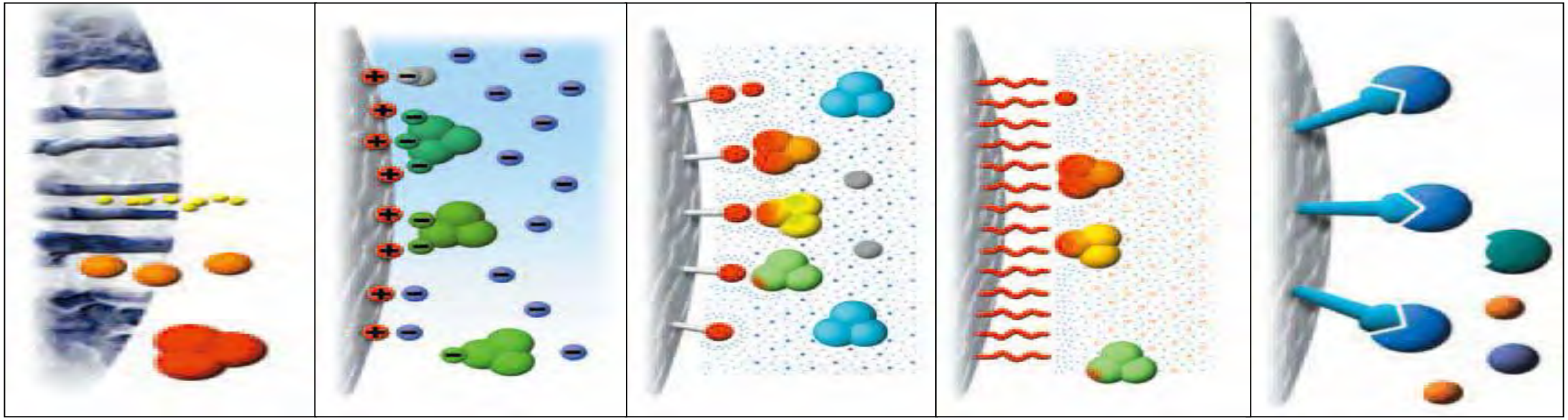
Application of Chromatography in Biomacromolecular Analysis

Suzhou Sepax Technology CO., LTD.

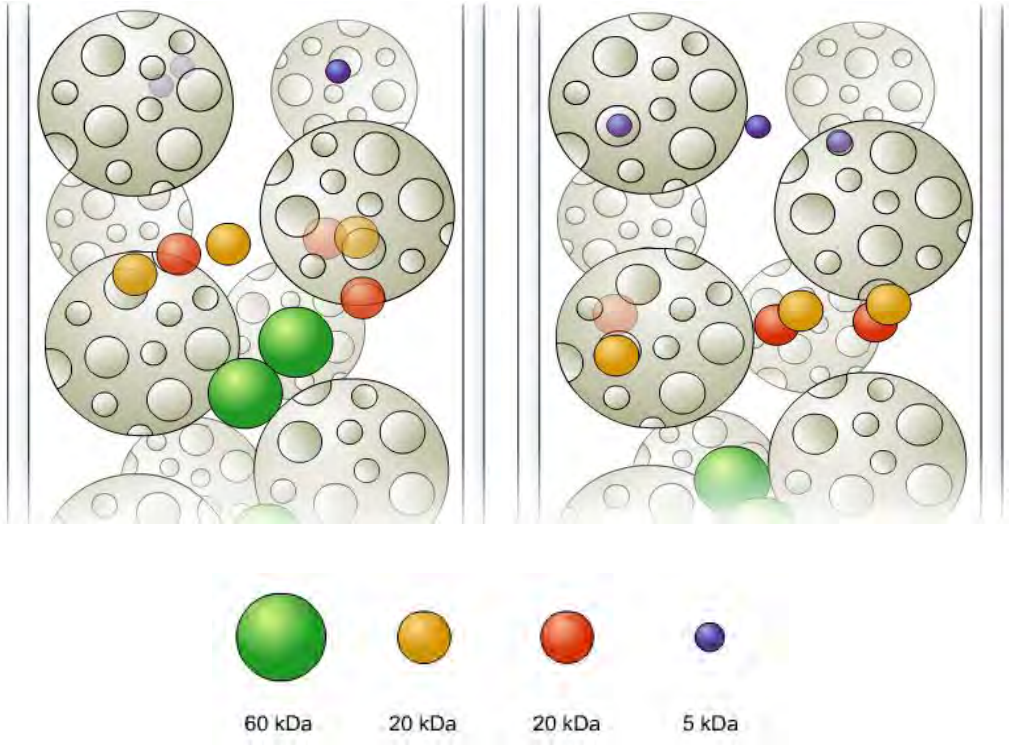
‘Bio’ Separation Methods in LC

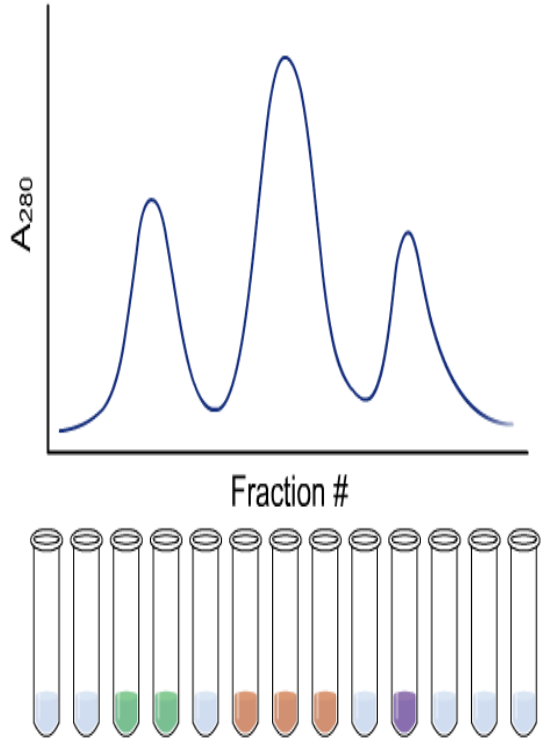


SEC IEX HIC Reverse Phase Affinity

SEC Separation mechanism

The separation is based on the relationship between the molecular weight of the separated component and the pore size of the stationary phase matrix. Mobile phase does not affect the distribution coefficient.





SEC Separation characteristics

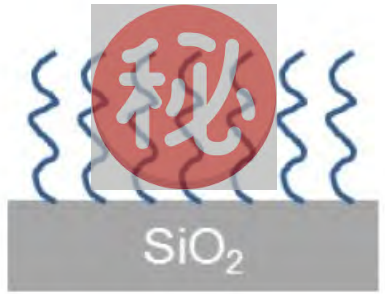
- Rapid analysis to improve detection efficiency
- High activity recovery for biomacromolecule samples
- Choose different pore sizes for different biological samples, with high selectivity
- The mobile phase is simple and environmentally friendly, and commonly used water-soluble salt solutions

Sepax SEC Column Selection

More Hydrophobic
Promote
hydrophobic interaction

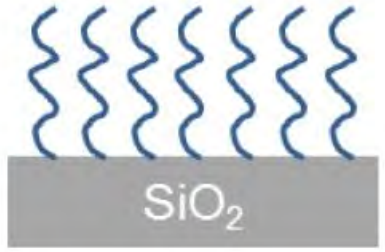
More Hydrophilic
Minimize
Secondary interaction

BioMix 04



Mis-paired and different forms of BsAbs

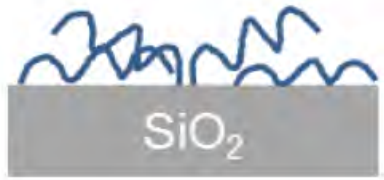
Unix
Zenix
SRT
SRT-10 02



Stand Up Monolayer

Versatile mAbs and Proteins

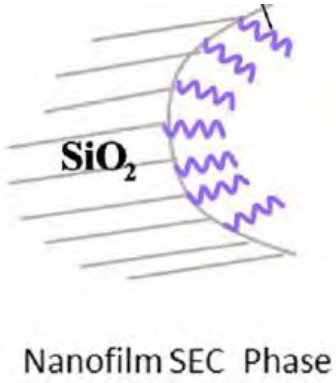
Unix-C
Zenix-C
SRT-C
SRT-10C 03



Lay Down Monolayer

Highly Hydrophobic/ Sticky Sample: ADC

Nanofilm 01



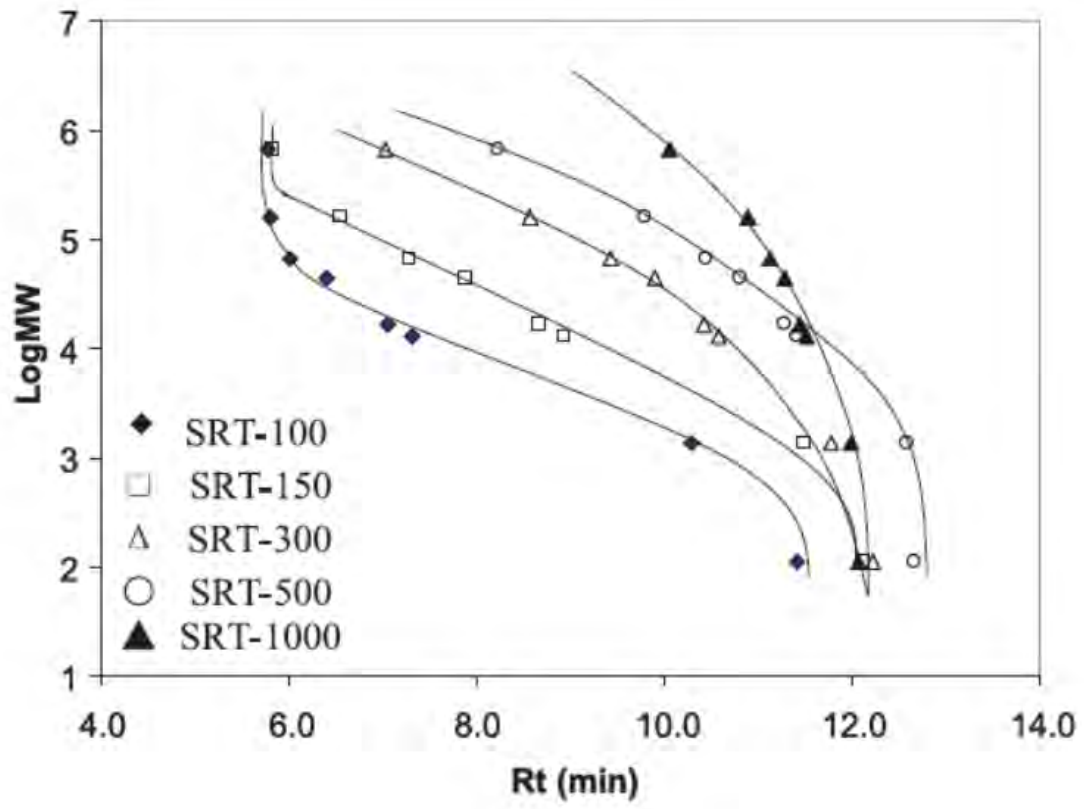
Some Membrane Protein Projects with Detergent

Mixed Mode: Similar MW with different hydrophobicity

Separation based on size difference; Aggregates, protein mixture....

Standard curve of SRT SEC

Select a chromatographic column whose sample molecular weight is within a good linear range



Columns: SRT, 4.6x300 mm, 5 µm
Mobile phase: 0.15 M phosphate buffer, pH 7.0
Flow rate: 0.35 mL/min
Detector: UV 214 nm
Injection volume: 3 µL
Sample: 1. Thyroglobulin, 670 kD
2. γ-Globulin, 158 kD
3. BSA, 66 kD
4. Ovalbumin, 44 kD
5. Myoglobin, 17.6 kD
6. Ribonuclease A, 13.7 kD
7. B12, 1.35 kD
8. Uracil, 120

Sepax water-soluble SEC

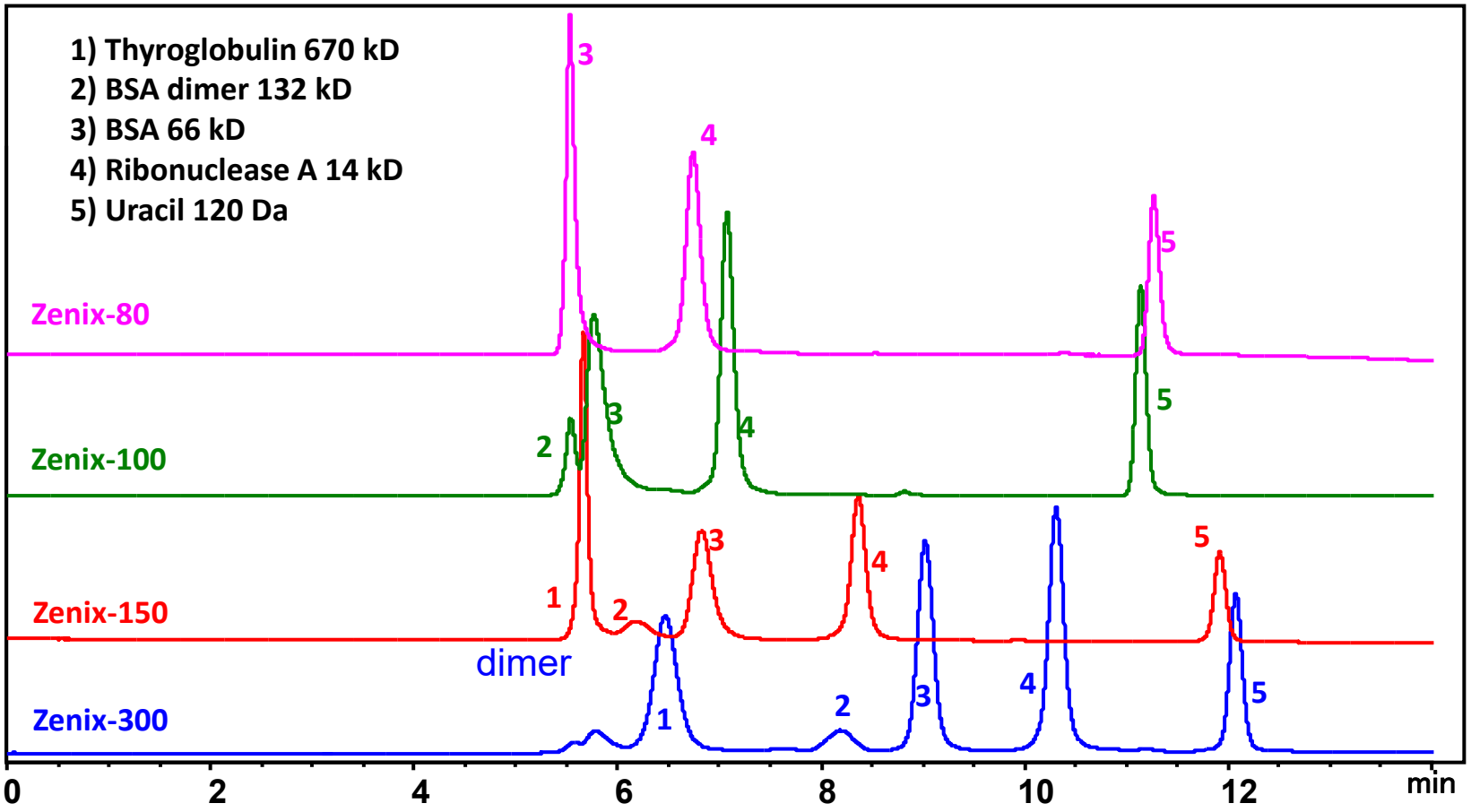
Type	Particle Size (μm)	Pore Size (Å)	Protein Separation range	Water-soluble polymer Separation range
SRT SEC-100	5	100	100-100000	500-10000
SRT SEC-150	5	150	500-150000	500-25000
SRT SEC-300	5	300	5000-1250000	1000-100000
SRT SEC-500	5	500	15000-5000000	2500-500000
SRT SEC-1000	5	1000	50000-7500000	5000-1500000
SRT SEC-2000	5	2000	>10000000	50000-〉2500000
Zenix SEC-80	3	80	< 10,000	500-5000
Zenix SEC-100	3	100	100-100,000	500-10000
Zenix SEC-150	3	150	500-150,000	500-25000
Zenix SEC-300	3	300	5,000-1,250,000	1000-100000

Protein standard separation on Zenix

Column: Zenix-SEC (3 μm, 80, 100, 150, 300 Å, 7.8 x 300 mm each)

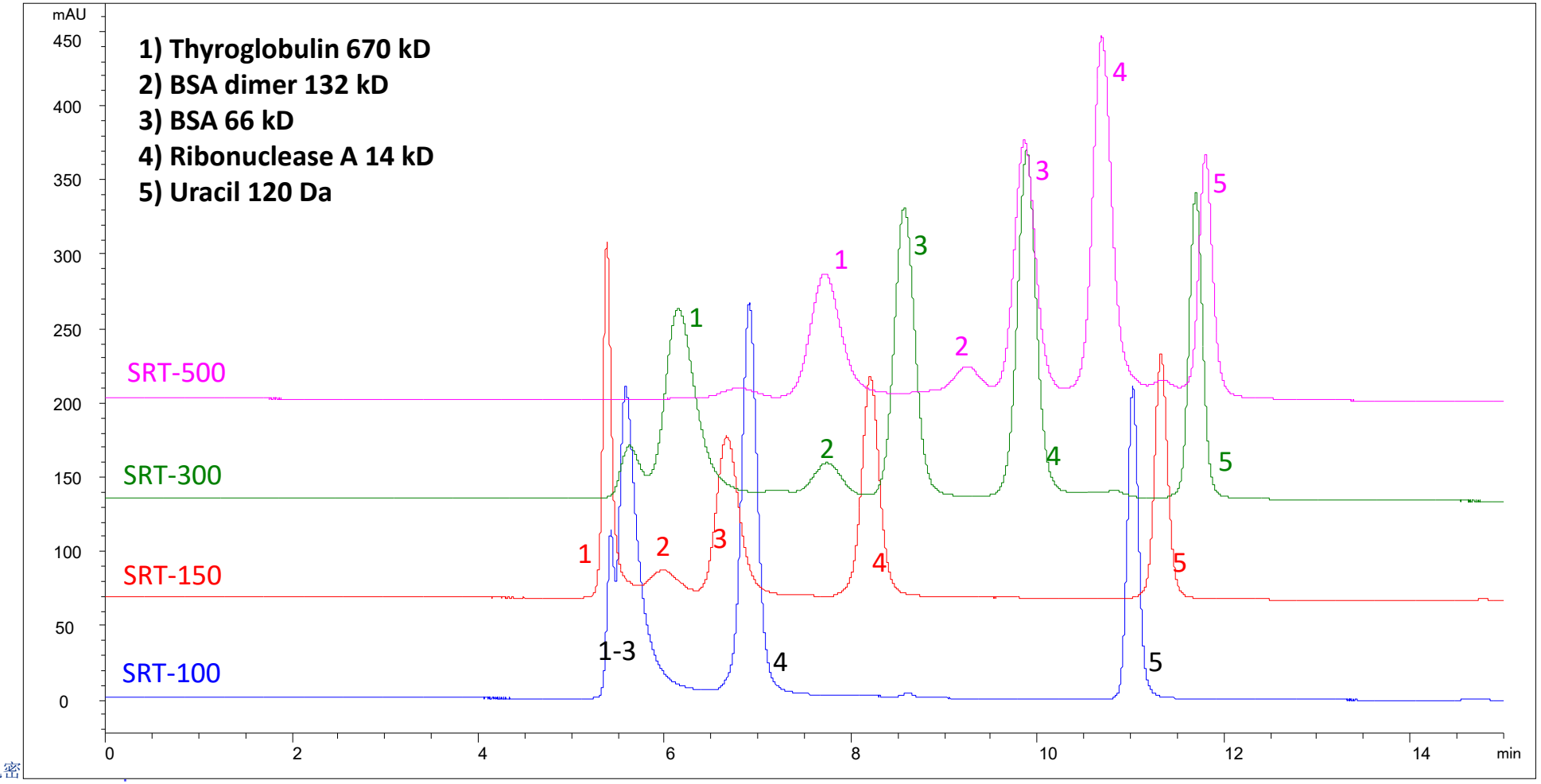
Mobile phase: 150 mM Phosphate buffer, pH 7.0

Flow rate: 1.0 mL/min Detector: UV 214 nm Column temperature: 25 ℃ Injection volume: 5 μL



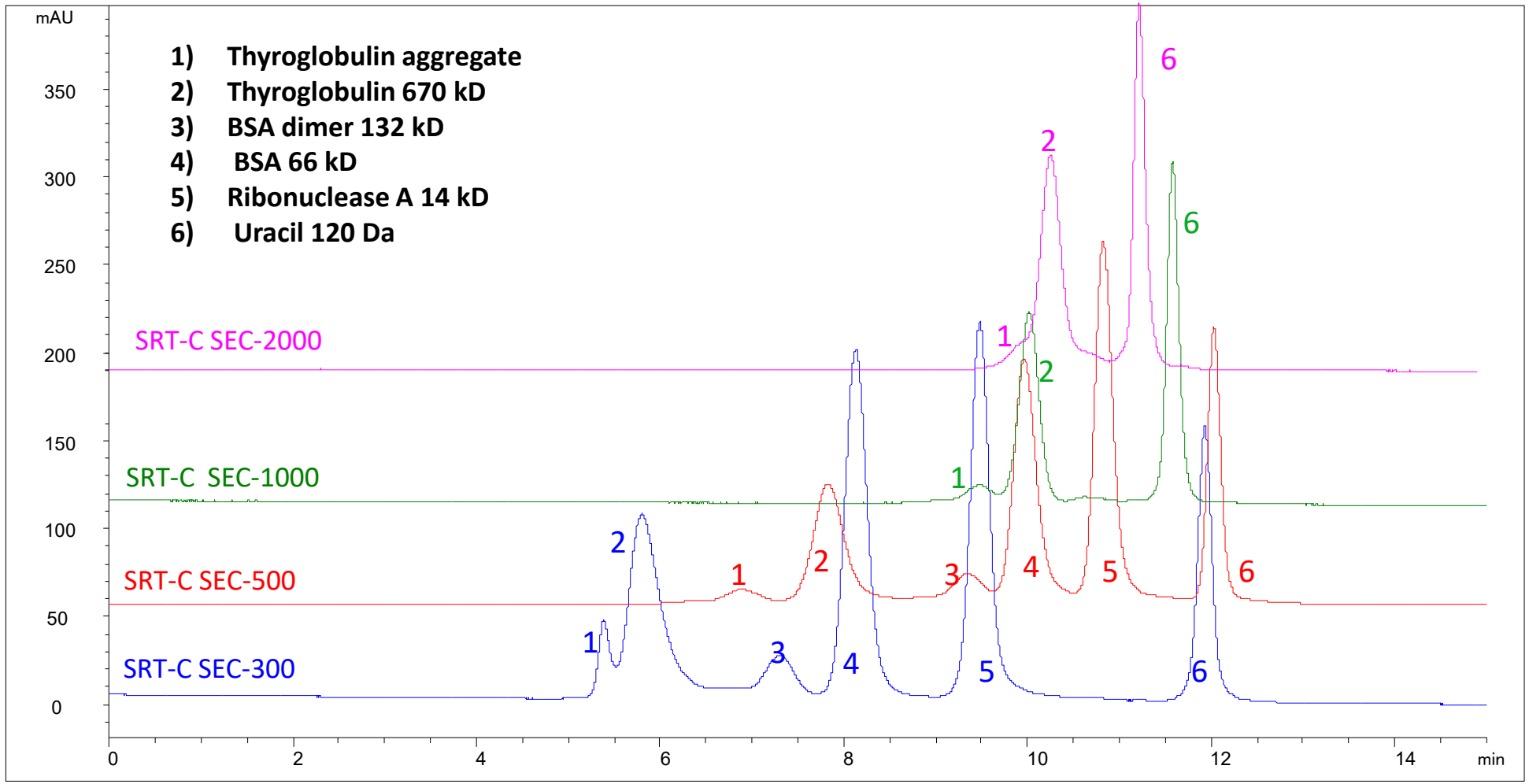
Protein standard separation on SRT SEC

Column: SRT-SEC (5 µm, 100, 150, 300, 500 Å, 7.8 x 300 mm each)
Mobile phase: 150 mM Phosphate buffer, pH 7.0
Flow rate: 1.0 mL/min; Detector: UV 214 nm; Column temperature: 25 ℃
Injection volume: 5 µL



Protein standard separation on SRT-C

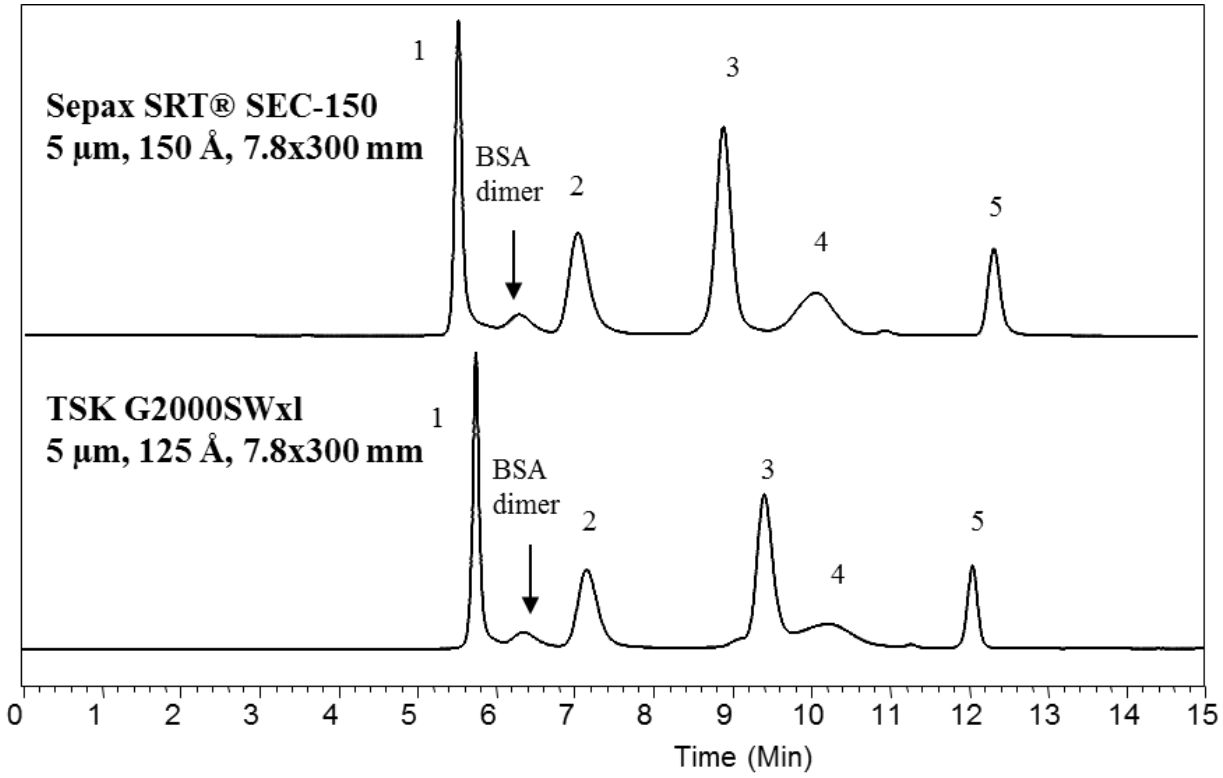
Column: SRT-C -SEC (5 μm, 300, 500, 1000, 2000 Å, 7.8 x 300 mm each)
Mobile phase: 150 mM Phosphate buffer, pH 7.0
Flow rate: 1.0 mL/min; Detector: UV 214 nm; Column temperature: 25 ℃
Injection volume: 5 μL

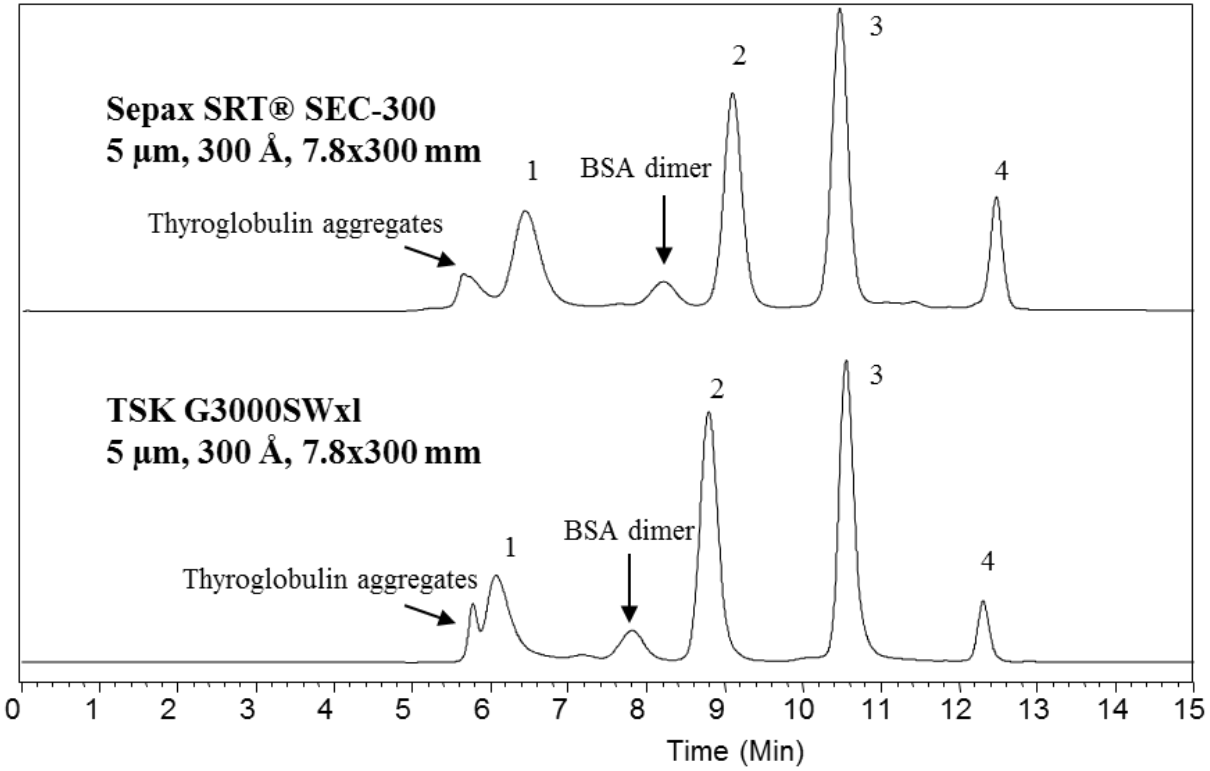


SEC columns comparison

Comparison of chromatograms of Sepax SRT and Tosoh TSK SWxl on protein mixture

1) Thyroglobulin, 2) BSA monomer, 3) Ribonuclease A, 4) Poly-DL-alanine, 5) Uracil





SEC column applications

1.Protein	SRT/-C，Zenix/-C，Unix/-C，Nanofilm
2.Viral vector	SRT 500，1000，2000
3.Antibody	SRT/Zenix/Unix 300
4.Nucleic acid	SRT/-C，Zenix/-C
5. Heparin/ polypeptide	SRT/Zenix 100、150、300
6.Cephalosporins	Mono GPC 50~100（Liposoluble）
7.Polymers	SRT/Zenix（water） Mono GPC（Liposoluble）
8.Lipids	Mono GPC 50，100，150，300

Selection based on two factors:

1.Solubility

2.Mocular weight

SEC method development

- Recommended mobile phase: phosphate, acetate, acetonitrile (TFA) system

  Common UV detection wavelengths： 214nm、280nm、260nm

- Recommended test methods for easily adsorbed samples：

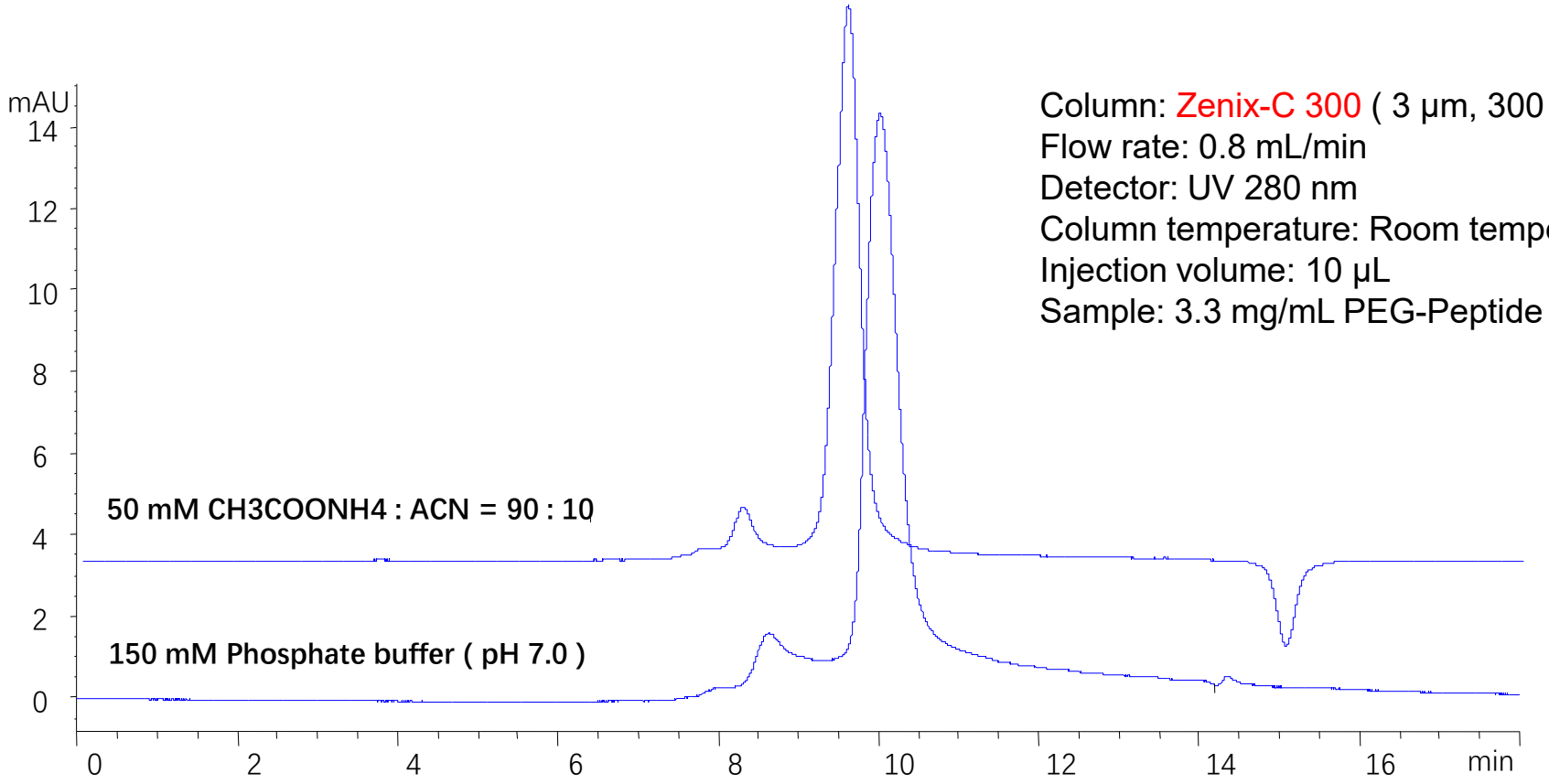
  High salt mobile phase： NaCl, Na_2SO_4, $NaClO_4$

  Adding modifiers: acetonitrile, isopropanol, guanidine hydrochloride

  Select Zenix-C series: to minimize the adsorption between protein and matrix

PEG modified polypeptide

SEC analysis

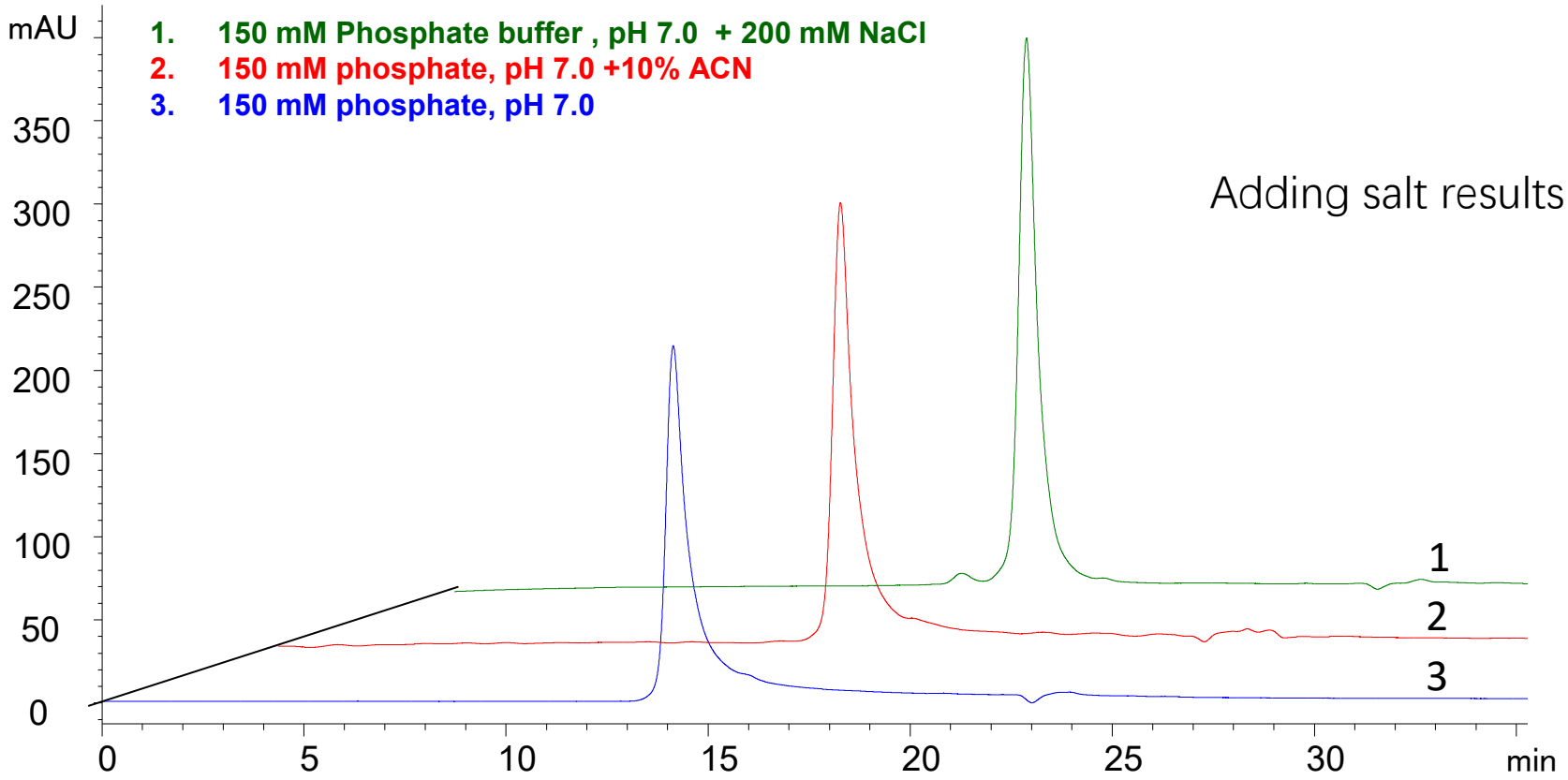


Column: Zenix-C 300 (3 μm, 300 Å, 7.8 x 300 mm)
Flow rate: 0.8 mL/min
Detector: UV 280 nm
Column temperature: Room temperature
Injection volume: 10 μL
Sample: 3.3 mg/mL PEG-Peptide in water

* Adding appropriate proportion of organic solvents to the mobile phase can reduce the secondary interaction between PEG modified proteins and the matrix, it will improve the peak shape.

Effect of Mobile Phase on the Separation of Fusion Proteins

Column: Zenix-C SEC-300 (3 µm, 300 Å, 7.8 x 300 mm)
Mobile phase: as indication
Flow rate: 0.5 mL/min; Detector: UV 214 nm; Column temperature: 25 ℃;
Injection volume: 10 µL; Samples: 1 mg/mL fusion protein, MW 170 kD, pI 6.8-7.0

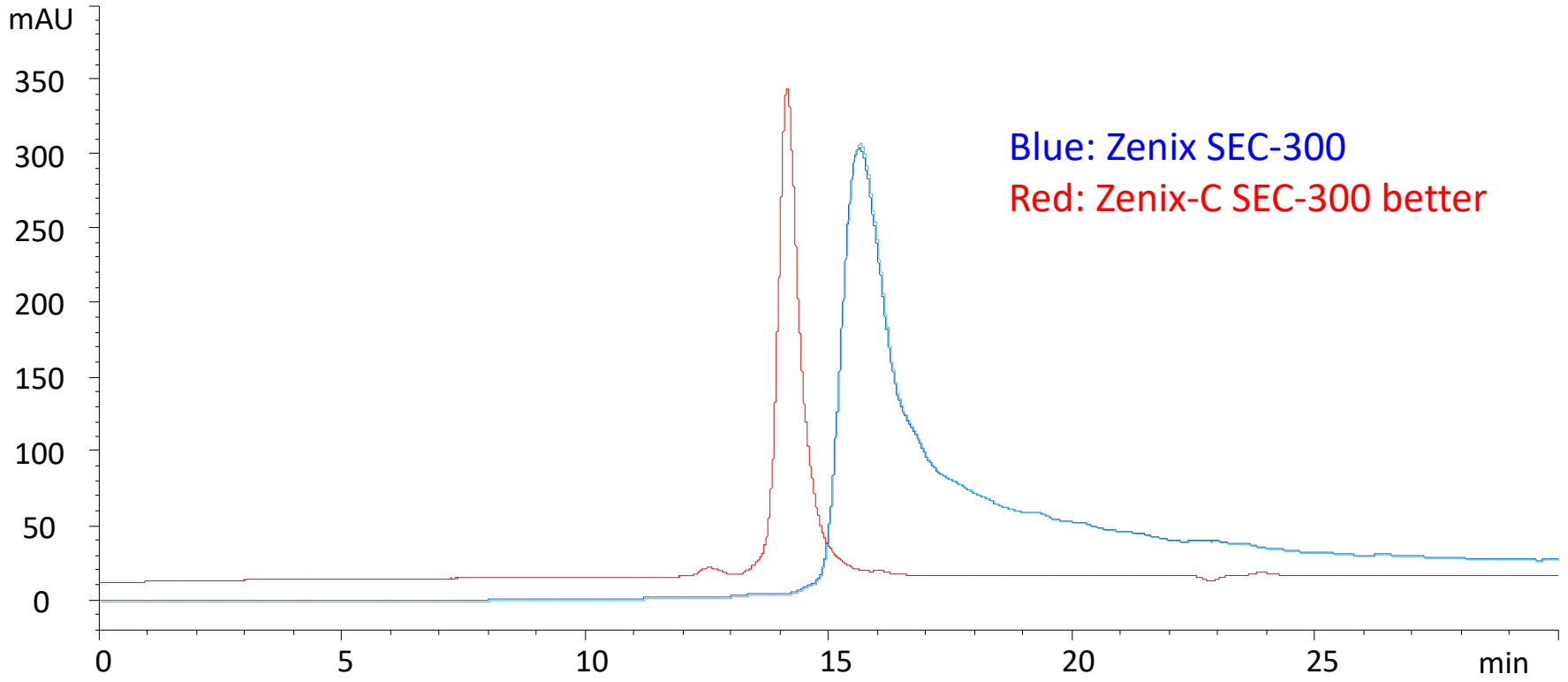


Adding salt results in better resolution

Separation of fusion proteins

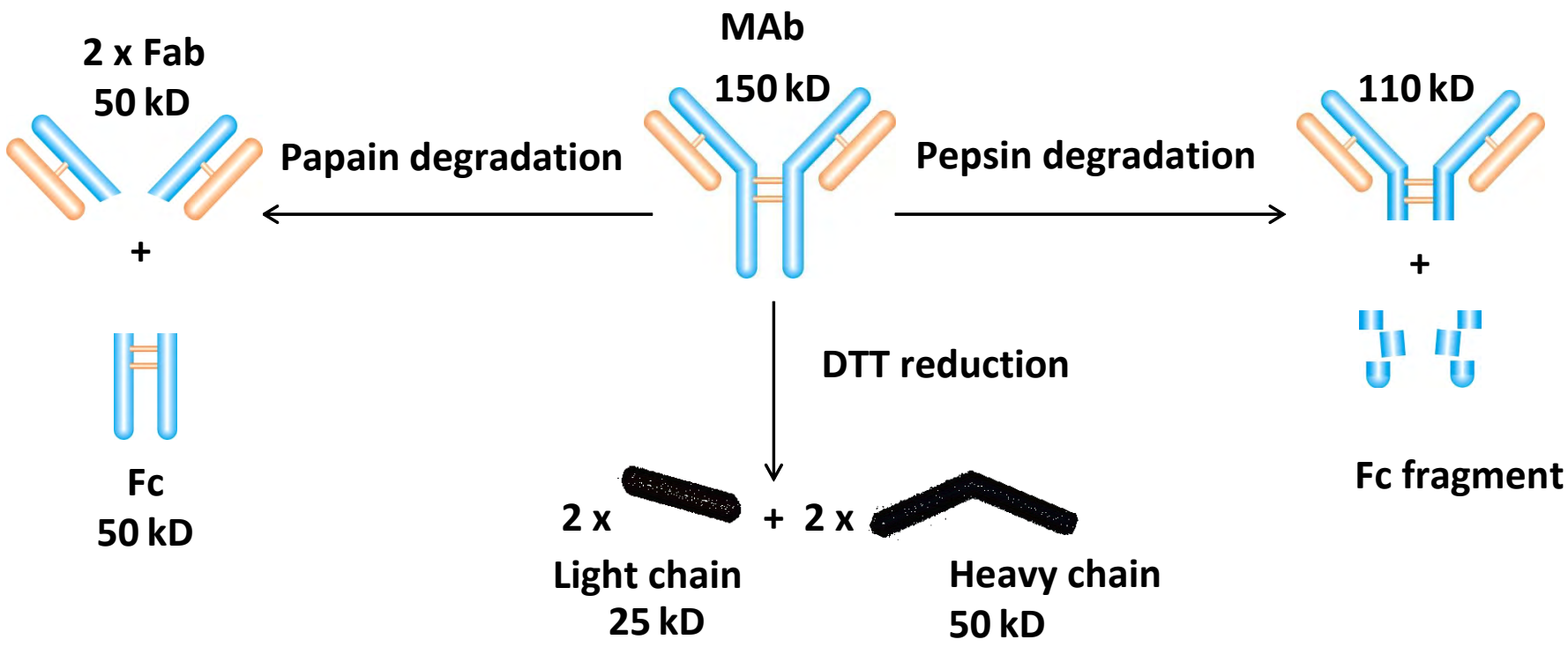
Column selection

Column: Zenix SEC-300, Zenix-C SEC-300 (3 μm, 300 Å, 7.8 x 300 mm)
Mobile phase: 150 mM Phosphate buffer (pH 7.0) + 200 mM NaCl;
Flow rate: 0.5 mL/min; Detector: UV 214 nm; Column temperature: 25 ℃;
Injection volume: 10 μL; Samples: 1 mg/mL fusion protein, MW 170 kD, pI 6.8-7.0



Antibody fragment analysis

Zenix-300 (3 mm, 300 Å)—SEC-MS

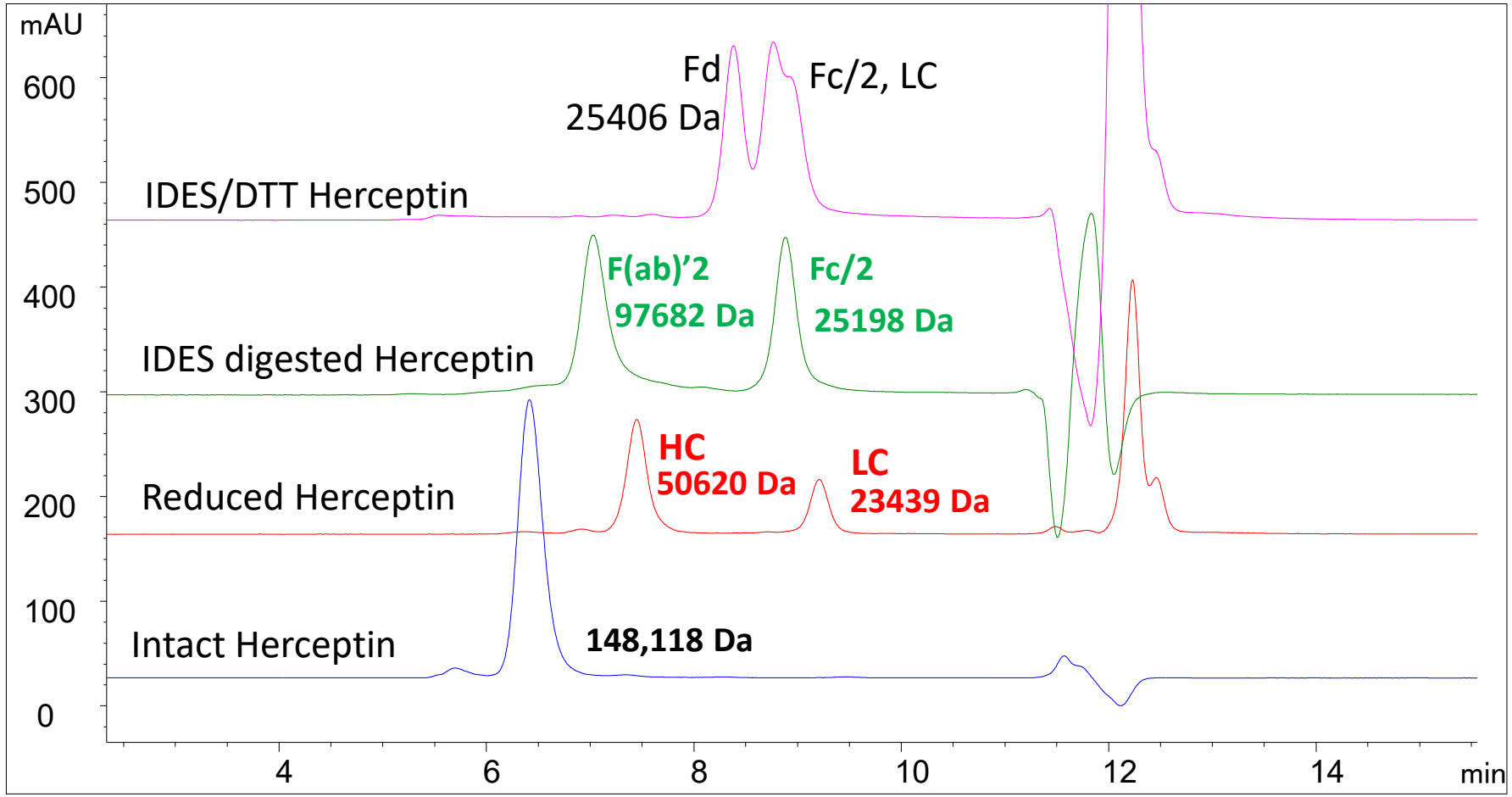


MAb degradation products

Herceptin and fragment Analysis on Zenix-C

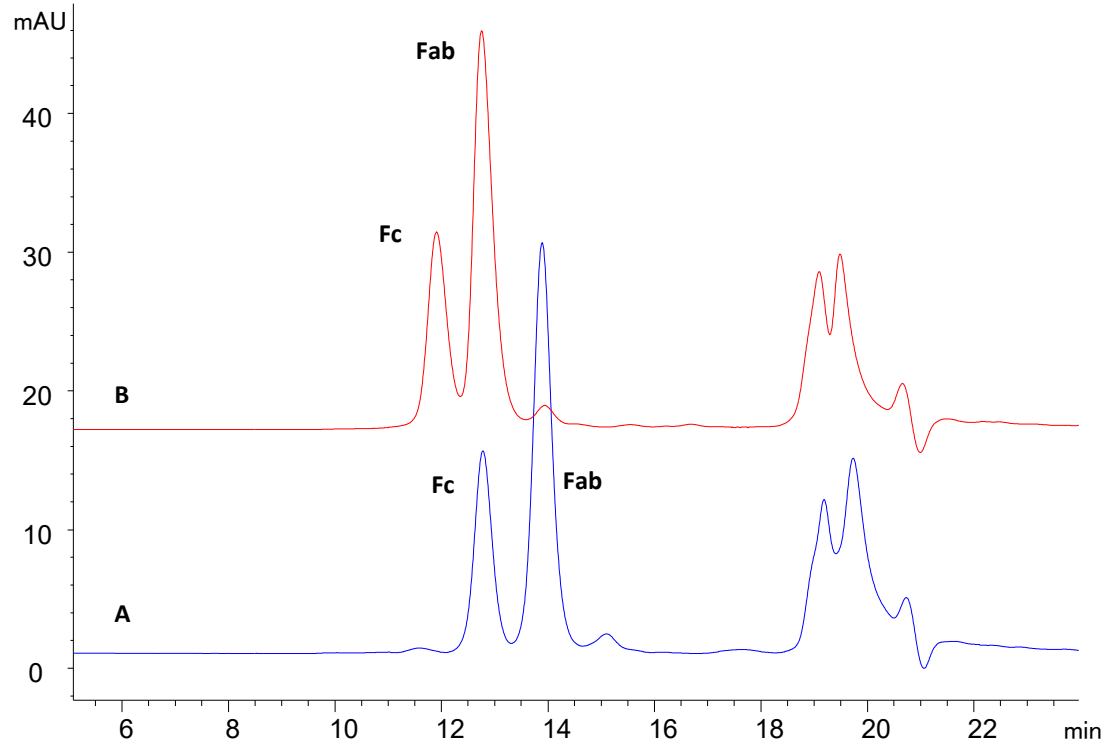
0.1% TFA, 0.1% formic acid and 20% ACN

Column: Zenix-C SEC-300 (3 µm, 300 Å, 4.6 x 300 mm)
Mobile phase: 0.1% TFA, 0.1% formic acid and 20% ACN
Flow rate: 0.35 mL/min Detector: UV 280 nm Column temperature: 25 ℃
Injection volume: 2 µg for each sample



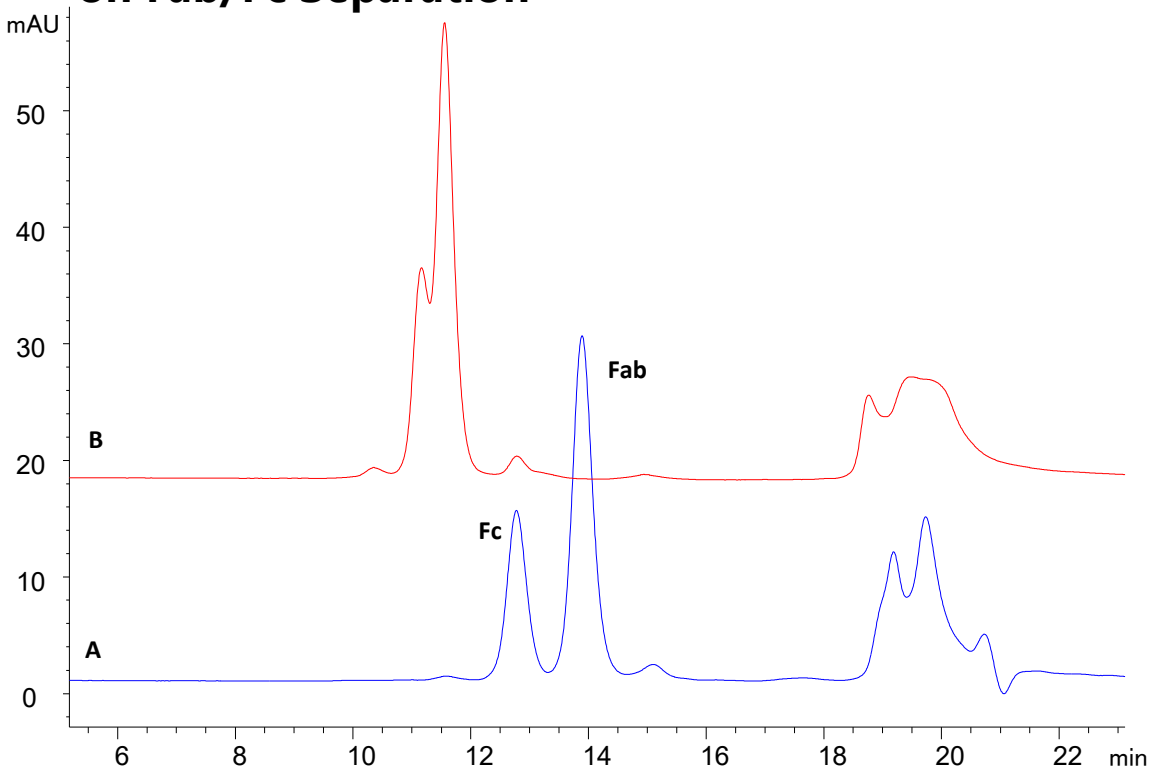
mAb fragment——Fab/Fc

Effect of TFA addition on separation under volatile mobile phase conditions



A. 0.02% TFA, 1% formic acid, 20% ACN
B. 1% formic acid, 20% ACN

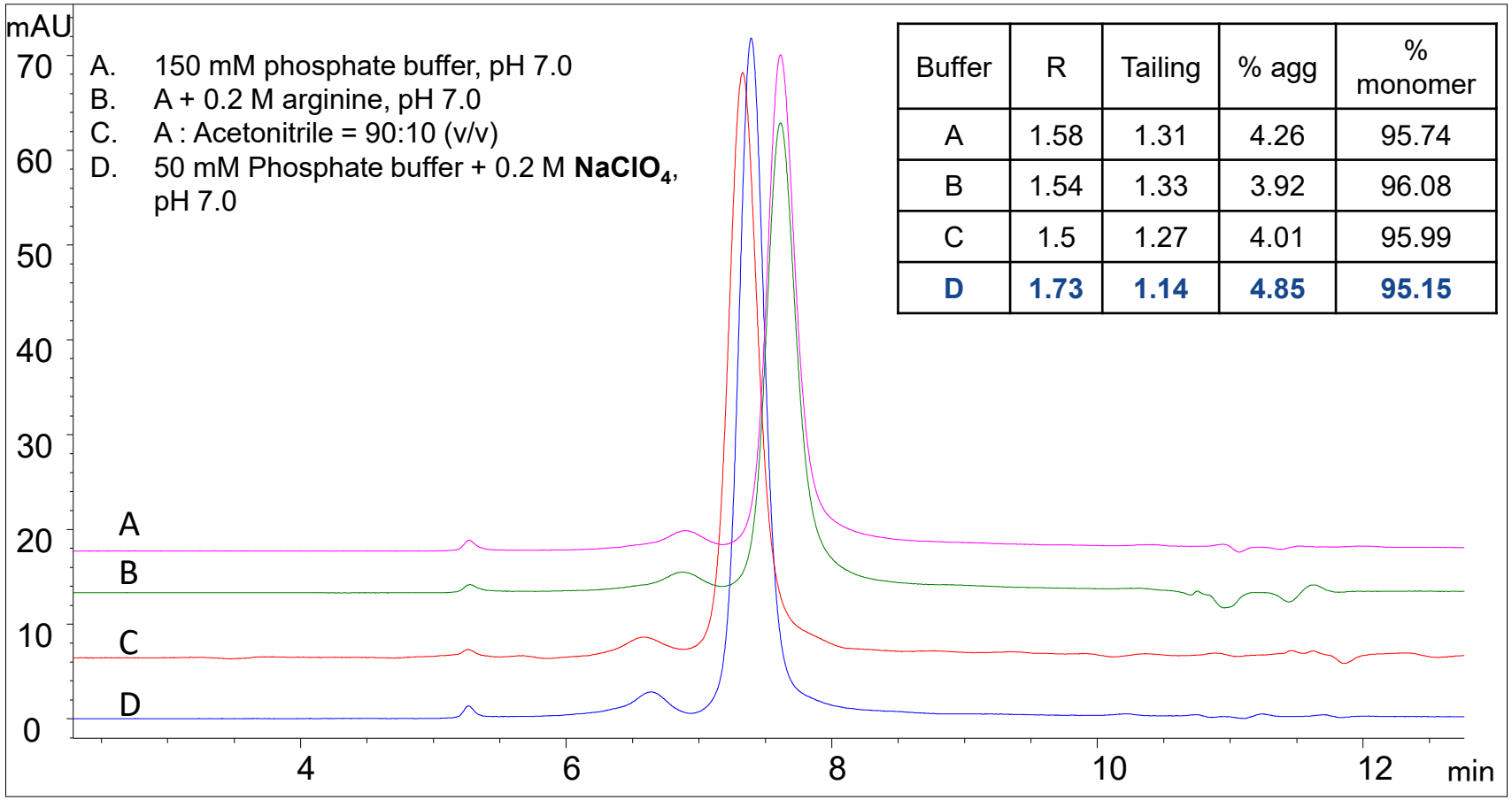
Effect of Acetonitrile Concentration on Fab/Fc Separation



A. 0.02% TFA, 1% formic acid, 20% ACN
B. 0.02% TFA, 1% formic acid, 50% ACN

Antibody drug conjugate Analysis on Zenix-C

Column: Zenix-C SEC-300 (3 µm, 300 Å, 7.8 x 300 mm)
Mobile phase: As indicated
Flow rate: 1 mL/min Detector: UV 280 nm Column temperature: 25 ℃
Injection volume: 20 µL Samples: 1.68 mg/mL ADC

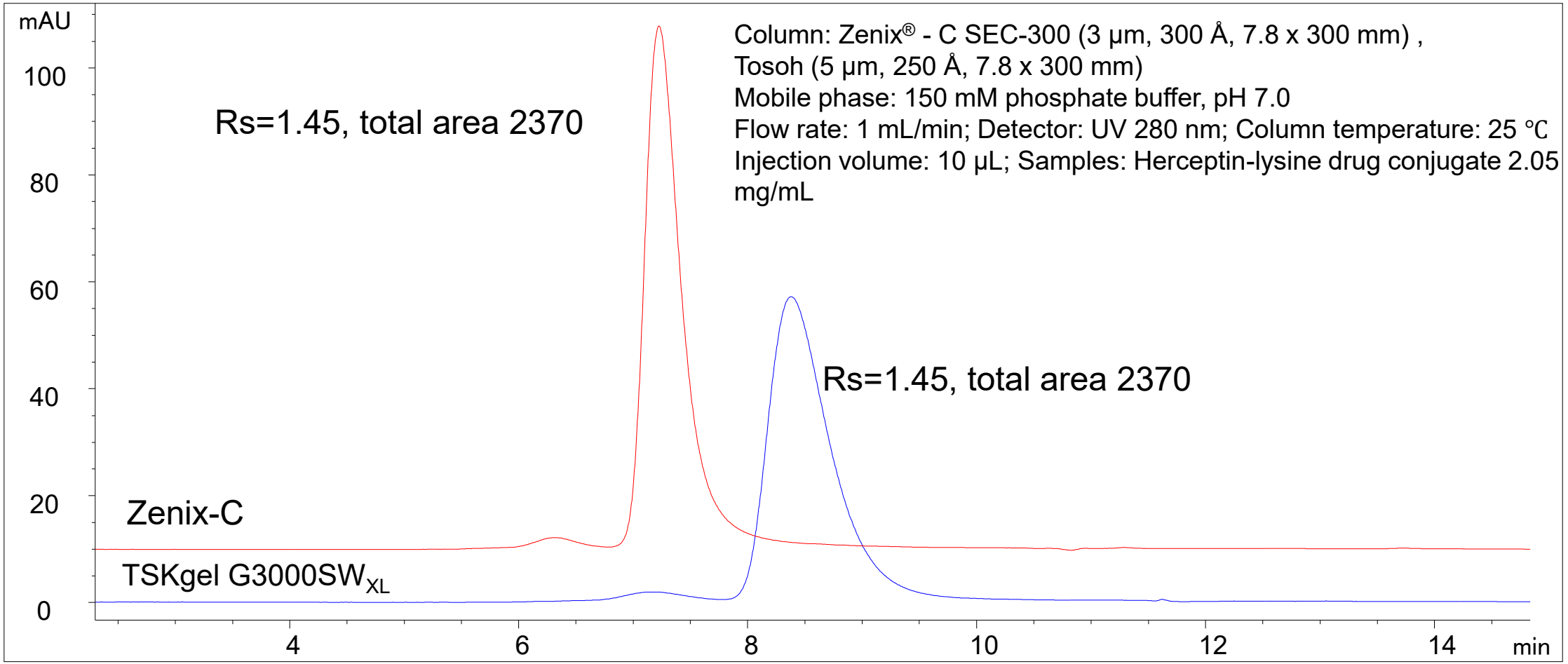


Buffer	R	Tailing	% agg	% monomer
A	1.58	1.31	4.26	95.74
B	1.54	1.33	3.92	96.08
C	1.5	1.27	4.01	95.99
D	**1.73**	**1.14**	**4.85**	**95.15**

With 10% acetonitrile and 200 mM $NaClO_4$, total protein recovery, resolution and tailing factor of monomer peak are improved.

Herceptin-Lysine Drug Conjugate Analysis

Zenix-C SEC-300 vs TSKgel G3000SWXL



TSKgel and Tosoh Bioscience are registered trademarks of Tosoh Corporation.
Comparative separations my not be representative of all applications.

