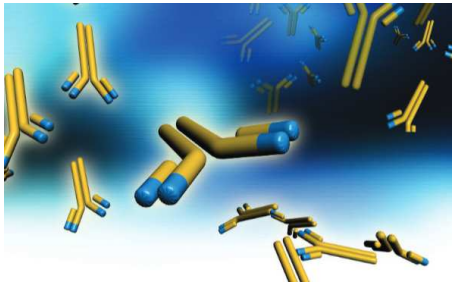
ThermoFisher SCIENTIFIC



UHPLC for Large Bio-Therapeutics

Ken Cook

EU Bio-separations Support Expert

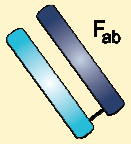
June 2015

The world leader in serving science

Various Types of Antibody-Based Therapeutics

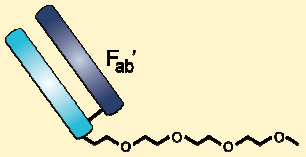
mAb Fab fragment

Cimzia (certolizumab pegol)
Lucentis (ranibizumab)



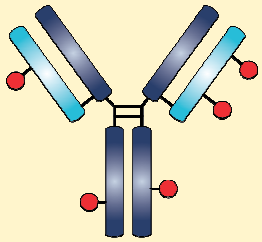
PEGylated mAb

Cimzia (certolizumab pegol)



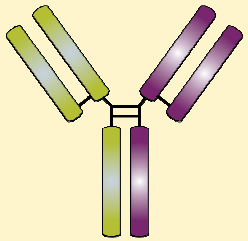
Antibody-drug conjugate

Kadcyla (trastuzumab emtansine)
Adcetris (brentuximab)



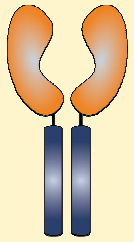
Bispecific mAb

Removab (catumaxomab)



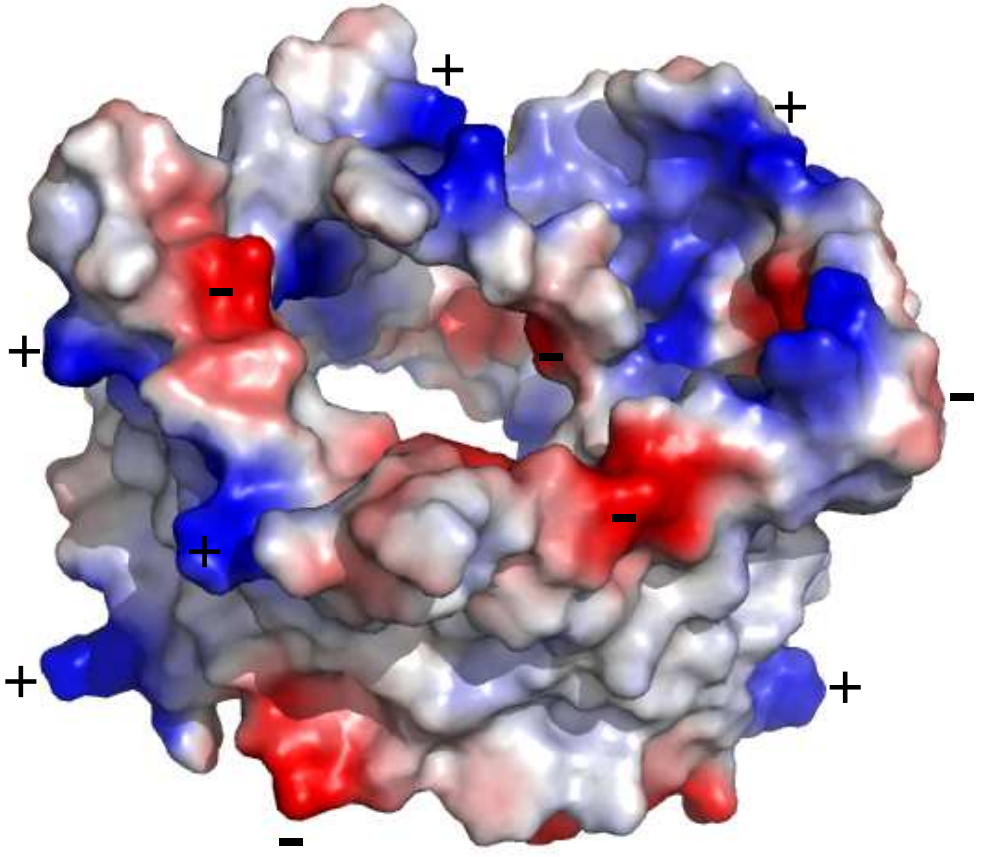
Fusion protein

Enbrel (etanercept)
TNF receptor-Fc fusion

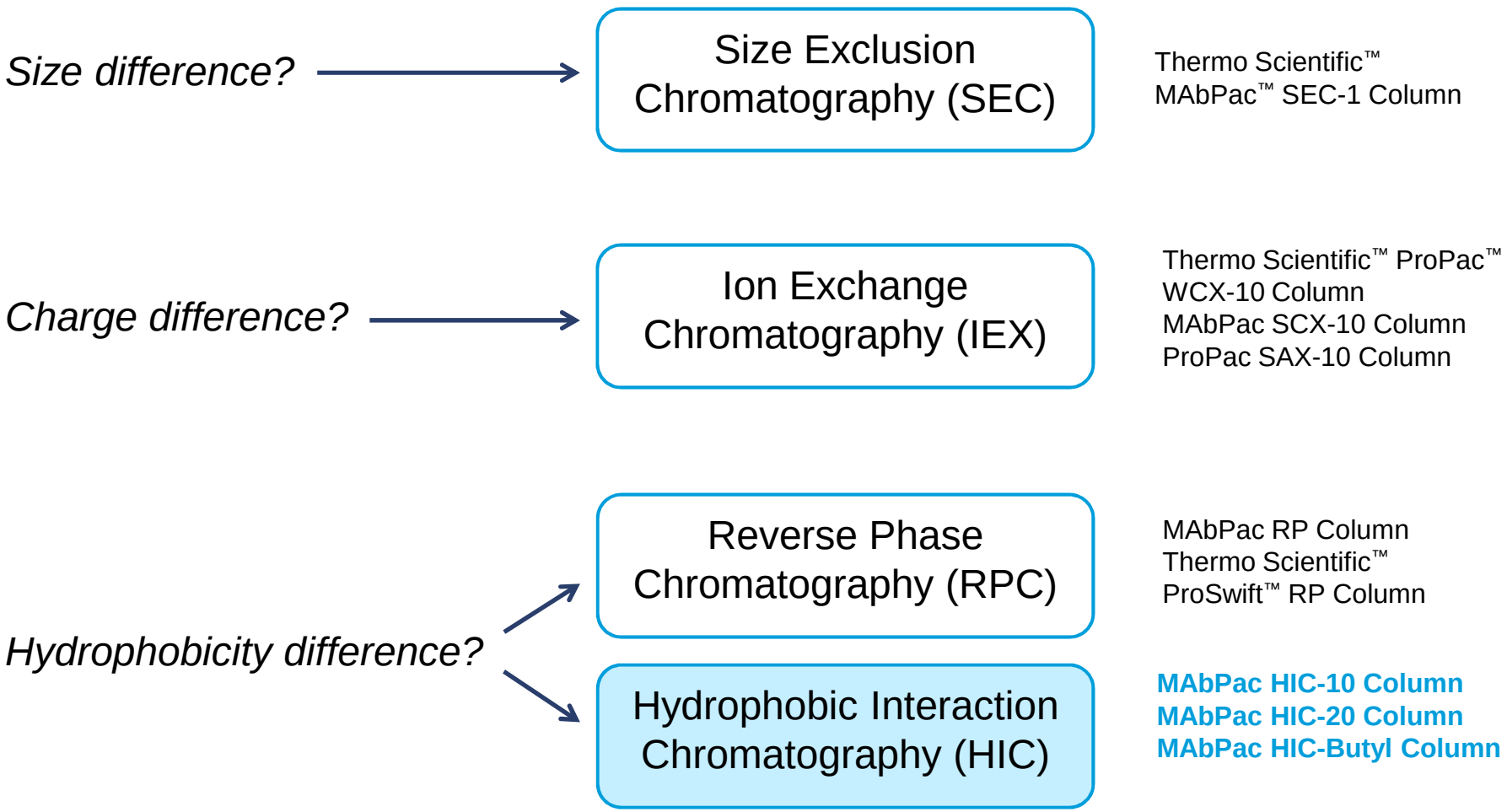


Properties of Proteins

- Properties:
  - Size
  - Charge
  - Hydrophobicity
  - Affinity or Recognition



Protein Separation by UHPLC



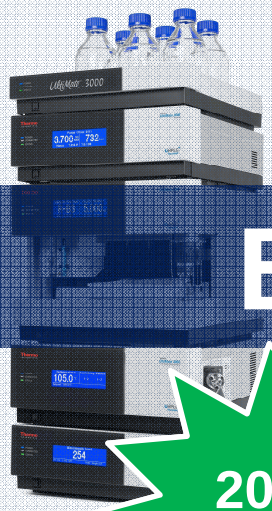
Requirements for Development of New Solutions

- High resolution – small changes in a large molecule
- Speed – many variations to analyse and many potential drug candidates
- Robust analysis
- Global methods to reduce method development time
- Multidimensional possibilities
- **New systems and new chemistries**

Biopharma UHPLC Portfolio

Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System

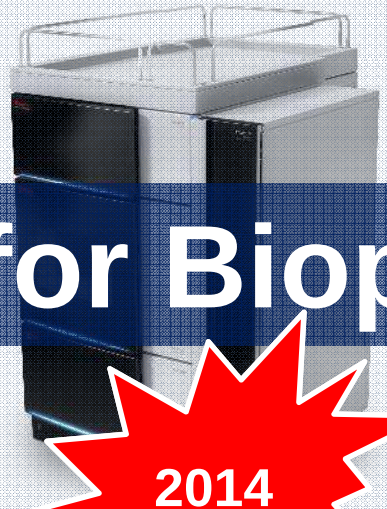
Established biocompatible UHPLC.
Key benefit: ***Proven***



2013

Thermo Scientific™ Vanquish™ System

Flagship system.
Key benefit: ***High end performance***



2014

Vanquish Flex System

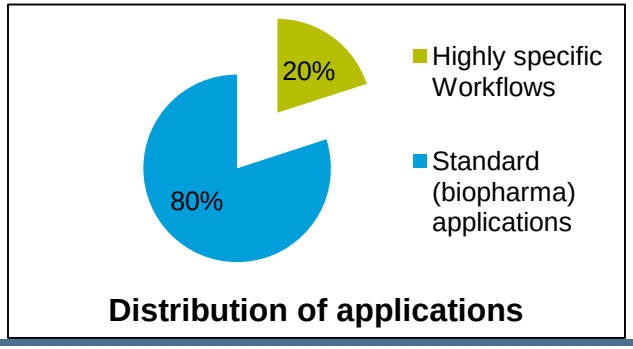
Designed for <u>all</u> biopharma labs. Key benefit: ***Flexibility***

2015

Built for Biopharma

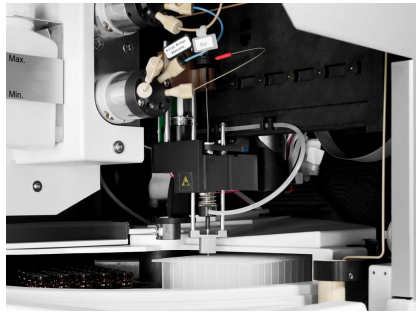
2015 Systems for Bio-Therapuetic Analysis

Vanquish Systems



UltiMate 3000
BioRS system

High Resolution, Cooled Fractionation



pH and Conductivity

System Biocompatibility by Default

UHPLC is now not limited to small molecules
Biomolecular analysis can benefit from the higher resolution and faster analysis this technique offers.

Thermo Scientific™ Viper™ Fingertight Fittings
MP35N 100um i.d. Finger tight and zero dead volume.

Vanquish Parallel Binary Pump
Independent piston drives and variable stroke volume, 1500 bar. – ultra low baseline noise

LightPipe™ Technology in the Vanquish DAD Detector
Provides you with an unmatched detection experience. Ultra-wide dynamic range, high sensitivity

Sampler
Zero maintenance ceramic injection valve, loop pre compression, 4 x 96 well plates, charger.

