A Chromatographic Separation of Biological Macromolecules

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Agilent
Trusted Answers

Outline

LC techniques for biomolecule analysis

Reversed phase

- Commonly used, wide column choice and denaturing conditions. Can be used for intact proteins or much smaller molecules, including peptides and amino acids.

Size exclusion

- Separation by size in solution (rather than molecular weight) under nondenaturing conditions, for example, aggregate analysis.

Ion exchange

- Separation of molecules differing in net charge, under nondenaturing conditions, for example, charge variants.

Hydrophobic interaction chromatography (HIC)

- Alternative to reversed-phase chromatography, capable of separating minor impurities arising from post-translational modifications, such as oxidation, under nondenaturing conditions.

Hydrophilic liquid interaction chromatography (HILIC)

- Typically used for very hydrophilic molecules; polar analytes, unlabeled amino acids, glycans.

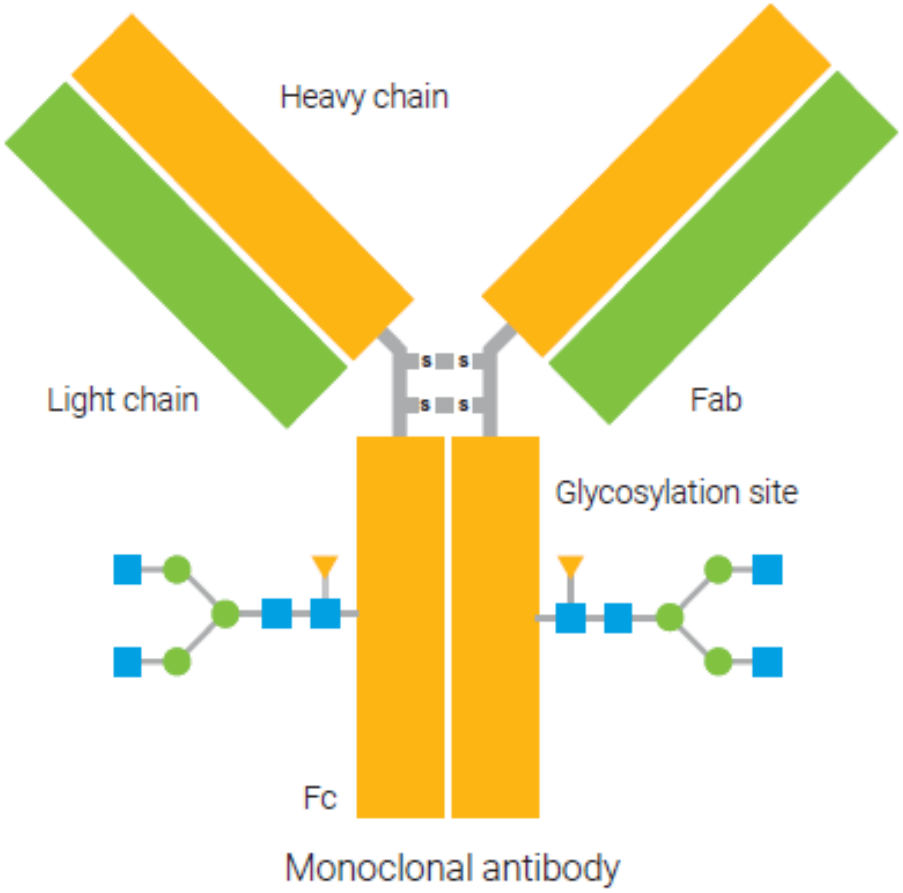
Affinity

- Biospecific binding to an immobilized ligand like Protein A.

What Is a Biomolecule?

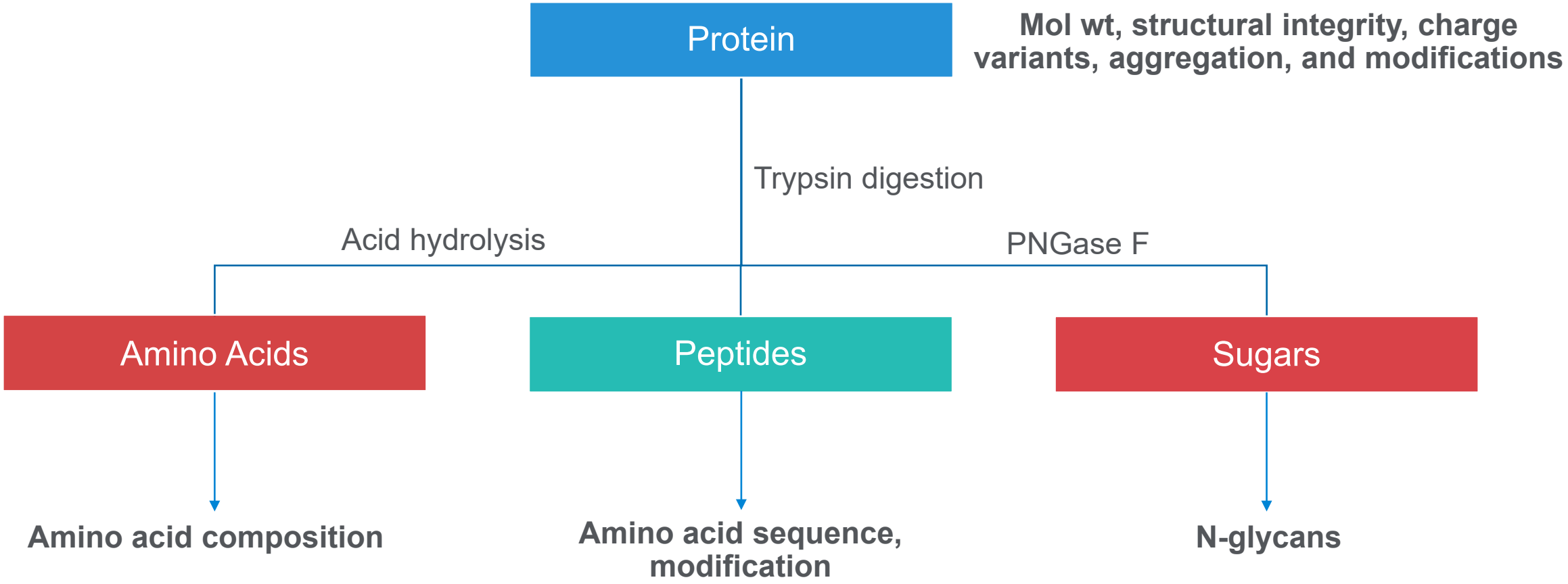
Biomolecules range in size from amino acids and small lipids to large polynucleotides, such as DNA or RNA.

- Proteins
- Peptides
- DNA/RNA oligonucleotides
- Amino acids



Protein Characterization

Different levels

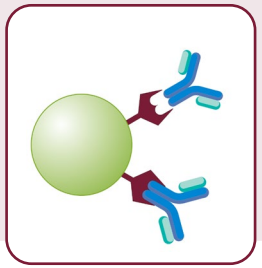


Biochromatography Separation Modes – LC Biocolumn Offerings

Titer Determination

Affinity

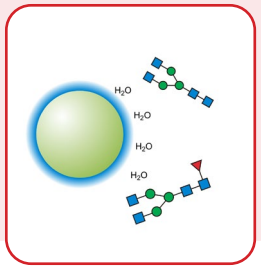
Isolation and quantitation of IgG



Glycan Analysis

HILIC

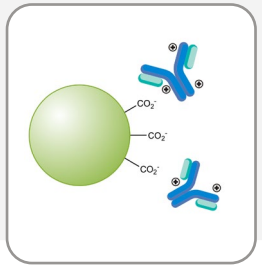
Fast, high-resolution, reproducible glycan separation



Charge Variant Analysis

Ion exchange

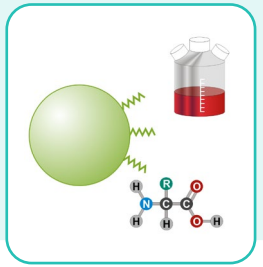
Enhances the accuracy and speed of separation



Amino Acid and Cell Culture

Small molecule (RP)

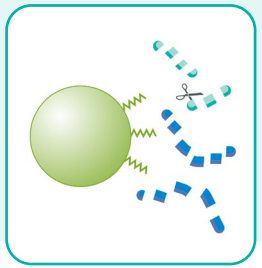
Delivers robust, high-resolution separations



Peptide Mapping

Reversed phase (RP)

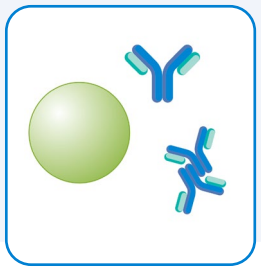
Reliably characterizes primary sequence and detects PTMs



Aggregate/Fragment

SEC

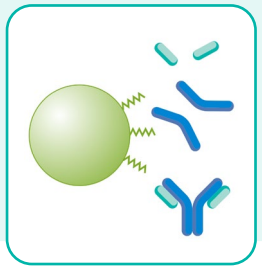
Solution for separating and analyzing intact proteins



Intact and Subunit Purity

Large molecule (RP)

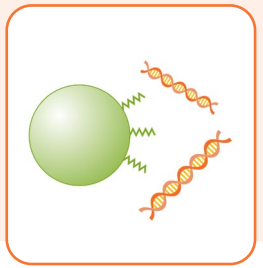
Selective options for every separation need



Oligonucleotide Analysis

Ion-pair RP or SAX

Delivers robust, high-efficiency separations for DNA/RNA



Reversed-Phase Particle Properties for Intact Proteins

Base particle chemistry – polymer or silica?

- Polymeric particles overcome the challenges associated with silica – poor stability at high pH and unreacted silanol groups – but have lower back pressure tolerance.

Bonded phase chemistry – C3, C4, C8, or diphenyl?

- The larger the protein, the shorter the alkyl chain should be
- Diphenyl offers a unique selectivity option
- Increased mol wt = decreased ligand size
  - < 70 kDa – C18
  - > 70 kDa – C8, C4, C3, diphenyl

Particle size – < 2 µm to 5 µm or higher

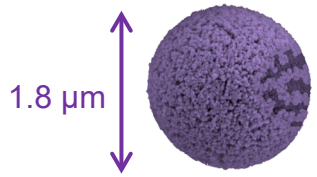
- Smaller particles give higher resolution, but also higher back pressure

Pore size – anywhere from 300-1000 Å

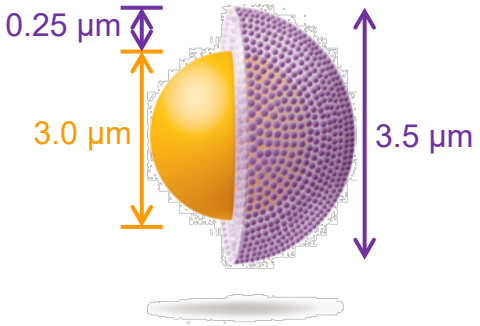
- Pores should be ≥ 3x the hydrodynamic radius of the protein
- 300 to 500 Å is most common, but 1000 Å is increasing in popularity

Particle morphology – totally porous or superficially porous?

- Totally porous gives maximum resolution, but also high back pressure
- Superficially porous gives almost as much efficiency at about half the back pressure



ZORBAX RRHD 300SB
300 Å pore size
0.9 µm diffusion depth



RP mAb
450 Å pore size
0.25 µm diffusion depth

Reversed-Phase Particle Properties for Intact Proteins

Start at low pH with simple aqueous/organic gradient (ZORAX 300A SB-C8 column)

- Mobile phase:
  - A: 95:5, H_2O:ACN with 0.1% TFA
  - B: 5:95, H_2O:ACN with 0.085% TFA
- Gradient: 5 to 70% in 60 min
- Temperature: 35 to 40
- Flow rate: 1.0 mL/min (5 µm, 4.6 mm id column)
- Ion pair agents: Typically, TFA is used, but for LC/MS, substitute with formic or acetic acid

Optimize organic modifier (in order of increasing polarity)

- Methanol → Acetonitrile → N-propanol → THF

Increase temperature

- Higher column temperature can improve resolution and recovery
- Check for temperature compatibility with respect to the specific column in use

Obtain best resolution by optimizing:

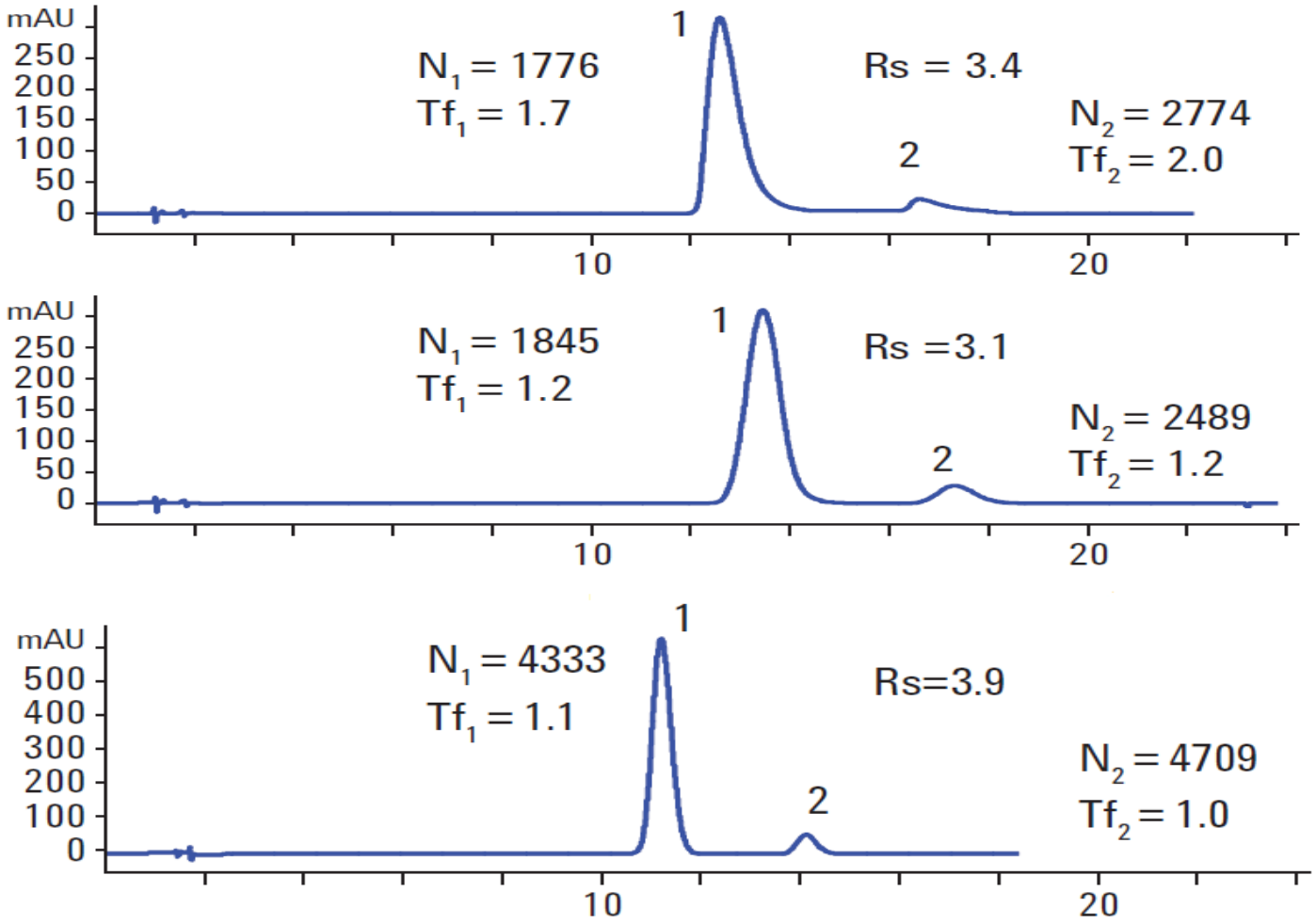
- Gradient steepness, bonded phase, temperature, column configuration

