2. **Zawartości IBO/MUS w grzybach świeżych (i suszonych) w zależności od części owocnika (cap, cap-flesh, cuticle, stem, stalk, base and spores)**

Ten temat jest szczególnie interesujący a najwięcej informacji dostarcza artykuł Tsunody z 1990 roku: **Changes in Concentration of Ibotenic Acid and Muscimol in the Fruit Body of Amanita muscaria during the Reproduction Stage**.
Ich wyniki zebrałem w Tabeli 2.1

Tabelka 2.1. Zawartości IBO/MUS w poszczególnych częściach świeżych owocników A. muscaria w zależności od fazy dojrzewania.

Sample no.	*Stage*	MUS (ppm)				IBO (ppm)			
		Cap	Stalk	Base	Whole body	Cap	Stalk	Base	Whole body
6	I	32	22	18	22	583	302	214	336
16	II	34	19	16	22	688	285	219	365
16	III	30	17	21	23	508	231	277	346
11	IV	28	16	23	21	438	246	385	347
13	V	26	13	21	21	364	223	348	312
	Mean	30	17	20	22	519	253	290	343

Rysunek z ich artykułu przedstawiający poszczególne fazy rozwoju grzyba A. muscaria:

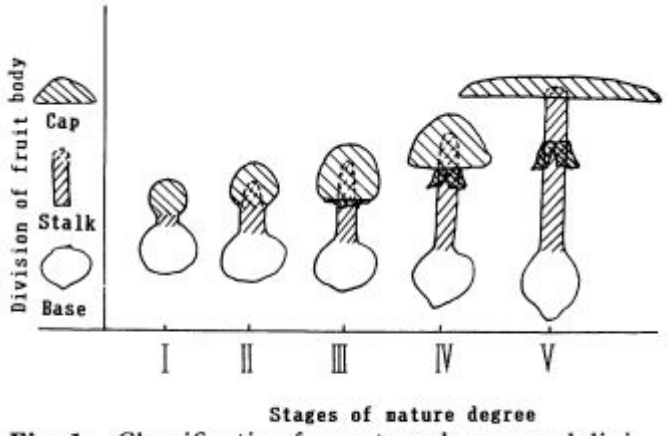

Fig. 1. Classification for mature degree, and division of fruit body, *A. muscaria*

Ich wyniki są bardzo ciekawe:
- większość IBO i MUS znaleziono w kapeluszach (Cap): 519 ppm IBO, 30 ppm MUS
- następnie w pochwie (Base): 290 ppm IBO, 20 ppm MUS
- najmniej znaleźli w trzonach (Stalk): 253 ppm IBO, 17 ppm MUS

Tu warto zaznaczyć, ze różnice zawartość IBO i MUS w poszczególnych częściach wcale nie są aż takie wielkie: **Wręcz średnie wartości ibotenu i muscymolu w trzonach i pochwach razem – <u>przewyższają te w kapeluszach</u> (!)** – oczywiście średnio licząc.

Następne wnioski –aczkolwiek nie stanowią meritum tego opracowania - też są bardzo ciekawe:

- Stężenia IBO i MUS w kapeluszu zwiększały się pod czas fazy I – II (maksimum), po czym stopniowo zmniejszały, aż do minimum w ostatnie fazie V **(czyli gdy owocnik jest najdojrzalszy !!!**) Podobnie w trzonie.
- jeżeli chodzi o pochwę (Base) to następował stopniowy wzrost stężenia IBO i MUS aż do maksimum w fazie IV
- poziomy IBO i MUS licząc cały owocnik (Whole body) **były prawie stałe podczas dojrzewania** (z lekka przewaga fazy II i III), chociaż wzrost masy całego owocnika był sześciokrotny – porównując do masy pochwy, która masa nie zmieniła się pod czas dojrzewania.

Następnie do omówienia dostępne są dwa artykuły Tsujikawy:

1. Determination of muscimol and ibotenic acid in Amanita mushrooms by high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry (2007)
2. Analysis of hallucinogenic constituents in Amanita mushrooms circulated in Japan (2006)

Tabelka zbiorcza wyników badan Tsujikawy z 2006 i 2007 roku.

Kenji Tsujikawa I inni 2007					
Amanita mushrooms naturally grown: 7 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 All samples were stored at 4 ◦C until analysis.					
Sample no.	***Amanita* sp.**	**MUS (ppm)**		**IBO (ppm)**	
		Cap	**Stem**	**Cap**	**Stem**
1	*A. muscaria-only cap*	381	-	623	-
2	*A. muscaria-only cap*	46	-	182	-
3	*A. muscaria-only cap*	317	-	528	-
4	*A. muscaria*	599	292	615	627
5	*A. muscaria*	204	82	785	1998
6	*A. muscaria-only cap*	859	-	1469	-
7	*A. muscaria*	1203	159	1839	751

Kenji Tsujikawa I inni 2006					
7 dried mushrooms sold as Amanita muscaria or Amanita pantherina (five A. muscaria and two A. pantherina) that are currently circulated in Japan were determined.					
Sample no.	***Amanita* sp.**	**MUS (ppm)**		**IBO (ppm)**	
		Cap (Cuticle)	**Cap (flesh)**	**Cap (Cuticle)**	**Cap (flesh)**
1	*A. muscaria*	239	425	84	527
2	*A. muscaria- only cap*	35	558	54	1366
3	*A. muscaria- only cap*	54	202	58	322
4	*A. muscaria- only cap*	<25	125	<10	<10
5	*A. muscaria- only cap*	297	774	187	732
6	*A. pantherina*	1304	3544	508	985
7	*A. pantherina- only cap*	929	1242	491	377

Widać, że – tam gdzie próbka trzonu jest dostępna - znaleziono zarówno IBO jak i MUS. Ich wartości trudno porównywać do badan Tsunody, gdyż są to próbki grzybów suszonych i metoda suszenia raczej na pewno miała wpływ na wartości IBO i MUS. W jednej próbce jest dwa i pół razy więcej ibotenu w trzonie niż w kapeluszu a w innej prawie tyle samo.
Zawartość muscymolu - na podstawie tych trzech próbek (badanie z 2007) – jest bardziej przewidywalna i jest zawsze mniejsza w trzonie a większa w kapeluszu: od dwa do siedmiu razy większa.
Ciekawsze jest badanie z 2006 roku, gdzie mamy wyniki zawartości IBO i MUS w samej skorce i w pozostałej części kapelusza. **Widać, że w miąższu znajduje się zdecydowanie więcej ibotenu i muscymolu niż w samej skorce. Od około 4 do 25 razy więcej ibotenu i od 2 do 16 razy więcej muscymolu.**

Ostatnim badaniem, które przedstawię krótko, jest badanie zawartości IBO i MUS w zarodnikach (Spores). Jest to badanie z 2004 roku, którego wyniki przedstawia poniższa tabelka:

FREDRIK C STØRMER & KAREL JANAK 2004
Ibotenic acid in Amanita muscaria spores and caps

Amanita muscaria was collected in three different localities in Akershus and Oslo County in 2001, so that variation due to age and locality difference could be properly assessed. The stems were removed from the mushrooms, and the caps were placed onto glass plates for 24 h to collect the spores. No moisture was observed during the spore drop. The caps and spores were and stored at -20ºC

Sample no.	*Amanita* sp.	% MUS in fresh (ppm)		% IBO in fresh (ppm)	
		Cap	Spores	Cap	Spores
1	*A. muscaria-only cap*	ND	ND	0.0078	0.0047
2	*A. muscaria-only cap*	ND	ND	0.0260	0.0061
3	*A. muscaria-only cap*	ND	ND	0.0160	0.0055

Dziwne że nie wykryli muscymolu w kapeluszach (?), natomiast wykryli iboten zarówno w kapeluszach jak i zarodnikach. Od 1,6 do 4,3 razy więcej ibotenu w kapeluszach niż w samych zarodnikach.

Wszystkie te dane są bardzo ciekawe i pokazują, że iboten i muscymol rozsiane są po całym owocniku i zasadne jest zbieranie muchomora w całości.
Ja zamierzam w sezonie 2020 nazbierać trochę trzonów (plus pochwy), ususzyć i zmiksować na proszek tak by ujednolicić zawartość IBO i MUS. Myślę, że jeżeli chodzi o spożycie to ambrozja będzie dobrym wyjściem. Zobaczymy jaka moc maja trzony :)

Pozdrawiam
Dobry Muchomor

Źródła:

1. Kenji Tsujikawa i inni 2006, Analysis of hallucinogenic constituents in Amanita mushrooms circulated in Japan
2. 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
3. Koujun TSUNODA i inni 1990, Changes in Concentration of Ibotenic Acid and Muscimol in the Fruit Body of Amanita muscaria during the Reproduction Stage
4. FREDRIK C STØRMER & KAREL JANAK 2004, Ibotenic acid in Amanita muscaria spores and caps

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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

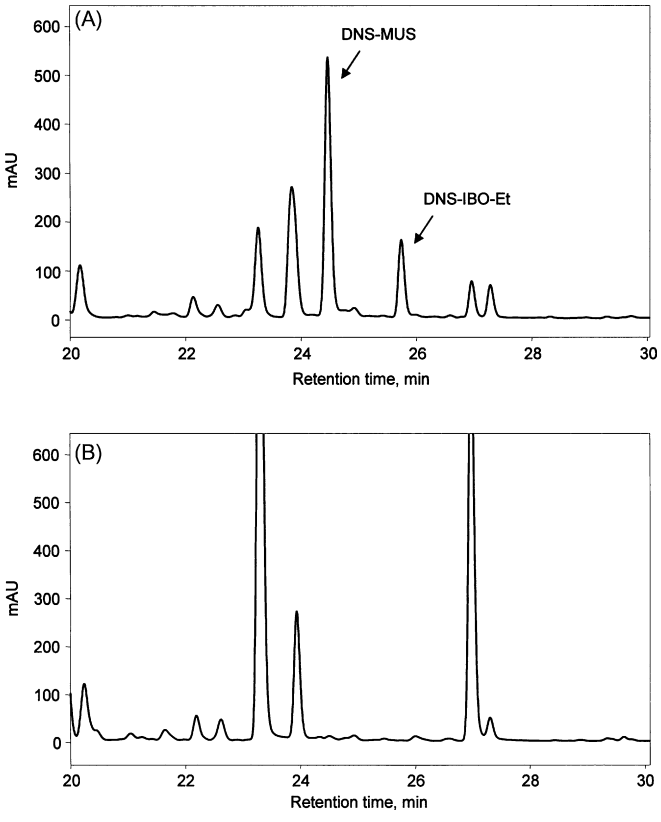

Fig. 1. Chromatograms obtained from *A. pantherina* (sample #5) extract (A) and blank mushroom (*Pleurotus ostreatus*) extract (B). Detection: 256 nm.

