A Protein’s Journey

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LC Application Engineer
June 11, 2020

Agilent
Trusted Answers

Outline

LC techniques for biomolecule analysis

Reversed phase

- Commonly used, wide column choice 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, e.g., aggregate analysis.

Ion exchange

- Separation of molecules differing in net charge, under nondenaturing conditions, e.g., 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)

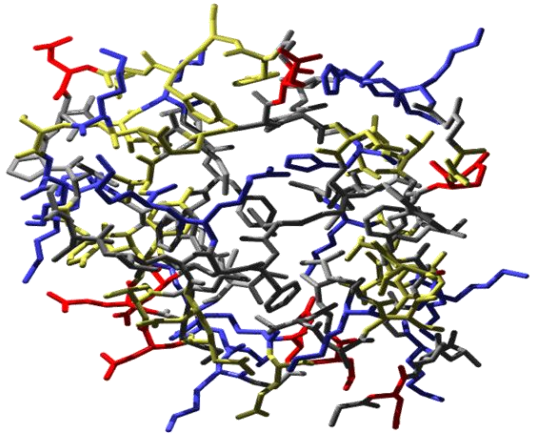
- Typically used for very hydrophilic molecules; polar analytes, unlabeled amino acids, glycans

Affinity

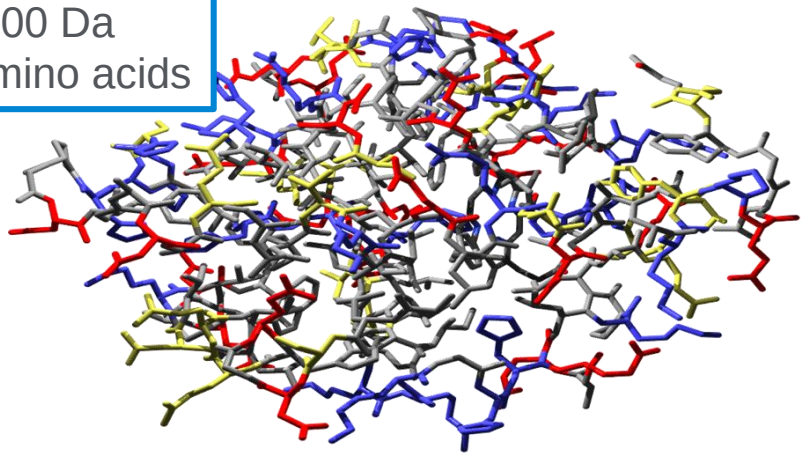
- Biospecific binding to an immobilized ligand like Protein A

Protein Molecules Come in Different Shapes and Sizes

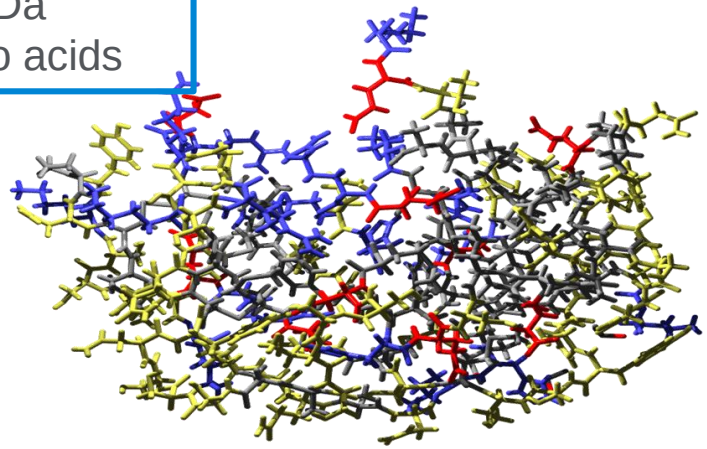
Cytochrome C
~ 12,000 Da
103 amino acids

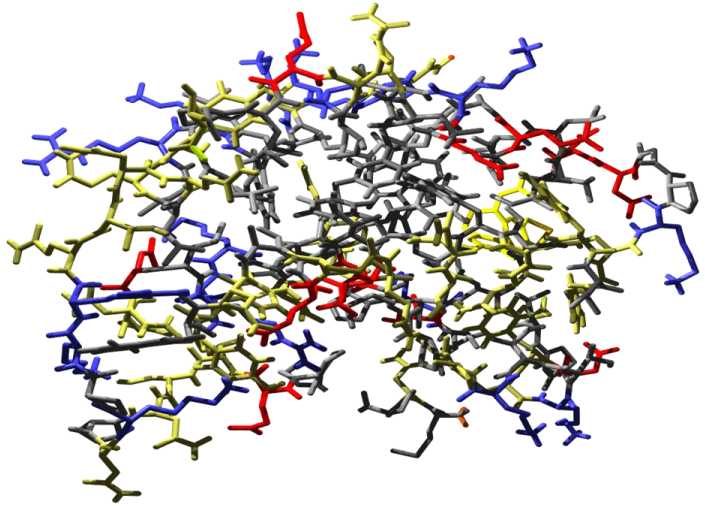


Myoglobin
~ 16,700 Da
153 amino acids



Ribonuclease A
~ 13,700 Da
124 amino acids

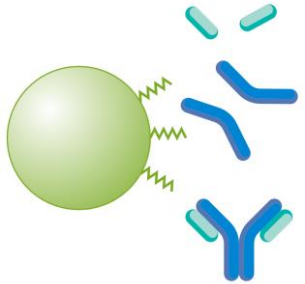
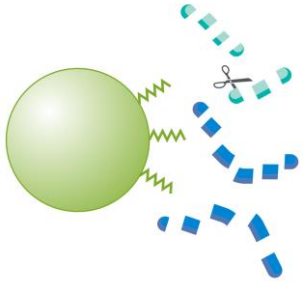
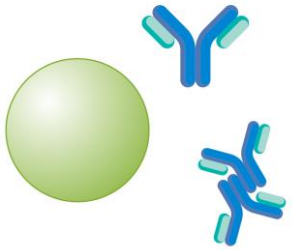
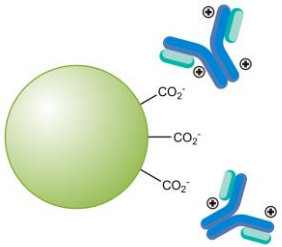
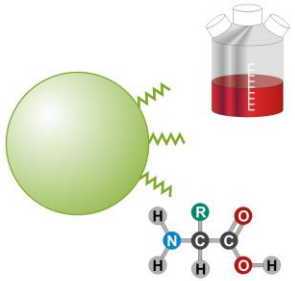
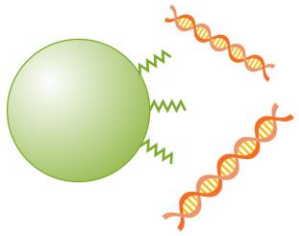
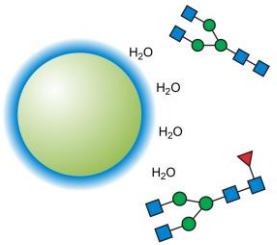
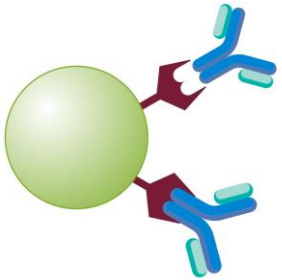




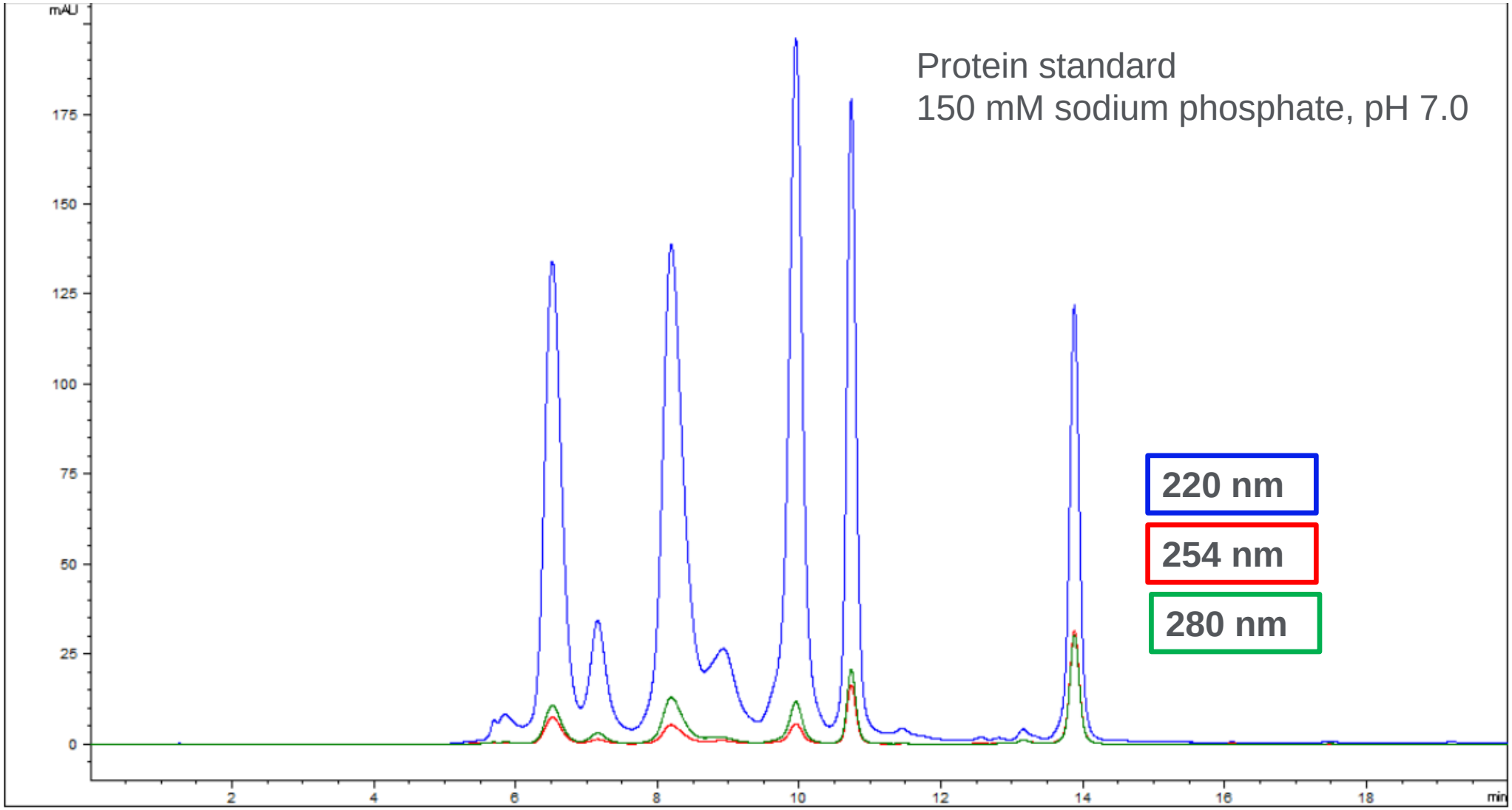
Lysozyme
~ 14,000 Da
129 amino acids

Understanding Proteins

LC tools available

Intact and fragmented protein analysis by reversed phase	Peptides and peptide mapping by reversed phase	Aggregated proteins and size exclusion	Charge variants and ion exchange
			
