1.0 Purpose

The purpose of this procedure is to define a method for the quantitative analysis and/or identification of choline in finished products and raw materials using HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure applies to choline quantification and identification. Some excipients and dietary ingredients used in the finished products can interfere with the analysis of choline. Alternate wavelengths can be used with justification if interferences are present. Many botanical extracts can have significant quantities of natural choline.

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 QC Laboratory Management to implement this procedure and to ensure that the procedure is being followed.

3.3 It is the responsibility of the QC Laboratory Management and Analytical Development to keep this procedure current with latest Ion Labs practices.

4.0 Definitions

4.1 **UV/VIS** – Ultraviolet and Visible Electromagnetic Spectrums

4.2 **ACN** – Acetonitrile

4.3 **Na_2HPO_4** – Sodium Phosphate Dibasic

4.4 **H_3PO_4** – Phosphoric Acid

4.5 **H_2O** – Water

4.6 **QC** – Quality Control

5.0 References

5.1 MV-LAB-13-053, Protocol, L-Choline Determination and Identification by HPLC

5.2 D-793, SOP, Cryogenic Grinding of Chewable Gels

6.0 Reagents, Supplies, Glassware and Equipment

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

6.1.1 H_2O

6.1.2 ACN

6.1.3 H_3PO_4

6.1.4 Choline, Choline Bitartrate, or Choline Chloride traceable standard

6.1.5 Na_2HPO_4

6.2 Supplies and Glassware

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

6.2.2 Mobile phase containers

6.2.3 Volumetric glass ware as required by standard and sample preparations

6.2.4 Pipette tips for adjustable pipettes

6.2.5 Plastic luer-lock syringes and 0.45 µm syringe filters

6.2.6 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

6.3.1 Analytical Balance

6.3.2 Stir Plate

6.3.3 Wrist Action Shaker

6.3.4 Vortex

6.3.5 Sonicator Bath

6.3.6 Adjustable Pipettes

7.0 Preparation of Mobile Phase, Diluent, Samples and Standards

7.1 Mobile Phase A - 0.048M Na_2HPO_4 in H_2O

7.1.1 Transfer 8.54 g $Na_2HPO_4 \cdot 2H_2O$ (or 6.81 g of anhydrous Na_2HPO_4) to a 1000-mL bottle.

7.1.2 Add about 950 mL H_2O.

7.1.3 Adjust to pH 2.5 with H_3PO_4.

7.1.4 Transfer to a 1000-mL volumetric flask, and dilute to volume with H_2O.

7.2 Mobile Phase B – 100% ACN

7.3 Diluent – Mobile Phase A

7.4 Standard Preparation

7.4.1 The linear range of the method is 0.01 mg/mL – 1.0 mg/mL. The Standard and Sample Preparations must be within this range.

7.4.2 All standards are prepared by weighing no less than the minimum weight of the analytical balance then bring up to two thirds their final volumes in an appropriate volumetric flask using Diluent. Mix on a wrist action shaker for 20 minutes then inspect to ensure complete dissolution. Sonication for 10 minutes can also be used to facilitate dissolution. Once the standard is fully dissolved, bring standard to final volume using Diluent.

7.4.3 To manage large volumes, the standard can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. **Equilibrate**

the solution to room temperature prior to performing further dilution. Dilutions can be made using volumetric glassware and/or adjustable pipettes. Final dilutions can be prepared in HPLC vials

7.4.4 Working standard concentrations will approximate the concentration expected to be found in the product being tested based on the sample dilution and calculated from the label.

7.5 Sample Preparation

7.5.1 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

7.5.2 For raw materials: weigh no less than 20 mg 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.

7.5.3 For solid and liquid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (capsules), serving size (powders) or tablet weight per dose, weigh no less than 50 mg of the pooled dosages into a suitably sized volumetric flask of no less than 25 mL to generate an analyte concentration that is within the validated linear range. Dilute to volume with Diluent, and sonicate for 10 min.

