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

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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. Ensure that the related substances procedure used on the Waters Alliance HPLC is adequately evaluated and validated.

1.1.2. Verify that the quantitative related substances procedure meets requirements for System Suitability, Accuracy, Precision, Intermediate Precision, Specificity, Linearity, Range, and Limit of Quantitation.

1.1.3. To establish solution stability for samples and standards.

1.1.4. To ensure that the proper reagents and testing materials are used and the correct documentation is provided for the evaluation.

2. SCOPE:

2.1. This Analytical Method Validation Protocol applies to the Trehalose Hydrate JP Assay using BioSpectra's Waters Alliance HPLC.

2.2. Related Substance Specifications:

Related Substance	Specification (%wt/wt) Max.
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 will be validated as a category II quantitative test.

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

3. RESPONSIBILITIES:

3.1. The Executive Director of Quality Control is responsible for the control, training, implementation and maintenance of this procedure.

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

3.3. The Quality Control Analysts performing this procedure, with help and training from the Quality Control Manager and Senior Chromatography Specialist 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. REFERENCE:

4.1. BSI-SOP-0098, Balance SOP,

4.2. BSI-SOP-0134, Pipette SOP

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

4.4. *Trehalose Hydrate JP Monograph*

4.5. *JP <2.01> Liquid Chromatography*

4.6. *USP <621> Chromatography*

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

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

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

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

5. PRE-VALIDATION REQUIREMENTS:

5.1. Equipment
  5.1.1. All equipment to be used in this Validation is in proper working order and with current calibrations.
5.2. Personnel
  5.2.1. All personnel who perform this Validation will be properly trained in accordance with the Analytical Method Validation Master Plan and this protocol.
5.3. Supplies
  5.3.1. All supplies used in the Validation will be clean and appropriate for their intended use.
5.4. Reagents
  5.4.1. All reagents will be current, meet required specifications, and suitable for their intended use.
5.5. Reference Standards
  5.5.1. All standards that will be used in this Validation are listed in the Materials and Equipment section.

6. MATERIALS AND EQUIPMENT:

6.1. All materials and equipment utilized in this Validation are outlined in this section.
  6.1.1. Analytical Balance
  6.1.2. Waters Alliance HPLC
6.2. Reagents
  6.2.1. HPLC Grade Water
6.3. Supplies
  6.3.1. Class A Volumetric Flasks
  6.3.2. Polypropylene Transfer Funnels
  6.3.3. Micropipettes
  6.3.4. Micropipette tips
  6.3.5. Transfer pipettes
  6.3.6. Screw top glass autosampler vials
  6.3.7. Screw top caps with septa
6.4. Reference Standards
  6.4.1. USP Traceable Maltotriose (Dextrose Impurity C)
  6.4.2. USP Traceable Glucose (Dextrose)
  6.4.3. USP Traceable Trehalose
6.5. Authentic Sample
  6.5.1. Trehalose Hydrate:
    6.5.1.1. Manufacturer: Biospectra, Inc.
    6.5.1.2. Lot: TE3250-003-1119
6.6. LC Column
  6.6.1. Rezex RNM-Carbohydrate Na+ (8%) 7.8mm x 300mm, 8µm
  6.6.2. Part number: 00H-0136-K0

7. GENERAL TESTING PROCEDURE:

7.1. Solution Preparation:

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

7.1.2. Diluent/Mobile Phase/Needle Wash: 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 50mg (±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 28mg (±10%) Trehalose, 30mg (±10%) Maltotriose, and 25mg (±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.

7.1.4.5. Solution Stability to be determined during validation.

7.1.4.5.1. 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 1.0mL of the calibration standard into a 50mL volumetric flask.

7.1.5.2. Fill to volume with 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.

7.1.6.7. Solution stability to be determined during validation.

7.2. System Setup:

7.2.1. 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

7.2.2. Column Care:

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

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

7.2.2.3. 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.

7.2.3. Column Conditioning/System Equilibration:

7.2.3.1. 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.

