BIOSPECTRA

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UREA ASSAY AND ORGANIC IMPURITY DETERMINATION BY LIQUID CHROMATOGRAPHY WITH UV DETECTION

TABLE OF CONTENTS

1. PURPOSE:

1.1. To provide Laboratory Analysts with a procedure for determining Assay and quantitating Organic Impurities by liquid chromatography with UV detection.

2. SCOPE:

2.1. This analytical method applies to the USP Urea Assay and Organic Impurity procedures on the Waters ACQUITY UPLC and Waters Alliance HPLC.

2.2. Impurity Specifications (Disregard any impurity peaks less than 0.05%)

Table 1: Acceptance Criteria

Name	Acceptance Criteria NMT (%)
Urea Related Compound A (RCA)	0.1
Any Individual Unspecified Impurity	0.1
Total Impurities	2.0

2.3. The Assay specification for urea is 98.0% - 102.0%.

3. RESPONSIBILITIES:

3.1. The Laboratory Technology Manager is responsible for the control, training, implementation and maintenance of this procedure.

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

3.3. Laboratory Analysts performing this procedure, with help and training from the Laboratory Technology Manager, 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. REFERENCES:

4.1. BSI-PRL-0531, Analytical Method Verification Protocol: Urea Assay via Liquid Chromatography with UV detection

4.2. BSI-RPT-0979, Analytical Method Verification Report: Urea Assay by Liquid Chromatography with UV detection

4.3. BSI-SOP-0098, Balance SOP

4.4. BSI-SOP-0126, Laboratory Notebooks

4.5. BSI-SOP-0134, Pipette SOP

4.6. BSI-SOP-0348, Waters Acquity UPLC H-Class Plus SOP

4.7. USP <1225> Validation of Compendial Procedures

4.8. USP <1226> Verification of Compendial Procedures

4.9. USP-NF Current

4.10. Waters 2695 Separations Module Operator's Guide

4.11. Waters 2489 UV/Visible Detector Operator's Guide

5. MATERIALS AND EQUIPMENT:

5.1. Analytical Balance

5.2. Microbalance

5.3. Weighing supplies:

5.3.1. Weighing boats/funnels and spatulas

5.4. Liquid Chromatographs:

5.4.1. Waters ACQUITY UPLC with TUV Detector

5.4.2. Waters Alliance HPLC with UV Detector

5.5. Reagents:

5.5.1. HPLC grade Water or equivalent

5.5.2. HPLC grade Acetonitrile or equivalent

5.5.3. Formic Acid or equivalent

5.6. Supplies:

5.6.1. Class A Volumetric Flasks.

5.6.2. Polypropylene transfer funnels or aluminum weighing boats

5.6.3. Analytical Balance

5.6.4. HPLC auto sampler vials and caps

5.6.5. Micropipettes

5.6.6. Micropipette Tips

5.6.7. Transfer pipettes

5.6.8. 10 mm Screw Thread Vial Convenience Kit

5.6.8.1. Note: due to the volatility of the diluent, do not use pre-slit HPLC caps.

5.7. Reference Standards:

5.7.1. USP Traceable Related Compound A Reference Standard

5.7.2. USP Traceable Urea Reference Standard

5.8. LC Column:

5.8.1. Ascentis Express OH5 90Å 15cm x 4.6 mm. 2.7µm

5.8.2. Part number: 53778-U

5.9. Guard Column:

5.9.1. Ascentis Express OH5 90Å 0.5cm x 4.6mm, 2.7µm

5.9.2. Part number: 53782-U

6. PROCEDURE:

6.1. Solution preparation:

6.1.1. Diluent:

6.1.1.1. Prepare a 90:10 Acetonitrile: HPLC Water

6.1.1.1.1. Combine 100 mL of HPLC water and 900 mL of Acetonitrile. Mix thoroughly and allow to equilibrate to RT.

6.1.1.1.2. Solution may be scaled as needed.

