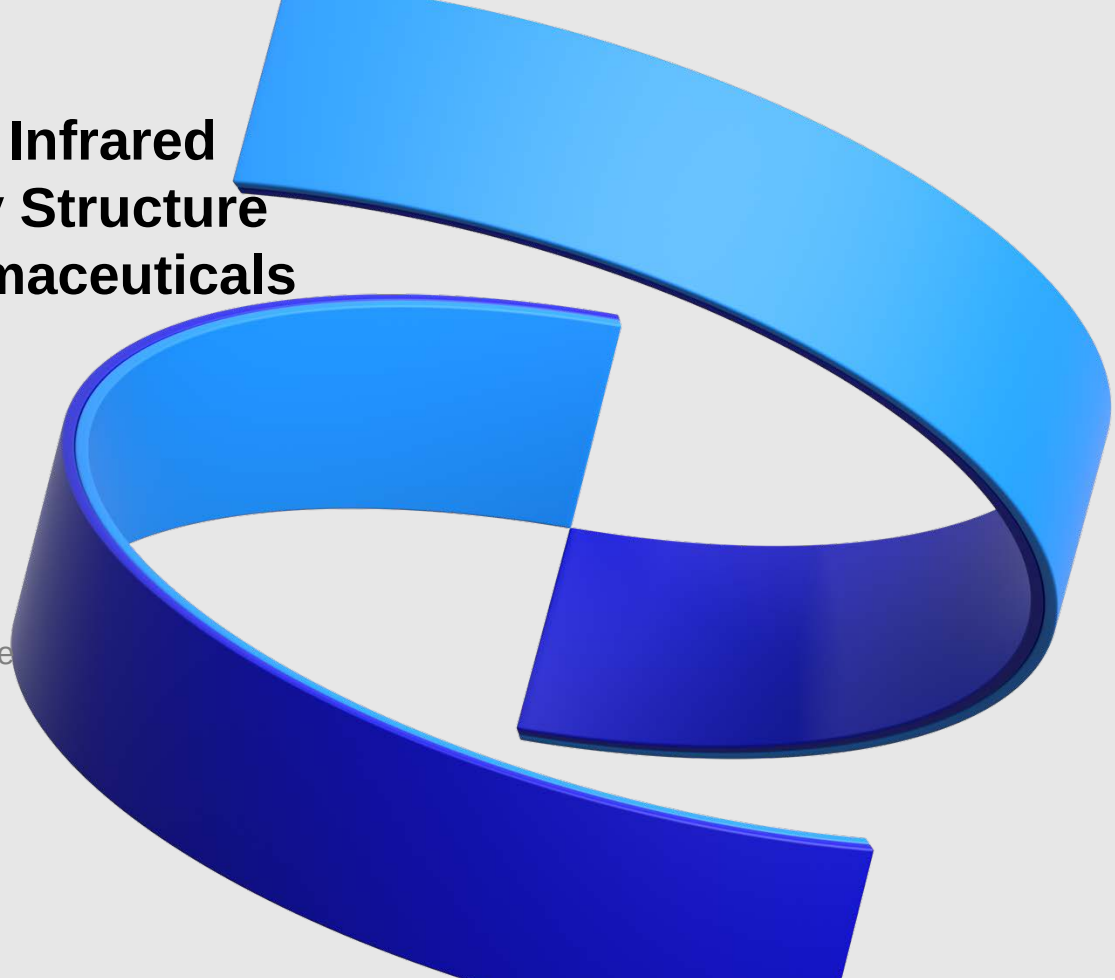
Automated High-throughput Infrared Spectroscopy for Secondary Structure Analysis of Protein Biopharmaceuticals



