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ANALYTICAL METHOD VALIDATION PROTOCOL: TRIS ORGANIC IMPURITIES VIA LIQUID CHROMATOGRAPHY WITH UV DETECTION

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1. PURPOSE:

1.1. The purpose of this protocol is to:

1.1.1. Ensure that the Tris Organic Impurity procedure used on the Waters Acquity UPLC is adequately evaluated and verified.

1.1.2. To provide the justification for the following method changes to BSI-SOP-0430: mobile phase preparation, column stationary phase, column dimensions, and injection volume.

1.1.3. Verify that the optimized Tris Organic Impurity procedure meets requirements for Linearity, Accuracy, Precision, Intermediate Precision, and Specificity.

1.1.4. To establish solution stability for samples and standards.

1.1.5. 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 TRIS Organic Impurities (OI) using BioSpectra's Waters Acquity HPLC.

2.2. Impurity Specifications:

TRIS – Active Pharmaceutical Ingredient – Impurity Specifications	
Name	Acceptance Criteria
Tris(hydroxymethyl)nitromethane	NMT 1 ppm
2-Nitropropane-1,3-diol	NMT 1 ppm
2-Nitroethanol	NMT 1 ppm
Any unspecified impurity	NMT 300 ppm
Total impurities	NMT 300 ppm

3. RESPONSIBILITIES:

3.1. The Senior Chromatography Specialist 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. The Senior Chromatography Specialist is responsible for writing the validation report.

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

4. REFERENCE:

4.1. BSI-RPT-0472, Analytical Method Validation Report: Limit of Tris (Hydroxymethyl) Nitromethane in Tris

4.2. BSI-RPT-0473, Analytical Method Validation Report: Limit of 2-Nitroethanol and 2-Nitropropane-1,3-diol in Tris

4.3. BSI-SOP-0098, Balance SOP

4.4. BSI-SOP-0134, Pipette SOP

4.5. BSI-SOP-0430, Tris Organic Impurities Via UPLC

4.6. BSI-SOP-0436, Analytical Methods Validation Master Plan

4.7. *USP <621> Chromatography*
4.8. *USP <1225> Validation of Compendial Procedures*
4.9. *USP <1226> Validation of Compendial Procedures*
4.10. *Waters ACQUITY UPLC TUV Detector Operator's Overview and Maintenance 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. Equipment:
6.1.1. Analytical Microbalance
6.1.2. Class A volumetric flasks
6.1.3. Waters ACQUITY UPLC H-Class Plus Instrument with UV Detector
6.1.4. LC Column
6.1.4.1. Luna Omega Polar C18, 250 x 4.6 mm, 3µm
6.1.4.2. Part Number: 00G-4760-E0
6.1.5. Eppendorf Autopipettes
6.1.6. Class A Volumetric Glassware
6.2. Reagents:
6.2.1. Water, UPLC grade or equivalent
6.2.2. Phosphoric Acid, 85%, HPLC Grade or equivalent
6.2.3. Potassium Phosphate Monobasic, HPLC Grade or equivalent
6.2.4. Acetonitrile, HPLC Grade or equivalent
6.3. Supplies:
6.3.1. HPLC vials and caps
6.3.2. Transfer pipettes
6.4. Authentic Sample:
6.4.1. Tris base
6.5. Reference Standards:
6.5.1. Tris(hydroxymethyl)nitromethane, Reagent grade
6.5.2. 2-Nitropropane-1,3-diol, Reagent grade
6.5.3. 2-Nitroethanol, NLT 97.0% purity

7. GENERAL TESTING PROCEDURE:

7.1. Glassware Cleaning:

7.1.1. This test method is making determinations at the ppb level. During the execution of this method, great care must be taken to assure cleanliness of all glassware.

7.1.2. Prior to use, glassware must be cleaned using the following general process:

7.1.2.1. Determine the glassware needed to perform the test and gather the glassware (e.g. 3 –100 mL volumetric flasks, 3 – stoppers, 2 – beakers, etc.).

