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The Determination of Psilocin and Psilocybin in Hallucinogenic Mushrooms by HPLC Utilizing a Dual Reagent Acidic Potassium Permanganate and Tris(2,2′-bipyridyl)ruthenium(II) Chemiluminescence Detection System

ABSTRACT: This paper describes a procedure for the determination of psilocin and psilocybin in mushroom extracts using high-performance liquid chromatography with postcolumn chemiluminescence detection. A number of extraction methods for psilocin and psilocybin in hallucinogenic mushrooms were investigated, with a simple methanolic extraction being found to be most effective. Psilocin and psilocybin were extracted from a variety of hallucinogenic mushrooms using methanol. The analytes were separated on a C_{12} column using a (95:5% v/v) methanol:10 mM ammonium formate, pH 3.5 mobile phase with a run time of 5 min. Detection was realized through a dual reagent chemiluminescence detection system of acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(II). The chemiluminescence detection system gave improved detectability when compared with UV absorption at 269 nm, with detection limits of 1.2×10^{-8} and 3.5×10^{-9} mol/L being obtained for psilocin and psilocybin, respectively. The procedure was applied to the determination of psilocin and psilocybin in three Australian species of hallucinogenic mushroom.

KEYWORDS: forensic science, HPLC, chemiluminescence detection, psilocin, psilocybin, hallucinogenic mushrooms, extraction

Psilocin and psilocybin are naturally occurring indoles in hallucinogenic mushrooms belonging to the genera of *Psilocybe* (1,2). Psilocin and psilocybin can be present in concentrations up to 0.5% and 2% m/m, respectively, in hallucinogenic mushrooms (3).

Several techniques have been employed to determine psilocin and psilocybin in hallucinogenic mushrooms for forensic analysis, with high-performance liquid chromatography (HPLC) being by far the most widely used analytical technique. Detection modes include ultraviolet (UV) (1,2,4–18), fluorescence (2,12,13,17,19,20), electrochemical (12), voltametric detection, (6,9), and mass spectrometry (2,5,13,18,20). Other techniques for the determination of psilocin and psilocybin in mushroom samples also include gas chromatography-mass spectrometry (21–23), capillary electrophoresis (3), infrared spectroscopy (8,23), thin layer chromatography (2,15,18,21,24), gas chromatography (6,25), ion mobility spectrometry (22), and flow injection analysis (26). A major focus of many of the studies listed above has been attempts to improve detection limits, as these compounds may only be present in trace quantities. Detection limits for various techniques mentioned above are shown in Table 1.

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Chemiluminescence detection for chemical analysis has attracted great interest in recent years due to its potential for very low limits of detection, and its application to detection for flow analysis and HPLC has been the subject of several reviews (27–33).

A dual acidic potassium permanganate and tris(2,2′-bipyridyl)-ruthenium(II) postcolumn chemiluminescent detection system for the determination of the opiate alkaloids morphine, codeine, oripavine, and thebaine in *Papaver somniferum* extracts was described by Lenehan et al. (34). Potassium permanganate was able to oxidize tris(2,2′-bipyridyl)ruthenium(II) to tris(2,2′-bipyridyl)ruthenium(III) and selectively react with the phenolic opiates morphine and oripavine to elicit chemiluminescence. The chemically generated tris(2,2′-bipyridyl)ruthenium(III) generated chemiluminescence with the nonphenolic opiates thebaine and codeine. A 1:1 mixture of potassium permanganate to tris(2,2′-bipyridyl)ruthenium(II) was demonstrated to generate chemiluminescence for all four alkaloids (34).

In a previous paper, we demonstrated the utility of acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III) chemiluminescence detection of psilocin and psilocybin respectively, utilizing flow injection analysis (26). This paper describes the determination of psilocin and psilocybin in hallucinogenic mushrooms using HPLC with a dual postcolumn chemiluminescence detection system. The results obtained were compared to those obtained using UV absorption detection. A comparison of four extraction methods to determine the concentration of psilocin and psilocybin from *Psilocybe subaeruginosa* using flow injection analysis with acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(II) chemiluminescence detection, respectively, is also discussed.

