Paper Chromatographic Determination of Muscarine in *Inocybe* Species

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A relatively simple, paper chromatographic method was developed for the quantitation of muscarine in dried mushroom tissue. Application of the procedure to 34 species of *Inocybe* revealed detectable quantities of muscarine ranging from 0.01 to 0.80% in approximately 75% of them. No significant relationship was found between distribution of muscarine and infrageneric taxonomy of the genus *Inocybe*.

THE GENUS *Inocybe* is composed of nondescript, generally brownish agarics, often with characteristic odors. Although recognition of a mushroom as an *Inocybe* is comparatively simple, species identification is extremely difficult, even when microscopic characteristics are considered. In 1924, Kauffman (1) recognized 105 North American species, but today this number is known to be very conservative. Unfortunately, no modern comprehensive treatment of domestic species is available; the best European monograph is that of Heim (2).

Not all species of *Inocybe* are toxic, but muscarine is the toxic principle found in all poisonous species which have been examined. Eugster (3) has reviewed the older literature on the occurrence of this compound in mushrooms and has pointed out that the concentrations reported by Loup (4) for *Inocybe* species are too high, being based on physiological tests compared with an impure muscarine standard. Isolation of muscarine from several European species of *Inocybe* has recently been carried out (5). Percentage concentrations, calculated on fresh-weight basis, have been reported for the following species (*sic*): *I. fastigiata* (Fr. ex Sch.) Quél., 0.01%; *I. patouillardi* (Bres.), 0.37%; and *I. umbrina* (Bres.), 0.003%.

Species of *Inocybe* are abundant in western Washington and pose an appreciable health hazard to the indiscriminate collector. It was of interest, therefore, to develop a procedure which would permit the rapid screening of small quantities of carpophore tissue in order to determine the relative toxicity of the species.

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EXPERIMENTAL

Inocybe Species Examined.—The naturally occurring carpophores used in this investigation were collected in western Washington during the fall and early winter of 1960 through the spring of 1961. One collection of *I. nigrescens* was obtained from the Portage Glacier area in Alaska. Representative herbarium specimens of each collection were deposited in the mycological herbarium of the University of Washington. Some of the species investigated have not been described in the literature; consequently, these are referred to by a herbarium number pending publication of their characteristics.

All collections from western Washington (Table I) were cleaned, dried in a forced-air drying oven at 48° for at least 72 hours, and stored in tightly closed glass containers until examined. The species of Alaskan origin which had been dried prior to shipment was redried and treated similarly. Each collection of any given species was maintained separately from other collections of the same species obtained at other times or places.

Extraction and Purification Procedures.—A number of different methods were investigated to determine their suitability for the quantitative extraction of quaternary ammonium compounds from the dried carpophores. Homogenization with 70% ethanol in a Waring Blendor, extraction of finely powdered carpophores by shaking for prolonged periods with 70% ethanol, and continuous extraction of finely powdered carpophores in a Soxhlet apparatus with 95% ethanol proved to be relatively inefficient extraction procedures. When carpophores (5.0 Gm.) were milled to a No. 40 powder, defatted, macerated for 1 hour with a mixture of absolute ethanol:10% ammonium hydroxide solution (19:1), and extracted in a Soxhlet apparatus with the same solvent for 33 hours, chromatographic examination of a subsequent extract of the marc failed to detect residual quaternary compounds.

Purification of the extract was effected by concentrating it under reduced pressure at 50° in a flash-evaporator until a thick syrupy residue (approximately 2 ml.) was obtained. This residue was dissolved in successive small volumes of distilled water which were combined (total 25 ml.). Sufficient sodium hydroxide was added to produce a concentration of 3 *N*, and 5.0 ml. of a 13% solution of ammonium reineckate in methanol was added to this alkaline solution. The mixture was then refrigerated at 5° for 10 to 12 hours, centrifuged, the supernatant liquid decanted, and the precipitate washed with cold *n*-propanol (three 2-ml. quantities) until the washings were colorless. The washed precipitate was then dissolved in successive small

