Clearing the Confusion: GPC, SEC, GFC – What , When, Why, and How?

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LC Columns & Consumables

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What we will cover……

What

- **Clarification of the terms**

Nomenclature

How is the separation accomplished – mode of separation

Key definitions

When & Why

Similarities & Differences

analysis goals - MWD, quantitation, aggregation

dissolution – what is my sample type & what is it soluble in?

How

Column Choices

Available chemistries

Sample type - polymer vs biomolecule

Method Considerations

Eluents, modifiers, buffers, etc

Calibrants & Calibration

Nomenclature

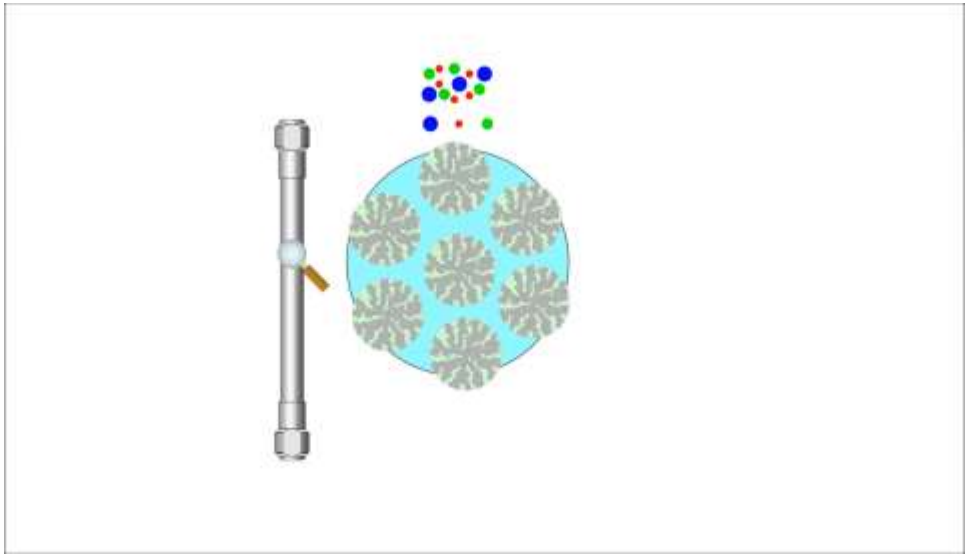
GPC - Gel Permeation Chromatography

SEC – Size Exclusion Chromatography

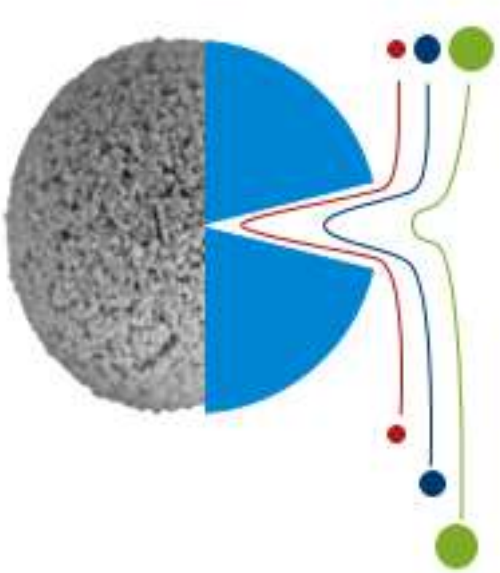
GFC – Gel Filtration Chromatography

What is GPC/SEC?

- A GPC/SEC column is packed with porous beads of controlled porosity and particle size
- Sample is prepared as a dilute solution in the eluent and injected into the system
- Large molecules are not able to permeate all of the pores and have a shorter residence time in the column
- Small molecules permeate deep into the porous matrix and have a long residence time in the column
- Sample molecules are separated according to molecular size, eluting largest first, smallest last

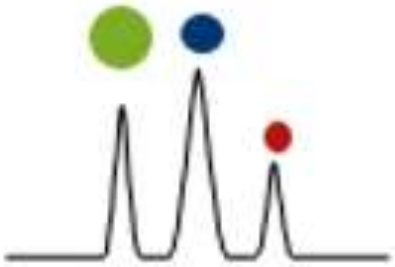


GPC/SEC/GFC



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

Smaller molecules spend longer in the pores and elute later.



Size in solution is related to retention time

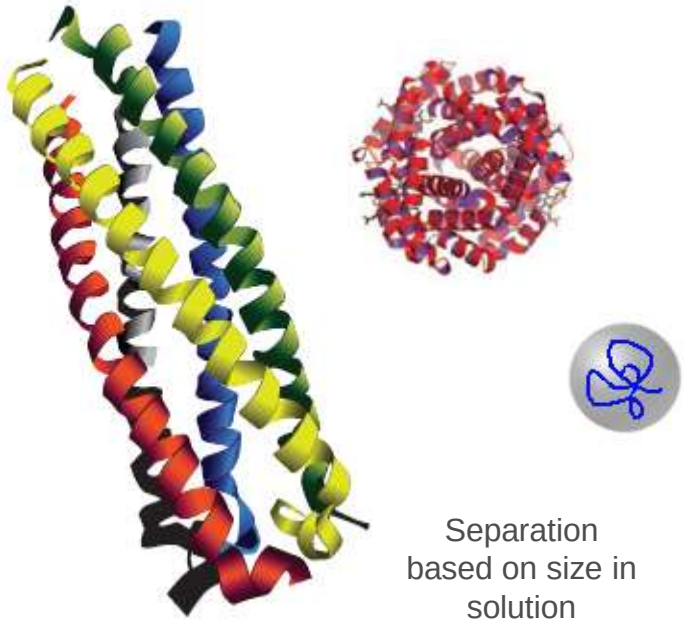
Some key definitions

- Column volume
- Hydrodynamic volume
- Interstitial volume
- Pore volume
- Exclusion limit / Void volume
- Total permeation
- Non-specific interaction

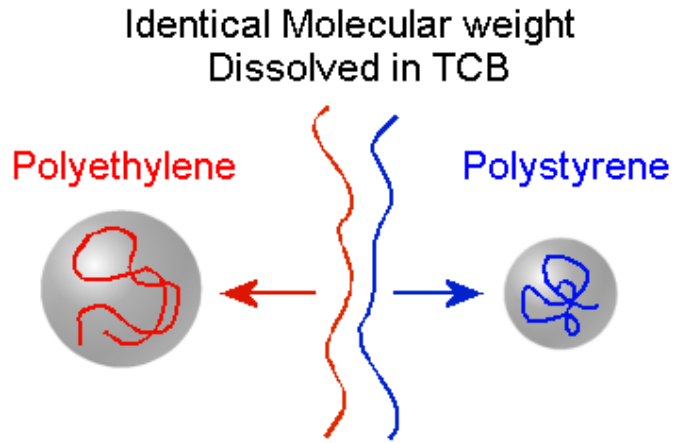
Column Dimensions: 7.8 x 300 mm
Column Volume = 14.3 mL

Hydrodynamic volume

- the size of a polymer/protein coil in solution
- Measure of molecular size in solution

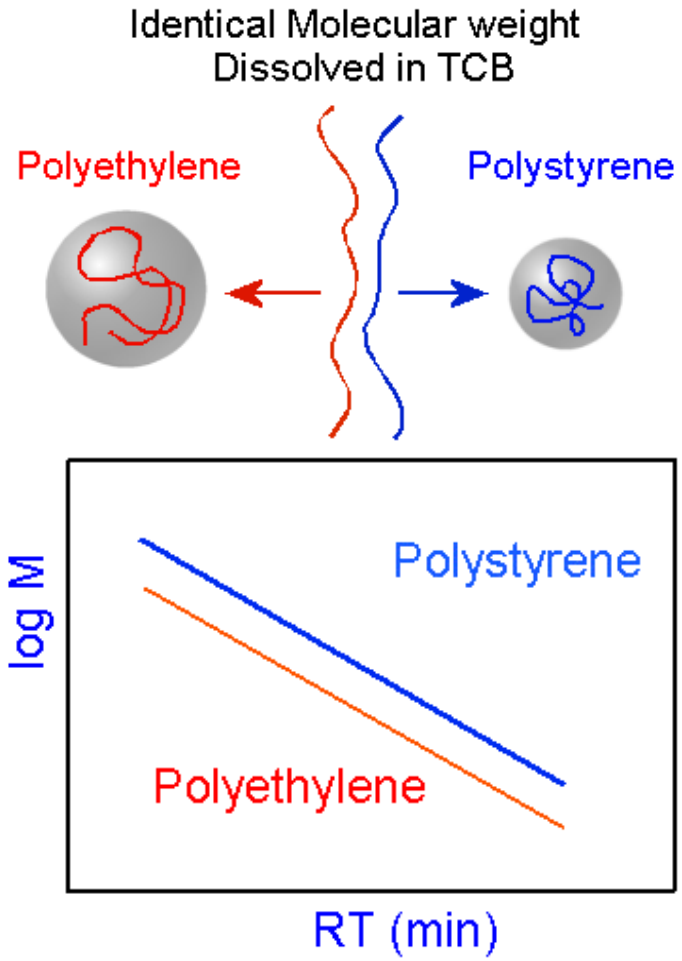


Separation based on size in solution (hydrodynamic volume)



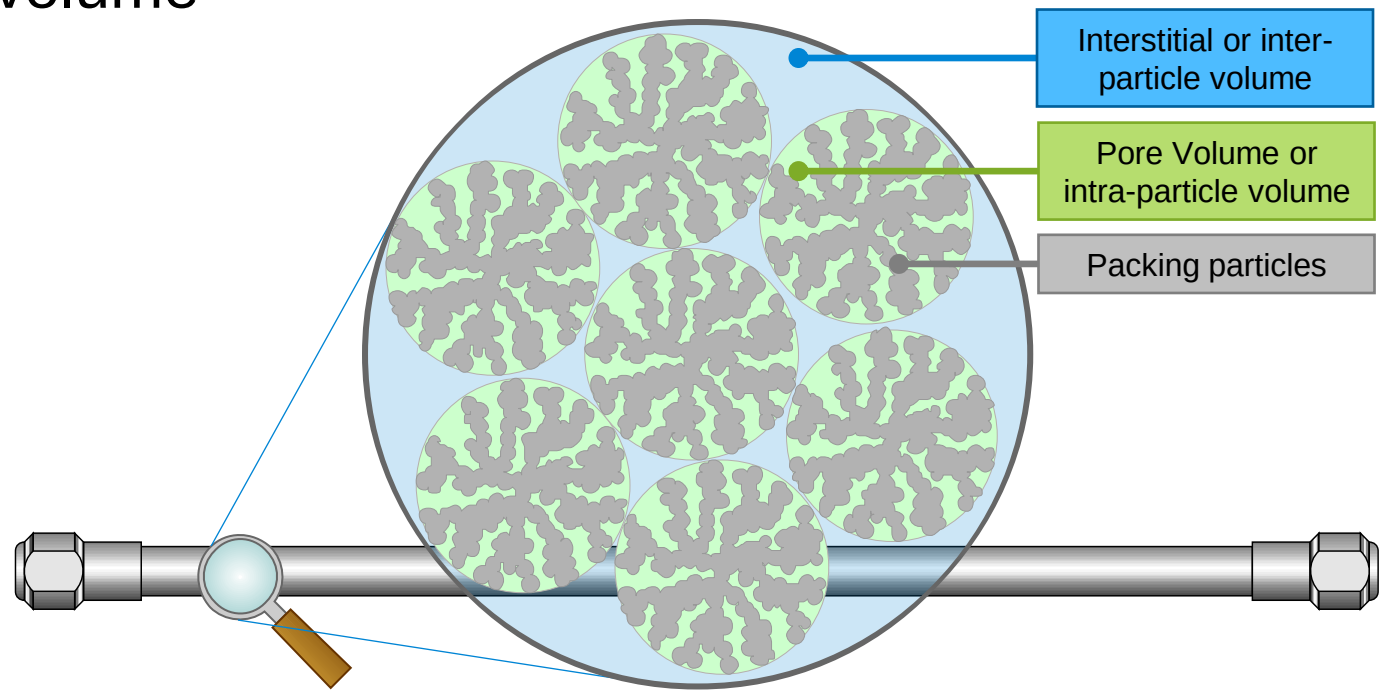
Hydrodynamic Volume…..expect differences

- Two different polymers will behave differently with solvent
- Column separates on basis of molecular size NOT molecular weight
- At any molecular weight, the two polymers will have different sizes in solution

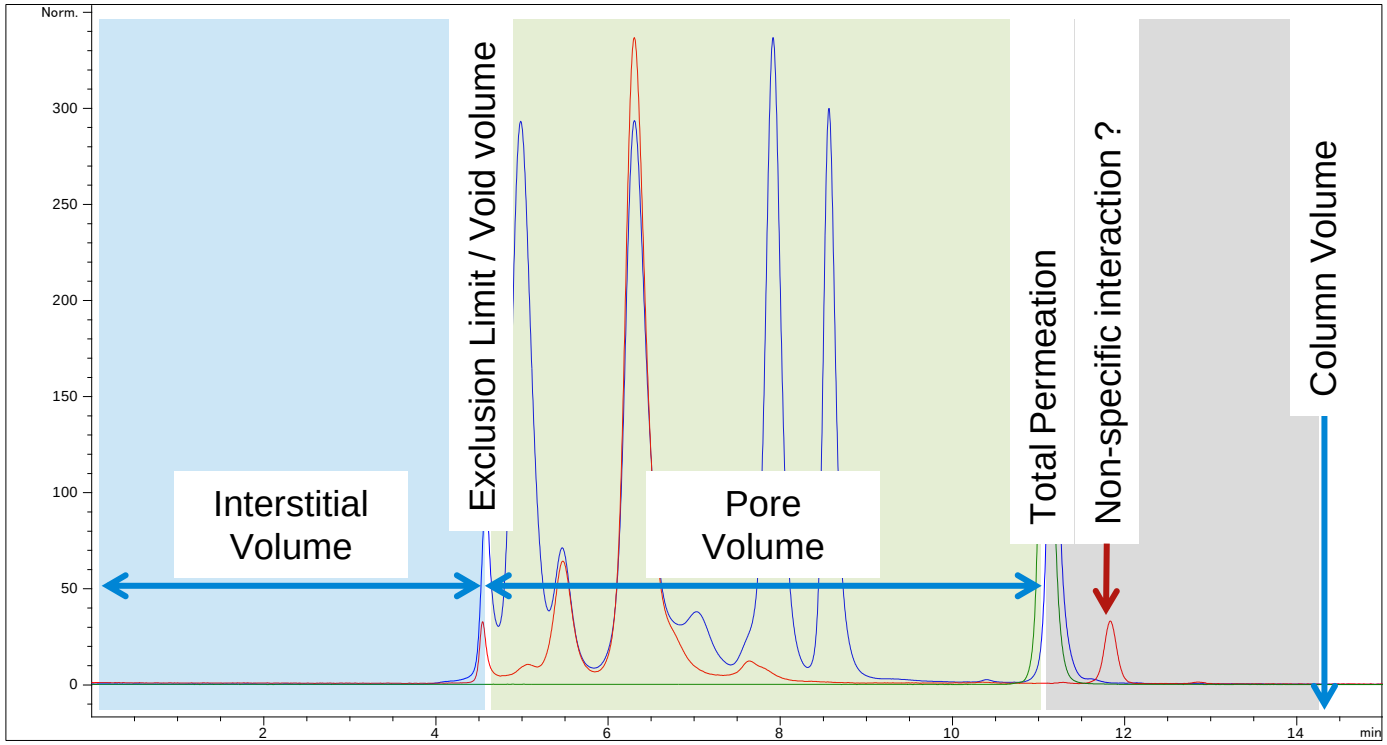


What are these inside the column ?

