BIOSPECTRA
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TRIS ORGANIC IMPURITIES VIA UPLC

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1. PURPOSE:

1.1. The purpose of this procedure is to ensure material meets the limit of organic impurities in Tris (tromethamine) by ultrahigh performance liquid chromatography (UPLC).

2. SCOPE:

2.1. This Analytical Method, Organic Impurities in Tris, using BioSpectra's Waters ACQUITY UPLC H-Class Plus, will quantitatively determine Tris (hydroxymethyl) nitromethane (THNM), 2- Nitroethanol (NE) and 2-Nitropropane-1,3-diol (NPD) in Tris.

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

4. REFERENCE:

4.1. BSI-PRL-0618, Analytical Method Validation Protocol: Tris Organic Impurity via Liquid Chromatography with UV Detection

4.2. BSI-RPT-1226, Analytical Method Validation Report: Tris Organic Impurities via Liquid Chromatography with UV Detection

4.3. BSI-SOP-0098, Balance SOP

4.4. BSI-SOP-0134, Pipette SOP

4.5. *USP <621> Chromatography*

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

4.7. *USP <1226> Validation of Compendial Procedures*

4.8. *Waters ACQUITY UPLC TUV Detector Operator's Overview and Maintenance Guide*

5. MATERIALS AND EQUIPMENT:

5.1. Equipment:
- 5.1.1. Analytical Microbalance
- 5.1.2. Class A volumetric flasks
- 5.1.3. Waters ACQUITY UPLC H-Class Plus Instrument with UV Detector
- 5.1.4. LC Column
  - 5.1.4.1. Luna Omega Polar C18, 250 x 4.6 mm, 3µm
  - 5.1.4.2. Part Number: 00G-4760-E0
- 5.1.5. Eppendorf Autopipettes
- 5.1.6. Class A Volumetric Glassware

5.2. Reagents:
- 5.2.1. Water, UPLC grade or equivalent
- 5.2.2. Phosphoric Acid, 85%, HPLC Grade or equivalent
- 5.2.3. Potassium Phosphate Monobasic, HPLC Grade or equivalent
- 5.2.4. Acetonitrile, HPLC Grade or equivalent

5.3. Supplies:
- 5.3.1. HPLC vials and caps
- 5.3.2. Transfer pipettes

5.4. Authentic Sample:
- 5.4.1. Tris base

5.5. Reference Standards:
- 5.5.1. Tris(hydroxymethyl)nitromethane, Reagent grade
- 5.5.2. 2-Nitropropane-1,3-diol, Reagent grade
- 5.5.3. 2-Nitroethanol, NLT 97.0% purity

6. PROCEDURE:

6.1. Glassware Cleaning:

6.1.1. 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.

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

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

6.1.2.2. 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.

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

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

6.1.2.4. Allow glassware to dry prior to use.

6.2. Solution Preparation:

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

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

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

6.2.2.2. Stir until fully dissolved.

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

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

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

6.2.3. Tris (hydroxymethyl) nitromethane Stock Standard (THNM) – 500 µg/mL

6.2.3.1. Accurately weigh 50 mg of tris (hydroxymethyl) nitromethane reference standard and transfer into a 100 mL volumetric flask.

6.2.3.2. Fill ~3/4 full with mobile phase and swirl to dissolve.

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

6.2.4. Tris(hydroxymethyl)nitromethane Intermediate Standard – 1.0 µg/mL

6.2.4.1. Pipette 200 µL of Tris(hydroxymethyl)nitromethane Stock solution into a 100 mL volumetric flask.

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

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

6.2.5. 2-Nitroethanol Stock Standard (NE) – 500 µg/mL
- 6.2.5.1. Add ~25 mL of mobile phase into a 250 mL volumetric flask.
- 6.2.5.2. Place the volumetric flask onto an analytical balance and tare.
- 6.2.5.3. Pipette 100 µL of 2-Nitroethanol reference standard into the flask and record the weight.
  - 6.2.5.3.1. The weight must be 127 mg (± 10%). If necessary, additional solution may be added dropwise to meet the minimum weight tolerance of the balance.
- 6.2.5.4. Fill to volume with mobile phase and mix thoroughly