7.5.4 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 pooled and homogenized dosages into a beaker. Use several small portions of Diluent to completely transfer the sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Dilute to volume, and sonicate for 10 min.

7.5.5 To manage large volumes, the standard can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. **Equilibrate**

the solution to room temperature prior to performing further dilution. Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials

7.5.6 If particulates remain in the final sample preparation, a portion may be centrifuged at 10,000 rpm for 5 min prior to HPLC analysis. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 3 – 4 mL.

8.0 Chromatographic Conditions

8.1 Gradient-Multistep

8.1.1

Time	%A	%B
0.00	98	2
6.00	98	2
6.1	20	80
8.1	20	80
14.00	98	2

8.1.2 Column- Luna C18(2), 5µm, 100 Å, 4.6mm x 250mm or equivalent

8.1.3 Flow Rate- 1.0mL/min

8.1.4 UV detection- 194nm

8.1.5 Injection volume- 20uL

8.1.6 Column Temperature- 30°C

8.1.7 Recommended 3-D Spectral Range- 190nm to 250nm

8.1.8 Retention Time

8.1.8.1 Choline ≈ 2.8 min

8.2 Recommended Sequence

8.2.1 Make at least 2 injections of the diluent.

8.2.2 Make five (5) injections of Standard Solution.

8.2.3 Make a single injection of each Sample Preparation.

8.2.4 Make a single injection of the Standard Solution after every six (6) samples and at the end of the run.

8.3 System Suitability

8.3.1 The %RSD of five (5) injections of Working Standard is NMT 5.0%.

8.3.2 The %RSD of all injections of Working Standard is NMT 5%.

8.4 Column Wash and Storage

8.4.1 Wash the column with H_2O/ACN (90/10) at 1mL/min for at least 15 min.

8.4.2 Wash the column with H_2O/ACN (50/50) at 1 mL/min for at least 15 min.

8.4.3 Store the column with H_2O/ACN (50/50).

9.0 Example calculations

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

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)

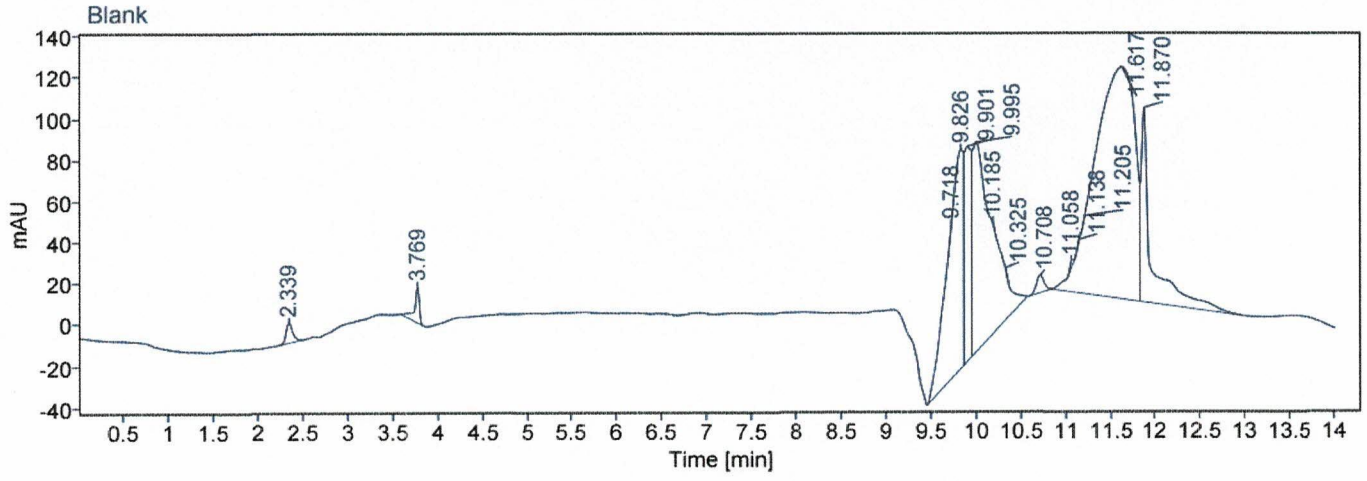
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

