

Higher-Order Structure (HOS) Characterization of USP Monoclonal Antibody Reference Standards



INTRODUCTION

Therapeutic monoclonal antibodies (mAbs) have become one of the fastest growing classes of drugs for the treatment of wide range of indications. The mAbs market is projected to expand from \$168 billion in 2021 to \$494 billion by the end of 2030.[1]

Manufacturing processes or degradation upon storage can alter the physicochemical properties of these proteins, leading to changes in higher-order structure (HOS) that can result in enhanced immunogenicity, increased aggregation, and loss of biological function. HOS is a critical quality attribute (CQA) that can impact the safety and efficacy of biopharmaceutical products. Monitoring HOS is, therefore, essential to ensure product quality and stability. Determining conformational changes of therapeutic antibodies throughout the development and manufacturing process enables an in-depth understanding of the impact of process conditions on protein quality and may lead to further improvement of product and process performance.[2]

Changes in HOS are especially important in comparability studies. The most common methods used to characterize protein structure are Circular Dichroism (CD) and Infrared (IR) Spectroscopy.

In this study, the structural differences of the [USP mAbs](#) were characterized on all three platforms in a multi-laboratory study. The resulting analysis and comparison across the platforms show that the two CD and the IR analyses can distinguish structural differences between the three mAbs and these mAbs can be utilized to demonstrate repeatability and reproducibility of the methods.

MATERIALS AND METHODS

The CD and MMS spectra of the USP mAbs were collected, employing the following instruments and associated parameters.

Jasco J-815 Spectropolarimeter

- ▶ *Far-UV spectra:* path length 0.1 mm, protein concentration 2 mg/mL (diluted in formulation buffer or dialyzed in phosphate buffer), wavelength range 190–250 nm, D.I.T 8 sec., scan speed 50 nm/min, eight accumulations, wavelength interval 0.2 nm.

Applied Photophysics Chirascan Q100

- ▶ *Far-UV spectra:* path length 0.1 mm, protein concentration 1 mg/mL (diluted in formulation buffer or buffer exchanged in phosphate buffer), wavelength range 190–260 nm, Time-per-point 2 sec., 3 repeats, step-size of 1 nm.

RedShift BioAnalytics AQS³pro

- ▶ Protein dialyzed overnight in its respective formulation buffer to 10 mg/mL (first set), modulation rate of 1 Hz and a back pressure of 5 Psi, triplicate measurements.
- ▶ Protein diluted to 2 mg/mL in its respective formulation buffer (second set), modulation rate of 1 Hz and a back pressure of 5 Psi, triplicate measurements.

RESULTS AND DISCUSSION

CD is the most often used to assess secondary and tertiary structure, and to monitor conformational changes caused by external factors such as pH, temperature, mutations, or binding interactions.

The spectrum signal and its variations are governed by the Beer-Lambert law, which states that a linear relationship exists between the absorption measurements of light at specific wavelengths (the optical density or OD) of the sample and the extinction coefficient of that sample. Protein concentration and cuvette pathlength are factors that govern the signal strength especially at lower wavelength regions where the energy of the incident light is very high. In addition, the signal is sensitive to the protein structural changes and more importantly the formulation buffer. The buffer should ideally neither contain CD-active compounds (e.g., histidine), nor contribute to the total absorbance (e.g., salt at high concentration).

Two sets of the three USP mAbs were prepared. The first set was diluted in formulation buffer and the second set was dialyzed in phosphate buffer overnight at 2–8°C. Far-UV CD spectra were then collected using 0.1mm cells on the J-815 Jasco system. For each USP mAb, six replicates were collected from a single preparation. Each spectrum was then subtracted from the corresponding blank and converted into mean residue ellipticity (MRE) (calculation not shown here). Conversion to MRE facilitates comparison of protein molecules having different molecular weights. The secondary structure estimation (SSE) comparison was made using MRE values. The resulting spectra of all six replicate measurements were plotted as a function of the wavelength and were overlaid as shown in Figure 1 and Figure 2. Monoclonal antibodies have a typical β -sheet structure with corresponding CD spectra characterized by a negative band centered at ~218 nm. The overlaid far-UV spectra of the six replicates of each USP mAb that inform about secondary structure appear to have similar profiles regardless of the buffer used.



