Determination of Psilocybin and Psilocin in Hallucinogenic Mushrooms by HPLC with Diode Array and MS Detection

by

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Glossary of Terms

Symbol		**Unit**
a	Length of perpendicular line drawn from center vertical line of the peak to right side of peak at 10% of the peak height	min
ACN	Acetonitrile	
API-ES	Atmospheric pressure ionization-electrospray	
α	Separation factor	
amu	Atomic mass unit	
b	Length (min) of perpendicular line drawn from center vertical line of the peak to left side of peak at 10% of the peak height	min
CID	Collision-induced-dissociation	
DL	Detection Limit	
GC-MS	Gas chromatography-mass spectrometry	
IS	Internal Standard	
k	Capacity factor	
LC/MS	Liquid Chromatography Mass Spectrometry	
M + H	Molecular ion plus hydrogen	
M + Na	Molecular ion plus sodium	
MS	Mass Spectrometer	
m/z	Mass to charge ratio	
MeOH	Methanol	
N	Theoretical plates	
P_{ase}	Phosphatase activity	µmole min^{-1}
pNPP	p-nitrophenate	
RP-HPLC	Reverse Phase High Performance Liquid Chromatography	
RP-HPLC-MS	Reverse Phase High Performance Liquid Chromatography Mass Spectrometry	
RP-HPLC-MS^2	Reverse Phase High Performance Liquid Chromatography Mass $Spectrometry^2$	
TCA	Trichloroacetic acid	
SID	Source-induced dissociation	
TFA	Trifluoroacetic acid	

t_r	Retention time	min
t_v	Void volume	min
UV	Ultraviolet	
V_f	Total assay volume	mL
v/v	Volume per volume ratio	
$w_{0.5}$	Width of peak at half height	min

Abstract

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Psilocin and psilocybin are the main active components in hallucinogenic mushrooms. However, their chromatographic quantification has been problematic due in part to the poor chromatographic separation of these compounds, co-elution of interfering compounds, and incomplete extraction of psilocin and psilocybin from mushroom samples. Therefore, a study was undertaken to optimize 1) the extraction of these compounds from mushrooms and 2) the quantification of psilocin and psilocybin by reversed phase high performance liquid chromatography (RP-HPLC) with diode array or mass spectrometry (MS) detection. The effects of the mobile phase on the presence of interfering compounds, the relative separation, and peak shape were examined for psilocybin, psilocin, bufotenine and tryptamine (internal standard). Factors examined included, buffer concentration, buffer type, buffer pH and organic phase type. These parameters all affected chromatographic separations, with the largest effect seen when the buffer type or pH were changed. A short and effective extraction procedure was also developed to quantify these analytes by RP-HPLC with diode array detection and to qualitatively identify them by RP-HPLC with mass spectrometry detection. The estimated detection limit of this method, with a signal-to-noise ratio of three, was 10 ng/mL for diode array detection and 0.1 ng/mL for MS detection of psilocin and psilocybin in methanolic mushroom extracts. Psilocin and psilocybin were stable for a minimum of one year when stored in an acidic methanolic solution that was sparged with high purity nitrogen gas, sealed in an amber vial and stored at -11 ^{o}C.

KEYWORDS: Forensic Science, psilocybin, psilocin, reverse phase high performance liquid chromatography, liquid chromatography mass spectrometry, hallucinogenic mushrooms, HPLC.

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CHAPTER 1

Introduction

Hallucinogenic mushrooms have been imbibed for centuries, especially in Indian cultures of Mexico and Central America. More recently in the twentieth century, especially during the 1960s, these “magic mushrooms” have made there way into pop culture. It is therefore not surprising that extensive research has been undertaken in the United States and around the world to identify the active components in hallucinogenic mushrooms. Studies have also been conducted to determine 1) mushroom species that contained hallucinogenic compounds (e.g., Gartz, 1994), 2) concentrations of active compounds in hallucinogenic mushrooms, and 3) the environmental and physiological conditions leading to the production of these compounds (Casale, 1985).

Two main psychoactive substances have been detected in hallucinogenic mushrooms, psilocybin (4-phosphoryloxy-N, N-dimethyltryptamine) and psilocin (4-hydroxy-N, N-dimethyltryptamine). Psilocybin and psilocin are controlled by federal and state laws. In New York, psilocybin and psilocin are classified as hallucinogens. Under this designation, all forensic laboratories in New York State are required to quantitate these compounds from mushroom material in order to determine penal law charges. One problem with this mandate, however, is that an accurate and sensitive technique to extract and quantify psilocybin and psilocin in mushrooms does not exist. This results from poorly characterized 1) extraction procedures, 2) stability assessments and 3) chromatographic methods. This may have led to many studies characterizing psilocin as a minor component in mushroom samples, when this may not have been the case. Samples may have contained psilocin, but it may not have been detected because it was not extracted or it degraded during sample storage or analysis.

Numerous analytical techniques have been developed to quantify psilocybin and psilocin in hallucinogenic mushrooms. The most common approach to quantify these compounds is by reversed phase liquid chromatography. It is surprising that, given the many analytical techniques that have been developed over the past thirty years, no one has systematically assessed or optimized the chromatographic conditions used to separate psilocybin and psilocin from each other or from other interferences that may be present in extracted mushrooms. It is even more surprising that no one has systematically examined extraction techniques, since the success of any analytical approach to quantify psilocin and psilocybin in mushrooms requires that these compounds be extracted reproducibly.

Given these limitations, there were two goals of the present study**.** The first goal was to optimize the chromatographic procedure for the quantification of psilocin and psilocybin in mushroom samples. The second goal was to determine the best solvent system to extract these compounds from ground mushroom samples and to determine the stability of psilocin and psilocybin in standards and mushroom extracts. To accomplish these goals, specific objectives of this study were to: 1) evaluate the effect of mobile phase buffer and organic phase on the chromatographic separation of psilocin and psilocybin (Chapter 3); 2) optimize the selectivity and detection limit of the chromatographic method for the analysis of hallucinogenic mushroom material for psilocybin and psilocin by employing diode array detection and mass spectrometry (Chapter 3 and 4); 3) conduct a kinetic analysis to evaluate the stability of psilocin and psilocybin standard solutions and mushroom extracts (Chapter 3); and 4) determine the best procedure to extract psilocybin and psilocin from ground mushroom samples (Chapter 4).

CHAPTER 2

Background

History. Hallucinogenic mushrooms have been used for centuries by many cultures for medicinal purposes and in religious ceremonies. Some of the earliest reports of mushroom use dates back over 2000 years ago by the Mesoamerican Indians, and perhaps even earlier, from 7000-9000 years ago, in the Saharan Desert. The identities of the compounds responsible for the mushrooms psychoactive properties were not identified until the 1950s by Hoffman et al. (1959). They were able to isolate and identify psilocybin and psilocin from the mushroom species *Psilocybe mexicana.* Now there are over one hundred mushroom species from the genera Psilocybe, Conocybe, Panaeolus, Inocybe, Pluteus, Copelandia, and Gymnopilus that are known to contain the psychoactive compounds psilocybin and psilocin (e.g., Casale, 1985; Gross, 2000; Rodriguez-Cruz, 2005). Many of these species commonly grow in moist, organic-rich environments such as found in animal dung and rice patties. In addition to psilocin and psilocybin, several other compounds have also been identified in mushrooms including baeocystin, nor-baeocystin which are demethylated derivatives of psilocybin, ergosterol, ergosteral peroxide, and α,α-trehalose (Hoffman et. al., 1959; Casale, 1985). In the United States, hallucinogenic mushroom use spiked in the 1960s as part of the "psychedelic" movement and this caused psilocybin and psilocin to be classified under schedule 1 of the controlled substance act. This became important to law enforcement and forensic laboratories as they had to develop methods to quantify these substances.

Methods of Analysis. Over the years there have been countless analytical techniques developed to identify and quantify psilocybin and/or psilocin in mushroom material. Techniques that were developed included thin layer chromatography (Beug and

Bigwood, 1981; Christiansen and Rasmussen, 1982; Vanhaelen-Fastre and Vanhaelen, 1984; Wurst et al., 1984; Wurst et al., 1992; Gross, 2000; Sarwar and McDonald, 2003), gas chromatography- mass spectrometry (Repke et al., 1977; Christiansen and Rasmussen, 1982; Wurst et al., 1984; Casale, 1985; Wurst et al., 1992; Keller et al., 1999; Gross, 2000; Sticht and Kaferstein, 2000; Sarwar and McDonald, 2003), ion mobility spectrometry (Keller et al. 1999), capillary zone electrophoresis (Pedersen-bjergaard et al., 1997), ultraviolet spectroscopy (Wurst et al., 1984; Lee, 1985; Wurst et al., 1992), infrared spectroscopy (Casale, 1985; Lee, 1985), high performance liquid chromatography with ultraviolet detection (White, 1979; Perkal et al., 1980; Thomson, 1980; Beug and Bigwood, 1981; Christiansen et al., 1981; Christiansen and Rasmussen, 1982; Sottolano and Lurie, 1983; Vanhaelen-Fastre and Vanhaelen, 1984; Wurst et al. 1984; Wurst et al., 1992; Gartz, 1993; Tsujikawa et al., 2003; Anastos et al., 2006), high performance liquid chromatography with fluorescence detection (Christiansen et al., 1981; Vanhaelen-Fastre and Vanhaelen, 1984; Saito et al., 2004), high performance liquid chromatography with electrochemical detection (Wurst et al. 1984; Kysilka and Wurst, 1990; Wurst et al., 1992; Lindenblatt et al., 1998; Hasler et al., 2002), and high performance liquid chromatography with electrospray mass spectrometric detection (Kamata et al., 2003; Saito et al., 2004; Kamata et al., 2005; Rodriguez-Cruz, 2005).

The earliest techniques employed gas chromatography as a technique for the detection of psilocybin and psilocin in mushroom samples (Repke et al., 1977; Christiansen and Rasmussen, 1982). A serious drawback to this approach was that psilocybin dephosphorylated to psilocin in the injection port. One technique commonly used to offset this problem was to derivatize psilocin and psilocybin with agents such as

N-(methyl-N-(tert-butyl-dimethylsilyl) trifluoroacetamide (Wurst et al. 1992) and N-methyl-N-trimethylsilyltrifluoroacetamide (Kellar et al., 1999; Sticht and Kaeferstein, 2000). However, derivatization is a time consuming technique and many derivatization reagents are hazardous.

The method of choice for mushroom analysis is reversed-phase high performance liquid chromatography (RP-HPLC), owing to the high water solubility of psilocin and psilocybin. In many RP-HPLC techniques, psilocybin and psilocin are separated employing ion pair chromatography, which makes it impossible to detect psilocybin and psilocin by mass spectroscopy. When an ion pair is used in the mobile phase, psilocybin and psilocin are detected by either fluorescence or absorbance. This presents some problems, as baeocystin is a demethylated derivative of psilocybin (Hoffman et al., 1959) and is a common component found in mushrooms. Baeocystin and psilocybin have nearly the same absorbance spectrum (Leung and Paul, 1968; Christiansen and Rasmussen, 1982) and these compounds co-elute in some chromatographic systems (Beug and Bigwood, 1981) making their quantification difficult.

When simpler mobile phases are used, especially ones with volatile buffers, RP-HPLC can be used in conjunction with mass spectrometry to obtain structural confirmation. This is a critical step in method development for mushroom analyses because forensic labs are required to positively identify illicit compounds by infrared spectroscopy or mass spectrometry. Only two RP-HPLC mass spectrometry methods have been published on the identification of psilocybin and psilocin in standards or mushroom extracts. Kamata et al. (2005) conducted the first detailed study of psilocin and psilocybin by RP-HPLC-MS andRP-HPLC-MS^2. They found both techniques to be highly sensitive (e.g., pg detection limit), but as might be expected tandem mass spectrometric detection also improved the selectivity of the method, especially when applied to mushroom extracts. Rodriquez-Cruz (2005) used RP-HPLC to separate psilocybin and psilocin and MS fragmentation techniques (collision-induced-dissociation (CID) and source-induced dissociation (SID)) for positive identification of these indole alkaloids. The CID and SID fragmentation "fingerprinting" techniques allow for structural confirmation similar to that obtained by electron impact MS.

Despite all the liquid chromatographic approaches that have been developed, nothing has been published that has systematically assessed or optimized the chromatographic conditions used to separate psilocybin and psilocin from each other or from other interferences that may be present in extracted mushroom samples. Two papers examined the effect of buffer pH on the separation of psilocybin and psilocin. Pedersen-Bjergaard et al. (1997) conducted a pH study for the background electrolyte (running buffer) in capillary zone electrophoresis to determine its effect on the separation of psilocybin, baeocystin and psilocin. Using a 10 mM borate-phosphate buffer, psilocin was separated from psilocybin and baeocystin at all pHs examined between 4 and 12.5, while psilocybin and baeocystin were not baseline resolved at any pH. The best separation was achieved at a high pH (11.5). Thomson (1980) examined the effect of phosphate buffer pH (4.6-7.5) on the separation of psilocin and psilocybin by RP-HPLC. As the mobile phase pH increased, Thomson observed that the retention time of psilocin more than doubled, while the retention time of psilocybin decreased by approximately 40%. Thomson also found that a pH < 7.0 resulted in good chromatographic separation, with psilocybin and psilocin resolved from several unknown interfering peaks. Other than these studies, no one has examined the effect of buffer type, buffer concentration, buffer additives (e.g., ion pair reagents), or organic phase type (or concentration) on the efficiency and selectivity of the chromatographic separation of psilocin and psilocybin. There have also been no studies done to examine the effect of column type (e.g., amine vs C_{18}) on the relative separation of these compounds.

Stability. Even though the literature is replete with studies that have examined the stability of psilocin and psilocybin in standard solutions and extracts, these studies tend to be qualitative providing no quantitative information such as rate constants and half-lives. Anastos et al. (2006) conducted a time course experiment (three time points) to examine the stability of psilocin and psilocybin over a period of 14 days. They concluded that protecting aqueous standards from laboratory lighting significantly prolonged the useful life of the standards, with aqueous solutions of both psilocin and psilocybin stable over a period of 7 days (i.e., no loss observed) when stored at room temperature. Beug and Bigwood (1981) found methanolic mushroom extracts could be stored in a freezer at –5 ^{o}C for over 1 yr with little change (quantitative information is not supplied in this paper). However, when extracts were stored at room temperature there was a complete loss of psilocin and some loss of psilocybin, albeit no quantitative values were listed. Christiansen et al. (1981) stated that their methanolic mushroom extracts containing 10% 1M ammonium nitrate were stable for several weeks (storage conditions not given), but again no quantitative results were reported.

One important factor that affects the stability of psilocin is that it is easily oxidized (Lindenblatt et al., 1998; Hasler et al., 2002; Anastos et al., 2006). Anastos et al.

(2006) pointed out that the indoles, serotonin and 5-hydroxyindooeacetic acid, were susceptible to oxidative degradation, but they never determined whether this was also true for other indoles such as psilocybin and psilocin. Hasler et al. (2002) reported that psilocin was readily oxidized in the body, but again no experimental results were presented to support this supposition.

Although past studies do not provide a quantitative assessment of the stability of psilocin and psilocybin in standards or extracts, they do indicate that light exposure and molecular oxygen can be important in controlling the stability of these compounds. Clearly, oxidation plays an important roll in the breakdown of psilocin in solution and psilocybin is easily dephosphorylated (Casale, 1985), but quantitative details are missing. Studies are needed to determine the effect of different parameters on the stability of psilocin and psilocybin. Parameters to consider include temperature, metal and oxygen concentrations, phosphatase enzyme activity in mushroom extracts, and mushroom to mushroom variability in stability (within and between different mushroom species).

Extraction. The ability to efficiently extract psilocybin and psilocin from mushroom material is probably the most important step in any method that is developed to quantify these compounds in hallucinogenic mushrooms. Methanol is the most common solvent used to extract psilocybin and psilocin from mushrooms (e.g., Perkal et al., 1980; Thomson, 1980; Beug and Bigwood, 1981; Sottolano and Lurie, 1983; Wurst et al., 1984; Lee, 1985; Gartz, 1994; Pedersen-Bjergaard et al., 1997; Gross, 2000; Tsujikawa, 2003; Kamata et al., 2005). In all of these methods, methanol extracted psilocybin from mushroom samples. Psilocin was generally not detected, however, either because it was in fact a minor component or because it was poorly extracted from mushrooms with methanol. The only exceptions to this general finding were Gartz (1994) who found that psilocin was the major component in the species *G. purpuratus* and *P. cyanescens*, Wurst et al. (1992) who detected more psilocin than psilocybin in *P. cyansescens*, and Tsujikawa et al. (2003) who found more psilocin than psilocybin in *Copelandia*. Many investigators noted other chromatographic peaks obtained from the methanol extracts. These interferences were often used as markers to optimize chromatographic methods.

Other extraction methods were employed to extract psilocybin and/or psilocin from mushrooms, but it was not always clear why these methods were developed. Methanol was used with aqueous ammonium nitrate or aqueous potassium nitrate to extract psilocybin from mushroom samples (Christiansen et al., 1981; Christiansen and Rasmussen, 1982). They found that psilocybin was the major component in *P. semilanceata*, a result that was comparable to what other investigators found using only methanol. Chloroform was used as an extraction solvent by Kellar et al. (1999), but no quantitative information was provided in this precolumn derivatization GC-MS method. A 50% ethanol and 50% water solution was used by Vanhaelen-Fastre and Vanhaelen (1984), and they determined psilocybin and baeocystin in *P. semilanceata*, but did not report results for psilocin. A solution of 75% ethanol with 25% water was used by Kysilka and Wurst (1990) and they increased the amount of psilocin extracted in *P. bohemica* ten fold, but it was still less than the amount of psilocybin extracted. Ethyl acetate was used by Saito et al. (2004) to extract psilocin, which was then dried with nitrogen gas and the psilocin derivatized with a fluorescent agent. Casale (1985) extracted mushrooms by boiling the sample with dilute acetic acid (pH 4). The extract was filtered and adjusted to pH 8 with concentrated ammonium hydroxide. This solution was extracted twice with diethyl ether. This method was used to identify psilocin, but no quantitative information was presented. Given the unknown extraction efficiencies of each of these methods and the different extraction conditions that were used, it was not possible to quantitatively compare results from different studies with respect to psilocin or psilocybin concentrations in mushrooms nor was it possible to determine the best extraction technique.

In addition to the wide range of extraction solvents that have been reported in the literature, there is no uniformity in the conditions used to extract psilocin and psilocybin from mushroom samples. In some methods, samples were not mixed and allowed to extract for 15 min (Lee, 1984), 1 h (Casale, 1985) or overnight (Christiansen et al., 1981; Gross, 2000; Kamata et al., 2005). In other methods, samples were stirred at room temperature for 12 h (Beug and Bigwood, 1981; Gartz, 1994); shaken by rotary inversion for 30 min (Christiansen et al., 1981; Christiansen et al., 1982); sonicated for 15 min (Pedersen-Bjergaard et al., 1997), 30 min (Tsujikawa et al., 2003; Saito et al., 2004) or 60 min (Sottolano and Lurie, 1983; Keller et al., 1999); roller mixed for 24 h (Thomson, 1980); mixed with a reciprocal shaker for 10-160 min (Kysilka and Wurst, 1990; Wurst et al., 1992); homogenized in methanol for 2 min (Perkal, et al., 1980; Wurst et al., 1984); or percolated through a micropercolator followed by maceration for 2 h (Vanhaelen-Fastre and Vanhaelen, 1984). As evident from this compilation of methods, there is considerable variation in the techniques and times used for mushroom extractions, but there is no consensus or study done to determine the best technique to apply to mushroom analyses. Some extraction techniques are very time consuming, while other extraction methods are very fast. Ideally, the extraction technique should be as short and efficient as possible to allow for maximum sample throughput and good recovery of both psilocin and psilocybin.

Another issue related to mushroom extraction, is the amount (and particle size—i.e., surface area) of mushroom sample that is extracted. There is a large range in sample sizes reported in the literature. For quantitative analysis, as little as 10 mg (Kysilka and Wurst,1990; Wurst et al., 1992; Gartz,1994; Tsujikawa et al., 2003) to as high as 600 mg of mushroom sample has been analyzed (Sottolano and Lurie, 1983). Qualitative procedures used even larger quantities of mushroom sample, usually ≥ ~2 grams (Lee, 1984; Casale, 1985). In almost all methods, dried mushroom samples were ground with a mortar and pestle prior to an extraction. However, no one has determined whether this approach leads to a homogenous sample or an efficient extraction of psilocin and psilocybin. A smaller sample size (10 mg) is likely less representative and less homogenous compared to what would be obtained from the same mushroom with a larger sample (ca. 500 mg), but again this has not been tested.

CHAPTER 3

Quantification of Mushroom-Derived Hallucinogenic Indoles Psilocin and Psilocybin by High Performance Liquid Chromatography

ABSTRACT

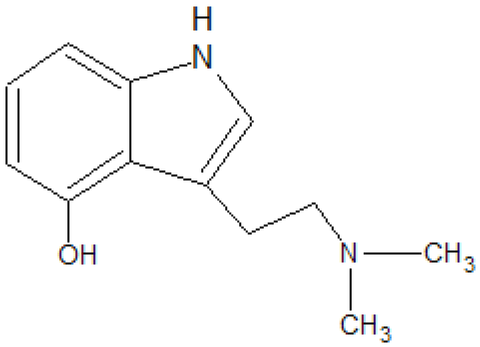
Psilocin and psilocybin are the main active components in hallucinogenic mushrooms. However, their chromatographic quantification has been problematic due in part to the poor chromatographic separation of these compounds, co-elution of interfering compounds, and incomplete extraction of psilocin and psilocybin from mushroom samples. Therefore, a study was undertaken to optimize the quantification of psilocin and psilocybin by reversed phase high performance liquid chromatography with diode array or mass spectrometry (MS) detection. This study evaluated the effect of the mobile phase on the presence of interfering compounds in the chromatogram, and on the relative separation and peak shape of psilocybin, psilocin, bufotenine and tryptamine. Factors that were examined included buffer concentration, buffer type, buffer pH and organic phase type. These parameters all affected chromatographic separations, with the largest effect seen when the buffer type or pH was changed. When all four compounds were considered, the worst chromatographic results were obtained (e.g., poor peak shapes) with 50 mM, pH 2.5 acetate, trifluoroacetate and ammonium acetate, while the best results were obtained with 50 mM trichloroacetate (pH 2.5). The estimated detection limit of this method, with a signal-to-noise ratio of three, was 10 ng/mL for diode array detection and 0.1 ng/mL for MS detection of psilocin and psilocybin in methanolic mushroom extracts. Psilocin and psilocybin in standards and mushroom extracts showed no loss after one year when stored in a solution of acidic methanol that was sparged with high purity nitrogen gas, sealed in an amber vial and stored at -11 ^{o}C.

INTRODUCTION

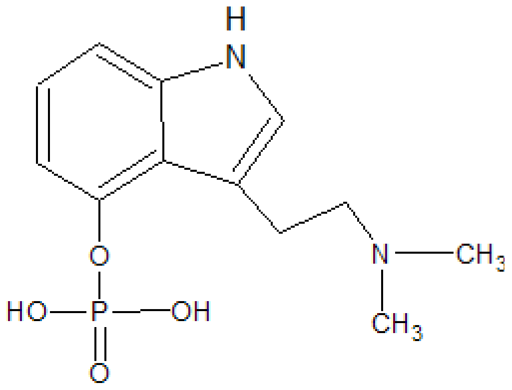
The use of hallucinogenic mushrooms dates back to at least 1500 BC where they were used in religious ceremonies in Mexico and Central America. Mushroom use remained in obscurity until 1953, when R. Gordon Wasson discovered the existence of a hallucinogenic mushroom cult in central Mexico. He obtained specimens of mushrooms that belonged to the genus *Psilocybe*. After successfully cultivating several species, Wasson isolated the active substances (Wasson, 1961). The two active compounds responsible for the hallucinogenic effects observed as a result of mushroom consumption were identified by Hoffmann et al. (1959) as psilocybin (4-phosphoryloxy-N, N-dimethyltryptamine) and psilocin (4-hydroxy-N, N-dimethyltryptamine).

