BIOSPECTRA
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ANALYTICAL METHOD VALIDATION REPORT: DETERMINATION OF RELATED SUBSTANCES FOR TREHALOSE BY HPLC WITH RI DETECTION

TABLE OF CONTENTS

1. PURPOSE:

1.1. The JP Trehalose Related Substances analytical method was modified to allow for the quantitation of related substances. The purpose of this protocol is to:

1.1.1. To ensure the Trehalose Related Substances analytical method validation, which was performed on the Waters Alliance HPLC, is adequately evaluated and validated.

1.1.2. To summarize the findings from the Trehalose Related Substances validation and demonstrate that the analytical method meets all requirements for: System Suitability, Accuracy, Precision, Specificity, Linearity, Limit of Quantitation, and Range.

2. SCOPE:

2.1. This analytical method validation report applies to the Trehalose Related Substances method using BioSpectra's Waters Alliance HPLC.

2.2. Related Substances Specifications:

Related Substance	Specification Max (%wt/wt)
Glucose (Impurity A)	0.5
Maltotriose (Impurity B)[1]	0.2, 0.5
Unspecified Impurity	0.03
[1]Multiple specifications listed for Impurity B	

2.3. The Trehalose Related Substances method was validated as a category II quantitative test.

2.4. The Analytical Method Validation Master Plan dictates that this report will include an assessment and conclusive statements of validation on the following: System Suitability, Accuracy, Precision, Specificity, Linearity, Limit of Quantitation, and Range.

3. RESPONSIBILITIES:

3.1. The Senior Chromatography Specialist, analysts and/or the Associate director of Product Life Cycle, if necessary, are responsible for completing the Method Validation Report using conclusions made from the results obtained from testing.

4. REFERENCE:

4.1. BSI-PRL-0566, Method Validation Protocol: Determination of Related Substances for Trehalose by HPLC with RI Detection

4.2. BSI-SOP-0098, Balance SOP

4.3. BSI-SOP-0134, Pipette SOP

4.4. BSI-SOP-0436, Analytical Method Validation Master Plan

4.5. *JP <2.01> Liquid Chromatography*

4.6. *Trehalose Hydrate JP Monograph*

4.7. *USP <621> Chromatography*

4.8. *USP <1225> Validation of Compendial Procedures*

4.9. *USP <1226> Verification of Compendial Procedures*

4.10. *Waters 2414 Refractive Index Detector Operator's Guide*

4.11. *Waters 2695 Separations Module Operator's Guide*

5. PRE-VALIDATION REQUIREMENTS:

5.1. Equipment

5.1.1. All equipment used in this Validation was in proper working order and within calibration. Serial numbers, date of last calibration, and the calibration due date for each instrument and equipment, where applicable, are included in the report.

5.2. Personnel

5.2.1. All personnel who executed this Validation were properly trained in accordance with the Analytical Methods Validation Master Plan.

5.3. Supplies

5.3.1. All supplies used in the Validation were clean and appropriate for their intended use. Suppliers and part numbers of all supplies are included in this report.

5.4. Reagents

5.4.1. All reagents were current and suitable for their intended use. The reagent name, lot number, manufacturer, date of opening, date of expiration, and part number are included in this report.

5.5. Reference Standards

5.5.1. All reference standards that were used in this Validation are listed in Section 6 and are traceable to nationally recognized standards, if available. The name of the reference standard, lot number, manufacturer, date of opening, date of expiration, and part number for reference standards used are included in this report.

6. MATERIALS AND EQUIPMENT:

6.1. All materials and equipment utilized in this Validation are outlined in this section.

6.2. Analytical Balance

6.2.1. Manufacturer: Sartorius
6.2.2. Model: Secura 124-1S
6.2.3. Serial Number: 29212172
6.2.4. Last Serviced: 04/20/22
6.2.5. Next Service: 10/2022

6.3. Analytical Balance

6.3.1. Manufacturer: Sartorius
6.3.2. Model: MSE224S
6.3.3. Serial Number: 24801744
6.3.4. Last Serviced: 4/20/22
6.3.5. Next Service: 10/22

6.4. Waters Alliance HPLC

6.4.1. 30cm Column Compartment
6.4.1.1. SN: K21SMC188G
6.4.1.2. Last PM: 03/03/22
6.4.1.3. Next PM due: 03/2023

6.4.2. e2695 Separations Module
6.4.2.1. SN: M21SM4744A
6.4.2.2. Last PM: 03/03/22
6.4.2.3. Next PM due: 03/2023

6.4.3. 2414 Refractive Index Detector
6.4.3.1. SN: M21214125M
6.4.3.2. Last PM: 03/03/22

6.4.3.3. Next PM due: 03/2023

6.5. Reagents

6.5.1. HPLC Grade Water (Milli-Q Purified Water)

6.5.1.1. Manufacturer: Millipore Sigma

6.5.1.2. Serial Number: F9SA14284H

6.5.1.3. Last Service: 06/14/22

6.5.1.4. Next Service: 06/2023

6.6. Supplies

6.6.1. Disposable Polypropylene Weighing Funnels

6.6.1.1. Supplier: TWD Scientific, LLC

6.6.1.1.1. Part Number: DPWF-PP1-S

6.6.2. Micropipettes

6.6.2.1. Model: 100µL - 1000µL

6.6.2.1.1. Supplier: Eppendorf

6.6.2.1.2. Serial Number: G26211D

6.6.2.1.3. Last Service: 05/23/22

6.6.2.1.4. Next Service: 11/30/22

6.6.2.2. Model: 100µL - 1000µL

6.6.2.2.1. Supplier: Eppendorf

6.6.2.2.2. Serial Number: R24330H

6.6.2.2.3. Last Service: 07/14/22

6.6.2.2.4. Next Service: 01/31/23

6.6.2.3. Model: 100µL - 1000µL

6.6.2.3.1. Supplier: Eppendorf

6.6.2.3.2. Serial Number: Q28940G

6.6.2.3.3. Last Service: 7/14/22

6.6.2.3.4. Next Service: 1/31/23

6.6.2.4. Model: 500µL - 5000µL

6.6.2.4.1. Supplier: Eppendorf

6.6.2.4.2. Serial Number: K53394I

6.6.2.4.3. Last Service: 06/13/22

6.6.2.4.4. Next Service: 12/31/22

6.6.2.5. Model: 500µL - 5000µL

6.6.2.5.1. Supplier: Eppendorf

6.6.2.5.2. Serial Number: L21310F

6.6.2.5.3. Last Service: 7/14/22

6.6.2.5.4. Next Service: 1/31/23

6.6.3. Micropipette Tips

6.6.3.1. Supplier: Eppendorf

6.6.3.2. Part Number: 0030071581

6.6.4. Transfer pipettes

6.6.4.1. Supplier: Fisher

6.6.4.2. Part Number: 13-711-9AM

6.6.5. Screw top glass autosampler vials and pre-slit caps

6.6.5.1. Supplier: Waters

6.6.5.2. Part Number: 186000307C

6.7. Reference Standards

6.7.1. USP Traceable Maltotriose (Dextrose Impurity C)

6.7.1.1. Supplier: Sigma Aldrich

6.7.1.2. Lot: LRAC6491

6.7.1.3. Expiration Date: 07/2024

6.7.1.4. Purity: 85.9%

6.7.1.5. Part Number: PHR2086-1G

6.7.1.6. CAS Number: 1109-28-0

6.7.1.7. Date of Opening: 07/19/22

6.7.1.8. Date of Opening: 08/30/22

6.7.2. USP Traceable Glucose (Dextrose)

6.7.2.1. Supplier: Sigma Aldrich

6.7.2.2. Lot: LRAC4110

6.7.2.3. Expiration Date: 11/2023

6.7.2.4. Purity: 99.9%

6.7.2.5. Part Number: PHR1000-1G

6.7.2.6. Date of Opening: 05/31/21

6.7.2.7. CAS Number: 50-99-7

6.7.3. USP Trehalose

6.7.3.1. Supplier: USP

6.7.3.2. Lot: R112L0

6.7.3.3. Expiration Date: Current lot

6.7.3.4. Purity: 90.2%

6.7.3.5. Part Number: 1673715

6.7.3.6. Date of Opening: 7/29/22

6.7.3.7. Date of Opening: 8/12/22

6.7.3.8. Date of Opening: 8/23/22

6.7.3.9. Date of Opening: 8/30/22

6.7.3.10. CAS Number: 6138-23-4

6.8. Authentic Sample

6.8.1. Trehalose Hydrate:

6.8.1.1. Manufacturer: BioSpectra, Inc.

6.8.1.2. Lot: TE3250-003-1119

6.9. LC Column

6.9.1. Rezex RNM-Carbohydrate Na+ (8%) 7.8mm x 300mm, 8µm

6.9.2. Supplier: Phenomenex

6.9.3. Part number: 00H-0136-K0

6.9.4. Analyst 1:

6.9.4.1. Serial Number: H22-107706

6.9.5. Analyst 2:

6.9.5.1. Serial Number: H22-183012

7. GENERAL TESTING PROCEDURE:

7.1. Solution Preparation: The following procedure was carried out on each day of analysis.

7.1.1. Thoroughly rinse all glassware with purified water and allow to fully dry.

7.1.2. Diluent/Mobile Phase/Needle Wash: HPLC Grade Water

7.1.3. Resolution Solution (5.0 mg/mL Maltotriose, 5.0 mg/mL Glucose, 5.0 mg/mL Trehalose)
- 7.1.3.1. Weigh and transfer 50 mg (±10%) each of Maltotriose, Glucose, and Trehalose reference standards into a 10mL volumetric flask.
- 7.1.3.2. Fill ~3/4 full with diluent and swirl to dissolve.
- 7.1.3.3. Fill to volume with diluent.
- 7.1.3.4. Mix by Inversion.

