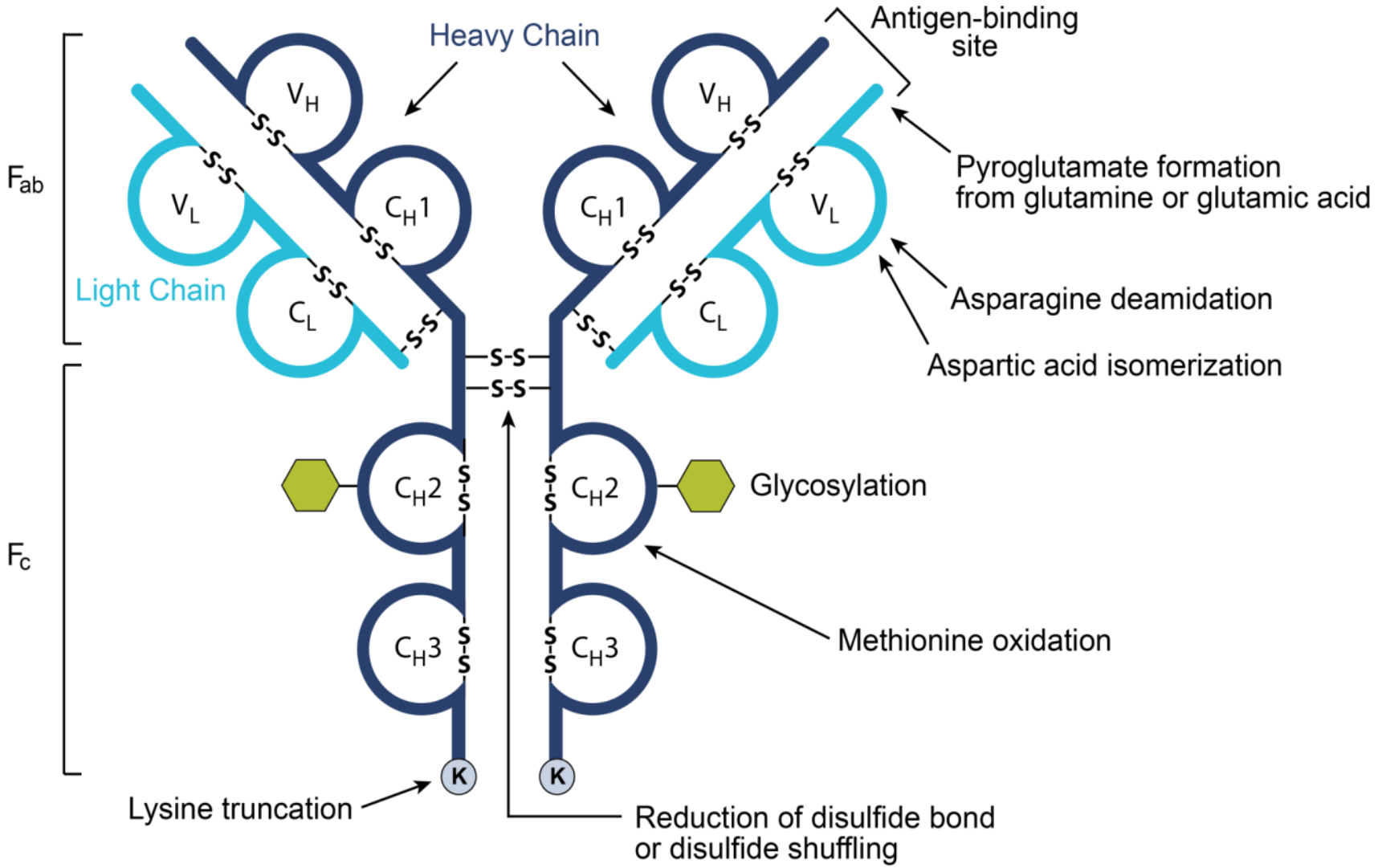
ThermoFisher SCIENTIFIC

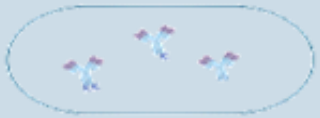
Taking charge variant analysis from research into the routine environment

Global BioPharma Summit

Structure of IgG and Typical Forms of Heterogeneity

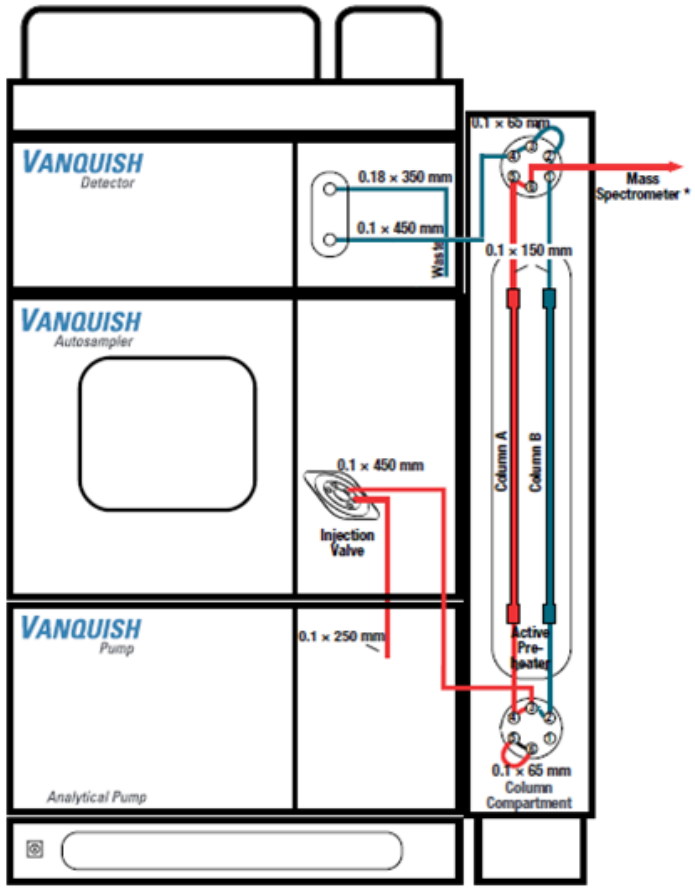


Bio-Column Selection Guide

Analysis		Description	Columns and Buffers	Detection
Titer		mAb capture, titer & screening	Thermo Scientific™ MAbPac™ Protein A	UV
Aggregate		Routine screening for aggregates and fragments	Thermo Scientific™ MAbPac™ SEC-1	UV & 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 & 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	UV and MS

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

Requirements of a UHPLC System for Charged Variant Analysis



pH and Conductivity

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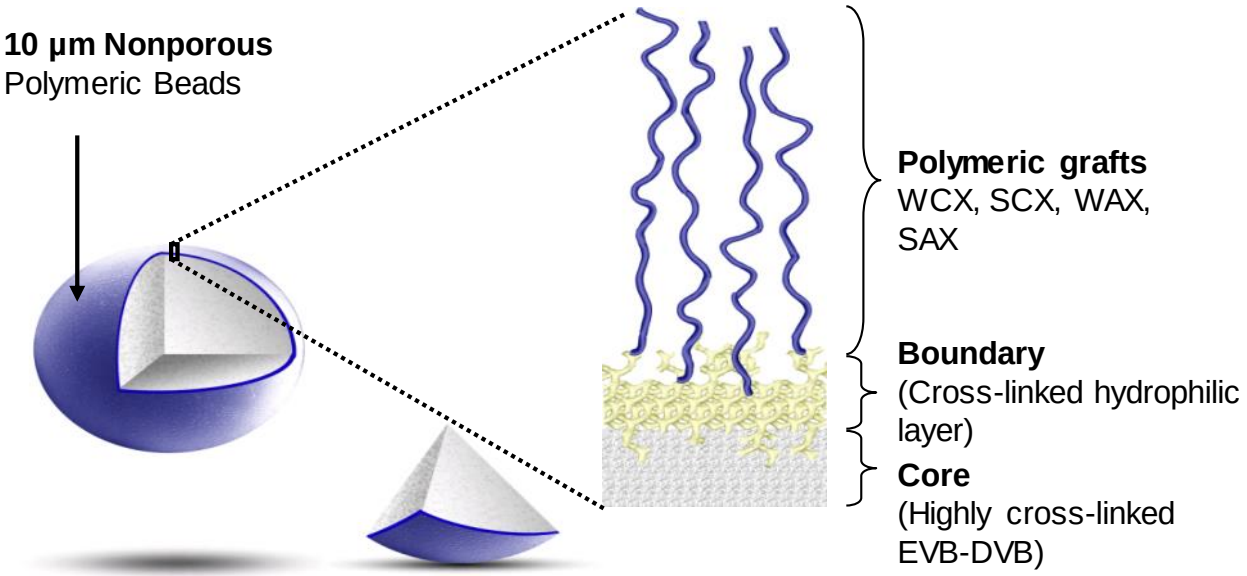
Charge Variant Analysis using Cation Exchange Chromatography (CEX)

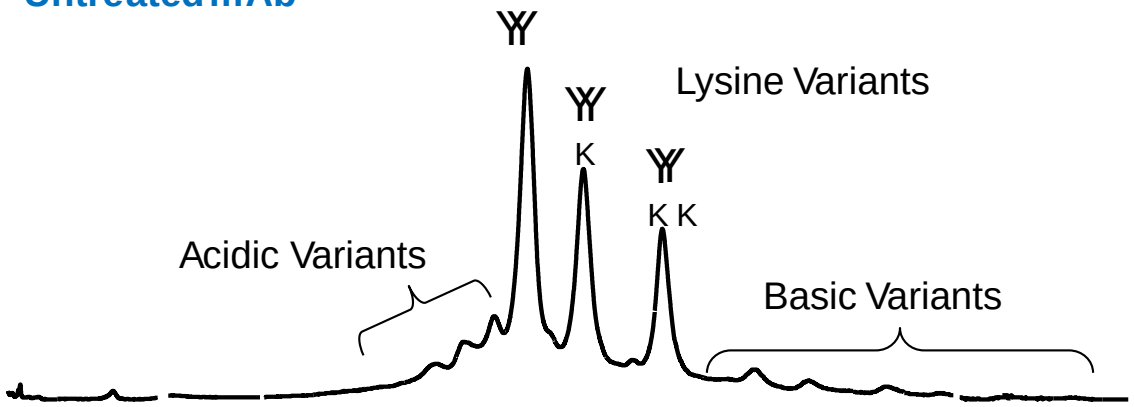
ProPac WCX-10 Column



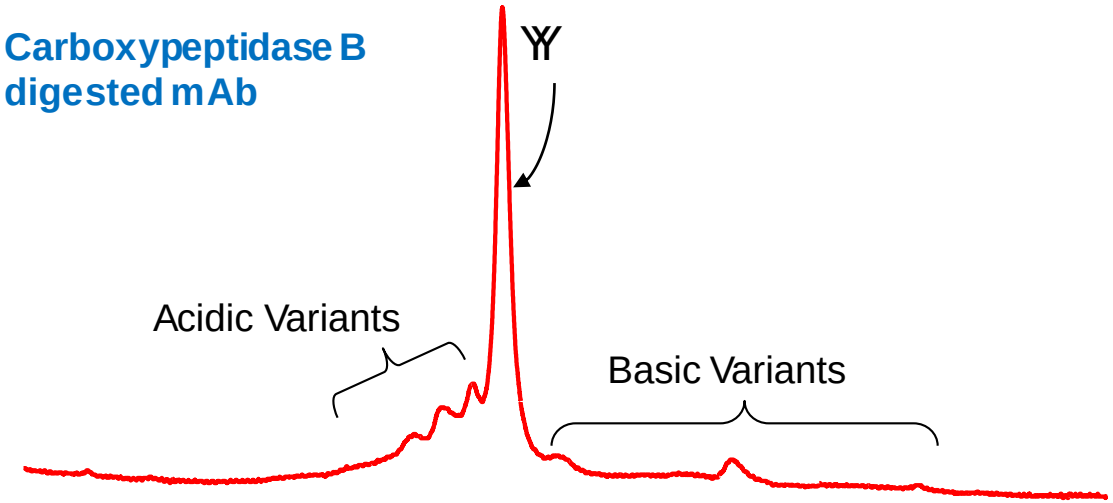
470 *Current Pharmaceutical Biotechnology*, 2008, *Vol. 9, No. 6*

by CEX [18]. Table 1 summarizes different modifications that were detected in antibodies by CEX. As seen in Table 1, ProPac WCX-10 column (Dionex Corp.), based on the polymeric tentacle resin [19], has become the gold standard for antibody analysis.

Untreated mAb



Carboxypeptidase B digested mAb



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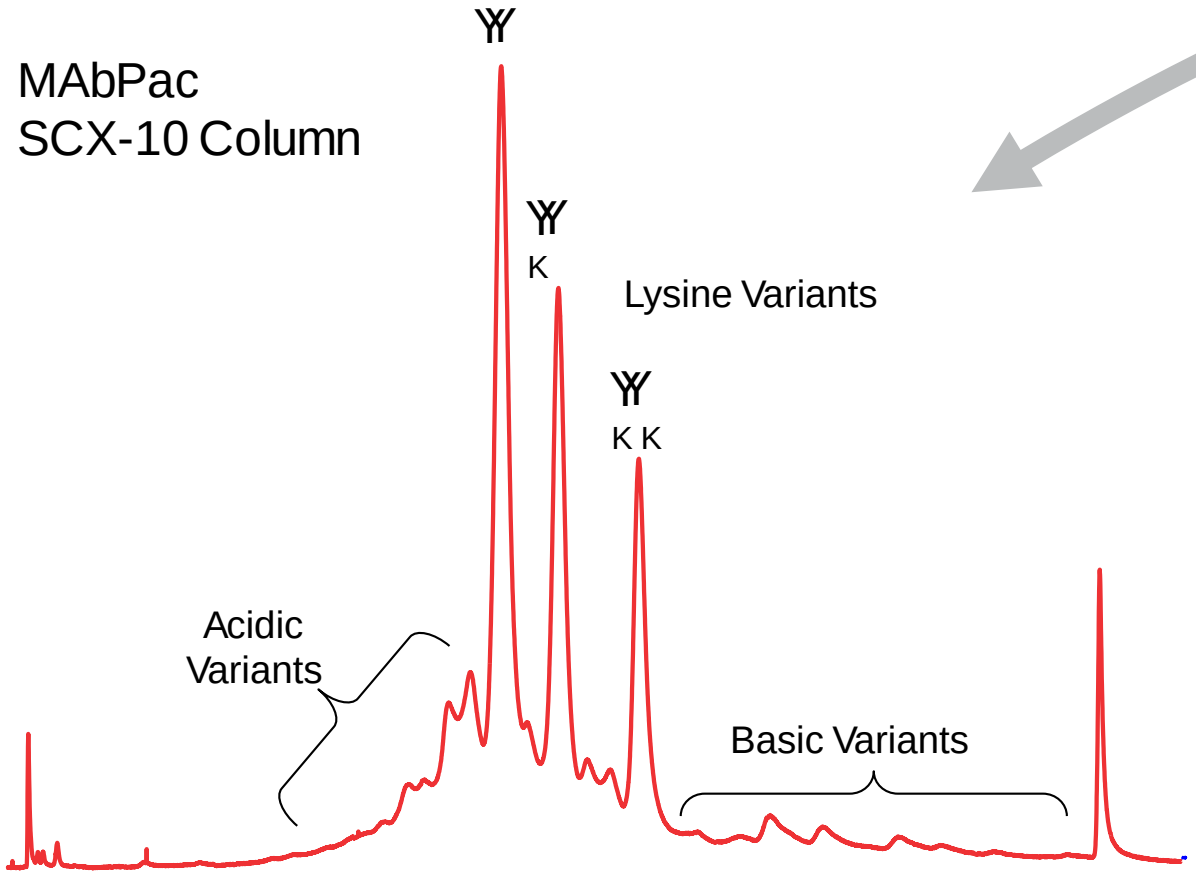
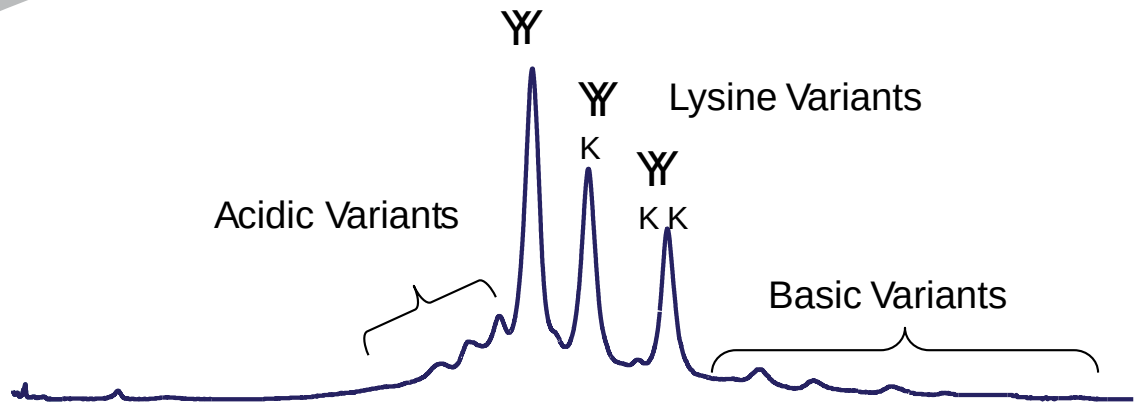
Next-generation CEX Column

