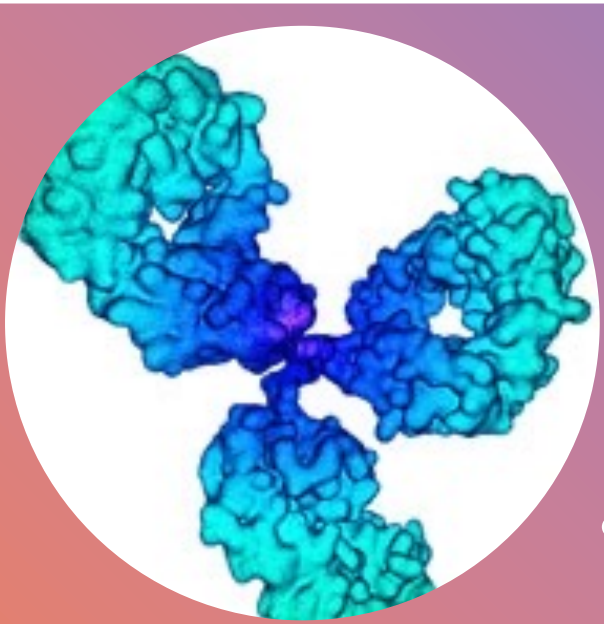
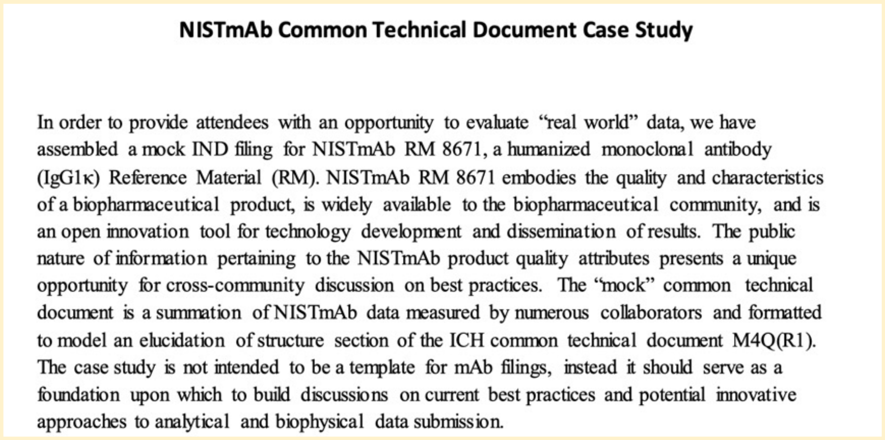


Advancing Development of Biotherapeutics: New Tools for Emerging Modalities

Presented by: Rina K. Dukor, Ph.D

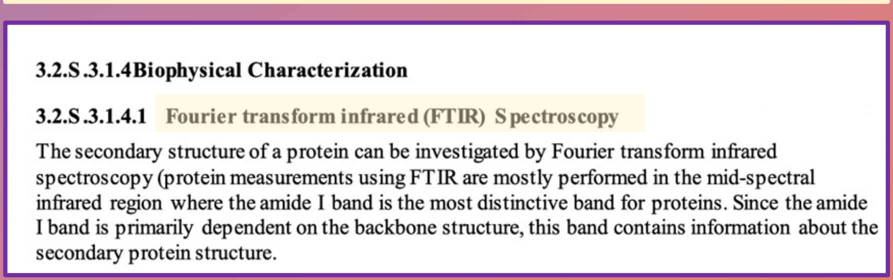
BioTools
20 YEARS OF INNOVATION

From HOS-2017: Biophysical Characterization of NIST mAb RM 8671 IgG1k



NISTmAb Common Technical Document Case Study

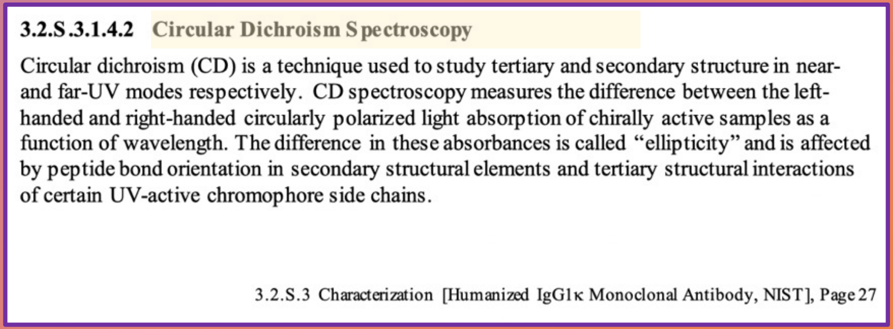
In order to provide attendees with an opportunity to evaluate "real world" data, we have assembled a mock IND filing for NISTmAb RM 8671, a humanized monoclonal antibody (IgG1κ) Reference Material (RM). NISTmAb RM 8671 embodies the quality and characteristics of a biopharmaceutical product, is widely available to the biopharmaceutical community, and is an open innovation tool for technology development and dissemination of results. The public nature of information pertaining to the NISTmAb product quality attributes presents a unique opportunity for cross-community discussion on best practices. The "mock" common technical document is a summation of NISTmAb data measured by numerous collaborators and formatted to model an elucidation of structure section of the ICH common technical document M4Q(R1). The case study is not intended to be a template for mAb filings, instead it should serve as a foundation upon which to build discussions on current best practices and potential innovative approaches to analytical and biophysical data submission.



3.2.S.3.1.4 Biophysical Characterization

3.2.S.3.1.4.1 Fourier transform infrared (FTIR) Spectroscopy

The secondary structure of a protein can be investigated by Fourier transform infrared spectroscopy (protein measurements using FTIR are mostly performed in the mid-spectral infrared region where the amide I band is the most distinctive band for proteins. Since the amide I band is primarily dependent on the backbone structure, this band contains information about the secondary protein structure.



3.2.S.3.1.4.2 Circular Dichroism Spectroscopy

Circular dichroism (CD) is a technique used to study tertiary and secondary structure in near- and far-UV modes respectively. CD spectroscopy measures the difference between the left-handed and right-handed circularly polarized light absorption of chirally active samples as a function of wavelength. The difference in these absorbances is called "ellipticity" and is affected by peptide bond orientation in secondary structural elements and tertiary structural interactions of certain UV-active chromophore side chains.

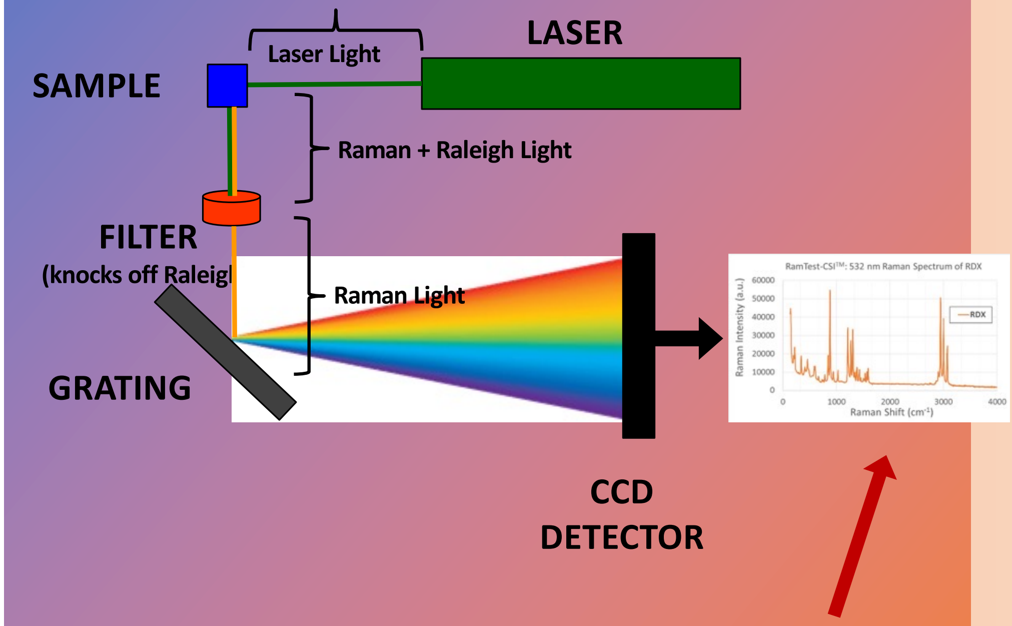
3.2.S.3 Characterization [Humanized IgG1κ Monoclonal Antibody, NIST], Page 27

Is there need / room for another technique?

Are CD / FTIR sensitive enough for mAbs and new modalities?

- **I would like to argue a YES!**

(Re)Introducing Raman / ROA



Raman scattering creates a **unique** fingerprint for a molecule

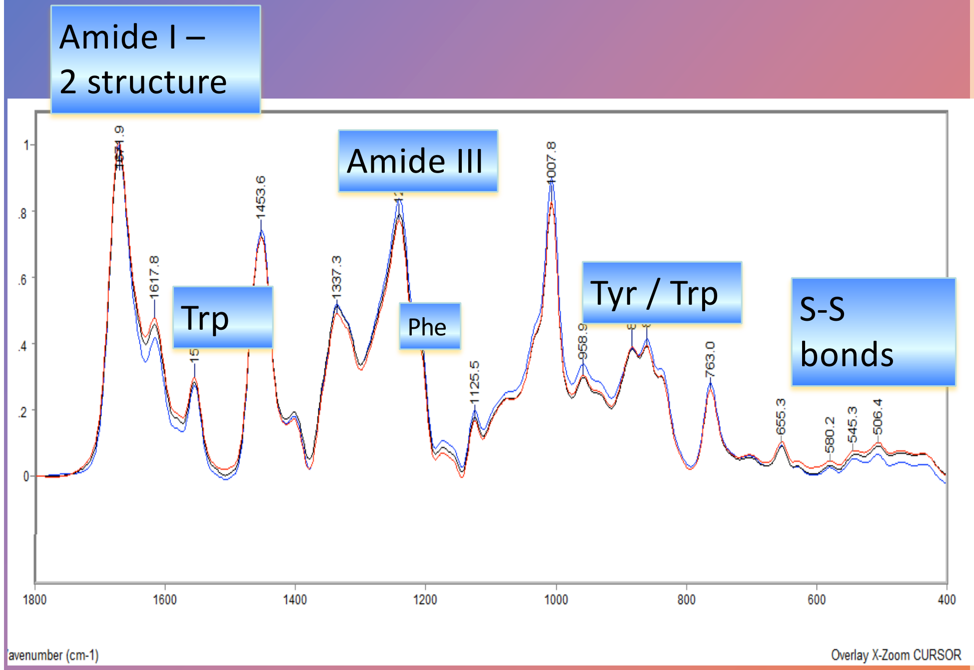
Many uses in Pharma for PAT, contaminant analysis, polymorphism

But very few for protein HOS

WHY??

Because previously no system on the market could EASILY measure high quality spectra at variety of concentrations

What is **Raman** and what are the advantages for mAbs / peptides / proteins?

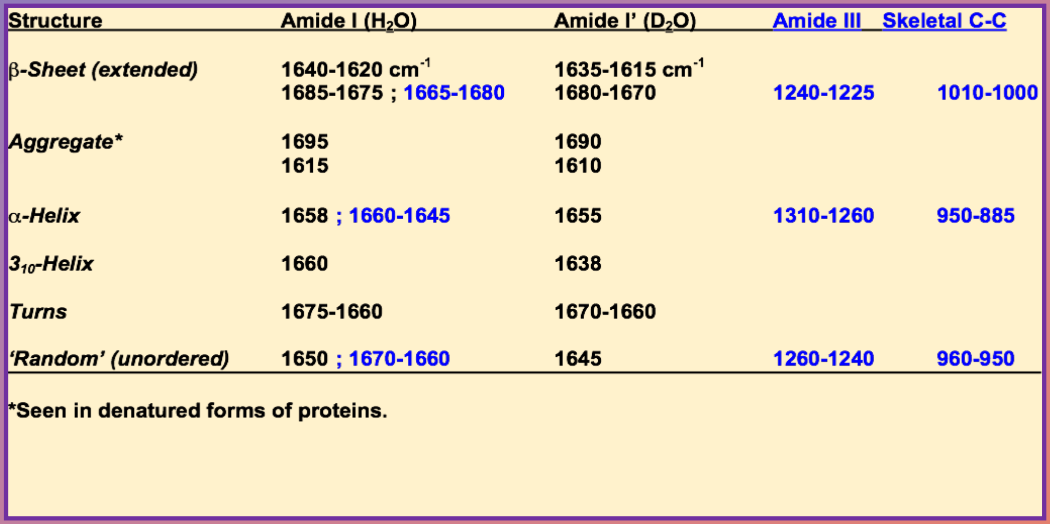


In very simple terms…

RAMAN is complimentary, 'like' FTIR – basically same fingerprint spectral region

BUT whereas FTIR is *only* sensitive to secondary structure, RAMAN provides detailed information on secondary structure, disulfide bonds, and all **aromatics**, thus providing secondary and tertiary structure at the same time

Marker Bands IR & RAMAN



Structure	Amide I (H_2O)	Amide I' (D_2O)	Amide III	Skeletal C-C
β-Sheet (extended)	1640-1620 cm^{-1} 1685-1675 ; 1665-1680	1635-1615 cm^{-1} 1680-1670	1240-1225	1010-1000
*Aggregate**	1695 1615	1690 1610		
α-Helix	1658 ; 1660-1645	1655	1310-1260	950-885
3_{10}-Helix	1660	1638		
Turns	1675-1660	1670-1660		
'Random' (unordered)	1650 ; 1670-1660	1645	1260-1240	960-950

*Seen in denatured forms of proteins.

