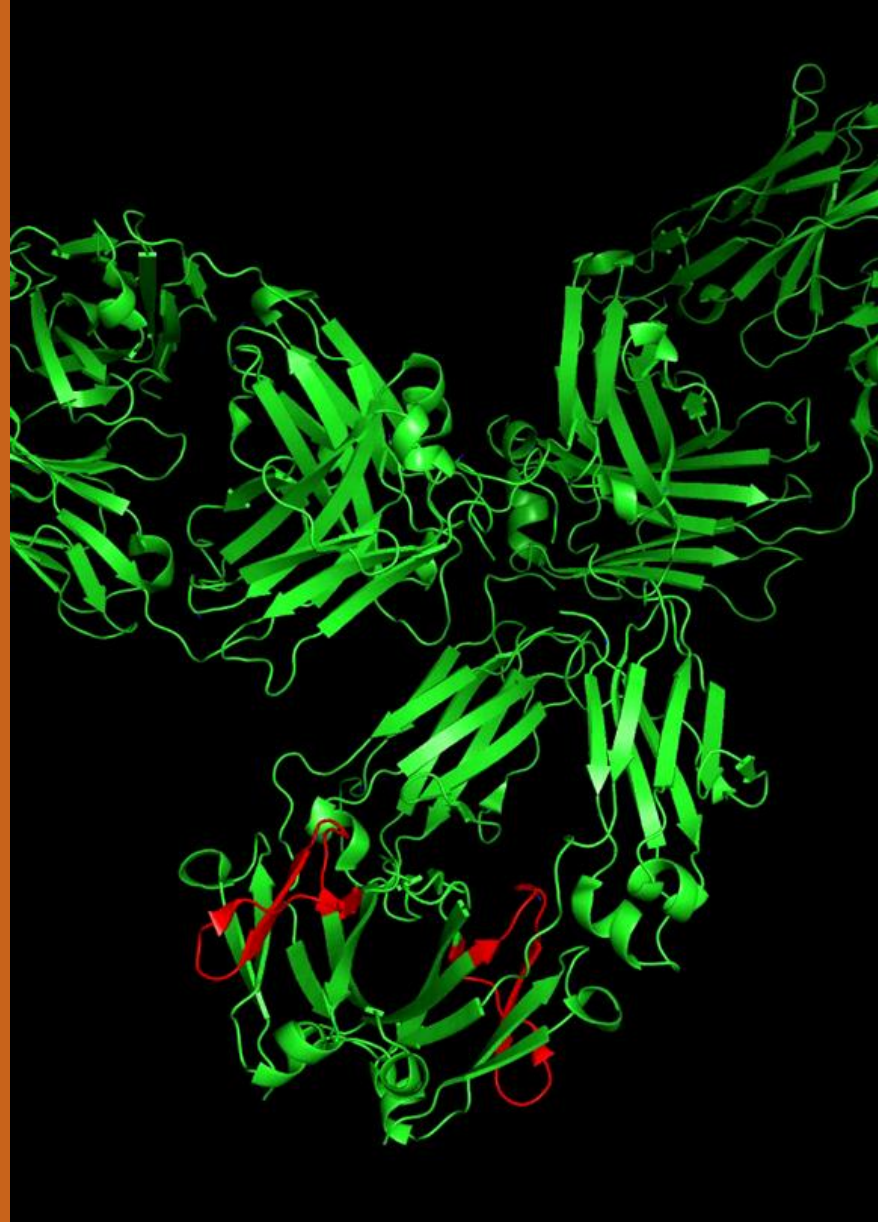
Higher Order Structure: Route to QC testing?



Carl Jone, UCB, Belgium

CASSS AT in Europe, Brussels, Belgium

14-17 March 2016

Inspired by patients.
Driven by science.

Introduction

Many quality attributes measured

- Why is HOS usually performed?

Why measure Higher Order Structure in QC?

- Regulatory request
- Are the current release and stability assays sufficient?
- Example of hGH

Comparison of Characterisation vs QC assays

Many HOS methods

- What HOS methods likely candidates for routine analysis?

Case Study

- Native Peptide maps

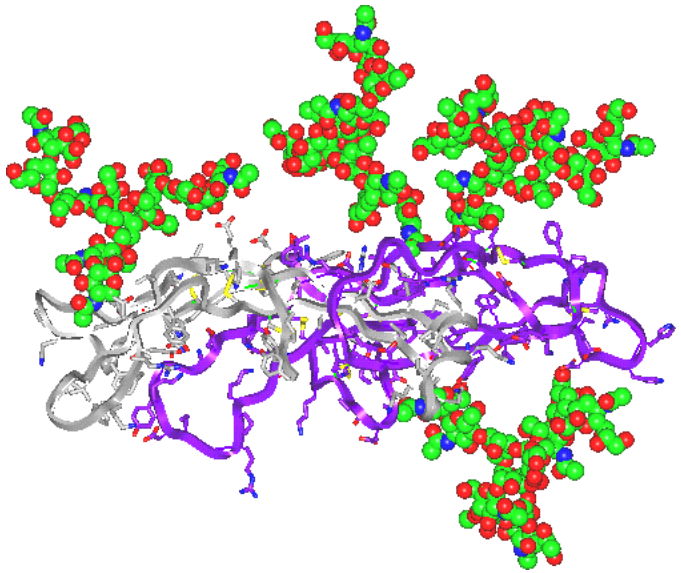
Conclusion

Many quality attributes measured

- Why is HOS usually performed?

There are many physicochemical methods and bioassays used to measure quality attributes of biopharmaceuticals.

- **Higher-order structure**
  - ***Circular Dichroism***
  - X-Ray Structure
  - ***NMR***
  - Epitope Detection
  - Specific Binding
  - ***FTIR***
  - ***Nmaps***
- **Structure / Sequence**
  - N- and C-terminus
  - Amino Acid Analysis
  - Peptide Mapping and Sequencing
  - Monosaccharide Analysis
  - Oligosaccharide Mapping
  - Mass Spectrometry
  - ***Disulphide linkage***
- **Assay**
  - OD, HPLC, AAA, Biacore, ELISA, IFMA, Bradford, Lowry, Bioassay
- **Activity**
  - Bioassay in vivo and in vitro
  - Specific binding assay
  - Gene reporter assay (immunogenicity)



- **Size & Aggregation**
  - ***SE-HPLC*** (also identity and assay)
  - SDS PAGE / Bioanalyser
  - ***AUC***
  - AF4
  - LLS
- **Purity**
  - RP-HPLC
  - SE-HPLC
  - Peptide mapping
  - SDS-PAGE
  - Field Flow Fractionation
  - Elisa (HCP)
  - Immunoblot
  - DNA assay
  - LAL test
  - Virus test
- **Identity**
  - N-Terminal Sequence
  - Peptide Mapping
  - Specific Bioassay
  - IEF
  - HPLC
- **Carbohydrate analysis**
  - ESI-MS (whole molecule)
  - MALDI-TOF (released carboh.)
  - Separation of labelled released carbohydrates (2-AA, 2-AB)
- **Surface charge**
  - IEF
  - CZE
  - **IEX-HPLC**
  - iCE280
  - Chromatofocusing

HOS evaluations in regulatory submissions

- **Release when there are aggregates**
- **Early characterisation studies. Structure-function relationships**
- **Supporting process development**
- **Comparability studies**
- **Stability –rare to include HOS studies**
- *Emily Shacter, FDA, CMC Strategy Forum 2011, Barcelona. Spain*

Why measure Higher Order Structure in QC?

- Regulatory request
- Are current release and stability assays sufficient?
- Example of hGH

Regulatory Expectations

Following a manufacturing process change, manufacturers should attempt to determine that higher order structure is maintained in the product.

ICH Q5E Comparability of Biotechnological product subject to changes in their manufacturing process, 2004

" Our current ability to predict the potency of biologics would be enhanced if we had improved ability to measure and quantify the correct three-dimensional structure, aberrant three-dimensional structrure and the distribution of the different three-dimensional structures"

Steven Kozlowski, Director, Office of Biotechnology Products, CDER, FDA, 2009 before the Committee on Science and technology, US House of Representatives

Regulatory Expectations (*17th CMC Strategy Forum Jan 2010)*

- It was acknowledged that some of the latest available technologies may not yet be amenable for measuring higher-order structure in a quality control (QC) setting.
- In line with QbD, higher-order structure analysis will increasingly become an expectation.
- But…
- Regulatory attendees confirmed that their agencies have not been requiring advanced higher-order structure studies for most investigational new drug (IND) submissions, unless they are necessary to establish comparability.

- *The Role of Higher-Order Structure in Defining Biopharmaceutical Quality, Wei et al, BioProcess International, 58-66, April 2011*

Are current release and stability assays sufficient? Thioether in hGH

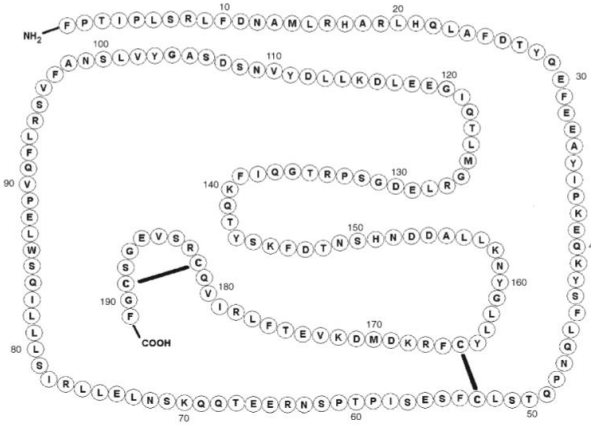


Figure 1. Primary structure of human growth hormone including the disulfide bridge pairing.

Table 2. Thioether Content Estimated by ES/MS Whole Molecule Analysis

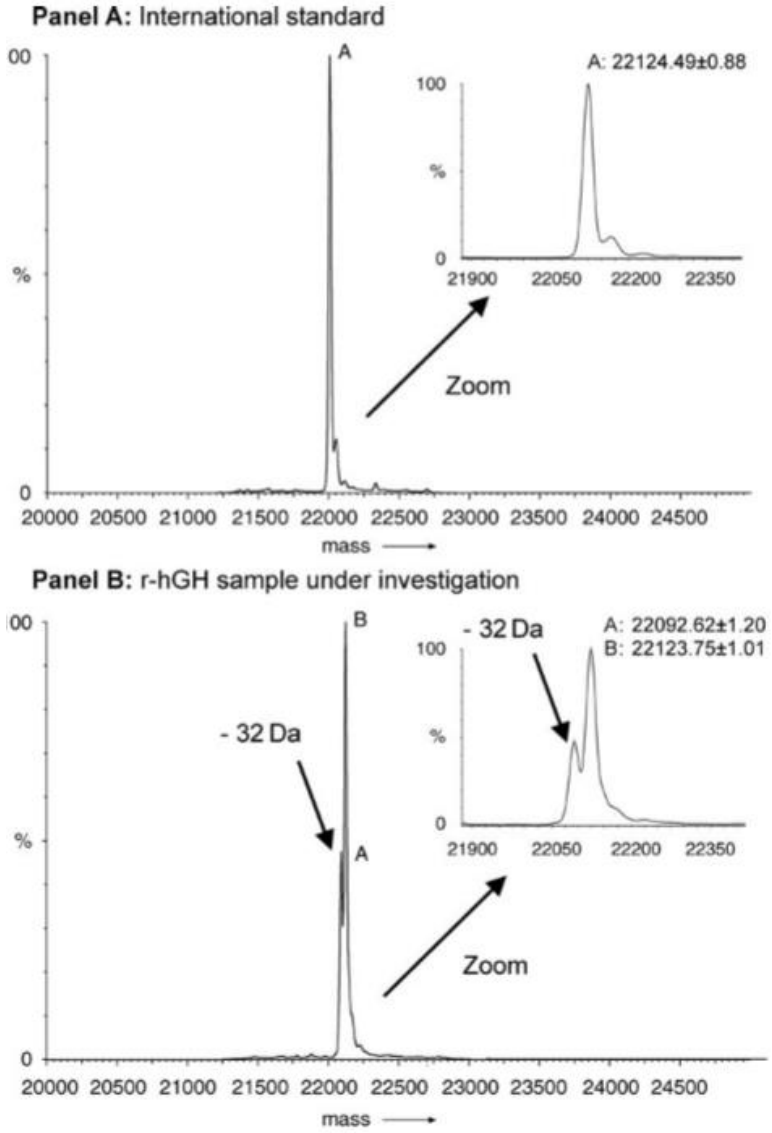
Product	Batch Code	% Thioether Variant
Hormotrop® (4 IU)	50897	32
Hormotrop® (4 IU)	50793	7
Hormotrop® (12 IU)	51026	6
Hormotrop® (12 IU)	50923	18
Yelit® (4 IU)	4684	10
Cryotropin® (4 IU)	50631	5
Saizen® (8 mg click.easy)	SC305D	Not detectable
Saizen® (8 mg click.easy)	SC310	Not detectable
NIBSC r-hGH	98/574	Not detectable
NIBSC p-hGH	80/505	Not detectable
EP r-hGH CRS	Batch 1	Not detectable

