CHEMICAL VARIATION IN *AMANITA*

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ABSTRACT.—The value of chemical characters in the taxonomy of mushrooms of the genus *manita* was investigated. Many specimens of different species were examined to determine the population variation in content of: a) tryptamines (bufotenine, 5-HT, and 5-HTP), b) cyclopeptides (amatoxins and phallotoxins), and c) isoxazoles (ibotenic acid and muscimol). Various species of *Amanita* were screened for the occurrence and for the amounts of toxins to evaluate their potential for toxicity. The three classes of compounds seem to be restricted to specific sections of the genus, supporting the sectional divisions made on morphologic grounds. No evidence of hybridization was found in sect. *Amanita*.

Fungi of the genus *Amanita* have had a long history of human toxicity. Three classes of toxins have been chemically characterized: tryptamines (1), cyclopeptides (2), and isoxazoles (3). For the purposes of chemical taxonomy and clinical toxicology it is of interest to determine which species of *Amanita* contain the different toxins. Since it cannot be assumed that all mushroom samples from a given species contain toxins, and since the divisions between closely related taxa are often vague, the examination of multiple collections of different *Amanitas* serves the dual purpose of defining taxa and clarifying population variability.

The major objectives of this study were: 1) to develop rapid analytical methods requiring smaller samples than previous procedures, 2) to examine a broad range of specimens, with as many samples as possible for each species, and 3) to demonstrate the range of variation in the chosen chemical traits within the population. As many samples as possible were screened for each class of compound, then the concentration of each individual toxin in the positive samples was quantitated.

The genus *Amanita* contains a number of "species complexes" of closely related taxa which would seem to have potential for unification. A working hypothesis of this research was that "facultative metabolism", or occasional production of toxins by a species, did not necessarily indicate separate sub-taxa, but rather was the result of genetic differences between individuals of the same species.

Bufotenine was isolated from *A. citrina* Schaeff. ex S. F. Gray [=*A. mappa* (Batsch. ex Lasch) Quel.] (1, 5), along with several other tryptamine compounds, and from *A. porphyria* (Alb. & Schw. ex Fr.) Secr. and *A. tomentella* Kromb. (6). Such 5-substituted tryptamines can be easily quantitated by the tlc method of Andary (7) using o-phthalaldehyde spray reagent (OPT).

Four hepatotoxic amanitins have been isolated from *A. phalloides* (Fr.) Secr. (8–11), along with several other octapeptides (12, 13). The orally nontoxic heptapeptide phallotoxins (14–18) and several minor cyclopeptides (19–21) have also been isolated from *A. phalloides*. Amatoxins have been detected in several species of *Amanita* (22–26) and in several other genera (27–30). Phallotoxins have never been detected outside of the genus *Amanita*. Various assays for the cyclopeptides have been developed recently (28, 31–34).

The isoxazoles ibotenic acid and muscimol were isolated as the CNS active compounds of *A. muscaria* (L. per Fr.) Hooker (35–38) and have been analyzed

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by paper chromatography, electrophoresis (39, 40), and silylation gas chromatography (41). None of these methods has been adequate for routine quantitation.

The isoxazoles have been used as chemotaxonomic markers in two studies (39, 40) and have been found in varieties of *A. muscaria* and *A. pantherina* (DC ex Fr.) Secr. and occasionally in *A. gemmata* (Fr.) Gill. The classic paper of Benedict, Tyler and Brady (39) described a series of chemical and morphologic intergrades of *A. pantherina* and *A. gemmata* which were interpreted as evidence of hybridization. Whether this happens with other species in the section *Amanita* is not yet known.

The toxicologic goal of this research was to identify the risk of toxicity inherent in different species of *Amanita* by the determination of toxin occurrence and toxin levels in different collections. Many suspect species were examined to make their potential for toxicity more definite.

RESULTS AND DISCUSSION

TRYPTAMINES.—Crude fungal extracts were screened by the Meixner test (42). Specimens which gave a red-brown color instantly, then gradually changed to blue, were considered to contain 5-substituted tryptamines.