MM SEC Chromatography to analysis Bispecific Antibodies

analytical chemistry

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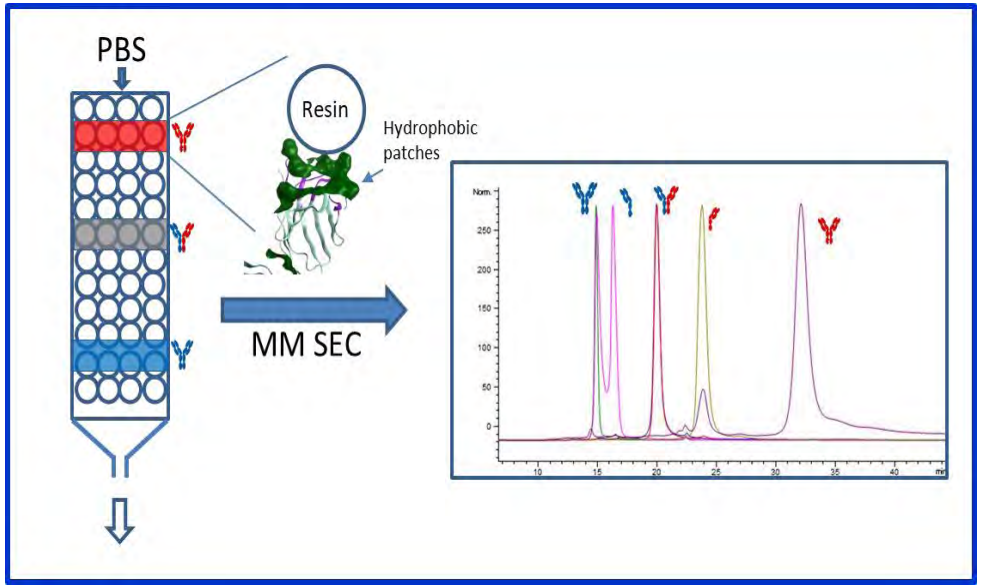
Article

Characterization of bispecific antibody production in cell cultures by unique mixed mode size exclusion chromatography

Haitao Jiang, Wei Xu, Ren Liu, Balrina Gupta, Bruce Kilgore, Zhimei Du, and Xiaoyu Yang

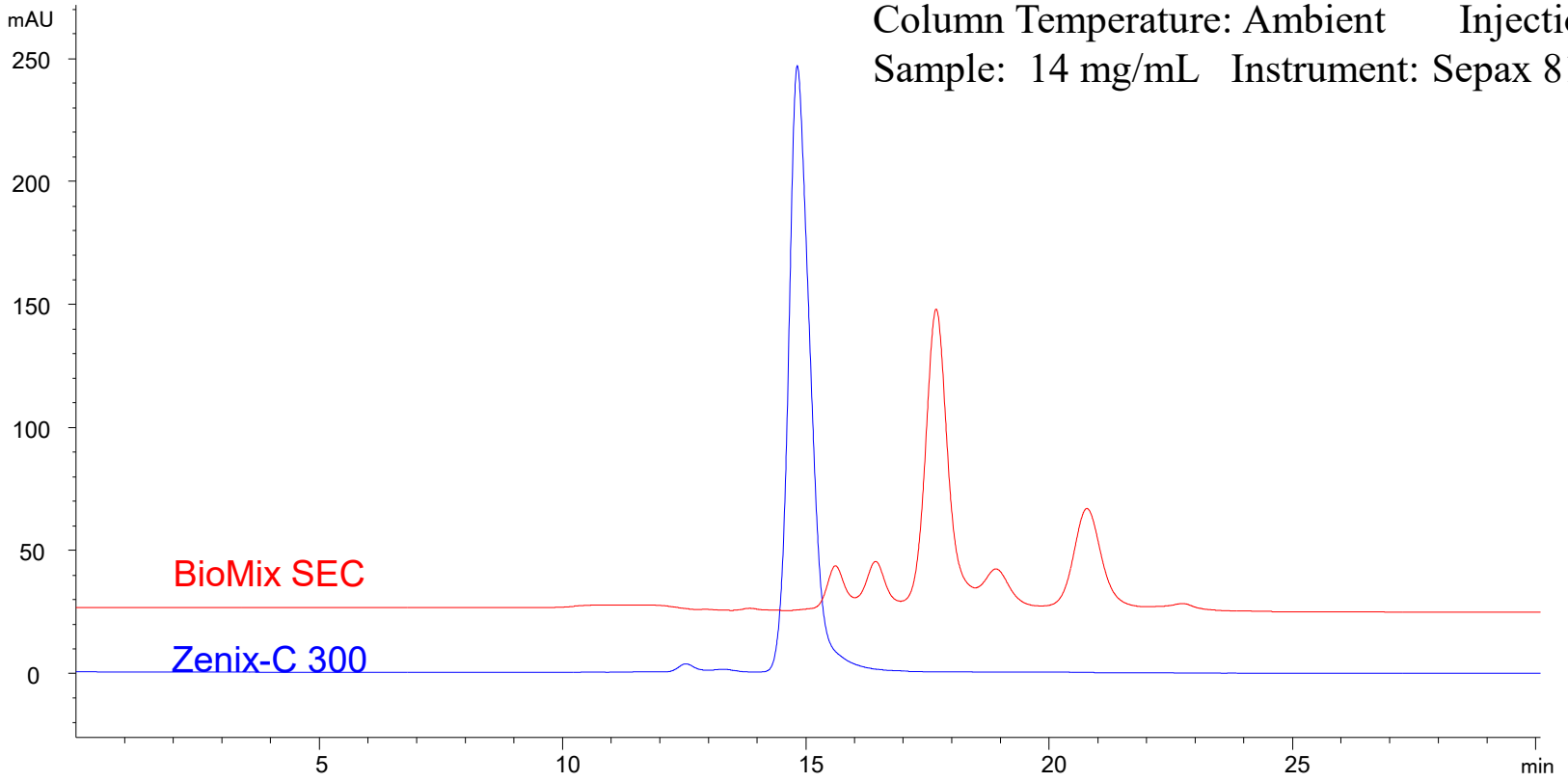
Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.0c01641 • Publication Date (Web): 04 Jun 2020



Mixed mode size exclusion chromatography coupled with multi-angle light scattering (MM SEC-MALS).
The MM SEC chromatography under native conditions was performed on Sepax BioMix™ SEC-300, 3 um, 7.8x300mm (P/N: 214300-7830) at 25 oC.

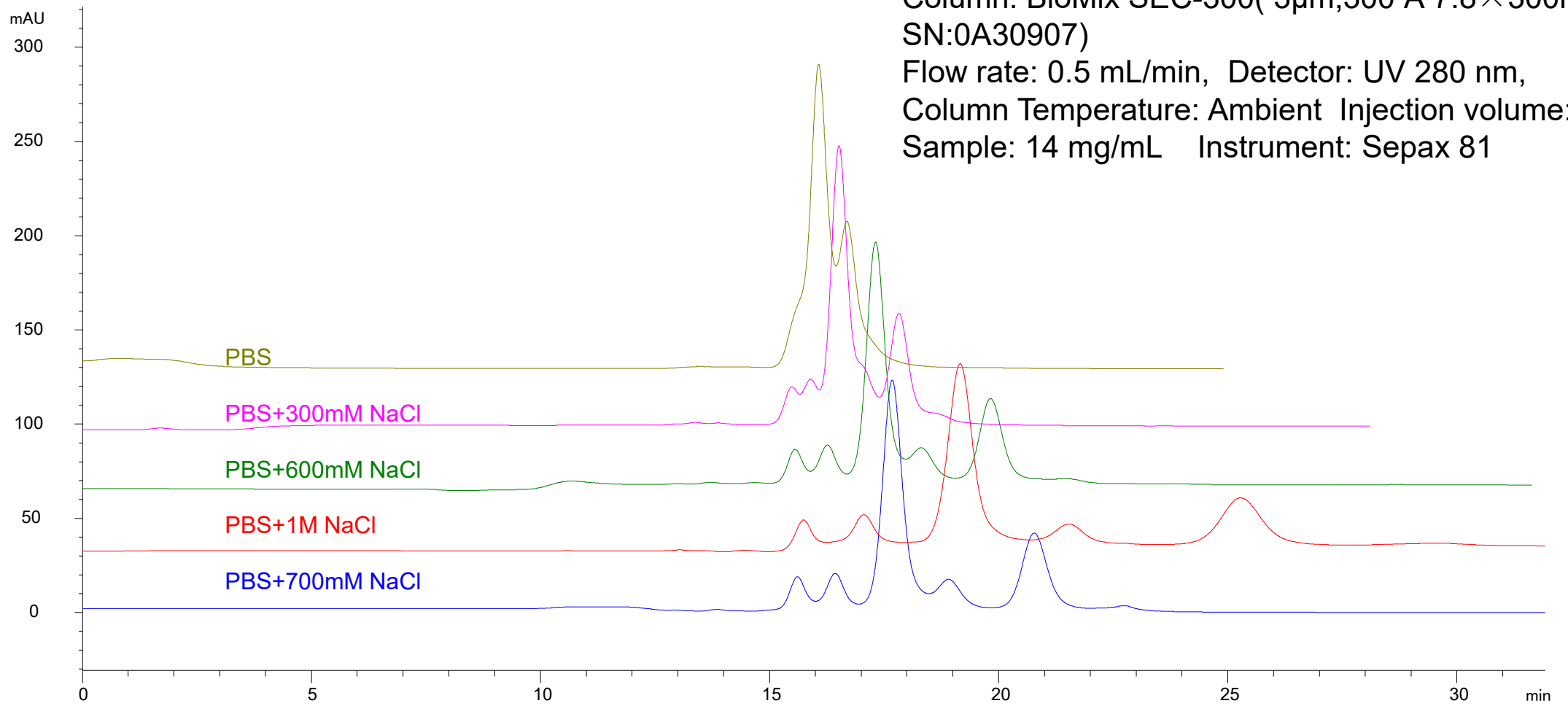
BsAb analysis--BioMix

Column: BioMix SEC-300(3μm,300 Å 7.8×300mm，SN:0A30907)
Column: Zenix-C SEC-300(3μm,300 Å 7.8×300mm，SN:2F38830)
Flow rate: 0.5 mL/min, Detector: UV 280 nm
Column Temperature: Ambient Injection volume: 4 μL
Sample: 14 mg/mL Instrument: Sepax 81



Effect of different salt conc. on BsAb analysis

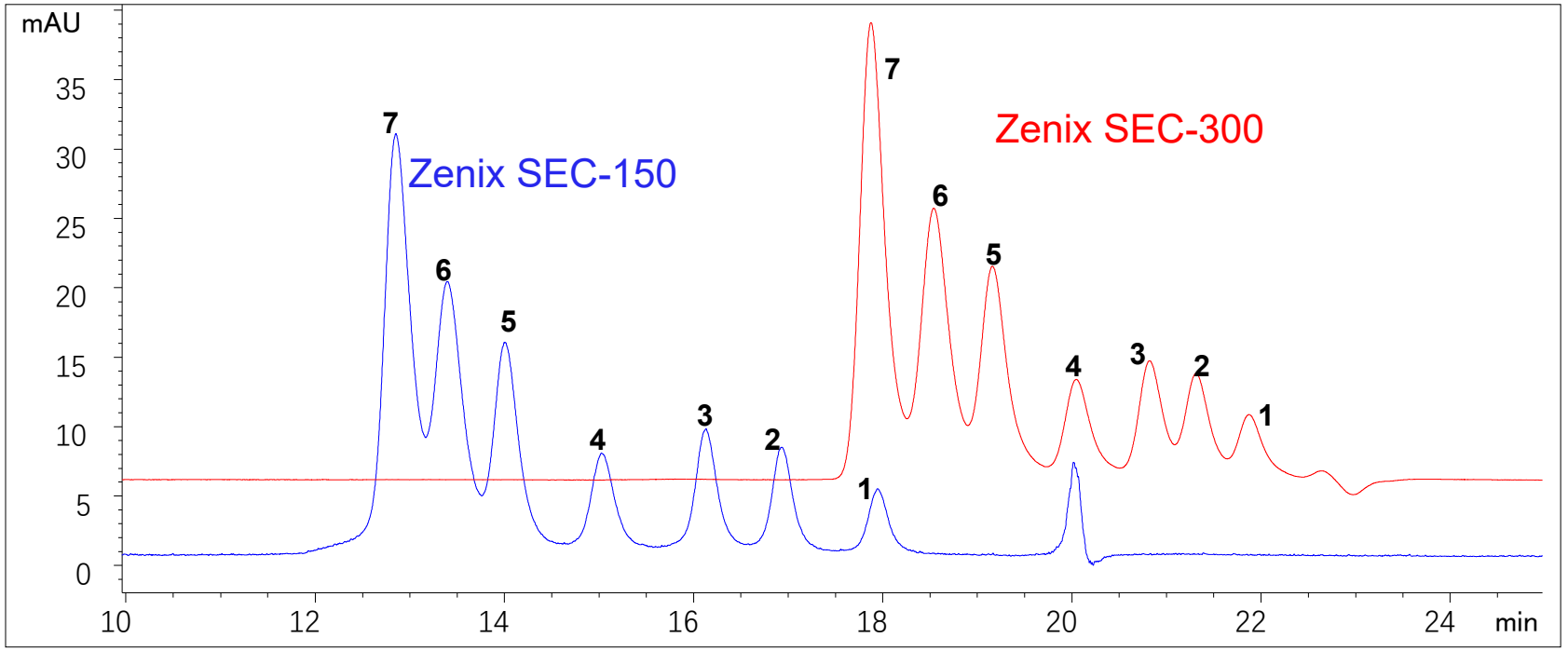
Column: BioMix SEC-300(3μm,300 Å 7.8×300mm, SN:0A30907)
Flow rate: 0.5 mL/min, Detector: UV 280 nm,
Column Temperature: Ambient Injection volume: 4 μL
Sample: 14 mg/mL Instrument: Sepax 81



Oligonucleotides separation

Different pore size Zenix SEC-150 vs Zenix SEC-300

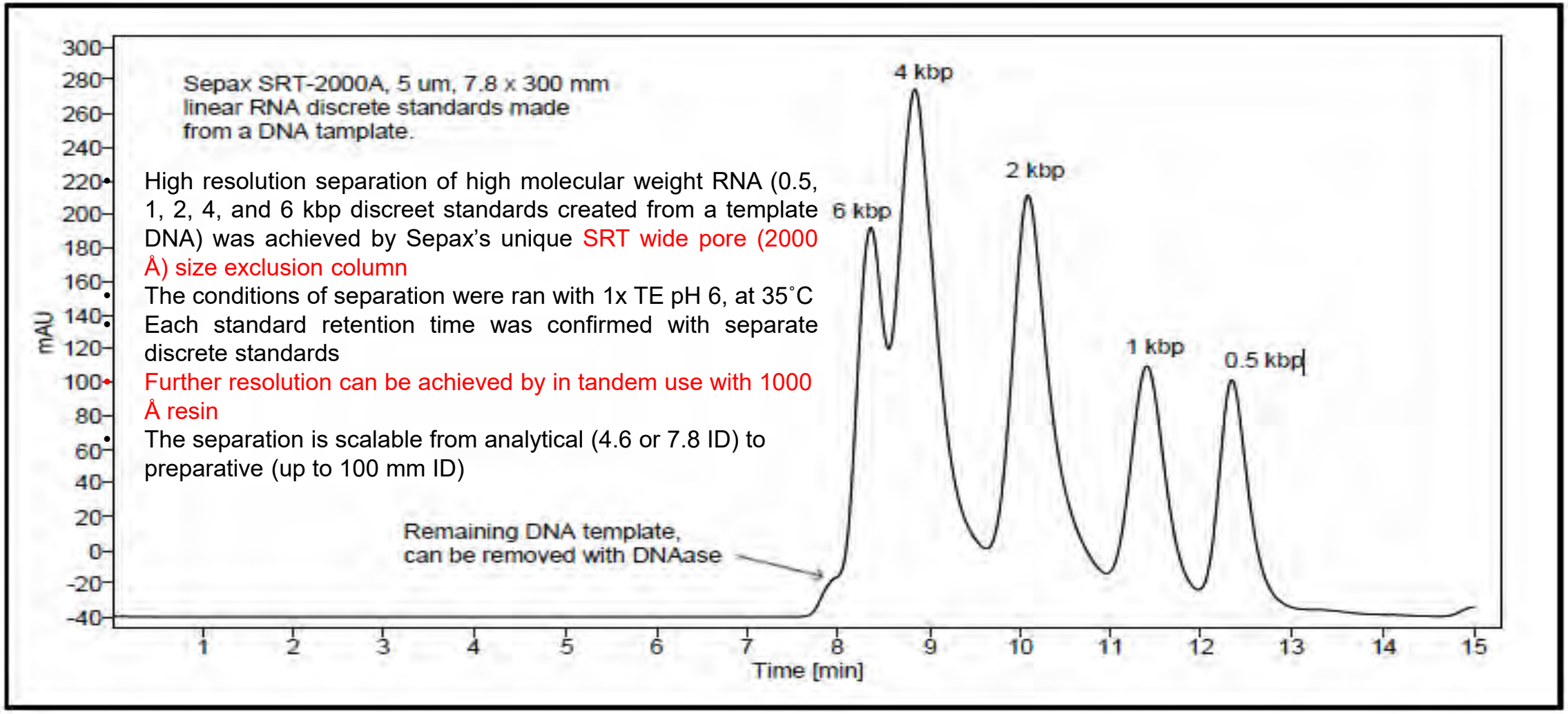
Column: Zenix™ SEC- 300, Zenix™ SEC-150, 7.8 x 300 mm
Mobile phase : 150 mM Phosphate buffer, pH 7; Flow rate: 0.5 mL/min;
Detector: UV 260 nm, Column temperature: 25 ℃; Injection volume: 30 µL; Pressure: 41 bar;
Sample: 1) dA10, 2) dA15, 3) dA20, 5) dA40, 6) dA50, 7) dA60,0.1 µM each in water
4) 5'-ATATCTACACGGCTACCCGTACCAATGCTGCTTCC-3' (35 nt)



300 Å gives better resolution for longer oligonucleotides (>30 nt), 150 Å separates poly dAs with baseline resolution for shorter oligos (<35 nt)

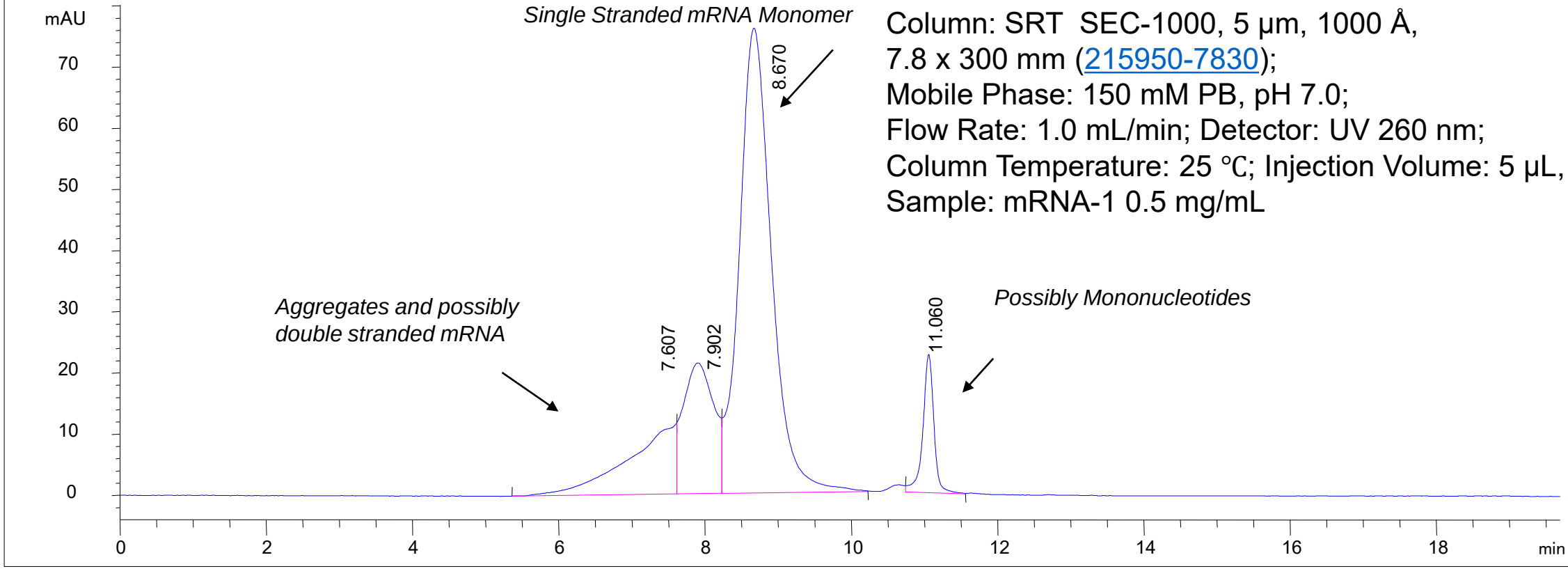
RNA——Wide pore SEC

Separation of RNA on Wide Pore SEC



RNA——Wide pore SEC

mRNA-1 on SRT SEC-1000

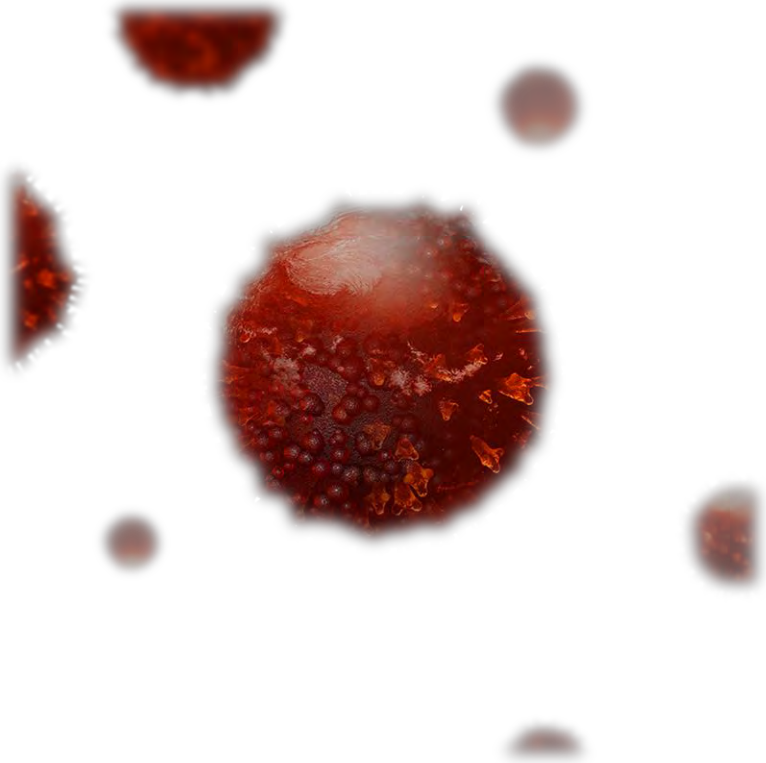


Column: SRT SEC-1000, 5 μm, 1000 Å,
7.8 x 300 mm (215950-7830);
Mobile Phase: 150 mM PB, pH 7.0;
Flow Rate: 1.0 mL/min; Detector: UV 260 nm;
Column Temperature: 25 ℃; Injection Volume: 5 μL,
Sample: mRNA-1 0.5 mg/mL

RT [min]	Height	Area	Area%	Plates	Tailing	Resolution
7.607	11	517.2	13.952	832	0.50	
7.902	21	623.2	16.812		-1.74	
8.670	76	2329.6	62.847	2055	1.23	
11.060	23	236.9	6.390	31503	0.78	4.71

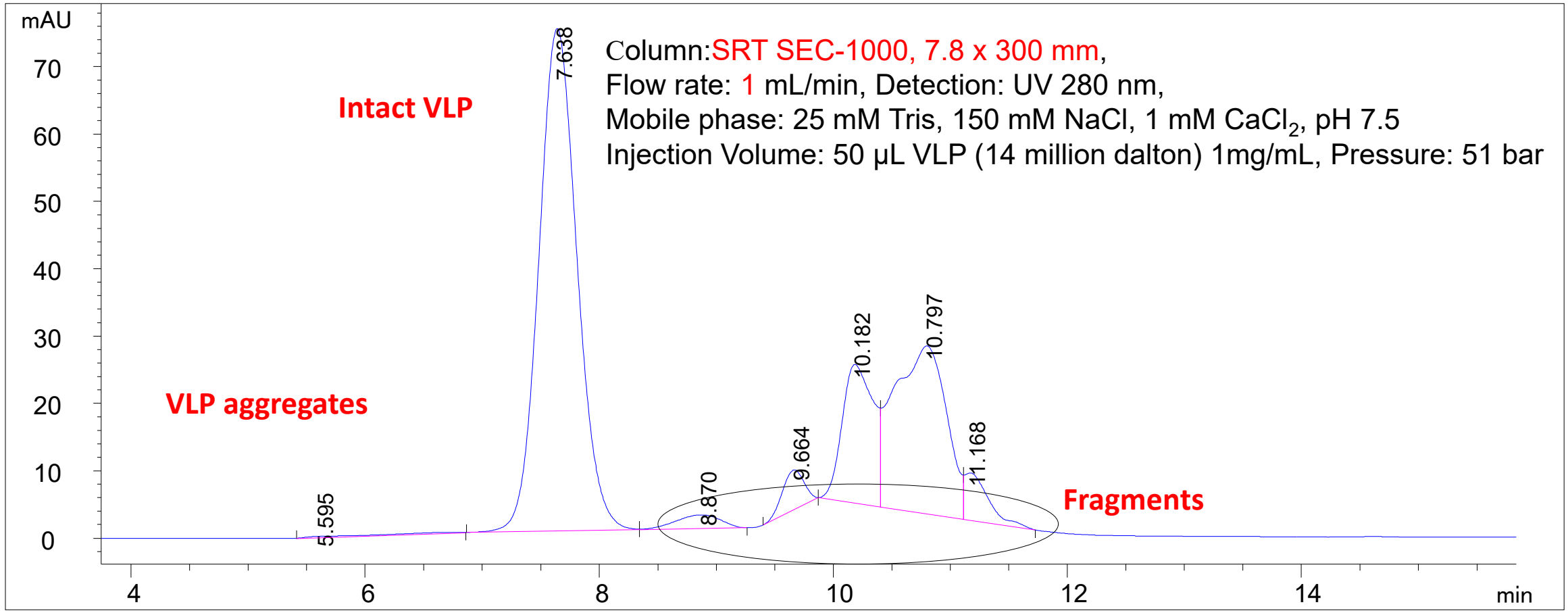
500 Å, 1000 Å, 2000 Å SEC application

- VLP、AAV
- PEGylated Protein (Large PEG > 30 KDa)
- IgM
- Virus
- mRNA



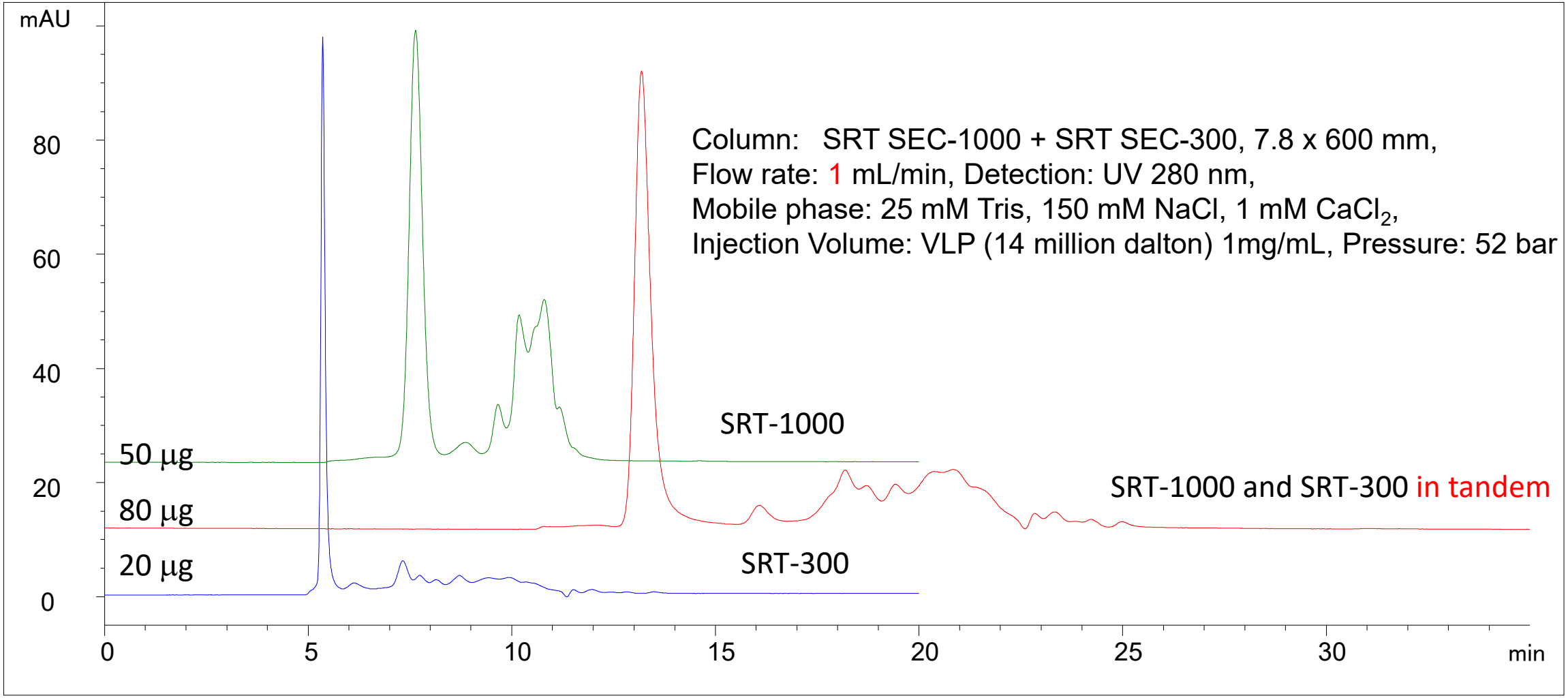
Vaccine and Gene Therapy Field

Virus Like Particle



#	Time	Area	Height	Width	Area%	Symmetry
1	5.595	13.8	2.3E-1	0.9976	0.445	0.145
2	7.638	1692.7	74.6	0.3488	54.702	0.893
3	8.87	54.6	2	0.4019	1.763	1.303
4	9.664	76.3	5.9	0.2052	2.466	1.203
5	10.182	388.1	20.6	0.2757	12.542	0.684
6	10.797	768.3	24.9	0.4397	24.828	1.57
7	11.168	100.7	7	0.2151	3.254	0.279

VLP separation SRT SEC-1000 & SEC-300



Dual Wavelength Detection of AAV

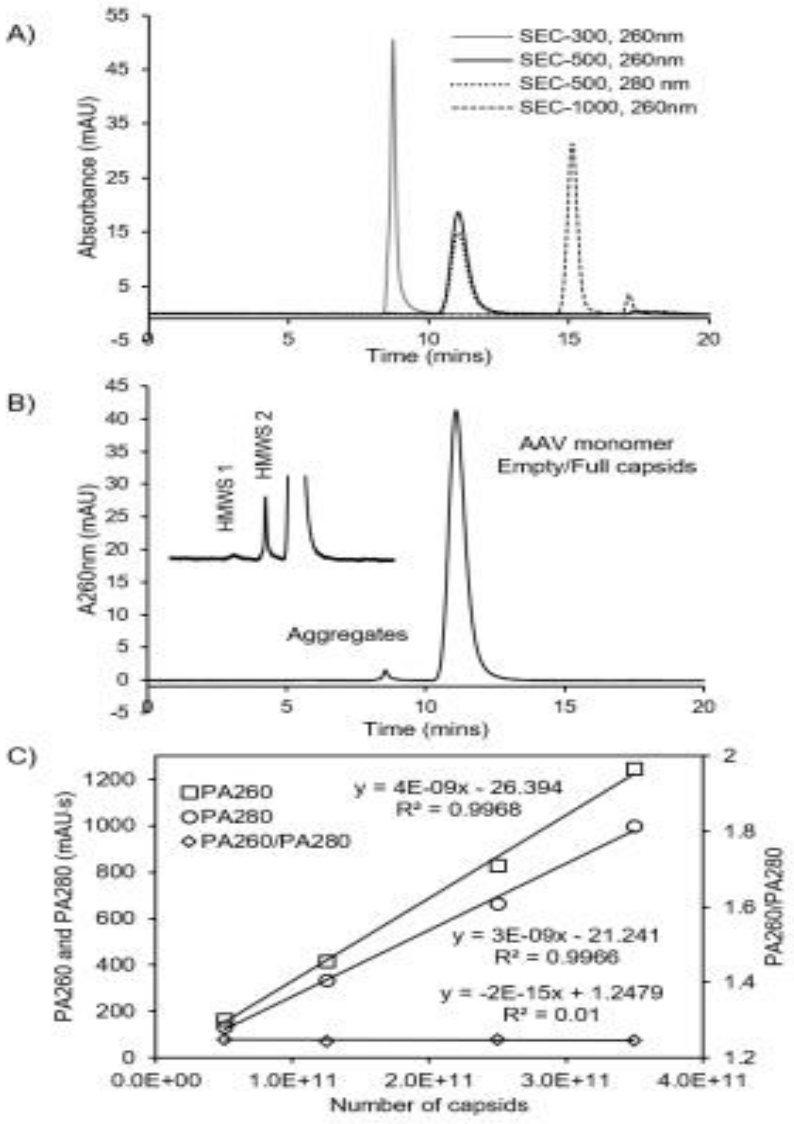
Size Exclusion Chromatography with Dual Wavelength Detection as a Sensitive and Accurate Method for Determining the Empty and Full Capsids of Recombinant Adeno-Associated Viral Vectors

He Meng*[1], Michelle Sorrentino[1], Denise Woodcock[2], Catherine O'Riordan[2], Vijender Dhawan[1], Marc Verhagen[1], and Claire Davies[1]

