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Determination of muscimol and ibotenic acid in *Amanita* mushrooms by high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry

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Abstract

A reliable analytical method was developed for the quantification and identification of muscimol (MUS) and ibotenic acid (IBO), the toxic constituents of *Amanita muscaria* and *Amanita pantherina*. MUS and IBO were extracted from mushrooms by aqueous methanol and derivatized with dansyl chloride (DNS-Cl). After extraction with ethyl acetate and evaporation of the solvent, the residue was ethylated with 1.25 M hydrogen chloride in ethanol. The resulting derivatives were quantified by high-performance liquid chromatography with UV detection and identified by liquid chromatography electrospray ionization tandem mass spectrometry. Calibration curves were linear in the range of 25–2500 ppm for MUS and 40–2500 ppm for IBO, respectively. This method was successfully applied to identify and quantify MUS and IBO in *Aminita* mushrooms naturally grown and circulated in the drug market.

Keywords: *Amanita muscaria*; *Amanita pantherina*; Muscimol; Ibotenic acid; Dansylation; HPLC; LC/MS

1. Introduction

Amanita muscaria and *Amanita pantherina* are toxic mushrooms grown in North America, Europe, Africa, and Japan [1]. Accidental poisoning has been caused by ingestion of these species in several countries, including Japan [2–4]. In recent years, it has been reported that young people in several countries have intentionally eaten *A. muscaria* to evoke hallucinations [5,6]. In Japan, not only *A. muscaria* but also *A. pantherina* can be purchased via the Internet or in "smoke shops".

The symptoms caused by *A. muscaria* are as follows: neuropsychiatric symptoms (dizziness, nervousness, euphoria, exhilaration, drowsiness, altered perceptions), gastrointestinal disturbance (nausea, vomiting, diarrhea), and muscular symptoms (muscle twitches, numbness in the limbs) [7]. These mushrooms each contain two major active constituents, muscimol (MUS) and ibotenic acid (IBO). Ingestion of purified MUS and IBO caused symptoms similar to those experienced after ingestion of either of these mushrooms [8,9].

There are several reports on the contents of MUS and IBO in *A. muscaria* and *pantherina*. Determination of MUS and IBO in mushrooms was performed using paper chromatography [10], high-performance liquid chromatography (HPLC) [11], single-column chromatography [12], and gas chromatography/mass spectrometry (GC/MS) [13,14].

Analysis of MUS and IBO by liquid chromatography/mass spectrometry (LC/MS) has been scarcely reported, because these compounds are not retained on reversed-phase columns without ion-pair reagents. Mohri et al. analyzed them by LC/MS on an octadecylsilyl column with a mobile phase containing heptafluoropropionic acid as a volatile ion pair reagent [15]. However, there were few application data in their report.

Precolumn derivatization with dansyl chloride (DNS-Cl) prior to HPLC is a major analytical technique for the assay of amino acids. DNS-Cl reacts with primary and secondary amino groups and provides very stable derivatives. Dansyla-

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tion improves the retention of amino acids to reversed-phase columns. Classically, the applications of dansyl derivatives generally employ UV or fluorescence detection. However, some reports used dansylation to enhance liquid chromatographic-mass spectrometric determination [16–18]. The objective of the present study was to quantify MUS and IBO under UV detection and to identify them by liquid chromatography-tandem mass spectrometry (LC/MS/MS).

2. Experimental

2.1. Specimens

2.1.1. Amanita mushrooms

All *Amanita* mushrooms used in this study were identified by macroscopic and microscopic examinations according to our methods previously reported [14]. All samples were stored at 4 °C until analysis.

(a) *Amanita* mushrooms naturally grown: Seven *A. muscaria* mushrooms were collected in Nagano Prefecture in October 2005. The fresh fruit bodies were dried in a desiccator until their weight had plateaued.
(b) *Amanita* mushrooms purchased in the drug market: These were dried mushrooms sold as *A. muscaria* (two samples) or *A. pantherina* (three samples). These samples were obtained via the Internet in August 2005.

2.1.2. Edible mushrooms

These were purchased at a supermarket in Chiba prefecture. Six kinds of edible mushrooms (*Lentinus edodes*, *Flammulina velutipes*, *Pleurotus ostreatus*, *Grifola frondosa*, *Pleurotus eryngii*, *Agaricus bisporus*) were used in this study.

