BIOSPECTRA
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ANALYTICAL METHOD VERIFICATION REPORT: CHOLESTEROL ASSAY VIA GAS CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION (GC-FID)

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1. PURPOSE:

1.1. The purpose of this report is to:
1.1.1. Provide performance data demonstrating that the Cholesterol Assay Via Gas Chromatography with Flame Ionization Detection (GC-FID) procedure is adequately evaluated and verified.
1.1.2. Provide proof that the Cholesterol Assay Via Gas Chromatography with Flame Ionization Detection (GC-FID) procedure meets requirements for System Suitability, Accuracy, Precision, and Specificity.
1.1.3. To ensure that the proper reagents and testing materials were used and the correct documentation is provided for the evaluation.

2. SCOPE:

2.1. This Analytical Method Verification Report applies to Cholesterol for Assay Via Gas Chromatography with Flame Ionization Detection (GC-FID).
2.2. The Cholesterol Assay Specification is 95.0% - 102.0% on the dried basis.

3. RESPONSIBILITIES:

3.1. The Senior Product Life Cycle Manager is responsible for the control, implementation and maintenance of this report.
3.2. The Laboratory Technicians, and/or qualified designees, were responsible for performing the testing stated in the protocol and for performing the verification.
3.3. The Laboratory Technicians, and/or qualified designees, performing the testing were responsible for completing the Analytical Method Verification Report using conclusions made from the results obtained from testing.

4. REFERENCES:

4.1. BSI-PRL-0830, Analytical Method Verification Protocol: Cholesterol Àssay Via Gas Chromatography with Flame Ionization Detection (GC-FID)
4.2. BSI-SOP-0098, Balance SOP
4.3. BSI-SOP-0126, Laboratory Notebooks
4.4. BSI-SOP-0134, Pipette SOP
4.5. BSI-SOP-0316, Shimadzu QP2010S GC/MS SOP
4.6. BSI-SOP-0436, Analytical Methods Validation Master Plan
4.7. ICH Q2, Analytical Validation Guidelines
4.8. Shimadzu QP2010S Operation Manual
4.9. USP <1225> Validation of Compendial Procedures
4.10. USP <1226> Verification of Compendial Procedures

5. PRE-VERIFICATION REQUIREMENTS:

5.1. Equipment
5.1.1. All equipment used in this Verification was in proper working order and within current calibrations. This is documented in the Materials and Equipment portion of the Analytical Method Verification Report.
5.2. Personnel
5.2.1. All personnel performing this Verification were properly trained on the analysis technique.
5.3. Supplies
5.3.1. All supplies used in the method verification were clean and appropriate for their intended use. A list of supplies used is included in the Materials and Equipment section of the Analytical Method Verification Report and is identified with the supplier and description.

5.4. Reagents

5.4.1. All reagents were current, met required specifications and were suitable for the intended use. A list of reagents used is included in the Analytical Method Verification Report and laboratory documentation. This includes: reagent name, lot number, manufacturer, date of opening (if applicable), part number, and expiration date (if applicable).

5.5. Reference Standards

5.5.1. Any standards used in this verification are listed in the Materials and Equipment section of the Analytical Method Verification Report. The name of the reference standard, lot number, manufacturer, date of opening, date of expiration, and part number are provided in the verification report and recorded during validation testing.

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. Gas Chromatograph

6.2.2.1. Shimadzu GC2010 with Flame Ionization Detection (GC-FID)

6.2.2.1.1. AOC-20i Liquid Injection Autosampler

6.2.2.2. GC Column:

6.2.2.2.1. Restek Rtx-1 GC Capillary Column, 30 m, 0.25 mm ID, 0.25 µm

6.2.2.2.1.1. Catalog Number: 10123

TABLE 1: EQUIPMENT AND INSTRUMENTATION

Equipment	Model / Part Number	Manufacturer	Serial Number	Calibration Due
Balance	Secura 124-1S	Sartorius	29212172	4/30/25
GC-FID with AOC-20i Liquid Injector	GC-2010	Shimadzu	20385050364	9/2025
GC Column	Rtx-1 30 m, 0.25 mm ID, 0.25 µm	Restek	1653299	Not Applicable

