RESTEK Pure Chromatography

Homogenization, Extraction, and Stability Testing by LC-UV of Psychoactive Alkaloids Found in Psychedelic Mushrooms

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Introduction

Microdosing is the practice of regularly administering very low doses of psychoactive substances. Recent studies have indicated microdosing shows promise for the management and treatment of anxiety, depression, and other mental health disorders. Emerging research has been focusing on microdosing psilocin and psilocybin, which are frequently found in psychedelic mushrooms. With recent decriminalization and legalization in certain areas of the Unites States, there is a growing interest among research and production labs to understand more about the stability and testing of these compounds. This work examines three distinct strains of *Psilocybe* mushrooms, focusing on the processes of homogenization, extraction efficiency, and extract stability. Analysis was performed using LC-UV monitoring a total of seven psychoactive alkaloids commonly found in psychedelic mushrooms.

Background

Some compounds found in psychedelic mushrooms exhibit non-specific binding (NSB) or non-specific adsorption (NSA) characteristics. This is described as any binding or adsorption that was not intended. Molecules tend to adhere to any exposed surfaces, in this case, metal surfaces. This can be problematic and cause chromatographic challenges since it can lead to poor peak shape, low recovery, and poor performance from HPLC systems. An HPLC system contains a significant amount of metal tubing, however upwards of 80% of the metal surface area comes from the column hardware. For this reason, an inert column and guard were used for this analysis. Figure 1, below, highlights the absence of peaks on stainless steel hardware, compared to that of inert hardware. two analytes that have phosphate groups known for their chelating properties.

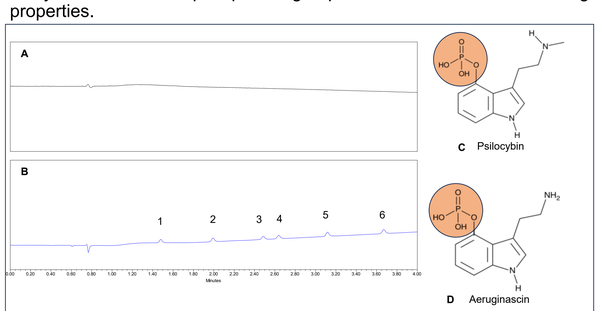


Figure 1: Chromatograms of first injection showing response of analytes using stainless steel hardware (**A**) versus inert hardware (**B**). Alongside structures of Psilocybin (**C**) and Aeruginascin (**D**) highlighting the chelating moiety.

Experimental Design

The following conditions were studied:

Mushroom Strain	• Avery's Albino • ODPE • Melmac
Drying Method	• Dehydrated • Lyophilized
Homogenization	• Standard grinding • Grinding with dry ice
Extraction Additives	• Acetic acid • Ammonium formate
Vortex Times	• 5, 10, 15, 20 minutes
Vortex Rate	• 500 RPM • 1000 RPM
Stability	• 6 month

Sample Preparation

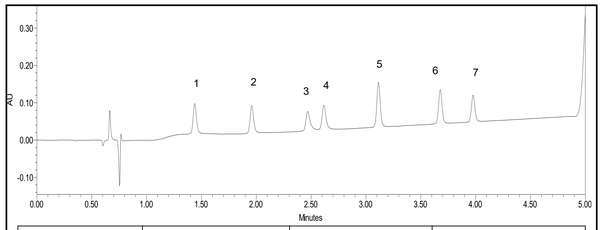
Dry Ice Homogenization	Standard Homogenization
1. Weigh 3000 - 5000 mg pre-dried mushroom	1. Weigh 3000 - 5000 mg pre-dried mushroom
2. Add to Robot Coupe® containing ~ quarter/fifty cent piece size of dry ice	2. Add to Robot Coupe® and pulse 10x
3. Pulse Robot Coupe® 10x	3. Weigh 500 mg into Environmental Express Tube
4. Weigh 500 mg into Environmental Express Tube	4. Add 20 mL extraction solvent
5. Add 20 mL extraction solvent	5. Vortex for pre-determined amount of time per experiment
6. Vortex for pre-determined amount of time per experiment	6. Add filter and push with plunger
7. Add filter and push with plunger	7. Remove plunger, aliquot sample into autosampler vial for analysis
8. Remove plunger, aliquot sample into autosampler vial for analysis	

The second sequential extraction was performed by pouring of supernatant into an appropriately sized container, and the filter removed. The pellet was then transferred to a filtration apparatus and vacuumed dry. The sample was then transferred into a new Environment Express Tube and steps 5-8 (Dry Ice) or 4-7 (Standard) were repeated from above.