MAbPac
SCX-10 Column

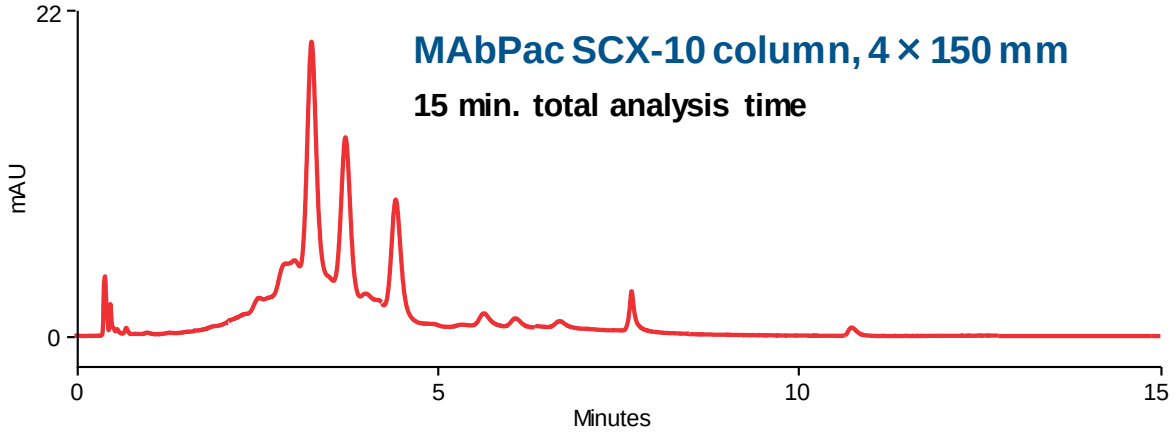


MAbPac SCX-10 column, 4×250 mm

60 min. total analysis time



ProPac WCX-10 column, 4×250 mm



MAbPac SCX-10 column, 4 × 150 mm

15 min. total analysis time

Improve resolution or sample throughput through column chemistry

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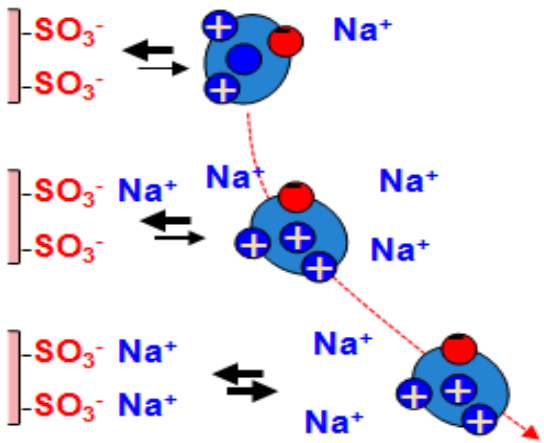
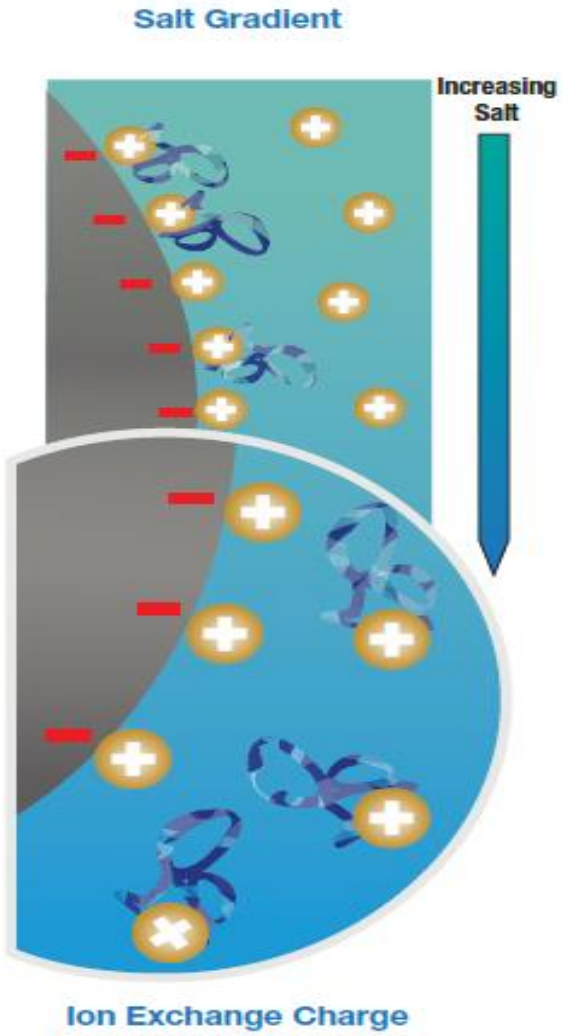
mAb Charge Variant Analysis by CEX Salt Elution

Cation Exchange of mAb

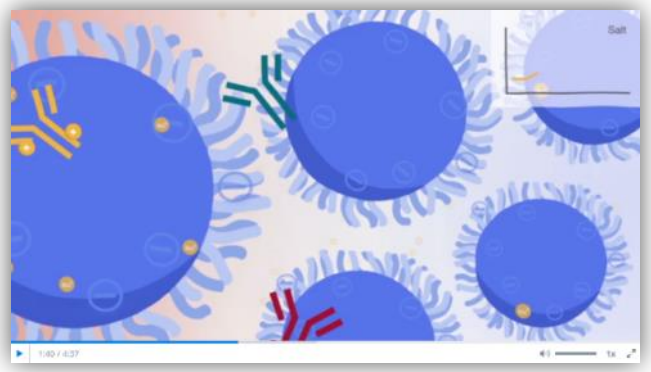
Elution with competing sodium ions from an NaCl gradient



Cation Exchange

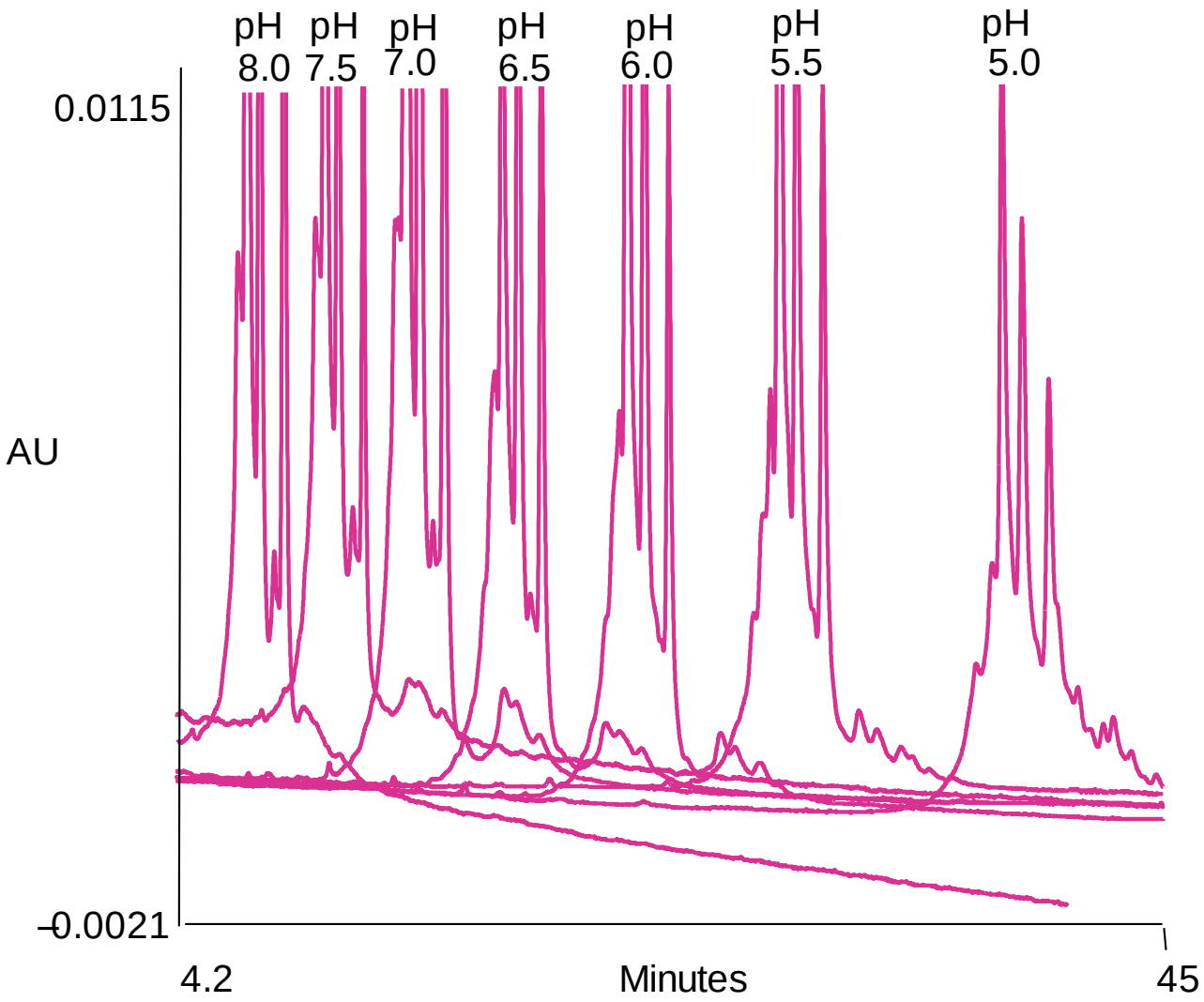


- Competition for Ion Exchange sites between the mAb and Na^+ ions
- This interaction happens all the way through the column
- The longer the column the better the resolution
- Surface exchange on a pelicular resin for high resolution



http://bit.ly/ChargeVariants

Effect of pH on elution of mAb (salt gradient)



Column:	ProPac WCX-10, 4 mm x 250 mm
Eluents :	A. 20 mM HEPES + 20 mM MES + 20 mM TAPS B: 20 mM HEPES + 20 mM MES + 20 mM TAPS + 250 mM NaCl

Gradient:

Min	%A	%B
–10	95	5
0	95	5
50	5	95
52	5	95
52.5	95	5

Temperature:	30 °C
Inj. Volume:	20 µL
Sample:	2.5 mg/mL mAb
Flow Rate:	1.0 mL/min
Detection:	280 nm

23746

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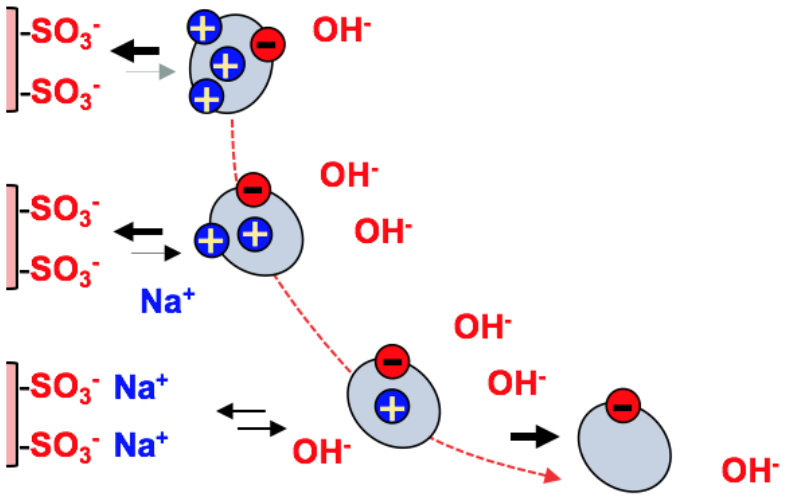
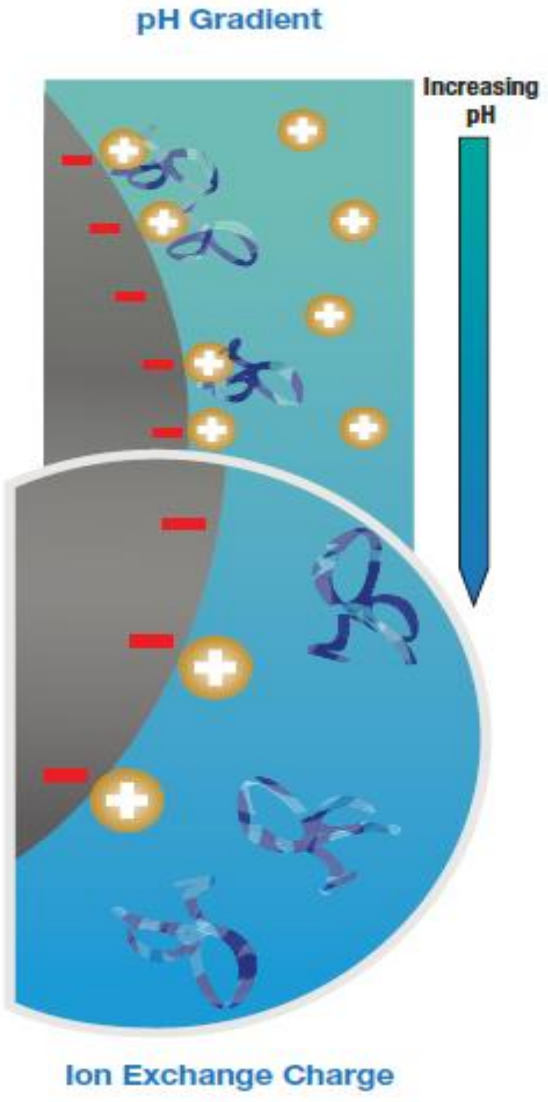
Mab Charge Variant Analysis by CEX pH Gradient Elution

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)

