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ANALYTICAL METHOD VERIFICATION REPORT: BIS-TRIS HCL WATER DETERMINATION VIA KARL FISCHER UTILIZING METROHM 907 AUTO TITRATOR

TABLE OF CONTENTS

1. PURPOSE:

1.1. The purpose of this Verification report is to:

1.1.1. Provide performance data demonstrating that the Karl Fischer procedure used on the Metrohm 907 Auto-Titrator for Bis-Tris HCl samples is adequately evaluated and verified.

1.1.2. Provide verification that the Karl Fischer procedure on the Metrohm 907 Auto-Titrator for Bis-Tris HCl at the set specification meets all requirements for Accuracy, Precision, Linearity, Range, Specificity, Intermediate Precision, Limit of Detection, and Limit of Quantitation.

1.1.3. The targeted range of the analysis is 1.0% w/w water maximum. Sample size was varied to work within the limits of 10mL maximum titration volume of the Metrohm 10mL auto-burette. Karl Fischer reagent Composite 5 generally titrates 5mg of water per 1mL of titrant, limiting the total titrated amount of water to at most 50mg. For the higher levels of water spike analysis, sample size was reduced in order to not over-run the burette capacity. Sample size therefore may be varied depending on specification(s) and will be investigated at varying levels in this verification protocol to thoroughly cover the range of moisture measurement required.

2. SCOPE:

2.1. This Analytical Method Verification Report applies to the Water Determination method used on the Metrohm 907 Auto-Titrator to determine the water content of Bis-Tris HCl.

2.2. The Water Determination of Bis-Tris HCl on the Metrohm 907 Auto-Titrator was verified as a Category II Quantitative method.

3. RESPONSIBILITIES:

3.1. The Associate Director of Product Life Cycle, or designee, was responsible for completing the Method Verification Report using conclusions made from the results obtained during testing.

3.2. The Laboratory Manager is responsible for the control, training, implementation, and maintenance of this report.

3.3. Trained chemists, or qualified designees, were responsible for performing the testing stated in the verification protocol.

4. REFERENCES:

4.1. BSI-RPT-1448, Analytical Method Verification: Bis-Tris HCl Water Determination via Karl Fischer Utilizing the Metrohm 907 Auto Titrator

4.2. BSI-SOP-0098, Balance SOP

4.3. BSI-SOP-0126, Laboratory Notebooks

4.4. BSI-SOP-0140, Standardization of Titrants

4.5. BSI-SOP-0143, Metrohm Titrando 907 Auto-Titrator SOP

4.6. BSI-SOP-0436, Analytical Methods Validation Master Plan

4.7. USP<1225>

4.8. USP<1226>

5. PRE-VERIFICATION REQUIREMENTS:

5.1. Equipment:

5.1.1. All equipment used in this Verification was in proper working order and with current calibrations. This will be documented in the Materials and Equipment portion of this report.

5.2. Personnel:

5.2.1. All personnel who executed this verification were properly trained in accordance with the Analytical Methods Validation Master Plan.

5.3. Supplies:
5.3.1. All supplies used in the Verification were clean and appropriate for the intended use. Supplies are listed in the Materials and Equipment section of this report.
5.4. Reagents:
5.4.1. All reagents were current and suitable for their intended use. The reagent name, lot number, manufacturer, CAS number, and expiration date are included in this report.
5.5. Reference Standards:
5.5.1. All reference standards used in this verification are listed in the Materials and Equipment section.

6. MATERIALS AND EQUIPMENT:

6.1. All materials and equipment utilized in this Verification are outlined in this section.
6.2. Equipment:
6.2.1. Analytical Balance
6.2.2. Double Pt-wire electrode
6.2.3. Metrohm 907 Auto-Titrator

Equipment				
Equipment	**Model / Part Number**	**Manufacturer**	**Serial Number**	**Calibration Due**
Auto-Titrator	907 Titrando	Metrohm	12155	7/24
Analytical Balance	Secura124-1S	Sartorius	29212172	10/31/23
Micropipette	0.5µL - 10µL Research Plus	Eppendorf	R32893H	11/30/23
			N27646F	12/31/23
Micropipette	2µL - 20µL Research Plus	Eppendorf	O31160G	1/31/24
	20µL - 200µL Research Plus	Eppendorf	O35990B	2/29/24
Calibrated Timer	14-649-17	Fisherbrand	221117200	1/5/24

6.3. Reagents:
6.3.1. Composite 5 (Titrant)
6.3.2. Formamide Dry
6.3.3. Methanol Dry
6.3.4. Purified Water
6.3.5. Bis Tris HCl (Dried, Minimum 20g, homogenized and stored over desiccant.)

