BIOSPECTRA
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URACIL ASSAY VIA LIQUID CHROMATOGRAPHY WITH UV DETECTION

TABLE OF CONTENTS

1. PURPOSE:

1.1. To provide Quality Control (QC) analysts with a procedure for determining assay for uracil by liquid chromatography with UV detection.

2. SCOPE:

2.1. This analytical method applies to the uracil assay analytical method using BioSpectra's Waters Alliance and Acquity liquid chromatographs.
2.2. Assay specification: 97.0% – 102.0%

3. RESPONSIBILITIES:

3.1. The Senior Chromatography Specialist and/or a qualified designee 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 from the Quality Control Manager if necessary, 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-ATM-0016, Uracil Testing Methods
4.2. BSI-PRL-0329, Analytical Method Validation Protocol: Uracil Assay via HPLC
4.3. BSI-PRL-0644, Analytical Method Transfer Protocol: Uracil Assay via Liquid Chromatography with UV Detection
4.4. BSI-RPT-0532, Analytical Method Validation Report: Uracil Assay Via HPLC
4.5. BSI-RPT-1256, Analytical Method Transfer Report: Uracil Assay via Liquid Chromatography with UV Detection
4.6. BSI-SOP-0098, Balance SOP
4.7. BSI-SOP-0126, Laboratory Notebooks
4.8. BSI-SOP-0134, Pipette SOP
4.9. BSI-SOP-0422, Empower 3 General Procedure
4.10. *Waters 2695 Separations Module Operator's Guide*
4.11. *Waters 2489 UV/Visible Detector Operator's Guide*
4.12. *ACQUITY UPLC TUV Detector Operator's Overview and Maintenance Guide*
4.13. *ACQUITY UPLC Quaternary Solvent Manager PLUS Series*

5. MATERIALS AND EQUIPMENT:

- 5.1. Equipment
  - 5.1.1. Analytical Balance
  - 5.1.2. Analytical Microbalance
  - 5.1.3. Calibrated Timer
  - 5.1.4. Liquid Chromatographs
    - 5.1.4.1. Waters Alliance HPLC or Acquity UPLC with UV-Vis Detector.
  - 5.1.5. Sonication Bath
- 5.2. Reagents
  - 5.2.1. HPLC Grade Water
  - 5.2.2. Potassium Phosphate Monobasic, HPLC Grade or equivalent
- 5.3. Supplies
  - 5.3.1. Class A Volumetric Flasks
  - 5.3.2. LC auto-sampler vials and caps
  - 5.3.3. Micropipettes
  - 5.3.4. Micropipette tips
  - 5.3.5. Polypropylene Transfer Funnels, Aluminum Weighing Boats, or equivalent
  - 5.3.6. Transfer pipettes
- 5.4. Reference Standards
  - 5.4.1. Uracil Certified Reference Standard (CRS)
- 5.5. LC Column
  - 5.5.1. Description: Luna C18(2), 150 x 3.9 mm, 5µm
  - 5.5.2. Supplier: Phenomenex
  - 5.5.3. Part Number: 00F-4252-C0

6. TESTING PROCEDURE:

6.1. Solution Preparation

6.1.1. 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.

6.1.2. Dry a suitable amount of sample in a calibrated oven for 3 hours as per the Loss on Drying procedure. The LOD sample may be utilized. Grind the dried sample if necessary to help facilitate dissolution.