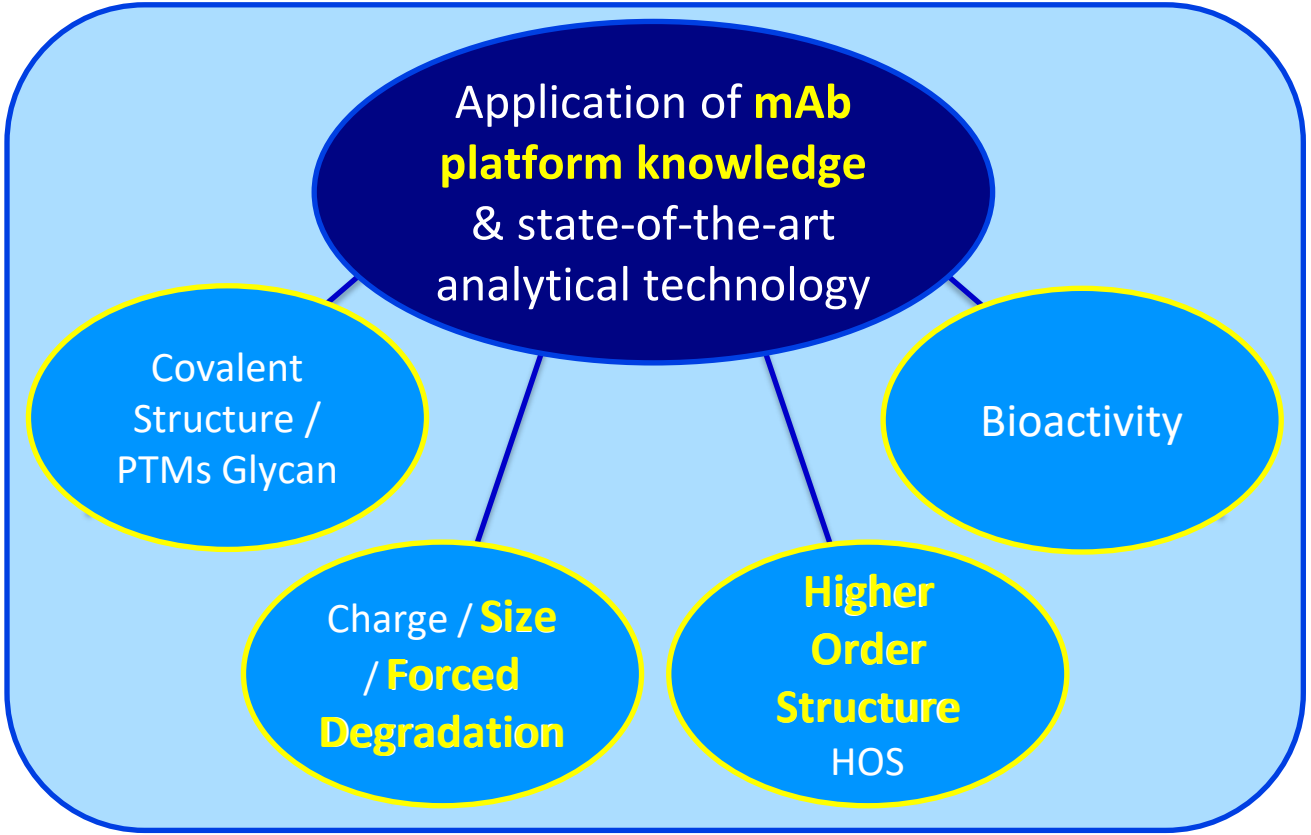
Lucy L. Liu

9th International Symposium on Higher Order Structure of Protein Therapeutics

Pfizer, BioTherapeutics Pharmaceutical Sciences

April 15th, 2021

Scope of Biophysical Characterization



State-of-the-Art Biophysical Techniques

Size

Higher Order Structure

Thermal Stability

Forced Degradation

Soluble Aggregates

Secondary Structure

Tertiary Structure

CD: Circular Dichroism **MM-IR**: Microfluidic Modulation Infrared (IR) Spectroscopy

Correct HOS is Important to Biological Function

Strategic alignment with CMC regulatory expectations

- Implementation of orthogonal, complementary, redundant biophysical techniques

Biophysical Testing List – formulated based on knowledge drawn from

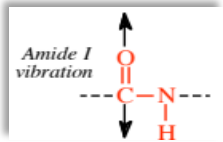
- FDA and EMA – Recommendations

  - AUC, SEC-MALS as additional, orthogonal sizing methods: assess soluble aggregates
  - For near-UV CD, far-UV CD, FTIR, DSC, provide details on method sensitivity
  - For HOS, additional state-of-the-art methods can be considered: FTIR, DSC, NMR
  - Describe / justify statistical methods used for comparability exercise

- Industry – Late-stage project related publications
- Our own – Past comparability work experience, subject matter expertise

Regulators' emphasis on the importance the 3-D protein structures and biological functions

Automated Amide-I IR System: MM-IR



Microfluidic **M**odulation Infrared (IR) **S**pectroscopy

MMS AQS^{3}

Specifically determine protein amide I band 1720-1580 cm^{-1}

- Routinely test with 1.4 mL sample for triplicate at 1 mg/mL protein concentration by 96-well-plate

Pre-optimized configuration with minimal tuning

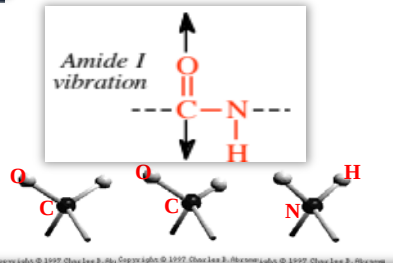
- Small Footprint – integrates with MM-IR
- Easy and intuitive to use

End-to-End **AQS**^{3}™ software: data acquisition, analysis, and reporting

MM-IR vs. FTIR

Manual FTIR
Whole Mid-IR 4000-400 cm^{-1} Region

Automated MM-IR (microfluidic modulation)
Spectral coverage 1720-1580 cm^{-1} Amide I Band only

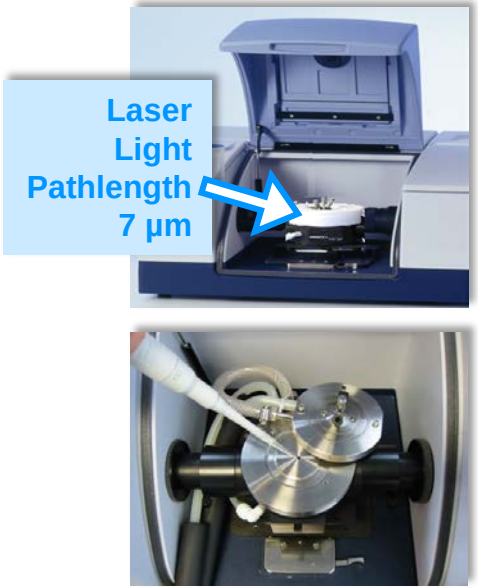


Both techniques cover *Amide-I*

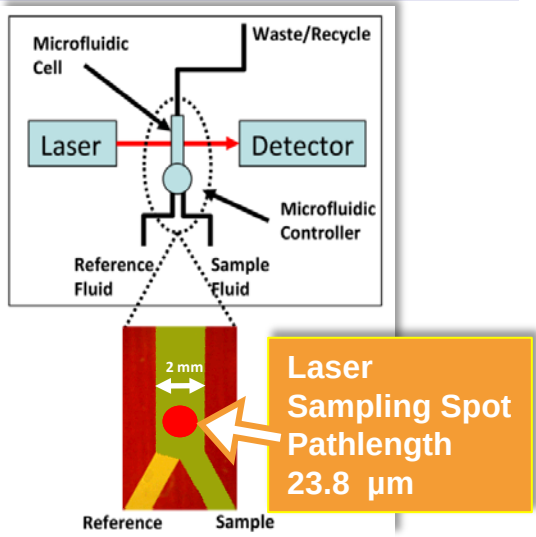
Automated MM-IR is specialized just for *Amide-I*

MM-IR vs. FTIR

Manual FTIR

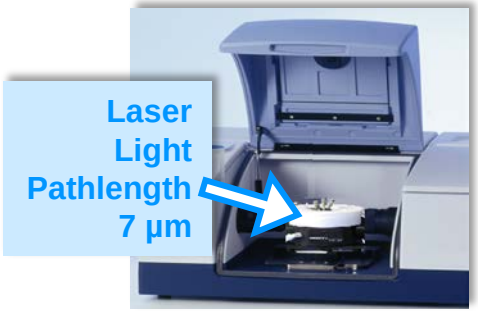


Automated MM-IR (microfluidic modulation)



