

# The Expanding Hyphenation of LC Techniques to MS for Intact Mass Characterization of Biopharmaceuticals During Development - Lessons Learned and The Road Ahead

Dan Bach Kristensen, Symphogen



**17<sup>th</sup>** Symposium on the  
Practical Applications of  
**Mass Spectrometry**  
in the Biotechnology Industry

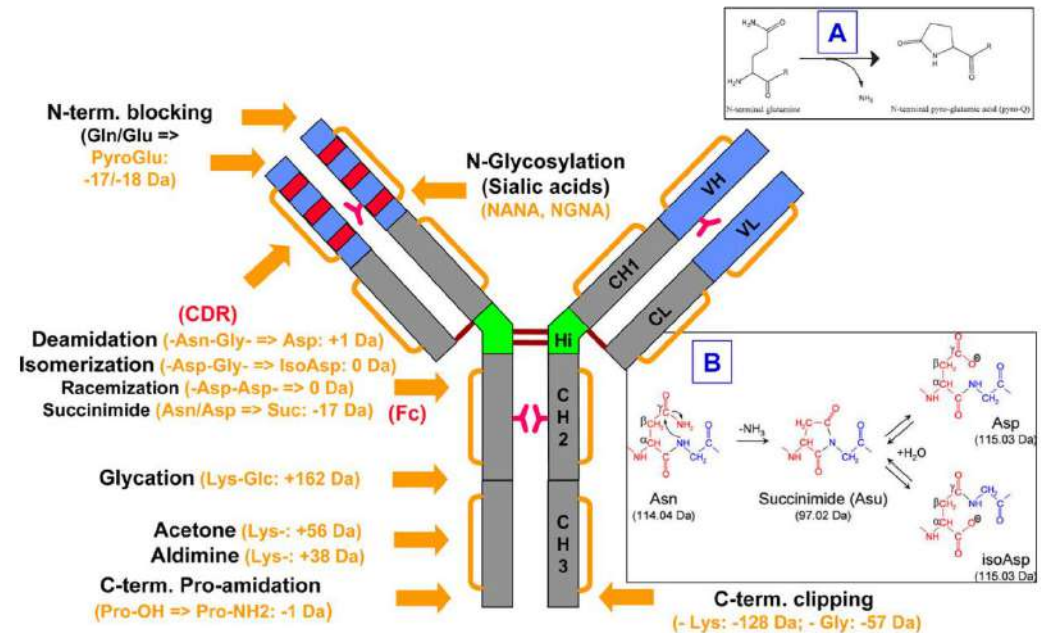
**VIRTUAL SYMPOSIUM**

SEPTEMBER 14-17



# The Biopharmaceutical Characterization Challenge

- Regulator requirement for understanding and controlling product variants (quality attributes), including impurities that impact safety and efficacy of drug
- <http://www.unimod.org/> ~1500 modifications described to date
  - oxidation
  - deamidation
  - isomerization
  - amino acid substitution/addition/deletion
  - glycosylation
  - glycation
  - ...and many others
- In reality a biopharmaceutical product is a mixture of *many* molecular variants (which change over time)
- Mass changes often accompany protein modifications, and consequently mass spectrometry is inherently powerful for the characterization of protein modifications

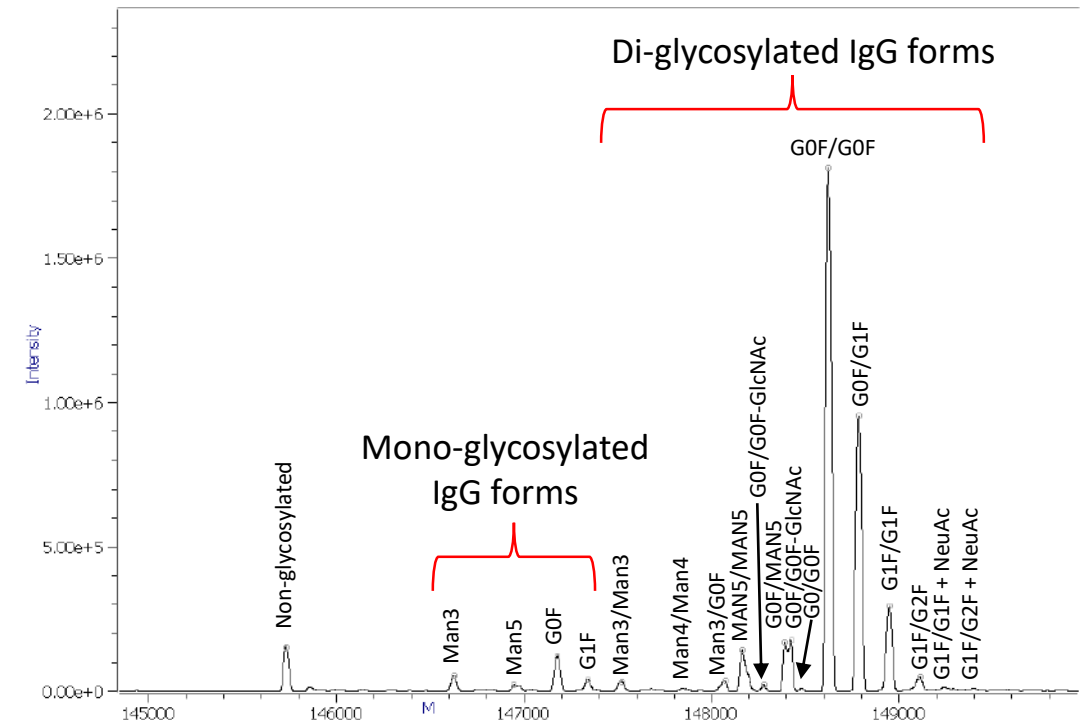


Beck A et al (2013), Anal. Chem., 85:715–736.

# Why Intact Mass Characterization by LC MS in Biopharmaceutical Development?

- Provides structural insight into proteoforms of large biopharmaceuticals (e.g. mAbs) with minimal sample handling and short analysis time
- Provides a powerful link to LC analyses performed on clinical batches in QC environment (release & stability)
- Traditionally intact mass analysis of large biopharmaceuticals by ESI MS was restricted to low resolution reversed-phase (RP) LC MS (e.g. formic acid/desalting)
- With the maturation of native LC MS platforms and improved solvents for intact RP LC MS the situation has changed radically in recent years...

Intact Mass Spectrum of IgG1



# Recent Publications on Native LC MS

## Charge Variant Analysis of Monoclonal Antibodies using Direct Coupled pH Gradient Cation Exchange Chromatography to High Resolution Native Mass Spectrometry

Florian Füssl, Ken Cook, Kai Scheffler, Amy Farrell, Stefan Mittermayr, and Jonathan Bones

*Anal. Chem.* 2018, 90, 7, 4669-4676

## Native size-exclusion chromatography-mass spectrometry: suitability for antibody-drug conjugate drug-to-antibody ratio quantitation across a range of chemotypes and drug-loading levels

Jay Jones<sup>a</sup>, Laura Pack<sup>b</sup>, Joshua H. Hunter<sup>c</sup>, and John F. Valliere-Douglass<sup>a</sup>

MABS 2020, VOL. 12, NO. 1

## Coupling Mixed-Mode Size Exclusion Chromatography with Native Mass Spectrometry for Sensitive Detection and Quantitation of Homodimer Impurities in Bispecific IgG

Yuetian Yan, Tao Xing, Shunhai Wang<sup>a</sup>, Thomas J. Daly, and Ning Li

*Anal. Chem.* 2019, 91, 11417–11424

## Native Hydrophobic Interaction Chromatography Hyphenated to Mass Spectrometry for Characterization of Monoclonal Antibody Minor Variants

Bingchuan Wei<sup>a</sup>, Guanghui Han<sup>a</sup>, Jia Tang<sup>a</sup>, Wendy Sandoval<sup>a</sup>, and Yonghua Taylor Zhang<sup>a</sup>

*Anal. Chem.* 2019, 91, 24, 15360-15364

## Native Reversed-Phase Liquid Chromatography: A Technique for LCMS of Intact Antibody–Drug Conjugates

Tse-Hong Chen<sup>a</sup>, Yun Yang<sup>a</sup>, Zhaorui Zhang<sup>a</sup>, Cexiong Fu<sup>a</sup>, Qunying Zhang<sup>a</sup>, Jon D. Williams<sup>a</sup>, and Mary J. Wirth<sup>a</sup>

*Anal. Chem.* 2019, 91, 2805–2812

## Detailed Characterization of Monoclonal Antibody Receptor Interaction Using Affinity Liquid Chromatography Hyphenated to Native Mass Spectrometry

Rabah Gahoual<sup>a</sup>, Anna-Katharina Heidenreich<sup>a</sup>, Govert W. Somsen<sup>a</sup>, Patrick Bulau<sup>a</sup>, Dietmar Reusch<sup>a</sup>, Manfred Wuhrer<sup>a</sup>, and Markus Habberger<sup>a</sup>

*Anal. Chem.* 2017, 89, 10, 5404-5412

## Glycoform-resolved FcγRIIIa affinity chromatography–mass spectrometry

Steffen Lippold<sup>a</sup>, Simone Nicolardi<sup>a</sup>, Elena Domínguez-Vega<sup>a</sup>, Anna-Katharina Heidenreich<sup>b</sup>, Gestur Vidarsson<sup>c</sup>, Dietmar Reusch<sup>b</sup>, Markus Habberger<sup>b</sup>, Manfred Wuhrer<sup>a</sup>, and David Falck<sup>a</sup>

MABS 2019, VOL. 11, NO. 7, 1191–1196



# Symphogen's Intact Mass Platform

## Versatile separation

Thermo Scientific  
Vanquish Duo UHPLC



Native MS  
[ SEC  
CIEX  
Protein A ]

RPC

Native & denatured MS  
Thermo Scientific Exploris 480



ID test by MS  
UV chromatography  
(relative peak areas)

Proteoform characterization  
Identity & relative  
quantitation by MS

## Data Processing



Thermo Scientific™  
Chromeleon™ Chromatography  
Data System (CDS)



PROTEIN METRICS  
Byos™  
Intact Mass™

# Symphogen's Subunit & Peptide Mapping Platform

## Sample preparation

Thermo Scientific  
KingFisher Duo Prime



Magnetic beads  
96 deep well plate

Subunit analysis  
FabRICATOR MagIC

20 min, 37°C,  $\pm$ TCEP



Peptide mapping  
SMART Digest Trypsin  
SMART Digest Pepsin  
SMART Digest Proteinase K  
SMART Digest Chymotrypsin