6.1.3. Mobile Phase and Diluent (0.68% Potassium Phosphate (0.68:100, W:V):

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

6.1.3.2. Stir until fully dissolved.

6.1.4. Assay Standard Stock Solution (1.0 mg/mL Uracil CRS):

6.1.4.1. Weigh and transfer 25 mg (± 10%) of Uracil CRS into a 25 mL volumetric flask.

6.1.4.2. Fill the volumetric flask approximately ~3/4 full with mobile phase.

6.1.4.3. Sonicate for 60 min with occasional swirling until the solution is fully dissolved.

6.1.4.3.1. If necessary, additional sonication is permitted to ensure complete dissolution

6.1.4.4. Allow the solution to equilibrate to room temperature

6.1.4.5. Fill to volume with mobile phase and mix by inversion.

6.1.4.6. Prepare in duplicate and label SS1 and SS2, respectively.

6.1.5. Assay Standard (50 µg/mL Uracil CRS):

6.1.5.1. Volumetrically pipette 5.0 mL of SS1 into a 100 mL volumetric flask.

6.1.5.2. Fill to volume with mobile phase and mix by inversion.

6.1.5.3. Label AS1.

6.1.6. Assay Check Standard (50 µg/mL Uracil CRS):

6.1.6.1. Volumetrically pipette 5.0 mL of SS2 into a 100 mL volumetric flask.

6.1.6.2. Fill to volume with mobile phase and mix by inversion.

6.1.6.3. Label AS2.

6.1.7. Stock Sample Solution (1.0 mg/mL Uracil):

6.1.7.1. Weigh and transfer 100 mg (± 10%) of Uracil into a 100 mL volumetric flask.

6.1.7.2. Fill the volumetric flask approximately ~3/4 full with mobile phase.

6.1.7.3. Sonicate for 60 min with occasional swirling until the solution is fully dissolved.

6.1.7.3.1. If necessary, additional sonication is permitted to ensure complete dissolution.

6.1.7.4. Allow the solution to equilibrate to room temperature

6.1.7.5. Fill to volume with mobile phase and mix by inversion.

6.1.8. Sample Solution (50 µg/mL Uracil):

6.1.8.1. Volumetrically pipette 5.0 mL of the *Stock Sample Solution* into a 100 mL volumetric flask.

6.1.8.2. Fill to volume with mobile phase and mix by inversion

6.2. Instrument Setup

6.2.1. Waters LC Method Parameters:

Parameter	Setting
Flow Type	Isocratic
Mobile Phase A	0.68% Potassium Phosphate
Needle Wash	Water
Flow Rate	1.0 mL/min
Run Time	5 min
Injection Volume	10 µL
Column Temperature (°C)	Ambient
Sample Temperature (°C)	Ambient
Detector Settings	
Detector	UV-Vis
Wavelength	266 nm
Sampling Rate	5

6.2.2. Injection Sequence:

Sample ID	Number of Injections
System Suitability	
Diluent (Mobile Phase)	≥1
AS1	5
AS2 (Standard Check)	2
Sample Injections	
Diluent	1
Samples	≤ 6 samples (1 injection each)
AS1 (QC Check)	1
Diluent	1
• Repeat the sample injection sequence if additional samples are to be analyzed • Samples may be substituted with diluent injections	

6.2.3. System Suitability Criteria:

System Suitability Parameter	Acceptance Criteria
The relative standard deviation of the uracil peak from the first five (5) injections of the AS1 solution.	NMT 0.73%
Average %Agreement between the first five (5) AS1 injections and the AS2 injections.	99% - 101%
The %Agreement between the first five (5) AS1 injections and each AS1 (QC Check).	99% - 101%
The USP Plates of the uracil peak from the first AS1 injection	NLT 2000
The Capacity Factor (K') of the uracil peak from the first AS1 injection	NLT 1.0
The USP Tailing factor of the uracil peak from the first AS1 injection.	NMT 1.5

6.3. Calculations

6.3.1. The following equations are calculated in the Empower software:

6.3.2. Percent Standard Agreement (AS2) = (R_{AS2}/R_{AS1}) x (C_{AS1}/C_{AS2}) x 100

6.3.2.1. R_{AS1} = average peak response of uracil from the first five (5) AS1 injections

6.3.2.2. R_{AS2} = peak response of uracil from the AS2 injection

6.3.2.3. C_{AS1} = Concentration of uracil in AS1 x Purity Factor

6.3.2.4. C_{AS2} = Concentration of uracil in AS2 x Purity Factor

6.3.2.5. **Empower custom field:** Control Percent Agreement

6.3.2.5.1. Sample Type: Control

6.3.2.5.2. Enter dilution factor in the "Alter Sample" window.

6.3.2.5.3. Enter Sample weight and purity in the "Amounts" tab.

6.3.3. Percent Standard Agreement (QC Check) = $(R_{AS1(QC)}/R_{AS1})$ x 100

6.3.3.1. R_{AS1} = average peak response of uracil from the first five (5) AS1 injections

6.3.3.2. $R_{AS1(QC)}$ = peak response of uracil from the AS1 (QC check) injections

