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Analysis and isolation of indole alkaloids of fungi by high-performance liquid chromatography

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ABSTRACT

An efficient analytical and isolation method was elaborated for biologically active tryptamines using a computer-aided liquid chromatographic–gas chromatographic system. The separation method includes a new efficient extraction procedure, optimization programme for high-performance liquid chromatographic separation, identification by diode-array detection and a spectrometric and electrochemical assay. The identification of indole alkaloids was confirmed by thin-layer and gas chromatography and mass spectrometry. The method was used for analysis and isolation of psychotropic substances in extracts from the fruit bodies of hallucinogenic fungi of genera *Psilocybe*, *Inocybe* and *Amanita* and in mycelial extracts from the species *Psilocybe bohemica*.

INTRODUCTION

Three decades after their discovery in fungi of the genus *Psilocybe* in Mexico, indole alkaloids of the tryptamine type [1,2] have become biochemically important drugs in psychotherapy and psychodiagnostics. Chromatogrpahic methods are widely used in studies of these psychotropic compounds found in biological materials. Special attention is paid to fungi of the genera *Psilocybe*, containing psilocybin and psilocin. Gas chromatography (GC) has been widely used for this purpose [3]; prior to analysis, however, poorly volatile and heat-labile tryptamines have to be chemically converted into acetate [4], enamine [5], isothiocyanate [6] or trimethylsilyl (TMS) derivatives [7]. A GC–mass spectrometric (MS) system has been used for the analysis of TMS derivatives of bufotenin [6], psilocybin and psilocin [8]. Tryptamine compounds are often separated and assayed by high-performance liquid chromatography (HPLC) with UV spectrophotometric [9–23], electrochemical (ED) [16,19–21] or fluorimetric [11,12,15,16,18] detection, and sometimes with MS identification [10,14,18].

Studies of indole alkaloids form part of research into the metabolism of aromatic amino acids in microorganisms and include a search for new efficient extraction and separation procedures, optimization of HPLC analysis, high identification fidelity and computer-aided evaluation of results. Some studies in this direction are reported in this paper.

EXPERIMENTAL

Chemicals

Analytical standards of indole alkaloids were ob-

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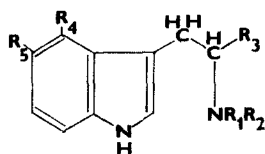

	R_1	R_2	R_3	R_4	R_5
Tryptamine	H	H	H	H	H
Tryptophan	H	H	COOH	H	H
Serotonin	H	H	H	H	OH
5-Hydroxy-N-methyltryptamine	Me	H	H	H	OH
5-Hydroxytryptophan	H	H	COOH	H	OH
Bufotenin	Me	Me	H	H	OH
Psilocin	Me	Me	H	OH	H
Psilocybin	Me	Me	H	H_2PO_4	H

tained from Sigma (St. Louis, MO, USA), except psilocybin and psilocin, which were obtained from Sandoz (Basle, Switzerland).

The silylation reagent N-(methyl-N-(*tert.*-butyldimethylsilyl) trifuoroacetamide (MTBSTFA) was purchased from Pierce Eurochemie (Oud-Beijerland, Netherlands). Other chemicals were of analytical-reagent grade from Lachema (Brno, Czechoslovakia).

Microbiological materials

Species of fungi of the genera *Psilocybe* and *Amanita* were collected at various places in Czechoslovakia and of the genus *Inocybe* in Germany. The fungus *Psilocybe bohemica* from our laboratory collection was cultivated at 24°C in a 300-ml flask containing 100 ml of complex medium on a reciprocal shaker (3 Hz, amplitude 50 mm).

Sample preparation

The dried fruit bodies of fungi of the genera *Psilocybe, Inocybe* or *Amanita* were cut and then completely homogenized in a glass mortar, whereas the samples of mycelium were only homogenized. Extraction was performed in 20-ml vials at a constant sample weight of 10 mg on a reciprocal shaker (1.2 Hz, amplitude 15 mm).

Psilocybin was extracted for 10 min with 0.50 ml of 70% methanol (saturated with potassium nitrate), whereas psilocin and bufotenin were extracted with water–ethanol (75:25) for 160 min. The crude extract was filtered through a 1-μm PTFE filter before injection into the HPLC apparatus.

