GPC/SEC Method Development:

It isn’t only about a sample’s size in solution

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Topics for Discussion :

GPC/SEC
– an overview of the separation mechanism and WHY do we use it

Sample Type
– what type of sample do we have and how they differentiate

Solvent
– considerations for solvent selection and why solvent choice IS important

Column Considerations & Selection
– SO MANY to choose from, how do you go about making your column selection
– how to maximize your column performance thru selection

Calibration
– which calibration standard to select & why
– its importance for data analysis & system reproducibility

Detector choice
– concentration detectors
– advanced detection

Terminology:

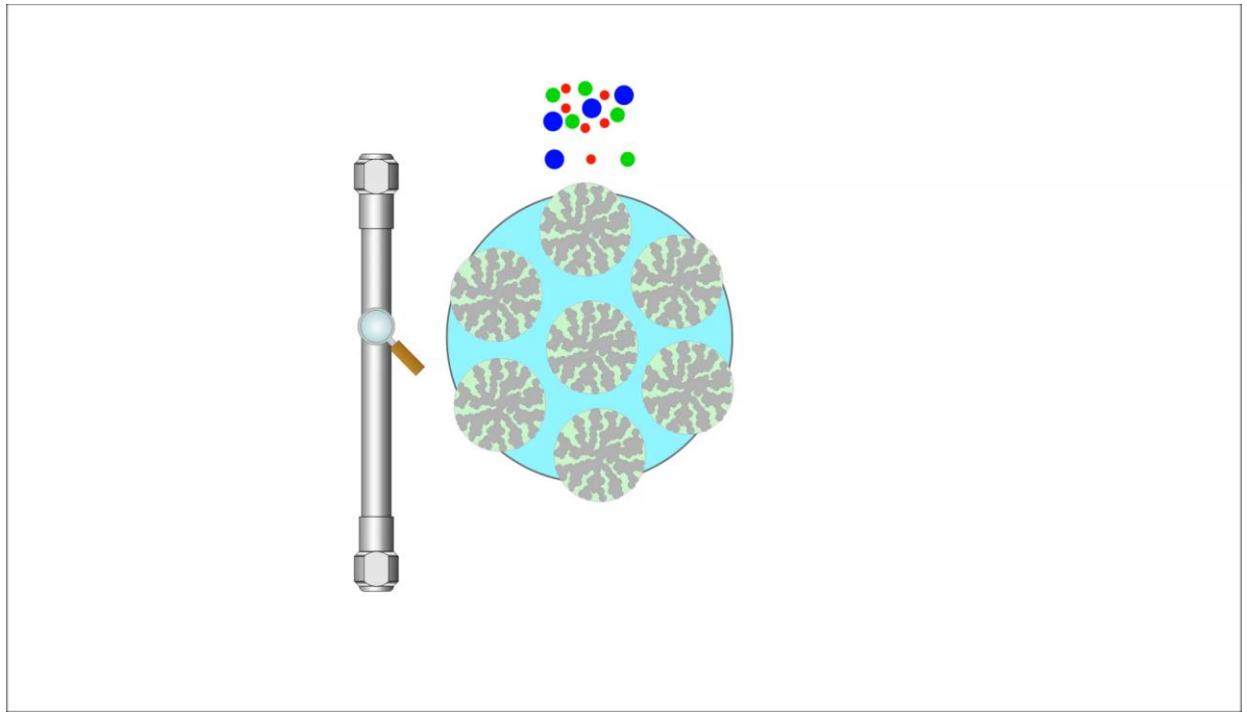
GPC/SEC refers to the chromatographic technique that separates compounds by their size.

Same technique, but different acronyms:

- **GPC** – Gel Permeation Chromatography
  - organic solvents like THF and methylene chloride
- **SEC** – Size Exclusion Chromatography
  - Primarily water and buffer
- **GFC** – Gel Filtration Chromatography
  - Water and buffer, common term for industrial purification step in the life sciences industry

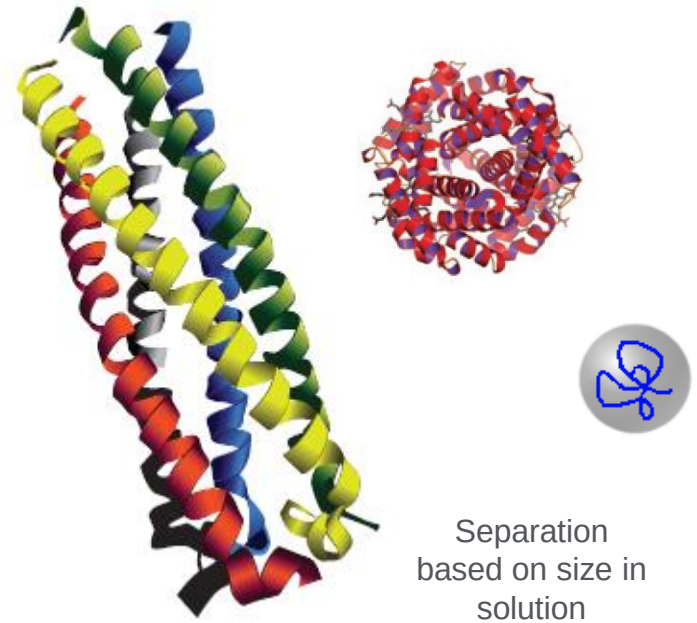
GPC/SEC Separation Mechanism

- 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

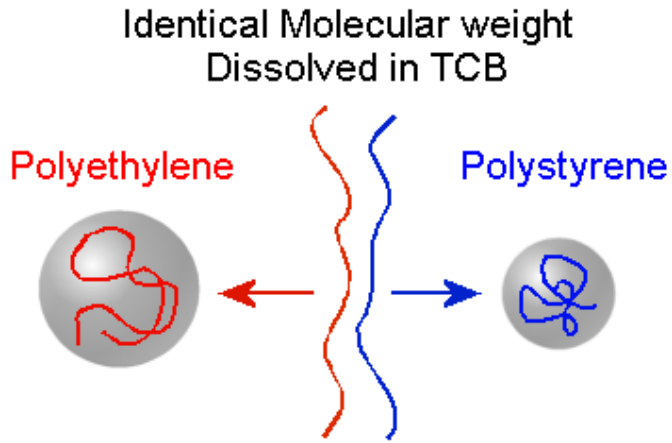


Hydrodynamic volume

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

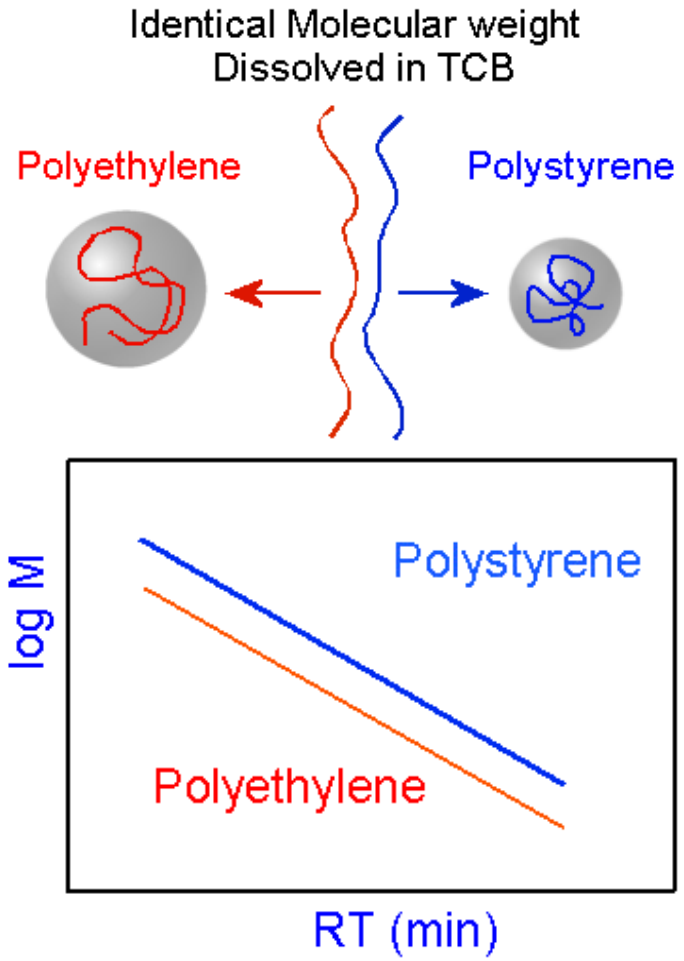


Separation based on size in solution (hydrodynamic volume)



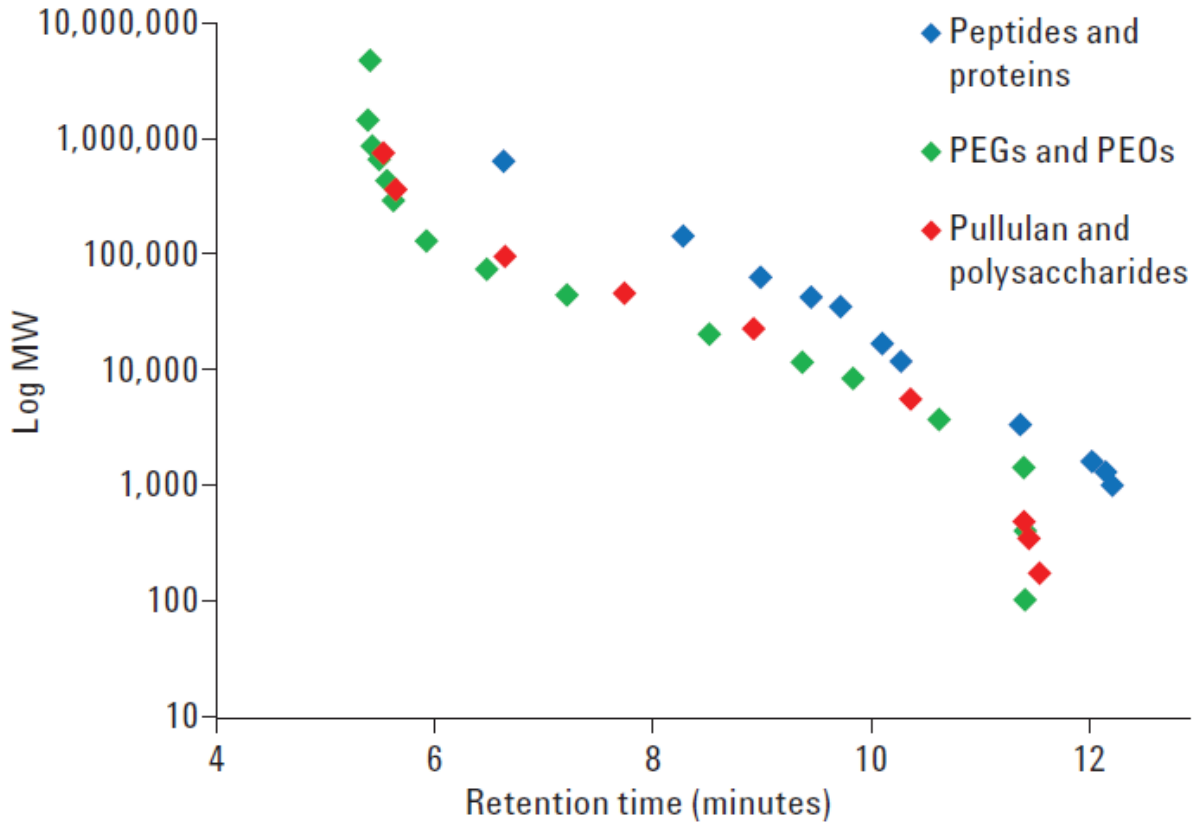
Hydrodynamic Volume…..expect differences

- 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



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’

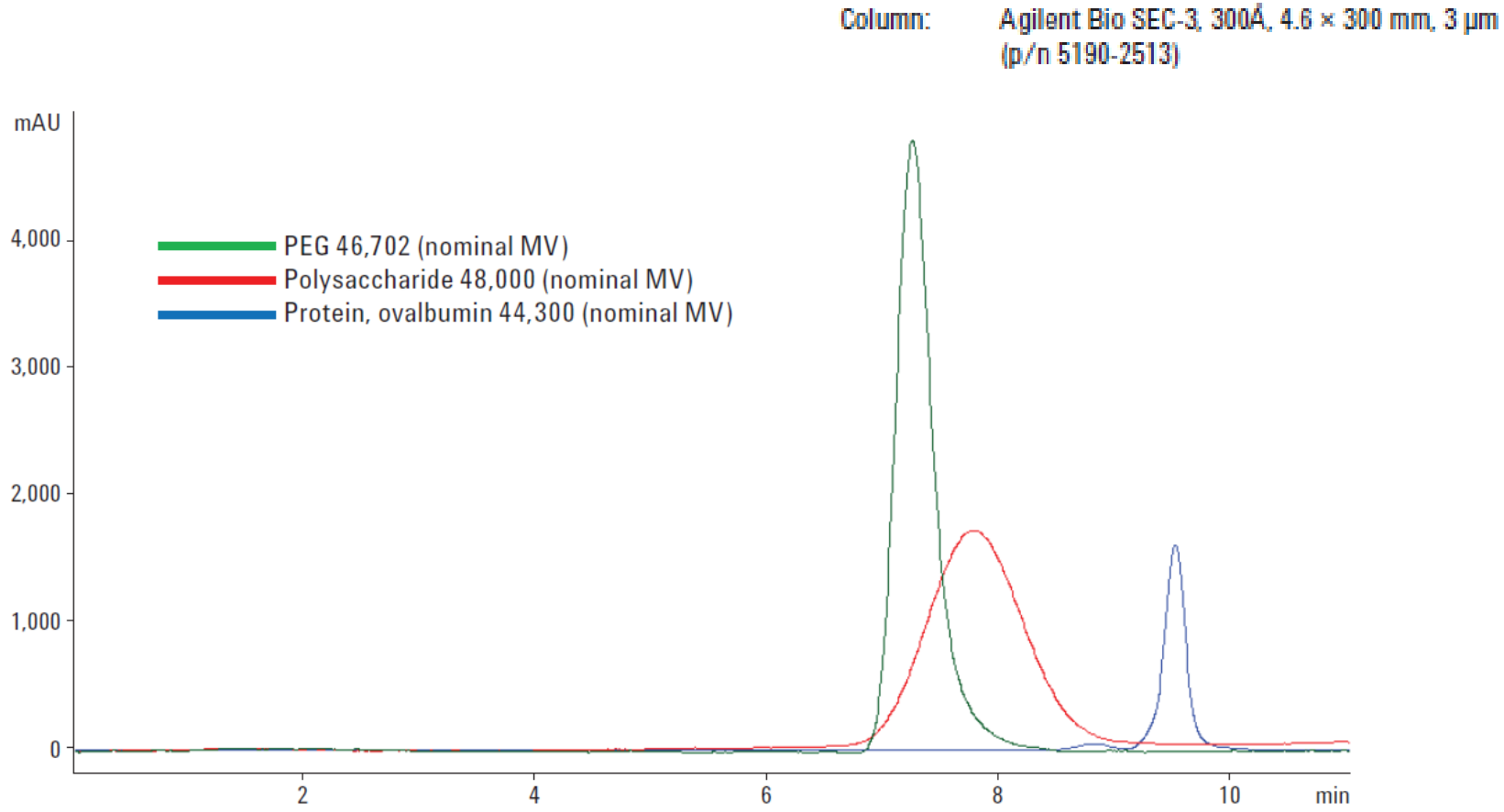


Figure 5. Overlay of chromatograms obtained for calibrants of similar molecular weight.