7.1.4. Calibration Standard Solution (0.25 mg/mL Trehalose, 0.25 mg/mL Maltotriose, 0.25 mg/mL Glucose)
- 7.1.4.1. Weigh and transfer 28 mg (±10%) Trehalose, 30 mg (±10%) Maltotriose, and 25 mg (±10%) Glucose reference standards into a 100 mL volumetric flask.
- 7.1.4.2. Fill ~3/4 full with diluent and swirl to dissolve.
- 7.1.4.3. Fill to volume with diluent.
- 7.1.4.4. Mix by inversion.
  - 7.1.4.4.1. Note: the amount of reference standard to be used may require adjustment based off CoA values. The final concentration for each analyte must be within ±10% of 0.25 mg/mL.

7.1.5. LOQ Solution: (0.005 mg/mL Trehalose, 0.005 mg/mL Maltotriose, 0.005 mg/mL Glucose)
- 7.1.5.1. Pipette 1.0 mL of the calibration standard solution into a 50 mL volumetric flask.
- 7.1.5.2. Fill to volume diluent.
- 7.1.5.3. Mix by inversion.
- 7.1.5.4. Prepare fresh.

7.1.6. Test Samples (50 mg/mL Trehalose anhydrous basis)
- 7.1.6.1. Weigh 550 mg (±5%) Trehalose Hydrate into a 10 mL volumetric flask.
- 7.1.6.2. Fill ~3/4 full with diluent.
- 7.1.6.3. With occasional swirling, allow the solids to fully dissolve.
- 7.1.6.4. Fill to volume with diluent.
- 7.1.6.5. Mix by inversion.
- 7.1.6.6. Perform a single injection.

7.2. System Setup:

Table 1: Waters Alliance HPLC Method Parameters

Parameter	Setting
Flow Type	Isocratic
Diluent	Water
Mobile Phase A	Water
Flow Rate	0.35 mL/min
Run Time	30 minutes
Injection Volume	20 µL
Stroke Volume	25 µL
Syringe Draw Rate	Normal
Pre-Column Volume	0.0
Needle Wash Time	Normal
Column Temperature (°C)	65 ± 1.0
Sample Temperature (°C)	25 ± 5.0
Detector	Refractive Index
Detector Temperature	40 °C
Sampling Rate	10
Filter Time	1.0
Sensitivity	4
Polarity	Positive

7.2.1. Column Care:

7.2.1.1. Avoid jostling and dropping the column as this might cause column shock.

7.2.1.2. Store the column in 100% HPLC grade water.

7.2.1.3. It is recommended to periodically back-flush the column in order to extend the lifespan and maintain an acceptable level of performance. Install the column in the reverse direction of flow, and bring the mobile phase flow rate up to 0.1 mL/min and allow to backflush overnight.

7.2.2. Column Conditioning/System Equilibration:

7.2.2.1. Install the column in the direction of flow, turn on the column oven and allow the temperature to stabilize at 65°C, then slowly bring the flow rate to 0.35 mL/min. Allow the column to equilibrate until a consistent pressure is observed.

7.2.2.2. Turn on the RI detector and allow to warm and stabilize at 40°C. It is recommended to allow the RI detector to stabilize for a few hours prior to initiating the analysis.

7.2.2.3. Purge the detector for at least 20 minutes before initiating an injection sequence.

7.2.2.3.1. Note: The 2414 Refractive Index detector's purge function must be manually disengaged prior to initiating the injection sequence.

Table 2: Injection Sequence

Sample ID	Number of Injections
System Suitability	
Diluent	≥1
LOQ	1
Resolution Solution	1
Calibration Standard	6
Samples[1]	
Diluent	1
Samples[2]	≤ 6
Calibration Standard	1
[1]Repeat the sample injection sequence if additional samples are to be analyzed. [2]Samples may be substituted with diluent injections.	

Table 3: System Suitability Parameters

System Suitability Parameter	Acceptance Criteria
%RSD of the peak area of Trehalose in the first six (6) *Calibration Standard Solution* injections	NMT 1.0%
%RSD of the peak area of Trehalose in all *Calibration Standard Solution* injections	NMT 1.0%
USP Resolution between Trehalose and Maltotriose in the *Resolution Solution* injection	NLT 1.5
USP Resolution between Trehalose and Glucose in the *Resolution Solution* injection	NLT 4
Signal to noise of the Trehalose peak in the LOQ injection	NLT 10

7.2.3. Calculations: the following equations will be calculated in the Empower software:

7.2.3.1. Maltotriose (%wt/wt, anhydrous basis) = $(R_U/R_{CS}) \times (C_{CS}/C_U) \times 100$

7.2.3.1.1. R_{CS} = Average peak area response of Maltotriose in all *Calibration Standard* injections

7.2.3.1.2. R_U = Peak area response of Maltotriose in the sample injection

7.2.3.1.3. C_{CS} = Concentration of Maltotriose in the *Calibration Standard* x Certified Purity

7.2.3.1.4. C_U = Concentration of Trehalose in the sample x 0.905

7.2.3.2. Glucose (%wt/wt, anhydrous basis) = $(R_U/R_{CS}) \times (C_{CS}/C_U) \times 100$

7.2.3.2.1. R_{CS} = Average peak area response of Glucose in all *Calibration Standard* injections

7.2.3.2.2. R_U = Peak area response of Glucose in the sample injection

7.2.3.2.3. C_{CS} = Concentration of Glucose in the *Calibration Standard* x Certified Purity

7.2.3.2.4. C_U = Concentration of Trehalose in the sample x 0.905

7.2.3.3. Unspecified impurities (%wt/wt, anhydrous basis) = $(R_U/R_{CS}) \times (C_{CS}/C_U) \times 100$

7.2.3.3.1. R_{CS} = Average peak area response of Trehalose in all *Calibration Standard* injections

7.2.3.3.2. R_U = Peak area response of any unspecified impurity in the sample injection

7.2.3.3.3. C_{CS} = Concentration Trehalose in the *Calibration Standard* x Certified Purity

7.2.3.3.4. C_U = Concentration of Trehalose in the sample x 0.905

8. VALIDATION SUMMARY:

Table 4: Summary of the validation performance parameters, acceptance criteria, and results.

Performance Parameters	Acceptance Criteria	Results
System Suitability	• %RSD of the peak areas of Trehalose in the first six (6) *Calibration Standard* injections is NMT 1.0% • %RSD of the peak areas of Trehalose in all *Calibration Standard* injections is NMT 1.0% • USP Resolution between Trehalose and Maltotriose in the Resolution Solution injection is NLT 1.5 • USP Resolution between Trehalose and Glucose in the Resolution Solution injection NLT 4 • Signal to Noise of the Trehalose peak in the LOQ injection is NLT 10	All system suitability requirements were met for each analysis
Specificity	• The USP Resolution between Trehalose and Maltotriose is NLT 1.5 in the 0.5% Level Accuracy and Precision Sample injection. • The Trehalose peak is visually resolved from diluent interference.	• USP Resolution = 1.7 • The Trehalose peak was visually resolved from diluent interference
Low-level Linearity 0.01% to 0.05%	• Report the y-intercept, slope, and residual sum of squares. • The correlation coefficient (r) is NLT 0.990. • Y-intercept bias is NMT 25.0%	• Y-intercept = -39.8 Slope = 411391 RSS = 97888 • Correlation Coefficient (r) = 0.999 • Y-intercept bias = 2.0%
Linearity 0.05% to 0.75%	• Report the y-intercept, slope, and residual sum of squares. • The correlation coefficient (r) is NLT 0.990. • Y-intercept bias is NMT 15.0%	Trehalose • Y-intercept = -208.1 Slope = 390195 RSS = 1957938 • Correlation Coefficient (r) = 1.000 • Y-intercept bias = 0.2% Maltotriose • Y-intercept = -169.1 Slope = 412325 RSS = 1340709 • Correlation Coefficient (r) = 1.000 • Y-intercept bias = 0.2% Glucose • Y-intercept = 61.5 Slope = 401006 RSS = 1942767 • Correlation Coefficient (r) = 1.000 • Y-intercept bias = 0.1%

Table 5: Summary of the validation performance parameters, acceptance criteria, and results.

Performance Parameters	Acceptance Criteria	Results	
Accuracy and Precision	• The % Recovery for each replicate at all levels for both Maltotriose and Glucose is 85%-115% • The %RSD at each level is NMT 10%	Maltotriose • %RSD 0.10% Level = 0.6 0.25% Level = 0.6 0.50% Level =0.4 0.75% Level = 0.4 • %Recoveries: 0.10% Level Replicate 1 = 91 Replicate 2 = 91 Replicate 3 = 91 0.25% Level Replicate 1 =94 Replicate 2 = 95 Replicate 3 = 95 0.50% Level Replicate 1 = 98 Replicate 2 = 98 Replicate 3 = 98 Replicate 4 = 97 Replicate 5 = 98 Replicate 6 = 98 0.75% Level Replicate 1 = 99 Replicate 2 = 98 Replicate 3 = 98	Glucose • %RSD 0.10% Level = 0.3 0.25% Level = 0.5 0.50% Level =0.3 0.75% Level = 0.3 • %Recoveries: 0.10% Level Replicate 1 = 94 Replicate 2 = 94 Replicate 3 = 94 0.25% Level Replicate 1 = 96 Replicate 2 = 96 Replicate 3 = 96 0.50% Level Replicate 1 = 99 Replicate 2 = 99 Replicate 3 = 99 Replicate 4 =98 Replicate 5 = 99 Replicate 6 = 98 0.75% Level Replicate 1 = 99 Replicate 2 = 99 Replicate 3 =99
Limit of Quantitation	• Maltotriose and Glucose: All acceptance criteria must be met for the 0.10% level from the Accuracy and Precision evaluation. • Trehalose: The %RSD of the peak areas is NMT 15% • Trehalose: The S/N for each injection is NLT 10 • Trehalose: The average %Recovery is between 75% - 125%	• Maltotriose and Glucose: All acceptance criteria were met for the 0.10% level • Trehalose %RSD = 9 • Trehalose S/N: Injection 1 = 28 Injection 2 = 28 Injection 3 = 27 Injection 4 = 29 Injection 5 = 28 Injection 6 = 27 • Trehalose average %Recovery: 99.5	
Range	• Range for specified related substances: The method should demonstrate suitable levels of precision, accuracy, and linearity from 20% to 150% of the 0.5% (wt/wt%) related substance specification • The working range of Trehalose: The method should demonstrate suitable linearity and from 33% to 167% of the 0.03% (wt/wt%) unspecified impurity specification and suitable precision at the 33% (0.01 %wt/wt) level.	• Range for specified related substances: All acceptance criteria for Accuracy and Precision, and Linearity were met. The range was established from 20% to 150% of the 0.5% (wt/wt%) related substance specification. • The working range of Trehalose: All acceptance criteria for Low-level linearity and LOQ were met. The working range was established from 33% to 167% of the 0.03% (wt/wt%) unspecified impurity specification.	

