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HPLC Analysis of Hallucinogenic Mushroom Alkaloids (Psilocin and Psilocybin) Applying Hydrophilic Interaction Chromatography (HILIC)

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Abstract

Hallucinogenic mushrooms containing psilocin and psilocybin psychoactive compounds are permanent offers on the black market palette. Reliable quantifications of psilocin and psilocybin are especially important task of forensic analysis because their results have significant effect on the hardness of judgement to be punished by the court. For quantification of psilocybin mainly HPLC procedures are applied because the compound is thermally labile and converts into psilocin by dephosphorylation at gas chromatographic conditions. The majority of HPLC procedures available in the scientific literature is based on reversed phase separation which is not advantageous particularly for the psilocybin because of its insufficient retention caused by the high polarity of the compound. The elution at the unretained section may even hinder the mass spectrometric detection of psilocybin because of the quenching of formed ions by other unretained co-eluting matrix components. To overcome drawbacks outlined previously the application of hydrophilic interaction chromatography (HILIC) is promising which allows analysis of polar compounds with appropriate retention and allowing mass spectrometric detection. The aim of the present study was to develop a procedure for quantification of psilocin and psilocybin in hallucinogen mushrooms applying HPLC separation and subsequent UV-photometric and mass spectrometric detection. For this reason, an isocratic hydrophilic interaction chromatographic (HILIC) phase system consisting of a zwitterionic stationary phase and a mixture of acetonitrile and formate buffer as mobile phase was applied. The developed system assures sufficient retention for both psilocin and psilocybin, baseline separation of the compounds from each other and from matrix components, too. Unique feature of the applied system that psilocybin elutes with a retention factor of approximately two times greater than that of psilocin which is a scientific novelty. The applicability of the proposed procedure is demonstrated via results obtained by analysis of mushrooms seized by police.

Keywords: Hallucinogenic mushrooms; Psilocin; Psilocybin; Hydrophilic interaction chromatography; HILIC; HPLC/MS

Introduction

Determination of psilocin and psilocybin is important task of forensic analysis. The two compounds are banned as narcotic substances in several countries including Hungary. In Hungary, the reliable quantification of controlled substances is of special importance because the penalty is highly dependent on the amount of pure controlled substance connected with the given criminal act. For analysis of the two hallucinogen compounds in mushrooms, researchers often used HPLC for quantification. However, the psilocybin is thermally not stable owing to dephosphorylation at elevated temperature effecting at gas chromatographic conditions. The chemical structures of the two compounds are shown in Figure 1. The figure shows that psilocybin is phosphate ester of psilocin being significantly more polar than psilocin owing to its phosphate group which causes its thermal lability. An international manual [1] for testing *Psilocybe* mushrooms, ammonia containing alkaline mobile phases with silica and octadecyl silica stationary phases, respectively, are suggested. Alkaline mobile phase (especially pH above 8) is not advantageous as far as the lifetime of the chromatographic column is concerned. Silica and silica based sorbents can be damaged due to partial dissolution of the silica at alkaline condition. The majority of HPLC procedures available in the scientific literature is based on reversed phase [2-6] separation which disadvantageous particularly for the psilocybin because of its insufficient retention caused by the high polarity of the compound. The flow-through may even hinder the mass spectrometric detection of psilocybin because of the quenching of formed ions by other unretained co-eluting matrix components. Ion-exchange chromatography may assure sufficient retention for psilocybin but the high salt contents in aqueous mobile phases are inconvenient for mass spectrometric

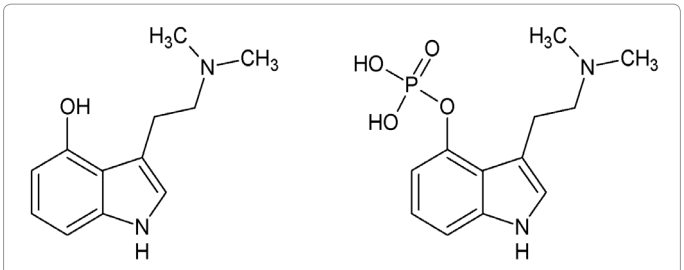

Figure 1: Chemical structures of psilocin (left) and psilocybin (right).