All 17 collections of *A. citrina* gave this reaction, and all showed appreciable amounts of bufotenine by tlc. Minor amounts of 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT) were present as well. Variations in bufotenine content (table 1) did not correlate with the location of the collection or the time of the year. It was clear, however, that the method of preservation affected the level of bufotenine. Samples dried at 55°, or old, weathered or otherwise damaged samples contained less than average amounts of bufotenine. Freeze drying is

TABLE 1. Tryptamine concentration of specimens of *Amanita*.

Species	Coll. #	St[a]	Date	Pr[b]	Habitat	Tryptamine conc.		
						M[c]	Bufo[d]	5-HTP[e]
A. porphyria	JAB#305	NJ	07/79	FD	Oak+Pine	R	0.1	2.2
	JAB# 18	NY	08/76	FD		R	0.2	5.1
A. citrina	KHM#A	MD	11/76	AD		R	0.4	0.5
	JAB#274	NJ	10/78	FD	Pine	R	1.6	0.2
	JAB#146	MI	09/75	AD		R	1.9	<0.1
	JAB#268	NJ	10/78	FD	Pine	R	1.9	0.3
	JAB#237	NJ	10/78	FD	Pine Barrens	R	2.2	0.2
	JAB# 19	NY	08/76	OD		R	2.6	<0.1
	JAB#137	PA	10/77	FD		R	3.1	<0.2
	JAB#106	NY	09/77	FD	Pine	R	3.2	<0.3
	JAB# 45	PA	10/76	OD		R	4.2	<0.1
	JAB#248	NJ	10/78	FD	Mixed Woods	R	4.4	0.6
	JAB#120	PA	10/77	FD	Beech+Hemlock	R	4.7	<0.2
	JAB#224	PA	10/78	FD	Oak	R	4.7	0.8
	JAB#249	NJ	10/78	FD	Oak+Pine	R	4.9	0.3
	JAB#105	NY	09/77	FD	Hemlock	R	5.9	<0.4
	JAB#344	PA	09/79	FD	Pine+Hardwood	R	6.7	0.9
	JAB#259	PA	10/78	FD	Conifer	R	7.2	<1.9
	JAB#128	PA	10/77	FD	Oak	R	7.5	<0.3

[a]State in which collection was made (Postal abbreviation).
[b]Preservation method used. FD = Freeze-dried, AD = Air-dried, OD = Oven-dried.
[c]Result of Meixner test. R = red color changing to blue (tryptamines).
[d]Bufotenine content, mg/g on dry wt. basis.
[e]5-Hydroxytryptophan content, mg/g on dry wt. basis. < indicates detection limit.

seen as a solution to this preservation problem. None of the other tryptamines reported by Tyler and Gröger (5) were found.

Both collections of *A. porphyria* examined gave the same Meixner reaction as *A. citrina*, but in this species 5-HTP was the major tryptamine present, with minor amounts of 5-HT and bufotenine. Several other taxa placed in section *Mappae* Gilbert by Singer but in a second stirps, *Brunnescens* (4) gave negative Meixner tests. Tlc examination of *A. brunnescens* Atk., *A. brunnescens* var. *pallida* Krieger, and *A. aestivalis* Singer (DMS #1915) showed no detectable tryptamines. A natively fluorescent compound with a low R_f in solvent system EMNW was seen in these species, perhaps identical with a compound reported but not characterized by Malak (43) from *A. brunnescens*.

Since only *A. citrina* and *A. porphyria* were found to contain the tryptamines, bufotenine, 5-HT and 5-HTP, Singer's use of these compounds to delimit two stirpes within the section *Mappae* seems to be valid. The fact that all 19 collections of these species contained easily detectable levels of the tryptamines indicates that this character may be valid for identification purposes, since the deadly species *A. virosa* Secr., *A. verna* (Bull. ex Fr.) Vitt., and *A. bisporigera* Atk. are easily confused in the field with *A. citrina*, and the deadly species do not contain these tryptamines. The levels of bufotenine found in *A. citrina* agree well with that reported by Andary *et al.* (7): 5.5 to 6.5 mg/g. The range found in the present study was 0.4 to 7.5 mg/g., with a mean of 4.9 mg/g. The two collections of *A. porphyria* studied differ significantly from those studied by Andary *et al.*