Oven
Adiabatic and forced air. 30cm column, active eluent preheating, column switching, ceramic valves.

In Solution Trypsin Digestion

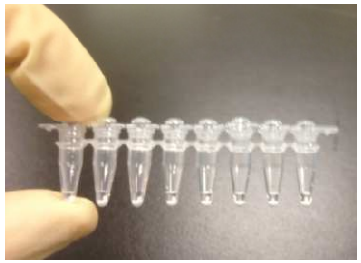
- Do a protein concentration assay
- Make up to 8M Urea
- Add DTT or TCEP at 70 C and wait for 30 minutes
- Make up Iodoacetate
- Add Iodoacetate and wait 15 minutes
- Dilute to 1 M Urea
- Make up Trypsin
- Add trypsin to vial and and wait for 4 hours
- Add more Trypsin and digest overnight
- Spin to remove particulates
- Possible SPE to remove detergents and Urea

Thermo Scientific Smart Trypsin Digestion

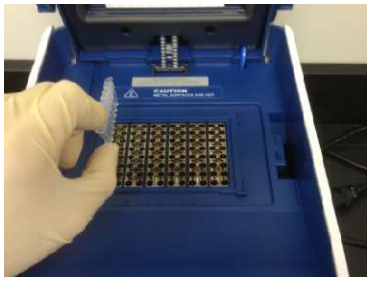
- Heat stable Trypsin
- 70 ºC heat denaturation of proteins so no need for detergents or urea
- Immobilized Trypsin in throw away PCR tube
- No autolysis
- Less steps and no clean up – much quicker and easier to use
- Less modifications
- More reproducible

Up to 96 Samples, ≤ 60 Minute No Pretreatment Necessary

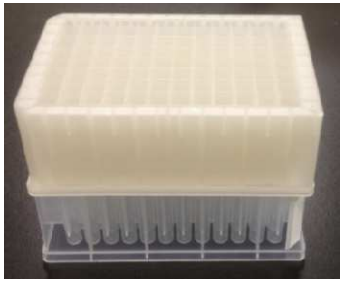
❶ Undigested Protein



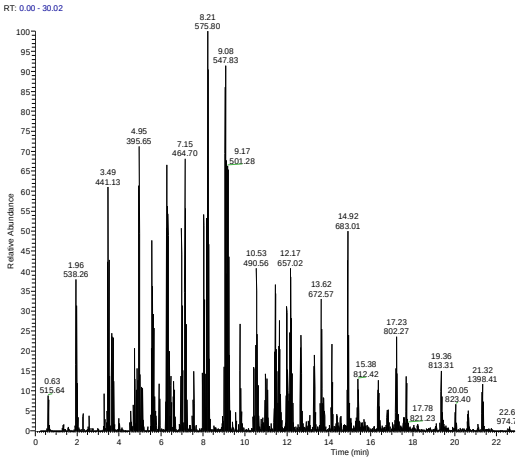
❷ ≤ 90 ºC, ≤60 Minutes



❸ Filter



LC/MS



- Complete digest of native protein in ≤ 60 minutes
- Pretreatment (reduction and alkylation) – optional
- Equipment required – thermal cycler
- Multiple patents pending

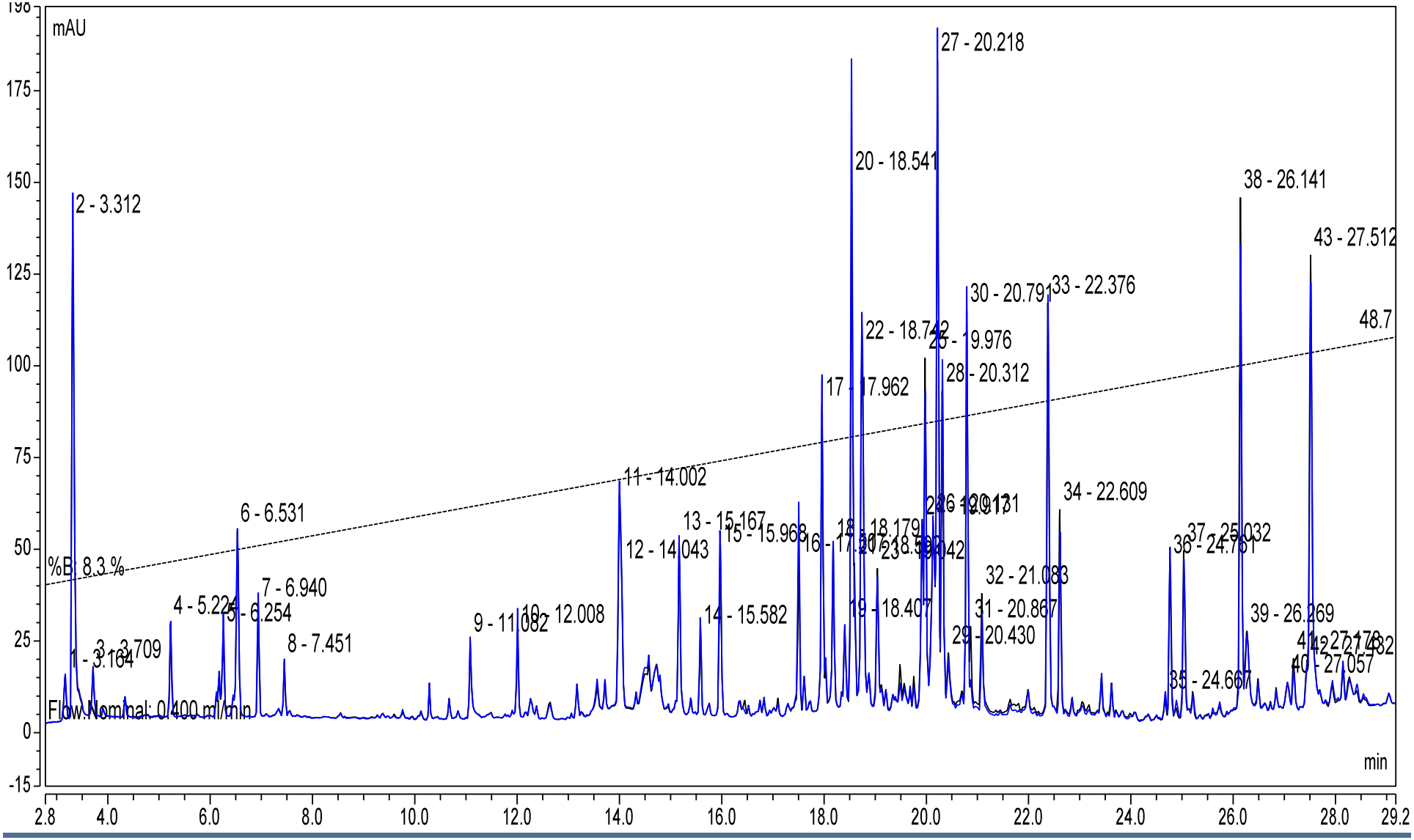
Time for Digestion

Overall time for digestion is faster

Actual hands on preparation time is drastically reduced

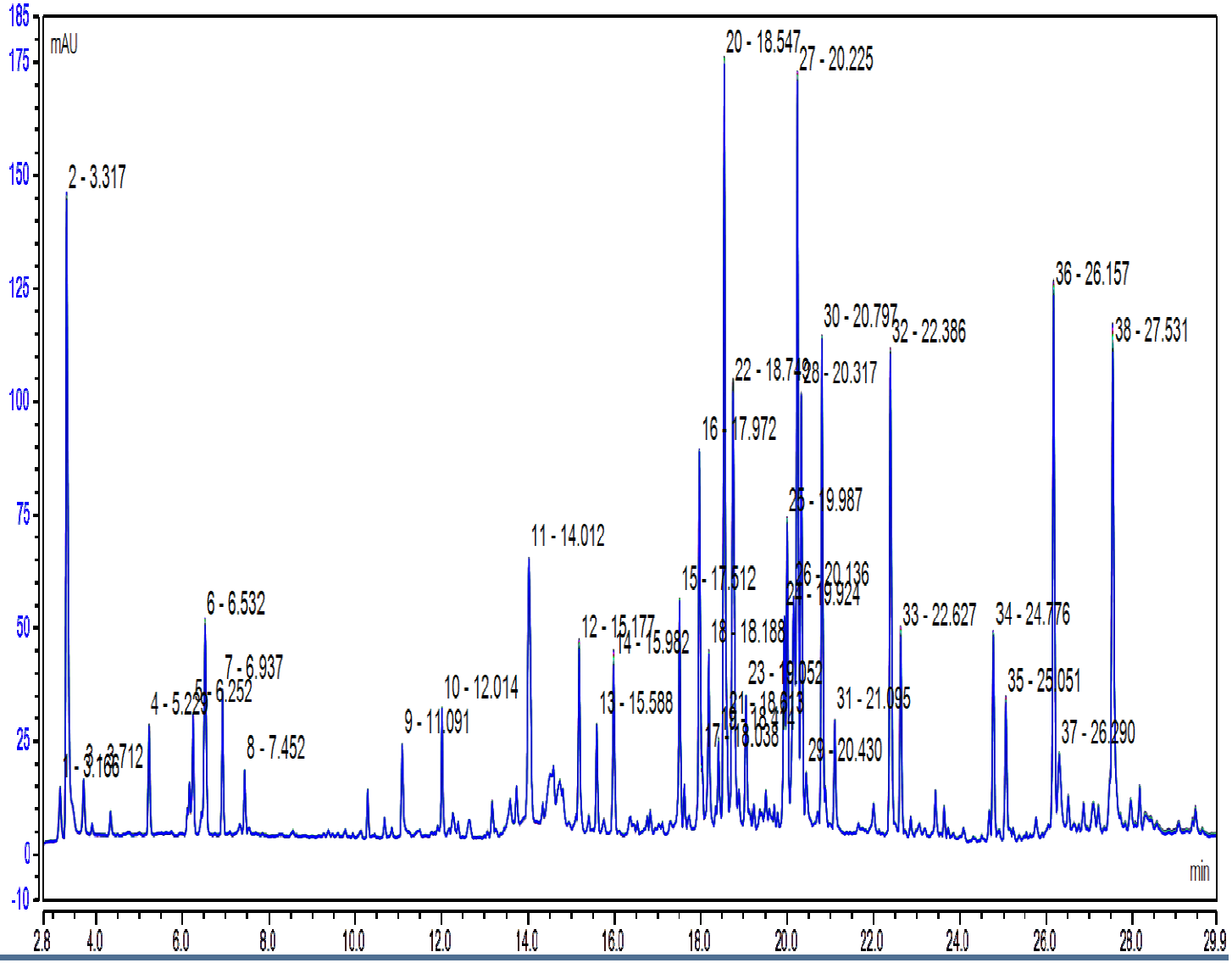
Less repetitive manual steps reduces inaccuracy

