1.0 Purpose

The purpose of this procedure is to describe a method for the quantitative analysis of amino acids in finished products and raw materials using derivitivization coupled with HPLC and UV/VIS spectrophotometry.

2.0 Scope

This procedure is applicable to the analysis of β-alanine, aspartic acid, carnosine, glutamine, isoleucine, leucine, lysine, phenylalanine, taurine, theanine, threonine, tyrosine, and valine using HPLC in the QC laboratory at Ion Labs.

3.0 Responsibility

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

3.2 It is the responsibility 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 personnel to keep this procedure aligned with current practices.

4.0 Definitions

4.1 **ACN** – Acetonitrile

4.2 **NaH_2PO_4** – Sodium phosphate monobasic

4.3 **HPLC** – High Performance Liquid Chromatography

4.4 **OPA** – Ortho-phthalaldehyde

4.5 **$Na_2B_4O_7.10H_2O$** – Sodium tetraborate decahydrate

4.6 **NaOH** – Sodium Hydroxide

4.7 **H_2O** – Water, ≥18.2MΩ·cm, 0.22µm filtered

4.8 **QC** – Quality Control

5.0 References

5.1 Henderson JW, Ricker RD, Bidlingmeyer BA, Woodward C. Rapid, Accurate, Sensitive, and Reproducible HPLC Analysis of Amino Acids. Agilent Technologies. 2000.

5.2 MV-LAB-13-062, Protocol, Amino Acid Determination using Derivitivization and HPLC with UV Spectroscopy

5.3 MV-LAB-14-055, Protocol, L-Theanine and β-alanine Determination using OPA Derivitivization coupled with HPLC and UV/VIS Spectroscopy

5.4 MV-LAB-18-008, Protocol, Glutamine, Threonine, Valine, Phenylalanine, Isoleucine, Lysine and Aspartic acid Determination using OPA Derivitization coupled with HPLC and UV/Vis Spectroscopy

5.5 MV-LAB-18-214, Protocol, Carnosine Determinations using OPA Derivitization coupled with HPLC and UV/Vis Spectroscopy.

6.0 Procedure

6.1 Reagents: all reagents are HPLC grade or better.

6.1.1 Reference standards for target analytes

6.1.2 H_2O

6.1.3 ACN

6.1.4 NaH_2PO_4

6.1.5 Methanol

6.1.6 NaOH

6.1.7 OPA - Agilent Part Number 5061-3335

6.1.8 2-Mercaptoethanol

6.1.9 $Na_2B_4O_7.10H_2O$

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 Tips for adjustable automatic pipets

6.2.5 10mL plastic luer lock syringe

6.2.6 0.2 or 0.45µm nylon syringe filters

6.2.7 Microcentrifuge tubes

6.2.8 Weigh paper or 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 Centrifuge

6.3.6 Wrist action shaker

6.3.7 Adjustable automatic pipets as required by standard and sample preparations

6.4 Mobile Phase and Buffer Preparation

6.4.1 Mobile Phase A (40mM NaH_2PO_4 in H_2O, pH= 7.8)- is prepared by dissolving 4.78g NaH_2PO_4 in 950ml H_2O, then titrating to pH 7.8 with NaOH solution. After pH adjustment, bring up to 1 L with H_2O.

6.4.2 Mobile Phase B (ACN: MeOH: H_2O (45:45:10))- is prepared by adding 450mL ACN, 450mL MeOH and 100mL H_2O to a 1L volumetric flask and mix well.

6.4.3 Diluent- H_2O.

6.4.4 0.2M Borate buffer- is prepared by dissolving 7.6274g of $Na_2B_4O_7$. $10H_2O$ into 90ml of H2O, titrating to pH= 10.2 with NaOH solution. After pH adjustment, bring up to 100ml with H_2O.

6.5 Stock Standard Preparation

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

6.5.2 Dissolve in and dilute to volume using Diluent.

6.6 Working Standard Preparation

6.6.1 Transfer 5.0 mL of the Stock Standard into a 25-mL volumetric flask.

6.6.2 Dilute to volume using Diluent.

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

6.7 Sample Preparation

6.7.1 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.

6.7.2 The linear range of the method for each analyte is listed in the table below. The concentration of the sample preparation must be within the linear range.

Analyte	**Linear Range (mcg/mL)**
Leucine	25 - 400
Taurine	10 - 600
Theanine	12 – 800
β-alanine, aspartic acid, carnosine, glutamine, isoleucine, lysine, phenylalanine, threonine, tyrosine, and valine	10 - 100

6.7.3 For raw materials: weigh no less than 25 mg into a suitably sized volumetric flask of no less than 50 mL volume to generate an analyte concentration that is within the validated linearity range. Dilute to 2/3 of the flask volume with Diluent, shake mechanically for 30 minutes, and dilute to volume using Diluent.

