Zawartości IBO/MUS w grzybach świeżych (Raw) a w grzybach suszonych (Dry)

Tutaj omówię dwie prace Tsujikawy i jedna Paliwody.
Paliwoda 2014: 38 próbek A. muscaria suszonych w temperaturze pokojowej, zebranych w południowo-zachodniej Polsce w okresie wrzesień – listopad w latach 2008-2011
Tsujikawa 2006: 5 próbek A. muscaria suszonych (nie wiadomo jak) zakupionych gdzieś w Japonii na ryneczku :)
Tsujikawa 2007: 7 próbek A. muscaria suszonych w temperaturze pokojowej, zebranych w okręgu Nagano w październiku 2005

Tabelka 3.1. Zawartości IBO/MUS w suszonych próbkach A. muscaria
Wszystkie wartości podane w ppm (części na milion).

Dry A. muscaria	Ilość próbek	IBO	MUS	IBO	MUS	IBO	MUS
		Min		Max		Mean	
Anna Poliwoda 2014	38	292	73	6565	3561	2341	1064
Kenji Tsujikawa 2006	5	10	46	2845	1052	781	422
Kenji Tsujikawa 2007	7	809	128	3837	1495	1988	694

Taka sama analiza jak w przypadku owocników świeżych została przeprowadzona:

Dla badania z 2014:

IBOmax = 22,5 x IBOmin (około 22 razy więcej)
MUSmax = 48,8 x MUSmin (około 49 razy więcej)
IBO = 2,2 x MUS (około 2 razy więcej)

Dla badania z 2006:

IBOmax = 284,5 x IBOmin (około 284 razy więcej)
MUSmax = 22,9 x MUSmin (około 23 razy więcej)
IBO = 1,8 x MUS (około 2 razy więcej)

Dla badania z 2007:

IBOmax = 4,7 x IBOmin (około 5 razy więcej)
MUSmax = 11,7 x MUSmin (około 12 razy więcej)
IBO = 2,9 x MUS (około 3 razy więcej)

A wiec dla trzech badań

IBOmax = 4,7 do 284,5 x IBOmin (około 5 do 284 razy więcej)
MUSmax = 11,7 do 48,8 x MUSmin (około 12 do 49 razy więcej)
IBO = 1,8 do 2,9 x MUS (około 2 do 3 razy więcej)

Można te wyniki zebrać w tabelce i zestawić z tymi uzyskanymi dla owocników świeżych (Raw)

Dry	Ilosc probek	IBOmax = X IBOmin	MUSmax = X MUSmin	IBO = X MUS
		X =	X =	X =
PALIWODA 2014	38	22	49	2
TSUJIKAWA 2006	5	284	23	2
TSUJIKAWA 2007	7	5	12	3
zakres z trzech badań		22 ÷ 284	12 ÷ 49	2 ÷ 3
Raw	**Ilosc probek**	**IBOmax = X IBOmin**	**MUSmax = X MUSmin**	**IBO = X MUS**
		X =	X =	X =
TSUNODA 1989	8	1.8	1.6	16.7
TSUNODA 1990	62	4.1	3.6	15.6
TSUNODA 1993	12	2.4	6.5	57.7
zakres z trzech badań		2 ÷ 4	2 ÷ 6	17 ÷ 58

Widać, że:
- zakres wartości minimalnych i maksymalnych zarówno IBO jak i MUS jest większy niż przy badanych owocnikach świeżych, natomiast
- zakres średnich wartości IBO do MUS jest wielokrotnie mniejszy i wynosi od 2 do 3, czyli ibotnenu jest nadal (średnio) więcej w owocnikach suszonych niż muscymolu, lecz zakres ten jest o wiele mniejszy i właściwie stały: średnio 2,5 razy więcej IBO niż MUS a nie 17 do 58 jak dla owocników świeżych.

Jednakże, analiza poszczególnych badan i próbek rzuca jeszcze ciekawsze światło na omawiane zagadnienie. Zacznijmy od polskiego badania z 2014:

Paliwoda 2014: 38 próbek A. muscaria suszonych w temperaturze pokojowej, zebranych w południowo-zachodniej Polsce w okresie wrzesień – listopad w latach 2008-2011

Tabelka 3.2. Zawartości IBO/MUS w suszonych(w temperaturze pokojowej) próbkach A. muscaria dla badania Paliwoda 2014

Anna Poliwoda I inni 2014 **Determination of muscimol and ibotenic acid in mushrooms of Amanitaceae by capillary electrophoresis**					
Sample no.	***Amanita* sp.**	**Substratum**	**Ecosystem**	**MUS**	**IBO**
1	*A. muscaria*	Soil	Fallow land	989	1710
2	*A. muscaria*	Soil	Lawn planted with trees/shrubs	1641	454
3	*A. muscaria*	Soil—humus layer	Woodland and scrub	480	3589
4	*A. muscaria*	Soil—humus layer	Woodland and scrub	0	0
5	*A. muscaria*	Soil	Lawn planted with trees/shrubs	143	3380

6	*A. muscaria*	Soil	Woodland and scrub	2436	3186
7	*A. muscaria*	Soil	Woodland	1221	0
8	*A. muscaria*	Soil	Woodland	374	6565
9	*A. muscaria*	Soil	Fallow land/field	339	4731
10	*A. muscaria*	Soil	Fallow land/field planted with trees/shrubs	1841	0
11	*A. muscaria*	Soil	Woodland and scrub	710	4444
12	*A. muscaria*	Soil	Woodland	1473	1536
13	*A. muscaria*	Soil	Woodland	335	1060
14	*A. muscaria*	Soil	Woodland	1124	671
15	*A. muscaria*	Soil	Woodland	1892	1704
16	*A. muscaria*	Soil	Woodland	1013	390
17	*A. muscaria*	Soil	Woodland	603	1759
18	*A. muscaria*	Soil	Woodland	528	2576
19	*A. muscaria*	Soil	Woodland	597	5240
20	*A. muscaria*	Soil	Woodland	3561	0
21	*A. muscaria*	Soil	Woodland	174	1455
22	*A. muscaria*	Soil	Parkland and scattered trees	0	0
23	*A. muscaria*	Soil	Allotment	450	646
24	*A. muscaria*	Soil	Woodland	2303	292
25	*A. muscaria*	Soil	Woodland and scrub	906	0
26	*A. muscaria*	Soil	Woodland and scrub	270	2040
27	*A. muscaria*	Soil	Woodland and scrub	725	3128
28	*A. muscaria*	Soil	Woodland and scrub	1991	1525
29	*A. muscaria*	Soil	Woodland and scrub	313	821
30	*A. muscaria*	Soil	Woodland and scrub	0	0
31	*A. muscaria*	Soil	Fallow land/ field planted with trees/shrubs	1387	2065
32	*A. muscaria*	Soil	Fallow land/ field planted with trees/shrubs	0	0
33	*A. muscaria*	Soil	Allotment	73	0
34	*A. muscaria*	Soil—humus layer	Mountain grassland	0	0
35	*A. muscaria*	Soil—humus layer	Mountain grassland	1945	865
36	*A. muscaria*	Soil—humus layer	Mountain grassland	634	6469
37	*A. muscaria var. muscaria*	Soil—humus layer	Woodland	508	920
38	*A. muscaria var. muscaria*	Soil—humus layer	Fallow land/ field planted with trees/shrubs	2159	0
			A. muscaria Max (detected)	3561	6565
			A. muscaria Min (detected)	73	292
			A. muscaria Mean	1064	2341

Wnioski:
- tylko w trzynastu próbkach (34%) znaleziono więcej muscymolu niż ibotenu,
- w sześciu z tych trzynastu nie znaleziono ibotenu w ogóle,
- w pięciu nie znaleziono ani ibotenu ani muscymolu (!)
- 60% próbek (pomijając te gdzie nie znaleziono nic) zawierało więcej ibotenu niż muscymolu

- maksymalna ilość muscymolu w próbce nr 20 (3561 ppm) przewyższała ilości ibotenu aż w dwudziestu jeden próbkach (!)
- w próbce numer 8 znaleziono aż 6565 ppm ibotenu i żadna inna próbka nawet nie zbliżyła się do tej wartości jeżeli chodzi o muscymol, tylko próbka nr 20 zawierała około połowę tej wartości (3561 ppm)
- minimalna zawartość ibotenu w próbce nr 24 (292 ppm) przewyższała ilości muscymolu tylko w czterech próbkach (!)
- najwięcej ibotenu i muscymoly znaleziono w próbkach pochodzących z ekosystemów: teren lesisty i górskie łąki co przedstawiono w tabelce poniżej

Tabelka 3.3. Zawartości IBO/MUS w suszonych próbkach A. muscaria w zależności od ekosystemu dla badania Paliwoda 2014

Ecosystem	MUS (ppm)		IBO (ppm)	
	min	max	min	max
Fallow land/field (planted with trees/shrubs)	0	2159	0	4731
Lawn planted with trees/shrubs	143	1641	454	3380
Woodland and scrub	0	2303	0	3589
Woodland	174	3561	0	6565
Mountain grassland	0	1945	0	6469

Jak sama autorka pisze:
Można założyć, że tak duże zróżnicowanie zawartości badanych halucynogennych izoksazoli może wynikać z szeregu czynników. Czynniki te są następujące:
- charakter środowiska, w którym wyhodowano analizowanego grzyba (rodzaj ekosystemu, warunki temperaturowe, wilgotność, pH gleby itp.),
- wiek grzybów i etap rozwojowy, a także sposób przechowywania próbek.
Podobny związek, a mianowicie zależność stosunku MUS / IBO od środowiska wzrostu opisali Michelot i Melendez-Howell w 2003 roku.
Zakłada się, że genotyp A. muscaria może również wpływać na różnice w stężeniu IBO w porównaniu z MUS (Tsujikawa 2007)"

Wspomnę jeszcze to co pisałem przy okazji omawiania zawartości IBO i MUS w świeżych owocnikach:
„Duże wahania zawartość IBO i MUS mogą zależeć od indywidualnych różnic okoliczności wzrostu" (Tsunoda 1990).
Zawartość substancji halucynogennych w owocniku grzyba zależy od licznych czynników środowiskowych, jak rodzaj gleby, miejsce występowania, a w szczególności pora zbioru (Benjamin 1992, Deja et al. 2014). Wiosną i latem muchomory mogą zawierać nawet dziesięciokrotnie więcej kwasu ibotenowego i muscymolu niż jesienią (Benjamin 1992). Festi i Bianchi (1992) także o tym wspominają: skutki biologiczne są powiązane z okresem zbioru, zebrane we wrześniu wywołują większe nudności a doświadczenie narkotyczne/wizjonerskie jest słabsze niż dla grzybów zebranych w sierpniu.

