ION Nutritional Labs	Standard Operating Procedure **Determination of Trimethylglycine (Betaine) by HPLC/UV**	**SOP Number** **D-784**	**Revision** **2**
		Effective Date 06/13/23	**Page** **Page 1 of 7**

Written by/ Date	**Reviewed by/ Date**	**Approved by/ Date**
SAS 06/12/23	06-12-23	06/12/23
Title: Analytical Development Scientist	**Title: Analytical Development Scientist**	**Title: Quality Control Director**

1.0 Purpose

This document describes the analytical procedure for the determination of trimethylglycine (betaine) in raw materials and finished products.

2.0 Scope

This procedure applies to the identification and quantification of trimethylglycine in raw materials and finished products.

3.0 Responsibility

3.1 It is the responsibility of QC 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 to keep this procedure current with the associated monographs and laboratory practices.

4.0 Definitions

4.1 **HPLC/UV** – High Pressure Liquid Chromatography with Ultraviolet Detection

4.2 **QC** – Quality Control

4.3 **AD** – Analytical Development

4.4 **TMG** – Trimethylglycine (Betaine)

4.5 **MeOH** – Methanol

4.6 **$NaH_2PO_4 \cdot 2H_2O$** – Sodium Phosphate Monobasic Dihydrate

4.7 **H_3PO_4** – 85% Phosphoric Acid

5.0 References

5.1 MV-LAB-19-026, Protocol, Validation of an Analytical Method for the Determination of Trimethylglycine (Betaine) using HPLC/UV

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

6.0 Supplies

6.1 Chemicals: All reagents are HPLC grade or better

6.1.1 MeOH

6.1.2 Sodium-1-nonanesulfonate

6.1.3 $NaH_2PO_4 \cdot 2H_2O$

6.1.4 H_3PO_4

6.1.5 TMG Reference Standard

6.2 Supplies and Glassware

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

6.2.2 Class A Volumetric glassware and/or adjustable pipettes and tips

6.2.3 Weigh paper or funnels

6.2.4 1.5 mL or 2.0 mL micro centrifuge tubes

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 Sonicator bath

6.3.4 Stir Plate

6.3.5 Centrifuge

7.0 Preparation of Mobile Phase, Diluent, Samples and Standards Equipment

7.1 Mobile Phase Preparation

7.1.1 Mobile Phase

7.1.1.1 Combine 1.09 g of sodium-1-nonanesulfonate, 2.96 g of $NaH_2PO_4 \cdot 2H_2O$, and 950 mL of water. Begin stirring and adjust the pH to 2.5 using H_3PO_4. Add 50 mL of MeOH and mix well.

7.1.2 Diluent

7.1.2.1 Water

7.2 Standard Prep

7.2.1 Accurately weigh and transfer about 50 mg of TMG reference standard into a 100-mL volumetric flask.

7.2.2 Dissolve in and dilute to volume with Diluent.

7.2.3 The standard preparation may be scaled as required.

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 for TMG is 0.08 mg/mL – 0.80 mg/mL. The concentration of the sample preparation must be within the linear range.

7.3.3 For solid and liquid dose finished products: at least 10 dosage units are pooled and ground by mortar and pestle. Based on the weight per dose and the label amount, weigh no less than 40 mg of the pooled dosages into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linearity range. Dilute the sample to 2/3 of the flask volume with water and shake

for 20 minutes to facilitate dissolution. Sonication for 10 minutes can also be used to assist dissolution. Equilibrate the sample to room temperature (if sonicated), and dilute to volume using water.

7.3.4 For liquid capsules: weigh no less than 5 dosage units and transfer them into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linearity range. Fill with water to about 80% of the flask volume. Sonicate for 10 minutes or until the capsules are completely dissolved. Allow the sample to equilibrate to room temperature. Add water until the solution reaches the flask neck, and add a minimal volume of methanol to disperse frothing. Dilute to volume using water.

7.3.5 For raw materials: weigh no less than 40 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 for the analyte being tested. Fill with water to about 80% of the flask volume, and sonicate for 10 minutes. Allow the sample to equilibrate to room temperature, and dilute to volume using water.

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. Fill with water to about 80% of the flask volume, and sonicate for 10 minutes. Allow the sample to equilibrate to room temperature, and dilute to volume using water.

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

7.3.8 Centrifuge a portion of the sample at 10,000 rpm for 5 min to remove particulates prior to HPLC analysis. Alternatively, the sample may be filtered through a 0.45 µm membrane discarding the first 3 – 4 mL.

