BIOSPECTRA
100 Majestic Way, Bangor, PA 18013 / www.biospectra.us

DETERMINATION OF RELATED SUBSTANCES FOR TREHALOSE BY HPLC WITH RI DETECTION

TABLE OF CONTENTS

1. PURPOSE:

1.1. To provide Analysts and other qualified personnel with a procedure to quantify the related substances of Trehalose Dihydrate by liquid chromatography with RI detection.

2. SCOPE:

2.1. This analytical method 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.

3. RESPONSIBILITIES:

3.1. The Director of Laboratory Services, or designee, is responsible for the control, training, implementation and maintenance of this procedure.

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

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

4. REFERENCE:

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

4.2. BSI-RPT-1106, Analytical Method Validation Report: Determination of Related Substances for Trehalose by HPLC with RI Detection

4.3. BSI-SOP-0098, Balance SOP

4.4. BSI-SOP-0134, Pipette SOP

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

4.6. *JP <2.01> Liquid Chromatography*

4.7. *Trehalose Hydrate JP Monograph*

4.8. *USP <621> Chromatography*

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

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

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

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

5. MATERIALS AND EQUIPMENT:

- 5.1. Analytical Balance
- 5.2. Analytical Microbalance
- 5.3. Waters Alliance HPLC
  - 5.3.1. 30cm Column Compartment
  - 5.3.2. e2695 Separations Module
  - 5.3.3. 2414 Refractive Index Detector
- 5.4. Reagents
  - 5.4.1. HPLC Grade Water
- 5.5. Supplies
  - 5.5.1. Disposable Polypropylene Weighing Funnels
  - 5.5.2. Micropipettes
  - 5.5.3. Micropipette Tips
  - 5.5.4. Screw top glass autosampler vials and caps
- 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 (dihydrate)
- 5.7. LC Column
  - 5.7.1. Rezex RNM-Carbohydrate Na+ (8%) 7.8mm x 300mm, 8µm
  - 5.7.2. Supplier: Phenomenex
  - 5.7.3. Part number: 00H-0136-K0

6. PROCEDURE:

6.1. Solution Preparation

6.1.1. Note: Ensure the amounts to be weighed are NLT the minimum weight tolerance of the balance. Solutions may be scaled as needed.

6.1.2. Diluent – Mobile Phase – Needle Wash: HPLC grade water

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

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

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

6.1.3.3. Fill to volume with diluent.

6.1.3.4. Mix by Inversion.

6.1.4. Calibration Standard Solution (0.25 mg/mL Trehalose, 0.25 mg/mL Maltotriose, 0.25 mg/mL Glucose)

6.1.4.1. Weigh and transfer 28 mg (±10%) Trehalose, 30 mg (±10%) Maltotriose, and 25 mg (±10%) Glucose reference standards into a 100 mL volumetric flask.

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

6.1.4.3. Fill to volume with diluent.

6.1.4.4. Mix by inversion.

6.1.4.4.1. 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.

6.1.4.5. Solution is stable for 11 days at normal laboratory conditions.

6.1.5. LOQ Solution: (0.005 mg/mL Trehalose, 0.005 mg/mL Maltotriose, 0.005 mg/mL Glucose)

6.1.5.1. Pipette 1.0 mL of the calibration standard solution into a 50 mL volumetric flask.

6.1.5.2. Fill to volume diluent.

6.1.5.3. Mix by inversion.

6.1.5.4. Prepare fresh.

6.1.6. Test Samples (50 mg/mL Trehalose anhydrous basis)

6.1.6.1. Weigh 550 mg (±5%) Trehalose Hydrate into a 10 mL volumetric flask.

6.1.6.2. Fill ~3/4 full with diluent.

6.1.6.3. With occasional swirling, allow the solids to fully dissolve.

6.1.6.4. Fill to volume with diluent.

6.1.6.5. Mix by inversion.

6.1.6.6. Perform a single injection.

6.1.6.7. Sample solutions are stable for 7 days at normal laboratory conditions.

6.2. 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

6.2.1. Column Care:

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

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

6.2.1.3. 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 0.1mL/min and allow to backflush overnight.

6.2.2. Column Conditioning/System Equilibration:

6.2.2.1. 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.

6.2.2.2. 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.