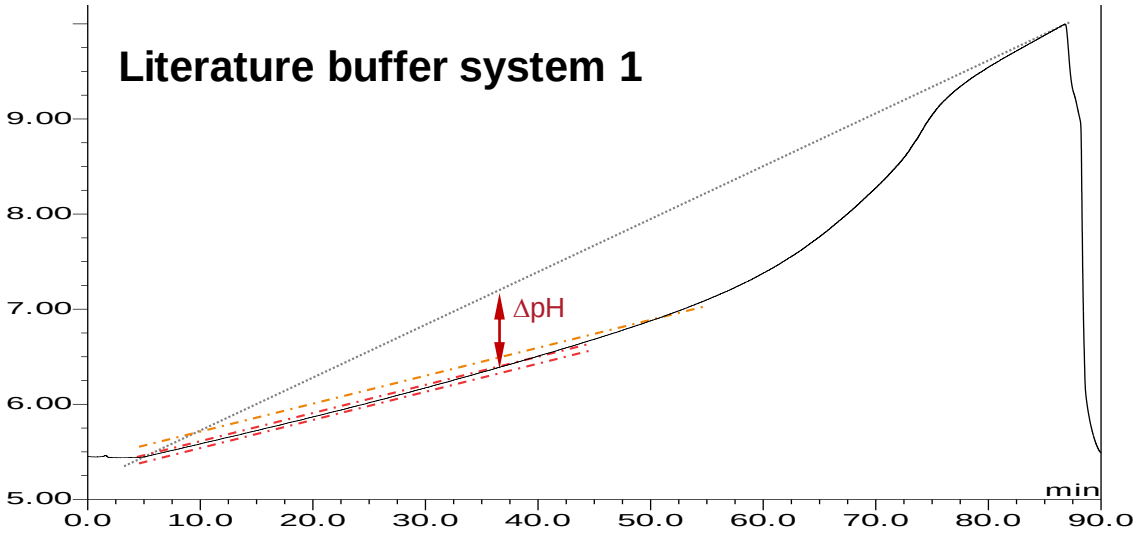


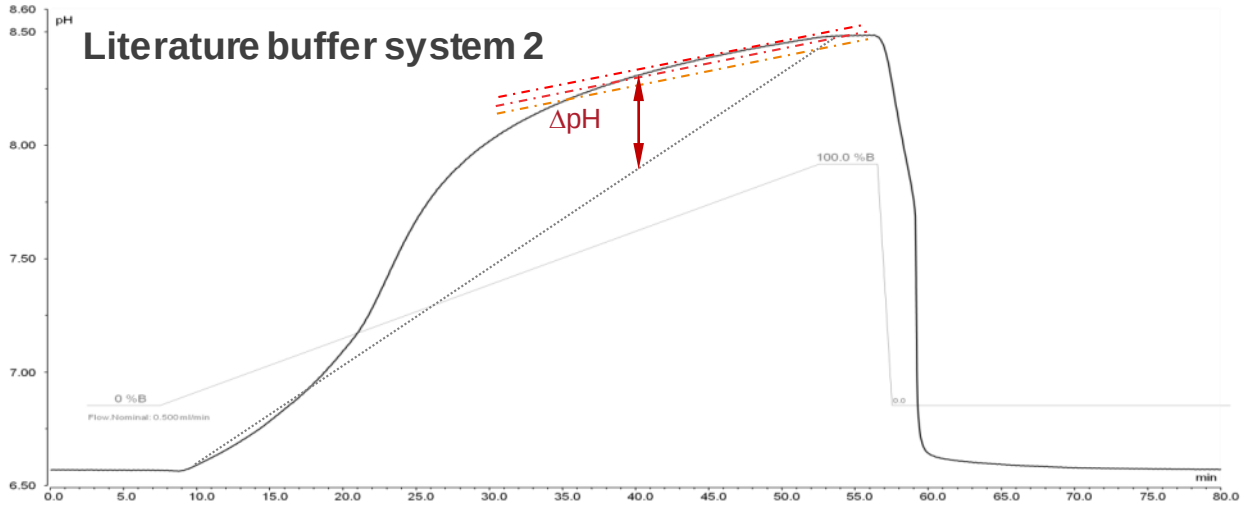


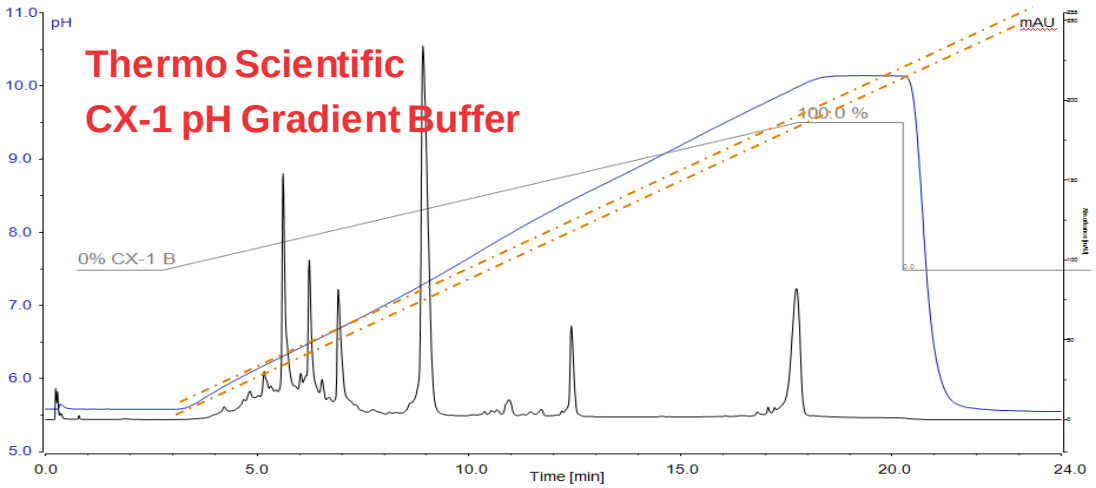
Isoelectric Focusing on a Cation Exchange Column

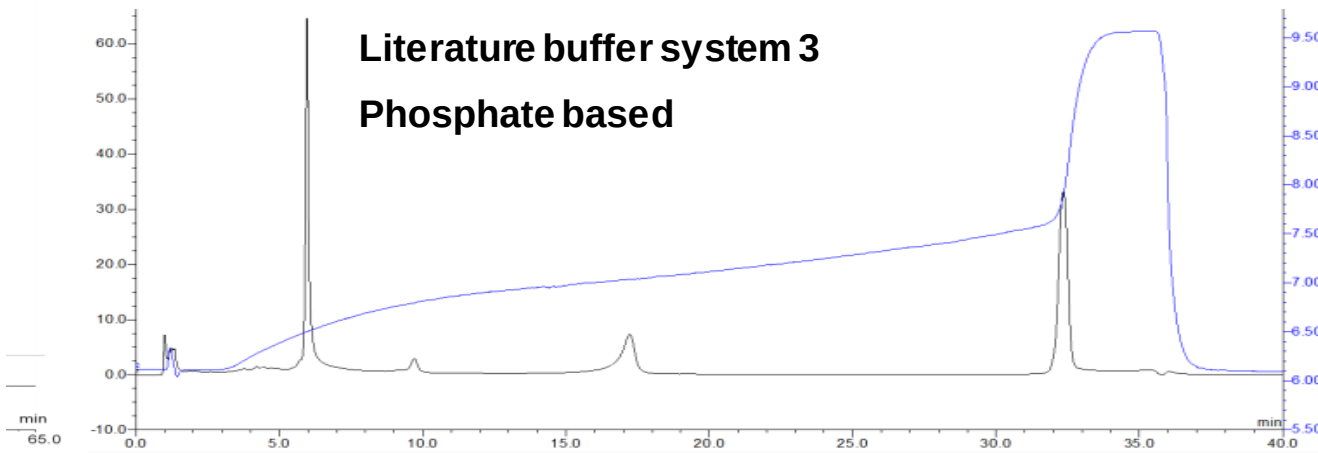
- mAb binds to cation exchange sites on the column
- A gradient of increasing pH is applied
- mAb is released from the exchange site when the net charge on the mAb is neutral
- This interaction happens once, then the mAb runs through the rest of the column
- Column length has little effect on the resolution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

Comparison of pH gradient buffer systems









MAbPac SCX-10 (5 µm) 4x50 mm

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Advantages

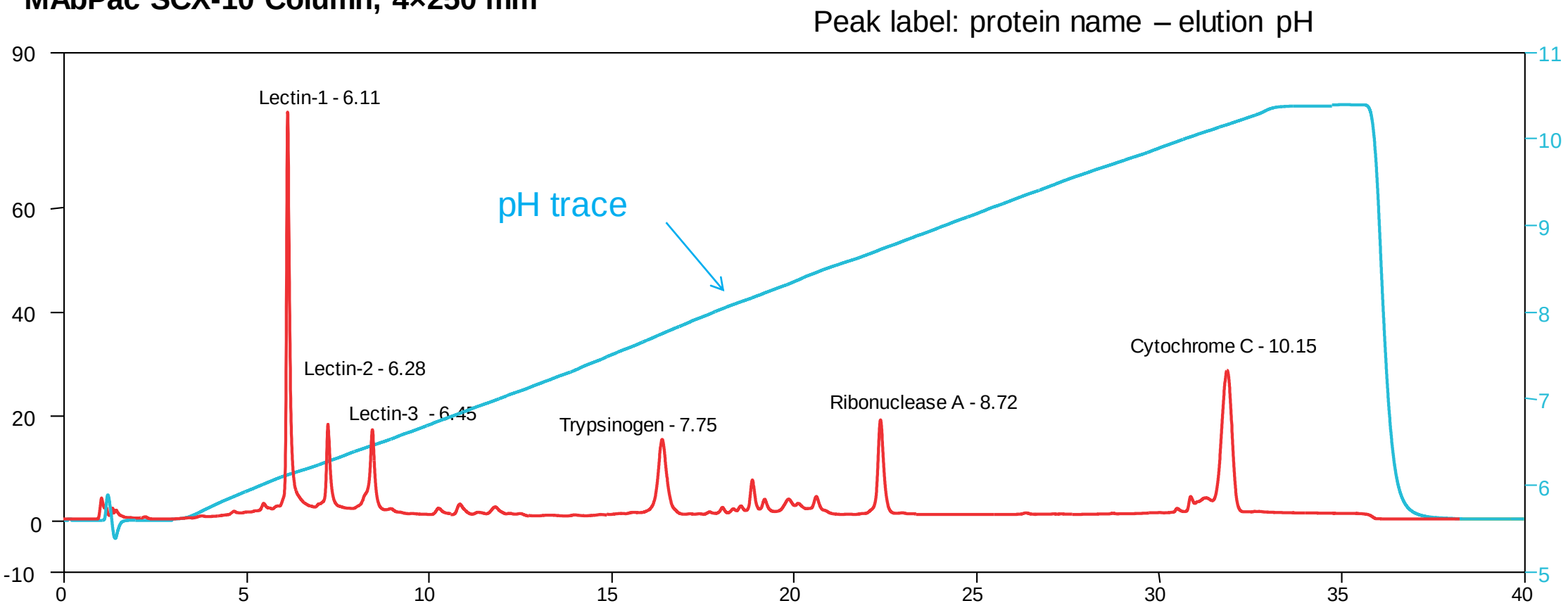
- **Platform** method ⇨ single method for wide range of mAbs
- Reduced method development and method transfer times
- Outperforms any other charge variant technology
- Less effect from column variability
- Transferability of method from development to QC

- 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

Protein Standard – Linear CX-1 pH Gradient Buffers

MAbPac SCX-10 Column, 4×250 mm



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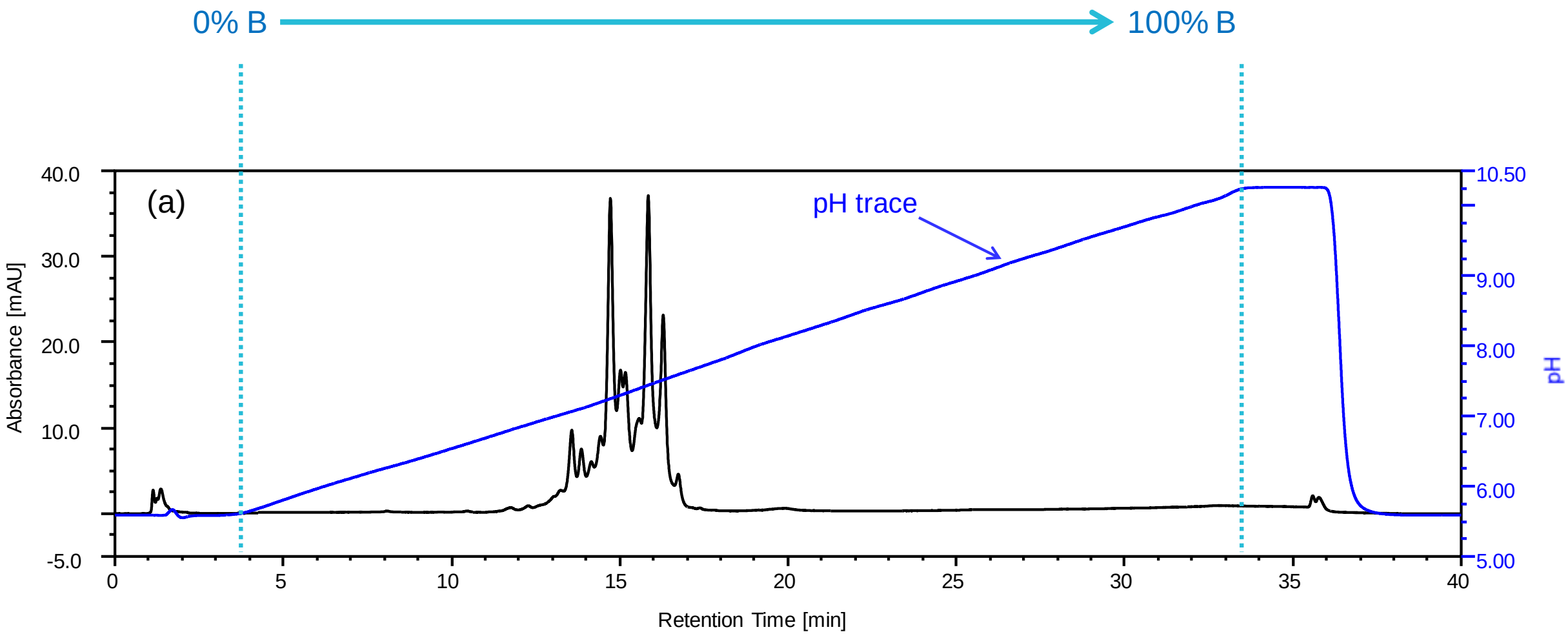
mAb Charge Variant Separation, 0–100% B



*The pH trace at elution was obtained with the Thermo Scientific™ UltiMate™ 3000 pH and Conductivity Monitoring Module (PCM-3000)

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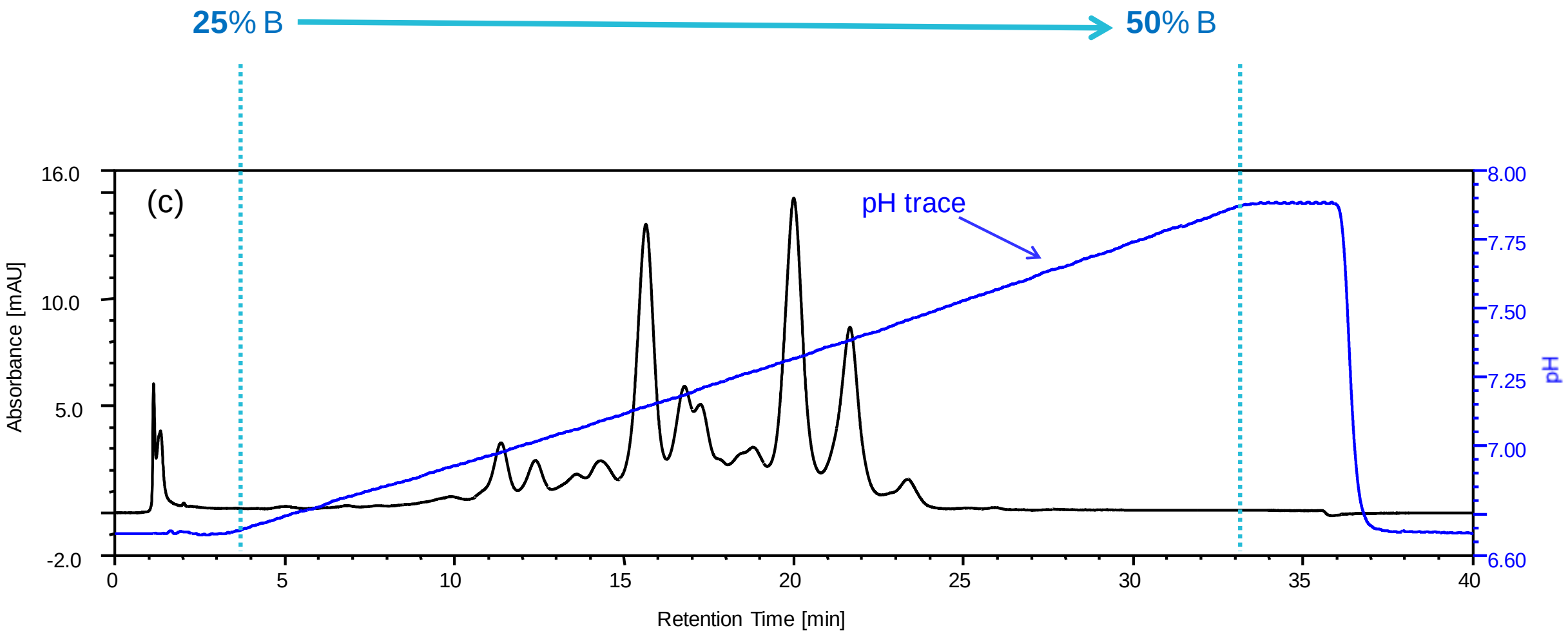
mAb Charge Variant Separation, 25–50% B



