



ThermoFisher
S C I E N T I F I C

Chromatographic Workflows for Biopharmaceutical Characterization

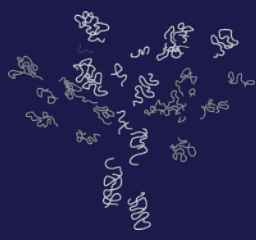
Dr. Ken Cook
European Bioseparation Sales Support Expert
Thermo Fisher Scientific, Hemel Hempstead/Germany

Humanized IgG antibody fragment (Fab) | 50,000 Daltons | VH, CH1
and VL, CL regions, linked by an intramolecular disulfide bond.

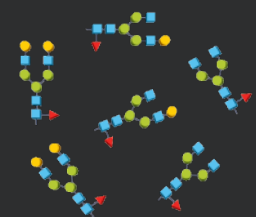
STRUCTURAL INSIGHTS

Fulfilling the Needs of Biotherapeutic Characterization

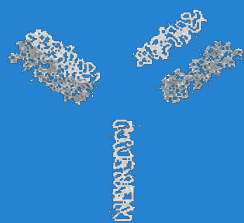
Peptide mapping



Released glycan analysis



Sub-unit analysis



Intact analysis



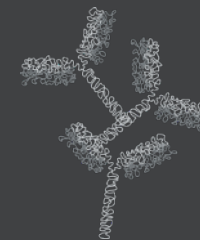
Glycan analysis



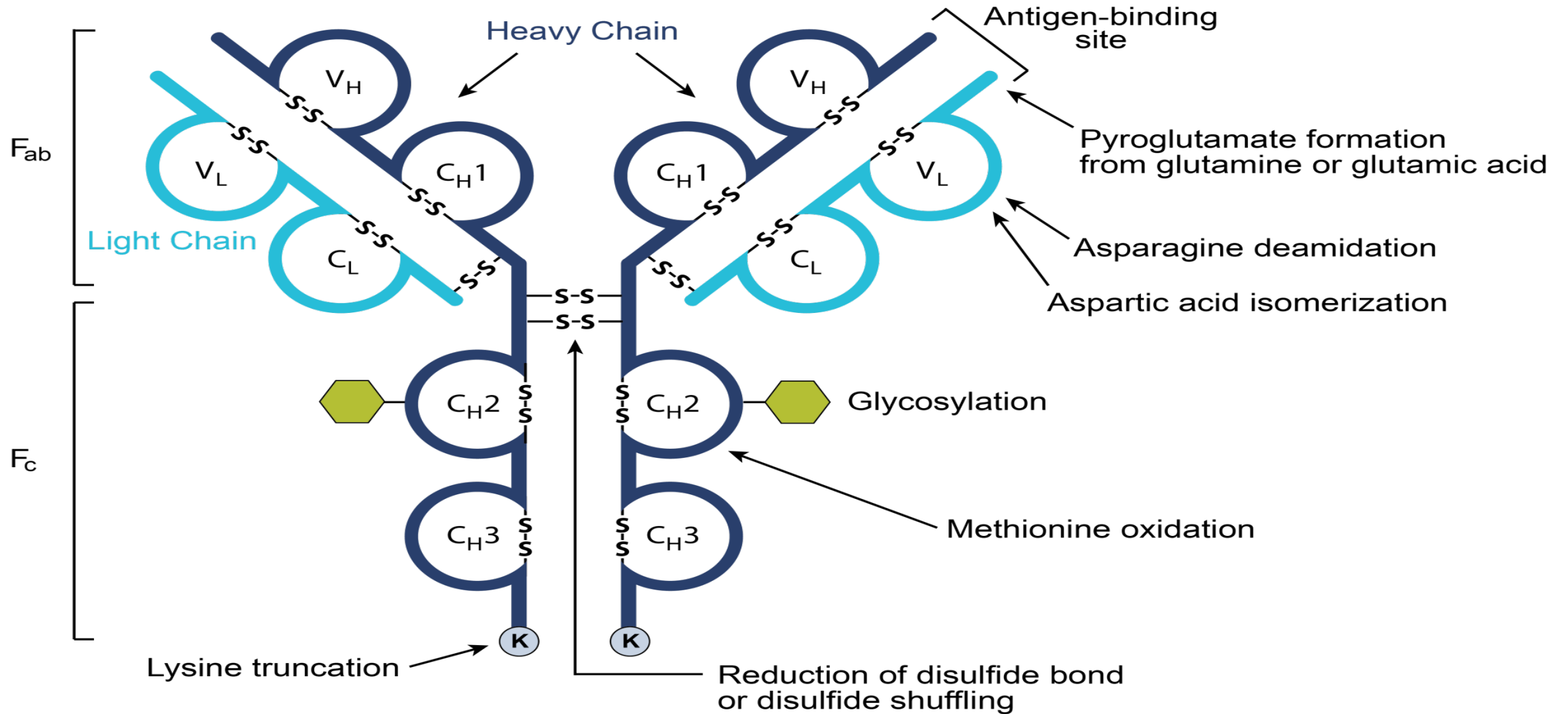
Charge variant screening











Aggregate screening



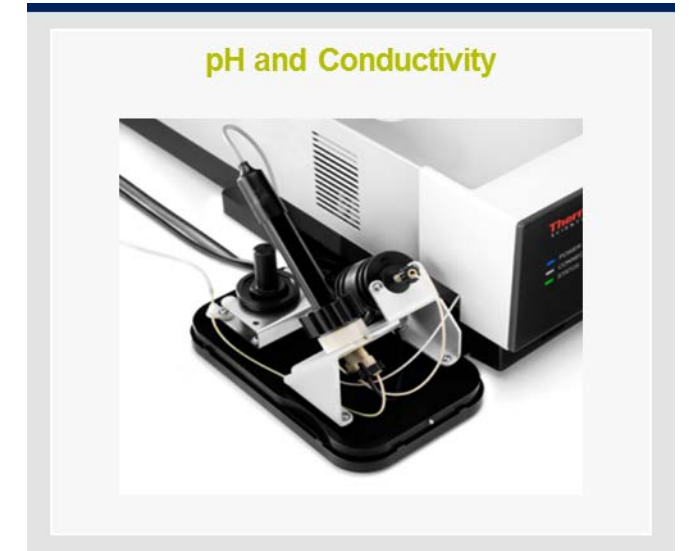
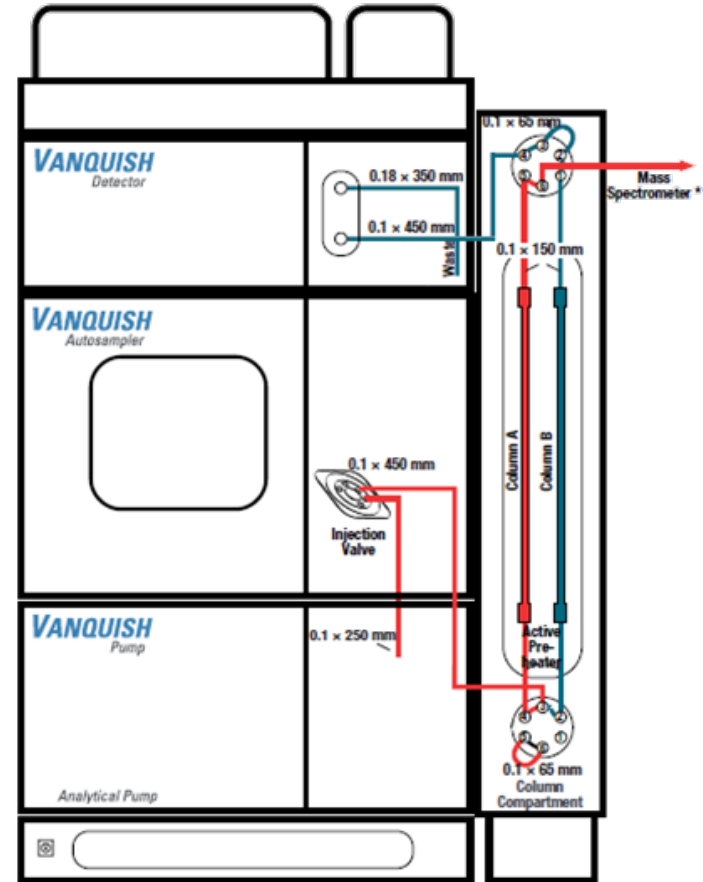
Structure of IgG and Typical Forms of Heterogeneity



BioColumn Selection Guide

| Analysis | | Description | Columns and buffers | Detection |
|--|---|---|--|-------------------------|
| Titer |  | mAb capture, titer and screening | Thermo Scientific™ MAbPac™ Protein A | UV |
| Aggregate |  | Routine screening for aggregates and fragments | Thermo Scientific™ MAbPac™ SEC-1 | UV and light scattering |
| Charge heterogeneity |  | Routine variant profiling including; lysine truncation, deamidation and acylation | Thermo Scientific™ MAbPac™ SCX-10 Thermo Scientific™ MAbPac™ SCX-10 RS Thermo Scientific™ ProPac™ WCX-10 Thermo Scientific™ CX-1 pH Gradient Buffer Kit | UV |
| Methionine and Tryptophan oxidation |  | Targeted analysis of methionine and tryptophan oxidation | Thermo Scientific™ MAbPac™ HIC-20 Thermo Scientific™ MAbPac™ HIC-10 Thermo Scientific™ ProPac™ HIC-10 | UV |
| Antibody drug conjugate (ADC) |  | Drug to Antibody ratios | Thermo Scientific™ MAbPac™ HIC-10 Butyl Thermo Scientific™ MAbPac™ HIC-20 Thermo Scientific™ MAbPac™ HIC-10 Thermo Scientific™ MAbPac™ RP | UV |
| Antibody drug conjugate (ADC) using MS |  | Drug to Antibody ratios and intact mass | Thermo Scientific™ MAbPac™ SEC-1 Thermo Scientific™ MAbPac™ RP Thermo Scientific™ Acclaim™ SEC-300 | |
| Intact or fragment mass |  | Intact, light (LC), heavy chain (HC) and fragment (Fab & Fc) analysis | Thermo Scientific™ MAbPac™ RP | UV and MS |
| Native mass |  | Intact native mass analysis | Thermo Scientific™ MAbPac™ SEC-1 Thermo Scientific™ Acclaim™ SEC-300 Thermo Scientific™ MAbPac™ SCX-10 RS | UV and MS |

Thermo Scientific™ Vanquish™ UHPLC Platform for Bio-Therapeutic Characterization

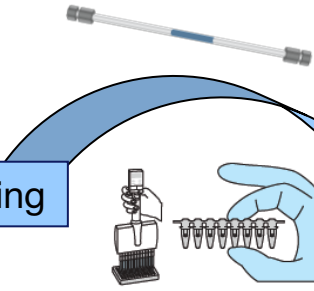


Thermo Scientific™ Q Exactive™ MS BioPharma Option

Thermo Scientific™ Acclaim™ Vanquish™ C18 column

Standard mode

Peptide mapping



Thermo Scientific™ SMART Digest™ kits

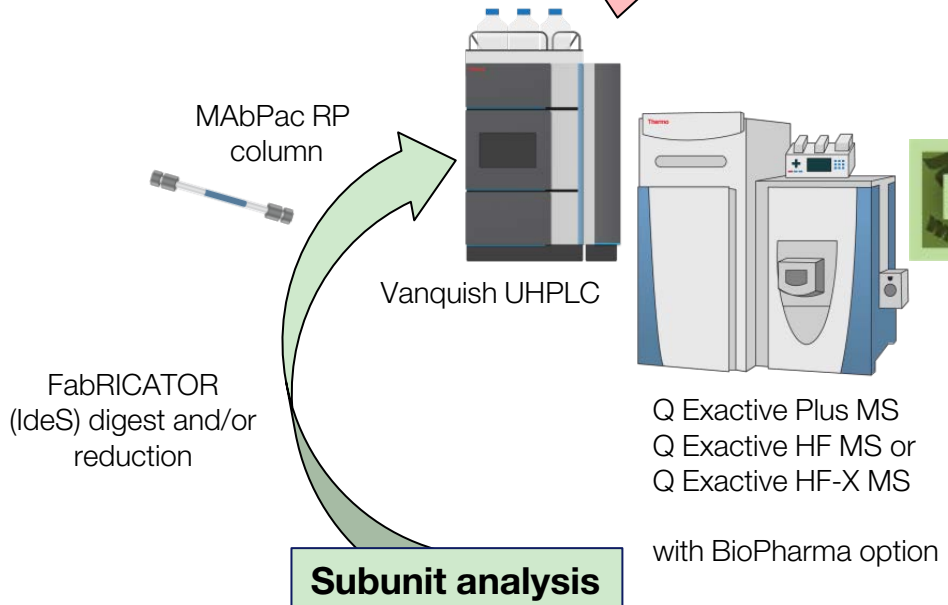
Thermo Scientific™ MAbPac™ RP column for denatured samples

Acclaim SEC & MAbPac SCX-10 for native

HMR mode

Intact mAb analysis native & denatured

The Q Exactive Plus and Q Exactive HF mass spectrometers equipped with the BioPharma Option provide three different modes to cover the three major workflows in BioPharma. Coupled to the inert Vanquish UHPLC system and the high resolution BioPharma columns

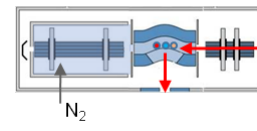


FabRICATOR (IdeS) digest and/or reduction

Subunit analysis

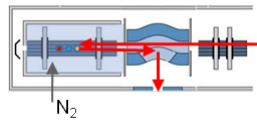
Protein mode

Standard Mode



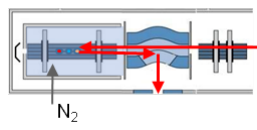
Trapping in Standard Mode: trapping gas pressure setting 1 (fixed on setting 1)

Protein Mode

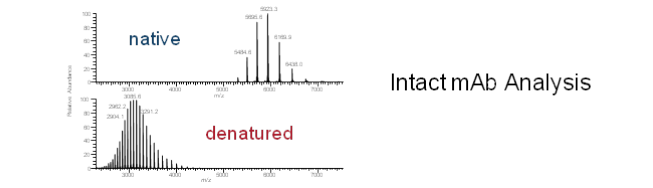
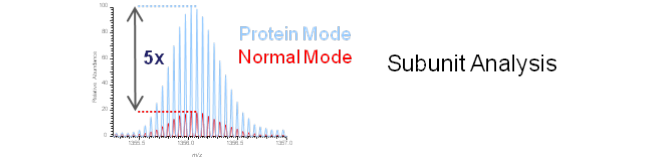
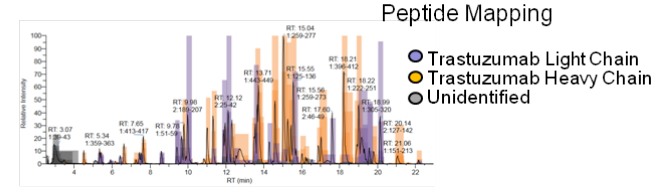


Trapping in Protein Mode: trapping gas pressure default setting 0.2 (range 0.2-1)

