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TREHALOSE ASSAY BY HPLC WITH RI DETECTION

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1. PURPOSE:

1.1. To provide Quality Control (QC) Analysts with a procedure for the determination of Trehalose Assay by HPLC with RI detection.

2. SCOPE:

2.1. This analytical method applies to the Trehalose Assay by HPLC with RI detection procedure on the Waters Alliance HPLC.

2.2. The analytical procedure described in this method is a modified version of the assay procedure outlined in the Trehalose Hydrate JP monograph.

2.3. Assay specification: 98.0% – 101.0%

3. RESPONSIBILITIES:

3.1. The Director of Laboratory Systems, Laboratory Analysts, and/or the QC Manager, if necessary, are responsible for the control, training, implementation and maintenance of this procedure.

3.2. The Laboratory analysts and/or qualified designee are responsible for performing the testing as stated in this procedure.

3.3. The Laboratory analysts performing this procedure, with help and training from the Laboratory Technology Manager, are responsible for documenting the results obtained from testing.

3.4. Safety: Standard laboratory safety regulations apply. Before working with any chemical, read and understand the Safety Data Sheet (SDS).

4. REFERENCES:

4.1. BSI-PRL-0562, Analytical Method Validation Protocol: Trehalose Assay via Liquid Chromatography with RI detection

4.2. BSI-RPT-1107, Analytical Method Validation Report: Trehalose Assay via Liquid Chromatography with RI detection

4.3. BSI-SOP-0098, Balance SOP

4.4. BSI-SOP-0126, Laboratory Notebooks

4.5. BSI-SOP-0134, Pipette SOP

4.6. USP-NF Current

4.7. *JP <2.01> Liquid Chromatography*

4.8. *Trehalose Hydrate JP Monograph*

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

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

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

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

5. MATERIALS AND EQUIPMENT:

- 5.1. Analytical Balance
- 5.2. Weighing supplies: Weighing boats/funnels and spatulas
- 5.3. Liquid Chromatograph
  - 5.3.1. Waters Alliance HPLC with RI Detector
- 5.4. Reagents
  - 5.4.1. HPLC grade Water or equivalent
- 5.5. Supplies
  - 5.5.1. Class A Volumetric Flasks.
  - 5.5.2. Polypropylene transfer funnels or aluminum weighing boats
  - 5.5.3. Analytical Balance
  - 5.5.4. HPLC auto sampler vials and caps
  - 5.5.5. Micropipettes
  - 5.5.6. Micropipette Tips
  - 5.5.7. Transfer pipettes
- 5.6. Reference Standards
  - 5.6.1. USP Traceable Maltotriose (Dextrose Impurity C)
  - 5.6.2. USP Traceable Glucose (Dextrose)
  - 5.6.3. USP Trehalose Primary Reference
    - 5.6.3.1. Alternatively, an in-house Trehalose secondary reference standard may be used.
- 5.7. LC Column:
  - 5.7.1. Rezex RNM-Carbohydrate Na+ (8%) 7.8mm x 300mm, 8µm
  - 5.7.2. Part number: 00H-0136-K0

6. PROCEDURE:

6.1. Solution Preparation:

6.1.1. All solutions may be scaled as needed.

6.1.2. Diluent: Water

6.1.3. Mobile phase: Water

6.1.4. Needle Wash: Water

6.1.5. Resolution Solution (5.0 mg/mL Maltotriose, 5.0 mg/mL Glucose, 5.0 mg/mL Trehalose)

6.1.5.1. Weigh and transfer 50 mg (±10%) each of Maltotriose, Glucose, and Trehalose reference standards into a 10 mL volumetric flask.

