HOS: The Journey from Characterisation to QC

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8th International Symposium on the Higher Order Structure of Protein Therapeutics

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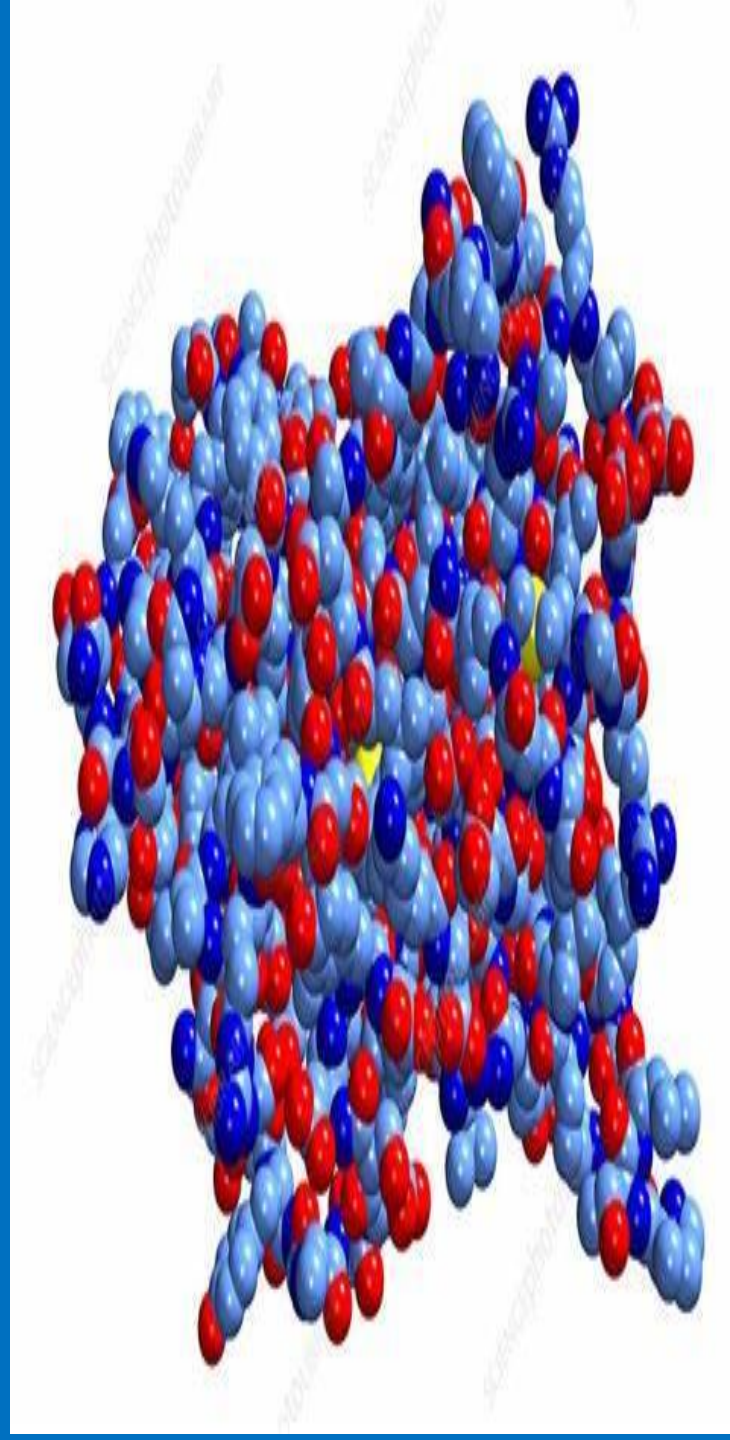


Introduction

Many quality attributes measured

- Which ones are critical?
- 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
- SAR
- Specifications

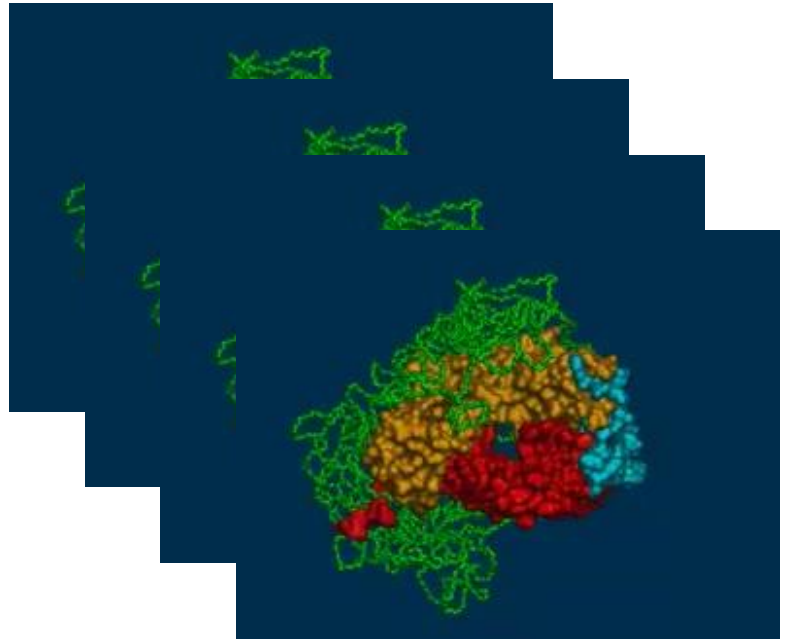
Future work & Conclusion

Many quality attributes measured

- Which ones are critical?
- Why is HOS usually performed?

Biopharmaceuticals spend most of their life in the Commercial World

- ***We know what we make***
- ***We make what we say we make***
- ***What we make is safe and efficacious***
- ***We make it consistantly***



But Biopharmaceuticals are complex….

Identity
Quantity
Structure (especially higher order structure)
Purity (Process and Product Related Impurities)
Post Translational Modifications – Glycosylation

Aggregation
Degradation
Stability
Comparability
In Process Controls
Structure-Activity relationships

- ***What Quality attributes are Critical?***
- ***What Quality attributes need to be routinely monitored?***

What is a Critical Quality Attribute (CQA)?

> A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality (ICH Q8)

> The identification of CQAs for complex products …typically possess such a large number of quality attributes that it might not be possible to fully evaluate the impact on safety and efficacy of each one (ICH Q11)

> Analytical methods that measure CQAs are key elements of the Control Strategy to ensure that the product is consistently manufactured to specifications that have been demonstrated to be safe and efficacious

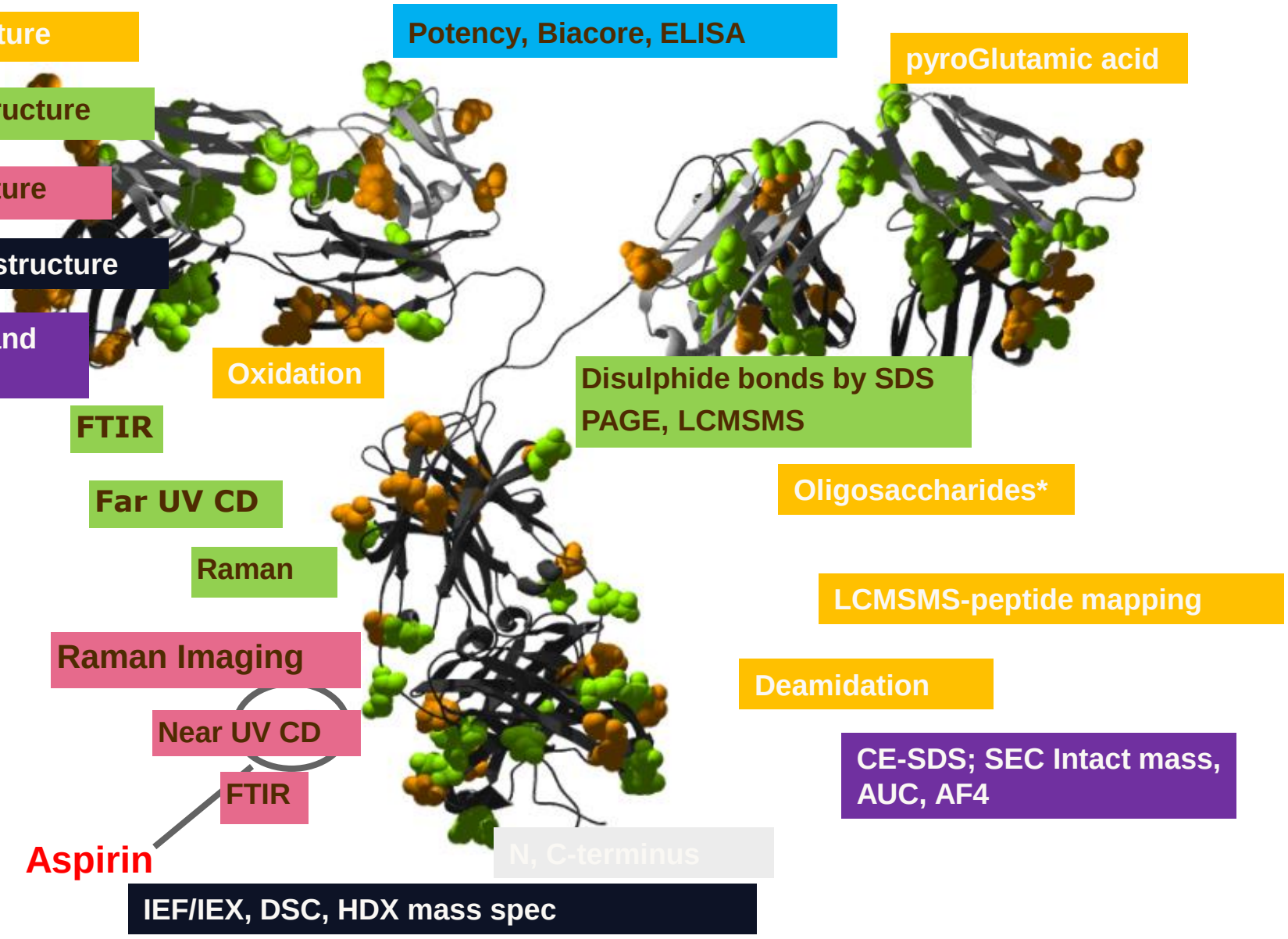
Analytical Development process: CQA to QC

- Identification of CQAs including those that need to be routinely monitored
- SAR

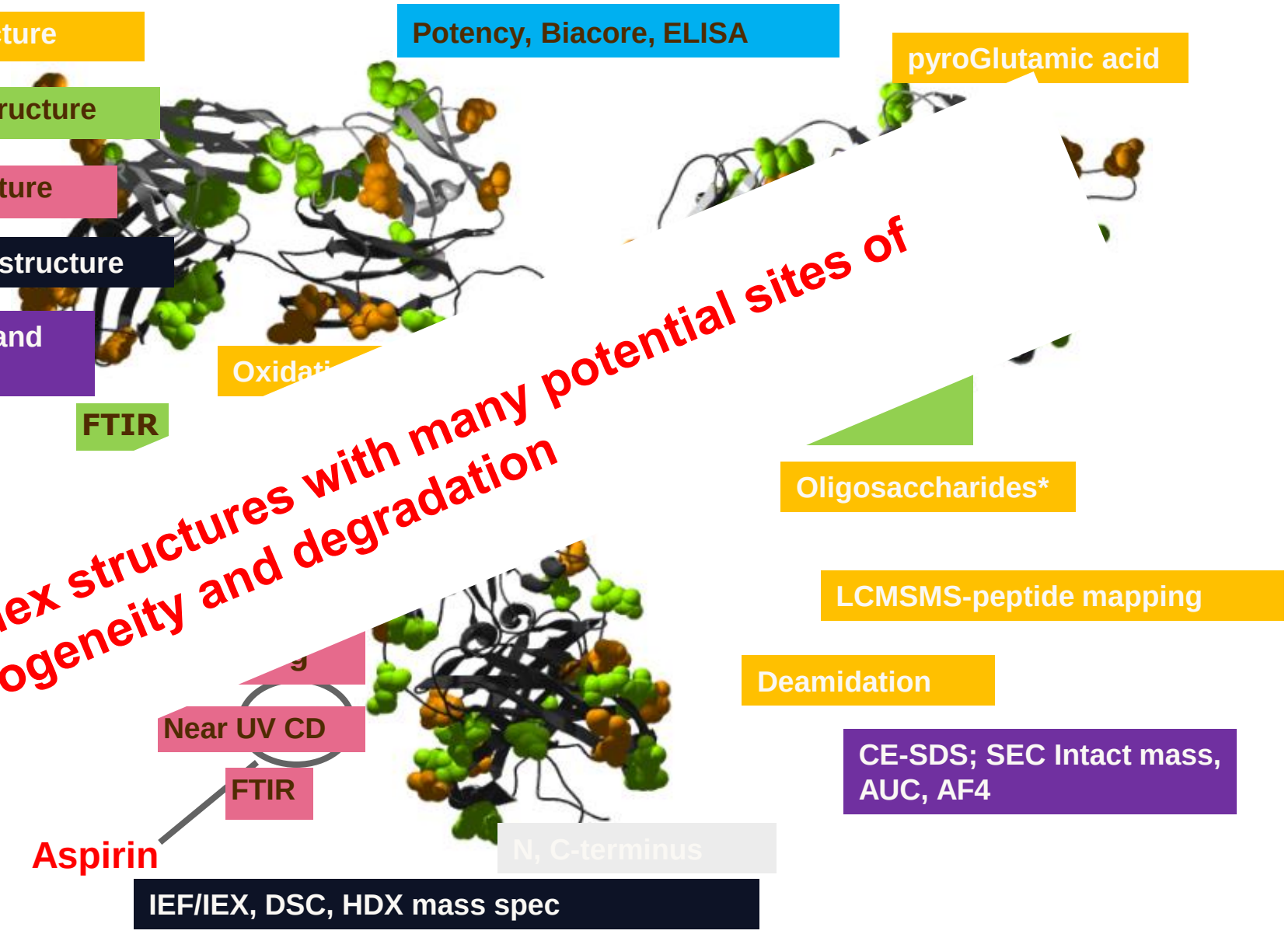
Robust “QC-able” Assays developed, validated and tech transferred to commercial QC

Routine use of assays for release and stability over commercial life of molecule

How do we measure Structure during Characterisation?



How do we measure Structure during Characterisation?



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
- Risks of conformational change
- 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*

Regulatory Expectations

IPQ INTERNATIONAL PHARMACEUTICAL QUALITY
Inside the Global Regulatory Dialogue

> **FDA May Ask for More Data on Higher Order Protein Structure in Biotech Applications. May 11th, 2011**

Very little of what we know about the higher order structure of proteins is applied in biotechnology submissions to the agency," Shacter pointed out. "This is not because methods are not available **– they are, and some of them are amenable to a QC environment.** But we still do not see them very much."
In turn, FDA is considering whether it is "time to raise the bar" on expectations for this kind of data.

