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GALACTOSE ASSAY AND RELATED SUBSTANCES VIA LIQUID CHROMATOGRAPHY WITH RI DETECTION

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1. PURPOSE:

1.1. To provide Quality Control (QC) Analysts with a procedure for determining Assay and Related Substances for Galactose by liquid chromatography with RI detection.

2. SCOPE:

2.1. This Analytical Method Verification applies to the Galactose USP Assay and USP Related Substances analytical methods using BioSpectra's Waters Alliance HPLC.
2.2. Assay specification: 98.0% – 102.0%
2.3. Related Substances: Disregard peaks less than 0.05%

Table 1: USP Galactose Acceptance Criteria

Name	Approximate Relative Retention Time	Acceptance Criteria
Lactose and 1,6-galactosyl-galactose	0.79	NMT 0.6 %
Galacturonic acid	0.89	NMT 0.6 %
Dextrose	0.93	NMT 0.6 %
Tagatose	0.96	NMT 0.6 %
Dulcitol	1.06	NMT 0.6 %
Arabinose	1.10	NMT 0.6 %
Any unspecified impurity	----	NMT 0.2%
Total impurities	----	NMT 1.0%

3. RESPONSIBILITIES:

3.1. The Laboratory Technology Manager is responsible for the control, training, implementation, and maintenance of this procedure.
3.2. The Laboratory Services Staff and/or the qualified designee is 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-0586, Analytical Method Verification Protocol: Galactose Assay and Related Substances Via Liquid Chromatography with RI Detection
4.2. BSI-RPT-1187, Analytical Method Verification Report: Galactose Assay and Related Substances Via Liquid Chromatography with RI Detection
4.3. BSI-SOP-0098, Balance SOP
4.4. BSI-SOP-0134, Pipette SOP
4.5. BSI-SOP-0436, Analytical Methods Validation Master Plan.
4.6. *USP Galactose*
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. MATERIALS AND EQUIPMENT:

5.1. All materials and equipment utilized in this Verification are outlined in this section.
- 5.1.1. Analytical Balance
- 5.1.2. Microbalance
- 5.1.3. Waters Alliance HPLC, or qualified HPLC

5.2. Reagents
- 5.2.1. HPLC Grade Water
- 5.2.2. 1 N Sulfuric Acid, Certified Grade

5.3. Supplies
- 5.3.1. Micropipettes
- 5.3.2. Micropipette tips
- 5.3.3. Transfer pipettes
- 5.3.4. Screw top glass autosampler vials and caps

5.4. Reference Standards
- 5.4.1. USP Traceable Galactose
- 5.4.2. USP Traceable Arabinose
- 5.4.3. Galacturonic Acid Monohydrate (NLT 97.0%)
- 5.4.4. USP Traceable Dextrose (Glucose)
- 5.4.5. USP Traceable Anhydrous Lactose

5.5. LC Columns
- 5.5.1. Two (2) SUPELCOGEL C-610H, 6% Crosslinked HPLC Columns in Tandem
- 5.5.2. Dimensions: 9µm particle size, 30 cm x 7.8 mm
- 5.5.3. Part Number: 59320-U

6. TESTING PROCEDURE:

6.1. Solution Preparation

6.1.1. Note: All solutions are to be thoroughly mixed after being prepared. Ensure the amounts to be weighed are NLT than the minimum weight tolerance of the balance. Solutions may be scaled as needed.

6.1.2. Mobile Phase (0.009 N Sulfuric Acid)
- 6.1.2.1. Dilute 9 mL of 1 N Sulfuric Acid to a final volume of 1000 mL using purified water.
- 6.1.2.2. Mix thoroughly.
- 6.1.2.3. Expires one week (7 days) after preparation.

6.1.3. Stock Impurity Reference Standard Solution - 1.0 mg/mL Related Substances
- 6.1.3.1. Accurately weigh 25 mg (±10%) of each impurity (See Below) and transfer into a 25 mL volumetric flask.
  - 6.1.3.1.1. Arabinose RS
  - 6.1.3.1.2. Galacturonic Acid
  - 6.1.3.1.3. Dextrose (Glucose) RS
  - 6.1.3.1.4. Anhydrous Lactose RS
- 6.1.3.2. Fill the flask ~3/4 full with mobile phase and swirl to dissolve.
- 6.1.3.3. Dilute to volume with mobile phase and mix well.

6.1.4. System Suitability Solution - 10.0mg/mL Galactose CRS, 0.2mg/mL Related Substances
- 6.1.4.1. Accurately weigh 250 mg (±10%) of USP Traceable Galactose Reference Standard and transfer to a 25.0 mL volumetric flask.
- 6.1.4.2. Pipette 5.0 mL of the above *Stock Impurity Reference Standard Solution* into the flask.
- 6.1.4.3. Fill the flask ~3/4 full with mobile phase and swirl to dissolve.
- 6.1.4.4. Fill to volume with mobile phase and mix thoroughly.
- 6.1.4.5. Solution Stability: 4 days when stored in stoppered clear glassware at room temperature.