6.1.5.2. Fill ~3/4 full with diluent and swirl to dissolve.

6.1.5.3. Fill to volume with diluent.

6.1.5.4. Mix by Inversion.

6.1.6. Assay Standard Solutions (10 mg/mL Trehalose anhydrous basis, duplicate)

6.1.6.1. Weigh and transfer 110 mg (±5%) Trehalose reference standard into a 10 mL volumetric flask.

6.1.6.2. Fill ~3/4 full with diluent and swirl to dissolve.

6.1.6.3. Fill to volume with diluent.

6.1.6.4. Mix by inversion.

6.1.6.5. Prepare in duplicate.

6.1.6.6. Label SS1 and SS2, respectively.

6.1.6.7. Stability: Six (6) days when stored, sealed with a fitted stopper, in clear glassware, at normal laboratory conditions.

6.1.7. Test Samples (10 mg/mL Trehalose anhydrous basis, duplicate)

6.1.7.1. Weigh and transfer 110 mg (±5%) Trehalose Hydrate into a 10 mL volumetric flask.

6.1.7.2. Fill ~3/4 full with diluent and swirl to dissolve.

6.1.7.3. Fill to volume with diluent.

6.1.7.4. Mix by inversion.

6.1.7.5. Prepare in duplicate for Finished Good products only.

6.1.7.5.1. Mother liquor, wet crystal, and raw material samples only need single replicate.

6.1.7.6. Perform a single injection for each replicate.

6.1.7.7. Stability: Six (6) days when stored, sealed with a fitted stopper, in clear glassware, at normal laboratory conditions.

6.2. Instrument Setup

6.2.1. 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	25µL
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

6.2.2. Column Care:

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

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

6.2.2.3. 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 0.1mL/min and allow to backflush overnight.

6.2.3. Column Conditioning/System Equilibration:

6.2.3.1. 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.

6.2.3.2. 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.

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

6.2.4. Injection Sequence:

Sample ID	Number of Injections
System Suitability	
Diluent	≥ 1
Resolution Solution	1
SS1	6
SS2	1
Samples[1]	
Diluent	1
Samples[2]	≤ 6
SS1	1
[1]Repeat the sample injection sequence if additional samples are to be analyzed. [2]Samples may be substituted with diluent injections.	

6.2.5. System Suitability:

System Suitability Parameter	Acceptance Criteria
%RSD of the peak area of Trehalose in the first six (6) SS1 injections.	NMT 0.42%
%RSD of the peak area of Trehalose in all SS1 injections.	NMT 0.42%
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
Standard %Agreement between the first six (6) SS1 injections and the SS2 injection	99.0% - 101.0%

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

6.2.6.1. Percent Agreement = $(R_{SS2}/R_{SS1}) \times (C_{SS1}/C_{SS2}) \times 100$

6.2.6.1.1. R_{SS1} = Average peak area of Trehalose from the first six (6) SS1 injections

6.2.6.1.2. R_{SS2} = Peak area of Trehalose from the SS2 injection

6.2.6.1.3. C_{SS1} = Concentration of SS1 x Certified Purity

6.2.6.1.4. C_{SS2} = Concentration of SS2 x Certified Purity

6.2.6.2. Assay Anhydrous Basis (%wt/wt) = $(R_U/R_{SS1}) \times (C_{SS1}/C_U) \times 100$

6.2.6.2.1. R_{SS1} = Average peak area of Trehalose from all SS1 injections

6.2.6.2.2. R_U = Peak area of Trehalose from the sample injection

6.2.6.2.3. C_{SS1} = Concentration of SS1 x Certified Purity

6.2.6.2.4. C_U = Concentration of the sample x 0.905

6.2.6.2.5. 0.905 = Hydrate correction factor (molar ratio between the anhydrous and dihydrate forms of Trehalose)

