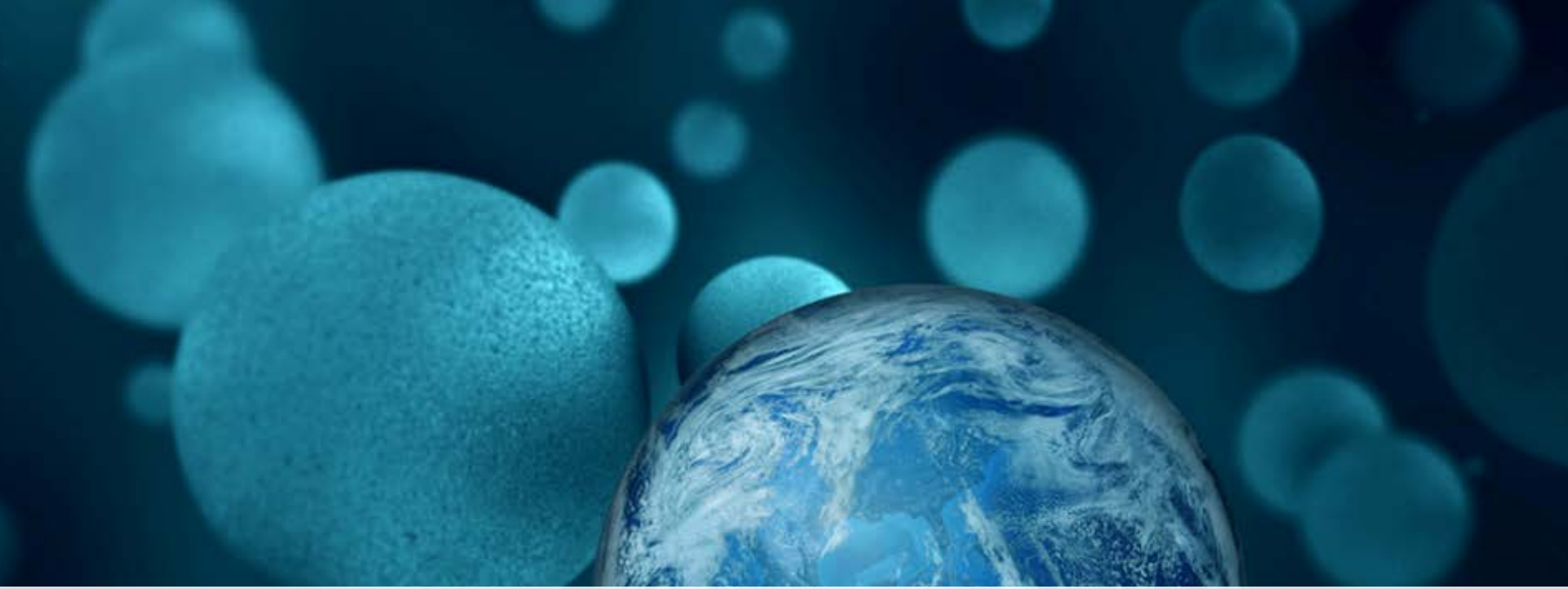


ThermoFisher SCIENTIFIC

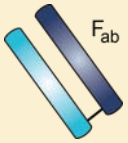
UHPLC and HRMS Methods for Bio-Therapeutic Protein Characterization, Speed, Resolution and the Pitfalls

Ken Cook
Thermo Fisher Scientific, Hemel Hempstead, United Kingdom

Types of Antibody-based Therapeutics

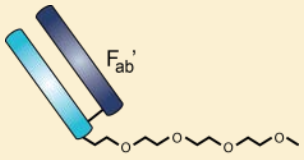
mAb Fab fragment

Cimzia (certolizumab pegol)
Lucentis (ranibizumab)



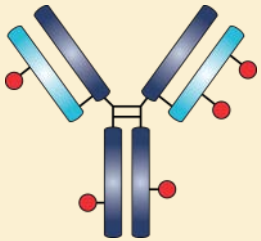
PEGylated mAb

Cimzia (certolizumab pegol)



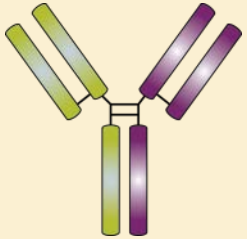
Antibody-drug conjugate

Kadcyla (trastuzumab emtansine)
Adcetris (brentuximab)



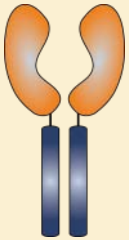
Bispecific mAb

Removab (catumaxomab)

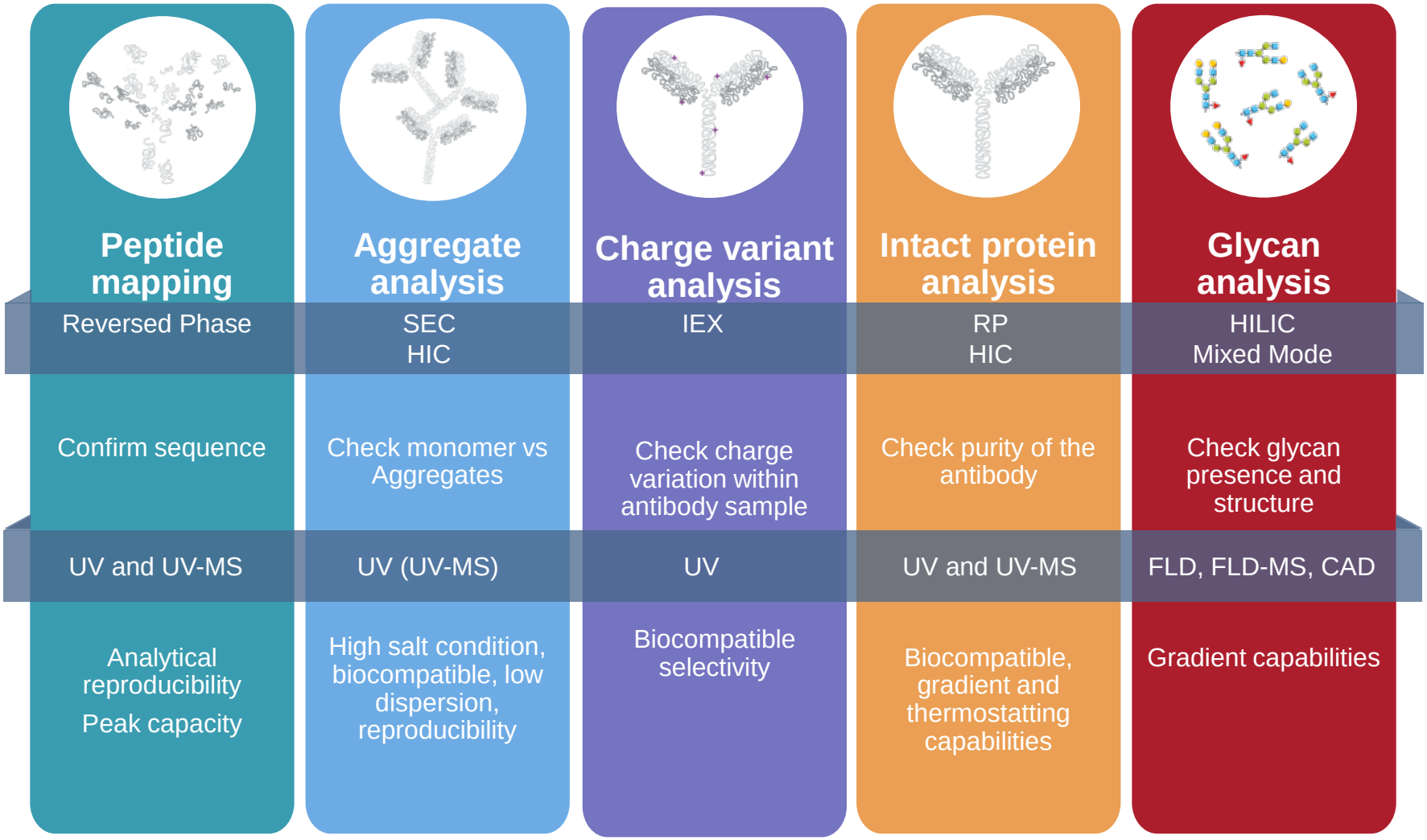


Fusion protein

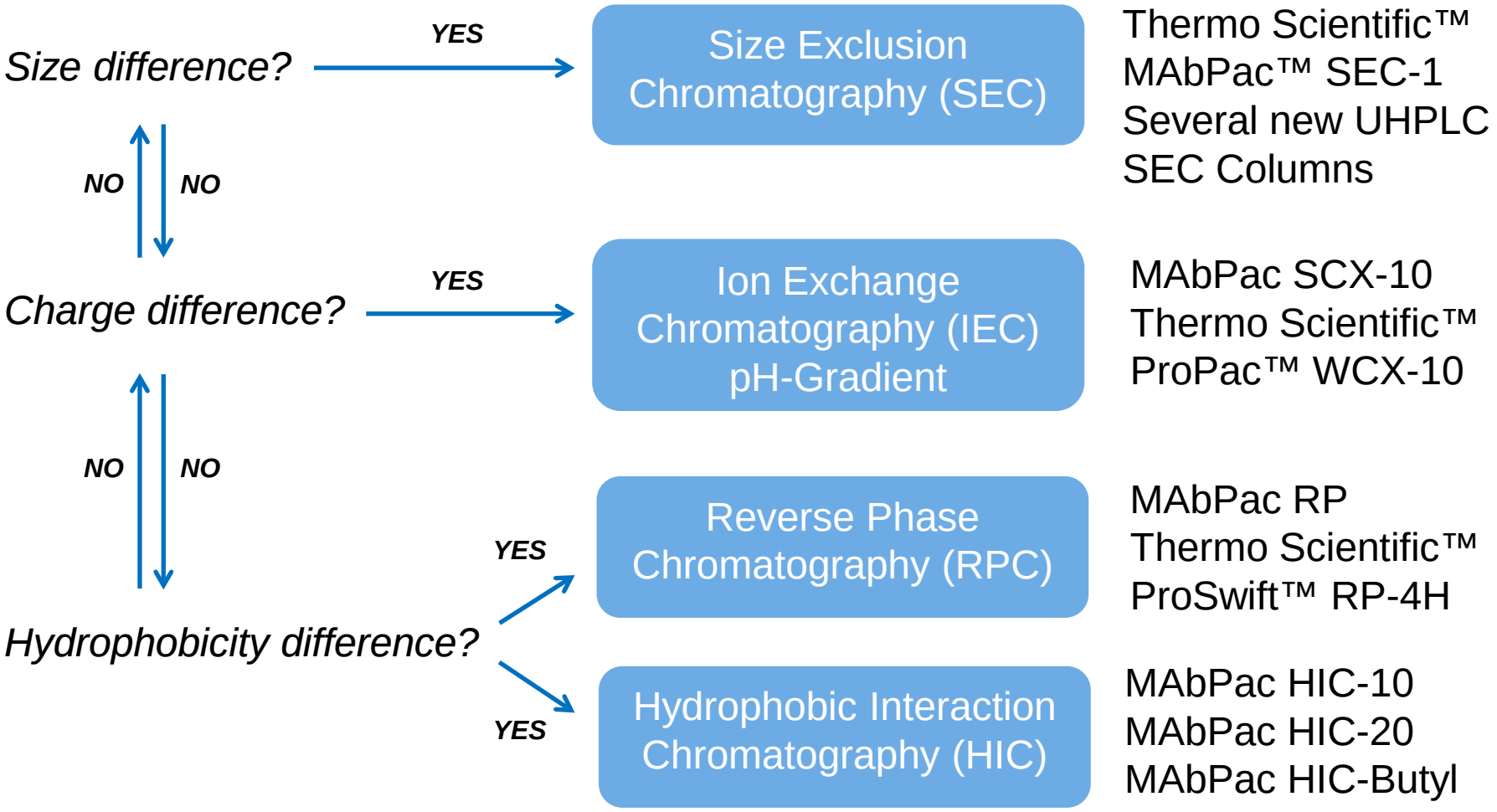
Enbrel (etanercept)
TNF receptor-Fc fusion