Paliwoda wspomina jeszcze że: „Zasadniczo zawartość obu substancji halucynogennych znacznie przekraczała stężenie zgłoszone przez Tsujikawę w grzybach z rodzaju A.

muscaria uprawianych naturalnie w Japonii", co jest dobrym wstępem do omówienia właśnie badań Tsujikawy.

Tabelka 3.4. Zawartości IBO/MUS w suszonych próbkach A. muscaria dla badań Tsujikawa 2006 i 2007

Kenji Tsujikawa I inni 2006			
Sample no.	***Amanita***	**MUS**	**IBO**
1	*A. muscaria*	612	612
2	*A. muscaria- only cap*	472	97
3	*A. muscaria- only cap*	254	342
4	*A. muscaria- only cap*	46	10
5	*A. muscaria- only cap*	1052	2845
Kenji Tsujikawa I inni 2007			
Sample no.	***Amanita***	**MUS**	**IBO**
1	*A. muscaria-only cap*	381	623
2	*A. muscaria-only cap*	46	182
3	*A. muscaria-only cap*	317	528
4	*A. muscaria*	891	1242
5	*A. muscaria*	286	2783
6	*A. muscaria-only cap*	859	1469
7	*A. muscaria*	1362	2590

Wnioski:
- tylko w 2 próbkach (18%) znaleziono więcej muscymolu niż ibotenu,
- aż 82% próbek zawiera więcej ibotenu niż muscymolu,
- jedna próbka zawiera tyle samo ibotenu co muscymolu,
- maksymalna ilość ibotenu (2845 ppm) przewyższa dwa razy maksymalna ilość muscymolu (1362 ppm),
- większość próbek przejawia tendencje gdzie ibotenu jest 2 do 2,5 razy więcej nic muscymolu.

Pomijając fakt, że badane grzyby z Polski zawierały więcej IBO i MUS (maksymalne wartości), niż te z Japonii, to bezwzględne wartości są podobne:
- 70% badanych próbek zawiera więcej ibotenu niż muscymolu w grzybach suszonych (w temperaturze pokojowej)
- 30% badanych próbek zawiera więcej muscymolu niż ibotenu w grzybach suszonych (w temperaturze pokojowej)

Sumując, spożywając muchomora suszonego w temperaturze pokojowej, mamy tylko 30% szansy, że znajdziemy tam więcej muscymolu niż ibotenu. Dlatego też, dalej omówię wydajniejsze sposoby suszenia, które zapewniają lepszy (dla zdrowia i samopoczucia przy spożyciu) stosunek tych dwóch substancji.

Ciąg dalszy nastąpi….:)

Źródła:

1. Anna Poliwoda i inni 2014, Determination of muscimol and ibotenic acid in mushrooms of Amanitaceae by capillary electrophoresis.
2. Kenji Tsujikawa i inni 2006, Analysis of hallucinogenic constituents in Amanita mushrooms circulated in Japan
3. Kenji Tsujikawa i inni 2007, Determination of muscimol and ibotenic acid in Amanita mushrooms by high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry

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Research Article

Determination of muscimol and ibotenic acid in mushrooms of Amanitaceae by capillary electrophoresis

In this study, the CZE method for rapid quantitative and qualitative determination of ibotenic acid and muscimol in *Amanita* mushrooms naturally grown in Poland was developed. The investigations included the species of *A. muscaria*, *A. pantherina*, and *A. citrina*, collected in southern region of Poland. The studied hallucinogenic compounds were effectively extracted with a mixture of methanol and 1 mM sodium phosphate buffer at pH 3 (1:1 v/v) using ultrasound-assisted procedure. The obtained extracts were separated and determined by CZE utilizing a 25 mM sodium phosphate running buffer adjusted to pH 3 with 5% content of acetonitrile v/v. The calibration curves for both analytes were linear in the range of 2.5–7000 μg/mL. The intraday and interday variations of quantitative data were 1.0 and 2.5% RSD, respectively. The recovery values of analyzed compounds were over 87%. The identities of ibotenic acid and muscimol were confirmed by UV spectra, migration time, and measurements after addition of external standard.

Keywords:
Amanitaceae / Capillary electrophoresis / Hallucinogens / Ibotenic acid / Muscimol
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1 Introduction

Amanita is the most popular genus of psychoactive basidiomycete fungus. It contains over 600 species of agarics and one of them is species *A. muscaria*, commonly known as the "fly agaric" or "fly amanita." This mushroom as a "cosmopolitan" one has been found in many regions of Europe, Asia, and North America. Because of its characteristic appearance (mushroom with a white-spotted red cap), it is impossible to confuse it with other fungi [1]. The other popular hallucinogenic representatives of *Amanita* genus are *A. pantherina* ("panther cap") and *A. citrina*, also known as the "false death cap" [2, 3]. In comparison to *A. muscaria*, *A. pantherina* has a gray-brown cap that is also covered with scattered white spots, whereas *A. citrina* has yellow or sometimes white cap with white stem, ring, and volva [3]. These species are not as popular as *A. muscaria* but they can be found in many countries. They usually grow in summer, and very often occur in the vicinity of *A. muscaria* (this applies mainly to the fungus of *A. pantherina*). The psychoactive effect of fly agaric is caused by its two main active constituents: ibotenic acid (IBO) and its breakdown product—muscimol (MUS). Besides *A. muscaria*, IBO and MUS were also isolated in large quantities from other species of *Amanita* genus, including *A. pantherina* [4], *A. strobiliformis* [5], *A. cothurnata* [6], and *A. gemmata* [7]. IBO (α-amino–3-hydroxy-5-isoxazoloacetic acid ($C_5H_6N_2O_4$)) and MUS (3-hydroxy-5-aminomethyl-1-isoxazole) belong to the group of isoxazole derivatives, rather uncommon chemical structures observed in natural products. The hallucinogenic effect of IBO in human beings is evoked at doses ranging from 50 to 100 mg, whereas MUS causes an equivalent effect at concentrations five times lower (at doses of 10–15 mg) [8]. Any attempts of drying (dehydration) IBO cause its decarboxylation, which yields quantitatively MUS. It has been previously proven by Eugster C. H., that IBO is known to be converted into MUS in the dried mushrooms, however this aspect has not been studied in detail [21]. Otherwise, Nielsen et al. described that IBO has the ability to undergo decarboxylation in the absence or presence of appropriate enzymes when biological samples are analyzed [9]. Therefore, MUS is suggested to be the primary substance responsible for hallucinogenic effects evoked in living organisms after having a meal of cooked mushrooms or gastric digestions. The full effect of intoxication is reached approximately 2–3 h after oral ingestions and usually persists for 6–8 h, but it strictly depends on the dosage of the consumed mushrooms and individual susceptibility. Both, MUS and IBO are able to cross the blood–brain barrier by active transport and then cause central nervous system (CNS) dysfunction [10]. These hallucinogenic constituents interfere with normal glutamate- and

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Abbreviations: IBO, ibotenic acid; **MUS**, muscimol

Colour Online: See the article online to view Figs. 2–4 in colour.

GABA-mediated (GABA is γ-aminobutyric acid) neurotransmissions [11]. IBO influences most of the excitatory synaptic neurotransmission as a conformation analog of glutamic acid in the mammalian brains [11]. The typical symptoms of IBO intoxication are related to the signs of central nervous system excitation such as seizures, muscle tremors, and hallucinations. On the other hand, MUS binds to the same site of the GABAA receptor complex as GABA itself. Considering the fact that GABA receptors are widely distributed in the brain, MUS alters neuronal activity in multiple regions, so its spectrum of effects is more intensive. The visual distortions, loss of equilibrium, mild muscle twitching, and hypersensitive perception are usually reported [8, 11]. Sometimes, the poisoning effect after MUS uptake has similar symptoms to those induced by atropinic plants such as *Datura stramonium* and therefore it was called "mycoatropinic" syndrome [11]. Also dryness of the mouth was observed in the case of some patients [11].

In recent years there has developed undesirable interest, especially among young people, in the intake of these hallucinogenic mushrooms to enjoy hallucination for recreational use. The 20th-century technological achievements (such as internet) make it incredibly easy to purchase hallucinogenic mushrooms without any limitations. The popularity of the so-called "smoke shops" led to situations that these psychoactive fungi can be bought just on the spot. Furthermore, *Amanita* mushrooms and their active ingredients are uncontrolled in the most countries that contributed to their spread. The growing interest in psychedelic mushrooms and their excessive consumption have become a serious medical problem of our time, resulting frequently in fatal effects [12, 13]. The risk of hallucinogenic mushrooms consumption results not only from their toxicity but the fact that they possess ambivalent potential means that they can evoke either positive or negative reactions. Therefore, the quantitative and qualitative measurement of the level of IBO and MUS is essential for monitoring poisoning status as well as for characterization of mushroom samples confiscated by the police authorities.

The determination of IBO and MUS in *Amanita* mushrooms was described in several reports [2, 4, 14–16]. Due to high polarity and amphoteric nature of both compounds, the studies included an application of 2D paper chromatography and TLC on silica gel with the application of ninhydrin as a visualization reagent, HPLC or LC with various detection modes like UV (HPLC-UV) [4, 15] and MS (HPLC-MS or LC-MS) [4, 14] as well as GC-MS [16, 17]. Considering the fact that both IBO and MUS are not retained on RP column, in some cases the requirement for derivatization with dansyl chloride or *N,O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane, before LC with MS/MS (LC-MS-MS) and GC-MS analysis, respectively, is necessary to be performed [2, 4]. However, an ion-interaction HPLC method or hydrophilic interaction described recently in literature permitted the simultaneous detection of MUS and IBO without any derivatization procedure [14]. The GC-MS analysis was also enforced by carrying out the derivatization step [16, 17].

