Short report validation (V076-22-05)

Content determination of psilocybin in various dried mushroom components as well as extracts using the PSILO-QTest rapid test set

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Introduction

The active ingredient content of psilocybin in organic materials is subject to large natural variations between the several hundred fungal species, natural site conditions, and between different developmental stages. Even between the different fungal components of a fruiting body, considerable variations can be observed, and even mushrooms cultivated under standardized laboratory conditions are subject to large variations in their active ingredient content.

The new PSILO QTest method can analyze the concentration of the active ingredient psilocybin within 30 minutes, making it the world's first and only rapid test. The basis for its operation is a linear colorimetric chemical reaction of psilocybin with the detection reagent developed by miraculix. This provides the basis for the first quantitative tests (QTests) for a wide variety of active ingredients By means of the enclosed color chart, the tests can be easily evaluated by eye. Correctly performed tests (with white background and under daylight) achieve a high precision for a color rapid test. In specific, this means that inexperienced test persons only misjudge by a maximum of one color field when evaluating by color chart. It was found that comparison with reference photos of the entire color series significantly simplified the evaluation. A spectrophotometric evaluation achieves an even higher precision of the results (~5% deviation from the HPLC analysis).

This is a voluntarily validated and standardized test procedure based on the pharmaceutical method validation guideline ICH Q2(R1), which we have extended to the QTests. This guideline describes the requirements of the analytical method for active ingredients that are subsequently to be used in human medicine and guarantees a safe dosage of these active ingredients, for example in pharmacies.

The PSILO-QTest was developed at the Friedrich Schiller University Jena at the Institute of Pharmacy, Chair of Pharmaceutical Microbiology, under the grant number 03EGSTH1189, supported by the German Federal Ministry of Economics and Climate Protection and the European Social Fund, in the course of the third-party funded project "Production of quantitative test systems of psychotropic agents" in the period from 2019 to 2021 under the project management of Dr. Felix Blei. The necessary permission for the handling and acquisition of the narcotics psilocybin/psilocin according to § 3 of the German Narcotics Act was granted for the Friedrich Schiller University Jena under the current BtM number 463 23 75 as well as previous for the premises of the Pharmaceutical Microbiology in Jena. The pure substances required for the project were purchased from LIPOMED GmbH and LGC Standards Ltd.

Summary

This extract from the validation summarizes the results of the quantitative test procedure for the concentration determination of psilocybin in organic materials or extracts. The aim of the validation is to demonstrate the suitability of the PSILO-QTest as a rapid test for the concentration determination of psilocybin in dried fungal components or extracts. The test system is an extraction with a subsequent determination of the concentration by means of a color test.

Acceptance criteria and test parameters

Parameter	Description and expected values	Acceptance criteria
Suitability test of the method of quantitative measurement of psilocybin (linearity).	Dilution series with psilocybin reference: stock solution psilocybin 1mg/ml (in extraction solution). ascending concentrations of 5, 10, 20, 30, 40, 50, 60, 70 µg PSB per reaction make for increasing color intensities, measurable in the spectrophotometer by OD at 590nm	Dilution series with psilocybin reference: stock solution psilocybin 1mg/ml (in extraction solution) n=6 Linear response with minimum or higher Pearson correlation coefficients R of 0.95
Correctness of the method	Sample: *Psilocybe cubensis* mycelium Alkaloid content of 1% PSB Sample: *Psilocybe cubensis* mycelium Alkaloid content of 0.3% PSB Sample: *Psilocybe cubensis* fruiting body Alkaloid content of 1.6% PSB Sample: *Psilocybe tampanensis* pseudosclerotia. Alkaloid content of 0.4 % PSB	Sample: *Psilocybe cubensis* mycelium (n=3) Alkaloid values from 0.9 - 1.1 % PSB Sample: *Psilocybe cubensis* mycelium (n=3) Alkaloid values from 0.27 - 0.33 % PSB Sample: *Psilocybe cubensis* fruiting bodies (n=3) Alkaloid values from 1.44 - 1.76 % PSB Sample: *Psilocybe tampanensis* pseudosclerotia (n=3). Alkaloid values from 0.36 - 0.44 % PSB
Specificity of the method	Reagent blank Extraction solution in detection reagent is incubated	Reagent blank (n=3) Concentration PSB: No PSB detectable
Method precision	Repeatability Measure the 8 concentrations of the dilution series in the plate reader 3 times in succession, determine the standard deviation Internal laboratory precision Measurement of the 8 concentrations of the dilution series in the plate reader on 2 different days by different analytes Measuring the same dilution series on another spectrophotometer	Repeatability No significant standard deviation for at least 3 concentrations x 3 replicates Internal laboratory precision No significant standard deviation when measured by second analyte Similarly linear curve when measured on second spectrophotometer
Detection and limits of quantification	Limit of quantification LOQ by visual inspection Mixtures with 0.02 mg - 0.04 mg - 0.08 mg - 0.16 mg PSB	Limit of quantification LOQ by visual inspection (n=3) Precision and accuracy guaranteed at lowest point of color evaluation scale (2mg PSB/g substance)