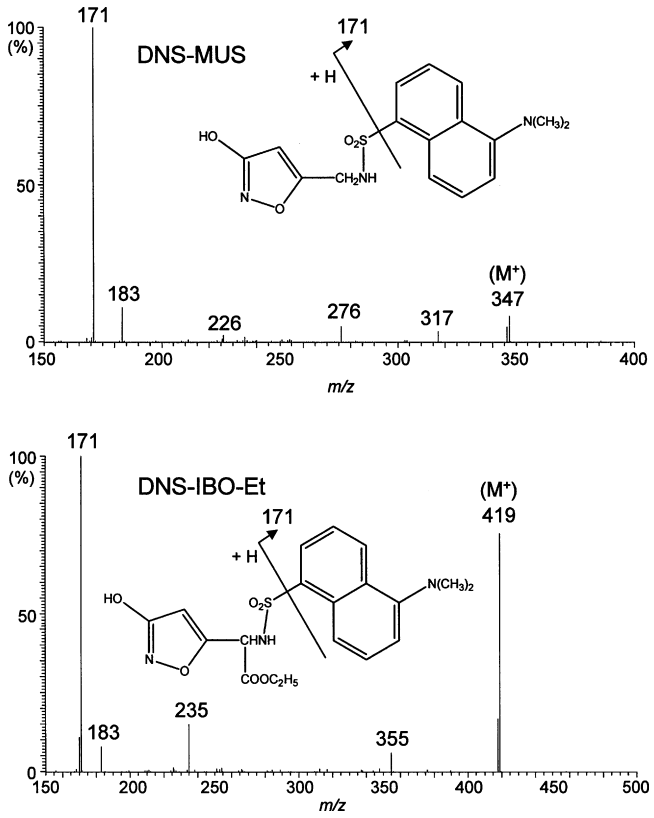

Fig. 2. Product ion spectra at *m/z* 347 from DNS-MUS and *m/z* 419 from DNS-IBO-Et.

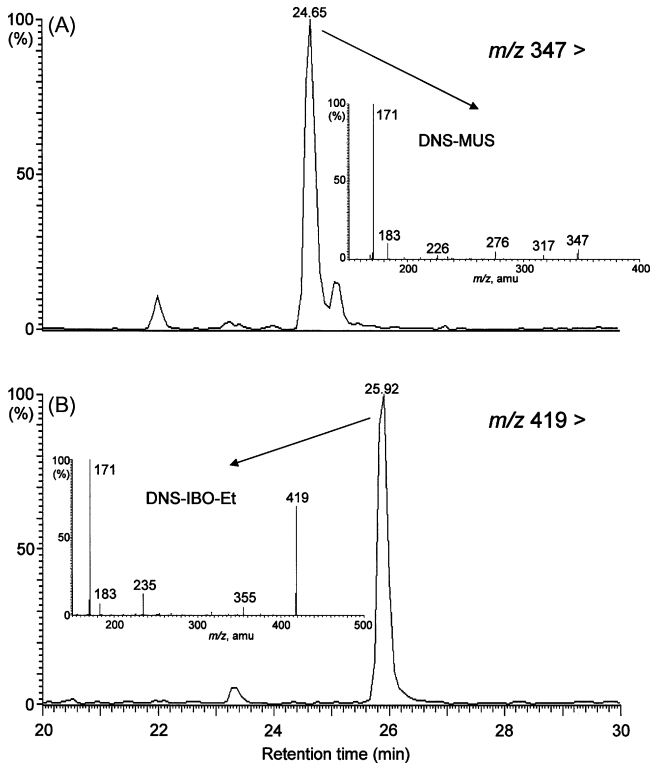

Fig. 3. LC/MS/MS analysis of the cap of *A. pantherina* (sample #5). (A): Total ion chromatogram in the product ion scan mode using *m/z* 347 and product ion spectrum at *m/z* 347 for the 24.65-min peak. (B): Total ion chromatogram in the product ion scan mode using *m/z* 419 and product ion spectrum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

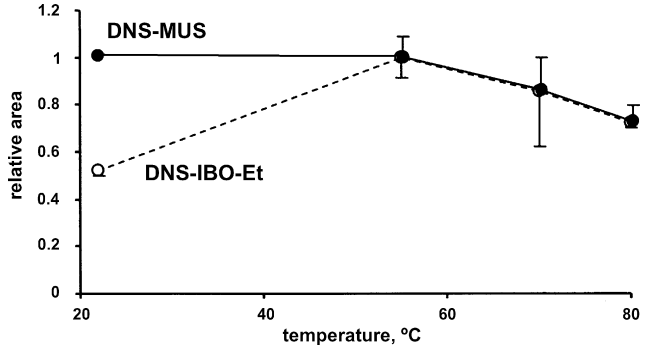

Fig. 4. Optimization of the reaction temperature for ethylation of DNS-IBO-Et. Each sample was heated for 60 min at 22 °C (room temperature), 55 °C, 70 °C, or 80 °C. Each datum indicates a relative peak area normalized to that of 55 °C. Each data point represents the mean ± SD of three determinations.

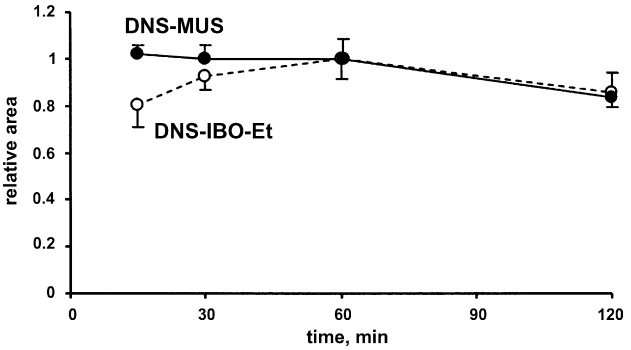

Fig. 5. Optimization of the reaction time for ethylation of DNS-IBO-Et. Each sample was heated at 55 °C for 15 min, 30 min, 60 min, or 120 min. Each datum is indicated as relative peak area normalized to that of 60 min. Each data point represents the mean ± SD of three determinations.

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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References

[1] R. Imazeki, T. Hongo, Books of Fungi in Japan Illustrated in Color, second ed., Hoikusya, Tokyo, Japan, 1977, p. 44, in Japanese.
[2] D.R. Benjamin, J. Toxicol. Clin. Toxicol. 30 (1992) 13.
[3] A. Oztekin-Mat, Ann. Pharm. Fr. 52 (1994) 260.
[4] Archives on the Occurrence of Food Poisoning in Japan, Ministry of Health, Labour and Welfare, Tokyo, Japan, 2002–2004.
[5] L. Satora, D. Pach, B. Butryn, P. Hydzik, B. Balicka-Slusarczyk, Toxicon 45 (2005) 941.
[6] J.H. Halpern, Pharmacol. Ther. 102 (2004) 131.
[7] M.R. Jacobs, K.O. Fehr, Drugs and Drug Abuse, second ed., Addiction Research Foundation, Toronto, 1987, p. 489.
[8] F.G. Waser, in: D.H. Efron, B. Holmstedt, N.S. Kline (Eds.), Ethnopharmacological Search for Psychoactive Drugs, U.S. Public Health Service Publication, Washington, DC, 1979, p. 419.
[9] W. Theobald, O. Büch, H.A. Kinz, P. Krupp, E.G. Stenger, H. Heimann, Arzneim. Forsch. 18 (1968) 311.
[10] R.G. Benedict, V.E. Tyler Jr., L.R. Brady, Lloydia 29 (1966) 333.
[11] K. Tsunoda, N. Inoue, Y. Aoyagi, T. Sugahara, J. Food Hyg. Soc. Jpn. 34 (1993) 12.
[12] M.G. Gore, P.M. Jordan, J. Chromatogr. 243 (1982) 323.
[13] D.B. Repke, D.T. Leslie, N.G. Kish, J. Pharm. Sci. 67 (1978) 485.
[14] K. Tsujikawa, H. Mohri, K. Kuwayama, H. Miyaguchi, Y. Iwata, A. Gohda, S. Fukushima, H. Inoue, T. Kishi, Forensic Sci. Int. 164 (2006) 172.
[15] H. Mohri, A. Gohda, S. Fukushima, Jpn. J. Sci. Tech. Iden. 8 (Suppl.) (2003) 69.
[16] Y. Li, H. Shi, S. Zhou, W.Z. Shou, X. Jiang, W. Naidong, J.H. Lauterbach, Rapid Commun. Mass Spectrom. 19 (2005) 3331.
[17] F. Beaudry, S.A. Guenette, A. Winterborn, J.F. Marier, P. Vachon, J. Pharm. Biomed. Anal. 39 (2005) 411.
[18] A. Gantverg, G. Elliott, J. Pineault, R. Demers, J. Chromatogr. B 803 (2004) 311.
[19] Y. Tapuhi, N. Miller, B.L. Karger, J. Chromatogr. 205 (1981) 325.
[20] K. Tsunoda, N. Inoue, Y. Aoyagi, T. Sugahara, J. Food Hyg. Soc. Jpn. 34 (1993) 18, in Japanese.
[21] T. Maruyama, N. Kawahara, T. Fukiharu, K. Yokoyama, Y. Makino, Y. Goda, J. Food Hyg. Soc. Jpn. 46 (2005) 49, in Japanese.
[22] D. Michelot, L.M. Melendez-Howell, Mycol. Res. 107 (2003) 131.
[23] K. Tsunoda, N. Inoue, Y. Aoyagi, T. Sugahara, J. Food Hyg. Soc. Jpn. 34 (1993) 153, in Japanese.

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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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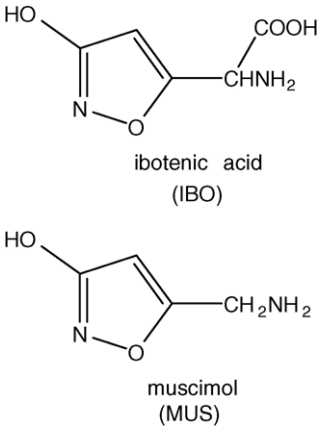

Fig. 1. Chemical structures of ibotenic acid (IBO) and muscimol (MUS).

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.