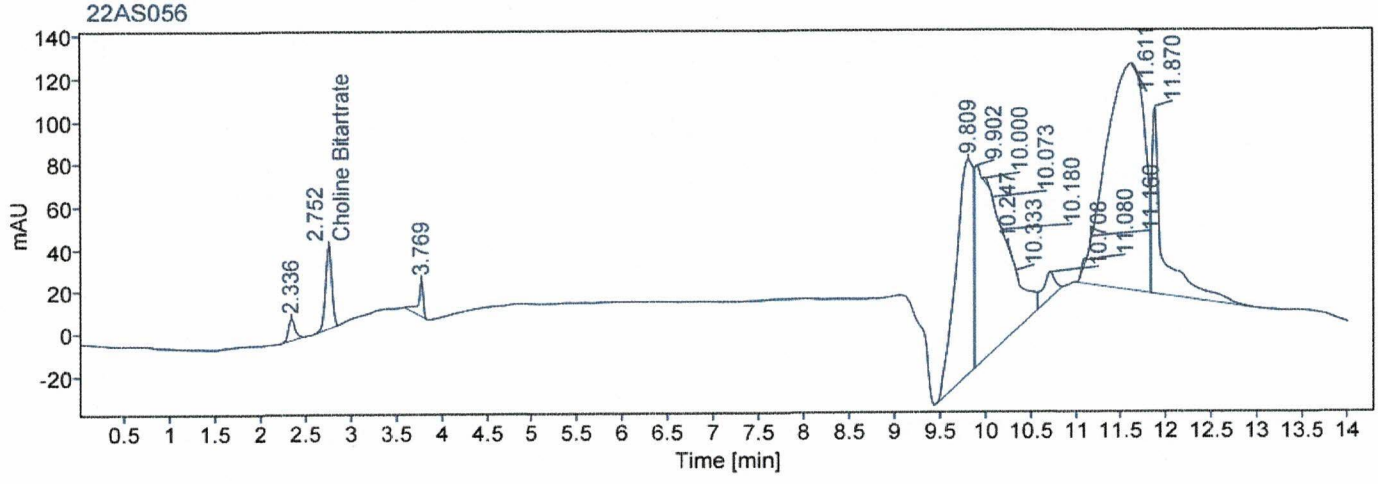
LA Label amount in mg per dose or 1 for raw materials