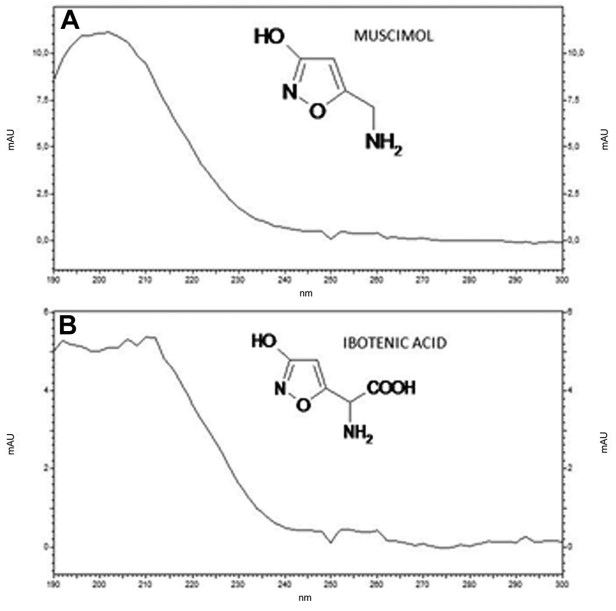

Figure 1. UV spectra (in MeCN:25 mM sodium phosphate buffer (5:95, v/v)) and molecular structures of MUS (A) and IBO (B).

According to the best of our knowledge no CE method has been reported for the determination of MUS and IBO in mushroom samples. CE is a analytical technique that was already used in analysis of narcotic drugs as well as hallucinogenic indoles from *Psilocybe semilanceata* that enabled determination of compounds with nonvolatile, polar, and ionic properties [18–20]. Thus, the aim of the present study was to develop a capillary zone electrophoretic method for the fast separation, identification, and quantification of isoxazol substances (IBO and MUS) in hallucinogenic mushrooms of *Amanita* species. The assay was focused on the mushrooms collected in the region of west-southern Poland.

2 Materials and methods

2.1 Chemicals

Standard substances of MUS and IBO were purchased from Abcam (Cambridge, UK) and prepared as 10 mg/mL stock solutions in a mixture (1:1 v/v) of methanol and 1 mM sodium phosphate buffer at pH 3. The UV spectra (in MeCN:25 mM sodium phosphate buffer (5:95, v/v)) and molecular structures of MUS (A) and IBO (B) are presented in Fig. 1. The 1 mM sodium phosphate buffer was prepared by dissolving the adequate amounts of sodium phosphate dibasic heptahydrate in water. The pH 3 was adjusted with 85% phosphoric acid (the final phosphate concentration was 3.2 mM). The stock solutions were protected from the light and stored at temperature 4°C. For CE measurements, the appropriate dilutions with a solution of methanol and 1 mM sodium phosphate aqueous solution at pH 3 were prepared with

analyte concentration in the range from 2.5 to 7000 μg/mL. The internal standard, tryptamine, was obtained from Sigma-Aldrich (Poznań, Poland). Methanol and acetonitrile of HPLC grade and sodium phosphate heptahydrate of analytical grade were purchased from Sigma-Aldrich (Poznań, Poland). Orthophosphoric acid (85%) and sodium hydroxide were also purchased from POCH (Gliwice, Poland). DI water was obtained from Milli-Q system (Millipore, MA, USA).

2.2 *Amanita* mushrooms

The dried samples of 41 mushroom samples were used in this study: 38 of *A. muscaria*, 1 of *A. pantherina*, and 2 of *A. citrina*. All analyzed material was collected in the southwestern Poland during the periods from September to November in 2008, 2009, 2010, and 2011. A microscopic examination of mushroom material was performed on a Nikon eclipse E-400 light microscope equipped with a Nikon digital camera (DS-Fi1). The obtained mushroom samples were dried at the room temperature and kept frozen at −18°C until analysis. The full characterization of analyzed mushrooms is presented in Table 1.

2.3 Extraction procedure

Dried mushrooms were grounded into a fine powder in a mortar. A total of 100 mg of the powdered sample was dissolved in 20 mL mixture of methanol and 1 mM sodium phosphate buffer at pH 3 (1:1 v/v). The extraction of studied analytes was performed by ultrasonic-assisted process (UAE) for 180 min. An extraction time of 180 min allowed to destroy a cell wall of examined mushrooms and to isolate IBO and MUS from mushrooms effectively. Ultrasonic conditions such as frequency and power were 42 kHz and 130 W, respectively. Then the extracts (19.5 mL each) were filtered gravitationally and evaporated to dryness on a rotary evaporator. The residue was dissolved in a mixture of methanol (1 mL) and 1 mM sodium phosphate buffer at pH 3 (0.5 mL) and analyzed by CE. The same procedure was applied for all studied mushrooms.

2.4 CE system and electrophoretic conditions

The electrophoretic analyses were performed on a PA800 plus (Beckmann Coulter, USA) equipped with photodiode array (PDA) detector. All analyses were performed at a wavelength of 214 nm. Separations were run in 57 cm × 75 μm fused-silica capillary with the detector window 7 cm from the outlet. Samples were injected at a pressure of 0.5 psi. Injection time was 5 s (hydrodynamic injection). Applied voltage was 30 kV. The analysis was conducted at 22 ± 0.1°C. At the beginning of each day, capillary was washed sequentially with 0.1 M NaOH (10 min) and water (2 min). Before each analysis capillary was conditioned as following: 0.1 M NaOH, distilled water (1 min) and running buffer (1 min). The running buffer was a mixture of MeCN:25 mM sodium phosphate buffer pH 3 (5:95 v/v) . The 25 mM sodium phosphate buffer was prepared by dissolving the adequate amounts of sodium phosphate dibasic heptahydrate in water. The pH 3 was adjusted with 85% phosphoric acid (the final phosphate concentration was 55.5 mM).

2.5 Calibration curve

The quantitative analyses of MUS and IBO were based on peak area measurements relative to the internal standard (tryptamine). The calibration curves for both analytes were linear in the range of 2.5–7000 μg/mL. Extracts of *Amanita* mushrooms and solutions of mixtures of IBO and MUS (25.0, 50.0, 100.0, 250.0, 500.0, 1000.0, 2500.0, and 5000.0 μg/mL) were analyzed for intraday ($n = 6$) and interday variations ($n = 6$). The LOD was determined at an S/N ratio of 3.3, while the LOQ was defined at an S/N ratio of 10.

3 Results and discussion

3.1 Optimization of CZE separation

In order to optimize experimental conditions for the simultaneous separation of IBO and MUS, major separation factors including type of running buffer, its pH and concentration as well as presence of organic modifier were investigated. Four BGE types (sodium phosphate, sodium tetraborate, sodium citrate and acetate) applied in the range of pH 3–11 and concentration range of 5–50 mM were investigated. Among these electrolytes best results were obtained with sodium phosphate (25 mM) in terms of peak shape, resolution, sensitivity, and time of analysis. Increasing the running buffer concentration from 5 to 50 mM resulted in increase of sensitivity. In the case of acetate and tetraborate BGE, the concentration <20 mM did not make the detection of studied analytes possible. The worst separation of studied analytes was observed when sodium acetate was used.

According to the zwitterionic nature of IBO and basic properties of MUS, their separation and migration times were expected to be highly dependent on pH of the BGE. pH of running electrolyte determined the analyte ionization and magnitude of the EOF. Therefore, the effect of pH on separation of IBO and MUS at a constant concentration of 25 mM sodium phosphate aqueous solution in pH range of 3–11 as BGE was studied. In Fig. 2 the variation of electrophoretic mobility values (μ_{eff}, (cm^2/Vs) × 10^{-6}) with changes of BGE pH are presented. It can be observed that the electroosmotic mobility was increased with increasing pH value. At all pH range, IBO appeared after the EOF marker, which implied that this analyte was negatively charged or is zwitterionic in applied running electrolyte. This phenomenon is directly related to pK_a values of IBO, which are 3.0, 5.04, 8.16 [21]. MUS, as an amine with pK_a values 4.8

Table 1. Detailed characterization of collected fungal material

Sample no.	*Amanita* sp.	Substratum	Ecosystem	MUS (μg/g)	IBO (μg/g)
1	*A. muscaria*	Soil	Fallow land	989 ± 160	1710 ± 90
2	*A. muscaria*	Soil	Lawn planted with trees/shrubs	1641 ± 330	454 ± 80
3	*A. muscaria*	Soil—humus layer	Woodland and scrub	480 ± 19	3589 ± 230
4	*A. muscaria*	Soil—humus layer	Woodland and scrub	Nd	Nd
5	*A. muscaria*	Soil	Lawn planted with trees/shrubs	143 ± 3	3380 ± 260
6	*A. muscaria*	Soil	Woodland and scrub	2436 ± 320	3186 ± 360
7	*A. muscaria*	Soil	Woodland	1221 ± 140	Nd
8	*A. muscaria*	Soil	Woodland	374 ± 70	6565 ± 230
9	*A. muscaria*	Soil	Fallow land/field	339 ± 50	4731 ± 240
10	*A. muscaria*	Soil	Fallow land/field planted with trees/shrubs	1841 ± 240	Nd
11	*A. muscaria*	Soil	Woodland and scrub	710 ± 20	4444 ± 300
12	*A. muscaria*	Soil	Woodland	1473 ± 170	1536 ± 150
13	*A. muscaria*	Soil	Woodland	335 ± 30	1060 ± 40
14	*A. muscaria*	Soil	Woodland	1124 ± 20	671 ± 50
15	*A. muscaria*	Soil	Woodland	1892 ± 70	1704 ± 270
16	*A. muscaria*	Soil	Woodland	1013 ± 40	390 ± 40
17	*A. muscaria*	Soil	Woodland	603 ± 14	1759 ± 50
18	*A. muscaria*	Soil	Woodland	528 ± 50	2576 ± 220
19	*A. muscaria*	Soil	Woodland	597 ± 19	5240 ± 50
20	*A. muscaria*	Soil	Woodland	3561 ± 250	nd
21	*A. muscaria*	Soil	Woodland	174 ± 14	1455 ± 140
22	*A. muscaria*	Soil	Parkland and scattered trees	Nd	Nd
23	*A. muscaria*	Soil	Allotment	450 ± 20	646 ± 10
24	*A. muscaria*	Soil	Woodland	2303 ± 170	292 ± 20
25	*A. muscaria*	Soil	Woodland and scrub	906 ± 50	Nd
26	*A. muscaria*	Soil	Woodland and scrub	270 ± 10	2040 ± 140
27	*A. muscaria*	Soil	Woodland and scrub	725 ± 60	3128 ± 210
28	*A. muscaria*	Soil	Woodland and scrub	1991 ± 270	1525 ± 140
29	*A. muscaria*	Soil	Woodland and scrub	313 ± 14	821 ± 60
30	*A. muscaria*	Soil	Woodland and scrub	Nd	Nd
31	*A. muscaria*	Soil	Fallow land/fidel plantem with trees/shrubs	1387 ± 90	2065 ± 45
32	*A. muscaria*	Soil	Fallow land/field planted with trees/shrubs	Nd	Nd
33	*A. muscaria*	Soil	Allotment	73 ± 4	Nd
34	*A. muscaria*	Soil—humus layer	Mountain grassland	Nd	Nd
35	*A. muscaria*	Soil—humus layer	Mountain grassland	1945 ± 130	865 ± 30
36	*A. muscaria*	Soil—humus layer	Mountain grassland	634 ± 110	6469 ± 570
37	*A. muscaria* var. *muscaria*	Soil—humus layer	Woodland	508 ± 33	920 ± 60
38	*A. muscaria* var. *muscaria*	Soil—humus layer	Fallow land/field planted with trees/shrubs	2159 ± 60	Nd
39	*A. citrina* var. *citrina*	Soil—humus layer	Coniferous plantation	259 ± 40	Nd
40	*A. citrina* var. *citrina*	Soil	Woodland	1339 ± 80	3575 ± 210
41	*A. pantherina*	Soil	Urban greenery (planted with shrubs)	1228 ± 30	3367 ± 350
Mean				1055	2432
Max (detected)				2436	6565
Min (detected)				73	292

