BIOSPECTRA
100 Majestic Way, Bangor, PA 18013 / www.biospectra.us

TROMETHAMINE API
STABILITY STRESS STUDY

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1. PURPOSE:

1.1. The purpose of this protocol and report is to report on two separate stress study results for Tromethamine (API) as per ICH guidance's Q1A(R2) & Q2(R1).
1.2. The purpose of stress testing was to provide evidence on how the quality of Tris API is affected after manipulations of temperature, humidity, pH, oxidation, and photolytic (light) stress.
1.3. To establish an analytical procedure for re-test period estimation for the Tris API and recommended storage conditions.
1.4. This Stress study shall be required to prove stability indicating property of Assay and organic impurity method as per ICH Q1A(R2) and USP <1225> Validation of Compendial Procedure.

2. SCOPE:

2.1. Establish stress conditions (acid, base, light, heat, humidity, and oxidation) and determine degradation pathway and analyze stressed samples in Assay by HPLC procedure and Related substances by HPLC method.
2.2. Establish Mass Balance & Peak Purity.

3. RESPONSIBILITIES:

3.1. The Executive Director of Quality Control is responsible for the control, implementation, training, and maintenance of this protocol and report.
3.2. The QC Analysts are responsible for performing the testing stated in this protocol and recording all results in the appropriate laboratory documentation.
3.3. The QC Laboratory Manager is responsible for the completion this protocol/report at the conclusion of the testing executed.

4. REFERENCES:

4.1. BSI-PRL-0425, Analytical Method Validation Tromethamine Assay via HPLC
4.2. BSI-RPT-0602, Analytical Method Validation Report: Tromethamine Assay via HPLC
4.3. BSI-RPT-0440, Analytical Method Validation Report: Limit of Tris(hydroxymethyl)nitromethane via HPLC
4.4. BSI-SOP-0098, Balance SOP
4.5. BSI-SOP-0250, HPLC Cleaning and Maintenance SOP
4.6. BSI-SOP-0126, Laboratory Notebooks SOP
4.7. BSI-ATM-0062, Tris Related Substances Analysis Method via HPLC
4.8. BSI-ATM-0007, Tris Testing Methods
4.9. ICH Q1A(R2) & Q2(R1)

5. EQUIPMENT:

5.1. Analytical Balance
5.2. Calibrated Oven
5.3. Perkin Elmer Flexar HPLC or equivalent

6. PROCEDURE:

6.1. Two Stress Studies were executed on TR1200-017-0220-PV.

6.2. Solution/Sample Preparation:

6.2.1. Diluent Preparation Instruction: (0.2N HCL**):** Transfer 200 mL of Hydrochloric acid 1M into a 1000 mL water. Mix well. Solubility is maintained at 500mg/25mL concentration.

6.2.1.1. Diluent Preparation**:** Transferred 250mL of HPLC grade water to a 1000mL volumetric flask. Diluted to volume with HPLC grade acetonitrile (Fisher, Lot 208069 Exp 3/31/26). Internal laboratory lot assigned as BSP27P80 Exp. 6/22.

6.2.2. Control sample: (Unstressed sample) Instruction: Weigh and transfer 500 mg Tromethamine in 25 mL flask. Add diluent and mix well till compete dissolution. Tromethamine concentration is 20000 ppm.

6.2.2.1. Sample Preparation**:** Weighed out 0.5005g of Tris and transferred to a 25mL volumetricflask. Dissolved with diluent lot BSP27P80 Exp. 6/22. Tris concentration is ~20,000ppm.

6.2.3. Blank Acid degradation: (1N HCL stress solution) Instruction**:** Transfer 2 mL of 1N HCL solution in 25mL flask and keep it aside for 2 hours in dark placeat room temperature. After 2 hours add 2mL of 1N NaOH solution to neutralize. Add analytical diluent and mix well till compete dissolution. Tromethamine concentration is nil.

6.2.3.1. Sample Preparation**:** Transferred 2mL of 1N HCl solution (Fisher, Lot 200538 Exp 3/22) to a 25mL volumetric flask and stored protected from light for 2 hours in a dark place at 20-25°C. After 2 hours added 2mL of 1N NaOH (Fisher, Lot 200538 Exp 3.22) solution to neutralize. Filled to volume with diluent and mixed well until complete dissolution. Tris concentration is nil.

6.2.4. Acid degradation: (1N HCL stress solution) Instruction**:** Weigh and transfer 500 mg Tromethamine in 25 mL flask. Added 2mL of 1N HCl solution and sonicate to dissolve it and keep it aside for 2 hours in dark place at room temperature. After 2 hours add 2mL of 1N NaOH solution to neutralize. Add diluent and mix well till compete dissolution. Tromethamine concentration is 20000ppm.

6.2.4.1. Sample Preparation: Weighed out 0.5007g of Tris and transferred to a 25mL volumetric flask. Transferred 2mL of 1N HCl solution (Fisher, Lot 200538 Exp 3/22) to the 25mL volumetric flask and stored protected from light for 2 hours in a dark place at 20-25°C. After 2 hours added 2mL of 1N NaOH (Fisher, Lot 201454 Exp. 4/22) solution to neutralize. Filled to volume with diluent and mixed well until complete dissolution. Tris concentration is~20000ppm.

6.2.5. Blank base degradation: (1N NaOH stress solution) Instruction**:** Transfer 2mL of 1N NaOH solution in 25mL flask and keep it aside for 2 hours in dark place at room temperature. After 2 hours add 2mL of 1N HCl solution to neutralize. Add diluent and mix well till compete dissolution. Tromethamine concentration is nil.

6.2.5.1. Sample Preparation: Transferred 2mL of 1N NaOH solution (Fisher, Lot 201454 Exp 4/22) toa 25mL volumetric flask and stored protected from light for 2 hours in a dark place at 20- 25°C. After 2 hours added 2mL of 1N HCl (Fisher, Lot 200538 Exp 3/22) solution to neutralize. Filled to volume with diluent and mixed well until complete dissolution. Tris concentration is nil.

6.2.6. Base degradation (1N NaOH stress solution) Instruction**:** Weigh and transfer 500 mg Tromethamine in 25 mL flask. Add 2 mL of 1N NaOH solution and sonicate to dissolve it and keep it aside for 2 hours in dark place at room temperature. After 2 hours add 2mL of 1N HCl solution to neutralize. Add diluent and mix well tillcompete dissolution. Tromethamine concentration is 20000 ppm.

6.2.6.1. Sample Preparation **s:** Weighed out 0.5000 grams of Tris and transferred to a 25mL volumetric flask. Added 2mL of 1N NaOH (Fischer, Lot 201454 Exp. 4/22) solution, dissolved, and stored protected from light for 2 hours in a dark place at 20-25°C. After 2 hours, added 2mL of 1N HCl (Fischer, Lot 200538 Exp 3/22) solution to neutralize. Filled to volume with diluent and mixed well until complete dissolution. Tris concentration is 20000 ppm.

6.2.7. Blank oxidative degradation (10% H_2O_2) Instruction: Transfer 2mL of 10% H_2O_2 solution in 25mL flask and keep it aside for 2 hours in dark place at room temperature. Add diluent and mix well till compete dissolution. Tromethamine concentration is nil.

6.2.7.1. Sample Preparation: Transferred 2mL of 10% H_2O_2 solution to a 25mL volumetric flask and stored protected from light for 2 hours in a dark place at 20-25°C. After 2 hours filled to volume with diluent and mixed well until complete dissolution. Tris concentration is nil.

6.2.8. Oxidative degradation: (10% H_2O_2) Instruction**:** Weigh and transfer 500 mg Tromethamine in 25 mL flask. Add 2 mL of 10% H_2O_2 solution and sonicate to dissolve it and keep it aside for 2 hours in dark place at room temperature.Add diluent and mix well till compete dissolution. Tromethamine concentration is 20000ppm.

Note: This solution shall be neutralized with equivalent concentration of Sodium thiosulfate solution or it can be used un-neutralized injecting in HPLC immediately.