- Interstitial volume
- Pore volume

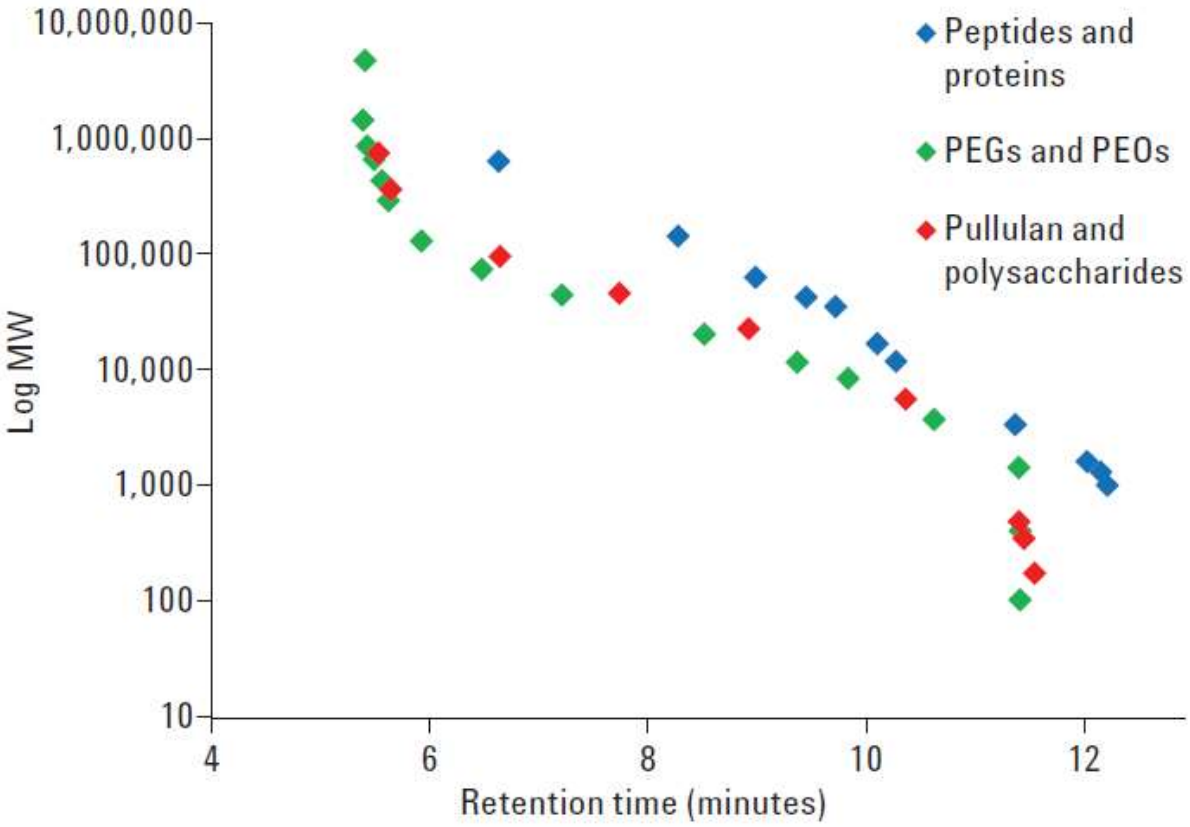


Regions on the chromatogram -



Example of Polymer vs Proteins…..

Column:	Agilent Bio SEC-3, 300Å, 4.6 × 300 mm, 3 µm (p/n 5190-2513)
Eluent:	150 mM Sodium phosphate buffer, pH 7
Flow rate:	0.35 mL/min
Detector:	RI for pullulan polysaccharides, PEGs and PEOs UV, 220 nm for proteins
System:	Agilent 1260 Infinity LC



Truly ‘size in solution’

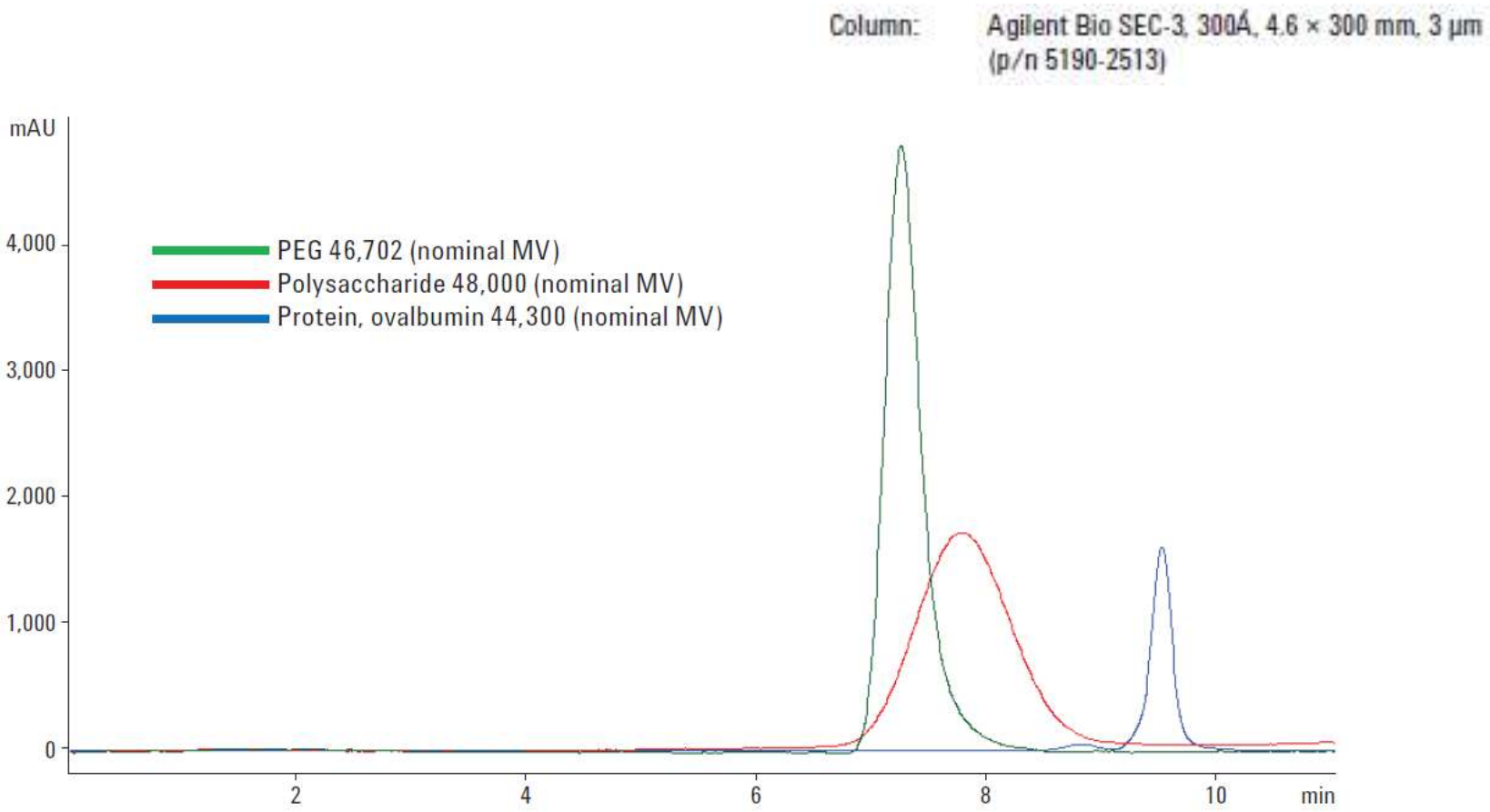


Figure 5. Overlay of chromatograms obtained for calibrants of similar molecular weight.

When & Why: Dissolution?

What is my sample and what is it soluble in?

Sample Type:

Polymer :

Organic -> THF, Tol, MeCl

Polar Organic -> DMF, DMSO

Aqueous -> water/buffer

Protein, mAb, etc :

Aqueous

Water/buffer

When & Why: Analysis goals?

What are you hoping to achieve from the analysis?

Sample Type:

Polymer analysis:

Determination of molecular weight

Determination of molecular weight distribution

Separation of polymers from impurities, sample clean up, etc

Protein Analysis:

Protein aggregation

Quantification of monomers, dimers, etc

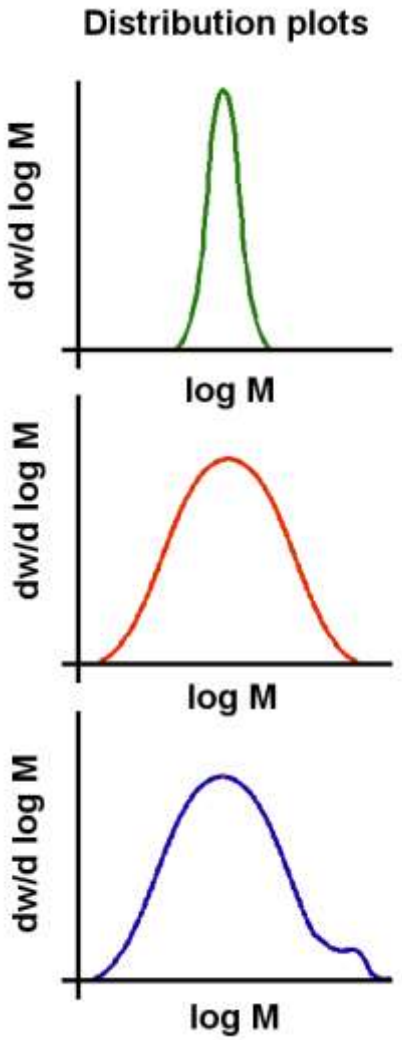
Separation of protein from impurities, excipients, etc

Measuring Molecular Weight

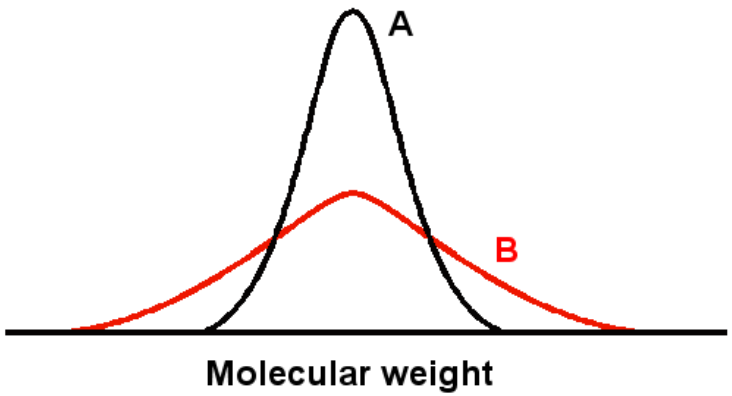
- There are many ways to measure molecular weights
- Examples include osmometry, centrifugation, and batch light scattering
- Each of these methodologies gives **a single measurement**, and average molecular weight
- For example, light scattering measures Mw, osmometry measures Mn and centrifugation measures Mz
- Although these methods give you a molecular weight, they do not describe a distribution
- The advantage of GPC and that it is a separation technique, and it is the only common technique that allows the measurement of the molecular weight distribution, not just a single average value

Shapes of Distributions

- Even for the same type of polymer, each of these distributions will describe a polymer that behaves differently
- The red and green plots are for low and high polydisperity materials
- The blue plot shows a high polydispersity material with a additional high molecular weight component
- Describing these distributions is not easy, especially if they are complex



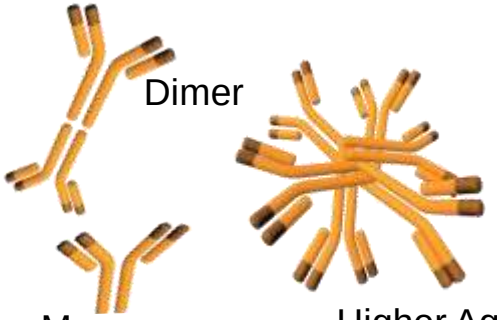
Why? - Effect of Polydispersity on a Polymer



- As the broadness of the distribution decreases the strength and toughness of the polymer increases
- However as the broadness of the distribution decreases the polymer becomes more difficult to process
- GPC provides key information to predict the processability and material properties of a polymer

	Strength	Toughness	Brittleness	Melt viscosity	Chemical resistance	Solubility
Increasing Mw	+	+	+	+	+	-
Decreasing distribution	+	+	-	+	+	+

WHY - Aggregates



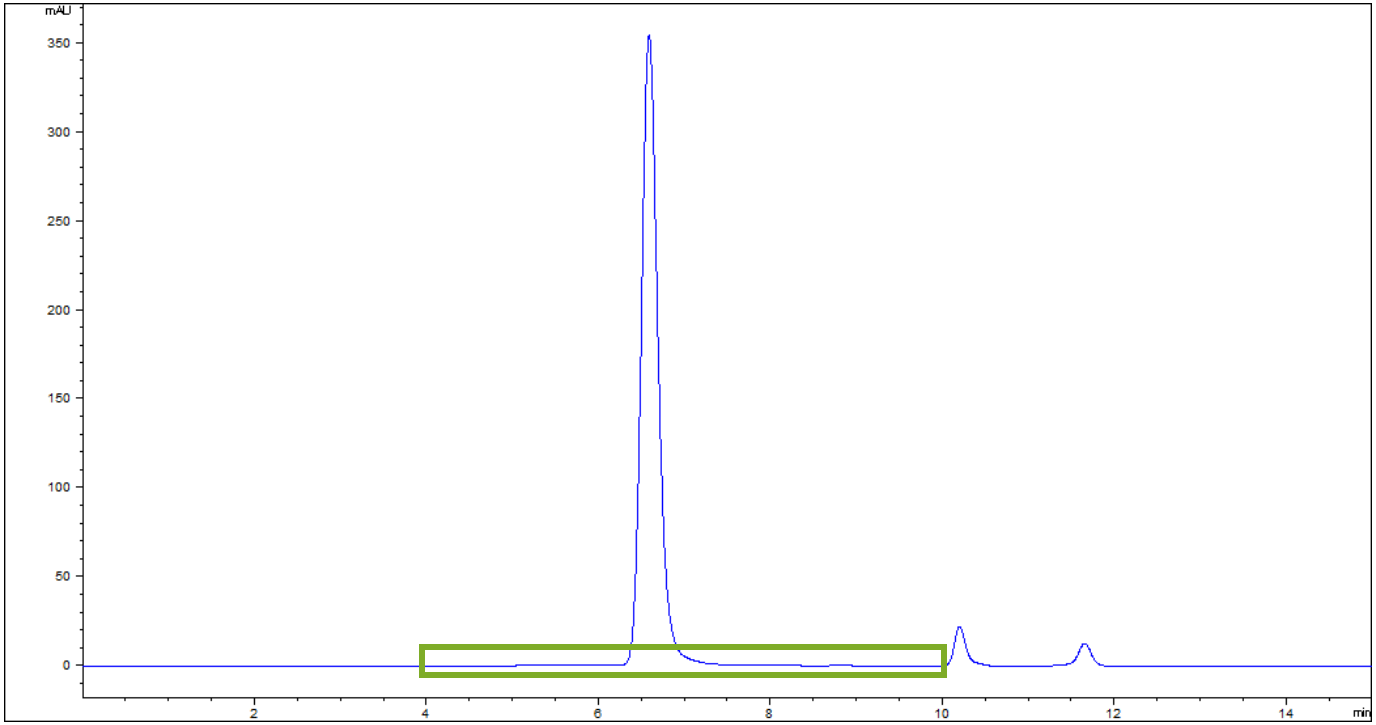
Monoclonal antibodies aggregate!

