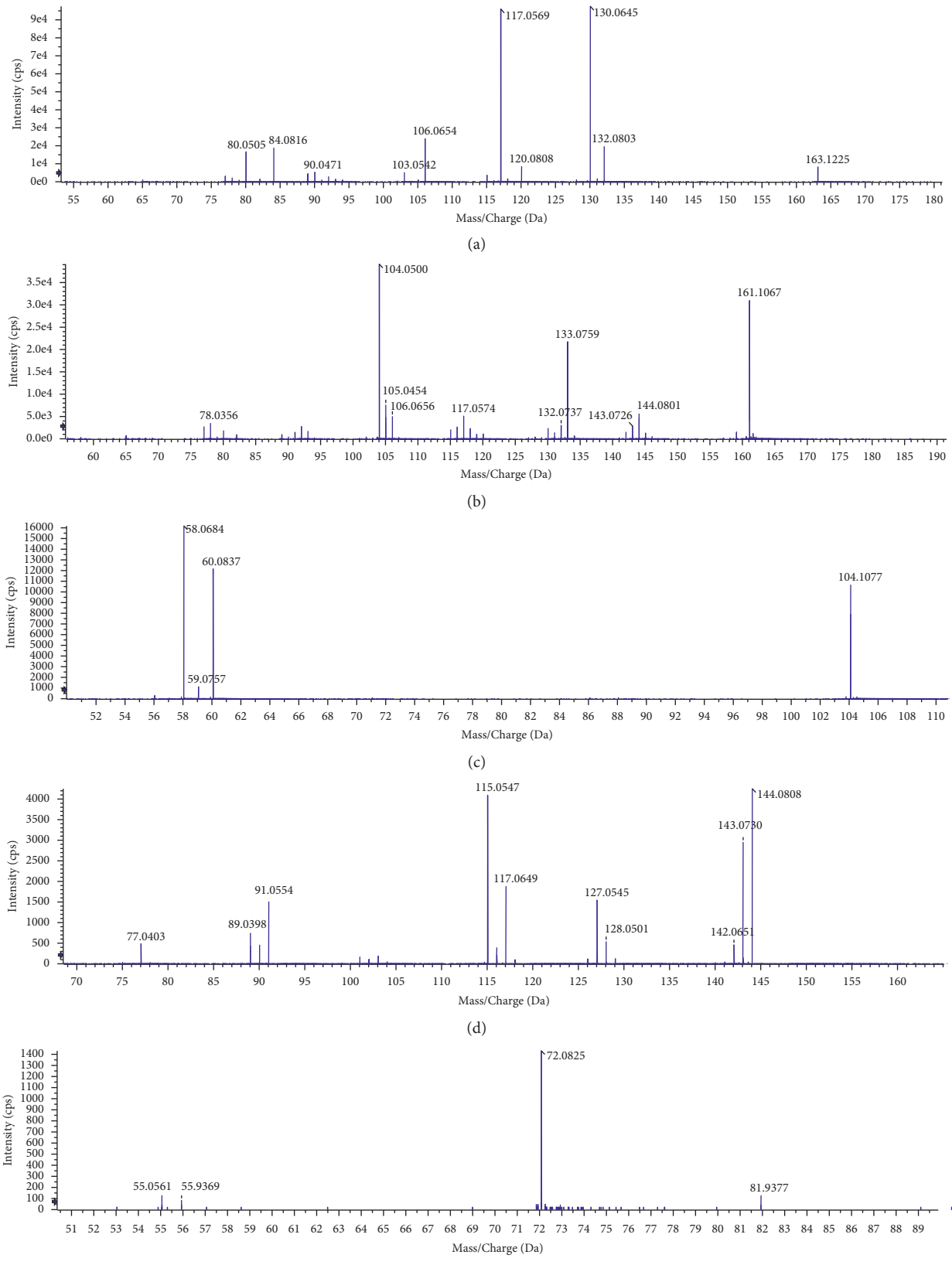

Figure 4: Continued.

