Botanical and Chemical Characterisation of a Forensic Mushroom Specimen of the Genus *Psilocybe*

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A sample of mushroom seized by the Royal Canadian Mounted Police in Vancouver was shown by morphological examination to be a species of Psilocybe, *a genus known to contain psychotomimetic principles. Chemical tests proved the presence of psilocybin* (1.5% *of the dried mushroom) as well as several other indole alkaloids. Psilocin could not be detected. Procedures suitable for routine examination of similar samples are described.*

Substances with psychotomimetic or hallucinogenic properties have been subject to abuse to a degree that special legislation to curb their sale, distribution or trafficking has been enacted in various countries. Thus, in Canada, mescaline and lysergic acid diethylamide (LSD) are controlled by the Food and Drug Act and Regulations. In the United States, these potent therapeutic agents as well as peyote, the natural product from which mescaline is derived, dimethyltryptamine (DMT), psilocin and psilocybin are subject to provisions of the Drug Abuse Control Amendments of the Food, Drug and Cosmetic Act which became effective on 17th May 1966. While reports on misuse and abuse of LSD are frequent and have found wide publicity in popular media of communication, reports on misuse of mushrooms containing psychotomimetics are less common. A recent police seizure in Vancouver, Canada, of a small sample of a mushroom stimulated the following investigation.

The elucidation of the chemistry and taxonomy of hallucinogenic mushrooms is largely due to the research work of Heim (1956, 1957 ab, 1958 ab, 1965) and Hofmann *et al.* (1958, 1959, 1963). The history of psychotomimetic mushrooms used in magico-religious ceremonies by the Indians of Mexico and Central America goes back to the second millenary B.C. (Hofmann 1960) and has been described in detail by Heim and Wasson (1958). These mushrooms, for which the Indians applied the summary name of "Teonanacátl", were found to belong mostly to the genus *Psilocybe* (Heim 1956, 1957 ab; Heim and Cailleux 1958). Their active principles, psilocybin and psilocin, were isolated and synthesised by Hofmann *et al.* (1958, 1959). *Psilocybe mexicana* Heim and *P. semperviva* Heim and Cailleux proved to be particularly rich in psychotomimetic substances and suitable for the growing of cultures in the laboratory. Psilocybin and/or psilocin were also reported to be present in one species of *Stropharia*, in one species of *Panaeolus* (Heim and Hofmann, 1958), in one species of *Conocybe* (Benedict *et al.* 1962), in *Psilocybe semilanceata* which occurs in Europe (Hofmann *et al.* 1963) and, recently in a *Copelandia* in France (Heim *et al.* 1966).

It is the purpose of this paper to describe methods of analysis for psilocybin in a seized mushroom sample.

Botanical Investigation

The lack of a ring and examination of microscopic characteristics, basidiospores and cystidiform hairs showed that the dry mushroom specimen was a *Psilocybe* within the ianthinospored Agaricales, family Strophariaceae, which Quélet has included in his genus *Geophila*.

Classification of the sample within the section *Caerulescentes* Sing. was suggested mainly by the presence of a papilla on the pileus and also by the relatively light spore pigmentation and the dark purple lamellae, together with the arched profile of the cap (light ochre under the microscope).

Psilocybes of the *Caerulescentes* section have been classified into a number of groups based on the size and profile of carpophores, the presence or absence of a papilla on the pileus, the existence or non-existence of cystidia with contents, and the form and dimensions of the basidiospores (Heim 1958 b). On this basis, in our sample, most of the hallucinatory species of Psilocybe previcusly described in Mexico or the U.S.A. were excluded. For example, it had the ovoid shape of *Psilocybe mexicana* Heim, one of the species used by certain Mexican Indians for magico-religious purposes (together with *P. caerulescens* Murr., *P. zapotecorum* Heim, *P. vunjensis* Sing. and Sm., *P. wassonii* Heim, *P. hoogshageni* Heim and *P. mixaeensis* Heim), but it exhibited a pronounced papilla and much larger ellipsoidal and non-navicular spores. *P. fagicola* Heim and Cailleux, discovered in a *Fagus mexicana* beech-grove in Zacatlamaya, north-east Mexico, is a cerulescent *Psilocybe* strongly resembling our specimen but its spores are small and navicular. Again, *P. semperviva* Heim and Cailleux is distinguished from our sample by its larger size, presence of cystidia with contents and smaller spores.