MM-IR vs. FTIR

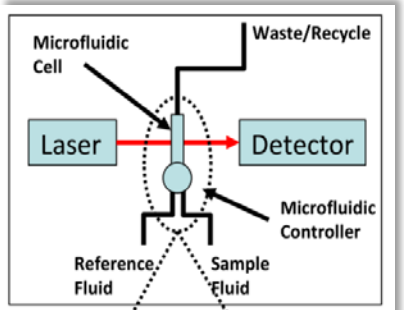
Manual FTIR

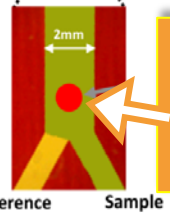


Automated MM-IR (microfluidic modulation)

Tunable
IR
Quantum
Cascade
Laser

QCL





Laser
Sampling Spot
Pathlength
23.8 µm

MM-IR made room temp detector possible by brighter laser QCL
(more user friendly, liquid N_2 cooling no longer needed)

MM-IR vs. FTIR

Manual FTIR

Baseline Drift

- FTIR measurements typically are taken on minute-intervals (depending on measurement time per test)

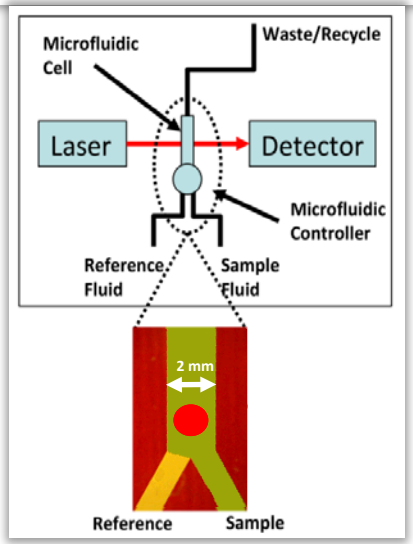
- Slight shift on peak positions and variation of peak intensities, from a last minute-interval test on reference to the next minute-interval test on sample, are commonly observed, causing S/N ratio of the processed sample spectrum to deteriorate, after performing the reference spectrum subtraction

Automated MM-IR (microfluidic modulation)

Core Feature

Solved Baseline Drift

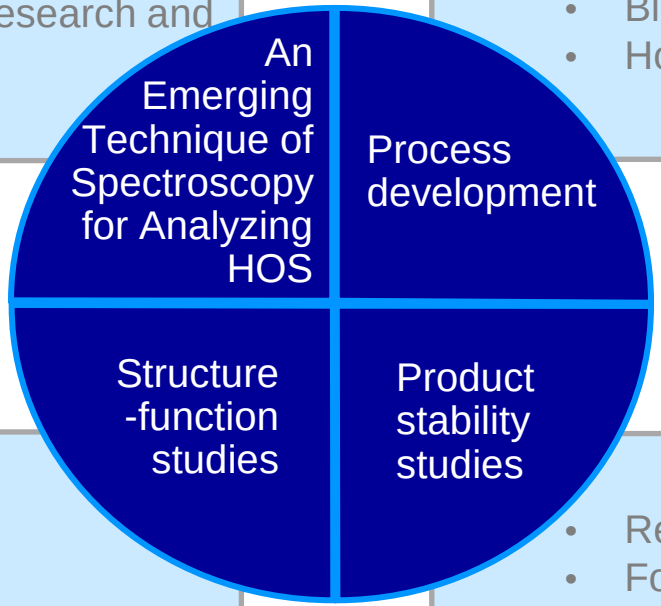
through modulated reference spectrum subtraction, which occurs every second – 1Hz (real-time)



Application of MM-IR in Biotherapeutics

- Early / late-stage research and development

- Bioreactor DOEs
- Hold time



Routine IR will increase process / product knowledge

- Define QAs
- QC isolates

- Real-time
- Forced degradation

MM-IR Applied for Characterization of Protein Secondary Structure

Instrument Design	Performance	Convenience	Case Studies
Auto-Referencing and Real-Time Buffer Subtraction The Broad Quantitation Linearity Range	The Sensitivity of MM-IR in Detecting Structural Differences The Robustness	System Suitability Test Matching Buffer Blank Normalization Prior to Area Overlapping Calc.	Comparability and Biosimilarity Assessment

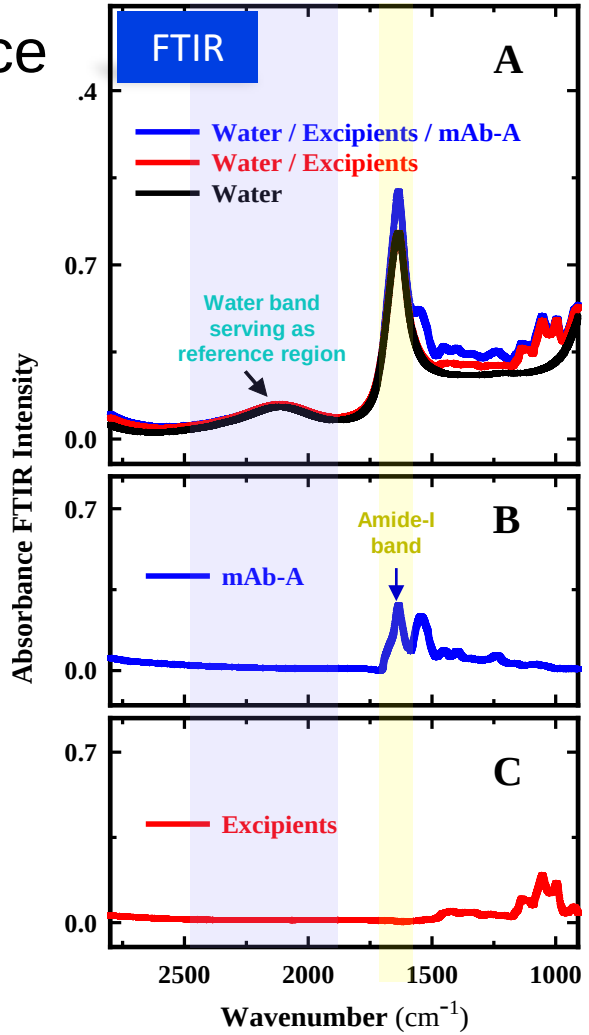
Cross-over Study FTIR vs. MM-IR in the Absence of Water Band Reference Region ~ 2100 cm^{-1}