detection. Moreover, applicability of classic normal phase adsorption HPLC systems are excluded owing to insufficient solubility of the target compounds in non-polar mobile phases used in normal phase systems. To overcome these drawbacks outlined previously the application of hydrophilic interaction chromatography (HILIC) seems to be promising which allows analysis of polar compounds with appropriate retention and is compatible with mass spectrometric detection. Gambarro at

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al. [7] applied HILIC/HPLC with mass spectrometric detection for analysis of psilocybin and psilocin mushrooms. They used silica-based column in gradient mode. Ncube et al. [8] tested several silica-based reversed phase columns and a HILIC column for HPLC separation of hallucinogenic alkaloids including psilocin but not including psilocybin. In our experiments silica-based columns are not preferred because they are liable irreversibly bond components from multi-component biological matrices, such as mushroom and plant material extracts. Irreversible bonding on the stationary phase decreases the column lifetime. In routine HPLC work the isocratic separation is more advantageous than gradient because the equilibration can be avoided before the next analysis. The aim of the present study was to develop a procedure for the reliable quantification of psilocin and psilocybin in hallucinogen mushrooms applying isocratic HILIC/HPLC separation and subsequent UV-photometric and mass spectrometric detection. The applicability of a phase system consisting of a polymer-based zwitterionic stationary phase and a mixture of acetonitrile and formate buffer as mobile phase was taken into consideration for the separation.

Materials and Methods

Chemicals, solvents and test materials

Psilocin as powder material, psilocybin as 1 mg/ml methanolic solution were purchased from the Cayman Chemical Company (USA). For preparation of calibration solutions methanol was used as solvent. The acetonitrile used as mobile phase component for HPLC separation was LiChrosolv grade solvent from Merck KGaA (Darmstadt, Germany). Double distilled water was used for preparation of buffer component of the mobile phase. Methanol used for the extraction was of analytical grade. The formic acid (98%) was GPR grade (VWR Prolabo), the ammonium formate was MS grade (Fluka). The mushroom samples used for the extraction experiments were confiscated by the Hungarian Police.

Instrumentation

Pulverizette 23 ball mill (Fritsch GmbH, Idar-Oberstein, Germany) and a commercially available electric coffee grinder were used for the homogenisation of the samples. The quasi counter current extraction was performed with a Shimadzu 10/A HPLC system using its pumps and column thermostat and an empty HPLC guard column (5 cm × 4.6 mm I.D.) as an extractor chamber. The LC/MS instrument consisted of an Agilent 1100 HPLC system (quaternary pump, diode array UV-detector, thermostated column compartment, autosampler) coupled with an electrospray ionization interface unit to an Esquire 6000 ion trap mass spectrometer (Bruker Daltonics).

Quasi counter current extraction of mushroom samples

About 50 mg amounts of air-dried and homogenized mushroom were weighed into the extractor chamber. The extractor chamber was equipped into the column compartment of the HPLC system and thermostated at 60°C. After equilibration of the set temperature (about 15 min to 20 min) the extraction was started with a flow rate of 0.5 ml/min and the extracted portions were collected for an hour in a 50 ml screw-cap vial. The psilocin and psilocybin contents of extracts were analysed by LC/MS. The completeness of the extraction was checked with preliminary experiments, by collecting and analysing about 1.7 ml extracted fractions separately. The 1.7 ml volumes are equal to ones reaching the neck level of 2 ml autosampler vials. According to results exhaustive extractions could be reached after the 6^{th} to 10^{th} 1.7 ml extraction segments which are less significantly than the collected 30 ml total extraction volumes, depending on the sample matrices. The extraction temperature was set to 60°C according to results of preliminary experiments which showed that elevated temperature assures quicker extraction than ambient temperature does.

