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ANALYTICAL METHOD VALIDATION REPORT: QUANTIFICATION OF SULFUR BY INDUCTIVELY COUPLED OPTICAL EMISSION SPECTROMETRY (ICP-OES) IN DEXTRAN SULFATE

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1. PURPOSE:

1.1. The purpose of this validation report is to establish documented evidence that the test protocol, BSI-PRL-0538 v. 1.0, for Sulfur Quantification in Dextran Sulfate performs according to BioSpectra Analytical Method Validation Master Plan and USP requirements.

2. SCOPE:

2.1. Applies to Dextran Sulfate (molecular weight 8000) and related products manufactured at BioSpectra.

2.2. Applies to the Avio 500 S/N 081S1905062 ICP-OES located in the Quality Control (QC) Laboratory at the BioSpectra Bangor, PA facility.

2.3. This report applies to the protocol validation for sulfur quantification in dextran sulfate, by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) performed at BioSpectra.

3. REFERENCES:

3.1. BSI-PRL-0538, Sulfur Quantification Method Validation Protocol
3.2. BSI-SOP-0362, Avio 500 ICP-OES SOP
3.3. BSI-SOP-0436, Analytical Method Validation Master Plan
3.4. USP <730> Plasma Spectrochemistry
3.5. USP <1730> Plasma Spectrochemistry—Theory and Practice

4. BACKGROUND:

4.1. This validation was executed as a Category I Quantitative Analytical Method.

4.2. The test protocol validation report includes the following parameters:
    4.2.1. Specificity
    4.2.2. Linearity and Range
    4.2.3. Robustness
    4.2.4. Accuracy by "Spiked Recovery"
    4.2.5. Precision (Repeatability)
    4.2.6. Intermediate Precision
    4.2.7. Standard and Sample Solution Stability

5. MATERIALS AND EQUIPMENT:

Table 1: Equipment				
Type	**Supplier**	**Model**	**Serial Number**	**Cal. Due**
Analytical Balance	Sartorius	MSE224S	36707108	10/2022
Automatic Pipette[1]	Rainin	E4-XLS (20-200 µL)	C016314640	06/30/22 12/31/22
Automatic Pipette[1]	Rainin	E4-XLS (100-1000 µL)	C016314969	06/30/22 12/31/22
Automatic Pipette[1]	Rainin	E4-XLS (0.5-5 mL)	C023506909	06/30/22 12/31/22
ICP-OES	Perkin Elmer	Avio 500	081S1905062	09/2022
Digestion Microwave	CEM	Mars 6	MY2255	09/22/22
Deionized Water System	Millipore	IQ-7005/ Element POD	F9SA14284H	06/2023

[1] Addition calibration due date from third experiment for intermediate precision as pipettes were serviced between experiments.

Table 2: Reagents					
Type	**Grade**	**Supplier**	**Catalog Number**	**Lot Number**	**Expiration**
70% Nitric Acid	Trace Metal	VWR	87003-261	1121090	09/29/23
70% Nitric Acid	Trace Metal	VWR	87003-261	1121110	12/08/23
70% Nitric Acid	Trace Metal	VWR	87003-261	1122020	02/16/24
Deionized water	Type 1 Ultrapure	In-House	N/A	N/A	N/A

5.1. Consumable Supplies
  5.1.1. SCP Digitubes® 15 mL, 50 mL and 100 mL
  5.1.2. Pipette Tips of various sizes

Table 3: Reference Standards				
Identification	**Manufacturer**	**Lot Number**	**Expiration**	**Concentrations / Elements**
Manganese Stock Standard N9303783	Perkin Elmer	25-71MNY1	10/30/22	Mn (1,000 µg/mL)
Scandium Stock Standard N9303798	Perkin Elmer	25-96SCY1	01/30/23	Sc (1,000 µg/mL)
Sulfur Stock Standard N9303796	Perkin Elmer	25-161SY1	09/30/22	S (1,000 µg/mL)
Yttrium Stock Standard N9303810	Perkin Elmer	25-126YY1	02/28/23	Y (1,000 µg/mL)

6. PROCEDURE:

6.1. All standards were prepared volumetrically from stock solutions purchased from certified vendors. If the vendor supplied stock standard was within 2% of the nominal value as per the certificate of analysis, then the nominal value was used to calculate the concentration of the standard. If the stock standard certificate of analysis value was greater than or less than 2% of the nominal value, then the certificate of analysis value was used for the stock standard concentration.

