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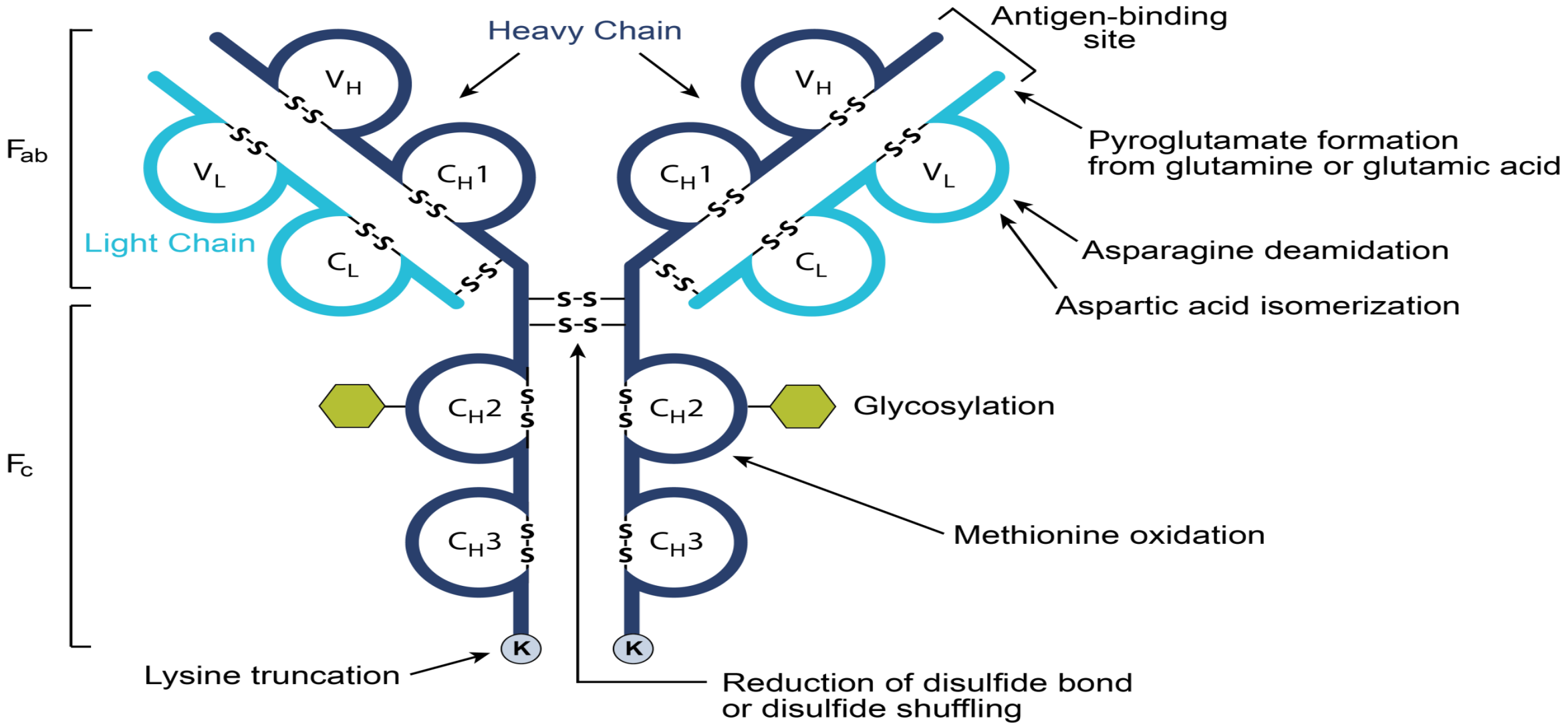
Taking aggregate analysis from research into the routine environment

Spring 2017 Bio-Pharma Summit

Structure of IgG and Typical Forms of Heterogeneity



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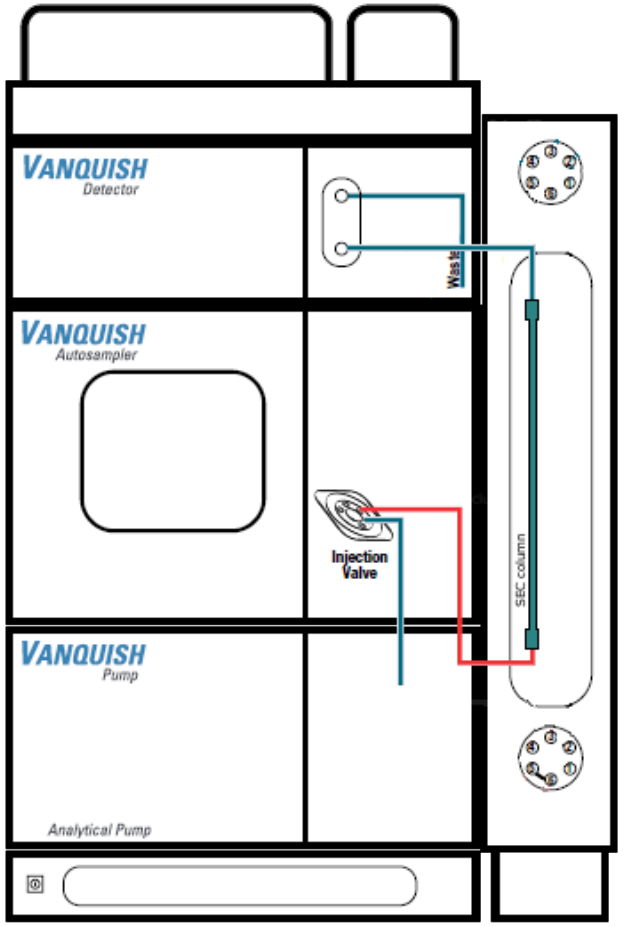
Bio-Column Selection Guide

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



Inlet tubing optimisation may be required for low flow SEC analysis

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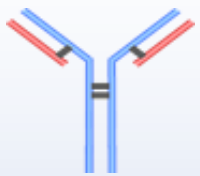
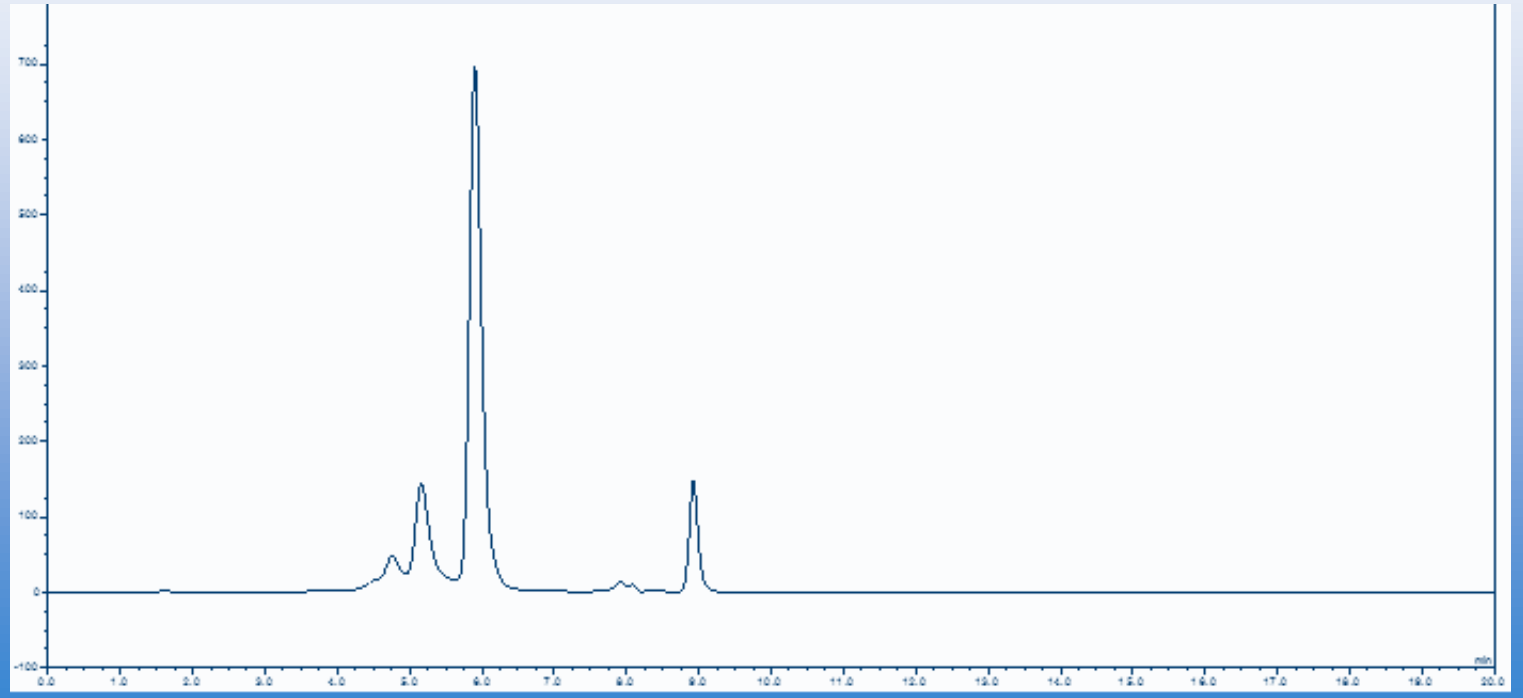
Routine screening and analysis for Aggregation

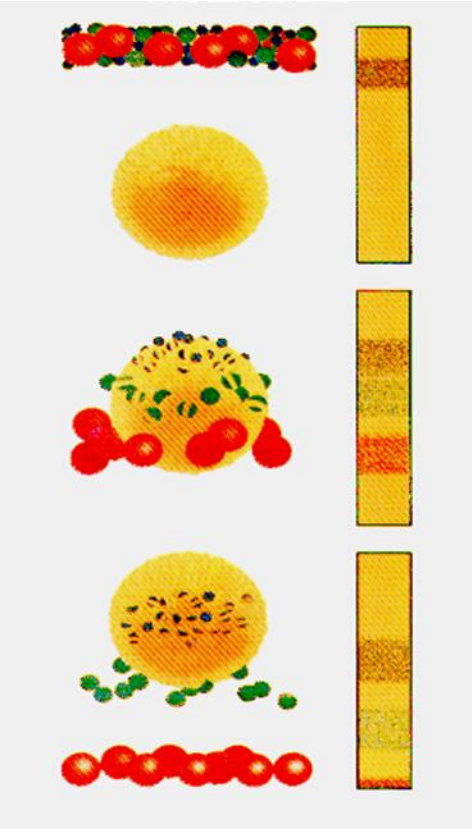
From screening of clones, to formulation and shelf life, Aggregation Analysis is essential at all stages.

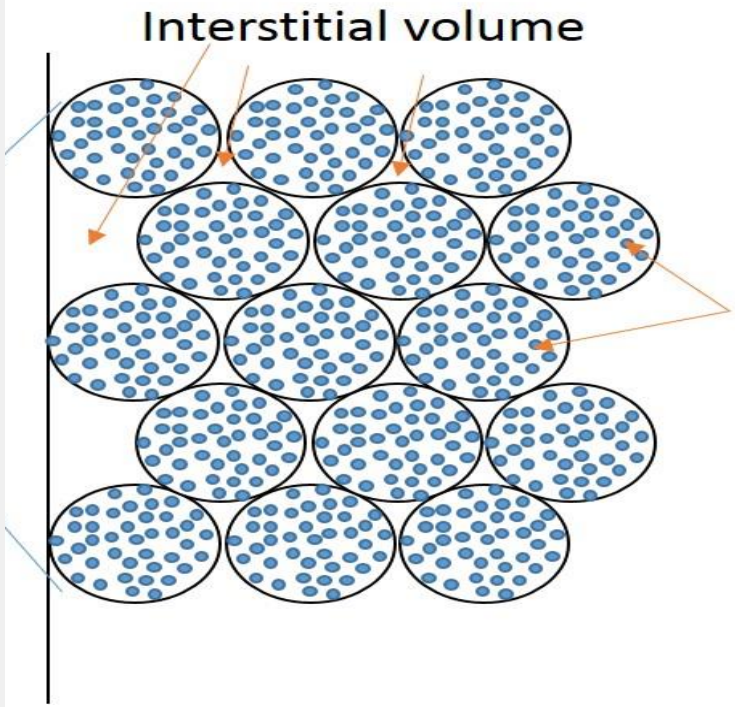


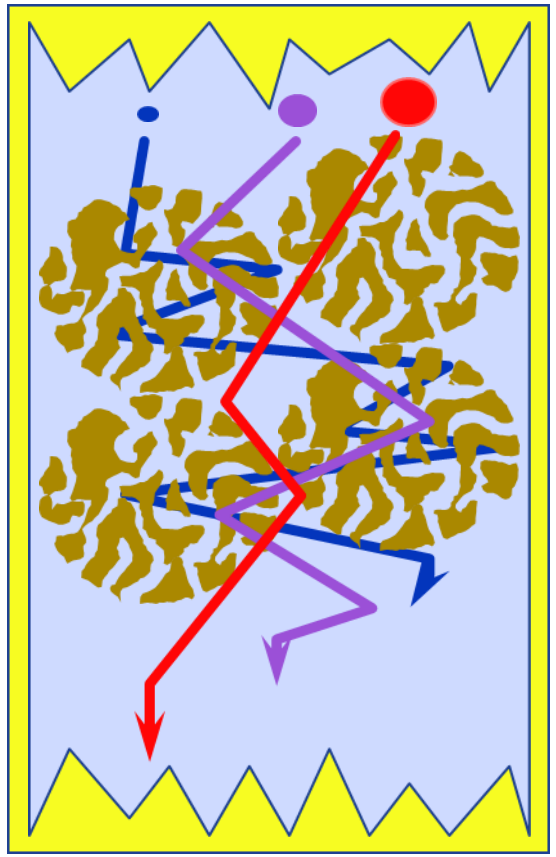
Clone Selection → R & D → Production → Formulation and life time