RAMAN S-S; S-H bonds

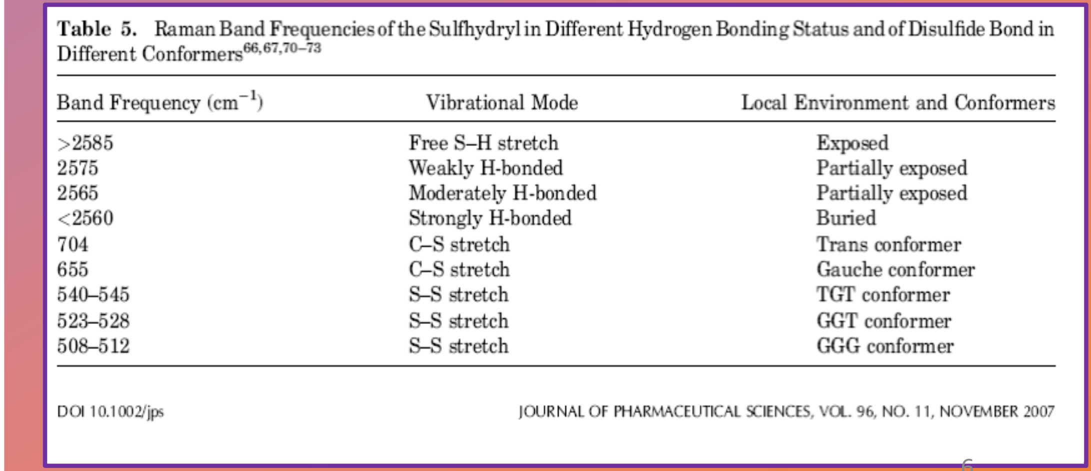


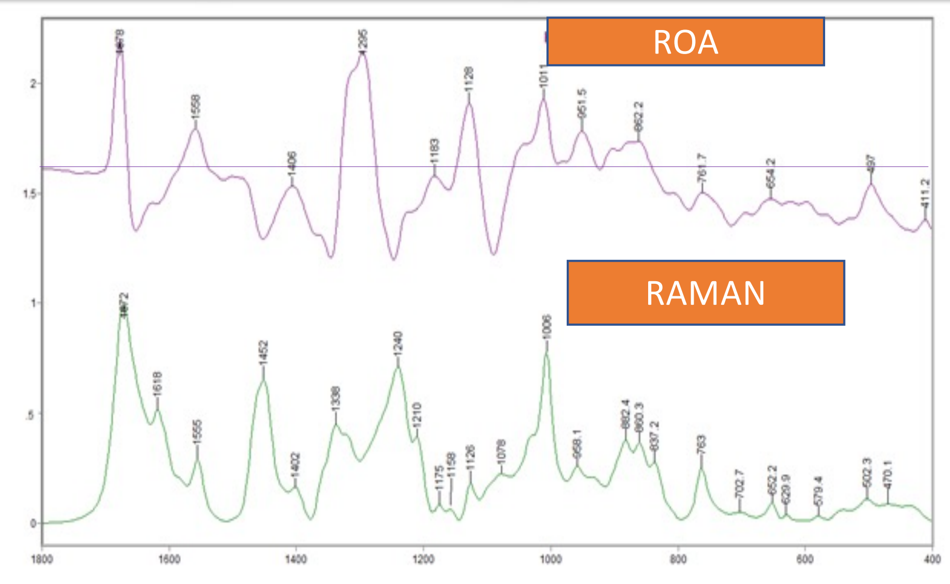
Table 5. Raman Band Frequencies of the Sulfhydryl in Different Hydrogen Bonding Status and of Disulfide Bond in Different Conformers[66,67,70–73]

Band Frequency (cm^{-1})	Vibrational Mode	Local Environment and Conformers
>2585	Free S–H stretch	Exposed
2575	Weakly H-bonded	Partially exposed
2565	Moderately H-bonded	Partially exposed
<2560	Strongly H-bonded	Buried
704	C–S stretch	Trans conformer
655	C–S stretch	Gauche conformer
540–545	S–S stretch	TGT conformer
523–528	S–S stretch	GGT conformer
508–512	S–S stretch	GGG conformer

DOI 10.1002/jps

JOURNAL OF PHARMACEUTICAL SCIENCES, VOL. 96, NO. 11, NOVEMBER 2007

ONE Measurement gives TWO spectra: RAMAN & ROA

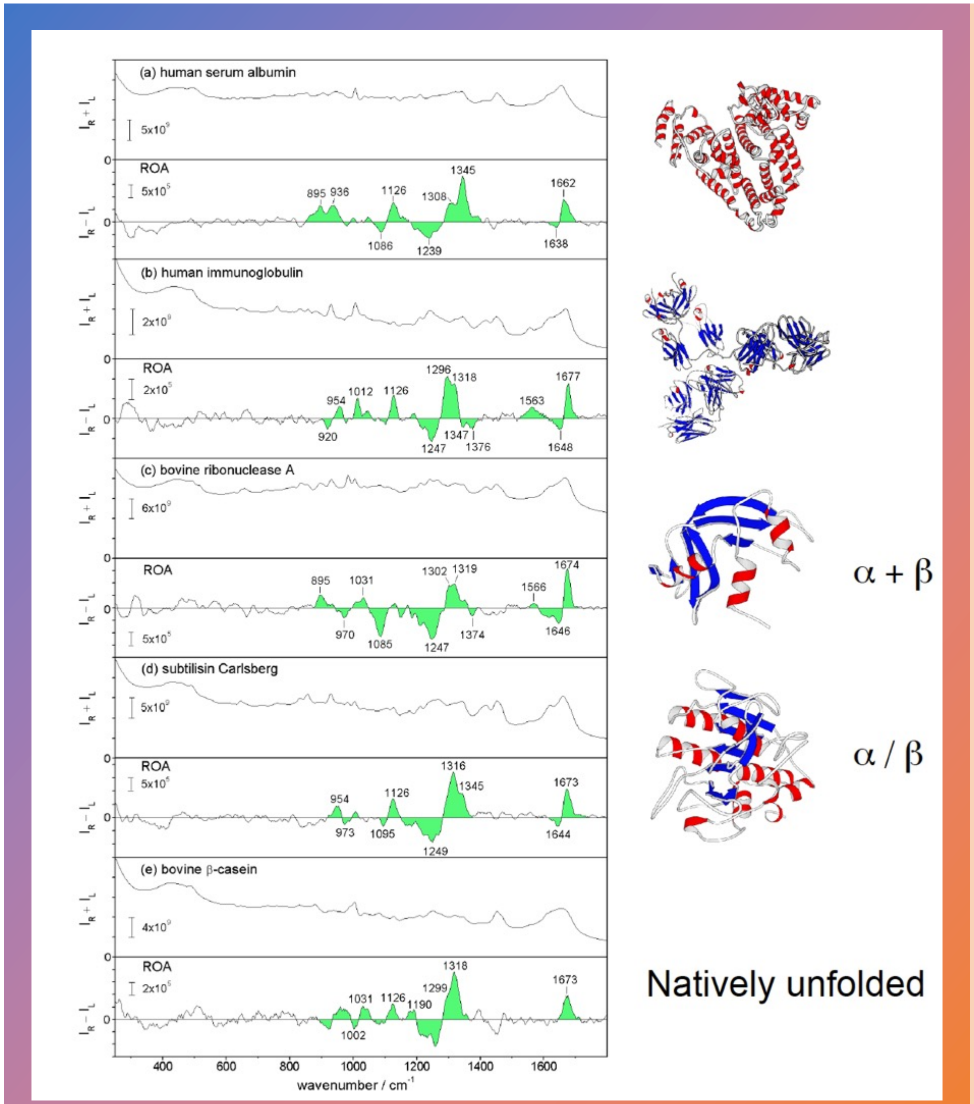


What is **ROA** and what are the advantages for mAbs / peptides / proteins?

In very simple terms…

ROA is combination of *'Raman' + 'CD'*

ROA spectra are dominated by peptide backbone bands and thus give more *direct* information about secondary and tertiary structure, combining all the advantages of Raman full spectral region & stereochemistry of CD.



Different Structural Forms show very different Raman / ROA spectra

As with other spectroscopic techniques such as FTIR & CD, the **unique spectra provide a snapshot of the fold**

L.D. Barron; The Biochemist, June, 2006, 27-31

RAMAN /ROA of Glycolytic-Structure in Proteins

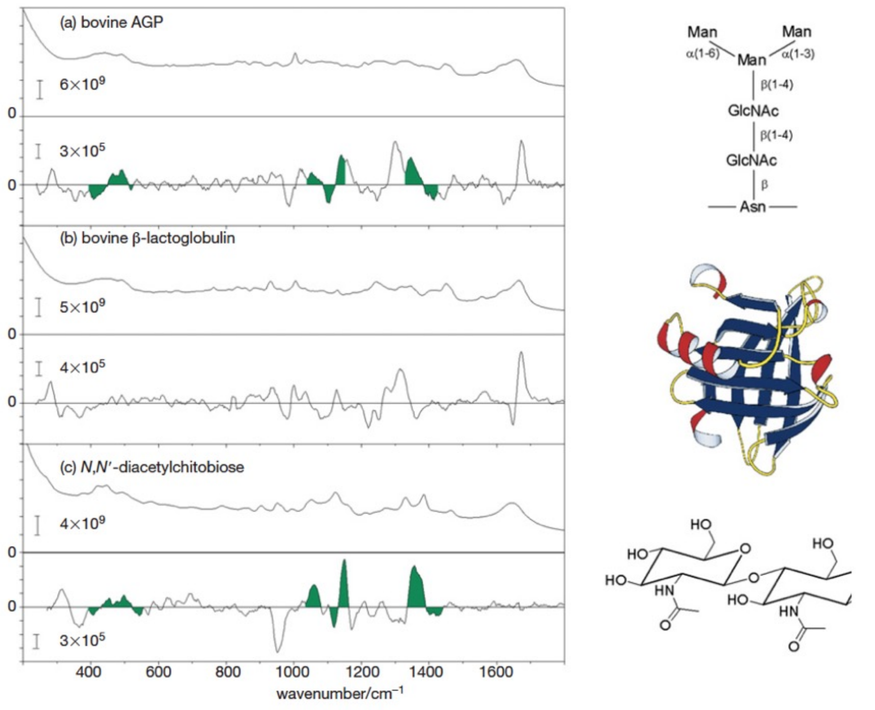
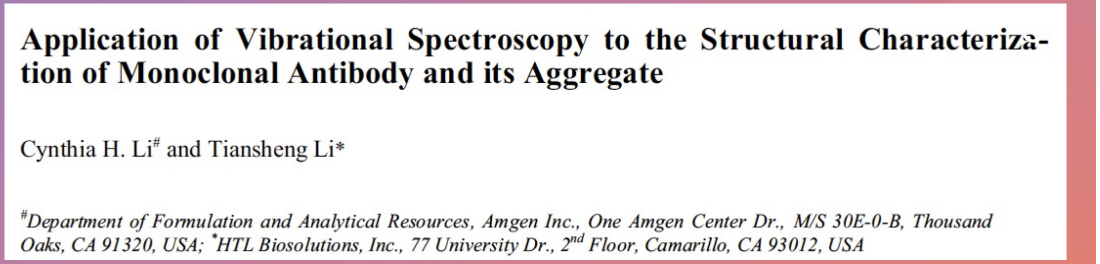


Figure 4. Backscattered SCP Raman ($I_R + I_L$) and ROA ($I_R - I_L$) spectra, all in aqueous solution, of (**a**) bovine AGP together with a diagram of the common pentasaccharide core, (**b**) bovine β-lactoglobulin together with its X-ray crystal structure and (c) *N,N*′-diacetylchitobiose. Some of the more prominent ROA bands provisionally assigned to carbohydrate structure in (a) are coloured green. Reproduced with permission from J. Am. Chem. Soc. (2005) 127, 6142–6143, ©2005 American Chemical Society

Sensitivity of ROA to Higher-Order Protein Structure

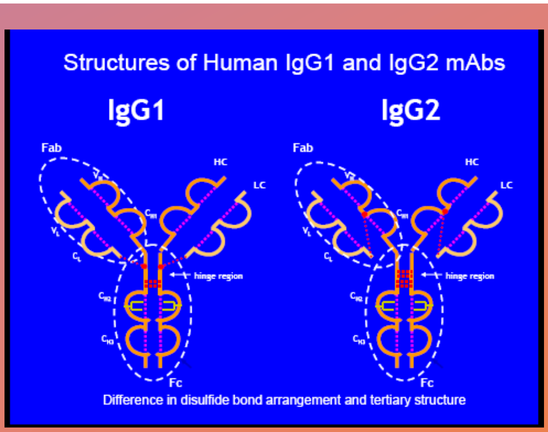


Application of Vibrational Spectroscopy to the Structural Characterization of Monoclonal Antibody and its Aggregate

Cynthia H. Li[#] and Tiansheng Li*

[#]Department of Formulation and Analytical Resources, Amgen Inc., One Amgen Center Dr., M/S 30E-0-B, Thousand Oaks, CA 91320, USA; [*]HTL Biosolutions, Inc., 77 University Dr., 2nd Floor, Camarillo, CA 93012, USA