30 min, 75°C,  $\pm$ TCEP



Thermo Scientific  
Vanquish Duo



RPC  
SEC  
CIEX

Thermo Scientific  
Exploris 480



Data Processing

  
PROTEIN METRICS

Byos™  
Intact Mass™

Thermo Scientific  
Vanquish Horizon



RPC

Thermo Scientific  
Orbitrap Fusion



  
PROTEIN METRICS

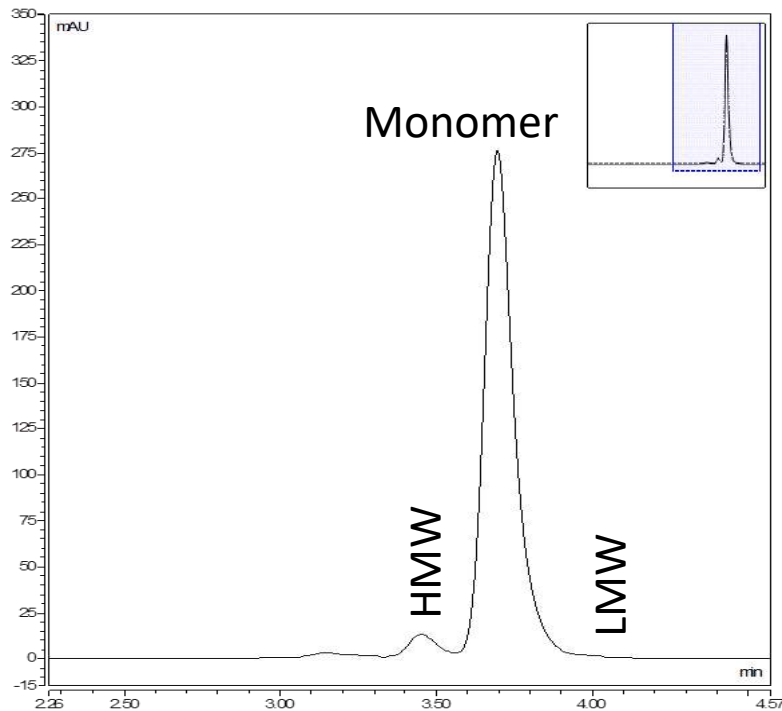
Byos™  
Byonic™  
Byologic™



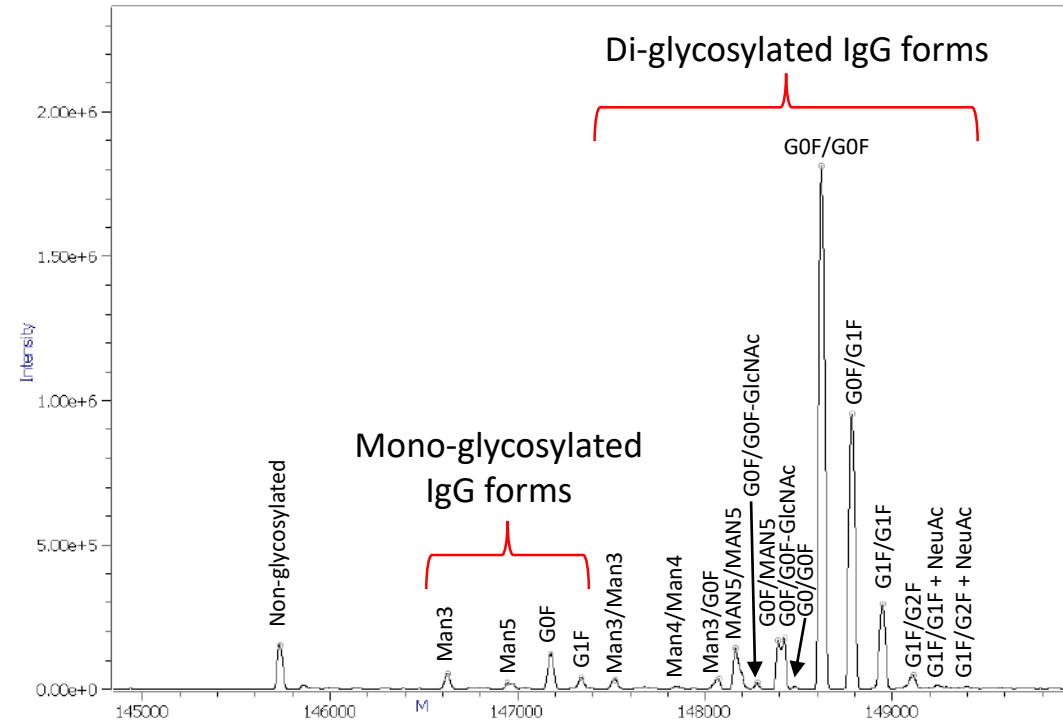
# Native SEC MS – Principal Intact Mass Platform at Symphogen

Multiple birds with one stone...

Monomer, aggregates, fragments from UV



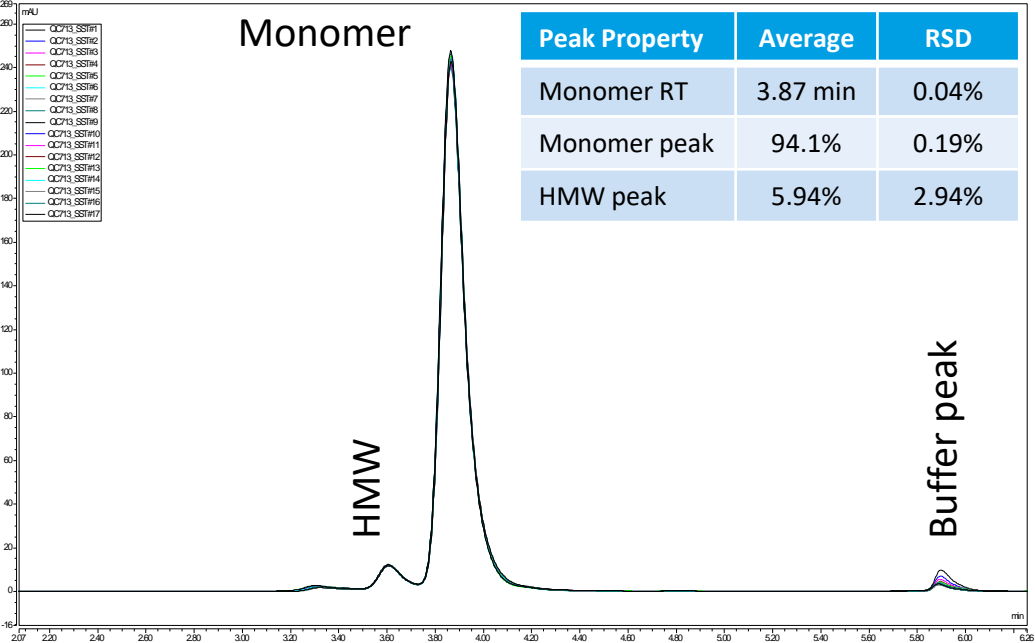
Identity and proteoforms from MS



# Native SEC MS – Principal Intact Mass Platform at Symphogen

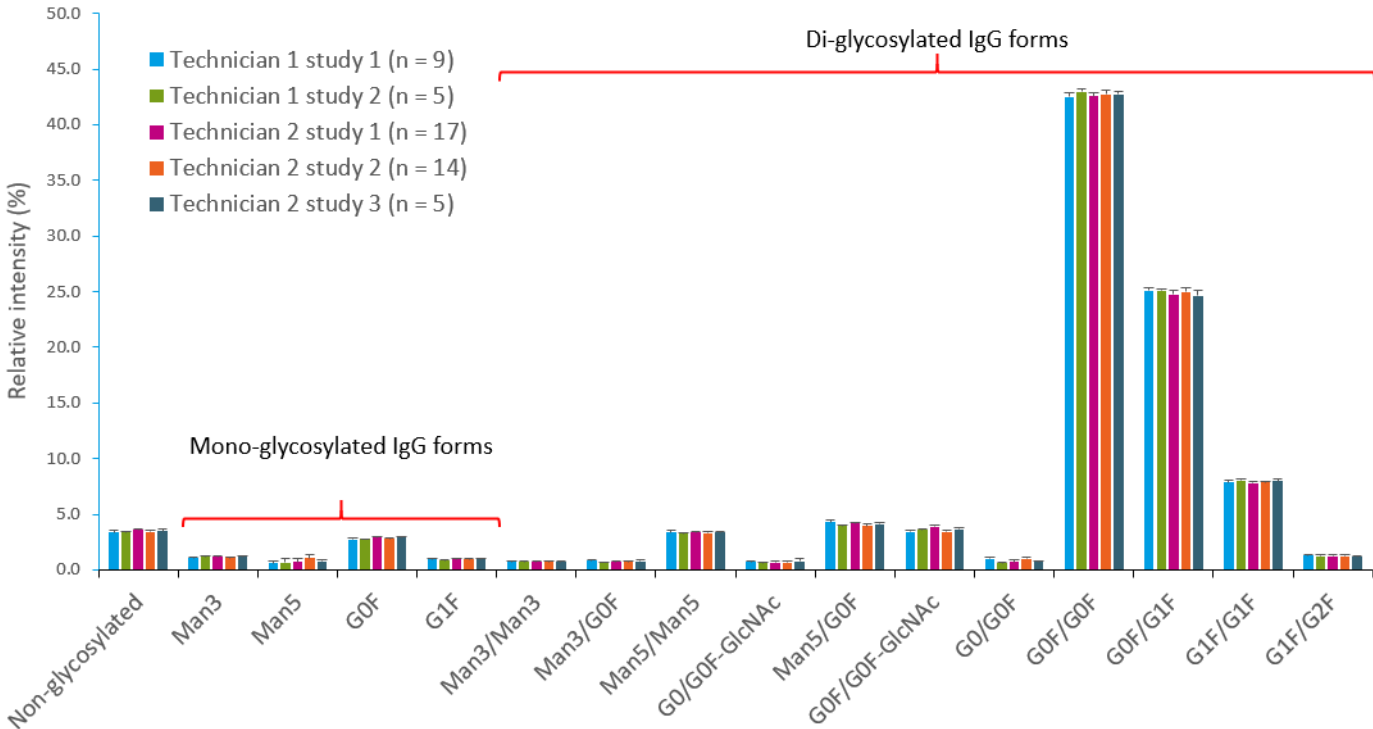
## Platform robustness

### LC robustness\*



\*Overlay of 17 reference (QC713) runs from a lead selection study (384 leads). The reference was analyzed once every 24<sup>th</sup> sample. A total of 413 native SEC runs performed in the study. Total analysis time was 56 hrs.

### MS robustness\*\*



\*\*Data collected from five independent lead selection studies over a six months period. Data collected by two lab technicians using four different SEC column lots.