Size Exclusion Chromatography



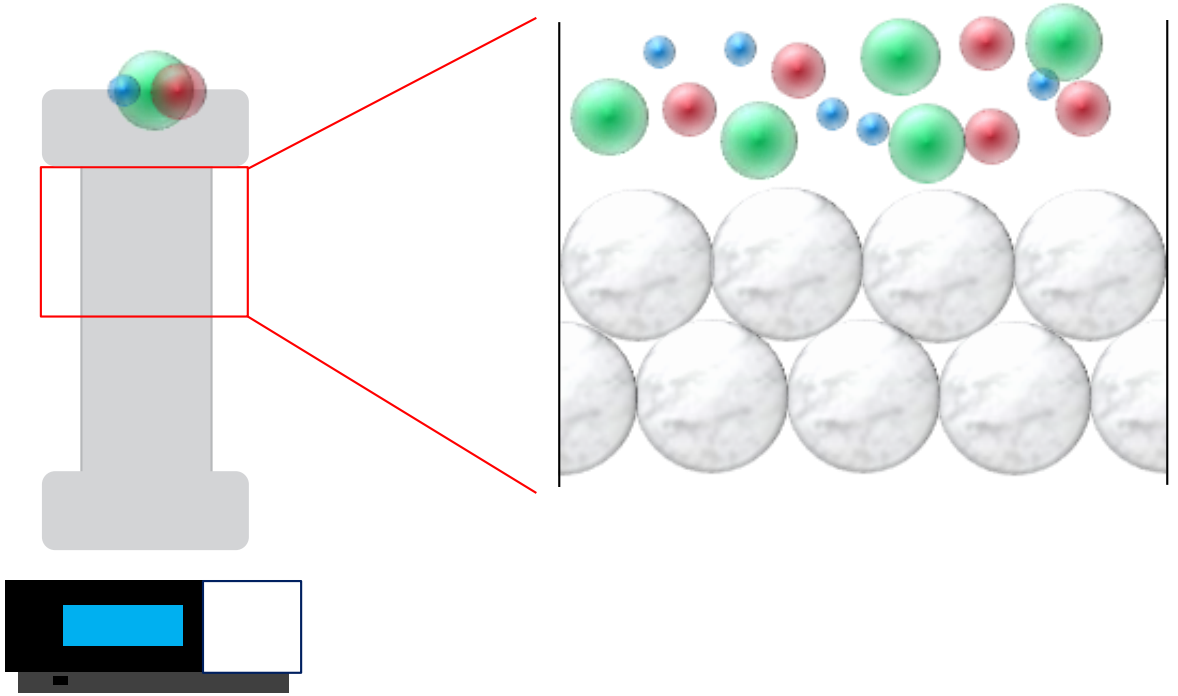


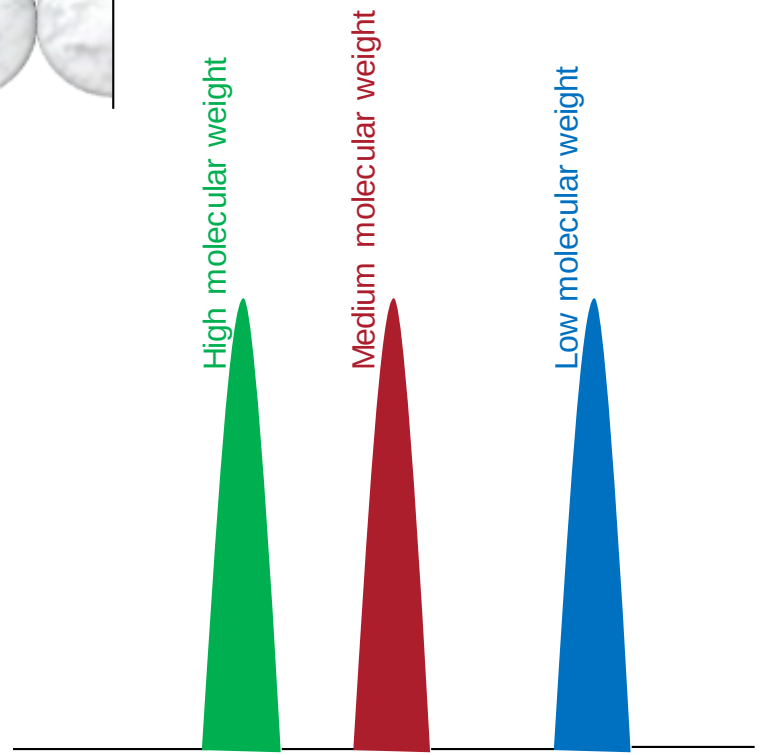


- Limits on diffusion control the separation
  - Pore size – controls the relative separation of proteins
  - Pore volume – controls the overall retention time of each species

Aggregation Analysis

- Typically using Size Exclusion Chromatography (SEC)
- MAbPac SEC-1
  - Silica, 5 µm, 300Å





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Physical Data on a MAbPac SEC column

Bonding Chemistry	**Diol**
Silica Substrate	Spherical, high-purity porous silica
Particle size	5 µm
Pore size	300 Å
Column housing	PEEK for 4.0 mm I.D. columns SST for 7.8 mm and 2.1 mm I.D. columns
Separation range for globular proteins	10,000 - 1,000,000
Exclusion limit for globular proteins	>1,000,000

The Hydrophilic boundary layer and the Pore Size are important factors

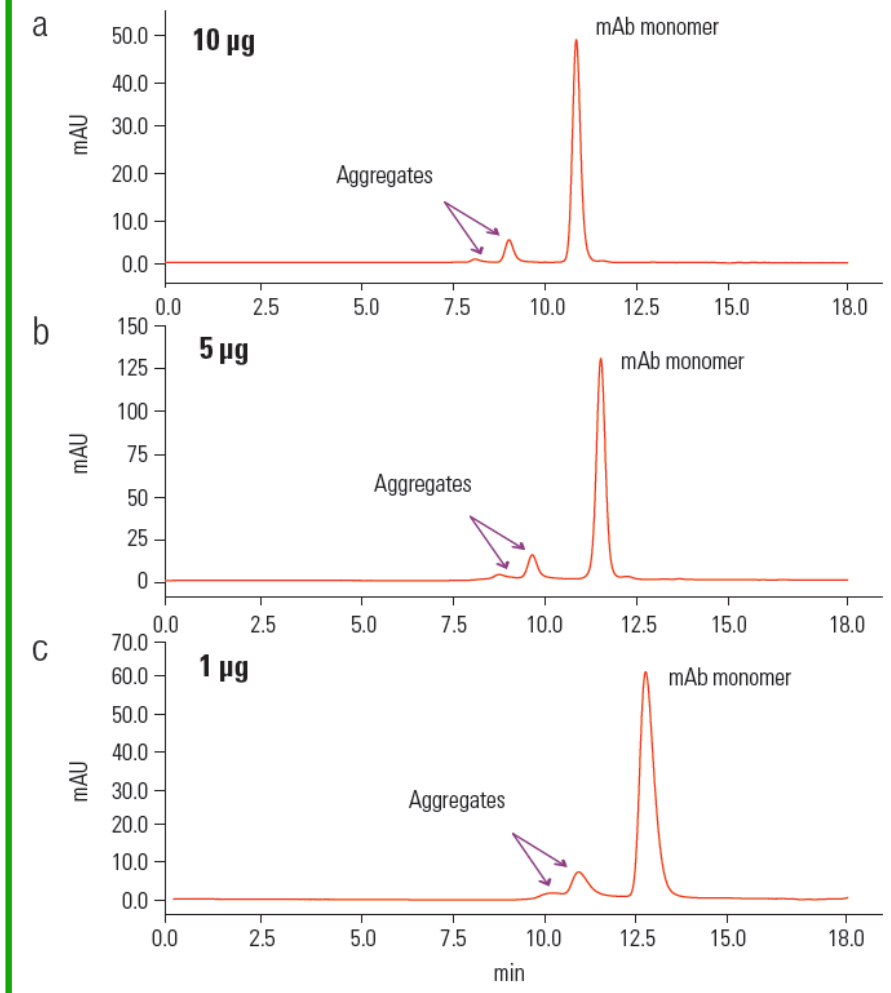
Column Formats Versus Target Applications

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

Column Scalability in SEC Analysis

- Range of MAbPac SEC-1 columns
  - 7.8 mm ID
  - 4.0 mm ID
  - 2.1 mm ID
- Highest resolution between monomer and dimer/trimer using 7.8 mm ID
- Easiest MS coupling using 2.1 mm ID



Column:	**MAbPac SEC-1**, 5 µm,
Format:	a. 7.8 × 300 mm
	b. 4.0 × 300 mm
	a. 2.1 × 300 mm
Mobile Phase:	50mM sodium phosphate pH 6.8, in 300 mM sodium chloride
Flow Rate:	a. 760 µL/min
	b. 200 µL/min
	c. 50 µL/min
Inj. Volume:	a. 10 µL
	b. 5 µL
	a. 1 µL
Temp.:	30 °C
Detection:	280 nm
Sample:	mAb (1 mg/mL)

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Optimizing Aggregation Analysis with UHPLC

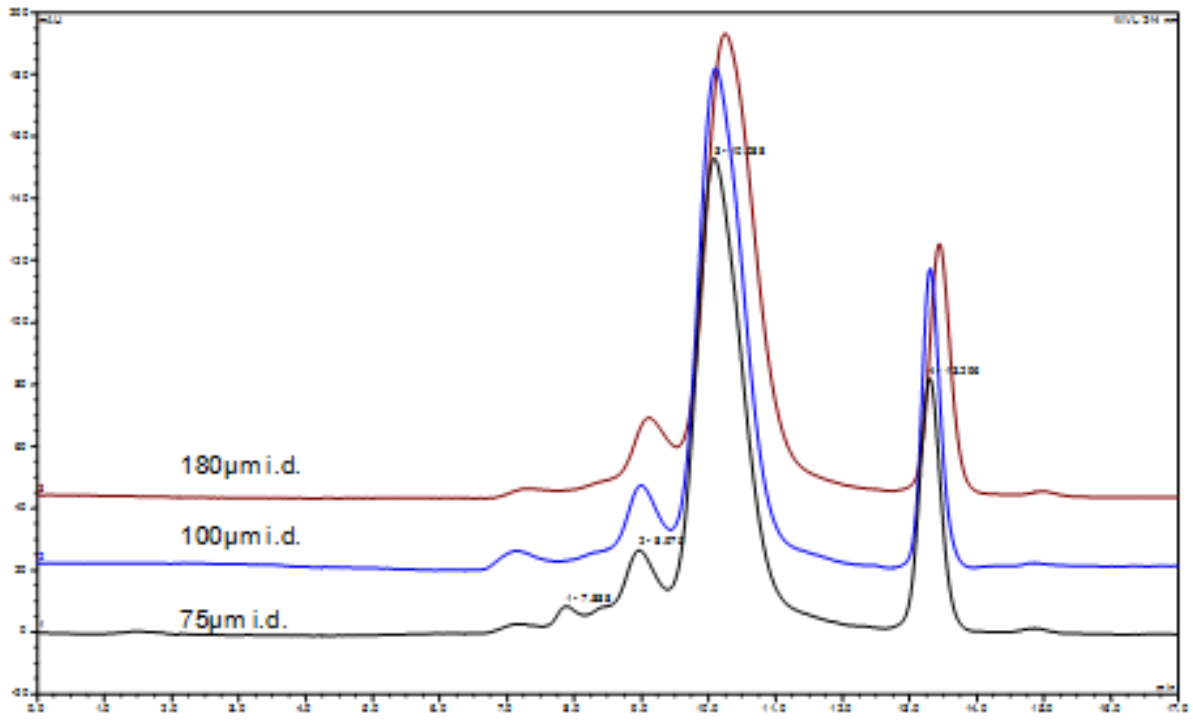
- UHPLC systems ideal for SEC
  - Optimized flow path without dead volumes
- Thermo Scientific Vanquish
  - Easily adaptable tubing
  - Viper connections
  - Bio-inert construction
  - Sample pre-compression routine
  - Adaptable flow cell volumes

Vanquish Platform

Viper Connection

4 x 300mm column

Optimize LC Set-up in front of SEC Column



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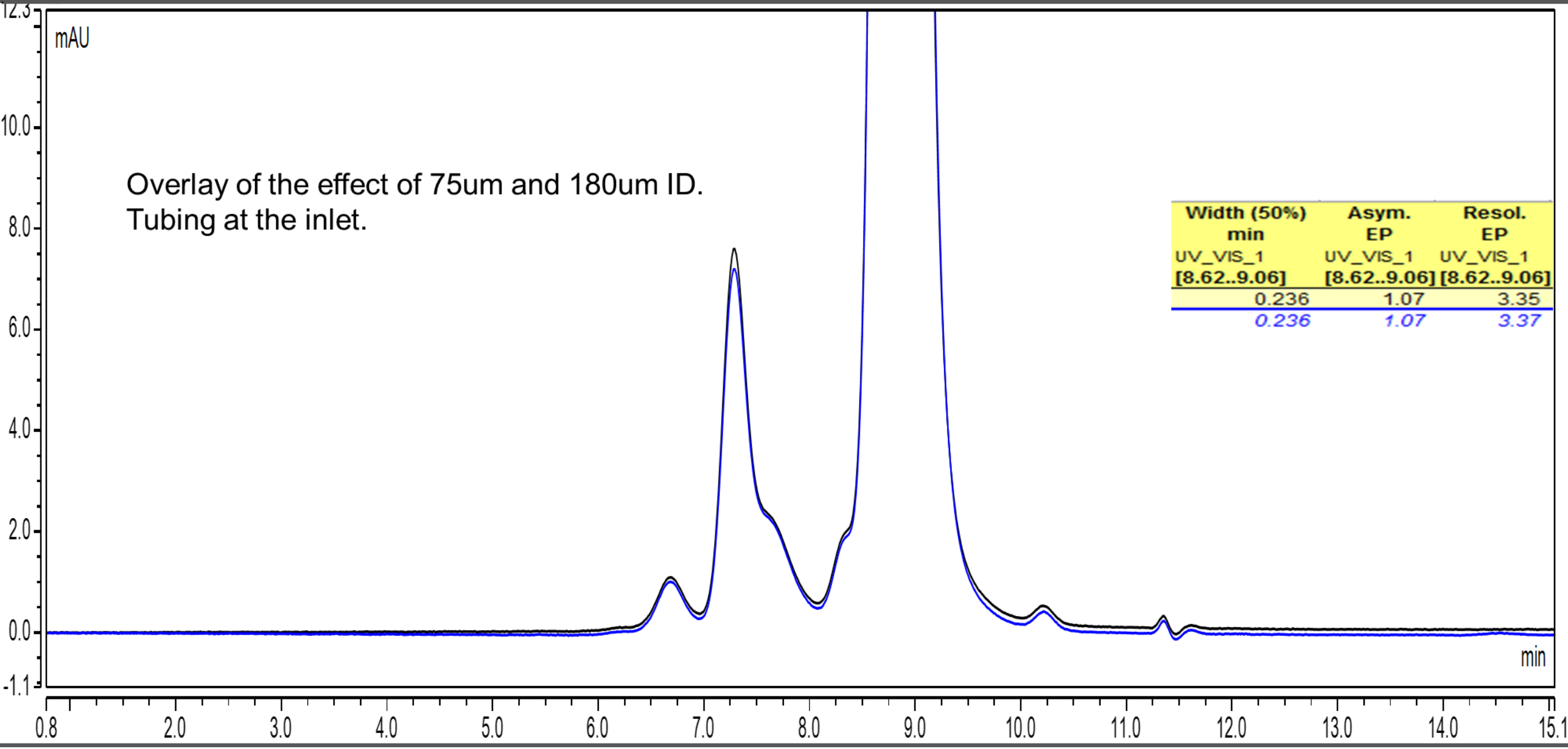
Effect of tubing on 7.8mm SEC [1.0 ml/min]