The Primary Goal of GPC is to Discover the MW Distribution

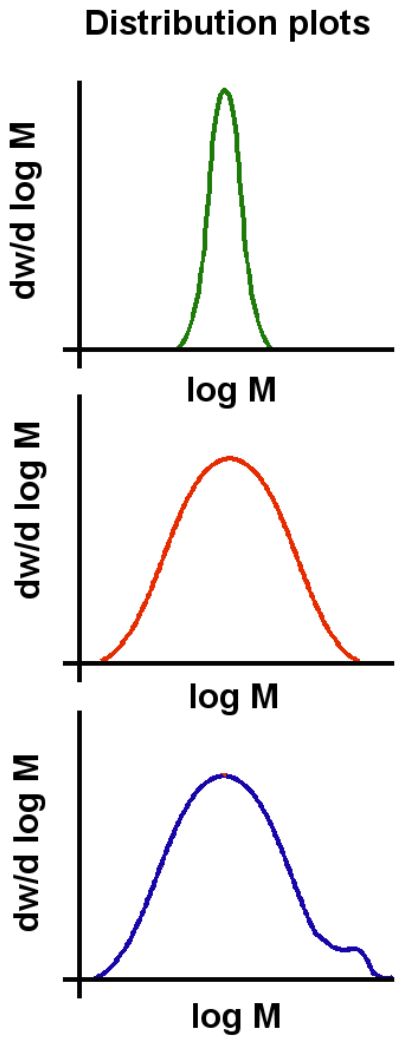
- Samples of synthetic polymers *always* contain polymer chains with a range of chain lengths
- One way to describe the length of the polymer chains is in terms of an average molecular weight, i.e the average of all the chain lengths in the sample

HOWEVER….

- Different samples of the same polymer can have the same average chain length but very different distributions of chain lengths depending on the method of production
- In polymer science it is the molecular weight *distribution* that is important

Example 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



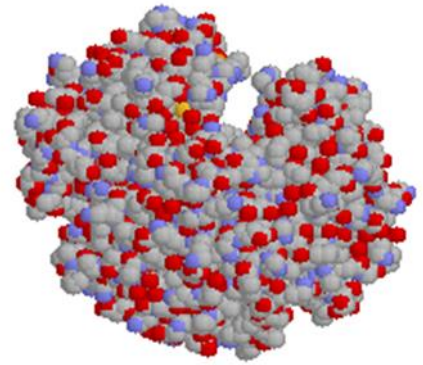
Reasons for Why GPC/SEC is done?

- **Plastics**
  - MW dictates polymer strength, flexibility, and physical properties

- **Sample cleanup**
  - Separates target molecules from large molecules that fragment in MS and cause interference

- **Water Soluble polymers**
  - MW impacts viscosity, surfactant effects, dissolution, and chemical characteristics

- **Biomolecules**
  - MW is often known
  - Similar separation to gel electrophoresis
  - Can be run on intact molecules
  - Aggregation can be dangerous



Sample Type – Polymer or BioMolecule

Polymers

Questions that you need to ask?

What type of polymer do I have?

Organic soluble → GPC

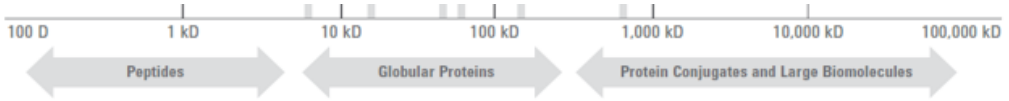
Aqueous soluble → SEC

BioMolecule

Questions that you need to ask?

What type of sample do I have?

Peptides
Proteins/Globular Proteins
mAbs
Protein Conjugates
Large BioMolecules



Solvents

- Selecting a solvent system is one of the first steps in developing a GPC method
- Some polymers or biomolecule samples are easy to dissolve, some are much harder
- The solvent conditions must be appropriate for the sample; to prevent any unwanted interactions between that of the sample with the packing particle. Interactions will give a false MW result
- Agilent's range GPC/SEC columns are available with phase chemistries that are optimized for all types of solvents that may be required: aqueous & organic, polar, and nonpolar solvents.

Solvent Considerations

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 to use for the analysis

Points of Consideration:

Sample type -?

Column to be used -?

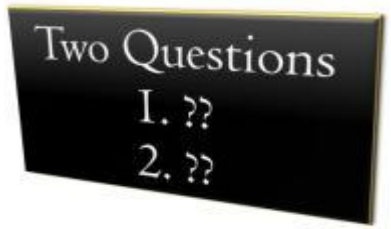
Solvent polarity	Solvent
6.0	Perfluoroalkane
7.3	Hexane
8.2	Cyclohexane
8.9	Toluene
9.1	Ethyl acetate
9.1	Tetrahydrofuran (THF)
9.3	Chloroform
9.3	Methyl ethyl ketone (MEK)
9.7	Dichloromethane
9.8	Dichloroethene
9.9	Acetone
10.0	0-Dichlorobenzene (o-DCB)
10.0	Trichlorobenzene (TCB)
10.2	m-Cresol
10.2	o-Chlorophenol (o-CP)
10.7	Pyridine
10.8	Dimethyl acetamide (DMAc)
11.3	n-Methyl pyrolidone (NMP)
12.0	Dimethyl sulfoxide (DMSO)
12.1	Dimethyl formamide (DMF)

- Solvents listed all compatible with Agilent organic GPC columns

Two Common Questions in GPC/SEC method development

Question 1:

- What solvent is your sample soluble in?



Type	Typical Solvents
Organic	• THF • Chloroform • Toluene • TCB
Mixed or Polar Organic	• THF/water • DMF • NMP
Aqueous	• Water • Buffer in water • Water/methanol (up to 50%)

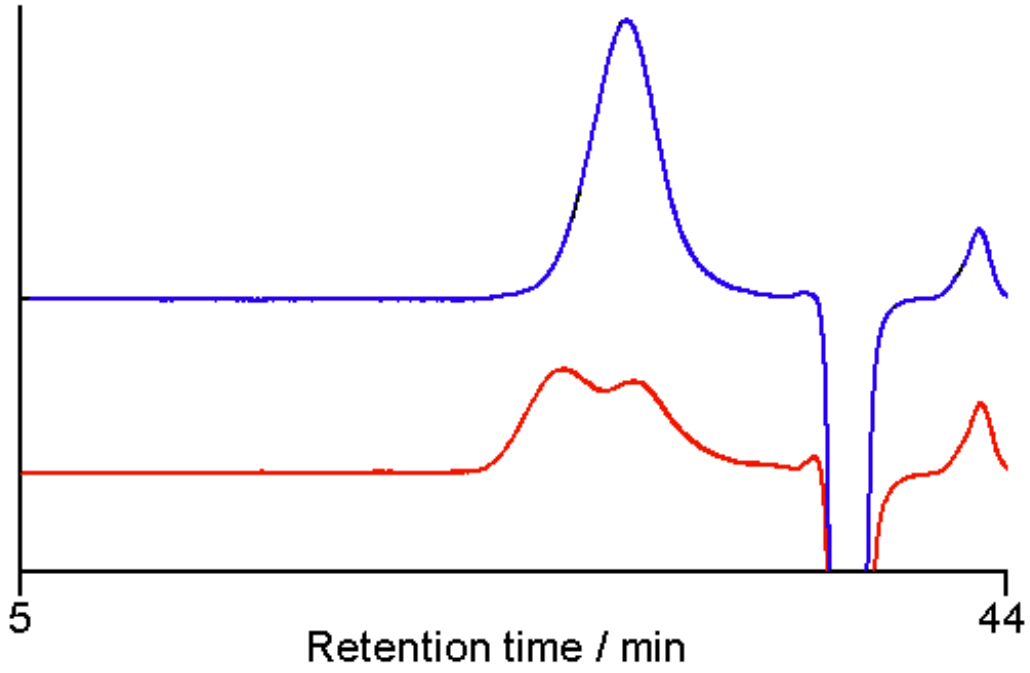
Additives can be employed:

- Minimize non-size exclusion interactions between the sample and the column
- Stabilize the solution of the polymer (ionic aggregation)