***Current Pharmaceutical Biotechnology*, 2009, *10*, 391-399**

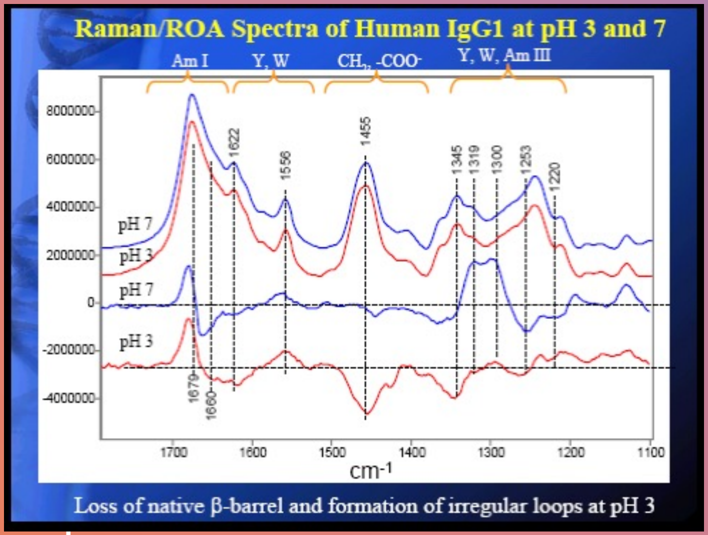


Structures of Human IgG1 and IgG2 mAbs

Difference in disulfide bond arrangement and tertiary structure

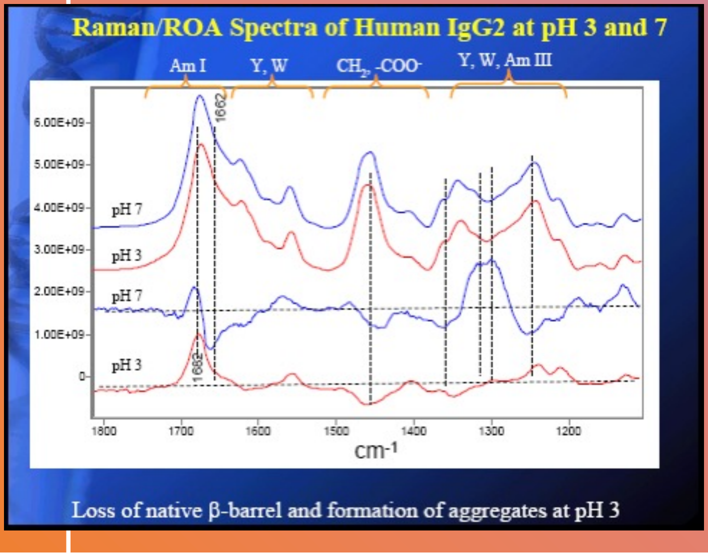
Work and slides courtesy of Dr. Tiansheng Li – Amgen, Inc.

Aggregation of Human IgG1 & IgG2 w/ pH



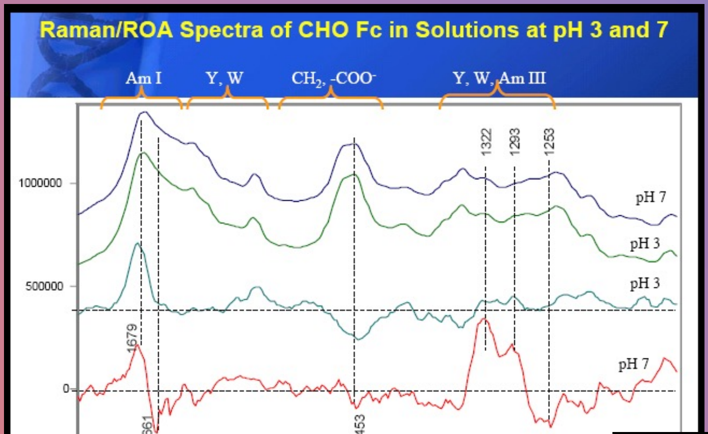
Raman/ROA Spectra of Human IgG1 at pH 3 and 7

Loss of native β-barrel and formation of irregular loops at pH 3



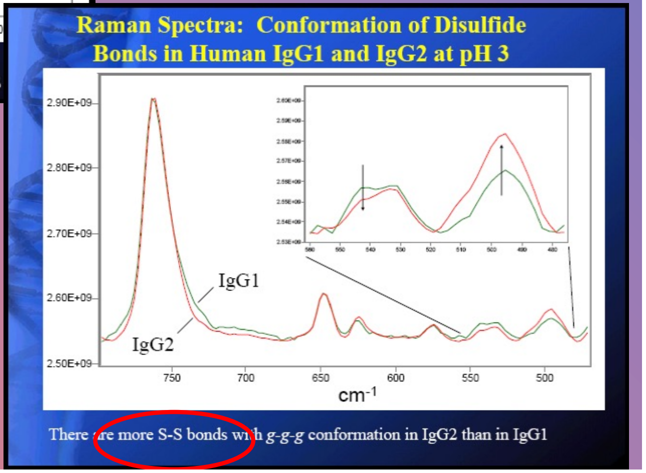
Raman/ROA Spectra of Human IgG2 at pH 3 and 7

Loss of native β-barrel and formation of aggregates at pH 3



Raman/ROA Spectra of CHO Fc in Solutions at pH 3 and 7

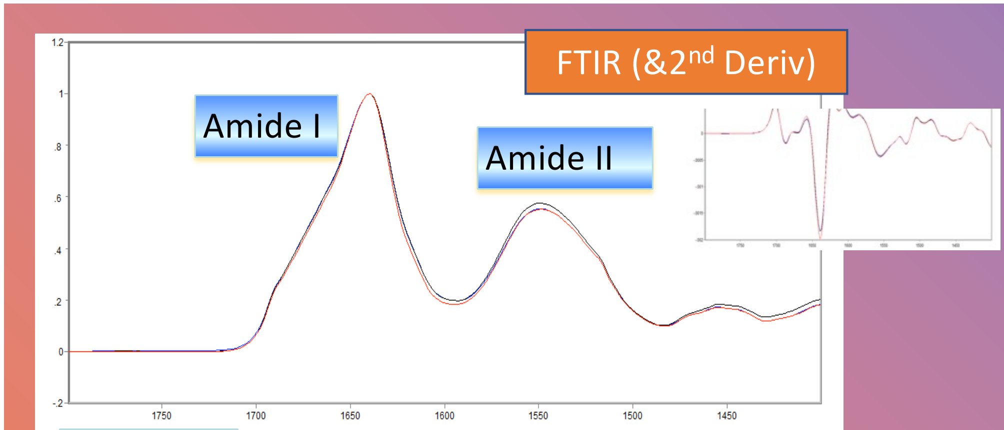
Loss of native β-barrel and formation of aggregate at pH 3

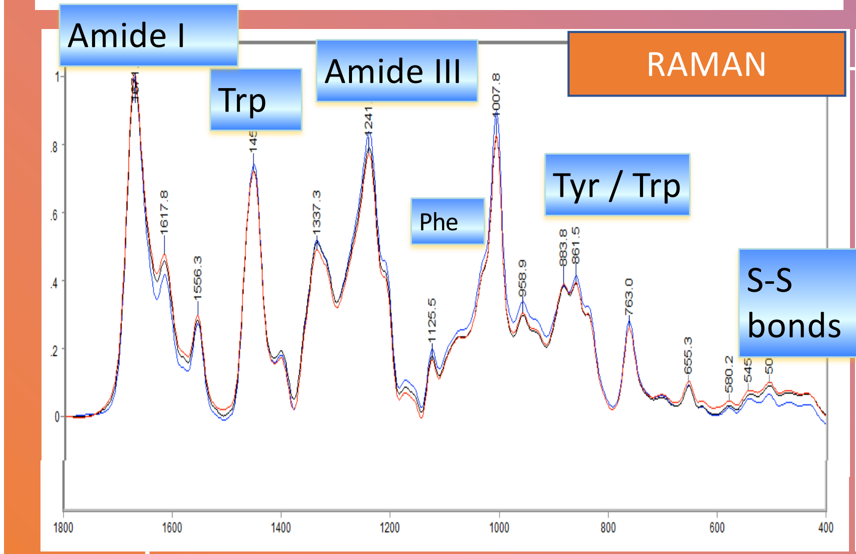


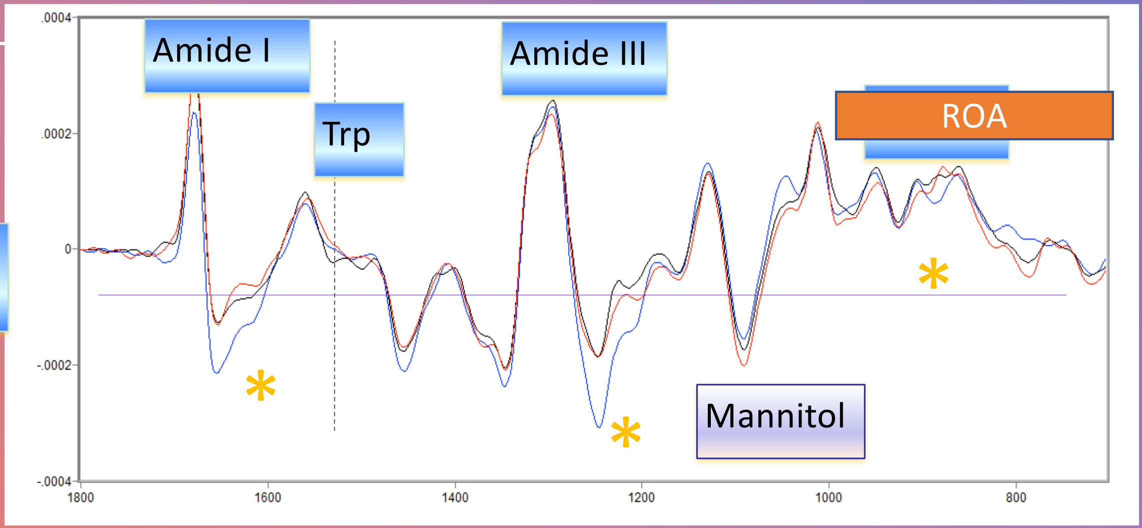
Raman Spectra: Conformation of Disulfide Bonds in Human IgG1 and IgG2 at pH 3

There are more S-S bonds with g-g-g conformation in IgG2 than in IgG1

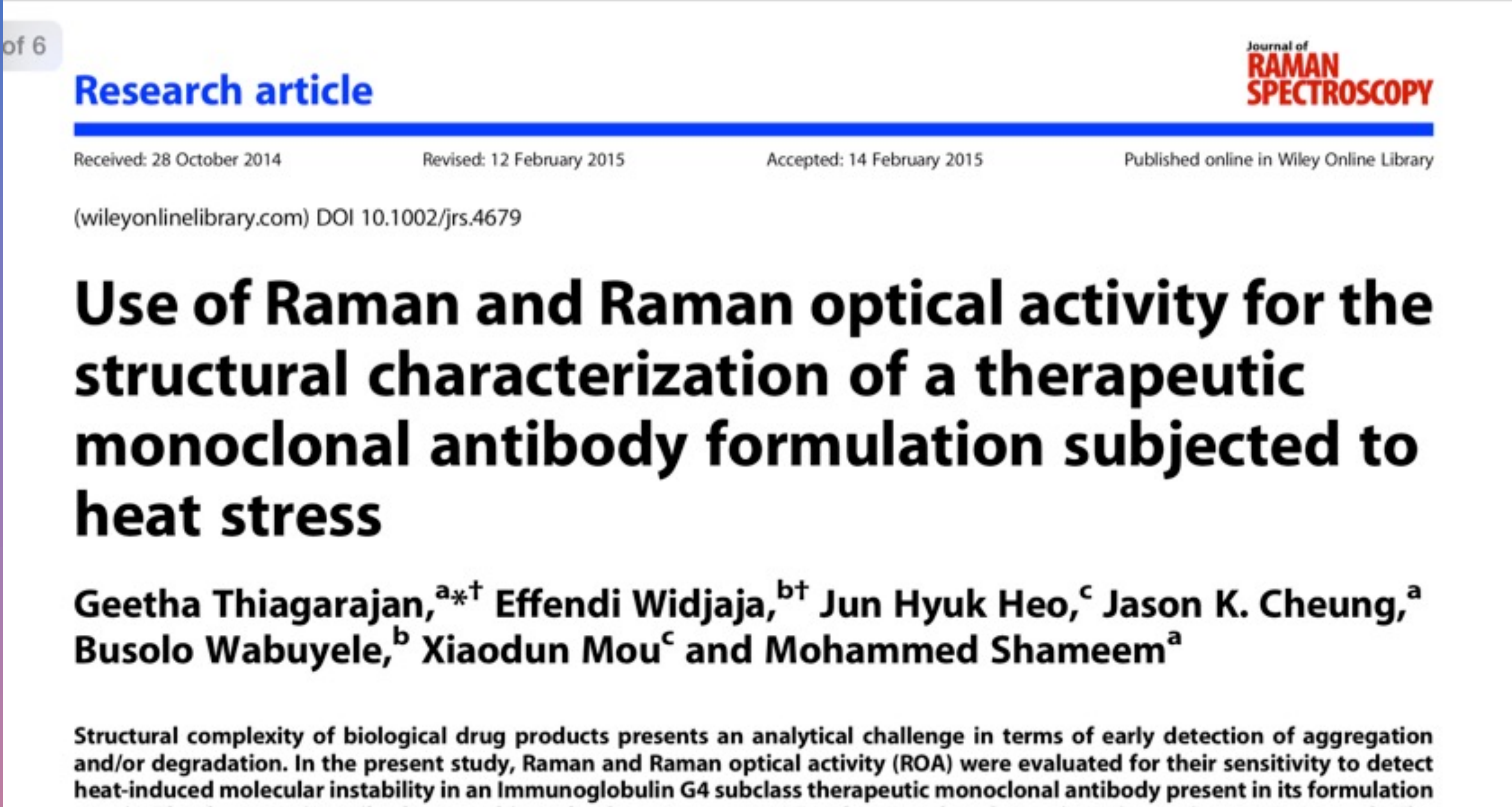
Three lots of commercial IgG1 with one lot identified as bad by customer