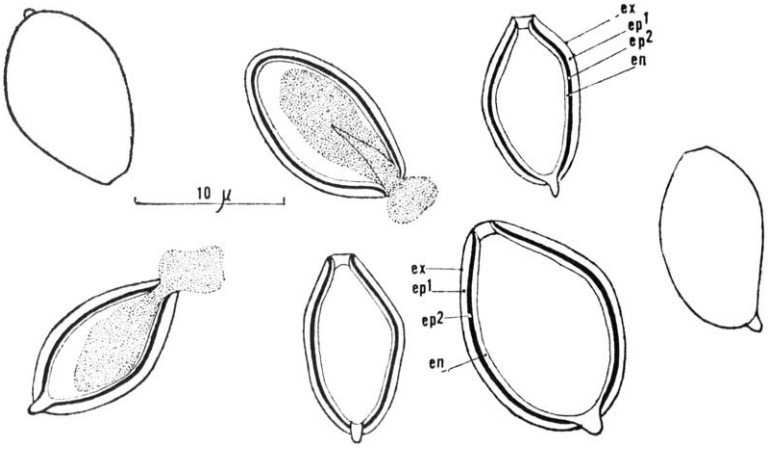

FIGURE 1.—Basidiospores of seized mushroom sample showing the membrane sequence: *ex*, exospore; *ep*^{1}, external epispore; *ep*^{2}, internal episporic layer, highly coloured in appearance; *en*, endospore.

The specimen has large spores measuring 11.6—13.3 (–16.6 –18)×6.8 – 7.6 (– 10.8) μ (Fig. 1), a significant observation since the number of species with large ovoidal or ellipsoidal spores is quite restricted in this section. *P. aztecorum* Heim, found exclusively in the high Popocatepetl area of Mexico, has spores measuring 10.5 – 15 (– 16) × 6.1 – 7.6 (– 8.3) μ, the largest of the North-American species. However, it has a larger cap than our specimen, not papillate or even subumbonate, with an irregular profile. The specimen appeared to be closest to *P. semilanceata* Fr., the only European *Psilocybe* in which psilocybin has been detected (Hofmann *et al.* 1963). This species, whose occurrence in the U.S.A. has been seriously questioned (Murrill 1923), has elongated, ovoidal cap with a rather long papilla and spores which measure 11 – 12.5 (– 14.2) × 6.5 – 7.5 (– 8.5) μ. On the other hand, it does have a very long, slender stalk. Another species which is quite close to the specimen is *P. squalidella* (Peck 1893; Murrill 1923). It has comparable spores, only a little smaller than those of our specimen (10.2 – 13.2 × 5.8 – 7.5 μ), and a slightly umbonate pileus. However, in more recent American publications on *Psilocybe* (Smith 1937, 1941; Singer and Smith 1958; Singer 1959) no species is described which is close to our specimen.

The incomplete and stunted fruit-bodies made an unambiguous botanical identification of the sample especially difficult. Evidence for the presence of indole compounds responsible for the psychotomimetic activity of *Psilocybe* mushrooms was therefore necessary.

Chemical Examination

Extraction. The mushroom sample (1.18 g) was found to contain 6.05% moisture by drying in a vacuum oven at 40°C. Dry material (100 mg) was ground in a mortar, transferred into an Erlenmeyer flask and extracted for a period of 5 hours with methanol (10 ml) using a mechanical shaker. After filtration and washing, the extract was protected from light and concentrated in a stream of nitrogen to exactly 1 ml.