Standard solutions

For preparation of the calibration curves 2 µg, 6 µg, 17 µg, 52 µg, 156 µg and 467 µg psilocin/ml and 31 µg, 62 µg, 125 µg, 250 µg, 500 µg and 1000 µg psilocybin/ml concentration solutions were prepared using methanol as solvent.

To determine limits of detection (LOD) the most dilute solutions were diluted until reaching the S/N ratio of 3 according to peak heights for UV detection and the smallest peaks consisting of still ten peak points which are minimum peak points needed to construct a chromatographic peak for mass spectrometric detection with selected ion monitoring.

To check the stability of standards in methanol the solutions were thermostated at 60°C for an hour and the peak areas were compared with those of non-thermostated ones.

HPLC conditions

Parameters of the HPLC separation were set as follows — Column: ZIC-pHILIC, zwitterionic phase (Merck, 150 mm × 4.6 mm I.D, d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80 v/v:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl; detection: diode array UV photometric in the wavelength range of 190 nm to 400 nm.

MS conditions

Features of the mass spectrometric detection were as follows — electrospray ionization in positive mode; pressure of nebulizer gas: 60 psi; flow of drying gas: 10 l/min; temperature of drying gas: 365°C; ion trap analyzer; compound stability: 10%; trap drive level: 25%, scan range: 40 Da to 400 Da.

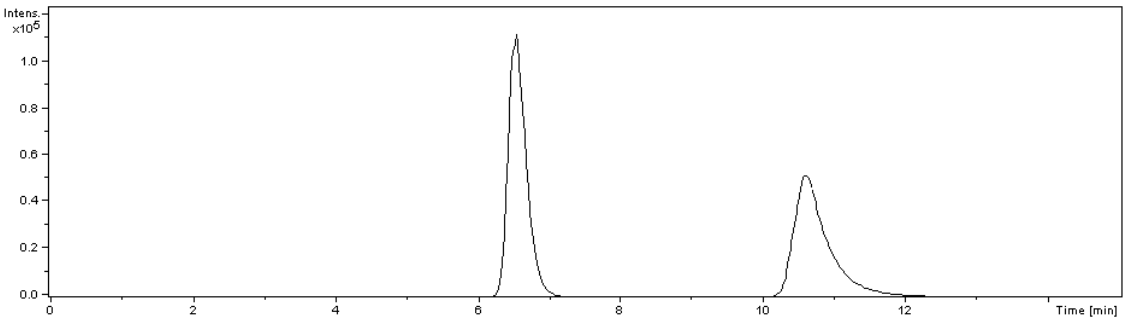
All experiments were run triplicate, except checking retention times where six parallel measurements were done.

Results and Discussion

Typical chromatograms obtained for a test mixture of psilocin and psilocybin and for a methanolic mushroom extract are shown in Figures 2 and 3 respectively. Baseline separation and good peak shapes can be achieved for the two alkaloids within 15 min. The reproducibility of retention times was within 0.5% expressed in relative standard deviation of measured data obtained with six parallel measurements.

