HBI Ion Labs	Standard Operating Procedure **Determination of Mannose by HPLC/UV**	**SOP Number** **D-1024**	**Revision** **1**
		Effective Date	**Page** **Page 1 of 11**
Written by/ Date MMA 10/09/25	**Reviewed by/ Date** AJS 10/09/25	**Approved by/ Date** Tracy Kolz 21-Oct-2025	
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(1) Franz Rolwagy, HBI Amerlab QC Manager approving in absence of Nicole Motley. FR 21-Oct-2025

1.0 Purpose

The purpose of this procedure is to define the method for the determination of mannose in raw materials and finished products by HPLC/UV.

2.0 Scope

This procedure applies to the determination of mannose by HPLC/UV in the QC laboratory at HBI Ion Labs.

3.0 Responsibility

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

3.2 It is the responsibility of QC Laboratory Management 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 Performance Liquid Chromatography with Ultraviolet Detection

4.2 **QC** – Quality control

4.3 **PMP** – 3-methyl-1-phenyl-2-pyrazoline-5-one

4.4 **HCl** – Hydrochloric acid

4.5 **NaOH** – Sodium hydroxide

4.6 **H_3PO_4** – Phosphoric acid (~85%)

4.7 **K_2HPO_4** – Potassium phosphate dibasic

4.8 **H_2O** – Deionized water (>18MΩ·cm)

4.9 **DAD** – Diode array detector

4.10 **ACN** – Acetonitrile

4.11 **MeOH** - Methanol

5.0 References

5.1 PRTCL-24-0015, Protocol, Validation of a Method for the Determination of Mannose by HPLC/UV.

5.2 Gonzalez NM, Fitch A., Al-Bazi J. (2020) Development of a RP-HPLC method for the determination of glucose in *Shewanella oneidensis* cultures utilizing 1-pheyl-3-methyl-5-pyrazolone derivatization, PLoS ONE 15(3).

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

6.0 Supplies

6.1 Chemicals

6.1.1 D-(+)-Mannose and D-(-)-Ribose reference standards

6.1.2 ACN

6.1.3 PMP

6.1.4 0.5 M HCl

6.1.5 0.5 M NaOH

6.1.6 H_3PO_4

6.1.7 K_2HPO_4

6.1.8 MeOH

6.1.9 Chloroform

6.1.10 H_2O

6.2 Glassware and Disposables

6.2.1 Volumetric glassware as required for standard and sample preparations

6.2.2 HPLC vials, 2mL with screw-cap enclosures and septa

6.2.3 Tips for adjustable pipettes

6.2.4 0.45 μm nylon or PVDF syringe filters

6.2.5 10-mL plastic syringes with luer lock fitting

6.3 Equipment

6.3.1 Suitable gradient HPLC system consisting of a pump, autosampler, column oven, and UV or DAD detector.

6.3.2 Analytical balance

6.3.3 pH meter

6.3.4 Water bath capable of maintaining temperature at 70°C

6.3.5 Wrist action shaker

6.3.6 Adjustable pipettes

7.0 Preparation of Mobile Phase, Extraction Solvent, Standards, Internal Standards, and Samples

7.1 Mobile Phase A (15 mM K_2HPO_4 in H_2O)

7.1.1 Transfer 2.61 g of K_2HPO_4 to a suitable container.

7.1.2 Add 1000 mL of H_2O and a stir bar.

7.1.3 Begin stirring and adjust to pH 7.2 using H_3PO_4.

7.2 Mobile Phase B (ACN)

7.2.1 Use 100% ACN.

7.3 Internal Standard Solution (12.5 mg/mL D-(-)-Ribose in H_2O)

7.3.1 Accurately weigh and transfer about 625 mg of D-(-)-Ribose to a 50-mL volumetric flask.

7.3.2 Dissolve in and dilute to volume using H_2O.

7.4 Derivatization Solution (0.5 M PMP in Methanol)

7.4.1 Accurately weigh and transfer about 870 mg of PMP to a 10-mL volumetric flask.

7.4.2 Dissolve in and dilute to volume using methanol.

7.5 Stock Standard (2.5 mg/mL D-(+)-Mannose + 2.5 mg/mL D-(-)-Ribose)

7.5.1 Accurately weigh and transfer about 62.5 mg D-(+)-Mannose into a 25-mL volumetric flask.

7.5.2 Add 5.0 mL of Internal Standard Solution.

7.5.3 Dissolve in and dilute to volume using H_2O.

7.6 Stock Sample Preparation (~2.5 mg/mL D-(+)-Mannose + 2.5 mg/mL D-(-)-Ribose)

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 of this method.