Table 1. Assessment of r-hGH Product Quality by Compendial Methods

Analytical Method	Expected Information	Dong-A, Lots 1–5	Merck Serono, Lots 1 and 2	BTG, Lot 1
RP-HPLC (EP & USP)	r-hGH related proteins (degraded forms)[a]	Conform	Conform	Conform
SE-HPLC (EP and USP)	Assay and purity profile (aggregate forms)	Conform	Conform	Conform
Peptide mapping (EP and USP)	r-hGH identity	Conform	Conform	Conform
CZE (EP)	Charged variants (related impurities)	Conform	Conform	Conform

Datola et al, ChemMedChem 2007, 2, 1181-1189
Lispi et al, Journal of Pharmaceutical Sci, 98, 12, 4511-4524, 2009

Are current release and stability assays sufficient? Thioether in hGH



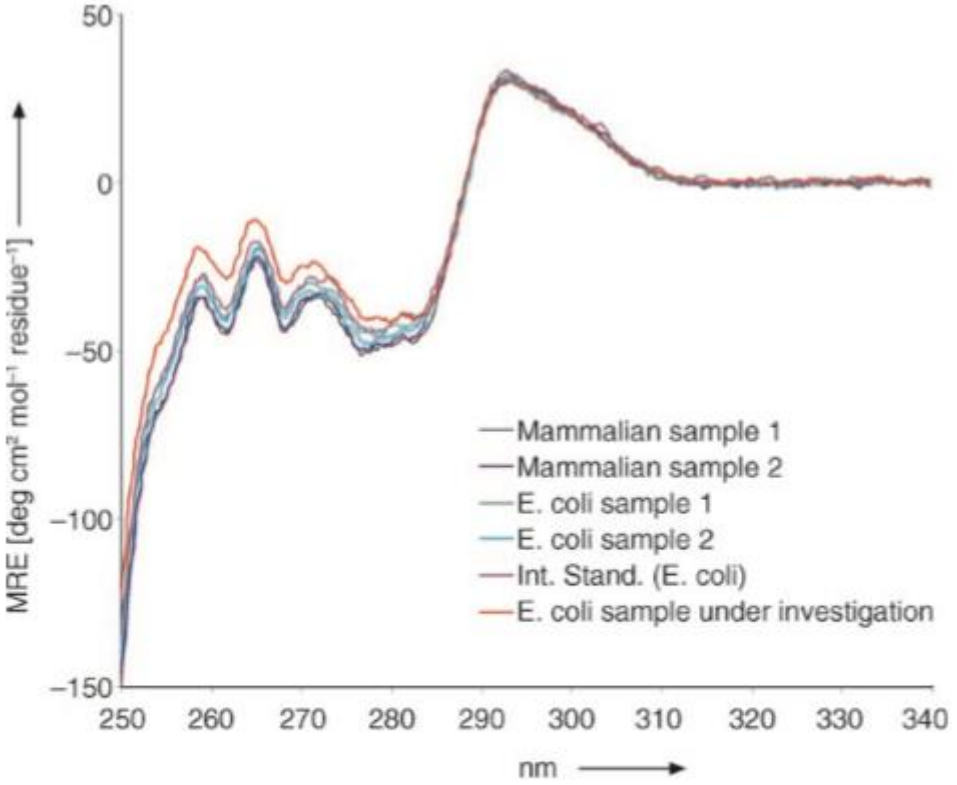


Figure 8. Near UV CD spectra of hGH samples expressed in *E. coli* and mammalian cells.

24, 2009

Comparison of Characterisation vs QC assays

Many HOS methods

- Usually ensemble methods
- What HOS methods likely candidates for routine analysis?
- Trouble with wavy lines
- Quantitative spectroscopy

Differences between Characterisation and QC assays

Characterisation	QC
Expensive equipment	Cheaper equipment
Complex interpretation	Simple Yes/No answer
Non Validated, Fit for purpose	Validated
Difficult to tech transfer	Tech transferable
Fit for purpose	Highly robust
Short term studies	Designed for long-term use (>10 years)
Highly specialist operators, rare skillset	Generalist operators
Speed and high throughput not primary driver	High throughput and speed essential

The problem with populations & ensemble methods

The problem with populations & ensemble methods

The problem with populations & ensemble methods

Typical HOS methods

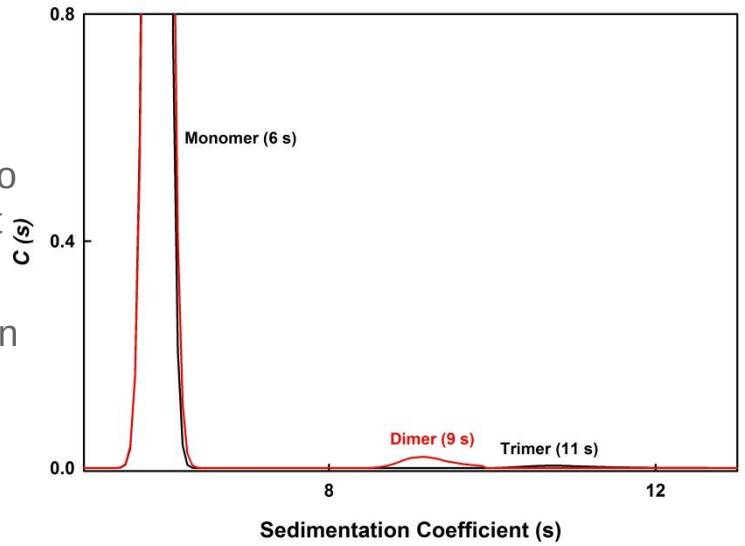
	Metric	Characterisation	QC
Circular dichroism	Secondary, Tertiary	✓	?
FTIR	Secondary	✓	?
AUC	Quaternary, aggregates	✓	✗
Intrinsic fluorescence	Tertiary	✓	?
DSC	Tertiary structure (Tm)	✓	✗
NMR	Tertiary, Quaternary	✓	?
AF4	aggregates	✓	✗
X-ray	Tertiary, Quaternary	✓	✗
Intact native MS	Tertiary, Quaternary	✓	✗ ?
HDX by LCMS	Tertiary	✓	✗
Peptide map LCMS	Tertiary	✓	✓

AUC good for aggregates – not QC friendly

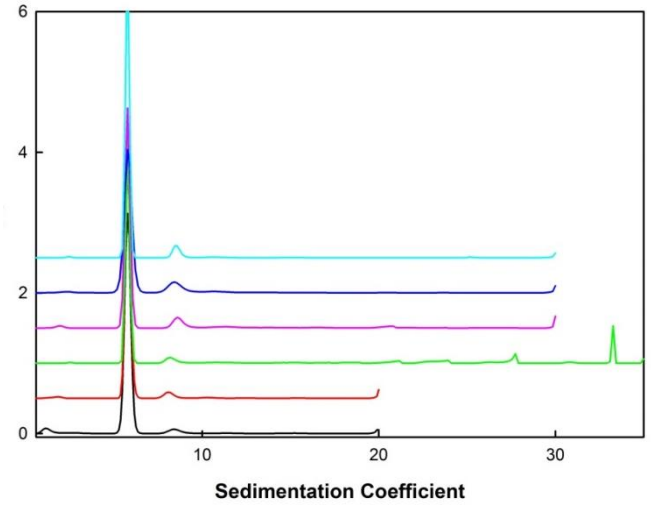
AUC characterises aggregation species in process and FDS studies

- (1) the corresponding *c(s)* distribution of Mab A, process 3 (red line) and process 2 (black line).
- Trimers were the predominate species in process 2 compared to process 3, where dimers predominated. This difference was not detected by SEC and not resolved using DLS.
- In a separate experiment, we demonstrated that the composition of the formulation was changing the aggregate stoichiometry.

1



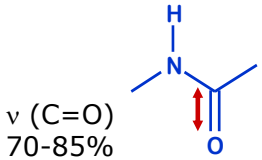
2



- AUC has also been used in our FDS studies (2).
- The amount and type of aggregation differ between different conditions, with 50°C (green line) showing a far larger species.
- Interestingly, the data suggests that the monomer confirmation remains similar under each condition and activity was not impacted (SPR data not shown).

Wavy Lines: Difficulty with spectroscopic methods (FT-IR)

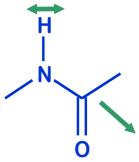
- FTIR applied to FDS of therapeutic mAb. Which modifications are responsible for structural changes?
- Normalised absorbance data set (overlay of 10 spectra).
- Obvious differences in FDS samples shown below. Broadening of Amide I peak observed for 50°C samples.