Risks of HOS confirmational change

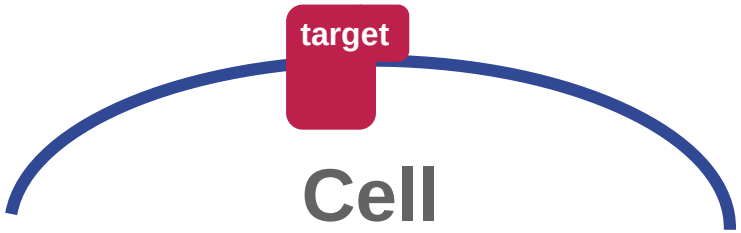
Changes in protein structure can result in changes to :

efficacy, stability, specificity and affinity.

Degradation by temperature, pH, Oxidative stress, agitation, light exposure,…

O_2

No drug efficacy



Could potentially affect drug safety – trigger disease progression
(increased potential for immunogenicity and loss of biological function**)**

“Stability indicating” methods

= Pool of physico/chemical & bioassay methods

Risk for the patient ?

Are current release and stability assays sufficient?

Thioether in hGH

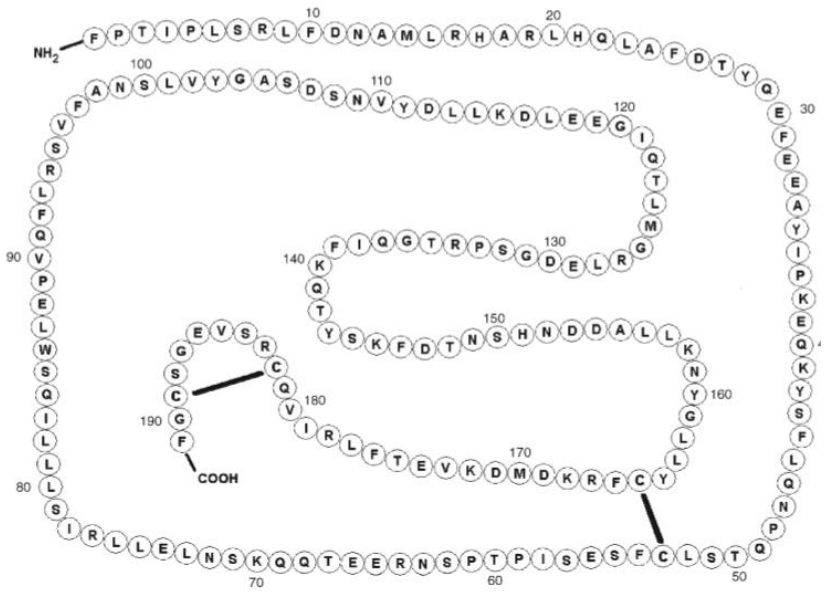


Figure 1. Primary structure of human growth hormone including the disulfide bridge pairing.

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

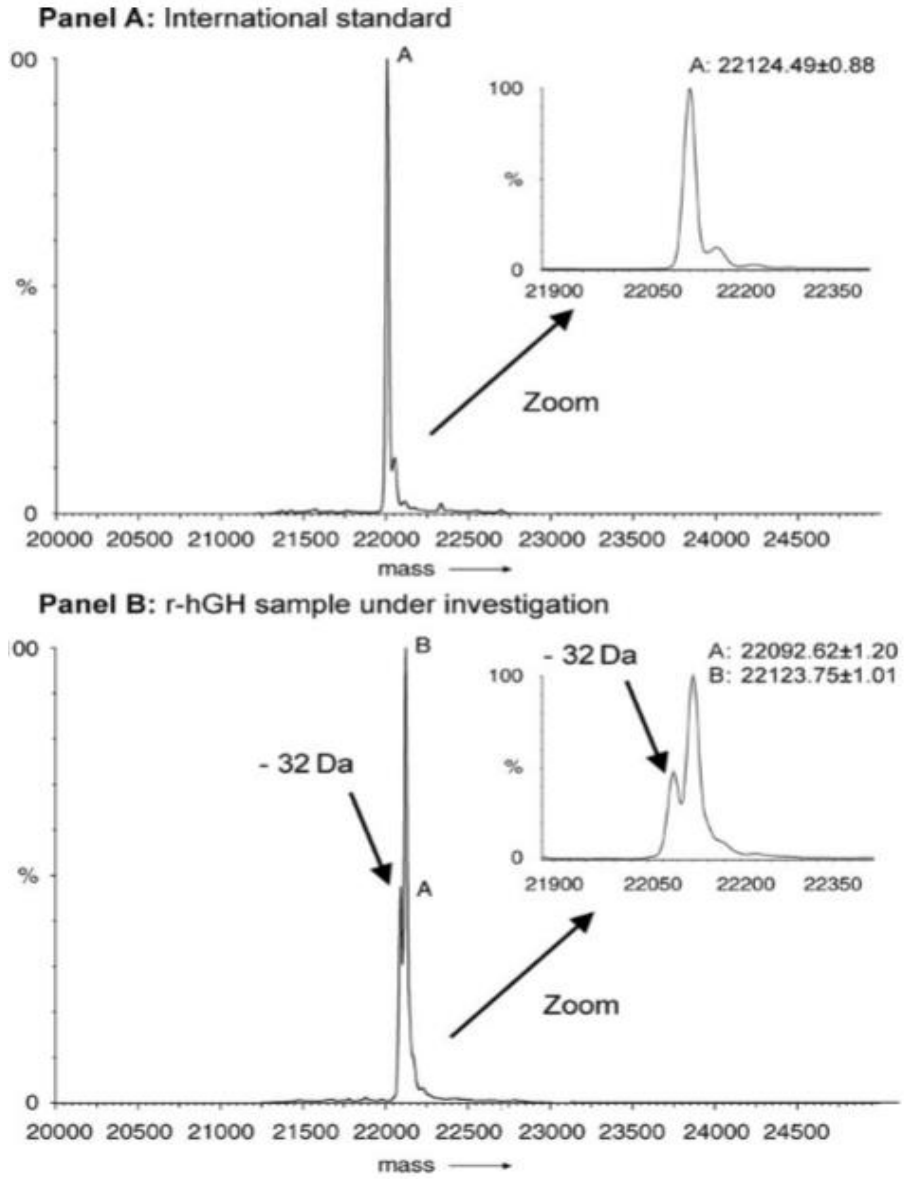
Table 1. Assessment of r-hGH Product Quality by Compendial Methods

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



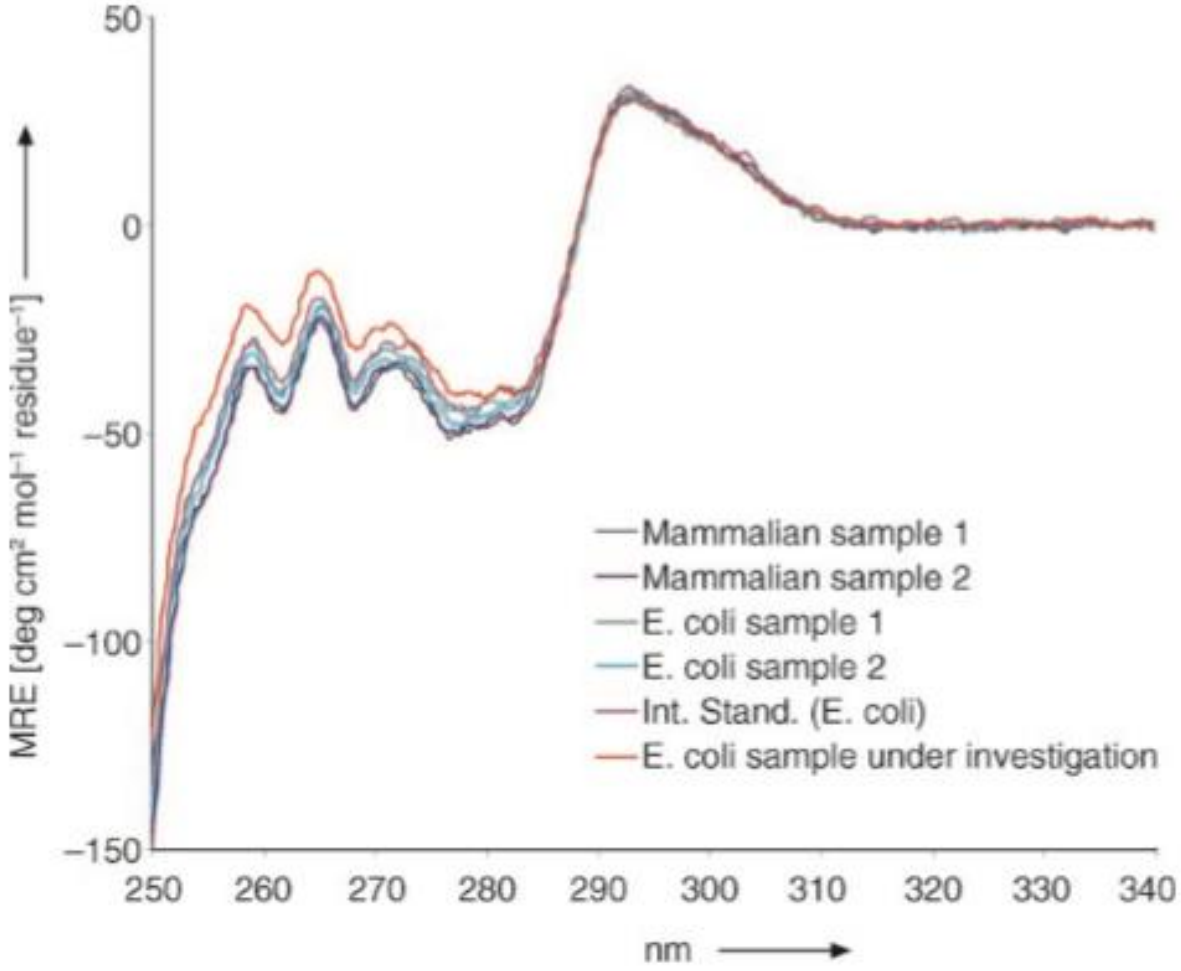


Figure 8. Near UV CD spectra of hGH samples expressed in *E. coli* and mammalian cells.

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 (Quantitative)
Non Validated, Fit for purpose	Validated
Difficult to tech transfer	Easy to tech transfer
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 (except for process support)	High throughput and speed essential

The problem with populations & ensemble methods

Many HOS methods: Current applicability in 2019

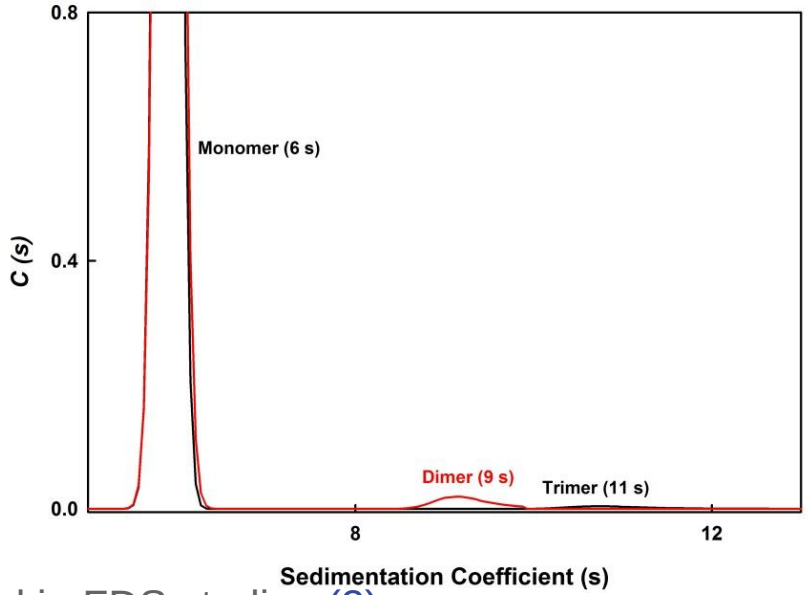
Cry EM	Metric	Characterisation	QC
Circular dichroism	Secondary, Tertiary	✓	?
FTIR	Secondary	✓	?
AUC	Quaternary, aggregates	✓	✗
Intrinsic fluorescence	Tertiary	✓	?
DSC	Tertiary structure (Tm)	✓	?
NMR	Tertiary, Quaternary	✓	?
AF4	Aggregates	✓	?
X-ray	Tertiary, Quaternary	✓	✗
Intact native MS	Tertiary,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

AUC good for aggregates – currently 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