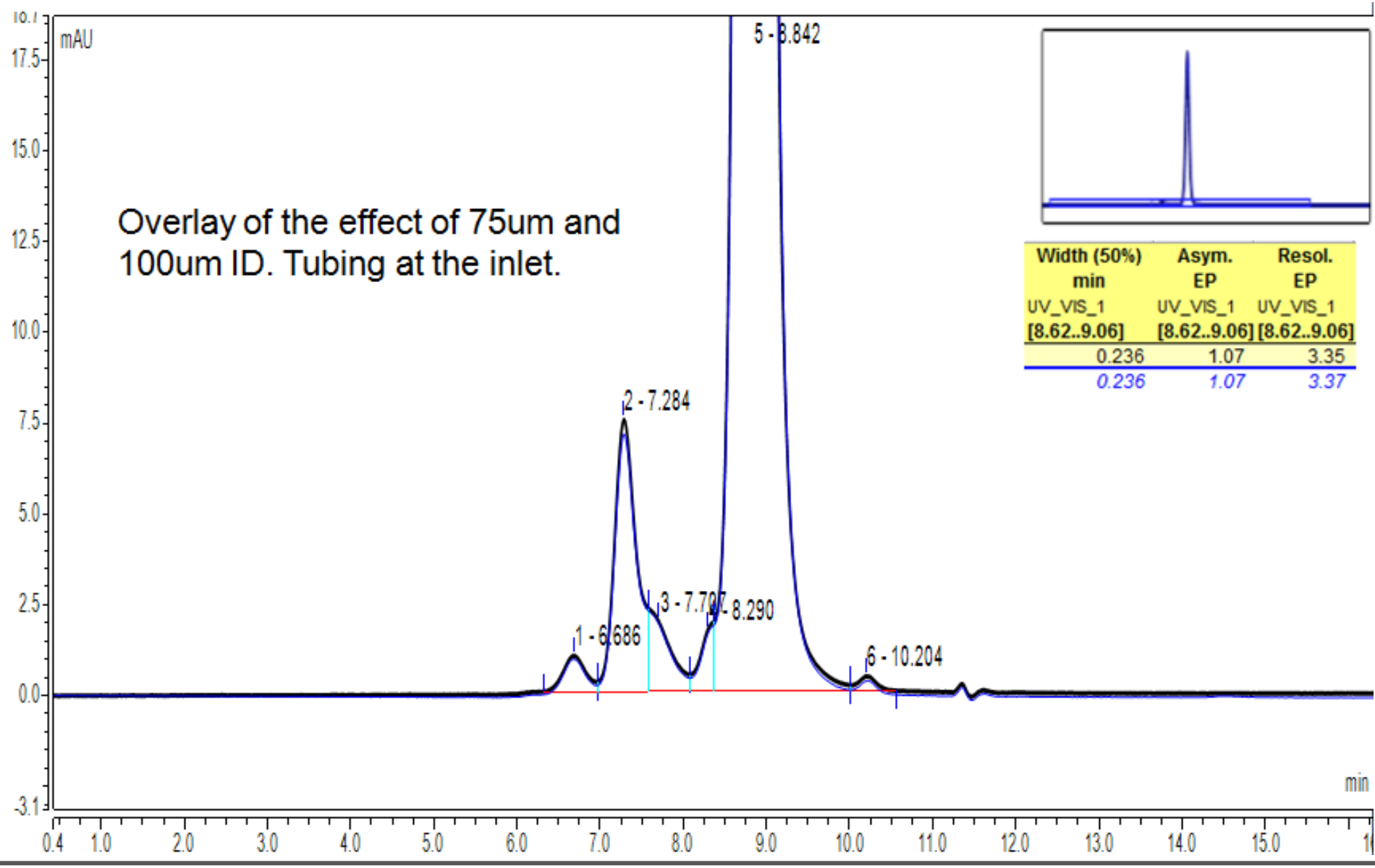
5 Fundamental Workflows



Protein and mAb Separation by U/HPLC



Effect of Tubing on 7.8mm SEC at 1.0 ml/min



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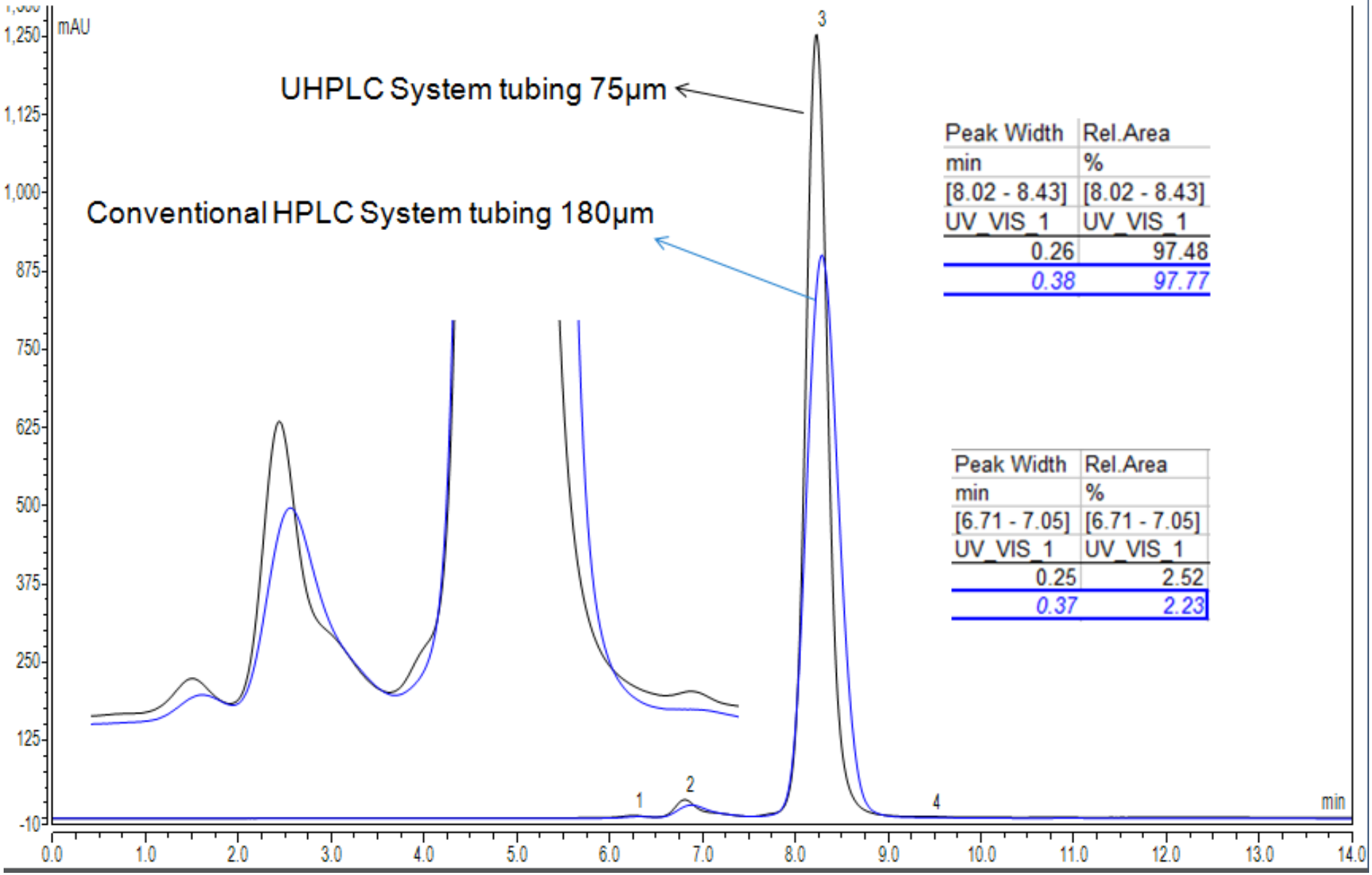
Bevazizumab mAb with a 4mm SEC Column at 0.3 ml/min

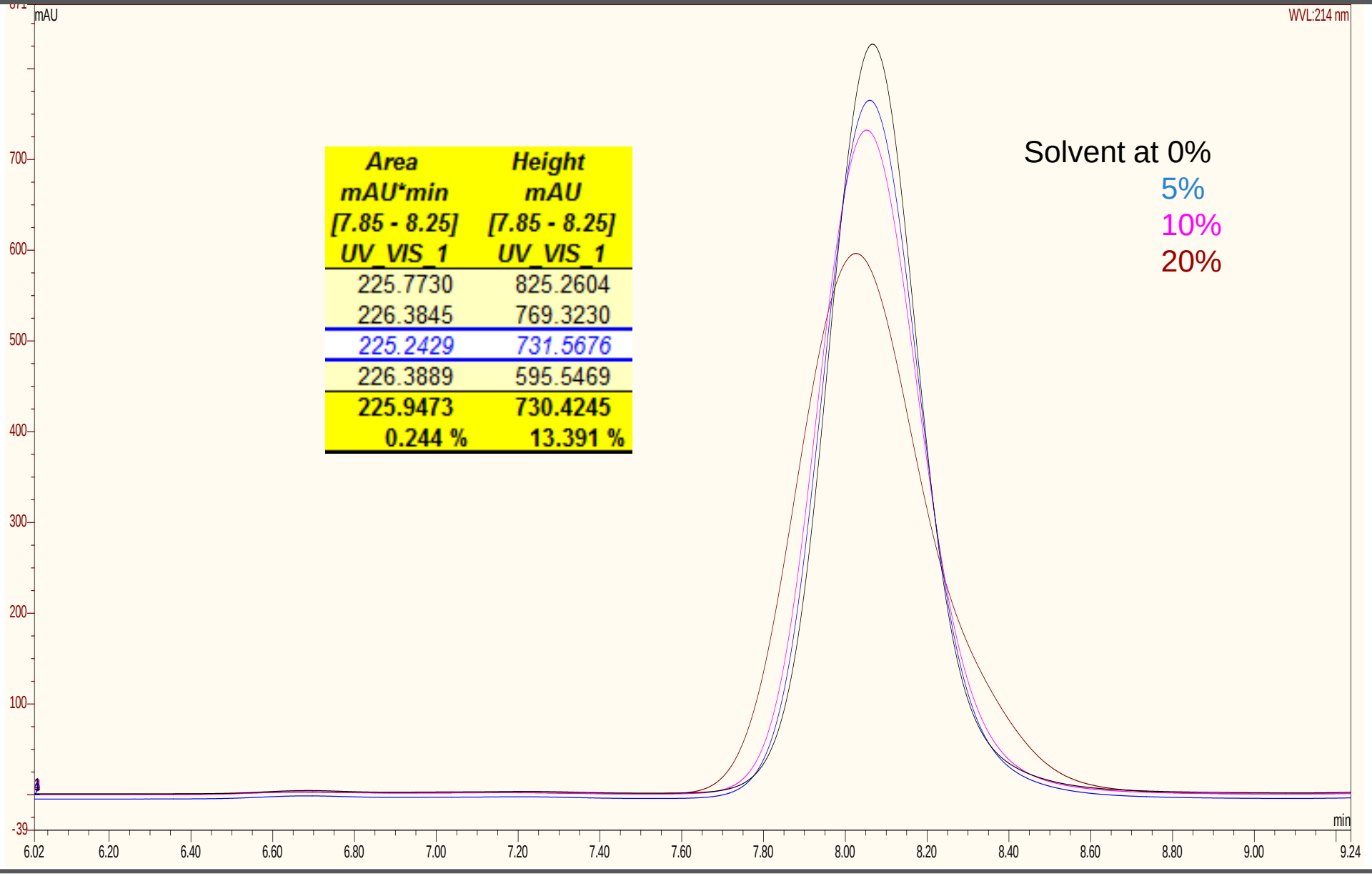


Peak Width	Rel.Area
min	%
[8.02 - 8.43]	[8.02 - 8.43]
UV_VIS_1	UV_VIS_1
0.26	97.48
0.38	*97.77*

Peak Width	Rel.Area
min	%
[6.71 - 7.05]	[6.71 - 7.05]
UV_VIS_1	UV_VIS_1
0.25	2.52
0.37	*2.23*

ThermoFisher SCIENTIFIC

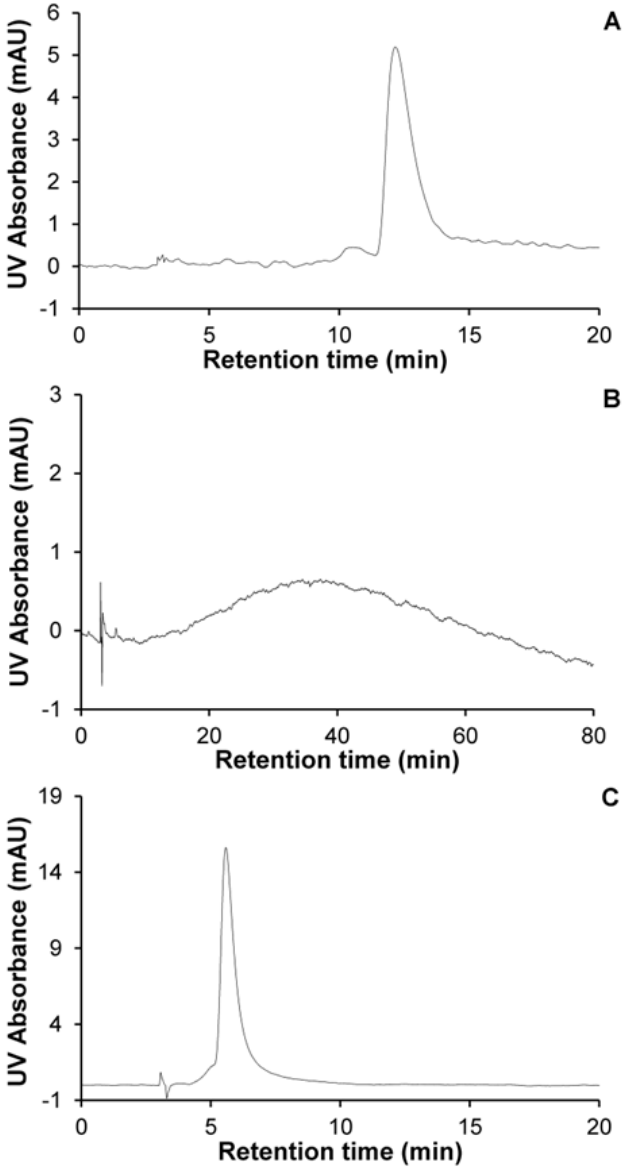
Effect of Solvent on SEC with Rituximab



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 %

ThermoFisher SCIENTIFIC

The Effect of Solvent Addition in HIC Chromatography



Lactalbumin has a strong calcium binding site.