Obtain best recovery by optimizing:

- Bonded phase, temperature, sample solubility

Pore Size

Efficiency and resolution



1. Porcine insulin
2. A-21 desamido insulin

Agilent ZORBAX SB-C18
80 Å

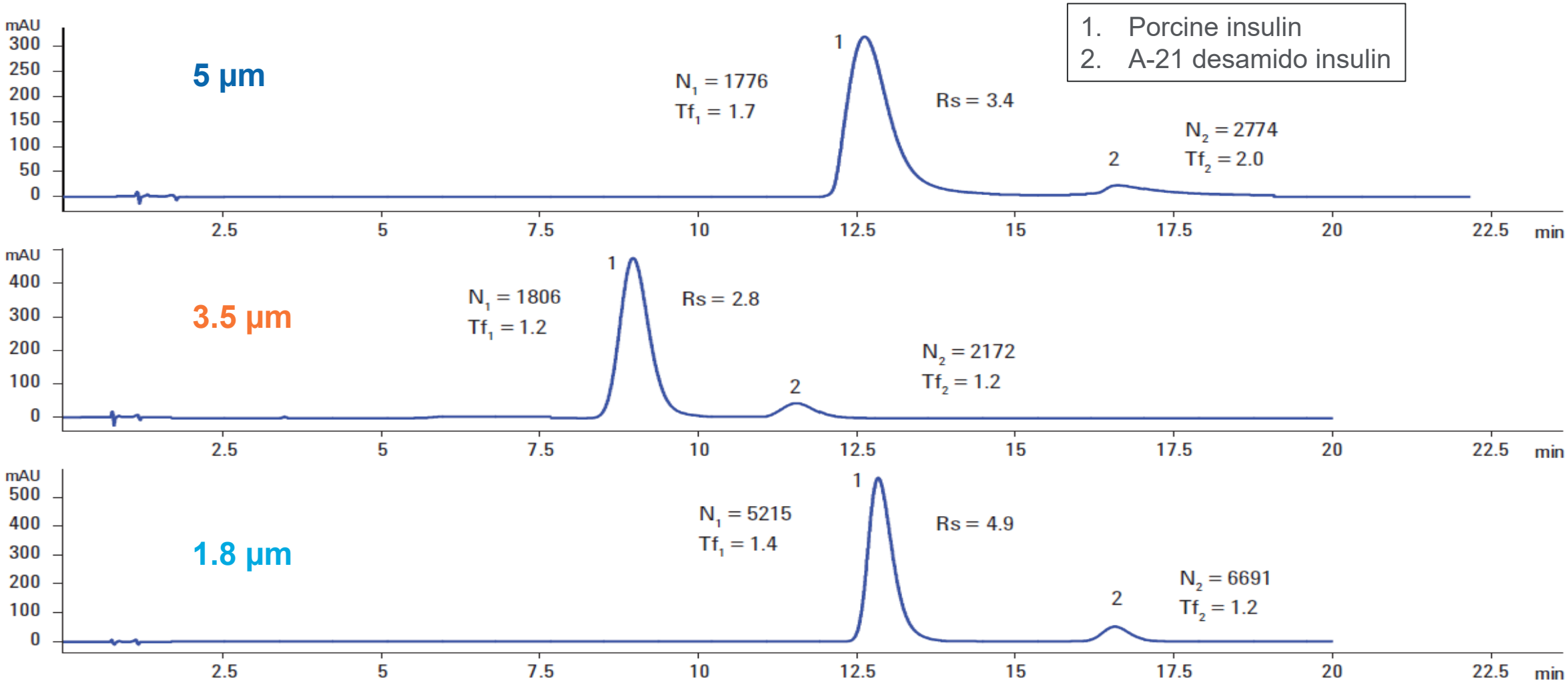
Agilent ZORBAX Eclipse Plus C18
95 Å

Agilent ZORBAX 300SB-C18
300 Å

Columns used were 4.6 x 150 mm, 5 µm, mobile phase: 74% A and 26% B, A: 0.2 M sulfate, pH 2.3, B: acetonitrile, 1.0 mL/min, 40 °C, 20 µL injection, DAD: 214 nm. Agilent publication: 5990-9028EN

Particle Size

Efficiency and resolution

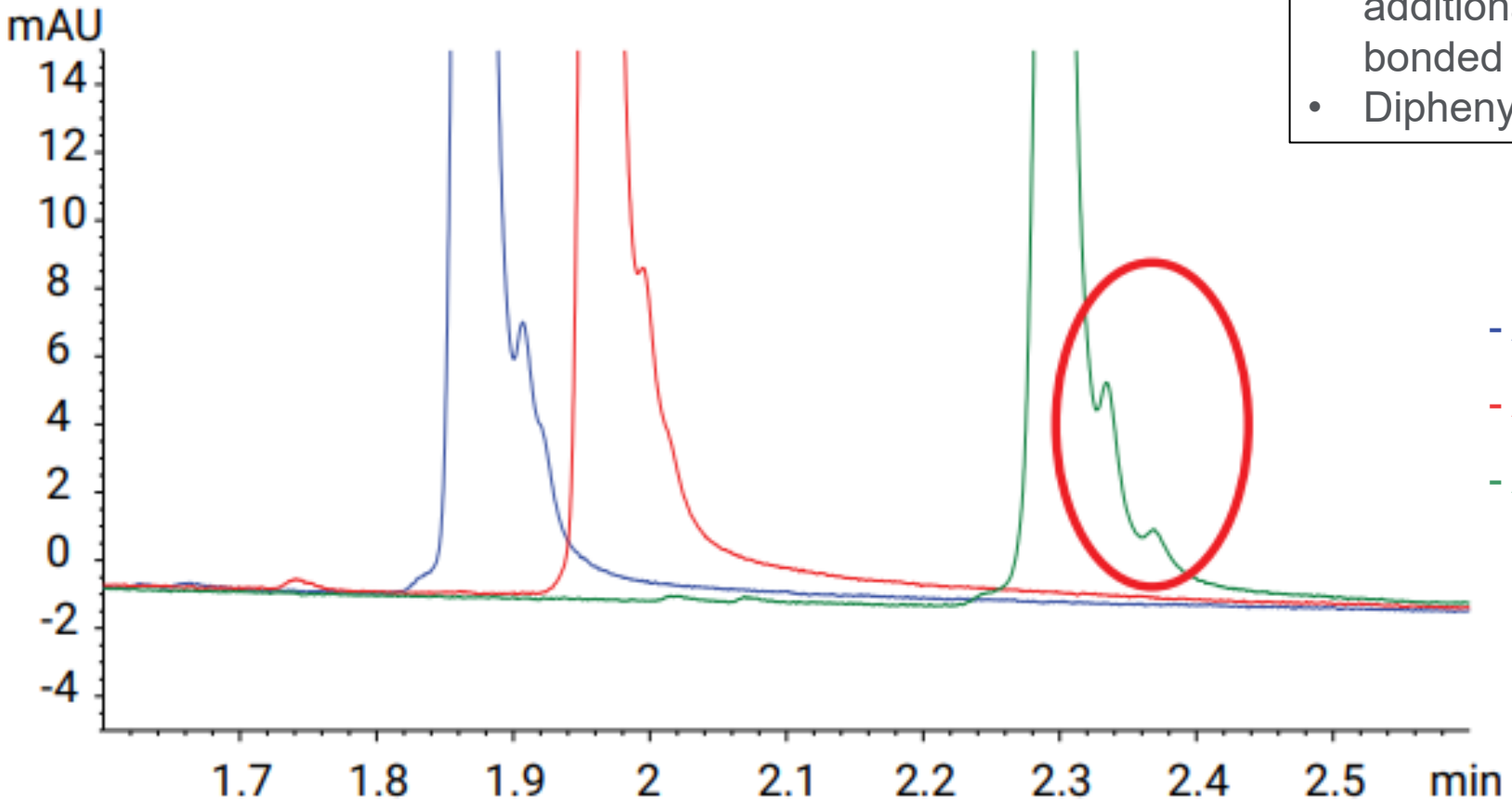


Columns used were ZORBAX SB-C18, 4.6 x 100 mm, mobile phase: 74% A and 26% B, A: 0.2 M sulfate, pH 2.3, B: acetonitrile, 1.0 mL/min, 40 °C, 20 µL injection, DAD: 214 nm. Agilent publication: 5990-9028EN

Bonded Phase Comparison – Intact mAb Analysis

Fast and high-resolution separation

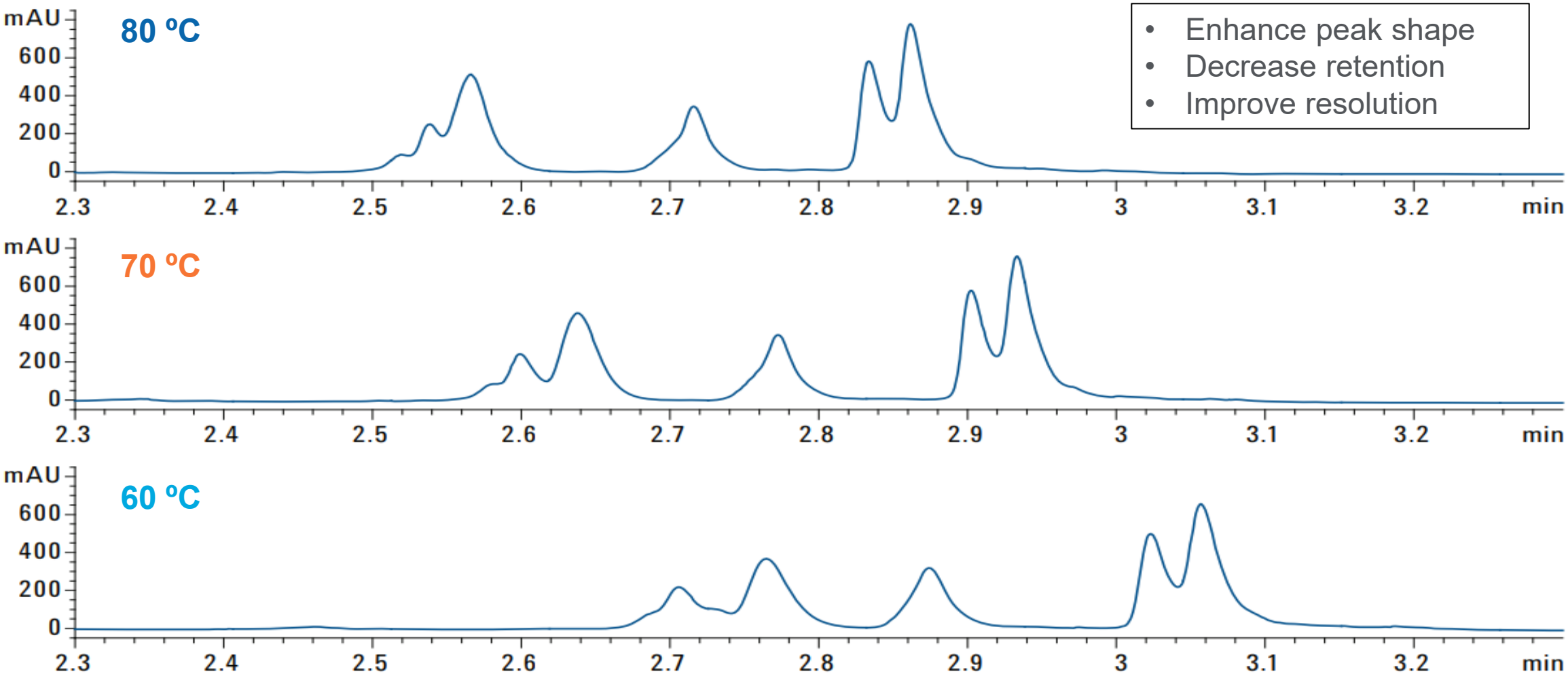
- AdvanceBio RP-mAb Diphenyl resolves additional fine detail compared to other bonded phases
- Diphenyl is unique to Agilent



- AdvanceBio RP-mAb C4
- AdvanceBio RP-mAb SB-C8
- AdvanceBio RP-mAb Diphenyl

Columns used were 2.1 x 100 mm, 3.5 µm, A: 0.1% TFA in water (98:2), B: IPA:ACN:Eluent A (70:20:10), 1.0 mL/min, gradient: 10-58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B, 80 °C, 5 µL injection, DAD: 254 nm. Agilent publication: 5994-1501EN

Temperature Dependance – Intact mAb Analysis



- Enhance peak shape
- Decrease retention
- Improve resolution

Columns used were AdvanveBio RP-mAb Diphenyl, 2.1 x 100 mm, 3.5 µm, A: 0.1% TFA in water, B: IPA:ACN:Eluent A (80:10:10), 1.0 mL/min, Gradient: 5-50% B in 5 min, 60 °C, 1 µL injection, DAD: 220 nm. Agilent publication: 5991-2032EN

Choosing a Column Chemistry

For LC/MS work, PLRP-S with formic acid is the first choice.