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Figure 1. Far-UV CD spectra obtained on a J-815 system of A) USP mAb001 B) USP mAb002 C) USPmAb003 in formulation buffer (FB) measured using 0.1mm cell

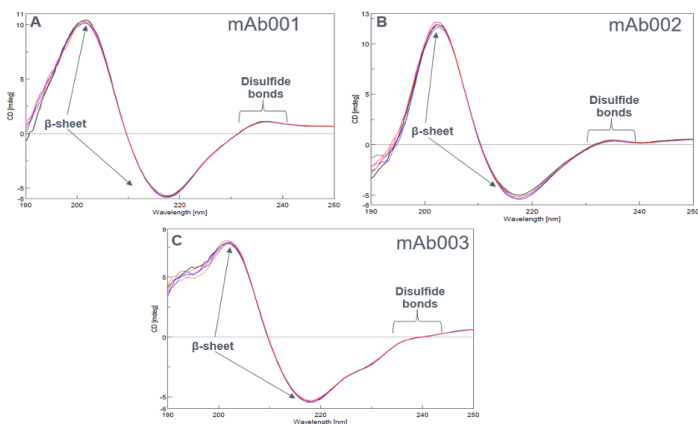
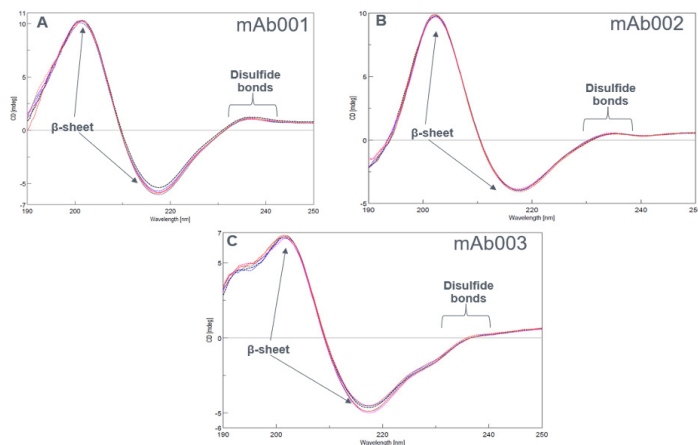


Figure 2. Far-UV CD spectra obtained on a J-815 system of A) USP mAb001 B) USP mAb002 C) USPmAb003 in phosphate buffer (PB) measured using 0.1mm cell



The Chirascan instrument is also a CD spectrometer used to determine the secondary, and tertiary structure of proteins by measuring the CD signal arising from the peptide bonds and aromatic sidechains, respectively, and disulfide bonds. Far-UV CD spectra of USP mAbs (mAb001, mAb002 and mAb003) that were buffer exchanged with phosphate buffer and diluted in their respective formulation buffers were collected using a 0.1mm flow cell on the Chirascan Q100 system. The baseline was corrected and then normalized by absorbance before comparison of the spectra. For each USP mAb, three replicates were collected from a single preparation and the resulting spectra were overlaid as shown in Figure 3 and 4. The overlaid far-UV spectra of the three replicates of each USP mAb that inform about the secondary structure appear to have similar profiles regardless of the buffer used, similar to the Jasco system.

