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Analysis of agaritine in mushrooms and in agaritine-administered mice using liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and specific method for quantifying a genotoxic hydrazine, agaritine, has been developed using liquid chromatography–electrospray ionization tandem mass spectrometry (MS). Synthetic agaritine was structurally assigned by ^{1}H, ^{13}C and two-dimensional nuclear magnetic resonance (NMR) analysis (heteronuclear multiple-bond correlation [HMBC] and heteronuclear multiple-quantum coherence [HMQC]), high-resolution fast-atom-bombardment (HR-FAB) MS. Agaritine was separated on an ODS column using 0.01% AcOH–MeOH (99:1) as an eluent with a simple solid-phase-extraction cleanup for mushroom samples and with acetonitrile and methanol deprotenization for plasma samples. There were no interference peaks in any of the mushrooms or mouse plasma samples. The recoveries of agaritine from the spiked mushroom samples and spiked mouse plasma were 60.3–114 and 74.4%, respectively. The intra-day precision values for the spiked mushrooms were 5.5 and 4.2%, and the inter-day precision values were 15.0 and 23.0%, respectively. The limit of quantification was 0.01 μg/g (in mushrooms) and 0.01 μg/ml (in plasma). A precursor ion scan confirmed that agaritine derivatives, which can exert a similar toxicity, were absent. These results indicate that this analytical method for quantifying agaritine could help to evaluate the risk of mushroom hydrazines to humans.

Keywords: Phenylhydrazine; Agaritine; Mushroom; Genotoxic; LC–MS–MS

1. Introduction

The cultivated mushroom *Agaricus bisporus* contains large quantities of aromatic hydrazines. Amongst these, the most abundant is agaritine [β-*N*-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine], the concentration of which is approximately 165–475 μg/g [1,2] or 1.7 mg/g in fresh mushrooms [3]. Toth and co-workers [4,5] demonstrated that the administration of uncooked mushrooms to mice induced a significant increase in the number of bone and forestomach tumors in both sexes, and in the occurrence of lung tumors in males [4,5]. Ethanolic and aqueous extracts from *A. bisporus* led to mutagenicity in the Ames test [6]. The direct-acting mutagenicity in this study was not attributed to agaritine, but rather to phenols and quinines that might behave as reactive oxygen species [7,8]. The direct mutagenicity was not affected by baking the mushrooms at 225 °C for 10 min [9]. Compounds leading to mutagenicity appear to be strong against heat. There are, however, also reports showing that agaritine does have a direct mutagenic effect. The mutagenicity of agaritine can be attributed directy to 4-(hydroxymethyl)phenylhydrazine (HMPH) and/or the 4-(hydroxymethyl)benzenediazonium ion (HMBD), both of which are formed by the eenzymatic degradation of agaritine that results in the loss of the γ-glutamyl group [10–13]. HMPH and HMBD are highly unstable and are carcinogenic [10,12,14].

Price et al. reported that agaritine was metabolized to compounds that covalently bond to proteins [15]. Shephard et al. demonstrated covalent bonding between agaritine and DNA [16]. These results have led to the hypothesis that mechanisms of the mutagenicity of agaritine and the *A. bisporus* mushroom might be similar.

A report evaluating the risk posed by phenylhydrazines in cultivated mushrooms (*A. bisporus*) to humans was recently published [17]. This report states that agaritine is thought to

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be converted to free hydrazine HMPH by γ-glutamyl transpeptidase, which is abundant in the kidney. This is consistent with a previous study that found that the mutagenicity of agaritine incubated with kidney homogenate and with kidney plus liver homogenates more than doubled and tripled, respectively [13]. These results indicate that agaritine might be converted to HMPH and then to HMBD, which can then be transformed into a radical compound that is potentially mutagenic. However, no evidence has been obtained of the presence of either HMPH or HMBD [13,18], as no suitable detection method had been identified and the molecules are unstable. Therefore, the *N*-acetyl derivative of HMPH must generally be used for toxicological studies.

