Method Development For Biomolecules

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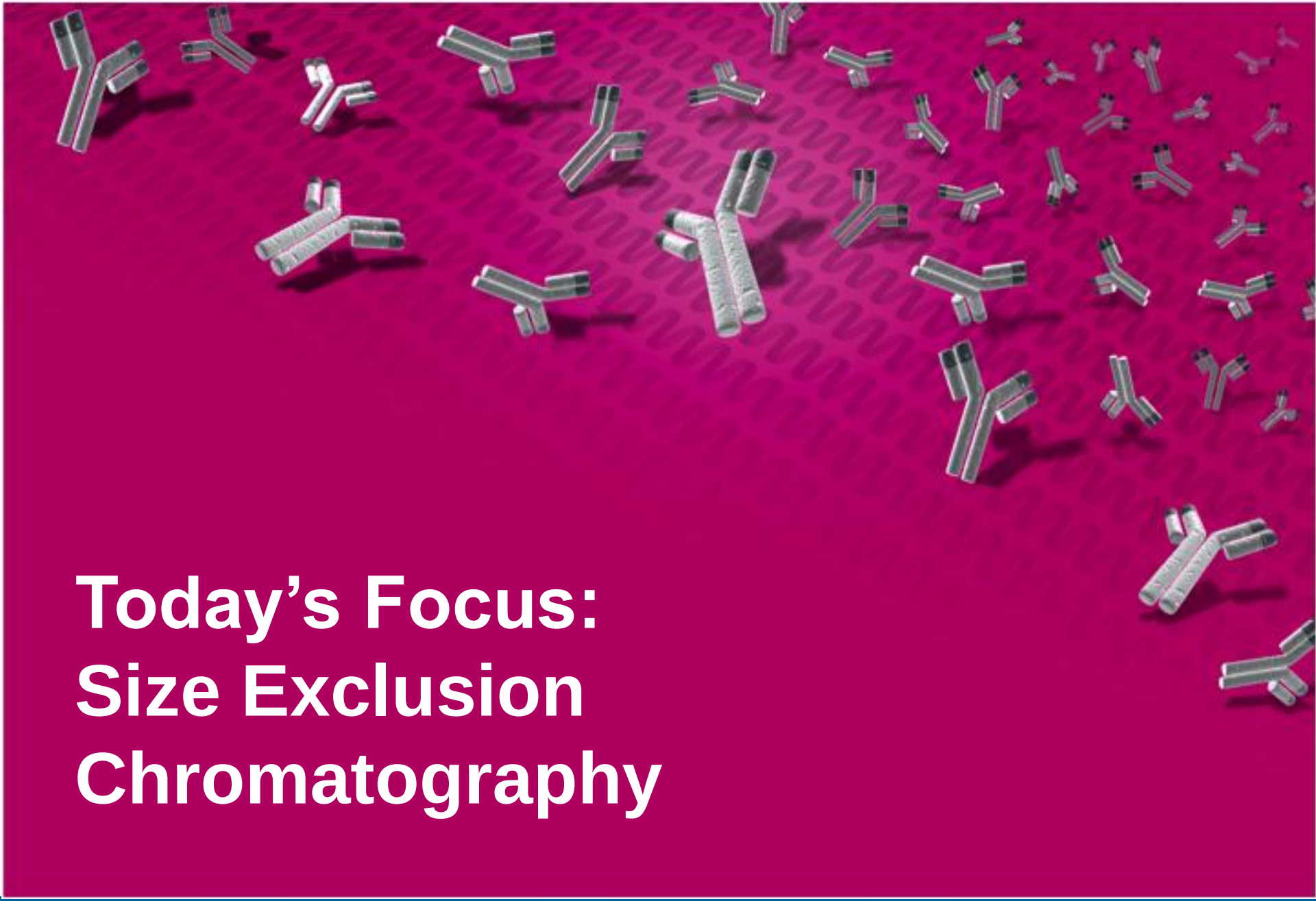
Where to Begin?

1. Define the separation goals
2. Choose the mode (SEC, IEX, or RP)
3. Choose the appropriate column
4. Make sure you have the right equipment.
5. Choose the appropriate sample and mobile phase conditions
6. Choose optimal flow rate and gradient slope for best separation.



Today's Focus: Size Exclusion Chromatography

First Things First!

Define the separation Goals:

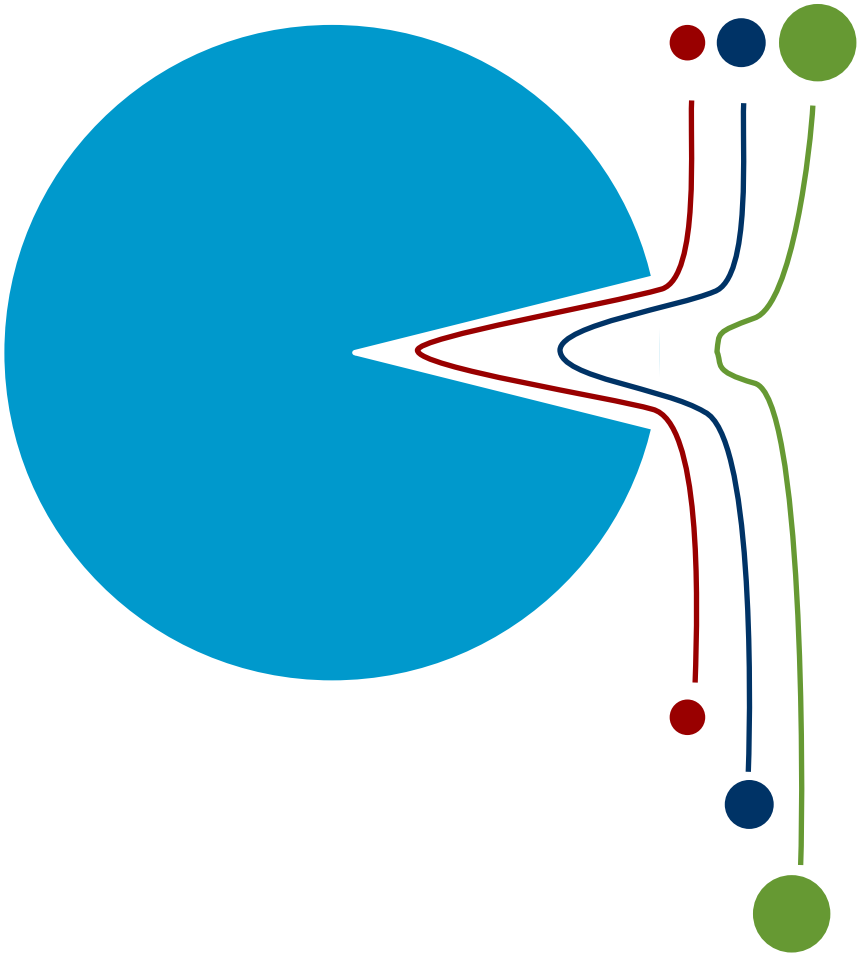
Aggregation or Stability Studies?