Reagents and Standards				
Reagent / Standard	**Lot ID**	**Manufacturer**	**CAS Number**	**Expiration Date**
Bis-Tris HCl	SLCK7508	Sigma Aldrich	124763-51-5	7/13/27
Purified Water	F9SA14284H	Millipore Sigma	7732-18-5	12/31/23
Composite 5	M0340	Honeywell	693-98-1 68007-08-9 7446-09-5 288-32-4 7553-56-2	1/18/25
Methanol	M234A	Honeywell	67-56-1	7/27/27
Formamide	M0660	Honeywell	75-12-7	2/19/25
Hydranal Water Standard	K0370	Honeywell	100-66-3	1/10/25

6.4. Supplies:
- 6.4.1. Syringe
- 6.4.2. Micropipettes
- 6.4.3. Micro Stir bars
- 6.4.4. Micropipette Tips (LoRetention)
- 6.4.5. Glass Weighing Spoon
- 6.4.6. Scoops

7. PROCEDURE:

7.1. Standardization Composite 5:
- 7.1.1. Standardize Karl Fischer titrant (Composite 5) using Standardization of Titrants, DCN: BSI-SOP-0140, as guidance as well as Metrohm instrument operation manual (electronic or print).

7.2. Bis Tris HCl Test Procedure:
- 7.2.1. Weigh approximately 1.0g of dried sample into a glass weighing spoon and tare the balance.
- 7.2.2. Transfer the sample to the Karl Fischer vessel by removing the rubber septum and adding the sample into the titration vessel.
  - 7.2.2.1. Do not leave the rubber septum open for longer than 20 seconds as this will allow moisture to enter the titration vessel.
- 7.2.3. Return the weighing spoon to the balance, making sure not to lose any sample that was left behind. Once the weight stabilizes, record the weight in the Tiamo Software.
- 7.2.4. Bis Tris HCl may not fully dissolve in the 50/50 Methanol/Formamide mix. Ensure that all sample that was added to the KF vessel is suspended in the solution.
- 7.2.5. The moisture content will then be determined by the KF titration using the Metrohm Titrando 907.

$$\%\ Moisture = \frac{(mL\ of\ Composite\ 5)\left(\frac{mg}{mL}of\ Composite\ 5\right)(0.1)}{Sample\ Weight\ (g)}$$

7.3. Verification Sample Preparations:
- 7.3.1. 0% Limit (Dried Sample):
  - 7.3.1.1. Weigh 1.0g of dried sample.
  - 7.3.1.2. Transfer to Karl Fischer vessel.
  - 7.3.1.3. Record weight by difference.
  - 7.3.1.4. Analyze the sample.
  - 7.3.1.5. Record result.
- 7.3.2. 0.10% Limit (10% Limit Spike, Low Level Analysis Method Verification):
  - 7.3.2.1. Weigh 1.0 g of dried sample.
  - 7.3.2.2. Transfer to Karl Fischer vessel.
  - 7.3.2.3. Record weight by difference.
  - 7.3.2.4. Pipette 1µL of Purified Water into vessel as quickly as possible to ensure environmental moisture does not enter vessel.
  - 7.3.2.5. Titrate to analyze the sample.
  - 7.3.2.6. Record result.
  - 7.3.2.7. Repeat in triplicate for LOQ, Linearity, and Range data.
- 7.3.3. 0.50% Limit (50% Limit Spike):
  - 7.3.3.1. Weigh 1.0 g of dried sample.
  - 7.3.3.2. Transfer to Karl Fischer vessel.
  - 7.3.3.3. Record weight by difference.

7.3.3.4. Pipette 5µL of Purified Water into vessel as quickly as possible to ensure environmental moisture does not enter vessel.
7.3.3.5. Titrate to analyze the sample.
7.3.3.6. Record result.
7.3.3.7. Analyze one time for Linearity.