HMR Mode



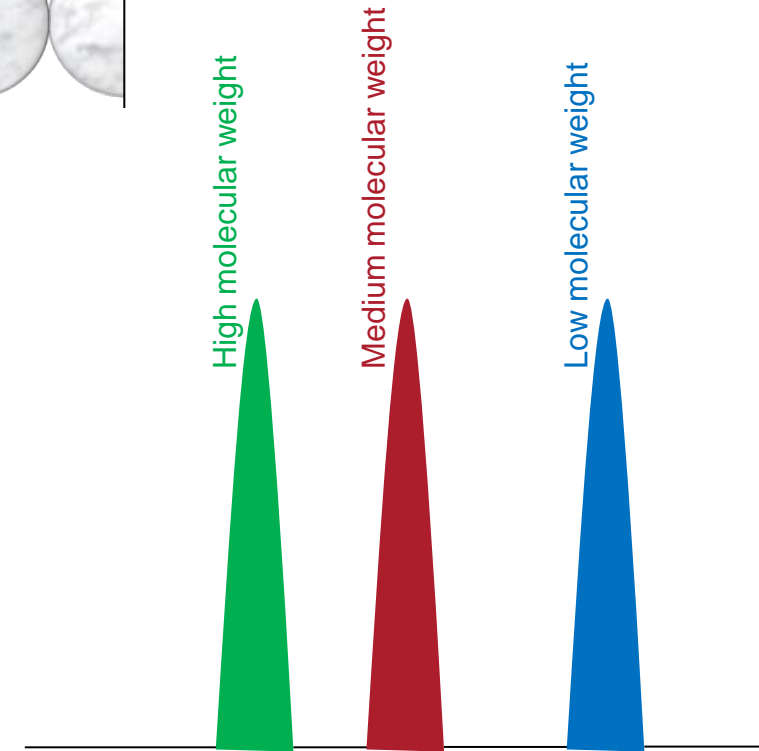
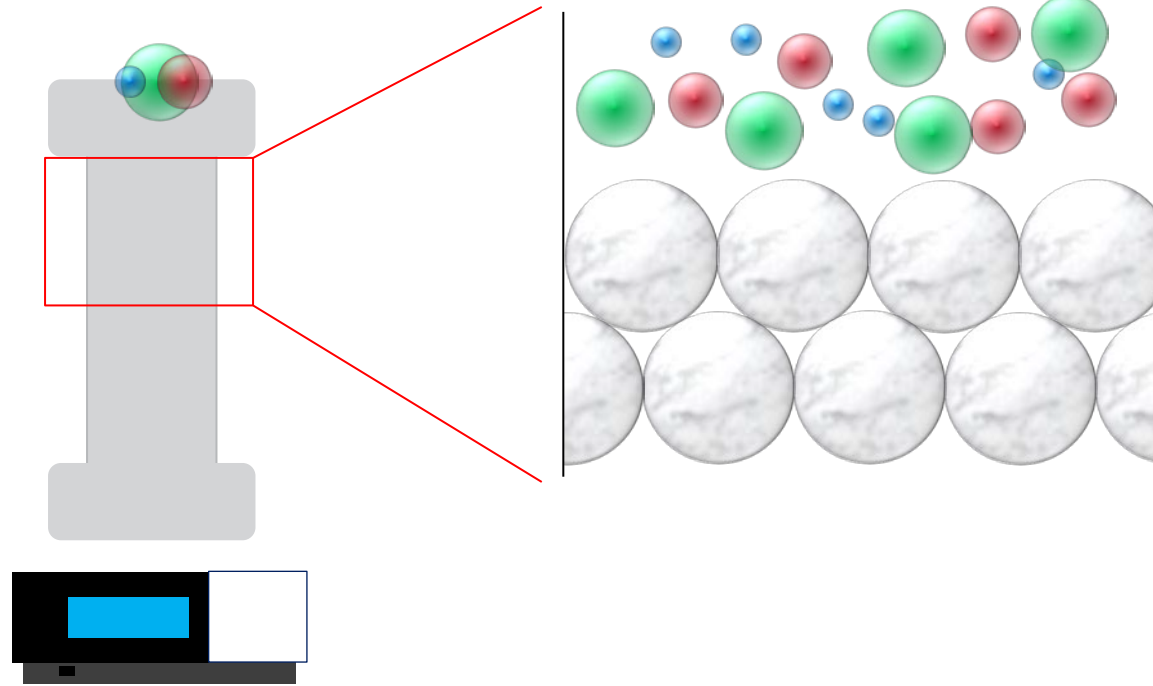
Trapping in HMR Mode: trapping gas pressure default setting 1 (range 1.0-1.5)



Aggregation Analysis



- Typically using size exclusion chromatography (SEC)
- MAbPac SEC-1
 - Silica, 5 μm , 300Å

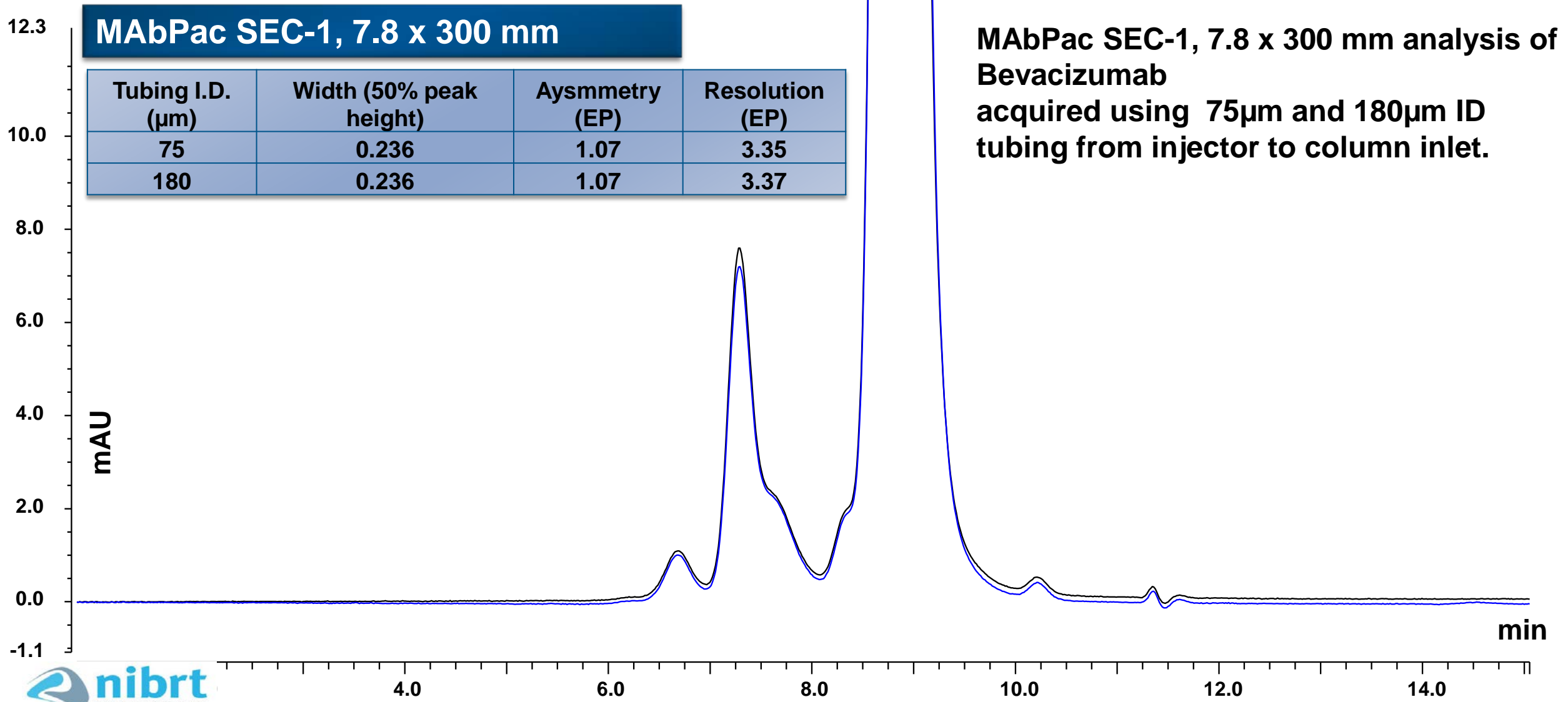


Column Formats Versus Target Applications

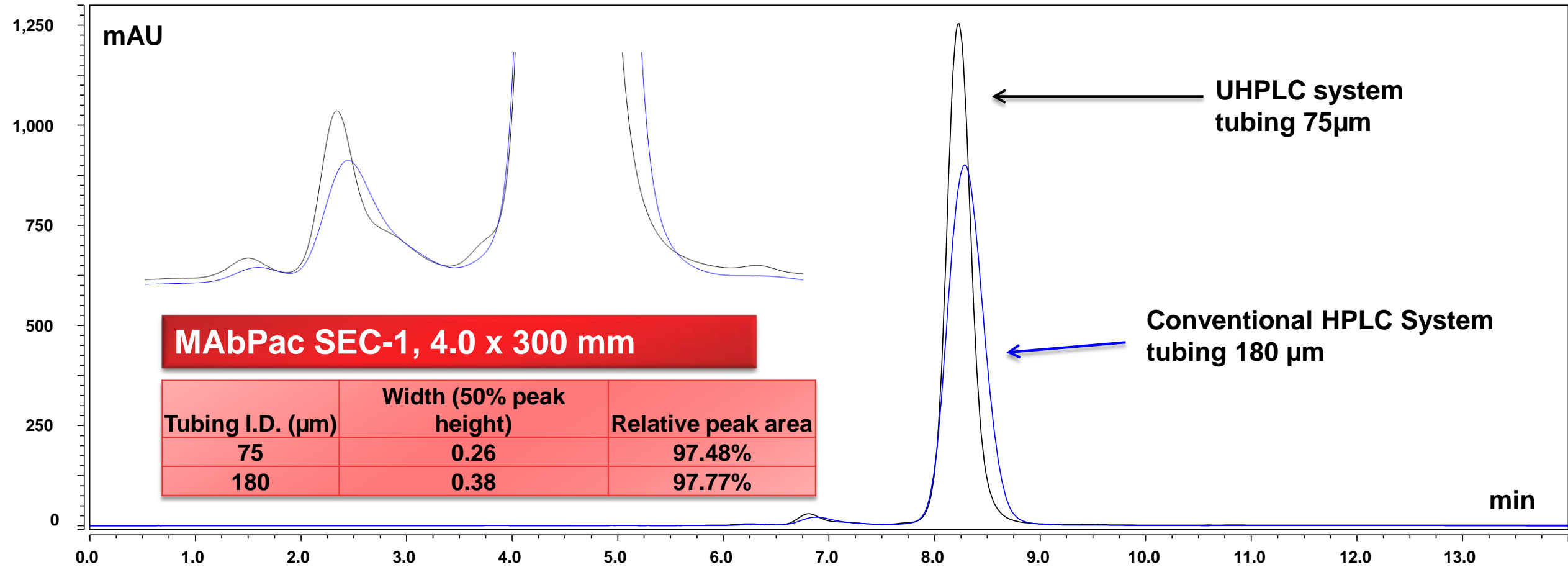
| Formats | Target application | Why is it important |
|------------------------------|--|---|
| 7.8 × 300 mm | Highest resolution separation of mAb and their aggregates. | Accurate quantification of mAb aggregates. Used in the batch QC release assay |
| 4.0 × 300 mm 4.0 × 150 mm | High resolution separation of mAb and their aggregates | The 4.0 × 300 mm column enables baseline separation of mAb monomer and dimer, required ¼ of sample comparing to the 7.8 × 300 mm column |
| 2.1 × 300 mm 2.1 × 150 mm | Designed for SEC-MS application | Low flow rate and low sample loading makes this format perfect for MS detection |

| Column ID (mm) | 2.1 | 4.0 | 7.8 |
|---------------------------------------|----------------|---------------------|--------------------|
| Flow rate (µL/min) | 50-75 | 200-300 | 760-1,000 |
| UV flow cell | Micro (180 nL) | Semi-micro (2.5 µL) | Analytical (11 µL) |
| Tubing ID (µm) | 50 | 75 | 150-250 |
| Sample loop size (Pull loop WPS) (µL) | 1 | 5 | 20 |

Effect of Pre-Column Tubing

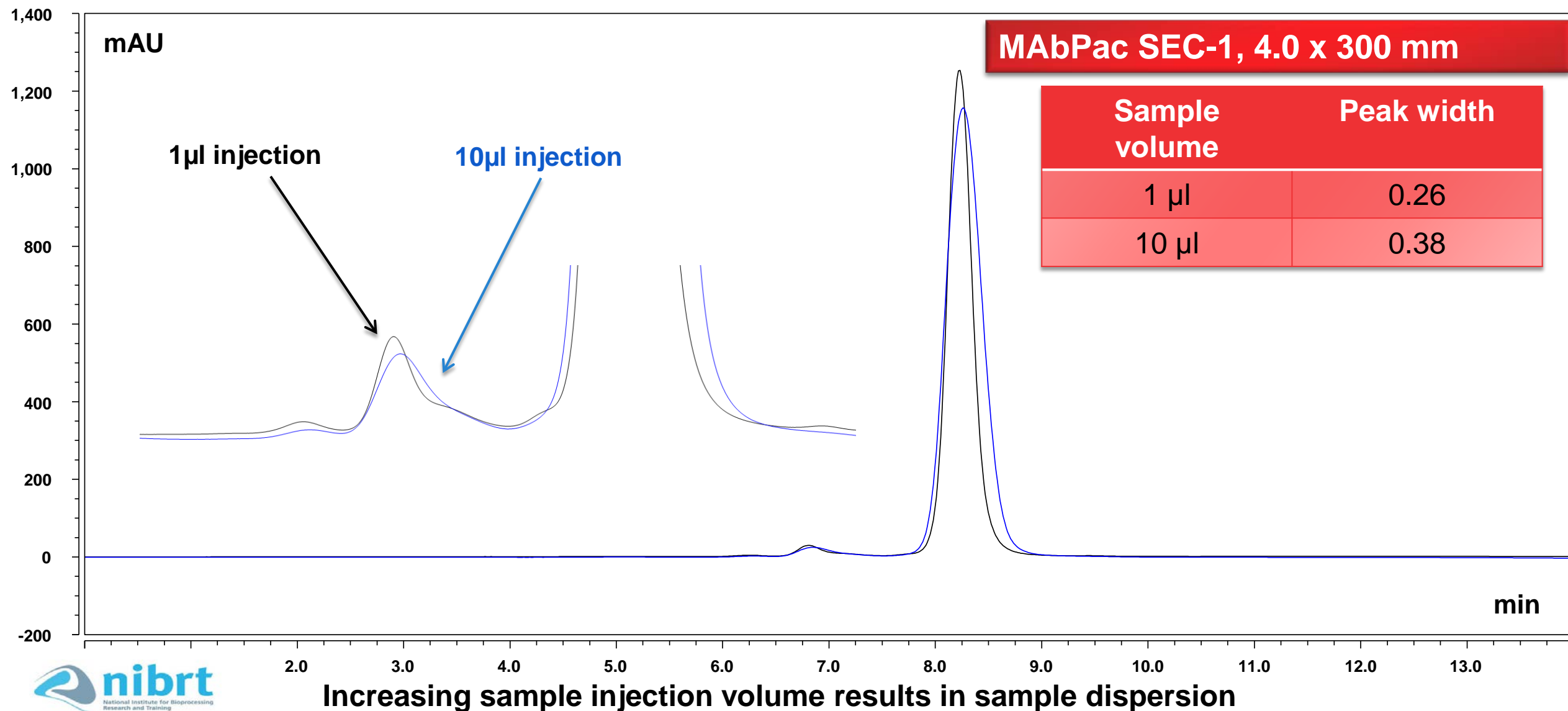


Effect of Pre-Column Tubing: Column 4mm

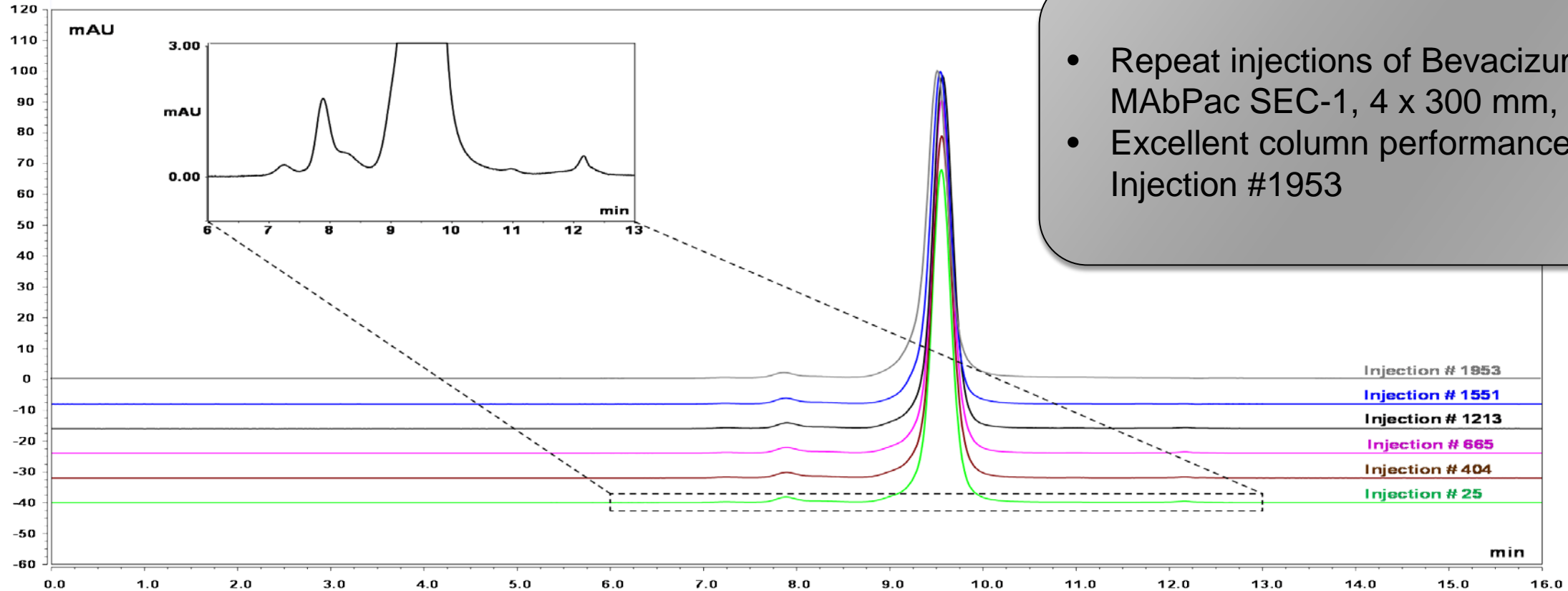


MAbPac SEC-1, 4.0 x 300 mm, analysis of Bevacizumab using Vanquish UHPLC chromatograms acquired using 75 μm and 180 μm ID tubing from injector to column inlet