7.2.3.2. 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.

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

7.2.4. Injection Sequence:

Sample ID	Number of Injections
System Suitability	
Diluent	≥ 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.	

7.2.5. System Suitability:

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

7.2.6. Calculations: the following equations will be calculated in the Empower software:
- 7.2.6.1. Maltotriose (%wt/wt, anhydrous basis) = (R_U/R_{CS}) x (C_{CS}/C_U) x 100
  - 7.2.6.1.1. R_{CS} = Average peak area response of Maltotriose in all *Calibration Standard* injections
  - 7.2.6.1.2. R_U = Peak area response of Maltotriose in the sample injection
  - 7.2.6.1.3. C_{CS} = Concentration of Maltotriose x Certified Purity
  - 7.2.6.1.4. C_U = Concentration of the sample x 0.905
- 7.2.6.2. Glucose (%wt/wt, anhydrous basis) = (R_U/R_{CS}) x (C_{CS}/C_U) x 100
  - 7.2.6.2.1. R_{CS} = Average peak area response of Glucose in all *Calibration Standard* injections
  - 7.2.6.2.2. R_U = Peak area response of Glucose in the sample injection
  - 7.2.6.2.3. C_{CS} = Concentration of Glucose x Certified Purity
  - 7.2.6.2.4. C_U = Concentration of the sample x 0.905
- 7.2.6.3. Unspecified impurities (%wt/wt, anhydrous basis) = (R_U/R_{CS}) x (C_{CS}/C_U) x 100
  - 7.2.6.3.1. R_{CS} = Average peak area response of Trehalose in all *Calibration Standard* injections
  - 7.2.6.3.2. R_U = Peak area response of any unspecified impurity in the sample injection
  - 7.2.6.3.3. C_{CS} = Concentration Trehalose x Certified Purity
  - 7.2.6.3.4. C_U = Concentration of the Trehalose in the sample x 0.905

8. VALIDATION PROCEDURE:

8.1. System Suitability:
- 8.1.1. Refer to the injection sequence (7.2.4) and the system suitability acceptance criteria (7.2.5)
- 8.1.2. System suitability will be carried out for each analysis. All acceptance criteria must be met for results to be considered valid.

8.2. Specificity:
- 8.2.1. Overlay the chromatograms from one (1) Diluent Injection, one (1) Resolution Solution injection, one (1) 0% Level Accuracy and Precision Injection, one (1) 0.5% Level Accuracy and Precision Solution injection, and one (1) Calibration Standard Injection
- 8.2.2. Acceptance Criteria:
  - 8.2.2.1. The Trehalose peak has a USP Resolution of NLT 1.5 in the 0.5% Accuracy and Precision injection.
  - 8.2.2.2. The Trehalose peak is visually resolved from diluent interference.

8.3. Low-level Linearity:

8.3.1. Linearity will be evaluated from 0.01% to 0.05% of the 50 mg/mL nominal sample concentration.

8.3.2. Low-level Stock Solution (1.0 mg/mL Trehalose anhydrous basis):

8.3.2.1. Weigh and transfer 55 mg of Trehalose reference standard into a 50 mL volumetric flask.

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

8.3.2.3. Fill to volume with diluent

8.3.2.4. Mix by inversion.

8.3.3. Low-level Linearity Solutions:

8.3.3.1. Per the table below, pipette low-level stock solution into separate volumetric flasks

8.3.3.2. Fill to volume with diluent.

8.3.3.3. Mix by inversion

8.3.3.4. Prepare a single replicate of each level.

8.3.3.5. Perform three (3) injections for each replicate.

8.3.4. Low-level Linearity Solution Table:

Low-Level Linearity (0.01% - 0.05%)			
	Impurity Sample Concentration (mg/mL)		50
Level (%)	Amount of Low-level stock to pipette (mL)	Volumetric Flask (mL)	Trehalose (anhydrous) Concentration (mg/mL)
0.05	2.50	100	0.025
0.04	2.00	100	0.020
0.03	1.50	100	0.015
0.02	1.00	100	0.010
0.01	0.50	100	0.005

8.3.5. Analyze the samples per the General Testing Procedure (Section 7). Plot the peak area response against concentration, perform a linear regression, and calculate the y-intercept bias with respect to the 0.01% Level. Plot the percent deviation against concentration.