Paper Chromatography. Methods described by Hofmann *et al.* (1963, 1966) were used.

Thin layer chromatography (TLC). 250 μ layers were applied to five TLC-plates (20 x 20 cm) using a commercial spreader (Shandon Scientific Co. Unoplan) and a slurry of MN Silica Gel G (30 g) and water (65 ml) for systems 1 and 2 and MN Silica Gel G (20 g), Kieselguhr G (10 g) and water (65 ml) for systems 3 to 5. The plates were activated for 1 hour at 110°C. and allowed to cool in a desiccator. Spots were applied to the cooled plates by means of a Hamilton syringe. The following TLC-systems were used:

1. MN Silica Gel G; acetone : piperidine 9 : 1.
2. MN Silica Gel G; acetone : ethylpiperidine 9 : 1.
3. MN Silica Gel G/Kieselguhr (2 : 1); n-propanol : 5% NH_4OH 2 : 1.
4. MN Silica Gel G/Kieselguhr (2 : 1); n-butanol : acetic acid : water 2 : 1 : 1.
5. MN Silica Gel G/Kieselguhr (2 : 1); benzene : methanol : 5% NH_4OH 10 : 15 : 2.

Elutions were made with methanol after chromatographing a band of the mushroom extract, spraying only the edges of the plate with detection reagent and scraping off the corresponding alkaloidal areas.

Detection reagents for TLC.

Dimethylaminobenzaldehyde (DMBA): DMBA (1 g) was dissolved in conc. HCl (10 ml) and ethanol (40 ml). Violet spots, indicative of indole-type alkaloids reached their maximal intensity about 5 minutes after spraying.

Ceric sulphate: Ceric ammonium sulphate (1%) was dissolved in N H_2SO_4. Following its application, the plate was heated at 110°C. for 5 minutes. Brown and grey spots of various hues developed in the mushroom extract.

Ammonium Molybdate: (1) 0.1 N NaOH. (2) Ammonium molybdate (1%) in water. (3) Stannous chloride (1%) in 10% HCl. Plates were sprayed twice with 0.1 N NaOH and allowed to dry each time at 110°C. They were then sprayed with the ammonium molybdate solution and again dried at 110°C. After the final spray with stannous chloride, azure-blue spots on a fading bluish background appeared with psilocybin.

Alkaline solution of Fast Blue B: Fast Blue B (15 mg) was dissolved in N NaOH (20 ml). The solution has to be freshly prepared. A red-brown spot appeared with known samples of psilocin.

Phosphate Spot Tests.

Reagents: (1) Ammonium molybdate (5 g) was dissolved in water (100 ml) and poured into conc. HNO_3 (100 ml). (2) Benzidine hydrochloride (0.05 g) was dissolved in glacial acetic acid (10 ml) and diluted to 100 ml with water. (3) Saturated sodium acetate in water.

Procedure: TLC eluates were evaporated in a small test tube to dryness. Conc. HCl (0.5 ml) was added and the test tube sealed. It was heated in an autoclave at 125°C./15 lbs./in^2 for one hour. The contents of the tube were then evaporated to dryness on a spot-test plate and one drop of each of the reagent solutions was added. A blue colour developed indicating the presence of phosphate.

Determination of total indole alkaloids.

Van Urk reagent: DMBA (125 mg) and saturated aqueous ferric chloride (0.05 ml) were dissolved in 65% H_2SO_4 (100 ml).

Procedure: Methanolic standard solutions of psilocybin and mushroom extract (corresponding to 10 mg dried mushroom) were evaporated to dryness in a stream of nitrogen and the residue treated with 0.1N H_2SO_4 (1 ml) and Van Urk reagent (2 ml). The solution was heated for one hour on the boiling water bath and left standing at room temperature. A blue coloration, developing gradually, stabilised four hours after addition of the reagents. It was measured in a Beckman DK-2 recording spectrophotometer, and the maximum at 550 mμ was read for the determination of total alkaloids.