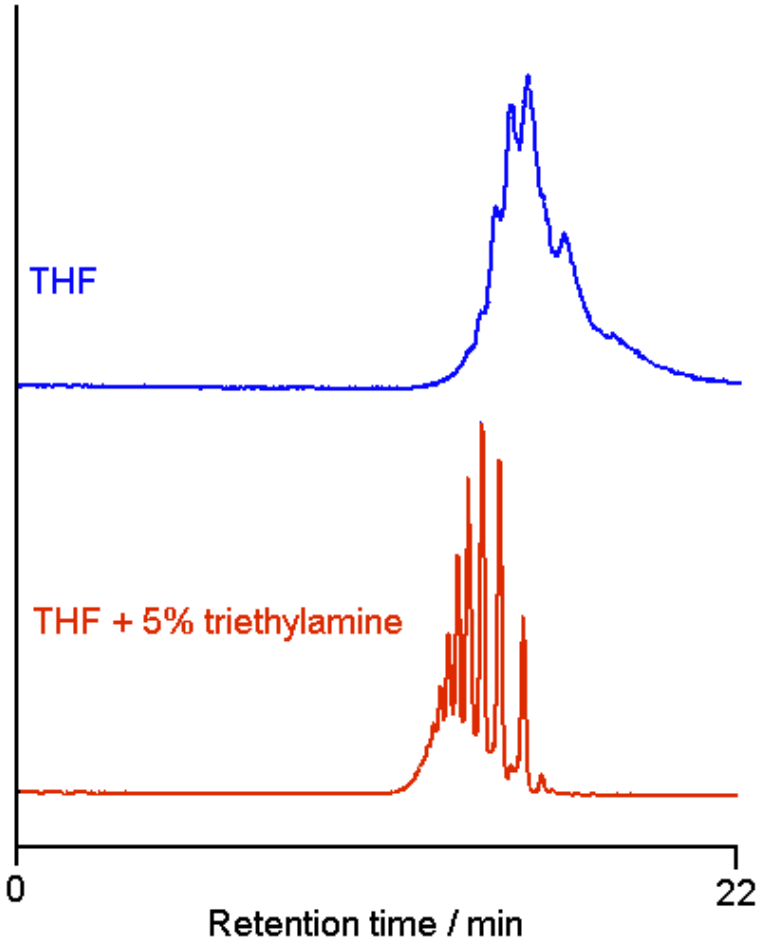
Starch Analysis

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

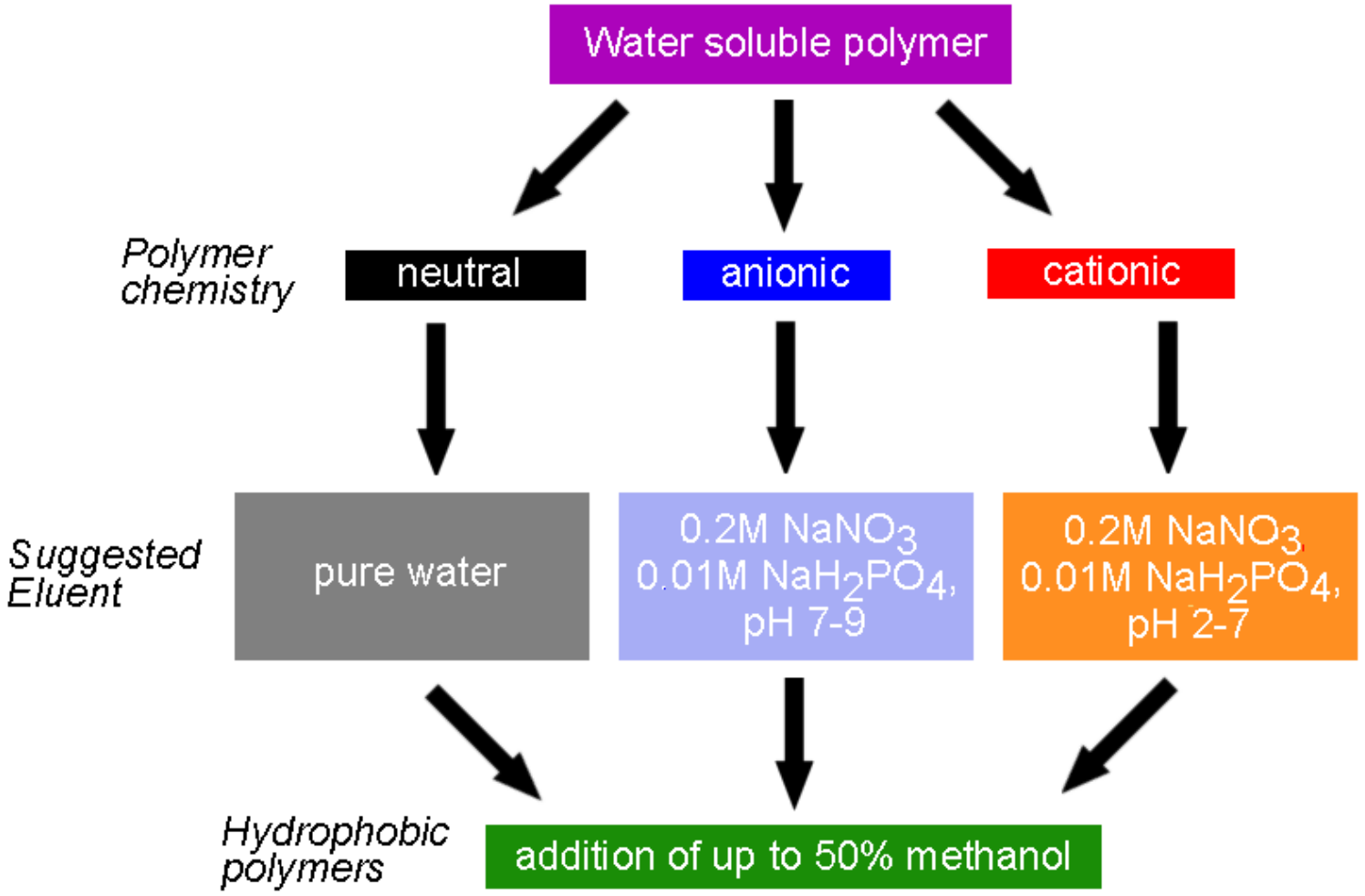


Hostavin N30

- Polymeric UV stabiliser containing secondary amine groups

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

Solvent Considerations and Optimizing for Aqueous SEC:



Guide to Eluent Selection for PL aquagel-OH Applications

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

Buffers and SEC: criteria for optimal mobile phase

The optimal eluent for the separation should be determined by the characteristics of the column stationary phase and the proteins/polymers 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).

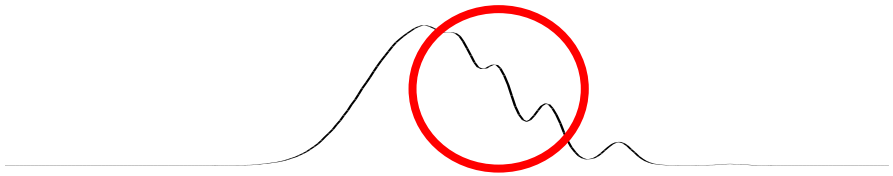
GPC/SEC Columns - Making a Choice

Questions to consider:

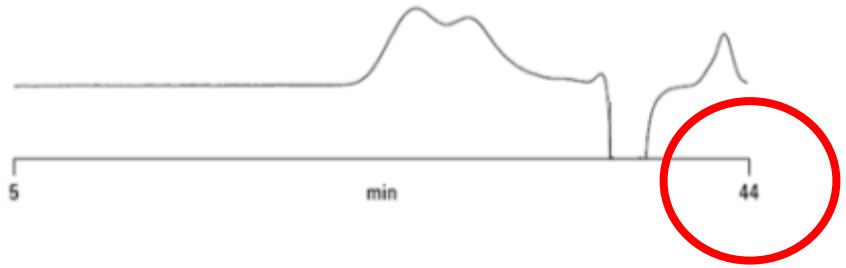
- What type of column chemistry
- Organic or Aqueous eluents being used
- What are your KEY requirements for your GPC/SEC analysis?
  i. Resolution is important
  ii. Reproducibility of sample chromatography and results
  iii. Speed of analysis and/or sample throughput is something to improve on

KEY requirement might be:

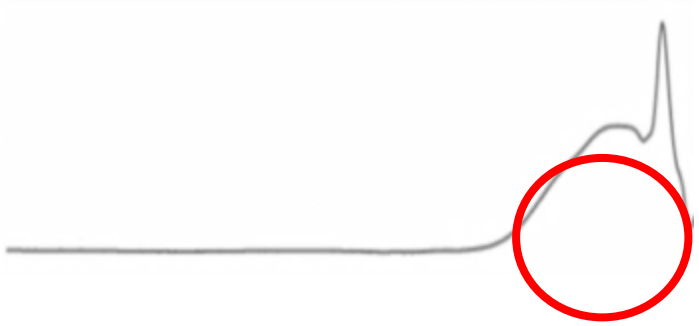
Resolution is too low



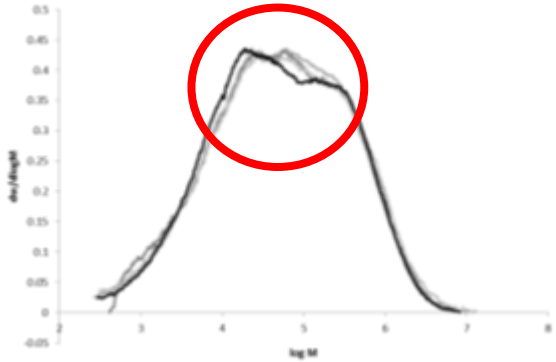
Analysis time is too long



Peak shapes are poor



Results are not reproducible

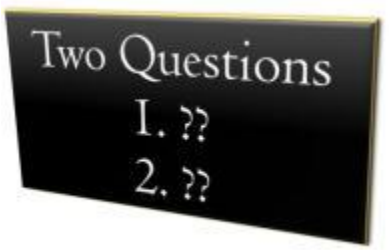


Two Common Questions in GPC/SEC method development

Question 2:

- What is the expected molecular weight range of your polymer or your protein sample?

MW	MW Range (g/mol or Da)
High	Up to several millions
Intermediate	Up to hundreds of thousands
Low	Up to tens of thousands
Very Low	A few thousand



Column Chemistries

Polymer chemistries:

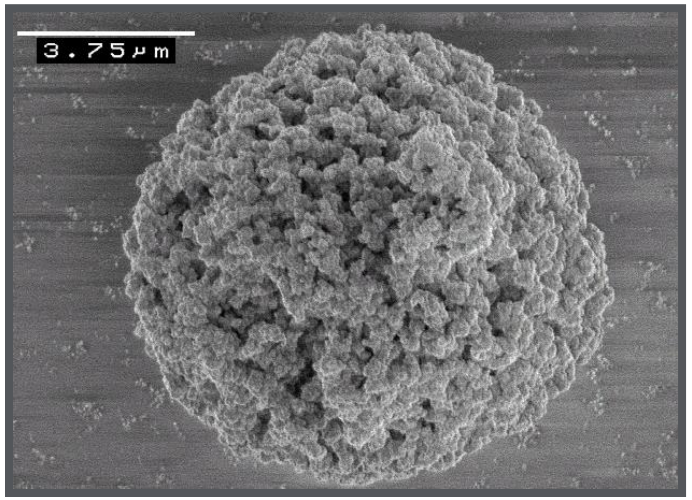
Common Types:

Polymethacrylate packings

Polyester copolymers

DVB, divinylbenzene

PS-DVB, polystyrene divinylbenzene



Silica Chemistries

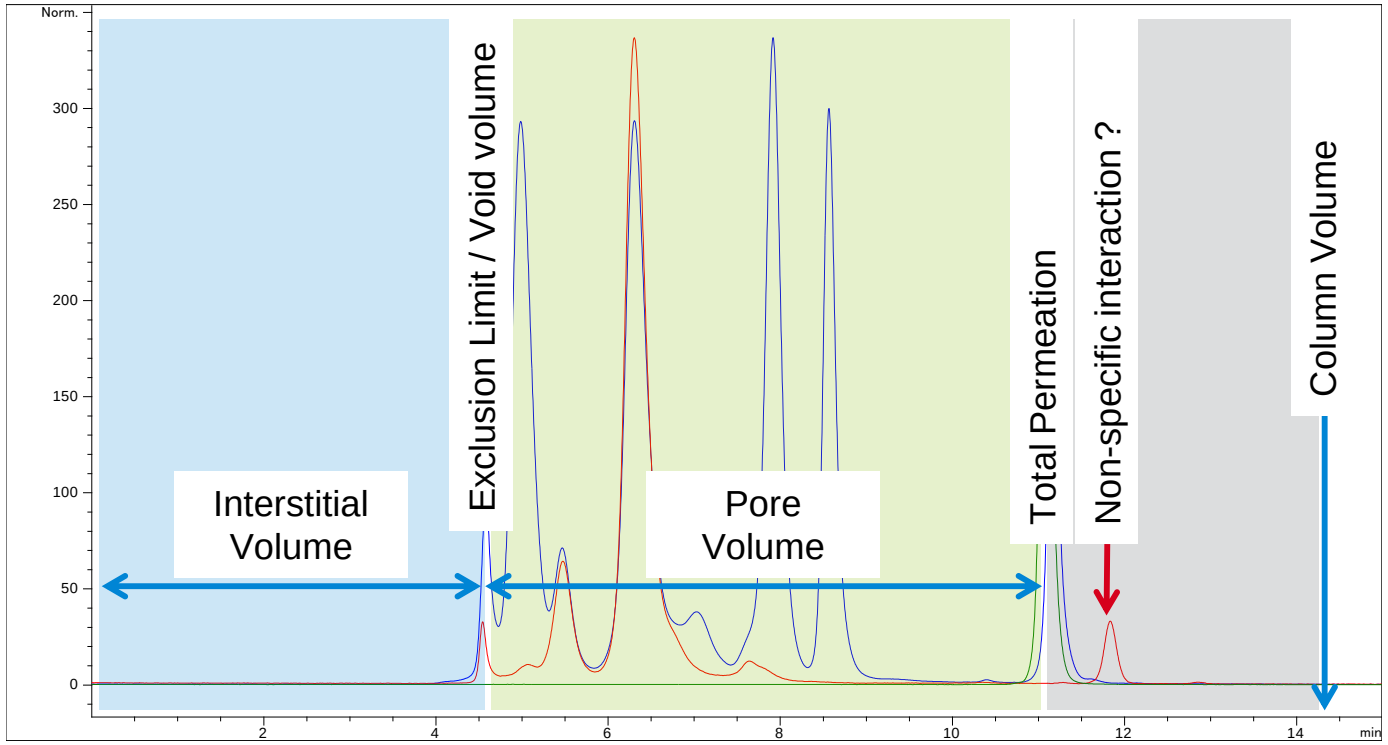
Common Types:

Diol

Surface modified hydroxyl

Surface modified polymeric

Regions on the chromatogram -



Column Selection:

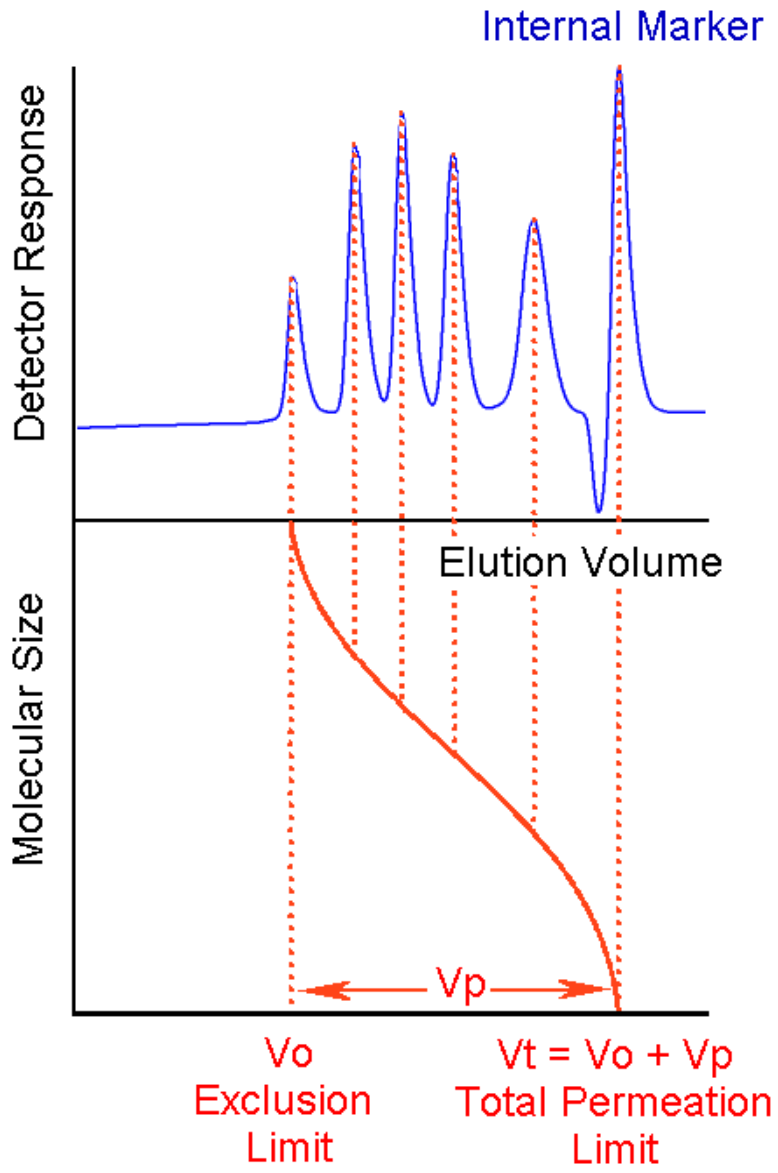
Choose the right pore size

- 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 to not be excluded.
- Provides for complete coverage for the MW range of your sample and for your calibration.standards
- 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