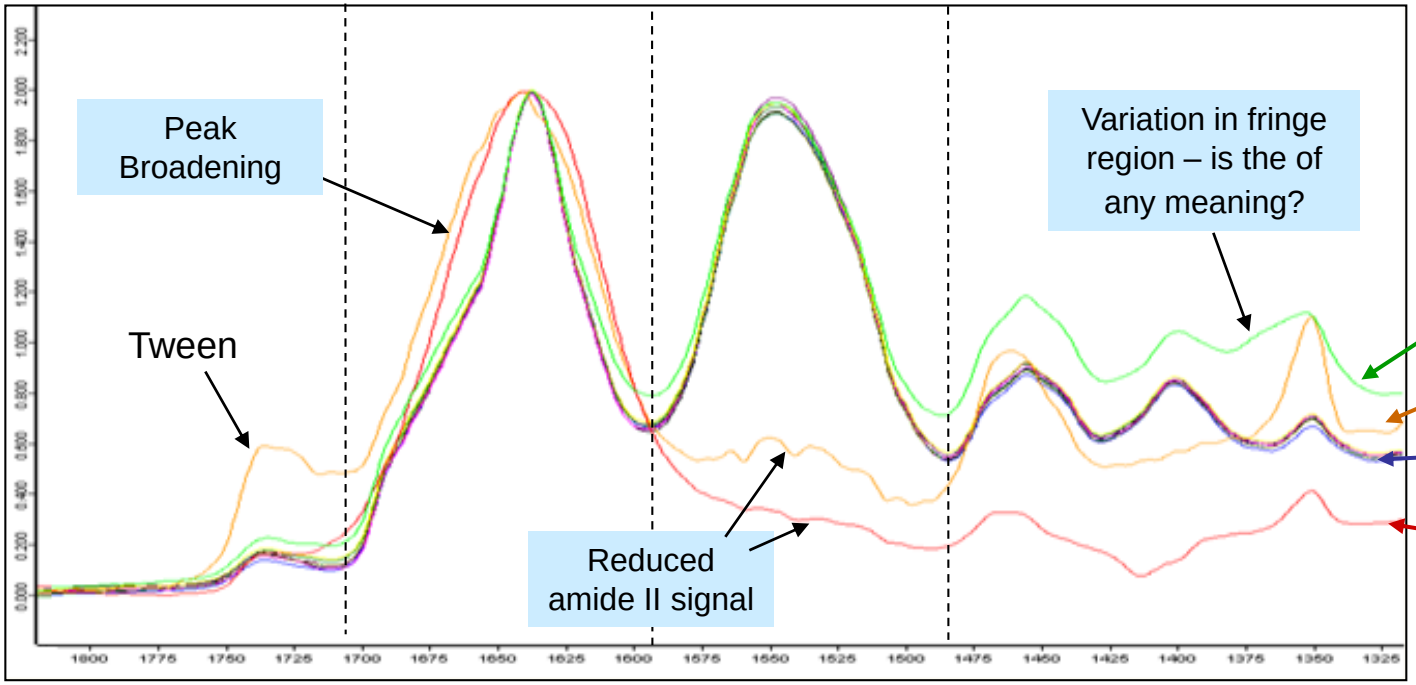
ν (C=O)
70-85%

Amide I

Amide II



δ (N-H) major
ν (C-N) minor



*All samples = 2-8°C day 0, wks 2 & 12; RT wks 2 & 12; 37°C wks 2 & 12; C-term Lys 37°C

Wavy Lines: Difficulty with spectroscopic methods (CD)

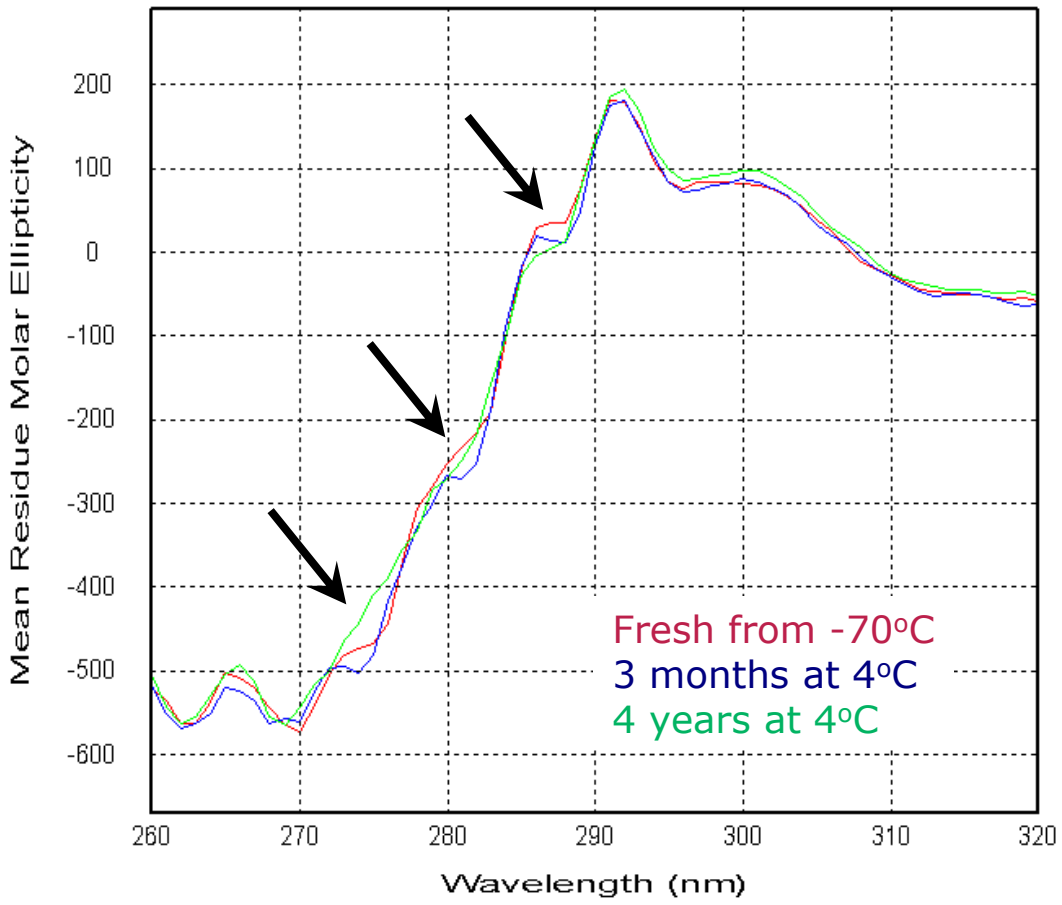
Near UV CD of a MAb aged at 4°C

Probing 3˚ and 4˚ structure

Samples similar

Samples vary subtly (arrows)

Question: would they be comparable in a characterisation study? Probably yes.



Quantitative Spectroscopy

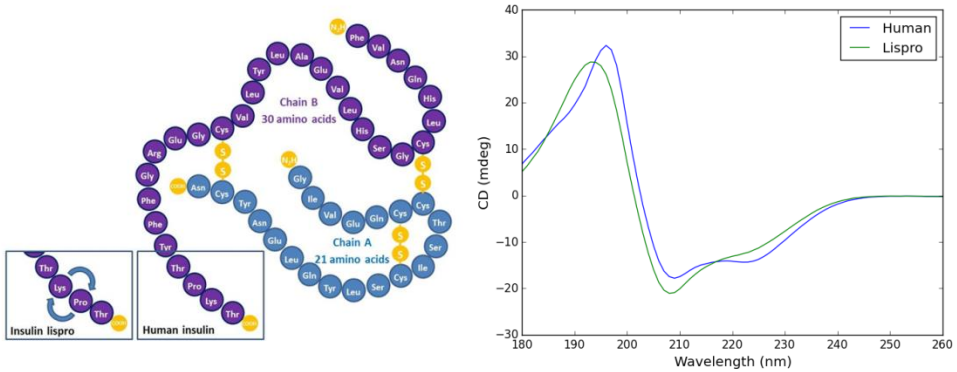
> ***Spectroscopy has lacked an objective means of comparing spectra, making it difficult to detect small differences in the data (and hence small differences in HOS).***

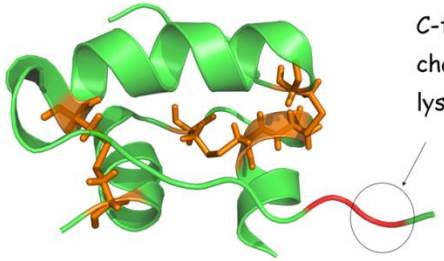
> ***For this reason a number of proposals have been put forward to make the comparison of CD spectra objective and quantitative (Bierau & Tranter, 2008) (Teska et al., 2013) (Dinh et al., 2014).***

Quantitative Circular Dichroism opportunities for QC?

Insulin study at APL (Marshall, 2015) provides PoC for Innovate proposal

- Lispro and human insulin differ by a switch of one amino acid and have different Far UV CD spectra.
- Using the WSD (Dinh *et al*., 2014), APL were are able to detect a statistical difference between insulin and an insulin + 2.5% Lispro-spiked sample.





C-terminus of B-chain and site of lys:pro reversal

Weighted spectrum difference

***Characteristic: WSD>0, 0 = identical*; does not normalise data**

$$\mathrm{WSD} = \sqrt{\sum_{i=1}^{n}\left[\frac{1}{n}\left(\frac{|x_i|}{|x_i|_{\mathrm{ave}}}\right)(x_i - y_i)^2\right]}$$

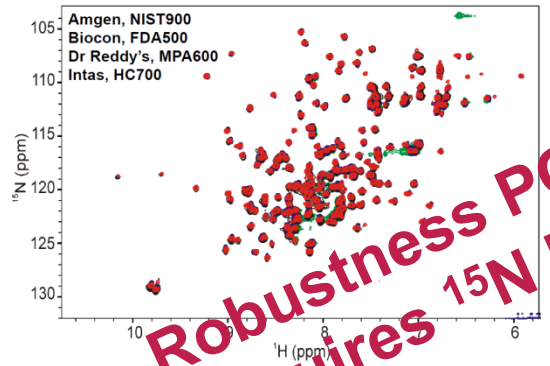
Dopant Concentration	WSD (average)	p-value (average)
0% (Control)	0.12	0.497
2.5%	0.20	0.00798
5%	0.25	5.00e-5
10%	0.51	9.59e-8

All p< 0.05 for non control dataset and >0.05 for control dataset

NMR opportunities for QC?

> NMR provide High Resolution and robust structural fingerprints data for NBE

Comparison of 4 Filgrastim Products: ^{1}H-^{15}N HSQC NMR Spectra at 4 sites



Nearly identical 'finger print' map b[illegible] the 4 samples/instruments/magnetic fields using com[illegible] acquisition and processing parameters

Robustness POC
Requires ^{15}N labelling

"In contrast to CD, IR or SEC, the NMR spectral fingerprint uniquely provides a combined readout of the primary and higher order structure of the protein at atomic resolution."

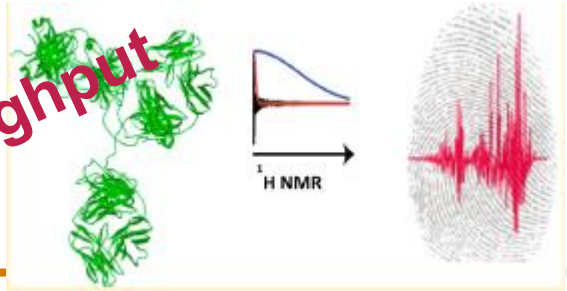
Profiling Formulated Monoclonal Antibodies by ^{1}H NMR Spectroscopy

Leszek Poppe,[†,*] John B. Jordan,[†] Ken Lawson,[‡] Matthew Jerums,[‡] Izydor Apostol,[‡] and Paul D. Schnier[†]

[†]Molecular Structure and Characterization and [‡]Process and Product Development, Amgen Inc., One Amgen Center Drive, Thousand Oaks, California 91320, United States

Amgen

High throughput



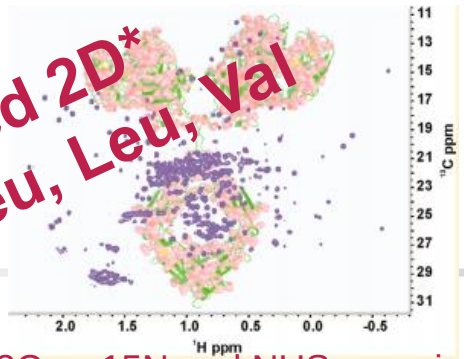
Mapping Monoclonal Antibody Structure by 2D ^{13}C NMR at Natural Abundance

Luke W. Arbogast, Robert G. Brinson, and John P. Marino*

Institute for Bioscience and Biotechnology Research, National Institute of Standards and Technology and the University of Maryland, 9600 Gudelsky Dr., Rockville, Maryland 20850, United States

