This paper reports an investigation into the temporal stability of aqueous solutions of psilocin and psilocybin reference drug standards over a period of fourteen days. This study was performed using high performance liquid chromatography utilising a (95.5% v/v) methanol: 10 mM ammonium formate, pH 3.5 mobile phase and absorption detection at 269 nm. It was found that the exclusion of light significantly prolonged the useful life of standards, with aqueous solutions of both psilocin and psilocybin being stable over a period of seven days.

Este trabajo refiere la investigación sobre la estabilidad temporal de soluciones de patrones de referencia de psilocina y psilocibina durante un período de catorce días. Este estudio se realizó usando cromatografía de líquidos de alta eficacia con fase móvil de metanol (95.5% v/v) y formato amónico 10mM, pH 3.5 y detección a 269 nm. Se encontró que la exclusión de luz prolongaba significativamente la vida útil de los patrones resultando estables las soluciones acuosas de psilocina y psilocibina en un período de siete días.


Cet article présente une investigation de la stabilité temporelle de la psilocine et de la psilocybine standard en solution aqueuse sur une période de quatorze jours. Cette étude a été faite en utilisant la chromatographie liquide à haute performance, en utilisant une solution (95/5% v/v) méthanol : 10mM de formiate d’ammonium, pH 3.5 comme phase mobile et la détection de l’absorption à 269 nm. Les résultats montrent que l’absence de lumière prolonge de manière significative la durée de vie des standards avec des solutions aquéuses aussi bien de psilocine que de psilocybine qui sont stables sur une période de sept jours.

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Introduction
Psilocybin (4-phosphoryloxy-N,N-dimethyltryptamine), and psilocin, its dephosphorylated precursor are found in hallucinogenic mushrooms, Figure 1. These tryptamines belong to the group of indole alkylamine hallucinogens derived from tryptophan [1]. Many psychoactive plants owe their activity to structures containing an indole nucleus [2]. This nucleus is also evident in serotonin (5-hydroxytryptamine) a neurotransmitter in the central nervous system, the synthetic drug LSD, and bufotenine (5-hydroxy-N,N-dimethyltryptamine) which is found in the skin of certain toads [2], Figure 1. Psilocybin is the only known natural indole with a 4-substitution to be isolated from plants and is converted to the less stable psilocin in vivo by alkaline phosphatases [3, 4].

HPLC is widely employed to determine concentrations of psilocin and psilocybin in hallucinogenic mushrooms [5-23]. An important issue with analysis for these compounds by HPLC is the reported lack of stability of standard solutions of psilocin and psilocybin due to oxidation upon exposure to air and light [3, 4, 24, 25]. However these reports provide no significant investigation of standard stability.

Psilocin and psilocybin standards are expensive, and as a result of synthetic challenges [26] are difficult to obtain in sufficient quantities for analytical research. Knowledge of their stability as analytical standards and the ability to extend their useful lifetime, is of importance to the forensic community. In this paper we provide the first report into the temporal stability of psilocin and psilocybin aqueous standard solutions with and without protection from ambient light over a period of 14 days.

Material and Methods

Chemicals and Reagents
Deionised water was from a MilliQ system (Millipore, North Ryde, NSW, Australia). Stock solutions of psilocin and psilocybin were purchased from Alltech (Sydney, Australia). Ammonium formate and methanol were purchased from BDH (Poole, UK). All reagents were used as supplied.

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).

High Performance Liquid Chromatography
Chromatographic analyses 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 was achieved using Hewlett Packard Chemstation Software (Agilent Technologies). All separations were accomplished using a Synergi 4u Max-RP C12 column of dimension 150 mm x 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 employing a flow rate of 0.5 μL min⁻¹. Mobile phases were filtered through a 0.45 μm membrane.

Stability study
The stability of a 10 μg mL⁻¹ aqueous solution of psilocin and psilocybin was investigated over a fourteen day period. Two different conditions at ambient temperature were investigated; (i) protection from light and (ii) no protection from light.

Figure 1  Chemical structures of the indoles (A) psilocin, (B) psilocybin, (C) serotonin, (D) LSD, (E) bufotenine
Protection from light involved taping black felt material so as to completely cover the auto-sampler and injection module. Each analyte was injected individually to view the degradation products. Continual individual injections of a 10 μg mL⁻¹ psilocin and a 10 μg mL⁻¹ psilocybin standard were performed throughout 14 days, to the schedule outlined in Table 1.

**Results and Discussion**

Under the described conditions for the HPLC separation both analytes were fully resolved with a total chromatographic time of less than five minutes. Figure 2 shows a typical chromatogram for a 10 μg mL⁻¹ psilocin and psilocybin mix.

