# Challenges of Developing an LC-MS Method for the Measurement of Psilocin and Psilocybin in Dog Plasma

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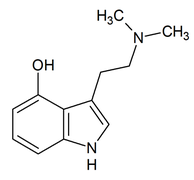
RESOLIAN BIOANALYTICS

**Best Poster Award**

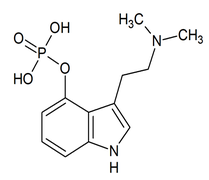
**Introduction -** Psilocybin is a compound found to naturally occur in the mushrooms commonly referred to as magic mushrooms due to their hallucinogenic effects. Psilocybin has recently been dosed together with psychiatric therapy with the aim of treating mental-health disorders such as severe depression and anxiety. Psilocin is the pharmacologically active metabolite of psilocybin

## Background information –

- Requested to develop an LC-MS method for the measurement of psilocybin and psilocin in dog plasma
- Anticipated analytical range 2.5 to 1250 ng/mL with a 20 µL sample volume
- Deuterated internal standards used
- Analytes known to be susceptible to oxidation and sensitive to light
- Chromatographic and stability challenges were encountered



Psilocin



Psilocybin

## Challenge 1- Instability of Psilocin

| Sample | Stabilisers | Stability Condition | %CV | %Accuracy |
|---|---|---|---|---|
| QC High | Ascorbic acid | 23 hours, +4°C and light protected | 2.4 | 35.4 |
| QC High | TCEP | 23 hours, +4°C and light protected | 2.5 | 79.8 |
| QC Med | 0.1 M DTT | 23 hours, +4°C and light protected | 3.6 | 88.8 |
| QC Med | None | 15 hours, +4°C and light protected | 1.7 | 83.1 |
| QC Med | 0.5 M DTT | 15 hours, +4°C and light protected | 0.04 | 99.0 |

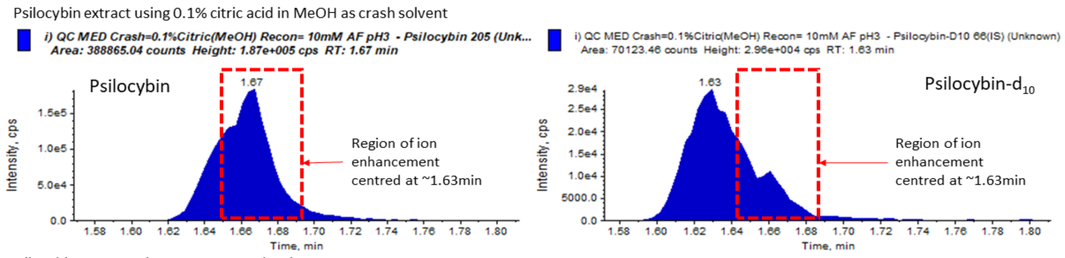
- Psilocybin was found to be stable in dog plasma without a stabiliser
- Psilocin was found to be unstable in dog plasma
- Ascorbic acid stabiliser was tested as an anti-oxidant as analytes are known to be susceptible to oxidation. It resulted in accelerated degradation of psilocin.
- TCEP (tris(2-carboxyethyl)phosphine) and DTT (Dithiothreitol) were tested for their disulphide bonds reduction ability in suspected protein/enzyme interaction with Psilocin. Results showed DTT to be more effective.
- Initially 0.1 M DTT was tested but the final concentration was increased to improve stability further

