LC-MS Solutions for Biopharmaceutical Characterisation

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UCD DUBLIN

NIBRT Overview

- World-class facility dedicated to address the training and research needs of the global biopharmaceutical industry based in Dublin, Ireland
- Competency based training experience in an environment that replicates modern industrial bioprocessing facilities
- Research with impact – developing solutions to address real challenges faced within the biopharmaceutical industry
- Proud to collaborate with Thermo Fisher Scientific to demonstrate the power of their world leading instrumentation, software and consumables for biopharmaceutical characterisation

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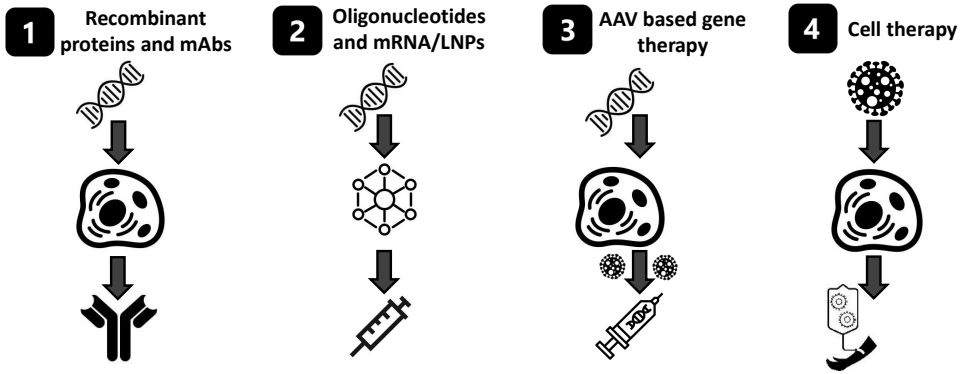
New NIBRT Initiatives in 2023

- Facility expansion opening in Q3.
- Dedicated suites for cell and gene therapy manufacture training activities.
- New research labs for expanded research teams.

Expansion will also contain a new early stage development facility designed to serve the research community funded by Science Foundation Ireland.



Science Foundation Ireland sfi For what's next

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Protein based biopharma workflows

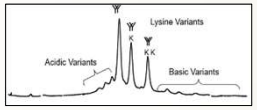
Intact Protein Analysis

- Intact mAbs
- ADC profiling
- Charge variant and aggregate analysis
- mAb subunit analysis

Intact mAb or ADC, e.g. in formulation buffer

SEC, SCX or RP separation

Sensitive Full MS detection of intact proteins in native or denated state

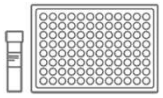


Spectral deconvolution using sliding windows feature for most sensitive deconvolution or species of high and low abundance

Peptide Analysis

- Peptide mapping
- HCP analysis
- Multi-Attribute Method (HR-MAM)

Intact mAb



Digestion with SMART™ digest-kit

High performance peptide separation

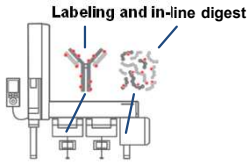
HRAM detection with fragmentation supporting identification

Software supporting sequence confirmation, PTM identification and quantitation, CQA monitoring, and HCP identification

HDX Analysis

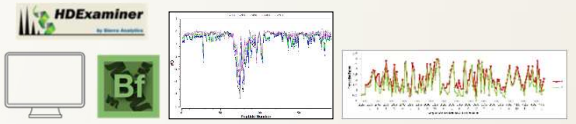
- Any Orbitrap-based system can be coupled to the H/D-X PAL/LC front end

Intact mAb



Deuterium-labelling over time course with in-line digestion followed by fast separation

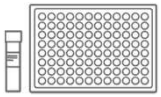
HRAM mass detection providing isotope pattern for monitoring of deuterium uptake.



Software solution for peptide identification, PTM analysis, top-down and bottom-up HDX data analysis.

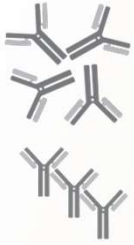
Large Molecule Bioanalysis

- Analysis on the peptide level
- Analysis on the protein level



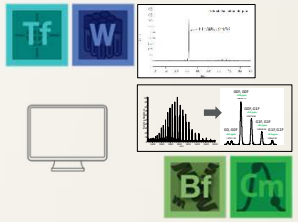
Digestion with SMART™ digest-kit

Enrichment by affinity capture with MSIA microcolumns

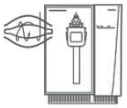
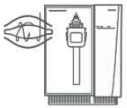
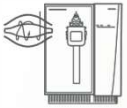
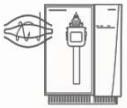


High performance peptide and protein separation

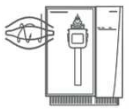
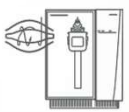
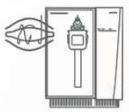
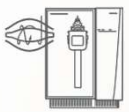
HRAM detection with
- Fragmentation supporting peptide ID
- Full MS for intact protein analysis



MAM workflows

					Acquisition	Processing	
Peptide mapping for characterization		Hypersil Gold		Vanquish Flex or Horizon		OE 480/240 or Orbitrap Tribrid	BioPharma Finder/ Proteome Discoverer
High throughput peptide mapping (large volume of samples)		Hypersil Gold		Vanquish Duo		OE 240	BPF
MAM (MS for monitoring)		Hypersil Gold		Vanquish Flex or Horizon		OE MX	BPF in dev/CM for monitoring
MAM (MS/MS for monitoring)		Hypersil Gold		Vanquish Flex or Horizon		OE 240	BPF in dev/GAP in monitoring

Protein based therapeutics workflows

						Data acquisition	Data processing	
Intact mass analysis (native/denatured)		MAbPac RP		Vanquish Flex or Horizon	/w BioPharma Option 	QE UHMR OE 480/240 Orbtrap Tribrid			BioPharma Finder
High throughput mass analysis/confirmation (large volume of samples)		MAbPac RP/SEC		Vanquish Duo	/w BioPharma Option 	OE 240 OE MX			BPF in dev/CM in late stage dev/QC
Charge variant analysis		IEX, CEX, SEC		Vanquish Flex or Horizon	/w BioPharma Option 	OE 240 OE MX			BPF in dev/CM in late stage dev/QC
mAb subunit analysis (intact mass/top down)		MAbPac RP		Vanquish Flex or Horizon		OE 240			BPF

Intact Protein Workflows

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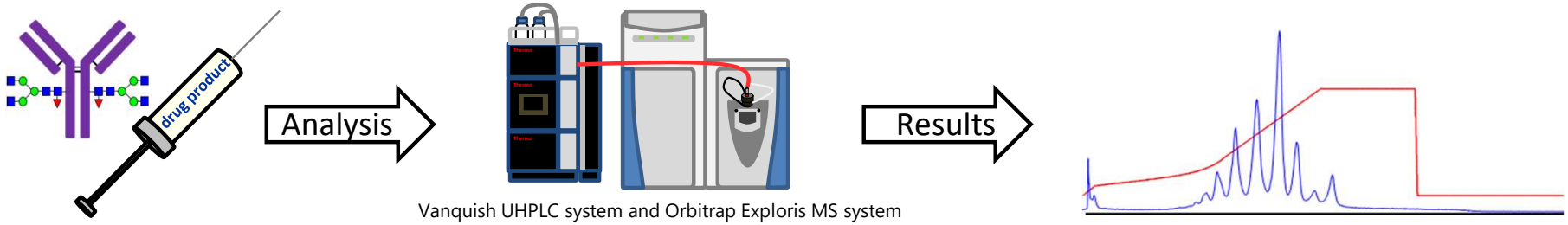
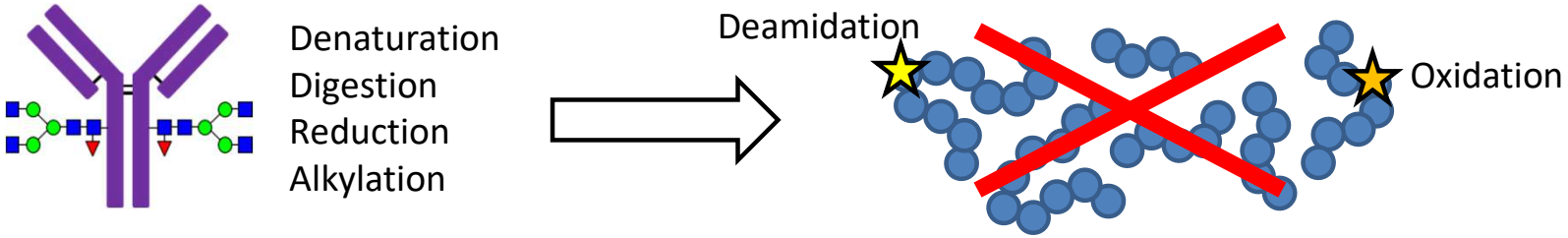
Why Intact Mass Analysis?

1. Quick and easy, no or very limited requirement for sample preparation

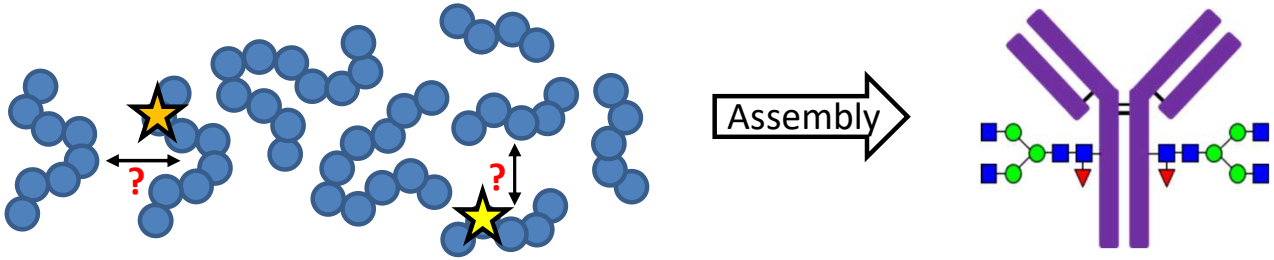


Vanquish UHPLC system and Orbitrap Exploris MS system

2. No sample preparation induced modifications



3. Assembly of differently modified residues into intact proteoforms known



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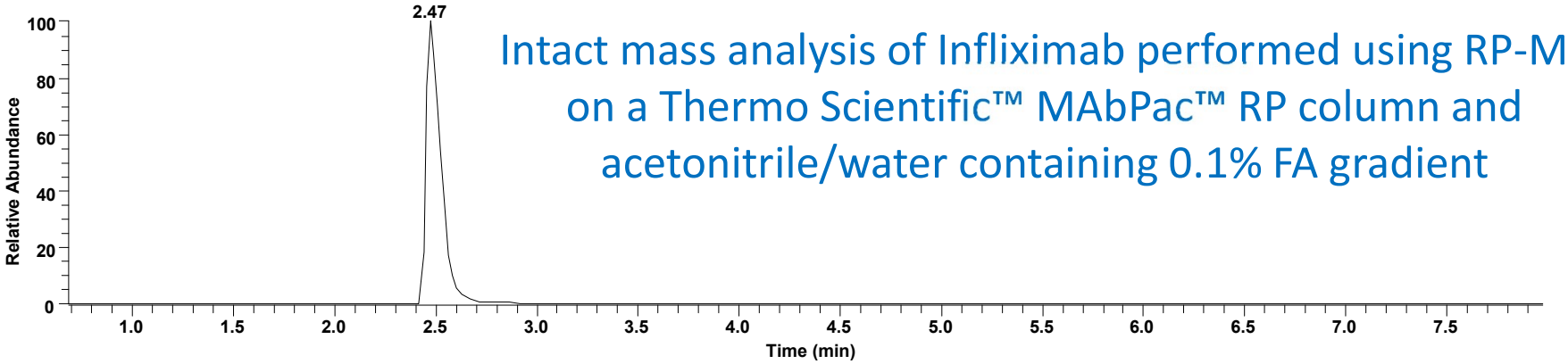
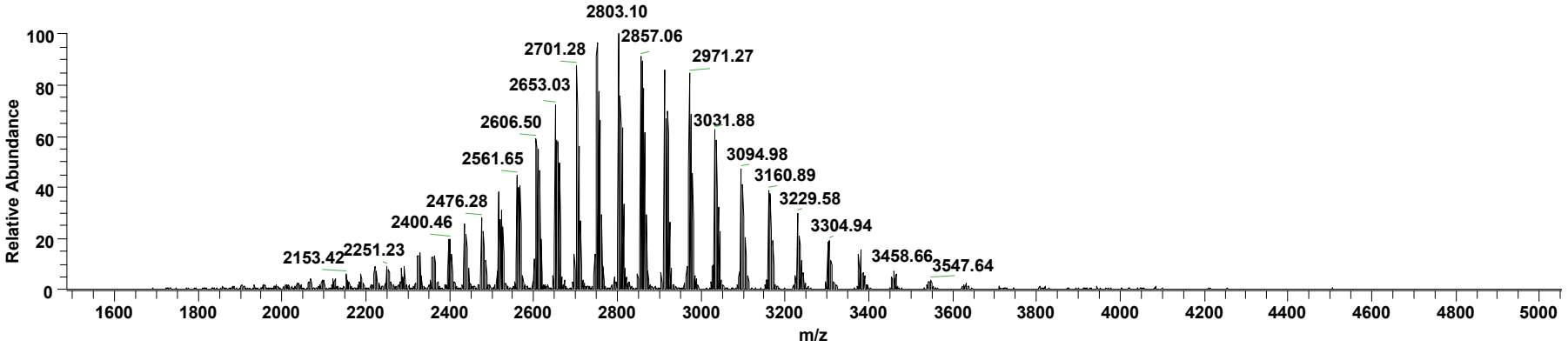
Intact Mass Analysis on Orbitrap Exploris 240 MS



Intact mass analysis of Infliximab performed using RP-MS on a Thermo Scientific™ MAbPac™ RP column and acetonitrile/water containing 0.1% FA gradient



Beautiful spectra collected for intact denatured infliximab with excellent resolution of the glycoforms present for each individual charge state, shown on next slide

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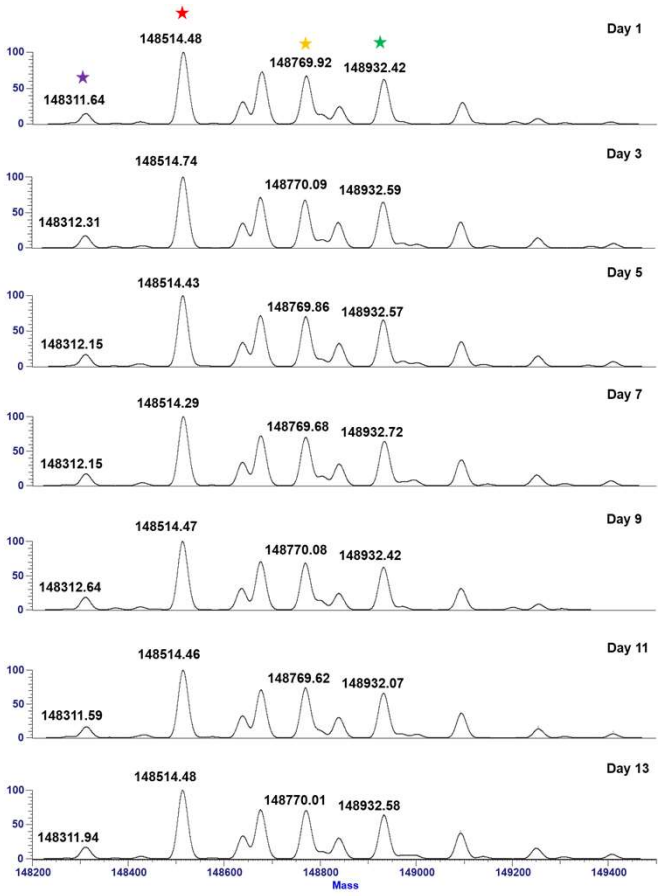
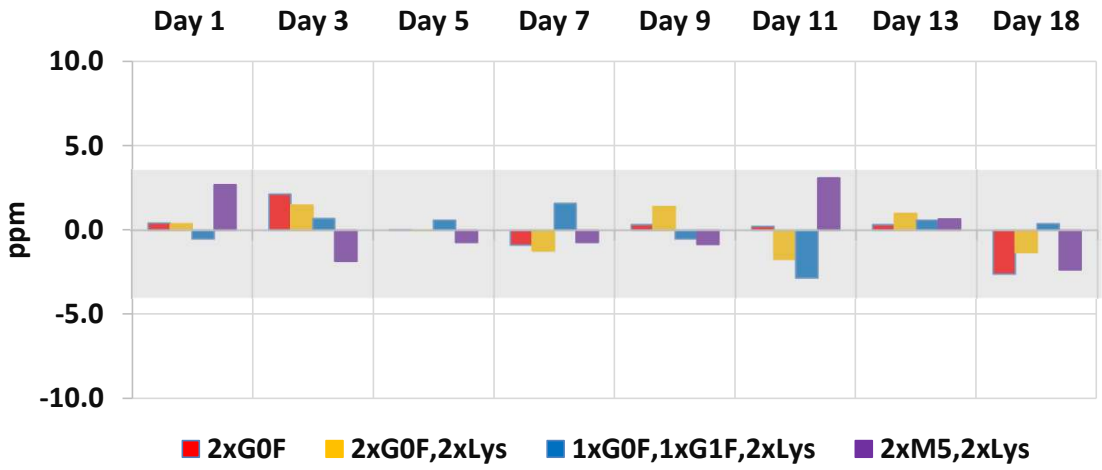
Evaluation of Mass Stability

Analysis was performed on a daily basis to evaluate the mass stability of the instrument without EASY-IC source calibration, data processing standardised in Biopharma Finder software



ppm mass accuracy determined and plotted to assess instrument stability on a day by day basis for up to 18 days with no calibration



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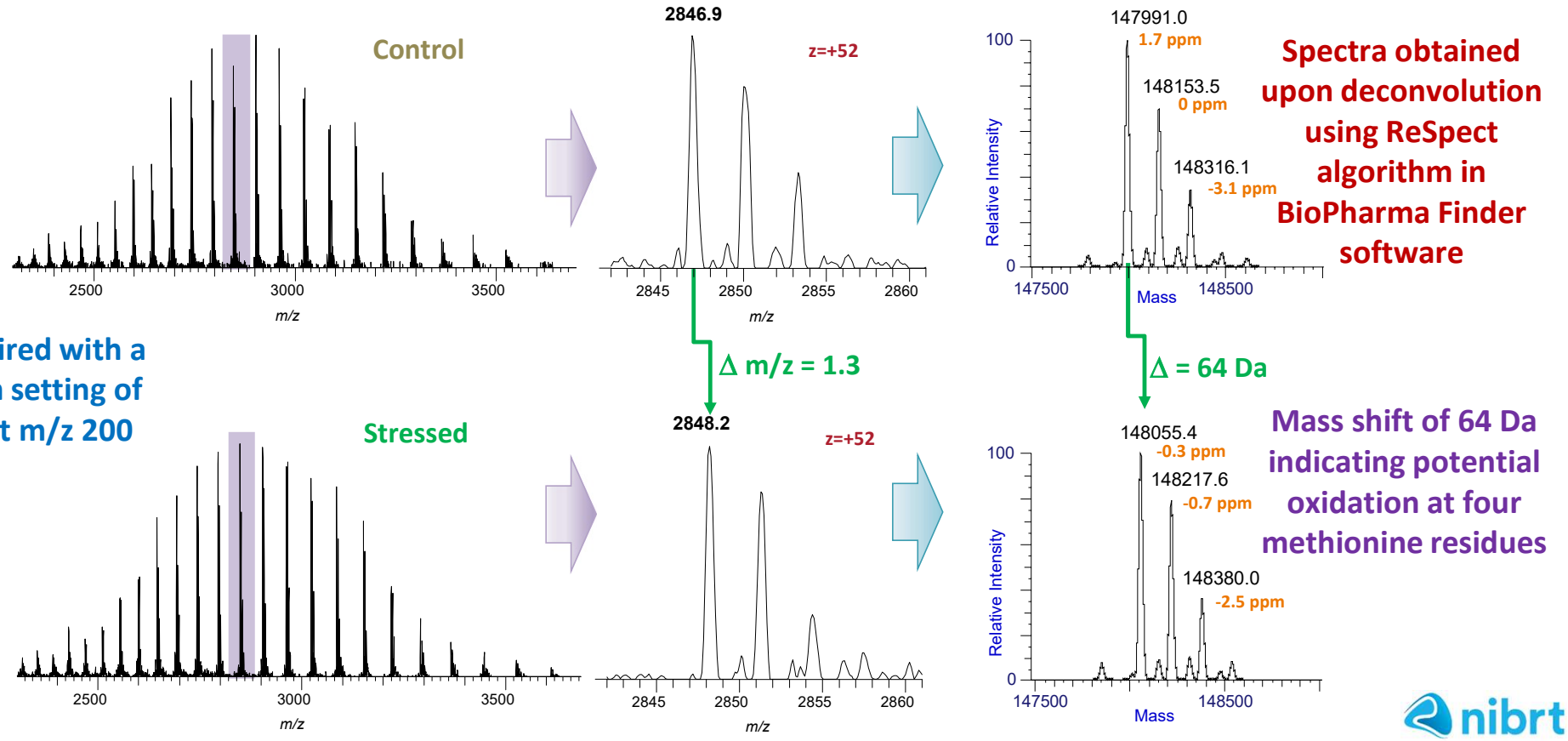
Intact Mass Analysis of Stressed Ipilimumab

Charge envelope of intact control and stressed Ipilimumab (500 ppm H_2O_2) and zoom of +52 charge state representing a baseline resolved glycoform pattern



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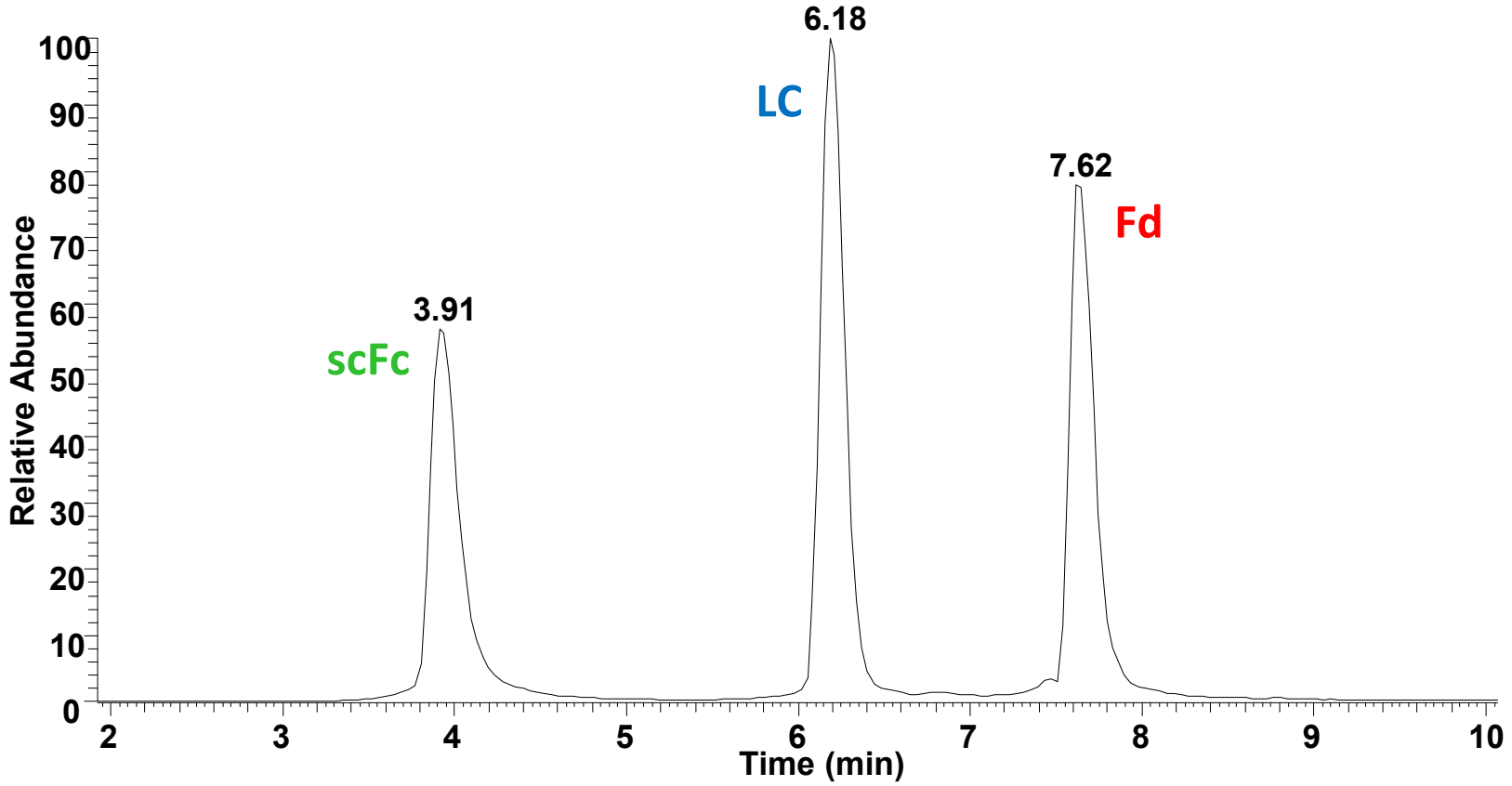
Analysis of Subunits following IdeS Digestion

Ipilimumab digested with IdeS protease followed by reduction, resulting subunits separated on MAbPac RP column on Vanquish Duo UHPLC coupled to Orbitrap Exploris 240 MS



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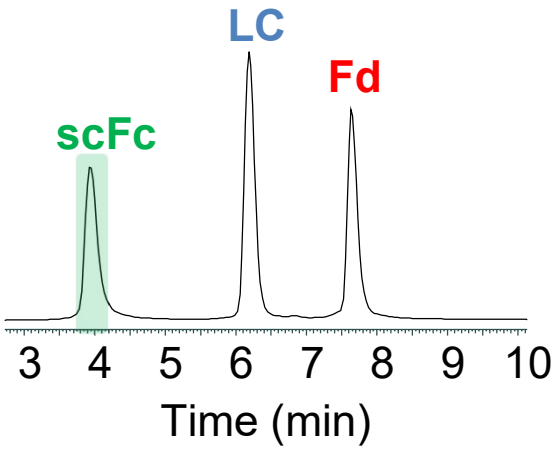
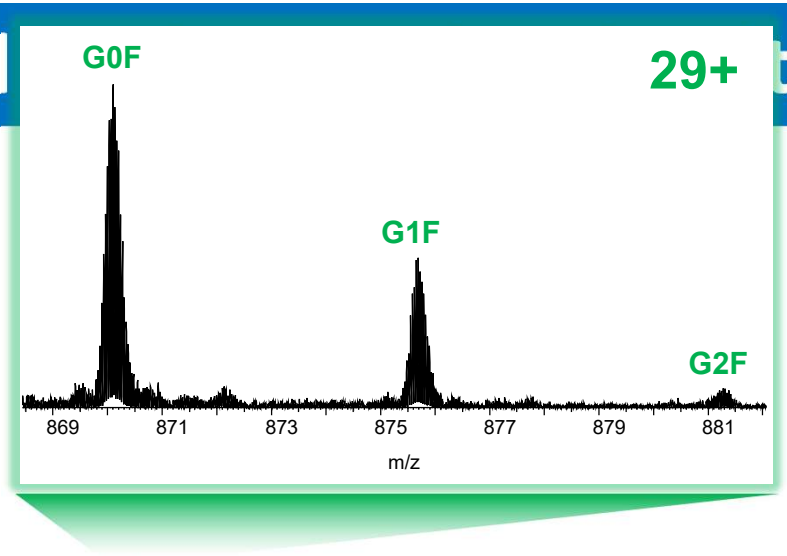
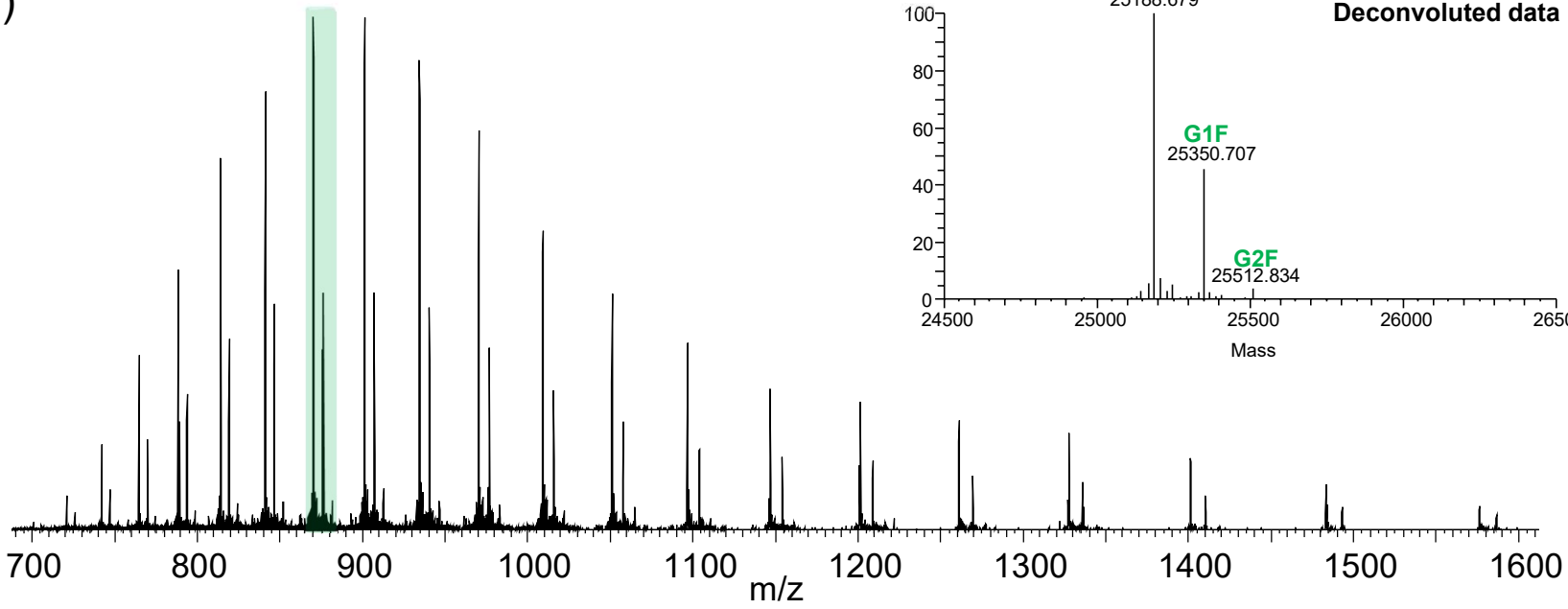
A More Detailed





Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing near baseline-resolved isotope patterns of the +29 charge state of the scFc glycoforms