TABLE I.—TIME AND PLACE OF COLLECTION OF THE SPECIES INVESTIGATED

Species of *Inocybe*[a]	Collection No.	Area of Collection (Washington)	Date
picrosma	FHPi-1	Friday Habor	11-6-60
1790[b]	UWP-1	U. of W. Campus[c]	12-1-60
1838	FHG-1	Friday Harbor	11-6-60
terrifera	OT-1	Olympia	5-20-61
geophylla	FHGG-1	Friday Harbor	11-6-60
lilacina	FHL-1	Friday Harbor	11-6-60
pudica	LPPu-1	Lincoln Park[c]	12-2-60
pudica	OP-1	Olympia	10-23-60
agglutinata	FHAg-1	Friday Harbor	11-6-60
3691	FHH-1	Friday Harbor	11-6-60
subdestricta	LFS-1	Lee Forest[d]	9-21-60
2147	OSu-1	Olympia	10-23-60
2147	A2147-1	U. of W. Arboretum[c]	11-14-60
3365	TSu-2	Tenino	4-1-61
griseolilacina	FHGr-1	Friday Harbor	11-12-60
3399	FHNo-1	Friday Harbor	11-12-60
3761	TO-1	Tenino	11-20-60
3761	OO-1	Olympia	10-27-60
2907	FHPo-1	Friday Harbor	11-6-60
1774	FHNo-2	Friday Harbor	11-12-60
3983	LPPg-1	Lincoln Park	11-2-60
lacera	TL-1	Tenino	11-20-60
lacera	FHLa-1	Friday Harbor	11-12-60
griseoscabrosa	FHGs-1	Friday Harbor	11-12-60
pallidipes	LPP-1	Lincoln Park	11-21-60
cinnamomea	FHC-1	Friday Harbor	11-12-60
sororia	FHS-1	Friday Harbor	11-6-60
sororia	FHS-2	Friday Harbor	11-6-60
sororia	FHS-3	Friday Harbor	11-12-60
hirsuta var. *maxima*	OHM-1	Olympia	10-27-60
mixtilis	TM-1	Tenino	10-30-60
xanthomelas	OXa-1	Olympia	11-12-60
nigrescens	FHN-1	Friday Harbor	11-12-60
nigrescens	FHN-2	Friday Harbor	11-6-60
nigrescens	ALN-1	Alaska[e]	7-10-61
albodisca	FHA-1	Friday Harbor	11-12-60
umbrina	UWU-1	U. of W. campus	12-1-60
1187	TPU-1	Tenino	10-30-60
4291	FHU-1	Friday Harbor	11-6-60
napipes	TN-1	Tenino	4-9-61
1540	FHNo-3	Friday Harbor	5-20-61
subexilis	FHSx-1	Friday Harbor	11-12-60

[a] Correct botanical designations of the named species are as follows: *I. picrosma* Stuntz, *I. terrifera* Kühn., *I. geophylla* (Fr.) Karst. var. *geophylla*, *I. lilacina* (Boud.) Kauff., *I. pudica* Kühn., *I. agglutinata* Peck, *I. subdestricta* Kauff., *I. griseolilacina* Lange, *I. lacera* (Fr.) Quél., *I. griseoscabrosa* (Peck) Earle, *I. pallidipes* Ellis and Everhart sensu Kauff., *I. cinnamomea* A. H. Smith, *I. sororia* Kauff., *I. hirsuta* var. *maxima* A. H. Smith, *I. mixtilis* (Britz.) Sacc. sensu Kühn., *I. xanthomelas* Boursier and Kühn. in Kühn., *I. nigrescens* Atk., *I. albodisca* Peck, *I. umbrina* Bres., *I. napipes* Lange, and *I. subexilis* (Peck) Sacc. [b] Deposited in the University of Washington mycological herbarium under this number (Stuntz No.). [c] Seattle. [d] University of Washington experimental forest, near Maltby. [e] Portage Glacier area, near Anchorage.

volumes of acetone (total 7 ml.) which were combined.

Quaternary reineckates contained in the acetone extract were converted to the respective chlorides (6) by adding 1.4 ml. of water, 5.0 ml. of a saturated solution of silver sulfate, and 0.5 ml. of a 20% aqueous solution of barium chloride. This mixture was cooled (12 hours), centrifuged, the supernatant liquid decanted and retained, and the precipitate washed with 5.0 ml. of cold water which was then added to the supernatant liquid. The combined solution was evaporated under reduced pressure at 50° in a flash evaporator and the residue dissolved in absolute ethanol (total volume 10 ml.). Purified extracts prepared in this manner were subjected to chromatographic examination.

