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Analysis of psilocybin and psilocin in *Psilocybe subcubensis* GUZMÁN by ion mobility spectrometry and gas chromatography–mass spectrometry

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Abstract

A new method has been developed for the rapid analysis of psilocybin and/or psilocin in fungus material using ion mobility spectrometry. Quantitative analysis was performed by gas chromatography–mass spectrometry after a simple one-step extraction involving homogenization of the dried fruit bodies of fungi in chloroform and derivatization with MSTFA. The proposed methods resulted in rapid procedures useful in analyzing psychotropic fungi for psilocybin and psilocin.
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Keywords: Psychedelic fungi; *Psilocybe subcubensis*; psilocybin ; psilocin; ion mobility spectrometry (IMS); gas chromatography–mass spectrometry (GC–MS); MSTFA

1. Introduction

The psychedelic effects of some species of the genus *Psilocybe* were first described by Wasson in 1957 [1,2]. Hofmann et al. [3] then isolated two hallucinogenic components of the tryptamine type, psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine), the main

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psychotropic compound, and psilocin (4-hydroxy-*N*,*N*-dimethyltryptamine). Four decades after their discovery in fruit bodies of *Psilocybe mexicana* Heim, indole alkaloids of the tryptamine type have become biochemically important drugs in psychotherapy and psychodiagnostics. At the same time recreational use of hallucinogenic fungi has become an increasing problem in several countries all over the world. The detection methods for psilocybin and psilocin in fungi include paper chromatography [4], thin layer chromatography [5–9], gas chromatography [10], gas chromatography–mass spectrometry [9,11–13], and mostly HPLC [5–9,13–22]. Detailed information on sample preparation or data on operation parameters using the IMS methodology have not been published in forensic literature so far. The purpose of this paper was to develop both a quick method based on the IMS technology for the rapid screening of fruit bodies of fungi for psilocybin and/or psilocin as well as the development of a reliable quantitative analysis method performed by GC–MS after derivatization with MSTFA. The origin of basidiocarps of *Psilocybe subcubensis* (the psychotropic species studied in this paper) is unknown. Collections of this fungus were confiscated by police in the course of a border patrol. This species naturally grows on dung or in rich pastures in subtropical to tropical climates. Under sterile or semisterile conditions, *Psilocybe subcubensis* is easily cultured [23].

2. Principle of IMS

Ion mobility spectrometry refers to the principles, practice and instrumentation for characterizing chemical substances through their gas-phase ion mobilities. IMS is an analytical technique that distinguishes ionic species on the basis of the differences in the drift velocity through a gas under an applied electrostatic field. It is a sensitive technique for the detection of trace organics under atmospheric pressure conditions. Fig. 1 depicts the schematic representation of the IONSCAN detection system. The sample, collected on a membrane filter for example, is heated to vaporization by the desorber. The neutral molecules of the vapor are carried in a stream of dried, filtered, ambient air through the heated transfer line into the reaction region. There, ionization is initiated by high energy electrons emitted from a ^{63}Ni beta-ray source. Every 20 ms the product ions (positive or negative) are gated with a pulse width of 0.02 ms into the heated drift region for mobility analysis. Under the influence of a controlled electric field and against a counterflow of ambient air drift gas the ions move to the collector electrode. The drift times required by the ions to reach the collector electrode are proportional to their masses, but inversely proportional to their characteristic reduced ion mobilities (K_0). For drug detection, the ion mobility spectrometer is operated in the positive mode. In this mode, the drift gas contains trace amounts of nicotinamide (NTA) used as both calibrant and reactant. In the reaction region, the protonated nicotinamide transfers a proton to the sample molecule according to Eq. (1):

$$[\mathrm{NTA}]\mathrm{H}^+ + \mathrm{M} \rightarrow \mathrm{NTA} + [\mathrm{M}]\mathrm{H}^+ \qquad (1)$$

This reaction only proceeds if the proton affinity of the sample molecule is greater then that of nicotinamide. This is true for nearly all drug molecules, which are thus