Overlay of Different mAb Digests on the Vanquish System



Overlay of 10 Runs of a Trypsin Digest mAb with Retention Time Precision

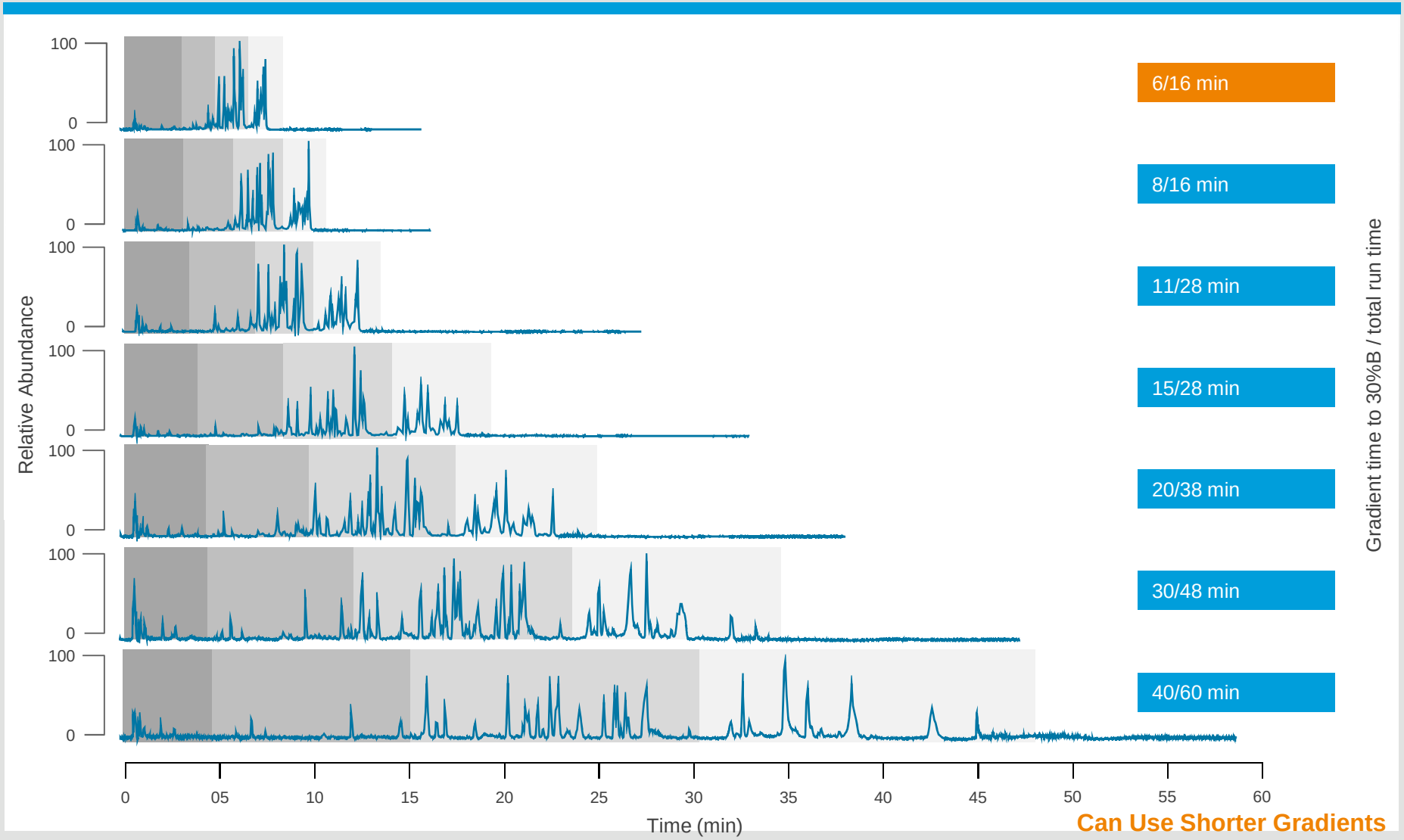
peak #	RT (min)	RT-RSD (%)
3	3.315	0.082
9	5.231	0.065
14	6.532	0.017
15	6.937	0.023
19	10.290	0.021
23	12.013	0.012
31	14.011	0.013
39	15.177	0.012
42	15.589	0.010
51	17.511	**0.007**
55	17.969	0.011
61	18.546	0.010
83	20.798	0.010
85	21.095	0.012
87	22.386	**0.009**
96	24.774	0.012
103	26.155	**0.009**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



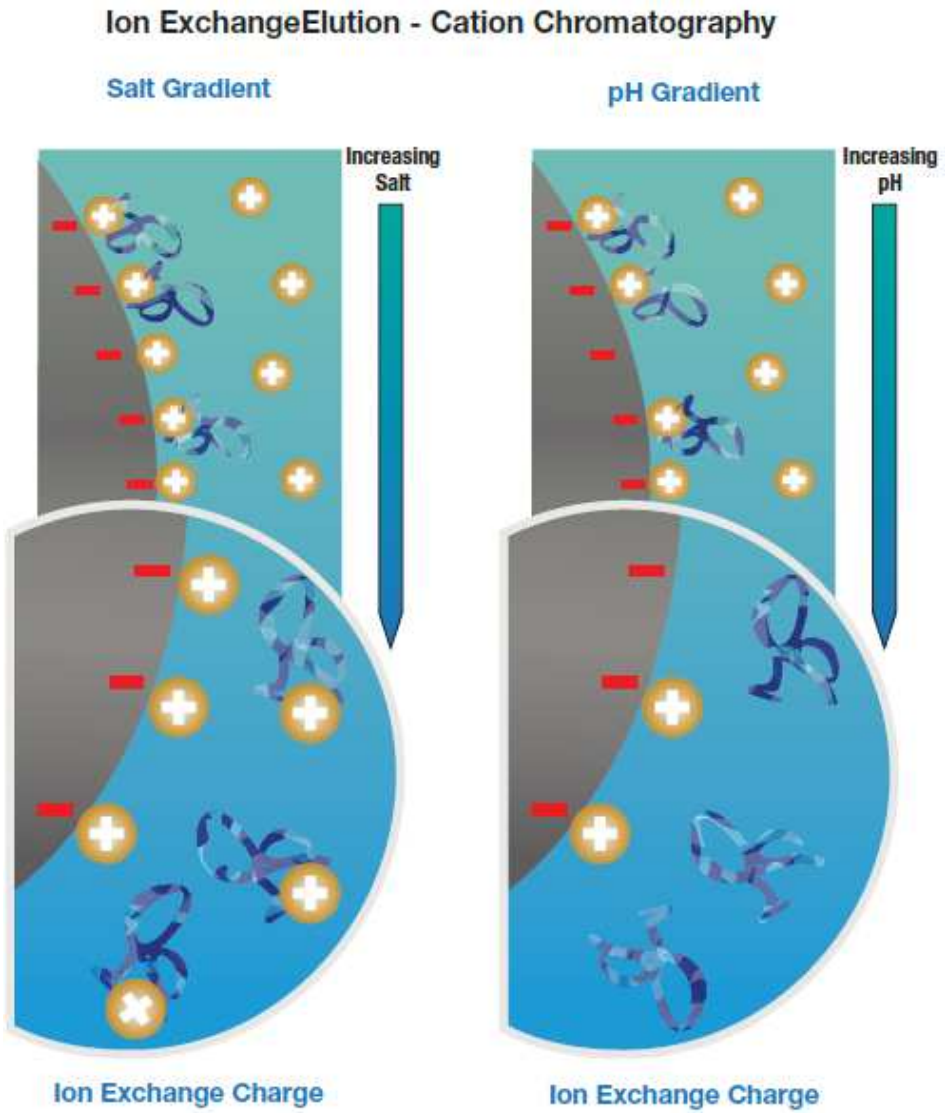
Slide 14

JH1 Please format with title-case text.
Jim Hegarty; 15.10.2014

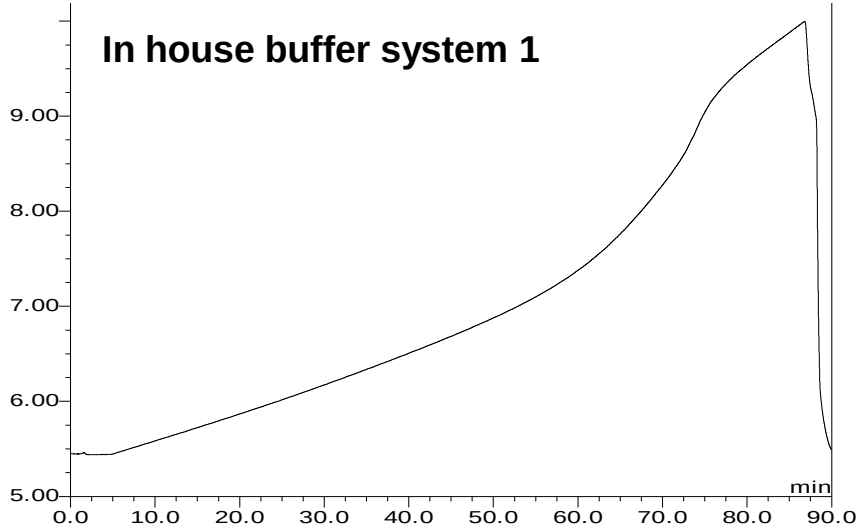
Peptide Maps of Herceptin Digest; 6-40min Gradients

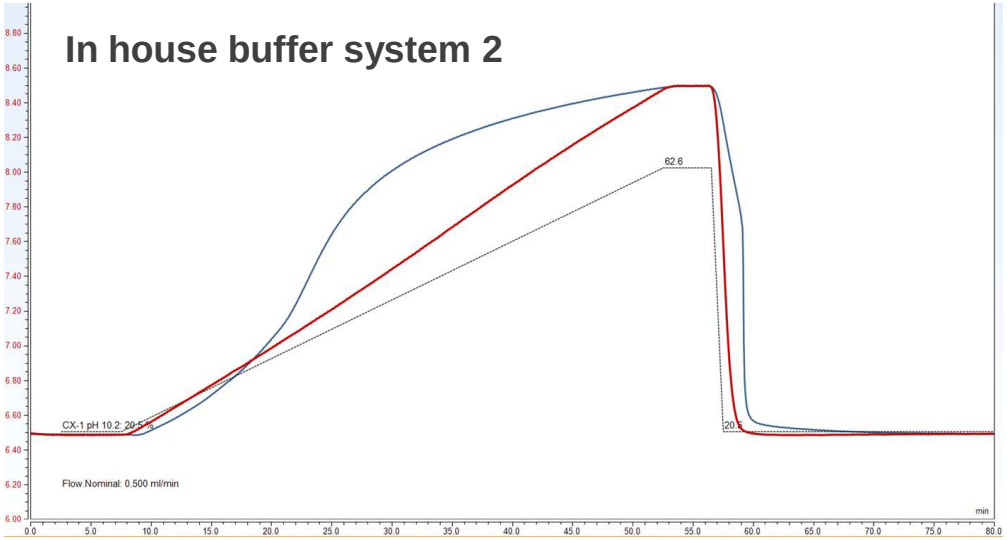


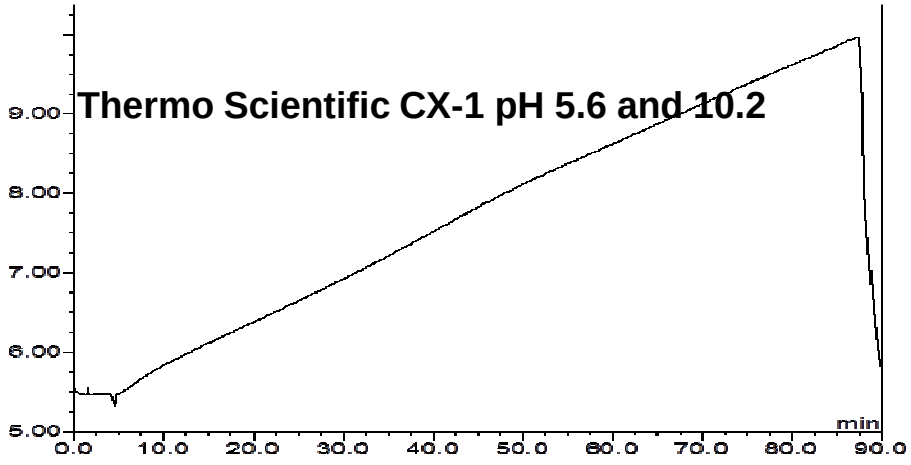
Mechanism of Salt and pH Elution of Proteins