6.3. Reagents and Standards

6.3.1. Cholesterol Certified Reference Standard

6.3.2. Heptane

6.3.3. Pregnenolone Isobutyrate Certified Reference Standard

TABLE 2: REAGENTS AND STANDARDS

Reagent / Standard	Lot Number	Manufacturer	Part Number	CAS Number	Expiration Date	Date of Opening
Cholesterol CRS	0000338967	Sigma Aldrich	C8667-5G	57-88-5	2/28/29	8/16/24
Heptane	SHBR0859	Sigma Aldrich	34873-1L	142-82-5	8/31/28	11/15/24
Pregnenolone Isobutyrate CRS	ID:00YCwS Batch: 8	EDQM	P2920000	Not Applicable	Current Lot	11/27/24

6.4. Supplies
- 6.4.1. Class A Volumetric Flask
- 6.4.2. Liquid Injection auto-sampler vials and caps
- 6.4.3. Transfer Pipettes
- 6.4.4. Weigh Boats/ Paper/ Funnels/ Pans
- 6.4.5. Weighing Spatulas

TABLE 3: SUPPLIES

Supply	Manufacturer	Part Number
Weigh Boat / Paper / Pan	TWD Scientific, LLC	DPWF-PP1-S
Spatula	LevGo	17211
Class A Volumetric Flasks	Pyrex	5642
Transfer Pipettes	Samco Scientific	204
Liquid Injection Auto-Sampler Vials and Caps	Fisherbrand	03-391-18

7. METHOD PARAMETERS:

7.1. GC-2010

7.1.1. Carrier Gas Saver: Off
7.1.2. Carrier Gas: Helium
7.1.3. Column Flow: 2.0 mL/min
7.1.4. Column Oven Temperature: 275 °C
7.1.5. Detector Temperature: 300 °C
7.1.6. Flow Control Mode: Pressure
7.1.7. High Pressure Injection: Off
7.1.8. Injection Mode: Split
7.1.9. Injection Volume: 1.0 µL
7.1.10. Injector Temperature: 285 °C
7.1.11. Linear Velocity: 50.4 cm/sec
7.1.12. Pressure: 254.0 kPa
7.1.13. Purge Flow: 3.0 mL/min
7.1.14. Split Ratio: 25.0
7.1.15. Splitter Hold: Off
7.1.16. Total Flow: 55.0 mL/min

7.2. Ready Checks

7.2.1. Column Oven: YES
7.2.2. SPL1: YES
7.2.3. FID1: YES
7.2.4. SPL1 Carrier: YES
7.2.5. SPL1 Purge: YES
7.2.6. APC1: YES
7.2.7. APC2: YES
7.2.8. APC3: YES
7.2.9. FID Makeup: YES
7.2.10. FID1 H2: YES
7.2.11. FID1 Air: YES
7.2.12. External Wait: NO
7.2.13. Auto Flame On: YES
7.2.14. Auto Flame Off: YES
7.2.15. Reignite: YES
7.2.16. Auto Zero After Ready: YES
7.2.17. Equilibrium Time: 3.0 min

8. PROCEDURE:

8.1. Solution Preparation:

8.1.1. **Note:** Solutions may be scaled as needed.

8.1.2. Diluent: Heptane

8.1.3. Assay Standard Solution (1.0 mg/mL Cholesterol; 1.0 mg/mL Pregnenolone Isobutyrate):

8.1.3.1. Accurately weigh 25 mg of Cholesterol CRS and 25 mg of Pregnenolone Isobutyrate, transfer to a 25 mL volumetric flask, dissolve in Heptane, fill to volume with Heptane, and mix well.

8.1.3.1.1. Prepare in duplicate, label as *Assay Standard Solution* and *Assay Standard Solution Check* respectively.

8.1.4. Assay Sample Solution (1.0 mg/mL Cholesterol; 1.0 mg/mL Pregnenolone Isobutyrate):

8.1.4.1. Accurately weigh 25 mg of Cholesterol sample and 25 mg of Pregnenolone Isobutyrate, transfer to a 25 mL volumetric flask, dissolve in Heptane, fill to volume with Heptane, and mix well.

8.2. Verification Solution Preparation:

8.2.1. Accuracy / Precision Sample Solutions:

8.2.1.1. Per the table below, accurately weigh Cholesterol CRS and 25 mg of Pregnenolone Isobutyrate, transfer to a 25 mL volumetric flask, dissolve in Heptane, fill to volume with Heptane, and mix well.