6.3.3.3. **Empower custom field:** Control Percent Agreement

6.3.3.3.1. Sample Type: Control

6.3.3.3.2. Enter dilution factor in the "Alter Sample" window.

6.3.3.3.3. Enter Sample weight and purity in the "Amounts" tab.

6.3.4. Assay (% Uracil) = (R_u/R_{AS1}) x (C_{AS1}/C_u) x 100

6.3.4.1. R_{AS1} = average peak response of uracil from the first five (5) AS1 injections

6.3.4.2. R_u = peak response of uracil from the sample

6.3.4.3. C_{AS1} = Concentration of AS1 x Purity Factor

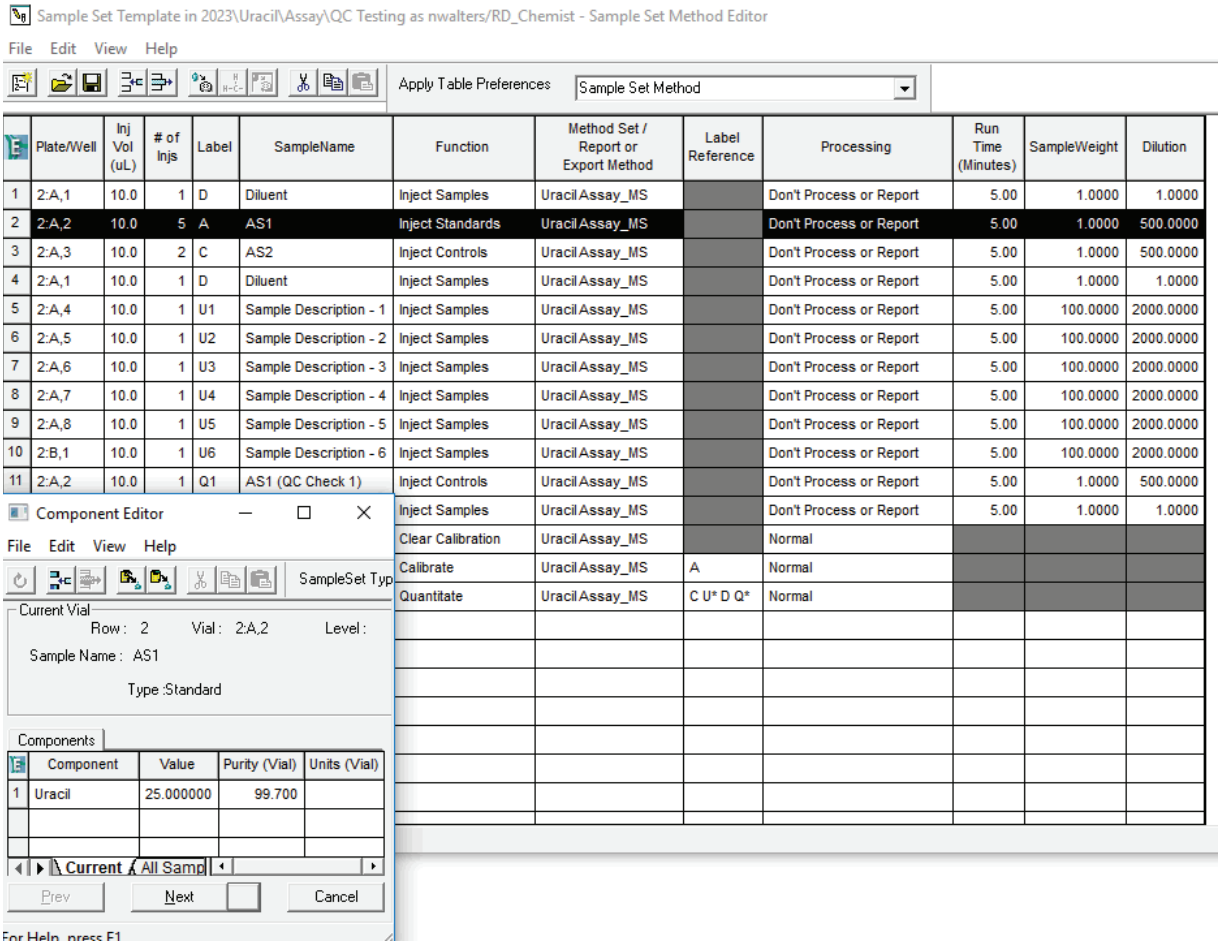
6.3.4.4. C_u = Concentration of the sample