Reports of tryptamines in other species of *Amanita* (7) may be due to artifacts in the analytical systems used. The report of 0.03–0.04 mg/g bufotenine from *A. phalloides* may be due to interference from amatoxins, since a crude methanolic extract was used. It was noted (see below) that amatoxins gave a pale greenish-yellow fluorescence when sprayed with OPT reagent, but that this fluorescence is also produced when the plate is sprayed with methanol-HCl (1:1) and heated. Hydrolysis of the amatoxin to liberate a fluorescent 6-hydroxytryptophan residue is thought to be involved. Conversely, the lengthy isolation procedures used by Tyler and Gröger (5) were probably responsible for a number of the tryptamines they reported from *A. citrina*.

CYCLOPEPTIDES.—The results for the Meixner test for amatoxins are summarized in a previous paper (42). Crude extracts of all species of *Amanita* in which any collection showed a positive Meixner test (blue) were screened by tlc before quantitation. The anisaldehyde reagent (33) and spraying with methanol-HCl (1:1) and heating at 100° for 15 min. were used to detect the amatoxins and phallotoxins. The results correlated well with the results of the Meixner test.

A typical chromatogram scan of amatoxins after reaction with the Pauly reagent is shown in fig. 1. Phallisin was not detectable in the solvent system used, but it is considered to occur only in small amounts. Phalloidin and γ-amanitin have similarly close R_f's, but these two toxins were easily distinguished by their different uv maxima. Results are presented below by species, and summarized in tables 2 and 3.

A. phalloides. This species was collected from three different locations at several different times. Several collections included many carpophores. The same overall pattern of toxins was found as by previous workers. The Cape May, New Jersey, location (JAB #262, 278, 373) was the same as that which provided DMS #1528 in a previous paper (32). By separately examining a number of carpophores from the same collection it was found that the ratio of α- to β-

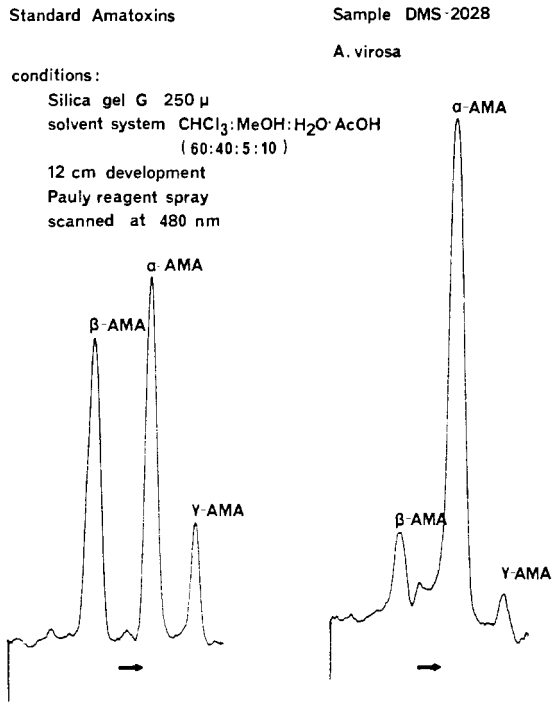

FIG. 1. Spectrodensitometric scan of tlc separation of amatoxins from *Amanita virosa* (DMS#2028); visualization reagent used was Pauly reagent, plate scanned at 480 nm.

TABLE 2. Cyclopeptide content of *A. phalloides* and *A. ocreata*.