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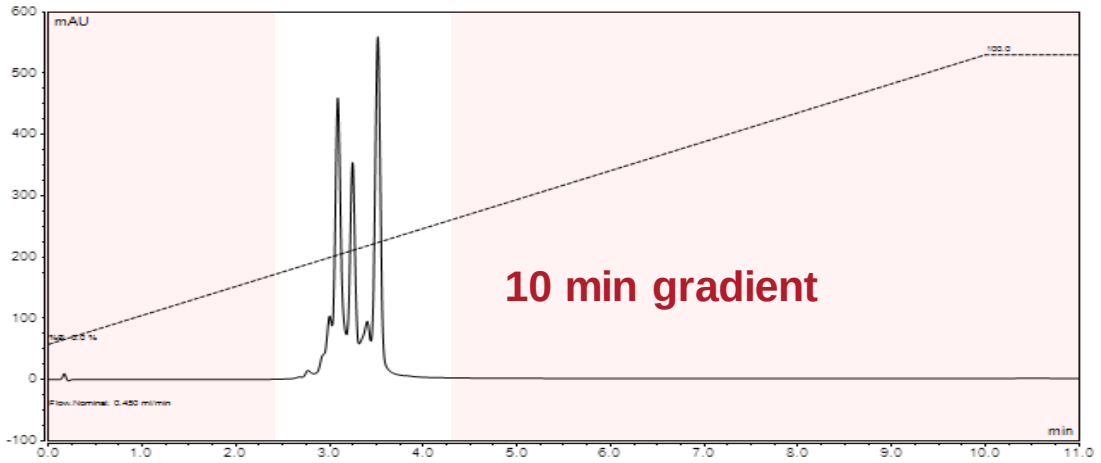
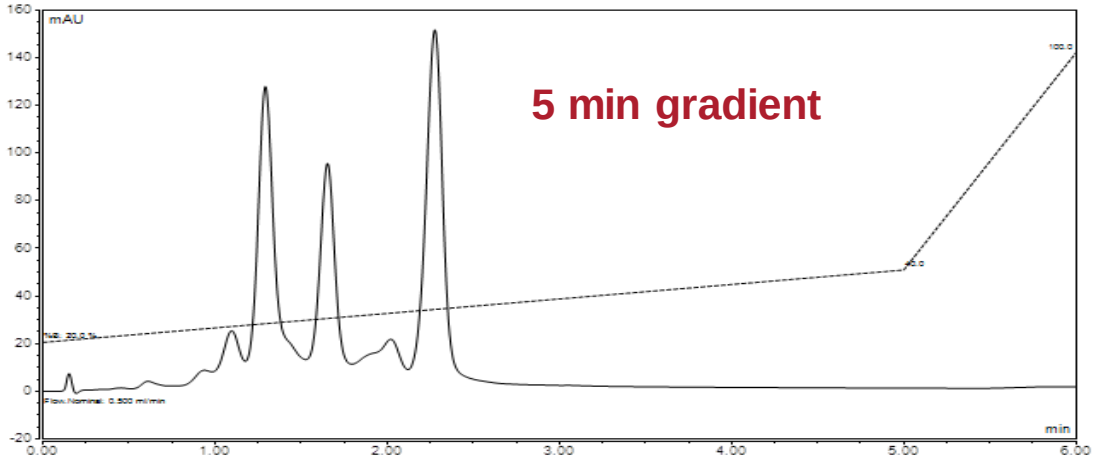
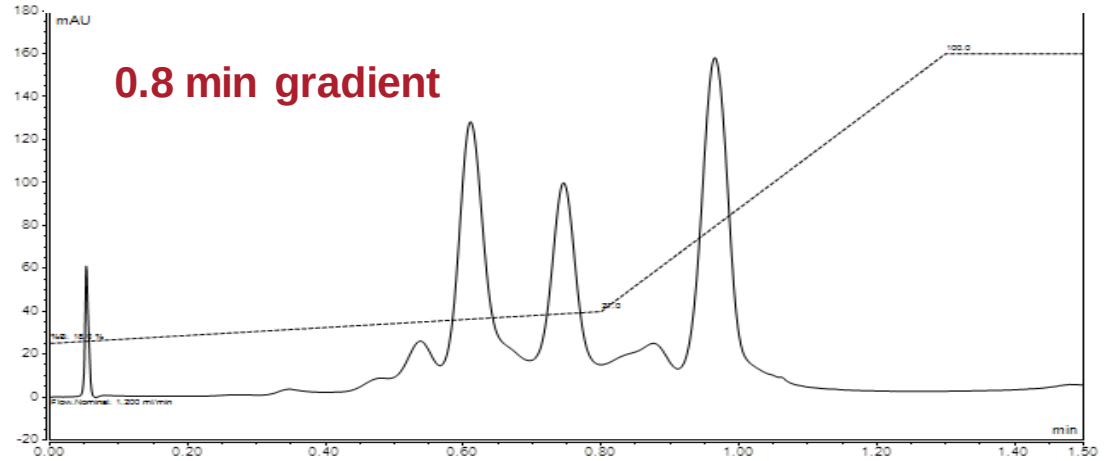
Infliximab – Vanquish System Ultra-fast Gradients







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

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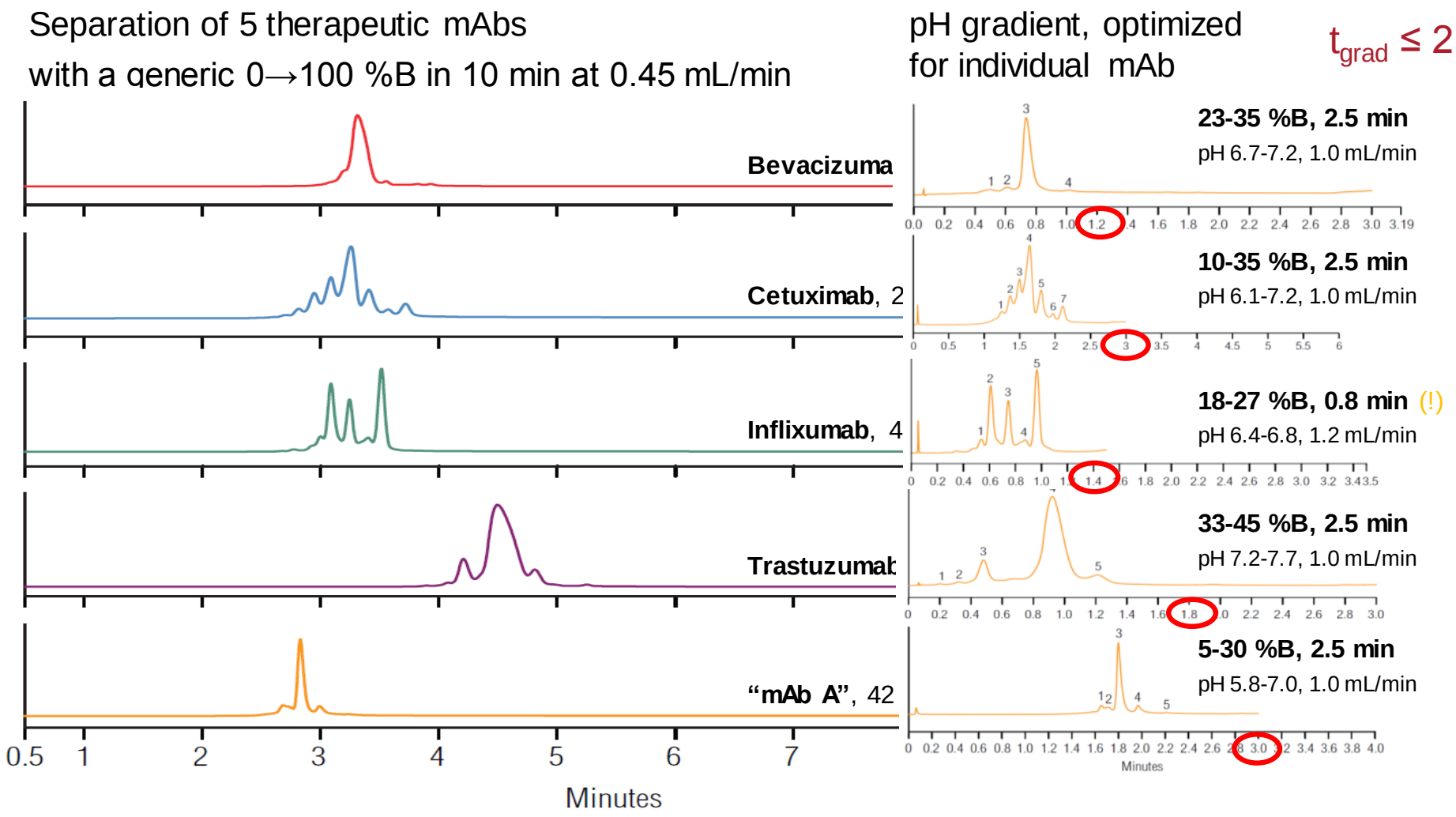
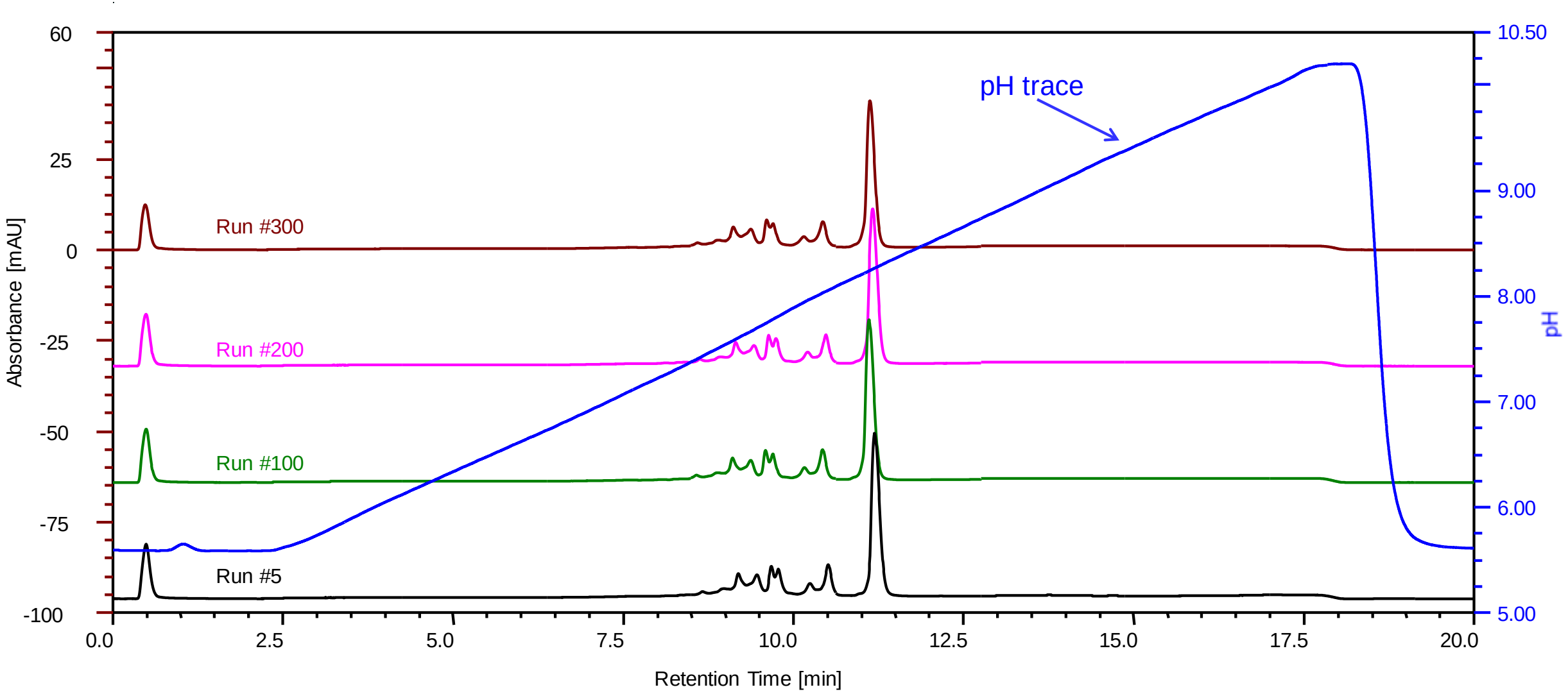
Fast, Generic and Linear pH Gradient – Vanquish UHPLC



***pH 5.6 to 10.2 in 10 minutes, MAbPac SCX-10** (5 µm), 2 x 50 mm*

Repeat Injections of Ribonuclease A: >300 Runs



Retention time reproducibility <0.8% RSD

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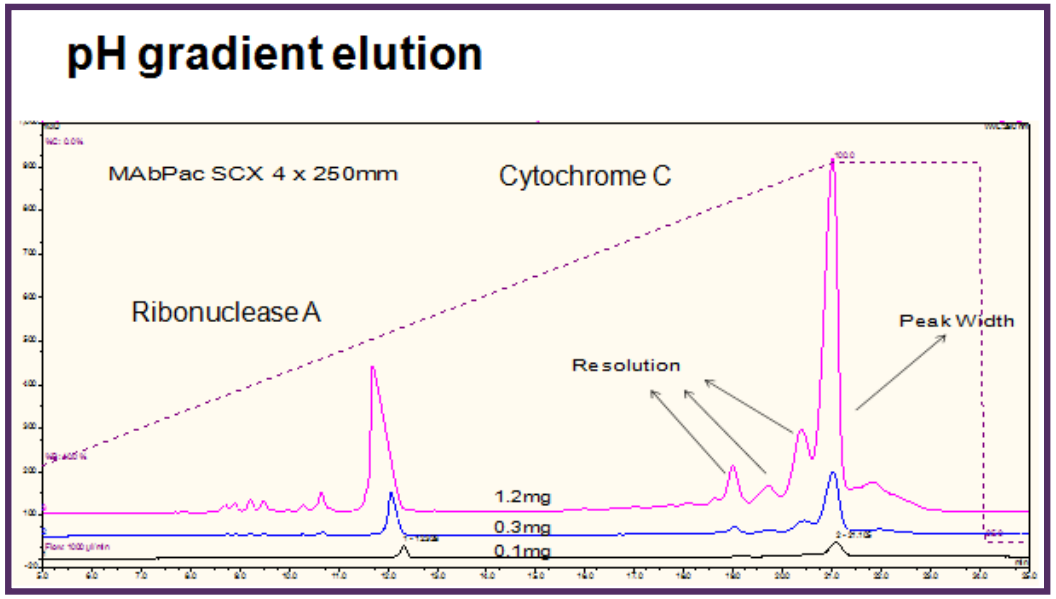
Effect on Loading Capacity and Peak Resolution