In the United States, both psilocybin and psilocin (Figure 3.1) are controlled by federal and state laws. For example, psilocybin and psilocin are defined as hallucinogenic substances listed under Schedule I of the Controlled Substance Act, and the New York State Penal Law further defines these two compounds by classifying them as hallucinogens. This requires that all forensic laboratories in New York State quantitate these compounds in hallucinogenic mushrooms.

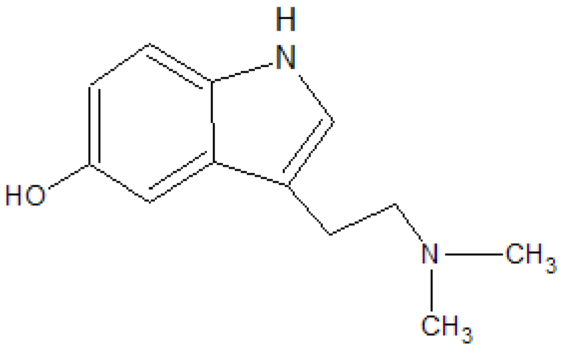
The first step to quantify psilocybin and psilocin in hallucinogenic mushrooms is to extract them from mushrooms into a chromatographically compatible solvent. Most published methods have relied on the use of methanol to extract psilocybin and psilocin (Hoffman, 1959; Perkal et al., 1980; Thomson, 1980; Beug and Bigwood, 1981; Sottolano and Lurie, 1983; Wurst et al., 1984; Lee, 1985; Gartz, 1993; Gross, 2000; Tsujikawa et al., 2003; Kamata et al., 2005). A few investigators have used methanol with added ammonium nitrate (Christiansen et al., 1981; Christiansen and Rasmussen,



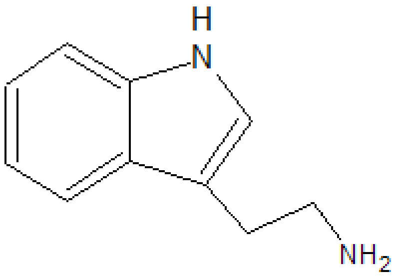
Psilocin
MW 204



Psilocybin
MW 284



Bufotenine
MW 204



Tryptamine
MW 160

Figure 3.1 General chemical structure of indole alkaloids examined in this study. The exact structure of each compound may change depending on the solution pH.

1982) or potassium nitrate (Wurst et al., 1992). Other solvents have been used as well including aqueous ethanol (Vanhaelen-Fastre and Vanhaelen, 1984; Kysilka and Wurst, 1990; Wurst et al., 1992), ethyl acetate (Saito et al., 2004), butyl chloride (Lee, 1985) and chloroform (Gross, 2000). Although many different extraction procedures have been employed, there has been no systematic study to examine the extraction efficiency or stability of psilocybin and psilocin in mushroom extracts. There has also been very little work done to identify potential interferences with co-extracted compounds such as baeocystin, a known chromatographic interference with psilocybin (Beug and Bigwood, 1981).

Once psilocybin and psilocin are extracted from mushrooms, they are quantified by chromatography. The early gas chromatographic (GC) techniques relied on the separation of underivatized psilocybin and psilocin. These GC methods are limited, however, owing to dephosphorylation of psilocybin to psilocin during the chromatographic analysis. Dephosphorylation was eliminated by employing derivatization agents such N-(methyl-N-(tert-butyl-dimethylsilyl) trifluoroacetamide (Wurst et al. 1992) N-methyl-N-trimethylsilyltrifluoroacetamide (Kellar et al., 1999; Sticht and Kaeferstein, 2000).

One of the most common techniques that has been used to quantify psilocybin and psilocin is reversed phase-high performance liquid chromatography (RP-HPLC), owing to the high water solubility of these compounds. Early RP-HPLC techniques relied on the use of mobile phase buffers with modifying agents or ion pair reagents such as 1-heptane sulfonic acid (Beug and Bigwood, 1981; Vanhaelen-Fastre and Vanhaelen, 1984) and certrimonium bromide (Thomson, 1980). Precolumn derivatization techniques have also been employed to modify psilocybin and psilocin prior to their RP-HPLC separation (Saito et al., 2004). However, these methods are relatively cumbersome and expensive, and prone to interferences that limit their detection in reversed phase-high performance liquid chromatography-mass spectrometry (RP-HPLC-MS). Most HPLC methods now rely on RP-HPLC separation of psilocybin and psilocin without precolumn derivatization by employing simple aqueous buffers such as acetic acid (Wurst et al., 1984 and 1992), ammonium acetate (Hasler et al., 2002), ammonium phosphate (Perkal et al., 1980), phosphoric acid (Sottolono et al., 1983), trifluoroacetic acid (TFA) (Saito et al., 2004) or ammonium formate (Tsujikawa et al., 2003; Kamata et al., 2005; Anastos et al., 2006). Published RP-HPLC techniques have generally used ultraviolet detection to quantify psilocybin and psilocin (White, 1979; Perkal et al., 1980; Beug and Bigwood, 1981; Christiansen et al., 1981; Sottolano and Lurie, 1983; Vanhaelen-Fastre and Vanhaelen, 1984; Wurst et al. 1984; Gartz, 1994; Thomson, 1998), although other detection systems have been used including fluorescence (Perkal et al., 1980; Christiansen et al., 1981; Saito et al., 2004), electrochemistry (Wurst et al., 1992; Lindenblatt et al., 1998; Hasler et al., 2002), and more recently electrospray ionization mass spectrometry (Saito et al., 2004; Kamata et al., 2005).

It is surprising that, given the many chromatographic approaches that have been developed over the past thirty years, no one has published a systematic assessment or optimization of the chromatographic conditions used to separate psilocybin and psilocin from each other or from other interferences that may be present in extracted mushrooms. Therefore, the goals of the present study were to: 1) evaluate the effect of mobile phase buffer and organic phase on the separation of psilocin and psilocybin, 2) optimize the selectivity and detection limit of the method for the analysis of hallucinogenic mushroom material for psilocybin and psilocin by employing mass spectrometry, and 3) evaluate the stability of psilocin and psilocybin in standard solutions and mushroom extracts that were used in this study.

METHODS

Chemicals. All chemicals were of the highest purity available (ACS grade or better) and, except where noted, were used without further purification. The acids (and their sodium salts) that were evaluated as mobile phase buffers in this study included acetic acid (Fisher Scientific, Houston, TX), formic acid (Sigma-Aldrich, St. Louis, MO), trifluoroacetic acid (TFA) (Spectrum Chemical, New Brunswick, NJ), trichloroacetic acid (TCA) (Spectrum Chemical, New Brunswick, NJ), citric acid (United States Biochemical Corp., Cleveland, OH), ammonium acetate (J.T. Baker, Phillipsburg, NJ) and phosphoric acid (EM Science, Cherry Hill, NJ). HPLC grade organic solvents methanol (MeOH) and acetonitrile (ACN) were purchased from Burdick and Jackson (Muskegon, MI); LC-MS solvents were Burdick and Jackson high purity or Fisher Scientific optima grade. Psilocybin and psilocin standards were purchased from Alltech Applied Sciences (State College, PA). Bufotenine and tryptamine (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO). High purity water used throughout this study was obtained from a US Filter DI water filtration system consisting of a 0.2 μm prefilter, carbon tank, two mixed ion exchange tanks, UV irradiation and sterilization, and a 0.2 μm final filtration. The resistivity of this water was $\geq$ 18.2 MΩ.

All mobile phase buffer solutions were 50 mM and adjusted to pH of 2.5 by the addition of a 50 mM solution of the sodium salt (of the appropriate acid) to a 50 mM solution of the acid, except for acetic acid and formic acid buffers that were pH-adjusted through dropwise addition of concentrated HCl. The pH study was accomplished employing a 50 mM citric acid solution that was pH-adjusted by addition of 50 mM tribasic citrate buffer. An Accumet AB15 pH meter from Fisher Scientific (Houston, Texas) was used to measure the pH of all solutions used in this study. It was calibrated with pH 4.0, 7.0, and 10.0 standard buffers from VWR (Westchester, PA). All mobile-phase buffers were vacuum-filtered through a 47 mm diameter, 0.45 μm Nylon filter (Millipore Corp., Bedford, MA) prior to use.

Apparatus. The HPLC consisted of a modular Hewlett Packard model 1100 system (Wilmington, DE) with an online vacuum degasser, quaternary gradient pump and high-speed proportioning valve, low-volume pulse dampener, automated liquid sampler (ALS) with 100 vial tray and programmable injection volumes from 0.1 μL to 1.8 mL, and a thermostated column compartment. The complete system was controlled by PC-based HP ChemStation software. Compounds were separated on a Hewlett Packard 4.6 x 250 mm Hypersil BDS C_{18} column, with 5 μm packing. Detection was accomplished by monitoring the absorbance with a Hewlett Packard diode array spectrophotometer set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. A second HPLC system was used for mass spectral detection of psilocybin and psilocin. This second system consisted of a Hewlett Packard model 1100 system (as described above), with a high pressure binary gradient pump and a HP model SL mass selective detector. For this HP system, a

Phenomenex Hydro-RP C_{18} column (3.0 x 150 mm) with 4 μm packing was used to separate psilocin and psilocybin.

Mass Spectrometer Settings. The Hewlett Packard series 1100 model SL mass spectrometer was outfitted with an atmospheric pressure ionization electrospray (API-ES) source. The API-ES was operated in positive polarity, with a scanning mass range from 50-320 amu. The fragmentor voltage was optimized for psilocybin and set at 130V. The spray chamber parameters were gas temperature 350 ^{o}C, drying gas flow rate 12.0 L min^{-1}, nebulizing pressure 60 psig, and capillary voltage 3250 V.

HPLC Conditions. Unless noted, chromatographic separations were performed by isocratic elution at 25 ^{o}C and at a flow rate of 1.0 mL min^{-1}. To optimize chromatographic separations, three types of experiments were conducted. The goal of these experiments was to test the effect of the mobile phase on the separation of psilocybin and psilocin. In the first experiment, the buffer type and organic phase were examined by varying the mobile phase composition from 5-25% organic phase (methanol or acetonitrile) and from 75%-95% 50 mM buffer (pH 2.5). Buffers that were examined included acetate, formate, phosphate, citrate, TFA, TCA and ammonium acetate. In the second experiment, the effect of buffer pH on chromatographic separations of psilocybin, bufotenine, psilocin and tryptamine was examined. For this study, the mobile phase consisted of 10% ACN and 90% 50 mM citric acid buffer, with an aqueous phase buffer pH ranging from 2.3 – 8.9. In the third experiment, the effect of buffer concentration on compound separation was performed using a citric acid buffer ranging from 1 to 100 mM, with separations carried out at a mobile phase composition of 10% ACN and 90% citric acid buffer (pH 2.5). All other chromatographic conditions remained the same as described above.

Analysis of psilocybin and psilocin by reversed-phase high performance liquid chromatography-mass spectrometry (RP-HPLC-MS) was performed using a gradient elution at 25 ^{o}C and a flow rate of 0.5 mL min^{-1}. The mobile phase consisted of (A) pH 2.5, 50 mM buffer and (B) methanol. The elution program was isocratic at 5% B from 0 to 3 min, 5 to 30% B from 3 to 5min, 30% B between 5 and 8 min, and 30 to 5% B from 8 to 9 min, followed by equilibration at 5% B from 9 to 18 min.

Sampling Procedure. Stock solutions of psilocin, psilocybin, bufotenine and tryptamine were made at 1.0 mg mL^{-1} in methanol containing 0.1% formic acid (v/v). Calibration standards were prepared from 1.0 mg mL^{-1} stock solutions of psilocin and psilocybin in the range of 0.01 – 0.20 mg mL^{-1}. An internal standard (IS) containing 0.5 mg mL^{-1} of tryptamine was prepared. Tryptamine was selected as the IS because it is structurally similar to psilocin and psilocybin and it was not found in the psilocybin-containing mushrooms used in this study. A 100 μL aliquot of this IS was added to all standards, controls and sample extracts that were placed in 2 mL amber borosilicate vials containing vial inserts and sealed with threaded caps containing Teflon-faced butyl rubber septa.

Mushroom samples were obtained from completed forensic case samples that were ordered for destruction and were obtained with a court order for research purposes. The mushroom samples were dried in a Lab-Line Imperial-V oven (Krackler Scientific, Albany N.Y.) overnight at 60 ^{o}C. The dried mushroom samples were ground with a hand cheese grater, poured into a cone, flattened and then cut into quarters. Opposite corners were selected and the above process was repeated. The second quartered samples were extracted according to Sottolano and Lurie (1983). Approximately 500 mg of sample was weighed out and placed in a 50 mL Erlenmeyer flask with 10 mL of vacuum-degassed methanol. The resultant solution was sonicated in an ultrasonic water bath (Sonicor model SC-5OT 50 Watt sonicator) for 1 h. The liquid was decanted and then centrifuged in a Dynac II centrifuge from Becton Dickinson (NJ) at 7500 RPM for 7 min. It should be noted that a better extraction procedure was developed after the completion of this study. The procedure that was adopted involved sequential extraction with 25 mL methanol followed by 25 mL of a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5) (Chapter 4). Extracted mushroom samples were prepared for analysis by pipetting 200 μL of extract into a 2 mL amber vial, followed by the addition of 200 μL of the IS dissolved in a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5).

Typically, samples were analyzed by HPLC soon after they were extracted (ca. 1-2 days). However, mushroom extracts and standards were stable for more than a year with no loss of psilocybin and psilocin if samples were deoxygenated with high purity N_2 gas and stored tightly sealed in amber vials at -11 ^{o}C.

Quantification and Peak Identification. The concentration of psilocybin and psilocin in mushroom material was quantified by linear regression analysis. Standard curves were generated by injecting 10 μL of several psilocin and psilocybin standards, with tryptamine added as an internal standard and plotting the ratio of analyte to internal standard versus concentration over the concentration range 0.005 - 0.20 mg mL^{-1}. Peaks were identified by their retention time and from the absorption spectrum obtained from the diode array detector or by the mass spectrum obtained from the mass selective detector consisting of the M + H peak (as well as the M + Na peak for psilocybin).

Detection Limit. The detection limit of the method for the determination of psilocybin and psilocin in mushroom material was estimated by taking two samples of ground mushroom that contained no psilocybin or psilocin, and adding a known, very low concentration of psilocybin and psilocin, which was then analyzed by RP-HPLC-MS. The concentration corresponding to an S/N ratio of three was then calculated to yield the detection limit of the method for a 10 μL injection.

RESULTS AND DISCUSSION

Optimization of Chromatographic Separation. The first goal of this study was to evaluate the effect of the mobile phase on the capacity factor (**k**) for psilocybin, bufotenine, psilocin and tryptamine:

$$\mathbf{k = (t_r - t_v) / t_v} \tag{3.1}$$

where $\mathbf{t_r}$ is the retention time (min) and $\mathbf{t_v}$ is the column dead time (void volume, min). The capacity factor was determined as a function of percent methanol and acetonitrile in the mobile phase (Figure 3.2). In general, the capacity factor for each compound decreased exponentially with an increase in organic phase concentration as expected (Dolan and Snyder, 1989). Acetonitrile was the stronger solvent compared to methanol resulting in smaller values of **k** at a given percentage of organic phase in the mobile

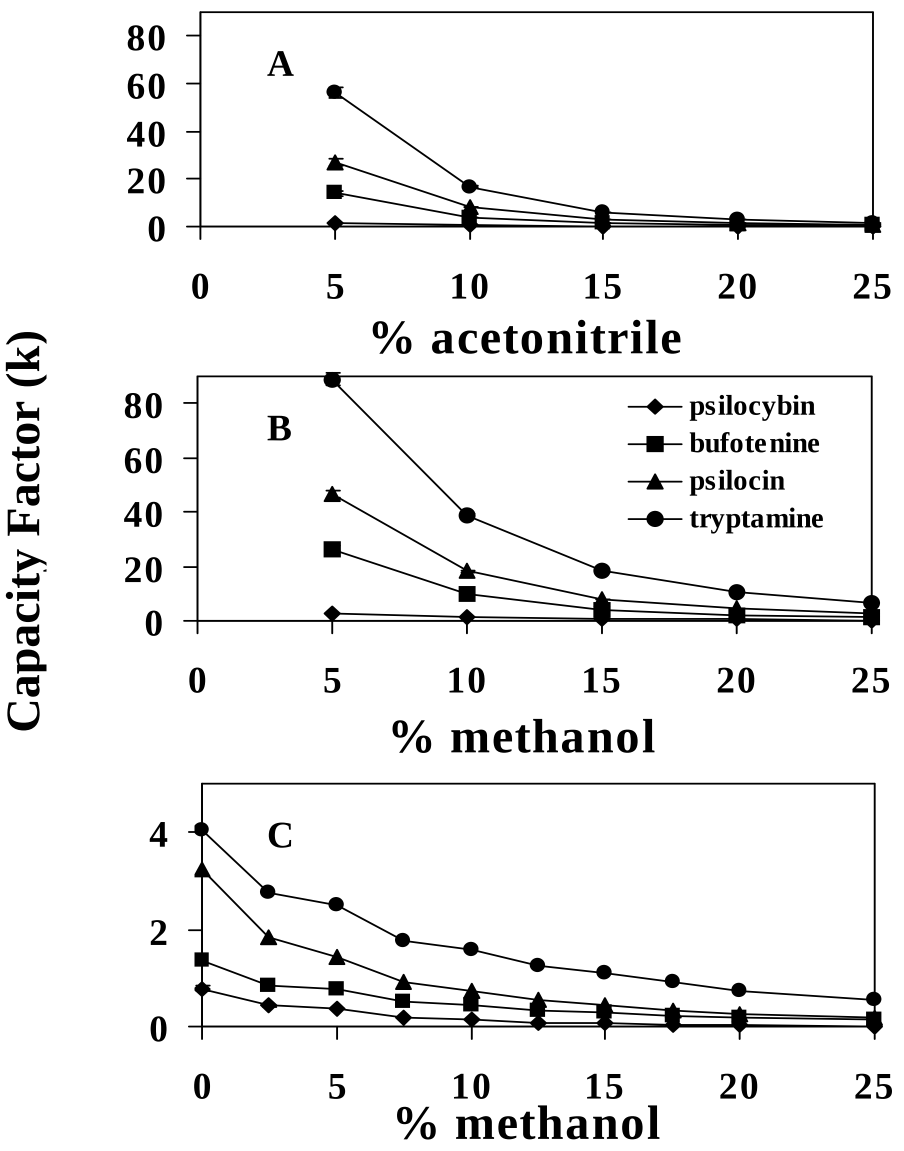


Figure 3.2 Effect of % organic phase on the capacity factor (k) of psilocybin, bufotenine, psilocin, and tryptamine as a function of percent acetonitrile or methanol in the mobile phase employing two mobile phase buffers: A) TCA, B) TCA, and C) phosphate. All buffers were pH 2.5 and 50 mM. Separations were performed by isocratic elution at 25 ^{o}C and a flow rate of 1 mL min^{-1}. Error bars denote the data range for duplicate injections of a standard, which for many compounds and conditions were smaller than the data points. Note difference in the y-scale between panels A-C.

phase, in accordance with relative solvent strengths of theses two solvents in RP-HPLC (Dolan and Snyder, 1989). For example, the value of k for psilocin was 18 in 10% methanol and only 8 in 10% acetonitrile (Figure 3.2, panels A and B). This trend was observed for all four compounds that were examined (data not shown for bufotenine and tryptamine). In addition to the organic component of the mobile phase, the mobile phase buffer also affected the capacity factor. In 10% methanol, the value of k was 18 for psilocin when TCA was used as the buffer compared to 0.4 in phosphate (Figure 3.2, panels B and C).

To further assess the effect of the buffer type on the chromatographic separation of psilocybin, bufotenine, psilocin and tryptamine, the organic phase composition was held constant at 20 % methanol and the buffer concentration was fixed at 50 mM (pH 2.5). For the seven different buffers that were examined, the mobile phase buffer had, in some cases, a dramatic effect on the selectivity of separation as evaluated by comparing the separation factor (α):

$$\alpha = \mathbf{k}_2/\mathbf{k}_1 \tag{3.2}$$

where $\mathbf{k}_1$ and $\mathbf{k}_2$ are the capacity factors for the earlier and later eluting compound, respectively. All compounds were baseline separated, but depending on the mobile phase composition (and specifically the buffer that was used) the separation factor varied over an order of magnitude from 1.7 to 40.7 (Table 3.1). For psilocin and either bufotenine or tryptamine, α changed very little between the different buffer types, ranging from 1.9 to 2.5. These small differences were observed even though the type of buffer used in the mobile phase resulted in some large changes in the capacity factor for psilocybin, bufotenine and tryptamine. This occurred because the capacity factor varied in the same

Table 3.1 Separation factor (α) for psilocin and psilocybin (p & pb), psilocin and bufotenine (p & b), psilocybin and psilocin (pb & p), psilocybin and tryptamine (pb & t), and psilocin and tryptamine (p & t). Compounds were separated at 25 °C and a flow rate of 1.0 mL min^{-1} by isocratic elution at 80% 50 mM buffer (pH 2.5) and 20% methanol.

Buffer	$\alpha_{\mathbf{p\ \&\ pb}}$	$\alpha_{\mathbf{p\ \&\ b}}$	$\alpha_{\mathbf{pb\ \&\ b}}$	$\alpha_{\mathbf{pb\ \&\ t}}$	$\alpha_{\mathbf{p\ \&\ t}}$
Phosphate	4.2	2.4	1.7	10.4	2.4
Citrate	4.5	2.3	1.9	11.3	2.4
Formate	5.6	2.1	2.5	13.2	2.3
Acetate	8.0	1.9	4.1	18.0	2.2
TCA	15.6	2.0	7.5	40.7	2.5
TFA	7.1	2.1	3.3	16.6	2.3
Ammonium acetate	16.1	2.0	7.8	31.5	1.9

proportion for all three compounds as a function of mobile phase type (Figure 3.3). This was not the case for psilocybin when compared to the other three compounds. Its capacity factor varied very little with mobile phase type (Figure 3.3), and therefore its separation factor relative to the other compounds varied tremendously depending on the buffer used. The lowest values of $\alpha_{p\ \&\ pb}$, $\alpha_{pb\ \&\ b}$ and $\alpha_{pb\ \&\ t}$ were observed when phosphate, citrate or formate were used in the mobile phase (Table 3.1); intermediate values of α were observed in acetate and TCA; and the highest values of α, approximately 3-4 times greater than the lowest values (e.g., 4.2-4.5 versus 15.6-16.1 for $\alpha_{p\ \&\ pb}$, see Table 3.1 column 2), were seen when TCA and ammonium acetate buffers were used. These results demonstrated that the selectivity changed significantly for psilocin relative to psilocybin when going from phosphate to TCA or ammonium acetate. This relative change in retention time (or capacity factor) can be used as one means to positively confirm the presence of psilocin in the chromatogram.

The mobile phase also strongly affected the chromatographic efficiency of psilocybin and psilocin, as determined from the theoretical plates for each compound. The number of theoretical plates (**N**) was calculated from the following equation:

$$\mathbf{N = 5.55(t_r / w_{0.5})^2} \qquad 3.3$$

where $\mathbf{t_r}$ is the retention time of the peak and $\mathbf{w_{0.5}}$ is the width of the peak at half height. The number of theoretical plates is an important criterion to evaluate in order to optimize the sensitivity of the method and the separation of psilocybin, bufotenine, psilocin and tryptamine. To assess the effect of the mobile phase type on N for each compound, standards were separated by isocratic elution at 20% methanol and 80% aqueous buffer (50 mM, pH 2.5). Seven buffers were tested, and the results are shown in Figure 3.4.