Species	Coll. #	Date	St[a]	Toxin levels mg/g:					
				Amanitins[b]			Phallotoxins[c]		
				α	β	γ	phsc-cd	phd	phn
A. ocreata.....	JAB#395	n.d.	CA	0.35	0.27	0.14	0.32	0.11	—
A. phalloides...	JAB#394	11/79	CA	1.61	1.59	0.72	0.63	0.56	0.06
"	JAB#262	10/78	NJ	0.51	0.84	0.17	0.52	0.29	—
"	JAB#278	11/79	NJ	0.34	0.96	0.07	0.31	0.29	0.08
"	JAB#389	10/79	PA	1.15	0.73	0.73	0.79	0.53	0.07
"	JAB#373-A (green)	10/79	NJ	0.70	2.45	0.19	0.57	0.35	0.10
	B (white)	10/79		2.82	1.29	1.58	0.36	0.33	0.11
(age series)	C (youngest)			2.43	1.28	0.92	0.67	0.36	—
	D			2.09	2.63	0.78	0.90	0.38	—
	E			0.53	1.93	0.29	0.72	0.23	—
	F			0.62	2.02	0.25	0.72	0.27	—
	G			2.09	1.30	1.10	0.58	0.42	0.15
	H (oldest)			1.45	0.91	0.74	0.50	0.21	—
	Means A–H			1.59	1.73	0.73	0.63	0.32	—

[a]State in which collection was made (Postal abbreviation).
[b]Amanitin levels on dry wt. basis.
[c]Phallotoxins on dry wt. basis. phsc-cd = Phallacidin + Phallisin, phd = Phalloidin, phn = Phalloin.

amanitin varied markedly from carpophore to carpophore. The toxin content of JAB #373 samples A–H is shown in table 2 along with data from other collections from the same location. The toxin content of a (predominant) green-colored carpophore was compared to that of a (rare) white one (samples A and B, respectively). Since sample B was a much younger specimen than A, the marked difference in toxin content could have been due to age, rather than color. Therefore, an age series of six more carpophores was selected and analyzed. No trend from younger to older emerged, but there was considerable variation in amatoxin content.

A. ocreata Pk. The only other chemical work reported on this California species was by Horgen *et al.* (22). They estimated that *A. ocreata* contained roughly one-fourth the amount of amatoxin as did *A. phalloides*, and our results confirm this. The single specimen contained 0.76 mg/g of amatoxins. This sample also appeared to contain the acidic phallotoxins and neutral phalloidin. This is the first report of phallotoxins in this species, and it is not at all surprising, considering its close relationship to *A. phalloides*.

A. virosa. No acidic phallotoxins were found in any of the three white species of *Amanita* examined. With *A. virosa*, the variation in toxin content noted by

TABLE 3. Cyclopeptide content of white *Amanita* species.

Species	Coll. #	St[a]	Date	Q[b]	10%[c] KOH	M[d]	Cyclopeptide Toxins: mg/g					
							Amanitins			Phallotoxins		
							α	β	γ	phsc -cd	phd	phn
A. virosa	Type A:											
	JAB # 366	NJ	10/79	1.10	Y	+	0.67	0.47	0.09	—	0.88	0.24
	DMS #2028	DE	10/79		W	+	0.79	0.15	0.11	<0.09	0.13	0.02
	JAB # 024	PA	08/76	1.0	W	+	0.80	0.11	0.15	—	0.05	—
	JAB # 125	PA	10/77		W	+	0.84	0.14	0.12	—	0.14	0.05
	JAB # 322	NJ	08/79	1.18	Y	+	0.96	0.31	0.18	—	1.00	—
	JAB # 353	NJ	09/79	1.31	Y	+	1.01	0.54	0.06	—	0.19	0.12
	DMS #2026	DE	10/79	1.2	Y	+	1.06	0.14	<0.12	<0.01	0.22	0.03
	DMS #2027	DE	10/79		Y	+	1.32	0.27	0.08	<0.06	0.22	0.04
	JAB # 334	NJ	08/79	1.22	Y	+	1.56	0.88	0.18	—	0.78	0.27
	JAB # 147	MI	09/77		Y	+	2.13	0.45	—	—	0.47	—
	Type B:											
	JAB # 103	NY	09/77		Y	−	—	<0.01	<0.05	—	0.73	0.38
	JAB # 243	NJ	10/78	1.16	Y	−	<0.03	<0.08	<0.03	—	1.24	0.49
	JAB # 216	NJ	09/78		W	−	<0.03	<0.07	<0.09	—	1.41	0.43
	JAB # 104	NY	09/77	1.0	Y	−	<0.04	<0.04	<0.04	—	1.82	0.58
	DMS #1993	NJ	09/78	1.0	Y	−	<0.04	<0.02	<0.04	—	1.42	0.45
	JAB # 198	NJ	09/78	1.0	Y	−	<0.11	<0.01	<0.12	—	1.30	0.24
	JAB # 275	NJ	10/78	1.11	Y	−	<0.17	<0.16	—	—	1.58	—
A. bisporigera	JAB # 148	MI	09/77		W	+	1.32	0.24	<0.06	—	0.50	—
	JAB # 183	NJ	08/78	1.0	Y	+	0.99	0.60	0.21	—	1.31	—
A. verna	JAB # 166	PA	07/78	1.7	—	−	<0.01	<0.02	<0.07	—	—	—