Figure 3. Far-UV CD spectra obtained on Chirascan Q100 of A) USP mAb001 B) USP mAb002 C) USPmAb003 in formulation buffer (FB) measured using a 0.1mm cell

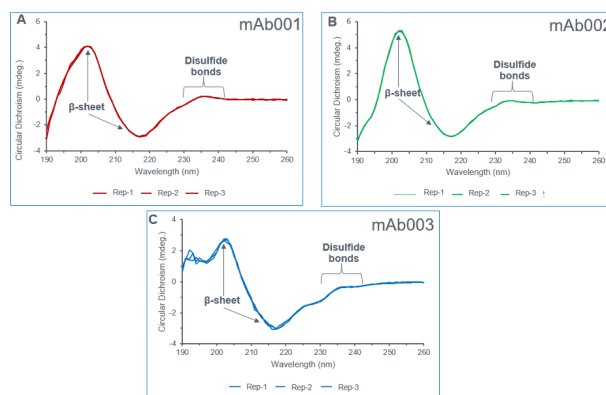
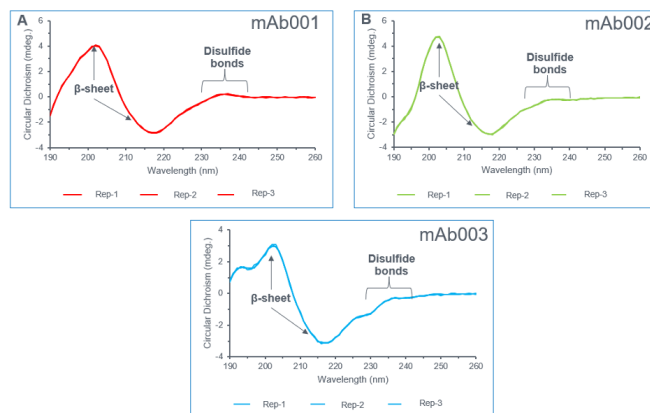


Figure 4. Far-UV CD spectra obtained on a Chirascan Q100 of A) USP mAb001 B) USP mAb002 C) USPmAb003 in phosphate buffer (PB) measured using 0.1mm cell



The buffer in the sample can have an enormous spectral contribution to the far-UV region of the CD spectrum and should not have high absorbance in the 190-250 nm region. Formulated protein samples containing high concentrations of Dithiothreitol (DTT), histidine or imidazole prevents collecting spectra below 200 nm. The actual percentage of different structures due to these excipients can lead to compromised results with questionable accuracy. This was seen on both CD platforms (J-815 and Chirascan Q100) with USP mAb003 formulated in histidine resulting in an increased absorbance which, in-turn, lowering the signal-to-noise ratio in the CD spectra particularly between 190 and 200 nm wavelengths (Figure 1C and 3C) as compared to phosphate buffer (Figure 2C and 4C).

An ideal buffer should have a low absorbance in the wavelength region being probed. In this case, the sample spectra were collected in the wavelength range of 190-250 nm and 190-260 nm using the J-815 system and the Chirascan Q100, respectively. When USP mAb003 was buffer exchanged or diluted in phosphate buffer, the absorbance was more suitable and, in-turn, CD spectra with an improved signal-to-noise ratio were observed (Figure 2C and 4C).

CD is a sensitive and nondestructive biophysical technique to study conformational changes of proteins in the solution-state. The asymmetric absorption of circularly polarized light by the peptide backbone in the far-UV region provides a way to assess total secondary structure of a protein. The unique sensitivity to changes in protein structure can be seen in an overlay spectra of the three USP mAbs in formulation buffer and phosphate buffer analyzed using the J-815 and Chirascan Q100 systems as shown in Figure 5. The instruments and methods are sensitive enough to capture the structural changes of each of the USP mAbs that occur due to differences in buffer composition (refer Fig. 6 & 7). In addition, the resulting data from the two CD instruments are also comparable for both set of samples.