Determination of psilocybin by UV-spectrophotometry.

The methanolic mushroom extract (200 μl; corresponding to 20 mg dry mushroom) was applied as a band to a thin layer plate. After chromatography in system 4, the band corresponding to psilocybin was eluted as described above. The eluate was protected from light and concentrated to 6 ml in a stream of nitrogen and its ultraviolet absorption spectrum recorded on a Beckman DK-2 spectrophotometer. The absorbance at λ max 267 mμ was used for quantitative measurements.

Results and Discussion

Separation of the psychotomimetic *Psilocybe* principles can be accomplished by thin layer chromatography in systems described earlier for the examination of ergoline alkaloids in Convolvulaceae (Genest 1965, Genest and Sahasrabudhe 1966) (System 1 and 2, Table 1). Other useful systems are those applied to the study of psilocybin in saprophytic cultures (Leung *et al.* 1965) (System 3–5, Table 1). The presence of psilocybin in extracts of the mushroom seizure was ascertained by paper chromatography (Hofmann 1966) and TLC, followed by application of various detection reagents as shown in Table 2. The DMBA, ceric sulphate and molybdate reagents gave spots of the same colour and Rf-value as standard psilocybin. The presence of phosphate in the psilocybin spot from the mushroom extract was verified by a spot test after elution and hydrolysis of the eluate. Further identification of the alkaloid was afforded by ultraviolet spectrophotometry of the mushroom extract following TLC and elution of the psilocybin spot. Maxima at 267 and 290 mμ and a shoulder at 280 mμ were found (Fig. 2). These data are in agreement with those for standard psilocybin (Hofmann *et al.* 1959). The sensitivities of the TLC detecting reagents for psilocybin and psilocin are given in Table 3. The dilution technique described earlier for the assay of muscarine in *Inocybe* species (Brown *et al.* 1962) and *Clitocybe dealbata* (Hughes *et al.* 1966) was applied to the DMBA-psilocybin spot of the mushroom extract and a value of approximately 1.5% psilocybin was obtained. This value was confirmed by paper chromatographic methods (Hofmann 1966). Since this method is based on subjective judgment of the observer, results based on measurement of the UV-maximum at 267 mμ, are considered to be more reliable. The psilocybin

TABLE 1

Rf-VALUES OF PSILOCIN AND PSILOCYBIN IN VARIOUS TLC-SYSTEMS

Compound	TLC-System 1	2	3	4	5
psilocin	0.59	0.45	0.73	0.54	0.54
psilocybin	0.00	0.00	0.14	0.33	0.04

TABLE 2

Rf-VALUES AND COLOURS OF ALKALOIDAL SPOTS OBTAINED WITH VARIOUS REAGENTS ON EXTRACT OF MUSHROOM SEIZURE IN TCL-SYSTEM 4

Rf	DMBA	Ceric sulphate	Fast Blue B	Molybdate
0.33	red–violet	yellow, grey halo	none*	azure blue
0.39	red–violet	yellow, grey halo		
0.43	red–violet	—		
0.48	—	brown–violet		
0.56	light blue	—		
0.60	blue	violet		
0.68	red–violet	light brown		
0.72	—	greyish		
0.81	—	grey		
0.87	—	brown		

*psilocin standard gave a red–brown spot

TABLE 3

LIMIT OF DETECTION (IN μg) OF PSILOCIN AND PSILOCYBIN FOLLOWING TLC

Reagent	Psilocin	Psilocybin
DMBA	0.2	0.15
Molybdate	—	2.0
Molybdate spot test	—	2.0
Ceric sulphate	0.5	0.1
Fast Blue B	0.1	—

