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Pascal Kintz, Jean-Sébastien Raul, Alice Ameline. Testing human hair after magic mushrooms abuse by LC-MS/MS: Pitfalls and limitations. *Forensic Chemistry*, 2021, 26, <10.1016/j.forc.2021.100364>. <hal-03626917>

**HAL Id: hal-03626917**

**<https://hal.science/hal-03626917v1>**

Submitted on 16 Oct 2023

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(Short communication)

## Testing human hair after magic mushrooms abuse by LC-MS/MS: pitfalls and limitations

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Conflict of interest: the authors declare no conflict of interest, financial or otherwise

### Abstract

Although it has been published during the last 15 years that hallucinogens derived from new psychoactive substances are increasingly abused, the old psychedelic agents are still used. Among these vegetal compounds, magic mushrooms are frequently mentioned. The major ingredient of the *Psilocybe* species is psilocybin, which is rapidly converted into psilocin in the human body. Therefore, psilocin represents the key target to document magic mushrooms abuse. The aim of the present work was to develop a specific method to identify psilocin in human hair by LC-MS/MS and review potential pitfalls. The method involves incubation of 10 mg of cut hair in 1 mL of acid methanol in presence of psilocin-d<sub>10</sub> for 2 hours at 4 °C. The chromatographic separation was performed using a reverse phase column HSS C18 with a gradient elution of 8 min. Linearity was verified from 1 to 200 pg/mg ( $r^2 = 0.9994$ ), after spiking blank hair with the corresponding amounts of psilocin. The limit of detection was estimated at 0.4 pg/mg. The precision was lower than 20 % and there was no interference with any extractable material present in hair. Psilocin was identified in the hair of a repetitive magic mushrooms consumer at 2.5 pg/mg (segment 0-1 cm), 4.4 pg/mg (segment 1-2 cm) and 5.4 pg/mg (segment 2-3 cm). Cold conditions during hair preparation and protection from the light were found mandatory to avoid psilocin degradation.

## Key words

Magic mushroom, psilocin, psilocybin, hair, LC-MS/MS

## 1. Introduction

During the last 15 years, an increasing abuse of new psychoactive substances (NPS) has been reported worldwide. Stimulants, narcotics and hallucinogens belong to this generic group of substances that are more and more popular among recreational drug abusers. The common property of these 1000 substances is their synthetic chemical preparation. As a consequence, it has been claimed that legislative answers are needed to fit against this international health hazard [1]. A review of screening methods for NPS in blood, urine, but also hair and oral fluid has recently published [2]. Analytical methods, including colorimetric, immunochemical and chromatographic / mass spectrometry techniques have been reviewed, clearly indicating that the high number of different chemical structures of NPS can be a challenge for toxicologists. Beside NPS, consumption of vegetal substances is sometimes presented as an environmental “green” alternative [3]. Although limited in term of frequency of use [4], magic mushrooms consumption is still a standard practice for abusers interested in psychedelic experiences [5]. Indeed, the species *Psilocybe* contain 2 main hallucinogenic compounds, i.e. psilocybin and psilocin.

The consumption of magic mushrooms will produce time and space deformation, associated to visual and hearing hallucinations [6]. The mushrooms can be consumed fresh (but rapidly after the harvest) or as dried products after rehydration. It is also possible to buy on-line, via e-shops, capsules containing powdered material. Psilocybin is a labile substance. Degradation can occur in fresh mushrooms, even stored at – 80 °C [7]. It is also very sensitive to light and air [8, 9]. In the body, due to alkaline phosphatase, psilocybin undergoes rapid conversion to its dephosphorylated analog, psilocin, which is pharmacologically active. In vitro, the addition of an organic acid, such as ascorbic acid [10] can prevent this transformation.

Therefore, it is not surprising that psilocin has been proposed as the target substance in hair to document repetitive use of magic mushrooms [11-13]. A literature review indicates that psilocin has been very rarely identified in the hair of abusers of magic mushrooms. Although Martin et al [10] and Shi et al [11] screened for psilocin in hair, they did not report any positive test result. Very recently, Zhou et al [12] found

psilocin in two authentic hair samples at 150 and 161 pg/mg. Psilocybin was not detected in the corresponding hair specimens. On the opposite, Pichini et al [13] identified psilocybin at 800 pg/mg in the hair of a psilocybe consumer, while no psilocin was found.

Because of these very limited data, sometimes controversial, it is of interest to present additional positive cases and to review the potential difficulties in testing for psilocin in hair.