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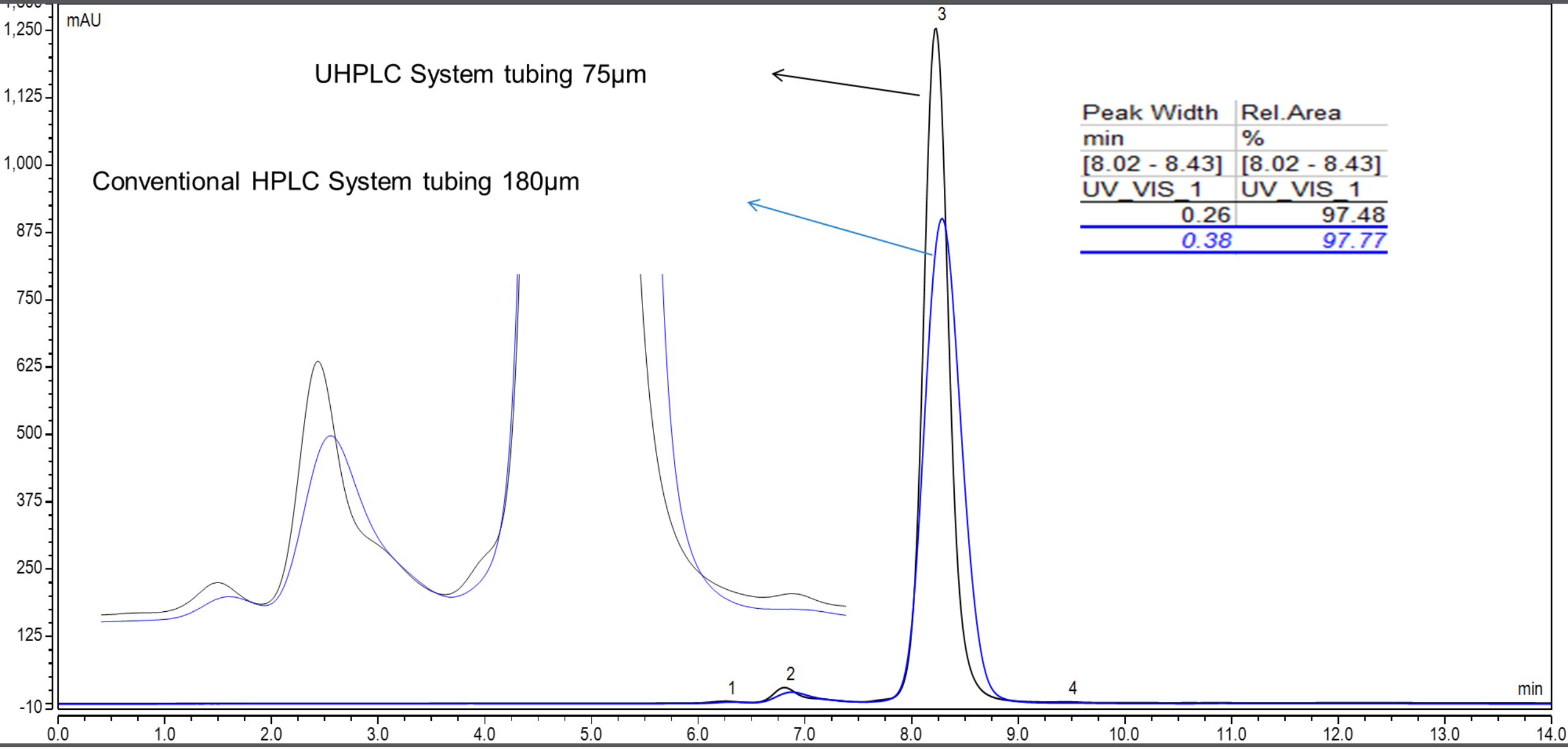
Effect of tubing with a 4mm SEC column [0.3 ml/min]



Peak Width	Rel.Area
min	%
[8.02 - 8.43]	[8.02 - 8.43]
UV_VIS_1	UV_VIS_1
0.26	97.48
0.38	*97.77*

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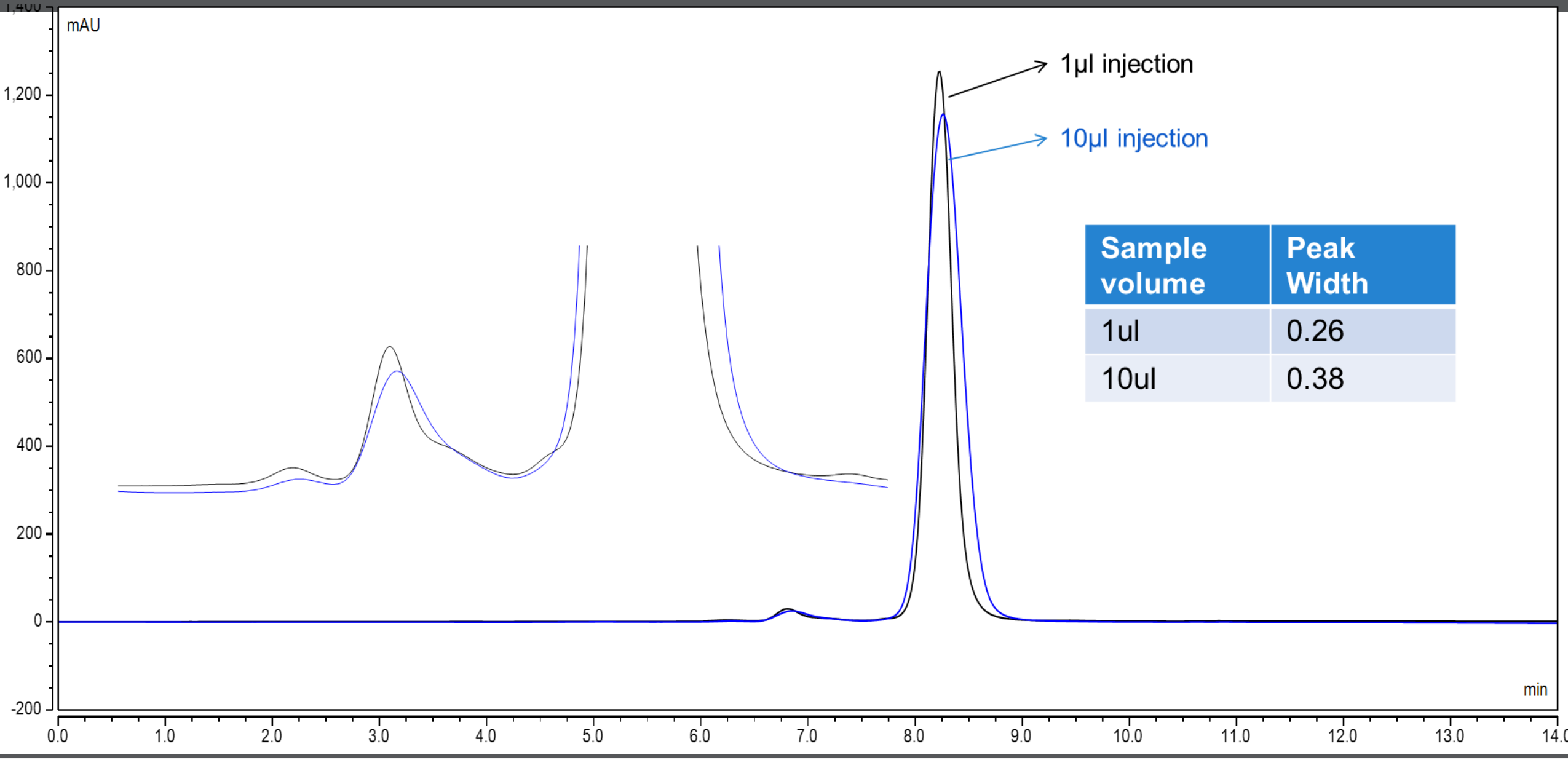
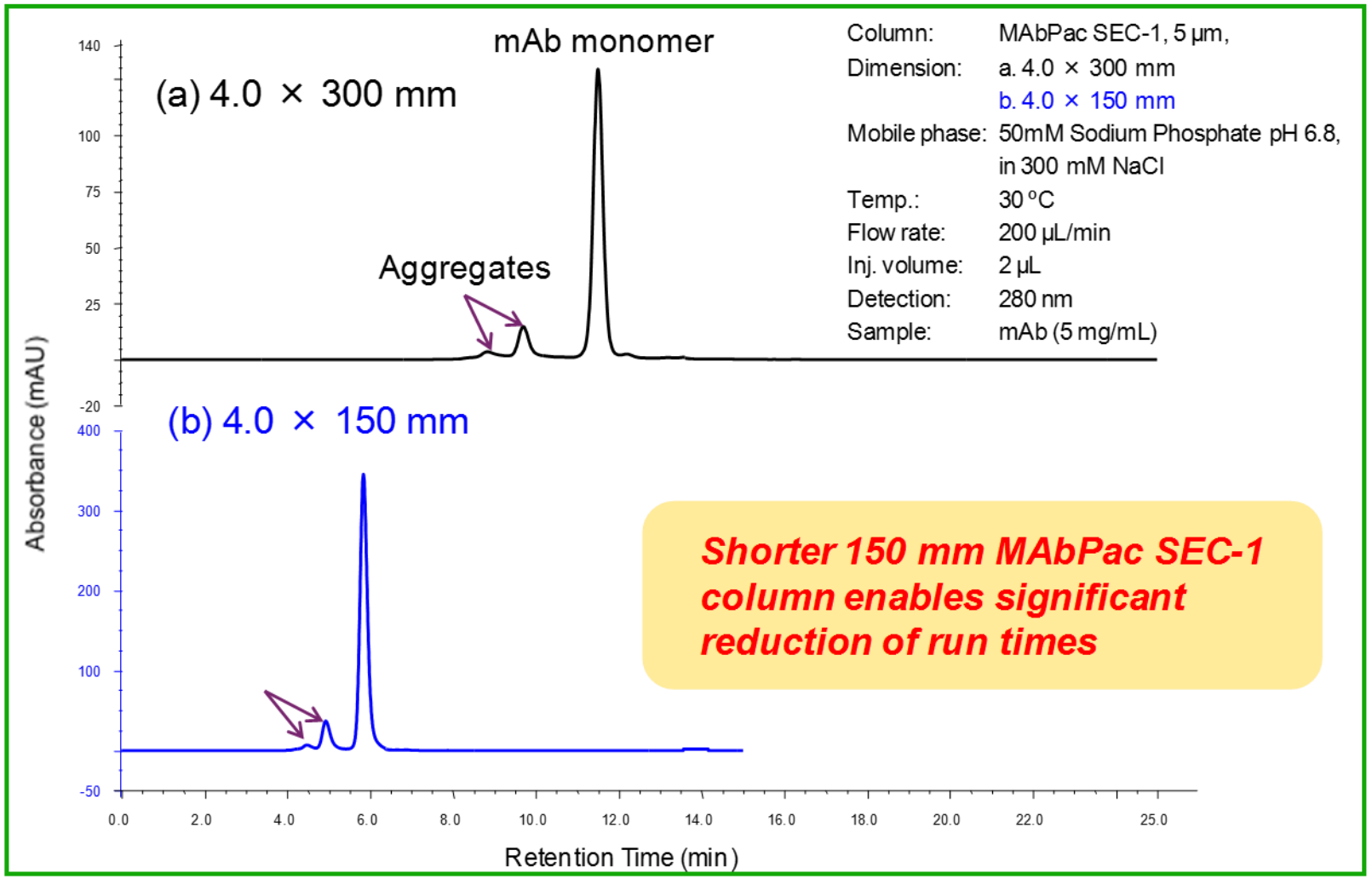
Changes in injection volume on 4mm SEC [diffusion before the analysis]



Sample volume	Peak Width
1ul	0.26
10ul	0.38

Reduced Runtimes in SEC

- Shorter column to reduces runtimes
- Increasing flow rate will negatively influence the resolution



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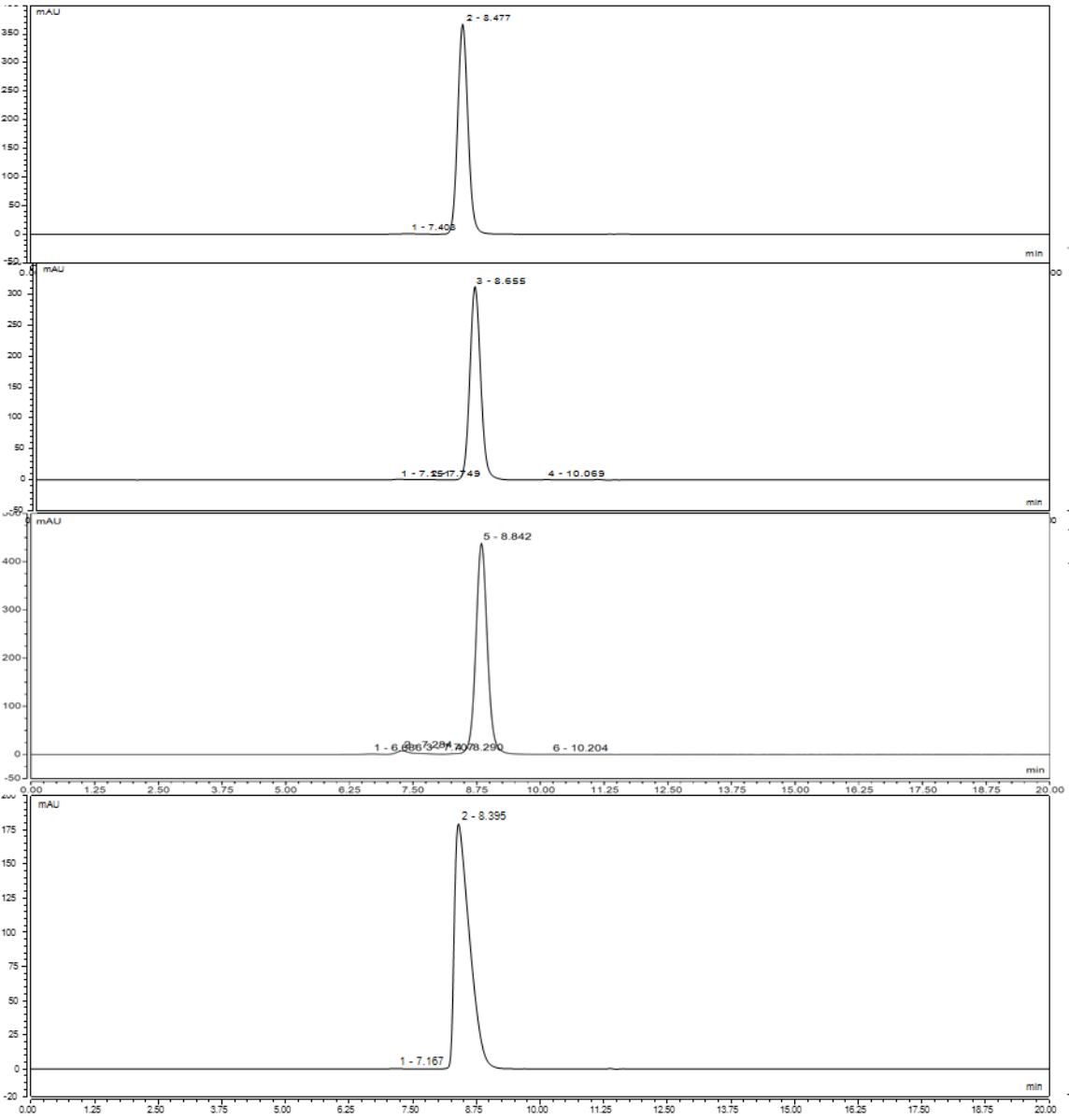
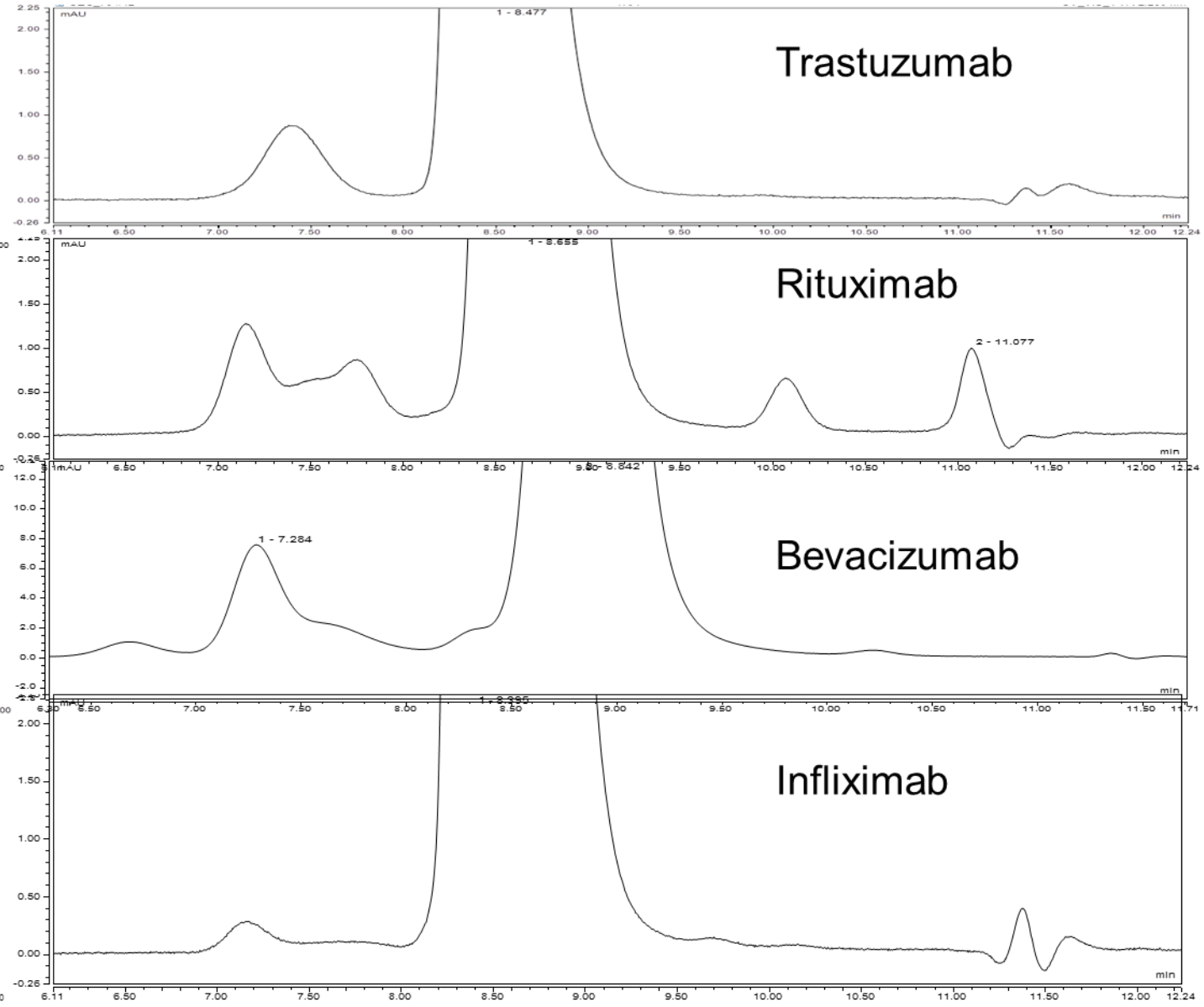
Top 4 Mab samples with the MAbPac SEC – Global Applicability





