Supporting information:

Stability of psilocybin and its four analogs in the biomass of the psychotropic mushroom *Psilocybe cubensis*

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Table of contents

[2 Materials and methods 2](#_Toc52869765)

[2.1 General experimental methods of the synthesis and characterization of mushroom tryptamines 2](#_Toc52869766)

[2.2 Cultivation of mushrooms and their drying and storage 5](#_Toc52869767)

[2.3 Blank matrices 6](#_Toc52869768)

[2.4 Preparation of samples and their extracts 6](#_Toc52869769)

[2.5 Instrumentation and conditions of UHPLC-MS/MS 7](#_Toc52869770)

[2.6 Method validation 8](#_Toc52869771)

[2.6.1 Specificity (selectivity) 8](#_Toc52869772)

[2.6.2 LOD, LOQ, and linearity 8](#_Toc52869773)

[2.6.3 Precision and accuracy 8](#_Toc52869774)

[2.6.4 Extraction recovery and matrix effect 8](#_Toc52869775)

[2.6.5 Stability 8](#_Toc52869776)

[2.7 Optimisation of the extraction procedure 8](#_Toc52869777)

[2.7.1 Effect of homogenization 8](#_Toc52869778)

[2.7.2 Extraction solvents 9](#_Toc52869779)

[2.8 Effect of temperature change 9](#_Toc52869780)

[3 Results and discussion 9](#_Toc52869781)

[3.1 Cultivation of mushrooms 9](#_Toc52869782)

[3.2 Instrumentation and validation of the results 9](#_Toc52869783)

[3.2.1 LOD, LOQ, and linearity 9](#_Toc52869784)

[3.2.2 Precision and accuracy 9](#_Toc52869785)

[3.2.3 Extraction recovery and matrix effect 10](#_Toc52869786)

[3.2.4 Stability 10](#_Toc52869787)

[3.5 Stability of tryptamines in dried fungal powder 11](#_Toc52869788)

# 2 Materials and methods

## 2.1 General experimental methods of the synthesis and characterization of mushroom tryptamines

Baeocystin and norbaeocystin were prepared by a modified literature method for synthesis of psilocybin.1 Used synthetic way is essentially the same as in a previous study.2 Although there is a published method for aeruginascin synthesis from baeocystin.2-3 Aeruginascin for this study was prepared according to the recent literature method from psilocybin.4 Advantages of this method are simple isolation and purification and higher yield of aeruginascin. Product yields were similar2 or better than previous studies.4 The structure of analyzed mushroom tryptamines is shown in Figure S 1. Reactions were performed using commercially obtained solvents. Unless otherwise stated, all commercially obtained reagents were used as received. Reactions were monitored by thin-layer chromatography (TLC) using EMD/Merck silica gel 60 F254-precoated plates (0.25 mm). Flash column chromatography was performed using pre-packaged RediSepRf columns on a CombiFlash Rf system (Teledyne ISCO Inc.). 1H and 13C NMR spectra were recorded on a Varian 300 (at 300 and 75 MHz, respectively) and are reported relative to internal CHCl3 (1H, δ = 7.26) and CDCl3 (13C, δ = 77.0). High-resolution mass spectra were acquired on an Agilent 6550 Q-TOF in ESI-positive mode.



Figure S 1: Schema of the synthesized mushroom tryptamines

**3-(2-Chloro-2-oxoacetyl)-1*H*-indol-4-yl acetate** (**1)** To a solution of 4-acetoxyindole (5.00 g, 28.6 mmol) in anhydrous diethyl ether (30 mL) with stirring in an ice bath was added oxalyl chloride (5.30 g, 3.7 mL, 41.8 mmol). After the addition was complete, the bright yellow suspension was stirred for 1 hour at 0°C. Hexane (60 mL) was added to this suspension, and the reaction flask was placed in a freezer for 3 hours. The resulting yellow crystal was collected from the solution by suction filtration, washed with diethylether/hexane mixture (1:2, 15 mL), and dried in vacuum. The collected yellow crystalline solid (7.17 g, 95 %) was used immediately without further characterization.