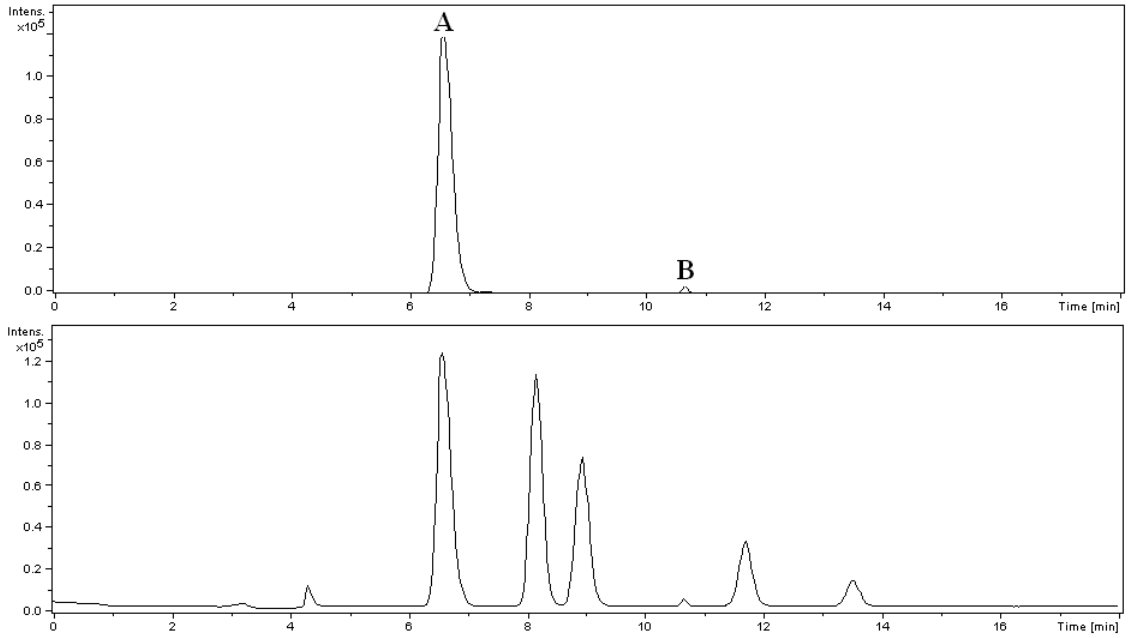
The peak purities were checked by evaluating both UV and MS spectra using those of standards and comparing them with those of ones measured in real samples. No overlapping components could be detected in case of psilocin and psilocybin, respectively, which indicates the selectivity of the applied procedure. UV and MS spectra of two compounds are shown in Figures 4 and 5 respectively. Figures show that UV spectrum of psilocin is very similar to that of psilocybin, small differences in the wavelength range of 280 nm to 300 nm can be observed. The mass spectra of two compounds show mass to charge ratio equal to protonated molecular ions without any additional ions indicating that no degradation and cluster formation take place during the analysis.

The calibration measurements were evaluated according to both UV signal at 220 nm and MS signal of molecular ions, i.e. at mass-to-charge ratio of 205 Da for psilocin and at 285 Da for psilocybin. The calibration curves were constructed by plotting the peak areas against the injected amounts for both compounds at both detector signals. Results show that in case of psilocin the relationship giving the best fit between the peak areas based on MS signal and injected amounts



Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, detection ESI (+ mode): MS, extracted ion monitoring (m/e 205 for psilocin, m/e 285 for psilocybin).

Figure 2: Typical chromatogram obtained by HPLC/MS analysis of a test mixture of psilocin (left) and psilocybin (right), test mixture: 5 µg psilocin/ml and 500 µg psilocybin/ml, solvent methanol.