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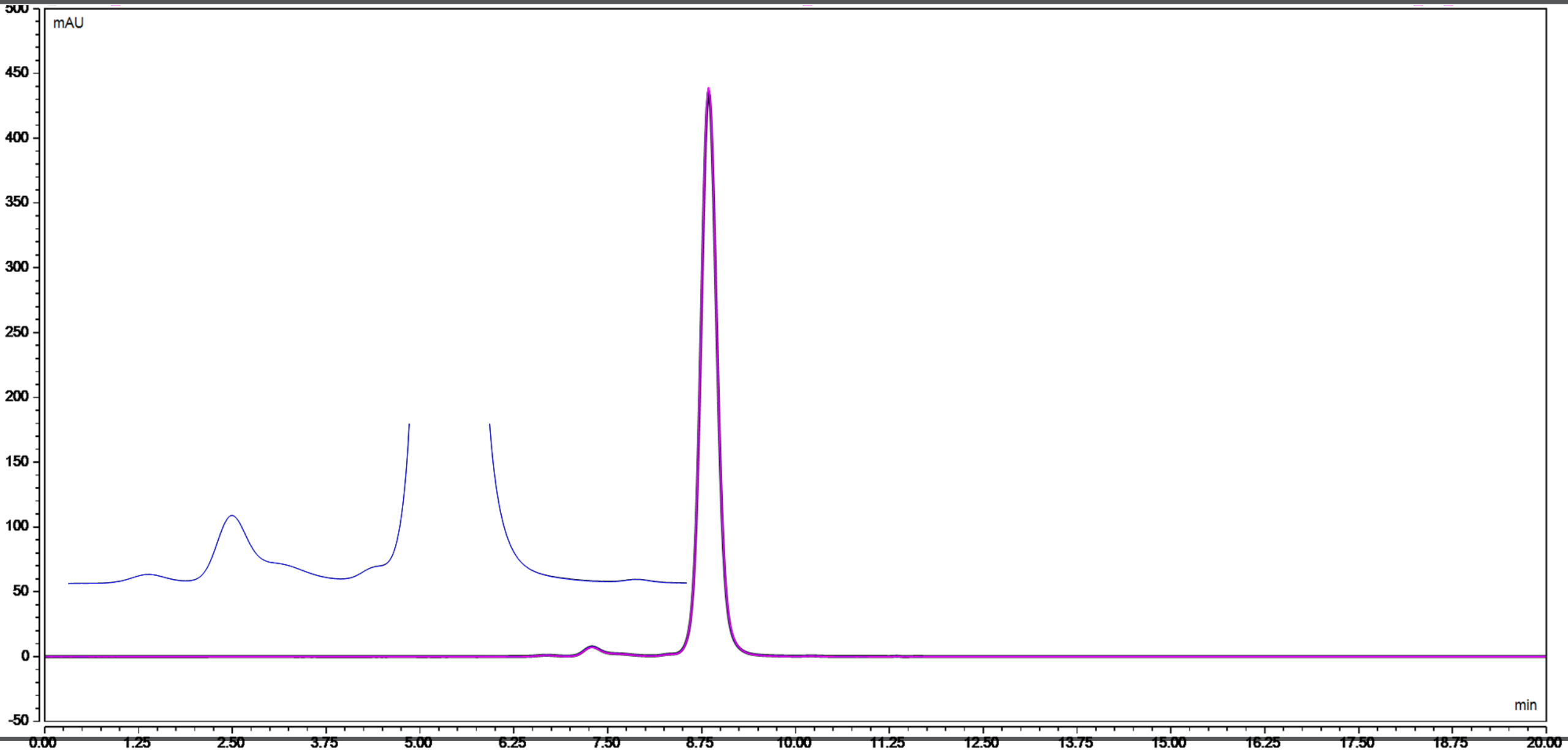
Overlay of 3 chromatography runs of Bevacizumab



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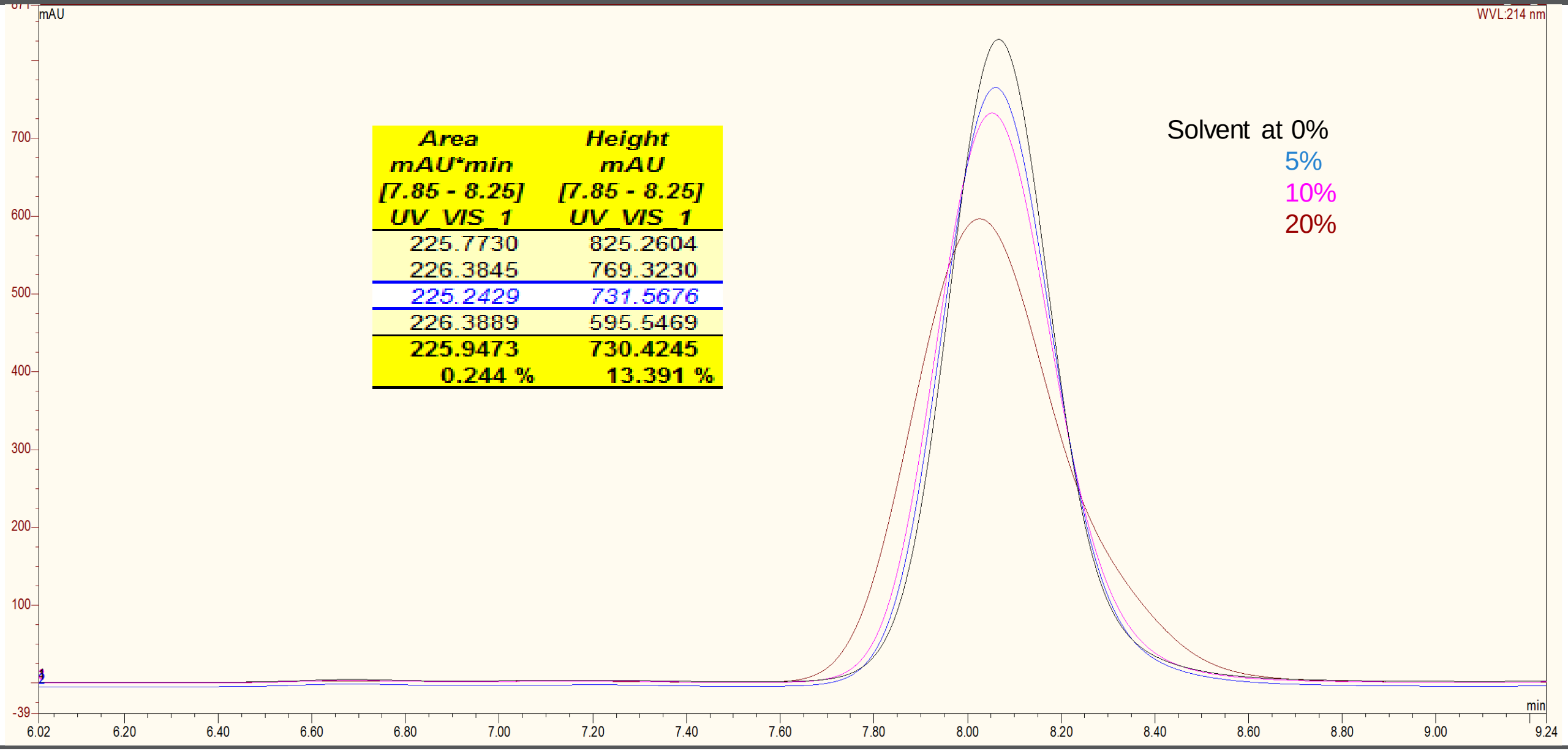
Effect of Solvent on SEC with Rituximab



Area mAU*min [7.85 - 8.25] UV_VIS_1	Height mAU [7.85 - 8.25] UV_VIS_1
225.7730	825.2604
226.3845	769.3230
225.2429	731.5676
226.3889	595.5469
225.9473	730.4245
0.244 %	13.391 %

Solvent at 0%
5%
10%
20%

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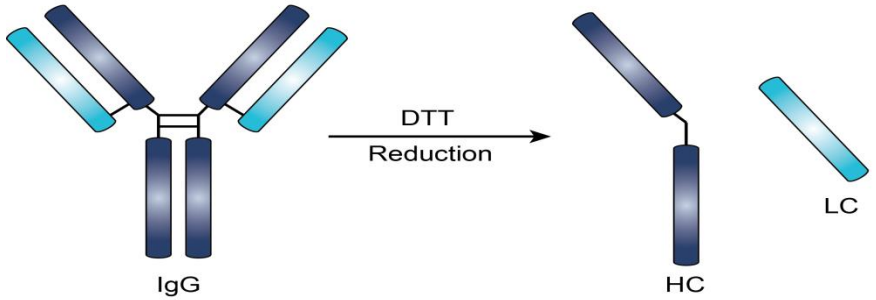
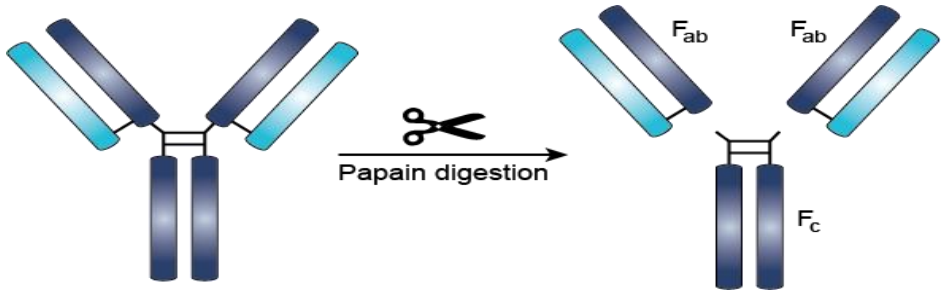
mAb Fragment Analysis

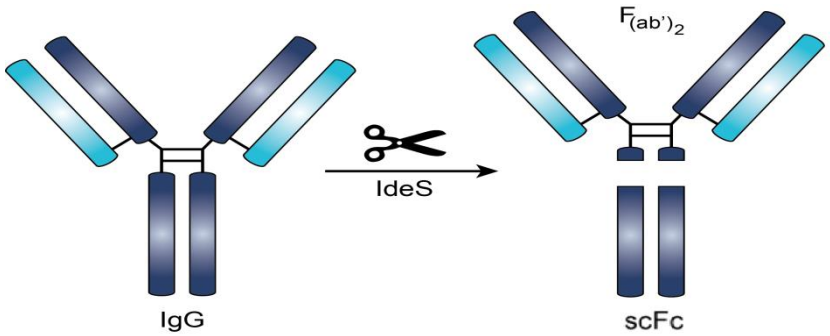
(a)