Auto-Referencing and Real-Time Buffer Subtraction

The Broad Quantitation Linearity Range

Instrument Design

- To obtain high-quality spectra for proteins in aqueous solution, the spectra of atmospheric water and water in protein sample must be adequately subtracted from observed protein spectrum
- Without a sample prep procedure by dialysis, a matching reference buffer was hard to obtain[1]

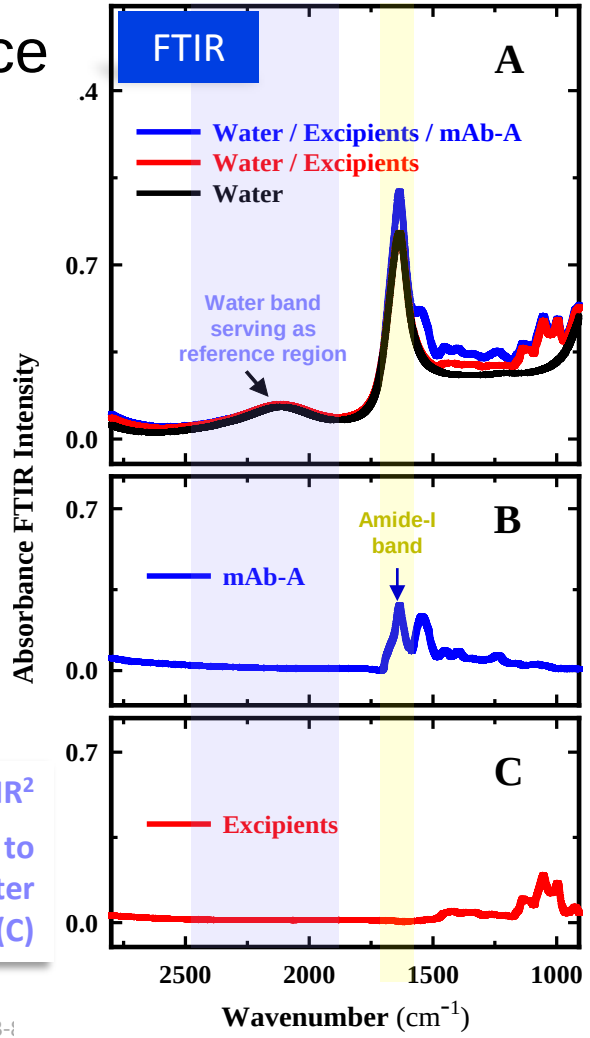


Cross-over Study FTIR vs. MM-IR in the Absence of Water Band Reference Region ~ 2100 cm^{-1}

Auto-Referencing and Real-Time Buffer Subtraction

The Broad Quantitation Linearity Range

Instrument Design



Water band subtraction by FTIR[2]

The arrow noted water band in (A), serving as the reference region to show whether a correct water subtraction is accomplished. If the water subtraction is correct, this region should be flat as shown in (B) and (C)

1.Dong A, Huang P, Caughey WS. Protein secondary structures in water from 2nd-derivative amide I IR spectra. Biochem. 1990; 29:3303-
2.Liu LL, Wang L, Zonderman J, Rouse JC, Kim HY. Automated, high-throughput IR spectroscopy for 2° structure analysis of protein biopharmaceuticals. JPharmSci, 2020; 109 (10): 3223-30.

Cross-over Study FTIR vs. MM-IR in the Absence of Water Band Reference Region ~ 2100 cm^{-1}

Auto-Referencing and Real-Time Buffer Subtraction

The Broad Quantitation Linearity Range

Instrument Design

Core Feature

Solved Baseline Drift

through modulated reference spectrum subtraction, which occurs every second – 1Hz (real-time)

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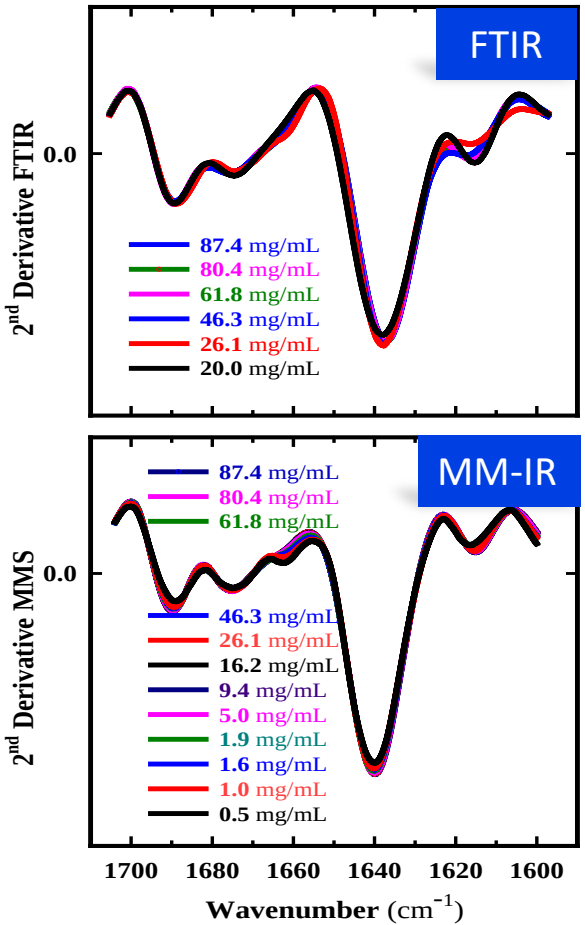
Cross-over Study FTIR vs. MM-IR in the Absence of Water Band Reference Region ~ 2100 cm^{-1}

Auto-Referencing and Real-Time Buffer Subtraction

The Broad Quantitation Linearity Range

Instrument Design

Despite different methods of water/ buffer subtraction, both FTIR and MM-IR show comparable 2nd derivative IR data.



Protein Concentration (mg/mL)	Mean Similarity (%) of Replicates
0.5	97.69
1.0	98.84
1.6	99.26
1.9	99.57
5.0	99.32
9.4	99.23
16.2[a]	99.17
26.1	99.06
46.3	99.00
61.8	98.99
80.4	98.94 (one rep)
87.4	98.92

[a] The spectral similarity scores were calculated by comparing the Area of Overlap (AO) of each replicate to that of the mean AO of the three replicates of mAb-A at 16.2 mg/mL. AO plots are not shown here.