- Best peak shape with formic acid, unique selectivity versus silica and alkyl column, excellent chemical and thermal stability

For LC/UV work, AdvanceBio RP mAb with TFA is the first choice.

- Excellent peak shape with TFA, multiple chemistries available (C4, C8, diphenyl)

Reminder 1: Try a different column for different selectivity to separate a key pair.

Polymeric	vs	"Regular" silica chemistry
PLRP-S		AdvanceBio RP mAb
AdvanceBio Desalting-RP		ZORBAX RRHD
		ZORBAX 300SB
		Poroshell 300

Reminder 2: TFA and formic also change selectivity.

It is acceptable to use formic acid with UV, and if needed it is ok to use TFA with MS

Agilent Biomolecule Columns Portfolio

Protein Therapeutics								Oligonucleotides		Vector Therapeutics		
Titer Determination	Aggregate Analysis	Intact Purity and PTM Analysis		Peptide Mapping and PTM Analysis	Charge Variant Analysis	Glycan Analysis	Amino Acid/Cell Culture Media Analysis	Purification and Impurity Analysis		Aggregation	Empty/Full	Capsid Identity
Affinity	Size Exclusion	Reversed phase > 150 Å	Hydrophobic Interaction	Reversed phase < 150 Å	Ion Exchange	Hydrophilic Interaction	Reversed phase < 150 Å	Reversed phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase
Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis	AdvanceBio Oligonucleotide	PL-SAX	Bio SEC-5	Bio SAX	ZORBAX RRHD 300 Å, 1.8 µm
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm	PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)				Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX							
	Bio SEC-5	ZORBAX 300SB 3.5, 5 and 7 µm			Bio-Monolith (QA, DEAE, SO3)							
	ZORBAX GF250 & GF450	Poroshell 300 5 µm										

Stainless steel (SS) column hardware

Solid PEEK or PEEK-lined SS bioinert column hardware

Hydrophobic Interaction Chromatography (HIC)

Protein purification applications

Separates protein variants based on differences in hydrophobicity

Utilizes nondenaturing mobile phase solutions to preserve the biological activity of the intact proteins (native state)

- Aqueous eluents with buffer and salt

Characterization tool:

- mAbs
- mAb variants
- Antibody Drug Conjugates (ADCs)

Novel Optimized HIC Chemistry

AdvanceBio HIC column

Optimum separation of intact mAb and ADCs

3.5 µm particle size

- Fully porous
- Maximizes column efficiency while keeping pressure within operating range of <200 bar

Large 450 Å pore size

- Effective mass transfer of larger molecules

Optimized column bonding density and surface area → increase in stationary phase hydrophobicity → low salt gradient can be used

HIC Operating Parameters

Stationary phase that is hydrophobic, but utilizes nondenaturing mobile phases.

Mobile phase contains a salt that encourages the protein to absorb onto the stationary phase without denaturing it.

- Ammonium sulfate, typically 1 to 2 M concentration

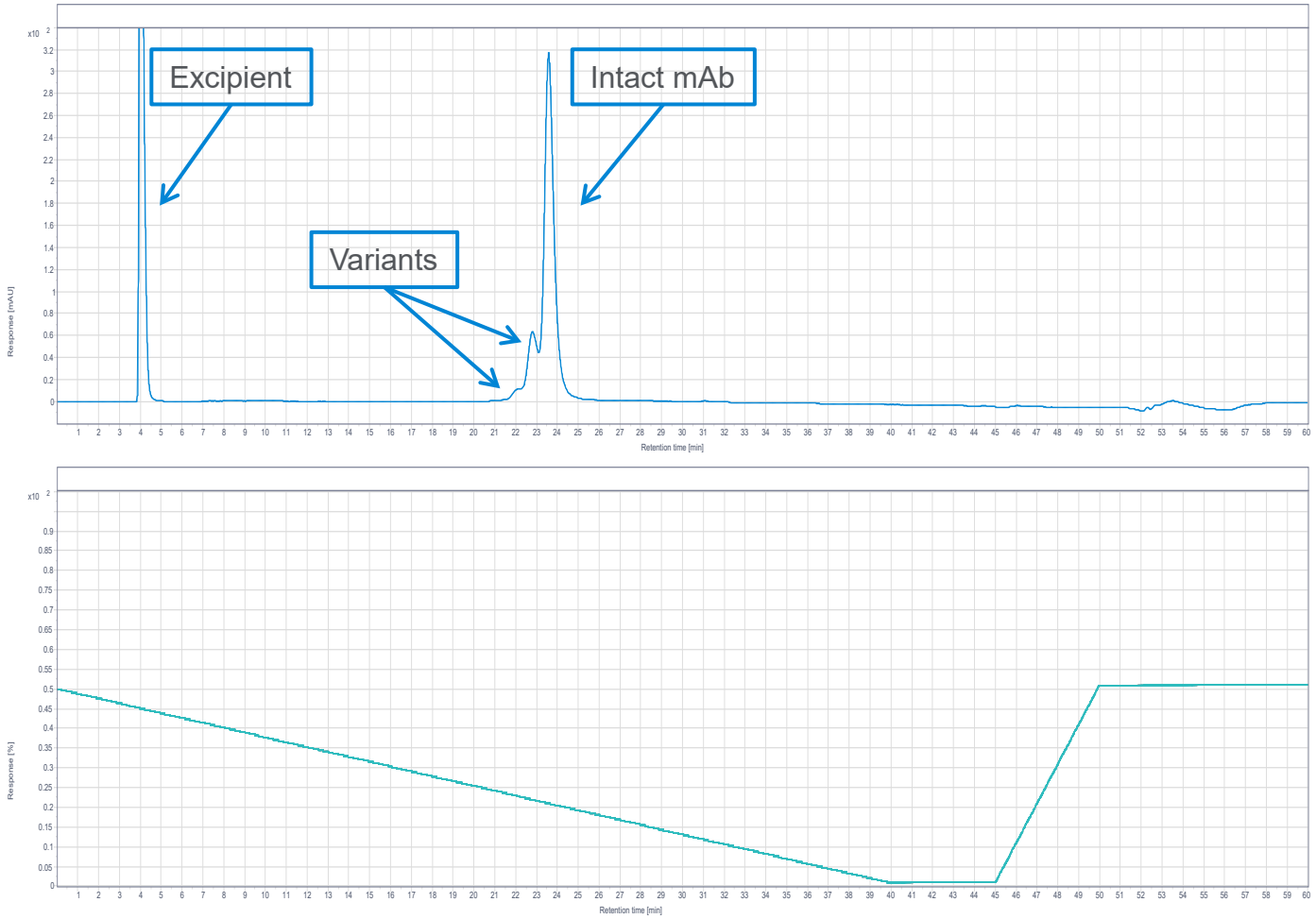
Buffer is used to stabilize pH and maintain protein solubility.

- Sodium phosphate, pH 7, typically 20 to 100 mM concentration

Gradient required

- High to low salt concentration (ammonium sulfate)
- 10 to 20 column volumes

HIC Separation of NIST mAb

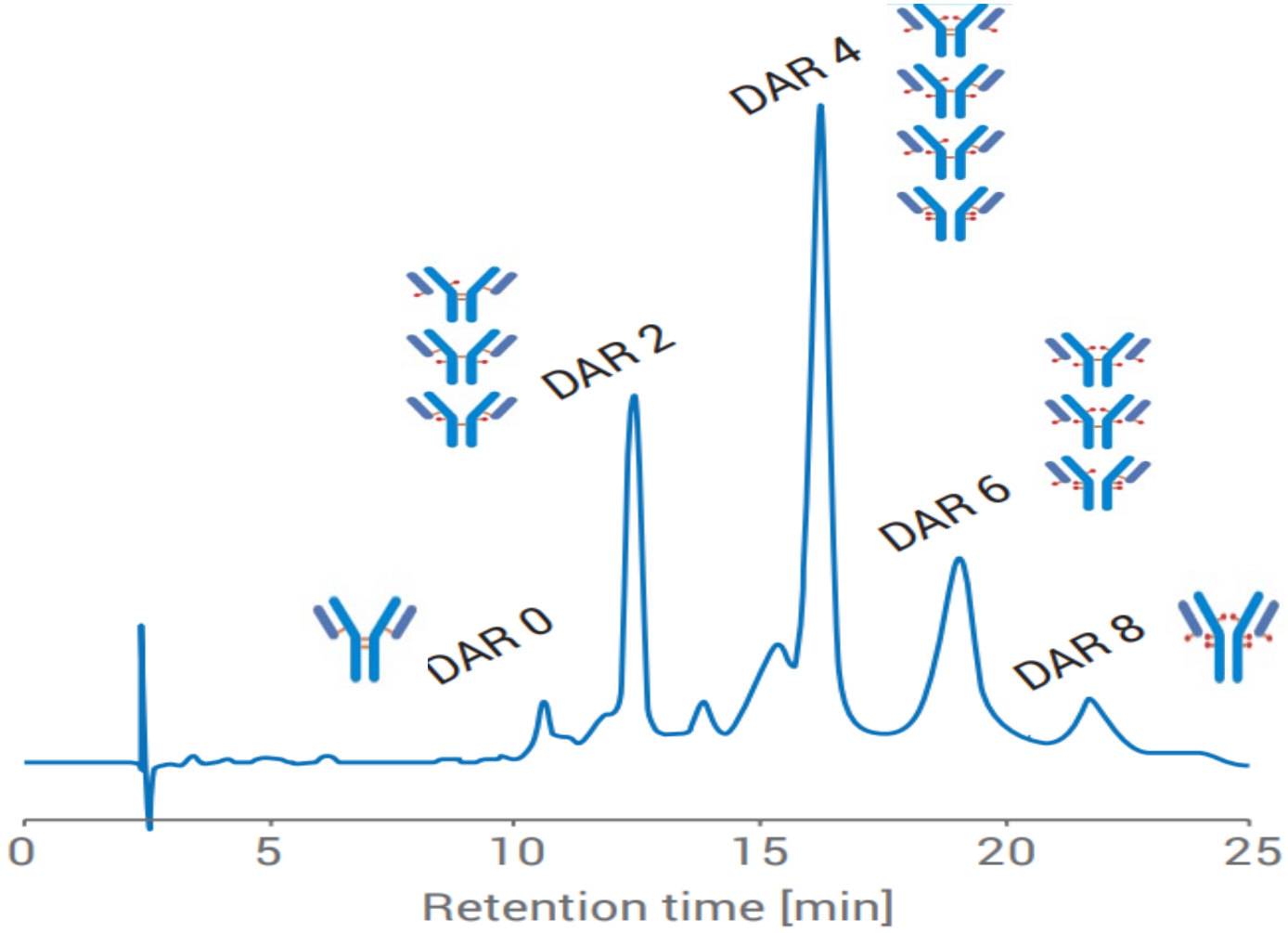


Gradient profile

Time (minutes)	%A	%B
0	50	50
40	100	0
45	100	0
50	50	50
60	50	50

Column used was AdvanceBio HIC, 4.6 x 100 mm, mobile phase: A: 50 mM sodium phosphate, pH 7.0, B: 2 M $(NH_4)_2SO_4$, 50 mM sodium phosphate, pH 7.0, 0.3 mL/min, 25 °C, injection: 5 µL of NIST mAB (RM 8671)

HIC Separation of ADCs (DAR Analysis)



Gradient profile

Time (minutes)	%A	%B	% C
0	45	50	5
15	75	0	25
20	75	0	25
21	45	50	5
31	45	50	5

Column used was AdvanceBio HIC, 4.6 x 100 mm, mobile phase: A: 50 mM sodium phosphate, pH 7.0, B: 2 M ammonium sulphate, 50 mM sodium phosphate, pH 7.0, C: IPA, 0.5 mL/min, 25 °C, injection: 5 µL, DAD: 220 nm, Agilent publication: 5994-0200EN

Agilent Biomolecule Columns Portfolio

Protein Therapeutics									Oligonucleotides		Vector Therapeutics		
Titer Determination	Aggregate Analysis	Intact Purity and PTM Analysis		Peptide Mapping & PTM Analysis	Charge Variant Analysis	Glycan Analysis	Amino Acid / Cell Culture Media Analysis		Purification & Impurity Analysis		Aggregation	Empty/Full	Capsid Identity
Affinity	Size Exclusion	Reversed Phase > 150 Å	Hydrophobic Interaction	Reversed Phase < 150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase < 150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase
Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis	AdvanceBio MS Spent Media	AdvanceBio Oligonucleotide	PL-SAX	Bio SEC-5	Bio SAX	ZORBAX RRHD 300Å, 1.8 µm
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm		PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)					Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX								
	Bio SEC-5	ZORBAX 300SB 3.5, 5 & 7 µm			Bio-Monolith (QA, DEAE, SO3)								
	ZORBAX GF250 & GF450	Poroshell 300 5 µm											

Stainless steel (SS) column hardware

Solid PEEK or PEEK-lined SS bioinert column hardware

Size Exclusion Chromatography (SEC)