Table 6: Summary of the validation performance parameters, acceptance criteria, and results.

Performance Parameters	Acceptance Criteria	Results		
Intermediate Precision	• For Analyst 2, the % RSD of the %Recoveries is NMT 10%. • For Analyst 2, the %Recoveries for all replicates are between 85% and 115%. • The %RSD for the combined Assay values (analyst 1 + analyst 2) %RSD is NMT 15.0%.	Maltotriose		Glucose
		• %RSD = 1 • %Recoveries: Replicate 1 = 99 Replicate 2 = 97 Replicate 3 = 99 Replicate 4 = 100 Replicate 5 = 100 Replicate 6 = 99 • Combined %RSD = 1.0		• %RSD = 2 • %Recoveries: Replicate 1 = 95 Replicate 2 = 94 Replicate 3 = 95 Replicate 4 = 96 Replicate 5 = 97 Replicate 6 = 98 • Combined %RSD = 1.9
Standard Solution Stability	• The percent agreement for the aged Calibration Standard solution is between 90.0% and 110.0%.	Maltotriose	Trehalose	Glucose
		• %Agreement: Day 11 = 99.3	• %Agreement: Day 11 = 100.4	• %Agreement: Day 11= 99.8
Sample Solution Stability	• 0.50% Level: The %Recovery of the aged sample solution is between 85.0% and 115.0% • 0.50% Level: The absolute difference between Day 0 and Day X is NMT 15%	Maltotriose		Glucose
		• %Recovery: Day 0 = 97.7 Day 7 = 99.3 • Absolute Difference: Day 7 = 2%		• %Recovery: Day 0 = 98.7 Day 7 = 96.4 • Absolute Difference: Day 7 = 2%
Empower Calculation Verification	• Hand calculated results and Empower results must be identical out to 5 decimal places.	• The hand calculated results and Empower results were identical out to 5 decimal places.		

9. VALIDATION RESULTS:

9.1. System Suitability:

9.1.1. System suitability was performed on each day of analysis per the analytical method procedure in section 7. All acceptance criteria were met on each day of analysis. See Table 7 for results.

Table 7: Summary of system suitability parameters and results for each analysis

System Suitability Parameter	Results (notebook reference, date)		
	MV8P98, 07/26/22	MV9P16, 08/25/22	MV9P22, 8/30/22
%RSD of the peak area of Trehalose in the first six (6) Calibration standard injections. Criterion: NMT 1.0%	0.1%	0.4%	0.3%
%RSD of the peak area of Trehalose in all Calibration standard injections. Criterion: NMT 1.0%	0.1%	0.3%	0.3%
USP Resolution between Trehalose and Maltotriose in the Resolution *Solution* injection. Criterion: NLT 1.5	1.7	1.7	1.8
USP Resolution between Trehalose and Glucose in the Resolution *Solution* injection. Criterion: NLT 4	6	6	6
Signal to noise of the Trehalose peak in the LOQ injection Criterion: NLT 10	28[1]	26	20
[1]This value is taken from the first LOQ injection performed during the LOQ evaluation (Section 9.6).			

9.2. Specificity:

9.2.1.' Specificity was assessed visually by overlaying one diluent, LOQ, resolution solution, calibration standard, 0% Level Accuracy and Precision, and 0.5% Level Accuracy and Precision sample injections. All major analytes were visually resolved from diluent interference, and a USP resolution of 1.7 was observed between Trehalose and Maltotriose in the 0.5% Level Accuracy and Precision Sample. It's important to note that coelution was observed between Maltotriose and the unspecified impurity (RRT 0.90) in the spiked accuracy and precision samples. With a resolution of approximately 0.6, there was enough separation to utilize a perpendicular drop from the valley of the peaks to the baseline, and all Accuracy and Precision criteria were met for Maltotriose. All acceptance criteria were met for specificity. Refer to Figures 1, 2, and 3 for the representative chromatograms.

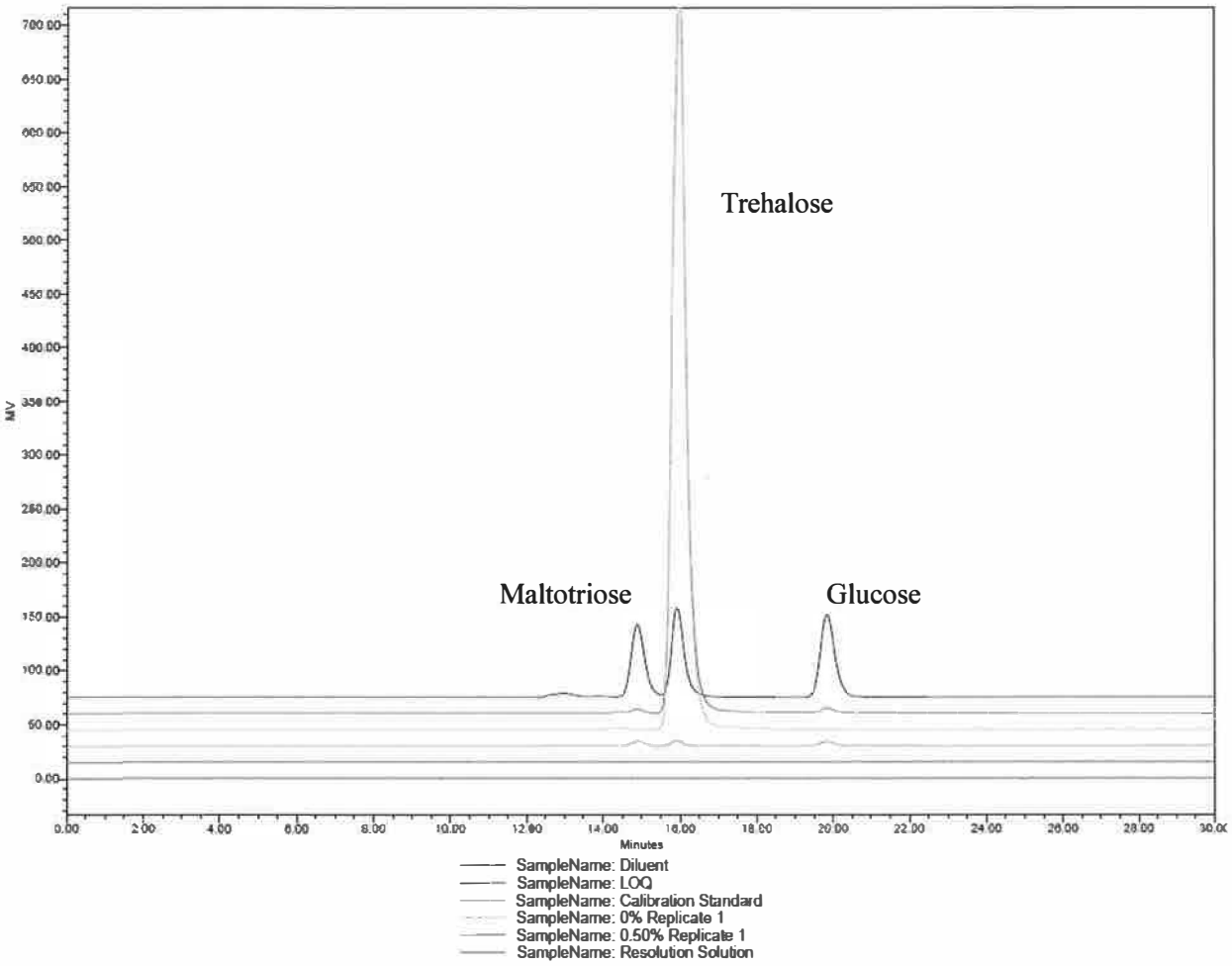

Figure 1: Specificity overlay (full view)