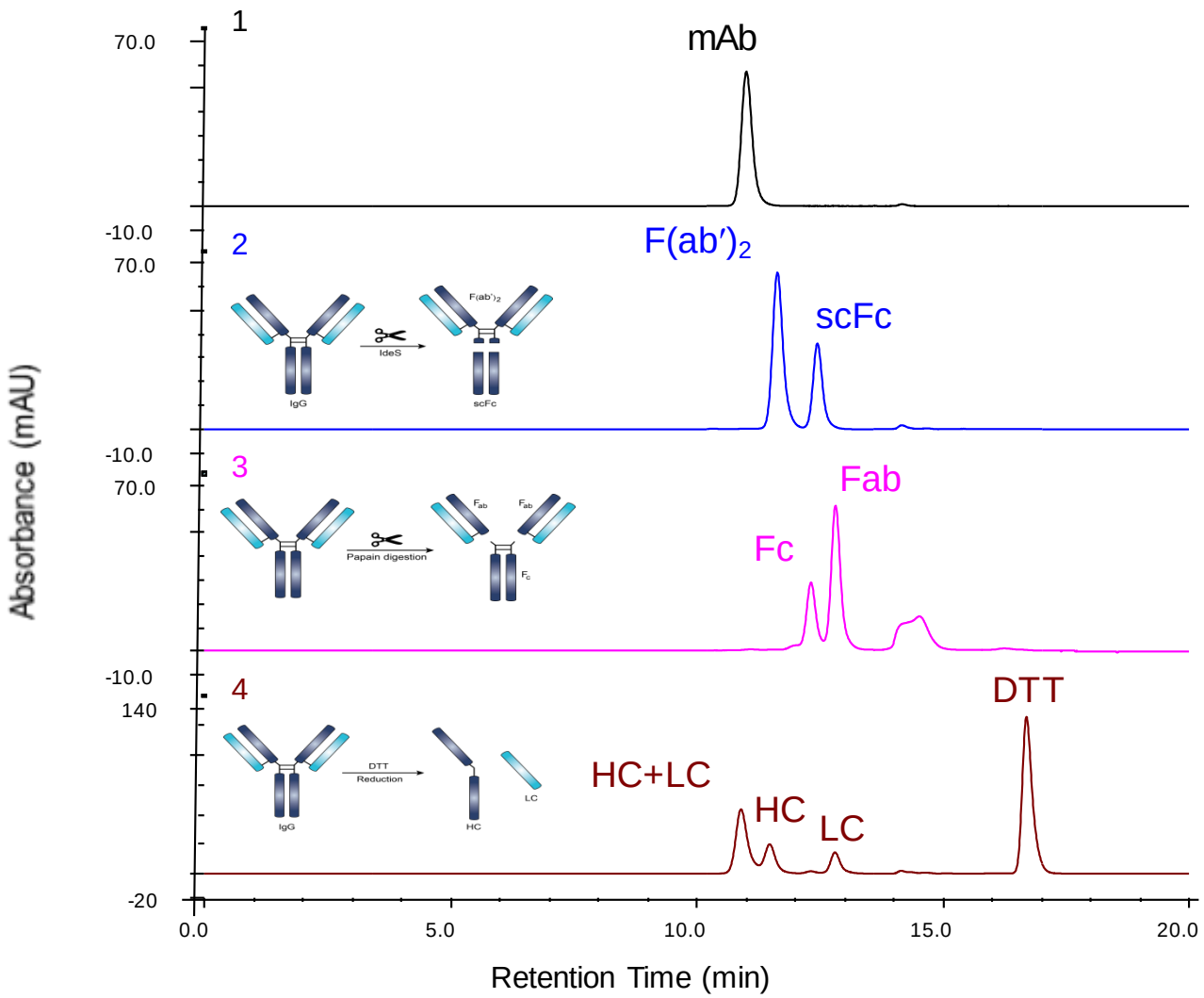
(b)



(c)



Separation of mAb Fragments



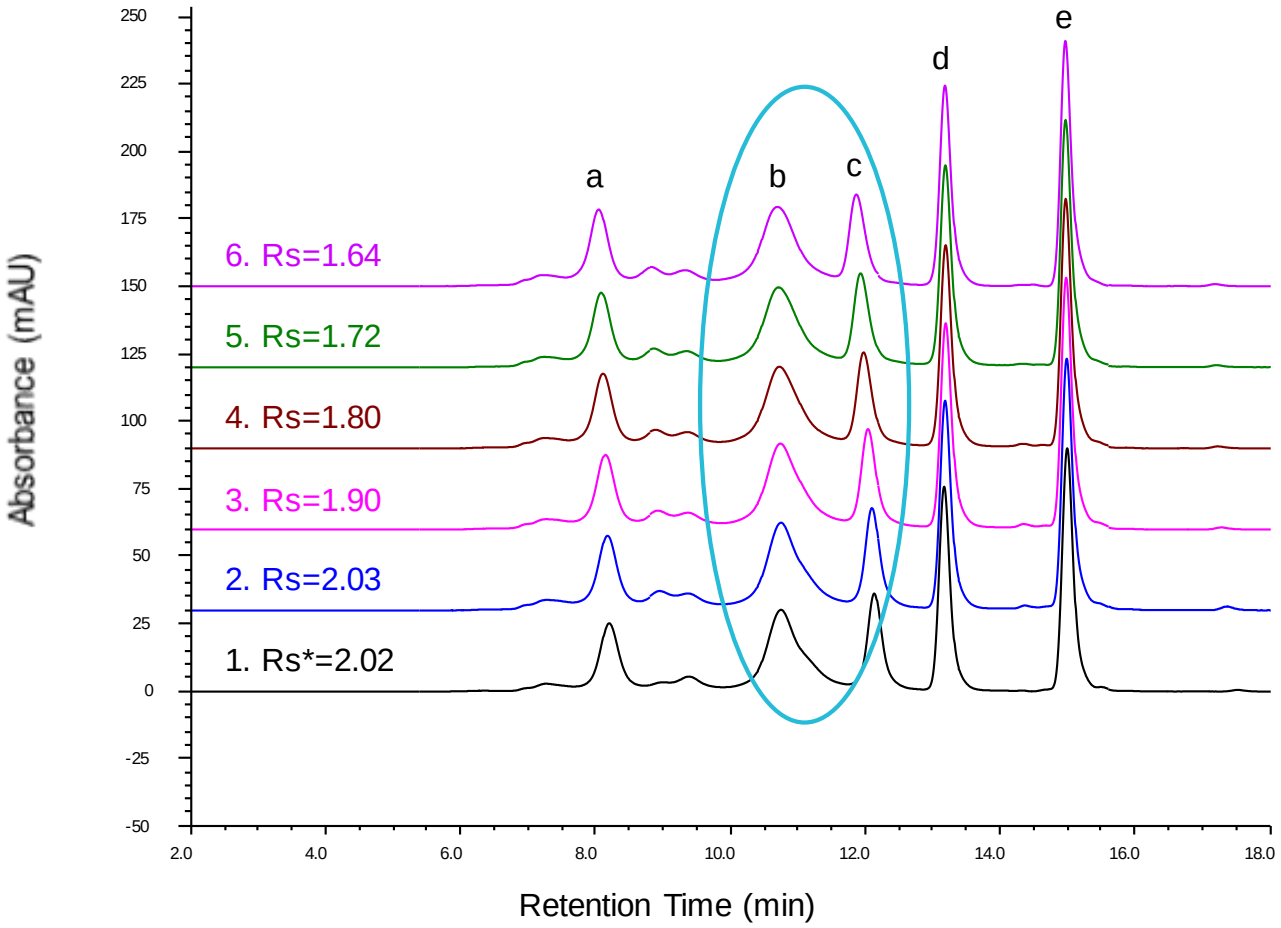
Column:	MAbPac SEC-1, 5 µm
Format:	7.8 ×300 mm
Mobile phase:	50 mM sodium phosphate, 300 mM NaCl, pH 6.8
Gradient:	Isocratic
Temperature:	30 ºC
Flow rate:	0.76 mL/min
Inj. volume:	10 µL
Detection:	UV (280 nm)
Sample:	1. Rituximab (5 mg/mL), inject 2 µL
	2. Rituximab + IdeS (2 mg/mL)
	3. Rituximab + Papain (2 mg/mL)
	4. Rituximab + DTT (2 mg/mL)

mAb fragments F(ab′)$_2$ and scFc, Fc and Fab, HC and LC are separated.

Using MAbPac & Acclaim SEC Columns for Protein Analysis with Orbitrap™ MS

- Reduced salt concentration and volatile buffers are key enabling technologies for introducing proteins in their native-folded state into the MS.
- Direct coupling to MS – Traditional silica-based SEC columns run in high salt concentrations. If the salt is removed the proteins then interact with active sites on the silica surfaces, which results in poor peak shape, changes in elution times and other undesirable effects.
- The MAbPac SEC column has a good hydrophilic boundary layer which allows salt concentrations as low as 50 to 20mM ammonium acetate to be used
- The Acclaim™ SEC is a proprietary mono-dispersed multi-pore resin made from a hydrophilic polymethacrylate resin. The hydrophilic nature of the resin allows for minimal salt to be used in the eluent system.
- Introducing the Thermo Scientific™ Exactive™ Plus EMR and Q-Exactive BioPharma – Allow the use of the MS for native protein analysis where the protein is still in its native folded state. Due to the folding, the charge state is lowered as only surface sites are available, resulting in a higher mass / charge which the new m/z ranges on these systems allow.

Impact of Ionic strength: MAbPac SEC-1

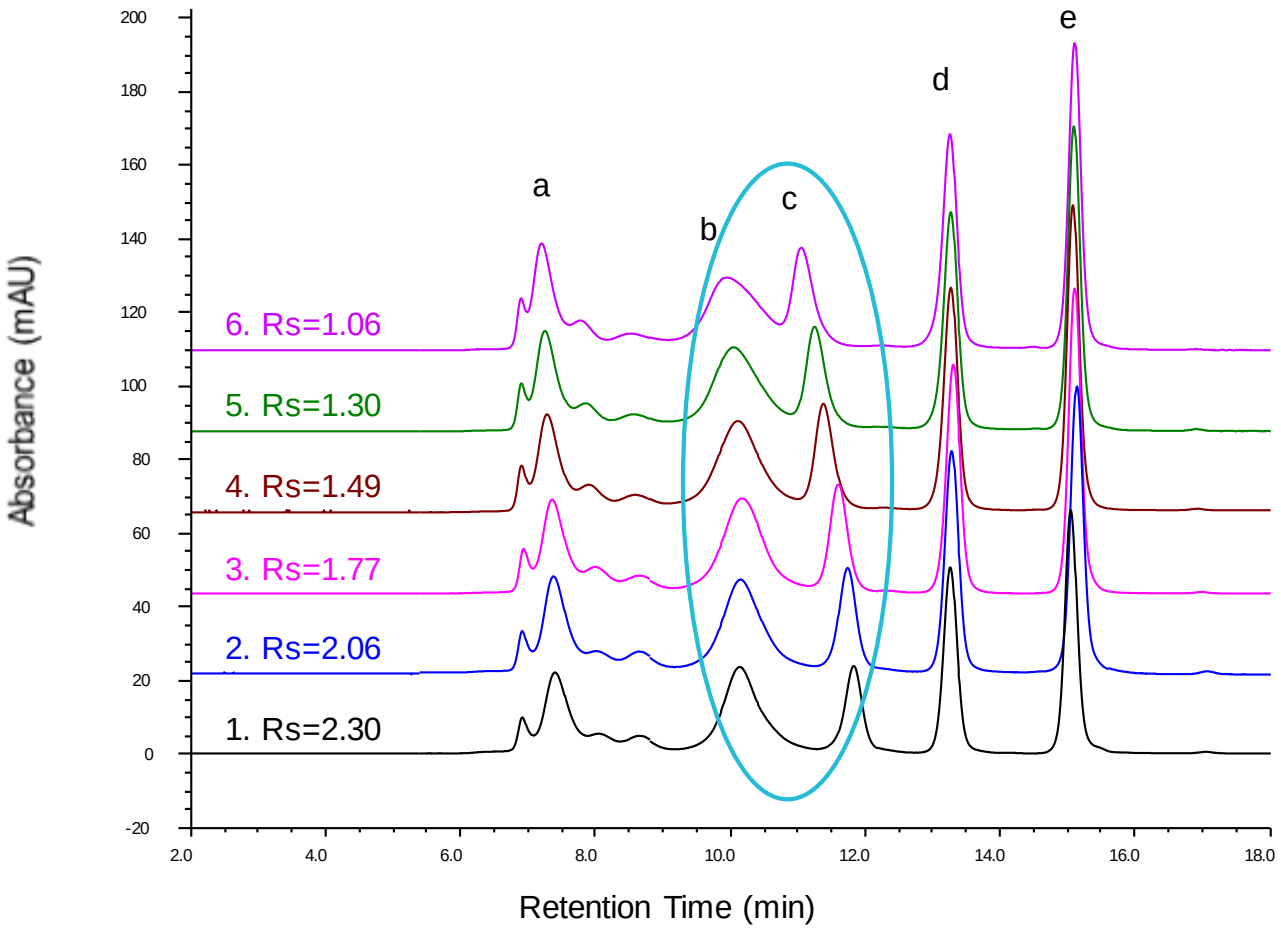


*Rs is the resolution between γ-globulin and ovalbumin

Column:	MAbPac SEC-1, 5 µm
Format:	7.8 ×300 mm
Mobile phase:	50 mM sodium phosphate, pH 6.8 and 1. 300 mM NaCl 2. 200 mM NaCl 3. 100 mM NaCl 4. 50 mM NaCl 5. 25 mM NaCl 6. 0 mM NaCl
Gradient:	Isocratic
Temperature:	30 ºC
Flow rate:	0.76 mL/min
Inj. volume:	20 µL
Detection:	UV (280 nm)
Sample:	SEC standard a. Thyroglobulin (bovine): 0.1 mg/mL b. γ-globulin (bovine): 0.1 mg/mL c. Ovalbumin (chicken): 0.1 mg/mL d. Myoglobin (horse): 0.05 mg/mL e. Vitamin B12: 0.01 mg/mL

MAbPac SEC-1 column has low secondary interaction, its separation is not sensitive to the mobile phase salt concentration.