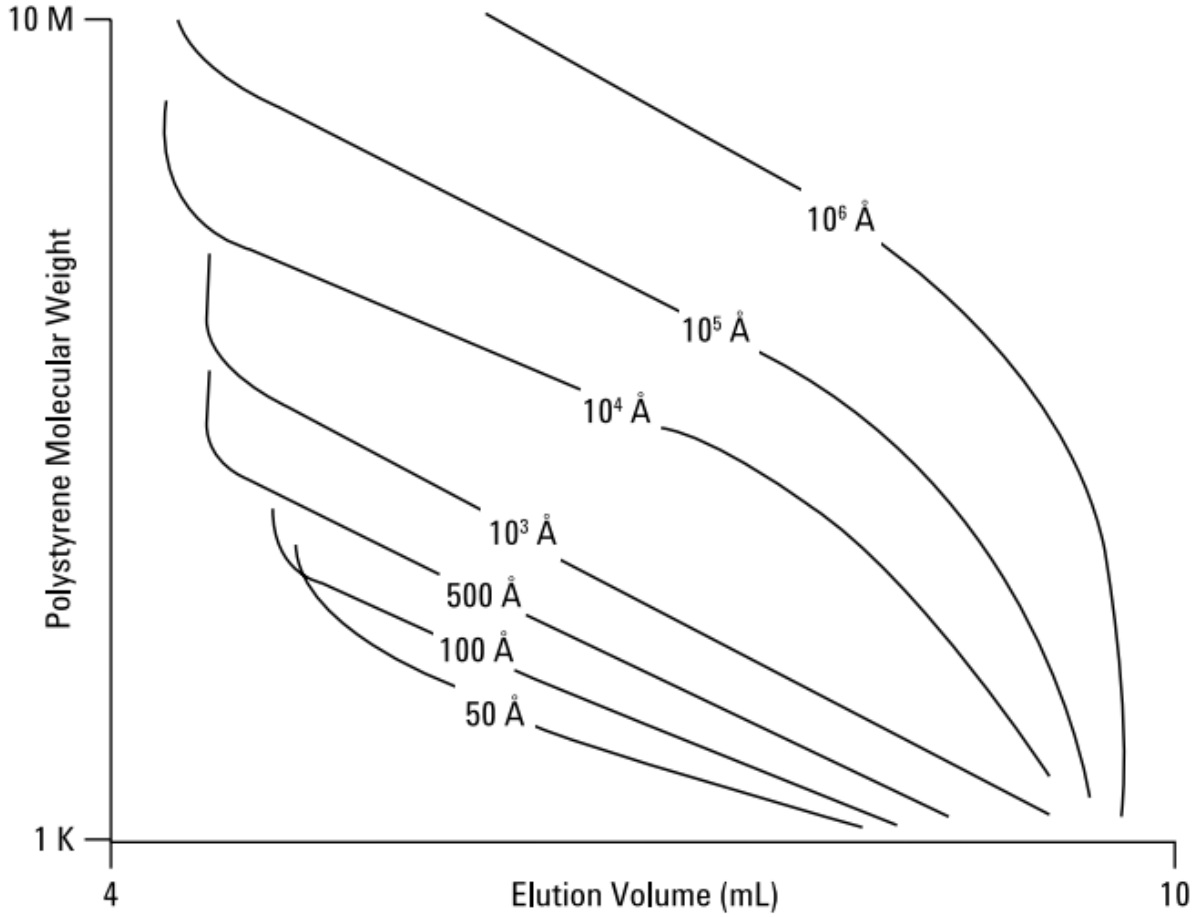
- The example chromatogram & calibration curve illustrates how different size molecules elute from the column
- Choose a pore size that allows you to work in the linear portion of the calibration curve.
- If two molecules have the same molecular weight but different size in solution they may be separated



Column Types: **Individual Pore Size**

- All particles have the same pore size
- Good separation, but narrow range of MW
- Very nonlinear curve; linear only over a very narrow MW range
- Oldest technology, but still popular, and useful for separating very small and very large compounds
- Wider MW range possible by combining different columns in series, but need to select carefully so not to have column ‘mismatch’

Calibration Curve

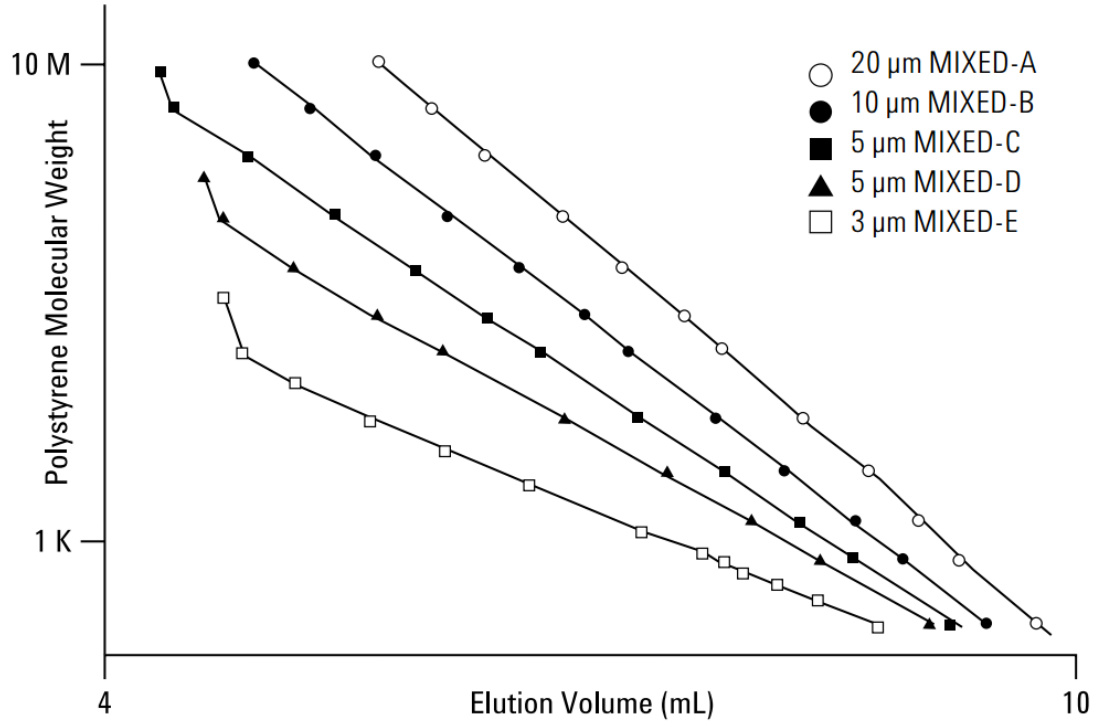


PLgel Individual Pore size calibration plots

Column Types: **MIXED**

- Individual Pore Size particles are mixed together/blended to make a linear curve
- Very wide ranges possible, but only a small amount of separation of each MW
- Linear curve makes chromatogram easy to read and analyze
- Most popular technology, well established and widely used
- Columns in series of same type are still linear

Column Family: PLgel

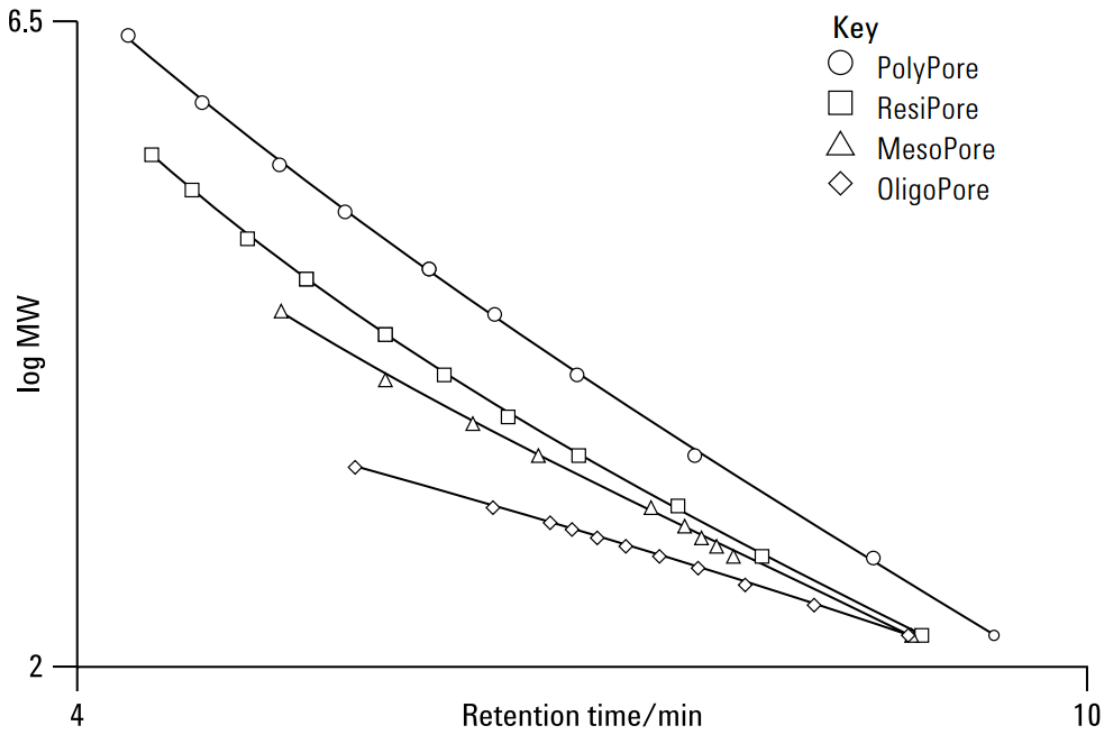


PLgel MIXED calibration plots

Column Types: Multi-Pore Particle

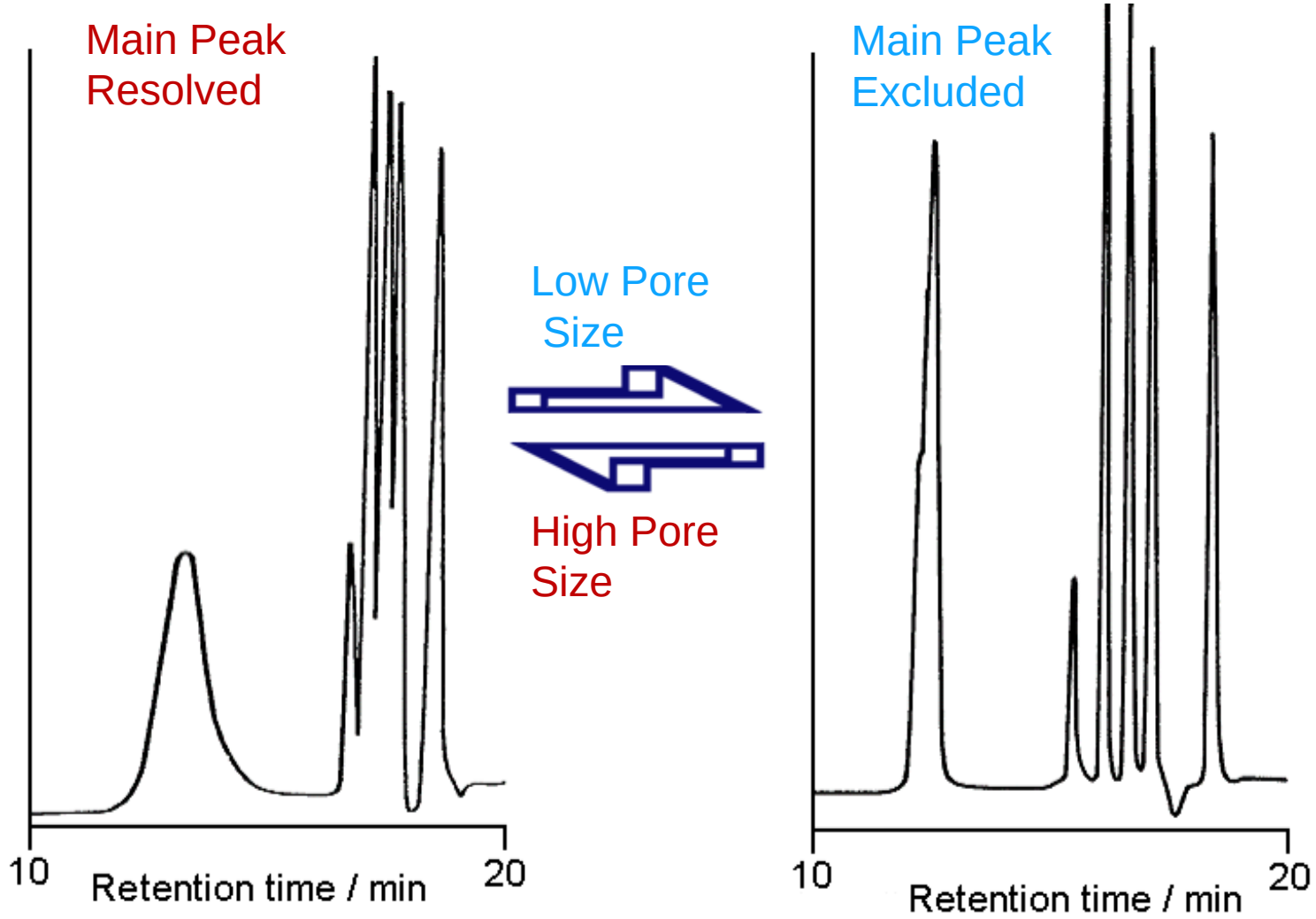
- Newest, fastest growing technology
- Each particle has multiple pore sizes
- Increased pore volume
- Highest resolution and efficiency
- Best performance for most common MW ranges