Agaritine was first isolated and identified by Levenberg [19,20] and Kelley et al. [21]. This L-glutamic acid-containing hydrazine is susceptible to oxidation in the air. The stability of this molecule was examined by Hajšlová et al., who demonstrated that agaritine degrades within 48 h in tap water and that the degradation appeared to be oxygen-dependent. The best analytical method reported to date for the detection of agaritine is based on high performance liquid chromatography (HPLC) equipped with an ultraviolet (UV) detector (237 or 254 nm) using an ODS column [1,2,13]. In this method, only the commonly eaten *A. bisporus*, which contains substantial amounts of agaritine, can be analyzed. Agaritine cannot be detected in other mushroom species that contain only small quantities of agaritine due to multiple unspecific peaks in the HPLC. For toxicological studies, mouse and rat plasma have been analyzed for agaritine and its metabolites by liquid scintillation counting after the administration of radio-labeled agaritine [22]. The addition of radio-labeled agaritine was necessary because the concentration of agaritine in the plasma samples was too low to be detected by a UV method. There are no previous reports of a sensitive and specific agaritine detection method that is applicable to both food and biological samples [22]. A widely applicable analytical method for the detection of agaritine and its metabolites is required in order to assess the risk posed by phenylhydrazine agaritine to humans. The presence of agaritine degradation products in food and agaritine metabolites in plasma from agaritine-administration in mice or rats, as the toxicities of these molecules, remains unclear, although the presence of unidentified agaritine metabolites were reported in both of these experimental cases [13,15].

In this study, an analytical method for the quantification of agaritine using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) for several edible and processed mushroom species and agaritine-administered mouse plasma is described. This method is comprised of an extraction with methanol, a simple solid-phase-extraction (SPE) cleanup for the mushroom samples or deproteinization by acetonitrile and methanol for the plasma samples, and electrospray mass spectrometry in the negative mode in conjunction with HPLC. An agaritine standard was synthesized and structurally elucidated using two-dimensional (2D) nuclear magnetic resonance (NMR) techniques such as heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC), as well as high-resolution (HR) fast-atom-bombardment (FAB) MS.

2. Experimental

2.1. Food samples

Fresh samples of *A. bisporus*, the Shiitake mushroom *Lentinus edodes*, the Maitake mushroom *Grifola frondosa*, and dried samples of the Himematsutake mushroom (*Agaricus blazei* Murill) were purchased from supermarkets in Tokyo, Japan. The Sugihiratake mushroom *Pleurocybella porrigens* was collected from in northern Japan. All of the fresh mushrooms were freeze-dried for 2 days using a vacuum freeze dryer (FD-81; EYELA, Kawasaki, Japan).

2.2. Chemicals

The methanol used in the sample preparation and in the LC/MS/MS analysis was obtained from Kanto Chemicals (Tokyo, Japan). MilliQ water was also used in this study. All of the other chemicals were of the highest grade available. Agaritine was synthesized according to the method described by Datta et al. with minor modifications [23]. The purity of the synthetic agaritine was >95% based on HPLC (254 nm). A standard stock solution was prepared in methanol and stored at below −20 °C before use.

2.3. Mouse plasma

Mouse plasma was taken from Slc:ddY mice (25–30 g, male, 8W; Japan SLC, Shizuoka, Japan) after agaritine administration (4.0 mg/kg mouse).

2.4. NMR Measurements

^{1}H, ^{13}C and 2D NMR (HMBC and HMQC) spectra were recorded on an ECA-500 (JEOL, Japan) in CD_3OD and $CDCl_3$ (^{1}H at 500 MHz and ^{13}C at 125 MHz), respectively. Chemical shifts (δ) are described in ppm using tetramethylsilane (TMS) as a reference. Coupling constants (J) are given in Hz. The samples to be measured were prepared under a nitrogen atmosphere to avoid oxidation in air.

2.5. HRMS

To identify the synthetic agaritine, FABMS in the positive mode (JMS-700; JEOL, Japan) was used.