TABLE 1—*Detection limits reported in the literature for the determination of psilocin and psilocybin.*

Technique	Detection Limits (mol/L)		Elution Time (min)		Reference
	Psilocin	Psilocybin	Psilocin	Psilocybin	
Ion mobility spectrometry	NR	1.4×10^{-4}	12	12	(22)
HPLC					(12)
(i) UV absorption	3.9×10^{-6}	3.5×10^{-6}	13	11	
(ii) fluorescence	9.7×10^{-6}	1.7×10^{-6}			
(iii) electrochemical	3.7×10^{-8}	1.7×10^{-6}			
HPLC-UV absorption	3.9×10^{-6}	3.5×10^{-6}	12	6.3	(7)
HPLC-UV absorption (ion pairing reagent)	4.9×10^{-6}	3.5×10^{-6}	5.91	1.94	(15)
HPLC-ESI-MS	6.2×10^{-7}	NR	23	NR	(20)
GC-MS	4.9×10^{-4}	3.5×10^{-4}	8	NR	(21)
TLC	1.5×10^{-4}	1.1×10^{-4}	NR	NR	(21)
Capillary electrophoresis	NR	3.1×10^{-6}	NR	7.0	(3)
Flow injection analysis	9.0×10^{-10}	3.5×10^{-10}	NR	NR	(26)

HPLC, high-performance liquid chromatography; UV, ultraviolet; GC-MS, gas chromatography–mass spectrometry; TLC, thin layer chromatography; NR, not reported.

Materials and Methods

Chemicals and Reagents

Deionized water and analytical grade reagents were used unless otherwise specified. Stock solutions of psilocin and psilocybin were purchased from Alltech (Sydney, Australia). 4-Hydroxyindole was purchased from Aldrich (Milwaukee, WI). Tris (2,2′-bipyridyl) ruthenium chloride hexahydrate (Strem Chemicals, Newburyport, MA) was dissolved in dilute sulfuric acid (0.05 M, BDH, Poole, U.K.). Potassium permanganate (Ajax Chemicals, Sydney, Australia) solutions were prepared daily in sodium polyphosphate solution (0.05% m/v, Aldrich, Castle Hill, Australia), with the pH adjusted to pH 2.0 with dilute orthophosphoric acid (BDH, Poole, U.K.). Ammonium formate and methanol were purchased from BDH. Dried mushroom samples of *Psilocybe subaeruginosa*, *Hypholoma aurantiaca*, and *Panaeolina foenisecii* were supplied by the Royal Botanical Gardens Melbourne, Australia.

Instrumentation

All pH measurements were made using a Jenko Electronics Model 6071 pH meter (CHK Engineering, Rozelle, Australia) using BDH calibration buffers (BDH, Crown Scientific, Rowville, Australia).

UV Detection

Chromatographic runs were performed using a Hewlett Packard 1100 LC series liquid chromatograph (Agilent Technologies, Forest Hill, Australia). Control of the HPLC pump, UV detection at 269 nm, and data acquisition were achieved using Hewlett Packard Chemstation Software (Agilent Technologies). All separations were accomplished using a Synergi 4 μm Max-RP C_{12} column of dimension 150 mm × 4.6 mm (Phenomenex, Sydney, Australia) and an injection volume of 10 μL. The HPLC solvent composition was 95:5 (% v:v) methanol:10 mM ammonium formate, pH 3.5. A flow rate of 0.5 mL/min was employed over 5 min. Mobile phases were filtered through a 0.45 μm membrane.

Postcolumn Mixed Reagent Instrumentation

The mixed reagent system used a Hewlett Packard 1100 series liquid chromatograph (Agilent Technologies) equipped with a Hewlett Packard analogue digital interface box (Agilent Technologies) for analogue input from the chemiluminescence detector, based on that of Lenehan et al. (34). All separations were accomplished using a Synergi 4 μm Max-RP C_{12} column of dimension 150 mm × 4.6 mm (Phenomenex, Sydney, Australia) and an injection volume of 10 μL. Control of the HPLC pump, UV detection at 269 nm, and data acquisition from the chemiluminescence detector were achieved using Hewlett Packard Chemstation Software (Agilent Technologies). A flow rate of 0.5 mL/min was employed with a run time of 5 min. The column eluate and chemiluminescence reagent were merged at a T-piece in front of a spiral flow cell comprising 0.5 mm i.d. PTFE mounted flush against a photomultiplier tube (THORN-EMI 9924BS, ETP Ltd., Salsbury, Australia) which was operated at 900 V, using a stable power supply (THORN-EMI Model PM28BN) via a voltage divider supply (Thorn EMI Model C611, ETP Ltd.) which monitored the resultant emission. The flow cell and photomultiplier tube were enclosed in a light tight housing. Delivery of the postcolumn reagent was achieved using a Gilson Minipuls 3 peristaltic pump (John Morris, Chatswood, Australia) with PVC pump tubing (1.85 mm i.d., A.I. Scientific, Clontarf, Australia) to propel both reagent streams at a flow rate of 2.5 mL/min. The potassium permanganate and tris (2,2′-bipyridyl) ruthenium (II) streams merged at a T-piece prior to merger with the column eluate.