Column	SRT SEC (300, 500, 1000A), 7.8 x 50mm & 7.8 x 300mm
Particle Size	5 µm
Sample	AAV5
Mobile Phase	Dulbecco PBS
Flow rate	0.75mL/min
Injection Vol	25µL
Concentration	5.0 x 10^{12} cp/mL
Wavelength	230, 260, 280mm

ratio of ~1.8 pure DNA
ratio of ~2.0 pure RNA

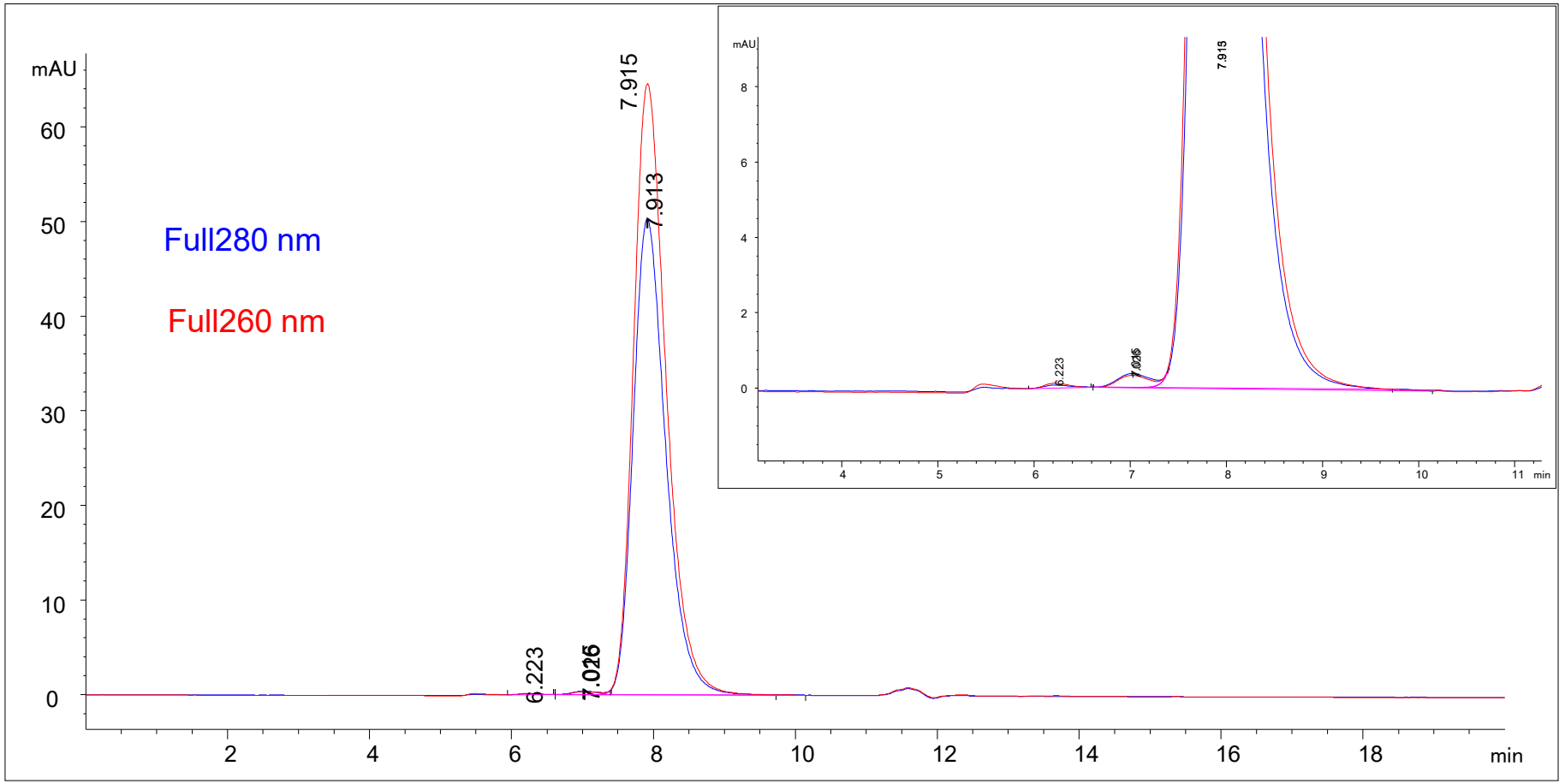
Monomeric heavy capsids ~ 1.3
light capsids had a ratio of ~ 0.6



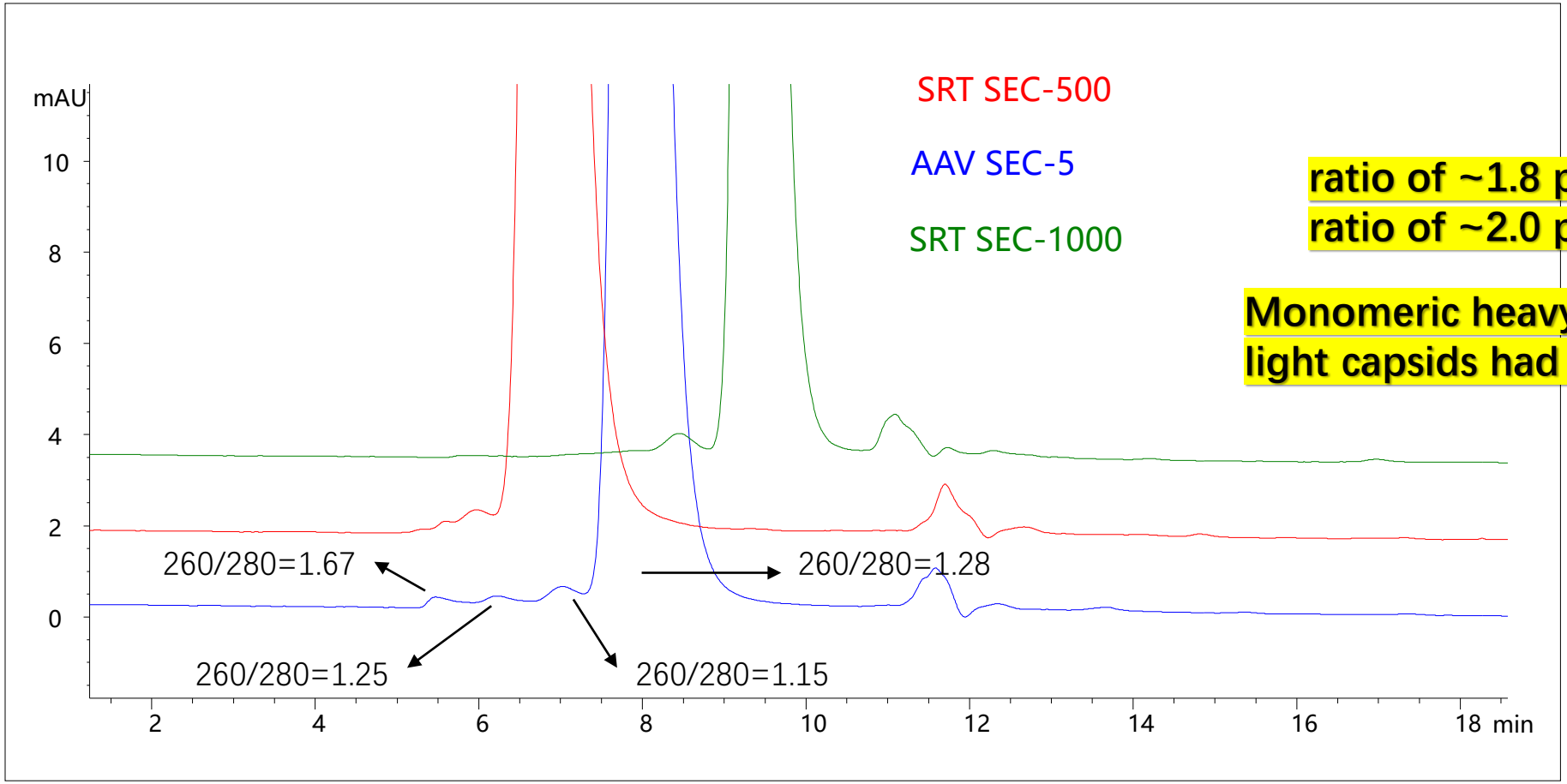
A) Chromatograms of SEC – Pore Size Selection
B) Monomer and aggregate assessed at 260/280nm
C) Curves of A260 and 280nm + ratio of peak area vs. Titer

AAV8 Full Analysis on AAV SEC-5 (4630)

Column: AAV SEC-5 (5μm, 4.6x300mm)
Mobile Phase: A: 1.8X DPBS + 10% Ethanol
Flow rate: 0.35mL/min, Column Temperature: 25℃ Column Pressure: 48bar
Injection volume: 20 μL Detector: FLD Excitation 280nm, Emission 330nm; UV 260nm, 280nm
Sample: AAV8 Full Instrument: Sepax 81 Date: 2022-06-13



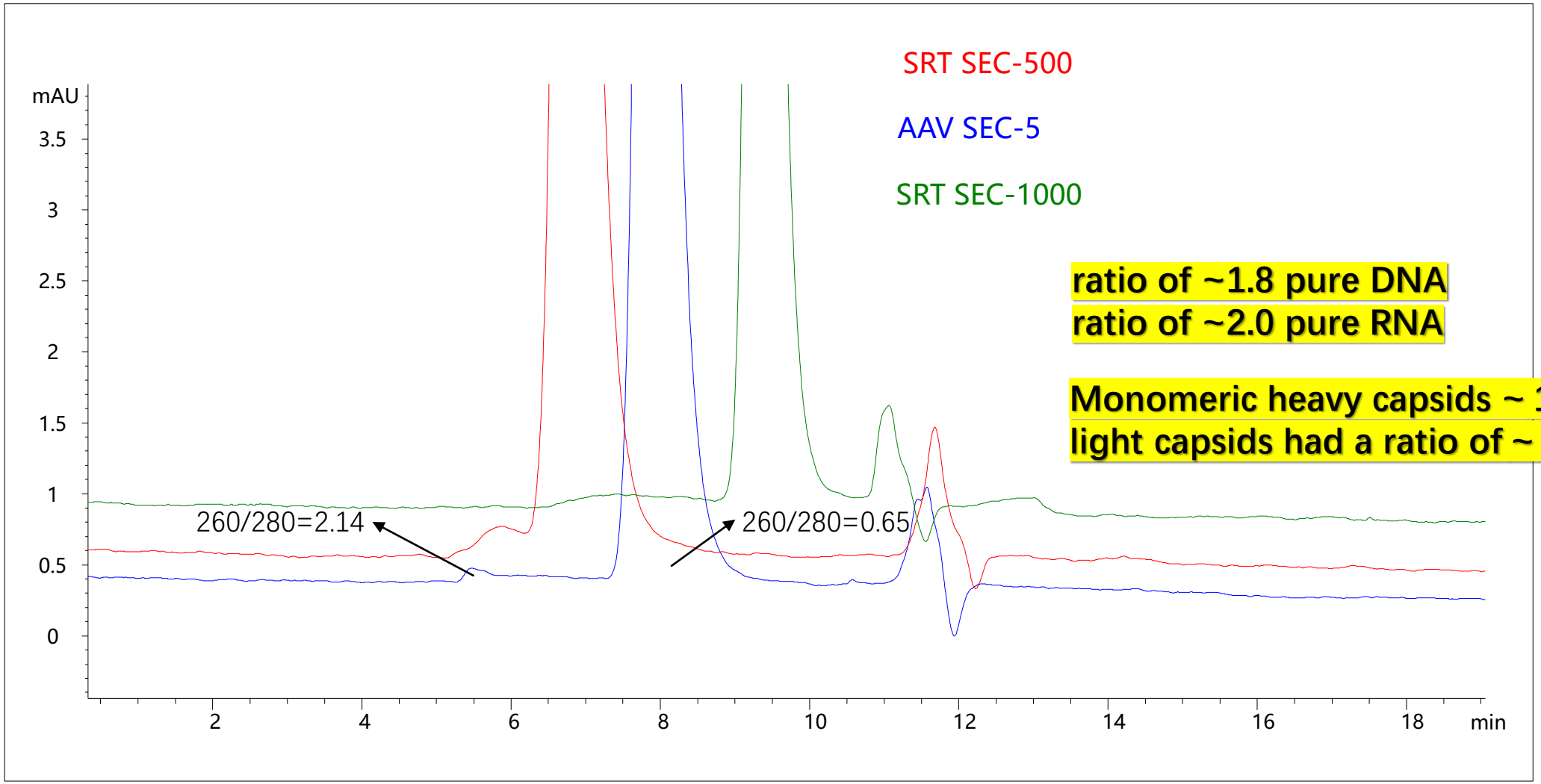
AAV8 full sample overlay (λ =260 nm)



ratio of ~1.8 pure DNA
ratio of ~2.0 pure RNA

Monomeric heavy capsids ~ 1.34
light capsids had a ratio of ~ 0.6

AAV8 Empty overlay （λ =280 nm)



ratio of ~1.8 pure DNA
ratio of ~2.0 pure RNA

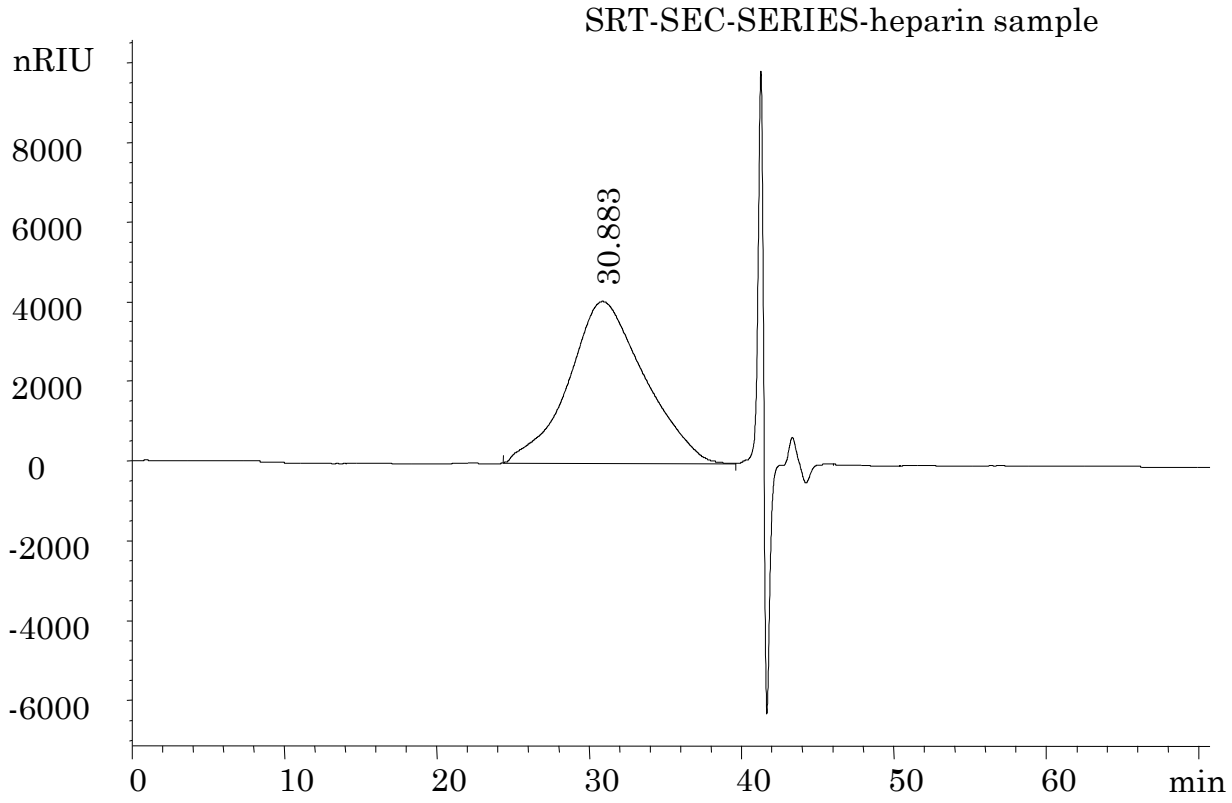
Monomeric heavy capsids ~ 1.34
light capsids had a ratio of ~ 0.6

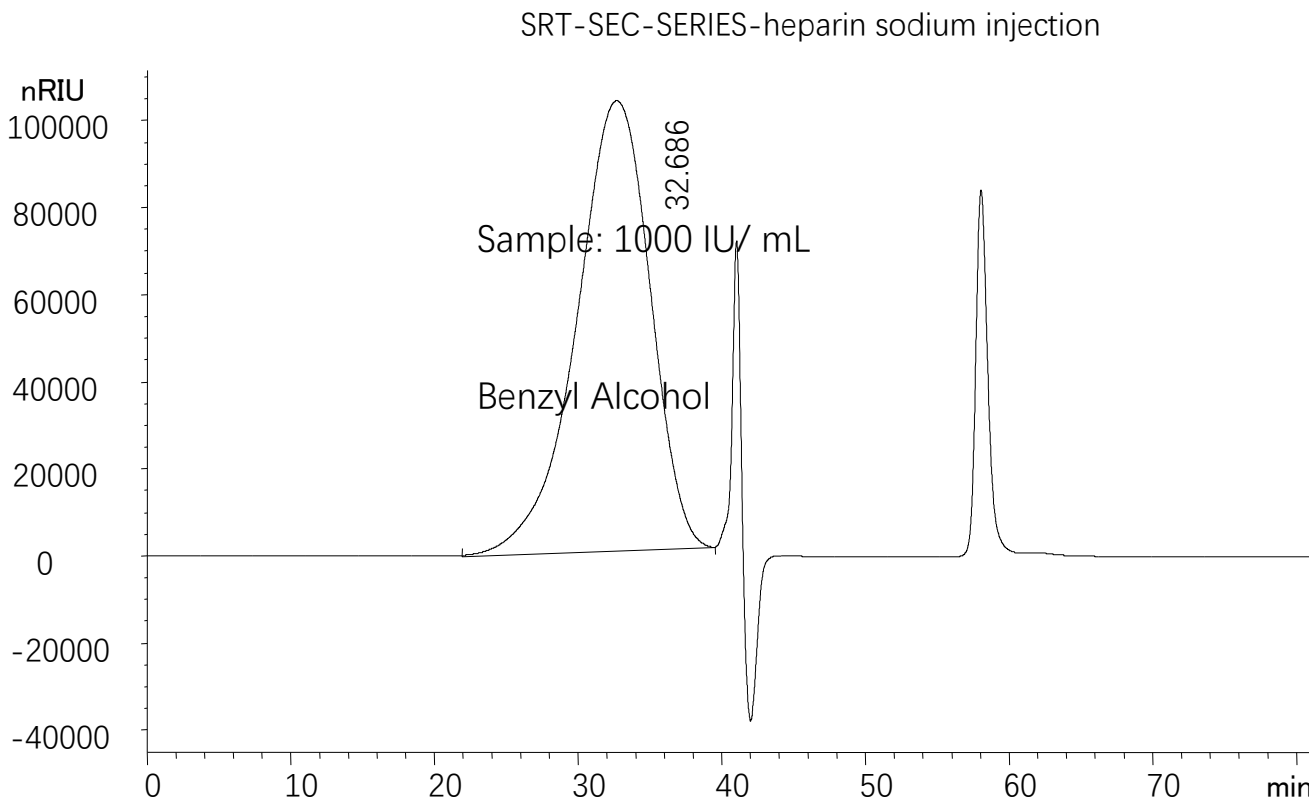
heparin—Heparin sodium

Molecular weight distribution of heparin sodium

(CP 2020 Version)

Column: SRT SEC-500 (5 μm, 500 Å, 7.8 x 50 mm)
SRT SEC-500 (5 μm, 500 Å, 7.8 x 300 mm)
SRT SEC-300 (5 μm, 300 Å, 7.8 x 300 mm)
Mobile phase: 0.1 M ammonium acetate, Flow rate: 0.6 mL/min,
Detector: RI (30℃), Column temperature: 30℃,
Injection volume: 25 μL, Sample: 5 mg/mL in mobile phase



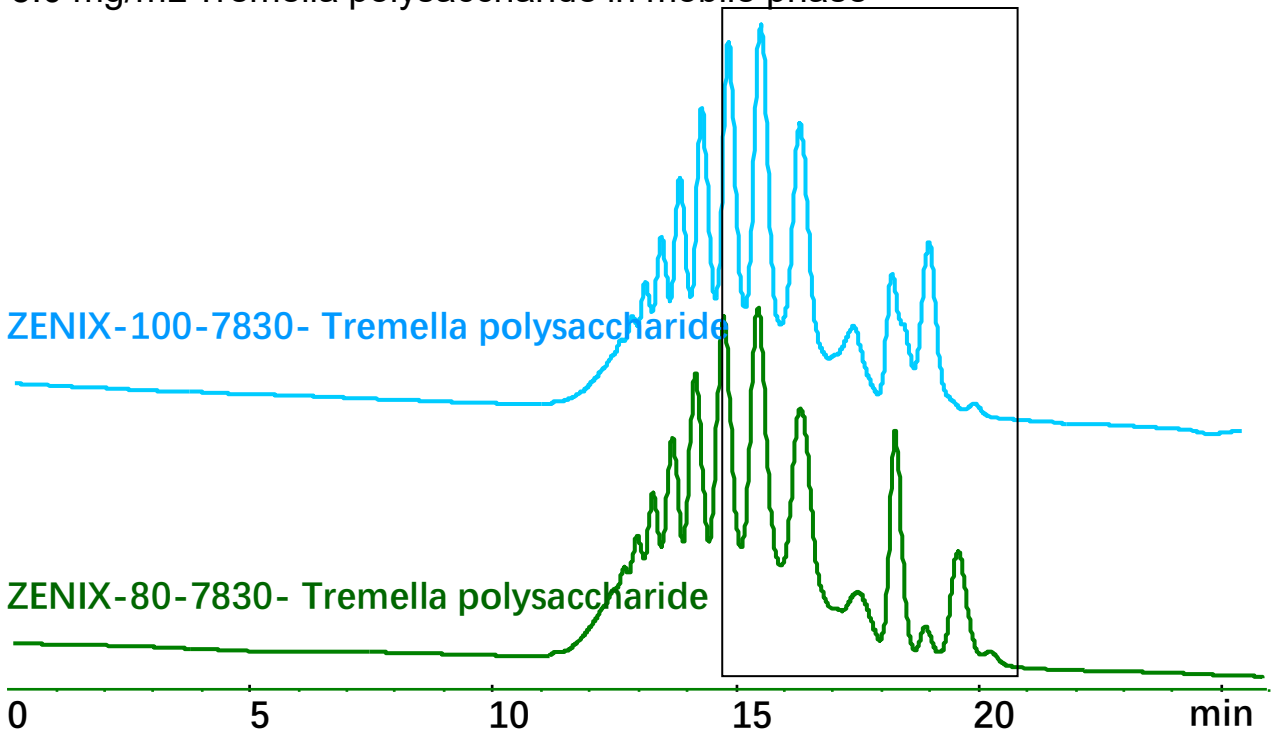


It fully meets the requirements of the test of molecular weight and molecular weight distribution under the item of heparin sodium [inspection] in the CP 2020.

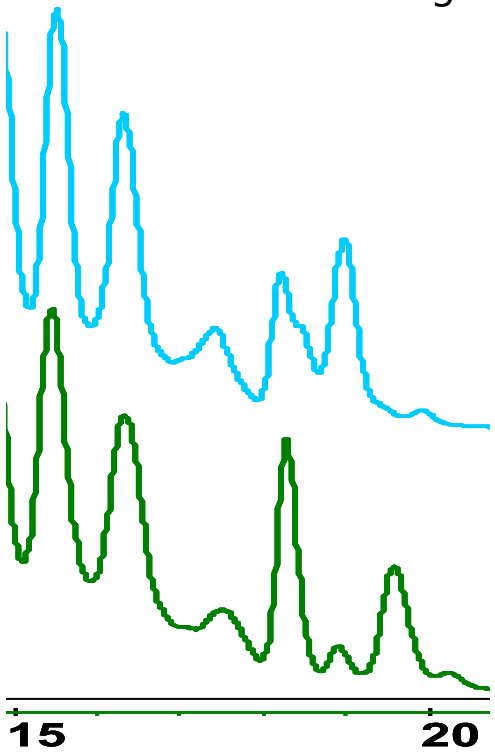
Water-soluble polymer - Tremella polysaccharide

Tremella polysaccharide analysis on Zenix SEC-80

Column: Zenix SEC-80 (3 μm, 80 Å, 7.8 x 300 mm)
Mobile phase: 0.7% Na_2SO_4
Flow rate: 0.5 mL/min
Detector: RI (35℃)
Column temperature: 35℃
Injection volume: 10 μL
Sample: 5.0 mg/mL Tremella polysaccharide in mobile phase



Tremella polysaccharides: Currently divided into five major categories, namely acidic heteropolysaccharides, neutral heteropolysaccharides, cell wall polysaccharides, extracellular polysaccharides, and acidic oligosaccharides.Molecular weight: below 5W



SEC columns with smaller pore size is superior in separation of smaller molecule

SEC Maintenance and cleaning

Storage: In daily use, Sepax SEC columns are recommended to be stored in 150 mM sodium phosphate buffer (pH 7.0); Or store it in 50 mM sodium phosphate buffer containing 0.02% (w/v) sodium azide (NaN3), and it is not recommended to store it in pure water.

Maintenance: After using the column every day, give a positive injection of 100ul of 6M Guanidine HCl

During the conversion of salt, water, and organic solvents, it is necessary to pay attention to pressure changes. We do not recommend using solvents with high viscosity such as Ethanol and IPA. At the beginning and end, it is necessary to slowly increase and decrease the flow rate to ensure that there is no significant change in pressure

Cleaning: After multiple uses, some samples may be adsorbed onto the inlet sieve plate or filler, and when accumulated to a certain extent, there will be a phenomenon of pressure rise accompanied by peak pattern broadening. At this time, it is necessary to clean the column.

The general process is to disconnect the detector, reverse connect the column, and rinse 10-15 times the column volume with cleaning solution at a flow rate below 50% of the maximum recommended flow rate.

Recommended cleaning solutions include:

0.5 M sodium sulfate solution (pH 3.0): suitable for cleaning after alkaline protein adsorption;

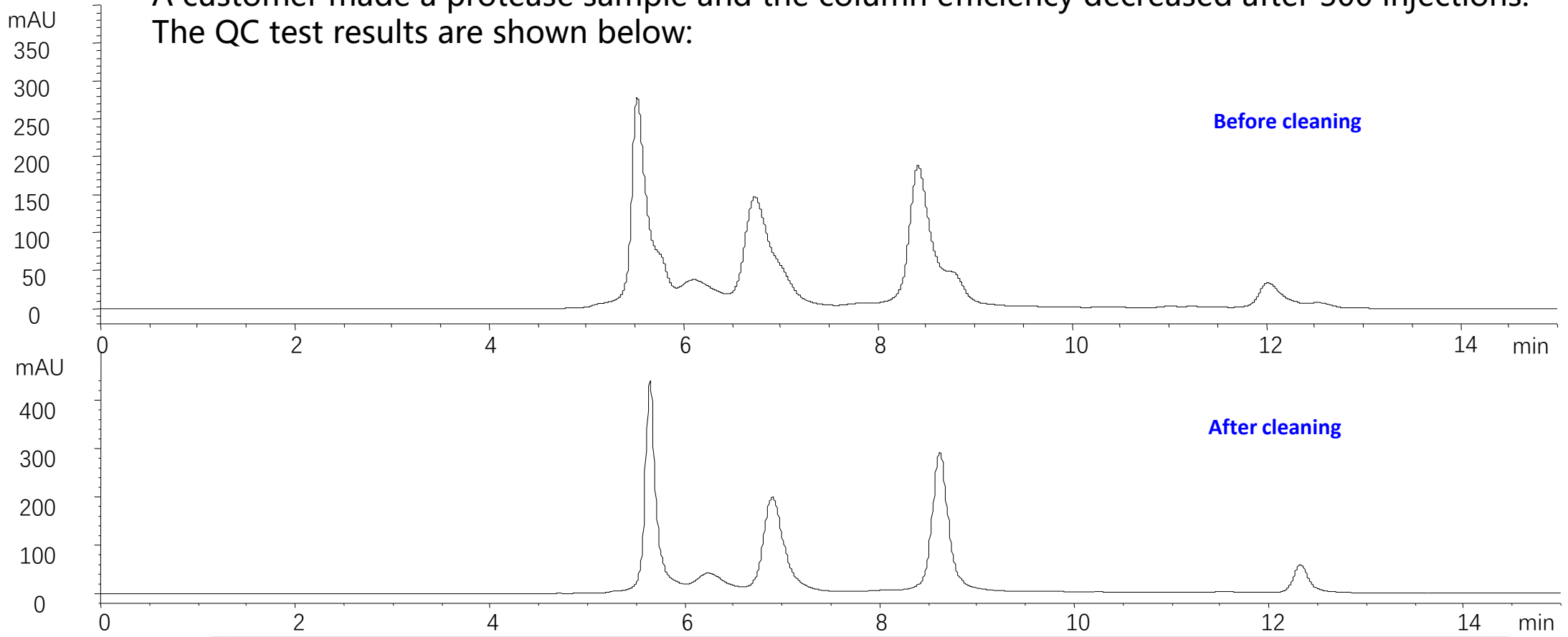
10% - 20% organic solvents (ethanol, isopropanol, acetonitrile): cleaning after adsorption of hydrophobic proteins;

4~6 M urea (pH 7.0): suitable for cleaning easily aggregated protein samples;

*Regardless of the cleaning method, ensure that the pH value of the cleaning solvent is within the allowable pH range of the chromatographic column

Before and after cleaning with 6M urea

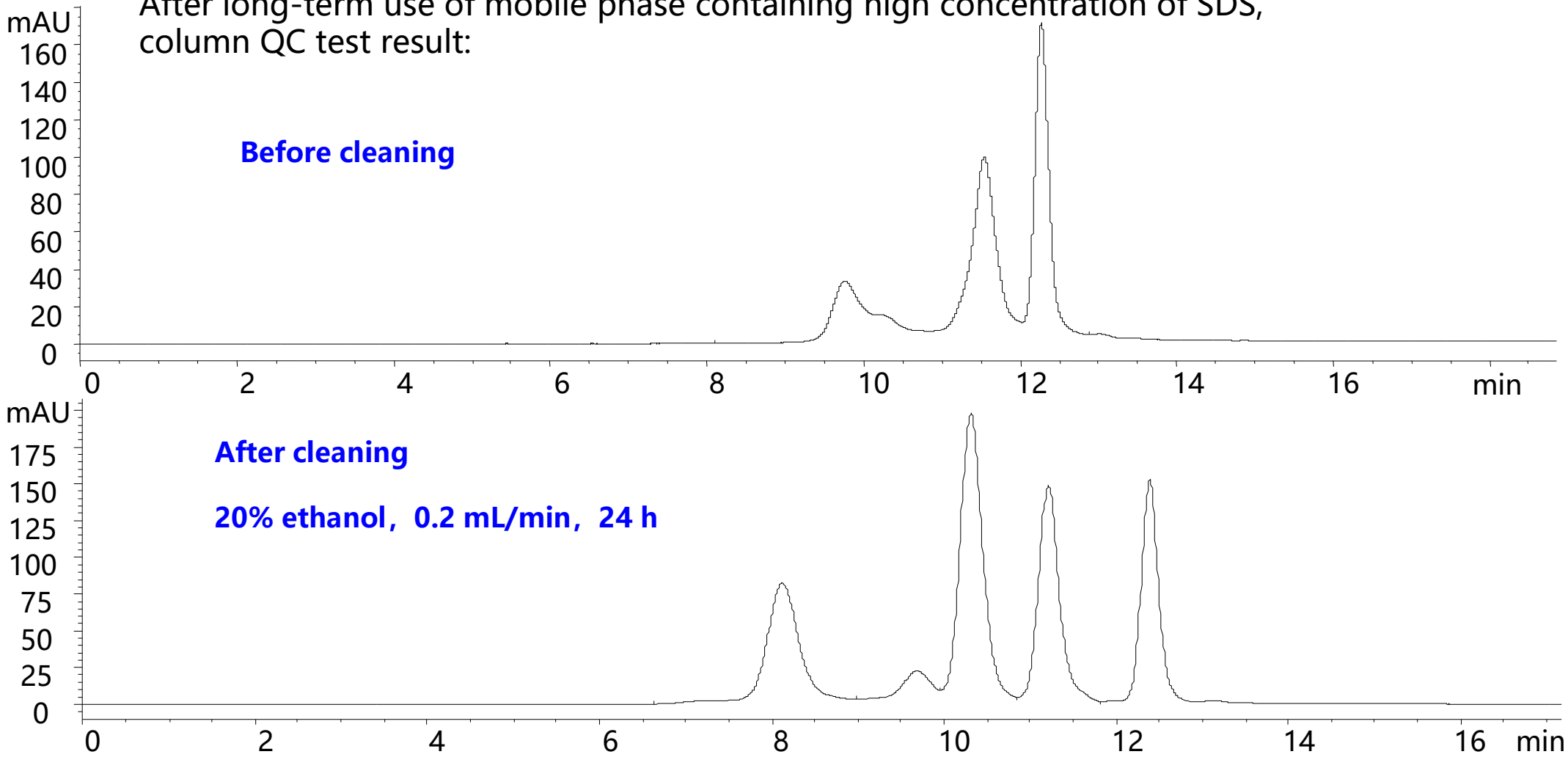
A customer made a protease sample and the column efficiency decreased after 300 injections. The QC test results are shown below:



procedure: 4 M urea (pH 7.0) —0.5 mL/min, 1 h→ Ultra pure water —0.5 mL/min, 1 h→ 150 mM PBS —1 mL/min, 2 h→ QC