2.6. LC/MS conditions

LC/MS/MS measurements were performed using a PE SCIEX (Concord, ON, Canada) model API 3000 triple-quadrupole mass spectrometer coupled to an Agilent 1100 series HPLC system with a G1315 photodiode-array detector (Palo Alto, CA). The HPLC system was equipped with a 3-μm Shiseido CAPCELL PAK AQ column (2.1 mm × 250 mm and

2.1 mm × 150 mm; Yokohama, Japan). The gradient conditions ranged from 99% water containing 0.01% AcOH with 1% MeOH to 90% MeOH with 10% water containing 0.01% AcOH. The flow rate was 0.2 ml/min and the column temperature was 35 °C.

The analytes were detected using electrospray ionization (ESI) in the negative mode. Multiple-reaction-monitoring (MRM) was performed using the characteristic fragmentation ions *m/z* 266 → 248 and 266 → 122 for agaritine. Optimization of the ionization and fragmentation conditions in the ESI mode for agaritine was achieved by the infusion and flow-injection analysis of agaritine. The optimization was performed several times to determine parameters such as the collision gas to be used and the focusing potential (FP). The parameters for the LC/MS/MS analysis of agaritine were as follows: ionspray voltage = −4500 V; collision gas = 6; focusing and entrance potentials = 60 and 20 V, respectively, and temperature = 500 °C. A switching valve led the column eluents to the mass spectrometer as the analytes were being eluted.

Precursor-ion and neutral-loss scans were performed to analyze the agaritine derivatives. The collision energy (CE) was changed from −2 to −40 to obtain better resolution. The data were acquired and calculated using Analyst 1.4.1 software (PE SCIEX).

2.7. Sample preparation

Freeze-dried mushrooms (1.0 g) and processed foods (1.0 g) made from *A. blazei* Murill were extracted with MeOH (3 × 30 ml) by shaking for 20 min. After filtration with a paper filter, the samples was evaporated to dryness and the residue was dissolved in 3 ml of 0.01% AcOH–MeOH (9:1). Bond Elut C_{18} cartridges (500 mg/3 ml; Varian, Palo Alto, CA) were conditioned with MeOH followed by 0.01% AcOH–MeOH (9:1). The sample solutions (1 ml) were loaded onto the cartridge and an additional 2 ml of 0.01% AcOH–MeOH (9:1) was added. The eluent was collected removing the yellow pigments and lipid-soluble materials. The final sample solutions (10 μl) were injected into the LC/MS/MS system. Agaritine was quantified using a linear calibration function that was established using the agaritine standard at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml (r^2 = 0.993–0.999).

Mouse plasma from agaritine-administered mice was prepared as follows; blood was collected 20 min after agaritine administration to mice and immediately placed on ice before centrifugation (10,000 rpm, 2 min). After centrifugation, the plasma (200 μl) was deproteinized by acetonitrile and methanol, and then diluted with mobile phase to 600 μl. Agaritine was quantified using a linear calibration function as described above (0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 μg/ml).

2.8. Accuracy and precision

The amount of agaritine recovered from the mushrooms spiked with agaritine (5 μg/g) using the above methods was determined. Intra-day precision was obtained by measuring three replicate samples that were spiked with 5 μg/g of the agaritine standard on the same day. Inter-day precision was estimated by measuring three replicate samples on different days. The quantification limit was determined using fortified samples based on an S/N ratio of 10:1.

The recovery of agaritine from mouse plasma spiked with agaritine (0.25 μg/ml) was determined based on the results from 11 mice.

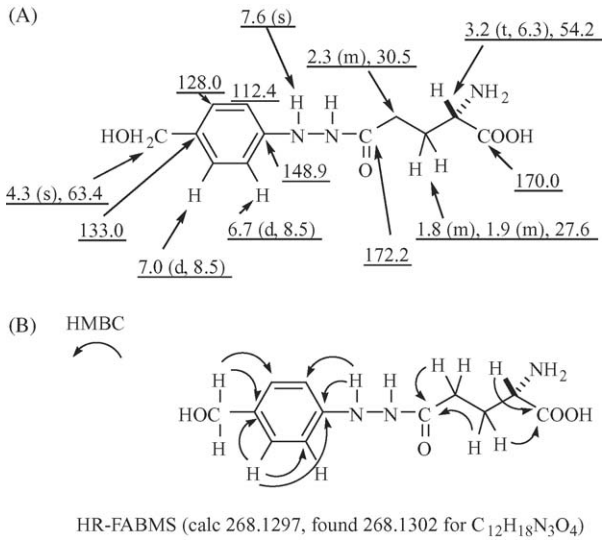

Fig. 1. Structure and NMR spectral data of agaritine. All signals were assigned by ^{1}H, ^{13}C and 2D NMR (HMBC and HMQC) spectra. (A) Chemical shifts (δ) of the synthesized agaritine; (B) HMBC correlation of the signals in agaritine. δ = 4.3 (s) and 7.6 (s) correspond to the HOH_2C– group on the benzene ring and NH– of the hydrazine group, respectively.