FTIR - no real differences between the three spectra; **Raman** - small differences;
ROA - the differences in the blue spectrum are visible and and are observed in secondary and tertiary structures



of 6

Research article

Journal of RAMAN SPECTROSCOPY

Received: 28 October 2014 Revised: 12 February 2015 Accepted: 14 February 2015

(wileyonlinelibrary.com) DOI 10.1002/jrs.4679

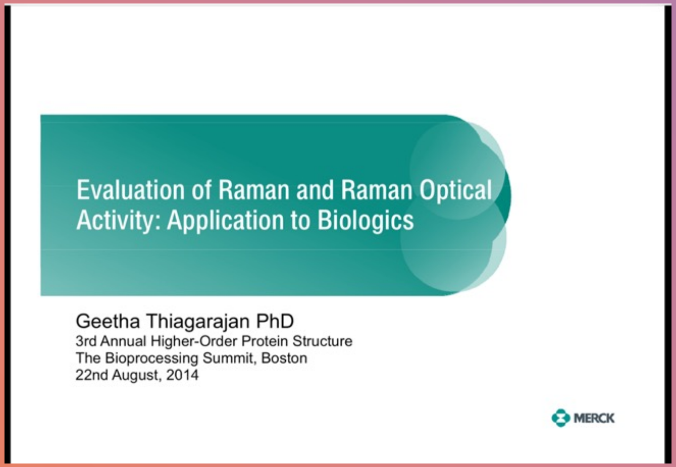
Use of Raman and Raman optical activity for the structural characterization of a therapeutic monoclonal antibody formulation subjected to heat stress

Geetha Thiagarajan,[a*†] Effendi Widjaja,[b†] Jun Hyuk Heo,[c] Jason K. Cheung,[a] Busolo Wabuyele,[b] Xiaodun Mou[c] and Mohammed Shameem[a]

Structural complexity of biological drug products presents an analytical challenge in terms of early detection of aggregation and/or degradation. In the present study, Raman and Raman optical activity (ROA) were evaluated for their sensitivity to detect heat-induced molecular instability in an Immunoglobulin G4 subclass therapeutic monoclonal antibody present in its formulation matrix. The therapeutic antibody was subjected to heat stress at 50 °C and was analyzed at various time points up to 1 month.

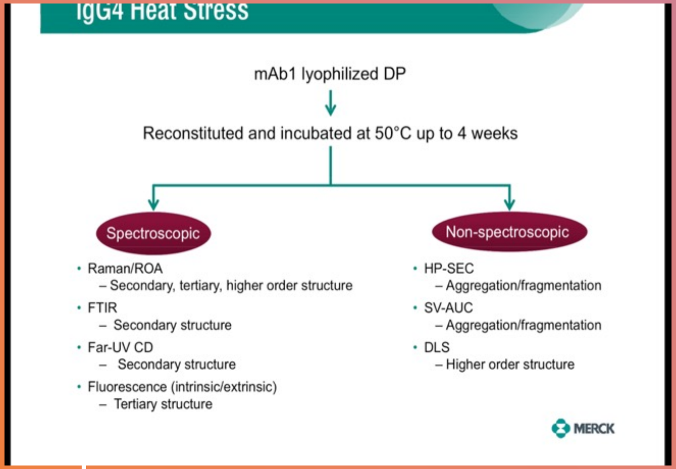
Comparison of Biophysical Methods:

Raman/ ROA is shown to be the most sensitive and a ‘predictor’ of stability



Evaluation of Raman and Raman Optical Activity: Application to Biologics

Geetha Thiagarajan PhD
3rd Annual Higher-Order Protein Structure
The Bioprocessing Summit, Boston
22nd August, 2014



IgG4 Heat Stress

mAb1 lyophilized DP

↓

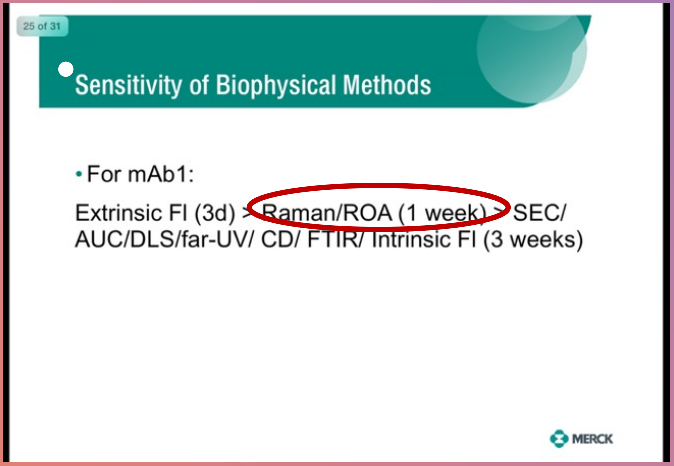
Reconstituted and incubated at 50°C up to 4 weeks

Spectroscopic

- Raman/ROA
  - Secondary, tertiary, higher order structure
- FTIR
  - Secondary structure
- Far-UV CD
  - Secondary structure
- Fluorescence (intrinsic/extrinsic)
  - Tertiary structure

Non-spectroscopic

- HP-SEC
  - Aggregation/fragmentation
- SV-AUC
  - Aggregation/fragmentation
- DLS
  - Higher order structure



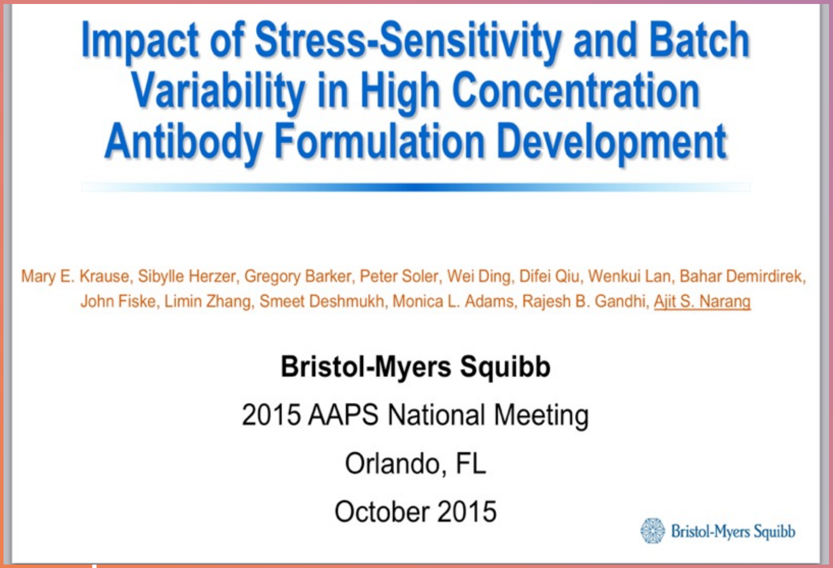
25 of 31

Sensitivity of Biophysical Methods

- For mAb1:

Extrinsic FI (3d) > Raman/ROA (1 week) > SEC/ AUC/DLS/far-UV/ CD/ FTIR/ Intrinsic FI (3 weeks)

Note: Raman/ ROA wasn’t measured on Day 3



Impact of Stress-Sensitivity and Batch Variability in High Concentration Antibody Formulation Development

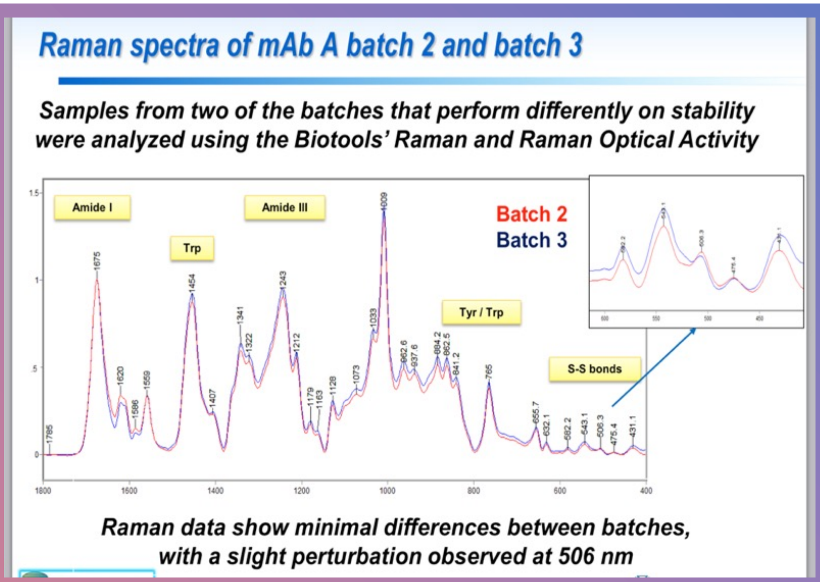
Mary E. Krause, Sibylle Herzer, Gregory Barker, Peter Soler, Wei Ding, Difei Qiu, Wenkui Lan, Bahar Demirdirek, John Fiske, Limin Zhang, Smeet Deshmukh, Monica L. Adams, Rajesh B. Gandhi, Ajit S. Narang

Bristol-Myers Squibb

2015 AAPS National Meeting

Orlando, FL

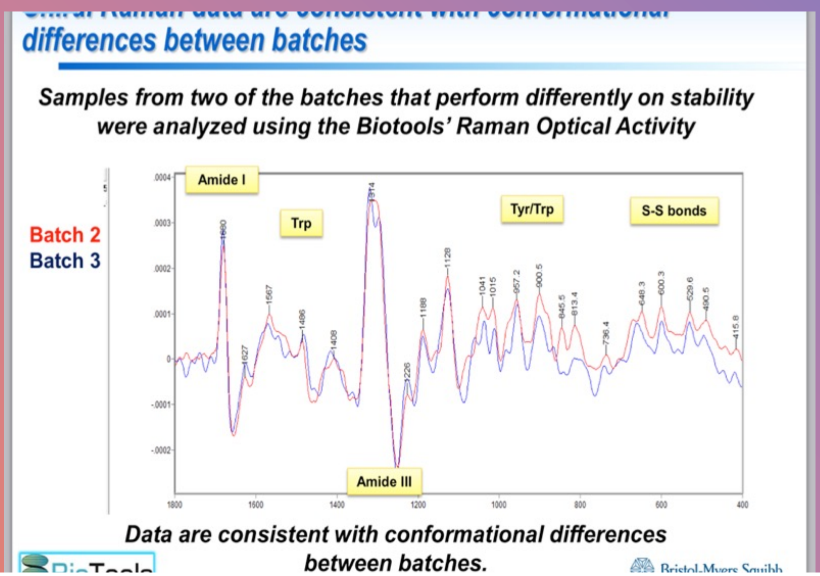
October 2015



Raman spectra of mAb A batch 2 and batch 3

Samples from two of the batches that perform differently on stability were analyzed using the Biotools' Raman and Raman Optical Activity

Raman data show minimal differences between batches, with a slight perturbation observed at 506 nm

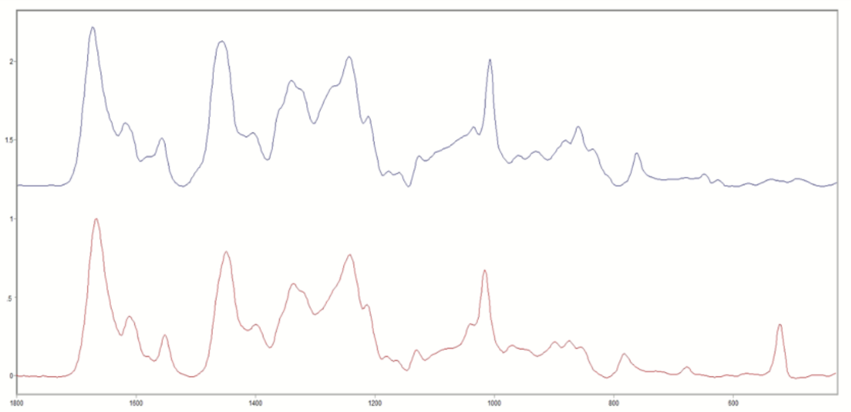


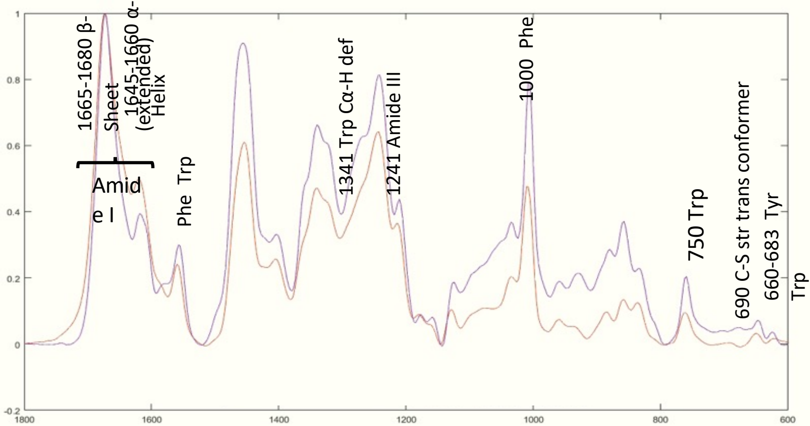
differences between batches

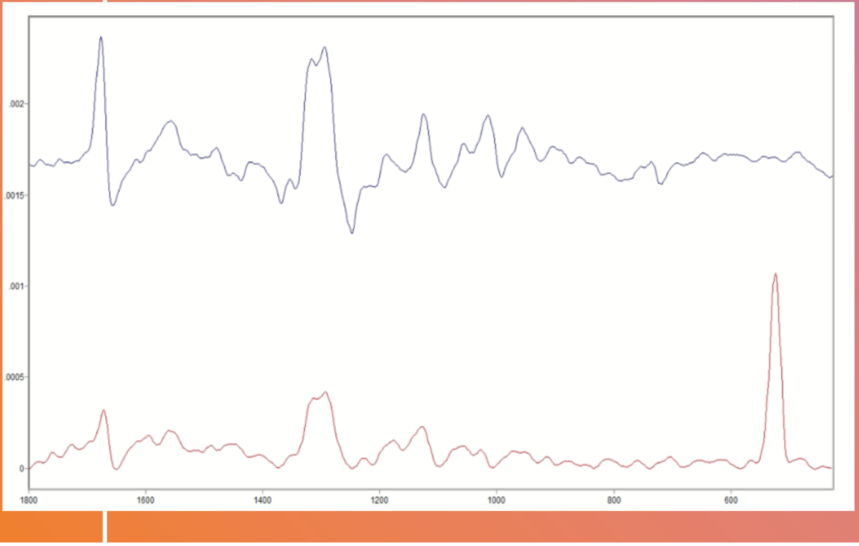
Samples from two of the batches that perform differently on stability were analyzed using the Biotools' Raman Optical Activity