Figure 5. Overlay of all 3 mAb's far-UV CD spectra obtained on a J-815 system for A) formulation buffer (FB) B) phosphate buffer (PB) and Chirascan Q100 for C) formulation buffer D) phosphate buffer (phos)

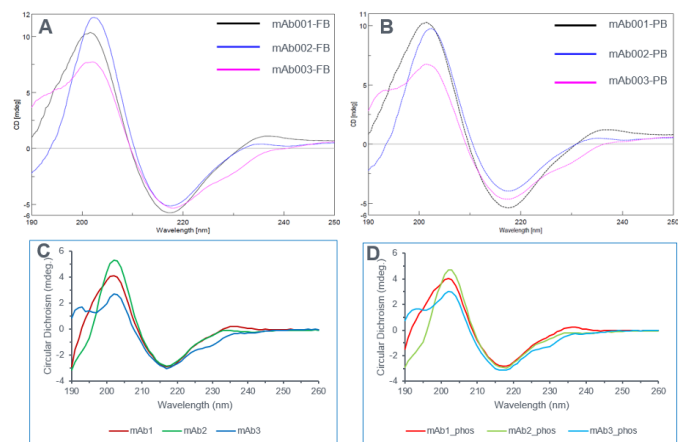


Figure 6. Far-UV CD spectra obtained on a J-815 system of A) USP mAb001 B) USP mAb002 C) USP mAb003 in phosphate buffer (PB) and Formulation buffer (FB) measured using a 0.1mm cells, six replicates in each buffer

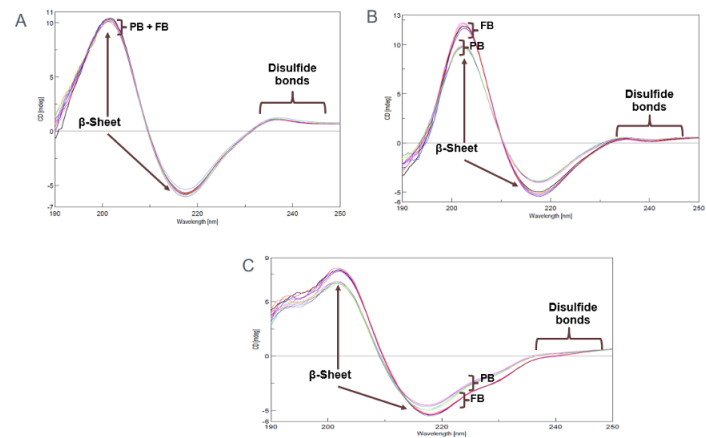
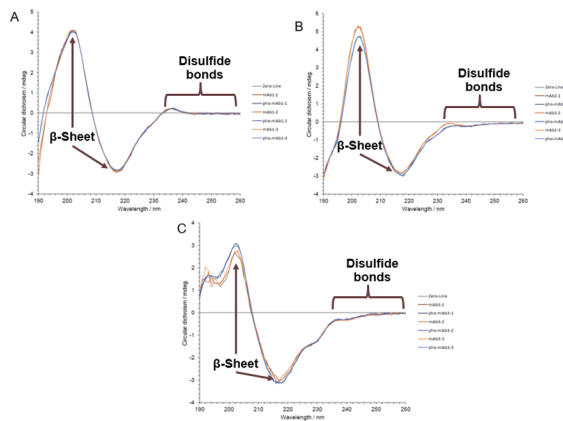


Figure 7. Far-UV CD spectra obtained on a Chirascan Q100 system of A) USP mAb001 B) USP mAb002 C) USP mAb003 in phosphate buffer (PB) and Formulation buffer (FB) measured using a 0.1mm cell, three replicates in each buffer



For far-UV measurements, all the CD spectra were compared one-on-one to have a complete population spectral variation and were evaluated statistically using weighted spectral difference (WSD) and the resulting values were plotted within ± 2 standard deviation (SD) of the reference.