Impact of Ionic strength: Alternative SEC Column

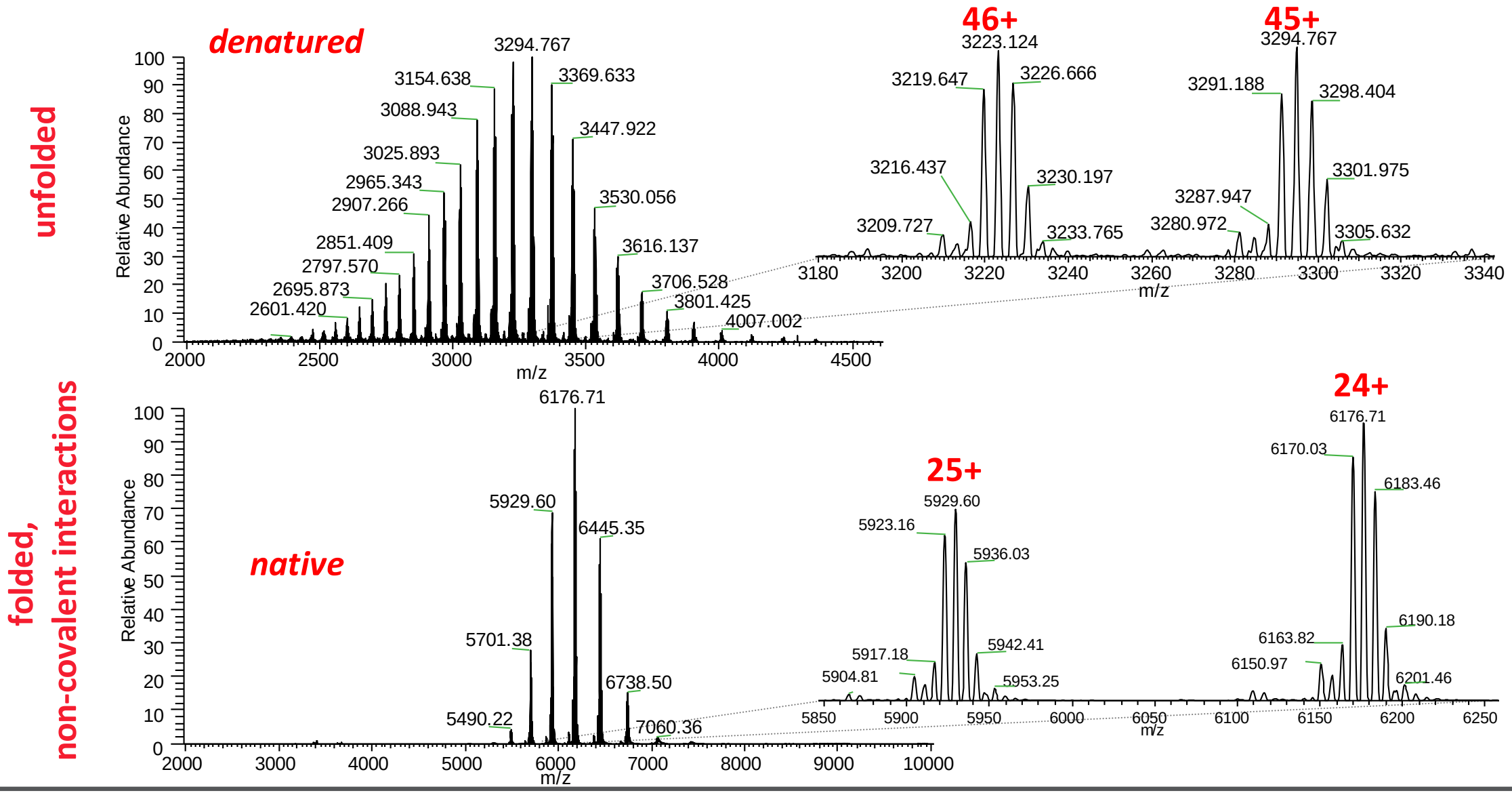


*Rs is the resolution between γ-globulin and ovalbumin

Column:	Competitor SEC, 5 µm
Format:	7.8 ×300 mm
Mobile phase:	50 mM sodium phosphate, pH 6.8 and 1. 300 mM NaCl 2. 200 mM NaCl 3. 100 mM NaCl 4. 50 mM NaCl 5. 25 mM NaCl 6. 0 mM NaCl
Gradient:	Isocratic
Temperature:	30 ºC
Flow rate:	0.76 mL/min
Inj. volume:	20 µL
Detection:	UV (280 nm)
Sample:	SEC standard a. Thyroglobulin (bovine): 0.1 mg/mL b. γ-globulin (bovine): 0.1 mg/mL c. Ovalbumin (chicken): 0.1 mg/mL d. Myoglobin (horse): 0.05 mg/mL e. Vitamin B12: 0.01 mg/mL

Many SEC columns have high secondary interaction, Separation is then very sensitive to the mobile phase salt concentration.

Analysis of Herceptin mAb, Full MS with SEC under native and denaturing conditions



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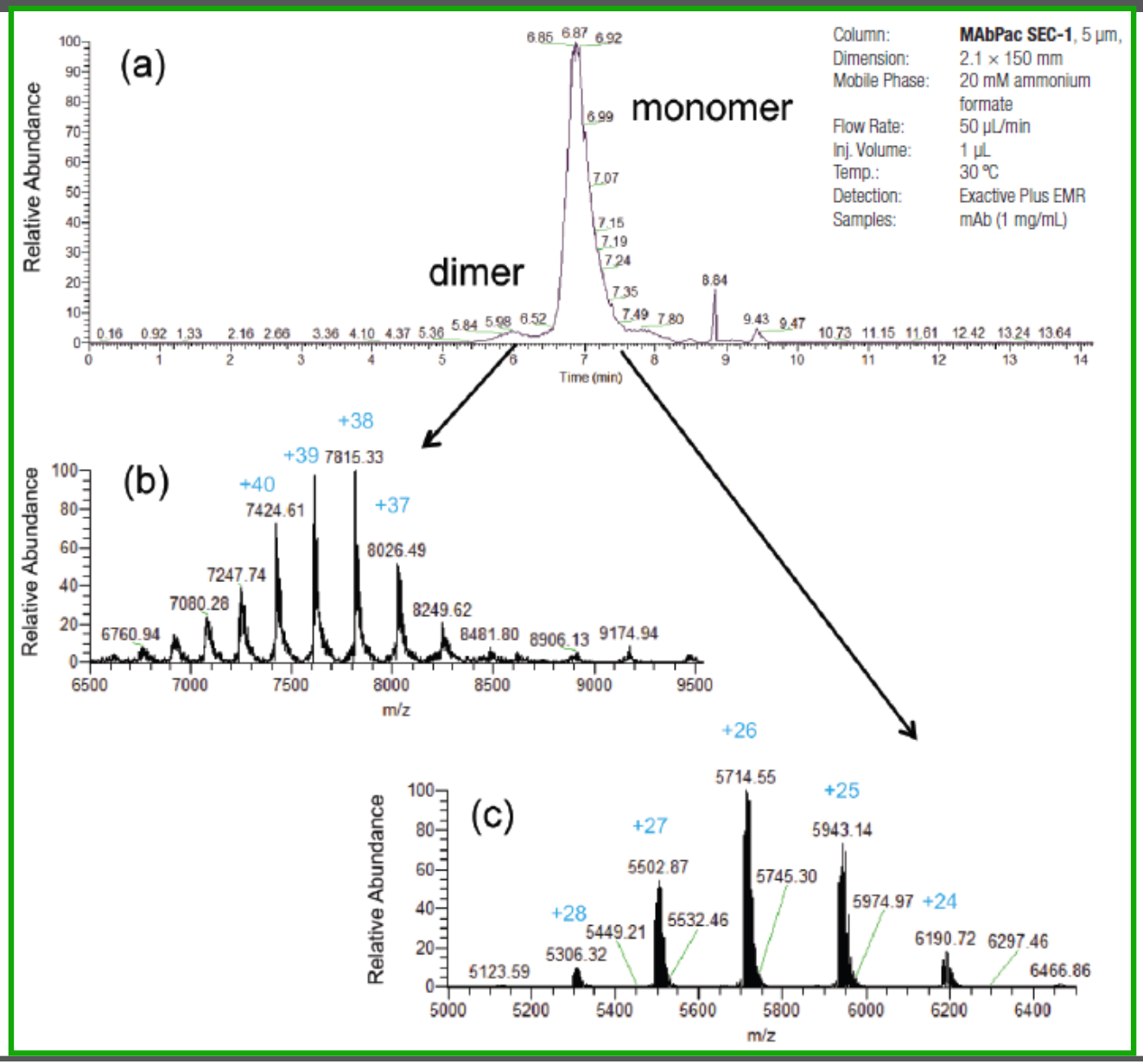
Direct Coupling to MS after SEC Separation

- MS friendly separation conditions
- Mass confirmation of the dimer



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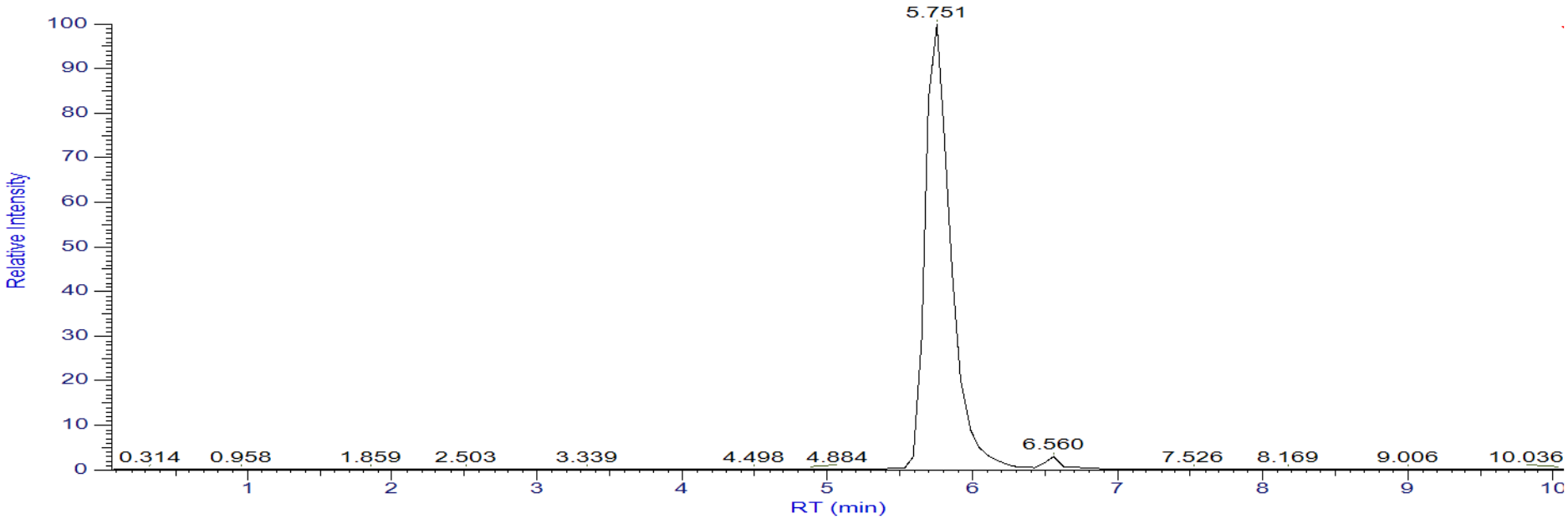

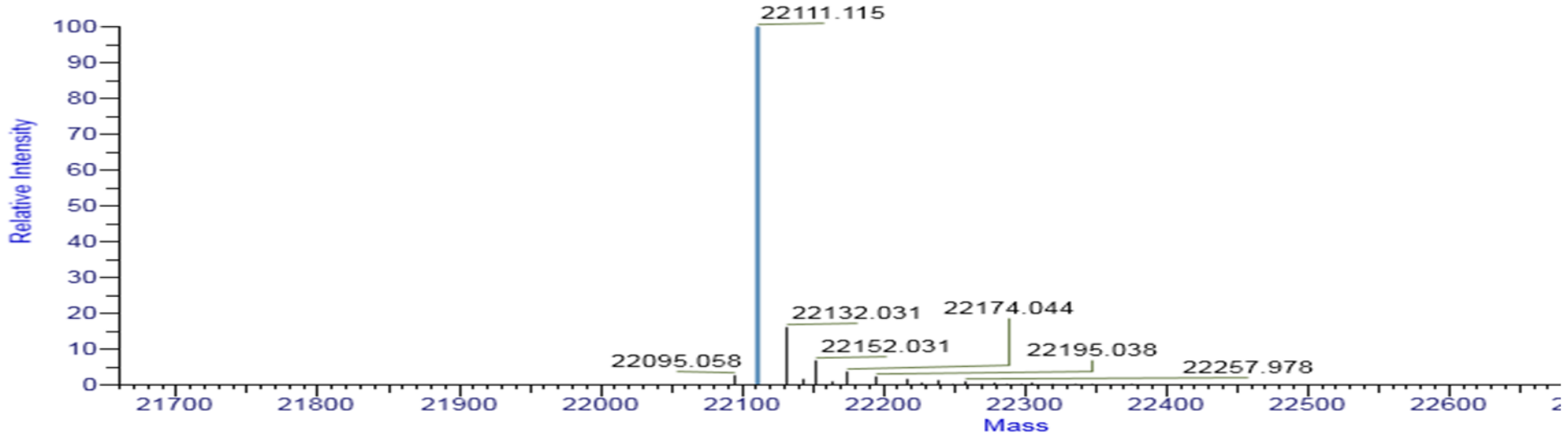

8.8 E7

50mM Am Acetate

Isotopically resolved

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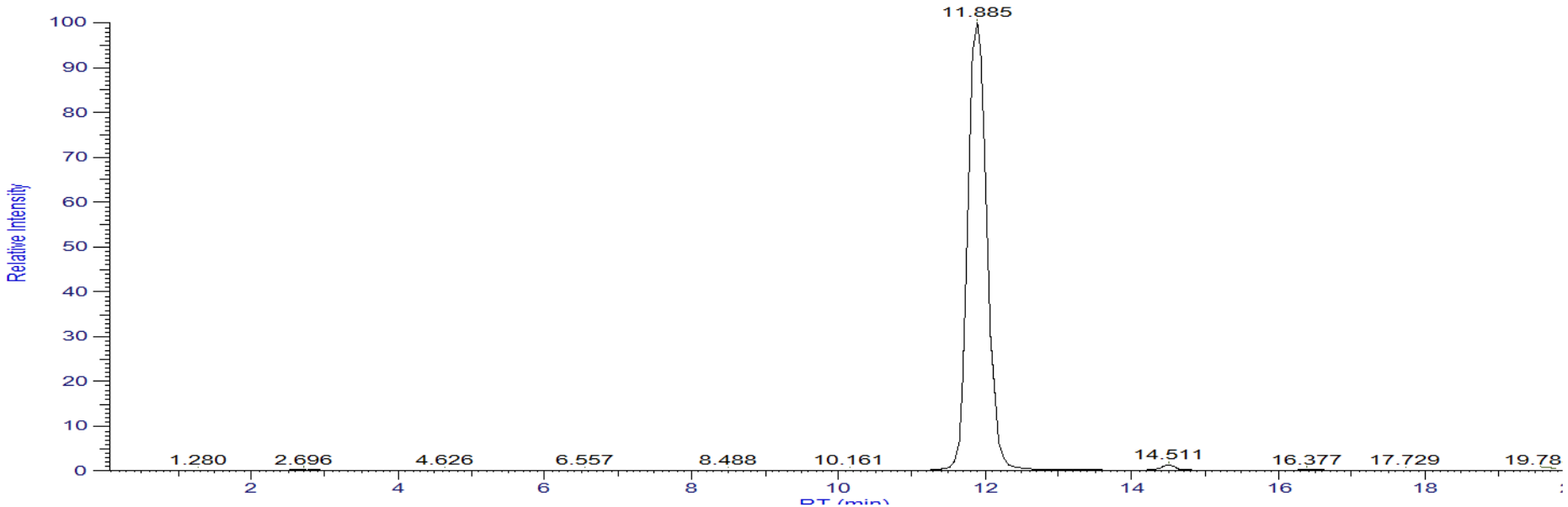
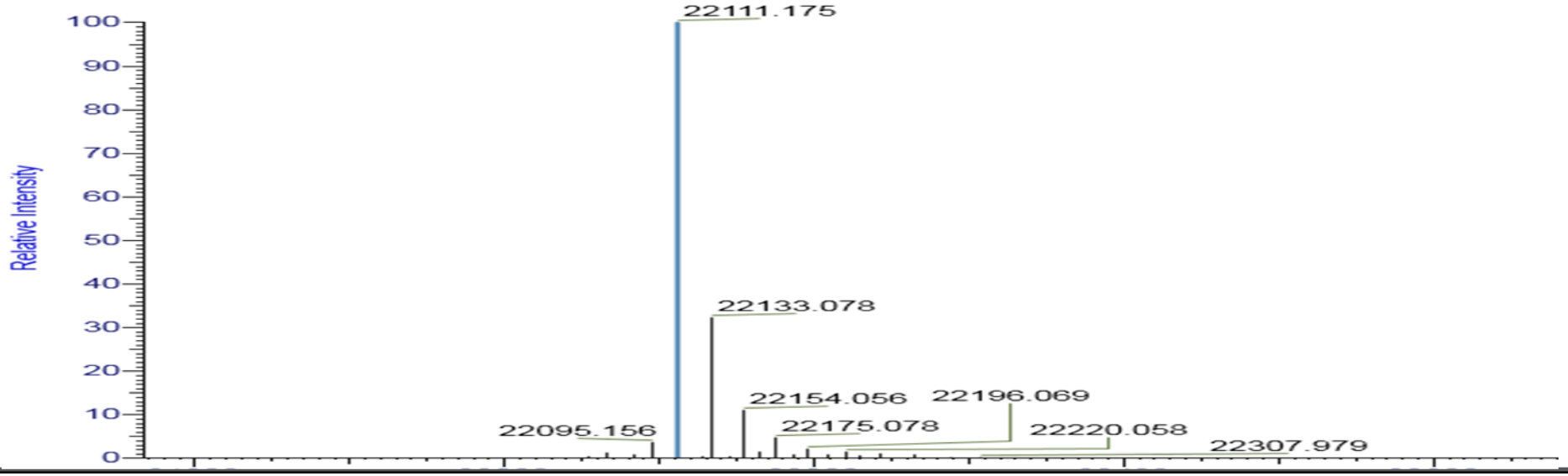
Somatotropin Acclaim SEC Isotopically resolved