Column Family: PlusPore



PlusPore calibration plots

Effect of Column Selection: Pore size



* Samples run using PLgel individual pore size columns

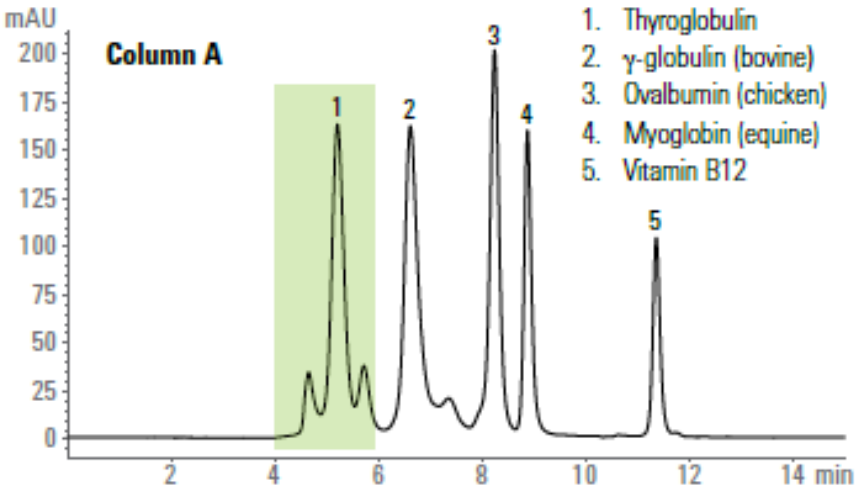
Importance of Pore Size Selection: calibrants

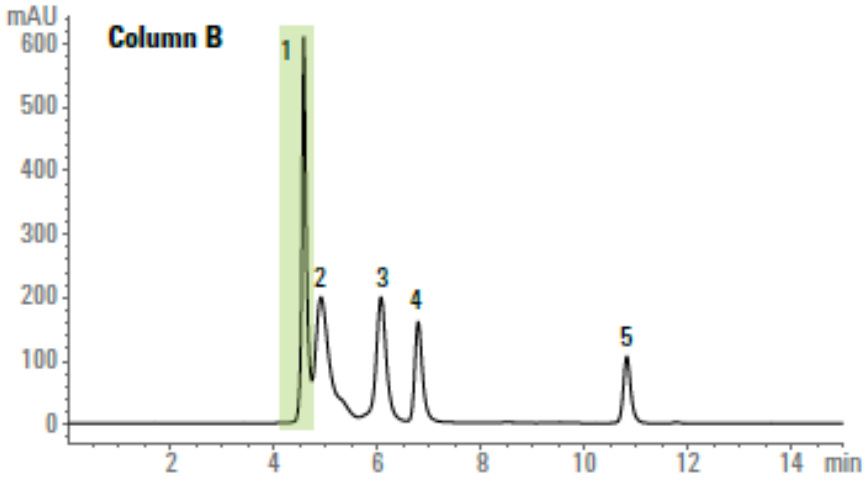
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM phosphate buffer, pH 7.0
Flow rate:	0.35 mL/min
Detector:	UV, 220 nm
Sample:	BioRad gel filtration standards mix

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)

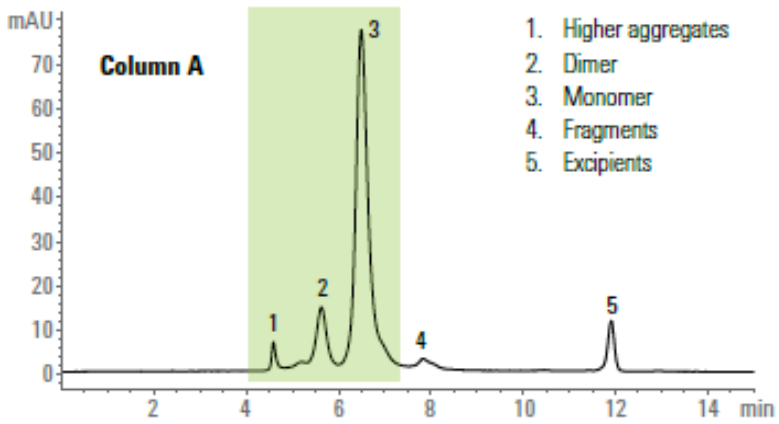
BioRad gel filtration standards mix

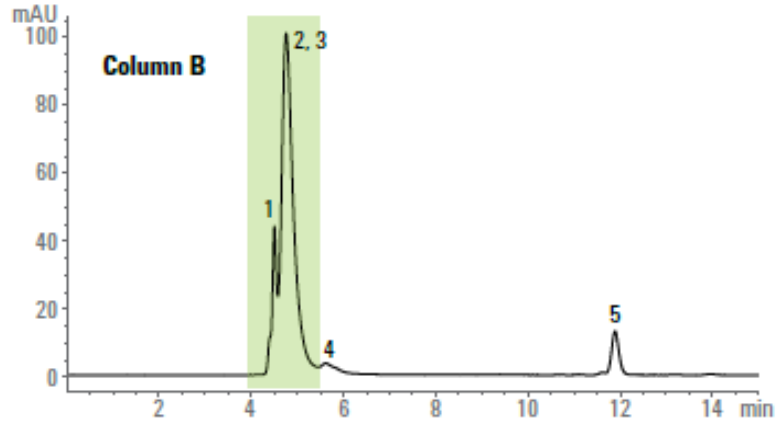




Importance of Pore Size Selection: sample

Polyclonal IgG separation



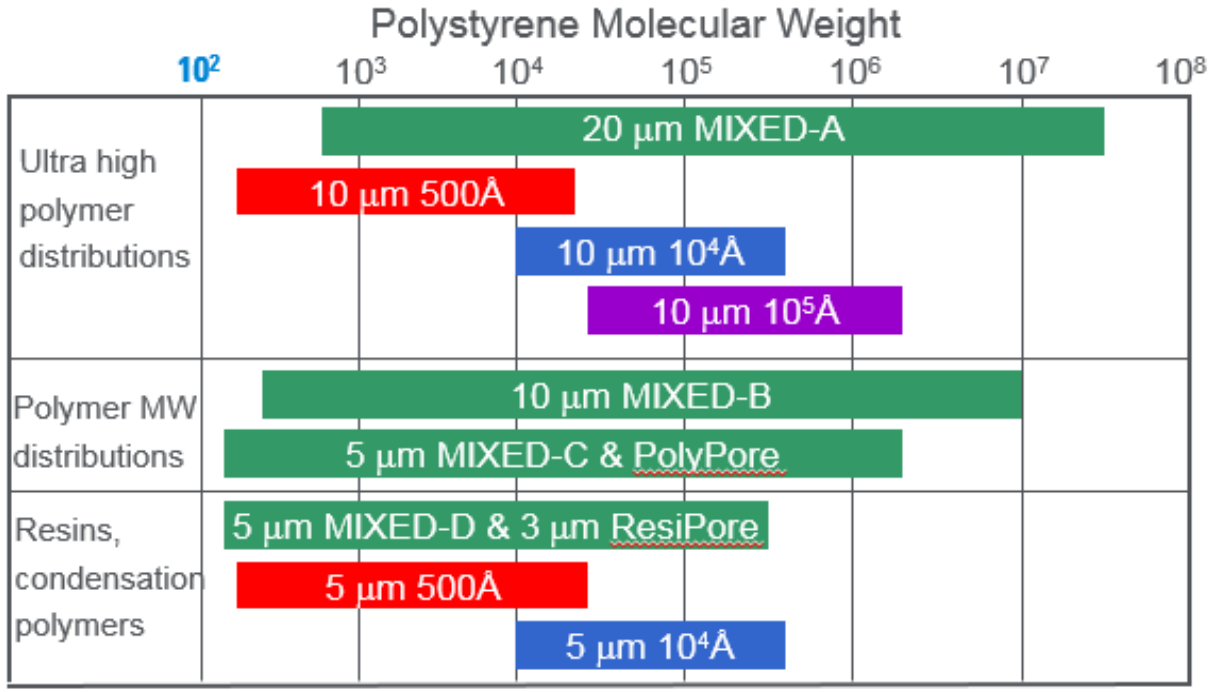


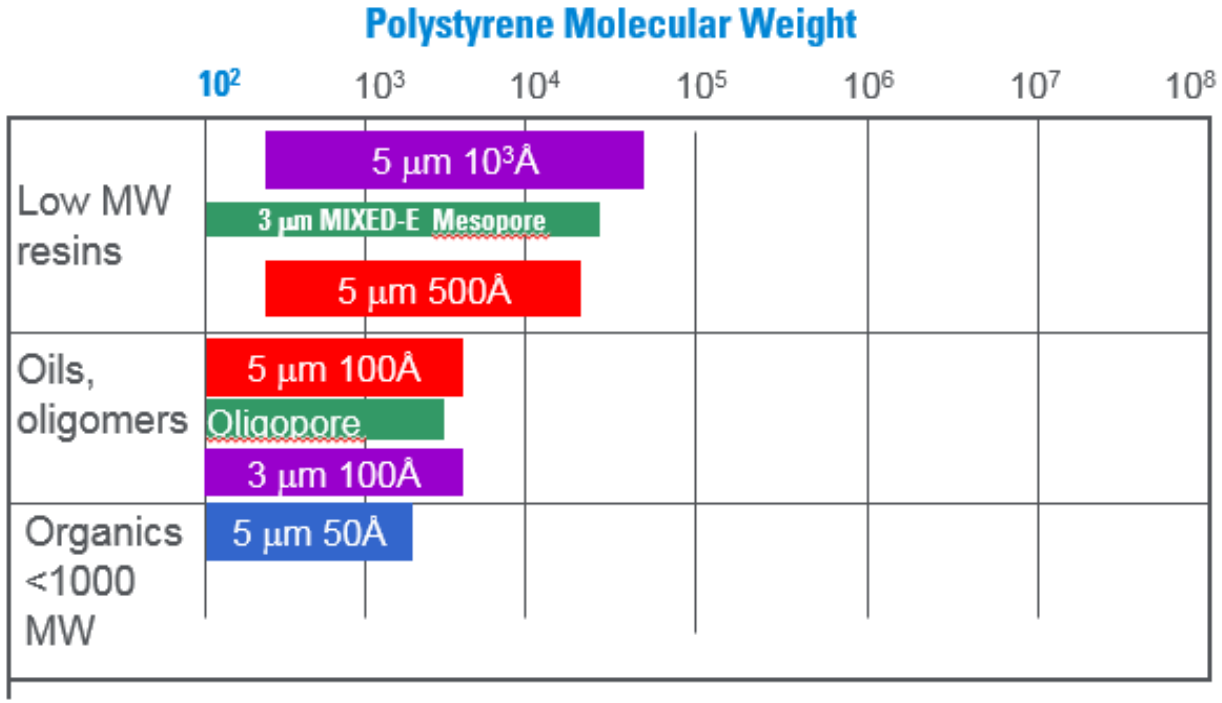
Column A: **AdvanceBio SEC 300Å**
4.6 x 300 mm, 2.7 µm (p/n PL1580-5301)

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

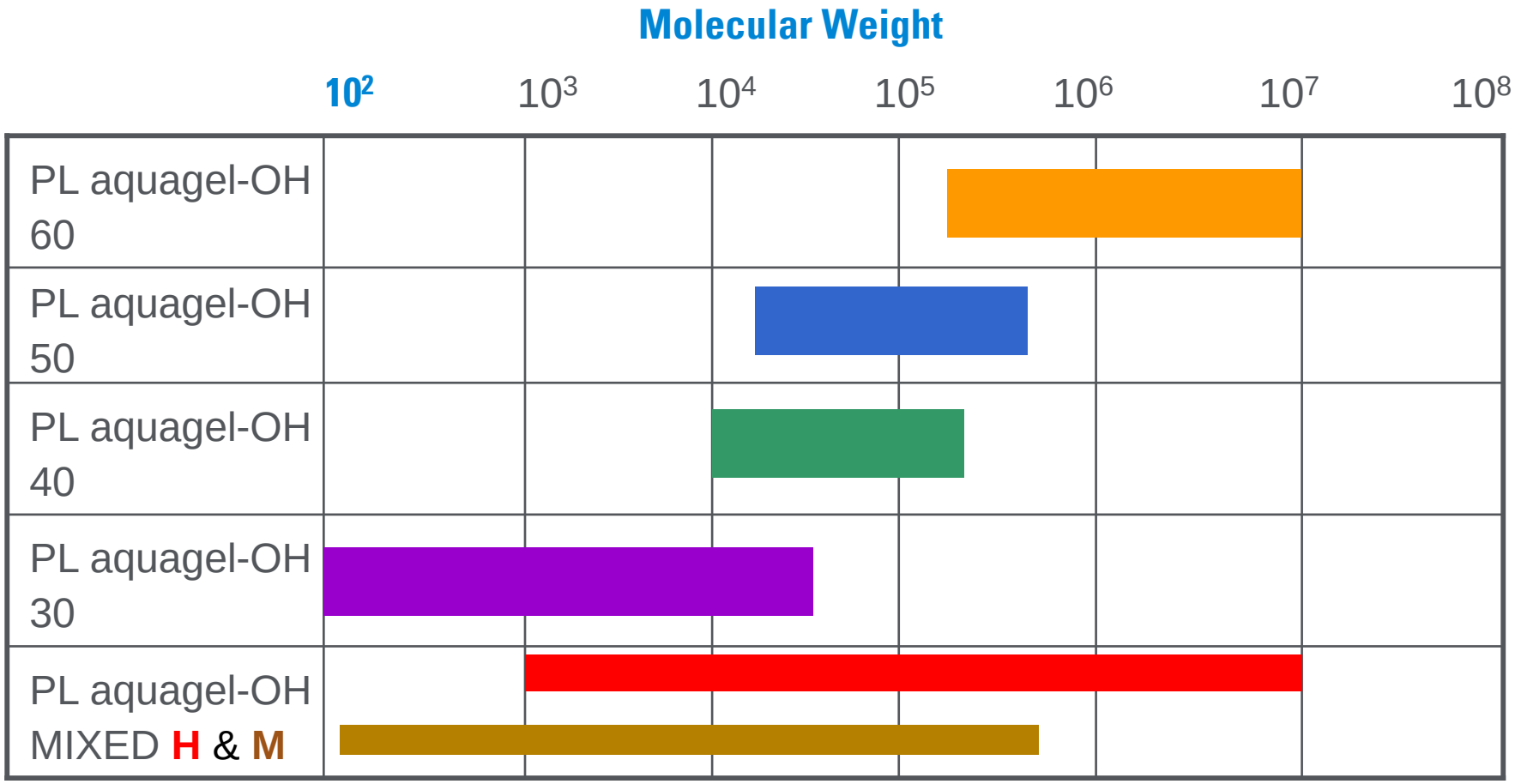
Agilent GPC Columns - Separation Ranges and Column Choices for Organic Soluble Polymers