Deconvoluted data

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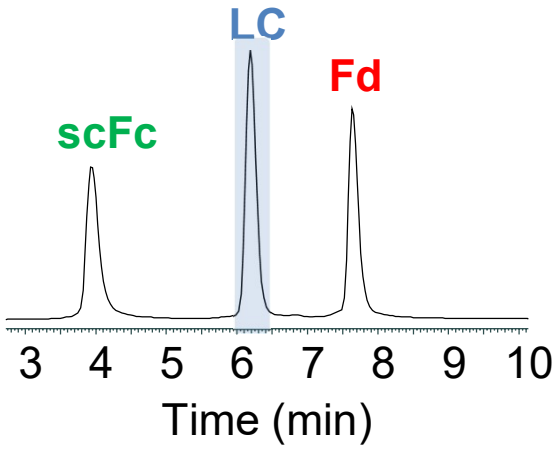
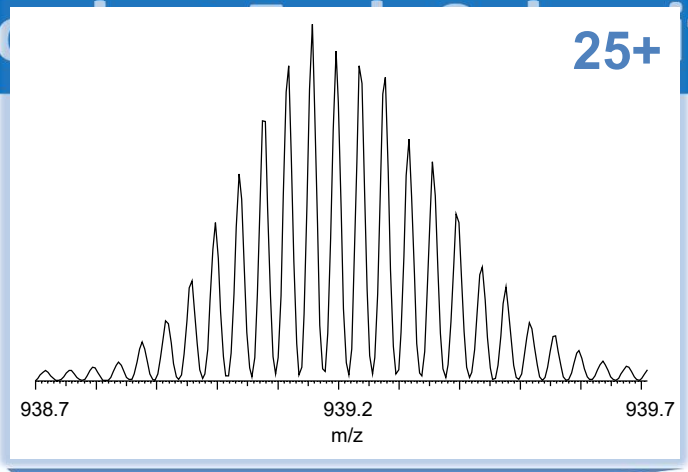
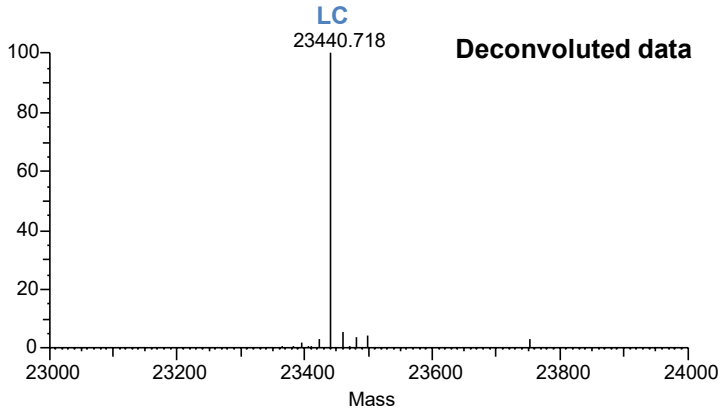
A More Detailed L t





Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing baseline-resolved isotope patterns of the +25 charge state of the light chain



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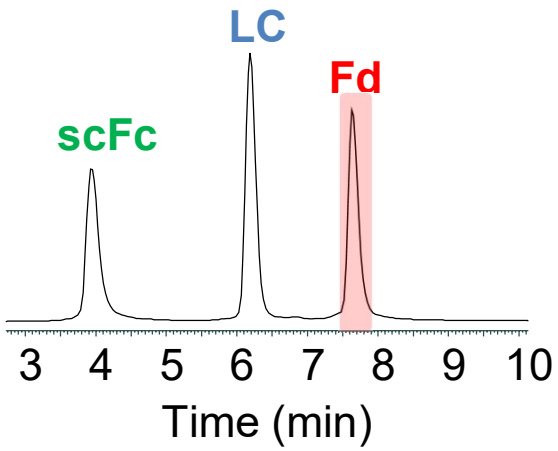
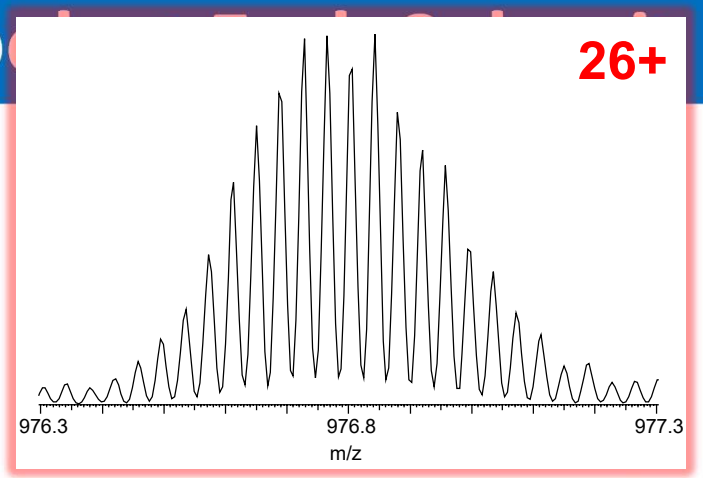
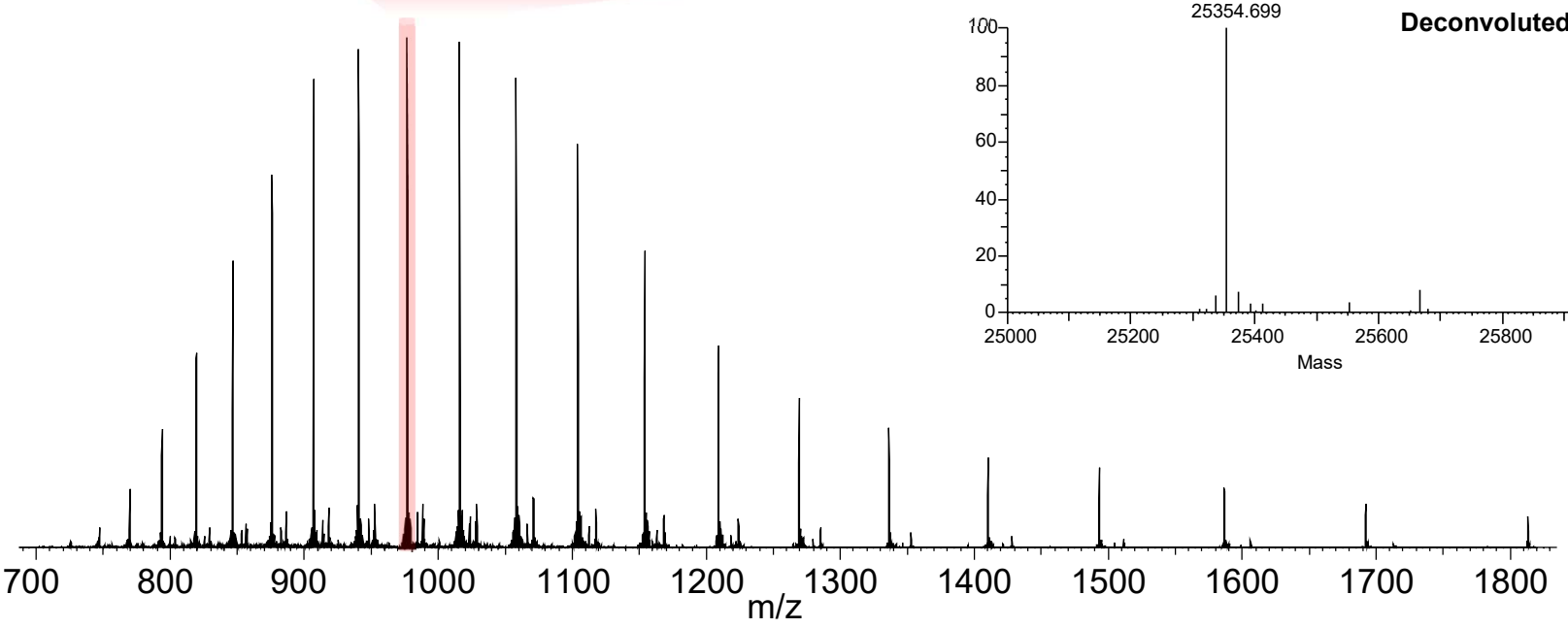
A More Detailed Lo





Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing baseline-resolved isotope patterns of the +26 charge state of the Fd



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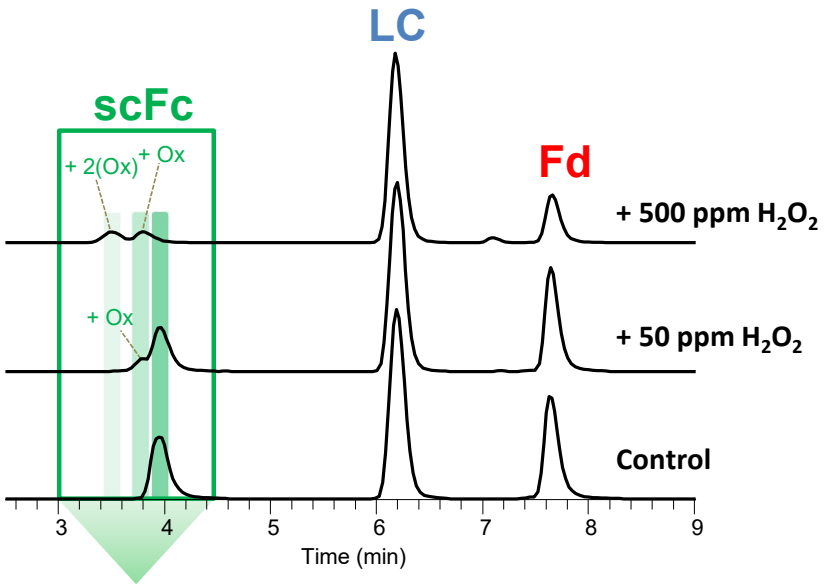
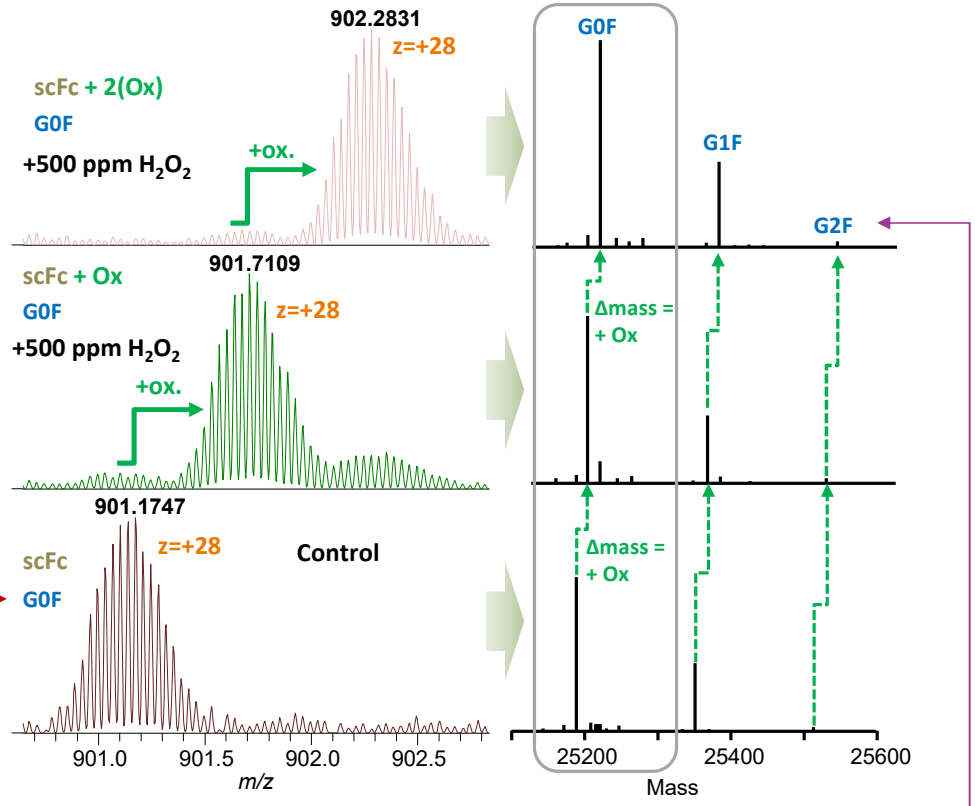
Forced Oxidation of Ipilimumab

We subjected Ipilimumab to forced oxidation using peroxide treatment and repeated subunit analysis following IdeS digestion and reduction





TIC of separated G0F glycoform of ipilimumab scFc for control and stressed samples (50 and 500 ppm H_2O_2)

Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing near baseline-resolved isotope patterns of the +28 charge state of the scFc G0F subunit

Deconvolution of the entire charge envelope including all scFc subunit glycoforms using Sliding Window Xtract algorithm

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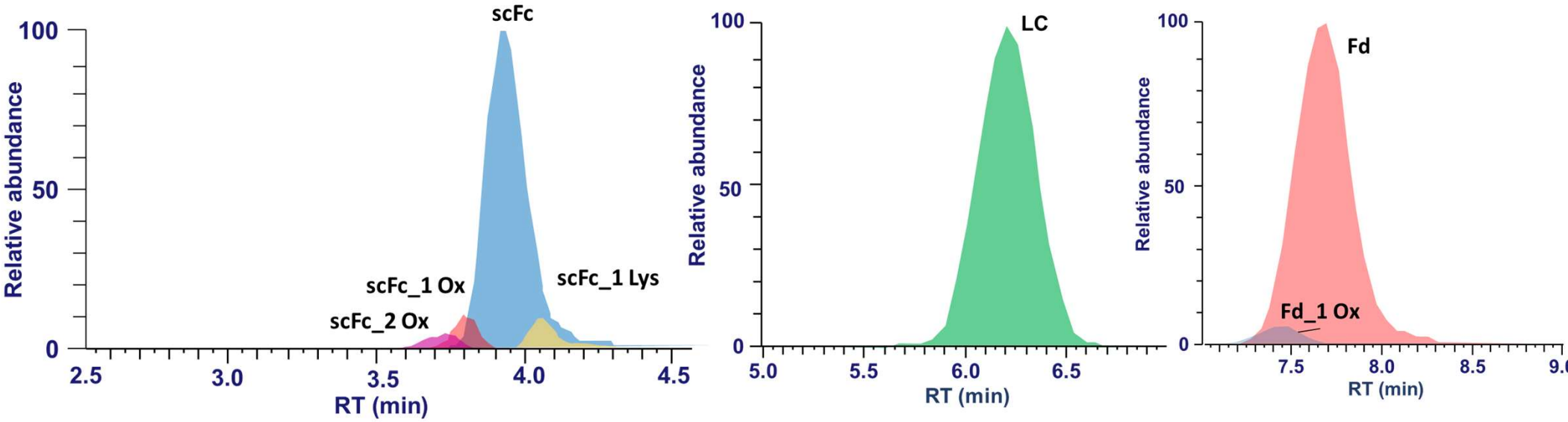
Forced Oxidation of Ipilimumab

Deconvolution of the subunit spectra revealed modifications on the heavy chain only, no oxidation on the light chain. An additional oxidation was noted on the Fd region of the heavy chain as well as those previously noted on the scFc region



Pseudo extracted ion chromatograms following sliding window deconvolution for all detected forms of the scFC, LC and Fd subunits

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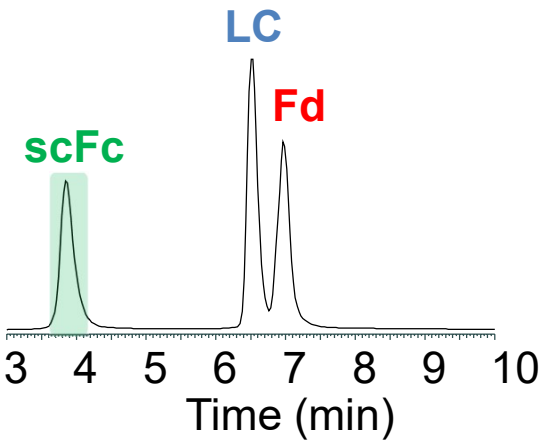
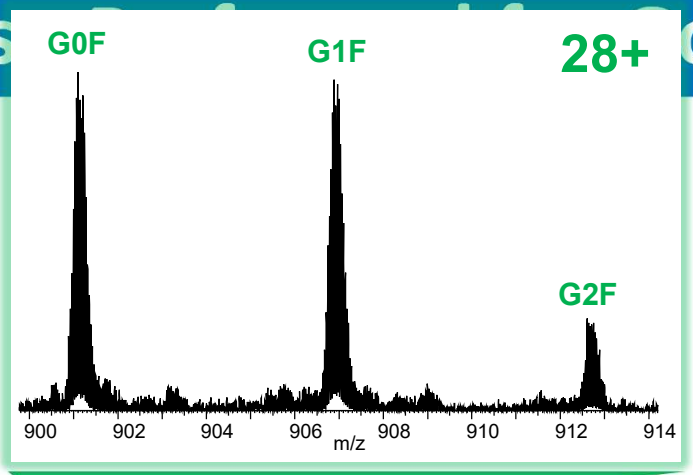
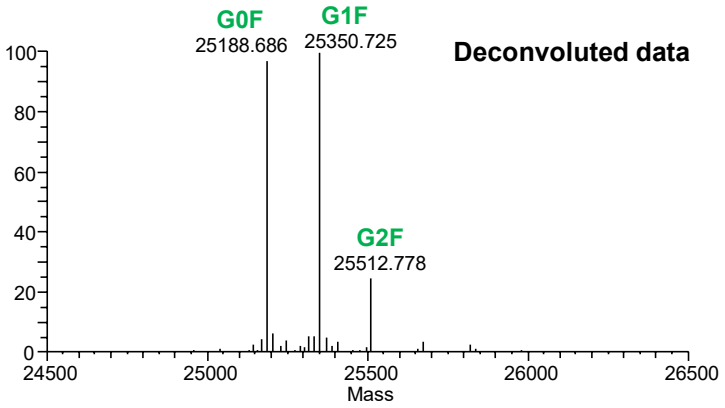
Subunit Analysis als olimumab





Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing near baseline-resolved isotope patterns of the +28 charge state of the scFc glycoforms



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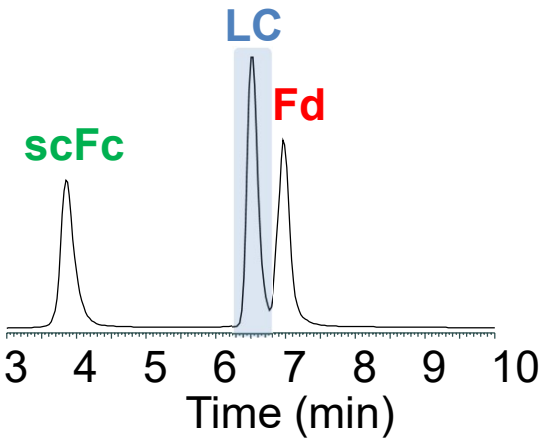
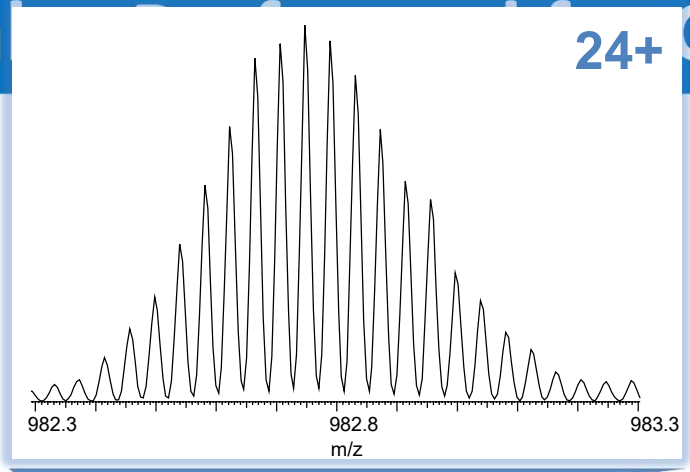
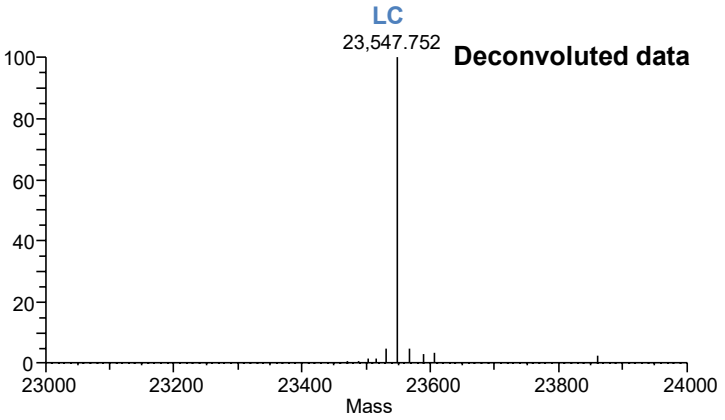
Subunit Analysis a Golimumab





Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing baseline-resolved isotope patterns of the +24 charge state of the light chain



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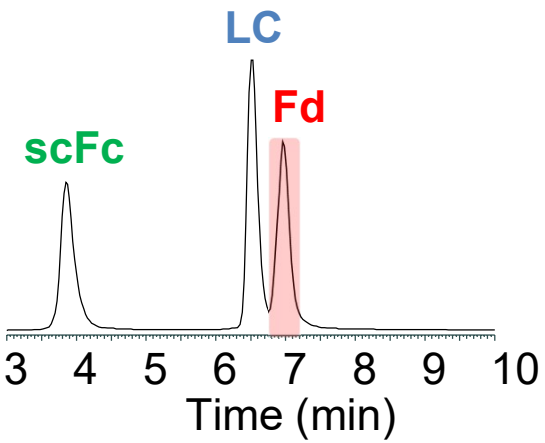
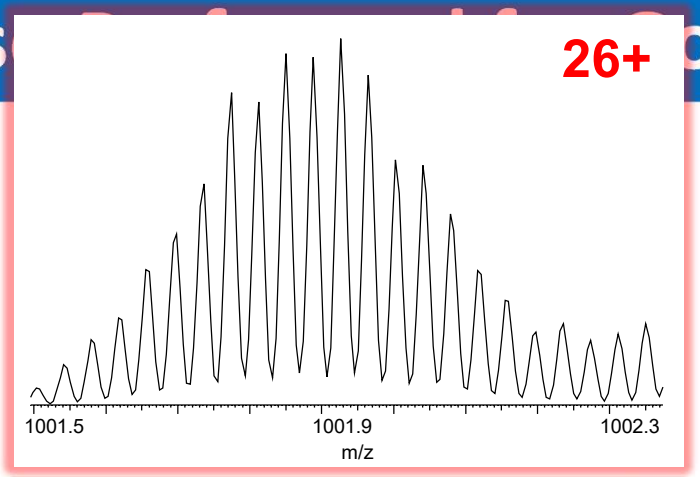
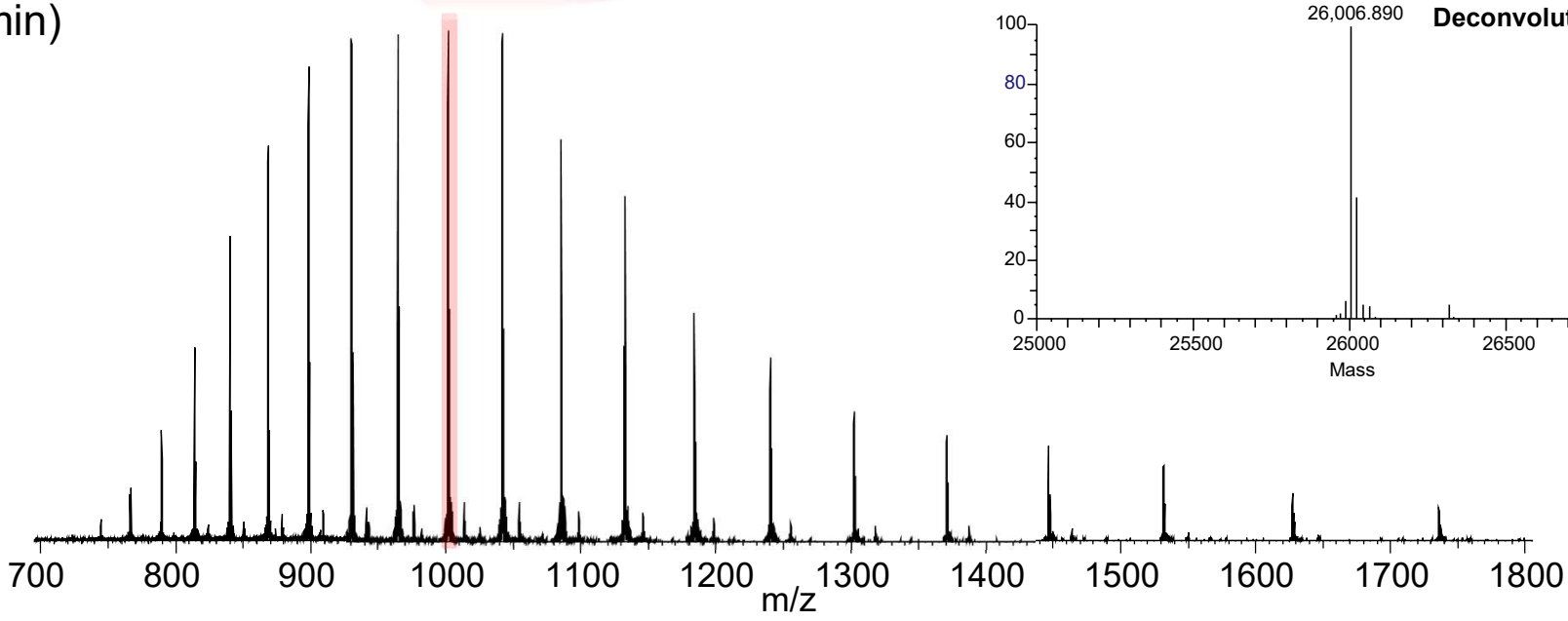
Subunit Analysis als olimumab





Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing baseline-resolved isotope patterns of the +26 charge state of the Fd



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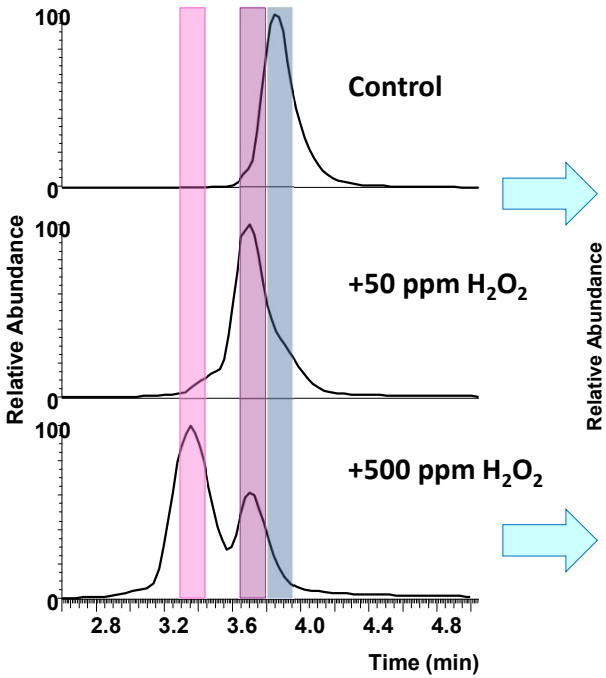
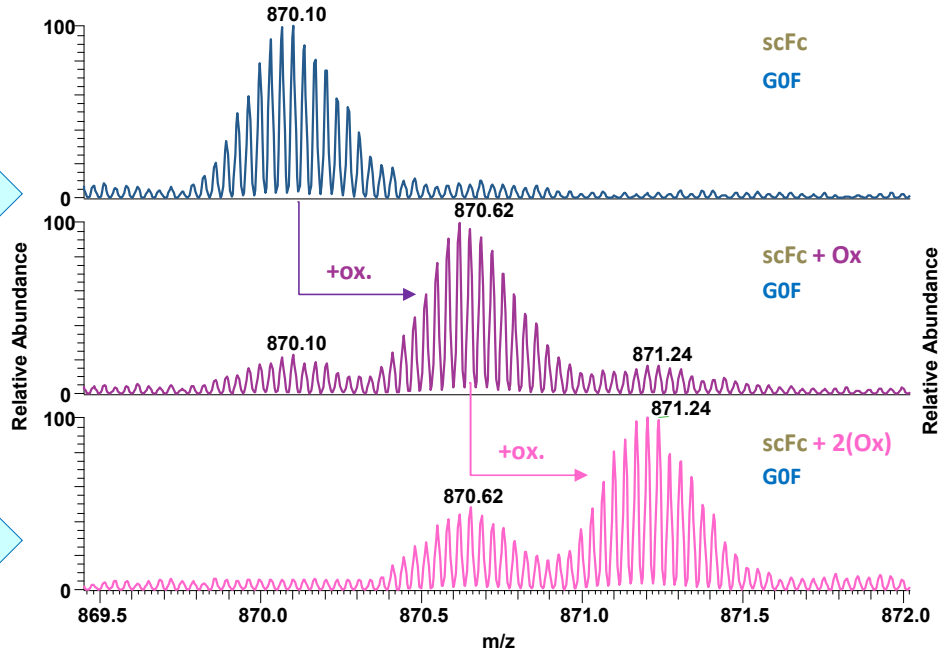
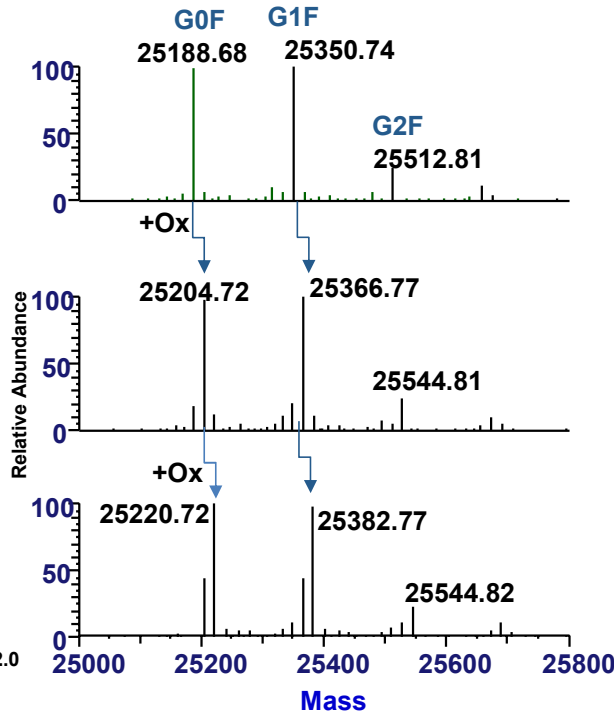
Forced Oxidation of Golimumab scFc Sub Unit



TIC of separated G0F glycoform of golimumab scFc for control and stressed samples (50 and 500 ppm H_2O_2)



Zoomed mass spectra acquired with Rs = 120,000 (at *m/z* 200) showing near baseline-resolved isotope patterns of the +29 charge state of the scFc G0F subunit



Deconvolution scFc subunit glycoforms using Sliding Window Xtract algorithm showing distinctive oxidation shifts

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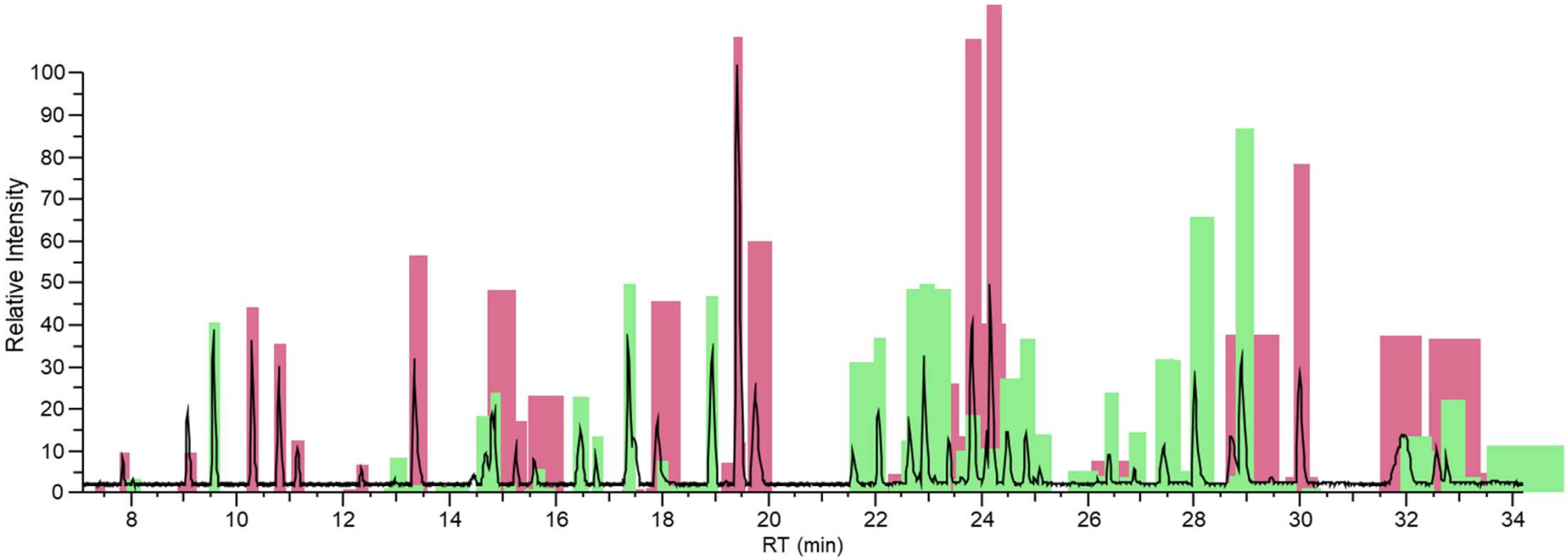
Peptide Mapping on Orbitrap Exploris 240 MS



Base Peak Chromatogram of denosumab (Prolia™) indicating the peptide origin to light or heavy chains indicated by red (light chain) and green (heavy chain)

Data generated by Tom Buchanan, European Applications Development Scientist at Thermo Fisher Scientific

Prolia is a trademark of Amgen, Inc.

