1.0 Purpose

The purpose of this procedure is to define a process for the quantitative analysis and/or identification of caffeine, theacrine and theobromine in finished products and raw materials using HPLC with UV/VIS detection

2.0 Scope

This procedure applies to the quantification and identification of caffeine, theacrine and theobromine.

3.0 Responsibility

3.1 It is the responsibility of QC and Analytical Chemists to follow this procedure.

3.2 It is the responsibility of Analytical Development and 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 to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **Caffeine** – 1,3,7 Trimethylxanthine

4.2 **Theacrine** – 1,3,7,9 tetramethyluric acid

4.3 **Theobromine** – 3,7-dimethylxanthine

4.4 **ACN** – Acetonitrile

4.5 **KH_2PO_4** – Potassium Phosphate Monobasic

4.6 **H_3PO_4** – Phosphoric Acid

4.7 **STD** – Standard

4.8 **QC** – Quality Control

5.0 References

5.1 MV-LAB-049, Protocol, Method Validation Caffeine (2011)

5.2 MV-LAB-18-065, Protocol, Method Validation Theacrine

5.3 MV-LAB-18-123, Protocol, Method Validation Theobromine

5.4 RPT-21-0025, Report, D-720 Estimation of Uncertainty

6.0 Reagents, Supplies, Glassware and Equipment

6.1 Reagents: all reagents are HPLC grade or better unless otherwise noted.

6.1.1 H2O- Millipore Water ($\geq$ 18 MΩ·cm)

6.1.2 ACN

6.1.3 KH2PO4

6.1.4 H3PO4

6.1.5 Caffeine (traceable reference standard with uncertainty of no more than 0.2%)

6.1.6 Theacrine reference standard

6.1.7 Theobromine reference standard

6.2 Supplies and Glassware

6.2.1 HPLC vials with screw cap enclosures

6.2.2 1L mobile phase container

6.2.3 Volumetric glassware

6.2.4 10mL Plastic luer-lock syringes

6.2.5 0.2µM or 0.45µM 25mm Nylon syringe filters

6.2.6 1.5mL and 2.0mL micro centrifuge tubes

6.2.7 Weigh paper and weigh boats

6.3 Equipment

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

6.3.2 Analytical Balance

6.3.3 Wrist Action Shaker

6.3.4 Vortex

6.3.5 Sonicator Bath

6.4 Mobile Phase Preparation

6.4.1 Buffer (0.1M KH_2PO_4 in H_2O, pH 3.2-3.3)

6.4.1.1 Transfer 13.609g of KH2PO4 to a 1-L bottle.

6.4.1.2 Add 950mL H2O.

6.4.1.3 Adjust the pH to 3.2 - 3.3 using H3PO4.

6.4.1.4 Dilute to volume using H_2O.

6.4.2 Mobile Phase

6.4.2.1 Transfer 250 mL of ACN to a 1-L bottle.

6.4.2.2 Add 750 mL of Buffer.

6.4.2.3 Filter through 0.45 µm membrane.

6.4.3 Diluent

6.4.3.1 Use H_2O as the Diluent.

7.0 Procedure

7.1 Standard Preparation

7.1.1 Use the actual purity from the CofA for caffeine, theacrine, or theobromine standard in the calculations.

7.1.2 All standards are prepared in duplicate (Std A and Std B).

7.1.3 Theacrine and theobromine standard preparations may be scaled down within the working range of the analytical balance.

7.1.4 Transfer 25 mg of reference standard into a 250-mL volumetric flask.

7.1.5 Dissolve in and dilute to volume using diluent. For theobromine, sonicate for 20 minutes after diluting to volume to facilitate dissolution.

