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DNA-based taxonomic identification of basidiospores in hallucinogenic mushrooms cultivated in "grow-kits" seized by the police: LC-UV quali-quantitative determination of psilocybin and psilocin

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ABSTRACT

The taxonomic identification of the biological material contained in the hallucinogenic mushrooms culture media, was carried out using a DNA-based approach, thus highlighting the usefulness of this approach in the forensic identification of illegal samples also when they are present as basidiospores mixed in culture media and spore-bearing fruiting body are not present.

This approach is very useful as it allows the unequivocal identification of potentially illicit material before the cultivation and it enables to stop the material to the Customs and to destroy it due to its dangerousness without cultivating the "grow-kits" and without instructing a criminal case.

In fact, even if psilocin and psilocybin and the whole mushrooms are illegal in many countries, there is no specific indication in the law about the so called "grow-kits", containing the spores.

To confirm the data obtained by the taxonomic identification, a simple, reliable, efficient LC-UV method, using tryptamine as internal standard, suitable for the forensic quali-quantitative determination of psilocin and psilocybin in hallucinogenic mushroom was optimized, validated and applied to the mushrooms grown after the cultivation of the grow-kits seized by the judicial authority, with the authorization of the Ministry of Health. A cation exchange column was used in a gradient elution mode (Phase A: 50 mM K_2HPO_4; 100 mM NaCl pH = 3 Phase B: methanol). The developed method was linear over the calibration range with a $R^2 > 0.9992$ for both the analytes. The detection and quantification limits were respectively 0.01 and 0.1 μg/mL for psilocybin and 0.05 μg/mL and 0.1 μg/mL for psilocin and the intra- and inter-day precision was satisfactory (coefficients of variation <2.0% for both the analytes).

The content of psilocybin in the mushrooms grown from the seized "grow-kits" ranged from 1.02 to 7.60 mg/g of dry vegetable material, while the content of psilocin from 0.415 to 8.36 mg/g.

1. Introduction

During the last decade, the recreational use of hallucinogenic mushrooms have become an increasing problem in Europe. The mushrooms are sold via the internet or in so-called "smart shops". They are supplied in a fresh or air-dried state or as powder incapsules for later use [1,2]. Additionally, "grow-kits" can be purchased which consist of inoculated substrate in plastic boxes. Most of the hallucinogenic fungi on the market belong to the *Psilocybe* genus. They mainly contain two hallucinogenic alkaloids, psilocin and its phosphorylated derivative psilocybin [1–9] which are controlled substances in many countries. These alkaloids are present at total concentrations of approximately 1–2% in dried mushrooms and the amount of the phosphorylated compound psilocybin usually exceeds the amount of psilocin [1–9].

Psilocybin (*O*-phosphoryl-4-hydroxy-*N*,*N*-dimethyltryptamine) and its active dephosphorylated metabolite psilocin (*N*,*N*-dimetyltryptamine) structurally belong to the group

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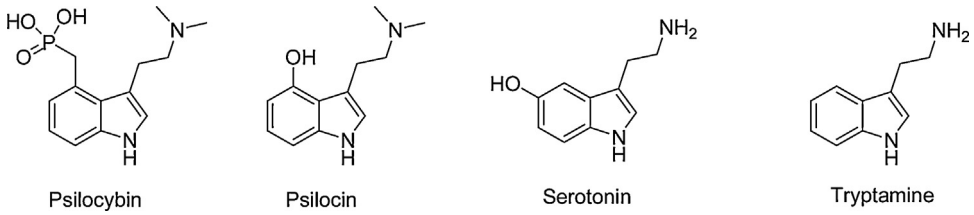

Fig. 1. Chemical structures of psilocybin, psilocin, serotonin and tryptamine.

of tryptamine/indolamine hallucinogens and are structurally related to serotonin [10–18] (Fig. 1).