6.3.4.5. **Empower custom field:** Assay

6.3.4.5.1. Sample Type: Unknown

6.3.4.5.2. Enter sample weight and Dilution factor in the "Alter Sample" window.

6.3.5. Example Alter Sample Window:



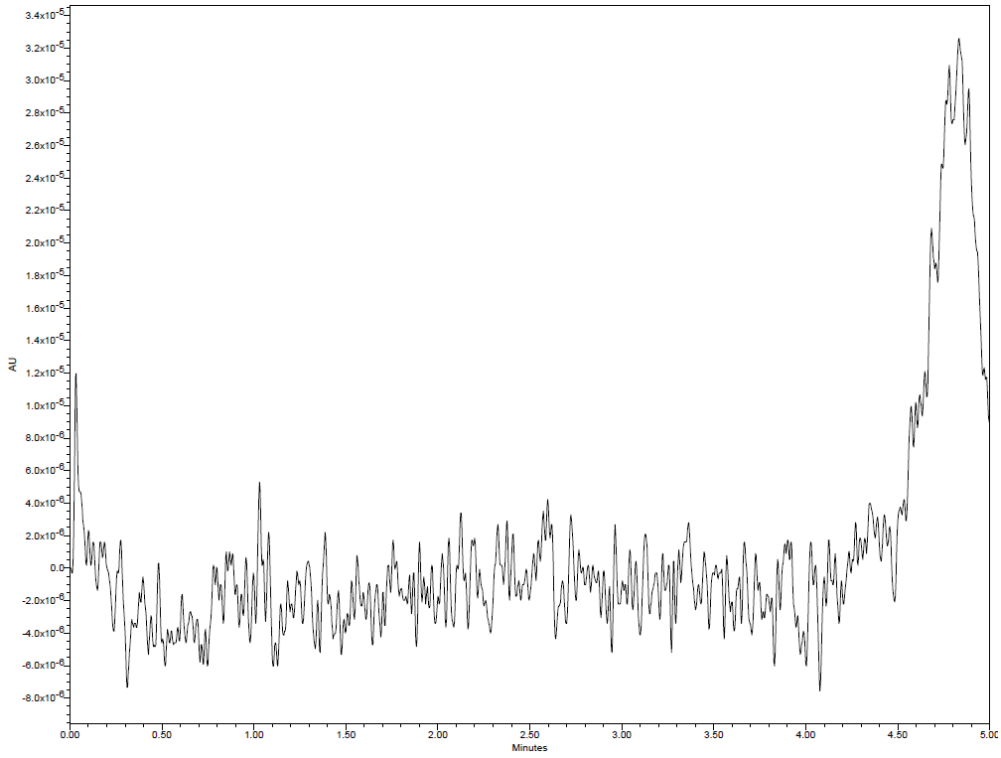
	Plate/Well	Inj Vol (uL)	# of Injs	Label	SampleName	Function	Method Set / Report or Export Method	Label Reference	Processing	Run Time (Minutes)	SampleWeight	Dilution
1	2:A,1	10.0	1	D	Diluent	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	1.0000	1.0000
2	2:A,2	10.0	5	A	AS1	Inject Standards	Uracil Assay_MS		Don't Process or Report	5.00	1.0000	500.0000
3	2:A,3	10.0	2	C	AS2	Inject Controls	Uracil Assay_MS		Don't Process or Report	5.00	1.0000	500.0000
4	2:A,1	10.0	1	D	Diluent	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	1.0000	1.0000
5	2:A,4	10.0	1	U1	Sample Description - 1	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	100.0000	2000.0000
6	2:A,5	10.0	1	U2	Sample Description - 2	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	100.0000	2000.0000
7	2:A,6	10.0	1	U3	Sample Description - 3	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	100.0000	2000.0000
8	2:A,7	10.0	1	U4	Sample Description - 4	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	100.0000	2000.0000
9	2:A,8	10.0	1	U5	Sample Description - 5	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	100.0000	2000.0000
10	2:B,1	10.0	1	U6	Sample Description - 6	Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	100.0000	2000.0000
11	2:A,2	10.0	1	Q1	AS1 (QC Check 1)	Inject Controls	Uracil Assay_MS		Don't Process or Report	5.00	1.0000	500.0000
						Inject Samples	Uracil Assay_MS		Don't Process or Report	5.00	1.0000	1.0000
						Clear Calibration	Uracil Assay_MS		Normal			
						Calibrate	Uracil Assay_MS	A	Normal			
						Quantitate	Uracil Assay_MS	C U* D Q*	Normal			

