Application Note
FEB 2022
AN-850-0120

Microfluidic Modulation Spectroscopy as a Non-Destructive Structural Characterization Technique

- ○ Biosimilars
- ○ mAbs
- ○ ADCs
- ○ AAVs
- ○ Ligand Binding
- ✓ Protein/Peptide Analysis
- ○ VLPs
- ○ Nucleic Acid
- ○ Fusion Proteins
- ✓ Enzyme Analysis

- ⬢ Aggregation
- ✓ Quantitiation
- ✓ Structure
- ⬢ Stability
- ✓ Similarity

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Introduction

Protein characterization includes primary, secondary, tertiary, and quaternary structural analysis, with secondary structure providing a direct representation of the spatial arrangement around the peptide backbone. Monitoring this level of protein folding is important for preserving protein critical quality attributes. Many of the tools typically involved in measuring protein structure are destructive techniques, meaning the samples cannot be recovered or reused once analyzed. For samples that are limited in quantity or precious in nature, the inability to further analyze them is a significant drawback. The AQS³pro, manufactured by RedShiftBio and powered by Microfluidic Modulation Spectroscopy (MMS), is a novel infrared spectroscopy tool that measures protein secondary structure in a non-destructive manner, thus enabling subsequent characterization and analysis.

By incorporating MMS early into the characterization workflow, sample integrity is preserved allowing the sample to be collected post-MMS analysis, reconcentrated, and further characterized using other techniques. This work validates MMS as a non-destructive and essentially consumption-free characterization tool purpose-built to provide insight into the higher order structural composition of biomolecules.

This study focusses on lysozyme as a test case to demonstrate robust protein analysis using MMS, successful re-collection post-analysis with some dilution, and intact structural integrity. Additionally, this study highlights the ability to reconcentrate post-MMS collected samples with a recovery rate of approximately 90% and similarity of >98% relative to the initial protein preparation.

Methods

Hen egg white lysozyme (Sigma #L6876) was prepared at 5 mg/mL in HPLC water and filtered through a 0.22 um filter. Six replicates were run for the initial sample preparation (designated as **5 mg/mL**) on the AQS³pro at 5 psi backing pressure and a modulation rate of 1 Hz, and the post-analysis sample stream was retrieved using the fraction collector port on the back of the system. The collected sample was then separated into two aliquots: aliquot 1 was run through the MMS system a second time without further treatment (designated as **collected**), and aliquot 2 was concentrated using 0.5 mL 10 kDa Pierce™ concentrators (designated as **concentrated**) prior to MMS re-analysis. All samples were analyzed on the AQS³pro using the AQS³delta control and processed using Data Analysis software.

Results

I. Raw Differential Absorbance and Quantitation:

The Raw Differential Absorbance plot (Figure 1A) shows the differences in absorbance observed between the three samples: 5 mg/mL, collected, and concentrated.

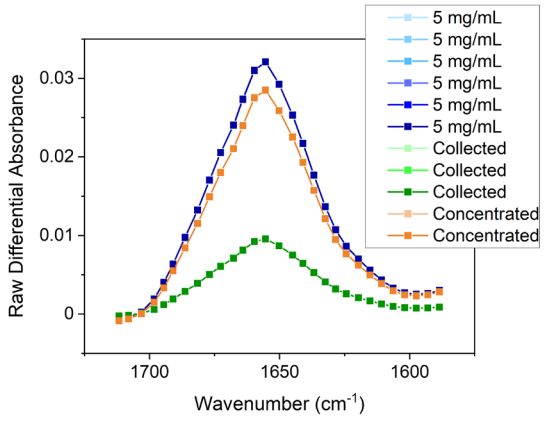

Figure 1A) Raw Differential Absorbance spectra of lysozyme at initial 5 mg/mL, collected, and concentrated.

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Results, continued

The collected sample is approximately three times more dilute than the initial 5 mg/mL sample since the AQS³pro modulates the flow of the sample with reference buffer into the flow cell following some dilution once retrieved from the fraction collector port. MMS is also a quantitative technique so the concentration was calculated for each sample with the resulting dilution curve shown for the three samples in Figure 1B below. As calculated by the quantitation algorithm, the initial prepared lysozyme sample of 5 mg/mL was diluted to 1.5 mg/mL as the collected sample, and the concentrated sample had a final concentration of 4.5 mg/mL, indicating a 90% recovery from the filter concentrators.

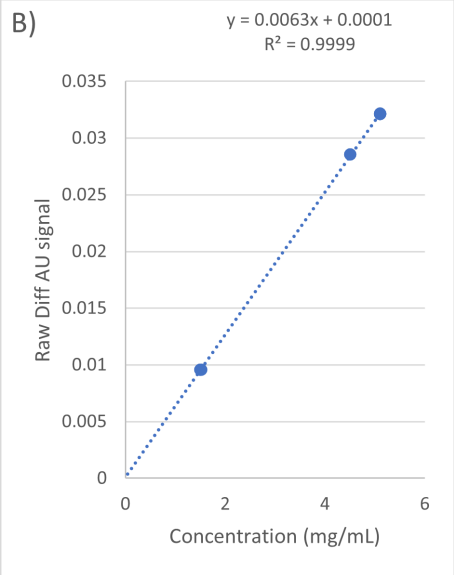

Figure 1B) Concentration curve based on the Differential Absorbance for the original 5 mg/mL sample (highest point), the collected sample (lowest point), and the concentrated sample (middle point).

II. Absolute Absorbance and Second Derivative: Figure 2A below, left shows the Absolute Absorbance plot in which the spectra have been normalized for buffer contribution and concentration. This plot demonstrates good overlap between the three processed samples. By calculating and comparing a second derivative for each, the features that make up the spectrum are highlighted in greater detail, as shown in Figure 2B below, right. These data plots confirm that the collected sample and the and concentrated sample have very consistent secondary structures to the original 5 mg/mL sample, and that the initial MMS analysis does not alter the structure of the lysozyme.