6.1.2. Mobile Phase:

6.1.2.1. Mobile Phase A: 0.1% Formic acid in water

6.1.2.1.1. Add 1 mL of formic acid to 1 L HPLC grade water and mix thoroughly

6.1.3. Mobile Phase B: Acetonitrile

6.1.4. Needle Wash: Use diluent listed above

6.1.5. Purge Solvent (ACQUITY Only): Use diluent listed above

6.1.6. RCA Stock Solution: (0.5 mg/mL RCA)

6.1.6.1. Weigh and transfer 5 mg (± 10%) biuret into a 10 mL volumetric flask.

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

6.1.6.3. Fill to volume with diluent.

6.1.6.4. Mix by inversion.

6.1.7. System Suitability Solution (10 mg/mL Urea, 0.01 mg/mL RCA)

6.1.7.1. Weigh and transfer 250 mg (± 5%) urea reference standard into a 25 mL volumetric flask

6.1.7.2. Pipette 500 µL of RCA Stock Solution into the common flask

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

6.1.7.4. Fill to volume with diluent

6.1.7.5. Cap and mix by inversion

6.1.8. Urea Stock Standard Solution (5 mg/mL Urea, single replicate)
- 6.1.8.1. Weigh and transfer 125 mg (± 5%) urea reference standard into a 25 mL volumetric flask
- 6.1.8.2. Fill ~3/4 full with diluent and swirl to dissolve
- 6.1.8.3. Fill to volume with diluent
- 6.1.8.4. Mix by inversion
- 6.1.8.5. Note: If performing both assay and organic impurity analyses, this solution may be substituted with SS1 or SS2 below

6.1.9. Standard Solution for Urea Assay (5 mg/mL Urea, duplicate)
- 6.1.9.1. Weigh and transfer 125 mg (± 5%) urea reference standard into a 25 mL volumetric flask.
- 6.1.9.2. Fill ~3/4 full with diluent and swirl to dissolve.
- 6.1.9.3. Fill to volume with diluent.
- 6.1.9.4. Mix by inversion.
- 6.1.9.5. Prepare in duplicate.
- 6.1.9.6. Label SS1 and SS2, respectively.
- 6.1.9.7. Stability: Two (2) calendar days when stored, sealed with a fitted stopper, in clear glassware, at normal laboratory conditions.

6.1.10. Organic Impurity Standard (0.01 mg/mL Urea, 0.01 mg/mL RCA, single replicate)
- 6.1.10.1. Pipette 200 µL of Urea Stock Standard Solution into a 100 mL volumetric flask.
- 6.1.10.2. Pipette 2.0 mL of RCA Stock Solution into the common flask
- 6.1.10.3. Fill to volume with diluent
- 6.1.10.4. Mix by inversion
- 6.1.10.5. Stability: One (1) calendar day when stored, sealed with a fitted stopper, in clear glassware, at normal laboratory conditions.

6.1.11. Assay Sample Solution (5.0 mg/mL, duplicate)
- 6.1.11.1. Weigh and transfer 125 mg (± 5%) of urea into a 25 mL volumetric flask.
- 6.1.11.2. Fill ~3/4 full with diluent and swirl to dissolve.
- 6.1.11.3. Fill to volume with diluent.
- 6.1.11.4. Mix by inversion.
- 6.1.11.5. Prepare in duplicate
- 6.1.11.6. Stability: Two (2) calendar day when stored, sealed with a fitted stopper, in clear glassware, at normal laboratory conditions.

6.1.12. Organic Impurity Sample Solution (10.0 mg/mL, single replicate)
- 6.1.12.1. Weigh and transfer 250 mg (± 5%) of urea into a 25 mL volumetric flask.
- 6.1.12.2. Fill ~3/4 full with diluent and swirl to dissolve.
- 6.1.12.3. Fill to volume with diluent.
- 6.1.12.4. Mix by inversion.
- 6.1.12.5. Stability: One (1) calendar day when stored, sealed with a fitted stopper, in clear glassware, at normal laboratory conditions.