WSD is an enhanced version of spectral difference, with an additional factor to magnify differences in spectral regions with higher signal intensity (both negative and positive signals).[3] In the formula (Figure 8) n is the number of data points in a spectral range, Y_A are the signals of the reference, $|Y_A|_{ave}$ is the average of the absolute signals of the reference, and Y_B are the signals of the sample.[3] To obtain the WSD, first the sample spectrum is subtracted by the reference spectrum, then the resulting difference is squared so that the negative values cannot cancel out the positive values. By taking each of the measurement as a reference spectrum, WSD was calculated with the remaining spectra in an iterative cycle. In this process the entire sample variation distribution was captured (15 data points for 6 replicates from J-815 system). The resulting WSD values were plotted, and their limits of variations were captured within $\pm 2SD$. A weighting based on the reference is then applied followed by normalizing the absolute value and then obtaining the weighted difference spectrum. Finally, the WSD is obtained by calculating the square root of the average intensity of the weighted difference spectrum. The mean of these WSD values and a quality range which is defined by twice the reference SD ($2xSD$ is somewhat arbitrary and does not penalize a small number of replicates) is plotted for each of the mAbs. The spectral results of the formulation buffer and phosphate buffer samples from each of the three USP mAbs were converted into numerical values using WSD, as a measure for similarity and plotted as shown in Figure 9 and 10.

Figure 8. Weighted spectral difference (WSD) formula

$$WSD = \sqrt{\sum_{i=1}^n \left[\left(\frac{1}{n} \right) \left(\frac{|Y_{Ai}|}{|Y_A|_{ave}} \right) (Y_{Ai} - Y_{Bi})^2 \right]}$$

WSD was employed to provide a more precise comparison of the spectral similarities and dissimilarities between the two buffer conditions. Majority of the data points regardless of the buffer conditions, are within the limits of variation of $\pm 2SD$ and data points within each buffer condition show similarity (Figure 9 and 10).

Figure 9. Statistical evaluation of USP mAbs far-UV measurements on J-815 system for A) USP mAb001 B) USP mAb002 C) USPmAb003 in phosphate buffer (Phos.B) and Formulation buffer (FB), one preparation, 6 replicates measured using a 0.1mm cell

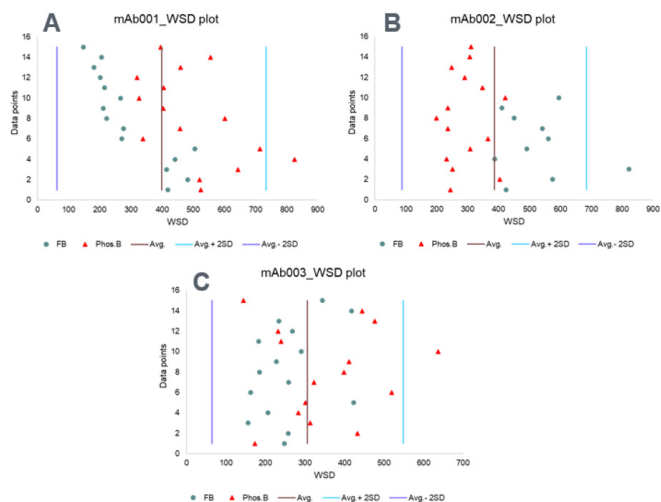
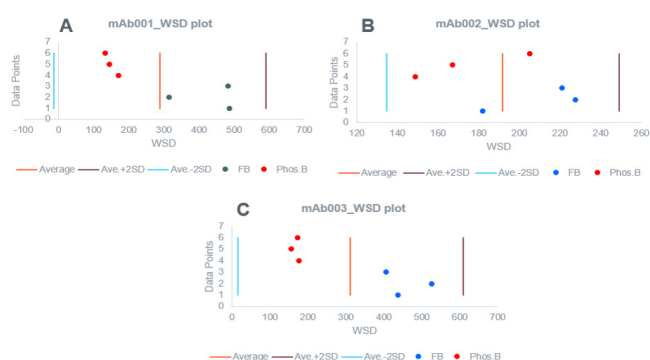
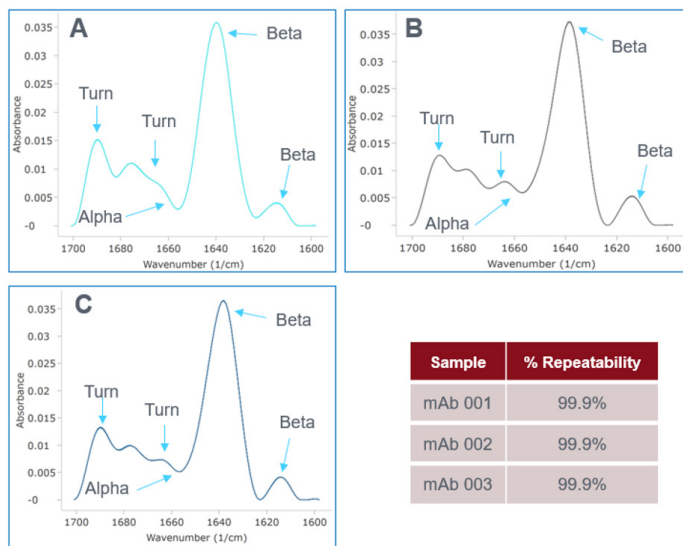