Nd: not detected.

and 8.4, appeared before the EOF signal in BGE of pH <6 and it was attributed to the protonation of one of its amine group. At pH between 6 and 7, MUS as a neutral molecule co-migrated together with EOF marker. The further increase in pH of running electrolyte resulted in increased migration time of MUS, which appeared after EOF signal (lower values of μ_{eff}). Under all applied separation conditions the studied analytes were well separated. However, in order to shorten the analysis time and maintaining acceptable separation, the 25 mM sodium phosphate running solution was adjusted to pH 3 in the final procedure.

In order to improve the resolution, the effect of organic modifiers added to solution of running electrolyte was studied. Their task was to prevent the absorption of analytes on

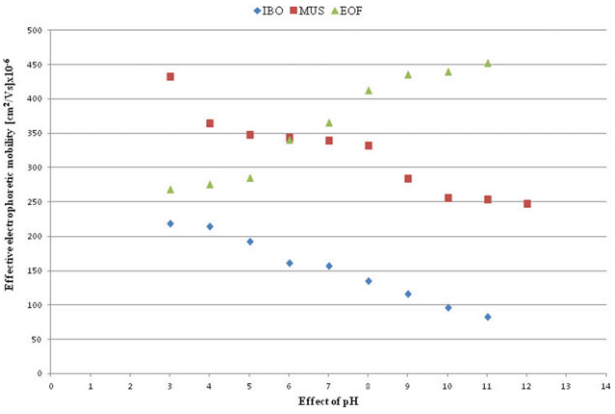

Figure 2. Effect of running buffer pH on the effective electrophoretic mobility of IBO, MUS, and EOF marker. Buffer: 25 mM sodium phosphate in pH range from 2 to 12.

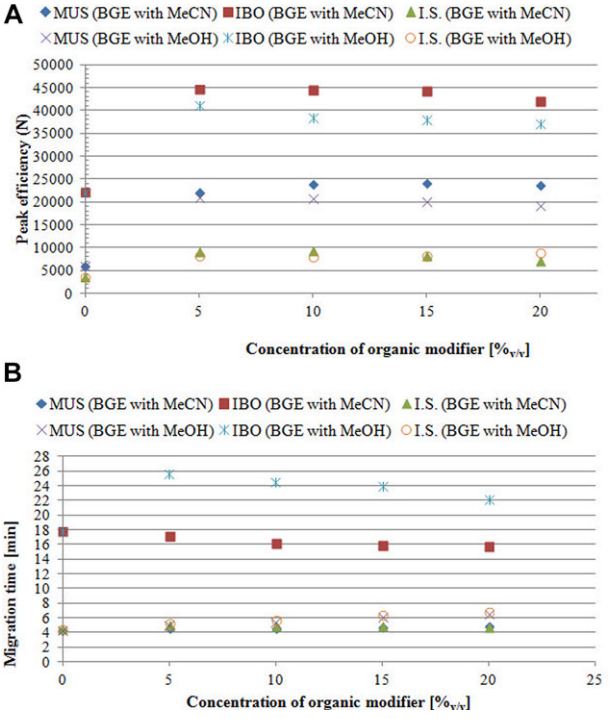

Figure 3. Effect of concentration of organic modifier in running electrolyte on peak efficiency (A) and migration times (B) of studied analytes. Electrophoretic conditions: 25 mM sodium phosphate (pH 3.0).

the walls of the capillary (especially in the case of complex sample matrices), improve their solubility, and change the BGE viscosity. Figure 3 illustrates the effects of methanol and acetonitrile concentration on peak efficiency and migration times of analyzed fungal hallucinogenic compounds.

From these results, the overall trend is the same for both solvents: a slight decrease of migration time with an increasing volume percent of the organic modifier, which is a result of this effect on EOF electrophoretic mobility. Under these conditions, an application of acetonitrile resulted in higher mobility of studied hallucinogens (shorter analysis time) and gave higher values of peak efficiency. These results can be attributed to the decrease in BGE viscosity and the value of dielectric constant (substance's ability to insulate charges from each other) that determines solvent polarity. Important parameter, in this case, is dielectric constant value, higher ε means higher polarity, and greater ability to stabilize charges. The ε for water is 80.1, whereas for acetonitrile and methanol the values are 37.5 and 32.7, respectively. The higher the values of dielectric constant of organic solvent added to aqueous solution of BGE, the better the solubility of studied isoxazoles was observed. It can be concluded that the mobility of IBO and MUS was influenced both by dielectric constant and viscosity of BGE in the applied CZE method. Therefore, the 5% v/v content of acetonitrile in a BGE (25 mM sodium phosphate aqueous solution, pH 3) was selected for the further CZE studies.

3.2 Method validation

To validate the method, a series of standard mixture solutions with concentrations from 2.5 to 7000 μg/mL were prepared and analyzed. The obtained calibration curves of the studied analytes exhibited good linearity and coefficient of determination (R^2) in the range of 0.9973–0.9981. The LODs, determined by spiking the standards into the blank mushrooms, were 1.5 μg/g mushroom tissue for IBO and 1.8 μg/g for MUS. The LOQs, measured by spiking procedure too, were 4.6 μg/g for IBO and 5.4 μg/g for MUS. The obtained sensitivity of developed CZE method is comparable to those of the well-established HPLC-UV and HPLC-MS methods described in the literature recently [4, 14]. Furthermore, in the mentioned HPLC-UV procedure [4], the derivatization step with dansyl chloride was mandatory in order to obtain the required detection limit.

The precision of the method was examined with the following set of experiments. First, intraday repeatability was investigated by analyzing six injections of the standard solutions at the concentrations of 25.0, 50.0, 100.0, 250.0, 500.0, 1000.0, 2500.0, and 5000 μg/mL for all analytes. The obtained data showed that RSDs of migration time and peak area were in the range of 2.4–4.7% and 4.3–7.2%, respectively. (The determination of RSD of retention time and peak area was performed by using internal standards). Interday reproducibility was also investigated in consecutive 3 days. The achieved results showed that interday RSDs ($n = 6$) of migration time and peak area were lower than 3.9 and 6.6%, respectively.

Finally, in order to investigate the reliability of the established method, the analysis of spiked samples were performed. Standard solutions of the investigated fungal hallucinogenic compounds (IBO and MUS) were added to the *Amanita* mushroom's homogenate (the negative samples, where IBO and MUS were not detected), at three concentrations of 50.0, 100.0, and 500.0 μg/g and analyzed by the developed CZE method. Recoveries were calculated through the peak area comparison and were in the range 87–95%.

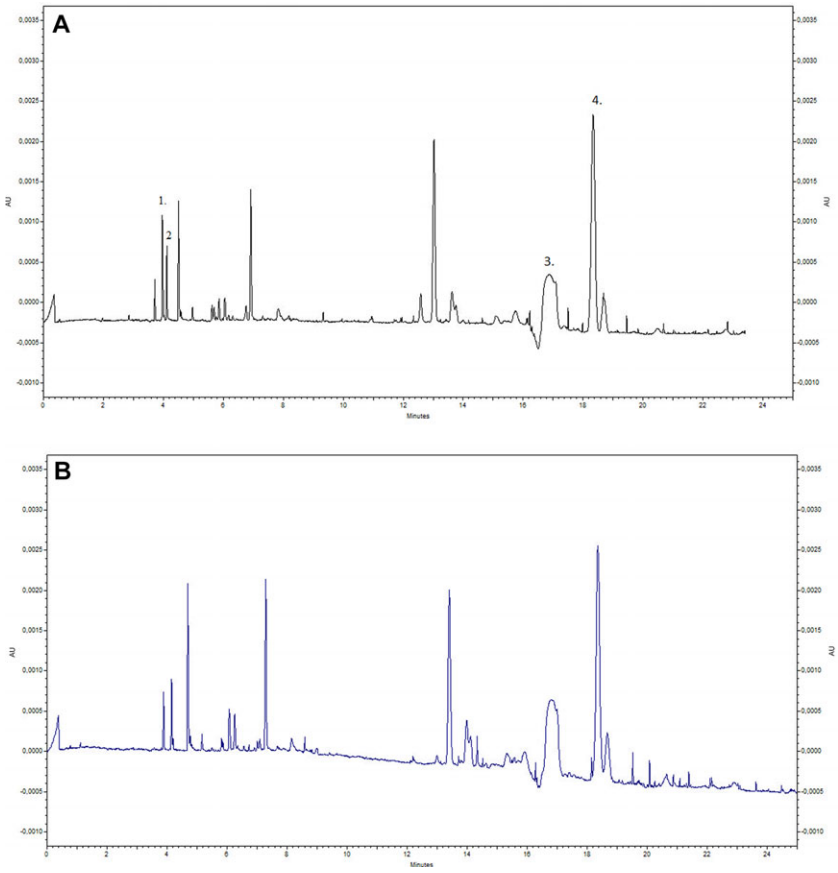

Figure 4. Electropherograms obtained from *A. muscaria* (sample no. 28) extract with addition of external standards (A) and blank extract (B). Detection: 214 nm. Peaks: 1, MUS; 2, I.S.; 3, EOF; 4, IBO.