Amino acids and cell culture medium analysis	**Oligonucleotides by ion pair reversed phase or SAX**	**Glycan analysis by HILIC chromatography**	**Affinity chromatography (and titer determination)**
			

Detector Wavelength



Reversed Phase

Commonly used, wide column choice, denaturing conditions

Bonded phase; C18, C8, C4, C3, diphenyl

- Increased MW = Decreased ligand size
  - < 70 kDa – C18
  - > 70 kDa – C8, C4, C3, diphenyl

Particle size

- Smaller particle size (e.g. 1.8 µm) will increase resolving power

Pore size

- 300 Å, 450 Å
  - Larger pore sizes available in PLRP-S

Separation Conditions

Initial

Mobile phase: A: 95% H2O/5% ACN, 01.% TFA; B: 5% H2O/95% ACN, 0.1% TFA

- If LC/MS is used, can substitute formic or acetic acid

Gradient: 0–60% B in 60 min

Temperature: 35–40^{o} C

Flow rate: 1 mL/min

Optimized

Organic

- MeOH<ACN<IPA<THF

Temperature

- Higher temperature can dramatically improve resolution and recovery
  - Check column stability

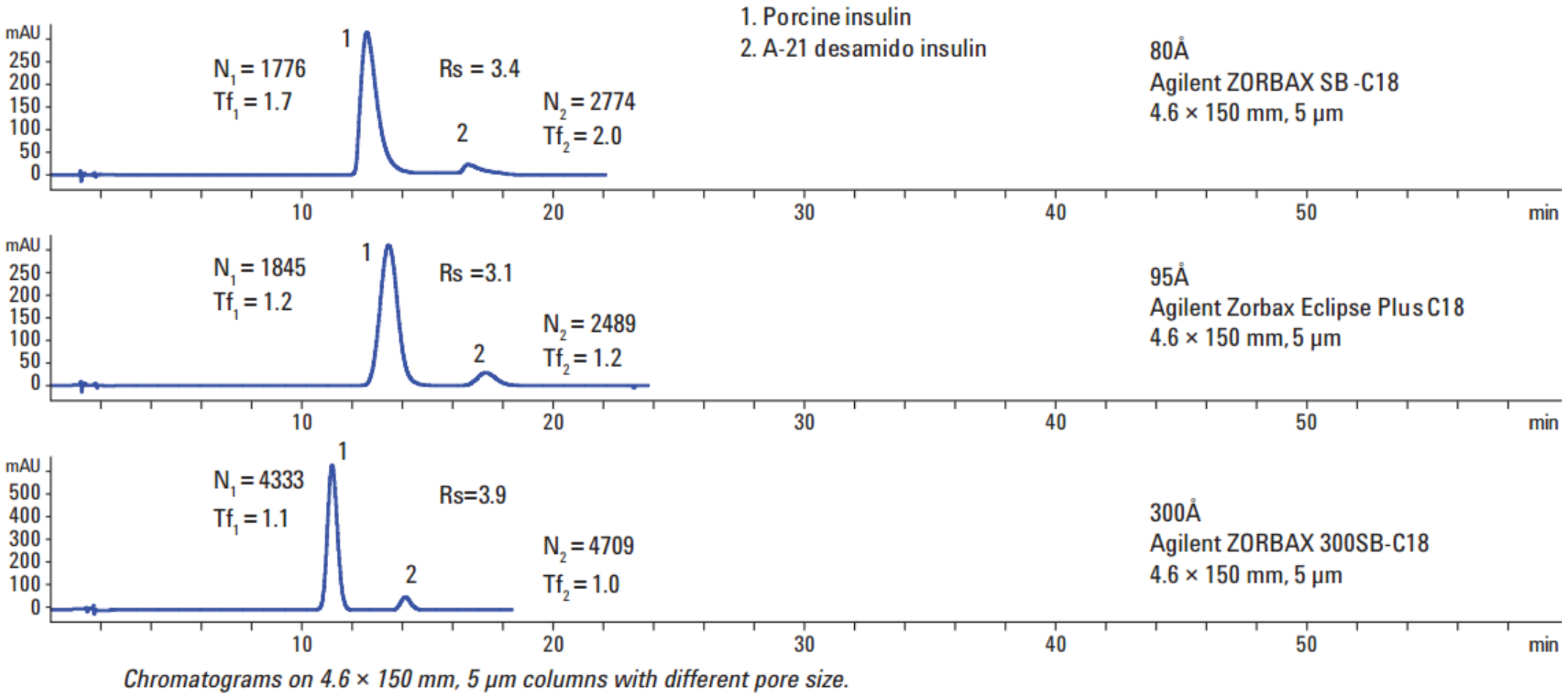
pH

- Try acidic first, then try mid or high pH
- Selectivity will change
  - Acidic AAs will become negatively charged
  - Basic AAs may lose their charge

Gradient

Pore Size

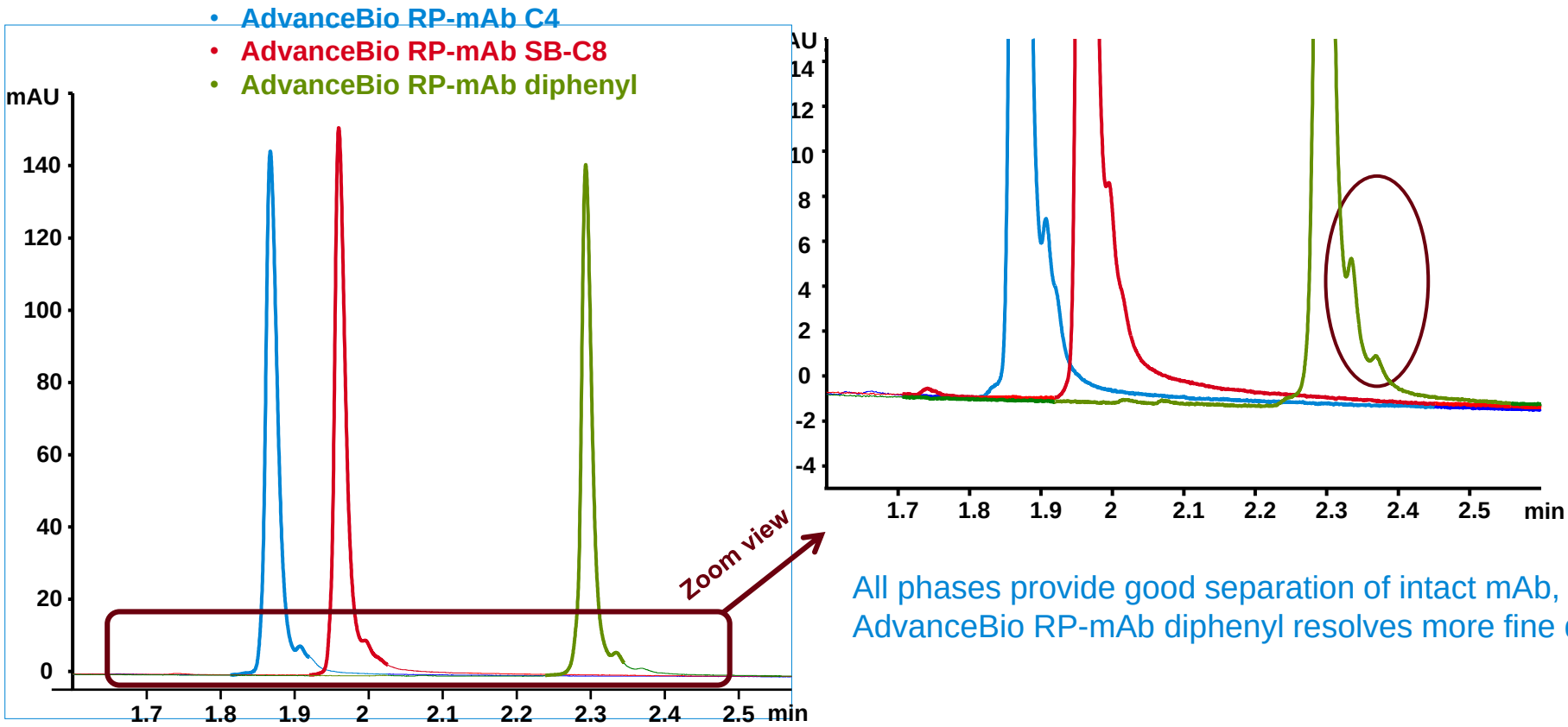
Effect on the efficiency of a large molecule



Chromatograms on 4.6 × 150 mm, 5 µm columns with different pore size.

