| HBI Ion Labs | Standard Operating Procedure<br>**Determination of D-Biotin by HPLC using UV/VIS Spectroscopy** | **SOP Number**<br>**D-789** | **Revision**<br>**3** |
|---|---|---|---|
| | | **Effective Date** | **Page**<br>**Page 1 of 8** |

| **Written by/ Date** | **Reviewed by/ Date** | **Approved by/ Date** |
|---|---|---|
| MMA 10/20/25 | AJS 10/20/25 | FR 21-OCT-2025 |
| **Title: QC Microbiologist II** | **Title: QC Laboratory Manager** | **Title: QA/QC Director** ① FR 21-OCT-2025 |

① Approved by Franz Roluang, QC Manager in absence of Nicole Molley. FR 21-OCT-2025

## 1.0 Purpose

The purpose of this procedure is to define the method for the quantification and identification of D-Biotin in raw materials and finished products by HPLC using UV/VIS Spectroscopy.

## 2.0 Scope

This procedure applies to the identification and quantification of Biotin in raw material and finished products in the QC laboratory at Ion Nutritional 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 of QC Laboratory Management to implement this procedure and to ensure that this 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 **HPLC/UV –** High pressure liquid chromatography with ultraviolet detection

4.2 **QC** – Quality Control

4.3 **ACN** – Acetonitrile

4.4 **$H_3PO_4$** – 85% Phosphoric acid

4.5 **$NaClO_4$** – Sodium perchlorate monohydrate

4.6 **$K_2HPO_4$** – Potassium phosphate dibasic

4.7 **DMSO** – Dimethylsulfoxide

4.8 **$H_2O$** – Water (≥18.2 MΩ·cm)

## 5.0 References

5.1 MV-LAB-19-143, Protocol, Validation of an Analytical Method for the Determination of D-Biotin by 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 Reference Standard

6.1.2 $H_2O$

6.1.3 ACN

6.1.4 $H_3PO_4$

6.1.5 $NaClO_4$

6.1.6 $K_2HPO_4$

6.1.7 DMSO

6.2 Glassware

6.2.1 Volumetric glassware as required for standard and sample preparations

6.2.2 HPLC vials, 12mm x 32mm with screw cap enclosures with septa

6.2.3 Scintillation vials

6.2.4 Mobile phase containers

6.3 Disposables

6.3.1 Centrifuge tubes

6.3.2 Plastic Luer-lock syringe

6.3.3 Nylon syringe filters, 0.45µm

6.3.4 Weigh paper

6.4 Equipment

6.4.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven and UV detector with a chromatographic data handling system

6.4.2 Analytical Balance

6.4.3 Centrifuge

6.4.4 Adjustable Pipette

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

7.1 Solution Preparation

7.1.1 Mobile Phase

7.1.1.1 Transfer about 0.915 g of $NaClO_4$ to a 1-L bottle, add 915 mL $H_2O$, 0.915 mL of $H_3PO_4$, and 85 mL ACN. Mix until dissolved.

7.1.2 Diluent

7.1.2.1 Transfer about 4.36 g of potassium phosphate dibasic to a 1-L bottle, add 100 mL DMSO and 900 mL $H_2O$. Mix until dissolved.

7.2 Standard Preparation

7.2.1 Stock Standard: Accurately weight and transfer about 32 mg of D-Biotin reference standard to a 100-mL volumetric flask. Dissolve in and dilute to volume with Diluent.

7.2.2 Working Standard: Transfer 0.75 mL of Stock Standard to a 50-mL volumetric flask. Dilute to volume with Diluent.

7.2.3 Note: Deviation from preparation above is acceptable as long as concentrations remain within linear range.

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 D-Biotin is 0.25 µg/mL – 60 µg/mL. The concentration of the sample preparation must be within the linear range.

7.3.3 For solid dose finished products: Combine and homogenize at least 10 dosage units. Based on the weight per dose and the label amount, weigh a portion of the homogenized sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Dilute the sample to 2/3 of the flask volume with Diluent and shake for 20 minutes. Sonicate for 5 minutes, and then shake again for an additional 15 minutes.

7.3.4 For liquids: use a TC pipet to transfer a portion of the sample into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Rinse the pipet several times using Diluent, and collect the rinses in the volumetric flask. Dilute to volume using Diluent.

7.3.5 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 homogenized sample into a suitably sized 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.

7.3.6 For raw materials: Based on the D-Biotin content, weigh a portion into a suitably sized volumetric flask to generate an analyte concentration that is within the validated linear range. Dilute the sample to 2/3 of the flask volume with Diluent and shake for 20 minutes. Sonicate for 5 minutes, and then shake again for an additional 15 minutes.

7.3.7 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 using Diluent to bring the analyte concentration into the linear range of measurement.

7.3.8 The final sample must be filtered or centrifuged before analyzing by HPLC.

7.3.8.1 For filtration: filter a portion through a 0.45µm nylon membrane discarding the first 2-3 mL of filtrate before collecting an aliquot for analysis.