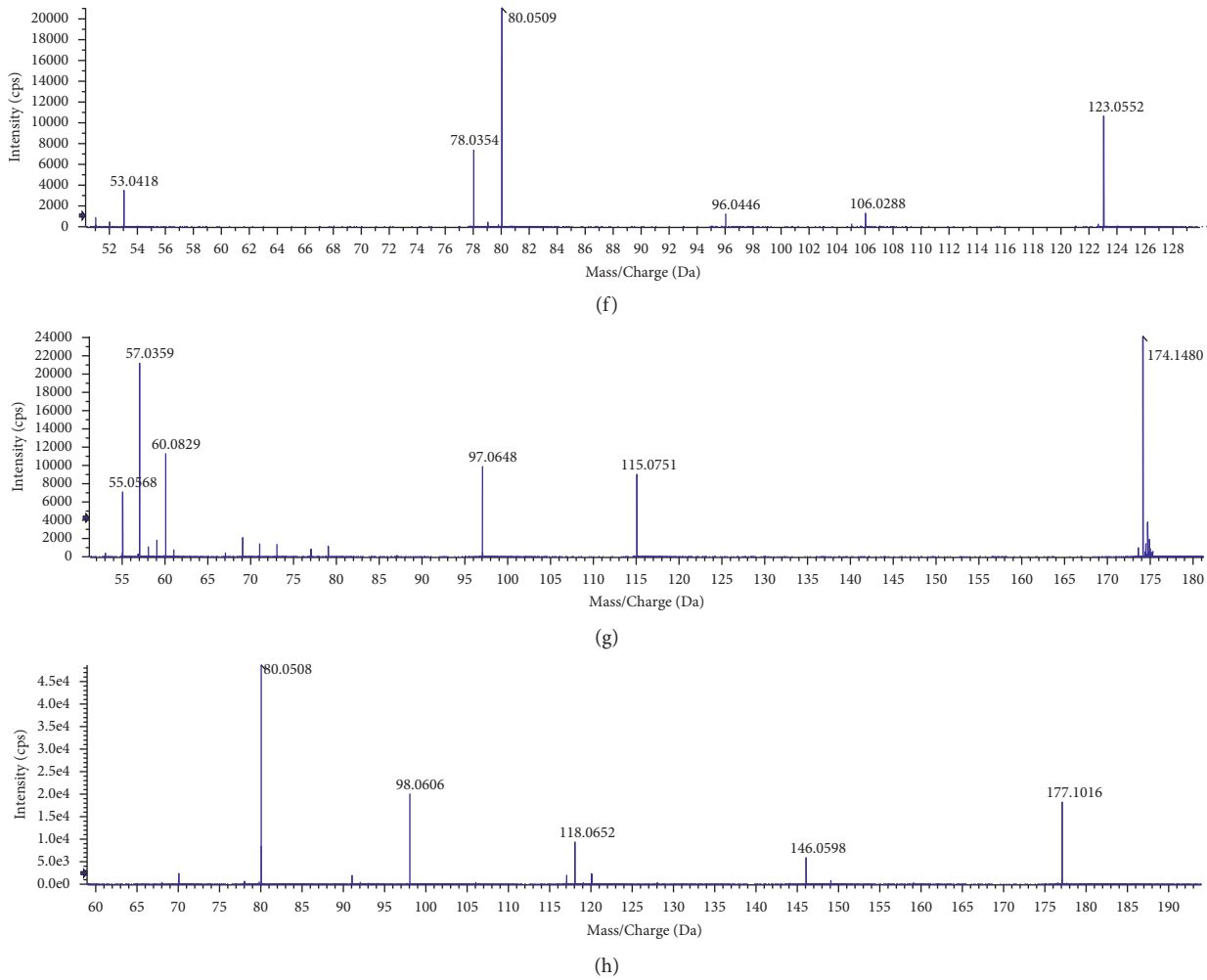

Figure 4: MS/MS spectra of the 8 endogenous alkaloids. (a) Nicotine. (b) Anabasine. (c) Choline. (d) Tryptamine. (e) Putrescine. (f) Nicotinamide. (g) Muscarine. (h) Cotinine.