[a]State in which collection was made.
[b]Length-breadth ratio of spores.
[c]Reaction with 10% KOH on cap cuticle. W = weak yellow, Y = strong yellow.
[d]Reaction on Meixner test. + = positive test for amanitins (blue).

several researchers (23, 24, 32) has been confirmed with a much larger number of collections. This variation may be grouped into two types: Type A contained amanitins and phalloidin; in Type B no amanitins were detected, but larger amounts of phalloidin and phalloin were noted.

For samples collected in the pine barrens region of New Jersey, the 5 collections made in different areas on different days all belonged to type B in 1978. The 4 collections made in 1979 all were type A. There is no obvious explanation for this variation at this time. In two collections made from the same mycelium in two successive years in Pennsylvania, (JAB #24, 125) the toxin content was almost identical. In addition, the overall phallotoxin content of type A of *A. virosa* (mean = 0.41 mg/g) was significantly lower than that of type B (mean = 1.36 mg/g)[2]. Recent work by Buku *et al.* (44) on this species indicates that type B of *A. virosa* contains amaninamide instead of the amanitins. Standards of this compound were not available to us, but it is plausible that it might chromatograph at the same R_f as phalloidin. Since its tryptophan residue is not hydroxylated, it would pass as a phallotoxin under the analytical system used in this study.

A. bisporigera. This species had been dubbed the "most toxic" *Amanita* by investigators who found up to 5.0 mg/g of amanitins in several samples (23). Our results for two samples indicate that it is not necessarily more toxic than other species, although both samples contained potentially lethal amounts of amatoxins, as well as phallotoxins. Additional validated collections must be analyzed before any generalizations can be made about this species.

A. verna. One sample showed no detectable toxins of either kind. Other workers (23, 32) have reported some collections of *A. verna* to be devoid of amatoxins in some instances, but there has been no analysis to demonstrate the lack of phallotoxins in most cases.

There does not seem to be any chemotaxonomic significance to the ratio of α- to β-amanitin in *Amanita*, as was previously supposed. It is possible that a non-enzymatic hydrolysis of α- to β-amanitin may occur during isolation, or that this process occurs in the developing carpophore.

Isoxazoles.—All samples of *A. muscaria* examined which were less than three years old, and which had not been oven-dried, showed some muscimol or ibotenic acid. *A. pantherina* var. *pantherina* and *A. pantherina* var. *multisquamosa* (Pk.) Jenkins (= *A. cothurnata* Atk.) both showed identifiable amounts of isoxazoles in the samples examined. More recently collected specimens showed a preponderance of ibotenic acid to muscimol, but in older samples, muscimol was the only isoxazole seen (table 4). Existing methods for isoxazole quantitation were found lacking, but no satisfactory alternative method was developed.

The occurrence of muscimol in two collections of *A. gemmata* is notable; 32 other collections did not contain detectable muscimol or ibotenic acid. The two positive specimens were morphologically indistinguishable from the other collections. This is the first report of isoxazoles from eastern *A. gemmata*.

One collection of *Amanita parcivolvata* Pk., a previously untested species in sect. *Amanita*, did not contain either ibotenic acid or muscimol. According to Jenkins (45), this species has only slight affinities with the isoxazole-containing species in the section.

