1.0 Purpose

This document describes the analytical procedure for the determination of Huperzine A, Theacrine, Caffeine & Methylliberine in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of Huperzine A, Theacrine, Caffeine & Methylliberine in raw materials and finished products. This method was validated under protocol PRTCL-22-0064.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical chemists who have verified their ability to execute this procedure to follow this procedure.

3.2 It is the responsibility of QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of QC Laboratory Management and/or Analytical Development Personnel to keep this procedure current with the associated monographs and laboratory practices.

4.0 Definitions

4.1 **QC** – Quality Control

4.2 **AcOH** – Acetic Acid

4.3 **MeOH** – Methanol

4.4 **ACS** – American Chemical Society

4.5 **HPLC** – High Performance Liquid Chromatography

4.6 **UV-Vis** – Ultraviolet-Visible (Detection)

5.0 References

5.1 PRTCL-22-0064, Protocol, Validation of an Analytical Method for the Determination of Huperzine A, Theacrine, Caffeine & Methylliberine by HPLC-UV

5.2 *Development of a Sensitive High Performance Liquid Chromatographic Method with Simple Extraction for Simultaneous Determination of Huperzine A and Huperzine B in the Species Containing Lycopodium Alkaloids.* Zhang et al., Journal of AOAC International Vol. 92, No. 4, 2009, pp. 1060-63.

6.0 Supplies

6.1 Chemicals – All reagents are ACS grade or better

6.1.1 Milli-Q Water

6.1.2 MeOH

6.1.3 AcOH

6.1.4 Huperzine A Reference Standard

6.1.5 Caffeine Reference Standard

6.2 Supplies and Glassware

6.2.1 HPLC vials, 12mm X 32mm with screw cap enclosures w/ septa

6.2.2 Volumetric glassware

6.2.3 Weigh paper

6.2.4 Syringes with 0.45µ nylon filters

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV-Vis detector with a chromatographic data handling system

6.3.2 Analytical Balance

6.3.3 Micro Analytical Balance

6.3.4 Wrist Action Shaker

6.3.5 Sonicator Bath

7.0 Procedure

7.1 Mobile Phase & Diluent Preparation

7.1.1 Mobile Phase

7.1.1.1 Mobile Phase A - Add 2ml AcOH to 1000ml water and mix well.

7.1.1.2 Mobile Phase B – MeOH.

7.1.2 Extraction Solvent / Diluent

7.1.2.1 20% MeOH (20:80 MeOH/H_2O)

7.1.2.2 Use MeOH to extract Huperzine A from raw material and finished product matrices. Prepare the final, working solution such that the composition is 20% MeOH.

7.1.2.3 Use 20% MeOH to extract Theacrine, Caffeine & Methylliberine raw materials. Use MeOH to extract Theacrine, Caffeine & Methylliberine from finished product matrices. Prepare the final, working solution such that the composition is 20% MeOH.

7.2 Standard Prep

7.2.1 Prepare Huperzine A stock at 0.05 mg/ml in MeOH. Sonicate briefly to dissolve, then dilute 1:5 with MeOH. Dilute a further 1:5 with water. (Note: Allow the heat of mixing generated when combining MeOH with water to dissipate and equilibrate to room temperature prior to volumetric glassware QS.)

7.2.2 Apply Relative Response Factors (RRFs) in Section 7.7.1 for quantitation of Theacrine and Methylliberine.

7.2.3 Alternatively, Theacrine and Methylliberine reference standard materials may be used.

7.2.4 Prepare Caffeine working standard at ~ 0.075 mg/ml in 20% MeOH.

7.2.5 Alternative standard preparations are acceptable as long as the preparations are within the linear range of this method.

7.3 Sample Preparation

7.3.1 Specific sample testing details are provided in each products profile. If a specific testing details section is not available, follow preparation procedure as described below, maintaining concentration within the linear range of this method.

7.3.2 The validated linear ranges for the analytical method are 0.47 – 3.15 µg/mL Huperzine A, 44.3 – 103.4 µg/mL Theacrine, 44.2 – 103.2 µg/mL Caffeine and 15.3 – 35.7 µg/mL Methylliberine.