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Peptide Mapping on Orbitrap Exploris 240 MS

Data searched using Biopharma Finder software, 100% sequence coverage obtained for denosumab light and heavy chains. Coloured bars represent identified peptides with colours indicating peptide intensities based on full MS spectra

Sequence Coverage Map

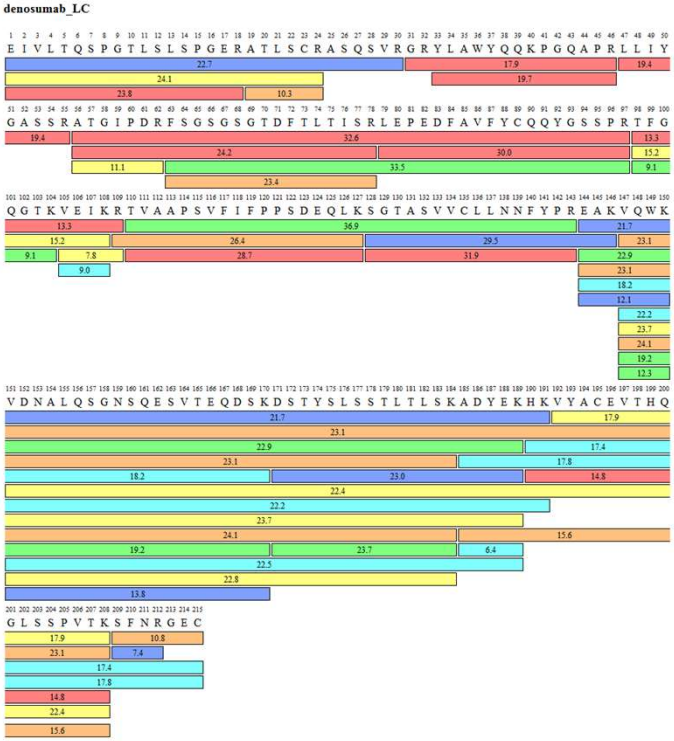
Created on 04/24/20 by tom.buchanan
Data Folder = C:\Users\tom.buchanan\Desktop\Desktop\Lab Data\MS Data\NIBRT\MM\Peptide Mapping\
Minimum MS Signal = 24000
Data File = Prolia_Reduced_1_20200317010642.raw
Protease = Trypsin

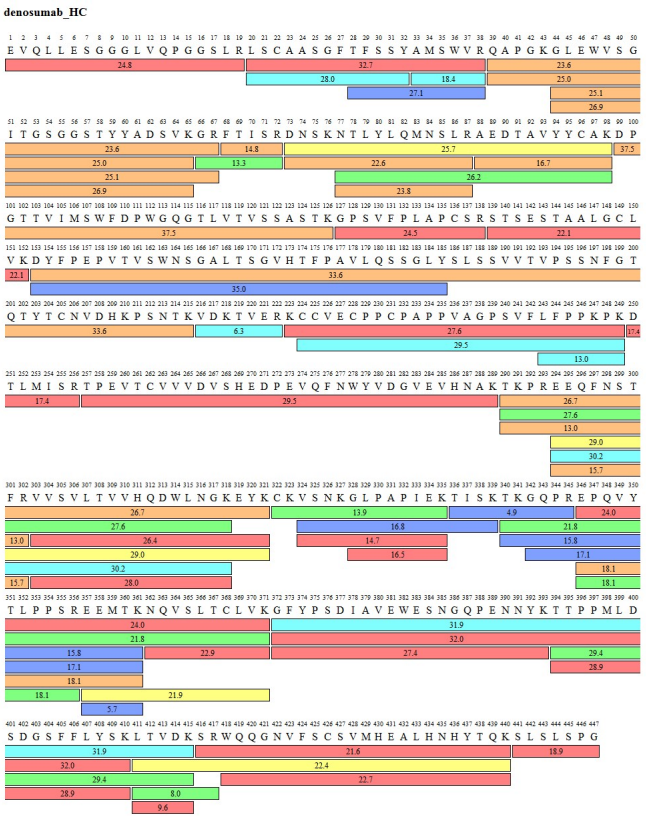
Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:denosumab_LC	391	45.4%	100.0%	63.38%
2:denosumab_HC	431	33.3%	100.0%	36.62%
Unidentified	2248	21.2%		

Minimum Recovery = 1%
Minimum Recovery of Overlapping Peptides = 0%
Minimum Confidence = 80
Maximum Mass = 7000

Color code for peptide recovery

>30.0%	>20.0%	>10.0%	>5.0%	>2.0%	>1.0%	>0.5%	>0.2%	>0.1%	>0.0%
good			fair			poor			





Data generated by Tom Buchanan, European Applications Development Scientist at Thermo Fisher Scientific

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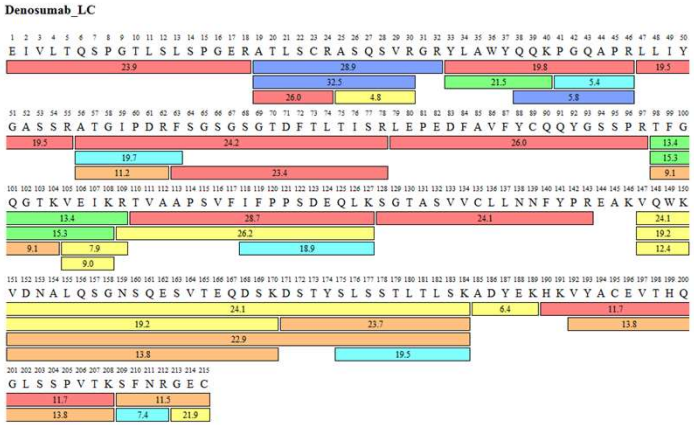
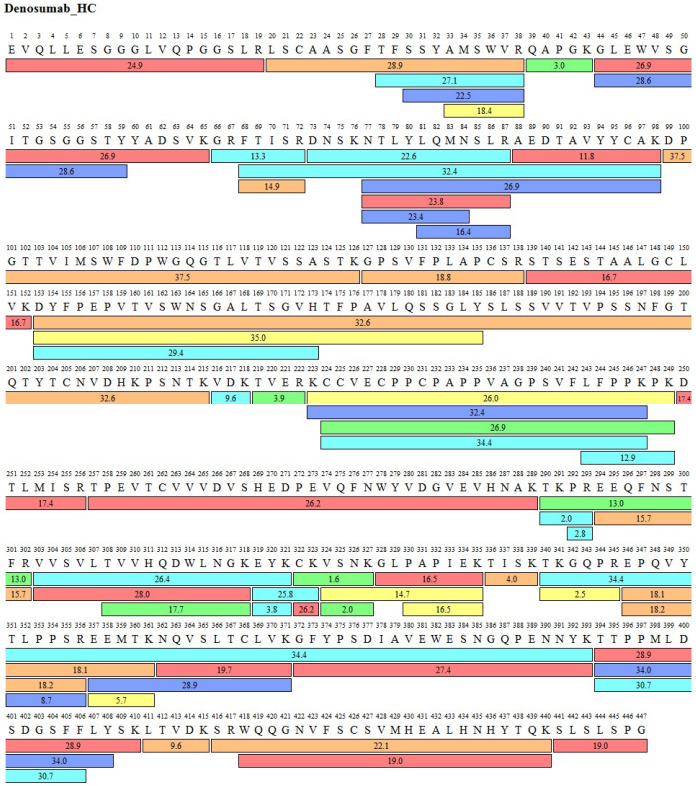
Peptide Mapping on Orbitrap Exploris 240 MS

Experiment was repeated using without reduction to keep disulphide bonds intact, coloured bars represent identified peptides intensities based on full MS spectra





Sequence Coverage Map

Created on 04/29/20 by tom.buchanan
Data Folder = C:\Users\tom.buchanan\Desktop\Desktop\Lab Data\MS Data\NIBRT\MM\Peptide Mapping\
Minimum MS Signal = 16000
Data File = Prolia_NonReduced_1_20200317043152.raw
Protease = Trypsin

Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:Denosumab_LC	492	23.9%	98.6%	50.20%
2:Denosumab_HC	625	31.0%	100.0%	49.80%
Unidentified	6810	45.1%		

Minimum Recovery = 1%
Minimum Recovery of Overlapping Peptides = 0%
Minimum Confidence = 0
Maximum Mass = 20000

Color code for peptide recovery

>50.0%	>20.0%	>10.0%	>5.0%	>2.0%	>1.0%	>0.5%	>0.2%	>0.1%	>0.0%
good			fair			poor			

Data generated by Tom Buchanan, European Applications Development Scientist at Thermo Fisher Scientific

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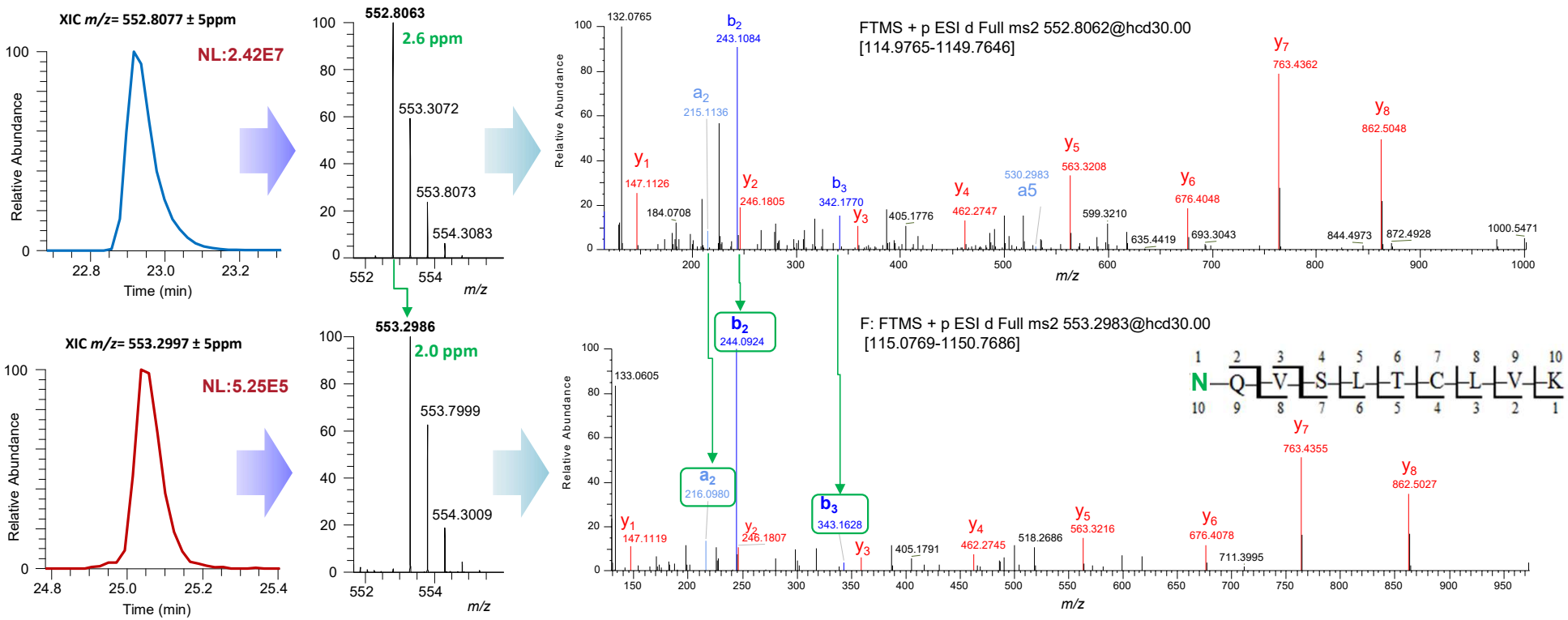
Low Level Peptide Modifications



Detection of deamidation at ^{366}N in denosumab heavy chain, extracted ion chromatograms, MS and MS/MS spectra indicated induced mass shift in b-ions

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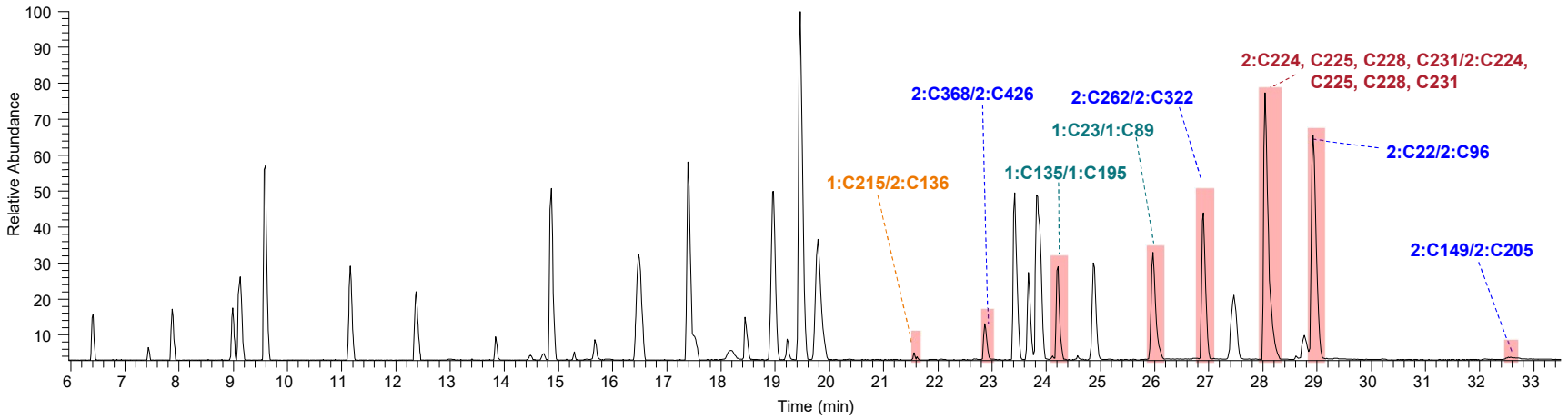
Disulphide Bond Mapping on Orbitrap Exploris 240 MS



S-S Bond Type		Peptide Sequence	Position	Δ ppm	RT
Intra Chain	LC1	ATLSCR / LEPEDFAVFYCQQYGSSPR	1:C23/1:C89	-1.2	25.95
	LC2	SGTASVVCLLNNFYPR / HKVYACEVTHQGLSSPVTK	1:C135/1:C195	-2.1	24.15
	HC1	LSCAASGFTFSSYAMSWVR / AEDTAVYYCAK	2:C22/2:C96	-2.7	28.95
	HC2	STSESTAALGCLVK / DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTK	2:C149/2:C205	-0.9	32.54
	HC3	TPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAK / CK	2:C262/2:C322	-0.6	26.76
	HC4	NQVSLTCLVK / WQQGNVFSCSVMHEALHNHYTQK	2:C368/2:C426	0.4	22.87
Inter Chain	LC-HC	GEC / GPSVFPLAPCSR	1:C215/2:C136	-2.5	21.86
	Hinge	KCCVECPPCPAPPVAGPSVFLFPPKPK / KCCVECPPCPAPPVAGPSVFLFPPKPK	2:C224, C225, C228, C231/ 2:C224, C225, C228, C231	-0.02	28.05

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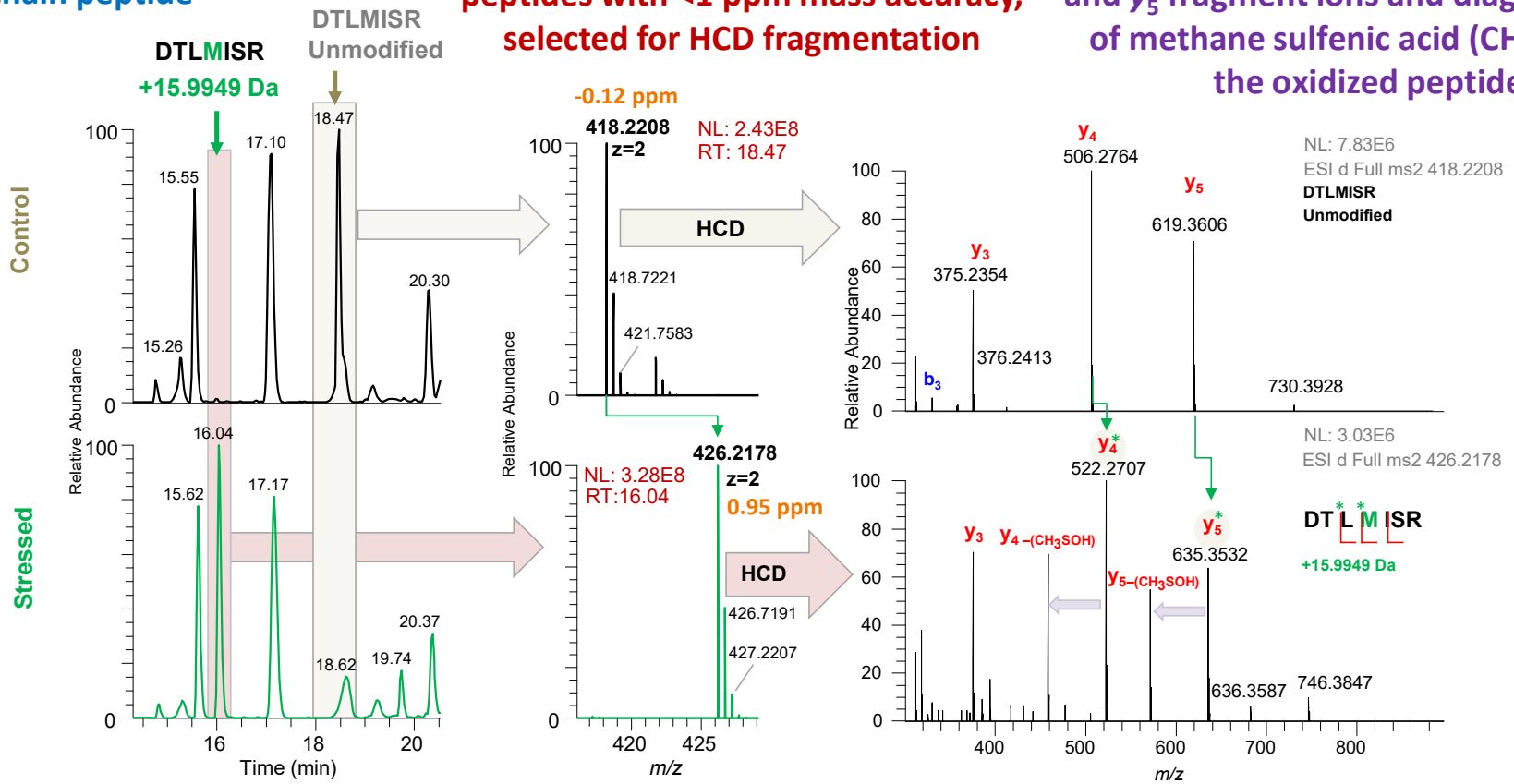
Back to Ipilimumab: Oxidation on the Peptide Level

TIC zoom of the control (upper) and stressed (lower) ipilimumab after digestion, highlighting 'DTLMISR' heavy chain peptide

Zoom spectra of DTLMISR displaying isotope patterns and relative abundances of the doubly-charged peptides with <1 ppm mass accuracy, selected for HCD fragmentation

MS/MS fragment ion spectra for mass range *m/z* 300 - 900. Identification supported by +15.9949 Da shifts for y_4 and y_5 fragment ions and diagnostic loss of methane sulfenic acid (CH_3SOH) in the oxidized peptide



Data generated by Tom Buchanan, European Applications Development Scientist at Thermo Fisher Scientific

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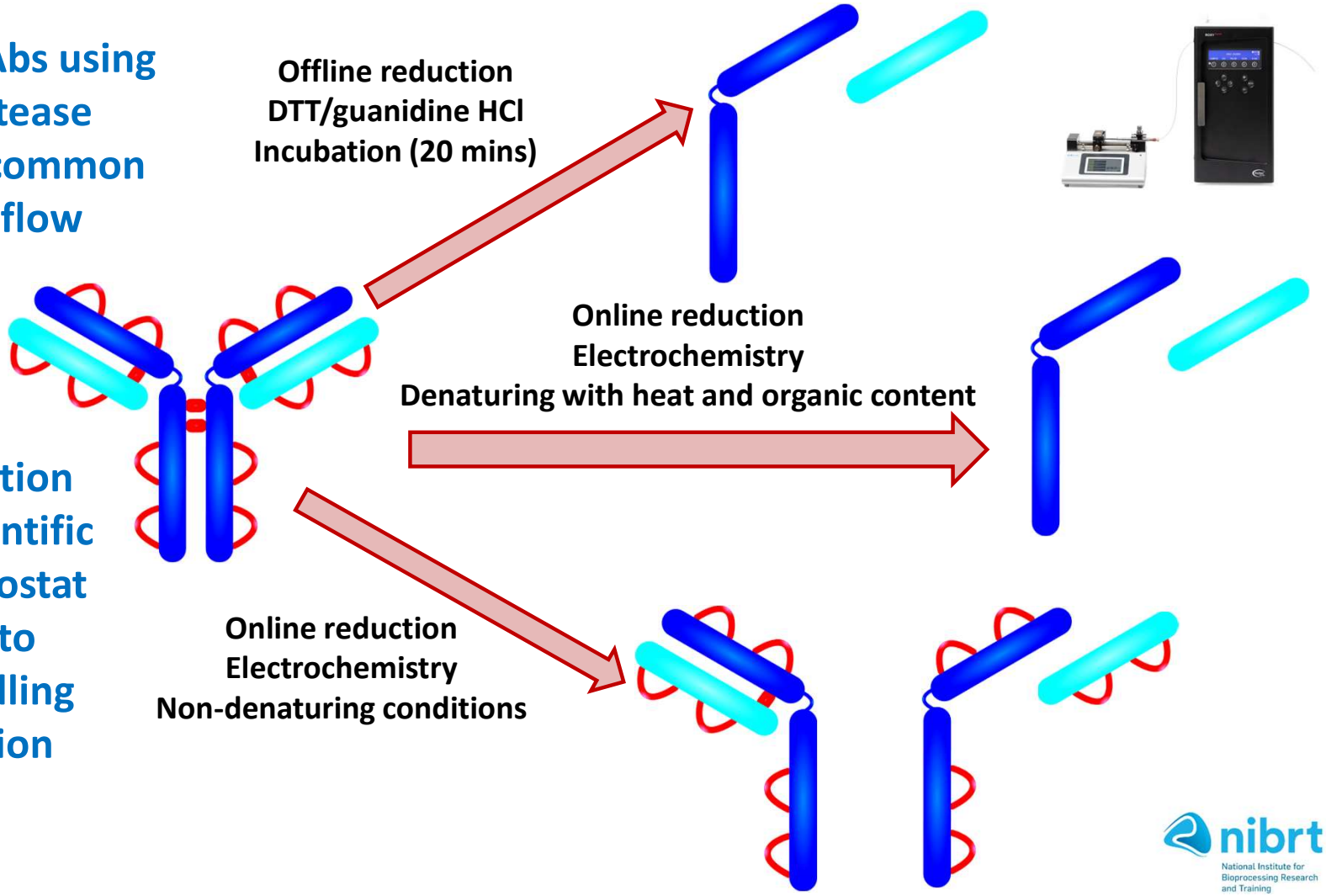
Sub unit analysis of mAbs is a pretty standard workflow

Sub unit analysis of mAbs using reduction, IdeS protease digestion or both is a common but laborious workflow

Use of online electrochemical reduction such as the Antec® Scientific ROXY™ Exceed potentiostat offers the potential to minimise sample handling and sample preparation steps



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Inline electrochemical reduction with ROXY™ Exceed

Thermo Scientific™ Vanquish™ Duo UHPLC

+

Antec® Scientific ROXY™ Exceed Potentiostat

+

2x

Thermo Scientific™ MAbPac™ Reversed Phase Column

+

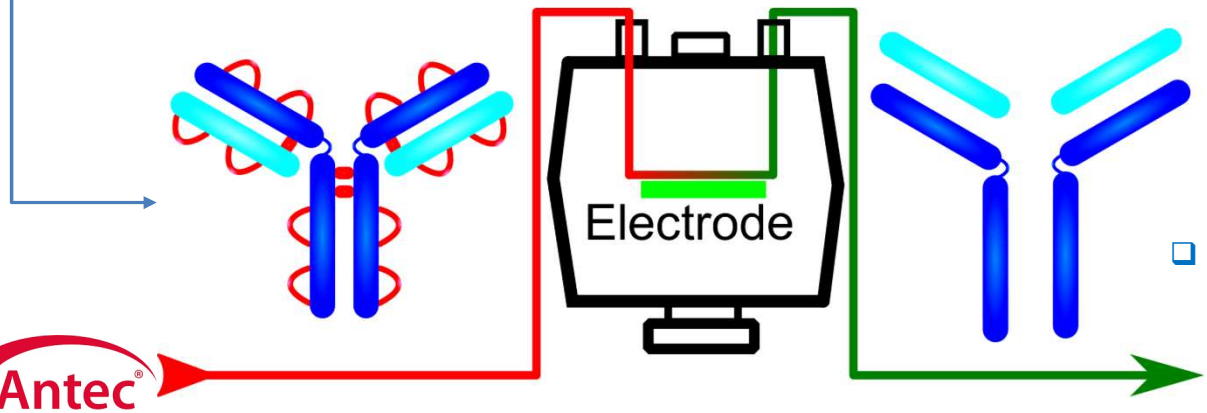
Thermo Scientific™ Q Exactive™ Plus MS

or

Thermo Scientific™ Orbitrap Exploris™ 240 MS

+

Thermo Scientific™ Chromeleon™ 7.2 CDS



- ROXY™ Exceed offers state-of-the-art electronics and generates voltage and current in the range of ± 4.9 V and 1 nA – 100 mA, respectively
- Different operational modes: DC, Scan and Pulse for high product yields

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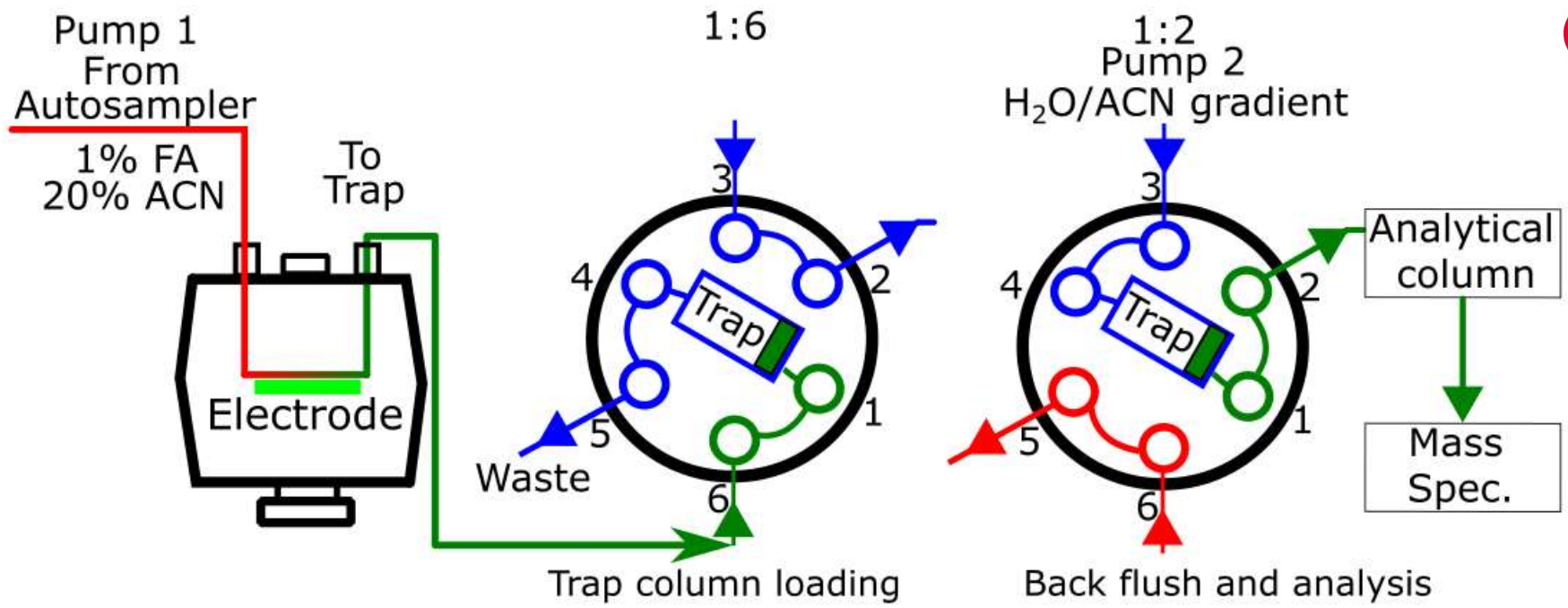
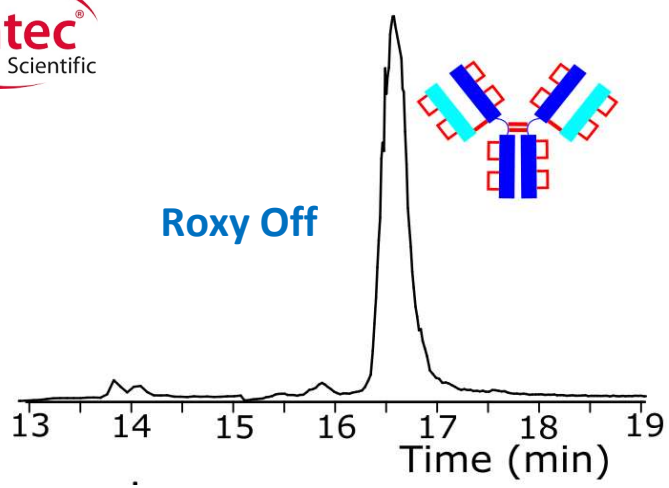
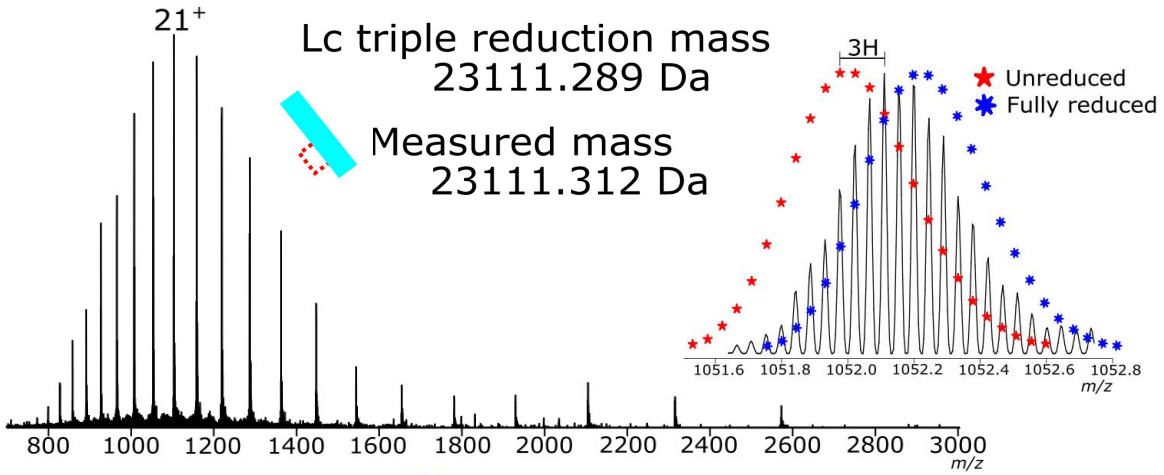
Coupling Roxy™ Exceed potentiostat inline to LC-MS