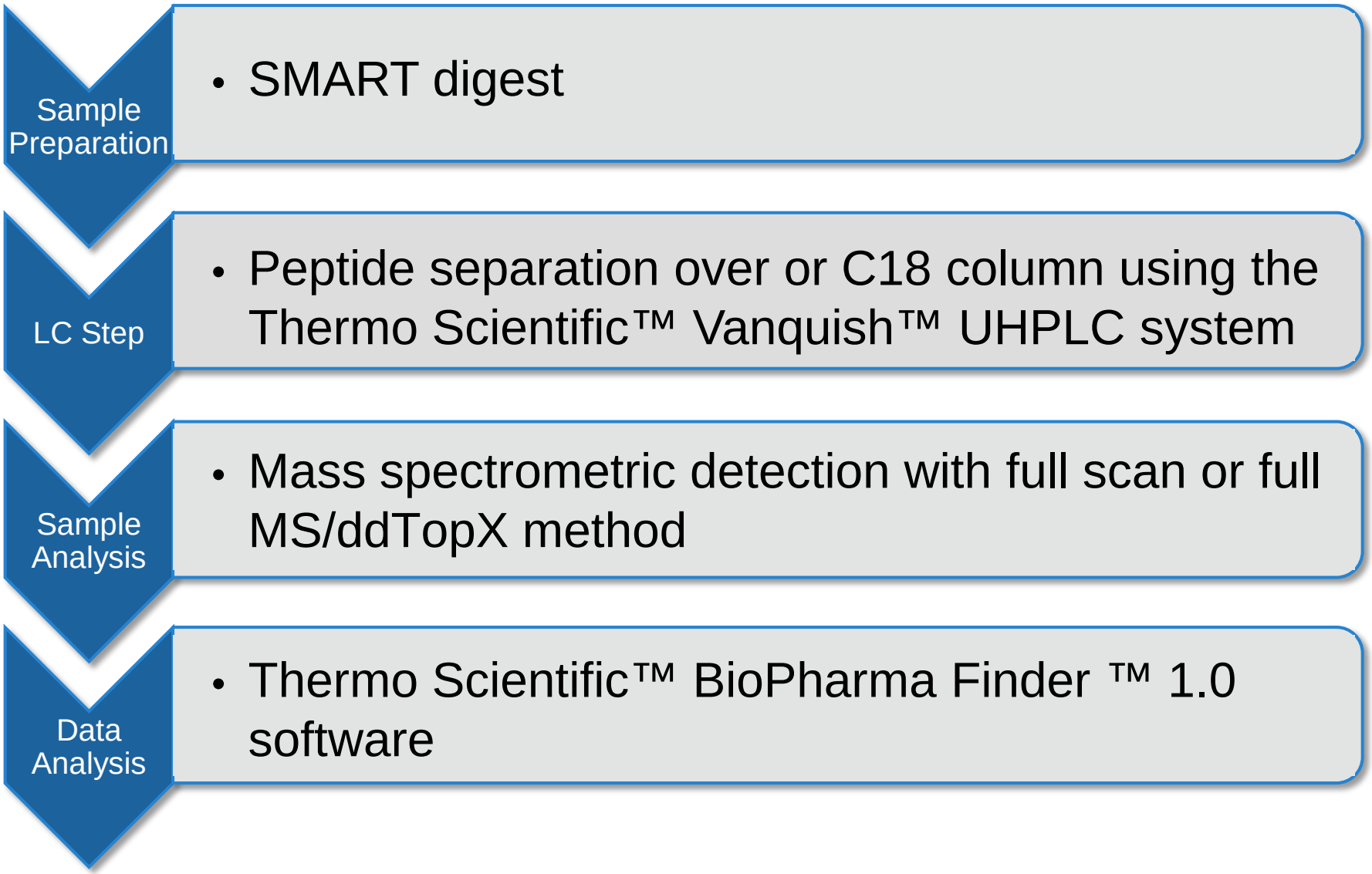
Conventional (isocratic) HIC

- Protein maintains 3D structure.

- Addition of 5% IPA
- Protein denaturation.
- Number of exposed hydrophobic groups increases.

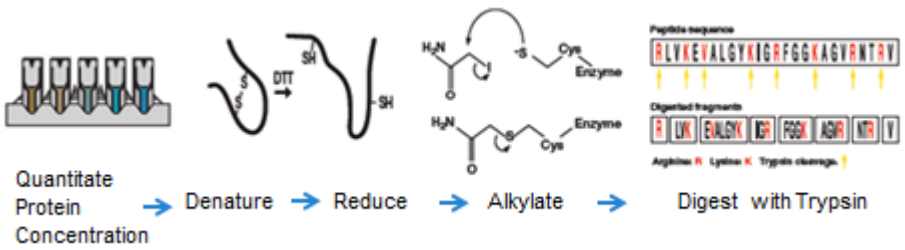
- **Addition of Ca^{2+} and IPA**
- 3D structure is maintained.
- Retention decreases due to partitioning and effect of using a stronger solvent

Peptide Mapping Workflow Overview



Thermo Scientific™ SMART Digest™ Kits

From this…



- Simple, three step protocol
- Quicker than in-solution digestion
- Reduces operator variability
- Increases reproducibility
- Higher throughput
- Ability to automate

To this…!

Heat Denaturation

Add protein sample to SMART Digest tube → Incubate and shake SMART Digest tube → Thermo Scientific™ SOLAμ™ SPE or centrifuge/filter (optional)

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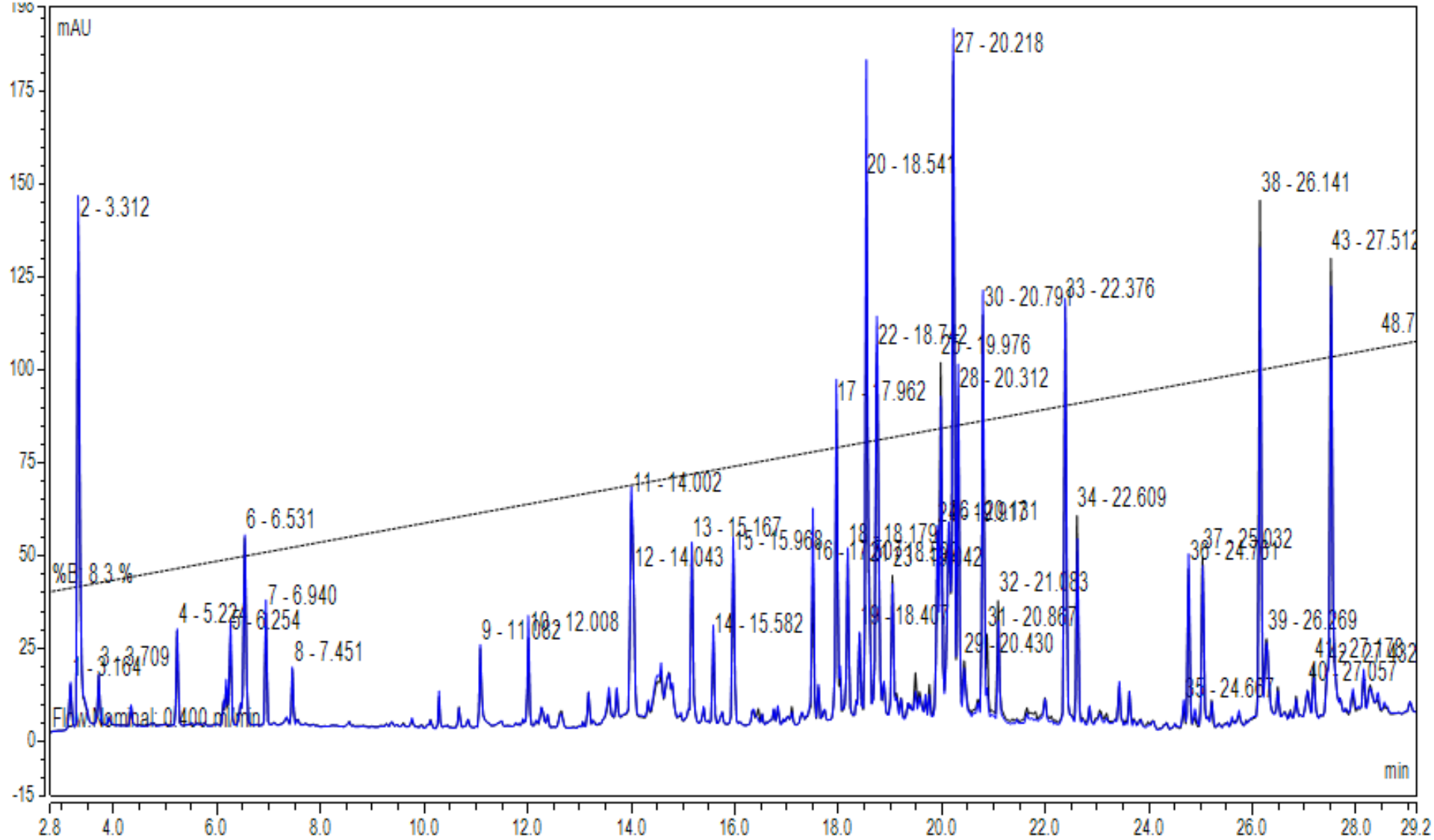
Overlay of different mAb Digests on the Vanquish System



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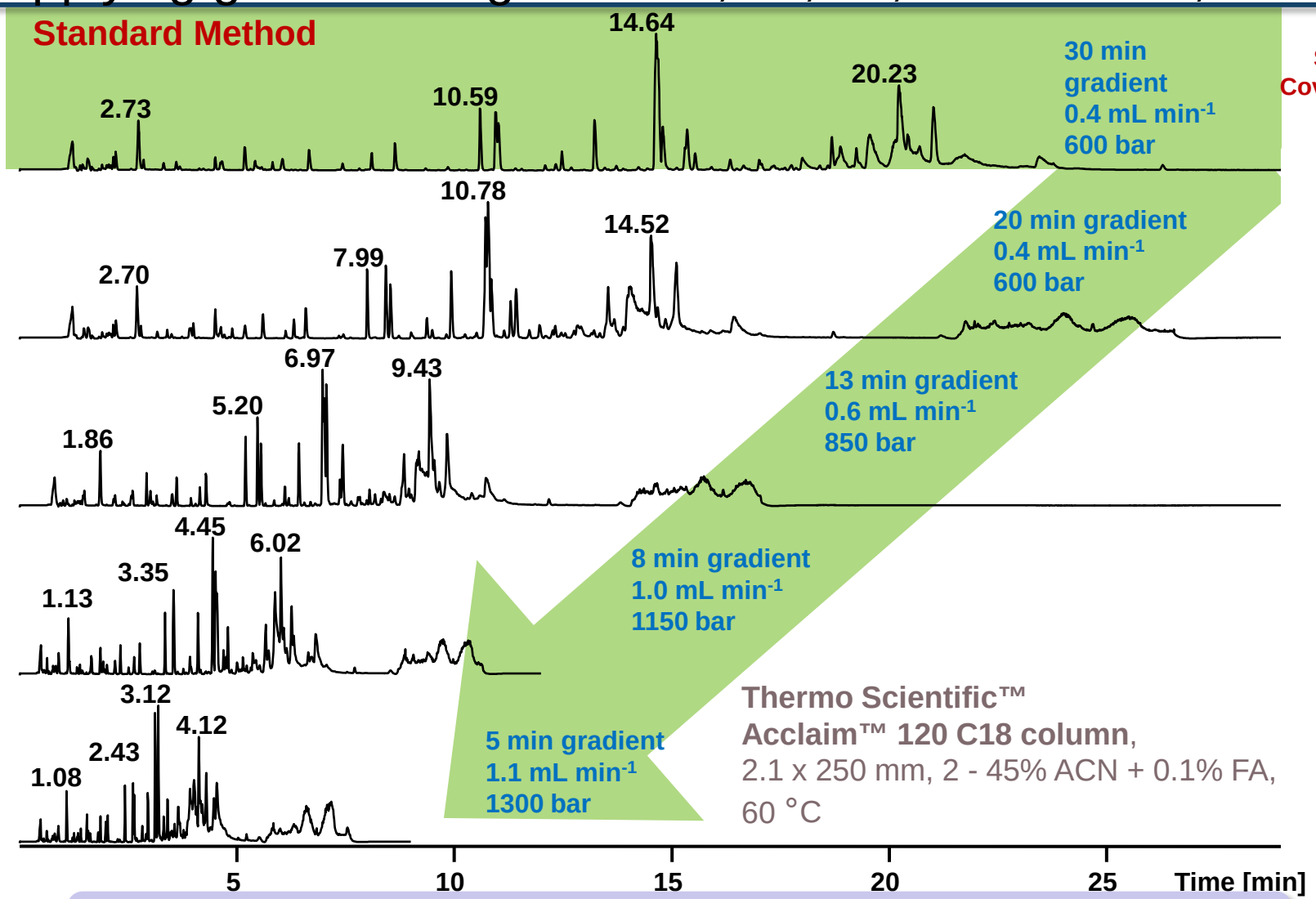
TICs obtained from Peptide Mappoing Experiments of Rituximab

Applying gradient lengths of 30, 20, 13, 8 and 5 min,



AN 1135: High-Throughput Peptide Mapping with the Vanquish UHPLC System and the Q Exactive HF MS

ThermoFisher SCIENTIFIC

Charged Variant Analysis Workflow Overview

Sample Preparation

- CX-1 pH Gradient Buffers, 10 X concentrated

LC Step

- MAbPac SCX RS columns

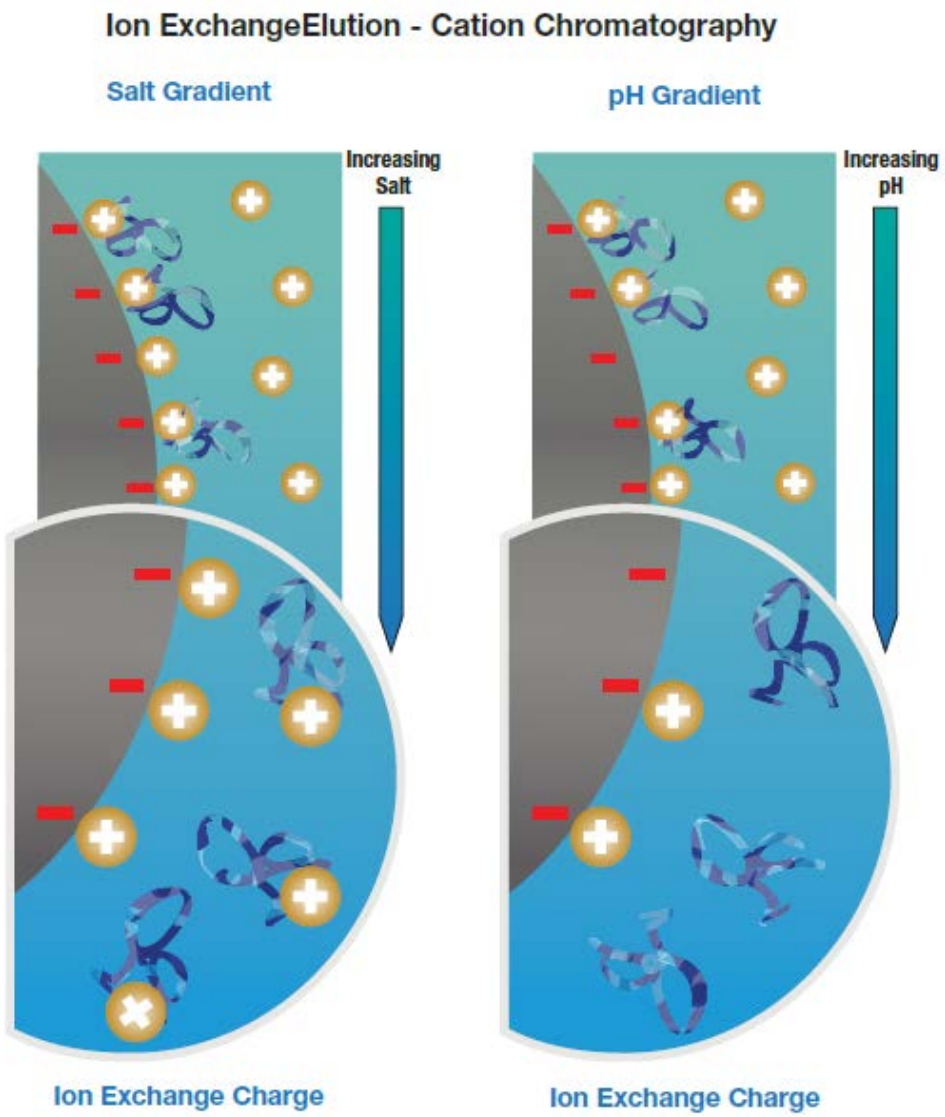
Sample Analysis

- Thermo Scientific ™ Vanquish™ Flex and UltiMate™ 3000 BioRS System
- Automated desalting on Polymeric RP