In this paper we applied the DNA-based approach [19] for the identification of the genus and species to the spores contained in the grow-kits in order to assess the presence of illegal mushrooms before the cultivation. In fact there is a controversial issue: the fungi belonging to the genus *Stropharia*, *Conocybe* and *Psilocybe* and the active principles psilocin and psilocybin are illegal, but the grow-kits containing the spores are not, so that persons trafficking with the grow boxes could not be prosecuted by the law. The DNA analysis on the spores, leads to an identification of the hallucinogenic species *Psilocybe cubensis* therefore, it could be of help for the judicial authority to bring to justice people involved in grow-kits trade. In fact it enables to stop the material to the Customs and to destroy it due to its dangerousness without cultivating the "grow-kits" and without instructing a criminal case.

To confirm the data obtained by the taxonomic identification it is important to confirm psilocin and psilocybin in hallucinogenic mushrooms for forensic pourposes; to this end several techniques have been employed with high-performance liquid chromatography (LC) being by far the most widely used analytical technique. Detection modes include ultraviolet (UV) fluorescence, electrochemical, voltametric detection, and mass spectrometry [18]; in this frame, we decided to apply a simple, reliable, efficient LC-UV method, using tryptamine as internal standard (IS) to the analysis of hallucinogenic mushroom grown after the cultivation, carried out after DNA analysis, of grow-kits seized by the judicial authority, with the authorization of the Ministry of Health.

2. Materials and methods

2.1. Seized material

12 "Grow kits", containing spores of different species of the genus *Psilocybe,* were seized and delivered to our laboratory by the judicial authority in February 2015. The grow boxes were seized at the Malpensa airport in Northern Italy and were composed of a bag with air filter and plastic tray filled with three layers of different materials: Growth substrate, containing the spores was withdrawn and DNA extraction and analysis was carried out.

2.2. DNA extraction from growth substrate and fungi

Hundred mg of growth substrate including spores or 100 mg of fungi after 20 days of incubation at 25 °C were processed for DNA extraction by means of UltraCleanTM Microbial DNA Isolation Kit (Mo Bio, Canada), according to manufacturer's instructions. After DNA quantification with a Smart SpecTM Plus Spectrophotometer (Bio-Rad Laboratories, Milan, Italy) and evaluation of 260/280 ratio, 50 ng of DNA were used for species identification based on the D1/D2 domain at the 5′ end of the large subunit (28S) rDNA, as previously well acknowledged for its ability to identify yeast to the species level [20]. The amplification was performed in a final volume of 25 μL, containing 0.5 μM of each primer (NL1: GCATATCAATAAGCGGAGGAAAAG; and NL4: GGTCCGTGTTTCAAGACGG) [19], 1 unit of DreamTaq (Fermentas, Thermo Scientific, Italy) and 50–70 ng of DNA. The reactions were performed in an automatic thermal cycler (Eppendorf Mastercycler, Italy) under the following conditions: initial denaturation at 94° C for 3 min; 36 cycles of 94° C for 2 min, 52 °C for 1 min, 72° C for 2 min; final exten-

