Application Note
July 2021
AN-850-0114

Biosimilar Structural Comparison of Commercially Sourced Reference Standards by MMS Rapidly Detects Subtle but Critical Differences to Correctly Predict Activity for Use in an ELISA Product

- [x] Research
- [x] Discovery
- [ ] Formulation and Development
- [x] Quality Control
- [ ] Manufacturing

- [x] Aggregation
- [ ] Quantitation
- [x] Stability
- [x] Structure
- [x] Similarity

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Introduction

An ELISA product manufactured by Biokit S.A.*, includes Bovine Serum Albumin (BSA) as a reference molecule that is periodically sourced from several commercial suppliers as required. It was determined that many of the BSA reference candidates did not exhibit acceptable activity after inclusion into the final product though all purchased samples shared the same name and identification and typically included acceptable supporting analytical data such as from HPLC and mass spectrometry. The inconsistency in activity causes delays, increases cost of manufacture, and adds an element of variability that is not acceptable to Biokit. Current in-house biophysical techniques fail to correctly identify the inactive candidates prior to product inclusion due to inconclusive results or a lack of sensitivity, thus a more accurate, reliable analytical method was sought.

This biosimilar reference study was performed using the AQS^{3}®pro system powered by Microfluidic Modulation Spectroscopy (MMS) and driven by AQS^{3}delta Analytics to successfully identify the 5 of 13 BSA biosimilar samples that retained acceptable activity upon inclusion in the ELISA product through structural comparison to a reference of known activity. Reference standards are important for the purpose of providing reliable and accurate comparison for biosimilars, both externally and as in-house controls. In practice therefore, reference standards are validated molecules against which biosimilar molecules are compared and must be reproducible in their own composition, potency, and secondary structure. Due to the extremely high sensitivity and reproducibility of MMS measurements, this study confidently detected <2% structural difference between samples to correctly predict activity amongst the commercially sourced materials. After preparation and loading into a well plate, data was acquired automatically and with little user interaction, and processing was performed using the AQS^{3}delta Analytics program. In addition to more detailed analyses and measurements provided by the AQS^{3}pro, a simple numerical output reporting the percent similarity of each sample's spectra relative to the active in-house standard reference was sufficient to correctly identify the active 5 candidates, providing the basis for Biokit to consider adopting MMS as a rapid screening method for material sourced commercially in the future.

Methods

Thirteen BSA samples were purchased from several different suppliers and prepared in phosphate-buffered saline (PBS) at a nominal concentration of 10 mg/mL. The 13 samples were compared against the gold-standard BSA reference sample 4112305 which was previously shown to have the correct activity in the final ELISA-based product. MMS analysis was performed automatically in triplicate for all 14 BSA samples using the RedShift BioAnalytics AQS^{3}pro system with a 24-well plate configuration. Sample and reference fluids were modulated through the microfluidic flow cell at a rate of 1 Hz with a backing pressure of 5 psi for all measurements. The differential absorbance spectra were collected and background subtracted real-time using reference spectra for water and PBS, resulting in averaged Absolute Absorbance (AbsAU) spectra for each sample. All results were processed automatically using AQS^{3}delta Data Analysis software. Gaussian curve fitting determined secondary structure content as part of this analysis and was applied to baseline-corrected second derivative absorbance spectra for each sample.

Results

I. Delta Plot and Stability Analysis: The AbsAU spectra were processed as second derivative spectra to enhance subtle differences in various regions across the Amide I band. These spectra were used to generate Delta and Stability plots (Figure 1), showing the spectral differences between the 13 biosimilars and gold-standard sample 4112305.