Working range of the test procedure	Standard curve The experiments carried out on the linearity of the measurement method define the working range, as expected the working range is from 2 - 24 mg PSB per gram of substance sample due to the color evaluation scale	Standard curve For the method of determining the uniformity of the content, the working range should normally be from 70% to Cover 130% of the test concentration
Robustness of the test procedure	Variation of incubation time with staining Only coarsely crushed substance sample Variation extraction time	Variation of incubation time with staining same staining with doubling of incubation time Only coarsely crushed substance sample same staining if sample material is only crushed with scissors Variation extraction time same coloration with doubled extraction time

Methods

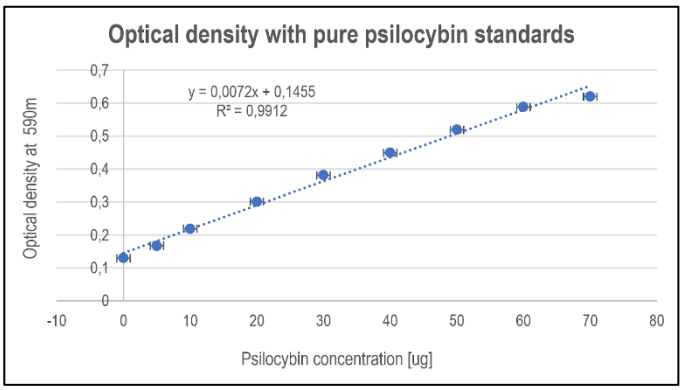
All measurements were performed at room temperature (21 °C), i.e. samples and reagents had an equally corresponding room temperature. The tests were carried out according to the instructions enclosed with the rapid test set, corresponding deviation is marked in the methods.

Sample	Dilution series with psilocybin reference: stock solution psilocybin 1mg/ml (in extraction solution)	Different fungal components with different PSB concentrations.	Reagent blank	Limit of quantification LOQ by visual inspection
PSB Content	5 µg PSB 10 µg PSB 20 µg PSB 30 µg PSB 40 µg PSB 50 µg PSB 60 µg PSB 70 µg PSB	*Psilocybe cubensis* mycelium Alkaloid content of 1% PSB *Psilocybe cubensis* mycelium Alkaloid content of 0.3% PSB *Psilocybe cubensis* fruiting body Alkaloid content of 1.6% PSB *Psilocybe tampanensis* Pseudosclerotia Alkaloid content of 0.4% PSB	0 mg/ml PSB in extraction solution	0.02 mg/g PSB in extraction solution 0.04 mg/g PSB in extraction solution 0.08 mg/g PSB in extraction solution 0.16 mg/g PSB in extraction solution
Sample quantity	70 µl	150 mg	150 mg	1 ml
Extraction volume	ad 70 µl	4 ml	4 ml	1 ml
Detection solution	330 µl	3 ml	3 ml	3 ml
Quantity	n = 6	n = 3	n = 1	n = 1

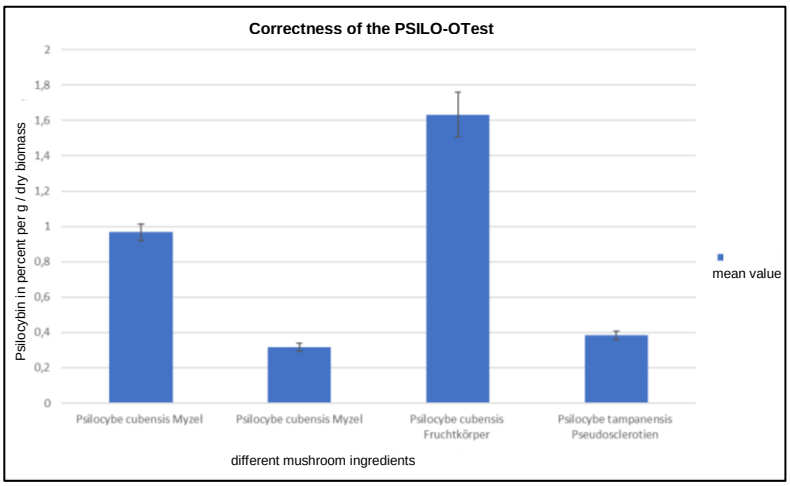
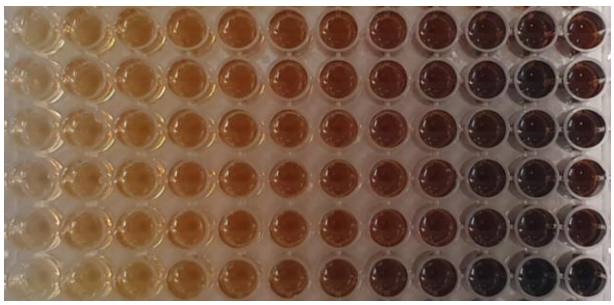
Results

<table>
<tr><th>Sample name</th><th>Result</th><th>Validation result</th></tr>
<tr><td>Dilution series with psilocybin reference: stock solution psilocybin 1mg/ml (in extraction solution)</td><td>The spectrophotometer measurement showed a markedly linear chemical response with Pearson correlation coefficients R of 0.99. This is very close to a perfect result and is absolutely within the acceptance criteria of validation.

