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

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1. PURPOSE:

1.1. The purpose of this report is to:

1.1.1. Ensure that the Tris Organic Impurity analytical validation, which was performed on the Waters Acquity UPLC was adequately evaluated.

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. To summarize the findings from the Tris Organic Impurity analytical validation and demonstrate that the analytical method meets all requirements for: System Suitability, Accuracy, Precision, Specificity, Linearity, Limit of Quantitation, Range, Solution Stability and Robustness.

2. SCOPE:

2.1. This Analytical Method Validation report applies to TRIS Organic Impurities (OI) using BioSpectra's Waters Acquity HPLC.

2.2. Total Organic 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

2.3. ***This method will no longer be utilized to analyze for Any Unspecified Impurity and will only be applicable for Specified Impurities analysis. BSI-RPT-1226, was revised to show this update. Refer to the section 10. Validation Status for more details.***

3. RESPONSIBILITIES:

3.1. The Director of Laboratory Services, or other qualified personnel, if necessary, are responsible for completing the Method Validation Report using conclusions made from the results obtained from testing.

4. REFERENCES:

4.1. BSI-ATM-0050, Analytical Method: Quantification of Formaldehyde by Derivatization with Pentafluorobenzylhydroxyl Amine by GC MS

4.2. BSI-ATM-0112, Tromethamine Unspecified Degradation Products Via GC-FID

4.3. BSI-PRL-0618, Analytical Method Validation Protocol: TRIS Organic Impurities via Liquid Chromatography with UV Detection

4.4. BSI-PRL-0768, Analytical Method Validation Protocol Addendum: TRIS Organic Impurities via Liquid Chromatography with UV Detection Robustness

4.5. BSI-RPT-0472, Analytical Method Validation Report: Limit of Tris(hydroxymethyl) nitromethane in Tris

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

4.7. BSI-SOP-0098, Balance SOP

4.8. BSI-SOP-0134, Pipette SOP
4.9. BSI-SOP-0430, Tris Organic Impurities Via UPLC
4.10. BSI-SOP-0436, Analytical Methods Validation Master Plan
4.11. *USP <621> Chromatography*
4.12. *USP <1225> Validation of Compendial Procedures*
4.13. *USP <1226> Validation of Compendial Procedures*
4.14. *Waters ACQUITY UPLC TUV Detector Operator's Overview and Maintenance Guide*

5. PRE-VALIDATION REQUIREMENTS:

5.1. Equipment
5.1.1. All equipment used in this Validation was in proper working order and within calibration. Serial numbers, date of last calibration, and the calibration due date for each instrument and equipment, where applicable, are included in the report.
5.2. Personnel
5.2.1. All personnel who executed this Validation were properly trained in accordance with the Analytical Methods Validation Master Plan.
5.3. Supplies
5.3.1. All supplies used in the Validation were clean and appropriate for their intended use. Suppliers and part numbers of all supplies are included in this report.
5.4. Reagents
5.4.1. All reagents were current and suitable for their intended use. The reagent name, lot number, manufacturer, date of opening, date of expiration, and part number are included in this report.
5.5. Reference Standards
5.5.1. All reference standards that were used in this Validation are listed in Section 6. The name of the reference standard, lot number, manufacturer, date of opening, date of expiration, and part number for reference standards used are included in this report.

6. MATERIALS AND EQUIPMENT:

6.1. Equipment:
6.1.1. Analytical Balance
6.1.1.1. Manufacturer: Sartorius
6.1.1.2. Model: Secura 124-1S
6.1.1.3. Serial Number: 29212172
6.1.1.4. Method validations except Robustness
6.1.1.4.1. Last Serviced: 10/28/22
6.1.1.4.2. Next Service: 04/2023
6.1.1.5. Method validation for Robustness
6.1.1.5.1. Last Serviced: 10/05/23
6.1.1.5.2. Next Service: 04/30/24
6.1.2. Analytical Balance
6.1.2.1. Manufacturer: Sartorius
6.1.2.2. Model: MSE224S
6.1.2.3. Serial Number: 24801744
6.1.2.4. Method validations except Robustness

6.1.2.4.1. Last Serviced: 10/28/22
6.1.2.4.2. Next Service: 04/2023
6.1.2.5. Method validation for Robustness
6.1.2.5.1. Last Serviced: 10/05/23
6.1.2.5.2. Next Service: 04/30/24
6.1.3. Analytical Microbalance
6.1.3.1. Manufacturer: AND Company
6.1.3.2. Model: BM-20
6.1.3.3. Serial Number: T1004421
6.1.3.4. Method validations except Robustness
6.1.3.4.1. Last Serviced: 11/08/22
6.1.3.4.2. Next Service: 04/2023
6.1.3.5. Method validation for Robustness
6.1.3.5.1. Last Serviced: 10/05/23
6.1.3.5.2. Next Service: 04/30/24
6.1.4. pH Meter
6.1.4.1. Manufacturer: FisherBrand
6.1.4.2. Model: Accumet XL200
6.1.4.3. Serial Number: XL94107780
6.1.4.4. Calibrated Daily Before Use
6.1.5. Class A volumetric flasks
6.1.6. Waters ACQUITY UPLC H-Class Plus Instrument with UV Detector
6.1.6.1. 30 cm Column Compartment
6.1.6.1.1. Serial Number: B19UPX416G
6.1.6.1.2. Method validations except Robustness
6.1.6.1.2.1. Last PM: 03/16/22
6.1.6.1.2.2. Next PM due: 03/2023
6.1.6.1.3. Method validation for Robustness
6.1.6.1.3.1. Last PM: 03/06/23
6.1.6.1.3.2. Next PM due: 03/31/24
6.1.6.2. Sample Manager FTN-H
6.1.6.2.1. Serial Number: K18FTP166G
6.1.6.2.2. Method validations except Robustness
6.1.6.2.2.1. Last PM: 04/13/22
6.1.6.2.2.2. Next PM due: 04/2023
6.1.6.2.3. Method validation for Robustness
6.1.6.2.3.1. Last PM: 03/06/23
6.1.6.2.3.2. Next PM due: 03/2024
6.1.6.3. Quaternary Sample Manager
6.1.6.3.1. Serial Number: K18QSP106A
6.1.6.3.2. Method validations except Robustness
6.1.6.3.2.1. Last PM: 04/13/22
6.1.6.3.2.2. Next PM due: 04/2023
6.1.6.3.3. Method validation for Robustness

6.1.6.3.3.1. Last PM: 03/06/23
6.1.6.3.3.2. Next PM due: 03/31/24
6.1.6.4. TUV Detector
6.1.6.4.1. Serial Number: J18TUV016A
6.1.6.4.2. Method validations except Robustness
6.1.6.4.2.1. Last PM: 04/13/22
6.1.6.4.2.2. Next PM due: 04/2023
6.1.6.4.3. Method validation for Robustness
6.1.6.4.3.1. Last PM: 03/06/23
6.1.6.4.3.2. Next PM due: 03/2024
6.1.7. LC Column
6.1.7.1. Luna Omega Polar C18, 250 x 4.6 mm, 3µm
6.1.7.2. Part Number: 00G-4760-E0
6.1.7.3. Serial Number (Analyst 1): H22-349771
6.1.7.4. Serial Number (Analyst 2): H22-349770
6.1.7.5. Serial Number (Robustness): H23-116554
6.1.7.6. Serial Number (Robustness): H22-349771
6.1.7.7. Serial Number (Robustness): H22-324548
6.1.8. Micropipettes
6.1.8.1. Model: 10 µL – 100 µL
6.1.8.1.1. Supplier: Eppendorf
6.1.8.1.2. Serial Number: N31016H
6.1.8.1.3. Last Service: 07/14/22
6.1.8.1.4. Next Service: 01/31/23
6.1.8.2. Model: 20 µL – 200 µL
6.1.8.2.1. Supplier: Eppendorf
6.1.8.2.2. Serial Number: N41555G
6.1.8.2.3. Last Service: 08/23/22
6.1.8.2.4. Next Service: 02/28/23
6.1.8.3. Model: 100 µL – 1000 µL
6.1.8.3.1. Supplier: Eppendorf
6.1.8.3.2. Serial Number: R24330H
6.1.8.3.3. Last Service: 07/14/22
6.1.8.3.4. Next Service: 01/31/23
6.1.8.4. Model: 100 µL – 1000 µL
6.1.8.4.1. Supplier: Eppendorf
6.1.8.4.2. Serial Number: O39512B
6.1.8.4.3. Last Service: 06/13/22
6.1.8.4.4. Next Service: 12/31/22
6.1.8.5. Model: 500 µL – 5000 µL
6.1.8.5.1. Supplier: Eppendorf
6.1.8.5.2. Serial Number: K53394I
6.1.8.5.3. Last Service: 06/13/22
6.1.8.5.4. Next Service: 12/31/22

6.1.8.6. Model: 1 mL – 10 mL
6.1.8.6.1. Supplier: Eppendorf
6.1.8.6.2. Serial Number: O34393G
6.1.8.6.3. Last Service: 11/15/22
6.1.8.6.4. Next Service: 05/31/23
6.1.8.7. Model: 30 µL – 300 µL
6.1.8.7.1. Supplier: Eppendorf
6.1.8.7.2. Serial Number: P53563H
6.1.8.7.3. Last Service: 06/20/23
6.1.8.7.4. Next Service: 12/31/23
6.1.8.8. Model: 100 µL – 1000 µL
6.1.8.8.1. Supplier: Eppendorf
6.1.8.8.2. Serial Number: R14419C
6.1.8.8.3. Last Service: 08/25/23
6.1.8.8.4. Next Service: 02/29/24

6.2. Reagents:
6.2.1. HPLC Grade Water (Milli-Q Purified Water)
6.2.1.1. Supplier: Millipore Sigma
6.2.1.2. Serial Number: F9SA14284H
6.2.1.3. Method validations except Robustness
6.2.1.3.1. Last Service: 06/14/22
6.2.1.3.2. Next Service: 06/2023
6.2.1.4. Method validation for Robustness
6.2.1.4.1. Last PM: 06/02/23
6.2.1.4.2. Next PM due: 06/30/24
6.2.2. Phosphoric Acid, 85%, HPLC Grade or equivalent
6.2.2.1. Supplier: Fisher
6.2.2.2. Lot Number: 190331
6.2.2.3. Date of Opening: 1/15/20
6.2.2.4. Expiration: 3/31/24
6.2.3. Potassium Phosphate Monobasic, HPLC Grade or equivalent
6.2.3.1. Supplier: Fisher
6.2.3.2. Lot Numbers: 207694, 215057, 217958
6.2.3.3. Date of Opening: 10/18/21, 03/01/22, 07/05/23
6.2.3.4. Expiration: 01/31/23, 10/31/23, 05/31/24