Extraction Procedures

Four extraction procedures for psilocin and psilocybin were investigated (3,16,23,35). The various extraction methodologies have been summarized in Table 2. In each experiment, 1 g of a common Australian hallucinogenic mushroom, *Psilocybe subaeruginosa*, was used for analysis. The extraction efficiencies for the two compounds were compared using flow injection analysis with potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III) chemiluminescence detection as described in our earlier paper (26).

Sample Preparation for HPLC

Working solutions of psilocin and psilocybin were prepared by appropriate dilution of stock solutions with methanol and the addition of 25 μg/mL of internal standard, 4-hydroxyindole, to each. A series of calibrations for psilocin and psilocybin were prepared over the range of 0.5–50 μg/mL for each analyte. Ground mushroom samples of *Psilocybe subaeruginosa*, *Hypholoma*

TABLE 2—*Various extraction methodologies investigated utilizing potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III) chemiluminescence detection.*

Method	Step 1	Step 2	Step 3	Step 4	Step 5
1: Casale (23)	Grind sample and mix with acetic acid, adjust to pH 4	Stand for 1 h, boil to 70°C, and cool	Collect filtrate, adjust to pH 8, and extract twice with diethyl ether	Dry over sodium sulfate, filter, evaporate	
2: Sarwar and McDonald (35)	Cover sample with acetic acid and grind	Add water and grind into slurry	Centrifuge slurry, collect supernatant, and neutralize sample	Extract with equal amount of chloroform, centrifuge, and collect organic layer	Evaporate
3: Pedersen-Bjergaard et al. (3)	Sonicate sample for 15 min in methanol	Collect supernatant and repeat procedure			
4: Thomson (16)	Grind sample and store in methanol overnight	Filter, wash in methanol	Reduce volume		

aurantiaca, and *Panaeolina foenisecii* were extracted in methanol for 24 h. Each species of mushroom was diluted with methanol 1:1 (v/v) and filtered with a 0.45 μm membrane prior to analysis.

Results and Discussion

Extraction Methods

The chemiluminescence responses from each extract utilizing acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III) are shown in Fig. 1.

Extraction numbers 3 and 4 were the only methods where both psilocin and psilocybin were detected. Similar concentrations of psilocin were observed using methods 1 and 2. Extraction methods 1 and 2 both required the use of aqueous acetic acid as the extraction solvent. This is a suitable extraction solvent as both psilocin and psilocybin are very soluble in dilute acetic acid, and very little other interfering substances are extracted (23). However, the heating of the acid extract in method 1 led to the dephosphorylation of psilocybin to psilocin. This was evident as no chemiluminescence was elicited upon reaction with tris(2,2′-bipyridyl)ruthenium(III), Fig. 1.

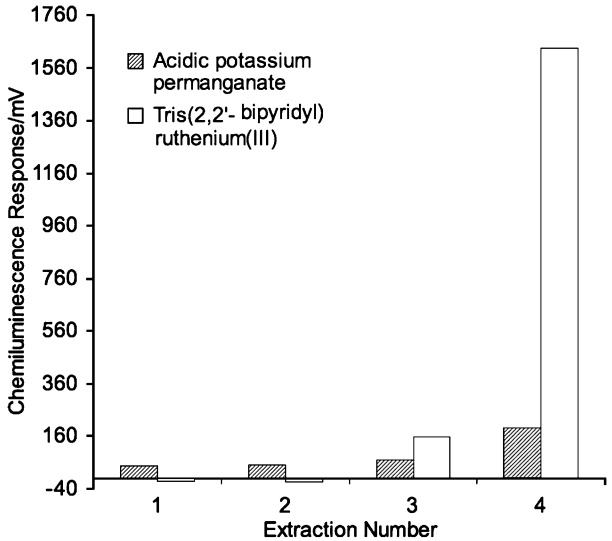

FIG. 1—*Acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III) chemiluminescence responses from the various extraction procedures.*