Separation of biomolecules based on size

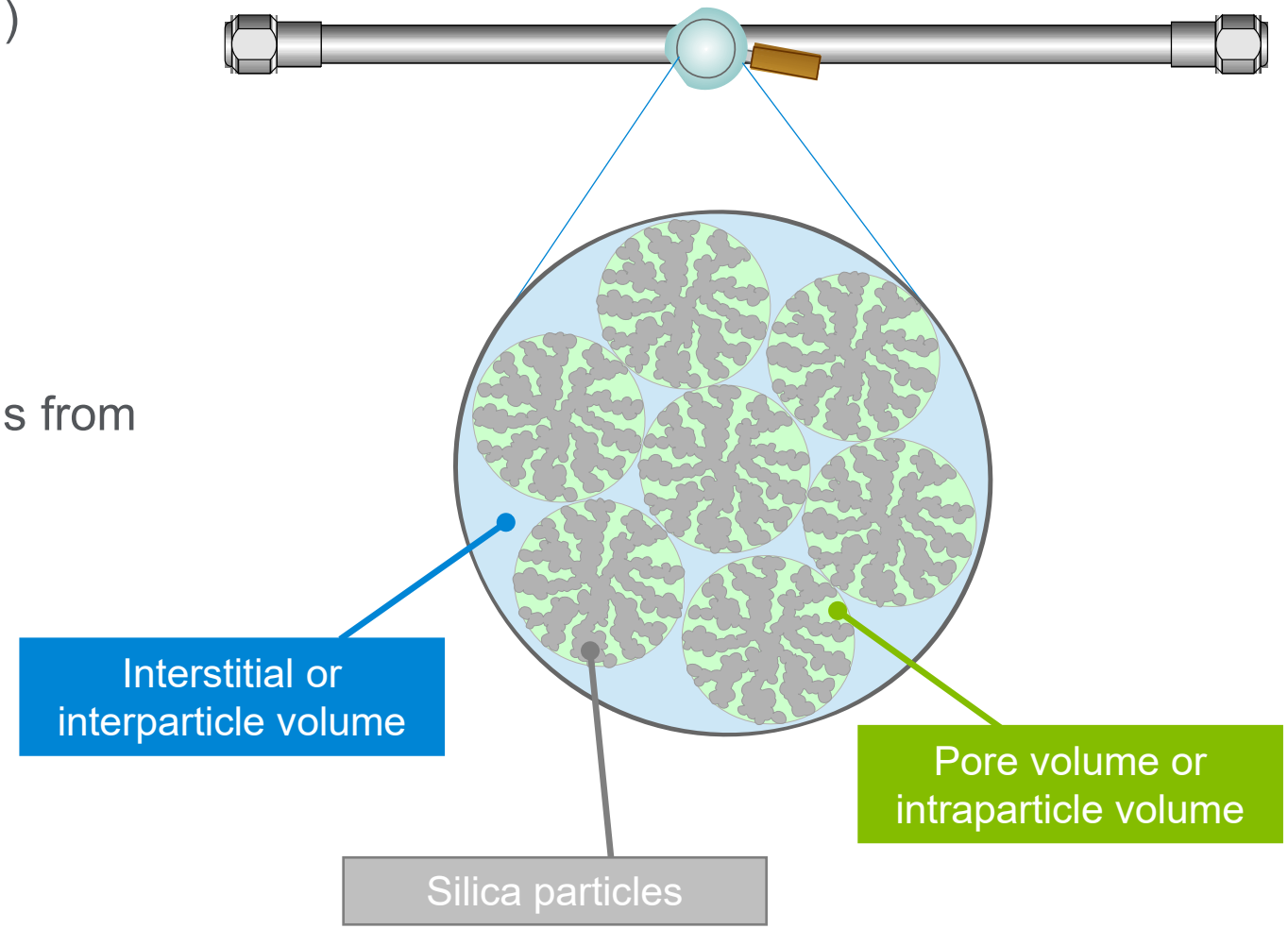
Separation by size under native (nondenaturing) mode

Relies on absence of interactions between the analyte and the stationary phase

Ideal for separating and analyzing intact proteins from contaminants, such as:

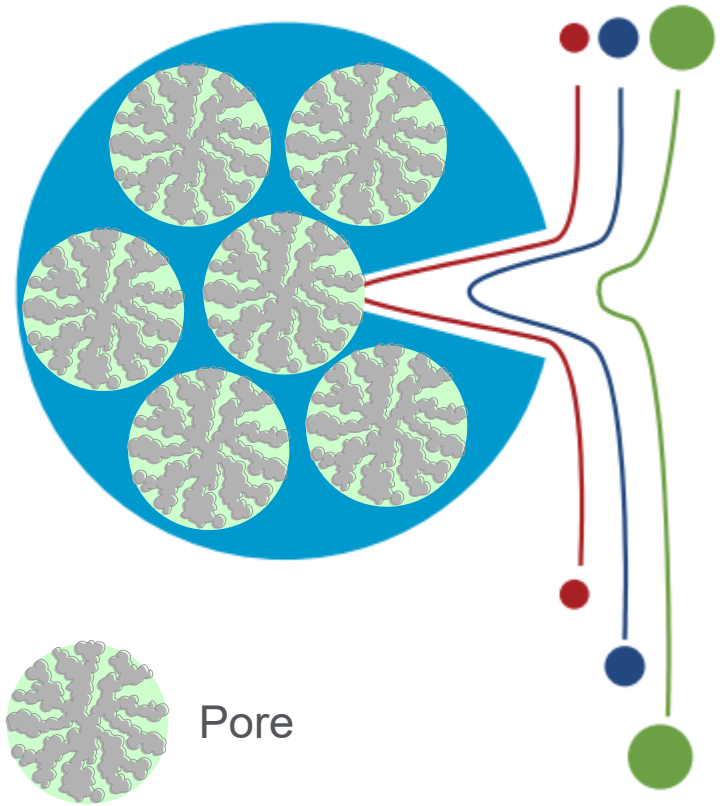
- Aggregates
- Excipients
- Cell debris
- Degradation impurities

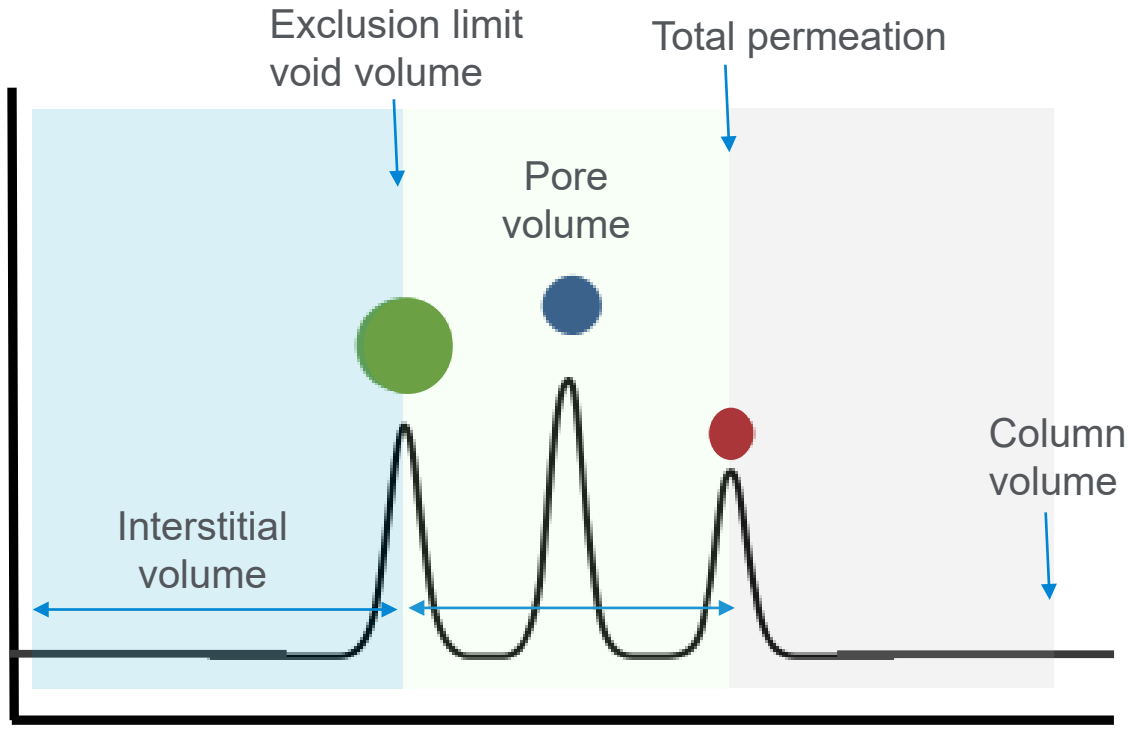
High salt concentration puts excessive wear on instrument parts



Size Exclusion Chromatography (SEC)

Separation by size under native (nondenaturing) mode





- Smaller molecules spend longer in the pores and elute later
- Larger molecules spend less time in the pores and elute sooner

Aggregation and Fragment Analysis (SEC)

Column selection parameters

Choose initial columns and conditions for size-based separation of biomolecules, aggregation analysis—peptides, polypeptides, and proteins

Peptides, polypeptides, proteins Mol Wt >0.1–1,250 kda	Peptides, polypeptides, proteins Mol Wt >0.1–10,000 kda

Select column based on molecular weight range and pore size

AdvanceBio SEC (2.7 µm)

Pore size	Mol Wt range, kDa
130 Å	0.1–120
300 Å	5–1,250

Bio SEC-3 (3 µm)

Pore size	Mol Wt range, kDa
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250

Bio SEC-5 (5 µm)

Particle size, µm	Flow rate, mL/min
100 Å	0.1–100
150 Å	0.5–150
300 Å	5–1,250
500 Å	15–5,000
1000 Å	50–7,500
2000 Å	>10,000

Aggregation and Fragment Analysis (SEC)

Recommended initial separation conditions

Columns:	AdvanceBio SEC Bio SEC (3 µm and 5 µm)
Mobile phase:	Phosphate buffer 150 mM, pH 7.0*
Gradient:	Isocratic in 15 to 60 min range
Temperature:	Recommended 10 to 30 °C, maximum 80 °C
Flow rate:	0.1 to 0.4 mL/min for 4.6 mm id columns 0.1 to 1.25 mL/min for 7.8 mm id columns 1.0 to 10.0 mL/min for 21.2 mm id columns
Sample size:	≤5% of total column volume

* Other aqueous buffers with high and low salt can be used

For additional information, see:
Resolve Protein Aggregates and Degradants With Speed and Confidence (publication 5991-2898EN)
www.agilent.com/search

Aggregation and Fragment Analysis (SEC)

Mobile phase selection

Minimize secondary interactions by adjusting the mobile phase composition

- pH
- Ionic strength
- Organic modifiers

Increase salt concentration

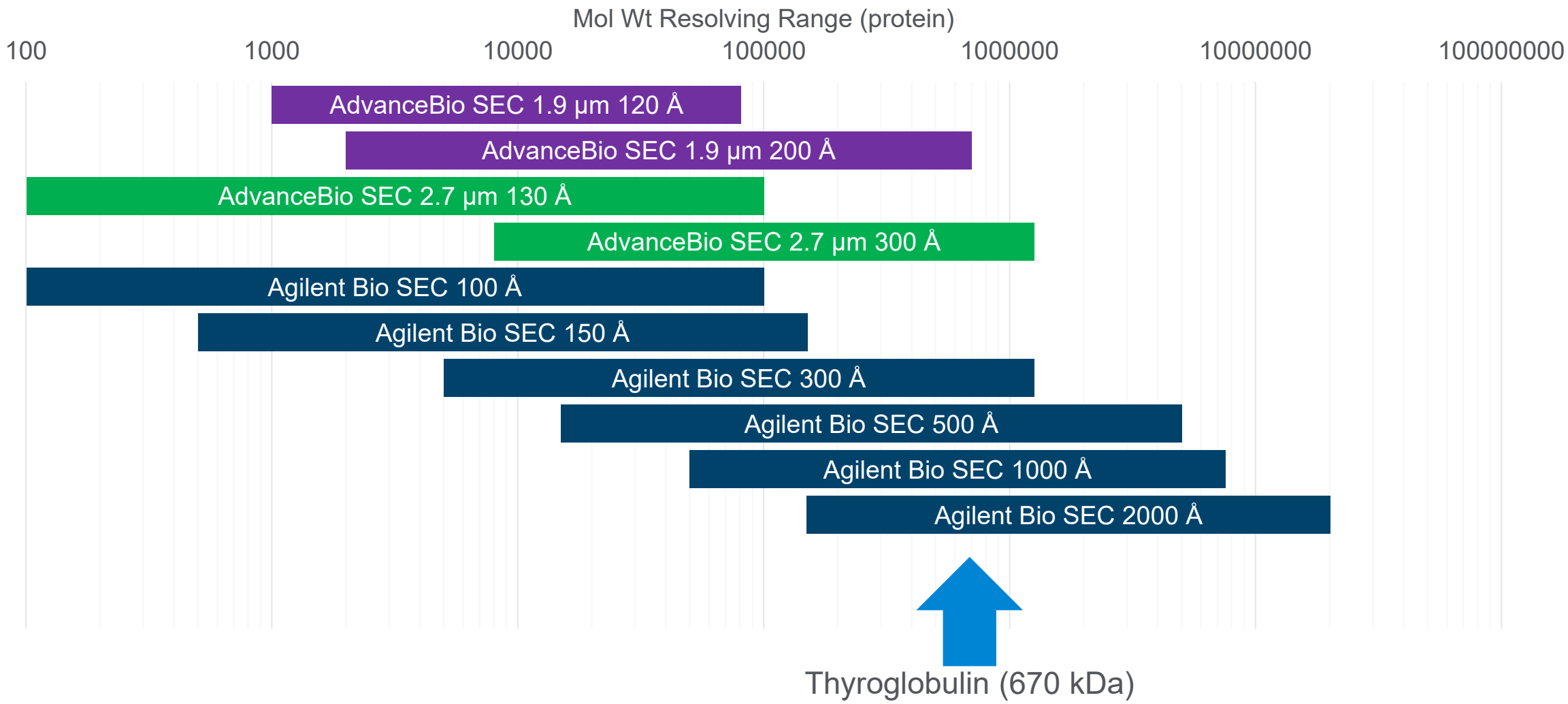
- 100 to 150 mM sodium chloride in 50 mM sodium phosphate, pH 7.0
- 100 to 150 mM sodium sulfate in 50 mM sodium phosphate, pH 7.0
- 50 to 100 mM urea in 50 mM sodium phosphate, pH 7.0

Make a fresh batch, filter, and promptly use the mobile phase.

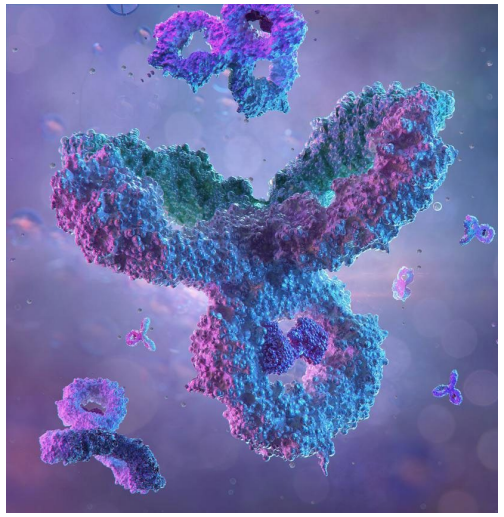
Buffers should maintain constant pH, but be wary of methods that state the pH can be +/- 0.2 pH units.

Avoid sudden changes in operating pressure (either as a result of changing flow rate, or as a result of changing viscosity).