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

6.2.2.3.1. 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

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

6.2.3.1. Maltotriose (%wt/wt, anhydrous basis) = (R_U/R_{CS}) x (C_{CS}/C_U) x 100

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

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

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

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

6.2.3.1.5. **Empower custom field:** Trehalose_Assay

6.2.3.1.5.1. Sample Type: Unknown

6.2.3.1.5.2. Enter the sample weight and dilution factor in the "Alter Sample" window.

6.2.3.2. Glucose (%wt/wt, anhydrous basis) = (R_U/R_{CS}) x (C_{CS}/C_U) x 100

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

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

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

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

6.2.3.2.5. **Empower custom field**: Trehalose_Assay

6.2.3.2.5.1. Sample Type: Unknown

6.2.3.2.5.2. Enter the sample weight and dilution factor in the "Alter Sample" window

6.2.3.3. Unspecified impurities (%wt/wt, anhydrous basis) = (R_U/R_{CS}) x (C_{CS}/C_U) x 100

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

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

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

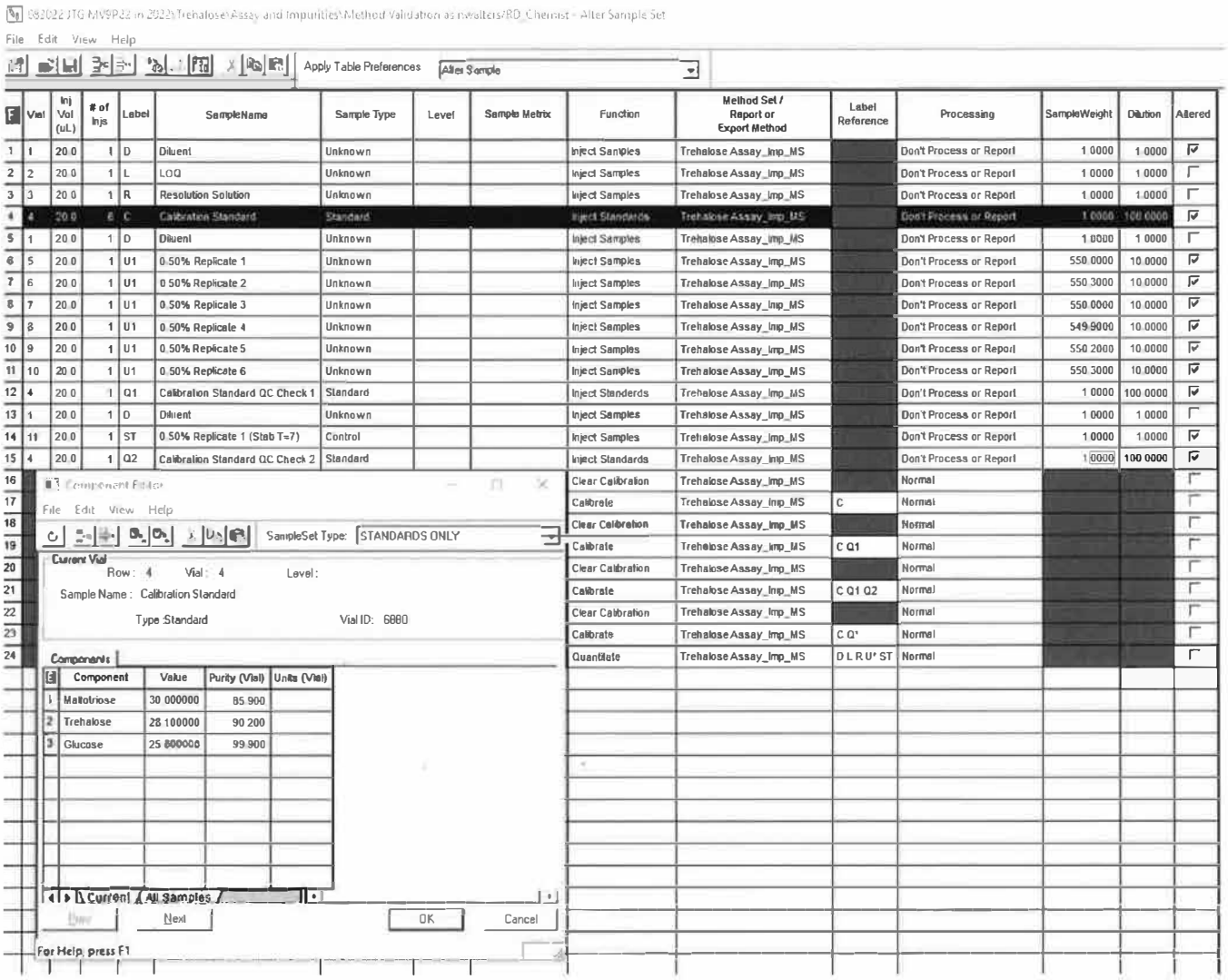
6.2.3.3.4. C_U = Concentration of Trehalose in the sample x 0.905