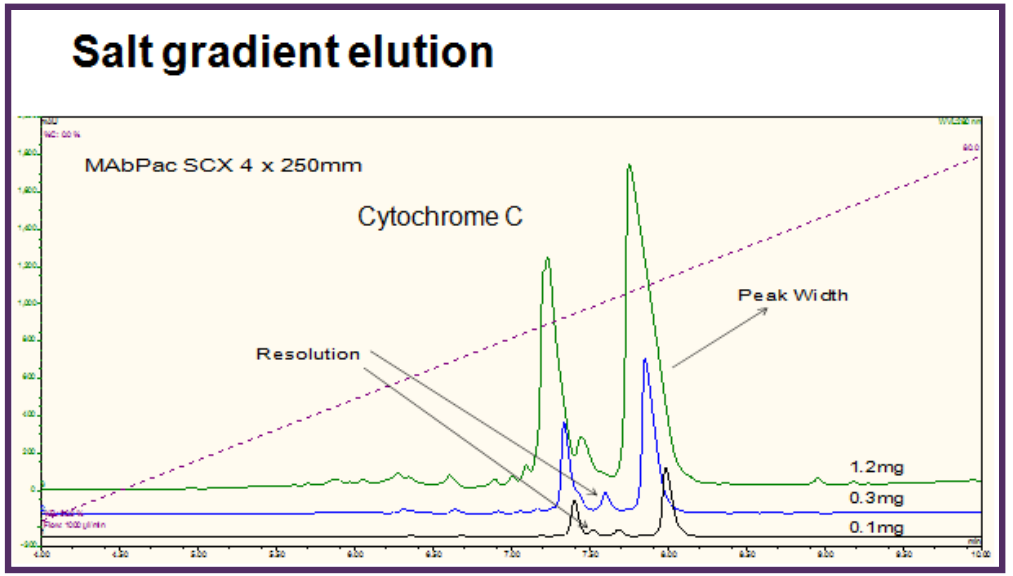
pH gradient

- Peak resolution increases with loading
- Isoelectric focusing
- Full column capacity can be used for loading without affecting peak resolution and capacity

Salt gradient

- Peak resolution lost with increased loading
- Separation occurs over complete column length
- Peak capacity easily exceeded and resolution lost





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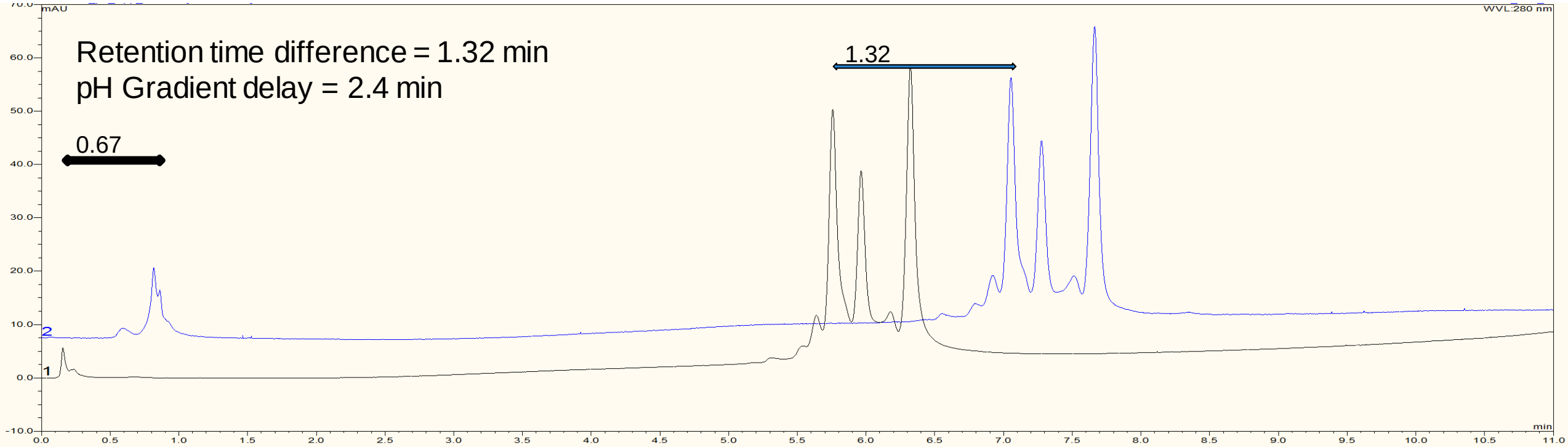
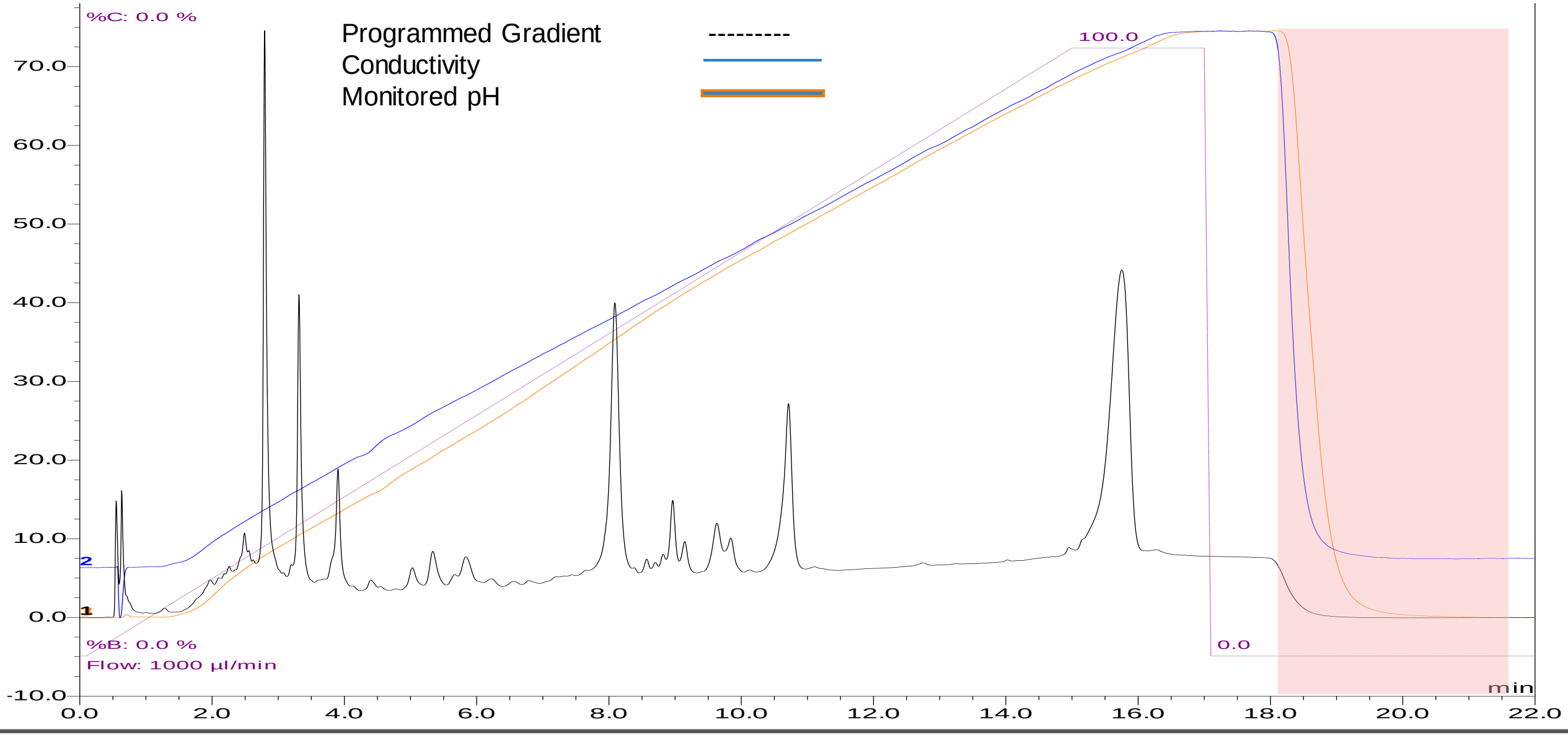
Peak Analysis of 50 and 250 mm Columns with pH Gradient Elution



	PW[HH] Peak 1	PW[HH] Peak 2	PW[HH] Peak 3	Resolution Peak 2 to 3	Elution pH Peak 1	Elution pH Peak 2	Elution pH Peak 3
50mm column	0.06	0.06	0.06	3.63	6.61	6.68	6.81
250mm column	0.06	0.06	0.06	3.73	6.81	6.9	7.04

Equilibration times on a 150mM long MAbPac SCX column



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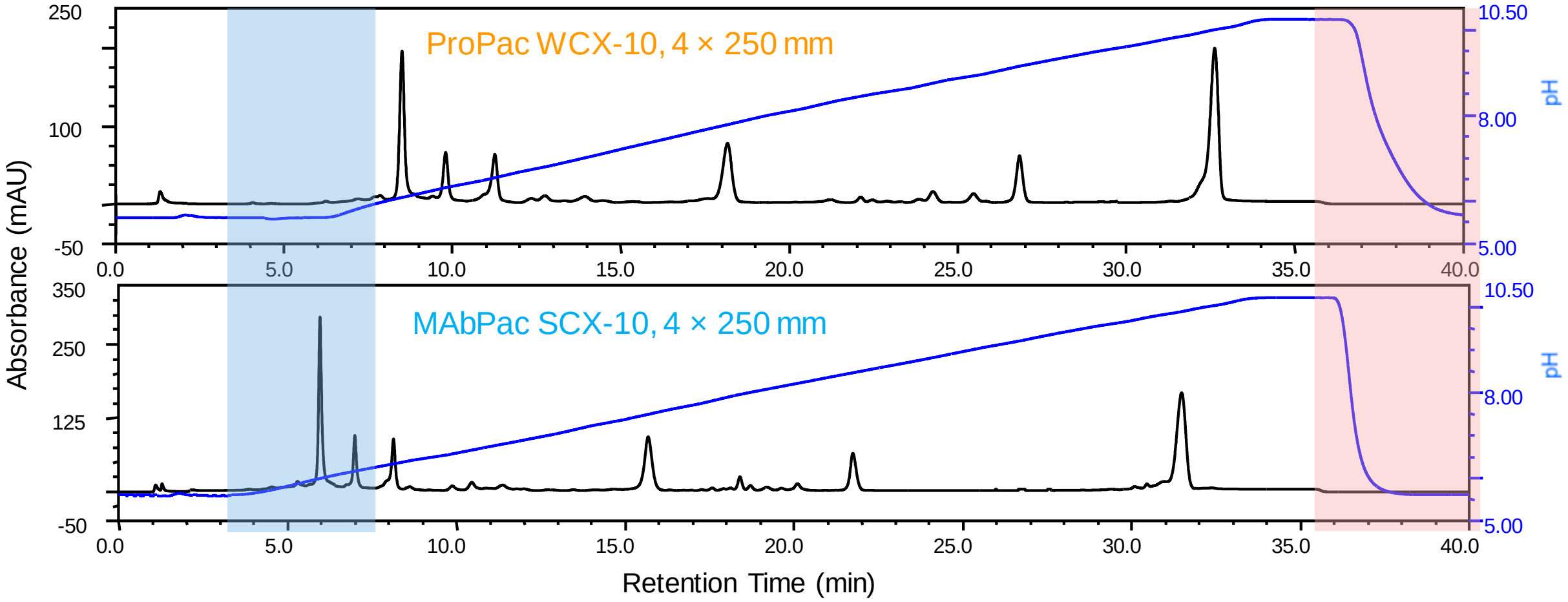
ProPac WCX-10 vs MAbPac SCX-10



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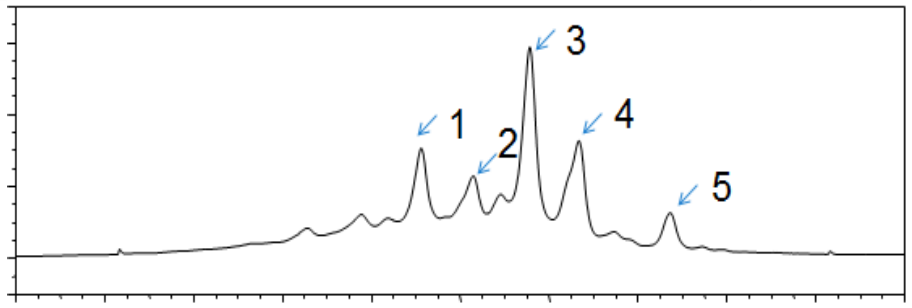
In-depth Charge Variant Characterization using CEX fraction collection and LC-MS