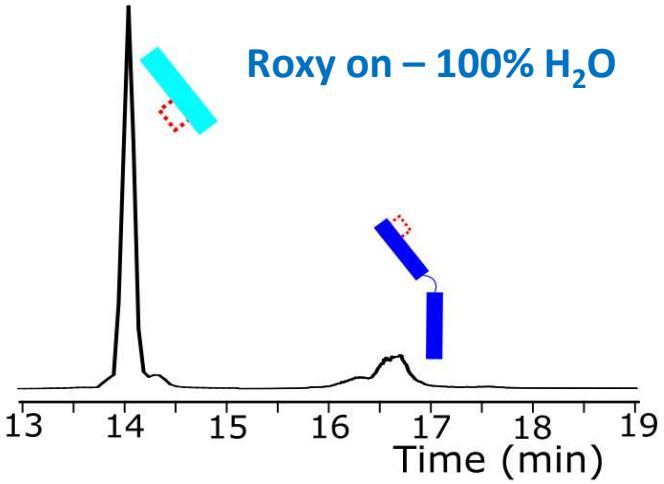


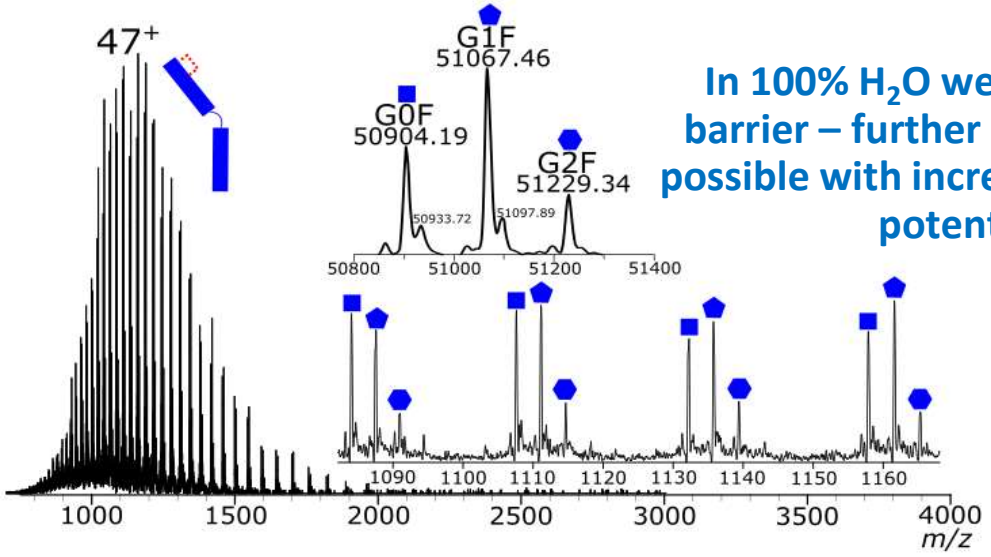
- Experimental design of inline electrochemical reduction using a dual pump and trap column setup
- Allows use of solvents optimised for best reduction conditions followed by best conditions for analysis

Optimisation of inline electrochemical reduction



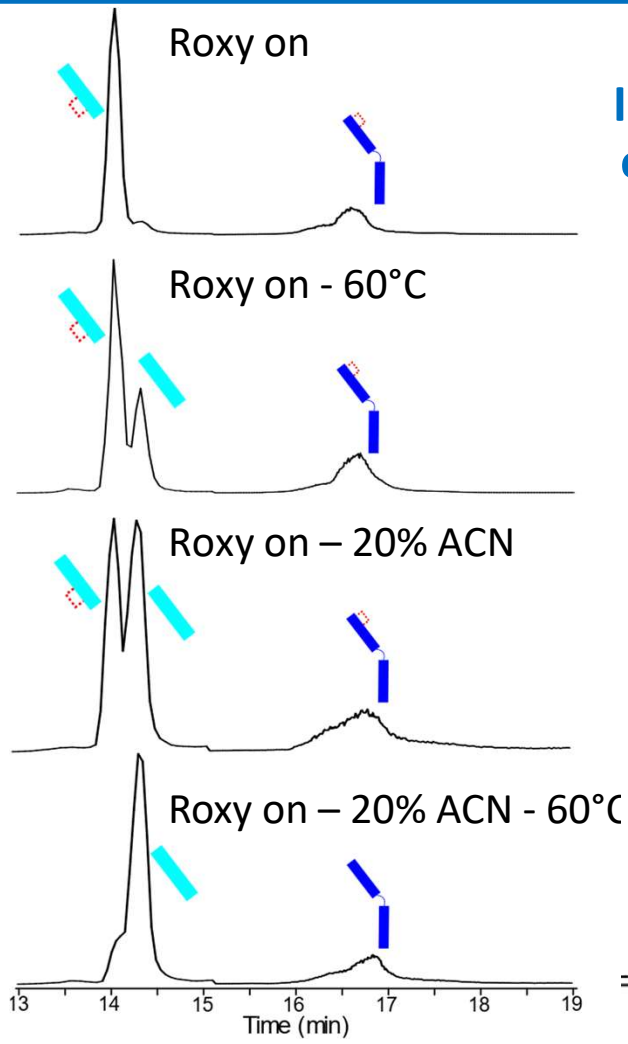




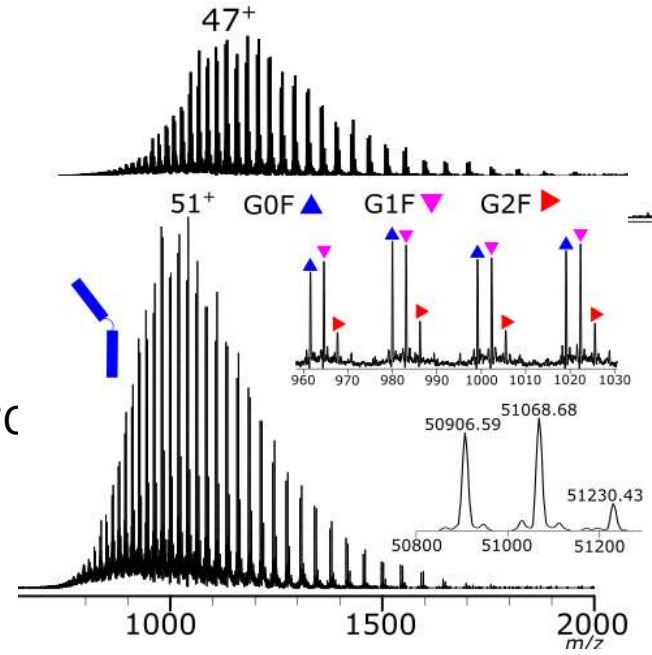


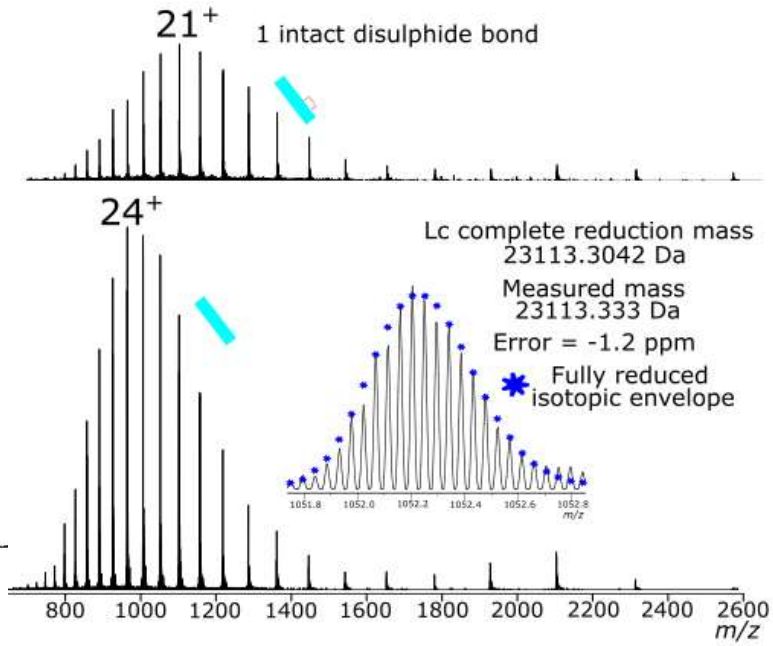
In 100% H_2O we hit a kinetic barrier – further reduction not possible with increased electrical potential

Optimisation of inline electrochemical reduction



Increasing temperature and organic content overcomes kinetic barrier and allows inter- and intra-chain reduction





Light Chain

Heavy Chain

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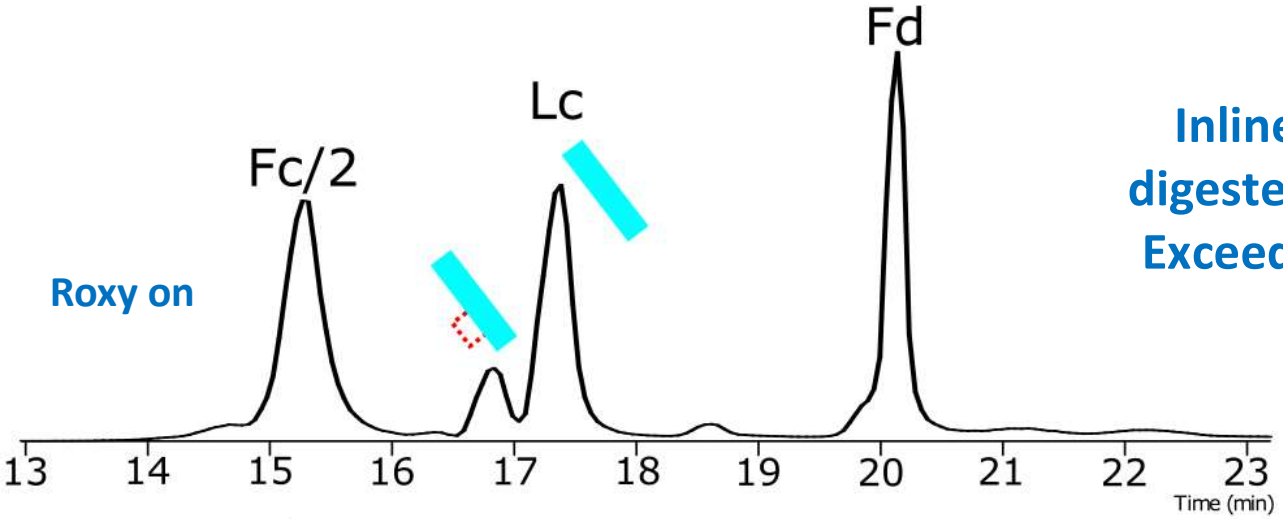
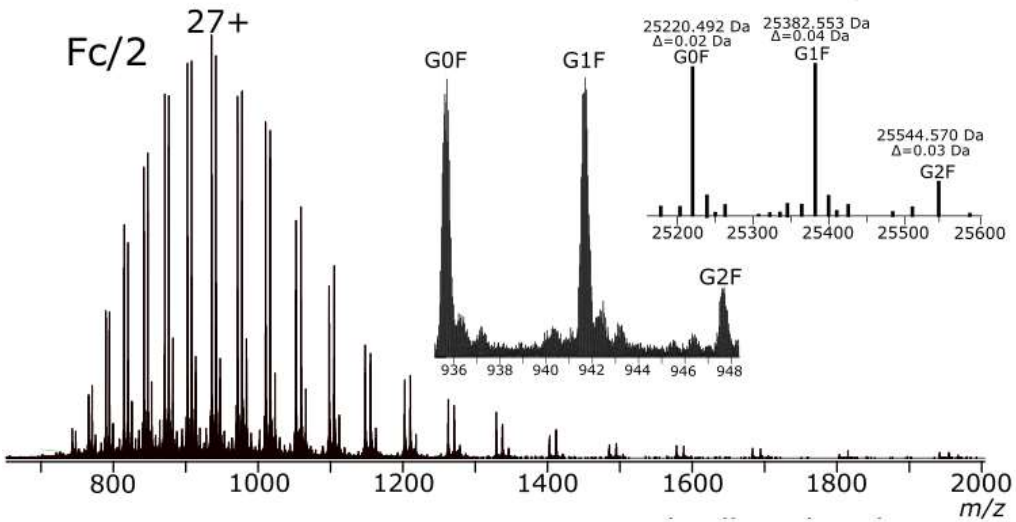
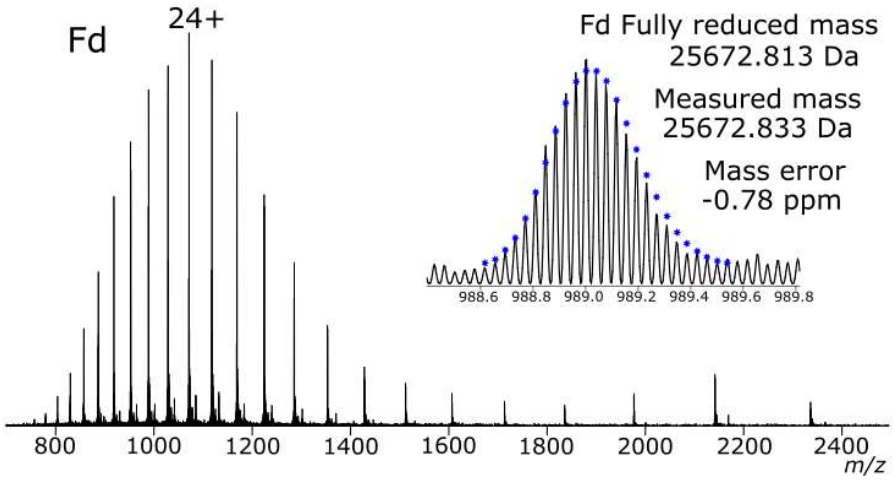
Combination with IdeS digestion



Inline reduction of IdeS digested mAb using ROXY™ Exceed potentiostat inline with LC-MS





Native Separations Coupled to Native MS

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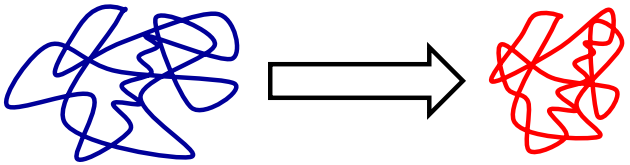
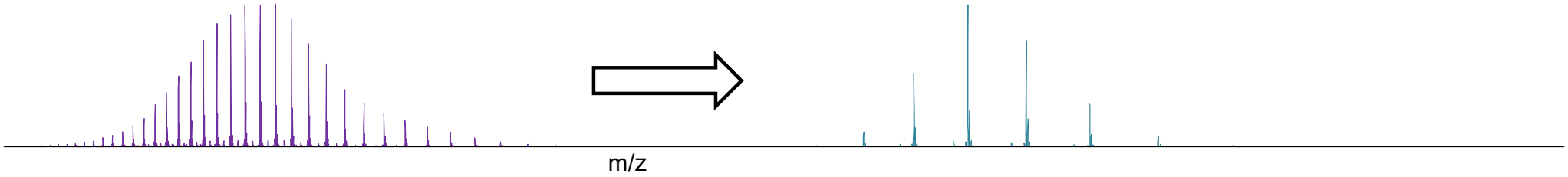
Why Native MS?

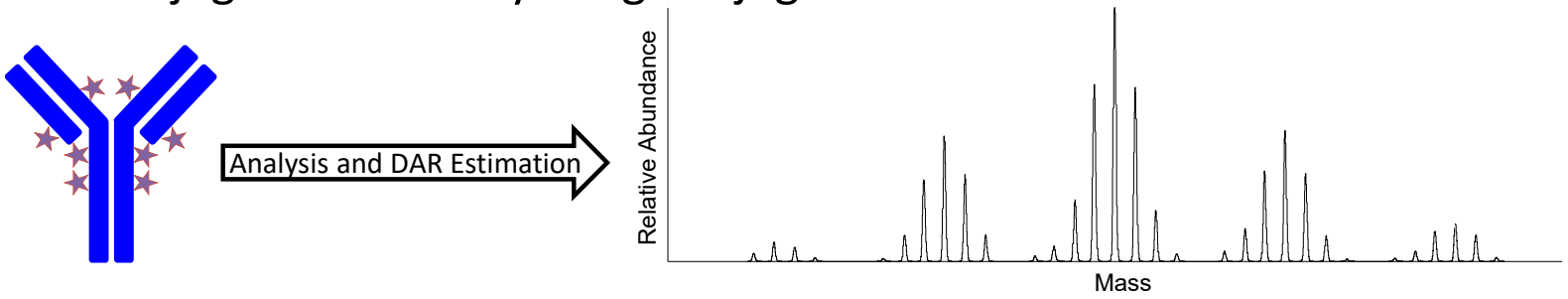
1. Preservation of higher order protein structure allows for structural analysis, e.g. structural changes upon pH change, ligand binding, complex formation, etc.



2. Spectra in the higher *m/z* range with increased spatial spectral resolution



3. Preservation of non-covalent interaction allows for the analysis of fragile molecules such as cysteine-conjugated antibody-drug conjugates



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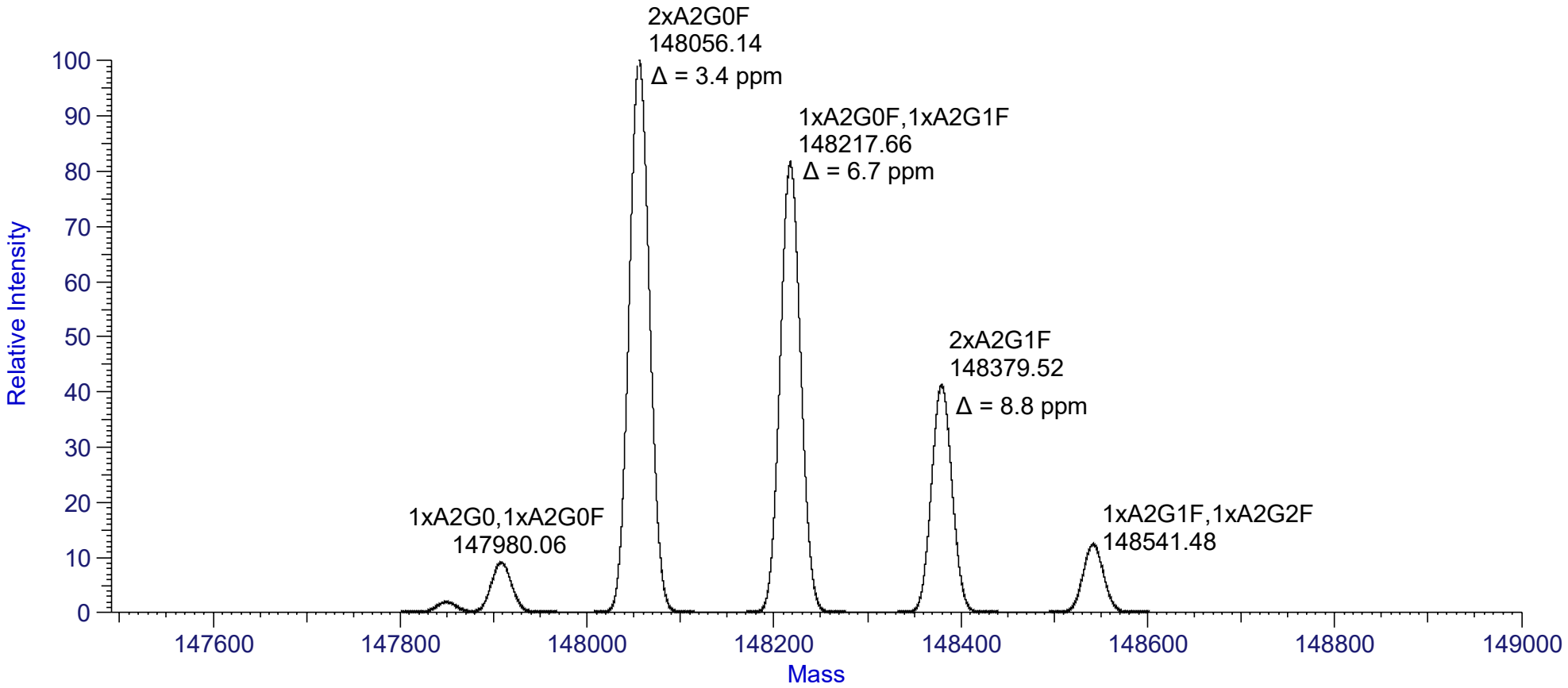
Native SEC-MS on Orbitrap Exploris 240 MS



Deconvoluted spectrum of trastuzumab from SEC-MS dilution series data, excellent mass accuracy across the glycoform distribution

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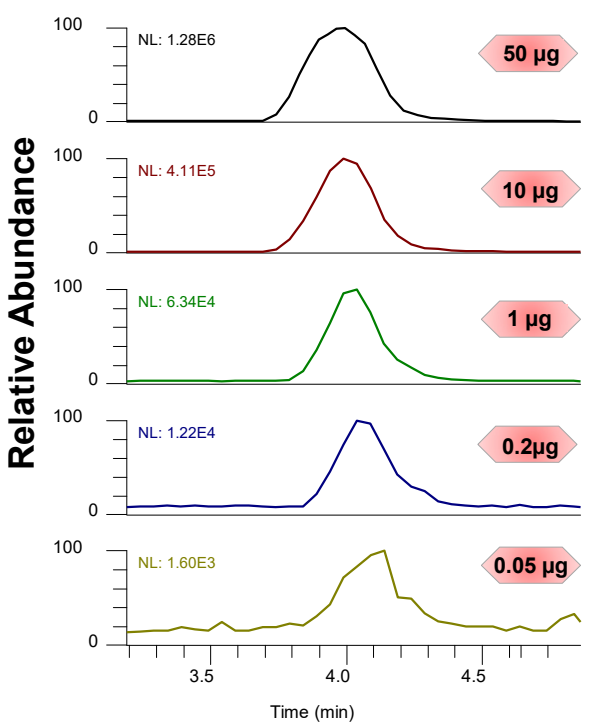
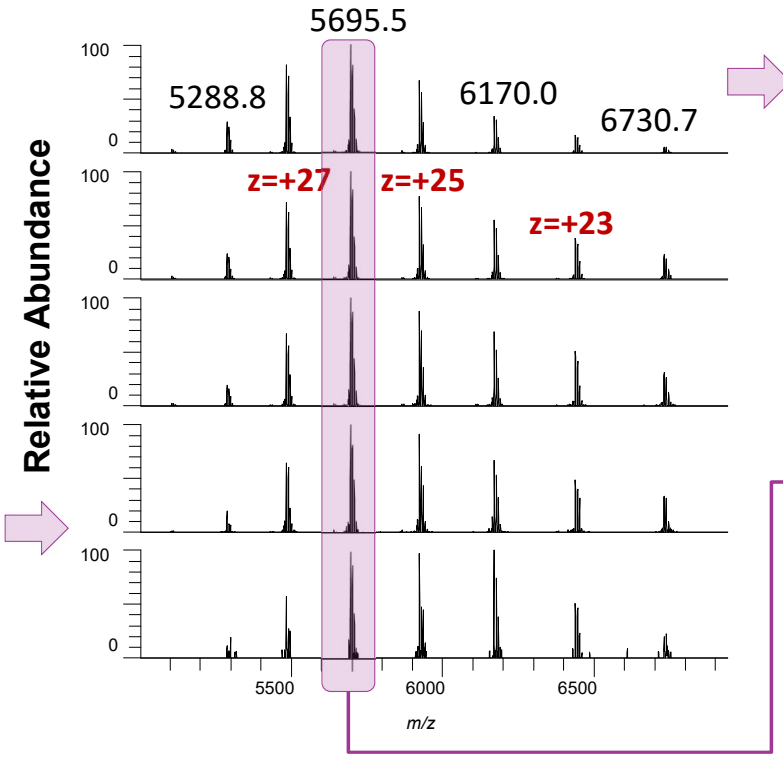
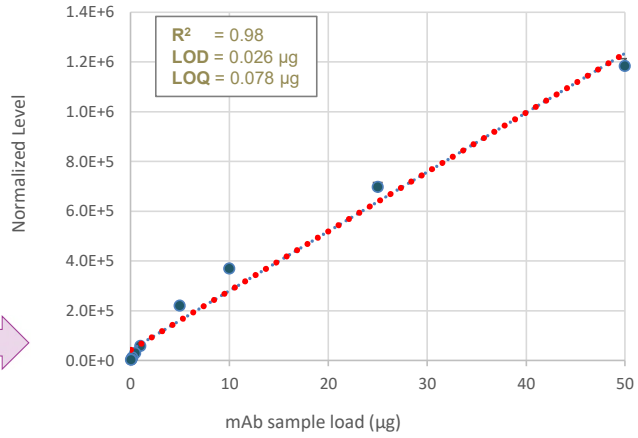
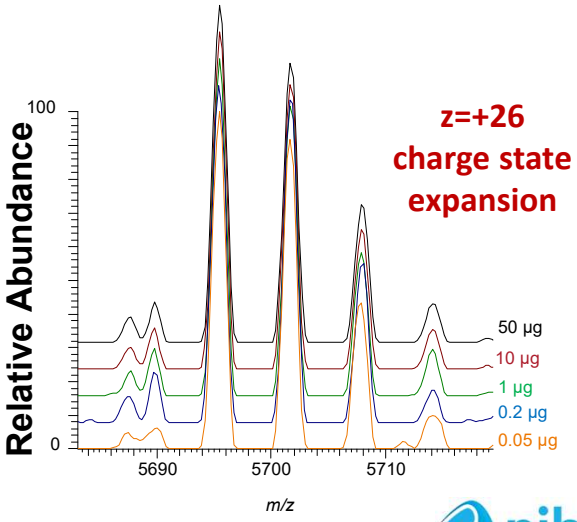
Native SEC-MS on Orbitrap Exploris 240 MS

Dilution series for trastuzumab using native SEC-MS, excellent instrument sensitivity and linearity. Beautiful native, deconvolutable spectra acquired even down to 50 ng on column!









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Particle Size and Chromatographic Performance

- Reducing the particle size of chromatographic stationary phases is well known to increase the efficiency of the separation due to reduced plate height.
- Advances in stationary phase formats have enabled the achievement of high chromatographic efficiency without the associated increase in pressure that arises when using particles with small diameters.
- However, stationary phase particles are often polydisperse, resulting in a range of particles, some smaller and some larger than the stated particle size, which effects column performance according to van Deemter.
- Monodisperse particle size distributions represent a more idealised stationary phase format, reducing diffusive effects and facilitating more reproducible mass transfer, together yielding increased chromatographic performance.

Introducing a Novel Monodisperse Particle Technology

- Thermo Scientific have developed new ion exchange phases based on a novel monodisperse particle technology – ProPac 3R Range.
- Columns are packed with a 3 µm monodisperse non-porous polymeric phase, coated with a hydrophilic polymeric layer comprising anion or cation exchange functionalities.
- Column features include:
  - Increased capacity,
  - Ability to use shorter formats to increase speed,
  - Increased chromatographic performance,
  - Excellently controlled chemistry of manufacture yielding tight batch-to-batch reproducibility.