**3-(2-(Benzyl(methyl)amino)-2-oxoacetyl)-1*H*-indol-4-yl acetate (2)** To a solution of **1** (3.58 g, 13.5 mmol) in dry THF (15 mL) was added solution of *N*-methylbenzylamine (2.06 g, 17.0 mmol) and triethylamine (2.4 mL, 17.1 mmol) in THF (10 mL) with stirring at 0°C. The reaction was stirred at ambient temperature for 2 h. After evaporation of THF the residue was taken up in dichloromethane (40 mL) and washed with 1 M HCl (2 x 20 mL) and water (20 mL). The organic phase was dried over anhydrous Na2SO4, the dichloromethane was evaporated, and the crude product was purified by flash chromatography (silica, DCM + MeOH 0 – 10 %). The product **2** was acquired as a white solid (4.30 g, 91 %), mp. 143-146°C (lit. 145-148 °C)4; 1H NMR\*(CDCl3, 300 MHz) *δ* 10.40 (1H, br s), 7.59 (1H, d, *J* = 3.5 Hz), 7.45−7.36 (5H, m), 7.15 (1H, t, *J* = 7.9 Hz), 7.08 (1H, d, *J* = 7.9 Hz), 6.94 (1H, d, *J* = 7.9 Hz), 4.65 (2H, s), 2.89 (3H, s), 2.51 (3H, s); 13C NMR\*(CDCl3, 75 MHz) *δ* 185.1, 171.0, 168.5, 144.3, 139.2, 138.1, 136.0, 128.9, 128.1, 127.8, 124.8, 118.3, 116.0, 113.5, 110.8, 50.0, 35.1, 21.6 (major rotamer); HRMS (ESI) calcd for C20H19N2O4+ 351.1340 [M + H]+,found 351.1344. \*Note: NMR spectra indicate a∼1:1.2 mix of rotamers, signals are reported for the major rotamer. All analytical data are in agreement with the literature.4

**3-(2-(Dibenzylamino)-2-oxoacetyl)-1*H*-indol-4-yl acetate (3)** Amide **3** was synthetized from chloride **1** (3.58 g, 13.5 mmol), dibenzylamine (3.40 g, 17.0 mmol) and triethylamine (2.4 mL, 17.1 mmol) analogously as amide **2**. The product **3** was acquired as a white solid (4.89 g, 85 %), mp. 140-142°C (lit. 139-141 °C)5; 1H NMR (CDCl3, 300 MHz) *δ* 10.21 (1H, d, *J* = 10.6 Hz), 7.67 (1H, d, *J* = 3.3 Hz), 7.45−7.30 (10H, m), 7.11 (1H, dd, *J* = 7.6, 8.2 Hz), 7.07 (1H, d, *J* = 8.2 Hz), 6.90 (1H, d, *J* = 7.6 Hz), 4.61 (2H, s), 4.37 (2H, s), 2.47 (3H, s); 13C NMR (CDCl3, 75 MHz) *δ* 185.2, 171.0, 168.7, 144.2, 139.1, 137.9, 136.1, 135.3, 128.9, 128.7, 128.3, 128.3, 128.0, 127.8, 124.8, 118.2, 116.0, 113.9, 110.8, 50.5, 46.2, 21.4; HRMS (ESI) calcd for C26H23N2O4+ 427.1653 [M + H]+, found 427.1669. NMR spectra are in agreement with the literature.4

**3-(2-(Benzyl(methyl)amino)ethyl)-1*H*-indol-4-ol (4)** To a suspension of LAH (1.41 g, 37.2 mmol) in anhydrous THF (35 mL) under an argon atmosphere has dropwise added a solution of amide **2** (3.30 g, 9.4 mmol) in anhydrous THF (30 mL) over 1 h, and then the reaction mixture was refluxed for 19 h. The reaction was cooled to 5°C, and Na2SO4·10 H2O (5 g) was added in portions. The suspension was left to stir for 1 h at room temperature. The reaction was then diluted with THF (60 mL) and filtered through a Celite pad by suction. The pad was washed with THF (50 mL). The organic solution was quickly concentrated in vacuo. The oily residue solidified after drying in a high vacuum for 2 days. The product **4** was acquired as a light brown solid (1.95 g, 74 %),: mp 49−52°C (lit. 51-53 °C)4; 1H NMR (CDCl3, 300 MHz) *δ* 7.90 (1H, br s), 7.32−7.22 (5H, m), 7.08 (1H, dd, *J* = 7.6, 8.2 Hz), 6.87 (1H, d, *J* = 8.2 Hz), 6.78 (1H, s), 6.63 (1H, d, *J* = 7.6 Hz), 3.65 (2H, s), 2.94 (2H, m), 2.76 (2H, m), 2.35 (3H, s); 13C NMR (CDCl3, 75 MHz) *δ* 151.7, 139.0, 135.4, 130.3, 128.4,127.8, 123.4, 121.0, 117.9, 114.3, 106.5, 102.9, 62.8, 59.2,42.2, 25.0; HRMS (ESI) calc for C18H21N2O+ 281.1649 [M + H]+, found 281.1661. All analytical data are in agreement with the literature.4