Injection Volume – Effect on SEC (4 mm)



Column Lifetime Stability Evaluation



- Repeat injections of Bevacizumab on MAbPac SEC-1, 4 x 300 mm, 5 μ m
- Excellent column performance up to Injection #1953

‘Inject until failure’ study performed on MAbPac SEC-1 column performed on Vanquish Flex quaternary with DAD Light Pipe detector using repeat injections of Bevacizumab, selectivity and reproducibility maintained for >1950 injections!

Peptide Mapping: Our Workflow Solution



New product

Thermo Scientific™ **SMART Digest™** offer extremely reproducible and rapid protein digestion

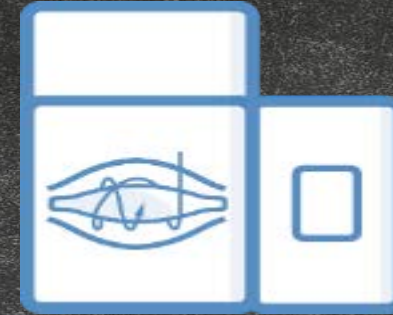


New product

Thermo Scientific™ **Vanquish™ UHPLC** is engineered for high resolution, reproducible peptide separations



Thermo Scientific™ **Acclaim™ 120 C18 column** is the perfect column choice to ensure sharp peaks during peptide mapping



Thermo Scientific™ **Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometers** are the gold standard for accurate mass measurement

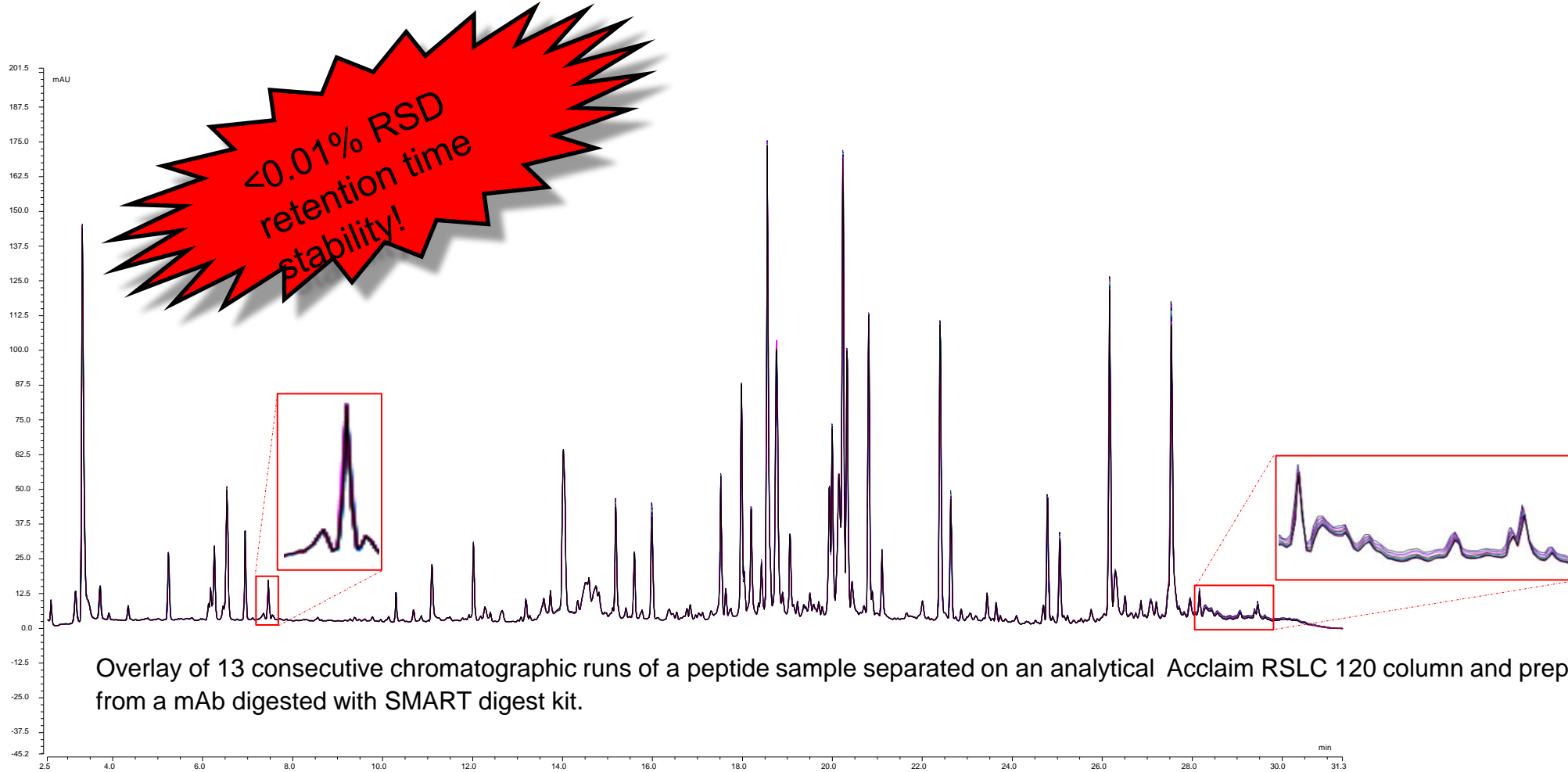


Brand new product

Thermo Scientific™ **BioPharma Finder™ software** is the perfect software tool for peptide identification and sequence mapping

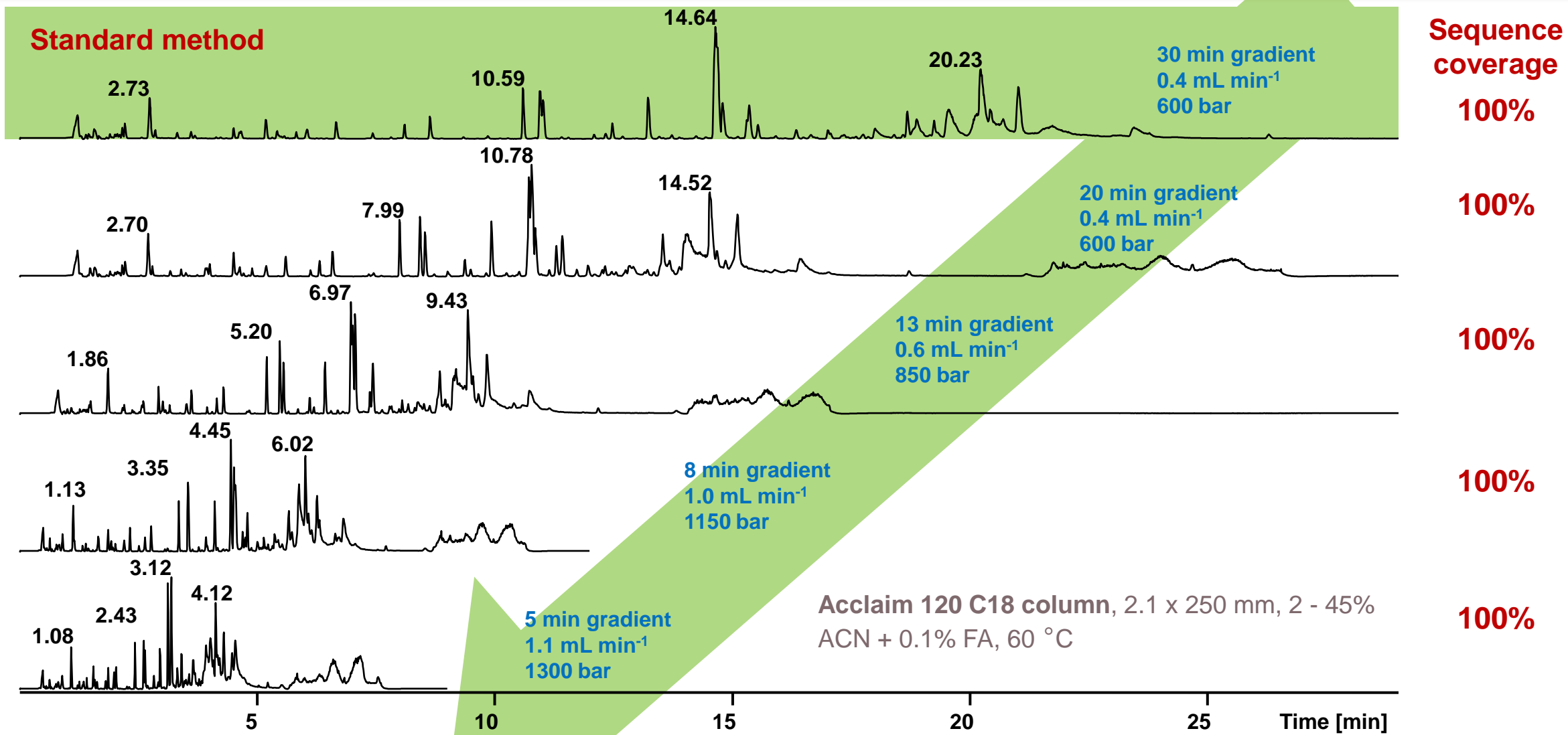
Vanquish UHPLC System: Retention Time Reproducibility

Retention time repeatability of a peptide separation



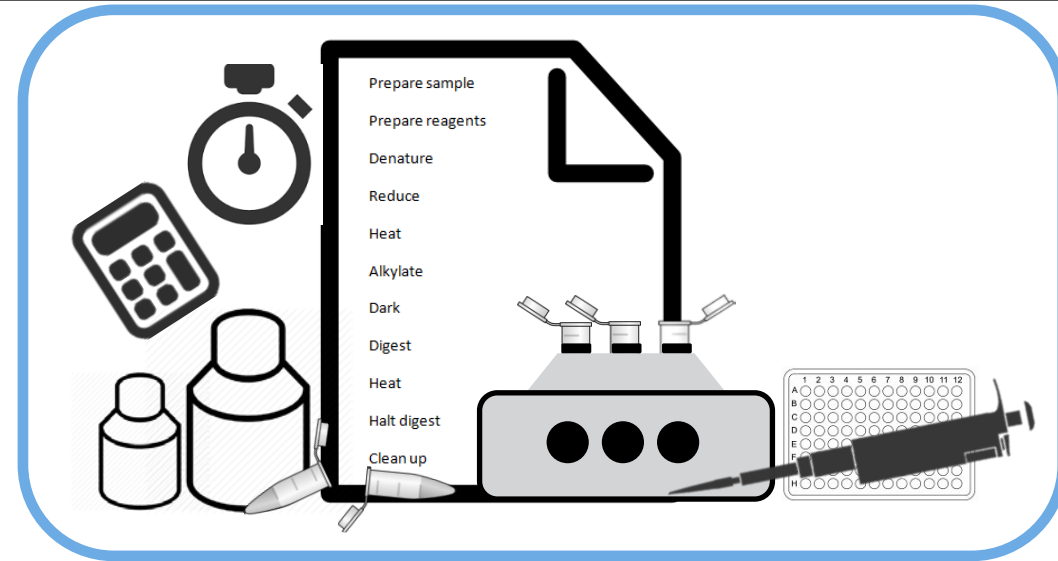
| Retention time repeatability | | |
|------------------------------|----------|--------------|
| peak # | RT (min) | RSD (%) |
| 3 | 3.315 | 0.082 |
| 9 | 5.231 | 0.065 |
| 14 | 6.532 | 0.017 |
| 15 | 6.937 | 0.023 |
| 19 | 10.290 | 0.021 |
| 23 | 12.013 | 0.012 |
| 31 | 14.011 | 0.013 |
| 39 | 15.177 | 0.012 |
| 42 | 15.589 | 0.010 |
| 51 | 17.511 | 0.007 |
| 55 | 17.969 | 0.011 |
| 61 | 18.546 | 0.010 |
| 83 | 20.798 | 0.010 |
| 85 | 21.095 | 0.012 |
| 87 | 22.386 | 0.009 |
| 96 | 24.774 | 0.012 |
| 103 | 26.155 | 0.009 |
| 106 | 26.155 | 0.009 |
| 109 | 27.529 | 0.010 |

Rituximab Analysis with Reducing Gradient

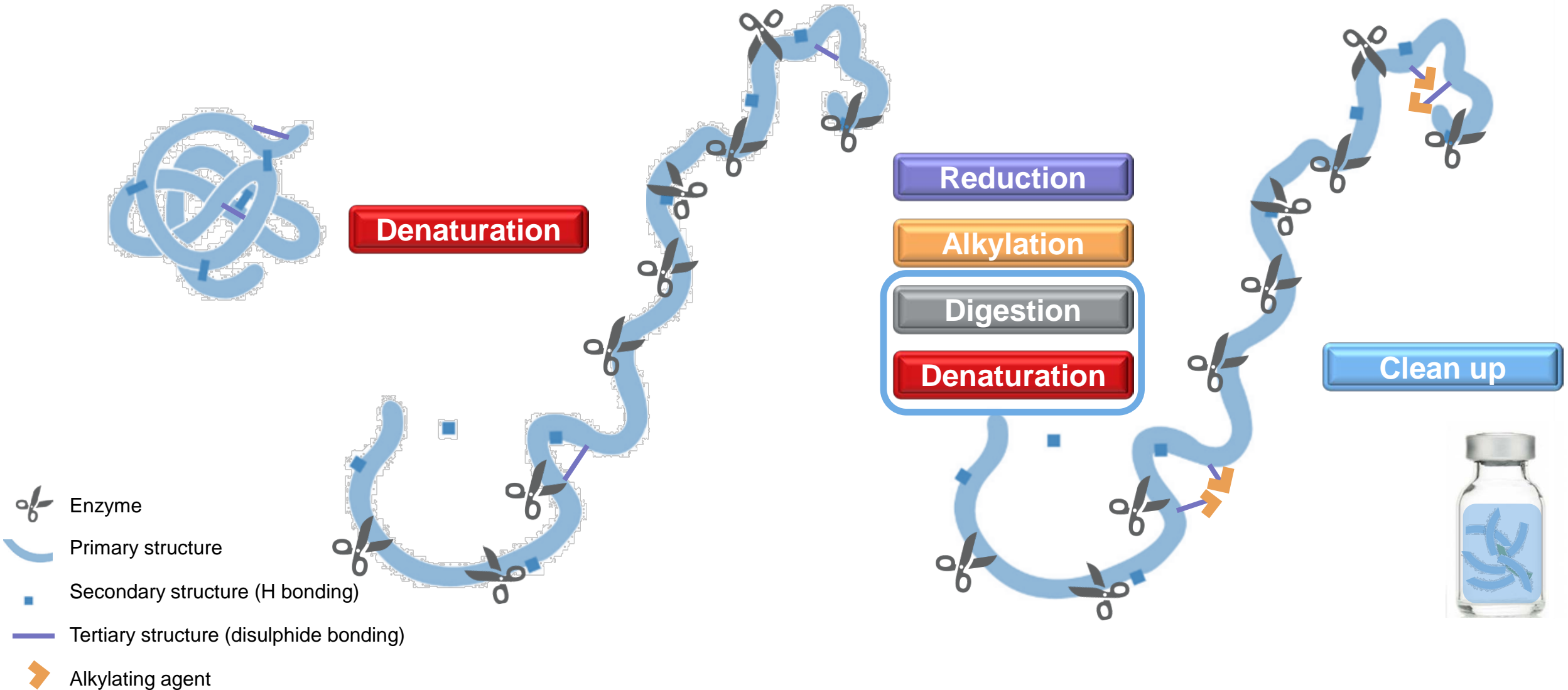


What is the Problem with Protein Digestion?