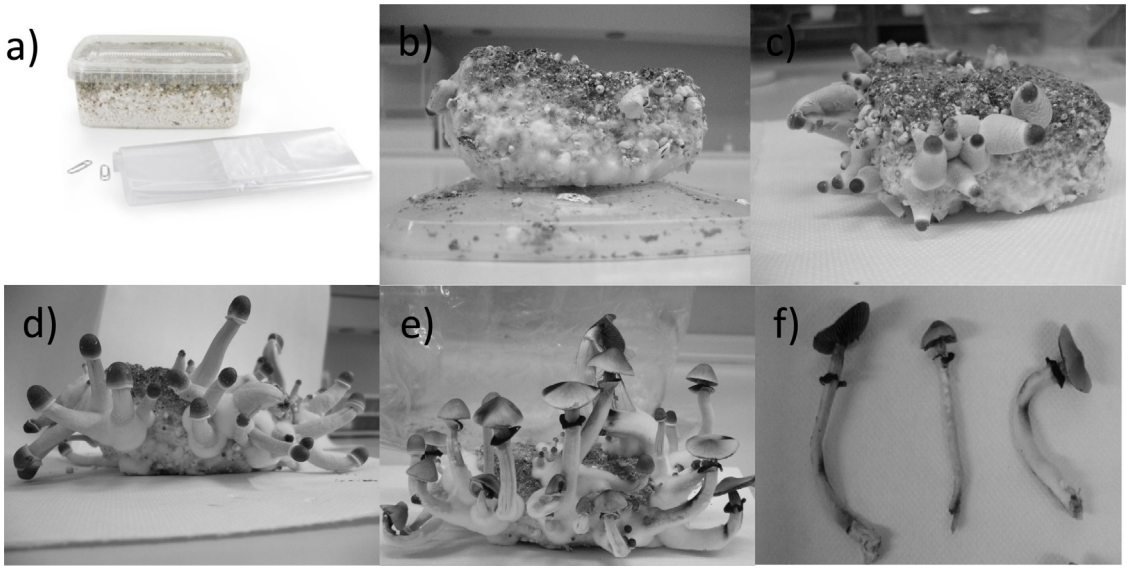

Fig. 2. (a) Grow box; (b) first day; (c) third day; (d) fifth day; (e) seventh day; (f) harvested mushrooms.

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Table 1
Accuracy.

Psilocybin		
Theoretical Concentration (μg/mL)	Experimental Concentration (μg/mL)	% E
4	4.009	0.23
4	4.013	0.34
4	4.014	0.35
8	7.902	−1.22
8	7.806	−2.41
8	7.806	−2.42
12	12.118	0,99
12	12.114	0.95
12	12.110	0.92
24	24.155	0.65
24	24.166	0.69
24	24.170	0.71
32	32.399	1.25
32	32.354	1.11
32	32.353	1.11
48	49.032	2.15
48	49.066	2.22
48	49.086	2.26
Psilocin		
2	2.029	1.49
2	2.023	1.18
2	2.031	1.57
3	2.967	−1.10
3	2.943	−1.90
3	2.942	−1.91
5	4.899	−2.01
5	4.926	−1.47
5	4.927	−1.44

Table 2
Precision. In bold the solution analyzed in different days for intra-day precision.

Psilocybin				
Day	Theoretical Concentration (μg/mL)	Experimental Concentration (μg/mL)	SD	%CV
1	**8**	**8.014**	0.1436	1.76
1	8	8.019		
1	8	8.013		
1	8	8.013		
1	8	8.127		
1	8	8.020		
1	8	8.227		
2	8	8.319		
3	8	8.313		
4	8	8.306		
5	8	8.316		
6	8	8.312		
1	**32**	**32.033**	0.3081	0.95
1	32	32.054		
1	32	32.058		
1	32	32.044		
1	32	32.082		
1	32	32.086		
1	32	32.541		
2	32	32.643		
3	32	32.651		
4	32	32.687		
5	32	32.658		
6	32	32.687		
Psilocin				
1	**3**	**2.993**	0.0547	1.87
1	3	2.972		
1	3	2.973		
1	3	2.968		
1	3	2.976		
1	3	2.972		
1	3	2.892		
2	3	2.866		
3	3	2.877		
4	3	2.868		
5	3	2.867		
6	3	2.867		

sion at 72° C for 7 min, holding at 4° C. The amplified products were then purified by means of UltraCleanTM PCR Clean-up DNA purification kit (Mo Bio, Canada) and finally sequenced by using primer NL1. The sequences were analyzed by use of the software Chromas 2.33 (Technelysium Pty Ltd., South Brisbane, Australia) and compared to the sequences reported in the GenBank using the BLAST algorithm.

Total DNA, directly extracted from culture medium suspected to contain spores of hallucinogenic fungi, was used in a PCR assay in order to amplify the internal transcribed region encompassing the *18S*- and the *28SrDNA* genes, and the PCR fragment obtained was subjected to sequence analysis. From all the four samples tested, sequence analysis and comparison allowed to unambiguously identify the biological materials present in the culture medium as belonging to the hallucinogenic species *P. cubensis* (Accession number LN830950).

2.3. Mushroom cultivation

After the DNA analysis to determine the genus and species of the spores, the cultivation of the hallucinogenic mushrooms was carried out, from grow boxes containing spores of the genus *Psilocybe*. The "grow kits" were composed of three layers of different materials: a perlite layer, which serves as a reservoir of water maintaining a high level of humidity in the box; a layer inoculated with mycelium and a thin vermiculite layer protecting the mycelium from dehydration. The cover was removed and the box was completely filled with warm water. The box was recovered and allowed to stand for 12 h. The tray was opened, the excess of water was removed and the box was inserted in the polyethylene bag, folding the top edge and securing it with staples. The kit was maintained at a temperature of 22–24 °C in a lighted place. After 20 days the first mushrooms began to sprout from the growth medium. In Fig. 2 the grow kits and the development of the mushrooms are depicted starting from the first day when we noticed the first little fungi.