- Aggregates can stimulate immune responses and may cause adverse effects
- These responses may impact safety and efficacy of a biopharmaceutical drug
- Aggregation can impact production process economics

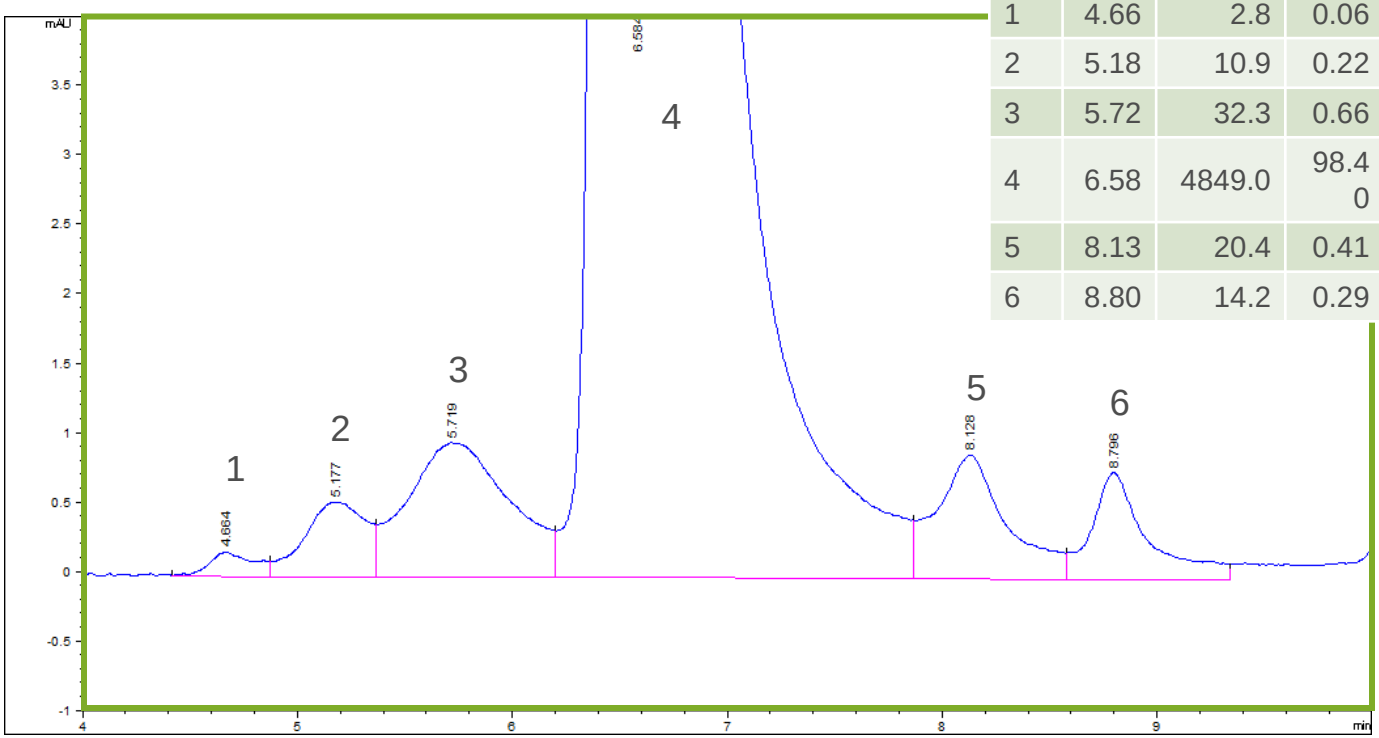
Manufacturing Processes
Steps and Products
- Fermentation
- Purification
- Formulation
- Storage
- Shipping
- Administration

Stress Conditions
- Heat
- Freeze-thaw
- Cross-linking
- Protein concentration
- Formulation change – pH, salt
- Chemical modification
- Mechanical stress / surface

WHY - Detecting and quantifying mAb aggregation



Detecting and quantifying mAb aggregation



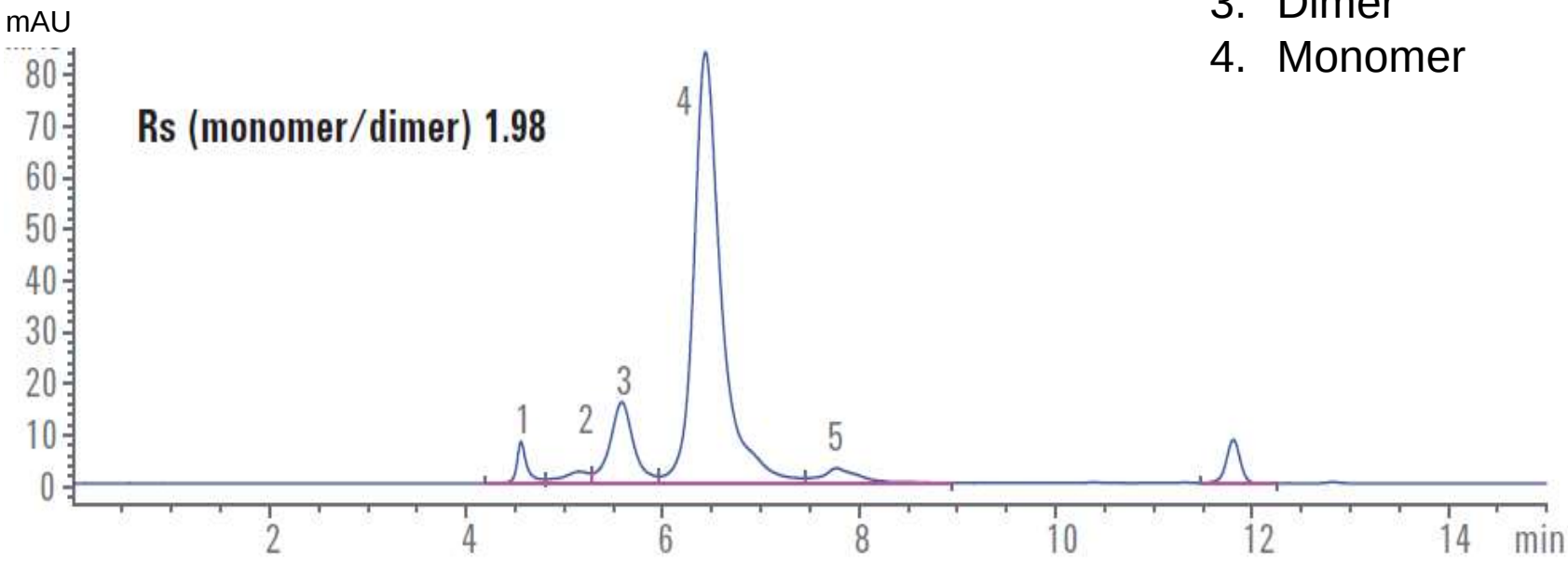
#	Time	Area	Area %
1	4.66	2.8	0.06
2	5.18	10.9	0.22
3	5.72	32.3	0.66
4	6.58	4849.0	98.40
5	8.13	20.4	0.41
6	8.80	14.2	0.29

Resolving for Monomer/Dimer:

Column: AdvanceBio SEC 300A
4.6 x 300 2.7um

Sample: IgG

3. Dimer
4. Monomer



HOW…..is it done?

Column Choices

Available chemistries

Sample type - polymer vs biomolecule

Selection of column - pore size, particle size, # of columns

Method Considerations

Choosing your eluent: organic, aqueous

When to use modifiers, buffers, etc

Calibrations

Polymer calibrants, Protein/Peptide standards

Column Chemistries

Polymer chemistries:

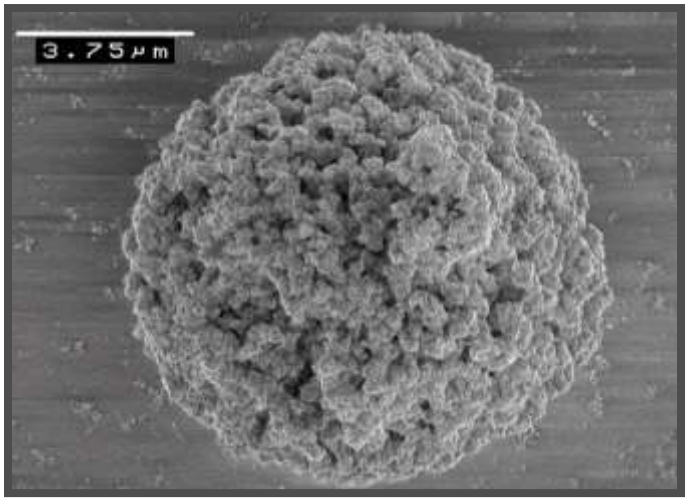
Common Types:

Polymethacrylate packings

Polyester copolymers

DVB, divinylbenzene

PS-DVB, polystyrene divinylbenzene



Silica Chemistries

Common Types:

Diol

Surface modified hydroxyl

Surface modified polymeric

Sample Type – Polymer vs BioMolecule

Polymers

Questions that you need to ask?

What is my sample soluble in?

What is the expected MW range?

Organic soluble → GPC

Aqueous soluble → SEC

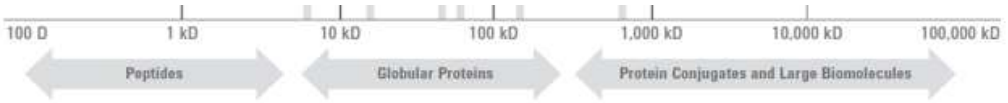
BioMolecule

Questions that you need to ask?

What type of sample do I have?

What is the expected MW for my sample?

Peptides
Proteins/Globular Proteins
mAbs
Protein Conjugates
Large BioMolecules



Column Selection:

Choose the right pore size

- It is essential to select a column that has pores sufficiently large enough to allow your molecule to permeate into the pore structure of the stationary phase and not be excluded.

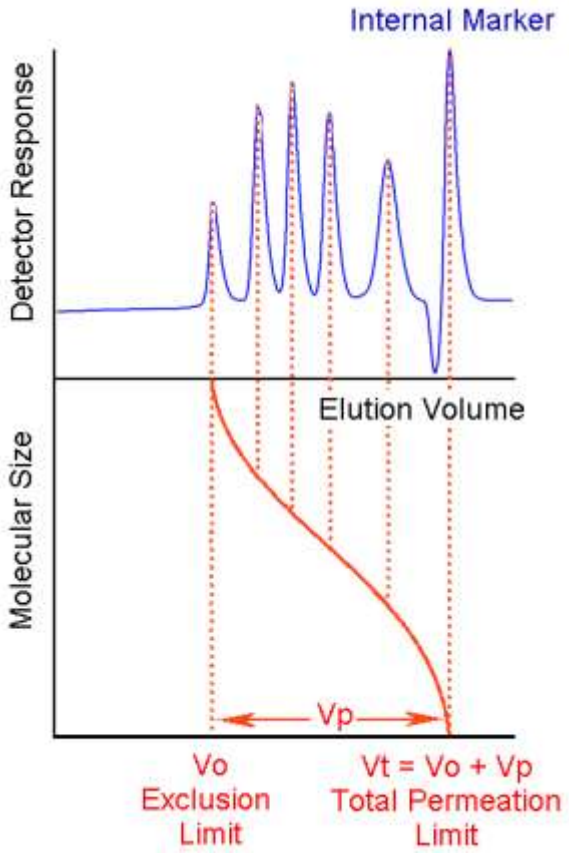
- Provides for complete coverage for the MW range of your sample and for your calibration.