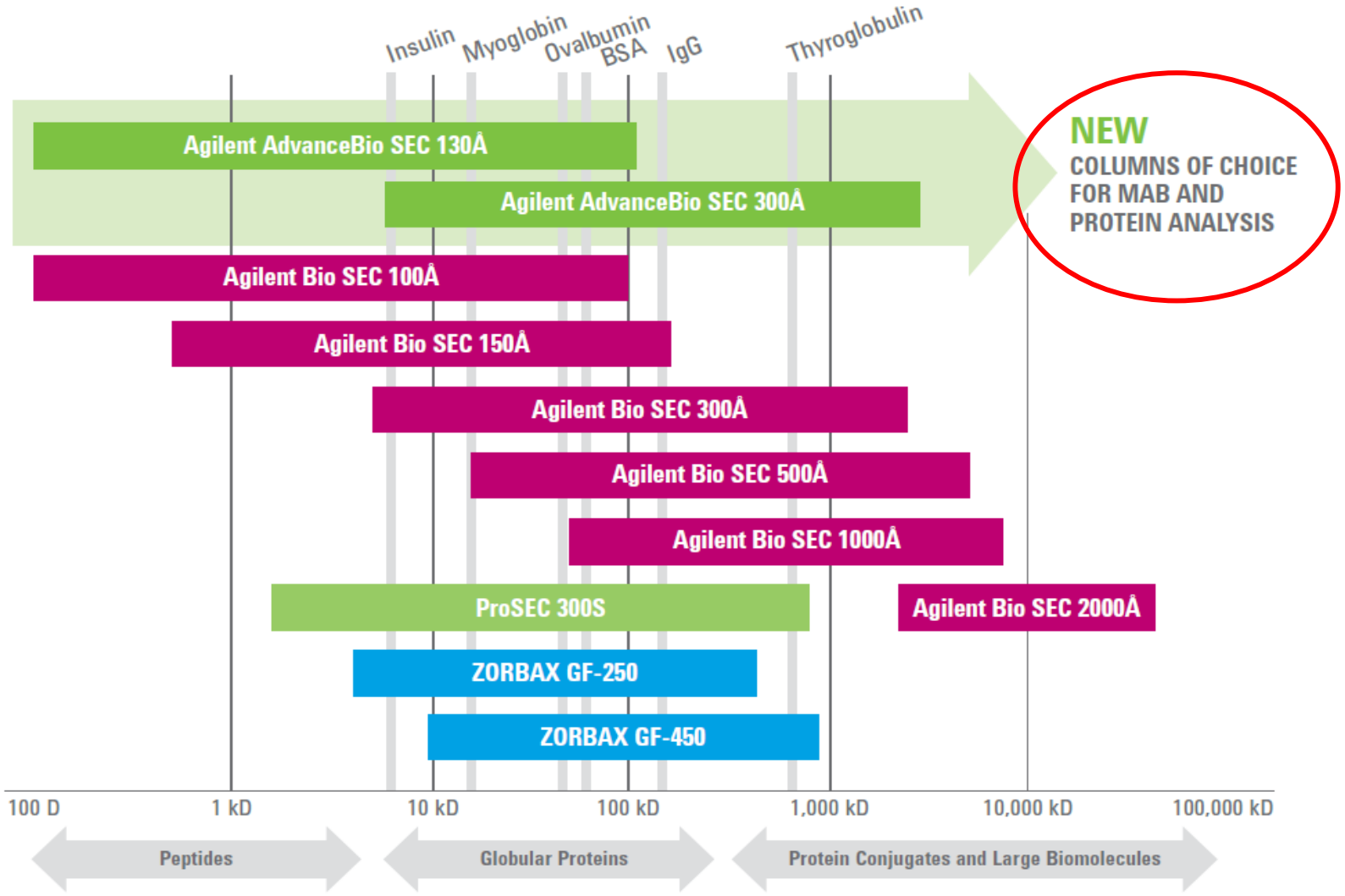


Column Family:
PLgel
PlusPore

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



Agilent SEC Columns – Peptides, Proteins, mAbs



Resolution in GPC/SEC

Running two columns in series, same pore size

- Increase pore volume, increases resolution

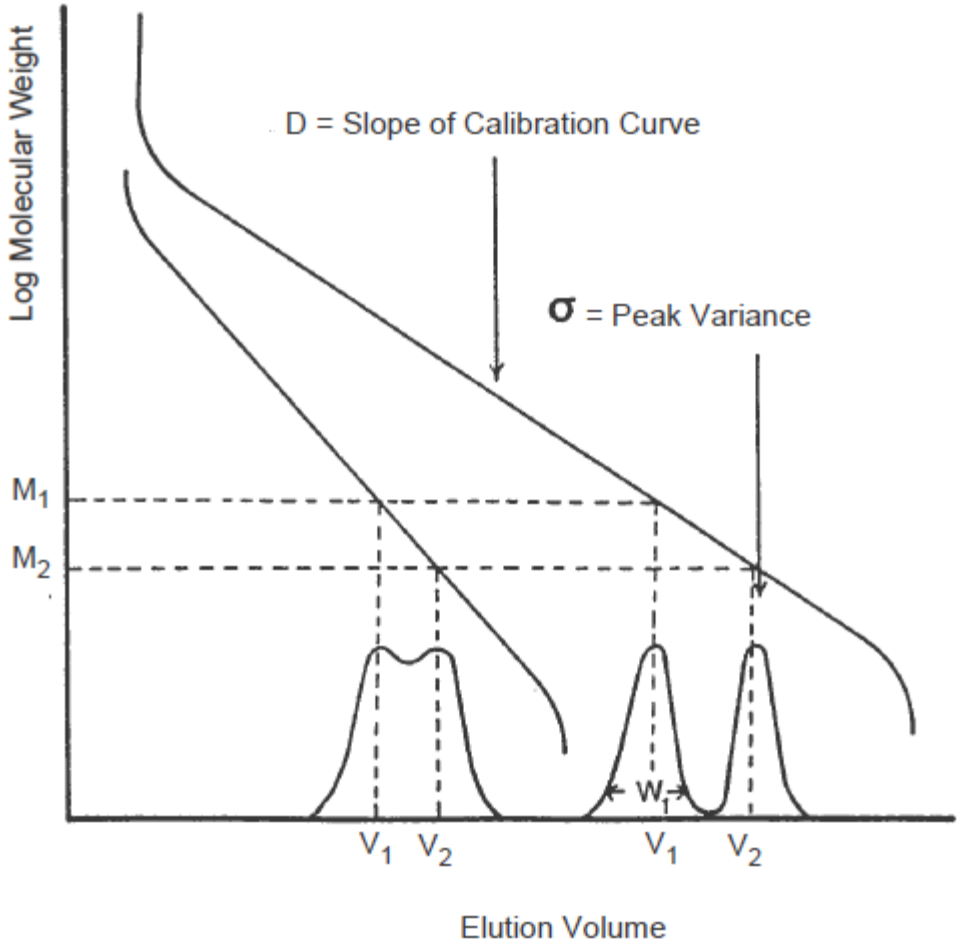
Running two columns in series, different pore size

- 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

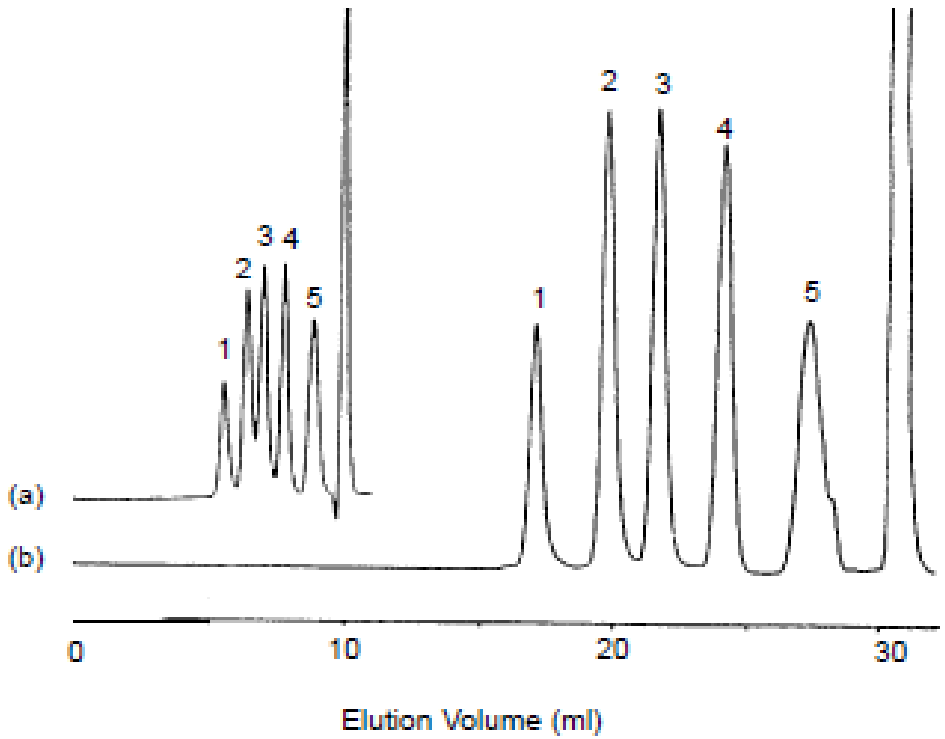


Ex: Effect of column length on resolution

Polystyrene Standards
(EasiCal)

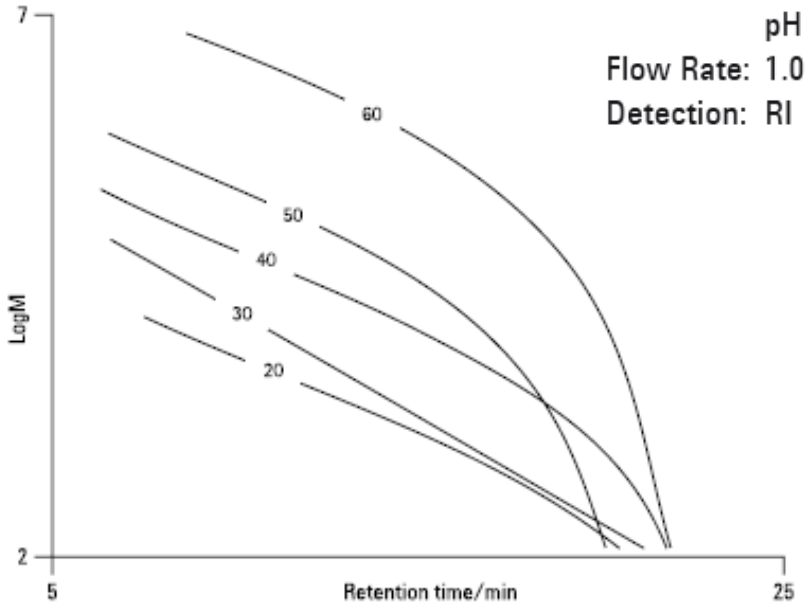
1. 3,040,000
2. 330,000
3. 66,000
4. 9,200
5. 580

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



Column in series: to extend resolving 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