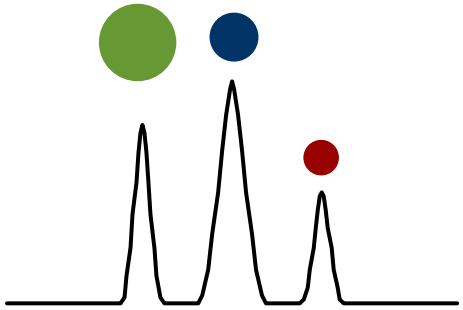
What is the molecular weight of your protein of interest?

What are you trying to separate it from?

The answers to these questions help you choose the appropriate column.

Size Exclusion Process

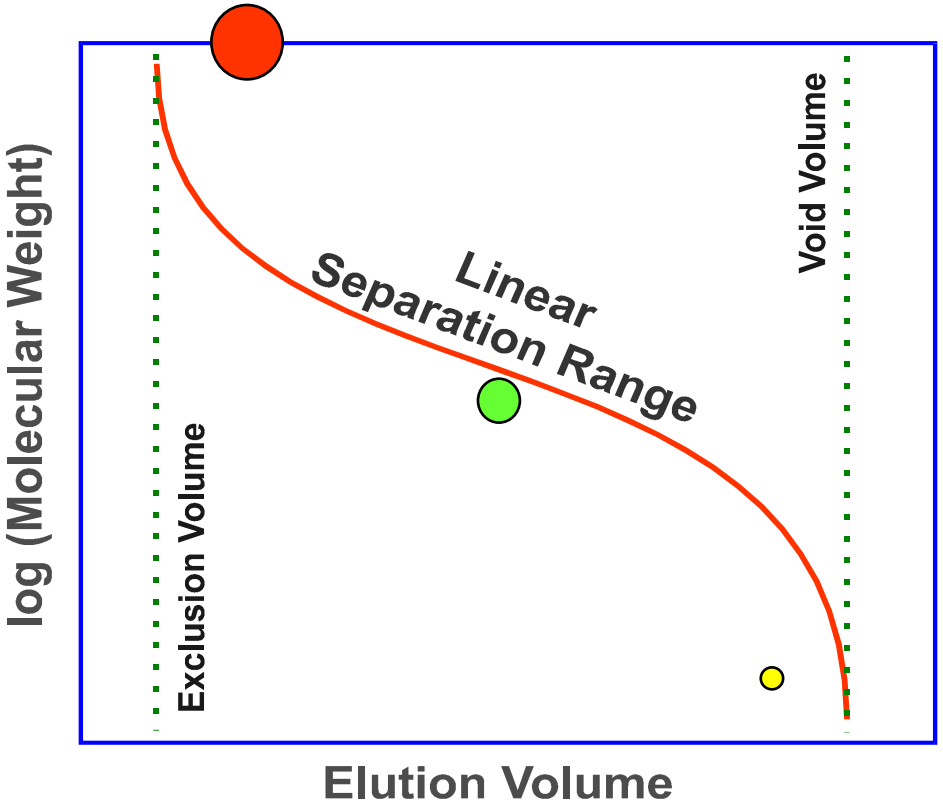


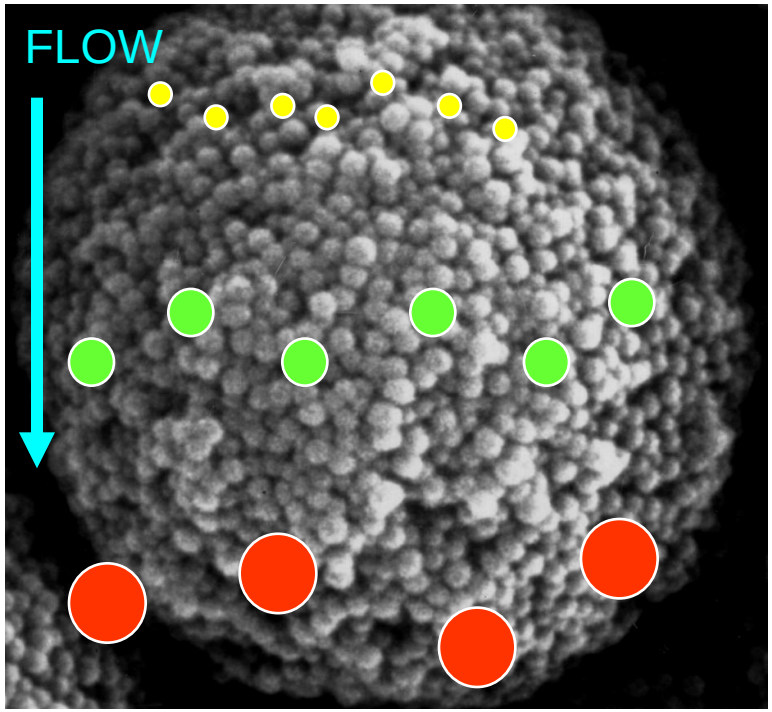


Smaller molecules spend longer in the pores and elute later.

Larger molecules spend less time in the pores and elute sooner.

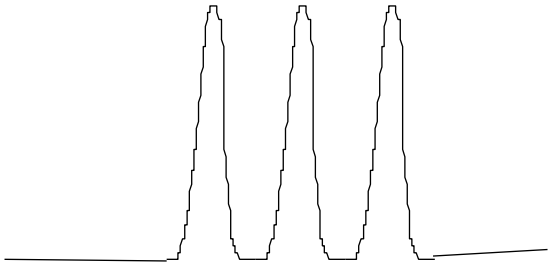
Mechanism of SEC Separation – Pore Size Determines Linear Separation Range





Some General Guidelines for SEC

1. SEC will only provide baseline separation of molecules with more than a 2 fold difference in MW or size.
2. Sample volume should be limited to below 5% of the total column volume.
3. When methods are to be validated, test for ruggedness with several different column lots, mobile phase preparations, and operators.