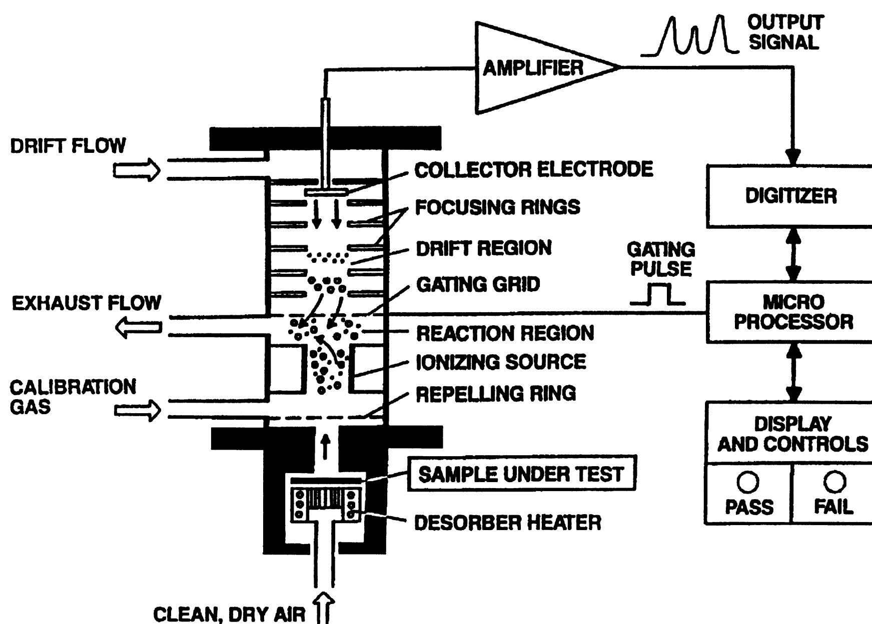


Fig. 1. Schematic representation of the IONSCAN 400 detection system.

detectable by IMS. The principles and theoretical background of ion mobility spectrometry has been extensively described elsewhere [24–30] and will not be discussed in this paper.

3. Mushroom analysis by IMS and GC–MS

3.1. Microbiological materials

The confiscated fruit bodies of the investigated species were determined as *Psilocybe subcubensis* Guzmán. The distinct annulus on the stem, the blueing of the whole fruit body and the size of the spores are characteristic for this species (see Fig. 2).

3.1.1. Description

Pileus 25–70 mm broad, up to 15 mm high, convex campanulate to subumbonate, plano-convex with only an obtuse umbo, smooth, young cream or buff, ochraceous towards the umbo, then brownish red (Me[1] 4A3, 4B4-5, 5B4, 5C4-6), with white spots, margin with whitish veil fragments, becoming patchily blueish (Me 24C2-3). Lamellae adnate or adnexed, slightly crowded, ochraceous at first, then greyish violet to dark

[1]Me = used colorcodex: A. Korneup and J.H. Wanscher in: Methuen Handbook of Color, 3rd ed., Methuen London Ltd., 1983.

Fig. 2. Dried fruit bodies of *Psilocybe subcubensis*.

violet brown (Me 4B3-4), with whitish subflocculose edges. Stipe 50–105×5–15 mm, subequal tapered upwards from a most distinctly swollen base, hollow, white to pale yellowish (Me 4A2, 3A2), with a white, persistent but fragile annulus, smooth to subflocculose, blueing (Me 24C2-3). Context whitish, blueing when cut, no distinct smell recorded. Spores 9.5–11.5×5.5–7.0 μm, subhexagonal in face view, (sub-)ellipsoid in side view, thick walled, with a distinct broad apical grem pore, dark yellowish brown. Basidia 4-spored, clavatecylindric. Cheilocystidia numerous, thin walled, hyaline, fusiform to sublageniform, up to 30 μm long. Pleurocystidia scarce, similar to Cheilocystidia. Cap cuticle of narrow, radially arranged, gelatinized, cylindric, hyaline to yellowish hyphae, clamp-connections present.

3.1.2. Collection examined

Locality and habitat unknown, exsiccat collection number TR 83-97, det. Th. Rücker. As a ‘‘negative’’ sample, fruit bodies of the species *Agrocybe praecox* (Pers.) Fayod were chosen.

3.1.3. Collection examined

Austria, Salzburg, Salzburg Stadt, Nonntal, 22 May 1990, det. Th. Rücker (TR 14-90). Macroscopic characters of *Agrocybe praecox* are very similar to *Psilocybe*

subcubensis but *Agrocybe praecox* does not contain the hallucinogenic components psilocybin or psilocin.