Data Analysis

- Thermo Scientific ™ Q Exactive™ Orbitrap Mass Spectrometer
- Full scan method

Charge Variant Analysis



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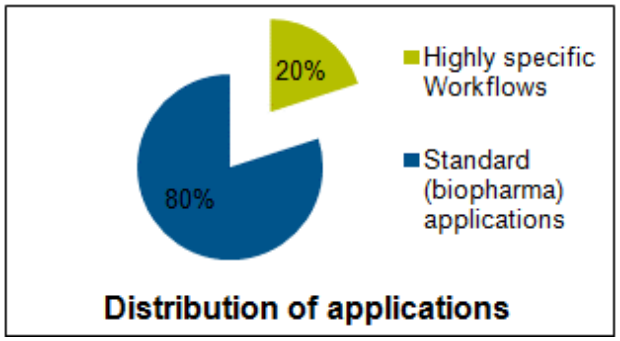
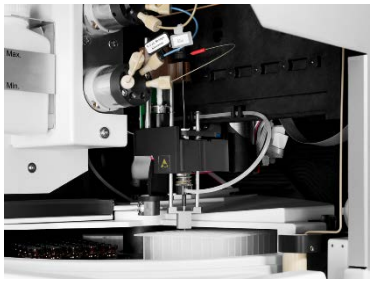
LC Systems for Bio-Therapeutic Protein Analysis

Vanquish™ UHPLC and Vanquish™ Flex UHPLC systems



UltiMate 3000 BioRS system



High Resolution, Cooled Fractionation

pH and Conductivity

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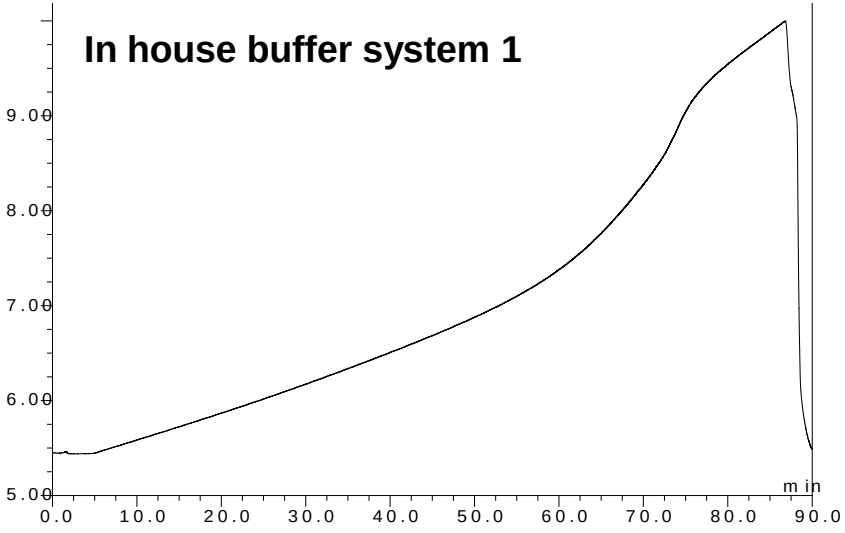
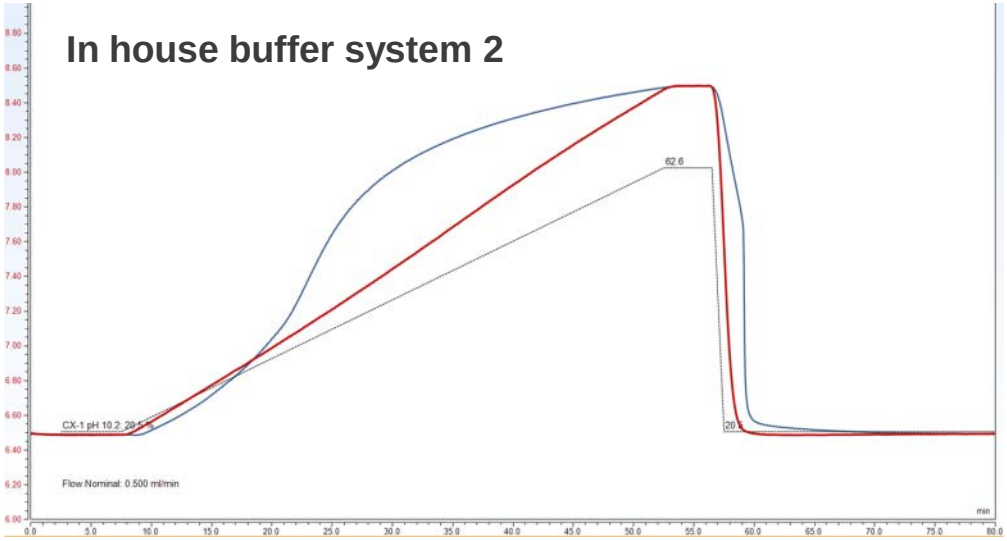
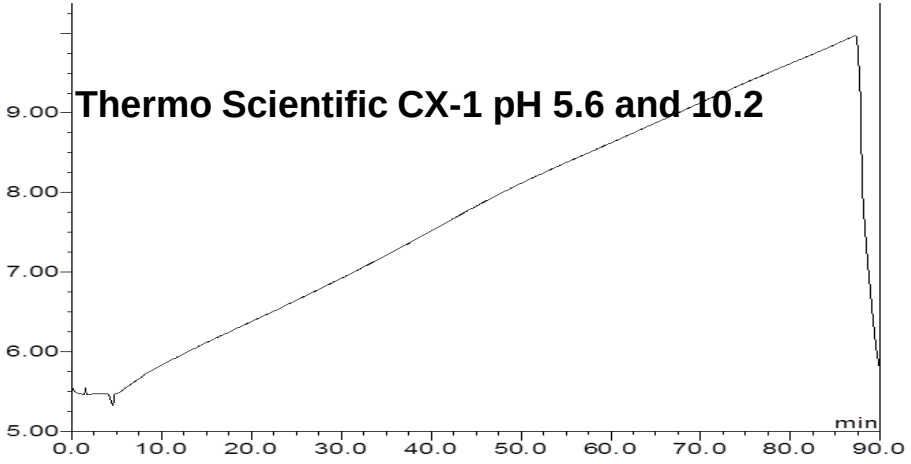
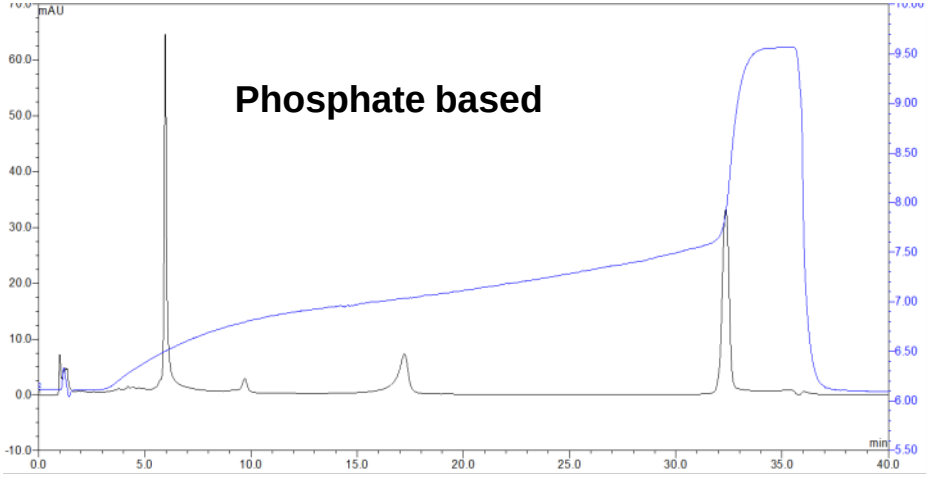
Comparison of pH Gradient Buffer Systems









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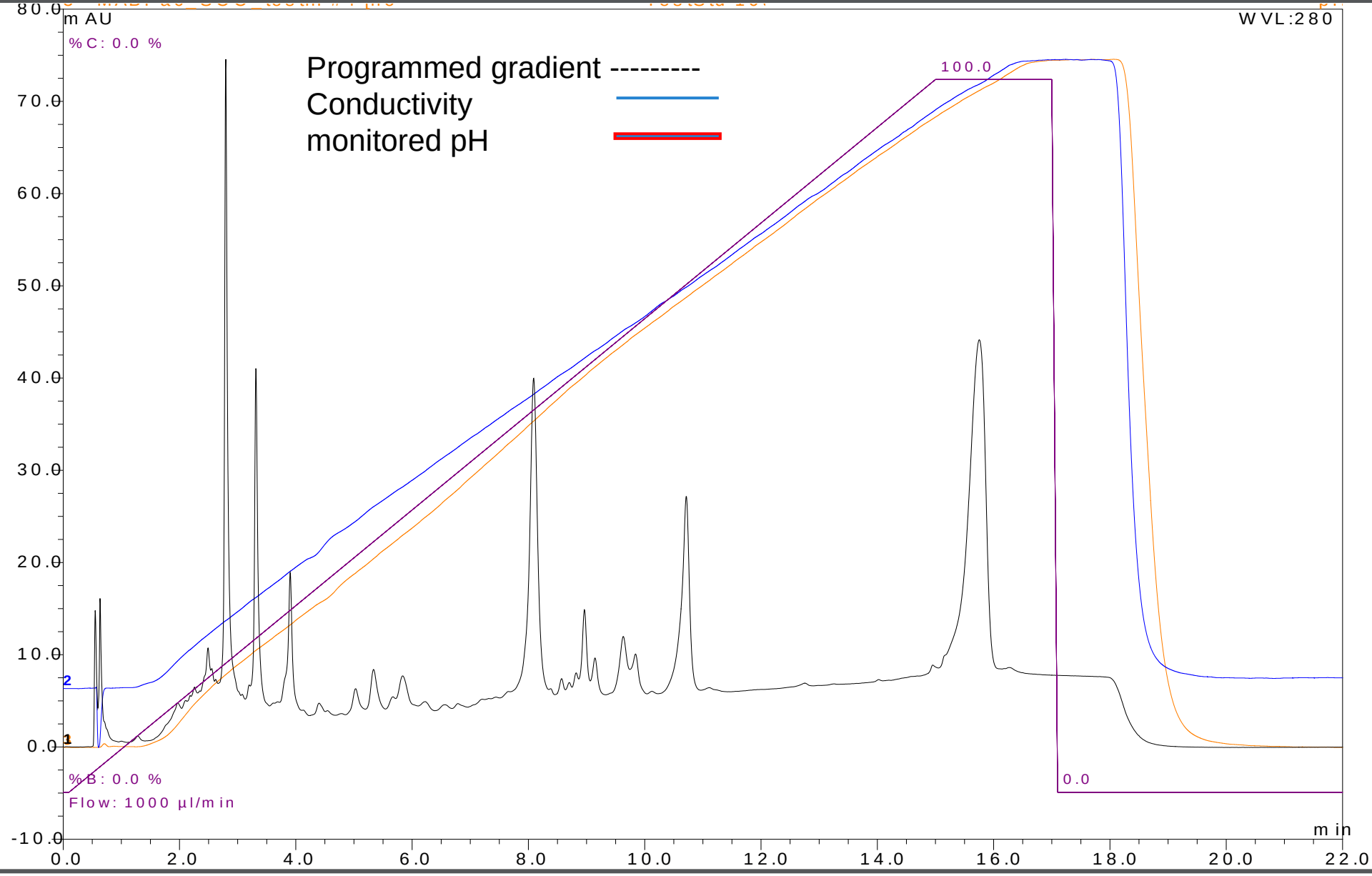
Equilibration Times on a 150mM long Column