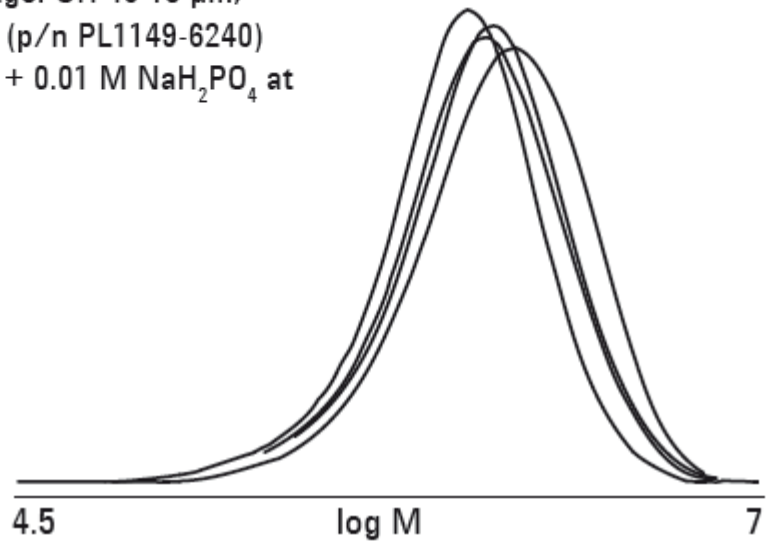
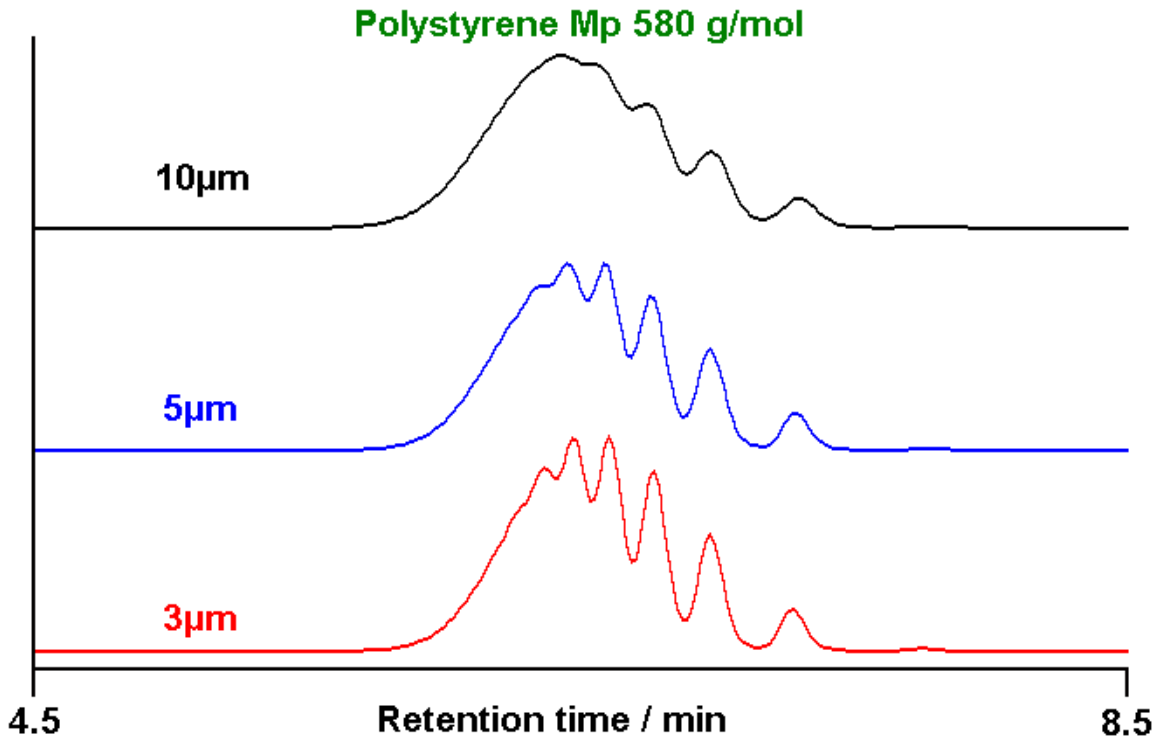


Figure 3. Overlay of the molecular weight distributions of four hyaluronic acid samples

Effect of Particle Size on Resolution

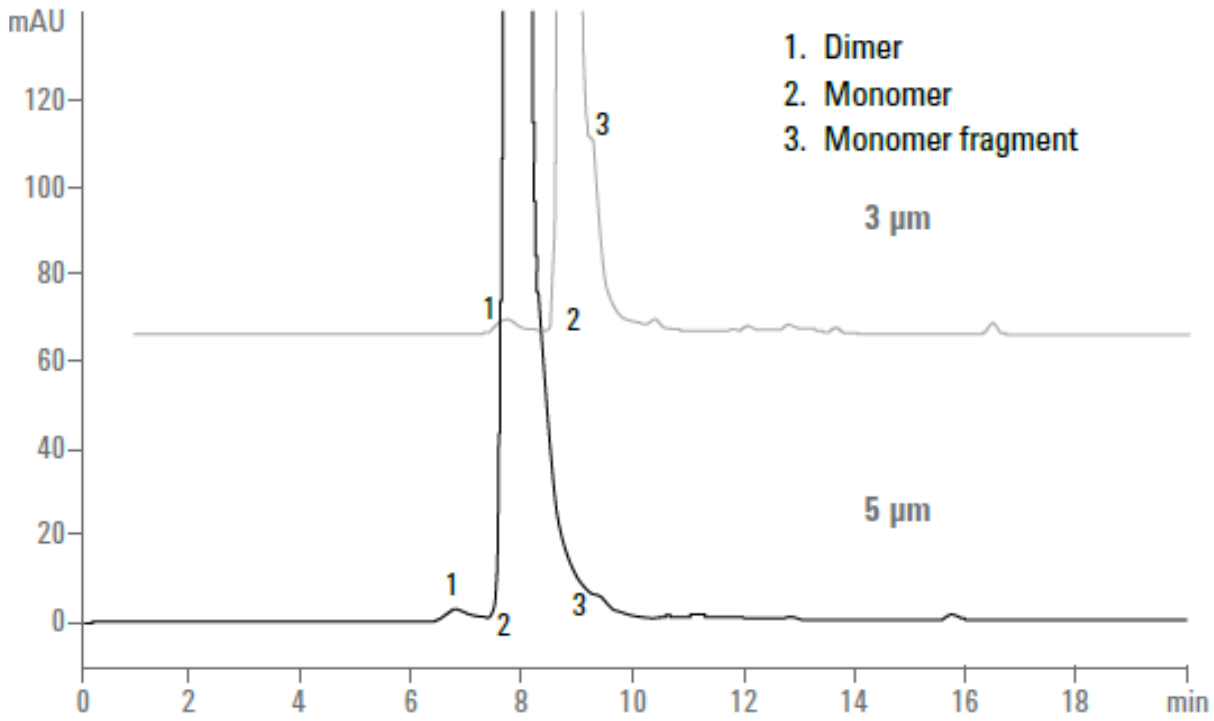
Column:	PLgel 100A 300x7.5mm
Eluent:	THF
Flow Rate:	1.0ml/min
Inj Vol:	20µl
Detector:	DRI



Comparison of 3um vs 5um:

Analysis of monoclonal antibody

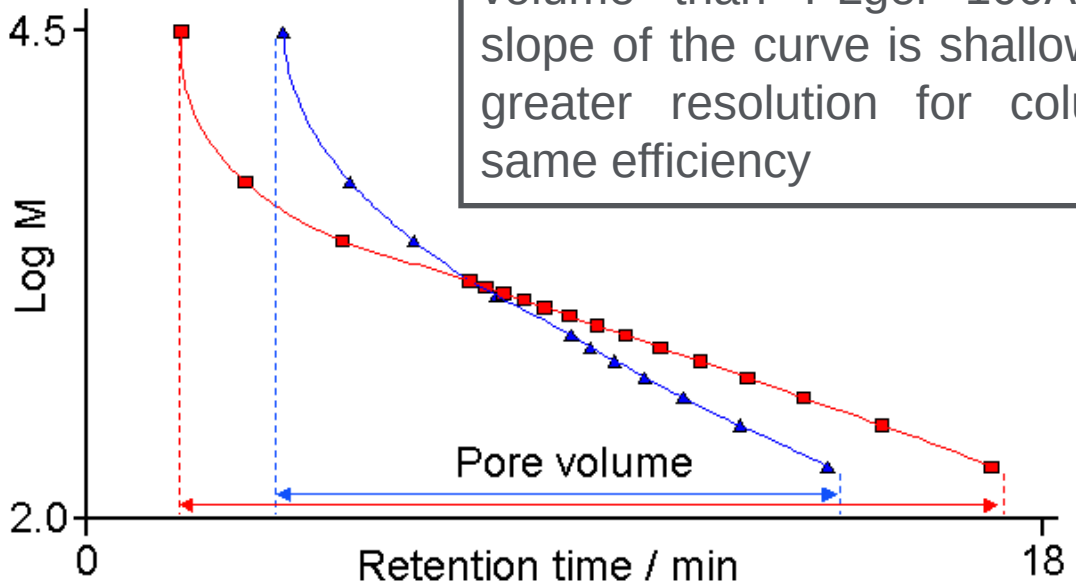
Column:	**Bio SEC-3, 300Å 7.8 x 300 mm, 3 µm (p/n 5190-2511)**
Column:	**Bio SEC-5, 300Å 7.8 x 300 mm, 5 µm (p/n 5190-2526)**
Instrument:	Agilent 1260 Infinity Bio-inert Quaternary LC System
Mobile phase:	150 mM sodium phosphate, pH 7
Flow rate:	1 mL/min
Detector:	UV, 220 nm
Sample:	Humanized monoclonal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



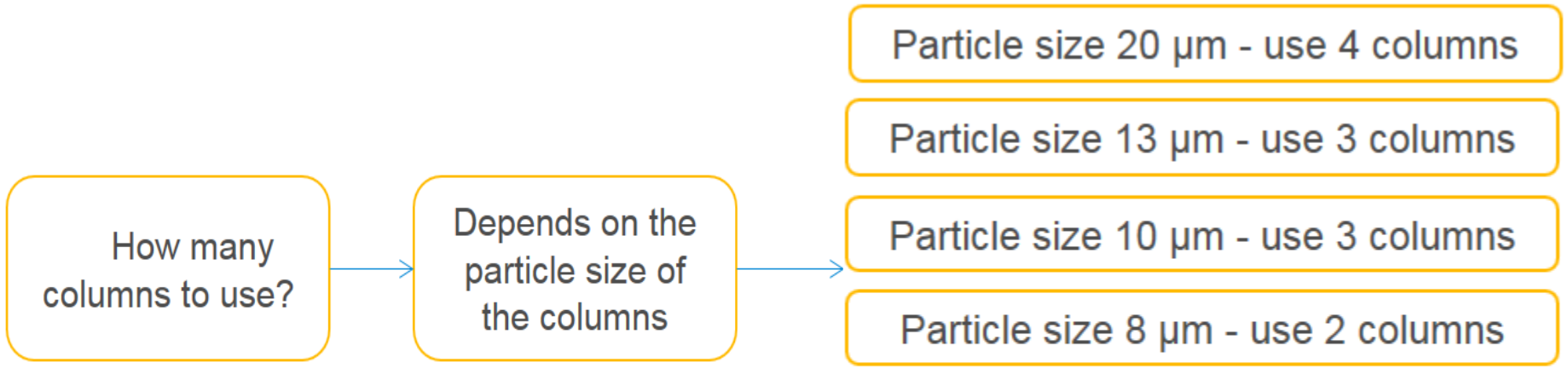
Effect of Increased Pore Volume

Columns	2xPLgel 3µm 100Å 300x7.5mm 2xOligoPore 300x7.5mm
Eluent	THF
Flow rate	1.0ml/min

Both columns have a similar exclusion limit but OligoPore has greater pore volume than PLgel 100Å. Hence the slope of the curve is shallower leading to greater resolution for columns of the same efficiency



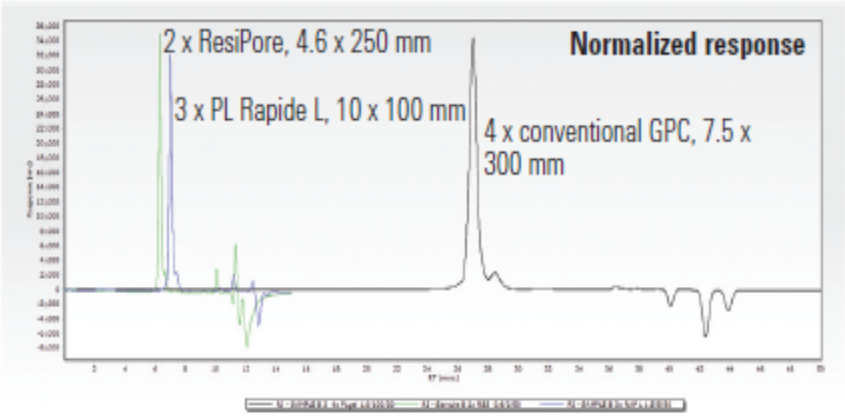
Guideline for # of columns to use:

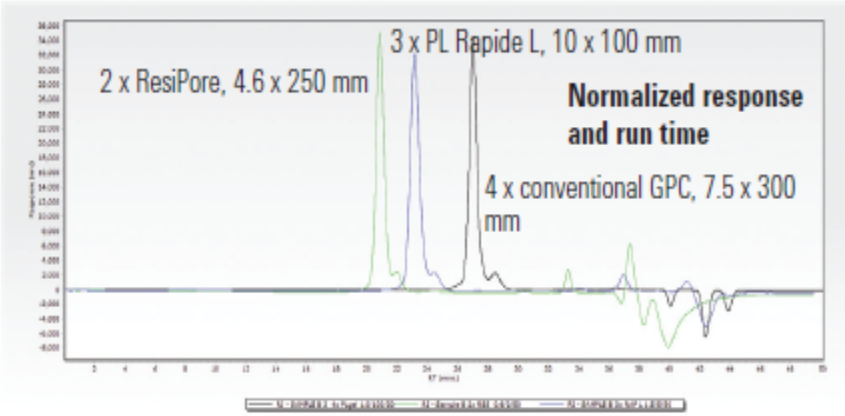


The greater the particle size of the media in the column (which is dependent on the expected mol. Wt of the samples), the lower the resolution and the more columns are required to maintain the quality of the results. For higher MW samples, larger particles are necessary to reduce the danger of shear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