Derivatization

Silylation was modified for derivatization of samples or standard compounds of the tyrptamine type: a dried sample of standard indole alkaloids (about 0.3 mg) was places in a glass ampoule, dissolved in a mixture of 0.1 ml of acetonitrile and 0.1 ml of MTBSTFA and heated in the sealed ampoule for 10 min at 90°C. The procedure yielded samples converted completely into *tert.*-butyldimethylsilyl (TBDMS) derivatives. After cooling, the samples were used directly for GC analysis.

Gas chromatography

GC analyses were performed on Sigma 3B gas chromatograph (Perkin-Elmer, Norwalk, CT, USA) with flame ionization detection (FID). TBDMS derivatives of indole alkaloids were separated on a 30 m × 0.25 mm I.D. SPB-1 fused-silica column (Supelco, Gland, Switzerland) with temperature programming from 250 to 300°C at 3°C/min. The injection port (split injector, splitting ratio 1:70) and detector were maintained at 300°C. Hydrogen was employed as the carrier gas at a flow-rate of 42 cm/s. Samples of 0.2–0.5 μl were introduced with a 1-μl Hamilton (Bonaduz, Switzerland) microsyringe.

Identification of the TBDMS derivatives of individual components of a mixture was carried out by comparing their retention characteristics with those of the standards. Detector signals were processed by Baseline 810 chromatographic software (Waters, Milford, MA, USA).

High-performance liquid chromatography

The liquid chromatograph consisted of a Model 3B high-pressure pump, which ensures programming of the mobile phase concentration and flow-rate, a Model 7105 injection valve (Perkin-Elmer) and a Waters model 990+ photodiode-array detector. A model 641 VA voltammetric detector (Metrohm, Herisau, Switzerland) was connected in series with a UV detector. The separations were performed on a 300 mm × 3.9 mm I.D. column (A) packed with μBondapak C_{18} (7 μm) and Guard Pak C_{18} (Waters), on a 250 mm × 4 mm I.D. column (B) packed with Silasorb SPH C_{18} (7.5 μm) (Lachema, Brno, Czechoslovakia) or on a 250 mm × 8 mm I.D. semi-preparative column (C) packed with Separon SGX C_{18} (7 μm] (Tessek, Prague, Czechoslovakia). The columns were eluted isocratically with 0.1 *M* citrate–phosphate buffer (pH 2.8)–ethanol (95:5) (column A) or methanol–water–acetic acid (5:95:1) (column B) or (10:90:1) (column C). The flow-rate was 1.0 ml/min and the columns were maintained at 25°C.

Sample doses were 1–5 μl of methanolic solution (concentration about 1 mg/ml) in the analytical column and 100–200 μl in the semi-preparative column. Samples of 200 μl were repeatedly injected on to the semi-preparative column and the fraction containing the analyte compound was collected in a 5-ml flask during elution. It was then evaporated under vacuum and the residue was used for identification by GC, MS and UV spectrometry. Standard compounds and samples were used for thin-layer chromatography (TLC) in 0.1-ml aliquots (about 20 μg of the compounds).

Qualitative analysis of psychotropic indole compounds was performed by comparing their elution volumes with those of reference samples. The results were confirmed by TLC, GC, MS and UV spectrometry. Indole alkaloids were determined by means of the internal normalization and external standard method. Detector signals were processed by the Baseline 810 software (Waters).

Thin-layer chromatography

Psilocybin, psilocin, bufotenin, etc., were identified on Silufol UV 254 foils eluted with *n*-butanol–water–acetic acid (24:10:10). Tryptamines were detected with Ehrlich reagent (2% *p*-dimethylaminobenzaldehyde in 1 *M* hydrochloric acid) and yielded red-violet spots after completion of the reaction.

Mass spectrometry

Mass spectra were measured with a Varian MAT model 311 instrument with ionization energy 70 eV, current 1 mA, ion source temperature 200°C and inlet temperature 35°C for psilocin, bufotenin and 140°C for psilocybin. The high-resolution peak-matching technique employed gave errors of ±5 ppm.