3.3 Analysis of *Amanita* mushrooms by the developed procedure

The applicability of the developed CE-PDA (photodiode array) method for the analysis of IBO and MUS in *Amanita* mushrooms naturally grown in Poland was studied. The samples of analyzed mushroom extracts were prepared based on the procedures described in Section 2.3 and analyzed by optimized CZE method. The identification of MUS and IBO peaks was confirmed by UV spectra, migration time, and external standard additive (Fig. 4). The calculated content of studied isoxazoles in the analyzed samples was presented in Table 1. The concentration of MUS determined in studied samples was in a range of 73–2436 μg/g, whereas that of IBO was in a range of 292–6565 μg/g. In the case of five samples (sample no. 4; 22; 30, 32 and 34) the presence of MUS and IBO was not observed. Generally, the content of IBO was higher in the studied samples than the amount of identified MUS. Among the 41 samples of analyzed mushrooms, in seven cases IBO was not identified or was below LOQ. The absence of IBO often correlated with high concentration of MUS (from 906 up to 3561 μg/g). Only in two analyzed samples, the concentration of MUS was small (73 and 259 μg/g for sample no. 33 and 39, respectively). It can be assumed that this large variation in the content of studied hallucinogenic isoxazoles might be a result of the number of factors. These factors are the following: the nature of the environment in which the analyzed mushroom was grown (the type of ecosystem, temperature conditions, humidity, soil pH, etc.), mushroom age, and developmental stage, as well as the way of sample storage. A similar relationship, namely the dependence of MUS/IBO ratio on the growth environment, was described by Michelot and Melendez-Howell in 2003 [11]. It is assumed that the genotype of *A. muscaria* can also influence the differences in concentration of IBO versus MUS [4]. Differentiation in the content of investigated hallucinogens, depending on the species can be also observed. From the obtained results, the lowest concentration values of IBO and MUS were determined in the mushrooms of *A. citrina*. Furthermore, the obtained, from CZE analysis, peak profiles of the analyzed extracts of *A. citrina* were characterized by the simplest composition (a small number of signals). For comparison, considering the number of signals observed in CE electropherograms for extracts of *A. muscaria* showed much greater complexity (data not shown).

Summing up, there are no data describing the determination of IBO and MUS concentration in mushrooms of *Amanita* sp. growing in Poland. Generally, the content of both hallucinogenic substances significantly exceeded the concentration reported by Tsujikawa et. al. [2,4] and Gonmori et. al. [14] in the mushrooms of genus *A. muscaria* grown naturally in Japan. In these studies, seven samples of *A. muscaria*

mushrooms were analyzed. The content of MUS ranged from 46 to 1362 μg/g, whereas the content of IBO ranged from 182 to 2983 μg/g.

4 Concluding remarks

This is the first application of electrophoretic technique for the determination of isoxazoles in mushroom material. The benefits of this method, in comparison to the previously described procedures, are good selectivity and sensitivity without requirement of derivatization step or sample pretreatment technique (e.g. SPE). Under the optimized conditions the investigated hallucinogens were separated within 20 min and detected with LOD equal to 1.5 μg/mL for IBO and 1.8 μg/mL for MUS. The obtained sensitivity of developed CZE method was comparable to these applying HPLC-UV and HPLC-MS procedures described in the literature. The proposed method enabled an effective and fast determination of MUS and IBO in *Amanita sp*. mushrooms, which makes it of choice in criminology and medicine.

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The authors have declared no conflict of interest.

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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

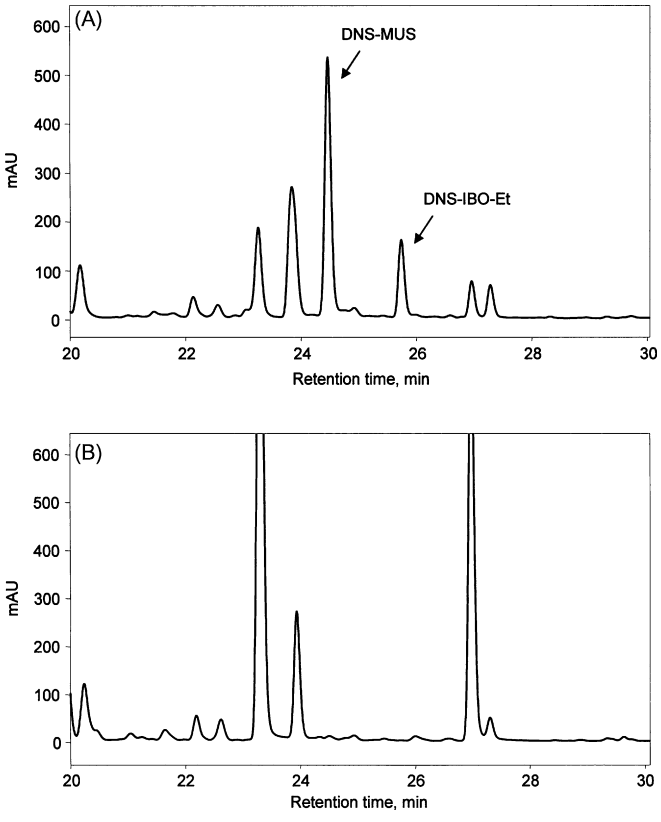

Fig. 1. Chromatograms obtained from *A. pantherina* (sample #5) extract (A) and blank mushroom (*Pleurotus ostreatus*) extract (B). Detection: 256 nm.

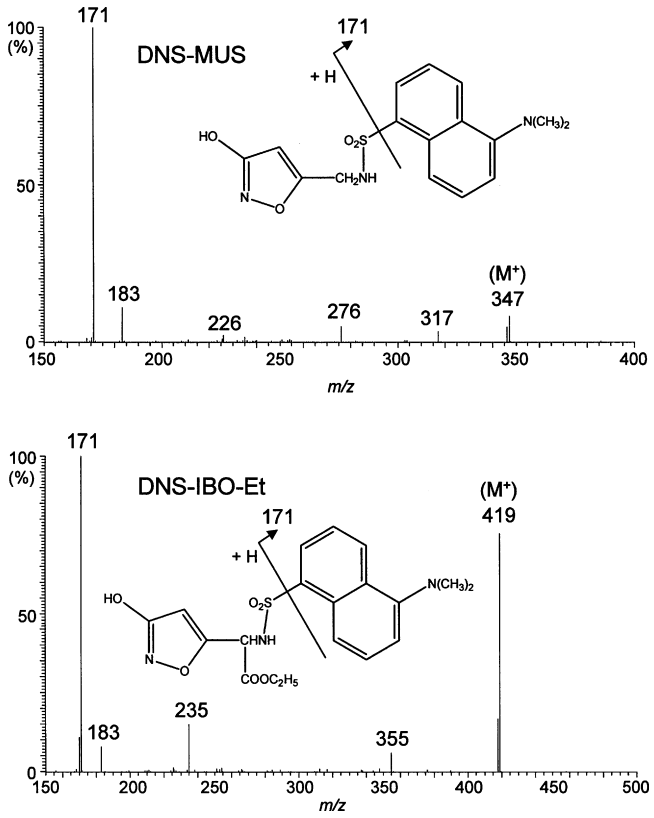

Fig. 2. Product ion spectra at *m/z* 347 from DNS-MUS and *m/z* 419 from DNS-IBO-Et.

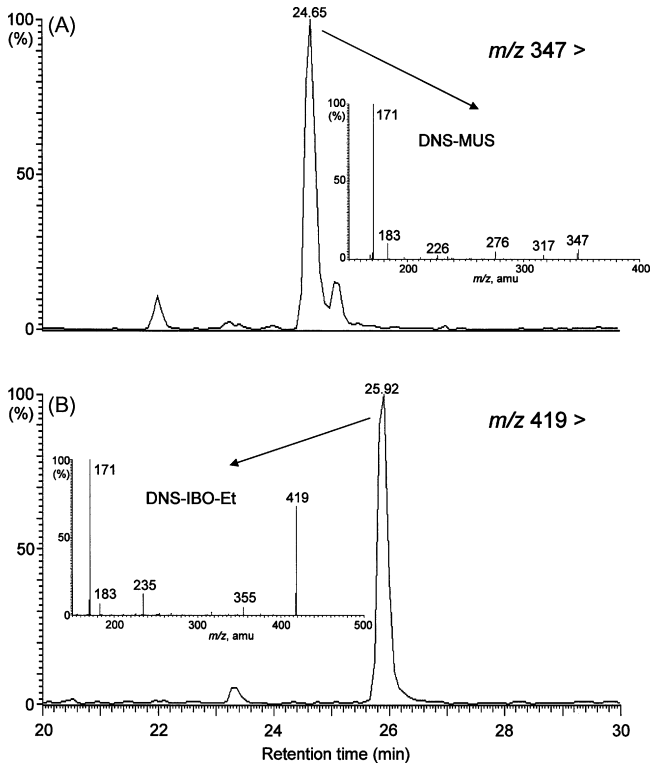

Fig. 3. LC/MS/MS analysis of the cap of *A. pantherina* (sample #5). (A): Total ion chromatogram in the product ion scan mode using *m/z* 347 and product ion spectrum at *m/z* 347 for the 24.65-min peak. (B): Total ion chromatogram in the product ion scan mode using *m/z* 419 and product ion spectrum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

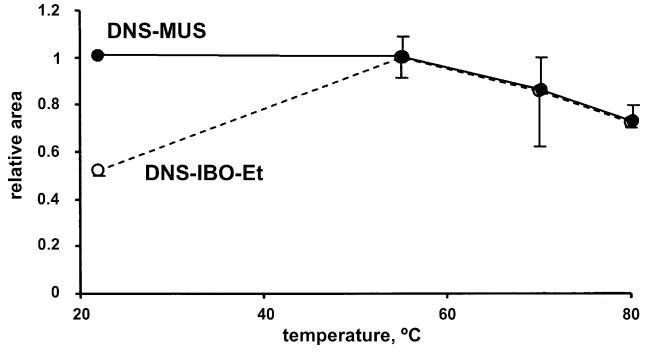

Fig. 4. Optimization of the reaction temperature for ethylation of DNS-IBO-Et. Each sample was heated for 60 min at 22 °C (room temperature), 55 °C, 70 °C, or 80 °C. Each datum indicates a relative peak area normalized to that of 55 °C. Each data point represents the mean ± SD of three determinations.