3.2. Sample preparation and IMS measurement

The cap and stem of a basidiocarp were cut in small pieces. A small piece of each part was placed on a Teflon membrane filter and inserted into the ion mobility spectrometer. Figs. 3 and 4 show the plasmagrams of a psilocybin and psilocin standard solution, respectively. Fig. 5 shows the plasmagram of the negative control *(Agrocybe praecox).* The typical plasmagrams of the mushroom cap and stem of *Psilocybe subcubensis* are depicted in Figs. 6 and 7.

3.3. Sample preparation and GC–MS measurement

Parts of fungal fruit bodies were lyophilized and the freeze-dried cap and stem were cut into small pieces and ground to a fine powder in a mortar. An accurately weighed amount of the corresponding powdered sample (50 mg) was extracted with 1 ml chloroform in an ultrasonic bath for 1 h. Centrifugation at 14 000 rpm for 10 min and filtration through a cotton filter resulted in a clear supernatant. A 0.5-ml volume of the filtrate from spiked mushroom samples was pipetted into a GC vial and evaporated to dryness at 50°C under a gentle stream of nitrogen. The same procedure was applied to the psychedelic mushroom material except that only 0.05 ml of the filtrate was evaporated. Each residue was dissolved in 30 μl MSTFA and heated for 30 mm at 70°C. After cooling, 1 μl of the sample was directly used for GC–MS analysis. Standards of known amounts of psilocybin and psilocin were prepared by spiking ''negative'' mushroom samples to obtain final concentrations of 0.1, 0.2, 0.9 mg psilocybin/g and 0.01, 0.1, 0.2 mg psilocin/g dry mass. Standard curves for psilocybin and psilocin fitted a linear model over the concentration range mentioned. The correlation coefficients calculated by linear regression analyses were 0.997 for both psilocybin and psilocin.

4. Experimental

4.1. Chemicals and reagents

Psilocybin and psilocin were supplied from Alltech (USA). Chloroform and *N*-Methyl-*N*-(trimethylsilyl)-2,2,2-trifluoroacetamide (MSTFA) were of analytical grade and were obtained from E. Merck (Darmstadt, Germany).

4.2. Instrumentation

A LYOVAC GT2 freeze-dryer (Leybold-Heraeus, Germany) was utilized to lyophilize the mushroom samples.

An IONSCAN model 400 ion mobility spectrometer (Barringer, Rexdale, Canada) was utilized in the positive mode. Teflon membrane filters with a thickness of 0.25 mm were utilized as filter substrates.

GC was performed on an HP 6890 Series gas chromatograph equipped with an HP 5973 mass selective detector. The TMS-derivatives were separated on a 30 m×0.25 mm i.d. HP-5 MS fused-silica capillary column (95% dimethyl–5% diphenyl polysiloxane) with a 0.25 μm film thickness with helium as the carrier gas at a constant flow rate of 1 ml/min. The splitless mode was used with 1 μl samples being injected. The operating conditions for the analyses were: injection port temperature 250°C; initial temperature 180°C, programming 20°C/min to final temperature, 320°C for 5 mm.

5. Results

A plasmagram of a psilocybin and psilocin standard solution in methanol is shown in Figs. 3 and 4. The drift times for the psychotropic substances psilocybin and psilocin were 11.834 and 11.822 ms, respectively. Due to thermal dephosphorylation of underivatized psilocybin in the inlet system of the ion mobility spectrometer (or gas chromatograph [31]), psilocybin is converted into psilocin. The plasmagram of the negative mushroom sample is shown in Fig. 5. Typical plasmagrams of cap and stem of *Psilocybe subcubensis* are depicted in Figs. 6 and 7, respectively. The peak detected was