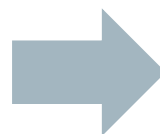


# Changing to a New MS Platform – Impact On Intact MS Data Quality?

Thermo Scientific  
Vanquish Duo UHPLC



Thermo Scientific™  
Q Exactive™ Plus with  
Biopharma Option



Thermo Scientific  
Vanquish Duo UHPLC



Thermo Scientific  
Exploris 480 with  
BioPharma Option

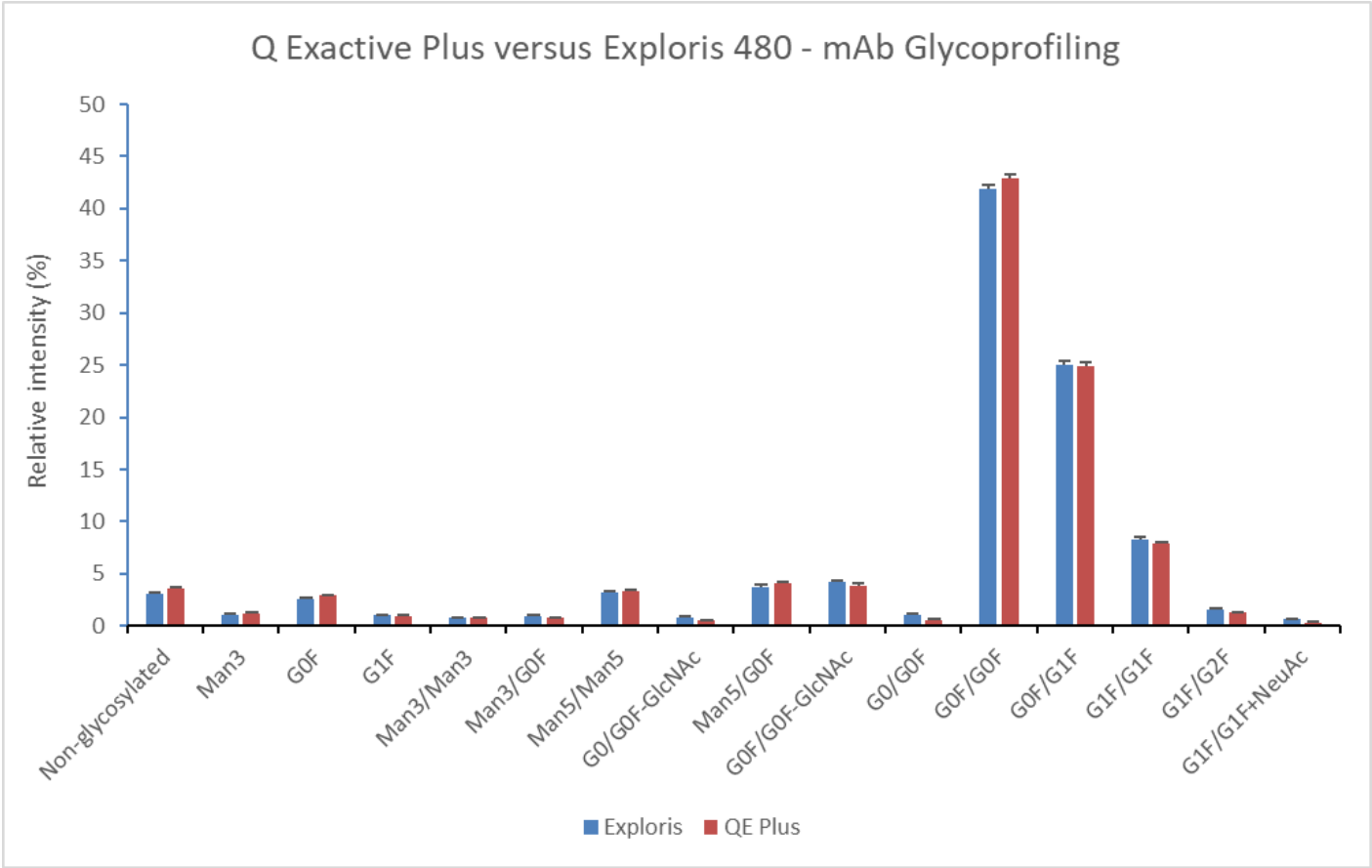


# Changing to a New MS Platform – Impact On Intact MS Data Quality?

Reference (QC713) results from two full lead selection studies (384 leads, 17 QC713 runs)

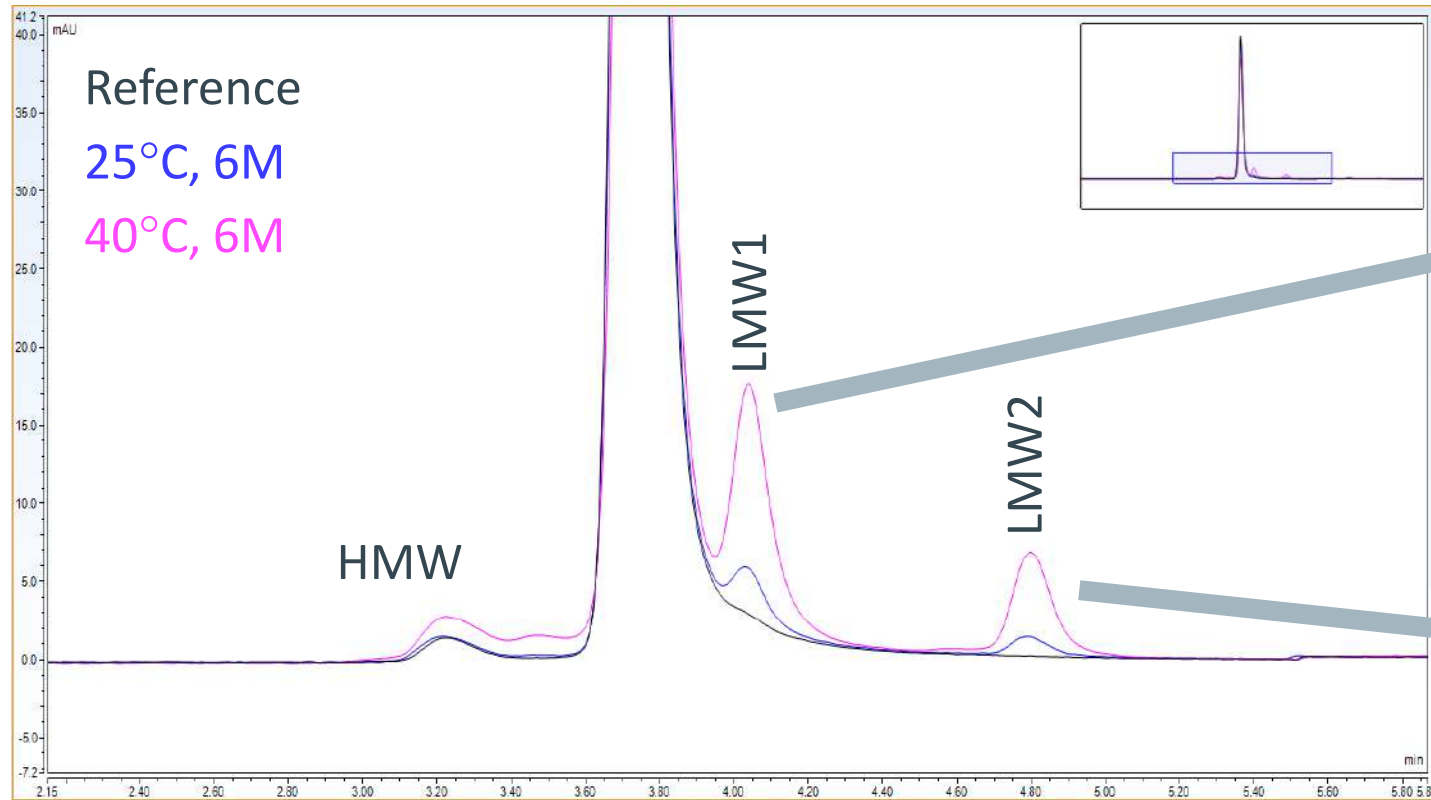
	Q Exactive Plus	Exploris 480
Load	10 µg	4 µg
Resolution	35,000	45,000
Acquisition date	Jan 2019	April 2020

Glycoform	Exploris %	QE Plus %
Non-glycosylated	3.1	3.6
Man3	1.1	1.2
G0F	2.6	2.9
G1F	1.0	0.9
Man3/Man3	0.8	0.8
Man3/G0F	1.0	0.8
Man5/Man5	3.3	3.4
G0/G0F-GlcNAc	0.8	0.5
Man5/G0F	3.8	4.1
G0F/G0F-GlcNAc	4.2	3.9
G0/G0F	1.1	0.5
G0F/G0F	41.9	42.9
G0F/G1F	25.0	24.9
G1F/G1F	8.2	7.9
G1F/G2F	1.6	1.3
G1F/G1F+NeuAc	0.6	0.3

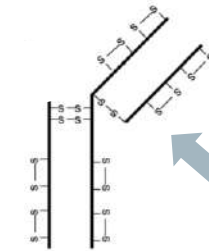
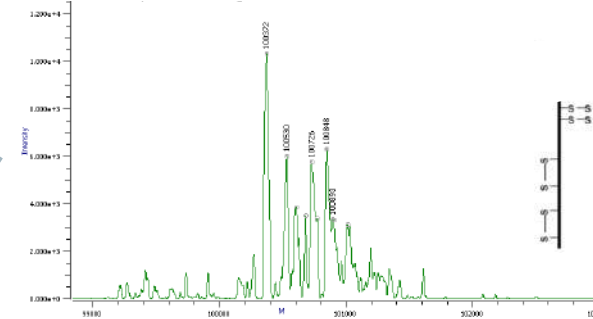


Overall glycoform distribution is highly similar despite different MS platforms and analysis conditions (load, MS resolution). Method transfer from Q Exactive Plus to Exploris 480 was successful.