6.2. Instrument Setup

6.2.1. Method Parameters

Table 2: Method Parameters

Parameter	Setting
Flow Type	Gradient Elution
Diluent	90:10, Acetonitrile: Water
Mobile Phase A	0.1% Formic Acid in Water

Table 2: Method Parameters	
Parameter	**Setting**
Mobile Phase B	Acetonitrile
Flow Rate	1.0 mL/min
Injection Volume	2 µL
Detector	UV - 195nm
Sample Temperature	10 °C
Column Temperature	30 °C
Column Compartment	30 cm
Run Time	15 min
Sampling Rate	10/sec

6.2.2. Gradient Table

Table 3: Gradient		
Time (min)	**Mobile Phase A (%)**	**Mobile Phase B (%)**
0.0	2.5	97.5
7.0	10.0	90.0
7.01	2.5	97.5
15.0	2.5	97.5

6.2.3. Injection Sequence

Table 4: Example Injection Sequence	
System Suitability Injections	
Diluent	≥ 2
System Suitability Solution	5
O.I. Standard[1]	3
SS1[2]	3
SS2[2]	1
Sample Injections	
Diluent	1
Samples[3]	≤ 10
System Suitability Solution	1

[1]O.I. Standard may be omitted if performing assay only.
[2]SS1 and SS2 may be omitted if performing O.I. only.
[3]Samples injections may be substituted with diluent injections.

6.2.4. System Suitability Criteria

Table 5: System Suitability Criteria	
System Suitability Parameter	**Acceptance Criteria**
Instrument Precision: The %RSD of the Urea peak in the first five (5) *System Suitability Solution* injections	NMT 1.0%
Instrument Precision (QC Checks): The %RSD of the Urea peak in all *System Suitability Solution* injections	NMT 1.0%
Resolution: The resolution between the RCA peak and urea peak in the first *System Suitability Solution*	NLT 1.5
Tailing Factor: The USP tailing of the Urea peak in the first SS1 injection	NMT 2.0
Standard Agreement: The percent agreement between the three (3) SS1 injections and SS2 injection	99% - 101%

6.3. Calculations:

6.3.1. Assay:

6.3.1.1. Result = $(r_u / r_{SS1}) \times (C_{SS1} / C_u) \times 100$

6.3.1.1.1. r_u = peak area response of urea from the *Sample Solution*

6.3.1.1.2. r_{SS1}= average peak area response of urea from all SS1 injections

6.3.1.1.3. C_{SS1}= concentration of urea in the SS1 solution

6.3.1.1.4. C_u = concentration of the *Sample Solution*

6.3.2. Percent Urea Related Compound A:

6.3.2.1. Result = $(r_u / r_{O.I.}) \times (C_{O.I.} / C_u) \times 100$

6.3.2.1.1. r_u= peak area response of RCA from the *Sample Solution*

6.3.2.1.2. $r_{O.I.}$= average peak area response of RCA from the three (3) *O.I. Standard injections*

6.3.2.1.3. $C_{O.I.}$= concentration of RCA in the *O.I. Standard*

6.3.2.1.4. C_u = concentration of the *Sample Solution*

6.3.3. Percent Unknown Impurity:

6.3.3.1. Result = $(r_u / r_{O.I.}) \times (C_{O.I.} / C_u) \times 100$

6.3.3.1.1. r_u= peak area response of an unknown impurity from the *Sample Solution*

6.3.3.1.2. $r_{O.I.}$= average peak area response of urea from the three (3*) O.I. Standard injections*

6.3.3.1.3. $C_{O.I.}$= concentration of urea in the *O.I. Standard*

6.3.3.1.4. C_u= concentration of the *Sample Solution*

6.3.4. Standard Agreement:

6.3.4.1. Result = $(r_{SS2} / r_{SS1}) \times (C_{SS1} / C_{SS2}) \times 100$

6.3.4.1.1. r_{SS2} = Peak area response of urea from the SS2 injection

6.3.4.1.2. r_{SS1} = average peak area response of urea from the three (3) SS1 injections

6.3.4.1.3. C_{SS1} = concentration of urea in the SS1solution

6.3.4.1.4. C_{SS2} = concentration of urea in the SS2 solution

6.3.5. Standard Concentration

6.3.5.1. Result = $(W_{Std} / DF) \times$ Purity

6.3.5.1.1. W_{Std} = Weight of Standard

6.3.5.1.2. DF = Dilution Factor (Total volume)

6.3.6. Sample Concentration

6.3.6.1. Result = W_{Smp} /DF

6.3.6.1.1. W_{Smp} = Weight of Sample

6.3.6.1.2. DF = Dilution Factor (Total volume)

6.4. Reporting

6.4.1. **Assay**: Calculate the % Urea for both replicates and report the average to 1 (one) decimal place.