6.4. Reporting

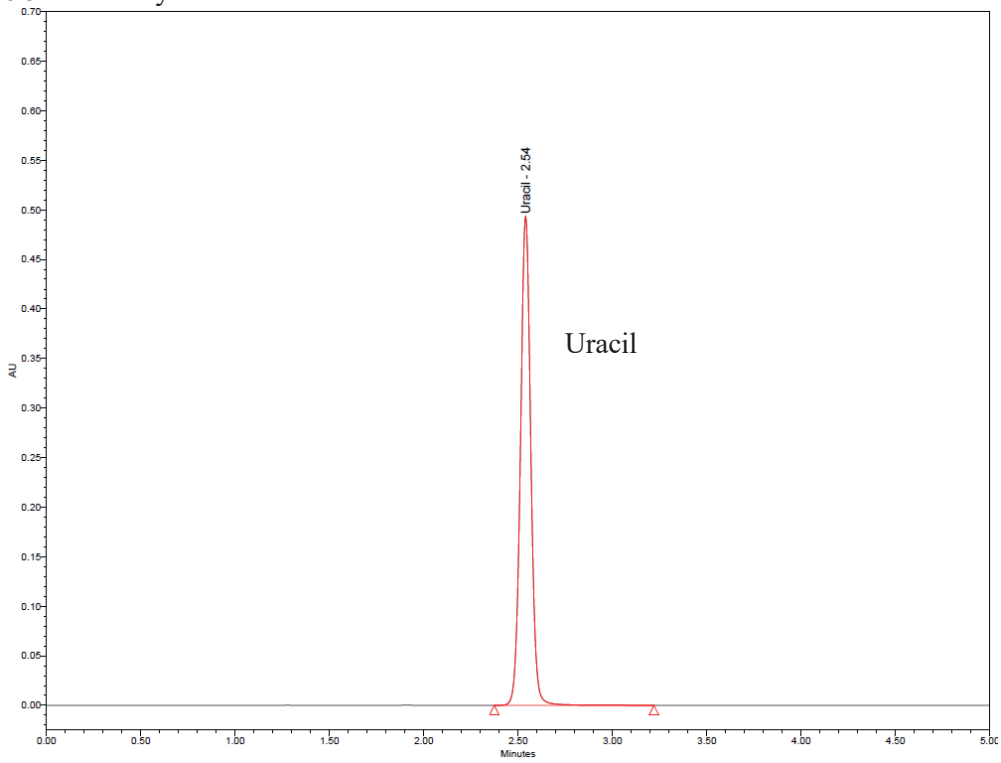
6.4.1. **Assay**: Calculate the % Uracil and report to a one (1) decimal place.

