1.0 Purpose

The purpose of this procedure is to define a method for the quantitative analysis and/or identification of L-citrulline in finished products and raw materials using HPLC coupled with UV/VIS detection.

2.0 Scope

This procedure has been validated to quantify and identify L-citrulline. Some excipients and dietary ingredients used in the finished products can interfere with the analysis of L-citrulline. λmax was used for the validation, but 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 and/or Analytical Development to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **ACN** – Acetonitrile

4.2 **H_3PO_4** – Phosphoric Acid

4.3 **H_2O** – Millipore Water, 18.2Ω

4.4 **QC** – Quality Control

4.5 **CofA** – Certificate of Analysis

4.6 **RT** – Room Temperature

5.0 References

5.1 MV-LAB-13-043, Protocol, L-Citrulline Determination by HPLC

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- Millipore Water

6.1.2 ACN

6.1.3 Phosphoric acid- ACS Grade

6.1.4 L-citrulline traceable standard

6.2 Supplies and Glassware

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

6.2.2 1L mobile phase container

6.2.3 10mL, 50mL, 100mL, 500mL, and 1L volumetric flasks

6.2.4 200µL, 1mL, and 10mL pipette tips

6.2.5 10mL Plastic luer-lock syringes

6.2.6 0.2µM or 0.45µM Nylon syringe filters

6.2.7 22mL screw cap vials

6.2.8 1.5mL and 2.0mL micro centrifuge tubes

6.2.9 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 Column- Acclaim 120-C18, 5µ, 120A, 4.6 X 250mm

6.3.3 Analytical Balance

6.3.4 Stir Plate

6.3.5 Wrist Action Shaker

6.3.6 Vortex

6.3.7 Sonicator Bath

6.3.8 200µL, 1mL, and 10mL Pipettes- adjustable

7.0 Preparation of Mobile Phase, Diluent, Samples and Standards

7.1 Mobile Phase A - 0.1% H_3PO_4 in H_2O

7.1.1 Transfer 1000 mL H_2O to a 1-L bottle.

7.1.2 Add 1.0 mL H_3PO_4, and mix well.

7.2 Mobile Phase B - 0.1% H_3PO_4 in ACN

7.2.1 Transfer 1000 mL ACN to a 1-L bottle.

7.2.2 Add 1.0 mL H_3PO_4, and mix well.

7.3 Diluent

7.3.1 Use Mobile Phase A

7.4 Stock Standard Preparation

7.4.1 Accurately weigh and transfer about 25 mg of reference standard into a 50-mL volumetric flask.

7.4.2 Dissolve in and dilute to volume using Diluent.

7.5 Working Standard Preparation

7.5.1 Transfer 5.0 mL of Stock Standard into a 50-mL volumetric flask.

7.5.2 Dilute to volume using Diluent.

7.5.3 Alternate standard preparations are acceptable provided that the Working Standard concentration is within the linear range listed below.

7.6 Sample Preparation

7.6.1 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.

7.6.2 The linear range of the method is 0.0005 mg/mL – 0.6 mg/mL. The concentration of the Sample Preparation must be within this range.

7.6.3 10 dosage units can be pooled and ground by mortar and pestle if necessary.

7.6.4 Based on the fill weight or tablet weight per dose weigh a portion of the pooled dosages to generate an analyte concentration that is within the validated linearity and solubility range for the analyte being tested. Raw materials samples can be prepared using 100% as purity.

7.6.5 Dilute the sample to the calculated volume with diluent, cap, and sonicate for 10 minutes to facilitate dissolution. Samples can be shaken on a wrist action shaker for 30 minutes at RT in two thirds their initial volume then brought up to final volume.

7.6.6 Remove sample from the sonicator and allow it to cool to RT. (Skip step if using wrist action shaker).

7.6.7 Samples can be dissolved in diluent at any volume starting from 10 mL. 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. The final diluted sample must be filtered or centrifuged before analyzing by HPLC.

7.6.8 For filtration, using the final large scale diluted sample withdraw up to 10 mL using a 10 mL plastic syringe then filter and discard at least the first 0.5 mL of sample before collecting. From the collected sample dilute as needed then add 1 mL to an HPLC vial for analysis.

7.6.9 For centrifugation using the final large scale diluted sample, fill an even number of 1.5 or 2.0 mL micro-centrifuge tubes and pellet insoluble matter for 5 minutes at 10,000rpm.