6.2.8.1. Sample Preparation: Weighed out 0.5000 grams of Tris and transferred to a 25mL volumetricflask. Added 2mL of 10% H_2O_2 solution, dissolved, and stored protected from light for 2hours in a dark place at 20-25°C. After 2 hours filled to volume with diluent and mixed well until complete dissolution. Tris concentration is 20,000ppm.

6.2.9. Oxidative degradation: (3% H_2O_2) Instruction**:** Weigh and transfer 500 mg Tromethamine in 25 mL flask. Add 2 mL of 3% H_2O_2 solution and sonicate to dissolve it and keep it aside for 2 hours in dark place at room temperature. After 2 hours add 2mL of 1N HCl solution to neutralize. Add diluent and mix well tillcompete dissolution. Tromethamine concentration is 20000 ppm.

Note: This solution shall be neutralized with equivalent concentration of Sodium thiosulfate solution or it can be used un-neutralized injecting in HPLC immediately.

6.2.9.1. Sample Preparation: Weighed out 0.5000 grams of Tris and transferred to a 25mL volumetricflask. Added 2mL of 3% H_2O_2 solution, dissolved, and stored protected from light for 2 hours in a dark place at 20-25°C. After 2 hours added 2mL of 1N HCl solution to neutralize. Filled to volume with diluent and mixed well until complete dissolution. Tris concentration is 20,000ppm.

6.2.10. Blank Hydrolytic stress degradation Instruction**:** Transfer 2 mL of water in 25mL flask and keep it on water bath for 2 hours at 50C. Add diluent and mix well till compete dissolution. Make up to mark with diluent. Tromethamine concentration is nil.

6.2.10.1. Sample Preparation**:** Transferred 2mL of Milli-Q Purified Water into a 25mL volumetric flask and placed it in a water bath for 2 hours at 50°C. After 2 hours, filled to volume with diluent and mixed well until complete dissolution. Tris concentration is nil.

6.2.11. Hydrolytic degradation Instruction**:** Weigh and transfer 500 mg Tromethamine in 25 mL flask. Add 2 mL of milli-Q water and sonicate to dissolve it and keep it on water bath for 2 hours at 50C. After 2 hours Add diluent and mix well till compete dissolution. Make up to mark with diluent. Tromethamine concentration is 20000 ppm.

6.2.11.1. Sample Preparation**:** Weighed out 0.5000 grams of Tris and transferred to a 25mL volumetric flask. Added 2mL of Milli-Q Purified Water, dissolved, and placed in a water bath for 2 hours at 50°C. After 2 hours filled to volume with diluent and mixed well until complete dissolution. Tris concentration is 20,000ppm.

6.2.12. Thermal degradation Instruction**:** Weigh and transfer 500 mg Tromethamine in petri dish, keep it in hot air oven at 105C for 3 hours. Then transfer entire quantity in to 25 mL flask. Make sure no spillage or left over in Petri dish. Add diluent and mix well till compete dissolution. Make up to mark with diluent. Tromethamine concentration is 20000 ppm.

6.2.12.1. Sample Preparation: Weighed out 0.5003 grams of Tris and transferred to a glass Petri Dish. Placed in the VWR Gravity Convection Oven at 105°C for 3 hours. After 3 hours, quantitatively transferred entire quantity of the Petri Dish to a 25mL volumetric flask, dissolved in diluent, and filled to volume with diluent mixing well until complete dissolution. Tris concentration is 20,000 ppm.

6.2.13. Light degradation: (1day, 5 days) Instruction: Weigh and transfer 500 mg Tromethamine in quartz cuvette cover it from top with proper closure or with parafilm to avoid oxygen entry/air circulation. Keep it under normal indoorday light for 1 day and 5 days at room temperature. Transfer the content of each cuvette following each timepoint into a 25mL flask. Add diluent and mix well till compete dissolution. Make up to mark with diluent. Tromethamine concentration is 20000 ppm. Analyze both 1 day and 5 days sample independently.

Note: Quartz cuvette is ideal for light induced degradation because it does not absorb UV light significantly. Borosilicate glass container absorbs UV light, limiting the overall exposure.

6.2.13.1. Sample preparation: Weighed out 0.5006 grams (1 Day) and 0.5002 grams (5 Day) of Tris, transferred to two separate quartz cuvettes (Supplier, Perkin Elmer) and covered both to prevent air circulation. Once the samples met their target Lux Hours target they were transferred to a25mL volumetric flask, dissolved in diluent, and filled to volume with diluent mixing well until complete dissolution. Tris concentration is 20,000ppm.

= *10176 lux x 24 hours x 5 days = 1.22 million lux hours* Indoor Light Levels

Calculating Illumination

Illumination can be calculated as
$E = \Phi_l \, C_u \, L_{LF} / A_l$

Where,
E = illumination (lux, lumen/m²) Φ_l = *luminance per lamp (lumen)* C_u = *coefficient of utilization*
L_{LF} = *light loss factor* A_l = *area per lamp (m²)*

Example - Illumination
10 lamps of *500 W (10600 lumens per lamp)* are used in an area of 10 m^2.With $C_u = 0.6$ and $L_{LF} = 0.8$ illumination can be calculated as
$E = 10$ (10600 lumens) x (0.6) x (0.8) / (5 m^2)

Measuring Illumination: Optionally commercially available Lux meter can also be utilized to measure the intensity.

6.2.14. Target: should be 1.2 Million Lux hours light degradation

6.2.15. The target exposure for degradation over a 5 day period is 1.2 million lux hours. The lux hours of exposure was calculated using the following equations (Lux was measured using the ILT10 Lux Meter

$$Lux\ Hours = Lux \times Duration\ (Hours)$$

Measure Lux Value = 36,500 Lux

$$1\ Day\ Exposure\ Lux\ Hour\ Target = \frac{1\ Day\ Exposure}{5\ Day\ Exposure} = \frac{x}{1{,}200{,}000\ Lux\ Hours}$$

X= 240,000 *Lux Hours*

$$1\ Day\ Exposure\ Duration = \frac{Lux\ Hours}{Lux} = \frac{240{,}000\ Lux\ Hours}{36{,}500\ Lux} = 6.6\ Hours$$

$$5\ Day\ Exposure\ Duration = \frac{Lux\ Hours}{Lux} = \frac{1{,}200{,}000\ Lux\ Hours}{36{,}500\ Lux} = 32.9\ Hours$$

6.2.16. Analysis of stressed samples:

6.2.17. System Suitability performed in accordance with Method**:**

Parameter	Setting
Column	SIELC Newcrom R1, 3.2 x 100mm, 3µm particle size
Mobile Phase	90% Water; 10% Acetonitrile; 0.1% Phosphoric Acid
Flow Rate	0.5mL/min
Column Temperature	25°C
Autosampler Temperature	25°C
Injection Volume	20µL
UV Wavelength	210nm
Run Time	5 minutes

System Suitability 2-Nitroethanol (9.5ppb)			
System Suitability	**Area**	**Average Area**	**%RSD**
System Suitability 1	1241	1247	1.1%
System Suitability 2	1227		
System Suitability 3	1259		
System Suitability 4	1259		
System Suitability 5	1249		

System Suitability 2-Nitropropane-1,3-diol (9.5ppb)			
System Suitability	**Area**	**Average Area**	**%RSD**
System Suitability 1	953	946	1.4

6.2.18 Procedure for Analysis: Related Substances of Tromethamine drug substance by UPLC (PDA Detector) (Inject stress solutions in Section 6.2 with appropriate dilutions).

Measure peak purity for each specified Impurities and Tromethamine peak. Calculate all three specified Impurities (2-Nitropropane-1, 3-diol, Tris(hydroxymethyl) nitromethane, 2-Nitroethanol), Any Unspecified Impurities, and total Impurities.