Kendrick B. S., Gabrielson, J. P., Solsberg, C. W., Ma, E., and Wang, L., "Determining Spectroscopic quantitation limits for misfolded structures", J Pharm Sci, 2020, 109 (1), 933-6

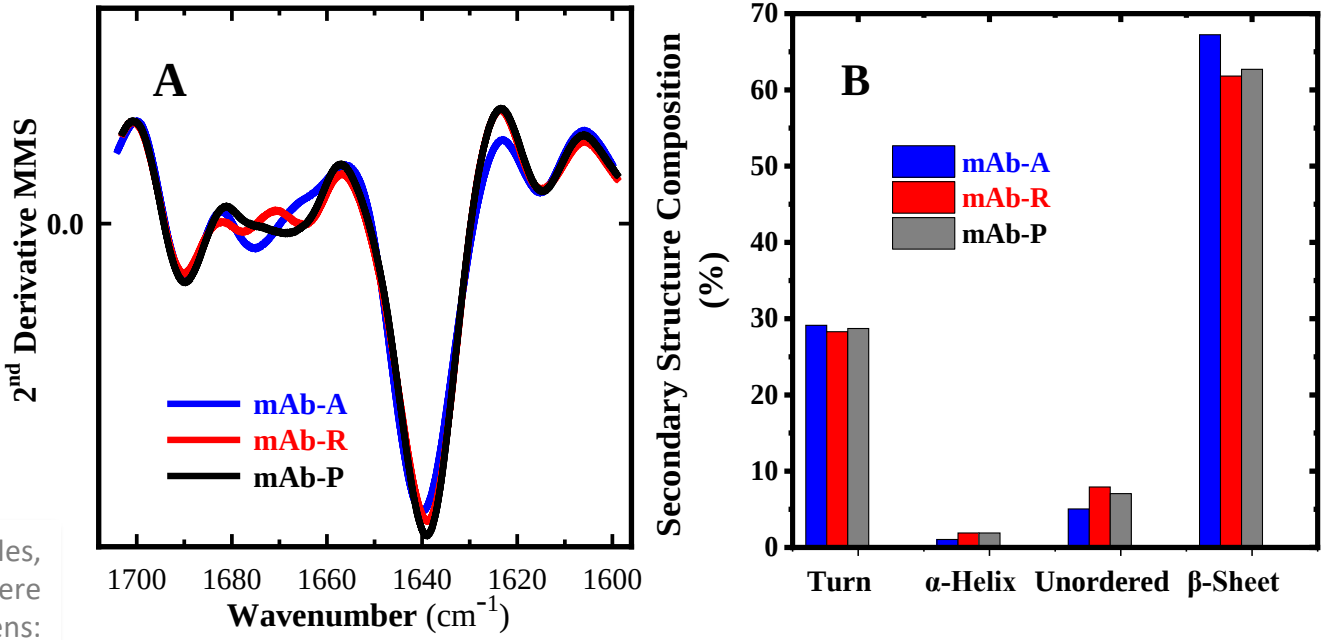
The Sensitivity of MM-IR in Detecting Structural Differences

The Sensitivity of MMS in Detecting Structural Differences

The Robustness

Performance

Subtle structural differences distinctively reflected in the region of 1680 - 1660 cm^{-1}, indicating some turn structure uniqueness of each mAb.



3 different therapeutic IgG1 molecules, mAb-A, mAb-P and mAb-R, which were designed to bind different antigens:
(A) The overlaid 2nd derivative MMS spectra
(B) the secondary structure composition (%)

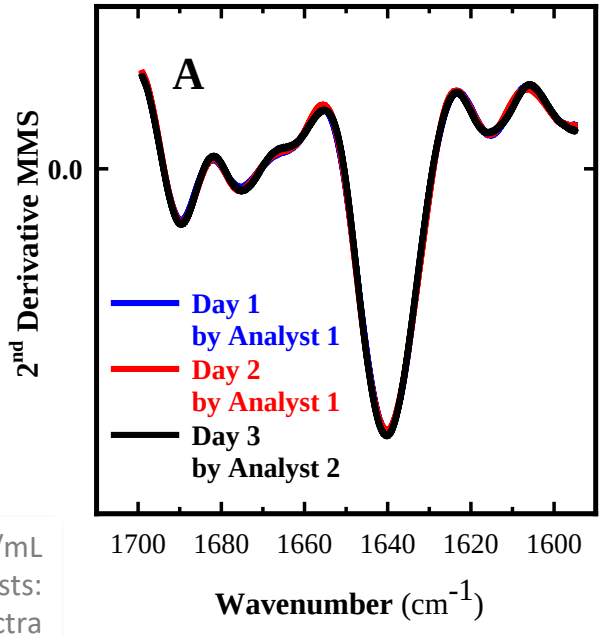
The Robustness of MM-IR

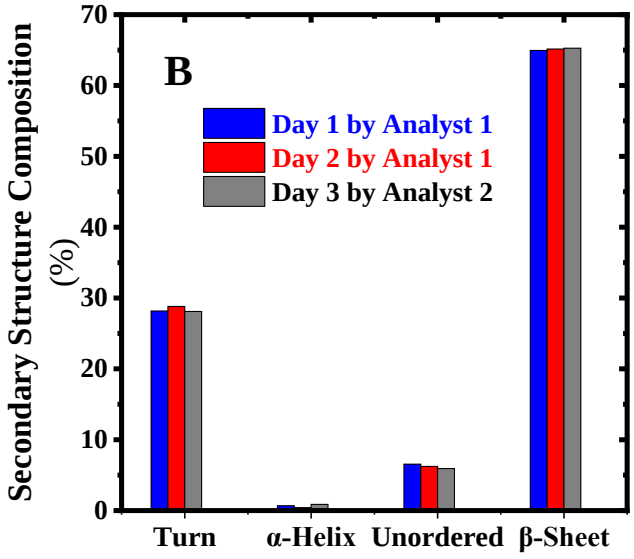
The Sensitivity of MMS in Detecting Structural Differences

The Robustness

Performance

Spectra superimposed across the entire amide I band and have similarity scores of 99%, demonstrating superior repeatability and robustness of MM-IR method.