2.4. LC-UV analysis

2.4.1. Reagents and standards

Psilocybin 1.0 mL (100 μg/mL) in methnol was purchased by Grace (Illinois, USA); Psilocin 99.3%, 1.0 mL (5 mg/mL) in methanol was from Cerilliant, (Texas, USA); tryptamine, luzindole and serotonin and all the other reagents and solvents of analytical grade were supplied by Sigma Aldrich (St. Louis USA) and were stored as required by the manufacturer. Water (18.2 MΩ cm^{-1}) was prepared by a Milly-Q System (Millipore, France). Tryptamine solution of the internal standard (IS) was prepared in methanol (100 μg/mL).

2.4.2. Extraction of the active principles

Each mushroom was divided into cap and stem. From each part 100 mg were withdrawn and suspended in 500 μL of methanol. 500 μL of IS (10 μg/mL) were added, the mixture was shaken on a rotary shaker for 45 min and macerated overnight. The mixture was centrifuged (4000 rpm) for 10 min and the supernatant filtered on a 0.45 μm membrane. Each extract was analyzed in triplicate.

2.4.3. Apparatus

Experiments were performed on a 1200 Series Liquid Chromatography System equipped with two chromatographic pumps (G1310A) and an auto sampler (G13229A), coupled to a UV detec-

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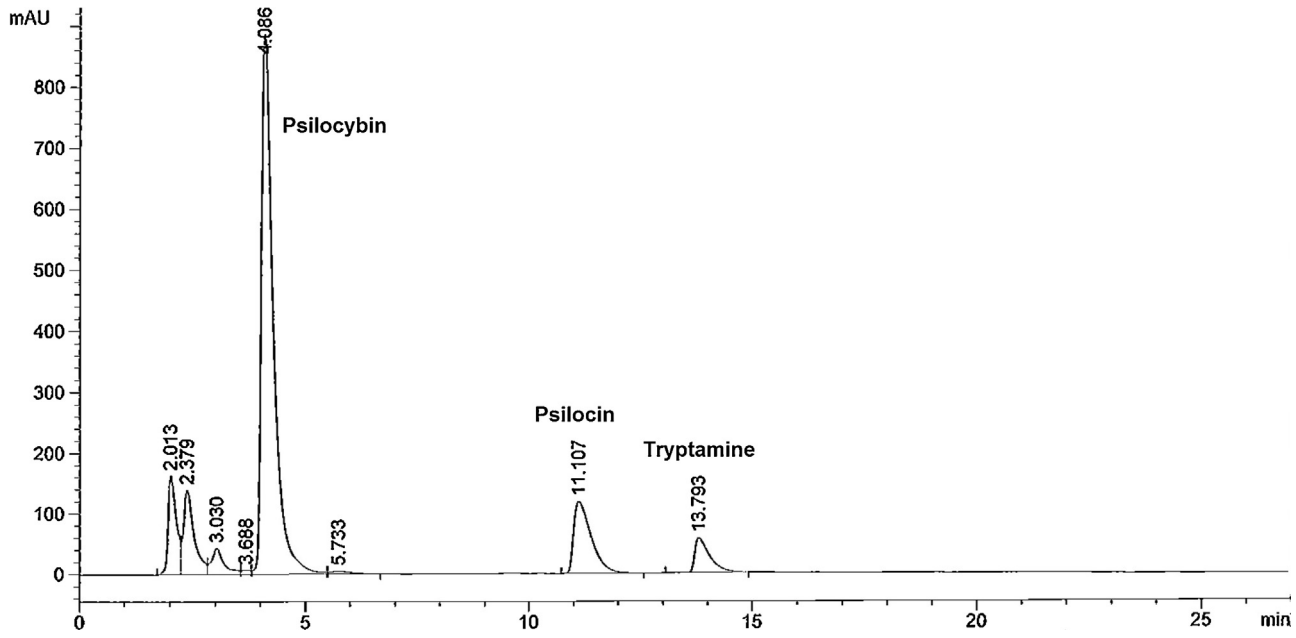

Fig. 3. Chromatogram of a mushroom sample; psilocybin 4.085 min; psilocin 11.107 min and tryptamine (IS) 13.793 min.