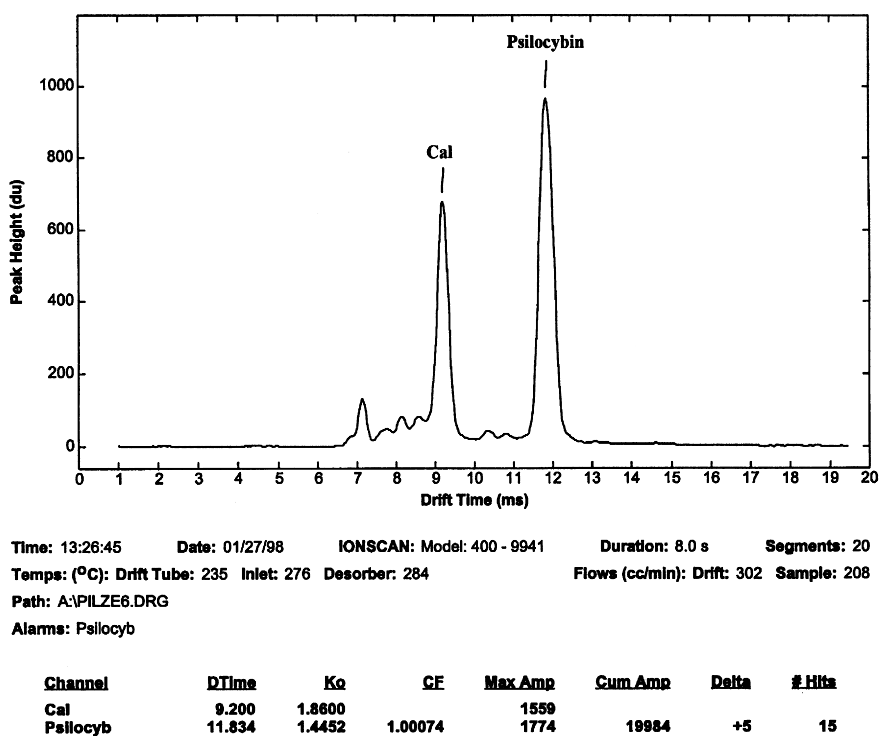


Fig. 3. Plasmagram of a psilocybin standard solution in methanol (DTime=11.834 ms (psilocybin)).

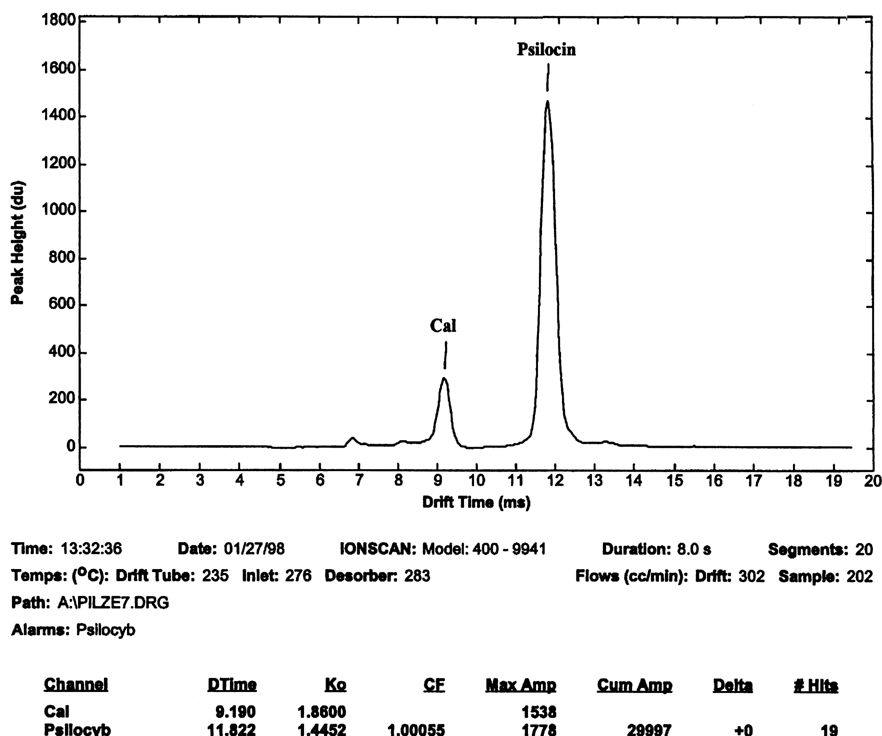


Fig. 4. Plasmagram of a psilocin standard solution in methanol (DTime = 11.822 ms (psilocin)).

identified as psilocybin on both reduced ion mobility (K_0) and drift time (DTime). Throughout the IMS experiments, the threshold was set at 50 digital units (du). The utilization of Teflon membrane filters was most effective for the analysis of the mushroom material. A desorber temperature of 288°C (also ideal for the detection of other illicit drugs) resulted in a maximum peak intensity for psilocybin and psilocin. Optimized operation parameters are summarized in Table 1.