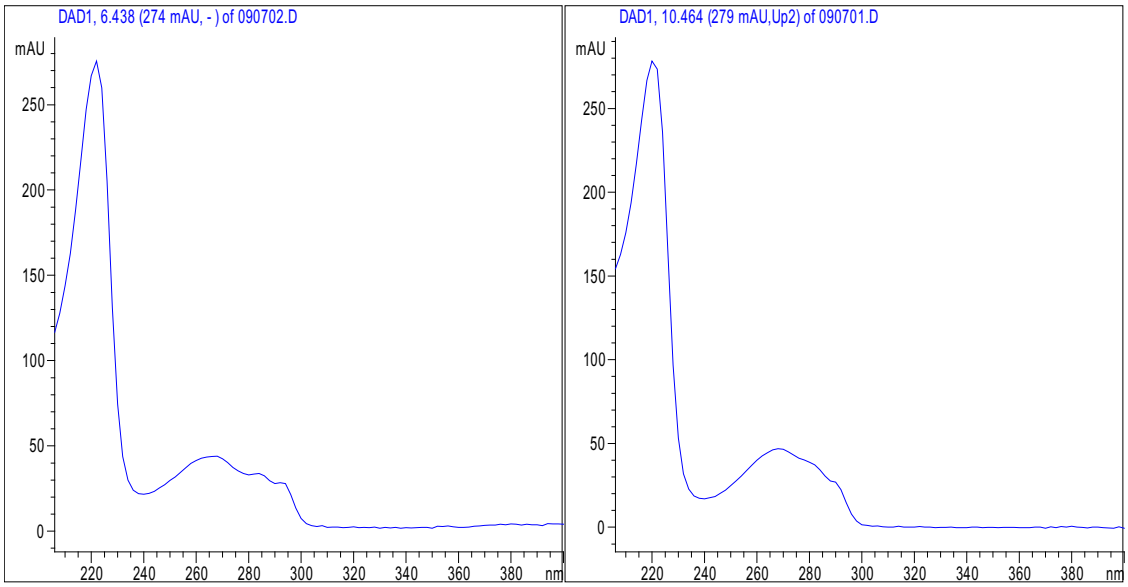
Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, detection ESI (+ mode): MS, extracted ion monitoring (m/e 205 Da for psilocin, m/e 285 Da for psilocybin) (up) and total ion monitoring (m/e 40 Da to 400 Da) (down).

Figure 3: Typical total ion (down) and extracted ion (up) chromatograms of molecular ion of psilocin (peak A) and psilocybin (peak B), respectively, obtained by HPLC/MS analysis of methanolic extract of a seized hallucinogenic mushroom.

is quadratic (Figure 6) and that is one between the peak areas based on UV signal and injected amounts is linear (Figure 7). The quadratic response of mass spectrometric ion-trap detectors is commonly known and accepted in quantitative work [9]. In case of psilocybin for the relationships between peak areas of MS signal and injected amounts (Figure 8) as well as between peak areas of UV signal and injected amounts linear correlation (Figure 9) were found in the range investigated.

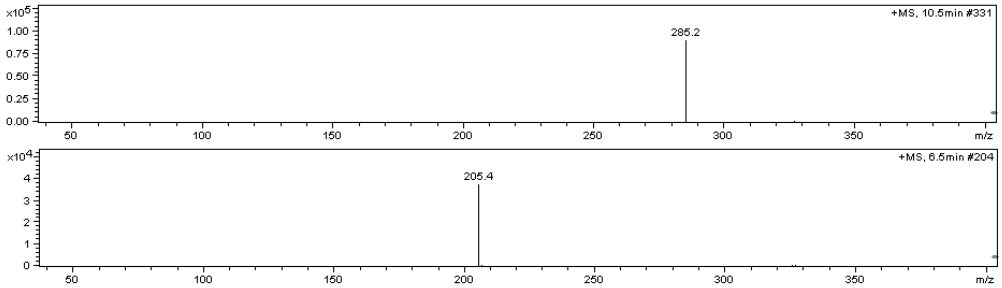
According to results of stability experiments, no degradation of the target compounds occurred at the set extraction conditions.

At UV detection, the limit of detections (LOD) were regarded as minimal injected amounts assuring peak heights exceeding the amplitude of background fluctuation three times and at MS detection as minimal injected amounts assuring peaks consisting of ten evaluable peak points at detection of molecular ions by extracted ion monitoring, respectively. In the latter case the fluctuation of the baseline is not measurable. The measured LOD values for the two compounds at the two detections signals are listed in Table 1. As data in Table 1 show, LOD values obtained with mass spectrometric detection are less by twenty-five times for psilocin and by sixty times for psilocybin, respectively, than those of ones measured with UV detection which finding is feasible. Psilocin has a response factor nearly hundred times higher than psilocybin has in mass spectrometric detection mode and twice higher in UV photometric detection which can be estimated from the average slopes of the calibration curves. The significant difference