7.1.2.2. Ensure that the mobile phase bottle does not have a plastic pouring ring. If so, remove it and ensure the bottle and bottle rim is thoroughly cleaned with water.

7.1.2.3. Thoroughly clean all glassware, including stoppers with ≥ 5x rinses with HPLC grade purified water

7.1.2.3.1. **NOTE: Do not use soap or detergents in the rinse solutions to clean any glassware used for this analytical method.**

7.1.2.4. Allow glassware to dry prior to use.

7.2. Solution Preparation:

7.2.1. 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.

7.2.2. Mobile Phase: 0.68% Potassium Phosphate (0.68:100, W:V), pH 2.0

7.2.2.1. Combine 6.80 g (± 5%) of potassium phosphate monobasic and 1000 mL of HPLC grade water.

7.2.2.2. Stir until fully dissolved.

7.2.2.3. Adjust pH to 2.00 (± 0.05) with phosphoric acid

7.2.2.4. Expires one week (7 days) after preparation.

Tris(hydroxymethyl)nitromethane (THNM) Standard Solutions	
Stock	500 µg/mL
Intermediate	1.0 µg/mL

7.2.3. Tris(hydroxymethyl)nitromethane Stock Standard (THNM) – 500 µg/mL
- 7.2.3.1. Accurately weigh 50 mg of tris(hydroxymethyl)nitromethane reference standard and transfer into a 100 mL volumetric flask.
- 7.2.3.2. Fill~3/4 full with mobile phase and swirl to dissolve.
- 7.2.3.3. Fill to volume with mobile phase and mix thoroughly.

7.2.4. Tris(hydroxymethyl)nitromethane Intermediate Standard – 1.0 µg/mL
- 7.2.4.1. Pipette 200 µL of Tris(hydroxymethyl)nitromethane Stock solution into a 100 mL volumetric flask.
- 7.2.4.2. Fill to volume with mobile phase and mix thoroughly.

2-Nitroethanol (NE) Standard Solutions	
Stock	500 µg/mL
Intermediate	1.0 µg/mL

7.2.5. 2-Nitroethanol Stock Standard (NE) – 500 µg/mL
- 7.2.5.1. Add ~25 mL of mobile phase into a 250 mL volumetric flask.
- 7.2.5.2. Place the volumetric flask onto an analytical balance and tare.
- 7.2.5.3. Pipette 100 µL of 2-Nitroethanol reference standard into the flask and record the weight.
  - 7.2.5.3.1. Should be ~127 mg (±10%)
- 7.2.5.4. Fill to volume with mobile phase and mix thoroughly

7.2.6. 2-Nitroethanol Intermediate Standard – 1.0 µg/mL
- 7.2.6.1. Pipette 200 µL of 2-Nitroethanol Stock solution into a 100 mL volumetric flask.
- 7.2.6.2. Fill to volume with mobile phase and mix thoroughly

2-Nitropropane-1,3-diol (NPD) Standard Solutions	
Stock	500 µg/mL
Intermediate	1.0 µg/mL

7.2.7. 2-Nitropropane-1,3-diol Stock Standard (NPD) – 500 µg/mL
- 7.2.7.1. Accurately weigh 50 mg of 2-Nitropropane-1,3-diol standard and transfer into a 100 mL volumetric flask.
- 7.2.7.2. Fill~3/4 full with mobile phase and swirl to dissolve.
- 7.2.7.3. Fill to volume with mobile phase and mix thoroughly

7.2.8. 2-Nitropropane-1,3-diol Intermediate Standard – 1.0 µg/mL
- 7.2.8.1. Pipette 200 µL of 2-Nitropropane-1,3-diol Stock solution into a 100 mL volumetric flask.
- 7.2.8.2. Fill to volume with mobile phase and mix thoroughly

Resolution Standard Solution		
Impurity ID	Solution Concentration	Corresponding Sample Concentration
THNM	0.02 µg/mL	1 ppm (µg/g)
NE	0.02 µg/mL	1 ppm (µg/g)
NPD	0.02 µg/mL	1 ppm (µg/g)