6.2.18.1 Result Conclusion: The method of analysis via UPLC for tris(hydroxymethyl)nitromethane, 2-nitropropane-1,3-diol, and 2-nitroethanol is specific for only these three compounds and is not expected or designed to detect unspecified impurities. Tromethamine elutes prior to separation and is not integrated or reported from this method. The sensitivity of the method is also specifically designed for these three compounds of interest at a level in solution of 11ppb max. The injections were made however the data is not considered viable since the stress method blanks contained a peak overlapping with the expected retention time of tris(hydroxymethyl)nitromethane.

6.2.19 Procedure for Analysis: Assay of Tromethamine by HPLC Requirements: (Inject stress solution in Section 6.2 with appropriate dilutions)

6.2.19.1 System Suitability performed in accordance with Assay Method:

6.2.19.2 Notes: The HPLC method of tris analysis is also qualified as alternative ID test to TLC plate identification and validated for detection of tris(hydroxymethyl)nitromethane at limit levels of 0.03% w/w in Tris. This method was the original method of organic impurities analysis to target the EP specified impurity tris(hydroxymethyl)nitromethane. The UPLC method was adopted when two other specified impurities introduced during processing were identified. The reason for the UPLC method of analysis being set at <1ppm limits was due to concern that the impurities in tris may have been mutagenic and the limits were set following ICH M7 guidelines. It was later discovered that the impurities are not mutagenic and should follow the ICH Q3 guidelines of <300ppm (0.03%) for unspecified impurities. Tris(hydroxymethyl)nitromethane routine analysis uses tris(hydroxymethyl)nitromethane as a standard and the response factor at 210nm in relationship to tris was measured to be substantially higher than the response factor that tris provides.

Peak Response Factor = Peak Response (mV) / Concentration (mg/L)

Analyte	Concentration (mg/L)	Peak Response (mV)	Peak Response Factor
Tris(hydroxymethyl)nitromethane	0.6	17804.40	29674.00
Tromethamine	20000	2330601.60	116.53

HPLC Assay Method Parameters

Column: Agilent Zorbax Part ID: 843300-908

Parameter	Setting
Flow Type	Isocratic
Mobile Phase	25% Water; 75% Acetonitrile
Flow Rate	1.0mL/min
Injection Volume	20µL
Detector	UV at 210nm
Detector Temperature	Ambient
Column Temperature	30°C
Run Time	15 minutes

System Suitability			
System Suitability	**Tris Area**	**Average Tris Area**	**%RSD**
System Suitability 1	2330601.60	2314336.68	2.3%
System Suitability 2	2374958.40		
System Suitability 3	2319823.80		
System Suitability 4	2227191.20		
System Suitability 5	2319108.40		

6.2.20 Acceptance Criteria:

6.2.20.1 Target degradation of about 1-5% (or even up to 15% is acceptable) in any of the condition.

6.2.20.2 Perform summation of Total Impurities and Assay value to calculate Mass Balance. Target should be > 95% of Control Sample Assay.

6.2.21 Summary of 1st Stress Study Results:

6.2.22 % w/w Assay Results

Assay Results					
Sample	Sample Weight (mg)	Final Volume (mL)	Sample Concentration (mg/mL)	Tris Area	Assay
TR1200-017-0220-PV Control (Unstressed) Sample	500.5	2500	2.002	2228040.20	96.20%
TR1200-017-0220-PV Acid Degradation	500.7	2500	2.003	1094034.40	47.22%
TR1200-017-0220-PV Base Degradation	500.0	2500	2.000	765343.96	33.08%
TR1200-017-0220-PV Oxidative (10% H2O2)	500.0	2500	2.000	1292904.21	55.88%
TR1200-017-0220-PV Oxidative (3% H2O2)	500.2	2500	2.001	1300103.04	56.17%
TR1200-017-0220-PV Hydrolytic Degradation	500.0	2500	2.000	2296931.40	99.28%
TR1200-017-0220-PV Thermal Degradation	500.3	2500	2.001	2254488.8	97.38%
TR1200-017-0220-PV Light Degradation 1 Day	500.6	2500	2.002	2305628.8	99.53%
TR1200-017-0220-PV Light Degradation 5 Days	500.2	2500	2.001	2303692.8	99.53%

6.2.23 **%** w/w Impurity Results

% w/w Impurity Results			
Tris Standard Value 2.00mg/mL =	**2314336.68 Area Count**		
Sample ID	Impurity Area	Sample Concentration (mg/mL)	% w/w Impurity
TR1200-017-0220-PV Control (Unstressed) Sample	3617.80	2.002	0.16
TR1200-017-0220-PV Acid Degradation	80352.20	2.003	3.47
TR1200-017-0220-PV Base Degradation	801797.16	2.000	34.64
QC Check NIST Std. 723e	0.00	2.000	0.00
TR1200-017-0220-PV Oxidative (10%H2O2) Degradation	9699593.54	2.000	419.11
TR1200-017-0220-PV Oxidative (3% H2O2) Degradation	2132155.56	2.001	92.08
TR1200-017-0220-PV Hydrolytic Degradation	4131.60	2.000	0.18
QC Check NIST Std. 723e	2856.40	2.000	0.12
TR1200-017-0220-PV Thermal Degradation	4828.80	2.001	0.21
TR1200-017-0220-PV Photolytic Degradation 1 Day	5251.20	2.002	0.23
TR1200-017-0220-PV Photolytic Degradation 5 Day	5093.60	2.001	0.22
QC Check NIST Std. 723e	3484.80	2.000	0.15

6.2.24 Chromatographic Purity Section:

6.2.25 Calculate Peak Purity for specified Impurities and Tromethamine peak.

Peak Purity = Ru/Rr

Where:

R_u = Peak Response of Target AnalyteR_t = Total Peak Response Detected

Chromatographic Purity		
Sample ID	% Impurity	% Tris Purity
TR1200-017-0220-PV Control (Unstressed) Sample	0.16	99.84
TR1200-017-0220-PV Acid Degradation	6.84	93.16
TR1200-017-0220-PV Base Degradation	37.21	62.79
TR1200-017-0220-PV Oxidative (10%H2O2) Degradation	88.24	11.76
TR1200-017-0220-PV Oxidative (3% H2O2) Degradation	62.12	37.88
TR1200-017-0220-PV Hydrolytic Degradation	0.18	99.82
TR1200-017-0220-PV Thermal Degradation	0.21	99.79
TR1200-017-0220-PV Photolytic Degradation 1 Day	0.23	99.77
TR1200-017-0220-PV Photolytic Degradation 5 Day	0.22	99.78

6.2.26 Analytical Instrumentation/Equipment/Reagents utilized for Protocol Execution:

Waters H-Class UPLC	
Identification	Serial number and due date
UV Detector	J18TUV016A, due 2/22
Sample Manager	FTN-K18FTP166G, due 2/22
Column Heater	K18CHA186g, due 2/22
Quaternary Solvent Manager	K18QSP106A, due 2/22
Perkin Elmer Flexar HPLC	
Identification	Serial number and due date
Pump	291S133111109F, due 6/22
Autosampler	293H32080804A, due 6/22
Column Oven	OVHF130915868, due 6/22
UV/Vis Detector	292S14031703F, due 6/22
Solvent Manager	260S13111110F, due 6/22
HPLC Column	
Identification	Serial number
Agilent ZORBAX Carbohydrate 5µm 4.633 IDx150mm	USAR006906
SIELC Newchrom R1, 3.2x100mm 3µm particle size	NRIQP2504
Analytical Balance	
Identification	Serial number and due date
Secura 124-1S	29212172, due 10/21
MSE 224S	24801744, due 10/21
Timers	
Identification	Serial number and due date
Fisher Scientific Traceable Four-Channel Countdown Alarm Timer/Stopwatch with Memory Recall	200299696 due 5/17/22 210012708 due 1/7/23

Pipettes	
Identification	Serial number and due date
Eppendorf Research Plus	G54479H due 11/21 I45595H due 11/21
Oven	
Identification	Serial number and due date
VWR Gravity Convection Oven Model:414005-106	1100001138R003 due 6/21
Water Bath	
Identification	Serial number and due date
Fisherbrand Isotemp Digital-Control Water Bath: Model 205	300004011 due 2/22
ILT10 Lux/Light Meter	
Serial Number/Calibration date	00370, Calibration date 6/3/21