Quantitative analysis of the mushroom material was performed by GC–MS after a one-step extraction involving homogenization and ultrasonification of the dried fruit bodies in chloroform. Unlike Repke et al. [11] who used BSTFA at 140°C and Haensel et al. [31] who utilized a BSTFA–TMCS–pyridine mixture at 100°C to form the TMS-derivatives, the use of MSTFA at 70°C proved to be the derivatization method of choice in our case. In contrast to BSTFA, the use of MSTFA resulted in a reduction of background noise. The MSD was operated in the SIM mode using the ions m/z 485, 455 and 442 for psilocybin and m/z 348, 291 and 290 for psilocin, respectively. The last ion listed for each compound was used for quantitation. Psilocybin and psilocin were found in an amount of 0.86% (8.6 mg/g) and 0.02% (0.2 mg/g) per dry mass in the cap of *Psilocybe subcubensis,* respectively. 0.8% psilocybin (8.0 mg/g) and 0.03% psilocin (0.3 mg/g) per dry mass could be found in the stem (see Table 2).

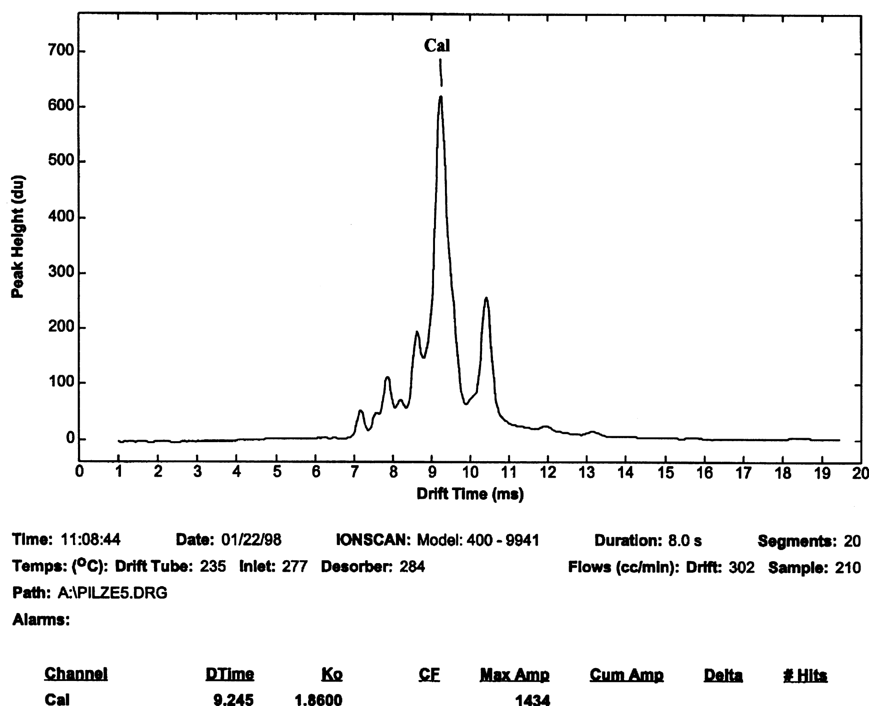
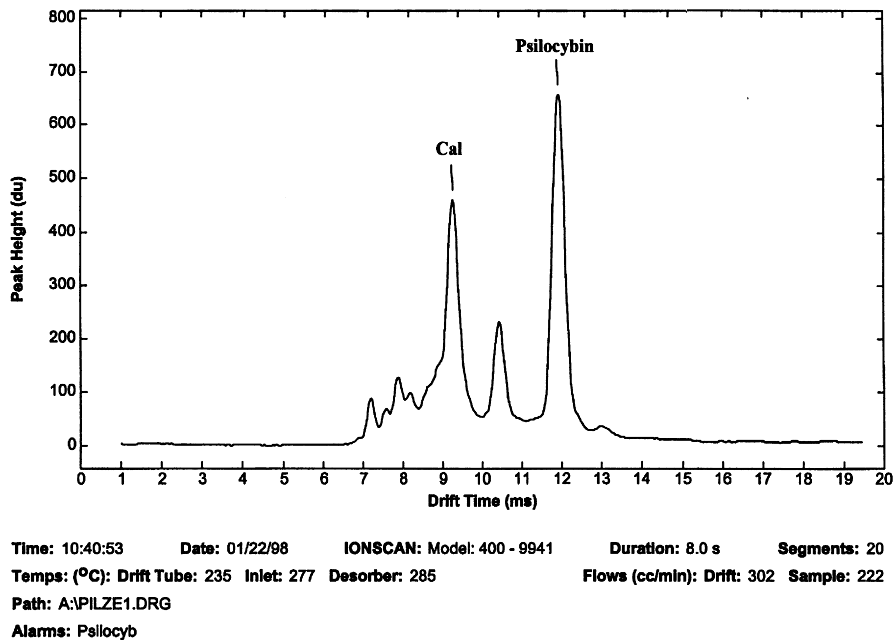