RESULTS AND DISCUSSION

Extraction

Extraction of psychotropic indole compounds from biological material (*Psilocybe bohemica*) has received scant attention in the literature on the analysis of these substances. The extraction is performed with mixtures of lower alcohols and water, sometimes with adjusted ionic strength or pH. The yield of psilocybin extraction by the commonly used methanol is only 80% of the amount obtained by extraction into 75% (v/v) methanol saturated with potassium nitrate (Fig. 1). Aqueous ethanol solutions are unsuitable for psilocybin extraction (maximum yield 36%). A 90% yield can be attained with 1% acetic acid in methanol. This system is suitable expecially for preparative purposes as both solvents can be evaporated.

The best extraction results with psilocin were obtained with 75% (v/v) aqueous ethanol, whereas

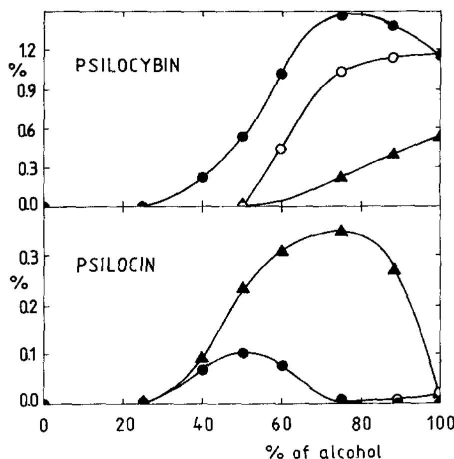

Fig. 1. Dependence of the determined amount of psilocybin and psilocin on the composition of the extracting agent: ● = methanol–water–saturated KNO_3; ○ = methanol–water; ▲ = ethanol–water.

methanol gave less than 10% of the amount recovered with 75% ethanol (Fig. 1). The optimum extraction systems for psilocybin and psilocin therefore differ.

A kinetic equation was used to calculate the time necessary for extraction of 99.9% of both psilocybin and psilocin. In contrast to literature data, complete recovery of psilocybin was found to require only 10 min and of psilocin 160 min [24].

Separation

The optimum composition of the mobile phase (amount of ethanol) for the chromatographic separation of tryptamines was determined by computer processing of experimental data on retention and on column efficiency, using an optimization and simulation program based on application of the so-called chromatographic optimization function (COF) [20]. Fig. 2 gives the conditions for the HPLC separation of individual tryptamines. Optimum separation of key tryptamines was achieved on the Silasorb SPH C_{18} column (B) with citrate–phosphate buffer containing 10% ethanol as mobile phase (Table I). Separation of indole alkaloids from *Amanita* fungus was done on a μBondapak C_{18} + Guard Pak column (A) with citrate–phosphate buffer containing 5% ethanol as mobile phase.

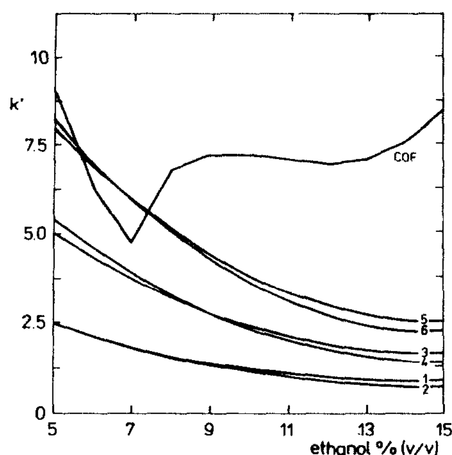

Fig. 2. Dependence of the capacity factor (k') and the chromatographic optimization function (COF) on the ethanol content in the mobile phase. 1 = Psilocybin; 2 = serotonin; 3 = tryptophan; 4 = bufotenin; 5 = tryptamine; 6 = psilocin.

Identification

The separation and identification of a mixture of indole alkaloids in extracts from fungi of genera *Psilocybe*, *Inocybe* and *Amanita* were first verified by TLC. The purity of individual compounds was demonstrated chromatographically (R_F) based on red-violet spots typical of tryptamines. Identifica-

TABLE I

RETENTION AND DETECTION DATA FOR THE INDOLE ALKALOIDS

Substance	Retention data		Detection limit (ng)		Relative standard deviation (%)[b]	
	GC (°C)[a]	HPLC (k')	UV	ED	UV	ED
5-Hydroxytryptophan	294.0	1.004	20	0.5	3.2	3.2
Psilocybin	–	1.004	20	1.0	2.8	3.5
5-Hydroxytryptamin (serotonin)	273.5	1.253	64	2.6	3.2	3.2
Bufotenin	263.5	1.931	34	1.0	3.1	3.5
Tryptophan	273.0	2.721	88	2.8	2.0	2.4
Tryptamine	260.	4.043	30	1.0	2.7	2.5
Psilocin	263.5	3.979	22	0.7	3.0	2.9
5-Hydroxy-N-methyltryptamine	277.0	–	–	–	–	–

[a] Temperature programmed from 200 to 300°C.
[b] Six parallel determinations.