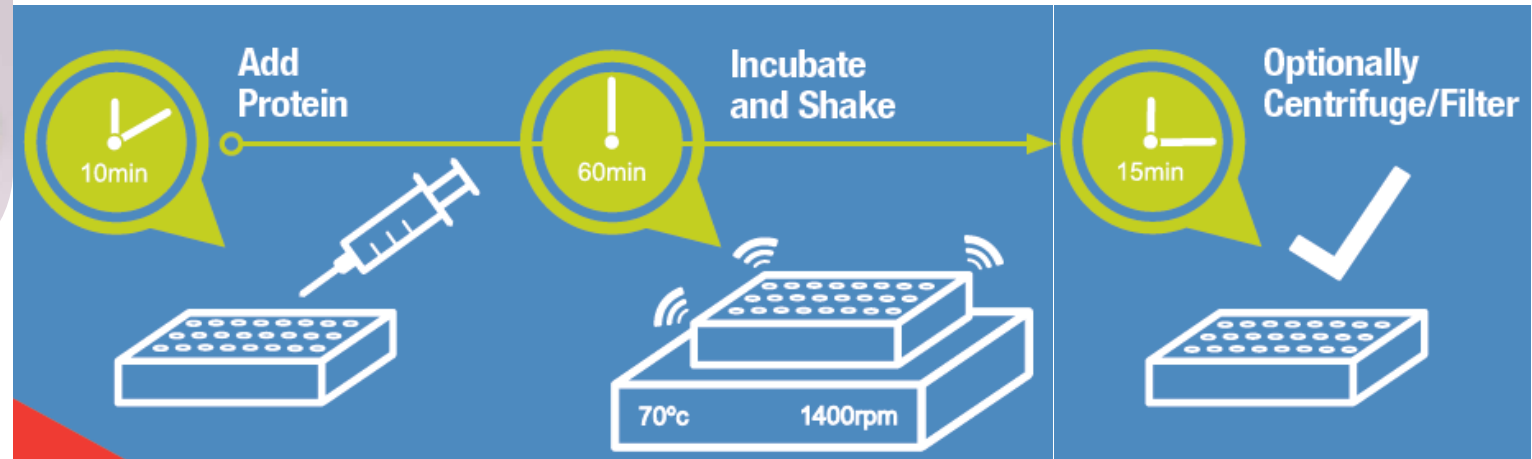
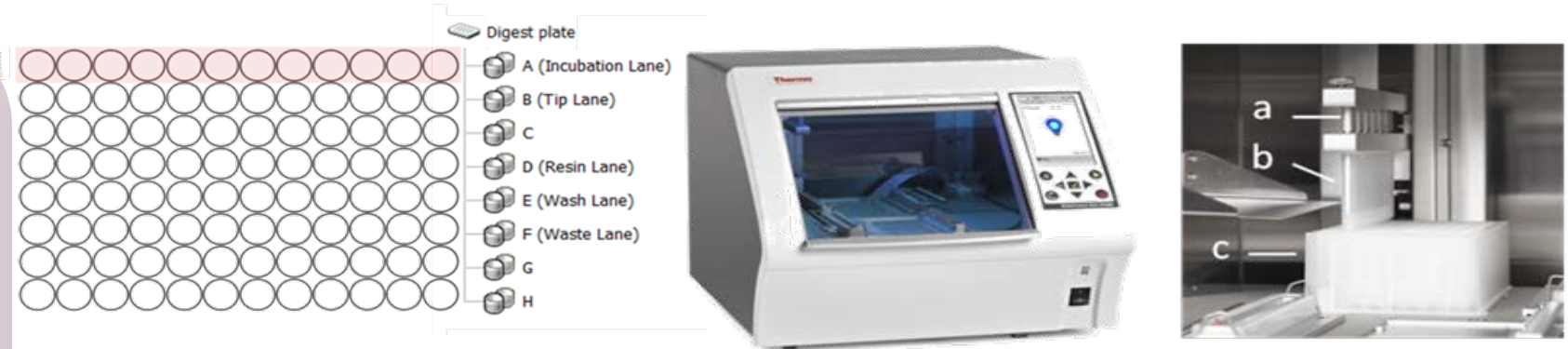
- Lengthy multi-step protocols
- Process-induced PTMs
- Reproducibility
- Throughput/Speed
- Method development ease and transfer



The Fundamental Five...or is that Two?



SMART Digest Kits

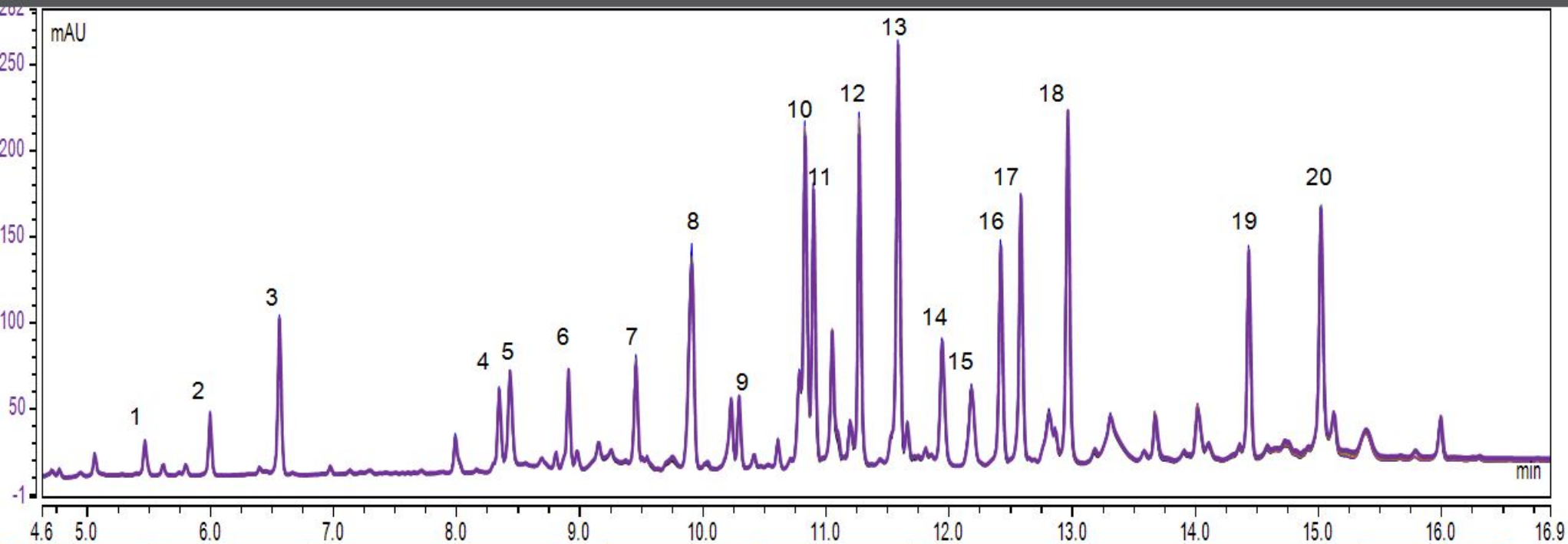


Denaturation

Digestion

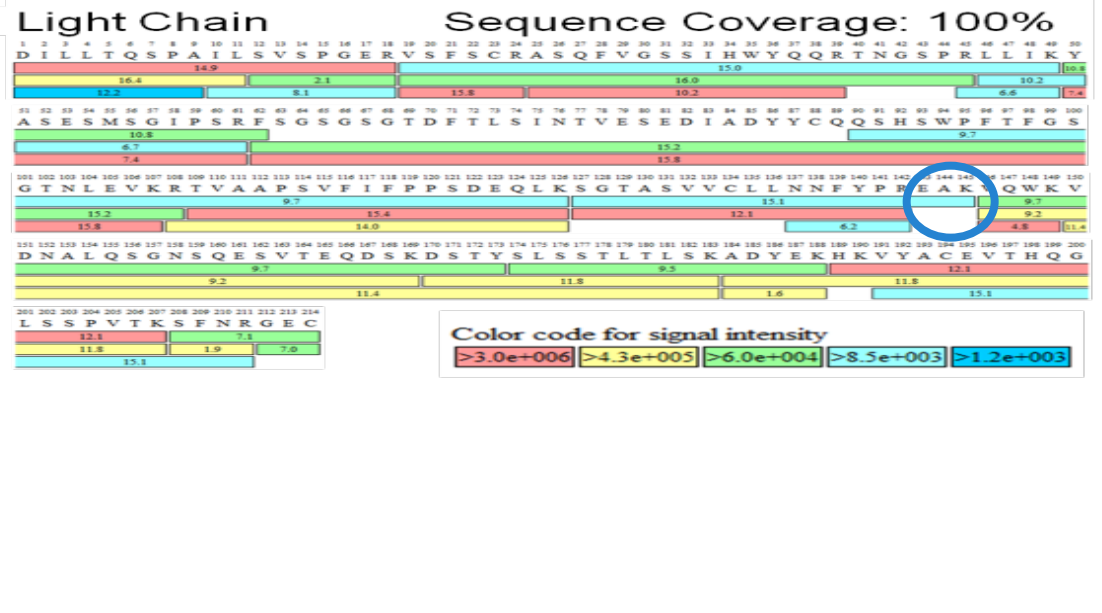
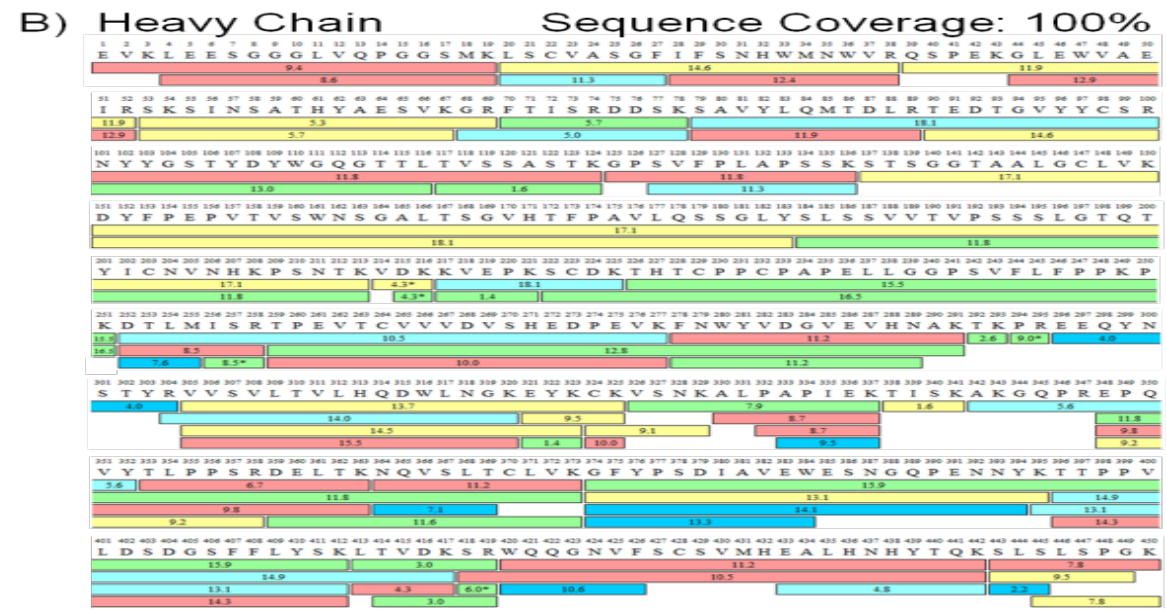
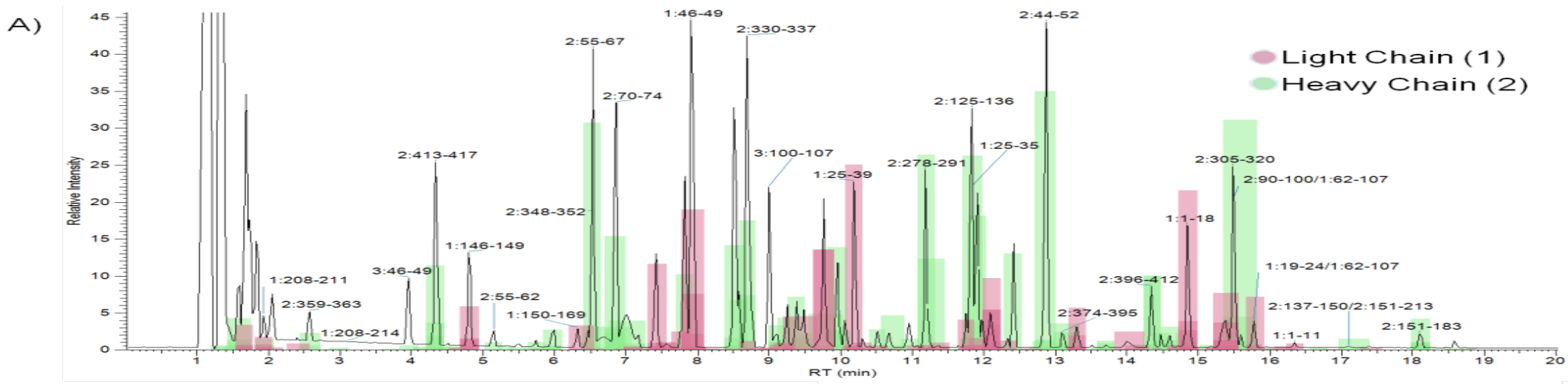
Clean up

Five Different Rituximab Digests by Five Different Seminar Attendees



| Peak 1 | Peak 2 | Peak 3 | Peak 4 | Peak 5 | Peak 6 | Peak 7 | Peak 8 | Peak 9 | Peak 10 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 2.54 | 2.41 | 1.89 | 3.39 | 3.53 | 2.16 | 4.41 | 2.10 | 2.10 | 3.65 |
| Peak 11 | Peak 12 | Peak 13 | Peak 14 | Peak 15 | Peak 16 | Peak 17 | Peak 18 | Peak 19 | Peak 20 |
| 1.96 | 3.51 | 3.72 | 2.26 | 2.91 | 1.97 | 3.28 | 2.62 | 3.16 | 1.20 |