- It is also essential to choose a pore size that is not too large

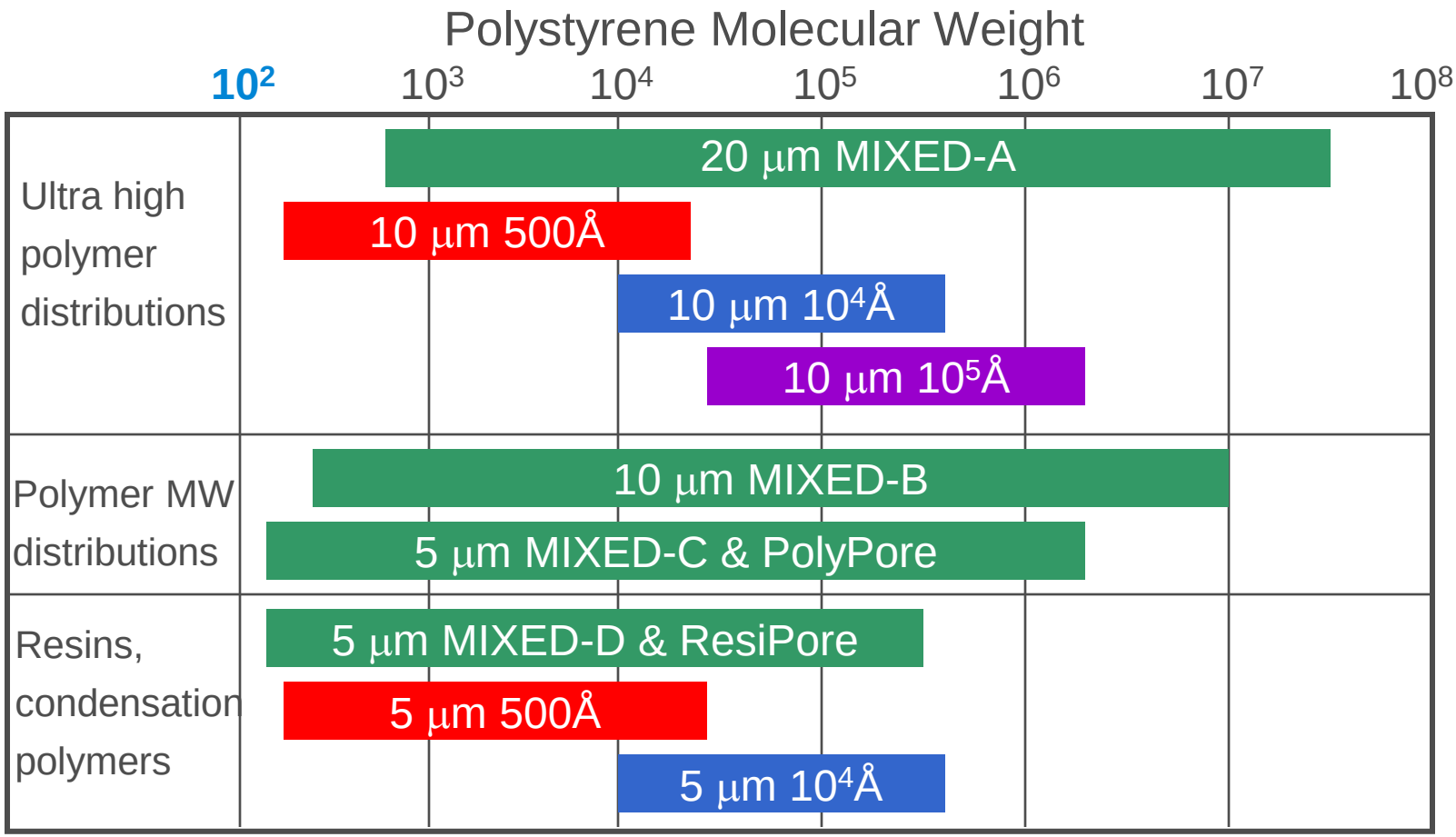
  Ex: For monoclonal antibodies the optimum pore size is around 300Å …

Choose The Right Pore Size

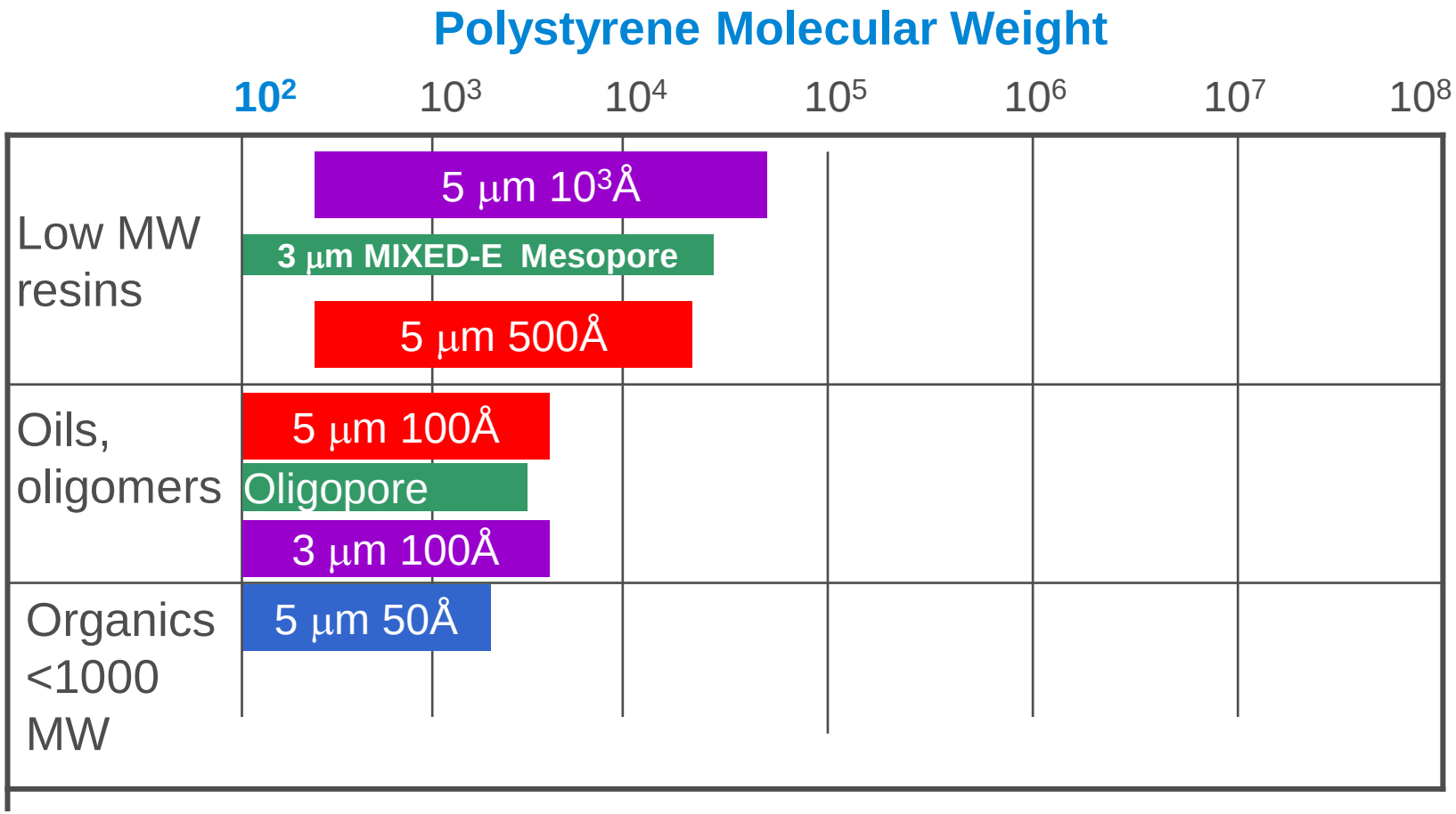
- The calibration curve describes how different size molecules elute from the column and can be used to determine molecular weight equivalents based on solution size
- Choose a pore size that allows you to work in the linear portion of the calibration curve.
- If two molecules have the same molecular weight but different size in solution they may be separated



Agilent PLgel Columns - Separation Ranges and Column Choices for Organic Soluble Polymers

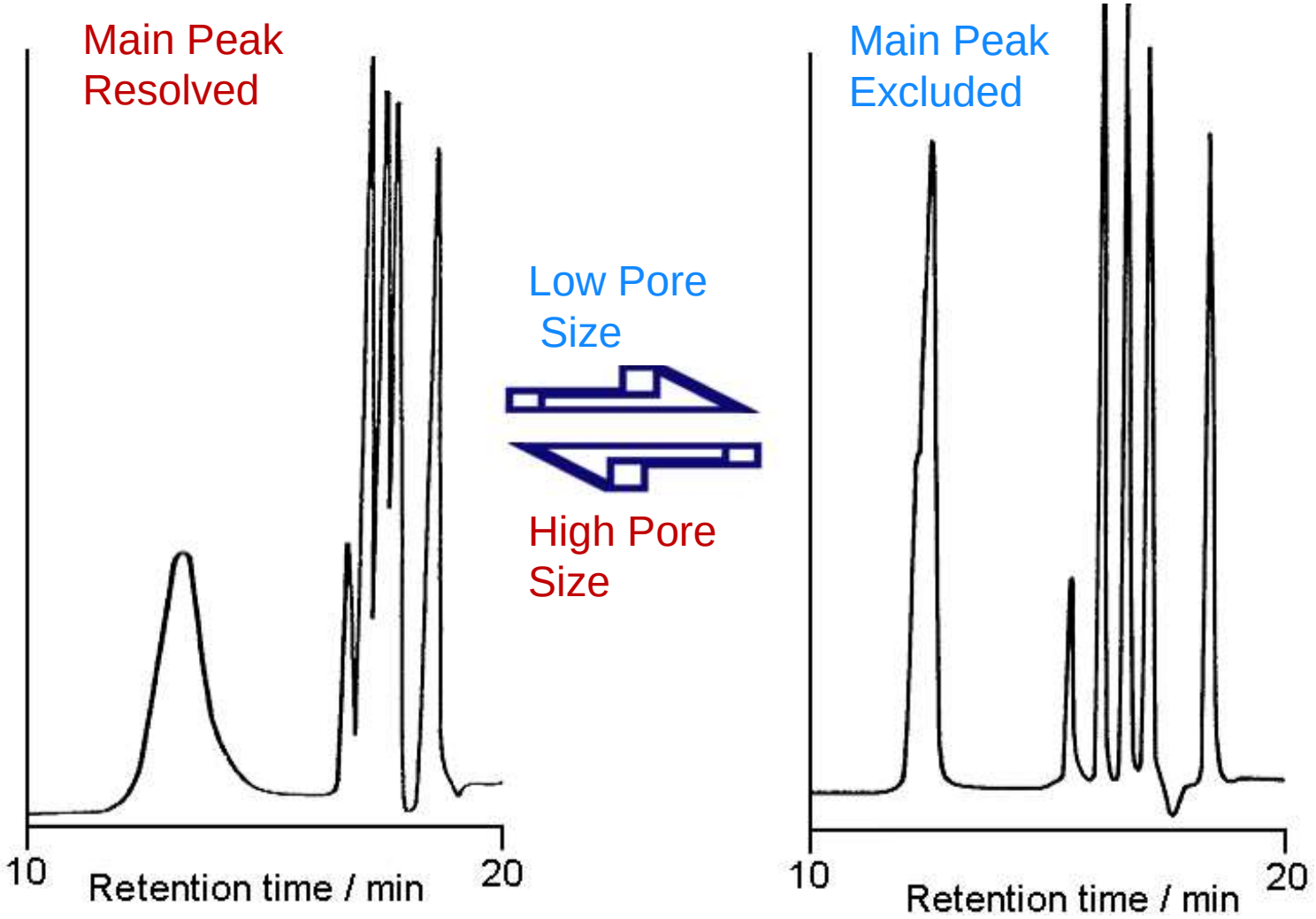


Agilent PLgel Columns-Separation Ranges and Column Choices for Organic Soluble Polymers

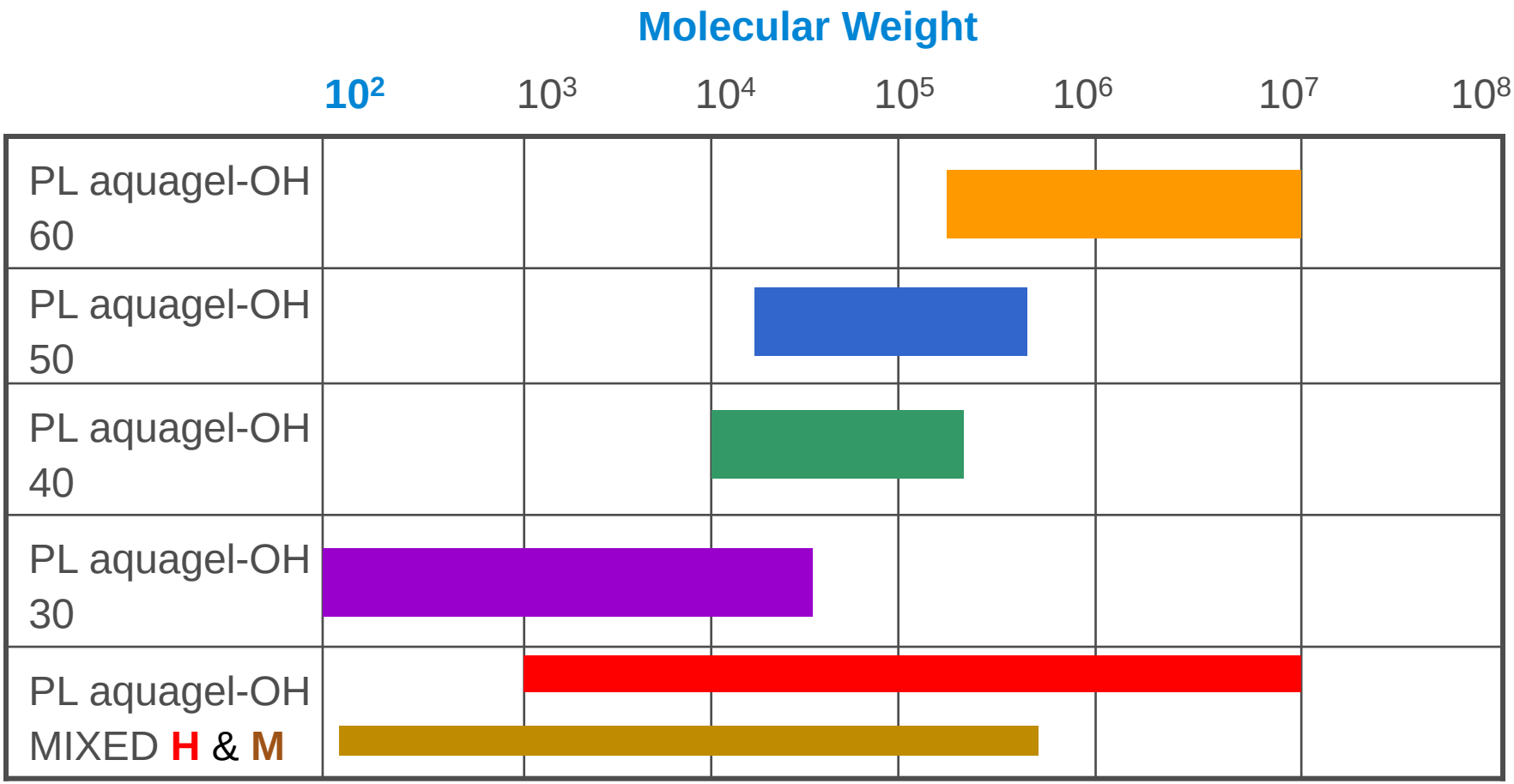


- PLgel columns can separate organic soluble polymers with MW from 100 – 20 x 10^6