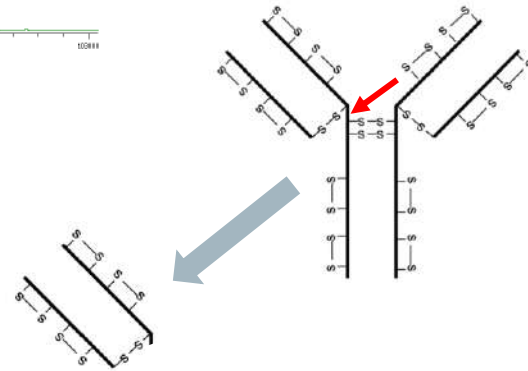
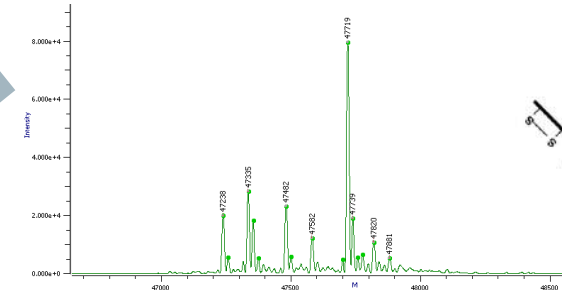
# Application Examples – Native SEC MS – Hinge Region Fragmentation in Stability



Main mass: 100372 Da



Main mass: 47719 Da

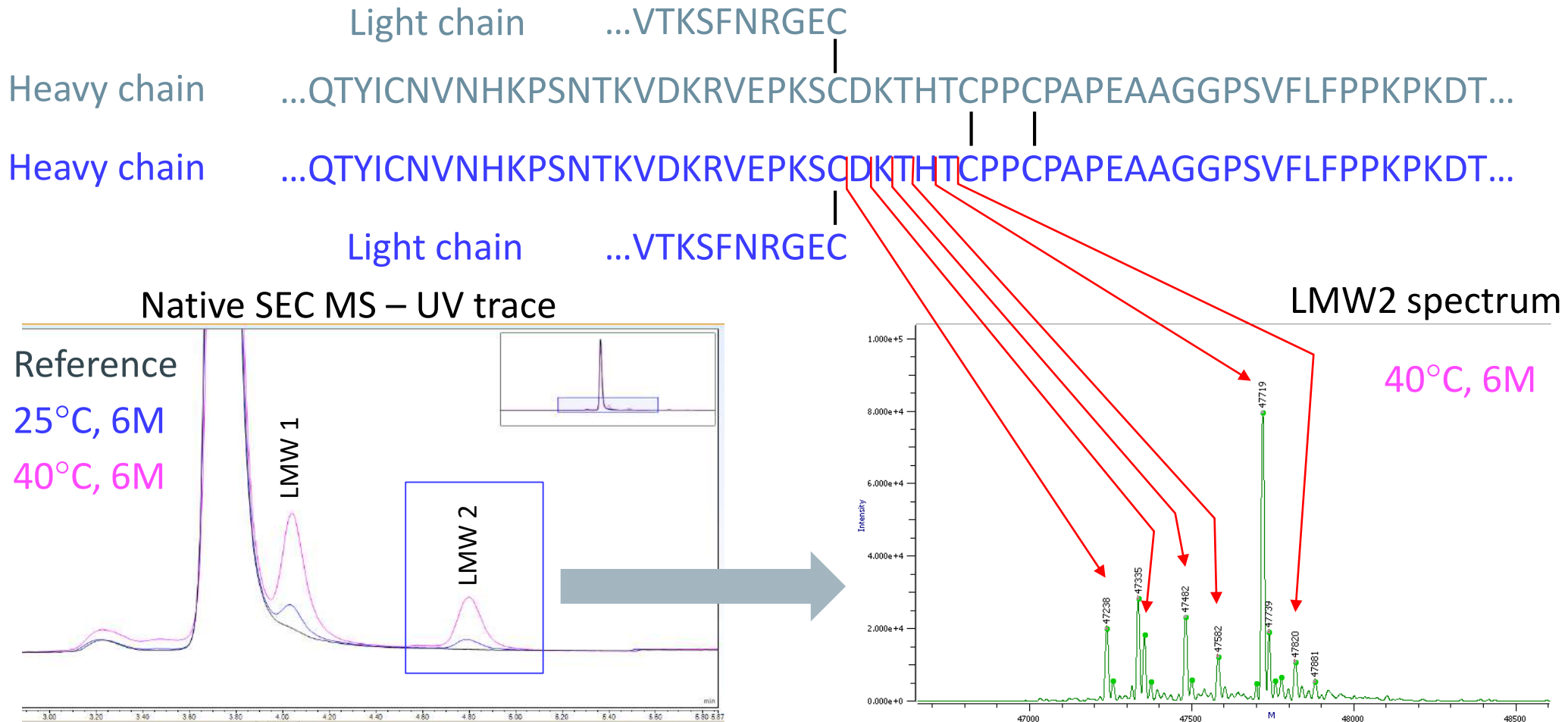


	HMW	Monomer	LMW1	LMW2
Reference	1.1%	98.8	0.2%	0%
25°C, 6M	1.2%	93.6%	4.4%	0.8%
40°C, 6M	3.1%	83.2%	10.3%	3.5%

SEC UV trace reveals rise of fragments in stressed samples. Native SEC MS results reveal fragmentation in hinge region between HC-HC interchain and HC-LC disulphide bonds.



# Application Examples – Native SEC MS – Hinge Region Fragmentation in Stability

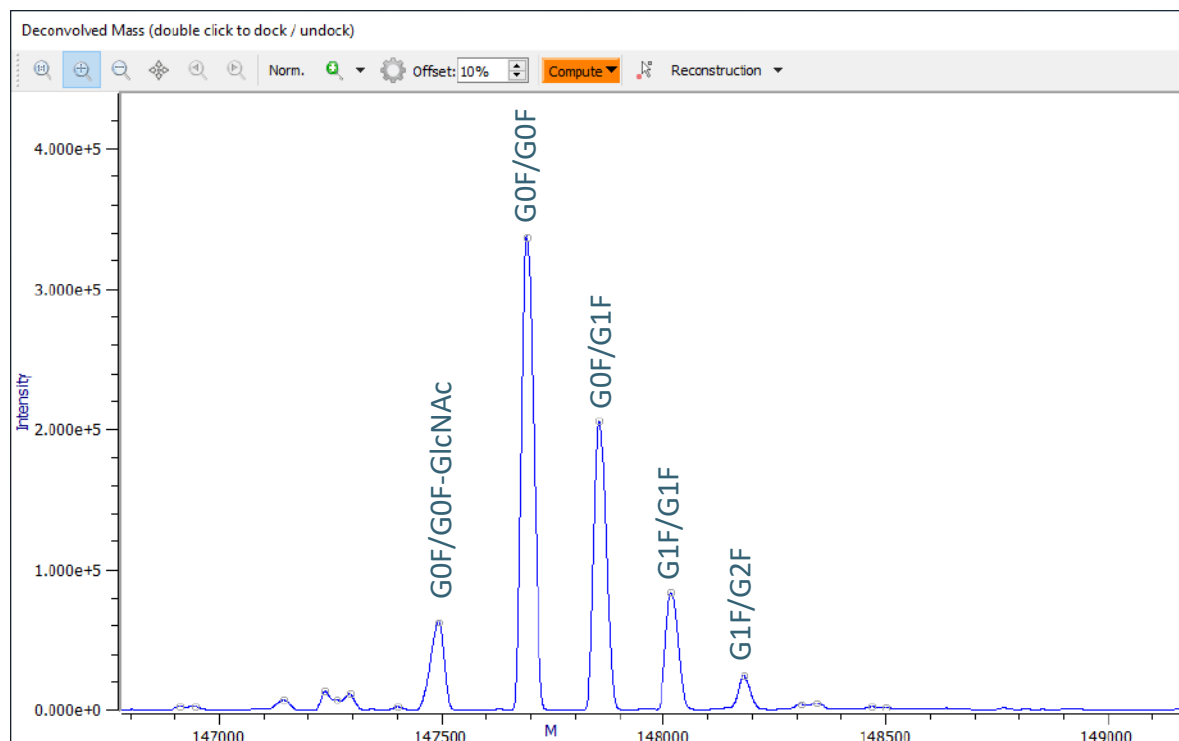


LMW2 deconvoluted spectrum confirms fragmentation at multiple residues in hinge region of stressed sample.

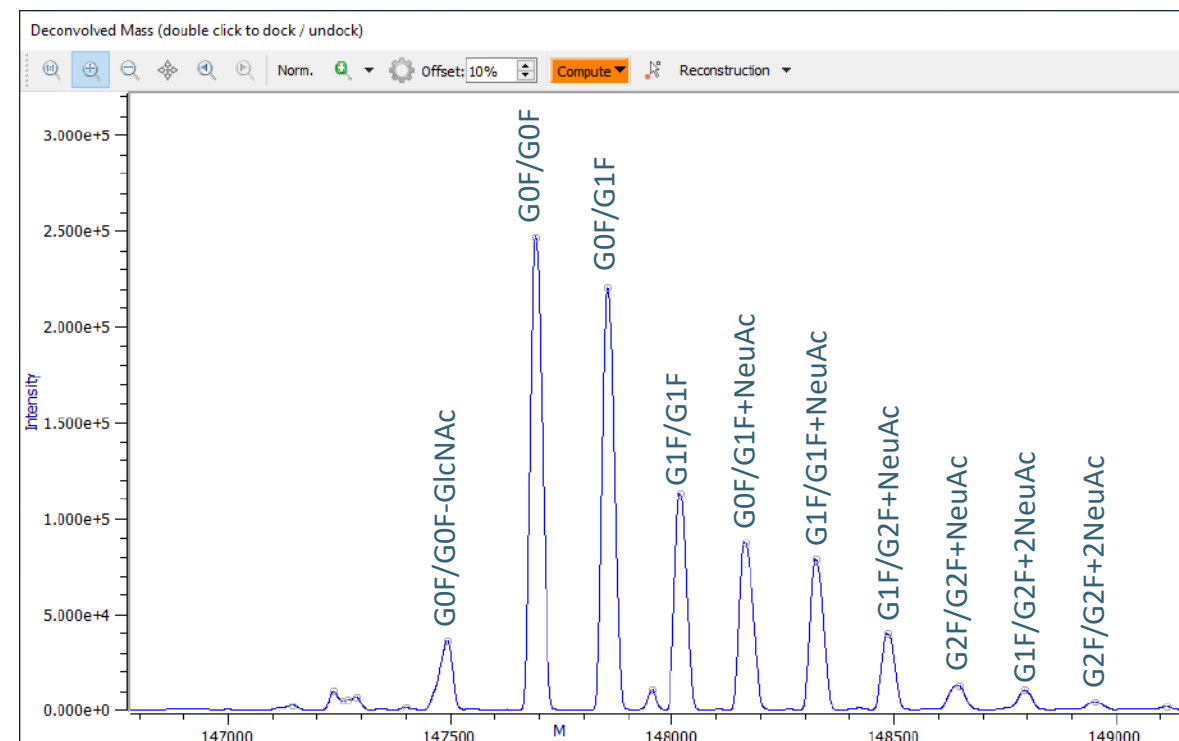
# Application Examples – Native SEC MS – Glycoprofiling During Clone Selection

In a typical clone selection study 24 clones are screened.

Clone 1 – Intact Mass Spectrum



Clone 18 – Intact Mass Spectrum



Glycoform distribution may vary significantly between clones. In this clone selection study clone 18 produced mAbs with exceptionally high level of sialic acid containing glycoforms.