Table 3
Content of the active principles.

	Mushroom	Psilocin			Psilocybin		
		Fresh		Dry	Fresh		Dry
		Concentration (μg/mL)	Concentration (mg/g)	Concentration (mg/g)	Concentration (μg/mL)	Concentration (mg/g)	Concentration (mg/g)
Cap	1	0.974	0.047	0.415	17.85	0.85	7.599
	2	19.391	0.939	8.358	2.37	0.11	1.023
	3	1.536	0.074	0.655	11.83	0.57	5.045
Stem	1	1.996	0.097	0.866	11.90	0.58	5.157
	2	3.885	0.184	1.640	5.06	0.24	2.137
	3	4.081	0.199	1.775	8.32	0.41	3.616

tor (G1365D). The system was managed by Chromstation software (Agilent Technologies, Santa Clara, CA, US).

2.4.4. Chromatographic conditions

Chromatographic column: Supelcosil TM LC-SCX, particle size 5 μm, 25 cm × 3,0 mm i.d., no cat. 58997C30 (SupelcoTM Analytical, Bellefonte, Pennsylvania, USA). Pre-column: Security Guard Cartridges C18 4 × 2.0 mm (Phenomenex™, Castel Maggiore, Italy); column temperature: 25 °C; flow rate: 0.7 mL/min; wavelength: 220 nm; injection volume: 20 μL; injection mode: partial loop fill (volume of the loop 100 μL);

Solvent A: NaCl 100 mM and KH_2PO_4 50 mM adjusted to pH 3 with 85% H_3PO_4; Solvent B: methanol; mobile phase: solvents were filtered under vacuum on 0.45 μm membrane filters and degassed by immersion in ultrasonic bath for 15 min before column conditioning; linear gradients: 0.0–6.0 min, 3% B; 6.0–10.0 min, 3–30% B; 10.0–18.0 min 30% B; 18.0–22.0 min 30–3% B; 22.0–27.0 min 3% B. Retention times: psilocybin 4.36 min; psilocin 11.09 min; tryptamine: 13.71 min.

2.5. Validation of the LC method

2.5.1. Specificity and choice of the internal standard

The LC method was validated for the identification and quantification of the analytes meeting the requirements of forensic analysis. In a previous work, we used 5-hydroxy-*N*,*N*-diethyltryptamine, as internal standard [19]. Unfortunately, in the case of UV detection this molecule had not the suitable chromatographic features (large and tailed peak). So other compounds were investigated such as luzindole, serotonin and tryptamine, which showed the best characteristics as IS. Tryptamine is a biosynthetic precursor of psilocin and psilocybin, but it is mainly concentrated in the mycelium. In the grown mushroom there is not an appreciable concentration of this analyte, so that endogenous tryptamine does not interfere with its use as IS.

2.5.2. Linearity

Due to the higher concentration in the vegetable material linearity of psilocybin was evaluated in two different concentration ranges: between 1 μg/mL and 16 μg/mL and between 16 and 64 μg/mL; in the case of psilocin linearity was assessed between 0.5 μg/mL and 20 μg/mL. In these ranges different non sequential concentrations were analyzed in triplicate: 1, 2, 4, 8, 12, 16 μg/mL and 16, 24, 32, 48, 64 μg/mL for psilocybin and 0.5, 1, 2, 3, 5, 10, 20 μg/mL for psilocin. All these standard solutions contained the IS at a concentration of 10 μg/mL. The linearity equations, obtained plotting the ratio between the area of the analyte and the area of the IS against concentrations were $y = 0.1652x - 0.0501$ ($R^2 = 0.9995$) and $y = 0.1640x - 0.1986$ ($R^2 = 0.9992$) for the two ranges of psilocybin and $y = 0.0705x - 0.0145$ ($R^2 = 1.0000$) for psilocin.