6.2. Internal Standard Solution

6.2.1. Added 100 µL of 1,000 µg/mL Scandium Stock Standard and 100 µL of 1,000 µg/mL Yttrium Stock Standard into a 15 mL Digitube®.

6.2.2. Diluted to a final volume of 10 mL using deionized water and mixed well.

6.2.3. Scaled proportionally as needed for use.

6.3. Calibration Standard Preparation

6.3.1. Prepared calibration blank and calibration standards, as per Table 4 below, using the 1,000 µg/mL sulfur stock standard and internal standard solution prepared above. Added stock standard to separate 50 mL Digitubes® followed by addition of internal standard. Added DI water to approximately 45 mL then added nitric acid. Diluted to final volume using DI Water.

Table 4: Calibration Standards

Identification	Element	Intermediate Standard (mL)	Internal Standard (mL)	Nitric Acid (mL)	Final Volume (mL)	Final Concentration (µg/L)
Cal Blank	S	0.000	0.100	2.5	50	0.0
2.5 ppm Std		0.125				2.5
5.0 ppm Std		0.250				5.0
10 ppm Std		0.500				10
15 ppm Std		0.750				15

6.4. Method Blank Preparation

6.4.1. Pipetted 4.0 mL of nitric acid into a clean 20 mL digestion vessel, placed a plug on the vessel, and properly torqued the vessel cap. Placed vessel in the microwave carousel then digested and completed preparation according to Section 6.6 below.

6.5. Sample Preparation

6.5.1. Weighed approximately 100 mg of sample into a clean 20 mL digestion vessel and added 4.0 mL of nitric acid.

6.5.2. Placed a plug on the vessels and properly torqued vessel cap before placing in the microwave carousel. Digested and completed sample preparation according to Section 6.6 below.

6.6. Microwave Digestion Procedure

6.6.1. Referred to BSI-SOP-0426 for general usages and guidelines for the Mars 6 Microwave Digestion System.

6.6.2. Prepared at least one method blank per digestion run. Method blank was prepared in the same manner as the samples without the addition of actual sample.

6.6.3. Digested the samples using the program listed in Table 5 below.

Table 5: Temperature Controlled Microwave Digestion Program

Power (Watts)	Percent Power	Ramp (Minutes)	Temperature (°C)	Hold (Minutes)
1800	100	15:00	150	10:00
1800	100	6:00	175	5:00

6.6.4. After digestion, the digestion vessels were placed in an ice bath and allowed the vessels to cool for approximately 40 minutes. Before opening, the vessels were turned sideways and slowly rotated in order to collect the condensation on the inside walls of the vessels.

6.6.5. Quantitatively transferred the vessel contents into a 50 mL Digitube® containing 5 mL of deionized water. Rinsed the bottom of the plug into the same 50 mL Digitube® using deionized water.

6.6.6. Rinsed the vessel an additional two times with deionized water and transferred each rinse to the 50 mL Digitube®. Diluted to final volume of 50 mL using deionized water and mixed well.

6.6.7. Performed an additional dilution for each sample by pipetting 1.0 mL of the digested sample solution into another 50 mL Digitube®.

6.6.8. Pipetted 100 µL of internal standard and diluted to 45 mL using deionized water.

6.6.9. Added 2.5 mL of nitric acid then diluted to final volume of 50 mL using deionized water and mixed well.

7. INSTRUMENT PROCEDURE:

7.1. Performed the ICP-OES daily performance check prior to beginning the analytical sequence. Refer to Avio 500 ICP-OES SOP DCN BSI-SOP-0362 for Daily Check procedures.

7.2. A calibration curve of no less than two standards and a blank was used. The calibration correlation coefficient (R) was ≥ 0.995.

7.3. All sample files were set up the according to the sequence described in Table 6.

7.4. Confirmed the calibration by analyzing the 10 ppm standard after the calibration. The calibration check must recover ±10% of the calculated theoretical concentration for single element determinations.