6.2.6. 2-Nitroethanol Intermediate Standard – 1.0 µg/mL
- 6.2.6.1. Pipette 200 µL of 2-Nitroethanol Stock solution into a 100 mL volumetric flask.
- 6.2.6.2. 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

6.2.7. 2-Nitropropane-1,3-diol Stock Standard (NPD) – 500 µg/mL
- 6.2.7.1. Accurately weigh 50 mg of 2-Nitropropane-1,3-diol standard and transfer into a 100 mL volumetric flask.
- 6.2.7.2. Fill ~3/4 full with mobile phase and swirl to dissolve.
- 6.2.7.3. Fill to volume with mobile phase and mix thoroughly

6.2.8. 2-Nitropropane-1,3-diol Intermediate Standard – 1.0 µg/mL
- 6.2.8.1. Pipette 200 µL of 2-Nitropropane-1,3-diol Stock solution into a 100 mL volumetric flask.
- 6.2.8.2. 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)

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

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

6.2.9.2. Fill to volume with mobile phase and mix thoroughly

6.2.9.3. Solution stability: 6 days when stored in clear glassware at normal laboratory conditions.

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)

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

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

6.2.10.2. Fill to volume with mobile phase and mix thoroughly

6.2.10.3. Solution stability: 6 days when stored in clear glassware at normal laboratory conditions.

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

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

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

6.2.11.2. Fill to volume with mobile phase and mix thoroughly

6.2.11.3. Solution stability: 6 days when stored in clear glassware at normal laboratory conditions.

6.2.12. Sample Preparation – 20 mg/mL Tris

6.2.12.1. **Note: Prior to preparing samples, ensure the sample manager and standards are equilibrated to 10°C per section 6.3.2.**

6.2.12.2. Weigh 1.0 g (± 5%) of Tris and transfer to a clean, dry 50 mL volumetric flask.
6.2.12.3. Fill the flask ~3/4 with mobile phase and swirl for ~30 sec until the sample is full dissolved.
6.2.12.4. Fill to volume with mobile phase and mix thoroughly.
6.2.12.5. Record the time of day
6.2.12.6. Transfer an aliquot to an HPLC vial, cap, and place onto the instrument for analysis.
6.2.12.6.1. **Note**: The samples must be injected NMT 2.5 hours after preparation. If necessary, the injection sequence (Section 6.3.3) may be initiated prior to preparing samples to meet timing requirements.

6.3. System Setup:

6.3.1. 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

6.3.2. Column Conditioning/System Equilibration:

6.3.2.1. Install the column and prime the system with mobile phase.
6.3.2.2. Slowly bring the flow rate up to 1.0 mL/min.
6.3.2.3. Turn on the sample compartment and allow to cool and stabilize at 10°C.
6.3.2.4. Turn on the column compartment and allow the column to warm and stabilize at 40°C.
6.3.2.5. Place the standard solutions onto the instrument and allow the standards to equilibrate to the 10 °C sample compartment (Approximately 30 min).
6.3.2.5.1. 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.
6.3.2.6. At the end of each analysis, clean the column using a gradient of purified water and acetonitrile.
6.3.2.6.1. Final storage solution: 65:35, Acetonitrile: Purified Water

6.3.3. Injection Sequence:

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

6.3.4. 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

6.3.5. Calculations: the following equation will be calculated in the Empower software:

6.3.5.1. Note: Disregard all peaks NMT than ½ the area of NPD in LOQ injection.

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

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

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

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

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

6.3.5.2.5. RRF = Relative Response Factor

Relative Response Factors	
Name	USP Relative Response Factor
2-Nitropropane-1,3-diol (NPD)	0.792
2-Nitroethanol (NE)	0.615
Any unspecified impurity	1.0