6.3. Reporting

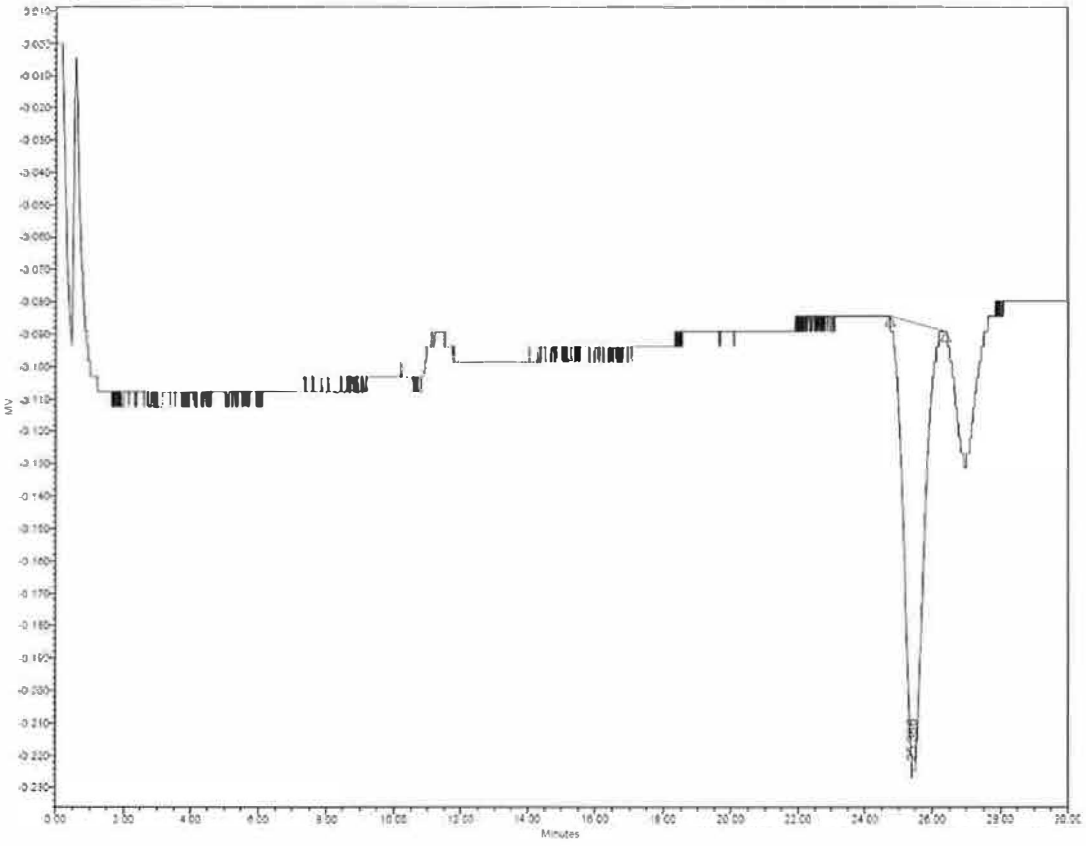
6.3.1. **Assay**: Calculate Assay on the Anhydrous Basis for both replicates and report the average to 1 (one) decimal place.