6.2.3.3.5. **Empower custom field:** Trehalose_Assay

6.2.3.3.5.1. Sample Type: Unknown

6.2.3.3.5.2. Enter the sample weight and dilution factor in the "Alter Sample" window

6.2.4. Example Alter Sample Window:



	Vial	Inj Vol (uL)	# of Injs	Label	SampleName	Sample Type	Level	Sample Matrix	Function	Method Set / Report or Export Method	Label Reference	Processing	SampleWeight	Dilution	Altered
1	1	20.0	1	D	Diluent	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	1.0000	☑
2	2	20.0	1	L	LOQ	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	1.0000	☐
3	3	20.0	1	R	Resolution Solution	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	1.0000	☐
4	4	20.0	6	C	Calibration Standard	Standard			Inject Standards	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	100.0000	☑
5	1	20.0	1	D	Diluent	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	1.0000	☐
6	5	20.0	1	U1	0.50% Replicate 1	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	550.0000	10.0000	☑
7	6	20.0	1	U1	0.50% Replicate 2	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	550.3000	10.0000	☑
8	7	20.0	1	U1	0.50% Replicate 3	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	550.0000	10.0000	☑
9	8	20.0	1	U1	0.50% Replicate 4	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	549.9000	10.0000	☑
10	9	20.0	1	U1	0.50% Replicate 5	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	550.2000	10.0000	☑
11	10	20.0	1	U1	0.50% Replicate 6	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	550.3000	10.0000	☑
12	4	20.0	1	Q1	Calibration Standard QC Check 1	Standard			Inject Standards	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	100.0000	☑
13	1	20.0	1	D	Diluent	Unknown			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	1.0000	☐
14	11	20.0	1	ST	0.50% Replicate 1 (Stab T=7)	Control			Inject Samples	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	1.0000	☑
15	4	20.0	1	Q2	Calibration Standard QC Check 2	Standard			Inject Standards	Trehalose Assay_Imp_MS		Don't Process or Report	1.0000	100.0000	☑
16									Clear Calibration	Trehalose Assay_Imp_MS		Normal			☐
17									Calibrate	Trehalose Assay_Imp_MS	C	Normal			☐
18									Clear Calibration	Trehalose Assay_Imp_MS		Normal			☐
19									Calibrate	Trehalose Assay_Imp_MS	C Q1	Normal			☐
20									Clear Calibration	Trehalose Assay_Imp_MS		Normal			☐
21									Calibrate	Trehalose Assay_Imp_MS	C Q1 Q2	Normal			☐
22									Clear Calibration	Trehalose Assay_Imp_MS		Normal			☐
23									Calibrate	Trehalose Assay_Imp_MS	C Q*	Normal			☐
24									Quantitate	Trehalose Assay_Imp_MS	D L R U* ST	Normal			☐

6.3. Reporting

6.3.1. **Related Substances** (Glucose and Maltotriose): Calculate the amount (%wt/wt, anhydrous basis) of each related substance ≥ the reporting limit (0.10%) in the sample solution

TABLE 4: RELATED SUBSTANCES REPORTING

Related Substance Reporting	
Result	**Reporting**
If < 0.10%	Report as < LOQ
If ≥ 0.10% and < 1.0%	Report to two (2) decimal places
If > 1.0 %	Report to one (1) decimal place

6.3.2. **Unspecified Impurities**: Calculate the amount (%wt/wt, anhydrous basis) of each related substance ≥ the LOQ (0.01%) in the sample solution.