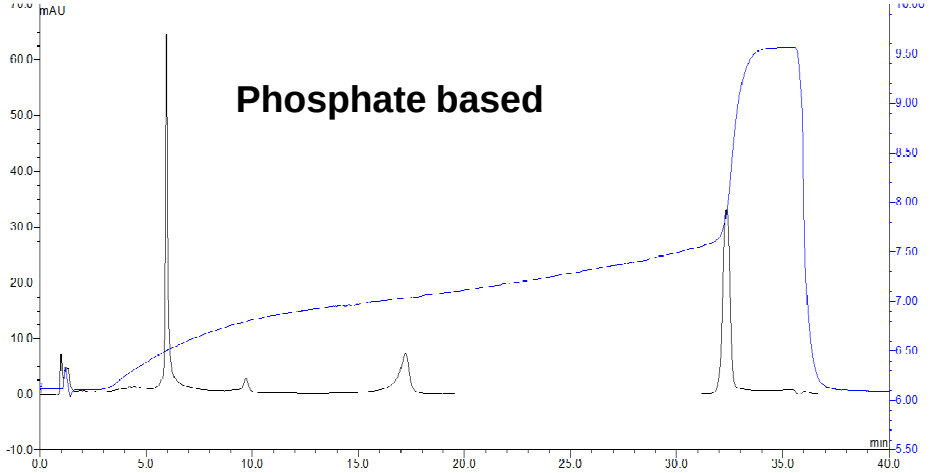


Comparison of pH Gradient Buffer Systems

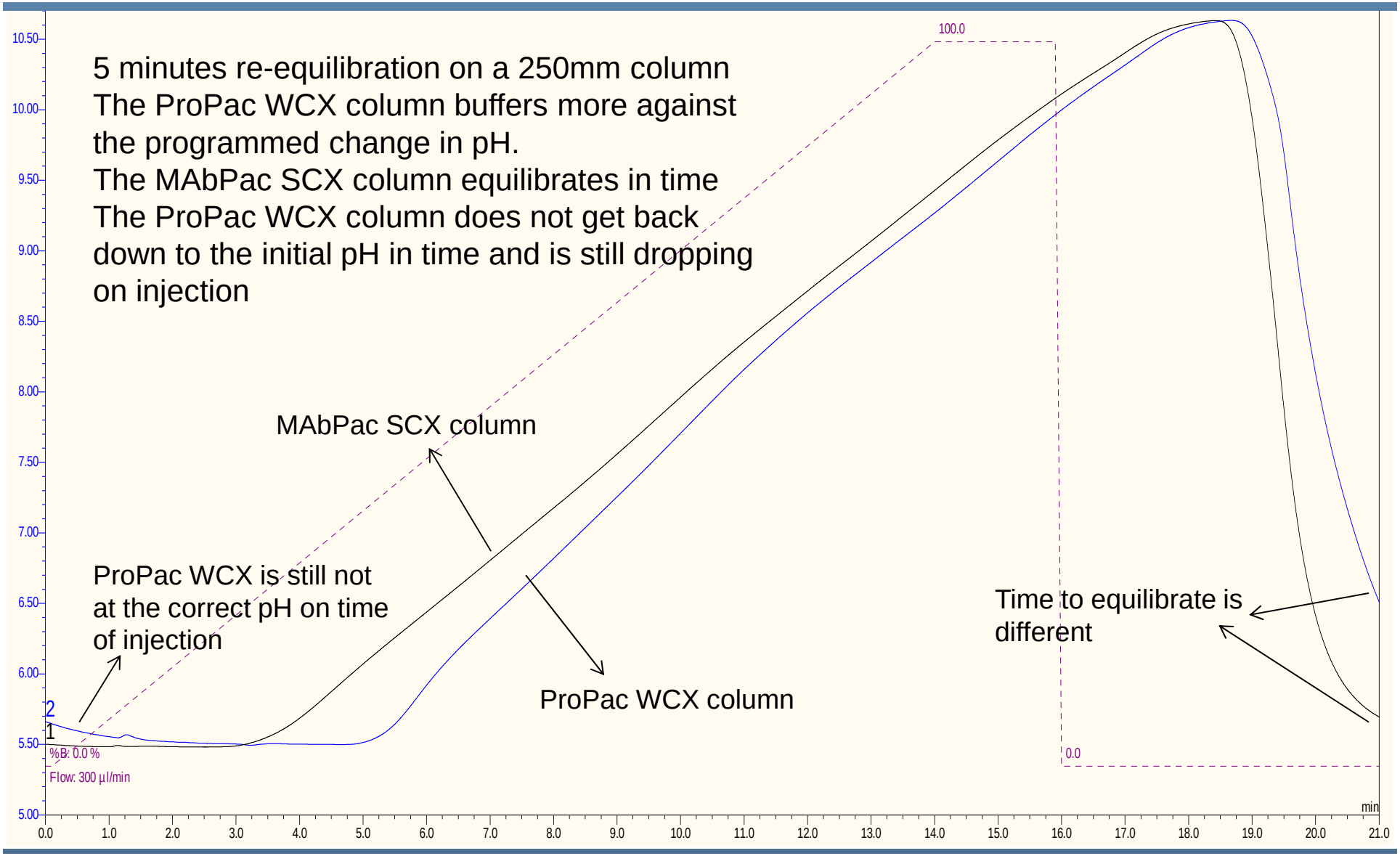








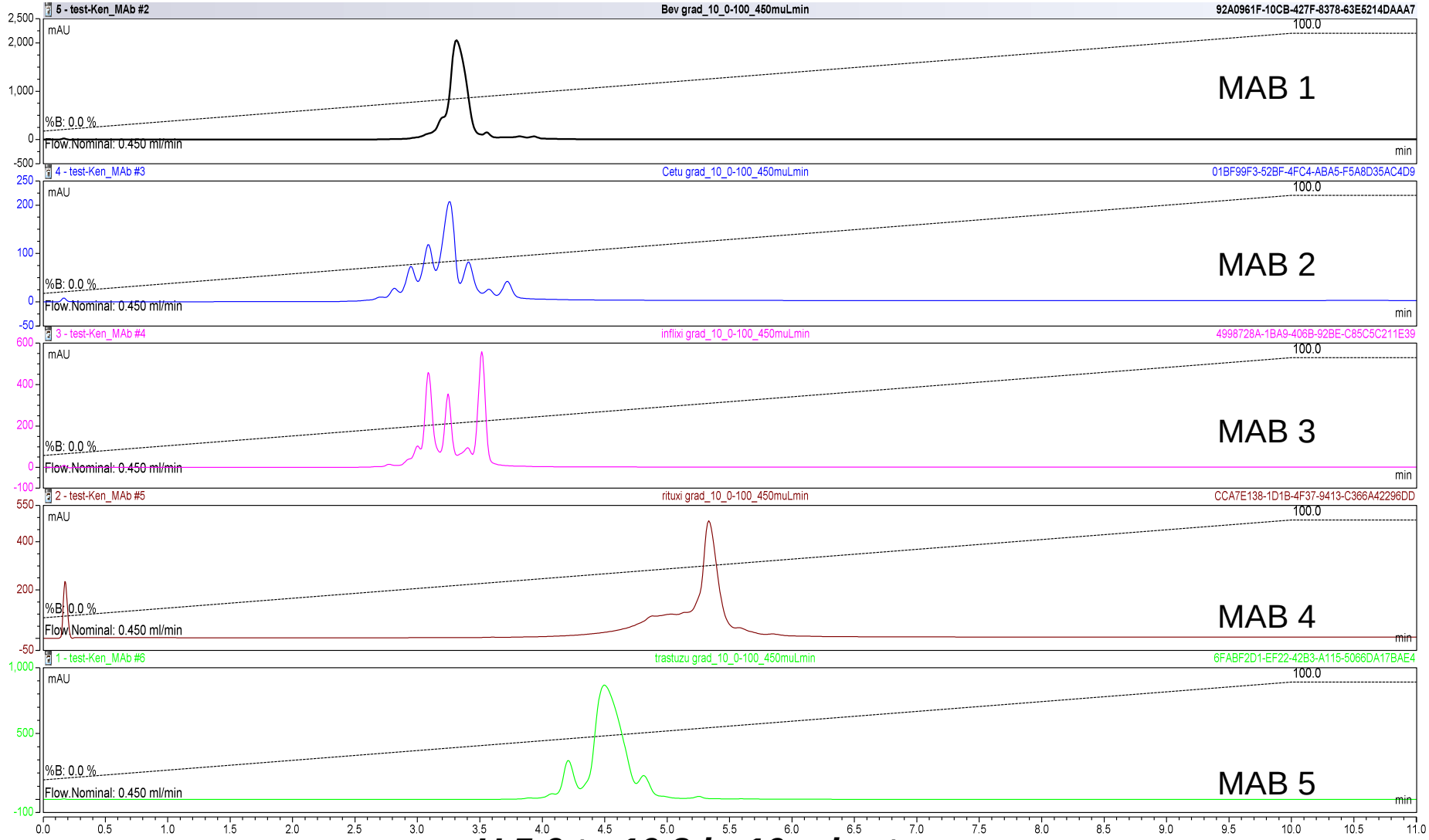
Re-equilibration Problems



Improving Charged Variant Analysis

- Speed of analysis – 60min to 6min or below?
- Resolution – No loss in resolution
- Time to develop the method
- Global applicability
- Robustness