The neutralization process required in extraction method 2 to keep the pH below pH 8.5, was to avoid base facilitated destruction of psilocybin. It is quite surprising that no chemiluminescence was elicited upon reaction with tris(2,2′-bipyridyl)ruthenium(III), Fig. 1. It has been reported that enzymes of the phosphatase type can be extracted with aqueous acetic acid in contrast to organic solvents (36), thus facilitating the dephosphorylation process.

The utilization of an ultrasonic bath for 15 min in extraction method 3 may not have allowed for complete extraction of the indoles. An extraction procedure with methanol generally requires up to 12 h at room temperature (15) or 1 h at 45°C (11) for complete extraction.

Extraction method 4 resulted in the greatest psilocin and psilocybin concentrations utilizing a simple extraction with methanol from *Psilocybe subaeruginosa*. Based on these findings, all subsequent experiments employed this extraction method.

The amount of time required for maximum indole extraction was also investigated. A methanolic extraction was performed over 72 h using flow injection analysis with chemiluminescence detection. The maximum indole response for both acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(III) chemiluminescence was observed at 48 h. As this response was not significantly greater than that at 24 h, this implied that indole extraction was essentially complete at 24 h.

Separation Conditions

As this method is to be applied to the liquid chromatography-mass spectrometry (LC-MS) determination of psilocin and psi-

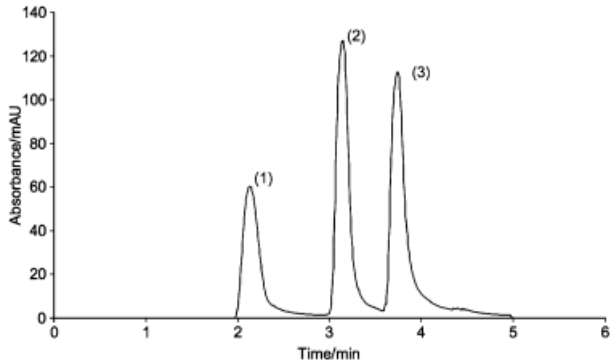

FIG. 2—*A test mixture containing (1) psilocin, (2) psilocybin, and (3) 4-hydroxyindole (internal standard).*

TABLE 3—*Analytical figures of merit using UV detection.*

Analyte	Retention Time (min)	Calibration Function*	Correlation Coefficient (r^2)	Limit of Detection (mol/L)	Limit of Quantitation (mol/L)	Peak Area RSD (%)†
Psilocin	2.2	$y = 13.71x - 1.96$	0.9992	4.9×10^{-7}	1.2×10^{-6}	1.7
Psilocybin	3.1	$y = 23.30 + 5.81$	0.9995	1.8×10^{-7}	4.0×10^{-7}	1.9

*y, peak area (mAu); x, concentration (mol/L).
†RSD calclulated from 10 replicates of a 5×10^{-5} mol/L test mixture.
UV, ultraviolet; RSD, relative standard deviations.

locybin in the future, a sufficiently volatile mobile phase able to vaporize in the LC-MS interface was required. Ammonium formate, a commonly employed mobile phase in LC-MS (37) was used for the HPLC separation of the hallucinogenic indoles.

Psilocin and psilocybin were well resolved employing a methanol:10 mM ammonium formate, pH 3.5 (95:5% v/v) mobile phase and separation on a C_{12} column. Analytes containing both an indole backbone and chemiluminescence properties were tested as candidates as internal standards; among them, 4-hydroxyindole was selected as the internal standard in terms of elution. A mix containing 25 μg mL^{-1} psilocin, psilocybin, and 4-hydroxyindole (IS) is shown in Fig. 2, with a total chromatographic time of less than 5 min. This is quite a rapid method when compared to others as mentioned in Table 1.