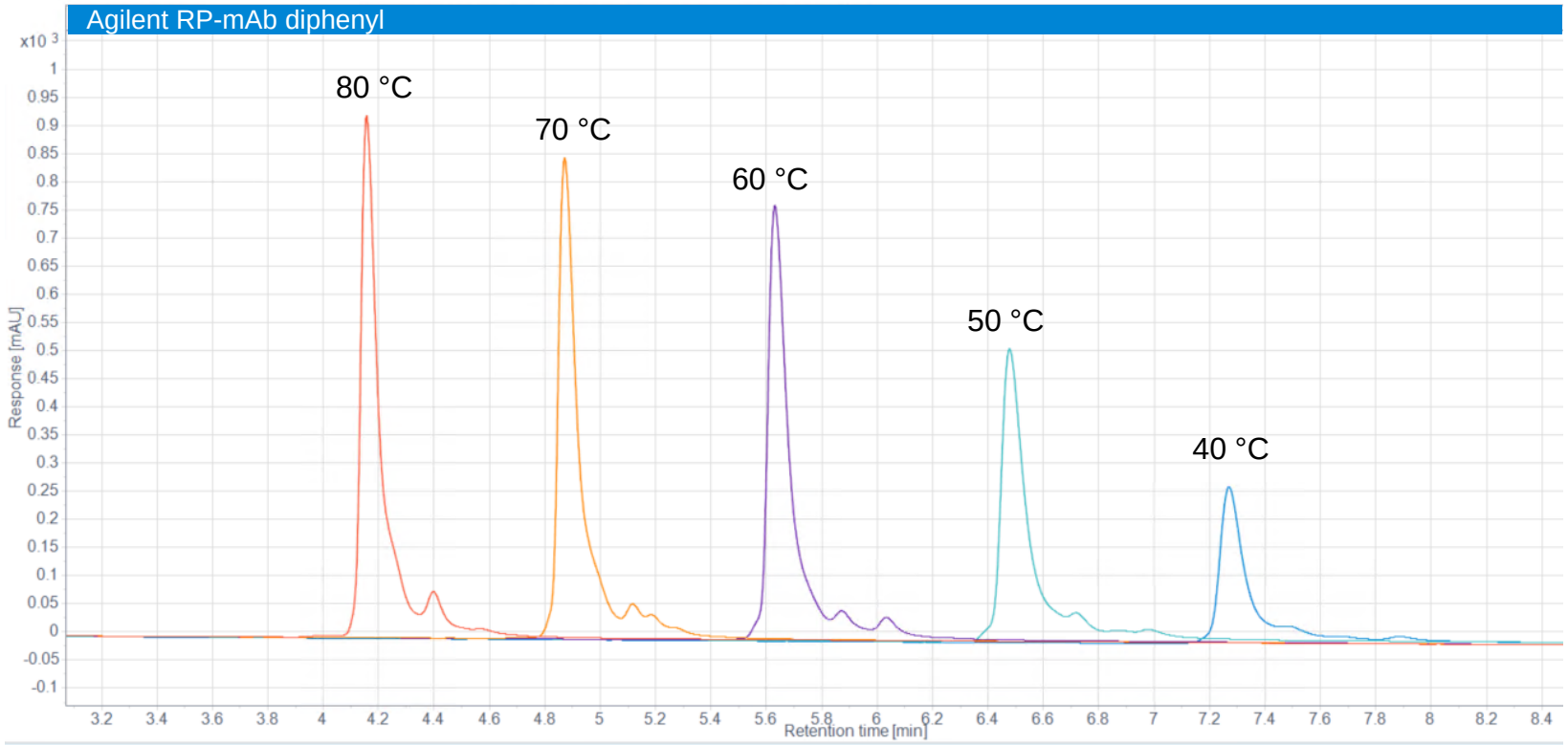
Bonding Chemistry

- AdvanceBio RP-mAb C4 provides a sharp peak and resolves fine detail in less than 2 minutes
- AdvanceBio RP-mAb diphenyl resolves more fine detail



All phases provide good separation of intact mAb, but AdvanceBio RP-mAb diphenyl resolves more fine detail

Effect of column temperature

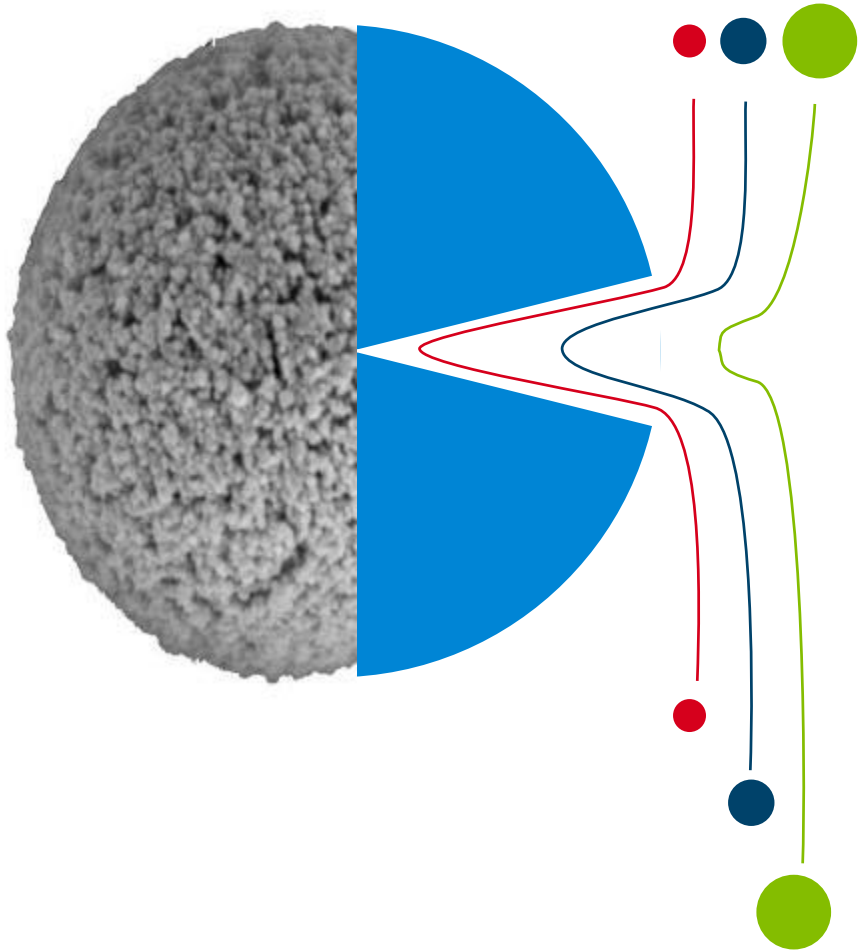


Increasing temperature:

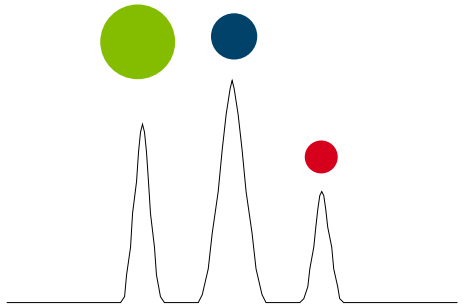
- Reduces mobile phase viscosity
- Improves mass transfer (for sharper peaks)
- Leads to shorter retention times
- Reduces operating pressure
- Can cause degradation of some proteins

Size Exclusion Chromatography

Separation by size in solution under nondenaturing conditions



Size in solution is related to retention time:

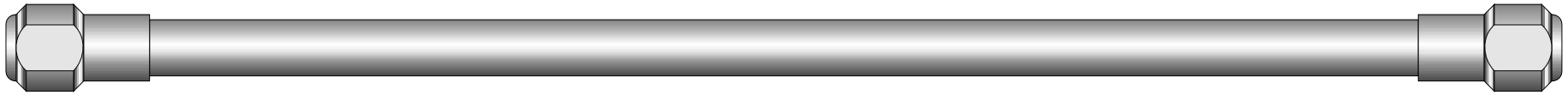


Smaller molecules spend longer in the pores and elute later

Larger molecules spend less time in the pores and elute sooner

Some Definitions

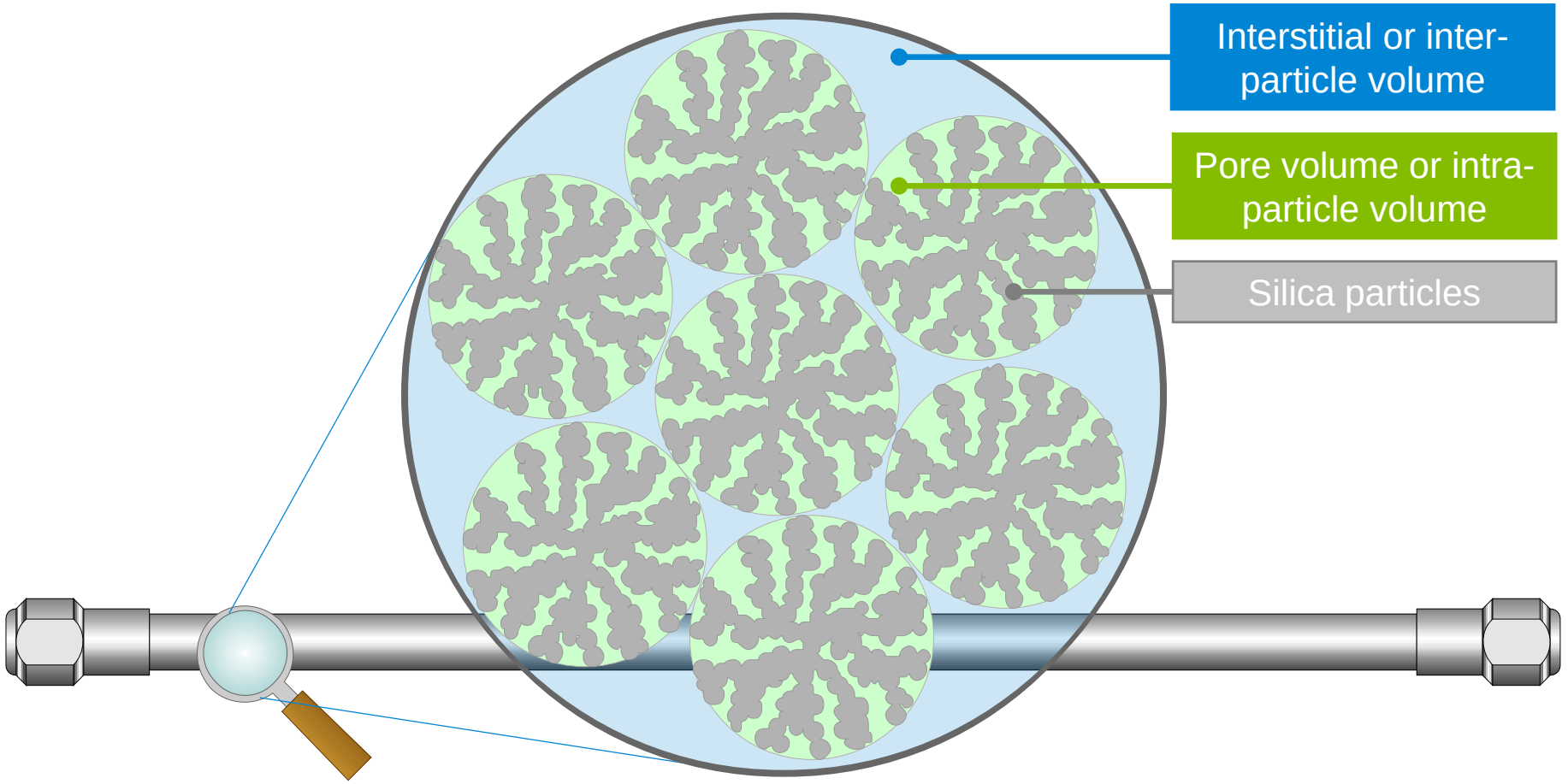
- Column volume
- Exclusion limit/void volume
- Interstitial volume
- Pore volume
- Total permeation
- Nonspecific interaction