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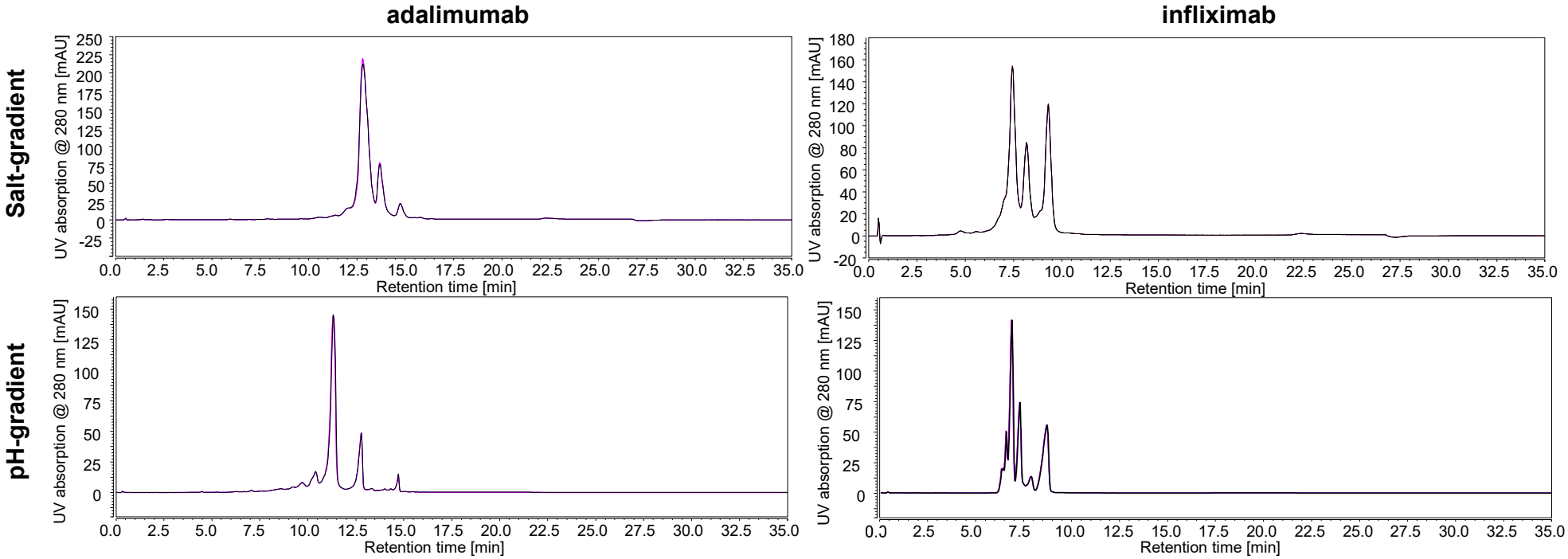
Chromatographic Reproducibility on ProPac 3R



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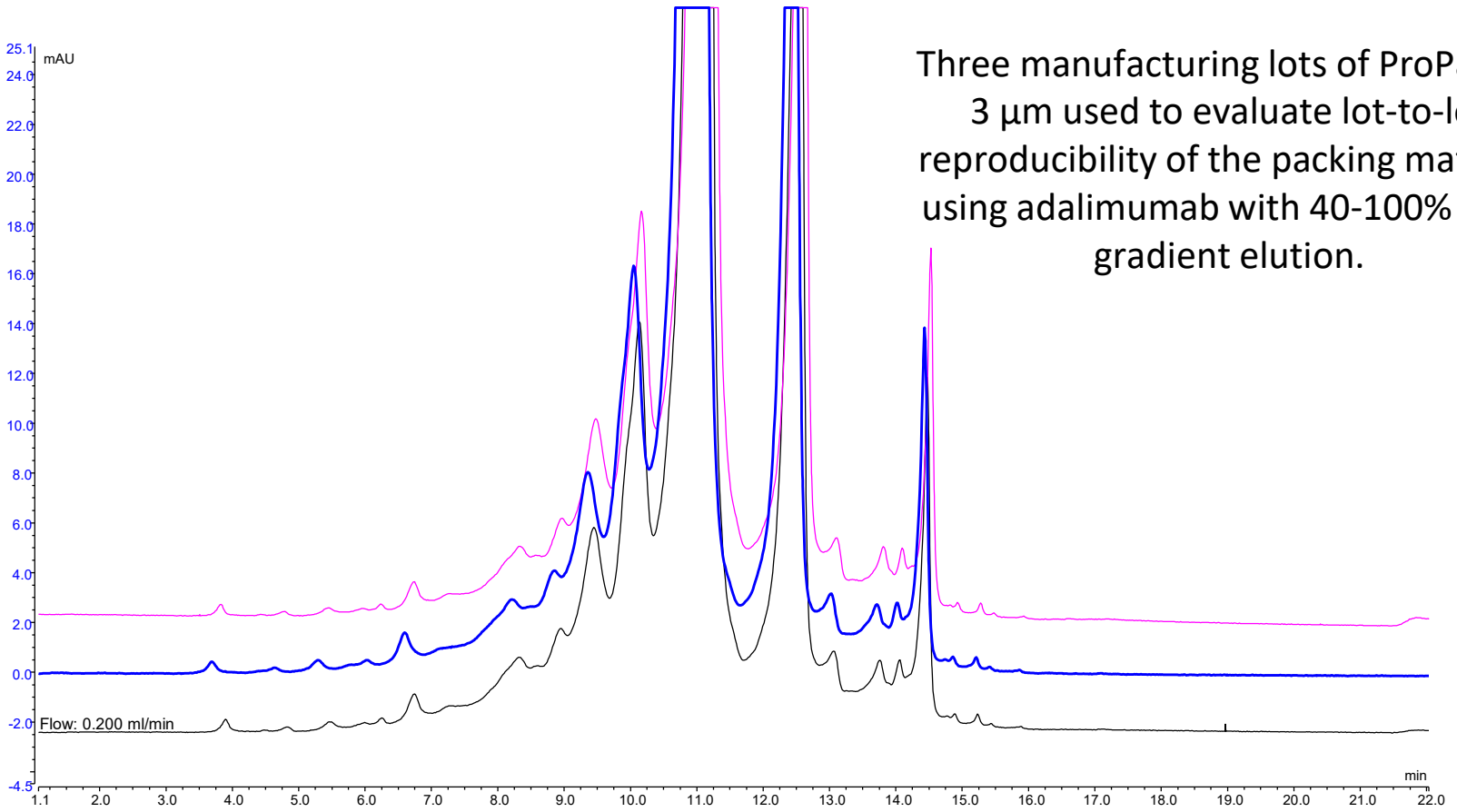
Lot-to-Lot Reproducibility of the Packing Material



Three manufacturing lots of ProPac 3R 3 µm used to evaluate lot-to-lot reproducibility of the packing material using adalimumab with 40-100% B pH gradient elution.

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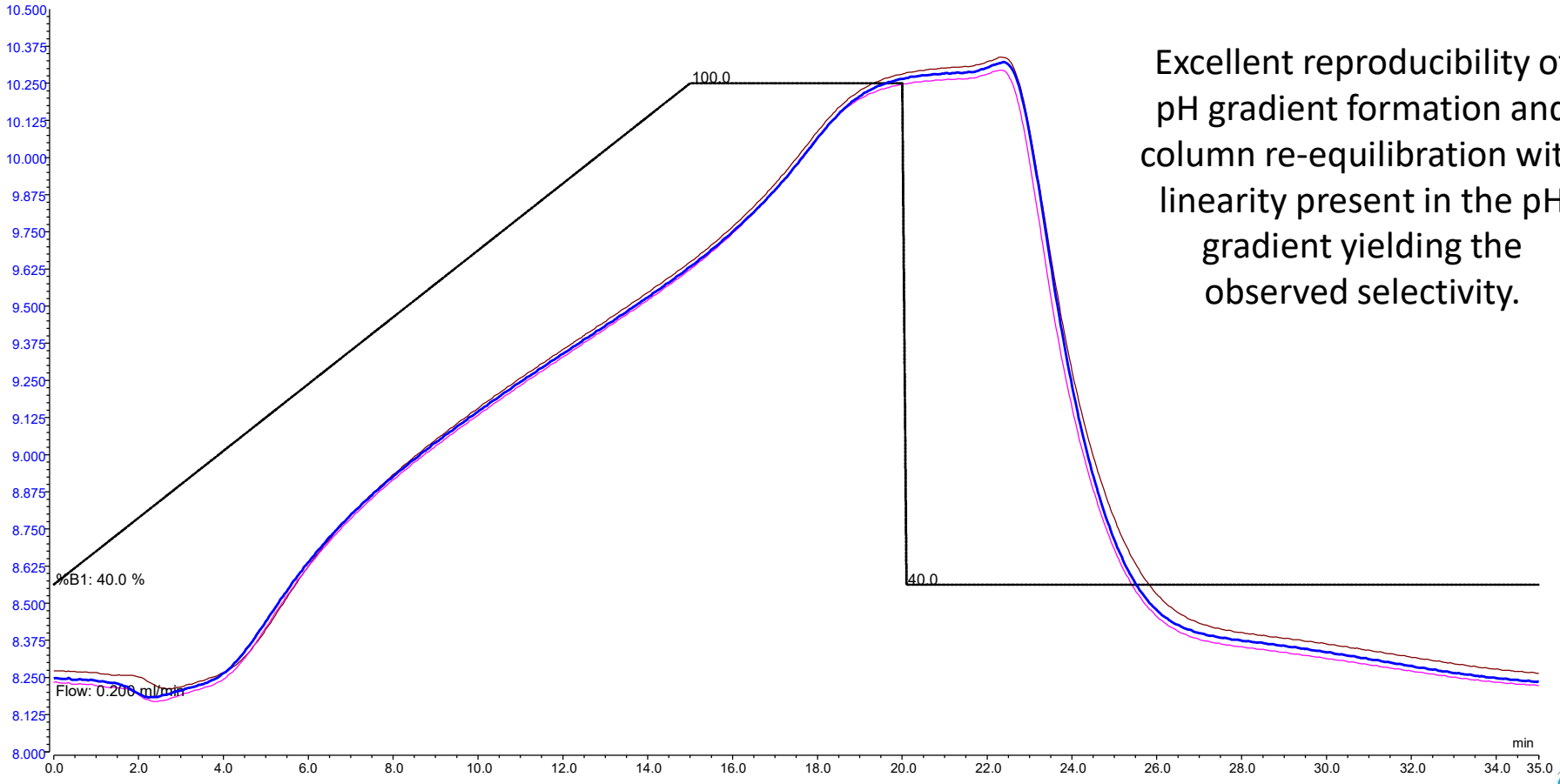
pH Gradient Reproducibility



Excellent reproducibility of pH gradient formation and column re-equilibration with linearity present in the pH gradient yielding the observed selectivity.

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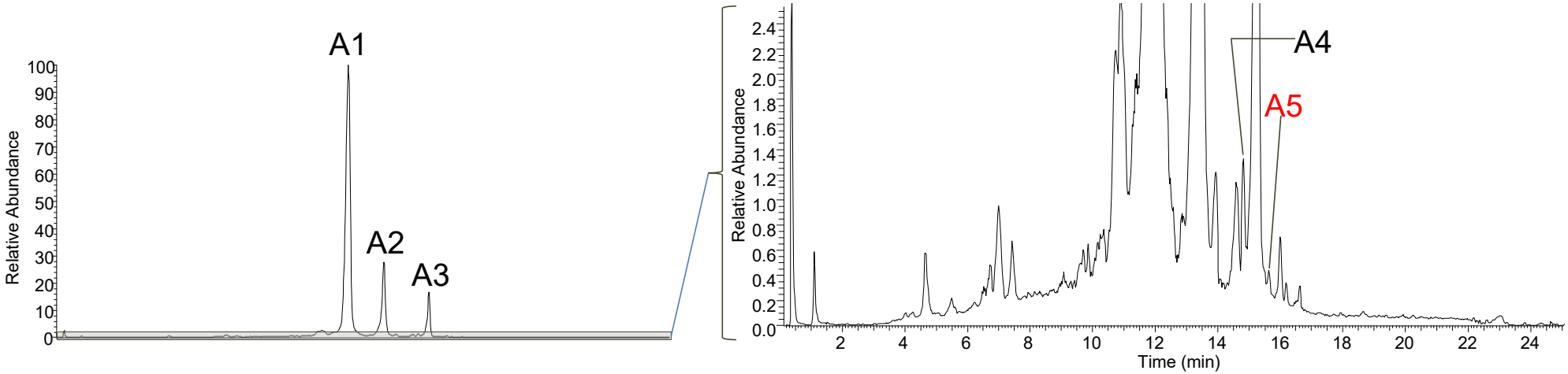
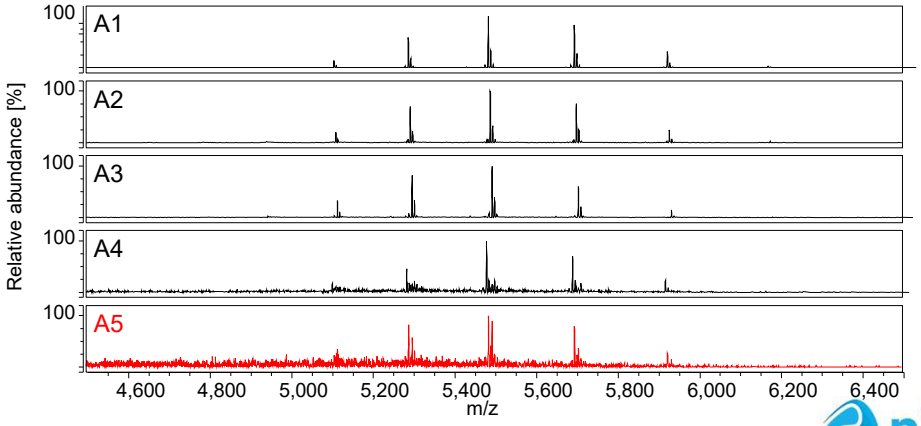
High Resolution: pH Gradient MS Coupling



- Sample amount 50 µg
- Excellent MS sensitivity
- Peaks can be clearly distinguished in the sub 1% relative abundance region
- Clear mass spectra obtained for all major peaks
- Even peaks with a relative abundance of 0.4% (A5) show spectral quality sufficient for deconvolution.



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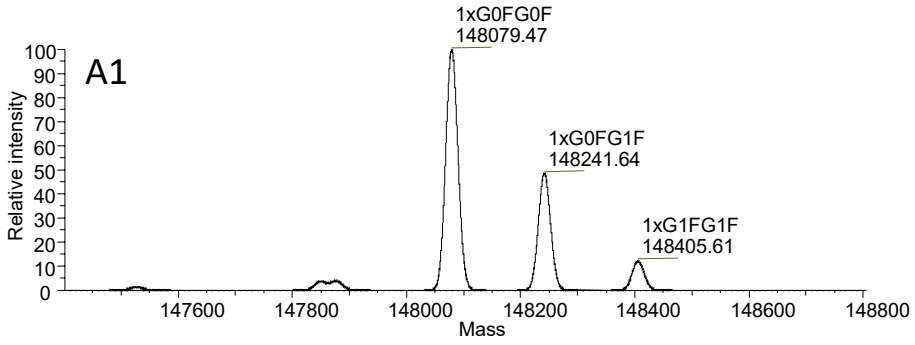
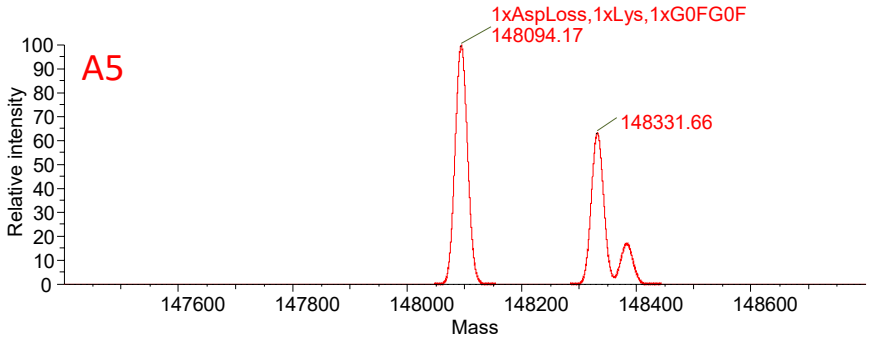
High Resolution: pH Gradient MS Coupling

Deconvolution of the data using the Sliding Window algorithm in BioPharma Finder facilitates assessment and annotation of peaks, from high to low abundance.





High abundant species, *e.g.,* A1 easily identified as des-C-term K form. Selectivity on SCX-MD 3 µm is so good that we now can identify lower abundant proteoforms of the molecule, tentative identification based on mass, confirmation by peptide mapping

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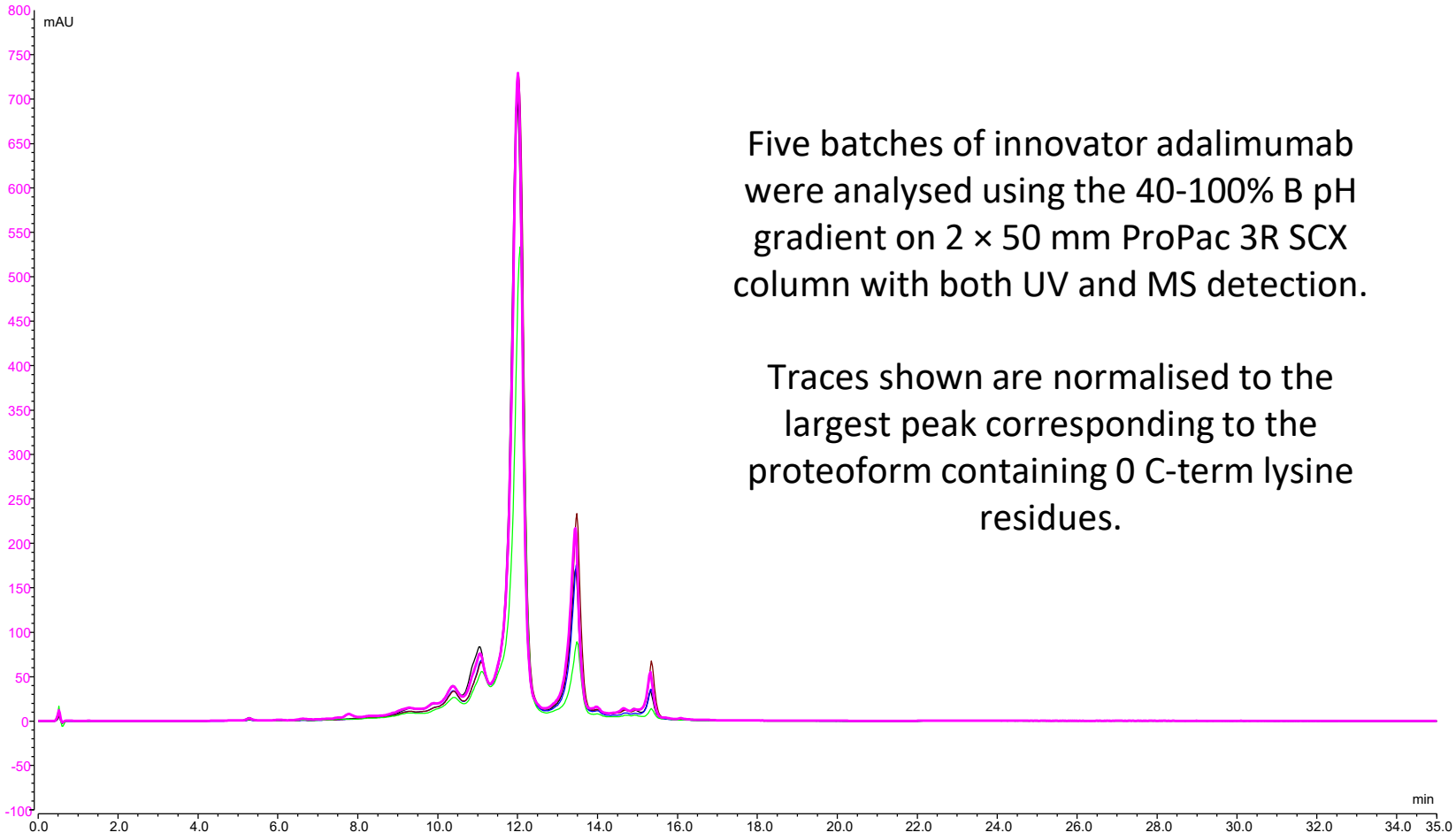
Comparability Assessment - Adalimumab



Five batches of innovator adalimumab were analysed using the 40-100% B pH gradient on 2 × 50 mm ProPac 3R SCX column with both UV and MS detection.

Traces shown are normalised to the largest peak corresponding to the proteoform containing 0 C-term lysine residues.

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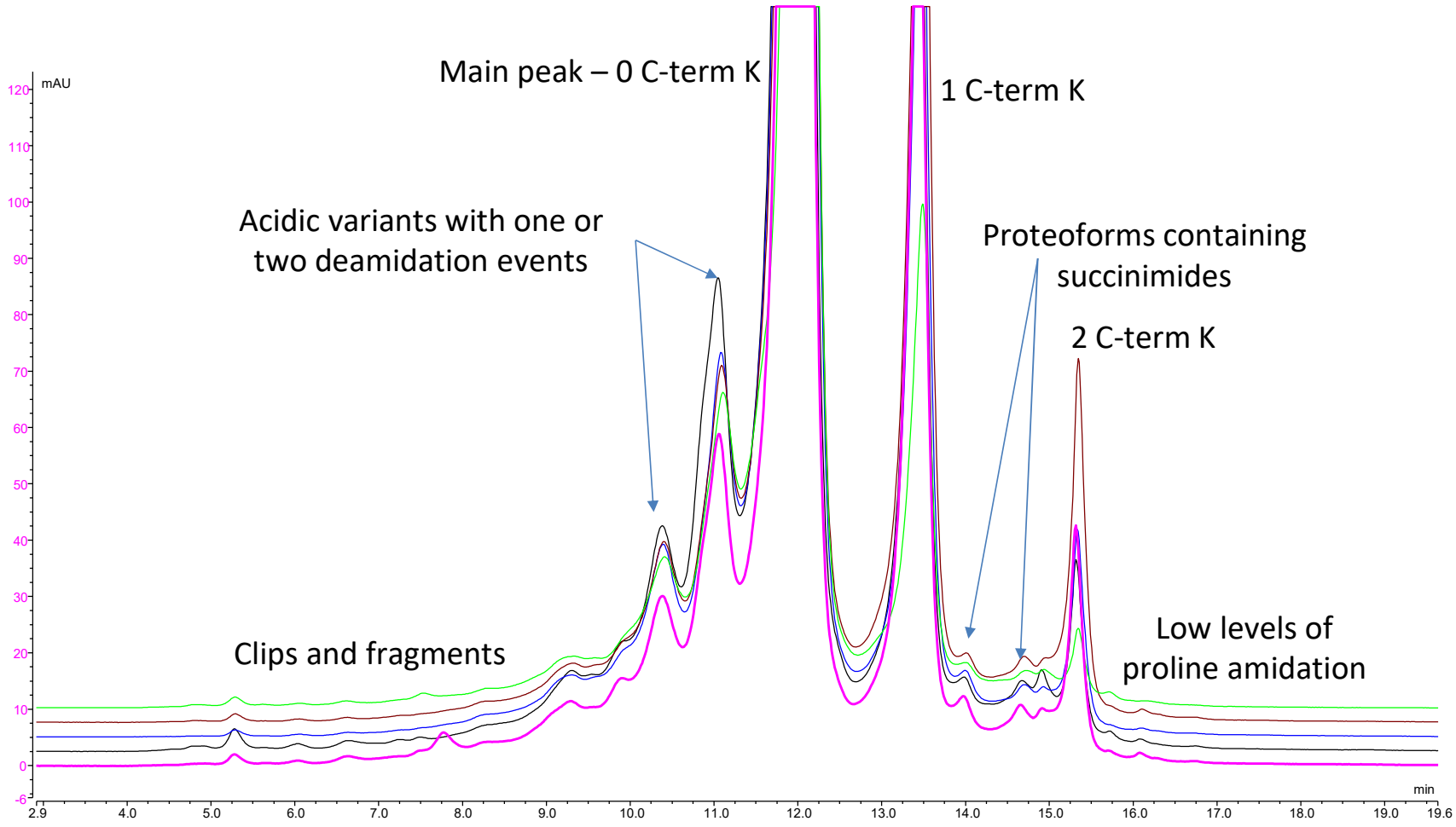
Baseline Zoom – Impressive Selectivity of SCX-MD 3µm



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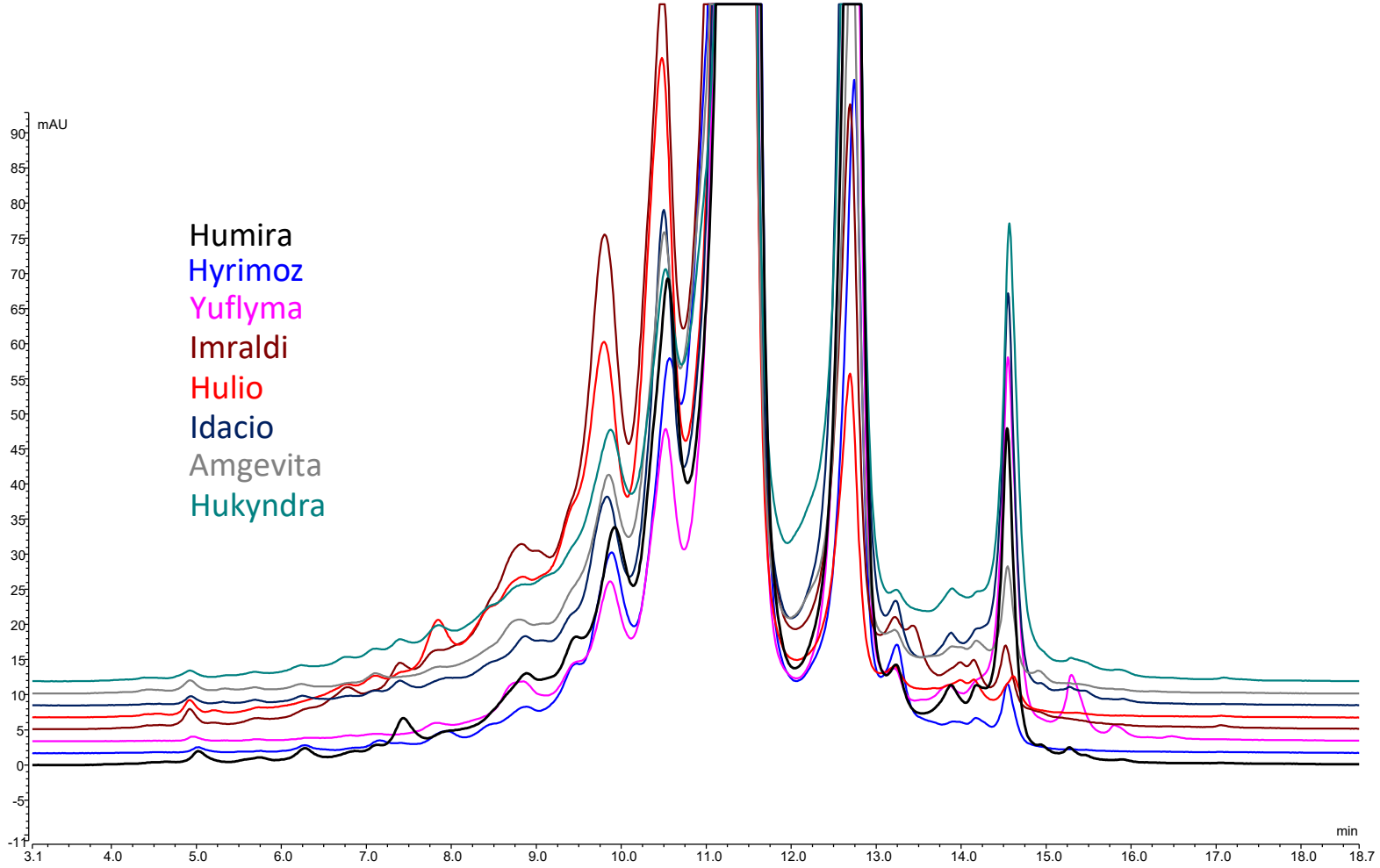
Comparability Assessment of Adalimumab Biosimilars



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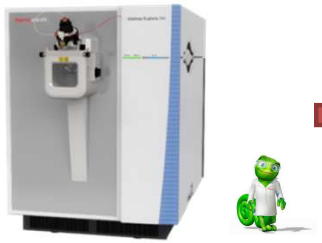
iMAM Data Processing

Carillo *et al.*, Eur J Pharm Biopharm, **2022**, *177*, 241-248.

Characterization

LC-MS



Thermo Scientific™ Vanquish™ UHPLC
Thermo Scientific™ Orbitrap Exploris™ 240

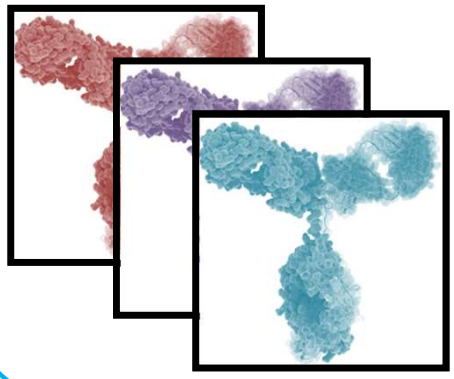
Deconvolution and search

Thermo Scientific™ BioPharma Finder™ v. 4.1

Processing method for target components monitoring

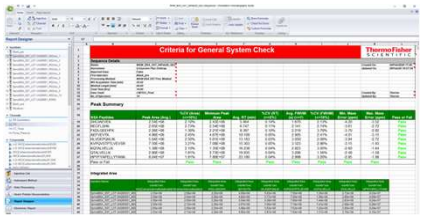
Thermo Scientific™ Chromeleon™ CDS

Monitoring

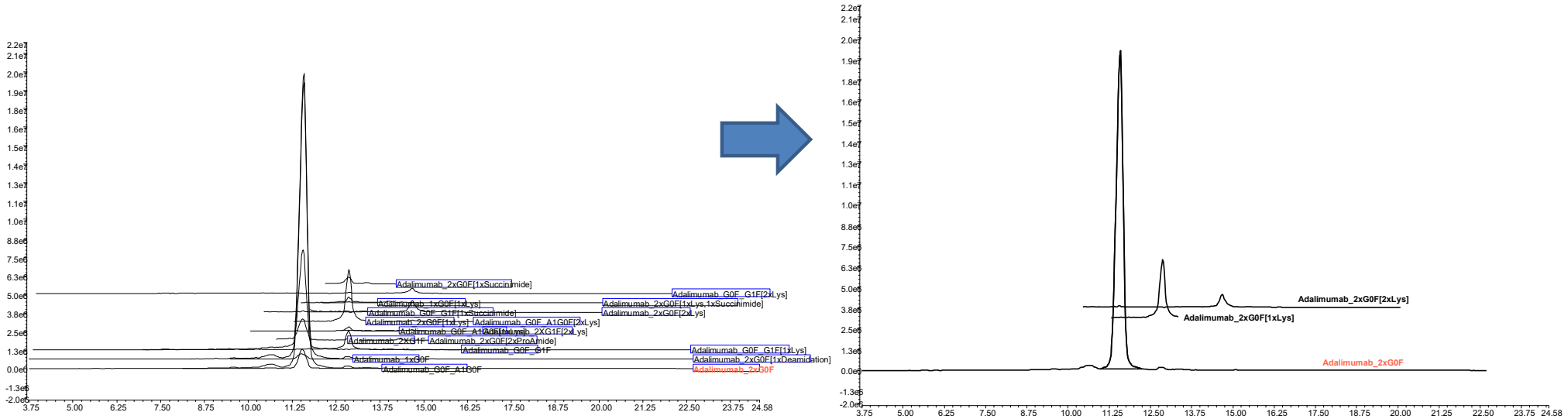


LC-MS

Compliant report generation and **new entities search**



Comparability Assessment of Adalimumab Biosimilars



iMAM processing of the resulting MS data enables the determination and quantitation of a large number of product quality attributes present on the intact level

Simplified output depicting only the C-term lysine variants used to compare the different adalimumab biosimilars

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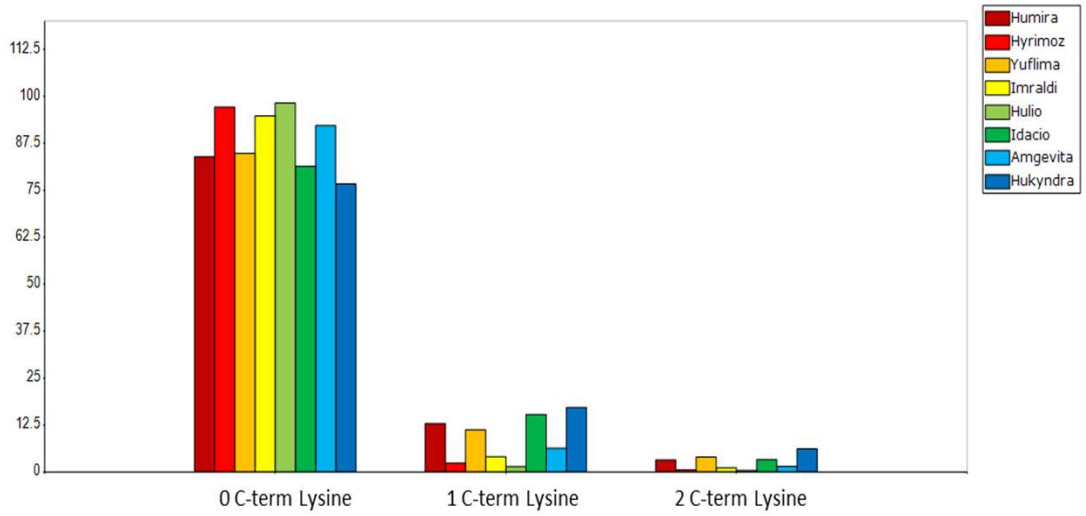
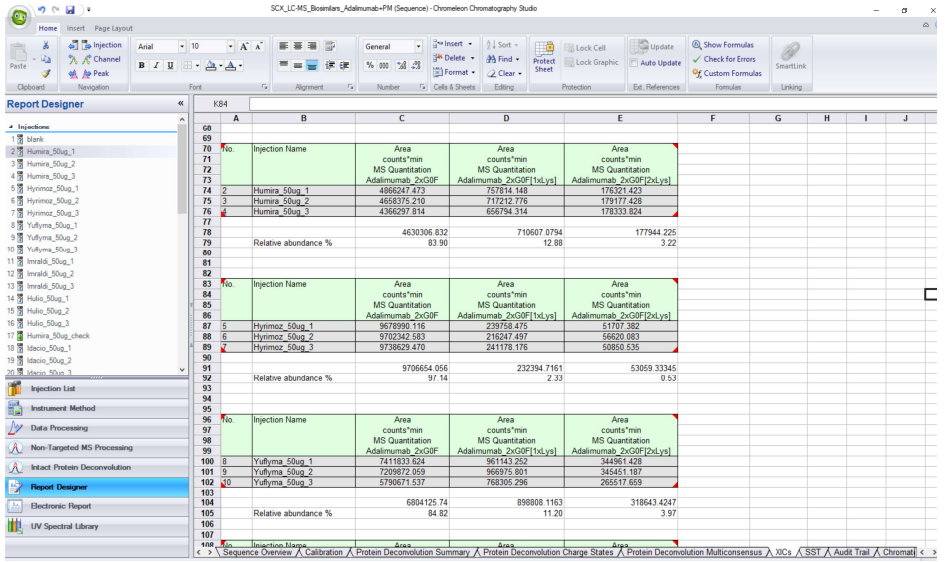
iMAM Processing of Adalimumab CEX-MS Data





Improved selectivity of ProPac 3R SCX improves iMAM processing allowing for more components to be identified and added to the processing method for targeted quantitation and reporting.