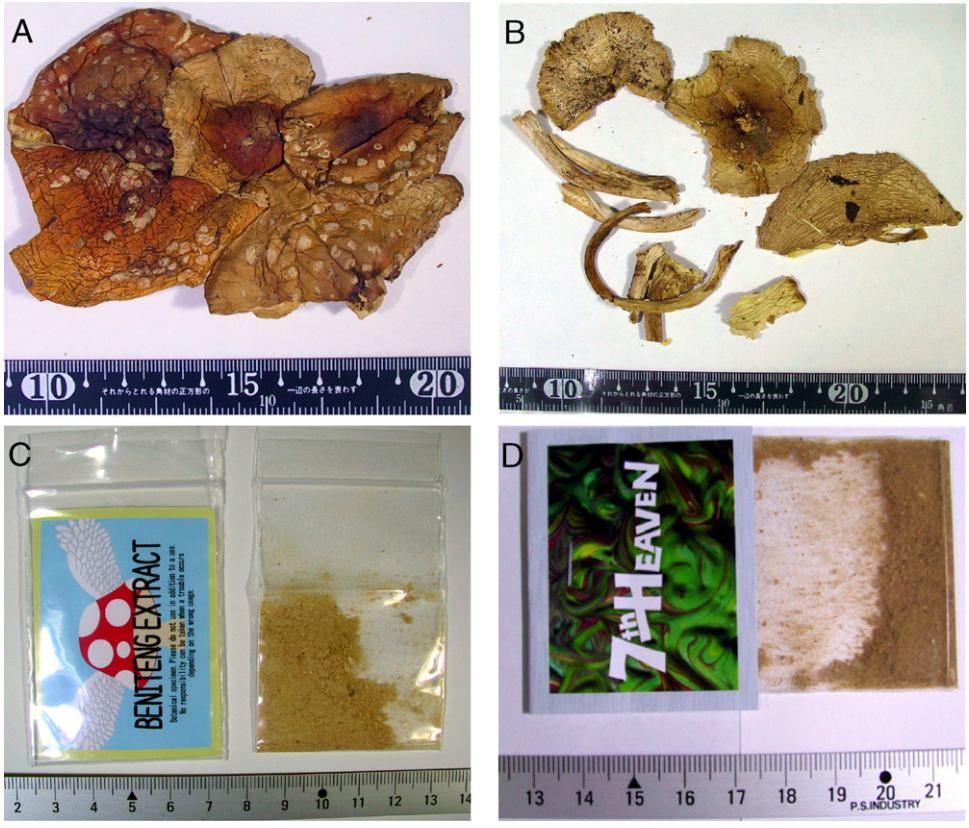

Fig. 2. Representative photographs of the samples: (A) *A. muscaria*; (B) *A. pantherina*; (C and D) "extracts purported to contain *A. muscaria*" products.

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*

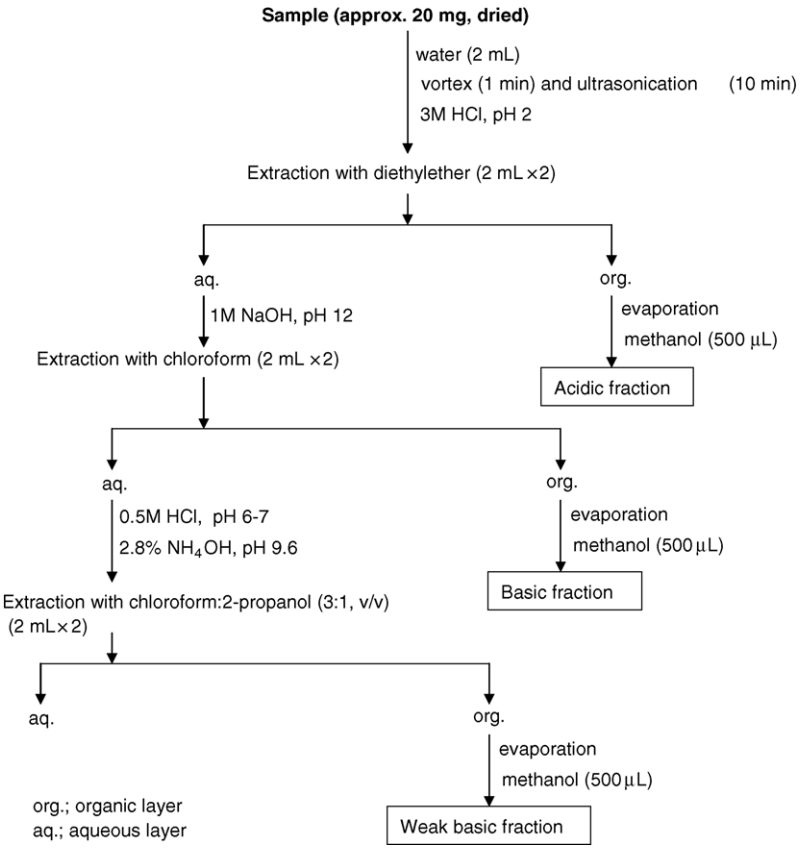

Fig. 3. Extraction procedures for other active constituents in "extracts purported to contain *A. muscaria*" products.

("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.

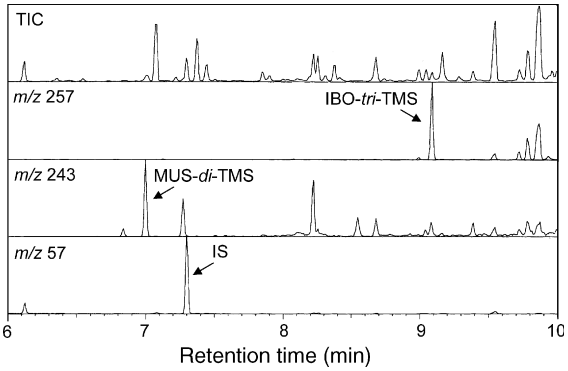

Fig. 4. TIC and mass chromatograms resulting from an *A. muscaria* (sample no. 1) in the scan mode.

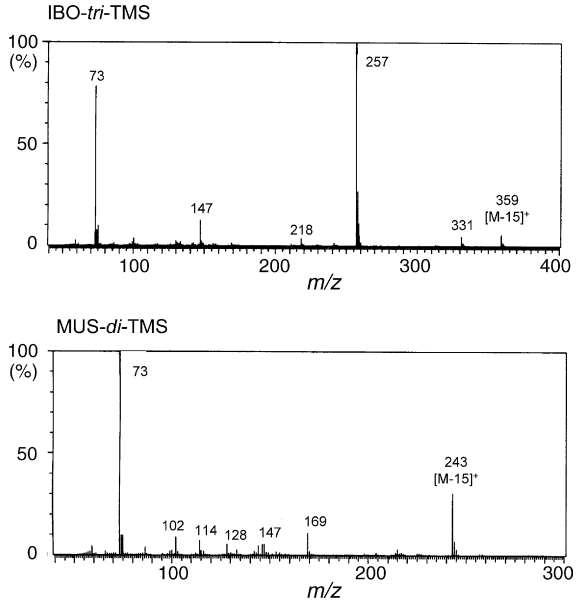

Fig. 5. EI mass spectra of IBO-*tri*-TMS and MUS-*di*-TMS.

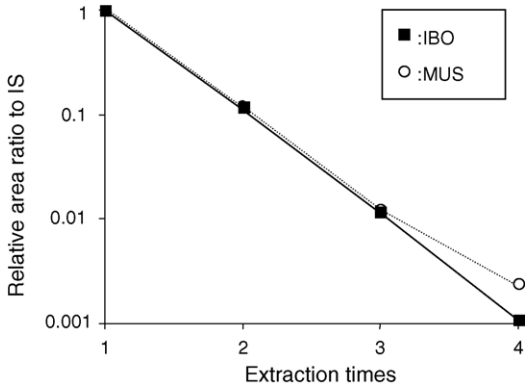

Fig. 6. Recovery of IBO and MUS in *A. muscaria* after repeated extraction. The *Y*-axis is relative area ratio to the first extract and the *X*-axis denotes extraction times. Each data point represents the mean of triplicate determinations.

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.

References

[1] M. Saar, Ethnomyocological data from Siberia and North-East Asia on the effect of *Amanita muscaria*, J. Ethnopharmacol. 31 (1991) 157–173.
[2] S. Hajicek-Dobberstein, Soma siddhas and alchemical enlightenment: psychedelic mushrooms in Buddhist tradition, J. Ethnopharmacol. 48 (1995) 99–118.
[3] J.H. Halpern, Hallucinogens and dissociative agents naturally growing in the United States, Pharmacol. Ther. 102 (2004) 131–138.
[4] J. Riba, M. Valle, G. Urbano, M. Yritia, A. Morte, M.J. Barbanoj, Human pharmacology of ayahuasca: subjective and cardiovascular effects, monoamine metabolite excretion, and pharmacokinetics, J. Pharmacol. Exp. Ther. 306 (2003) 73–83.
[5] Y. Kuroki, The latest tendency of uncontrolled substances inquires to JPIC, Jpn. J. Toxicol. 17 (2004) 241–243 (in Japanese).
[6] T.A. Verdoorn, R. Dingledine, Excitatory amino acid receptors expressed in *Xenopus* oocytes: agonist pharmacology, Mol. Pharmacol. 34 (1988) 298–307.
[7] E.O. Nielsen, A. Schousboe, S.H. Hansen, P. Krogsgaard-Larsen, Excitatory amino acid: studies on the biochemical and chemical stability of ibotenic acid and related compounds, J. Neurochem. 45 (1985) 725–731.
[8] E.J. Nestler, S.E. Hyman, R.C. Malenka, Molecular Pharmacology: A Foundation for Clinical Neuroscience, McGraw-Hill, New York, 2001, p. 10.
[9] F.G. Waser, The pharmacology of *Amanita muscaria*, in: D.H. Efron, B. Holmstedt, N.S. Kline (Eds.), Ethnopharmacological Search for Psychoactive Drugs, U.S. Public Health Service Publication, Washington, DC, 1979, pp. 419–439.
[10] C.H. Eugster, Isolation structure and synthesis of central active compounds from *Amanita muscaria* (L. ex Fr.) Hooker, in: D.H. Efron, B. Holmstedt, N.S. Kline (Eds.), Ethnopharmacological Search for Psychoactive Drugs, U.S. Public Health Service Publication, Washington, DC, 1979, pp. 416–419.
[11] W.S. Chilton, The course of an intentional poisoning, McIlvanea 2 (1975) 17–18.
[12] R.G. Benedict, V.E. Tyler Jr., L.R. Brady, Chemotaxonomic significance of isoxazole derivatives in *Amanita* species, Lloydia 29 (1966) 333–341.
[13] K. Tsunoda, N. Inoue, Y. Aoyagi, T. Sugahara, Simultaneous analysis of ibotenic acid and muscimol in toxic mushroom, *Amanita muscaria*, and analytical survey on edible mushrooms, J. Food Hyg. Soc. Jpn. 34 (1993) 12–17.
[14] K. Tsunoda, N. Inoue, Y. Aoyagi, T. Sugahara, Changes in concentration of ibotenic acid and muscimol in the fruit body of *Amanita muscaria* during the reproduction stage (food hygienic studies of toxigenic basidiomycotina II), J. Food Hyg. Soc. Jpn. 34 (1993) 18–24 (in Japanese).
[15] K. Tsunoda, N. Inoue, Y. Aoyagi, T. Sugahara, Change in ibotenic acid and muscimol contents in *Amanita muscaria* during drying, storing or cocking, J. Food Hyg. Soc. Jpn. 34 (1993) 153–160 (in Japanese).
[16] M.G. Gore, P.M. Jordan, Microbore single-column analysis of pharmacologically active alkaloids from the fly agaric mushroom *Amanita muscaria*, J. Chromatogr. 243 (1982) 323–328.
[17] D.B. Repke, D.T. Leslie, N.G. Kish, GLC–mass spectral analysis of fungal metabolites, J. Pharm. Sci. 67 (1978) 485–487.
[18] R. Watling, Hallucinogenic mushrooms, J. Forensic Sci. Soc. 23 (1983) 53–66.
[19] R. Imazeki, T. Hongo, Books of Fungi in Japan Illustrated in Color, second ed., Hoikusya, Tokyo, Japan, 1977, p. 5 (in Japanese).
[20] R. Imazeki, T. Hongo, Books of Fungi in Japan Illustrated in Color, second ed., Hoikusya, Tokyo, Japan, 1977, p. 44 (in Japanese).
[21] D.T. Jenkins, R.H. Petersen, A neotype specimen for *Amanita muscaria*, Mycologia 68 (1976) 463–469.
[22] W. Theobald, O. Büch, H.A. Kinz, P. Krupp, E.G. Stenger, H. Heimann, Pharmacological and experimental investigations of two components of the fly agaric, Arzneim. Forsch. 18 (1968) 311–315.
[23] B.L. Roth, Hallucinogens, Pharmacol. Ther. 101 (2004) 131–181.
[24] J. Ott, Phamepena-psychonautics: human intranasal, sublingual and oral pharmacology of 5-methoxy-*N*,*N*-dimethyltryptamine, J. Psychoactive Drugs 33 (2001) 403–407.
[25] D.E. Brush, S.B. Bird, E.W. Boyer, Monoamine oxidase inhibitor poisoning result from Internet misinformation on illicit substances, J. Toxicol. Clin. Toxicol. 42 (2004) 191–195.
[26] Drug Package Insert of Atropine Sulfate Injection, Tanabe Seiyaku Co., Ltd.
[27] Drug Package Insert of PAN-SCO Injection, Opium Alkaloids and Scopolamine Injection, Takeda Pharmaceutical Co., Ltd.