8.2.1.2. Prepare the six (6) replicates and perform a single injection of each replicate.

TABLE 4: ACCURACY / PRECISION SAMPLE SOLUTIONS

Analysis Level (%)	Replicates	Cholesterol Weight (mg)	Final Volume (mL)	Cholesterol Concentration (mg/mL)
100	6	25	25	1.0

8.3. Injection Sequence:

TABLE 5: INJECTION SEQUENCE

Sample ID	Number of Injections
System Suitability	
Diluent	≥1
Assay Standard Solution	5
Assay Standard Solution Check	2
Diluent	1
Samples	
Samples	≤6 (1 Injection Each)
Assay Standard Solution (QC Check)	1
Diluent	1
Repeat the sample injection sequence if additional samples are to be analyzed. Samples may be substituted with diluent injections.	

8.4. **System Suitability Criteria:**

TABLE 6: SYSTEM SUITABILITY CRITERIA	
System Suitability Parameter	**Acceptance Criteria**
The Relative Standard Deviation (%RSD) of the peak response ratio of Cholesterol to Pregnenolone Isobutyrate (Internal Standard) in the first five (5) injections of the *Assay Standard Solution.*	NMT 2.0%
The average % Agreement between the first five (5) *Assay Standard Solution* injections and each *Assay Standard Solution (QC Check)* injection.	98% - 102%
The average % Agreement between the first five (5) *Assay Standard Solution* injections and the *Assay Standard Solution Check* injections.	98% - 102%
The resolution between the Pregnenolone Isobutyrate (Internal Standard) and Cholesterol all *Assay Standard Solutions*.	NLT 10

8.5. **Calculations:**

8.5.1. Assay (As-Is)

$$\%\ Cholesterol\ (\text{As-Is}) = \frac{R_U}{R_S} \times \frac{C_S}{C_U} \times 100$$

8.5.1.1. R_U = Peak response ratio of Cholesterol to the internal standard (peak response of Cholesterol/ peak response of the internal standard) from the *Assay Sample Solution.*

8.5.1.2. R_S = Peak response ratio of Cholesterol to the internal standard (peak response of Cholesterol/ peak response of the internal standard) from the first five *Assay Standard Solution* injections.

8.5.1.3. C_S = Concentration of Cholesterol CRS x Certified Purity in the *Assay Standard Solution* (mg/mL).

8.5.1.4. C_U = Concentration of Cholesterol in the *Assay Sample Solution* (mg/mL).

8.5.2. Assay (Dried Basis)

$$\%\ Cholesterol\ (\text{Dried Basis}) = \frac{\%\ Cholesterol\ (\text{As-Is}) \times 100}{100 - Loss\ On\ Drying\ (\%)}$$

8.5.3. Percent Agreement

$$\%\, Agreement = \frac{R_C}{R_S} \times \frac{C_S}{C_C} \times 100$$

8.5.3.1. R_C = Peak response ratio of Cholesterol to the internal standard (peak response of Cholesterol/ peak response of the internal standard) from the *Assay Standard Solution Check.*

8.5.3.2. R_S = Peak response ratio of Cholesterol to the internal standard (peak response of Cholesterol/ peak response of the internal standard) from the first five *Assay Standard Solution* injections.

8.5.3.3. C_S = Concentration of Cholesterol CRS x Certified Purity in the *Assay Standard Solution* (mg/mL).

8.5.3.4. C_C = Concentration of Cholesterol CRS x Certified Purity in the *Assay Standard Solution Check* (mg/mL).

9. PERFORMANCE PARAMETERS:

9.1. **System Suitability:**

9.1.1. System Suitability will be assessed by injecting the *Diluent*, *Assay Standard Solution*, and *Assay Standard Solution Check* as per the *Injection Sequence Table* above and determining the Relative Standard Deviation (%RSD), Percent Agreement, and Resolution.

9.1.2. Acceptance Criteria:

TABLE 7: SYSTEM SUITABILITY CRITERIA

System Suitability Parameter	Acceptance Criteria
The Relative Standard Deviation (%RSD) of the peak response ratio of Cholesterol to Pregnenolone Isobutyrate (Internal Standard) in the first five (5) injections of the *Assay Standard Solution*.	NMT 2.0%
The average % Agreement between the first five (5) *Assay Standard Solution* injections and each *Assay Standard Solution (QC Check)* injection.	98% - 102%
The average % Agreement between the first five (5) *Assay Standard Solution* injections and the *Assay Standard Solution Check* injections.	98% - 102%
The resolution between the Pregnenolone Isobutyrate (Internal Standard) and Cholesterol in all *Assay Standard Solution*.	NLT 10

9.2. **Accuracy:**

9.2.1. Accuracy will be assessed at 100% of the nominal assay concentration of 1.0 mg/mL on the dried basis. The assay (as-is) will be compared to the certified purity value of the reference standard by calculating the Percent Recovery.

$$Percent\ Recovery\ (\%) = \frac{\%\ Cholesterol\ (As\text{-}Is)\ (\%)}{CoA\ Purity\ Value\ (\%)} \times 100$$

9.2.2. Acceptance Criteria:

9.2.2.1. The Percent Recovery of all replicates are between 98% and 102%.

9.3. **Precision:**

9.3.1. Precision will be assessed at 100% of the nominal assay concentration of 1.0 mg/mL on the dried basis by preparing six (6) replicates and calculating the Standard Deviation and Relative Standard Deviation (%RSD).

