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.
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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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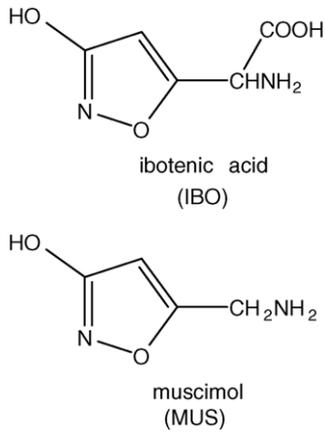 *E-mail address:* tujikawa@nrips.go.jp (K. Tsujikawa).

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.
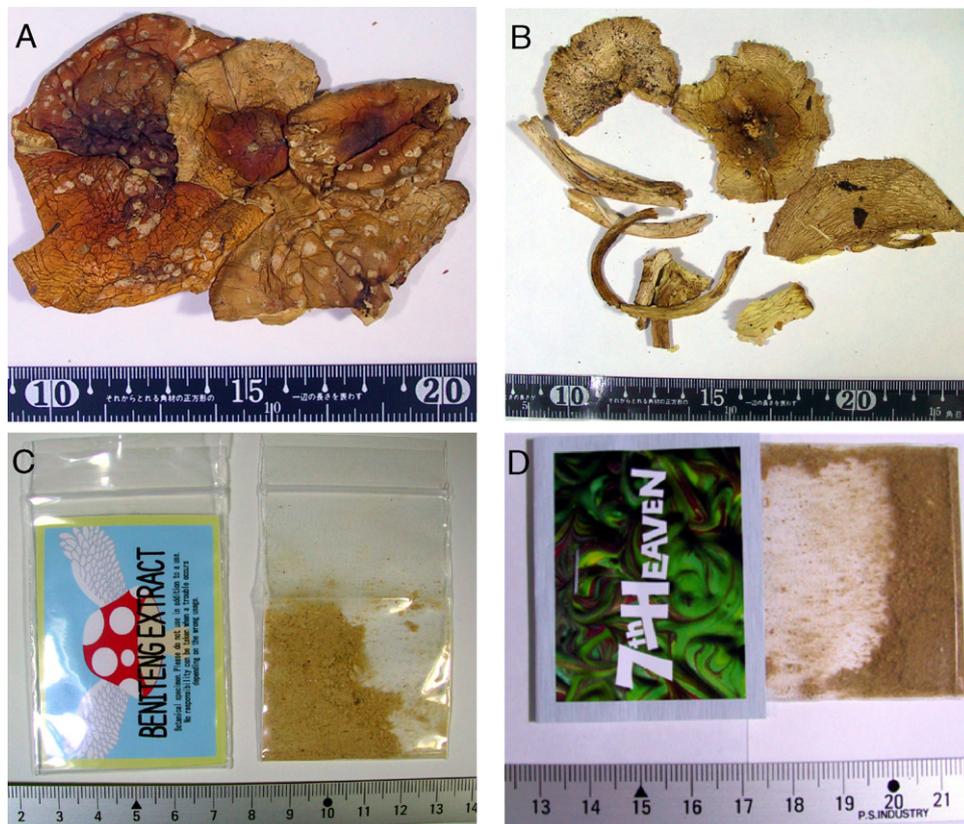

Fig. 2. Representative photographs of the samples: (A) *A. muscaria*; (B) *A. pantherina*; (C and D) ''extracts purported to contain *A. muscaria*'' products.