NIST

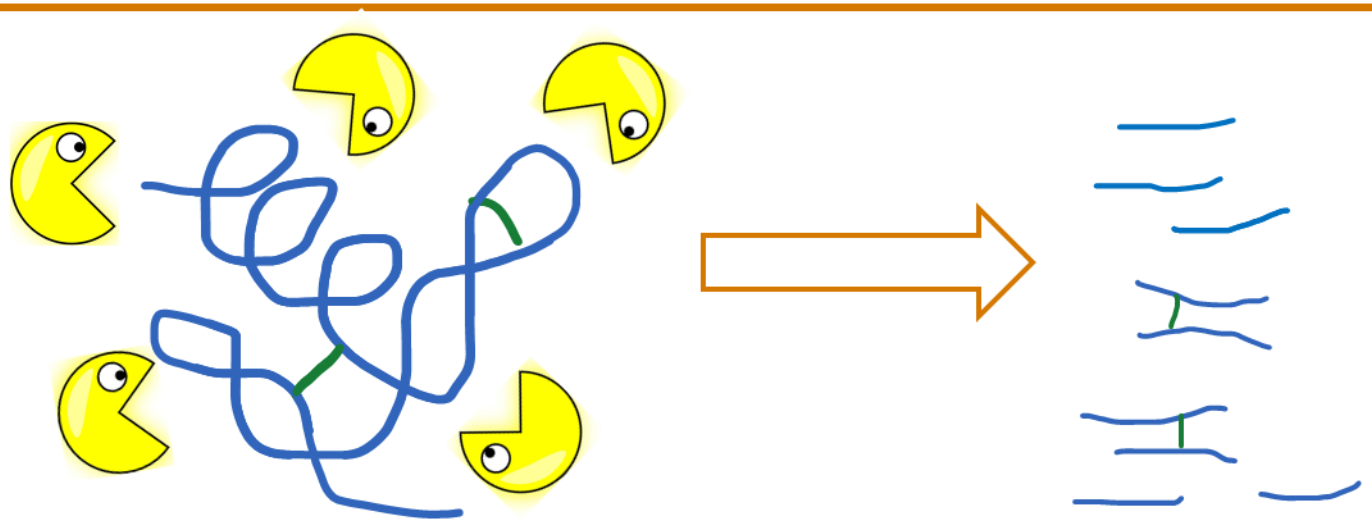
High speed 2D*
Me of iLeu, Leu, Val



*Thanks to higher sensitivity of 13C vs 15N and NUS experiments

Classical peptide map vs Native peptide maps

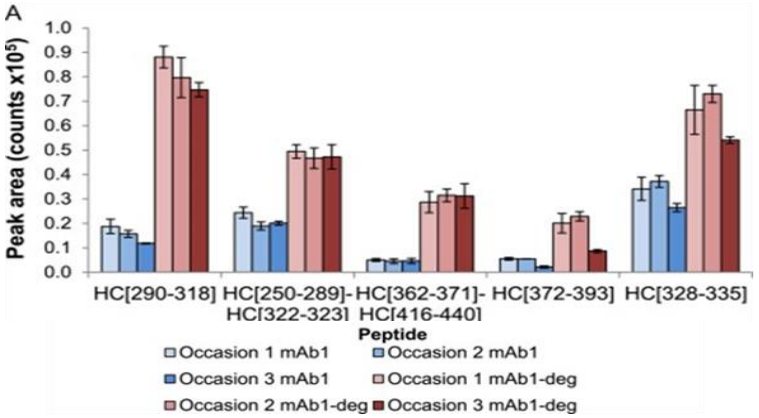
Loss of Higher Order Structure

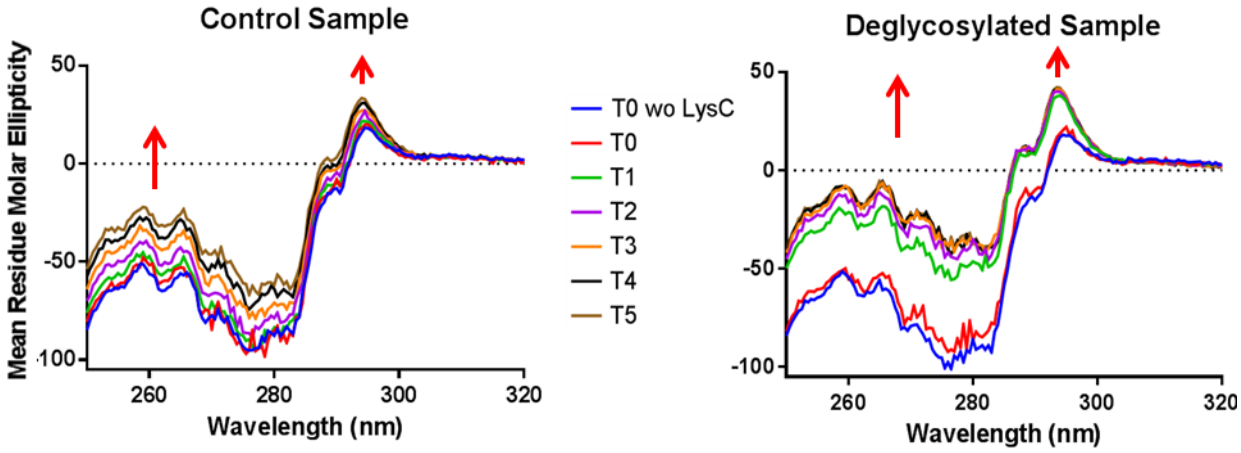


Native peptide map: Comparison on Mab and deglycosylated Mab

Perrin et al, Journal of Pharmaceutical and Biomedical Analysis, 123, 162-172, May 2016

- **The results are repeatable**
- **Simpler that HDX**



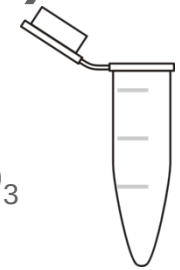


Evaluation in Development lab

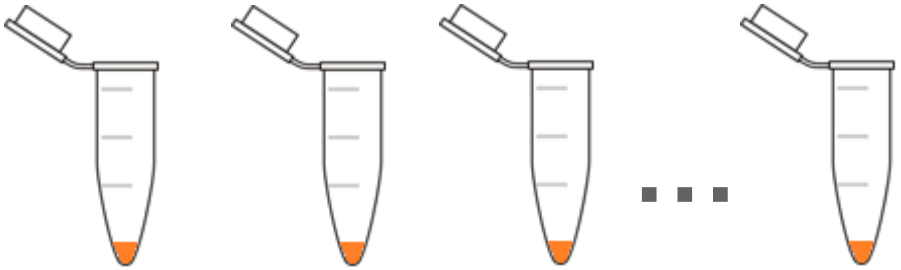
Method (detection by MALDI)

200µg Mab + 20µg trypsin ➔ ratio 1/10

Incubation @ 37°C – 0.1M NH_4HCO_3



20µL of digest / time point



T_{0min} 180µL TFA 1% (quench)

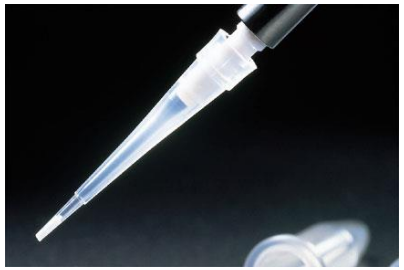
T_{5min} 180µL TFA 1% (quench)

T_{15min} 180µL TFA 1% (quench)

T_{240min} 180µL TFA 1% (quench)

Each time point sample is desalted thanks to:

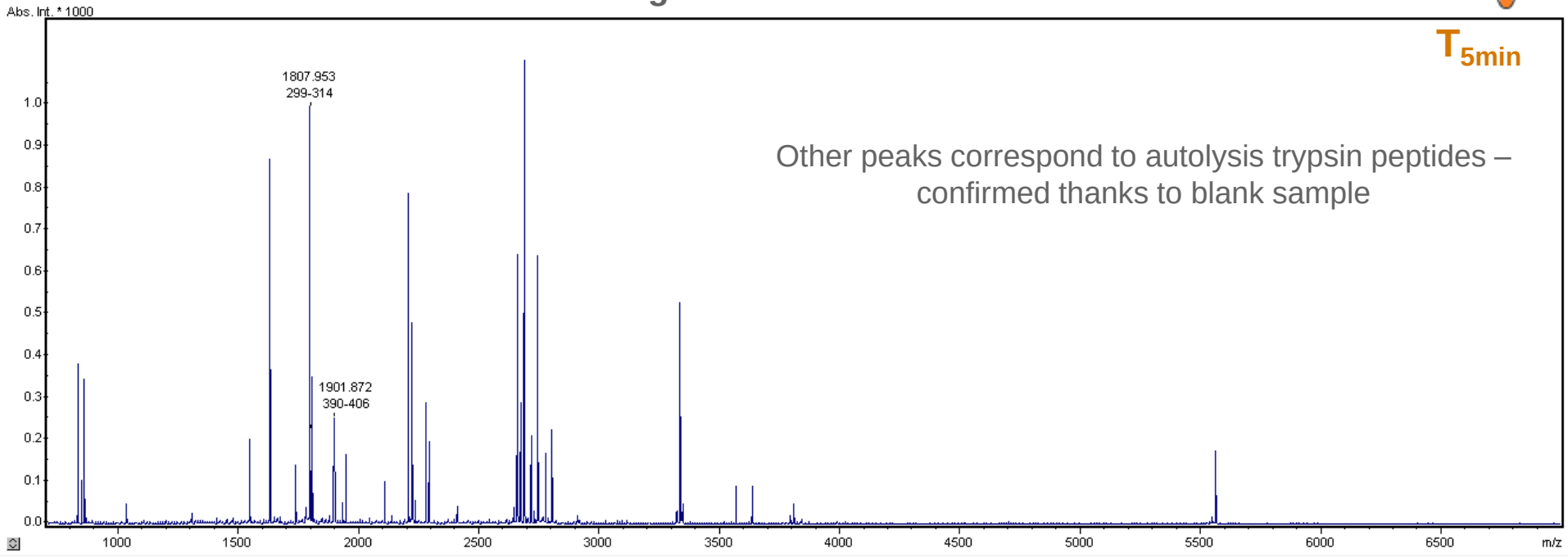
- **ZipTip C18 (peptides analysis)**



Evaluation in Development lab

T5min

Results – 700 to 7k m/z mass range

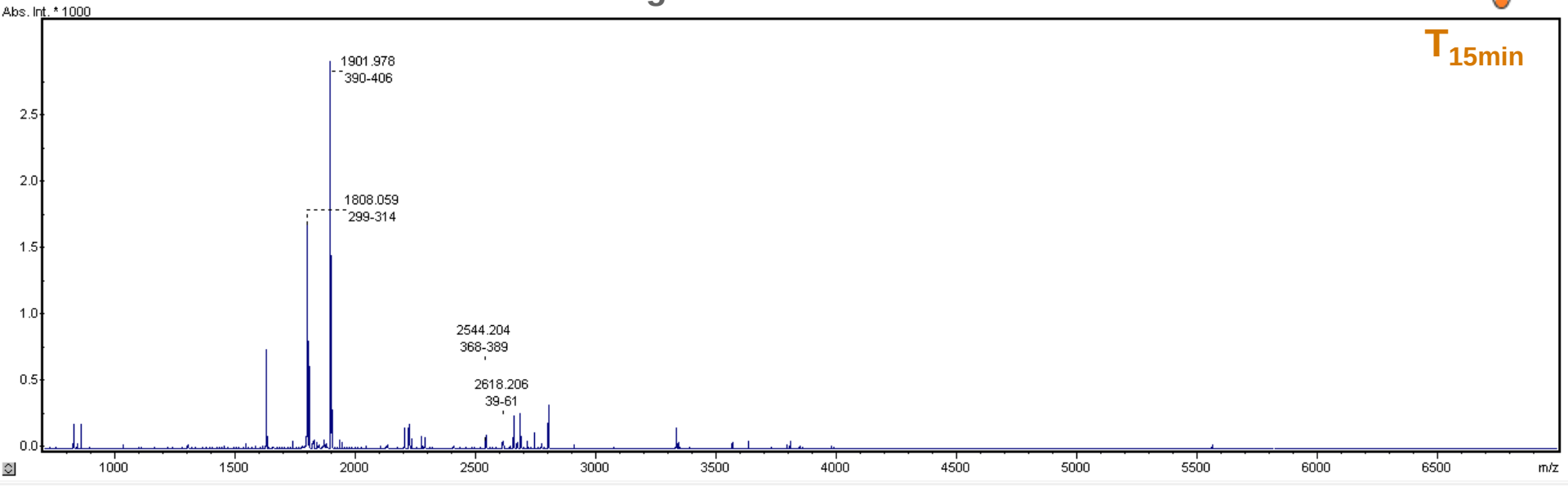


Other peaks correspond to autolysis trypsin peptides – confirmed thanks to blank sample

