1.0 Purpose

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

2.0 Scope

This procedure applies to the quantification and identification of Arginine and Methionine. Some excipients and dietary ingredients used in the finished products can interfere with the analysis of Arginine and Methionine. Other wavelengths can be used to measure area if interferences are present.

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 QC Laboratory Management/Analytical Development to keep the procedure current with Ion Labs practices.

4.0 Definitions

4.1 **ACN** – Acetonitrile

4.2 **HCl** – Hydrochloride

4.3 **H_2O** – Deionized water (≥ 18.2 MΩ·cm)

4.4 **QC** – Quality Control

4.5 **CofA** – Certificate of Analysis

5.0 References

5.1 MV-LAB-14-012, Protocol, Open validation protocol for amino acids and amines.

5.2 MV-LAB-13-040, Protocol, L-Arginine Determination by HPLC

5.3 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.

6.1.1 H_2O

6.1.2 ACN

6.1.3 Ammonium Acetate

6.1.4 Arginine reference standard (HCL or α-ketoglutarate)

6.1.5 Methionine reference standard

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 glassware as required by standard and sample preparations

6.2.4 50 mL and 100 mL beakers

6.2.5 Tips for adjustable pipettes

6.2.6 Micro centrifuge tubes

6.2.7 Plastic luer lock syringe

6.2.8 0.2 or 0.45µm nylon syringe filters

6.2.9 Screw cap vials (scintillation)

6.2.10 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 Vortex

6.3.4 Stir Plate

6.3.5 Wrist action shaker

6.3.6 Micro-centrifuge

6.3.7 Adjustable pipettes

7.0 Procedure

7.1 Mobile Phase and Buffer Preparation

7.1.1 Mobile Phase A – 20 mM Ammonium acetate in H_2O

7.1.1.1 Transfer 1.54 g of ammonium acetate to a 1-L bottle.

7.1.1.2 Add 1000 mL H_2O, and mix well.

7.1.2 Mobile Phase B - ACN

7.1.3 Diluent - Mobile Phase A

7.2 Standard Preparation

7.2.1 All Standards are prepared by weighing no less than the minimum weight of the analytical balance. Dissolve standard in two-thirds its final volume in an appropriately sized volumetric flask using Diluent. Mix on the wrist action shaker for 30 minutes then inspect to ensure complete dissolution. Once standard is fully dissolved, bring standard to final volume before using.

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

can be made using volumetric glassware and/or adjustable pipettes. Final dilutions can be prepared in HPLC vials.

7.2.3 Specific 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.

7.3 Sample Preparation

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

7.3.2 The linear range of the method for arginine is 0.1 mg/mL – 1.6 mg/mL. HCl or ketoglutarate salt.

7.3.3 The linear range of the method for methionine is 0.005 mg/mL – 0.8 mg/mL.

7.3.4 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. Add Diluent to 2/3 of the flask volume, shake mechanically for 30 min, and dilute to volume using Diluent.

7.3.5 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. Add Diluent to 2/3 of the flask volume, shake mechanically for 30 min, and dilute to volume using Diluent.

7.3.6 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. Add Diluent to 2/3 of the flask volume, shake mechanically for 30 min, and dilute to volume using Diluent.

7.3.7 To manage large volumes, the sample can be initially prepared at a higher concentration and further diluted into the linear range using Diluent. Dilutions can be made using volumetric glassware and/or adjustable pipettes. Dilutions can be prepared in HPLC vials

7.3.8 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.

7.4 Test Conditions

7.4.1 Gradient-Isocratic

7.4.1.1

Time	%A	%B
0.00	98	2
8.00	98	2

7.4.2 Column- Acclaim 120 C18, 5 µm, 4.6 X 250 mm or equivalent

7.4.3 Flow Rate- 1.0 mL/min

7.4.4 UV detection- Arginine: 206 nm, Methionine: 210 nm

7.4.5 Injection volume- 20 uL

7.4.6 Column Temperature- 40 °C

7.4.7 Recommended 3-D Spectral Range- 200 nm to 300 nm

7.5 Retention Times

7.5.1 Arginine: 2.7 min

7.5.2 Methionine: 4.1 min

7.6 Recommended Sequence

7.6.1 Make at least 2 injections of a Blank (Diluent).

7.6.2 Make five injections of the Working Standard.

7.6.3 Make a single injection of each Sample Preparation.

7.6.4 Make a single injection of the Working Standard after every six samples and at the end of the run.

7.7 System Suitability

7.7.1 %RSD of five consecutive injections of the Working Standard is NMT 5.0%.

7.7.2 %RSD of all injections of the Working Standard is NMT 5%.