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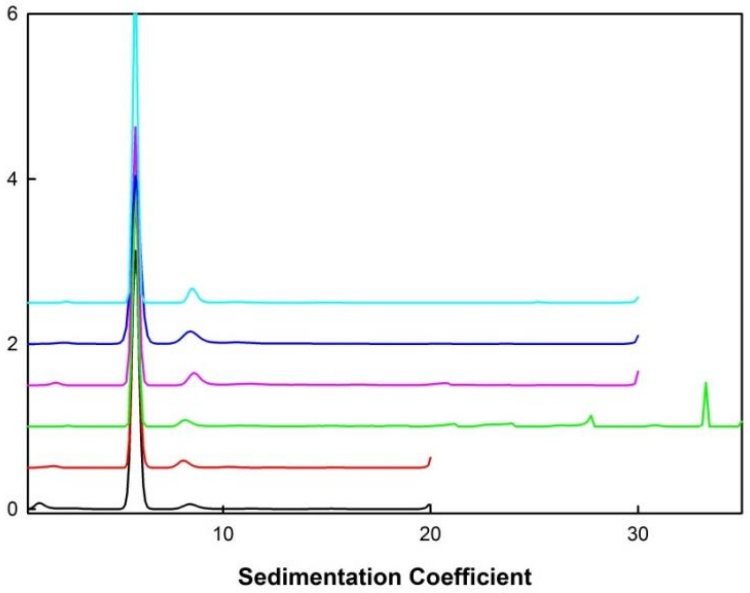
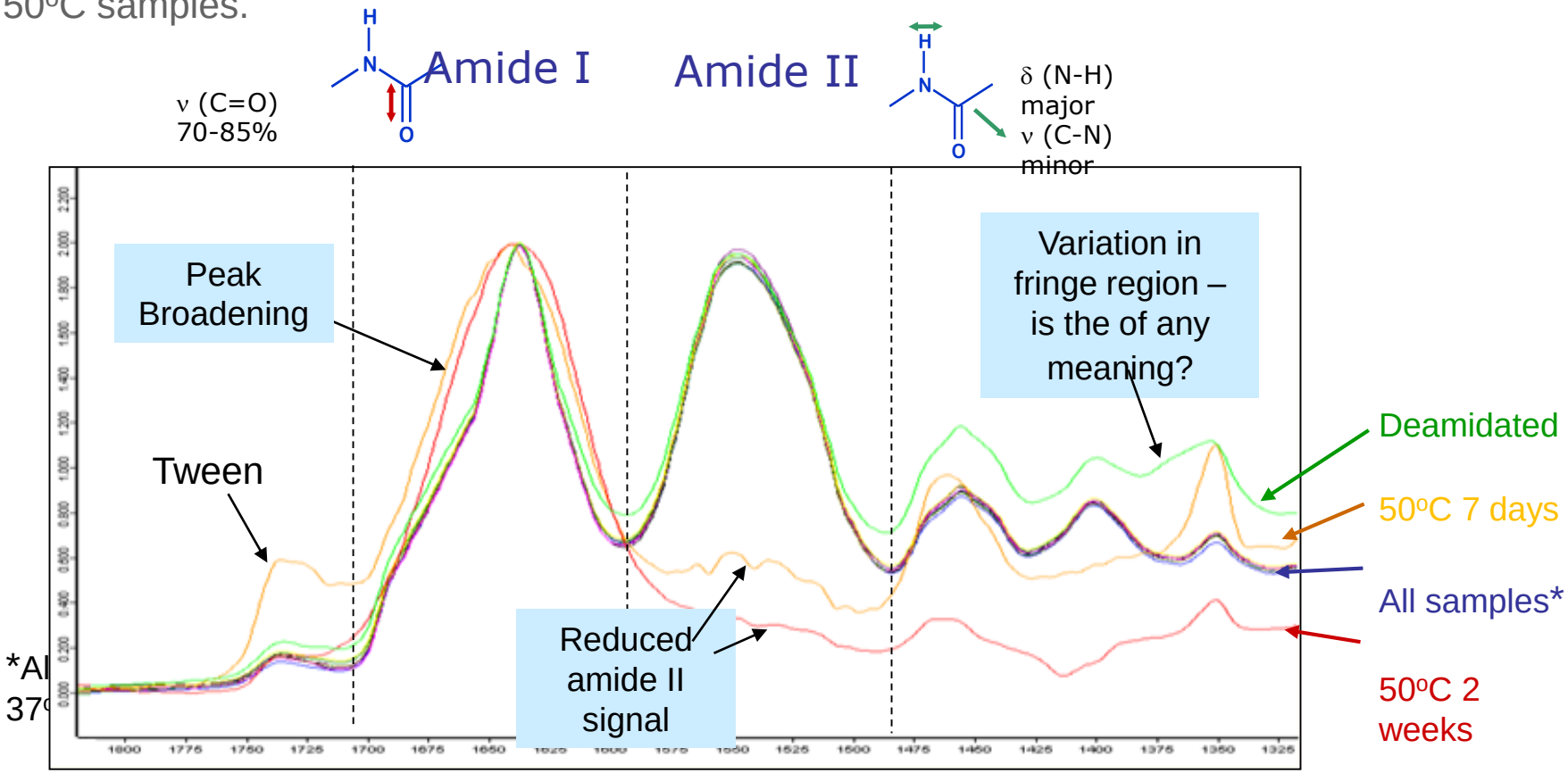


- AUC has also been used in FDS studies (2).
- The amount and type of aggregation differ between different conditions, with 50°C (green line) showing a far larger species.
- Interestingly, the data suggests that the monomer confirmation remains similar under each condition and activity was not impacted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



Wavy Lines: Difficulty with spectroscopic methods (CD)

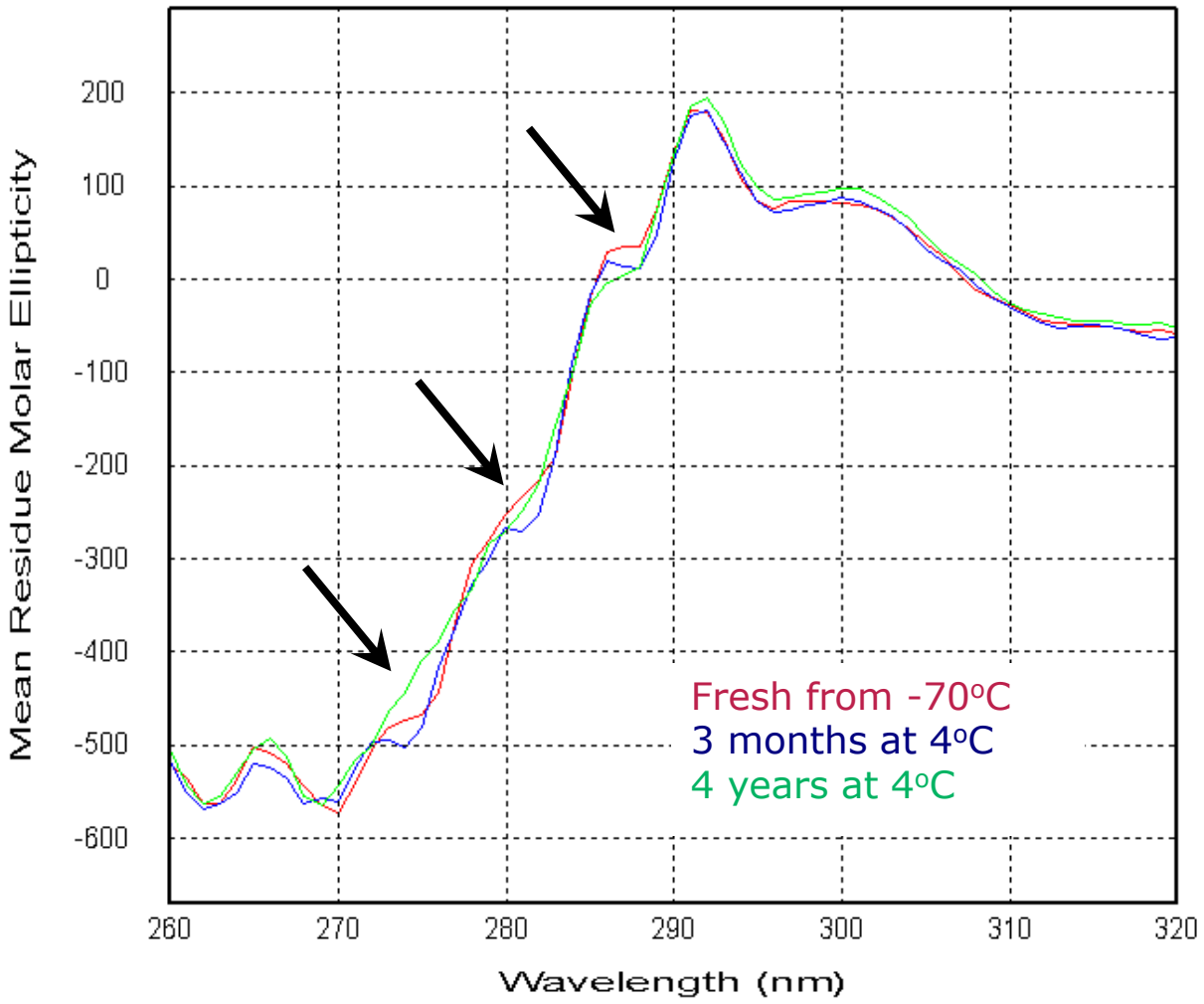
Probing 3˚ and 4˚ structure

Samples similar

Samples vary subtly (arrows)

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

Near UV CD of a MAb aged at 4ºC



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).***

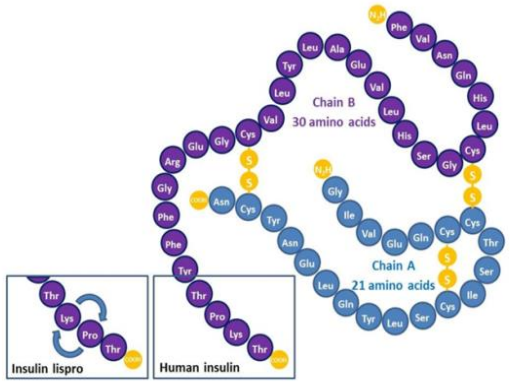
> ***For this reason a number of proposals have been put forward to make the comparison of CD spectra objective and quantitative (Bierau & Tranter, 2008) (Teska et al., 2013) (Dinh et al., 2014).***

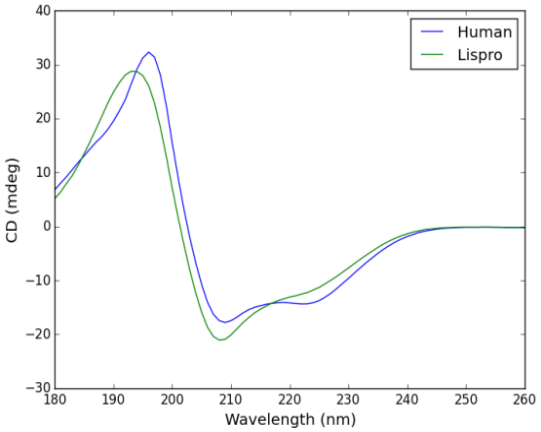
> ***Comparison of National measurement labs around the world showed significant variability in measurements.*** *International comparability in spectroscopic measurements of protein structure by circular dichroism: CCQM P59 Jascindra Ravi et al, Metrologia · January 2014*

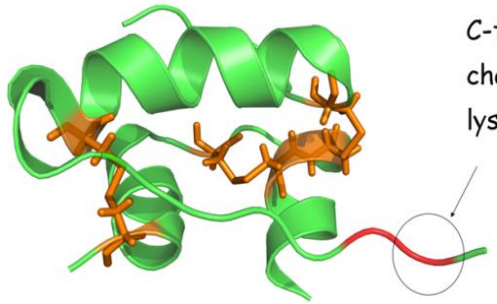
Quantitative Circular Dichroism opportunities for QC?

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**

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

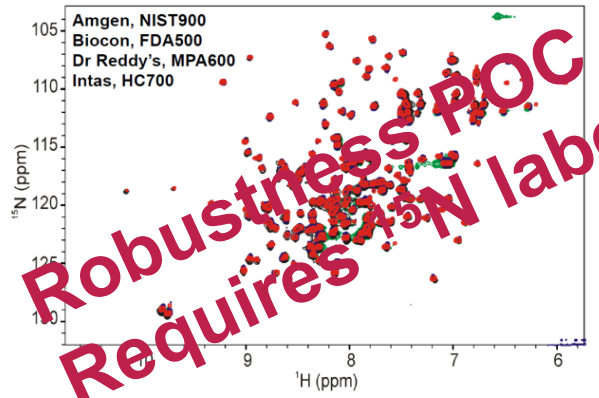
Dopant Concentration	WSD (average)	p-value (average)
0% (Control)	0.12	0.497
2.5%	0.20	0.00798
5%	0.25	5.00e-5
10%	0.51	9.59e-8

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



Robustness POC
Requires ^{15}N labelling

Nearly identical 'finger print' map between the 4 samples/instruments/magnetic fields using comparable acquisition and processing parameters

"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."

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

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

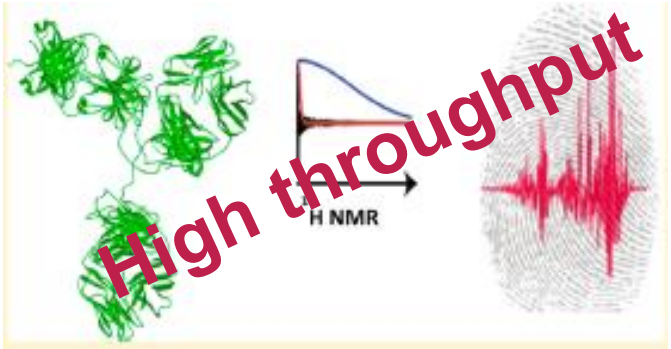
Institute for Bioscience and Biotechnology Research, National Institute of Standards and Technology and the University of Maryland, 9600 Gudelsky Dr., Rockville, Maryland 20850, United States

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

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

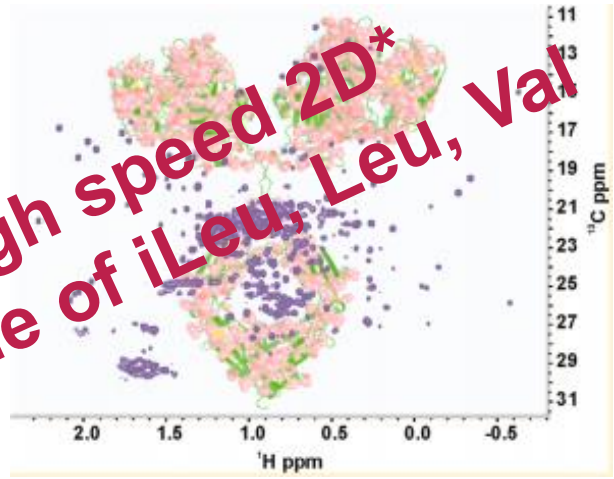
[†]Molecular Structure and Characterization and [‡]Process and Product Development, Amgen Inc., One Amgen Center Drive, Thousand Oaks, California 91320, United States

Amgen



High throughput

NIST



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

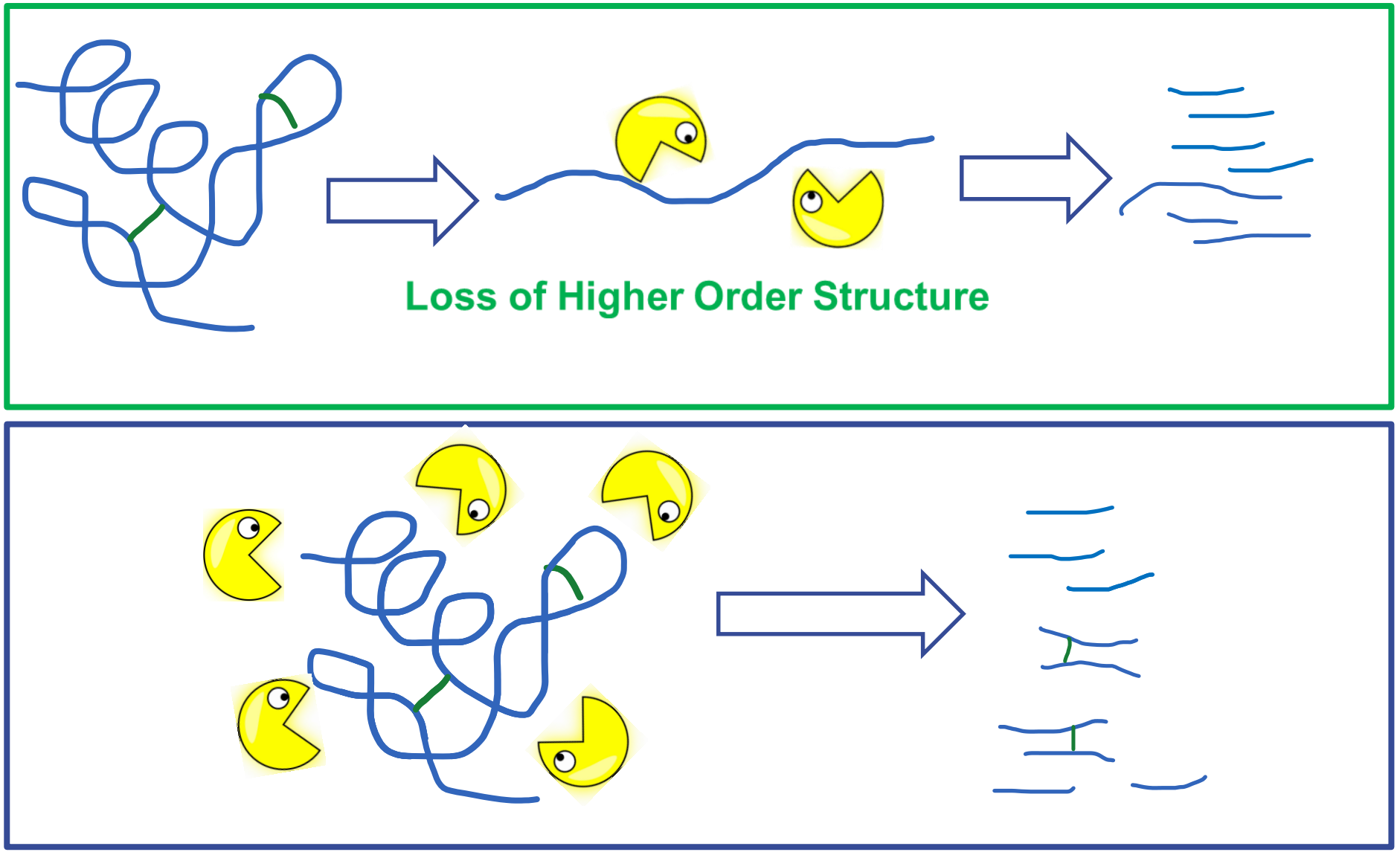
*Thanks to higher sensitivity of 13C vs 15N and NUS experiments

"Everything that can be invented has been invented."