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 super-natant 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 × (mean back calculated concentration − nominal concentra-tion)/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 pur-ported 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 mush-room 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*
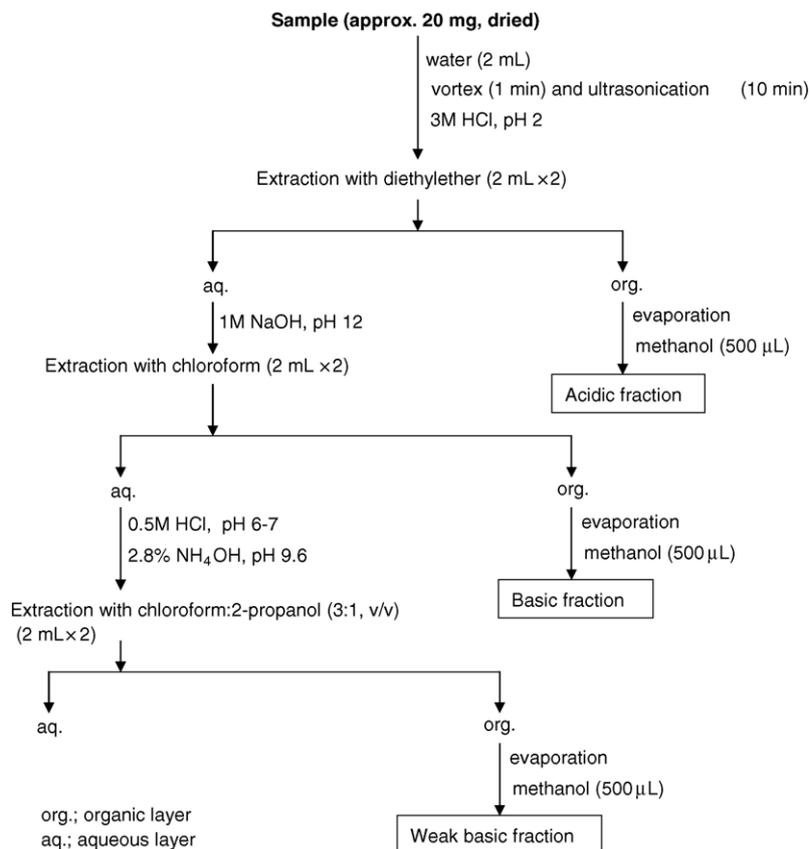
Fig. 3. Extraction procedures for other active constituents in "extracts purported to contain *A. muscaria*" products.

("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.
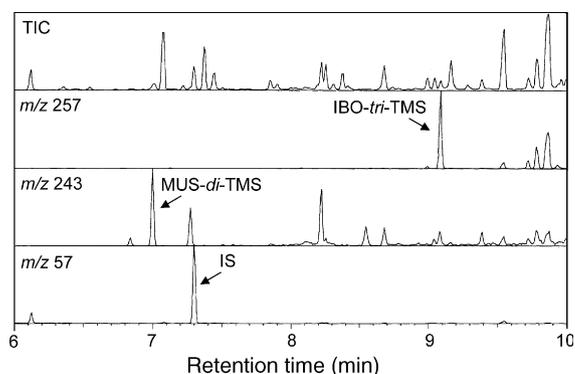

Fig. 4. TIC and mass chromatograms resulting from an *A. muscaria* (sample no. 1) in the scan mode.
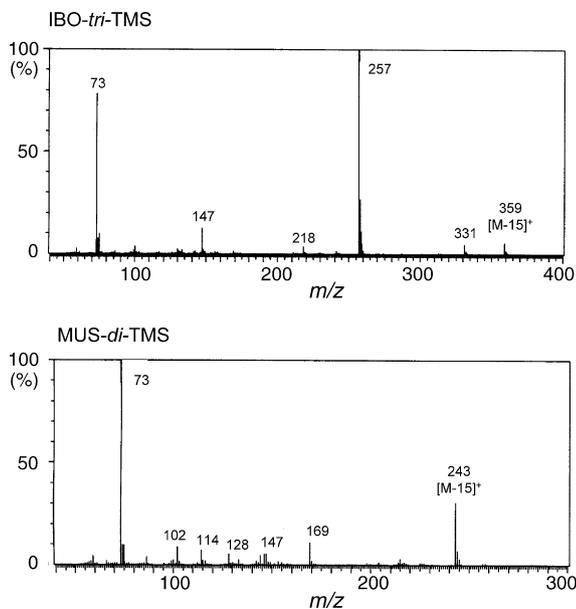
IBO-*tri*-TMS

MUS-*di*-TMS

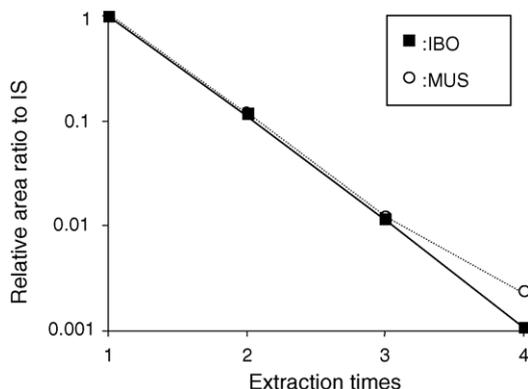Fig. 5. EI mass spectra of IBO-*tri*-TMS and MUS-*di*-TMS.

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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