Chemical Details			
Name	CAS #	Lot #	Retest/Expiry Date
Tris Bio FUISA Grade	77-86-1	TR1200-017-0220-PV	2/28/22
Acetonitrile HPLC Grade	75-05-8	208069	3/26
1N HCl, Certified Grade	7647-01-0, 7732-18-5	200538	3/22
1N NaOH, Certified Grade	7732-18-5, 1310-73-2	201454	4/22
Hydrogen Peroxide 30%, Certified ACS	7722-84-1, 7732-18-5	175412	9/21
Tris(hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$ Acidimetric Standard, NIST Standard Reference Material	77-86-1	SRM723e	12/28/23

6.2.27 Mass Balance Table (95%)

Mass Balance Table			
Sample ID	% w/w Impurity	% w/w Tris	% Total (NLT 95%)
TR1200-017-0220-PV Control (Unstressed) Sample	0.16	96.20	96.36
TR1200-017-0220-PV Acid Degradation	3.47	47.00	50.47
TR1200-017-0220-PV Base Degradation	34.64	33.08	67.72
TR1200-017-0220-PV Oxidative (10%H2O2) Degradation	419.11	55.88	474.99
TR1200-017-0220-PV Oxidative (3% H2O2) Degradation	92.08	56.17	148.25
TR1200-017-0220-PV Hydrolytic Degradation	0.18	99.28	99.46
TR1200-017-0220-PV Thermal Degradation	0.21	97.38	97.59
TR1200-017-0220-PV Photolytic Degradation 1 Day	0.23	99.53	99.76
TR1200-017-0220-PV Photolytic Degradation 5 Day	0.22	99.53	99.75

6.2.28 Conclusion of Study**:** Unexpectedly low and high mass balances (red) were achieved during the chemically induced stress tests. The tris standard used to quantitate unknown values has very low UV absorptivity characteristics. It was noted that the oxidized stress solutions were yellow in color, the color change suggests that the species in solution after oxidative stress may have very different absorptivity properties than tromethamine. The acid and base samples showed poor peak quality and shape, suggesting the chromatographic method was affected by the introduction of acids and bases. Previously studied as a free base, tromethamine during the acid and base stress, acts as a buffer it is unknown how UV absorptivity is affected.

Addendum: Stress Test Study on Tris API (Forced Degradation) Mild Condition

7. SCOPE:

7.1. Subject Tris API to acid, base, oxidation, thermal, and light induced degradation.

7.2. Target is 5-20% degradation. Mild conditions will be evaluated to avoid the formation of secondary products of degradation.

7.3. Report Mass Balance.

8. PROCEDURE:

Samples to be Prepared:

8.1. Control (Unstressed) Sample

8.1.1. Sample Preparation: Lot TR1200-017-0220-PV, 200.4mg of sample was transferred to a 100mL volumetric flask. The sample was dissolved and diluted to volume with diluent (75:25 Acetonitrile: Water) for a final concentration of 2.00mg/mL.

8.2. Acid Degradation Instruction: Add 2mL of 0.2N HCl on 500 mg Tris and keep for 2 hours at RT in dark, followed by neutralization with 0.2N NaOH. Prepare solution as per the test method and inject.

8.2.1. Sample Preparation: Added 2mL of 0.2N HCl on 500.5 mg DS and stirred to dissolve. Capped, placed away for 2 hours at room temperature in the dark, followed by neutralization with 0.2N NaOH. Pipetted 0.800mL of the 250mg/mL degradation sample and transferred to a 100mL volumetric flask for a final concentration of 2.00mg/mL. The sample was dissolved and diluted to volume with diluent (75:25 Acetonitrile: Water).

8.3. Base Degradation: Add 2mL of 0.2N NaOH on 500 mg Tris and keep for 2 hours at RT in dark, followed by neutralization with 0.2N HCl. Prepare solution as per the test method and inject.

8.3.1. Sample Preparation: Added 2mL of 0.2N NaOH on 500.4 mg DS and stirred to dissolve. Capped, placed away for 2 hours at room temperature in the dark, followed by neutralizationwith 0.2N HCl. Pipetted 0.800mL of the 250mg/mL degradation sample and transferred to a 100mL volumetric flask for a final concentration of 2.00mg/mL. The sample was dissolved and diluted to volume with diluent (75:25 Acetonitrile: Water).

8.4. Oxidative Degradation: Add 2 mL of 0.5% H2O2 on 500 mg Tris and keep for 2 and 4 hours at RT. Prepare solution as per the test method. In the HPLC sequence, after establishing system suitability, consider injecting oxidized samples first if the solutions are not quenched(i.e. to arrest any further oxidation).

8.4.1. Sample Preparation: Oxidative Degradation (2 Hour): Added 2 mL of 0.5% H_2O_2 on 500.1 mg DS and stirred to dissolve. Kept for 2 hours at room temperature protected from light. Pipetted 0.800mL of the250mg/mL degradation sample and transferred to a 100mL volumetric flask for a final concentration of 2.00mg/mL. The sample was dissolved and diluted to volume with diluent (75:25 Acetonitrile: Water).

8.4.2. Sample Preparation: Oxidative Degradation (4 Hour): Added 2 mL of 0.5% H_2O_2 on 500.1 mg DS and stirred to dissolve. Kept for 2 hours at room temperature protected from light. Pipetted 0.800mL of the250mg/mL degradation sample and transferred to a 100mL volumetric flask for a final concentration of 2.00mg/mL. The sample was dissolved and diluted to volume with diluent (75:25 Acetonitrile: Water).

8.5. Summary of Mild Conditions Results:

HPLC Assay Method Parameters

Column: Agilent Zorbax Part ID: 843300-908

Parameter	Setting
Flow Type	Isocratic
Mobile Phase	25% Water; 75% Acetonitrile
Flow Rate	1.0mL/min
Injection Volume	20µL
Detector	UV at 210nm
Detector Temperature	Ambient
Column Temperature	30°C
Run Time	15 minutes

% w/w Assay Results			
Tris Standard Value 2.00mg/mL =	**2204974.64 Area Count**		
Sample ID	Tris Area	Sample Concentration (mg/mL)	% w/w Tris
TR1200-017-0220-PV Control (Unstressed) Sample	2249633 .60	2.002	102.03
TR1200-017-0220-PV Mild Acid Degradation	3162904 .53	2.002	143.48
TR1200-017-0220-PV Mild Base Degradation	870254. 82	2.002	39.49
TR1200-017-0220-PV Mild Oxidative 2 Hours H2O2 Degradation	1883506 .40	2.000	85.51
TR1200-017-0220-PV Mild Oxidative 4 Hours H2O2 Degradation	1890827 .80	2.000	85.84
QC Check NIST Std. 723e	2270806 .40	2.002	102.99

Chromatographic Purity Evaluation:

Calculated Peak Purity for specified Impurities and Tromethamine peak:

$$\text{Peak Purity} = R_u/R_T$$

Wher
e: R_u = Peak Response of Target AnalyteR_t
= Total Peak Response Detected

Chromatographic Purity		
Sample ID	% Impurity	% Tris Purity
TR1200-017-0220-PV Control (Unstressed) Sample	0.21	99.79
TR1200-017-0220-PV Acid Degradation	14.63	85.37
TR1200-017-0220-PV Base Degradation	30.42	69.58
TR1200-017-0220-PV Oxidative 2 Hours H2O2 Degradation	11.75	88.25
TR1200-017-0220-PV Oxidative 4 Hours H2O2 Degradation	12.31	87.69
QC Check NIST Std. 723e	0.00	100.00