6.3. Supplies:
6.3.1. Disposable Polypropylene Weighing Funnels
6.3.1.1. Supplier: TWD Scientific, LLC
6.3.1.2. Part Number: DPWF-PP1-S
6.3.2. 10mm Screw Top Vials, 2 mL 10 mm x 32 mm and Pre-Slit Lid
6.3.2.1. Supplier: Fisher
6.3.2.2. Part: 03-391-18
6.3.3. Transfer pipettes
6.3.3.1. Supplier: Fisher
6.3.3.2. Part Number: 13-711-9AM

6.3.4. Authentic Tris Base Sample:
6.3.4.1. Supplier: BioSpectra, Inc.
6.3.4.2. Lot Number: TRIS-0122-00137

6.4. Reference Standards:
6.4.1. Tris(hydroxymethyl)nitromethane, Reagent grade
6.4.1.1. Supplier: Sigma-Aldrich
6.4.1.2. Lot: MKBV8368V, MKCP1920
6.4.1.3. CAS Number: 126-11-4
6.4.1.4. Expiration Date: 6/18/23, 06/08/28
6.4.1.5. Purity: 99%
6.4.1.6. Part Number: 108189-500G
6.4.1.7. Date of Opening: 06/18/18, 06/08/23
6.4.2. 2-Nitropropane-1,3-diol, Reagent grade
6.4.2.1. Supplier: Aaron Chemicals
6.4.2.2. Lot: AR2022-950434
6.4.2.3. CAS Number: 1794-90-7
6.4.2.4. Expiration Date: 02/26/27, 11/30/27
6.4.2.5. Purity: 95%
6.4.2.6. Part Number: AR-3815
6.4.2.7. Date of Opening: 11/30/22
6.4.3. 2-Nitroethanol, NLT 97.0% purity
6.4.3.1. Supplier: Acros Chemicals
6.4.3.2. Lot: A0399937
6.4.3.3. CAS Number: 625-48-9
6.4.3.4. Expiration Date: 8/31/24
6.4.3.5. Purity: 99.9%
6.4.3.6. Part Number: 397960050
6.4.3.7. Date of Opening:10/19/21, 12/12/23

7. GENERAL TESTING PROCEDURE:

NOTE: The validation was carried out using the following general testing procedure on each day of analysis.

7.1. Glassware Cleaning:
7.1.1. This test method is making determinations at the parts per billion (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 2.00

7.2.2.1. Combine 6.80 g (± 5%) of potassium phosphate monobasic and 1,000 mL of HPLC grade.

7.2.2.2. Stir until solid is fully dissolved.

7.2.2.3. Adjust pH to 2.00 (± 0.05) with using 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 SUMMARY:

Table 1: Summary of the Validation Performance Parameters, Acceptance Criteria, and Results

Performance Parameters	Acceptance Criteria	Results
System Suitability	• %RSD of the peak area of THNM in the first six (6) *Calibration Standard* injections is NMT 5% • %RSD of the peak area of THNM in all *Calibration Standard* injections is NMT 5% • USP Resolution between THNM and NPD in the *Resolution Standard* injection is NLT 0.9 • USP Resolution between NE and THNM in *Resolution Standard* injection is NLT 1.2. • USP S/N value of each specified impurity in the *LOQ Standard* injection is NLT 10 • Baseline interference (peak area) at the retention times corresponding THNM, NPD, and NE in the *Mobile Phase* injection is NMT 1/2 the peak areas corresponding to THNM, NPD, and NE in the LOQ injection	All system suitability requirements were met for each analysis
Specificity	• The THNM peak has a USP Resolution of NLT 0.9 in the 100% Level Accuracy and Precision Sample injection. • The NE peak has a USP Resolution of NLT 1.2 in the 100% Level Accuracy and Precision Sample injection. • The Mobile phase chromatogram meets the interference system suitability criterion.	• THNM USP Resolution = 0.9 • NE USP Resolution = 1.5 • The mobile phase injection was free of interference and met the interference system suitability criterion.
Calibration Linearity (THNM) 50% to 150%	• Report the y-intercept, slope, and residual sum of squares. • The correlation coefficient (r) is NLT 0.990. • Y-intercept bias is NMT 25.0%	• Y-intercept = -5.3 Slope = 30276 RSS = 2608 • Correlation Coefficient (r) = 0.998 • Y-intercept bias = -0.9%
Impurity Linearity 50% to 150%	• Report the y-intercept, slope, and residual sum of squares. • The correlation coefficient (r) is NLT 0.990. • Y-intercept bias is NMT 25.0%	2-Nitropropane-1,3-diol (NPD) • Y-intercept = 51.8 Slope = 38205 RSS = 796 • Correlation Coefficient (r) = 0.999 • Y-intercept bias = 6.5% • Relative Response Factor = 0.792 Tris(hydroxymethyl)nitromethane (THNM) • Y-intercept = -11.7 Slope = 29870 RSS = 1639 • Correlation Coefficient (r) = 0.996 • Y-intercept bias = -2.1% 2-Nitroethanol (NE) • Y-intercept = -4.9 Slope = 49244 RSS = 1893 • Correlation Coefficient (r) = 0.999 • Y-intercept bias = -0.5% • Relative Response Factor = 0.615

Table 2: Summary of the Validation Performance Parameters, Acceptance Criteria, and Results.

Performance Parameters	Acceptance Criteria	Results		
Accuracy and Precision	• The % Recovery for each replicate at the 50% level for each individual impurity is between 50.0% and 150.0% • The % Recovery for each replicate at the 100% and 150% level for each individual impurity is between 80.0% and 120.0% • The %RSD at the 100% level is NMT 15.0%	NPD	THNM	NE
		• %RSD 50% Level = 1.9 100% Level = 2.1 150% Level = 3.1 • %Recoveries: 50% Level (LOQ) Replicate 1 =116.7 Replicate 2 = 113.7 Replicate 3 = 112.6 100% Level Replicate 1 = 118.7 Replicate 2 = 111.8 Replicate 3 = 115.3 Replicate 4 = 114.9 Replicate 5 = 115.9 Replicate 6 = 112.9 150% Level Replicate 1 = 104.8 Replicate 2 = 111.4 Replicate 3 = 107.3	• %%RSD 50% Level = 7.3 100% Level = 2.9 150% Level = 2.2 • %Recoveries: 50% Level (LOQ) Replicate 1 = 93.5 Replicate 2 = 99.0 Replicate 3 = 108.0 100% Level Replicate 1 = 98.0 Replicate 2 = 94.5 Replicate 3 = 97.3 Replicate 4 = 100.6 Replicate 5 = 100.3 Replicate 6 = 93.7 150% Level Replicate 1 = 106.0 Replicate 2 = 110.6 Replicate 3 =107.7	• %RSD 50% Level = 7.2 100% Level = 2.7 150% Level = 1.3 • %Recoveries: 50% Level (LOQ) Replicate 1 = 91.6 Replicate 2 = 85.9 Replicate 3 = 79.4 100% Level Replicate 1 = 84.3 Replicate 2 = 87.4 Replicate 3 = 90.5 Replicate 4 = 88.1 Replicate 5 = 88.1 Replicate 6 = 84.5 150% Level Replicate 1 = 94.6 Replicate 2 = 95.7 Replicate 3 =93.3
Limit of Quantitation	• The %RSD of the peak areas for all known impurities is NMT 10%. • The USP S/N for all impurities in each injection is NLT 10.	NPD	THNM	NE
		• %RSD = 2.5 • USP S/N: Injection 1 =23 Injection 2 =23 Injection 3 =23 Injection 4 = 22 Injection 5 =22 Injection 6 =22	• %RSD = 3.4 • USP S/N: Injection 1 = 17 Injection 2 = 18 Injection 3 = 18 Injection 4 = 18 Injection 5 = 18 Injection 6 = 18	• %RSD = 5.6 • USP S/N: Injection 1 = 31 Injection 2 = 31 Injection 3 = 30 Injection 4 = 31 Injection 5 = 30 Injection 6 = 30
Range	• Range for specified related substances: The method should demonstrate suitable levels of precision, accuracy, and linearity from 50% to 150% of the 1ppm (µg/g) organic impurity specification.	• The method demonstrated acceptable precision, accuracy and linearity for all impurities from 50% to 150% of the 1 ppm organic impurity specification		

Table 3: Summary of the Validation Performance Parameters, Acceptance Criteria, and Results.