8.0 HPLC Parameters

8.1 Column: Inertsil ODS-3, 5µm, 4.6mm X 150mm

8.2 Column Temperature: 40 °C

8.3 Flow rate: 1.5 mL/min

8.4 Wavelength: 210 nm

8.5 Injection Volume: 10 µL

8.6 Run Time: 7 minutes

8.7 Recommended 3-D Spectral Range (for Identification)- 190nm to 400nm

8.8 Recommended Sequence

8.8.1 Make at least 2 injections of the diluent.

8.8.2 Make five (5) injections of Standard Solution.

8.8.3 Make a single injection of each Sample Preparation.

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

8.9 System Suitability Requirements

8.9.1 The %RSD of the first five (5) standard injections is NMT 5.0%.

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

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

9.0 Example Calculation

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

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

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

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

LA Label amount in mg (use 1 for raw materials)

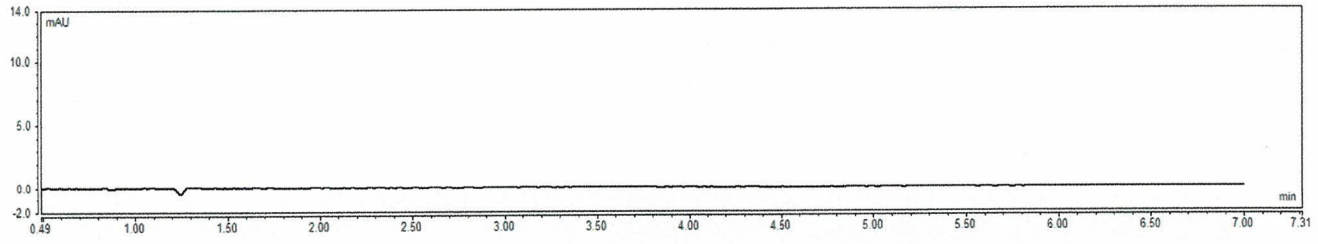
10.0 Column Wash and Storage

10.1 Rinse the column with Water / MeOH (80/20).

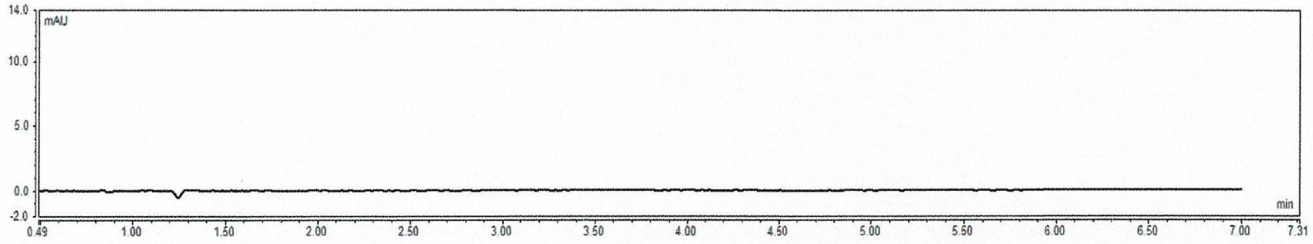
10.2 Store the column in Water / MeOH (50/50).

11.0 Chromatograms

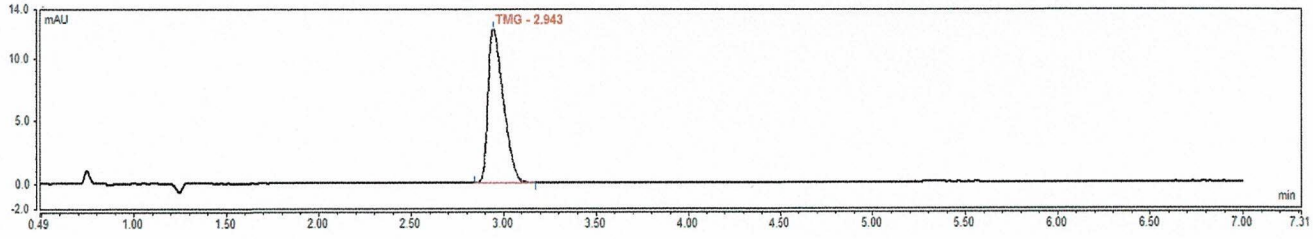
11.1 Typical Diluent Chromatogram



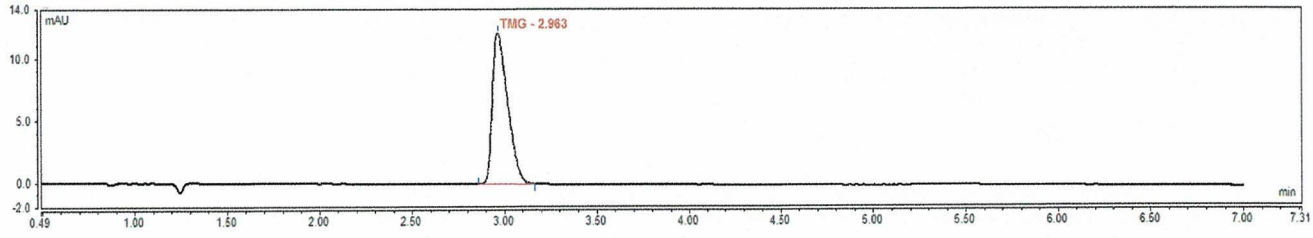
11.2 Typical Working Standard Chromatogram



11.3 Typical Raw Material Sample Chromatogram



11.4 Typical Finished Product Sample Chromatogram



12.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	06/26/19	New	N/A	S. Sassman
1	06/24/22	Updated logo and format.	CC-22-0293	K. Burris
2	06/06/23	Minor edits for clarity, add instructions to follow test details containing product specific sample prep, add sample prep instructions for gummies, remove salt correction from example calculation. Update logo and format.	CC-23-0265	S. Sassman