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Results, continued

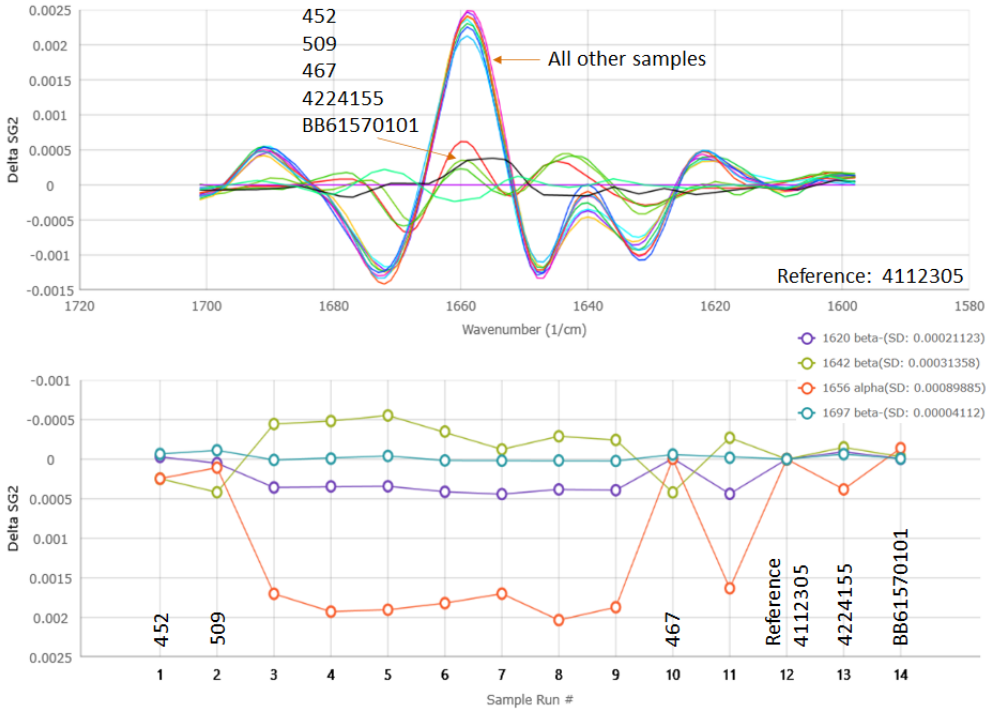

Figure 1: Delta plot and Stability analysis of 13 BSA samples compared to a control. Top: Delta plot showing second derivative spectra for each biosimilar subtracted from reference sample (4112305) to highlight spectral differences. Bottom: Stability analysis tracks signals corresponding to anti-parallel β-sheet, parallel β-sheet, and α-helix for each sample.

Delta plot analysis generates differential second derivative spectra, each subtracted from the chosen reference, and highlights spectral differences in this case between the 13 biosimilars and the gold-standard control 4112305 (Figure 1, top). Two different populations are immediately evident, particularly at 1656 cm^{-1} which corresponds to α-helical content, in which 5 of the samples stand apart from the other 8.

Stability plot analysis (Figure 1, bottom) tracks secondary structure-related spectral features across a sample set and in this case clearly also highlights the same 5 samples (452, 509, 467, 4224155, and BB61570101) as in Figure 1 (top) that are structurally most similar to the control. Delta plot analysis, monitoring the α-helical signal at 1656cm^{-1}, gave the best differentiation shown as an orange line in Figure 1 (bottom). The five samples most similar to the control in both analyses were verified to be active in the Biokit ELISA product (data not shown).

II. Similarity (Area of Overlap): As part of processing spectra, AQS^{3}delta analytics automatically calculates the concentration of a sample based on IR absorbance of the peptide or protein backbone, while similarity analysis mathematically compares spectra with one another by area of overlap comparison[1] through generation of a percent similarity score (Table 1).

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Sample Name	Given Concentration mg/mL	Calc. Concentration mg/mL	Similarity %
452	10	8.4	98.88
509	10	9.3	98.92
651	10	8.9	97.84
707	10	8.7	97.77
720	10	9	97.89
735	10	9.5	97.68
113	10	9	97.51
119	10	8.8	97.64
116	10	8.6	97.66
467	10	8.7	98.67
737	10	9.5	97.76
Reference 4112305	10	8.5	100.00
4224155	10	10.4	99.50
BB61570101	10	9.7	99.57

Table 1: Sample concentrations and similarity comparisons relative to reference sample 4112305, by area of overlap analysis.

Reference molecule 4112305, previously verified to be functional in the ELISA assay, was used as the comparative reference for similarity analysis thus resulting in a similarity score of 100.00% (in yellow, Table 1). All other samples are compared to this reference to determine how similar each sample is in terms of its overall spectrum by area of overlap analysis. To confirm that observed differences are significant, measurements of each sample were made in triplicate and the internal variance of the replicates for each sample was calculated to be <0.5% (data not shown). Therefore, differences greater than the 0.5% inter-sample variance, when calculated relative to the reference, can be considered significant.