6.4.1.1. If any replicate has a result that is OOS, an OOS checklist will be issued to evaluate further.

6.4.1.2. If the replicates vary by more than ± 2% of each other, no results will be averaged or reported until evaluated by the Laboratory Technology Manager to determine if the results are valid/reportable or if any further action is required.

Table 6: Impurity Reporting

Result	Reporting
If < 0.05%	Report as < LOQ
If ≥ 0.05% and < 1.0%	Report to two (2) decimal places
If ≥ 1.0%	Report to one (1) decimal place

6.5. Example Chromatograms and Integrations

6.5.1. Diluent:

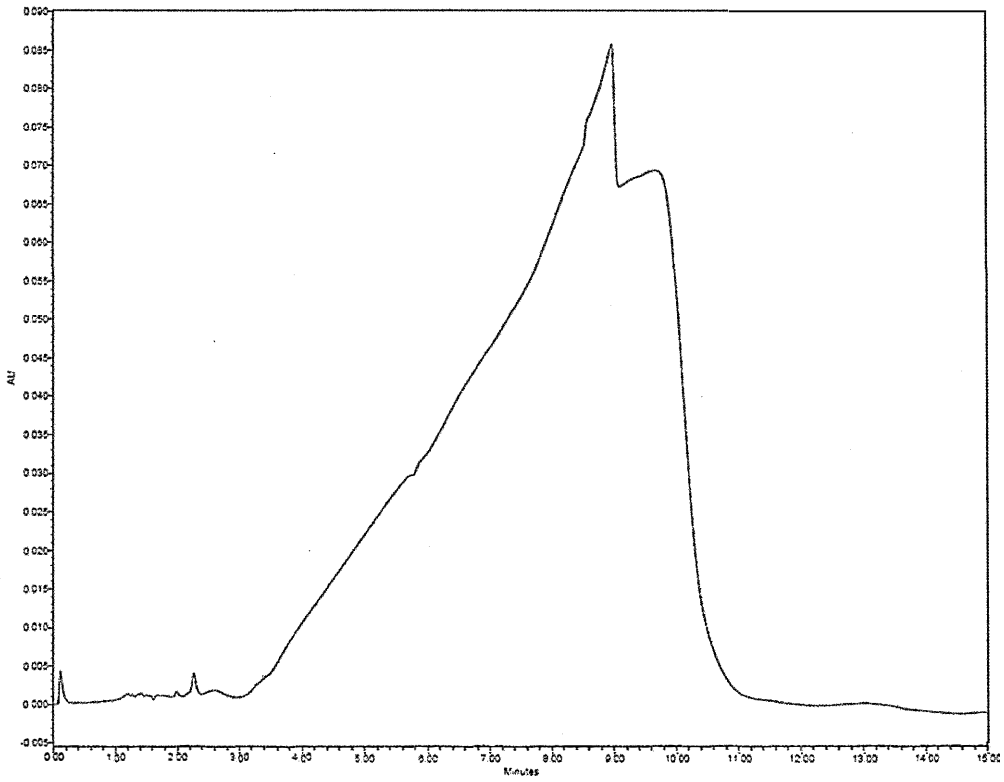

Figure 1: Diluent Chromatogram

6.5.2. Organic Impurity Standard – Full View

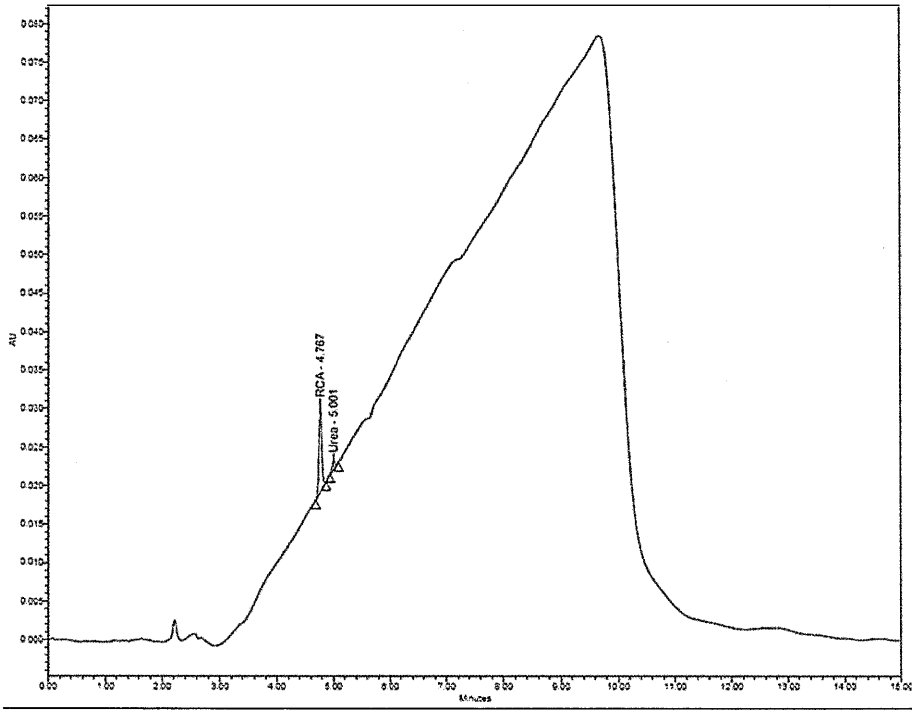

Figure 2: Organic Impurity Standard Chromatogram