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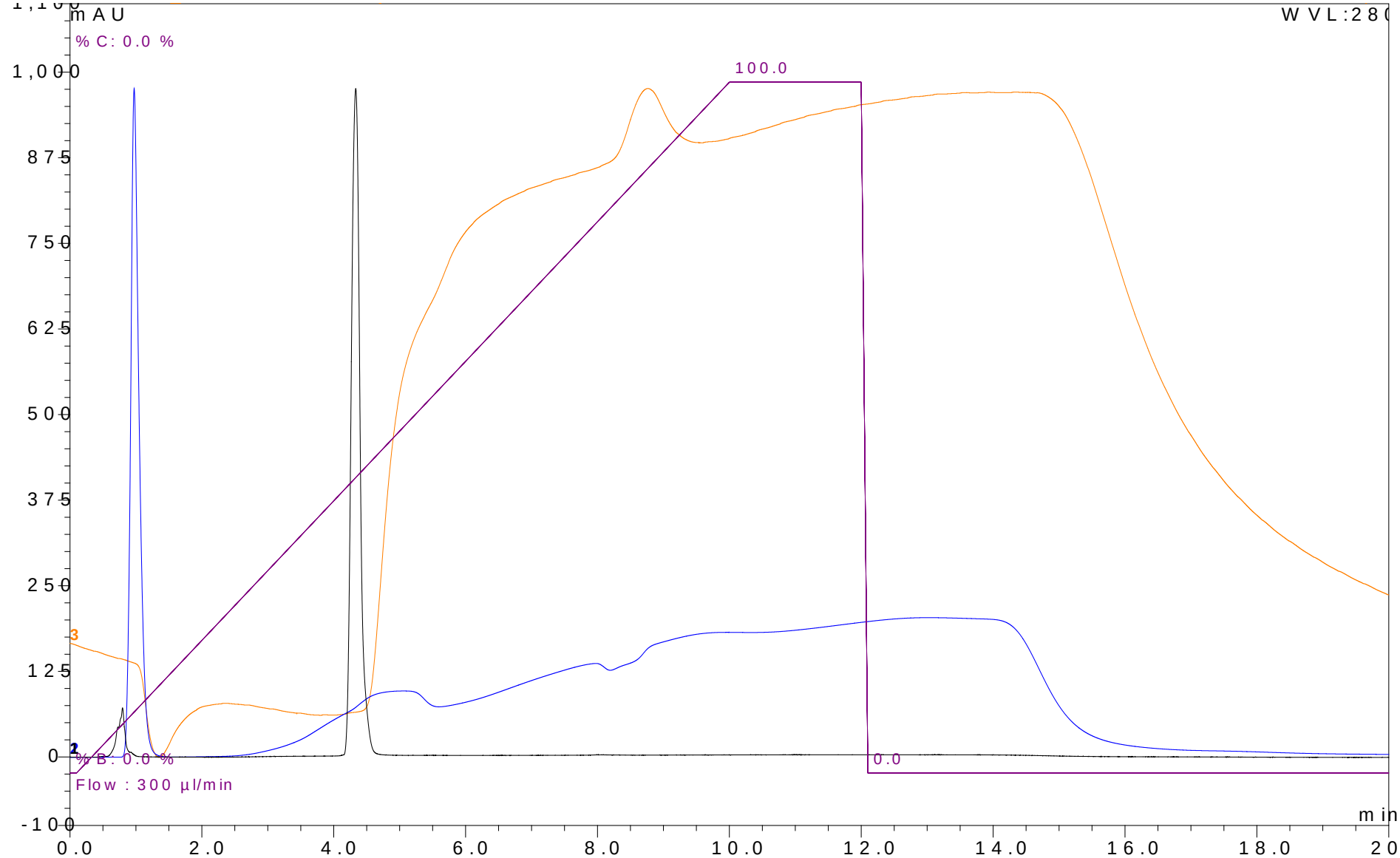
Linear Gradient Cetuximab „Bad Buffering“



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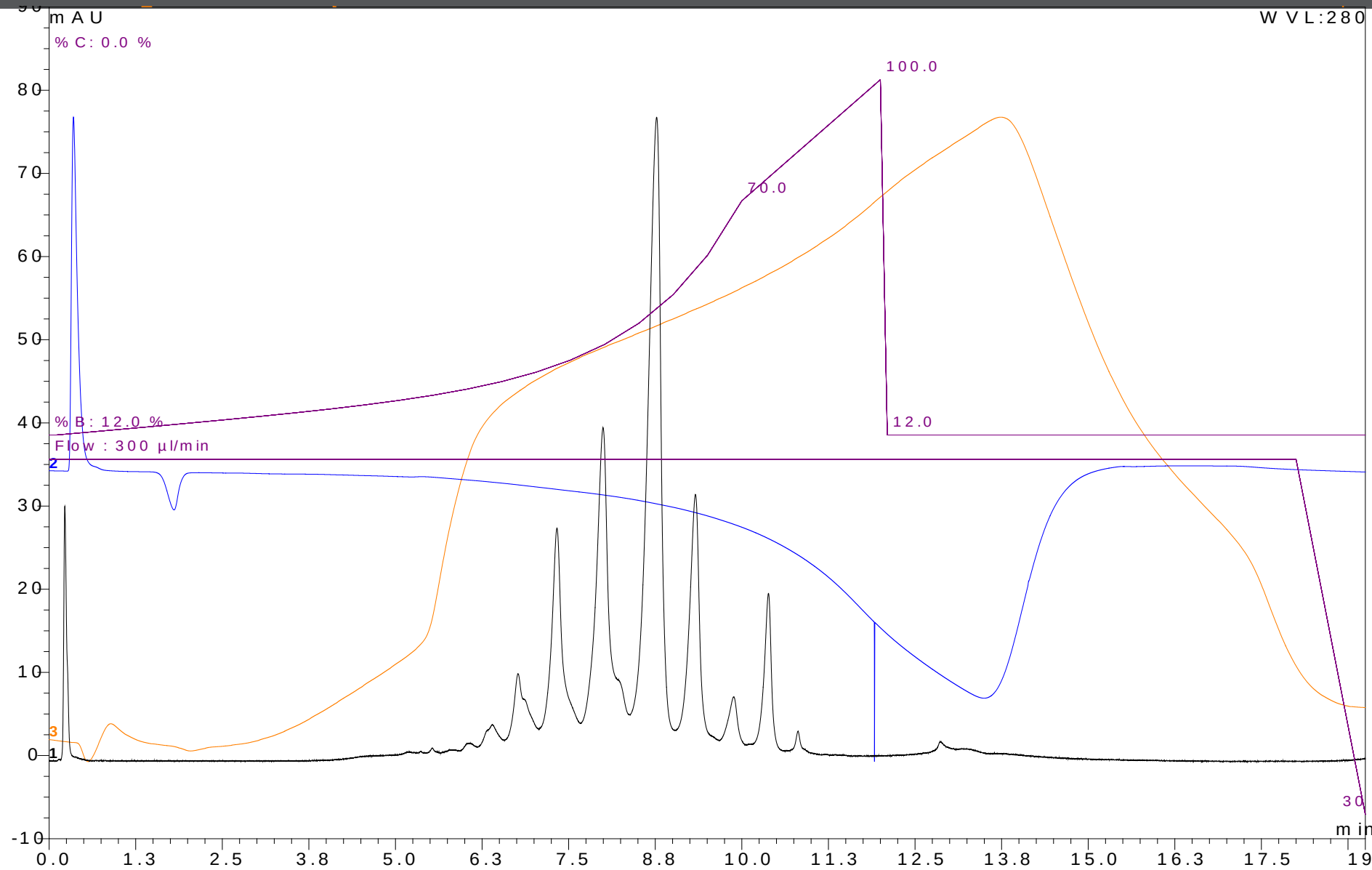
pH Profile using a Home Made Buffer System on a 50mm Column



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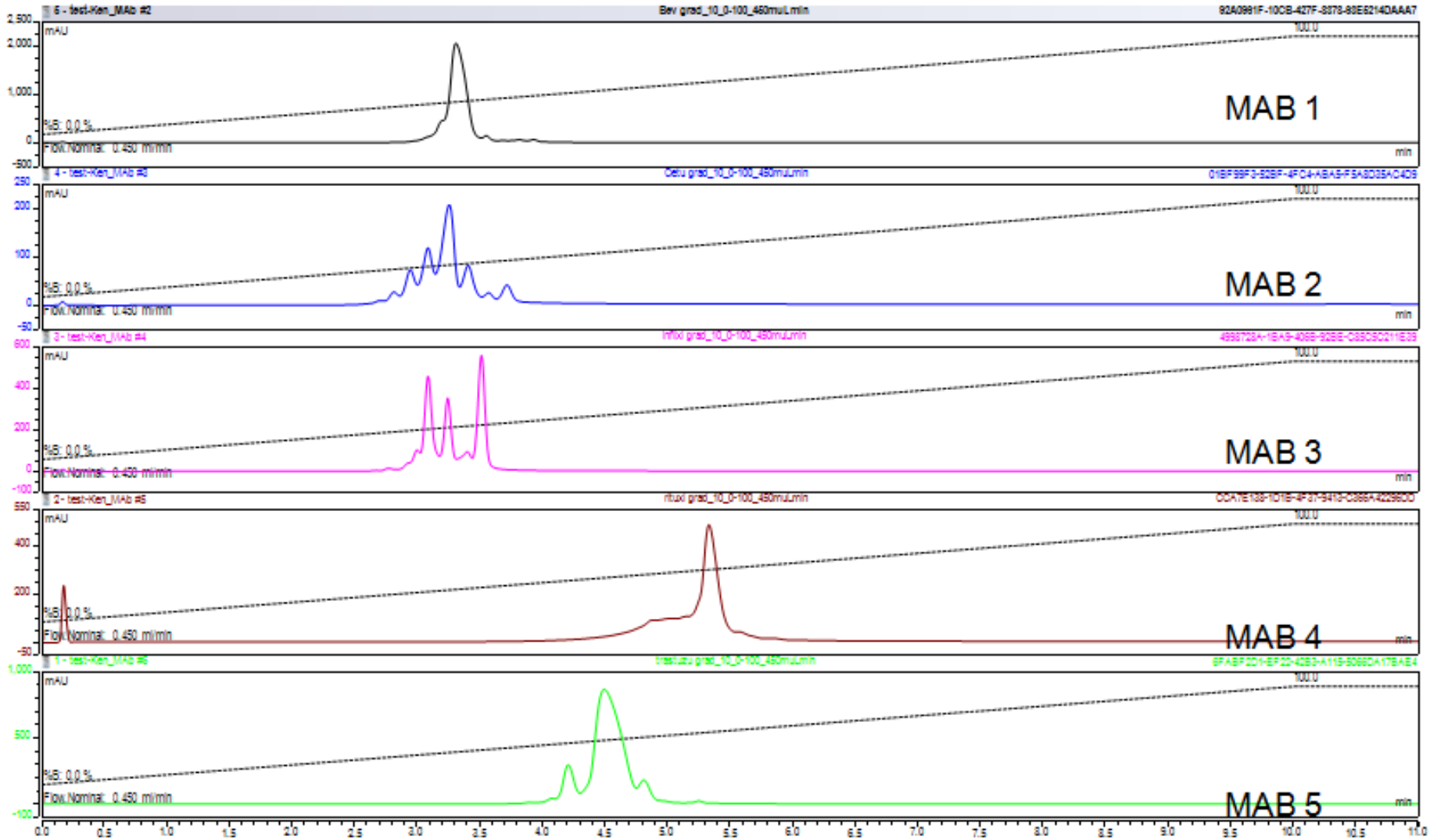
Generic Linear pH Gradient with the Vanquish System



pH 5.6 to 10.2 in 10 minutes

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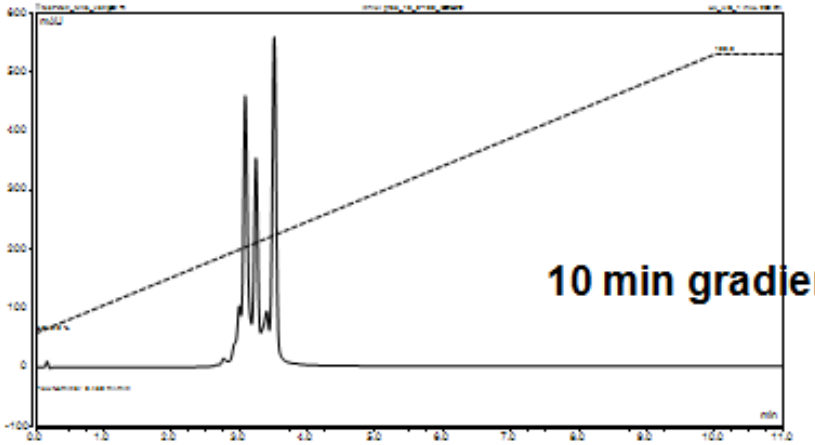
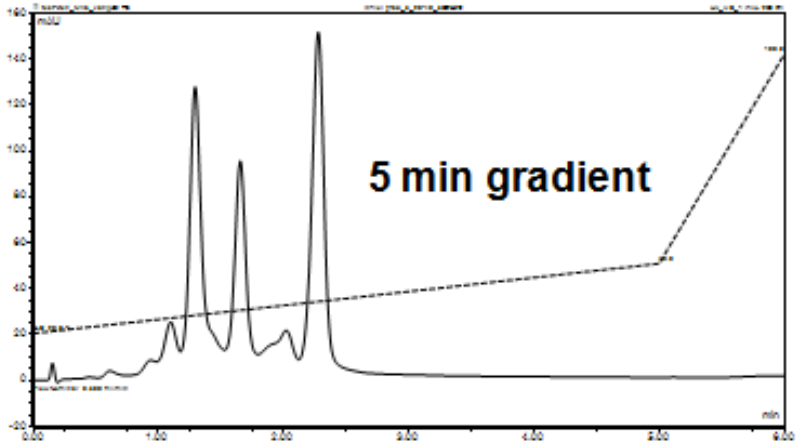
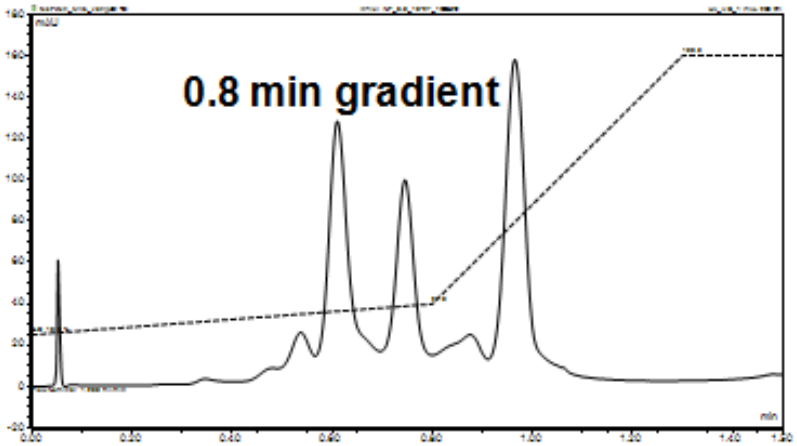
Example 2/5: Infliximab – Vanquish System fast Gradients







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

Intact and native Protein Workflow Overview

Sample Preparation

- Protein A, IEC pH-gradient, HIC, SEC, enzymes

LC Step

- Vanquish Flex and UltiMate 3000 BioRS System
- Automated desalting on Polymeric RP