Generic Linear pH Gradient with the Vanquish System

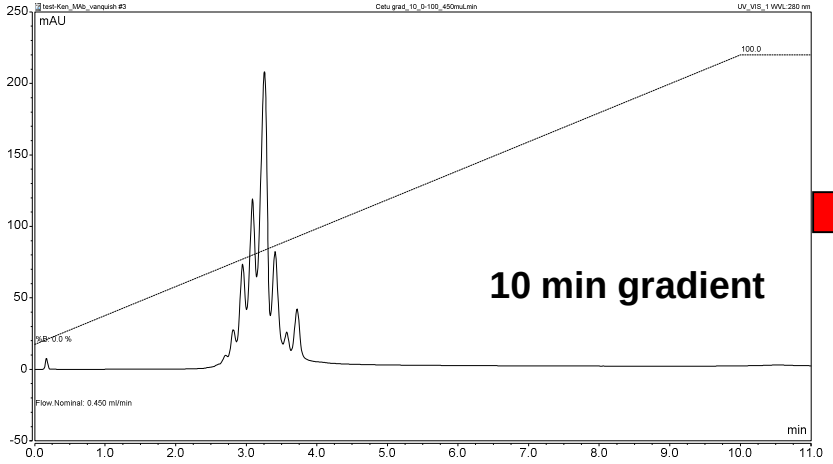


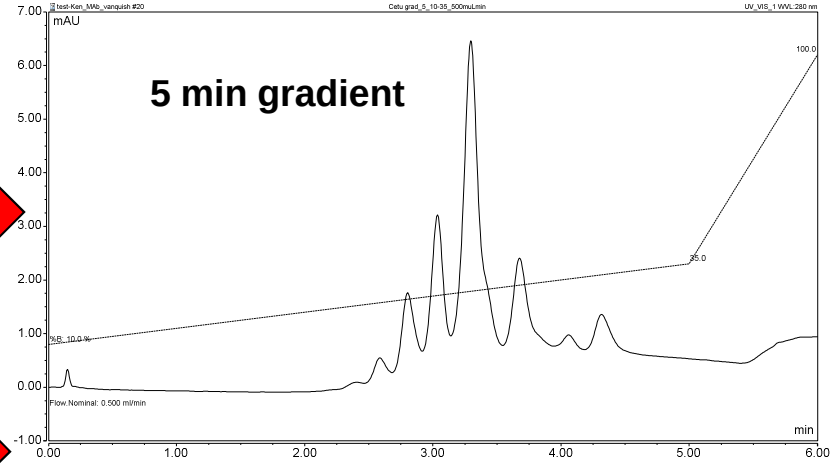
pH 5.6 to 10.2 in 10 minutes

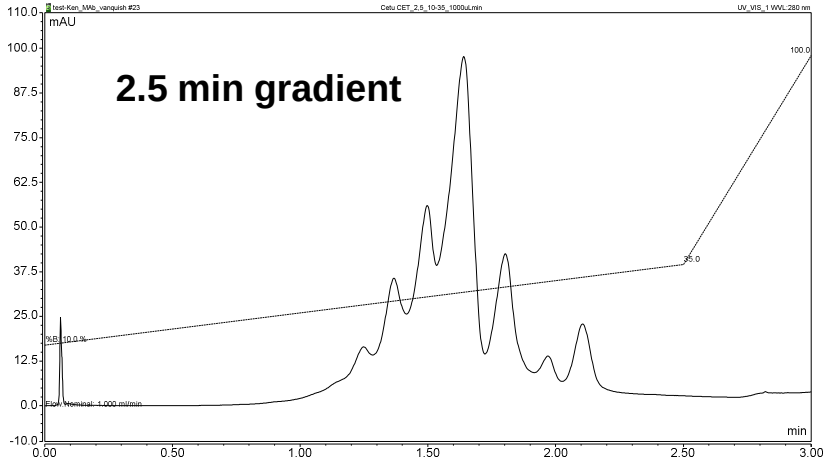
Slide 20

JH3 Please format with title-case text.
Jim Hegarty; 15.10.2014

Example 1/5: Cetuximab – Vanquish System Fast Gradients





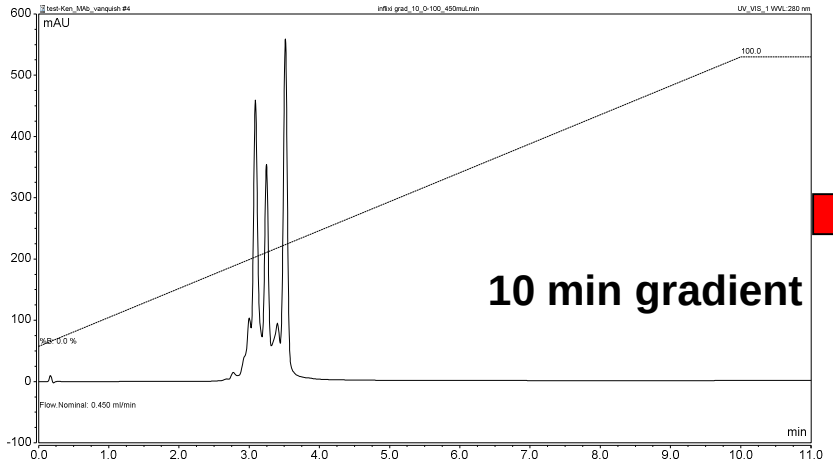


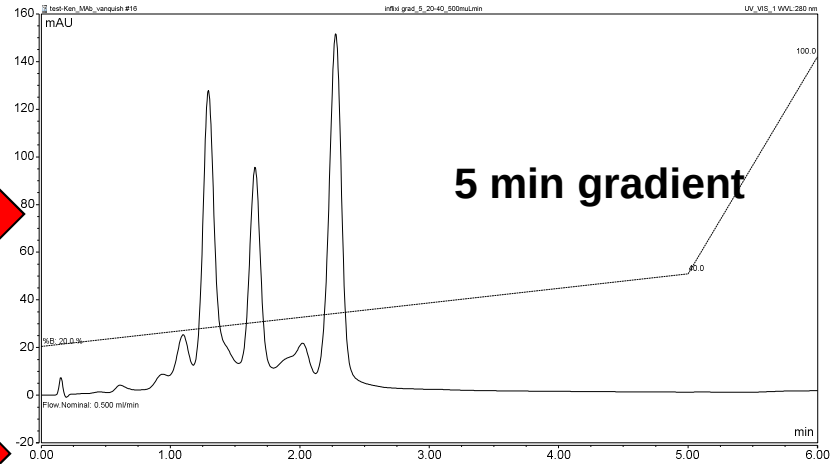
3 steps method development

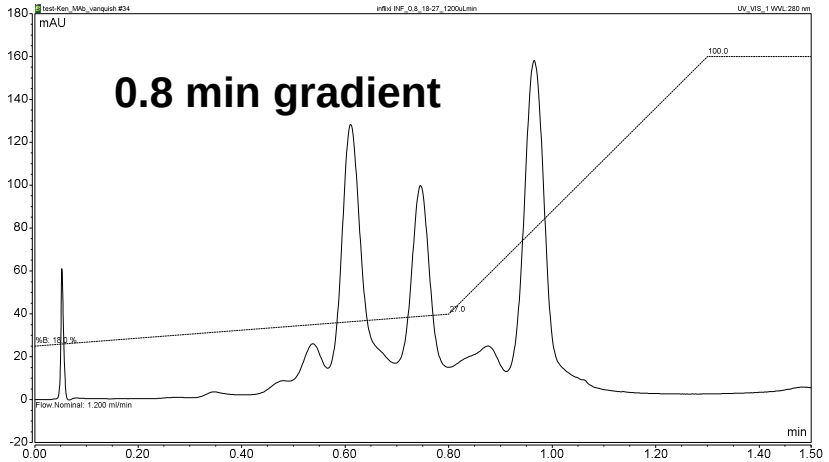
1. 10 minutes 0→100% B in 10 minutes
2. 10→35% B in 5 minutes
3. 10→35% B in 2.5 minutes

Number of charge variants and resolution maintained for 2.5 min gradient

Example 2/5: Infliximab – Vanquish System Fast Gradients





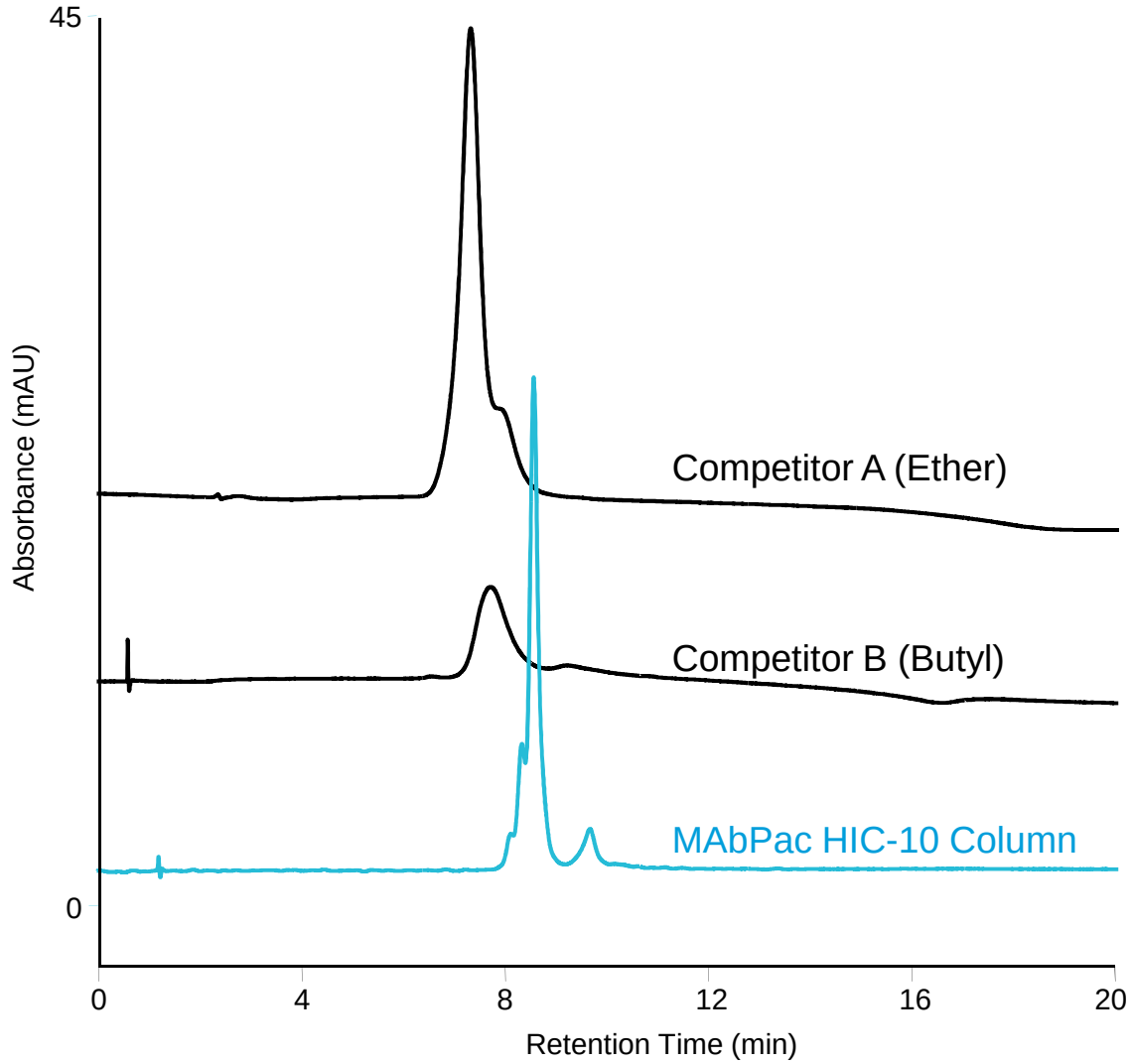


3 steps method development

1. 10 minutes 0→100% B in 10 minutes
2. 20→40% B in 5 minutes
3. 18→27% B in 0.8 minutes

Number of charge variants and resolution maintained for sub-minute gradient

Hydrophobic Interaction Chromatography

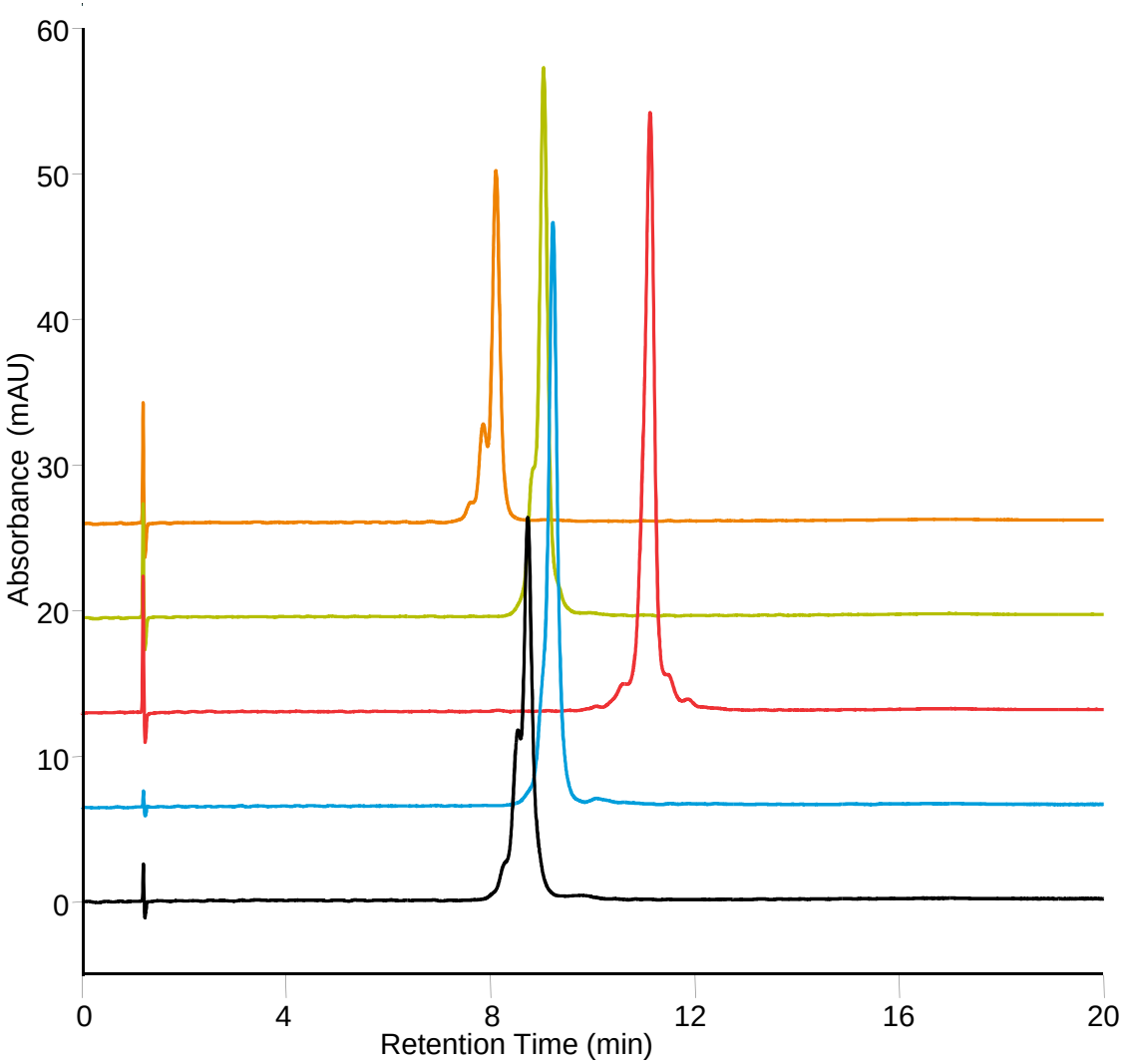


Column:	MAbPac HIC-10, 4.6 × 100 mm Competitor A (Ether), 7.5 × 75 mm Competitor B (Butyl), 4.6 × 100 mm
Mobile phase A:	2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B:	100 mM sodium phosphate, pH 7.0
Gradient:	

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

Temperature:	30 ºC
Flow rate:	1.0 mL/min
Inj. volume:	2 µL (4 mg/mL) Competitor A (Ether): 4 µL
Detection:	UV (280 nm)
Sample:	mAb