Screening showed no ibotenic acid or muscimol in collections outside the section *Amanita*. Specimens of *A. vaginata* (Fr.) Vitt. and *A. flavoconia* Atk. were notable for their low content of any fluorescamine-reacting compounds.

[2]By Student's t-Test, at $P<0.005$ level.

TABLE 4. Isoxazole content of *Amanita* species, as determined by two-dimensional tlc.

Species	Coll.	St[a]	Yr	Pr[b]	Habitat	Isoxazoles[c]
A. gemmata	JAB #11	PA	75	FD		M
"	JAB #181	PA	78	FD	Hardwood	M
"	(10)[d]	PA, NJ, DE	76–79	FD	Spruce, Pine	—
A. muscaria var. *alba*	JAB #151	MI	77	FD		—
A. muscaria var. *formosa*	(22)[e]	NJ, PA, DE, CO, NY	75–79	OD, SI, FD	Pine	M
"	(7)[f]	NJ, PA, DE, NC	76, 77, 79	FD	Pine	I, M
"	(5)[g]	PA, NY, DE, MI	75, 77	OD, AD, FD		—
A. muscaria var. *muscaria*	JAB #7	AZ	75	AD		—
A. pantherina var. *multisquamosa*	JAB #150	MI	77	FD		M
	JAB #330	NJ	79	FD	Pine, Oak	I, M
A. pantherina var. *pantherina*	JAB #160	WA	78	FD		M
A. parcivolvata	JAB #406	WV	80	AD	Oak	—

[a]State in which collection was made (postal abbrev.).
[b]Preservation method: OD = Oven-dried, FD = Freeze-dried, SI = Silica gel, AD = Air-dried.
[c]M = muscimol present, I = ibotenic acid present, — = neither isoxazole detected.
[d]JAB #43, 173, 203, 210, 335, 356, 368, 376, 380, DMS # 2009.
[e]JAB #9, 10, 27, 38, 40, 41, 69, 84, 134, 204, 207, 208, 209, 250, 260, 284, 287, 355, 364, 386, DMS #1999, 2007.
[f]JAB #46, 47, 48, 112, 300, 377, 393.
[g]JAB #1, 2, 3, 153, 288.

All of the taxa of section *Amanita* recognized by Jenkins in his monograph (45) which have been thoroughly tested have contained muscimol or ibotenic acid in at least some specimens, and the case of *A. gemmata* bears further examination to determine whether this is a genetic phenomenon or not. The production of muscimol in *A. gemmata* is apparently not dependent on a particular mycorrhizal host, since one of the positive collections was made under pine, and the other under hardwoods.

CONCLUSIONS

A large number of collections of the more common species of *Amanita* were examined in this study, for example: *A. muscaria* var. *formosa* (39 collections), *A. gemmata* (34), *A. citrina* (20), *A. virosa* (17), *A. vaginata* (33), *A. rubescens* (Pers. ex Fr.) S. F. Gray (13), and *A. phalloides* (5 collections but many individual carpophores). This makes it possible to define which species vary in toxin content and which species are relatively invariant.

A system of "total analysis" of *Amanita* specimens can be arranged to test for all three classes of toxin in a single tissue sample. The Meixner test is performed on a small fragment of cap tissue, then a sample of freeze-dried tissue is extracted with 50% ethanol. If the Meixner test shows the presence of tryptamines, the solution or an aliquot can be concentrated for direct tlc analysis. The rest of the solution is extracted with chloroform to remove lipids, concentrated, taken up in water, then passed through an XAD-4 column. The water wash contains the amino acids and isoxazoles, and the subsequent ethanol eluate of the column contains the amatoxins and phallotoxins. The aqueous wash is made acidic and loaded onto an IR-120+ ion exchange column, which is then eluted with ammonium

hydroxide solution. This eluate contains the free amino acids and isoxazoles, which can be concentrated for two-dimensional tlc.

Overall there is evidence for consolidation of taxa in the section *Phalloideae*, less so in sections *Mappae* and *Amanita*. The results of this work enable the sections to be defined more clearly in chemical terms. Perhaps the most striking finding is that each class of toxin is limited to a single section of the genus, and that there may be many species of *Amanita* which are entirely safe to eat if properly identified.