6.5.3. Organic Impurity Standard - Zoomed View

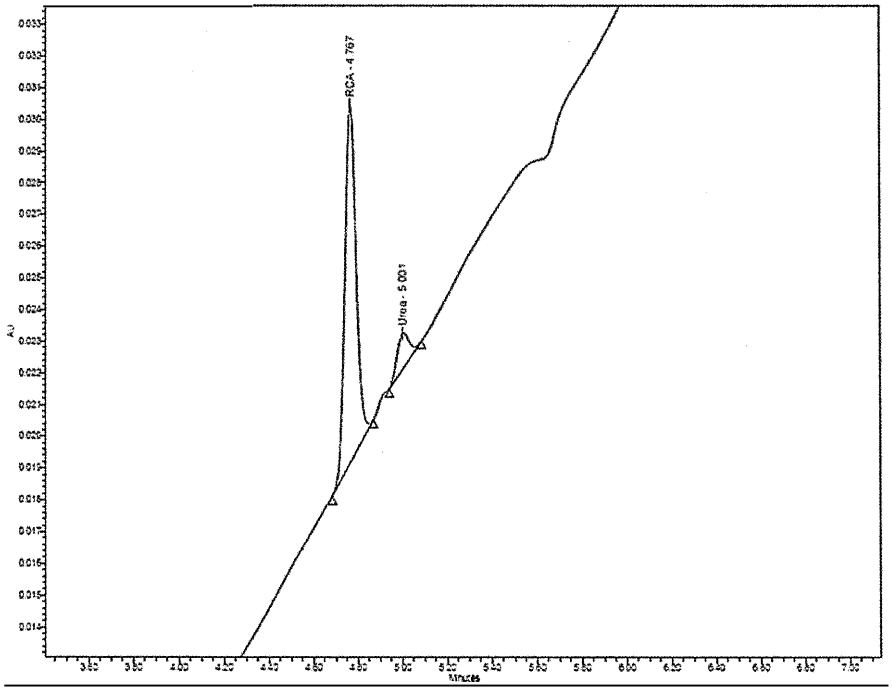

Figure 3: Organic Impurity Standard Zoomed Chromatogram

6.5.4. System Suitability Solution – Full View

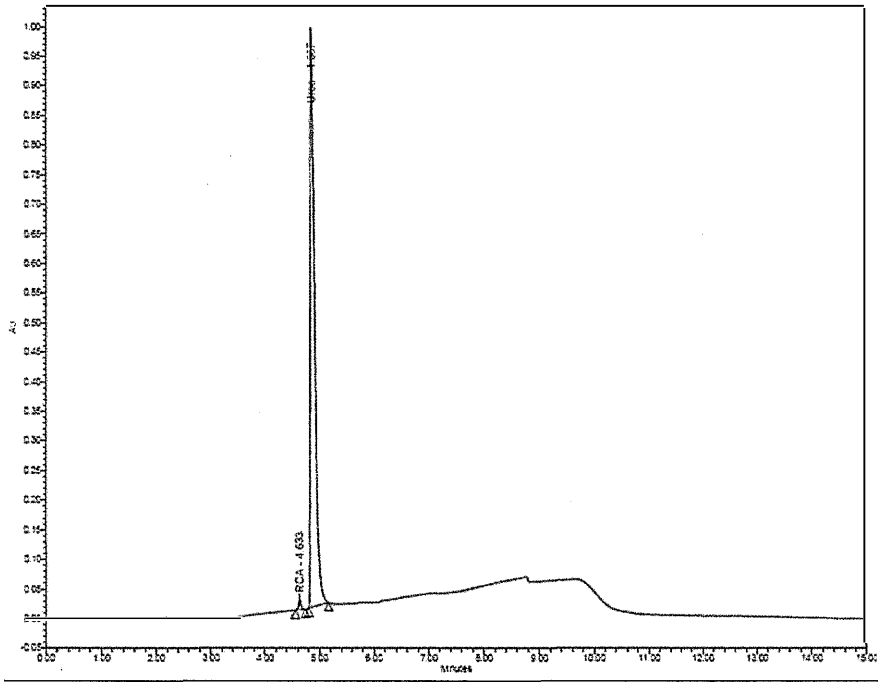

Figure 4: System Suitability Solution Chromatogram

6.5.5. System Suitability Solution – Zoomed View

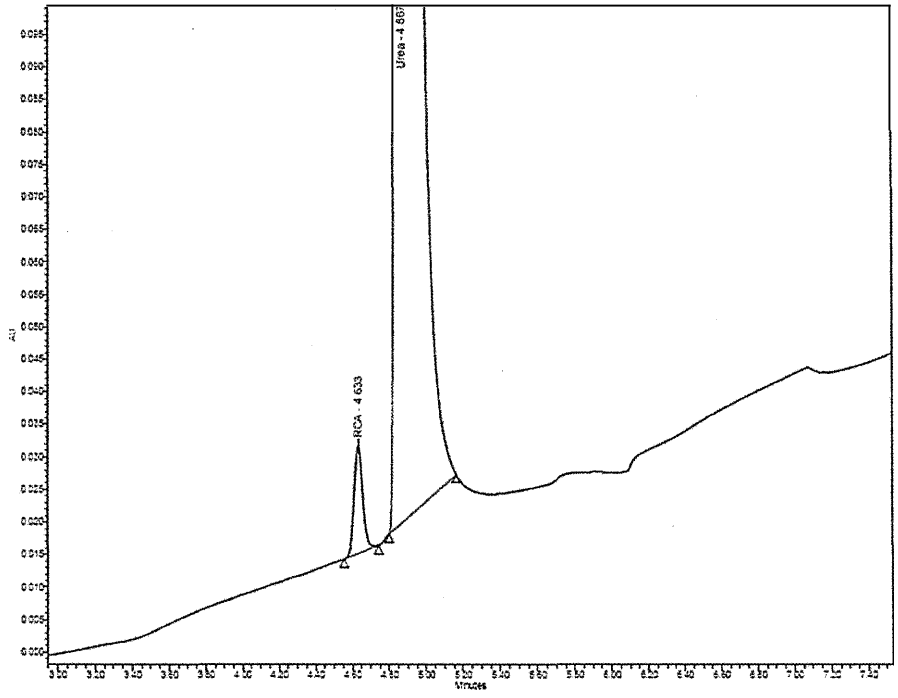