4.0 E8

5mM Amm Acetate

Increased response with lower salt

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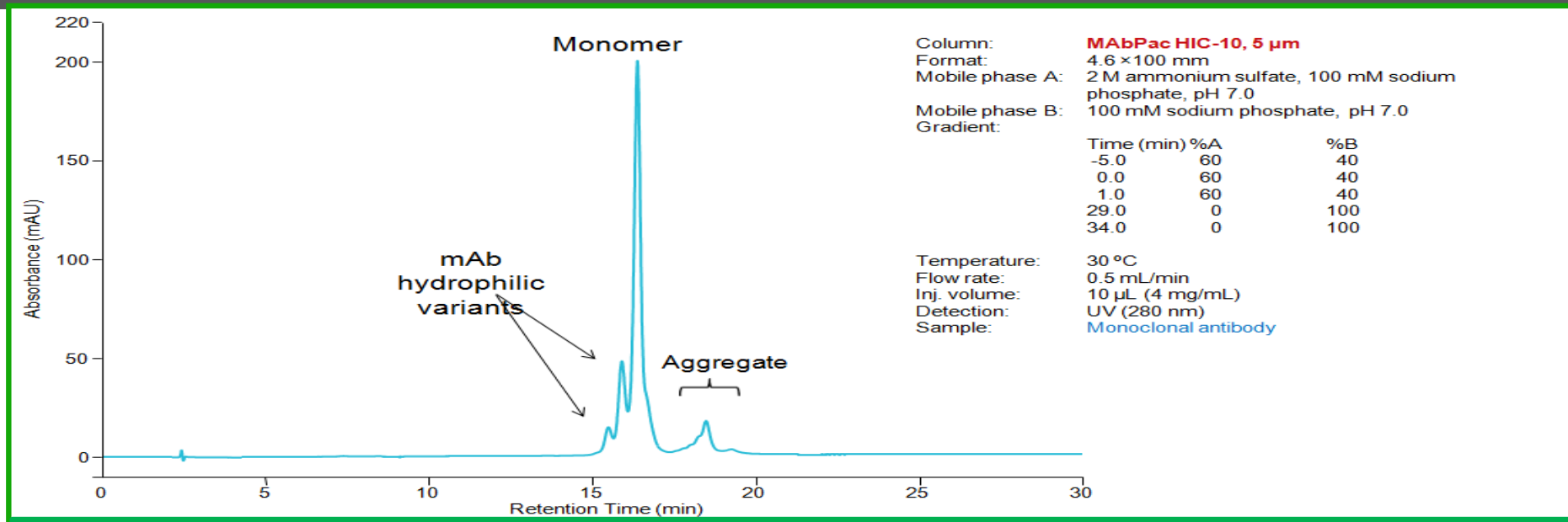
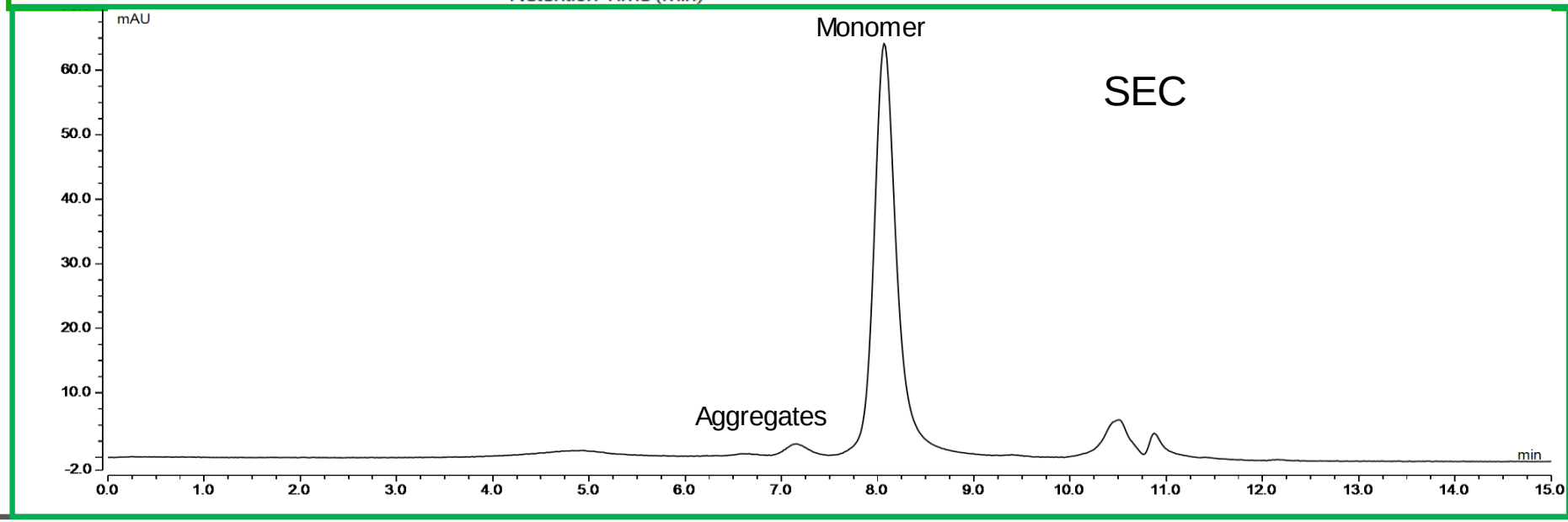
Aggregation Screening Using HIC

- Alternative separation mechanism
- Higher resolution
- No longer size based separation





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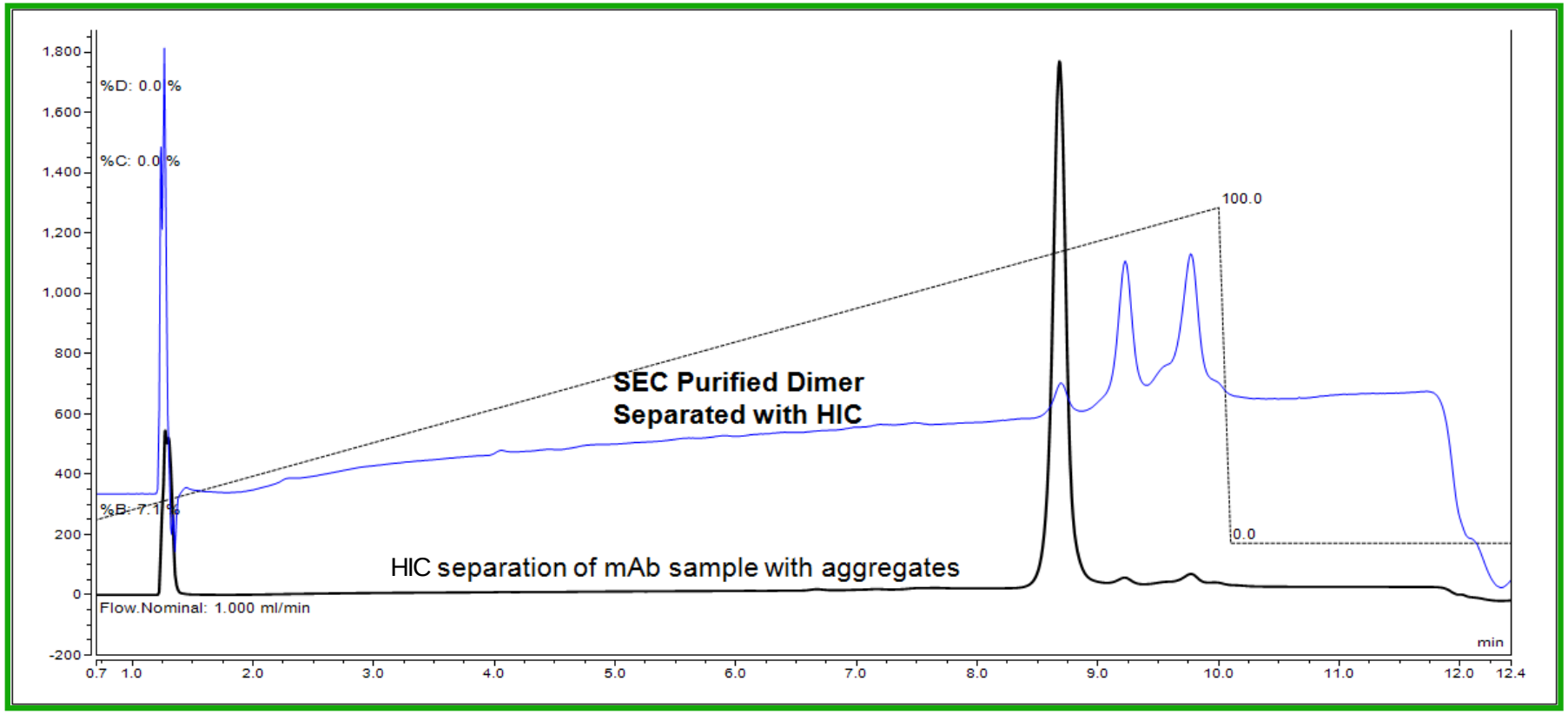
Separation of SEC-purified Dimer on MAbPac HIC-10

- SEC followed by HIC
- Purified SEC dimer gives multiple peaks in HIC



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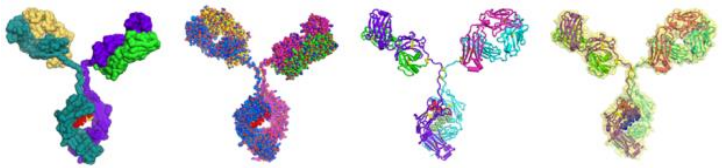
Example Applications of SEC for Aggregation Analysis

Applications

- NIST mAb reference standard
- Biosimilar candidates vs. approved drug
- Column lifetime stability study
- Preparative CEX for glycan profiling of subunits

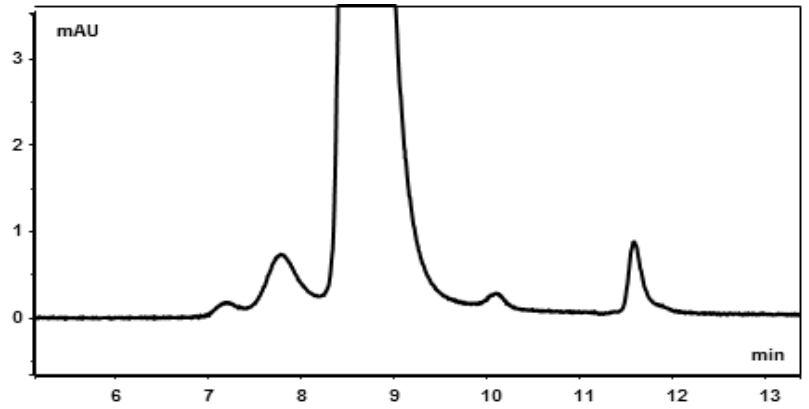
1. Size Exclusion Analysis – NIST mAb 8671

NIST mAb
Reference Material 8671
(Lot# 14HB-D-002)
Humanized IgG1κ



National Institute of Standards and Technology
U.S. Department of Commerce

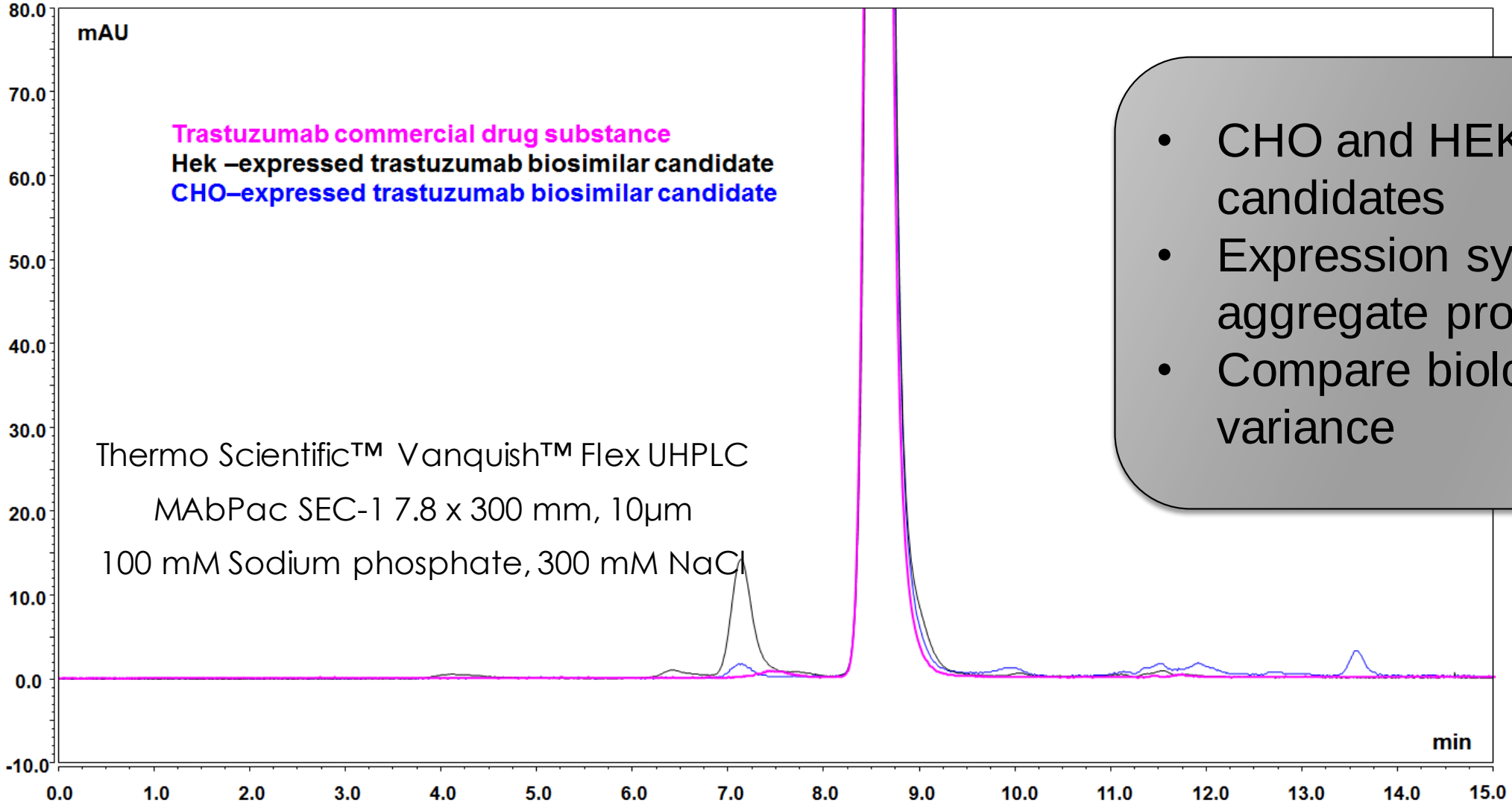
Thermo Scientific™ Vanquish™ Flex UHPLC
MAbPac SEC-1 7.8x300 mm, 10µm
100 mM Sodium phosphate, 300 mM NaCl