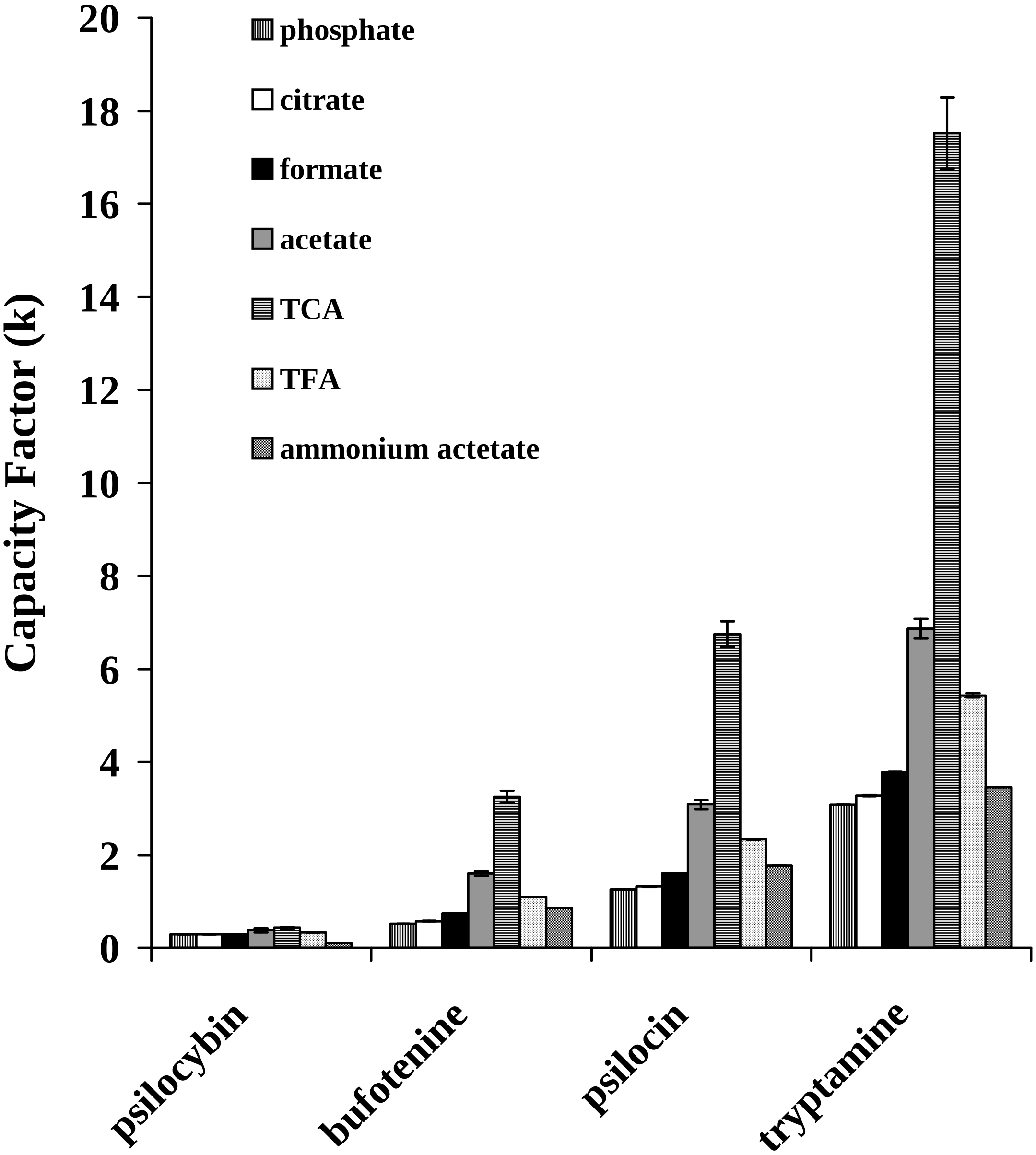


Figure 3.3 The average capacity factor (k) for each compound plotted for each buffer type used in the mobile phase. Each mobile phase consisted of 20% methanol and 80% 50 mM, pH 2.5 buffer. Analyses were performed at 25 ^{o}C and a flow rate of 1.0 mL min^{-1}. The column was a Hypersil C_{18} (4.6 x 250 mm, 5 μm packing) and the HP diode array detector was set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. Error bars denote the data range for duplicate injections of a standard.

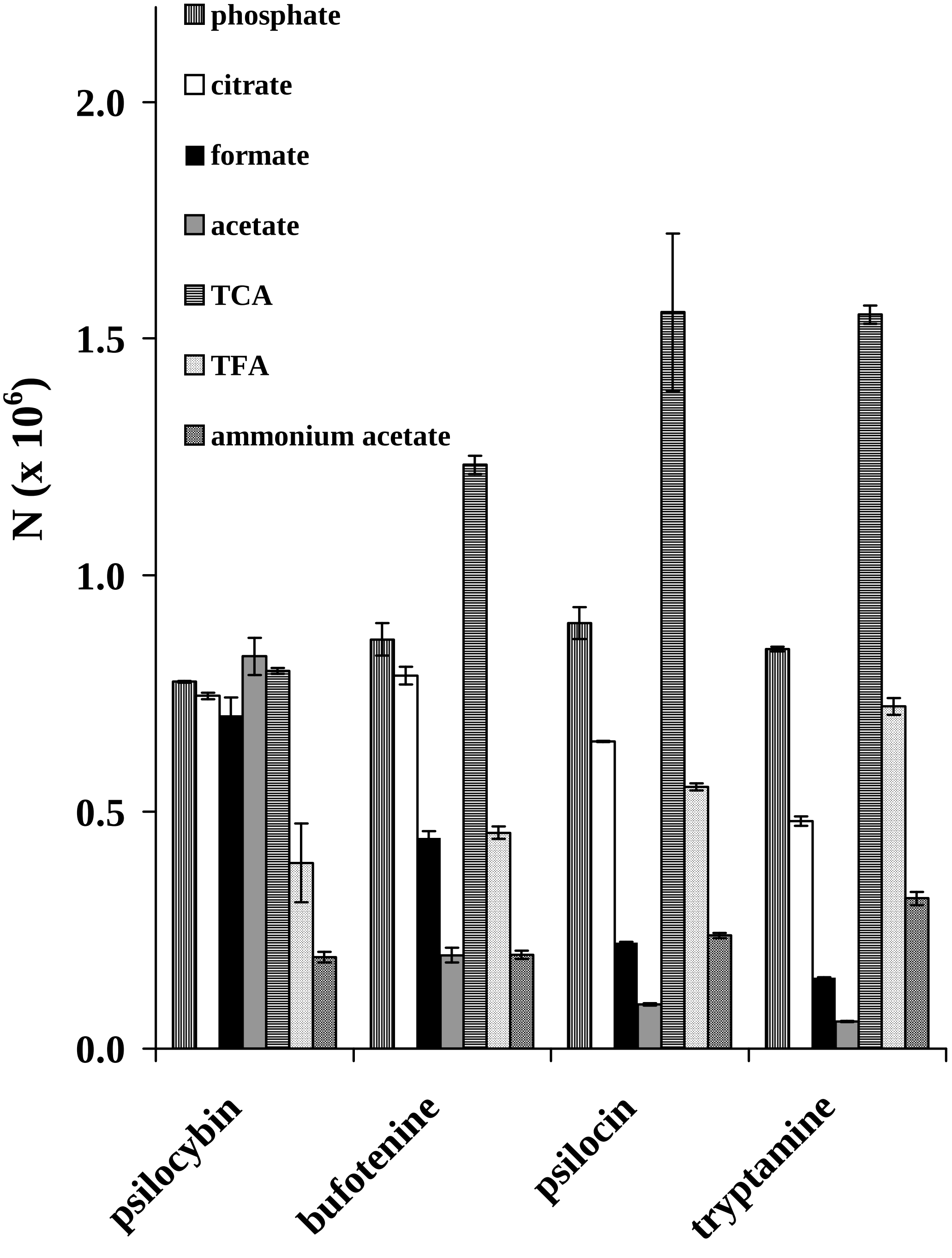


Figure 3.4 The average number of theoretical plates (N) calculated for each compound plotted for each buffer type used in the mobile phase. Each mobile phase consisted of 20% methanol and 80% 50 mM, pH 2.5 buffer. Analyses were performed at 25 °C and a flow rate of 1.0 mL min^{-1}. The column was a Hypersil C_{18} (4.6 x 250 mm, 5 μm packing) and the HP diode array detector was set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. Error bars denote the data range for duplicate injections of a standard.

Theoretical plates varied by more than two orders of magnitude, ranging from approximately 580 to 15600 plates. These values of N, in the 10^2-10^4 range, are typical of what is expected in analytical systems employing a standard, silica-based reversed phase HPLC column with 5 μm packing. What is interesting, however, is how much N varied with buffer-type, especially for the latter eluting peaks bufotenine, psilocin and tryptamine. Overall, these three compounds showed very similar trends in N as a function of mobile phase buffer type (Figure 3.4). The highest number of theoretical plates and therefore the best efficiencies (narrowest peaks) were observed when TCA was used as the buffer in the mobile phase (12330 – 15560 plates). Intermediate efficiencies were obtained in citrate and phosphate, while the worst efficiencies were observed in an acetate-buffered mobile phase (ca. 580-1970 plates).

The earliest eluting peak, psilocybin, showed a very different trend in N with buffer-type. For this compound, very little difference in N was observed for the different buffers (for citrate, phosphate, formate, acetate and TCA, values ranged between 7030-8290 plates), except that much lower values were observed in TFA (3920 plates) and ammonium acetate (1930 plates) (Figure 3.4). It is also interesting to note that N was the highest for psilocybin with a sodium acetate-buffered mobile phase compared to the other compounds where acetate yielded the lowest values of N. This probably was attributed to the low values of k observed for psilocybin (not enough time for the peak to broaden).

The large variations in N that were observed here, particularly for psilocin and tryptamine, demonstrated that there are strong interactions of the buffer with the analytes either directly or indirectly through interactions with the stationary-phase silanol groups.

The poor efficiencies that were observed in some cases were indicative of poor peak shapes observed in the HPLC chromatograms. In general, the best peak shapes (and higher efficiencies) were observed in a TFA-buffered mobile phase, while the worst peak shapes were observed when acetate was used as the mobile phase buffer (Figure 3.5). Poor peak shapes can be quantitatively evaluated by examining the effect of the mobile phase buffer type on the asymmetry factor for the four compounds of interest (Figure 3.6). The asymmetry factor (**T**) for each compound was calculated from the following equation:

$$\mathbf{T = b/a} \qquad 3.4$$

where **a** is the length (min) of a perpendicular line drawn from the center vertical line of the peak to the right side of the peak and **b** is the length (min) from the center line of the peak to left side of the peak. The values **a** and **b** are determined at 10% of the peak height. Ideally, well-shaped symmetrical chromatography peaks have an asymmetry factor between 0.9 and 1.1 (Dolan and Snyder, 1989). In the present study, psilocin, bufotenine and tryptamine exhibited the largest variations in T as a function of mobile phase type. In acetate, which was the worst buffer examined, very poor asymmetry factors were observed for these compounds (4, 8 and 13 respectively). Asymmetry factors were nearly as bad in formate, while they were generally acceptable in the other buffers. The TFA buffer uniformly gave the best symmetrical peaks for all compounds, ranging from an asymmetry factor of 0.7 for psilocybin and 0.9 for bufotenine. In contrast to psilocin, bufotenine and tryptamine, the asymmetry factor for earliest eluting peak, psilocybin, varied very little among the different buffers, with values of T approximately 1 in citrate, phosphate, formate and TCA, and 0.7 in acetate, TFA and

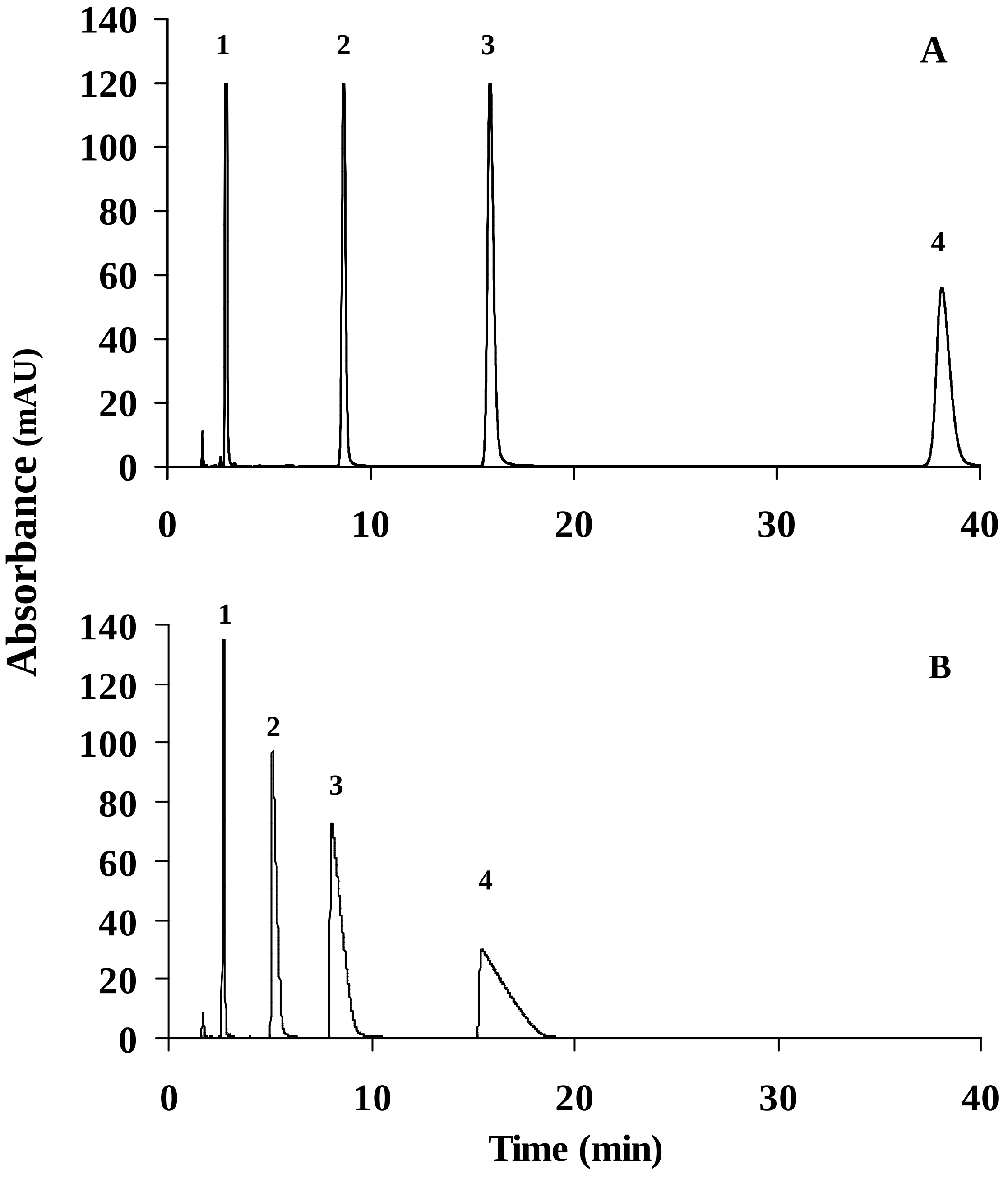


Figure 3.5 Chromatograms showing the peak shape and separation of (1) psilocybin, (2) bufotenine, (3) psilocin and (4) tryptamine obtained by isocratic elution in 90% 50 mM (pH 2.5) buffer and 10% methanol. Two buffers were used: A) TCA and B) acetate. Analyses were performed at 25 ^{o}C and a flow rate of 1.0 mL min^{-1}. The column was a Hypersil C_{18} (4.6 x 250 mm, 5 μm packing) and the diode array detector was set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. The injection volume was 4 μL.

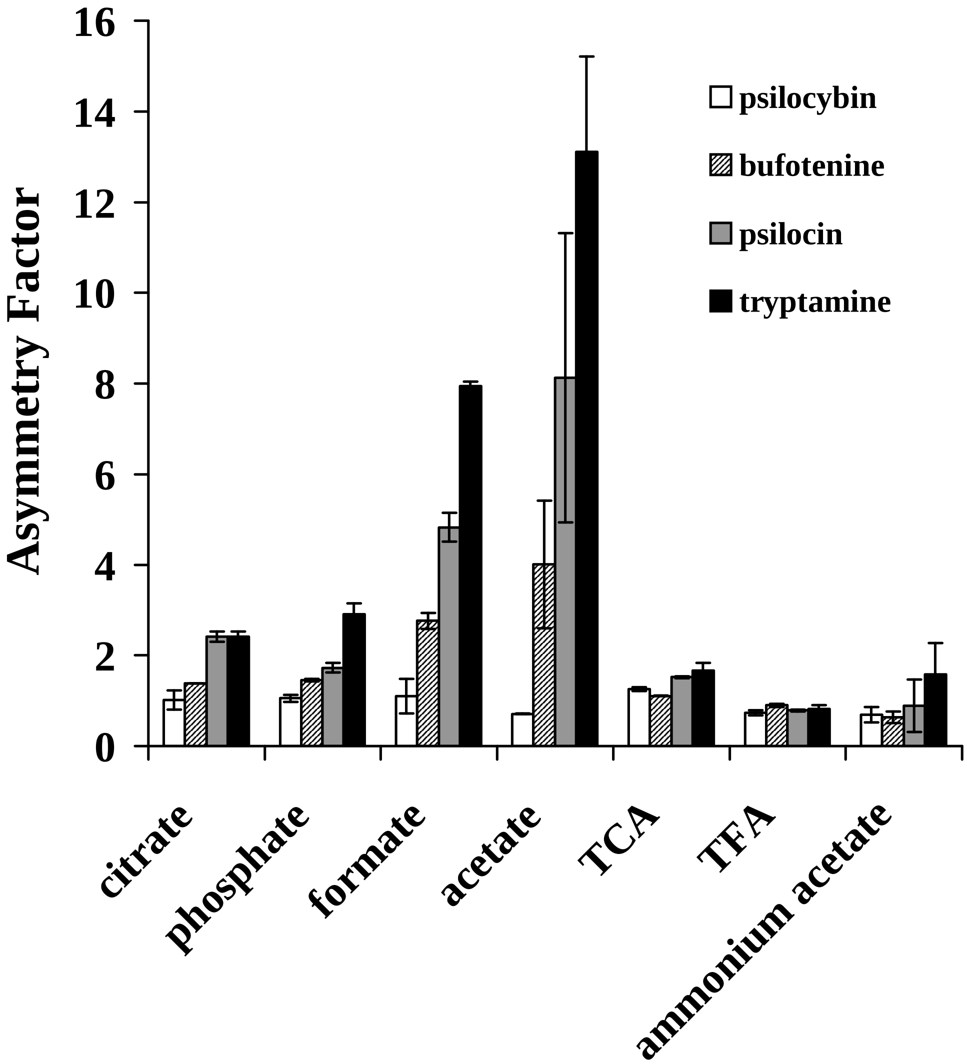


Figure 3.6 Asymmetry factor calculated at 10% of the peak height for each compound in all buffers. Error bars denote the data range for duplicate injections of a standard.

ammonium acetate. This finding is not surprising given that psilocybin eluted near the void volume and therefore did not appreciably interact with the stationary phase.

Buffer pH and Concentration. To assess the effect of buffer pH on the selectivity of the separation of psilocin, bufotenine, tryptamine and psilocybin, the capacity factor for each compound was plotted as a function of the pH of the citrate buffer (Figure 3.7). In general, the k for psilocybin was the least affected by pH, showing only a slight decrease in the capacity factor with increasing pH. The other three compounds showed a nearly constant capacity factor up to pH 3.5, followed by a nonlinear decrease in k as the pH increased from 3.5 to 6.0. At a pH above 6.0, the capacity factor for each of these three compounds increased. The biggest change was seen for tryptamine and psilocin, which varied by more than a factor of two.

When comparing relative retention times, it can be seen that the selectivity of the method changed very little in this pH study up to pH 6. However, as the pH increased above pH 6, relative retention times changed. In particular, the separation of bufotenine and psilocin increased, while the separation of tryptamine and psilocin decreased. Tryptamine’s capacity factor showed the largest change across the pH range examined here, almost doubling between pH 6 and 9. Based on these results, it will be possible to change relative retention times through a change in the buffer pH, which can be used as further confirmation of compound identity.

The effect of buffer concentration on capacity factors was determined employing citrate. As shown in Figure 3.8, no difference in capacity factor of the four compounds was observed at citrate concentrations greater than 25 mM. However, when the citrate

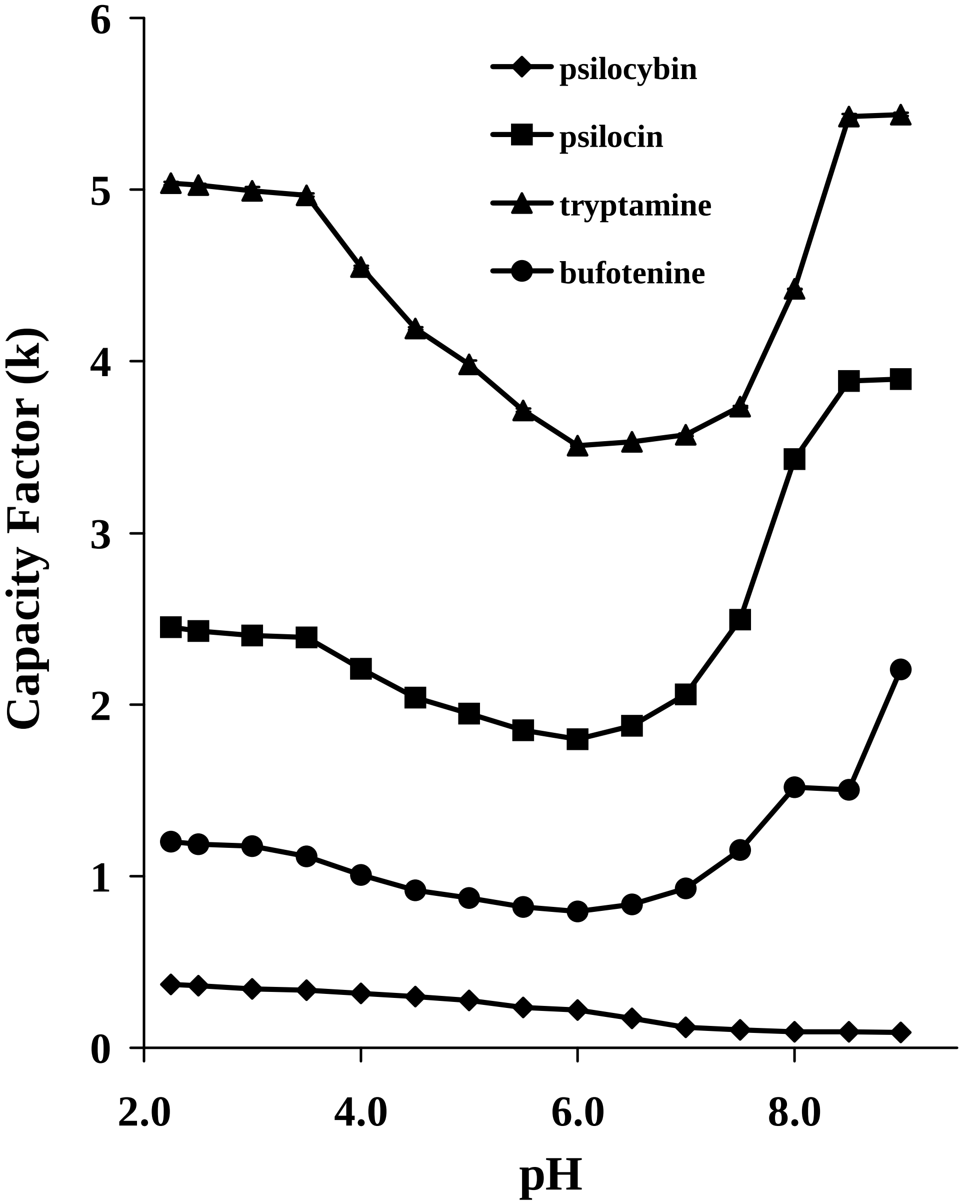


Figure 3.7 Effect of buffer pH on the capacity factor (k) of bufotenine, psilocin, psilocybin, and tryptamine. All separations were performed isocratically in 90% 50 mM citrate buffer and 10% acetonitrile. Analyses were performed at 25 °C and a flow rate of 1.0 mL min^{-1}. The column was a Hypersil C_{18} (4.6 x 250 mm, 5μm packing) and the HP diode array detector was set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. Error bars denote the data range for duplicate injections of a standard, which for all compounds and pHs were smaller than the data points.