7.5. The check standard was verified after each calibration. A re-analysis of the check standard was to be performed a minimum of once every 10 samples and at the end of the analytical run.

7.6. Each bracketing standard must recover ±10% of the calculated theoretical concentration for each wavelength. Additionally, the drift (calculated as absolute difference) between the bracketing standard checks must be NMT 10% for each Target wavelength.

7.7. The sample concentration is calculated as:

$$\text{Conc. } (\mu g/g) = \frac{\text{Solution Conc. } (\mu g/L) \times \text{Solution vol. } (L) \times \text{Dilution Factor}}{\text{Sample Mass } (g)}$$

Table 6: Example Sample Analysis Sequence

ID	Type	Level
Cal Blank	Cal Blank	Level 1
2.5 ppm Std	Cal Std	Level 2
5.0 ppm Std	Cal Std	Level 3
10 ppm Std	Cal Std	Level 4
15 ppm Std	Cal Std	Level 5
Cal Blank Check	QC Check	N/A
10 ppm Check Std 1	QC Check	N/A
Method Blank	Sample	N/A
Sample(s) 10 or less	Sample	N/A
10 ppm Check Std 2	QC Check	N/A

7.8. Instrument Setup and Parameters

7.8.1. Instrument settings are only listed as guidelines. Settings may be changed in order to accommodate changes in sample matrix or hardware configurations.

7.8.2. The Gas Flows for Plasma, Auxiliary, and Nebulizer were set at 12 mL/min, 0.20 mL/min, and 0.60 mL/min, respectively.

Table 7: ICP-OES Parameters

ICP-OES System	Perkin Elmer Avio 500 Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) with Syngistix Software
Points per Peak	3
Replicates	3
Viewing Distance	15.0
Nebulizer Gas	Argon
Shear Gas	Compressed Air + Nitrogen
Sample Rinse	Rinse-1: 30 sec at 1.0 mL/min 5.0% HNO_3 (or as applicable to mitigate carry over)

Table 8: Linear Range and Corresponding Mode

Element	**Mode**	**Wavelength (nm)**	**Linear Range (µg/mL)**
S	Axial	181.975 180.669	1.0-20

7.9. Linearity and Range

7.9.1. The ICP-OES linearity study included standards equivalent to the following concentrations: 1.0 ppm, 2.5 ppm, 5.0 ppm, 7.5 ppm, 10 ppm, 12.5 ppm, 15 ppm, and 20 ppm. Each standard was prepared in triplicate and analyzed against the calibration curve using the calibration standards prepared above in Section 6.3. The average standard recovery for each level of the three replicates was then determined.

7.9.1.1. Acceptance Criteria:

7.9.1.1.1. The mean standard recovery for sulfur at each of the linearity levels must be in the range of 80% -120%.

Table 9: Linearity Standard Preparation

Description	Intermediate Standard (mL)	Internal Standard (mL)	Nitric Acid (mL)	Final Volume (mL)
Cal Blank	N/A	0.100	2.5	50
1.0 ppm Std	0.050	0.100	2.5	50
2.5 ppm Std	0.125	0.100	2.5	50
5.0 ppm Std	0.250	0.100	2.5	50
7.5 ppm Std	0.375	0.100	2.5	50
10 ppm Std	0.500	0.100	2.5	50
12.5 ppm Std	0.625	0.100	2.5	50
15 ppm Std	0.750	0.100	2.5	50
20 ppm Std	1.000	0.100	2.5	50

Table 10: Linearity Percent Recovery Results

Wavelength »	S 181.975 (% Recovery)	S 180.669 (% Recovery)
1.0 ppm Mean	98	97
2.5 ppm Mean	98	98
5.0 ppm Mean	99	99
7.5 ppm Mean	100	99
10 ppm Mean	98	98
12.5 ppm Mean	98	98
15 ppm Mean	98	98
20 ppm Mean	99	98

All wavelengths meet Linearity acceptance criteria of 80% - 120%.