Charles H. Duell, 1899.

Director of the U.S. Patent Office.

Classical peptide map vs Native (Limited proteolysis) peptide maps



Native Peptide Mapping (NMAP)

Background

Objective

- To identify tertiary structure changes more finely than spectroscopic methods (which show an average signal)

Principle

- Enzymatic digestion in native conditions: no unfolding/reducing step
- Protease only digests available/exposed peptides
- Order and rate of digestion, and proteolytic resistance will provide information related to tertiary structure

Native Peptide Mapping

Method / sample preparation

IgG4 (sample vs control)

⇩

Digestion with LysC

⇩

Incubation at 37°C

⇩

Separation of digestion products by UPLC

⇩

Products detection by ESI-MS or UV

⇩

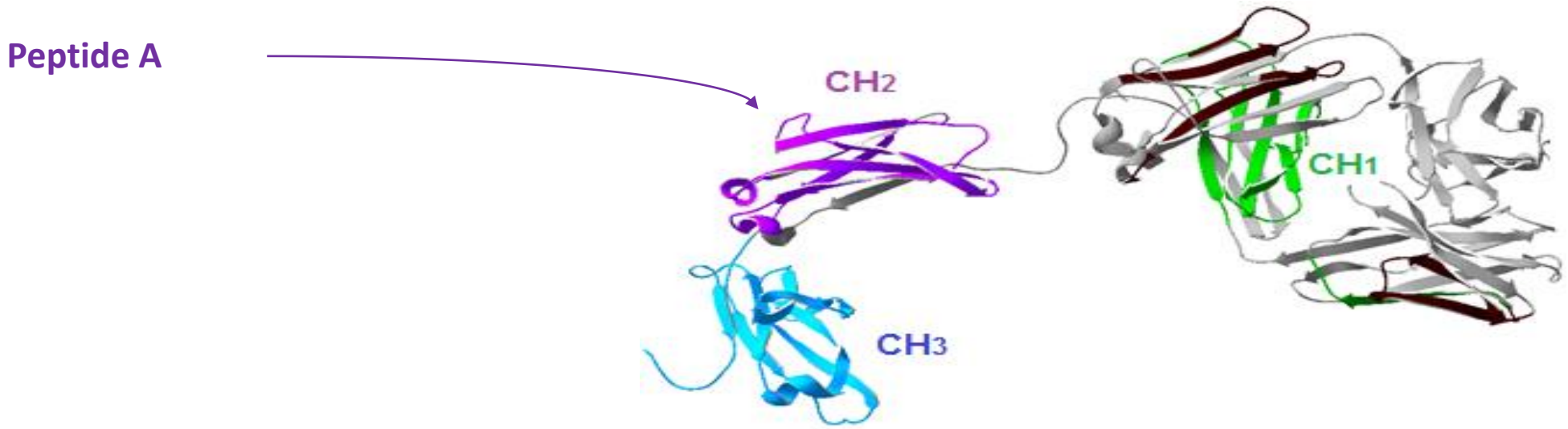
Identification of released peptides

Native Peptide Mapping

Analysis of results

- **Integration of peaks corresponding to each peptide**
- **Comparison between sample and control profiles**