Independent mAb-A samples at 1.0 mg/mL analyzed on different days by different analysts:
(A) Overlaid 2nd derivative MMS spectra
(B) the secondary structure composition (%)

System Suitability Test (SST)

System Suitability Test
Matching Buffer Blank

Normalization Prior to
Area Overlapping Calc.

Convenience

Purpose

Ensure instrumental/method variability was minimized for a more accurate/precise qualitative assessment of protein HOS comparability with regards to their secondary structure composition

In terms of specificity, the IR spectra demonstrate: the secondary structure of samples are mostly β-sheet and have similar spectral features to published FTIR spectra for IgG1 mAbs

IR is a sensitive method for detecting potential changes in secondary structure based on the comparison of properly folded and fully denatured protein

Establish the SST with a commercial standard protein HEWL (Lysozyme from hen egg white)

On top of a system check protocol which is part of system suitability and definitely needed

Signal-to-noise
Wavenumber accuracy
Electronics performance

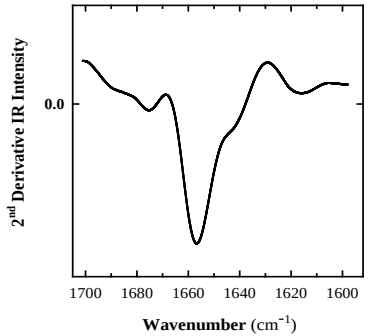
SST with HEWL ensures the system produces the same reference IR spectrum each time before analyzing the test samples as well as produces the same secondary structure composition

SST by HEWL

Standard (Sigma# **L6876**) HEWL, Lysozyme from hen egg white (90% protein and 10% acetate salt)

Recon in Water

- Water as blank
  - **Acetate salt interference not accounted for**

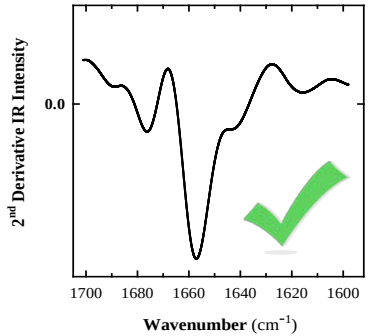


Replicates	Mean ± SD[a]			
	α-Helix	*β-Sheet*	*Turn*	*Unordered*
1, 2, 3	**42.9** ± 0.3	**20.8** ± 0.3	**26.8** ± 0.2	**9.5** ± 0.2
4, 5, 6	**42.9** ± 0.3	**21.3** ± 0.3	**27.0** ± 0.5	**8.8** ± 0.06

a. Standard deviations of triplicates

Recon in Water and Dialyze

- Water final dialysate as blank
  - **A matching buffer blank used as a reference - exactly matches the buffer composition in which the sample is prepared**

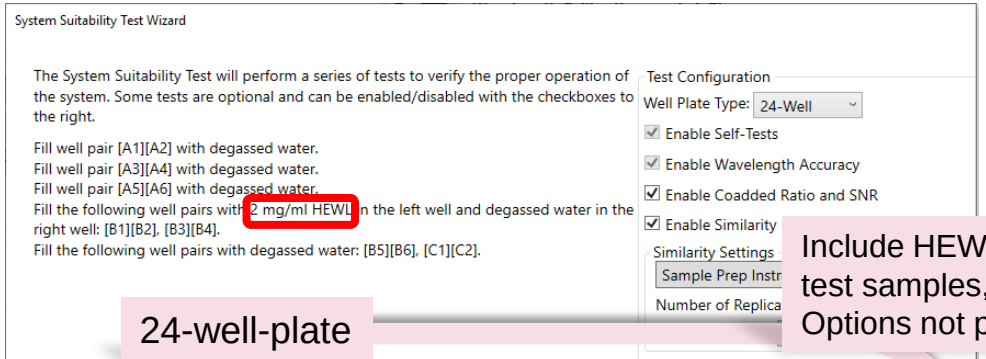


Replicates	Mean ± SD[a]			
	α-Helix	*β-Sheet*	*Turn*	*Unordered*
1, 2, 3	**48.4** ± 0.7	**21.8** ± 0.5	**24.0** ± 0.7	**5.8** ± 0.6
4, 5, 6	**48.7** ± 0.7	**21.0** ± 0.8	**24.0** ± 0.9	**6.3** ± 0.6

a. Standard deviations of triplicates

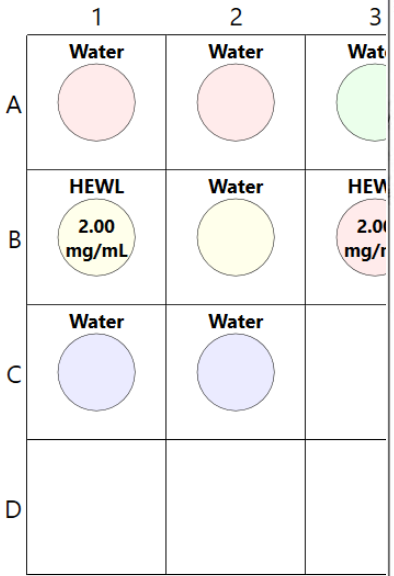
Dialysis is required for the perfect buffer matching algorithm to perform accurate background correction