8.3.6. Calculations:

8.3.6.1. Y-intercept bias (%) = (Y-intercept/average 0.01% Level peak area) x 100

8.3.7. Acceptance Criteria:

8.3.7.1. Report the y-intercept, slope, and residual sum of squares.

8.3.7.2. Correlation Coefficient: NLT 0.990

8.3.7.3. Y-intercept bias: NMT 25.0%

8.4. Linearity:

8.4.1. Linearity will be evaluated from 0.05% to 0.75% of the 50 mg/mL nominal sample concentration, which corresponds to 10% to 150% of the 0.5% impurity specification limit.

8.4.2. Impurity Linearity Stock Solution (2.5 mg/mL Trehalose (anhydrous), 2.5 mg/mL Maltotriose, 2.5 mg/mL Glucose)

8.4.2.1. Weigh and transfer 140mg (±5%) Trehalose, 150mg (±5%) Maltotriose, and 125 mg (±5%) Glucose reference standards into a 50 mL volumetric flask.

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

8.4.2.3. Fill to volume with diluent.

8.4.2.4. Mix by inversion

8.4.3. Linearity Solutions:

8.4.3.1. Per the table below, pipette the Impurity Linearity Sock Solution into separate volumetric flasks

8.4.3.2. Fill to volume with diluent.

8.4.3.3. Mix by inversion

8.4.3.4. Prepare a single replicate of each level.

8.4.3.5. Perform three (3) injections for each replicate.

8.4.4. Linearity Solution Table:

Linearity (0.05% - 0.75%)			
	Impurity Sample Concentration (mg/mL)		50
Level (%)	Amount of Linearity Stock to pipette (mL)	Volumetric flask (mL)	concentration (µg/mL)
0.75	1.50	10	375
0.625	1.25	10	313
0.5	1.00	10	250
0.25	0.50	10	125
0.125	0.25	10	63
0.0625	1.25	100	31
0.05	1.00	100	25

8.4.5. Analyze the samples per the General Testing Procedure (Section 7). For all analytes, plot the peak area response against concentration, perform a linear regression, and calculate the y-intercept bias with respect to the 0.5% Level. Plot the percent deviation against concentration.

8.4.6. Calculations:

8.4.6.1. Y-intercept bias (%) = (Y-intercept/average 0.5% Level peak area) x 100

8.4.7. Acceptance Criteria:

8.4.7.1. Report the y-intercept, slope, and residual sum of squares.

8.4.7.2. Correlation Coefficient: NLT 0.990

8.4.7.3. Y-intercept bias: NMT 15.0%

8.5. Accuracy and Precision:

8.5.1. Accuracy and precision samples will be evaluated from 20% to 150% of the 0.5% Impurity specification. Known quantities of Glucose and Maltotriose will spiked into nominal sample concentration of Trehalose authentic sample

8.5.2. Impurity Stock Solution (2.5 mg/mL Maltotriose, 2.5 mg/mL Glucose)

8.5.2.1. Weigh and transfer 150mg (±5%) Maltotriose, and 125 mg (±5%) Glucose reference standards into a 50 mL volumetric flask.