7.6.2 The validated linear range of the method is 1 mg/mL – 5 mg/mL based on the concentration of mannose in the Stock Sample. The Stock Sample preparation must be within the linear range of the method.

7.6.3 Ensure that the sample is homogeneous prior to weighing.

7.6.3.1 For capsules, combine the fill material from at least 10 dosage units and homogenize in a mortar and pestle if necessary.

7.6.3.2 For tablets, combine at least 10 dosage units and homogenize in a mortar and pestle.

7.6.3.3 For chewable gels (gummies), homogenize at least 10 dosage units as outlined in D-793.

7.6.4 Based on the raw material manufacturer's assay value or finished product label claim, accurately weigh and transfer a sufficient amount of sample to deliver about 125 mg of D-(+)-Mannose to a 50-mL volumetric flask.

7.6.5 Add 5.0 mL of methanol and swirl to completely wet the sample.

7.6.6 Add 10.0 mL of Internal Standard Solution.

7.6.7 Add about 20 mL of H_2O.

7.6.8 Shake on a wrist-action shaker for 20 min.

7.6.9 Dilute to volume with H_2O, and shake vigorously.

7.7 Derivatization Reaction and Cleanup (Option 1)

7.7.1 Transfer 1.6 mL of Stock Standard into a 15-mL glass centrifuge tube.

7.7.2 Transfer 1.6 mL of Stock Sample into a separate 15-mL glass centrifuge tube.

7.7.3 Add 1.6 mL of 0.5 NaOH to each of the standard/sample tubes.

7.7.4 Vortex briefly to mix.

7.7.5 Add 1.6 mL of Derivatization Solution to each of the standard/sample tubes.

7.7.6 Vortex briefly to mix.

7.7.7 Incubate in a water bath at 70°C for 90 min, shaking by hand every 30 min.

7.7.8 Equilibrate to room temperature.

7.7.9 Add 1.6 mL of 0.5 M HCl to each of the standard/sample tubes.

7.7.10 Add 2.0 mL of chloroform to each of the standard/sample tubes.

7.7.11 Vortex for about 5 sec to mix well.

7.7.12 Right away vent sample to release pressure.

7.7.13 Repeat steps 7.7.11 – 7.7.12 until pressure stops building up.

7.7.14 Centrifuge at 4000 rpm for 3 min to separate the layers.

7.7.15 Use a glass pipet to remove and discard the bottom (chloroform) layer.

7.7.16 Repeat steps 7.7.10 – 7.7.15 twice for a total of 3 washes.

7.8 Derivatization Reaction and Cleanup (Option 2)

7.8.1 Transfer 1.6 mL of Stock Standard into a 15-mL glass centrifuge tube.

7.8.2 Transfer 1.6 mL of Stock Sample into a separate 15-mL glass centrifuge tube.

7.8.3 Add 1.6 mL of 0.5 NaOH to each of the standard/sample tubes.

7.8.4 Vortex briefly to mix.

7.8.5 Add 1.6 mL of Derivatization Solution to each of the standard/sample tubes.

7.8.6 Vortex briefly to mix.

7.8.7 Incubate in a water bath at 70°C for 90 min, shaking by hand every 30 min.

7.8.8 Equilibrate to room temperature.

7.8.9 Add 1.6 mL of 0.5 M HCl to each of the standard/sample tubes.

7.8.10 Quantitatively transfer reaction mixture from standard/sample tubes to separate separatory funnels with at least 2 mL of chloroform

7.8.11 Shake and vent well to release pressure, let layers separate

7.8.12 Discard bottom (chloroform) layer

7.8.13 Repeat steps 7.7.11 – 7.7.12 with at least 2 mL of chloroform twice more for a total of 3 washes.

7.8.14 Transfer remaining (top) layer to scintillation vials

7.9 HPLC Sample Preparation

7.9.1 Transfer 1.0 mL of each reaction mixture after chloroform washing into separate 100-mL volumetric flask.

7.9.2 Dilute to volume using H_2O.

7.9.3 Filter a portion using a 0.45 µm syringe filter (nylon or PVDF), discarding the first 2-3 mL before collecting a portion in an HPLC vial for analysis.