# Application Examples – Native SEC MS – Glycoprofiling During Clone Selection

Sialic acid containing glycoforms

Clone	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F	G2F/G2F	G0F/G1F+NeuAc	G1F/G1F+NeuAc	G1F/G2F+NeuAc	G2F/G2F+NeuAc
Clone 1	45	27	11	3	1	0	1	0	0
Clone 2	47	26	9	2	0	0	0	1	0
Clone 3	32	33	20	7	1	0	1	1	0
Clone 4	41	28	11	3	1	0	1	0	0
Clone 5	50	28	11	3	0	0	0	0	0
Clone 6	48	29	11	3	0	0	0	0	0
Clone 7	38	31	13	4	1	0	1	0	0
Clone 8	43	28	11	3	1	0	0	0	0
Clone 9	43	29	13	4	1	0	1	0	0
Clone 10	40	30	13	4	1	1	1	0	0
Clone 11	43	31	13	4	1	0	1	0	0
Clone 12	39	32	16	5	1	0	1	1	0
Clone 13	37	32	17	5	1	0	1	1	0
Clone 14	59	22	6	1	0	0	0	0	0
Clone 15	40	32	16	5	1	0	1	1	0
Clone 16	50	24	10	3	1	0	1	0	0
Clone 17	36	33	16	4	1	0	1	1	0
Clone 18	25	23	12	7	5	9	8	4	1
Clone 19	52	22	7	2	0	0	1	0	0
Clone 20	37	33	16	5	1	0	1	0	0
Clone 21	50	28	10	2	0	1	1	0	0
Clone 22	48	24	10	3	1	0	1	0	0
Clone 23	35	33	17	5	1	0	1	1	0
Clone 24	36	33	16	5	1	0	1	1	0

Clone 18 was disregarded for the project since sialic acid containing glycoforms are undesirable in the drug product. However, clone 18 was kept with the aim of establishing a sialic acid rich reference mAb.



# Tips & Tricks – Native SEC MS

- 1. Column choice is critical since buffer with weak ion strength and buffer capacity is used**
  1. Waters ACQUITY UPLC BEH200 SEC, 4.6 x 150 mm, 1.7  $\mu\text{m}$  (cat# 186005225)
- 2. Prepare 25 mM ammonium acetate pH 5.4 by adding chemicals directly to purchased 1 L MS grade water bottle**
  1. Add 1.93 g ammonium acetate + 220  $\mu\text{L}$  acetic acid (100%), dissolve by swirling
  2. Use directly (do not filter), discard after one week
- 3. Add clean ion transfer tube regularly (weekly)**

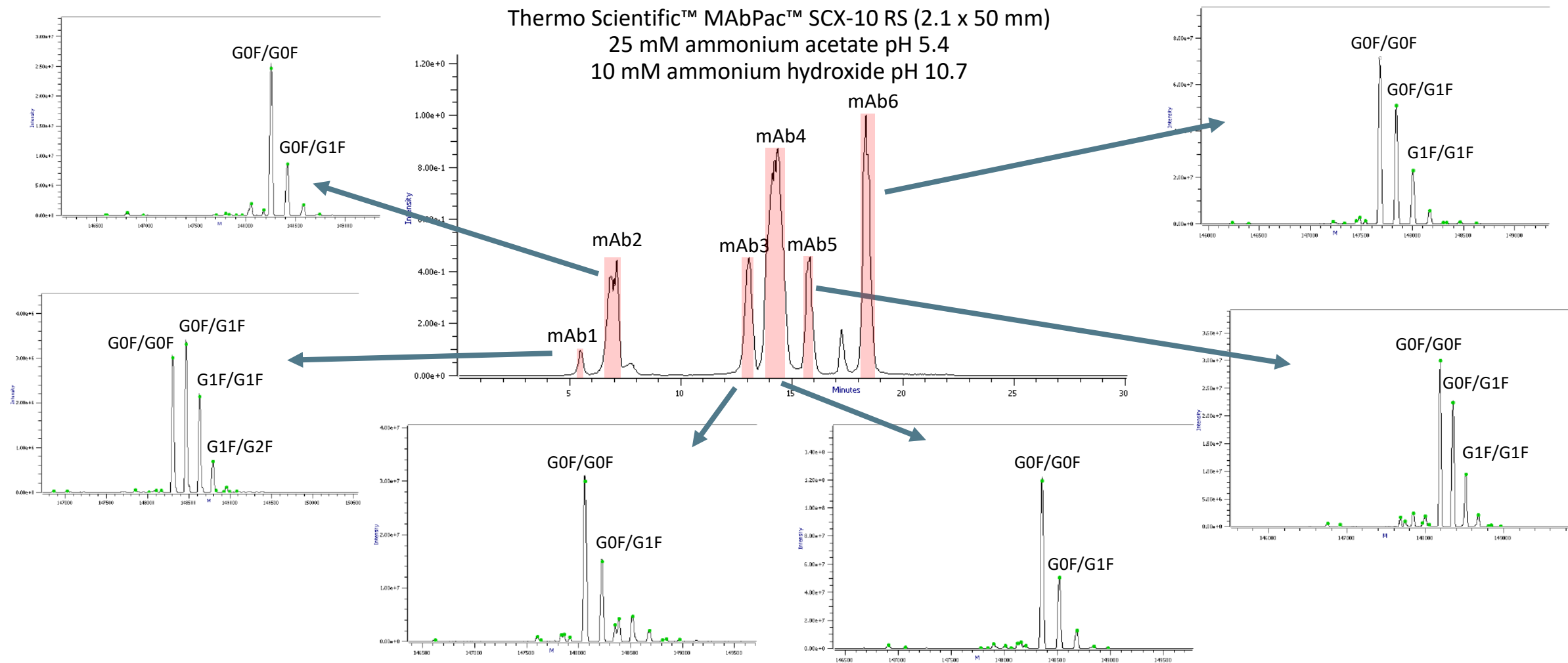
# Application Examples – Native CIEX MS Analysis of Complex mAb Mixture

## Native CIEX MS

Thermo Scientific™ MAbPac™ SCX-10 RS (2.1 x 50 mm)

25 mM ammonium acetate pH 5.4

10 mM ammonium hydroxide pH 10.7



Good chromatographic resolution and excellent MS data for all mAbs in mixtures!



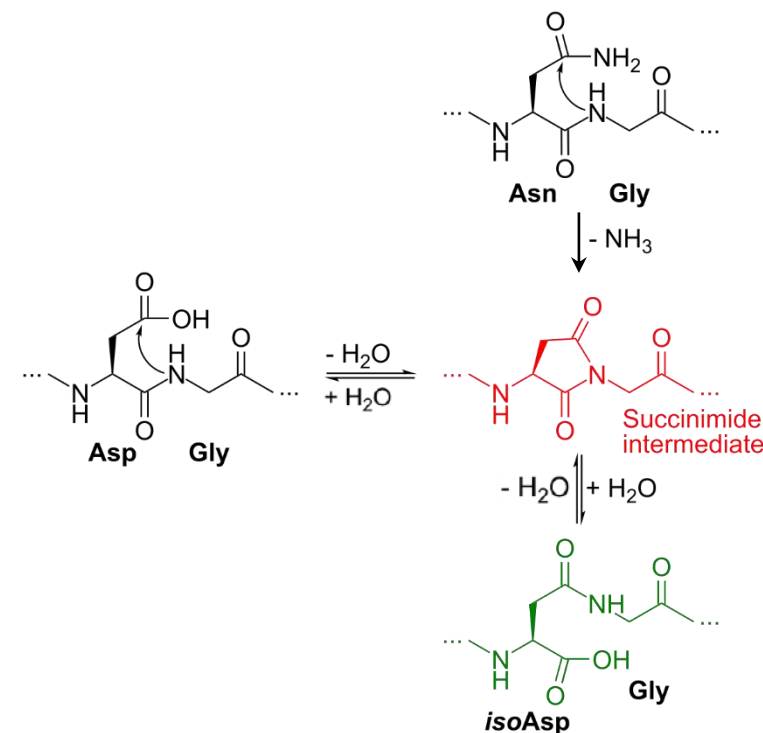
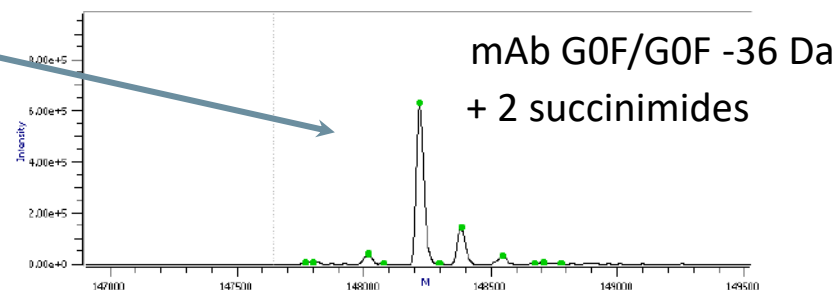
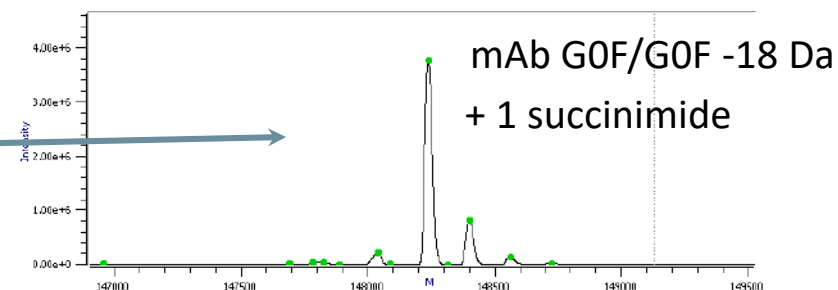
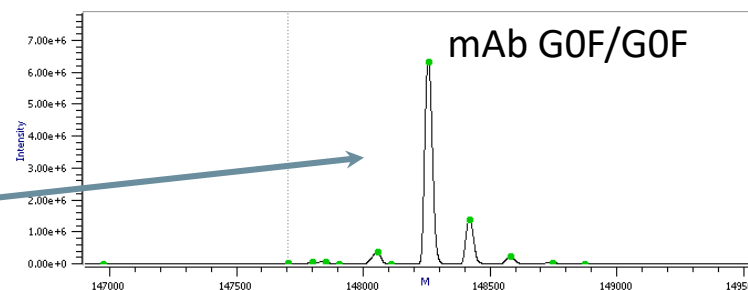
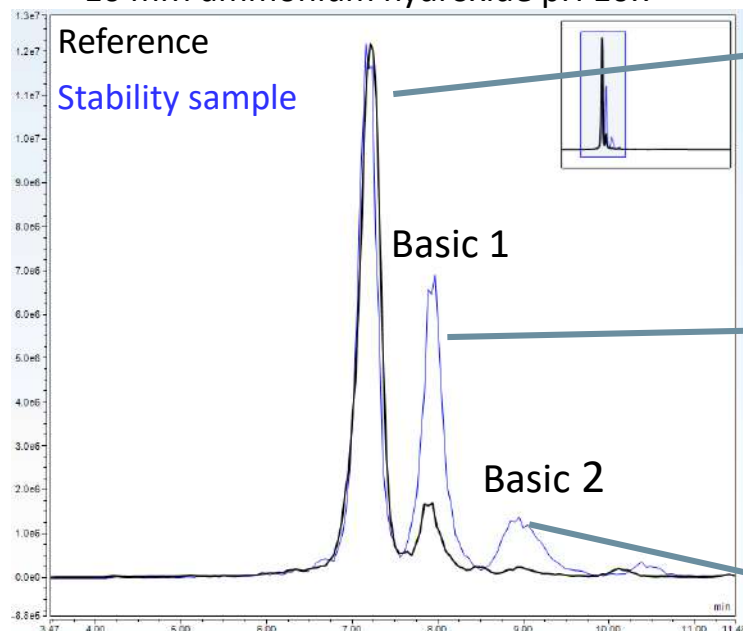
# Application Examples – Native CIEX MS Succinimide Formation in Stability

## Native CIEX MS

Thermo Scientific MAbPac SCX-10 RS (2.1 x 50 mm)

25 mM ammonium acetate pH 5.4

10 mM ammonium hydroxide pH 10.7



Native CIEX MS reveals accumulation of succinimide intermediate in stability sample.