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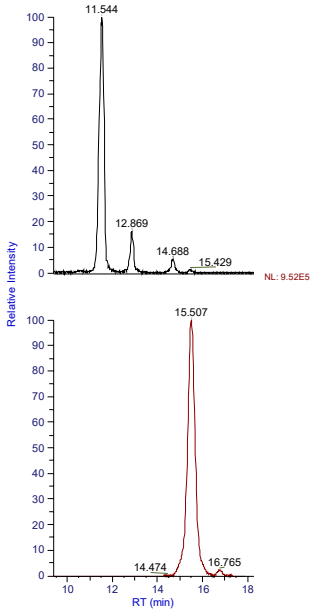
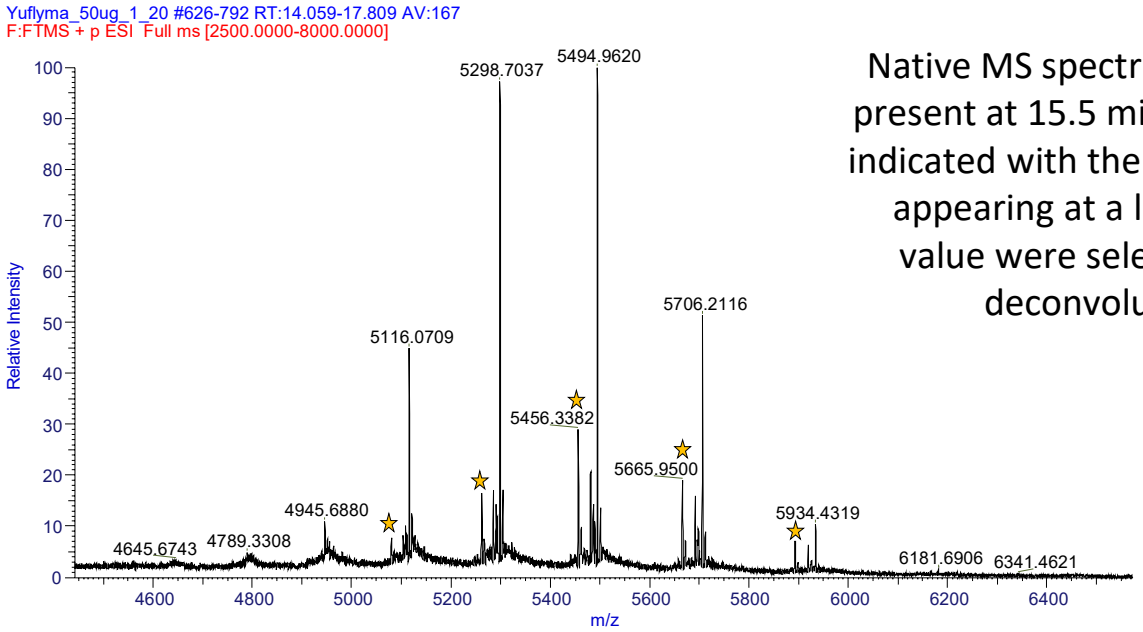
An Additional Basic Peak Present in Yuflyma





Native MS spectrum of peak present at 15.5 mins, features indicated with the yellow stars appearing at a lower m/z value were selected and deconvoluted

Yuflyma was found to contain an extra peak in the basic region of the chromatogram, at 15.5 minutes, eluting later than any other peak present in the innovator or other adalimumab biosimilars.

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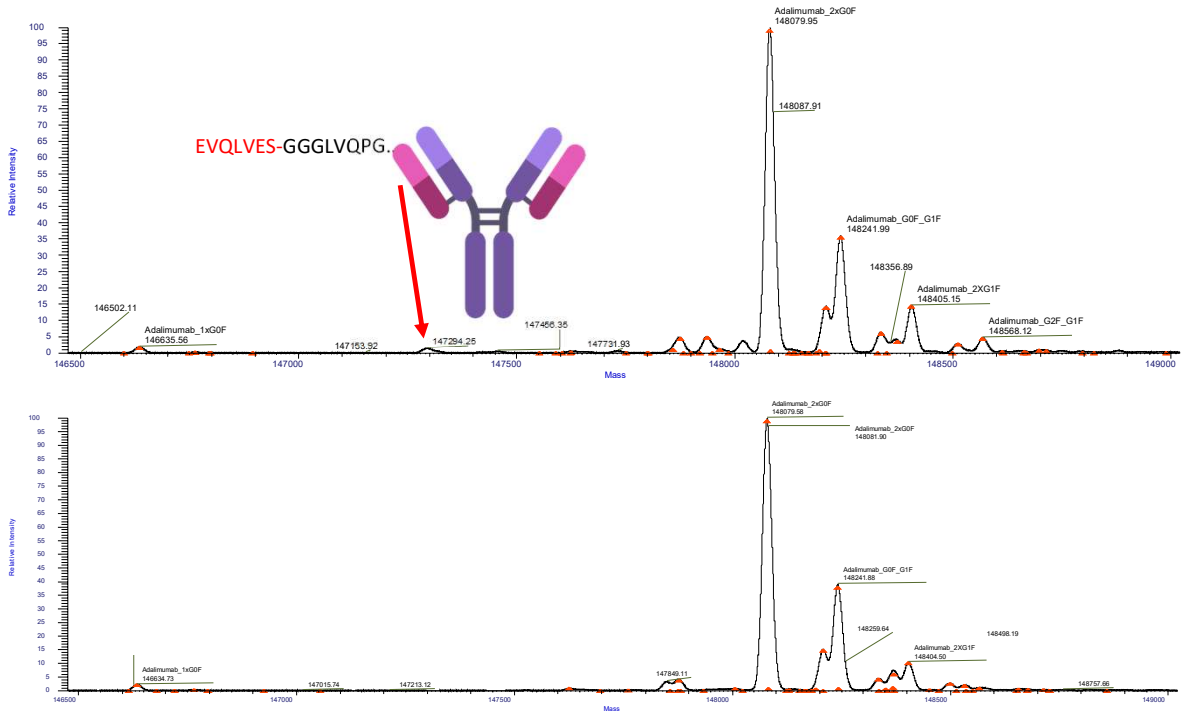
An Additional Basic Peak Present in Yuflyma



Deconvoluted mass spectra of Yuflyma and the innovator product were compared.

An additional peak at 147,294.25 Da was observed in Yuflyma.

Working the numbers, this lower mass species potentially corresponds to a truncation on the heavy chain N-terminus.

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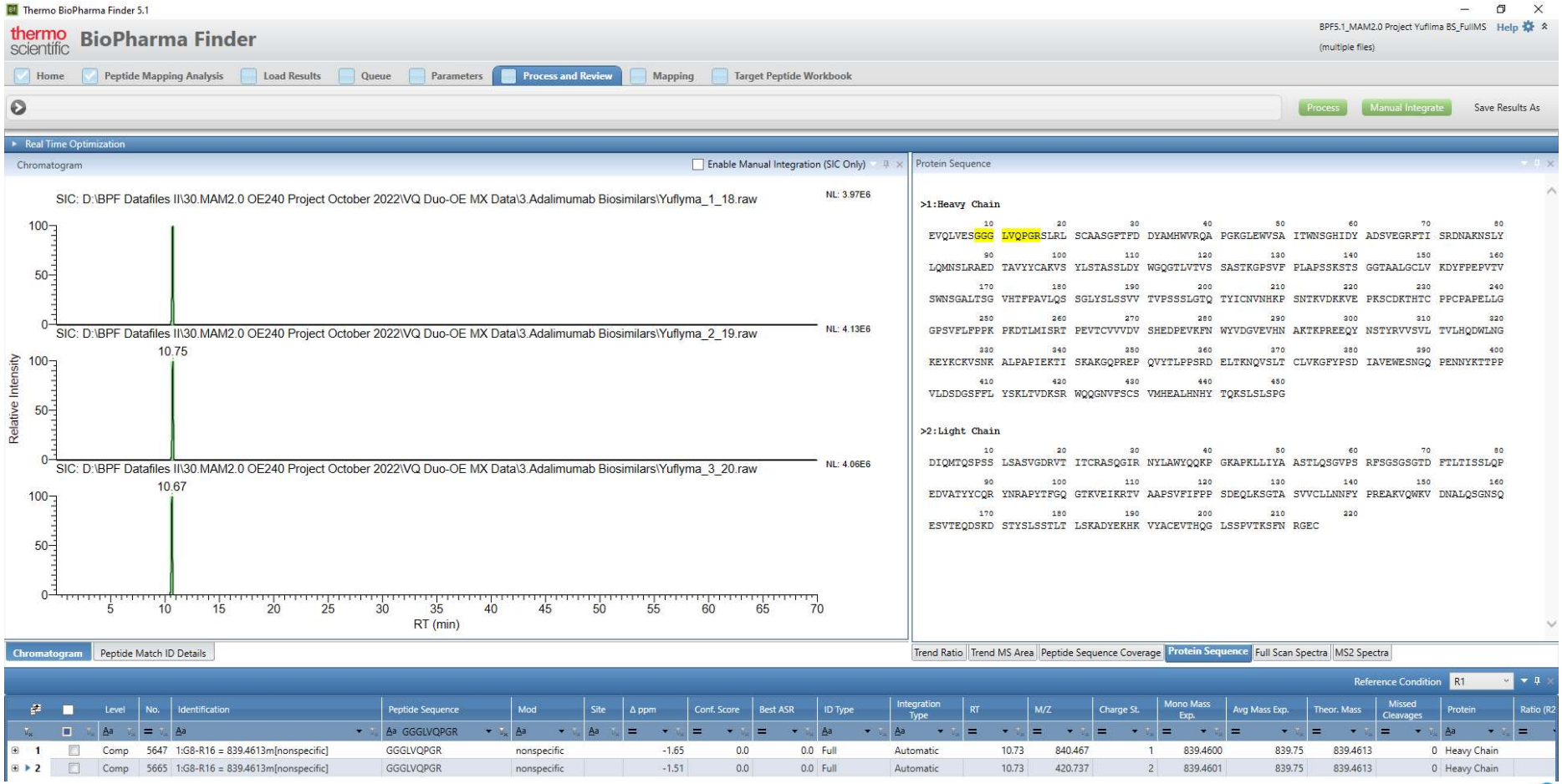
Confirmation of HC N-term Cleavage by Pep Mapping



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Coupling ProA affinity chromatography to high res MS

Protein A affinity chromatography is a key step in the purification of mAbs and Fc fusion proteins

We also routinely use the Thermo Scientific™ MAbPac™ Protein A Antibody Analysis and Purification HPLC Column for rapid titre determination by LC-UV

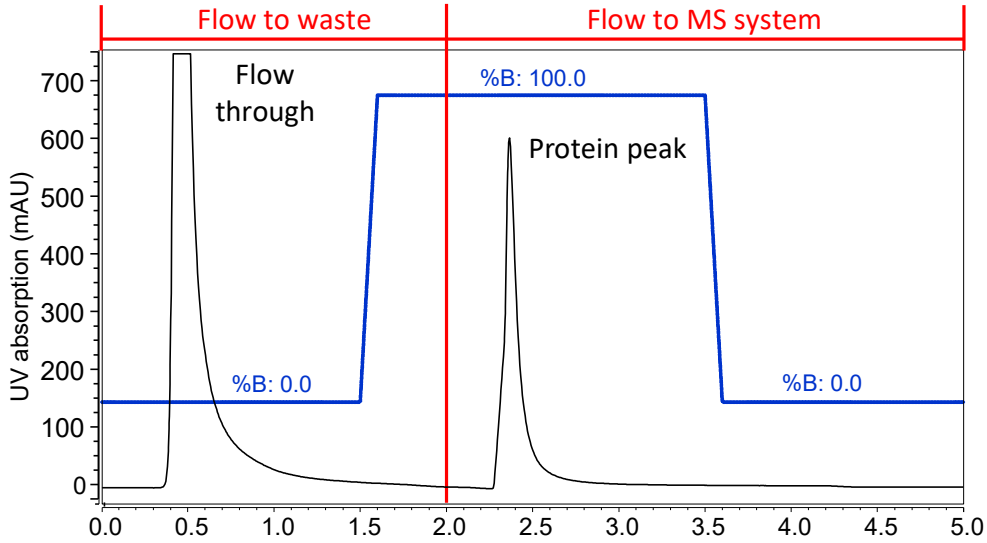
analytical chemistry Anal. Chem. 2015, *87*, 2023-8 Editors' Highlight
pubs.acs.org/ac

Inline Protein A Mass Spectrometry for Characterization of Monoclonal Antibodies

Kenneth M. Prentice, Alison Wallace, and Catherine M. Eakin*,†

Department of Analytical Sciences, Amgen Inc., 1201 Amgen Court West, Seattle, Washington 98119, United States

Previous report from Amgen on ProA coupling to MS but used solvent make up flow for denatured intact mass analysis



We wanted to explore if we could couple ProA to high resolution Orbitrap MS under native conditions using volatile buffers for rapid titre and characterisation

Image Credits:
Column image: https://www.sartorius.com/en/products/process-chromatography/chromatography-columns
ProA 3D ribbon structure: https://www.rcsb.org/structure/1BDD

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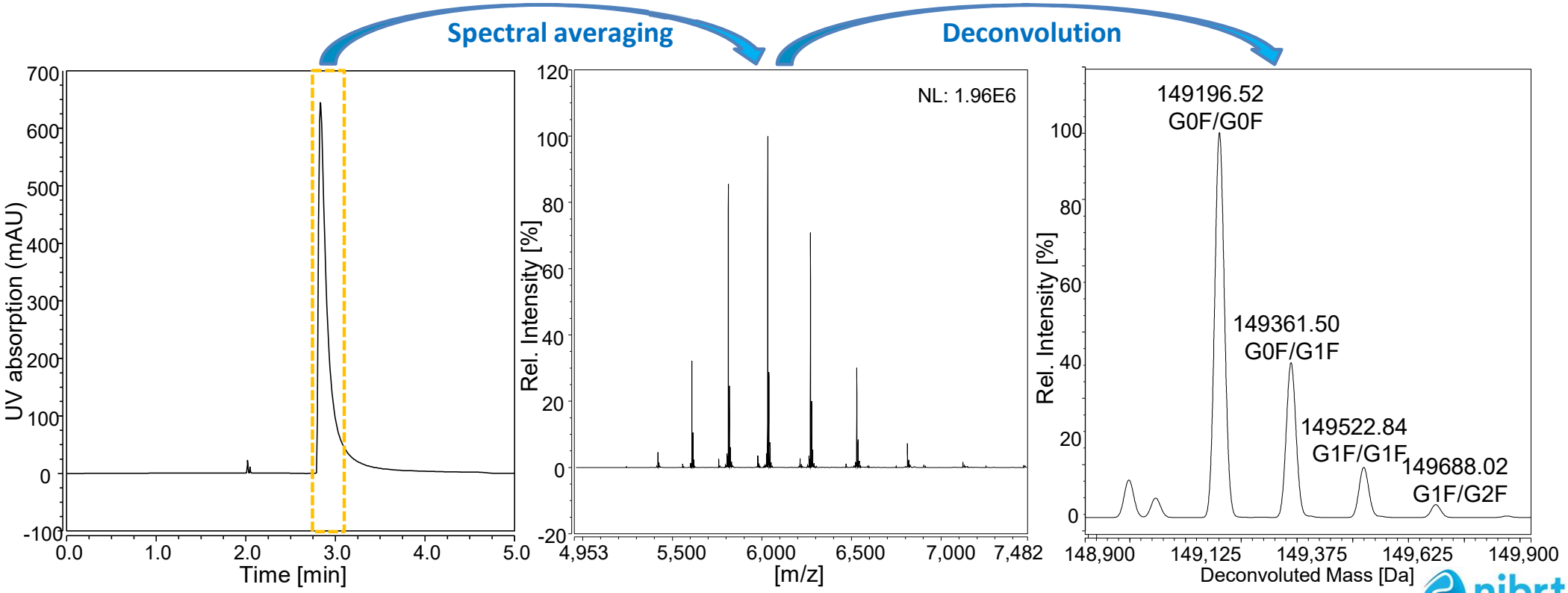
ProA-MS using MAbPac™ Protein A coupled to QE-

Protein A affinity chromatography of bevacizumab using a MAbPac™ Protein A column on a Thermo Scientific™ Vanquish™ Flex UHPLC coupled to a Q Exactive™ UHMR Hybrid Quadrupole-Orbitrap™ Mass Spectrometer



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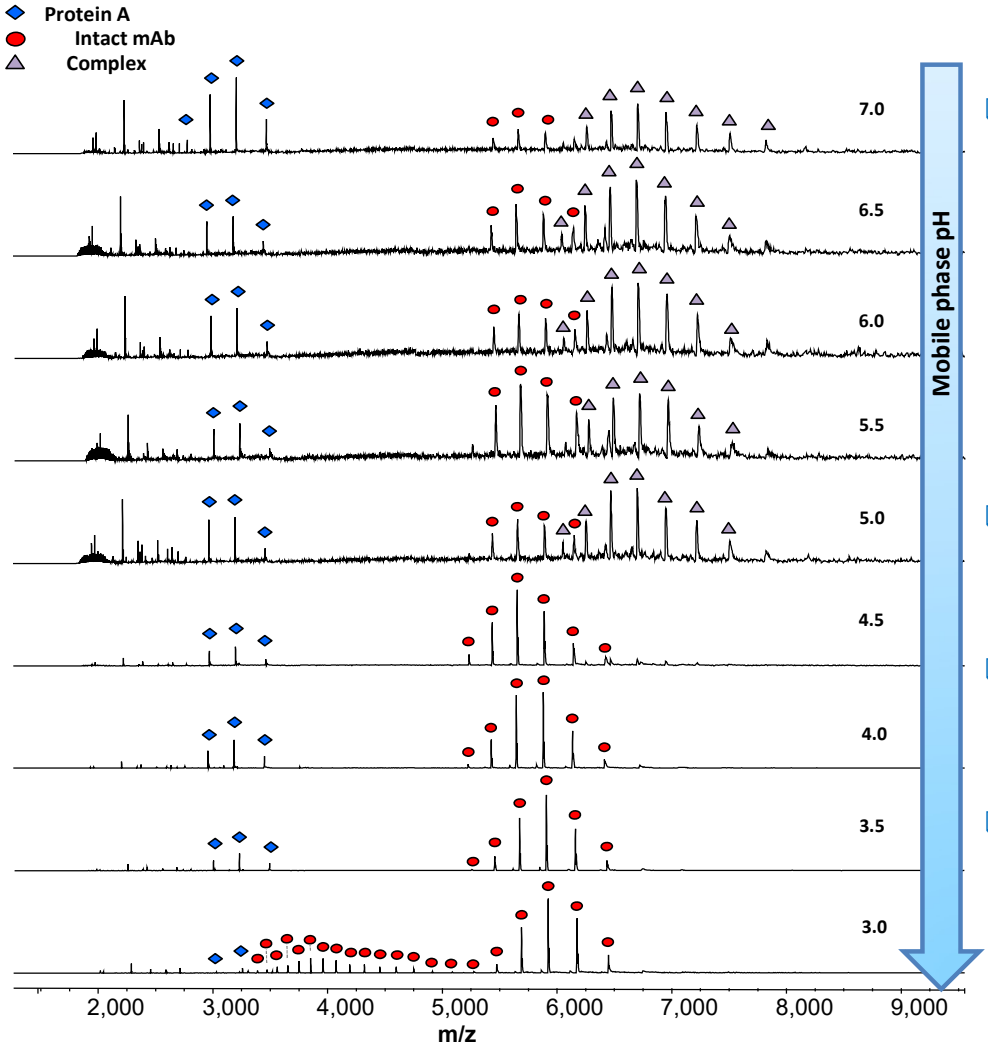
Investigating the pH dependency of the interaction



- Considering that native like MS spectra were observed at low pH, the pH dependence and stoichiometry of the mAb /ProA interaction was investigated using SEC-MS on a Thermo Scientific™ MAbPac™ SEC-1 size exclusion chromatography column
- Stoichiometry appeared consistently at a 1:1 mAb / ProA complex
- Clear pH dependency of the interaction observed until pH lowered to below pH 4.5
- Under classic acidic buffer elution conditions, mAb appears to undergo a reversible confirmational shift

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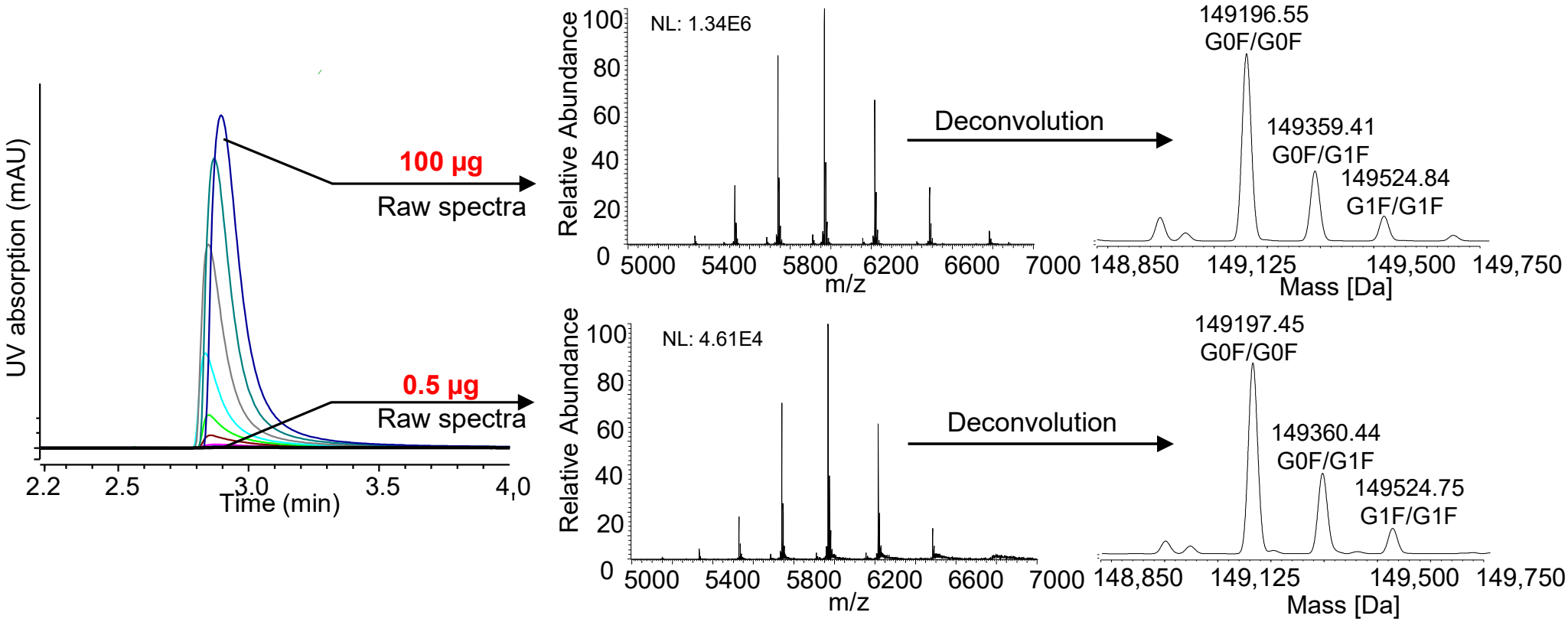
Method performance assessment



Linearity assessment of mAb spiked into cell culture media at levels from 100 µg to 0.5 µg, excellent response and sensitivity and mass accuracy was maintained at < 25 ppm even at the lowest spiking levels evaluated

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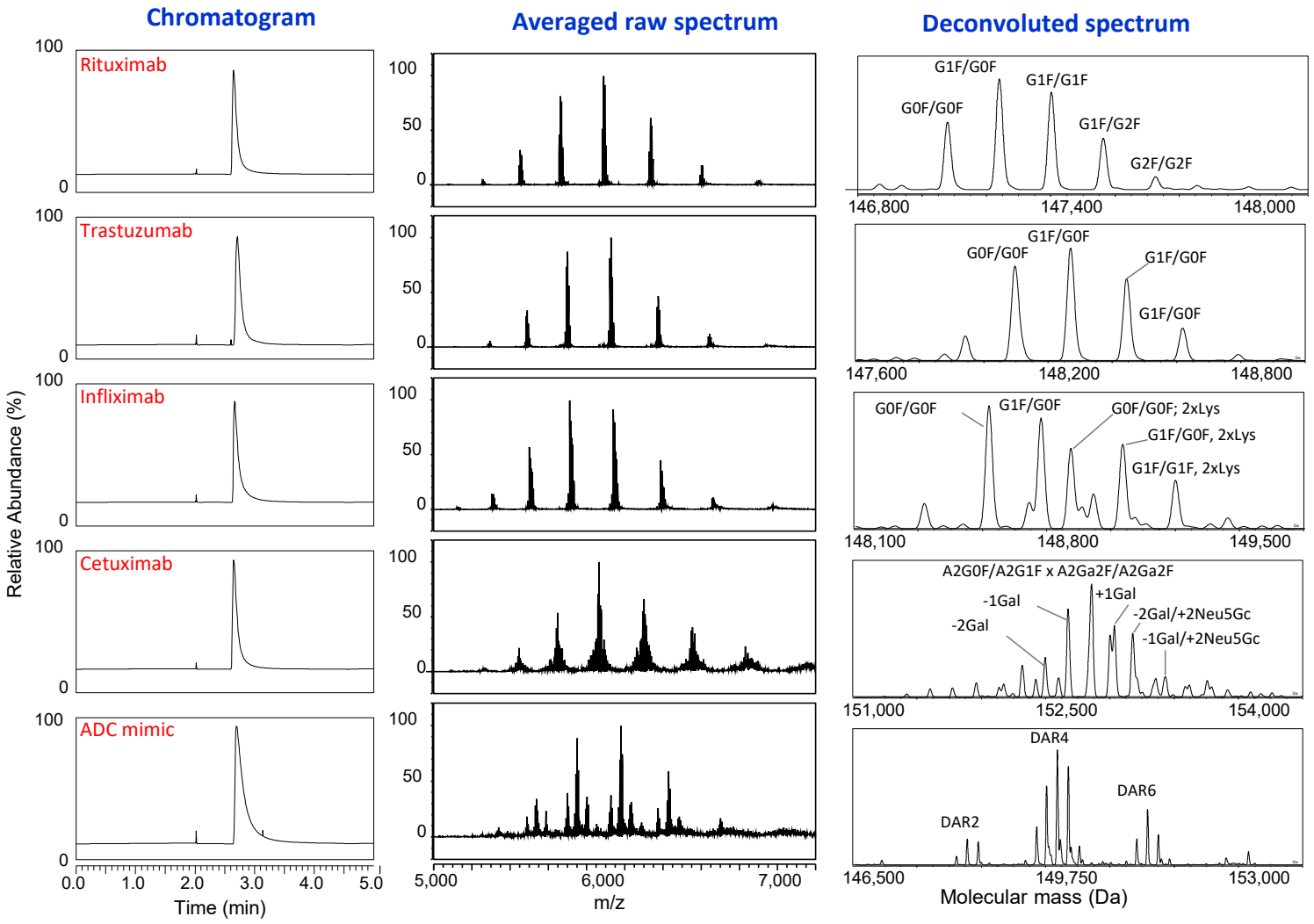
Application to various mAbs and antibody samples



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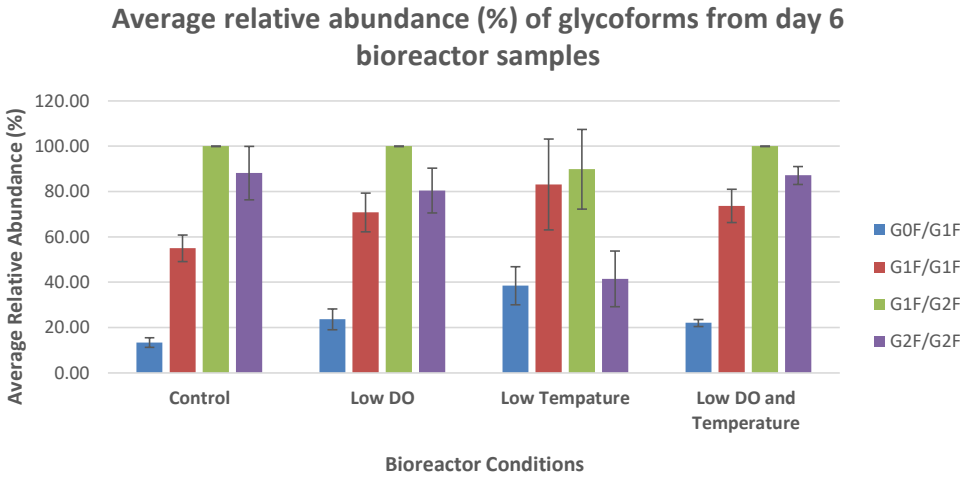
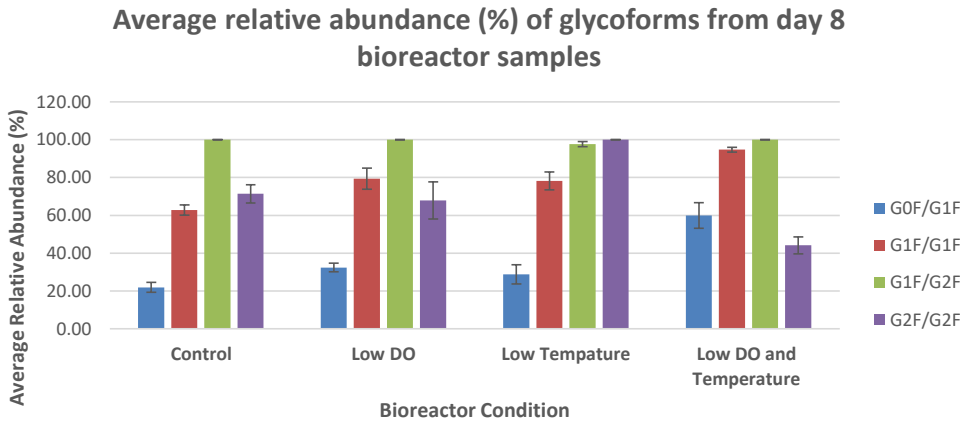
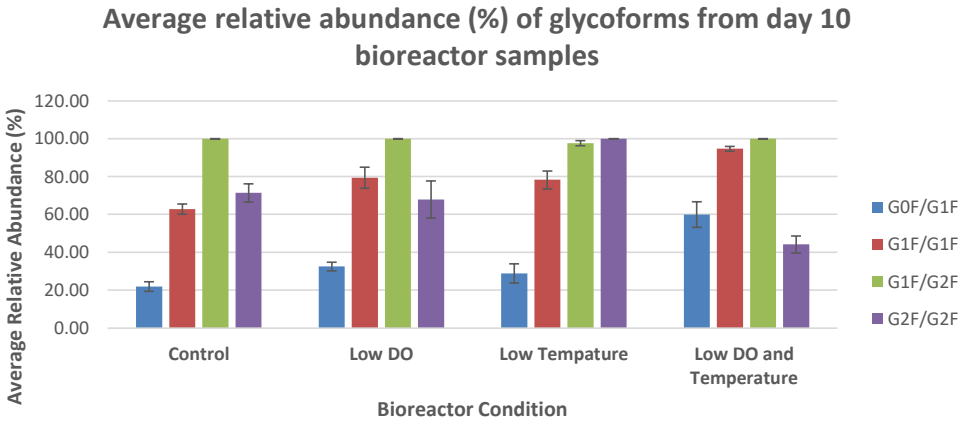
Application for at-line process monitoring







- ProA-MS applied at-line to samples collected from IgG1 producing bioreactors
- Method provided a rapid insight into glycoform abundances and alterations over various days
- Lowering temperature had the biggest impact on glycosylation, move towards more complete galactosylation

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Characterisation Workflows on Vanquish Neo

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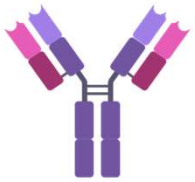
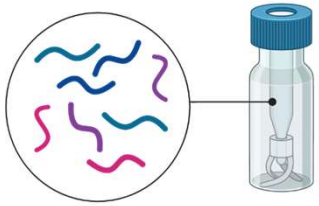
PQA Analysis of NIST mAb using nano LC-MS/MS

Sample Preparation

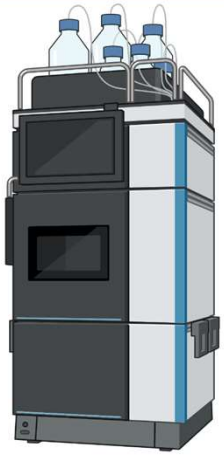


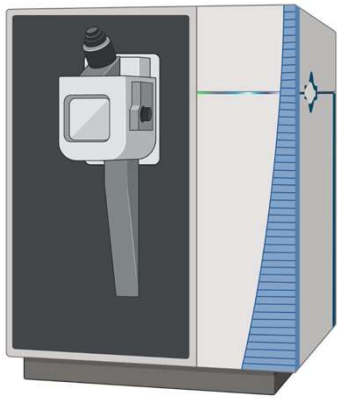
NIST mAb

In solution digest:
- Reduction
- Alkylation
- Clean up
- Trypsin 30min @ 37°C



nano LC-MS/MS





Column	EasySpray 50cm x 75um
Flow rate	250 nl/min, 45C
Gradient	2-35% ACN in 55 min, 40-45% in 5 min

Acquisition	DDA Top 20
MS1	60k Res, m/z 200-2000, RF Lens 40%, AGC 100%, max IT 25 ms
MS2	Isolation window 1.2 m/z, NCE 28, 15k Res, AGC 50%, max IT 50 ms
	10s dyn excl, 10ppm tol, charge state 2-7, intensity threshold 5.0e3

Challenge: Detection of low abundant quality attributes!