9.3.2. Acceptance Criteria:

9.3.2.1. Standard Deviation: Report.

9.3.2.2. Relative Standard Deviation (%RSD): NMT 2.0%.

9.4. **Specificity:**

9.4.1. Specificity will be demonstrated by overlaying the chromatograms from one (1) *Diluent* injection, one (1) *Assay Standard Solution* injection, and one (1) *Accuracy / Precision Sample Solution* injection.

9.4.2. Acceptance Criteria:

9.4.2.1. The resolution between Pregnenolone Isobutyrate (Internal Standard) and Cholesterol is NLT 10 in the *Accuracy / Precision Sample Solution*.

9.4.2.2. The Pregnenolone Isobutyrate (Internal Standard) and Cholesterol peaks are visually resolved from diluent interference.

10. DOCUMENTATION PROCEDURES:

10.1. All data sheets, including notebooks, were signed and dated by the employee executing the Protocol. Pages may be copied and uploaded as supporting material in Master Control.
10.2. All testing equipment was calibrated. Ensured that there is a certificate on file or appropriate standards were used if calibration is required.
10.3. Any critical changes that were made to the analytical procedure are noted in this document with supporting evidence for the change.

11. VALIDATION SUMMARY:

TABLE 8: VERIFICATION SUMMARY

Performance Parameter	Acceptance Criteria	Results
System Suitability	• The %RSD of the peak response ratio of Cholesterol to Pregnenolone Isobutyrate (Internal Standard) in the first five (5) injections of the *Assay Standard Solution* are NMT 2.0% • The average % Agreement between the first five (5) *Assay Standard Solution* injections and each *Assay Standard Solution (QC Check)* injection is 98% - 102%. • The average % Agreement between the first five (5) *Assay Standard Solution* injections and the *Assay Standard Solution Check* injections is 98% - 102%. • The resolution between the Pregnenolone Isobutyrate (Internal Standard) and Cholesterol in all *Assay Standard Solution* injections is NLT 10.	***Assay Standard Solution* %RSD** • %RSD: 0.2% **QC Check % Agreement** • QC Check 1: 100% • QC Check 2: 100% ***Assay Standard Solution Check* % Agreement** • % Agreement: 101% **Resolution** • Resolution: 14
Accuracy	• The Percent Recovery of all replicates are between 98% and 102%.	**100% Level** • Replicate 1: 100% • Replicate 2: 99% • Replicate 3: 98% • Replicate 4: 99% • Replicate 5: 99% • Replicate 6: 102%
Precision	• Standard Deviation: Report • %RSD: NMT 2.0%	**100% Level** • Standard Deviation: 1.438% • %RSD: 1.46%
Specificity	• The resolution between Pregnenolone Isobutyrate (Internal Standard) and Cholesterol is NLT 10 in the *Accuracy / Precision Sample Solution*. • The Pregnenolone Isobutyrate (Internal Standard) and Cholesterol peaks are visually resolved from diluent interference.	• Resolution: 13.7 • Pregnenolone Isobutyrate and Cholesterol peaks visually resolved from diluent interference.

12. VALIDATION RESULTS:

12.1. **System Suitability**

12.1.1. System Suitability was assessed by injecting the *Diluent*, *Assay Standard Solution*, and *Assay Standard Solution Check* as per the injection sequence below and determining the Relative Standard Deviation (%RSD), Percent Agreement, and Resolution.

TABLE 9: INJECTION SEQUENCE

Sample ID	Number of Injections
System Suitability	
Diluent	≥1
Assay Standard Solution	5
Assay Standard Solution Check	2
Diluent	1
Samples	
Samples	≤6 (1 Injection Each)
Assay Standard Solution (QC Check)	1
Diluent	1
Repeat the sample injection sequence if additional samples are to be analyzed. Samples may be substituted with diluent injections.	