Column Pore Size Choice Study

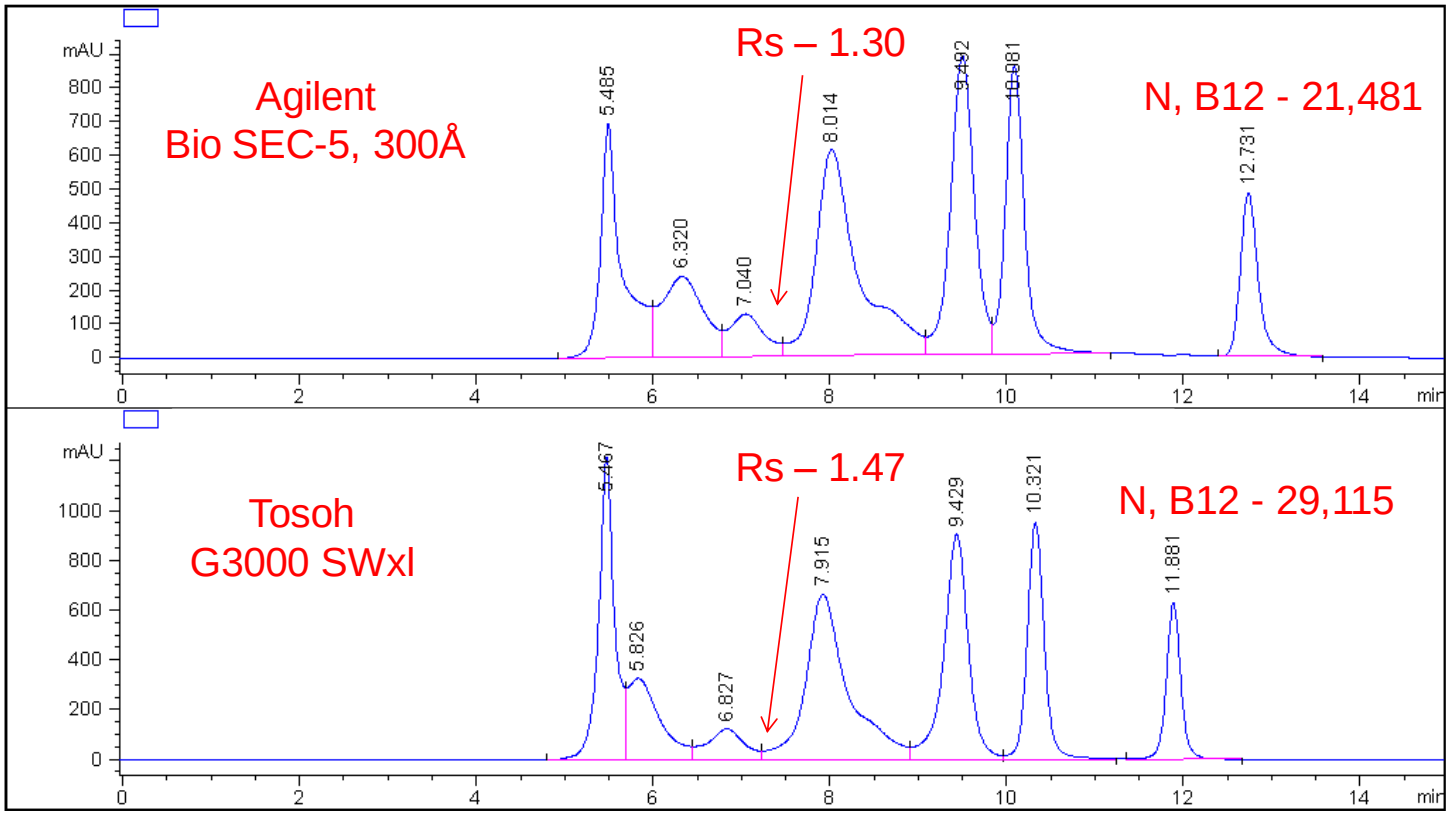
Bio-Rad Size Exclusion Standards

1. Thyroglobulin- 670 KD
2. Gamma Globulin – 320KD and 150 KD
3. Ovalbumin – 44.3 KD
4. Myoglobin- 17 KD
5. Vitamin B12 - 1,350 Da