# Tips & Tricks – Native CIEX MS

## **1. Column dimension is critical since buffers with weak ion strength and buffer capacity is used**

1. Use small CIEX column for faster equilibration and better gradient control
2. Thermo Scientific: MabPac SCX-10 RS 50\*2.1mm (cat# 082675)

## **2. Prepare solvents by adding chemicals directly to purchased 1 L MS grade water bottle**

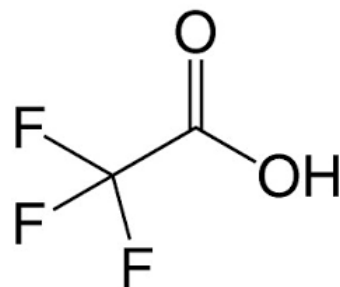
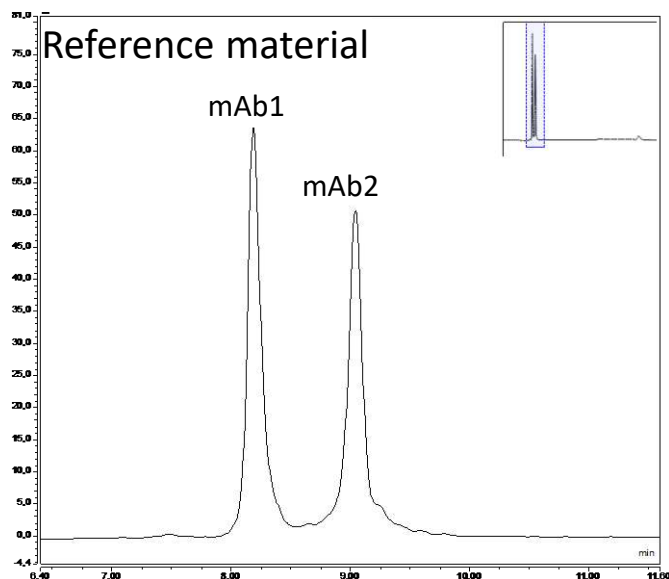
1. Solvent A (25mM Ammonium acetate pH 5.4): add 1.93 g ammonium acetate + 220  $\mu$ L acetic acid (100%), dissolve/mix by swirling
2. Solvent B (10mM Ammonium hydroxide pH 10.7): add 10 mL ammonium hydroxide (1N), mix by swirling
3. Use directly (do not filter), discard after one week
4. PS: commercial solvents available from Waters (IonHance CX-MS)

## **3. Add clean ion transfer tube regularly (weekly)**

# Application Examples – Intact RP LC MS – Difluoroacetic Acid To The Rescue

## Trifluoroacetic acid (TFA)

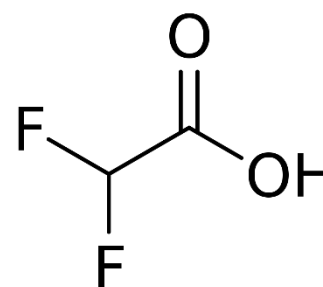
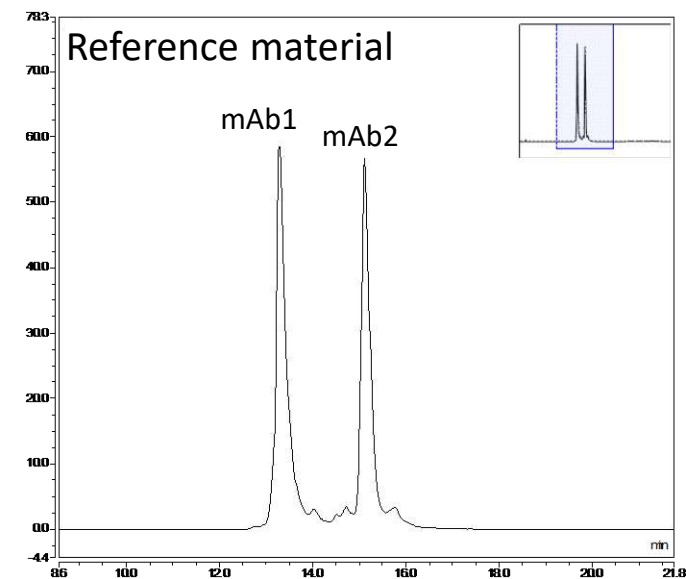
UV, QC RPC method



Conventional ion-pairing reagent in RPC  
Excellent chromatographic performance  
Poor (intact) MS performance due to ion suppression  
Pre-mixed solvent readily available

## Difluoroacetic acid (DFA)

UV, generic RPC MS method

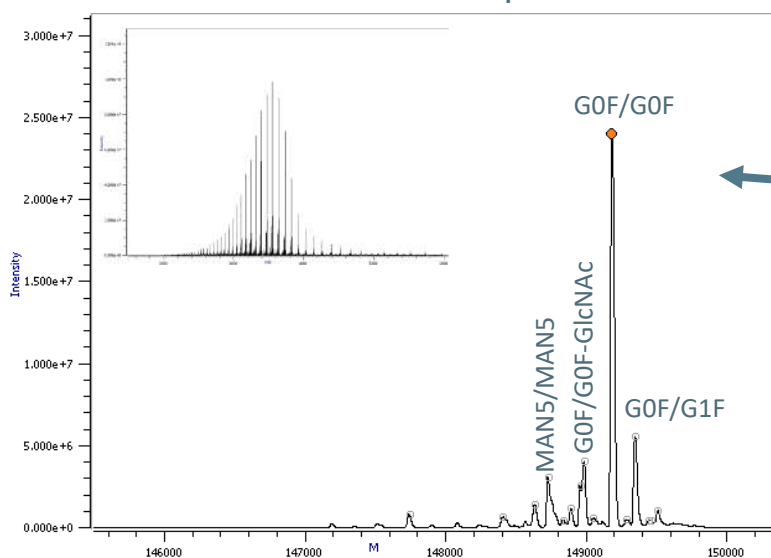


Excellent RPC performance  
Excellent MS performance (from intact to pepmap)  
Expensive, no pre-mixed solvents currently available

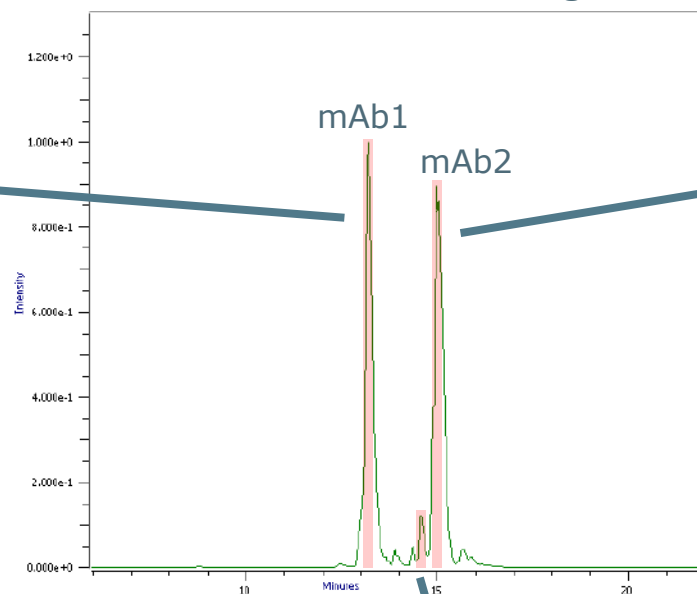
# Application Examples – Intact & Subunit RP LC MS of mAb Mixture

## Intact RPC MS of two mAb product

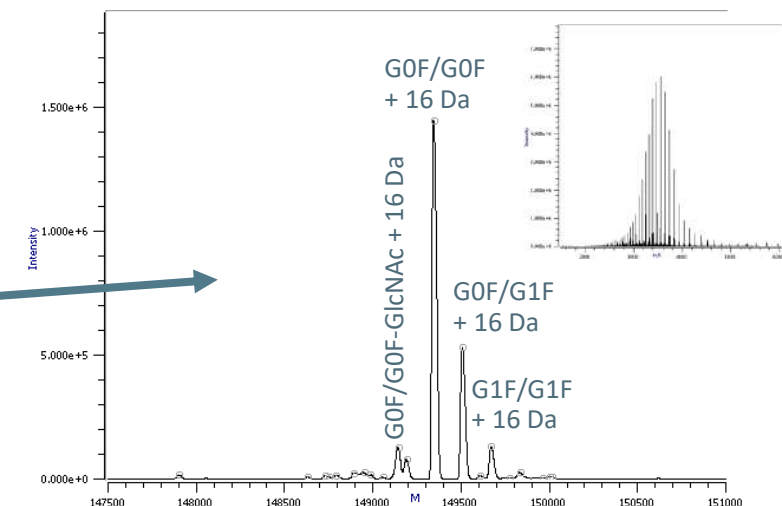
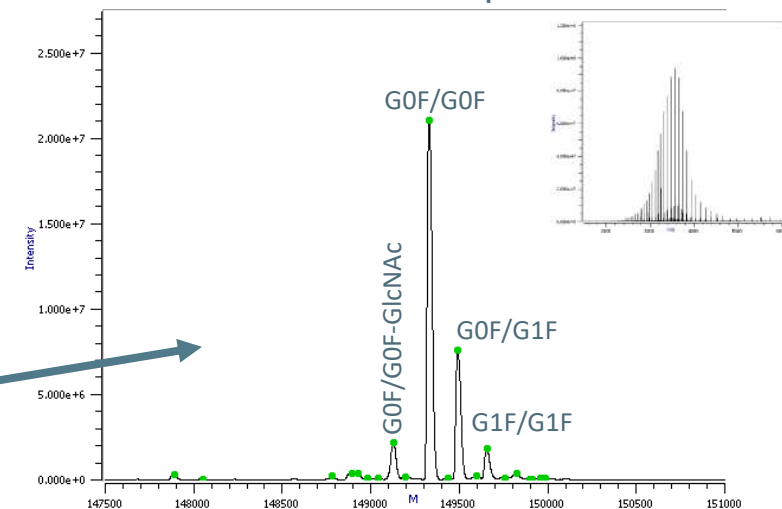
Raw & deconvoluted spectrum for mAb1