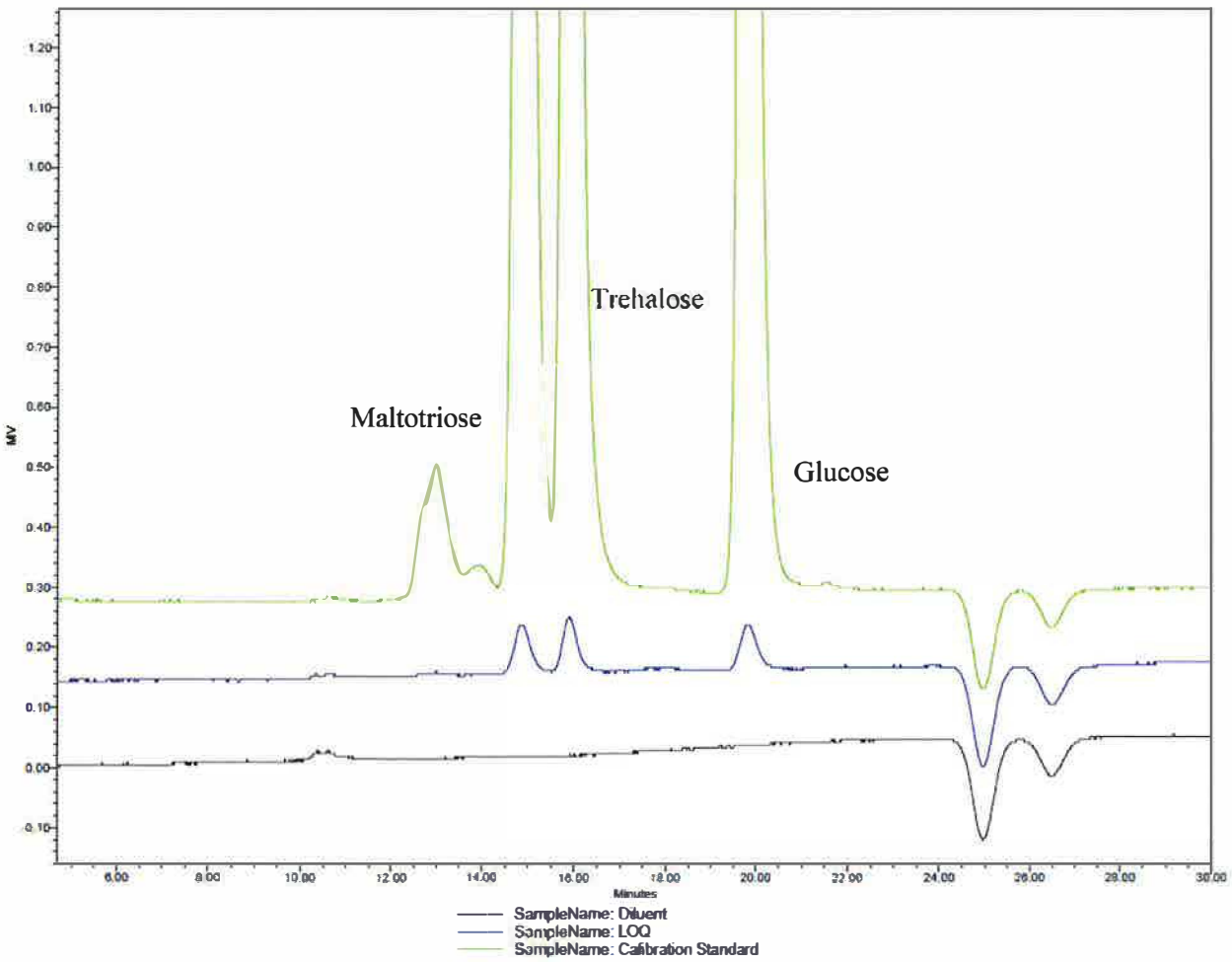

Figure 2: Specificity overlay (expanded baseline)

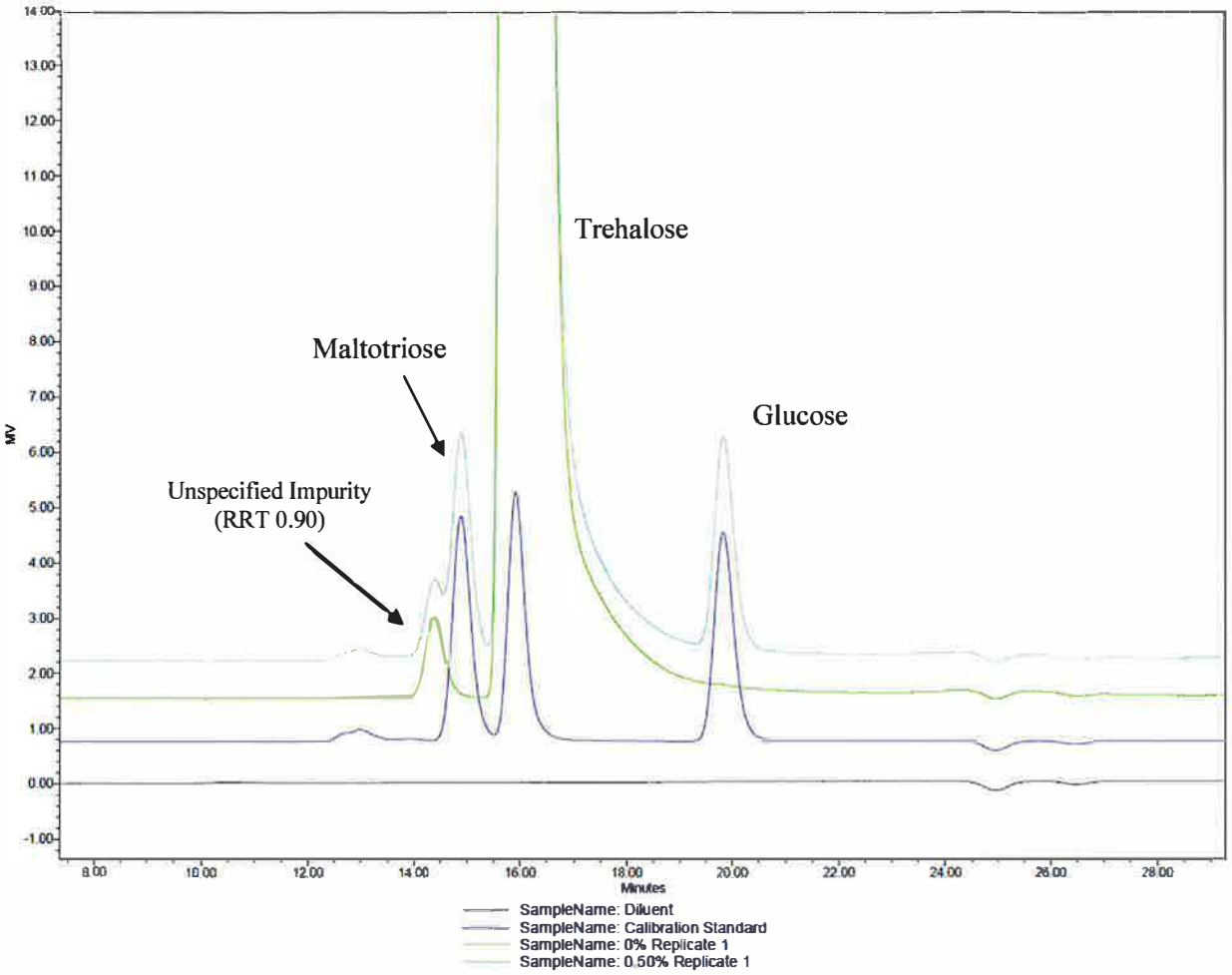

Figure 3: Specificity overlay (expanded baseline)

9.3. Accuracy and Precision (0.1% - 0.75% of the 50 mg/mL Trehalose anhydrous basis test concentration)

9.3.1. Accuracy and precision were evaluated at 20% (n = 3), 50% (n = 3), 100% (n = 6), and 150% (n = 3) of the 0.5% related substance specification. Using a stock solution, each replicate was spiked into a Trehalose authentic sample with varying levels of Maltotriose and Glucose and the Percent Recoveries were calculated. Additionally, neat Trehalose samples were prepared to assess for Glucose and Maltotriose for the Percent Recovery calculation. All acceptance criteria were met for Accuracy and Precision and the results are summarized in Tables 8, 9, and 10.

Table 8: Unspiked Trehalose blank

Trehalose Blank (0% Level)	
Analyte	Amount Measured
Maltotriose	None Detected
Glucose	None Detected

Table 9: Maltotriose Accuracy and Precision (spiked samples) Summary

Maltotriose Accuracy and Precision (20% to 150%)					
Sample Name	Amount Added[1](mg/mL)	Amount Measured (mg/mL)	% Recovery[1] (%)	Acceptance Criteria	Pass/Fail
20% Level (0.1% Trehalose test concentration)	0.0516	0.047	91	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.0516	0.047	91		
	0.0516	0.047	91		
Average		0.047	91		
%RSD		0.6			
50 % Level (0.25% Trehalose test concentration)	0.129	0.121	94	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.129	0.122	95		
	0.129	0.122	95		
Average		0.122	95		
%RSD		0.6			
100% Level (0.5% Trehalose test concentration)	0.258	0.252	98	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.258	0.253	98		
	0.258	0.252	98		
	0.258	0.250	97		
	0.258	0.252	98		
	0.258	0.252	98		
Average		0.252	98		
%RSD		0.4			
150% Level (0.75% Trehalose test concentration)	0.387	0.383	99	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.387	0.381	98		
	0.387	0.381	98		
Average		0.382	98		
%RSD		0.4			

[1]The table shows rounded values for the "Amount Added" column. %Recovery values were calculated with unrounded "Amount Added" values.

Table 10: Glucose Accuracy and Precision (spiked samples) Summary

Glucose Accuracy and Precision (20% to 150%)					
Sample Name	Amount Added[1] (mg/mL)	Amount Measured (mg/mL)	% Recovery[1] (%)	Acceptance Criteria	Pass/Fail
20% Level (0.1% Trehalose test concentration)	0.0502	0.047	94	%RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.0502	0.047	94		
	0.0502	0.047	94		
Average		0.047	94		
%RSD		0.3			
50 % Level (0.25% Trehalose test concentration)	0.126	0.120	96	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.126	0.121	96		
	0.126	0.121	96		
Average		0.121	96		
%RSD		0.5			
100% Level (0.5% Trehalose test concentration)	0.251	0.248	99	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.251	0.249	99		
	0.251	0.248	99		
	0.251	0.247	98		
	0.251	0.248	99		
	0.251	0.247	98		
Average		0.248	99		
%RSD		0.3			
150% Level (0.75% Trehalose test concentration)	0.377	0.374	99	RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.377	0.373	99		
	0.377	0.372	99		
Average		0.373	99		
%RSD		0.3			

[1]The table shows rounded values for the "Amount Added" column. %Recovery values were calculated with unrounded "Amount Added" values.

9.4. Low-level Linearity (Target Range: 0.01% - 0.05%)

9.4.1. Low-level linearity was performed from 33% to 167% of the unspecified impurity specification of 0.03%. A stock solution was prepared and sub-diluted to 5 levels ranging from 0.01% to 0.05% of the nominal sample concentration. Each solution was injected in triplicate and the response was plotted against concentration. A linear regression was performed and the % Deviation was plotted against concentration and is shown in Figure 5. Additionally, the residuals were calculated and plotted against concentration and appear to be randomly distributed as shown in Figure 6. The y-intercept bias was calculated with respect to the average peak area response at the proposed LOQ (0.01%). All acceptance criteria were met and are summarized in Table 12.