2 HC peptides identified – HC299-314 & HC390-406
Results confirmed in a second experiment (same strategy – different day)

Evaluation in Development lab

Results – 700 to 7k m/z mass range

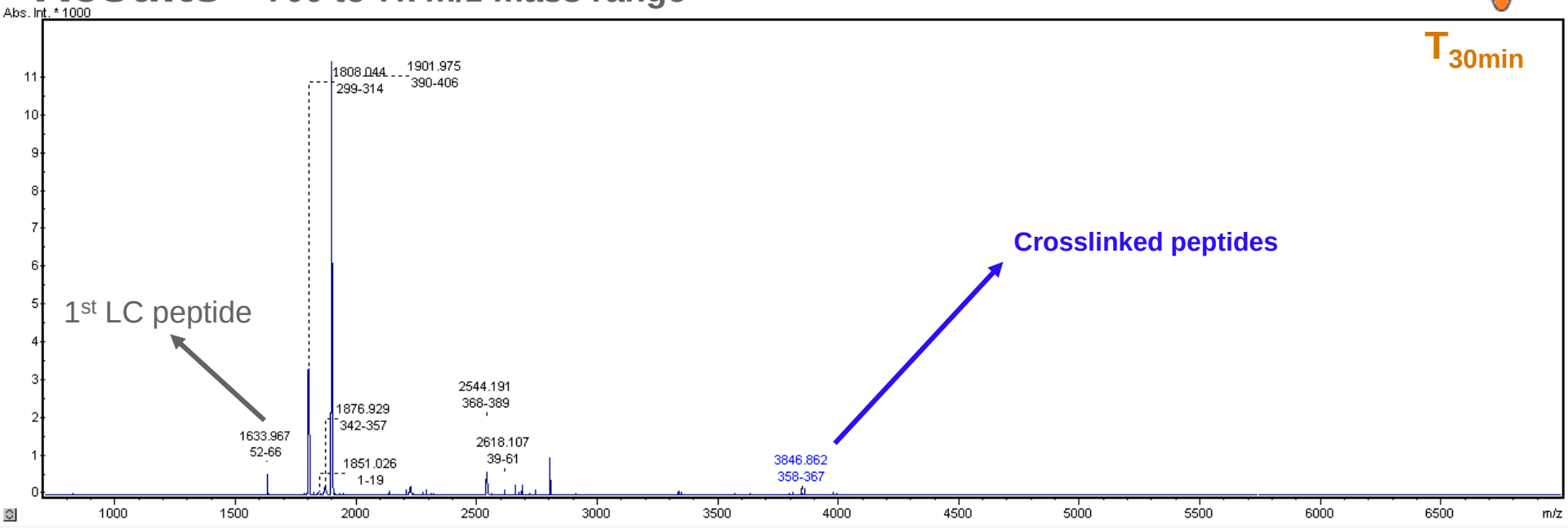


2 new HC peptides (HC 38-61 and HC 368-388) identified after 10 additional min incubation

Evaluation in Development lab

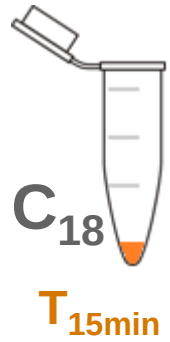
Results – 700 to 7k m/z mass range

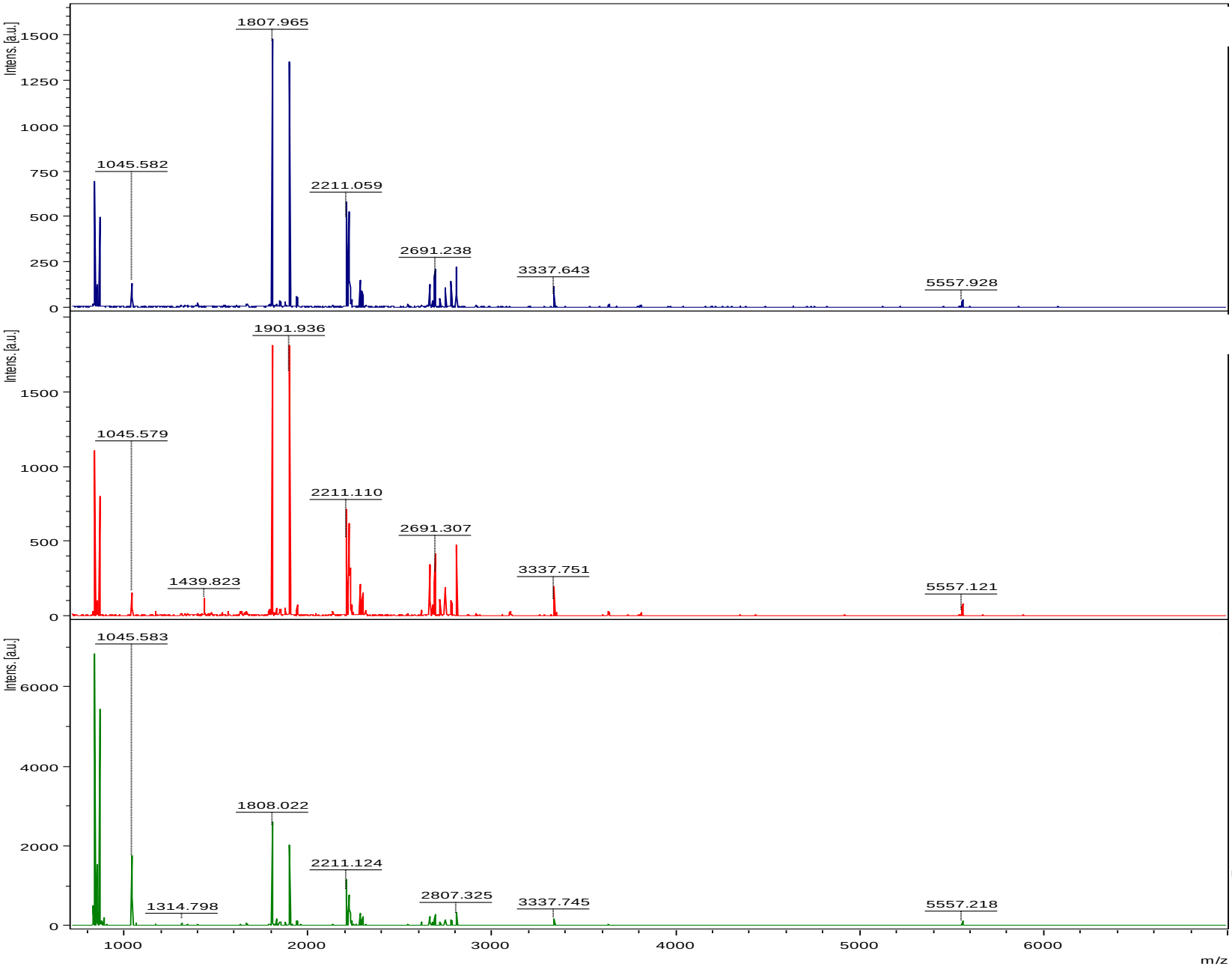
T_{30min}



New cleavage sites 4 HC peptides and 1 LC (HC 1-19, 342-358, HC 359-367, 414-436, LC 52-66) become accessible for the trypsin

Repeatability test – sample n=3

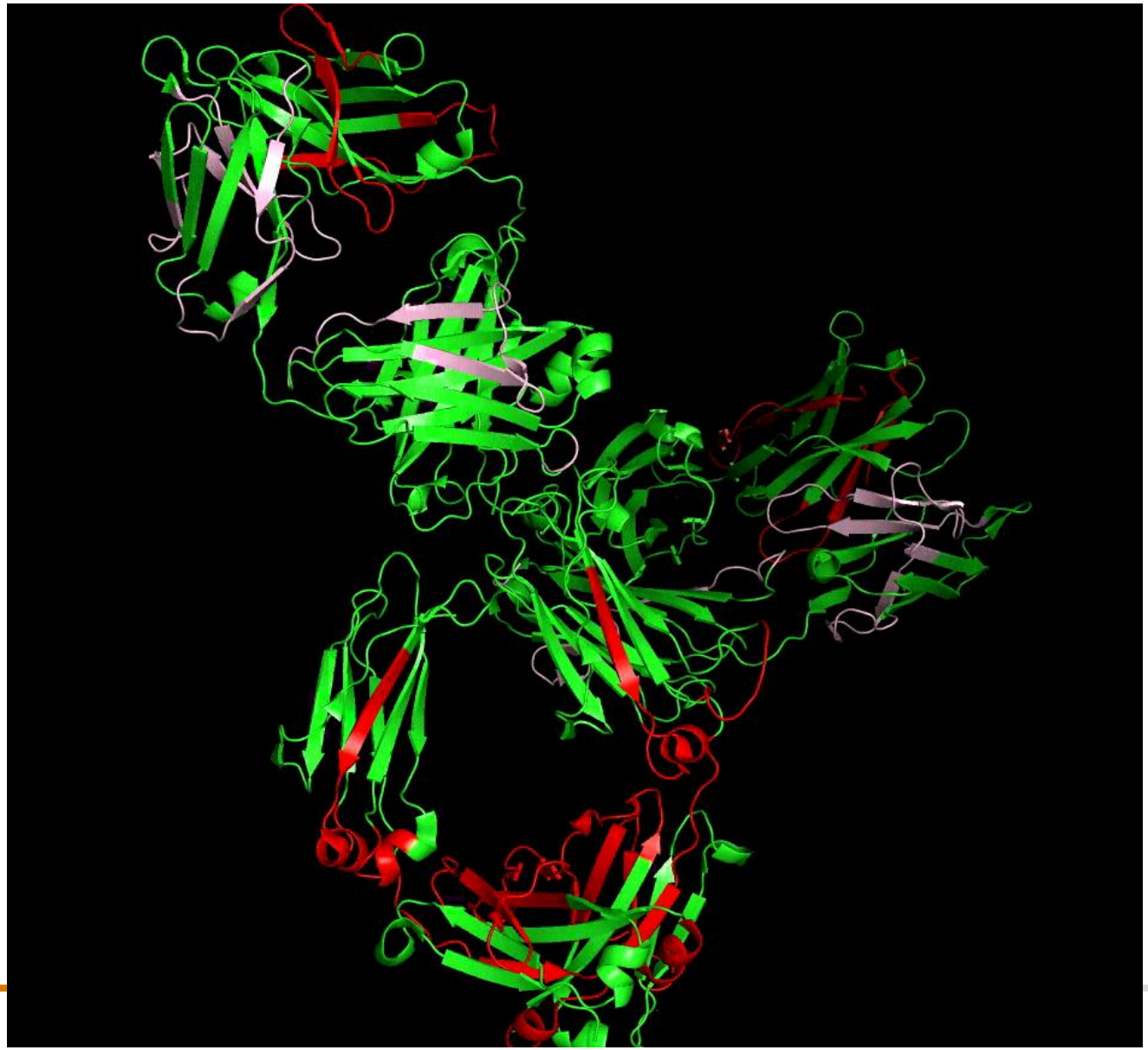




Assignment
+
Structure
Next slides

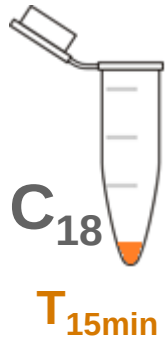
Repeatability test – sample n=3

3D structure



Red = HC

Pink = LC

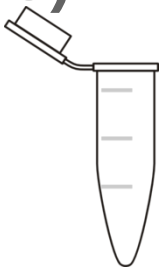


Native peptide map: Structure-function study

Method (MALDI & LC UV/MS)