3. Results and discussion

3.1. Agaritine

Agaritine is not commercially available so this compound must be synthesized for research purposes. However, a complete structural assignment for this compound has not been reported to date.

We fully assigned the structure of agaritine that we synthesized. The structure and chemical shifts (δ) of agaritine are shown in Fig. 1A. The characteristic signals are δ = 4.3 (singlet) and 7.6 (singlet), corresponding to the HOH_2C– group on the benzene ring and the –NH– of the hydrazine group, respectively. The agaritine structure was confirmed by ^{1}H, ^{13}C, 2D NMR (HMQC and HMBC), and HR-FABMS experiments. The HMBC correlations around the benzene ring are shown in Fig. 1B, which supports the agaritine structure. HR-FABMS result confirmed the identity of agaritine ($[M+H]^+$, calc. 268.1297; found 268.1302).

3.2. LC/MS/MS

The protonated $[M+H]^+$ (*m/z* 268) and deprotonated $[M-H]^-$ (*m/z* 266) molecular ions of agaritine were detected using ESI. Peaks at *m/z* 250 in the positive-ion and 248 in the negative-ion modes, corresponding to the expected fragmenta-

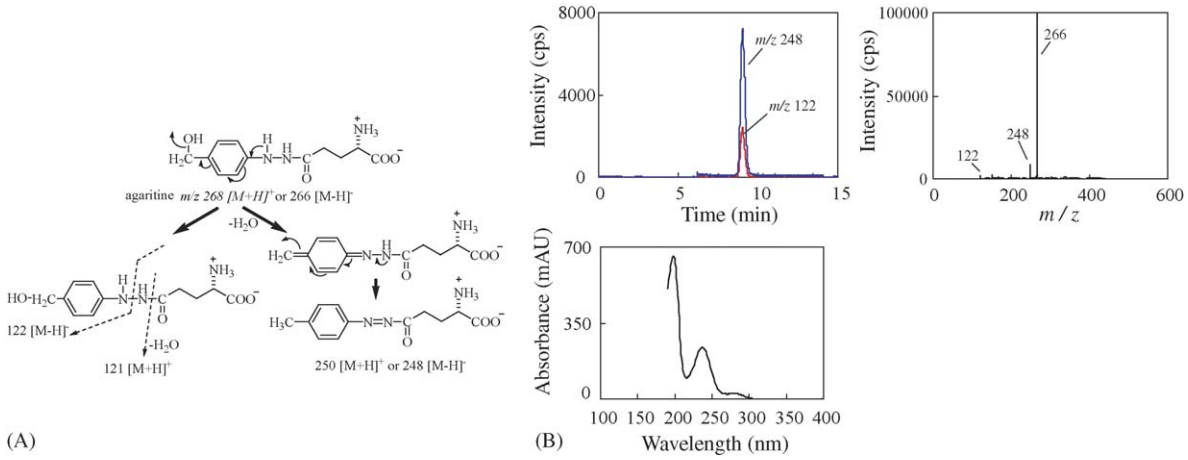

Fig. 2. Fragmentation mechanisms of agaritine in the positive- and negative-ion mode, MRM chromatograms of agaritine, and mass and UV spectra of agaritine. (A) The first fragmentation (loss of H_2O over the benzene ring) occurrs easily. High collision energy causes the second fragmentation, resulting in the loss of the glutamyl moiety; (B) MRM (*m*/*z* 266–248 and 266–122) chromatograms of the agaritine standard (0.05 μg/ml); (C) mass spectrum of the agaritine standard. Two fragment ions are observed (*m*/*z* 122 and 248); (D) UV spectrum of the standard.