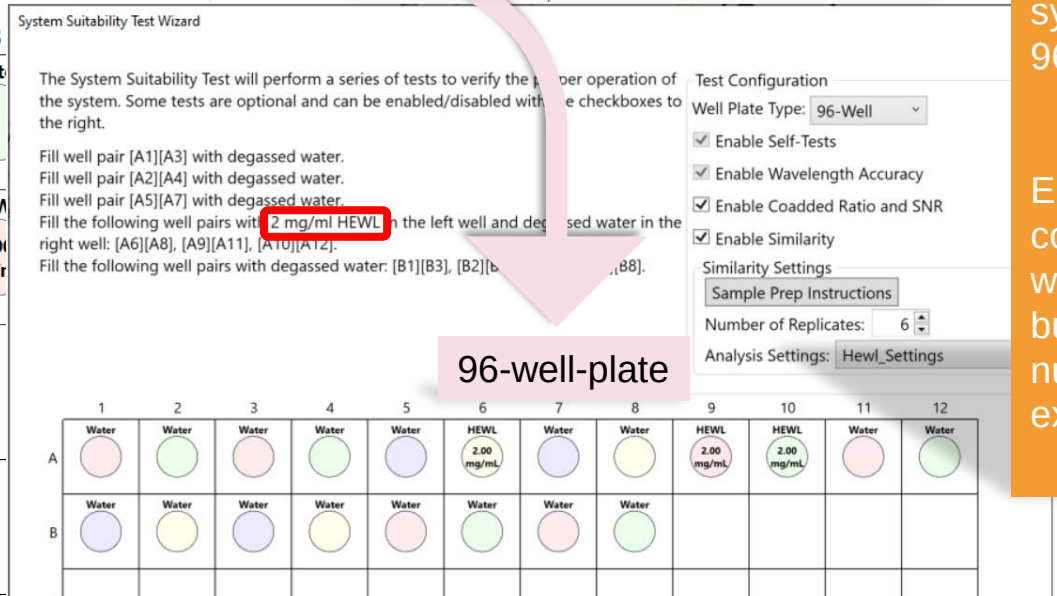
Changed to 96-Well-Plate and Updated SST Test Wizard



24-well-plate

Include HEWL on the plate with the test samples, or it is run separately
Options not possible for 24-well-plate





96-well-plate

Worked with vendor to design and upgrade system to accommodate 96-well-plate testing.

Enabled to load a large comparability sample set with control samples and buffer blanks since the number of wells required exceeded 24.

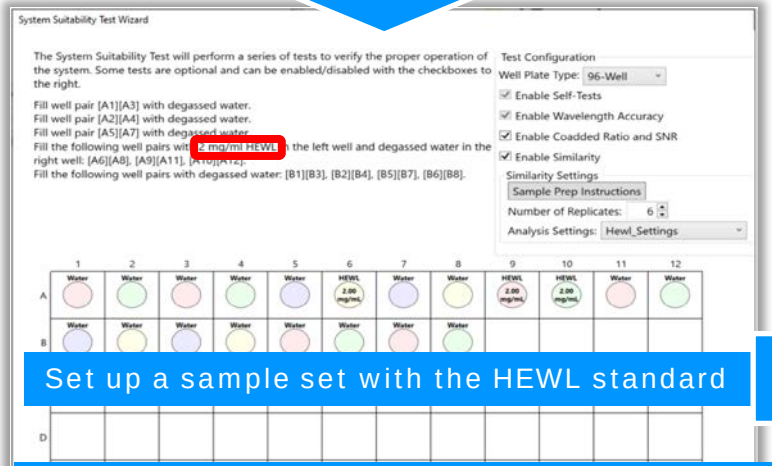
SST by HEWL (Need to be Made Fresh)

System Suitability Test
Matching Buffer Blank

Convenience

Standard (Sigma# **L6876**) HEWL (90% protein & 10% acetate salt)

Recon in Water and Dialyze



Set up a sample set with the HEWL standard

Show the reference spectrum of HEWL & the corresponding reference values for secondary structure composition, which helps colleagues know everything is working

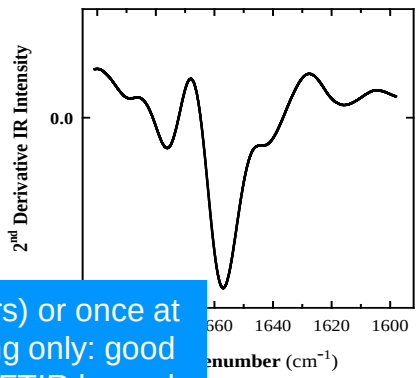


Figure. The 2nd Derivative IR Spectrum

sate as blank

Table 1. The Spectral Similarity

Replicate	Similarity (%)
1	98.5
2	99.2
3	98.6
4	99.0
5	98.2
6	99.2
Av	**99.8**
± Standard Devi	± 0.4

Precision ≥98%

Table 2. The Secondary Structure Composition (%)

Replicates	Mean ± SD[a]			
	α-Helix	*β-Sheet*	*Turn*	*Unordered*
1, 2, 3	**46.4** ± 0.9	**21.8** ± 0.5	**25.7** ± 0.8	**5.7** ± 0.5
4, 5, 6	**46.6** ± 0.3	**21.0** ± 0.8	**25.5** ± 0.4	**6.1** ± 0.7

a. Standard deviations of triplicates

Question: Run HEWL once at the beginning (good for 24 hrs) or once at beginning, middle & end because there is drift? A: Beginning only: good for 3 months. Because MM-IR overcomes the drift issue of FTIR by real time buffer subtraction with its microfluidic modulation auto-referencing

Utilize Area of Overlap Spectral Similarity Scoring Technique

System Suitability Test
Matching Buffer Blank

Normalization Prior to Area Overlapping Calc.

Convenience

AO is derived from second derivative IR spectra after normalized against concentration (so the concentration dependence is eliminated).
AO is used to calculate the structure similarity of different samples.

AO Initial use & subsequent improvements

Weighted Spectral Difference (WSD)

$$r = \frac{\sum (x_i y_i)}{\sqrt{\sum x_i^2 \sum y_i^2}}$$

The SD and WSD formulas are as follows:

$$SD = \sqrt{\sum_{i=1}^{n} \left[\left(\frac{1}{n}\right) (y_{Ai} - y_{Bi})^2 \right]} \quad (1)$$

$$WSD = \sqrt{\sum_{i=1}^{n} \left[\left(\frac{1}{n}\right) \left(\frac{|y_{Ai}|}{|y_A|_{ave.}}\right) (y_{Ai} - y_{Bi})^2 \right]}, \quad (2)$$

where y_A and y_B are signals of the reference and sample spectra, respectively, with n data points in a spectral range of interest, and the term $\left[\left(\frac{1}{n}\right) \left(\frac{|y_{Ai}|}{|y_A|_{ave.}}\right) \right]$ can be expressed as $\frac{|y_{Ai}|}{\sum_{i=1}^{n} |y_{Ai}|}$.

AO spectra norm. concept similar to:

Bruker Vector Normalization

Vector normalization – This method calculates the average y-value of the spectrum. The average value is subtracted from the spectrum decreasing the mid-spectrum to y = 0. The sum of the squares of all y-values is calculated and the spectrum is divided by the square root of this sum.