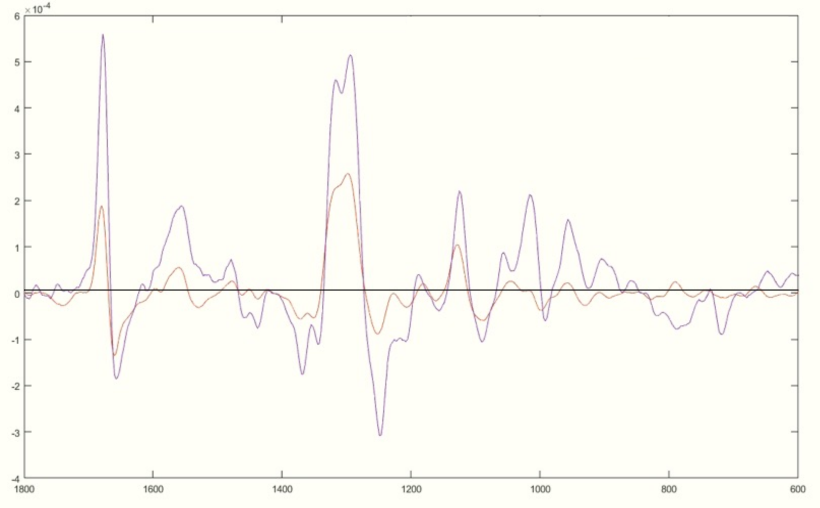
Data are consistent with conformational differences between batches.

Comparison of NIST IgG1(blue) and a different mAb (red)









BioTools
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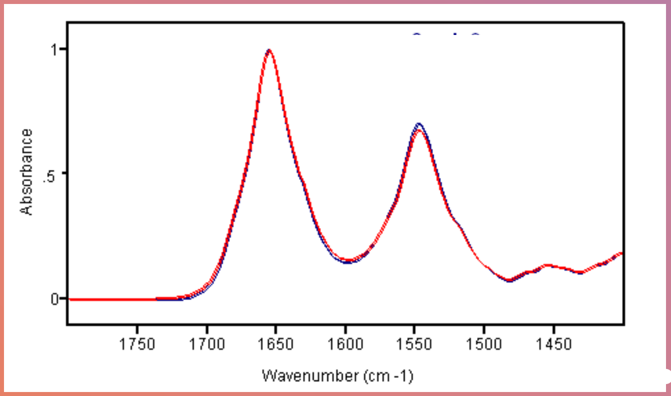
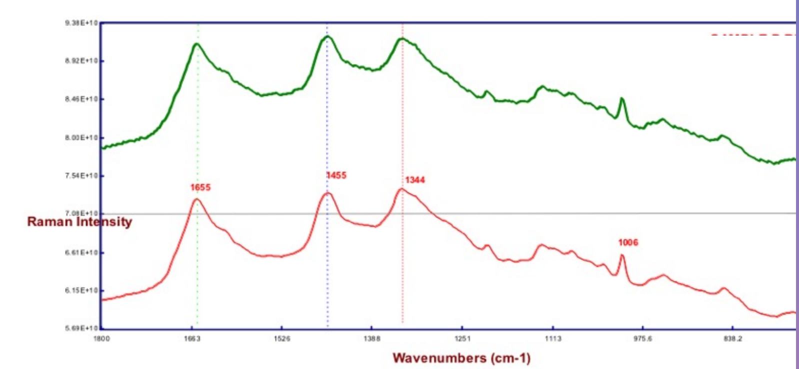
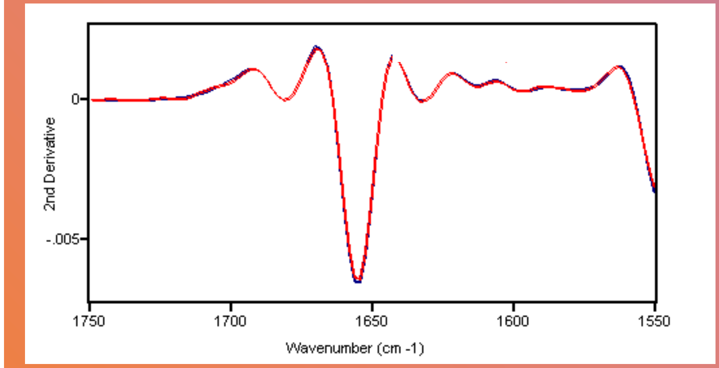
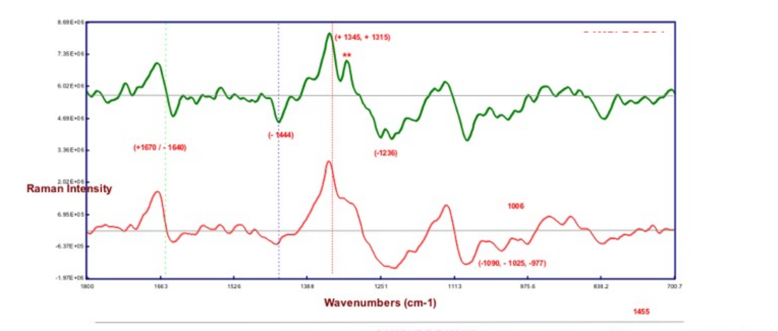
Fusion protein; one lot shows a problem – is there a structural change?

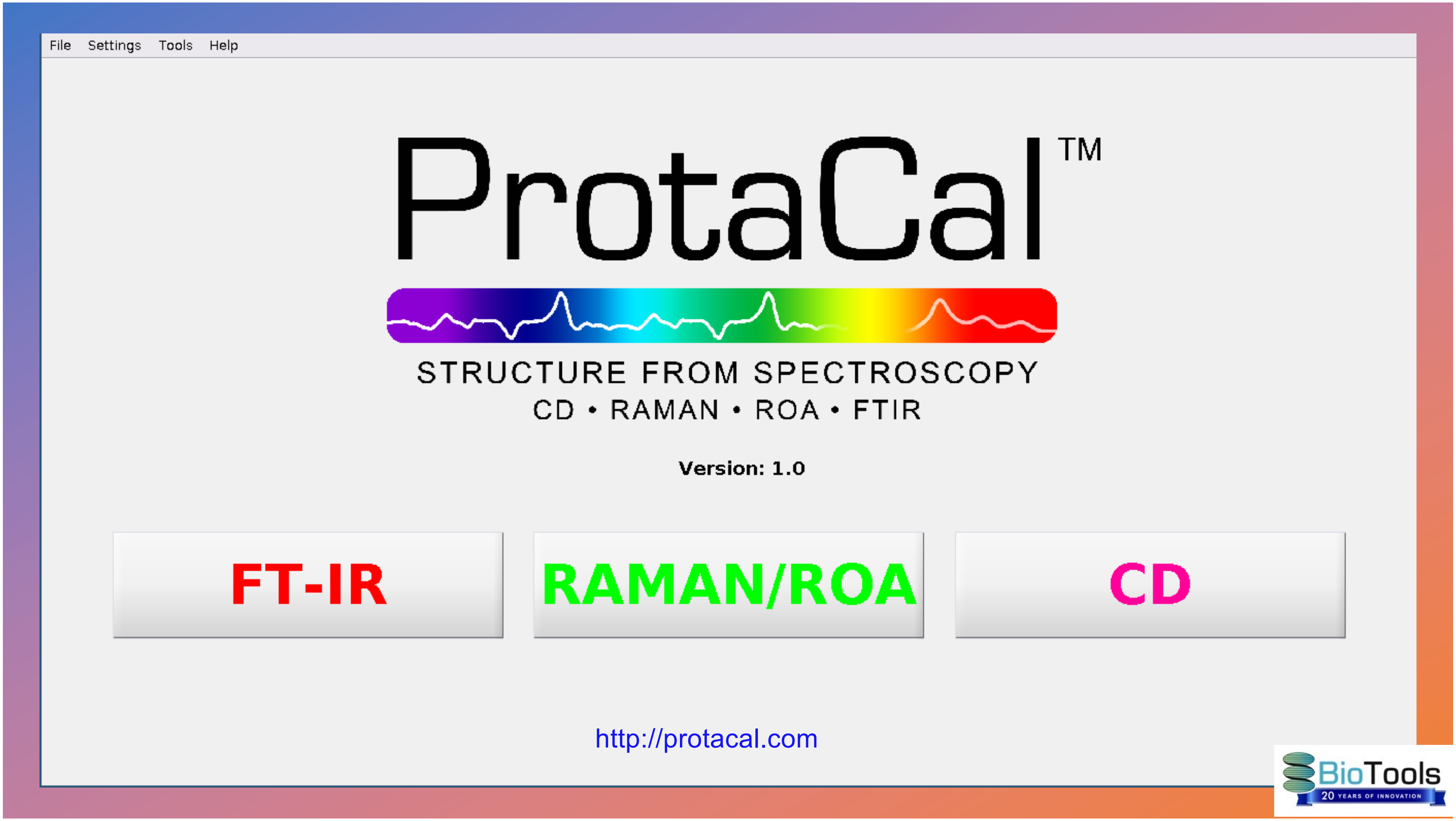








Structural Change is observed in ROA only:
Unfolding / presence of PLPII



ProtaCal™

STRUCTURE FROM SPECTROSCOPY
CD • RAMAN • ROA • FTIR

Version: 1.0

http://protacal.com

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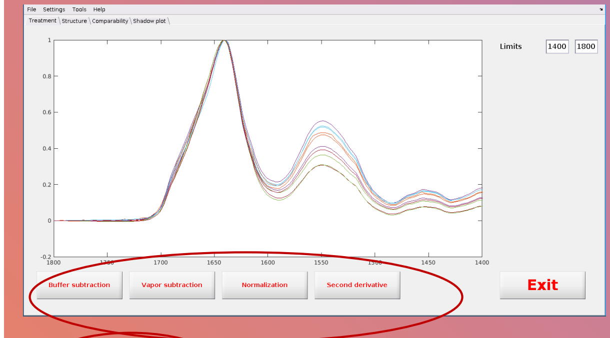
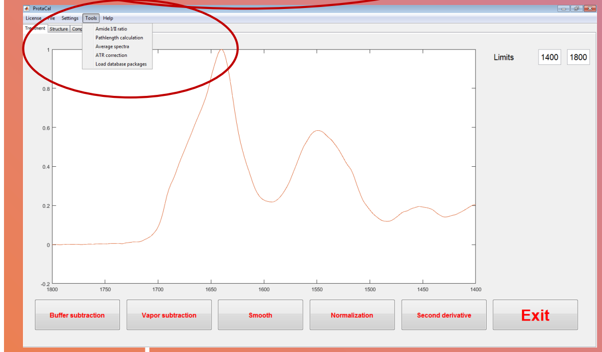
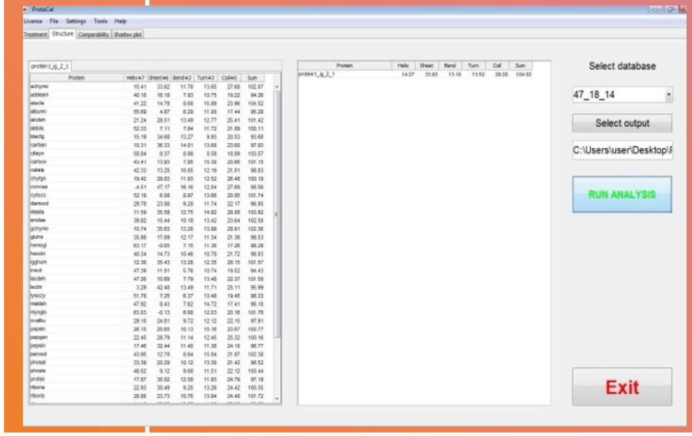
20 YEARS OF INNOVATION