→ Number of peaks / Peak intensity / Identification and location of peptides

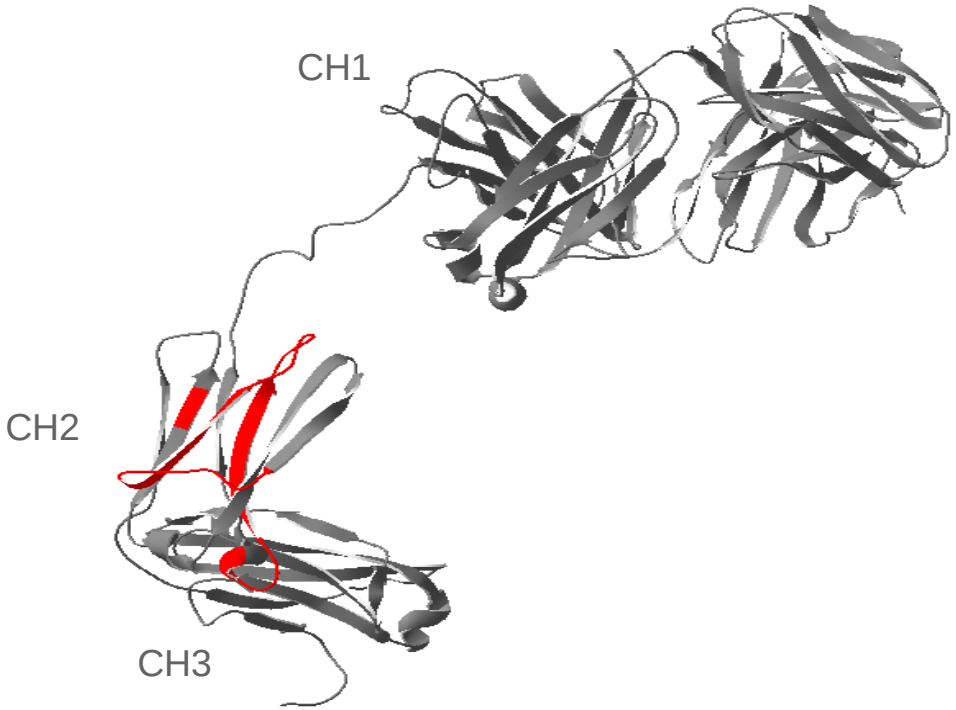


- **Comparison to orthogonal techniques**

Time point evaluation

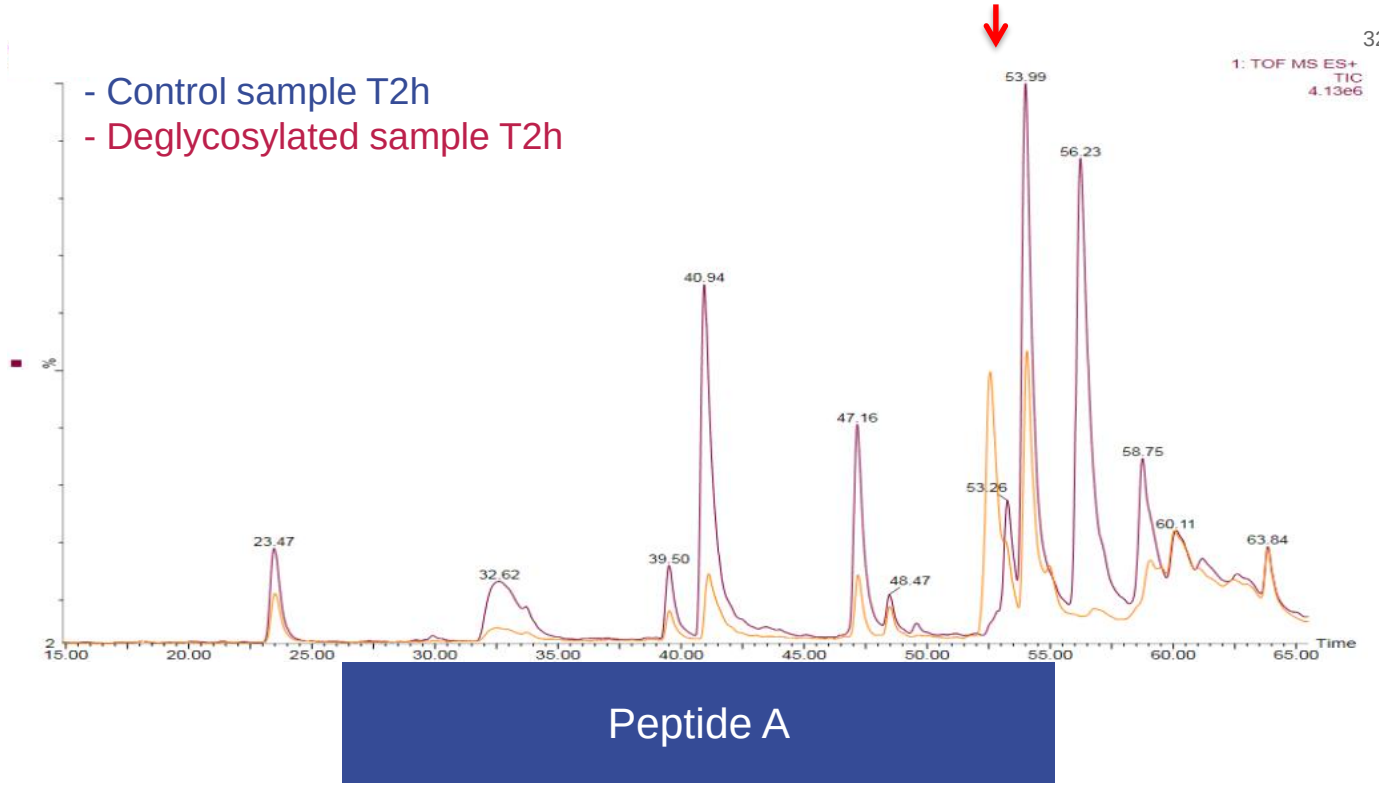
NMAP - LC-MS

- Located in CH2 domain

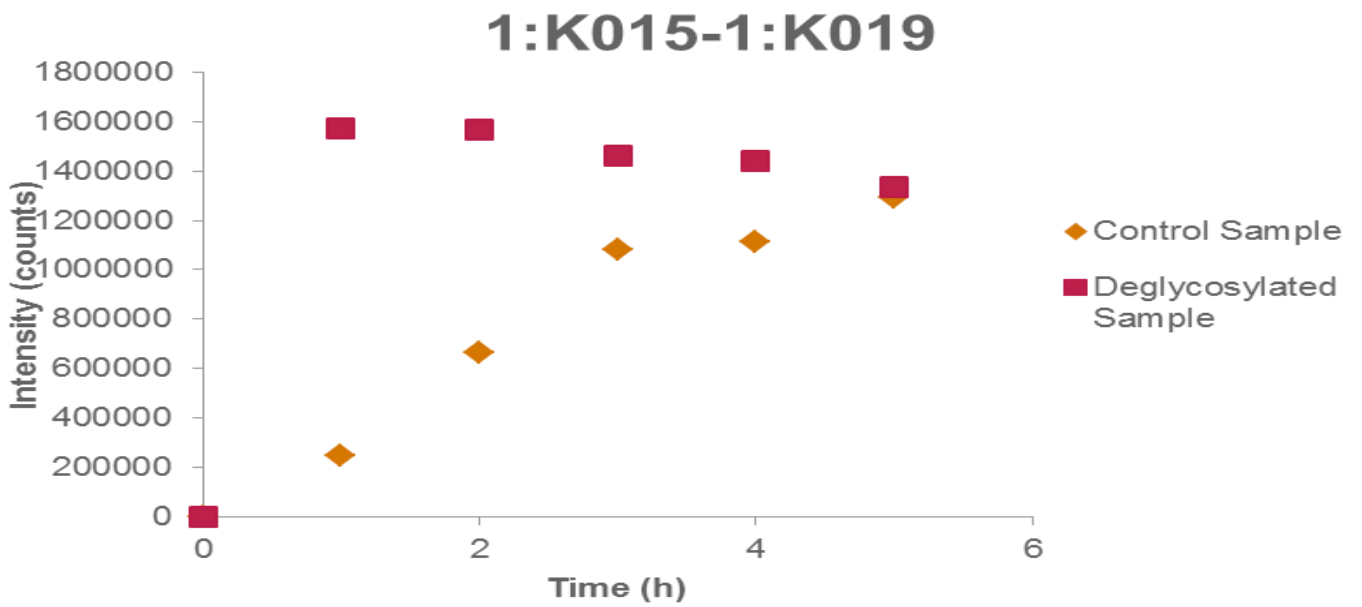


Nmap LC-MS TIC

- Control sample T2h
- Deglycosylated sample T2h

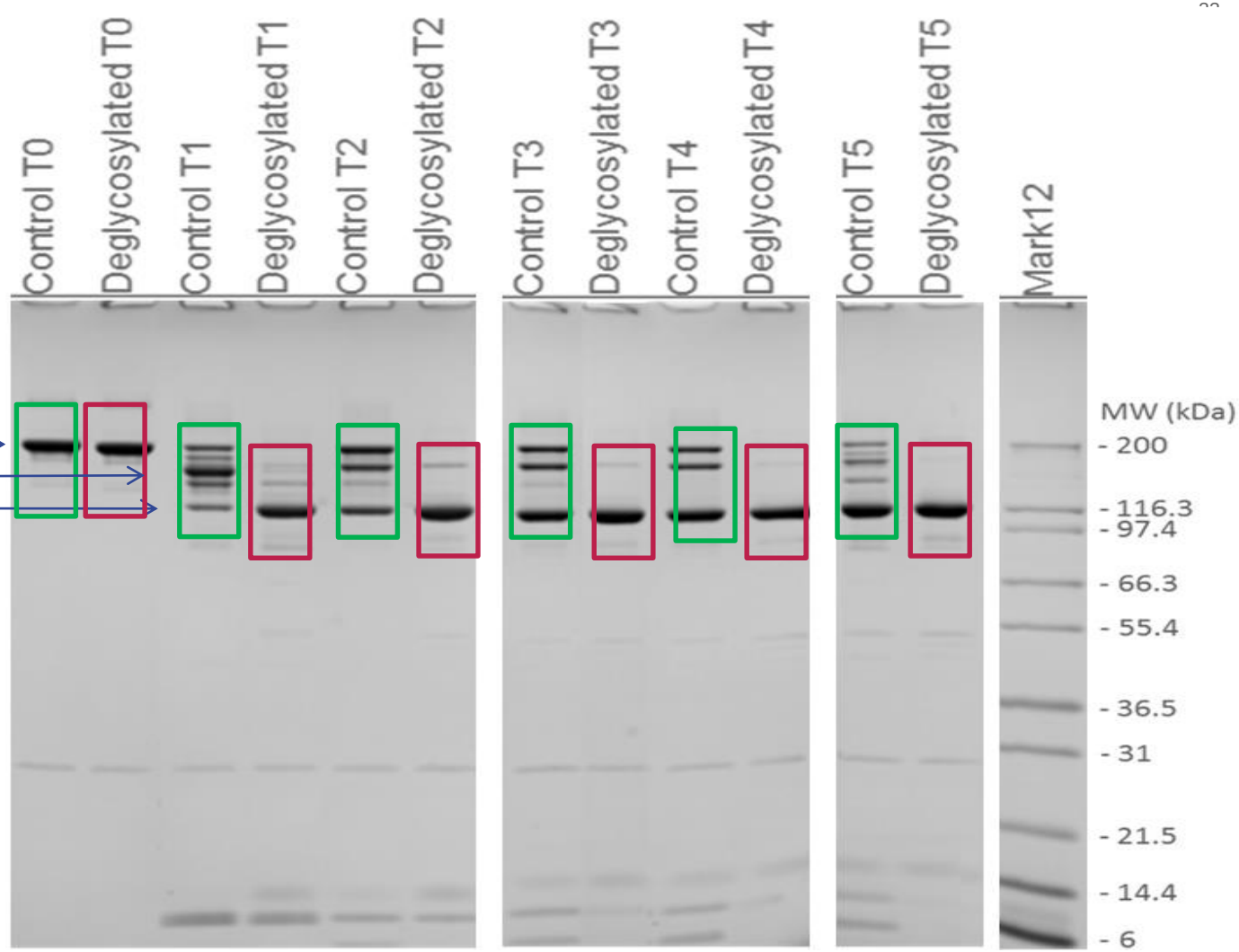


Peptide A



Time point evaluation

Non-reducing SDS-PAGE



Monomer

Monomer – ½ Fc

$F(ab)_2$

■ **Deglycosylated sample vs Control sample:**

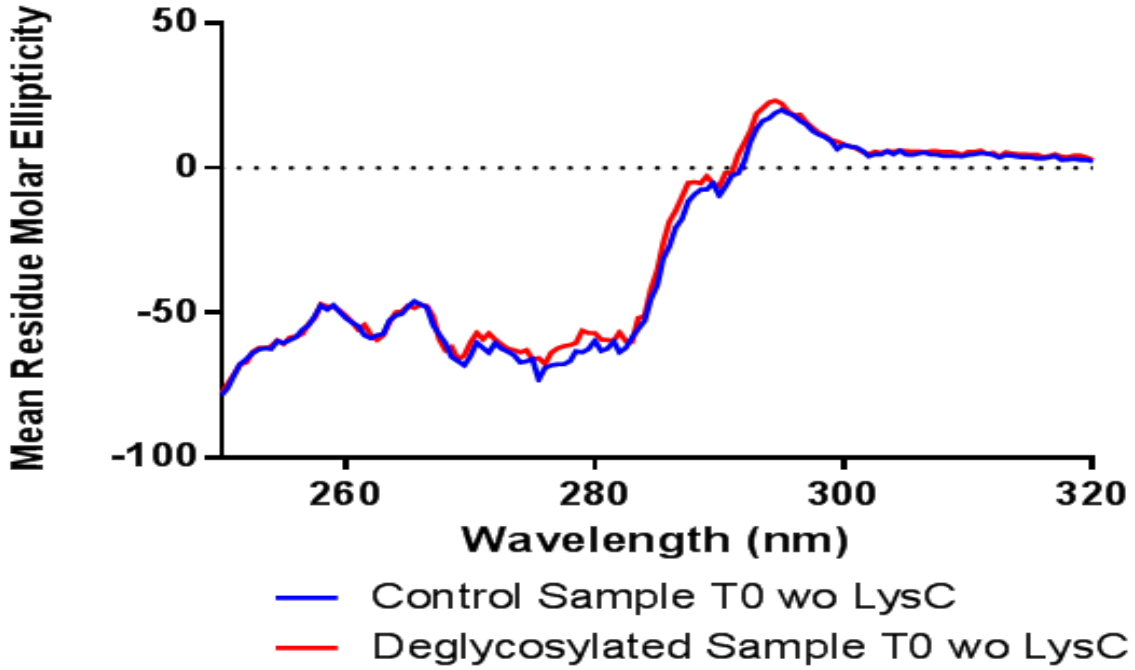
- Very low levels of monomer and monomer – ½ Fc
- Increase in $F(ab)_2$

■ **Digestion occurs faster in the deglycosylated sample**

Time point evaluation

Near-UV Circular Dichroism

- Spectra overlay prior digestion

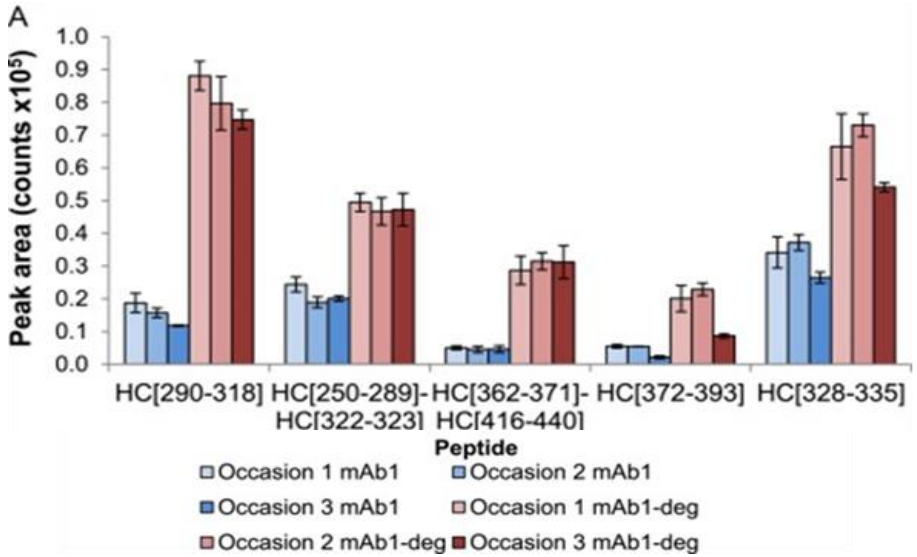


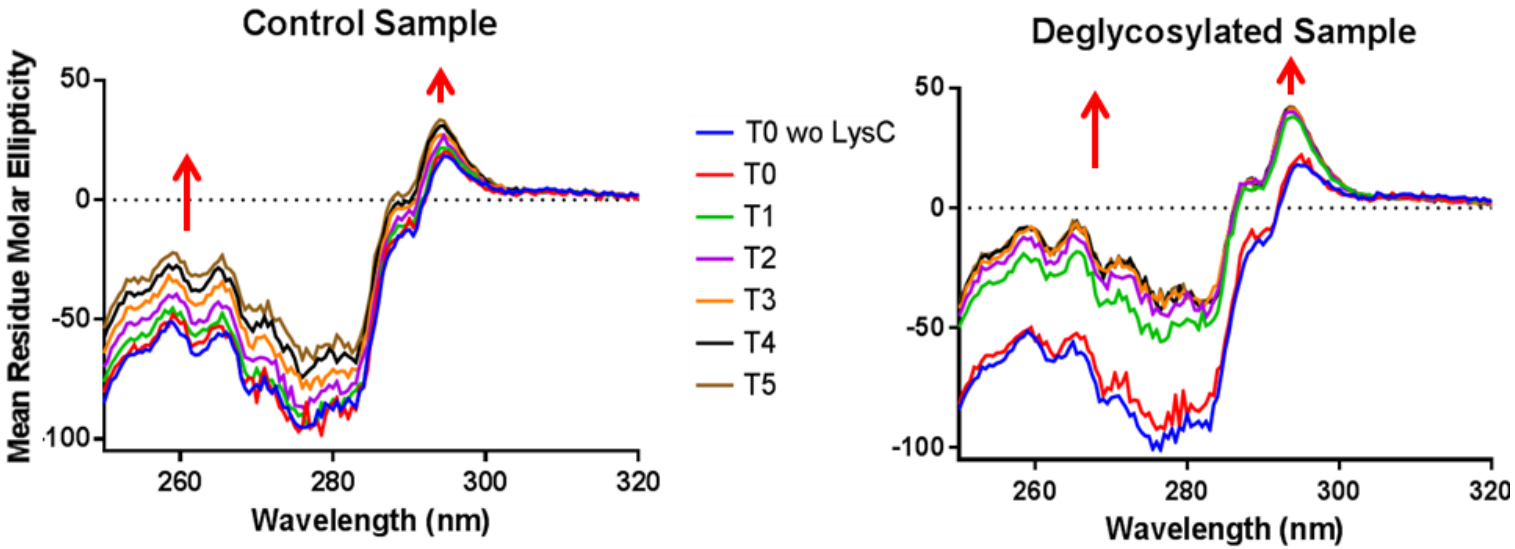
- **No major differences detected at T0**

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

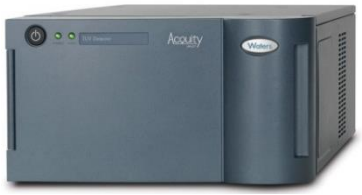




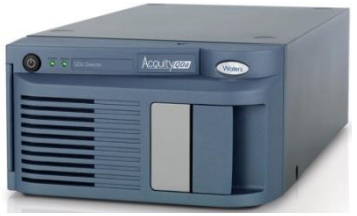
Implementation of the method to a UPLC-UV-MS system – « QC system »

« 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

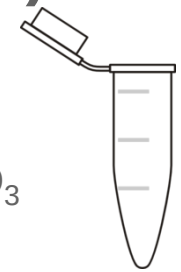
Sample → UPLC-UV-MS → RT determination (mass) UV area integration → Quantitation

Evaluation in Development lab

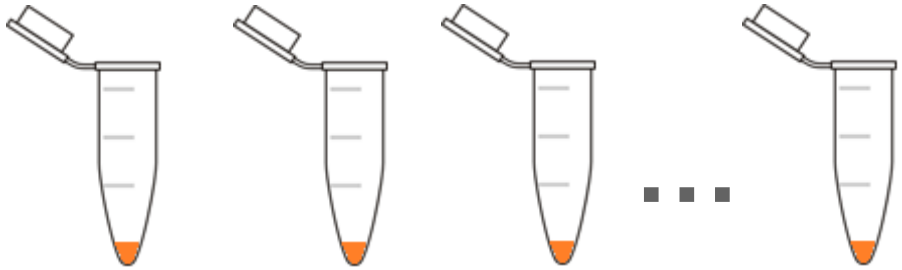
Method (detection by MALDI)

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)

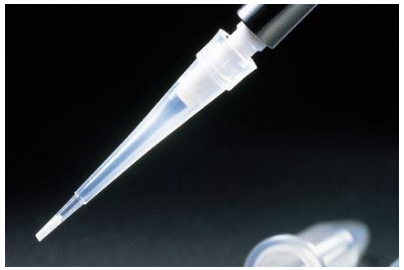
T_{5min} 180µL TFA 1% (quench)

T_{15min} 180µL TFA 1% (quench)

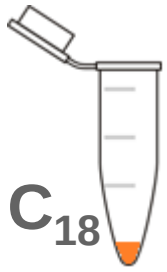
T_{240min} 180µL TFA 1% (quench)

Each time point sample is desalted thanks to:

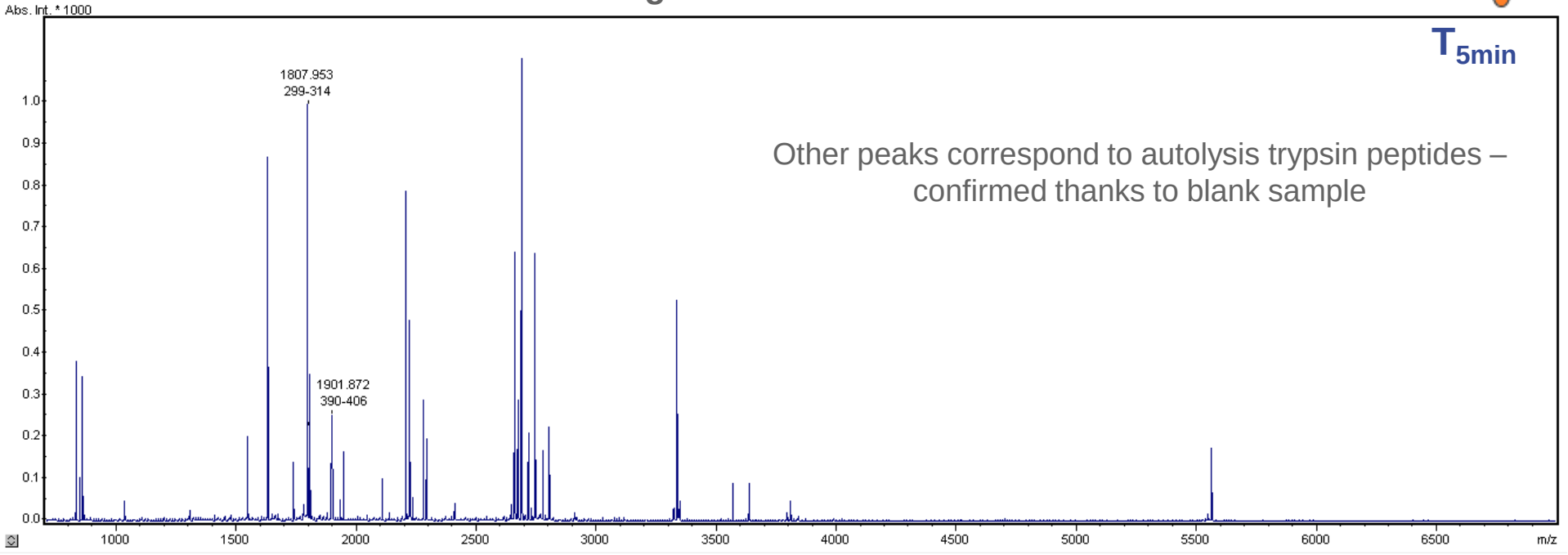
- **ZipTip C18 (peptides analysis)**



Evaluation in Development lab



Results – 700 to 7k m/z mass range

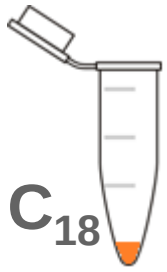


T_{5min}

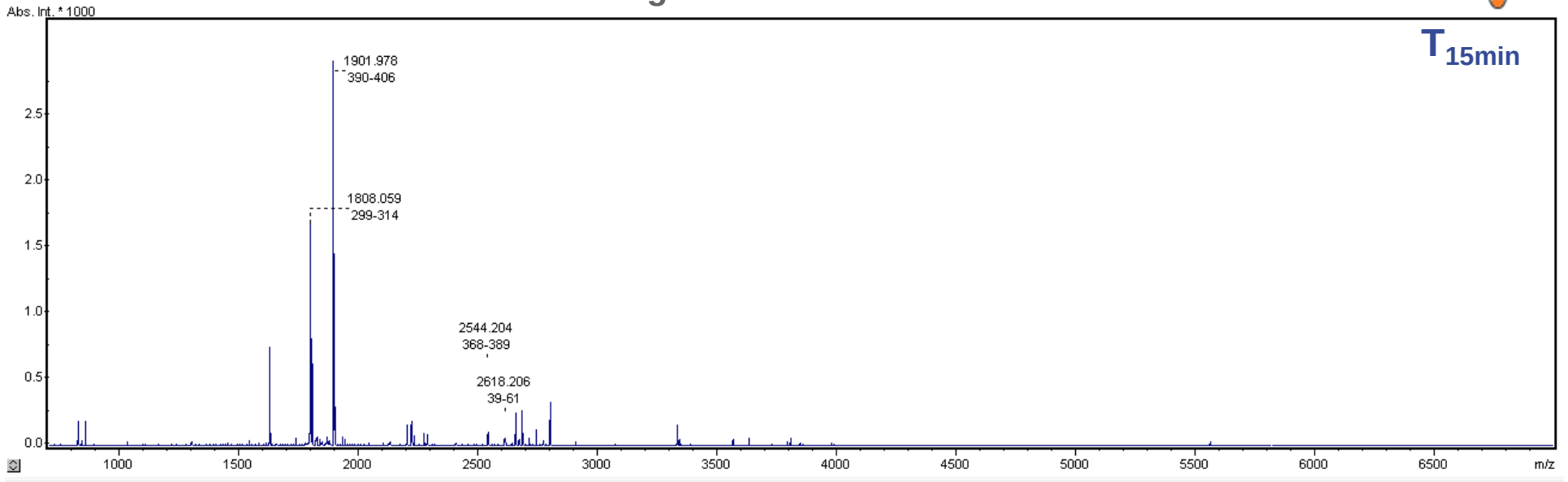
Other peaks correspond to autolysis trypsin peptides – confirmed thanks to blank sample

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

Evaluation in Development lab

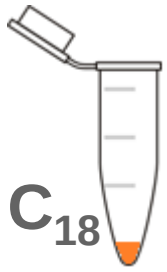


Results – 700 to 7k m/z mass range



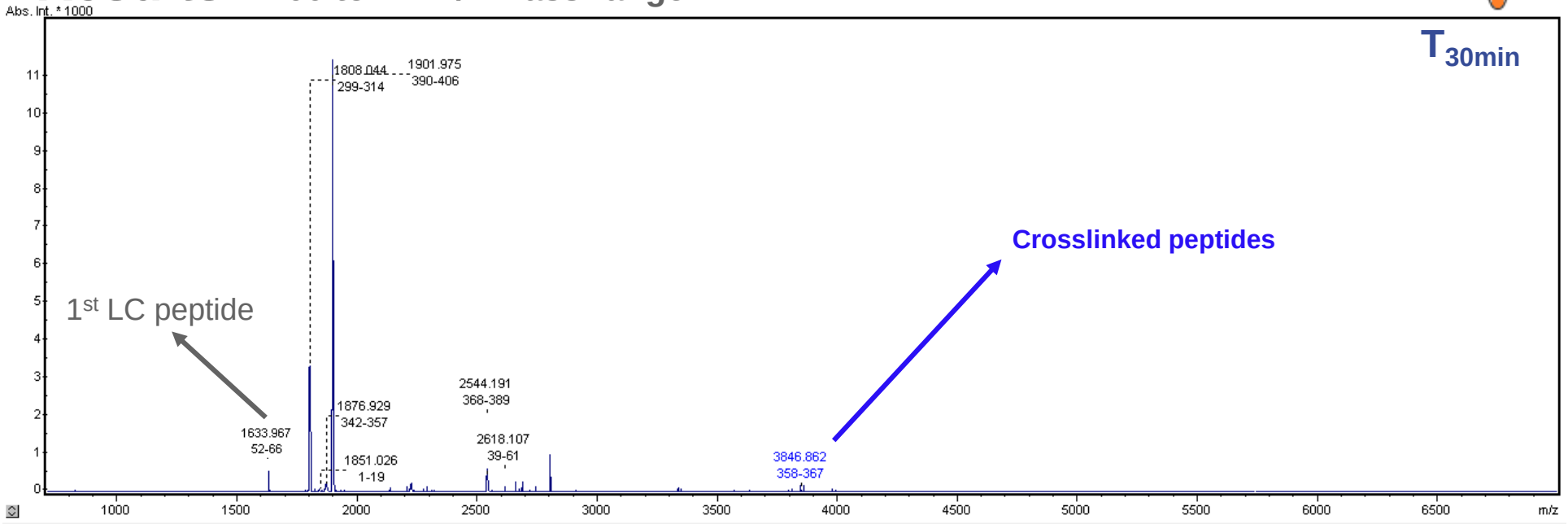
2 new HC peptides (HC 38-61 and HC 368-388) identified after 10 additional min incubation

Evaluation in Development lab



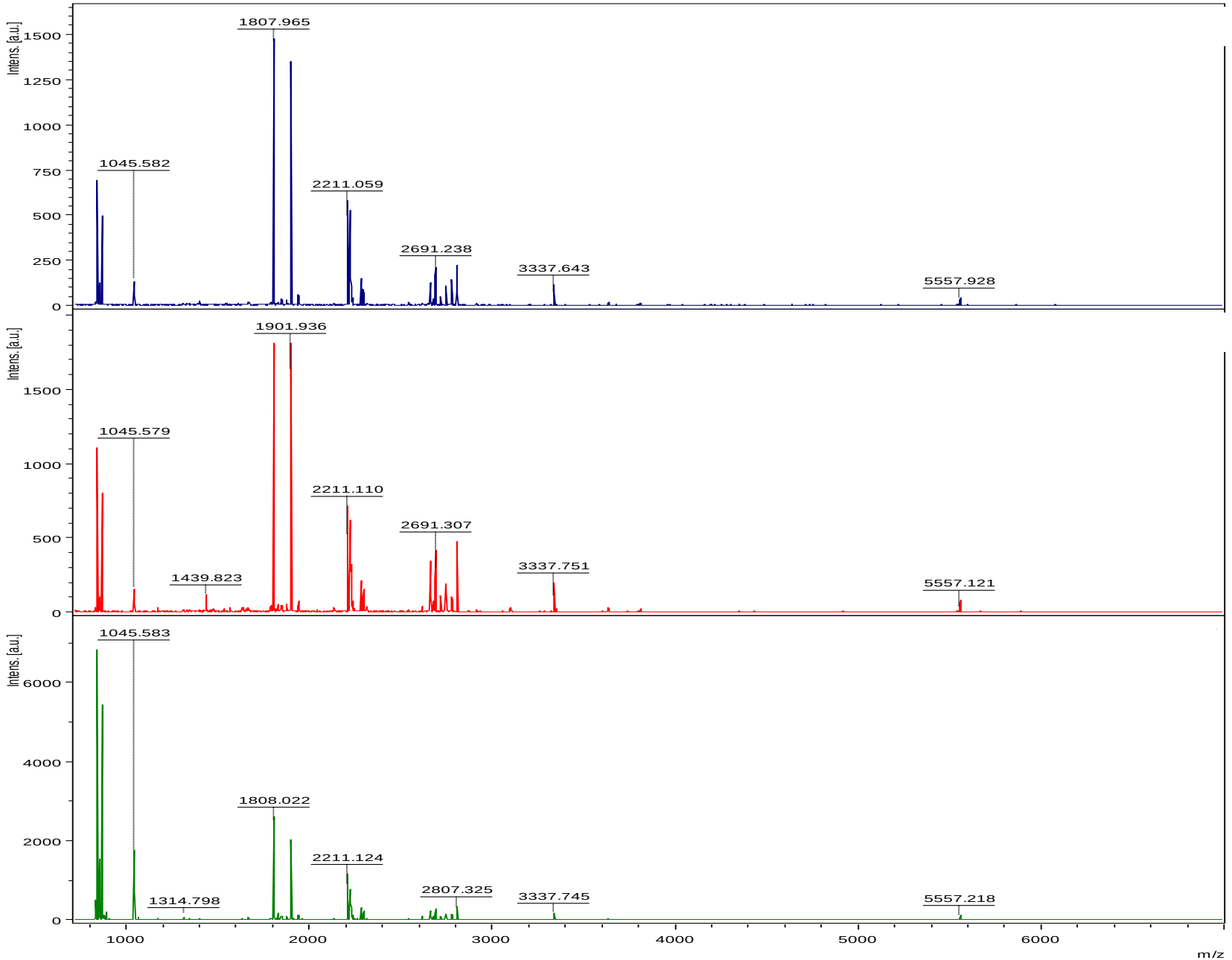
T_{30min}

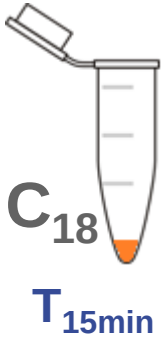
Results – 700 to 7k m/z mass range



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

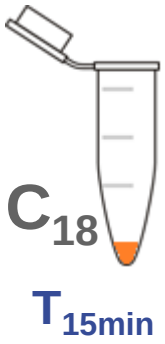




T_{15min}

Assignment
+
Structure
Next slides

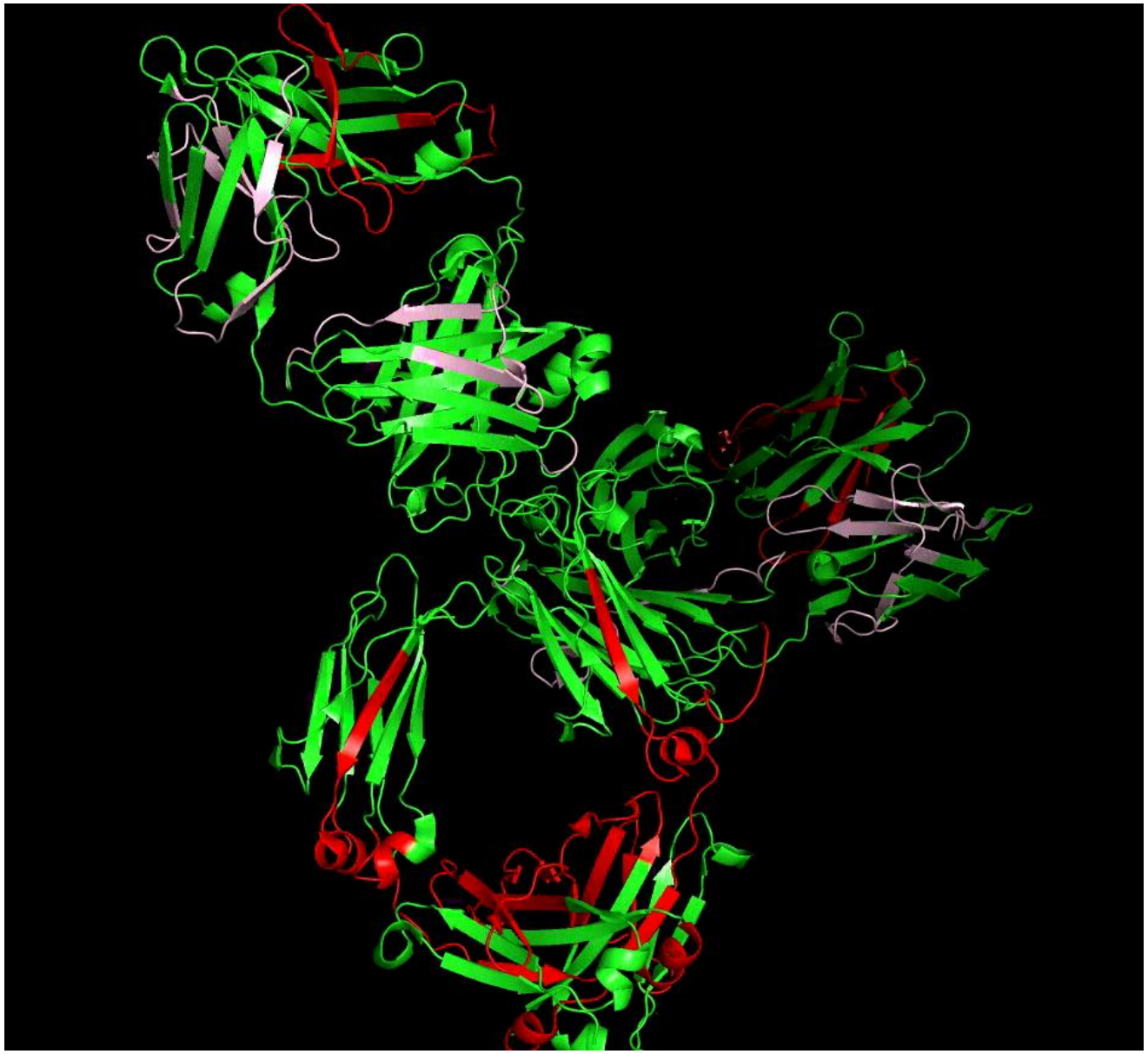
Repeatability test – sample n=3



3D structure

Red = HC

Pink = LC

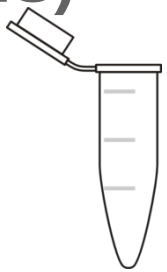


Native peptide map: Structure-function study

Method (MALDI & LC UV/MS)

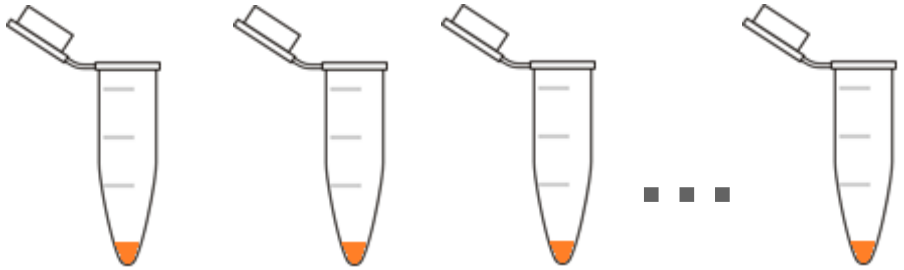
200µg Mab + 20µg trypsin ➔ ratio 1/10

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



8µL of digest / time point

Time point (min)
0
5
15
30
60
280



T_{0min} 42µL TFA 1% (quench)

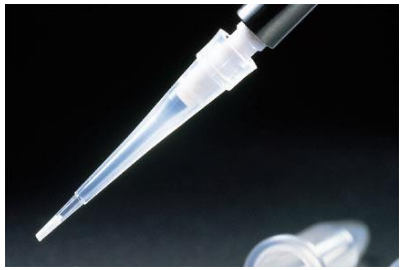
T_{5min} 42µL TFA 1% (quench)

T_{15min} 42µL TFA 1% (quench)

T_{280min} 42µL TFA 1% (quench)

Each time point sample is desalted thanks to:

- **ZipTip C18 (peptides analysis)**



Structure-Activity relationship by forced degradation studies

Intentional degradation at conditions more severe than accelerated conditions.