7.2.9. Resolution Standard Solution – 0.02 µg/mL Known Impurities (1 ppm with respect to the nominal 20 mg/mL Tris base sample solution)

7.2.9.1. Pipette 1.0 mL each of the THNM, NE, and NPD intermediate standard solutions into the same 50 mL volumetric flask

7.2.9.2. Fill to volume with mobile phase and mix thoroughly

7.2.9.3. Solution stability: To be determined during validation

LOQ Standard Solution		
Impurity ID	Solution Concentration	Corresponding Sample Concentration
THNM	0.01 µg/mL	0.5 ppm (µg/g)
NE	0.01 µg/mL	0.5 ppm (µg/g)
NPD	0.01 µg/mL	0.5 ppm (µg/g)

7.2.10. LOQ Solution – 0.01 µg/mL Known Impurities (0.5 ppm with respect to the nominal 20 mg/mL Tris base sample solution)

7.2.10.1. Pipette 500 µL each of the THNM, NE, and NPD intermediate standard solutions into the same 50 mL volumetric flask

7.2.10.2. Fill to volume with mobile phase and mix thoroughly

7.2.10.3. Solution stability: To be determined during validation

Calibration Standard Solution		
Impurity ID	Solution Concentration	Corresponding Sample Concentration
THNM	0.02 µg/mL	1 ppm (µg/g)

7.2.11. Calibration Standard – 0.02 µg/mL THMN (1 ppm with respect to the nominal 20 mg/mL Tris sample solution)

7.2.11.1. Pipette 1.0 mL of the THNM Intermediate Standard Solution into a 50 mL volumetric flask

7.2.11.2. Fill to volume with mobile phase and mix thoroughly

7.2.11.3. Solution stability: To be determined during validation

7.3. Sample Preparation and Analysis

7.3.1. Sample – 20 mg/mL Tris

7.3.1.1. Weigh 1.0 g (± 5%) of Tris on an appropriately sized weighing dish

7.3.1.2. Zero the balance and print

7.3.1.3. Transfer to a clean, dry 50 mL volumetric flask.

7.3.1.4. Return the weighing dish to the balance and print the negative weight.

7.3.1.5. Set the samples aside.

7.3.1.5.1. **Note:** Due to the chemical stability profile of THNM in solution, it is crucial that the volumetric flasks used for sample preparation are clean and dry.

7.3.2. Ensure the sample compartment and column compartment are equilibrated to 10 °C and 40 °C, respectively.

7.3.3. Initiate the System suitability Injection sequence per section 7.4.3. Ensure System suitability parameters meet acceptance criteria prior to injecting samples. The sequence may be paused if additional time is needed to assess the system suitability parameters.

7.3.4. Sample Dilution and Injection:

7.3.4.1. Follow the sample injection sequence outlined in Section 7.4.3

7.3.4.2. Within 10 min of the injection, fill the flask ~3/4 with mobile phase and swirl for ~30 sec until the sample is full dissolved.

7.3.4.3. Fill to volume with mobile phase and mix thoroughly.

7.3.4.4. Transfer an aliquot to an HPLC vial, cap, and place onto the instrument for analysis.

7.3.4.4.1. **Note**: The samples must be injected NMT 10 min after the addition of solvent. The injection sequence may be paused to meet stability timing requirements.