7.7 Test Conditions

7.7.1 Gradient-Isocratic

7.7.1.1 Column- Acclaim 120-C18, 5µ, 120A, 4.6 X 250mm, or equivalent

7.7.1.2 Flow Rate- 1.0mL/min

7.7.1.3

Time	%A	%B	Gradient type
0.00	98	2	0
6.00	98	2	0

7.7.1.4 UV detection- 195nm

7.7.1.5 Injection volume- 20uL

7.7.1.6 Column Temperature- 40°C

7.7.1.7 Recommended 3-D Spectral Range- 200nm to 700nm

7.8 Recommended Sequence

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

7.8.2 Make five injections of the Working Standard.

7.8.3 Make a single injection of each Sample Preparation.

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

7.9 System Suitability

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

7.9.2 The %RSD of all standard injections is NMT 5%.

7.10 Column Wash and Storage

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

7.10.2 Rinse the column with at least 10 mL of H_2O / ACN (50/50).

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

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

7.11.1 $$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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)

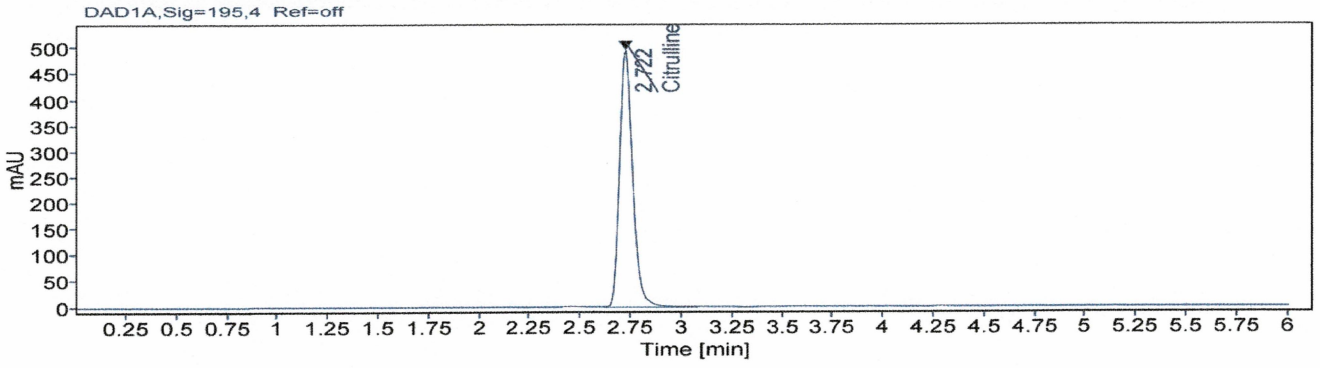
V_{spl} Volume of the sample preparation accounting for dilutions in mL

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.

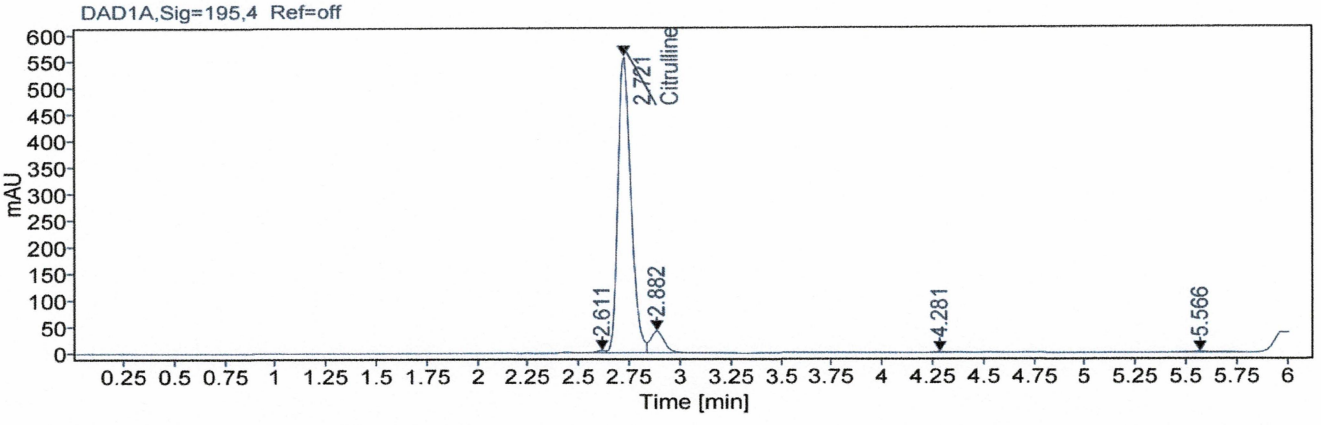
LA Label amount in mg per dose or 1 for raw materials

8.0 Example Chromatograms

8.1 Working Standard



8.2 Sample



10.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	07/23/13	New	13-614	B. Johns
2	01/05/16	Update SOP format. Update content to conform to present method SOP Structure	16-0020	N. Zhang
3	01/29/19	Scheduled review. Update responsibilities and sample prep	19-0114	J. Maignan
4	03/09/22	Update to reflect current practices. Simplify mobile phase preparation., Add recommended sequence section. Replace requirements section with system suitability. Update example calculation for consistency with current methods.	CC-22-0109	S. Sassman
5	02/22/23	Simplify standard preparation, add instruction to check the product profile for test details, remove language requiring in-process validation for new products.	CC-23-0097	S. Sassman