

## Single Method for the Separation and Detection of Psilocybin, Related Tryptamines, and Beta-Carbolines Found in Psychedelic Mushrooms

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### Abstract

Psychedelic mushrooms have recently gained attention for their use in treating illnesses. Two classes of active compounds, tryptamines which includes psilocybin, and beta-carbolines which are in a class of known antidepressants, have been found in these mushrooms. The aim of this study was to separate a panel of six tryptamines and four beta-carbolines in a single chromatographic method. The compounds included in this panel act as Lewis bases, which are known to interact with the metal oxide layers found within standard stainless-steel HPLC systems. MaxPeak High Performance surfaces (HPS) Technology has been found to resolve problems associated with analyte metal-oxide interactions.<sup>1</sup> This is seen in the chromatographic results by way of increased peak area and height by up to 426% and 882%, respectively. In this paper we describe a method to separate a panel of ten tryptamines and beta-carbolines using an Arc™ Premier System, in combination with the Empower™ Sample Set Generator (SSG), and an XSelect™ Premier HSS T3 Column.

### Benefits

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- Significant improvement in chromatographic performance when using MaxPeak HPS Technology versus standard stainless-steel HPLC systems
  - A greener method, developed using more environmentally friendly methanol as the strong solvent
  - A single 15-minute method for the baseline separation of a panel of tryptamines and beta-carbolines
  - Rapid creation of instrument methods and sample sets using the Empower Sample Set Generator
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## Introduction

In recent years, there has been a renewed focus on the study of compounds found in psychedelic mushrooms. Most studies have focused on the pharmaceutical potential of the tryptamine psilocybin, specifically its use in treating disorders including cluster headaches, anxiety, depression, obsessive-compulsive disorder, post-traumatic stress disorder, and substance abuse.<sup>2</sup> Beyond the pharmaceutical potential of these mushrooms their legalization for recreational use has also been a rising trend in some geographical regions.<sup>3</sup>

Proper dosing through the consumption of raw mushrooms can be difficult due to the vast differences in the quantity of active compounds that are found in various genera, species, variations, and growing conditions.<sup>4</sup> Additionally adverse reactions including muscle weakness have been anecdotally reported when consuming psychedelic mushrooms. This effect has been speculated to be caused by aeruginascin, one of psilocybin's related tryptamines.<sup>5</sup> The beta-carbolines found in these mushrooms are a type of mono-amine oxidase inhibitor (MAOI). MAOIs are generally prescribed as antidepressants but can also affect the metabolism of tryptamines.<sup>6</sup> The potential good and bad synergistic effects of this mixture of compounds has yet to be thoroughly investigated. To help facilitate clinical research, safety, and accuracy of dosing a simple fast method for the separation of these compounds is needed.

In the work presented here an HPLC method was developed for the separation of ten tryptamine and beta-carboline compounds. Chemical structures of these compounds are shown in Figure 1. The Empower SSG was used to automate the instrument method creation for the optimization step of the method development process. Crucially, Waters™ MaxPeak HPS Technology was utilized to substantially reduce the risks associated with analyte metal-oxide interactions.

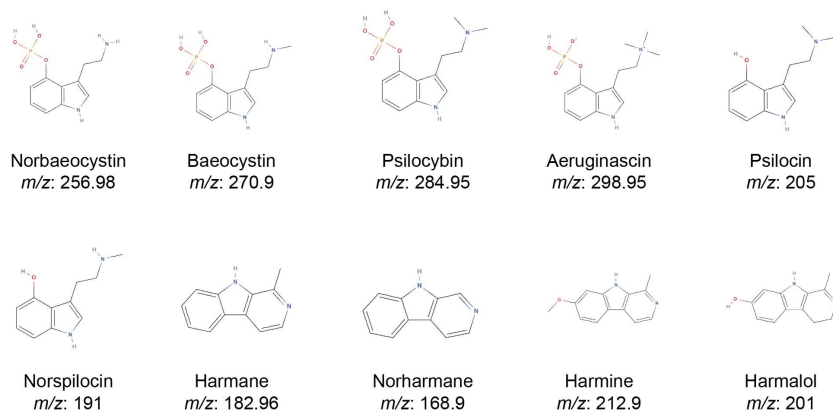


Figure 1. Chemical structures and corresponding *m/z* of all ten compounds used in this study.