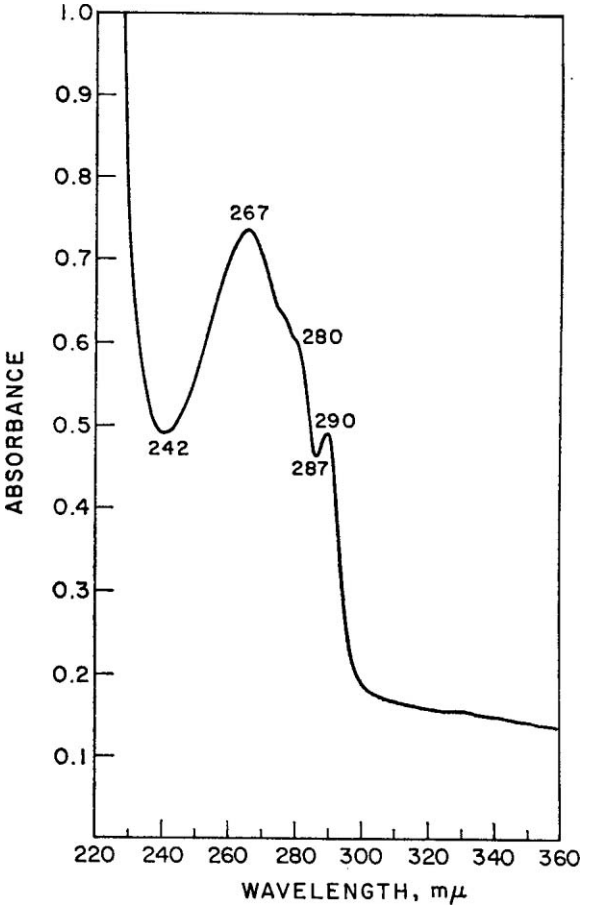

FIGURE 2.—UV spectrum of eluate of psilocybin spot from mushroom extract in methanol.

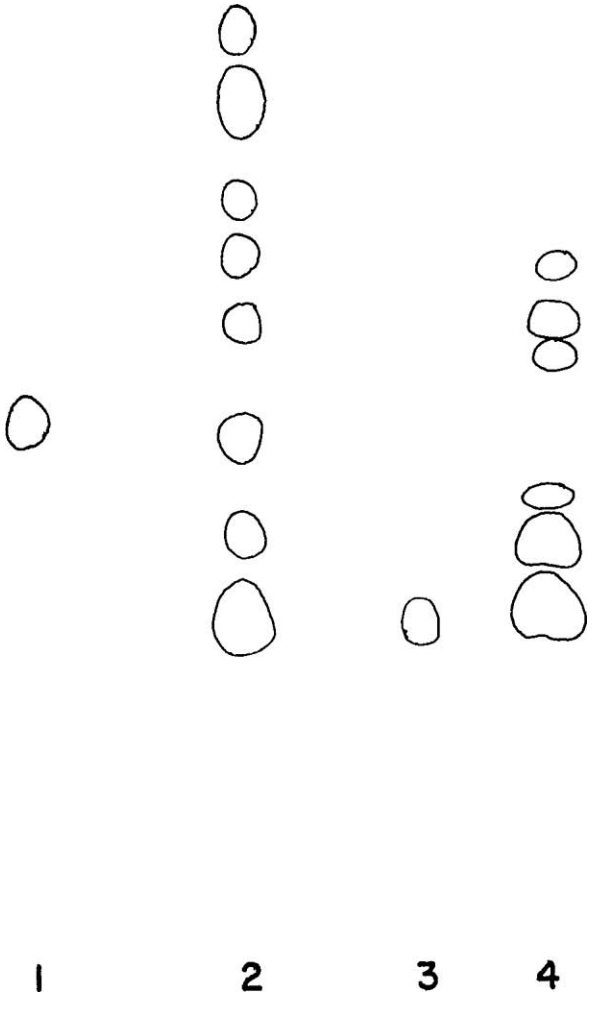

FIGURE 3.—Chromatogram of mushroom extract in TLC system 4: 1. psilocin standard—ceric sulphate spray. 2. mushroom extract—ceric sulphate spray. 3. psilocybin standard—ceric sulphate spray. 4. mushroom extract—DMBA spray.