Table 11: Low-level Linearity injection summary (0.01% - 0.05%)

Level (%)	% Specification (0.03%)	Trehalose (anhydrous) Concentration (mg/mL)	Average Peak Area
0.05	167	0.0249	10198
0.04	133	0.0199	8141
0.03	100	0.0149	6151
0.02	67	0.0100	4018
0.01	33	0.0050	2018

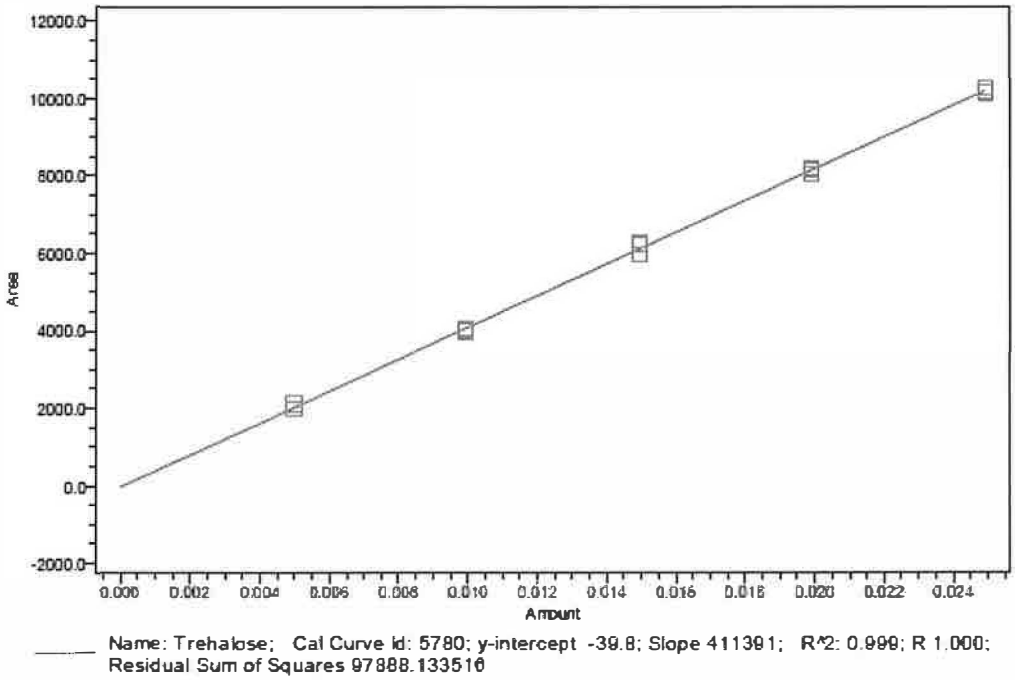

Figure 4: Trehalose response plotted against concentration (0.01% - 0.05%)

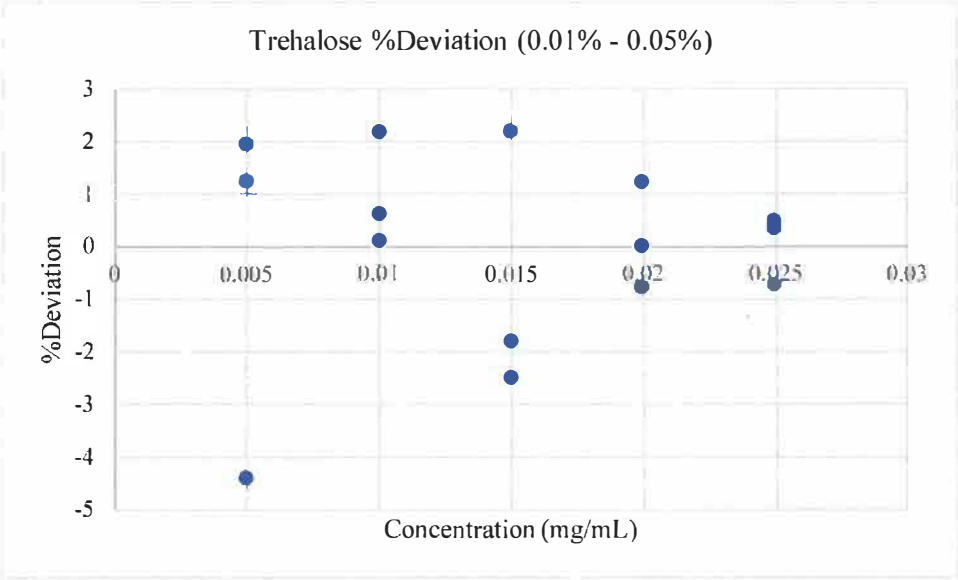

Figure 5: Trehalose Low-level Linearity - %Deviation plotted against concentration (0.01% - 0.05%)

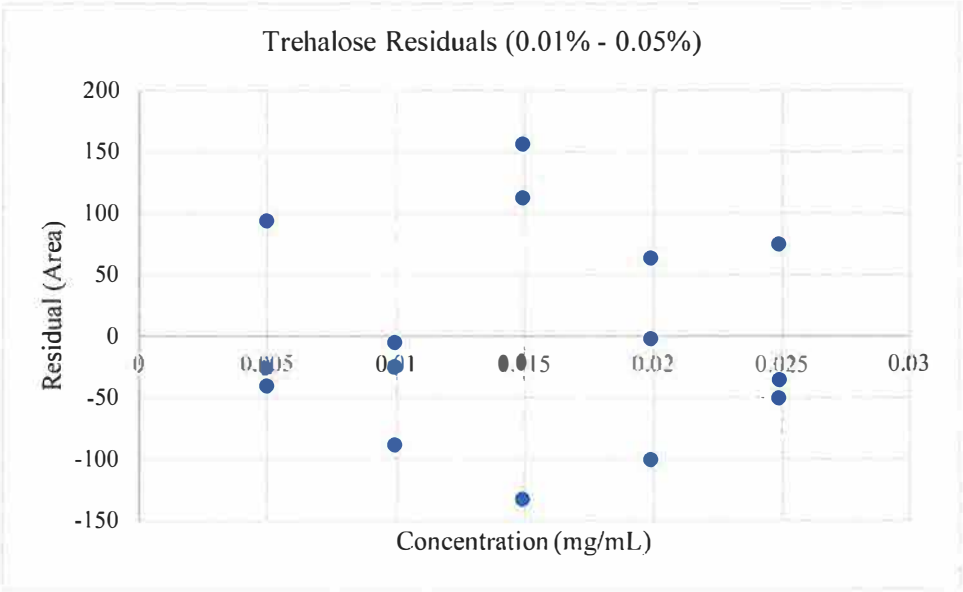

Figure 6: Trehalose Low-level Linearity - Residuals plotted against concentration (0.01% - 0.05%)

Table 12: Low-level linearity - linear regression and result summary

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	411391	Report	Not Applicable
Y-intercept	-39.8	Report	Not Applicable
Y-intercept Bias	2.0%	NMT 25.0%	Pass
Residual Sum of Squares	97888	Report	Not Applicable
R	0.999	NLT 0.990	Pass

9.5. Linearity (Target Range: 0.05% - 0.75%)

9.5.1. Linearity was evaluated for Maltotriose, Trehalose, and Glucose from approximately 10% to 150% of the 0.5% impurity specification limit. A stock solution was prepared and sub-diluted to 7 levels ranging from approximately 0.05% to 0.75% of the 50 mg/mL Trehalose sample concentration. Each solution was injected in triplicate, and the response was plotted against concentration. A linear regression was performed and the % Deviation was plotted against concentration and is shown in Figures 8, 11, and 14. Additionally, the residuals were calculated and plotted against concentration and appear to be randomly distributed as shown in Figures 9, 12, and 15. The y-intercept bias was calculated with respect to the average peak area response at 0.5% level. All acceptance criteria were met and are summarized in Tables 14, 16, and 18.

9.5.2. Trehalose Linearity

Table 13: Trehalose Linearity injection summary (0.05% - 0.81%)

Target Level (%)	Actual Level (%)	% Specification (0.5%)	Trehalose (anhydrous) Concentration (mg/mL)	Average Peak Area
0.750	0.812	162	0.406	158626
0.625	0.677	135	0.338	131594
0.500	0.542	108	0.271	105336
0.250	0.271	54	0.135	52579
0.125	0.135	27	0.068	25791
0.063	0.068	14	0.034	13140
0.050	0.054	11	0.027	10708

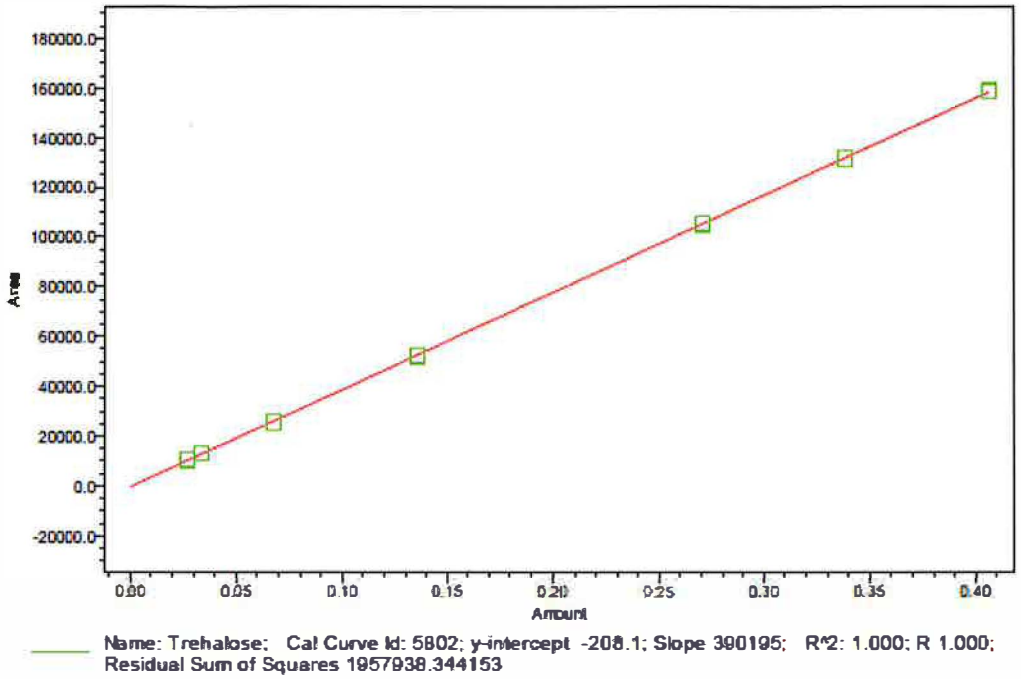

Figure 7: Trehalose response plotted against concentration (0.05% - 0.81%)