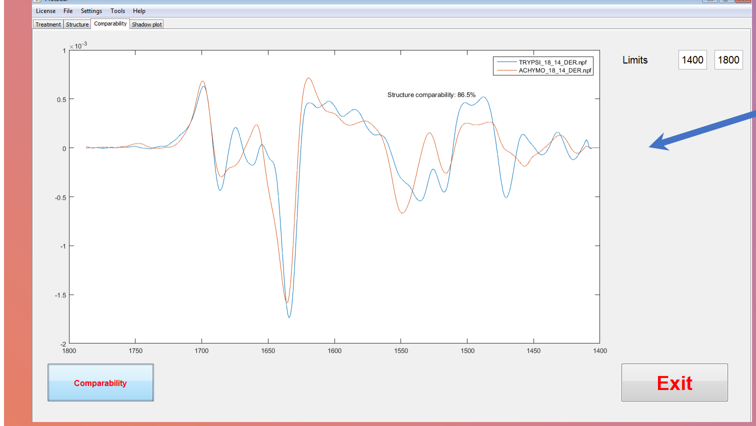
- Complete Spectral Processing (single or multiple files)

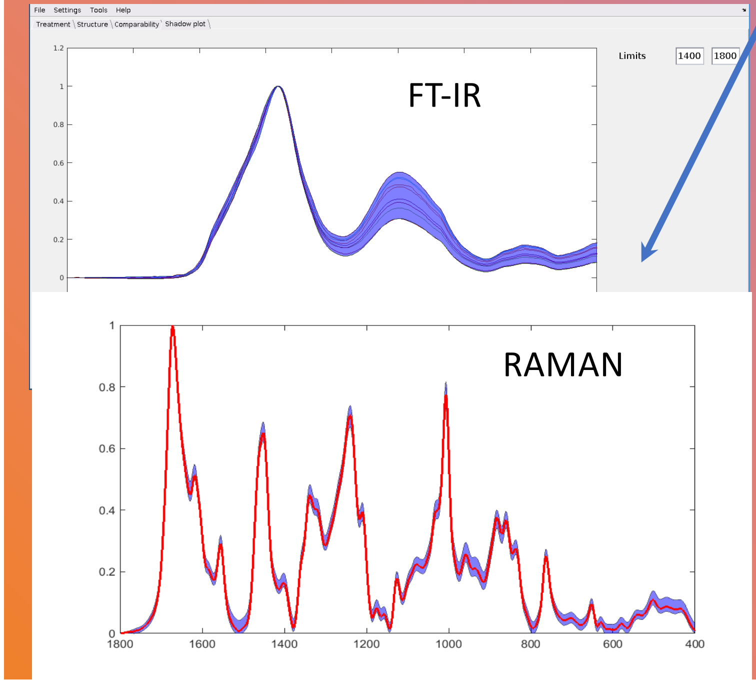
- Lots of Tools

- Secondary Structure Calculation based on ~50 proteins in the transmission database

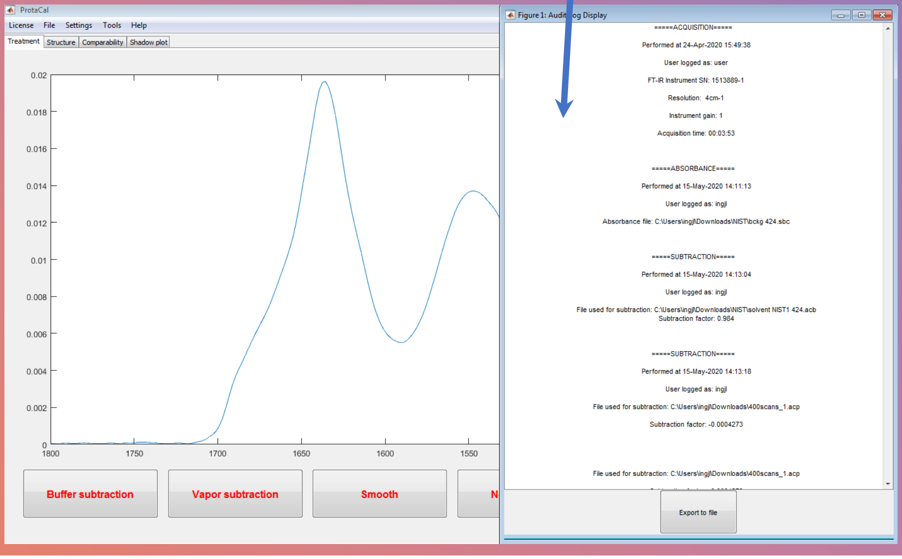
- Now includes NIST standard antibody!!

- ATR databases available for liquid and solid forms





- Comparability (based on area of overlap)
- Shadow Plot – provides an error of measurement / analysis
- 21CFR compliance: Audit Log for all functions



```
=====ACQUISITION=====

Performed at 24-Apr-2020 15:49:38

User logged as: user

FT-IR Instrument SN: 1513889-1

Resolution:  4cm-1

Instrument gain: 1

Acquisition time: 00:03:53


=====ABSORBANCE=====

Performed at 15-May-2020 14:11:13

User logged as: ingjl

Absorbance file: C:\Users\ingjl\Downloads\NIST\bckg 424.sbc


=====SUBTRACTION=====

Performed at 15-May-2020 14:13:04

User logged as: ingjl

File used for subtraction: C:\Users\ingjl\Downloads\NIST\solvent NIST1 424.acb
Subtraction factor: 0.984


=====SUBTRACTION=====

Performed at 15-May-2020 14:13:18

User logged as: ingjl

File used for subtraction: C:\Users\ingjl\Downloads\400scans_1.acp

Subtraction factor: -0.0004273



File used for subtraction: C:\Users\ingjl\Downloads\400scans_1.acp
```

New Modalities:
DNA / RNA
Viruses / AAV

RAMAN/ ROA Applications for Nucleic Acids based molecules

- Very rich history on characterization of viruses and nucleic acids; *can differentiate different viruses and different types of NA.*
- Can detect base-stacking and sugar phosphate modes

Marker Bands IR & RAMAN

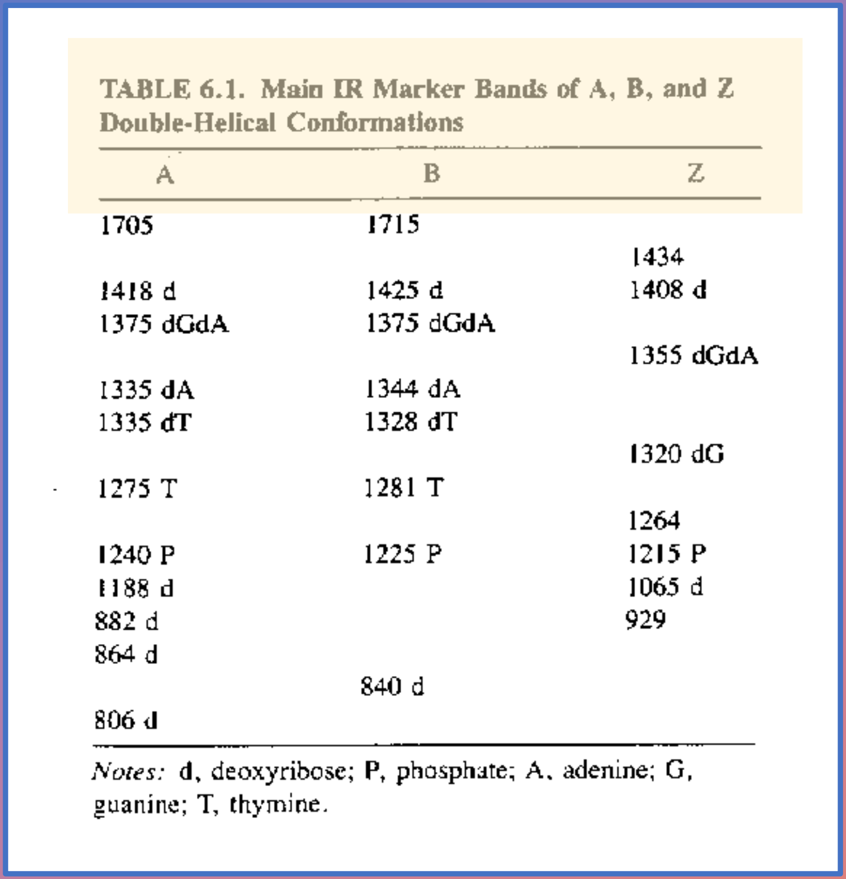


TABLE 6.1. Main IR Marker Bands of A, B, and Z Double-Helical Conformations

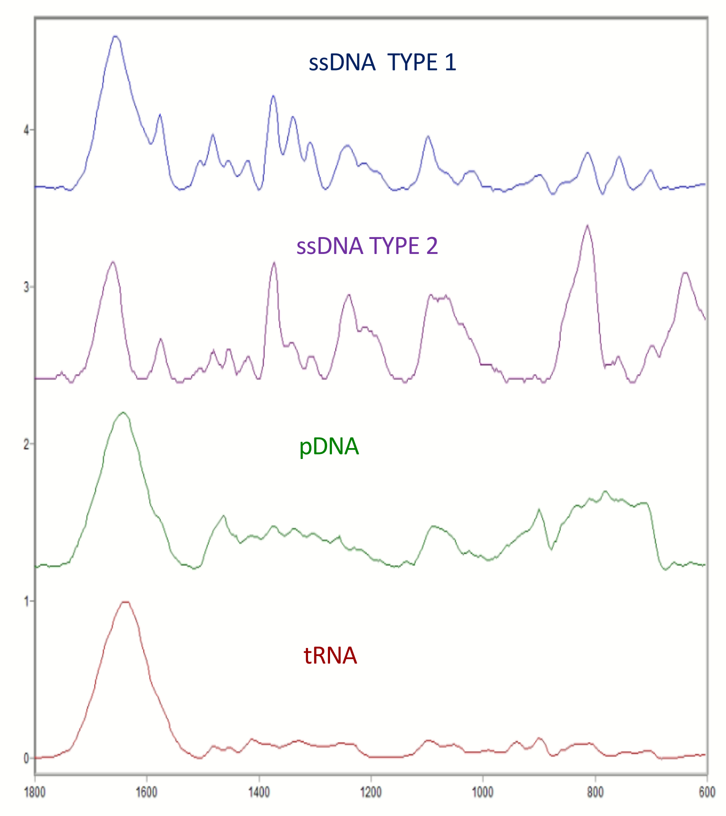
A	B	Z
1705	1715	
		1434
1418 d	1425 d	1408 d
1375 dGdA	1375 dGdA	
		1355 dGdA
1335 dA	1344 dA	
1335 dT	1328 dT	
		1320 dG
1275 T	1281 T	
		1264
1240 P	1225 P	1215 P
1188 d		1065 d
882 d		929
864 d		
	840 d	
806 d		

Notes: d, deoxyribose; P, phosphate; A, adenine; G, guanine; T, thymine.

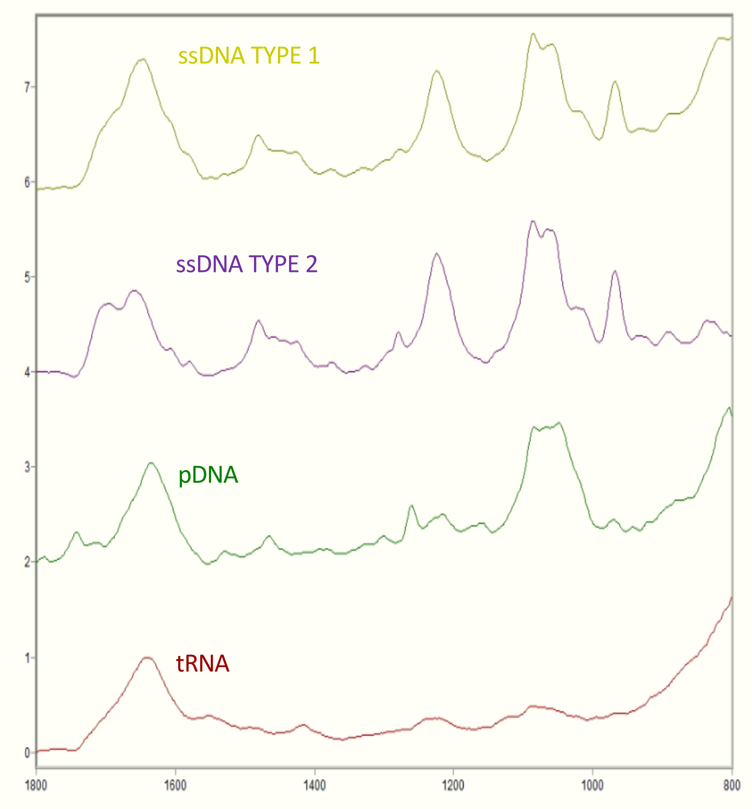
DNA, cm^{-1}	Assignments
1669	Thynine (O2)
1578	Purine stretching
1513	Adenine
1488	Guanine (N7)
1462	Deoxyribose
1376	Thymine (CH$_3$), purine
1339	Purine stretching
1304	Adenine
1256	Adenine, cytosine
1179	Thymine, cytosine
1094	Phosphodioxy stretching
895	Deoxyribose
838	Phosphodiester stretching
788	Phosphodiester stretching
753	Thymine
683	Guanine