## **2. Materials and methods**

### 2.1. Chemicals and reagents

Ammonium formate 99% was provided by Alfa Aesar (Schiltigheim, France). Formic acid was provided by VWR Prolabo (Fontenay-sous-Boise, France). Acetonitrile for LC-MS grade, methanol and dichloromethane were purchased from Merck (Molsheim, France). Psilocin and psilocin-d<sub>10</sub> was purchased from Cerilliant (Round Rock, TX, USA). Drugs were diluted to appropriate concentrations with acetonitrile, using a working 1 mg/L solution for psilocin and a 0.1 mg/L for psilocin-d<sub>10</sub>.

### 2.2. Hair specimens

Validation of the method was achieved using blank hair obtained from laboratory personal, after verification of the absence of any chromatographic response at the retention time of psilocin and its deuterated analog. This material was used to spike the vials at appropriate concentrations, since there is no reference hair material available for psilocin.

The method was applied by analysing the hair of a 38-year old male that was a possible consumer of magic mushrooms. The hair of the subject was light brown in color and 3 cm in length, oriented root-tip. Given it was a forensic request ordered by a Judge, the test was not relevant for any ethical review committee.

### 2.3. Hair preparation

After decontamination with 2 baths of 5 mL of dichloromethane for 2 min, the hair strand was segmented in 3 x 1 cm segments. Each segment was cut into very small pieces, less than 1 mm. Psilocin was extracted from 10 mg of cut hair in the presence of 2 ng of psilocin-d<sub>10</sub> used as internal standard, after incubation in a dark glass vial

covered with a foil of aluminum for 2 hours at 4 °C in 1 mL methanol + 1 % formic acid and horizontal agitation. After centrifugation, the supernatant was collected and carefully evaporated to dryness by a stream of nitrogen, while the dark vial was maintained in cold water. Finally, the residue was reconstituted in 30  $\mu$ L of 5 mM ammonium formate buffer adjusted at pH 3. The vials were kept at 4 °C while on the injection carousel of the auto-sampler.

#### 2.4. LC-MS/MS instrumentation

Chromatography was performed on an Acquity class I ultra-high performance liquid chromatography and separation was achieved using a Waters Acquity HSS C18 column (150 x 2.1 mm x 1.8  $\mu$ m) maintained at 50°C in a thermostatically controlled oven. A gradient elution was performed using formate buffer adjusted to pH 3 (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The flow rate was 0.2 mL/min. Elution profile: initial concentration of mobile phase A (95 %) held for 1 min, down to 80 % at 5 min, followed by a return to the initial conditions at 6.5 min, maintained for the last 1.5 min. An injection volume of 2  $\mu$ L was used in all cases. The detection of the molecules was performed on a Xevo TQD triple quadrupole mass spectrometer from Waters (Milford, MA, USA). Ionization was achieved using electrospray in the positive ionization mode (ES+).

The following conditions were found to be optimal for the analysis of psilocin and the internal standard: capillary voltage at 1.5kV; source block temperature at 149°C; desolvation gas nitrogen heated at 600°C and delivered at a flow rate of 1000L/h. In order to establish appropriate multiple reaction monitoring conditions, the cone voltage was adjusted to maximize the intensity of the protonated molecular ion and collision induced dissociated of both species was performed. Collision energy was adjusted to optimize the signal for the quantification transition of psilocin: m/z 205.2 > 58.1 (cone voltage at 20 V and collision energy at 18 eV), the confirmation transition: m/z 205.2 > 160.1 (cone voltage at 20 V and collision energy at 15 eV) and the most abundant transition of the internal standard: m/z 215.2 > 66.1 (cone voltage at 20 V and collision energy at 18 eV). MassLynx 4.1 software was used for quantification.

### **3. Results and discussion**

During the initial tests with an authentic hair specimen collected from a magic mushroom consumer, no psilocybin was observed. For this reason, and also because the literature review indicated that only psilocin is identified in human hair, the authors decided to focus on psilocin only, with no attempt to identify the instable psilocybin. Given psilocin is also known to be labile, it was essential that all the analytical procedure was achieved during one working day.

As there is no hair reference material for psilocin, the whole validation was achieved with spiked hair. Blank hair obtained from laboratory scientists tested negative (n=8). A unique donor, with brown hair, was used for the verification of the linearity over the range 1 to 200 pg/mg. The response of the detector was found linear, with a correlation coefficient of 0.9994. The limit of detection, corresponding to three times the background noise and the lower limit of quantification, corresponding to the first point of calibration were 0.4 pg/mg and 1 pg/mg, respectively. Internal quality control (QC) samples, at 5 and 100 pg/mg, analysed in eight independent tests, were used to establish the precision of the method. Intra-day precisions CVs were 15.9 and 13.8 % for low and high QC samples, respectively. Inter-day precisions CVs, over six consecutive days, were 18.3 and 16.1 % for low and high QC samples, respectively. There was no apparent interference between psilocin and the internal standard and any extractable element present in hair. Matrix effect was investigated after extraction of fifteen blank hair specimens, spiked at 10 pg/mg of psilocin and compared to a corresponding solution at the same concentration in methanol and was found to be less than 25 %. Analytes were stable at least 12 hours in the auto-sampler refrigerated at + 4 °C. Because of the well-known chemical instability of psilocin, it is mandatory to use its deuterated analog during the analytical process, as this can minimize the degradation consequences.