Base Peak Chromatogram



Raw & deconvoluted spectrum for mAb2

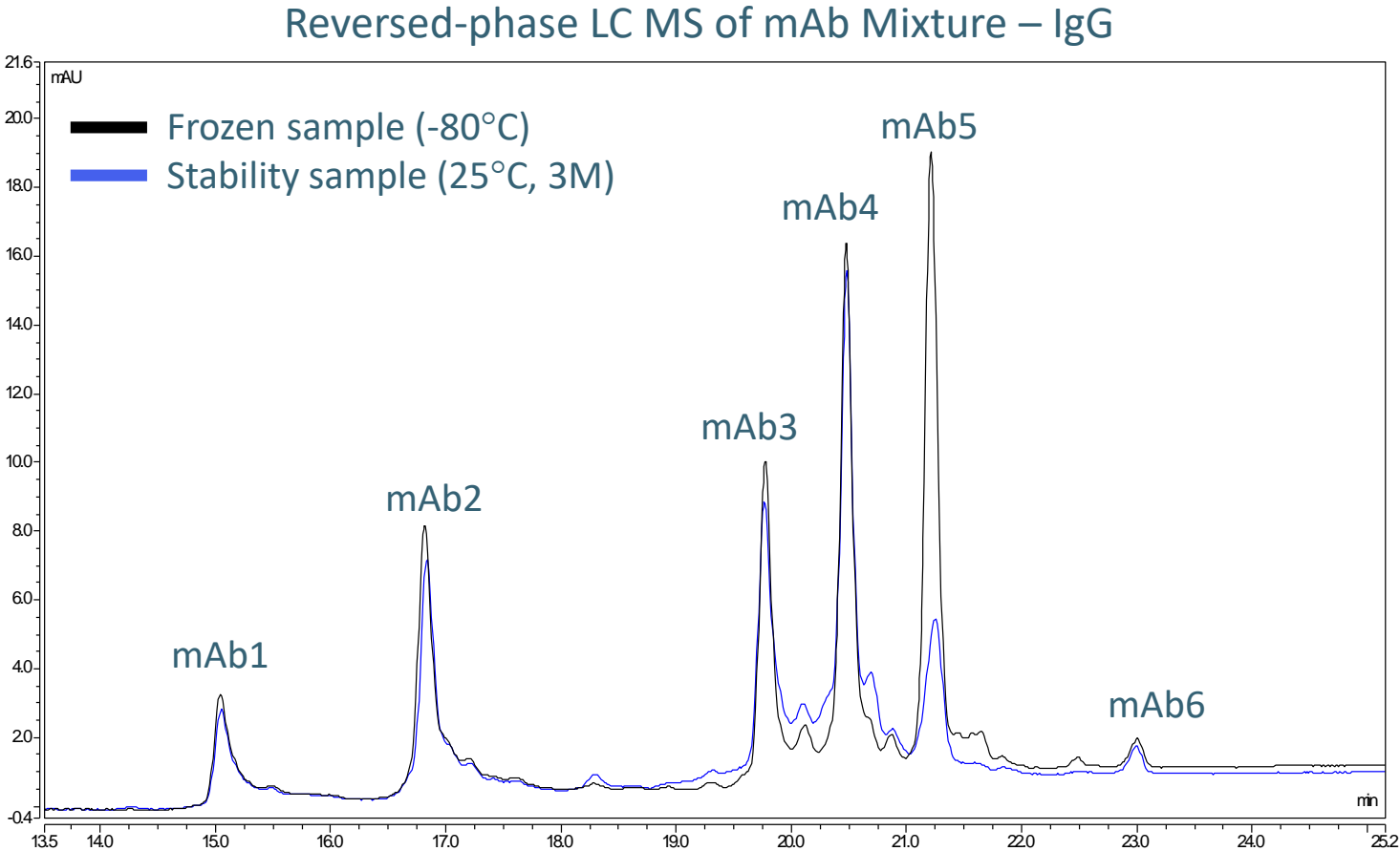


Excellent chromatographic and Orbitrap MS performance observed with DFA for intact mAbs!

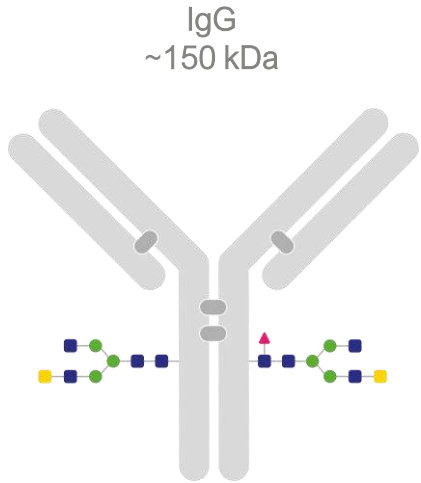




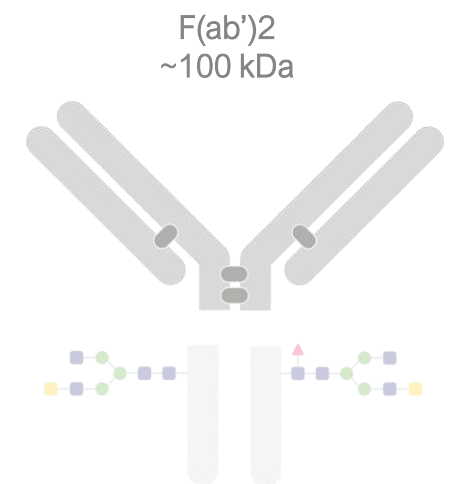
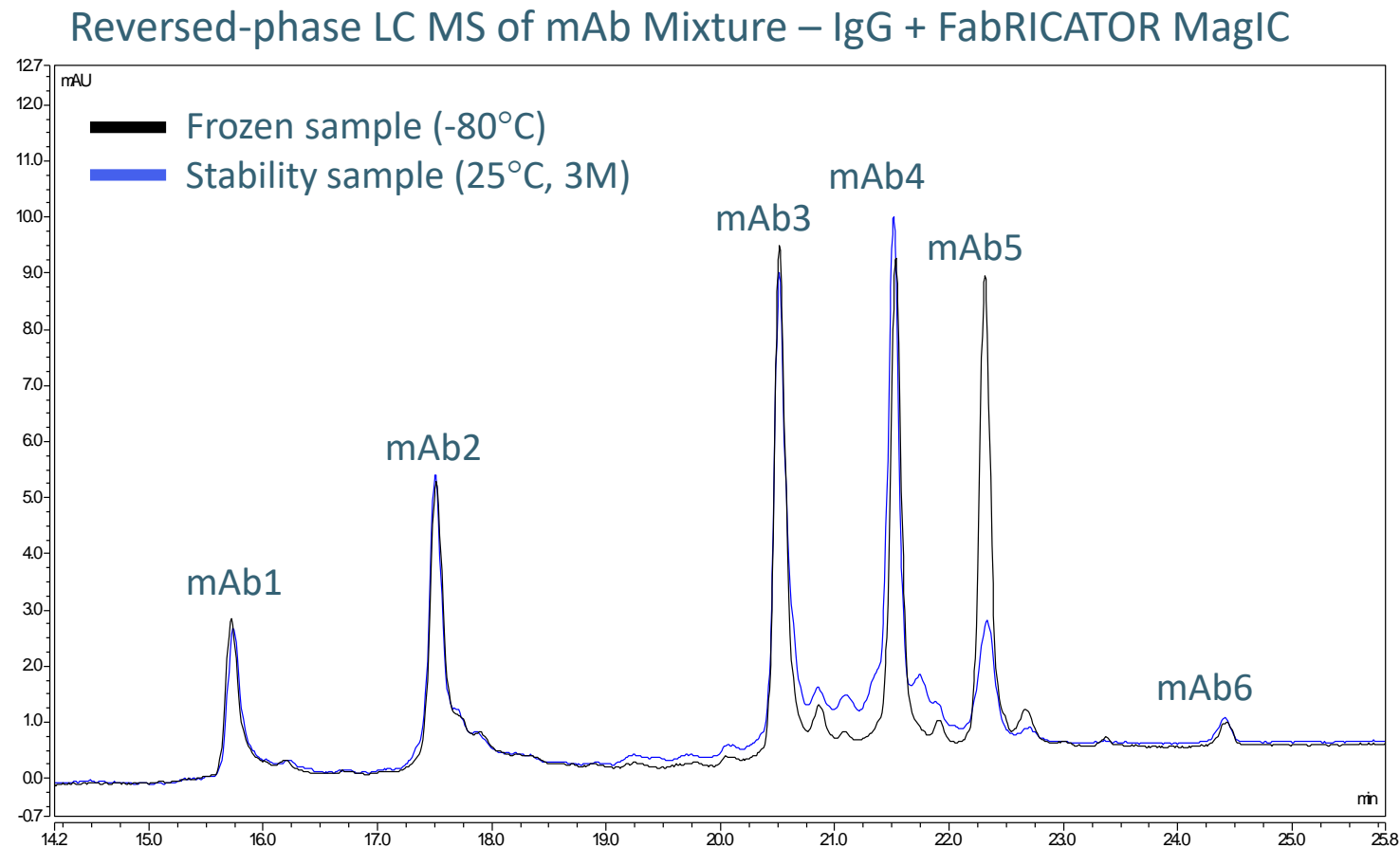
# Case Study: Tryptophan Oxidation in Complex Antibody Mixture



A clear drop in mAb5 intensity observed in stability sample.