tion pattern (loss of H_2O over the benzene ring), were observed. The high collision energy gave additional fragment ions that were observed at *m*/*z* 121 in the positive and 122 in the negative modes, which are the result of the loss of the glutamyl group. The agaritine standard and mushroom samples spiked with agaritine were analyzed in both the positive and the negative modes to compare the sensitivity, specificity and baseline stability of the two modes. Based on these results, MRM at both *m*/*z* 248 and *m*/*z* 122 in the negative mode was used for the quantification of agaritine, as the background noise level in the negative mode was lower than that in the positive mode, and the baseline was more stable. The peak area and height ratio of *m*/*z* 248 to *m*/*z* 122 was a constant value. This was then used as away to confirm that the observed peaks were agaritine. The fragmentation mechanisms of agaritine are illustrated in Fig. 2A. Representative mass chromatograms at *m*/*z* 248 and 122, along with mass and UV spectra of the agaritine standard (0.05 μg/ml) are shown in Fig. 2.

3.3. Agaritine in mushrooms

To date, the agaritine content has been determined by HPLC coupled to a UV detector (237 or 254 nm). *A. bisporus* contain large amounts of agaritine (165–475 μg/g [1,2] or 1.7 mg/g fresh mushroom [3]) and these concentrations permit agaritine detection by the HPLC–UV method, although the specificity is poor. We initially attempted to determine the presence of agaritine using HPLC equipped with a DAD detector in several species of mushrooms such as *A. bisporus*, *A. blazei* Murill, *L. edodes*, *G. frondose* and *P. porrigens*. Agaritine was detected in two *Agaricus* samples by UV (254 nm) and at least one peak overlapped with that of agaritine in the DAD data (190–400 nm scan; data not shown). It was clear that the agaritine peak on the sample chromatograms was not a single compound. Furthermore, the presence of agaritine could not be determined in the other species spiked with 5.0 μg/g due to the presence of large interference peaks (Fig. 3). The chromatograms presented in red show UV (237 nm)-monitored traces of two mushrooms spiked with agaritine. Compared to the chromatograms in black (nonspiked mushrooms), there is no significant difference between the two. Around the retention time (RT = 9 min) at which agaritine is supposed to be eluted, large interference peaks exist. This indicates that agaritine determination by UV detection is problematic in mushrooms.

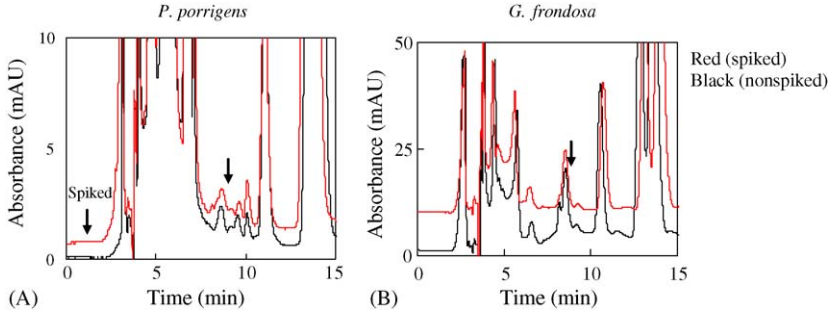

Fig. 3. Typical chromatograms of two mushroom species. Freeze-dried mushrooms were extracted with methanol and cleaned up using a C_{18} cartridge. The samples were analyzed with a UV detector (254 nm). Chromatograms of *P. porrigens* and *G. frondose* are shown in black. Chromatograms of two mushrooms spiked with the agaritine standard (5.0 μg/g) are in red. Small amounts of agaritine (less than μg/g level) could not be detected by the UV method. The spiked agaritine is predicted to appear at the retention times indicated by the arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