For this dataset, the 13 biosimilars scored between 97.51% and 99.57% in similarity relative to the reference molecule, highlighting the importance of making accurate measurements with high reproducibility and high confidence to ensure even very similar samples can be differentiated from one another. From the similarity analysis, two groups can clearly be distinguished: the 5 samples that are highlighted in blue in Table 1 which are ≥98.67% similar, and the remaining 8 samples which are ≤97.89% in similarity. All results for the samples are larger in magnitude than the differences observed internally for the replicates and are therefore considered to be significant. Subsequent product testing confirmed that this similarity score analysis alone is sufficient to predict the activity of the biosimilar candidates in the ELISA assay, as all 5 samples in group 1 of this analysis were found to be active, while the remaining 8 were deemed inactive to varying degrees.

III. Higher Order Structure (HOS): Higher-order structure plots generated by AQS^{3}delta analytics (Figure 2) show the percentage breakdown of secondary structures present in each sample based on deconvolution of IR spectra acquired in the Amide I band. In this case, the composition of four types of secondary structure were calculated using a well-established model by Dong et al[2], which demonstrated less than 2% structural differences between all samples (Figure 2). AQS^{3}delta analytics automatically deconvolutes second derivative spectra using Gaussian curve-fitting, a well-established method for IR analysis.[3-5] This process is fully automated, and enables the user to choose different fitting models if required or to create a custom model if desired.

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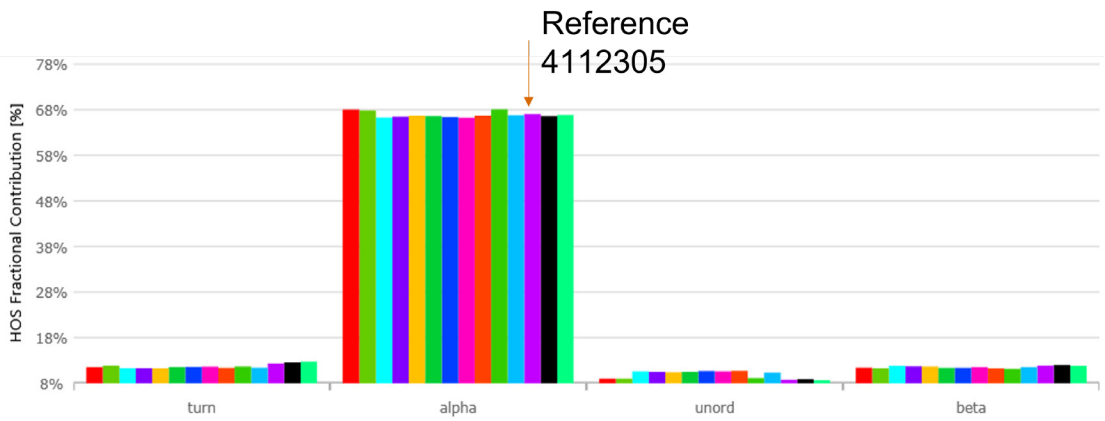

Figure 2: Combined Higher-Order Structure Plots generated in AQS³delta Data Analysis, comparing % turn, α-helix, unordered, and β-sheet secondary structures for 13 BSA samples from different manufacturers and batches relative to a known control sample (4112305). Very small differences were observed between samples but are valid due to the extreme sensitivity and reproducibility of MMS data.

Figure 3 shows the individual structural plots based on the combined HOS plot shown in Figure 2, with adjusted scaling to highlight the small but significant differences that were observed between the majority of samples other than 4224155, most pronounced in α-helix and unordered structures. Of these features, the fractional contribution of unordered structure most clearly discriminates between the two groups and corresponds to the same 5 samples identified in the Delta Plot and Stability analysis shown in Figure 1. This same population of 5 samples also demonstrates the highest degree of α-helical structure. Both the unordered and α-helix results correlate with the highest activity measured in the ELISA product, indicating that the lost α-helical region in the remaining samples is likely critical to the binding required for assay activity, and must be preserved in future reference samples.