7.10. Robustness

7.10.1. Three (N=3) samples were prepared for analysis at five separate levels. The triplicate sample preparation of the 100% preparation was used for recovery calculations. The solutions were analyzed by ICP-OES as per the protocol and by a single analyst.

$$\% \text{ Recovery} = \frac{\text{Concentration of replicate x 100}}{\text{Theoretical concentration Based on 100\% Preparation}}$$

7.10.1.1. Acceptance Criteria

7.10.1.1.1. The mean recovery at each of the four alternative preparation levels must be in the range of 95.0-105.0% from the 100% preparation level.

7.10.2. Robustness Sample Preparation

7.10.2.1. Weighed the appropriate amount of sample into a clean 20 mL digestion vessel.

7.10.2.2. Added 4.0 mL of nitric acid then properly torqued cap and placed vessel in microwave carousel. Digested samples according to Section 6.6.

7.10.2.3. After digestion, vessels were placed in an ice bath for approximately 40 minutes. Before transferring, the vessels were turned sideways and slowly rotated to collect condensation on the inside of the vessel walls.

7.10.2.4. Quantitatively transferred the contents into a 50 mL Digitube® containing 5 mL of deionized water.

7.10.2.5. Rinsed the vessel an additional two times using deionized water and transferred each rinse to the 50 mL Digitube®. Diluted to final volume of 50 mL and mixed.

7.10.2.6. Performed an additional dilution by pipetting 1.0 mL of the digested sample solution into another 50 mL Digitube® along with 100 µL of internal standard then diluted to 45 mL using deionized water.

7.10.2.7. Added 2.5 mL of nitric acid then diluted to final volume of 50 mL using deionized water.

Table 11: Robustness Sample Solution Preparations

Description	Sample Amount (mg)	Internal Standard (mL)	Nitric Acid (mL)	Final Volume (mL)
Method Blank	N/A	0.100	2.5	50
80% Preparation	80	0.100	2.5	50
90% Preparation	90	0.100	2.5	50
100% Preparation	100	0.100	2.5	50
110% Preparation	110	0.100	2.5	50
120% Preparation	120	0.100	2.5	50

Table 12: Robustness Results
(Mean percent recovery of triplicate preparations)

Wavelength »	S 181.975 (% Recovery)	S 180.669 (% Recovery)
80% Prep	100.0	100.6
90% Prep	100.1	100.0
110% Prep	98.6	99.0
120% Prep	97.6	97.5

All wavelengths met robustness acceptance criteria of 95.0-105.0%.

7.11. Accuracy by "Spiked Recovery"

7.11.1. Three (N=3) samples were prepared for analysis at three separate levels in triplicate to fulfill 9 different preparations. The 100% preparation replicate 1 from the robustness study was used for recovery calculations as this sample was used for the spiking study. The solutions were analyzed by ICP-OES as per the protocol and by a single analyst.

$$\% \text{ Recovery} = \frac{\text{Concentration of spiked replicate x 100}}{\text{Theoretical concentration of Spike + Concentration of 100\% Replicate}}$$

7.11.1.1. Acceptance Criteria

7.11.1.1.1. The mean recovery at each of the three preparation levels must be in the range of 95.0% to 105.0% from the 100% preparation level.

7.11.2. Accuracy Sample Preparation

7.11.2.1. Samples were prepared from the first replicate of the digested sample that was prepared at the 100% preparation in section 7.10.2.5.

7.11.2.2. Performed an additional dilution by pipetting 1.0 mL of the digested sample into another 50 mL Digitube® along with 100 µL of internal standard.

7.11.2.3. Pipetted sulfur stock standard according to Table 13 below then diluted to 45 mL using deionized water.

7.11.2.4. Add 2.5 mL of nitric acid then diluted to final volume of 50 mL using deionized water.

Table 13: Accuracy Sample Solution Preparations

Description	Sample Amount (mL)	Sulfur Stock Std Amount (mL)	Internal Standard (mL)	Nitric Acid (mL)	Final Volume (mL)
2.5 ppm Spike Level	1.0	0.125	0.100	2.5	50
5.0 ppm Spike Level	1.0	0.250	0.100	2.5	50
7.5 ppm Spike Level	1.0	0.375	0.100	2.5	50

Table 14: Accuracy Results
(Mean percent recovery of triplicate preparations)

Wavelength »	S 181.975 (% Recovery)	S 180.669 (% Recovery)
2.5 ppm Spike Level	98.1	98.1
5.0 ppm Spike Level	99.7	98.9
7.5 ppm Spike Level	98.8	97.9

All wavelengths met accuracy acceptance criteria of 95.0-105.0%.