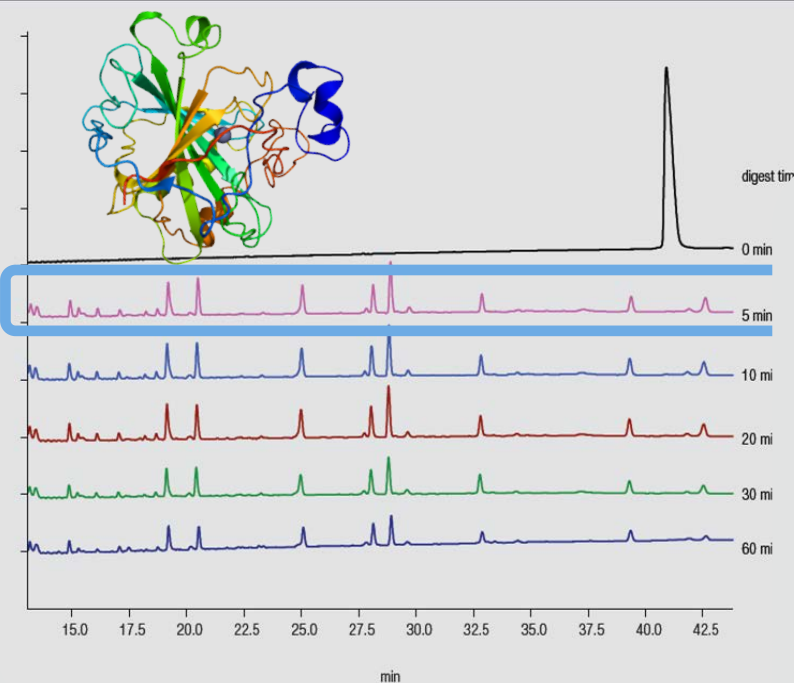
Sequence Coverage Map from Infiximab Using the Magnetic SMART Digest



Three Reasons to Change to SMART Digestion

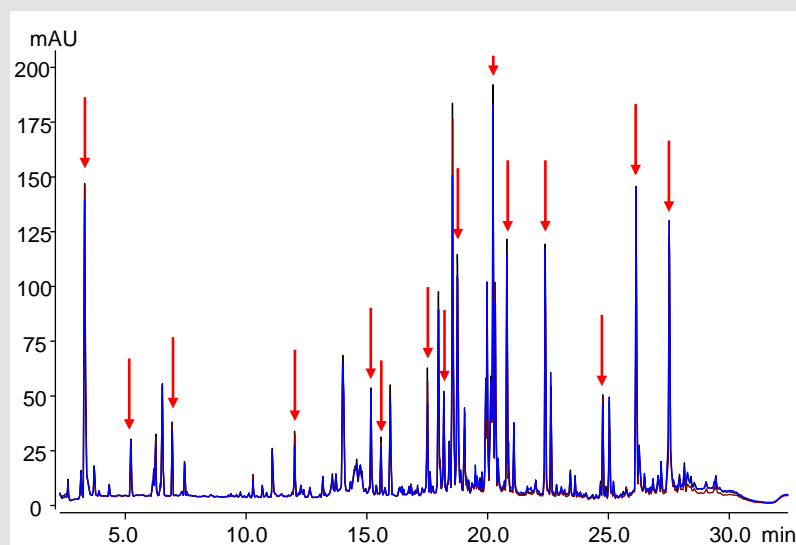
Carbonic Anhydrase, 29 KDa

Time course experiment for digestion optimization



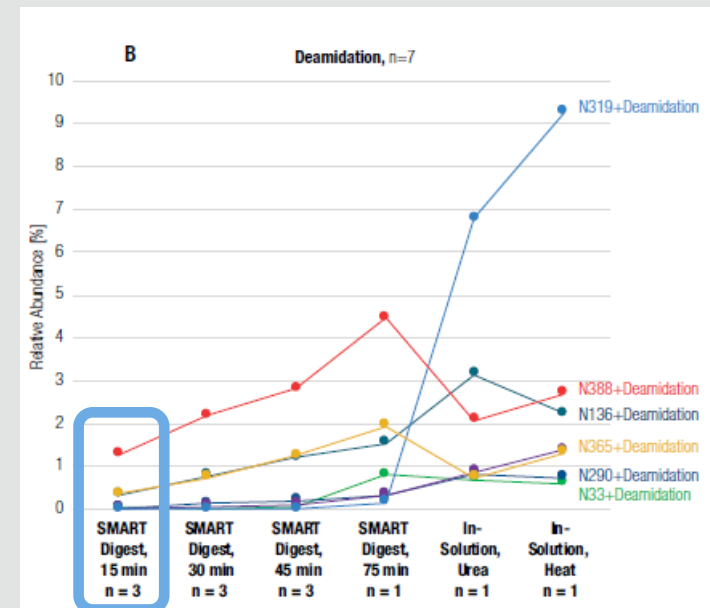
**Fast and flexible
method development**

Automation!



**RSDs: RT 0.024 %; Peak Area 2.82 % (n=5 users,
based on 15 peaks)**

Standardized reproducibility

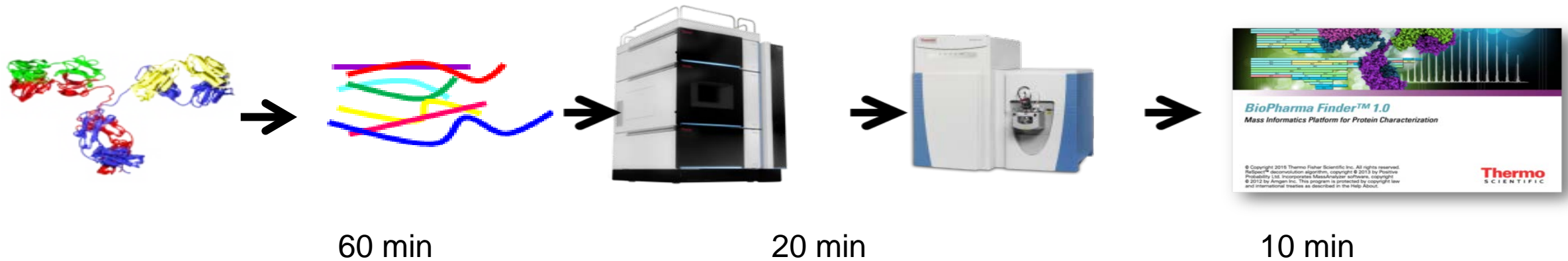


Reduced complexity

APPLICATION NOTE

SMART Digest Compared to Classic In-Solution Digestion of Rituximab for In-Depth Peptide Mapping Characterization

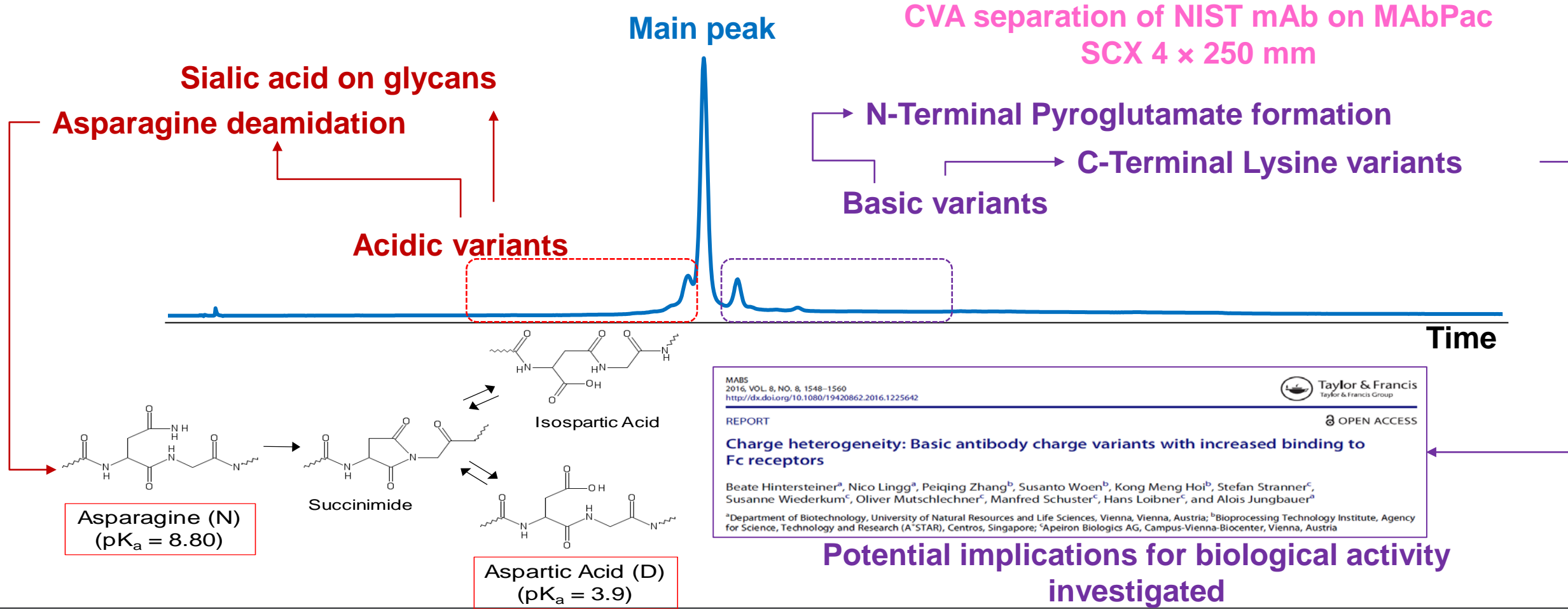
Overview of the Peptide Mapping Workflow



- Combining all the components of this peptide mapping work flow a complete analysis can be done in less than 90 minutes.
- Can be set up and verified by MS very quickly.
- A relatively inexperienced analyst can obtain reproducible results from UV with simple sample preparation and walk up UHPLC analysis.

Sources of Charge Variation on MAbs

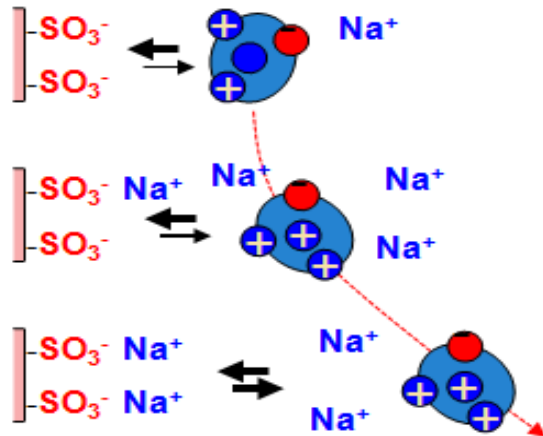
Considering the complexity of industrial bioprocessing and the number of conditions a mAb is exposed to, certain amino acids can become modified which in turn modifies the charge of the protein.



MAb Charge Variant Analysis by CEX Salt Elution

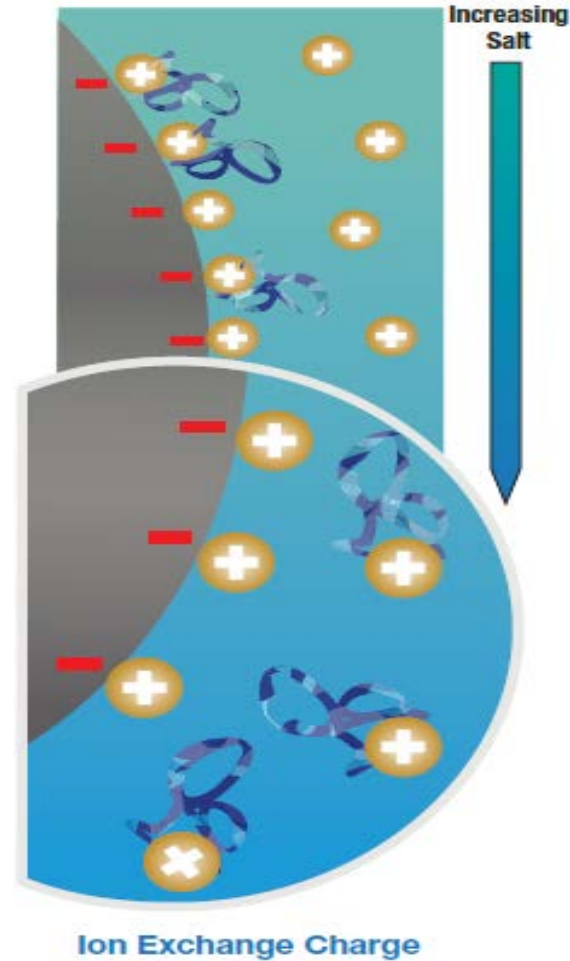
Cation exchange of MAb

Elution with competing sodium ions from an NaCl gradient

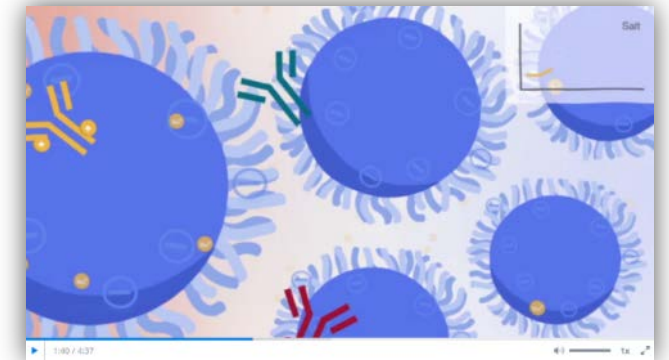


Cation exchange

Salt Gradient



- Competition for ion exchange sites between the MAb and Na^+ ions
- This interaction happens all the way through the column.
- The longer the column the better the resolution
- Surface exchange on a pellicular resin for high resolution

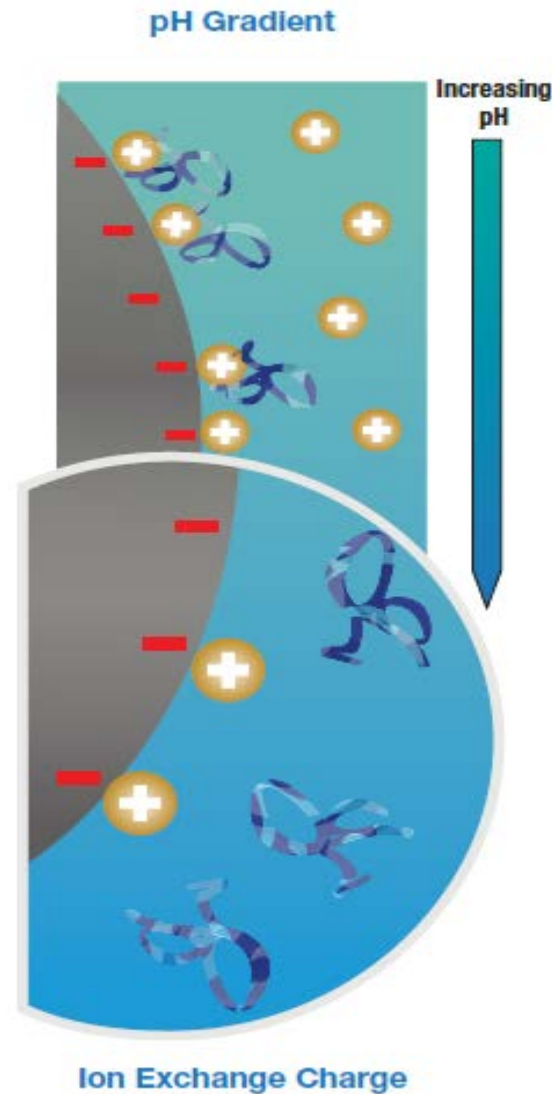
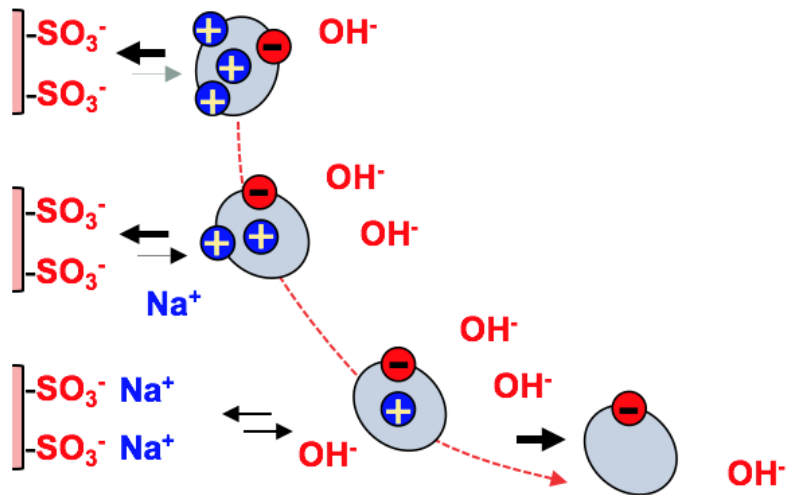