Sample Analysis

- Q Exactive Orbitrap Mass Spectrometer
- Full scan method

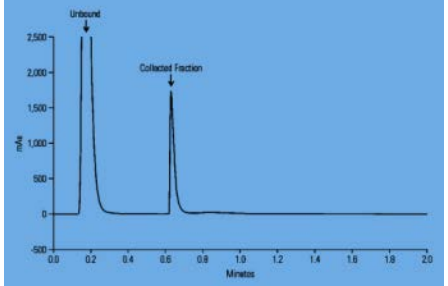
Data Analysis

- BioPharma Finder 1.0 software

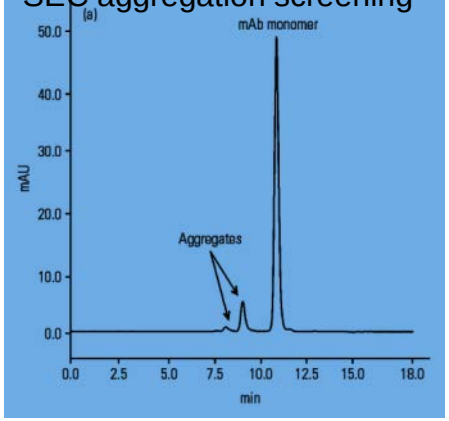
Wide Range of Bioanalytical Characterization Methods

Initial separation

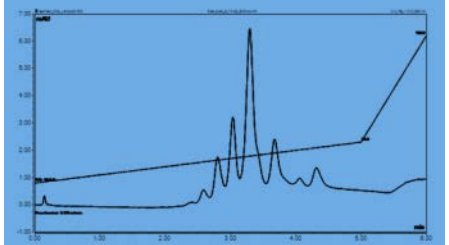
Protein A capture of IgG



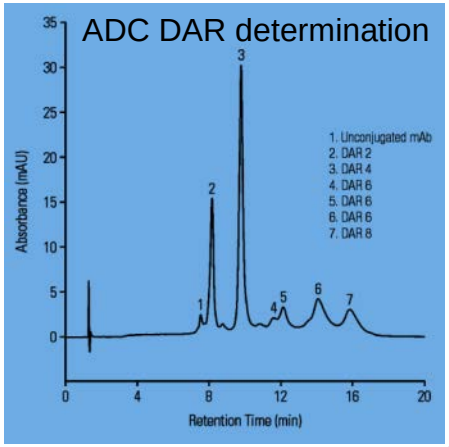
SEC aggregation screening



IEC pH-gradient charge variant analysis

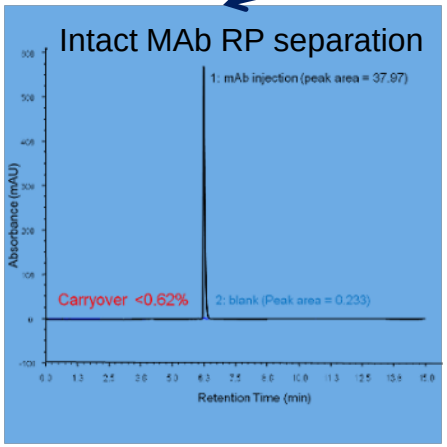


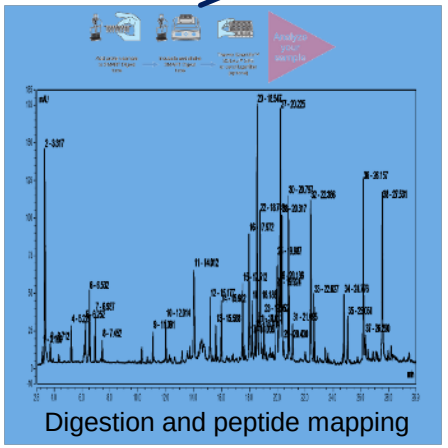
ADC DAR determination



MS Transfer

Intact MAb RP separation





Digestion and peptide mapping

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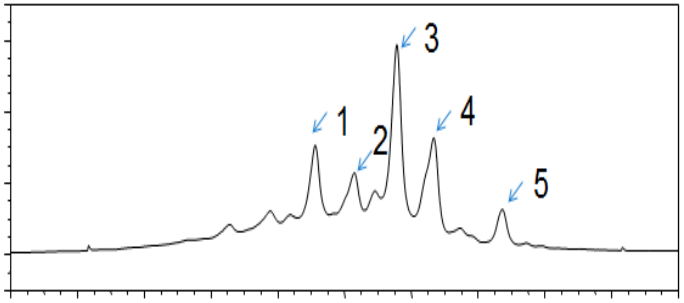
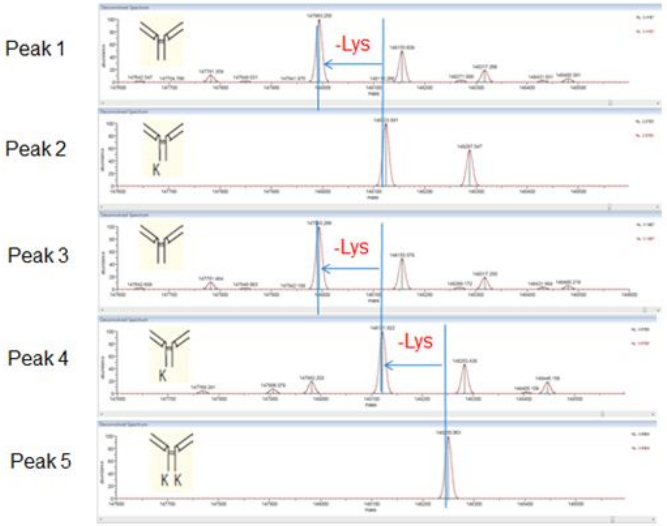
In-depth HRAM Charge Variant Characterization

1st dimension: IEX pH gradient + fraction collection



2nd dimension: Polymer RP-LC/MS



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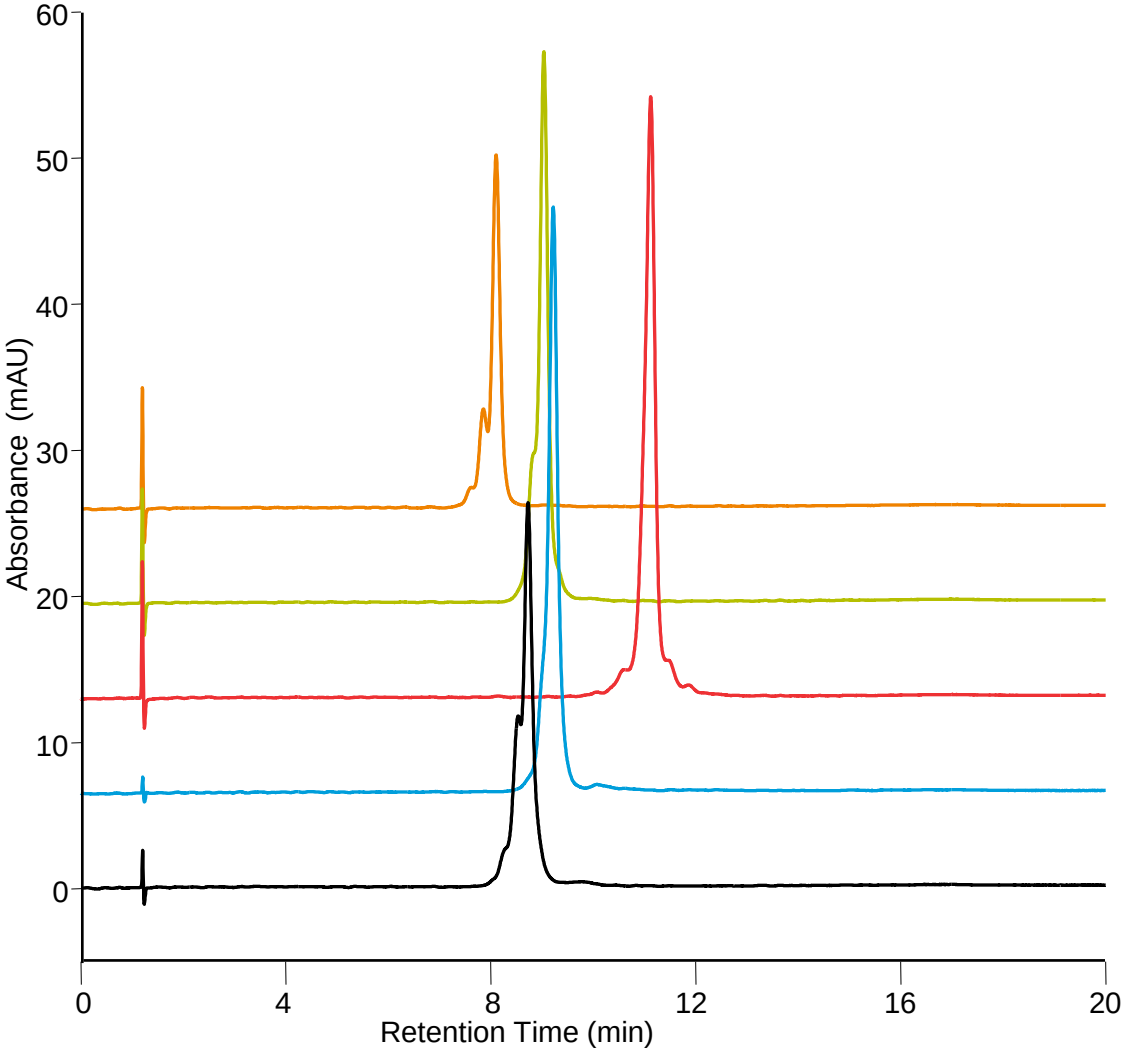
Global Analysis of Intact mAbs



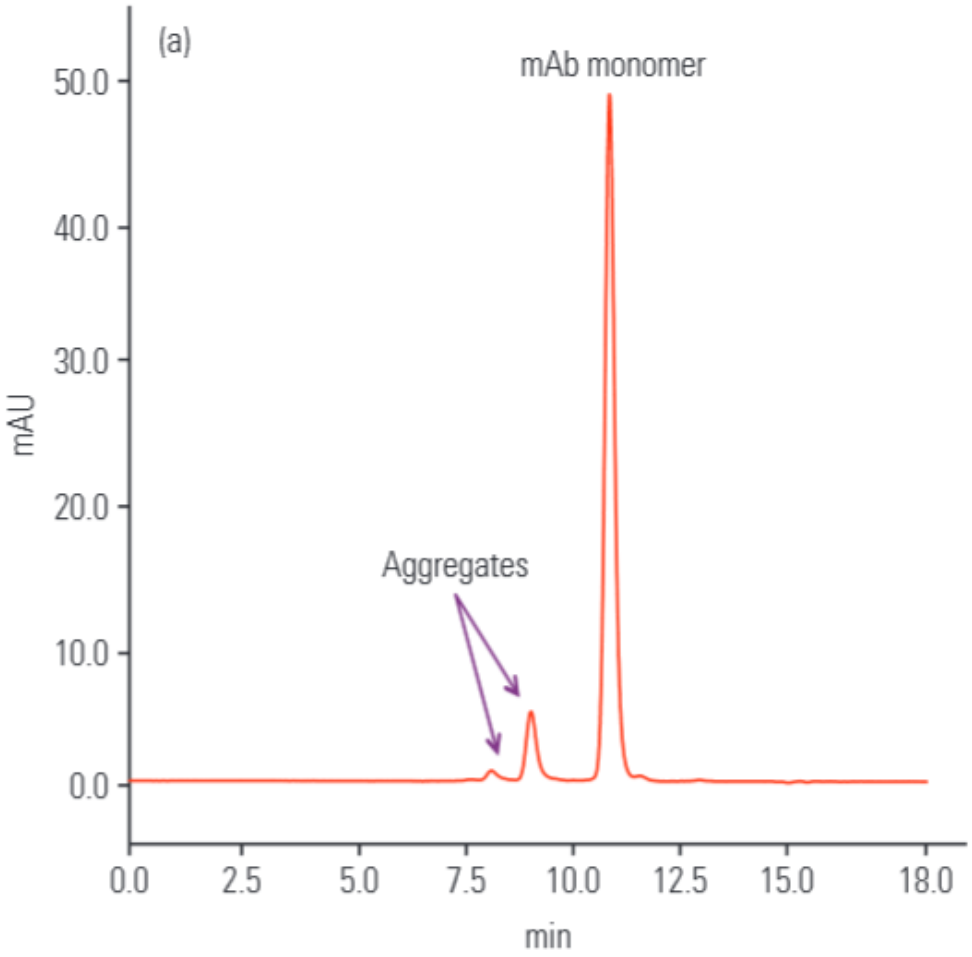
Column:	MAbPac HIC-10, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B:	100 mM sodium phosphate, pH 7.0