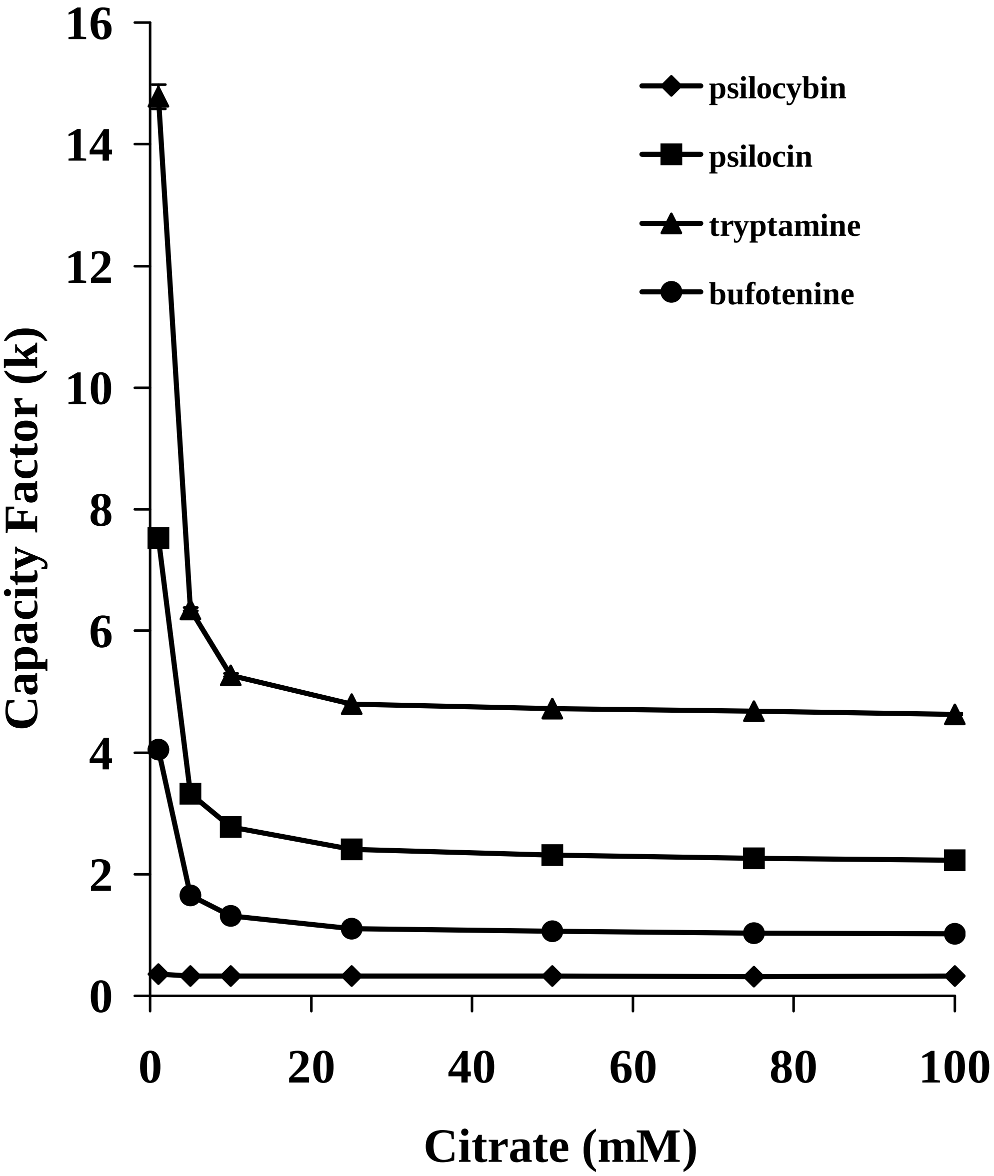


Figure 3.8 Effect of buffer concentration on the average capacity factor of bufotenine, psilocin, psilocybin, and tryptamine in pH 2.5, citrate buffer. All separations were performed isocratically in 90% aqueous buffer and 10% acetonitrile. Analyses were performed at 25 ^{o}C and a flow rate of 1.0 mL min^{-1}. The column was a Hypersil C_{18} (4.6 x 250 mm, 5 μm packing) and the HP diode array detector was set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. Error bars denote the data range for duplicate injections of a standard, which for all compounds and citrate concentrations were smaller than the data points.

concentration was too low <10 mM, k significantly increased nonlinearly for all compounds examined, except for the early eluting psilocybin. Presumably, citrate suppressed interactions of the analytes with the hydrophilic silanol moieties on the stationary phase at buffer concentrations greater than approximately 10 mM, while at lower buffer concentrations the analytes interacted with these silanol groups greatly increasing their interactions with the column and causing a large increase in k.

Gradient Elution and MS Detection. In addition to evaluating the mobile phase buffers and organic phase, the chromatographic method was optimized for the qualitative and quantitative identification of psilocin and psilocybin in mushroom extracts by performing a gradient elution to reduce the analysis time, and by employing MS detection to improve the selectivity of the method from possible co-eluting interferences. In general, all buffers except ammonium acetate and acetate yielded adequate separations of our compounds with no interferences detected by MS. When ammonium acetate or acetate were used in the mobile phase, psilocybin could not be separated from an unidentified mushroom component when employing either a gradient or isocratic elution. Furthermore, when these two compounds co-eluted, the UV spectra were nearly the same (ca. peaks at 260 and 266 nm, Figure 3.9) such that they could not be easily resolved with the diode array detector.

When the mass spectrometer was used as the detector, the single peak that was seen in the total ion chromatogram (Figure 3.10A) was easily deconvolved by viewing the extracted mass spectrum (Figure 3.11) generated from the total ion chromatogram. The extracted spectrum showed the (M + H) peak for psilocybin (*m/z* 285) and the

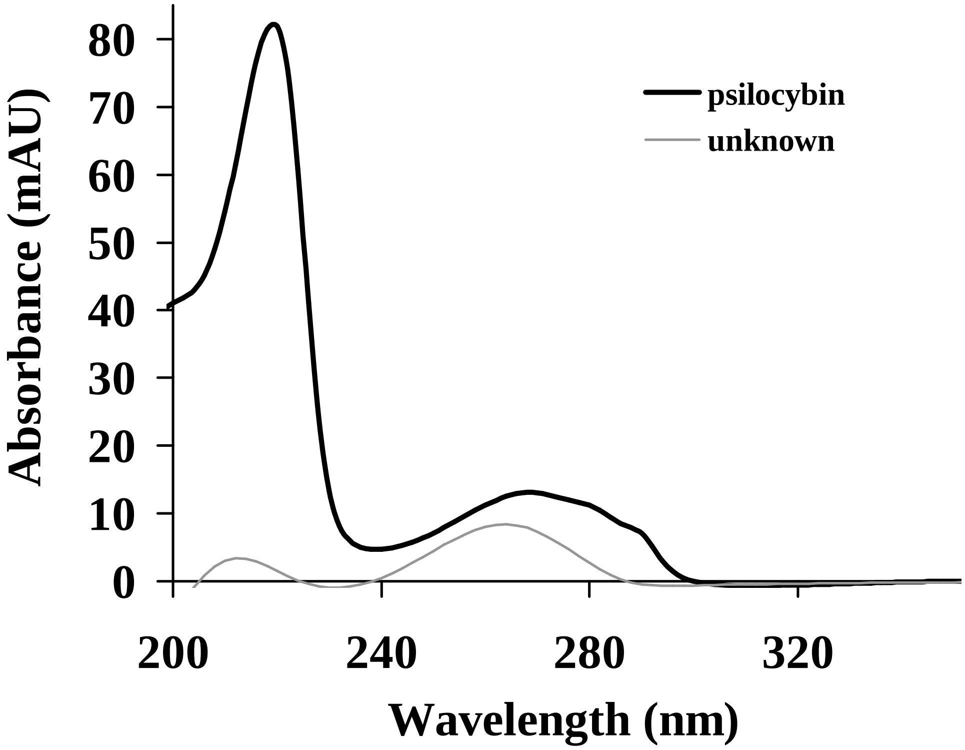


Figure 3.9 Ultraviolet spectrum of psilocybin and the unknown peak. UV spectra were obtained from the HPLC chromatogram at 6.93 min and 7.09 min in Figure 3.10B. Spectrophotometer settings are given in the Methods section.

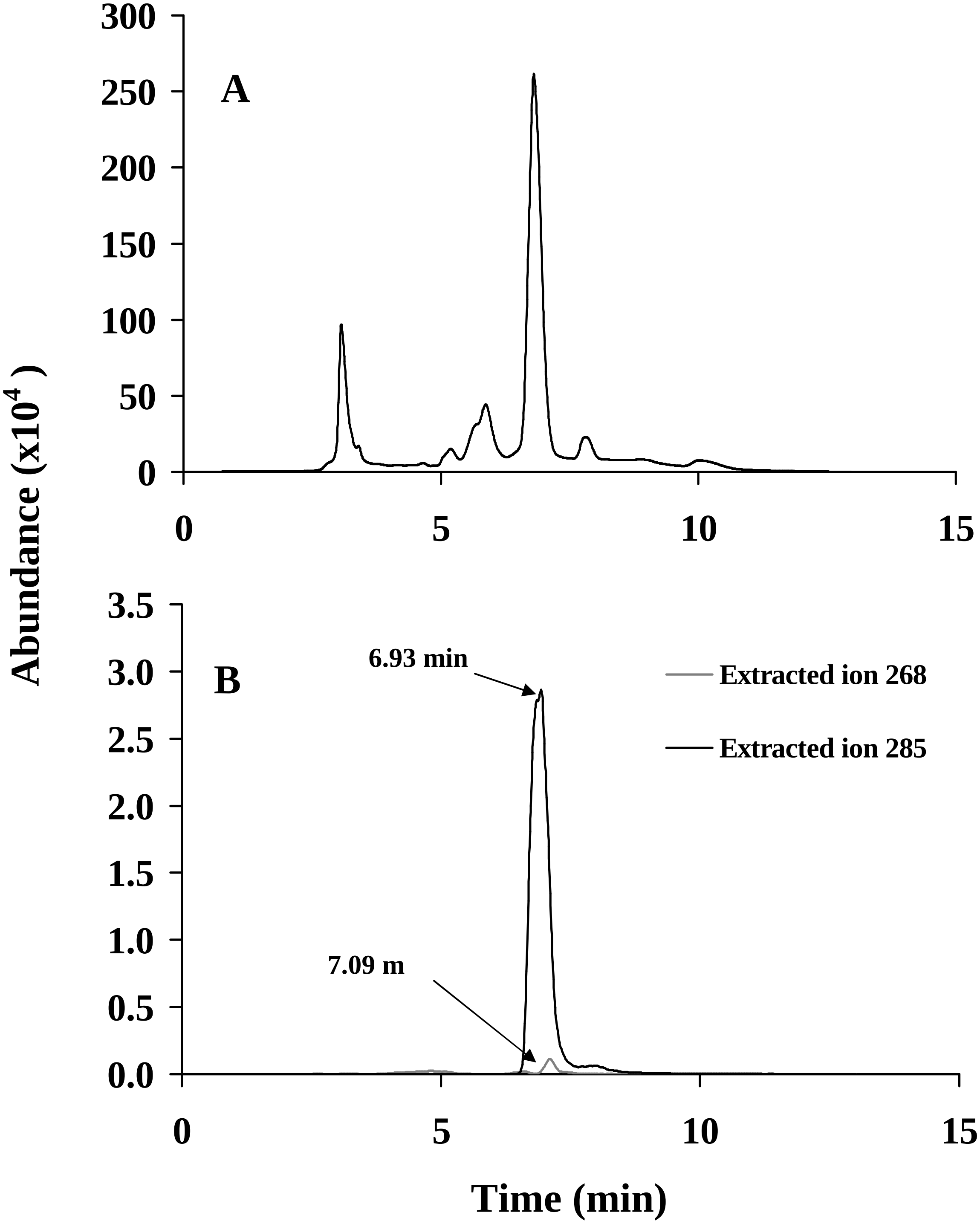


Figure 3.10 A) Total ion chromatogram of a methanolic mushroom extract analyzed by gradient elution with 0.1% ammonium acetate buffer and methanol. Chromatographic and gradient elution conditions are given in the Methods section. B) Extracted ion chromatogram of mushroom material for molecular weights of 268 and 285, corresponding to the unknown and psilocybin. See Figure 3.12 for mass spectra corresponding to the co-eluting peaks at 6.93 and 7.09 min.

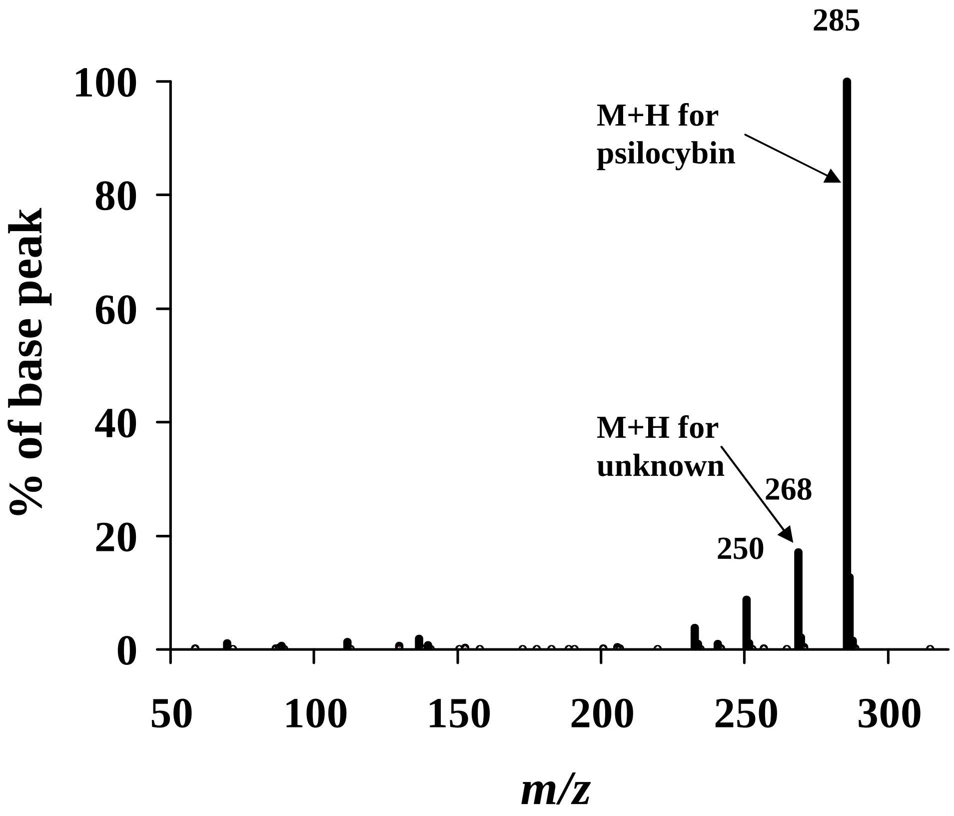


Figure 3.11 Mass spectrum of peak obtained from the HPLC chromatogram at 7.0 min in Figure 3.10A. Mass spectrometer conditions are given in the Methods section.

unknown peak (*m/z* 268), which strongly suggested that the single peak in the total ion chromatogram contained at least two co-eluting compounds. Co-eluting compounds were selectively resolved by first doing an extracted ion chromatogram for *m/z* 285 and *m/z* 268 (Figure 3.10B), and then identifying the mass spectrum generated for each extracted peak (Figure 3.12). In the mass spectrum for psilocybin, both the (M+H) peak and the (M+Na; *m/z* 307) molecular ions were observed, albeit the Na adduct was fairly minor at <10% of the base peak. For the unknown, the (M+H) peak was the main molecular ion that was observed, with a fragment at *m/z* 136 also detected. No *m/z* 270 (M+H) peak was observed in the total ion chromatograms indicating that baeocystin, a common component in mushrooms, was not present in our extracts.

In contrast to ammonium acetate or acetate, the other buffers allowed for the separation of psilocybin and psilocin from common interferences found in hallucinogenic mushroom material, with no co-eluting interferences noted during MS analysis. The optimized chromatographic gradient elution separations that were obtained employing TFA and TCA buffers are shown in Figure 3.13. The chromatogram for citrate is not shown because it is nearly identical to the chromatogram obtained using a TFA-buffered mobile phase (Figure 3.13A). Although not examined here, the chromatographic analysis employing MS detection can be further improved by lowering the TFA, citrate or TCA buffer concentration considerably without a change in selectivity (e.g., Figure 3.8), while at the same time significantly lowering the background noise.

During the optimization of the LC/MS method in our study, psilocybin was the only compound that was difficult to detect by MS owing to co-eluting interferences (as discussed above) and poor MS ionization. The abundance of the M+H peak for

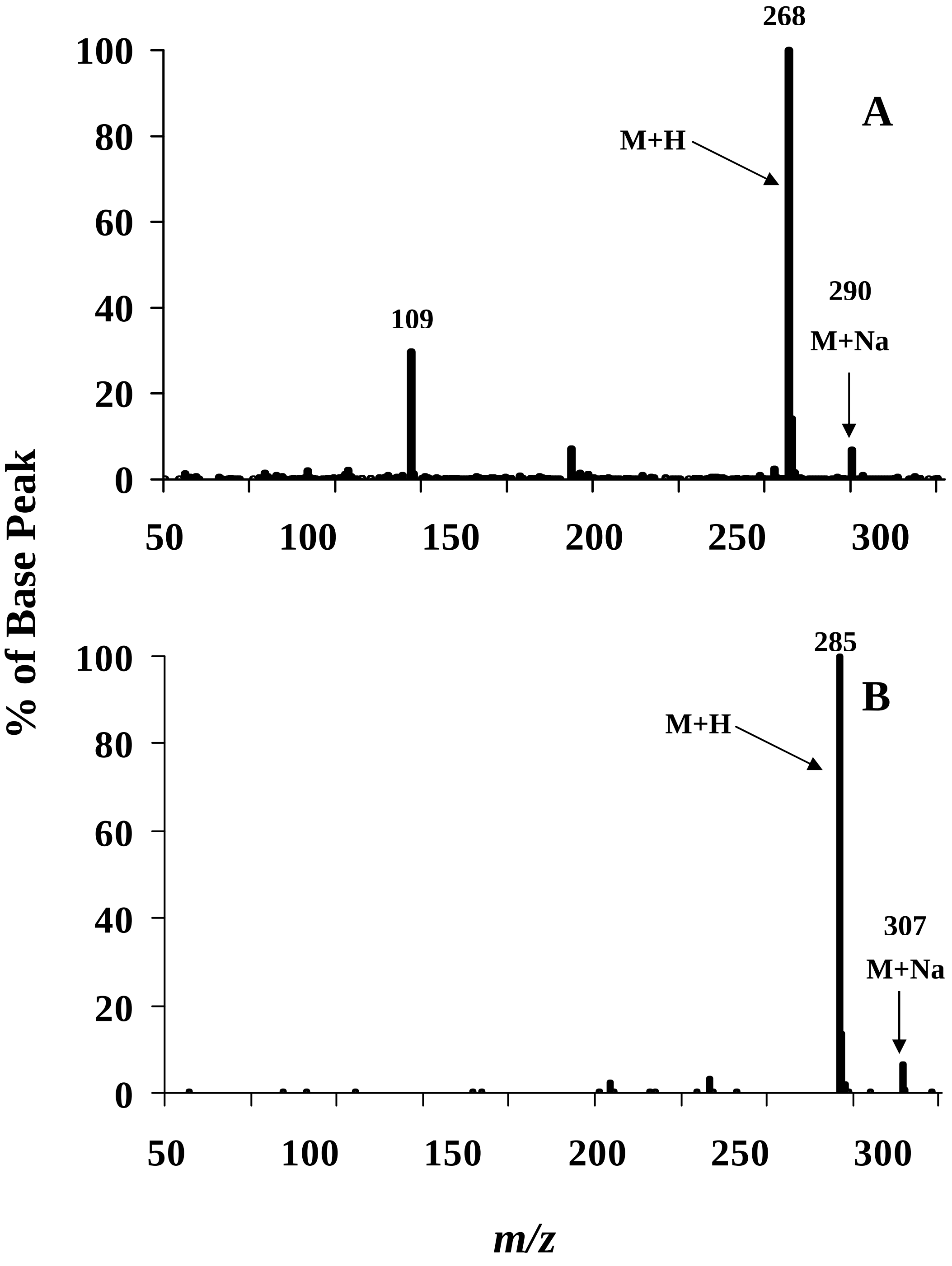


Figure 3.12 Mass spectrum of A) the unknown and B) psilocybin. Mass spectra were obtained from the HPLC chromatogram at 6.93 min and 7.09 min in Figure 3.10B. Mass spectrometer conditions are given in the Methods section.

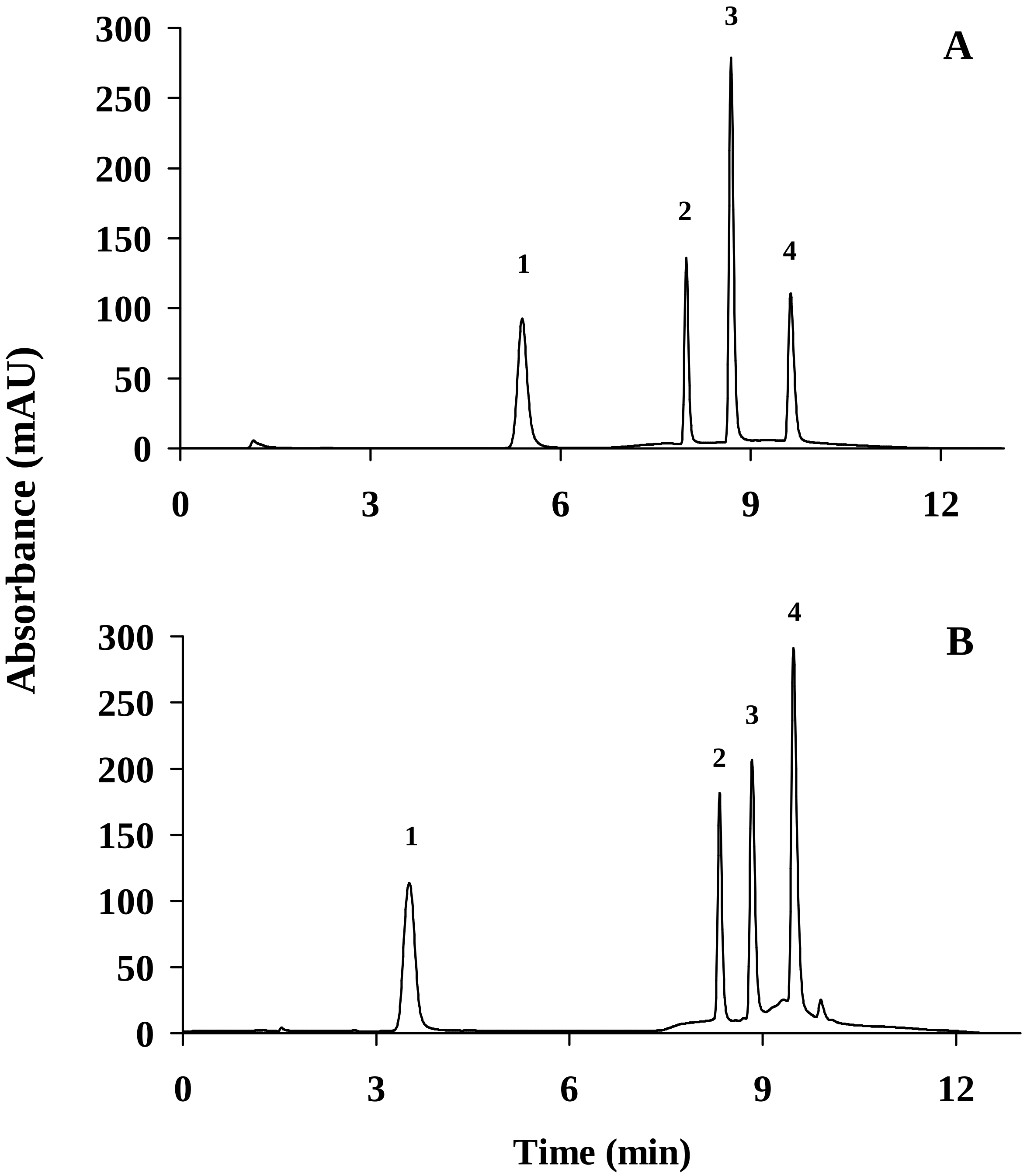


Figure 3.13 Optimized gradient elution chromatograms for (1) psilocybin, (2) bufotenine, (3) psilocin and (4) tryptamine. Mobile phases consisted of panel A) 50 mM, pH 2.5 TFA and methanol, and panel B) 50 mM TCA (pH 2.5) and methanol. The gradient elution program is given in the text and is the same for both mobile phases. Analyses were performed at 25 ^oC and a flow rate of 1.0 mL min^{-1}. The column was a Hypersil C_{18} (4.6 x 250 mm, 5 μm packing) and the diode array detector was set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. The injection volume was 4 μL. Gradient elution conditions are given in the Methods section.

psilocybin was substantially less than that observed for the other three compounds, which were easily detected in the mass spectrometer irrespective of the mobile phase employed. The lower detection of psilocybin by MS relative to the other three compounds results from the inability of this compound to maintain a positive charge in the spray chamber due to the phosphate moiety (Dr. Zavitsanos, Agilent Technologies, personal communication). Therefore, the MS was optimized for psilocybin by flow injection analysis (FIA) in the RP-HPLC-MS system by bypassing the HPLC column and adjusting the fragmentor and capillary voltages to optimize the M+H peak for this compound. Subsequently, all compounds were quantified employing RP-HPLC-MS employing the optimized MS conditions for psilocybin. Results obtained with the optimized MS parameters and employing the different mobile phase buffers are shown in Table 3.2. Under optimal conditions, psilocybin had an MS response similar to psilocin, bufotenine and tryptamine, except when TFA was used in the mobile phase. When the mobile phase contained TFA, psilocybin's response was lower than the other compounds by more than a factor of ten. The reason for this difference, which was only seen with TFA, is the well documented condition of ion suppression (Lim and Lord, 2002).