Analytical Method

Analytical Column:	**Force Inert Biphenyl, 100 mm x 3 mm, 3 µm**
Guard Column:	Force Inert Biphenyl EXP Guard Cartridge, 5 x 3.0 mm
Mobile Phase A:	Water, 10 mM ammonium formate, 0.1% formic acid
Mobile Phase B:	Methanol, 0.1% formic acid
Column Temperature:	50 °C
Injection Volume:	2 µL
Flow Rate:	0.8 mL/min
Detector:	UV
Wavelength:	222 nm

Conditions:	Time (min)	%B
	0.00	5
	4.00	35
	4.01	95
	5.00	95
	5.01	5
	8.00	5



Peak #	CAS#	Analyte	t_R (min)
1	214220-59-7	Norbaeocystin	1.43
2	21420-58-6	Baeocystin	1.95
3	114264-95-8	Aerugenascin	2.48
4	520-52-5	Psilocybin	2.62
5	28363-70-4	Norpsilocin	3.10
6	5220-53-6	Psilocin	3.66
7	262285-41-6	4-OH-TMT	3.98

Results

Each mushroom type was analyzed for each of the drying methods: dehydration and lyophilization. Mushrooms were pre-dried and shipped via AJNA BioSciences, shown below in Figure 2.

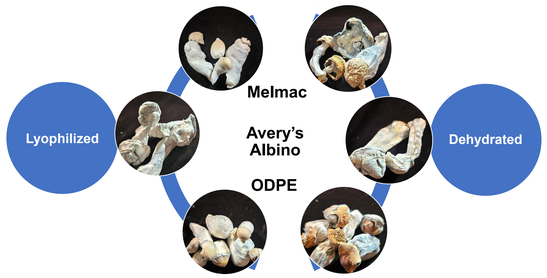


Figure 2: Picture comparison of mushroom strains and drying methods.

To determine the efficiency of sample preparation techniques, a sequential extraction was performed, samples analyzed, and data processed.

Homogenization and Vortex Time: Homogenization and vortex time were completed in tandem, with each sample prepared following described protocol only changing the length of time for vortex. Using ODPE and Avery's Albino mushroom data, graphs below show comparison results from 5- 10-, 15- and 20- minute vortex times.

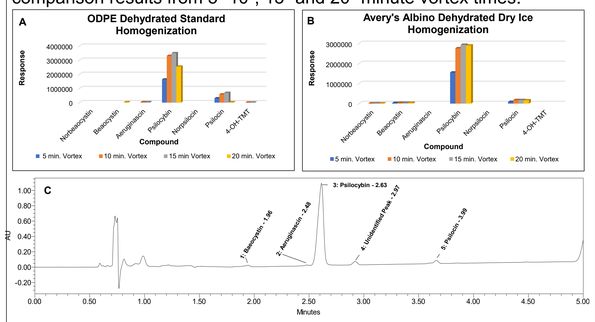


Figure 3: Description of homogenization and vortex time experiment, (**A**) ODPE dehydrated standard homogenization and (**B**) Avery's Albino dehydrated dry ice homogenization. (**C**) Example chromatogram of ODPE mushroom.

Vortex Rate: Vortex rate was compared, by preparing samples following prescribed protocol. Samples were then placed onto Glas-Col© shaker and vortex for 15 minutes at either 500 or 1000 RPM.

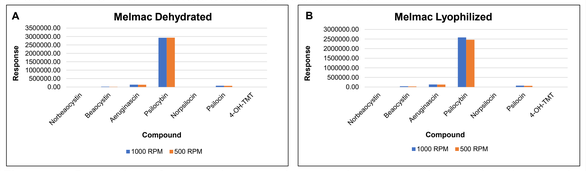


Figure 4: Description of vortex time experiment and accompanying graphs showing the difference between (**A**) Melmac dehydrated and (**B**) Melmac lyophilized mushroom strains at specified vortex rates.