Varying the ammonium formate concentration had no significant effect on the separation or absorbance response of both indoles. Decreasing the methanol content resulted in longer analysis times and a decrease in resolution. No analytes were observed within 5 min when a methanol content of 20% or below was employed.

Using the above separation conditions, analytical figures of merit were determined for psilocin and psilocybin, and these have been summarized in Table 3. The limit of detection for each analyte was determined as the lowest concentration giving a response of three times the baseline noise. The limit of quantitation was determined as a response of 10 times the baseline noise.

The concentration of psilocin and psilocybin in mushroom extracts were determined, and chromatograms of the three species of mushrooms *Psilocybe subaeruginosa*, *Hypholoma aurantiaca*, and *Panaeolina foenisecii* are shown in Figs. 3–5, respectively. The *Psilocybe subaeruginosa* extract was the only species of mushroom to contain trace amounts of psilocin at 2.2 min. A peak observed at 2.8 min in the extracts of *Hypholoma aurantiaca* and *Panaeolina foenisecii* has yet to be identified. It has been reported that methanol also coextracts endogenous substances such as baeocystin and norbaeocystin (19,23). Further investigations are required to confirm their identity.

Postcolumn Dual Chemiluminescence Detection

A mobile phase consisting of methanol:10 mM ammonium formate, pH 3.5 (95:5, %, v/v) and the effect of the postcolumn reagent flow rate between 1.0 to 3.0 mL/min was evaluated by replicate injections of a 50 μg/mL psilocin and psilocybin standard. The optimal flow rate using a dual chemiluminescent reagent system was investigated to generate the maximum signal. A previous paper established the optimal flow rate conditions for psilocin and psilocybin using the chemiluminescent reagents potassium permanganate and tris(2,2′-bipyridyl)ruthenium(II) individually (26). The flow rate yielding the most intense emission was observed at 2.5 mL/min. Operating at lower flow rates resulted in broad peaks, while operating at flow rates above 2.5 mL/min did not results in greater peak heights or areas for both indoles. The analytical figures of merit obtained for psilocin and psilocybin under these conditions are detailed in Table 4.

Detection limits of 4.9×10^{-7} and 1.2×10^{-8} mol/L were obtained for psilocin using UV and chemiluminescence detection, respectively. Detection limits of 1.8×10^{-7} and 3.5×10^{-9} mol/L were obtained for psilocybin using UV and chemiluminescence detection respectively. The detection limits attained using chemiluminescence detection were significantly lower than those obtained with UV detection, demonstrating the utility of chemiluminescence detection for application to trace analysis of these hallucinogenic compounds. The detection limits attained here were also lower than those previously reported for other separation techniques discussed in Table 1.

The limits of detection observed using a postcolumn HPLC detection system were inferior to those achieved in our previous paper using flow injection analysis using either of the chemiluminescence reagents autonomously (26). The dual postcolumn

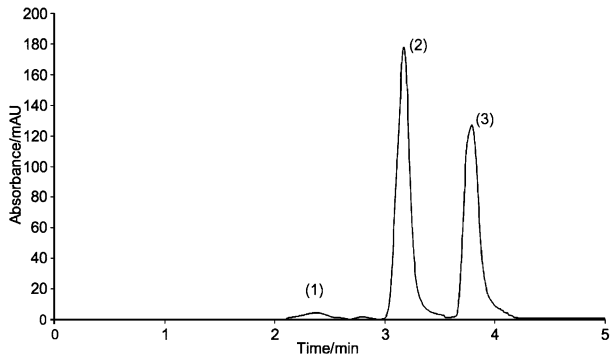

FIG. 3—*Chromatogram of a Psilocybe subaeruginosa extract containing (1) psilocin, (2) psilocybin, and (3) 4-hydroxyindole (internal standard).*

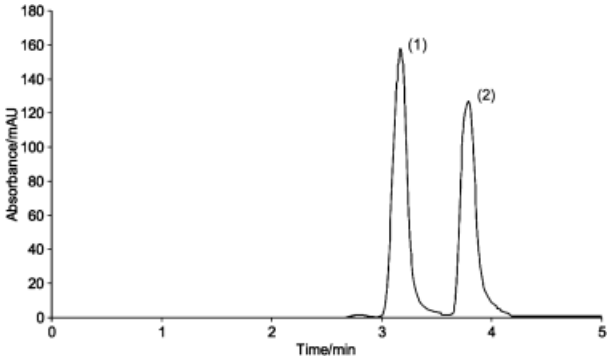

FIG. 4—*Chromatogram of a Hypholoma aurantiaca extract containing (1) psilocybin and (2) 4-hydroxyindole (internal standard).*