% w/w Impurity Results			
Tris Standard Value 2.00mg/mL =	**2204974.64 Area Count**		
Sample ID	Impurity Area	Sample Concentration (mg/mL)	% w/w Impurity
TR1200-017-0220-PV Control (Unstressed) Sample	4761.60	2.002	0.22
TR1200-017-0220-PV Mild Acid Degradation	541910.27	2.002	24.55
TR1200-017-0220-PV Mild Base Degradation	380547.58	2.002	17.24
TR1200-017-0220-PV Mild Oxidative 2 Hours H2O2 Degradation	250690.00	2.000	11.37
TR1200-017-0220-PV Mild Oxidative 4 Hours H2O2 Degradation	265392.00	2.000	12.04
QC Check NIST Std. 723e	0.00	2.002	0.00

Mass Balance Table			
Sample ID	% w/w Impurity	% w/w Tris	% Total (NLT 95%)
TR1200-017-0220-PV Control (Unstressed) Sample	0.22	102.03	102.25
TR1200-017-0220-PV Mild Acid Degradation	24.55	143.48	168.03
TR1200-017-0220-PV Mild Base Degradation	17.24	39.49	56.73
TR1200-017-0220-PV Mild Oxidative 2 Hours H2O2 Degradation	11.37	85.51	96.88
TR1200-017-0220-PV Mild Oxidative 4 Hours H2O2 Degradation	12.03	85.84	97.87

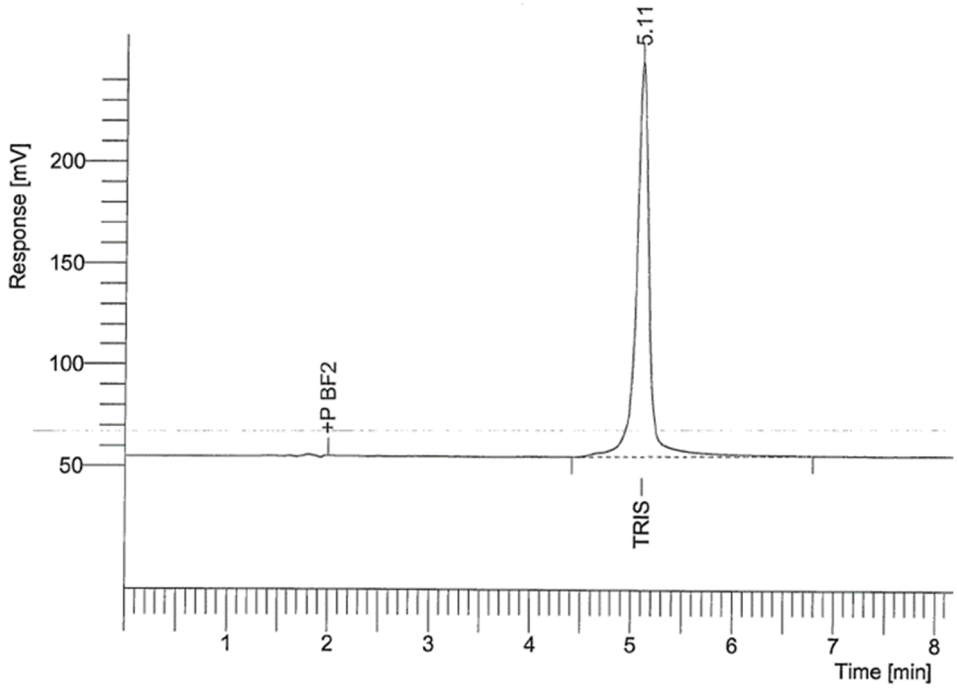
7.4. Conclusion of Study**:** Mass Balance was achieved with the oxidative stress samples in both 2- and 4-hour oxidation incubation cases. Unexpectedly low and high mass balances (red) were achieved during the acid and base induced stress tests. The acid and base treated samples continued to demonstrate poor peak quality and shape, suggesting the chromatographic method was affected by the introduction of acids and bases. Tris is compatible with anhydrous HCl and forms a salt, Tris hydrochloride, this buffer when used in crystalline form is usually combined with sodium hydroxide to make a robust and stable buffer in the physiological pH range. The stability with tris and acid is also suggested since the United States NIST (National Institute of Standards and Technology) adopted the material as a national acidimetric standard. In addition to this, no clear degradation peaks were seen in the chromatograms for both acid and base stress samples. The parent peak appears split and unresolved with no additional peaks generated. It is

concluded that since tris is compatible with both strong acids and bases and will not demonstrate degradation from such manipulations, however, chromatography separation is affected due to a buffer's inherent interaction and equilibrium with H+.

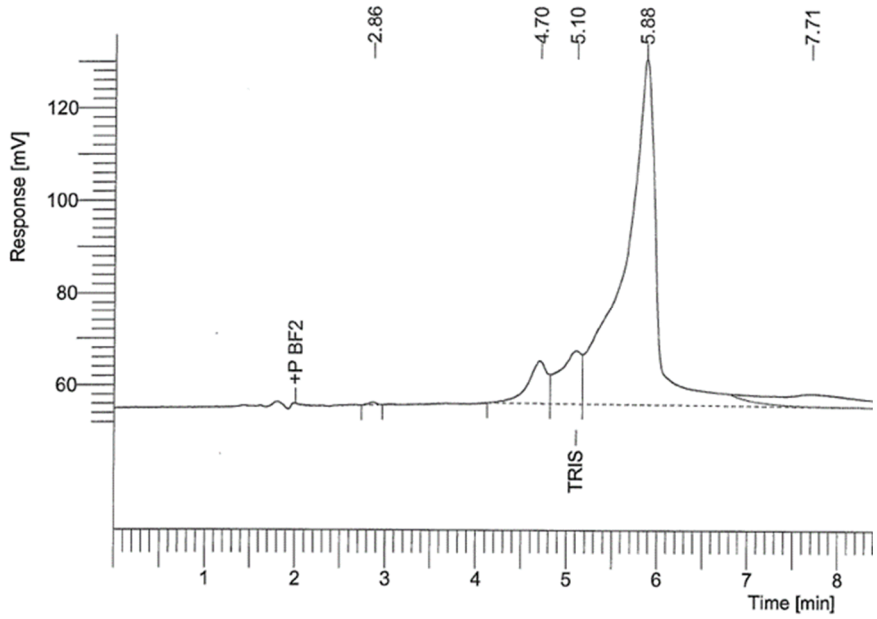
An acid gradient study was performed to verify the elution change and peak splitting due to HCl addition. Tris hydrochloride was analyzed to obtain a reference retention time for the tris hydrochloride peak, then tris was acidified in a series of increasing gradients (25%, 50%, 75%, and 100% Molar Equivalents of HCl). The acidified results from the 75% and 100% acidified solution matched tris hydrochloride in terms of retention time and peak characteristic while the less acidified samples (buffered) demonstrated split peaks similar to the peaks obtained from the stress study procedure for the acid and base stress injections. The poor chromatography after the 100% Tris Hydrochloride injection on the tris base is suspected to be due to the influence of H+ with the Zorbax Carbohydrate column. The size of splitting was directly proportionate to the amount of HCl spiked. Refer to BR19 pp 15-16; performed on 1/5/22.