7.3.4.5. Repeat steps 7.3.1 through 7.3.4 for additional samples.

7.4. System Setup:

7.4.1. Waters Acquity LC Method Parameters:

Parameter	Setting
Flow Type	Isocratic
Mobile Phase A	0.68% Potassium Phosphate pH 2.00
ACQUITY Solvent and Sample Manager	
Flow Rate	1.0 mL/min
Run Time	6 min
Injection Volume	20 µL
Column Temperature (°C)	40 ± 1
Sample Temperature (°C)	10 ± 1
ACQUITY TUV Detector	
Detection Wavelength	210 nm
Sampling Rate	10 Points/Sec

7.4.2. Column Conditioning/System Equilibration:

7.4.2.1. Install the column and prime the system with mobile phase.

7.4.2.2. Slowly bring the flow rate up to 1.0 mL/min.

7.4.2.3. Turn on the sample compartment and allow to cool and stabilize at 10°C.

7.4.2.4. Turn on the column compartment and allow the column to warm and stabilize at 40°C.

7.4.2.5. Place the standard solutions onto the instrument and allow the standards to equilibrate to the 10 °C sample compartment (Approximately 30 min).

7.4.2.5.1. In order to maintain the 10 °C sample compartment temperature. Load standards and samples onto the instrument as quickly as possible. Do not leave the sample compartment door open for extended periods.

7.4.2.6. At the end of each analysis, clean the column using a gradient of purified water and acetonitrile.

7.4.2.6.1. Final storage solution: 65:35, Acetonitrile: Purified Water

7.4.3. Injection Sequence:

Sample ID	Number of Injections
System Suitability[1]	
Mobile Phase	≥ 2
LOQ Solution	1
Resolution Solution	1
Calibration Standard	6
Samples[2]	
Mobile Phase	1
Samples[2]	≤ 6
QC Check (Calibration Standard)[3]	1

[1]Ensure system suitability met requirements prior to injecting the samples. If necessary, pause the injection sequence after the final calibration standard injection to evaluate system suitability.

[2]Samples are to be injected within 10 minutes of adding solvent. If necessary, pause the injection sequence, dilute the sample, place onto the instrument, and re-initiate the injection sequence.

[3]A calibration standard must be injected once every six (6) samples, or, if the injection sequence was paused, within 90 minutes of the previous calibration standard.

7.4.4. System Suitability:

System Suitability Parameter	Acceptance Criteria
%RSD of the peak area of THNM in the first six (6) *Calibration Standard* injections.	NMT 5%
%RSD of the peak area of THNM in all *Calibration Standard* injections.	NMT 5%
USP Resolution between THNM and NPD in the *Resolution Standard* injection.	NLT 0.9
USP Resolution between NE and THNM in *Resolution Standard* injection.	NLT 1.2
USP S/N value of each specified impurity in the *LOQ Standard* injection	NLT 10
Baseline interference (peak area) at the retention times corresponding THNM, NPD, and NE in the *Mobile Phase* injection.	NMT 1/2 the peak areas corresponding to THNM, NPD, and NE in the LOQ injection

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

7.4.5.1. Note: Ignore all peaks NMT than ½ the area of NPD in LOQ injection.

7.4.5.2. Impurity Result (ppm) = $(R_U \times RRF)/R_{CS} \times (C_{CS}/C_U)$

7.4.5.2.1. R_{CS} = Average peak area of THNM from all Calibration Standard Injection injections

7.4.5.2.2. R_U = Peak area of each individual impurity from the sample injection

7.4.5.2.3. C_{CS} = Concentration of the calibration standard (µg/mL) x Purity

7.4.5.2.4. C_U = Concentration of TRIS in the sample (g/mL)

7.4.5.2.5. RRF = Relative Response Factor

7.4.5.2.5.1. RRFs to be determined during validation

8. VALIDATION PROCEDURE:

8.1. System Suitability:

8.1.1. Refer to the injection sequence (7.4.3) and the system suitability acceptance criteria (7.4.4).

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) Mobile Phase Injection, one (1) Resolution Solution injection, one (1) Calibration Standard injection, one (1) 100% Level Accuracy and Precision Sample injection, one (1) Tris Blank Injection, and one (1) LOQ injection.