**3-(2-(Dibenzylamino)ethyl)-1*H*-indol-4-ol (5)** To a suspension of LAH (1.35 g, 35.6 mmol) in anhydrous THF (26 mL) under an argon atmosphere has dropwise added a solution of amide **3** (3.00 g, 7.0 mmol) in anhydrous THF (25 mL) over 1 h, and then the reaction mixture was refluxed for 19 h. The reaction was cooled to 5°C, and Na2SO4·10H2O (5 g) was added in portions. The suspension was left to stir for 1 h at room temperature. The reaction was then diluted with THF (50 mL) and filtered through a Celite pad by suction. The pad was washed with THF (40 mL). The organic solution was quickly concentrated in vacuo. The oily residue solidified after drying in a high vacuum for 2 days. The product **5** was acquired as a light brown solid (1.88 g, 75 %): mp 53−56°C (lit. 52-55 °C)4; 1H NMR (CDCl3, 300 MHz) *δ* 7.82 (1H, br s), 7.35−7.20 (10H, m), 7.08 (1H, t, *J* = 7.9 Hz), 6.86 (1H, d, *J* = 7.9 Hz), 6.72 (1H, s), 6.67 (1H, d, *J* = 7.6 Hz), 3.75 (4H, s), 2.83 (4H, m); 13C NMR (CDCl3, 75 MHz) *δ* 151.4, 138.7, 135.8, 130.4, 128.3, 127.5, 123.4, 120.6, 117.9, 114.5, 106.5, 103.0, 59.3, 55.0, 25.2; HRMS (ESI) calc for C24H25N2O+ 357.1962 [M + H]+, found 357.1971. All analytical data are in agreement with the literature.4

**3-(2-(Methylamino)ethyl)-1*H*-indol-4-ol (6, Norpsilocin)**. Intermediate **4** (150 mg, 5.35 mmol) was dissolved in MeOH (20 mL). Then 10% Pd/C was added (50 mg), and the flask was sealed, then purged with hydrogen gas via a balloon. After stirring for 8 h the reaction was determined to be complete by TLC. The catalyst was filtered on a pad of Celite and washed with MeOH (50 mL). The blue-gray filtrate was concentrated, then purified by flash chromatography with 90:10:1 CHCl3/MeOH/NH4OH to provide the target compound as a off-white solid (0.83 g, 80 %): mp 185−187°C (lit. 184-186 °C)4; 1H NMR (CDCl3, 300 MHz) *δ* 7.95 (1H, s), 7.08 (1H, t, J = 7.9 Hz), 6.93−6.84 (2H, m), 6.60 (1H, d, J = 7.6 Hz), 3.02 (2H, dt, J = 5.2, 3.1 Hz), 2.99−2.92 (2H, m), 2.50 (3H, s); 13C NMR (CDCl3, 75 MHz) *δ* 151.9, 138.9, 123.5, 121.0, 118.2, 113.9, 106.9, 102.8, 52.8, 35.9, 26.8; HRMS (ESI) calcd for C11H16N2O+ 191.1179 [M + H]+, found 191.1185. All analytical data are in agreement with the literature.4

**Dibenzyl[2-(4-oxyindol-3-yl)ethyl]methylammonio-4-*O*-benzyl phosphate (7)** To a solution of **4** (1.00 g, 3.6 mmol) in anhydrous tetrahydrofuran (40 mL) at -78°C was added 2.5 M *n*-butyllithium in hexanes (1.49 mL, 3.71 mmol) with stirring. After stirring for 5 min, tetrabenzylpyrophosphate (2.08 g, 3.85 mmol) was added all at once to the mixture. Stirring was continued for 1 h while the temperature was allowed to warm to 0°C. Stirring was continued for 2 h at 0°C and then aminopropyl silica gel (ca. 2 g) was added to the reaction mixture, and then the mixture was diluted with ethyl acetate and filtered through a Celite pad by suction. The filtrate was concentrated in vacuo, redissolved in dichloromethane, and let sit for 1 week. The precipitated white substance was collected by filtration and washed with dichloromethane to obtain **7** (1.64 g, 85 %) as a white powder: mp 215−217°C; 1H NMR (CD3OD, 300 MHz) *δ* 7.57−7.42 (10H, m), 7.29−7.18 (5H, m), 7.12−7.08 (3H, m), 7.00 (1H, t, *J* = 7.6 Hz), 4.93 (2H, d, *J* = 6.2 Hz), 4.79 (2H, d, *J* = 12.9 Hz), 4.53 (2H, d, *J* = 12.9 Hz), 3.56 (4H, m), 2.96 (3H, s); 13C NMR (CD3OD, 75 MHz) *δ* 147.5, 141.1, 139.0, 134.2, 131.8, 130.2, 129.3, 129.1, 128.9, 128.7, 124.4, 123.3, 120.2, 110.1, 109.0, 108.2, 71.6, 69.3, 69.2, 51.7, 21.8; HRMS (ESI) calc for C32H33N2O4P+ 541.6072 [M + H]+, found 541.6080.