Choosing the Correct Pore Size: Mol wt Resolving Ranges (SEC)



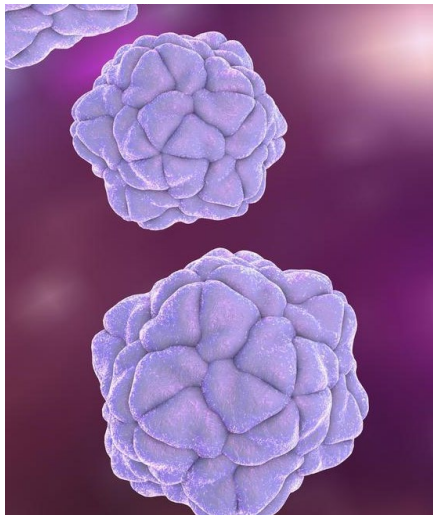
Biotherapeutics Size?



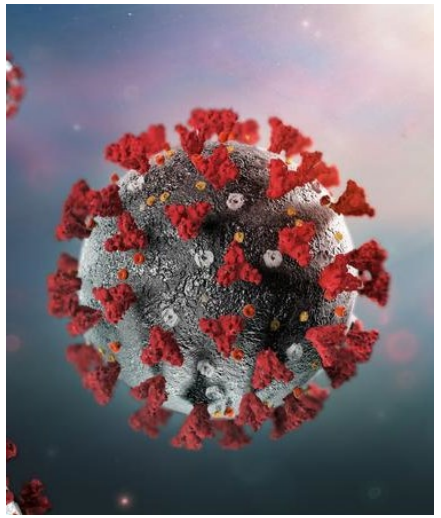
mAb
- ~150 kDa
- 5 nm hydrodynamic radius

mRNA
- ~ 1000 – 5000 nt
- ~ 320 – 1,600 kDa
- 300 – 1,500 nm chain length
- 100 – 200 nm diameter LNP

AAV
- ~ 3,800 kDa
- VP1:VP2:VP3 (5:5:50)
- ~ 25 – 30 nm diameter



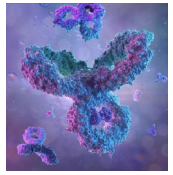
Viruses
- ~ 250 – 400 nm diameter



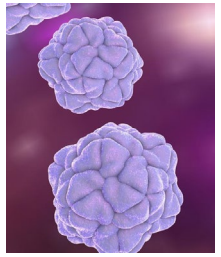
Pore Size Selection (SEC)

Suggested Guidelines

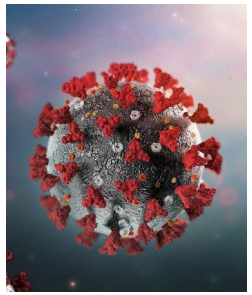
The pore size should be 3x larger than the **diameter** of the molecule you are interested in



- mAb (hydrodynamic radius ~ 5 nm); optimum pore size is about 3 x 10 nm
- 300 Å



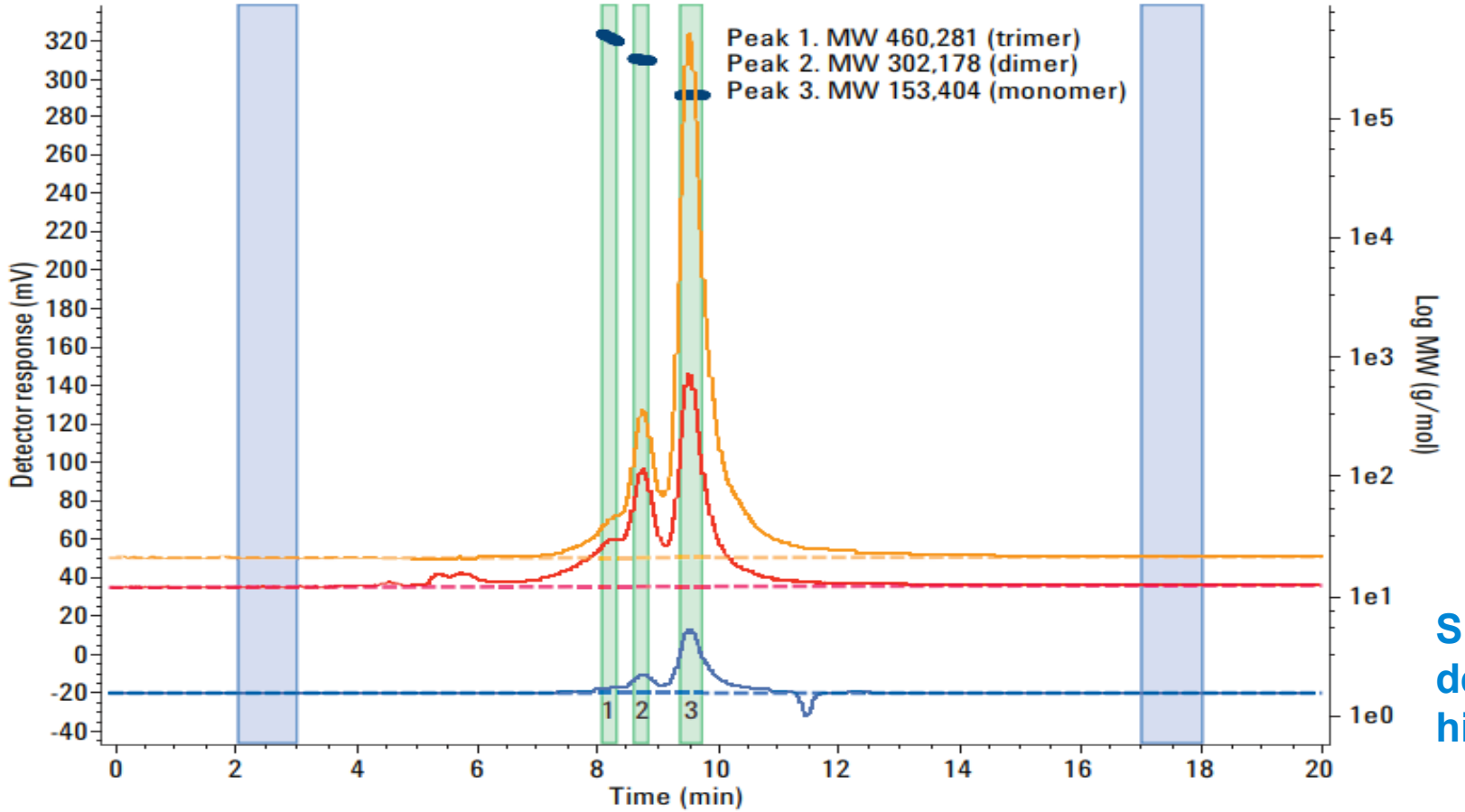
- AAV (diameter ~ 25 – 30 nm); optimum pore size is about 3 x 30 nm
- 500 – 1000 Å



- LNP (diameter ~ 100 – 200 nm); optimum pore size is about 3 x 200 nm; **6000 Å**
- VLP (diameter ~ 250 – 400 nm); optimum pore size is about 3 x 400 nm; **1.2 µm**

Multidetector Approach

Light scattering detector in combination with a concentration detector, such as UV or RI

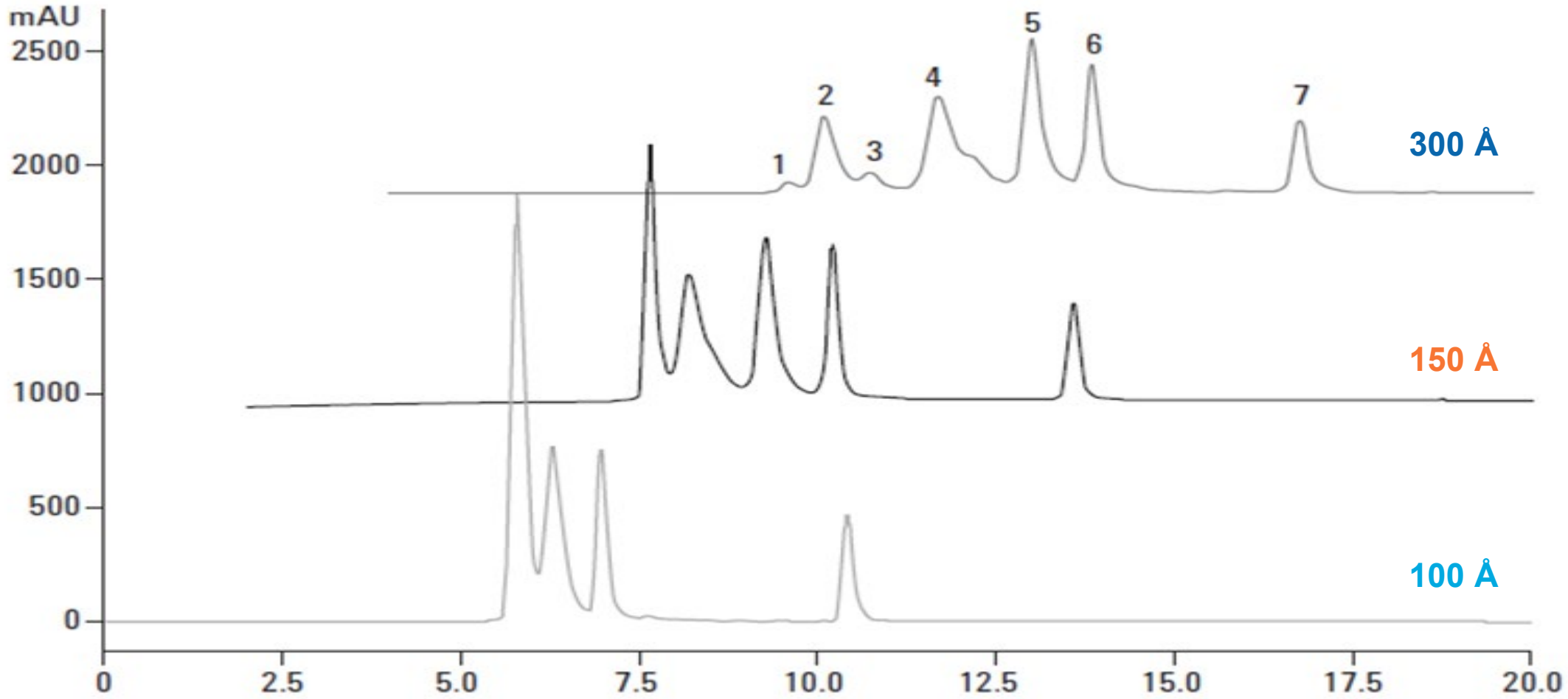


- UV 280 nm
- Light scattering 90°
- Refractive index

Signal from the light scattering detector was more responsive to higher molecular weight molecules

Column used was Agilent Bio SEC-5, 7.8 x 300 mm, stainless steel; A: 50 mM sodium phosphate, 250 mM NaCl, pH 7.0, isocratic elution, 1.0 mL/min, 30 °C, 1.0, 2.0 and 4.0 mg/mL of Bovine Y-globulin. Agilent publication: 5991-1400EN

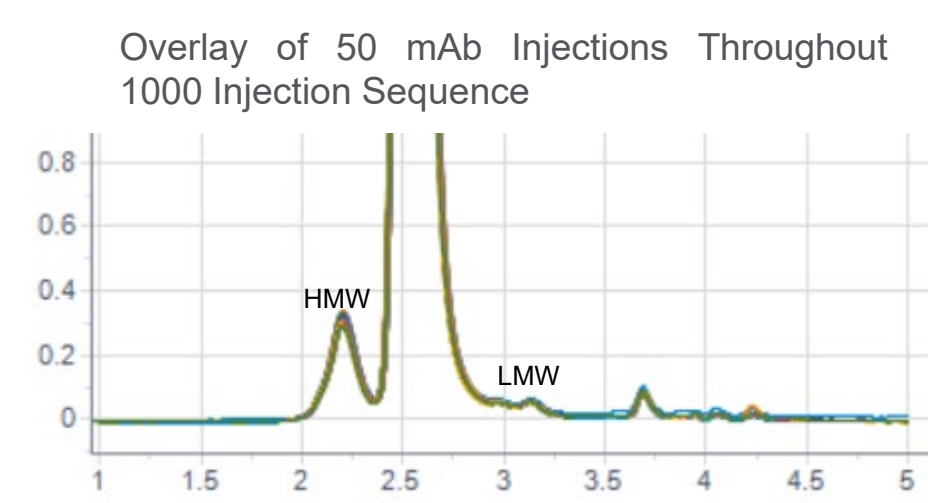
Effect of Pore Size on Resolution (SEC)

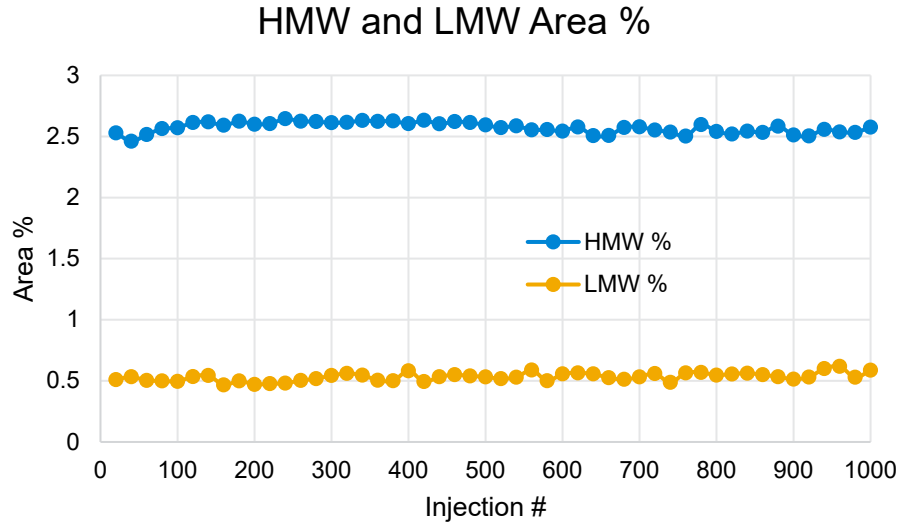


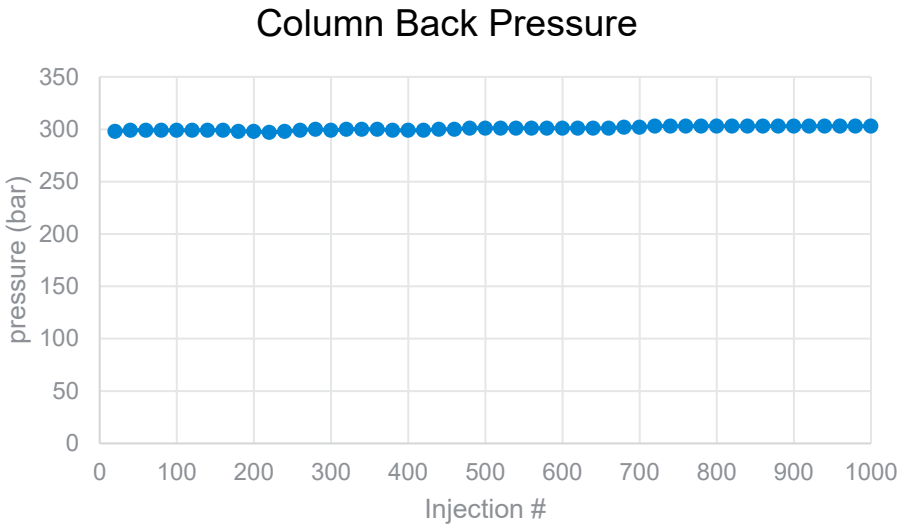
Columns used was AdvanceBio SEC 3.0 µm, 4.6 x 300 mm, mobile phase: 100 mM sodium phosphate with 150 mM sodium chloride, pH 6.8, 0.35 mL/min, gradient: 10 – 58% B in 4 min, 1 min wash at 95% B, 1 min re-equilibration at 10% B, DAD: 220 nm.
Agilent publication: 5994-0974EN