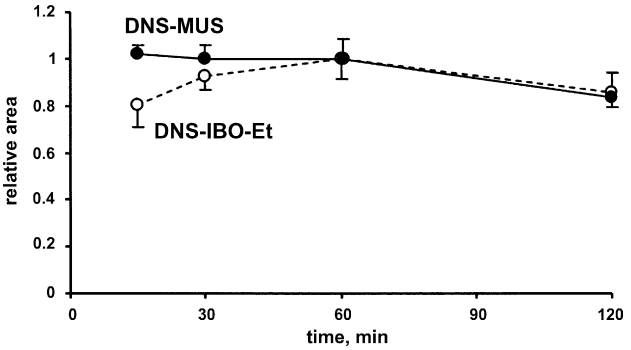

Fig. 5. Optimization of the reaction time for ethylation of DNS-IBO-Et. Each sample was heated at 55 °C for 15 min, 30 min, 60 min, or 120 min. Each datum is indicated as relative peak area normalized to that of 60 min. Each data point represents the mean ± SD of three determinations.

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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Analysis of hallucinogenic constituents in *Amanita* mushrooms circulated in Japan

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Abstract

The constituents of seven mushrooms sold as *Amanita muscaria* or *Amanita pantherina* (five *A. muscaria* and two *A. pantherina*) and four ''extracts purported to contain *A. muscaria*'' products that are currently circulated in Japan were determined. All mushroom samples were identified as *A. muscaria* or *A. pantherina* by macroscopic and microscopic observation. The dissociative constituents, ibotenic acid (IBO) and muscimol (MUS), were extracted with 70% methanol twice and determined by gas chromatography/mass spectrometry. The IBO (as the hydrate)/MUS contents were in the range of <10–2845 ppm/46–1052 ppm in the cap of *A. muscaria* and 188–269 ppm/1554–1880 ppm in the cap of *A. pantherina*. In the caps, these compounds had a tendency to be more concentrated in the flesh than in the cuticle. On the other hand, the IBO/MUS contents in the stem were far lower than in the caps. In the ''extracts purported to contain *A. muscaria*'' products, IBO/MUS were detected below the lower limit of calibration curve (<10 ppm/<25 ppm) or not detected. However, these samples contained other psychoactive compounds, such as psychoactive tryptamines (5-methoxy-*N,N*-diisopropyltryptamine and 5-methoxy-*N,N*-dimethyltryptamine), reversible monoamine oxidase inhibitors (harmine and harmaline) and tropane alkaloids (atropine and scopolamine), which were not quantified. This is the first report of the chemical analysis of *Amanita* mushrooms that are circulated in the drug market.

Keywords: *Amanita muscaria*; *Amanita pantherina*; Ibotenic acid; Muscimol; GC/MS

1. Introduction

Amanita muscaria, known by the name ''fly agaric,'' is a psychotropic mushroom that is traditionally used for religious or recreational purposes in Siberia, North-East Asia and India [1,2]. This mushroom has been recently reported as being used as an intoxicant in several countries [3,4]. In Japan, not only *A. muscaria* but also *Amanita pantherina*, a dissociative mushroom similar to *A. muscaria*, are circulated via the Internet or in ''smoke shops.'' In addition, ''extracts purported to contain *A. muscaria*'' products are also in circulation. In 2003, the Japan Poison Information Center received four cases of intoxication caused by ''extracts purported to contain *A. muscaria*'' products [5].

A. muscaria and *A. pantherina* contain two dissociative constituents, ibotenic acid (IBO) and muscimol (MUS) (Fig. 1). IBO is a powerful agonist of the *N*-methyl-D-aspartic acid (NMDA) receptor [6]. Nielsen et al. reported that IBO was converted by decarboxylation to MUS in mouse brain homogenates [7]. MUS, which acts as a potent $GABA_A$ agonist [8], has more potent neuropharmacological activity [9–11].

There are several reports on the contents of IBO/MUS in *A. muscaria* and *A. pantherina* in natural products. Determination of IBO/MUS in mushrooms was performed using paper chromatography [12], high performance liquid chromatography [13–15], single-column chromatography [16] and gas chromatography/mass spectrometry (GC/MS) [17]. However, an analysis of samples that are circulated in the drug market has not yet been reported. In this study, we report on the chemical analysis of *Amanita* mushrooms and ''extracts purported to contain *A. muscaria*'' products that are circulated in Japan.

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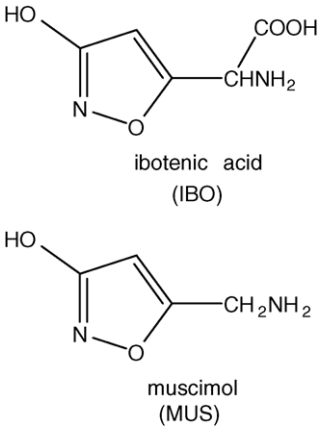

Fig. 1. Chemical structures of ibotenic acid (IBO) and muscimol (MUS).

2. Experimental

2.1. Samples and chemicals

Eleven samples were used in this study; seven were dried mushrooms sold as *A. muscaria* or *A. pantherina* (five *A. muscaria* (see Fig. 2A) and two *A. pantherina* (see Fig. 2B)), and four were "extracts purported to contain *A. muscaria*" products (see Fig. 2C and D). These samples were obtained from "smoke shops" or via the Internet in Japan.

IBO hydrate was obtained from Biosearch Technologies (Novato, CA, USA). MUS was obtained from Sigma (St. Louis, MO, USA). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 10% trimethylchlorosilane (TMCS) was obtained from Pierce Chemical Co. (Rockford, IL, USA). All other chemicals used in the experiments were of analytical grade.

2.2. Optical microscopic examination

A microscopic examination was performed using the method reported by Walting [18]. A 2.5% potassium hydroxide solution was used as a swelling agent and to return the dried tissues to their previous state. Small pieces of the gills were cut from the fruit-body and mounted on a glass slide, while directly in the 2.5% potassium hydroxide solution. After covering with a glass cover-slip, it was tapped with a rubber-tipped pencil to separate the tissues from each other. Observation was carried out using a biological microscope. The sizes of the spores were measured with Image J (Wayne Rasband, National Institute of Health, USA) and an average of 12 spores was measured for each sample.

Melzer's staining reaction was used for detecting amyloid, pseudoamyloid, or nonamyloid of the spores. Staining was performed using the method described in Ref. [19]. Small pieces of the gills were cut from the fruit-body and mounted on a glass slide, while directly in the 2.8% ammonia solution. After washing by water, the samples were swelled in the Melzer's reagent (composition as follows: 0.5 g of iodine, 1.5 g of potassium iodide, 22 g of chloral hydrate and 20 g of water) and were observed under a biological microscope.

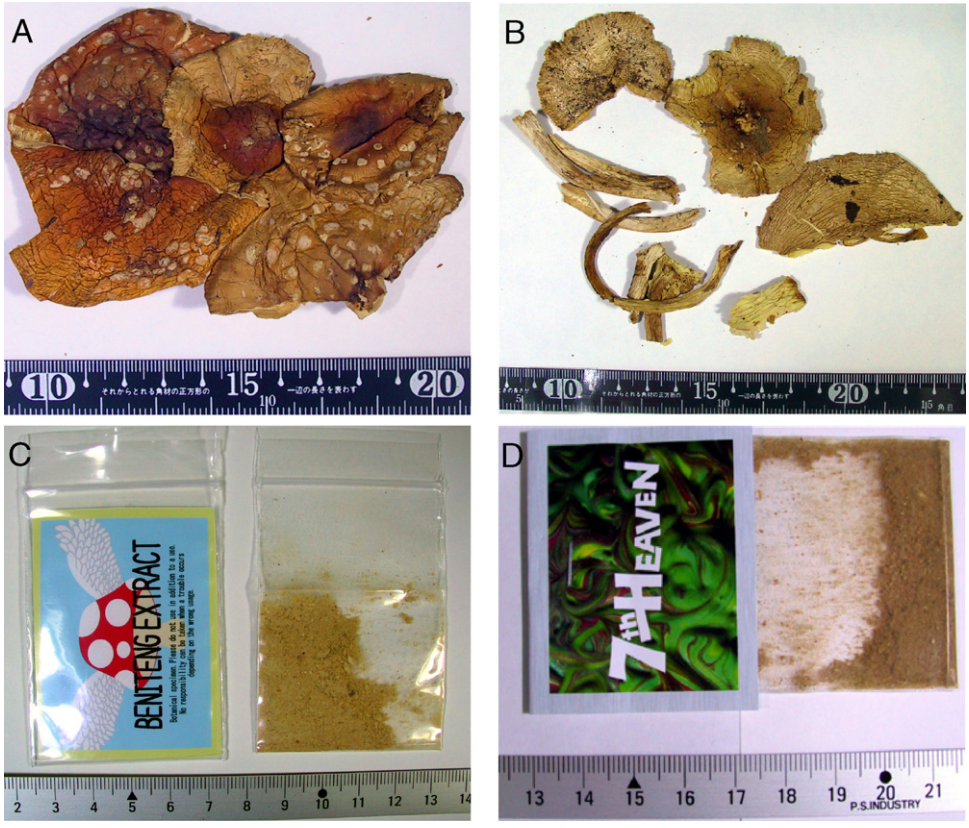

Fig. 2. Representative photographs of the samples: (A) *A. muscaria*; (B) *A. pantherina*; (C and D) "extracts purported to contain *A. muscaria*" products.

2.3. Chemical analysis

2.3.1. Extraction procedure of IBO and MUS

For the extraction of IBO and MUS, a previously reported method [13] was used with minor modifications as follows. The dried mushrooms were cut into the sections of caps and stems, and the cuticle and flesh in a part of the caps were also separated. Each section was ground to a fine powder in a mortar. Fifty milligrams of the powdered mushrooms or ''extracts purported to contain *A. muscaria*'' products were extracted twice with 2 mL of 70% aqueous methanol by shaking for 1 min followed by ultrasonication for 5 min. After centrifugation at 3000 rpm for 3 min, 200 μL of the supernatant was transferred to a separate vial and the solution evaporated to dried under a stream of nitrogen. The residues were derived by reaction with a mixture of 50 μL of BSTFA containing 10% TMCS and 50 μL of ethyl acetate containing 20 μg/mL *n*-pentadecane as an internal standard (IS) at 80 °C for 30 min. A 1 μL aliquot was used for gas chromatography/mass spectrometry.