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

8.5.2.3. Fill to volume with diluent.

8.5.2.4. Mix by inversion

8.5.3. Accuracy and Precision Solutions:

8.5.3.1. Weigh and transfer 550mg of Trehalose Authentic Sample into separate 10 mL volumetric flasks.

8.5.3.2. Per the table below, pipette *Impurity Stock Solution* into each flask.

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

8.5.3.4. Fill to volume with diluent.

8.5.3.5. Mix by inversion

8.5.3.6. Perform a single injection for each solution.

8.5.4. Accuracy and Precision Solution Table:

Accuracy and Precision						
Level	Replicates	Impurity Stock to pipette (mL)	Trehalose added (mg)	Final Volume (mL)	Maltotriose/Glucose (mg/mL)	Anhydrous Trehalose Concentration (mg/mL)
0%	2	0	550	10	0	50
0.10%	3	0.2			0.05	
0.25%	3	0.5			0.125	
0.50%	6	1.0			0.25	
0.75%	3	1.5			0.375	

8.5.5. Analyze the samples per the General Testing Procedure (Section 7). Calculate the % Recovery and %RSD for each analyte at all levels.

8.5.6. Calculations:

8.5.6.1. % Recovery = $A_M/(A_{Added}+A_0)$ x 100

8.5.6.1.1. A_M = amount of related substance measured

8.5.6.1.2. A_{Added} = amount of related substance added

8.5.6.1.3. A_0 = average amount of analyte measured in the 0% Spike

8.5.7. Acceptance Criteria:

8.5.7.1. The % Recovery for each replicate at all levels for both Maltotriose and Glucose is 85%-115%

8.5.7.2. The %RSD at each level is NMT 10%

8.6. Limit of Quantitation:

8.6.1. Analyze the LOQ solution (section 7.1.5) per the General Testing procedure (Section 7) as a sample and inject six (6) times. Calculate the %RSD of the peak area response, USP S/N, and % Recovery for the Trehalose peak only.

8.6.1.1. Note: The LOQ (reporting limit) for maltotriose and glucose will be assessed in section 8.5.

8.6.2. Calculations:

8.6.2.1. %Recovery: Refer to Section 8.8.4.1

8.6.2.1.1. Note: the equation is identical to % Agreement.

8.6.3. Acceptance Criteria:

8.6.3.1. Maltotriose and Glucose:

8.6.3.1.1. All acceptance criteria are met for the 0.10% level from Section 8.5.

8.6.3.2. Trehalose:

8.6.3.2.1. The %RSD of the peak areas is NMT 15%

8.6.3.2.2. The S/N for each injection is NLT 10

8.6.3.2.3. The average %Recovery is between 75% - 125%

8.7. Intermediate Precision:

8.7.1. A different analyst (Analyst 2) will repeat Accuracy and Precision evaluation described in Section 8.5 at the 0.50% level only (n=6). Perform the analysis on a different day with a different column, separately prepared diluent, mobile phase, and standard solutions.

8.7.2. Analyze the samples per the General Testing Procedure (Section 7). Calculate the % Recovery and % RSD of the assay values (6 injections). Combine the results with the results obtained during Accuracy and Precision and calculate the %RSD of the % Recovery values (12 injections).

8.7.3. Calculations: Refer to Section 8.5.

8.7.4. Acceptance Criteria:

8.7.4.1. Analyst 2 results must meet all acceptance criteria outlined in section 8.5.

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

8.8. Solution Stability:

8.8.1. Set aside one (1) 0.50% Level Authentic Sample Test Solution from Precision of Authentic Samples or Intermediate Precision and one (1) Calibration Standard solution. Store the solutions stoppered in the original glassware at normal lighting and laboratory conditions.

8.8.2. Store the solutions for at least one day before assessing solution stability.

8.8.3. Analyze the aged solutions as samples against freshly prepared standards per the General Testing Procedure (Section 7).

8.8.3.1. Calculate % Agreement for Maltotriose and Glucose in the standard solution.

8.8.3.2. Calculate the % Recovery and the absolute difference for Maltotriose and Glucose between Day 0 and Day X for the sample solution.