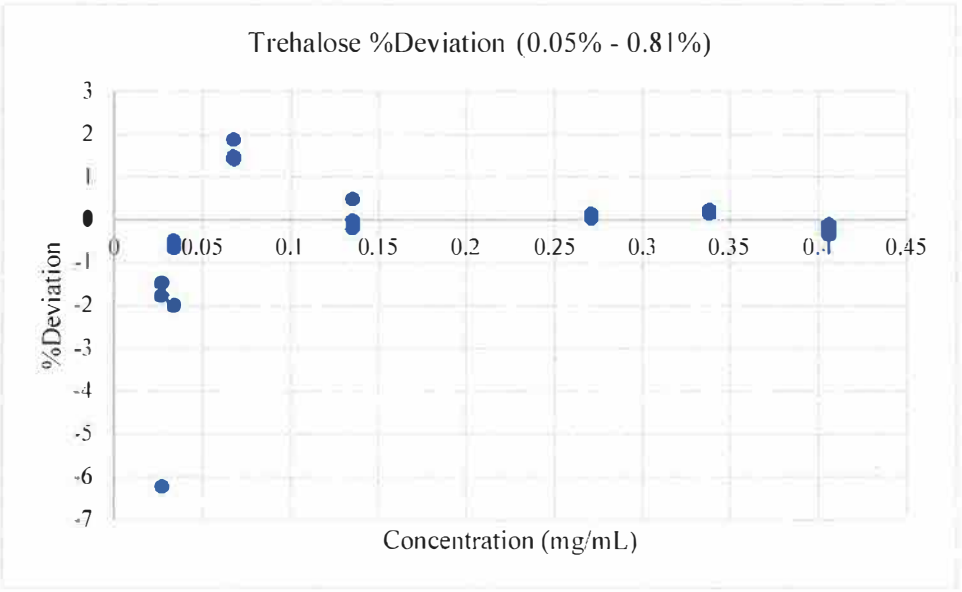

Figure 8: Trehalose Linearity - %Deviation plotted against concentration (0.05% - 0.81%)

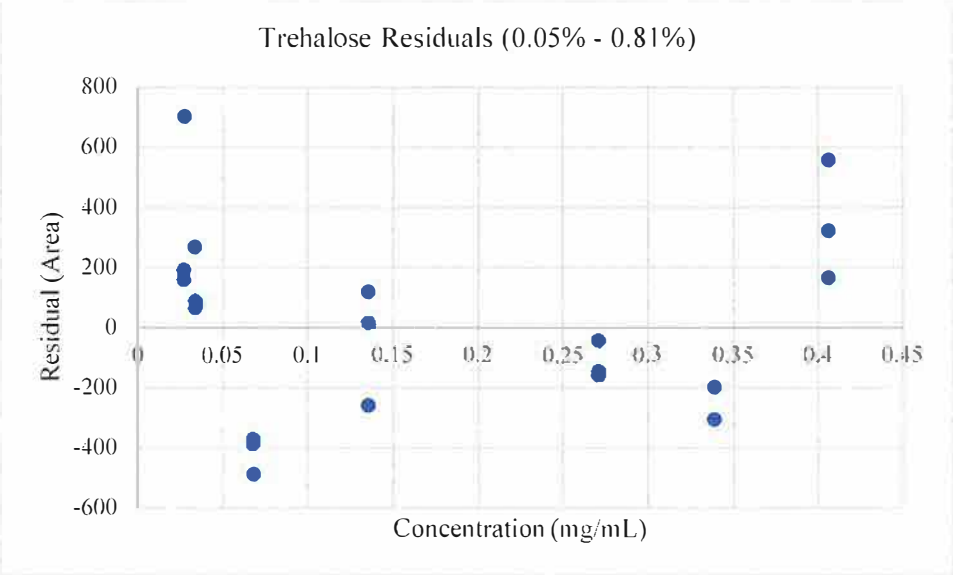

Figure 9: Trehalose Linearity – Residuals plotted against concentration (0.05% - 0.81%)

Table 14: Trehalose Linearity - linear regression and result summary

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	390195	Report	Not Applicable
Y-intercept	-208.1	Report	Not Applicable
Y-intercept Bias	0.2%	NMT 15.0%	Pass
Residual Sum of Squares	1957938	Report	Not Applicable
R	1.000	NLT 0.990	Pass

9.5.3. Maltotriose Linearity

Table 15: Maltotriose Linearity injection summary (0.05% - 0.72%)

Target Level (%)	Actual Level (%)	% Specification (0.5%)	Maltotriose Concentration (mg/mL)	Average Peak Area
0.750	0.722	144	0.361	148975
0.625	0.602	120	0.301	123647
0.500	0.481	96	0.241	99047
0.250	0.241	48	0.120	49344
0.125	0.120	24	0.060	24280
0.063	0.060	12	0.030	12414
0.050	0.048	10	0.024	10033

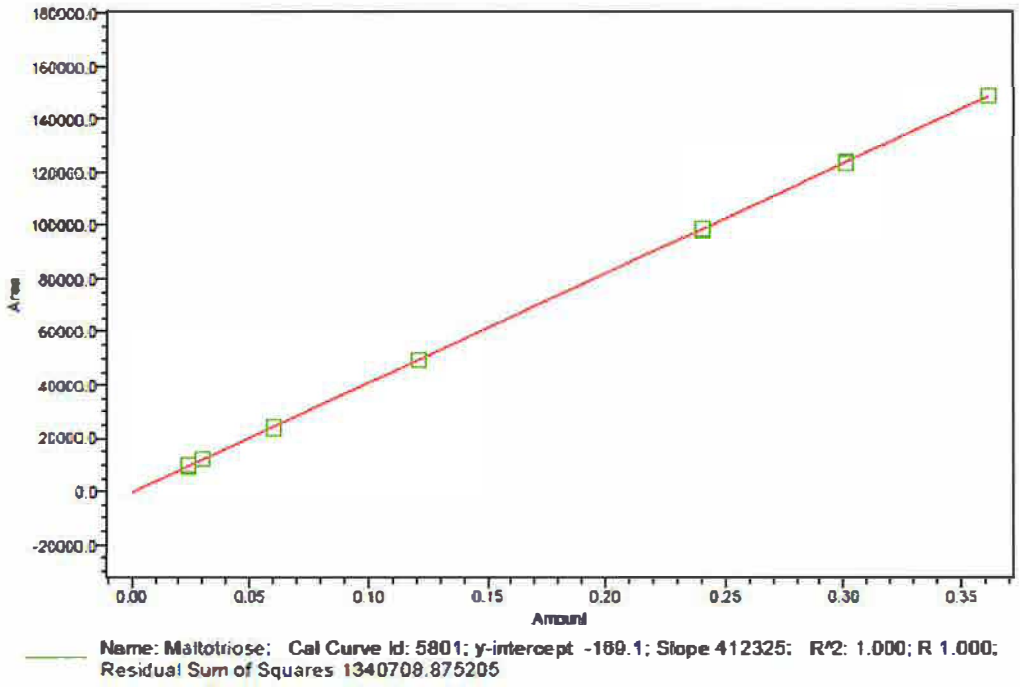

Figure 10: Maltotriose response plotted against concentration (0.05% - 0.72%)

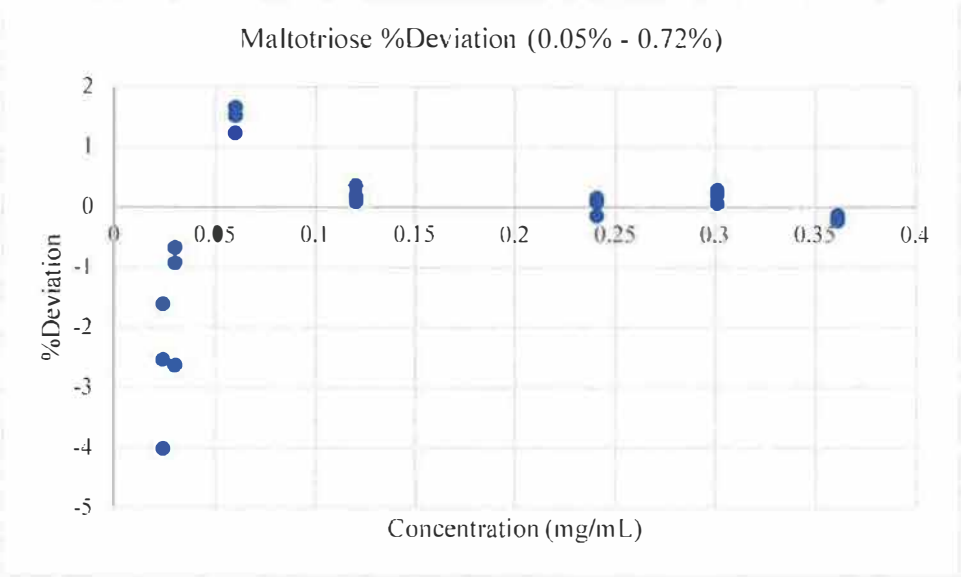

Figure 11: Maltotriose Linearity - %Deviation plotted against concentration (0.05% - 0.72%)