**Dibenzyl (3-(2-(dibenzylamino)ethyl)-1*H*-indol-4-yl) phosphate (8)**. To a solution of **5** (1.69 g, 3.96 mmol) in anhydrous tetrahydrofuran (50 mL) at -78 °C was added 2.5 M *n*-butyllithium in hexanes (1.65 mL, 4.12 mmol) with stirring. After stirring for 5 min, tetrabenzylpyrophosphate (2.30 g, 4.28 mmol) was added all at once to the mixture. Stirring was continued for 1 h while the temperature was allowed to warm to 0 °C. Stirring was continued for 2 h at 0 °C and then aminopropyl silica gel (ca. 3 g) was added to the reaction mixture, and then the mixture was diluted with ethyl acetate and filtered through a Celite pad by suction. The filtrate was concentrated in vacuo, redissolved in dichloromethane, and let sit for 2 weeks. No zwitterionic product was precipitated, so the crude product **8** (2.32 g, 95 %) which was acquired as an amber amorphous solid was used immediately without further characterization.

**3-(2-(Methylamino)ethyl)-1*H*-indol-4-yl dihydrogen phosphate (9, Baeocystin)**. Intermediate **7** (1.00 g, 1.85 mmol) was dissolved in MeOH (40 mL) and water (10 mL) and 10% Pd/C was added (100 mg). The flask was evacuated and then refilled with hydrogen gas five times via a balloon. After stirring for 5 h, the reaction was filtered on a pad of Celite. The filter cake was washed with additional water (10 mL). The filtrate was concentrated under reduced pressure to about 5 mL. Subsequently, 1 M NaOH was added dropwise cautiously to bring the pH to 4−5. After a short time, baeocystin was spontaneously crystallized out of the solution. Acetone (20 mL) was added in support of crystallization, and the resulting precipitate was collected by filtration to provide the target compound as a fine white solid (0.43 g, 85 %): mp 238−240°C (lit. 242-244 °C)4; 1H NMR (D2O, 300 MHz) *δ* 7.23 (1H, d, *J* = 8.2 Hz), 7.15 (1H, s), 7.13 (1H, dd, *J* = 7.6, 8.2 Hz), 6.99 (1H, d, *J*= 7.6 Hz), 3.31 (2H, m), 3.23 (2H, m), 2.65 (3H, s); 13C NMR (D2O, 75 MHz) *δ* 145.8, 138.6, 124.2, 122.4, 118.5, 108.9, 108.4, 107.7, 50.5, 32.7, 22.9; HRMS (ESI) calc for C11H16N2O4P+ 271.0842 [M + H]+, found 271.0846. All analytical data are in agreement with the literature.4

**3-(2-Aminoethyl)-1*H*-indol-4-yl dihydrogen phosphate (10, Norbaeocystin)**. Intermediate **8** (2.00 g, 3.24 mmol) was dissolved in MeOH (150 mL) and water (50 mL) and 10% Pd/C was added (300 mg). The flask was evacuated and then refilled with hydrogen gas five times via a balloon. After stirring for 24 h the reaction was filtered through a pad of Celite. The filter cake was washed with additional water (30 mL). The filtrate was concentrated under reduced pressure to dryness. The resulting tan solid was purified by flash chromatography (C-18, 5% MeOH/H2O → 100%MeOH). The collected fractions were concentrated to approximately 8−10 mL and the pH was adjusted to 4−5 by the dropwise addition of 1 M NaOH. After a short time, norbaeocystin was spontaneously crystallized out of solution. Acetone (40 mL) was added to this suspension, and the resulting precipitate was collected by filtration to provide compound **10** as a fine white solid (0.65 g, 78 %): mp. 211−214°C (lit. 210-212 °C)4; 1H NMR (D2O, 300 MHz) *δ* 7.24 (1H, d, *J* = 8.2 Hz), 7.18 (1H, s), 7.14 (1H, dd, *J* = 7.6, 8.2 Hz), 7.01 (1H, d, *J*= 7.6 Hz), 3.31 (2H, m), 3.25 (2H, m); 13C NMR (D2O, 75 MHz) *δ* 146.7, 138.5, 123.8, 122.6, 118.6, 109.00, 108.7, 107.00, 41.0, 23.8; HRMS (ESI) calc for C10H14N2O4P+ 257.0686 [M + H]+, found 257.0691. All analytical data are in agreement with the literature.4