6.1.5. Sensitivity Solution - 5.0 µg/mL (0.05%) Related Substances
- 6.1.5.1. Pipette 0.5mL above *Stock Impurity Reference Standard Solution* into a 100 mL volumetric flask.
- 6.1.5.2. Dilute to volume with mobile phase and mix well.
- 6.1.5.3. Solution Stability: 4 days when stored in stoppered clear glassware at room temperature.

6.1.6. Assay Standard Solution - 10.0 mg/mL Galactose CRS
- 6.1.6.1. Accurately weigh 250 mg (±10%) of USP Traceable Galactose Reference Standard and transfer into a 25.0 mL volumetric flask.
- 6.1.6.2. Fill the flask ~3/4 full with mobile phase and swirl to dissolve.
- 6.1.6.3. Dilute to volume with mobile phase and mix well.
- 6.1.6.4. Prepare in duplicate label AS1 and AS2, respectively.
- 6.1.6.5. Solution Stability: 4 days when stored in stoppered clear glassware at room temperature.

6.1.7. Sample Solution - 10.0 mg/mL Galactose

6.1.7.1. Accurately weigh 250 mg (±10%) of Galactose sample and transfer to a 25.0 mL volumetric flask.

6.1.7.2. Fill the flask ~3/4 full with mobile phase and swirl to dissolve.

6.1.7.3. Dilute to volume with mobile phase and mix thoroughly.

6.1.7.4. Prepare a single replicate.

6.1.7.5. Solution Stability: 4 days when stored in stoppered clear glassware at room temperature.

6.2. Instrument Setup

Table 2: Waters Alliance HPLC Method Parameters

Parameter	Setting
Flow Type	Isocratic
Diluent	0.009N Sulfuric acid
Mobile Phase A	0.009N Sulfuric acid
Needle Wash	Water
Flow Rate	0.25 mL/min
Run Time	70 minutes
Injection Volume	25 µL
Stroke Volume	25 µL
Syringe Draw Rate	Normal
Pre-Column Volume	0.0
Needle Wash Time	Normal
Column Temperature (°C)	35 ± 1.0
Sample Temperature (°C)	Ambient
RI Detector Settings	
Detector	Refractive Index
Detector Temperature	40 °C
Sampling Rate	5
Filter Time	1.0
Sensitivity	4
Polarity	Positive

6.2.1. Chromatographic System

6.2.1.1. Flush HPLC system with purified water, place 0.009N Sulfuric Acid in the mobile phase reservoir (A) and prime the lines.

6.2.1.2. Install Two (2) Supelcogel C610H, 7.8mm x 30cm columns in tandem and slowly bring flow up to 0.25mL/min. Allow the column to equilibrate until a consistent pressure is observed.

6.2.1.3. 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

6.2.1.4. Purge RID reference cell for at least 30 minutes prior to initiating the injection sequence.

6.2.1.4.1. Note: The purge function must be manually disengaged prior to initiating the injection sequence.

Table 3: Injection Sequence:

Sample ID	Number of Injections
System Suitability	
Diluent	≥1
Sensitivity	1
System Suitability	1
AS1	6
AS2	1
Sample Injections	
Diluent	1
Samples	≤ 6
AS1 (QC Check)	1

- Repeat the sample injection sequence if additional samples are to be analyzed
- Samples may be substituted with diluent injections
- The Sensitivity injection may be omitted if performing Assay testing only
- The AS2 injection may be omitted if performing Related Substances testing only

Table 4: System Suitability Criteria:

System Suitability Parameter	Acceptance Criteria
The relative standard deviation of the galactose peak from the first six (6) injections of the AS1 solution.	NMT 0.85%
The relative standard deviation of the galactose peak from all injections of the AS1 solution.	NMT 0.85%
USP Resolution between Lactose and Galacturonic acid in the *System Suitability Solution* injection.	NLT 3.0
USP Resolution between Galacturonic acid and Dextrose (Glucose) in the *System Suitability Solution* injection.	NLT 1.5
USP Resolution between Dextrose (Glucose) and Galactose in the *System Suitability Solution* injection.	NLT 2.0
USP Resolution between Galactose and Arabinose in the *System Suitability Solution* injection.	NLT 3.0
Signal-to-noise Ratio for Lactose, Galacturonic Acid, Dextrose, and Arabinose peaks in the *Sensitivity Solution* injection.	NLT 10
Standard %Agreement between the first six (6) AS1 injections and the AS2 injection.	99% - 101%