Adapted from Jakes et al, JASMS, 2021 - https://pubs.acs.org/doi/pdf/10.1021/jasms.0c00432
Millán-Martín et al, Anal & Bioanal Chem, 2020 - https://link.springer.com/article/10.1007/s00216-020-02809-z

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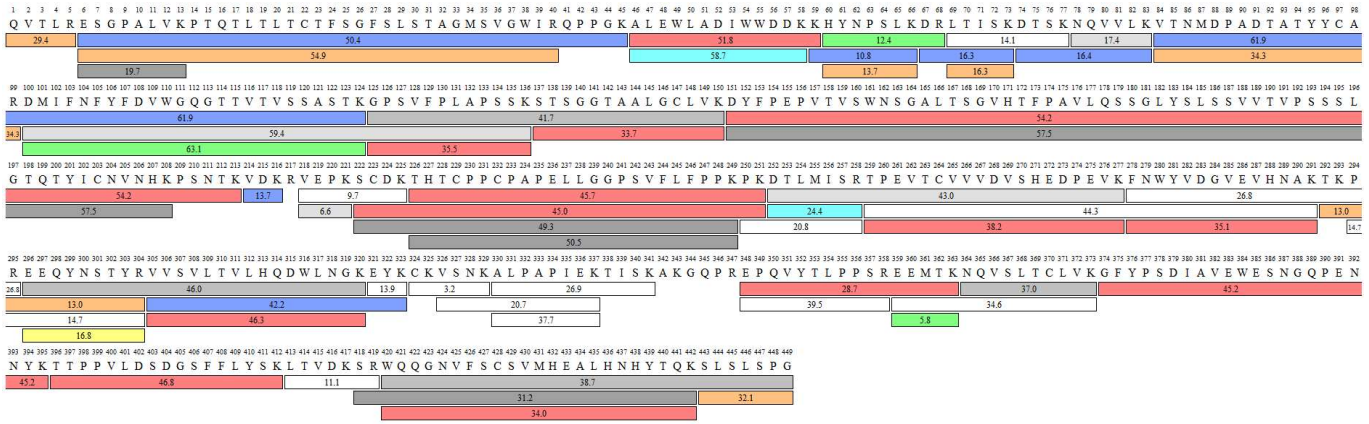
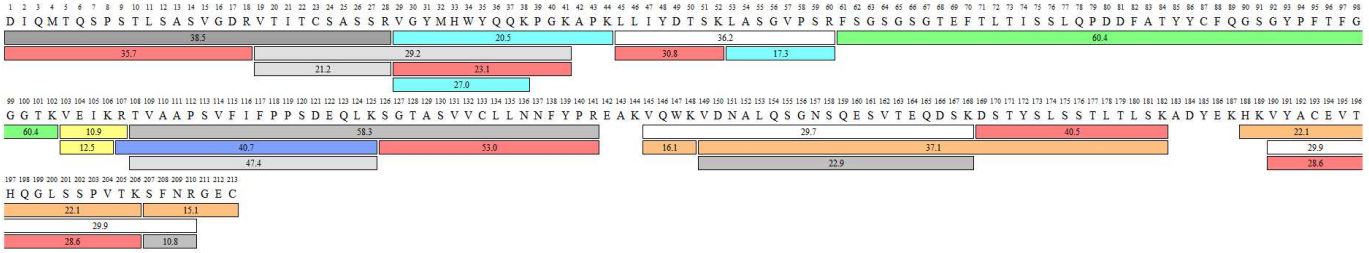
Sequence Coverage

NIST mAb injection	Sequence Coverage [%]		Number of MS peaks	
	Light Chain	Heavy Chain	Light Chain	Heavy Chain
1000 ng	96.24	98.44	229	813
750 ng	100.00	98.44	210	805
500 ng	100.00	98.44	200	776
250 ng	100.00	98.00	198	714
100 ng	100.00	97.10	167	584
50 ng	100.00	97.10	132	489
10 ng	98.59	96.21	73	274

NIST mAb Heavy Chain



NIST mAb Light Chain



Sequence coverage map, NIST mAb dilution series: 10 ng injections in triplicate, Easyspray 50 cm column (without trap).

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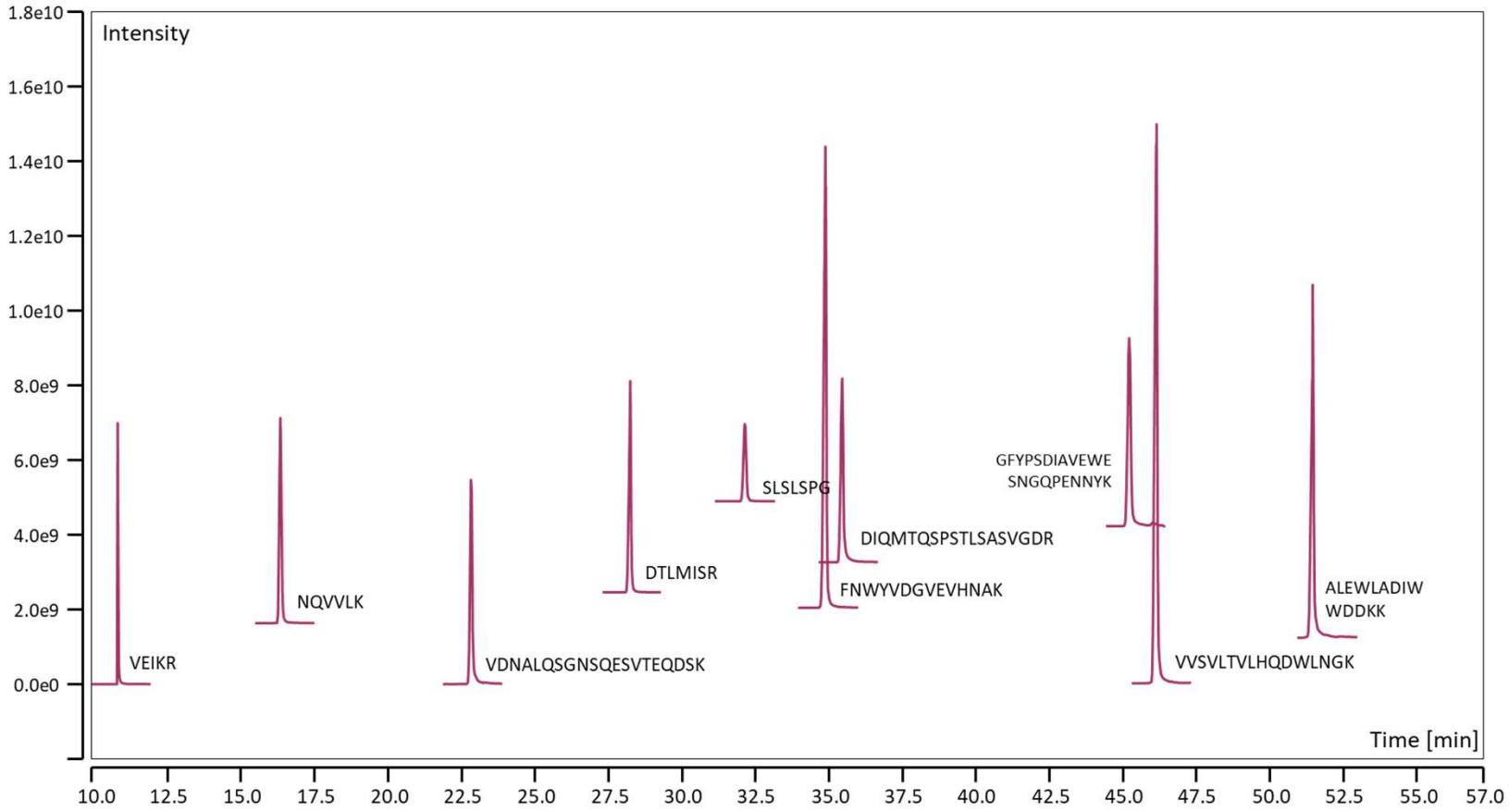
10 peptides selected for evaluation of NIST mAb



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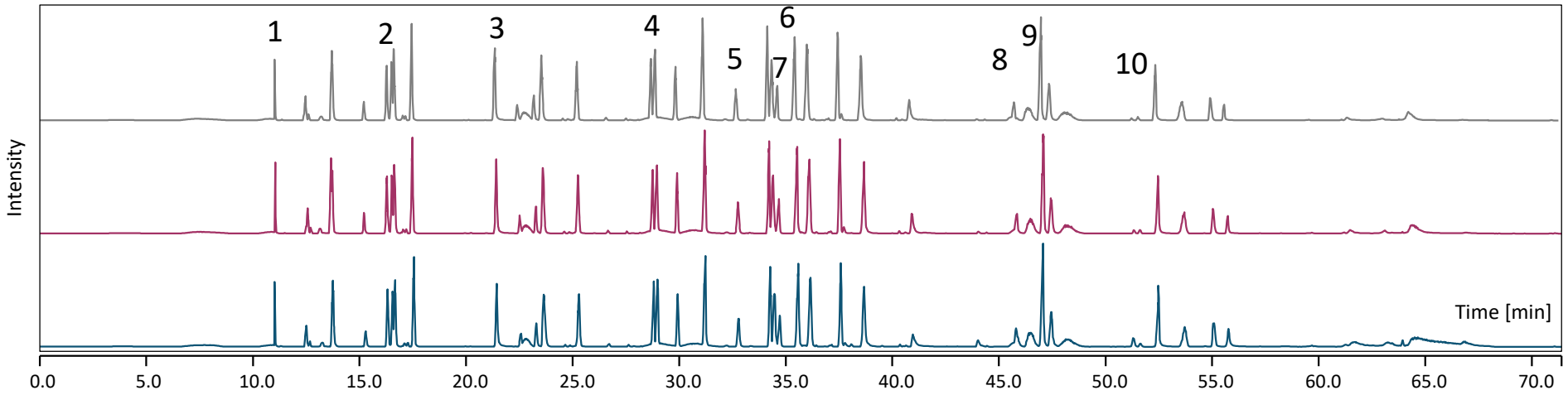
Reproducibility



	Peptide Sequence	Peak Area (Avg.)	%CV (Area) (<=10%)	Avg. RT (min)	%CV (RT) (<=2%)	Avg. FWHM (s)	%CV (FWHM) (<=10%)	Min. Mass Error (ppm)	Max. Mass Error (ppm)
1	VEIKR	1.15E+10	0.41%	10.81	0.13%	1.5	2.84%	-1.42	0
2	NQVVLK	2.86E+10	1.55%	16.32	0.12%	4.92	3.27%	-0.57	0.04
3	VDNALQSGNSQESVTEQDSK	3.26E+10	0.63%	22.81	0.11%	5.18	4.72%	-1.14	-0.34
4	DTLMISR	2.88E+10	0.68%	28.21	0.11%	4.95	4.88%	-0.19	0.54
5	SLSLSPG	1.43E+10	0.70%	32.12	0.09%	6.16	3.77%	0	0
6	DIQMTQSPSTLSASVGDR	3.34E+10	0.25%	35.43	0.08%	5.38	5.42%	0.11	1.01
7	FNWYVDGVEVHNAK	6.77E+09	1.30%	34.86	0.12%	5.89	3.34%	0.4	1.13
8	GFYPSDIAVEWESNGQPENNYK	3.14E+10	4.07%	44.93	0.07%	6.94	2.87%	-0.73	0.04
9	VVSVLTVLHQDWLNGK	8.82E+10	6.31%	46.16	0.05%	5.78	2.66%	-0.22	0.59
10	ALEWLADIWWDDKK	5.14E+10	8.08%	51.47	0.08%	5.03	2.22%	-1.36	0.07

* 100 ng injections in triplicate, Easyspray 50 cm column (without trap).

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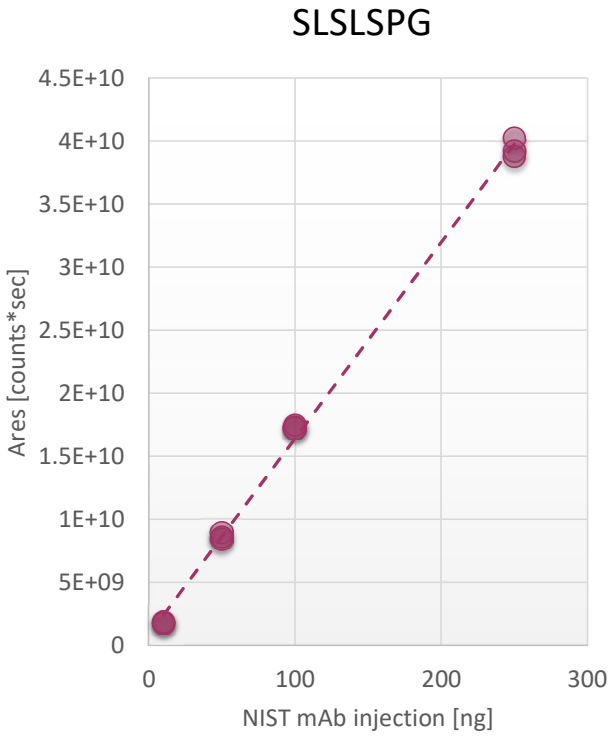
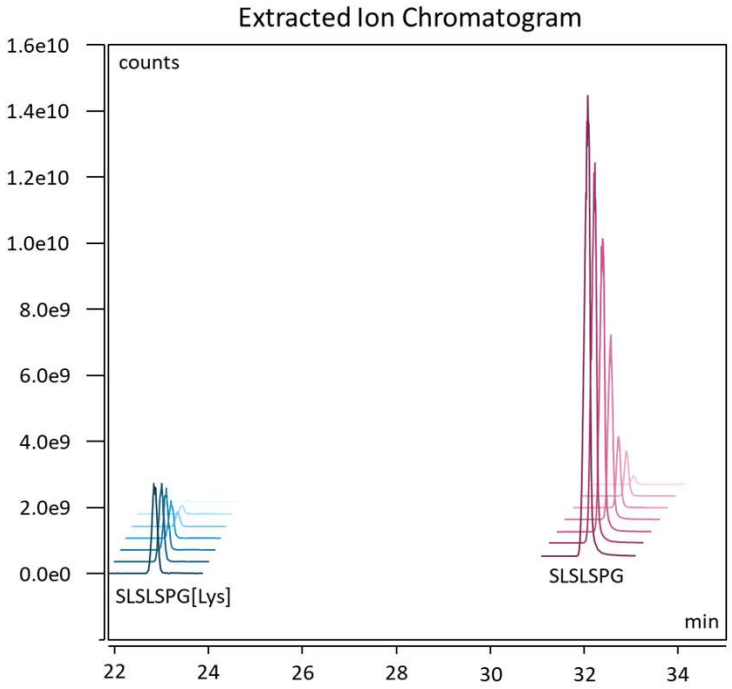
Robustness and Sensitivity



σ	3.13E+08	
Slope	1.56E+08	
R^2	0.9979	
LOD	6.63	ng
LOQ	20.10	ng



NIST mAb dilution series: 1000-10 ng injections in triplicate, Easyspray 50 cm column (without trap).
Calibration curve range 250 – 10 ng (due to poor peak shape at high injection amounts).

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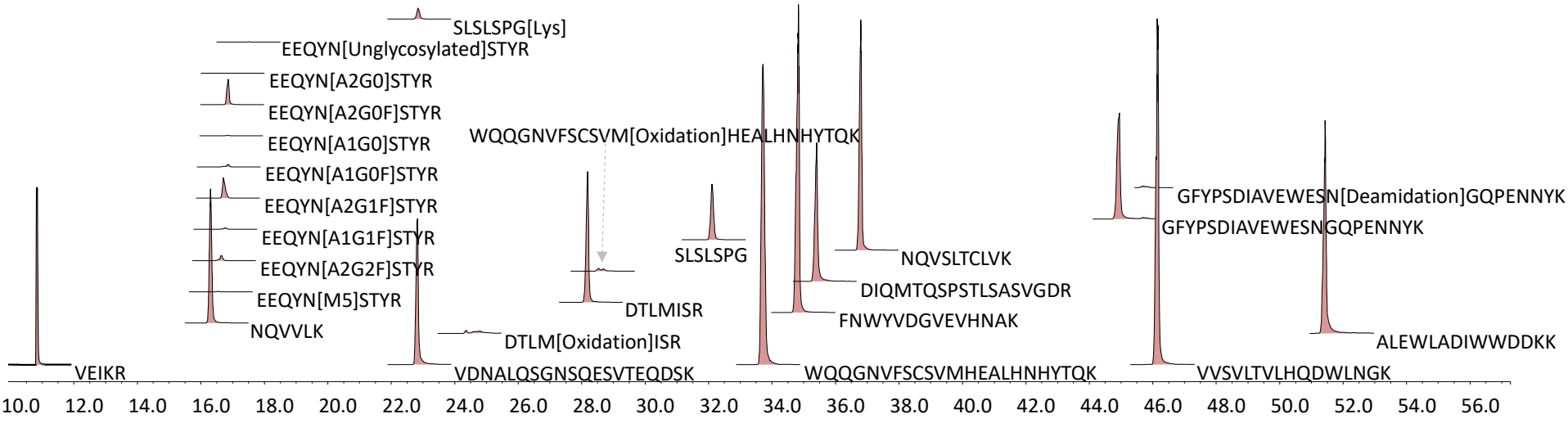
PQA Analysis



Peptide	Description	%Mod. (Avg.)	%CV (Mod.) (<=15%)	Pass or Fail
GFYPSDIAVEWESNGQPENNYK	Deamidation	1.88	12.39%	Pass
DTLMISR	Oxidation	1.89	1.61%	Pass
WQQGNVFSCSVMHEALHNHYTQK	Oxidation	1.36	0.92%	Pass
SLSLSPG	C-term Lys	16.15	0.65%	Pass
EEQYN[A2G1F]STYR	N-Glycan	40.56	0.50%	Pass
EEQYN[A2G0F]STYR	N-Glycan	38.75	0.86%	Pass
EEQYN[A2G2F]STYR	N-Glycan	9.56	1.89%	Pass
EEQYN[A1G0F]STYR	N-Glycan	5.23	2.41%	Pass

NIST mAb 100ng replicates, n=6

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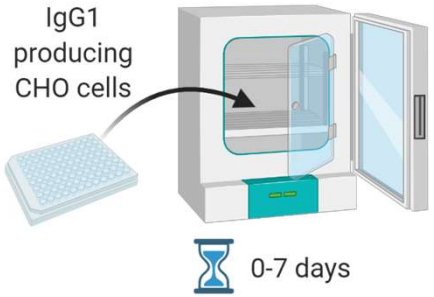
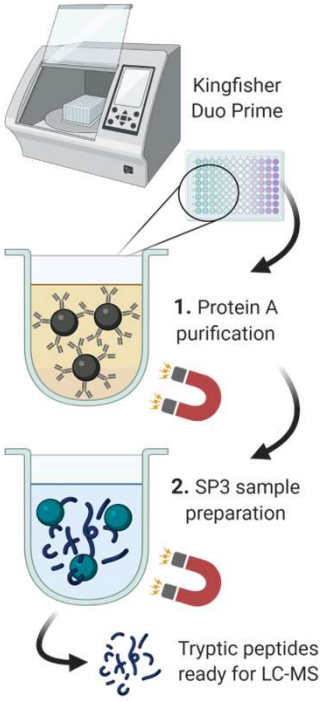
Nano-LC based MAM workflow for cell culture screening

Cell Culture*

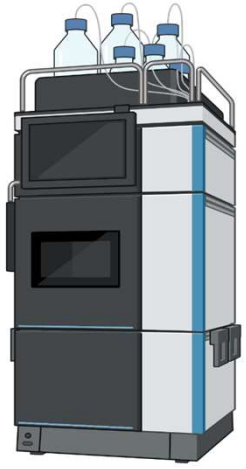


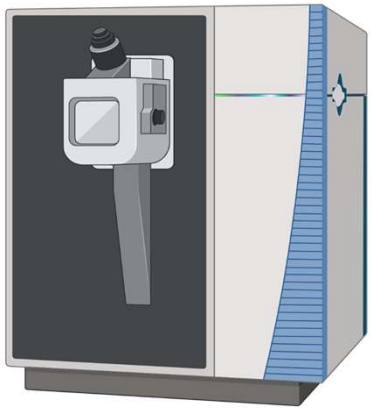
- Time course (day 3 vs. 5)
- Productivity (high vs. low)

Sample Preparation*



nano LC-MS/MS





Column	EasySpray 50cm x 75um; 500 ng injection
Flow rate	250 nl/min, 45C
Gradient	2-35% ACN in 55 min, 40-45% in 5 min

Acquisition	DDA Top 20
MS1	60k Res, m/z 200-2000, RF Lens 40%, AGC 100%, max IT 25 ms
MS2	Isolation window 1.2 m/z, NCE 28, 15k Res, AGC 50%, max IT 50 ms
	10s dyn excl, 10ppm tol, charge state 2-7, intensity threshold 5.0e3

Challenge: Limited sample amount!

* Proof of concept experiment – IgG1 was spiked in at concentrations based on previously published cellular productivity. SP3 digestion was performed in triplicate.

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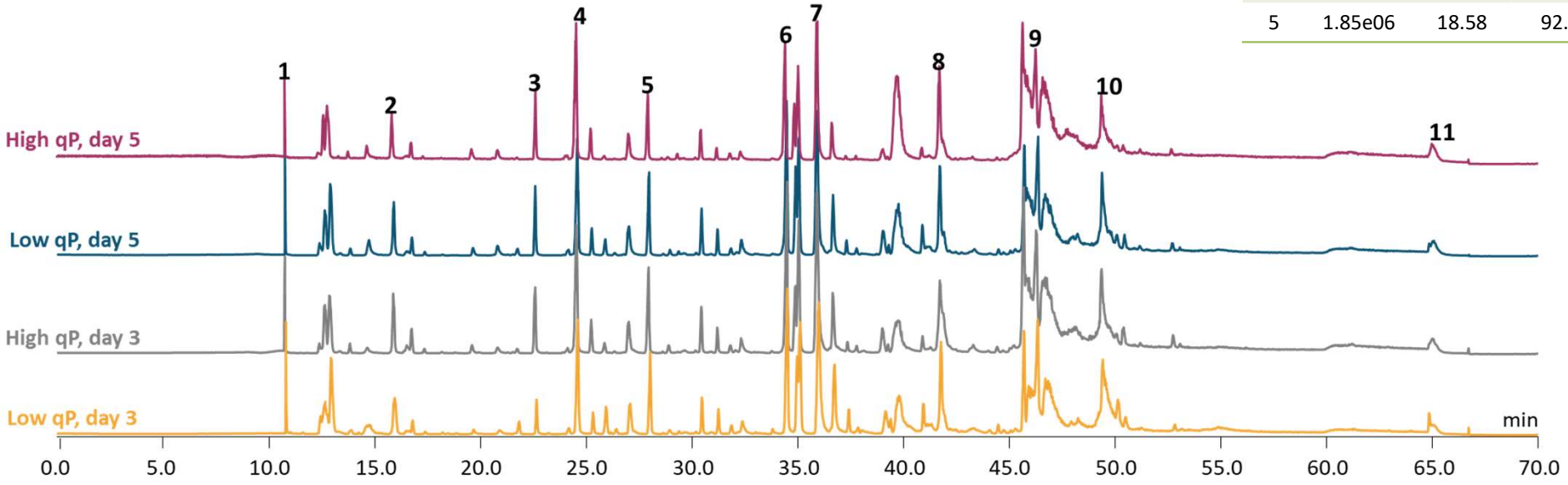
RT precision across 12 injections

Day	Cells/mL	qP low (10pg)	qP high (50pg)
3	1.02e06	6.13	30.65
5	1.85e06	18.58	92.91



Retention time (min)

Peptide	ADYEK	VQWK	SLSLSPG[Lys]	ALPAPIEK	DTLMISR	FNWYVDGVE VHNAK	VDNALQSGNSQESVTEQ DSKDSTYSLSSTLTLSK	VVSVLTVLHQD WLNGKEYK	GLEWIGAIYPGN GDTSYNQK	Q[NH3 loss]IVLSQ SPAILSASPGEK	STYYGGDWYFNVW GAGTTVTVSAASTK
Average RT	10.78	15.9	22.66	24.58	27.99	34.51	36.69	41.75	46.32	49.38	65.27
%CV (RT)	0.25%	0.33%	0.13%	0.13%	0.13%	0.12%	0.11%	0.08%	0.08%	0.04%	0.05%
Pass or Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass

3 biological replicates per condition, 500 ng injections

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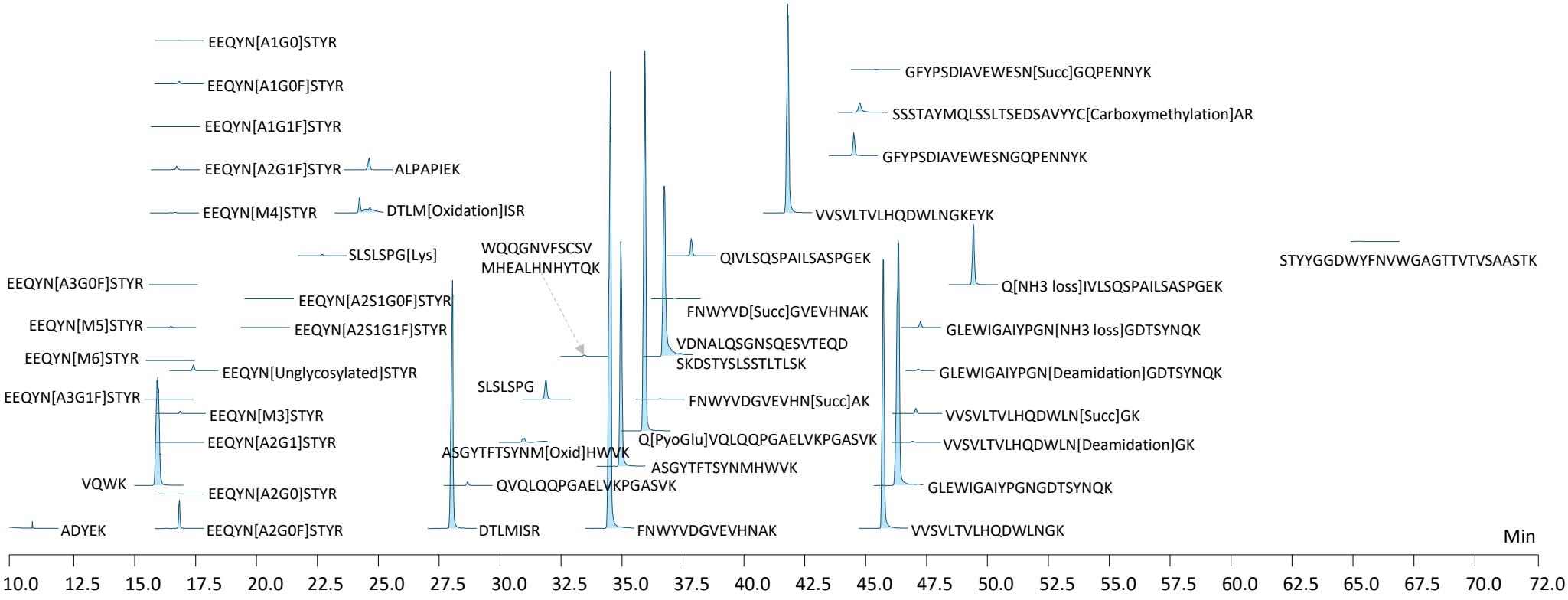
PQA Analysis