1st dimension: IEX pH gradient + fraction collection

Thermo Scientific™
UltiMate™ 3000
BioRS

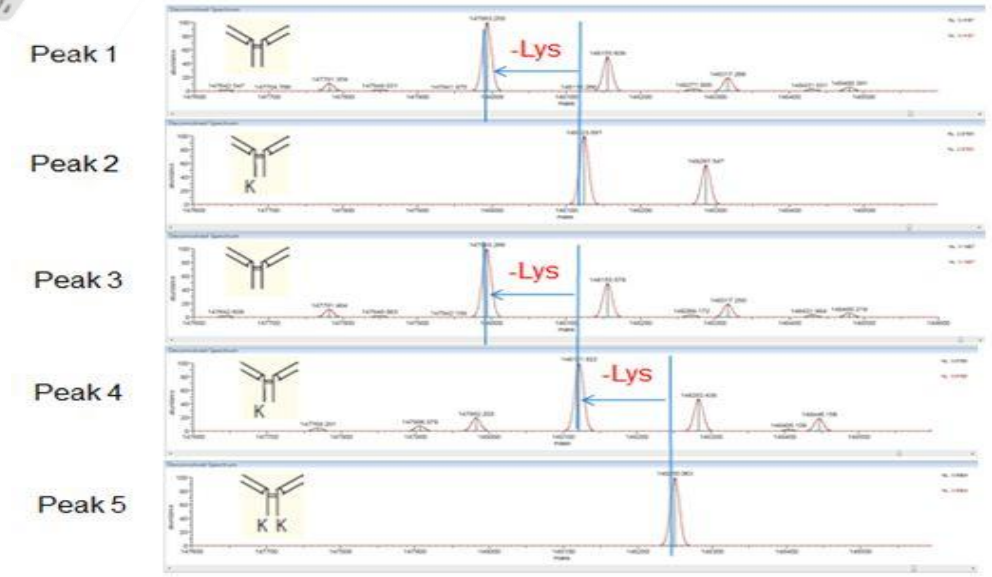
CX-1 pH Gradient Buffers
MAbPac SCX



2nd dimension: Polymer RP-LC/MS

MAbPac RP

Thermo Scientific™ Q
Exactive™ BioPharma



Thermo Scientific™ BioPharma Finder™ Software

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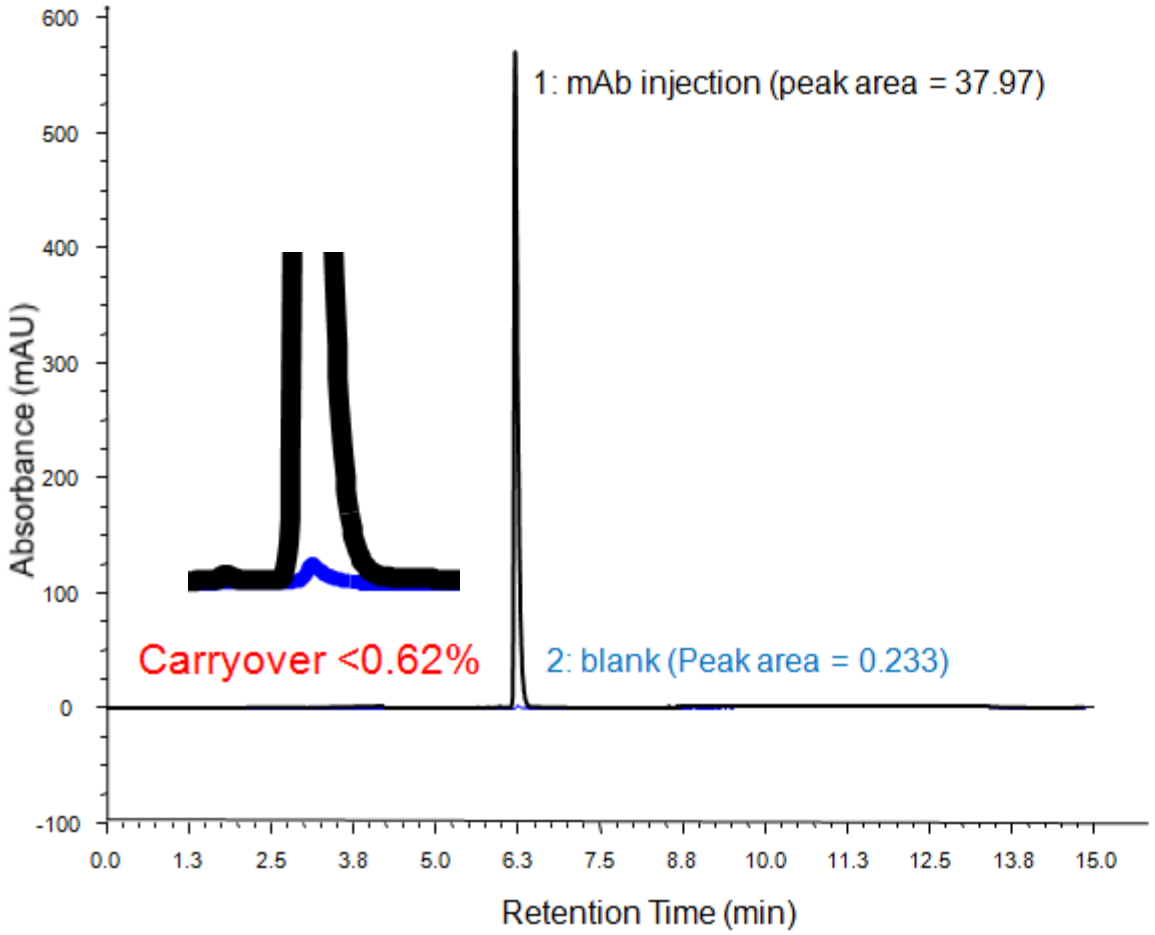
Minimized Carryover using Polymeric MAbPac RP



Column:	MAbPac RP, 4 µm
Format:	3 ×50 mm
Mobile phase A:	H_2O/TFA (99.9 : 0.1 v/v)
Mobile phase B:	MeCN/ H_2O/TFA (90: 9.9 :0.1 v/v/v)

Gradient:

Time (min)	%A	%B
0.0	100	0
1.0	100	0
11.0	0	100
12.0	0	100
14.0	100	0
15.0	0	100

Temperature:	80 ºC
Flow rate:	0.5 mL/min
Inj. volume:	5 µL
Detection:	UV (280 nm)
Sample:	mAb (5 mg/mL)

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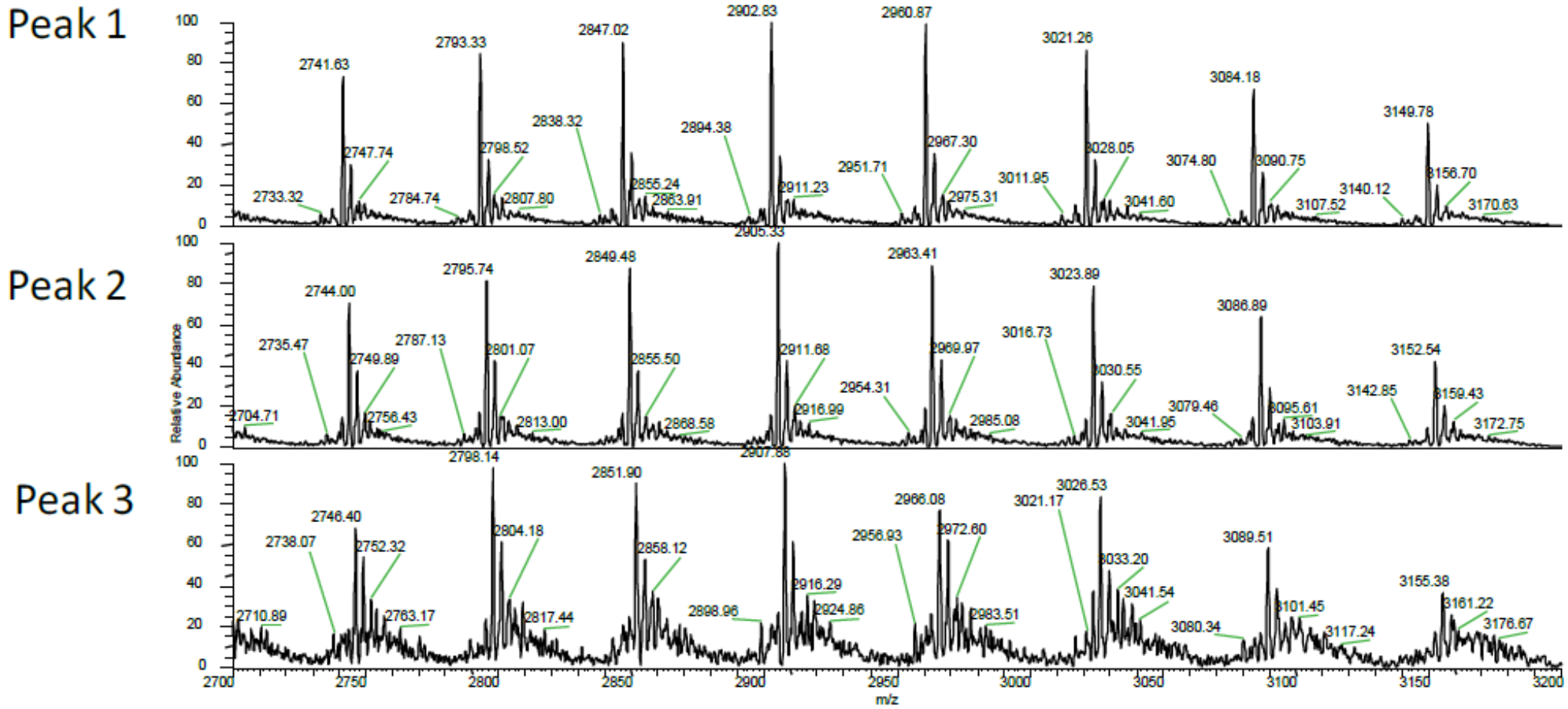
Full Scan MS Spectra from Q Exactive



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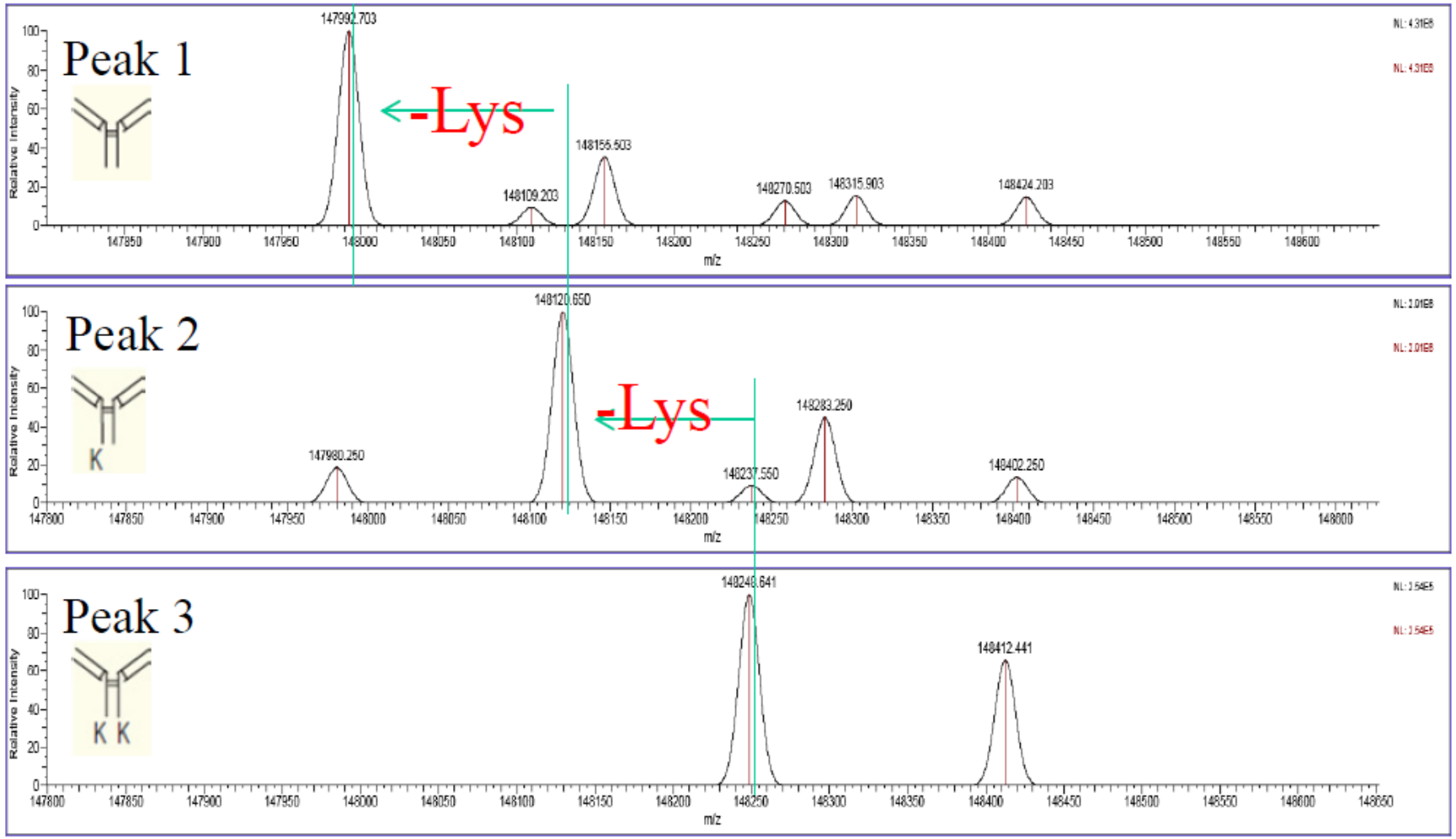
Deconvolved Spectra



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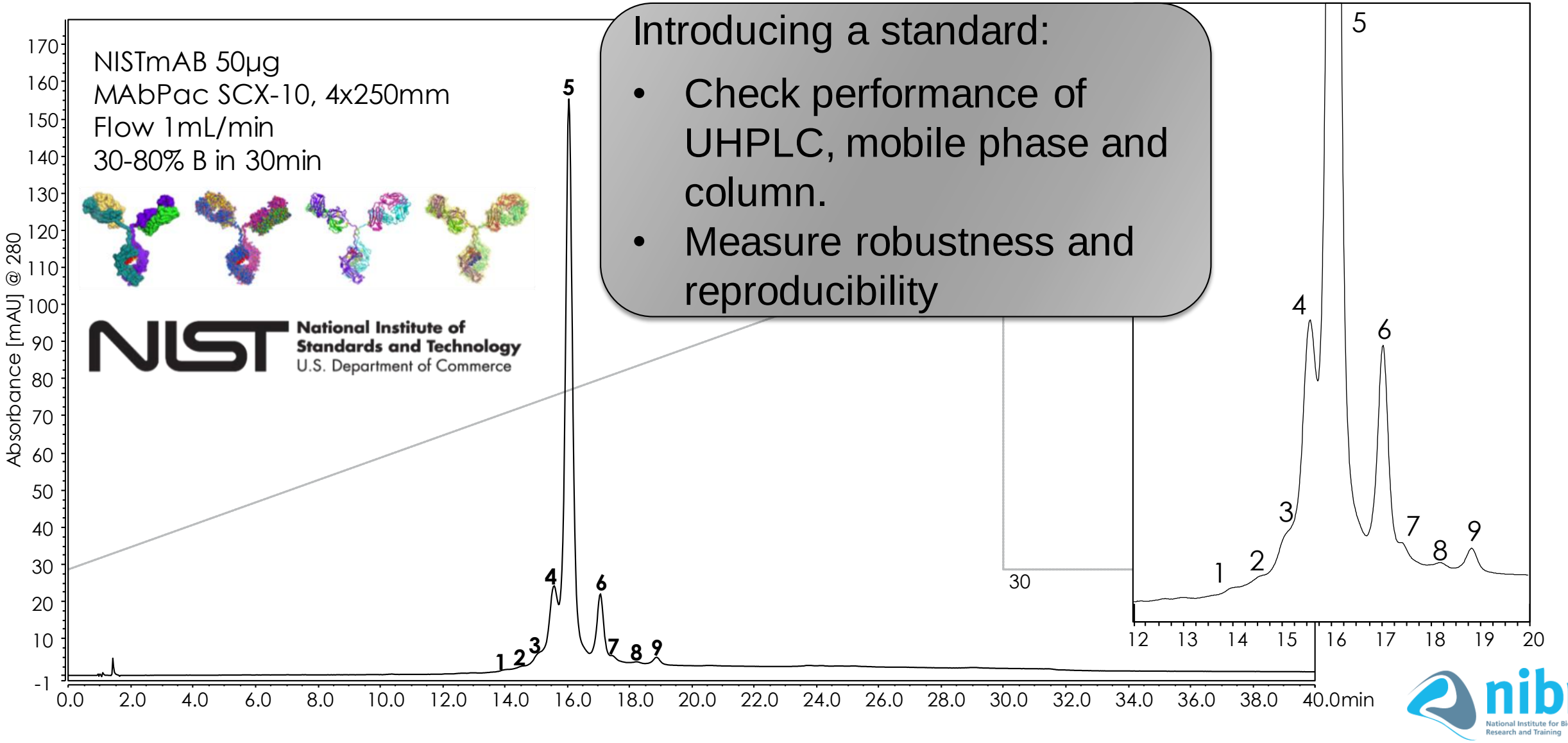
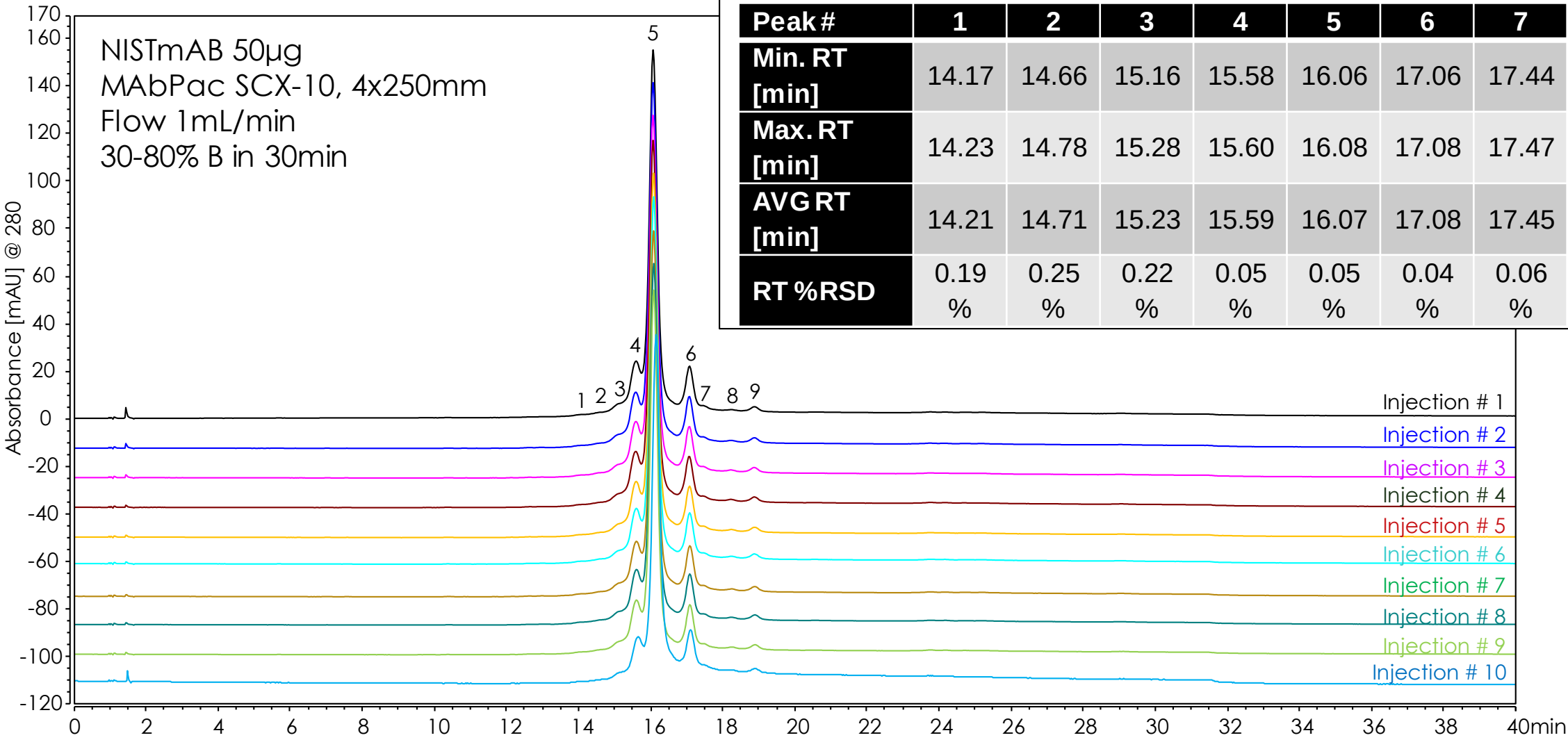
Optimized Conditions