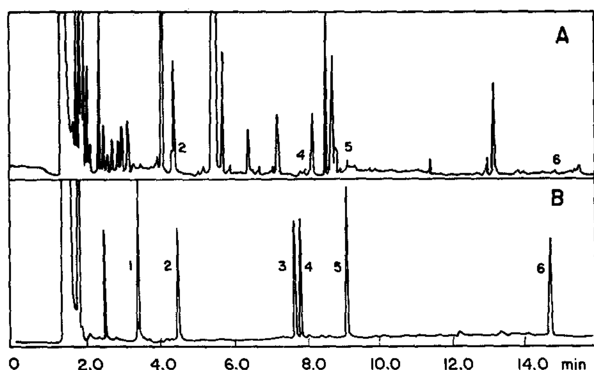

Fig. 3. GC of (A) the extract from fruit bodies of the fungus *Amanita citrina* (A) and (B) a mixture of the hallucinogen standards (TBDMS derivatives). 1 = Tryptamine; 2 = bufotenin; 3 = tryptophan; 4 = serotonin; 5 = 5-hydroxy-N-methyltryptamine; 6 = 5-hydroxytryptophan.

tion of a mixture of indole alkaloids from *Amanita* fungi was further confirmed by GC of their TBDMS derivatives, together with customary indole standards. The separation was performed on an SPB-1 capillary column with temperature programming from 250 to 300°C at 3°C/min. Chromatographic verification of their purity (Fig. 3, Table I) showed no separation of isomeric compounds (bufotenin and psilocin). This does not complicate the identification of the two compounds, however, as the probability of their mutual presence in any biological material is negligible.

The HPLC-separated mixture of indole alkaloids was then identified by recording the UV spectra of

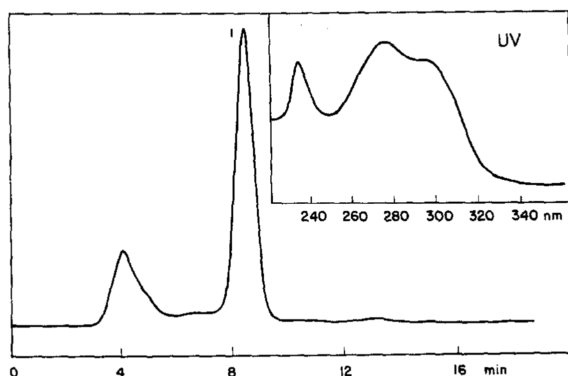

Fig. 4. HPLC of the extract from fruit bodies of the fungus *Amanita citrina* (Vestec, 1987). 1 = Bufotenin. Inset, UV spectrum of bufotenin.

individual compounds during the separation process itself using a diode-array detector (Table I). Fig. 4 shows a chromatogram of bufotenin with the UV spectrum of bufotenin obtained from the computer.

The UV spectra of psychotropic substances agree fully with literature data [25].

Determination

The rapid HPLC screening method on Silasorb SPH C_{18} (B) with citrate–phosphate buffer (pH 2.8)–ethanol (90:10) mobile phase does not ensure complete separation in all instances. A combination of chromatographic separation with selective ED can separate all components of a mixture of indole alkaloids. Hydroxylated tryptamines and tyrptophan can be selectively detected at a working electrode potential of +0.60 V (*vs.* Ag–AgCl), whereas the half-wave potential of other substances are higher than +0.80 V [21]. The detection limits with the UV photometric detector are of the order of units to tens of nanograms, and with the electrochemical detector units to tenths of nanograms in the inlet (Table I). Quantification of the mixture components was done by the external standard method and evaluation of peak heights. As indicated by the relative standard deviation (s_r), this screening method is less exact than common direct methods ($s_{r,\,uv}$ = 3.4–6.3%; $s_{r,\,ED}$ = 7.2–9.1%).