8.2.2. Acceptance Criteria:

8.2.2.1. The THNM peak has a USP Resolution of NLT 0.9 in the 100% Level Accuracy and Precision Sample injection.

8.2.2.2. The NE peak has a USP Resolution of NLT 1.2 in the 100% Level Accuracy and Precision Sample injection.

8.2.2.3. The Mobile phase chromatogram meets the interference system suitability criterion outlines in section

8.3. Calibration Standard Linearity

8.3.1. Tris(hydroxmethyl)nitromethane calibration standard will be evaluated from 50% to 150% of the 1 ppm impurity specification. The solutions will be prepared by diluting the THNM intermediate standard with mobile phase. The slope of the line will be used to calculate relative response factors for 2-Nitroethanol and 2-Nitropropane-1,3-diol.

8.3.2. Linearity solution preparation:

8.3.2.1. Pipette the amount specified in the table below into separate 50 mL volumetric flasks.

8.3.2.2. Fill to volume with mobile phase and mix well.

8.3.2.2.1. Note: the solutions **do not** need to be injected within 10 minutes of preparation.

8.3.3. Calibration Standard Linearity – Sample Preparation Table:

Calibration Standard – Linearity (50% - 150%)					
Level	Concentration (µg/mL)	Concentration ppm (µg/g)	THNM Intermediate standard (1.0 µg/mL) Amount to Pipette (mL)	Final Volume (mL)	Diluent
50	0.010	0.50	0.50	50	Mobile Phase
75	0.015	0.75	0.75		
100	0.020	1.00	1.00		
125	0.025	1.25	1.25		
150	0.030	1.50	1.50		

8.3.4. Inject each level in triplicate. Plot peak area against concentration, perform a linear regression, calculate the y-intercept bias with respect to the 100% Level, and plot the residuals.

Calculations:

8.3.4.1. Y-Intercept bias (%) = (Y-Intercept/average 100% Level peak area) x 100

8.3.5. Acceptance Criteria:

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

8.3.5.2. Correlation Coefficient: NLT 0.990

8.3.5.3. Y-intercept bias: NMT 25.0%

8.4. Impurity Linearity

8.4.1. All known impurities will be evaluated from 50% to 150% of the 1 ppm impurity specification. The intermediate standards prepared throughout section 7 will be spiked into a nominal 20 mg/mL Tris base solution. The relative response factors will be calculated using the slopes of 2-Nitropropane -1,3-diol and 2-Nitroethanol.

8.4.2. Tris Stock Solution – 20 mg/mL

8.4.2.1. Weigh and transfer 20 g (± 5%) of Tris Base into a 1000 mL volumetric flask.

8.4.2.2. Fill ~3/4 full with diluent and swirl for ~30 seconds until the sample is fully dissolved.

8.4.2.3. Fill to volume with diluent and mix thoroughly.

8.4.3. Linearity Solution preparation

8.4.3.1. Pipette the amount specified in the sample preparation tables into separate 50 mL volumetric flasks.

8.4.3.2. Fill to volume with the *Tris Stock Solution* and mix thoroughly

8.4.3.2.1. The 2-Nitropropane-1,3-diol and 2-Nitroethanol linearity solutions **do not** need to be injected within 10 minutes of adding the *Tris Stock Solution.*

8.4.3.2.2. **Inject all Tris(hydroxymethyl)nitromethane linearity solutions within 10 min of adding the *Tris Stock Solution*.**

8.4.4. 2-Nitropropane-1,3-diol Linearity – Sample Preparation Table:

2-Nitropropane-1,3-diol – Linearity (50% - 150%)					
Level	Concentration (µg/mL)	Concentration ppm (µg/g)	NPD Intermediate standard (1.0 µg/mL) Amount to Pipette (mL)	Final Volume (mL)	Diluent
50	0.010	0.50	0.50	50	Tris Stock (20 mg/mL)
75	0.015	0.75	0.75		
100	0.020	1.00	1.00		
125	0.025	1.25	1.25		
150	0.030	1.50	1.50		

8.4.5. 2-Nitroethanol Linearity – Sample Preparation Table:

2-Nitroethanol – Linearity (50% - 150%)					
Level	Concentration (µg/mL)	Concentration ppm (µg/g)	NE Intermediate standard (1.0 µg/mL) Amount to Pipette (mL)	Final Volume (mL)	Diluent
50	0.010	0.50	0.50	50	Tris Stock (20 mg/mL)
75	0.015	0.75	0.75		
100	0.020	1.00	1.00		
125	0.025	1.25	1.25		
150	0.030	1.50	1.50		

8.4.6. Tris(hydroxymethyl)nitromethane Linearity – Sample Preparation Table:

Tris(hydroxymethyl)nitromethane – Linearity (50% - 150%)					
Level	Concentration (µg/mL)	Concentration ppm (µg/g)	THNM Intermediate standard (1.0 µg/mL) Amount to Pipette (mL)	Final Volume (mL)	Diluent
50	0.010	0.50	0.50	50	Tris Stock (20 mg/mL)
75	0.015	0.75	0.75		
100	0.020	1.00	1.00		
125	0.025	1.25	1.25		
150	0.030	1.50	1.50		

8.4.7. Perform a single injection for each replicate. Plot peak area against concentration, perform a linear regression, calculate the y-intercept bias with respect to the 100% Level. Calculate the relative response factors for NPD and NE.

8.4.8. Calculations:

8.4.8.1. Y-Intercept bias (%) = (Y-Intercept/average 100% Level peak area) x 100

8.4.8.2. Relative Response Factor = M_{Std}/M_{Smp}

8.4.8.2.1. Where:

8.4.8.2.2. M_{Std} = Slope of THNM from Section 8.3.

8.4.8.2.3. M_{Smp} = Slope of NPD and NE from Section 8.4

8.4.9. Acceptance Criteria:

8.4.9.1. Report the y-intercept, slope, and residual sum of squares for each impurity.

8.4.9.2. For each impurity, the correlation coefficient is NLT 0.990

8.4.9.3. For each impurity, the Y-intercept bias is NMT 25.0%

8.4.9.4. Report the relative response factors for NPD and NE.

8.5. Accuracy and Precision:

8.5.1. Accuracy and precision samples will be evaluated from 50% to 150% of the 1 ppm Impurity specification. Tris base will be weighed out into individual volumetric flasks and will be spiked with known quantities of impurities. For the 50% and 150% levels, a single impurity will be evaluated as the quantities for the remaining two specified impurities will be held constant at the 1 ppm specification. For the 100% Level, all impurities will be evaluated, and the data obtained will be used as Analyst 1 for Intermediate Precision.

8.5.2. Tris Base Blank Samples (n = 2)

8.5.2.1. Carryout the sample preparation and injection procedure outlined in Section 7.3.

8.5.3. Accuracy and Precision Sample Preparation and Analysis:

8.5.3.1. Sample weigh out – 20 mg/mL Tris

8.5.3.1.1. Weigh 1.0 g (± 5%) of Tris on an appropriately sized weighing dish

8.5.3.1.2. Zero the balance and print

8.5.3.1.3. Transfer to a clean, dry 50 mL volumetric flask.

8.5.3.1.4. Return the weighing dish to the balance and print the negative weight.

8.5.3.1.5. Set the samples aside.

8.5.3.1.5.1. **Note:** Due to the chemical stability profile of THNM in solution, it is crucial that the volumetric flasks used for sample preparation are clean and dry.

8.5.3.2. Ensure the sample compartment and column compartment are equilibrated to 10 °C and 40 °C, respectively.

8.5.3.3. Initiate the System suitability Injection sequence per section 7.4.3. Ensure System suitability parameters meet acceptance criteria prior to injecting samples. The sequence may be paused if additional time is needed to assess the system suitability parameters.

8.5.3.4. Sample Dilution and Injection:

8.5.3.4.1. Follow the sample injection sequence outlined in Section 7.4.3

8.5.3.4.2. Within 10 min of the injection, pipette the amount of impurity intermediate standards specified in the sample preparation tables into the flask.