Fig. 5. Plasmagram of fruit bodies of *Agrocybe praecox* (negative control).

6. Discussion

The IMS methodology has to date been successfully utilized for the detection of prescription and illicit drugs [26,30,32–34], explosives [35,36] as well as for the detection of cocaine, clenbuterol, methamphetamine and designer drugs in human hair [37–40]. Detailed information on sample preparation or data on operation parameters using the IMS methodology have not been published in forensic literature so far. In applying IMS technology, the authors managed to establish a rapid, simple and sensitive screening method for the detection of psilocybin and/or psilocin in fruit bodies of confiscated psychedelic fungi. The limit of detection for psilocybin was found to be 40 ng absolute at a threshold value of 2 digital units (du), the minimum allowable amplitude value. Quantification of psilocybin and psilocin was achieved with GC–MS after derivatization with MSTFA at 70°C. Our results are in accordance with the findings of Wurst et al. [5] and Kysilka et al. [14] who also found lower concentrations of psilocybin in the stem and mycelium than in the cap of the hallucinogenic fungus. Both authors analyzed *Psilocybe bohemica* Šebek (a doubtful species of the *Psilocybe cyanescens group*) grown in Czechoslovakia. To the best of our knowledge, no data on the content of psilocybin and psilocin in *Psilocybe subcubensis* have been published in forensic literature so far. *P. subcubensis* is macroscopically very similar to *P. cubensis*

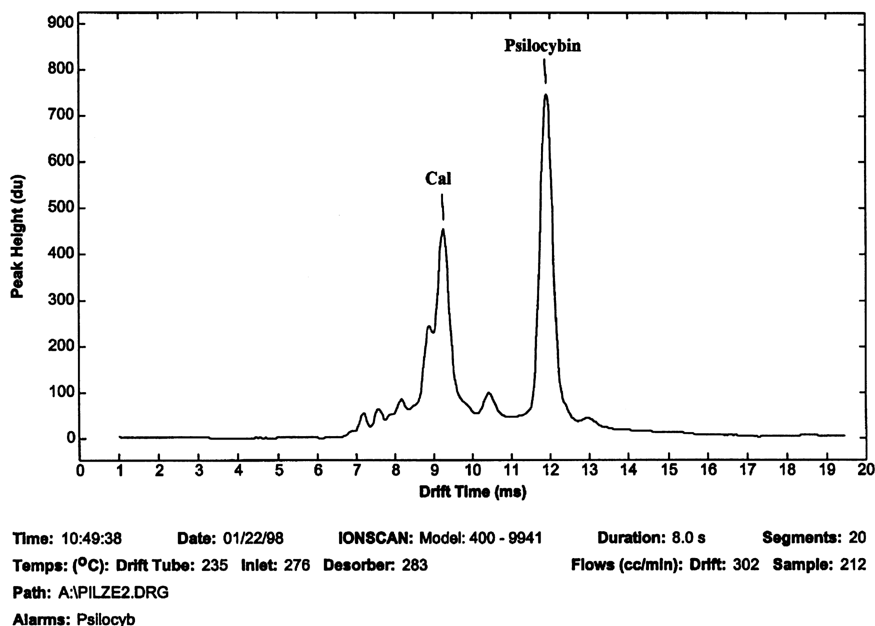


Channel	DTime	Ko	CF	Max Amp	Cum Amp	Delta	# Hits
Cal	9.263	1.8600		1552			
Psilocyb	11.915	1.4452	1.00080	1250	13722	+0	18

Fig. 6. Plasmagram of caps of *Psilocybe subcubensis* (DTime = 11.915 ms (psilocybin)).