12.1.2. Acceptance Criteria:

12.1.2.1. Refer to the "System Suitability Results" Table.

12.1.3. Results:

12.1.3.1. All acceptance criteria were met for System Suitability. Results are summarized in the "System Suitability Results" Table.

TABLE 10: SYSTEM SUITABILITY RESULTS

System Suitability Parameter	Acceptance Criteria	Result
The Relative Standard Deviation (%RSD) of the peak response ratio of Cholesterol to Pregnenolone Isobutyrate (Internal Standard) in the first five (5) injections of the *Assay Standard Solution*.	NMT 2.0%	0.2%
The average % Agreement between the first five (5) *Assay Standard Solution* injections and each *Assay Standard Solution (QC Check)* injection.	98% - 102%	QC Check 1: 100% QC Check 2: 100%
The average % Agreement between the first five (5) *Assay Standard Solution* injections and the *Assay Standard Solution Check* injections.	98% - 102%	101%
The resolution between the Pregnenolone Isobutyrate (Internal Standard) and Cholesterol in all *Assay Standard Solution*.	NLT 10	14

12.2. **Accuracy**

12.2.1. Accuracy was assessed at 100% of the nominal assay concentration of 1.0mg/mL on the dried basis. The assay (as-is) was compared to the certified purity value of the reference standard by calculating the Percent Recovery.

$$Percent\ Recovery\ (\%) = \frac{\%\ Cholesterol\ (As\text{-}Is)\ (\%)}{CoA\ Purity\ Value\ (\%)} \times 100$$

12.2.2. Acceptance Criteria:

12.2.2.1. The Percent Recovery of all replicates are between 98% and 102%.

12.2.3. Results:

12.2.3.1. All acceptance criteria were met for Accuracy. Results are summarized in the "Accuracy Results" table.

TABLE 11: ACCURACY RESULTS

						Cholesterol CoA Purity (%):	99%
						Loss on Drying (%):	0.0%
Sample ID	**Replicate**	**Cholesterol Concentration (mg/mL)**	**Cholesterol Peak Area**	**Internal Standard Peak Area**	**Cholesterol / Internal Standard Ratio**	**% Cholesterol**	**Percent Recovery (%)**
Assay Standard Solution	1	1.006	456792	419106	1.08992	[redacted]	[redacted]
	2		455954	418252	1.09014		
	3		453623	417369	1.08686		
	4		455541	418613	1.08822		
	5		454709	418418	1.08673		
Accuracy/ Precision Sample Solution	1	1.012	455961	419084	1.08799	99.4	100
	2	1.024	456289	420780	1.08439	97.9	99
	3	1.020	459091	431075	1.06499	96.5	98
	4	1.032	463552	424546	1.09188	97.8	99
	5	1.032	464848	424059	1.09619	98.2	99
	6	1.024	465619	417506	1.11524	100.7	102

12.3. **Precision**

12.3.1. Precision was assessed at 100% of the nominal assay concentration of 1.0 mg/mL on the dried basis by preparing six (6) replicates and calculating the Standard Deviation and Relative Standard Deviation (%RSD).

12.3.2. Acceptance Criteria:

12.3.2.1. Standard Deviation: Report.

12.3.2.2. Relative Standard Deviation (%RSD): NMT 2.0%.

12.3.3. Results:

12.3.3.1. All acceptance criteria were met for Precision. Results are summarized in the "Precision Results" table.

TABLE 12: PRECISION RESULTS

Sample ID	Replicate	%Cholesterol	Standard Deviation (%)	%RSD
Accuracy/Precision Sample Solution	1	99.36	1.438	1.46
	2	97.87		
	3	96.49		
	4	97.78		
	5	98.16		
	6	100.65		

12.4. **Specificity**

12.4.1. Specificity was demonstrated by overlaying the chromatograms from one (1) *Diluent* injection, one (1) *Assay Standard Solution* injection, and one (1) *Accuracy / Precision Sample Solution* injection.

12.4.2. Acceptance Criteria:

12.4.2.1. The resolution between Pregnenolone Isobutyrate (Internal Standard) and Cholesterol is NLT 10 in the *Accuracy / Precision Sample Solution*.

12.4.2.2. The Pregnenolone Isobutyrate (Internal Standard) and Cholesterol peaks are visually resolved from diluent interference.