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2.5.3. Limit of quantification (LOQ) and limit of detection (LOD)

The LOQ was determined as the lowest concentration having a signal to noise ratio of at least 10. The LOQ was 0.1 μg/mL for both the analytes.

The LOD was estimated as three times the signal to noise ratio. The LOD was determined by progressively diluting the solution prepared for the determination of the LOQ and resulted to be 0.01 μg/mL for psilocybin and 0.05 μg/mL for psilocin

2.5.4. Accuracy

Accuracy was evaluated calculating the percentage error between the experimental and the theoretical concentrations at six different levels (4, 8, 12, 24, 32 and 48 μg/mL) for psilocybin and at three different levels (2, 3 and 5 μg/mL) for psilocin. Each concentration was analyzed in triplicate.

$$\%E = (\text{experimental concentration} - \text{theoretical concentration}) / \text{theoretical concentration} \times 100$$

Experimental concentration was obtained by interpolation of the linearity equations.

2.5.5. Precision

Intra-day precision was evaluated analyzing on the same day six different standard solutions at a concentration of 8 and 32 μg/mL for psilocybin and 3 μg/mL for psilocin and calculating standard variation (SD) and percentage coeffiecient of variation (% CV).

Inter-day precision was assessed analyzing one solution for each concentration in six different days (Table 2).

3. Results and discussion

In the case of the mushrooms suspected to contain hallucinogenic active principles, it is very useful to assess the genus and species. Sometimes samples seized by the police are dried or powdered and the morphological characteristics are not recognizable. Therefore, an unambiguous identification by a phylogenetic approach could be very helpful, especially in the cases in which active principles could be degraded due to an incorrect storage of the vegetable material. Moreover, the DNA-based identification of mushrooms starting from spores is particularly important because it allows to establish the presence of illegal biological material, before the development of the spore-bearing fruiting body. This approach could be of help for the judicial authority when dealing with this kinds of grow-boxes, that are not considered illicit because they do not contain "yet" the illegal active principles. In fact it enables to stop the material to the Customs and to destroy it due to its dangerousness without cultivating the "grow-kits" and without instructing a criminal case.

After the identification by a phylogenetic approach, it is also mandatory to determine the presence of the hallucinogenic active principles, in the grown mushrooms. To this end we optimized a LC-UV method useful for the forensic determination of these compounds.

Once established the suitable IS for the analysis of the active principles contained in the hallucinogenic mushrooms, the analytical conditions were carefully studied and optimized and the resulting analytical method was validated, resulting adequate for the levels of psilocin and psilocybin usually found in the mushrooms [5–8].

The analytical method was endowed with a good level of accuracy, evaluated calculating the percentage error between the experimental and the theoretical concentrations, in fact the%E values were included between −2.5% and +2.0% (Table 1). Moreover the low%CV values demonstrated that the analytical method showed a good precision (Table 2).

Three mushrooms were harvested seven days after the appearance of the first little fungi, when the development was complete. The three mushrooms were divided into cap and stem and the active principles extracted as described in the Materials and methods section. A typical chromatogram obtained from a mushroom sample is reported in Fig. 3.

The quantitative analysis gave the results reported in Table 3. The content of psilocybin ranged from 1.02 to 7.60 mg/g of dry vegetable material, while the content of psilocin from 0.415 to 8.36 mg/g, in accordance with literature data [5–8]. These analyses confirmed the identification of the hallucinogenic species *P. cubensis* carried out by means of the DNA-based approach.

4. Conclusions

In this paper we describe a DNA-based approach for the identification of the genus and species of the spores contained in the grow-kits seized on the illegal market. In this way, the presence of illegal mushrooms can be established before the cultivation, thus allowing the judicial authority to bring to justice people involved in grow-kits trade, even if the grow kits are not illegal. In this way it is possible to stop the material to the Customs and to destroy it, due to its dangerousness, without cultivating the "grow-kits" and without instructing a criminal case. Moreover, a simple, reliable, efficient LC-UV method, suitable for the forensic confirmation of psilocin and psilocybin in hallucinogenic mushroom grown after the cultivation of grow-kits is described.

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