Two Similar Columns

Agilent Bio SEC-5

Tosoh TSKgel G3000 SWxl



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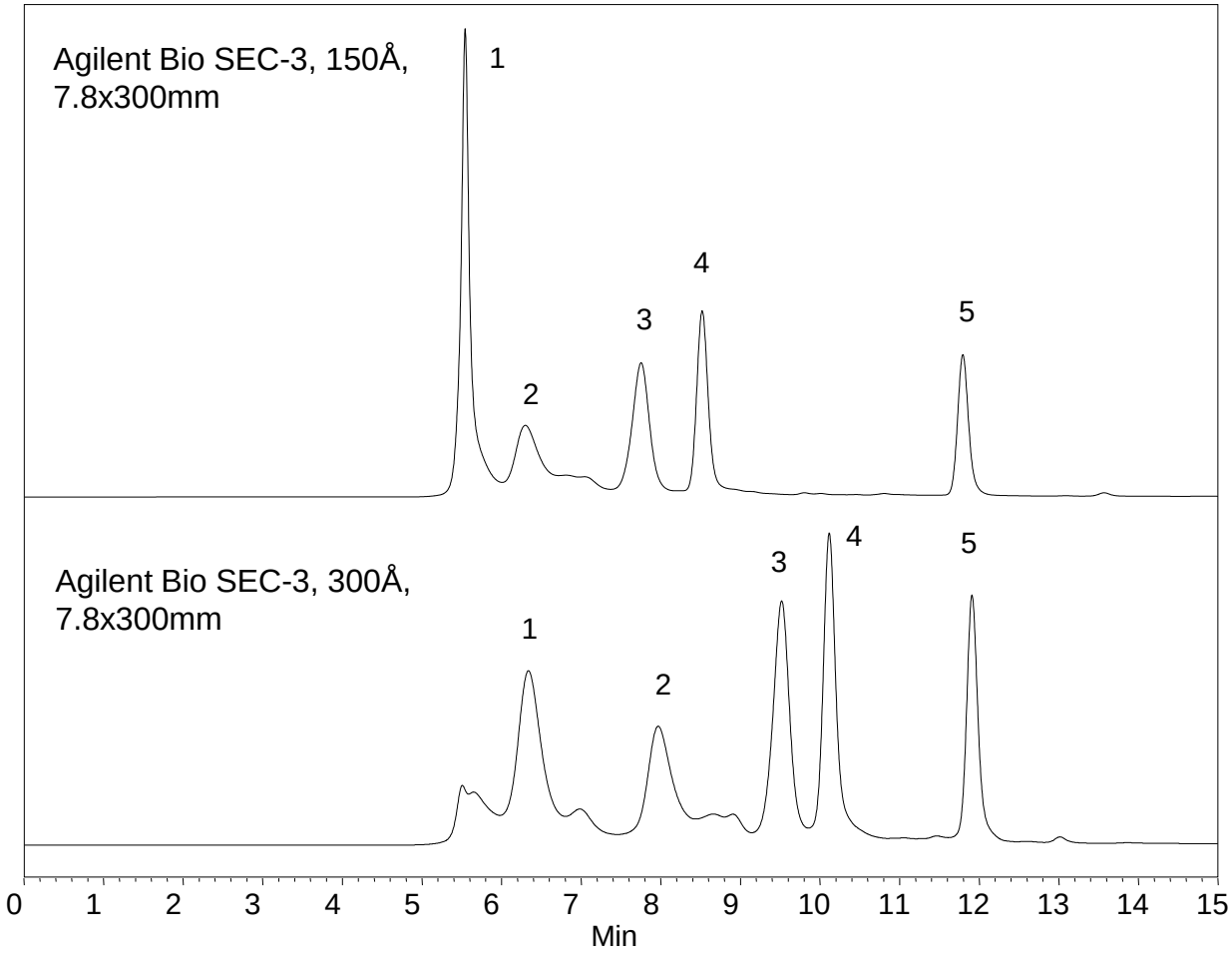
Agilent Bio SEC of Different Pore Size



Columns: Bio SEC-3, 150Å, 7.8x300mm & Bio SEC-3 300Å, 7.8x300mm

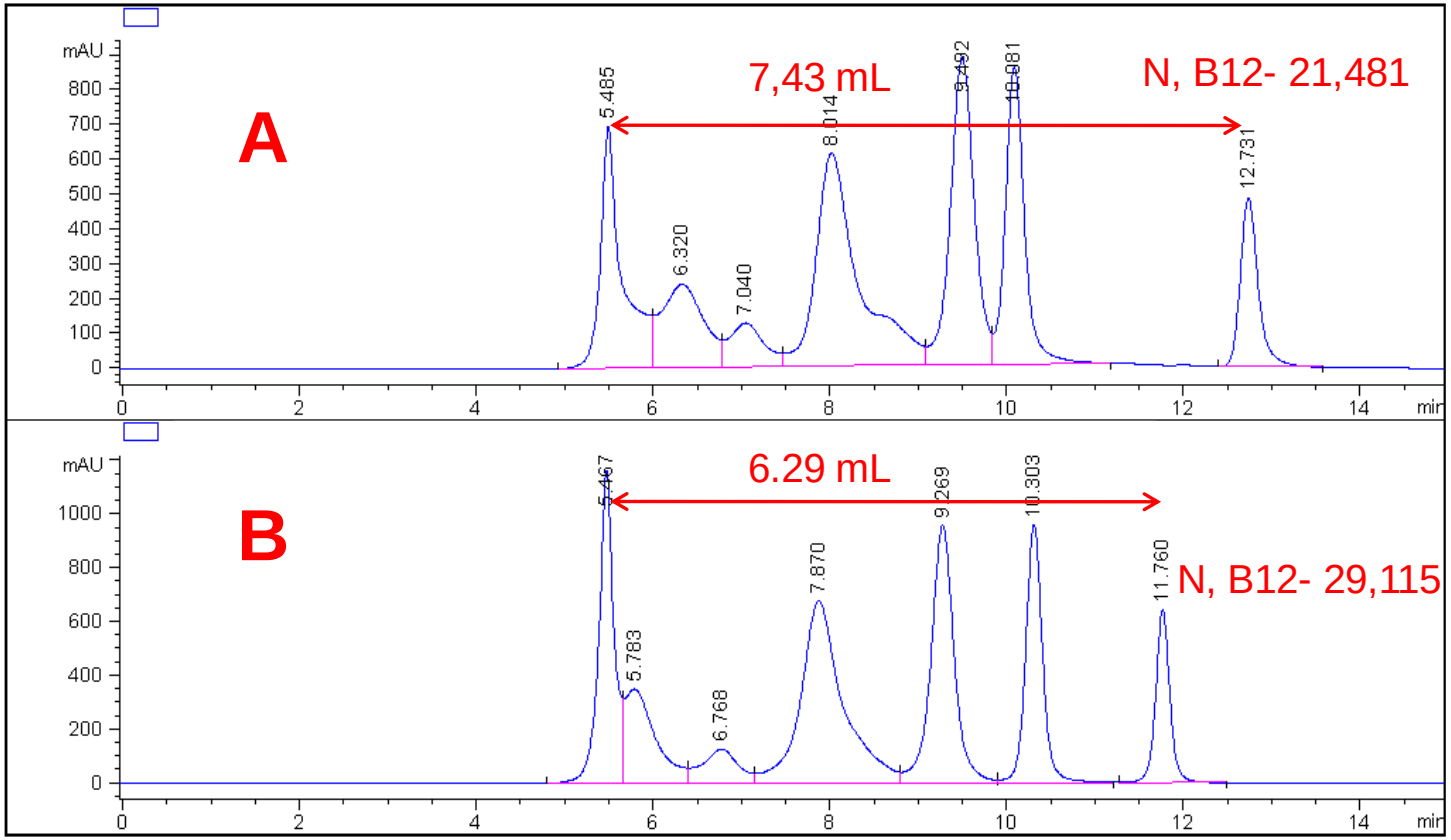
Sample:

1) Thyroglobulin, 670 kD
2) γ-Globulin, 158 kD
3) Ovalbumin, 44 kD
4) Myoglobulin, 16.9 kD
5) Vitamin B12, 1355 D.