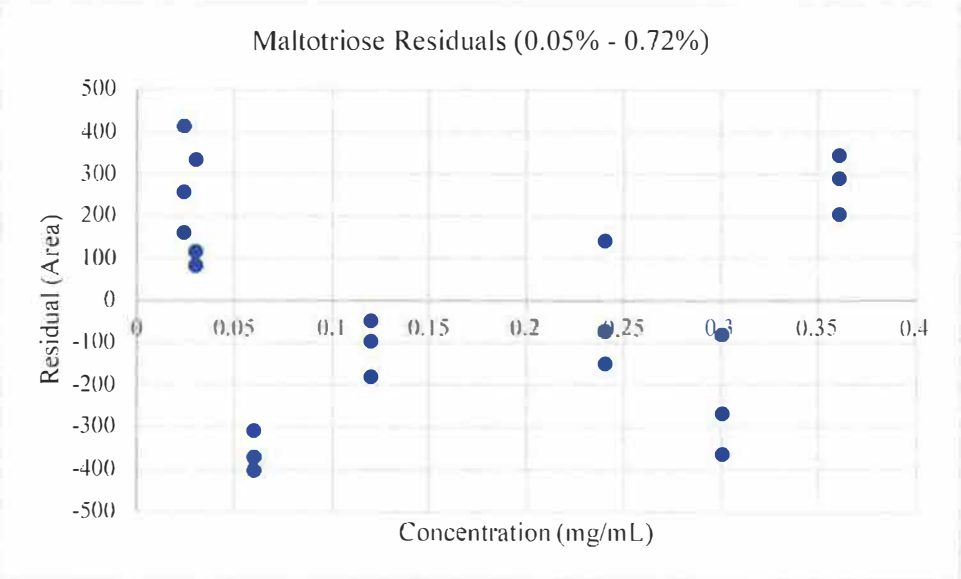

Figure 12: Maltotriose Linearity - Residuals plotted against concentration (0.05% - 0.72%)

Table 16: Maltotriose Linearity - linear regression and result summary

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	412325	Report	Not Applicable
Y-intercept	-169.1	Report	Not Applicable
Y-intercept Bias	0.2%	NMT 15.0%	Pass
Residual Sum of Squares	1340709	Report	Not Applicable
R	1.000	NLT 0.990	Pass

9.5.4. Glucose Linearity

Table 17: Glucose Linearity injection summary (0.05% - 0.75%)

Target Level (%)	Actual Level (%)	% Specification (0.5%)	Glucose Concentration (mg/mL)	Average Peak Area
0.750	0.752	150	0.376	151170
0.625	0.626	125	0.313	125231
0.500	0.501	100	0.251	100603
0.250	0.251	50	0.125	50017
0.125	0.125	25	0.063	24950
0.063	0.063	13	0.031	12864
0.050	0.050	10	0.025	10325

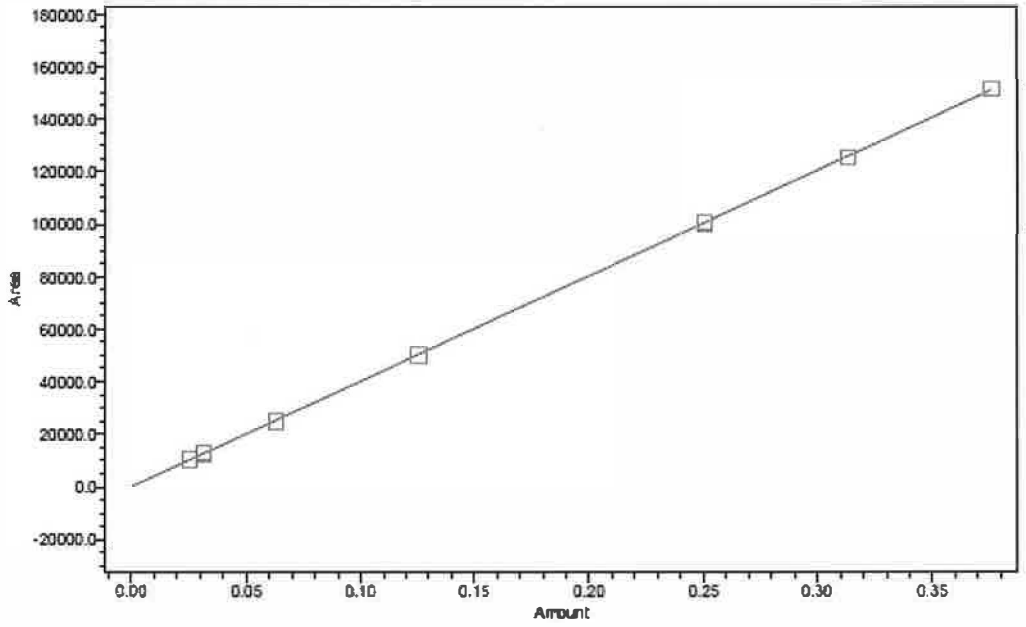

Figure 13: Glucose response plotted against concentration (0.05% - 0.75%)

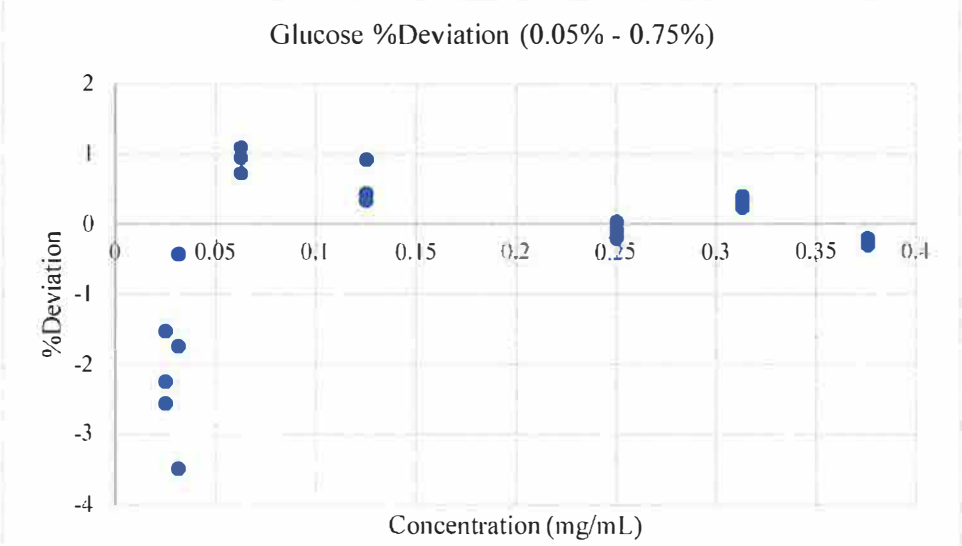

Figure 14: Glucose Linearity - %Deviation plotted against concentration (0.05% - 0.75%)

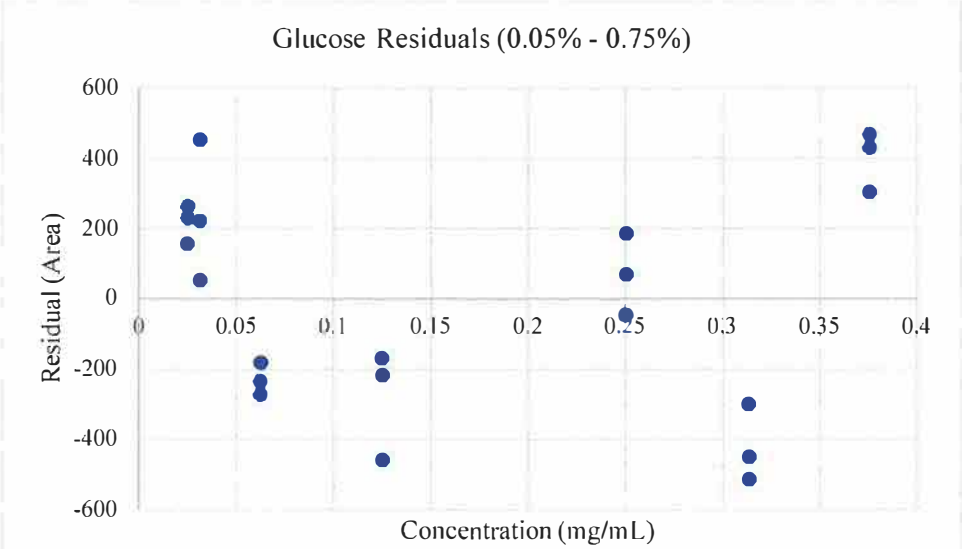

Figure 15: Glucose Linearity - Residuals plotted against concentration (0.05% - 0.75%)

Table 18: Glucose Linearity - linear regression and result summary

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	401006	Report	Not Applicable
Y-intercept	61.5	Report	Not Applicable
Y-intercept Bias	0.1%	NMT 15.0%	Pass
Residual Sum of Squares	1942767	Report	Report
R	1.000	NLT 0.990	Pass

9.6. Limit of Quantitation

9.6.1. An LOQ solution from Section 7.1.5 was prepared and six (6) injections were performed. The %RSD of peak area, USP S/N, and % Recoveries were calculated for the Trehalose peak. The LOQ (reporting limit) for Maltotriose and Glucose was assessed in section 9.3 Accuracy and Precision. All acceptance criteria were met and are summarized in Table 19.

Table 19: Trehalose LOQ injection summary (0.01%)

LOQ (0.01%)					
Sample	USP S/N	Area	%Recoveries	Acceptance Criteria	Pass/Fail
LOQ (0.005 mg/mL)	28	2079	98.4	%RSD of the peak areas is NMT 15% The USP S/N for each injection is NLT 10 The average %Recovery is between 75% - 125%	Pass
	28	2001	94.7		
	27	1931	91.4		
	29	2310	109.3		
	28	2374	112.3		
	27	1921	90.9		
Mean		2103	99.5		
%RSD		9			

9.7. Intermediate Precision

9.7.1. A different analyst (Analyst 2) repeated the accuracy and precision evaluation at the 0.50% Level (n = 6) described in Section 9.3. The analysis was performed on a different day with a different column, separately prepared diluent, mobile phase, and standard solutions. The %Recoveries were calculated, combined with analyst 1, and the %RSD of the %Recovery values was calculated. All acceptance criteria were met and are summarized in Tables 20 and 21.