8.5.3.4.3. Fill the flask ~3/4 with mobile phase and swirl for ~30 sec until the sample is full dissolved.

8.5.3.4.4. Fill to volume with mobile phase and mix thoroughly.

8.5.3.4.5. Transfer an aliquot to an HPLC vial and place onto the instrument for analysis.

8.5.3.4.5.1. **Note**: The samples must be injected NMT 10 min after the addition of solvent. The injection sequence may be paused to meet stability timing requirements.

8.5.4. 2-Niropropane-1,3-diol Accuracy and Precision Samples (n=3 for each level)

2-Nitropropane-1,3-diol – Accuracy and Precision – 50% and 150% Levels							
		NPD Intermediate standard (1.0 µg/mL)	THMN Intermediate standard (1.0 µg/mL)	NE Intermediate standard (1.0 µg/mL)	Tris Base		
Level (%)	Concentration (µg/mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to weigh (g)	Final Volume (mL)	Diluent
50	0.01	0.50	1.00	1.00	1.0	50	mobile phase
150	0.03	1.50					

8.5.5. 2-Nitroethanol Accuracy and Precision Samples (n=3 for each level)

2-Nitroethanol – Accuracy and Precision – 50% and 150% Levels							
		NE Intermediate standard (1.0 µg/mL)	THMN Intermediate standard (1.0 µg/mL)	NPD Intermediate standard (1.0 µg/mL)	Tris Base		
Level (%)	Concentration (µg/mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to weigh (g)	Final Volume (mL)	Diluent
50	0.01	0.50	1.00	1.00	1.0	50	mobile phase
150	0.03	1.50					

8.5.6. Tris(hydroxymethyl)nitromethane Accuracy and Precision Samples (n=3 for each level)

Tris(hydroxymethyl)nitromethane – Accuracy and Precision – 50% and 150% Levels							
		THMN Intermediate standard (1.0 µg/mL)	NE Intermediate standard (1.0 µg/mL)	NPD Intermediate standard (1.0 µg/mL)	Tris Base		
Level (%)	Concentration (µg/mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to weigh (g)	Final Volume (mL)	Diluent
50	0.01	0.50	1.00	1.00	1.0	50	mobile phase
150	0.03	1.50					

8.5.7. 100% Level Accuracy and Precision Samples (n=6)

All Impurities– Accuracy and Precision – 100% Levels							
		THMN Intermediate standard (1.0 µg/mL)	NE Intermediate standard (1.0 µg/mL)	NPD Intermediate standard (1.0 µg/mL)	Tris Base		
Level (%)	Concentration (µg/mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to Pipette (mL)	Amount to weigh (g)	Final Volume (mL)	Diluent
100	0.02	1.00	1.00	1.00	1.0	50	mobile phase

8.5.8. Perform a single injection for each replicate. Using the RRFs from the previous linearity studies, calculate the impurity values in ppm, the %Recoveries, and the %RSD of the %Recoveries for all analytes.

8.5.9. Calculations:

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

8.5.9.1.1. A_M = amount of impurity measured

8.5.9.1.2. A_{Added} = amount of impurity added

8.5.9.1.3. A_0 = average amount of analyte measured in the Blank samples

8.5.10. Acceptance Criteria:
- 8.5.10.1. The %Recovery for each replicate at the 50% level for each individual impurity is between 50.0% and 150.0%.
- 8.5.10.2. The % Recovery for each replicate at the 100% and 150% levels for each individual impurity is 80.0%-120.0%.
- 8.5.10.3. The %RSD at the 100% level is NMT 15.0%.

8.6. Intermediate Precision:

8.6.1. Repeat the Accuracy and Precision exercise outlined in Section 8.5 for the 100% level only. Using the RRFs from the previous linearity studies, calculate the impurity values in ppm, the %Recoveries, and the %RSD of the %Recoveries for all analytes. Combine the %Recovery values with those obtained by Analyst 1 and calculate the Combined %RSD.