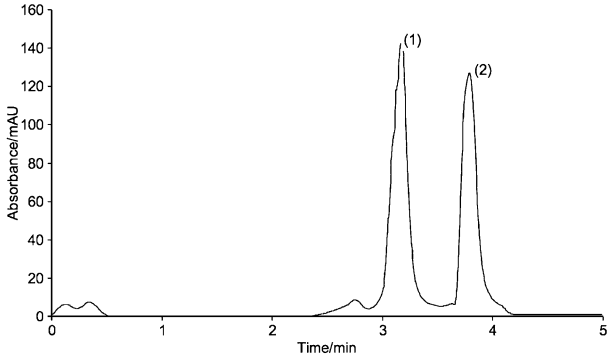

FIG. 5—*Chromatogram of a Panaeolina foenisecii extract containing (1) psilocybin and (2) 4-hydroxyindole (internal standard).*

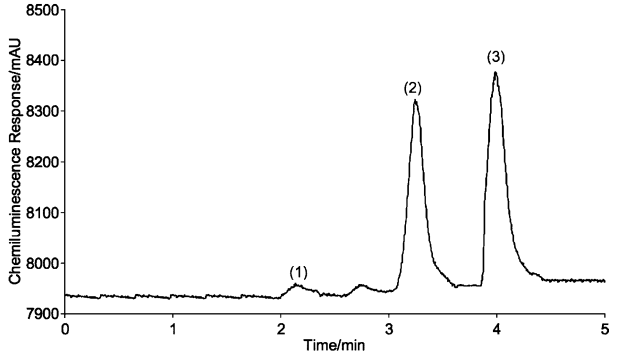

FIG. 6—*Chromatogram of a Psilocybe subacruginosa extract employing a dual postcolumn chemiluminescence detection system containing (1) psilocin, (2) psilocybin, and (3) 4-hydroxyindole (internal standard).*

chemiluminescence detection system delivered a detection limit of 1.2×10^{-8} and 3.5×10^{-9} mol/L for psilocin and psilocybin, respectively. Detection limits of 9.0×10^{-10} and 3.5×10^{-10} mol/L^{-1} for psilocin and psilocybin, respectively, were reported in our previous paper (26). Inferior postcolumn chemiluminescence detection limits for opiates when compared to flow injection analysis results were also observed by Lenehan et al. (34), who postulated that each eluting analyte may be reacting competitively with both chemiluminescence reagents, thus reducing the sensitivity of this system. Additionally, quenching of chemiluminescence is a phenomenon observed with the employment of various organic solvents. Abbott et al. (38) observed that acetonitrile completely quenched postcolumn chemiluminescence from acidic potassium permanganate upon reaction with morphine in body fluids. A low methanol content was found to cause some quenching, thus a content of 95%, as employed in this system, is likely to have caused quenching of the chemiluminescence (38). As reducing the methanol content did have an affect upon resolution, and given that psilocybin was present in mushroom extracts at levels well above the detection limits (see below), the effect of quenching was not considered to be an issue.

Postcolumn dual chemiluminescence chromatograms of the species *Psilocybe subaeruginosa*, *Hypholoma aurantiaca*, and *Panaeolina foenisecii* can be seen in Figs. 6–8, respectively. The chromatograms for all three species of mushrooms indicated the presence of psilocybin at 3.2 min. Psilocin was evident in trace amounts only in the *Psilocybe subaeruginosa*. There are several unidentified compounds that elicit chemiluminescence with the dual reagent. The chromatogram for *Panaeolina foenisecii*, Fig. 8, resulted in the presence of unknown peaks at 0.8 and 2.8 min. This unknown peak at 2.8 min was also evident in the *Psilocybe subacruginosa* extract, Fig. 6. As mentioned previously, methanol extractions also coextract endogenous substances such as baeocystin and norbaeocystin (23) where the structures of these molecule are quite similar to that of psilocybin, and therefore most