Standard Stability. Anastos et al. (2006) showed that exposure of aqueous psilocin and psilocybin standards to laboratory lighting caused them to degrade very rapidly (pH not reported). If standards were stored in the dark at room temperature, psilocybin and psilocin were stable for a week, after which compound degradation was noted. Based on these results, all samples and standards in the present study were stored in amber vials. When stored in these vials, psilocybin, bufotenine and tryptamine standards (1mg/mL)

Table 3.2 Mass spectral abundance for the M+H peak for psilocybin, psilocin, bufotenine and tryptamine in each mobile phase using the optimized mass spectrometer settings. Mass spectrometer parameters were: positive polarity, scanning from 50-320 *m/z*, capillary 3250 V, fragmentor 130 V and gain 1. Spray chamber parameters were: gas temperature 350 ^{o}C, drying gas 12 L min^{-1}, and nebulizing pressure 60 psig.

Mobile Phase Buffer	**Abundance (x 10^7)**			
	Psilocybin	**Psilocin**	**Bufotenine**	**Tryptamine**
Ammonium acetate	4.60	4.81	3.80	4.40
TCA	3.66	3.02	3.34	3.90
TFA	0.23	4.05	4.79	3.52
Citrate	3.33	3.76	3.16	3.65
Formate	2.36	3.30	2.85	3.56
Acetate	2.05	2.54	2.56	2.76

were stable in air-saturated methanol for a year at 4 ^{o}C, while psilocin completely degraded after a few weeks. However, if the air-saturated, methanolic standards were stored at room temperature (ca. 20 ^{o}C), psilocin completely degraded after a few days, bufotenine and tryptamine completely degraded after a few weeks, and psilocybin was stable for a minimum of four months.

In addition to standards, the stability of psilocin was also tested in air-saturated methanolic mushroom extracts, since it was reported that psilocin was stable for months when extracts were stored at room temperature (Beug and Bigwood, 1981). In the present study, air-saturated mushroom extracts were stable for no more than two weeks when stored at room temperature, and the stability varied, depending on the causative agent for the breakdown (extract to extract variability in psilocin stability was not tested). This finding agreed with results obtained with methanolic standards (*vide supra*), but was significantly different from Beug and Bigwood's 1981 results.

The decomposition of psilocin in methanol (and in water, Anastos et al., 2006) was likely caused by oxidation by dissolved oxygen. This supposition is based on the fact that indole alkaloid rings, particularly psilocin, are susceptible to oxygen degradation (Lindenblatt et al., 1998; Hasler et al., 2002; Anastos et al., 2006). Psilocybin does not degrade very quickly in air-saturated solutions possibly due to the phosphate group stabilizing the ring. To test the hypothesis that oxygen affects the stability of psilocin standards, the stability of psilocin in air-saturated water and methanol, and deaerated water was determined as a function of storage time at room temperature. The pH of the aqueous solutions was not determined, but should be close to pH 7. Deaerated samples were obtained by vacuum, which was repeated each time an aliquot was taken for analysis. Results of this study are shown in Figure 3.14, as first order kinetic plots, which implicitly assume that the oxidation of psilocin with oxygen followed psuedo first order kinetics. In this kinetic study, no loss of psilocybin was observed (results not shown). As shown in Figure 3.14, degradation of psilocin followed first order kinetics in all three storage solutions, but the rate of degradation varied widely as seen through a comparison of half lives. The half life of psilocin was less than 4 days in air-saturated water increasing to over a year when solutions were deaerated (Table 3.3). However, it is likely that traces of oxygen were still present in "deaerated" solutions, since vacuum deaeration does not completely remove oxygen and samples were briefly exposed to air when vials were capped. As might be expected, this would lead to a very slow loss of psilocin. One way to avoid this problem would be to sparge solutions with high purity nitrogen or argon after vials are sealed to remove residual oxygen. The addition of formic acid to the methanol significantly increased the solubility of psilocin in the methanol, but had no affect on its stability. Psilocin and psilocybin standards and mushroom extracts showed no loss after one year when stored in a solution of acidic methanol that was purged with high purity nitrogen gas, sealed, and stored at -11 ^{o}C.

Detection Limit. The detection limit (DL) for psilocin and psilocybin was estimated with aqueous standards and by addition of standards to mushroom samples that were known to contain no psilocin or psilocybin. Standards (diluted in the mobile phase) were added at concentrations that were near the expected DL for the method, as determined by mass spectroscopy. The DL for psilocin and psilocybin was 3.9 and 24 pg, respectively, for a 4 μL injection. These results are very similar to detection limits estimated by

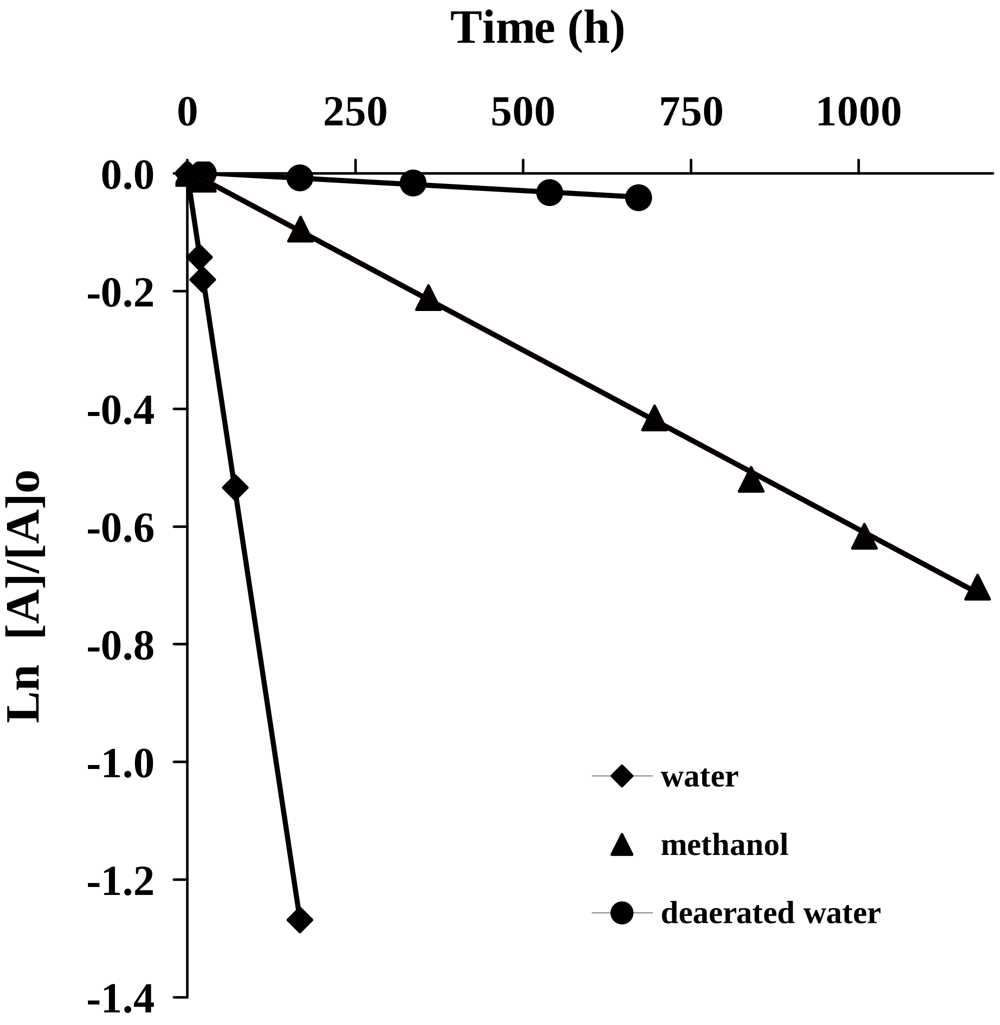


Figure 3.14 First order kinetic plot showing the loss of psilocin in air-saturated high purity laboratory water and methanol, and deaerated high purity laboratory water. The water used for this study was at a circumneutral pH and not buffered. $[A]_t$ is the concentration of psilocin at time t and $[A]_o$ is the initial concentration of psilocin. The solid lines represent the best fit line obtained from linear regression analysis. Regression results are given in Table 3.3.

Table 3.3 Slope, coefficient of variation, rate constant, and calculated half-life for psilocin in water, methanol and deaerated water.

Extraction Solvent	**Slope (h^{-1}, x10^{-4})**	**k (s^{-1}, x10^{-7})**	**r^2**	**$\tau_{1/2}$ (h)**
Water	-75	21	1.000	92
Methanol	-6.0	1.7	0.999	1,155
Deaerated Water	-0.6	0.017	0.992	11,500

Kamata et al. (2005) (ca. 1-25 pg, 5 μL injection). When mushroom extracts were analyzed, the baseline noise increased and this decreased the DL of the method substantially relative to that obtained with simple aqueous standards. For the analysis of mushroom extracts, the DL was ~10 ng for both psilocin and psilocybin employing the diode array detector (at 267 nm) and ~1 ng when MS detection was used. The DL obtained using the diode array detector was very similar to that obtained by Beug and Bigwood (1981) employing fixed wavelength detection at 254 nm (ca. 10 ng for psilocybin and psilocin, 10 μL injection).

SUMMARY

After thoroughly investigating and evaluating the effect of the mobile phase on the separation of psilocin, psilocybin, bufotenine and tryptamine it was determined that the best results were obtained when the mobile phase contained methanol and 50 mM TCA (pH 2.5). Using gradient elution, the analysis of mushroom extracts was completed in 18 min, and by utilizing mass spectrometry as a detector, psilocybin and psilocin were unequivocally identified and quantified in hallucinogenic mushrooms. It is recommended that both UV and MS detection be employed to quantify psilocybin in mushroom extracts, owing to the expected variable contamination of these extracts with baeocystin and other interfering compounds.

CHAPTER 4

Extraction and Identification of Mushroom-Derived Hallucinogenic Indoles Psilocin and Psilocybin by RP-HPLC with MS Identification.

ABSTRACT

Hallucinogenic mushrooms have been studied by several analytical techniques, since the late 1950s, with a focus on the quantification of the indole alkaloids, psilocybin and psilocin. These studies have generally shown that psilocybin was the main hallucinogenic compound detected in psychedelic mushrooms while psilocin was only a minor constituent. Our results suggest that this is not necessarily the case. We conducted an extraction study of hallucinogenic mushroom material and found that two extractions were needed to extract psilocybin and psilocin from hallucinogenic mushrooms. Optimal extraction was obtained for both compounds when 300 mg of ground mushroom was extracted for 2 h with 25 mL of methanol by rotary inversion followed by a second extraction of the same mushroom material with a solution of 50:50 (v/v) methanol and 50 mM citrate buffer (pH 2.5). Extracts were separated by reversed phase high performance liquid chromatography (RP-HPLC) employing a Phenomenex Hydro-RP C_{18} column (3.0 x 150 mm, 4 μm packing), and detected with an Agilent 1100 mass spectrometer. Optimal separation and detection were obtained employing a gradient elution with a mobile phase that consisted of methanol and 50 mM ammonium formate buffer (pH 3.5). The total analysis time for this chromatographic method was 18 min.

INTRODUCTION

"Magic mushrooms" or "Shrooms" are the street names for mushrooms that contain the hallucinogenic compounds psilocybin and psilocin. These compounds are known as indole alkaloids and are present in several different species of mushrooms from four families: Strophariaceae, Bolbitiaceae, Coprinaceae, and Cortinariaceae (Gross, 2000). There are several other components similar to psilocybin and psilocin found in hallucinogenic mushrooms including baeocystin and nor-baeocystin, which are demethylated derivatives of psilocybin (Hoffman et al., 1959). Analytical methods that are developed to identify and quantify psilocybin and psilocin in mushroom material must be able to separate these compounds from potentially interfering compounds such as baeocystin that may also be present in the mushrooms.

Several papers have been published on the extraction and quantification of psilocybin and psilocin from mushroom material using a variety of analytical methods. For qualitative identification, techniques such as ultraviolet and infrared spectroscopy have been employed (Casale, 1985; Lee, 1985) as well as gas chromatography-mass spectrometry (GC-MS) (Casale, 1985; Gross, 2000). Dephosphorylation occurs in gas chromatographic techniques resulting in the conversion of psilocybin to psilocin. This conversion is eliminated by employing derivatization agents such as N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA) (Kellar et al., 1999; Sticht and Kaeferstein, 2000). One of the most common techniques cited in the literature for the quantification of psilocybin and psilocin is reversed phase- high performance liquid chromatography (RP-HPLC) employing a variety of mobile phases and detection by either UV-Vis spectroscopy or fluorescence (White, 1979; Perkal et al., 1980; Thomsom, 1980; Beug and Bigwood, 1981; Christiansen et al., 1981; Sottolano and Laurie, 1983; Vanhaelen-Fastre and Vanhaelen, 1984; Wurst et al., 1984; Hasler et al., 1997; Lindenblatt et al., 1998; Saito et al., 2004). We recently evaluated and optimized the chromatographic separation of psilocybin and psilocin from extracted mushroom material and examined the long-term storage stability of these compounds in different extraction solvents (Chapter 3).

Although the chromatographic separation of these compounds in mushroom extracts was optimized, two important aspects of mushroom analysis remained. First, there have been only two reports employing RP-HPLC with mass spectrometric detection for the analysis of mushrooms, and neither of these studies was optimized for the detection of both psilocybin and psilocin. Saito et al. (2004) determined the psilocin concentration in rat plasma and mushroom material employing a precolumn derivatization procedure and RP-HPLC separation with fluorometric or electrospray mass spectrometric detection. However, they did not determine psilocybin in these samples. In a separate study, Kamata et al. (2005) quantified both psilocin and psilocybin in mushroom extracts by reversed phase HPLC-MS and HPLC-MS^2, but they did not evaluate or optimize their method.

Second, no one has conducted a systematic and quantitative study of the extraction efficiency of both psilocybin and psilocin from ground mushroom material employing one of the many extraction procedures published in the literature. Some investigators make no mention of the extraction efficiency during the quantification of psilocybin and/or psilocin in hallucinogenic mushrooms (Thomson, 1980; Wurst et al., 1984; Gross, 2000; Saito et al., 2004; Kamata et al., 2005), while others only determined extraction efficiencies for psilocybin. Sottolano and Lurie (1983) determined that sonication of several ground mushroom samples in methanol for 50 min yielded from 0.1 – 0.5 % (weight extracted/weight mushroom x 100) of the psilocybin extracted. Christiansen et al. (1981) determined that from 0.2 – 2.0 % (w/w x 100) of the psilocybin was extracted with two extractions of the dried mushroom material using10% aqueous 1 M ammonium nitrate and 90% methanol (v/v).

A few extraction studies were conducted to determine both psilocin and psilocybin in mushroom extracts. Beug and Bigwood (1981) extracted psilocybin and psilocin from ground mushroom samples for 12 h at room temperature with methanol and continual stirring. The mushroom samples they analyzed contained between 1.5 mg/g to 8.5 mg/g of psilocybin and between 0 and 5.9 mg/g of psilocin, which corresponded to 0.15 – 0.85% and 0.0 – 0.59 %, respectively, on a weight basis (w/w x 100). Gartz (1994) qualitatively compared methanol, ethanol, and aqueous solutions of methanol and ethanol, as well as acetic acid for the extraction of psilocybin and psilocin from dried mushroom samples. Gartz determined that methanol was the best extraction solvent, albeit no details regarding extraction procedures or extraction efficiencies were reported. Wurst et al. (1992) determined extraction efficiencies of psilocybin and psilocin from the fruiting bodies of mushroom samples extracted with aqueous solutions of methanol and ethanol. They reported quantitative (or near quantitative) extraction efficiency of psilocin and psilocybin, from mushroom material but with different extraction solvents. In particular, all of the psilocin (and none of the psilocybin) was extracted from samples with 75% aqueous ethanol, while nearly all of the psilocybin was extracted from 1% aqueous acetic acid. Wurst et al. (1992) concluded that two different extraction procedures were needed to optimize the extraction for both psilocin and psilocybin. In a later study, Gartz (1994) suggested that the high extraction efficiency for psilocin that was obtained by Wurst et al. (1992) may have resulted from the enzymatic conversion of psilocybin to psilocin during the extraction procedure, although this is unlikely given that enzymes are typically denatured in 75% aqueous ethanol.

Some investigators re-extracted mushroom samples and found very little psilocybin and psilocin present in the second extract. Based on this result, they concluded that the psilocybin and psilocin were efficiently extracted in the first extraction (Perkal et al., 1980; Vanhaelen-Fastre and Vanhaelen, 1984).

Mushroom extractions are routinely performed in forensic labs worldwide where caseloads are heavy and rapid sample throughput is critical. Therefore, it is important that the extraction procedure used to isolate psilocybin and psilocin from mushrooms is simple, selective and rapid. In this paper, the main extraction solvent that is published in the literature (methanol) is examined along with other, less common solvents to determine the effect of solvent type on the extraction efficiency of psilocybin and psilocin from hallucinogenic mushroom material. Based on the results of this study, a short and effective extraction procedure is presented to qualitatively identify both psilocybin and psilocin by RP-HPLC with mass spectrometric detection.

METHODS

Chemicals. All chemicals were of the highest purity available (ACS grade or better) and, except where noted, were used without further purification. The buffers used in this study consisted of trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) (Spectrum

Chemical, New Brunswick, NJ), citric acid (United States Biochemical Corp., Cleveland, OH), and ammonium formate (Sigma-Aldrich, St. Louis, MO). HPLC grade methanol and acetonitrile were purchased from Burdick and Jackson (Muskegon, MI), and Optima HPLC grade ethanol, acetone, 1-propanol and ethyl acetate were obtained from Fisher Scientific (Houston, Texas). Psilocybin and psilocin standards were purchased from Alltech Applied Sciences (State College, PA). Bufotenine and tryptamine (internal standard, IS) were purchased from Sigma-Aldrich (St. Louis, MO). Tryptamine was selected as the IS because it is structurally similar to psilocin and psilocybin and it is not found in psilocybin-containing mushrooms. High purity water used throughout this study was obtained from a US Filter DI water filtration system consisting of a 0.2 μm prefilter, carbon tank, two mixed ion exchange tanks, UV irradiation and sterilization, and 0.2 μm final filtration. The resistivity of this water was $\geq$ 18.2 MΩ.

All mobile phase buffers were 50 mM and adjusted to pH 2.5 by adding an appropriate amount of a 50 mM solution of the sodium salt to a 50 mM solution of the acid, except for ammonium formate that was made at a concentration of 15 mM and pH 3.5; the pH was adjusted with a 15 mM solution of formic acid. All buffer solutions were vacuum-filtered through a 0.45 μm Nylon filter (Millipore Corp., Bedford, MA) prior to use. Buffer pH was determined with an Accumet AB15 pH meter from Fisher Scientific (Houston, Texas) that was calibrated with standard pH 4.0, 7.0, and 10.0 buffers from VWR (Westchester, PA).

Apparatus. The HPLC consisted of a modular Hewlett Packard model 1100 system (Wilmington, DE) with an online vacuum degasser, quaternary gradient pump and high-speed proportioning valve, low-volume pulse dampener, automated liquid sampler (ALS) with 100 vial tray and programmable injection volumes from 0.1 µL to 1.8 mL. Unless otherwise noted, a 4 µL injection volume was used for this study. Compounds were separated on a temperature controlled (25 °C) Phenomenex Hydro-RP C_{18} column (3.0 x 150 mm) with 4 µm packing. Detection was accomplished by monitoring the absorbance with a Hewlett Packard diode array spectrophotometer set at 267 ± 10 nm, with the reference set at 360 ± 50 nm. The complete system was controlled by PC-based HP ChemStation software.

A second HPLC system was used for mass spectral detection of psilocybin and psilocin. This second system consisted of a Hewlett Packard model 1100 system (as described above), with a high pressure binary gradient pump and a HP model SL mass selective detector.

The Hewlett Packard series 1100 model SL mass spectrometer (Wilmington, DE) was outfitted with an atmospheric pressure ionization electrospray (API-ES) source. The API-ES was operated in positive polarity, with a scanning mass range from 50-320 amu. Unless noted, the optimal settings used in this study were gas temperature 350 °C, drying gas flow rate 12.0 L min^{-1}, nebulizing pressure 60 psig, gain 1, and capillary voltage 3250 V. The fragmentor voltage was optimized for psilocybin and set at 130V.

HPLC Conditions. All chromatographic separations for buffer evaluations were performed by gradient analysis at 25 °C and at a flow rate of 0.5 mL min^{-1}. The mobile Phase A consisted of the aqueous buffer and mobile phase B was 5% (v/v) aqueous methanol. Unless noted, the gradient elution employed was: 5% B for 3 min, 5 to 30% B from 3 to 5 min, 30% B from 5 to 8 min, 30 to 5% B from 8 to 9 min, and 5% B from 9 to 13 min, followed by a 9 min equilibration at the original starting conditions. When TCA was used as the aqueous buffer (A), the following elution gradient was used: 10% B for 4 min, 10 to 30% B from 4.0 to 4.1 min, 30 to 60% B from 4.1 to 7.0 min, 60% B from 7 to 8 min, 60 to 10% B from 8 to 9 min, followed by a 9 min equilibration at the original starting conditions. The total analysis time for either of these two gradient elution programs was 18 min.

Sampling Procedure. Stock solutions of psilocin, psilocybin, bufotenine and tryptamine were made at 1.0 mg mL^{-1} in methanol containing 0.1% formic acid (v/v). Calibration standards were prepared from 1.0 mg mL^{-1} stock solutions of psilocin and psilocybin in the range of 0.01 – 0.20 mg mL^{-1}. An internal standard (IS) containing 0.5 mg mL^{-1} of tryptamine was prepared in a 50:50 (v/v) solution of methanol and 50 mM, pH 2.5 citrate buffer. A 100 μL aliquot of this IS was added to all standards, controls, and sample extracts that were placed in 2 mL amber borosilicate vials containing vial inserts and sealed with threaded caps containing Teflon-faced butyl rubber septa.