<http://bit.ly/ChargeVariants>

MAb Charge Variant Analysis by CEX pH Gradient Elution

pH gradient elution

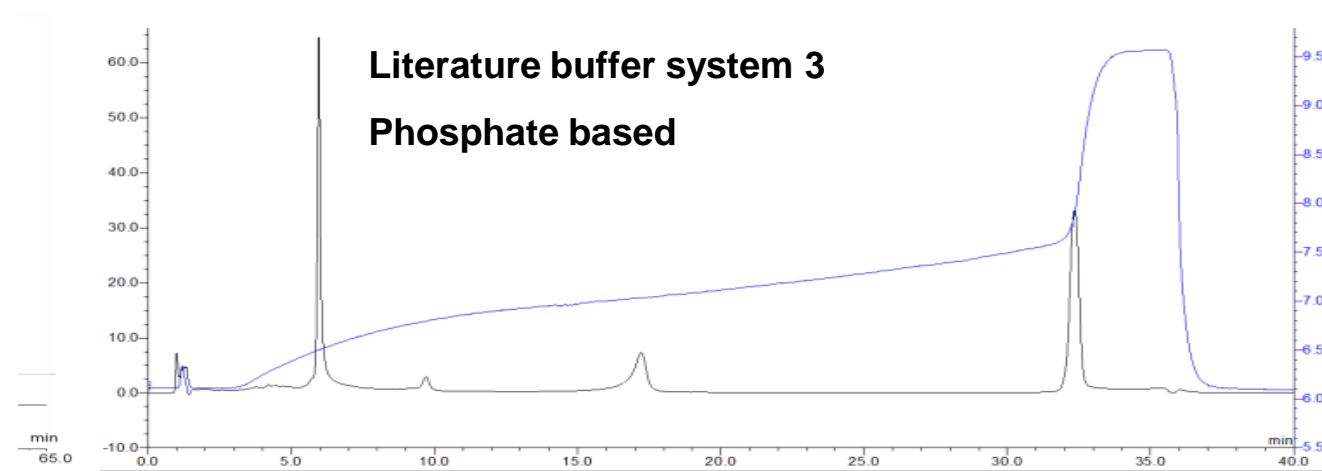
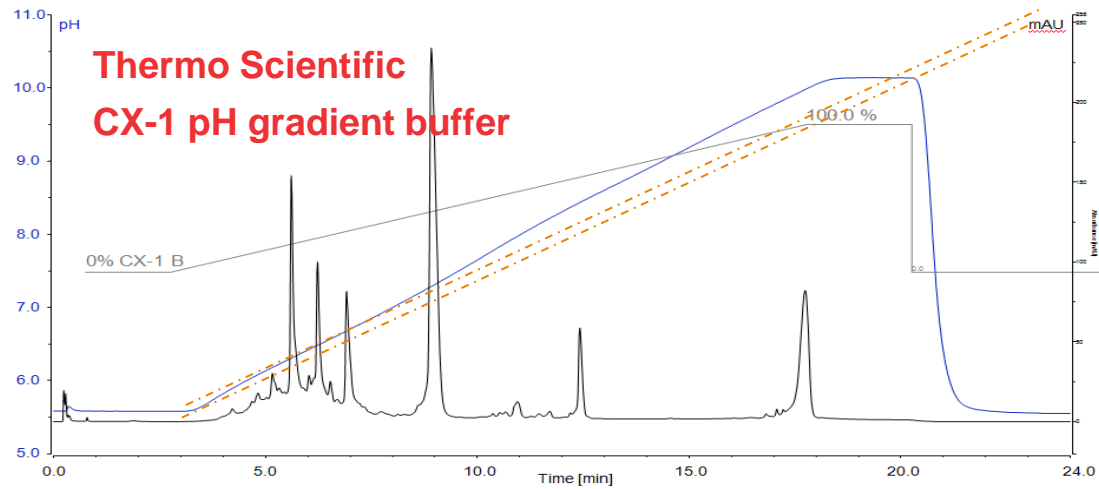
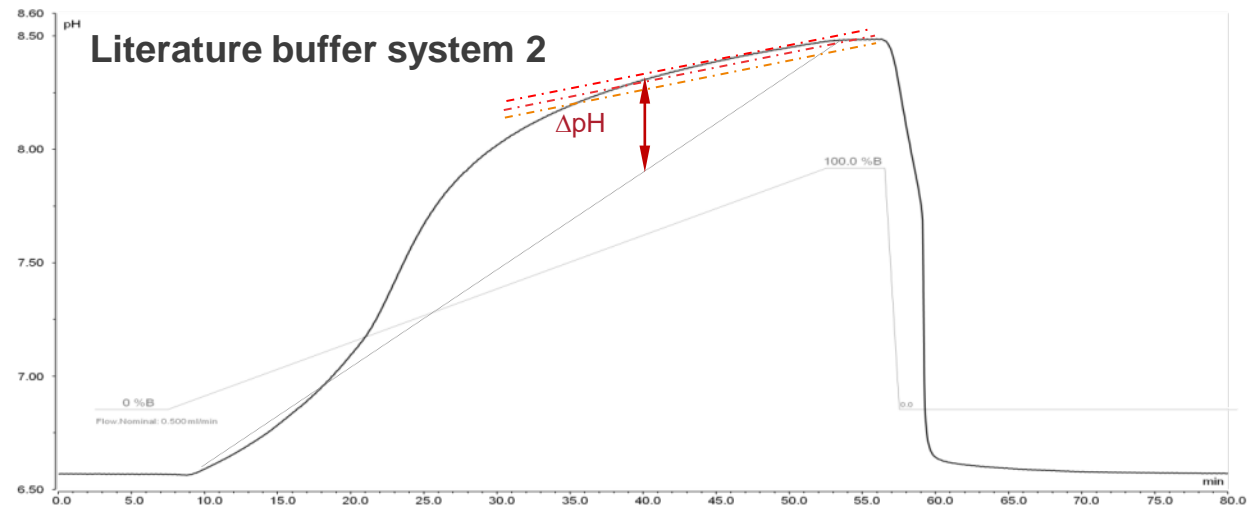
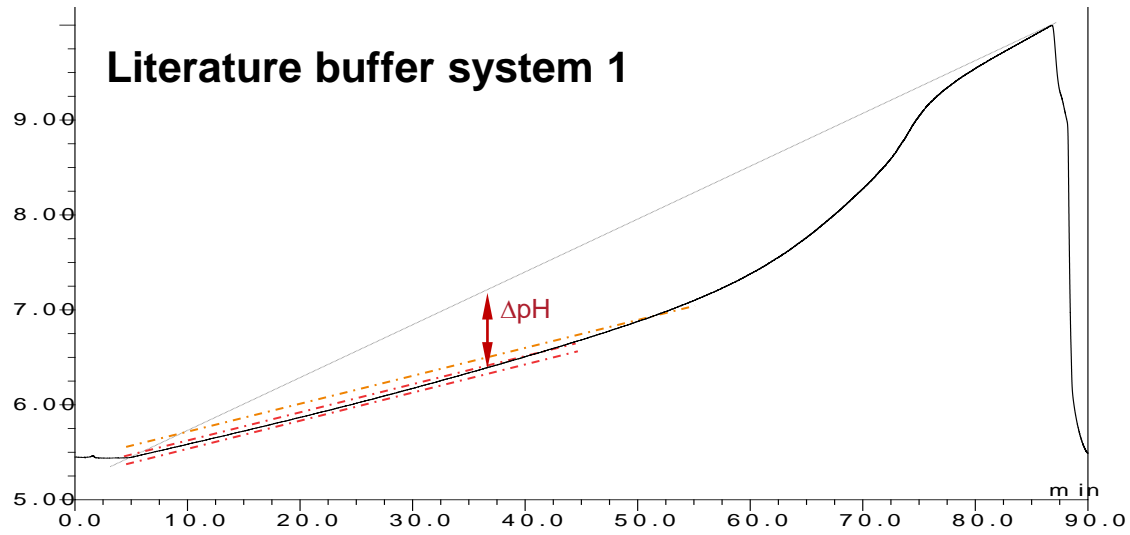
- Based on pI of protein
- Loss of retention with progressing pH gradient, depending on pI
- “Single” binding event, trapping at $pH < pI$ (for CEX)



Isoelectric Focusing on a cation exchange column

- MAb binds to cation exchange sites on the column.
- A gradient of increasing pH is applied.
- MAb is released from the exchange site when the net charge on the mAb is neutral.
- This interaction happens once, then the mAb runs through the rest of the column.
- Column length has little effect on the resolution.
- This is a concentrating technique.
- Surface exchange on a pellicular resin for high resolution and low buffering capacity effects

Comparison of pH Gradient Buffer Systems



MABPac SCX-10 (5 μ m) 4x50 mm

Advantages

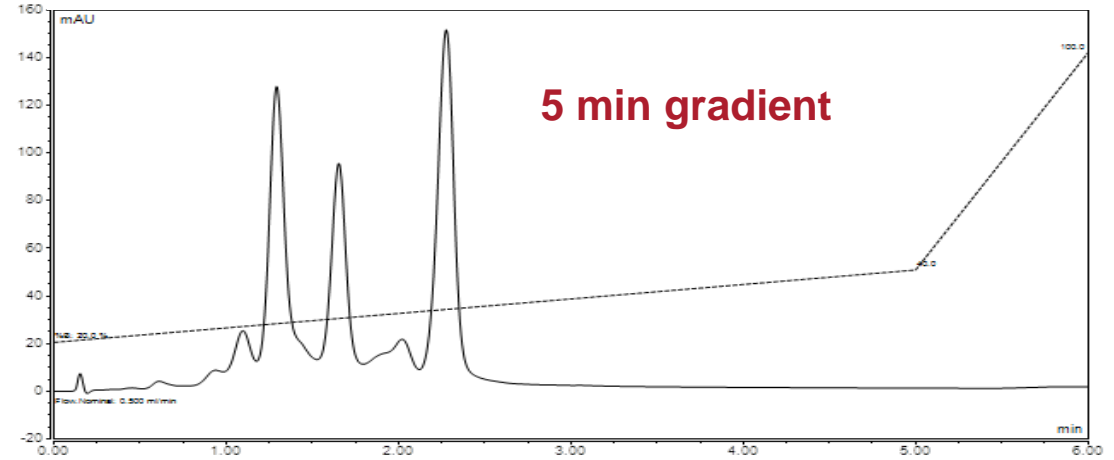
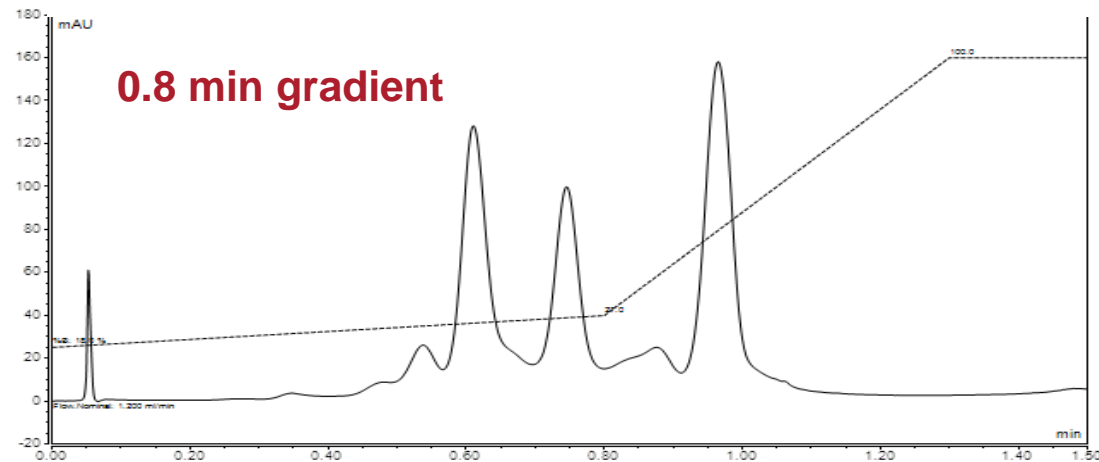
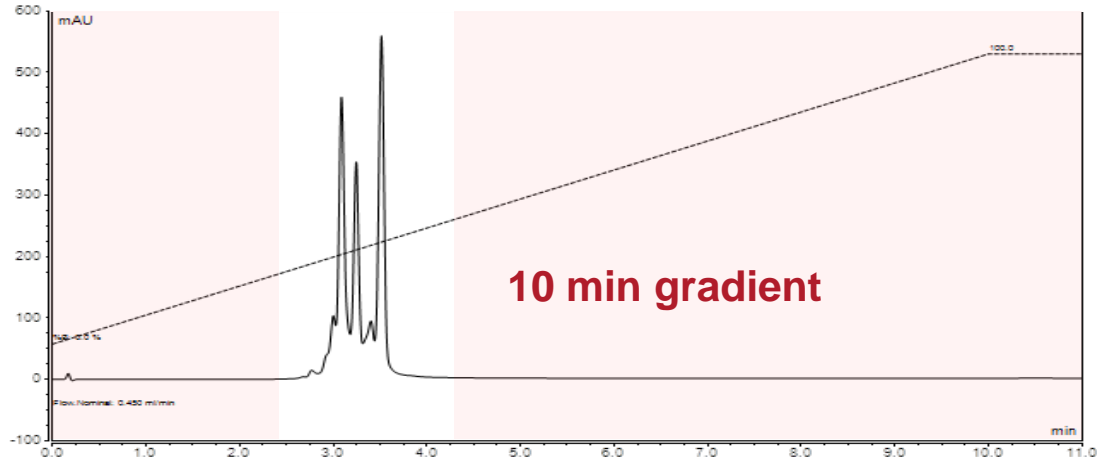
- **Platform** method \Rightarrow Single method for wide range of mAbs
- Reduced method development and method transfer times
- Outperforms any other charge variant technology
- Less effect from column variability
- Transferability of method from development to QC



- Dilute buffers 10-fold with DI water
- A linear pH gradient (pH 5.6 - 10.2) is generated by running a linear pump gradient from 100% Buffer A to 100% Buffer B
- Generic, fast & high-resolution!

| | Buffer A | Buffer B |
|--------------------|-----------|-----------|
| pH | 5.6 | 10.2 |
| Form | Liquid | Liquid |
| Concentrate | 10X | 10X |
| Shipping condition | Room Temp | Room Temp |
| Storage condition | 4 ~ 8 °C | 4 ~ 8 °C |