**Stability Studies without protection from light**

Standard solutions of psilocin and psilocybin have been reported to lack stability due to oxidation upon exposure to air and light [3, 4, 24, 25]. This is a significant impediment to successful analytical use. In order to establish the magnitude of the problem, 10 μg mL⁻¹ aqueous solutions of psilocin and psilocybin was subjected to repeated analyses without the benefit of protection from ambient light. The resulting chromatograms reveal a number of overlapping multiple peaks, (Figures 3 and 4). This degradation of the peaks occurred very rapidly, to the point that duplicate chromatograms of un-degraded psilocin and psilocybin were unobtainable.

These observations are similar to those reported for serotonin and 5-hydroxyindoleacetic acid by Jakubovic et al. [27]. Exposure to air and light was found to be detrimental to the stability of these indoles, however the presence of light in the absence of air appeared to have little effect. A minimal loss of response after eight days was observed for these indoles when kept in the dark [27].

Attempts to reduce degradation of the psilocin and psilocybin involved (i) introducing nitrogen into the vial, (ii) placing each standard in ice prior to injection and (iii) refrigeration of the standards until required for analysis. This was unsuccessful in preventing degradation with multiple peaks still being observed after replicate analyses of a sample prepared using the above procedures.

**Table 1**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Injections per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 1</td>
<td>12</td>
</tr>
<tr>
<td>1-2</td>
<td>6</td>
</tr>
<tr>
<td>2-3</td>
<td>4</td>
</tr>
<tr>
<td>3-4</td>
<td>2</td>
</tr>
<tr>
<td>4-72</td>
<td>1</td>
</tr>
<tr>
<td>72-336</td>
<td>1 per day</td>
</tr>
</tbody>
</table>

**Figure 3** Chromatogram of a 10μg mL⁻¹ standard of psilocin with exposure to light

**Figure 4** Chromatogram of a 10μg mL⁻¹ standard of psilocybin with exposure to light
Figure 6  Stability profile of a 10μg mL⁻¹ psilocybin standard over 14 days

Figure 7  Stability profile of a 10μg mL⁻¹ psilocin standard over 14 days
The dephosphorylation of psilocybin to psilocin, on column traces of psilocin were detected in the chromatographic conditions protonation at C3 produces the 3H-indolium cation (Figure 5) as the major species; this has been confirmed in solution spectroscopically. 3H-indolium cations are electrophilic and will react with unprotonated indoles to form acid dimerisation and trimerisation products. It would not be unreasonable to suggest that psilocin and psilocybin undergo similar reactions. Unfortunately the quantities available in the current study were insufficient to allow complete characterization; isolation of larger amounts of degradation products is currently being pursued.

**Stability Studies with protection from light**

In order to determine whether protection from light provided enough stability to allow analytical use of psilocin and psilocybin standards, individual standards were analysed over a period of 14 days with protection from light. This simply required covering the auto-sampler and injection module of the HPLC with black felt and carefully securing the sides with adhesive tape to ensure a light tight environment. As can be seen from the results (Figures 6 and 7), this simple precaution was extremely beneficial with respect to the stability of the indoles.

Psilocybin standards were found to be very stable under these conditions for seven days compared with those without protection from ambient light. A peak at approximately four minutes was observed after seven days of analysis, hence the slight decrease in the psilocybin peak height as shown in Figure 6. By day 14, a peak at eight minutes was observed, and the peak height of psilocybin was now approximately two thirds the initial response. The two peaks observed at four and eight minutes are unknown and further investigations are required to confirm their identity. Only trace amounts of psilocin (at 2.2 minutes) were observed under these conditions from Day 1.

Psilocin was also found to be stable under these conditions for seven days. A peak at approximately four minutes was observed after seven days of analysis, hence the decrease in peak height as observed in Figure 7. This unidentified peak observed at approximately four minutes in Figures 6 and 7 is likely to be of a similar nature and further investigations are required to confirm its identity. Trace amounts of degradation products were observed at 14 days of analysis, with the peak height observed at day 14 now approximately half the peak height initially observed.

These results are in line with earlier studies by Jakubovic et al. [27] for related biogenic indole amines and illustrates that aqueous standard solutions of psilocybin and psilocin are stable for one week of analysis using lightproof conditions.

**Conclusion**

From the data presented it is clear that light is detrimental to the stability of aqueous solutions of psilocin and psilocybin. It is also clear that the onset of this light induced decomposition of the standards is rapid upon exposure. We would therefore propose that when carrying out analysis of these compounds that the solutions should be protected from light at all times, including when awaiting analysis in an auto-sampler. This can be simply achieved as in our procedure here by covering the auto-sampler, alternatively individual vials could be foil wrapped. The breakdown products observed when these indoles are exposed to light are proposed to be dimers or trimers of the indoles psilocin and psilocybin, this last aspect is currently under investigation.

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**References**

Stability of Psilocin and Psilocybin