Chromatographic Procedures.—A number of different solvent systems, including those employed by previous investigators (7, 8) for the separation of muscarine and other quaternary compounds, were examined to determine their suitability for use in a quantitative chromatographic procedure.

Satisfactory separation of muscarine and choline and discrete spots with minimum tailing were achieved when sheets of Whatman No. 1 filter paper, which had been previously dipped in MacIlvaine's buffer (9) at pH 4.5 and air dried, were formed with a wash liquid composed of *n*-butanol:methanol:water (10:3:2). Reference choline, acetylcholine, and muscarine exhibited average R_f values in this system of 0.09, 0.23, and 0.28, respectively. Since the alkaline extraction procedure employed resulted in the hydrolysis of any acetylcholine present in the mushroom tissues, this compound was not detected in the extracts and did not interfere with muscarine determination.

Quantitation of muscarine was carried out by spotting, in duplicate, suitable quantities (3–100 μl.) of the purified extract on a buffered chromatographic sheet. After formation, the sheet was air dried and sprayed uniformly with Thies and Reuther's reagent (10).

Preliminary investigations had revealed that the sensitivity of this reagent for muscarine varied somewhat according to the length of time following

its preparation from the stock solution but preceding its use. It was determined that maximum sensitivity was reached between 8 and 10 hours after mixing; consequently, the reagent was always prepared this length of time prior to use. Experiments with reference quantities of muscarine also revealed that following formation in the above system, detection of 5 mcg. of muscarine was equivocal, but 6 mcg. could be detected with certainty. This finding is in agreement with the report of Eugster (3).

After spraying with the reagent, the sheet was dried and subjected to careful visual inspection to determine the minimum amount of extract which formed a detectable orange-red spot. This observation was then verified by respotting, in duplicate, the same quantity on an additional sheet together with quantities of extract slightly smaller than, and slightly larger than, this amount. Actual quantities applied varied according to the concentration of muscarine in the extract. If the results of subsequent chromatograms verified the initial observations, the quantity of extract producing a just-detectable muscarine spot was considered equivalent

TABLE II.—CONCENTRATIONS OF MUSCARINE IN *Inocybe* SPECIES

Species of *Inocybe*	Collection No.	Mushroom Concentration of Purified Extract Gm./ml.	Extract Equivalent to 6 mcg. Muscarine,[a] μl.	Muscarine Content, %
picrosma	FHPi-1	1.35	50	<0.009[b]
		1.35	50	<0.009[b]
1790	UWP-1	1.17	50	<0.01[b]
		1.15	50	<0.01[b]
1838	FHG-1	1.93	12	0.03
		1.92	12	0.03
terrifera	OT-1	3.94	14	0.01
		5.29	10	0.01
geophylla	FHGG-1	0.47	8	0.16
		0.47	8	0.16
lilacina	FHL-1	0.26	6	0.38
		0.27	6	0.37
pudica	OP-1	0.90	4	0.17
		0.90	4	0.17
pudica	LPPu-1	0.69	7	0.12
		0.60	8	0.13
agglutinata	FHAg-1	0.38	5	0.31
		0.31	6	0.32
3691	FHH-1	0.51	15	0.08
		0.51	15	0.08
subdestricta	LFS-1	0.45	6	0.22
		0.45	6	0.22
2147	OSu-1	1.40	6	0.07
		1.35	6	0.07
2147	A2147-1	0.32	10	0.18
		0.35	10	0.17
3365	TSu-2	1.57	50	<0.009[b]
		1.50	50	<0.009[b]
griseolilacina	FHGr-1	0.33	11	0.17
		0.36	10	0.17
3399	FHNo-1	1.44	8	0.05
		1.45	8	0.05
3761	TO-1	1.82	3	0.11
		1.78	3	0.11
3761	OO-1	0.15	5	0.80
		0.16	5	0.75
2907	FHPo-1	0.25	8	0.30
		0.27	8	0.28
1774	FHNo-2	1.77	6	0.06
		1.80	6	0.06
3983	LPPg-1	0.95	5	0.13
		0.97	5	0.12
lacera	TL-1	1.44	5	0.08
		1.44	5	0.08
lacera	FHLa-1	0.40	18	0.08
		0.40	18	0.08
griseoscabrosa	FHGs-1	0.75	30	<0.02[b]
		0.95	30	<0.02[b]
pallidipes	LPP-1	0.48	8	0.16
		0.48	8	0.16
cinnamomea	FHC-1	1.43	14	0.03
		1.43	14	0.03
sororia	FHS-1	0.35	6	0.28
		0.36	6	0.28
sororia	FHS-2	0.86	4	0.17
		0.73	5	0.17
sororia	FHS-3	0.75	6	0.13
		0.75	6	0.13