Infliximab – Vanquish System Ultra-fast Gradients



3 step method development

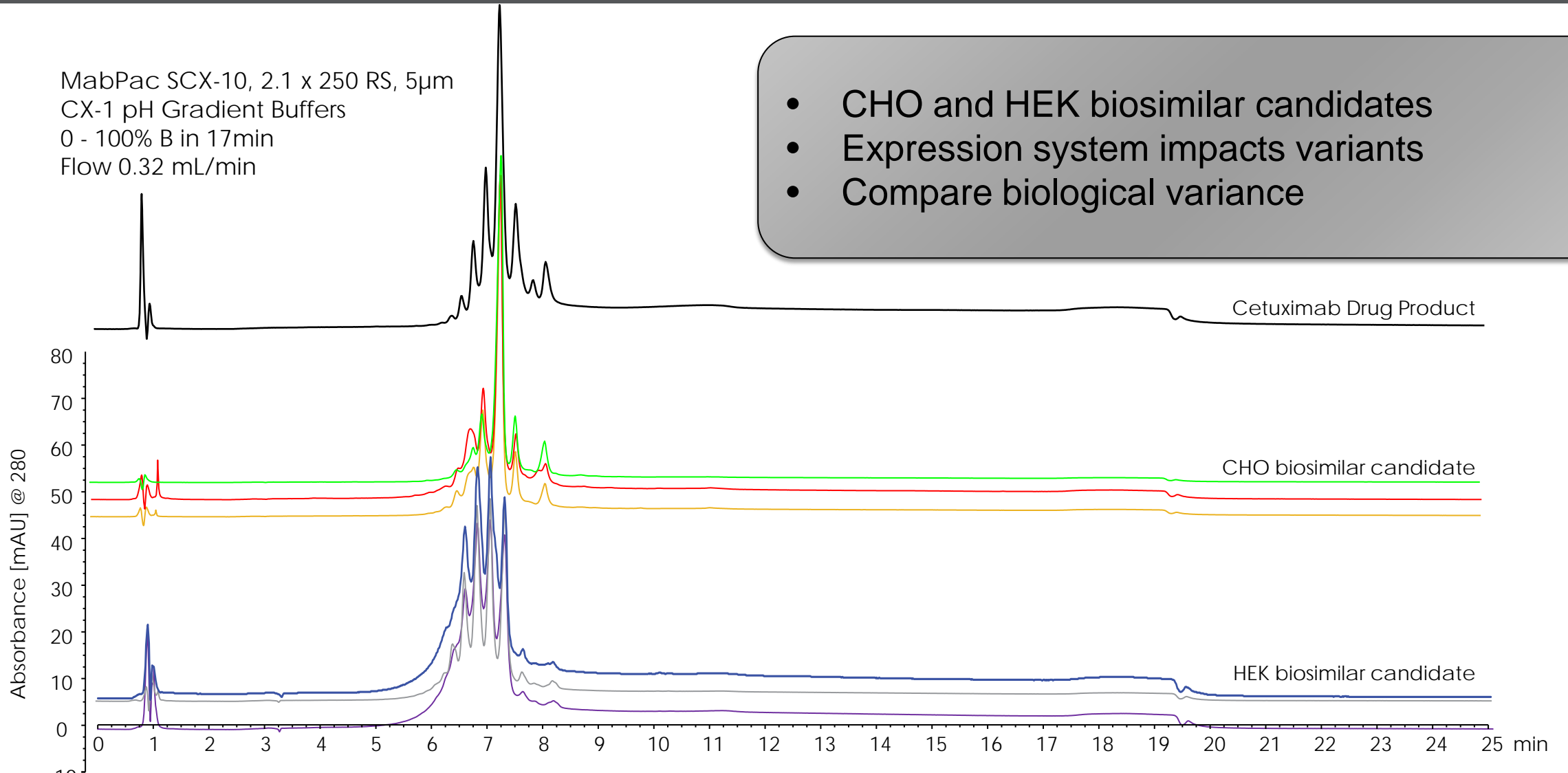
1. **Full gradient run**
10 minutes 0→100% B
2. **Adjusted pH window**
20→40% B in 5 minutes
3. **Flow and shape optimized**
18→27% B in 0.8 minutes

Resolution and number of charge variants maintained in sub-minute gradients

Different Charge Variants

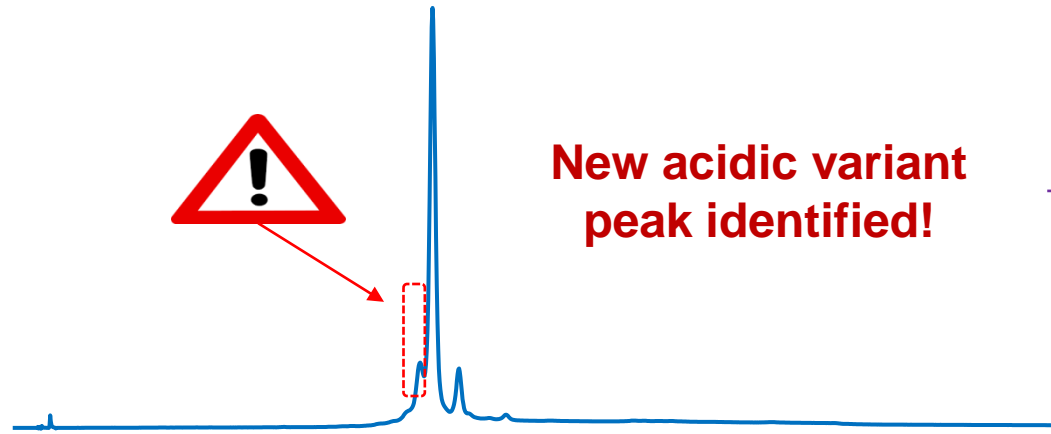
MabPac SCX-10, 2.1 x 250 RS, 5µm
CX-1 pH Gradient Buffers
0 - 100% B in 17min
Flow 0.32 mL/min

- CHO and HEK biosimilar candidates
- Expression system impacts variants
- Compare biological variance

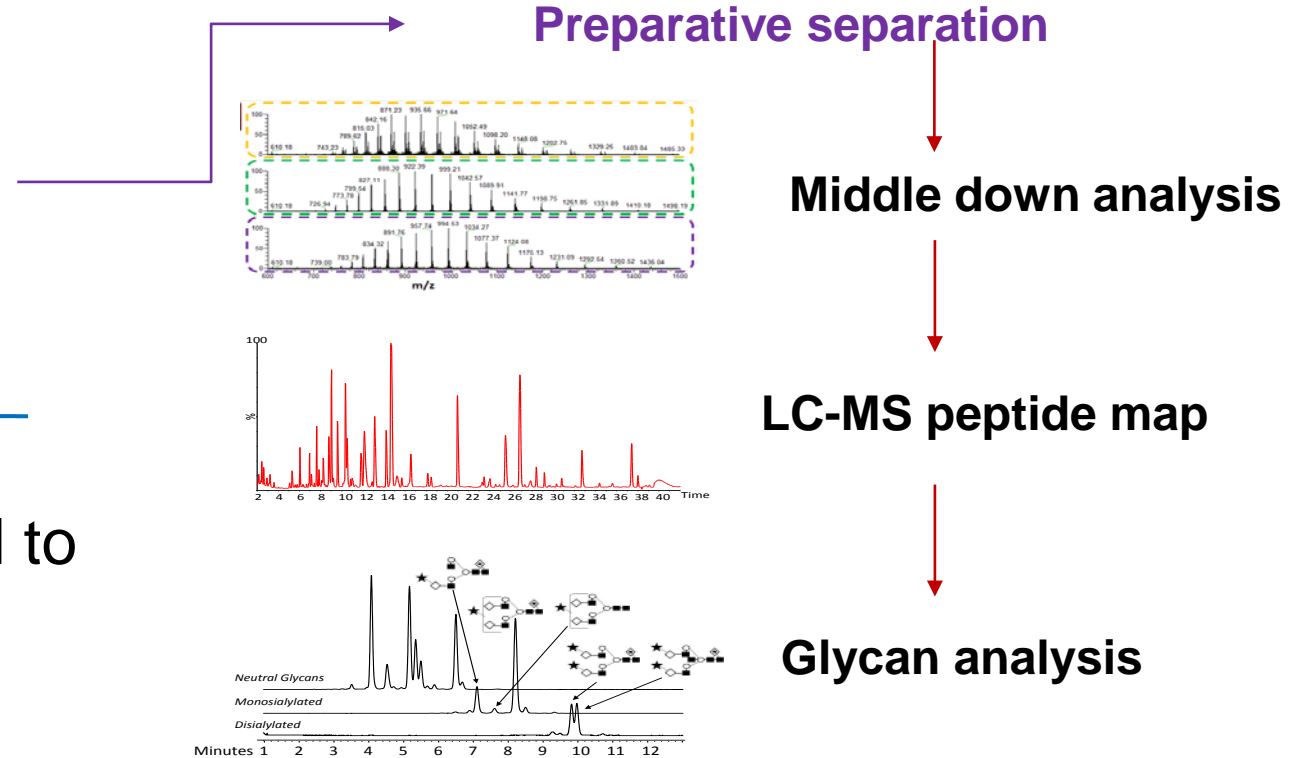


Something Different in the CVA Pattern – Multistep Strategy

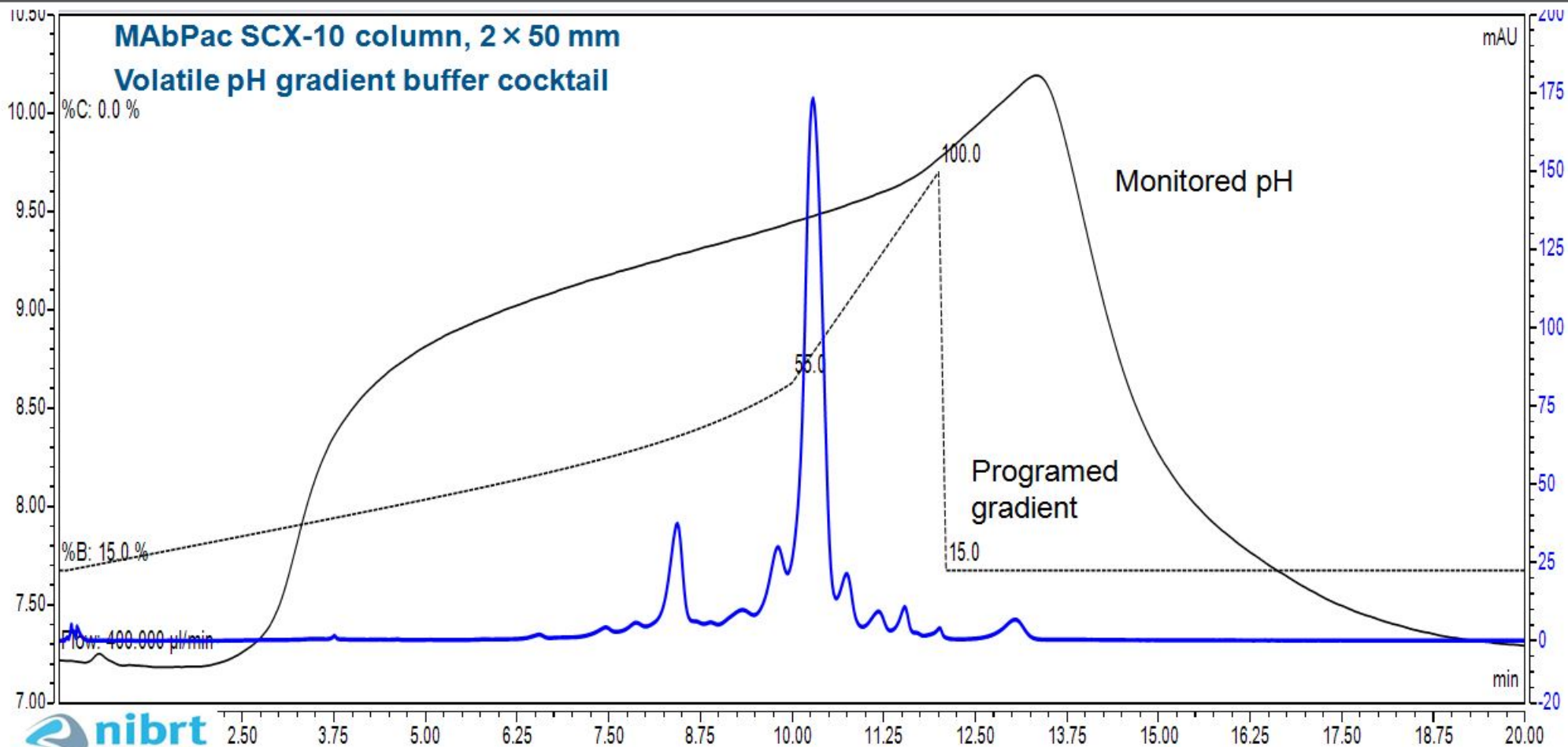
Differences in CVA patterns – Peak areas or appearance of new peaks, require investigation to determine root cause analysis.



Multi-step analytical strategy required to identify the induced posttranslational modification, location within primary sequence and potential on activity

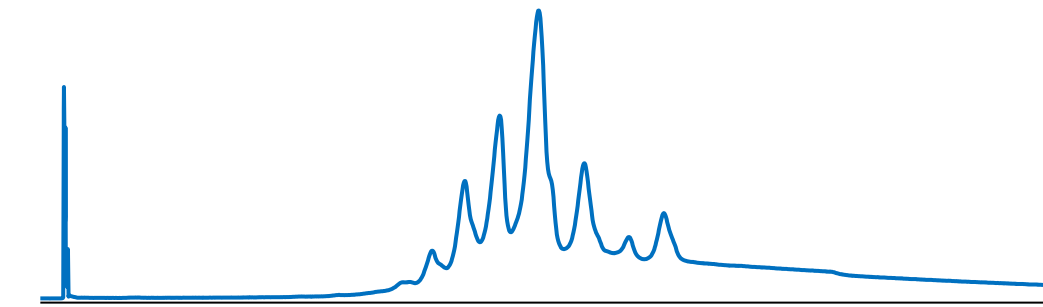


Trastuzumab Elution with MS Friendly Eluents



MS Friendly Eluents Required – Direct coupling of CVA to MS

Matching performance of offline CVA-UV and online CVA-MS – Combination of MS friendly eluents and non-linear gradient curves to generate linear pH elution gradients

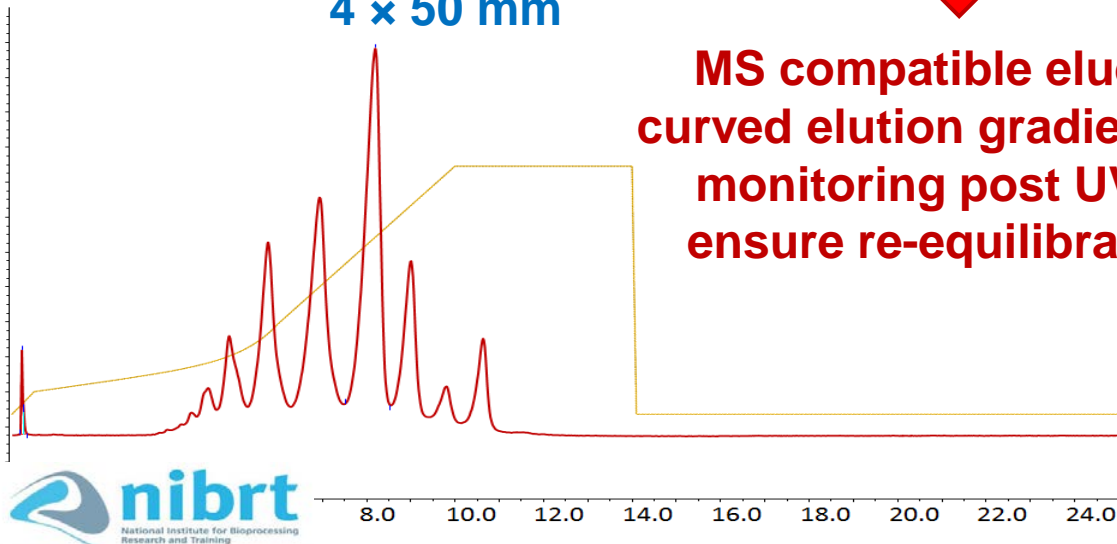
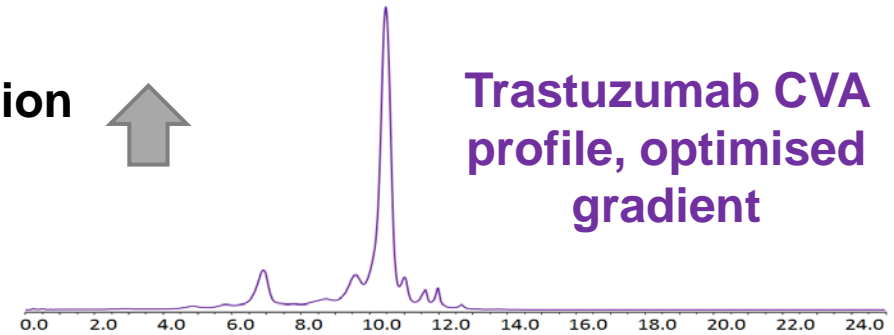