Reproducibility NIST mAb



Peak #	1	2	3	4	5	6	7	8	9
Min. RT [min]	14.17	14.66	15.16	15.58	16.06	17.06	17.44	18.22	18.87
Max. RT [min]	14.23	14.78	15.28	15.60	16.08	17.08	17.47	18.25	18.90
AVG RT [min]	14.21	14.71	15.23	15.59	16.07	17.08	17.45	18.24	18.88
RT %RSD	0.19 %	0.25 %	0.22 %	0.05 %	0.05 %	0.04 %	0.06 %	0.07 %	0.06 %

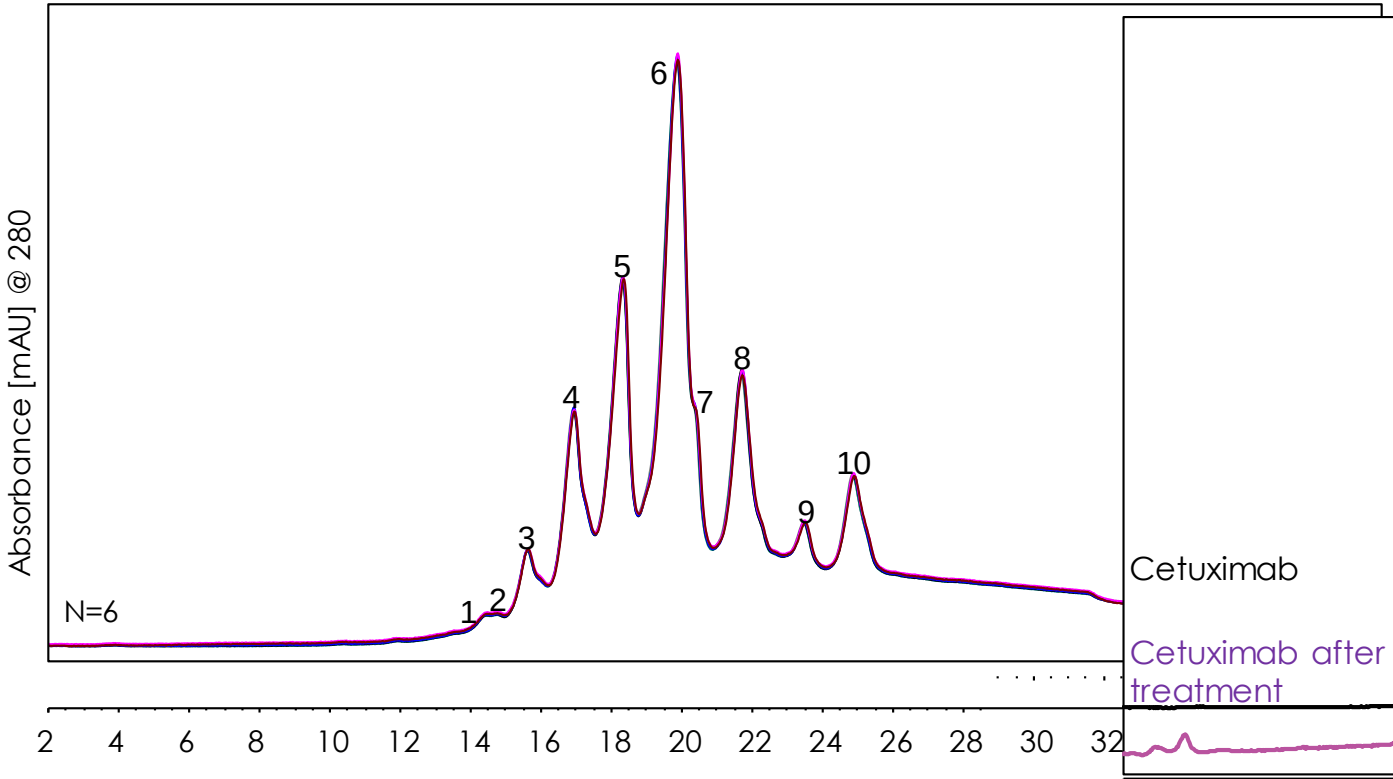
nibrt
National Institute for Bioprocessing Research and Training

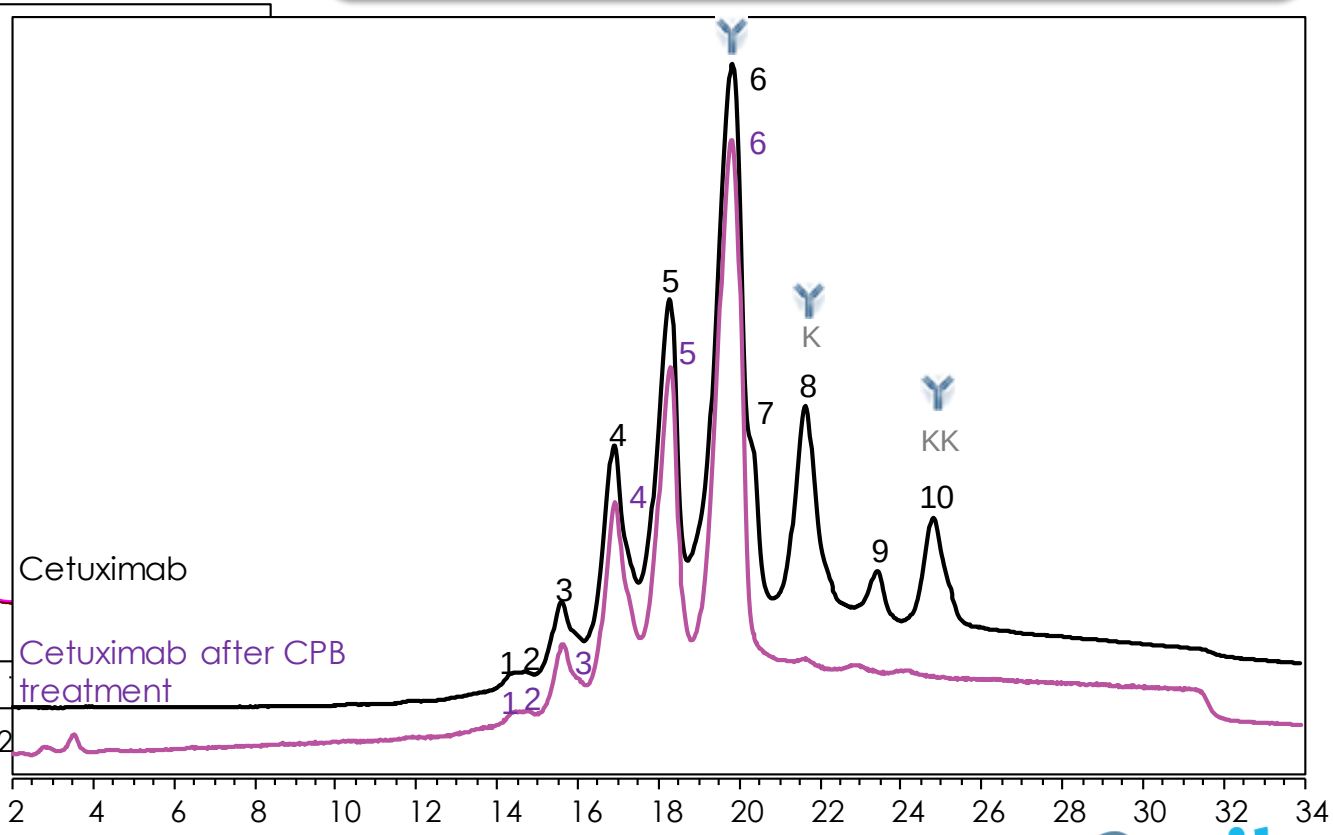
ThermoFisher SCIENTIFIC

Cetuximab Commercial Drug Product

Peak #	1	2	3	4	5	6	7	8	9	10
Minutes	14.50	14.75	15.64	16.94	18.33	19.89	20.42	21.72	23.48	24.89
RT RSD %	0.44	0.13	0.05	0.08	0.08	0.07	0.28	0.08	0.19	0.05