7.4.1. Example Peak Shapes and % Molar Equivalent HCl addition:

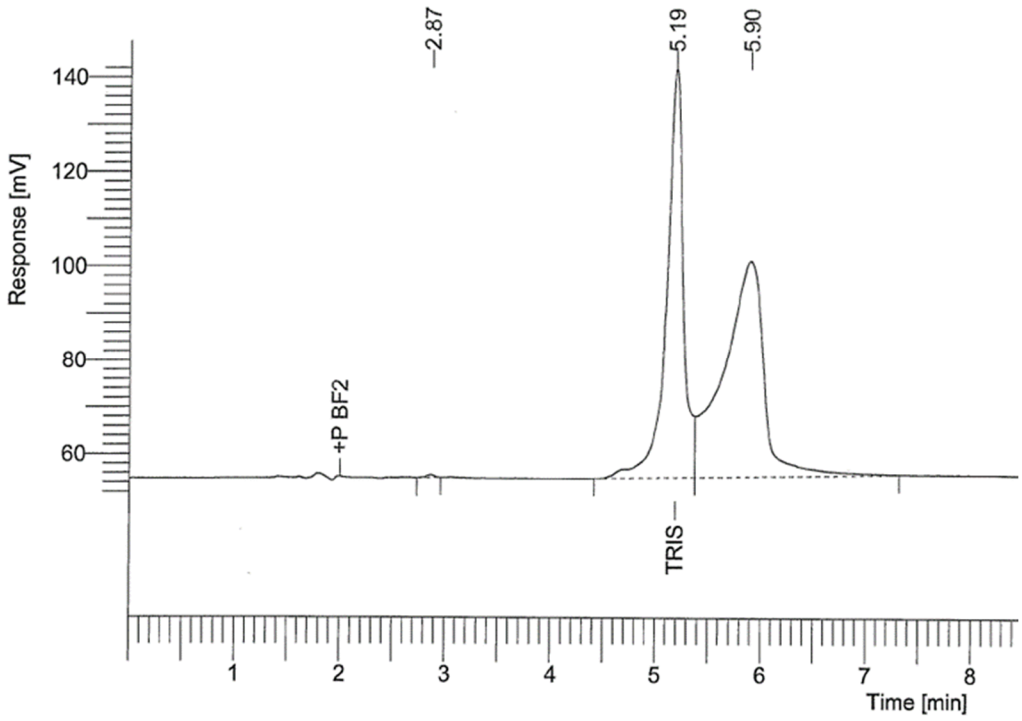
7.4.1.1. Tris Hydrochloride: 20mg/mL in diluent RT = 5.11min



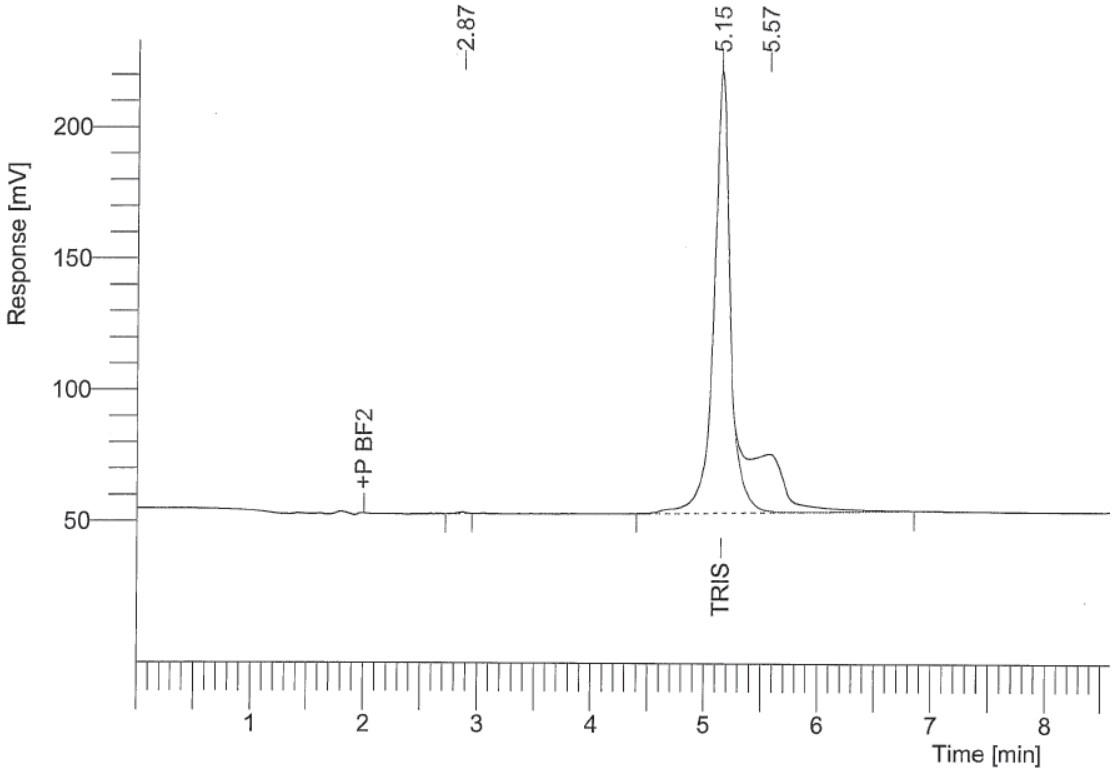
7.4.1.2. Tris Base: 20mg/mL in diluent RT = 5.88min