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Ibotenic acid in *Amanita muscaria* spores and caps

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Summary: Ibotenic acid (α-amino-3-hydroxy-5-isoxazole acetic acid) was separated from spores and caps from *Amanita muscaria* by reversed phase high performance liquid chromatography and identified by flow injection analysis with mass spectrometric detection. The keto and enol tautomers of ibotenic acid were separated and their ratio of 96:4 in favour of the enol form was determined. On average the ibotenic acid content was 0.0054±0.0010% of the spores and 0.017±0.010% in fresh caps. Muscimol, the decarboxylated product from ibotenic acid, was neither detected in spores nor in caps. 50 nanomol of ibotenic acid, muscimol or extracts from spores or caps did not inhibit the growth of *Bacillus subtilis*.

Keywords: *Amanita muscaria*. basidiospores, ibotenic acid, muscimol, neurotoxin, tautomerism

Introduction

Fly agaric (*Amanita muscaria* (L.: Fr.) Pers.) is one of our most handsome and mysterious toadstools. It grows in symbiosis with arboreal trees, such as birch, pine or fir in Europe and America. Poison extracted from this mushroom has traditionally been used for the destruction of flies and other insects – hence its name. Ibotenic acid, a-amino-3-hydroxy-5-isoxazoleacetic acid, is the principal toxin in *A. muscaria*. It is a neurotoxic substance found in *A. muscaria* and *A. pantherina* (DC.: Fr.) Krombh. Because of the acidic property of the isoxazole moiety, it is similar to glutamic acid and mimics its effect in animals. It causes motor depression, ataxia, and changes in mood, perceptions and feelings. Muscimol, 3-hydroxy-5-amino methylisoxazole, which is the decarboxylated product from ibotenic acid, shows structurally resemblance to GABA (g-amino butyric acid) and imitates the action of GABA, as an inhibitory neurotransmitter in the central nervous system in animals and humans.

Several mycotoxins have been detected in the conidia of moulds, for instance aflatoxins (Wicklow & Shotwell, 1983) aurasperone C and fumigaclavine (Palmgren & Lee, 1986), trichothecene mycotoxins (Sorensen *et al*., 1987), fumonisins and AAL-toxin (Abbas & Riley, 1996), and citrinin and minor amounts of ochratoxin A (Størmer *et al*., 1998). The presence of ochratoxin A in dust collected from households and from cowsheds (Richard *et al*., 1999, Skaug, Eduard & Størmer, 2001) indicates that fungal spores containing mycotoxins may pose a respiratory problem for humans as well as for animals. Occupational respiratory diseases associated with exposure to mushroom spores have been reported in recent years, but no toxins have been described. (Kamm *et al*., 1991; *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinus edodes*, Moinard *et al*.,1991; *Poria megalopora*, Matsui *et al*., 1992; *Lentinus edodes*, Ishii *et al*., 1994; *Pholiota nameko*, Inage *et al*., 1996; *Pholiota nameko*.) Working on a mushroom farm carries a significant risk for chronic cough from inhalation of spores from *Hypsizigus marmoreus* (Tanaka *et al*., 2002).

Orellanine, a toxin responsible for serious intoxications has recently been quantified in spores from *Cortinarius orellanus* Fries and *C. rubellus* Cooke (Koller *et al*., 2002). Prior to this study no report had described quantification of toxins in spores from Basidiomycota, despite their frequent association with poisoning and the increased interest on natural toxins and human health. In this work we have determined the amount of ibotenic acid in spores and caps from *A. muscaria*.

Materials and methods

Collection of fungal material and spore isolation- *Amanita muscaria* was collected in three different localities in Akershus and Oslo County in 2001, so that variation due to age and locality difference could be properly assessed. The stems were removed from the

mushrooms, and the caps were placed onto glass plates for 24 h to collect the spores. No moisture was observed during the spore drop. The sampled spores contained approximately 95% basidiospores, as evaluated by microscopic examination. The caps and spores were and stored at -20ºC. Spore volume can be calculated as that of an ellipsoid (Gross, 1972, Meerts, 1999) with spores of *A. muscaria* species with average length and width 10.5x8.5 µm having a spore volume of approximately 400 μm^3. Assuming a density of 1 g/cm^3, this corresponds to a spore weight of 400 pg.

Extraction of ibotenic acid and muscimol- The spore material was diluted to 5 mg/ml corresponding to $1.3x10^7$ spores/ml in water containing 0.1% formic acid and stored 10 min at room temperature. The solution was filtered, and 1.5 ml of the clear solution was freeze dried, dissolved in 0.5 ml water and used in the analysis. Prolonged storage, freezing and thawing, or 15 min with ultrasound treatment, did not increase the content of ibotenic acid released from the spores. At least three caps from each locality were powdered in liquid N_2 and added twice its weight with water. After centrifugation for 15 min at 15000 g and 4ºC, the clear supernatant was removed and stored at -20ºC.

High-pressure liquid chromatography (HPLC)- The HPLC equipment used for analysis consisted of a Perkin-Elmer series 4 HPLC pump, a Hewlett Packard 11040 photodiode array detector, and a 7125-075 Rheodyne injector with a variable volume loop. Waters (115x13 mm) C-18 preparative column was used for the ibotenic acid analysis. The mobile phase, flow rate of 0.5 ml/min, consisted of water-acetonitrile-methanol (65:20:15 v/v) containing sodium dodecylsulfate (2.1 mM) and phosphoric acid (4 mM) (Tsunada *et al*. 1993). All analyses were carried out at laboratory temperature. Ibotenic acid in extracts was tentatively confirmed by retention of standard at 8.2 min, and the amounts of ibotenic acid in the sample was determined by comparison with a standard curve. Zorbax SB-Aq (4.6x150 mm) was used for the muscimol analysis with flow rate of 1.0 ml/min and the same mobile phase as described above with a retention time of 12.8 min (sensitivity 1 nmol).

Liquid chromatography-Mass spectrometry (LC-MS)- Mass spectra were obtained with a VG Platform quadruple mass spectrometer (Fisons Instruments, VG Bio-tech, Altrincham, UK) equipped with an atmospheric pressure electro spray ionisation source. Characteristic ions for identification of ibotenic acid were determined by flow injection analysis (FIA) with mass spectrometric detection using electro spray in positive mode. Standard solution of the ibotenic acid at a concentration of 0.1 µg/ml in methanol-water (1:3 v/v) was injected into VG Platform at a flow of 10 µl/min. Total ion current mass spectra were recorded for mass ions in the range of 50–250 at a 3.75 kV and 0.35 kV for Capillary and HV lens, respectively, at a Skimmer Lens voltage of 5 V and at a Cone voltage optimised in the range of 10-70 V. The temperature of the electro spray source was set to 80ºC. Positive ionisation of ibotenic acid was examined at a pH of 2.0 adjusted by formic acid and at a pH of 6.1 adjusted by ammonium/ammonium acetate solution. To confirm the findings of ibotenic acid in spores and caps, water extracts of samples acidified with formic acid (0.1% v/v) were injected by Rheodyne 7125 injector using 20 µl loop on an XTerra™ MS C_{18} column (5 µm; 2.1x100 mm) (Waters) provided with Sentry™ Guard Column (2.1x10 mm). Composition of the mobile phase delivered by gradient pump (Perkin-Elmer) at a flow of 0.5 ml/min was programmed from water/methanol (19:1 v/v) to acetonitrile/water/methanol (18:1:1 v/v) in 1 min and then it was held for 7 min. Analysis was monitored by variable UV detector (Hewlett-Packard 1050) at 254 nm and ibotenic acid was selectively detected by VG Platform quadruple mass spectrometer equipped with an electro spray atmospheric pressure ionisation source. Parameters controlling mass spectrometric detection in single ion recording mode for masses at 53.7 and 91.0 were set as given above. Analysis was performed twice with cone voltages 50 V and 60 V, respectively. Chromatograms were acquired and result evaluated using Masslynx™ 3.5 software (Micro mass UK Lim.).

Results

The amount of ibotenic acid in spores of *Amanita muscaria*, based on the detemination from three different localities, varied from 0.0047 to 0.0061%with an average of 0.0054±0.0010 (Table 1). The amounts of ibotenic acid in caps (wet weight) from the same localities varied from 0.0078 to 0.0260%

Table 1 Ibotenic content of spores and caps from *A. muscaria*:

Location	% IBO in spores[a]	% IBO in fresh caps
1	0.0047	0.0078
2	0.0061	0.0260
3	0.0055	0.0160

[a]Assuming no water content

with an average of 0.017±0.010% (0.17% dry weight based on the assumption that 90% of the mushroom content is water) from the measurement from the three different localities. Muscimol was not detected in extracts from either spores or caps.