Carboxypeptidase B (CPB) digest

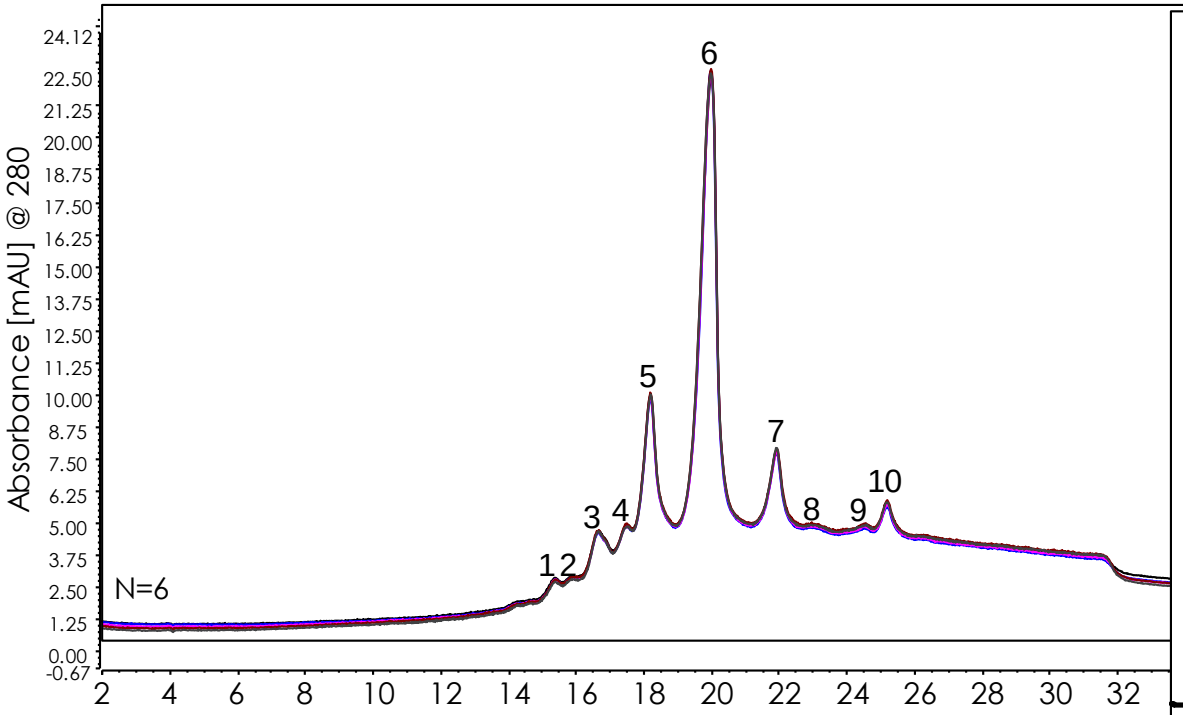


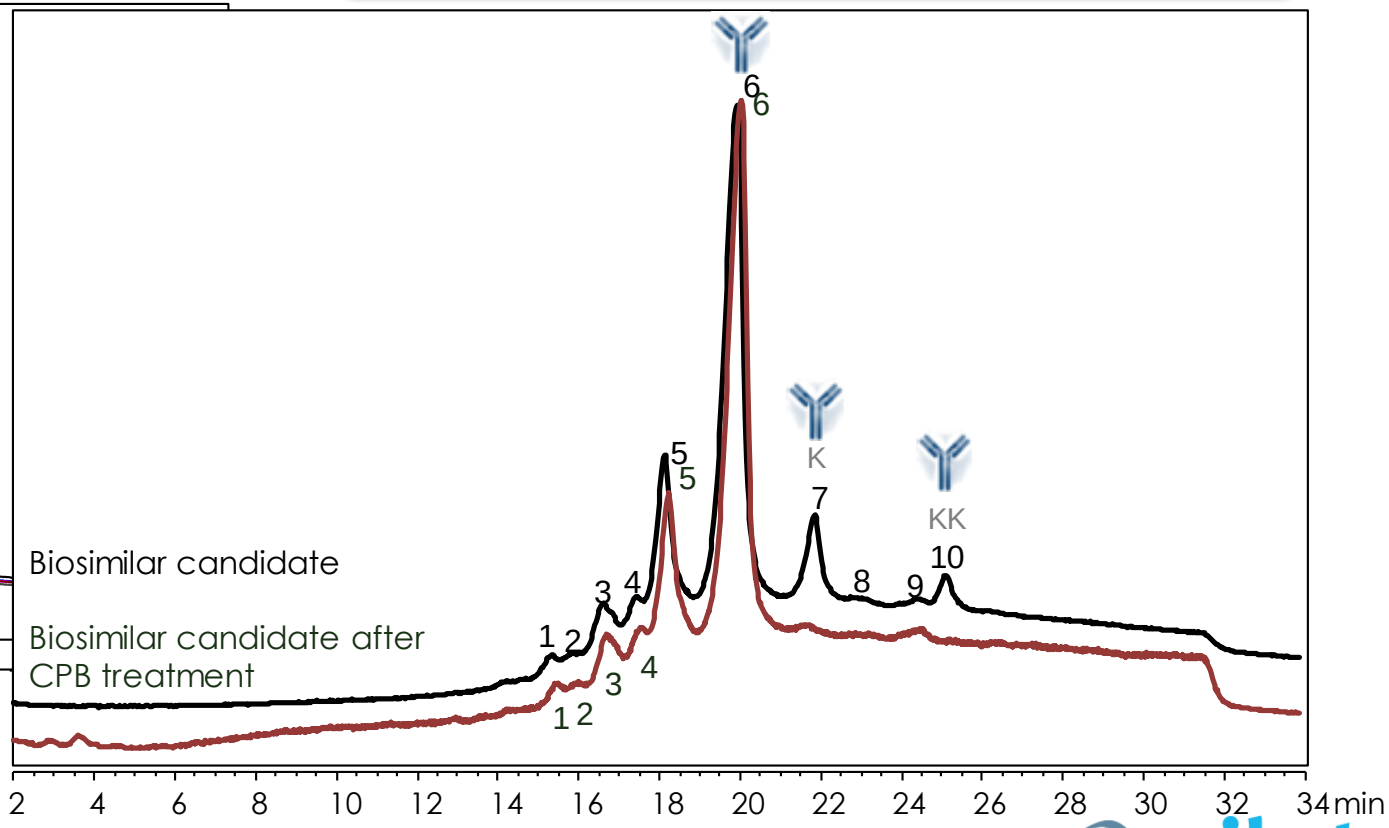


Biosimilar Candidate

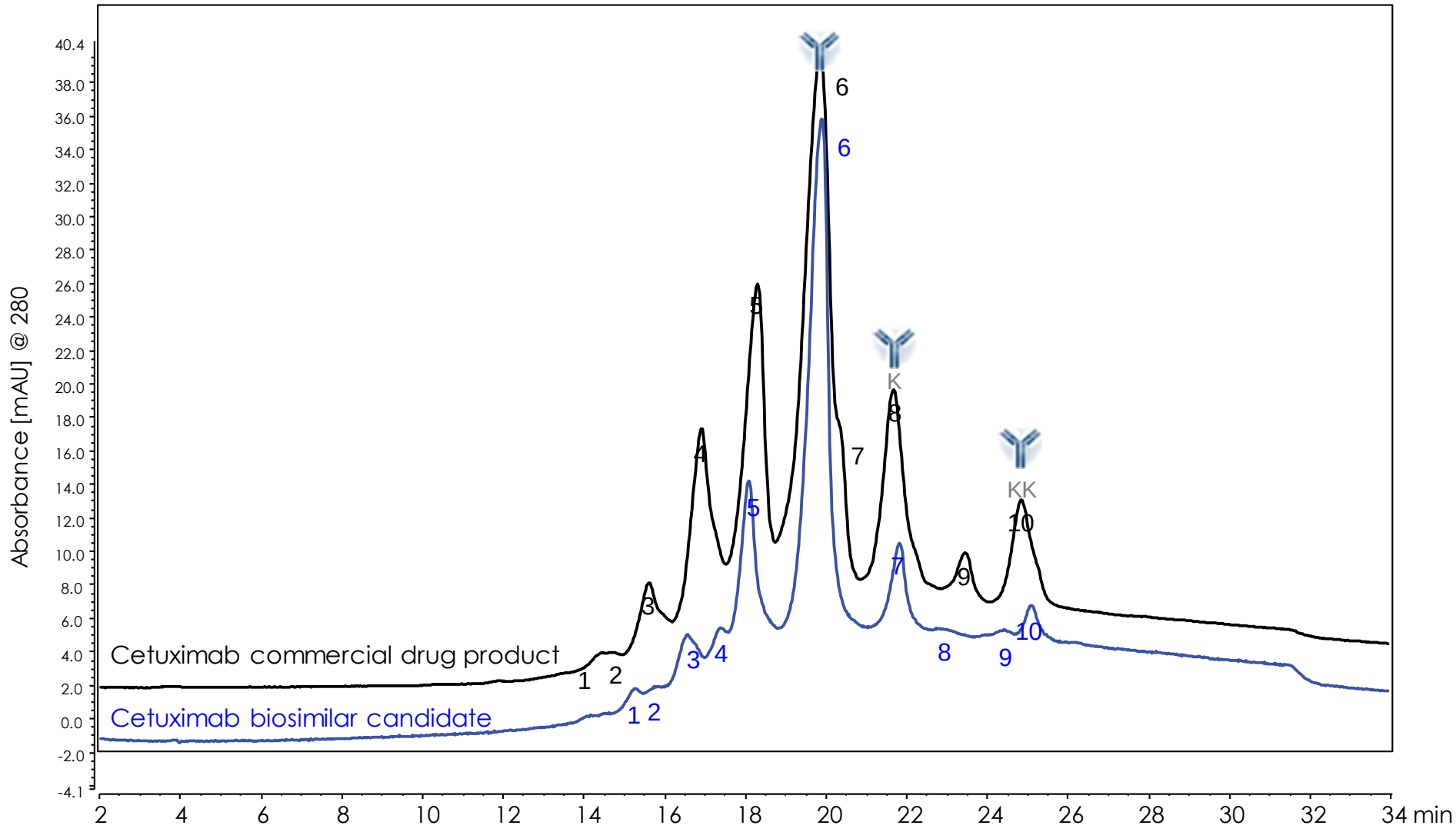
Peak #	1	2	3	4	5	6	7	8	9	10
Minutes	15.38	16.18	16.65	17.51	18.19	19.97	21.91	22.94	24.52	25.17
RT RSD %	0.10	0.65	0.08	0.13	0.04	0.03	0.01	0.20	0.11	0.04

Carboxypeptidase B (CPB) digest





Drug Product vs. Biosimilar Candidate



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Compare Expression Systems

Potential causes of variation	Chinese Hamster Ovary	Human Embryonic Kidney	Drug Product (NS0 Cell line)
Transfection	Transient	Transient	Stable
Culture Duration	8 days	6 Days	N/D
DNA construct	Human	Human	Chimeric mouse/human
Purification	Purified from culture media into neutralized Acetic Acid	Purified from culture media into neutralized Acetic Acid	Diafiltrated into formulation buffer prior to purification
Viability at harvest	~93%	~70%	N/D
Viable Cell count at harvest	(Mean) $6.9x10^6$ cells/mL	(Mean) $8.3x10^6$ cells/mL	N/D
Culture Volume	50 mL	50 mL	10,000-12,000 L

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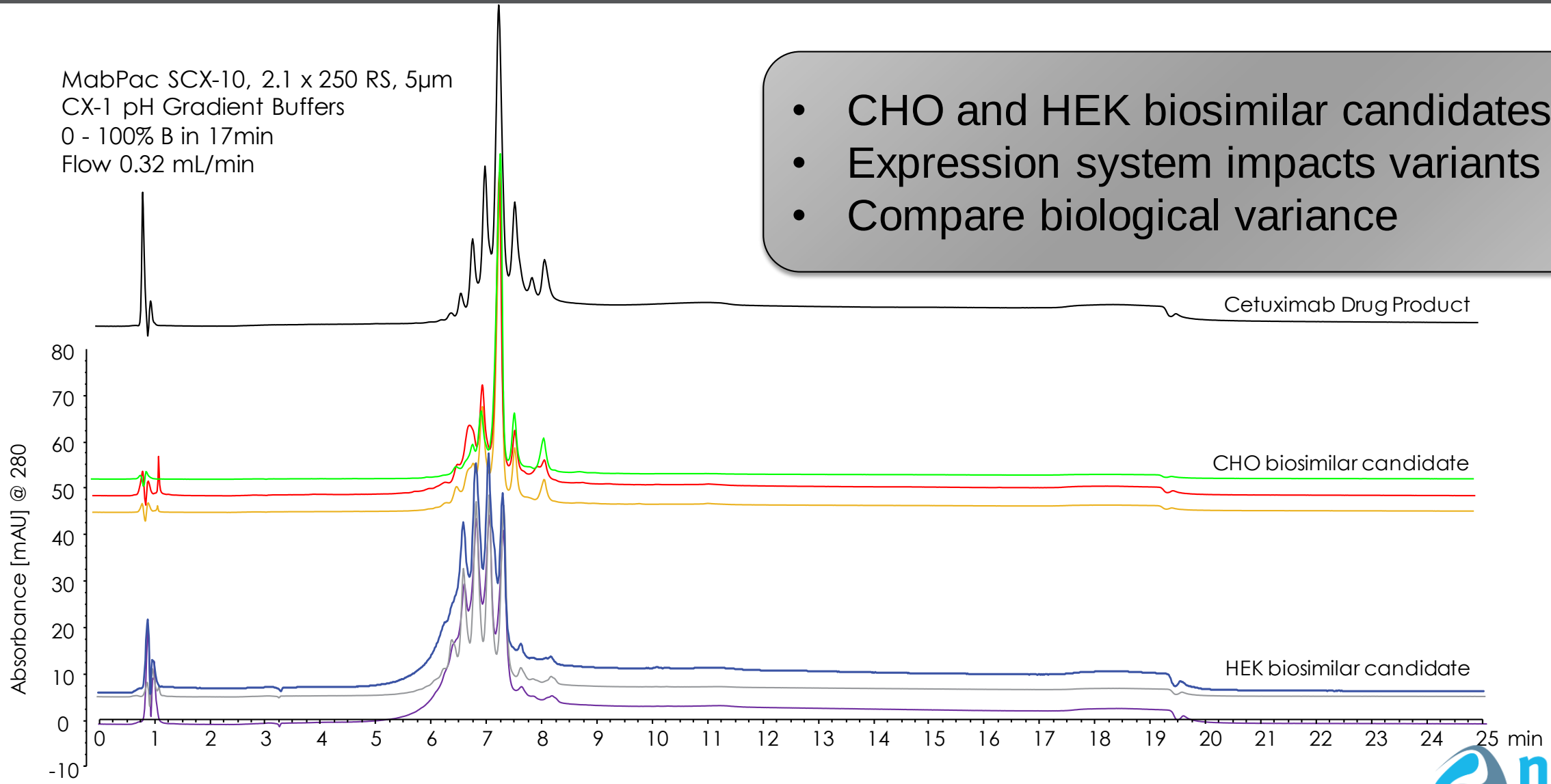
Different Charge Variants



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

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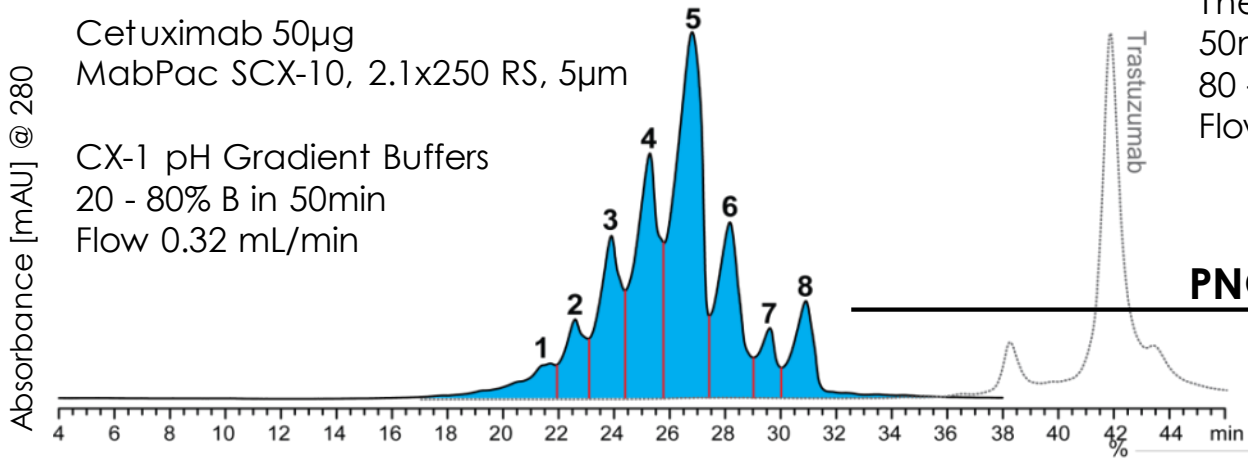
Preparative Chromatography

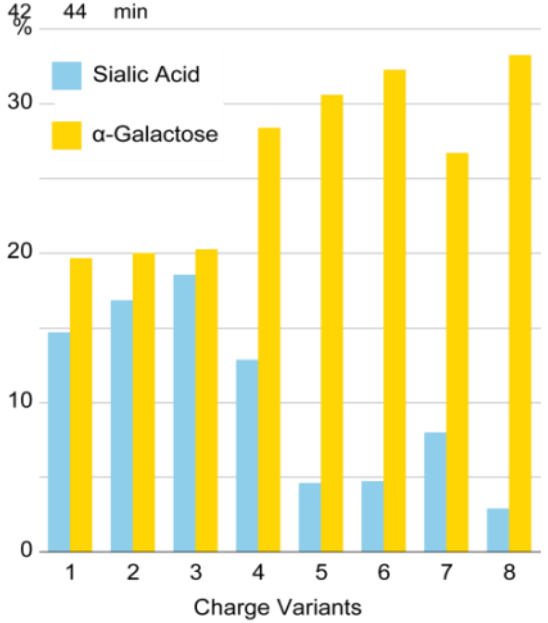
Cetuximab 50µg
MabPac SCX-10, 2.1x250 RS, 5µm

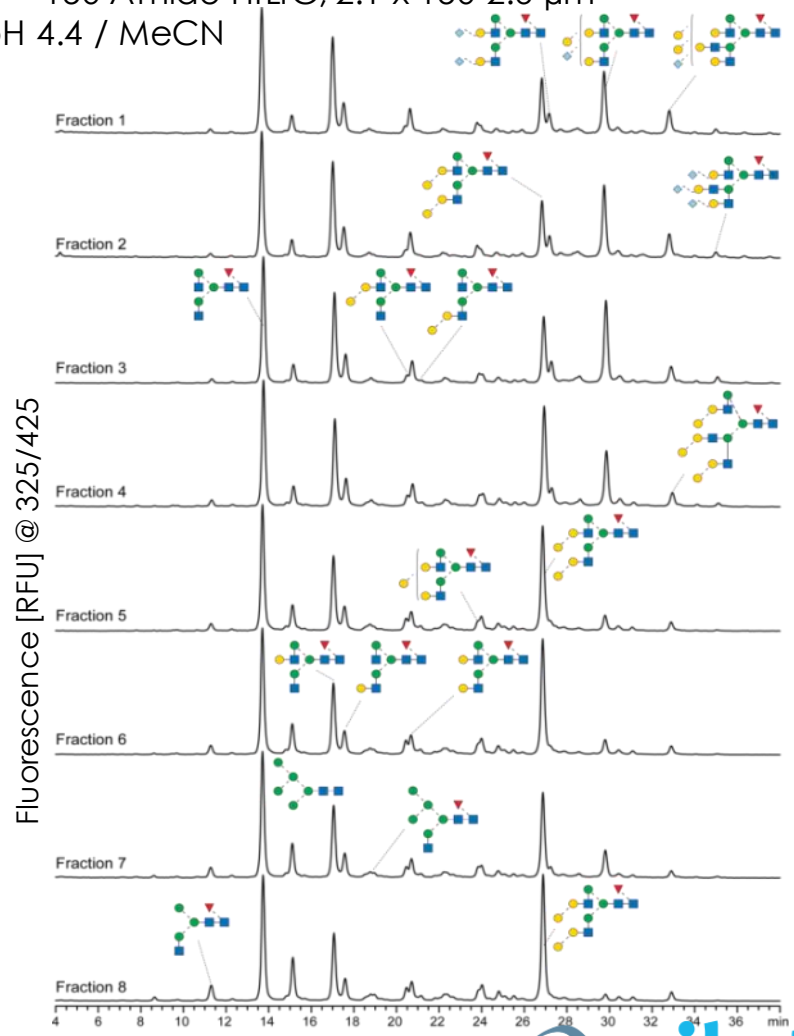
CX-1 pH Gradient Buffers
20 - 80% B in 50min
Flow 0.32 mL/min



Thermo Scientific™ Accucore™ 150 Amide HILIC, 2.1 x 150 2.6 µm
50mM Ammonium Formate, pH 4.4 / MeCN
80 – 60% B in 40min
Flow: 1 mL/min

PNGase F





Fraction collection using pH gradient buffer elution

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Conclusions: Charge Variant Analysis

- Charge variant analysis can be achieved with salt or pH gradient elution
- New column chemistries are available that can provide increased speed and resolution
- pH gradient elution can provide several advantages including; Global applicability, Increased speed, fast method development, High loading capacity, less column dependence, Easy method transfer
- Collection of variant peaks can be easily desalted on-line for further analysis by MS
- Protein expression in different cell lines resulted in altered charge variants
- Charge variants peak collection and individual *N*-glycan analysis can identify sialylation patterns.

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