7.3.8.2 For centrifugation: centrifuge for 5 min at 10,000 rpm.

## 8.0 Test Conditions

8.1 HPLC Parameters

8.1.1 Column: YMC-Pack Pro C8, 3 µm, 4.6 mm x 150 mm

8.1.2 Column Temperature: 30 °C

8.1.3 Flow rate: 1.2 mL/min

8.1.4 Wavelength: 200 nm

8.1.5 Injection Volume: 50 µL

8.1.6 Run Time: at least 20 minutes

8.1.7 Recommended Spectral Range (for Identification)- 200 nm to 600 nm

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 ten (10) sample injections or at the end of a run.

8.3 System Suitability Requirements

8.3.1 The %RSD of the first five (5) standard injections is NMT 2.0%.

8.3.2 The %RSD of all standard injections is NMT 2%.

8.3.3 The average (n=5) tailing factor is NMT 1.5.

8.4 Retention Times

8.4.1 D-Biotin: about 16 min

8.5 Recommended System Setup

8.5.1 Purge channel A with $H_2O$/methanol (90/10) at 5 mL/min for at least 5 min.

8.5.2 Rinse the column with $H_2O$/methanol (90/10) at 1 mL/min for at least 5 min.

8.5.3 Purge channel A with Mobile Phase at 5 mL/min for at least 5 min.

8.5.4 Begin flow of Mobile Phase through the column at 1 mL/min.

8.6 Column Wash and Storage

8.6.1 Rinse the column with $H_2O$ / ACN (90/10)

8.6.2 Store the column in $H_2O$ / ACN (50/50)

## 9.0 Calculations

9.1 $$\%\ \text{assay} = \frac{R_u}{R_s} \times \frac{Wt_{std} \times P}{V_{std}} \times \frac{SS}{SA} \times \frac{V_{spl}}{LA} \times 100$$

$R_u$ Sample peak area

$R_s$ Mean standard peak area

$Wt_{std}$ Weight of the 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)

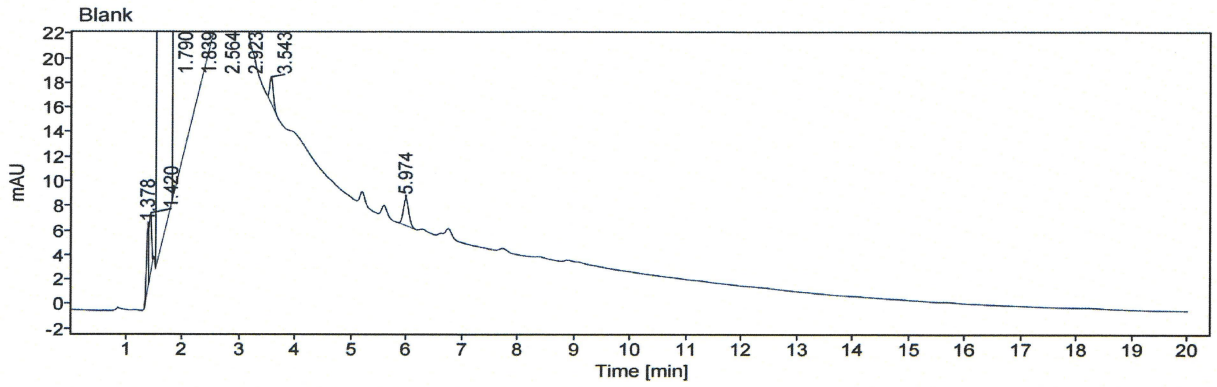
$SS$ Serving size: Average weight of ten dosage units in mg for tablets and capsules, volume of a single serving from the theoretical formula in mL for liquids, or 1 for raw materials.

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

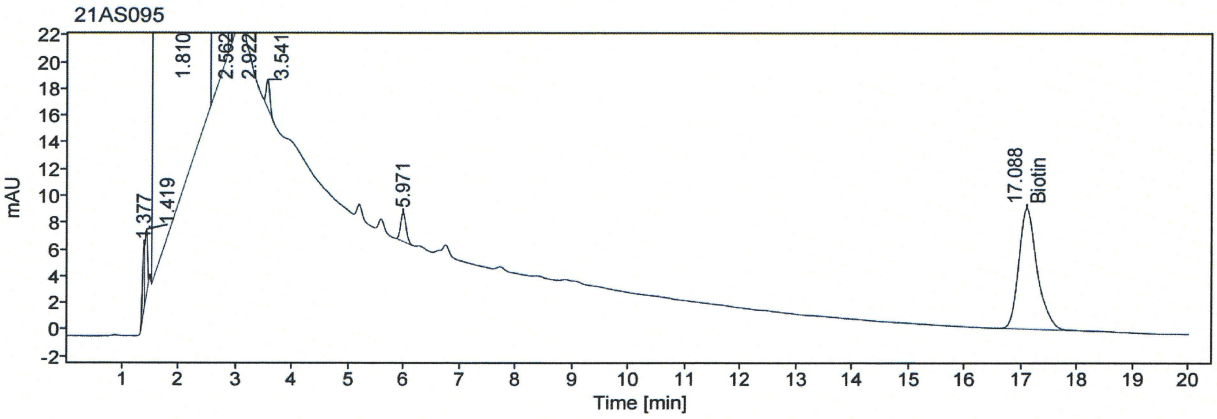
$LA$ Label amount in mg (use 1 for raw materials)