6.3.1.1. If any replicate has a result that is OOS, an OOS checklist will be issued to evaluate further.

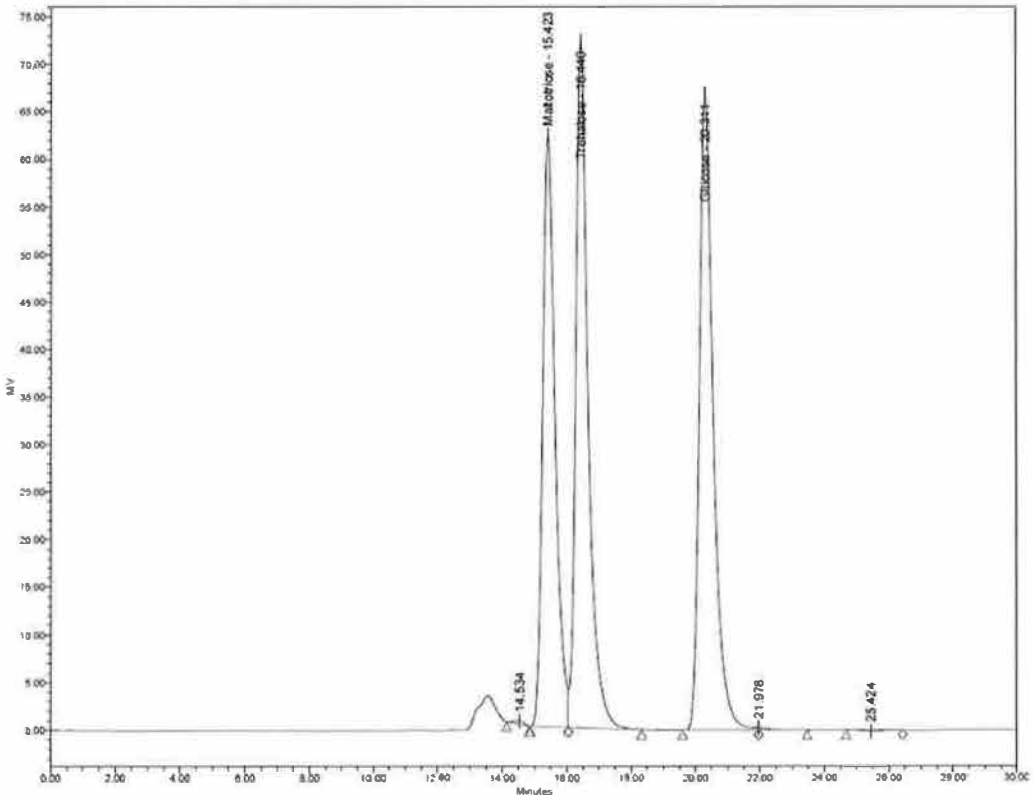
6.3.1.2. If the replicates vary by more than ±2%, no results will be averaged or reported until evaluated by the QC manager to determine if the results are valid/reportable or if any further action is required.

6.4. Example Chromatograms and Integrations

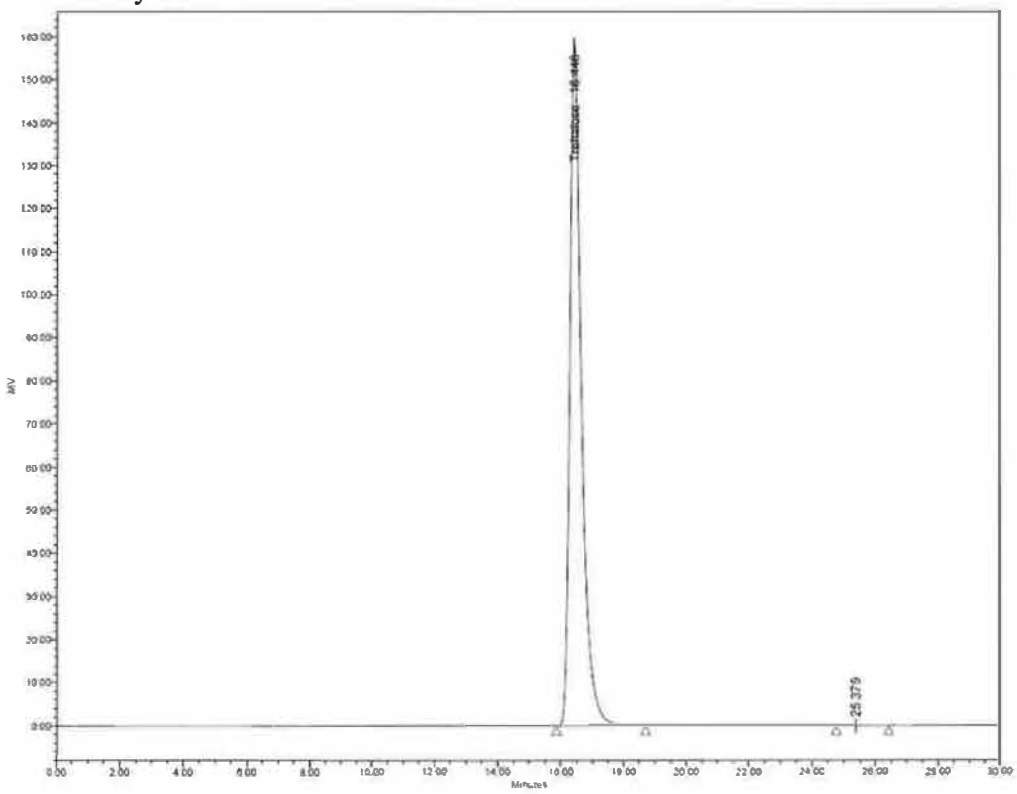
6.4.1. Diluent:



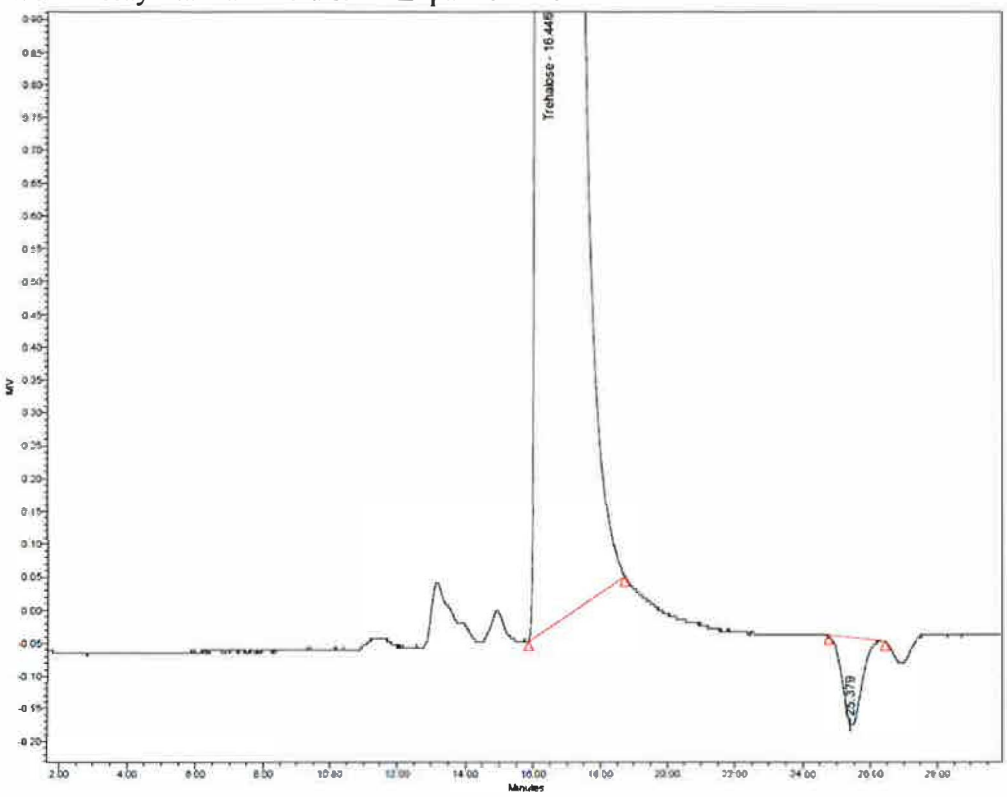
6.4.2. Resolution Solution – Full View



6.4.3. Assay Standard Solution – Full View



6.4.4. Assay Standard Solution – Expanded View



6.5. Example Integration Parameters for Empower software

6.5.1. Ensure integrations for samples and standards are similar enough for accurate quantitation

6.5.2. Integration parameters may be adjusted in order to achieve similar integrations as shown in Section 6.4.