10.0 Example Chromatography and UV Spectrum

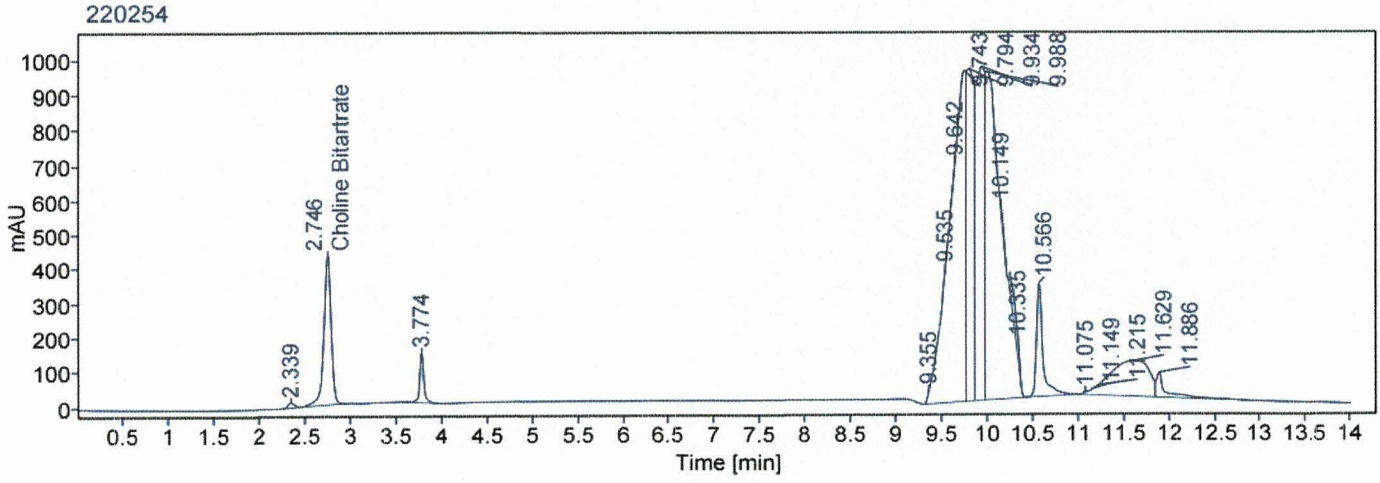
10.1 Blank



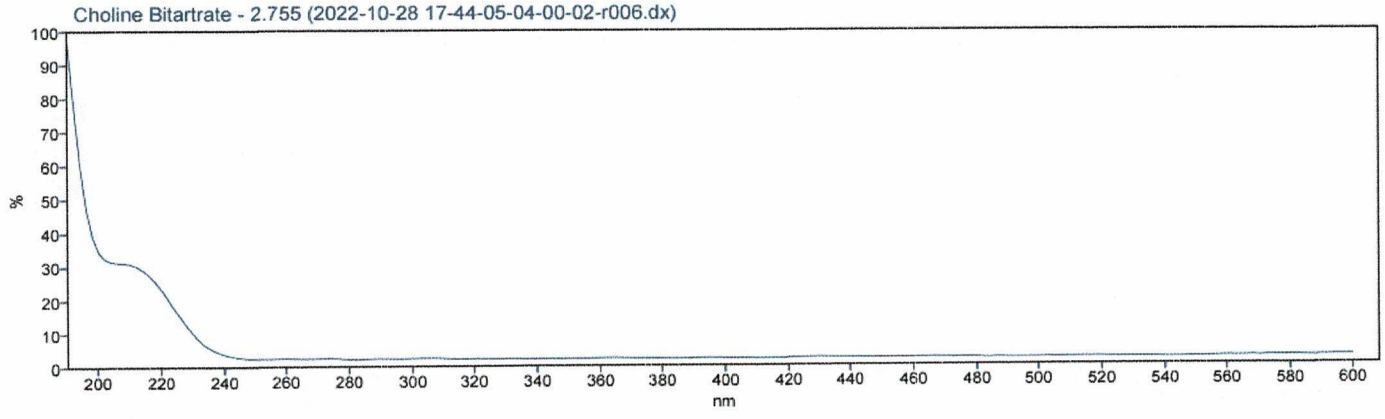
10.2 Standard



10.3 Finished Product Sample



10.4 UV Spectrum



11.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	08/19/13	New	13-705	B. Johns
2	01/05/16	Biennial review: updated SOP Format. Updated content to conform to present method SOP structure.	16-0025	N. Zhang
3	02/04/19	Scheduled review: updated responsibilities and corrected typos.	19-0113	J. Maignan
4	04/11/22	Update to reflect current practices and for clarity. Simplify mobile phase prep. Add retention time. Add system suitability requirements including bracketing standard. Narrow the range for spectral match. Add column wash and storage.	CC-22-0172	S. Sassman
5	05/17/23	Removed unnecessary information and align with current SOP format, add instruction to follow test details containing product specific sample preparation. Added specific sample prep instructions for different dosage forms. Added example chromatography and spectrum. Changed logo.	CC-23-0225	S. Sassman