Performance Parameters	Acceptance Criteria	Results		
Intermediate Precision	• For Analyst 2, the % RSD of the %Recoveries is NMT 15.0%. • For Analyst 2, the %Recoveries for all replicates are between 80.0% and 120.0%. • The %RSD for the combined Assay values (analyst 1 + analyst 2) %RSD is NMT 20.0%.	NPD • %RSD = 4.5 Replicate 1 =97.9 Replicate 2 = 101.8 Replicate 3 = 108.8 Replicate 4 = 99.4 Replicate 5 = 95.8 Replicate 6 = 99.7 • Combined %RSD = 7.7	THNM • %RSD = 4.5 Replicate 1 = 96.4 Replicate 2 = 100.2 Replicate 3 = 107.1 Replicate 4 = 97.8 Replicate 5 = 94.3 Replicate 6 = 98.1 • Combined %RSD = 3.7	NE • %RSD = 4.5 Replicate 1 = 92.4 Replicate 2 = 96.1 Replicate 3 = 102.7 Replicate 4 = 93.8 Replicate 5 = 90.4 Replicate 6 = 94.1 • Combined %RSD = 5.7
Standard Solution Stability	• For all impurities, the %Agreement between the aged and fresh Calibration Standard/Resolution solutions is between 80.0% and 120.0%. • For all impurities, the %Agreement between the aged and fresh LOQ standard solution is between 50.0% and 150.0%. • The S/N of each impurity in the aged LOQ standard chromatogram is NLT 10.	NPD **Resolution Solution** • %Agreement: Day 3 = 97.4 Day 6 = 99.4 **LOQ Solution** • %Agreement: Day 3 = 101.6 Day 6 = 94.6 S/N Day 3 = 20 S/N Day 6 = 17	THNM **Resolution Solution** • %Agreement: Day 3 = 84.4 Day 6 = 102.8 **LOQ Solution** • %Agreement: Day 3 = 94.9 Day 6 = 103.0 S/N Day 3 = 17 S/N Day 6 = 16 **Calibration Standard** • %Agreement: Day 3 = 98.8 Day 6 = 101.9	NE **Resolution Solution** • %Agreement: Day 3 = 91.2 Day 6 = 104.2 **LOQ Solution** • %Agreement: Day 3 = 94.6 Day 6 = 110.7 S/N Day 3 = 24 S/N Day 6 = 25
Sample Solution Stability	• The solution will be considered stable until 20% degradation (THNM) is observed	THNM • % Change: The %Change never exceeded 20%. The solution is considered stable for 2.5 hours when stored in an HPLC vial at 10°C.		
Robustness	• All system suitability parameters are met	• All conditions pass system suitability except for column temperature and flow rate of 1.2 mL/minute. Column temperature of 40°C is critical to meet system suitability requirements.		

9. VALIDATION PROCEDURE:

9.1. System Suitability:

9.1.1. System suitability was carried out on each day of analysis. All proposed acceptance criteria were met. The results are summarized in Table 4.

Table 4: Summary of the System Suitability Parameters and Results for Each Reportable Analysis

System Suitability Parameter	Results (notebook reference, date)			
	MV10P49, 12/12/22	MV10P53, 12/13/22	MV11P17, 12/16/22	MV10P65, 12/19/22
%RSD of the peak area of THNM in the first six (6) *Calibration Standard* injections. Criterion: NMT 5%	2%	4%	2%	4%
%RSD of the peak area of THNM in all *Calibration Standard* injections. Criterion: NMT 5%	2%	3%	3%	4%
USP Resolution between THNM and NPD in the *Resolution Standard* injection. Criterion: NLT 0.9	1.0	1.0	1.1	0.9
USP Resolution between NE and THNM in *Resolution Standard* injection. Criterion: NLT 1.2	1.5	1.5	1.5	1.5
USP S/N value of each specified impurity in the *LOQ Standard* injection[1] Criterion: NLT 10	NPD = 21 THNM = 19 NE = 29	NPD = 23 THNM = 19 NE = 31	NPD = 23 THNM = 19 NE = 27	NPD = 16 THNM = 15 NE = 23
Baseline interference (peak area) at the retention times corresponding THNM, NPD, and NE in the *Mobile Phase* injection. Criterion: NMT 1/2 the peak areas corresponding to THNM, NPD, and NE in the LOQ injection	Pass	Pass	Pass	Pass

[1]The noise value may be measured from the blank injection or a stable region of the LOQ chromatogram.

9.2. Specificity:

9.2.1. 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 were overlaid. The 100% Level Accuracy and Precision Sample injection shows USP resolutions values of 0.9, and 1.5 for THNM and NE. No interference was observed at the retention times of NPD, THNM, or NE in the mobile phase (Figure 1). All acceptance criteria were met.

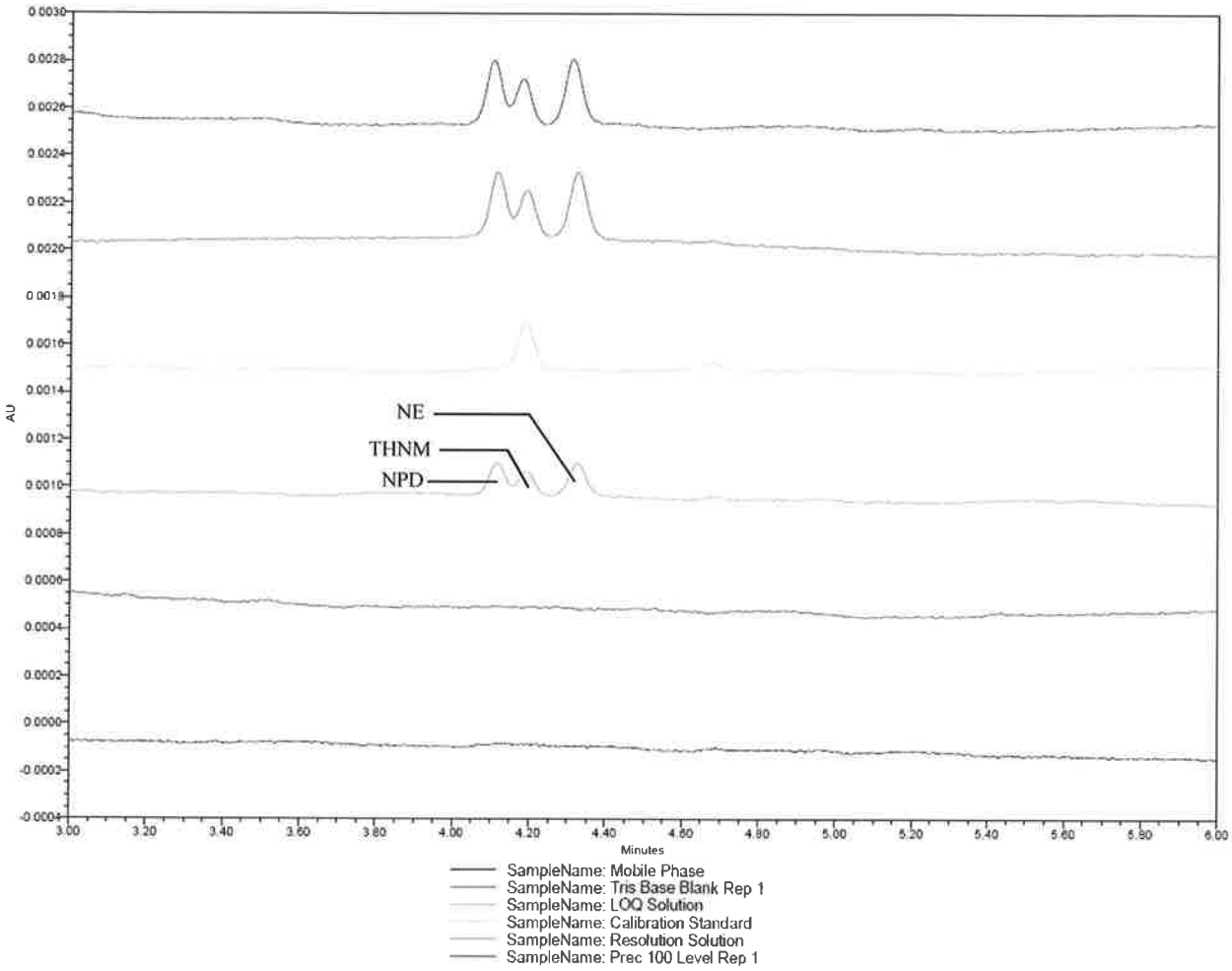


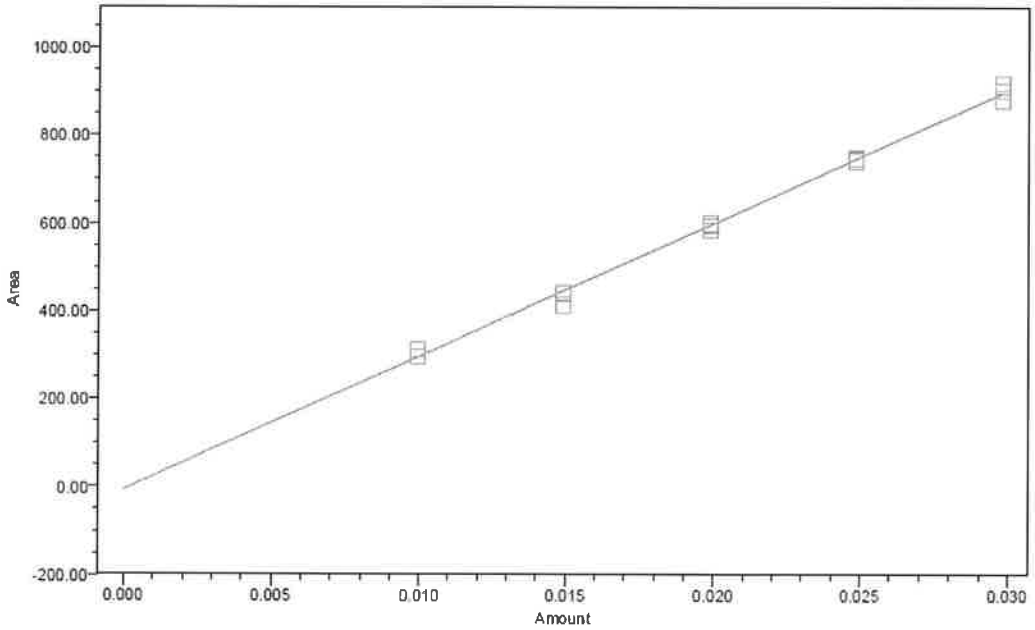
Figure 1: Specificity Overlay

9.3. Calibration Standard Linearity:

9.3.1. Tris(hydroxymethyl)nitromethane calibration standard was evaluated from 50% to 150% of the 1 ppm impurity specification. A stock solution was prepared and sub-diluted with mobile phase to 5 levels ranging from 0.01 µg/mL to 0.03 µg/mL. Each solution was injected in triplicate and the response was plotted against concentration. A linear regression was performed and the residuals were plotted against concentration and appear to be randomly distributed as is shown in Figure 3. The y-intercept bias was calculated with respect to the average peak area response at the proposed 100% Level. All acceptance criteria were met and the results are summarized in Tables 5 and 6. The slope was used to calculate relative response factors for 2-Nitroethanol (NE) and 2-Nitropropane-1,3-diol (NPD) in section 9.4.