6.3. Calculations

6.3.1. The following equations will be calculated in the Empower software:

6.3.2. Percent Agreement = (R_{AS2}/R_{AS1}) x (C_{AS1}/C_{AS2}) x 100

6.3.2.1. R_{AS1} = average peak response of galactose from the first six (6) AS1 injections

6.3.2.2. R_{AS2} = peak response of Galactose from the AS2 injection

6.3.2.3. C_{AS1} = Concentration of Galactose in AS1 x Purity Factor

6.3.2.4. C_{AS2} = Concentration of Galactose in AS2 x Purity Factor

6.3.2.5. **Empower custom field:** Control_PercentAgreement

6.3.2.5.1. Sample Type: Control

6.3.2.5.2. Enter dilution factor in the "Alter Sample" window.

6.3.2.5.3. Enter Sample weight and purity in the "Amounts" tab.

6.3.3. Assay As-Is (% Galactose) = (R_u/R_{AS1}) x (C_{AS1}/C_u) x 100

6.3.3.1. R_{AS1} = average peak response of galactose from all AS1 injections

6.3.3.2. R_u = peak response of Galactose from the sample

6.3.3.3. C_{AS1} = Concentration of AS1 x Purity Factor

6.3.3.4. C_u = Concentration of the sample

6.3.3.5. **Empower custom field:** Assay

6.3.3.5.1. Sample Type: Unknown

6.3.3.5.2. Enter sample Sample weight and Dilution factor in the "Alter Sample" window.

6.3.4. Assay Anhydrous Basis (USP/EP) (% Galactose) = % Assay as-is Basis / (100 – % Water Content)

6.3.5. Related Substances (% Area) = $R_u/(R_{SPL}$ x RRF) x 100

6.3.5.1. R_u = peak response of each individual impurity in the sample solution

6.3.5.2. R_{SPL} = peak response of Galactose in the sample solution

6.3.5.3. RRF = Relative Response Factor

6.3.5.3.1. See below for the response factors for the known Related Substances.

6.3.5.3.2. Disregard any peak due to the solvent and the peak at the relative retention time of approximately 0.64.

Table 5: Relative Response Factors

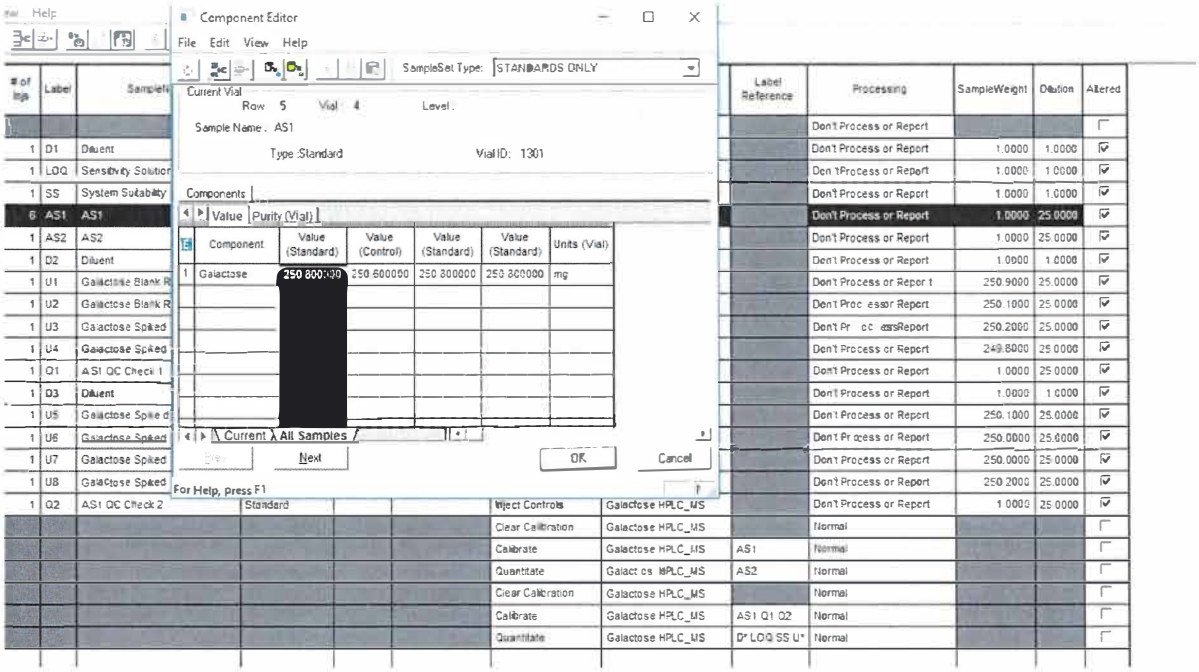
Name	USP Relative Response Factor
Lactose and 1,6-galactosyl-galactose	0.95
Galacturonic acid	0.88
Dextrose	0.99
Tagatose	0.96
Dulcitol	0.96
Arabinose	0.95
Any unspecified impurity	1.0