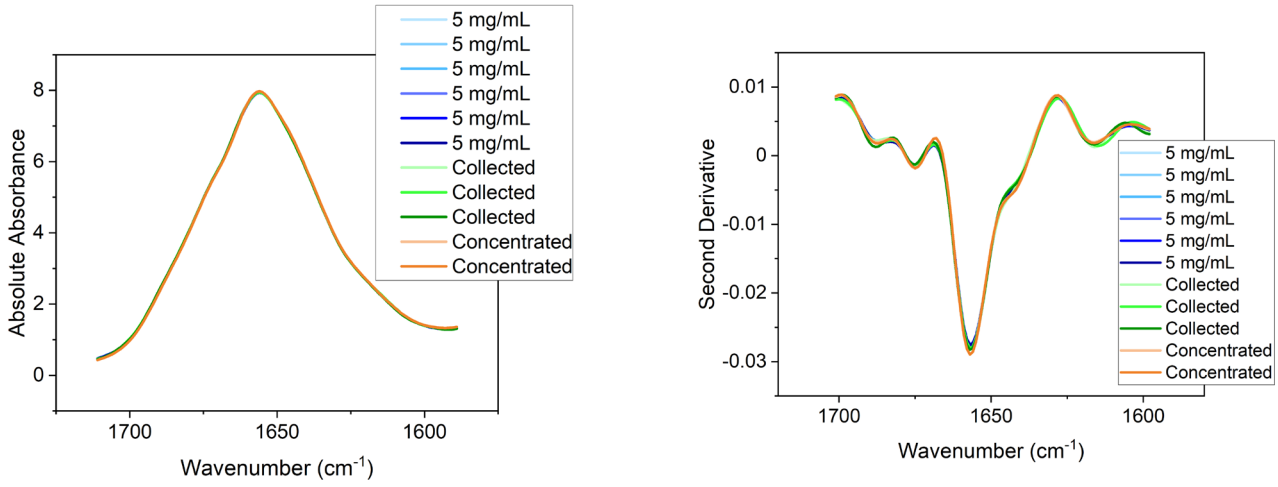

Figure 2A) The Absolute Absorbance plot has been normalized for buffer contribution and concentration. B) The Second Derivative plot shows the features making up the spectrum.

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Results, continued

III. Similarity by Area of Overlap: One method for quantifying the similarity among samples is to compare them using the Area of Overlap. This is calculated using the averaged (ave), inverted, and baseline-corrected second derivative plots (Figure 3). Table 1 below shows the results for replicate-to-replicate similarity and the similarity when compared to the orginal 5 mg/mL lysozyme sample as the control. All of the similarity values are above 98%, showing excellent repeatability and no detectable changes in the structure.

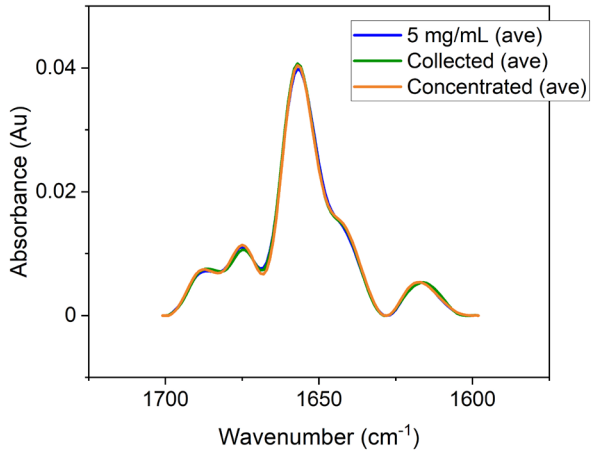

Figure 3) Similarity plot derived from the inverted and baseline subtracted second derivative.

Sample	Replicate-to-replicate % Similarity	% Similarity vs 5 mg/mL *
5 mg/mL initial*	99.62 +/- 0.09	100
Collected	98.81 +/- 0.05	98.54
Concentrated	99.37	98.48

Table 1) Percentage of Repeatability and Similarity (Area of Overlap).

IV. HIgher Order Structure (HOS): Finally, the Higher Order Structure (HOS) was quantitated by Gaussian curve fitting of the Similarity plot. Figure 4 below shows the percent contribution of each secondary structural element for the three samples. There are no significant changes in secondary structure after re-collection and re-concentration, demonstrating that analyzing samples by MMS does not alter the secondary structure, and samples can be collected and re-used.

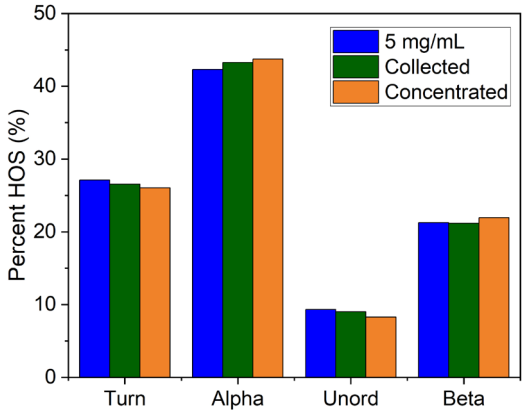

Figure 4) HOS results for the initial 5 mg/mL, recollected, and reconcentrated samples.

Conclusions

MMS is a highly-reproducible, highly-sensitive, and non-destructive secondary structure characterization technique that allows for re-collection of samples post-MMS analysis for use in further characterization. In this application note, the structure of lysozyme was demonstrated to be unaffected by MMS analysis, re-collection and re-concentration, enabling precious samples to be conserved and reused.

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