2.2. Chemicals

IBO hydrate was obtained from Biosearch Technologies (Novato, CA, USA). MUS was obtained from Sigma (St. Louis, MO, USA). DNS-Cl was obtained from Wako Pure Chemical Industries (Osaka, Japan). Hydrogen chloride (1.25 M) in ethanol was obtained from Fluka (Buchs, Switzerland). All other chemicals used in the experiments were of analytical grade.

2.3. Standard solutions

IBO hydrate and MUS were dissolved in distilled water to provide final concentrations of 1 mg/ml as stock solutions. Working standard solutions (0.5–100 μg/ml) used for calibrations were prepared by serial dilution with distilled water. These solutions were stored at −20 °C and were stable for 2 months.

2.4. Treatment of samples for analysis and derivatization procedures

2.4.1. Procedure for extracting MUS and IBO

The procedure for extracting MUS and IBO followed the method previously reported [14]. (In reference [14], MUS and IBO were determined by gas chromatography/mass spectrometry after trimethylsilyl derivatization.) The dried mushrooms were cut into sections of caps and stems. Each section was ground to a fine powder in a mortar. Two milliliter of a mixture of methanol/water (7:3, v/v) was added to 50 mg of the powder in a tapered test tube, followed by shaking for 1 min at room temperature and ultrasonication for 5 min at room temperature. After centrifugation at 3000 rpm for 3 min at room temperature, the supernatant was transferred to another glass test tube. The residue was extracted once more with 2 ml of the aforementioned mixture, shaked, ultrasonicated, centrifuged, and transferred in the same way. One hundred microliter of combined extract was transferred to a separate glass vial and the solution was evaporated under a stream of nitrogen until dry at 50 °C.

2.4.2. Dansylation

Dansylation was performed using the method described by Tapuhi et al. [19] with minor modifications. The reaction temperature and time were optimized in the preliminary study. After the dried residues were redissolved in 100 μl of borax solution (25 mM, adjusted to pH 9.5 with 100 mM NaOH), 50 μl of DNS-Cl solution (20 mM in acetonitrile, freshly prepared) was added to the sample solution and mixed. The mixture was allowed to react for 90 min at room temperature. The reaction was stopped by the addition of 10 μl of ethanolamine solution (2 v/v% in the aforementioned borax solution).

2.4.3. Ethylation

In the preliminary study, DNS-IBO could not be separated from the intrinsic matrices of the mushrooms (data not shown). Therefore, the samples were ethylated following dansylation to convert DNS-IBO to DNS-IBO ethyl ester (DNS-IBO-Et).

One milliliter of the borax solution was added to the dansylated solution after it was transferred to a tapered test tube. Three milliliter of ethyl acetate was added to the solution, followed by shaking for 5 min at room temperature and then centrifugation for 3 min at room temperature. The upper ethyl acetate layer was transferred to another tube using a disposable glass pipette. The aqueous layer was extracted twice more with 6 ml (3 ml × 2) of ethyl acetate, shaked centrifuged, and transferred in the same way. The combined ethyl acetate (total 9 ml) was evaporated under a stream of nitrogen until dry at 50 °C. The residues were derivatized by 100 μl of 1.25 M hydrogen chloride in ethanol at 55 °C for 60 min. The reaction was stopped by evaporation of the reagent under a stream of nitrogen at 55 °C. The residues were reconstituted in 100 μl of ethanol-water (1:1, v/v), and filtered through 0.45 μm membrane (UltrafreeMC, Millipore, Bedford, MA, USA) before HPLC analysis. A 20 μl aliquot was used in the analysis. Samples were placed in an autosampler (4 °C) for less than 24 h.

2.5. Calibration curve

Pleurotus ostreatus was selected as a blank mushroom with which to construct calibration curves of MUS and IBO, because it exhibits no interfering peaks near the peaks of DNS-MUS and DNS-IBO-Et. The mushroom was weighed and extracted

as described in Section 2.4.1. The resulting solution was used as blank solution for preparing MUS and IBO spiked solution to construct the calibration curves.