6.3.5.4. **Empower custom field:** USP_PercentArea

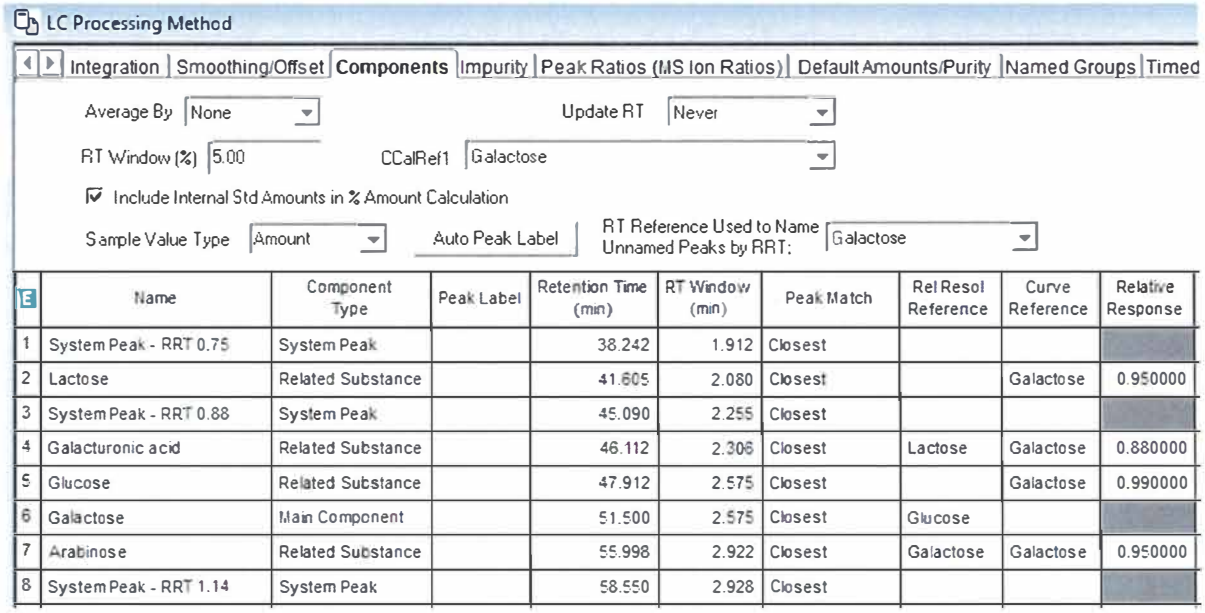
6.3.5.4.1. Sample Type: Unknown

6.3.5.4.2. Enter RRF into the "Relative Response" column in the processing method.

6.3.6. Example Alter Sample Window:



6.3.7. Processing Method Component Tab:



	Name	Component Type	Peak Label	Retention Time (min)	RT Window (min)	Peak Match	Rel Resol Reference	Curve Reference	Relative Response
1	System Peak - RRT 0.75	System Peak		38.242	1.912	Closest			
2	Lactose	Related Substance		41.605	2.080	Closest		Galactose	0.950000
3	System Peak - RRT 0.88	System Peak		45.090	2.255	Closest			
4	Galacturonic acid	Related Substance		46.112	2.306	Closest	Lactose	Galactose	0.880000
5	Glucose	Related Substance		47.912	2.575	Closest		Galactose	0.990000
6	Galactose	Main Component		51.500	2.575	Closest	Glucose		
7	Arabinose	Related Substance		55.998	2.922	Closest	Galactose	Galactose	0.950000
8	System Peak - RRT 1.14	System Peak		58.550	2.928	Closest			