7.3.4. 0.80% Limit (80% Limit Spike):
7.3.4.1. Weigh 1.0 g of dried sample.
7.3.4.2. Transfer to Karl Fischer vessel.
7.3.4.3. Record weight by difference.
7.3.4.4. Pipette 8µL of Purified Water into vessel as quickly as possible to ensure environmental moisture does not enter vessel.
7.3.4.5. Titrate to analyze the sample.
7.3.4.6. Record result.
7.3.4.7. Repeat in triplicate.

7.3.5. 1.00% Limit (100% Limit Spike):
7.3.5.1. Weigh 1.0g of dried sample.
7.3.5.2. Transfer to Karl Fischer vessel.
7.3.5.3. Record weight by difference.
7.3.5.4. Pipette 10µL of Purified Water into vessel as quickly as possible to ensure environmental moisture does not enter vessel.
7.3.5.5. Titrate to analyze the sample.
7.3.5.6. Record result.
7.3.5.7. Repeat in triplicate.

7.3.6. 1.20% Limit (120% Limit Spike):
7.3.6.1. Weigh 1.0 g of dried sample.
7.3.6.2. Transfer to Karl Fischer vessel.
7.3.6.3. Record weight by difference.
7.3.6.4. Pipette 12µL of Purified Water into vessel as quickly as possible to ensure environmental moisture does not enter vessel.
7.3.6.5. Titrate to analyze the sample.
7.3.6.6. Record result.
7.3.6.7. Repeat in triplicate.

8. PERFORMANCE PARAMETERS:

8.1. This method was verified as a Category II Quantitative test.

8.2. System Suitability, Accuracy, Precision, Linearity, Range, Specificity, Intermediate Precision, Limit of Detection, and Limit of Quantitation were assessed.

8.3. **System Suitability:**

8.3.1. System Suitability was assessed by standardizing Composite 5 in triplicate as per the Standardization of Titrants SOP. The average of the triplicate standardizations was reported as the titer value.

8.3.2. Acceptance Criteria:

8.3.2.1. A Relative Standard Deviation (%RSD) of NMT 2% between triplicate standardizations.

8.4. **Accuracy:**

8.4.1. Accuracy was assessed over twelve (12) determinations at four (4) concentration levels. Accuracy was reported as the percent recovery. The data was assessed by calculating the percent recovery for each determination or each concentration level.

$$\%\ Recovery = \frac{Result\ (\%)}{Spike\ Value\ (\%) + 0\%\ Level\ (\%)} \times 100$$

8.4.2. Acceptance Criteria:

8.4.2.1. Percent Recovery (%) between 80% and 120%.

8.5. **Precision:**

8.5.1. Precision was assessed over twelve (12) determinations at four (4) concentration levels. Precision was reported as the Relative Standard Deviation (%RSD) at each concentration level.

$$\text{Relative Standard Deviation (\%RSD)} = \frac{\text{Standard Deviation}}{\text{Average}} \times 100$$

8.5.2. Acceptance Criteria:

8.5.2.1. %RSD for each spike level is NMT 20%.

8.6. **Linearity:**

8.6.1. Linearity was assessed by plotting the Water Spike (µL Water) against the Average Instrument Response (mL Titrant) at each Water Spike Level and preforming a linear regression. Reported the Slope, Y-Intercept, and Correlation Coefficient (r^2).

8.6.2. Acceptance Criteria:

8.6.2.1. Slope: Report

8.6.2.2. Y-Intercept: Report

8.6.2.3. Correlation Coefficient (r^2): NLT 0.95.

8.7. **Range:**

8.7.1. The range of the analytical method is determined by the highest concentration of analyte that produce suitable results for precision, accuracy and linearity and the Limit of Quantitation value

8.7.2. Acceptance Criteria:

8.7.2.1. 0.50% to 1.20% minimum.

8.8. **Specificity:**

8.8.1. Karl Fischer analysis is inherently selective to water, by meeting requirements for accuracy and precision, specificity will be demonstrated.

8.8.2. Acceptance Criteria:

8.8.2.1. Requirements for Accuracy and Precision are met.

8.9. **Intermediate Precision:**

8.9.1. A separate analyst will perform the 100% Limit (1% Spike) analyses in triplicate on a different day in which they perform a separate standardization.

8.9.2. The standard deviation and relative standard deviation (%RSD) of the individual and combined runs were calculated at the 100% Limit.