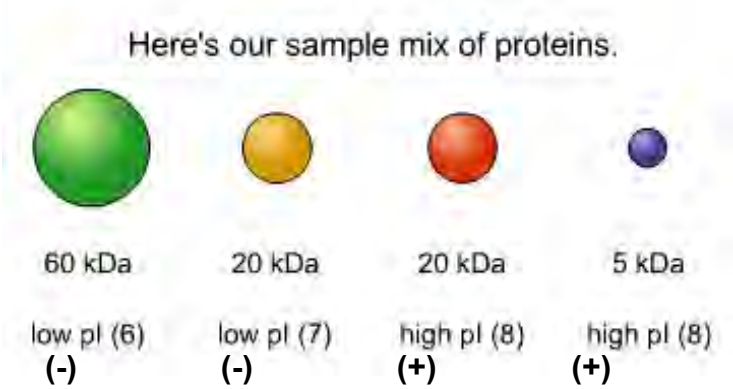
Before and after cleaning SRT SEC-500 with 20% ethanol

After long-term use of mobile phase containing high concentration of SDS, column QC test result:



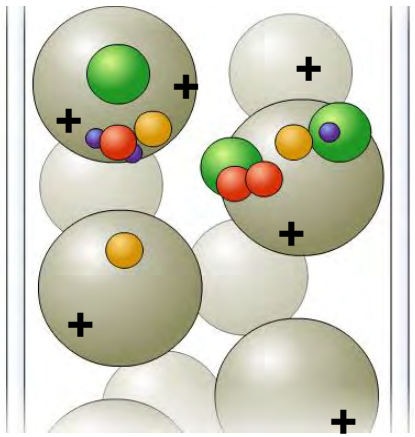
IEX Separation Mechanism

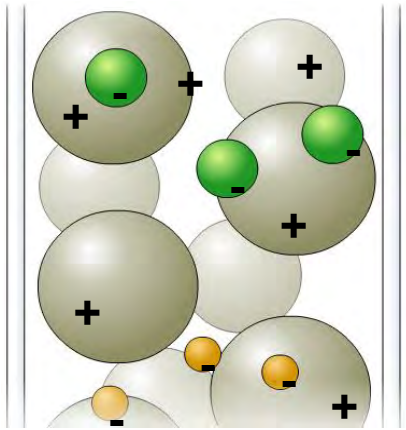
Separation is achieved based on competition between sample ions and mobile phase ions to occupy sites with opposite charges on the ion exchanger

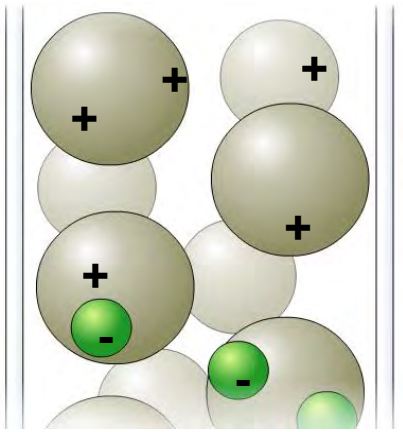


If pH mobile phase =7.2

Then charge of the proteins:

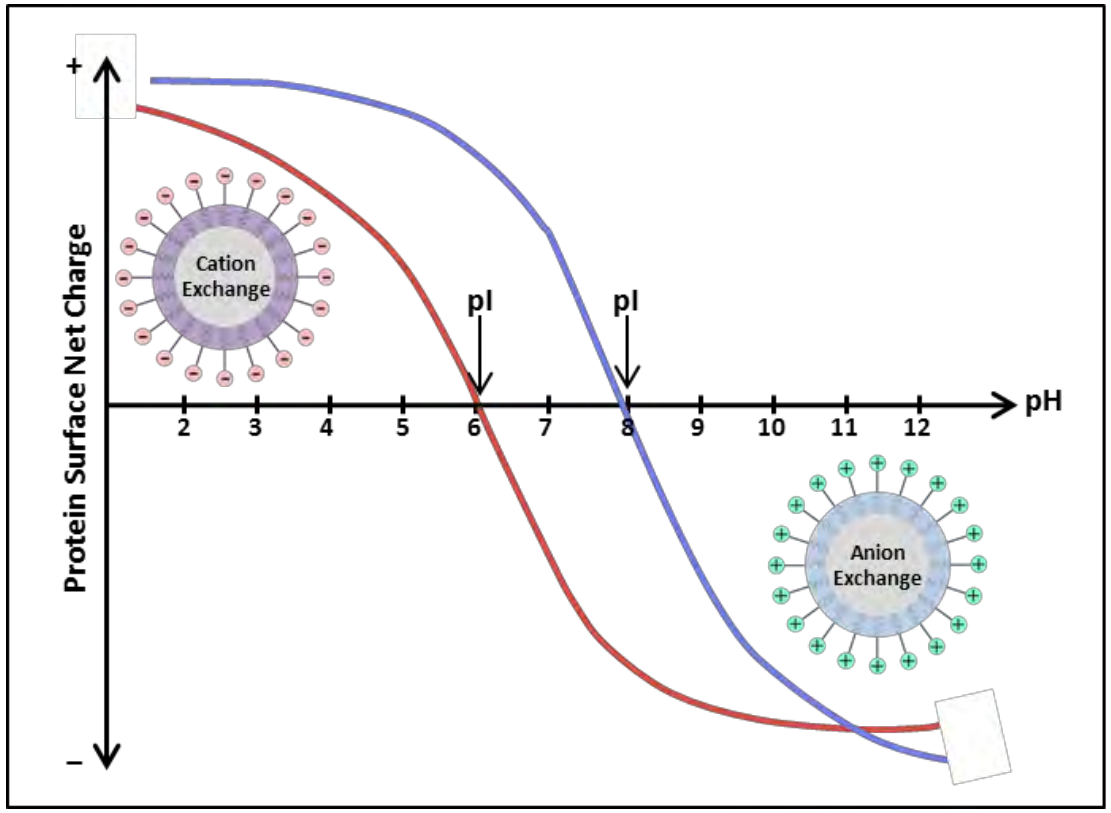






IEX Separation characteristics

- Using gradient elution a buffer salt system as the mobile phase
- A small amount of organic modifiers can be added to change the separation selectivity
- The retention value and separation degree of components are mainly regulated by controlling the pH value and ionic strength of the mobile phase
- On cation exchange columns, only proteins with a pI greater than the pH of the mobile phase have retentions on the column, while proteins with a pI less than or equal to the pH of the mobile phase have no retentions. Even if there is a difference in their pI, they are directly flushed out as solvent peaks at the same time. The peak sequence of the retained proteins is the first out peak with a small pI; The opposite is true on anion exchange columns.



IEX Separation solution

Sepax IEX

Proteomix

Antibodix

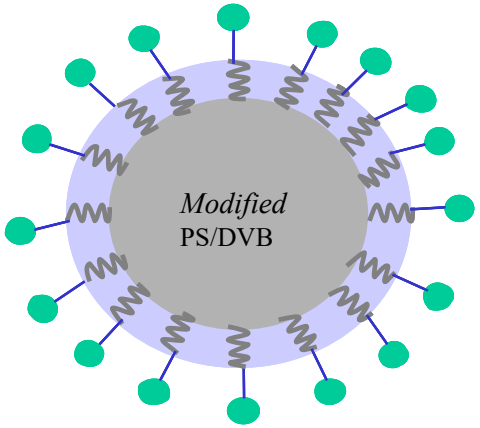
WAX

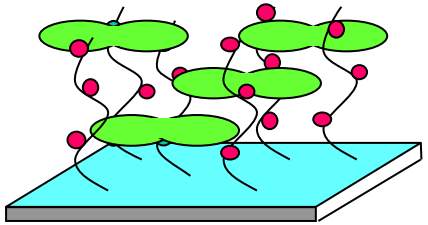
SAX

WCX

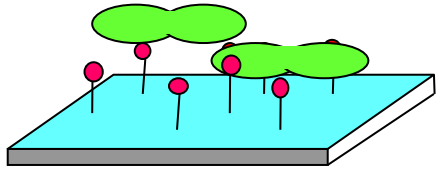
SCX

Patented surface hydrophilic film modification and graft bonding technology
Prevent protein adsorption and increase exchange capacity





Graft modification



Monolayer modification

Sepax IEX selection

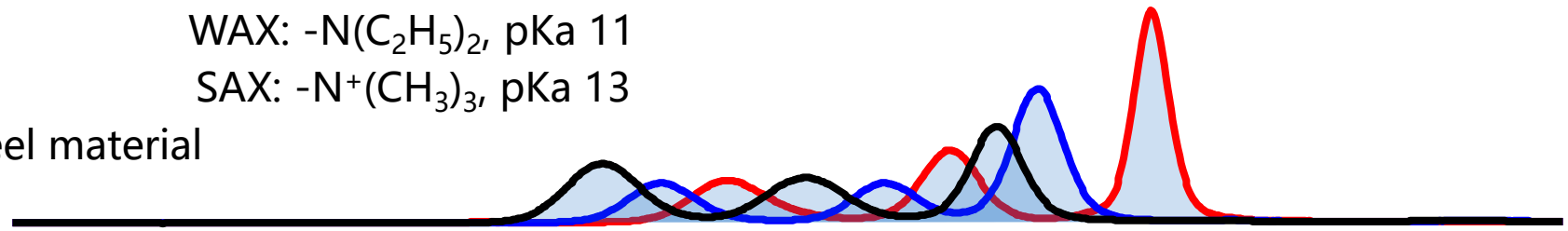
Type	Matrix	Partical size（μm）	pH range	Applicable Samples
Proteomix SCX	PS/DVB	1.7,3,5,10	2-12	pI＞7 protein，polypeptide
Proteomix WCX				
Proteomix SAX				pI＜7 protein，polypeptide DNA、RNA、Oligonucleotides、saccharides
Proteomix WAX				
Proteomix CV-1				Special column for BsAb analysis
Antibodix™				Special column for mAb analysis

•Functional group:

WCX: -COOH, pka 4.75　　WAX: $-N(C_2H_5)_2$, pKa 11

SCX: $-SO_3H$, pKa < 1.0　　SAX: $-N^+(CH_3)_3$, pKa 13

•Column: PEEK, Stainless steel material



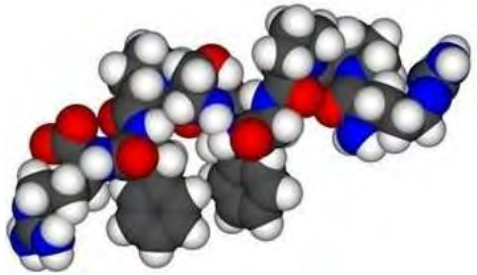
IEX column application

① Study on Charge Variants of Proteins and Antibodies

② Study on the structure of protein samples before and after glycosylation

③ Oligonucleotide fragment analysis

④ Analysis of oligosaccharides with different polymerization units



Sample requirements for IEX testing:

There is a difference in charge or pI between the target analyte and other components or impurities that may exist.

The sample components have good mutual solubility in the selected mobile phase system.

Selection of column for IEX analysis:

Select a suitable column type based on the difference in sample charge type and pI(SCX/WCX/SAX/WAX)

Select the appropriate matrix and pore size according to the resolution requirements for sample separation purposes

IEX column selection

1.Protein	Proteomix WCX/SCX/WAX/SAX
2.Antibody	Antibodix WCX / Proteomix WCX、SCX
3.Polypeptide	Proteomix WCX/SCX/WAX/SAX
4.Nucleic Acid	Proteomix SAX
5.Water-soluble Polymer	Proteomix SCX/WCX(base) WAX/SAX(acid)

Selection based on two factors:

1. Acidity and alkalinity of the sample
2. Isoelectric point of protein(pI)

IEX Method development

Selection of buffer solution:

- With sufficient buffer capacity within the selected pH range, the smaller the ionic strength, the better. The concentration of the initial buffer solution should be as low as possible, such as 10 mM
- Use anionic buffers when using CEX column, and use cations when using AEX column, to avoid unnecessary ion exchange processes
- Determine which buffer system to use based on different mobile phase pH conditions. For example, when the mobile phase pH is 3-5, ammonium formate buffer solution is used; When the mobile phase pH is 4-6, sodium acetate or potassium acetate buffer solution is used; When the mobile phase is pH 6-8, sodium phosphate or potassium phosphate buffer solution is used. In addition, the pH range of Tris hydrochloric acid buffer is 7-9, and the pH range of Tris phosphoric acid buffer is 6-9

Selection of elution method:

- Change the pH of the buffer solution to change the protein from adsorption to desorption. For example, in anion exchange, decrease the pH of the mobile phase to positively charge the negatively charged protein adsorbed on the column to achieve desorption
- Increase the ionic strength of the buffer and replace strongly adsorbed molecules from the ion exchanger
- Simultaneous changes in buffer pH and ionic strength

Buffers for CEX chromatography

pH interval	Salt	Counter-ion	pKa (25 °C)
1.4–2.4	Maleic acid	Na+	1.92
2.6–3.6	Methyl malonic acid	Na+ or Li+	3.07
2.6–3.6	Citric acid	Na+	3.13
3.3–4.3	Lactic acid	Na+	3.86
3.3–4.3	Formic acid	Na+ or Li+	3.75
3.7–4.7	Succinic acid	Na+	4.21
5.1–6.1	Succinic acid	Na+	5.64
4.3–5.3	Acetic acid	Na+ or Li+	4.75
5.2–6.2	Methyl malonic acid	Na+ or Li+	5.76
5.6–6.6	MES	Na+ or Li+	6.27
6.5-7.9	MOPS	Na+ or Li+	7.20
5.5–7.7	Phosphate	Na+	7.20
7.0–8.0	HEPES	Na+ or Li+	7.56
7.8–8.8	BICINE	Na+	8.33

Ref: Handbook of chemistry and physics, 93rd edition, CRC, 2012–2013.

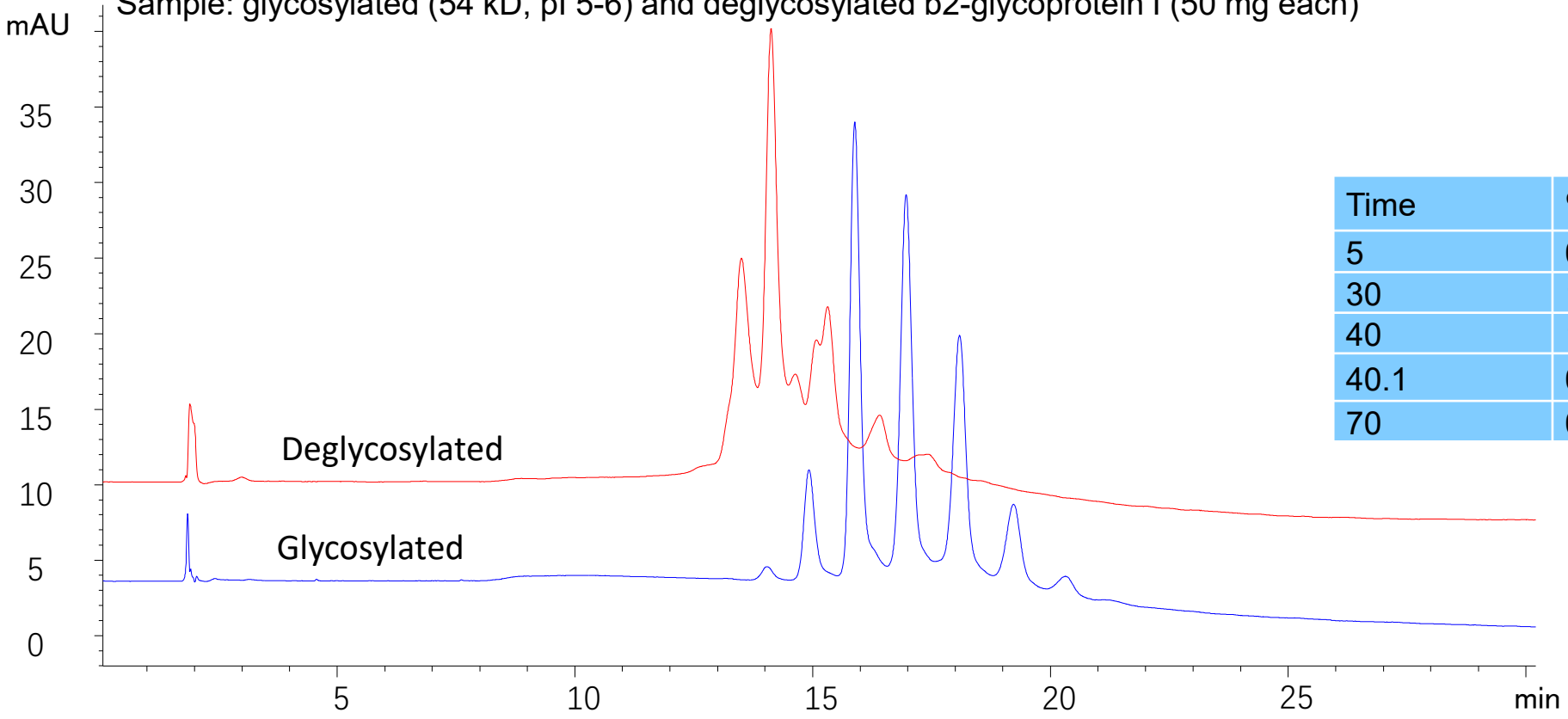
Buffers for AEX Chromatography

pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25°C)
4.5-5.3	N-Methylpiperazine*	20	Cl^-	4.75
4.8-6.0	Piperazine*	20	Cl^- or $HCOO^-$	5.68
5.5-6.0	L-Histidine*	20	Cl^-	6.15
5.8-7.0	bis-Tris*	20	Cl^-	6.48
6.4-7.3	bis-Tris propane*	20	Cl^-	6.80
6.5-7.9	MOPS*	20	Cl^-	7.28
7.3-8.2	Triethanolamine*	20	Cl^- or CH_3COO^-	7.76
7.5-8.8	Tris*	20	Cl^-	8.06
8.0-8.5	N-Methyldiethanolamine*	20		8.54
8.0-8.5	N-Methyldiethanolamine*	50	Cl^- or CH_3COO^-	8.54
8.4-9.4	Diethanolamine*	20 at pH 8.4 50 at pH 8.8	Cl^-	8.88
8.5-9.0	1,3-Diaminopropane*	20	Cl^-	8.64
9.0-10.0	Ethanolamine*	20	Cl^-	9.50
9.5-9.8	Piperazine*	20	Cl^-	9.73
9.7-10.0	CAPS*	20	Cl^-	10.40
9.8-10.3	1,3-Diaminopropane*	20	Cl^-	10.47
10.6-11.6	Piperidine*	20	Cl^-	11.12

IEX analysis——glycoprotein

Overlay of SAX on β2-Glycoprotein

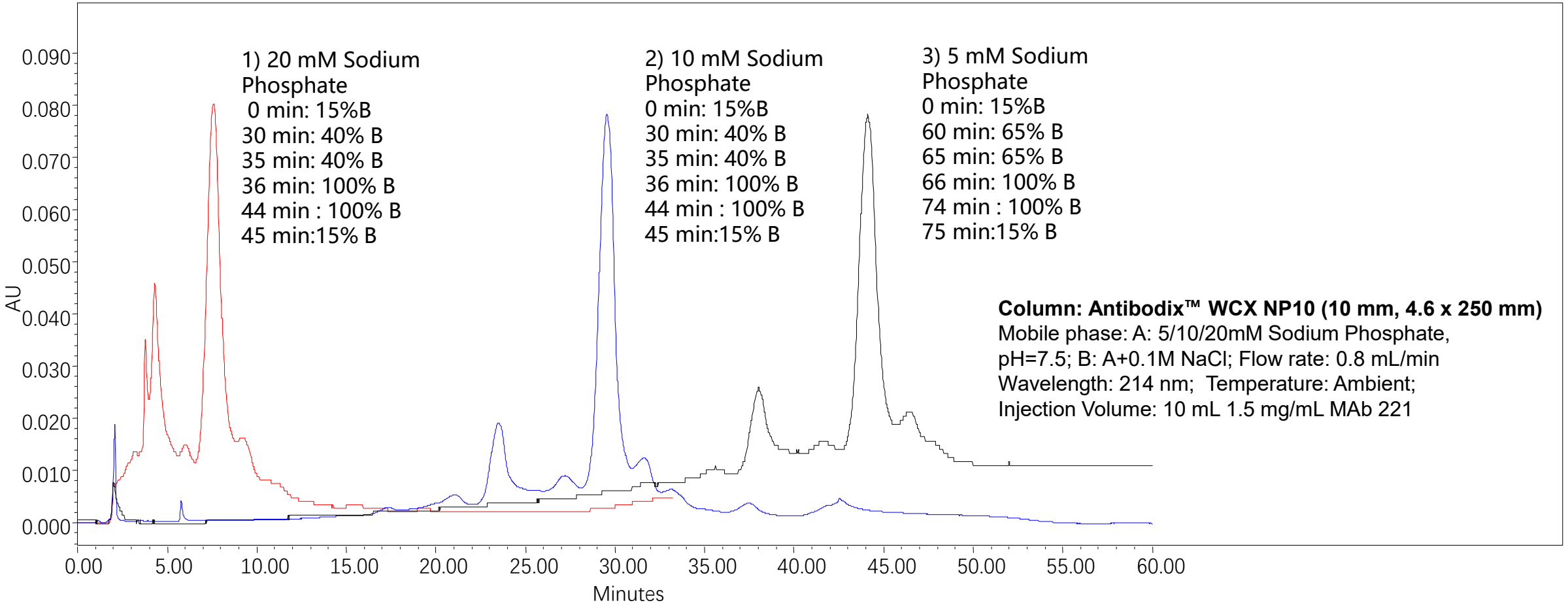
Column: Proteomix® SAX NP5 4.6 x 250 mm
Mobile phase:
A: 20 mM Piperazine + 20 mM Triethanolamine + 20mM Bis-tris propane + 20mM N-methylpiperazine pH 9.7 (titrate with HCl)
B: A at pH 3.7;
Detection: UV 280 nm; Column temperature: 30˚C;
Sample: glycosylated (54 kD, pI 5-6) and deglycosylated b2-glycoprotein I (50 mg each)



Time	% B
5	0
30	100
40	100
40.1	0
70	0

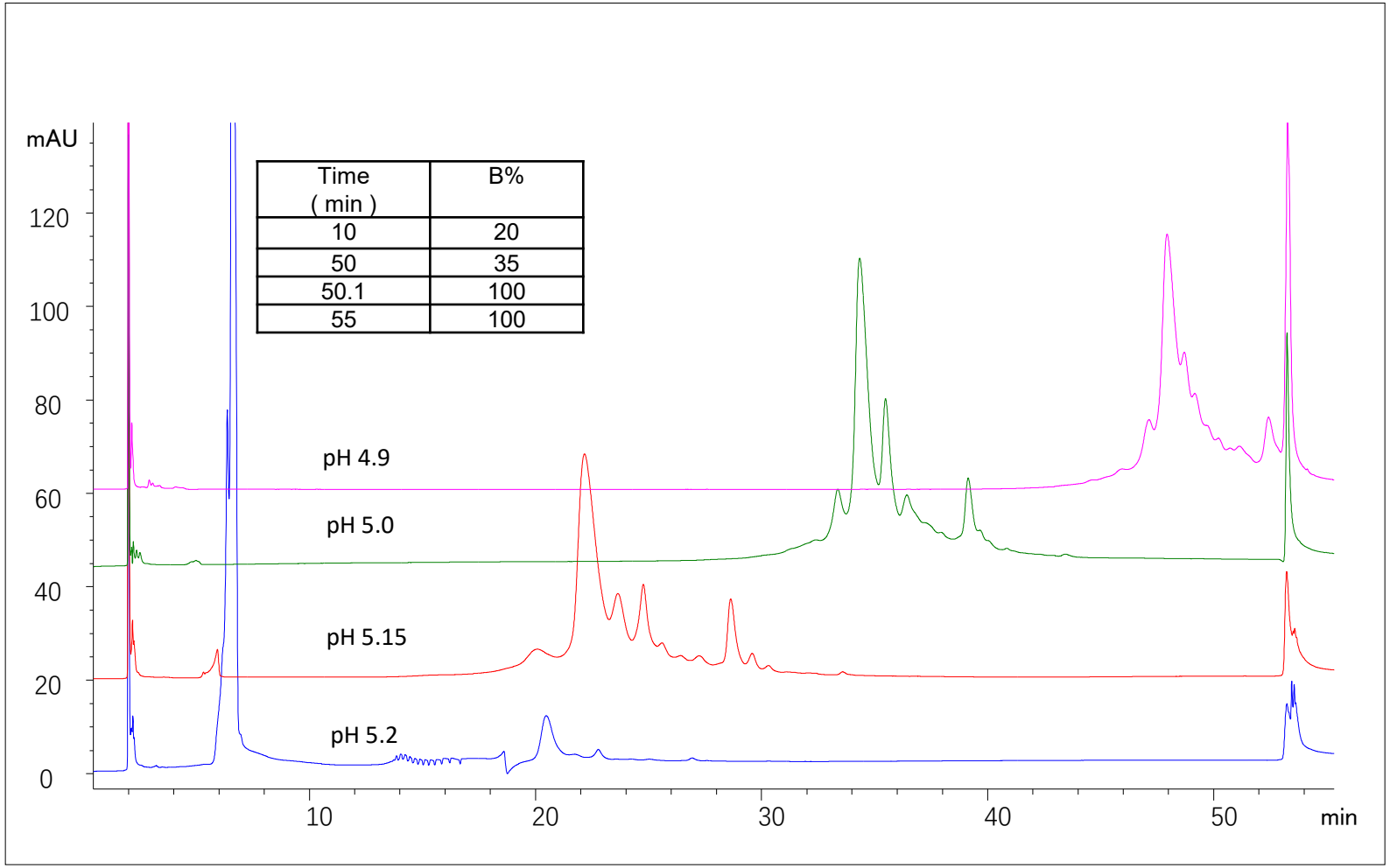
mAb Analysis under different buffer

Same pH, Different buffer A concentrations



mAb analysis——different pH buffer

MAb separation-pH Dependent



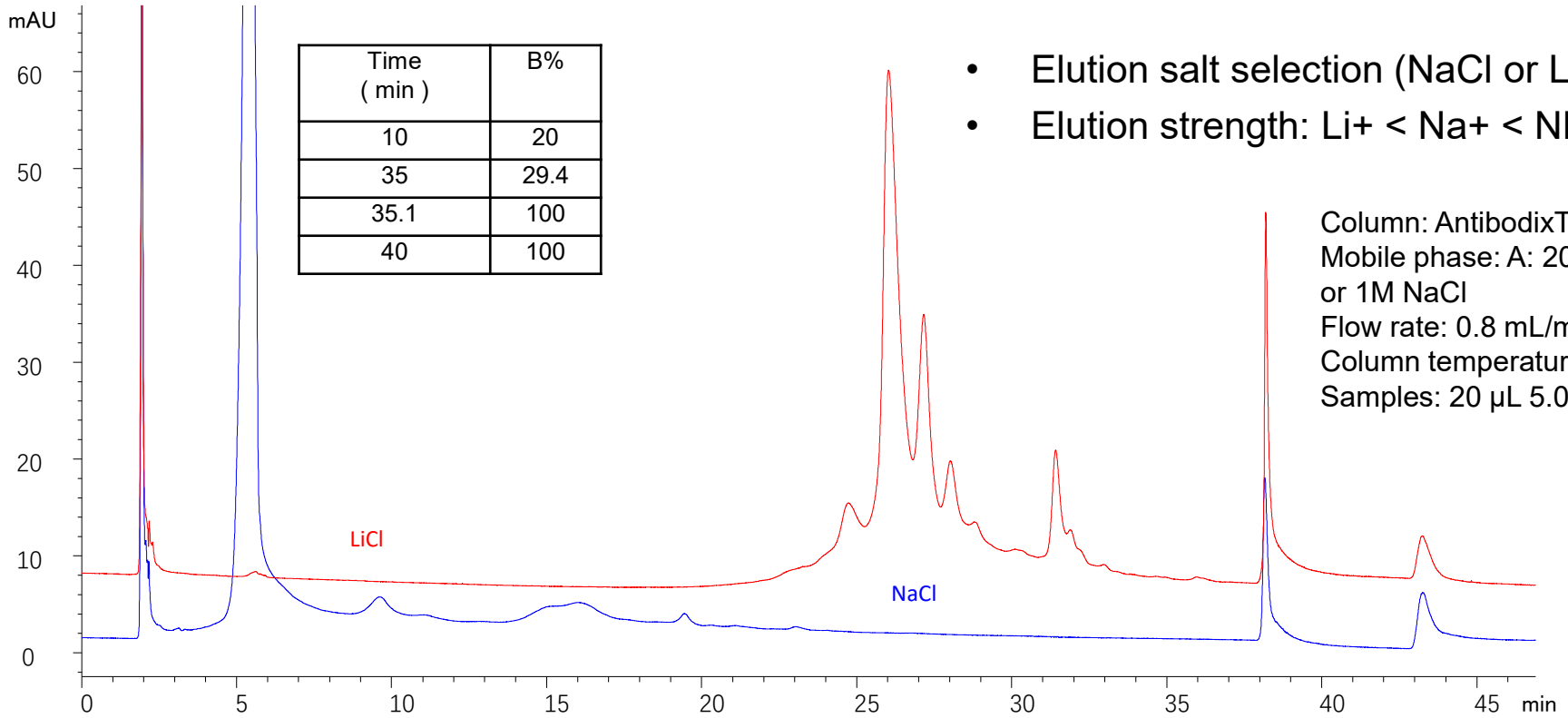
Time (min)	B%
10	20
50	35
50.1	100
55	100

Column: Antibodix WCX NP5
(5 μm, 4.6 x 250 mm)
Mobile phase: A: 20 mM NaAc, pH 5.15, B: A + 1 M LiCl
Flow rate: 0.8 mL/min;
Detector: UV 280 nm;
Column temperature: 30 ℃ ;
Pressure: 139 bar;
Samples: 20 μL 5.0 mg/mL MAb 321 in Tris buffer

Ensuring test stability in IEX analysis: mobile phase preparation, reagent source, validation

mAb analysis——different salt type

LiCl vs. NaCl on Antibodix WCX



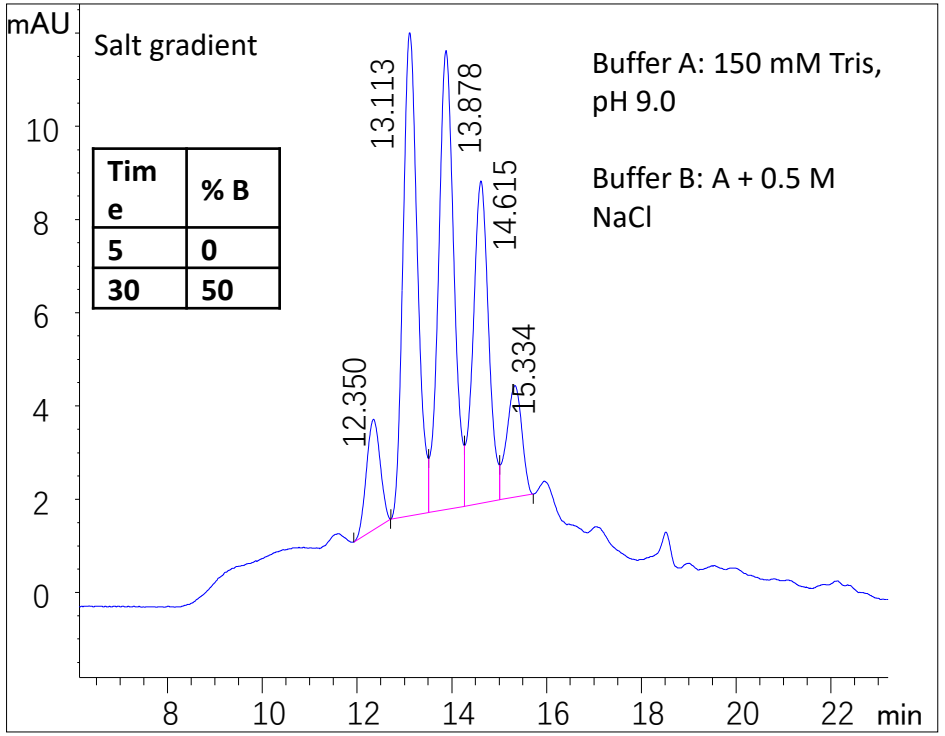
Time (min)	B%
10	20
35	29.4
35.1	100
40	100