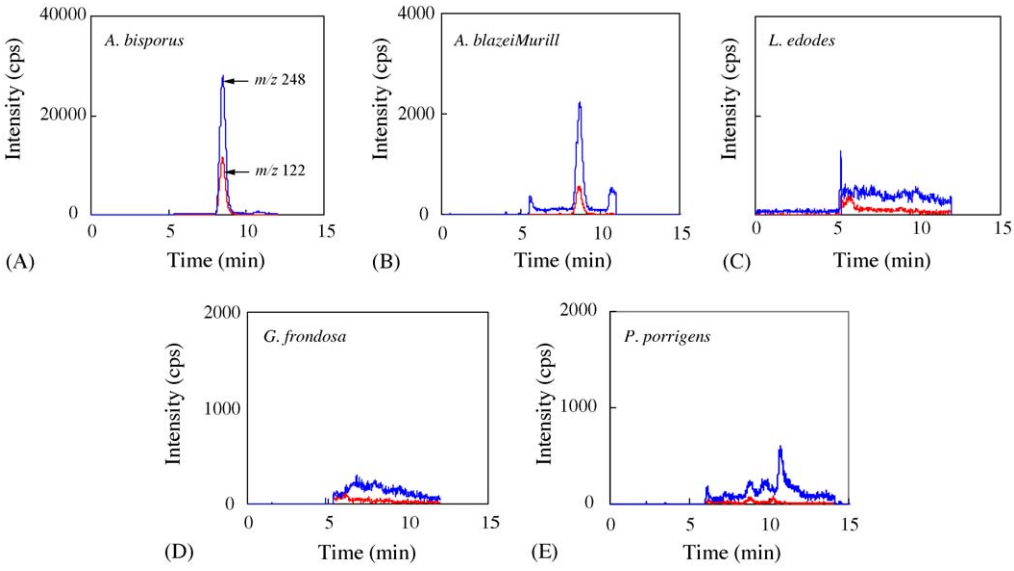

Fig. 4. Representative MRM chromatograms of five mushroom species. Two fragment ions were monitored simultaneously (*m/z* 266–248 in blue, 266–122 in red). None of the mushrooms, with the exception of the *Agaricus* spp., contained agaritine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Thus, an analytical method using LC/MS/MS has been developed to determine agaritine with specificity and sensitivity. Five mushrooms, *A. bisporus*, *A. blazei* Murill, *L. edodes*, *G. frondose*, *P. porrigens*, were analyzed. There were no peaks hindering agaritine determination (Fig. 4). Only *Agaricus* spp. Were found to contain agaritine, and it was not detected in the other species. *A. bisporus* and *A. blazei* Murill had an agaritine content of 198 μg/g wet and 2,017 μg/g dry. The limit of quantification (LOQ) of this method was 0.01 μg/g, although the mobile phase of only 1% organic solvent (methanol) was disadvantageous to the sensitivity during the MS analysis. The peak height ratio of *m/z* 248 to *m/z* 122 was always constant between the agaritine standard and the samples. This indicates that this LC/MS/MS method is highly sensitive and specific for agaritine determination, and can be applied to other foods and biological samples such as plasma.

In addition to these results, the presence of an agaritine derivative that consists of HMPH condensed with aspartic acid (agaritine-Asp) was investigated using a precursor ion scan of 122 and MRM (*m/z* 252–234 and 252–122). Agaritine-Asp may exhibit a similar toxicity to agaritine, because it is also capable of generating HMPH. A precursor ion scan of dried *A. blazei* Murill revealed three peaks (Fig. 5A). Peak 2 was identified as agaritine (Fig. 5D), while the other two peaks could not be identified (Fig. 5C and E). MRM experiments confirmed that the remaining two peaks were not agaritine-Asp. If agaritine-Asp was present, a peak would appear at both *m/z* 252–234 (in blue) and 252–122 (in red) at the same retention times as peaks 1 and 3 in the precursor ion scan. The fourth peak (RT = 18 min) in Fig. 5A was background noise, which was also observed in the control sample (solvent alone). The results of neutral loss scans (losses of 130 for agaritine-Asp and 144 for agaritine) confirmed that agaritine-Asp was not present (data not shown). Based on these results, only *Agaricus* spp. mushrooms synthesize agaritine, which arise from the condensation of HMPH with glutamic acid. This is the first time that agaritine derivatives have been analyzed.