LC-MS experiments were performed in order to confirm the findings of ibotenic acid in the spores and caps of *A. muscaria*. Using FIA with mass spectrometric detection at a positive electro spray ionisation, ibotenic acid gave an intense molecular ion M^+ at m/z=158.0 and even more intense ion $(M\text{-}OH)^+$ at m/z=140.9 at both pH=2.0 and pH=6.1. The presence of ibotenic acid was confirmed in all samples from both spores and caps using these specific masses. LC-MS using monitoring of the molecular ion M^+ resulted in two poorly resolved and tailing peaks with retention time of 0.98 min and 1.12 min respectively for both the standard of ibotenic acid as well as the extracts of the spores and caps. Selective ion monitoring revealed two clear peaks that were identified as keto and enol tautomers of ibotenic acid. The peak less retained on C-18 reverse phase column gave in electro spray atmospheric pressure ionisation predominantly ion at m/z=91 which corresponds to an adduct of the enol form of the protonated ibotenic acid with a sodium ion. The next intense ion in this peak is the one with m/z=82 that correspond to sodium adduct with a dehydroxylated form of the enol form of ibotenic acid. The more retained peak eluting in between 1.06 min and 1.23 min with a maximum at 1.12 min has been identified as the keto form of the ibotenic acid. The mass spectrum recorded at the maximum of the peak revealed an intense ion at m/z=53.7 (triple protonated molecular ion of the ibotenic acid), and a minor ion at m/z=71 (double charged; protonated and dehydroxylated molecular ion of the ibotenic acid) and at m/z=102 (molecular cluster with two sodium ions). The prevailing enol tautomer was evaluated to make about 95% of the compound.

Using selective ion monitoring at m/z=53.7 and at m/z=91.0, tautomers of ibotenic acid were identified in caps of the mushrooms. Ratio of keto and enol tautomers of ibotenic acid in the extracts was evaluated to be about 4:96 in favour of the hydroxy form. Neglecting minor ions (m/z=82 for enol- form, and m/z=71 and m/z=102 for keto- form) should not result in relative deviation higher then 10%.

We measured the effect of spore and cap extracts upon growth of *Bacillus subtilis*. Extracts from 0.5 mg spores or 12.5 mg caps (wet weight) did not inhibit the growth of *B. subtilis*. The extracts tested stimulated bacterial growth. 50 nmol of either ibotenic acid or muscimol had no effect upon growth of the organism.

Discussion

In two earlier investigations the content of ibotenic acid and muscimol in the caps (wet weight) from *A. muscaria* were determined to 0.0519% and 0.0253% (Tsunoda *et al*., 1993) and 0.099% and 0.038% respectively (Gennaro, Giascosa & Angelino, 1997). We determined the ibotenic acid in samples from three different localities with an average of 0.017% (Table 1). These values are lower than earlier described. Different environments and different strains of the mushroom may explain the discrepancy. The amount of toxin present may also be dependent on the age of the collected species, which may also be reflected in the variation in our values. Muscimol was not detected in our samples, probably due to the pre-treatment of the caps with liquid nitrogen followed by extraction, which did not allow the decarboxylase enzymes to act upon ibotenic acid.

The ibotenic acid content in the spores was in the range from 0.0047% to 0.0061%, indicating that the toxin content was less variable than we determined in the cap extracts. This may be due to the less variation in the water content in the spores which we assume are in the dry state and that the presence of ibotenic acid is not depending on the spore age.

Regarding keto and enol tautomerism, imidazolols are reported to prefer the hydroxy structure (Elguero *et al*., 1976). Our results show that the enol tautomer of ibotenic acid comprises 95% of the compound in the pH range 2-6, i.e. at physiological pH.

The orellanine content of basidiospores of *Cortinarius orellanus* and *C. rubellus* was previously determined to be 0.3% and 0.1% respectively. Orellanine (25 nmol) inhibited growth of *B. subtilis*. The amount of spore extracts corresponding to the presence of 25 nmol orellanine inhibited bacterial growth and indicate that the toxin could have a function in the germination process (Koller *et al*., 2002).

Average ibotenic acid content per *A. muscaria* spore is 0.005%. The function of ibotenic acid in the spores is dubious due to the small content, and no inhibition of *B. subtilis* growth was observed with 50 nmol ibotenic acid, muscimol or spore extracts. The growth was rather stimulated in the presence of the spore extract. These could be nutrients or trace elements that enabled bacterial growth. It is possible that other extraction methods could release compound(s) that would inhibit the growth of *B. subtilis*. We have not been aware of any report concerning a bacteriostatic effect of ibotenic acid.

Spores from *Aspergillus ochraceus* and *Penicillium verrucosum* contain ochratoxin A, and spore extracts from these organisms inhibited growth of *B. subtilis* (Skaug *et al*., 2001) which indicates that these toxins could play a role in the germination process (Størmer & Høiby, 1996). It has been suggested by the same authors that ochratoxin A and citrinin from spores could affect the iron uptake of other competing organisms and the presence of citrinin in the range of 8-24% of the spore weight in *P. verrucosum* make it obvious that this compound has an important role in the survival and/or the germination process. It was suggested that citrinin could protect the spores against UV radiation (Størmer *et al*., 1998).

References

Abbas, H.K. & Riley, R.T. (1996). The presence of phytotoxicity of fumonisins and AAL-toxin in *Alternaria alternata. Toxicon* **34**:133-136.

Elguero, J. Marzin, C. Katritzky, A.R. & Linda, P. (1976). The tautomerism of heterocycles. *Advances in heterocyclic chemistry*. Supplement 1.

Gennaro, M.C. Giascosa, D. & Angelino, S. (1997). Hallucinogenic species in *Amanita muscaria*: Determination of muscimol and ibotenic acid by ion-interaction HPLC. *Journal of liquid chromatography & related technologies* **20**:413-424.

Gross, G. (1972). Kernzahl und sporenvolumen bei einigen Hymenogasterarten. *Zeitschrift für Pilzkunde* **38**:109-158.

Inage, M. Takahashi, H. Nakamura, T. Masakane, I. & Tomoike, H. (1996). Hypersensitivity pneumonitis induced by spores of *Pholiota nameko. Internal Medicine* **35**: 301-304.

Ishii, M. Kikuchi, A. Kudoh, K. Konishi, K. Mohri, T. Tamura, M. & Tomichi, N. (1994). Hypersensitivity pneumonitis induced by inhalation of mushroom (*Pholiota nameko*) spores. *Internal Medicine* **33**:683-685.

Kamm, Y.J.L. Folgering, H.T.M. van den Bogart, H.G.G. & Cox, A.(1991). Provocation test in extrinsic allergic alveolitis in mushroom workers. *Netherlands Journal of Medicine* **38**:59-64.

Koller, G.E.B. Høiland, K. Janak, K. & Størmer, F.C. (2002). The presence of orellanine in spores and basidiocarp from *Cortinarius orellanus and Cortinarius rubellus. Mycologia* **94**: 752-756.

Matsui, S. Nakazava, T. Umegae, Y. & Mori, M. (1992). Hypersensitivity pneumonitis induced by Shitake mushroom spores. *Internal Medicine* **31**:1204-1206.

Meerts, P. (1999). The evolution of spores in Agarics: do big mushrooms have big spores? *Journal of Evolutionary Biology* **12**: 61-165.

Moinard, J. Vergeret, J. Tuon de Lara, J.M. & Taytard, A. (1991). A new form of mushroom workers lung. *Revue des maladies respiratoires* **8**: 308-310.

Palmgren, M.S. & Lee, L.S. (1986). Separation of mycotoxin containing sources in grain dust and determination of their mycotoxin potential. *Environmental Health Perspectives* **66**:105-108.

Richard, J.L. Plattner, R.D. May, J. & Liska, S.L. (1999). The occurrence of ochratoxin A in dust collected from a problem household. *Mycopathologia* **146**:99-103.

Skaug, M.A. Eduard, W. & Størmer, F.C. (2001). Ochratoxin A in airborne dust and fungal conidia. *Mycopathologia* **151**:93-98.

Sorenson, W.G. Frazer, D.G. Jarvis, B.B. Simpson, J. & Robinson, V.A. (1987). Trichothecene mycotoxins in aerosolized conidia of *Stachybotrys atra. Applied and Environmental Microbiology* **53**:1370-1375.

Størmer, F.C. & Høiby, E.A. (1996). Citrinin, ochratoxin A and iron. Possible implications for their biological function and induction of nephropathy. *Mycopathologia* **134**:103-107.

Størmer, F.C. Sandven, P. Huitfeldt, H.S. Eduard, W & Skogstad, A. (1998). Does the mycotoxin citrinin function as a sun protectant in conidia from *Penicillium verrucosum? Mycopathologia* **142**:43-47.

Tanaka, H. Saikai, T. Sugawara, H. Takeya, I. Tsunematsu, K. Matsuura, A. & Abe, S. (2002). Workplace-related chronic cough on a mushroom farm. *Chest* **122**:1080-1085.

Tsunoda, K. Inoue, N. Aoyagi, Y. & Sugahara, T. (1993). Simultaneous analysis of ibotenic acid and muscimol in toxic mushroom, Amanita muscaria and analytical survey on edible mushrooms. *Journal of the Food Hygienic Society of Japan* **34**:12-17.

Wicklow, D.T. & Shotwell, O.L. (1983). Intrafungal distribution of aflatoxins among conidia. *Canadian Journal of Microbiology* **29**:1-5.

生殖世代におけるベニテングタケ中のイボテン酸及びムシモールの濃度変化[*1]

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Changes in Concentration of Ibotenic Acid and Muscimol in the Fruit Body of *Amanita muscaria* during the Reproduction Stage[*1]

([*1]Food Hygienic Studies of Toxigenic Basidiomycotina. II)

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Ibotenic acid (IBO) and muscimol (MUS) in the fruit body of *Amanita muscaria* during the reproduction stage were investigated. The mean levels of IBO and MUS throughout the fruit were $\bar{x}$: 343 ppm and $\bar{x}$: 22 ppm, respectively; most was detected in the cap of the fruit (IBO $\bar{x}$: 519 ppm, MUS $\bar{x}$: 30 ppm), then in the base (IBO $\bar{x}$: 290 ppm, MUS $\bar{x}$: 20 ppm), with the smallest amount in the stalk (IBO $\bar{x}$: 253 ppm, MUS $\bar{x}$: 17 ppm).

The concentrations of IBO and MUS in the cap decreased gradually after increasing early on, and those in the stalk decreased gradually, where as in the base there was an increase; the levels in the whole body were nearly constant during maturation. Since the changes were similar in lone and colonial mushrooms, difference of mushroom-growing location had no influence on the concentrations of IBO and MUS. Also, difference of size of the fruit body had no influence on the concentrations of IBO and MUS. The large variations of the IBO and MUS contents may depend on individual differences of growth circumstances. Although the fruit body grew to about 6 times the weight of the base during maturation, the concentration of IBO remained nearly constant.

Detection of MUS may reflect the enzymatic decarboxylation of IBO before the analysis, since the changes in the concentration of MUS paralleled those of IBO at a lower level.