2.3.2. Calibration curve

Calibration curves for IBO/MUS were constructed by plotting the blank-subtracted peak area ratio of the target compound to IS versus the concentration of IBO/MUS. Blank-subtraction was performed by subtracting the peak area ratio of the blank from that of the samples. An entire cap of sample no. 4 (*A. muscaria*) was used as blank, because it contained lower concentrations of IBO/MUS than the other *Amanita* mushrooms determined in this study. IBO/MUS were added to the blank extract in the following blank-subtracted final concentrations: IBO hydrate (10, 25, 50, 150, 400 ppm) and MUS (25, 50, 150, 400, 1000, 2000 ppm). The regression parameters for the slope, intercept and correlation coefficient were calculated by weighted ($1/x$) linear regression using the Correlation2-2 freeware (http://homepage3.nifty.com/m_nw/j-frame.htm).

The determination of IBO/MUS in the blank sample (no. 4) was performed by the standard addition method. Calibration curves were constructed by adding standards to the blank extract and plotting the blank-subtracted peak area ratio of the target compound to IS versus the concentration of IBO/MUS. The final concentrations of adding standards are as follows: IBO hydrate (0, 10, 25, 50 ppm) and MUS (0, 25, 50, 150 ppm). The regression parameters were calculated by the above mentioned software.

Tests to determine the precision and accuracy of the method were performed using the blank mushroom extract that had been spiked with IBO hydrate and MUS. The concentrations spiked were as follows: 20, 80, 300 ppm for IBO hydrate and 40, 300, 1500 ppm for MUS, respectively. The accuracy of the assay was evaluated by percent deviation (%DEV) from the nominal concentration using the formula: $\%DEV = 100 \times$ (mean back calculated concentration − nominal concentration)/nominal concentration. Intra- and inter-assay precision is expressed as the coefficient of variation (CV, %) of the experimental values at each concentration.

2.3.3. Extraction procedure for drug screening analysis

For other active ingredients (e.g. abused drugs, natural pharmacologically-active compounds, etc.) in ''extracts purported to contain *A. muscaria*'' products, general drug screening was performed by a modified Stas-Otto's isolation method, as summarized in Fig. 3. After the extraction procedure, a 1 μL aliquot was used for GC/MS.

2.3.4. Apparatus and chromatographic conditions

GC/MS analysis was performed with a GCMS-QP5050A (Shimadzu, Kyoto, Japan) equipped with a DB-5 ms capillary column (30 m length, 0.25 mm i.d. and 0.25 μm film thickness, J&W). The temperature of the injector and the interface was set at 250 °C. The oven temperature was held at 100 °C (for IBO/MUS) or 50 °C (for general drug screening) for 1 min, then increased to 300 °C at 15 °C/min and held for 5 min. Helium was used as the carrier gas (head pressure at 72.3 kPa at 100 °C or 67.5 kPa at 50 °C, total flow 53.0 mL/min). The mass spectrometer was operated under the electron ionization (EI) mode at an ionization energy of 70 eV. For qualification, the analysis was performed in the scan mode (mass range: *m/z* 40–450 for IBO/MUS, and *m/z* 40–600 for general drug screening). For quantification, the MS was programmed for selected ion monitoring (SIM) detection of *m/z* 257 (IBO), *m/z* 243 (MUS) and *m/z* 57 (IS).

3. Results and discussion

3.1. Morphological examinations

For identification of the species of the mushrooms sold as *A. muscaria* or *pantherina*, we performed macroscopic and microscopic examination. The morphologic features of mushroom samples are summarized in Table 1. Some important morphological features such as stems and gill attachment could not be obtained from the samples, because there were no stems in most of the packages of the mushroom samples. Therefore, identification of species in this study was mainly performed by macroscopic features of caps and microscopic features of spores. Morphologic features of the samples were almost fully in accordance with the description of *A. muscaria* and *A. pantherina* in Refs. [20,21]. The color of caps of the mushrooms sold as *A. muscaria* tended to be yellowish in comparison with the references, but we presumed that this was caused by fading during drying. Judging comprehensively, all mushroom samples could be identified as *A. muscaria* and *A. pantherina* as described on the package of the samples.

3.2. Chemical examinations

Fig. 4 shows total ion chromatogram (TIC) and mass chromatograms resulting from an *A. muscaria* (sample no. 1) in the scan mode. The peaks for IBO-*tri*-TMS, MUS-*di*-TMS and IS had retention times of 9.1, 7.0 and 7.3 min, respectively. The peaks of IBO-*tri*-TMS and MUS-*di*-TMS were not detected in the extract of edible mushrooms like *Lentinus edodes* (''shiitake''), *Flammulina velutipes* (''enokitake'') and *Pleurotus ostreatus*

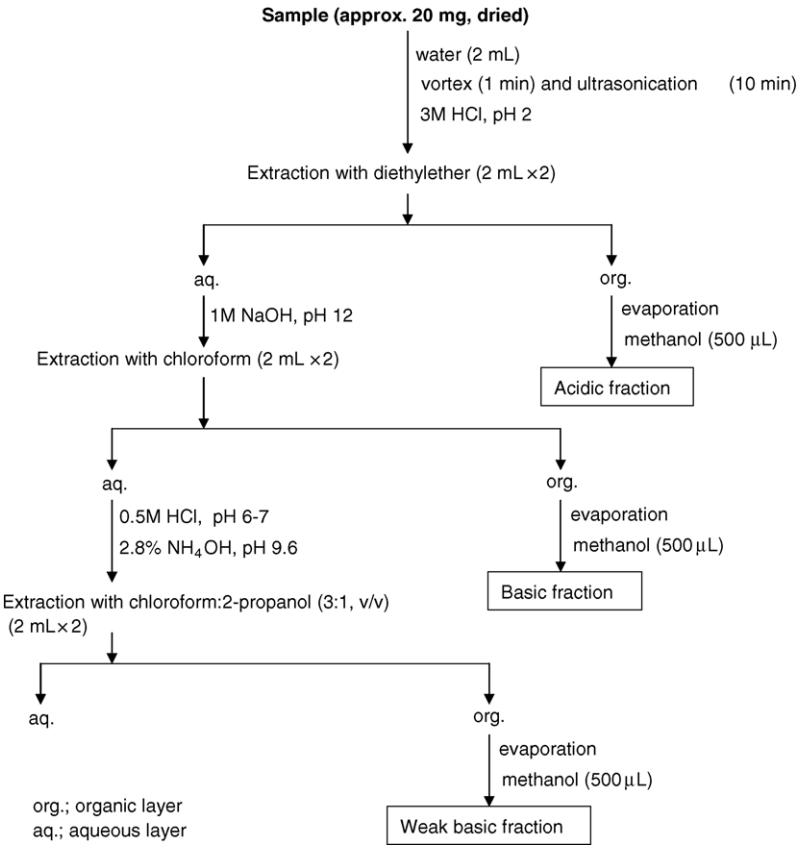

Fig. 3. Extraction procedures for other active constituents in "extracts purported to contain *A. muscaria*" products.

("hiratake"). Each derivative was stable for at least 10 h at room temperature. The mass spectra of IBO-*tri*-TMS and MUS-*di*-TMS are shown in Fig. 5. The fragmentations of these mass spectra were previously reported by Repke et al. [17].

Table 1
Morphologic characteristics of *A. muscaria* and *A. pantherina* used in this study

Species	Description
A. muscaria	Macroscopic feature *Cap*: 4–10 cm broad, plane shape, pale brown to orange color, strewn with whitish-brown warts *Gill*: whitish-brown color *Subcuticle*: white color
	Microscopic feature *Spores*: 9.5–9.9 μm × 6.6–7.0 μm, colorless, elliptical-ovate, smooth surface and nonamyloid *Basidia*: colorless, 4-sterigmate
A. pantherina	Macroscopic feature *Cap*: 3.5–6.5 cm broad, plane shape, gray-brown color, strewn with whitish-brown warts *Gill*: whitish-brown color *Subcuticle*: white color
	Microscopic feature *Spores*: 10.5–11.4 μm × 7.2–7.4 μm, colorless, elliptical-ovate, smooth surface and nonamyloid *Basidia*: colorless, 4-sterigmate

Repke et al. performed trimethylsilylation at 140 °C for 30 min with BSTFA, and they reported that shorter reaction times or lower reaction temperature resulted in the presence of variable amounts of a partially derivatized product, presumably IBO-*di*-TMS [17]. In the present study, by adding 10% TMCS to the BSTFA, IBO could be completely converted to IBO-*tri*-TMS at 80 °C for 30 min.

In the early stage of the experiment, IBO/MUS in the mushroom samples were extracted four times with 70% aqueous methanol to investigate the efficiency of the extraction. As shown in Fig. 6, almost all of IBO/MUS were recovered from the mushrooms in two extractions. Hereafter, two extractions were used for the quantitative analysis of IBO/MUS.

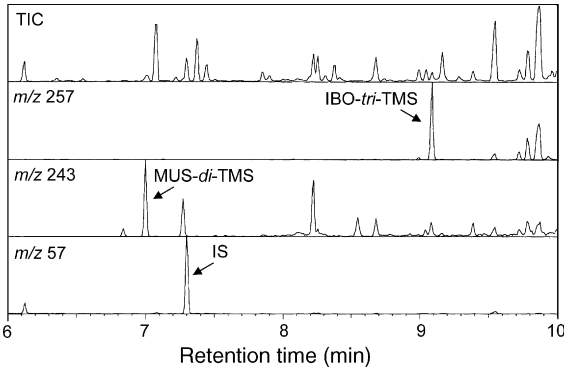

Fig. 4. TIC and mass chromatograms resulting from an *A. muscaria* (sample no. 1) in the scan mode.

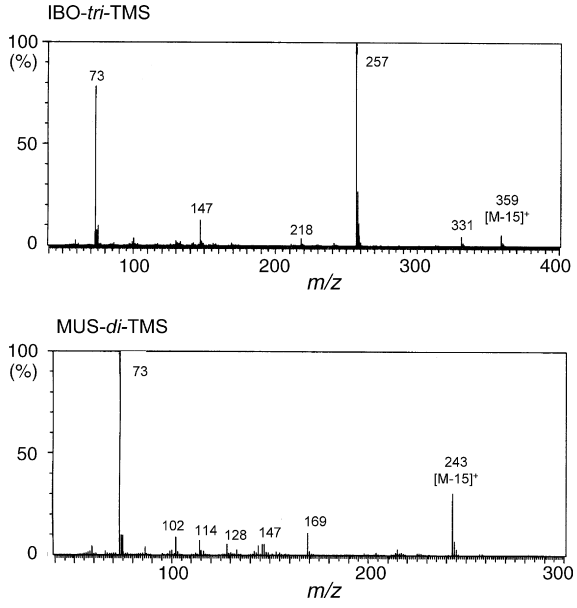

Fig. 5. EI mass spectra of IBO-*tri*-TMS and MUS-*di*-TMS.