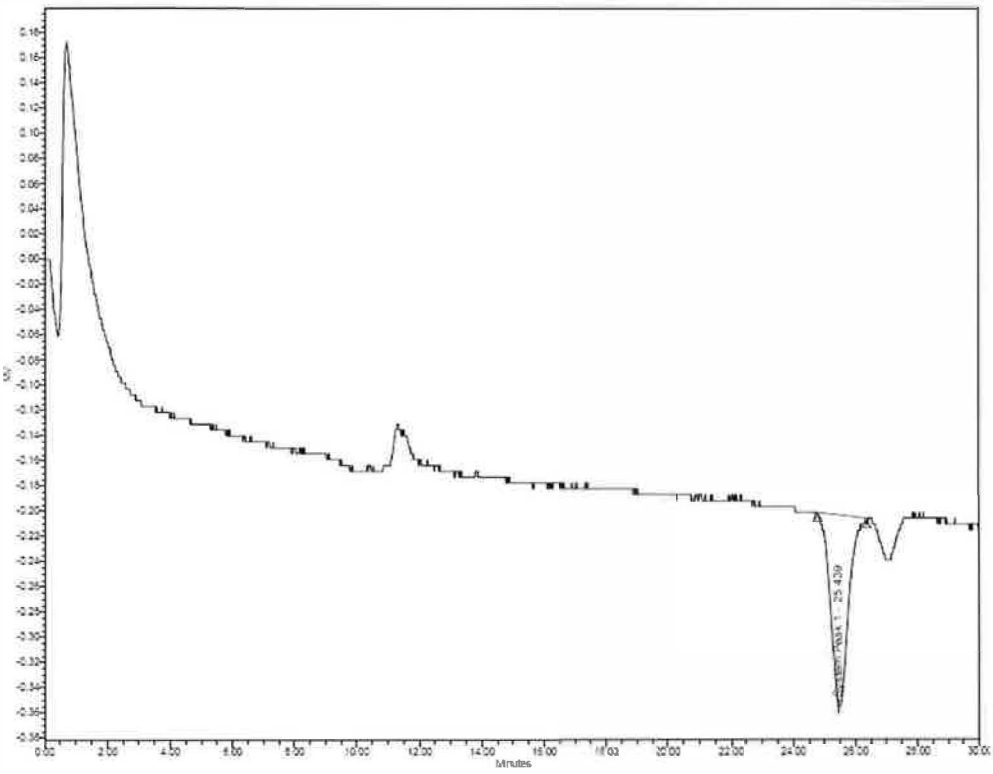
6.3.2.1. Disregard any peaks with an area less than the Trehalose peak in the LOQ injection.

TABLE 5: UNSPECIFIED IMPURITY REPORTING

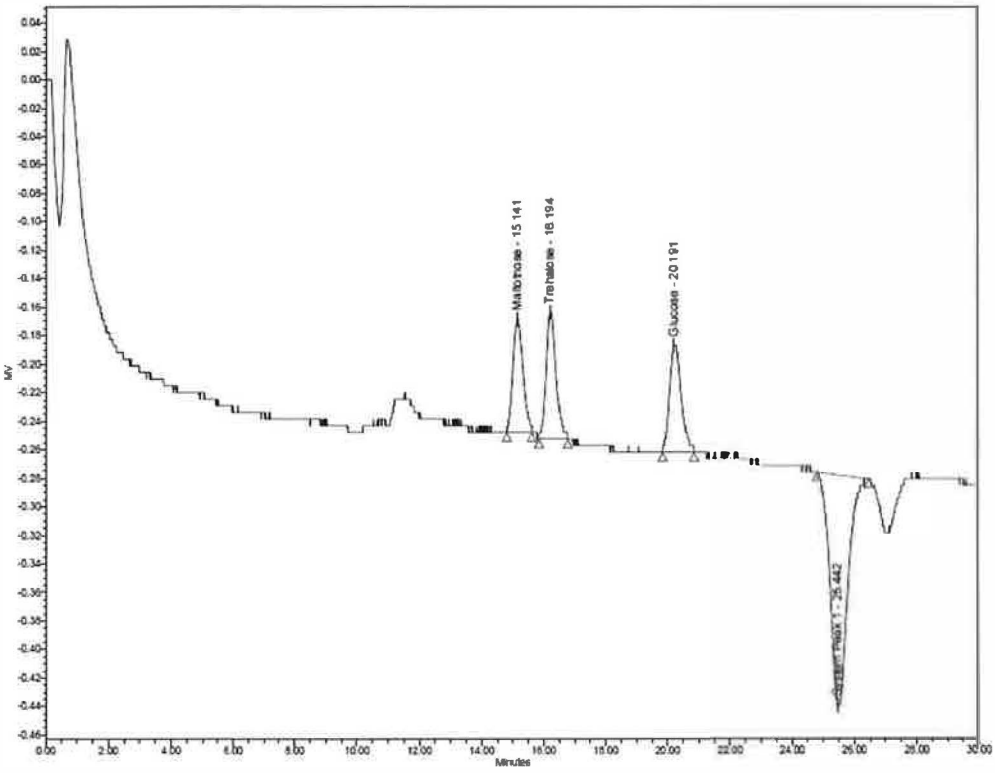
Unspecified Impurity Reporting	
Result	**Reporting**
If ≥ 0.01% and < 1.0%	Report to two (2) decimal places
If > 1.0 %	Report to one (1) decimal place