8.9.3. Acceptance Criteria:

8.9.3.1. Standard Deviation: Report

8.9.3.2. The Relative Standard Deviation (%RSD) for Analyst II and Combined (Analyst I and II) results is NMT 25%.

8.10. **Limit of Detection:**

8.10.1. The Limit of Detection (LoD) will be expressed as:

$$\text{Limit of Detection (LoD)} = \frac{3.3\sigma}{S}$$

8.10.2. Acceptance Criteria:

8.10.2.1. Report the Limit of Detection.

8.11. **Limit of Quantitation:**

8.11.1. The Limit of Quantitation (LoQ) will be expressed as:

$$\text{Limit of Quantitation (LoQ)} = \frac{10\sigma}{S}$$

8.11.2. Acceptance Criteria:

8.11.2.1. Report the Limit of Quantitation.

9. VALIDATION SUMMARY:

Validation Summary		
Performance Parameters	**Acceptance Criteria**	**Results**
System Suitability	• Standardized Composite 5 in triplicate and reported the average titer value. • %RSD of NMT 2% is required between triplicate runs.	Analyst I • Average Titer Value = 4.9452 mg/mL • %RSD = 0.15% Analyst II • Average Titer Value = 4.7991 mg/mL • %RSD = 0.65%

Validation Summary		
Performance Parameters	**Acceptance Criteria**	**Results**
Accuracy	• The Percent Recovery for all replicates is between 80% - 120%.	0.10% Spike • Replicate 1 = 121% • Replicate 2 = 121% • Replicate 3 = 107% 0.80% Spike • Replicate 1 = 101% • Replicate 2 = 99% • Replicate 3 = 100% 1.00% Spike • Replicate 1 = 100% • Replicate 2 = 100% • Replicate 3 = 103% 1.20% Spike • Replicate 1 = 101% • Replicate 2 = 94% • Replicate 3 = 99%
Precision	• The %RSD for each spike level is NMT 20%.	0.10% Spike • %RSD = 7% 0.80% Spike • %RSD = 1% 1.00% Spike • %RSD = 2% 1.20% Spike • %RSD = 3%
Linearity	• Plotted instrument response (mL of Composite 5) vs. Spike Level (µL of Water). • Reported the slope, y-intercept, and calibration coefficient (r^2). • The calibration coefficient (r^2) must be NLT 0.95.	• Slope = 0.1988 • Y-Intercept = 0.1381 • Calibration Coefficient (r^2) = 0.9998
Range	• Range is the highest concentration of analyte that produces suitable results for accuracy, precision, and linearity and the LoQ value.	• The range was established from 0.50% to 1.20% w/w Water Content.
Specificity	• Specificity was demonstrated by meeting requirements for accuracy and precision.	• Requirements for accuracy and precision were met.

Validation Summary		
Performance Parameters	**Acceptance Criteria**	**Results**
Limit of Detection (LoD)	• Report the Limit of Detection (LoD).	• 0.069% w/w Water Content.
Limit of Quantitation (LoQ)	• Report the Limit of Quantitation (LoQ).	• 0.21% w/w Water Content.
Intermediate Precision	• Report the Standard Deviation and %RSD of Analyst II and Combined (Analyst I and II) results. • The %RSD for Analyst II and Combined (Analyst I and II) is NMT 25%.	Analyst II • Standard Deviation = 0.010% • %RSD = 1% Combined (Analyst I and II) • Standard Deviation = 0.046% • %RSD = 4%

10. VALIDATION RESULTS:

10.1. **Standardization / System Suitability:**

10.1.1. System Suitability was assessed by standardizing Composite 5 in triplicate as per the Standardization of Titrants SOP. The average of the triplicate standardizations was reported as the titer value. All acceptance criteria were met and are summarized in the *Standardization / System Suitability Tables.*

10.1.2. Acceptance Criteria:

10.1.2.1. A Relative Standard Deviation (%RSD) of NMT 2% between triplicate standardizations.

Analyst 1 Standardization / System Suitability			
Replicate	**Purified Water (g) or (µL)**	**Endpoint (mL)**	**Titer Value (mg/mL)**
1	1.0737	2.1740	4.9389
2	0.9857	1.9899	4.9534
3	1.0018	2.0265	4.9434
		Average Titer Value (mg/mL):	4.9452
		%RSD:	0.15%

Analyst 2 Standardization / System Suitability			
Replicate	**Purified Water (g) or (µL)**	**Endpoint (mL)**	**Titer Value (mg/mL)**
1	30	6.2986	4.7630
2	30	6.2259	4.8186
3	30	6.2297	4.8156
		Average Titer Value (mg/mL):	4.7991
		%RSD:	0.65%

10.2. **Accuracy:**

10.2.1. Accuracy was assessed over twelve (12) determinations at four (4) concentration levels. Accuracy was reported as the percent recovery. The data was assessed by calculating the percent recovery for each determination or each concentration level.