Extraction Additive: Extract additives were analyzed by preparing two separate extract solvents. Both containing 0.5% (v/v) of respective additive in methanol and following prescribed protocol.

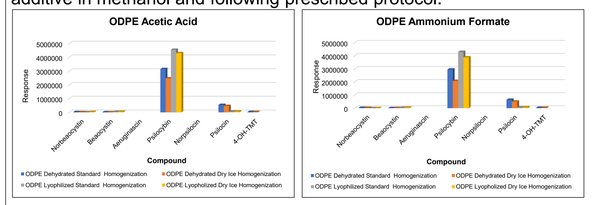


Figure 5: Description and data for ODPE mushroom strain showing the extract additive effects on extraction.

Stability Testing: After the initial sample aliquot, prepared extracts in Environmental Express Tubes from 15-minute vortex times, were placed and stored in a refrigerator for 6 months, at which time another aliquot of extract was removed from tube and analyzed.

Avery's Albino Dehydrated 6-month Extract Stability										
	Standard Homogenization					Dry Ice Homogenization				
Time Frame	Original		6-month			Original		6-month		
Analyte	Average Area Response	% RSD	Average Area Response	% RSD	% Diff	Average Area Response	%RSD	Average Area Response	%RSD	% Diff
Norbaeocystin						10617	5.62			
Baeocystin	49858	17.74	53972	5.16	**7.92**	41608	6.39	72786	2.46	**54.51**
Aerugenascin			13591	18.87				13655	4.4	
Psilocybin	3339454	3.22	3806436	0.51	**13.07**	2760119	6.54	4859262	1.31	**55.10**
Norpsilocin										
Psilocin	102020	6.68	85021	0.9	**18.18**	177207	7.32	138640	2.32	**24.42**
4-OH-TMT										

Avery's Albino Lyophilized 6-month Extract Stability										
	Standard Homogenization					Dry Ice Homogenization				
Time Frame	Original		6-month			Original		6-month		
Analyte	Average Area Response	% RSD	Average Area Response	% RSD	% Diff	Average Area Response	%RSD	Average Area Response	%RSD	% Diff
Norbaeocystin										
Baeocystin	24932	27.01	22589	7.7	**9.86**	15621	32.61	16163	11.25	**3.41**
Aerugenascin			9935	13.81				13157	4.94	
Psilocybin	3081044	5.14	3305721	0.6	**7.04**	2434298	1.66	2722087	0.88	**11.16**
Norpsilocin										
Psilocin	99175	6.7	21759	3.28	**128.03**	132901	6.71	58857	2.31	**77.23**
4-OH-TMT										

Figure 6: Description and data tables describing procedure and results for extract stability testing using Avery's Albino strain as example.

Discussion/Conclusion

- **Mushroom Strain**
  - Each mushroom variety contains a different profile and concentration of psychoactive alkaloids.
- **Drying Method**
  - Dehydrated drying method shows best response if analyzing fruiting bodies immediately.
  - Lyophilized drying method had longer shelf life of fruiting body and shows better stability at 6 months when extracted.
- **Homogenization**
  - Standard homogenization demonstrates best response.
  - Dry ice homogenization shows more inconsistent results, likely attributed to the moisture from the dry ice.
- **Extraction Additives**
  - Acetic acid improves extraction of minor tryptamines such as baeocystin, norbaeocystin, and aeruginascin.
  - Acetic acid provides additional stability for both psilocin and psilocybin.
  - Using more than 0.5% acetic acid (v/v) can result in reduction of extraction efficiency.
- **Vortex Experiments**
  - 5-minute vortex showed inconsistent extraction of analytes.
  - 15-minute vortex showed best extraction of analytes without degradation.
  - 1000 RPM provided more consistent extraction efficiencies compared to 500 RPM.
  - Conversion of psilocybin to psilocin occurs when vortexed longer than 15 min.
- **Stability**
  - At 6 months, additional extraction of minor alkaloids can be observed, but degradation of psilocin occurs.

Final Sample Preparation Recommendation:

Weigh 500 mg homogenized mushroom into Environmental Express tube → Add 20 mL MeOH, 0.5%(v/v) Acetic Acid → Vortex 1000 RPM 15 minutes → Add filter, push with plunger → Remove plunger, aliquot sample into autosampler vial for analysis