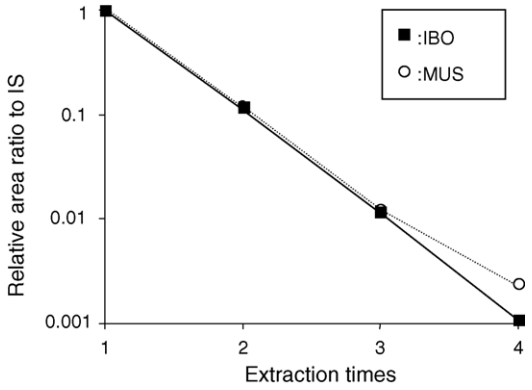

Fig. 6. Recovery of IBO and MUS in *A. muscaria* after repeated extraction. The *Y*-axis is relative area ratio to the first extract and the *X*-axis denotes extraction times. Each data point represents the mean of triplicate determinations.

Initially, we attempted to construct calibration curves by adding IBO/MUS standards to extracts of edible mushrooms such as *L. edodes*, *F. velutipes* and *P. ostreatus*. However, this approach was not feasible for IBO because of matrix differences. Therefore, we selected *A. muscaria* sample no. 4 as a blank mushroom, because it contained lower levels of IBO/MUS than the other *Amanita* mushroom samples. The calibration curves were linear over the concentration range of 10–400 ppm for IBO (as hydrate) and 25–2000 ppm for MUS with correlation coefficients that were routinely greater than 0.99. Samples that were found to contain IBO or MUS in excess of the upper limits of linearity were reanalyzed after dilution with the blank extract. Table 2 shows accuracy and intra- and inter-assay precision data. The intra-assay accuracy was between −6.3 and 4.5% deviation from nominal values. The CV for the intra- and inter-assay was between 3.5 and 12.8% at three concentrations of the two analytes.

Table 2
Summary of analytical accuracy and precision

	Nominal concentration (ppm)					
	IBO			MUS		
	20	80	300	40	300	1500
Accuracy ($n = 5$)						
Mean observed concentration (ppm)	20.9	82.6	303.4	37.5	291.9	1488.2
%DEV	4.5	3.2	1.1	−6.3	−2.7	−0.8
Precision ($n = 5$)						
Intra-assay (CV, %)	9.8	4.4	12.3	10.1	3.7	6.9
Inter-assay (CV, %)	7.9	6.6	12.8	11.3	3.5	7.0

In applying this quantification procedure, it is necessary to be careful for the following reasons:

(i) Application of this method is limited in the case of obtaining an *Amanita* mushroom which contained much lower levels of IBO/MUS than others.
(ii) Correction of the blank may confound calculation of the IBO/MUS concentrations in the samples.
(iii) The lower limits of calibration curves were dependent on the IBO/MUS levels in the blanks. There is the possibility of evaluation as ‘‘below the lower limits of calibration curves’’ despite detecting IBO/MUS peaks clearly, in case of using the highly-concentrative mushroom as a blank.

Table 3 summarizes the IBO/MUS contents in the dried mushroom samples. The total contents of IBO/MUS in the caps were <10–2845 ppm/46–1052 ppm in *A. muscaria* and 188–269 ppm/1554–1880 ppm in *A. pantherina*. Some reports have appeared in regard to IBO/MUS contents in naturally growing *A. muscaria* and *A. pantherina*. Benedict et al. reported that the IBO content was in the range of 0.17–0.18% in dried *A. muscaria* [12]. Tsunoda et al. reported that the IBO/MUS contents in the dried Japanese *A. muscaria* caps were in the range of 192–1260 ppm/13–58 ppm [14]. The IBO contents in our samples were in general agreement with the previously reported data, however, our MUS data were higher than the

Table 3
IBO and MUS contents of *Amanita* mushrooms

Sample no.	IBO (ppm)		MUS (ppm)	
	Cap	Stem	Cap	Stem
A. muscaria				
1	612	ND	286	ND
2	97	–	472	–
3	342	–	254	–
4	<10	–	46	–
5	2845	–	1052	–
A. pantherina				
1	188	<10	1880	64
2	269	–	1554	–

(ND) not detected; (–) no sample.

Table 4
IBO and MUS contents in the cuticle and flesh of caps of *Amanita* mushrooms

Sample no.	IBO			MUS		
	Concentration (ppm)		*B*/*A* ratio	Concentration (ppm)		*B*/*A* ratio
	Cuticle (*A*)	Flesh (*B*)		Cuticle (*A*)	Flesh (*B*)	
A. muscaria						
1	84	527	6.3	239	425	1.8
2	54	1366	25.2	35	558	15.9
3	58	322	5.6	54	202	3.7
4	<10	<10	–	<25	125	>5.0
5	187	732	3.9	297	774	2.6
A. pantherina						
1	508	985	1.9	1304	3544	2.7
2	491	377	0.8	929	1242	1.3

(–) *B*/*A* ratio could not be calculated.

previous reports. Drying *A. muscaria* in the sun or with a heater caused an increase in MUS in the mushroom by decarboxylation of IBO, but a lot of IBO was lost [15]. MUS is not biogenic and can be regarded as IBO artifact. We speculate that *A. muscaria* sold in the drug market were dried in the sun or with a heater to increase the MUS content. Concerning the IBO concentration in *A. pantherina,* our results were lower than the findings reported by Benedict et al. (4600 ppm in an American *A. pantherina*) [12].

On the other hand, there were no stems in the packages of most of the mushroom samples, and the IBO/MUS levels in stems were far lower than that in caps. This tendency is in agreement with findings reported by Tsunoda et al. [14] who found lower concentrations of IBO/MUS in the stem than in the cap of *A. muscaria*.

The thresholds for observation of central nervous system disturbances in humans were 30–600 mg of IBO or about 6 mg of MUS [9]. In another reports, effects were measurable about 1 h after ingestion of 50–90 mg of IBO or 7.5–10 mg of MUS in human volunteers. These effects continued for 3–4 h, with some residual effects lasting as much as 10–24 h in some subjects [10,11]. The symptoms caused by ingestion of purified IBO/MUS in volunteers were as follows: hallucination, delirium, muscular spasm and sleep [9,22]. Some parts of the symptoms caused by IBO were presumed to be attributed to MUS derived from IBO by its decarboxylation.

Judging from the MUS concentration in the *Amanita* mushrooms used in this study, it is estimated that the ingestion of approximately 7–30 g of *A. muscaria* caps (except for sample no. 4) or approximately 4–5 g of *A. pantherina* caps would be sufficient to cause central nervous effects. The former amount is consistent with the ''recommended dosage'' (1–30 g of dried *A. muscaria* caps) claimed on the Erowid Internet site (http://www.erowid.org/plants/amanitas/amanitas.shtml). Minimum units circulated in Japan (10 g for *A. muscaria* and 4 g for *A. pantherina*) are also approximately equal to the estimated amounts.

Moreover, we separately determined the IBO/MUS contents in the cuticle and in the flesh of the caps of mushrooms (Table 4). Our findings indicated that the flesh contained a higher concentration than the cuticle in most samples. This result was in agreement with findings reported by Gore and Jordan [16] and Erowid's claim that the material just under the cuticle of *A. muscaria* was the most ''active'' portion.

In Japan, ''extracts purported to contain *A. muscaria*'' products rather than dried mushrooms are mainly circulated. However, judging from their amounts (approximately 0.3–0.5 g) in one package (Table 5), their IBO/MUS contents were too low (below the lower limit of calibration curves or not detected) to evoke dissociative effect. On the other hand, other psychoactive chemicals such as hallucinogenic tryptamine derivatives (5-methoxy-*N,N*-diisopropyltryptamine (5-MeO-DIPT) and 5-methoxy-*N,N*-dimethyltryptamine (5-MeO-DMT), reversible monoamine oxidase (MAO) inhibitors (harmine and harmaline) and tropane alkaloids (atropine and scopolamine)) were detected in these products by the modified Stas-Otto's method (Table 5). However, these chemicals were not quantified. These compounds were presumed to be artificially added, because these are not contained in the *Amanita* mushrooms. 5-MeO-DIPT has been controlled by the Narcotics and Psychotropics Control Law in Japan since 2005. The other chemicals contained in these products are not controlled as of yet.

Table 5
IBO/MUS contents and other chemicals contained in the ''extracts purported to contain *A. muscaria*''

Sample no.	Concentration (ppm)		Other contents
	IBO	MUS	
1	ND	<25	5-MeO-DIPT
2	<10	<25	5-MeO-DIPT
3	<10	<25	5-MeO-DIPT, harmaline, harmine, atropine
4	ND	<25	5-MeO-DMT, 5-MeO-DIPT, harmaline, harmine, atropine, scopolamine, caffeine

(ND) not detected; 5-MeO-DIPT, 5-methoxy-*N,N*-diisopropyltryptamine; 5-MeO-DMT, 5-methoxy-*N,N*-dimethyltryptamine.

5-MeO-DMT, which is an orally-inert tryptamine derivative [23], will be orally psychoactive by coadministration with MAO inhibitors such as harmine and harmaline [24]. This is very dangerous for public health because a severe intoxication case caused by the combination of 5-MeO-DMT and harmine was reported [25]. Moreover, MAO inhibitors may potentiate pharmacological effects of tropane alkaloids [26,27]. We therefore conclude that psychotic symptoms caused by the ingestion of these products can be attributed to multiple effects of added psychoactive chemicals.

4. Conclusion

This is the first report on chemical analysis about *Amanita* mushrooms and ‘‘extracts purported to contain *A. muscaria*’’ products circulated in the drug market. This study indicated that *Amanita* mushrooms contained high enough levels of IBO/MUS to cause central nervous effects, and that ‘‘extracts purported to contain *A. muscaria*’’ products contained other psychoactive chemicals (e.g. hallucinogenic tryptamines) in place of IBO/MUS. These results will be very useful for comprehension of drugs circulated in the Japanese illicit drug markets.

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