7.3.3 Extract sufficient sample (based on the raw material manufacturer assay value / finished product profile) with Extraction Solvent in order to generate a concentration that is within the validated linear range. Shake mechanically at ~60% volume for 10 minutes. QS to volume and sonicate for 10 minutes. Make any final dilutions, then filter a 5ml aliquot for analysis, discarding the first 3-4ml of filtrate.

7.4 HPLC Parameters

7.4.1 Column: Phenomenex Luna $C_{18}(2)$, 4.6 x 250mm, 100Å, 5µm (Or Equivalent)

7.4.2 Column Temperature: 30°C

7.4.3 Flow rate: 1.0 mL/min

7.4.4 Mobile Phase – Gradient:

Time, min	%A	% B
0	82	18
6	82	18
15	30	70
15.1	82	18
20	82	18

7.4.5 Wavelength:

7.4.5.1 Huperzine A - 310 nm

7.4.5.2 Theacrine, Caffeine & Methylliberine - 287 nm

7.4.6 Injection Volume:

7.4.6.1 Huperzine A - 15 µL

7.4.6.2 Theacrine, Caffeine & Methylliberine - 5 µL

7.4.7 Run Time: 20 minutes

7.4.7.1 If desired, it is permissible to employ a 10 minute isocratic run time at 18% Mobile Phase B for Huperzine A standard injections.

7.4.8 Recommended 3-D Spectral Range (for Identification): 225nm - 350nm

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the Diluent.

7.5.2 Make at least five (5) injections of Working Standard.

7.5.3 Make a single injection of each Sample Preparation.

7.5.4 Make a single injection of the Working Standard after every ten (10) sample injections or at the end of the run.

7.6 System Suitability Requirements

7.6.1 The %RSD of five (5) consecutive standard injections is NMT 2%.

7.6.2 The %RSD of all standard injections is NMT 3%.

7.6.3 If present, any interference in the diluent should be subtracted out of the sample and standard peak areas.

7.7 Example calculations for determining % Assay / LC:

7.7.1 $\% = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times 100$ x (SS/(LA x RRF))

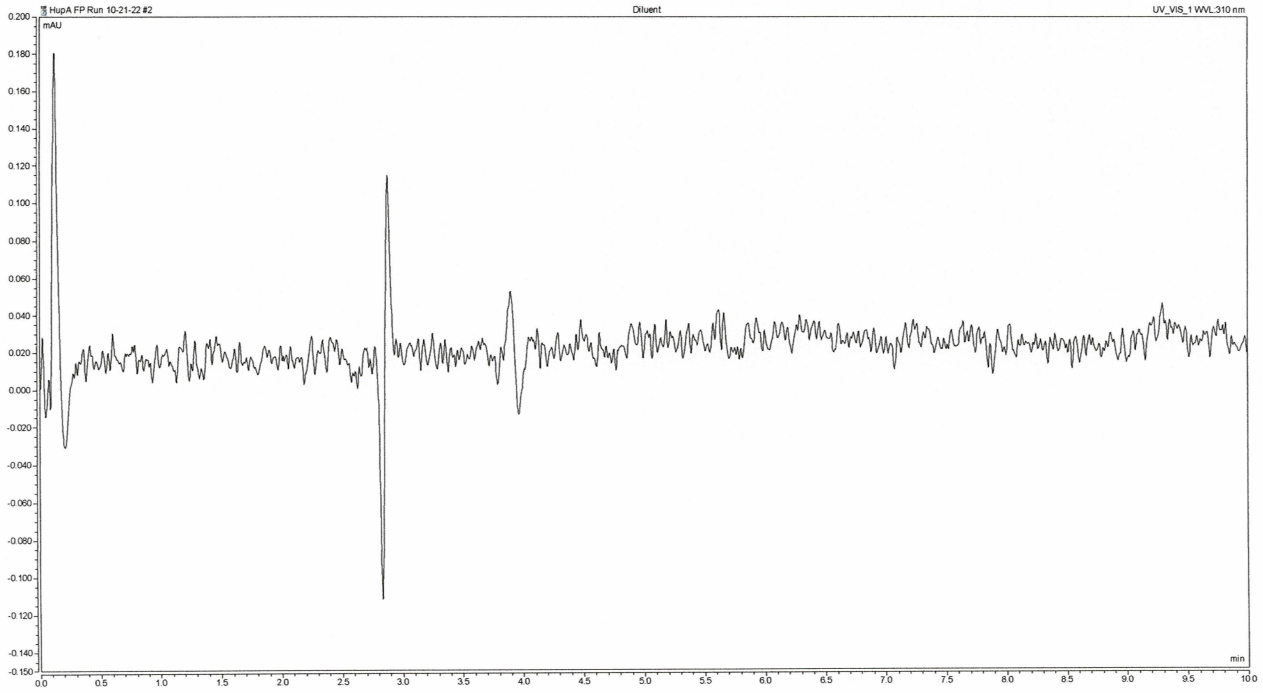
R_u	Sample peak area
R_s	Mean (n=All) standard peak area
Wt_{std}	Weight of the reference standard, mg
V_{std}	Volume of the standard preparation accounting for dilutions, ml
P	Purity of the reference standard in decimal format
SA	Sample amount, mg
V_{spl}	Volume of the sample preparation accounting for dilutions, ml
RRF	Equal to 0.694 for Theacrine when using Caffeine as Reference Standard. Equal to 0.763 for Methylliberine when using Caffeine as Reference Standard.
SS	Use average dosage weight for solids or use serving size specified in Product Profile for powders and liquids. Use "1" for raw materials.
LA	Label Amount specified in Product Profile. Use "1" for raw materials.