METHODS[3]

SPECIMENS.—Fungal specimens were collected in various parts of the U.S., but especially in southeastern Pennsylvania and in the pine barrens region of New Jersey. Field notes were made recording location and pertinent details of color and morphology. A section of cap tissue was used to make a spore print, and to perform the Meixner test (42). The rest of each collection was frozen and freeze-dried whole. The dry samples were stored in zip-lock plastic bags. Voucher specimens are deposited in the Farlow Herbarium, Harvard University. Representative specimens were identified by D. T. Jenkins.

Data from these observations and field notes were stored in a computer file for comparison and retrieval. Use of a standard sorting program made it possible to group specimens by any of the data parameters in the file, such as location or species.

TRYPTAMINE ASSAY.—Samples of cap tissue (200–300 mg) were weighed and ground with acid-washed sand in a mortar and pestle, then shaken overnight in Erlenmeyer flasks with 50 ml of 50% methanol. The solutions were filtered through Whatman #1 paper, and the methanol was removed by evaporation in a sample concentrator at 60°. The aqueous solutions remaining were frozen, freeze-dried, and taken up in 4 ml of 50% methanol. Storage in a freezer caused precipitation of solids, but comparative tlc analysis of the cool supernatant and the warmed solution of the solids showed no difference of bufotenine content.

Solvent systems EMNW (ethyl acetate-methanol-ammonium hydroxide-water; 80:15:5:5) and BAWE (butanol-acetic acid-water-ethyl acetate; 10:30:30:100) were developed 15 cm from the point of sample application. Plants were dried in a fume hood for 15 min. then sprayed with o-phthalaldehyde reagent (OPT) and heated at 100° for 15 min. (7).

Plates were read on the densitometer in fluorescence mode. Bufotenine, 5-hydroxytryptamine (5-HT), and 5-hydroxytryptophan (5-HTP) all produced an intense blue fluorescence with this treatment, quickly changing to an equally intense yellow fluorescence, which was used for quantitative measurements. Limits of detection were between 10 and 20 ng for all three hydroxytryptamines. Standard solutions were made up at concentrations of 1.0, 0.1, and 0.01 mg/ml in methanol, and a standard curve generated for bufotenine which showed that fluorescence emission was a linear function of concentration between 50 and 500 ng. Accordingly, all samples were spotted in volumes which produced spots within this range. Recovery of standard bufotenine from spiked samples was 80–90%.

CYCLOPEPTIDE ASSAY.—Cap tissue (20 to 1500 mg) was selected from a single carpophore, weighed and ground with sand, then mixed with 100 ml of 50% alcohol (ethanol or methanol), and shaken overnight. The solution was filtered, and the solids washed with 20–30 ml of 50% alcohol. The filtrate was extracted with 100 ml of chloroform, and the chloroform layer back-extracted twice with 50 ml each of 50% alcohol. The alcohol layers were combined and concentrated at 55°, then diluted to 50 ml with distilled water. The aqueous solution was applied to a minicolumn (12 x 100 mm) of cleaned XAD-4 Amberlite resin which had been equilibrated with distilled water; 100 ml of distilled water was used to wash the column, and the washings discarded. The column was connected to a clean suction flask and 2–3 column volumes of 95% ethanol were pulled through the column; the column was eluted under gravity with more ethanol. The eluate was concentrated, taken up in 3–4 ml of 50% ethanol, then filtered and made to 4.0 ml.

Plates were developed with solvent system CMAW #6 (chloroform-methanol-acetic acid-water; 60:40:5:10) for 12 cm, allowed to dry in a hood 15–20 min., then scanned in the densitometer at 290 nm. The plate was then sprayed with Pauly reagent (33) and scanned again at

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[3]Silica gel G plates, 250 μ with a preadsorbent area, Analtech, Inc., Newark, Delaware were used for thin layer chromatography. Quantitative determinations were made on samples in quadruplicate on a Schoeffel model SD 3000 spectrodensitometer. Gas chromatography was performed using open tubular capillary columns as well as packed columns on a Varian 3700 gas chromatography, OV-101 columns. Hplc was performed on a Perkin Elmer model 601 chromatograph using an LC-55 variable wavelength detector.