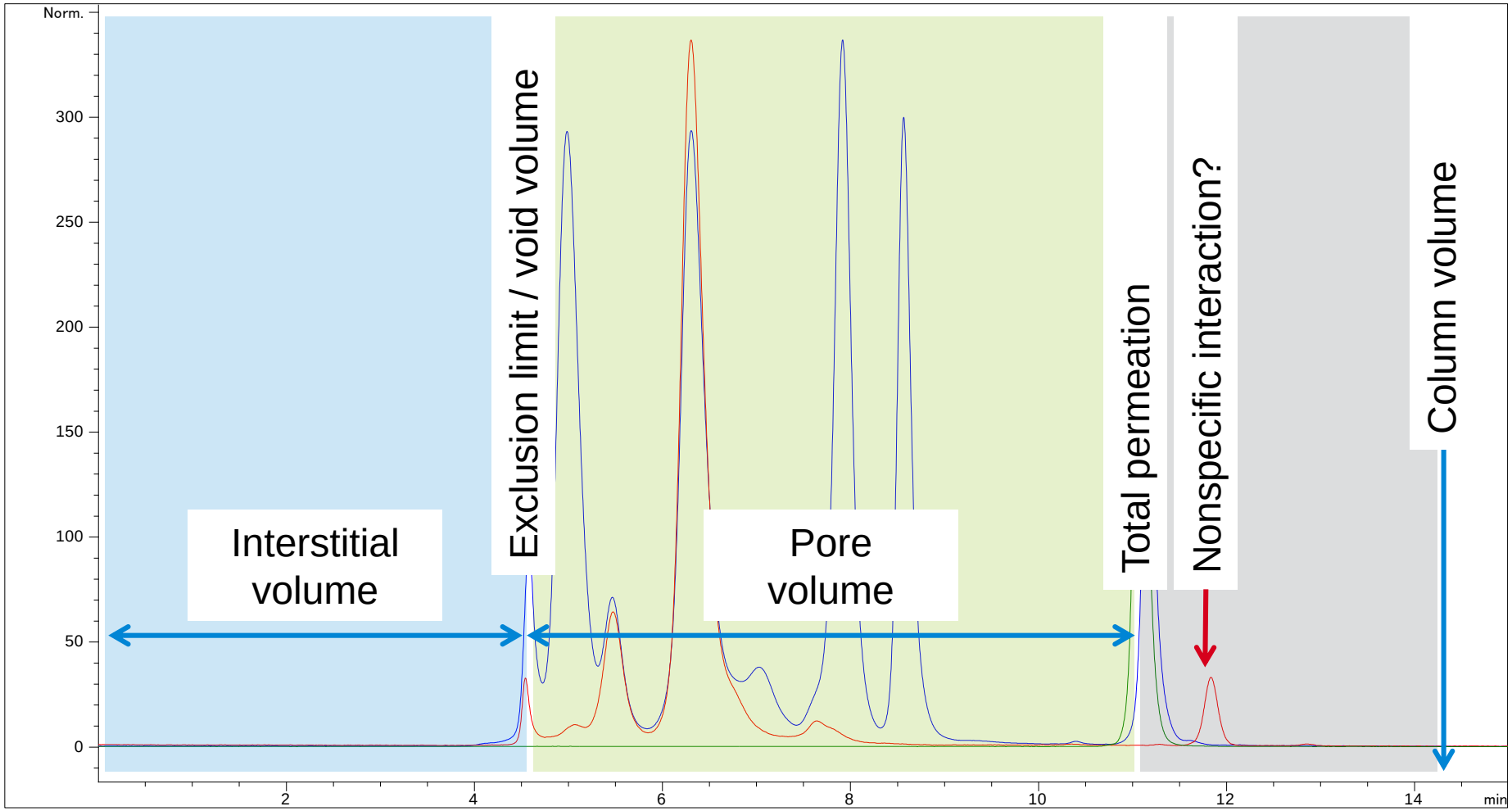
Column dimensions: 7.8 x 300 mm
Column volume = 14.3 mL

What are these inside the column ?

- Interstitial volume
- Pore volume



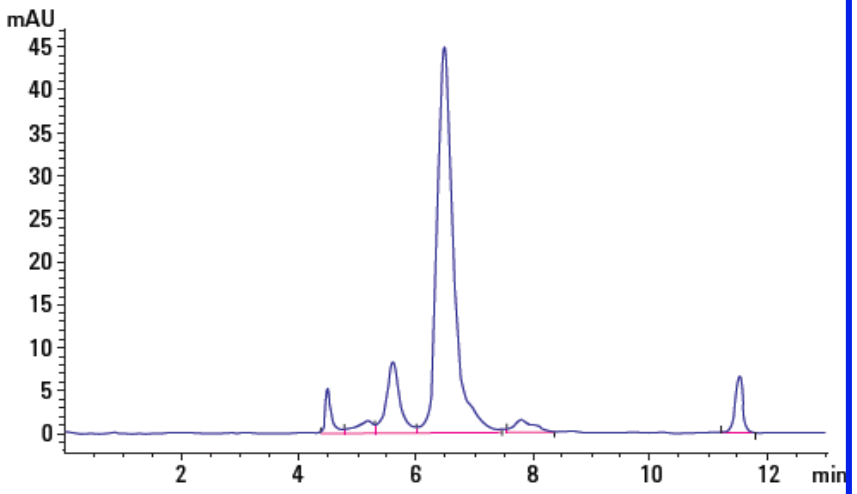
What are these regions on a chromatogram?



Operating parameters

Recommended starting conditions

Column:	Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 x 300 mm (p/n PL1180-5301)
Flow rate:	1 mL/min
Mobile phase:	150 mM phosphate buffer, pH 7.0
Wavelength:	220 nm
Temperature:	ambient
Injection volume:	5 µL
Sample:	IgG



Parameter	Conditions
Mobile phases	Aqueous buffers 150 mM phosphate buffer, pH 7.0 Aqueous organic mixes
pH	2–8.5
Operating temp	20–30 ˚C (recommended) 80ºC (maximum)
Operating pressure	<200 bar per column (recommended) 400 bar (maximum)
Flow rate	0.1–2.0 mL/min for 7.8 mm id 0.1–0.7 mL/min for 4.6 mm id
Protein resolving ranges	0.1–100 kD for 130 Å 5–1,250 kD for 300 Å

Common SEC Challenges

Insufficient/incorrect pore sizes can reduce resolution

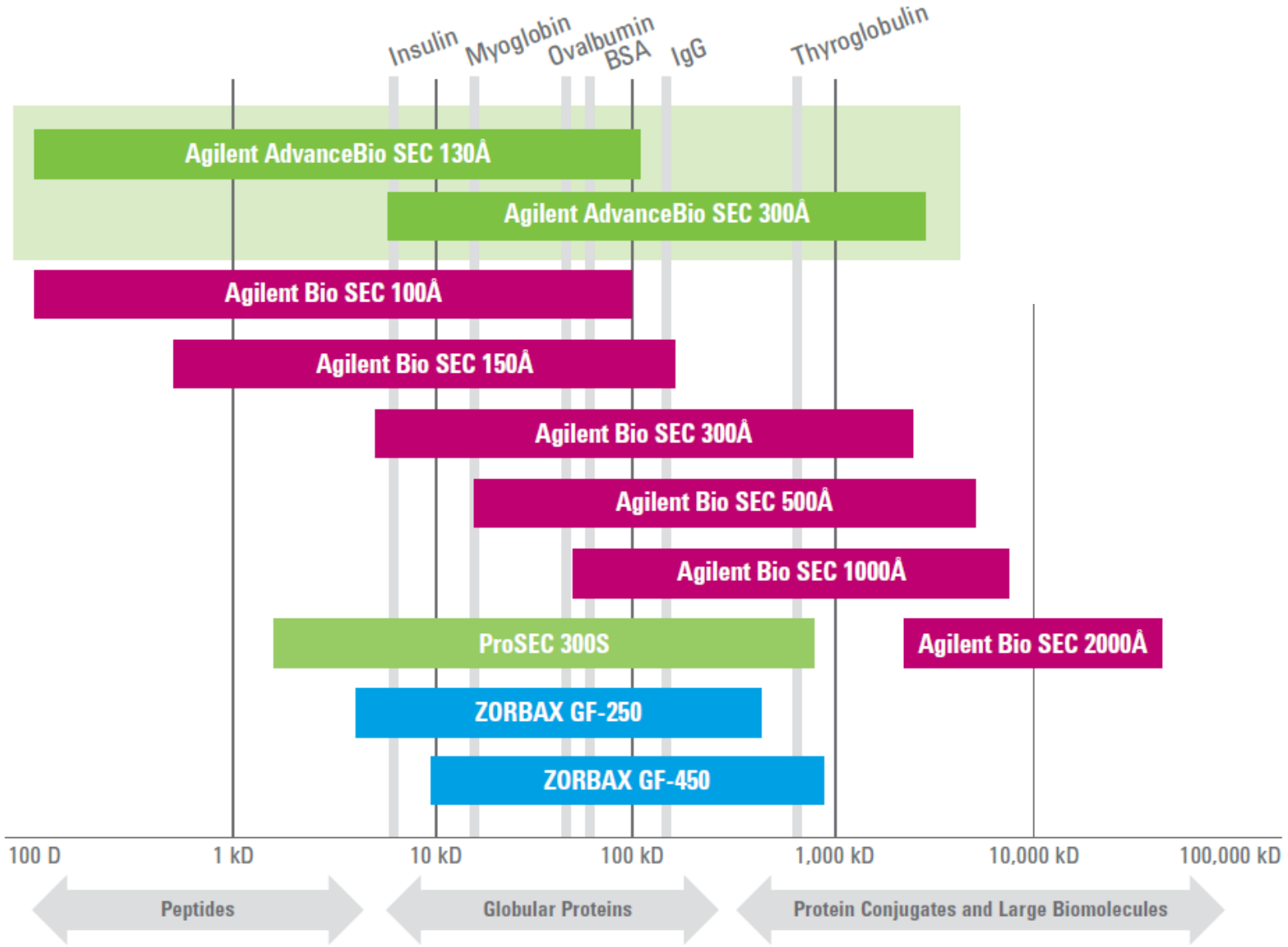
Nonspecific interactions contribute to loss of sample, lead to inconsistent results, rework

SEC is typically slow

Consistent and robust results

High-salt conditions puts excessive wear on instrument, parts

Agilent SEC Pore Size Selection Table



IEX for Proteins

Separation of molecules differing in net charge under nondenaturing conditions

Gradient required.