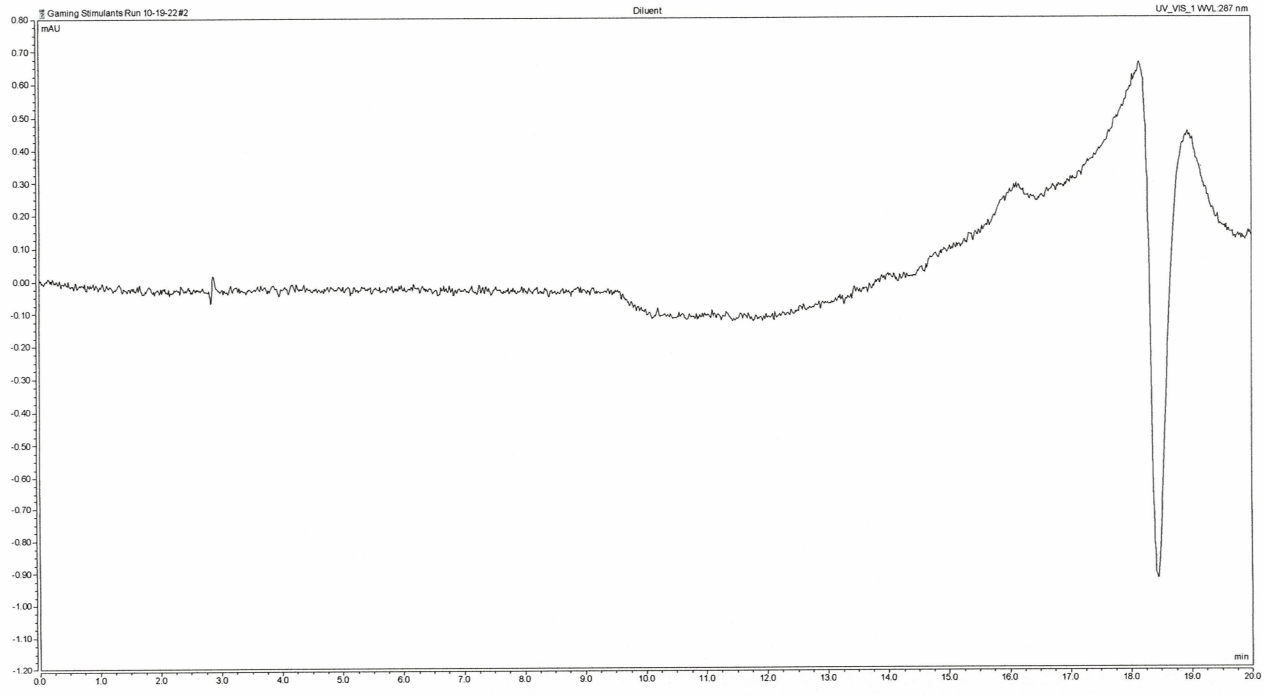
7.8 System Wash, Column Wash and Column Storage