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

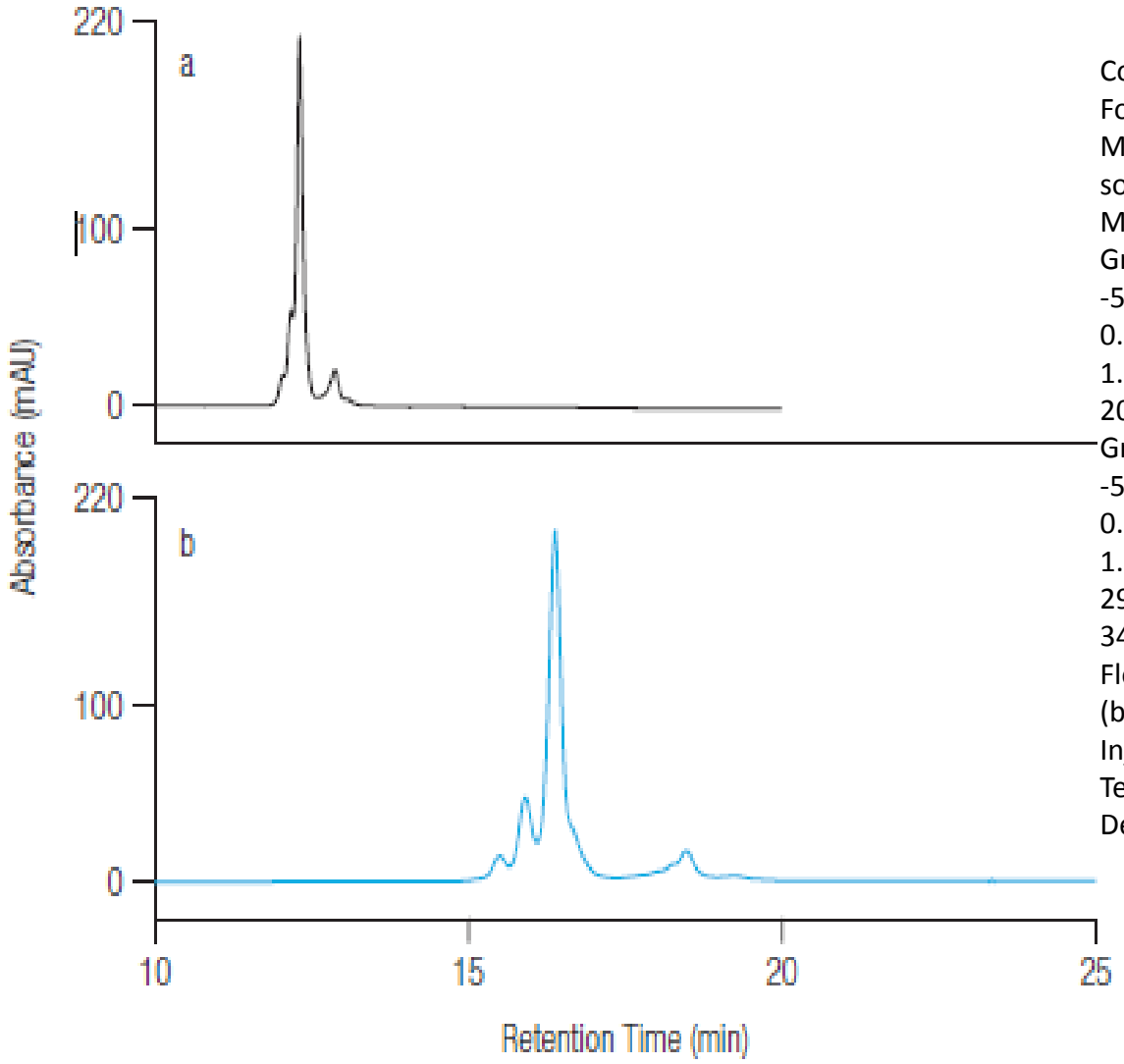
Temperature:	30 ºC
Flow rate:	1.0 mL/min
Inj. volume:	2 µL (4 mg/mL)
Detection:	UV (280 nm)
Sample:	mAb1 mAb2 mAb3 mAb4 mAb5

Aggregate Screening using the 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)

Method Speed up of mAb Aggregates with HIC Column



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)

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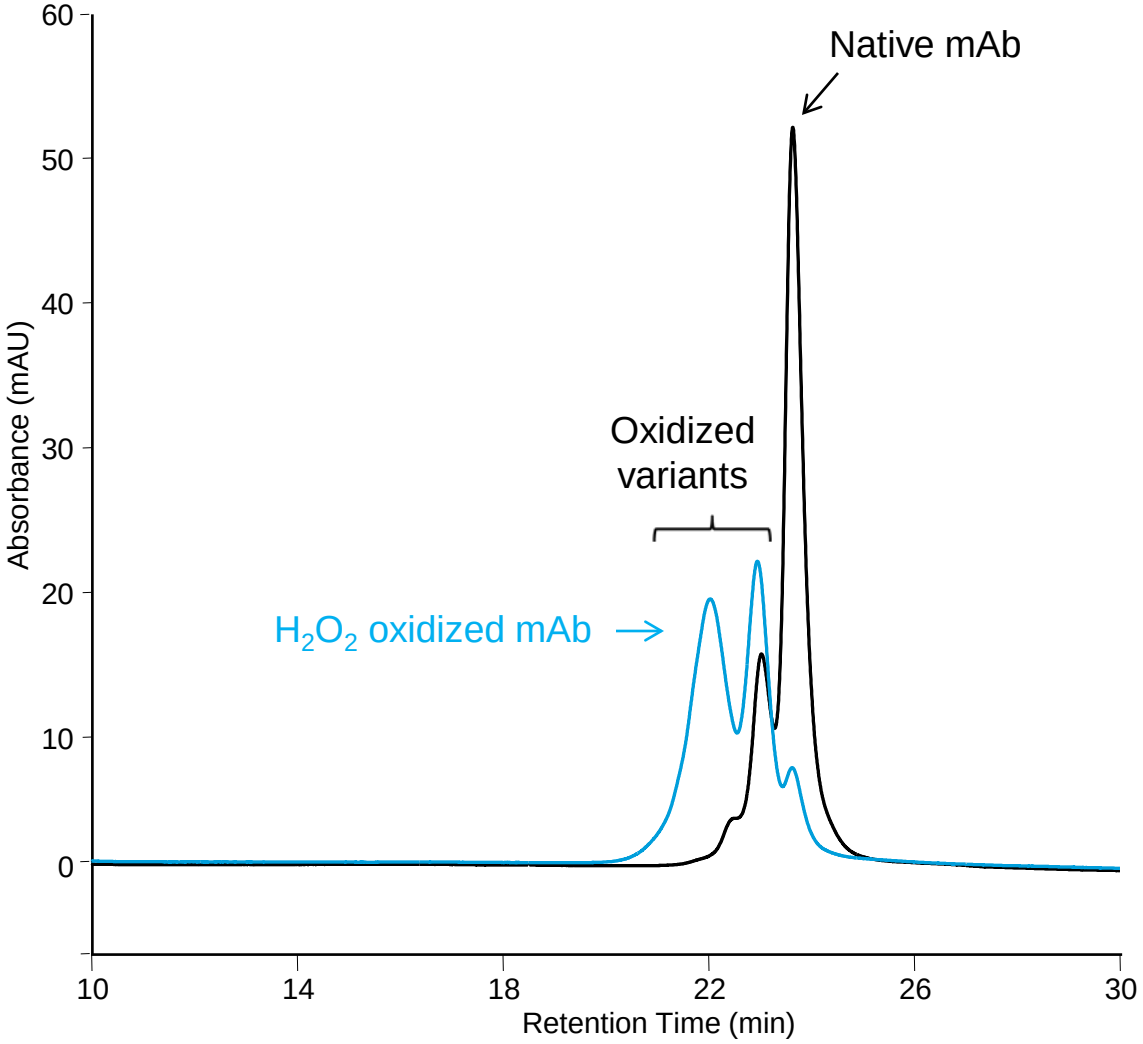
Separation of Oxidized mAb on MAbPac HIC-20

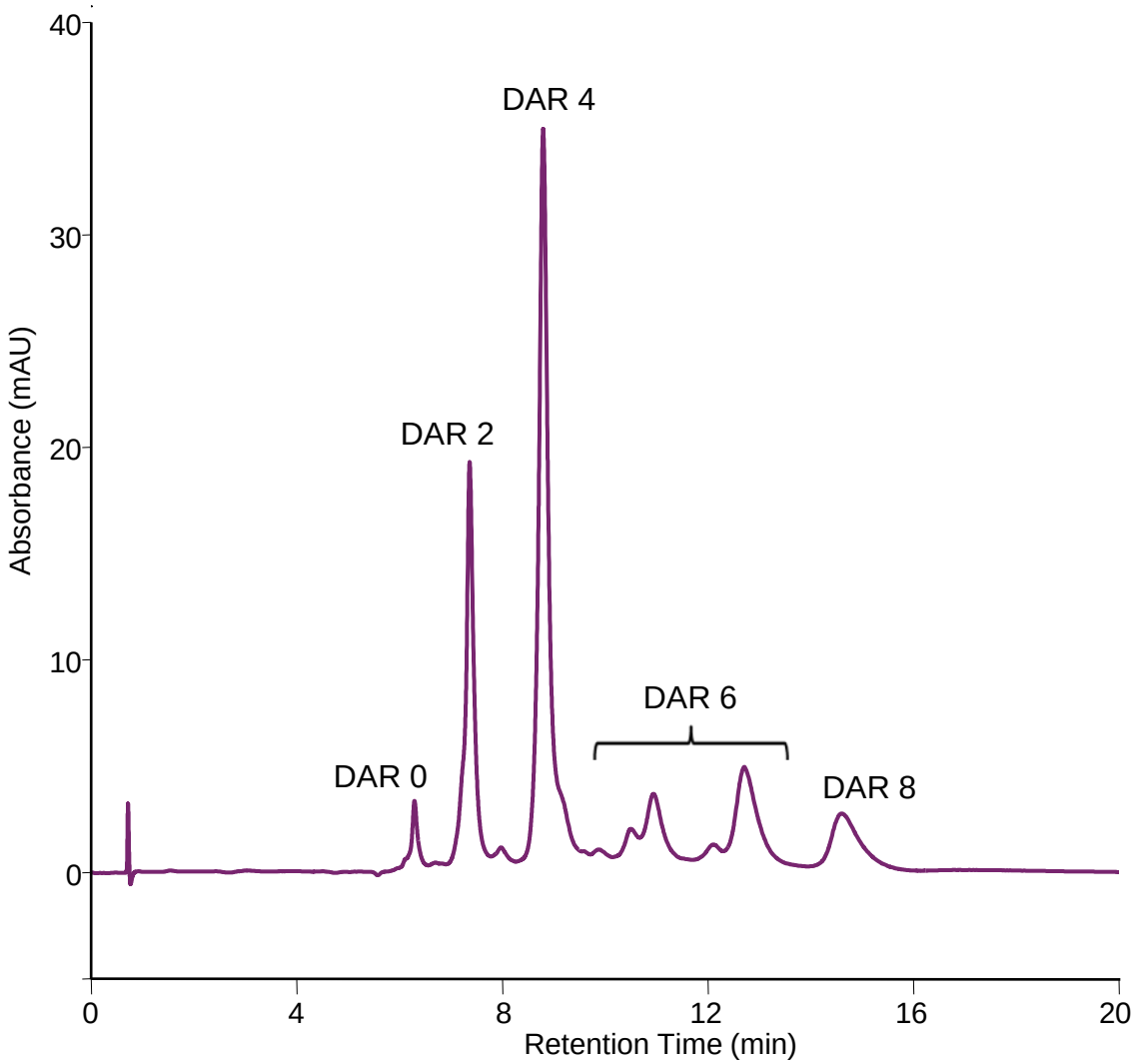


Column:	**MAbPac HIC-20, 5 µm**
Format:	4.6 × 250 mm
Mobile phase A:	2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0
Mobile phase B:	100 mM sodium phosphate, pH 7.0
Gradient:	

Time (min)	%A	%B
-6.0	50	50
0.0	50	50
2.0	50	50
30.0	0	100
35.0	0	100

Temperature:	30 ºC
Flow rate:	0.5 mL/min
Inj. volume:	Untreated mAb: 20 µL (1.25 mg/mL) Oxidized mAb: 20 µL (1.25 mg/mL)
Detection:	UV (280 nm)
Sample:	Untreated mAb H_2O_2 oxidized mAb

Separation of cys-linked ADC on MAbPac HIC-Butyl

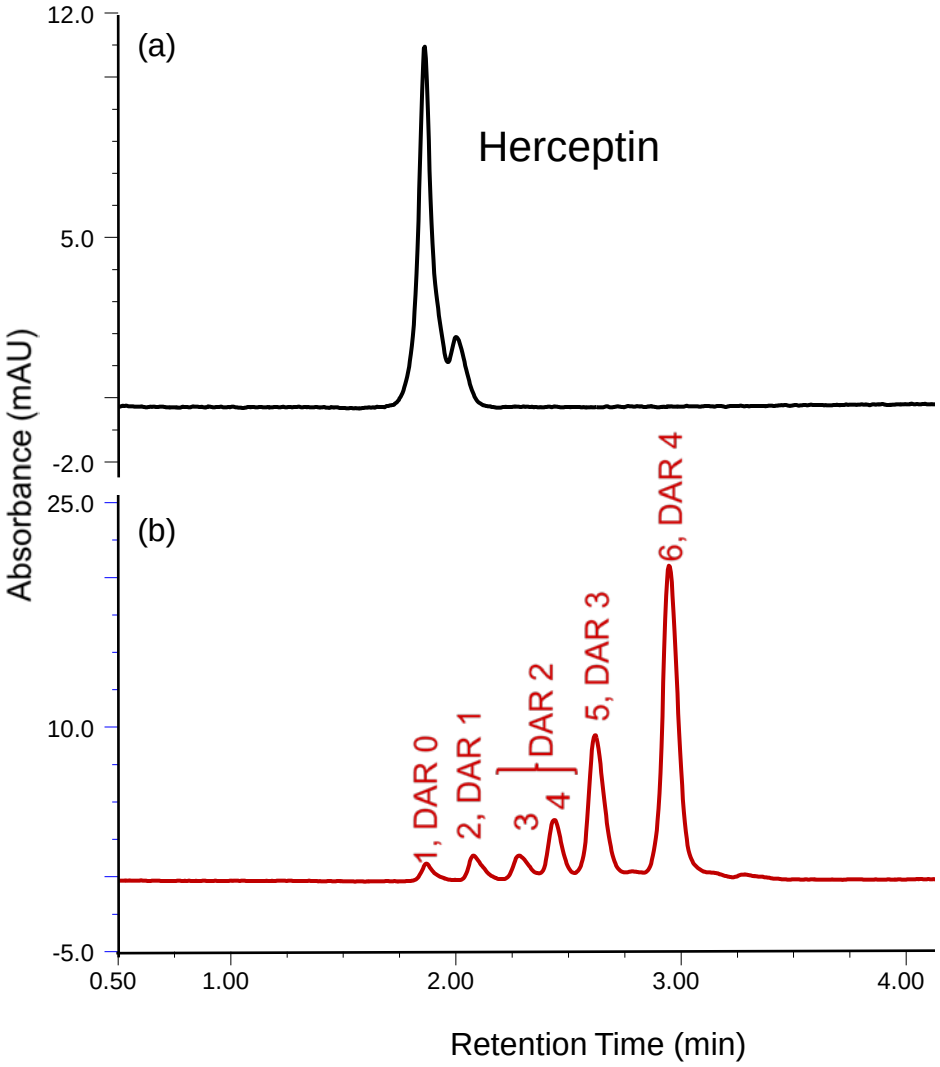


Column:	MAbPacHIC-Butyl, 5 µm
Format:	4.6 × 100 mm
Mobile phase A:	1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0 / isopropanol (95:5 v/v)
Mobile phase B:	50 mM sodium phosphate, pH 7.0 / isopropanol (80:20 v/v)
Gradient:	