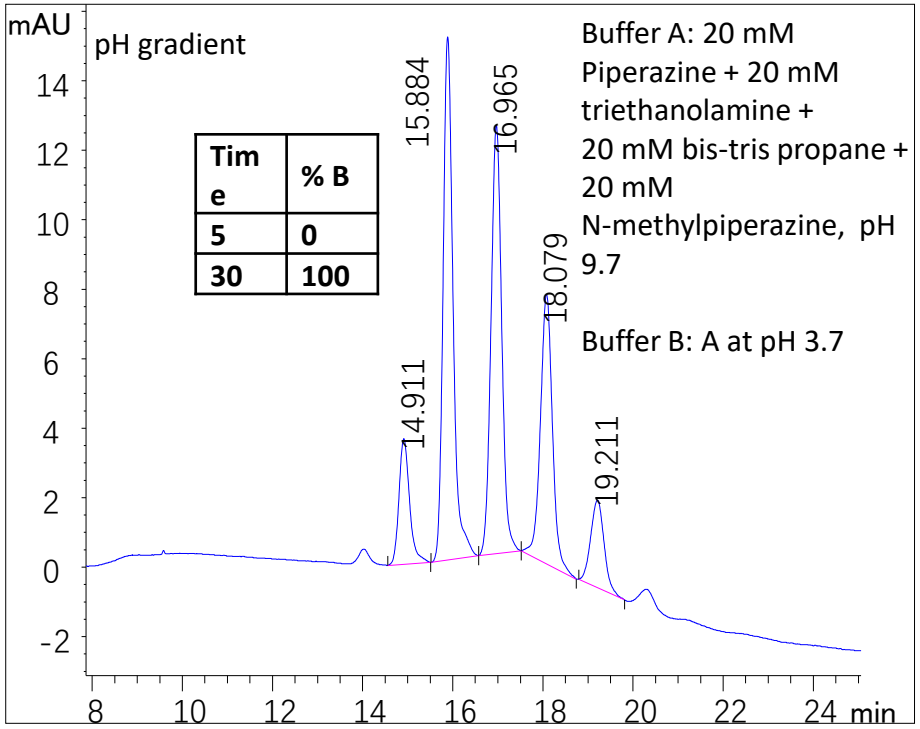
- Elution salt selection (NaCl or LiCl)
- Elution strength: Li+ < Na+ < NH4+ < K+

Column: AntibodixTM WCX NP5 (5 µm, 4.6 x 250 mm)
Mobile phase: A: 20 mM NaAc, pH 5.15, B: A + 1 M LiCl or 1M NaCl
Flow rate: 0.8 mL/min; Detector: UV 280 nm
Column temperature: 30 ℃ ; Pressure: 139 bar
Samples: 20 µL 5.0 mg/mL MAb 321 in Tris buffer

Glycoprotein - Different Elution Methods

Comparison of Separation Effects under Salt Gradient and pH Gradient





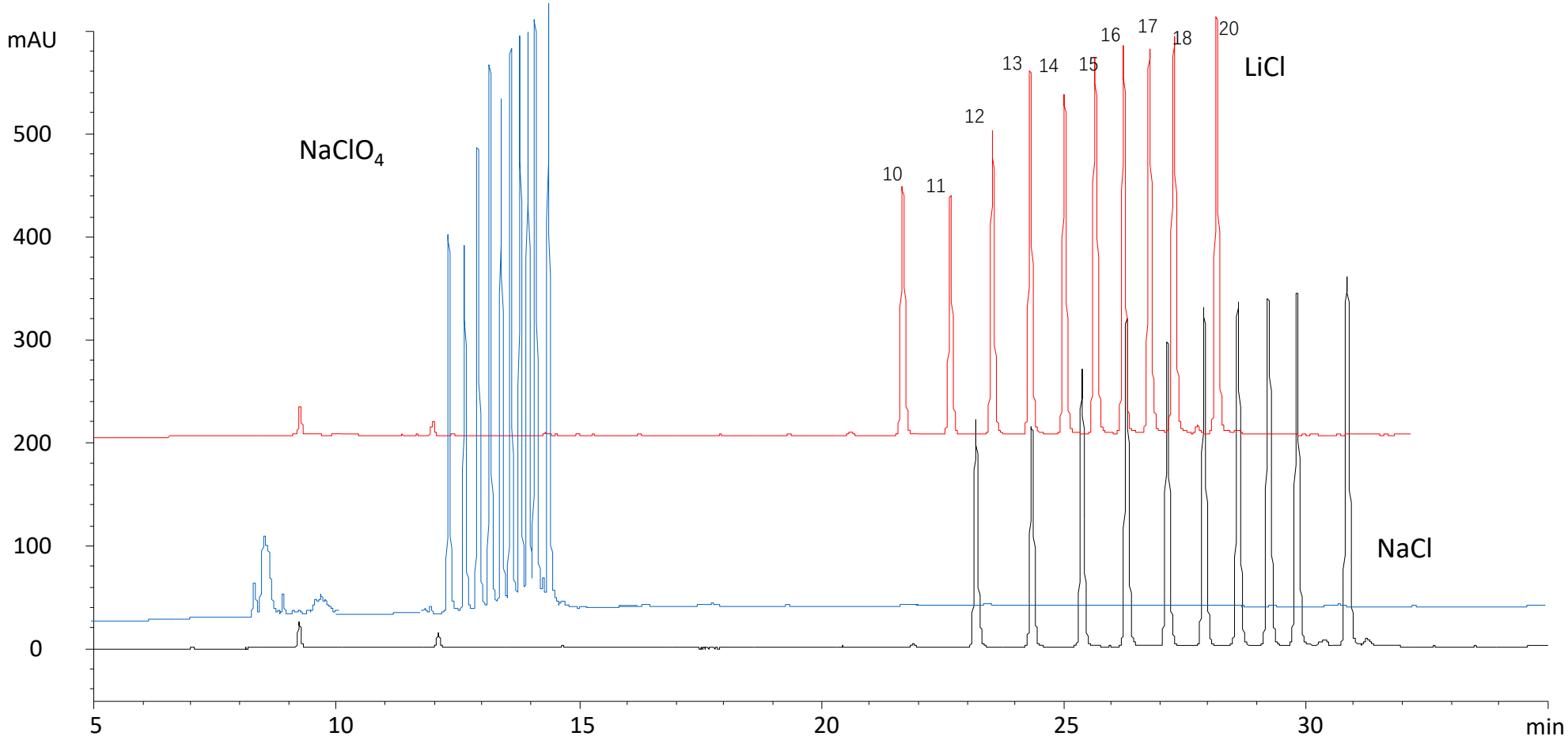
RT [min]	Height	Area	Plates	Tailing	Resolution
12.35	2	48	7914	1.16	
13.11	10	218	8927	1.17	1.37
13.88	10	223	8881	-1.35	1.33
14.62	7	158	9750	-1.45	1.25
15.33	2	54	9688	1.09	1.18

RT [min]	Height	Area	Plates	Tailing	Resolution
14.91	4	60	20620	1.41	
15.88	15	231	26689	1.38	2.41
16.97	12	203	24419	1.07	2.62
18.08	8	144	23470	0.79	2.45
19.21	3	52	20394	0.84	2.24

Nucleic Acids in Different Buffer Salts

Effect of buffer salts on oligonucleotide separation

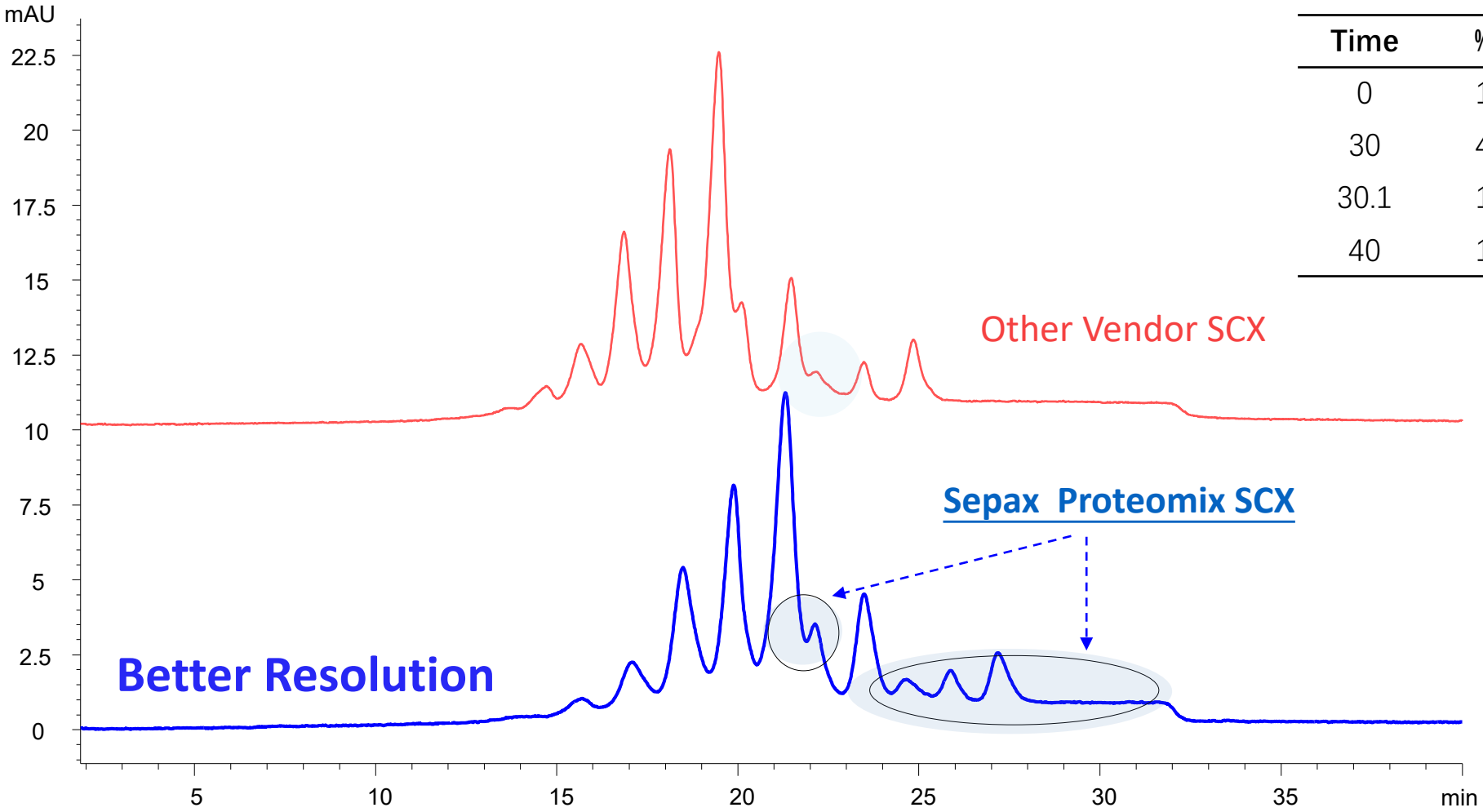
Column: Proteomix® NP5 SAX (5 µm, 4.6 x 250 mm)
Mobile phase: A: 20 mM Tris, pH 8.0, B: A + 0.5 M NaCl/$NaClO_4$/LiCl
Flow rate: 0.5 mL/min; Gradient: 0→100% B in 30 minutes;
Pressure: 73 bar;Detector: UV 260 nm;
Column temperature: 25 ℃;Samples: Mixture of poly $dA_{10\text{-}18}$, dA_{20}, 10 µM each in water; Injection volume: 5 µL



Erbitux–Proteomix SCX vs Vendor SCX

Columns: Proteomix SCX-NP5 PEEK, 5μm, 4.6 x 250 mm; Other Vendor SCX PEEK, 5μm, 4.0 x 250 mm;
Mobile phase: CX-1 pH Buffer A; B: CX-1 pH Buffer B; Flow velocity: 480 cm/h, Detector: UV 280 nm,
Column temperature: 30 ℃ ; Samples: Erbitux (2 mg/mL), Injection Volume: 10 μL

Time	%B
0	10
30	40
30.1	10
40	10



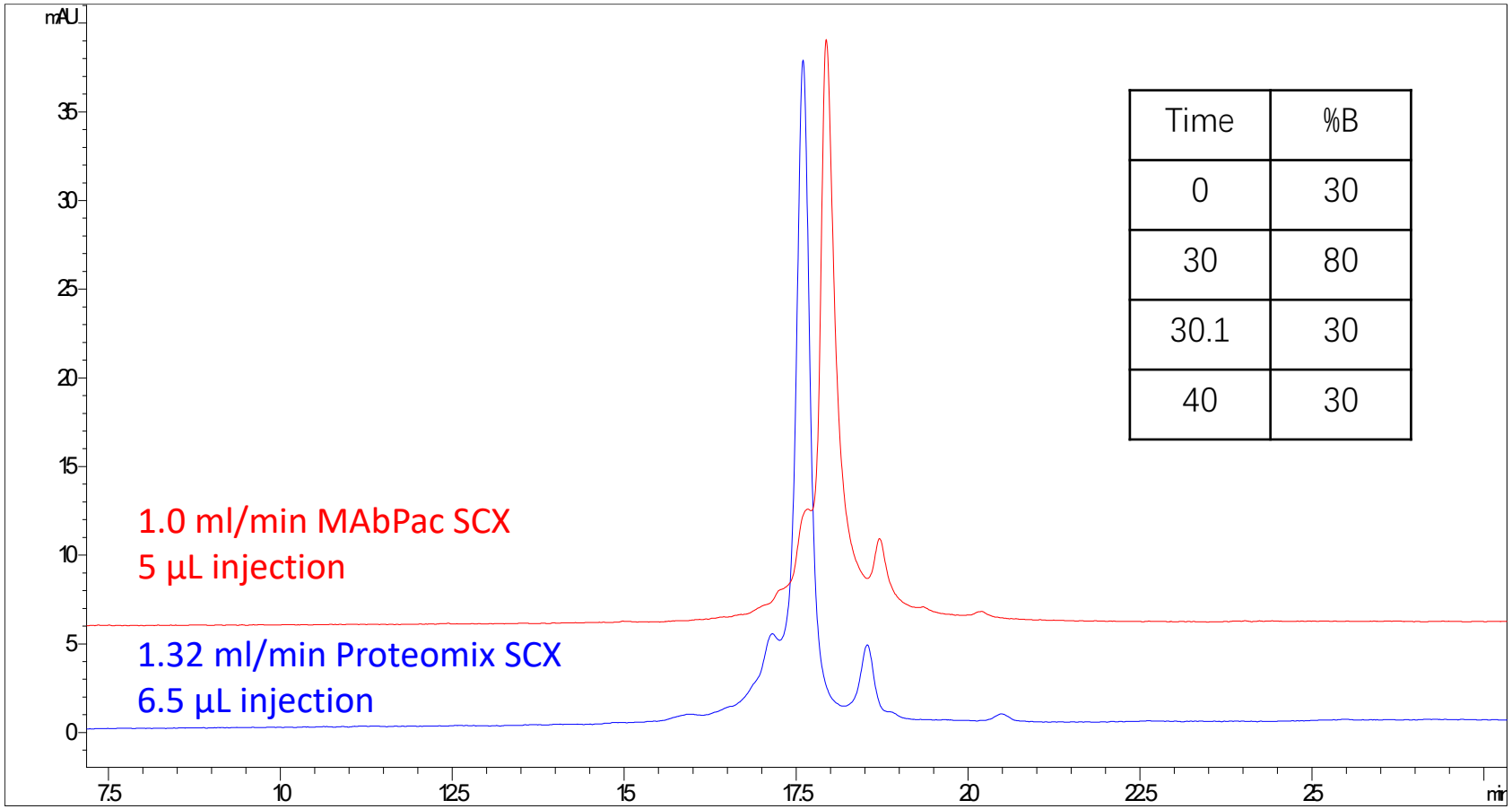
NIST Mab-Proteomix SCX vs MAbPac SCX

Column: See figure below.

Mobile Phase A: CX1 pH Buffer A (Thermo Scientific) B: CX1 pH Buffer B (Thermo Scientific)

Flow Rate: See figure below.; Detector: UV 280 nm; Column temperature: 30 ℃;

Sample: NIST (2 mg/mL): Injection Amount: See figure below

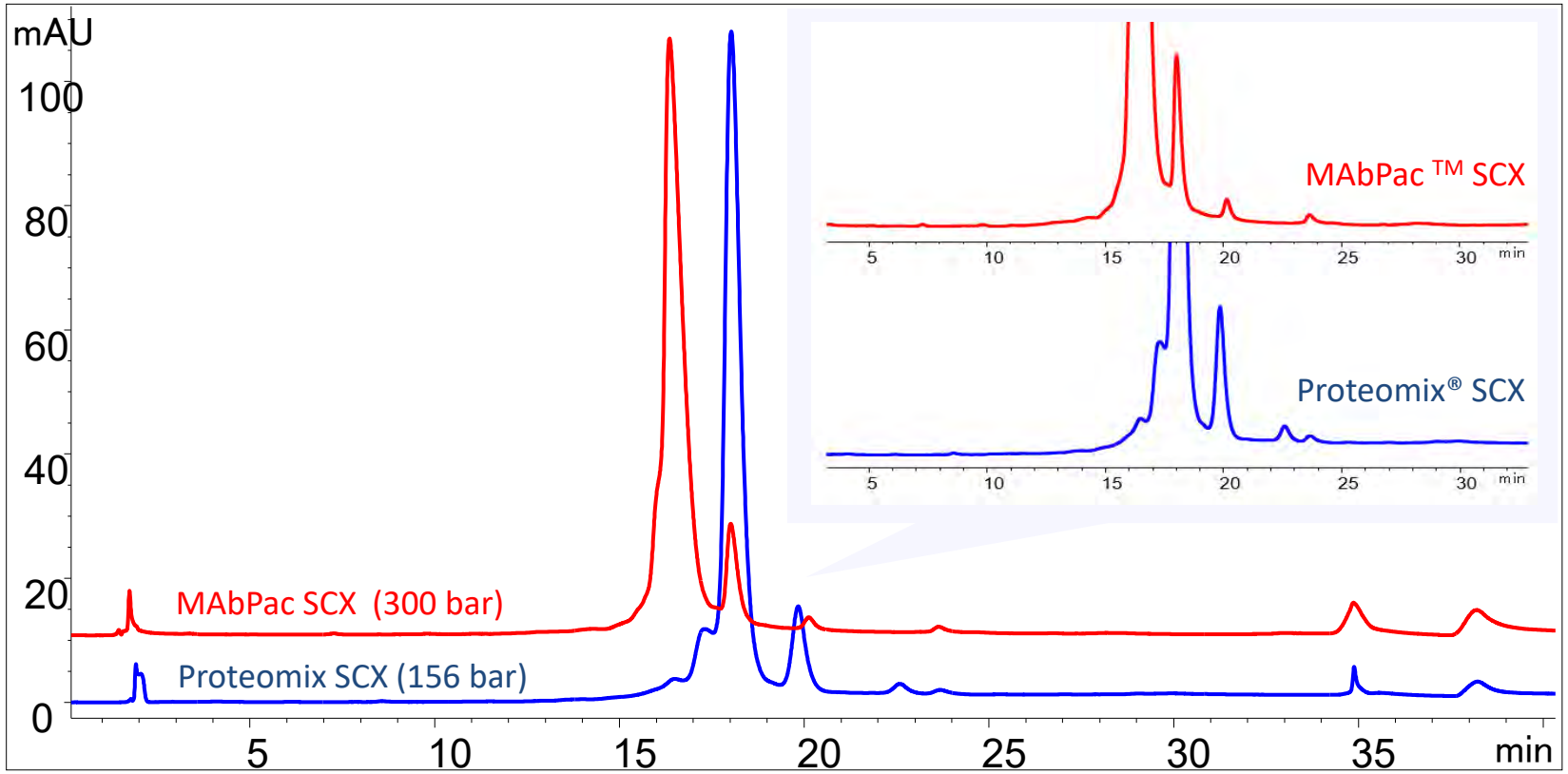


Time	%B
0	30
30	80
30.1	30
40	30

NIST Mab analysis on SCX

NIST Mab -Proteomix SCX vs. MAbPac SCX –Salt Gradient

Column: Proteomix SCX-NP5 PEEK(5 μm, 4.6 x 250 mm) , MAbPa SCX
Mobile phase: A: 10 mM phosphate, pH 7.0, B: A+ 0.5 M NaCl;
Flow rate: 0.75 mL/min for MAbPac™ SCX, 1 mL/min for Proteomix®, Detector: UV 280 nm, Column temperature: 25 ℃,
Samples: 10 mg/mL NIST mAb, 60 μg (pI 9.18, in 12.5 mM histidine, pH 6.0), 45 μg for MAbPac™ SCX

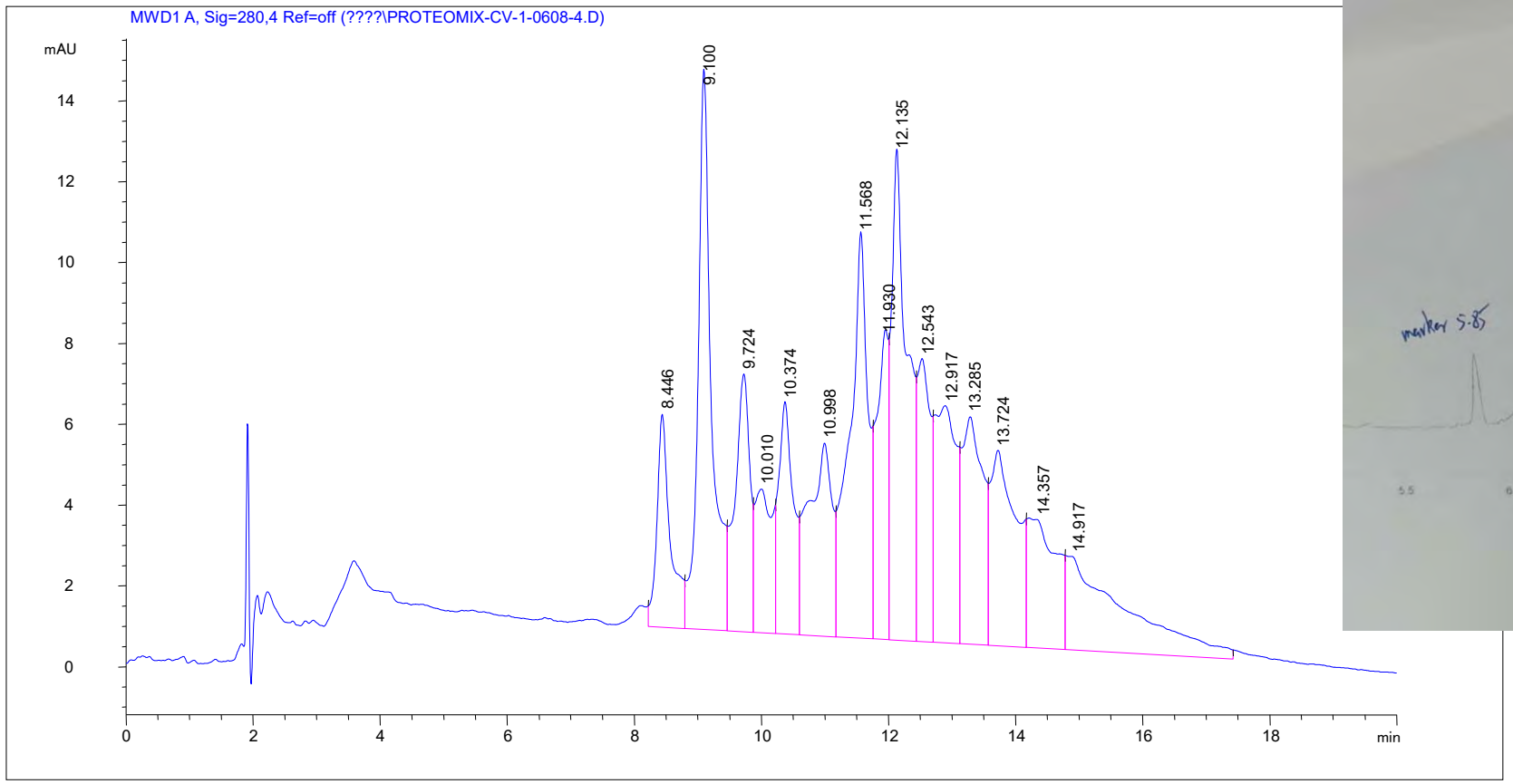


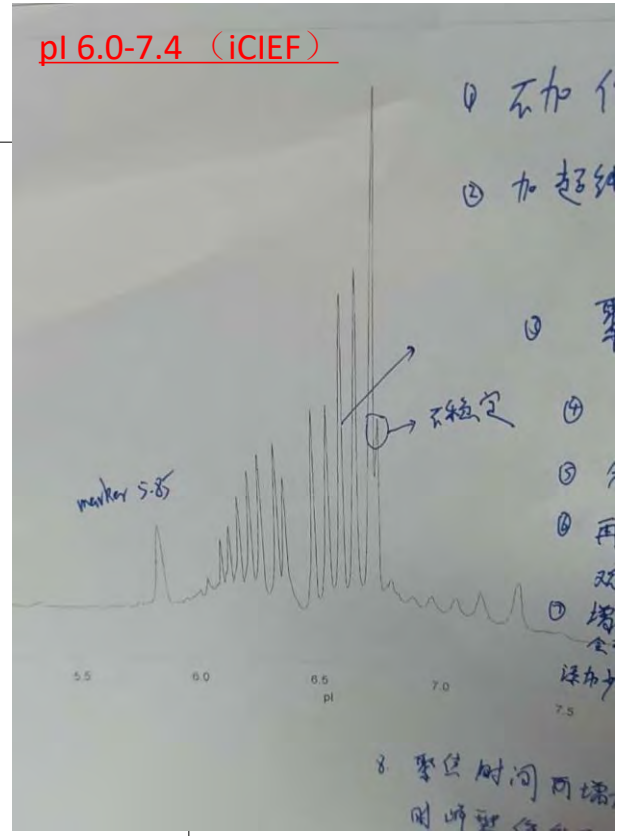
Time (min)	B%
2	10
32	23
32.5	100
38	100
38.5	10
60	10

Proteomix® SCX outperforms MAbPac™ SCX in both acidic and basic regions.

BsAb case — Proteomix CV-1

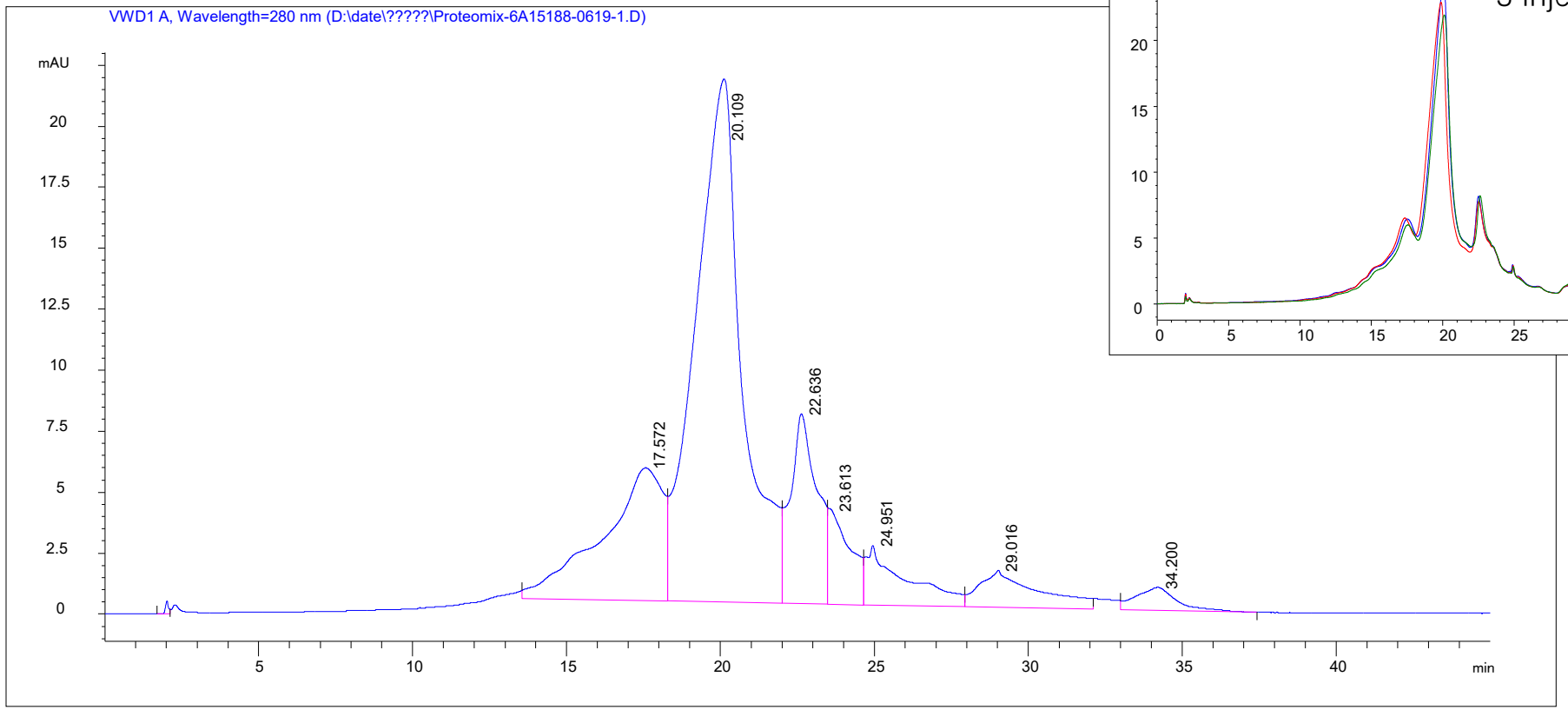
Column: Proteomix **CV-1**(5μm, 4.6×250mm)
Mobile phase:
Flow rate: 0.8 mL/min Detector: UV 280 nm Column : 30℃
Injection volume: 15 μL Sample: protein 2 mg/mL Column
Pressure：186 bar Instrument: Sepax 36





BsAb - Proteomix SCX

Column: Proteomix SCX-NP5 (5μm, 4.6×250mm)
Flow rate: 0.8 mL/min Detector: UV 280 nm Column Temperature : 30℃
Injection volume: 10 μL Sample: 4.8 mg/mL Column Pressure：131 bar
Instrument: Sepax 81 Date: 2020-06-19



IEX Maintenance & regeneration

IEX column storage:

In daily use, the Sepax IEX chromatographic column can be stored in the mobile phase A of the QC test. If it is not used for a long time, it is recommended to add 0.1% NaN_3 to phase A for storage to prevent bacterial growth.

IEX column cleaning:

After multiple uses, some samples may be adsorbed onto the inlet filter or resin, and when accumulated to a certain extent, there will be a phenomenon of pressure rise accompanied by peak pattern broadening. At this time, it is necessary to clean the column. The general process is to disconnect the detector, reverse connect the column, and rinse 10-15 times the column volume with cleaning solution at a flow rate below 50% of the maximum recommended flow rate.