7.8.1 Wash and store the column in 75:25 ACN / Water

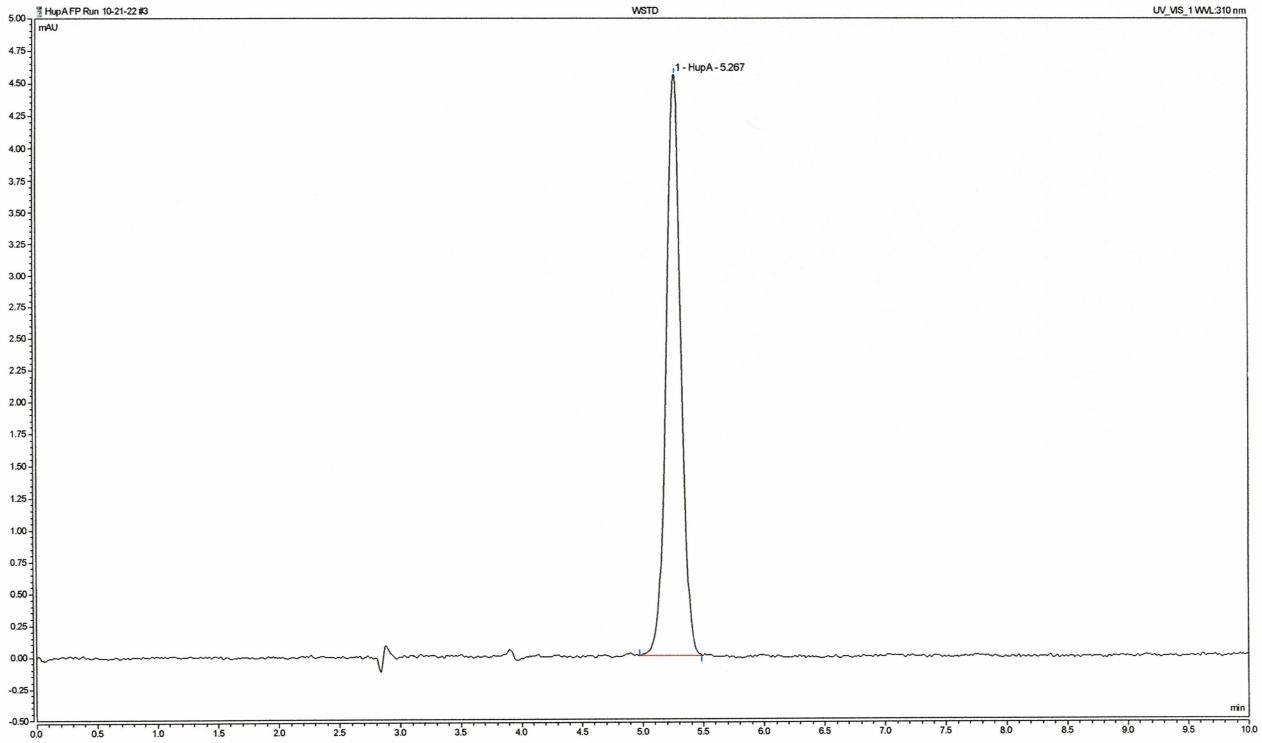
8.0 Chromatograms

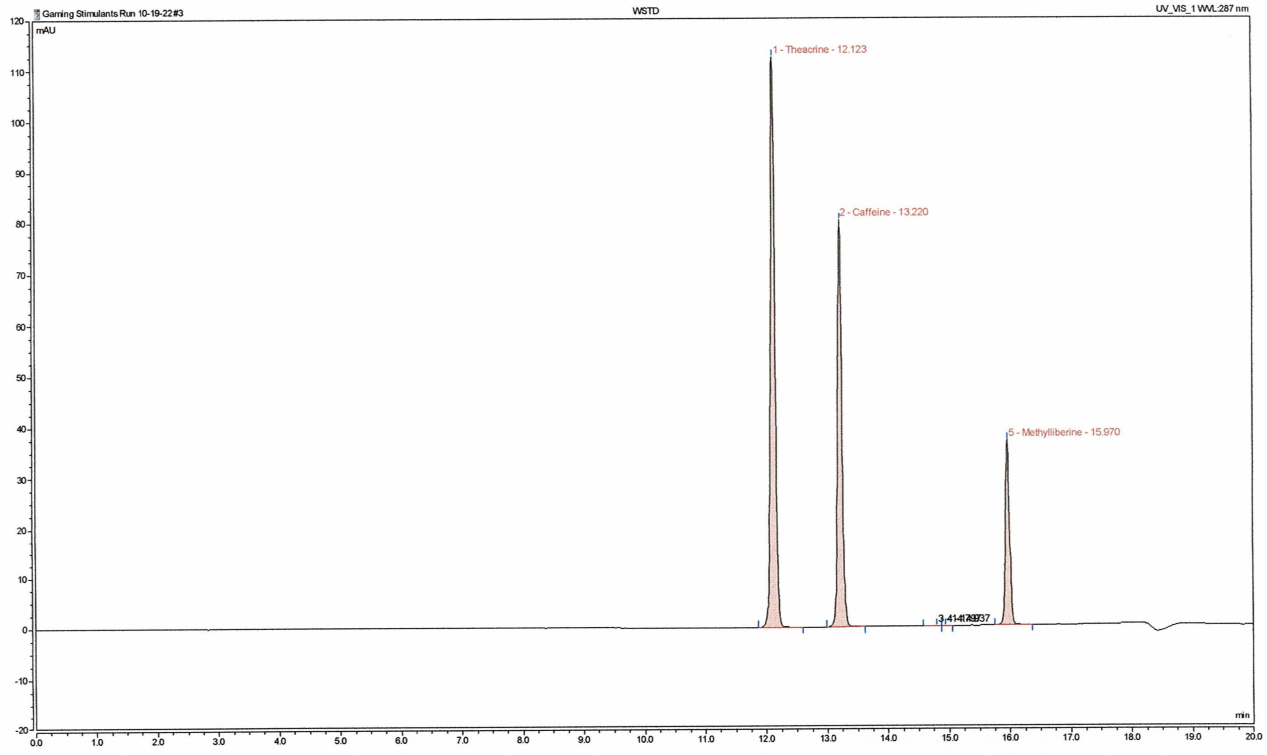