The calibration curves were constructed by an external standard method. The regression parameters for the slope, intercept, and correlation coefficient were calculated by weighted ($1/x$) linear regression using Correlation2-2 freeware (http://homepage3.nifty.com/m_nw/j-frame.htm).

2.6. Validation procedure

Four replicates of blank samples were used to calculate the limit of detection (LOD) and the limit of quantification (LOQ) under UV detection. The LOD and LOQ were expressed as $3\delta/S$ and $10\delta/S$, respectively (δ: the standard deviation of the blank responses, S: the slope of the calibration curve).

Tests to determine the precision and accuracy of this procedure were performed using the standard spiked blank mushroom. The concentrations spiked were as follows: 40, 400, and 2000 ppm for MUS and 60, 400, and 2000 ppm for IBO. Intra- and inter-assay precision was also evaluated by analyzing three kinds of *Amanita* mushroom extracts repeatedly.

The accuracy of the assay was evaluated by percent deviation (%DEV) from the nominal concentration using the formula: [%DEV = 100 × (mean back-calculated concentration − nominal concentration)/nominal concentration]. Intra- and inter-assay precision was expressed as the coefficient of variation (CV, %) of the experimental values at each concentration.

2.7. Recovery experiments

The known amounts (10, 25, and 100 μg) of MUS and IBO were spiked into 50 mg of fine powder of the cap of *A. muscaria*. The concentrations were determined by the validated method.

2.8. Apparatus and chromatographic conditions

The HPLC system consisted of an LC-10ADvp series (including a degasser, a binary pump, and an autosampler; Shimadzu, Kyoto, Japan) liquid chromatograph equipped with an SPD-M10ADvp diode array detector set at 256 nm. Chromatographic separation was performed with a Symmetry C18 column (150 mm × 2.1 mm, 3.5 μm, Waters, Milford, MA, USA) maintained at 40 °C. The mobile phase was 10 mM ammonium acetate/acetonitrile with a constant flow rate of 0.2 ml/min. The acetonitrile percentages were: 0–1 min, 30%; 1–25 min, linearly from 30 to 90%; 25–30 min, 90%; 30–31 min, linearly from 90 to 30%; 31–46 min (equilibration step), 30%.

The effluent from the diode array detector was injected into an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface in the positive mode. The main mass conditions were: capillary voltage: 30 V, tube lens offset: 55 V, spray voltage: 6 kV, capillary temperature: 300 °C, sheath gas flow: 72 l/h. The mass data were collected in the product ion scan mode. The MS/MS conditions were: collision energy: 14%, precursor ions: m/z 347 for DNS-MUS and m/z 419 for DNS-IBO-Et.

3. Results and discussion

3.1. Chromatographic separation under UV and MS/MS detection

Fig. 1 illustrates a typical UV chromatogram obtained from the extract of an *A. muscaria* and blank mushroom (*Pleurotus ostreatus*) extract. The peaks for DNS-MUS and DNS-IBO-Et had retention times of 24.4 min and 25.7 min, respectively. These peaks were separated from each other without any interfering peaks. However, a small peak having a retention time close to those of the two compounds was detected from some edible mushrooms such as *Flammulina velutipes*, *Grifola frondosa*, and *Agaricus bisporus*.

Identification of DNS-MUS and DNS-IBO-Et peaks detected under UV was based on their product ion spectra. The base peaks of the mass spectra of the two derivatives under scan mode were m/z 347 for DNS-MUS and m/z 419 for DNS-IBO-Et (data not shown). These ions, which corresponded to M^+, were selected as the precursor ions for the two derivatives. The product ion spectra of the two derivatives are shown in Fig. 2. For identification, specific fragment ions were selected as diagnostic ions as follows: DNS-MUS (m/z 317, 276, 226, 183) and DNS-IBO-Et (m/z 355, 235, 183). The fragment ion m/z 171 is common to dansyl derivatives and not specific to DNS-MUS