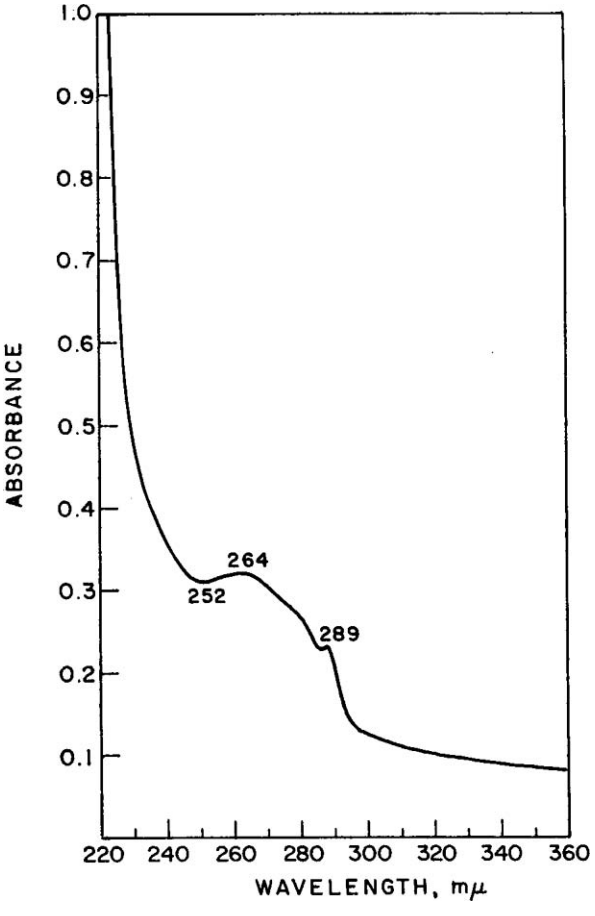

FIGURE 4.—UV spectrum of eluate of DMBA-positive spot (Rf 0.39 in TLC system 4) in methanol.

content *of the mushroom seizure* assayed by the UV-method was estimated to be 1.46%.

The reproduction of a chromatogram (Fig. 3) indicates that, in addition to psilocybin, several other compounds were present in the mushroom extract. The reaction with the DMBA-reagent, for example, gave indications that the mushroom extract contained at least five other indole-type alkaloids (Table 2). The Fast Blue B spray showed the absence of phenolic alkaloids. This finding and TLC-results in other solvents confirmed that the DMBA-positive spot at Rf 0.56 in system 4 is not identical with psilocin. The ceric sulphate reagent, widely used for the identification of vinca-alkaloids (Farnsworth 1964), revealed another four spots (Table 2). The most abundant indolic material, after psilocybin, had an Rf-value of 0.39 in system 4. It was eluted and its UV-spectrum measured (Fig. 4). Maxima were observed at 264 and 289 mμ. The total content of indole-type alkaloids as measured by colorimetry after treatment of the mushroom extract with Van Urk's reagent was 2.1% (expressed as psilocybin).

The identification of phosphate accomplished in the hydrolysate of the psylocybin spot as well as by direct application of the molybdenum blue test after TLC is of particular diagnostic value for forensic purposes. Apart from psilocybin, no other naturally occurring indole compound containing phosphate is known. The alkaloidal level in this *Psilocybe* is uncommonly high since values reported to date for psilocybin or psilocin or both, in hallucinogenic mushrooms, are generally below 1%. The highest content of psilocybin found previously in a mushroom was 0.6% (*P. semperviva*); those of *P. aztecorum* and *P. semilanceata* have been reported to be 0.02 and 0.25%, respectively (Hofmann *et al.* 1963). However, the active constituents of mushroom species will vary depending on geographic locale, climatic conditions, time of collection and state of preservation of the mushroom. Possibly, therefore, the seizure represents a new, as yet undescribed, species. Confirmation of this assumption must await further results from laboratory culture work.

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