longer. In relation to the aqueous phase, the results showed 20 mmol/L NH_4OAc as the aqueous solution showed better separation and elution capabilities. Considering that under the positive mode, the addition of formic acid could increase the ionization of the compounds, which improved the separation efficiency and the intensity of the mass spectrometry signal. Therefore, 20 mmol/L NH_4OAc solution containing 0.1% (v/v) formic acid and acetonitrile containing 0.1% (v/v) formic acid were selected as the mobile phase for subsequent experiments.

3.2.2. Optimization of Mass Spectrometry Parameters. The TripleTOF™ 5600$^+$ instrument has a CDS automatic calibration infusion system. The reference spray of the DuoSpray™ ion source was used to input the calibration solution for automatic system calibration. The DuoSpray™ ion source has two types: ESI and atmospheric pressure chemical ionization source (APCI). In this experiment, the ESI was selected as the detection ion source, and the APCI was used as the calibration ion source. Through the automatic calibration system, automatic batch calibration was performed to ensure the accurate mass of the system was stable for a long time.

The results showed that compounds have a higher response in positive ionization mode, so the positive ionization mode was used for detection. Under TOF MS mode, the experiment investigated the response of target compounds under different declustering potentials (50–300 V), with the findings that at the fragmentor voltage of 80 V, compound response was the highest; relatively low fragmentor voltage was unfavorable for ion transmission, and overly high fragmentor voltage would cause the compound to fragment within the source. The accurate mass, retention time, and isotope ratio was obtained. Figure 3 shows the extracted ion chromatograms of 8 alkaloids (500 ng/mL). The accurate mass deviations of target compounds were less than 5.0×10^{-6} (Table 1). The MS/MS spectra of the target were used for the final confirmation of the initial screening results of the accurate mass (Figure 4).

3.3. Method Validation

3.3.1. Linearity and Sensitivity. As Table 2 shows, the linear range was studied by preparing a calibration curve with a concentration range of 0.2–500 μg/L for each compound,

Table 2: Linearity, LODs, and LOQs of the 8 endogenous alkaloids.

Compound	Linear equation	Linear range (μg/L)	R^2	LOD (mg/kg)	LOQ (mg/kg)
Nicotine	$Y = 10091.33111X + 3329.87164$	0.5–500	0.99866	0.005	0.010
Anabasine	$Y = 11037.04373X + 23487.04699$	0.2–500	0.99844	0.002	0.004
Choline	$Y = 14658.46420X - 5.77788 \times 10^4$	0.5–500	0.99785	0.005	0.010
Tryptamine	$Y = 946.35326X + 3594.74788$	5.0–500	0.99876	0.050	0.100
Putrescine	$Y = 483.76213X + 2103.10951$	10.0–500	0.99876	0.100	0.200
Nicotinamide	$Y = 2672.35197X - 3753.02170$	0.5–500	0.99899	0.005	0.010
Muscarine	$Y = 28863.69968X - 3.26291 \times 10^5$	0.2–500	0.99932	0.002	0.004
Cotinine	$Y = 6622.74425X + 3.13607 \times 10^4$	0.5–500	0.99928	0.005	0.010

Table 3: Linearity, LODs, and LOQs of the 8 endogenous alkaloids.