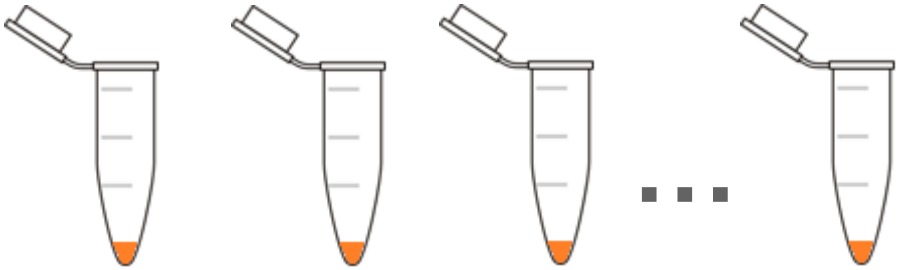
200µg Mab + 20µg trypsin ➔ ratio 1/10

Incubation @ 37°C – HBSS buffer due to Bioassay experiment



Time point (min)
0
5
15
30
60
280

8µL of digest / time point



T_{0min} 42µL TFA 1% (quench)

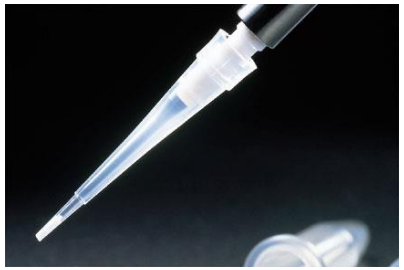
T_{5min} 42µL TFA 1% (quench)

T_{15min} 42µL TFA 1% (quench)

T_{280min} 42µL TFA 1% (quench)

Each time point sample is desalted thanks to:

- **ZipTip C18 (peptides analysis)**



Native peptide map: Structure-function study

3 Forced degradation studies samples were analyzed:

- Acidic pH stress
- Temperature stress
- Oxidative stress

Comparison with ➔ reference standard

Stress description:

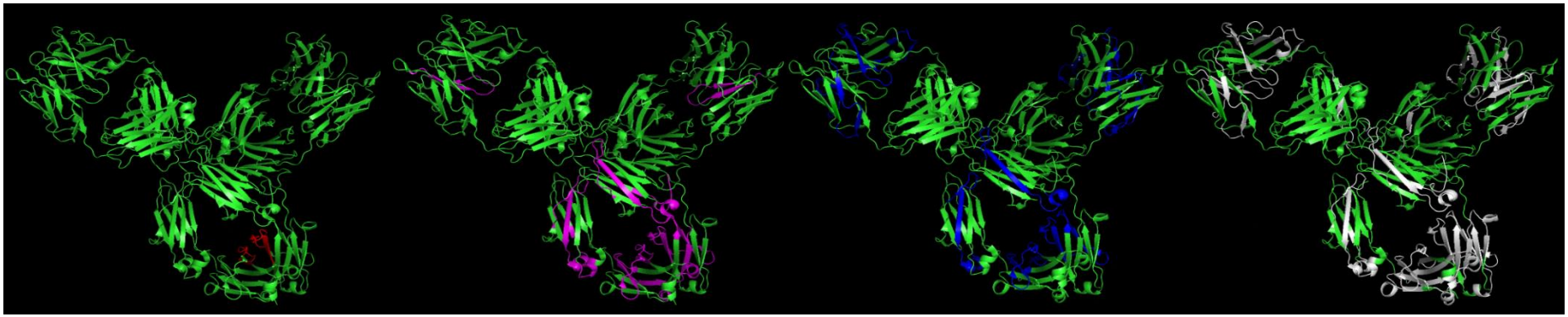
Acidic stress ➔ Incubation @ pH 3 during 14 days @ 5-8°C

Temperature stress ➔ Incubation @ 50°C during 14 days

Oxidative stress ➔ Incubation with 0.1% H_2O_2 @ 5-8°C

Goal: Correlate structural study based on limited digestion MALDI-MS analysis with biological activity

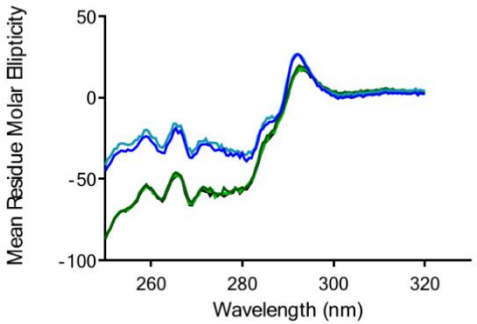
Comparison of stressed Mabs after 5 min proteolysis



Ref Std | Oxidative stress | Temperature stress | Acidic pH stress

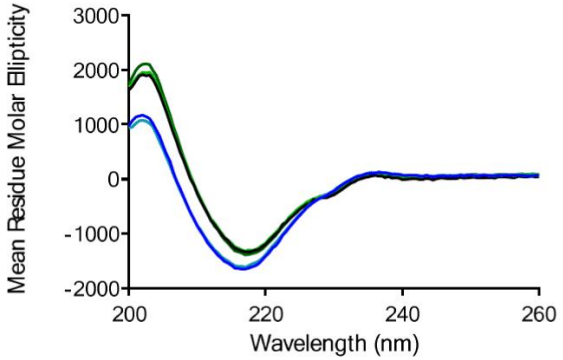
Enzyme accessability

Focus on acidic pH stressed sample



Near UV CD spectra

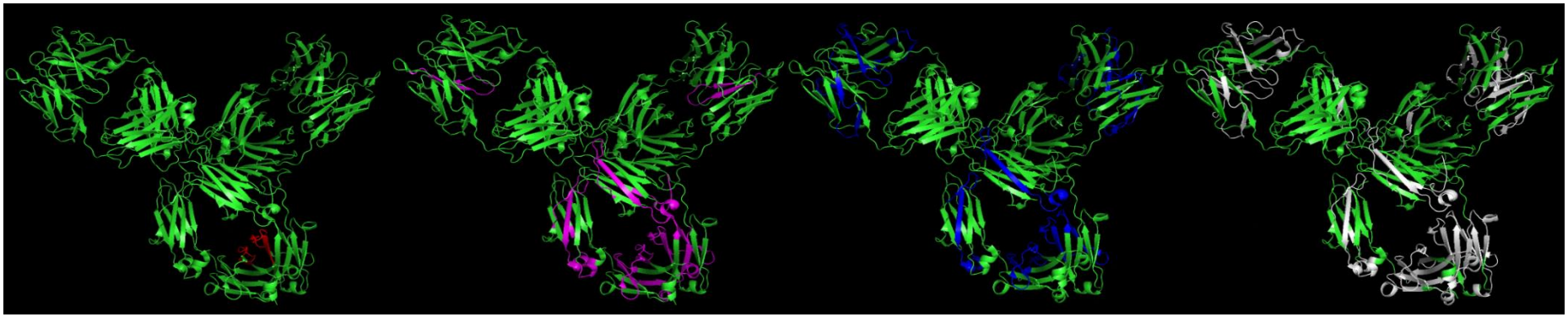
Ref std
Control 1
Control 2
Acid pH stress 1
Acid pH stress 2



Far UV CD spectra

This is indicative of significant change in secondary and tertiary structure in stressed samples

Comparison of stressed Mabs after 5 min proteolysis



Ref Std

Oxidative stress

Temperature stress

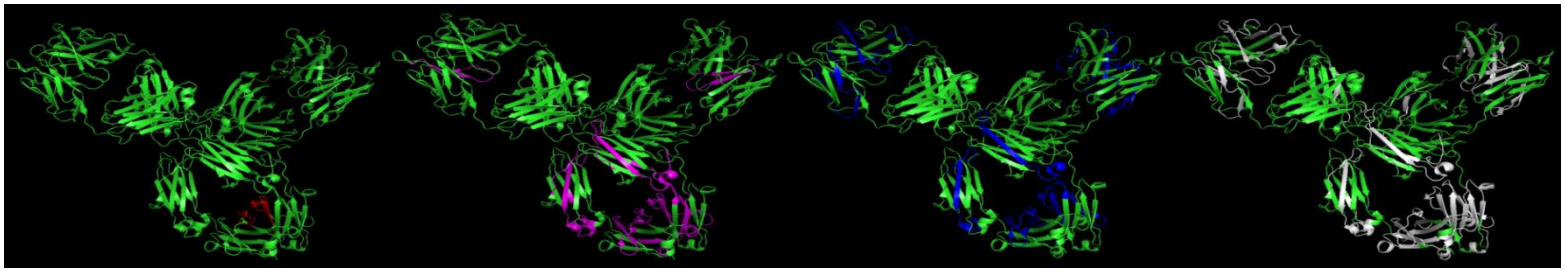
Acidic pH stress

Enzyme accessability

Biological activity: Ref Std vs stressed Mabs

ELISA	↑	X	X
Cell based assay	↑	=	↓

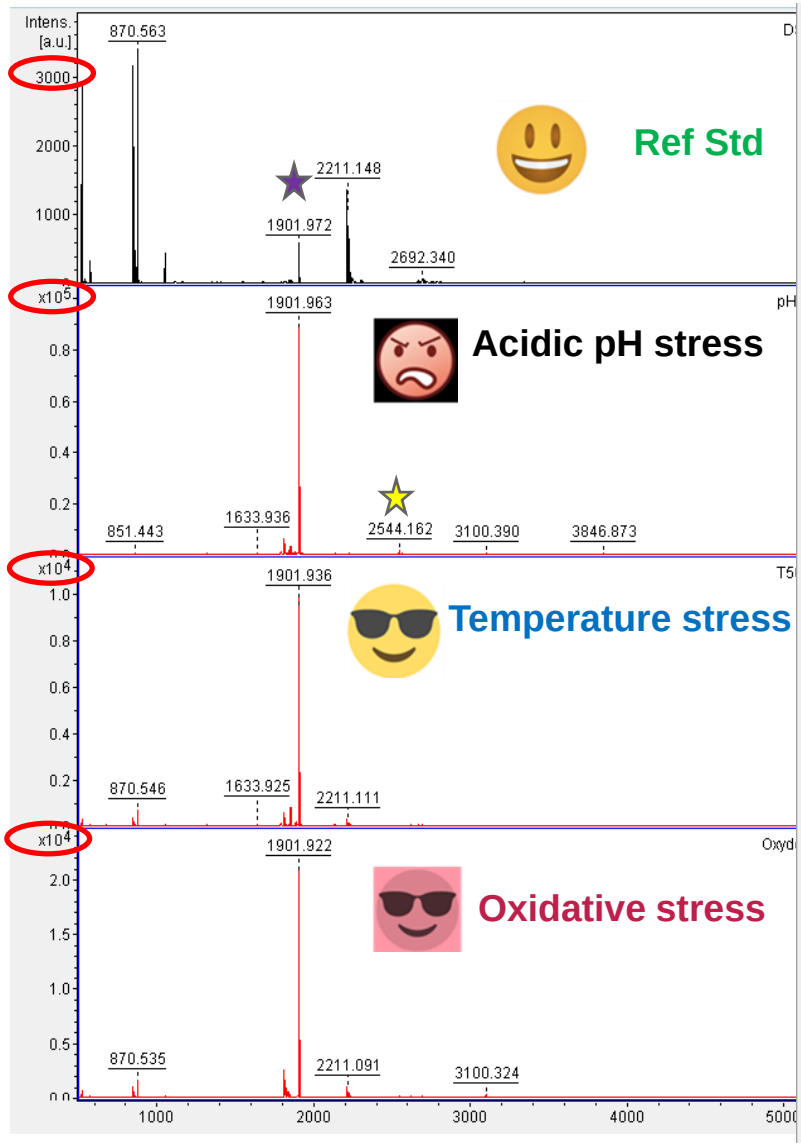
Comparison of stressed Mab vs Ref standard at 5 min trypsin digestion