Table 5: Calibration Standard Linearity Injection Summary

Calibration Standard Linearity – THNM - 50% - 150%		
Level (% of 1ppm Specification)	Concentration (µg/mL)	Average Peak Area
50	0.010	307.1
75	0.015	432.8
100	0.020	593.9
125	0.025	746.8
150	0.030	902.4



Name: THNM; Cal Curve Id: 5027; R 0.998; Y-Intercept -5.26110; Slope 30275.50084; Residual Sum of Squares 2608.276100

Figure 2: THNM Calibration Standard Linearity and Regression Analysis

Table 6 - THNM Calibration Standard Linearity – Linear Regression and Result Summary.

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	30276	Report	Not Applicable
Y-intercept	-5.3	Report	Not Applicable
Y-intercept Bias	-0.9 %	NMT 25.0%	Pass
Residual Sum of Squares	2608	Report	Not Applicable
R	0.998	NLT 0.990	Pass

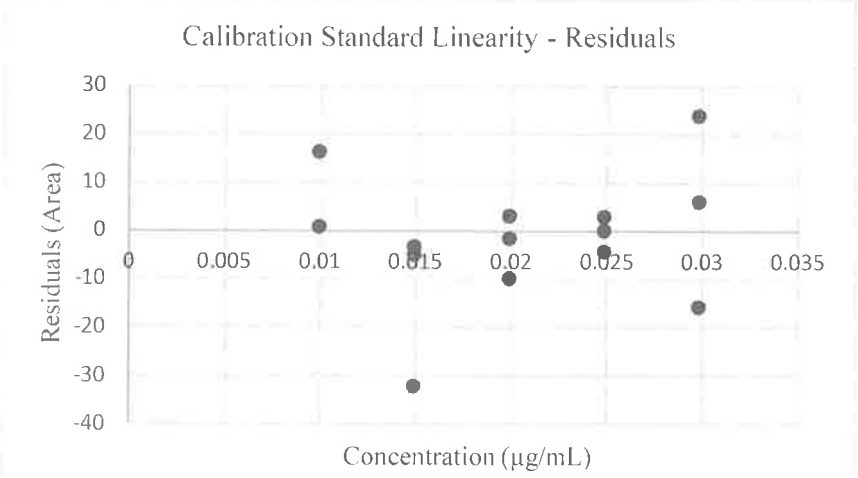


Figure 3: THNM Linearity - Residuals Plotted Against Concentration

9.4. Impurity Linearity:

9.4.1. Tris(hydroxymethyl)nitromethane, 2-Nitroethanol, and 2-Nitropropane-1,3-diol impurity standards were evaluated from 50% to 150% of the 1 ppm impurity specification. A stock solution was prepared and sub-diluted with mobile phase to 5 levels ranging from 0.01 µg/mL to 0.03 µg/mL. Each solution was injected once, the response was plotted against concentration and a linear regression was performed for all analytes (Figures 4, 5 and 6). The y-intercept bias was calculated with respect to the peak area response at the proposed 100% Level. All acceptance criteria were met and the results are summarized in Tables 7 through 12.

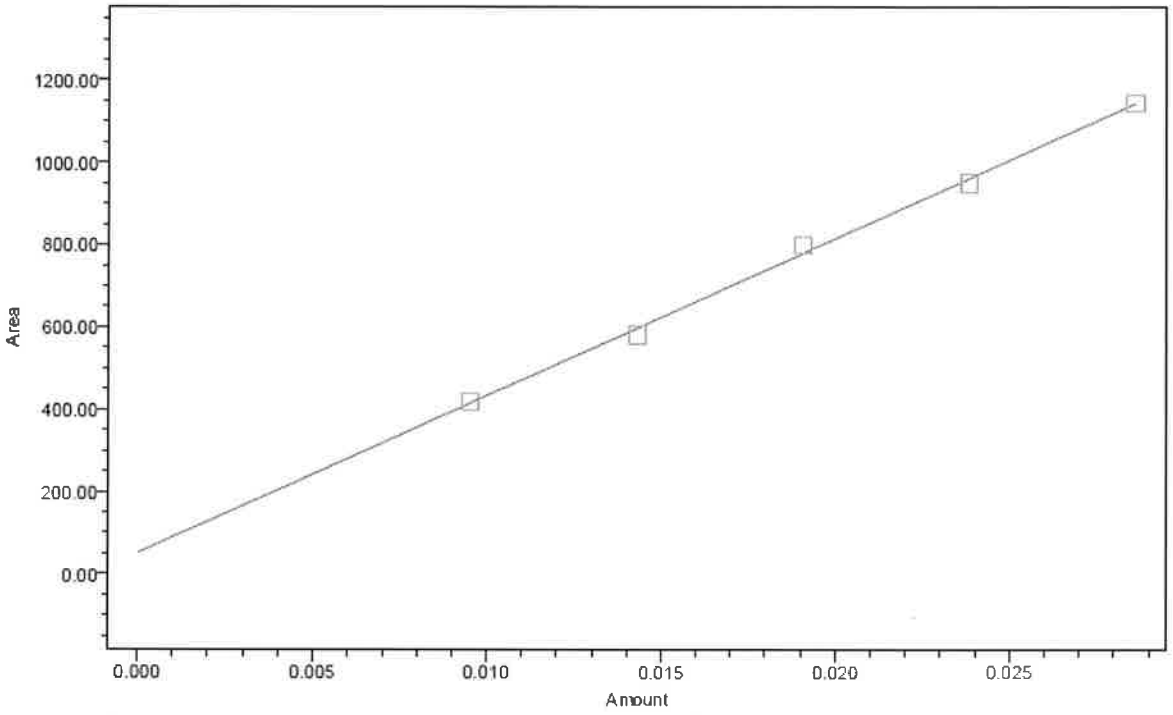
Table 7: NPD Linearity Injection Summary

Impurity Linearity – NPD – 50% - 150%				
Level (% Specification)	Solution Concentration (µg/mL)	Sample Concentration[1] (ppm, µg/g)	Peak Area	Tris Base Concentration (mg/mL)
50	0.010	0.48	421.4	20.01
75	0.014	0.71	582.0	
100	0.019	0.95	799.7	
125	0.024	1.19	951.4	
150	0.029	1.43	1147.5	

[1]Sample concentration refers is the ratio between the *'Solution Concentration'* and *'Tris Base Concentration'*

Table 8: NPD Linearity – Linear Regression and Result Summary.

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	38205	Report	Not Applicable
Y-intercept	51.8	Report	Not Applicable
Y-intercept Bias	6.5%	NMT 25.0%	Pass
Residual Sum of Squares	796	Report	Not Applicable
R	0.999	NLT 0.990	Pass
Relative Response Factor	0.792	Report	Not Applicable



Name: NPD; Cal Curve Id: 5017; R 0.999; Y-Intercept 51.79167; Slope 38205.37136; Residual Sum of Squares 795.810813

Figure 4: NPD Linearity Plot and Regression Analysis

Table 9: THNM Linearity Injection Summary

Impurity Linearity – THNM – 50% - 150%				
Level (% Specification)	Solution Concentration (µg/mL)	Sample Concentration (ppm)	Peak Area	Tris Base Concentration (mg/mL)
50	0.010	0.50	285.2	20.01
75	0.015	0.75	455.6	
100	0.020	0.99	553.8	
125	0.025	1.24	720.5	
150	0.030	1.49	895.0	

[1]Sample concentration refers is the ratio between the *'Solution Concentration'* and *'Tris Base Concentration'*

Table 10: THNM linearity – linear regression and result summary.

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	29870	Report	Not Applicable
Y-intercept	-11.7	Report	Not Applicable
Y-intercept Bias	-2.1%	NMT 25.0%	Pass
Residual Sum of Squares	1639	Report	Not Applicable
R	0.996	NLT 0.990	Pass

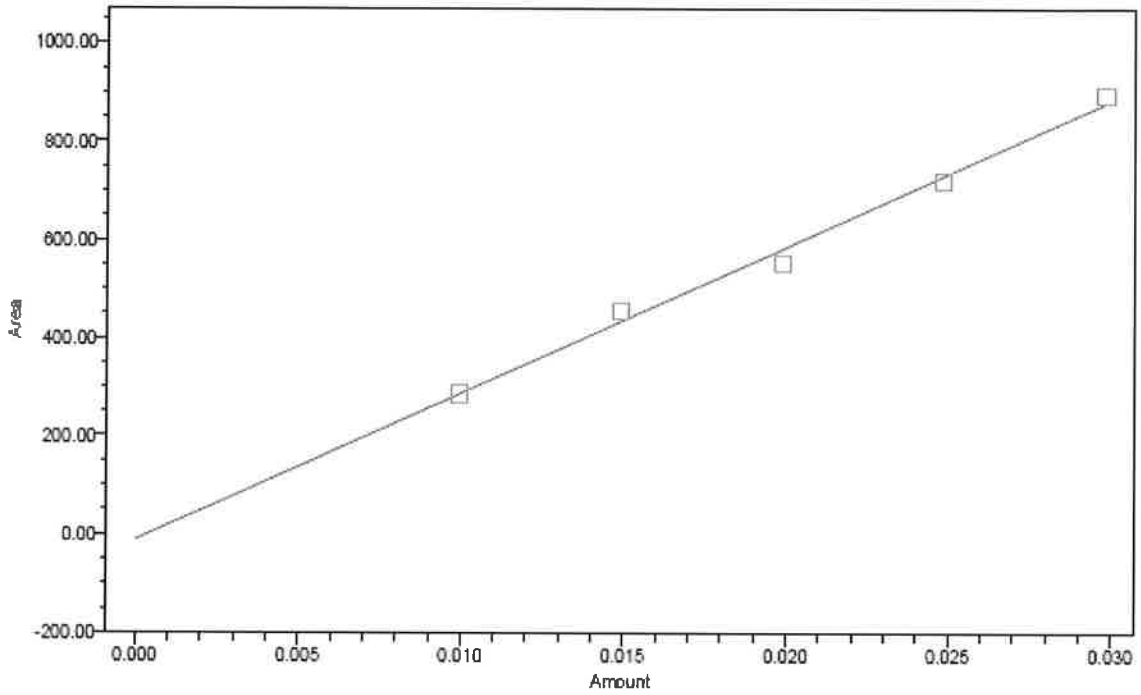


Figure 5: THNM Linearity Plot and Regression Analysis

Table 11: NE Linearity Injection Summary

Impurity Linearity – NE – 50% - 150%				
Level (% Specification)	Solution Concentration (µg/mL)	Sample Concentration (ppm)	Peak Area	Tris Base Concentration (mg/mL)
50	0.010	0.52	525.0	20.01
75	0.016	0.78	764.2	
100	0.021	1.04	995.1	
125	0.026	1.30	1260.1	
150	0.031	1.56	1559.1	