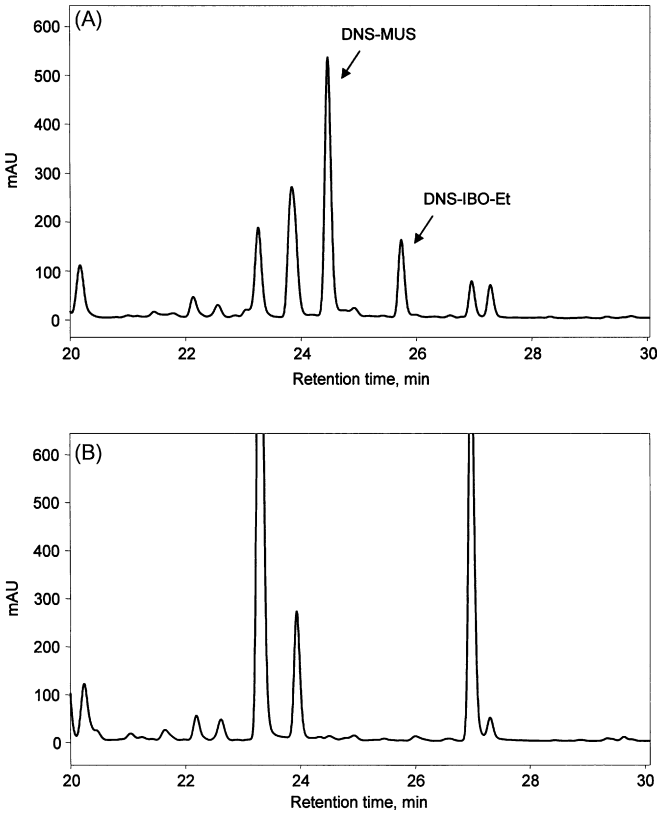

Fig. 1. Chromatograms obtained from *A. pantherina* (sample #5) extract (A) and blank mushroom (*Pleurotus ostreatus*) extract (B). Detection: 256 nm.

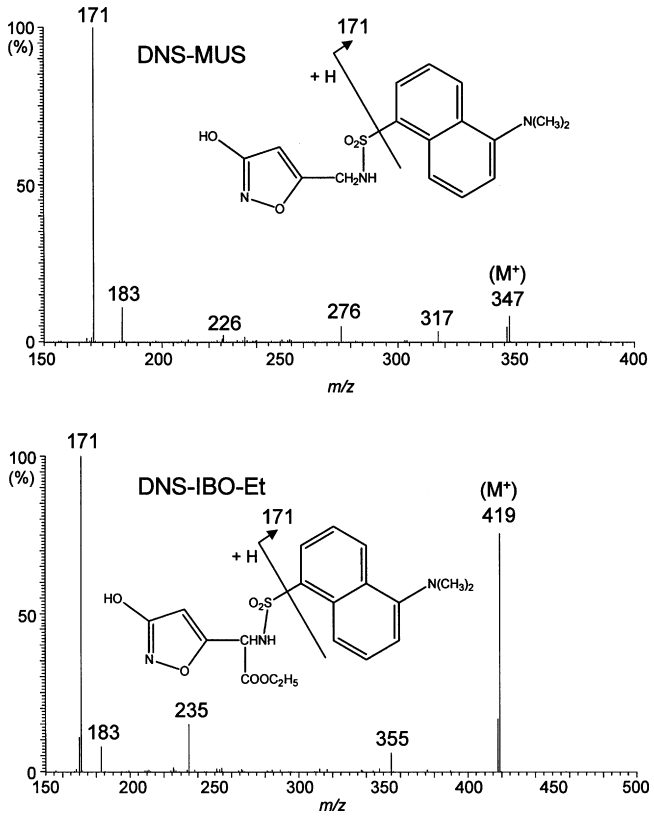

Fig. 2. Product ion spectra at *m/z* 347 from DNS-MUS and *m/z* 419 from DNS-IBO-Et.