Table 20: Maltotriose - Intermediate Precision (spiked samples) Summary

Maltotriose Intermediate Precision					
Sample Name	Amount Added[1] (mg/mL)	Amount Measured (mg/mL)	% Recovery[1]	Acceptance Criteria	Pass/Fail
0.50% Level (Analyst 2)	0.258	0.255	99	%RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.258	0.251	97		
	0.258	0.254	98		
	0.258	0.257	100		
	0.258	0.257	100		
	0.258	0.256	99		
Average		0.255	99		
%RSD		1.0			
0.50% Level (Analyst 1)	0.258	0.252	98	The combined %RSD of the %Recovery values is NMT 15.0%	Pass
	0.258	0.253	98		
	0.258	0.252	98		
	0.258	0.250	97		
	0.258	0.252	98		
	0.258	0.252	98		
(Analyst 1+2) Combined Average			98		
(Analyst 1+2) Combined %RSD			1.0		

[1]The table shows rounded values for the "Amount Added" column. %Recovery values were calculated with unrounded "Amount Added" values.

Table 21: Glucose - Intermediate Precision (spiked samples) Summary

Glucose Intermediate Precision					
Sample Name	Amount Added[1] (mg/mL)	Amount Measured (mg/mL)	% Recovery[1]	Acceptance Criteria	Pass/Fail
0.50% Level (Analyst 2)	0.251	0.240	95	%RSD is NMT 10% %Recovery for each replicate is 85% - 115%	Pass
	0.251	0.236	94		
	0.251	0.238	95		
	0.251	0.242	96		
	0.251	0.243	97		
	0.251	0.241	98		
Average		0.240	95		
%RSD		1			
0.50% Level (Analyst 1)	0.251	0.248	99	The combined %RSD of the %Recovery values is NMT 15.0%	Pass
	0.251	0.249	99		
	0.251	0.248	99		
	0.251	0.247	98		
	0.251	0.248	99		
	0.251	0.247	98		
(Analyst 1+2) Combined Average			97		
(Analyst 1+2) Combined %RSD			1.9		

[1]The table shows rounded values for the "Amount Added" column. %Recovery values were calculated with unrounded "Amount Added" values.

9.8. Solution Stability:

9.8.1. A 0.5% Level Accuracy and Precision spiked sample and calibration standard solution were stored in the original glassware at normal lighting and laboratory conditions. These solutions were analyzed on day 7 and day 11, respectively, against freshly prepared standards. For the spiked sample, the absolute difference in %Recovery values for Maltotriose and Glucose was calculated against day 0. The % agreement of the calibration standard was calculated against freshly prepared standards on day 11. All acceptance criteria were met and are summarized in Tables 22 and 23.

9.8.1.1. The Calibration Standard Solution is stable for 11 days when stored stoppered in clear glassware at normal laboratory conditions.

9.8.1.2. The Sample Test Solutions are stable for 7 days when stored stoppered in clear glassware at normal laboratory conditions.

Table 22: Calibration Standard solution stability summary

Calibration Standard Solution Stability					
Timepoint (day)	% Agreement			Acceptance Criteria	Pass/Fail
	Maltotriose	Trehalose	Glucose		
11	99.3%	100.4%	99.8%	The % Agreement is between 90.0% and 110.0%	Pass

Table 23: Sample Solution Stability summary

Sample Solution Stability						
Timepoint (day)	%Recovery		Absolute Difference		Acceptance Criteria	Pass/Fail
	Maltotriose	Glucose	Maltotriose	Glucose		
0	97.7%	98.7%			• The % Recovery of the aged solution is between 85.0% and 115.0% • The absolute difference between Day 0 and Day X and aged solution is NMT 15%	NA
7	99.3%	96.4%	2%	2%		Pass

9.9. Empower Custom Fields Verification:

9.9.1. All results for Intermediate Precision were hand calculated and compared to calculations performed by Empower. The acceptance criterion was met for all results. The results are summarized in Table 24.

Table 24: %wt/wt anhydrous basis custom field verification summary

Related Substances (%wt/wt, anhydrous basis) Custom Field Verification					
Maltotriose		Glucose			
Empower Calculated Result (%)	Hand Calculated Result (%)	Empower Calculated Result (%)	Hand Calculated Result (%)	Acceptance Criterion	Pass/Fail
0.51289	0.51289	0.48178	0.48178	Hand calculated results and Empower results must be identical out to 5 decimal places	Pass
0.50389	0.50389	0.47416	0.47416		
0.51010	0.51010	0.47786	0.47786		
0.51741	0.51741	0.48683	0.48683		
0.51692	0.51692	0.48891	0.48891		
0.51444	0.51444	0.48455	0.48455		

10. VALIDATION STATUS:

10.1. The method "Determination of Related Substances for Trehalose by HPLC with RI Detection" is considered validated and suitable for use at the BioSpectra Bangor, PA facility. All acceptance criteria for System Suitability, Accuracy and Precision, Intermediate Precision, Specificity, Low-level linearity, Linearity, and LOQ were met. For the specified related substances, the range was established from 20% to 150% of the 0.50% (wt/wt%) related substance specification. Standards and Samples are considered stable for 11 days and 7 days, respectively, when stored stoppered in clear glassware at normal laboratory conditions.

10.2. Critical Changes, Discrepancies, or Failures

10.2.1. Typographical error in "Intermediate Precision" of BSI-PRL-0566 Revision 1.0.

10.2.1.1. Section 8.7.4.2 states: The combined assay anhydrous basis (analyst 1 + analyst 2) %RSD is NMT 15.0%.

10.2.1.2. This acceptance criterion should state: the combined **%Recovery** (analyst 1 + analyst 2) %RSD is NMT 15.0%.

10.2.1.2.1. This change is justified as assay on the anhydrous basis is not calculated in this protocol, and Section 8.7.2 instructs the analyst to calculate %Recovery and combine with the values obtained by Analyst 1.

10.2.2. Exclusion of Range in the Analytical Method Validation Protocol (BSI-PRL-0566 v. 1.0).

10.2.2.1. Range was excluded from Section 8 Validation Procedure in the Analytical Method Validation Protocol (BSI-PRL-0566 v. 1.0).

10.2.2.2. Range is included in Section 8 Validation Summary of the Analytical Method Validation Report. Acceptance Criteria are outlined in Section 8. For specified related substances the method should demonstrate suitable levels of precision, accuracy, and linearity from 20% to 150% of the 0.5% (wt/wt%) related substance specification. For Trehalose the method should demonstrate suitable linearity and from 33% to 167% of the 0.03% (wt/wt%) unspecified impurity specification and suitable precision at the 33% (0.01 %wt/wt) level.

All acceptance criteria were met for range. For specified related substances the range was established from 20% to 150% of the 0.5% (wt/wt%) related substances specification. For Trehalose the range was established from 33% to 167% of the 0.03% (wt/wt%) unspecified impurity specification.

10.2.3. MV8P98 – Linearity

10.2.3.1. **Discrepancy Description –** The amount of Trehalose and Maltotriose weighed out for the Linearity stock solution was outside the specified weight range in the protocol. The protocol states to weigh and transfer 150 mg (±5%) of Maltotriose and 140 mg (±5%) of Trehalose for the Impurity stock solution. The analyst inadvertently weighed ~150 mg of Trehalose and ~140 mg of Maltotriose, and both samples were outside the ±5% tolerance.

The final concentrations of Maltotriose in the linearity solutions were calculated to be within 4% of the target concentration (see Table 15). The resulting concentration range assessed for Maltotriose was from 10% to 144% of the 0.5% known Impurity specification, which exceeded the upper 120% range requirement specified in ICH Q2.

Additionally, the established range was further demonstrated by successfully completing Accuracy and Precision from 20% (reporting limit) to 150% of the 0.50% specification.

The concentration of the Trehalose linearity solutions were ~8% higher than the target concentrations (see Table 13), however, Low-level linearity was also carried out for Trehalose. Considering both linearity evaluations, a working range of~2% to ~162% of the 0.50% related substance specification was evaluated, which exceeded the required minimum range specified in ICH Q2.

10.2.3.2. **Conclusion and Resolution** –The data was considered valid as the range established for Maltotriose exceeds the ICH requirements. The linearity range for Trehalose was sufficient and justify its use as a calibration standard for the quantitation of unknown impurities.

10.2.4. MV9P03 – Accuracy and Precision

10.2.4.1. **Discrepancy Description** – The LOQ solution was not prepared or injected, therefore system suitability was not fully assessed for the Accuracy and Precision evaluation. Section 7.2.5 of the protocol states that a signal to noise ration of the Trehalose peak in the LOQ injection must be NLT 10, however, the LOQ injection was inadvertently omitted from the injection sequence outlined in Section 7.2.4 of protocol. As such the analyst did not prepare and inject the LOQ solution.

10.2.4.2. **Conclusion and Resolution** – Since system suitability was not adequately assessed, all results were considered invalid and the analysis was reperformed. All subsequent notebook write-ups included the LOQ injection in the correct injection order as shown in Table 2.

10.2.5. MV9P08- Accuracy and Precision

10.2.5.1. **Discrepancy Description** – The RI detector purge valve was inadvertently left open for the duration of the injection sequence. The open purge valve resulted in large negative peaks as the sample solutions continuously flowed through both the sample flow cell and reference cell. It should be noted that the 2414 Refractive Index detector's purging function must be manually engaged and disengaged for the detector to function correctly. A precautionary note was added to Section 7.2.2.3.

10.2.5.2. **Conclusion and Resolution** –The data was considered invalid as the chromatograms generated contain no viable data. The analysis was repeated.

10.3. Laboratory Notebook References

10.3.1. MV8P98 – MV9P02: Linearity and Low-level Linearity

10.3.2. MV9P03 – 07: Accuracy and Precision (System Suitability Failure)

10.3.3. MV9P08 – 12: Accuracy and Precision (System Suitability Failure)

10.3.4. MV9P16 – 21: Accuracy and Precision, Solution Stability

10.3.5. MV9P22 – 27: Intermediate Precision, Solution Stability

10.3.6. MV10P01 – 02: Empower Custom Field Verification