7.10 Instrument Method Parameters

7.10.1 Column: Phenomenex Kinetex XB-C18, 4.6 x 150 mm, 2.6 µm or equivalent

7.10.2 Flow Rate: 1.0 mL/min

7.10.3 Run Time: 25 min

7.10.4 Gradient

Time (min)	%A	%B
0.0	88	12
12.0	80	20
15.0	35	65
20.0	35	65
20.1	88	12
25.0	88	12

7.10.5 Injection Volume: 5 µL

7.10.6 Column Temperature: 30 °C

7.10.7 Wavelength: 245 nm

7.10.8 Suggested 3-D Spectral Range (for Identification): 210 nm – 400 nm

7.11 Recommended Sequence

7.11.1 Make at least 1 injection of a Blank (H_2O).

7.11.2 Make 5 injections of the Working Standard.

7.11.3 Make a single injection of each Working Sample.

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

7.12 System Suitability Requirements

7.12.1 The %RSD for five consecutive injections of Working Standard is NMT 3%.

7.12.2 The %RSD for all injections of Working Standard is NMT 5%.

7.12.3 No significant (>0.5%) interference is present in the Blank injection.

7.13 Column Wash and Storage

7.13.1 Wash the column at 1 mL/min with H_2O/ACN (90/10).

7.13.2 Wash the column at 1 mL/min with H_2O/ACN (50/50).