7.12. Specificity

7.12.1. Specificity was demonstrated by using a method blank and a calibration blank for ICP-OES analysis. The method blank was prepared during the accuracy/robustness portions and prepared according to the protocol. This is not a duplicate preparation of the calibration blank.

7.12.2. The solutions were analyzed as per the analytical method and the intensities of the method blank, calibration blank, 2.5 ppm standard, and 15 ppm standard are reported to show no matrix interference is observed at all three wavelengths. The counts for the method blank and the standards are corrected by the software compared to that of the calibration blank.

7.12.2.1. Acceptance Criteria:

7.12.2.1.1. The lack of a significant interference or by any other element in the spiked blank solution or the solution matrix itself will indicate the specificity of the method.

Table 15: Specificity Results

Wavelength »	**S 181.975**	**S 180.669**
Blank (CPS)	20	50
Method Blank (CPS)	14	18
2.5 ppm Std (CPS)	2035	3596
15 ppm Std (CPS)	12316	21547

7.13. Precision

7.13.1. Repeatability

7.13.2. All solutions for the Repeatability test were prepared by a single analyst.

7.13.3. Six sample solutions were prepared at the 100% Preparation Concentration as described in the sample preparation, Section 6.5, and the microwave digestion, Section 6.6, above. For ICP-OES analysis, the three 100% preparation sample solutions from the robustness section were also included in the analysis for the precision portion.

7.13.4. The concentrations in Table 16 below are reported in ppm in calibration units based on the calibration curve, which is a factor of 25,000 less than the actual amount of sulfur present in the 100% target preparation.

7.13.4.1. Acceptance Criteria:

7.13.4.1.1. The %RSD for the 100% preparation must be NMT 5.0% for each wavelength.

Table 16: Precision Results
(Mean concentration of N=6 100% preparations)

Wavelength »	**S 181.975**	**S 180.669**
100% Mean Concentration	7.051	6.979
%RSD	2.1	2.0

All wavelengths meet Precision RSD% acceptance criteria of NMT 5.0%.

7.14. Intermediate Precision

7.14.1. A second and third analyst, on different days from the performance of the Repeatability experiment, prepared and analyzed the Intermediate Precision solutions. Six additional sample solutions were prepared at the 100% preparation level as shown in Section 6.5 for ICP- OES analysis (this fulfilled a total of three experiments as described in USP <730> as “different day” and “different analyst”).

7.14.2. The concentrations are reported in ppm in calibration units based on the calibration curve, which is a factor of 25,000 less than the actual amount of sulfur present in the 100% target preparation.

7.14.2.1. Acceptance Criteria:

7.14.2.1.1. The %RSD for the 100% preparation from three analysts (N=18) must be NMT 8.0% for each wavelength.

Table 17: Intermediate Precision Results
(Mean concentration of N=18 100% preparations)

Wavelength »	**S 181.975**	**S 180.669**
100% Mean Concentration	7.376	7.348
%RSD	4.0	4.6

All wavelengths meet the Intermediate Precision %RSD acceptance criteria of NMT 8.0%.

7.15. Sample and Standard Stability

7.15.1. The 2.5 ppm and 15 ppm calibration standards were analyzed as samples against calibration curves constructed from freshly prepared calibration standards at T=1 day, T=3 days, and T=6 days from the date of preparation (T=0).

7.15.2. One sample solution prepared at the target 100% preparation from the Robustness experiment was used for sample stability. The sample solution was analyzed against calibration curves constructed from freshly prepared calibration standards at time points T=1-day, T=3 days, and T=6 days (days from the date of preparation).

7.15.2.1. Acceptance Criteria:

7.15.2.1.1. The recovery of each wavelength must be within the range of 90% to 110% recovery of the T = 0 results for the calibration standards.

7.15.2.1.2. The recovery of each wavelength must be within the range of 90% to 110% recovery of the T = 0 results for the sample solution.