Comparison of different forms of DNA

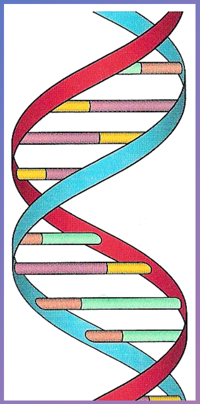
RAMAN

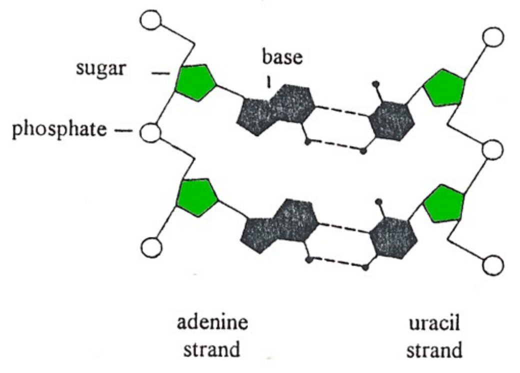


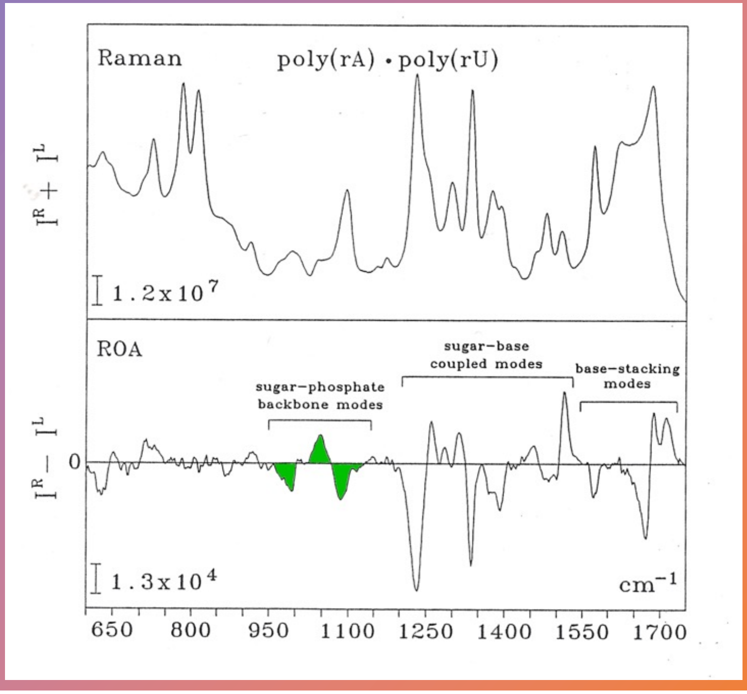
FT-IR



A-type RNA Double Helix: Raman (top) & ROA (bottom)

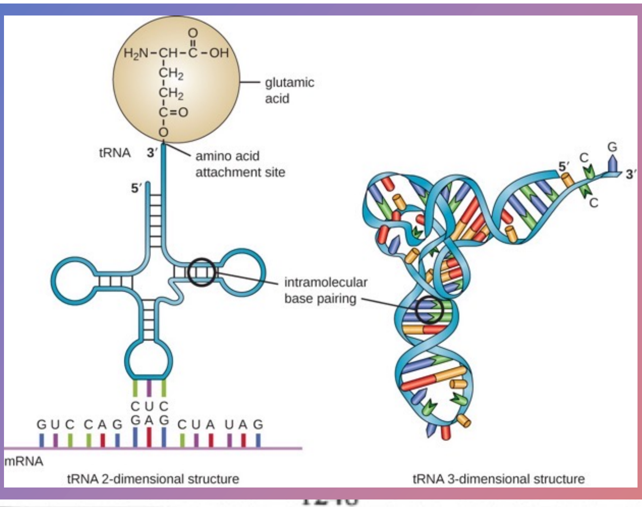






- L.D. Barron, L. Hecht, E.W. Blanch, A.F. Bell 2000, Progress in Biophys & Mol. Bio. **73**, 1-49
- A.F. Bell, L. Hecht, L.D. Barron 1998. *J. Am. Chem. Soc.* **120**, 5820.

tRNA Double Helix: Raman (top) & ROA (bottom)



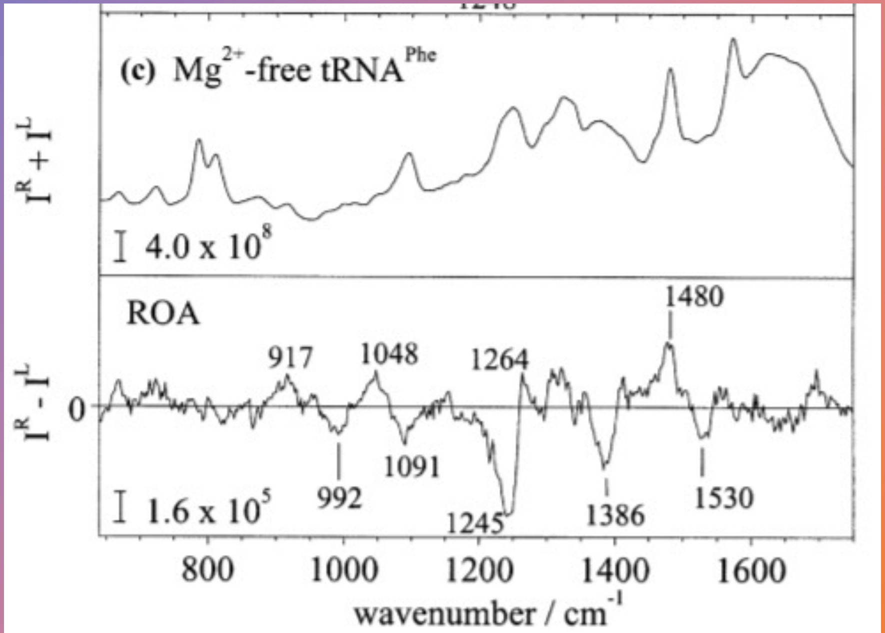
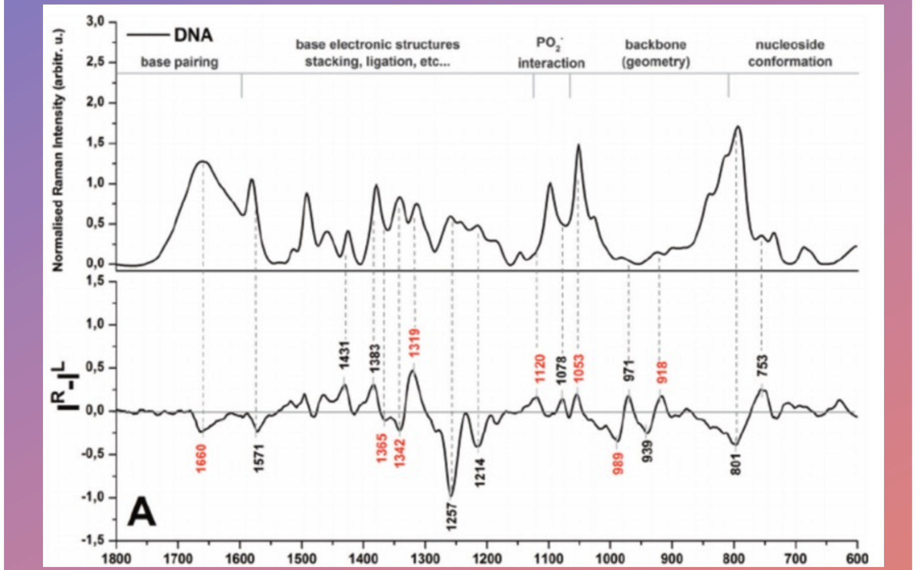


Image source for tRNA: https://courses.lumenlearning.com/suny-mcc-microbiology/chapter/structure-and-function-of-rna/

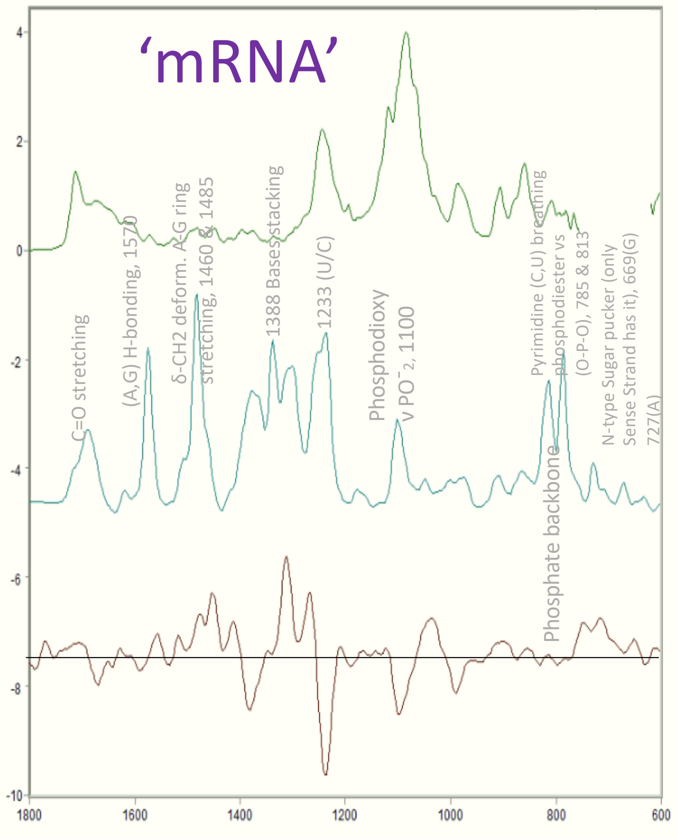
Source: https://www.researchgate.net/publication/10883622_Vibrational_Raman_Optical_Activity_of_Proteins_Nucleic_Acids_and_Viruses

Comparison of DNA / RNA:
note the incredible richness of Raman / ROA spectra

DNA



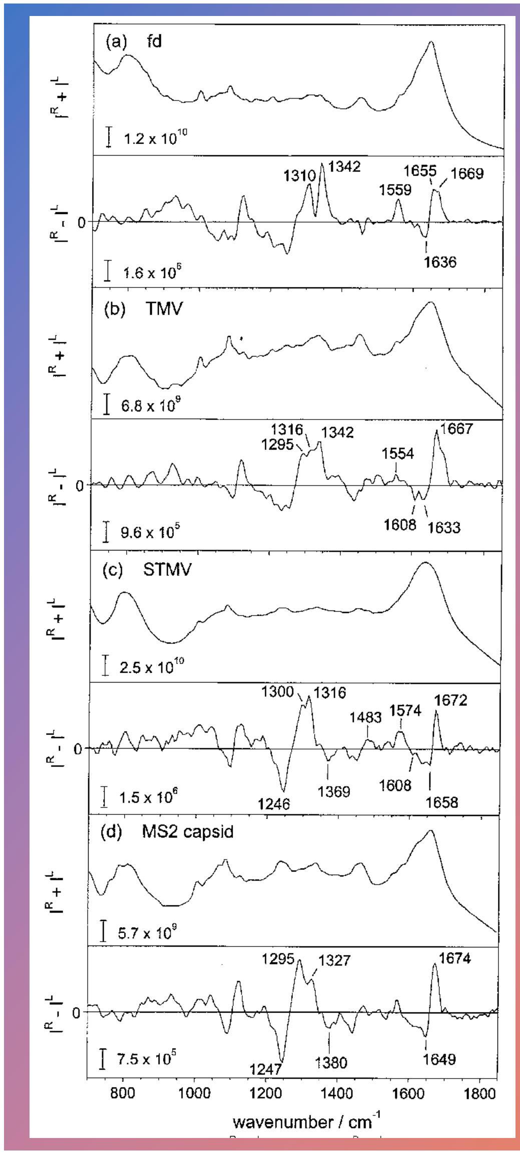
Source: https://www.researchgate.net/figure/Backscattered-SCP-Raman-and-Raman-optical-activity-ROA-spectra-of-DNA-A-and-the-DNA_fig1_283741138

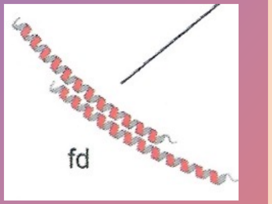


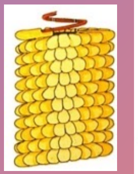
FT-IR

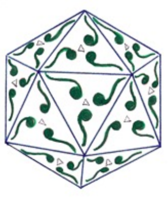
RAMAN

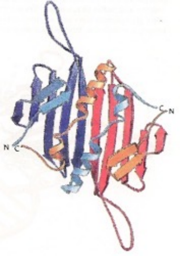
ROA











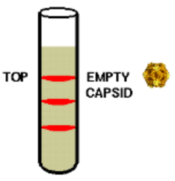
Raman & ROA of Viruses

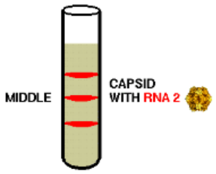
- Most virus types (filamentous, cylindrical and icosahedral) provide excellent ROA spectra.
- Coat protein folds can often be simply ‘read off’’
- ROA can ***simultaneously*** probe the structures of the protein and nucleic acid components of an intact virus

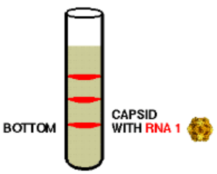
E.W. Blanch, L. Hecht, C.D. Syme, V. Volpetti, G.P.Lomonossoff, K. Nielsen, L.D. Barron 2002. *J. Gen. Virol.* **83**, 2593.