6.4. Example Chromatograms and Integrations.

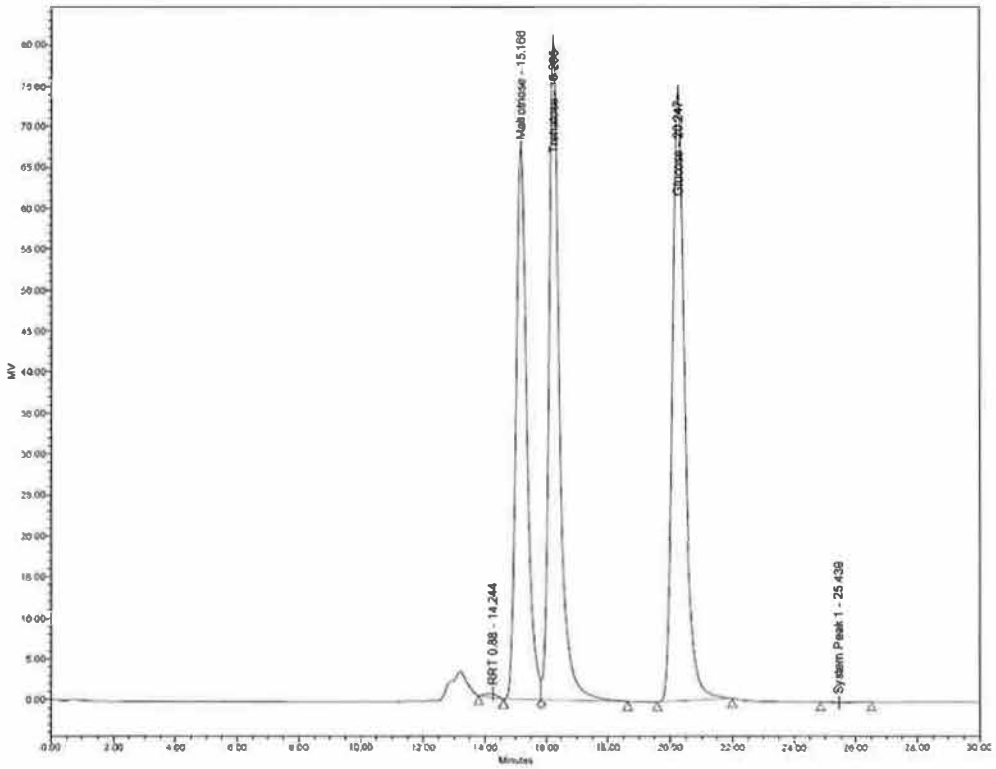
6.4.1. Diluent



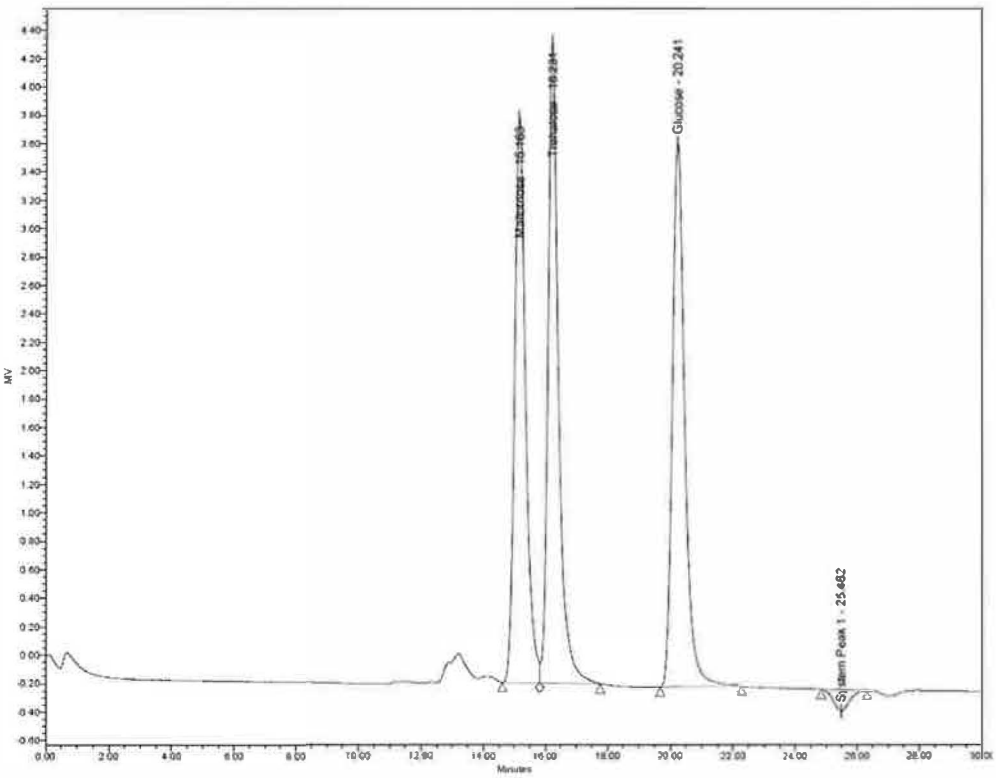
6.4.2. LOQ



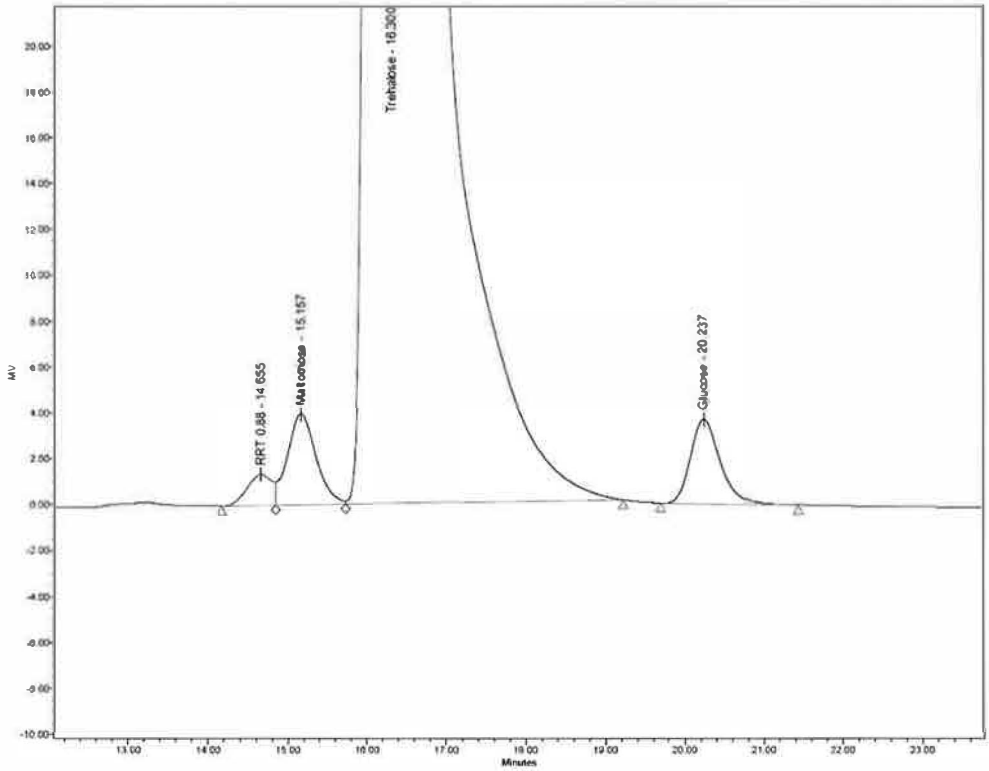
6.4.3. Resolution



6.4.4. Calibration Standard



6.4.5. Spiked Sample – Impurity Peak Integrations



6.5. Integration Parameters for Empower Software.

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

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

6.5.3. Example Integration Events

Integration | Smoothing/Offset | Components | Impurity | Peak Ratios (MS Ion Ratios) | Default Amounts/Purity | Named Groups | Timed Groups

Integration Algorithm: ApexTrack

Apex Detection

Start (min)	14.035	End (min)	
Peak Width (sec)	42.43	Detection Threshold	

Peak Integration

Liftoff %	0.000	Touchdown %	0.020
Minimum Area	1500	Minimum Height	0

	Time (min)	Type	Value	Stop (min)
1	0.000	Allow Negative Peaks		
2	18.000	Valley to Valley		