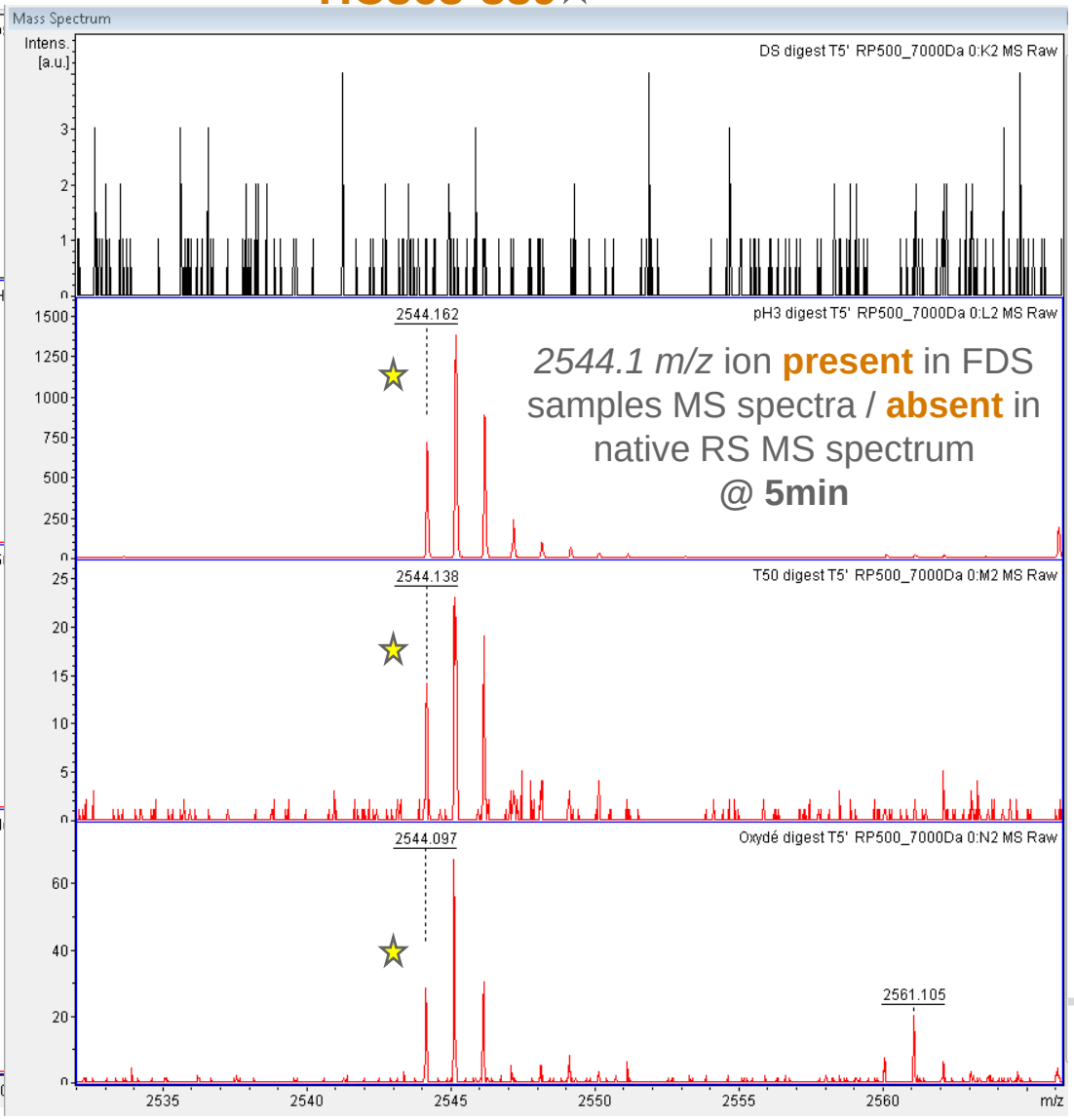
	Ref Std	Oxidative stress	Temperature stress	Acidic pH stress
HC 1-19			x	x
HC 39-61			x	x
HC-44-61				x
HC 44-65				x
HC 77-87				x
HC122-133=LC217-219				x
HC246-252		x		x
HC286-298		x		x
HC290-298				x
HC299-314 ☆		x	x	x
HC299-317		x		x
HC342-357		x		x
HC358-367				x
HC368-389 ☆		x	x	x
HC 390-406 ★	x	x	x	x
HC414-436				x
LC1-18			x	x
LC36-50			x	x
LC51-66				x
LC52-66				x

At 5min peptide HC368-389 is released from stressed Mab

HC390-406 ★



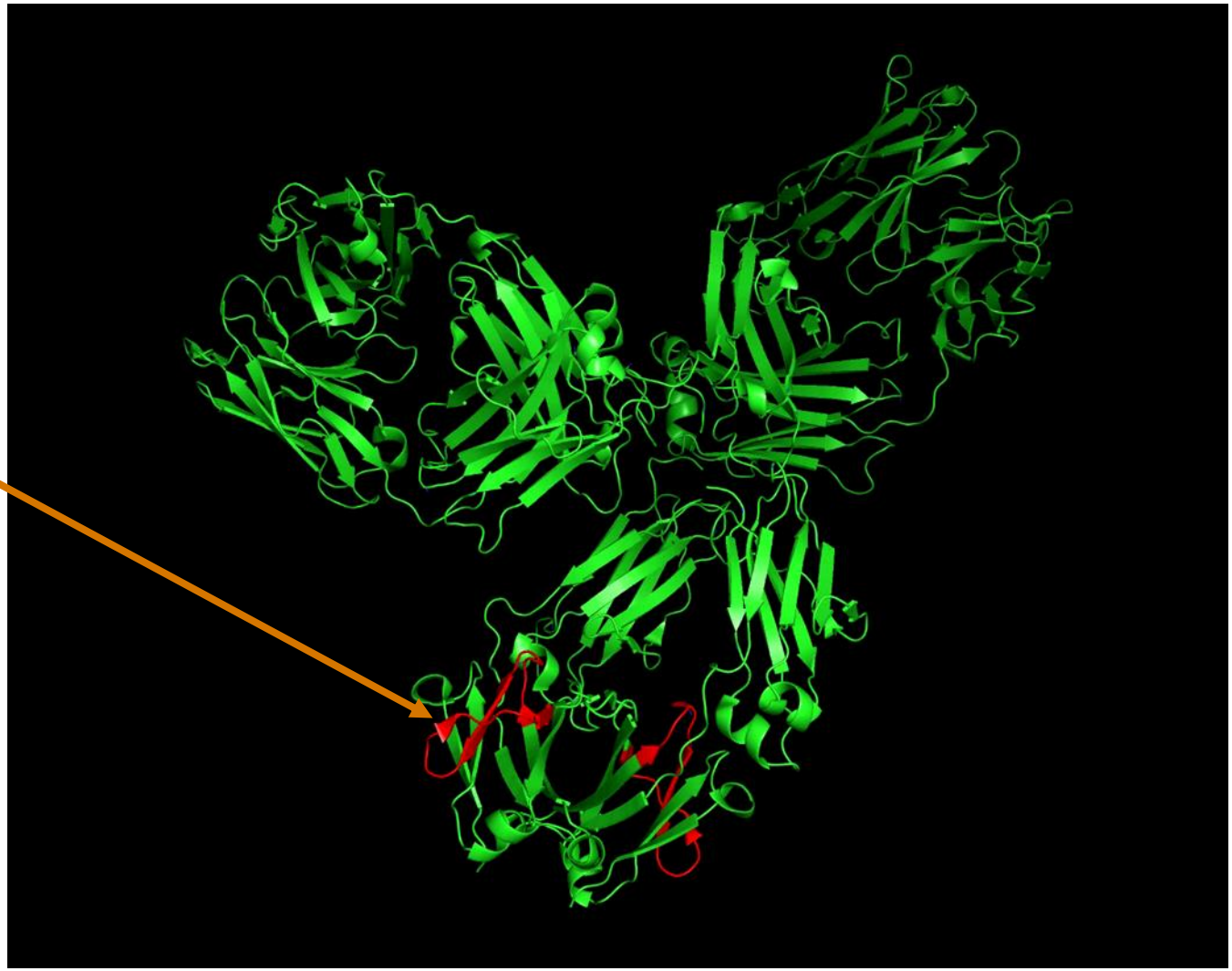
HC368-389☆



Location of HC 368-389 peak digested from stressed peptide

HC 368-389

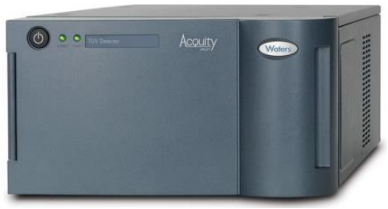
Mass 2544 m/z ion



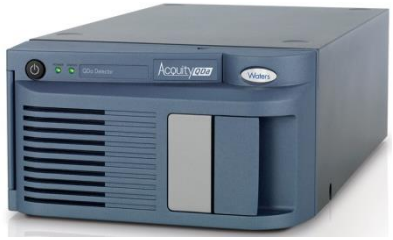
Implementation of the method to a UPLC-UV-MS system – « QC system »