The vector norm of the result spectrum is 1: $\sum_{i=1}^{NPT} (x_i)^2 = 1$

1. Kendrick BS, Dong A, Allison SD, Manning MC, Carpenter JF. Quantitation of area of overlap b/w second-derivative amide I IR spectra to determine the structural similarity of a protein in different states. J Pharm Sci. 1996; 85:155-8.
2. Dong A, Huang P, Caughey WS. Protein secondary structures in water from second-derivative amide I infrared spectra. Biochemistry. 1990; 29:3303-8.
3. Yang H, Yang S, Kong J, Dong A, Yu S. Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. Nat Protoc. 2015; 10:382-96.
4. Dinh NN, Winn BC, Arthur KK, Gabrielson JP. Quantitative spectral comparison by weighted spectral difference for protein higher order structure confirmation. Anal Biochem. 2014;464:60-2.

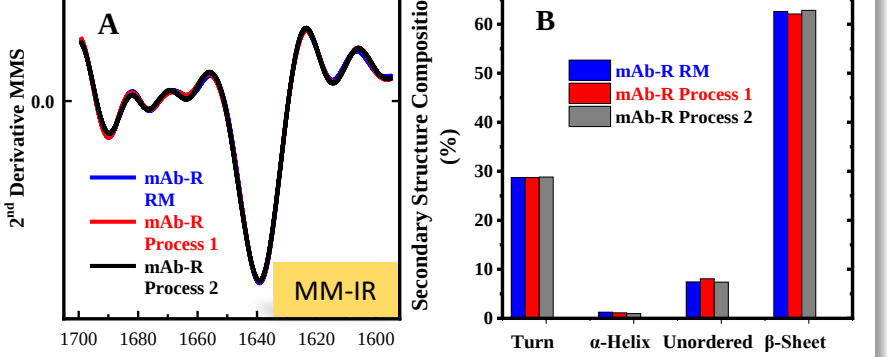
Case Studies

Comparability &

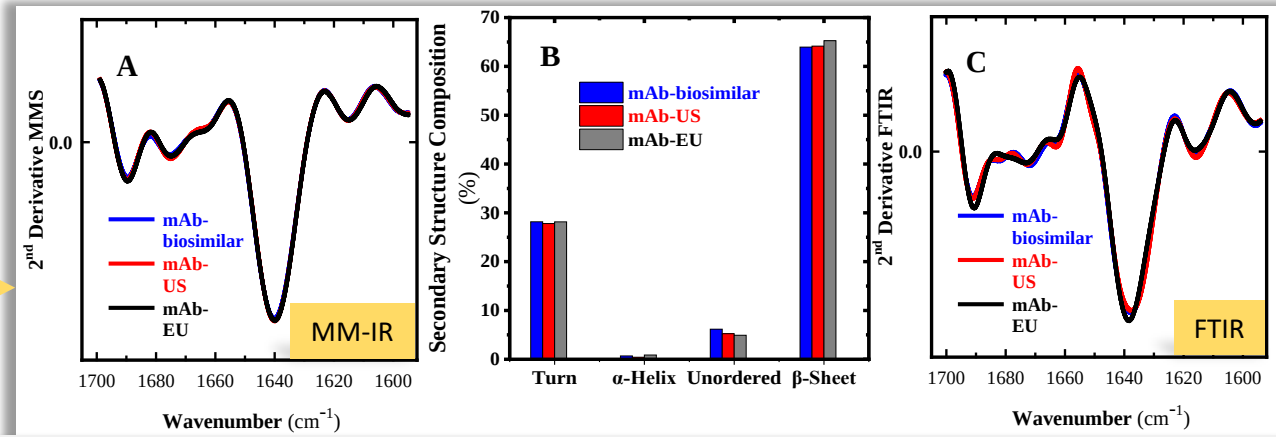
Biosimilarity Assessment

Case Studies

The MM-IR spectra of the respective samples under comparison are closely matched and the secondary structure compositions (%) are very consistent



HOS comparability study of mAb-R DS from 2 different manufacturing processes analyzed side-by-side with RM (A) Overlaid 2nd derivative MM-IR spectra (at 1 mg/mL); (B) Secondary structure composition (%)



HOS similarity study of mAb-biosimilar MM-IR and FTIR (A) Overlaid 2nd derivative MM-IR spectra (at 1 mg/mL). The biosimilar is **99.1%** similar in structure (using **spectral similarity** via AO calculation) when compared to both US & EU originator products. (B) Secondary structure composition (%); (C) Overlaid 2nd derivative FTIR spectra (at 10 mg/mL)

Summary

- MM-IR can measure protein samples from high concentrations to very low concentrations, & provide high quality, comparable data across a wide concentration range of 1 mg/mL - 87.4 mg/mL
- Our data indicates: MM-IR is a powerful protein characterization tool for secondary structure assessment of biopharmaceuticals, demonstrating high accuracy, linearity, sensitivity, & reproducibility, as well as a readout of discrete secondary structure elements
- Similar to far-UV CD and FTIR, MM-IR appears applicable to modalities beyond mAbs & has great potential to become the primary characterization tool to routinely elucidate & monitor the secondary structure product quality attribute (PQA) in protein therapeutics
- In today's fast-paced biotherapeutics laboratories, where each therapeutic project team maintains very aggressive development timelines, ease-of-use & fast turnaround are becoming as important as high data quality. As a high-throughput automated IR instrument, MM-IR is found to bring increased resolution, sensitivity, stability & efficiency for next-level biotherapeutics analysis (*J of Pharm Sci, 2020; 109 (10): 3223-30).*

Acknowledgements

Analytical Research & Development

Hai-Young Kim
Jason C. Rouse
Sharon Polleck
Victor Beaumont
Clifford Entrican
Zhaojiang Lu
Thomas Lerch
Matthew Thompson
Ling Gu
David J. Cirelli
Qin Zou
Mellisa M. Ly
Lisa Marzilli
Meg Ruesch

Libo Wang
Jeffrey Zonderman
Peter Guyette
Valerie Ivancic
Holly Lombardo
Frank Yuan
Eugene Ma
Sean Veale
Michael Getman
John Linnan