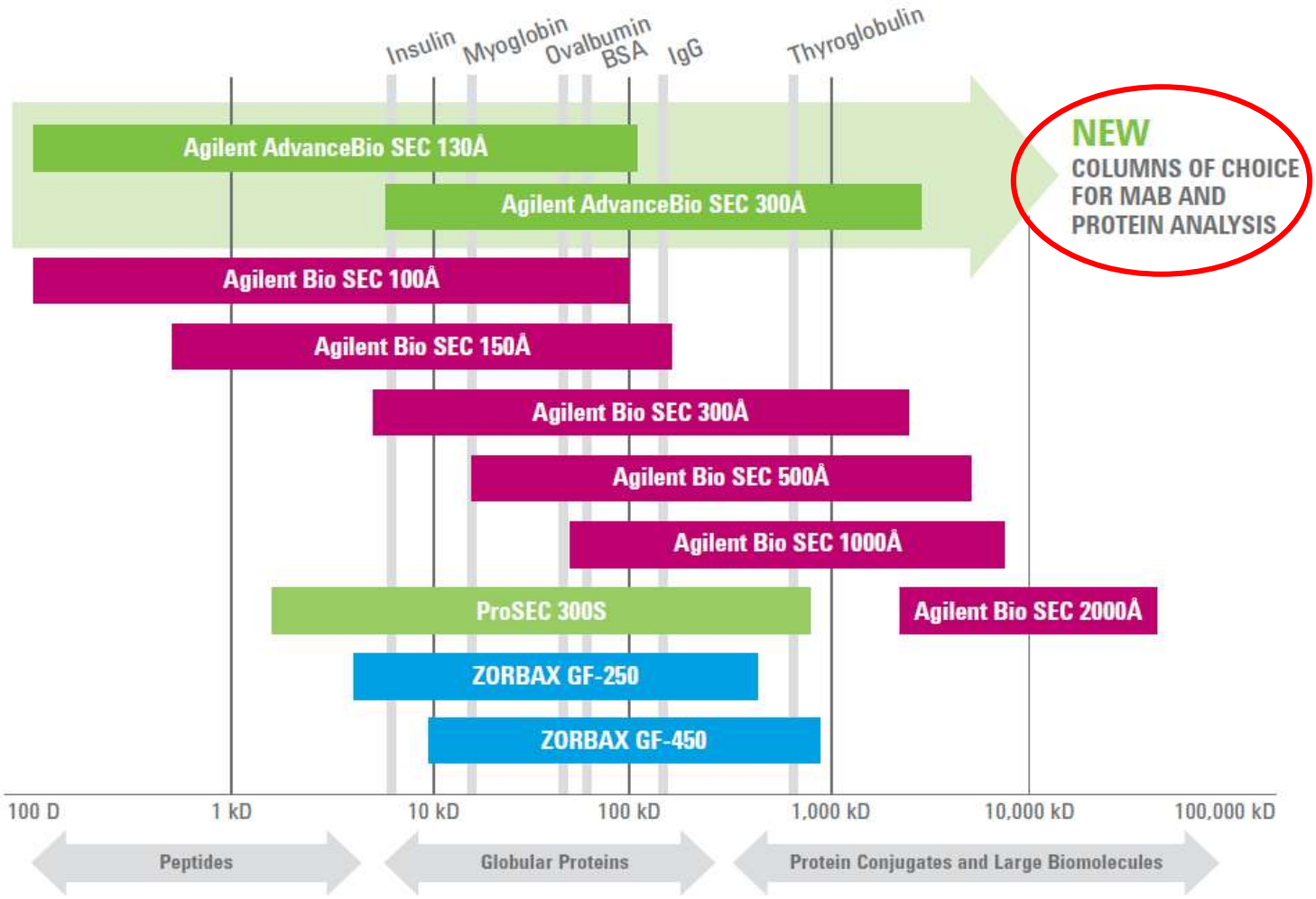
Effect of Column Selection: Pore size



Agilent PL aquagel-OH Columns for the SEC Analysis of Water Soluble Polymers



Agilent SEC pore size selection



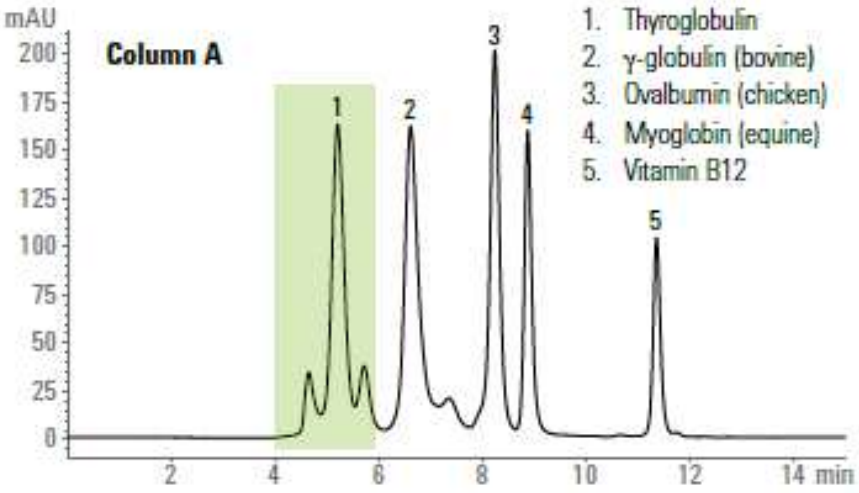
Importance of Pore Size Selection: calibrants

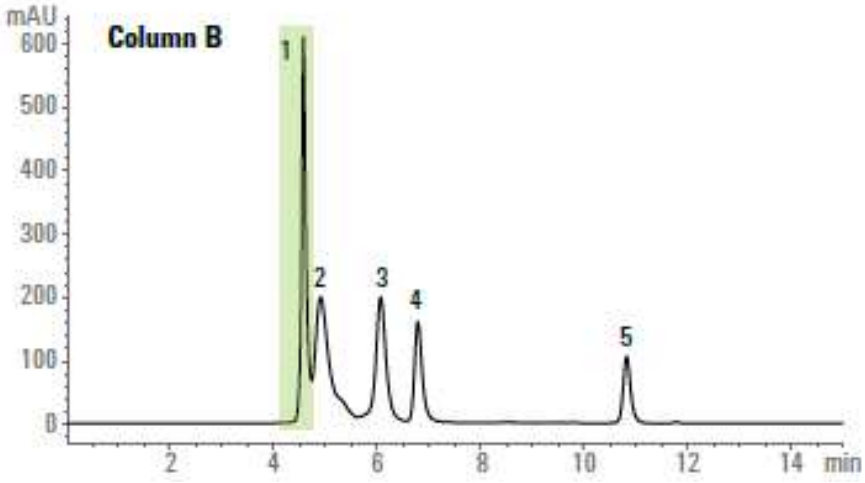
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm
Sample:	BioRad gel filtration standards mix

Column A: **AdvanceBio SEC 300Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)

Column B: **AdvanceBio SEC 130Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)

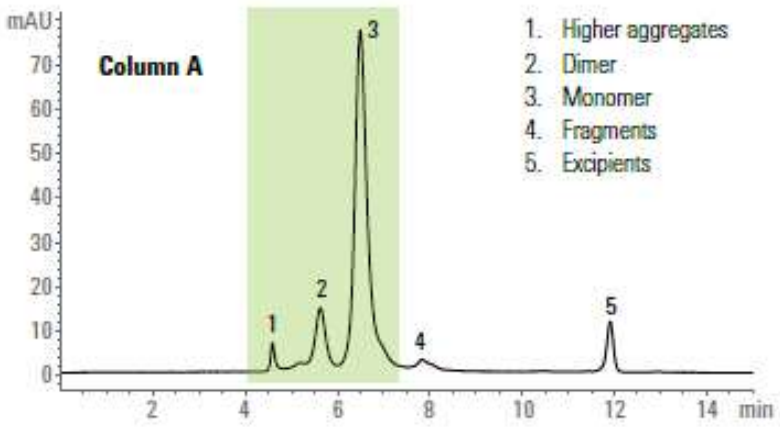
BioRad gel filtration standards mix

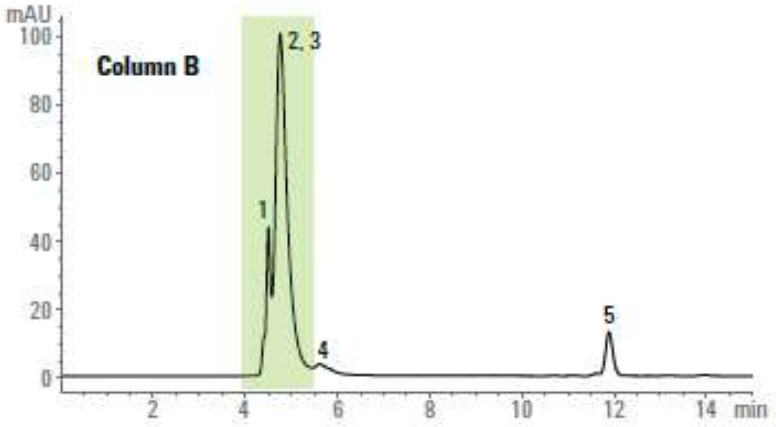




Importance of Pore Size Selection: sample

Polyclonal IgG separation





Column A: **AdvanceBio SEC 300Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)

Column B: **AdvanceBio SEC 130Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5350)

Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm
Sample:	Polyclonal IgG

Resolution in GPC/SEC

Running two columns in series, same pore size

- Increase pore volume, increases resolution

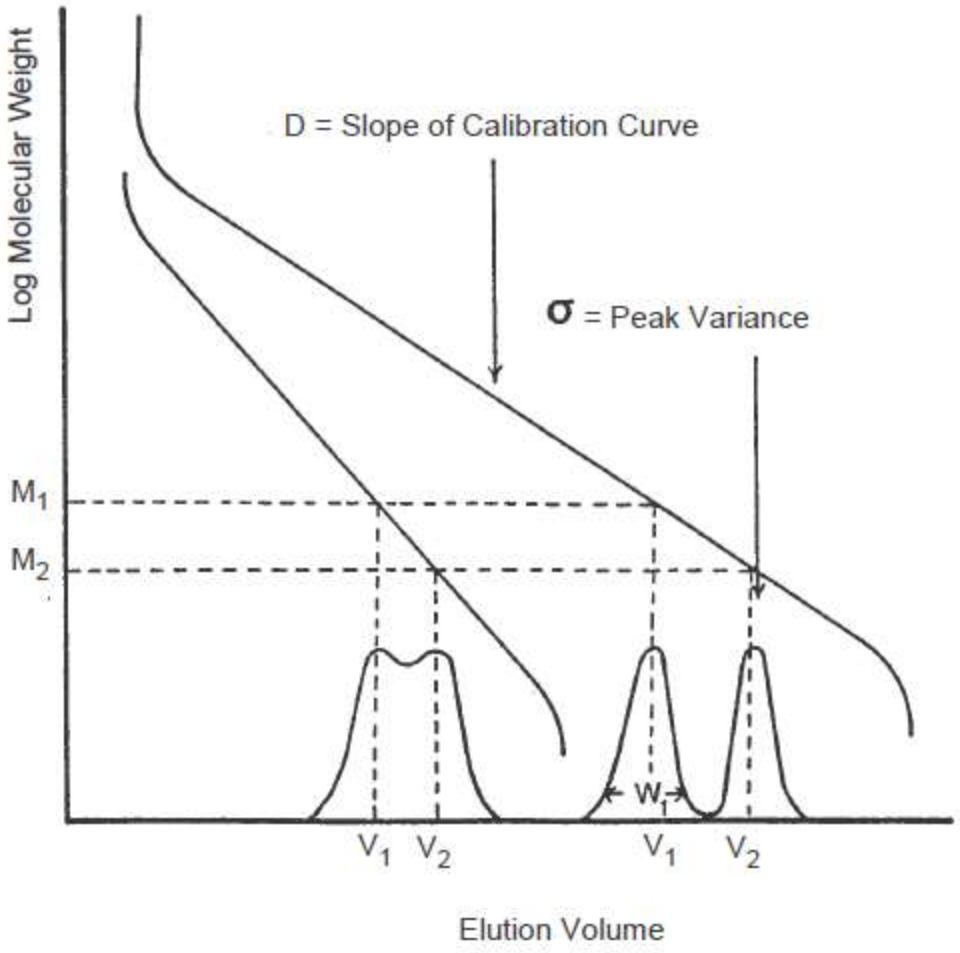
Running two columns in series, different pore size

- extends the resolving range and enables analysis of multiple attributes in one run