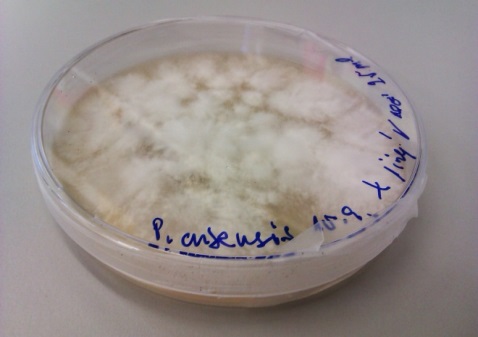
**3-(2-(Trimethylammonio)ethyl)-1*H*-indol-4-yl hydrogen phosphate (11, Aeruginascin).** Psilocybin (100 mg, 0.35 mmol) was suspended in MeOH (1 mL); then concentrated NH4OH (approximately 4 drops) was added dropwise until solids dissolved and the pH of the solution was 9−10. Methyl iodide (0.3 mL, 4.8 mmol) was added, and the reaction was protected from light and stirred overnight. A white precipitate formed throughout the reaction. The precipitated solid was collected by filtration and washed sparingly with cold methanol. The collected solid was slurried in MeOH (1 mL) containing 1 drop of ammonium hydroxide. The resulting crystalline solid was collected by filtration to provide aeruginascin (74 mg, 69 %): mp 174−177°C (lit. 176−178 °C)4; 1H NMR (D2O + 4 % HCO2H, 300 MHz) *δ* 7.22 (1H, d, *J* = 8.2 Hz), 7.13 (1H, dd, *J* = 8.2, 7.6 Hz), 7.12 (1H, s), 7.03 (1H, d, *J* = 7.6 Hz) 3.52−3.46 (2H, m), 3.31−3.26 (2H, m), 3.12 (9H, s); 13C NMR (D2O + 4 % HCO2H, 75 MHz) *δ* 146.8, 139.5, 125.1, 123.5, 119.3, 109.9, 108.8, 107.3, 68.5, 54.1, 21.2; HRMS (ESI) calc for C13H20N2O4P+ 299.1155 [M + H]+, found 299.1159. All analytical data are in agreement with the literature.4

## 2.2 Cultivation of mushrooms and their drying and storage

Mushroom basidiospores (spore print) of *P. cubensis* (Earle) Singer used for cultivation were donated anonymously. The basidiospores were inoculated to Petri dishes with sterilized agar (autoclave 5050MLV, Tuttnauer Co. Ltd., Beit Shemesh, Israel) with malt extract (Honeywell, Fluka™, USA) in a Flowbox Biohazard box EF/S 4 (Clean Air Techniek B.V., Utrecht, Netherlands).6-7 Mycelium of *P. cubensis* grew on the agar in an incubator WTC Binder B28 (Binder GmbH, Tuttlingen, Germany) at 28°C for one week. Then, the mycelium was inoculated to jars with a sterilized hydrated wheat substrate and incubated at 28°C.8 The mycelium grew through the entire contents of the jars within two weeks.7 Subsequently, the jars with mycelium were inserted into a sterilized terrarium with hydrated perlite. This mycosystem was covered up with a transparent stretch foil and cultivated at 28°C in a thermostat Lab Companion IS-971R (Jeio Tech Co. Ltd., Daejeon, Korea). The light diode with a 12-hour alternating cycle (light/dark) was added to the terrarium for one month and the temperature was lowered to 22°C, because at 28°C fructification was not successful. These conditions (light and 22°C) were responsible for the fruit body production (Figure S 2)*.*6, 9-11

Mature caps of *P. cubensis* were placed on aluminum foil and the spore print was collected after 3 days.7, 11 To document the investigated mushroom, a herbarium specimen was deposited in the Mycological Department, National Museum, Prague (collection PRM 946994). The morphological identification of the mushroom was confirmed by DNA sequencing according to Borovička et al. (2015).12 The ITS rDNA sequence was submitted to the GenBank database (MK910206).