12.4.3. Results:

12.4.3.1. All acceptance criteria were met for Specificity. Results are summarized in the "Specificity Results" table.

TABLE 13: SPECIFICITY RESULTS

Acceptance Criteria	Result
The resolution between Pregnenolone Isobutyrate (Internal Standard) and Cholesterol is NLT 1.5 in the *Accuracy / Precision Sample Solution*.	13.7
The Pregnenolone Isobutyrate (Internal Standard) and Cholesterol peaks are visually resolved from diluent interference.	PASS

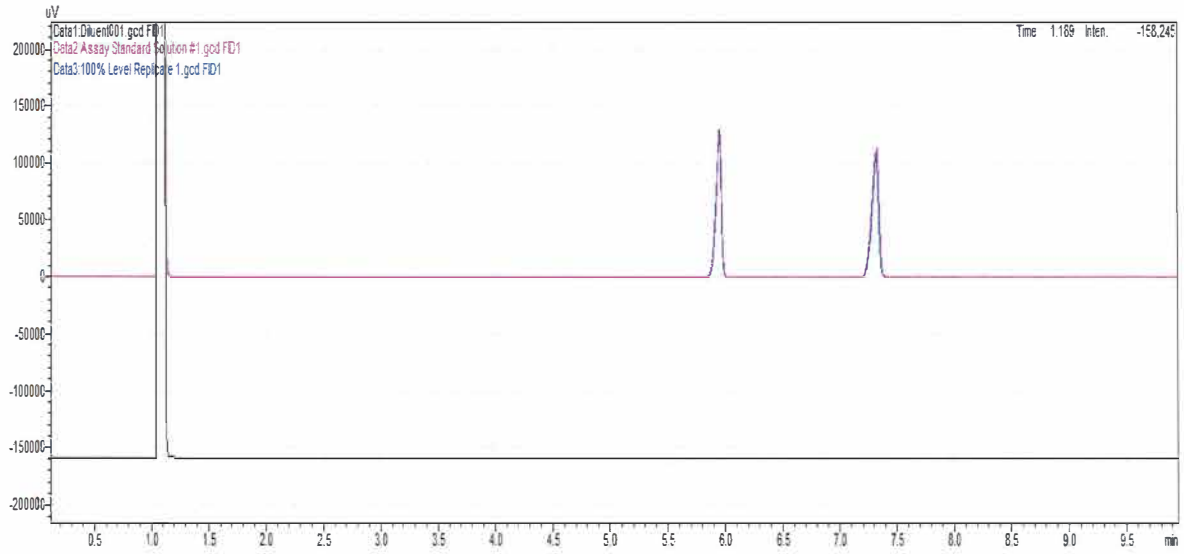

FIGURE 1: SPECIFICITY OVERLAY OF A DILUENT, ASSAY STANDARD SOLUTION, AND ACCURACY / PRECISION SAMPLE SOLUTION SHOWING THE HEPTANE PEAK AT ~1 MINUTE, THE PREGNENOLONE ISOBUTYRATE PEAK AT ~ 6 MINUTES, AND THE CHOLESTEROL PEAK AT ~7.3 MINUTES.

13. CONCLUSION:

13.1. **Performance Summary:**

TABLE 14: PERFORMANCE SUMMARY	
Method Performance Indicator	**Result**
System Suitability	Pass
Accuracy	Pass
Precision	Pass
Specificity	Pass

13.2. **Statement of Verification:** The method of analysis of Cholesterol Assay via Gas Chromatography with Flame Ionization Detection (GC-FID) is considered a verified method of analysis at the BioSpectra Inc. Majestic facility and is reviewed and approved by the review and approval team.

13.3. **Excursions or Critical Changes to Method Verification Protocol:**

13.3.1. **Critical Change – Assay (As-Is) and Percent Agreement Calculations –** The Analytical Method Verification Protocol states that R_S is the "Peak response ratio of Cholesterol to the Internal Standard (peak response of Cholesterol / peak response of the Internal Standard) from the *Assay Standard Solution*" this was clarified in the Analytical Method Verification Report to state "R_S = Peak response ratio of Cholesterol the Internal Standard (peak response of Cholesterol / peak response of the Internal Standard) from the first five *Assay Standard Solution* injections".

13.3.2. **Critical Change – Accuracy Specification –** In the Analytical Method Verification Protocol, Section 9 Performance Parameters, the Accuracy acceptance criteria is "The Percent Recovery of all replicates are between 98% and 102%". In Section 11.5, the accuracy acceptance criteria states "The Percent Recovery of all replicates are between 98.0% and 102.0%". Additionally, the Certificate of Analysis for the Cholesterol used has a purity value of 99%, which was used for the Percent Recovery calculations. The specification of 98% to 102% will be used for accuracy, this will be updated in the Analytical Method Verification Report.