7.8 Column Rinse and Storage

7.8.1 Rinse the column with at least 15 mL of H_2O/ACN (90/10).

7.8.2 Rinse the column with at least 15 mL of H_2O/ACN (10/90).

7.8.3 Store the column in H_2O/ACN (10/90).

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

7.9.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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

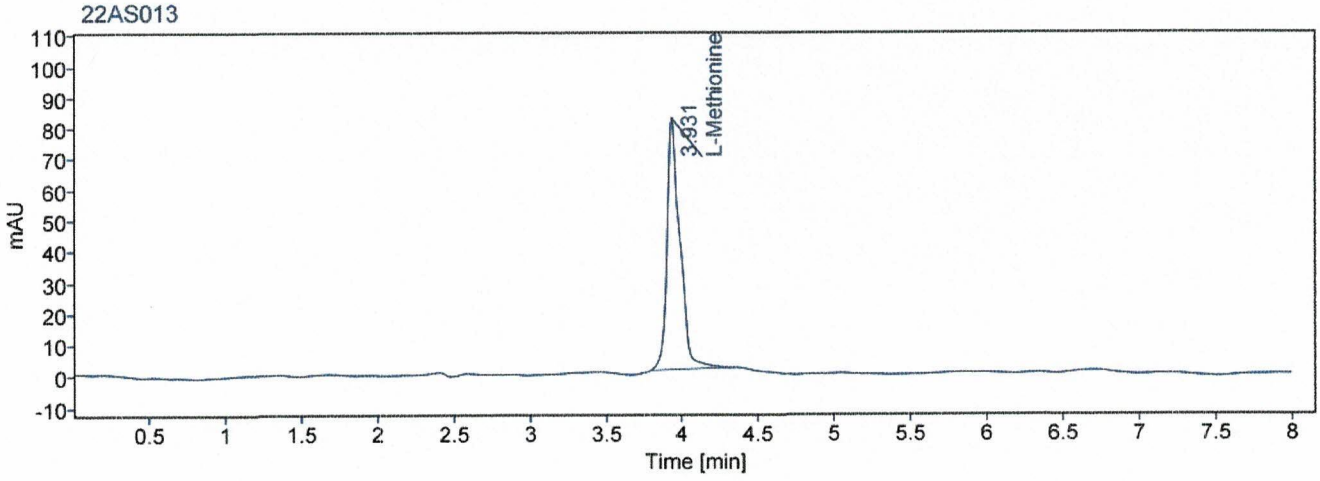
V_{spl} Volume of the sample preparation accounting for dilutions in mL

SS Serving size: Weight of a single dosage unit in mg, or 1 for raw materials.

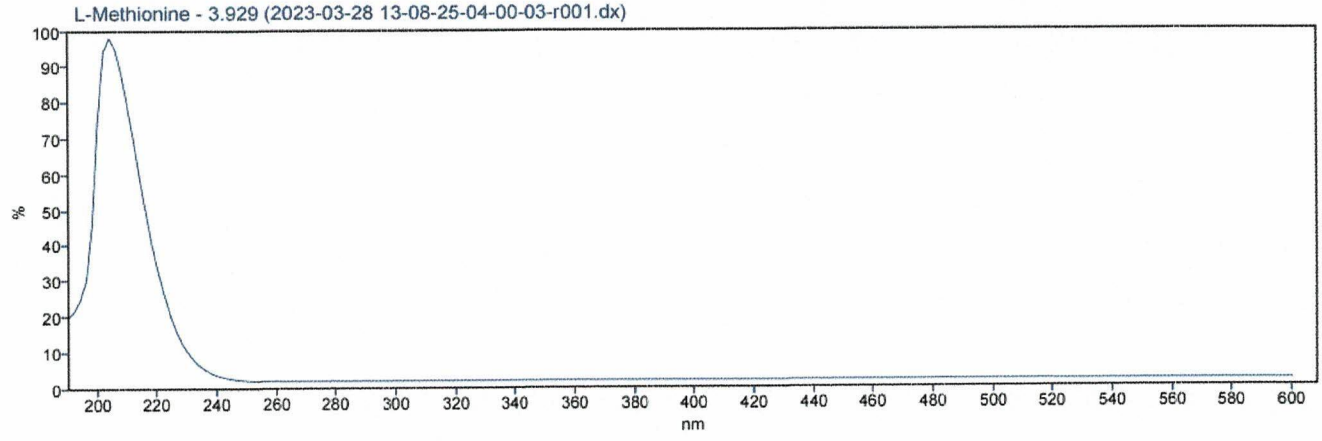
LA Label amount in mg per dose or 1 for raw materials