6.4. Reporting

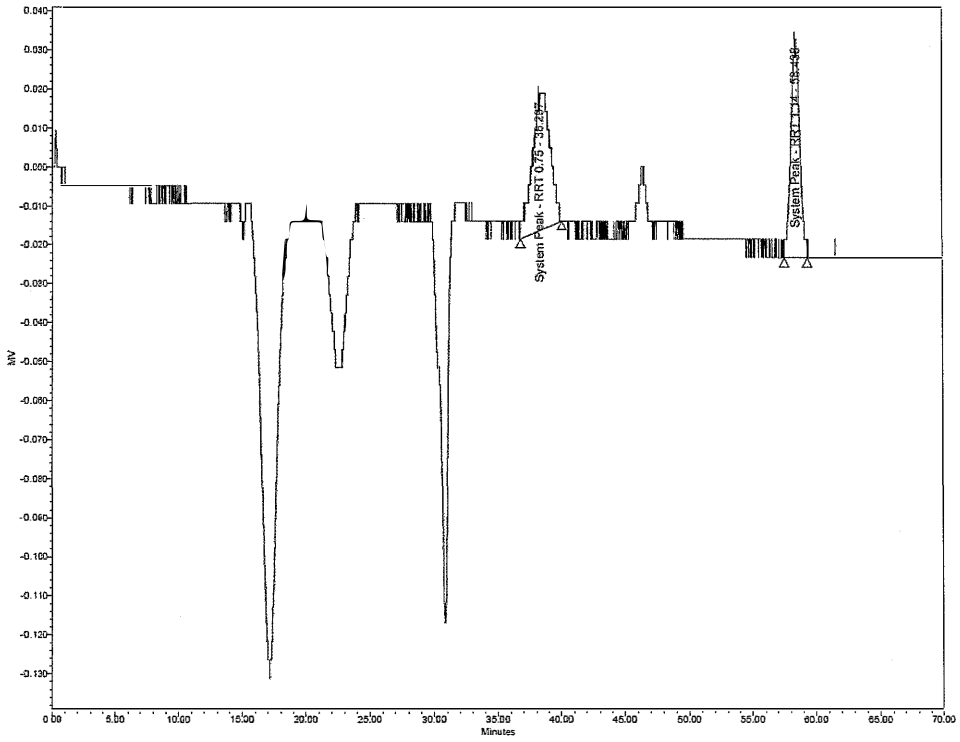
6.4.1. **Assay**: Calculate the % Galactose and report to a one (1) decimal place.

6.4.2. **Related Substances**: Calculate the % Area of each related substance ≥ the reporting limit (0.05%) in the sample solution.

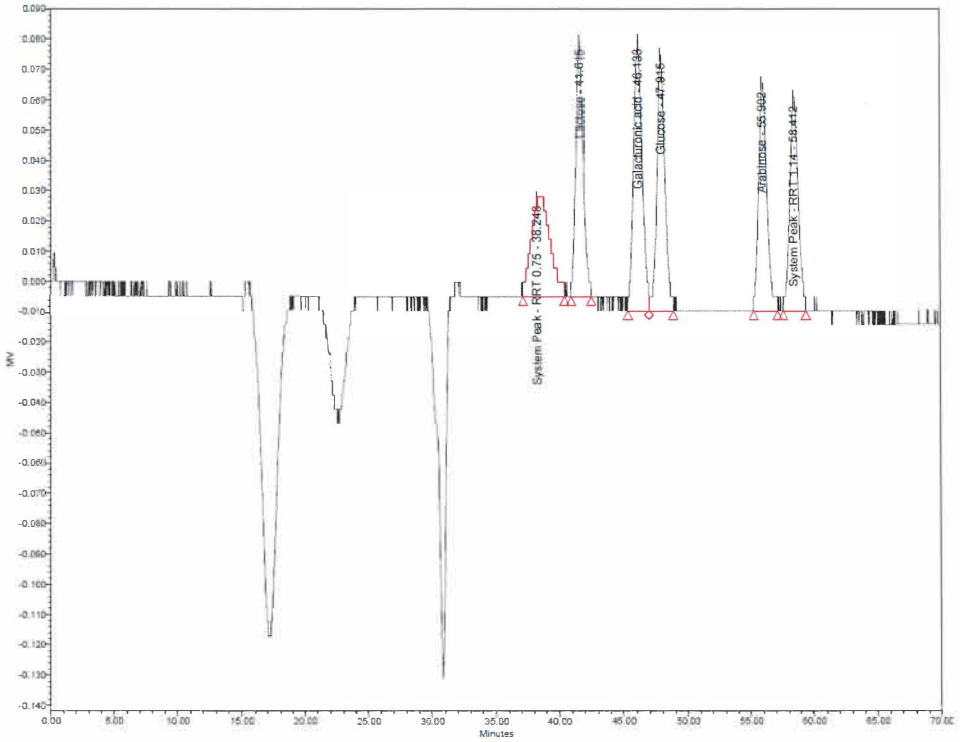
Table 6: Related Substance Reporting	
Result	**Reporting**
If < 0.05%	Report as < LOQ
If ≥ 0.05% and < 1.0%	Report to two (2) decimal places
If > 1.0 %	Report to one (1) decimal place