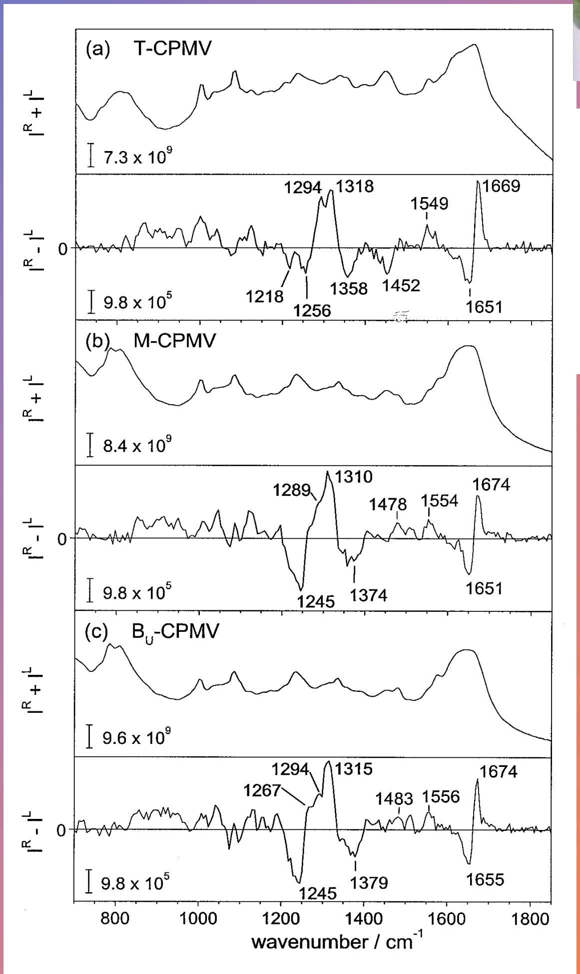
BioTools
20 YEARS OF INNOVATION

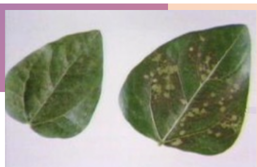
Cowpea Mosaic Virus (CPMV)

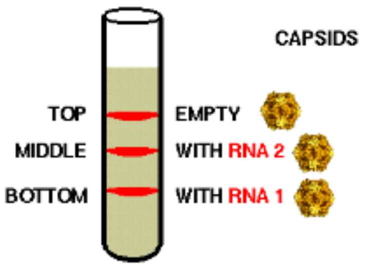


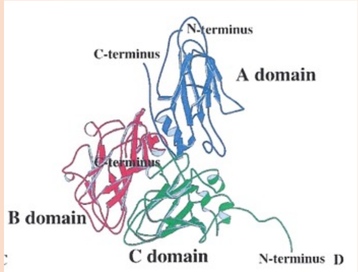


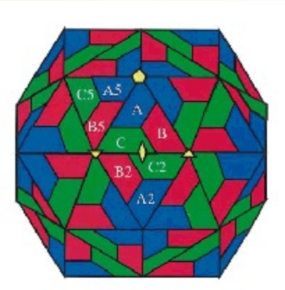








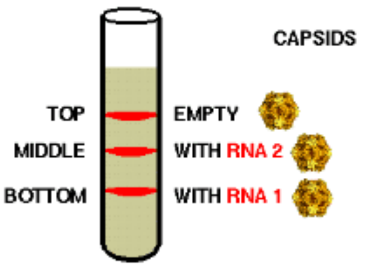




- Type member of the comovirus group of plant viruses.
- Preparations separate into three bands by centrifugation on CsCl density gradient.
- Bipartite genome of separately encapsidated RNA-1 and RNA-2 molecules.

E. W. Blanch, L. Hecht, C. D. Syme, V. Volpetti, G. P. Lomonossoff, K. Nielsen and L. D. Barron. *J. Gen. Virol.* **83**, 2593 (2002).

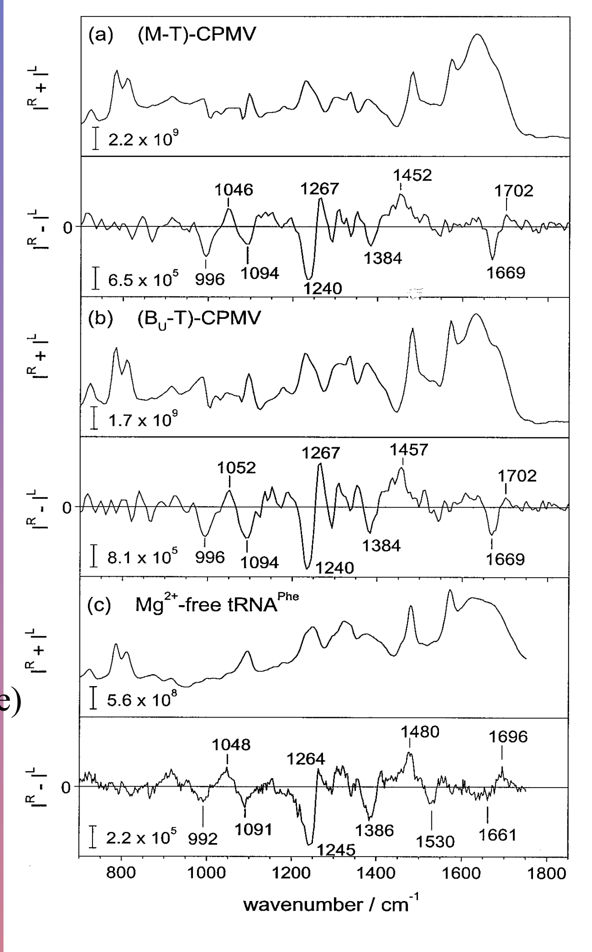
Cowpea Mosaic Virus (CPMV)



RNA-2

RNA-1

$\text{tRNA}^{(\text{Phe})}$



- Subtraction of the ROA spectrum of the top component (empty protein capsid) from those of the middle and bottom components provides ROA spectra of the RNA-1 and RNA-2 cores.
- The ROA of RNA-1 and RNA-1 are almost identical and are very similar to ROA of tRNA^{Phe}.

E. W. Blanch, L. Hecht, C. D. Syme, V. Volpetti, G. P. Lomonossoff, K. Nielsen and L. D. Barron. *J. Gen. Virol.* **83**, 2593 (2002).

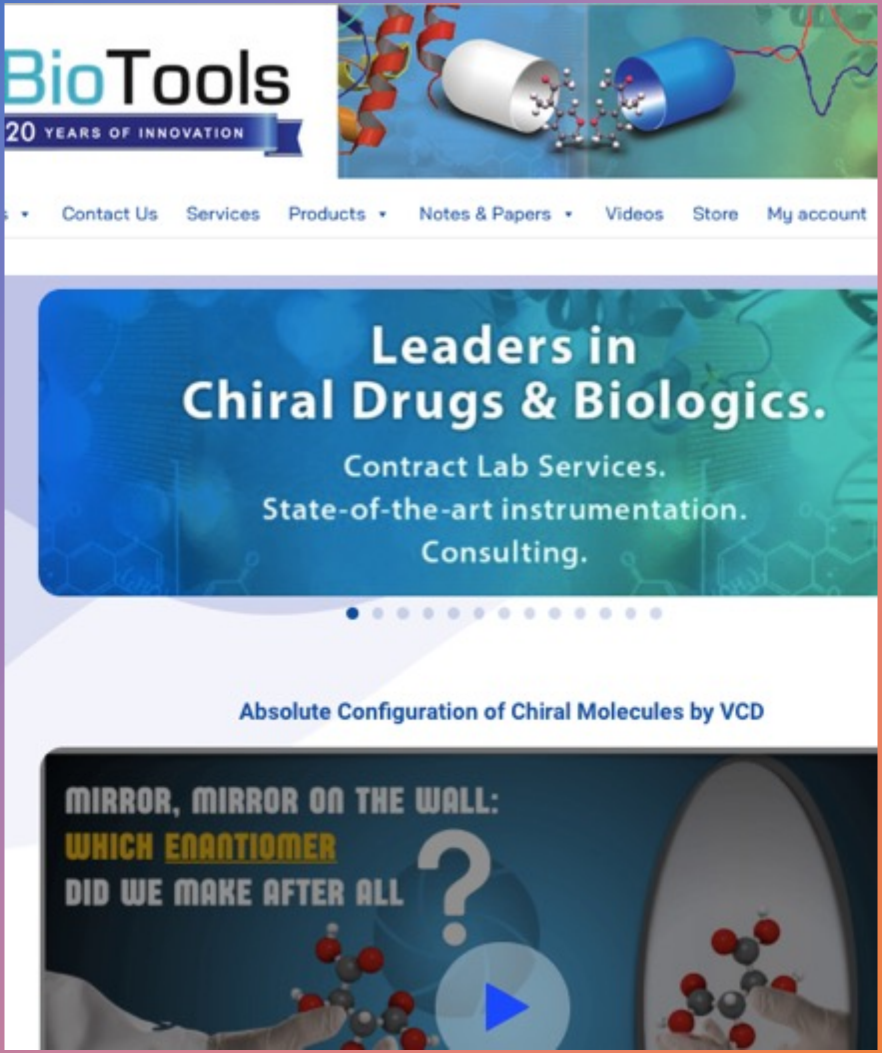
Bio & Advanced Therapeutics

"Forget enlightenment. I want you to concentrate on the structure of the protein molecule."

Structure is very sensitive to perturbation of any kind and to physical state

and structure is related to *function….*

Must know – folding, stressed degradation, *stability and comparability*



Leaders in
Chiral Drugs & Biologics.

Contract Lab Services.
State-of-the-art instrumentation.
Consulting.

Absolute Configuration of Chiral Molecules by VCD

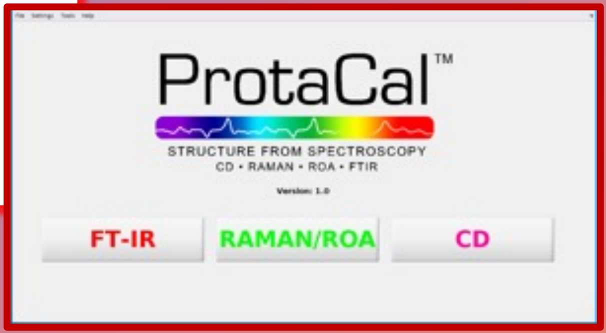
MIRROR, MIRROR ON THE WALL:
WHICH ENANTIOMER
DID WE MAKE AFTER ALL ?

Who are we?

- Co-founded by Dr. Rina Dukor and Professor Laurence Nafie in 2000;
- dedicated to helping customers solve real problems and bringing challenging groundbreaking techniques to market
- First to commercialize a dedicated solution for protein structure elucidation based on FTIR spectroscopy known as PROTA. (i.e. brought FTIR to biopharma market) that includes largest protein dbase; BioCell transmission cell & temperature controller
- First company to commercialize VCD for structure elucidation of chiral molecules (Chiral***IR***)
- First company to commercialize ROA – recipient of R&D 100 Award (Chiral***RAMAN***)

FT-IR

Protein Structure



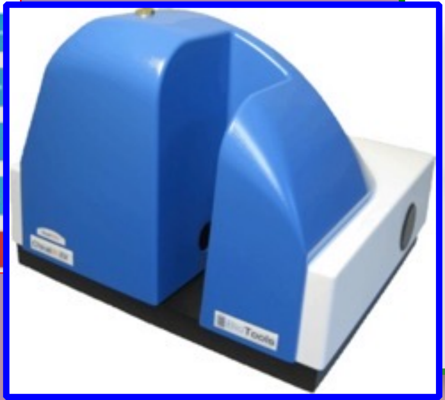
ProtaCal™

STRUCTURE FROM SPECTROSCOPY

CD • RAMAN • ROA • FTIR

Version: 1.0

VCD/IR



ROA/RAMAN

HAND-HELD RAMAN

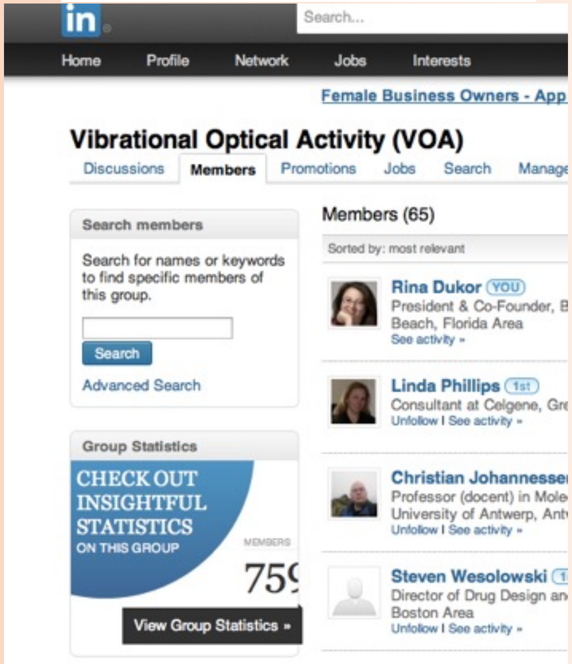
Acknowledgments

BioTools' dedicated employees & interns over the last 20 years

ALL our customers and collaborators for inspiring us to innovate

YOU for learning about BioTools techniques & products!

SOCIAL MEDIA



Female Business Owners - App

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MEMBERS 75

Members (65)

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Consultant at Celgene, Gre
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Professor (docent) in Mole
University of Antwerp, Ant
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Boston Area
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Thank you!