Fruiting bodies



Fructification

Mycelium



Spore print

Figure S 2: Cultivation process *P. cubensis* fruiting bodies

## 2.3 Blank matrices

Noble mold *Penicillium candidum* from a Président camembert type cheese was scraped with a scalpel and used as a blank matrix for the experiments with mycelium. Fruiting bodies of *Agaricus bisporus* sold commercially, both fresh and dried, were used as blank fungal biomass (matrix). These blank matrices were enriched using a Hamilton microsyringe with freshly prepared analytical standards of psilocin, psilocybin, baeocystin, norbaoecystin, and aeruginascin. After vortexing the prepared enriched blank matrices for half an hour, the samples were extracted like any other. The enriched material was also used as the blank and for method validation and matrix-matched calibrations. This analytical process of spiked blank matrices is standard according to the European Medicines Agency (EMA).13 The results are reported as an average value of all measurements.

## 2.4 Preparation of samples and their extracts

The basidiospores from mature caps (spore prints) and the fruiting bodies (lyophilized and dried) were homogenized using ceramic mortar and pestle. Approximately 10.0 ± 0.5 mg of a homogenous fungal powder was weighed into the test tubes with analytical balance ABT 220-5DM (Kern, Germany). For the mycelium and the fresh and frozen fungal fruiting bodies, approximately 50.0 mg of a) whole fungal parts and of b) chopped fungal parts were weighed and desiccated.

In an attempt to prevent degradation of the psychoactive tryptamines of *P. cubensis*, the extraction solvents were purged with a stream of nitrogen, and sets of dark testing tubes were covered with an aluminum foil. The samples were extracted with 1 mL of 0.5 % (v/v) acetic acid in methanol. The resulting suspensions were vortexed at 13 × *g* at 20°C for 1 hour. Then, the testing tubes were centrifuged for 10 min at 857 × g at 20°C. The samples were ultrafiltrated using an Eppendorf tube with 0.2 µm PTFE microfilter (Ciro, USA) in a centrifuge (15.256 × g, 5 min, 20°C).

The remaining mushroom pellets were then re-extracted with 1 mL methanol and ultrafiltrated using the same conditions as described above. Brown glass vials (Assistent-Glaswarenfabrik Karl Hecht, Germany) were used for a mixture of 100 µL final supernatant and of 900 µL solvent of 10 mmol.L-1 ammonium formate with 0.1 % formic acid in methanol/water (1/9, v/v). These samples were further diluted (10×, 100×, and 1000× diluted) due to different concentrations of analytes. The prepared extracts were then analyzed using UHPLC-MS/MS.

## 2.5 Instrumentation and conditions of UHPLC-MS/MS

Table S 1: Characteristics of the dMRM method

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Analyte** | **Retention time (min)** | **Precursor ion (m/z)** | **Fragmentor (V)** | **Product ion (m/z)** | **Collision energy (eV)** |
| **Aeruginascin** | 1.69 | 299.1 | 90 | 240.0 | 18 |
| 115.1 | 50 |
| **Baeocystin** | 1.88 | 271.1 | 97 | 240.0 | 14 |
| 115.1 | 54 |
| **Norbaeocystin** | 1.55 | 257.1 | 70 | 240.0 | 14 |
| 115.1 | 54 |
| **Psilocin** | 3.13 | 205.1 | 80 | 160.1 | 18 |
| 132.1 | 30 |
| 115.1 | 46 |
| **Psilocybin** | 1.99 | 285.1 | 85 | 205.1 | 14 |
| 115.1 | 54 |
|  |  |  |  |  |  |

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Figure S 3: Chromatogram of tryptamine analytes

## 2.6 Method validation

### 2.6.1 Specificity (selectivity)

Specificity was assessed by comparing the chromatograms of six different negative controls of extracted blank matrices of mushrooms *Agaricus bisporus* with the corresponding mushroom samples enriched with the standard solution with 5ng.mL-1 of aeruginascin, baeocystin, psilocin, psilocybin, and norbaeocystin.

### 2.6.2 LOD, LOQ, and linearity

LOD and LOQ for aeruginascin, baeocystin, psilocin, and psilocybin were determined using the software Agilent MassHunter Quantitative Data Analysis according to the noise ratio S/N (3 for LODs and 10 for LOQs).The linearity of the method was determined with fortified samples (n = 7) at concentrations of 0.5; 1; 10; 50; 100; 250 and 500 ng.mL-1.

### 2.6.3 Precision and accuracy

Precision and accuracy were evaluated by analyzing standard mushroom samples, which were spiked at three concentration QC levels 20, 50, and 200 ng.mL-1 of all five quantified analytes on five replicates. The concentration of each sample was determined using the calibration curve. Precision was expressed using the relative standard deviation, and accuracy was defined as the relative deviation in the determined concentration of the closeness of agreement between measurement samples obtained from multiple samplings of the same homogenous sample. Precision is expressed as the relative standard deviation (% RSD). The mean and standard deviation of the response are calculated for each concentration to determine the % RSD. The precisions were required within maximally 20 %, and accuracies were required not to exceed ±15 %.13-14

### 2.6.4 Extraction recovery and matrix effect

The extraction recovery and matrix effect were verified five independent replicates at a low, medium, and high concentration levels (20, 50, and 200 ng.mL-1). The experiments consisted of two sets. The first set of blank mushroom samples (*Agaricus bisporus* species) was fortified on the same final concentration as the second set of QC levels which were prepared as analytes spiked with a mixture of 10 % (v/v) methanol. The recovery and matrix effects were determined by comparing the peak areas of these two sets.