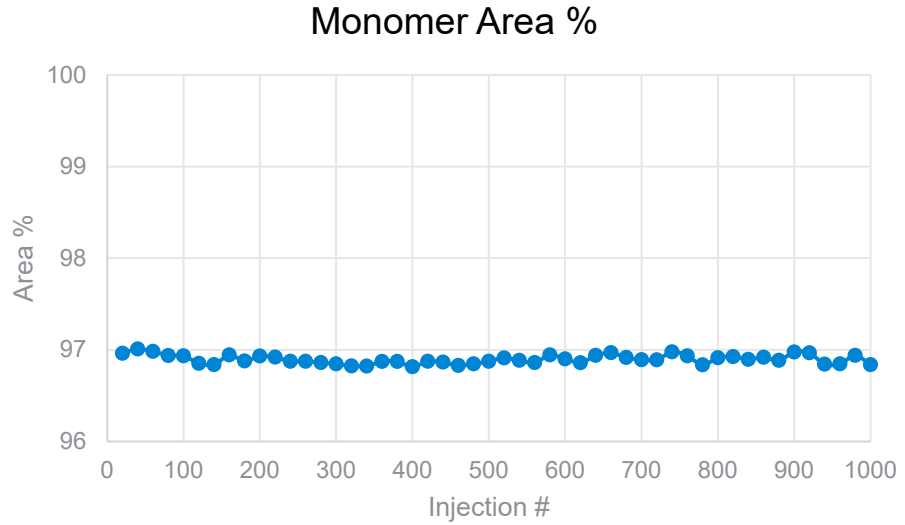
Lifetime (SEC)

AdvanceBio SEC 200 Å 1.9 µm, 4.6 x 150 mm, flow rate: 0.5 mL/min
1000 continuous injections of bovine IgG and SigmaMAb









Robustness over 1000 injections with reproducible mAb area % for HMW, monomer and LMW peaks and stable column back pressure.

Agilent Biomolecule Columns Portfolio

Protein Therapeutics									Oligonucleotides		Vector Therapeutics		
Titer Determination	Aggregate Analysis	Intact Purity & PTM Analysis		Peptide Mapping & PTM Analysis	Charge Variant Analysis	Glycan Analysis	Amino Acid / Cell Culture Media Analysis		Purification & Impurity Analysis		Aggregation	Empty/Full	Capsid Identity
Affinity	Size Exclusion	Reversed Phase > 150 Å	Hydrophobic Interaction	Reversed Phase < 150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase < 150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase
Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis	AdvanceBio MS Spent Media	AdvanceBio Oligonucleotide	PL-SAX	Bio SEC-5	Bio SAX	ZORBAX RRHD 300Å, 1.8 µm
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm		PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)					Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX								
	Bio SEC-5	ZORBAX 300SB 3.5, 5 & 7 µm			Bio-Monolith (QA, DEAE, SO3)								
	ZORBAX GF250 and GF450	Poroshell 300 5 µm											

Stainless steel (SS) column hardware

Solid PEEK or PEEK-lined SS bioinert column hardware

Hydrophilic Interaction Liquid Chromatography (HILIC)

Commonly used for retention and separation of polar compounds

Polar stationary phase (Si) and polar mobile phase (H_2O/ACN)

Water forms an aqueous-rich layer that is adsorbed to the surface of the polar stationary phase

Polar analytes have stronger interactions while partition into the aqueous-rich layer, leading to increase in retention time

- Retention of the analytes decreases as the polarity of the mobile phase increases
- Nonpolar to polar elution

Mobile phase can have high organic content and volatile salts (ammonium acetate or ammonium formate)

- Efficient desolvation → MS (ESI) detection

Applications: glycan analysis, hydrophilic peptides, sugars, and underivatized amino acids

AdvanceBio Glycan Mapping Column (HILIC)

Unique hydrophilic bonding

Fast analysis time

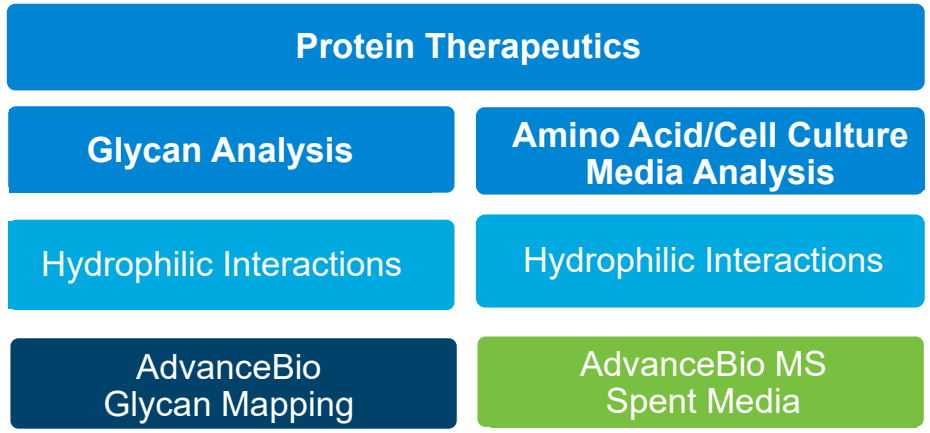
1.8 µm fully porous particles for highest performance

2.7 µm superficially porous particles for lower pressures

Ideal for all UHPLC/HPLC instruments

MS-compatible mobile phases

- ACN and H_2O
- Volatile salts – ammonium acetate or ammonium formate



Additional resources

Amino Acid Analysis “How-to” guide

Application finder

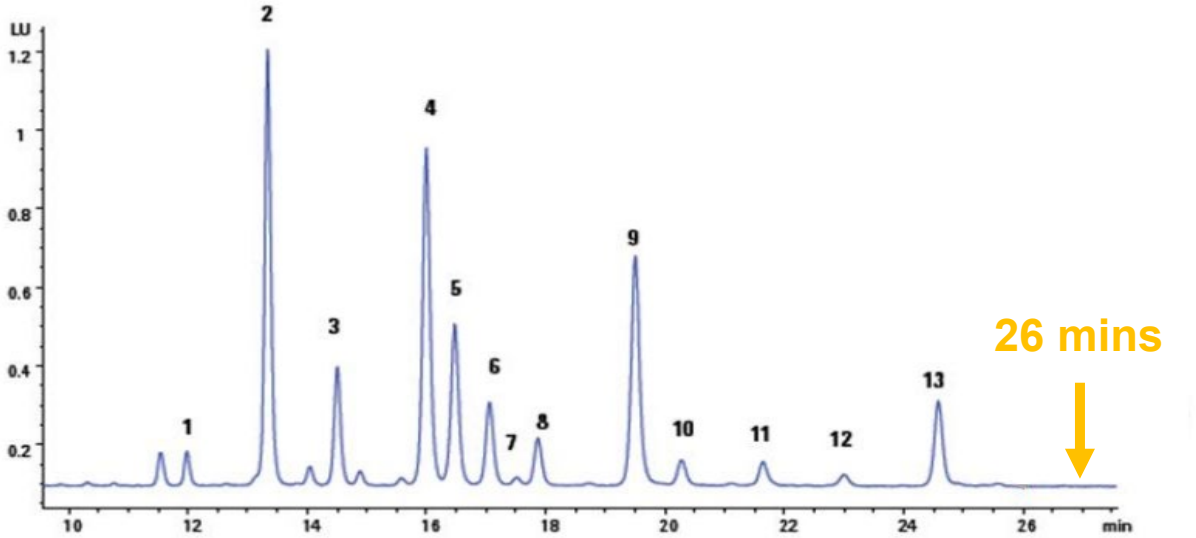
AdvanceBio e-seminar series

HIC Separation of Glycan by Fluorescence Detection (HILIC)

1260 Infinity
Bio-inert HPLC

+

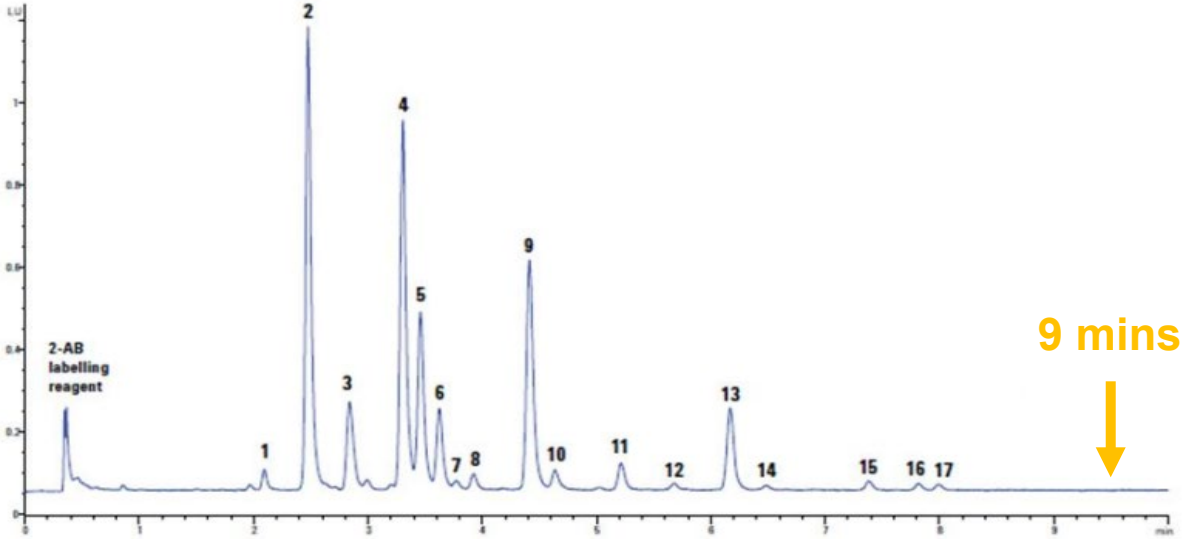
AdvanceBio glycan mapping 2.7 µm



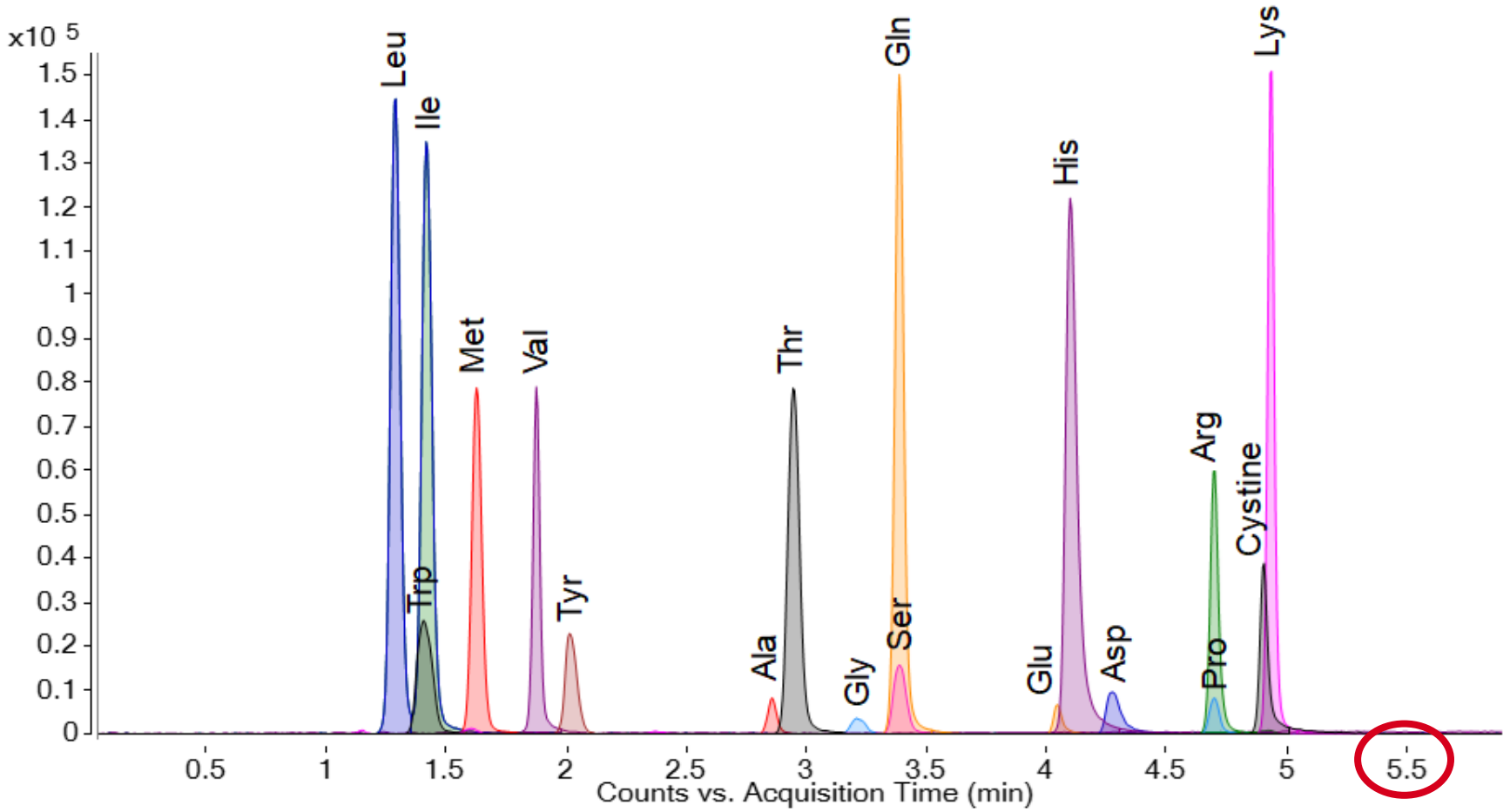
1290 Infinity UHPLC

+

AdvanceBio glycan mapping 1.8 µm



High Throughput LC/MS Analysis of Amino Acids with an AdvanceBio MS Spent Media Column (HILIC)