OPT reagent (o-phthalaldehyde) was obtained from Calbiochem-Behring Corp., bufotenine courtesy of J. M. Chao, Burlington Co., NJ Forensic Lab., other tryptamines from Sigma Chemical Co., XAD-4 Amberlite resin from Mallinckrodt Chemical Co., fluorescamine from Roche Diagnostics, BSTFA from Pierce Chemical Co.

480 nm. Phallotoxins were quantitated by native ultraviolet absorbance, and amatoxins by their reaction with Pauly reagent. The response was found to be linear for these compounds in the range 50 to 2000 ng.

HPLC OF AMATOXINS AND PHALLOTOXINS.—High pressure liquid chromatography (hplc) was done by means of a normal phase separation on a silica column. Solvent gradient programming was found to be necessary to achieve a useful separation, starting at 75% chloroform/25% of methanol-1.0 N acetic acid (10:1) and finishing at 65% chloroform after 30 min. Solvent flow was 1.0 ml/min, detection at 300 nm.

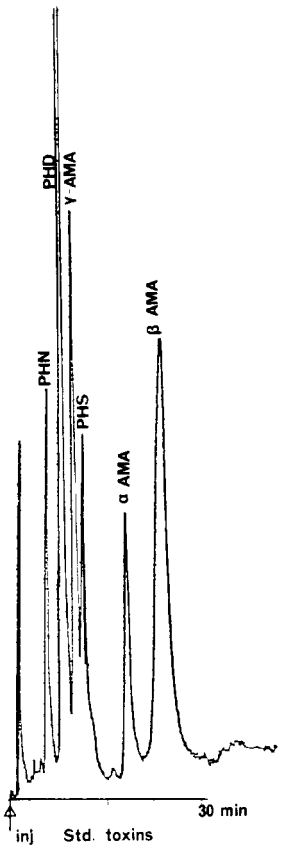

FIG. 2. High pressure liquid chromatographic separation of standard amatoxins and phallotoxins. AMA=amanitins, PHN=phalloin, PHD=phalloidin, PHS=phallisin.

ISOXAZOLE ANALYSIS.—The gas chromatographic procedure of Repke *et al.* (41) was applied to crude methanolic extracts of *A. muscaria* without success. Hplc was frustrated by the poor uv absorbance of ibotenic acid and muscimol. A tlc procedure with detection of the isoxazoles by fluorescamine spray reagent was developed. Solvent systems CMAW #2 (chloroform-methanol-acetic acid-water; 50:50:5:10) and BAWE (butanol-acetic acid-water-ethyl acetate; 10:30:30:100) gave useful separations in a sandwich tank. Interfering compounds with these solvent systems dictated the use of an ion exchange purification procedure.

ISOXAZOLE PURIFICATION.—Between 500 and 2000 mg of cap tissue was ground with sand, extracted one hour with 100 ml of 50% ethanol on a shaker, then filtered by suction and washed thoroughly with solvent. The alcoholic solution was evaporated at 48°, and the residue was taken up in 30 ml of distilled water; 10 ml of 0.5 N acetic acid was added, and the solution loaded on a 12 x 150 mm column of Amberlite IR-120+ resin. The column was washed with 100 ml of distilled water, then eluted with 100 ml of 1 in 10 ammonium hydroxide solution. The basic eluate was evaporated, dissolved in 3–4 ml of 50% ethanol, filtered, and made to 5.0 ml

with solvent. Controls showed that the purification procedure did not appreciably decarboxylate ibotenic acid to muscimol. Good recovery (70–100%) of standard muscimol and ibotenic acid was achieved from spiked samples; however, interfering amino acids were detected in some samples when ninhydrin spray reagent was applied.

Two dimensional tlc with solvent systems CMAW #6 and BAWE eliminated the interference, allowing qualitative detection of the isoxazoles. Ninhydrin was chosen as a more specific detection reagent than fluorescamine. Fifty-four purified samples were screened for isoxazoles.

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