Mushroom samples were obtained from completed forensic case samples that were ordered for destruction and were obtained with a court order for research purposes. The mushroom samples were dried overnight at 60 ^{o}C in a Lab-Line Imperial-V oven (Krackler Scientific, Albany N.Y.). The dried mushroom samples were ground with a hand cheese grater, poured into a cone, flattened and then cut into quarters. Opposite corners were selected and the above process was repeated. The second quartered samples were extracted according to Sottolano and Lurie (1983). Approximately 300-500 mg of ground hallucinogenic mushroom material was weighed out and placed in two capped vials and stored at room temperature until extracted.

Mushroom Extraction. Psilocybin and psilocin were extracted from 300-500 mg ground mushroom samples with agitation using either a rotary mixer model 099A (Glas-Col, Terre Haute, IN) at a setting of 50% (50 rpm) or an ultrasonic water bath (Sonicor model SC-5OT 50 Watt sonicator, Copiogue, NY) with a built in timer. These two agitation methods were compared to the extraction obtained by simply letting the sample sit on the bench top without mixing. Different extraction solvents were examined, in addition to three different extraction volumes (10, 25 and 50 mL). The different extraction solvents that were tested included ethanol, ethyl acetate, acetone and isopropyl alcohol. In addition to testing these solvents, an experiment was conducted to examine the extraction efficiency using from 0-100% (v/v) methanol and 50 mM aqueous citrate buffer (pH 2.5). In all cases, mushroom samples were extracted until no psilocybin or psilocin was detected in the extract. After mushroom samples were extracted, the extracts were syringe-filtered through an inline 0.45 μm Nylon filter (National Scientific) employing a polypropylene syringe. Filtered extracts were prepared for analysis by pipetting 200 μL of the extract into 2 mL amber vials followed by addition of 200 μL of an internal standard solution.

Quantification and Peak Identification. Typically, samples were analyzed by RP-HPLC soon after they were extracted (ca. hours). However, sample extracts could be stored for weeks if needed with no loss of psilocybin or psilocin when the extracts were stored deaerated in tightly capped amber vials at -11 ^{o}C.

The concentration of psilocybin and psilocin in mushroom material was quantified by linear regression analysis. Standard curves were generated by injecting 10 μL of several psilocin and psilocybin standards with tryptamine added as an internal standard and plotting the ratio of analyte to internal standard versus concentration over the concentration range 0.005 - 0.20 mg mL^{-1}. Peaks were identified by their retention time and absorption spectrum obtained from the diode array detector or by the spectrum obtained from the MS detector for the M + H peak (as well as the M + Na peak for psilocybin).

Phosphatase Activity. The stability of psilocybin in mushroom extracts may partly depend on whether phosphatase is also extracted, since psilocybin is easily dephosphorylated to form psilocin (Horita and Weber, 1961). Therefore, a study was conducted to determine the phosphatase activity in four different mushroom extracts: 1) methanol, 2) a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5), 3) 15 mM ammonium formate (pH 6.4), and 4) 50 mM citrate (pH 2.5). In addition to testing the phosphatase activity in these single extractions, we also examined the activity in the extracts obtained from the sequential extraction with methanol followed by a second extraction with a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5). Four milliliters of each extraction solution was tested for enzyme activity; the ammonium formate and citrate extracts were diluted 1:10 in either ammonium formate or citrate, respectively. To test for phospahtase activity, 100 μL of 70 mM p-nitrophenol was added to the 4 mL extract in an acid-washed 10 mL, 1cm diameter borosilicate test tube. Immediately following mixing, 500 µL of each solution was removed and 5.0 mL 0.2 M NaOH was added to stop the reaction. After 5, 10, 15 and 30 min another 500 µL aliquot of the pNPP-extract was removed and stopped with the addition of 5.0 mL 0.2 M NaOH. The absorbance of each 5.5 mL sample (i.e., 500 µL pNPP-extract and 5 mL base) was determined at 405 nm in a 1 cm test tube employing a Spectronic 2000 spectrophotometer. For each type of pNPP-extract that was tested, parallel blanks, containing the solvent or buffer used for the extraction, were also examined. The concentration of the *p*-nitrophenate product **[**pNPP**]** was determined using Beer's Law and a molar absorption coefficient of 18300 $M^{-1}cm^{-1}$ at 405 nm. After accounting for dilution of the original enzyme solution, the phosphatase activity (P_{ase}, µmole pNPP produced per minute) was calculated according to Verjee, (1969):

$$\mathbf{P_{ase} = \frac{[pNPP]V_f}{t}} \tag{4.1}$$

where **t** is the incubation time (min) and $\mathbf{V_f}$ is the total assay volume (i.e., 0.0055L). Phosphatase activity was normalized to protein content (P_{ase} /mg protein). The protein content was determined according to Bradford (1976). One milliliter of each extraction solution was placed in a microcentrifuge tube and vacuum centrifuged to dryness. Samples and blanks were reconstituted in 50 mM citrate buffer (pH 2.5), except for the ammounium formate extract which was reconstituted in 15 mM ammonium formate buffer (pH 6.4). To each of three aliquots (100, 250 and 500 µL) taken for each reconstituted sample, 5 mL of Bradford reagent was added, samples were shaken, followed by a 20 min incubation at room temperature. The absorbance was then measured for each aliquot in the Spectronic 2000 at 595 nm that was referenced with the appropriate blank. A standard curve was generated with bovine serum albumin used to calibrate absorbance readings.

RESULTS AND DISCUSSION

MS Optimization. Psilocin, bufotenine, and tryptamine were easily detected by MS with a large M+H peak. The MS response for psilocybin, on the other hand, was relatively small and very sensitive to changes in MS settings including the fragmentor voltage, capillary voltage, and gain. Therefore, the psilocybin response was optimized by quantifying the abundance of the M+H peak (*m/z* 285) at different MS settings (Table 4.1). To optimize the MS settings, 4 μL of a 125 μg L^{-1} methanolic standard of psilocybin was injected into the RP-HPLC mass spectrometry system and eluted isocratically. Several mobile phases were examined, and all yielded the same results shown in Table 4.1. As seen in Table 4.1, the optimal settings were at a fragmentor voltage of 130 V, capillary voltage of 3250 V, and gain of 5. For routine analyses, the gain was set at 1 because a lower gain substantially increased the lifetime of the electron multiplier, and the background noise was significantly lower at the lower gain. With these optimized MS settings, the peak height of psilocybin was approximately half of that observed for the other analytes in the total ion chromatogram while the areas were nearly the same (Figure 4.1). The corresponding mass spectrum for psilocybin is shown in Figure 4.2; the MS spectra for the other three compounds are also shown for comparison. As can be seen from these spectra, all compounds primarily showed the presence of the M+H peak: psilocybin, *m/z* 285; bufotenine, *m/z* 205; psilocin, *m/z* 205; and tryptamine, *m/z* 161. The mass spectrum for psilocybin contained a second significant peak (*m/z* 307), corresponding to the sodium adduct (M+Na) (Figure 4.2A).

Table 4.1 Peak area of the protonated molecular ion M+H (m/z 285) for psilocybin at different mass spectrometer settings. When the fragmentor voltage was examined, the capillary voltage and gain were set at 3000 V and 1, respectively. When the capillary voltage was examined, the fragmentor voltage and gain were set at 130 V and 1, respectively. When the gain was examined, the fragmentor voltage and capillary voltage were set at 130 and 3250 V, respectively.

Fragmentor Voltage		**Capillary Voltage**		**Gain**	
V	**Area (x 10^5)**	**V**	**Area (x 10^5)**		**Area (x 10^5)**
40	5.05	2000	6.37	1	6.76
65	6.58	2250	7.27	2	12.96
90	7.86	2500	7.20	3	16.12
115	7.46	2750	7.28	4	19.90
130	7.92	3000	7.31	5	34.02
140	7.86	3250	7.38		
165	5.19	3500	7.07		
190	2.16	3750	7.04		
215	0.38				

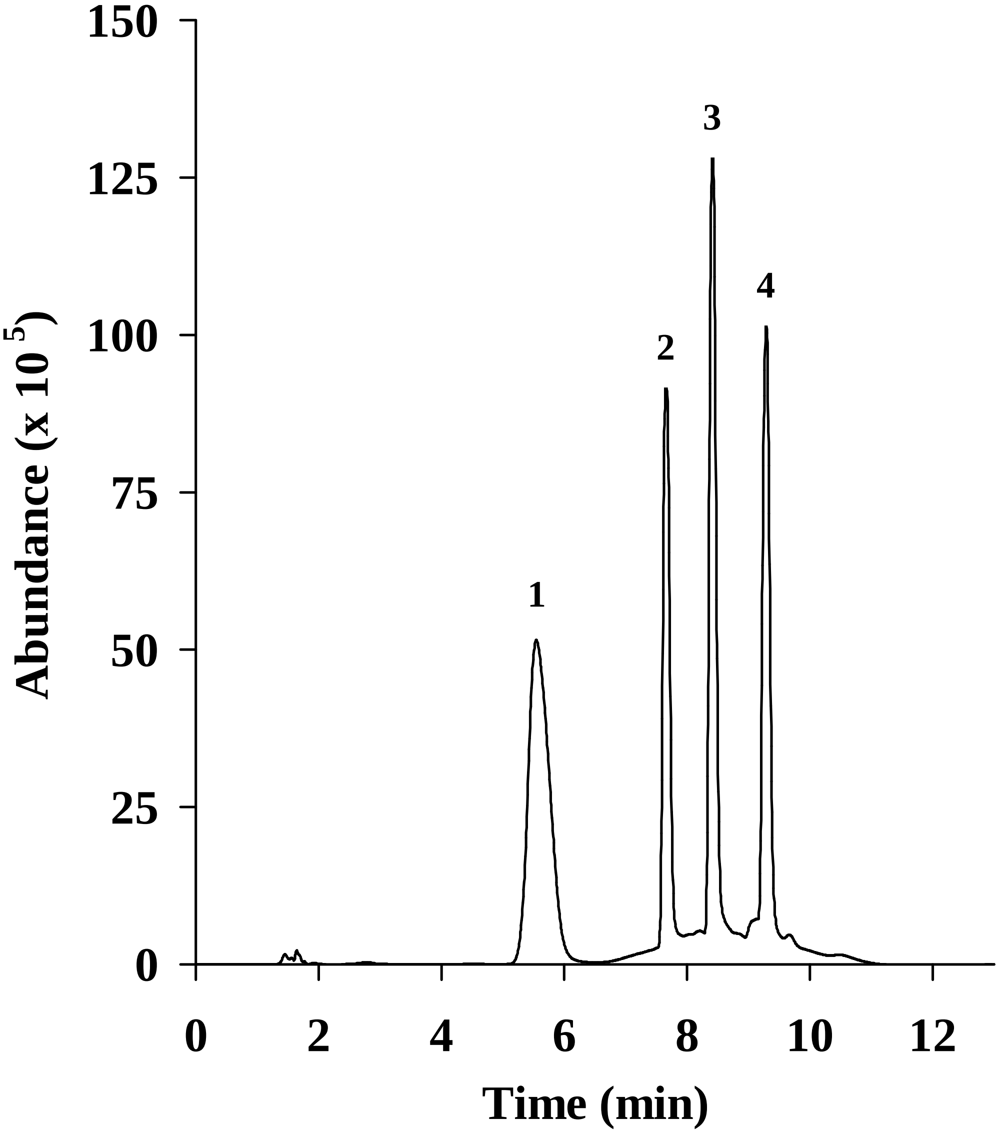


Figure 4.1 Optimized MS total ion chromatogram for (1) psilocybin, (2) bufotenine, (3) psilocin and (4) tryptamine standards employing a gradient elution with a Phenomenex Hydro RP C_{18} column (3.0 x 150 mm, 4 µm packing). The mobile phase consisted of A) 15 mM, pH 3.5 ammonium formate and B) 5% (v/v) aqueous methanol. The gradient employed was: 5% B for 3 min, 5 to 30% B from 3 to 5 min, 30% B from 5 to 8 min, 30 to 5% B from 8 to 9 min, and 5% B from 9 to 18 min. Analyses were performed at 25 ^{o}C and a flow rate of 0.5 mL min^{-1}. The injection volume was 4 µL. Mass spectrometer parameters were: positive polarity, scanning from 50-320 *m/z*, capillary 3250 V, fragmentor 130 V and gain 1. Spray chamber parameters were: gas temperature 350 ^{o}C, drying gas 12 L min^{-1}, and nebulizing pressure 60 psig.

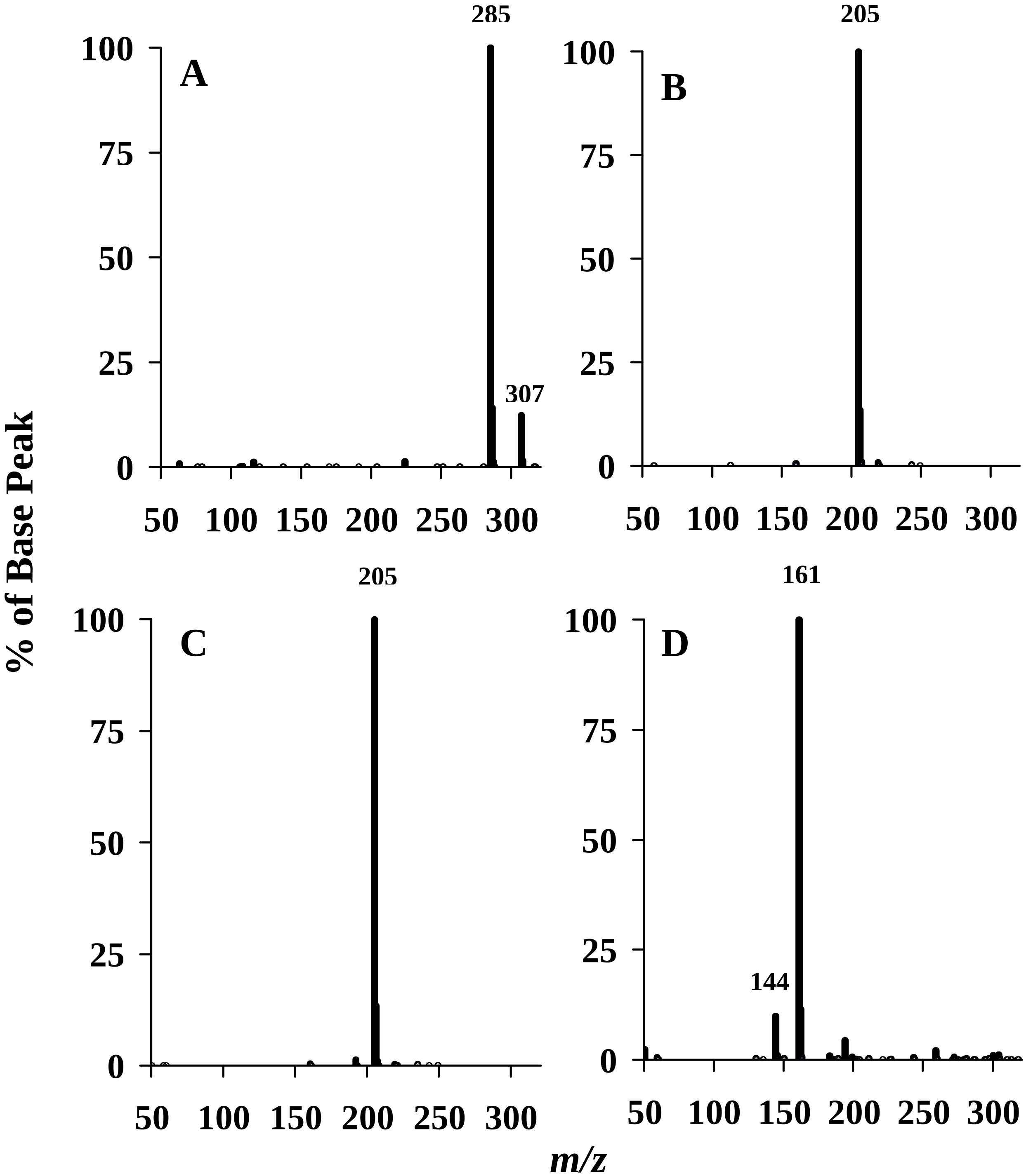


Figure 4.2 Mass Spectra of (A) psilocybin, (B) bufotenine (C) psilocin, and (D) tryptamine generated from the chromatographic peaks depicted in Figure 4.1, with the fragmentor voltage set at 130 V. Other mass spectrometer parameters are listed in Figure 4.1.

The optimized MS settings resulted in a small amount of fragmentation for tryptamine, as observed by the appearance of a fragment ion at *m/z* 144 (Figure 4.2D).

Increasing the fragmentor voltage increased the degree of fragmentation for each compound due to collision-induced dissociation (CID). To illustrate this point, the mass spectra of the four compounds at a fragmentor voltage of 130 V (Figure 4.2) were compared to the mass spectra obtained at a fragmentor voltage of 250 V (Figure 4.3). At 250 V, the M+H peak for bufotenine, psilocin and tryptamine disappeared, while several large peaks were observed for psilocybin, with the two main peaks corresponding to the M+H molecular ion (*m/z* 285) and a fragment at *m/z* 240. Although mass spectra were dominated by fragmentation, unique mass spectra were nonetheless obtained for the positional isomers bufotenine and psilocin at a fragmentor voltage of 250 V. For bufotenine, the base peak was observed at *m/z* 160, with abundant fragments found at *m/z* 105, 115, and 133, all $\geq$ 25% of the base peak (Figure 4.3B). By comparison, the base peak for psilocin was at *m/z* 115, with only one other significant fragment detected (*m/z* 160) at $\geq$ 25% of the base peak.

The fragment ion pattern that we observed for psilocin (*m/z* 205) and psilocybin (*m/z* 285 and 307) at a low fragmentor voltage (Figure 4.2) was very similar to that obtained by Rodriguez-Cruz (2005). In the Rodriguez-Cruz study, the main ion for psilocin was positioned at *m/z* 205, with two other smaller fragments observed at *m/z* 160 and 206. These two fragments were present in our mass spectrum but at a very low abundance (0.5% for *m/z* 160 and 13% for *m/z* 206). For psilocybin, Rodriguez-Cruz observed the same fragment ions of *m/z* 285 and 307. At higher voltages and using SID experiments, Rodriguez-Cruz detected a base peak for psilocybin at *m/z* 285 and a large secondary peak at *m/z* 307, which was approximately 45% of the base peak. Several significant

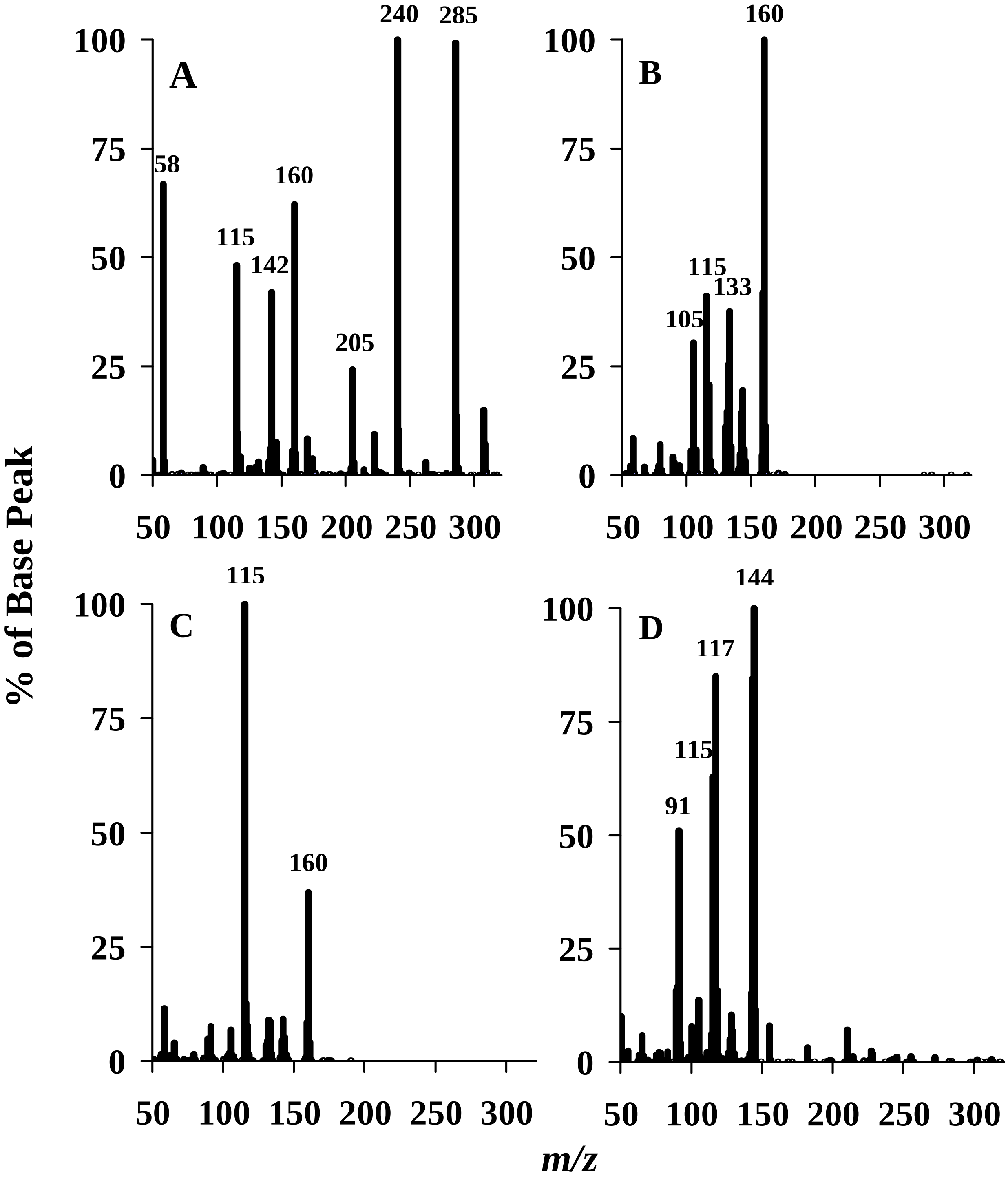


Figure 4.3 Mass Spectra of (A) psilocybin, (B) bufotenine (C) psilocin, and (D) tryptamine generated from the chromatographic peaks depicted in Figure 4.1, with the fragmentor voltage set at 250 V. Other mass spectrometer parameters are listed in Figure 4.1

fragments were also observed at *m/z* 115, 142, 160, 205 and 240. Our results for psilocybin at a higher fragmentor voltage and using CID, showed the same fragmentation pattern. The two main peaks were centered at *m/z* 285 and 240 (Figure 4.3A). The *m/z* 307 peak was at approximately 15%, of these two peaks. We also detected abundant fragments at *m/z* 115, 142,160 and 205. The one difference that was observed between these two studies was that we detected a significant fragment at *m/z* 58, which was not an important fragmentation species in the Rodriguez-Cruz study.

Good agreement was also observed for psilocin. Rodriguez-Cruz observed a base peak at *m/z* 160, with a substantial fragment at *m/z* 115 (~80% of the base peak). The other two main ions generated were at *m/z* 132 and 142; minor peaks were observed at *m/z* 105 (5%) and *m/z* 117 (20%). Our results for psilocin showed the base peak centered at 115, with the second main fragment at *m/z* 160 (Figure 4.3C). Other less abundant fragments were seen at *m/z* 105, 117, 132 and 142 (~10% each). Even though the fragmentation pattern was similar for these two studies, there were differences in the relative abundance of the fragments. This was likely due to differences in collision induced disassociation and source induced disassociation for these two studies. However, since the MS fragmentation patterns in the CID spectra are the nearly same, they can be used as a fingerprint for compound-specific identification.