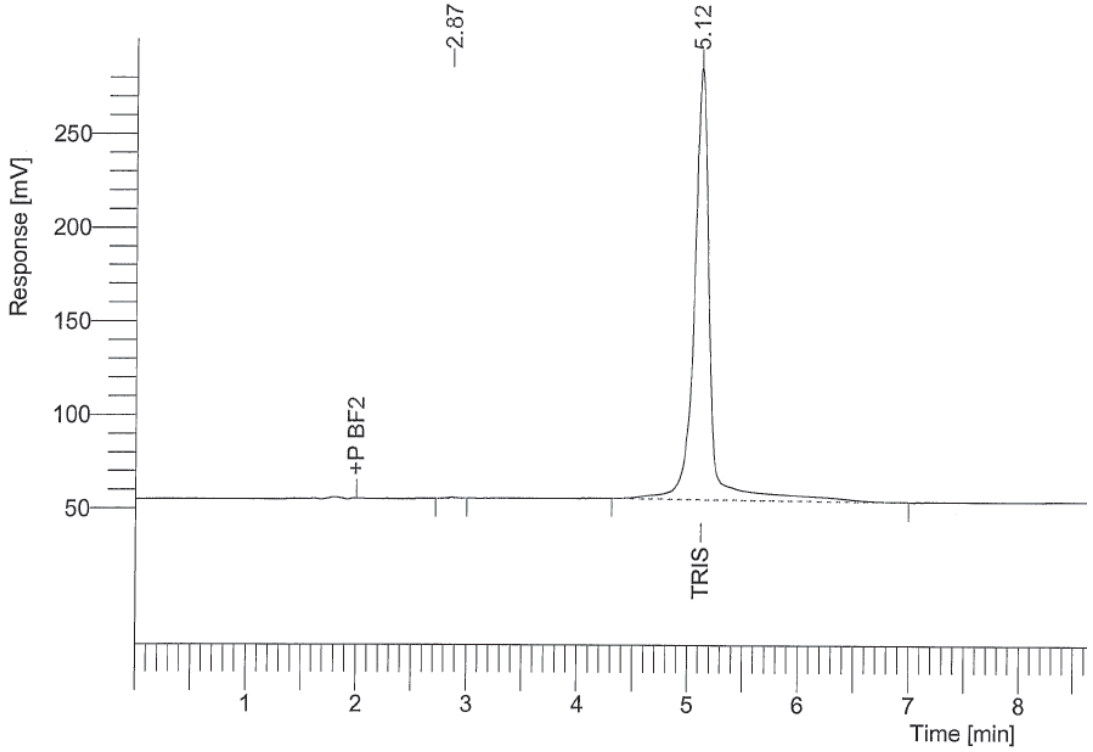
7.4.1.3. 25% Acidified Tris Base



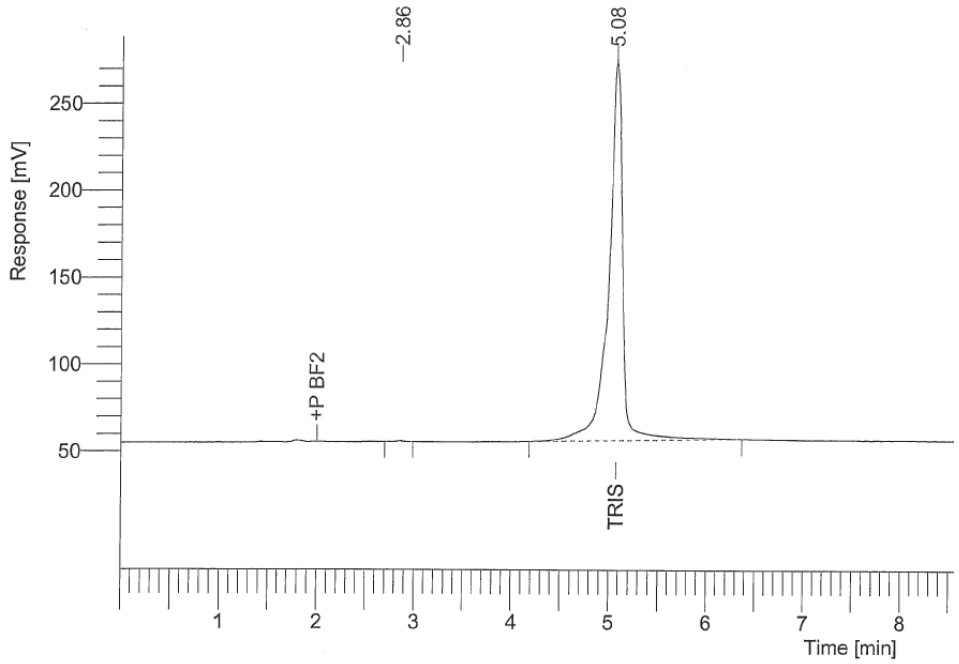
7.4.1.4. 50% Acidified Tris Base



7.4.1.5. 75% Acidified Tris Base



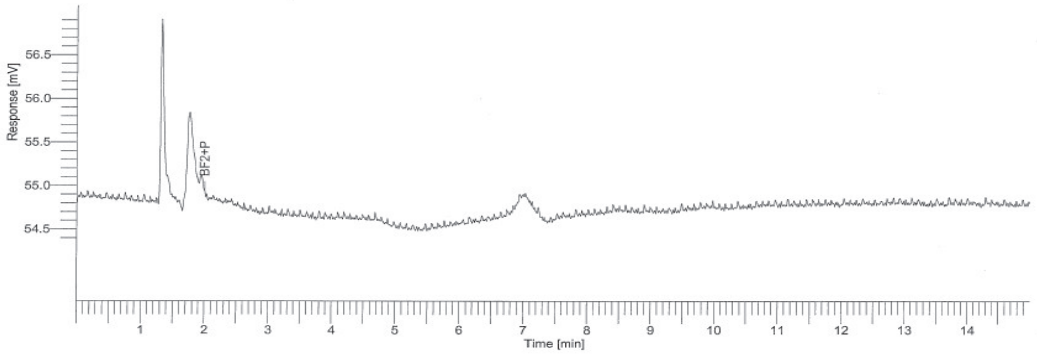
7.4.1.6. 100% Acidified Tris Base



Example Chromatograms from Study:

Blank Chromatogram

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
Tris	-	4.700	0.00
			0.00

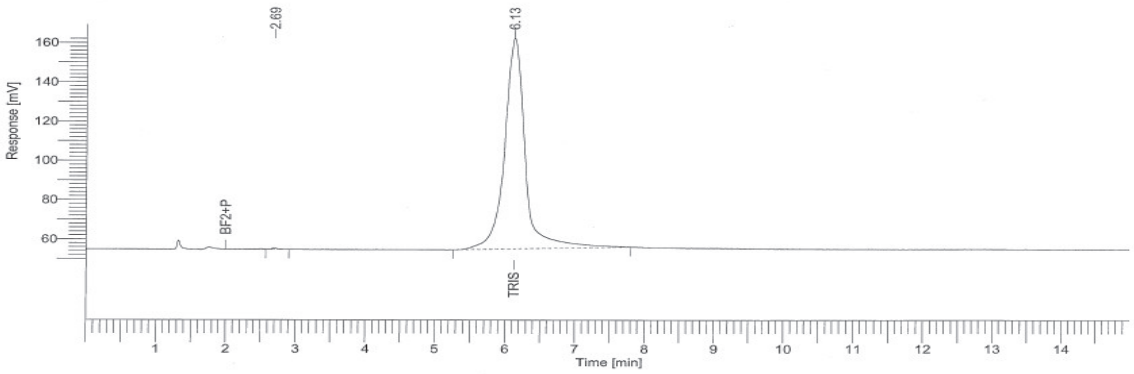
Missing Component Report

Component	Expected Retention (Calibration File)
Tris	4.700

BioSpectra Inc.

Control Sample Chromatogram

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.692	3617.80
Tris	2	6.128	2228040.20
			2231658.00

Missing Component Report

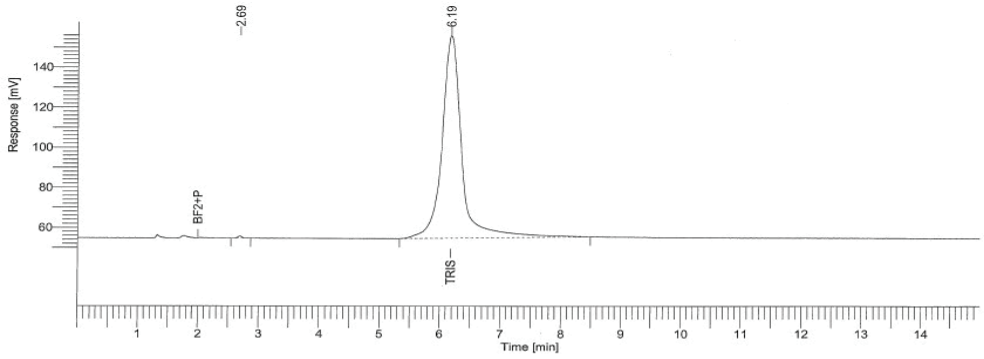
Component	Expected Retention (Calibration File)

All components were found

BioSpectra Inc.

5 Day UV Stress Sample

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.693	5093.60
Tris	2	6.186	2303692.80
			2308786.40

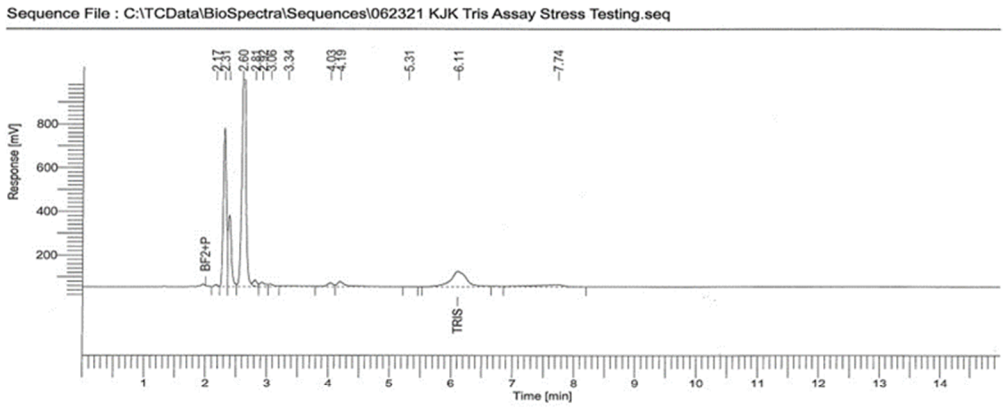
Missing Component Report

Component	Expected Retention (Calibration File)

All components were found

BioSpectra Inc.