6.4. Reporting:

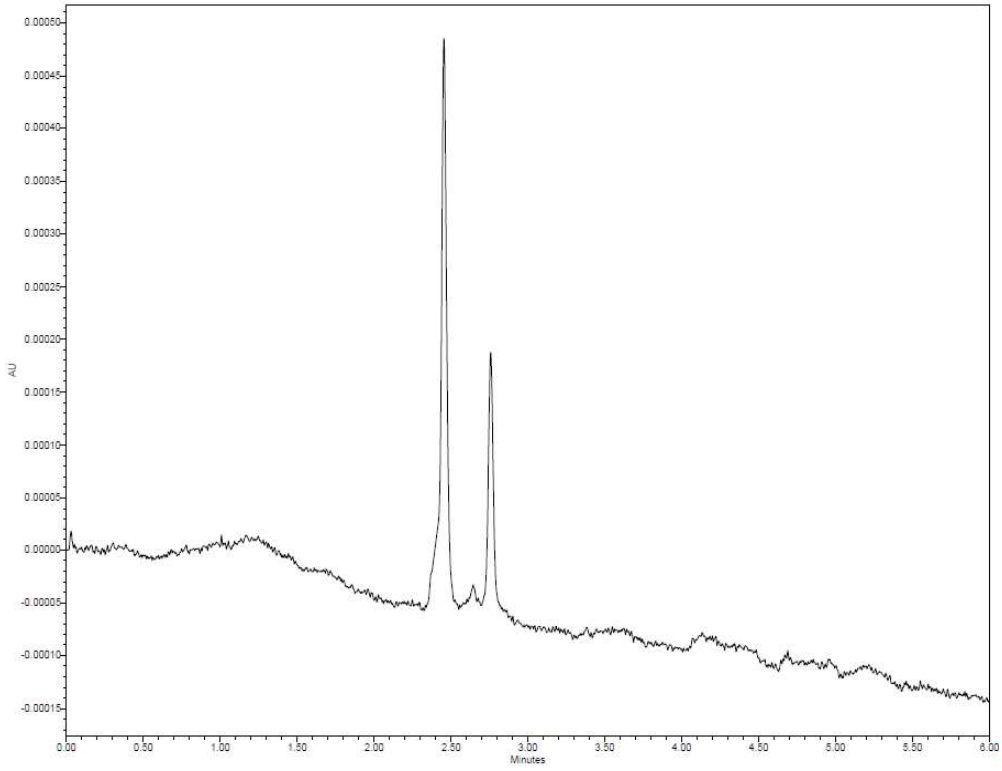
6.4.1. **Organic Impurities:** Calculate the amount of each impurity (ppm) and sum the total of all impurities ≥ LOQ (0.5 ppm).

6.4.2. For unspecified impurities, report the relative retention time (RRT) with respect to the retention time of the THNM peak. (RRT of THNM is = 1.0).

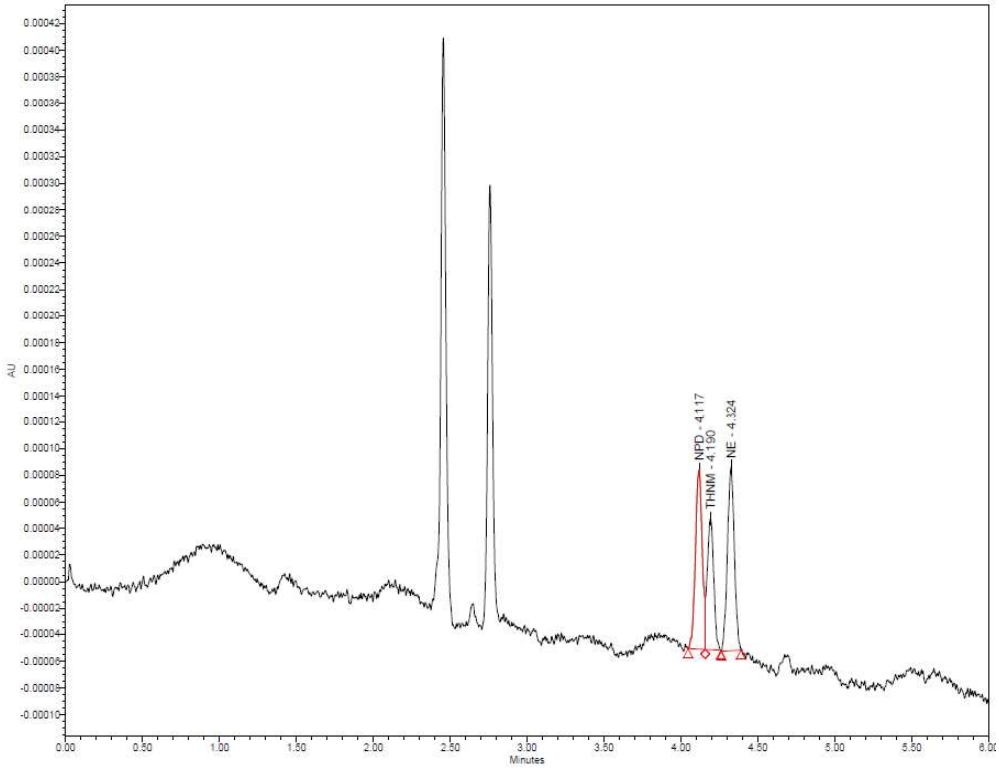
Impurity Reporting	
Result	**Reporting**
If < 0.5 ppm	Report as < LOQ
If ≥ 0.5 ppm	Report to one (1) decimal place