While useful information was obtained by optimizing the MS settings using methanolic standards, it was also important to determine if the optimal settings were appropriate for extracted mushroom samples. Therefore, a dry mushroom sample was first extracted with 25 mL of methanol by rotary inversion (50 rpm) followed by a second extraction with a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5). No bufotenine or IS were added to this mushroom sample. The two extracts were analyzed using the optimized RP-HPLC mass spectrometry parameters, and the resulting chromatograms are shown in Figure 4.4. The mass spectra generated for each peak from the chromatogram are depicted in Figure 4.5. From the chromatograms, it is evident that methanol yielded a high percentage of psilocybin but no psilocin, while the methanol-citrate solution extracted psilocin but no psilocybin. Although numerous peaks were detected in both chromatograms, the mass spectra generated for psilocybin and psilocin from the mushroom extracts at a fragmentor voltage of 130 and 250 V (Figure 4.5) were the same as the spectra generated for the standards (Figure 4.2 and 4.3). This provided excellent confirmation that peaks 1 and 2 in the chromatogram shown in Figure 4.4 were psilocybin and psilocin, with no significant interfering compounds masking their identification.

Extraction. The extraction of psilocybin and psilocin from ground mushroom material was first determined with methanol using an ultrasonic water bath (Sonicor model SC-5OT 50 Watt sonicator), a rotary invertor, and bench top incubation with no mixing. Methanol extraction was examined initially because it has been the extraction solvent of choice in many past studies focused on quantification of psilocybin and psilocin in mushroom material. In my study, when methanol was used to extract these compounds from mushrooms, psilocin was extracted very poorly, irrespective of the extraction volume used (data not shown), in large part because the batch of mushroom used to test methanol contained very little psilocin (ca. 0.005 mg mL^{-1}).

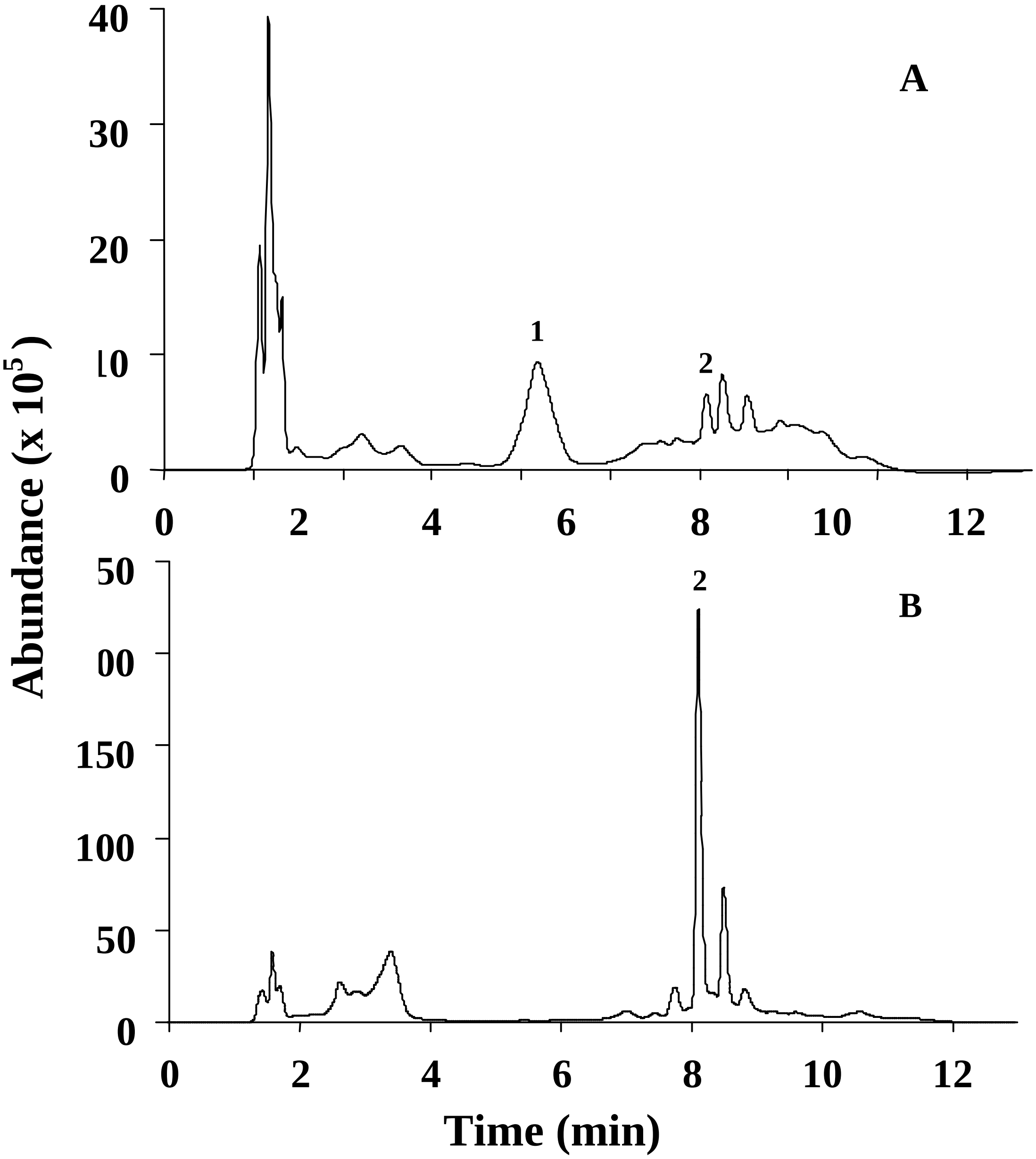


Figure 4.4 Optimized MS total ion chromatogram of mushroom material sequentially extracted with A) 25 mL methanol followed by B) a solution of 12.5 mL of methanol and 12.5 mL of 50 mM, pH 2.5 citrate buffer. Compounds were separated by gradient elution on a Phenomenex Hydro RP C_{18} column (3.0 x 150 mm, 4 µm packing). Peaks: (1) psilocybin and (2) psilocin. The mobile phase consisted of A) 15 mM, pH 3.5 ammonium formate and B) 5% (v/v) aqueous methanol. The gradient employed was: 5% B for 3 min, 5 to 30% B from 3 to 5 min, 30% B from 5 to 8 min, 30 to 5% B from 8 to 9 min, and 5% B from 9 to 18 min. Analyses were performed at 25 ^{o}C, and a flow rate of 0.5 mL min^{-1}. The injection volume was 4 µL. Mass spectrometer parameters were: positive polarity, scanning from 50-320 *m/z*, capillary 3250 V, fragmentor 130 V, and gain 1. Spray chamber parameters were: gas temperature 350 ^{o}C, drying gas 12 L min^{-1}, and nebulizing pressure 60 psig.

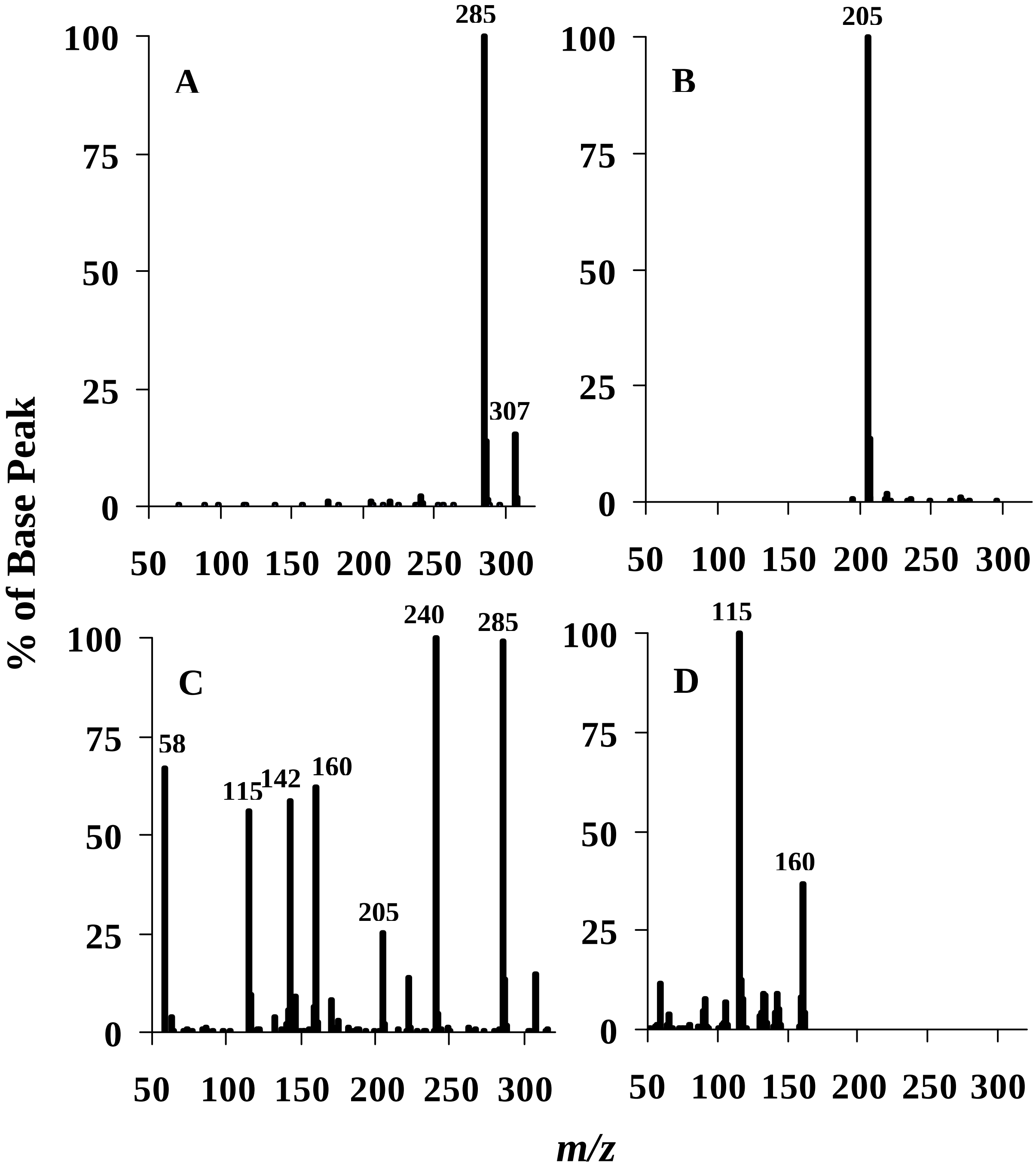


Figure 4.5 Mass Spectra generated from the chromatogram depicted in Figure 4.4 (A) psilocybin at a fragmentor voltage 130 V, (B) psilocin at fragmentor voltage 130 V, (C) psilocybin at fragmentor voltage 250 V, and (D) psilocin at fragmentor voltage 250 V. Other mass spectrometer conditions are listed in the Methods section.

In addition to methanol, other extraction solvents were examined to determine their ability to extract both psilocybin and psilocin from hallucinogenic mushrooms. Solvents examined included acidic methanol (i.e., 0.1% (v/v) formic acid in methanol), ethanol, isopropyl alcohol, ethyl acetate and a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5). Extractions were carried out with 300 mg of ground mushroom placed in 25 mL of each solvent. Samples were then secured to the rotary inverter and extracted for 2 h. Results of this study are presented in Table 4.2. For psilocybin, methanol was the best extraction solvent resulting in the highest percentage extracted (0.270%) closely followed by acidic methanol (0.249% extracted). As previously observed, psilocin was extracted very poorly from mushroom material with methanol (or acidic methanol (ca. 0.072%). The only solvent system that was found to extract significant quantities of psilocin was from the sequential method using methanol followed by a 50:50 (v/v) solution of methanol and citrate buffer (0.339% extracted). Using this method, approximately five times more psilocin was extracted from the ground mushroom samples compared to that obtained with methanol or acidic methanol. While aqueous solutions of methanol extracted psilocybin and psilocin from ground mushroom samples, neither compound was extracted after 2 h with ethanol, 1-proponol or ethyl acetate (Table 4.2). By comparison, Gartz (1994) found that extracting mushroom samples with methanol and continually stirring did not extract psilocybin in many mushroom species until samples were extracted for 6-12 h. Kysilka and Wurst (1990) used 90% methanol with 1% acetic acid and found that it extracted most of the psilocybin from the ground mushroom sample. They also found that 75% (v/v) aqueous ethanol yielded the highest mass of psilocin extracted from mushroom samples. In our

Table 4.2 Average percent (± standard deviation) of psilocybin and psilocin ((mg extracted / mg of mushroom material) x 100) extracted from 300 mg of mushroom material in 25 mL of each solvent after 2 h of rotary inversion (50 rpm). Each extraction was done in triplicate (n=3). BDL = below detection limit.

	Psilocybin	**Psilocin**
Ethanol	BDL	BLD
1-Propanol	BDL	BDL
Ethyl acetate	BLD	BDL
Acidic methanol*	0.249 (0.003)	0.079 (0.005)
Methanol	0.270 (0.004)	0.072 (0.004)
Citric acid and methanol**	0.203 (0.003)	0.339 (0.005)

*****0.1% formic acid in methanol (v/v).

******Sequential extraction: First extraction was with methanol followed by a second extraction of the same mushroom material with a 50:50% (v/v) solution of methanol and 50 mM, pH 2.5 citrate buffer. Each extract was analyzed separately by RP-HPLC and the amount of psilocin or psilocybin detected in both extracts was added to yield the total amount extracted.

study, no psilocybin or psilocin was extracted with ethanol. The basis for these contrasting results is not known, but may be related to the water content of the extracting solvent. Given the significance of these contrasting results, further research is warranted.

As discussed above, the sequential extraction method yielded the best extraction of psilocin from mushroom samples. Based on this finding, a study was conducted to determine the effect of % citrate buffer in methanol on the extraction of psilocybin and psilocin from mushroom samples. For this study, 500 mg mushroom and 25 mL citrate-methanol solution were extracted for 2 h by rotary inversion. Large differences were seen in the amount of psilocin extracted when going from 100% methanol to 100% citrate buffer (Figure 4.6). The best extraction was obtained with a 50:50 solution of citrate-methanol (0.75 mg extracted), while no psilocin was extracted from 100 % citrate buffer and very little was extracted from methanol (0.1 mg). The amount extracted at 50% was nearly twice the amount extracted with 25 and 75% citrate-methanol solutions. For psilocybin, the amount extracted at $\leq$ 25 % citrate was more than an order of magnitude higher than observed at $\geq$ 50 % citrate. As a result of these differences, it was concluded that overall the 50:50 % (v/v) citrate-methanol solution yielded the best extraction of both psilocin and psilocybin, even though this solvent system did not yield optimal results for psilocybin. Additionally, since methanol gave the best extraction results for psilocybin, a two-step extraction procedure was adopted to maximize the extraction of both psilocybin and psilocin from mushroom material.

The effect of extraction time on the amount of psilocin and psilocybin extracted from mushrooms was determined by extracting 300 mg mushroom with 25 mL of solvent

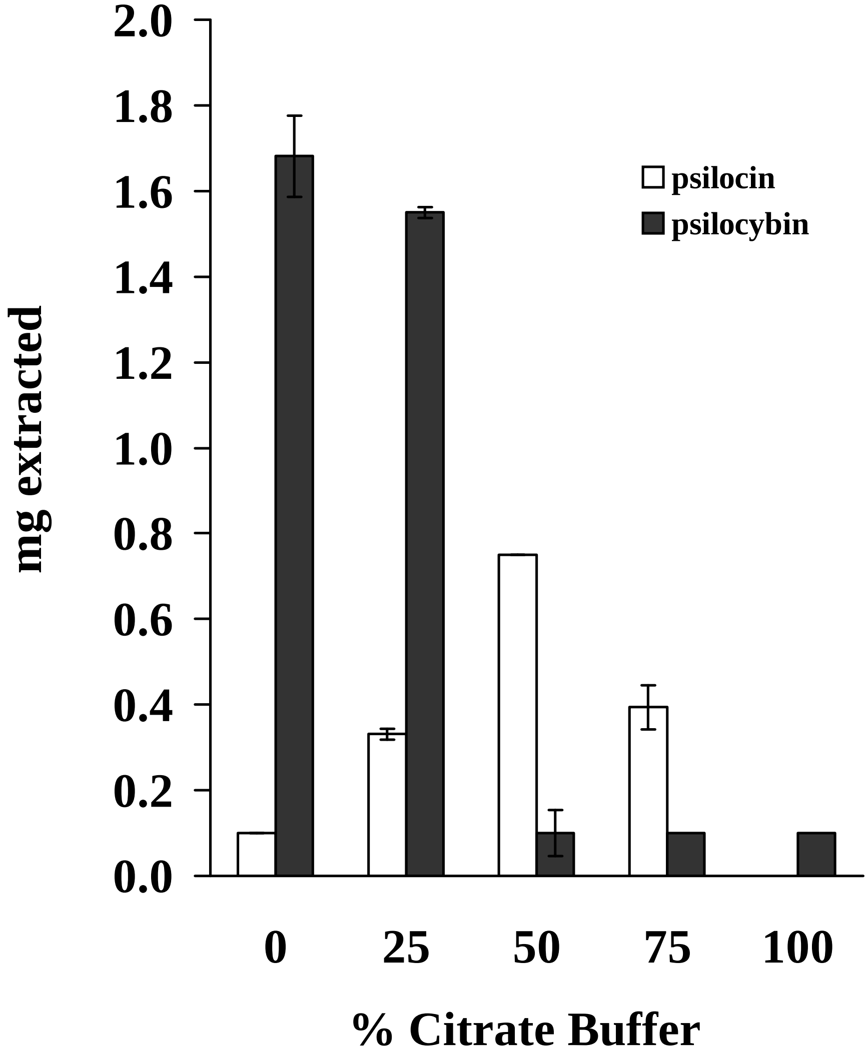


Figure 4.6 Mass (mg) of psilocybin and psilocin extracted from 500 mg ground mushroom material using different extraction solutions containing citrate buffer and methanol. Samples were extracted for 2 h with 25 mL of solution by rotary inversion (50 rpm). The extraction solution consisted of methanol and different percentages of 50 mM, pH 2.5 citrate buffer. Error bars denote the standard deviation of two different samples injected twice each (n=4).

using rotary inversion (set at 50 rpm). Two solvent systems were tested, methanol and a 50:50% (v/v) solution of methanol and 50 mM citrate (pH 2.5). Results of this study are shown in Figure 4.7. In general, psilocin and psilocybin were completely extracted from mushroom samples after approximately 2 h, with a larger amount of psilocybin extracted (ca. 0.18 %) compared to psilocin (ca. 0.12 %). For psilocin, the extraction was nearly the same in methanol versus that obtained in the 50:50 (v/v) methanol – citrate (50 mM, pH 2.5) solution.

In my study, only 51% of the psilocybin was extracted in the first 1 h (Figure 4.7). By contrast, Sottolano and Lurie (1983) found that approximately 95% of total amount of psilocybin was extracted from 600 mg ground mushroom in an ultrasonic water bath with 10 mL in less than 1 h. The basis for this difference is not known but may be related to the type species of mushroom analyzed, particle size extracted or method of extraction Using a 2 h extraction, sonication, rotary inversion and bench top incubation were compared with respect to the relative amount of psilocybin extracted from mushroom samples. Psilocin was not tested, since the mushroom sample used for this study contained no psilocin above the detection limit. Using a 2 h extraction period, all three extraction procedures yielded nearly the same amount of psilocybin extracted when 25 or 50 mL of methanol was used to extract samples, except for the 50 mL rotary inversion sample which was higher (ca. 0.34% extracted) (Figure 4.8). On a percent mass basis ((mg psilocybin extracted/mg mushroom sample)*100), the average extraction was 0.339%, while the range was 0.304% - 0.369%. The least amount of psilocybin was extracted with 10 mL methanol and benchtop incubation with no mixing. For this

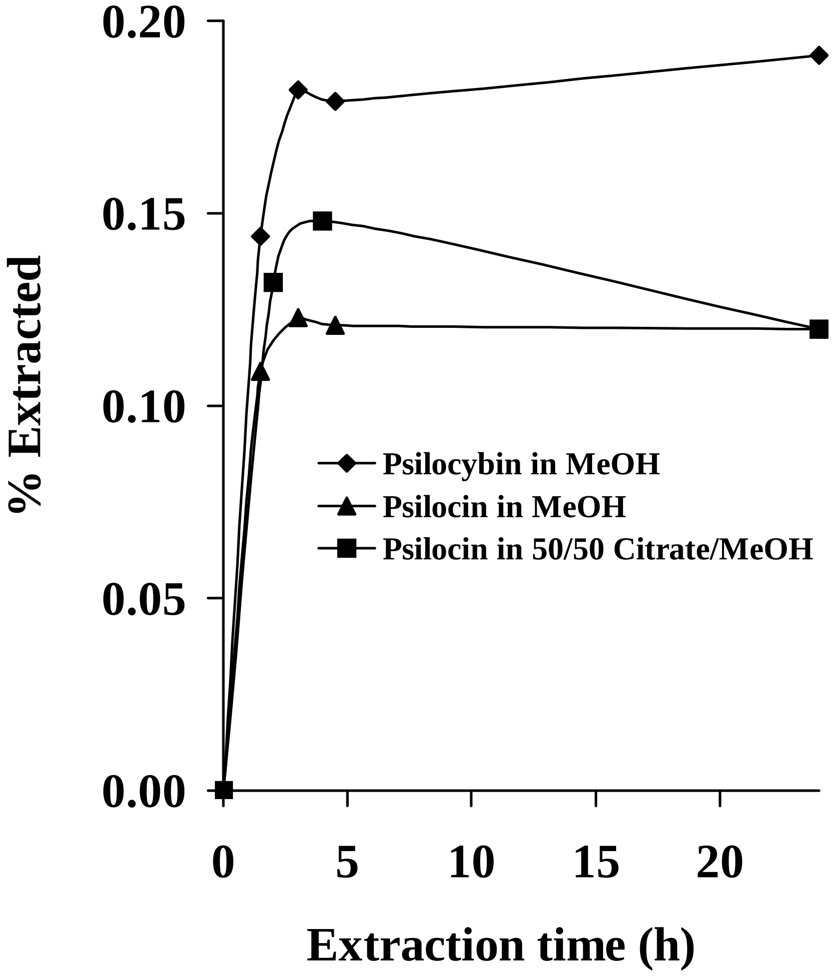


Figure 4.7 Percent psilocybin and psilocin extracted from 300 mg ground mushroom ((mg extracted/mg mushroom)*100) using rotary inversion (50 rpm) as a function of time. Mushroom samples were extracted with 25 mL of either methanol or a 50/50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5). Error bars denote the standard deviation (n=4), which for all samples and extraction times were smaller than the size of the data symbols.