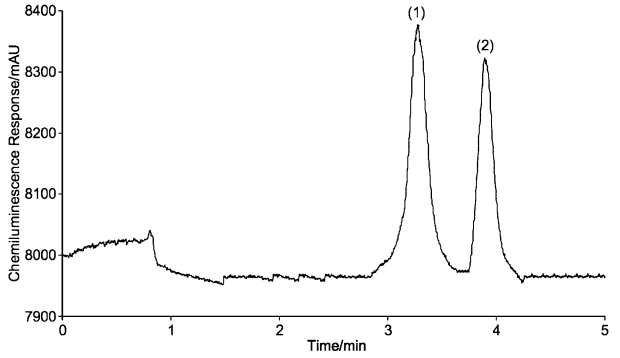

FIG. 7—*Chromatogram of a Hypholoma aurantiaca extract employing a dual postcolumn chemiluminescence detection system containing (1) psilocybin and (2) 4-hydroxyindole (internal standard).*

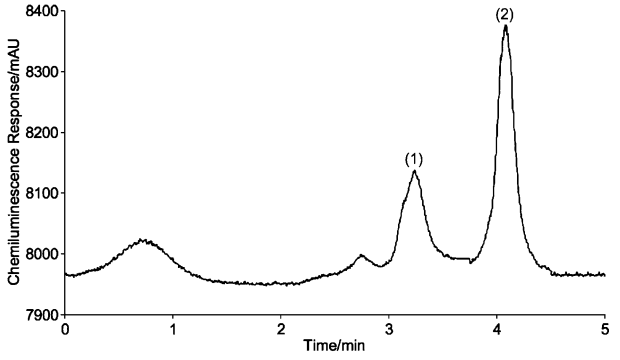

FIG. 8—*Chromatogram of a Panaeolina foenisecii extract employing a dual postcolumn chemiluminescence detection system containing (1) psilocybin and (2) 4-hydroxyindole (internal standard).*

TABLE 4—*Analytical figures of merit employing a dual postcolumn HPLC detection system.*

Analyte	Calibration Function*	Correlation Coefficient (r^2)	Limit of Detection (mol/L)	Limit of Quantitation (mol/L)	Peak Area RSD (%)†
Psilocin	$y = 21.89x - 1.90$	0.9989	1.2×10^{-8}	2.5×10^{-8}	2.3
Psilocybin	$y = 49.70 + 3.34$	0.9918	3.5×10^{-9}	1.0×10^{-8}	2.1

*y, peak area (mAu); x, concentration (mol/L).
†RSD calculated from 10 replicates of a 5×10^{-5} mol/L test mixture.
HPLC, high-performance liquid chromatography; RSD, relative standard deviations.

TABLE 5—*Comparison of results using both UV and a dual chemiluminescence (CL) detection system in extract samples.*

Species of Mushroom	Psilocin (% mg/g)		Psilocybin (% mg/g)	
	UV	CL	UV	CL
Psilocybe subacruginosa	0.011	0.019	1.12	1.07
Hypholoma aurantiaca	N/D	N/D	0.99	0.97
Panaeolina foenisecii	N/D	N/D	0.73	0.68

UV, ultraviolet; N/D, not detected.

likely to elicit chemiluminescence upon reaction with tris(2,2′-bipyridyl)ruthenium(III).

A comparison of the concentration of psilocin and psilocybin in three species of mushrooms, from both sets of results are summarized in Table 5. Good agreement between the two detection systems for psilocin and psilocybin are exhibited where the species were detectable by UV absorption.

Conclusion

A dual chemiluminescence detection system consisting of acidic potassium permanganate and tris(2,2′-bipyridyl)ruthenium(II) for the postcolumn detection of psilocin and psilocybin in extracts of *Psilocybe subaeruginosa*, *Hypholoma aurantiaca*, and *Panaeolina foenisecii* was demonstrated. Chemiluminescence detection gave improved detection limits when compared to UV absorption detection. A comparison of UV absorption and chemiluminescence detection for the determination of psilocin and psilocybin in mushroom extracts were in good agreement where the levels of these compounds were high enough to allow UV absorption detection. Psilocin and psilocybin were isolated from the hallucinogenic mushroom *Psilocybe subaeruginosa* in greatest amounts when methanol was used as the extraction solvent. This proved to be the simplest extraction method of the four investigated and an extraction time of at least 24 h was required for maximum results.

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