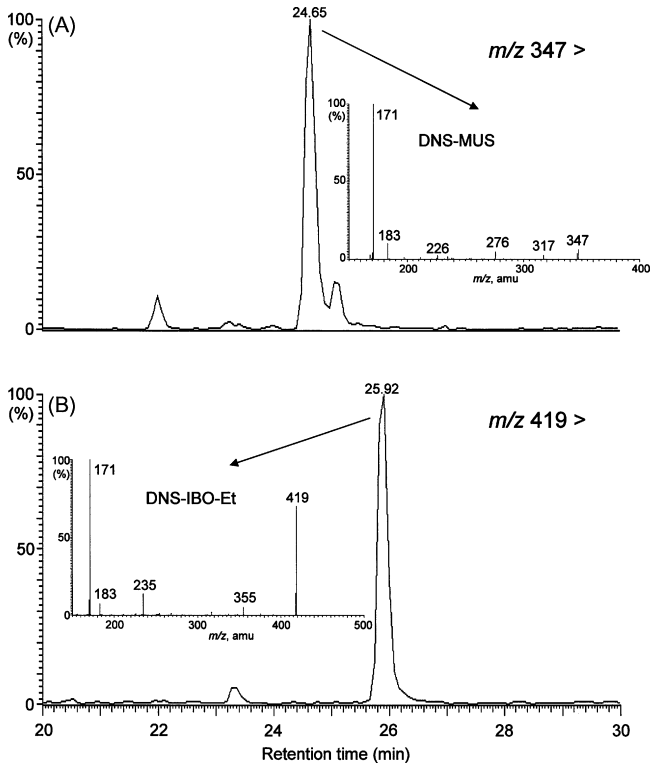

Fig. 3. LC/MS/MS analysis of the cap of *A. pantherina* (sample #5). (A): Total ion chromatogram in the product ion scan mode using *m/z* 347 and product ion spectrum at *m/z* 347 for the 24.65-min peak. (B): Total ion chromatogram in the product ion scan mode using *m/z* 419 and product ion spectrum at *m/z* 419 for the 25.92-min peak.

and DNS-IBO-Et. Therefore, this fragment ion was not used for identification.

Fig. 3 illustrates typical chromatograms obtained from a cap of *A. pantherina* in the product ion scan mode. The product ion spectra obtained from peaks eluting at 24.7 min and 25.9 min matched those of DNS-MUS and DNS-IBO-Et (shown in Fig. 3), respectively. This confirmed the presence of MUS and IBO. On the other hand, the extract of the aforementioned edible mushrooms did not give the matched spectra at the same retention time as the two derivatives. Therefore, quantification using HPLC (UV detection) was necessary following identification by LC/MS/MS.

3.2. Optimization of the ethylation conditions

For the evaluation of optimal ethylation conditions for the dansylated samples, reaction temperature (Fig. 4) and time (Fig. 5) were tested using *A. muscaria* extract. Judging from peak areas and their variations of DNS-MUS and DNS-IBO-Et, the optimized temperature and time were 55 °C and 60 min, respectively. Degradation of DNS-MUS was not indicated under this optimized ethylation condition.

3.3. Calibration curve

Calibration curves were established with *y* for the peak area of each derivative and with *x* for the concentration (ppm) of the analyte in the mushrooms. In the preliminary study, the slopes of the calibration curves prepared by the external standards method and the standard addition method were almost the same (3754.3 versus 3673.7 for MUS and 1254.3 versus 1391.8 for IBO). Therefore, in this study, all samples were quantified using external calibration. Calibration curves were linear in the range of 25–2500 ppm for MUS and 40–2500 ppm for IBO, respectively. The linear regression equations ($n = 5$, mean ± SD) obtained were $y = (3544.4 \pm 196.0)x - (12292.6 \pm 8573.6)$ for MUS and $y = (1321.6 \pm 258.2)x + (18348.2 \pm 15653.2)$ for IBO. The correlation coefficients were routinely greater than 0.998.

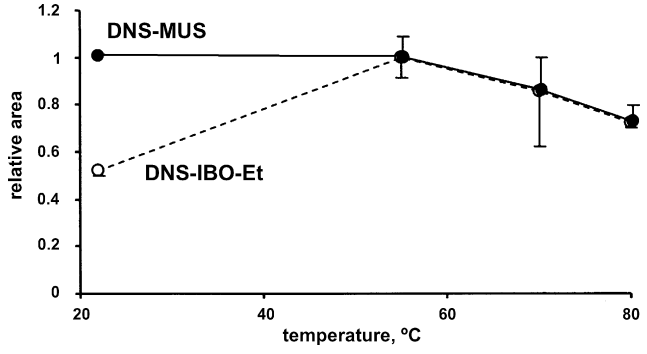

Fig. 4. Optimization of the reaction temperature for ethylation of DNS-IBO-Et. Each sample was heated for 60 min at 22 °C (room temperature), 55 °C, 70 °C, or 80 °C. Each datum indicates a relative peak area normalized to that of 55 °C. Each data point represents the mean ± SD of three determinations.