Since more than 30 years, hair testing for drugs is presented as the best approach to document possible repetitive drug exposure, but also after a single incident that happened some time ago when there is no interest to collect blood or urine as the drug is gone by body elimination. Pitfalls in hair analysis for drugs have been extensively reviewed [14-16]. Two types of problems of been identified, including 1. the analytical step its-self, and 2. the interpretation of the data. With respect to the analytical approach, the major issue is the hydrolysis of the compounds of interest in

strong alkaline medium used to destroy the keratinous matrix. This was observed for example for cocaine, heroin, mephedrone and benzodiazepines. Switching to acid extraction or buffer extraction helped to resolve this limitation. In the case of psilocin testing in hair, additional restraints were observed. As the drug is instable in aqueous solution [9], one cannot use buffers or diluted acids or bases to extract it from the solid hair matrix. Therefore, only direct solvent extraction responds to this difficulty. As acetonitrile does not swell the hair and precipitates the protein, this solvent was not used. Methanol with 1 % formic acid appeared to be the best compromise, as acid methanol was recommended since the early 2000 for a great variety of compounds [17] and also retained by Martin et al for psilocin [10]. Due to the lack of reference material, it was not possible to establish the true extraction recovery, as it is a non-sense to spike methanol with psilocin, evaporate the methanol, reconstitute the extract and inject it. Nevertheless, stability verification was achieved by comparing the area of psilocin obtained after injection of 100 pg from the standard solution and 100 pg after following the whole extraction process. A loss of 6-9 % was observed during six independent tests. In addition, protection from light and working at low temperature have been mentioned to obtain best results for psilocin analysis [9]. As a consequence, dark extraction vials covered with aluminum were used to minimize light degradation. Finally, as it was observed during the method development a massive loss of psilocin chromatographic response (more than 80 %) after hair pulverization in a ball mill and ultrasounds incubation, the final extraction protocol banned these two methods producing heat, which are generally presented as the best choice to enhance the extraction rate of a drug from the hair matrix [16, 18]. In our procedure, hair specimens were only cut into very short segments less than 1 mm and incubation achieved by horizontal agitation in a refrigerator at + 4 °C without sonication.

With respect to the interpretation of the data, there is also a limitation due to the lack of suitable literature. Psilocin testing in hair seems undervalued as the drug is generally included in screening procedures for hallucinogens but never identified [11]. For forensic purposes, the authors received a hair strand collected from a possible magic mushrooms consumer. Segmental analysis on 3 x 1 cm demonstrated the presence of psilocin in the range 2.5 to 5.4 pg/mg (Table 1). The chromatogram of the proximal segment (0 to 1 cm) is presented in Figure 1, clearly

demonstrating suitable sensitivity with a good signal to noise ratio even at very low concentration.

The complicated step is the interpretation of these concentrations. Obviously, because all 3 segments tested positive for psilocin at different concentrations, one can interpret these findings as corresponding to repetitive exposures to magic mushrooms. However, given the absence of any controlled study in the literature, one cannot anticipate the frequency and doses of consumption. The authors can accept that some parameters about the detection of psilocin in hair have not been addressed: dose necessary to give a positive result, dose-concentrations relationship, contamination or passive exposure, influence of cosmetics including shampoos during normal hygiene practice, interferences, variable incorporation into hair, external factors that may have an impact, etc. The minimum dose that had to be ingested by a subject in order to get a “positive” detection in one of the hair sections tested remains unknown.

In the scientific literature, only Zhou et al [12] identified psilocin (at 150 and 161 pg/mg) in the hair of two magic mushrooms consumers. Obviously, these concentrations are much higher than those measured in our case. The reasons of such different levels can be explained by several hypotheses: 1. The two subjects have consumed much more mushrooms than our subject; 2. although not indicated, one can anticipate that the two subjects were from China where the population has mostly black hair. It has been accepted that melanin, the hair color pigment, is the place where drug can bound in hair. When exposed to identical conditions, black hair will incorporate much more drug when compared to brown and blond hair [16]. Our subject was light brown in color. Differences of the incorporation rate of zolpidem due to the influence of hair color have been established when hair specimens were collected in France and in China [19]; 3. something happened after drug incorporation (cosmetic treatment, environmental influence) that has caused a decrease in drug concentration [20].