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Key words: ベニテングタケ *Amanita muscaria*; イボテン酸 ibotenic acid; ムシモール muscimol; 幻覚性のキノコ hallucinative mushroom; キノコ mushroom; 食中毒 food poisoning; 毒キノコ toxic mushroom

緒　言

前報[1]では，中枢神経に作用し，精神錯乱や幻覚を引き起こす毒キノコ・ベニテングタケ[2,3]に含まれる生理活性物質のイボテン酸 (IBO)[4)~7)] 及びムシモール (MUS)[4,7)~11)]の分析方法について報告した．その分析法を用いて，ベニテングタケ中の IBO 及び MUS の濃度分布や生長に伴う濃度変化などについて調べた．風味豊かな山菜や野生キノコがもてはやされる昨今，無農薬，低カロリー，抗腫瘍性などの健康や薬理効果を期待した

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り，旨いキノコといって悪酔性や幻覚性あるいは下痢性の野生キノコ[4),12)~16)]を喫食し，食中毒[3),17)]の原因にもなっている．また毒キノコの鑑別法や知識は，地方でさまざまに言い伝えられ[9),16),18)]根拠の乏しいものも少なくない．そこで，キノコの毒性は子実体の部位や大きさ，成熟度，生育場所などの違いが予想されたので，食中毒予防の観点から，一地方で喫食されているベニテングタケについて調べたので報告する．

実験方法

1. 試料，試薬及び装置器具

1.1　試料

1987～1989年，長野県八ガ岳原村，菅平及び白馬村他，周辺に自生していたベニテングタケを採取して，ドライアイスボックスに入れ，凍結しないようにして持ち帰り，4°で保存した．

試料A：群生していた1株32本の子実体

試料B：採取年月日，群落及び生育場所が異なる孤立に生育していた子実体30本

1.2　試薬及び装置

試薬：IBO及びMUSの標準溶液：精製したIBO[1)]とシグマ社製MUSを5, 10, 20, 30, 50 ppmの5段階に70 vol%メタノールで調製した．

メタノール及びアセトニトリル：HPLC用　和光純薬工業(株)製

ドデシル硫酸ナトリウム及びリン酸他：試薬特級

1.3　装置及び器具

高速液体クロマトグラフ(HPLC)：ポンプCCPE，検出器UV-8010，インテグレータSC-8010東ソー(株)製

ホモジナイザー：Urtra-Turrax T-25 Janke & Kunkel社製．

1.4 HPLCの測定条件

Analytical condition of HPLC

Column: IRIKA RP-18T (ODS, 5 μm) 4.0 mm×25 cm

Mobile phase: pH 2.2, 3 m*M* sodium dodecyl sulfate·20 m*M* phosphoric acid solution–methanol–acetonitrile (65 : 15 : 20, v/v)

Flow rate: 0.6 ml/min, Column temp.: 45℃; Wave length: 210 nm

Sensitivity: 0.02 Aufs.; Injection vol: 5 μl

2. 試料の調製

担子菌類の生殖世代における子実体の成熟の程度は一般的に菌傘及び菌柄部などの形態的な生長が目安にされていることから，菌傘部の開化状態とそれに伴う菌柄部の生長などによって，ベニテングタケ試料A及び試料Bを次のようにクラス分けした．それをFig. 1に示した．

ステージI (St-I)：菌傘及び菌柄部はともに生長していないつぼみ状の子実体

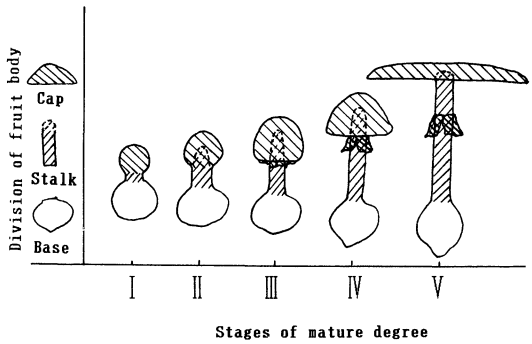

Fig. 1. Classification for mature degree, and division of fruit body, *A. muscaria*

ステージII (St-II)：菌柄部が少し伸び，菌傘部は開かずつぼみ状で，ヒダがまだ見えない子実体

ステージIII (St-III)：菌傘部は3分程開き，ヒダを覆っているツバが一部はがれてヒダが部分的に観察され，菌柄部が3分程伸びた子実体

ステージIV (St-IV)：菌傘部は6～7分程開き，ツバが完全にはがれてヒダが露出し，菌柄がよく伸びた子実体

ステージV (St-V)：菌傘部は平に又は反り返り，完全に開いた子実体

クラス分けした試料の子実体を菌傘，菌柄及び菌基部に3分割し，それぞれに重さを測定した．

3. 試験溶液の調製

細断した試料12.5 gにメタノール25 mlを加えて，4°でいったん保存した．保存した試料をホモジナイザーで均質化し，ろ紙でろ過した後，70%メタノールで残留物を洗いながら50 mlに定容した．その溶液をメンブランフィルターでろ過し，試験溶液とした．それを適宜に蒸留水で希釈して，その10 μlをHPLCに供した．

結果及び考察

1. 試料中のIBOについて

生育環境の異なる試料Aと試料Bを熟度別に5ステージに分けた子実体の菌傘，菌柄及び菌基部の部位について，IBO濃度を測定し，その変化を調べた．試料Aの結果をFig. 2に，試料BをFig. 3に示した．

1.1　分割した部位中のIBO濃度

(1)　試料A

全ステージにおける部位別のIBOの平均濃度 ($\bar{x}$: ppm) 及び最小最大濃度 (min ppm～max ppm) は菌傘が $\bar{x}$: 499 (221～1,140) で，菌柄は $\bar{x}$: 222 (149～322)，菌基部は $\bar{x}$: 336 (138～632) で，全体としては $\bar{x}$: 336 (214～582) であった．

(2)　試料B

全ステージにおける部位別のIBOの平均濃度 ($\bar{x}$: ppm) 及び最小最大濃度 (min ppm～max ppm) は，菌傘部が $\bar{x}$: 507 (192～1,260) で，菌柄部が $\bar{x}$: 270 (112～637)，菌基部は $\bar{x}$: 282 (92～555) で，全体としては $\bar{x}$:

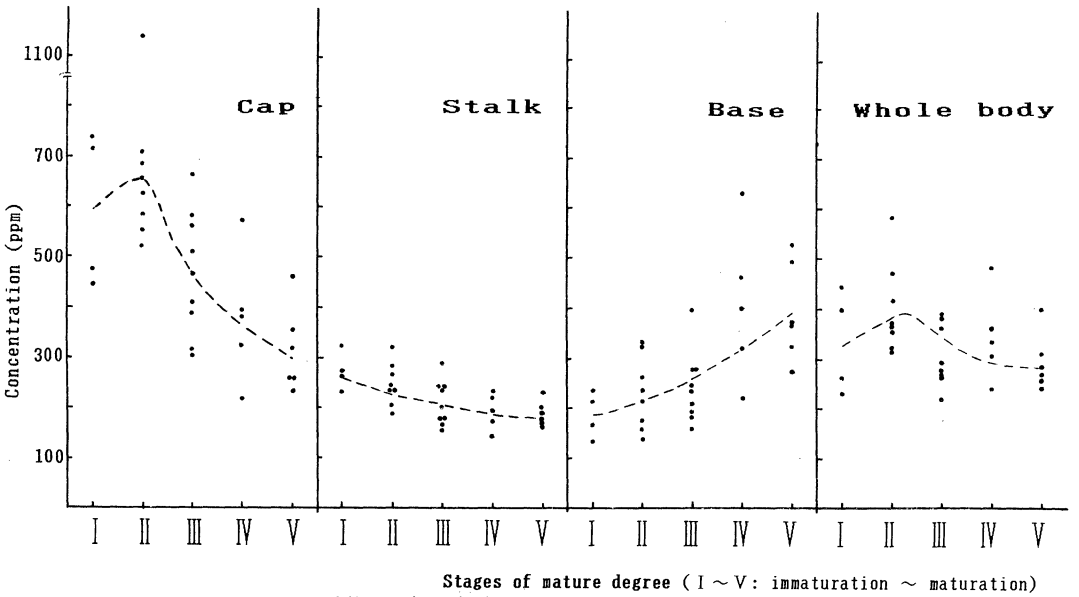

Fig. 2. Concentration of ibotenic acid in the parts for the stages, *A. muscaria* in a colony

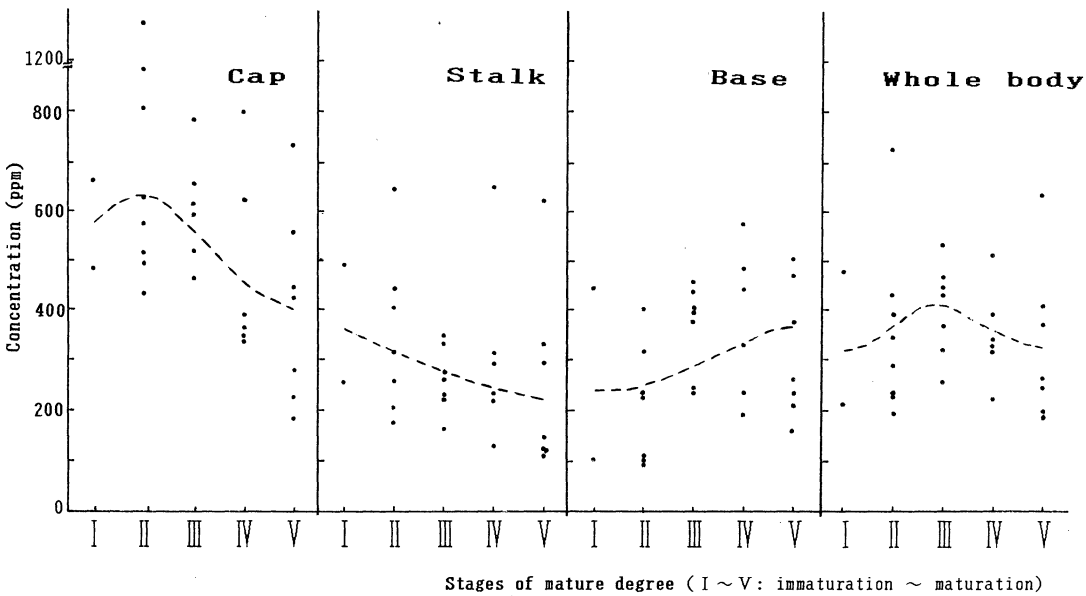

Fig. 3. Concentration of ibotenic acid in the parts for the stages, lone *A. muscaria*

333 (176~714) であった.

試料Bは, 試料Aに比べて, IBO濃度の変動幅は大きいが, 平均濃度は近似し, 両試料間の違いは特に認められなかった. 試料A及びB共に, IBOを最も多く含む部位は菌傘部で, ついで菌基部そして菌柄部の順で, 子実体部位の違いによるIBO濃度の差が認められた.