7.13.3 Store the column in H_2O/ACN (50/50).

7.14 Example Calculation

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

R_u Sample relative response

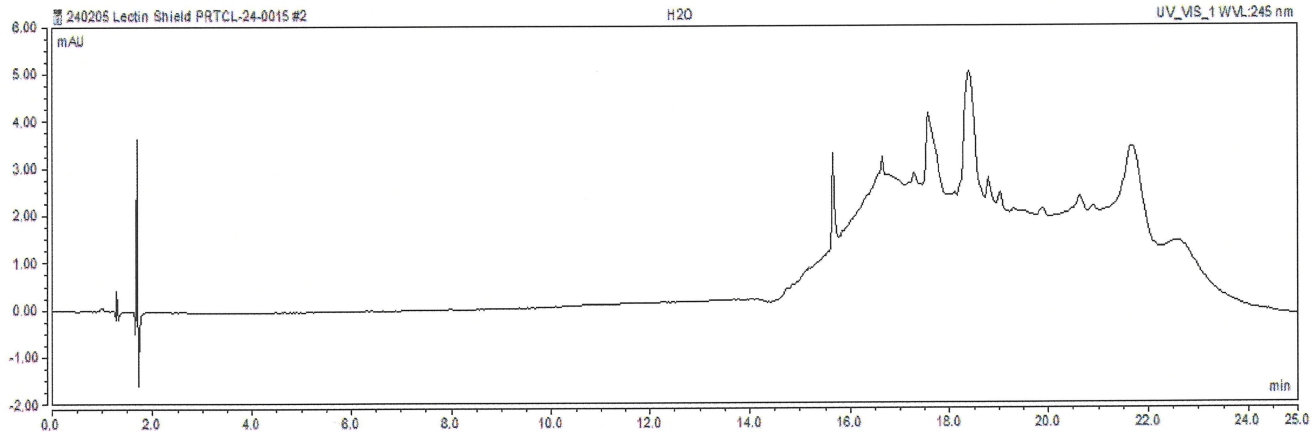
R_s Mean standard relative response

Wt_{std} Weight of reference standard in mg

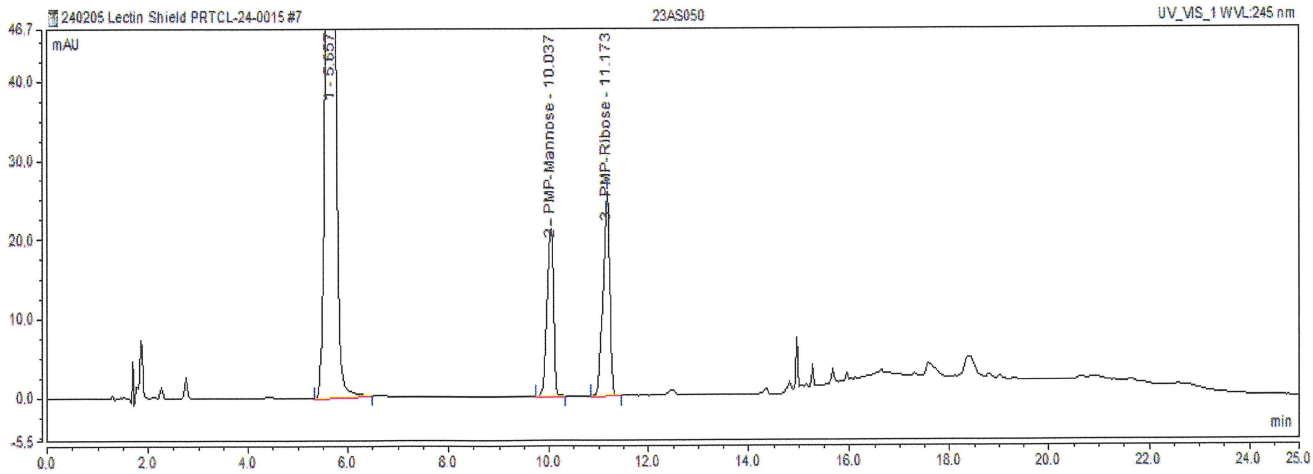
V_{std}	Volume of the standard preparation accounting for dilutions in mL
P	Purity of the reference standard in decimal format
SS	Serving size: Weight of a single dosage unit in mg or 1 for raw materials.
Spl_{wt}	Sample weight in mg
V_{spl}	Volume of the sample preparation accounting for dilutions in mL
LA	Label amount in mg per dose or 1 for raw materials

8.0 Example Chromatograms

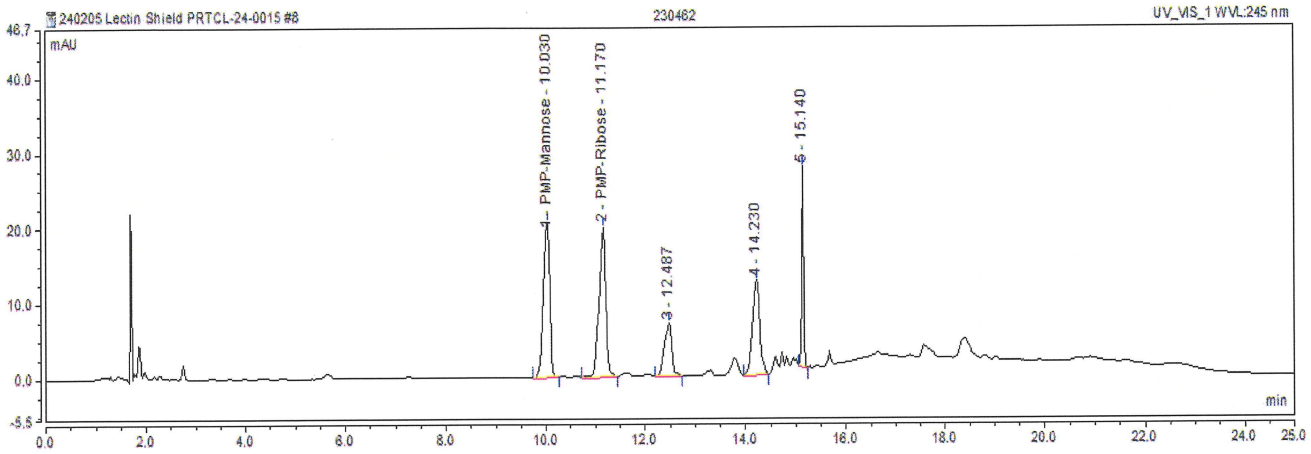
8.1 Blank



8.2 Standard



8.3 Sample



9.0 Revision History

Revision	Date	Description of Changes	CCR #	By
0	04/30/24	New procedure.	N/A	S. Sassman
1	10/01/25	Added use of separatory funnel as an acceptable method for derivatization step 7.8. Added clarifying steps to 7.7.	CC-25-0372	M. Autrey