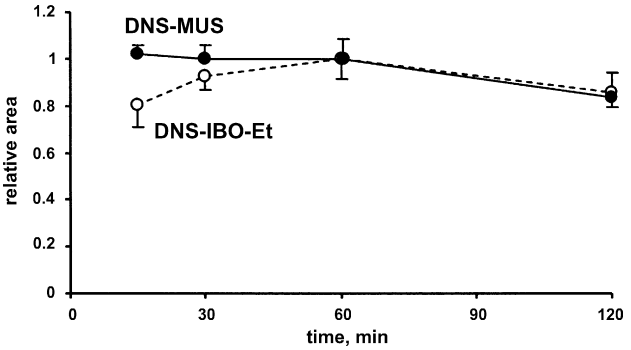

Fig. 5. Optimization of the reaction time for ethylation of DNS-IBO-Et. Each sample was heated at 55 °C for 15 min, 30 min, 60 min, or 120 min. Each datum is indicated as relative peak area normalized to that of 60 min. Each data point represents the mean ± SD of three determinations.

Table 1
Analytical accuracy and precision evaluated using standard spiked blank mushrooms

Concentration known (ppm)	Concentration found (ppm)	CV (%)	%DEV
MUS			
Intra-assay (n = 6)			
40	41.3 ± 2.6	6.4	3.2
400	420 ± 22	5.2	5.1
2000	1988 ± 81	4.1	−0.6
Inter-assay (n = 3)			
40	39.4 ± 3.4	8.7	−1.5
400	394 ± 11	2.9	−1.4
2000	1987 ± 35	1.8	−0.6
IBO			
Intra-assay (n = 6)			
60	52.6 ± 5.4	10.2	−12.4
400	441 ± 28	6.3	10.3
2000	1978 ± 156	7.9	−1.1
Inter-assay (n = 3)			
60	60.7 ± 2.0	3.4	1.2
400	416 ± 7.4	1.8	3.9
2000	1901 ± 127	6.1	−5.0

3.4. Method validation

The intra- and inter-assay precision and accuracy determined using the blank mushroom extract that had been spiked with MUS and IBO are indicated in Table 1. The CV for the intra- and inter-assay was between 1.8 and 10.2% at three concentrations for two analytes. The accuracy of the intra- and inter-assay was between −12.4 and 10.3% deviation from nominal values.

Table 2 indicates intra- and inter-assay precision evaluated using *Amanita* mushroom extracts. The CV for the intra- and inter-assay was between 1.8 and 13.4% for three samples.

The lower limits of identification were defined as the lowest concentration obtained at the same relative intensities of the aforementioned diagnostic ions of product ion spectra as the standards. The lower limits of identification were 25 ppm for both MUS and IBO. Under UV detection, the calculated LODs/LOQs of MUS and IBO were 1.4 ppm/4.6 ppm for MUS and 7.8 ppm/25.9 ppm for IBO. These LODs and LOQs were considered adequate for the purposes of the present study.

Table 2
Precision evaluated using *Amanita* mushrooms

Sample	Intra-assay		Inter-assay	
	Concentration (ppm)	CV (%)	Concentration (ppm)	CV (%)
MUS				
A	353 ± 21	5.9	354 ± 24	6.9
B	1242 ± 22	1.8	1267 ± 81	6.4
C	46.2 ± 1.2	2.7	44.2 ± 5.9	13.4
IBO				
A	571 ± 25	4.5	580 ± 53	9.1
B	917 ± 18	1.9	943 ± 116	12.3
C	ND		ND	

n = 3, mean ± SD.

The stabilities of DNS-MUS and DNS-IBO-Et in the autosampler after completing the ethylation procedure were studied for a period of 24 h. No relevant degradation was observed, with differences from initial concentrations (400 ppm) lower than 5%.