Use a packing with a smaller particle size

- Decrease particle size, increase column efficiency

Resolution in GPC – add a column to improve resolution

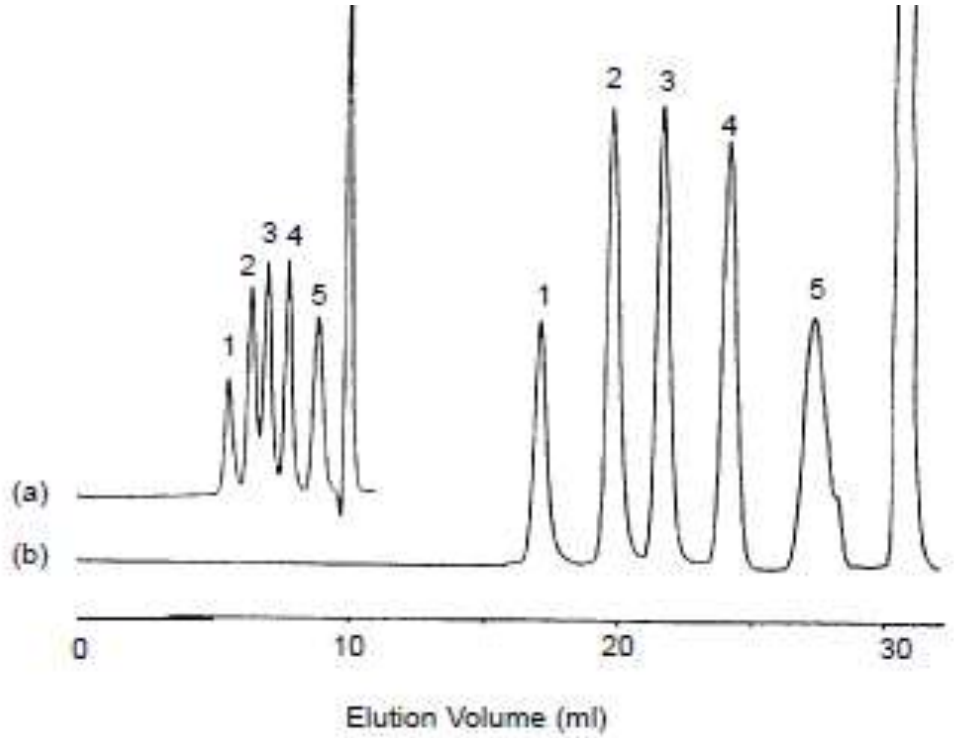


Ex: Effect of column length on resolution

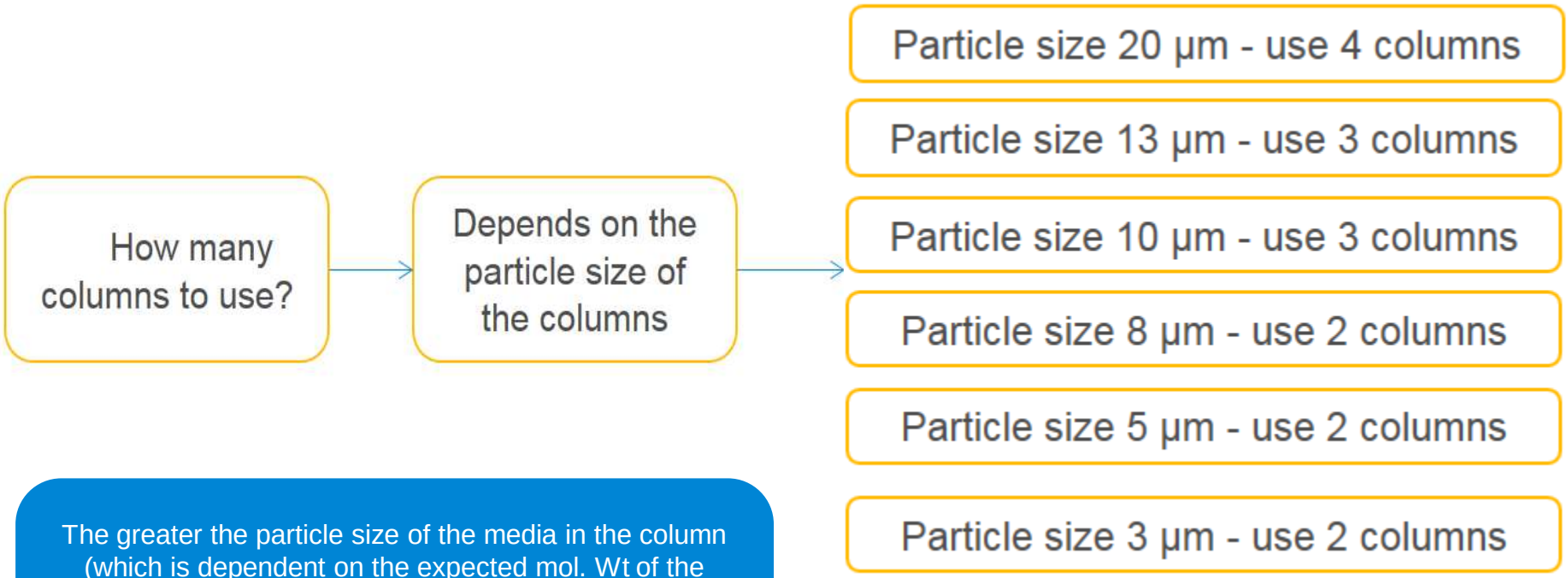
Polystyrene Standards
(EasiCal)

1. 3,040,000
2. 330,000
3. 66,000
4. 9,200
5. 580

Columns:	1xPLgel 10µm MIXED-B, 300x7.5mm (1110-6100)
	3xPLgel 10µm MIXED-B, 300x7.5mm (1110-6100)
Eluent:	THF
Flow Rate:	1.0ml/min
Detector:	RI



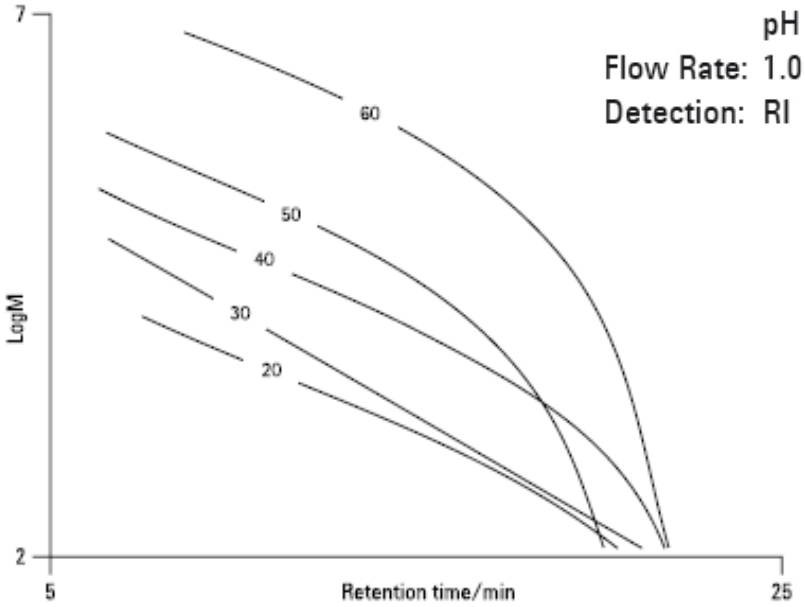
Guideline for # of columns to use:



The greater the particle size of the media in the column (which is dependent on the expected mol. Wt of the samples), the lower the resolution and the more columns are required to maintain the quality of the results. For higher MW samples, larger particles are necessary to reduce the danger of shear degradation of samples.

Column in series: to extend MW range

PL Aquagel OH columns
Individual Pore Sizes



Conditions

Samples:	Four samples of hyaluronic acid
Columns:	1 x PL aquagel-OH 60 15 µm, 300 x 7.5 mm (p/n PL1149-6260) + 1 x PL aquagel-OH 40 15 µm, 300 x 7.5 mm (p/n PL1149-6240)
Eluent:	0.2 M $NaNO_3$ + 0.01 M NaH_2PO_4 at pH 7
Flow Rate:	1.0 mL/min
Detection:	RI

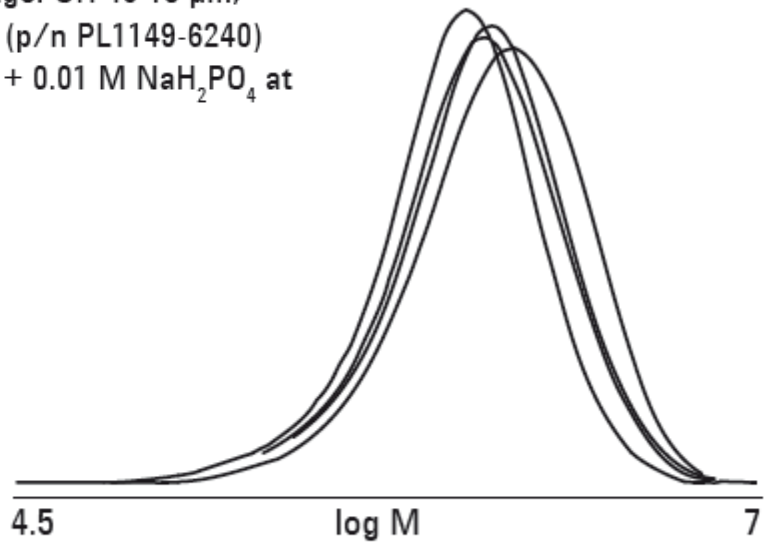
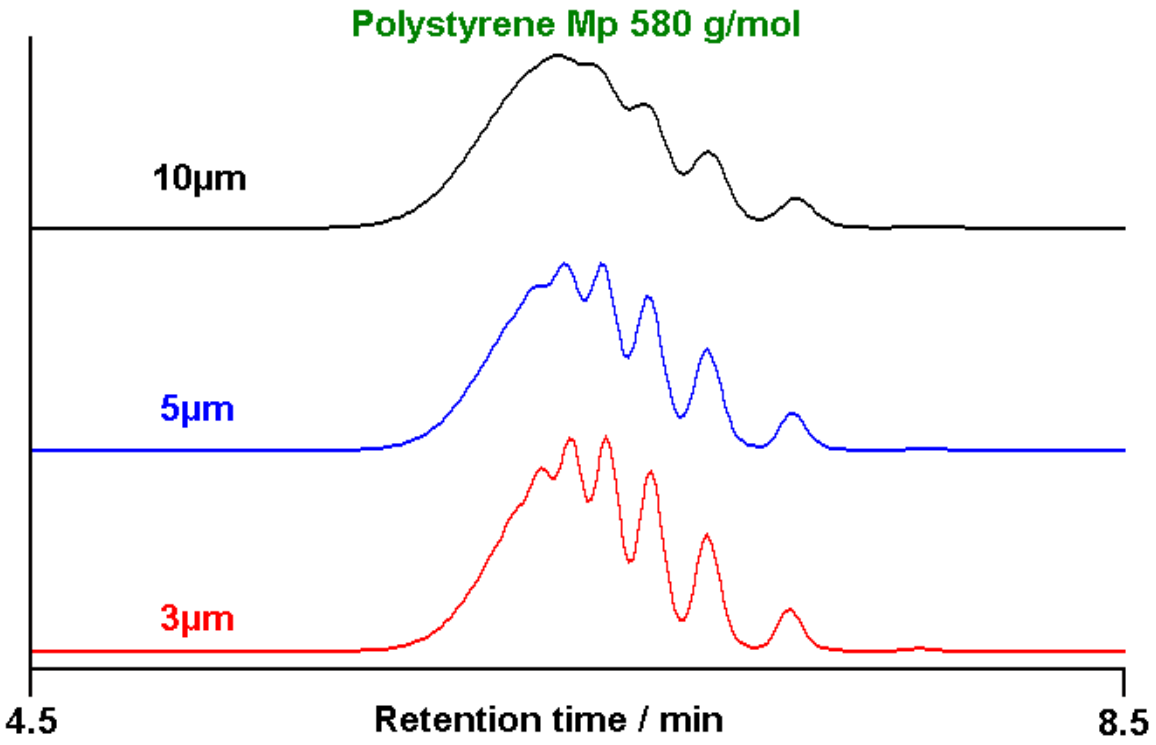


Figure 3. Overlay of the molecular weight distributions of four hyaluronic acid samples

Effect of Particle Size on Resolution

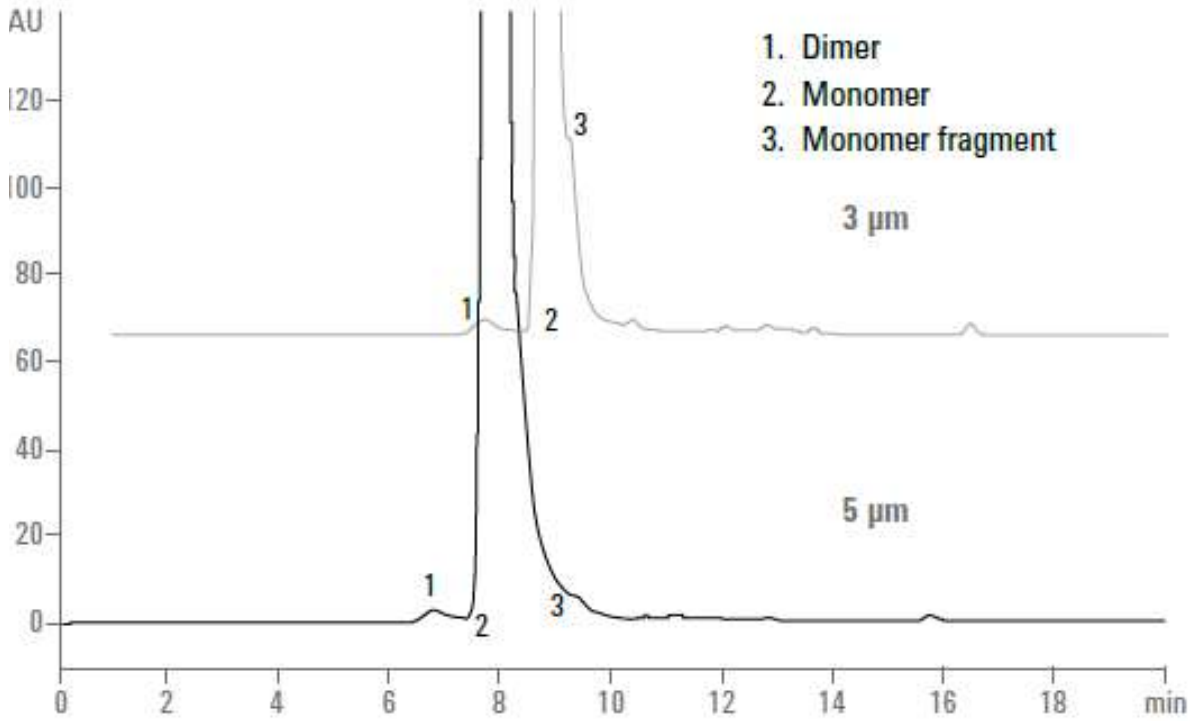
Column:	PLgel 100A 300x7.5mm
Eluent:	THF
Flow Rate:	1.0ml/min
Inj Vol:	20µl
Detector:	DRI



Comparison of 3um vs 5um:

Analysis of monoclonal antibody

Column:	**Bio SEC-3, 300Å 7.8 x 300 mm, 3 µm (p/n 5190-2511)**
Column:	**Bio SEC-5, 300Å 7.8 x 300 mm, 5 µm (p/n 5190-2526)**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM sodium phosphate, pH 7
Flow rate:	1 mL/min
Detector:	UV, 220 nm
Sample:	Humanized monoclonal antibody



Method Considerations: Eluent choices

Remember…..GPC/SEC/GFC is a non-interactive separations technique

In selecting the solvent or the mobile phase conditions for the sample and separation, of utmost importance, the size exclusion mechanism must be maintained

Simply because a sample is soluble in a particular solvent, it does not mean that it will be the suitable solvent for one to use for the analysis

Points of Consideration:

Sample type -?

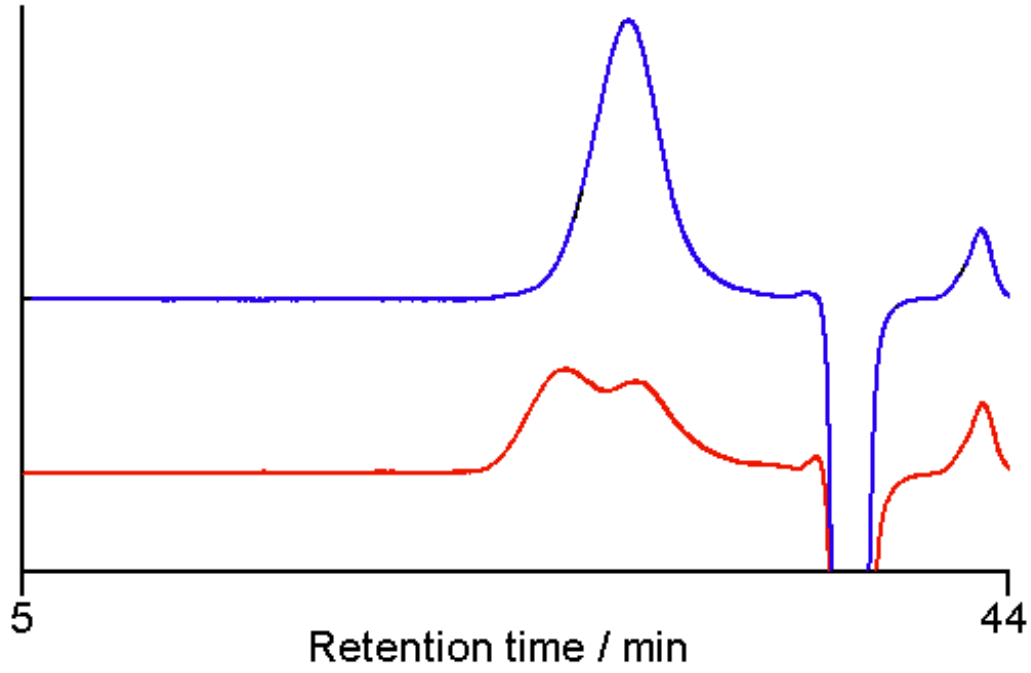
Column to be used -?