Protein interaction based on accessible surface charges and corresponding electrostatic interaction with column stationary phase.

- Degree of retention dependent on strength and number of interactions.

Separation based on differences in degree of charge.

Sample is injected in a mobile phase buffer with a low salt concentration

- Binds proteins to the column.

Typically eluted at constant pH with increasing salt gradients (mobile-phase ionic strength).

- Displaces proteins from stationary phase.

Higher charge proteins bind more strongly.

- Increased salt gradient needed to elute.

Typical mobile phase will contain NaCl.

Technique does not denature.

General Guidelines

The general rule for choosing an IEX column for proteins.

- 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

IEX Conditions

Buffer/Ionic Strength

Certain ionic strength required to sustain the column function

Usually minimal of 10–20 mM required

Greater than 30 mM may prevent adsorption

Commonly used salts are NaCl, KCl, and Acetate

Elution salt is typically 400–500 mM

Buffer and pH Selection

Phosphate, tris, MES, and ACES buffers are commonly used

For cation exchange, pH of 4–7

For anion exchange, pH of 7–10

Typical Starting Buffers

Anion exchange

Buffer A =
20 mM Tris, pH = 8.0

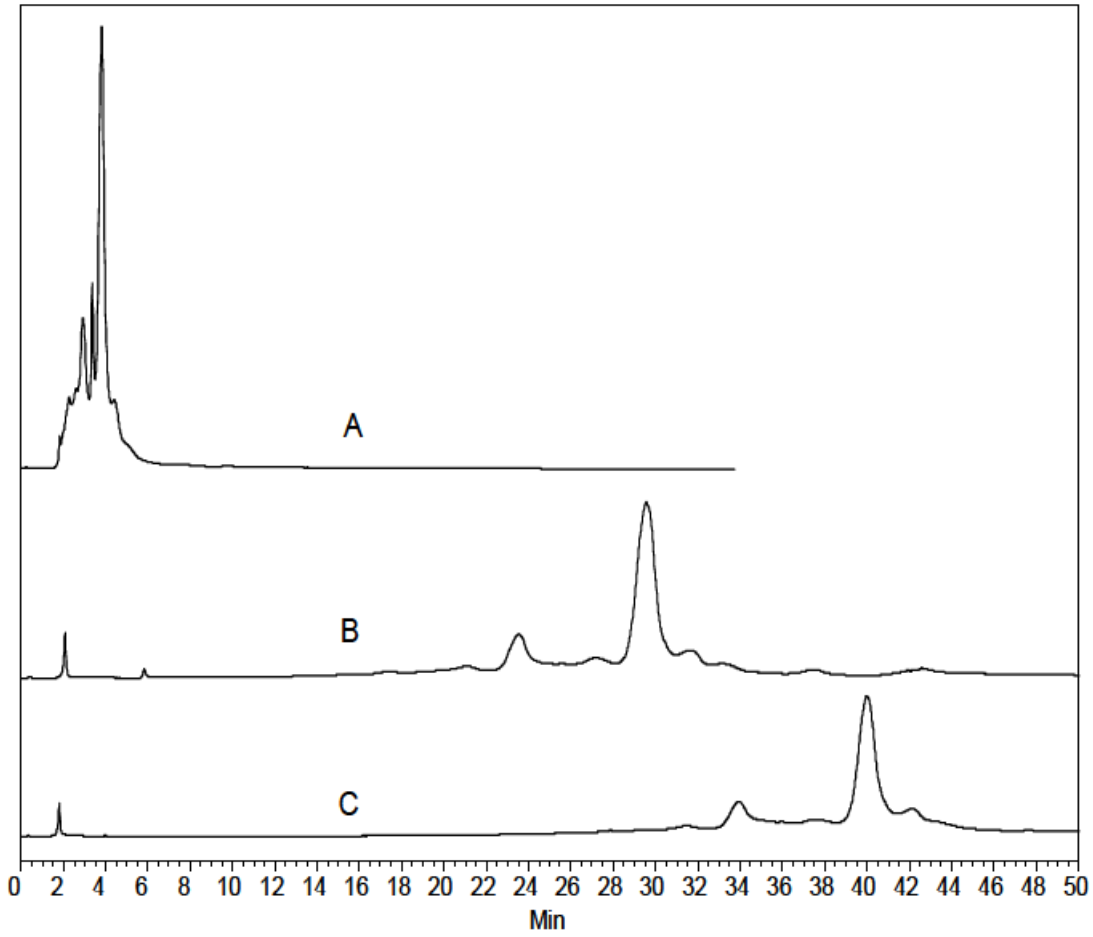
Buffer B =
20 mM Tris, 1 M NaCl, pH = 8.0

Cation exchange

Buffer A =
30 mM sodium acetate, pH = 4.5

Buffer B =
30 mM sodium acetate, 1 M NaCl, pH = 4.5

Impact of Buffer Concentration



Column: Agilent Bio MAb, NP10, 4.6 x 250mm

Mobile phase:

A: Phosphate buffer, pH 7.5

B: A + 0.1M NaCl

Initial buffer concentration:

A = 20 mM phosphate

B = 10 mM phosphate

C = 5 mM phosphate

Gradient: 15-65%B in 60 min

Flow rate: 0.8 mL/min

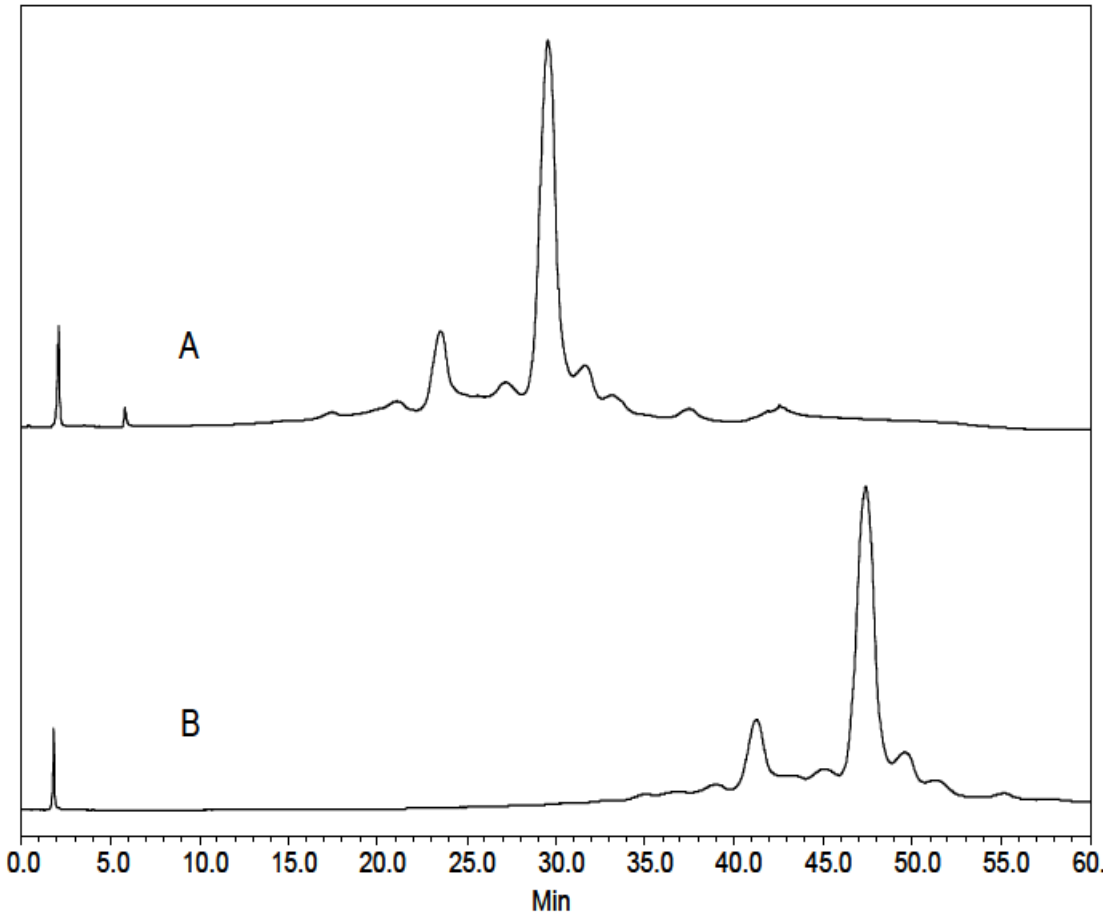
Sample: Monoclonal Antibody

Injection: 10 □L (1.5 mg/mL)

Temperature: 25o C

Detection: UV 214 nm

Effect of pH



Column: Agilent Bio MAb, NP10, 4.6 x 250 mm

Mobile phase:

A: 10 mM phosphate;

B: A + 0.1 M NaCl

pH:

A = pH 7.5

B = pH 7.0

Gradient: 15–65%B in 60 min

Flow rate: 0.8 mL/min

Sample: Monoclonal Antibody

Injection: 10 µL (1.5 mg/mL)

Temperature: 25 °C

Detection: UV 214 nm

Hydrophobic Interaction Chromatography

Separates molecules based on differences in hydrophobicity.