Figure 10. Statistical evaluation of USP mAbs far-UV measurements on Chirascan Q100 for A) USP mAb001 B) USP mAb002 C) USPmAb003 in phosphate buffer (Phos.B) and Formulation buffer (FB), one preparation, 3 replicates measured using a 0.1mm cell



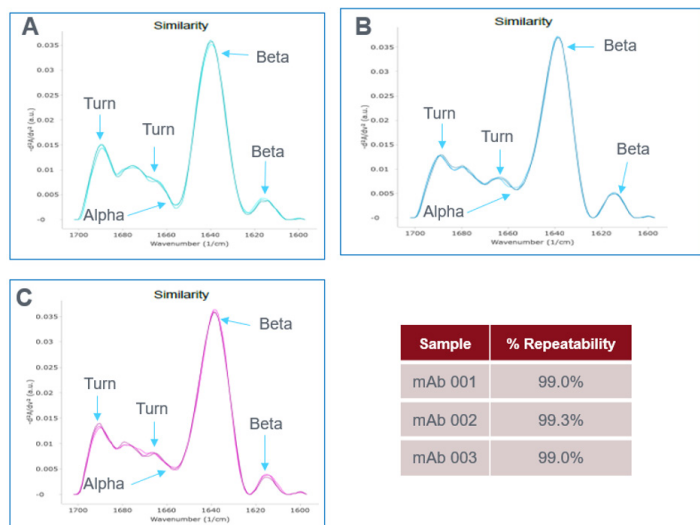
Microfluidic Modulation Spectroscopy (MMS) is an infrared spectroscopy tool for determination of protein secondary structure, which is an alternative to conventional mid-IR and far-UV CD techniques. USP mAbs (mAb001, mAb002, and mAb003) were dialyzed in their respective formulation buffers to two different concentrations: 10 mg/mL and 2 mg/mL. Differential absorbance spectra were collected automatically for the triplicate measurements from a multi-well plate at a modulation rate of 1 Hz and a back pressure of 5 Psi and analyzed using the AQS³delta Analytics software. The output for MMS is in absorbance of IR light in the amide I band. To calculate the percent similarity, the second derivative spectra for these samples were first calculated, then inverted and baseline subtracted. The area of overlap was then calculated for the resulting plot to quantitate percent similarity. As shown in Figure 11 and 12, all three mAb structures regardless of the concentration of the samples (10 mg/mL or 2mg/mL) are primarily composed of β -sheet resulting in a major peak at 1640 cm^{-1} . Consistent spectra's were obtained with overall repeatability greater than or equal to 99% for all the three mAbs

Figure 11. Overlay of mAbs 001 (A), 002 (B) and 003 (C) on AQS³pro MMS system at 10 mg/mL.