#### **4. Conclusion**

There are several issues that still deserve substantial research and discussion within the scientific community, before a definitive interpretation of either a positive or negative results can be safely given to medical staff in case of addiction survey or to lawyers or judges in case of forensic requests. Specifically, the following scenarios

have to be considered: 1. passive exposure vs. active consumption, 2. mindful versus unaware intake, and 3. sporadic versus repetitive use. On the opposite to the classic drugs of abuse, such as cannabis, cocaine, amphetamines and opiates, whose incorporation, analysis and interpretation in human hair has been extensively published, the analysis for active ingredients of magic mushrooms presents numerous limitations. Therefore, only experienced hair analysts should interpret results from psilocin hair tests, particularly when a negative result is obtained.

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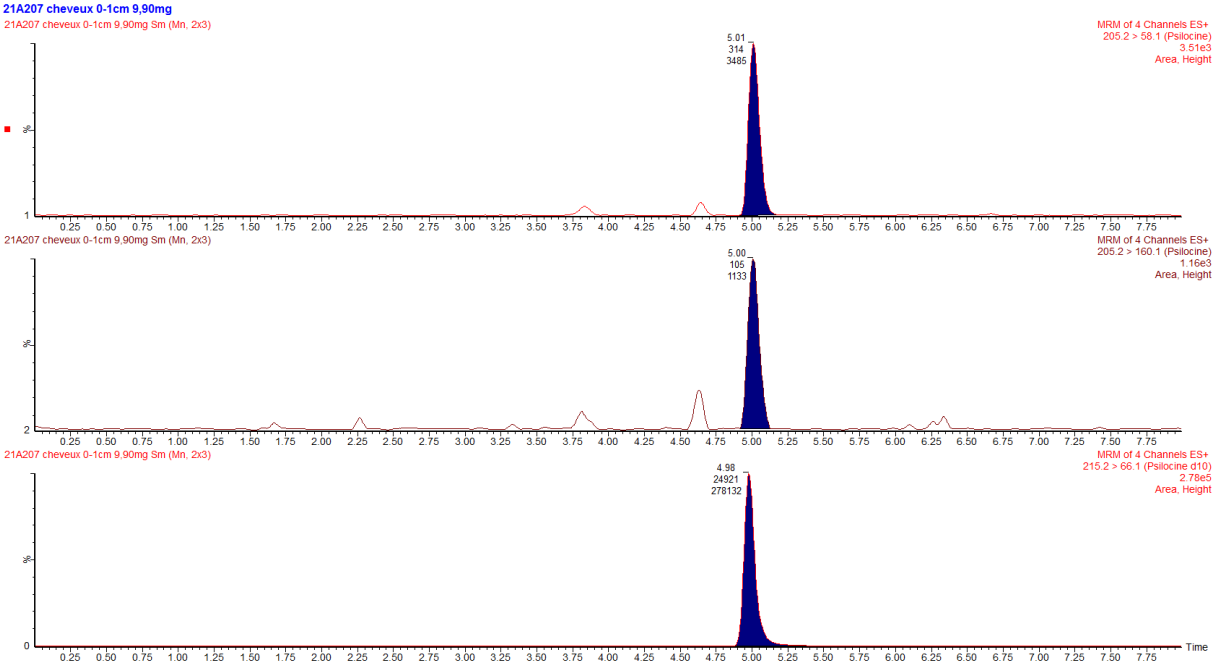
Table 1

Segmental analysis of the hair of a magic mushrooms consumer

<b>Segment</b>	<b>Psilocin concentration</b>
0 to 1 cm	2.5 pg/mg
1 to 2 cm	4.4 pg/mg
2 to 3 cm	5.4 pg/mg

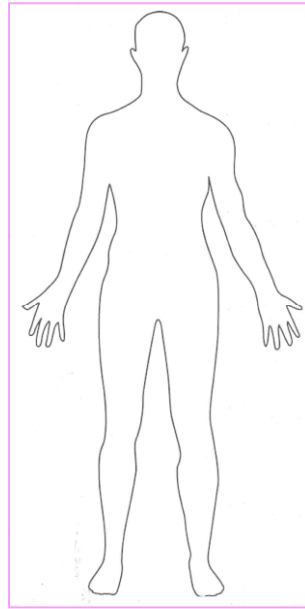
Figure 1

Chromatogram obtained after extraction of the 0 to 1 cm hair segment. From top to bottom: 2 transitions of psilocin and 1 transition of the internal standard. Psilocin concentration was 2.5 pg/mg





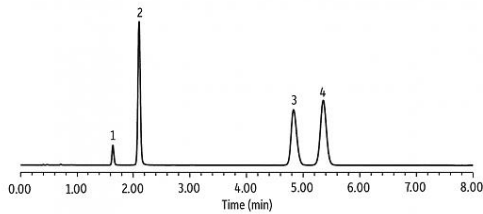
Magic mushrooms abuse



Hair preparation, including wash



Disposition in the body, including hair



Repetitive exposures will produce a chromatographic signal



Chromatographic analysis