[1]Sample concentration refers is the ratio between the *'Solution Concentration'* and *'Tris Base Concentration'*

Table 12: NE linearity – Linear Regression and Result Summary

Parameter	Value	Acceptance Criteria	Pass/Fail
Slope	49244	Report	Not Applicable
Y-intercept	-4.9	Report	Not Applicable
Y-intercept Bias	-0.5%	NMT 25.0%	Pass
Residual Sum of Squares	1893	Report	Not Applicable
R	0.999	NLT 0.990	Pass
Relative Response Factor	0.615	Report	Not Applicable

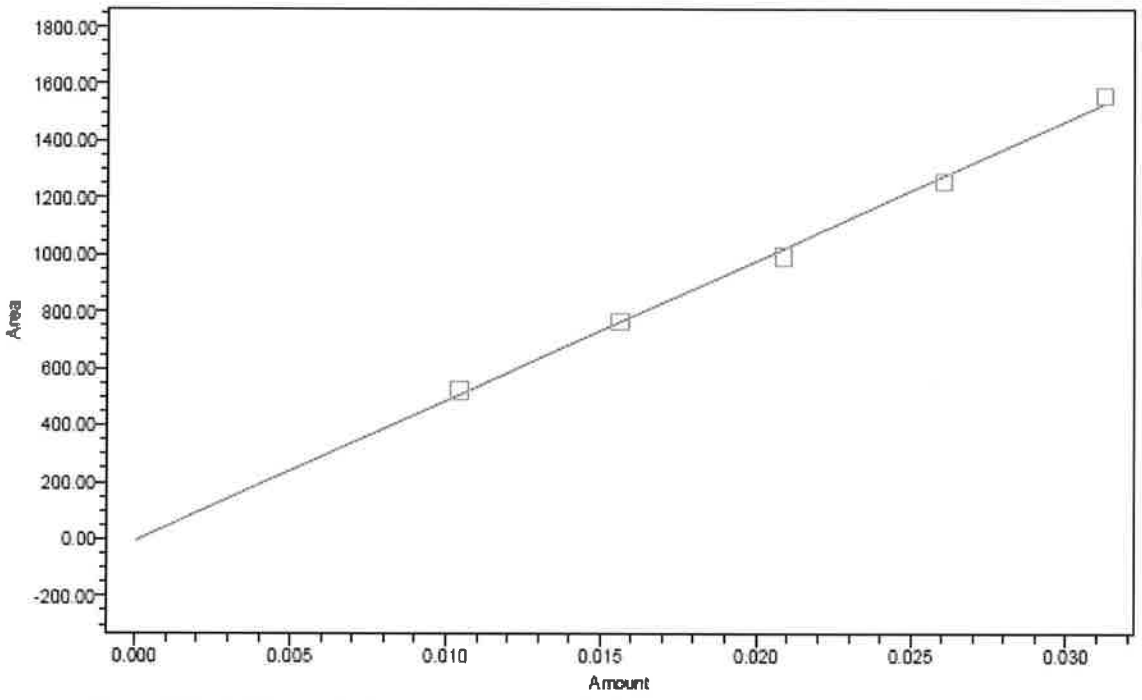


Figure 6: NE linearity Plot and Regression Analysis

9.5. Accuracy and Precision:

9.5.1. Accuracy and precision samples were evaluated from 50% to 150% of the 1 ppm impurity specification. Similar to the routine procedure described in Section 7.3, Tris base was weighed and transferred into individual volumetric flasks; however, each sample replicate was spiked with the impurity standards prior to dissolving and diluting with mobile phase. Each known impurity was evaluated at 50%, 100%, and 150% in the presence of the two other known impurities held constant at the 1ppm specification. All samples were injected once and within 10 minutes of preparation. Using the RRFs from the previous linearity studies (Section 9.3 and 9.4), the ppm values, % Recoveries, and the %RSDs of the %Recoveries were calculated. All Acceptance criteria were met and the results are summarized in Tables 13 through 16.

9.5.2. The data obtained at the 100% level (all impurities spiked at 1 ppm) was used as Analyst 1 for Intermediate Precision (Section 9.6)

Table 13: Tris Blank Impurity Summary

Tris Base Blank (0% Level)	
Analyte	Amount Measured (ppm)
NPD	None Detected
THNM	None Detected
NE	None Detected

Table 14: NPD - Accuracy and Precision Summary

2-Nitropropane-1,3-diol (NPD) – Accuracy and Precision – 50% to 150%					
Sample Name	Amount Added (ppm)	Amount Measured (ppm)	% Recovery (%)	Acceptance Criteria	Pass/Fail
50% Level (0.5 ppm tris base test concentration)	0.472	0.550	116.7	%Recovery for each replicate is 50.0% - 150.0%	Pass
	0.470	0.534	113.7		
	0.472	0.531	112.6		
		Average	114.3		
		%RSD	1.9		
100% Level (1.0 ppm tris base test concentration)	0.947	1.124	118.7	RSD is NMT 15.0% %Recovery for each replicate is 80.0% - 120.0%	Pass
	0.947	1.059	111.8		
	0.948	1.093	115.3		
	0.945	1.086	114.9		
	0.945	1.095	115.9		
	0.945	1.067	112.9		
		Average	114.9		
		%RSD	2.1		
150% Level (1.5 ppm tris base test concentration)	1.420	1.489	104.8	%Recovery for each replicate is 80.0% - 120.0%	Pass
	1.413	1.574	111.4		
	1.418	1.522	107.3		
		Average	107.9		
		%RSD	3.1		
Note: For all levels, NE and THNM were spiked in at the 1 ppm specification.					

Table 15: THNM - Accuracy and Precision Summary

Tris(hydroxymethyl)nitromethane (THNM) – Accuracy and Precision – 50% to 150%					
Sample Name	Amount Added (ppm)	Amount Measured (ppm)	% Recovery (%)	Acceptance Criteria	Pass/Fail
50% Level (0.5 ppm tris base test concentration)	0.495	0.463	93.5	%Recovery for each replicate is 50.0% - 150.0%	Pass
	0.495	0.490	99.0		
	0.494	0.534	108.0		
		Average	100.2		
		%RSD	7.3		
100% Level (1.0 ppm tris base test concentration)	0.987	0.967	98.0	RSD is NMT 15.0% %Recovery for each replicate is 80.0% - 120.0%	Pass
	0.987	0.932	94.5		
	0.988	0.961	97.3		
	0.985	0.991	100.6		
	0.985	0.988	100.3		
	0.985	0.924	93.7		
		Average	97.4		
		%RSD	2.9		
150% Level (1.5 ppm tris base test concentration)	1.488	1.577	106.0	%Recovery for each replicate is 80.0% - 120.0%	Pass
	1.410	1.560	110.6		
	1.418	1.527	107.7		
		Average	108.1		
		%RSD	2.2		
Note: For all levels, NE and NPD were spiked in at the 1 ppm specification.					

Table 16 NE - Accuracy and Precision Summary

2-Nitroethanol (NE) – Accuracy and Precision – 50% to 150%					
Sample Name	Amount Added (ppm)	Amount Measured (ppm)	% Recovery (%)	Acceptance Criteria	Pass/Fail
50% Level (0.5 ppm tris base test concentration)	0.518	0.475	91.6	%Recovery for each replicate is 50.0% - 150.0%	Pass
	0.515	0.442	85.9		
	0.514	0.408	79.4		
Average			85.6		
%RSD			7.2		
100% Level (1.0 ppm tris base test concentration)	1.044	0.880	84.3	RSD is NMT 15.0% %Recovery for each replicate is 80.0% - 120.0%	Pass
	1.044	0.913	87.4		
	1.045	0.945	90.5		
	1.042	0.918	88.1		
	1.042	0.917	88.1		
	1.042	0.881	84.5		
Average			87.1		
%RSD			2.7		
150% Level (1.5 ppm tris base test concentration)	1.548	1.465	94.6	%Recovery for each replicate is 80.0% - 120.0%	Pass
	1.548	1.482	95.7		
	1.554	1.450	93.3		
Average			94.5		
%RSD			1.3		
Note: For all levels, NPD and THNM were spiked in at the 1 ppm specification.					

9.6. Intermediate Precision:

9.6.1. Analyst 2 performed the Accuracy and Precision exercise at the 100% Level on a different day with a different column using separately prepared solutions. Using the RRFs from the previous linearity studies (Section 9.3 and 9.4), the ppm values, % Recoveries, the %RSD of the %Recoveries, and the pooled %Recoveries were calculated. All Acceptance criteria were met and the results are summarized in Tables 17 through 19.

Table 17: NPD - Intermediate Precision Summary

2-Nitropropane-1,3-diol (NPD) – Intermediate Precision – 100% Level					
Sample Name	Amount Added (ppm)	Amount Measured (ppm)	% Recovery (%)	Acceptance Criteria	Pass/Fail
100% Level (Analyst 2)	0.968	0.947	97.9	%RSD is NMT 15.0% %Recovery for each replicate is 80.0% - 120.0%	Pass
	0.971	0.988	101.8		
	0.966	1.050	108.8		
	0.951	0.945	99.4		
	0.951	0.911	95.8		
	0.959	0.956	99.7		
		Average	100.5		
		%RSD	4.5		
100% Level (Analyst 1)	0.947	1.124	118.7	The combined %RSD of the %Recovery values is NMT 20.0%	Pass
	0.947	1.059	111.8		
	0.948	1.093	115.3		
	0.945	1.086	114.9		
	0.945	1.095	115.9		
	0.945	1.067	112.9		
		(Analyst 1+2) Combined Average	107.7		
		(Analyst 1+2) Combined %RSD	7.7		
Note: For all levels, NE and THNM were spiked in at the 1 ppm specification.					