Total Column Pore Volume Comparison

(A) Agilent Bio SEC-5

(B) Tosoh TSKgel G3000SWxl



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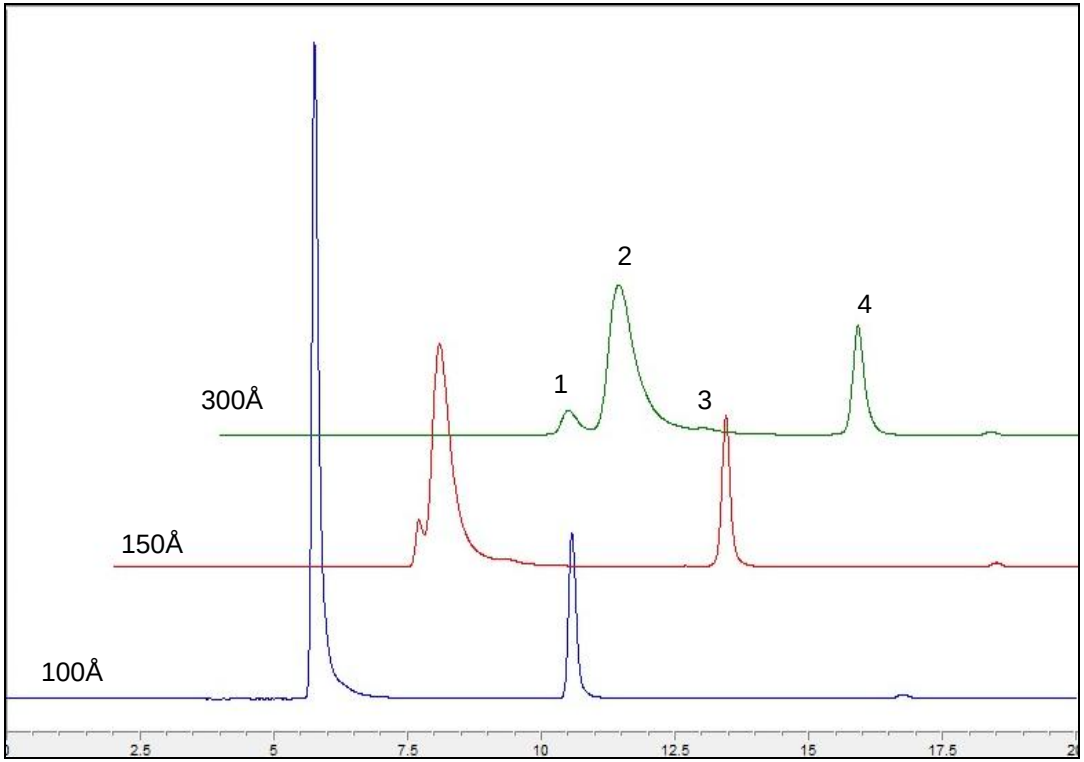
Pore Size Choice for Antibody Analysis

Columns:	Agilent Bio SEC-3 100Å, 150Å & 300Å 3µm 4.6x300mm
Eluent:	50mM Na_2HPO_4, 50mM NaH_2PO_4 + 0.15M NaCl, pH6.8
System:	Agilent 1260 Infinity Bio-Inert LC System
Detector:	UV@220nm
Flow rate:	0.35ml/min
Sample:	Mouse IgG



1. Dimer
2. Monomer
3. Monomer Fragment
4. Azide

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Determine Optimal Mobile Phase Conditions

- What conditions give you the best results?
- What additives may be required to reduce non-specific interactions?
- What is the optimal pH?
- What is the optimal flow rate?

Mobile Phase Considerations:

- Mobile phase should contain buffer/salt (to overcome ionic interactions).
- Mobile phase should not contain too much buffer/salt (to prevent hydrophobic interactions).
- Mobile phase should not alter the analyte (cause degradation / aggregation etc.).
- Mobile phase should be made up fresh and used promptly (bacterial growth is rapid in dilute buffer stored at room temperature).
- Buffer shelf life < 7 days unless refrigerated.
- Mobile phase should be filtered before use. Particulates may be present in water (less likely) or in buffer salts (more likely).

A Note About Pre-Made PBS:

Note: PBS is typically around 10mM phosphate, pH 7.2, 0.8% NaCl (150mM).

Our preference is to use 150mM phosphate buffer, pH 7.0 to avoid use of salt (NaCl).

Other possibilities: sodium sulfate instead of sodium chloride (but remember 0.15M Na_2SO_4 is twice the ionic strength of 0.15M NaCl).

100mM Phosphate Buffer

(Mixing NaH_2PO_4 and Na_2HPO_4 stock solution)

x mL 0.2M Na_2HPO_4	y mL 0.2M NaH_2PO_4	z mL H_2O	200 mL 0.1M pH, 25°C
4.0	46.0	50.0	**5.8**
6.15	43.85	50.0	**6.0**
9.25	40.75	50.0	**6.2**
13.25	36.75	50.0	**6.4**
18.75	31.25	50.0	**6.6**
24.5	25.5	50.0	**6.8**
30.5	**19.5**	**50.0**	**7.0**
36.0	14.0	50.0	**7.2**
40.5	9.5	50.0	**7.4**
43.5	6.5	50.0	**7.6**
45.75	4.25	50.0	**7.8**
47.35	2.65	50.0	**8.0**

Stock Solutions:

0.2M Na_2HPO_4 = 28.39 g/L Na_2HPO_4 (anhydrous) or 71.64 g/L $Na_2HPO_4.12H_2O$

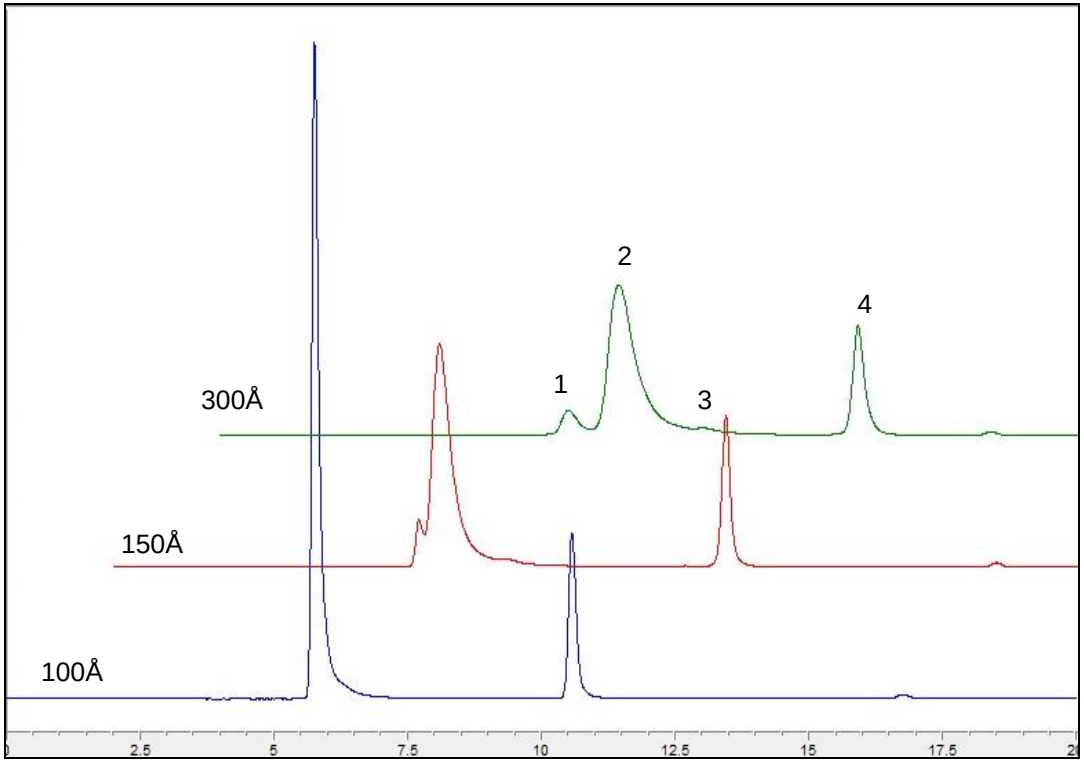
0.2M NaH_2PO_4 = 31.21 g/L $NaH_2PO_4.2H_2O$

RECOMMENDED STARTING CONDITIONS

- 150 Mm phosphate buffer, pH 7.0
- Flow rate of 0.1-1.25 ml/min for 7.8 mm id columns
- Isocratic for 30 minutes
- Temperature 20-30 C. Higher temperatures can be used, (see manufacturer's spec.)

Non-Specific Interaction Example

Columns:	Agilent Bio SEC-3 100Å, 150Å & 300Å 3µm 4.6x300mm
Eluent:	50mM Na_2HPO_4, 50mM NaH_2PO_4 + 0.15M NaCl, pH6.8
System:	Agilent 1260 Infinity Bio-Inert LC System
Detector:	UV@220nm
Flow rate:	0.35ml/min
Sample:	Mouse IgG



1. Dimer
2. Monomer
3. Monomer Fragment
4. Azide

EXAMPLES OF ADDITIVES TO REDUCE NONSPECIFIC INTERACTIONS

- 100-150mM NaCl
- 100-150mM NaSO4
- 50-100mM urea
- Guanidine hydrochloride can also be used
- 5-10% ethanol
- 5% DMSO

About Particle Size, Dimensions, and Flow Rate

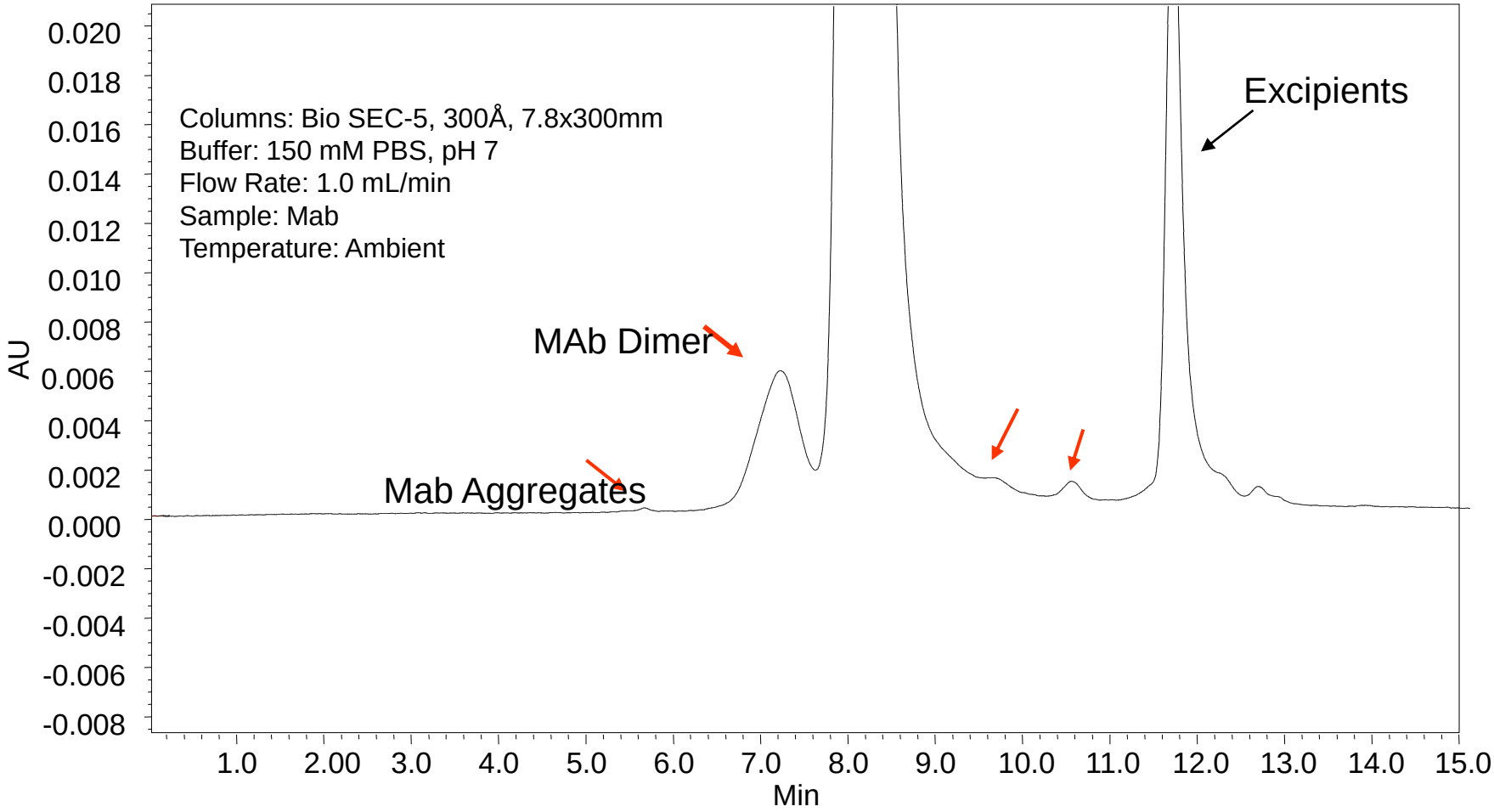
2 WAYS TO IMPROVE SEC RESOLUTION

With SEC, there are two ways to improve efficiency/resolution:

1. Increase column length
2. Decrease column particle size

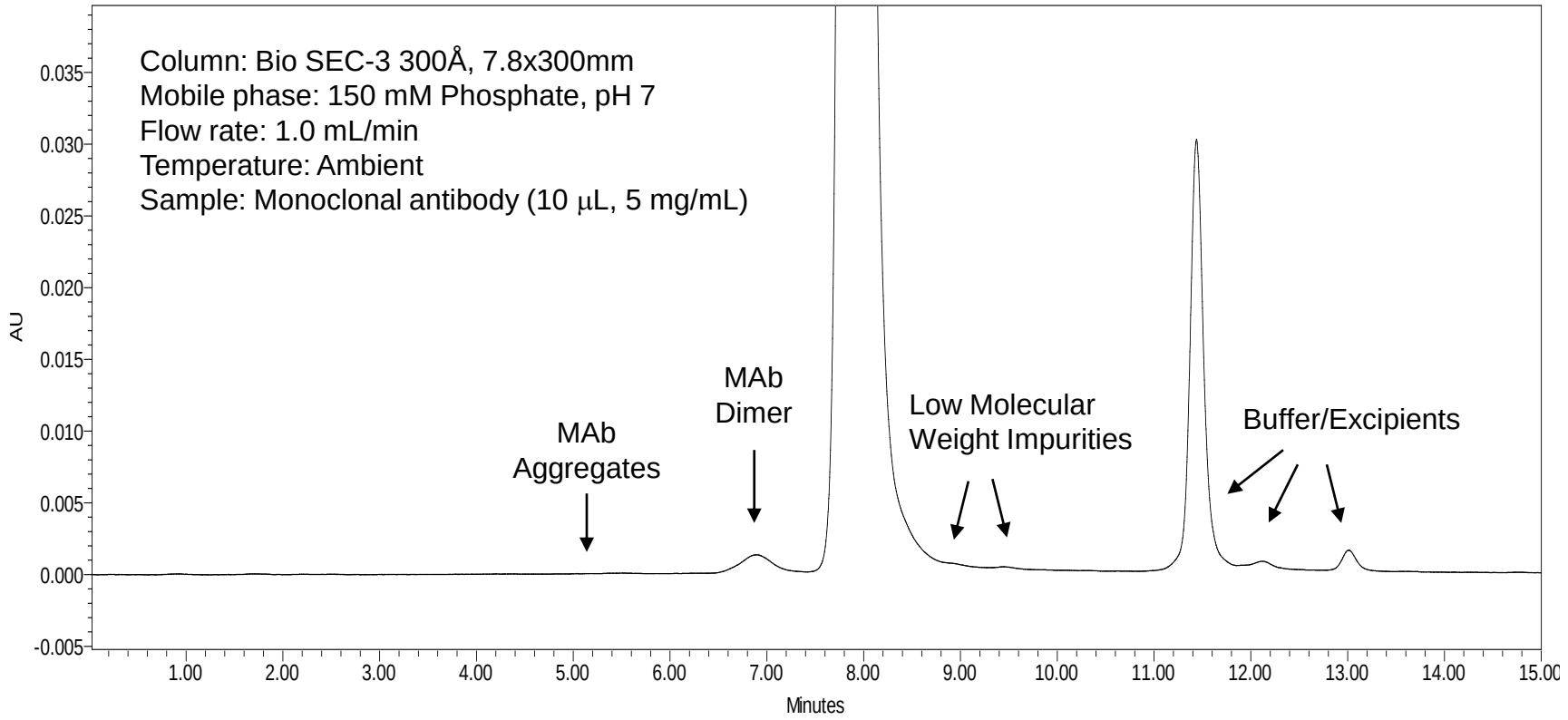
Agilent Bio SEC-5

Monoclonal Antibody Aggregation Monitoring



Size Exclusion

Monitoring Aggregation and Impurities of Monoclonal Antibodies



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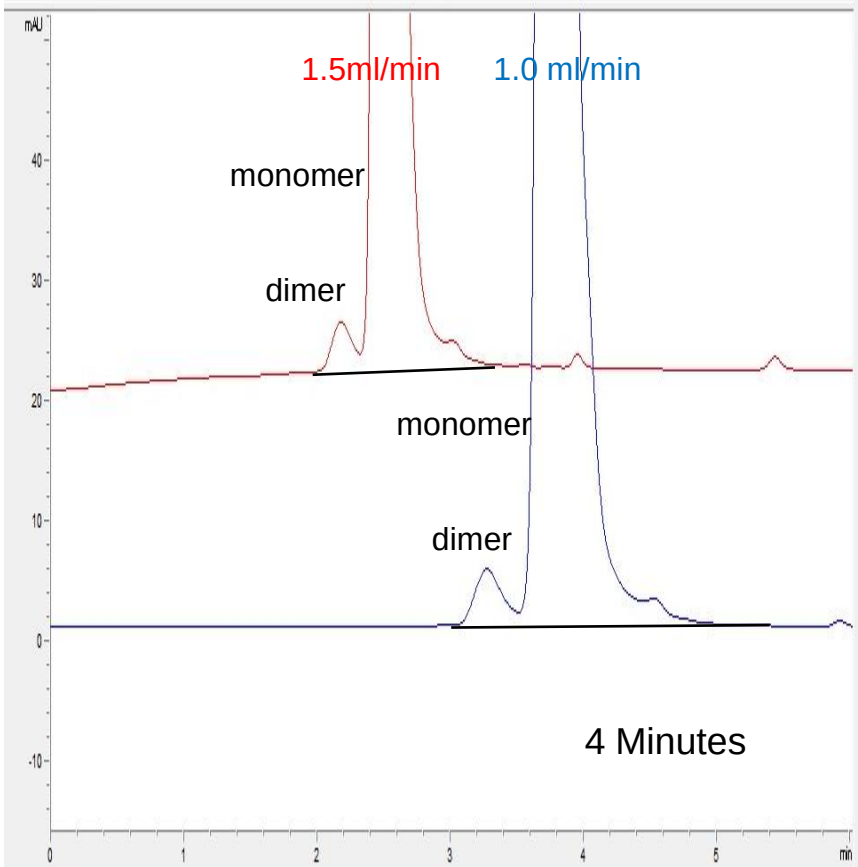
INCREASE THROUGHPUT/REDUCE TIME