## Experimental

### Sample Description

Psilocybin and psilocin dissolved in 1:1 acetonitrile:water were individually obtained from Sigma Aldrich (Darmstadt, Germany). Baeocystin, aeruginascin, norbaeocystin, norpsilocin, harmane, harmine, norharmane, harmalol were obtained from Caymen Chemicals (Michigan, USA)

A panel of tryptamines and beta-carbolines was prepared from standards to imitate active compounds that are found in psychedelic mushrooms. These compounds are generally extracted using methanol.<sup>7</sup> To reflect this, a panel of all ten compounds was prepared in 5% water, 5% acetonitrile, and 90% methanol. Psilocybin, psilocin, baeocystin, aeruginascin, norbaeocystin, norpsilocin, and harmalol were prepared at 0.05 mg/mL. Harmane, harmine, and norharmane were prepared at 0.025 mg/mL.

### LC Conditions

LC system:

Arc Premier QSM-r

Detection: PDA 2998 @ 254 nm

Vials: 1 mL Total Recovery Vial, p/n: 186000385DV

Column(s): XSelect Premier HSS T3 Column, 100 Å, 2.5 µm, 4.6 x 100 mm p/n: 186009859

Column temperature: 25 °C

Sample temperature: 15 °C

Injection volume: 1 µL

Flow rate: 0.75 ml/min

Mobile phase A: 0.1% Formic Acid in DI Water

Mobile phase B: 0.1% Formic Acid in Methanol

## Gradient Table

Time (min)	Flow rate (mL/min)	%A	%B	Curve
0	0.75	88	12	6
1	0.75	88	12	6
10	0.75	45	55	6
10.2	0.75	5	95	6
12	0.75	5	95	6
12.1	0.75	88	12	6
15	0.75	88	12	6

## MS Conditions

MS system:	ACQUITY QDa™ Mass Detector
Ionization mode:	Positive
Acquisition range:	100–400
Capillary voltage:	0.8 Kv
Cone voltage:	15 v

## Data Management

Chromatography software:	Empower 3.8.0
MS software:	Empower 3.8.0
Informatics:	Empower 3.8.0

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## Results and Discussion

### Method Development

Four column chemistries with varying selectivity properties were screened during the method development process. These columns included: XBridge™ Premier BEH C<sub>18</sub>, XSelect Premier CSH C<sub>18</sub>, XSelect Premier CSH phenyl hexyl, and XSelect Premier HSS T3. All columns were 2.5 µm, 4.6 x 100 mm. Screening runs utilized both the PDA and the QDa mass detector for peak identification. Data showed that the XSelect Premier HSS T3 Column had the best chromatographic peak shape and the best retention of analytes. Due to the superior performance, the XSelect Premier HSS T3 Column was selected for further method optimization.

The optimization of the method utilized the Empower SSG to investigate parameters such as gradient time,

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strong solvent percent, temperature, flow rate, and strong solvent type. To use this application a user must create a CSV spreadsheet that can be imported into the SSG. Upon following the prompts, the application will create a sample set to your specifications. This can reduce the errors and time associated with creating instrument methods and sample sets individually.

When analyzing the optimization data, the second and third chromatographic peak representing aeruginascin and baecocystin were identified as the critical pair. In the final method these two compounds were fully baseline resolved to a USP resolution of 1.95, as can be observed in Figure 2. This was accomplished by using a relatively low column temperature of 25 °C, a flow rate of 0.75 mL/min, and a faster gradient time of nine minutes from 12–55% methanol. When comparing methanol to acetonitrile, better retention and chromatographic performance were observed. Methanol was therefore selected for the final method with the additional benefit of being a “greener” solvent.