(Earle) Singer, thus accounting for possible cases of mistaken identity with *P. cubensis* in the past.

In addition, *Psilocybe subcubensis* was not described before 1978 [50]. Data about the content of psilocybin and psilocin in mushrooms of *Psilocybe cubensis* were first reported by Repke et al. [11] and later by Borner and Brenneisen [15] and Gartz [8,9]. According to these authors the amount of psilocybin varies from 0.17 to 1.07%. The content of psilocin is reported within the range of 0.11–0.42% per dry mass. Our investigations of fruit bodies of *Psilocybe subcubensis* show a similar content of the major component psilocybin, whereas the psilocin level seems to be much lower. Psilocybin and psilocin concentrations found in the fungi under investigation are depicted in Table 2. Differences in the psilocybin and psilocin content of fruit bodies of *Psilocybe* species are to be expected since the amount of active components present in the corresponding mushrooms undoubtedly depends on factors such as species, time of collection, climatic conditions, preservation of the material as well as the availability of soluble nitrogen and phosphorus in the soil [17]. The use of "real" negative mushroom material is essential for quantitative analyses. In our spiking experiment, we utilized the *Agrocybe praecox* which are similar in habitus and stature to *Psilocybe subcubensis*. The typical plasmagram of *Agrocybe praecox* is shown in Fig. 5. Beug and Bigwood [7] and



Channel	DTime	Ko	CF	Max Amp	Cum Amp	Delta	# Hits
Cal	9.251	1.8600		1497			
Psilocyb	11.900	1.4452	1.00059	1353	15321	-4	18

Fig. 7. Plasmagram of stems of *Psilocybe subcubensis* (DTime = 11.900 ms (psilocybin)).

Borner and Brenneisen [15] both used *Panaeolina foenisecii* (Pers.) Schroet. whereas Christiansen et al. [18] utilized *Panaeolus rickenii* Hora as negative mushroom material. As for *Panaeolina foenisecii,* there are some contradictory reports in the literature

Table 1
IONSCAN operation parameters

Parameter	Setting
Desorber temperature [°C]	288
Inlet temperature [°C]	279
Drift tube temperature [°C]	235
Drift flow [cm^3/min]	300
Sample flow [cm^3/min]	200
Stand-by drift flow [cm^3/min]	51
Gate width [ms]	20
Scan period [ms]	20
Drift tube length [cm]	7
Shutter grid width [ms]	0.2
Threshold [du]	50
Drift gas	Dried air
Carrier gas	Dried air
Calibrant/reactant	Nicotinamide

Table 2
Concentration of psilocybin and psilocin in *Psilocybe subcubensis* determined by GC/MS

Fruit body	Psilocybin % (mg g^{-1})	Psilocin % (mg g^{-1})
Cap	0.86 (8.6)	0.02 (0.2)
Stem	0.80 (8.0)	0.03 (0.3)

concerning toxicity and content of active components. In *Panaeolina foenisecii* grown in Italy, North America and Great Britain, psilocybin could be found [41–44]. Other papers state that no active hallucinogenic components could be found in *Panaeolina foenisecii* [45–49]. To avoid any uncertainty about the presence of active components in *Panaeolina foenisecii,* we decided to use fruit bodies of *Agrocybe praecox* as negative fungal biomass. Examinations on the content of psilocybin and psilocin performed by IMS and GC–MS revealed that these active components are not present in the fungi used as negative control.

The purpose of this paper was to illustrate the potential of ion mobility spectrometry as a highly sensitive analytical device, especially in drug analysis. It could be shown that IMS can be adopted as a routine tool in the daily work of a forensic chemist. On the other hand, we also managed to establish a new, simple and reliable quantitative GC–MS method which is not as time-consuming and laborious as the methods already referred to in this paper.

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