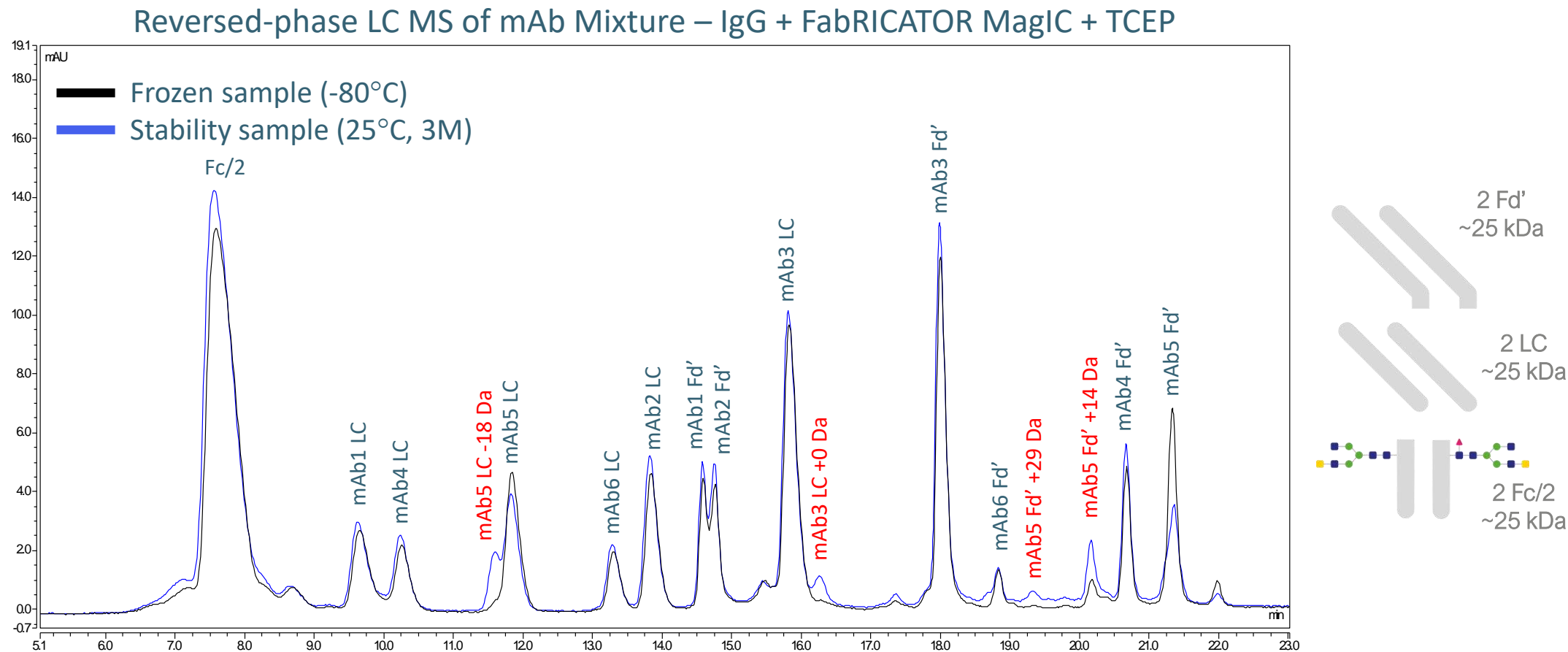


# Case Study: Tryptophan Oxidation in Complex Antibody Mixture



A clear drop in mAb5 Fab2 intensity observed, confirming that the change takes place in Fab2 region.

# Case Study: Tryptophan Oxidation in Complex Antibody Mixture



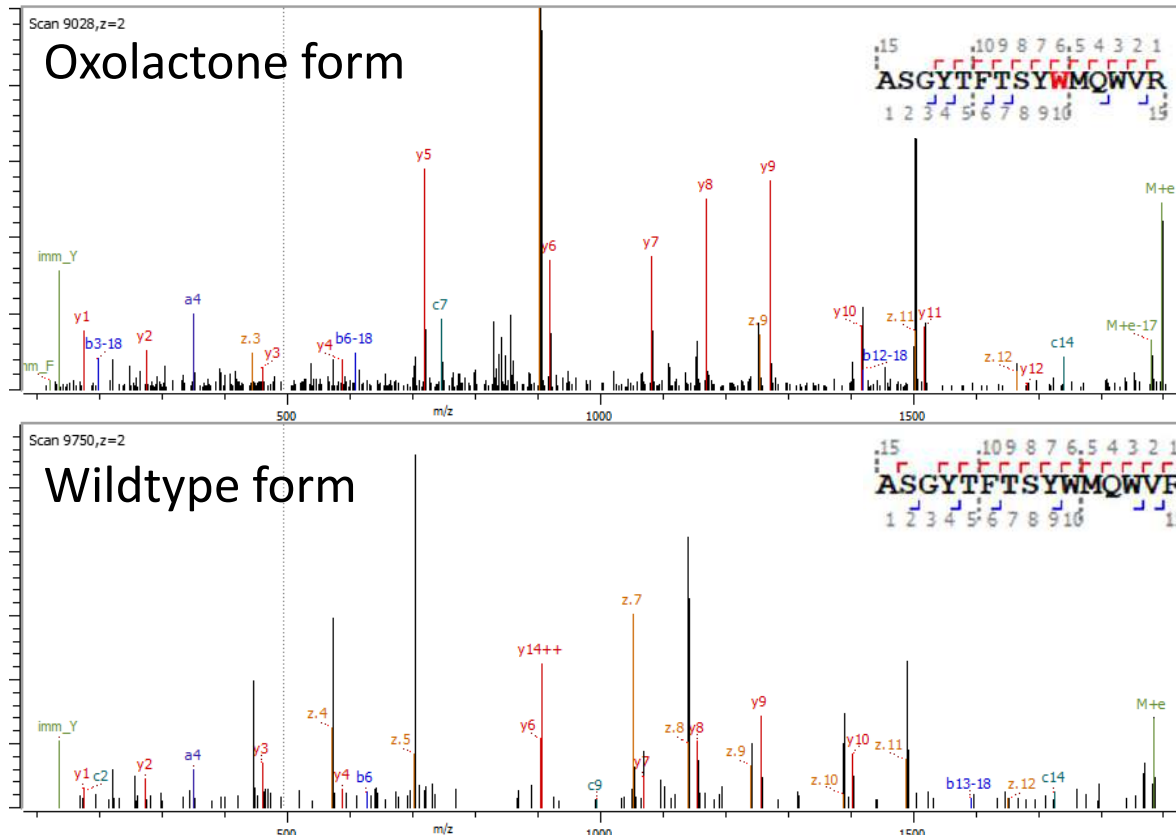
Drop in mAb5 was caused by a change in the Fd' region (resulting predominantly in a ~14 Da mass shift). Succinimide (-18 Da) of mAb5 LC and isomerization of mAb3 LC also observed.



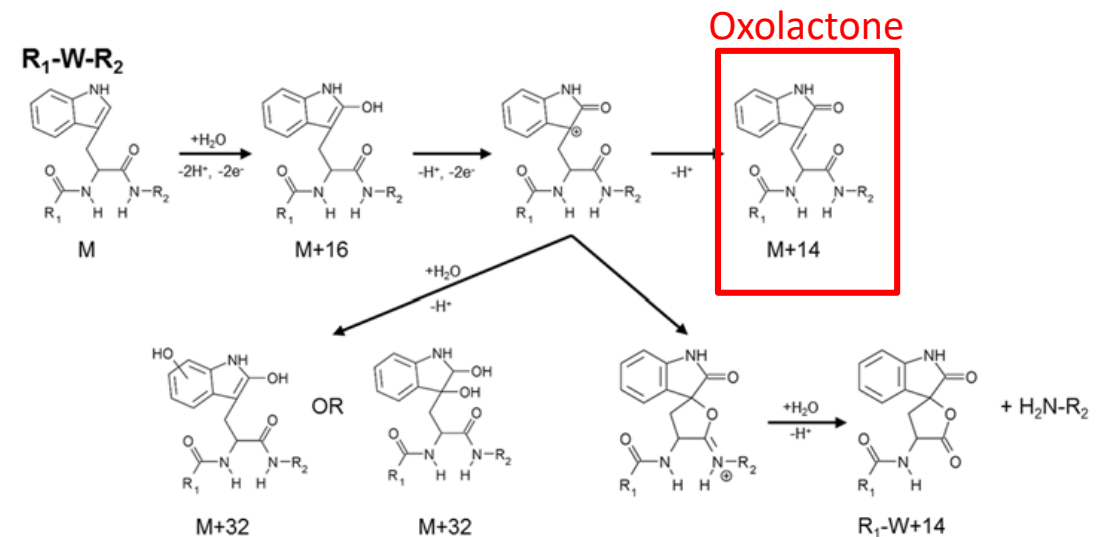
# Case Study: Tryptophan Oxidation in Complex Antibody Mixture

## Site specific information from peptide mapping by LC MS/MS

mAb heavy chain W33 oxidation - MS/MS (EThcD)



### Tryptophan oxidation pathways



Van den Brink, Floris. (2016). Microreactor for electrochemical conversion in drug screening and proteomics. 10.3990/1.9789036541459.

Peptide mapping confirmed that heavy chain W33 was an oxidation hotspot. Main oxidation forms were oxolactone (+14 Da) and dioxolactone (+ 30 Da), in line with intact RP LC MS observations.



# Tips & Tricks – Intact PRC MS

1. For intact and subunit RPC MS DFA is essential for excellent chromatographic performance *AND* good MS performance
2. DFA quality is very critical for good RPC MS performance
  1. Waters IonHance Difluoroacetic Acid (cat# 186009201)
3. DFA also highly suitable for biopharmaceutical peptide mapping by LC MS (less suitable for proteomics)
4. A good generic starting point when working with antibodies (intact, subunit, reduced subunit):
  1. LC conditions: 26-40 %B (30 min); 0.3 ml/min; column oven 55°C
    1. Thermo Scientific: MAbPac RP; 4 µm; 2.1\*150 mm (cat#303270)
  2. MS setting (QE Plus): HMR – Full MS; In-source CID 80 eV; resolution 35.000; scan range 2000 – 6000 m/z

# Future MS Plans & Trending MS Technologies

## Imaging Capillary Isoelectric Focussing (iCIEF) MS

Intabio Blaze



AES CEInfinite



## Capillary Electrophoresis MS

908 Devices ZipChip



Ideas for intact MS at Symphogen in the future...

- Intact MS in QC
  - ID test
  - Glycoform distribution test
- Native affinity MS as a tool for structure function studies
- ...and much more...

# Summary

- Intact mass analysis of large biopharmaceuticals has become a highly versatile characterization tool
- Multiple LC techniques are now routinely hyphenated to MS for intact mass analysis
- Robust intact mass data with a very high effectively resolution can be obtained on the Orbitrap platform
- Effective method transfer and consistent intact mass data demonstrated between different Orbitrap MS instruments
- Case studies demonstrated the power of intact mass analysis for characterization of:
  - Glycoform distribution
  - Hinge region fragmentation
  - Succinimide formation
  - Oxidation, including oxidation of W in mAb CDR

# Acknowledgements



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Annette Vinther Heydenreich  
Pernille Foged Jensen



Ilker Sen  
Stephane Bahraoui  
Pierre Alleman



Florian Füssl  
Jonathan Bones



Tom Buchanan  
Ken Cook  
Frank Steiner  
Krisztina Radi  
Kyle D'Silva