1.2 成熟に伴う部位中のIBO濃度の変化

(1) 試料A

i. 菌傘部: IBOの平均濃度が $\bar{x}$: 594 ppm のSt-Iから, $\bar{x}$: 684 ppm のSt-IIにいったん増加し, $\bar{x}$: 318 ppm のSt-Vへ漸次減少する傾向を示した.

ii. 菌柄部: 平均濃度 $\bar{x}$: 272 ppm のSt-Iから $\bar{x}$: 193 ppm のSt-Vへ緩やかに漸次減少する傾向を示した.

iii. 菌傘部: 平均濃度 $\bar{x}$: 189 ppm のSt-Iから $\bar{x}$: 394 ppm のSt-Vに漸次増加する傾向を示した.

iv. 子実体全体: 平均濃度 $\bar{x}$: 332 ppm のSt-Iから $\bar{x}$:

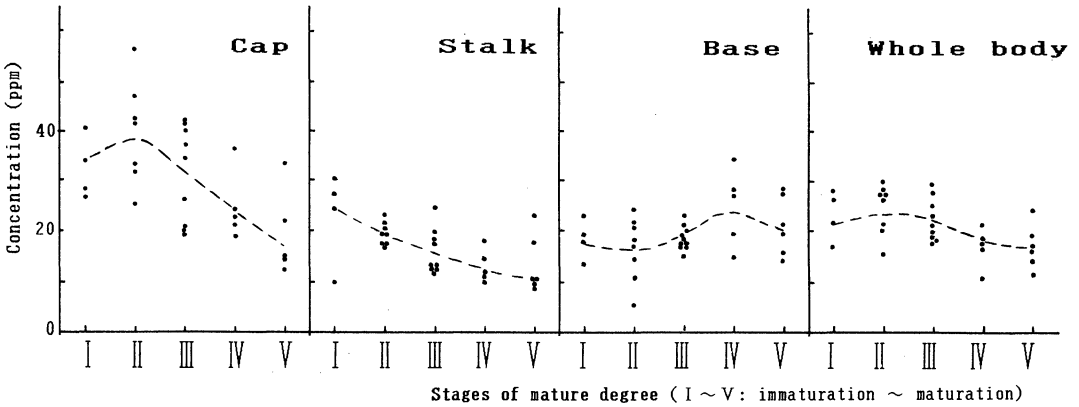

Fig. 4. Concentration of muscimol in the parts for the stages, *A. muscaria* in a colony

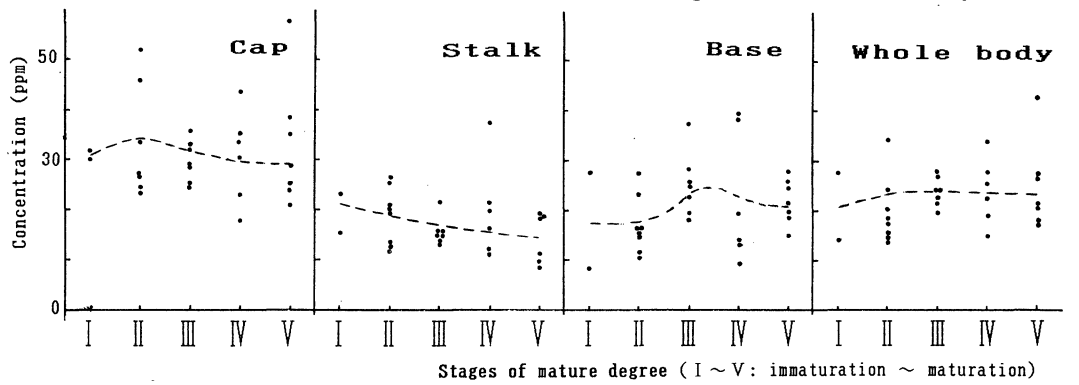

Fig. 5. Concentration of muscimol in the parts for the stages, lone *A. muscaria*

397 ppm の St-II に増加し，$\bar{x}$: 293 ppm の St-V へ漸次減少し，300 ppm 程の濃度を維持するような傾向を示した．

(2)　試料 B

i．菌傘部：IBO の平均濃度が $\bar{x}$: 561 ppm の St-I から，$\bar{x}$: 691 ppm の St-II にいったん増加し，$\bar{x}$: 403 ppm の St-V へ漸次減少する傾向を示した．

ii．菌柄部：平均濃度 $\bar{x}$: 363 ppm の St-I から $\bar{x}$: 249 ppm の St-V へ緩やかに漸次減少する傾向を示した．

iii．菌基部：平均濃度 $\bar{x}$: 266 ppm の St-I から $\bar{x}$: 310 ppm の St-V に漸次増加する傾向を示した．

iv．子実体全体：平均濃度 $\bar{x}$: 343 ppm の St-I から $\bar{x}$: 395 ppm の St-III に増加した後，$\bar{x}$: 329 ppm の St-V へ減少するような傾向を示すが，約 350 ppm を維持するような傾向であった．

成熟に伴う子実体中の IBO 濃度の変化は部位によって異なるものであった．そして，菌基部から菌傘部に IBO を濃縮後，St-II 以後の菌傘部や菌柄部の急激な生長に備えるものと思われた．ステージに対して，試料 A と試料 B 中の IBO は同じような濃度変化を示し，両試料間の明瞭な差は認められなかった．

2.　試料中の MUS について

上記「1.　試料中の IBO について」と同様に MUS 濃度を測定し，その変化を調べた．試料 A の結果を Fig. 4 に，試料 B を Fig. 5 に示した．

2.1　分割した部位中の MUS 濃度

(1)　試料 A

全ステージにおける部位別の MUS の平均濃度 ($\bar{x}$: ppm) 及び最小最大濃度 (min ppm～max ppm) は菌傘部が $\bar{x}$: 29 (13～56) で，菌柄部は $\bar{x}$: 16 (8～30)，菌基部は $\bar{x}$: 19 (5～34) で，全体としては $\bar{x}$: 22 (11～30) であった．

(2)　試料 B

子実体部位中の MUS の平均濃度 ($\bar{x}$: ppm) 及び最小最大濃度 (min ppm～max ppm) は，菌傘が $\bar{x}$: 29 (17～58) で，菌柄は $\bar{x}$: 16 (8～37)，菌基部は $\bar{x}$: 19 (7～38) で，全体としては $\bar{x}$: 21 (14～43) であった．

試料 A 及び試料 B 共にわずかであるが，MUS 濃度に部位差が認められた．その濃度レベルは試料 A が IBO の 1/15 ほどで，試料 B の濃度レベルは 1/20 ほどであった．

2.2　成熟に伴う部位中の MUS 濃度の変化

(1)　試料 A

Table 1. Concentration of IBO and MUS in Each Part of Fruit Bodies, *A. Muscaria*

Stage*1	No.*2 (n=62)		Cap (ppm)			Stalk (ppm)			Base (ppm)			Whole body (ppm)		
			Mean	Max	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min
I	6	IBO*3	583	742	443	302	478	235	214	430	103	336	471	215
		MUS*4	32	40	28	22	30	10	18	27	8	22	27	14
II	16	IBO	688	1260	429	285	632	173	219	388	92	365	714	194
		MUS	34	56	20	19	26	11	16	27	5	22	34	14
III	16	IBO	508	772	312	231	338	156	277	444	158	346	521	214
		MUS	30	41	19	17	24	12	21	37	15	23	29	17
IV	11	IBO	438	787	221	246	637	131	385	632	190	347	503	221
		MUS	28	43	17	16	37	10	23	38	9	21	33	12
V	13	IBO	364	734	192	223	621	112	348	523	159	312	669	176
		MUS	26	58	13	13	22	8	21	28	14	21	43	12
Mean		IBO	519			253			290			343		
		MUS	30			17			20			22		

*1 Stage: Stage of maturation; *2 No.: Number of sample; *3 IBO: Ibotenic acid; *4 MUS: Muscimol

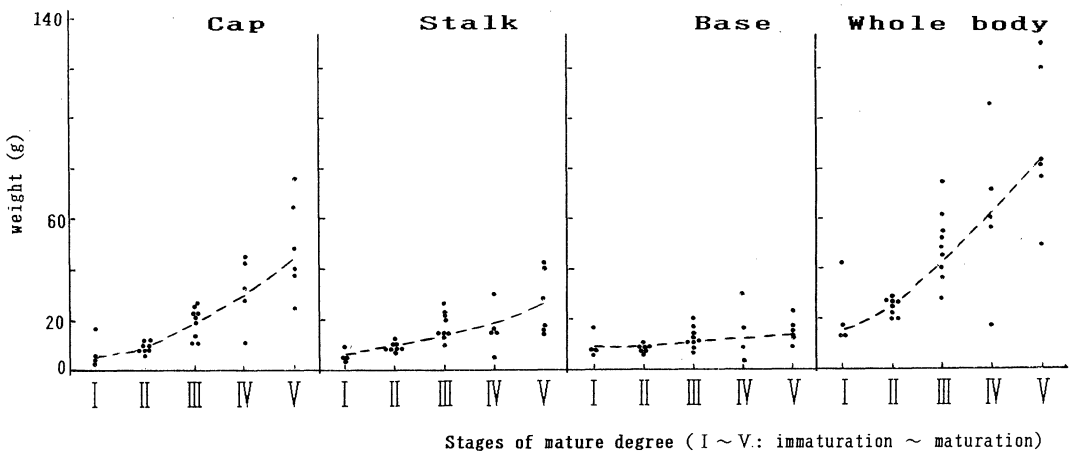

Fig. 6. Increase of weight of the parts for the stages, *A. muscaria* in a colony

i. 菌傘部: MUS の平均濃度 $\bar{x}$: 32 ppm の St-I から $\bar{x}$: 37 ppm の St-II に増加し, $\bar{x}$: 19 ppm の St-V へ漸次減少する傾向を示した.

ii. 菌柄部: 平均濃度 $\bar{x}$: 23 ppm の St-I から $\bar{x}$: 13 ppm の St-V へ漸次減少する傾向を示した.

iii. 菌基部: 平均濃度 $\bar{x}$: 18 ppm の St-I から $\bar{x}$: 25 ppm の St-IV に漸次増加し, $\bar{x}$: 21 ppm の St-V へ減少する傾向を示した.

iv. 子実体全体: 平均濃度 $\bar{x}$: 22 ppm の St-I から $\bar{x}$: 24 ppm の St-III へ若干増加し, $\bar{x}$: 18 ppm の St-V へ減少する傾向を示したが, むしろ全ステージに対しほぼ一定であった.

(2) 試料 B

i. 菌傘部: MUS の平均濃度 $\bar{x}$: 30 ppm の St-I から $\bar{x}$: 32 ppm の St-II に増加した後, $\bar{x}$: 30 ppm の St-IV へわずかに減少する傾向を示した.

ii. 菌柄部: 平均濃度 $\bar{x}$: 19 ppm の St-I から $\bar{x}$: 14 ppm の St-V へ漸次減少する傾向を示した.

iii. 菌基部: 平均濃度 $\bar{x}$: 18 ppm の St-I から $\bar{x}$: 25 ppm の St-III に漸次増加した後, $\bar{x}$: 21 ppm の St-V へ減少する傾向を示した.

iv. 子実体全体: 平均濃度 $\bar{x}$: 21 ppm の St-I から $\bar{x}$: 24 ppm の St-V へ若干増加する傾向を示したが, その変化はほとんど一定であった.