7.2 Sample Preparation

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

7.2.2 The sample preparation must be within the linear range of the method:

7.2.2.1 Caffeine: 2 – 200 mcg/mL

7.2.2.2 Theacrine: 10 – 300 mcg/mL

7.2.2.3 Theobromine: 10 – 100 mcg/mL

7.2.3 Theobromine has a limited solubility and should be prepared at a concentration of 0.3mg/mL or less. It should also be sonicated for at least 20 minutes to ensure solubility before further dilutions or analysis.

7.2.4 Some samples, especially those containing extended release or botanically sourced caffeine materials, may require sonication for up to 30 minutes for complete extraction. Alternatively, such samples may be heated at up to 90°C with stirring to extract extended release caffeine.

7.2.5 For raw materials: Accurately weigh and transfer a portion into a suitably sized volumetric flask of no less than 25 mL volume to generate an analyte concentration that is within the validated linearity range. Dilute to volume with Diluent, and sonicate for 10 min.

7.2.6 For solid dose finished products: Combine and homogenize no less than 20 dosage units. Based on the label claim and fill weight (for capsules) or tablet

weight per dose, accurately weigh and transfer a portion of the homogenized sample into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linearity range. Dilute to volume with Diluent, and sonicate for 10 min.

7.2.7 For liquid dose finished products: Use a TC pipet to transfer no less than 2.0 mL of the product into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linearity range. Wipe the outside of the pipet, and rinse the pipet three times with Diluent collecting the rinses in the volumetric flask. Dilute to volume using Diluent.

7.2.8 For chewable gels (gummies): homogenize at least 10 dosage units according to the procedure outlined in D-793 Cryogenic Grinding of Chewable Gels. Quickly weigh a portion of the homogenized sample into a volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linearity range. Dilute to volume with Diluent, and sonicate for 10 minutes.

7.2.9 To manage large volumes the sample can be initially dissolved in a smaller volume that is within the solubility range and a portion further diluted to bring the analyte concentration into the linear range of measurement. Ensure that the stock sample is equilibrated to room temperature prior to performing further dilution.

7.2.10 The final sample must be filtered or centrifuged before analyzing by HPLC.

7.2.10.1 For filtration: filter a portion through a 0.45µm nylon membrane discarding the first 2-3 mL of filtrate before collecting an aliquot for analysis.

7.2.10.2 For centrifugation: centrifuge for about 5 min at about 10,000 rpm.

7.3 HPLC Test Conditions- Isocratic

7.3.1 Time- 7.0 minutes

7.3.2 Flow Rate- 1.0 mL/min

7.3.3 UV detection- 275nm Caffeine and Theobromine; 254nm Theacrine

7.3.4 Injection Volume- 20uL

7.3.5 Column Temperature- 35°C

7.3.6 Column- Acclaim™ 120 C18 5µM 120Å, 4.6mm X 250mm

7.3.7 Recommended 3-D Spectral Range- 200nm to 700nm.

7.4 Approximate Retention Times

7.4.1 Caffeine 3.9 min

7.4.2 Theacrine 3.7 min

7.4.3 Theobromine3.2 min

7.5 Recommended Sequence

7.5.1 Make at least 2 injections of the diluent.

7.5.2 Make five (5) injections of Working Std A.

7.5.3 Make two (2) injections of Working Std B.

7.5.4 Make a single injection of each Sample Preparation.

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

7.6 System Suitability Requirements

7.6.1 The %RSD of the first five (5) standard injections is NMT 3.0%

7.6.2 The % recovery of Working Standard A, using Working Standard B is 97-103%.

7.6.3 The %RSD of all Working Std A injections is NMT 3%.

7.7 Example calculations for determining finished product % label or raw material % purity

7.7.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

R_u Sample peak area

R_s Mean standard peak area

Wt_{std} Weight of reference standard in mg

V_{std} Volume of the standard preparation accounting for dilutions in mL

P Purity of the reference standard in decimal format

SA Sample amount in mg (solids) or mL (liquids)

V_{spl} Volume of the sample preparation accounting for dilutions in mL

SS Serving size: Weight of a single dosage unit in mg for tablets and capsules, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