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

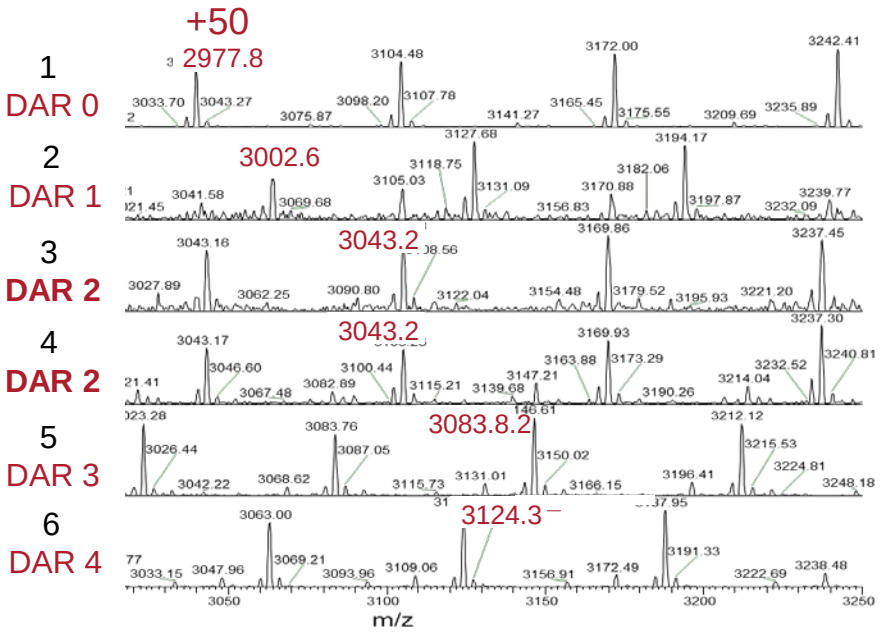
Temperature:	25 ºC
Flow rate:	1.0 mL/min
Inj. volume:	5 µL (5 mg/mL)
Detection:	UV (280 nm)
Sample:	Cys-conjugated ADC mimic

RP Separation of Unmodified mAbs and ADCs



Column:	MAbPac RP, 4 µm
Format:	2.1 × 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)

MS raw data



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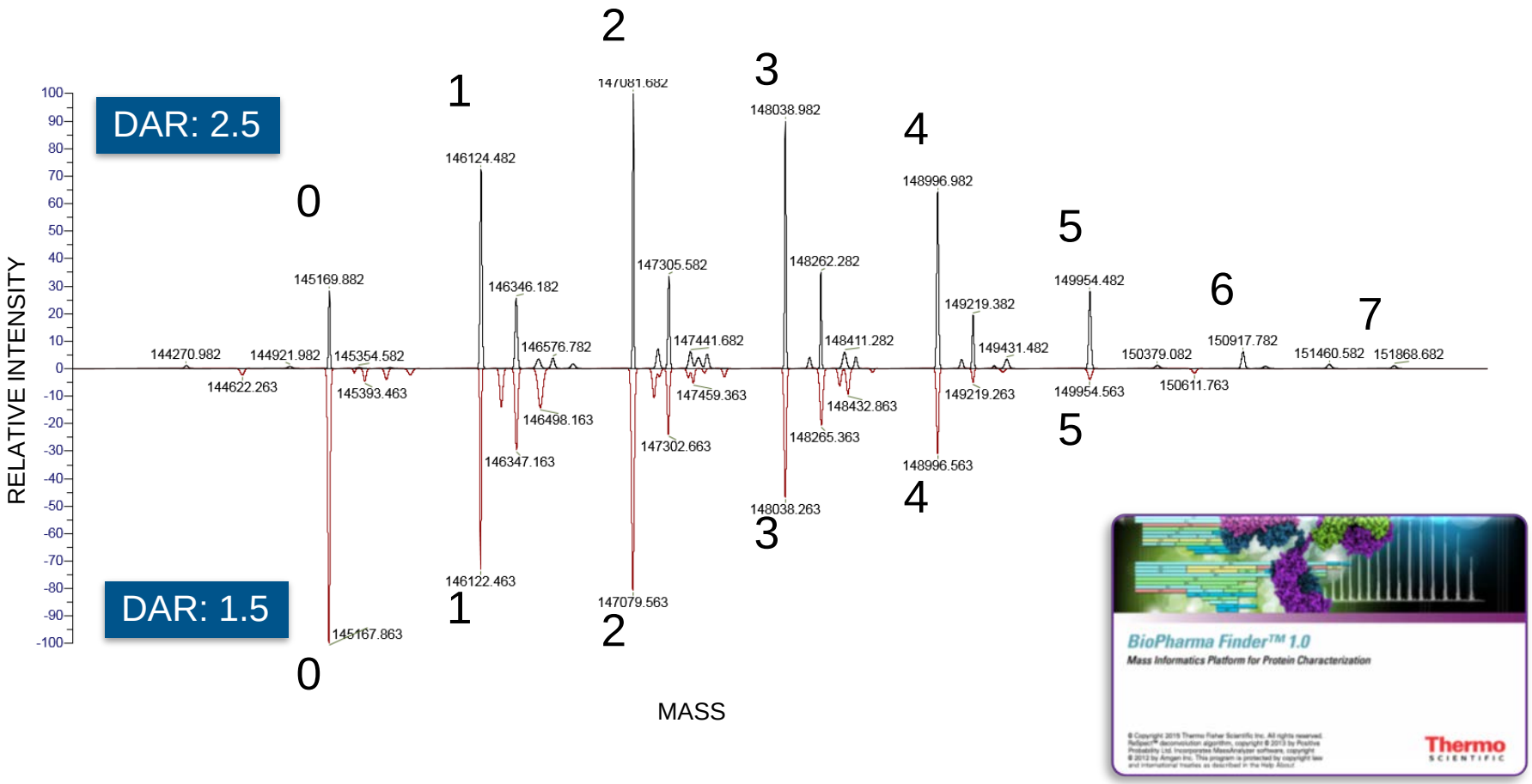
Comparison of two different Levels of Drug-Antibody Ratio (DAR)

Mirror plot of two ADC samples



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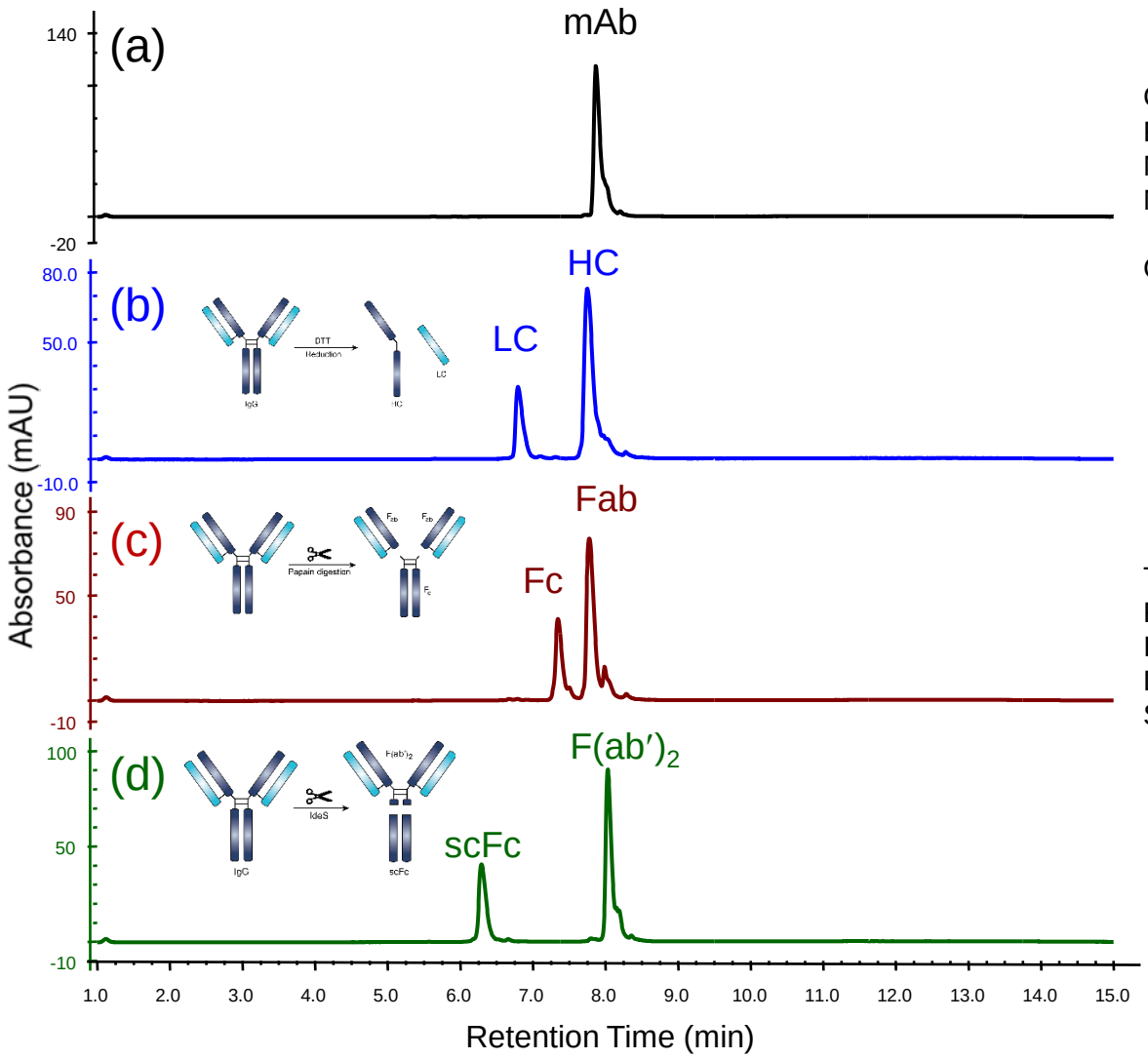
mAb and mAb Fragments Analysis – Reversed Phase



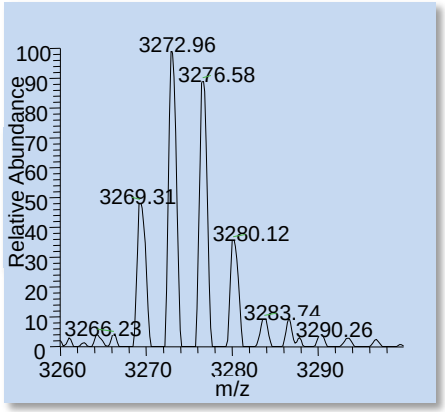
Column:	MAbPac RP, 4 µm
Format:	3 × 50 mm
Mobile phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)

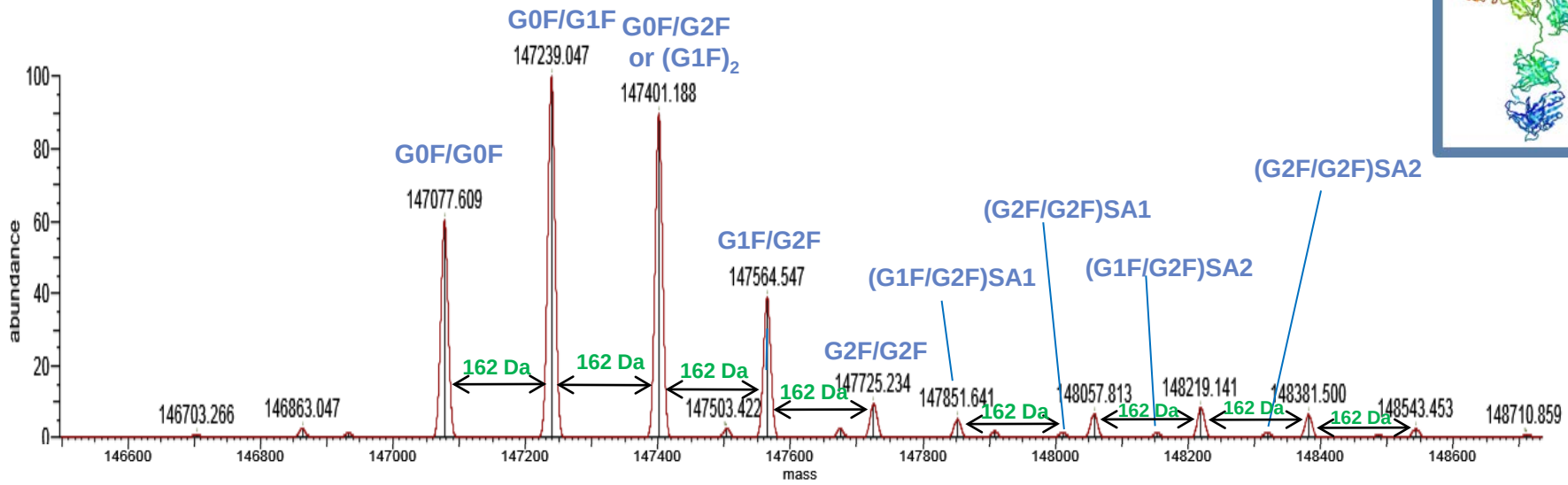
Gradient:

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Temperature:	80 ºC
Flow rate:	0.5 mL/min
Inj. volume:	5 µL
Detection:	UV (280 nm)
Sample:	(a) trastuzumab (5 mg/mL)
	(b) trastuzumab + DTT (4 mg/mL)
	(c) trastuzumab + Papain (2 mg/mL)
	(d) trastuzumab + IdeS (2 mg/mL)

Full MS Spectrum of an Intact Antibody (mAb) Rituximab





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