6.5. Example Chromatograms and Integrations

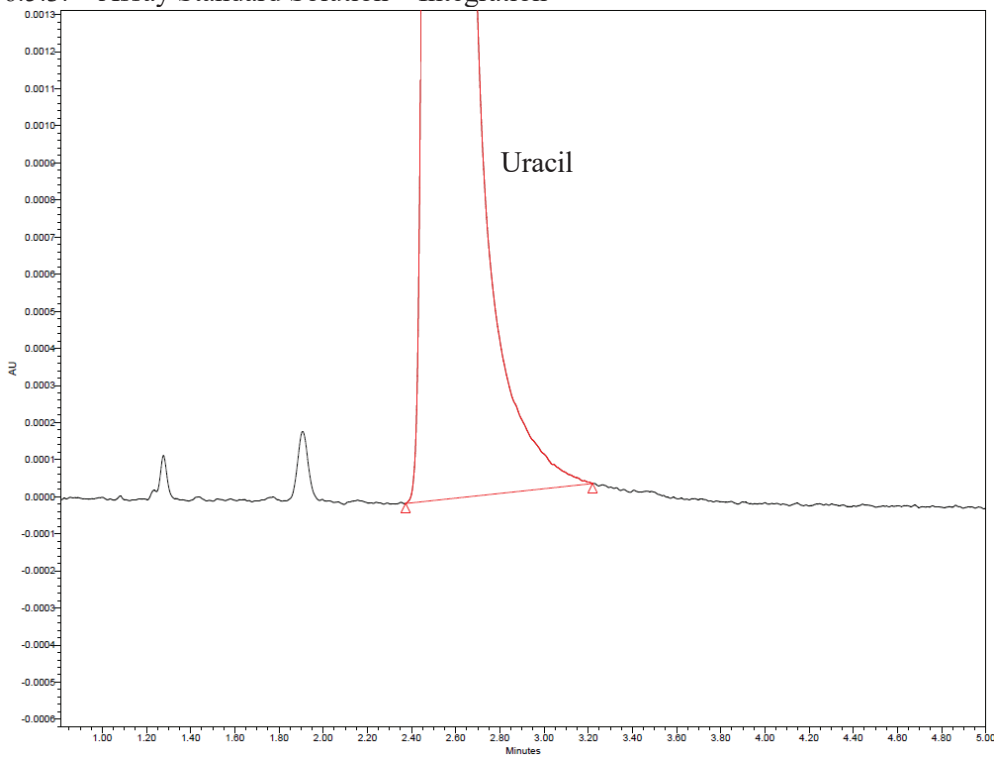
6.5.1. Diluent



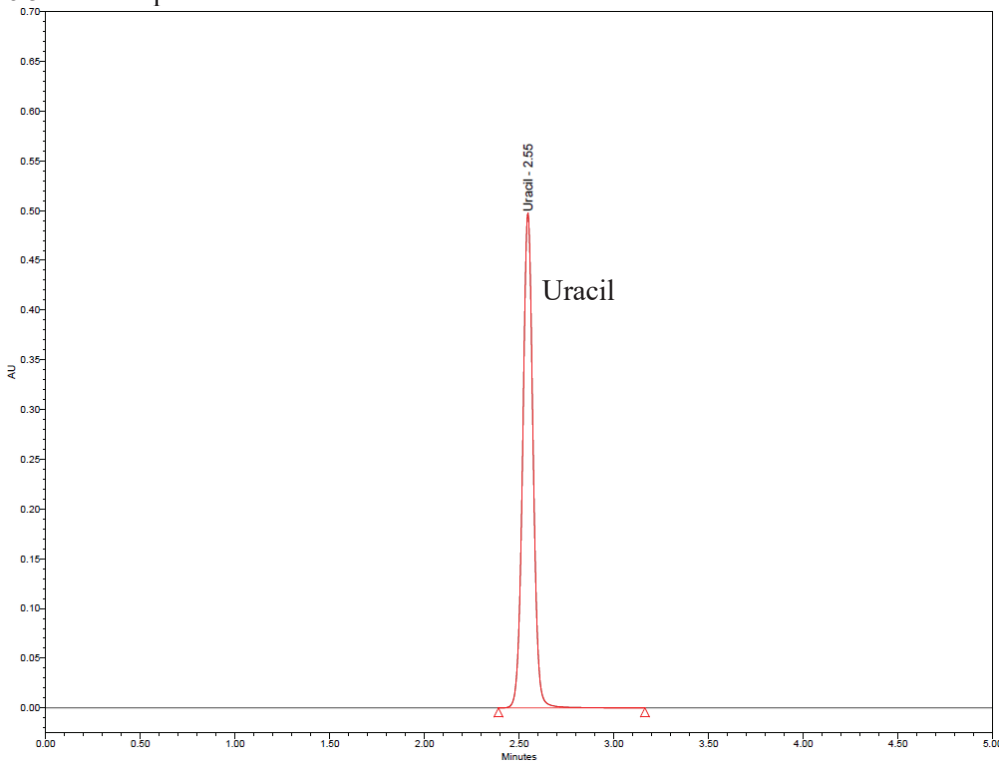
6.5.2. Assay Standard Solution