Differences in the chromatographic behaviours of psilocybin and psilocin make it necessary to use two different mobile phases for the assay of either substance. The preparative application dictates that the mobile phase must be easy to evaporate completely. Its pH cannot be adjusted with a buffer but with an easy-to-evaporate organic acid. The indole alkaloids in extracts from genera *Amanita, Psilocybe* and *Inocybe* were analysed by an exact HPLC method on column A (μBondapak C_{18}) with citrate–phosphate buffer (oH 2.8)–ethanol (90:10 or 95:5) as the mobile phase, and psilocybin and psilocin on column B (Silasorb SPH C_{18}) with methanol–water–acetic acid (5:95:1 or 10:90:1) as the mobile phase.

A series combination of spectrophotometric and electrochemical detection was also used. Psilocybin was determined from the UV detector (267 nm) recording and psilocin from the electrochemical detector trace (Fig. 5). Table I show that this method

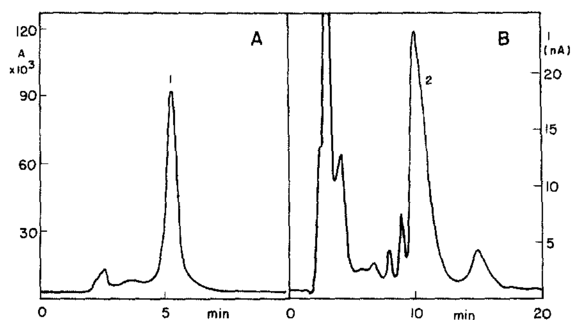

Fig. 5. HPLC of indole alkaloids with (A) UV photometric and (B) electrochemical detection. 1 = Psilocybin; 2 = psilocin.

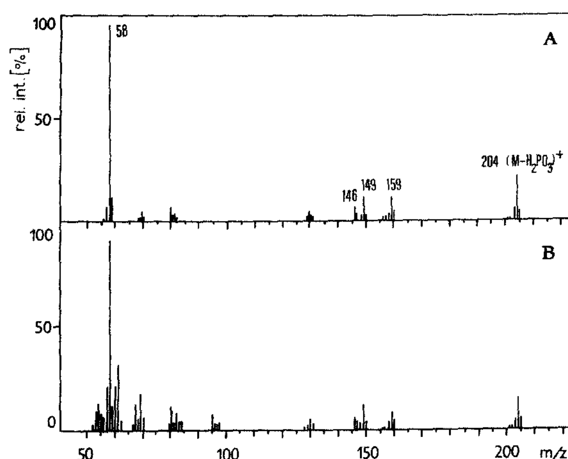

Fig. 6. Mass spectra of an extract of *Psilocybe bohemica*. (A) Psilocybin standard; (B) indole alkaloid isolated from the extract.

yields substantially better relative standard deviations than the screening method.

Isolation

The lipophilic fraction of biological material was removed by extraction into light petroleum (b.p. 40–60°C) (psilocin, bufotenin) or a mixture of light petroleum and chloroform (psilocybin). Psilocin was extracted with 75% aqueous ethanol whereas psilocybin was extracted with 1% acetic acid in methanol.

Isolation of tryptamines was done on a semi-preparative chromatographic column. Psilocybin was chromatographed on a column of Separon SGX C_{18} (C) with methanol–water–acetic acid (5:95:1) as the mobile phase. Psilocin and bufotenin were separated under analogous conditions on the same column but with methanol–water–acetic acid (10:90:1) as the mobile phase. Isolated psilocybin, psilocin and bufotenin were identified by MS. Fig. 6 shows the mass spectrum of an extract from biological material.