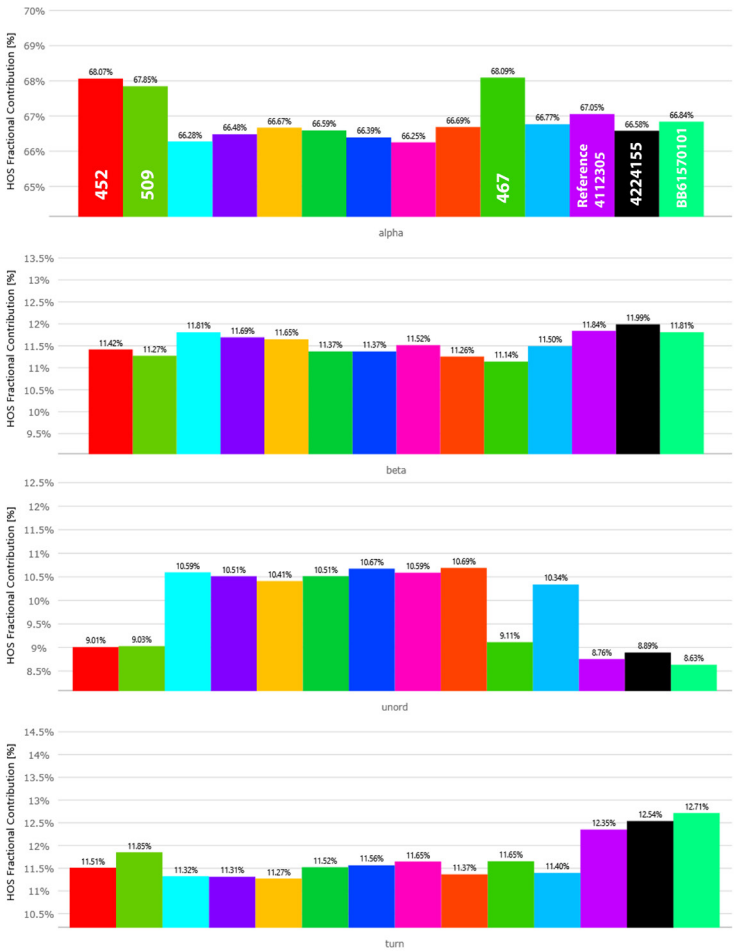

Figure 3: Expanded Higher-Order Structure plots of those shown in Figure 2, showing percent α-helix, β-sheet, unordered, and turn structures present in each biosimilar and reference sample after spectral deconvolution using a model described by Dong et al.[2]

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Conclusions

MMS is a revolutionary new infrared-based technology that combines a tunable quantum cascade laser with a novel microfluidic flow cell design, allowing for active referencing during data collection to generate secondary structure information that is higher in sensitivity, reproducibility, and quality than traditional FTIR and CD technologies.[6] Interfering water absorbances are also accounted for entirely using this method, which is often troublesome for IR-based protein analysis techniques. The advances provided by MMS technology enable a level of performance that provides confident structural differentiation between samples that was not previously attainable, here confidently identifying <2% differences in secondary structure between samples. This critical and precise information can be obtained at an early stage of development or manufacture to guide important product decisions to streamline the analytical workflow.

In this study, 13 commercially sourced BSA samples were compared to a control reference standard of known activity with the intention to use biosimilar structural analysis to qualify which suppliers and batches provided materials that would function correctly in an ELISA-based product prior to production. In-house biophysical techniques failed to provide sufficient information to make this assessment, yet MMS very accurately and repeatably measured <2% differences in secondary structure, most notably in α-helix and unordered types, and correctly enabled the prediction that 5 of the 13 samples would exhibit acceptable activity in the final product. Similarity, Stability, and Delta analyses, among additional structural metrics, are automatically generated by AQS^{3}delta analytics and were used to provide orthogonal results to confidently identify active candidates from large datasets, most simply as percent spectral similarity to the reference standard. MMS therefore provides a simple standard assay to validate reference candidates before inclusion into a product, preventing costly product mistakes at an early stage that can lead to delays and an increased cost of manufacture.

References

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