Cetuximab CVA analysis, CX-1 pH gradient buffers on MAbPac SCX 10 4 x 50 mm

MS hyphenation ready



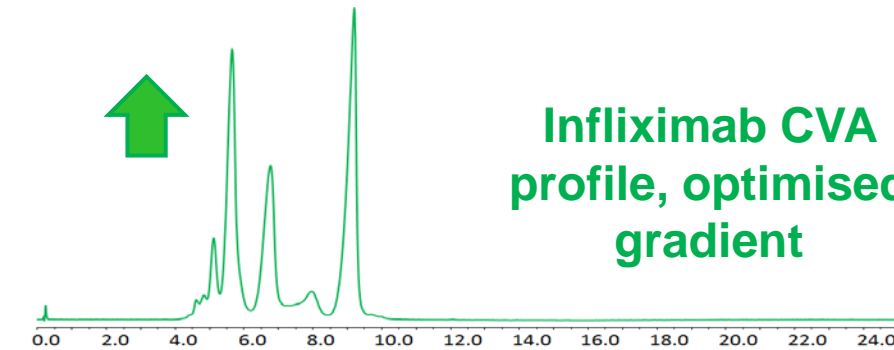
Trastuzumab CVA profile, optimised gradient



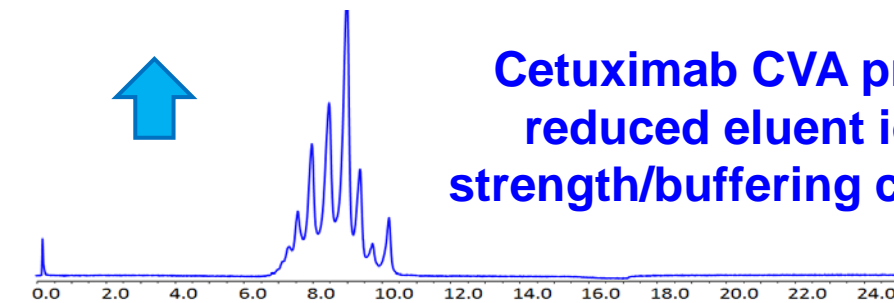
MS compatible eluent, curved elution gradient, pH monitoring post UV to ensure re-equilibration



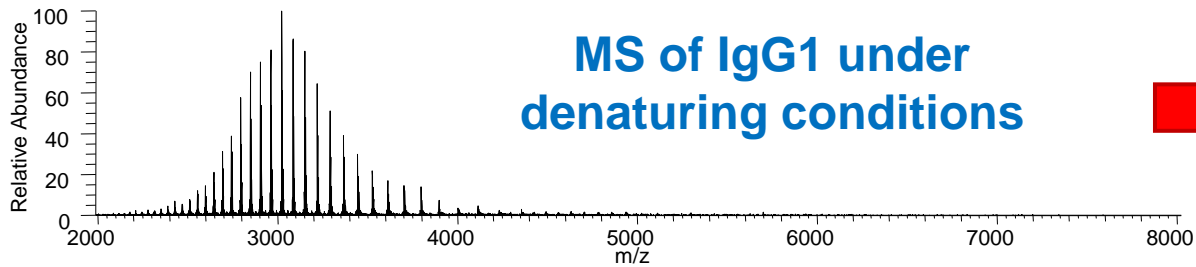
Infliximab CVA profile, optimised gradient



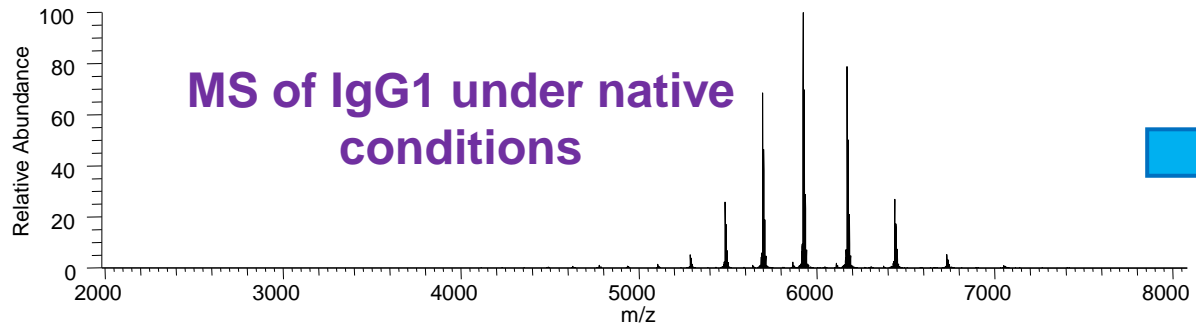
Cetuximab CVA profile, reduced eluent ionic strength/buffering capacity



Coupling to High Resolution Native MS



Reversed phase chromatography or other separation modes containing organic solvents

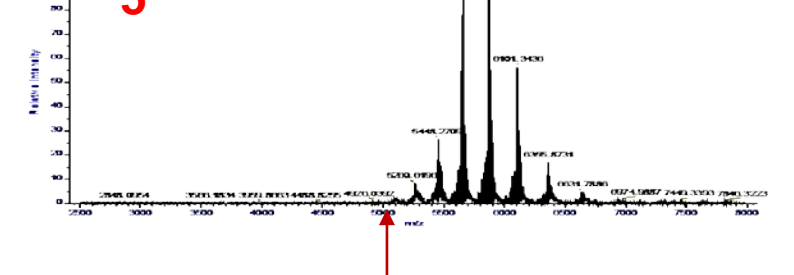
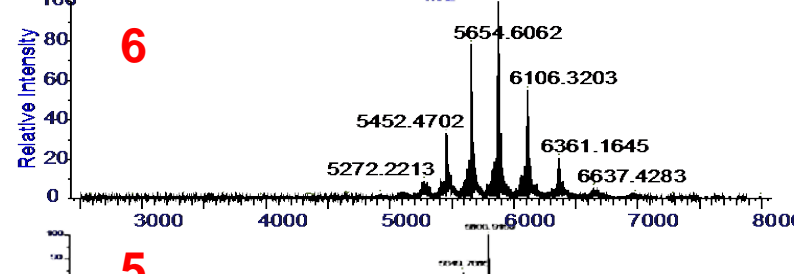
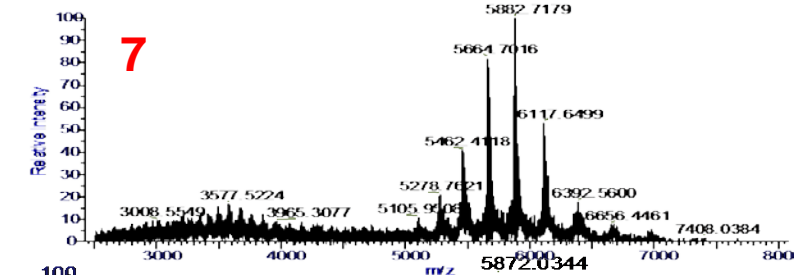
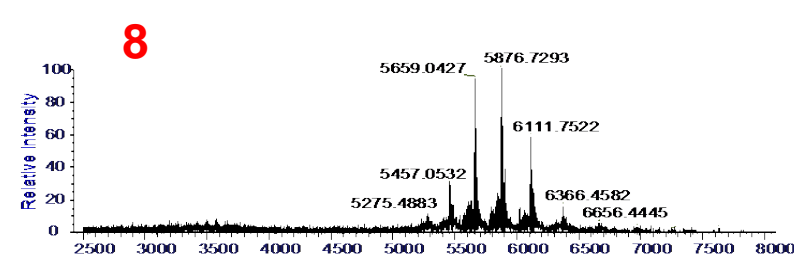
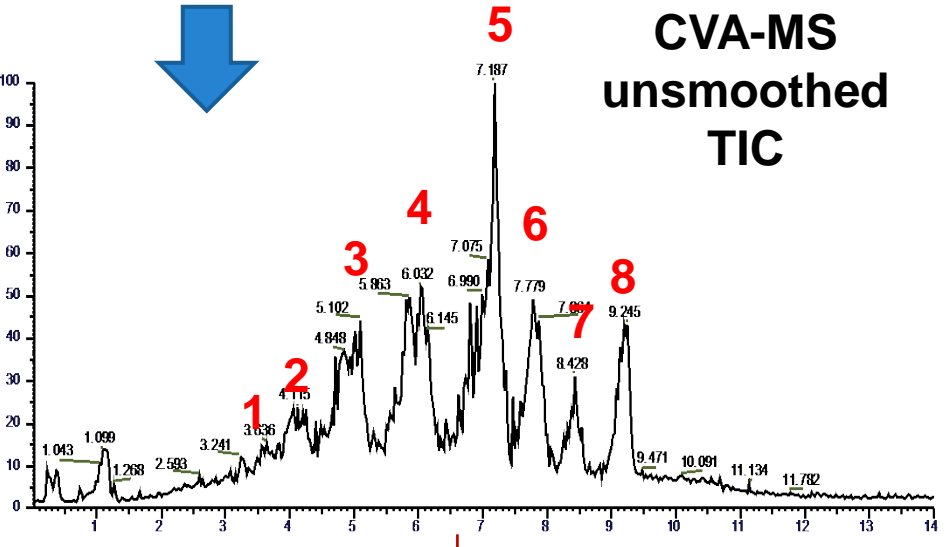
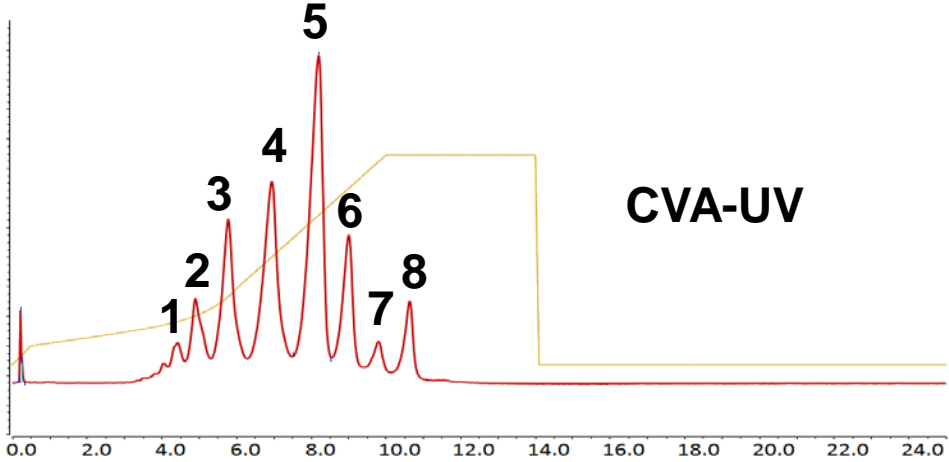
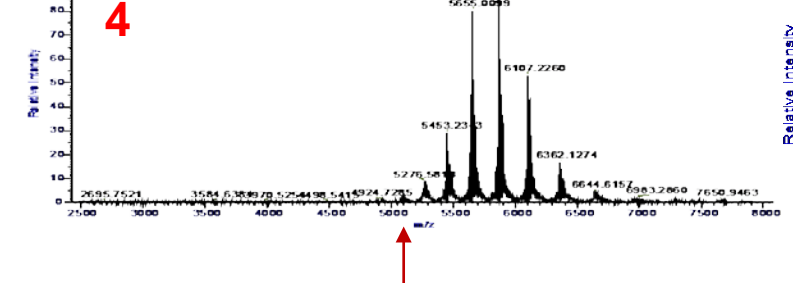
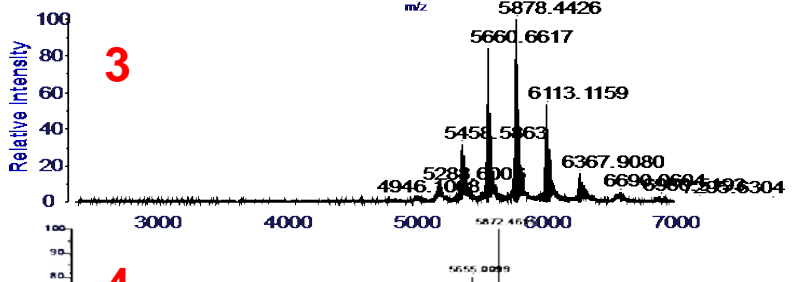
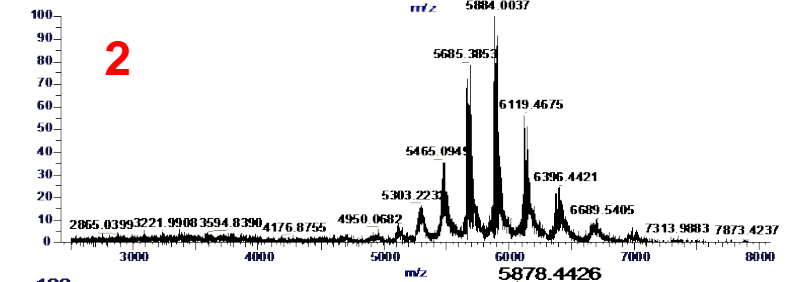
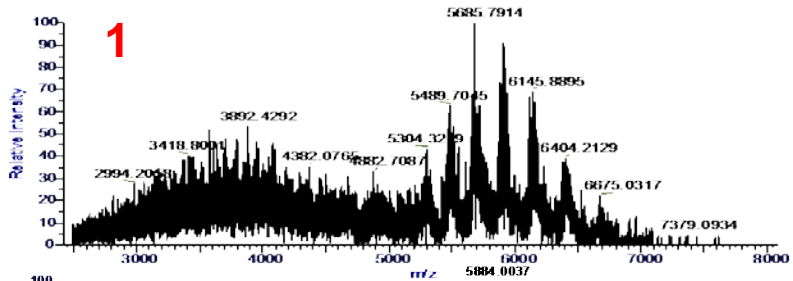


Traditional protein chromatography: SEC, now also CEX, AEX

- MS analysis of mAbs under native conditions is powerful, reflection of surface charge present in solution
- Greater spatial spectral resolution in native MS spectra
- Requires optimisation of source parameters: temperature, gas flow, in source CID for desolvation

Native CVA-MS of Cetuximab

Separation of Cetuximab proteoforms on MAbPac SCX using developed pH gradient elution



Conclusions

Game changing automation of protein digestion for ultimate precision and reproducibility in a robust peptide mapping workflow;

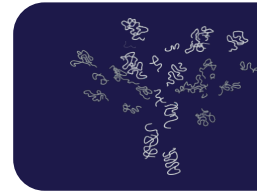
pH gradient elution of proteins for CVA has several advantages including; global applicability, increased speed, fast method development, high loading capacity, easy method transfer, new native MS compatibility;

Inert UHPLC with a sensitive high resolution mass spectrometer enables several critical workflows.

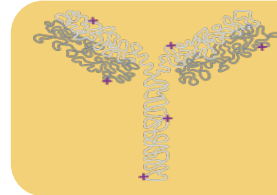
Industry specific Thermo Scientific™ Biopharma Finder™ software;

New workflows enabling characterization of several attributes in one injection provides ease of use and time saving – peptide mapping and CVA/MS.

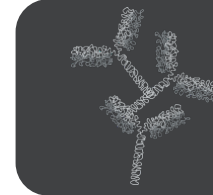
Native MS now has SEC and ion exchange.



Peptide mapping



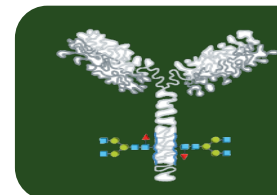
Charge variant screening



Aggregate screening



Intact analysis



Glycan analysis



Do you have additional questions or do you want to talk to an expert from Thermo Fisher Scientific?

Please send an E-Mail to analyze.eu@thermofisher.com and we will get back to you.