3.5. Recovery experiments

The results were shown in Table 3. The recoveries of MUS and IBO were between 95.4 and 101.1%, and the CV was between 1.8 and 5.5%. The developed sample preparation procedure showed high recovery.

3.6. Key points to improve the method ruggedness

In this study, quantitative experiments were performed using an external standard method. This reason was that we were not able to find the appropriate compound as an internal standard. Generally, quantification precision of the external standard method is somewhat lower than that of the internal standard method.

One of the considered points to maintain the ruggedness of quantitation was replication in triplicate of the liquid–liquid extraction after dansylation was three. This purpose was to reduce variation of recovery in the liquid–liquid extraction. Another important point was to quantify MUS and IBO by HPLC-UV, not by LC/MS. The sensitivity of HPLC-UV is more stable than that of LC/MS, since it may suffer from phenomena

Table 3
Recoveries of MUS and IBO added to *A. muscaria*

Spiked (μg/g samples)	Recovery (%)	CV (%)
MUS		
200	96.0 ± 3.7	3.8
500	95.4 ± 2.4	2.5
2000	101.1 ± 1.8	1.8
IBO		
200	99.4 ± 5.4	5.5
500	100.9 ± 2.5	2.4
2000	97.6 ± 4.8	4.9

n = 5, mean ± SD.

Table 4
MUS and IBO contents of *A. muscaria* naturally grown

Sample	MUS (ppm)		IBO (ppm)	
	Cap	Stem	Cap	Stem
1	381	–	623	–
2	46	–	182	–
3	317	–	528	–
4	599	292	615	627
5	204	82	785	1998
6	859	–	1469	–
7	1203	159	1839	751
Mean	516	178	863	1125
Max.	1203	292	1839	1998
Min.	46	82	182	627

(–): no sample.

such as ion suppression and contamination of the sample cone which caused deterioration of the sensitivity.

3.7. Method application

The method developed herein was applied to determine the contents of MUS and IBO in *Amanita* mushrooms naturally grown and in those purchased on the drug market. The concentrations in the samples are shown in Table 4 (naturally grown) and Table 5 (purchased on the drug market).

In the naturally grown *A. muscaria*, the mean of the MUS contents in the caps and stems was approximately ten times that reported by Tsunoda et al. [20], and the mean of the IBO contents in the caps and stems also tended to be higher than that reported by those authors.

In the *Amanita* mushrooms purchased on the drug market, the MUS/IBO levels in the stems of many samples were not detected or were below the limit of quantification, unlike the case with the ones naturally grown. Maruyama et al. reported that the genotype of *A. muscaria* was different between "naturally grown in Japan" and "purchased on the Japanese drug market" [21]. They also suggested that *A. muscaria* purchased on the drug market of Japan was imported from abroad. Michelot and Melendez-Howell described in their review that the MUS/IBO contents depended on the growing environment [22]. Therefore, we think that the differences in distribution of the active constituents between "naturally grown in Japan" and "purchased on the drug market" were caused by the genotype and/or the growing environment.

Table 5
MUS and IBO contents of *Amanita* mushrooms circulated in the drug market

Sample no.	Species	MUS (ppm)		IBO (ppm)	
		Cap	Stem	Cap	Stem
11	*A. muscaria*	40	62	<40	<40
12	*A. muscaria*	1318	ND	1277	ND
13	*A. pantherina*	332	<25	165	ND
14	*A. pantherina*	302	<25	ND	ND
15	*A. pantherina*	1233	109	843	<40

ND: not detected.

Tsunoda et al. reported that MUS and IBO in the mushrooms were stable for 90 days under dry condition at room temperature [23]. All our samples were sufficiently dried and were stored in plastic bags at 4 °C. These were analyzed within 3 months of acquisition. Therefore, it is unlikely that MUS and IBO decomposed in storage.

In conclusion, we developed a reliable method for MUS and IBO using HPLC under UV detection for quantification and LC/MS/MS for identification, respectively. The method was based on dansylation and ethylation of the analytes. The benefits of our method in comparison with previous reports are good retention and selectivity without ion-pair reagents and enhanced sensitivity of detection under UV and ESI-MS. This method was effective for the analysis of MUS and IBO in *Amanita* mushrooms.

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