Global Analysis of Intact mAbs



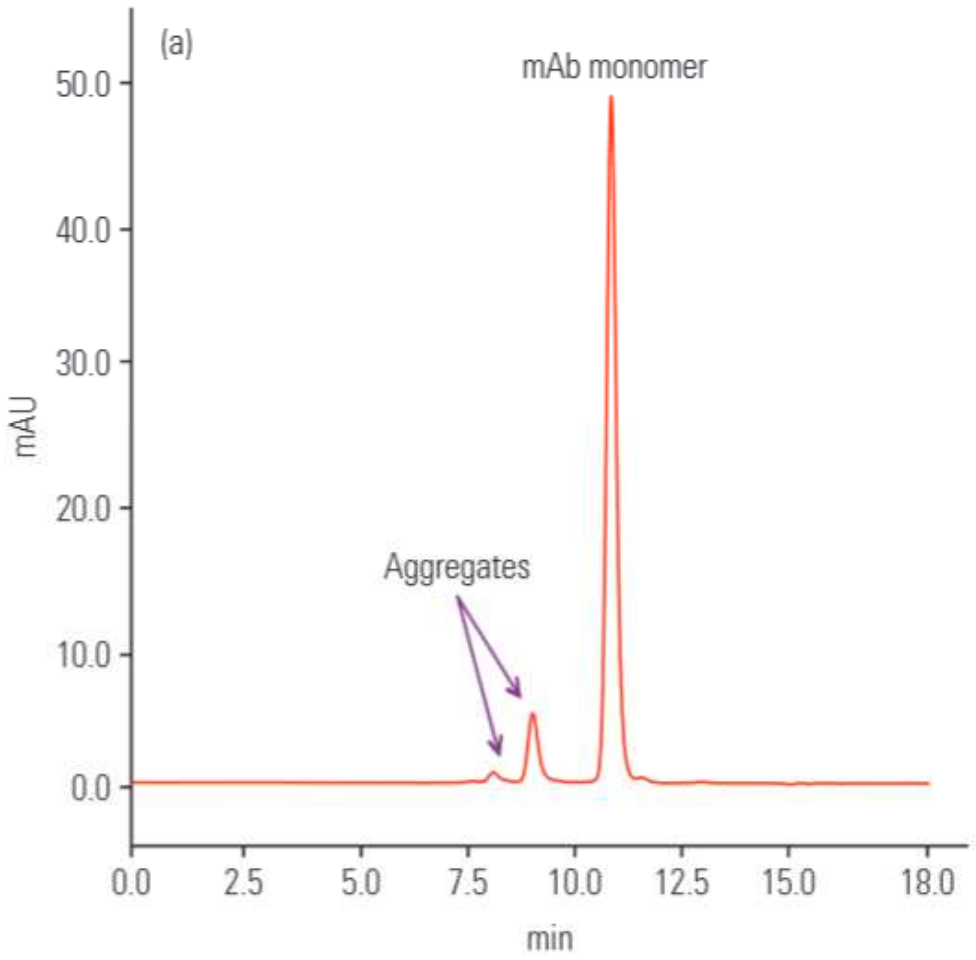
Column:	MAbPac HIC-10, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B:	100 mM sodium phosphate, pH 7.0

Gradient:

Time (min)	%A	%B
-5.0	60	40
0.0	60	40
1.0	60	40
15.0	0	100
20.0	0	100

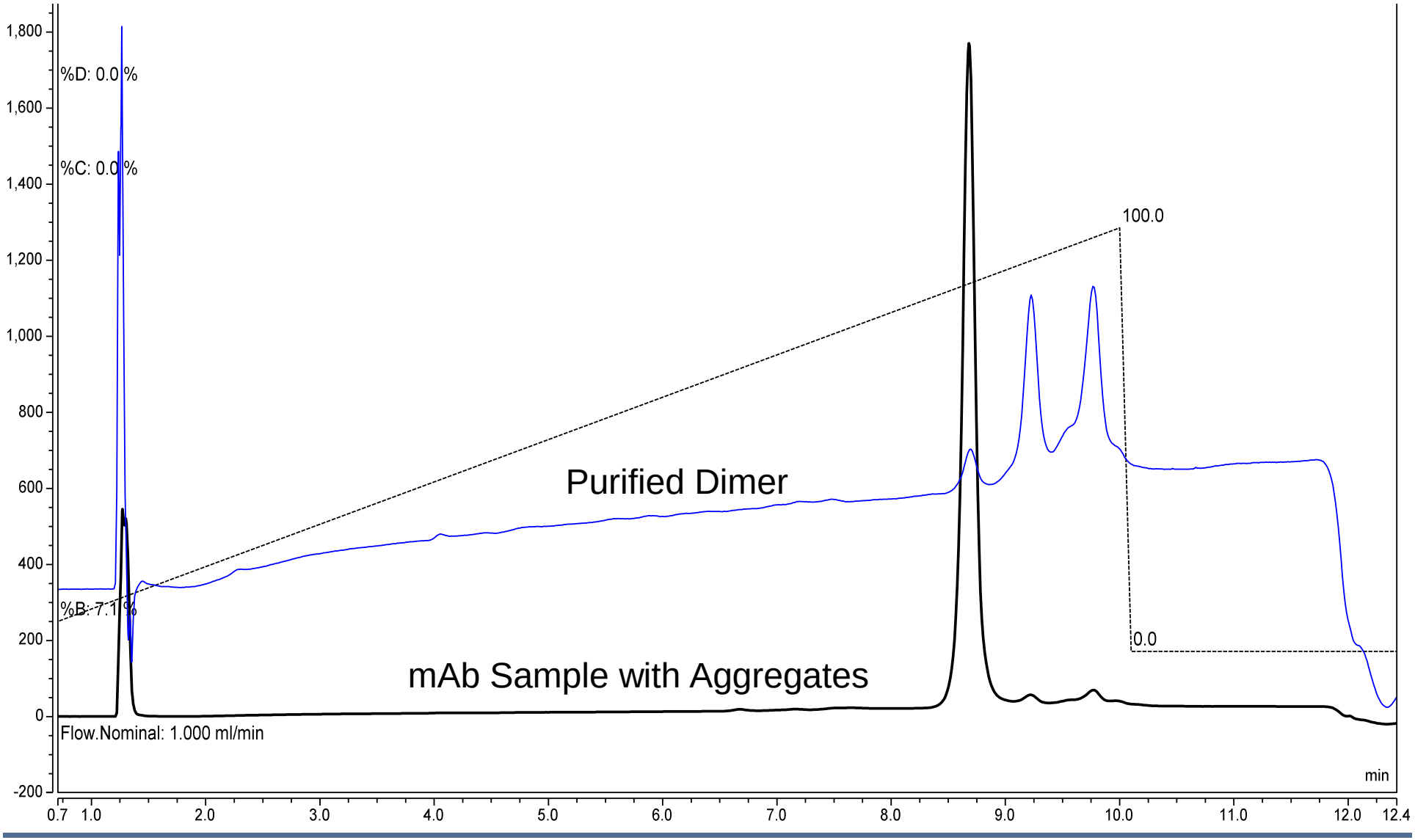
Temperature:	30 ºC
Flow rate:	1.0 mL/min
Inj. volume:	2 µL (4 mg/mL)
Detection:	UV (280 nm)
Sample:	mAb1
	mAb2
	mAb3
	mAb4
	mAb5

Aggregate Screening using MAbPac SEC-1 Column

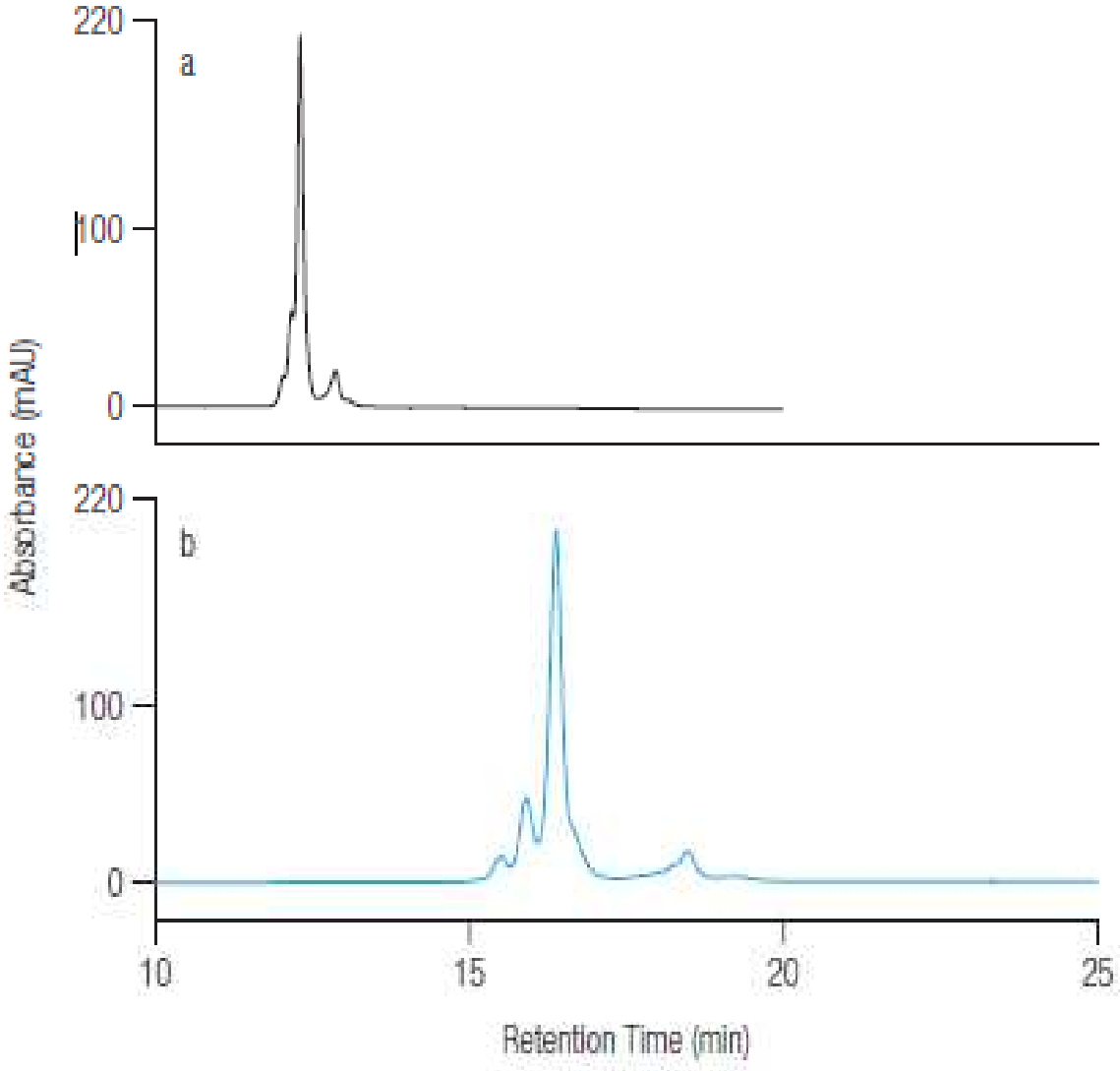


Column:	**MAbPac SEC-1**, 5 µm,
Dimension:	7.8 × 300 mm
Mobile Phase:	50 mM sodium phosphate pH 6.8, in 300 mM sodium chloride
Flow Rate:	760 µL/min
Inj. Volume:	10 µL
Temp.:	30 °C
Detection:	280 nm
Sample:	mAb (1 mg/mL)