Gradient profile

Time (minutes)	%B
0	97
2	90
5	70
5.5	70
5.6	97
7.5	97

Column used was AdvanceBio Spent Media, 2.1 x 50 mm, mobile phase: A: 10% of 200 mM ammonium formate, pH 3.5 plus 90% water, B: 10% of 200 mM ammonium formate, pH 3.5 plus 90% mM ACN, 0.5 mL/min

Agilent Biomolecule Columns Portfolio

Protein Therapeutics									Oligonucleotides		Vector Therapeutics		
Titer Determination	Aggregate Analysis	Intact Purity & PTM Analysis		Peptide Mapping & PTM Analysis	Charge Variant Analysis	Glycan Analysis	Amino Acid/Cell Culture Media Analysis		Purification & Impurity Analysis		Aggregation	Empty/Full	Capsid Identity
Affinity	Size Exclusion	Reversed Phase > 150 Å	Hydrophobic Interaction	Reversed Phase < 150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase < 150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase
Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis	AdvanceBio MS Spent Media	AdvanceBio Oligonucleotide	PL-SAX	Bio SEC-5	Bio SAX	ZORBAX RRHD 300Å, 1.8 µm
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm		PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)					Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX								
	Bio SEC-5	ZORBAX 300SB 3.5, 5 & 7 µm			Bio-Monolith (QA, DEAE, SO3)								
	ZORBAX GF250 & GF450	Poroshell 300 5 µm											

Stainless steel (SS) column hardware

Solid PEEK or PEEK-lined SS bioinert column hardware

Ion Exchange Chromatography (IEX)

Separation of biomolecules based on differences in ionic charge

Nondenaturing technique (does not require organic solvents but can be used as additives; 50% or less)

Protein function groups → differences in charge

- Acidic groups: C-terminal carboxylic acids, acidic side chains, and glycosylated proteins
- Basic groups: N-terminal amines and basic side chains

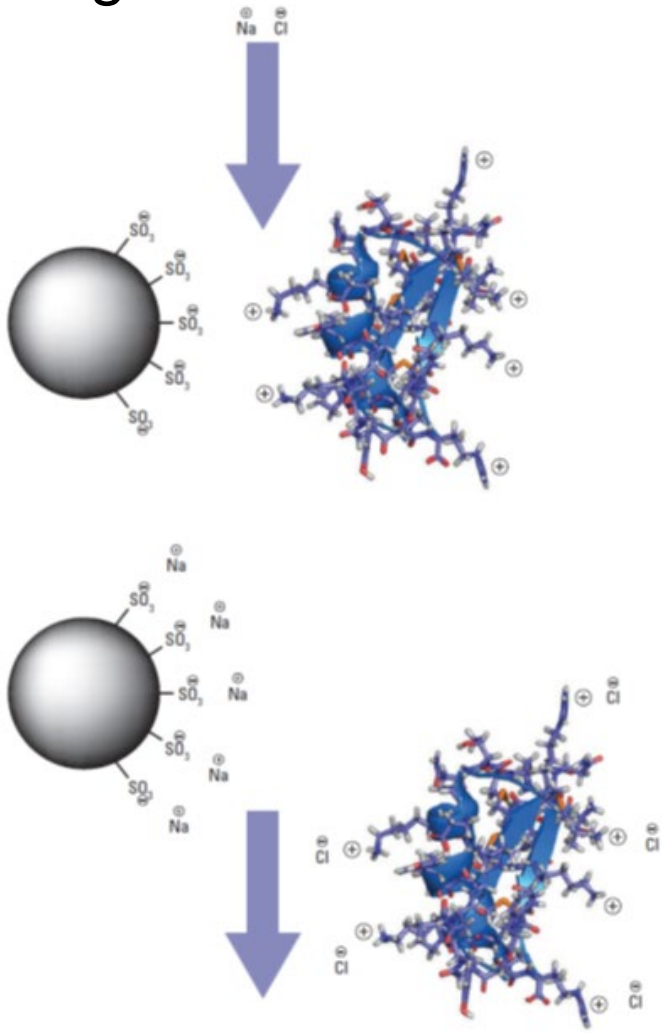
Overall charge of the protein depends on the pH of the solution

- Mobile phase must maintain a controlled pH throughout the separation
- Aqueous buffers are used as eluents

Increasing salt gradients, NaCl (mobile phase ionic strength)

- Displaces proteins from stationary phase.

Size consideration: mAbs are typically 150 kDa → chromatographic interactions will only occur with surface charges



Guidelines for IEX

The general rule for choosing a Bio IEX column

- Acidic proteins: SAX or WAX
- Basic proteins: SCX or WCX

Consider the isoelectric point (pI) of your protein when choosing the pH of your mobile phase

- If pH > pI, your protein will have a net negative charge
- If pH < pI, your protein will have a net positive charge

The pH of your starting buffer should be 0.5 to 1 pH unit from your pI

- Above pI for anion-exchange
- Below pI for cation-exchange

If your pI is unknown

- Start with pH 6.0 for cation exchange
- Start with pH 8.0 for anion exchange

Developing an Effective IEX Method

Sample preparation

Column media choice

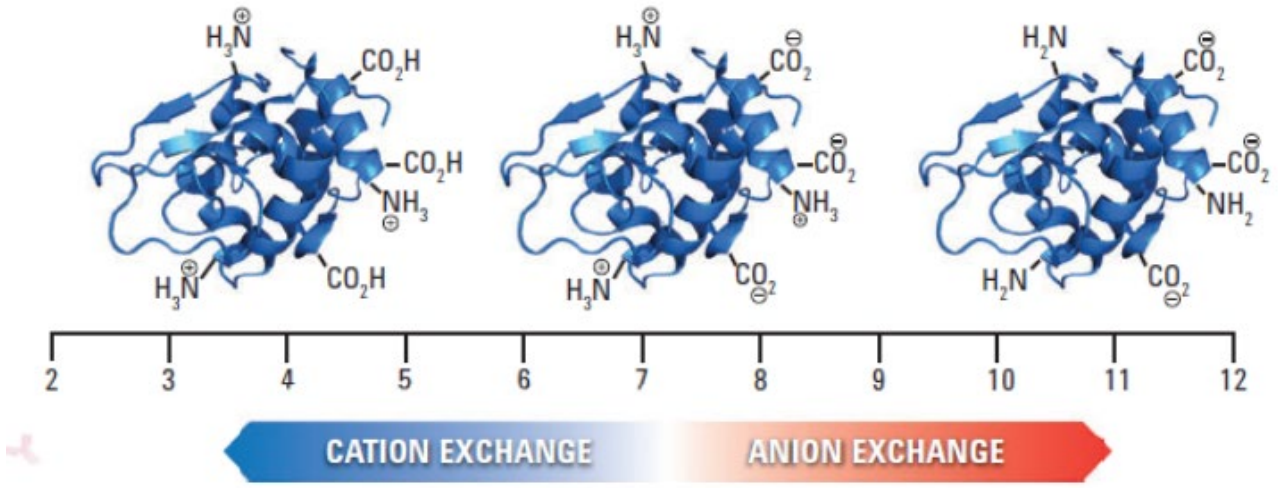
- The choice between anion and cation exchange depends on the isoelectric point of the protein(s) of interest.
- Strong ion exchangers are a good first choice, with weak ion exchange offering a difference in selectivity if it is required.

Column selection

- Pore size, particle size, column length, and column id

Mobile phase

Column conditioning and equilibration



Developing an Effective IEX Method

Mobile phase selection

Common buffers: phosphate, tris, MES, and ACES

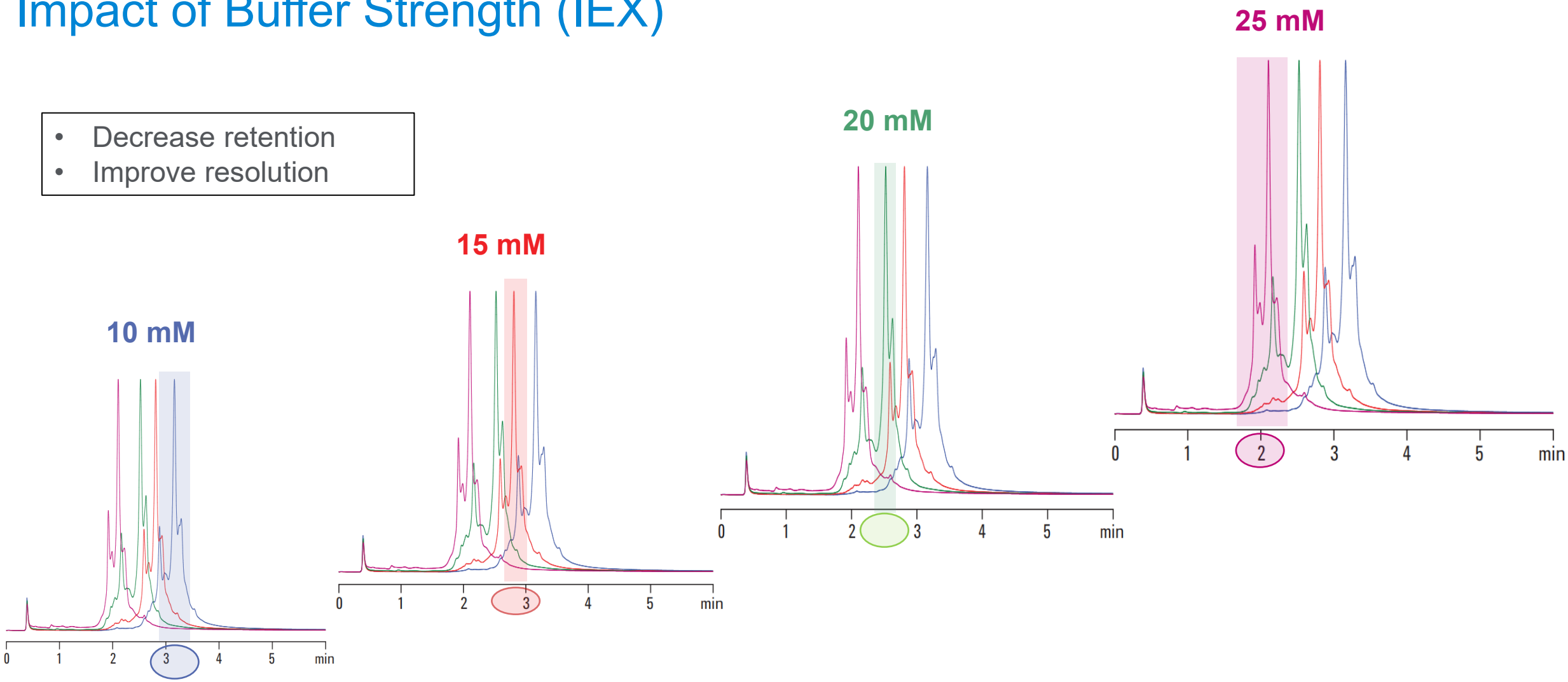
- Cation exchange, pH of 4 to 7
  - Phosphate buffer, 20 mM
  - Low background absorbance at 210 nm
- Anion exchange, pH of 7 to 10
  - Tris-HCl buffer, 20 mM

Ionic strength

- Certain ionic strength required to sustain the column function
  - Greater than 30 mM may prevent adsorption
- Eluent A: pH adjusted buffer and eluent B: eluent A + salt
  - Commonly used salts are NaCl, KCl, and acetate
  - Elution salt is typically 400 to 500 mM
  - Readjust the pH after adding salts

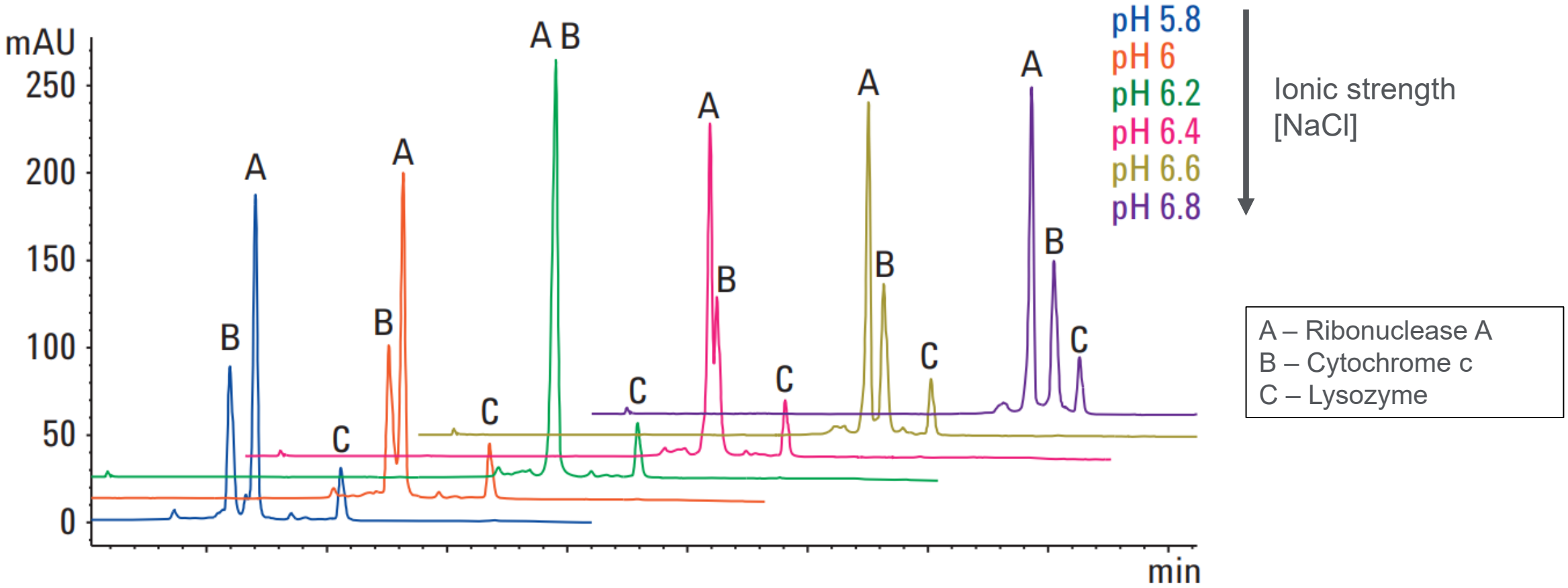
Impact of Buffer Strength (IEX)