### 2.6.5 Stability

The stability of all analytes solutions was evaluated by using five replicates at concentration levels of 10 ng.mL-1 and 100 ng.mL-1. The samples were left in the LC autosampler at 5°C for five days.

## 2.7 Optimization of the extraction procedure

### 2.7.1 Effect of homogenization

The conditions of homogenization (processed × unprocessed mushrooms) were verified before extraction. The homogenization effect was compared in two mushroom sets: The first set consisted of dried mushrooms and the second set contained fresh mushrooms.

The first dried mushroom set consisted of nine individual fruiting bodies that were divided into the two halves. The first half mushrooms of this set were separately homogenized in a mortar with a pestle on the fungal powder, which was weighed at an equal weight (10±0.5 mg). The second half of the dried individual fruiting bodies were weighed in whole pieces (1-3) to an identical weight. The second fresh mushroom set was prepared analogously, just instead of mushroom powder, the mushrooms were carefully cut into many small pieces. The extracts of the first and second parts of the samples were compared.

### 2.7.2 Extraction solvents

First of all, different extraction solvents for tryptamine alkaloids were tested – methanol, 50 % (v/v) methanol, deionized water, ethanol, 75 % (v/v) ethanol, isopropanol, and 25 mmol.L-1 acetate buffer (pH 4.5). The samples were prepared and measured in two independent replicates and the experiment consisted of 1 mL of extraction solvent per 10 mg of the matrix (*P*. *cubensis*). Meanwhile, methanol was chosen as the best extraction solution for tryptamine analytes. The second part of the experiment was to evaluate the effect of  acidification with formic acid (0.1 %, 0.2 %, 0.5 % and 1.0 %, v/v) or acetic acid (0.1 %, 0.2 %, 0.5 % and 1.0 %, v/v). The extraction efficiency was verified using a homogeneous mushroom powder (10 mg ± 0.1 mg), which was extracted with different solvents.

## 2.8 Effect of temperature change

All of the experiments were prepared in five replicates, where the dried fungal powder was placed in tubes in a thermostat at 25°C, 50°C, 75°C, 100°C, 125°C, and 150°C for 30 minutes. Subsequently, an extraction solvent was added to the samples, after tempering the tubes with the fungal powder to laboratory temperature. Then the tubes with suspensions were vortexed at 13 × *g* at 20°C for 15 minutes. The yield of analytes decreased when the samples were heated above 25°C.

# 3 Results and discussion

## 3.1 Cultivation of mushrooms

Perlite was used instead of vermiculite due to its water absorption function to maintain the humidity.6-7, 9-11. Culturing temperatures were determined experimentally. The most difficult task was to achieve the fructification of mushrooms, which we did not succeed until after the third attempt. A key aspect was setting the mode on the 12-hour light/dark diode and lowering the thermostat temperature from 28°C to 22°C. Several fungal cultivation studies recommend lowering the temperature to a similar level to achieve the fructification of the fruiting bodies.6, 15 If the temperature does not work, it is possible to try a cold shock (4°C) for a few days, which may trigger the subsequent fructification even at room temperature.10

## 3.2 Instrumentation and validation of the results

The introduction of the method was inspired by already published studies.16-21 The validation steps were verified by criteria from analytical studies according to which the validation method is acceptable. 22-25

### 3.2.1 LOD, LOQ, and linearity

LOD values were established for aeruginascin, baeocystin, psilocin, and psilocybin at 0.1 ng.mL-1,and norbaeocystin at 1.0 ng.mL-1. LOQs for all analytes except norbaeocystin were evaluated at 0.5 ng.mL-1 and for norbaeocystin at 5.0 ng.mL-1. Calibration curves were constructed using standards in a concentration range of 0.5 to 500 ng.mL-1 with the Pearson correlation coefficient ≥ 0.999.