Table 18: Sample and Standard Stability (% Recovery)

Wavelength »	**S 181.975 (% Recovery)**			**S 180.669 (% Recovery)**		
	T=1	**T=3**	**T=6**	**T=1**	**T=3**	**T=6**
2.5 ppm Std	101	101	102	101	100	100
15 ppm Std	101	100	101	100	100	101
100% Prep Sample	102	99	100	103	100	101

All wavelengths met the acceptance criteria of 90% to 110% recovery for stability solutions for standards and samples.

8. DEVIATIONS:

8.1. In the validation protocol, the %Recovery calculation listed for the Accuracy by "Spiked Recovery" study in Section 7.11.1 is the same calculation used for the robustness section. The calculation is corrected in the validation report in Section 7.11.1 In addition, only the concentration of the sample that is used for the spiking study is used for the calculation as opposed to the triplicate sample preparation as indicated in the validation protocol. This is deemed acceptable as the recovery would be based upon the single replicate that is used for the study as opposed to the average of samples.

8.2. The method validation protocol (BSI-PRL-0538) was mistakenly designed as a Category II Impurity test, as opposed to a Category I Assay. Since the sulfur present in Dextran Sulfate 8000 is not an impurity, this report has been updated to reflect the following changes in acceptance criteria to harmonize with USP <730>:

8.2.1. Accuracy & Robustness acceptance criterion changed from 70-150% to 95.0-105.0%. This acceptance criterion was still met.

8.2.2. Precision acceptance criterion changed from NMT 20% to NMT 5.0%. This acceptance criterion was still met.

8.2.3. Intermediate Precision criterion changed from NMT 25% to NMT 8.0%. This acceptance criterion was still met.

8.2.3.1. Due to wording in USP <730> referring to a minimum of "three experiments" with regard to Intermediate Precision, an additional six precision samples were prepared and analyzed by a third analyst on 08/26/22. The Intermediate Precision acceptance criterion is still met with N=18 measurements.

8.2.4. General correlation coefficient requirements for calibration changed from NLT 0.99 to NLT 0.995. This requirement was met for all portions of this method validation.

9. CONCLUSION:

9.1. The test method for sulfur quantification in dextran sulfate has been validated. The Method was found to be:

9.1.1. Specific: The calibration blank and method blank did not show any significant interference for all analyzed wavelengths.

9.1.2. Linear: 1.0 ppm through 20 ppm of calibration standards. Mean percent recovery ranged from 97% to 100%.

9.1.3. Robustness: From 80% to 120% preparation of target preparation with mean percent recoveries ranging from 97.5% to 100.6%. All wavelengths analyzed met acceptance criteria within the specified range.

9.1.4. Accurate: The method is able to accurately quantify sulfur by spiked recovery in a sample solution with mean percent recoveries ranging from 97.9% to 99.7%. All wavelengths analyzed met acceptance criteria within the specified range.

9.1.5. Precise: Closeness of agreement demonstrated between six sample preparations by percent RSDs of 2.0% and 2.1% for each wavelength.

9.1.6. Rugged: Satisfactory precision was demonstrated between three sets of six sample preparations performed on different days and by different analysts. The percent RSDs were 4.0% and 4.6% for each wavelength.

9.1.7. Stable: With respect to stability of solutions, the sample solution for target preparation was shown to be stable for 6 days for all validated wavelengths analyzed using this protocol. The calibration standard preparations are shown to be stable for 6 days.

10. NOTEBOOK REFERENCE:

Table 19: Notebook Reference

STUDY		NOTEBOOK REFERENCE
Specificity		ICPOES2 pages 24-27, ICPOES4 page 23
Linearity and Range		ICPOES2 pages 24-27
Robustness and Precision		ICPOES2 pages 24-27, ICPOES4 page 23
Accuracy by "Spiked Recovery"		ICPOES2 pages 24-27, ICPOES4 page 23
Intermediate Precision Analyst 2		ICPOES2 pages 28-30
Intermediate Precision Analyst 3		ICPOES4 pages 20-22
Solution Stability	Day-0	ICPOES2 pages 24-27
	Day-1	ICPOES2 pages 28-30
	Day-3	ICPOES2 page 31
	Day-6	ICPOES2 pages 34-35