Standard series with PSB Standard:
 

Standard curve with compensation line to determine the correlation coefficient:
 
</td><td>Validation passed</td></tr>
<tr><td>Different fungal components with different PSB concentrations</td><td>For the four different mushroom samples, which were previously measured for their psilocybin content on the HPLC using a reference substance, an optical evaluation was carried out in parallel by 3 different scientists (analytes) using the enclosed color chart according to the instructions.

<table>
<tr><th>Substance sample</th><th>Result by means of HPLC standard curve</th><th>Deviation in percent to HPLC</th></tr>
<tr><td>Psilocybe cubensis mycelium</td><td>Mean value: 1 % PSB</td><td>3.3 %</td></tr>
<tr><td>Psilocybe cubensis mycelium</td><td>Mean value: 0.3 % PSB</td><td>5,3 %</td></tr>
<tr><td>Psilocybe cubensis fruiting body</td><td>Mean value: 1.6 % PSB</td><td>2 %</td></tr>
<tr><td>Psilocybe tampanensis Pseudosclerotia</td><td>Mean value: 0.4 % PSB</td><td>4,2 %</td></tr>
</table></td><td>Validation passed</td></tr>
</table>

	Substance samples measured on the HPLC as well as by eye (from left to right): *Psilocybe cubensis* mycelium with 1 % PSB, *Psilocybe cubensis* mycelium with 0.3% PSB, *Psilocybe cubensis* fruiting bodies with 1.6% PSB, *Psilocybe tampanensis* pseudosclerotia ("truffles") with 0.4% PSB  	
Method precision	In the repeatability as well as the internal laboratory precision, no relevant or statistically significant deviations were found even when sextuplicates were prepared, and even in a single measurement using cuvettes, a linear curve progression identical to the standard curve could be demonstrated 	**Validation passed**
Reagent blank	Result: No psilocybin detectable, neither optically by eye nor by measurement on a spectrophotometer.	**Validation passed**

Limit of quantification LOQ by visual inspection	Samples with very low active ingredient contents were measured for analysis of the limits of quantification. Started (from left to right): 0.02 %, 0.04 %, 0.08 %, 0.16 % and 0.2 % psilocybin. Even in the lowest concentration, a discoloration was already visible to the eye in comparison with the reagent blank (not shown in the photo). By measuring the reference standard, we know that the measurement method already provides linear results in these ranges. So even well below the specified measuring range, the PSILO- QTest already delivers valid results: 	**Validation passed**
Working range of the test procedure	By measuring the standard series with the spectrophotometer, it was possible to accurately characterize the linear range of the measurement method, which is far above the working range of the measurement method. In the optical comparison, the test persons also showed themselves to be able to analyze samples precisely in the entire working range (0.2% - 2.4%). The naturally occurring active ingredient values of the fungi containing psilocybin also lie in this range. Only a few cultivars show significantly higher values. Here, however, valid results could be obtained again by halving the biomass and subsequent extrapolation.	**Validation passed**
Robustness of the test procedure	The variation of incubation time in the water bath was doubled, no significant differences in staining were found in the comparison. Only coarsely ground substance samples could even be drawn up much faster with the enclosed sterile filter, and showed the same color intensity as the same but very finely ground biomass. Doubling the extraction time also showed no significant changes in the indicated drug concentration	**Validation passed**

Rating

The tested PSILO-QTest from miraculix is safely suitable for the quantification of psilocybin in various fungal components. The experiments have shown the linear relationship of the optical density as a function of the psilocybin concentration present over the entire measurement range. The results are directly proportional to the present concentration in the sample and therefore comply with the necessary validation guidelines. The laboratory test showed a maximum deviation of 5% in the psilocybin concentration of the samples to be evaluated by eye compared to the real values measured by HPLC.. The analyses were performed on different days and by different analytes, and proved to be absolutely accurate in these experiments. This is a thoroughly robust test method, which provides absolutely reliable measurement results even when the incubation or extraction times are extended or when the biomass is only coarsely crushed. The very low detection limits are also striking; a significantly detectable discoloration of the detection reagent was already seen from 200 ng active ingredient content. The working range of the test method is between 0.2 % - 2.4 % based on the color chart included for evaluation, thus the working range covers the majority of naturally occurring active ingredient values and the validation confirms a reliable quantification in this working range.

During validation, the PSILO QTest procedure proved to be easy to handle and perform, and fast and reliable in its evaluation. All acceptance criteria of the validation plan were met. The method is suitable for the concentration determination of psilocybin in different fungal materials.