試料 A 及び試料 B 共に, MUS は IBO に比べて低レベルであるが, 成熟に伴う子実体部位中の濃度変化が認められた. それは IBO の濃度変化に近似し追随的で, 二次的な生成が伺われ, ベニテングタケの採取から分析までの過程において酵素的な IBO の脱炭酸生成が推測された.

3. 試料 A 及び試料 B について

試料 A 及び試料 B の総数 62 本について, 部位別に

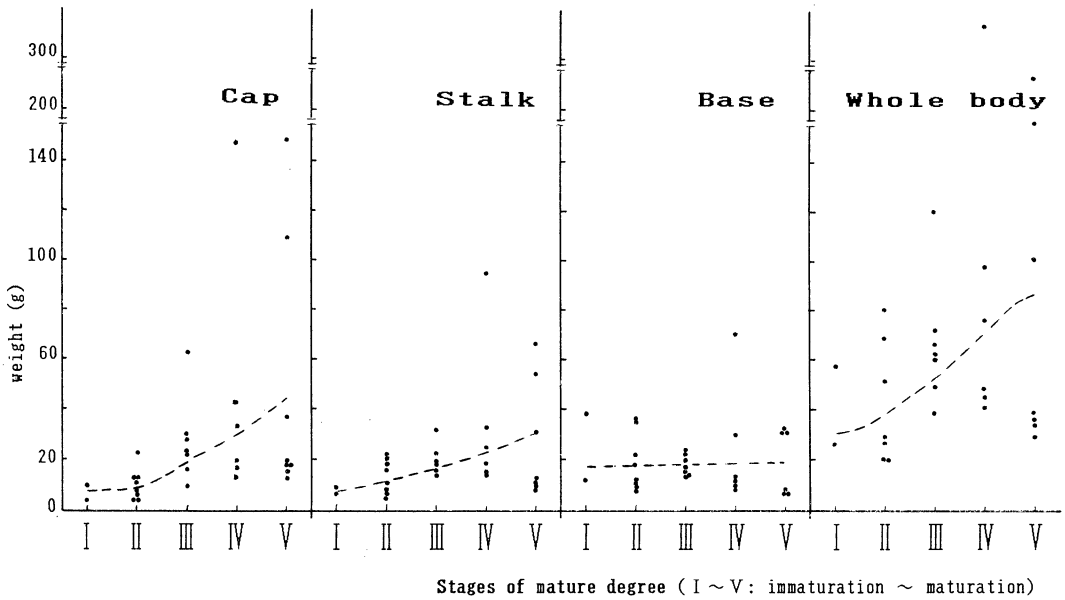

Fig. 7. Increase of weight of the parts for the stages, lone *A. muscaria*

IBO及びMUS濃度をTable 1に示した．両者を最も多く含む部位の濃度（平均濃度 $\bar{x}$: ppm）は菌傘部全ステージのIBO $\bar{x}$: 519, MUS $\bar{x}$: 30，次いで菌基部全ステージのIBO $\bar{x}$: 290, MUS $\bar{x}$: 20，菌柄部全ステージのIBO $\bar{x}$: 253, MUS $\bar{x}$: 17の順であった．

子実体全体ではIBO $\bar{x}$: 343, MUS $\bar{x}$: 22の濃度を示した．子実体部位の違いによるIBO濃度の差は著しかったが，MUSの濃度差はわずかであった．

4. 成熟に伴う子実体重量の変化

試料Aと試料Bを熟度別及び部位別に重さをひょう量し，その変化を調べた．試料Aの結果をFig. 6に，試料BをFig. 7に示した．

4.1. 分割した部位の重量変化

(1) 試料A

i．菌傘部：平均重量 $\bar{x}$: 7 gのSt-Iから $\bar{x}$: 8 g St-IIにわずかに増加した後，$\bar{x}$: 43 gのSt-Vへ急激に増加して約6倍に達した．

ii．菌柄部：平均重量 $\bar{x}$: 5 gのSt-Iから $\bar{x}$: 26 gのSt-Vへ，約5倍に急激に増加した．

iii．菌基部：平均重量はSt-IからSt-Vまで，ほぼ $\bar{x}$: 12 g程度で増減しない傾向であった．

iv．子実体全体：平均重量は $\bar{x}$: 22 gのSt-IからSt-V ($\bar{x}$: 90 g) へ約4倍に増加した．

(2) 試料B

i．菌傘部：平均重量 $\bar{x}$: 7 gのSt-Iから $\bar{x}$: 10 gのSt-IIにわずかに増加した後，$\bar{x}$: 51 gのSt-Vへ急激に増加して約7倍に達した．

ii．菌柄部：平均重量 $\bar{x}$: 8 gのSt-Iから $\bar{x}$: 28 gのSt-Vへ，約4倍弱に増加した．

iii．菌基部：ステージに対して，約20 gを維持するように増減しない傾向であった．

iv．子実体全体：平均重量は $\bar{x}$: 42 gのSt-Iから St-V ($\bar{x}$: 97 g) へ約2.5倍に増加した．

各ステージにおいて，子実体間の大きさに固体差があるものの菌基部を除いて，菌傘及び菌柄部の成熟度と大きさは密接な関係にあった．成熟度を大きさ順に置き換えた場合のIBO及びMUSの濃度変化は子実体部位の成熟のステージに近似していた．

4.2 大きさの異なる子実体中のIBO及びMUSの濃度

ステージごとに，子実体部位別の重量に対するIBO及びMUS濃度の変化を調べた．その結果，いずれのステージにおいても特異的な傾向は認められず，同じ成熟度で大きさの異なる子実体中の両物質の濃度変動は天候などの生育環境による個体差と推測された．

4.3 成熟に伴う重さの変化

成熟のステージに対して，試料Aと試料Bの部位別重量変化及びIBO, MUSの濃度変化は共に同じ傾向を示すことから，菌傘や菌柄が大きいほど濃度は低く，菌基部は逆に高くなる傾向であった．子実体は菌基部の生長終了後，菌傘や菌柄部を伸長し，最も成熟したSt-Vの子実体は菌基部の約6倍の重さに生長するものであった．

5. 生育場所の異なる子実体の違い

上記したように，群生試料Aと孤立の試料Bはステージに対するIBO及びMUSの濃度変化及び子実体重量の増加は共に同じような傾向を示したことから，場所による違いは認められないものであった．

6. 成熟に伴うIBOの供給

子実体は成熟するにつれ，菌基部の約5倍の重さにまで生長するが，この時点で子実体全体のIBO濃度は一定傾向であることから絶対量として，成熟に伴い5倍程のIBOが生成されていると推計される．成熟に伴う，菌傘や菌柄部中のIBO濃度の変化は各部位の生長に伴う重量増加率に関係すると考えられた．

ま と め

ベニテングタケ中の生理活性物質IBO及びMUSを毒性物質の指標として，子実体部位，生長度や大きさ及び採取地などの違いについて調べた．

1. IBO及びMUSを最も高濃度に含む部位は菌傘部で，ついで菌基部，菌柄部の順であった．部位による濃度差が認められた．

2. 成熟に伴い子実体中のIBO及びMUS濃度は，菌傘部ではSt-IIから減少し，菌柄部ではSt-Iから減少し，菌基部ではSt-Iから増加し，全体では一定傾向であった．

3. 大きさと成熟度は密接な関係にあり，大きさ，すなわち生長とIBO及びMUSの濃度変化は，成熟に伴うものであった．また，菌傘部や菌柄部は大きいほどIBO及びMUS濃度は低く，菌基部は逆に高いことが認められた．

4. 菌傘や菌柄部の著しい生長のため，成熟に伴うIBOの濃度は減少し，ほとんど生長しない菌基部ではIBOの産生もしくは供給により増加傾向を示しているものと予想された．

5. 孤立の子実体と群生子実体の成熟に伴うIBO及びMUS濃度の変化は，生育場所の違いによる両者の違いを認めず，その濃度変動は他の天候などの生育環境の違いから生ずる個体差と考えられた．

6. 同じ成熟度で大きさが異なる子実体のIBO及びMUSの濃度は注目する特異な傾向は認められず，その濃度変動は個体差と考えられた．

7. IBOの絶対量は成熟に伴って，各部位ともに著しく増加し，産生供給されているものと考えられた．またIBOの脱炭酸生成物MUSはIBO濃度に比べ，低レベルで1/15ほどに検出された．

文 献

1) Tsunoda, K., Inoue, N., Aoyagi, Y., Sugahara, T.: J. Food Hyg. Soc. Japan **34**, 12～17 (1993).
2) 宇田川俊一，椿 啓介，堀江義一，三浦宏一郎，箕浦久兵衛，山崎幹夫，横山竜夫，渡辺昌平："菌類図鑑（上）" (1978)，講談社.
3) 今関六也，本郷次雄："原色日本新菌類図鑑(I)" p. 115～136 (1987)，保育社.
4) Johnston, G. A. R., Curtis, D. R., de Grout, W. C., Puggan, A. W.: Biochem. Pharmacol. **17**, 2,488～2,489 (1968).
5) 竹本常松，中島 正，横部哲朗：薬誌. **84**, 1,232～1,233 (1964).
6) Bowder, K., Drysdale, A. C., Mogey, G. A.: Nature (London) **206**, 1,359 ～1,360 (1965).
7) Core, M. G., Jordan, P. M.: J. Chromatogr. **243**, 323～328 (1982).
8) Onda, M., Fukushima, H., Akagawa, M.: Chem. Pharm. Bull. **12**, 751 (1964).
9) Bowden, K., Drysdale, A. C.: Tetrahedron Lett. **12**, 727～728 (1965).
10) Muller, G. F. R., Eugster, C. H.: Helv. Chim. Acta **48**, 910～926 (1965).
11) Eugster, C. H., Muller, G. F. R., Good, R.: Tetrahedron Lett. **23**, 1,813～1,815 (1965).
12) 菅原龍幸：調理科学 **2**, 10～21 (1984).
13) Wieland, Th.: Science **159**, 946～952 (1968).
14) 山崎幹夫：医学の歩み **112**, 910～916 (1980).
15) 山浦由夫，込山茂久，福原守雄，高畠英伍，橋本 隆：食衛誌，**24**, 459～464 (1983).
16) 柴田承二編："生物活性天然物質" p. 262～267 (1978)，医歯薬出版.
17) 厚生省生活衛生局食品保健課編：全国食中毒事件録 (1975～1984).
18) 辺野喜正夫，細貝祐太郎，春田三佐夫，菅原龍幸編："原色食品衛生図鑑" p. 60～68 (1981)，建帛社.