3.4. Agaritine in mouse plasma

The analytical method for mushrooms was applicable for mouse plasma. The agaritine content in the plasma was analyzed using MRM at *m/z* 266–248 given the higher sensitivity and lower background level was lower. Agaritine was detected (0.06 μg/ml) in the mouse plasma 20 min after agaritine administration (4.0 mg/kg). As shown in Fig. 6, agaritine was determined in mouse plasma without any unspecific peaks in the MRM chromatogram. Together with the results of the mushroom samples, it is clear this LC/MS/MS method would be very useful in quantifying agaritine in both mushrooms and plasma.

3.5. Accuracy, precision and recovery

Following the development of this new method for the determination of agaritine, the novel technique was validated. Table 1 summarizes the recoveries of agaritine from the spiked mushroom samples. The recoveries of agaritine from *Agaricus* product, *L. edodes* and *P. porrigens* were 114, 60.3 and 91.6, respectively. A matrix standard was used in the *P. porrigens* samples due to a suppression of the ionization in the *P. porrigens* samples.

Table 1
Recoveries of agaritine from three mushrooms and *Agaricus* product ($n = 3$)

	Recoveries (%)
Product A from *Agaricus blazei* Murill	114
Product B from *Agaricus blazei* Murill	92
Shiitake (*Lentinula edodes*)	60.3
Sugihiratake (*Pleurocybella porrigens*)	91.6