LA Label amount in mg per dose or 1 for raw materials

7.8 Reporting Results for Caffeine

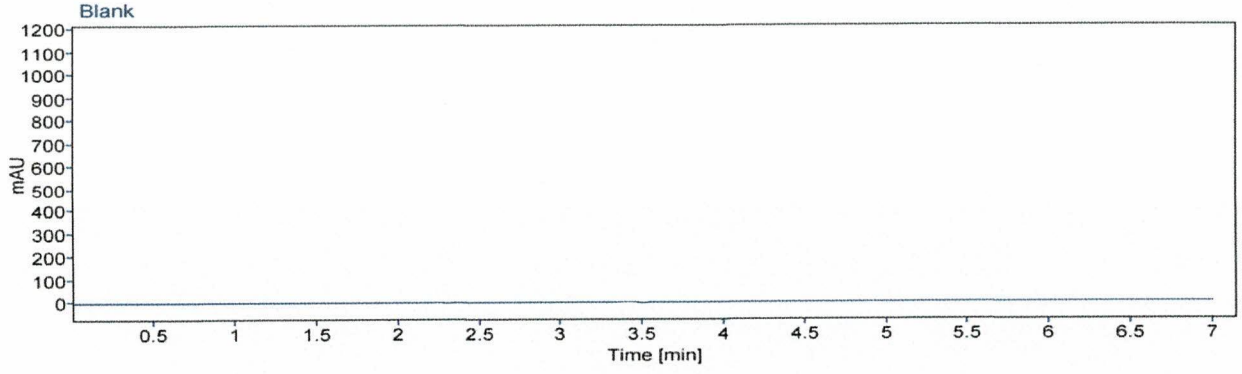
7.8.1 The expanded uncertainty of the method for caffeine is 2.9% with a coverage factor of 2.

7.8.2 Results should include the expanded uncertainty of the method along with the coverage factor in the following format:

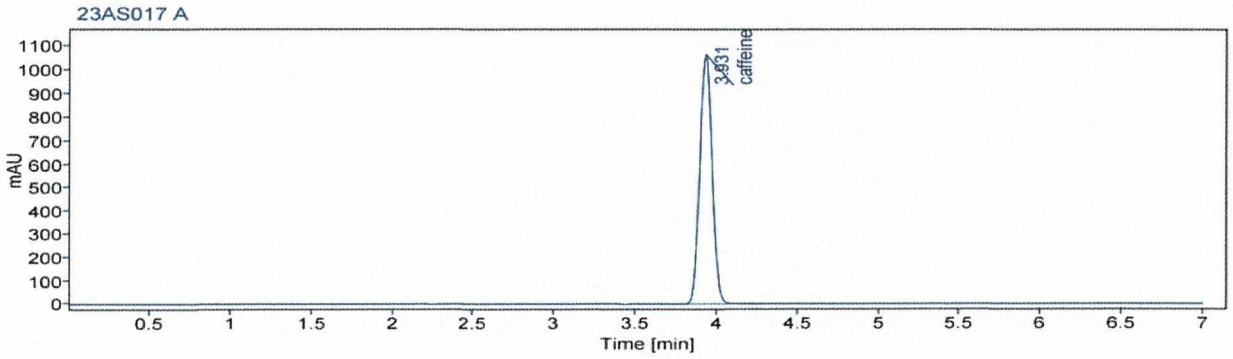
7.8.2.1 105% of label claim, U = ± 2.9% k = 2