10.2.2. The 0.10% Spike level did not show acceptable percent recovery with two (2) replicates giving a percent recovery of 121%. All other spike levels showed acceptable percent recovery results. All results are summarized in the *Accuracy Table.*

$$\text{Percent Recovery (\%)} = \frac{Result\ (\%)}{Spike\ Level\ (\%) + 0\%\ Spike\ Result\ (\%)} \times 100$$

10.2.3. Acceptance Criteria:
10.2.3.1. Percent Recovery (%) between 80% and 120%.

Accuracy			
Spike Level (%)	**Replicate**	**Water Result (%)**	**Percent Recovery (%)**
0%	1	0.04	Not Applicable
0.10%	1	0.17	121
	2	0.17	121
	3	0.15	107
0.80%	1	0.85	101
	2	0.83	99
	3	0.84	100
1.00%	1	1.04	100
	2	1.04	100
	3	1.07	103
1.20%	1	1.25	101
	2	1.17	94
	3	1.23	99

10.3. **Precision:**

10.3.1. Precision was assessed over twelve (12) determinations at four (4) concentration levels. Precision was reported as the Relative Standard Deviation (%RSD) at each concentration level. All acceptance criteria were met and are summarized in the *Precision Table.*

10.3.2. Acceptance Criteria:
10.3.2.1. %RSD for each spike level is NMT 20%.

$$\text{Relative Standard Deviation (\%RSD)} = \frac{\text{Standard Deviation}}{\text{Average}} \times 100$$

Precision			
Spike Level (%)	**Replicate**	**Water Result (%)**	**%RSD**
0.10%	1	0.17	7
	2	0.17	
	3	0.15	
0.80%	1	0.85	1
	2	0.83	
	3	0.84	
1.00%	1	1.04	2
	2	1.04	
	3	1.07	
1.20%	1	1.25	3
	2	1.17	
	3	1.23	

10.4. **Specificity:**

10.4.1. Specificity was demonstrated by meeting requirements for accuracy and precision.

10.4.2. Specificity results are summarized in the *Specificity Table*. Acceptance criteria were met at all analysis levels except for the 0.10% Spike Level.

Specificity	
Acceptance Criteria	**Result**
All requirements for accuracy were met.	Pass
All requirements for precision were met.	Pass

10.5. **Limit of Detection (LoD) / Limit of Quantitation (LoQ):**

10.5.1. The Limit of Detection (LoD) will be expressed as:

$$\text{Limit of Detection (LoD)} = \frac{3.3\sigma}{S}$$

10.5.1.1. Where:

10.5.1.1.1. σ = Average Standard Deviation of the Water Content Result (%).

10.5.1.1.2. S = Slope of the Water Content Result (%) versus the Water Spike Level (%).

10.5.2. The Limit of Quantitation (LoQ) will be expressed as:

$$\text{Limit of Quantitation (LoQ)} = \frac{10\sigma}{S}$$

10.5.2.1. Where:

10.5.2.1.1. σ = Average Standard Deviation of the Water Content Result (%).