Table 18 THNM - Intermediate Precision Summary

Tris(hydroxymethyl)nitromethane (THNM) – Intermediate Precision – 100% Level					
Sample Name	Amount Added (ppm)	Amount Measured (ppm)	% Recovery (%)	Acceptance Criteria	Pass/Fail
100% Level (Analyst 2)	0.983	0.947	96.4	%RSD is NMT 15.0% %Recovery for each replicate is 80.0% - 120.0%	Pass
	0.986	0.988	100.2		
	0.981	1.050	107.1		
	0.966	0.945	97.8		
	0.966	0.911	94.3		
	0.974	0.956	98.1		
Average			99.0		
%RSD			4.5		
100% Level (Analyst 1)	0.987	0.967	98.0	The combined %RSD of the %Recovery values is NMT 20.0%	Pass
	0.987	0.932	94.5		
	0.988	0.961	97.3		
	0.985	0.991	100.6		
	0.985	0.988	100.3		
	0.985	0.924	93.7		
(Analyst 1+2) Combined Average			98.2		
(Analyst 1+2) Combined %RSD			3.7		
Note: For all levels, NE and NPD were spiked in at the 1 ppm specification.					

Table 19: NE - Intermediate Precision Summary

2-Nitroethanol (NE) – Intermediate Precision – 100% Level					
Sample Name	Amount Added (ppm)	Amount Measured (ppm)	% Recovery (%)	Acceptance Criteria	Pass/Fail
100% Level (Analyst 2)	1.025	0.947	92.4	%RSD is NMT 15.0% %Recovery for each replicate is 80.0% - 120.0%	Pass
	1.028	0.988	96.1		
	1.023	1.050	102.7		
	1.007	0.945	93.8		
	1.008	0.911	90.4		
	1.016	0.956	94.1		
Average			94.9		
%RSD			4.5		
100% Level (Analyst 1)	1.044	0.880	84.3	The combined %RSD of the %Recovery values is NMT 20.0%	Pass
	1.044	0.913	87.4		
	1.045	0.945	90.5		
	1.042	0.918	88.1		
	1.042	0.917	88.1		
	1.042	0.881	84.5		
(Analyst 1+2) Combined Average			91.0		
(Analyst 1+2) Combined %RSD			5.7		
Note: For all levels, NPD and THNM were spiked in at the 1 ppm specification.					

9.7. Limit of Quantitation:

9.7.1. The LOQ solution was prepared and injected six (6) times. All analytes met the acceptance criteria for %RSD and USP S/N. See Tables 20 through 22 for results. For all impurities, the Limit of Quantitation will be set to 0.5ppm with respect to the 20 mg/mL Tris base samples solution.

Table 20: NPD - LOQ Injection Summary

2-Nitropropane-1,3-diol - LOQ				
Sample	USP S/N	Area	Acceptance Criteria	Pass/Fail
LOQ (0.01 µg/mL)	23	409	%RSD of the peak areas is NMT 10% The USP S/N for each injection is NLT 10	Pass
	23	415		
	23	392		
	22	392		
	22	408		
	22	394		
Mean		402		
%RSD		2.5		

Table 21: THNM - LOQ Injection Summary

Tris(hydroxymethyl)nitromethane - LOQ				
Sample	USP S/N	Area	Acceptance Criteria	Pass/Fail
LOQ (0.01 µg/mL)	17	312	%RSD of the peak areas is NMT 10% The USP S/N for each injection is NLT 10	Pass
	18	306		
	18	285		
	18	291		
	18	295		
	18	302		
Mean		299		
%RSD		3.4		

Table 22: NE - LOQ Injection Summary

2-Nitroethanol - LOQ				
Sample	USP S/N	Area	Acceptance Criteria	Pass/Fail
LOQ (0.01 µg/mL)	31	540	%RSD of the peak areas is NMT 10% The USP S/N for each injection is NLT 10	Pass
	31	532		
	30	460		
	31	512		
	30	509		
	30	527		
Mean		514		
%RSD		5.6		

9.8. Solution Stability:

9.8.1. Sample solution:

9.8.1.1. One 100% Level Accuracy and Precision sample prepared per section 9.5 (Section 8.5 of the validation protocol) was injected 20 times with the first injection occurring within 10 min of preparation. For THNM only, the %change in response was plotted vs time (min). All injections show less than a 20% change in response. The solution is considered stable for 2.5 hours when stored in an HPLC vial at 10 °C. See Table 23 and Figure 7 for results.

9.8.1.2. Acceptance Criteria:

9.8.1.2.1. The solution will be considered stable until 20% degradation is observed.

Table 23: Sample Solution Injection Summary

%Change of Tris(hydroxymethyl)nitromethane – Sample Solution Stability				
Date Acquired	Injection Number	Time (min)	Area	%Change
12/16/2022 1:51:55 PM EST	1	0 (Initial)	589.64142	
12/16/2022 1:59:01 PM EST	2	7	615.03197	4
12/16/2022 2:06:06 PM EST	3	14	599.38456	2
12/16/2022 2:13:11 PM EST	4	21	614.81030	4
12/16/2022 2:20:17 PM EST	5	28	637.86997	8
12/16/2022 2:34:31 PM EST	6	42	615.65820	4
12/16/2022 2:41:39 PM EST	7	49	603.30415	2
12/16/2022 2:48:47 PM EST	8	56	596.79962	1
12/16/2022 2:55:56 PM EST	9	63	645.98573	10
12/16/2022 3:03:04 PM EST	10	70	604.63979	3
12/16/2022 3:17:19 PM EST	11	84	578.20419	-2
12/16/2022 3:24:24 PM EST	12	91	664.35752	13
12/16/2022 3:31:30 PM EST	13	98	603.24669	2
12/16/2022 3:38:38 PM EST	14	105	616.82473	5
12/16/2022 3:45:43 PM EST	15	112	607.96491	3
12/16/2022 4:00:00 PM EST	16	126	642.63900	9
12/16/2022 4:07:08 PM EST	17	133	593.44952	1
12/16/2022 4:14:14 PM EST	18	140	613.95168	4
12/16/2022 4:21:20 PM EST	19	147	641.35143	9
12/16/2022 4:28:28 PM EST	20	154	617.56393	5

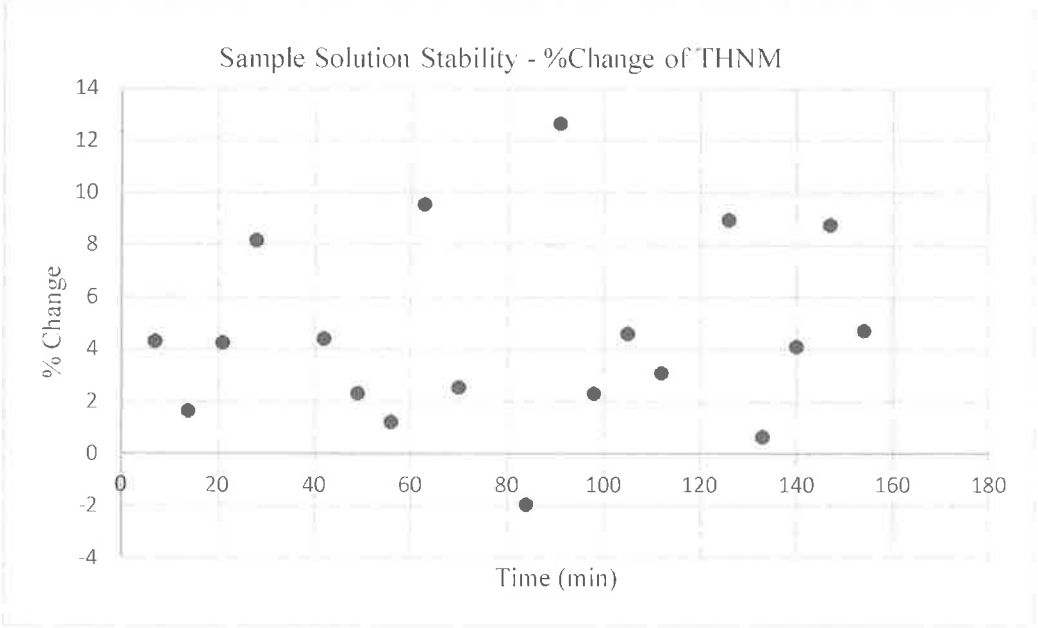


Figure 7: Sample Solution Stability %Change of THMN Plotted Against Time

9.8.2. Standard Solutions:

9.8.2.1. One (1) Calibration Standard solution, one (1) LOQ Standard solution, and one (1) resolution solution were stored in clear glassware at normal laboratory conditions. These solutions were analyzed against freshly prepare solutions, and the %Agreement was calculated for all applicable impurities. All standard solutions are considered stable for 6 days when stored in clear, stoppered volumetric flask at normal laboratory conditions.