138 total components (considering all charge states), 29 attributes monitored



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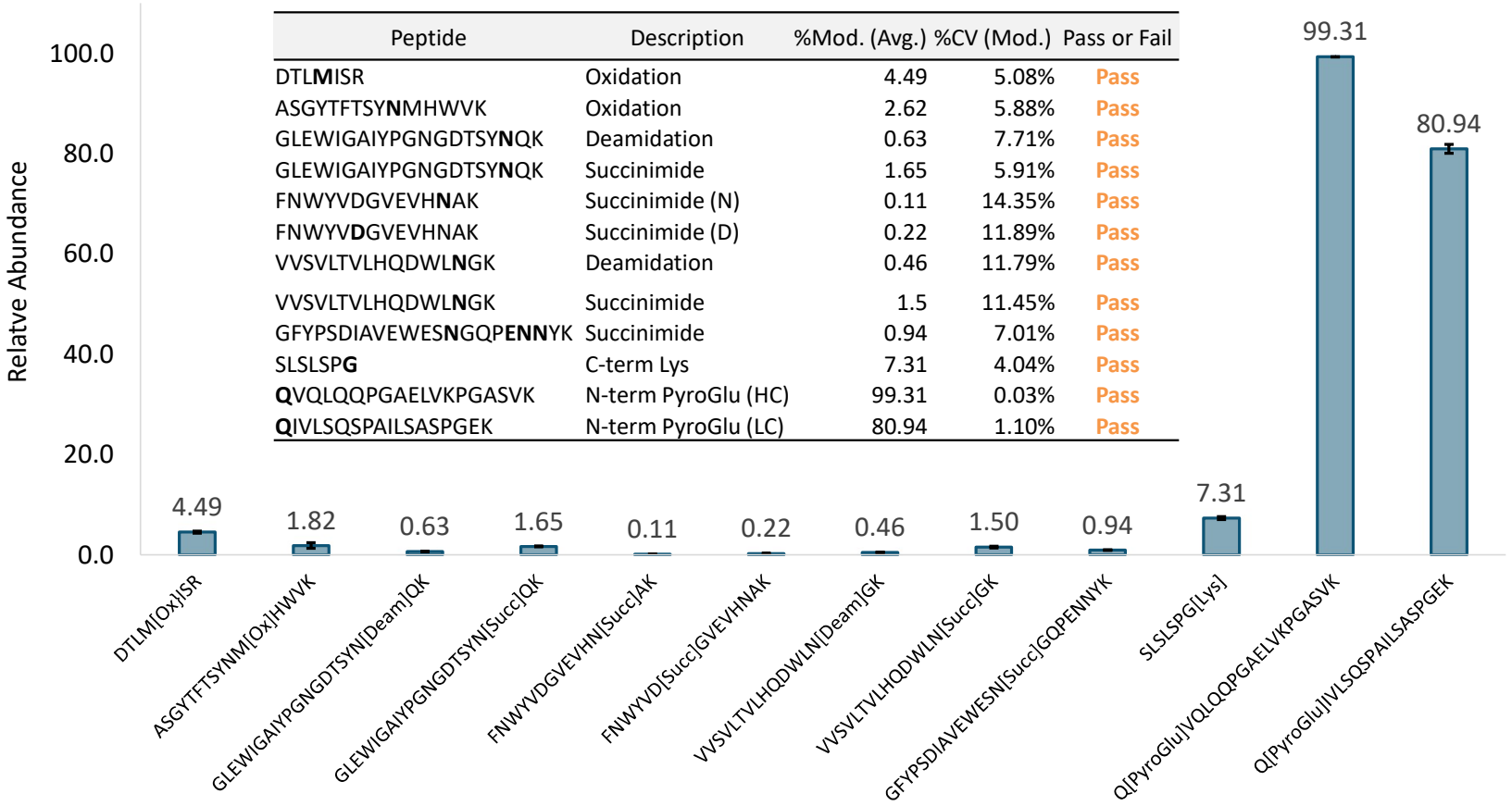
PQA Analysis

IgG1 Quality Attributes (n=12)

Peptide	Description	%Mod. (Avg.)	%CV (Mod.)	Pass or Fail
DTL**M**ISR	Oxidation	4.49	5.08%	Pass
ASGYTFTSY**N**MHWVK	Oxidation	2.62	5.88%	Pass
GLEWIGAIYPGNGDTSY**N**QK	Deamidation	0.63	7.71%	Pass
GLEWIGAIYPGNGDTSY**N**QK	Succinimide	1.65	5.91%	Pass
FNWYVDGVEVH**N**AK	Succinimide (N)	0.11	14.35%	Pass
FNWYV**D**GVEVHNAK	Succinimide (D)	0.22	11.89%	Pass
VVSVLTVLHQDWL**N**GK	Deamidation	0.46	11.79%	Pass
VVSVLTVLHQDWL**N**GK	Succinimide	1.5	11.45%	Pass
GFYPSDIAVEWES**N**GQP**ENN**YK	Succinimide	0.94	7.01%	Pass
SLSLSP**G**	C-term Lys	7.31	4.04%	Pass
QVQLQQPGAELVKPGASVK	N-term PyroGlu (HC)	99.31	0.03%	Pass
QIVLSQSPAILSASPGEK	N-term PyroGlu (LC)	80.94	1.10%	Pass



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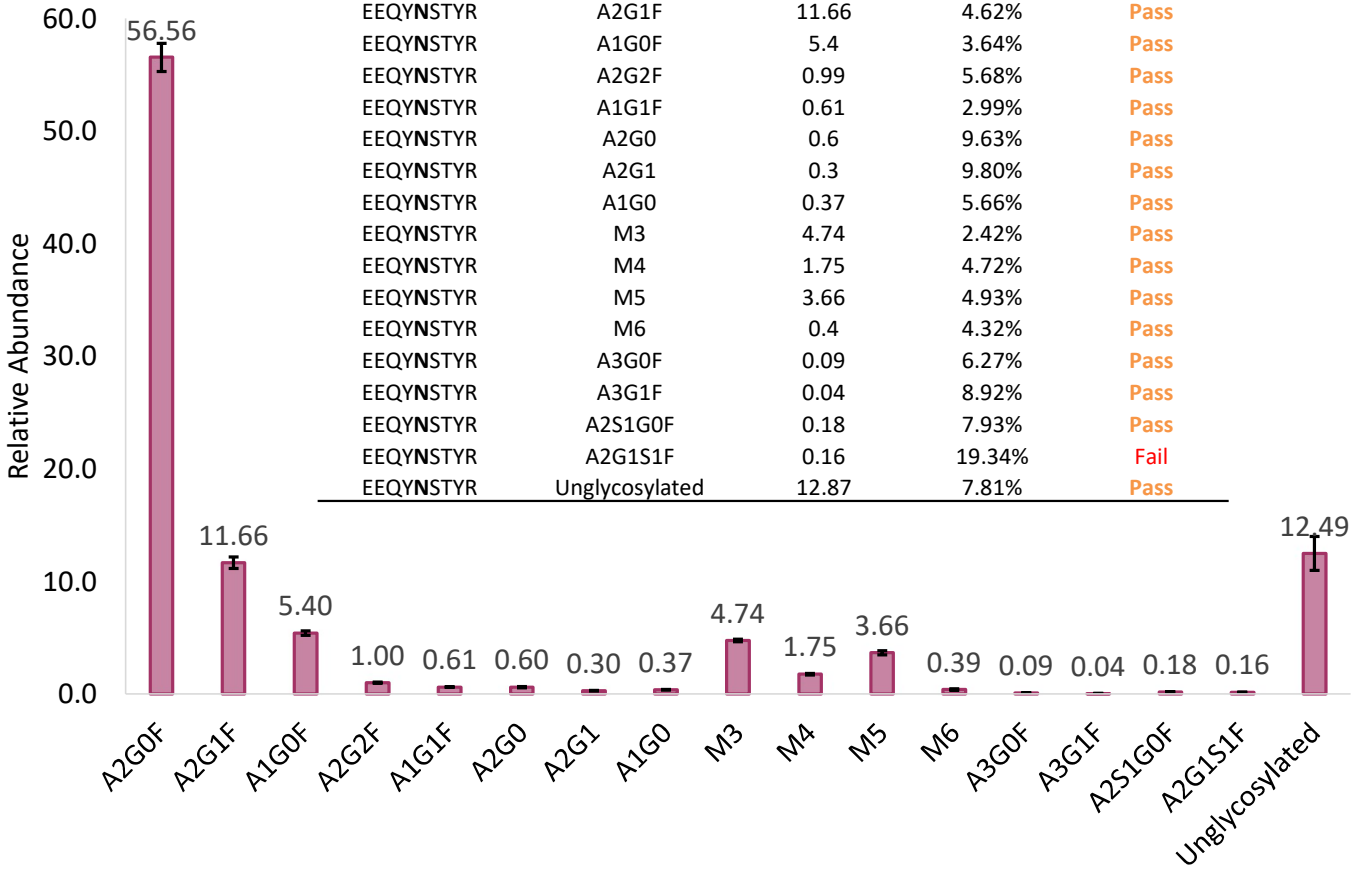
PQA Analysis

N-Glycosylation (n=12)

Peptide	Description	%Mod. (Avg.)	%CV (Mod.)	Pass or Fail
EEQYNSTYR	A2G0F	56.56	2.31%	Pass
EEQYNSTYR	A2G1F	11.66	4.62%	Pass
EEQYNSTYR	A1G0F	5.4	3.64%	Pass
EEQYNSTYR	A2G2F	0.99	5.68%	Pass
EEQYNSTYR	A1G1F	0.61	2.99%	Pass
EEQYNSTYR	A2G0	0.6	9.63%	Pass
EEQYNSTYR	A2G1	0.3	9.80%	Pass
EEQYNSTYR	A1G0	0.37	5.66%	Pass
EEQYNSTYR	M3	4.74	2.42%	Pass
EEQYNSTYR	M4	1.75	4.72%	Pass
EEQYNSTYR	M5	3.66	4.93%	Pass
EEQYNSTYR	M6	0.4	4.32%	Pass
EEQYNSTYR	A3G0F	0.09	6.27%	Pass
EEQYNSTYR	A3G1F	0.04	8.92%	Pass
EEQYNSTYR	A2S1G0F	0.18	7.93%	Pass
EEQYNSTYR	A2G1S1F	0.16	19.34%	Fail
EEQYNSTYR	Unglycosylated	12.87	7.81%	Pass



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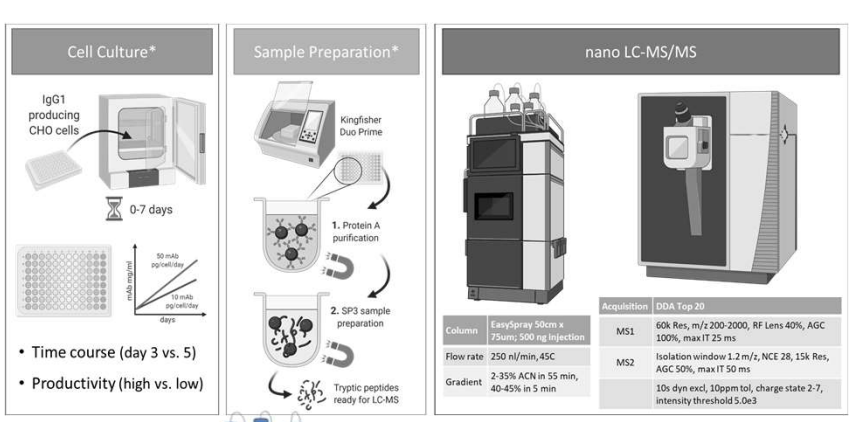
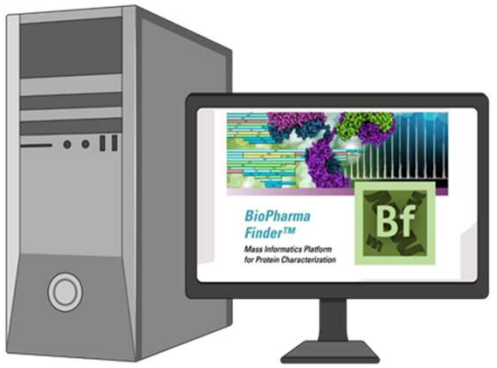
Nano-LC workflow for HCP detection



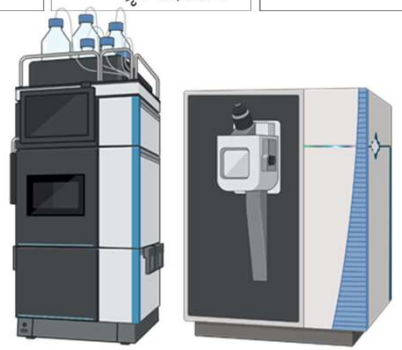


m/z	Retention Time	Charge
313.1450	9.80	3
1559.4260	12.93	2
839.4050	34.52	2
497.0170	35.93	4
603.3320	49.42	3

Generate exclusion list

Full Scan
MIPS
Intensity
Charge state
Dyn Excl
Targeted Mass Exclusion
ddMS²

20 Scans



Re-inject samples using modified method - 5 µg injection

Challenge: Dynamic range!
Low concentration of HCPs vs. high abundant mAb derived peptides.

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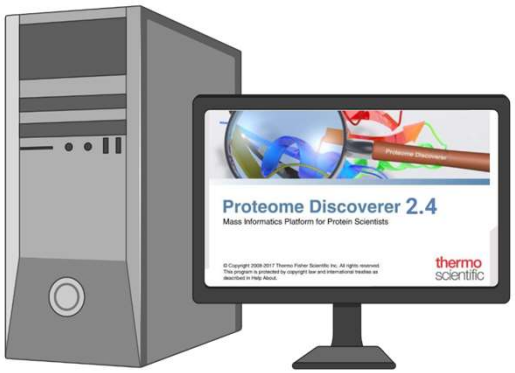
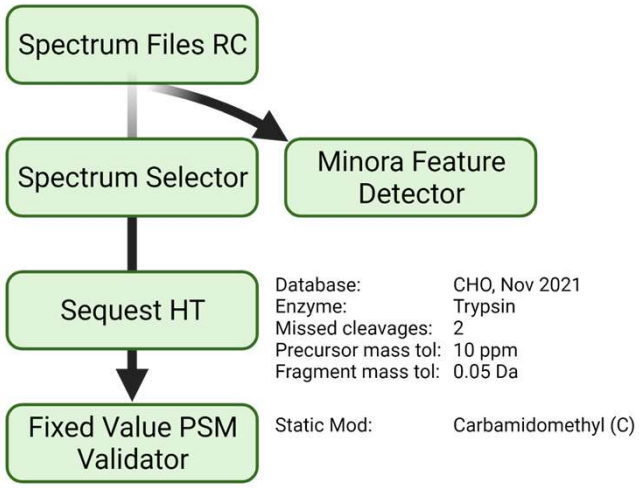
Host Cell Protein (HCP) detection in IgG1 samples





- ✓ **141 detected HCPs (min. of 2 unique peptides)**
- ✓ **65 HCPs quantified (LFQ, no missing values)**

Accession	Description	# Unique Peptides
G3H0L9	Cathepsin B	12
G3HNJ3	Clusterin	22
G3HMD1	Glyceraldehyde-3-phosphate dehydrogenase	6
G3IDL7	Heat shock cognate 71 kDa protein	4
G3H354	Heat shock protein HSP 90-alpha	10
G3HC84	Heat shock protein HSP 90-beta	9
G3H2T4	Histone H2B	4
G3GYP9	Peroxiredoxin-1	7
G3HC31	Protein S100	2

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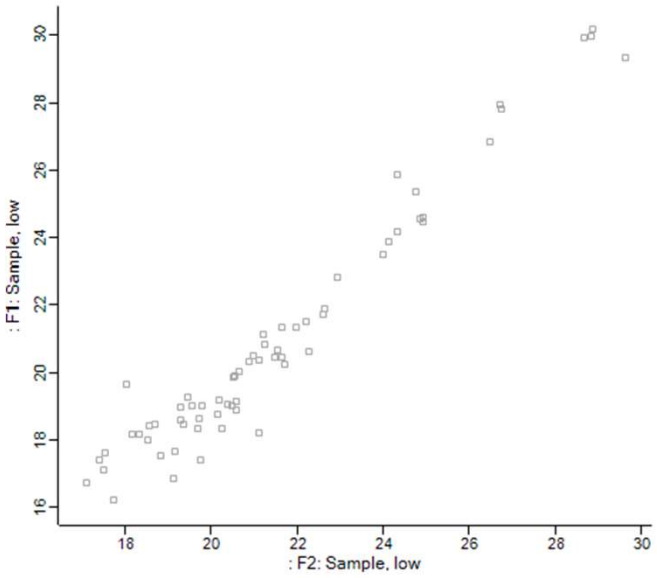
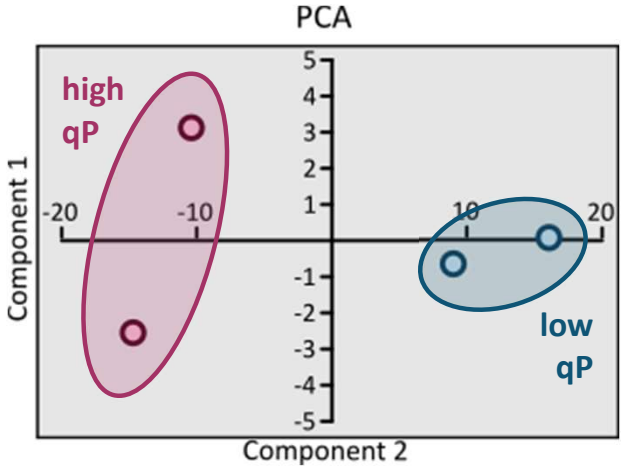
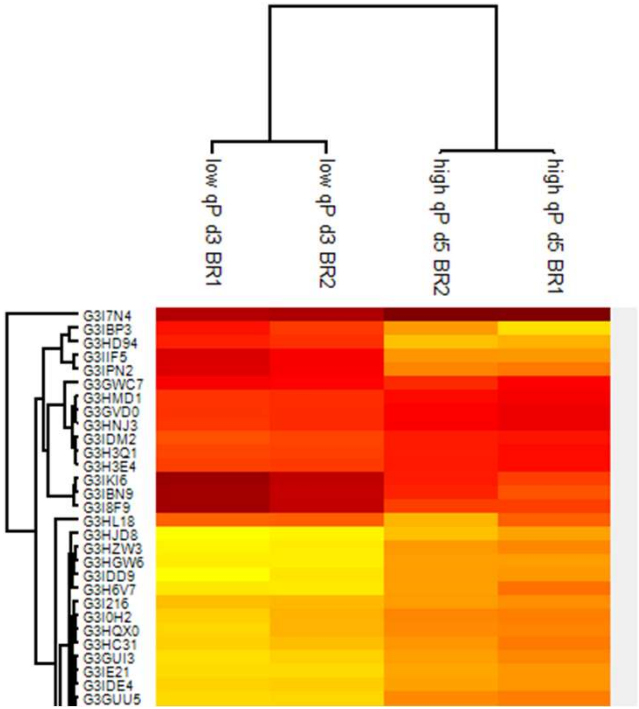
Host Cell Protein (HCP) detection in IgG1 samples

Pearson correlation = 0.973







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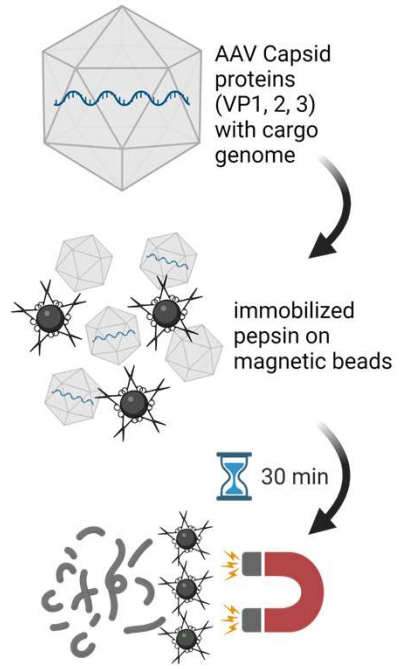
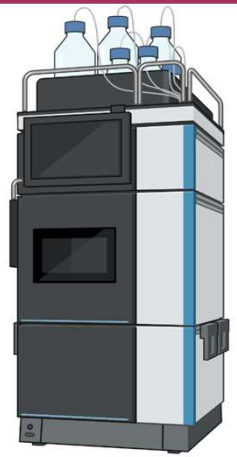
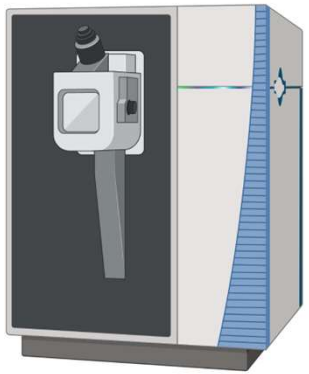
AAV Peptide Mapping Workflow

Sample Preparation



LC-MS/MS Method





Column	EasySpray Neo 50cm x 75um + Neo C18 trap
Flow rate	250 nl/min, 45C
Gradient	2-60% ACN in 20 min vs. 2-60% ACN in 60 min

Acquisition	DDA Top 15
MS1	60k Res, m/z 200-2000, RF Lens 40%, AGC 100%, max IT 25 ms
MS2	Isolation window 1.2 m/z, NCE 28, 15k Res, AGC 50%, max IT 50 ms
	15s dyn excl, 10ppm tol, charge state 2-8, intensity threshold 2.0e3

Challenge: low concentration and price of AAV samples!

Adapted from Guapo et al, J. Pharm. Biomed, 2021 - https://www.sciencedirect.com/science/article/pii/S0731708521005380

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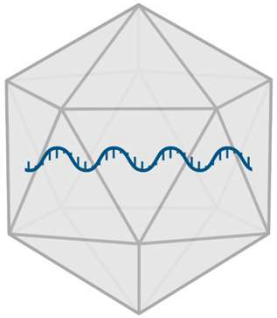
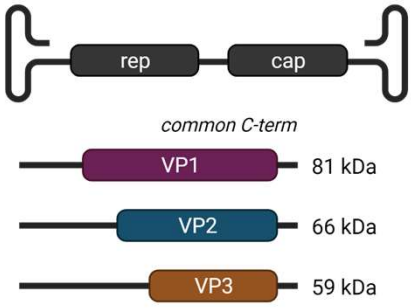
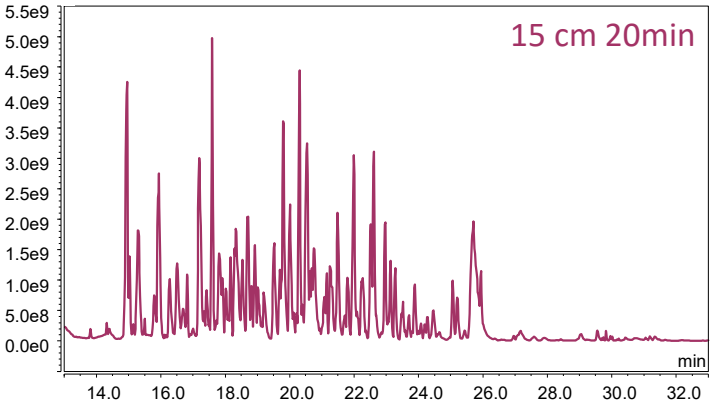
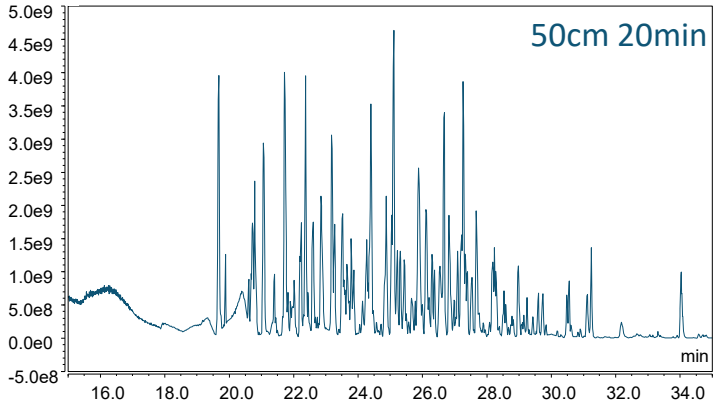
AAV Peptide Mapping Workflow

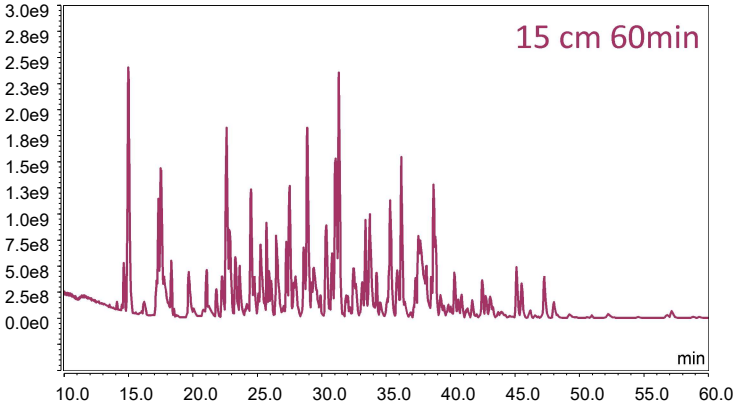


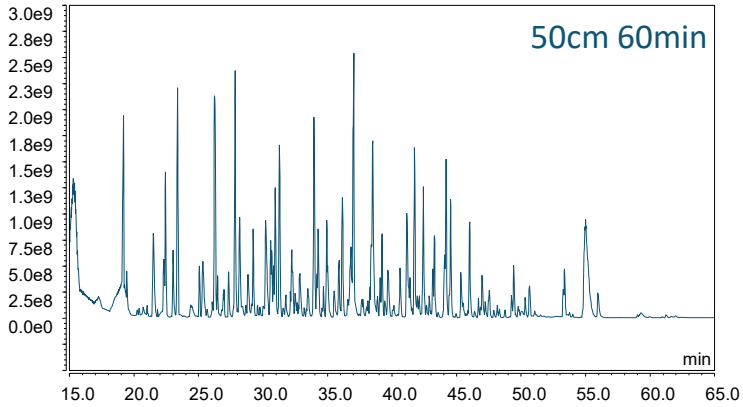
VP1:VP2:VP3
1:1:10
60 copies











200ng injections, 3 technical replicates – 50 cm vs. 15 cm EasySpray Neo Column

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Sequence Coverage of VPs

Method	VP1	VP2	VP3
50cm 20min	100	100	100
50cm 60min	99.4	100	100
15cm 20min	100	100	100
15cm 60min	98.3	98	100

VP1 – Sequence Coverage Map

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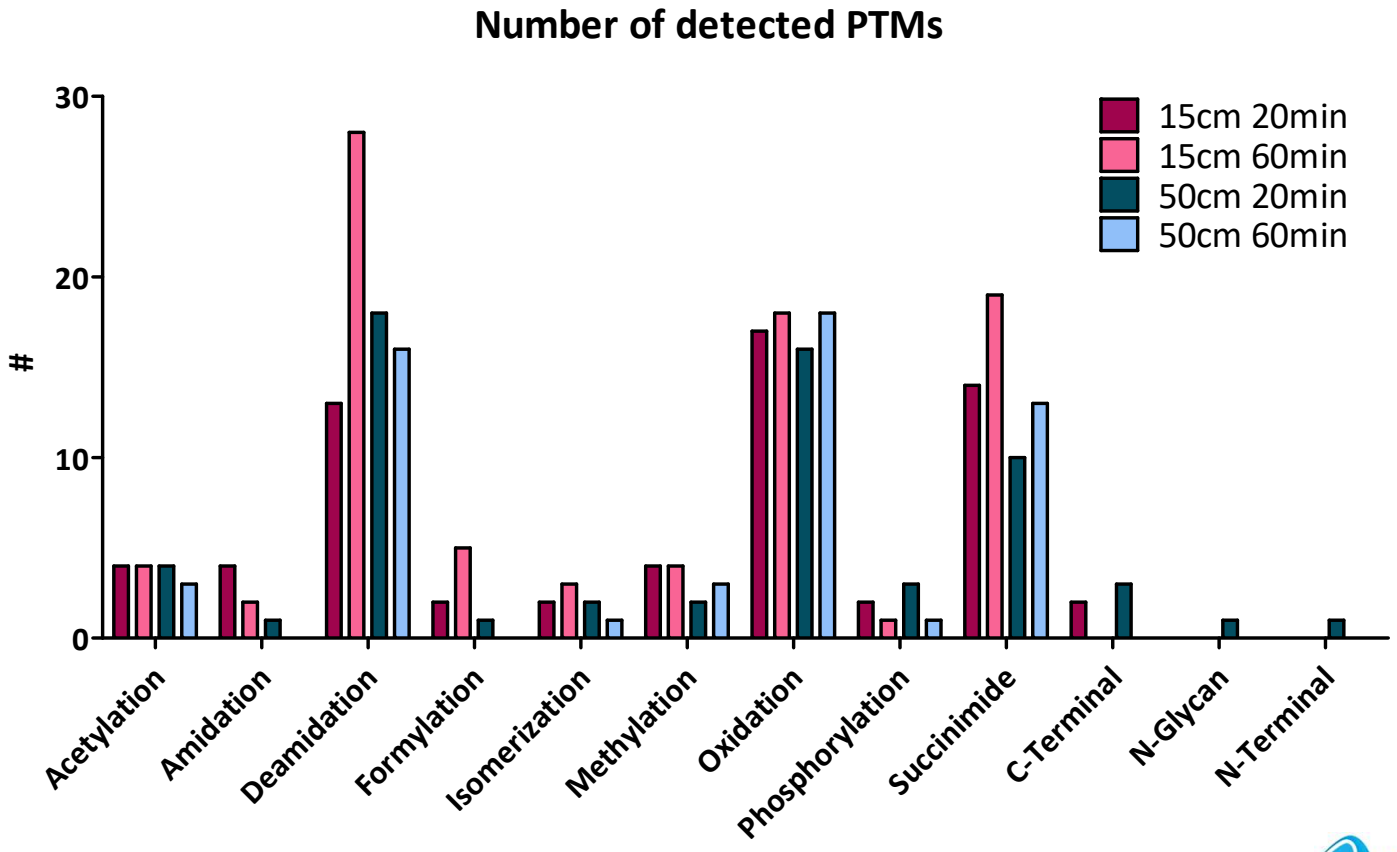
Monitoring potential quality attributes

Method	PTMs
15cm 20min	57
15cm 60min	55
50cm 20min	57
50cm 60min	55



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Summary / Take home messages

System preparation:

Vanquish Neo significantly **reduces required hands-on time**

User friendly interface and built in leak tests facilitate **easy change of trap-elute/direct injection workflows**

Performance:

Vanquish Neo hyphenated to Exploris 480 enables **highest reproducibility and robust performance**

High sensitivity at low flow rates facilitates of various biotherapeutics even at low sample concentrations in-depth analysis

Ease of obtaining information rich, **high-confidence** results

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