### 3.2.2 Precision and accuracy

RSDs at the concentration of 20 ng.mL-1 were specifically 1 % for baeocystin and 3 % for the other analytes. RSDs at the concentration of 50 ng.mL-1 were 1 % for norbaeocystin and psilocin, 3 % for aeruginascin and baeocystin, and 4 % for psilocybin, and RSDs at the concentration of 200 ng.mL-1 were exactly 3 % for psilocin, 5 % for psilocybin, 6 % for norbaeocystin, 7 % for aeruginascin, and 10 % for baeocystin.

### 3.2.3 Extraction recovery and matrix effect

Extraction recoveries and matrix effect values were 86-94 % for norbaeocystin, 96-102 % for aeruginascin, 88-93 % for baeocystin, 88-96 % for psilocybin, and 100-113 % for psilocin.

### 3.2.4 Stability

Published studies have shown that fungal tryptamines are unstable in aqueous extracts.21 Methanol is the safest extraction solvent for retaining the indole derivatives.26-27 Therefore, methanol was used for the preparation of standard stock solutions. These were stored in the freezer at -20°C. The stability of two calibration levels was evaluated to verify the possibility to leave the samples in the LC autosampler during the sequence. The concentrations of studied analytes ranged from 1 % to 5 %. For each experiment, new calibration solutions were prepared, and the solutions were considered to be sufficiently stable.

## 3.5 Stability of tryptamines in dried fungal powder

Table S 2: Effect of storage on concentration in dried fungal powder after 15 months

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Conc. of analytes (wt.%) in initial fungal powder** | | | | |
|  | **Norbeocystin** | **Aeruginascin** | **Baeocystin** | **Psilocybin** | **Psilocin** |
|  | 0.031 | 0.007 | 0.210 | 1.512 | 0.044 |
|  |  |  |  |  |  |
|  | **Conc. of analytes (wt.%) after 1 week of storage** | | | | |
|  | **Norbeocystin** | **Aeruginascin** | **Baeocystin** | **Psilocybin** | **Psilocin** |
| Light, 20°C | 0.030 | 0.005 | 0.183 | 0.964 | 0.017 |
| Dark, 20°C | 0.029 | 0.007 | 0.197 | 1.310 | 0.020 |
| Fridge, 4°C | 0.028 | 0.006 | 0.177 | 1.182 | 0.017 |
| Freezer, - 20°C | 0.025 | 0.005 | 0.161 | 1.084 | 0.017 |
| Freezer, - 80°C | 0.026 | 0.006 | 0.162 | 0.992 | 0.018 |
|  |  |  |  |  |  |
|  | **Conc. of analytes (wt.%) after 1 month of storage** | | | | |
|  | **Norbeocystin** | **Aeruginascin** | **Baeocystin** | **Psilocybin** | **Psilocin** |
| Light, 20°C | 0.009 | 0.003 | 0.036 | 0.724 | 0.005 |
| Dark, 20°C | 0.008 | 0.003 | 0.034 | 0.850 | 0.006 |
| Fridge, 4°C | 0.008 | 0.003 | 0.034 | 0.758 | 0.006 |
| Freezer, - 20°C | 0.008 | 0.003 | 0.034 | 0.793 | 0.006 |
| Freezer, - 80°C | 0.008 | 0.003 | 0.034 | 0.774 | 0.006 |
|  |  |  |  |  |  |
|  | **Conc. of analytes (wt.%) after 2 months of storage** | | | | |
|  | **Norbeocystin** | **Aeruginascin** | **Baeocystin** | **Psilocybin** | **Psilocin** |
| Light, 20°C | 0.005 | 0.001 | 0.017 | 0.669 | 0.003 |
| Dark, 20°C | 0.007 | 0.002 | 0.030 | 0.817 | 0.006 |
| Fridge, 4°C | 0.007 | 0.002 | 0.029 | 0.704 | 0.005 |
| Freezer, - 20°C | 0.006 | 0.002 | 0.026 | 0.723 | 0.006 |
| Freezer, - 80°C | 0.007 | 0.002 | 0.028 | 0.700 | 0.005 |
|  |  |  |  |  |  |
|  | **Conc. of analytes (wt.%) after 15 months of storage** | | | | |
|  | **Norbeocystin** | **Aeruginascin** | **Baeocystin** | **Psilocybin** | **Psilocin** |
| Light, 20°C | 0.006 | 0.003 | 0.036 | 0.445 | 0.009 |
| Dark, 20°C | 0.006 | 0.003 | 0.037 | 0.477 | 0.015 |
| Fridge, 4°C | 0.006 | 0.003 | 0.040 | 0.448 | 0.010 |
| Freezer, - 20°C | 0.006 | 0.003 | 0.040 | 0.453 | 0.009 |
| Freezer, - 80°C | 0.006 | 0.003 | 0.040 | 0.423 | 0.025 |

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