TABLE II

CONTENTS OF PSYCHOTROPIC COMPONENTS IN FRUIT BODIES OF THE GENUS *AMANITA*

Species	Locality	Year	Compound[a] (%)				
			OH-MeTPA	OH-TRP	BUF	TPA	TRP
A. citrina	Pribram	1987				0.06	
A. citrina	Vestec	1987			1.058		
A. citrina	Helfenburg	1988			0.593		
A. citrina	Vestec	1988			1.424		
A. citrina	Talin	1889	0.039		1.899		0.025
A. citrina	Krenicna	1989		0.033	0.678		
A. citrina		1989		0.593	1.693		
A. citrina	Neveklov	1989		0.099	0.332		
A. citrina	Vestec	1989			0.414		0.007
A. porphyria	Lounovice	1987			0.374		
A. porphyria	Vestec	1989	0.072		0.617		
A. rubescens	Vestec	1989			0.018		
A. rubescens	Krenicna	1989			0.020		

[a] TRP = Tryptophan; TPA = tryptamine; BUF = bufotenin; OH-MeTPA = 5-hydroxy-N-methyltryptamine; OH-TRP = 5-hydroxytryptophan.

TABLE III

CONTENTS OF PSYCHOTROPIC COMPOUNDS IN FRUIT BODIES OF THE GENERA *PSILOCYBE* AND *INOCYBE*

Species	Locality	Year	Psilocybin (%)	Psilocin (%)
P. bohemica	Frenstat	1981	0.46	0.02
P. bohemica	Sazava	1982	1.14	0.07
P. bohemica	Sazava	1983	0.64	0.48
P. semilanceata	Praha	1980	1.05	0.12
P. semilanceata	Krasna Lipa	1982	0.91	0.09
P. semilanceata	Spiska N. Ves	1986	0.76	0.09
P. cyanescens	Mason County, WA, USA	1984	0.00	0.45
P. cyanescens	Horni Bradlo	1986	0.10	0.47
I. aeruginascens	Potsam, Germany	1982	0.33	–
I. aeruginascens	Potsdam	1983	0.34	–
I. aeruginascens	Potsdam	1984	0.38	–
I. aeruginascens	Potsdam	1986	0.03	0.02

Application

The above procedures were used for the separation, identification, isolation and determination of indole alkaloids in extracts from the fruit bodies of fungi from the genera *Psilocybe*, *Inocybe* and *Amanita* and mycelial extracts from the fungus *Psilocybe bohemica*. Table II summarizes the results of analysis of three *Amanita* species collected recently (1987–89) at various localities in Czechoslovakia and Estonia. Two species, *A. citrina* and *A. porphyria*, contain bufotenin (0.3–1.9%) as the major component whereas other tryptamines and tryptophan are present at trace levels. *A. porphyria* has a lower bufotenin content (0.3–0.7%), *A. citrina* from Estonia has a high bufotenin level (1.9%). *A. rubescens* contains no bufotenin, similar to one finding in *A. citrina*.

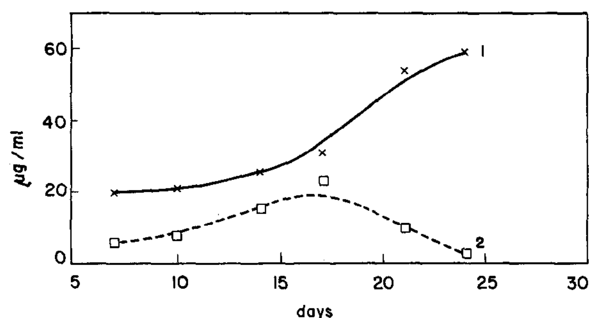

Fig. 7. Production of indole alkaloids during submerged cultivation of *Psilocybe bohemica*. 1 = Psilocybin; 2 = psilocin.

Comparison of *Amanita* and *Psilocybe* fungi collected in the 1980s in Czechoslovakia and in the USA (Table III) showed similar contents of the major component psilocybin (0.1–1.4%) but psilocin levels in *Psilocybe* are considerably lower (0.02–0.5%). Species of the genus *Inocybe* (collected in Germany) exhibit low psilocybin levels (0.03–0.4%) and negligible psilocin contents (0.02%). The extract from *Inocybe aeruginascens* (1986) contained another compound with a retention time shorter than that of psilocybin but with an essentially identical UV spectrum. It was probably baeocystin, in accordance with the data of Gartz [26].

Chromatographic analysis of psilocybin and psilocin was used during the culturing of the fungus *Psilocybe bohemica* (Fig. 7). Isolation of both compounds from fruit bodies and mycelia was verified by HPLC. The psilocybin recovery was 75–80% of the orginal amount and with psilocin it was 80–90%.

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