Compound	Background (mg/kg)	Added (mg/kg)	Recovery (%)	RSD (%)
		0.010	87.6	1.93
Nicotine	0.160	0.030	85.2	3.90
		0.100	90.4	1.62
		0.004	101.5	4.15
Anabasine	0.111	0.012	85.9	0.30
		0.040	81.1	1.63
		0.010	89.2	1.45
Choline	301	0.030	92.3	0.44
		0.100	88.7	0.13
		0.100	83.3	2.10
Tryptamine	ND	0.300	82.4	6.82
		1.00	82.3	1.89
		0.200	80.1	7.59
Putrescine	ND	0.600	89.2	3.08
		2.00	88.3	4.21
		0.010	82.5	3.38
Nicotinamide	7.01	0.030	80.1	2.58
		0.100	91.7	1.43
		0.004	97.5	3.45
Muscarine	ND	0.012	91.3	1.68
		0.040	88.8	0.06
		0.010	85.4	4.54
Cotinine	ND	0.030	87.3	7.58
		0.100	90.5	2.19

∗ND means not detected.

Table 4: Content of the 8 endogenous alkaloids in actual samples.

Compound	*Boletus albus* peck	*Boletus rubellus* krombh	*Boletus impolitus*
Nicotine (mg/kg)	0.491	1.45	3.41
Anabasine (mg/kg)	ND	ND	ND
Choline (mg/kg)	147	131	142
Tryptamine (mg/kg)	115	9.15	45.0
Putrescine (mg/kg)	108	90.6	82.6
Nicotinamide (mg/kg)	9.34	6.68	3.94
Muscarine (mg/kg)	0.215	0.0329	0.0483
Cotinine (mg/kg)	ND	ND	ND

and a good linear relationship with correlation coefficients (R^2) higher than 0.9990 was achieved for 8 endogenous alkaloids in their respective linear range. The LODs of 8 endogenous alkaloids were in the range of 0.002–0.100 mg/kg. The LOQs of 8 endogenous alkaloids were in the range of 0.004–0.200 mg/kg. LOD and LOQ for the methods of determination of 8 endogenous alkaloids in the tea are shown in Table 2.

3.3.2. Recovery and Precision. The recoveries of analytes were evaluated by adding the standard solutions with three different concentration levels to the known amounts of fungus samples. The data of recovery and precision are given in Table 3; the average recoveries of 8 endogenous alkaloids were in the range between 80.1% and 101.5%. The RSDs were in the range of 0.06%–7.59%.

3.4. Application to Actual Samples. In order to investigate the content of 8 endogenous alkaloids in *Boletus*, three *Boletus* samples from the local supermarket were analyzed using the developed method in this study. Their detection results are shown in Table 4. The compositions and contents of alkaloids were different in 3 *Boletus* samples. Anabasine and cotinine were not detected. The contents of muscarine in 3 *Boletus* samples were the lowest, which were 0.215, 0.0329, and 0.0483 mg/kg, respectively. The contents of choline in 3 *Boletus* samples were the highest, which were 147, 131, and 142 mg/kg, respectively.

4. Conclusions

In this experiment, a rapid and sensitive UPLC-Q-TOF/MS method was developed to analyze 8 endogenous alkaloids in the *Boletus* sample. The analytes were determined by Q-TOF/MS in TOF MS and IDA-MS/MS mode. In TOF MS mode, the target compounds qualified by the retention time, accurate mass, isotope distribution, and isotope abundance ratio of the target, and quantitated by the peak area of the excimer ion peak. In IDA-MS/MS mode, the target compounds were further confirmed by the ion fragment information under the corresponding collision energy. The linearity, sensitivity, accuracy, and precision of the method were investigated. The method has simple sample processing, high sensitivity, and high analysis efficiency. It is suitable for the rapid detection of alkaloids in batches of *Boletus* samples and can be used for quality control and formulation experiments of *Boletus* production. This method also provides a reference for the determination of various alkaloids in tobacco, tea, and other samples.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YZ was responsible for the conceptualization of the study; JM, QL, SF, LH, LS, DW, and HZ investigated the study; JM, QL, LH, LS, and HZ reviewed the study; JM, SF, and DW were involved in the discussion; JM and YZ were responsible for writing, reviewing, and editing the original draft; YZ was involved in the project administration and funding acquisition.

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