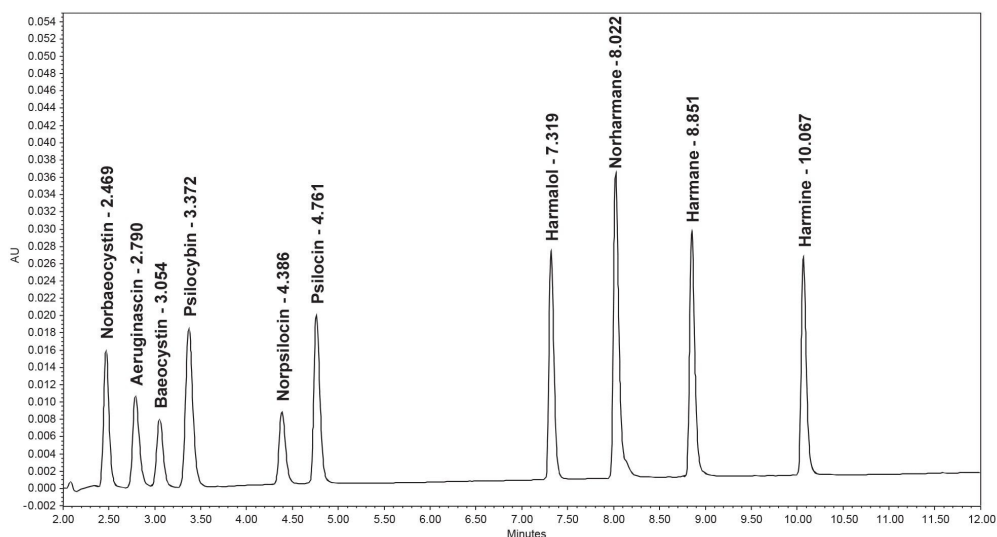


Figure 2. Representative chromatogram produced from the final method run on the Arc Premier System and columns featuring MaxPeak HPS Technology.

Chromatographic USP resolution, measured at base, produced values near two for all known compounds. Peak tailing was found to be adequate for all peaks. Norharmine was the only compound with a tailing factor greater than 1.5. The resolution and tailing of each compound run on the Arc Premier System and columns featuring MaxPeak HPS Technology can be found in Table 1.

Chromatographic peak characteristics		
Component	Resolution	Tailing
Aeruginascin	2.56	1.43
Baeocystin	1.95	1.17
Harmalol	23	1.34
Harmane	7.8	1.39
Harmine	11.84	1.36
Norbaeocystin	NA	1.24
Norharmane	6.77	1.62
Norpsilocin	7.38	1.23
Psilocin	3.02	1.32
Psilocybin	2.27	1.2

*Table 1. Chromatographic peak resolution and tailing of the final method run on the Arc Premier System.*

## MaxPeak HPS Versus Standard Surface Technology

To assess the impact of MaxPeak HPS Technology, the same analysis was run on a standard stainless-steel ACQUITY Arc System with a standard column. The resulting chromatogram can be seen in Figure 3. Using MaxPeak HPS Technology, the compounds in this analysis saw an increase in chromatographic peak height and area when compared to standard stainless-steel. Notably, the norharmane peak saw substantially increased height, area, and a decrease in tailing from 2.3 to 1.6 when using MaxPeak HPS Technology.