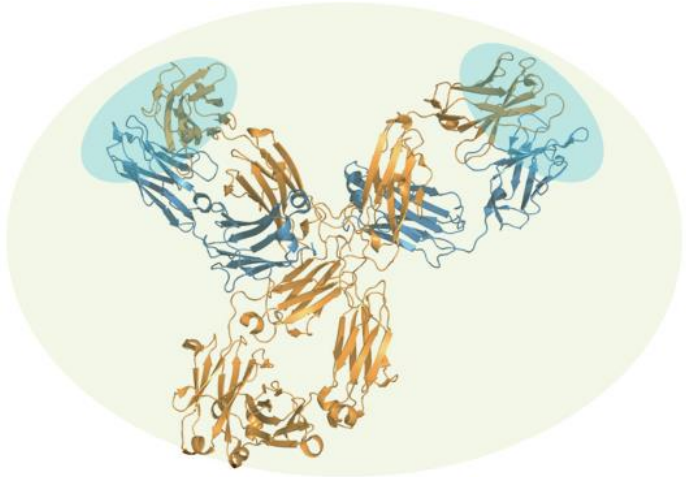
➔ forced degradation study ≠ shelf-life stability study

Concept Paper « Forced Degradation Studies for Therapeutic Proteins », EBE, March 2015

The analysis of FDS samples will help us to reveal the relationship between a change in
higher order structure and biological activity

⇩ **Native peptide mapping**

⇩ **Bioassay**



« At which point will a change in HOS form a real risk for the patient ? »

Native peptide map: Structure-Activity study

3 Forced degradation studies samples were analyzed:

- Acidic pH stress
- Temperature stress
- Oxidative stress

Comparison with ➔ reference standard

Stress description:

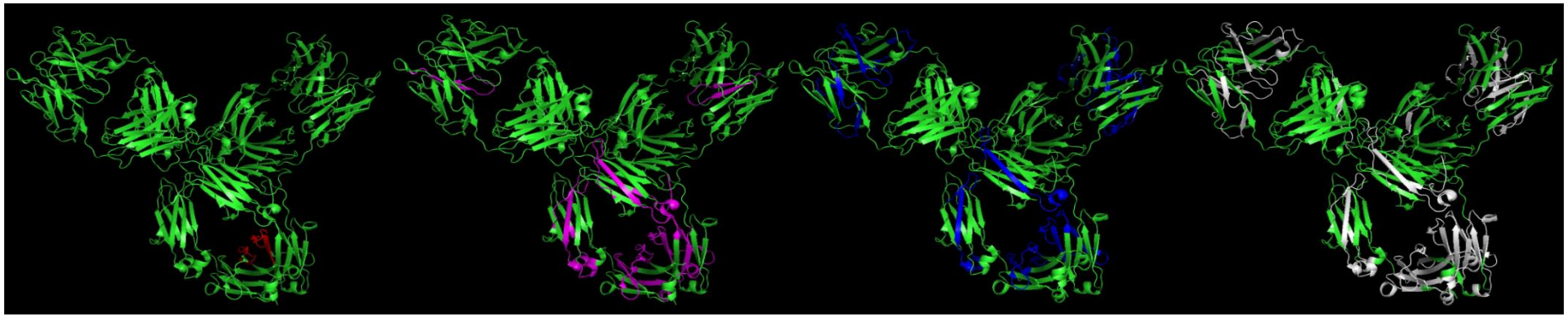
Acidic stress ➔ Incubation @ pH 3 during 14 days @ 5-8°C

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 nalysis with biological activity

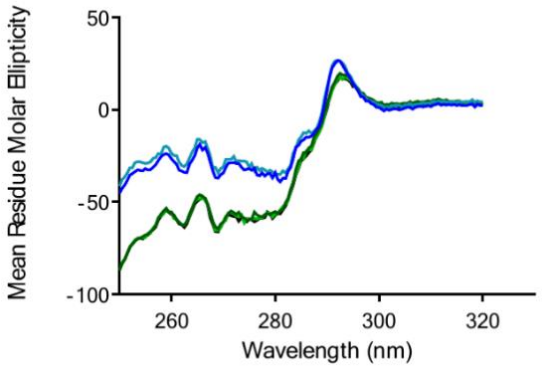
Comparison of stressed Mabs after 5 min proteolysis



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

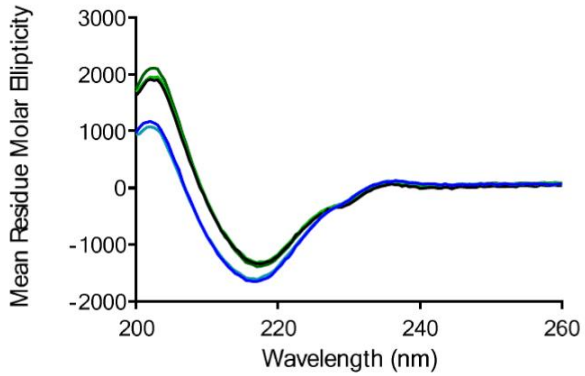
Enzyme accessability

Focus on acidic pH stressed sample



Near UV CD spectra

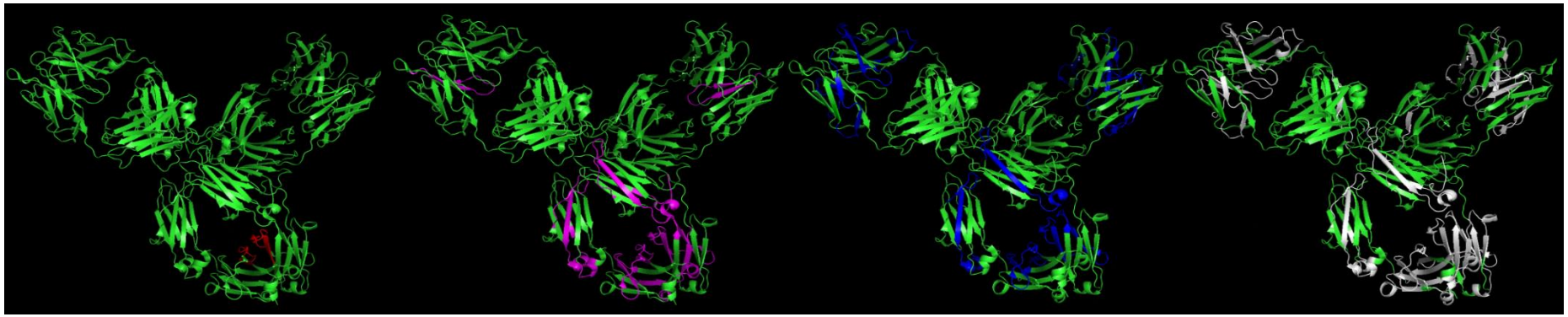
Ref std
Control 1
Control 2
Acid pH stress 1
Acid pH stress 2



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

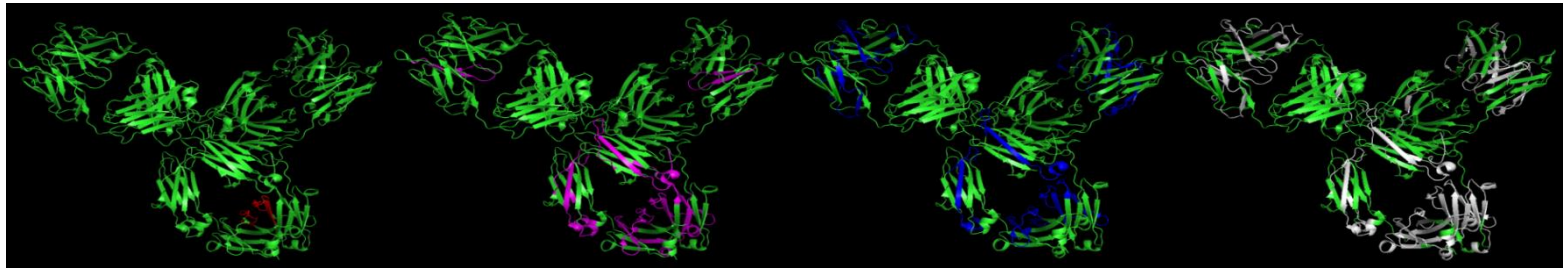
Acidic pH stress

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



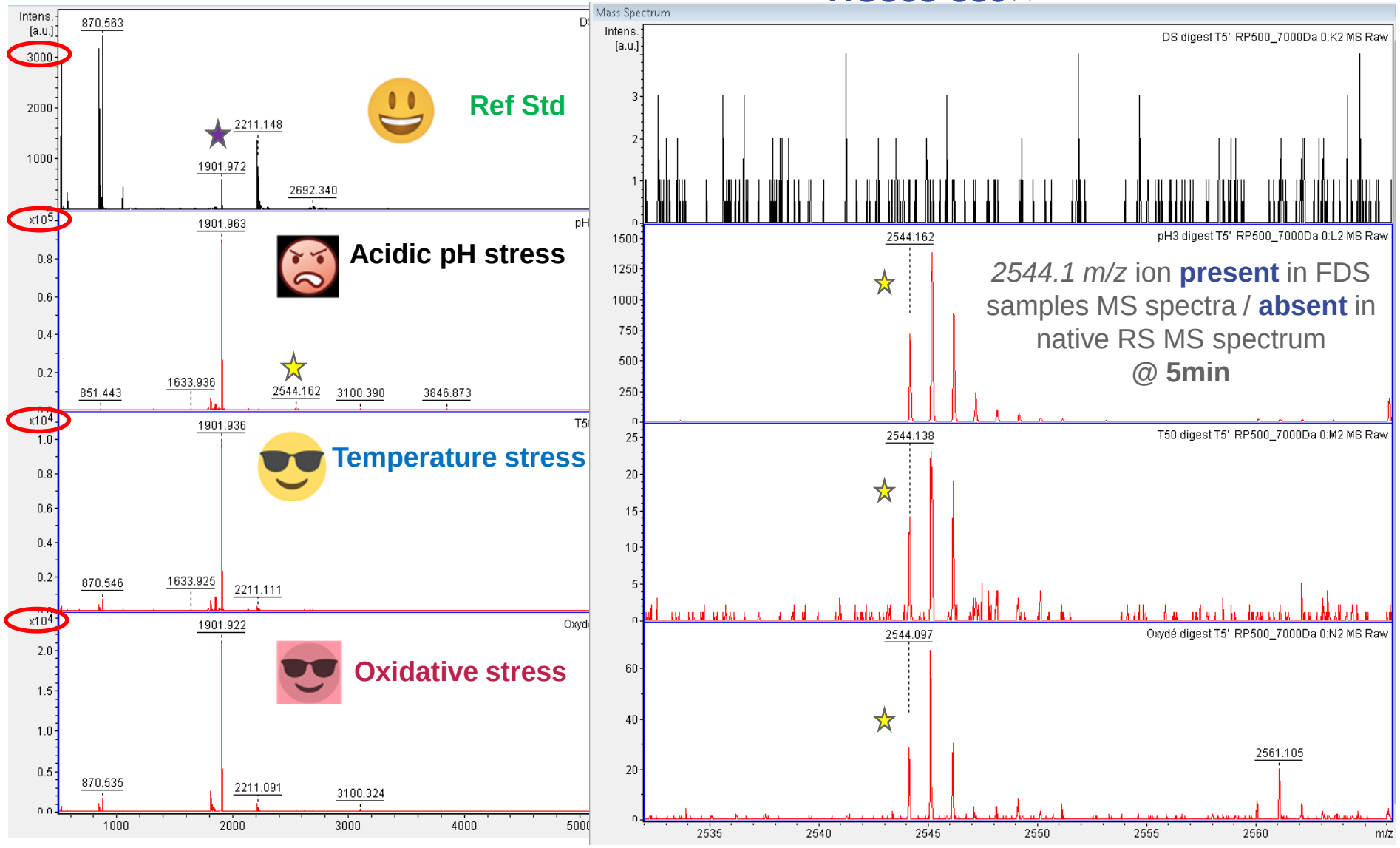
Ref Std | Oxidative stress | Temperature stress | Acidic pH stress

Peptide	Unstressed	Ox stress	Temp stress	Acid stress
HC 1-19			✓	✓
HC 39-61			✓	✓
HC 44-61				✓
HC 44-65				✓
HC 77-87				✓
HC 122-133-LC 217-219				✓
HC 246-252		✓		✓
HC 286-298		✓		✓
HC 299-314 ★		✓	✓	✓
HC 299-317		✓		✓
HC 342-357		✓		✓
HC 358-367				✓
HC 368-389 ★		✓	✓	✓
HC 390-406 ★	✓	✓	✓	✓
HC 414-436				✓
LC 1-18			✓	✓
LC 36-50			✓	✓
LC 51-66				✓
LC 52-66				✓