TABLE II (*continued*)

Species of *Inocybe*	Collection No.	Mushroom Concentration of Purified Extract Gm./ml.	Extract Equivalent to 6 mcg. Muscarine,[a] µl.	Muscarine Content, %
hirsuta var. *maxima*	OHM-1	1.67	30	<0.01[b]
		1.60	30	<0.01[b]
mixtilis	TM-1	0.63	10	0.10
		0.66	9	0.10
xanthomelas	OXa-1	1.45	30	<0.01[b]
		2.15	30	<0.009[b]
nigrescens	FHN-1	1.93	30	<0.01[b]
		1.93	30	<0.01[b]
nigrescens	FHN-2	0.63	30	<0.03[b]
		0.62	30	<0.03[b]
nigrescens	ALN-1	0.71	100	<0.01[b]
		0.71	100	<0.01[b]
albodisca	FHA-1	2.10	30	<0.01[b]
		2.00	30	<0.01[b]
umbrina	UWU-1	0.73	3	0.27
		0.75	3	0.27
1187	TPU-1	0.97	6	0.10
		0.97	6	0.10
4291	FHU-1	1.43	4	0.11
napipes	TN-1	0.14	6	0.71
		0.14	6	0.71
1540	FHNo-3	0.63	4	0.24
		0.65	4	0.23
sibexilis	FHSx-1	1.07	30	<0.01[b]
		1.20	30	<0.02[b]

[a] If muscarine was not detected, this was the largest volume spotted. [b] Muscarine not detected. The maximum quantity which could be present but remain undetected has been derived by calculation.

to 6 mcg. of muscarine. From these data, the concentrations of muscarine in the dried carpophores were calculated. If the maximum quantity of purified extract spotted did not produce a detectable muscarine spot, the maximum concentration which could be present and remain undetected was calculated. These data are reported in Table II.

In order to determine the relative efficiency and accuracy of the entire procedure (extraction, purification, and chromatographic estimation), 7.4 mg. of muscarine was added to a sample (4.41 Gm.) of *I. nigrescens*, a species in which muscarine could not be detected. This control sample was treated as previously described, and the amount of muscarine present in the purified extract was found to be equivalent to 7.0 mg., approximating a 95% recovery of the added compound.

Pharmacological estimations of the muscarine content of certain of the identical collections employed in this investigation have been carried out by Malone, *et al.* (11). With the exception of the assays of *I. napipes*, which apparently contains other potent muscarine-like agents or potentiators, the results of the biological and chromatographic assays were in general agreement.

DISCUSSION

Comparison of the muscarine contents of the species investigated in this study with proposed infrageneric divisions of *Inocybe* based on classical morphological and histological criteria (2) reveals little in the way of a definite relationship between muscarine content and taxonomic position. Certain generalizations may be made, such as the probable occurrence of appreciable muscarine concentrations in species centered about the stirps geophylla Heim of the section Viscosae Heim (*I. geophylla* var. *geophylla*, *I. lilacina*, *I. pudica*, *I. agglutinata*) or the various stirpes of the section Gibbosporae Heim (*I. umbrina*, *I. napipes*, Nos. 1187, 4291, 1540). There are, however, cases in which no correlation is possible such as the easily detectable occurrence of muscarine in *I. mixtilis* but its apparent absence in the closely related *I. xanthomelas* and *I. nigrescens*.

While muscarine has been shown to be of widespread occurrence in the genus *Inocybe*, its occurrence within the various subdivisions of the genus is apparently not entirely predictable on taxonomic grounds. This does not lessen the significance of the compound as a taxonomic criterion in those species in which it occurs, but it does reduce its relative importance as a genus characteristic. Some evidence obtained in this investigation by analysis of different collections of the same species indicates that the capacity of *Inocybe* species to accumulate muscarine may be influenced by certain hereditary (infraspecific races) or environmental factors.

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