8.6.2. Acceptance Criteria:
- 8.6.2.1. For Analysts 2: The % Recovery for each replicate at the 100% level for all impurities is 80.0%-120.0%.
- 8.6.2.2. For Analyst 2: The %RSD of the 100% level is NMT 15.0%
- 8.6.2.3. For the combined %Recoveries (Analyst 1 + Analyst 2): The %RSD of the 100% level for all impurities is NMT 20.0%.

8.7. Limit of Quantitation

8.7.1. The LOQ solution (Section 7.2.10) will be prepared in the mobile phase to enhance solution stability. This solution will be used during the system suitability test (SST) of routine analyses to evaluate the sensitivity of the instrument. During routine analyses, the detection threshold of the processing method will be set to ½ the area of the Tris(hydroxymethy)nitromethane peak.
- 8.7.1.1. Note: Accuracy and Precision of the LOQ will be assessed in Section 8.5.

8.7.2. Perform 6 Injections of the LOQ solution (Section 7.2.10).

8.7.3. Acceptance Criteria:
- 8.7.3.1. The %RSD of for all impurities is NMT 10%.
- 8.7.3.2. The USP S/N for all impurities in each in injection is NLT 10.

8.8. Solution Stability:

8.8.1. Sample solution:
- 8.8.1.1. Prepare one (1) 100% level Accuracy and Precision sample per section 8.5 and perform 20 injections of the same solution (bracket every 5 injections with a calibration standard). Plot the %change in response vs time (min) for THMN.
- 8.8.1.2. Acceptance Criteria:
  - 8.8.1.2.1. The solution will be considered stable until 20% degradation is observed.

8.8.2. Standard Solutions:
- 8.8.2.1. Store one (1) Calibration Standard solution, one (1) LOQ Standard solution, and one (1) Resolution Standard solution in the original glassware at normal lighting and laboratory conditions.
  - 8.8.2.1.1. The solutions should be stored for at least one day before assessing solution stability.
  - 8.8.2.1.2. Analyze the aged solutions against freshly prepared standards and calculate the %Agreement between the fresh and aged solutions.

8.8.2.2. Calculations:

8.8.2.2.1. Percent Agreement of the calibration standard = (R_{CSA}/R_{CS}) x (C_{CS}/C_{CSA}) x 100

8.8.2.2.1.1. R_{CS} = average peak response of THMN the all *Calibration Standard* Injections

8.8.2.2.1.2. R_{CSA} = peak response of THMN aged *Calibration Standard* Injection

8.8.2.2.1.3. C_{CS} = Concentration of THMN in in the fresh *Calibration Standard*

8.8.2.2.1.4. C_{CSA} = Concentration of THMN in in the Aged *Calibration Standard*

8.8.2.2.2. Percent Agreement of the Resolution/LOQ solution = (R_{SA}/R_{SF}) x (C_{SF}/C_{SA}) x 100

8.8.2.2.2.1. R_{SA} = Peak response of each impurity in the aged standard (Reso/LOQ) solution.

8.8.2.2.2.2. R_{SF} = Peak response of each impurity in the fresh standard (Reso/LOQ) solution

8.8.2.2.2.3. C_{SF} = Concentration of each impurity in the fresh standard (Reso/LOQ) solution

8.8.2.2.2.4. C_{SA} = Concentration of each impurity in the aged standard (Reso/LOQ) solution

8.8.2.2.3. % Change of THMN= $(R_A\text{-}R_i)/R_i$ x 100

8.8.2.2.3.1. R_A = Peak response of THMN in the aged sample solution.

8.8.2.2.3.2. R_i = Initial peak response of THMN in the first injection

8.8.2.3. Acceptance Criteria:

8.8.2.3.1. For all impurities, the %Agreement between aged and fresh calibration standards is between 80.0% and 120.0%

8.8.2.3.2. For all impurities, the %Agreement between aged and fresh LOQ standards is between 50% and 150.0%

8.8.2.3.3. The S/N of each impurity in the aged LOQ standard chromatogram is NLT 10.

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.