nibrt
National Institute for Bioprocessing Research and Training

ThermoFisher SCIENTIFIC

2. Trastuzumab Drug substance versus Biosimilar



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

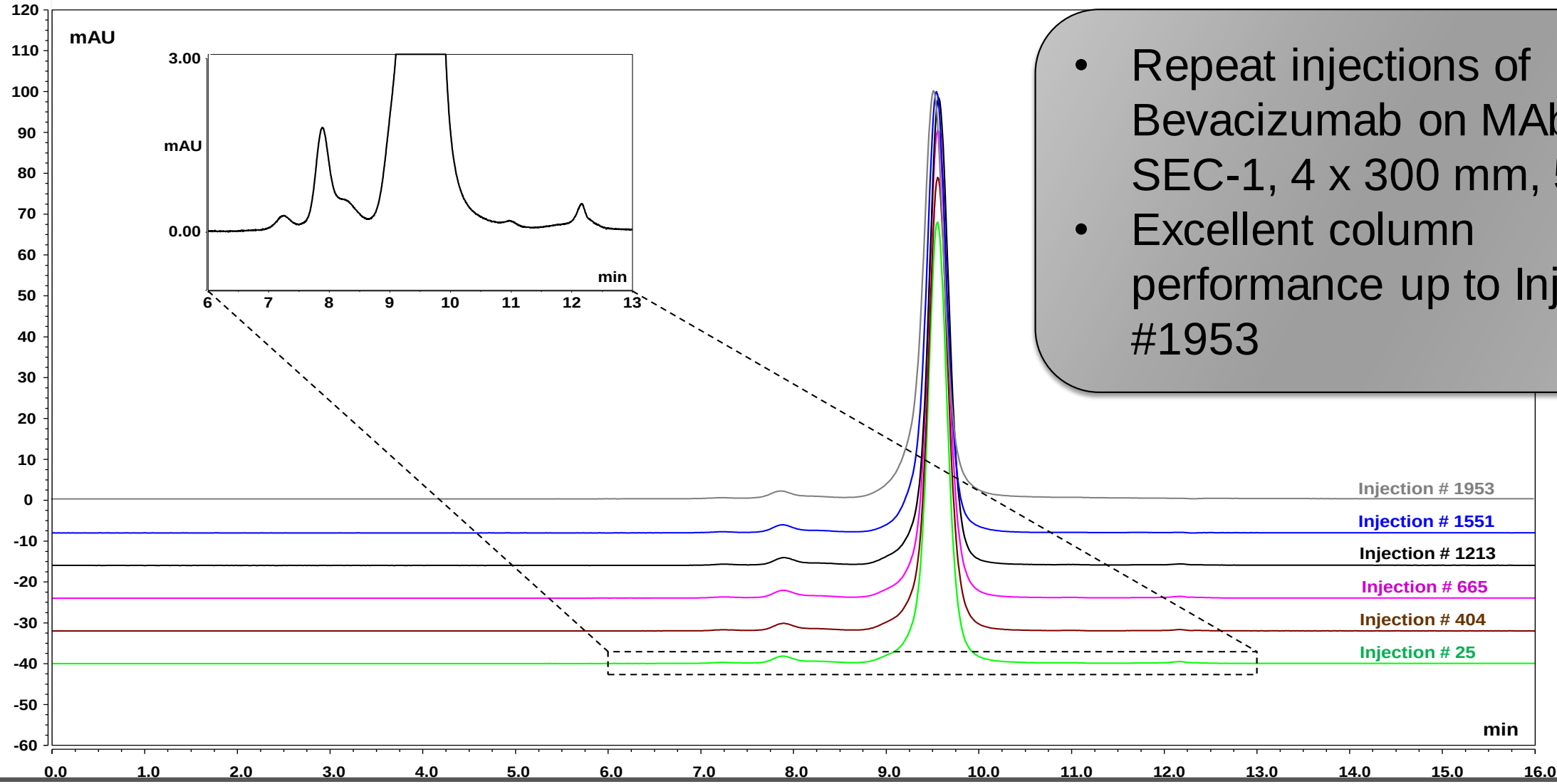
nibrt
National Institute for Bioprocessing Research and Training

ThermoFisher SCIENTIFIC

2. Compare Expression Systems

Potential causes of variation	Chinese Hamster Ovary	Human Embryonic Kidney	Drug Product (CHO Cell line)
Transfection	Transient	Transient	Stable
Culture Duration	8 days	6 Days	N/D
DNA construct	Human	Human	Chimeric mouse/human
Purification	Protein A chromatography, multimodal AEX	Protein A chromatography, multimodal AEX	Protein A chromatography, AEX, CEX, UF/DF
Viability at harvest	~90%	~70%	N/D
Viable Cell count at harvest	(Mean) 7.0 $x10^6$ cells/mL	(Mean) 7.7 $x10^6$ cells/mL	N/D
Culture Volume	50 mL	50 mL	12,000 L

3. Column Lifetime Stability Evaluation



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

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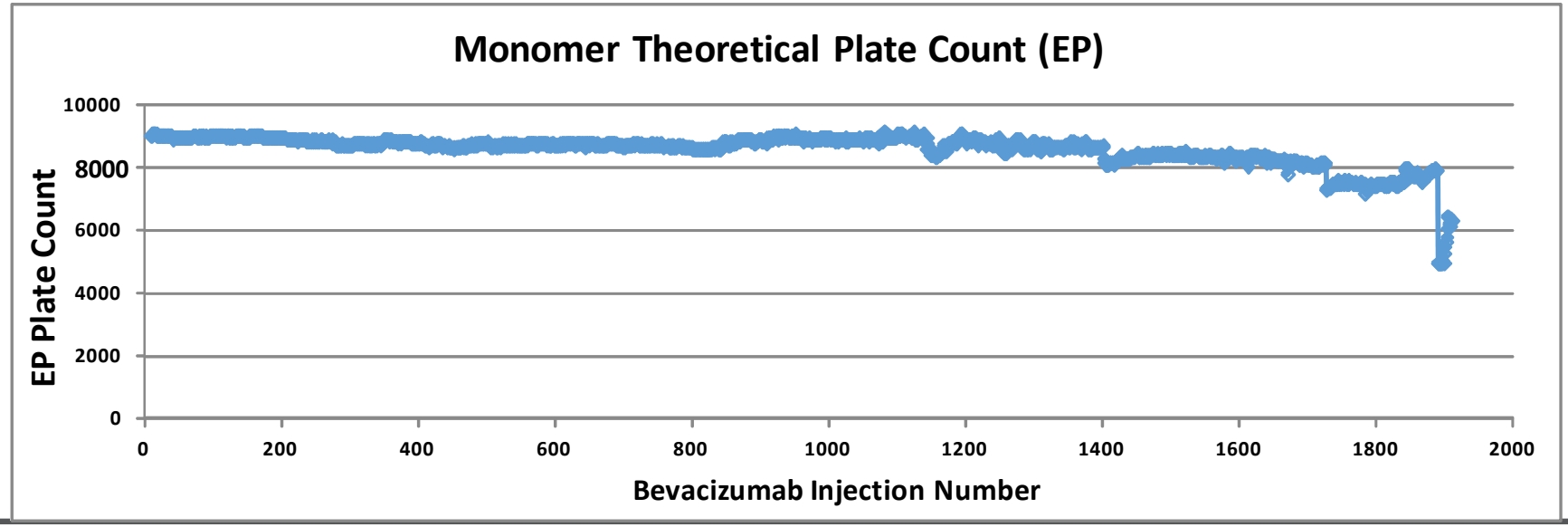
3. Column Lifetime Stability Evaluation

On Column Injection #	Retention Time (min)	Monomer Relative Peak Area (%)	Monomer Peak Width @ 50% Height (min)	Asymmetry (EP)	Theoretical Plates (EP)
25	9.554	96.96	0.237	0.92	9032
404	9.558	97.21	0.240	0.92	8797
665	9.558	96.96	0.241	0.93	8736
1213	9.567	97.27	0.243	0.89	8604
1551	9.544	96.99	0.244	0.88	8460
1953	9.524	96.30	0.255	0.89	7737



85% initial column efficiency following 1866 mAb injections (1953 on-column injections)

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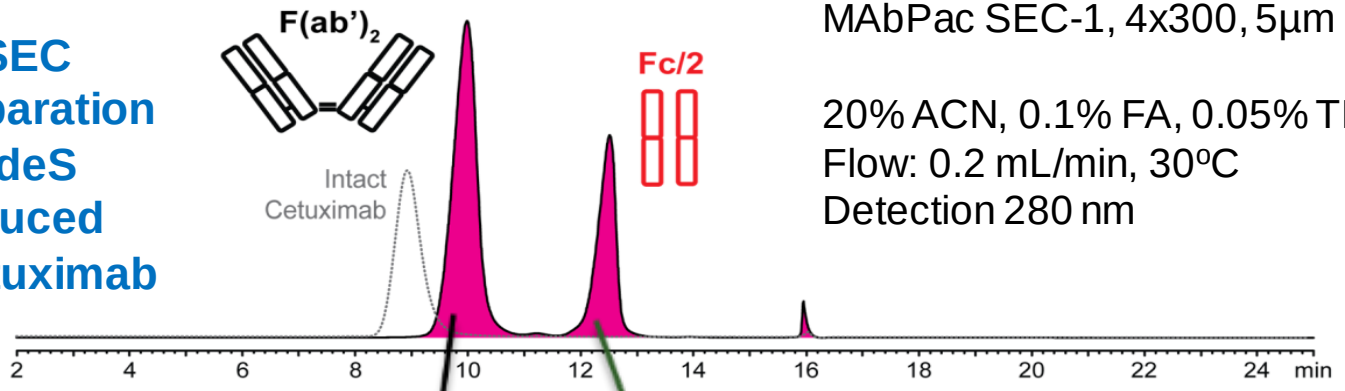
4. Preparative Chromatography

1. SEC separation of IdeS reduced Cetuximab

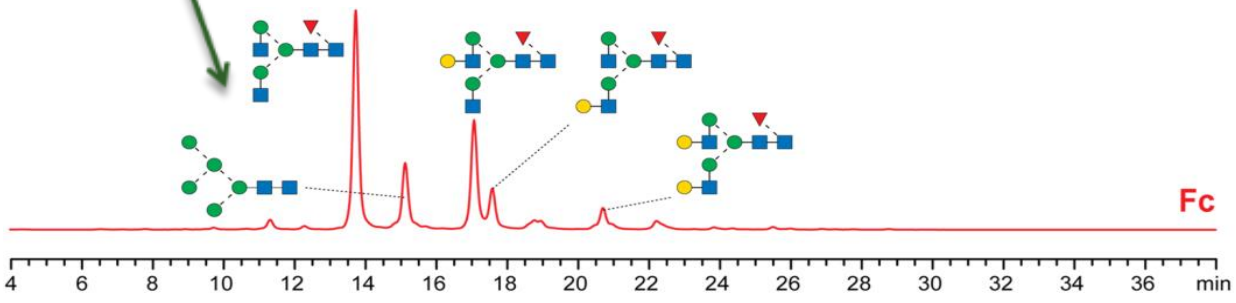


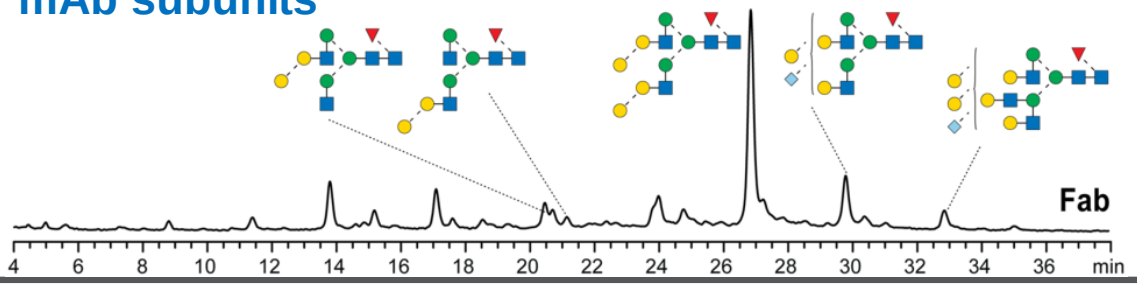
MAbPac SEC-1, 4x300, 5µm

20% ACN, 0.1% FA, 0.05% TFA
Flow: 0.2 mL/min, 30°C
Detection 280 nm

PNGase F

2. Analysis of N-glycans released from mAb subunits





	Retention time (min)	Fc	Fab
FA1	11.30	x	x
FA2	13.72	x	x
M5	15.12	x	x
FA2[6]G1	17.05	x	x
FA2[3]G1	17.57	x	x
FM5A1	18.76	x	x
FA2G1Ga1	20.46		x
FA2G2	20.70	x	x
FM5A1G1	22.25	x	x
FA2G2S1	23.84		x
FA2G2Ga1	24.00		x
FM5A1G1Ga1	24.77		x
FA2G2Ga2	26.88		x
FA2G2S2	27.25		x
FA3G3Ga1	27.82		x
FA2G2Ga1S1	29.80		x
FA3G3Ga2	30.44		x
FA3G3Ga3	32.89		x
FA3G3S3	35.05		x

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

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Summary

- Biotherapeutics such as mAbs may undergo degradation processes that impact drug safety, quality and efficacy
- Important to monitor aggregation using size-exclusion chromatography
- Protein expression in different cell lines resulted in altered aggregation profile
- SEC peak collection enables localization of *N*-glycans present on different regions of the protein

Summary: Aggregation Monitoring Workflow

SEC is the most popular analytical technique for Aggregate analysis.

Moderate flow rate, and larger column ID provide the best results with standard HPLC systems.

UHPLC systems are ideal with their optimized flow path.

Not all SEC columns are the same: pore size, hydrophilic coating and lifetime need to be considered.

Alternative techniques that can confirm aggregate analysis include IEX, HIC and native MS.

MAbPac SEC-1 columns offer high resolution, reproducible aggregation determination, MS compatibility, and exhibit long column life-time stability.