Solvent polarity	Solvent
0.0	Perfluoroalkane
2.3	Hexane
8.2	Cyclohexane
8.9	Toluene
9.1	Ethyl acetate
9.1	Tetrahydrofuran (THF)
9.3	Chloroform
9.3	Methyl ethyl ketone (MEK)
9.7	Dichloromethane
9.8	Dichloroethene
9.9	Acetone
10.0	1,2-Dichlorobenzene (o-DCB)
10.0	Trichlorobenzene (TCB)
10.2	m-Cresol
10.2	o-Chlorophenol (o-CP)
10.7	Pyridine
10.8	Dimethyl acetamide (DMAc)
11.3	n-Methyl pyrrolidone (NMP)
12.0	Dimethyl sulfoxide (DMSO)
12.1	Dimethyl formamide (DMF)

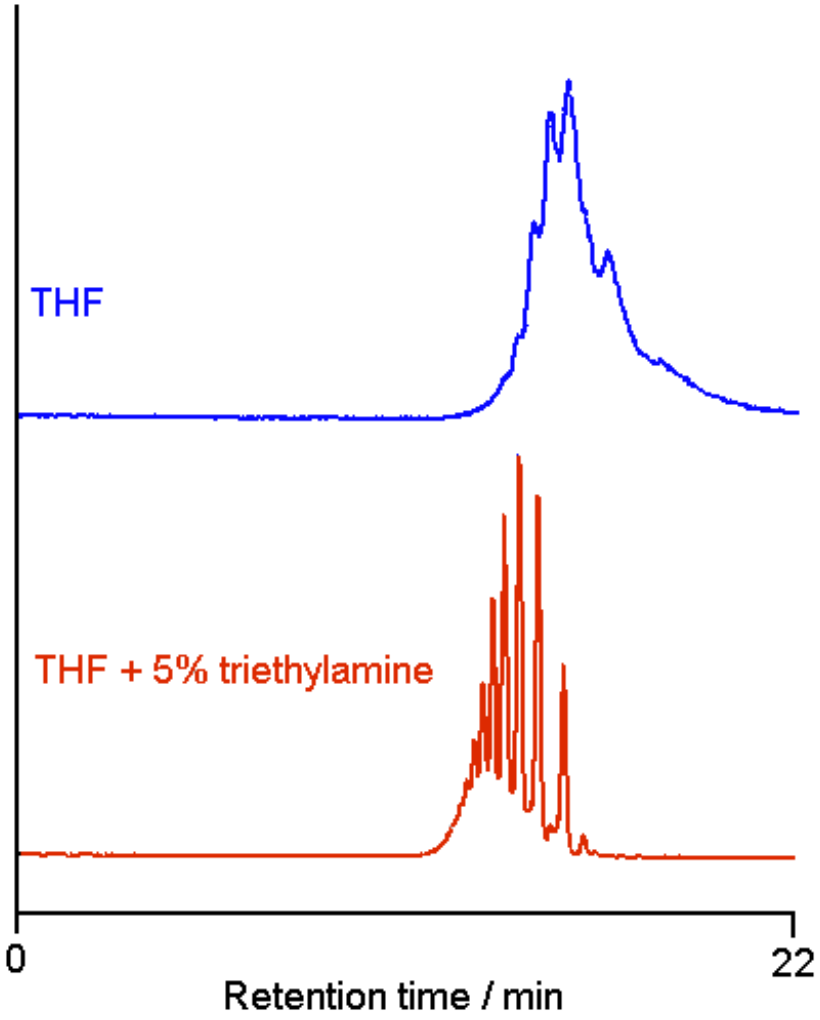
Starch Analysis

Column:	**4xPLgel 20µm MIXED-A 300x7.5mm**
Eluent:	**DMSO + 5mM $NaNO_3$**
Flow Rate:	**1.0 ml/min**
Temp:	**80°C**
Detector :	**DRI**

Addition of salt is often required for polar organic solvents to suppress ionic interaction effects



Eluent Modification in Organic GPC

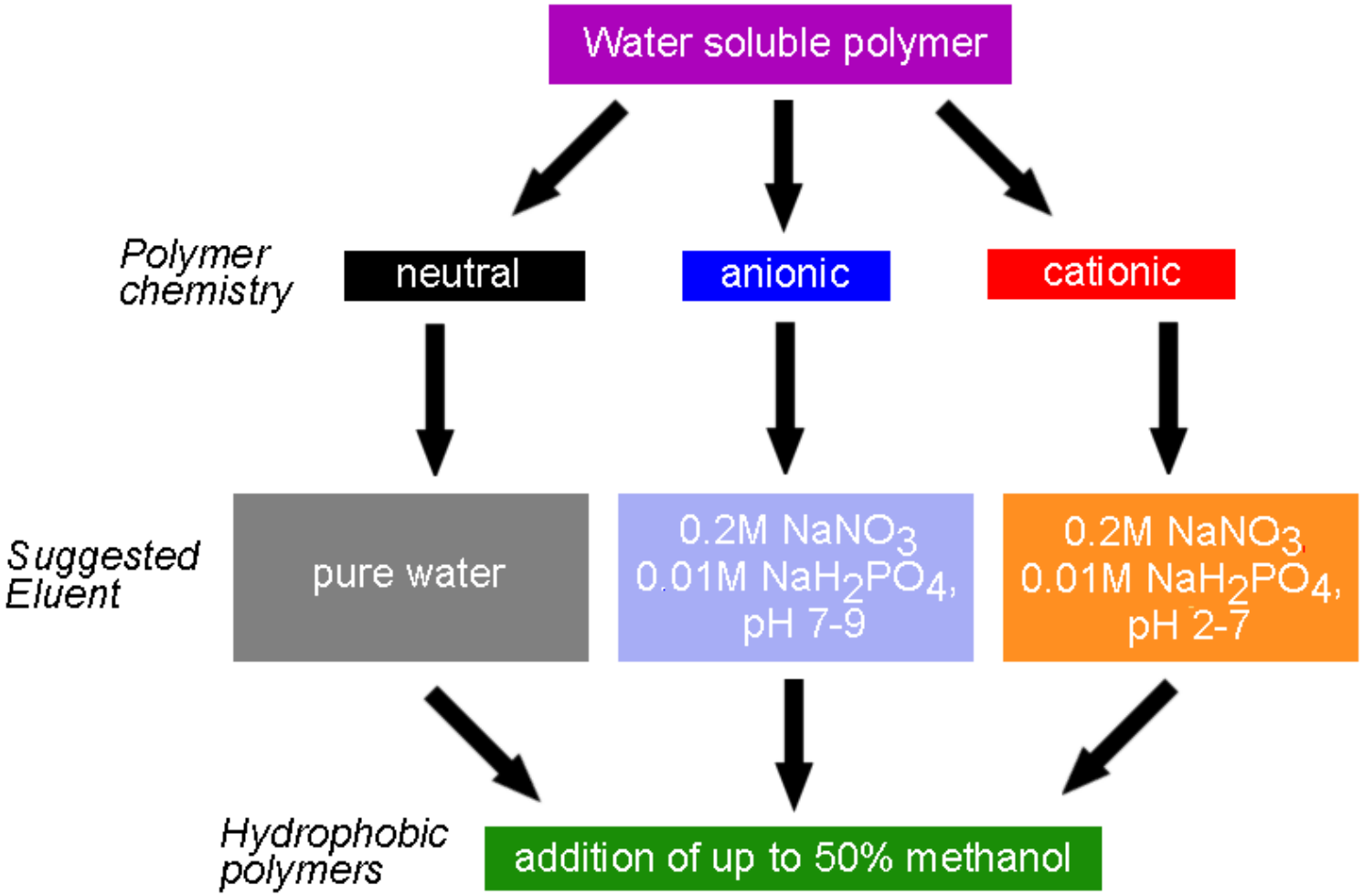


Hostavin N30

Polymeric UV stabiliser containing secondary amine groups

Column: 2xPLgel 3µm MIXED-E
Flow Rate: 1.0ml/min
Detector: PL-ELS 1000

PL aquagel-OH Eluent Modification



Buffers and Bio Sec: Choose the Right Mobile Phase

The optimal eluent for the separation should be determined by the characteristics of the column stationary phase and the proteins to be analyzed so that non specific interactions are minimized

- Mobile phase should contain enough 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).

Recommended Starting Conditions

For AdvanceBio SEC Columns we recommend starting with 150mM Sodium phosphate, pH 7.0

Peptides, polypeptides, proteins, mAbs
MW >0.1-1,250 kDa

AdvanceBio SEC (2.7 µm)	
Pore Size	**MW Range (kDa)**
130Å	0.1-100
300Å	5-1,250

Recommended Initial Separation Conditions

Column: AdvanceBio SEC or Agilent Bio SEC-5
Mobile phase: 150 mM phosphate buffer, pH 7.0*
Gradient: Isocratic in 10-30 min range
Temperature: Recommended: 10-30 °C, Maximum: 80 °C

Flow rate: 0.1-0.4 mL/min for 4.6 mm id columns
0.1-1.25 mL/min for 7.8 mm id columns
Sample size: ≤ 5% of total column volume
*Other aqueous buffers with high and low salt can be used

Buffer concentration and ionic strength can impact retention time, peak shape, and resolution
Adjustments can be made depending on your sample requirements

Calibrating your column:

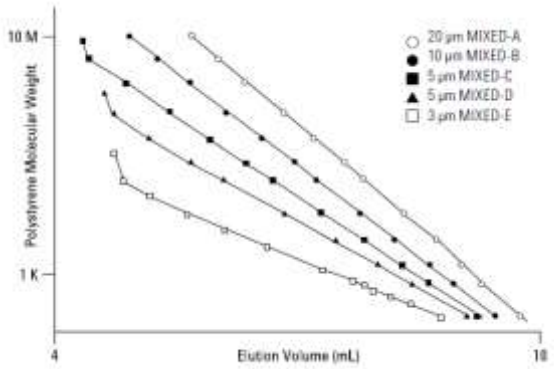
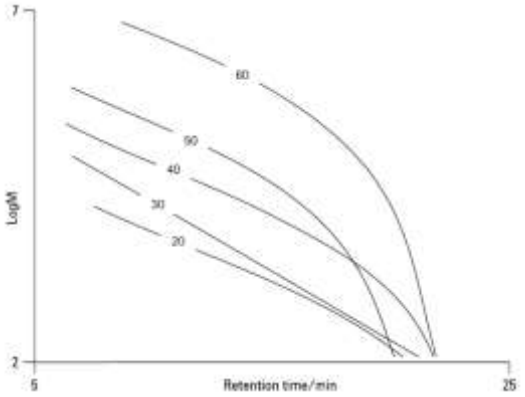
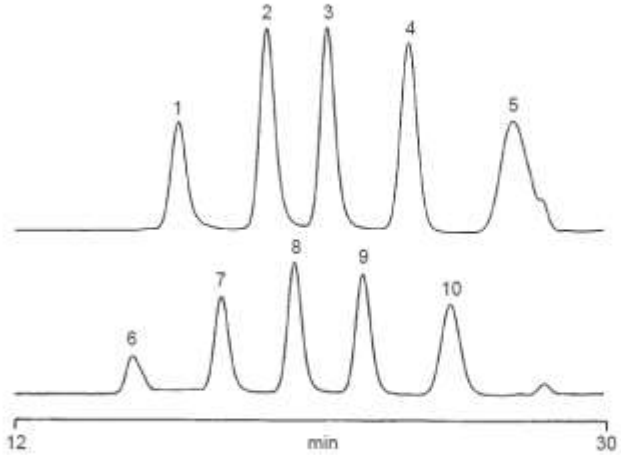


Figure 1. *PLgel MIXED gel calibration curves*



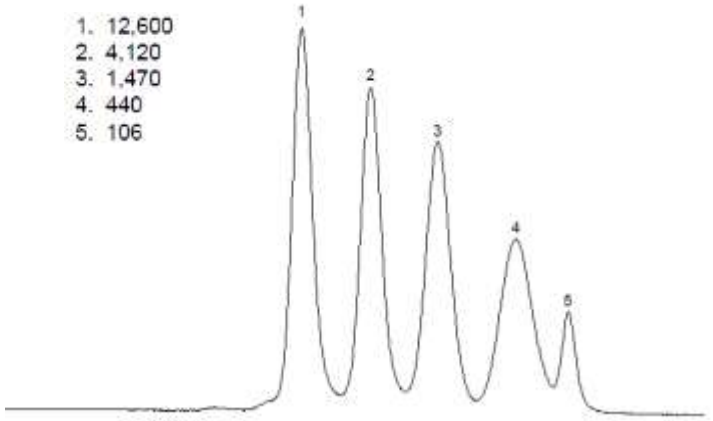


1.	3,040,000
2.	330,000
3.	66,000
4.	9,200
5.	580
6.	8,500,000
7.	1,030,000
8.	156,000
9.	28,500
10.	3,250

Column:	3xPLgel 10µm MIXED-B, 300x7.5mm (1110-6100)
Eluent:	THF
Flow Rate:	1.0ml/min
Detector:	UV, 254nm

Columns:	2xPL aquagel-OH 30 8µm, 300x7.5mm (1120-6830)
Eluent:	Water
Flow Rate:	1.0ml/min
Detector:	RI