« Development » stage
Peptides identification

UPLC
Peptides separation



TUV detector
Quantification



QDa mass detector
Peptides monitoring

Strategic plan

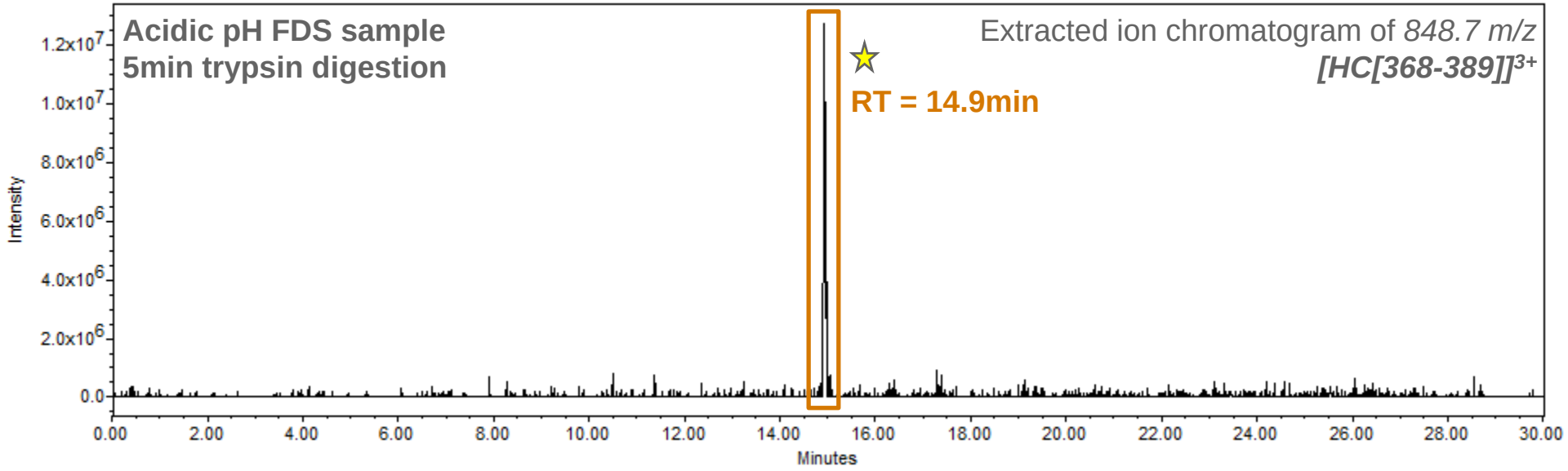
Development stage

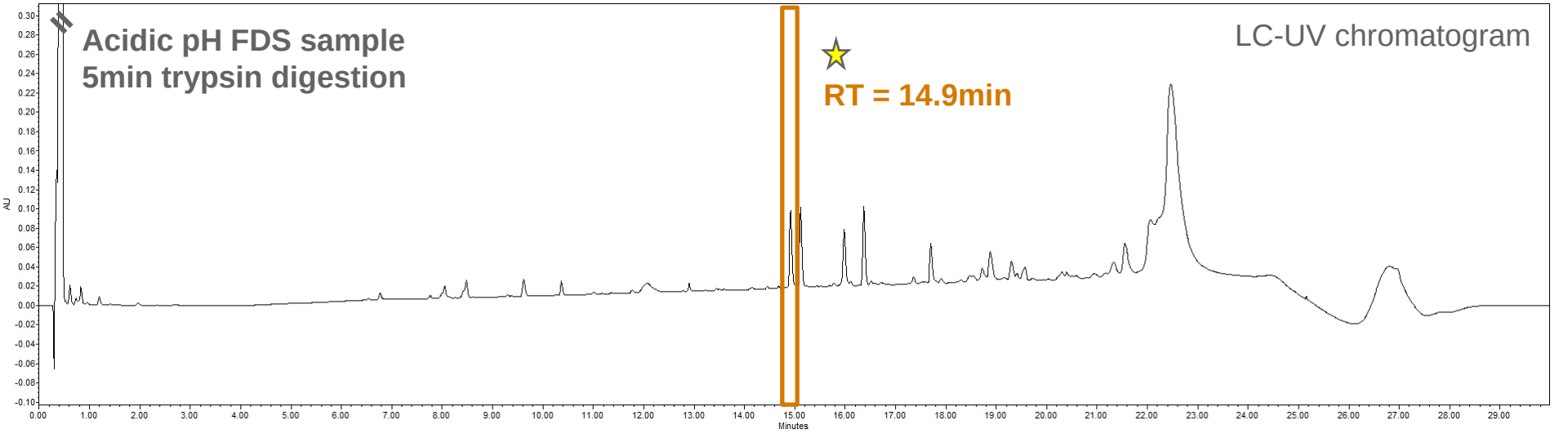
Sample → MALDI-MS → Identification investigation if necessary → Mass list

Routine stage

Sample → UPLC-UV-MS → RT determination (mass) UV area integration → Quantitation

Results HC[368-389] *2544 m/z* LC-UV-MS analysis

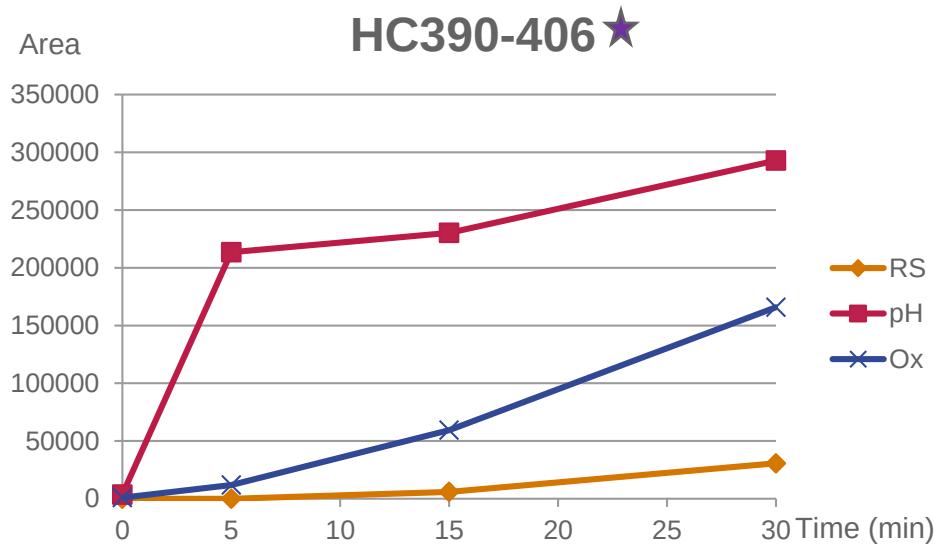




Rate of peptide release is correlated with enzyme accessability to MAb

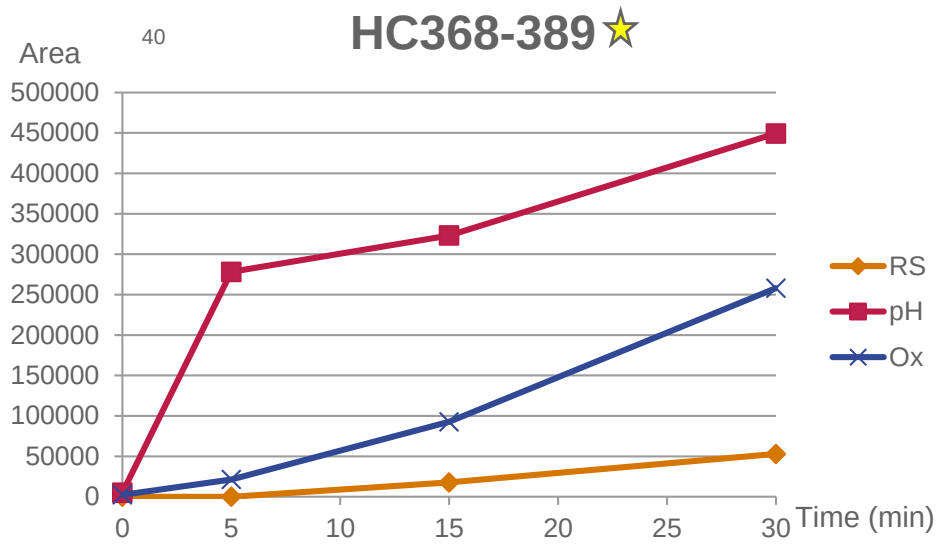
HC390-406

Time (min)	RS	pH	Ox
0	0	3709	945
5	0	213516	11890
15	5953	230268	59407
30	30705	292890	165817
280	245009	212689	355567



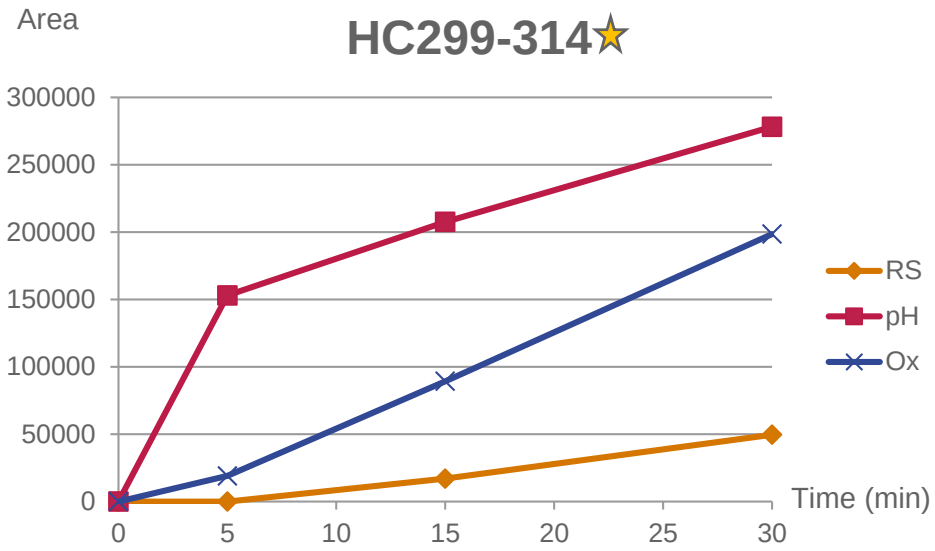
HC368-389

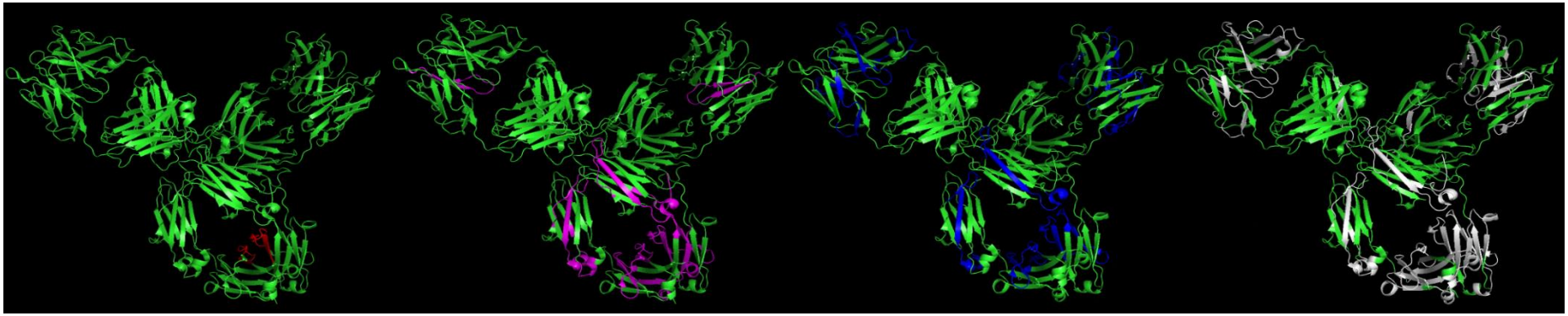
Time (min)	RS	pH	Ox
0	0	4904	2405
5	0	278171	21374
15	17682	323147	92714
30	52910	449305	257932
280	592579	615322	940413



Rate of peptide release is correlated with enzyme accessability to MAb

HC299-314			
Time (min)	RS	pH	Ox
0	0	0	0
5	0	152993	19019
15	17000	207558	89252
30	49606	278122	198489
280	328985	333298	522029





Ref Std | Oxidative stressed | Temperature stressed | Acidic pH stressed

Enzyme accessability

Summary

- Currently there is no request from regulatory authorities for more information on HOS than before
- This will probably change with more QbD
- Most HOS methods will remain characterisation methods
- Quantitative spectroscopic methods may be amenable to QC
- NMR (currently used for NCE) may be suitable for QC
- In our experience to date, Native peptide mapping is a good candidate for QC testing allowing batch-to-batch or routine HOS analysis
- Mass spec is a suitable detection method for QC

Thanks to

Analytical Development

- Annick Gervais
- Michel Degueldre
- Sandrine Van Leugenhaeghe

Bioassay Development

- Gael Debauve
- Eglantine Girot
- Anemie Wielant

QC

- Mathieu Benoit

Characterisation

- John O'Hara
- Camille Perrin
- Will Burkitt
- Xavier Perraud
- Olly Durrant

Thanks!

Questions?