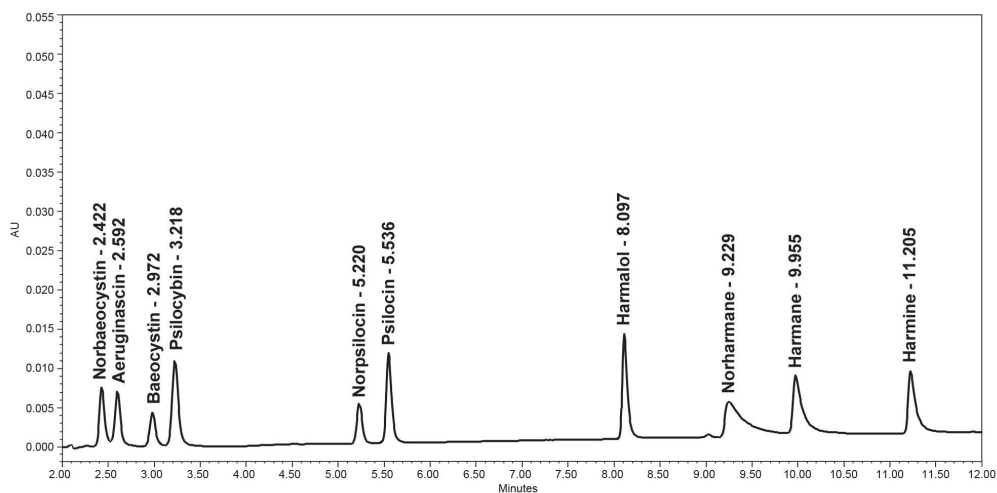


Figure 3. Representative chromatogram obtained with the final method on the ACQUITY Arc System containing standard stainless-steel hardware and columns.

Ten replicate injections of this method were simultaneously run on both HPLC configurations. The results of this experiment can be seen in Table 2 and Table 3. Calculations show this technology can reduce the relative standard deviation of chromatographic peak area and height, leading to a more reproducible method.

Chromatographic peak area MaxPeak HPS vs. stainless steel HPLC configurations						
Component	Average area response (10 injections)			Average area % RSD (10 injections)		
	HPS	Stainless-steel (SS)	HPS area/SS area	HPS	Stainless-steel (SS)	HPS/SS
Aeruginascin	56966	30311	188%	0.8	1.4	57%
Baeocystin	41167	19294	213%	0.6	1.8	33%
Harmalol	99810	52215	191%	0.4	1.8	22%
Harmane	112157	49021	229%	0.6	2.3	26%
Harmine	95821	44069	217%	0.6	1.9	32%
Norbaeocystin	72071	31817	227%	1.1	1.5	73%
Norharmine	148642	34896	426%	0.6	7.1	8%
Norpsilocin	40189	21097	190%	0.6	2	30%
Psilocin	88751	46678	190%	0.4	1.8	22%
Psilocybin	101713	52794	193%	0.4	1.8	22%

Table 2. Comparison of chromatographic peak areas on standard stainless-steel surfaces versus MaxPeak HPS surfaces.



Chromatographic peak height MaxPeak HPS vs. stainless steel HPLC configurations						
Component	Average height response (10 injections)			Average height % RSD (10 injections)		
	HPS	Stainless-steel (SS)	HPS height/SS height	HPS	Stainless-steel (SS)	HPS/SS
Aeruginascin	10882	7130	153%	0.5	1.4	36%
Baeocystin	8176	4330	189%	0.4	1.4	29%
Harmalol	26570	13461	197%	0.5	1.8	28%
Harmine	28455	7396	385%	0.7	2.4	29%
Harmine	25370	7895	321%	0.6	2.1	29%
Norbaeocystin	16316	7778	210%	0.4	1.4	29%
Norharmine	35524	4026	882%	0.7	2.6	27%
Norpsilocin	8388	5198	161%	0.4	1.8	22%
Psilocin	19638	11499	171%	0.4	1.8	22%
Psilocybin	18580	11023	169%	0.4	1.5	27%

Table 3. Comparison of chromatographic peak height on standard stainless-steel surfaces versus MaxPeak HPS surfaces.

## Conclusion

A rapid 15-minute method with baseline resolution was created to separate ten compounds that are present in psychedelic mushrooms. These compounds, which have recently been studied for their pharmaceutical potential includes psilocybin, related tryptamines, and beta-carbolines. This method was successfully run on the Arc Premier HPLC System with an XSelect Premier HSS T3 Column in combination with methanol as the strong solvent. The use of methanol instead of acetonitrile provides a much more environmentally friendly HPLC method. A substantial improvement in general chromatographic properties was observed when using the MaxPeak HPS Technology compared to standard stainless-steel HPLC configurations. Using MaxPeak HPS resulted in an increase in chromatographic peak area and height by up to 426% and 882% respectively.

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