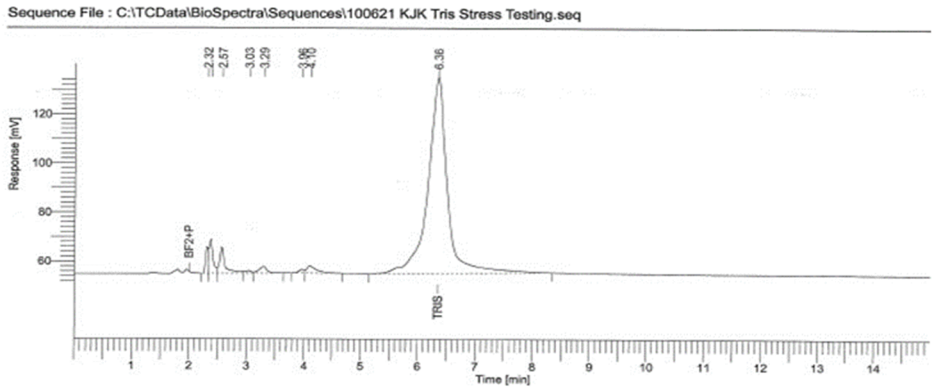
10% H_2O_2 Stress Sample Chromatogram



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.169	28163.66
	2	2.306	2638617.53
	3	2.387	1221537.97
	4	2.600	4682748.70
	5	2.806	128896.80
	6	2.918	120705.67
	7	3.056	70462.33
	8	3.340	80154.92
	9	4.032	121820.11
	10	4.193	248874.49
	11	5.310	7920.44
Tris	12	6.112	1292904.21
	13	7.743	349690.92
			10992497.75

0.5% (Mild) H_2O_2 Stress Sample Chromatogram

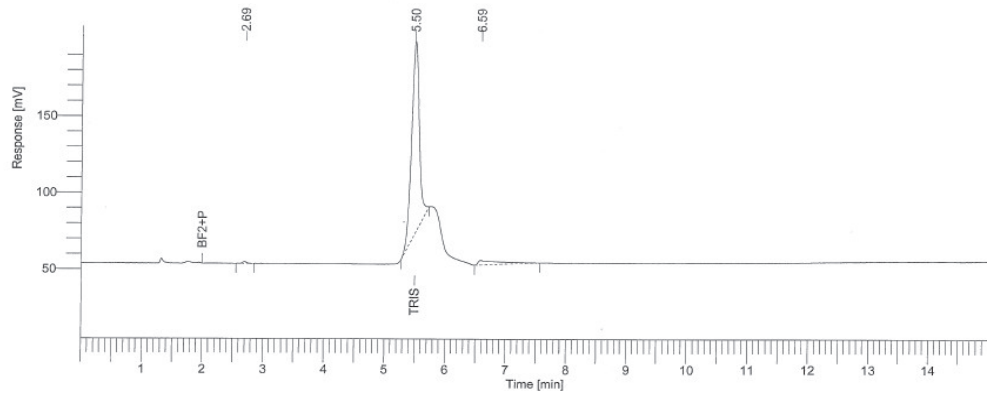


Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.320	33650.48
	2	2.382	67709.25
	3	2.573	71123.31
	4	3.032	7787.97
	5	3.292	24821.78
	6	3.957	9176.44
	7	4.102	36420.76
Tris	8	6.357	1883506.40
			2134196.40

Acid Stress Sample Chromatogram

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.693	5215.20
Tris	2	5.499	1094034.40
	3	6.590	75137.00
			1174386.60

Missing Component Report

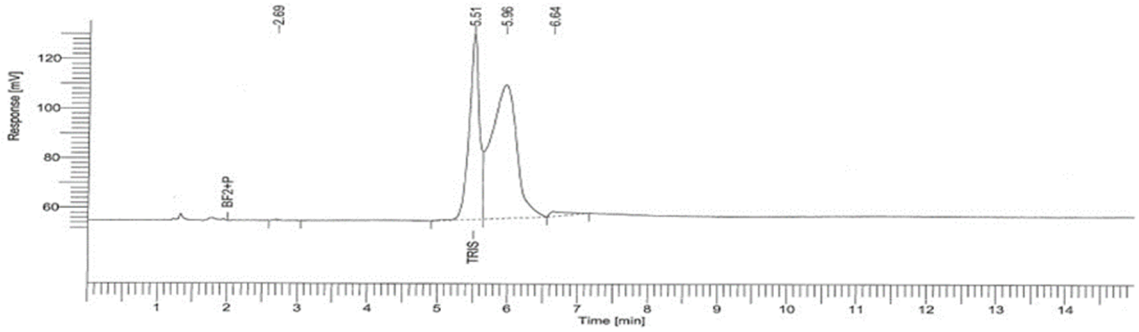
Component	Expected Retention (Calibration File)

All components were found

BioSpectra Inc.

Base Stress Sample Chromatogram

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.692	2735.60
Tris	2	5.507	765343.96
	3	5.963	1352840.24
	4	6.643	33717.60
			2154637.40

Missing Component Report

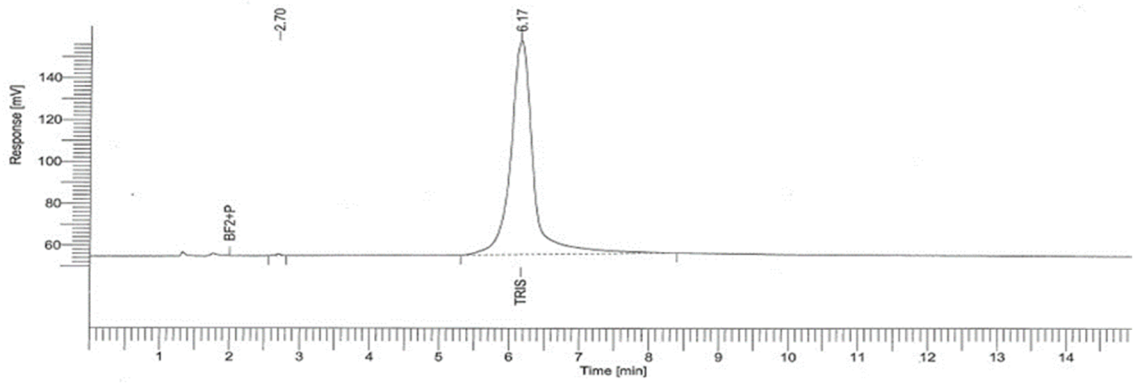
Component	Expected Retention (Calibration File)

All components were found

BioSpectra Inc.

Hydrolytic Stress Sample Chromatogram

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.695	4131.60
Tris	2	6.165	2296931.40
			2301063.00

Missing Component Report

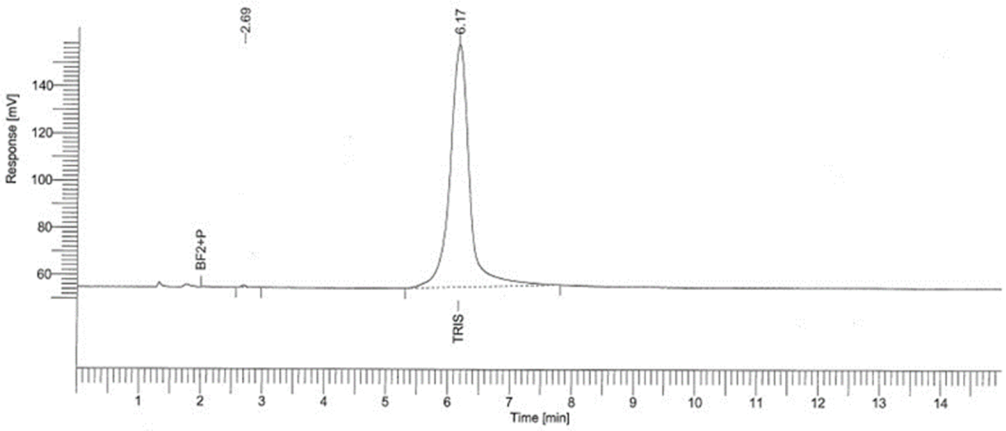
Component	Expected Retention (Calibration File)

All components were found

BioSpectra Inc.

Thermal Stress Sample Chromatogram

Sequence File : C:\TCData\BioSpectra\Sequences\062321 KJK Tris Assay Stress Testing.seq



Tris HPLC Identification Report

Component Name	Peak #	Time [min]	Area [µV·s]
	1	2.694	4828.80
Tris	2	6.169	2254488.80
			2259317.60

Missing Component Report

Component	Expected Retention (Calibration File)

All components were found

BioSpectra Inc.