Figure 5: System Suitability Solution Zoomed Chromatogram

6.5.6. Assay Standard Solution

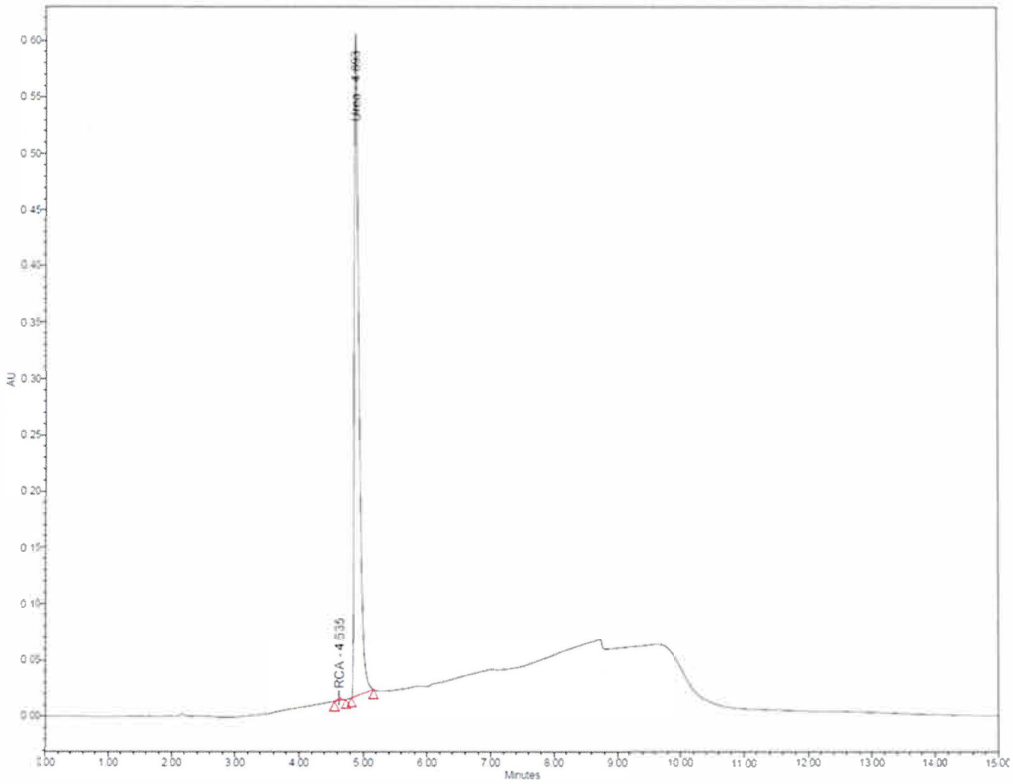

Figure 6: Assay Standard Solution Chromatogram

6.6. Example Integration Parameters for Empower software

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

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

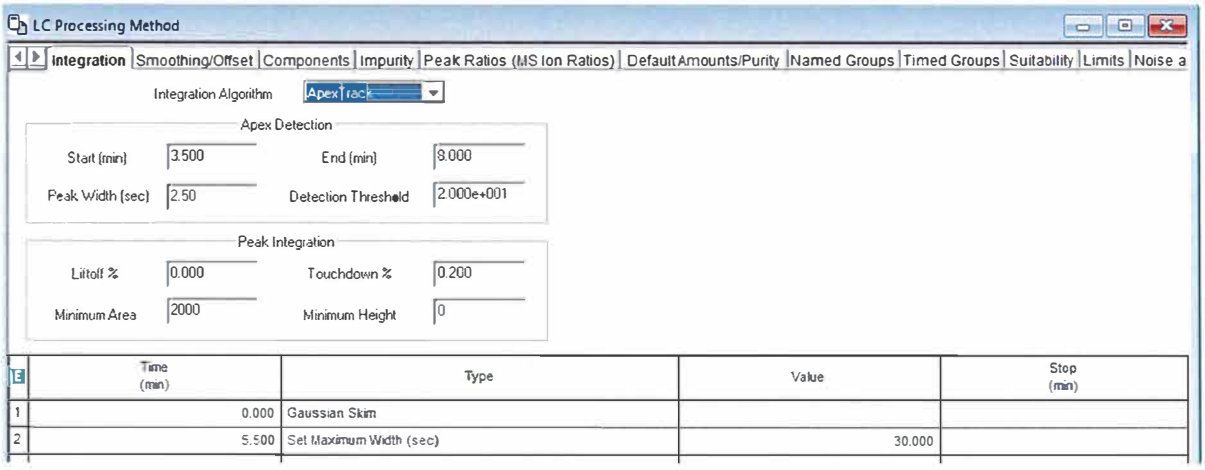

Figure 7: Integration Parameters