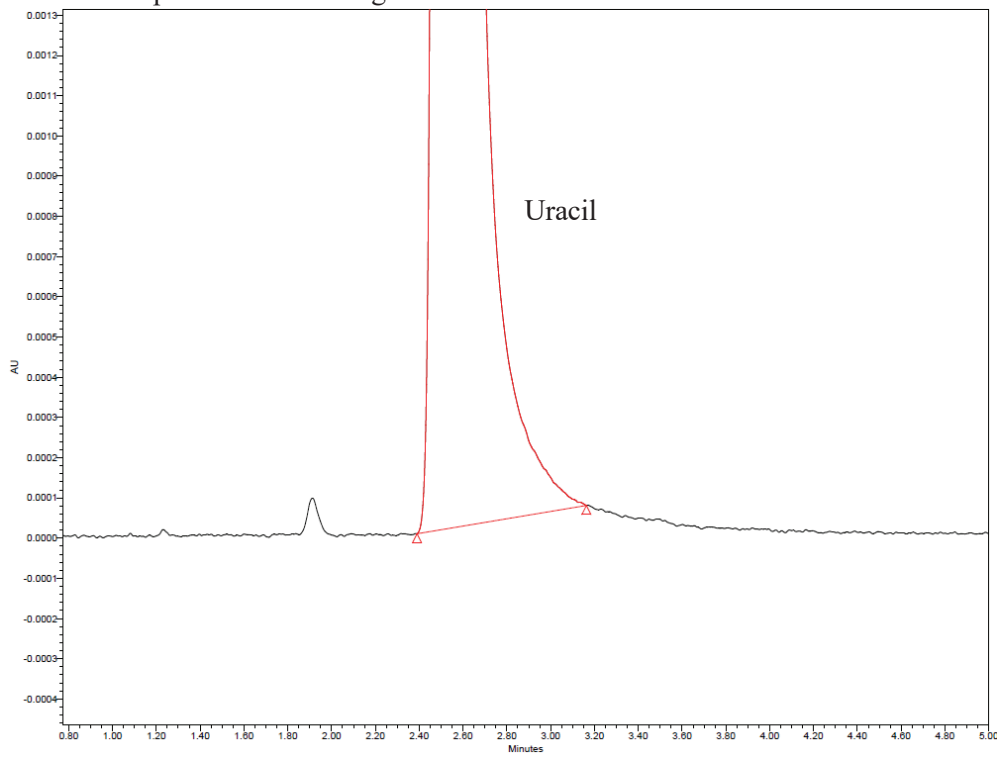
6.5.3. Assay Standard Solution – Integration



6.5.4. Sample Solution



6.5.5. Sample Solution – Integration



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