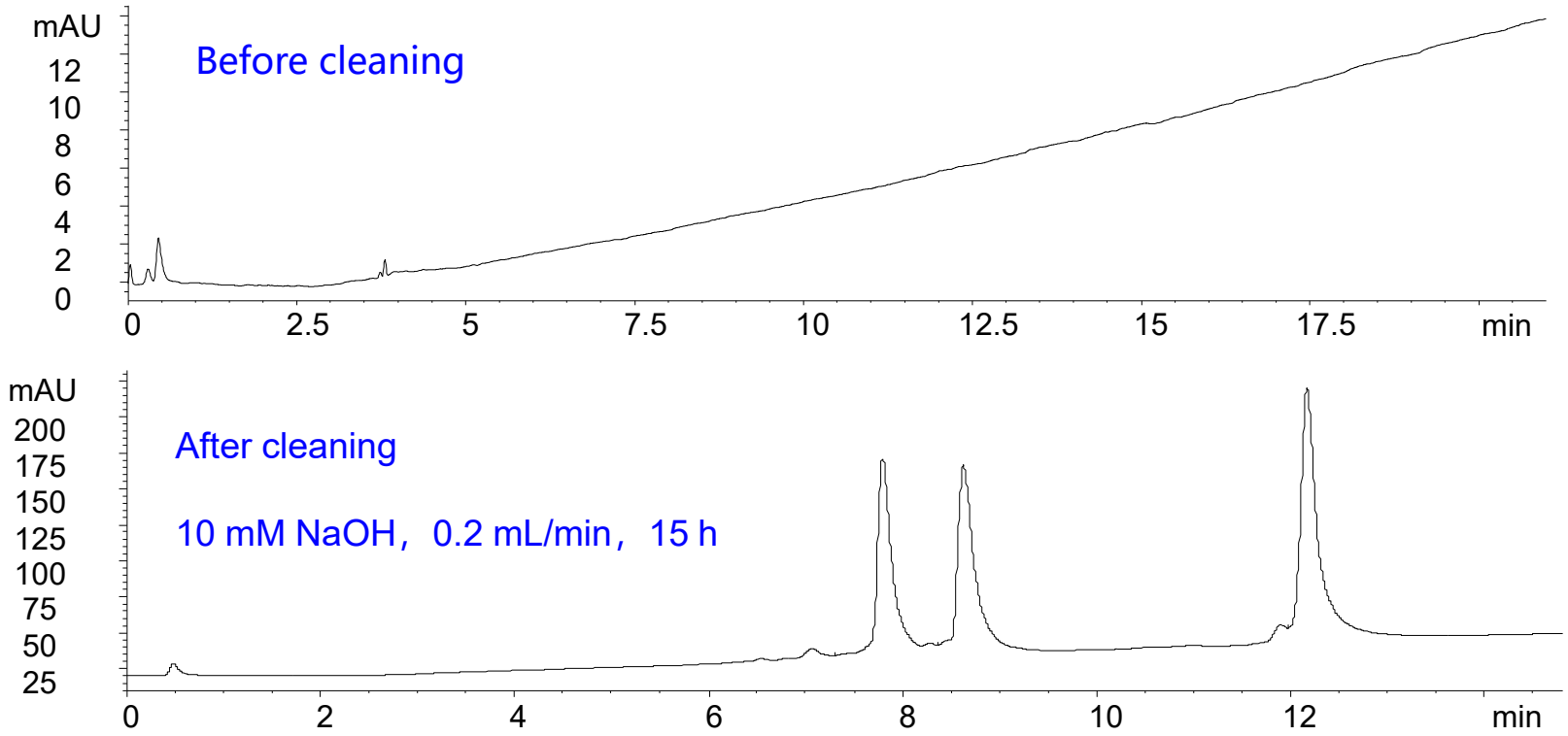
Recommended cleaning solution :

➢Proteomix CEX column: NaOH solution of less than 10 mM, 7 M guanidine hydrochloride, 10 mM EDTA · 2Na, or 50 mM phosphate buffer solution of 1.0 M NaCl (pH 10);

➢Proteomix AEX column: 75% acetonitrile solution with 150 mM potassium nitrate (pH 2), 0.1% TFA, 1 M HCl, 10 mM EDTA · 2Na, or 6 M urea

IEX Maintenance & regeneration

Before and after cleaning Proteomix SCX with 10 mM NaOH

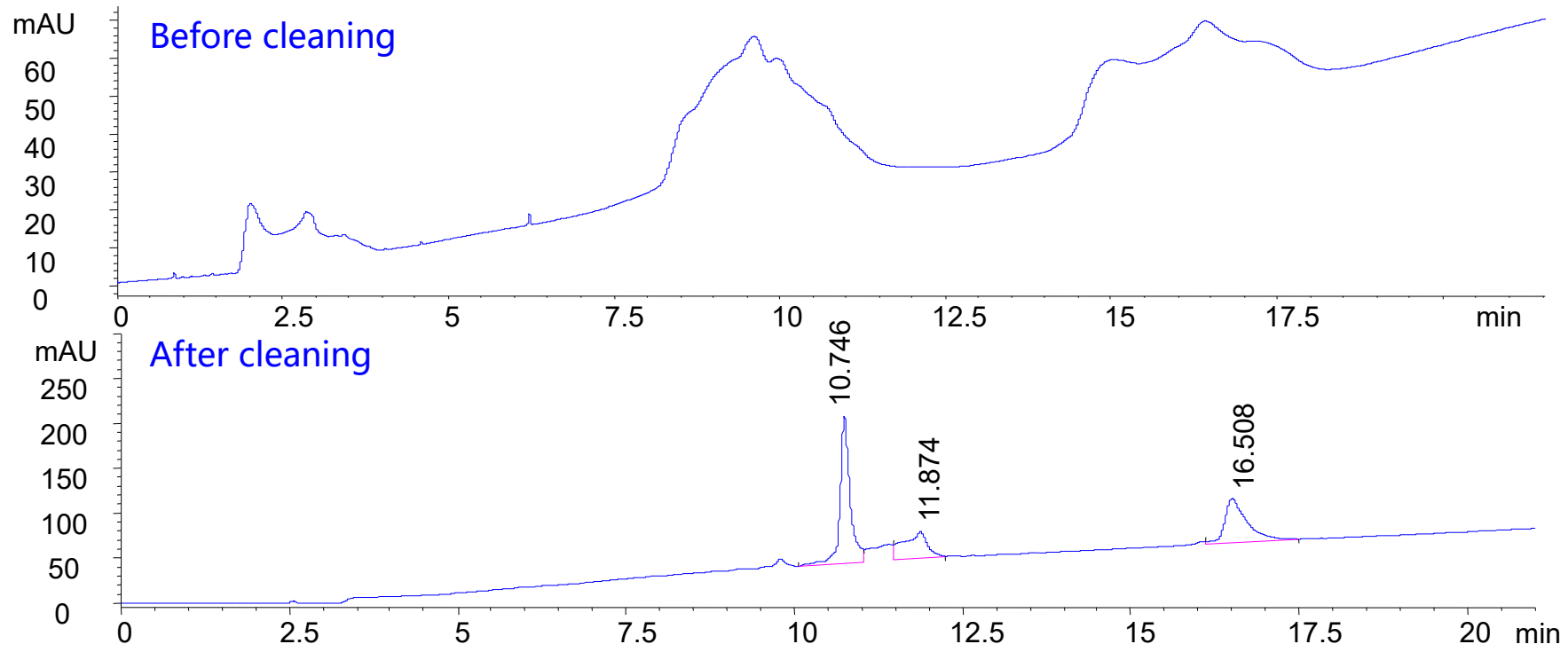


The IEX column was used for the detection of honey samples, and no peaks were found after long-term use. Use 10 mM NaOH solution for cleaning and retest. The sample peaks normally

IEX Maintenance & regeneration

Before and after cleaning Proteomix SCX with 7 M Guanidine hydrochloride

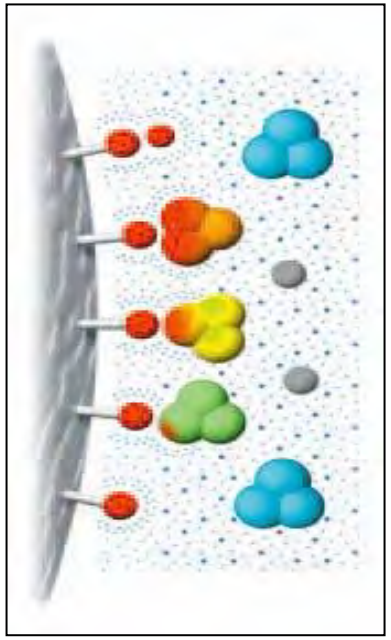
Using IEX column for semi preparative purification of a protein sample has resulted in a decrease in column efficiency after prolonged use. After the column is returned, the QC test shows that the peak is abnormal. inject 7 M guanidine hydrochloride solution to clean and analyze again. The peak is normal.



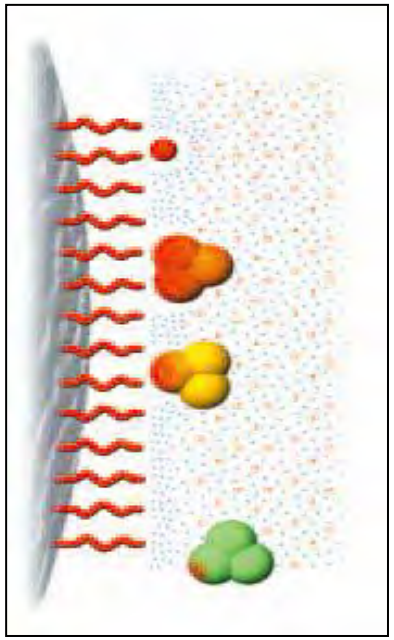
HIC and RP separation mechanism

Common of HIC and RP:

Based on the differences in the hydrophobicity of each component and the size of the interaction between the hydrophobic groups in the stationary phase matrix.



Hydrophobic interaction

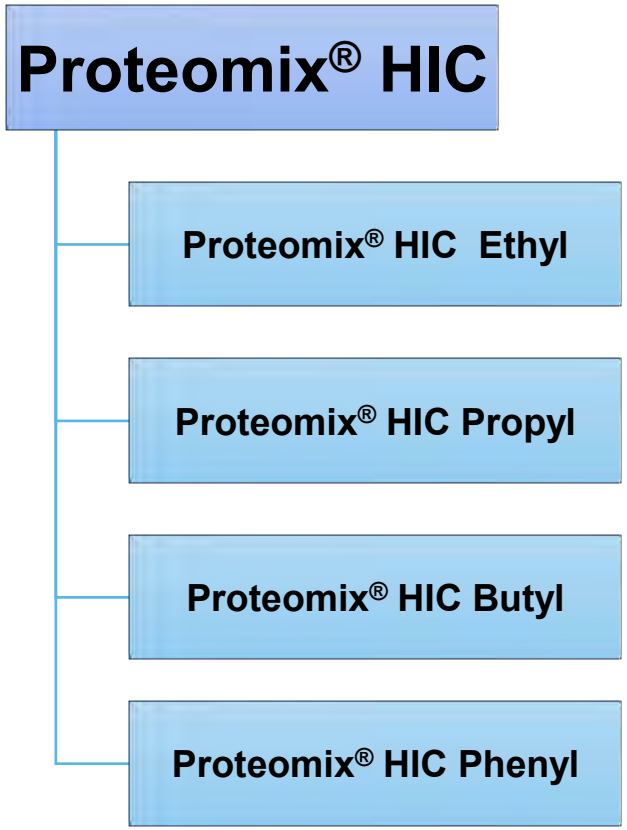


Reverse phase

Difference

Compared to HIC, the stationary phase of RP has stronger hydrophobicity. Therefore, when eluting, RP requires the use of organic solvents, such as methanol, acetonitrile, and HIC can be eluted using a milder salt phase. This elution environment can maintain the stability of biological macromolecules.

Sepax HIC column selection



Application:

- ✓ Purity analysis of proteins and polypeptides (including synthetic polypeptides);
- ✓ Analysis of DAR value of antibody coupled drugs (non denatured conditions)

Sample requirement:

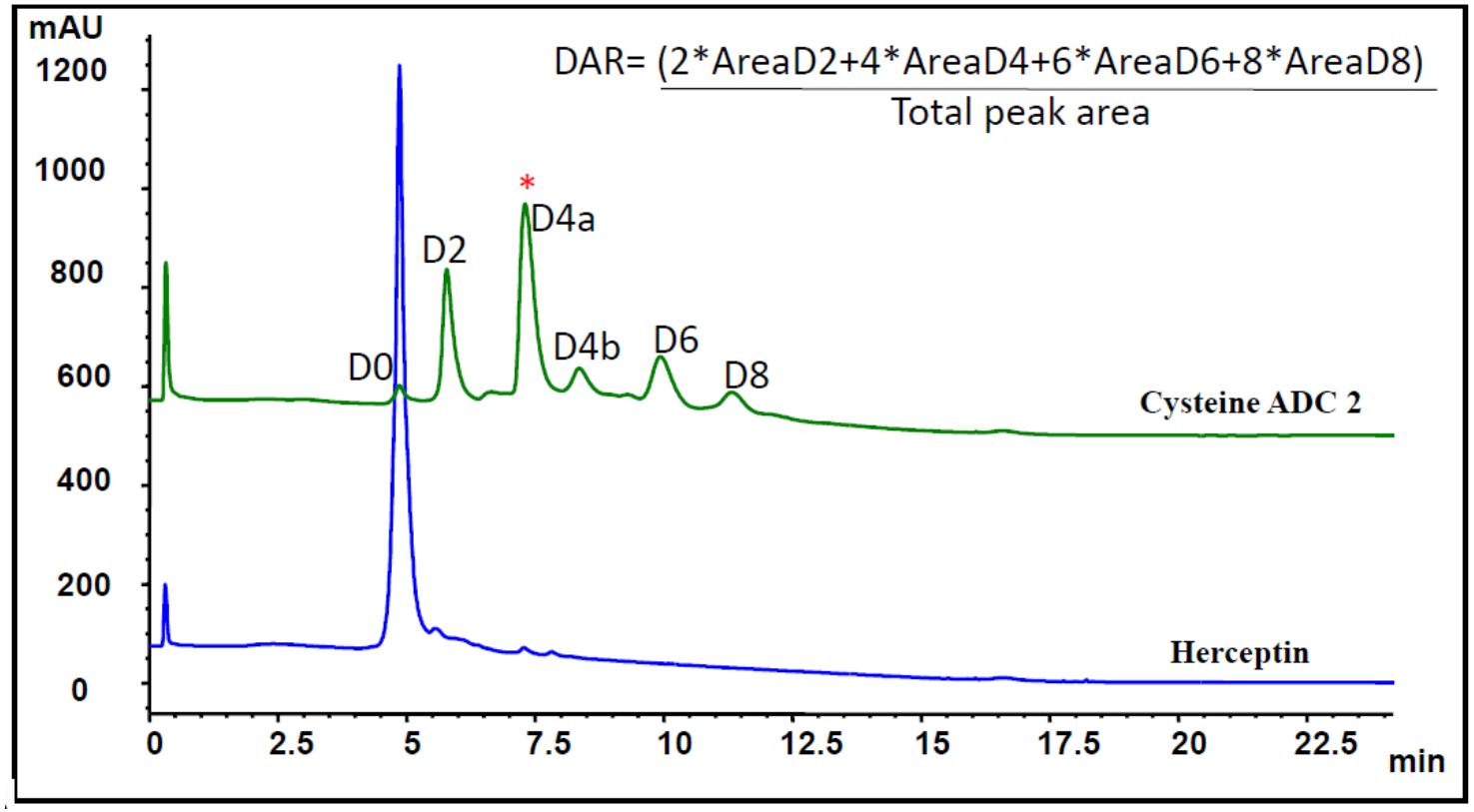
- ✓ Target analyte and other components or impurities may exist hydrophobic difference
- ✓ Sample components have good mutual solubility in selected mobile phase system

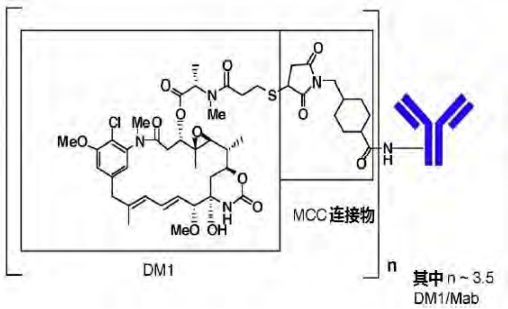
Column selection:

- ✓ If sample is unstable in an organic solvent system, HIC mode is preferred
- ✓ Determining the hydrophobicity of HIC columns

Hercetin-cysteine ADC DAR analysis

Column: Proteomix HIC Butyl-NP5 (5 µm, 4.6 x 35 mm)
Mobile phase: A: 2 M ammonium sulfate in 0.025 M sodium phosphate, pH 7.0,
B: 0.025 M sodium phosphate pH 7.0, C : 100% IPA
Flow rate: 0.8 mL/min Detector: UV 214 nm Column temperature: 25 ℃
Sample: Herceptin ADC, 1 mg/mL in 25 mM sodium phosphate Injection: 10 µL

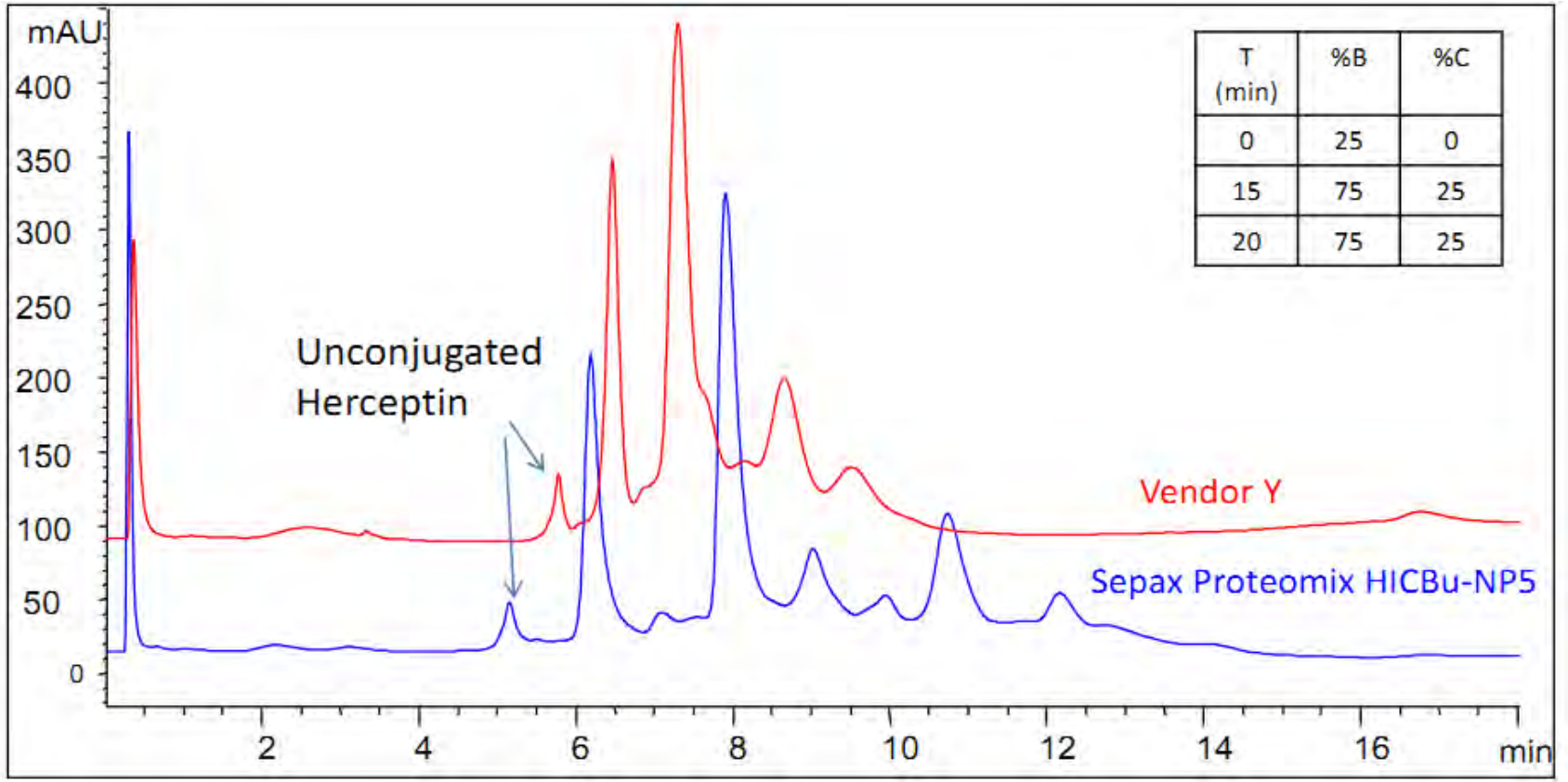




Using Proteomix HIC Butyl column can separate ADC samples with different DAR values and be used to calculate drug loading.

Hercetin-cysteine ADC separation

Column: Proteomix HIC Butyl-NP5 (5 μm, 4.6 x 35 mm) VS Vendor Y butyl
Mobile phase: A: 2 M ammonium sulfate in 0.025M sodium phosphate, pH 7.0,
B: 0.025 M sodium phosphate pH 7.0, C : 100% IPA ;
Flow rate: 0.8 mL/min Detector: UV 214 nm Column temperature: 25 ℃
Sample: ADC, 1mg/mL in 1M ammonium sulfate Injection: 10 μL

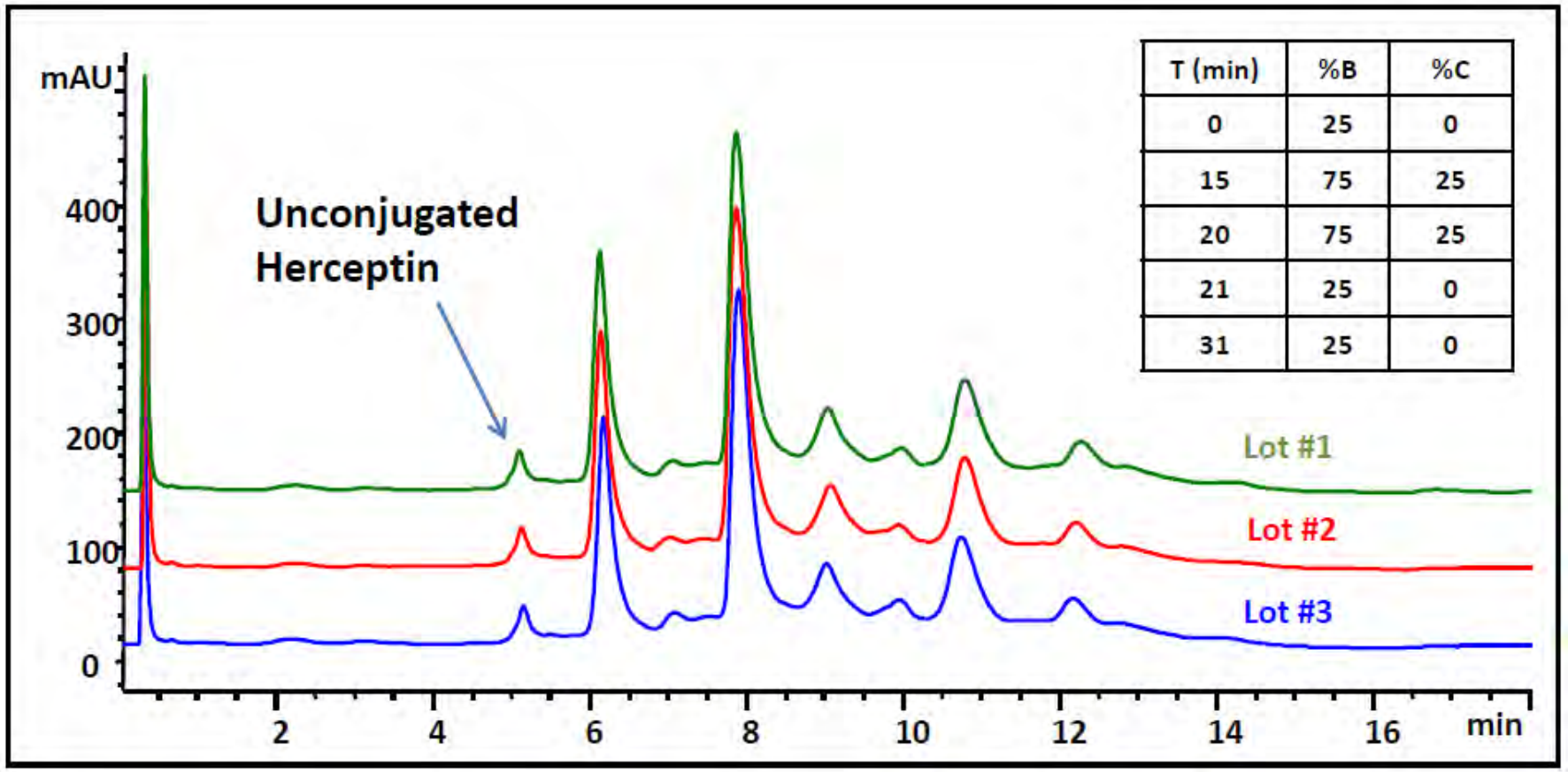


T (min)	%B	%C
0	25	0
15	75	25
20	75	25

Sepax Proteomix HIC Butyl VS Vendor Y butyl, better separation

Lot to lot consistency testing on Proteomix HIC

Sepax Proteomix HIC Butyl-NP5 for Herceptin-cysteine ADC separation Three resin

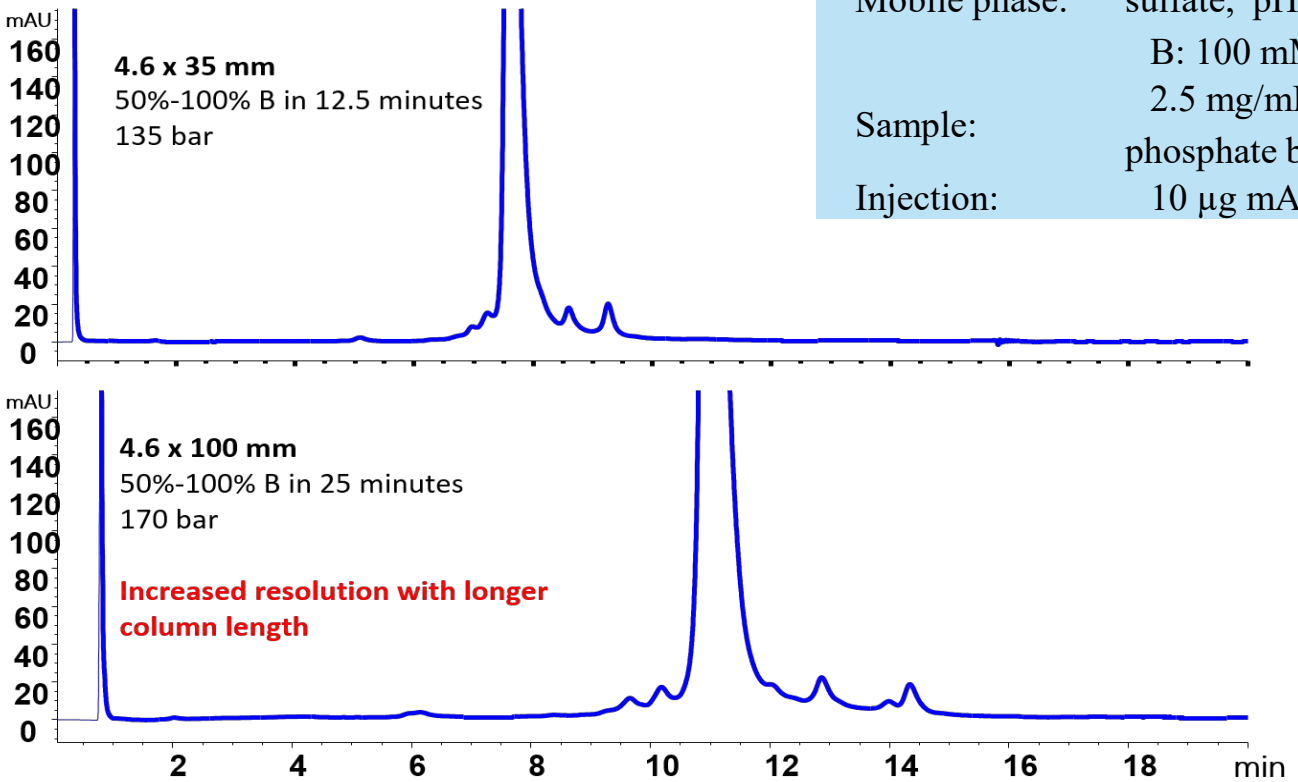


T (min)	%B	%C
0	25	0
15	75	25
20	75	25
21	25	0
31	25	0

The reproducibility of three Proteomix HIC Butyl columns with different resin batches was tested, and the peak positions and shapes of each chromatographic peak can overlap

Rituximab analysis on Proteomix HIC Butyl

Column:	*Proteomix*® HIC Butyl NP5 (4.6 x 35 mm), (4.6 x 100 mm),
Flow rate:	0.8 mL/min
Detector:	UV 214 nm
System:	UHPLC
Temperature:	25 ˚C
Mobile phase:	A: 100 mM sodium phosphate buffer, 2 M ammonium sulfate, pH 7.0 B: 100 mM sodium phosphate buffer, pH 7.0;
Sample:	2.5 mg/mL rituximab in 500 mM ammonium sulfate, 25 mM phosphate buffer
Injection:	10 µg mAb for 4603, 20 µg for 4610



Sepax RP columns

Proteomix® RP Polymer Matrix

Proteomix® RP-300（pore size：300 Å）
Proteomix® RP-500（pore size：500 Å）
Proteomix® RP-1000（pore size：1000 Å）

Silica Matrix

Bio-C18、C8、C4（pore size：200Å/300 Å）
HP-C18（endurance of 100% aqueous phase)
BR-C18（endurance of pH1.5~10.5）
….

Proteomix RP, based on highly crosslinked polystyrene/divinylbenzene (PS/DVB) resins, hydrophobic interactions are achieved through phenyl and surface modified alkyl groups.

Advantages of polymer matrix:

Compared to silica, more suitable for the separation of biological molecules and has a wide pH range (1-14)

High temperature resistance: up to 80℃

Efficient separation and specific selectivity of protein molecules and related protein fragments such as mAb and ADC;

Application of RP

Analysis of DAR value of ADC(polymer matrix, organic phase mobile phase)

Peptide map analysis, small molecule drug content analysis, etc. (silica matrix)

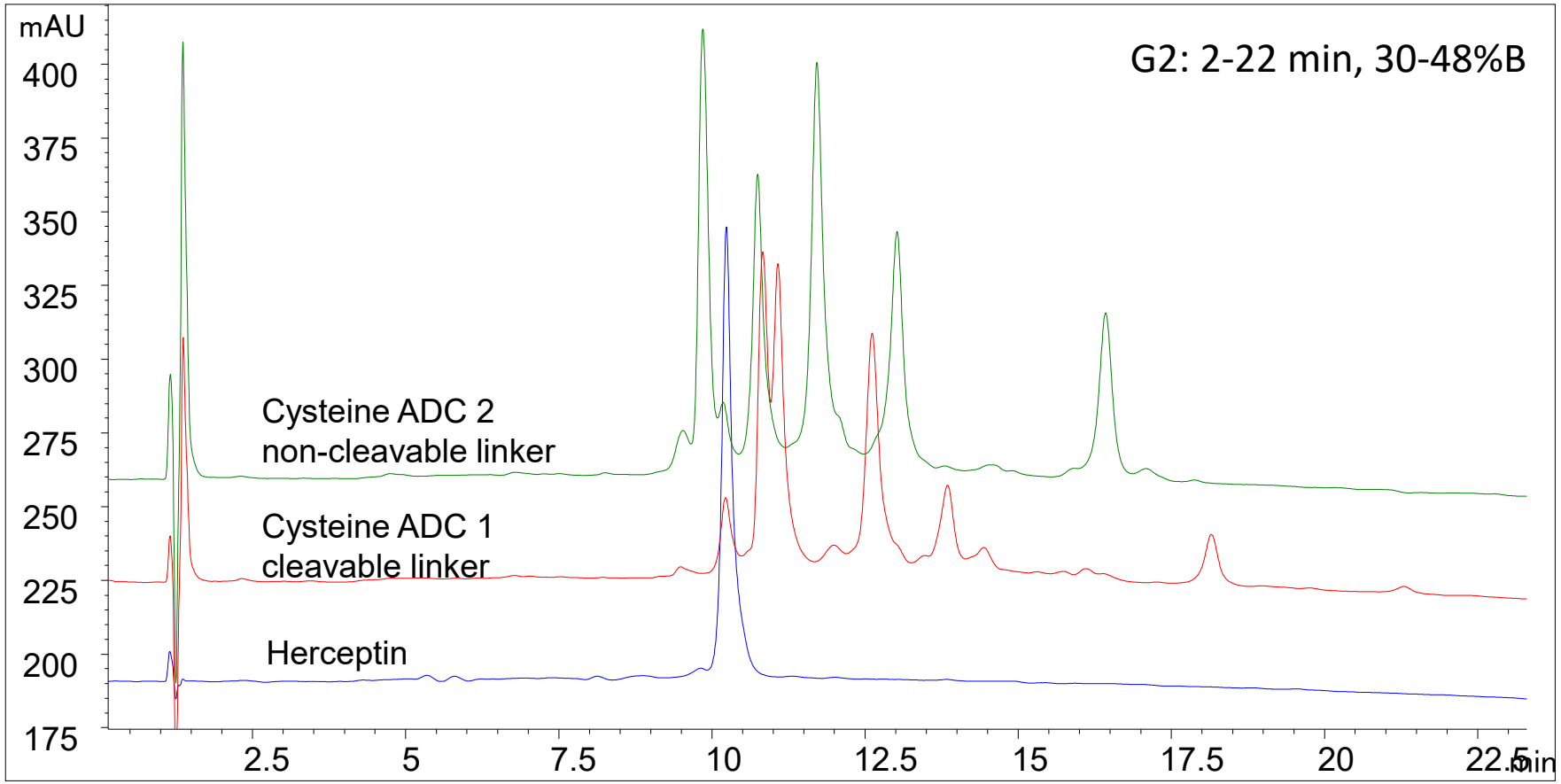
Herceptin and its ADCs Separation

Column: Proteomix RP-1000 (5 μm, 1000 Å, 4.6 x 100 mm)

Mobile phase: A: 0.1% TFA in water; B: 0.1% TFA in 100% ACN; Flow rate: 1.0 mL/min;

Detector: UV 210 nm; Column temperature: 80 °C Injection volume: 10 μL

Sample: Herceptin and ADCs 1 mg/mL diluted in 0.1% TFA



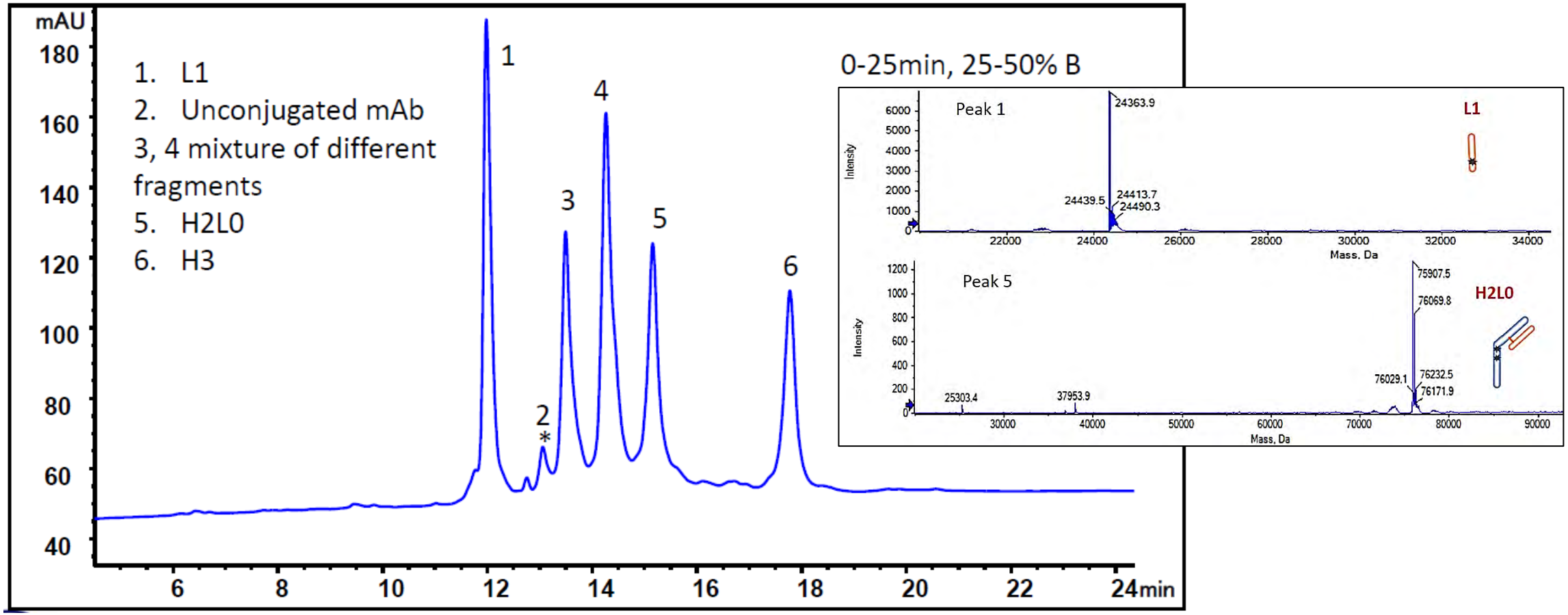
Herceptin Cysteine ADC Separation-MS

Column: Proteomix RP-1000 (5 μm, 1000 Å, 2.1 x 50 mm)