8.0 Example Chromatography and Spectra

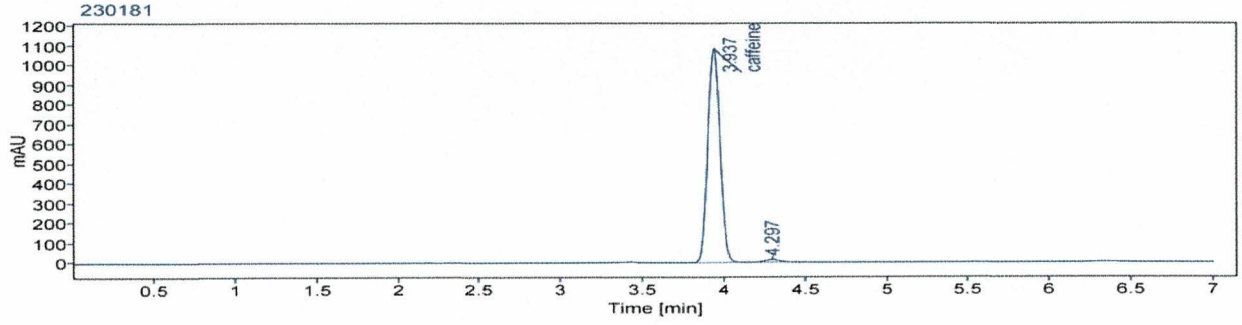
8.1 Blank



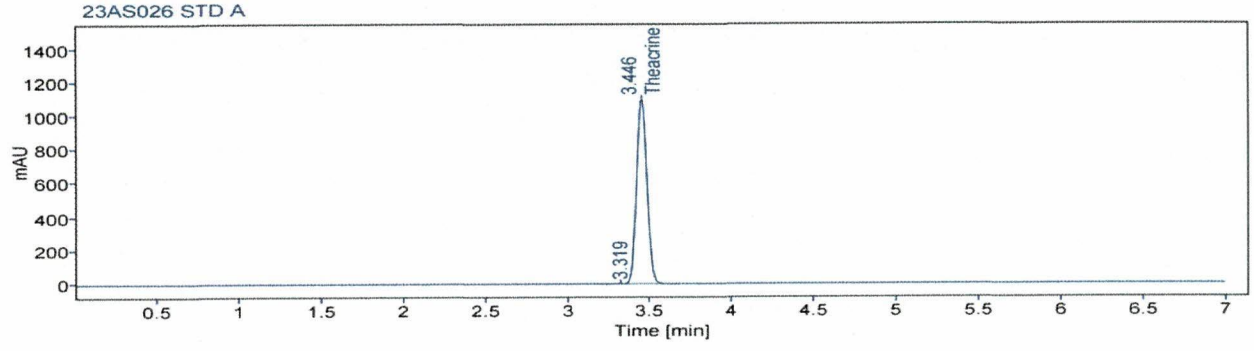
8.2 Caffeine Working Standard



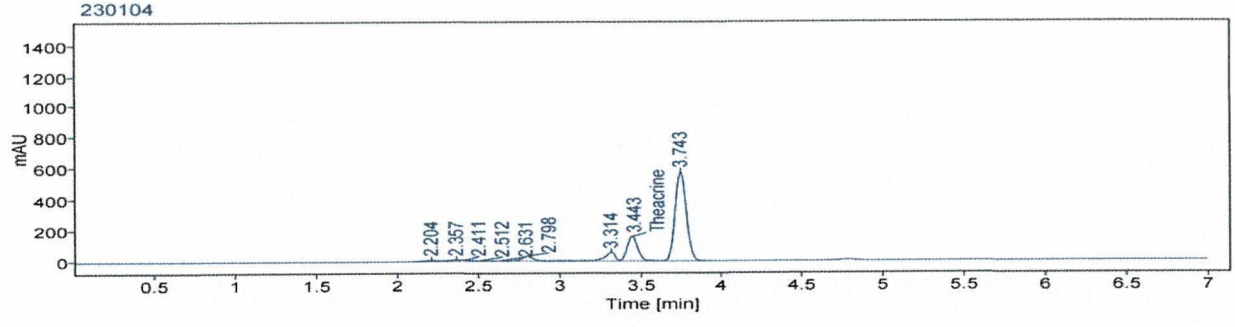
8.3 Caffeine Finished Product Sample



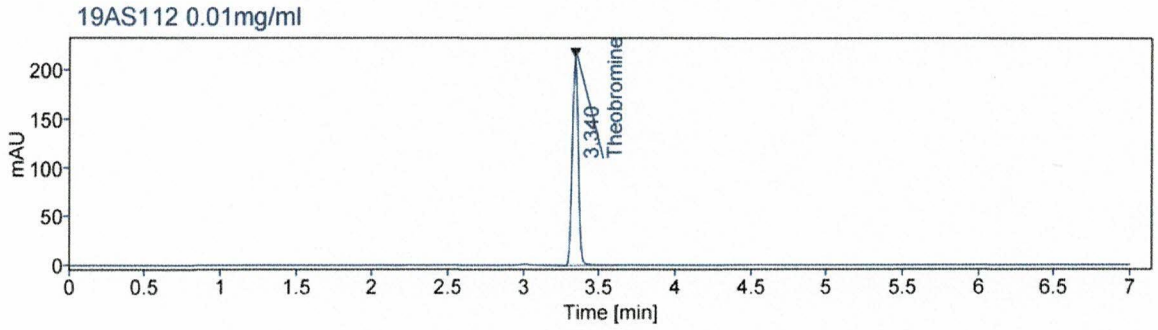
8.4 Theacrine Working Standard



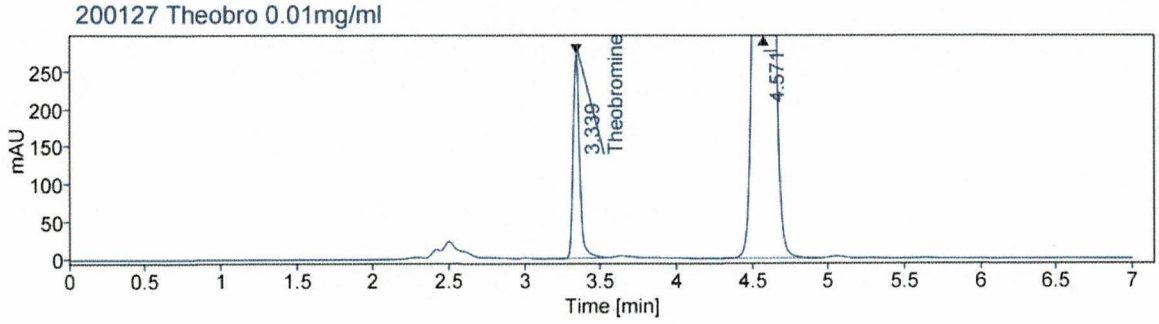
8.5 Theacrine Finished Product Sample



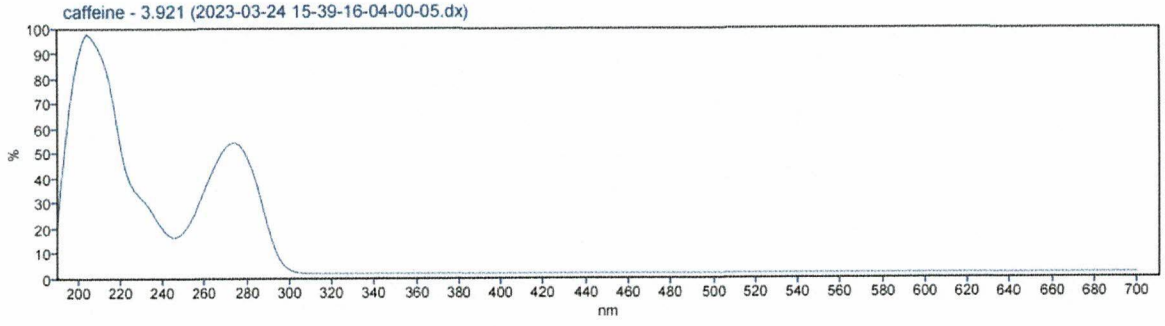
8.6 Theobromine Working Standard



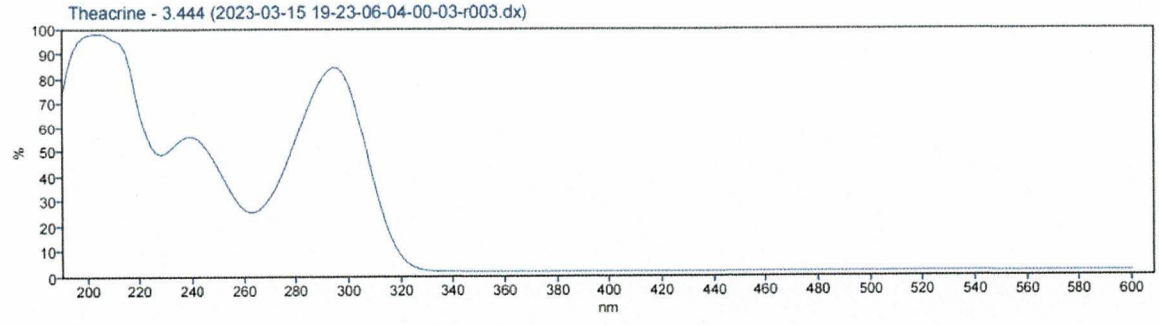
8.7 Theobromine Finished Product Sample



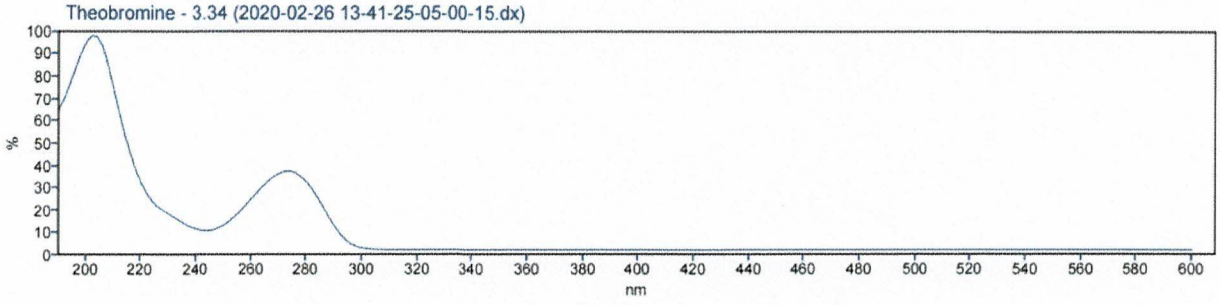
8.8 Caffeine UV Spectrum



8.9 Theacrine UV Spectrum



8.10 Theobromine UV Spectrum



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	01/20/12	New	-	-
1	03/07/13	Added Attachment 1, adjusted flow rate for sensitivity, added linear range of sensitivity for λmax, simplified calculations, separated mobile phase components, added spectral analysis, updated format, changed title.	-	-
2	09/03/13	Added HPLC, added equation, content adjustments, standardized HPLC format.	13-766	B. Johns
3	01/05/16	Updated SOP to reflect current method formatting.	16-0017	N. Zhang
4	07/03/18	Updated SOP and added Theacrine to method.	18-0180	J. Maignan
5	08/10/18	Updated SOP and added Theobromine to method.	19-0004	J. Maignan
6	12/02/19	Updated sample preparation section to include large serving size instructions. Minor clarifications throughout document.	19-0913	J. Sassman
7	02/08/22	Revised to include ISO 17025 requirements	CC-22-0058	S. Sassman
8	04/04/23	Add instruction to follow test details in the product profile for standard and sample preparation, modify sample preparation section to outline specific instructions for different sample types, add example chromatography and spectrum.	CC-23-0179	S. Sassman