HIC separation of MAb and Purified Dimer

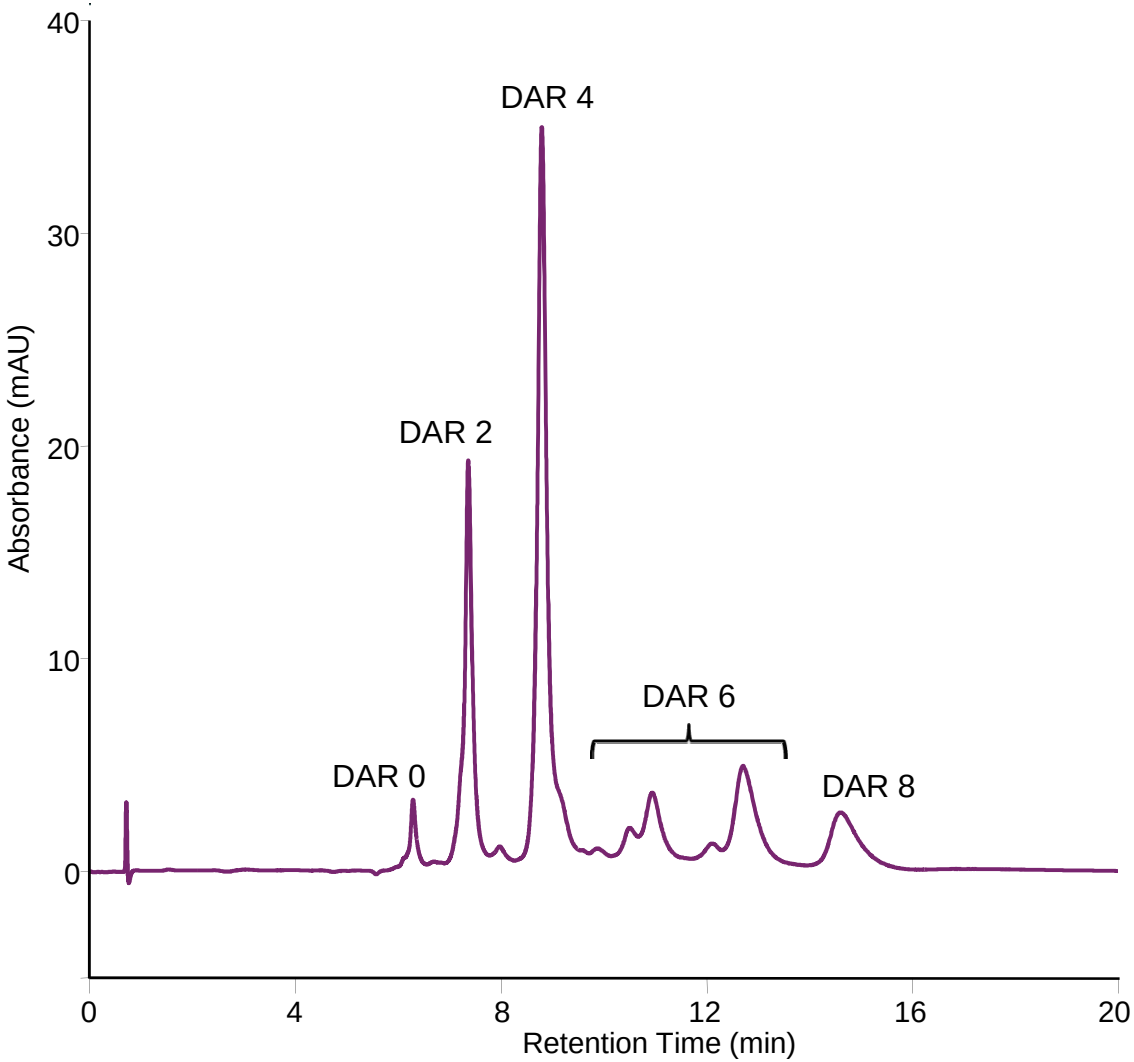


Method Speed up of Mab Aggregates with HIC



Column: **MAbPac HIC-10**, 5 µm
Format: 4.6 × 100 mm
Mobile Phase A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile Phase B: 100 mM sodium phosphate, pH 7.0
Gradient (a Time (min) %A %B
-5.0 60 40
0.0 60 40
1.0 60 40 15.0 0 100
20.0 0 100
Gradient (b) Time (min) %A %B
-5.0 60 40
0.0 60 40
1.0 60 40
29.0 0 100
34.0 0 100
Flow rate: (a) 1.0 mL/min
(b) 0.5 mL/min
Inj. Volume: 10 µL (4 mg/mL)
Temp.: 30 ºC
Detection: UV (280 nm)

Separation of Cys-Linked ADC on MAbPac HIC-Butyl

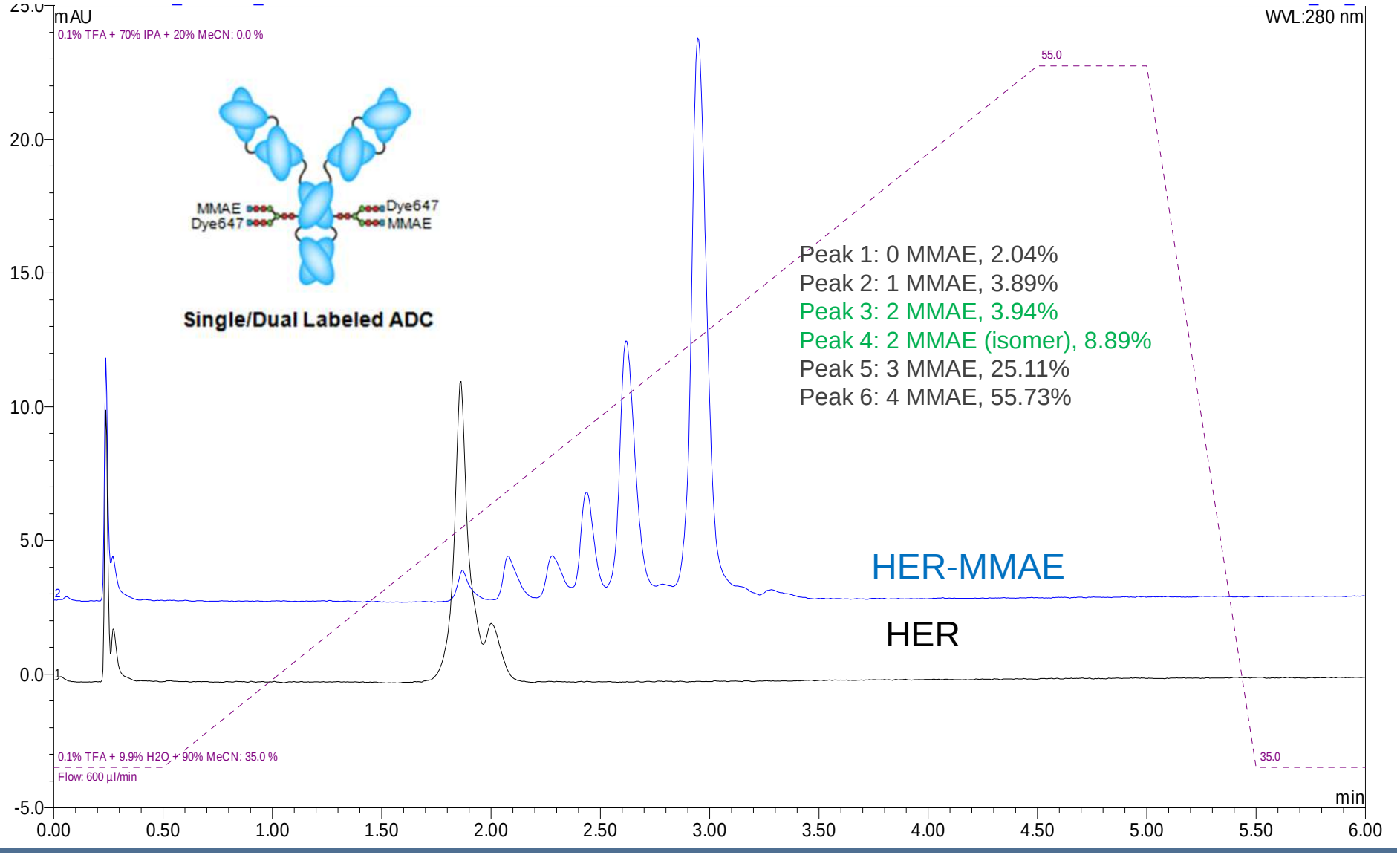


Column:	MAbPacHIC-Butyl, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)
Mobile phase B:	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
Gradient:	

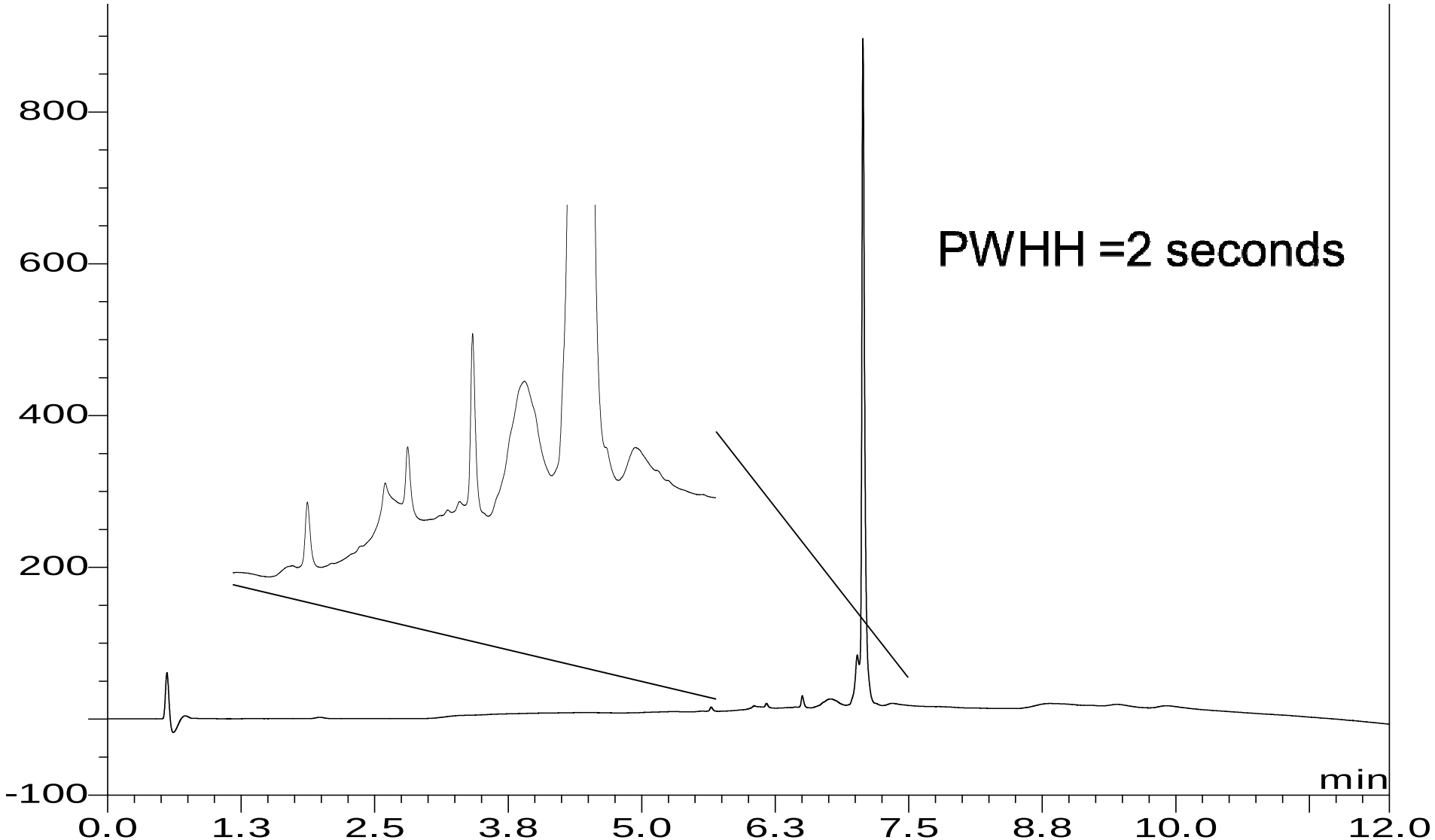
Time (min)	%A	%B
-5.0	100	0
0.0	100	0
1.0	100	0
15.0	0	100
20.0	0	100

Temperature:	25 ºC
Flow rate:	1.0 mL/min
Inj. volume:	5 µL (5 mg/mL)
Detection:	UV (280 nm)
Sample:	Cys-conjugated ADC mimic

6 min gradient, MAbPac RP Column with ADC Mimic



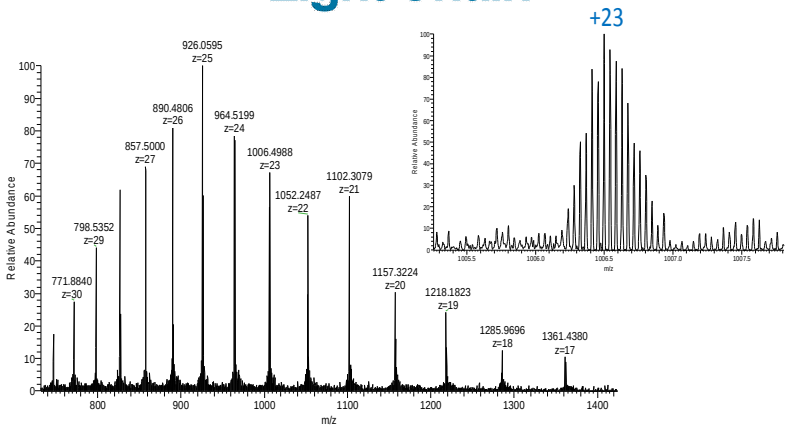
Cetuximab Separation on a MABPac RP Reverse Phase Column