10.5.2.1.2. S = Slope of the Water Content Result (%) versus the Water Spike Level (%).

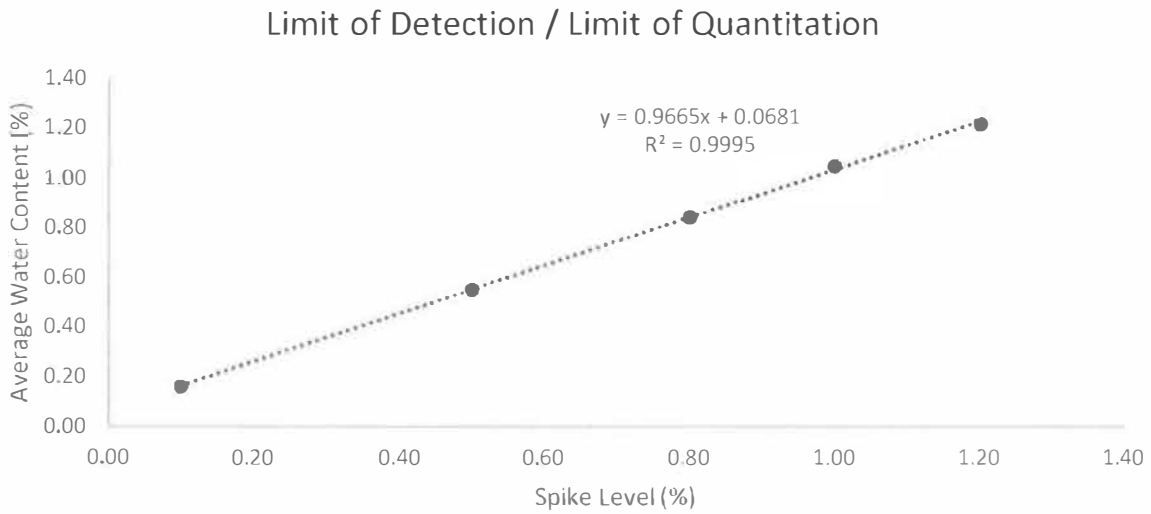

Figure 1: Limit of Detection / Limit of Quantitation graph plotting Water Spike Level (%) versus Average Water Content (%)

Limit of Detection / Limit of Quantitation			
Average Standard Deviation of the Water Content Result, σ (%)	**Slope of the Water Content Result (%) Versus the Water Spike Level (%), S**	**Limit of Detection (LoD)**	**Limit of Quantitation (LoQ)**
0.0201	0.9665	0.069%	0.21%

10.6. **Linearity:**

10.6.1. Linearity was assessed by plotting the Water Spike (µL Water) against the Average Instrument Response (mL Titrant) at each Water Spike Level and preforming a linear regression. Reported the Slope, Y-Intercept, and Correlation Coefficient (r^2). All acceptance criteria were met and are summarized in the *Linearity Table*.

10.6.1.1. Note: 10mg water spike is equivalent to 1.0% w/w water in the test sample.

10.6.1.2. 10mg/1000mg sample weight = 1.0%.