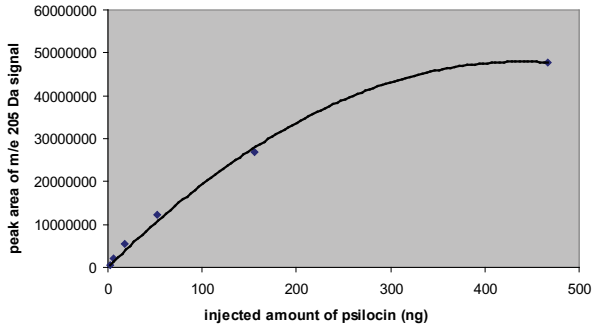
Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, diode array detection in the range of 190 nm to 400 nm.

Figure 4: UV spectrum of psilocin (left) and psilocybin (right) obtained by HPLC/UV analysis of compounds, respectively.



Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, detection ESI (+ mode): MS, scan range 40 Da to 400 Da.

Figure 5: MS spectrum of psilocybin (up) and psilocin (down) obtained by HPLC/MS analysis of compounds, respectively.

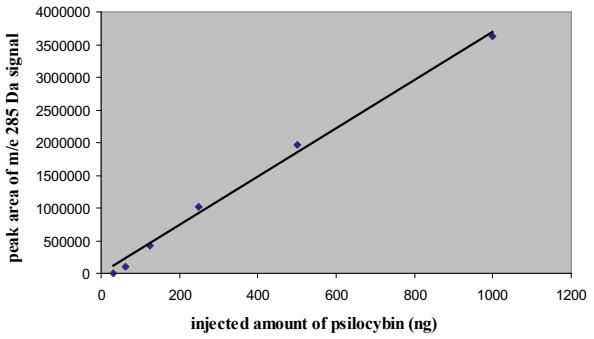


Correlation equation: $y=-248.57\ x^2+218318\ x$; correlation coefficient: 0.998, where y is the peak area, x is the injected amount in ng. Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, detection ESI (+ mode): MS, scan range 40 Da to 400 Da.

Figure 6: Calibration curve for psilocin constructed according to peak areas of molecular ion (m/e 205 Da) and injected amounts (ng) obtained by HPLC/MS analysis.

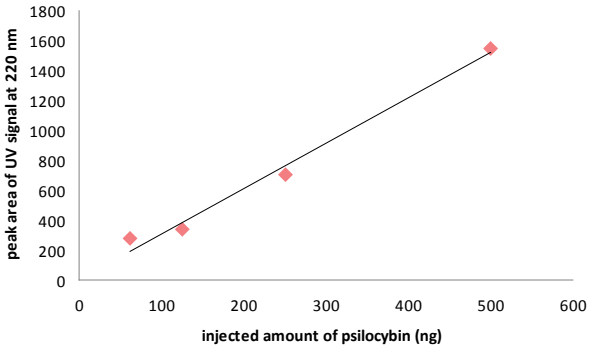
Correlation equation: $y=8.2746\ x$; correlation coefficient: 0.999, where y is the peak area, x is the injected amount in ng. Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, UV photometric detection at 220 nm.

Figure 7: Calibration curve for psilocin constructed according to peak areas of UV signal at 220 nm and injected amounts (ng) obtained by HPLC analysis with UV photometric detection.



Correlation equation: y=3691.4 x; correlation coefficient: 0.997, where y is the peak area, x is the injected amount in ng. Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, detection ESI (+ mode): MS, scan range 40 Da to 400 Da.

Figure 8: Calibration curve for psilocybin constructed according to peak areas of molecular ion (m/e 285 Da) and injected amounts (ng) obtained by HPLC/MS analysis.



Correlation equation: y=3.0464 x; correlation coefficient: 0.993, where y is the peak area, x is the injected amount in ng. Chromatographic conditions — Column: ZIC-pHILIC (150 mm × 4.6 mm I.D., d_p 5 µm); mobile phase: MeCN - 40 mmol formate buffer (pH 3.5) 80:20 v/v; flow rate: 0.5 ml/min; temperature: 40°C; injection volumes: 1 µl, UV photometric detection at 220 nm.