Table 24: Calibration Standard - Solution Stability Summary

Calibration Standard Solution Stability - %Agreement			
Timepoint (day)	% Agreement THNM	Acceptance Criteria	Pass/Fail
3	98.8	The % Agreement is between 80.0% and 120.0%	Pass
6	101.9		

Table 25: Resolution Solution: Solution Stability Summary

Resolution Solution Stability - %Agreement					
Timepoint (day)	% Agreement			Acceptance Criteria	Pass/Fail
	NPD	THNM	NE		
3	97.4	84.4	91.2	The % Agreement is between 80.0% and 120.0%	Pass
6	99.4	102.8	104.2		

Table 26: LOQ Solution: Solution Stability Summary - %Agreement

LOQ Solution Stability - %Agreement					
Timepoint (day)	% Agreement			Acceptance Criteria	Pass/Fail
	NPD	THNM	NE		
3	101.6	94.9	94.6	The % Agreement is between 50.0% and 150.0%	Pass
6	94.6	103.0	110.7		

Table 27: LOQ Solution: Solution Stability Summary - USP S/N

LOQ Solution Stability – USP S/N					
Timepoint (day)	% Agreement			Acceptance Criteria	Pass/Fail
	NPD	THNM	NE		
3	20	17	24	NLT 10	Pass
6	17	16	25		

9.9. Robustness:

9.9.1. Prepare System suitability solutions as per Section 7.2 and documented as per protocol BSI-PRL-0768. Evaluate each robustness condition in the table below:

Table 28: Robustness Study Conditions

Variable	Low	Target	High
Flow Rate	0.8 mL/min	1.0 mL/min	1.2 mL/min
Buffer Concentration (Potassium Phosphate)	0.65%	0.68%	0.71%
Column Temperature	35 °C	40 °C	45 °C
Detection Wavelength	205 nm	210 nm	215 nm
Column [1]	Luna Omega Polar C18, 250 x 4.6 mm, 3µm	Luna Omega Polar C18, 250 x 4.6 mm, 3µm	Luna Omega Polar C18, 250 x 4.6 mm, 3µm
[1] To be run at target conditions			

9.9.2. Results:

Table 29: Robustness Study Results

Variable	Low	Result	Target	Result	High	Result
Flow Rate	0.8 mL/min	Passes system suitability	1.0 mL/min	Passes system suitability	1.2 mL/min	Does not Passes system suitability
Buffer Concentration (Potassium Phosphate)	0.65%	Passes system suitability	0.68%	Passes system suitability	0.71%	Passes system suitability
Column Temperature	35°C	Does not Passes system suitability	40 °C	Passes system suitability	45 °C	Does not Passes system suitability
Detection Wavelength	205 nm	Passes system suitability	210 nm	Passes system suitability	215 nm	Passes system suitability
Column	Luna Omega Polar C18, 250 x 4.6 mm, 3µm Serial Number: H23-116554	Passes system suitability	Luna Omega Polar C18, 250 x 4.6 mm, 3µm Serial Number H22-349771	Passes system suitability	Luna Omega Polar C18, 250 x 4.6 mm, 3µm Serial Number H22-324548	Passes system suitability

9.9.3. Conclusion:

9.9.3.1. Column temperature is a critical component in achieving system suitability. It is important to ensure the column temperature is set to 40 °C. In addition, a flow rate of 1.2 mL/min resulted in a system suitability failure, it is critical to the resolution between NE and THNM to keep the flow rate at 1.0 mL/min.

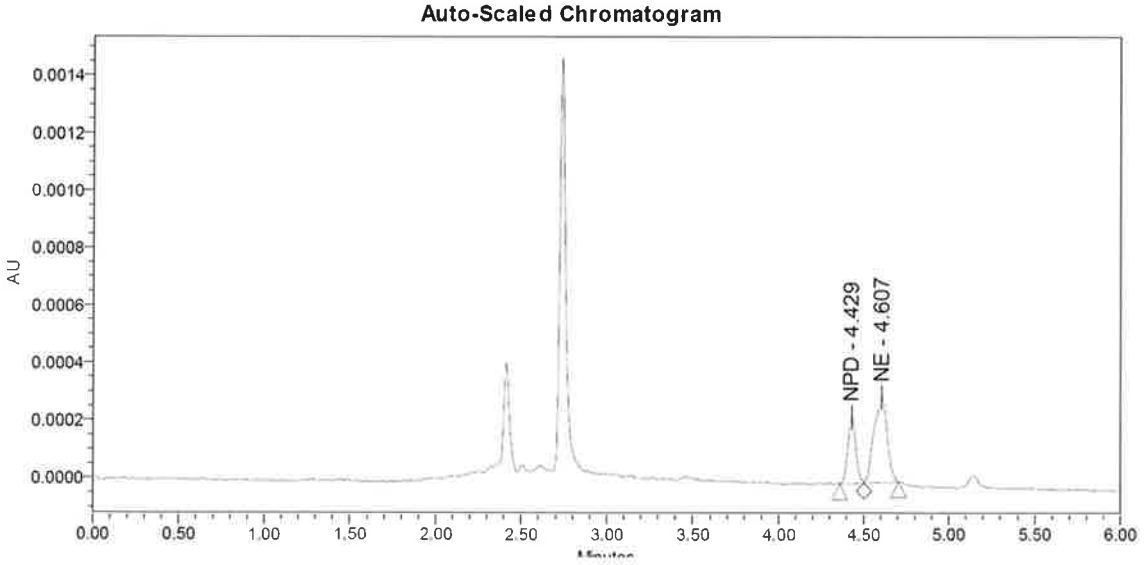


Figure 8: Example Chromatogram of Robustness Run at 35°C Showing Failing Resolution Requirement

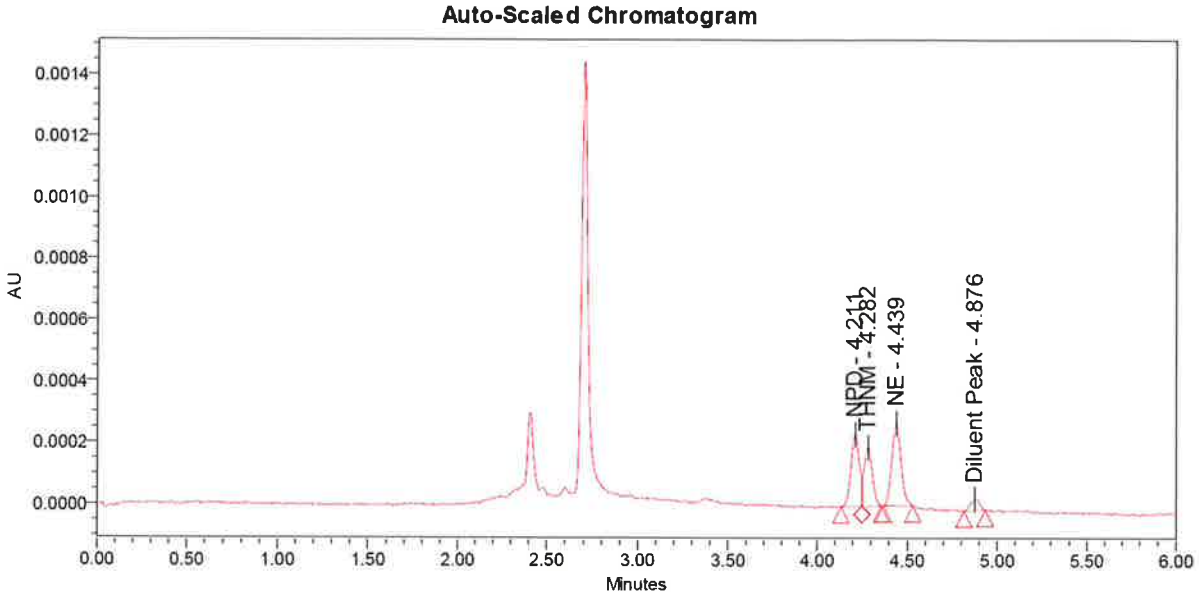


Figure 9: Example Chromatogram of Robustness Run at 45°C Showing Failing Resolution Requirement

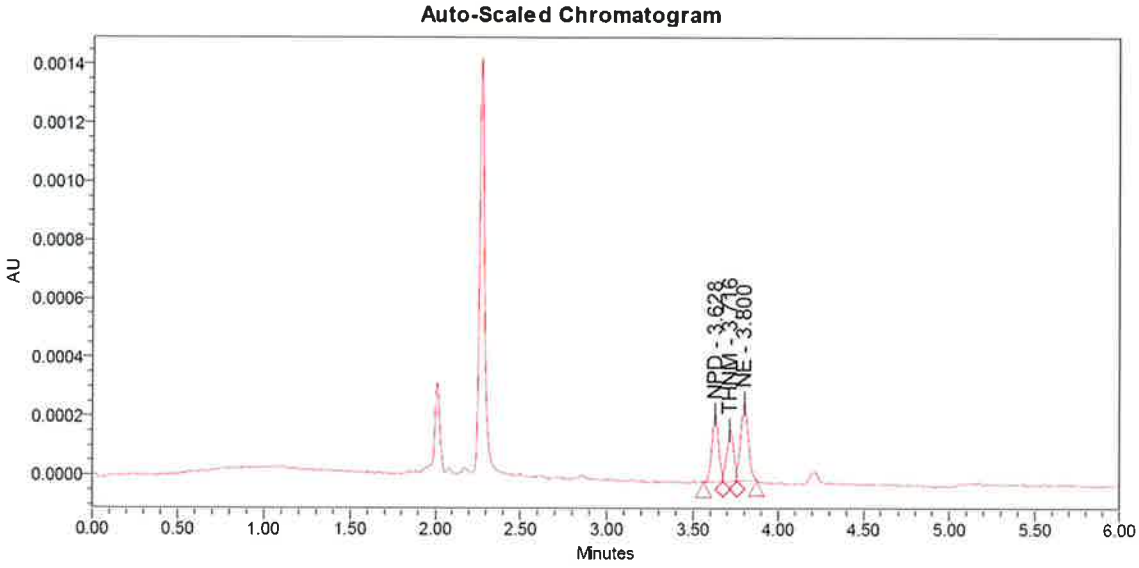


Figure 10: Example Chromatogram of Robustness Run at 1.2 mL/minute Showing Failing Resolution Requirement

10. VALIDATION STATUS:

10.1. The method "Tris Organic Impurities via UPLC" is considered validated and suitable for use at the BioSpectra Bangor, PA facility. All acceptance criteria for System Suitability, Accuracy and Precision, Intermediate Precision, Specificity, Linearity, and LOQ were met. The range was established from 50% to 150% of the 1 ppm impurity specification. Standards are considered stable for 6 days when stored stoppered in clear glassware at normal laboratory conditions. Samples are considered stable for 2.5 hours when stored in in capped HPLC vials at 10 °C.