6.5. Example Chromatograms and Integrations

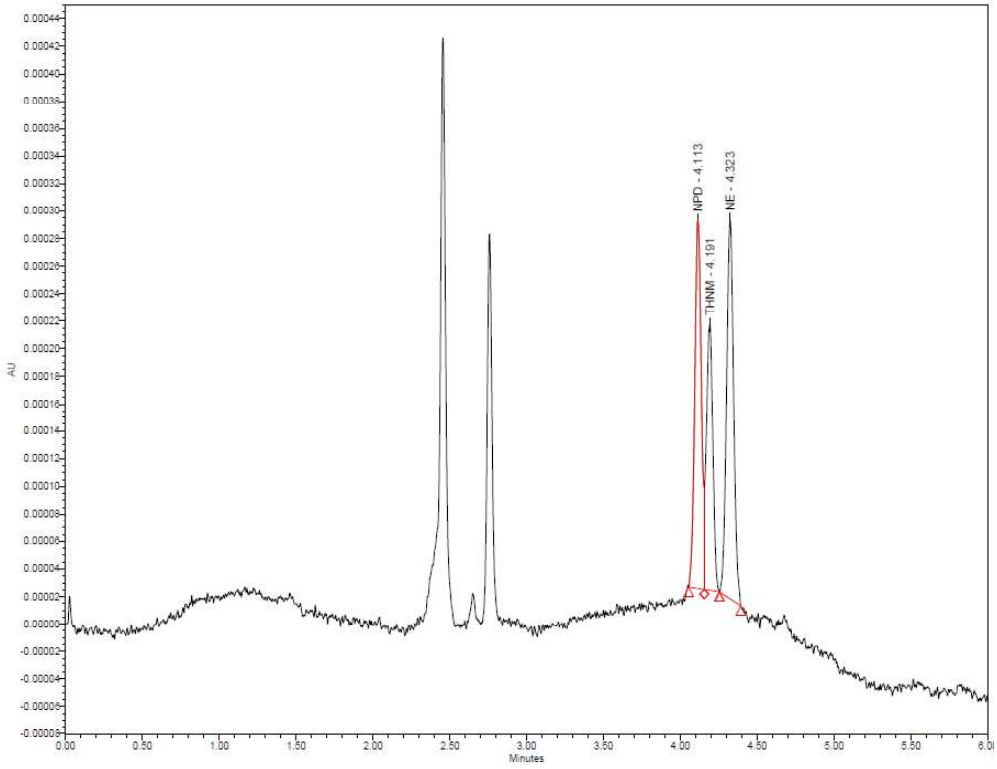
6.5.1. Mobile Phase



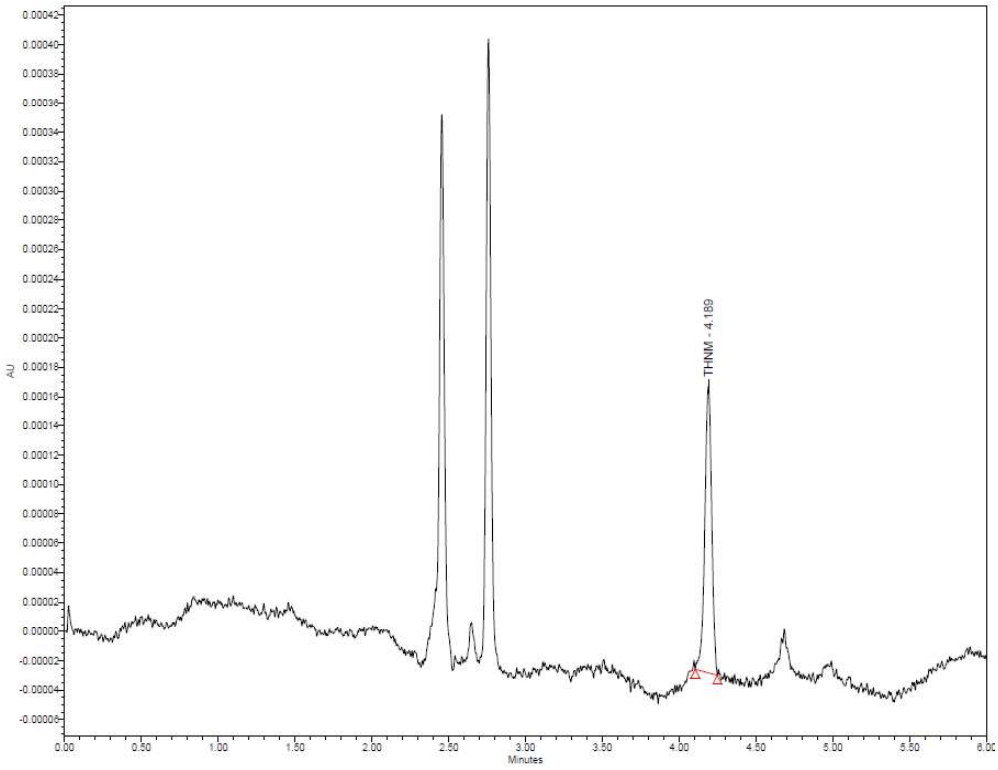
6.5.2. LOQ Solution



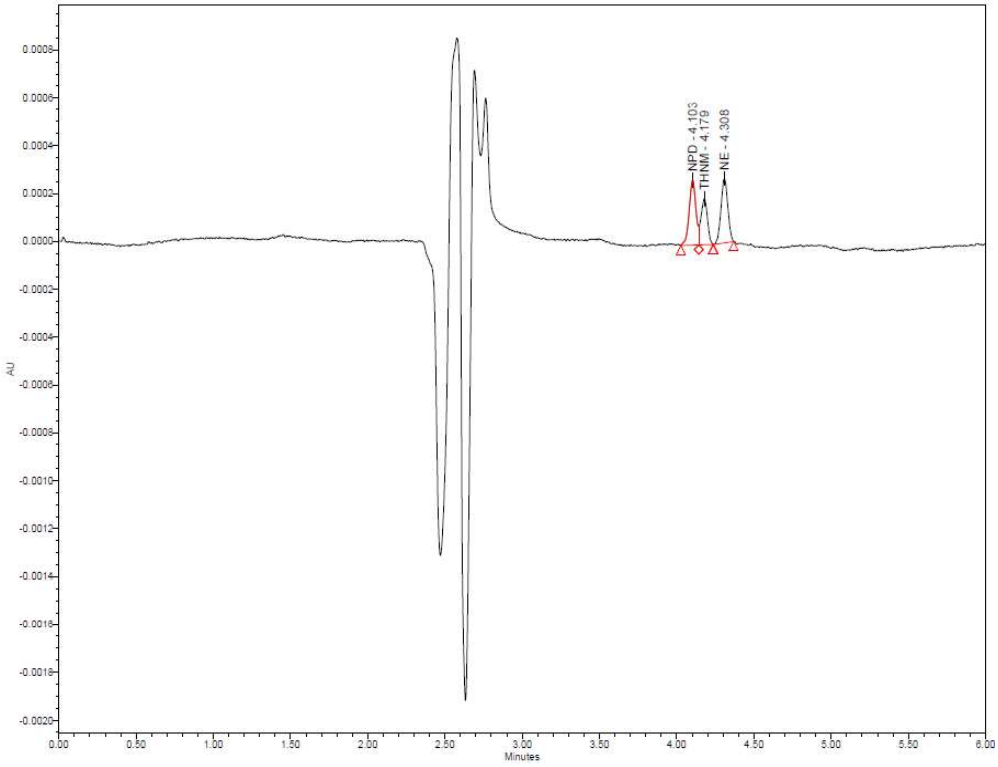
6.5.3. Resolution Solution



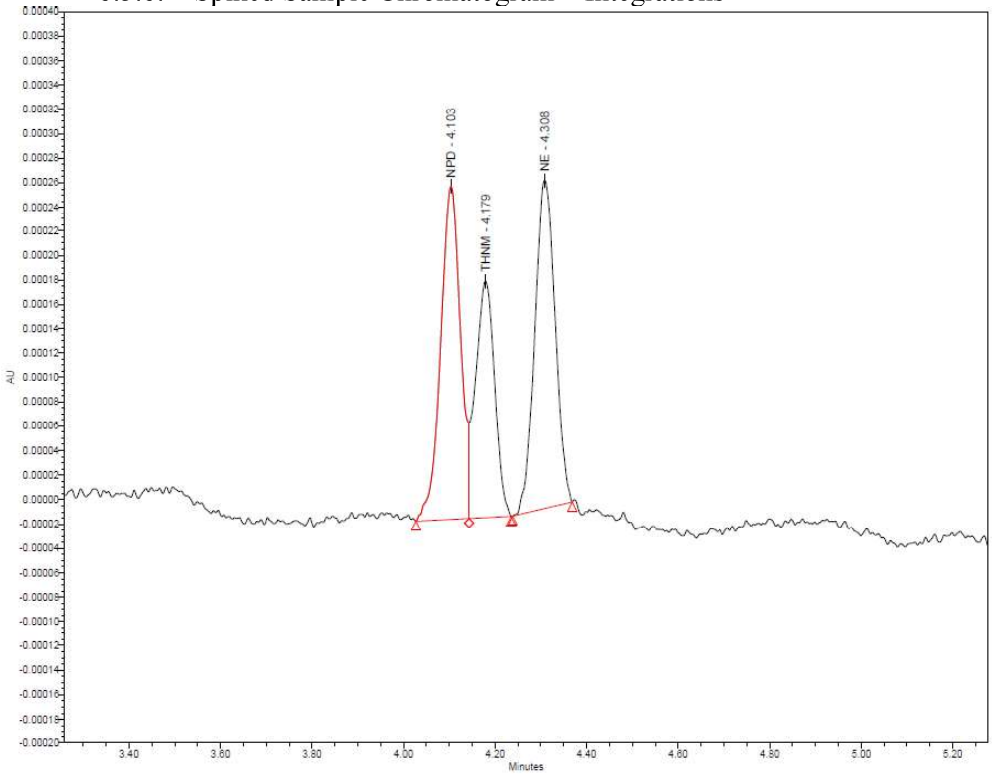