8.0 Example Chromatography and UV Spectra

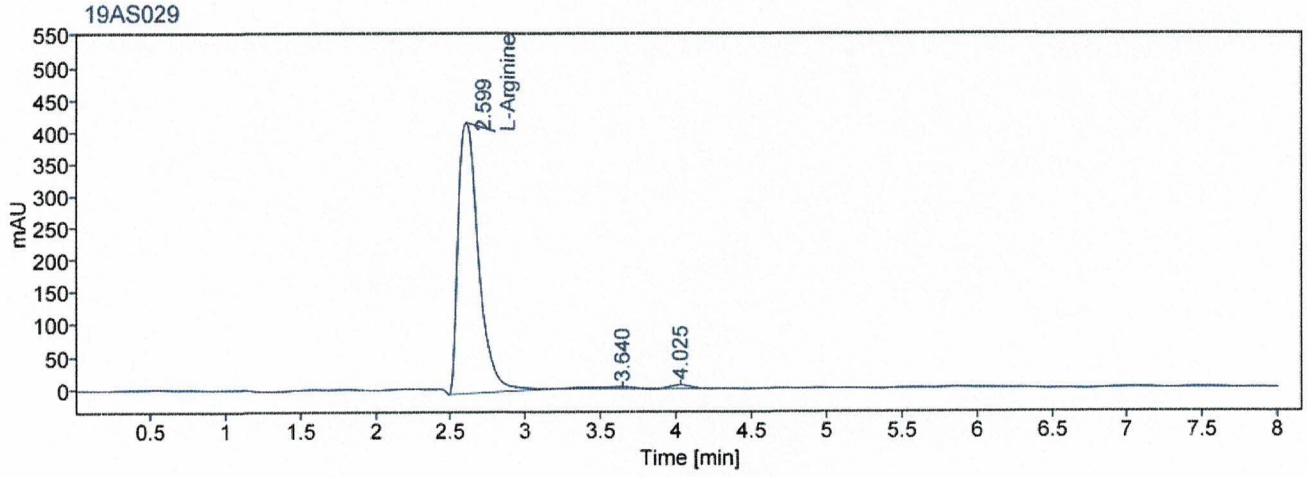
8.1 Methionine Chromatogram



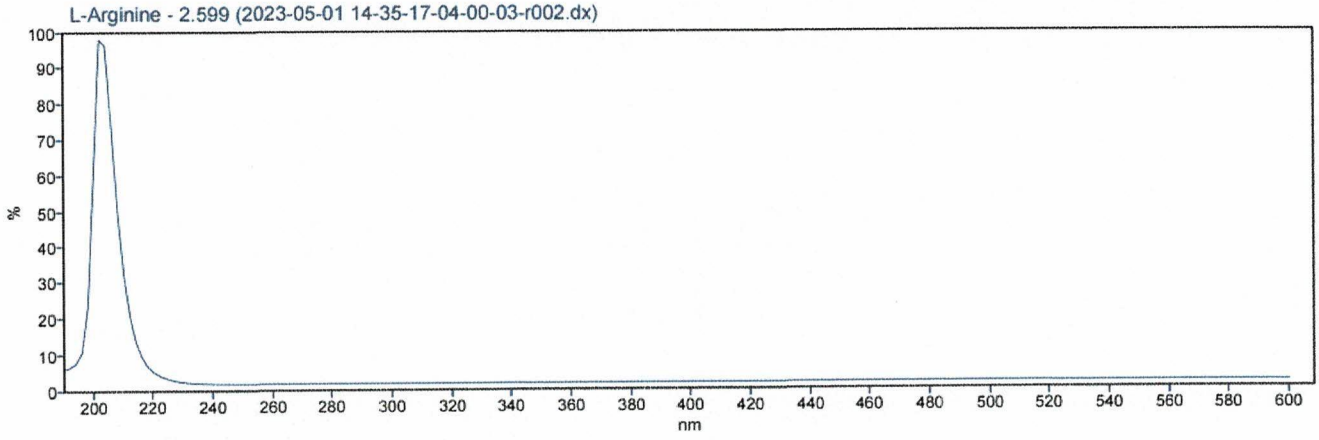
8.2 Methionine UV Spectrum



8.3 Arginine Chromatogram



8.4 Arginine UV Spectrum



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	06/27/13	New	13-0498	B. Johns
2	01/06/15	Updated SOP format. Added Methionine.	15-0007	X. Shao
3	01/02/19	Scheduled review: updated SOP format, stability requirement, weight requirement, and number of pooled tablets.	19-0009	J. Maignan
4	03/10/22	Updated for consistency with current methods. Added system suitability section. Added recommended sequence. Added column rinse and storage.	CC-22-0108	S. Sassman
5	05/05/23	Removed unnecessary information and align with current SOP format. Added instruction to follow test details containing product specific sample preparation, add specific sample prep instructions for different dosage forms. Added example chromatography and spectrum. Changed logo.	CC-23-0231	S. Sassman