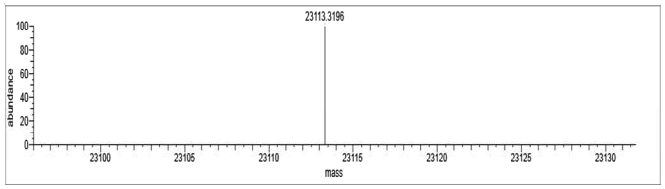
Accurate Monoisotopic Mass Measurement Using Ultra High Resolution

LC-MS of Antibody Light Chain and Heavy Chain

Light Chain

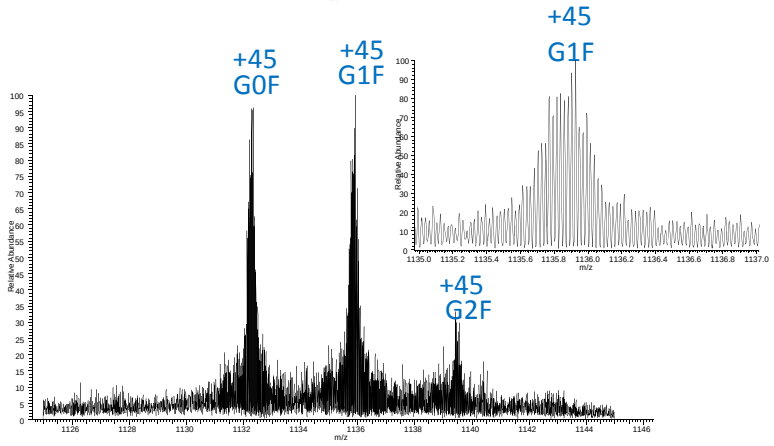


0.7 ppm



Thermo Scientific Q Exactive™ Plus MS, 140K

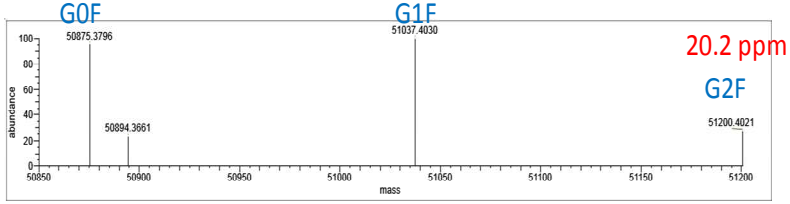
Heavy Chain



2.3 ppm G0F

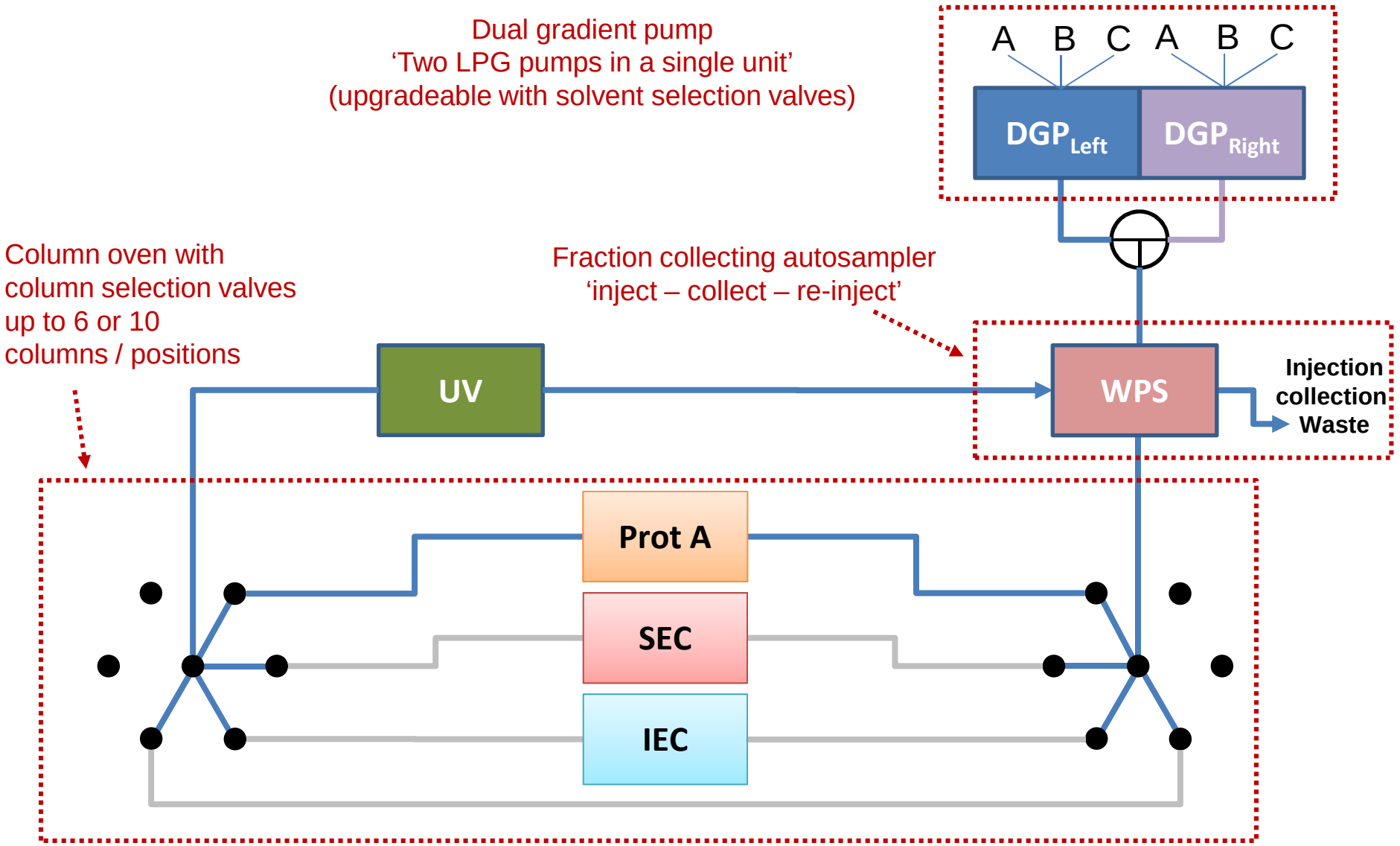
1.8 ppm G1F

20.2 ppm G2F



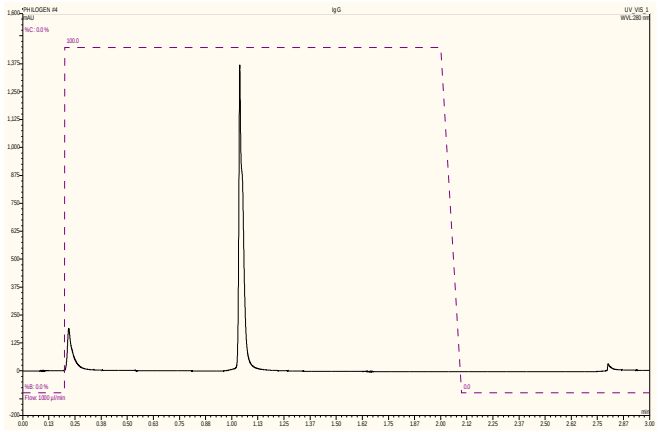
Q Exactive Plus MS, 280K, protein mode

System Configuration for Multiple Chemistries



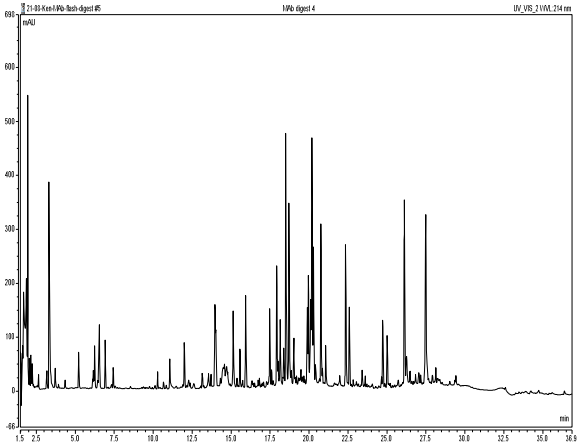
Wide Range of Bio Analytical UHPLC Characterization Methods

mAb Capture



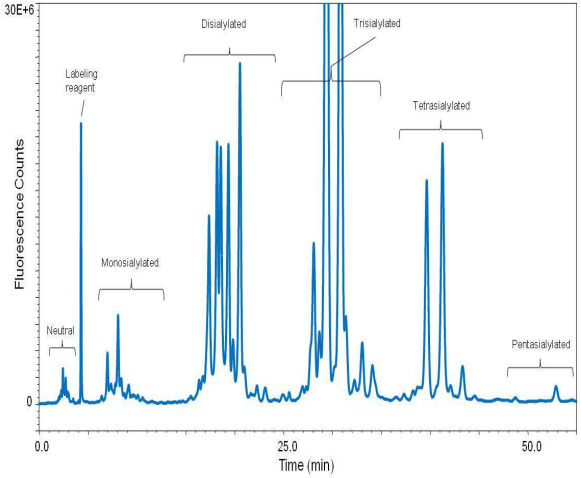
2 minutes

Peptide Mapping

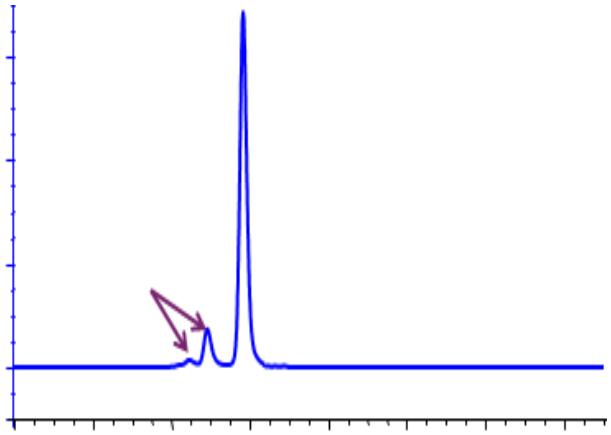


60 minute digest

Glycan Analysis

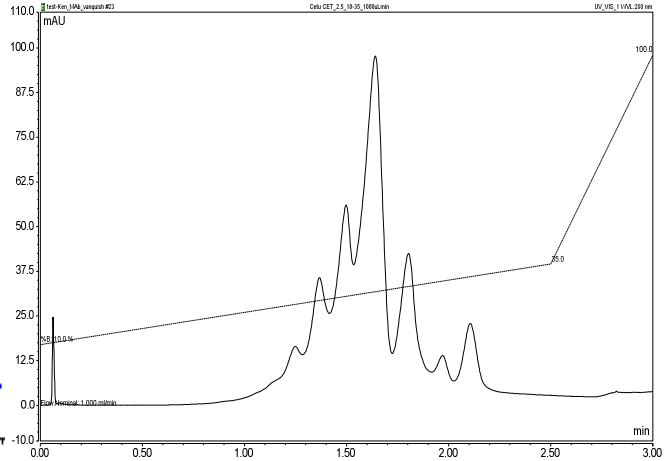


Aggregate Analysis



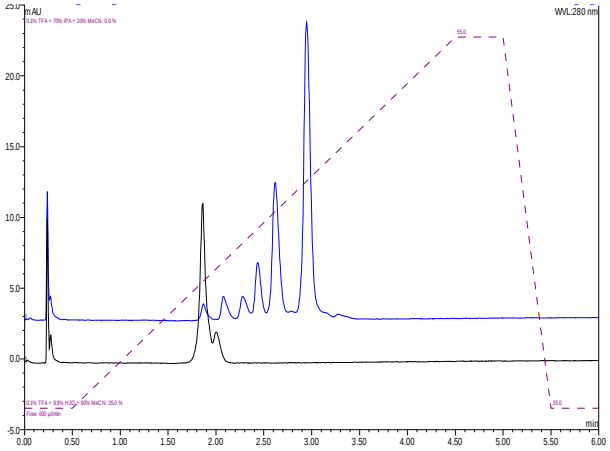
4 minutes

Charged Variant Analysis



2 minutes

ADC Analysis



6 minutes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