Smaller particle size columns allow you to reduce time and improve throughput by:

1. allowing you to use shorter column lengths without losing resolution;
2. allowing you to also increase flow rate because you have more resolution room.

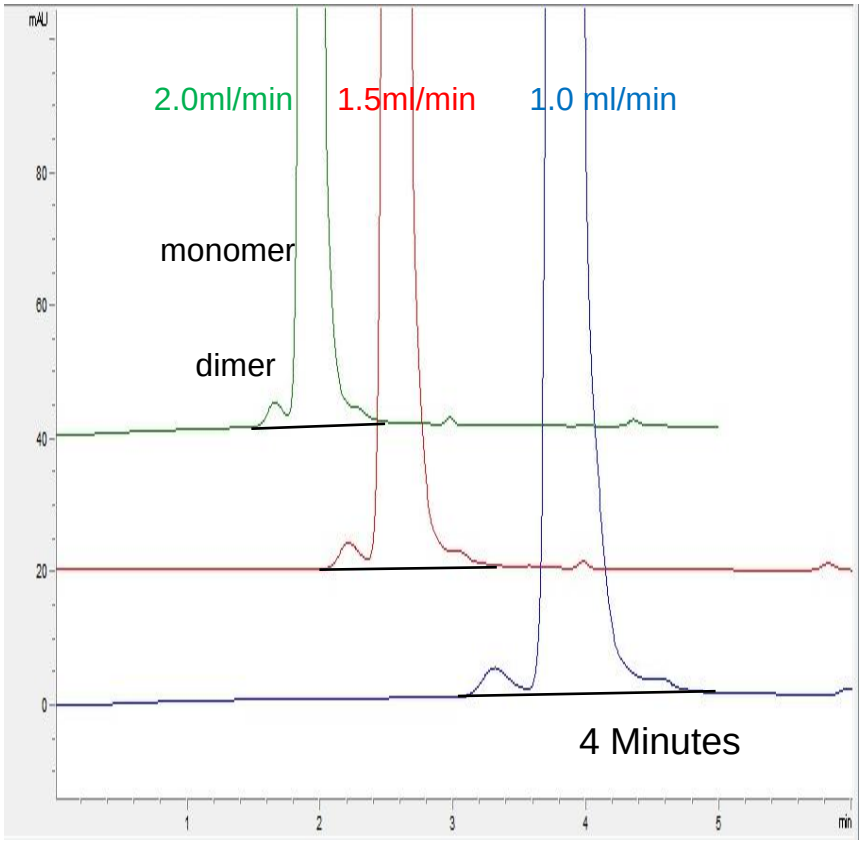
Fast SEC



Column: Agilent Bio SEC-3, 7.8 x150mm
Sample: mAb (2mg/ml)
Injection: 5ul
Flow rate: 1.0 and 1.5ml/min (56 bar , 75 bar)
Eluent : 150mM sodium phosphate
Detection: 220nm

Flow Rate	Resolution Monomer/Dimer	Monomer Efficiency	Percentage Dimer
1.0ml/min	1.58	3,684	0.65
1.5ml/min	1.31	2,574	0.70

Fast SEC



Column: Agilent Bio SEC-3, 7.8 x150mm
Sample: mAb (2mg/ml)
Injection: 5ul
Flow rate: 1.0, 1.5 and 2ml/min (56 bar , 75 bar, 105 bar)
Eluent: 150mM sodium phosphate + 100mM Na-sulfate
Detection: 220nm

Flow Rate	Resolution Monomer/Dimer	Monomer Efficiency	Percentage Dimer
1.0ml/min	1.53	3,510	0.64
1.5ml/min	1.43	2,502	0. 47
2.0ml/min	1.13	1,917	0.64

Agilent Offers Choice

Agilent Bio SEC-5

High Resolution Size Exclusion Columns

- 5µm Particle
- 100Å, 150Å, 300Å, 500Å, 1000Å, 2000Å pore sizes
- High stability and long lifetime
- Great reproducibility

For High Resolution

Agilent Bio SEC-3

High Efficiency and High Resolution

- Unique, 3μm particle
- 100Å, 150Å, 300Å pore sizes
- Highest resolution
- Highest efficiency
- Faster SEC separations

Choosing the Right Equipment

Bio-Inert liquid pathways?

Detectors?

Pressure capabilities?

The Agilent 1260 Infinity bio-inert quaternary LC

The New Standard in Bioanalysis

100% bio-inert

- ✓ Precious sample never touch metal surfaces
- ✓ Extended pH range 1-13 (shortterm 14)
- ✓ High salt tolerance: 2M salt, 8 M urea
- ✓ No stainless steel in mobile phase flow path
- ✓ New capillary technology

UHPLC capability

- ✓ 600 bar

Superior Ease of Use and Robustness

- ✓ Active seal wash
- ✓ Quaternary buffer mixing
- ✓ Superior Bio-HPLC columns for biotherapeutic characterization
- ✓ Column compartment for up to 30 cm columns

The choice for both bioanalytical and biopurification up to 10 ml/min

Using the 1260 Infinity LC for Protein SEC

Agilent 1260 Infinity Multi Detector Suite

Agilent 1260 MDS RI Detector

Agilent 1260 MDS Viscometer

Agilent MDS Light Scattering Detector

Add a Light Scattering Detector for Calibration-free Molecular Weight Determination

CONCLUSION

- Agilent has the columns, equipment, and consumables to help you achieve your SEC method development goals.

- Let us know how we can help you choose what works best for you!

Thank you!

Questions?