8.1 Typical Diluent Chromatograms



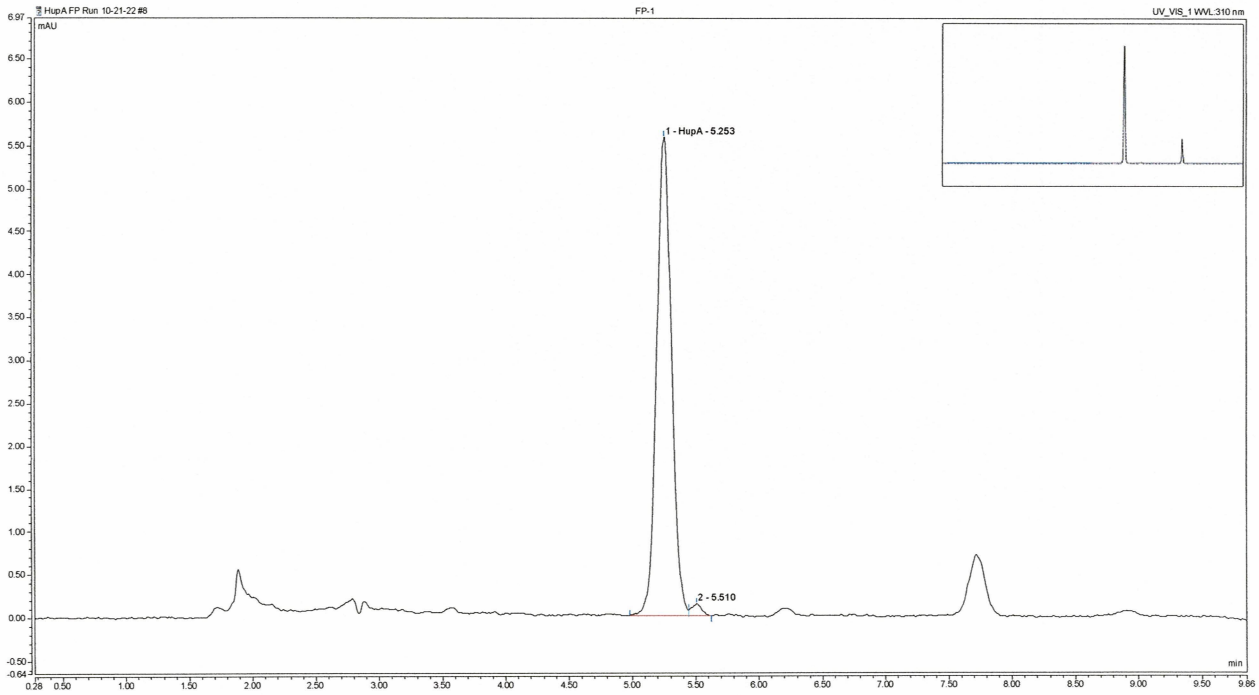


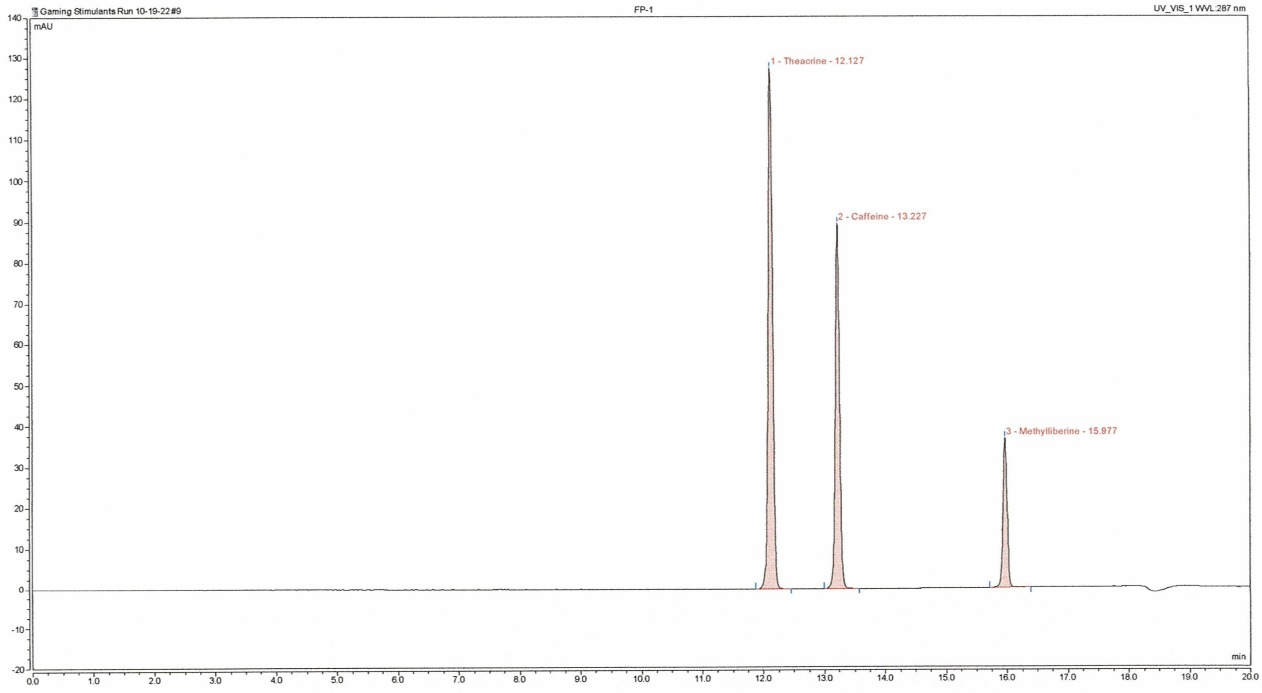
8.2 Typical Standard Chromatograms





8.3 Typical Finished Product Chromatograms





9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	11/10/22	New procedure.	N/A	C. Perry