8.8.4. Calculations:

8.8.4.1. % Agreement: $= (R_U/R_{CS}) \times (C_{CS}/C_{CSA}) \times 100$

8.8.4.1.1. R_{CS} = Average peak area response of the analyte (Maltotriose or Glucose) in all Calibration Standard injections

8.8.4.1.2. R_U = Peak area response of analyte of interest in the sample injection

8.8.4.1.3. C_{CS} = Concentration of the analyte (Maltotriose or Glucose) in the calibration standard x certified purity

8.8.4.1.4. C_{CSA} = Concentration of the sample x Certified Purity

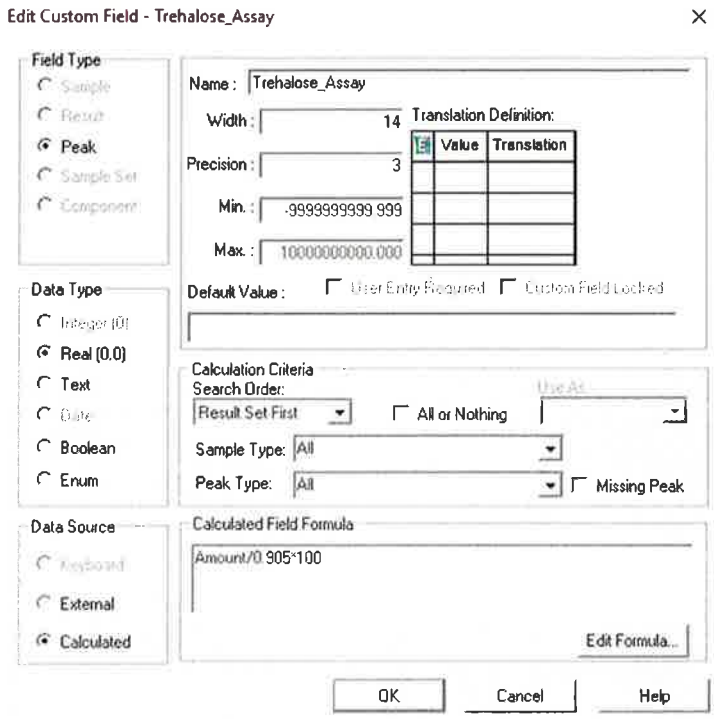
8.8.4.2. % Recovery: Refer to Section 8.5.6.1
8.8.4.3. Absolute Difference = |Day 0 – Day X|
8.8.4.3.1. Day 0 = fresh solution %Recovery value
8.8.4.3.2. Day X = aged solution %Recovery value

8.8.5. Acceptance Criteria:
8.8.5.1. Calibration Standard: The percent agreement for the aged CS solution is between 90.0% and 110.0%
8.8.5.2. 0.50 % Level: the % Recovery of the aged sample solution is between 85.0% and 115.0% for Maltotriose and Glucose
8.8.5.3. The absolute difference between Day 0 and Day X is NMT 15%.

8.9. Empower Custom Fields Verification:

8.9.1. In order to fully automate calculations within Empower software, the following equations will require custom fields. These custom fields will be verified during Intermediate Precision.

8.9.2. Related Substance (%wt/wt anhydrous basis) Calculation:



8.9.3. Ensure the custom fields have been entered into the Empower software as shown in figure 8.9.2.

8.9.4. Hand calculate the %wt/wt Anhydrous Basis for all Analyst 2 samples using equations 7.2.6.1 and 7.2.6.2. Compare the hand calculated results to the results calculated by Empower.

8.9.5. Acceptance criteria:

8.9.5.1. Hand calculated results and Empower results must be identical out to 5 decimal places.

9. DOCUMENTATION PROCEDURES:

9.1. A method validation report will be drafted after successful execution of this protocol summarizing method validation status and performance.

9.2. All data sheets and notebook pages are to be signed and dated by the analyst executing the Protocol. Pages should be copied and uploaded as supporting material into the Master Control documentation management system to be attached to the report.

9.3. All equipment and instrumentation used in the execution of this protocol must be calibrated. Ensure that there is a certificate on file or appropriate standards are used if calibration is required.

9.4. Any critical changes made to the method validation protocol must be noted in the Validation Report with supporting evidence for the change.