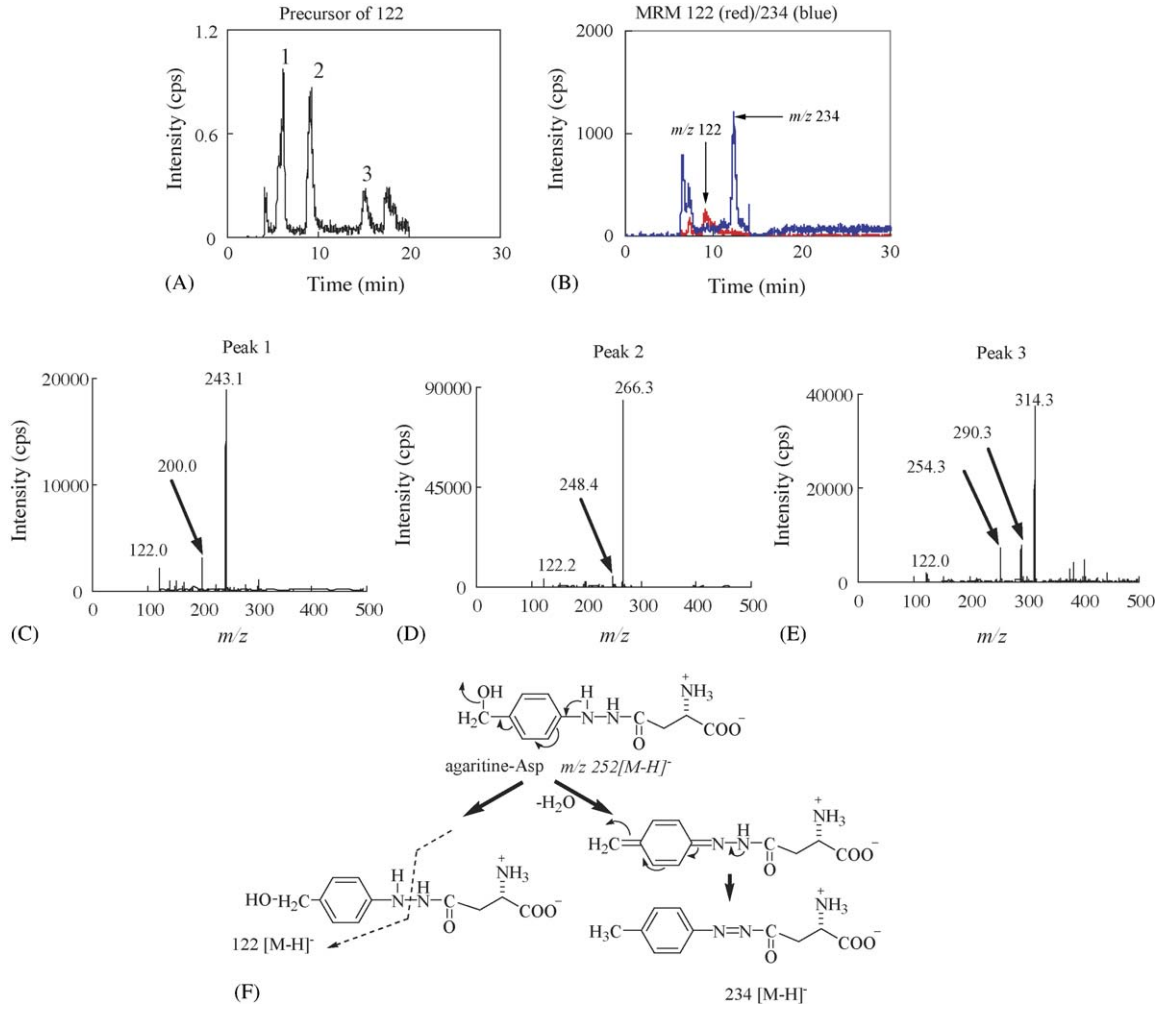

Fig. 5. A precursor ion scan of *m/z* 122 to investigate the agaritine derivatives. The fragment ion of *m/z* 122 was produced by cleavage of the N—N bond and elimination of the glutamyl moiety. The fragment ion is not dependent on the right part of agaritine like glutamic acid (F). Thus, agaritine-Asp will be detected by the precursor ion scan if it is present. An MRM analysis (*m/z* 252–234 in blue, 252–122 in red) was carried out to confirm the presence or absence of agaritine-Asp. Collision energies of −20 and −30 eV were required to obtain these results. (A) Precursor ion scan (*m/z* 122) of *A. blazei* Murill; (B) MRM chromatograms; (C–E) Mass spectra of peaks 1–3 in the chromatogram shown in (A); (F) fragmentation of agaritine-Asp. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Accuracy and precision for the determination of agaritine in mushroom samples (data are based upon assay of triplicate on three different days)

	Added (μg/ml)	Founded (μg/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Accuracy (%)
Product C from *Agaricus blazei* Murill	5.0	4.4	4.2	15.0	−11.5
Maitake mushroom (*Grifola frondose*)	5.0	4.1	5.5	23.0	−18.1

Table 2 shows the intra- and inter-day accuracy and precision values for two mushrooms. The intra-day precisions values for the *Agaricus* product (Product C) and *G. frondosa* were 4.2 and 5.5%, and the inter-day precisions values for the two species were 15.0 and 23.0%, respectively. The accuracy varied from −11.5 to −18.1%. These values were acceptable for the labile hydrazine agaritine in mushrooms.

The recovery of agaritine from agaritine-administered mice is shown in Table 3.

Table 3
Recoveries of agaritine from agaritine-administered mice ($n = 11$)

	Recoveries (%)
Mouse plasma	73 ± 4.4

The value is shown as mean ± S.D.

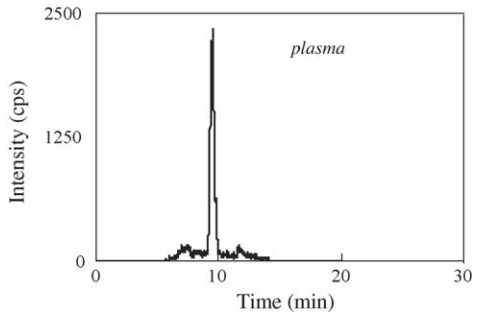

Fig. 6. A representative MRM chromatogram of mouse plasma 20 min after agaritine administration. The fragment ion of *m/z* 248 was monitored to quantify the agaritine in agaritine-administered mice.

4. Conclusion

The LC/MS/MS method described here demonstrated a high sensitivity and specificity for the quantification of agaritine in both various species of mushrooms and deproteinized mouse plasma. Even a trace amount of agaritine in both samples can be determined using this method. Additionally, we investigated for the first time agaritine-Asp, which is an agaritine derivative, and HMPH, which is a degradation product. No evidence was found of the presence of these compounds in the mushrooms in this study. The high specificity and versatility of this method make it a valuable tool for further identification-based research.

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