6.5.4. Calibration Standard Solution



6.5.5. Spiked Sample Chromatogram (Full scale)



6.5.6. Spiked Sample Chromatogram – Integrations

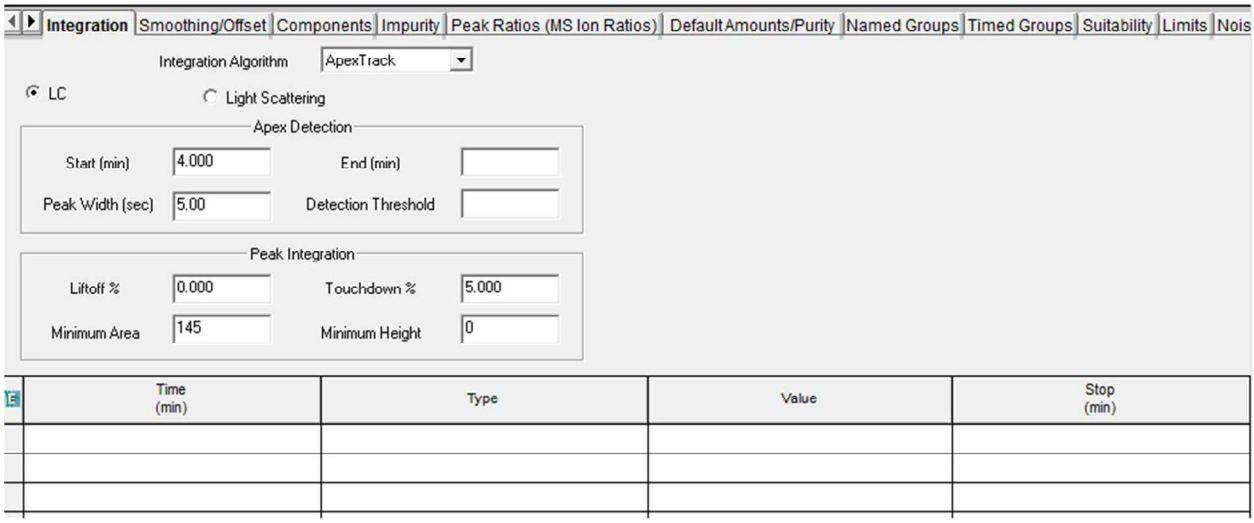


6.6. Integration Parameters for Empower software

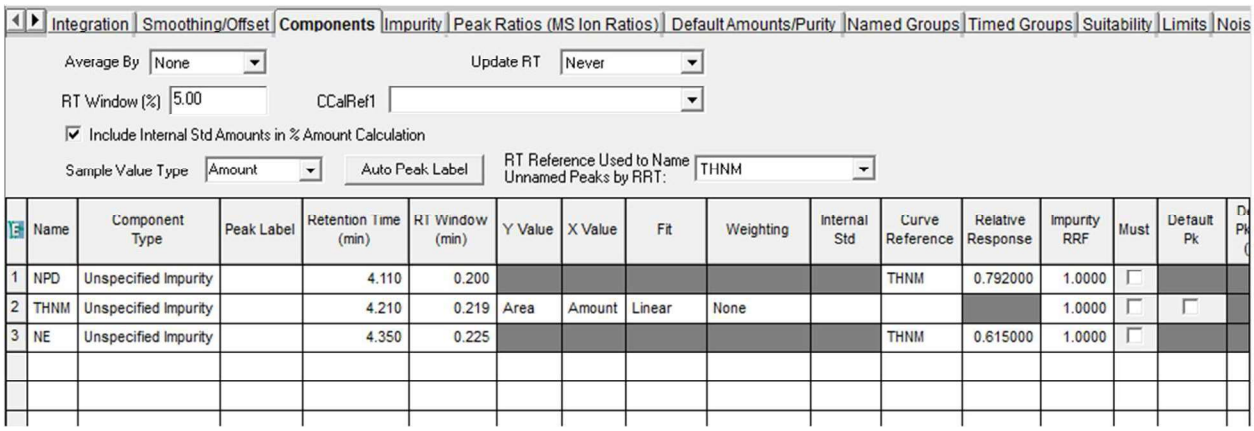
6.6.1. Ensure integrations for samples and standards are similar for accurate quantitation.

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

6.6.3. Example Integration Events:



6.6.4. Example Processing Method Component Tab



	Name	Component Type	Peak Label	Retention Time (min)	RT Window (min)	Y Value	X Value	Fit	Weighting	Internal Std	Curve Reference	Relative Response	Impurity RRF	Must	Default Pk
1	NPD	Unspecified Impurity		4.110	0.200						THNM	0.792000	1.0000	☐	
2	THNM	Unspecified Impurity		4.210	0.219	Area	Amount	Linear	None				1.0000	☐	☐
3	NE	Unspecified Impurity		4.350	0.225						THNM	0.615000	1.0000	☐	