Mobile phase: A: 0.1% TFA in water B: 0.1% TFA in 100% ACN

Flow rate: 0.4 mL/min Detector: UV 210 nm-MS Column temperature: 80 ˚C;

Column pressure: 45 bar Sample: ADC diluted in water Injection volume: 3 μL cysteine ADC 2



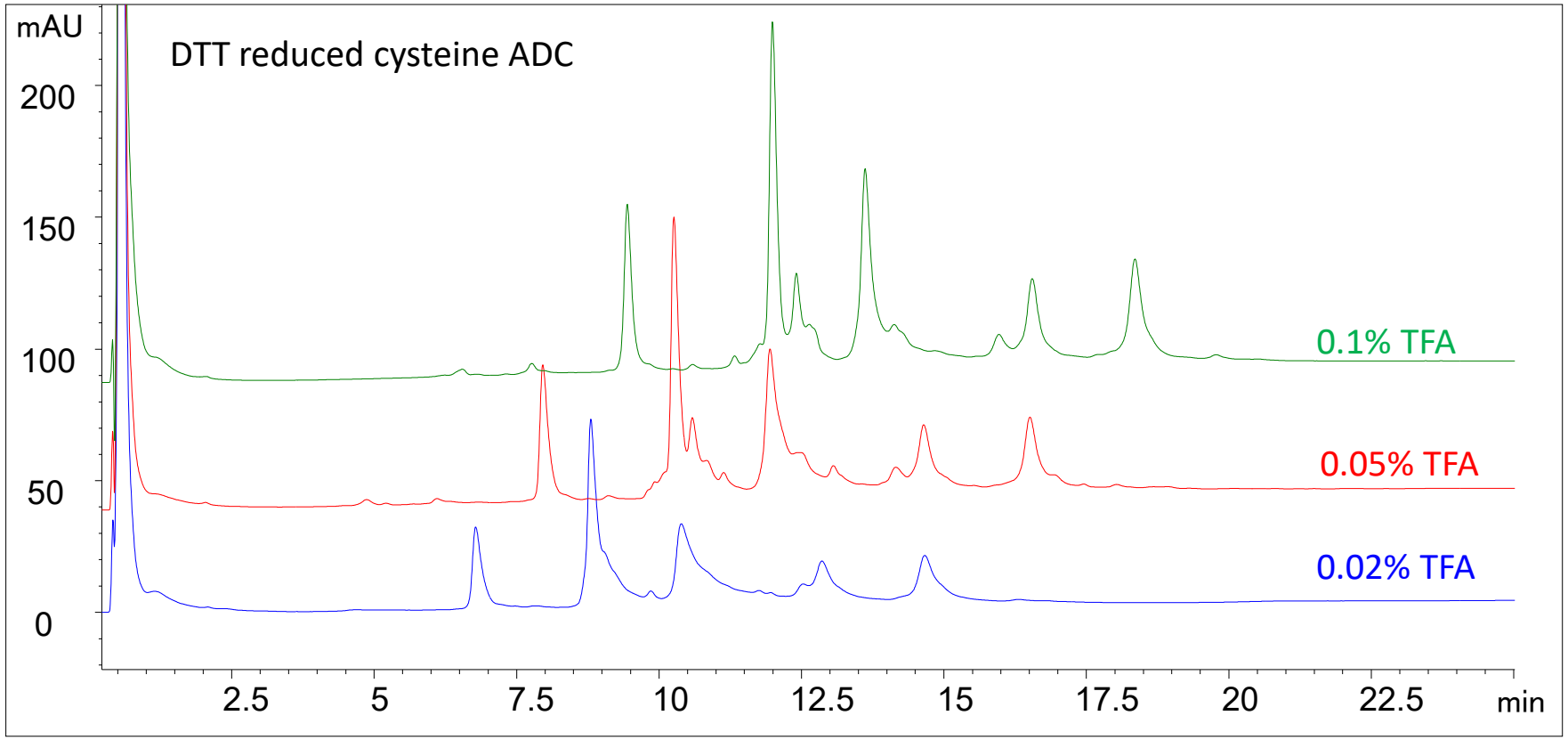
TFA effect on the ADC fragments separation

Column: Proteomix RP-1000 (5 μm, 1000 Å, 2.1 x 50 mm)

Mobile phase: A: X % TFA in water; B: X % TFA in 100% ACN;

Flow rate: 0.4 mL/min; Detector: UV 214 nm; Column temperature: 80 ˚C; Column pressure: 45 bar;

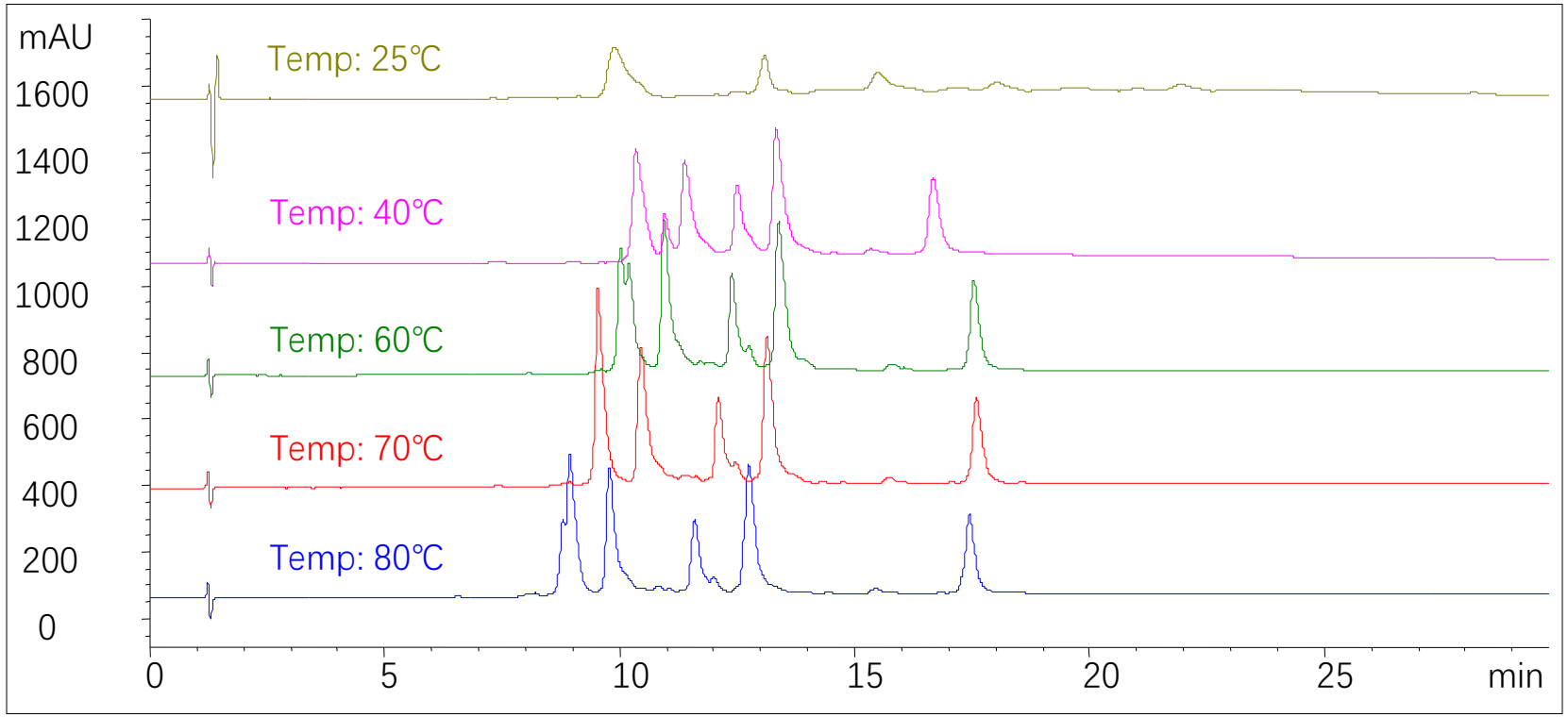
Injection: 3 μL for DTT reduced Herceptin cysteine ADC 0.1 and 0.05% TFA runs, 2 μL for 0.02% TFA



Under reversed phase conditions, higher content of TFA can better separate ADC samples after DTT reduction treatment, with significantly improved column efficiency and resolution

Column temperature effect on ADC separation

Column: Proteomix® RP-1000 (5 μm, 1000 Å, 4.6 x 100 mm);
Mobile phase: A: 0.1% TFA in water; B: 0.1% TFA in 100% ACN;
Flow rate: 1.0 mL/min Detector: UV 210 nm Column temperature: 25~80 ˚C;
Sample: Herceptin Cysteine ADC 2 1 mg/mL diluted in 0.1% TFA; Injection volume: 20 mL

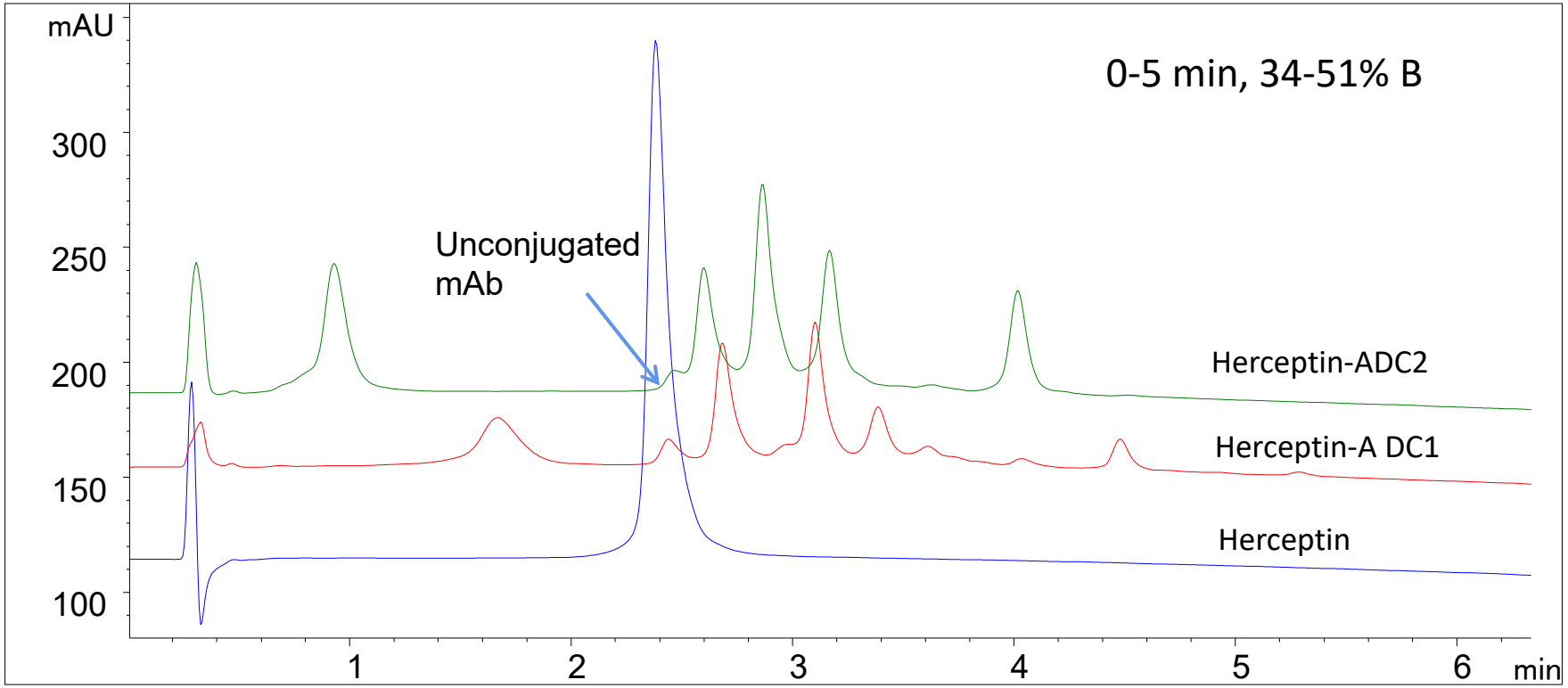


Time (min)	B%
0	32
2	32
22	48

Increasing temperature will promote changes in protein conformation, and conformational effects will increase peak broadening

Herceptin and its ADCs-fast analysis

Column: Proteomix RP-1000 (5 μm, 1000 Å, 2.1 x 50 mm)
Mobile phase: A: 0.1% TFA in water; B: 0.1% TFA in 100% ACN;
Flow rate: 0.6 mL/min; Detector: UV 210 nm; Column temperature: 80 ˚C; Column pressure: 70 bar;
Sample: Herceptin, ADC 1 and ADC 2; Injection volume: 0.5 μL for Herceptin, 1 μL for ADC 1 and ADC 2

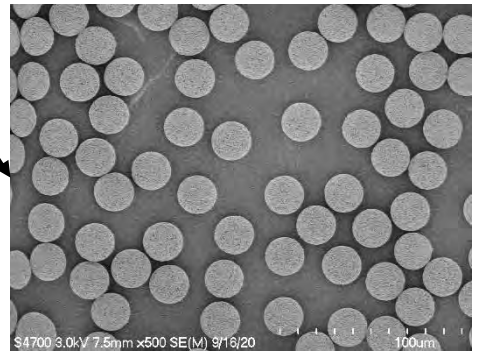


Proteomix RP-1000 for ADC sample quick analysis

Affinity column - ProAqa Excel

ProAqa Excel technical specifics

ProAqa Excel	Technical Specifics
Matrix	PS/DVB(20 μm, 1000-2000 Å)
Ligand	Recombination Protein A
Size (inner diameter × Length)	2.1 mm x 30 mm
Column material	PEEK or stainless steel
Maximum pressure	200 bar
pH range	1.2-13.0
Maximum flow rate	5 mL/min
Recommended flow rate	1-3 mL/min
CIP	0.1 M NaOH
Binding buffer pH	6.6-7.5
Life scale	>2000 injection
Standard injection	10 μL
IgG Detection concentration	0.029-40 mg/mL*
LOD	0.29 μg



ProAqa Excel SEM image, 20 μm
D90/D10<1.3

*(*UV 280 nm: 0.029 -7.500 mg/mL, UV 300 nm: 0.117-40.000 mg/mL)*

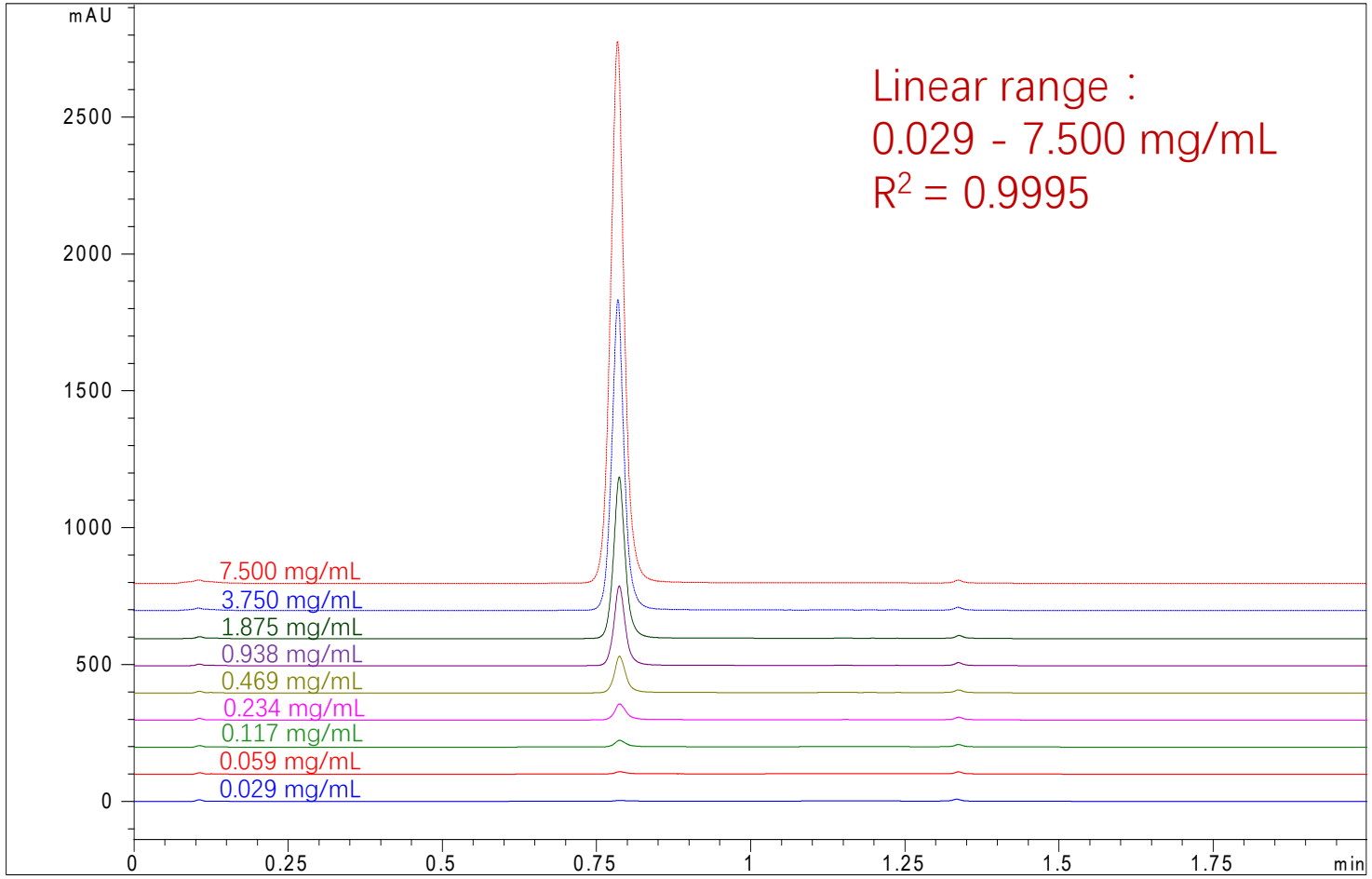
ProAqa Excel column

ProAqa Excel Affinity column introduction

This column is specifically designed for rapid and accurate antibody quantification during cell line screening or upstream bioprocess optimization and quality control processes. The resin is composed of uniform PS/DVB with an average particle size of 20 μm and a pore size of 1000-2000 Å coupled with a recombinant protein A ligand, which can bind to Fc containing immunoglobulins other than IgG_3, and is suitable for the determination of Fc immunoglobulin content in the supernatant of CHO cell culture at different levels.

- Rapid determination of antibody quantification
  - Flow rate upto 3 mL/min
  - The total cycle (including balance) time can be less than 0.5 min
- Linear response range up to 40 mg/mL :
  - UV 280 nm: 0.029 - 7.50 mg/mL, LOD 0.294 μg
  - UV 300 nm: 0.117 - 40.0 mg/mL
- Durability: > 2000 injection
- Excellent inter batch consistency
- Compatible with HPLC, UPLC, and FPLC systems

ProAqa Excel Column Test Linear Range and Detection Limit - UPLC

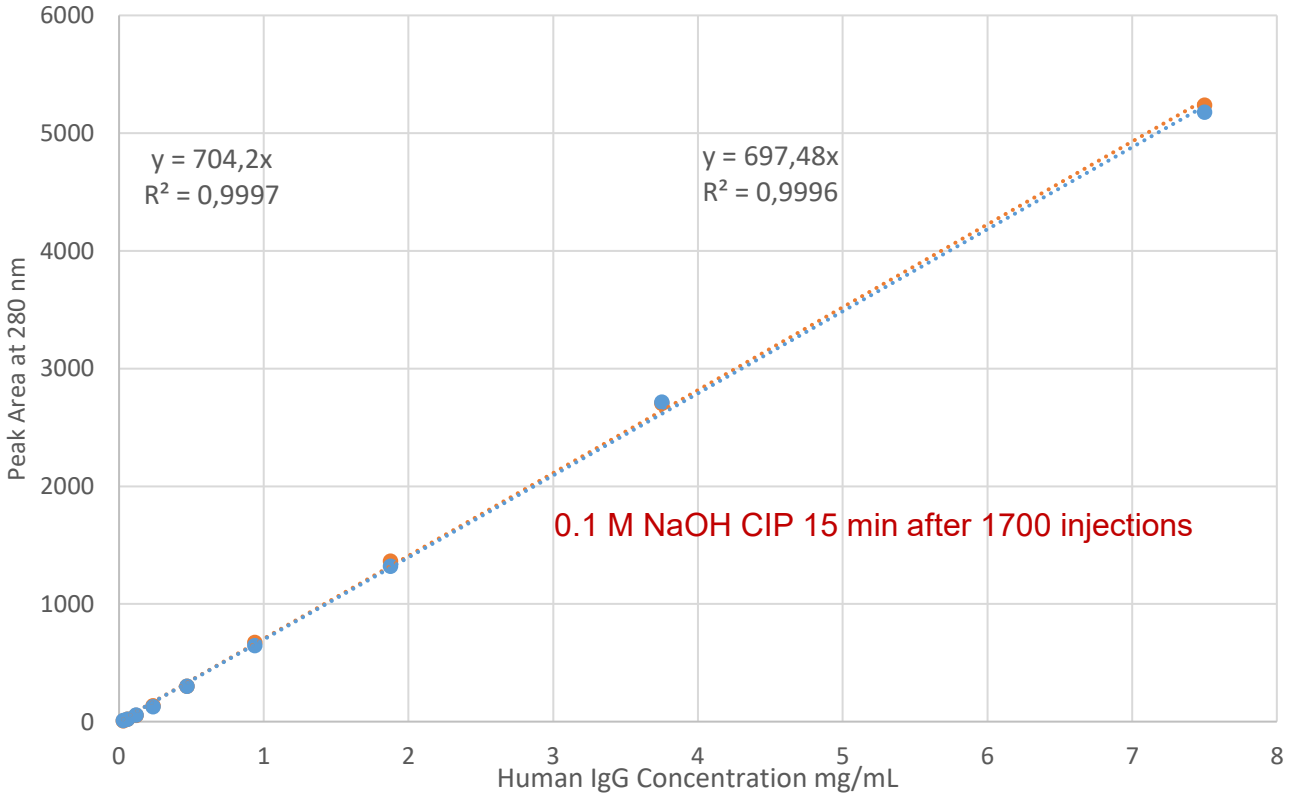


时间(min)	%B
0	0
0.1	0
0.11	100
0.60	100
0.61	0
2.00	0

ProAqa Excel

Column: ProAqa Excel 2.1×30 mm; Analyzer: Agilent 1260; Temperature: 25 °C;
Mobile phase: A: 50 mM Sodium phosphate, 150mM NaCl, pH 7.0; B: 100 mM glycine, pH 2.5;
Flow rate: 1 mL/min; Detection wavelength: 280 nm; Sample: hIgG solution; Injection: 10 µL

ProAqa Excel durability and linear range studies



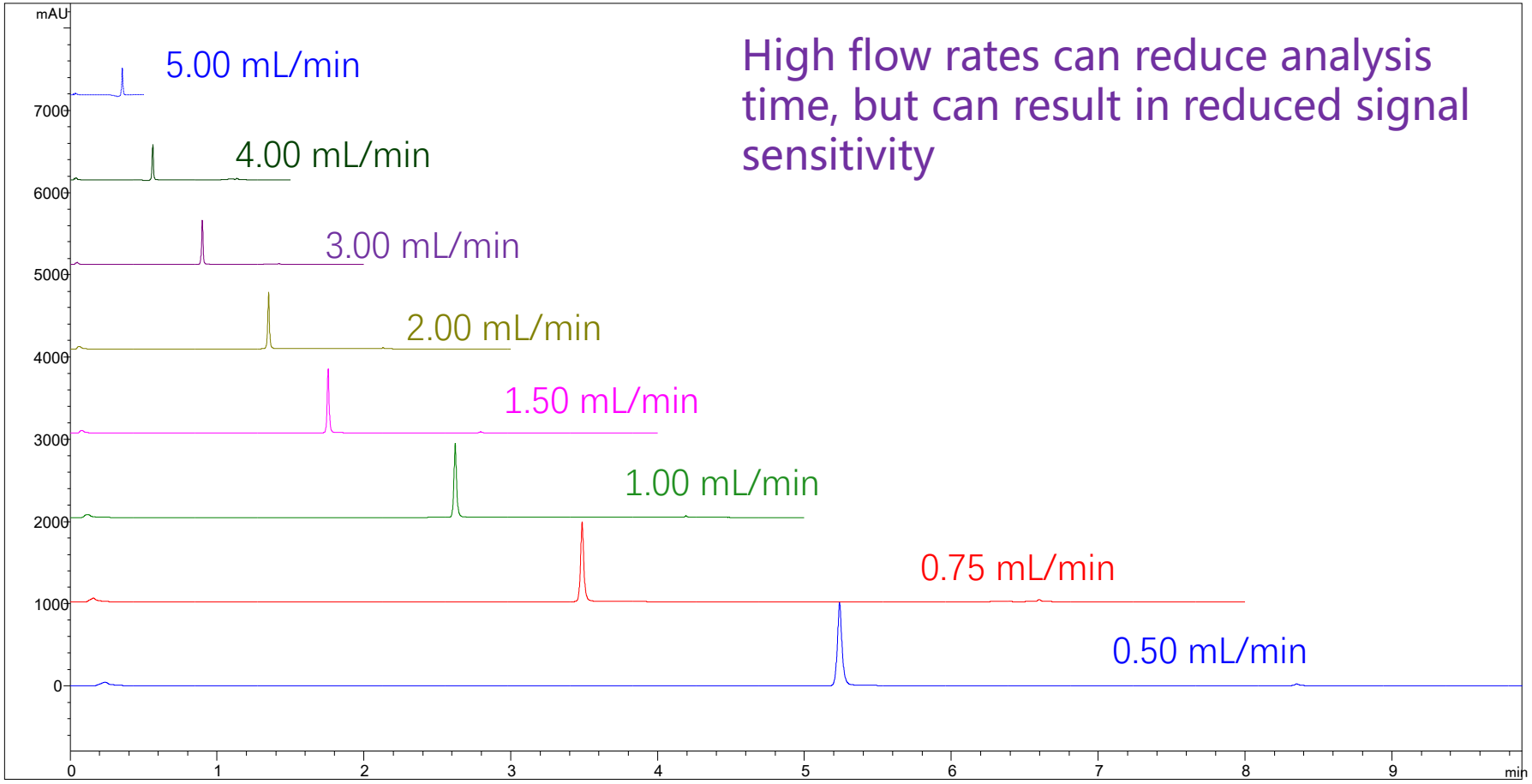
Injection #	Concentration(mg/mL)
1	0.029
2	0.059
3	0.117
4	0.234
5	0.469
6	0.938
7	1.875
8	3.750
9	7.500

RT(min)	%B
0	0
0.10	0
0.11	100
0.60	100
0.61	0
2.00	0

As shown in the figure, there is no significant change in the hIgG calibration curve. The column still maintains its high load and excellent linearity

ProAqa Excel flow rate study

Column: ProAqa Excel 2.1×30 mm SS; Analyzer: Agilent 1260; Temperature: 23 °C;
Mobile phase: A: 50 mM Sodium phosphate, 150 mM NaCl, pH 7.0, B: 100 mM glycine, pH 2.5;
Flow rate: as below; Detection wavelength: 280 nm; Sample: 2.0 mg/mL hIgG; injection:10 μL;

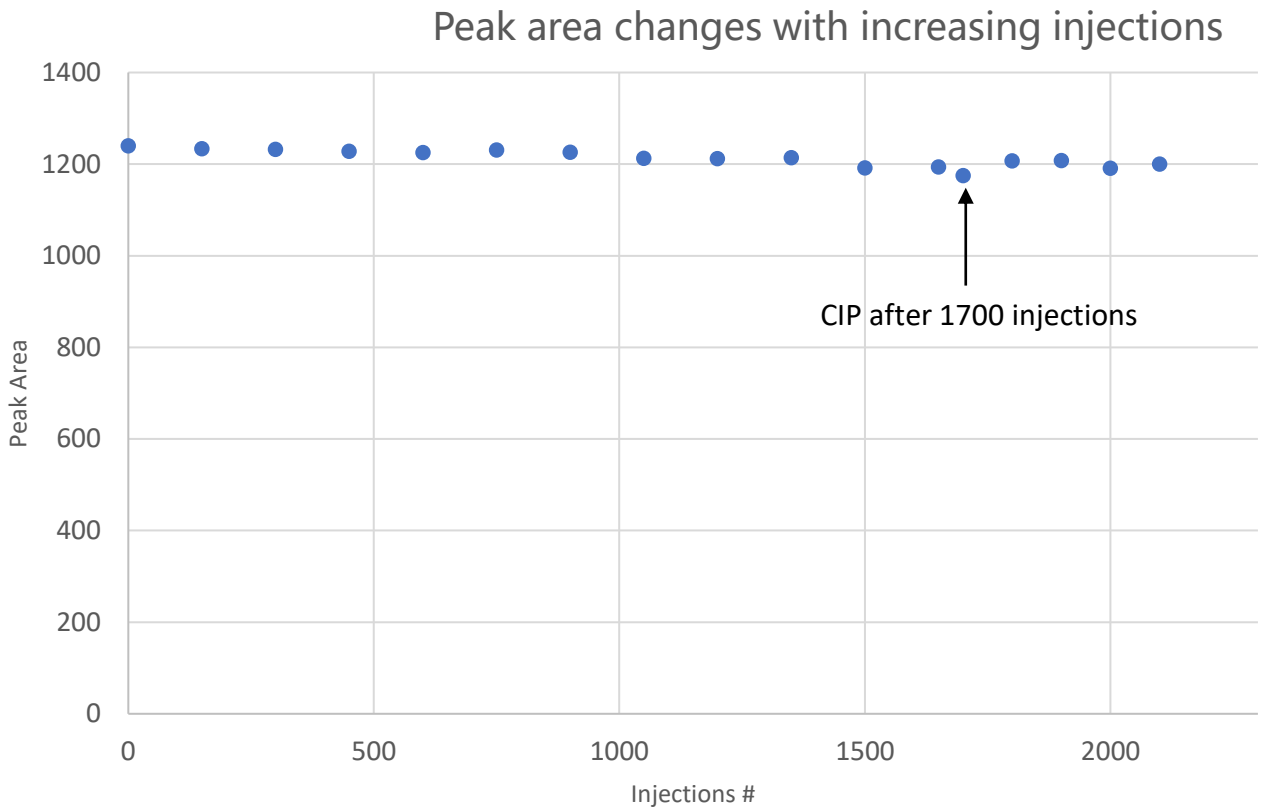


High flow rates can reduce analysis time, but can result in reduced signal sensitivity

ProAqa Excel lifetime test

Column: ProAqa Excel 2.1×30 mm; Analyzer: Agilent 1260; Temperature: 25 °C;
Mobile phase: A: 50 mM Sodium phosphate, 150mM NaCl, pH 7.0; B: 100 mM glycine, pH 2.5;
Flow rate: 1 mL/min; Detection wavelength: 280 nm; Sample: hIgG solution; Injection: 10 µL

Injection#	Peak area	Change rate%
0	1240	100.0
150	1234	99.5
300	1232	99.4
450	1228	99.0
600	1225	98.8
750	1231	99.3
900	1226	98.9
1050	1213	97.8
1200	1212	97.7
1350	1214	97.9
1500	1192	96.1
1650	1194	96.3
1700	1175	94.8
1800	1207	97.3
1900	1208	97.4
2000	1191	96.0
2100	1200	96.8



RT(min)	%B
0	0
0.10	0
0.11	100
0.60	100
0.61	0
2.00	0

The column is very durable, there are no significant changes in retention time, peak area, and symmetry after 2100 injections. (using 0.1 M NaOH CIP 15 min after 1700 injections)

Welcome to Visit Sepax

Thank you for watching!