1. 12,600
2. 4,120
3. 1,470
4. 440
5. 106

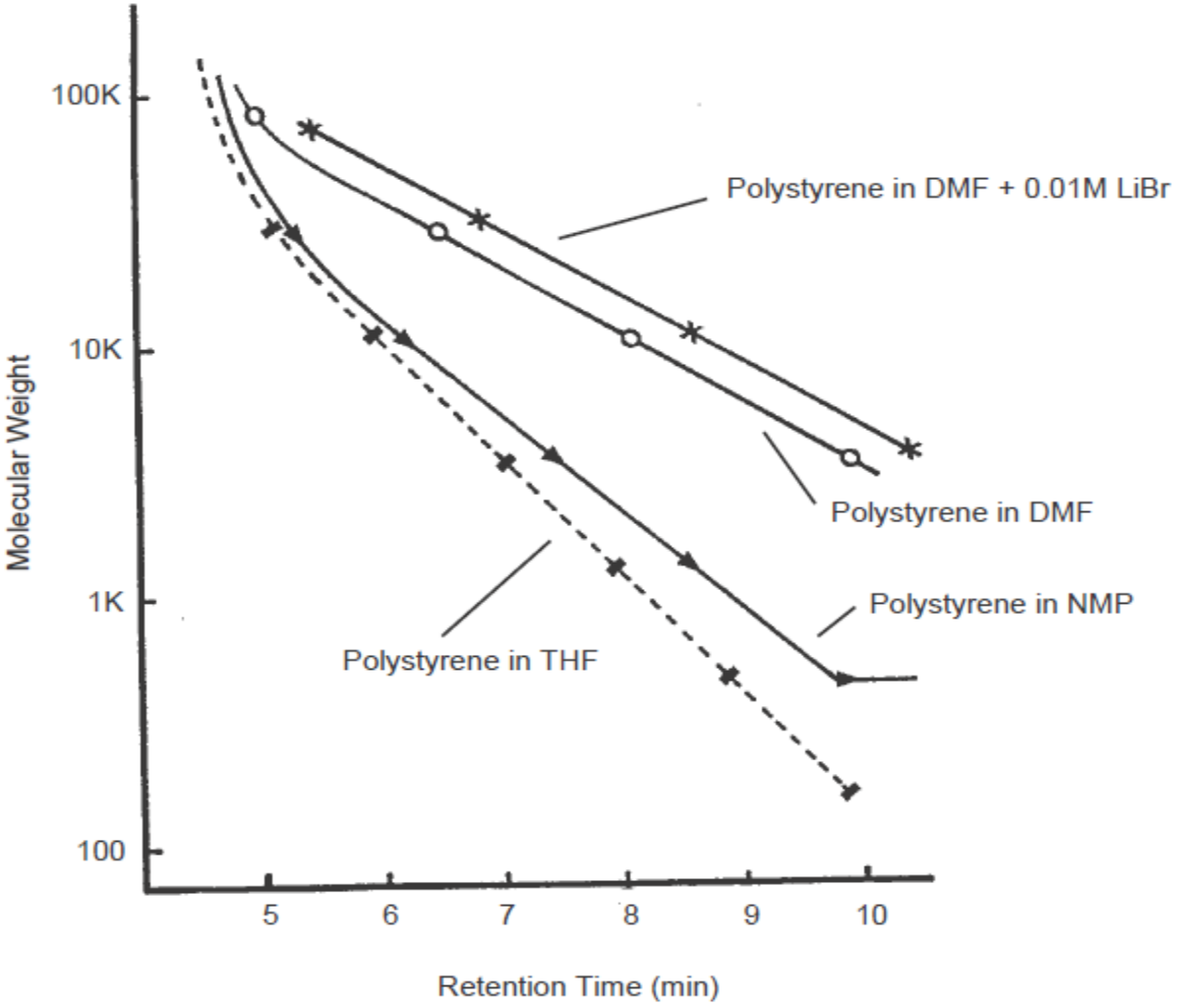


What Calibration standards are appropriate:

Question
What is the eluent?

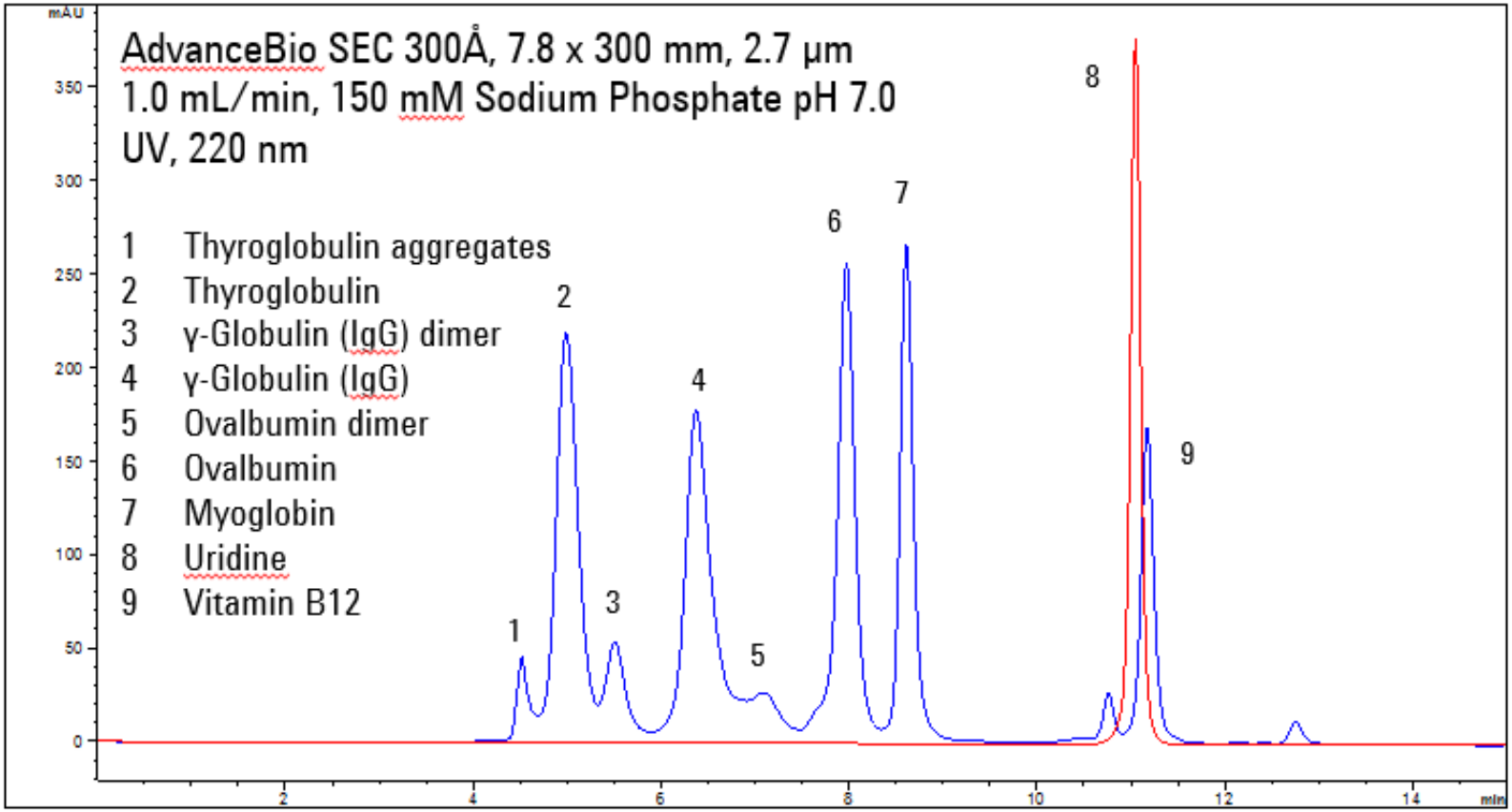
Answer	Recommendation
Water or water buffer with up to 50% methanol	**Polyethylene glycol (PEG)/ oxide (PEO)** or polysaccharides (SAC)
Typical organic solvent such as THF, chloroform, toluene	**Polystyrene (PS)** or polymethylmethacrylate (PMMA)
Organic/water mixtures or polar organics such as DMF, NMP	**Polyethylene glycol/oxide** or polymethylmethacrylate

Calibrations Standards and Importance of Solvent Selection

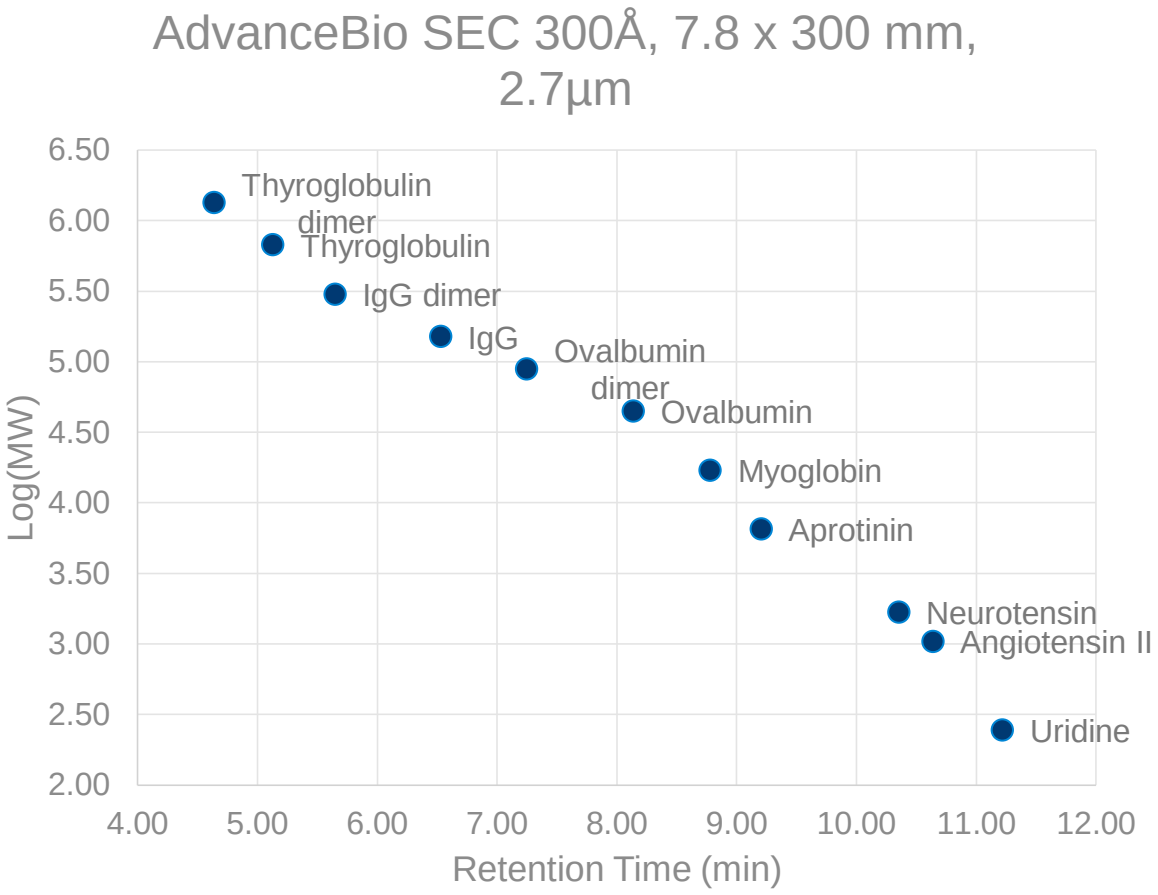


Ex: PS/DVB columns are excellent in many solvents, but remember that although the column may be used in certain solvents this does not mean SEC will occur - the example here is polystyrene standards running in NMP, DMF, etc.

SEC Chromatogram of proteins



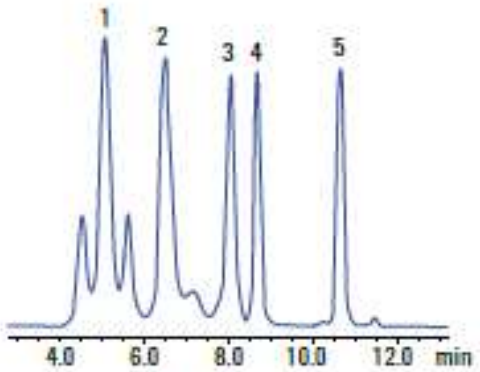
Creating a calibration curve



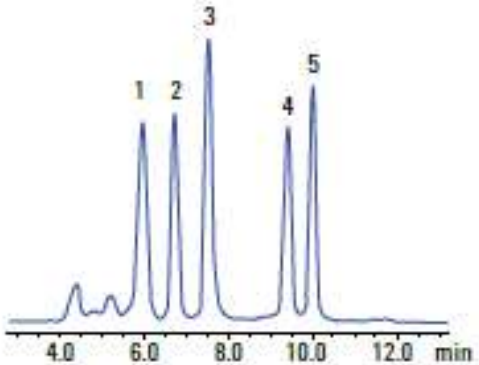
Protein or Peptide	MW	Log(MW)	RT (min)
Thyro Aggs	1340000	6.13	4.64
Thyroglobulin	670000	5.83	5.13
IgG Dimer	300000	5.48	5.65
γ-Globulin	150000	5.18	6.53
Oval Dimer	88600	4.95	7.25
Ovalbumin	44300	4.65	8.14
Myoglobin	16950	4.25	8.78
Aprotinin	6511	3.81	9.21
Neurotensin	1672	3.22	10.36
Angiotensin-II	1040	3.02	10.64
Uridine	244	2.39	11.22

Calibrating Your Column:

Agilent AdvanceBio SEC Protein Standards



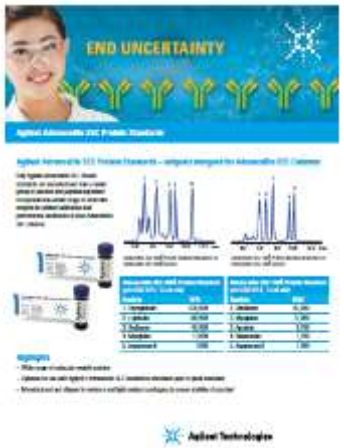
AdvanceBio SEC 300Å Protein Standard separation on AdvanceBio SEC 300Å column



AdvanceBio SEC 130Å Protein Standard separation on AdvanceBio SEC 130Å column

AdvanceBio SEC 300Å Protein Standard (p/n 5190-9417, 1.5 mL vial)	
Analyte	**MW**
1. Thyroglobulin	670,000
2. γ-globulin	150,000
3. Ovalbumin	45,000
4. Myoglobin	17,000
5. Angiotensin II	1,000

AdvanceBio SEC 130Å Protein Standard p/n 5190-9416, 1.5 mL vial)	
Analyte	**MW**
1. Ovalbumin	45,000
2. Myoglobin	17,000
3. Aprotinin	6,700
4. Neurotensin	1,700
5. Angiotensin II	1,000



Publication # 5991-6426EN

★ uniquely designed for AdvanceBio SEC Columns

Summary

What	I need to separate my sample by size: GPC, SEC, GFC. What is the type of sample -> polymer, protein,
WHEN	Analysis req. MWD or MW information of polymers Separating & quantitation of proteins, mAbs, aggregation
WHY	Other separation techniques not suitable. SEC preferred technique
HOW	Column Choices Method Considerations Calibrations

Agilent Resource Information

Agilent Resource Center: http://www.agilent.com/en-us/promotions/agilentresources

Biomolecule Separations, AdvanceBio SEC : http://www.agilent.com/en-us/products/liquid-chromatography/lc-columns/biomolecule-separations/advancebio-sec

GPC/SEC Solutions for Accurate, Reproducible Polymer Analysis : http://www.agilent.com/en-us/products/gpc-sec

THANK YOU FOR ATTENDING

ANY QUESTIONS??