6.7.4 For solid dose finished products: Combine and homogenize no less than ten dosage units. Based on the label claim and fill weight (for capsules) or tablet weight per dose, weigh no less than 25 mg of the pooled dosages into a suitably

sized volumetric flask of no less than 50 mL to generate an analyte concentration that is within the validated linearity range. Dilute the sample to 2/3 of the flask volume with Diluent, shake mechanically for 30 minutes, and dilute to volume using Diluent.

6.7.5 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.

6.7.6 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 glass beaker. Dissolve the sample in Diluent, transfer the dissolved sample 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 using small portions of Diluent to ensure complete transfer, and dilute to volume with Diluent.

6.7.7 Perform additional dilutions as required using Diluent

6.7.8 Filter an aliquot of the sample solution through a 0.45 µm membrane discarding the first 2 - 3 mL of filtrate before collecting a portion in a vial for analysis.

6.7.8.1 Alternatively, the solution may be centrifuged to remove particulates provided that the final solution is clear.

6.7.9 For samples that have multiple amino acids, care should be made to assure the concentrations are as low as possible due to the competitive reaction for the derivatization reagent.

6.8 Test Conditions

6.8.1 Gradient-Isocratic (For Taurine)

Time	%A	%B
0.00	70	30

11	70	30

6.8.2 Gradient-Isocratic (For Leucine)

Time	%A	%B
0.00	45	55
12	45	55

6.8.3 Gradient-Isocratic (For L-Theanine/ β-alanine/Tyrosine)

Time	%A	%B
0.00	70	30
16	70	30

6.8.4 Gradient-linear (For Glutamine, Threonine, Valine, Phenylalanine, Isoleucine, Lysine, Aspartic acid and Carnosine).

Time	%A	%B
0.00	100	0
1.90	100	0
18.1	43	57.0
18.6	0	100
22.3	0	100
23.2	100	0
26.0	100	0

6.8.5 Column- ZORBAX Eclipse AAA, 3.5µ, 120A, 4.6 X 250mm

6.8.6 Flow Rate- 1.0mL/min

6.8.7 Wavelength- 338nm

6.8.8 Column Temperature- 40°C

6.8.9 Recommended 3-D Spectral Range- 200nm to 500nm

6.8.10 Auto-injector program

6.8.10.1 Taurine, Leucine: injection volume- 5µL

Draw 10.0 uL from vial A(borate buffer), 200 ul/min speed

Draw 0.0 uL from vial B (H_2O), 200 ul/min speed

Draw 5.0 uL from vial C (amino acid solution), 200 ul/min speed

Mix 15.0 uL in air, max speed, 5 times

Wait 1.00 min

Draw 0.0 uL from vial B (H_2O), 200 ul/min speed

Draw 30.0 uL from vial D (OPA), 200 ul/min speed

Mix 45.0 uL in air, max speed, 5 times

Inject

Needle wash in vial B (H_2O), 1 times

Valve bypass

6.8.10.2 L-Theanine, β-alanine, Tyrosine Glutamine, Threonine, Valine, Phenylalanine, Isoleucine, Lysine, Aspartic acid and Carnosine: injection volume- 2.5 µL

Draw 5.0 uL from vial A(borate buffer), 200 ul/min speed

Draw 0.0 uL from vial B (H_2O), 200 ul/min speed

Draw 2.5 uL from vial C (amino acid solution), 200 ul/min speed

Mix 7.5 uL in air, max speed, 5 times

Wait 0.5 min

Draw 0.0 uL from vial B (H_2O), 200 ul/min speed

Draw 5.0 uL from vial D (OPA), 200 ul/min speed

Mix 12.5 uL in air, max speed, 6 times

Draw 32 uL from vial B (H_2O), 200 ul/min speed

Mix 44.5 uL in air, max speed, 8 times

Inject

Wait 0.5min

Valve bypass

Note: This program is an example; the positions for the reagents can be changed based on needs.

6.9 Recommended Sequence

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

6.9.2 Make five injections of the Working Standard.

6.9.3 Make a single injection of each Sample Preparation.

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

6.10 System Suitability

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

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

6.11 Column Wash and Storage

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

6.11.2 Rinse the column with H_2O / ACN (50/50) at 1 mL/min for at least 10 min.

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

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

6.12.1 $$\% \text{ assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{V_{spl}}{SA} \times \frac{SS}{LA} \times 100$$

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

7.0 Revision History

Revision	Date	Description of Changes	CCR #	By
1	10/14/13	New	13-907	X. Shao
2	03/25/15	Changed SOP format. Added L-Theanine.	15-0225	B. Johns
3	03/26/19	Scheduled review. added new analytes.	19-0240	J. Maignan
4	03/16/23	Update for clarity, ease of use, and consistency with current methods and lab practices.	CC-23-0141	S. Sassman