HIC is used for

- Separating proteins
- Separating variants (impurities) from individual proteins
- Separating antibody drug conjugate species

Unlike reversed phase, which denatures proteins, HIC conditions maintain proteins in their intact, native (and therefore active) state.

What Is Needed for HIC ?

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 – 2 M concentration

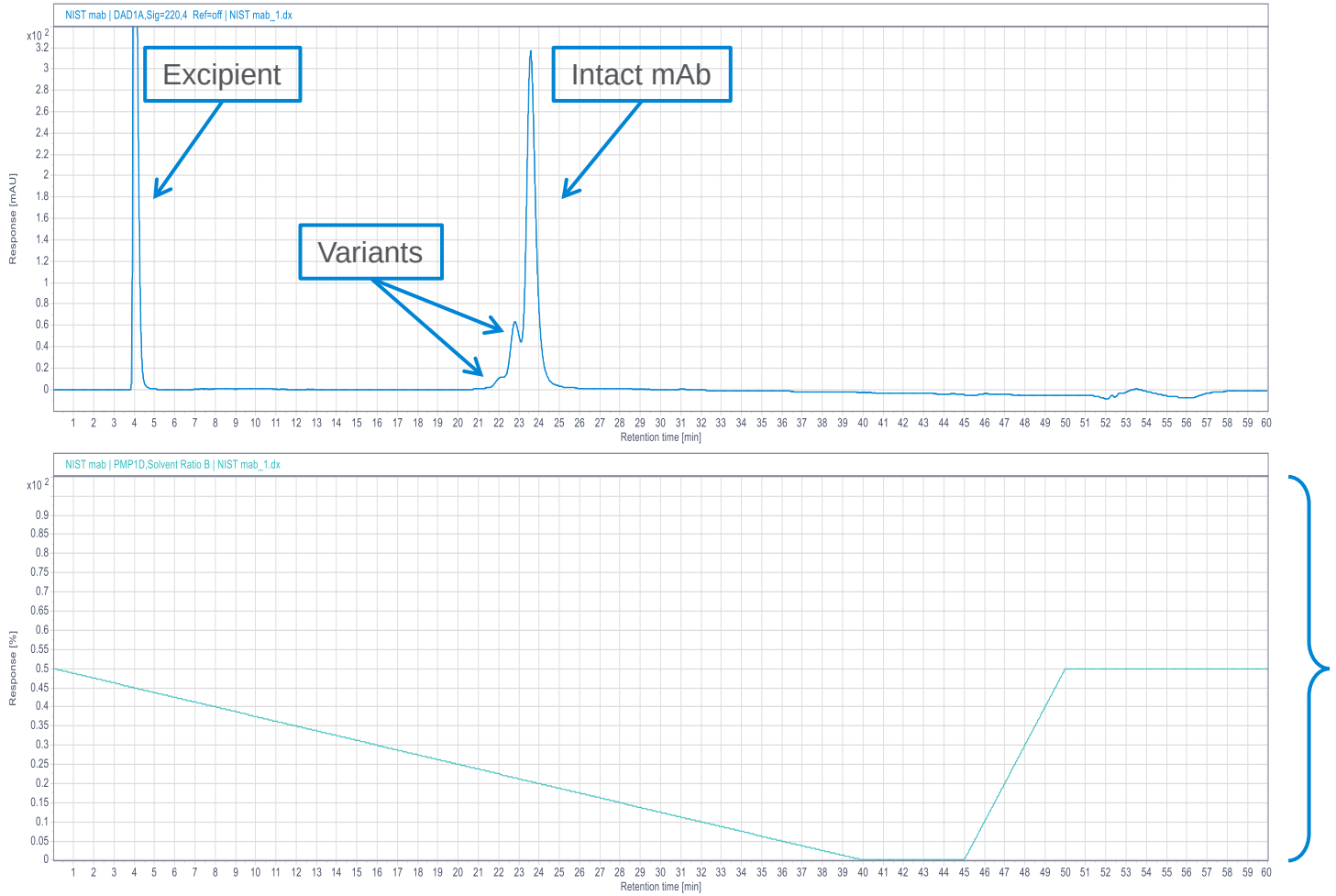
Mobile phase contains a buffer salt to ensure consistent pH and to keep the protein dissolved.

- Sodium phosphate, pH 7, typically 50 – 100 mM concentration

Gradient elution from high to low salt concentration

- 10 – 20 column volume gradients are ideal

HIC Separation of NIST mAb (RM 8671)



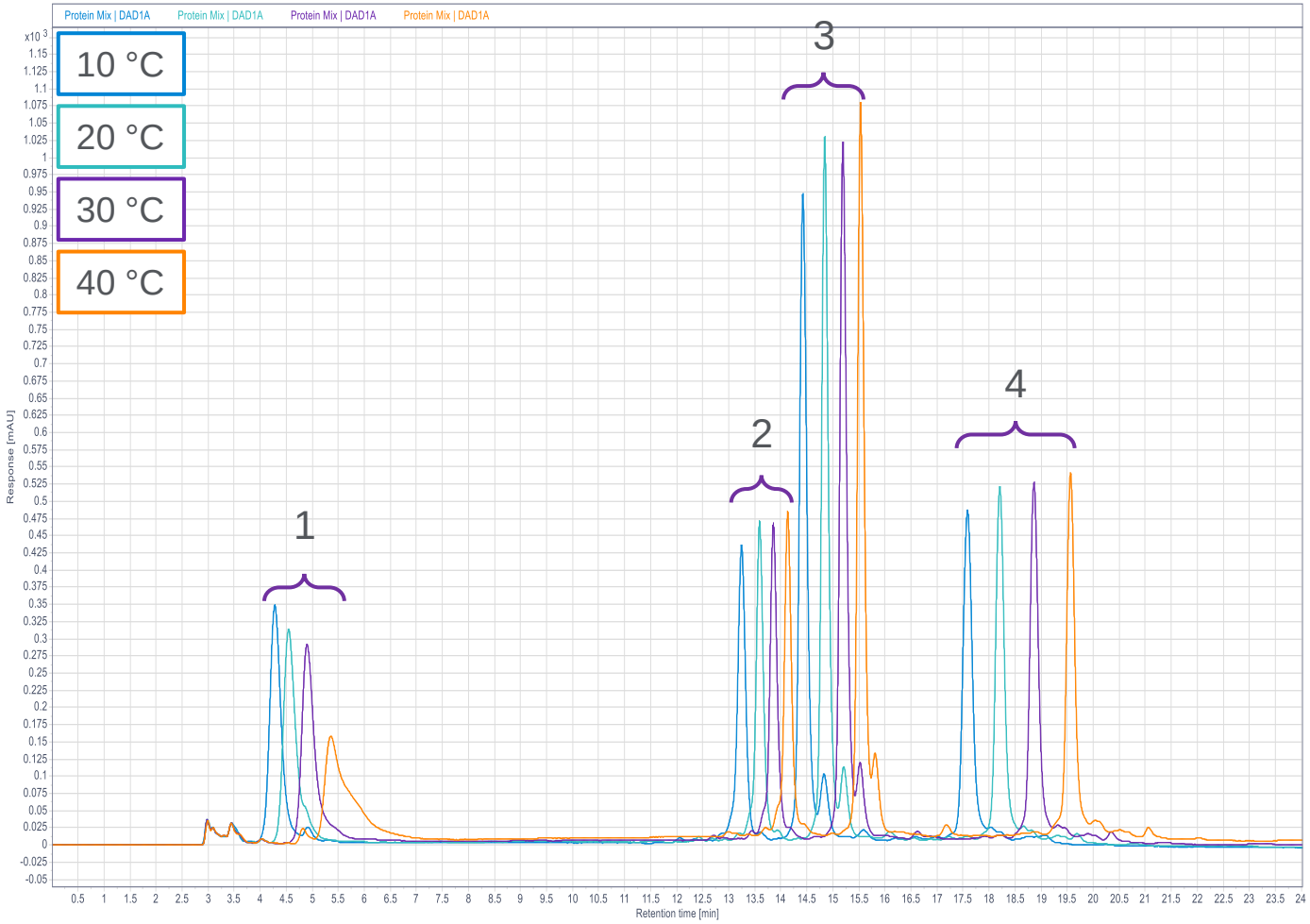
Method conditions

Column: AdvanceBio HIC 4.6 x 100 mm
Eluent A: 50 mM NaPO, pH 7.0
Eluent B: 2 M $(NH_4)_2SO_4$, 50 mM NaPO, pH 7.0
Flow rate: 0.3 mL/min
Temperature: 25 °C
Injection: 5 µL (1 mg/mL)
Sample: NIST mAb (RM 8671)

Gradient profile

Time	%A	%B
0	50%	50%
40	100%	0%
45	100%	0%
50	50%	50%
60	50%	50%

Effect of Column Temperature in HIC



Increasing temperature:

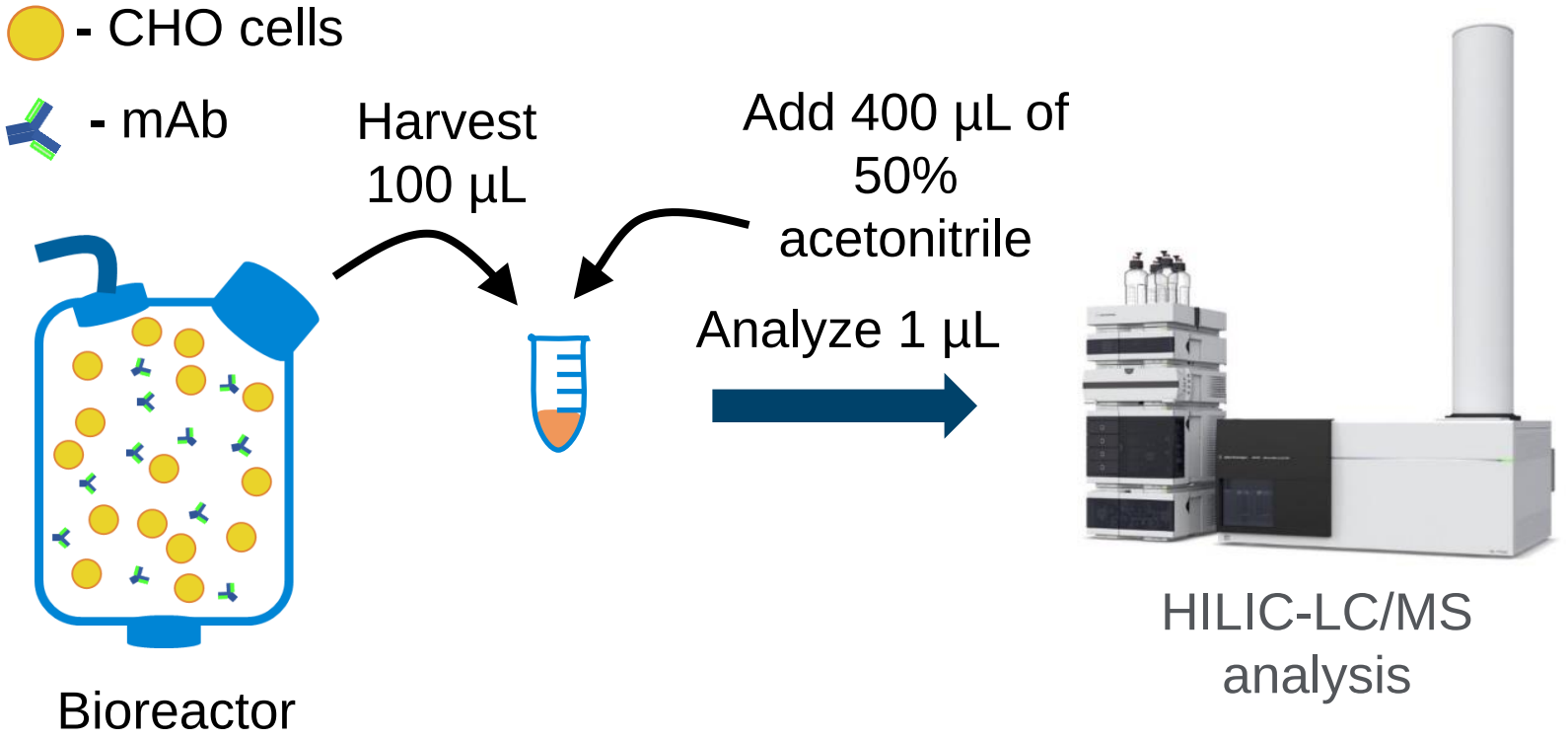
- Reduces mobile phase viscosity
- May lead to loss in resolution
- Leads to longer retention times
- Reduces operating pressure
- Can cause degradation of some proteins

1 Cytochrome C
2 Ribonuclease A
3 Lysozyme
4 α-Chymotrypsinogen A

HILIC

Typically used for very hydrophilic molecules like sugars, but also unlabeled amino acids.

LC/MS of underivatized amino acids and other metabolites



… a fast and simple approach to profiling cell culture media

AdvanceBio MS Spent Media Column

HILIC column for LC/MS analysis of underivatized amino acids

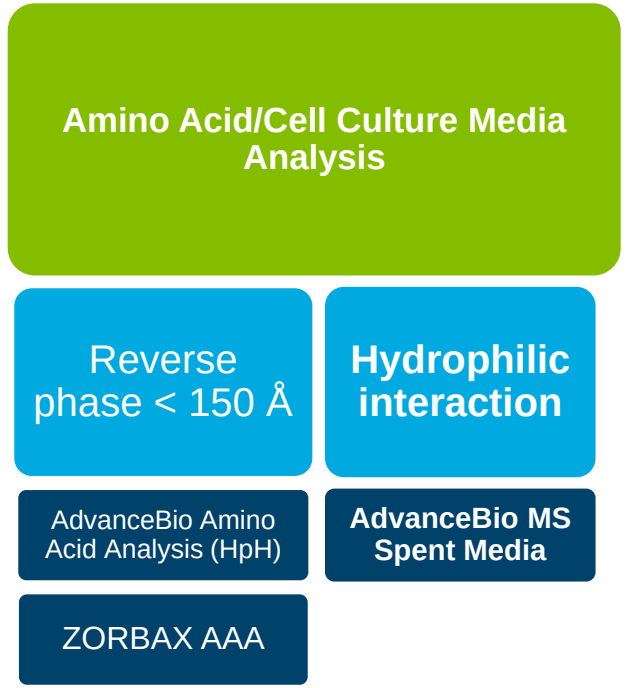
- 2.7 µm Poroshell particle
  - Superficially porous for high efficiency at moderate backpressures

PEEK-lined stainless steel hardware

- Inert flow path for:
  - Maximum recovery and best peak shape of “sticky” compounds

MS-compatible mobile phases

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



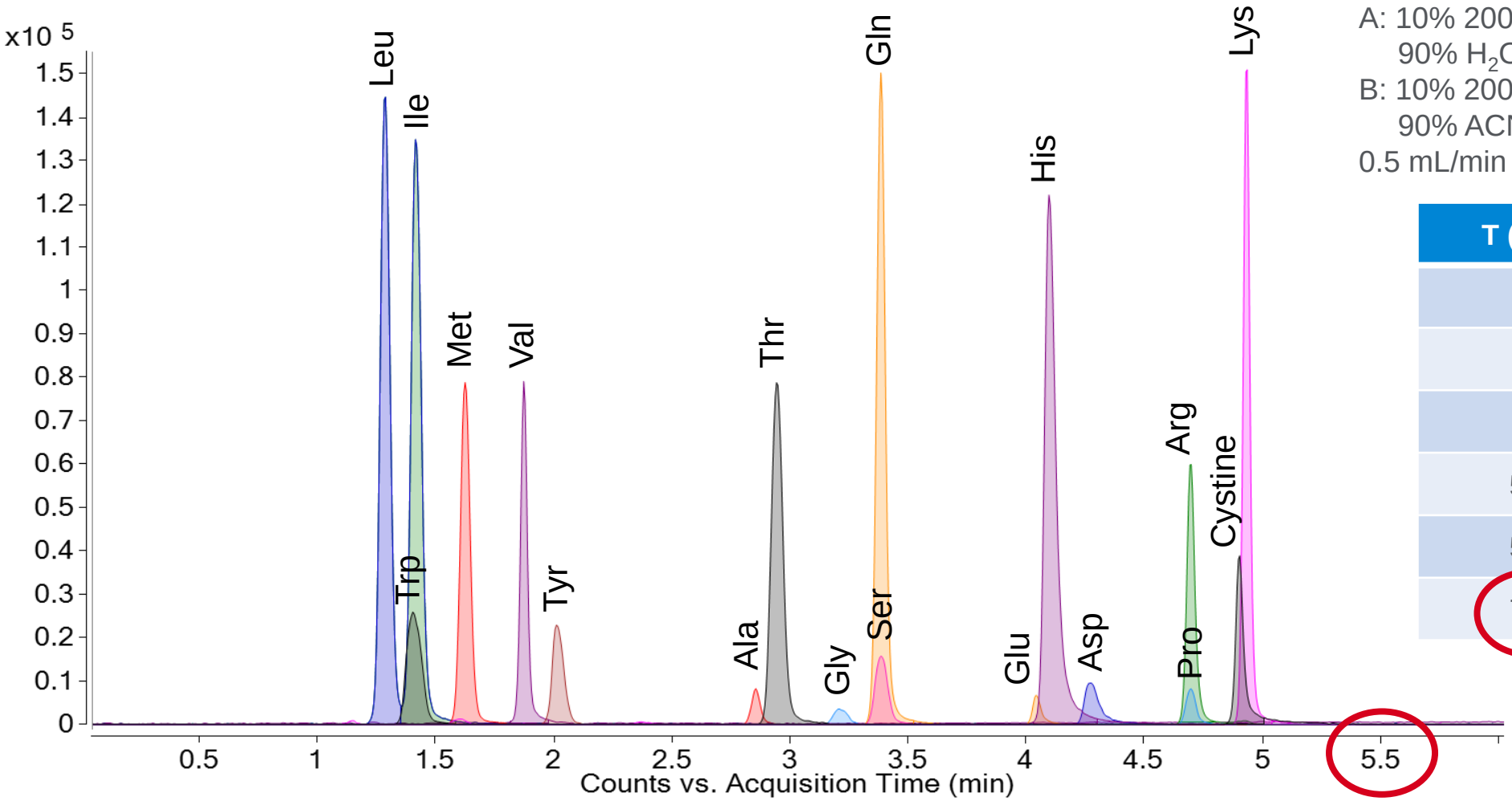
Additional resources:

Amino Acid Analysis “How-to” guide

Application finder

AdvanceBio e-seminar series

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

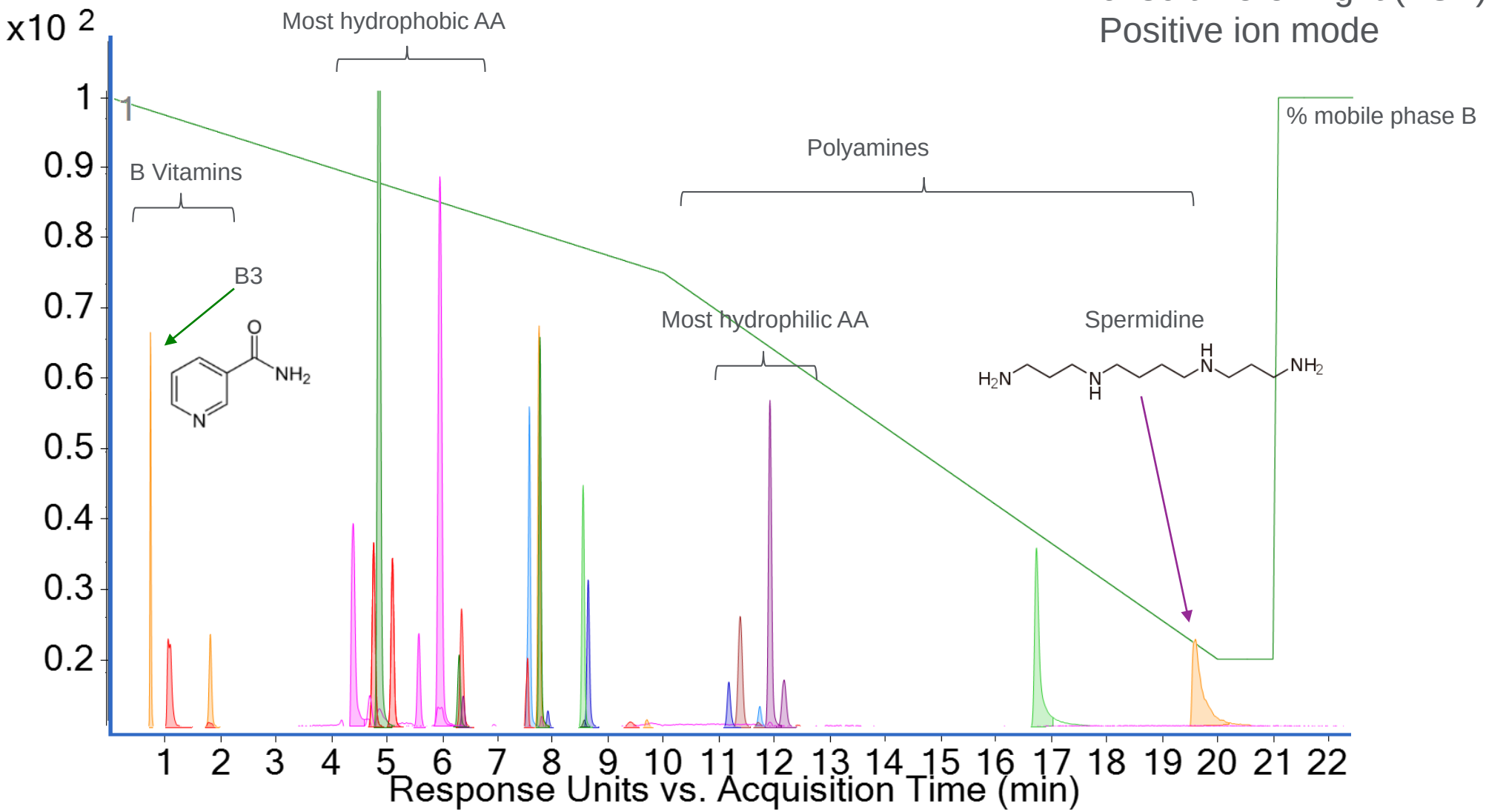


AdvanceBio MS Spent Media
2.1 x 50 mm
A: 10% 200 mM ammonium formate pH 3.5, 90% H_2O
B: 10% 200 mM ammonium formate pH 3.5, 90% ACN
0.5 mL/min

T (min)	%B
0	97
2	90
5	70
5.5	70
5.6	97
7.5	97

Survey a Wide Range of Spent Media Metabolites

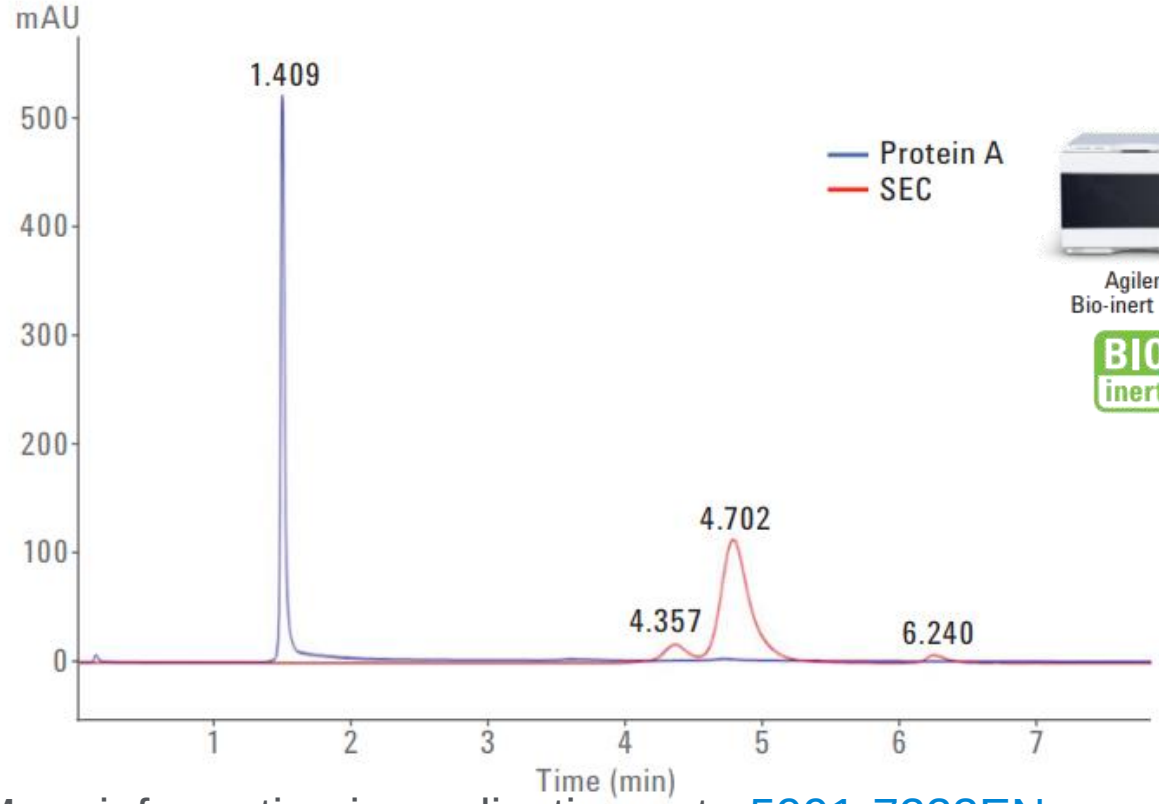
1260 Infinity II bio-inert LC
6230 time-of-flight (TOF) LC/MS
Positive ion mode

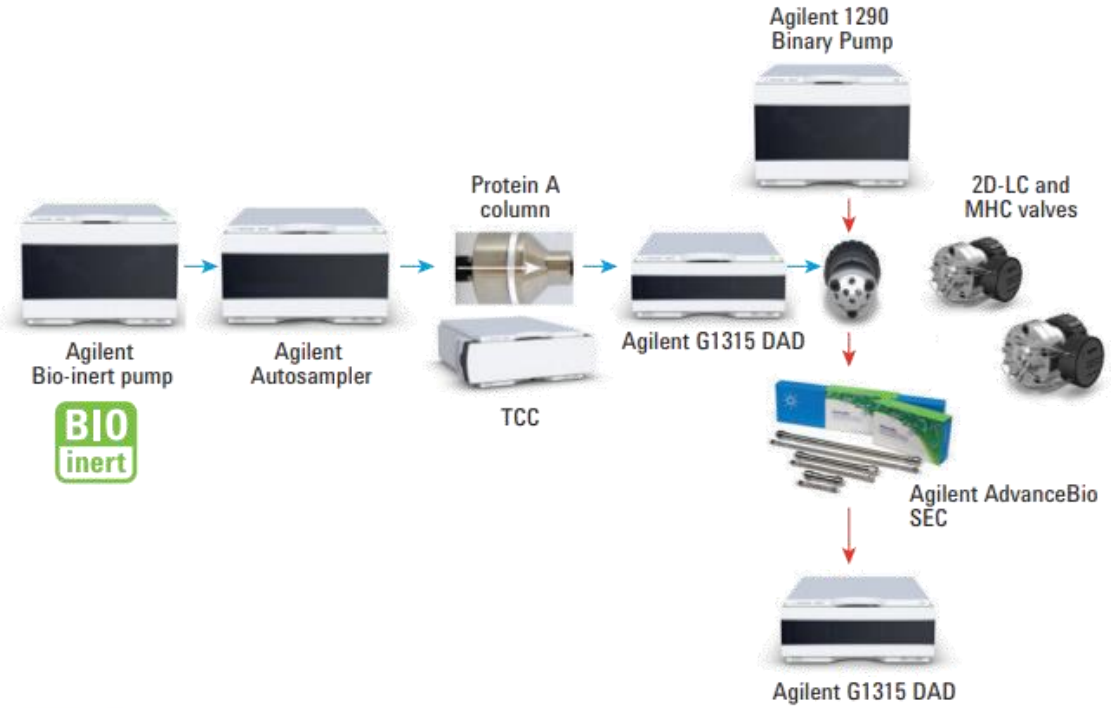


Affinity

Bio specificity for a defined ligand

2D-LC: Protein A capture followed by SEC analysis





More information in application note [5991-7223EN]

HPLC and Biomolecules

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

… so do Agilent Biocolumns!

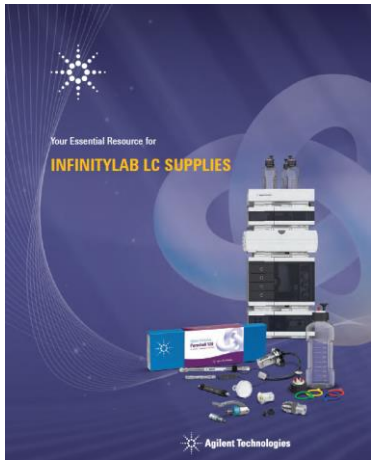
Agilent Biomolecule Columns

Agilent Biomolecule HPLC Columns

Titer determination	Aggregate analysis	Intact purity and PTM analysis		Sequence Variant and PTM analysis	Charge variant analysis	Glycan analysis	Amino acid /cell culture media analysis	
Affinity	Size exclusion	Reverse phase >150 Å	Hydrophobic interaction	Reverse phase < 150 Å	Ion exchange	Hydrophilic interaction	Reverse phase < 150 Å	Hydrophilic interaction
Bio-Monolith Protein A	AdvanceBio SEC	PLRP-S	AdvanceBio HIC	AdvanceBio Peptide Plus	Bio MAb	AdvanceBio Glycan Mapping	AdvanceBio Amino Acid Analysis (HpH)	AdvanceBio MS Spent Media
Bio-Monolith Protein G	Bio SEC-3	AdvanceBio RP mAb		AdvanceBio Peptide Mapping	Bio IEX (SAX, WAX, SCX, WCX)	ZORBAX RRHD 300-HILIC 1.8 µm	ZORBAX AAA	
	Bio SEC-5	ZORBAX RRHD 300 Å, 1.8 µm			PL SCX, SAX			
		ZORBAX 300SB			Bio-Monolith (QA, DEAE, SO3)			
		Poroshell 300						

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

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

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration, and QuEChERS

Option 4 for Spectroscopy Supplies

Option 5 for Standards

gc-column-support@agilent.com

lc-column-support@agilent.com

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