10.6.2. Acceptance Criteria:

10.6.2.1. Slope: Report

10.6.2.2. Y-Intercept: Report

10.6.2.3. Correlation Coefficient (r^2): NLT 0.95.

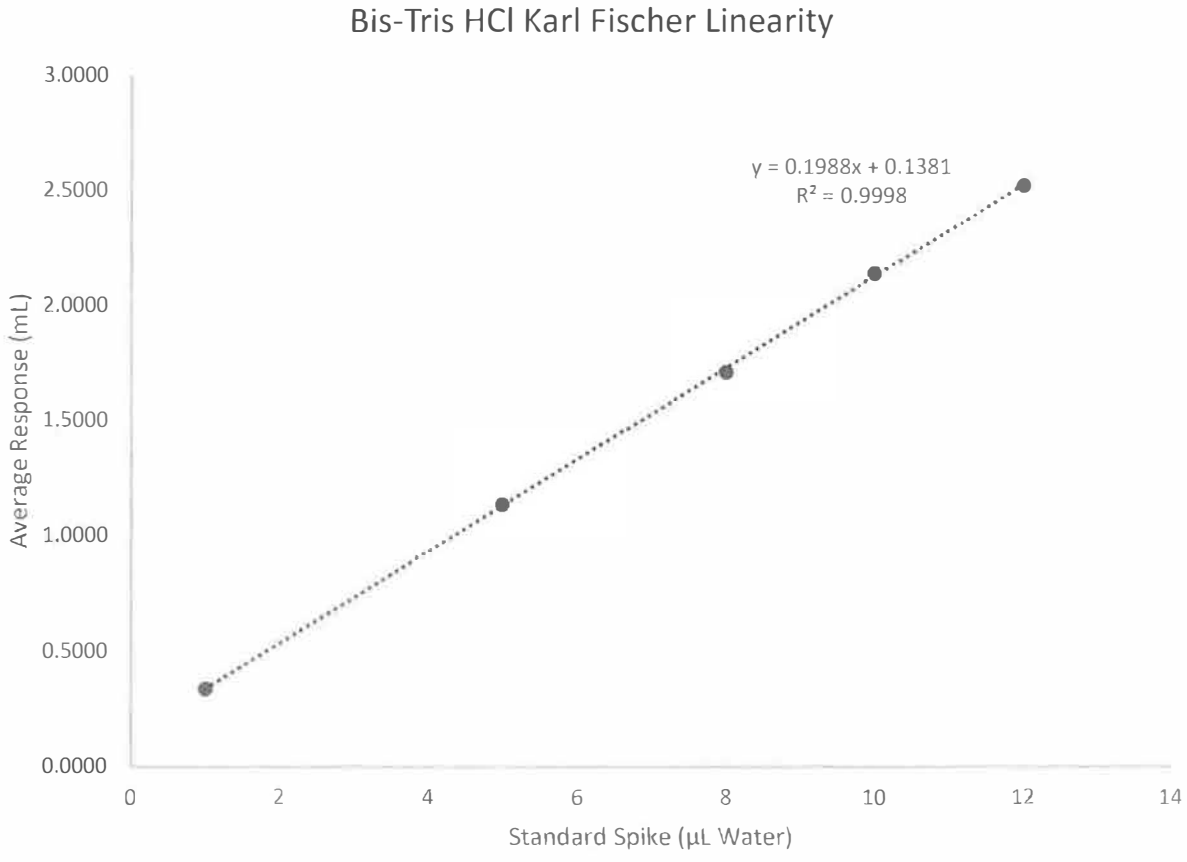

Figure 2: Bis-Tris HCl Karl Fischer Linearity plotting Water/Standard Spike (µL Water) versus Average Instrument Response (mL Titrant)

Linearity					
Spike Level (%)	**Water Spike (µL Water)**	**Average Instrument Response (mL Titrant)**	**Slope**	**Y-Intercept**	**Correlation Coefficient (r^2)**
0.10%	1	0.3378	0.1988	0.1381	0.9998
0.50%	5	1.1370			
0.80%	8	1.7117			
1.00%	10	2.1387			
1.20%	12	2.5230			

10.7. **Range:**

10.7.1. The range of the analytical method is determined by the highest concentration of analyte that produce suitable results for precision, accuracy and linearity and the LoQ value.

Range of Analysis: 0.50% - 1.20%

10.8. **Intermediate Precision (Analyst II):**

10.8.1. A separate analyst will perform the 100% Limit (1% Spike) analyses in triplicate on a different day in which they perform a separate standardization.

10.8.2. The standard deviation and relative standard deviation (%RSD) of the individual and combined runs were calculated at the 100% Limit.

10.8.3. All acceptance criteria were met and are summarized in the *Intermediate Precision Table.*

10.8.4. Acceptance Criteria:

10.8.4.1. Standard Deviation: Report

10.8.4.2. The Relative Standard Deviation (%RSD) for Analyst II and Combined (Analyst I and II) results is NMT 25%.

Intermediate Precision					
Spike Level (%)	**Analyst**	**Replicate**	**Water Result (%)**	**Standard Deviation**	**%RSD**
1.00%	I	1	1.04	0.017	2
		2	1.04		
		3	1.07		
	II	1	1.13	0.010	1
		2	1.12		
		3	1.14		
			Combined:	0.046	4

11. CONCLUSION:

11.1. Performance Summary:

Performance Summary	
Method Performance Indicator	**Result**
Standardization / System Suitability	Pass
Accuracy	Pass
Precision	Pass
Linearity	Pass
Range	0.50% - 1.20%
Specificity	Pass
Intermediate Precision	Pass
Limit of Detection	0.07%
Limit of Quantitation	0.21%

11.2. Statement of Verification: The method of analysis of Water in Bis Tris HCl by means of Karl Fischer Titration in samples is considered a Verified method of analysis at all BioSpectra facilities and is approved for use.

11.3. Critical Changes, Deviations, or Failures

11.3.1. **Failure – 0.10% Spike Level Accuracy –** The 0.10% Spike Level did not give an acceptable percent recovery. Two of the replicates gave percent recoveries of 121%, the acceptance criteria is 80% to 120%. This failure is reflected in the range of the analysis, which is 0.50% - 1.20%.