10.2. Critical Changes, Discrepancies, or Failures

10.2.1. **Method update**: BSI-PRL-0618 v. 1.1

10.2.1.1. During the validation of BSI-PRL-0618 v. 1.0, multiple accuracy and specificity parameters were outside the acceptance criteria

Table 30: BSI-PRL-0618 v. 1.0 - Accuracy and Precision Summary (MV10P43)

Analyte	USP Resolution 100% Level	Acceptance criteria
THNM	0.9	NLT 0.9
NE	1.0	NLT 1.2

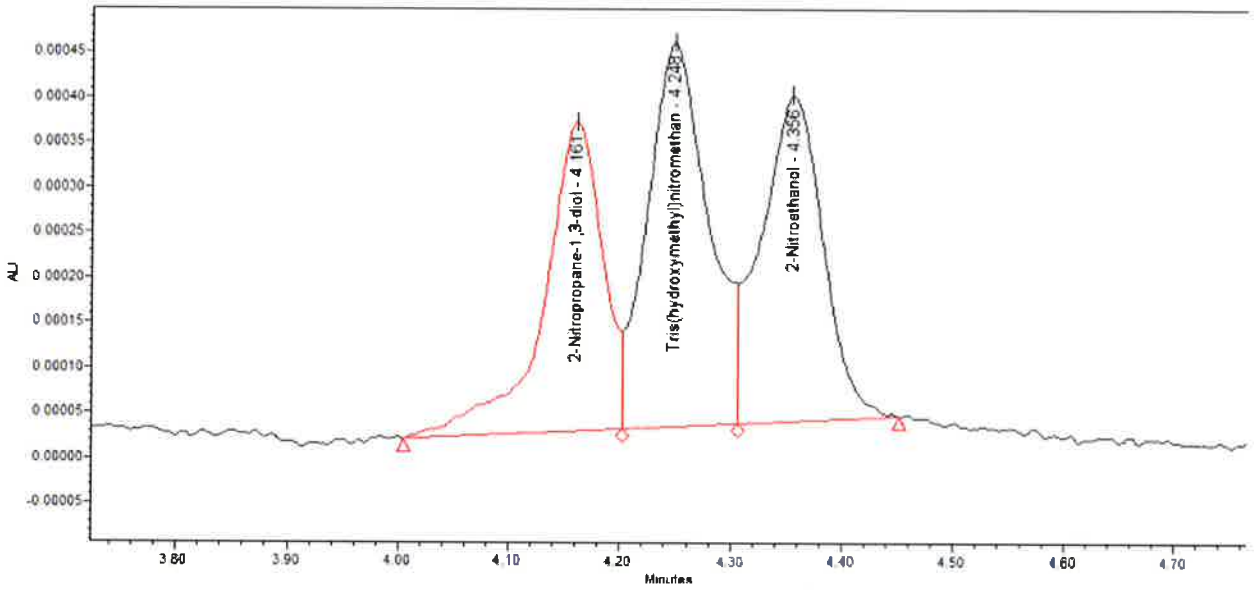


Figure 11: Example Chromatogram of the 100% Level Accuracy and Precision Level.

The figure shows the severity of the peak broadening and the poor integrations that resulted in accuracy values well outside acceptance criteria.

10.2.1.2. **Investigation:** excessive peak broadening to NPD and NE was observed in samples containing Tris. The severity of peak broadening resulted in poor resolution between all peaks. The excessive coelution resulted in integrations that would have produced %Recovery values well outside the limits for all impurities. It was discovered that there was a severe pH disparity between samples and standards. A phosphate buffer was added to the mobile phase/diluent and the pH was adjusted to 2.00. The addition of the phosphate buffer maintained a lower pH for samples containing tris and drastically improved peak shape.

10.2.1.3. **Resolution:** BSI-PRL-0618 v. 1.0 was revised to have the updated mobile phase and the all validation procedures were repeated. The following references contain data that will not be reported: MV10P32, MV10P36, MV10 P39, MV10P40, MV10P43.

10.2.2. % Recovery Failure for NE at the 100% Level (Ref MV10P58):

10.2.2.1. Recoveries for NE did not meet the acceptance criteria for %Recovery (80.0-120.0%).

10.2.2.2. **Investigation:** A pipetting error was suspected to have occurred when preparing the Intermediate Standard. The intermediate standard was re-prepared with the existing stock and was injected along with the original intermediate standard (SSM: 121522 JTG TRIS OI_Invest). The original intermediate standard showed a peak area that was ~ 80% of the peak area observed with the re-prepared intermediate standard, which is proportional to the %recoveries calculated. As such, a pipetting error was attributed to the low %recoveries for NE.

Table 31: NE %Recovery Summary

Sample ID	%Recovery
100% Level - Rep 1	78.0
100% Level - Rep 2	81.6
100% Level - Rep 3	74.1
100% Level - Rep 4	80.2
100% Level - Rep 5	81.3
100% Level - Rep 6	81.4
Mean	79.4
%RSD	3.7

10.2.2.3. **Resolution:** All results were invalidated and the analysis was repeated.

10.2.3. S/N calculation update (Ref. MV11P17)

10.2.3.1. During Intermediate Precision, the Noise value for USP S/N was calculated using a stable region of the LOQ chromatogram, whereas, all other analyses utilized the baseline noise in the mobile phase blank chromatogram. The mobile phase chromatogram from Intermediate precision (Analyst 2) showed a peak near the retention time of NPD. This peak had no impact on the analysis with the exception of the S/N calculation. The proximity of the peak in the mobile phase injection resulted in an inflated noise value used to calculate S/N for the NPD peak. This resulted in an unrealistic USP S/N value for NPD. As such, the S/N was calculated using a stable region of the LOQ chromatogram, which aligned with the S/N values from previous analyses. For future analyses, if closely eluting peaks are observed in the mobile phase injection, it will be acceptable to calculate noise from a stable region of the LOQ chromatogram.

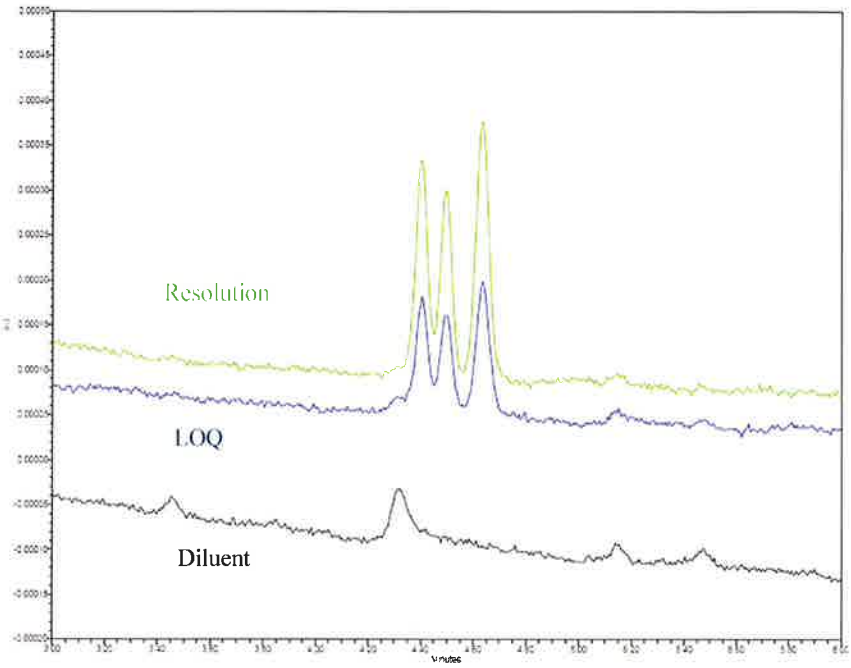


Figure 12: Intermediate Precision Chromatogram Overlay Showing the Relative Size and Proximity of the Mobile Phase Peak

10.2.4. Solution Stability typographical error (Ref MV10P65, MV11P17)

10.2.4.1. The Solution Stability procedure included the resolution solution in the evaluation; however, the acceptance criterion was inadvertently omitted. The following acceptance criteria was assigned to the resolution solution, which is identical to the LOQ solution.

10.2.4.1.1. For all impurities, the %Agreement between aged and fresh **Resolution Solution** is between 80.0% and 120.0%.

10.2.5. Routine sample preparation procedure update

10.2.5.1. Since 2.5 hours of solution stability was established for samples, the sample preparation procedure will be updated to allow for more flexibility. The analyst will no longer be required to prep the samples immediately before the injection.

10.3. Laboratory Notebook References

10.3.1. MV10P49 – Linearity, LOQ

10.3.2. MV10P53 – Accuracy and Precision

10.3.3. MV10P58 – Precision, Solution Stability (not reported)

10.3.4. MV10P65 – Precision, Solution Stability

10.3.5. MV11P17 – Intermediate Precision, Solution Stability

10.3.6. MV12P11,18 – Robustness

10.4. In accordance with BCC23-54, the analysis for Unspecified Impurities will no longer be performed with this Method. A new stability indicating method was validated for Unspecified Impurities utilizing the GC-FID. The following impurities will utilize this method for analysis only.

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

10.5. Organic Impurity analysis will be performed on 3 different methods:

10.5.1. BSI-SOP-0430 will be used for the detection of the specified impurities

10.5.2. BSI-ATM-0050 will be used for the detection of Formaldehyde

10.5.3. BSI-ATM-0112 will be used for the detection of Unspecified Impurities

10.6. Total Impurities will be reported as ≤300ppm for the final result as long as the Specified Impurities are less than or equal to the specification and Unspecified Impurities is less than or equal to the specification.

Signature Manifest

Document Number: BSI-RPT-1226 **Revision:** 1.3

Title: Analytical Method Validation Report: TRIS Organic Impurities via Liquid Chromatography with UV Detection

Effective Date: 08 Feb 2024

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Analytical Method Validation Report: TRIS Organic Impurities via Liquid Chromatography with UV Detection

Change Request

Name/Signature	Title	Date	Meaning/Reason
Virginia Pena (VIRGINIA.PENA)	Document Control Specialist	19 Jan 2024, 08:24:06 AM	Approved
Amy Yencho (AMY.YENCHO)	Vice President, Laboratory Services	19 Jan 2024, 08:54:05 AM	Approved

Originator and Peer Review Collaboration Workspace

Name/Signature	Title	Date	Meaning/Reason
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Departmental Approval

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Amy Yencho (AMY.YENCHO)	Vice President, Laboratory Services	25 Jan 2024, 02:45:30 PM	Approved

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Wayne Talamonti (WAYNE.TALAMONTI)	Director of Laboratory Services	25 Jan 2024, 01:36:29 PM	Approved

Quality Approval

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Hannah Kuchmas (HANNAH.KUCHMAS)	Senior Quality Manager, Rockdale & McConnell	08 Feb 2024, 02:05:25 PM	Approved

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