**Resolution: Dog plasma stabilised with 0.5 M DTT at a ratio of 10:1 (v/v) DTT reduces disulphide bonds.**

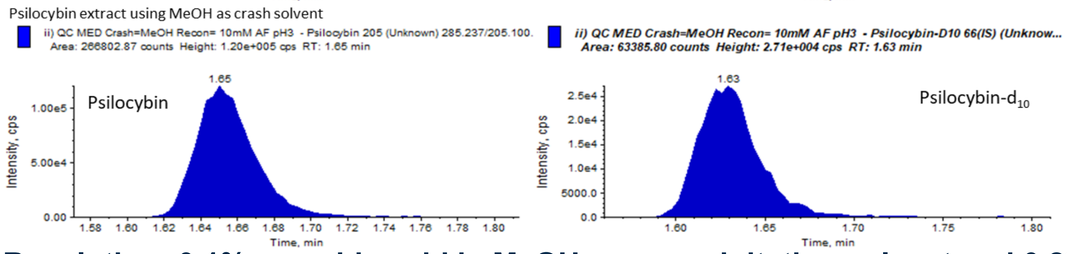
## Challenge 2- Extraction –

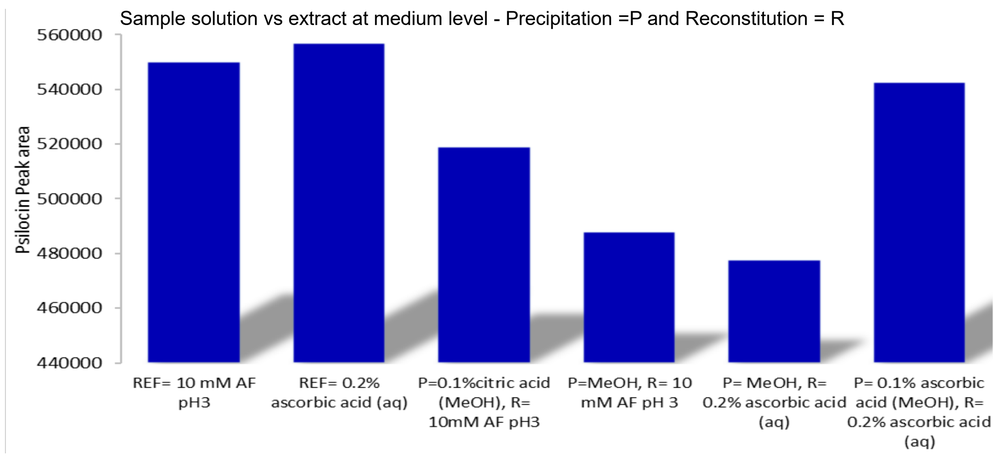
- When 0.1% citric acid in MeOH was used a precipitation solvent, the psilocybin response was ~50% greater in the extracted samples compared to the reference solution
- This suggested an ion enhancement effect from the citric acid, or a matrix component selectively extracted by the citric acid
- Experience from rat methodology has shown citric acid can interfere with poorly retained peaks due to elution in the same chromatographic region

Psilocybin extract using 0.1% citric acid in MeOH as crash solvent




Psilocybin extract using MeOH as crash solvent



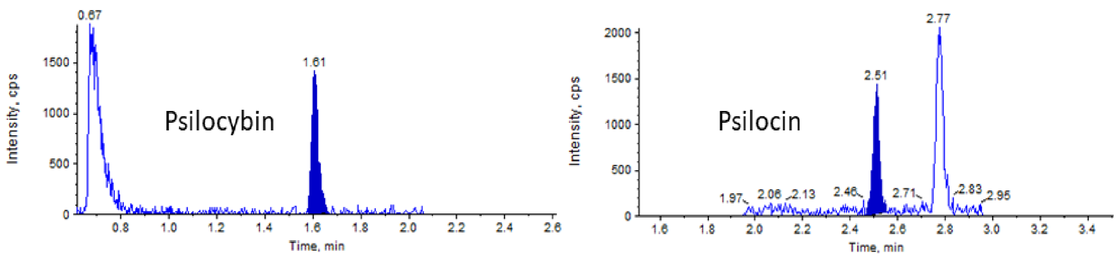

- Low response of psilocin in MeOH precipitation solvent. Either instability, matrix effect or low recovery.
- Extract reinjection after 16 hrs and 5 days storage at +4°C comparable to a reference solution when using 0.2% ascorbic acid (aq) as reconstitution solvent
- Suspected oxidation in 10 mM ammonium formate pH 3 (aq) when exposed to air for extended periods
- Higher concentrations of ascorbic acid during extraction were found to promote the interconversion of psilocybin to psilocin

**Resolution: 0.1% ascorbic acid in MeOH as a precipitation solvent and 0.2% ascorbic acid (aq) as a reconstitution solvent**

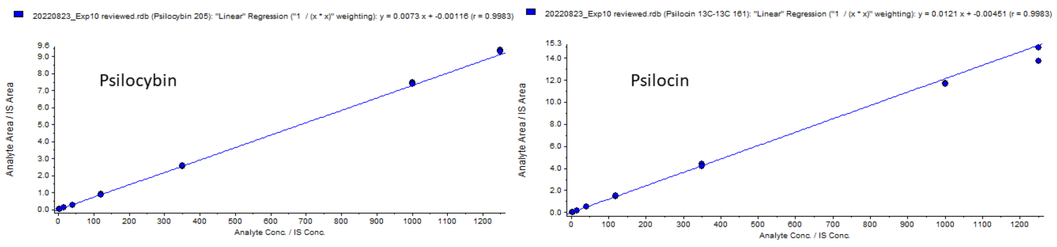
## Challenge 3 – Chromatography

- Due to the polar nature of psilocin and psilocybin HILIC chromatography was initially developed
- Initial HILIC chromatography used a Waters Acquity BEH Amide 1.7 µm 50 x 2.1 mm with mobile phase of 10 mM ammonium formate (aq) pH 3 and ACN:200 mM ammonium formate (aq) pH 3 (95:5, v/v)
- Linearity assessment in solution performed. Psilocin was found to be linear. Psilocybin was found to be non-linear. Insolubility in ACN was suspected

Final chromatography; LLOQ sample




Regression curve with final condition;




- Reverse phase chromatography investigated using a Waters HSS T3 column 1.7 µm 50 x 2.1 mm and ACN as the organic mobile phase
- Post gradient flush step (90%ACN) brought deterioration of psilocybin peak shape in the next injection. This was not the case when removed from gradient, highlighting poor compatibility from psilocybin with high %ACN
- MeOH was used instead for a gradient increase of 4% to 21% over 2.6 min

**Resolution: Reverse phase chromatography with MeOH and 10 mM ammonium formate (aq) pH 3 mobile phases**

**Conclusion –** A robust and selective LC-MS/MS method was developed for the analysis of psilocin and psilocybin in dog plasma after management of three analytical challenges encountered: unexpected instability of psilocin, extraction and chromatographic optimisation.