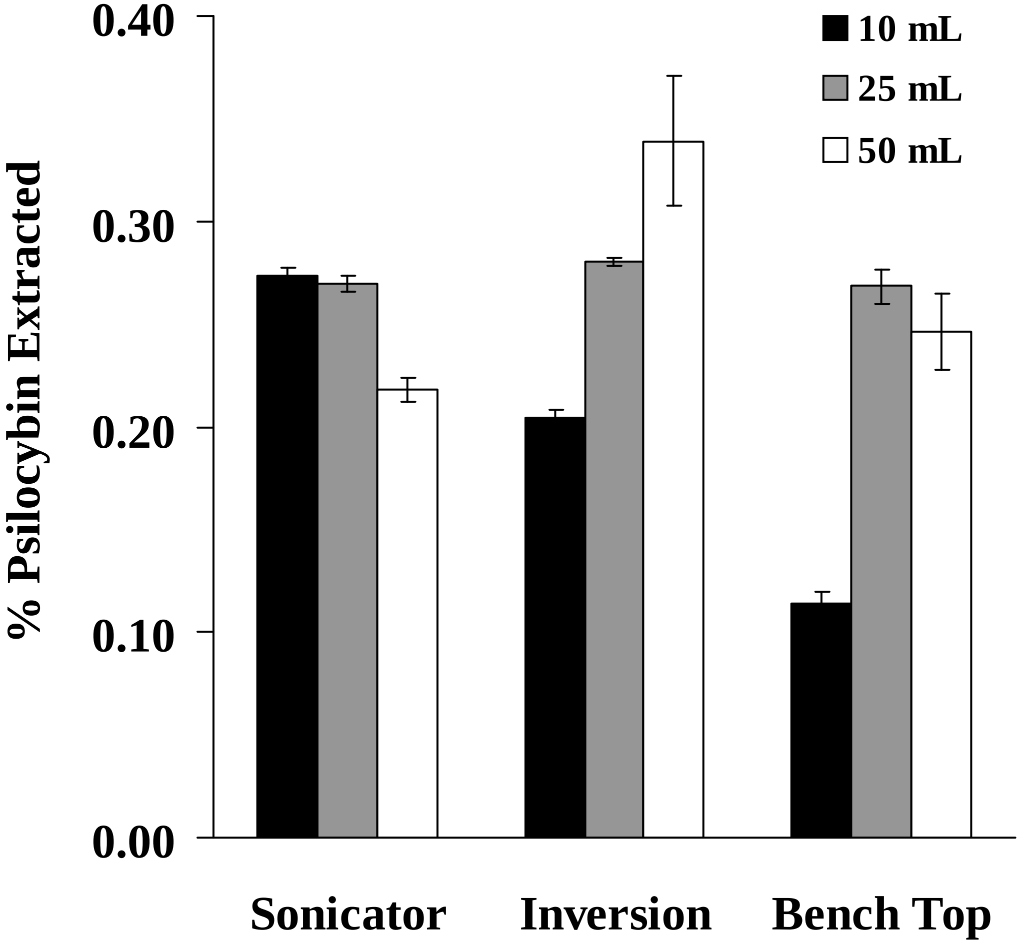


Figure 4.8 Percent psilocybin extracted ((mg psilocybin extracted/mg mushroom) x 100) in 2 h with different volumes of methanol from 500 mg of ground mushroom employing ultrasonic water bath (Sonicor model SC-5OT 50 Watt sonicator), rotary inversion (50 rpm), and bench top incubation with no mixing. Error bars denote the standard deviation (n=4). The mushroom sample used for this study contained no psilocin.

incubation, less than 50% of the psilocybin was extracted relative to the percent mass that was extracted with 25 or 50 mL of methanol.

One drawback to sonication is that the sample is heated during the extraction procedure. Sottolano and Lurie (1983) showed that sonication increased the temperature of samples up to 45^{o}C, which may be a problem for psilocybin since it is heat sensitive and can be easily dephosphorylated to psilocin. Sottolano and Lurie (1983) monitored the thermal breakdown with a separate psilocybin standard; no thermal breakdown was detected during the extracting procedure. In my study, a 4 ^{o}C water bath was used to control the sample temperature and avoid the thermal breakdown of psilocybin. As shown in Figure 4.8, there is no evidence for significant breakdown of psilocybin resulting from sonication, albeit the amount extracted with 50 mL was somewhat lower than obtained with a 25 or 50 mL extraction by inversion or benchtop. Nonetheless, sonication is not recommended as it may lead to significant free radical production and loss of psilocybin and psilocin, depending on the power output of the sonicator.

Mushroom Mass Extracted. In order to quantitatively analyze mushroom material, a representative sample of the mushroom material is needed. Most published methods ground mushroom samples using a mortar and pestle. The powder obtained after grinding is then used to extract psilocin and psilocybin from mushroom samples. Generally, only a very small sample is analyzed (ca. 10 – 25 mg; e.g., Vanhaelen-Fastre and Vanhaelen, 1984; Wurst et al., 1984; Saito et al., 2004; Kamata et al., 2005). This sample size may not be representative of the material being tested.

In my study, I ground mushroom material with a cheese grater because this resulted in mushroom material with uniform consistency. Analysis of 300-500 mg of the ground sample resulted in very reproducible results between samples, generally less than 5% difference. Nonetheless, a study was conducted to determine if this sample-size range yielded representative results. To determine the effect of sample size on the amount of psilocin and psilocybin extracted, from 100 - 500 mg of ground mushroom material was extracted using the two-step extraction procedure. Samples were first extracted with 25 mL methanol for 2 h using rotary inversion (50 rpm) followed by a 2 h extraction with a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5). As may be expected, the amount of psilocin and psilocybin extracted increased linearly with amount of mushroom material extracted (Figure 4.9A), and when extract results were normalized to the mass of mushroom it was observed that the amount extracted was independent of sample size within experimental error (Figure 4.9B).

Phosphatase Activity. As discussed previously, psilocin is generally reported to be a minor constituent in mushrooms. In cases where it has been detected in mushroom extracts, Gartz (1993) suggested that this may be an artifact of the extraction procedure "caused by the enzymatic destruction of psilocybin, which is common in different species (of mushrooms) by using water-containing organic solvents." To test this hypothesis, I determined the phosphatase activity in several different mushroom extracts. As shown in Table 4.3 no activity was detected in the methanol extracts. This is not surprising, as methanol is known to denature (and therefore deactivate) proteins. The advantage of

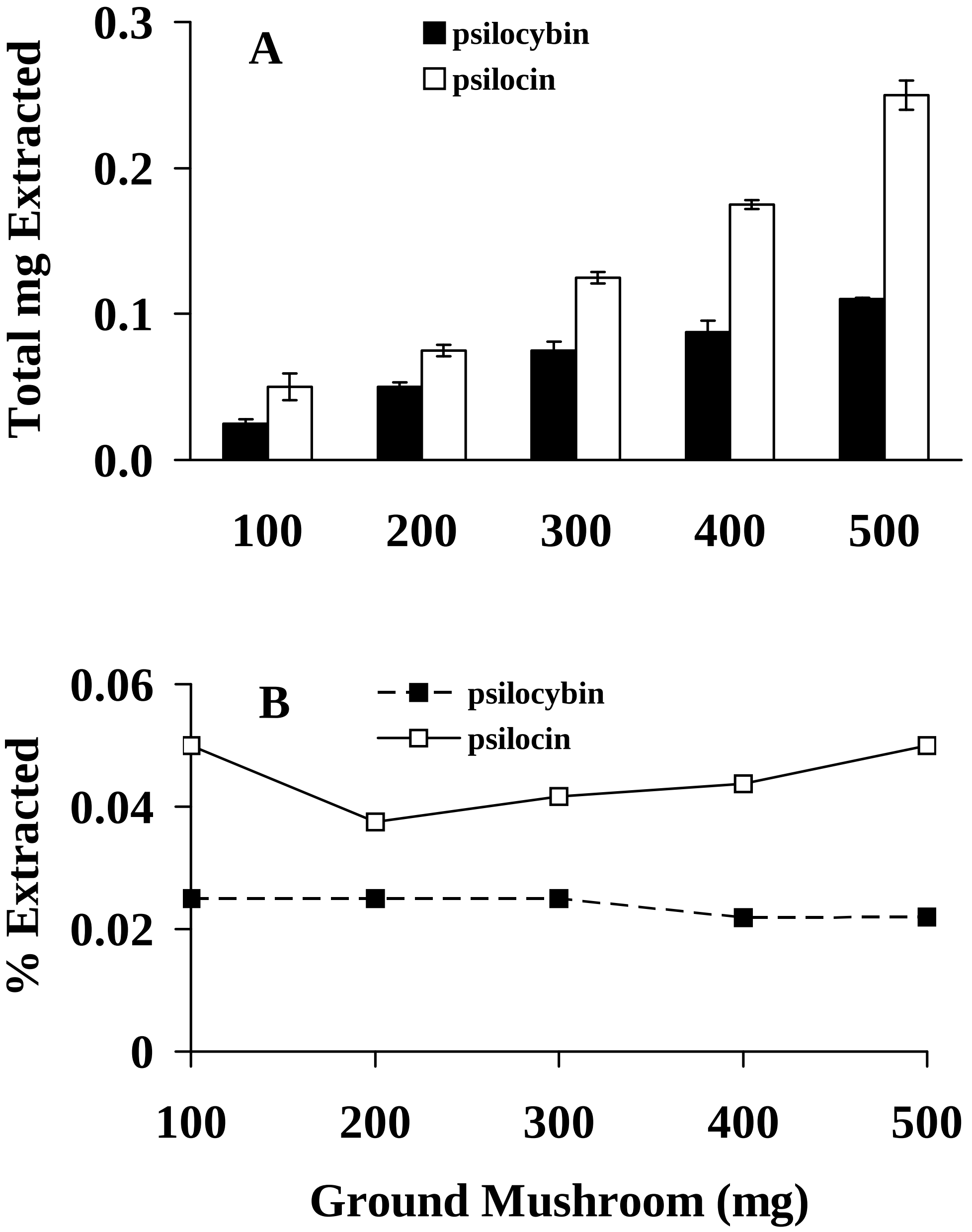


Figure 4.9 A) Total mass (mg) of psilocybin and psilocin extracted from ground mushroom material using rotary inversion (50 rpm). B) Percent total mass extracted normalized to mushroom sample size. Samples were sequentially extracted with 25 mL methanol followed by a solution of 12.5 mL of methanol and 12.5 mL of 50 mM citrate buffer (pH 2.5). Each extract was analyzed separately by RP-HPLC and the amount of psilocin or psilocybin detected in both extracts was added to yield the total amount extracted. Error bars denote the standard deviation (n=4).

Table 4.3 Phosphatase acitivity and specific activity in 25 mL mushroom extracts that were rotary inverted (50 rpm) for 2 h. The µmole *p*-nitrophenate produced per minute was determined by measuring the absorbance at 405 nm. Protein content was determined following the procedure described by Bradford (1976). BDL = below detection limit.

	Activity (µmol min^{-1})	**Specific Activity (µmol min^{-1}/µg protein)**
Methanol	BDL	BDL
15 mM Ammonium formate	0.361	0.158
50 mM, pH 2.5 Citrate	0.065	6.335
MCA*	0.003	BDL
50:50**	0.007	BDL

*Extraction with methanol followed by a second extraction with 50:50% (v/v) solution of methanol and 50 mM, pH 2.5 citrate buffer. The phosphatase activity was only measured in the second extract.

**Single extraction with a 50:50% (v/v) solution of methanol and 50 mM, pH 2.5 citrate buffer.

extracting mushroom material with methanol is that the majority ($\geq$99%) of the psilocybin is extracted in this fraction where it is not expected to degrade (i.e., dephosphorylate), since the phosphatase should be denatured and therefore inactive. After mushroom samples were extracted with methanol, this was followed by a second extraction with a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5). Phosphatase activity was detected in this aqueous methanolic extract as well as the other aqueous extracts examined in this study. While the activity that was detected was low, this could still lead to substantial loss of psilocybin and production of psilocin in stored samples, supporting Gartz's supposition. However, if the two-step extraction procedure is employed and the extracts are stored separately (as was done in this study), then the enzymatic conversion of psilocybin to psilocin is not expected to be a significant problem because essentially all the psilocybin is extracted in methanol (Table 4.2).

Case Study Comparison. Since the beginning of this study, the amount of psilocin and psilocybin quantified from mushroom material was recorded. However, during this time (ca. 5 years), three different extraction procedures were used. The first method used was that of Sottalano and Lurie (1983) wherein mushroom samples were extracted for 1 h using 10 mL of methanol and sonication (power not reported) in a 20 ^{0}C water bath (method A). This method was replaced by sequential extraction of 500 mg of mushroom for 1 h by rotary inversion, with 25 mL methanol followed by 25 mL of a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5) (method B). Finally, the optimized extraction procedure that was adapted consisted of extraction of 300 mg of mushroom sample for 2 h by rotary inversion with 25 mL methanol followed by a second

2 h extraction with 25 mL of a 50:50 (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5) (method C). These three extraction procedures were compared by pooling all the extraction results for each method. Results of this comparison are striking, as shown in the box and whisker plot (Figure 4.10). The Sottolano and Lurie method on average gave a very poor mass % extracted ((mg extracted/mg mushroom sample)*100) for psilocin (0.01%) and psilocybin (0.079%) compared to the 1 h sequential extraction of these compounds, (0.119%) and (0.159%) respectively. However, as noted previously (Figure 4.7), a 1 h extraction time was not sufficient to completely extract psilocin and psilocybin. It was therefore not surprising that the 2 h extraction yielded an even higher % mass extracted for psilocin and psilocybin. The percent mass extracted for psilocin and psilocybin in the 2 h extraction was nearly double that for the 1 h extraction, and nearly thirty times higher for psilocin and four times higher for psilocybin compared to the Sottolano and Lurie method (Figure 4.10). Although these results indicate that the two-step 2 h extraction yielded much more psilocin and psilocybin compared to the other extraction procedures, these results should only be evaluated qualitatively (i.e., general trend), since they are based on the analysis of different mushroom samples and as such are not directly comparable.

Percent Mass of Psilocybin and Psilocin Extracted. The amount of psilocybin and psilocin extracted with our optimized method was compared to published values (Table 4.4). The mass percent of psilocybin extracted in this study was comparable to published findings. At the high end, the amount of psilocybin extracted (0.91 w/w%) was lower than that observed in several published findings, which ranged between 1.0 and 2.0

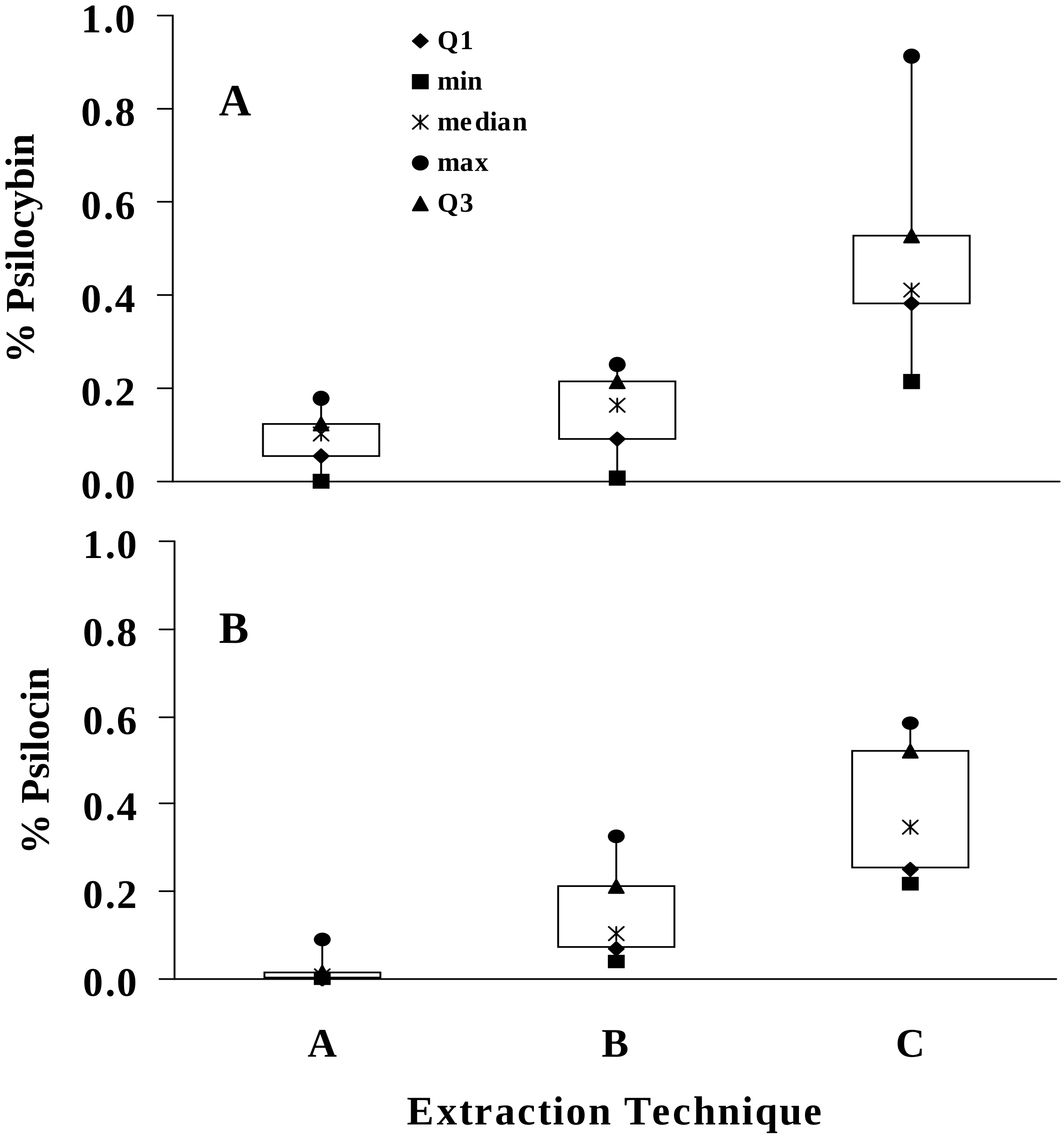


Figure 4.10 Box and Whisker plot showing the percentage of psilocybin and psilocin extracted ((mg extracted/mg of mushroom) x100) in separate forensic samples (cases) analyzed over several years using three different extraction methods. Method **A** (n=29 cases) is that of Sottolano and Lurie (1983). Method **B** (n=14 cases) is the sequential extraction procedure (500 mg ground mushroom extracted with 25 mL MeOH for 1 h by rotary inversion followed by a 1 h extraction with a 50:50 (v/v) solution of methanol and 50 mM, pH 2.5 citrate buffer. Method **C** (n=11 cases) is sequential extraction with methanol for 2 h by rotary inversion followed by a 2 h extraction with a 50:50 (v/v) solution of methanol and 50 mM, pH 2.5 citrate buffer. A case denotes a set of mushroom samples submitted to the Onondaga County Center for Forensic Sciences, Syracuse New York, for analysis to support criminal charges.

Table 4.4 Weight percent (w/w %) of psilocybin and psilocin in mushroom samples. NA denotes that the species of mushroom analyzed was not reported.

Reference	**Psilocybin (w/w %)**	**Psilocin (w/w %)**	**Species**
Perkal et al. (1980)	0.0 – 0.21		*P. subaeruginosa*
Beug and Bigwood (1981)	0.15 – 0.85	0.0 – 0.59	*P. baeocystis*
Christiansen et al. (1981)	0.55 – 1.96		*P. semilanceata*
	0.17 – 1.08		*P. semilanceata*
Christiansen and Rasmussen (1982)	0.55 – 1.0		
	0.2 – 2.0		*P. semilanceata*
Sottolano and Lurie (1983)	0.1 – 1.5		NA
Wurst et al. (1984)	0.33 – 1.05	0.04 – 0.68	*P. semilanceata*
	0.25 – 1.14	0.02 – 0.07	*P. bohemica*
Kysilka and Wurst (1990)	1.23	0.44	*P. bohemica*
Wurst et al. (1992)	0.46 – 1.14	0.02 – 0.48	*P. bohemica*
	0.76 – 1.05	0.09 – 0.12	*P. semilanceata*
	0.0 – 0.10	0.45 – 0.47	*P.cyanescens*
	0.03 – 0.38	0.0 – 0.02	*P. aeruginasens*
Gartz (1994)	0.32 – 0.98	0.0 – 0.51	NA
Keller et al. (1999)	0.86	0.02	*P. subcubensis*
Tsujikawa et al. (2003)	0.37 – 1.30	0.14 – 0.42	*P. cubensis*
	0.08 – 0.22	0.43 – 0.76	*Copelandia sp.*
Kamata et al. (2005)	0.02 – 0.38	0.06 – 0.14	NA
This study	0.09 – 0.91	0.23 – 0.58	NA

w/w% (Christiansen et al., 1981; Christiansen and Rasmussen, 1982; Sottolano and Lurie, 1983; Wurst et al., 1984; Kysilka and Wurst, 1990; Wurst et al., 1992; and Tsujikawa et al., 2003). Some of these differences may be attributed to differences associated with extracting caps versus stems or whole mushroom samples (this study), as caps tend to contain more psilocybin than the mushroom as a whole (Wurst et., 1984; Tsujikawa et al., 2003). Secondly, it could be that the species of mushrooms we used in this study (not determined) contained lower concentrations of psilocybin than species such as *P. cyanescens* and *P. semilanceata*. In contrast to the results obtained for psilocybin, the mass of psilocin extracted in my study (0.23 - 0.58 w/w%) was comparable to or exceeded that obtained in most other studies, except for Tsujikawa et al. (2003) (0.43 – 0.76 w/w%). It should be noted that Wurst et al. (1984) detected some high values using an aqueous acid and ethanol solution and a single extraction step to extract mushroom samples, which was optimal for the extraction of psilocin but not necessarily for psilocybin. Again, these differences (i.e., lower psilocin and higher psilocybin) could also be attributed to the species of mushrooms analyzed in this study compared to other studies.

SUMMARY

One of the most significant findings of this study is that there is a considerable amount of psilocin detected in hallucinogenic mushrooms. This was obtained largely by optimizing the extraction procedure. The widely used method of extraction employing methanol readily extracted psilocybin from ground mushroom samples, however, much more variable, and lower, % mass extracted results were obtained for psilocin (see Figure 4.6 and Table 4.2). In order to maximize the amount of psilocybin and psilocin extracted from hallucinogenic mushroom material, a two-step extraction procedure is recommended, with methanol used to extract psilocybin followed by extraction with a 50:50 % (v/v) solution of methanol and 50 mM citrate buffer (pH 2.5) to extract psilocin. These extracts, which are stored separately, can be filtered and analyzed directly by RP-HPLC with mass spectrometry to unequivocally confirm the presence of psilocybin and psilocin. Samples can also be analyzed by RP-HPLC with diode array detection to obtain quantitative information regarding the content of psilocybin and psilocin present in the mushroom samples.

CHAPTER 5

Thesis Summary

One of the main findings of this thesis was that psilocin can be a major component in hallucinogenic mushrooms, in contrast to what is published in the literature. Although not directly comparable, owing to mushroom to mushroom differences in psilocin and psilocybin content, my results suggest that the main reason why psilocin is generally not detected in mushrooms is because it is not extracted very well with methanol. As noted in my thesis, much better extractions were obtained with an aqueous solution of methanol and citrate buffer. The optimal extraction procedure involved two extraction steps. In the first step, mushroom material was combined with methanol, which extracted most of the psilocybin. The second extraction was with an aqueous citrate-methanol solution, which extracted most of the psilocin. No phosphatase activity was detected in the methanol extract that contained most of the phosphatase-labile psilocybin; and very little activity was found in the citrate-methanol extract, which contained very little psilocybin.

The ability to use mass spectrometry as a detector allowed for the unequivocal identification of psilocybin and psilocin in mushroom extracts. Mass spectrometry also allowed for the identification of other compounds present in hallucinogenic mushroom extracts, although, these were not identified in this study. Mass spectrometry has long been the preferred technique for compound identification, and by coupling it with RP-HPLC it is now possible to detect psilocybin and psilocin by their M+H peak and fragmentation pattern. This was not possible with direct GC-MS analysis due to the thermal lability of psilocybin.

Future research should continue to focus on the extraction technique for mushroom analysis because this is the main limitation to the quantification of psilocin and psilocybin in mushrooms. In this regard, studies should be conducted to examine the effect of mushroom particle size on the extraction efficiency of psilocin and psilocybin. Currently, the mortar and pestle approach is too crude and likely has resulted in mushrooms with very different particle size distributions in the literature. It is expected that increased surface area corresponding to a decrease in particle size should lead to higher extraction efficiencies.

It will also be important to determine the effect of mushroom sample storage on the stability of psilocin and psilocybin. Typically, mushroom caps and stems are stored whole at room temperature in a plastic bag for weeks to years. No one has studied the stability of psilocin or psilocybin under these conditions. In the only stability study done to date, Beug and Bigwood (1981) found that freeze-dried mushroom samples were stable when stored at -5 ^{o}C for over 2 years.

As important, the forensic community must adapt a widely available mushroom standard that can be used to test not only particle size effects but also the effect of different solvents on the extraction efficiency of psilocin and psilocybin from mushrooms. It will also be important to understand how physiological conditions and environmental factors affect the psilocin and psilocybin content of different mushroom species, and how mushroom sample storage affects concentrations of these psychoactive compounds. With the development of an efficient extraction procedure, it will also be possible to study the psilocin and psilocybin content of different components of the mushroom (caps, stems, gills, veil, mycelium).

CHAPTER 6

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