- Decrease retention
- Improve resolution



Columns used were Bio WCX and Bio SCX, 3.0 µm, 4.6 x 50 mm, mobile phase: A: water, B: 1.5 M HCl, C: 40 mM NaH_2PO_4, D: 40 mM Na_2HPO_4, gradient: pH 5.0 to 7.0, 10 to 26 mM buffer strength, 0 to 500 mM NaCl, 0 to 15 min, 500 mM NaCl, 15 to 20 min, 1.0 mL/min, injection: 5 µL of IgG mAb, DAD: 220 nm. Agilent publication: 5991-3775EN

Impact of Buffer pH and Ionic Strength – WCX



Column used was Bio mAb, 5.0 µm, 4.6 x 50 mm, PEEK, mobile phase: A: water, B: 1.5 M HCl, C: 40 mM NaH_2PO_4, D: 40 mM Na_2HPO_4, pH adjustment: combine appropriate proportions of C and D, 1.0 mL/min, injection: mixture of A-C proteins, PBS, pH 7.4, DAD: 220 nm.
Agilent publication: 5991-3775EN

Agilent Biomolecule Columns Portfolio

Protein Therapeutics									Oligonucleotides		Vector Therapeutics		
Titer Determination	Aggregate Analysis	Intact Purity & PTM Analysis		Peptide Mapping & PTM Analysis	Charge Variant Analysis	Glycan Analysis	Amino Acid / Cell Culture Media Analysis		Purification and Impurity Analysis		Aggregation	Empty/Full	Capsid Identity
Affinity	Size Exclusion	Reversed Phase > 150 Å	Hydrophobic Interaction	Reversed Phase < 150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase < 150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase
Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb/Bio IEX 5 µm	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis	AdvanceBio MS Spent Media	AdvanceBio Oligonucleotide	PL-SAX	Bio SEC-5	Bio SAX	ZORBAX RRHD 300Å, 1.8 µm
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm		PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)					Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX								
	Bio SEC-5	ZORBAX 300SB 3.5, 5 & 7 µm			Bio-Monolith (QA, DEAE, SO3)								
	ZORBAX GF250 & GF450	Poroshell 300 5 µm											

Stainless steel (SS) column hardware

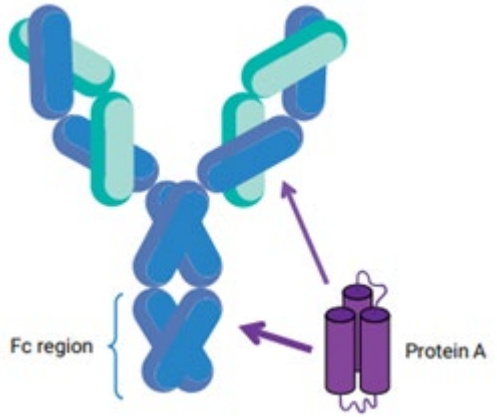
Solid PEEK or PEEK-lined SS bioinert column hardware

Affinity Chromatography

Purification technique for a specific molecule or group of molecules from a complex matrix

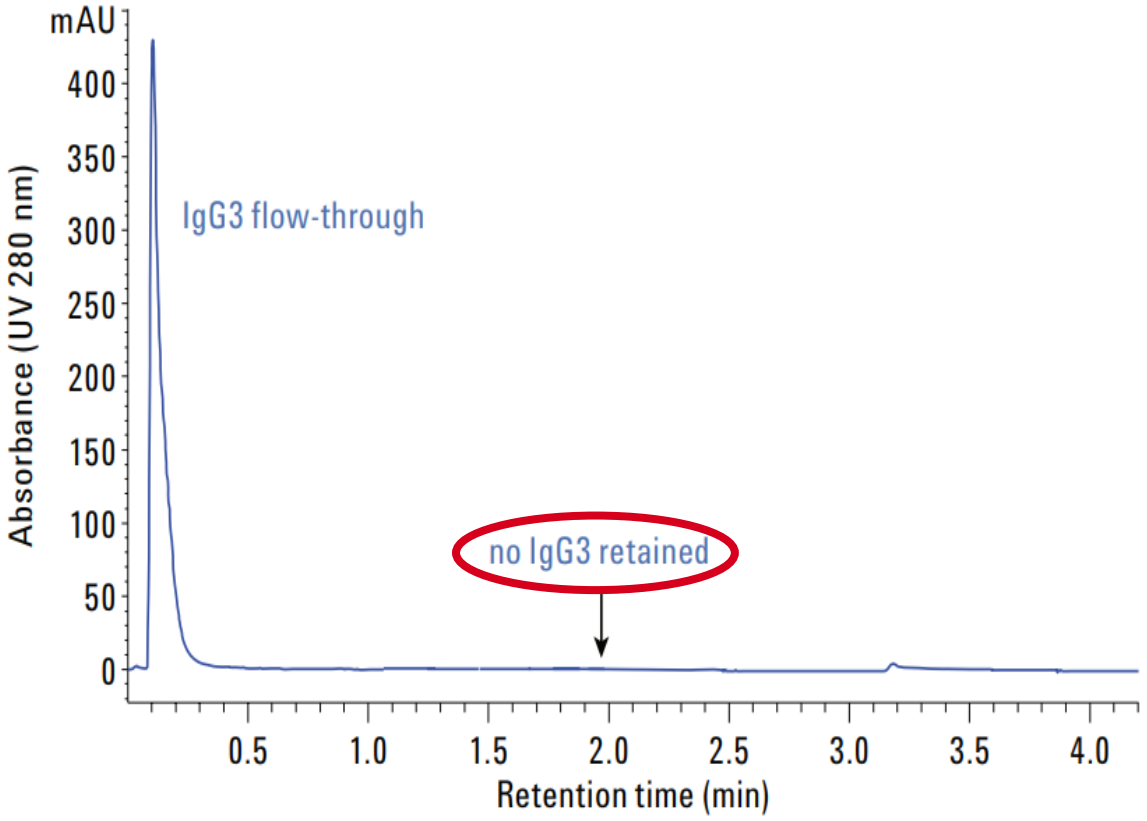
Relies on reversible interactions between two molecules, referred to as affinity ligand and target molecules, examples include:

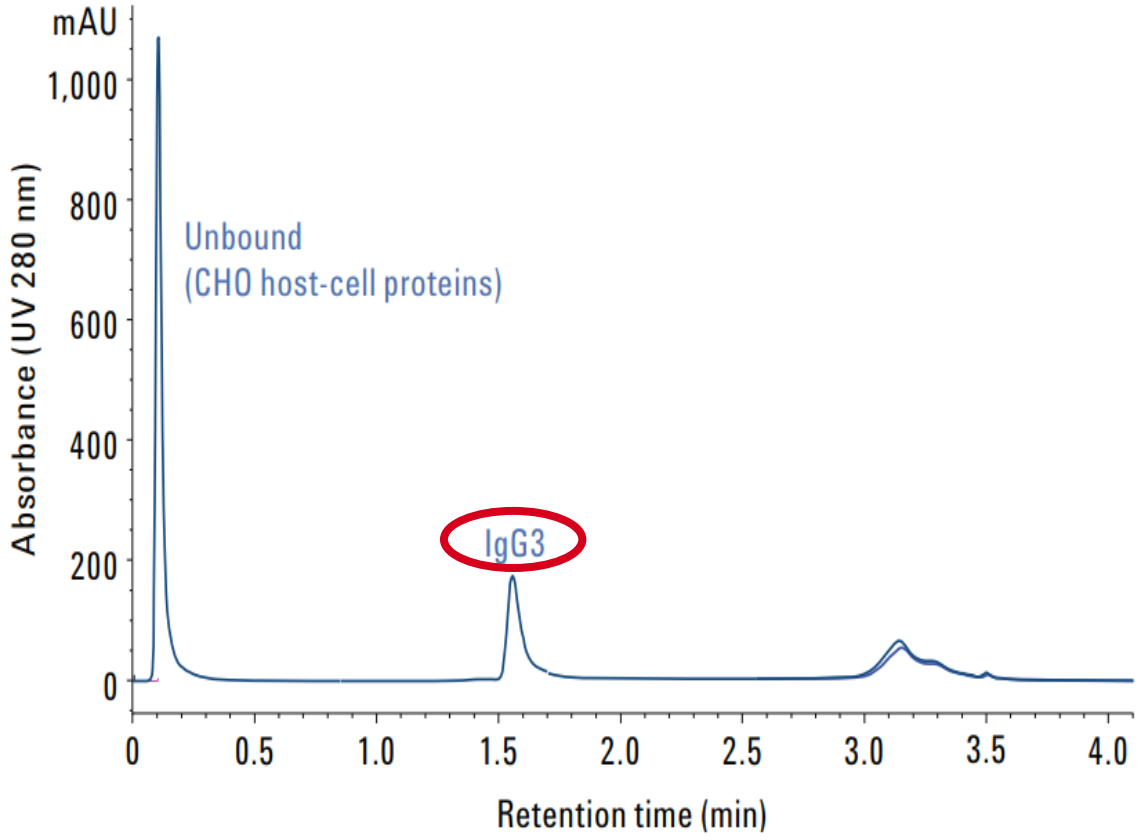
- Enzyme and substrate
- Receptor and ligand
- Antibody and antigen
- One of the interacting molecules (affinity ligand) is immobilized to a surface of the stationary phase while the analytes of interest (target molecules) are in the mobile phase
- mAbs → titer determination
  - Target molecules: Immunoglobulins (IgGs) form different sources (humans, mouse)
  - Affinity ligands: Protein A or G
    - Protein A for IgG except class 3
    - Protein G, alternate selectivity



Affinity Biochromatography

IgG3 selectivity





Columns used were Agilent Bio-Monolith Protein A and G, 5.2 diameter, 4.95 mm long, binding buffer, A: 50 mM sodium phosphate, pH 7.4, eluting buffer, B: 0.1 M citric acid, pH 2.0. Agilent publication: 5991-6094EN

Agilent Biomolecule Columns Portfolio

Protein Therapeutics									Oligonucleotides		Vector Therapeutics		
Titer Determination	**Aggregate Analysis**	**Intact Purity & PTM Analysis**		**Peptide Mapping & PTM Analysis**	**Charge Variant Analysis**	**Glycan Analysis**	**Amino Acid / Cell Culture Media Analysis**		**Purification & Impurity Analysis**		**Aggregation**	**Empty/Full**	**Capsid Identity**
Affinity	Size Exclusion	Reversed Phase > 150 Å	Hydrophobic Interaction	Reversed Phase < 150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase < 150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase
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Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm		PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)					Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX								
	Bio SEC-5	ZORBAX 300SB 3.5, 5 & 7 µm			Bio-Monolith (QA, DEAE, SO3)								
	ZORBAX GF250 & GF450	Poroshell 300 5 µm											

Stainless steel (SS) column hardware

Solid PEEK or PEEK-lined SS bioinert column hardware

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Bio-Monolith rProtein A	AdvanceBio SEC 1.9 µm	PLRP-S 1000 Å 5 µm	AdvanceBio HIC	AdvanceBio EC-C18	Bio mAb/Bio IEX 5 µm	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis	AdvanceBio MS Spent Media	AdvanceBio Oligonucleotide	PL-SAX	Bio SEC-5	Bio SAX	ZORBAX RRHD 300 Å, 1.8 µm
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S		AdvanceBio Peptide Mapping	Bio mAb (WCX)		ZORBAX Eclipse AAA 3.5 µm		PLRP-S	Bio SAX		Bio SAX	
Bio-Monolith Protein G	AdvanceBio SEC 2.7 µm	AdvanceBio RP mAb 450 Å		AdvanceBio Peptide Plus	Bio IEX (SAX, WAX, SCX, WCX)					Bio SAX			
	Bio SEC-3	ZORBAX RRHD 300 Å, 1.8 µm		ZORBAX RRHD 300 Å, 1.8 µm	PL SCX, SAX								
	Bio SEC-5	ZORBAX 300SB 3.5, 5 and 7 µm			Bio-Monolith (QA, DEAE, SO3)								
	ZORBAX GF250 and GF450	Poroshell 300 5 µm										Stainless steel (SS) column hardware	Solid PEEK or PEEK-lined SS bioinert column hardware

Biochromatography

Biomolecules come in different shapes and sizes, with different surface characteristics…

… so do Agilent Biocolumns

Resources for Support

Tech support www.agilent.com/chem/techsupport

Agilent Product Catalogs, www.agilent.com/en/promotions/catalog

- InfinityLab Supplies catalog (5991-8031EN)

Resource page www.agilent.com/chem/agilentresources

- Quick reference guides
- Catalogs, column user guides
- Online selection tools, how-to videos

Agilent University http://www.agilent.com/crosslab/university

YouTube – Agilent Channel

Your local FSE and Specialists

Agilent service contracts

Contact Agilent Chemistries and Supplies Technical Support

Available in the U.S. and Canada, 8am to 5pm all time zones

Web chat: Product pages of Agilent.com

1-800-227-9770 option 3, option 3:

- Option 1 – GC and GC/MS columns and supplies
  gc-column-support@agilent.com
- Option 2 – for LC and LC/MS columns and supplies
  lc-column-support@agilent.com
- Option 3 – for sample preparation, filtration, and QuEChERS
  spp-support@agilent.com
- Option 4 – for spectroscopy supplies
  spectro-supplies-support@agilent.com
- Option 5 – for standards
  chem-standards-support@agilent.com
- Option 6 for ProZyme products
  pzi.info@agilent.com

Thank you

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Trusted Answers