Figure 9: Calibration curve for psilocybin constructed according to peak areas of UV signal at 220 nm and injected amounts (ng) obtained by HPLC analysis with UV photometric detection.

Compound	LOD for UV photometric detection at 220 nm (ng)	LOD for mass spectrometric detection at m/e of molecular ion 205 Da for psilocin, 285 Da for psilocybin (ng)
Psilocin	5	0.2
Psilocybin	30	0.5

Table 1: Limit of detection (LOD) values as injected amounts for psilocin and psilocybin for UV photometric (220 nm) and mass spectrometric detection of molecular ions, respectively.

for mass spectrometric detection can be interpreted according to better ionization ability of psilocin in positive mode than that of psilocybin's one. Supposedly, psilocybin could be measured with higher sensitivity in negative ionization mode but the acidic mobile phase is not an appropriate medium for this reason. In alkaline medium the psilocin could not be detected because it is not able to undergo ionization at all.

The main advantage of this HILIC system that both psilocin and psilocybin elute with appropriate retention exceeding significantly the unretained section and no quenching of the matrix components disturbs the mass spectrometric detection of the two compounds. The developed system assures baseline separation of the compounds from each other and from matrix components, too. Unique feature of the applied system that psilocybin elutes with a retention factor of two times greater than that of psilocin which is a scientific novelty.

Compound	Mushroom No. 1	Mushroom No. 2
Psilocin (% w/w)	0.252 ± 0.011	1.244 ± 0.058
Psilocybin (% w/w)	trace	trace

Table 2: Psilocin and psilocybin contents of hallucinogenic mushrooms seized by police from Hungarian black market (results of three parallel measurements, including three separate sampling and three separate HPLC analysis).

The applicability of the proposed procedure is demonstrated with results obtained by analyses of two mushroom samples seized by the police from the Hungarian black market. The measured alkaloid contents of samples with some statistical data calculated from results of three parallel measurements, including three separate samplings and three separate HPLC/MS analyses are shown in Table 2. Data show that both mushrooms contain mainly psilocin with traces of psilocybin. This indicates that investigated mushrooms should have been under storage for a time before the seizure as the majority of psilocybin has been converted into psilocin during the storage. The relative standard deviation of the quantification is below 5% for which is acceptable for forensic analysis. Evaluation according to UV signal could not be performed because at alkaloid levels of the investigated mushrooms no measurable peaks were found. For UV photometric detection the ratio of mushroom amount to volume of extraction solvent should be increased by a factor of sixty or concentration of extract should be done by a similar degree prior to the analysis.

Conclusion

For quantification of psilocin and psilocybin in hallucinogen mushrooms an isocratic hydrophilic interaction chromatographic (HILIC) phase system consisting of a polymer-based zwitterionic stationary phase and a mixture of acetonitrile and format buffer as mobile phase with subsequent UV-photometric and mass spectrometric detection was applied. The developed system assures sufficient retention for both psilocin and psilocybin, baseline separation of the compounds from each other and from matrix components within 15 min. Unique feature of the applied system that psilocybin elutes with a retention factor of two times greater than that of psilocin which is a scientific novelty. Application of the proposed system assures reliable quantification without disturbance of any of overlapping matrix component. The developed procedure is selective for the two target compounds which were confirmed by checking of peak purities applying mass spectrometry. The sample preparation consists of few steps having minimal error sources which assure reliable results.

The applicability of the proposed procedure is demonstrated via results obtained by analysis of mushrooms seized by police. For psilocin 0.2 ng, for psilocybin 0.5 ng limits of detection (LOD) values could be reached with mass spectrometric detection and 5 ng and 30 ng values with UV photometric detection, respectively. The relative standard deviations of the determinations did not exceed the 5%.

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