## 10.0 Example Chromatography and Spectrum

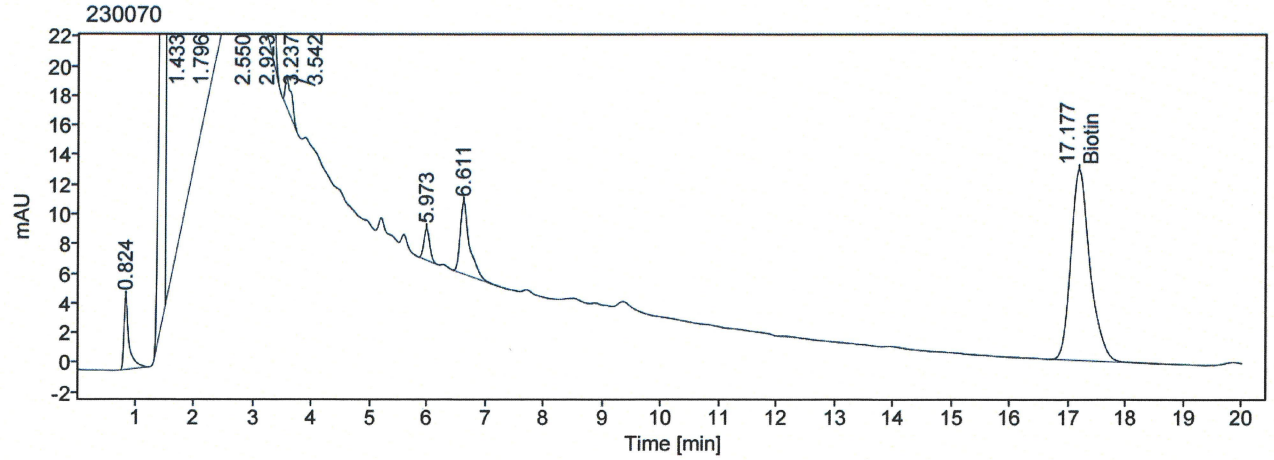
### 10.1 Blank



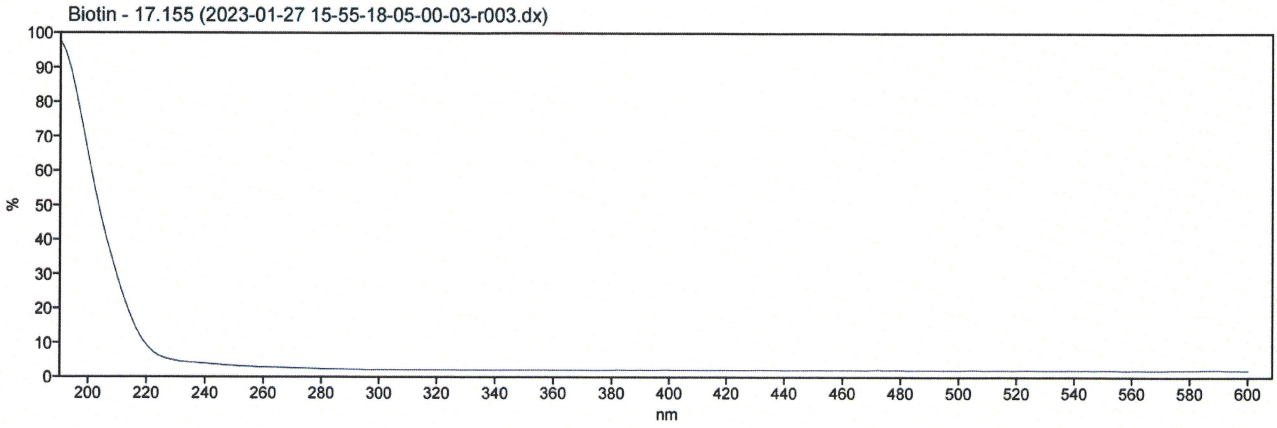

### 10.2 Working Standard




### 10.3 Finished Product Sample



10.4 Biotin UV Spectrum




## 11.0 Revision History

| Revision | Date | Description of Changes | CCR # | By |
|---|---|---|---|---|
| 0 | 02/11/20 | New | N/A | S. Sassman |
| 1 | 04/10/23 | Minor edits for consistency with current methods, add instruction to follow product specific test details, add example chromatography, add recommended system start up and shut down. | CC-23-0176 | S. Sassman |
| 2 | 08/02/24 | Added note to allow flexibility in standard prep. | CC-24-0353 | D. Hashmi |
| 3 | 10/15/25 | Added note to allow for preparations to be anywhere inside the linear range of Biotin | CC-25-0421 | M. Autrey |