6.5. Example Chromatograms and Integrations

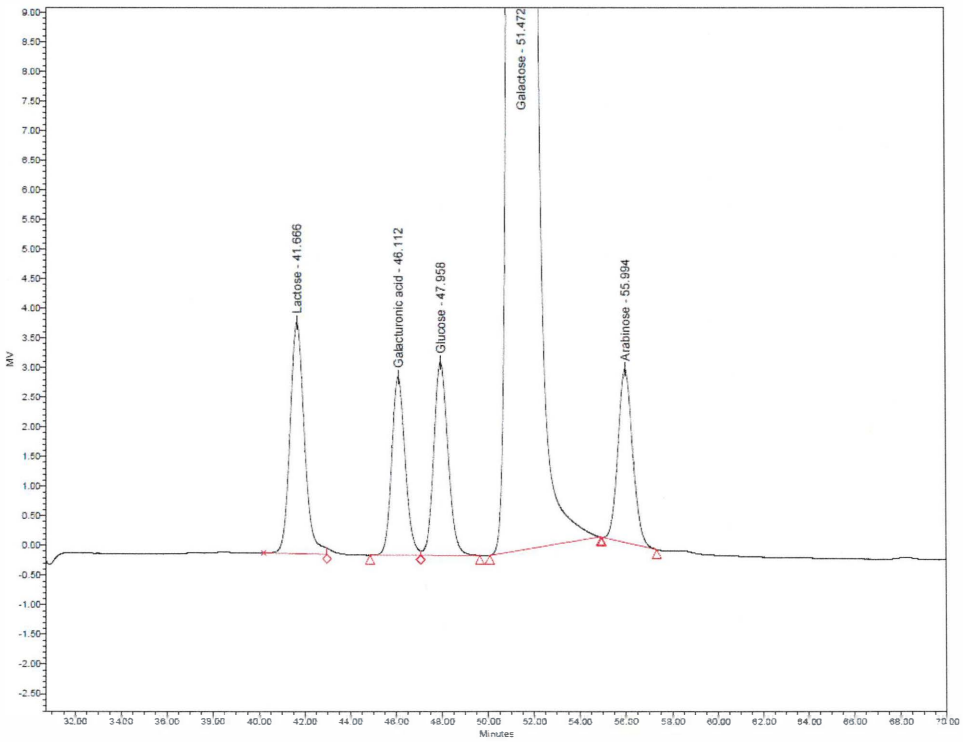
6.5.1. Diluent



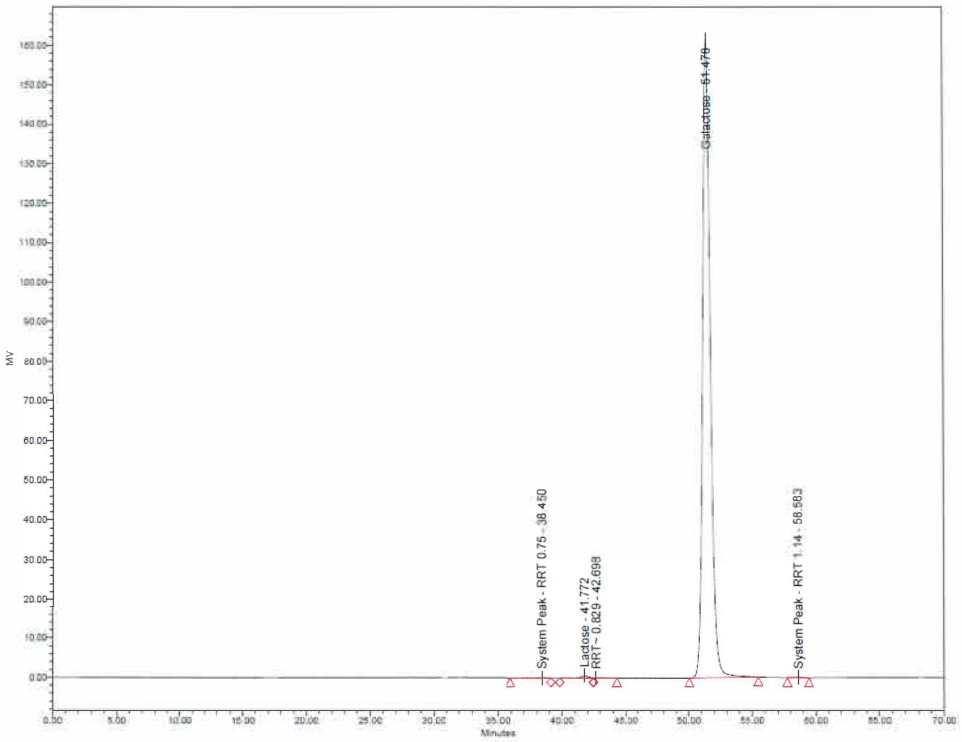
6.5.2. Sensitivity Solution:



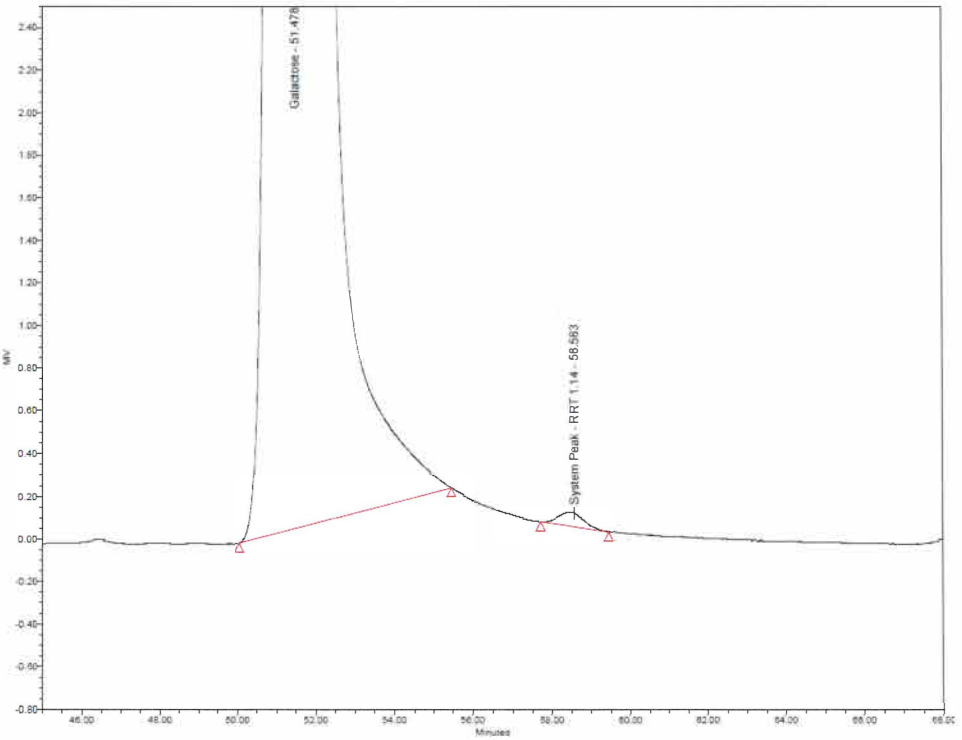
6.5.3. System Suitability Solution



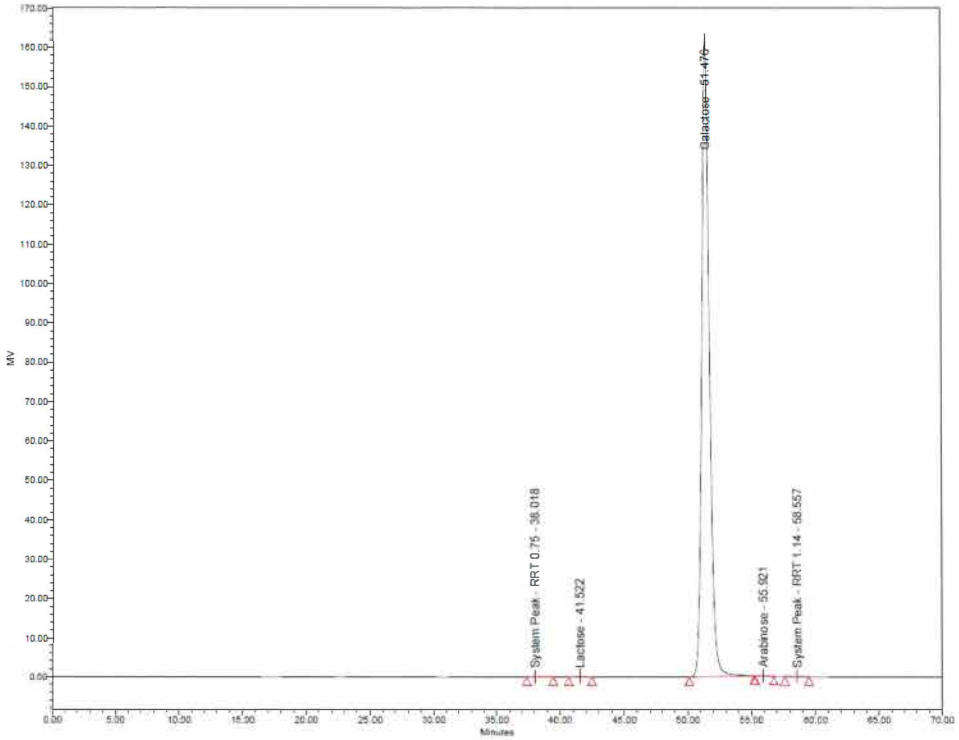
6.5.4. Assay Standard Solution



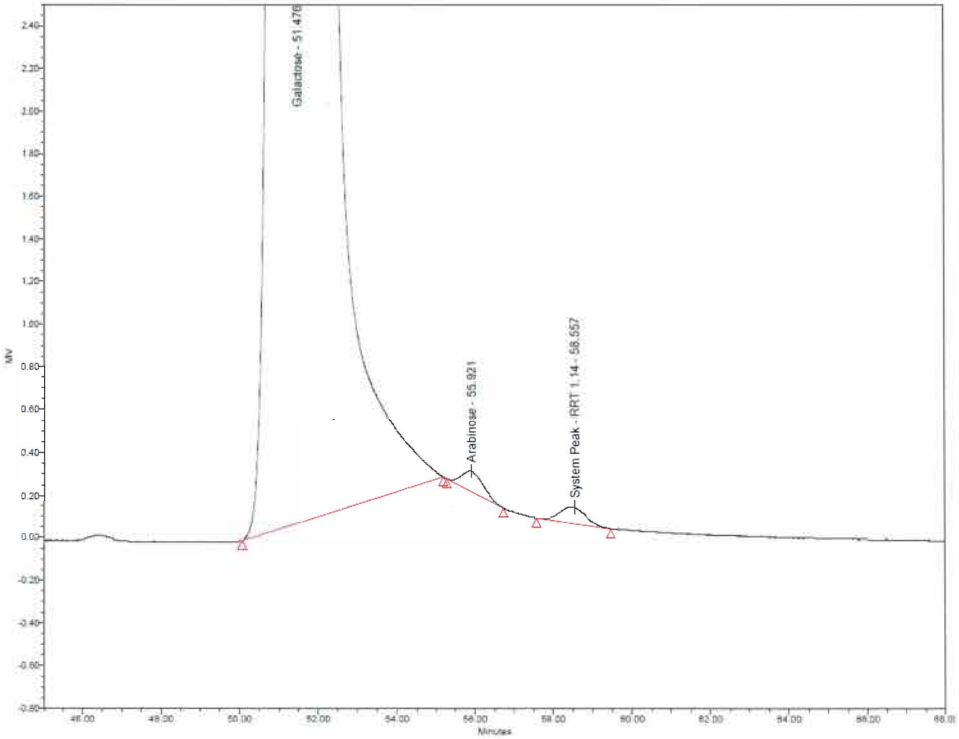
6.5.5. Assay Standard Solution – Galactose Peak



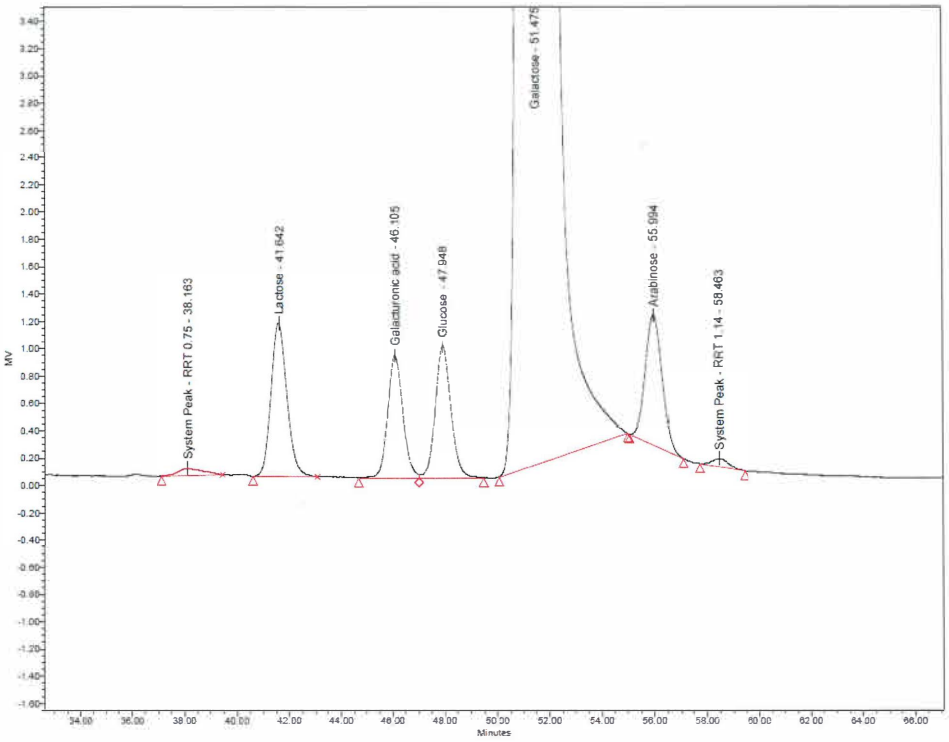
6.5.6. Sample Solution



6.5.7. Sample Solution – Galactose Peak



6.5.8. Spiked Sample Solution – Related Substances



6.6. Integration Parameters for Empower software

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

6.6.2. Integration parameters and component times may be adjusted in order to achieve similar integrations as shown in Section 6.5.

6.6.3. Ensure the components include the correct relative responses for the %Area custom field calculation.

6.6.4. Example Integration Events