Sample	% Repeatability
mAb 001	99.9%
mAb 002	99.9%
mAb 003	99.9%

Figure 12. Overlay of mAbs 001 (A), 002 (B) and 003 (C) on AQS³pro MMS system at 2 mg/mL



As shown in Figure 13, all 3 mAb structures are primarily composed of β -sheet structure, ~60% in β -sheets and ~30% of turns in the HOS calculations. In addition, all 3 mAb samples at 2 mg/mL were structurally similar to 10 mg/mL samples and no major structural differences were observed when comparing neat samples vs. dialyzed samples as shown in Figure 14. The study also shows that the MMS technology demonstrates high repeatability and high sensitivity at low and high concentrations.

Figure 13. Overlay of mAbs 001, 002 and 003 on AQS³pro MMS system at 2 and 10 mg/mL

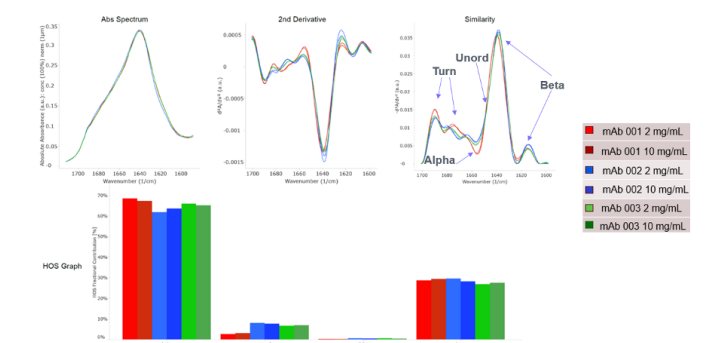
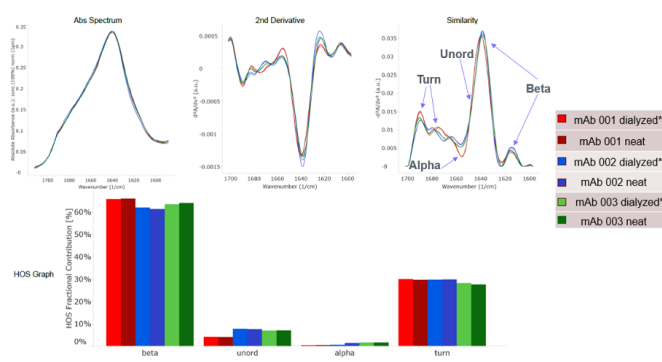
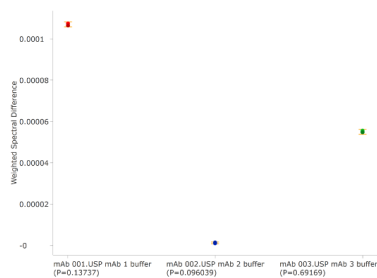


Figure 14. Overlay of mAbs 001, 002 and 003 on AQS³pro MMS system at 2 mg/mL neat vs. dialyzed



In addition, minor differences between the three mAbs makes it clear that they have distinguishable unique structures. The WSD values were calculated by inserting the values from the second derivative plot into the same formula described above for comparisons of all three mAbs. As shown in Figure 15, when using mAb 002 as reference, resulting in a WSD that is close to zero, mAb 001 and mAb 003 are statistically differentiated. The points on the plot also demonstrate error bars that are 2X the SD.

Figure 15. WSD plot using mAb 002 as control



CONCLUSION

The USP mAb Reference Standards provide users with options to select the best fit-for-purpose RS for their specific analytical needs. The changes in physicochemical properties of these proteins due to manufacturing processes or degradation upon storage can lead to changes in HOS. CD is an established characterization technique that can be used successfully to detect conformational changes in monoclonal antibodies. MMS is a newer infrared spectroscopy technology which can also be used for protein structural analysis. In this study, we have demonstrated that these technologies can be applied with high reproducibility and accuracy to monitor protein's higher-ordered structural quality attributes, specifically to quantify changes in secondary structures. Having well characterized RS can help manufacturers to optimize and demonstrate consistent instrument performance. Monitoring antibody structure changes is essential to safety and efficacy of the final drug product and ultimately, final product quality, and both CD and MMS may be valuable tools for this purpose.

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