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

HC390-406 ★

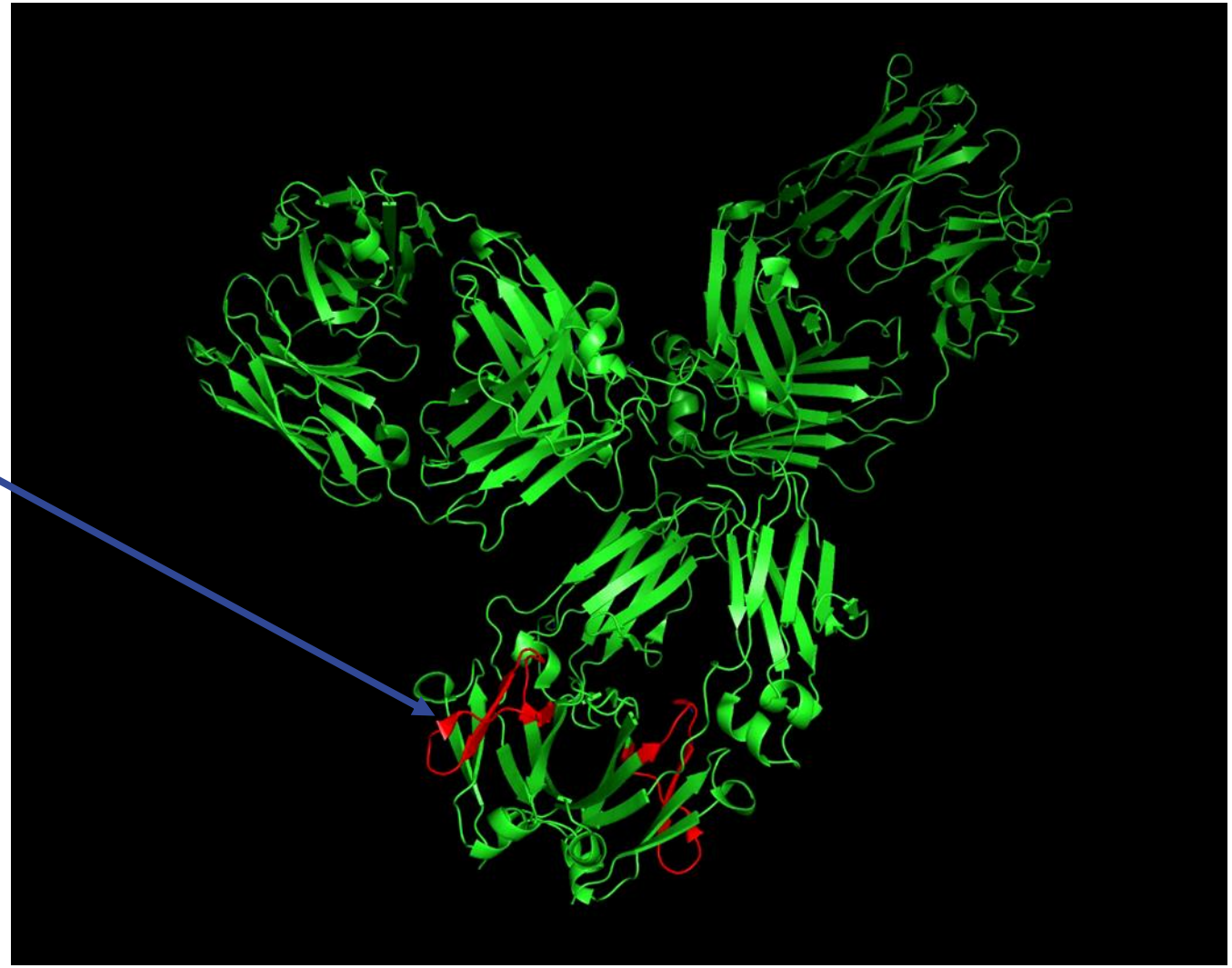
HC368-389☆



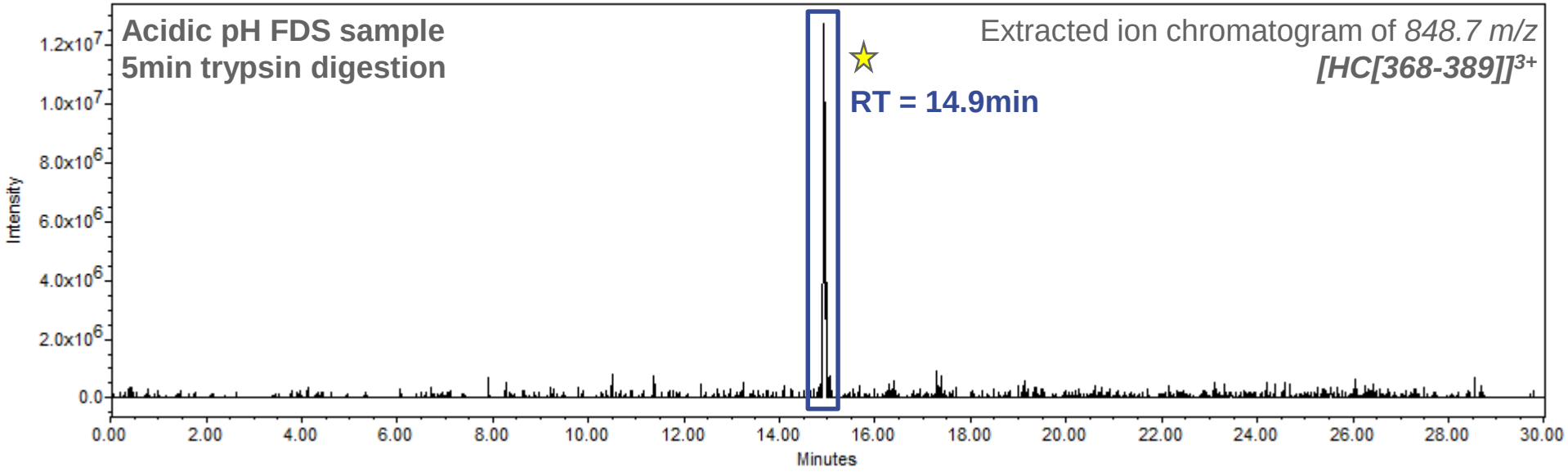
Location of HC 368-389 peak digested from stressed peptide

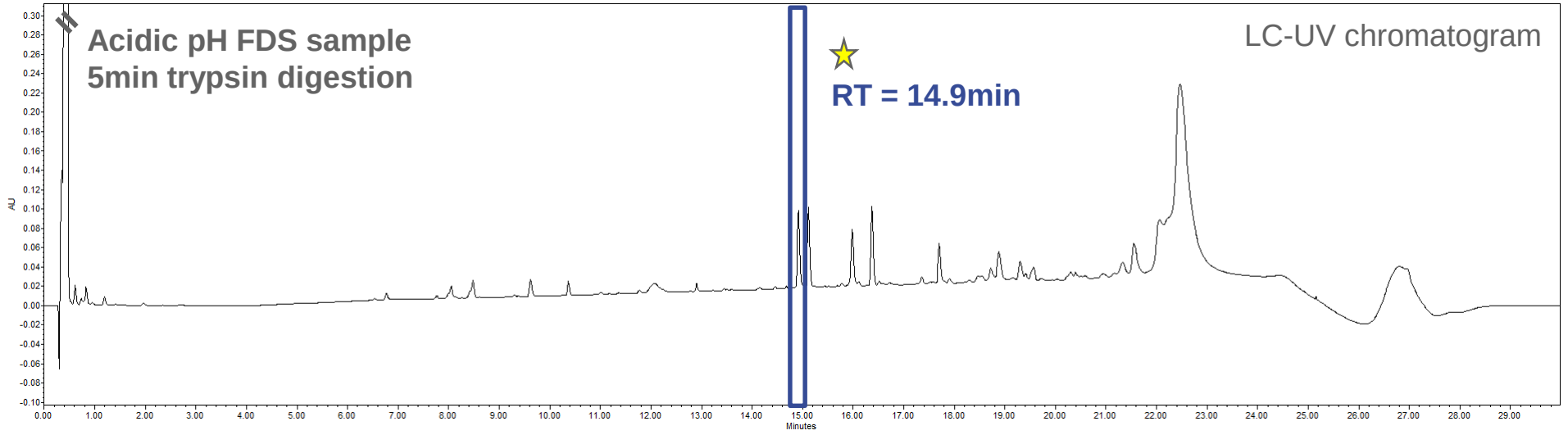
HC 368-389

Mass 2544 m/z ion



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

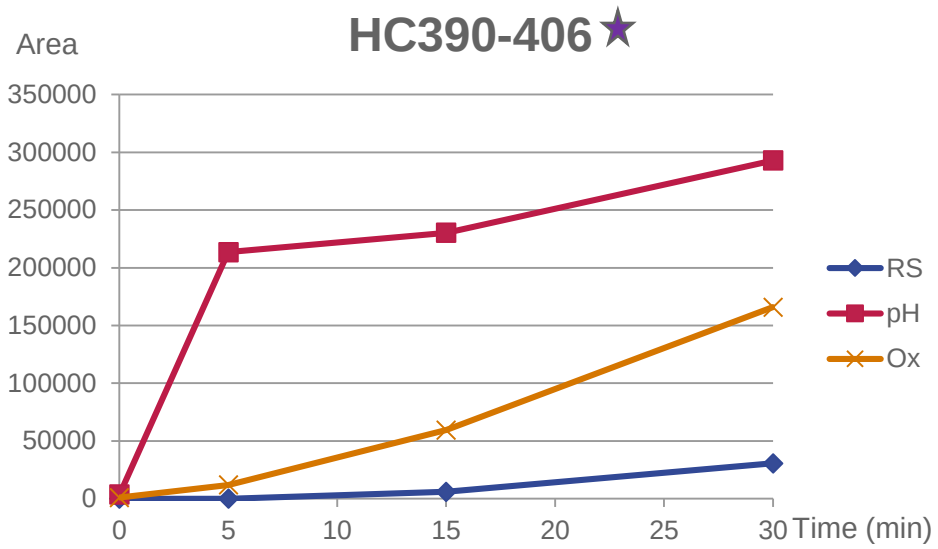




Rate of peptide release is correlated with enzyme accessability to MAb

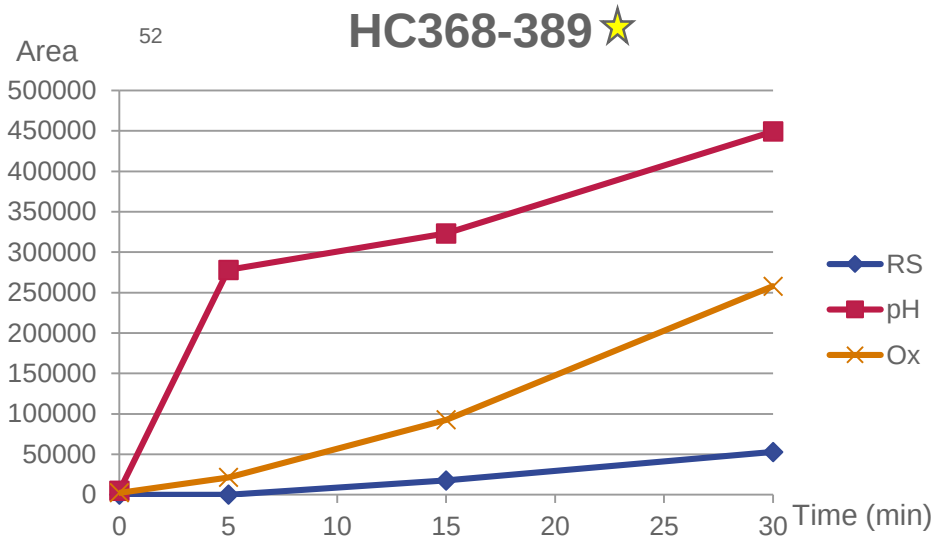
HC390-406

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

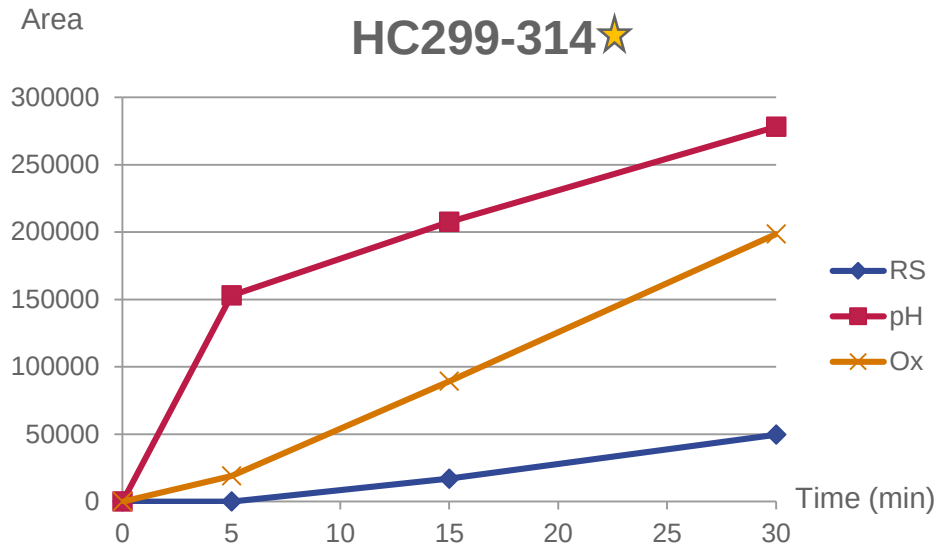
Time (min)	RS	pH	Ox
0	0	4904	2405
5	0	278171	21374
15	17682	323147	92714
30	52910	449305	257932
280	592579	615322	940413

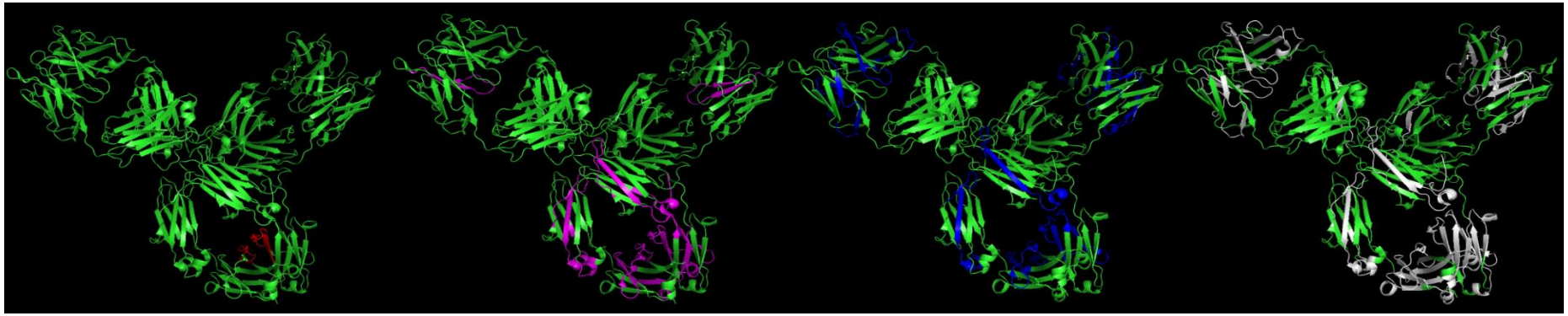


Rate of peptide release is correlated with enzyme accessability to MAb

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





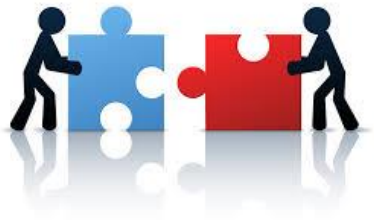
Ref Std | Oxidative stressed | Temperature stressed | Acidic pH stressed

Enzyme accessability

Further development

- Generate more data to support the link between bioassay and the native peptide mapping (HOS) results.
- Know-how on molecule degradation is built from the projects (past and present).
- Predictive tool for drug candidate engineering/screening.
- Evaluate implementation of the native peptide mapping in a QC environment (as part of stability package) -> anticipate regulators expectations in term of product knowledge.
- Tool to further characterize the correlation between structure – activity and… immunogenicity (patient safety) ?

In conclusion…



- Excellent highly resolving methods exist and are being developed to interrogate HOS for product characterisation.
- Current release and stability assays (physico-chemical assays and bioassays) may not be sufficient to identify changes in HOS that impact a product's safety and/or efficacy.
- Understanding the differences between Characterisation and QC needs will help the development of better HOS assays and instrumentation.
- For example Native Peptide mapping is:
  - A simple HOS method that is QC friendly
  - Has been used (during FDS) to identify changes in HOS that affect biological activity
- Understanding Structure-Activity Relationships can support clinically relevant specification setting.

Many Thanks to my former colleagues at UCB, in particular

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