Fast GPC : improving speed for analysis without sacrificing resolution

Comparison for Conventional Columns vs Cols for Fast GPC





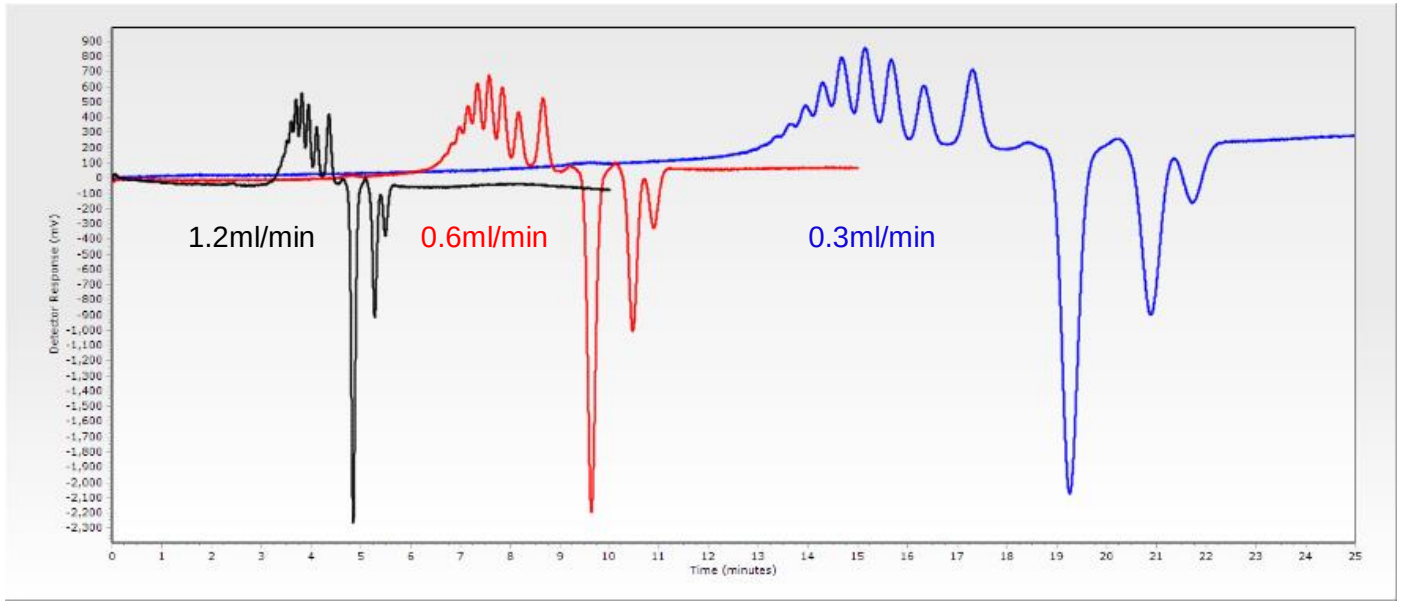
Throughput is increased by more than 3x

Columns	Peak 2 retention time (min)	Run time (min)
4 x conventional 7.5 x 300 mm	28.46	50
3 x PL Rapide L 10 x 100 mm	7.41	15
2 x ResiPore 4.6 x 250 mm	6.66	15

Without sacrificing separation quality

Columns	Resolution (Rs)	Selectivity (α)	Area %	Height %
4 x conventional 7.5 x 300 mm	1.2	1.05	8	7
3 x PL Rapide L 10 x 100 mm	1.1	1.06	7	7
2 x ResiPore 4.6 x 250 mm	1.1	1.05	8	8

Polystyrene Mw 580 – Oligopore 250x4.6mm

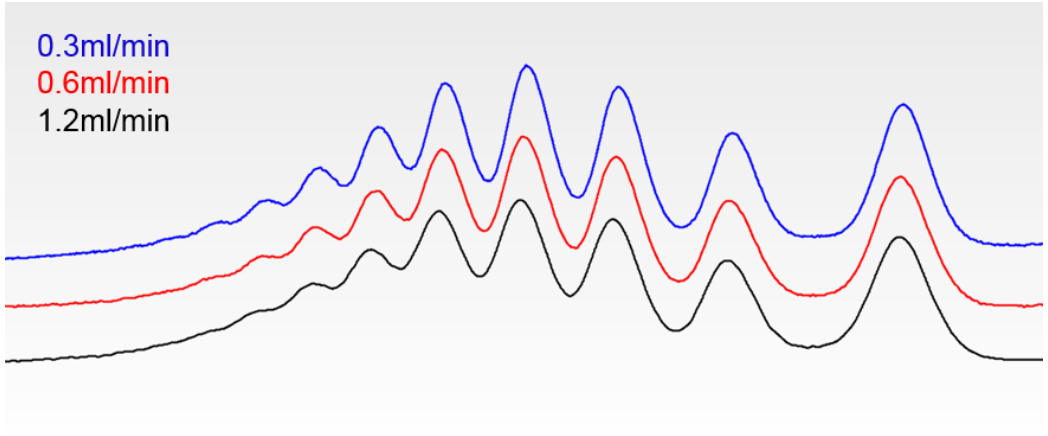


MW Range: up to 3,300 (g/mol)

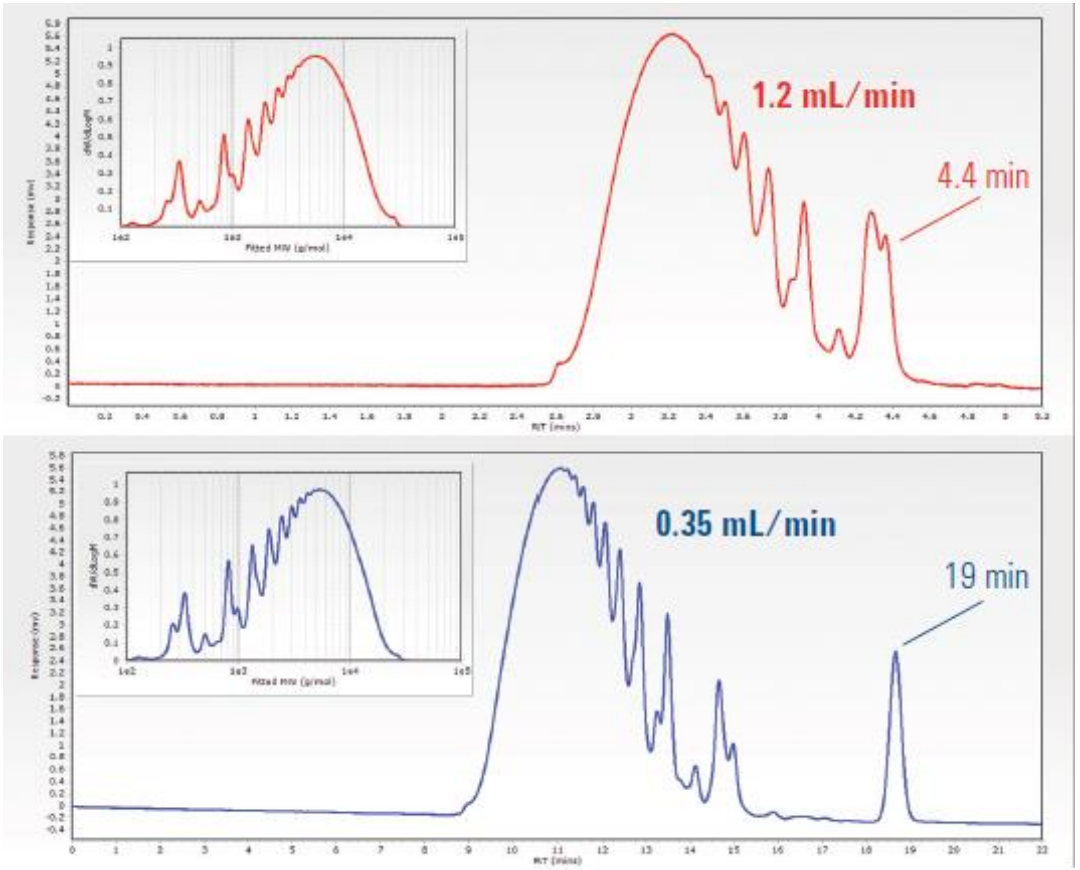
Nominal Particle Size: 6 µm

Typical Efficiency: >55,000 p/m

Different flow rates overlaid to show that faster doesn't sacrifice resolution. The chromatograms have been normalised to better illustrate the differences



High Speed MesoPore Columns



Conditions

Column:	2 x MesoPore, 4.6 x 250 mm (PL1513-5325)
Sample:	Epoxy resin
Eluent:	THF
Flow rate:	0.35 and 1.2 mL/min
Inj vol:	4 µL
System:	1260 Infinity GPC/SEC System, UV, 254 nm

Easy Method Transfer from Standard to rapid GPC on MesoPore 250x4.6mm GPC columns

MW Range: up to 25,000 (g/mol)

Nominal Particle Size: 3 µm

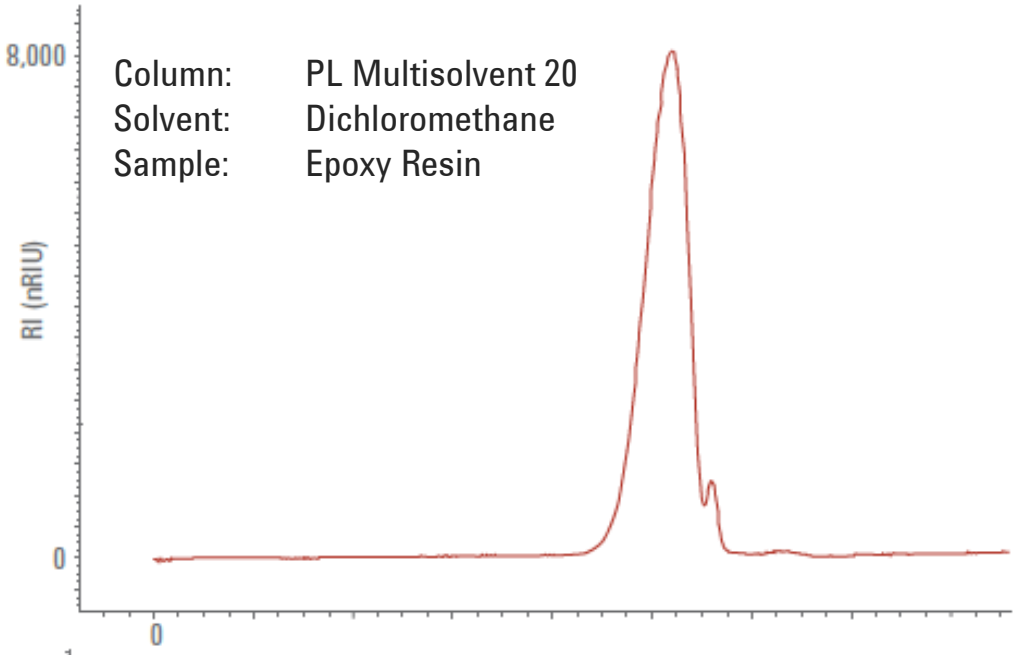
Typical Efficiency: >80,000 p/m

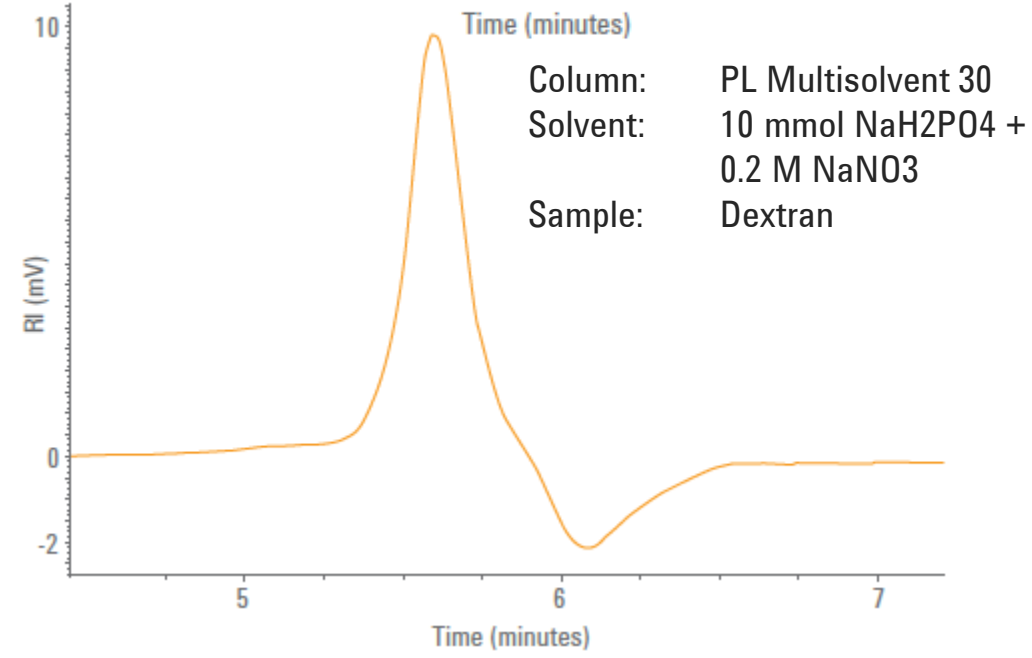
Column Family: **PL Multisolvent**

Description	MW range
Infintiy Lab PL MultiSolvent 20	**Up to 30K**
Infinity Lab PL MultiSolvent 30	**3K to 100K**

Available in:
4.6 & 7.8 mm ID
500mm & 150mm lengths

- Newest addition to Agilent's GPC/SEC line
- Rigid Silica backbone handles diverse solvents & solvent switching:
- ex: Buffer, Water, THF, Chloroform, Dichloromethane
- Small particle size, 2.7um allows for improved speed & resolution
- High efficiency silica core with inert polymeric coating – eliminates unwanted secondary interactions
- Ideal for mixed samples, new method development, and Fast GPC

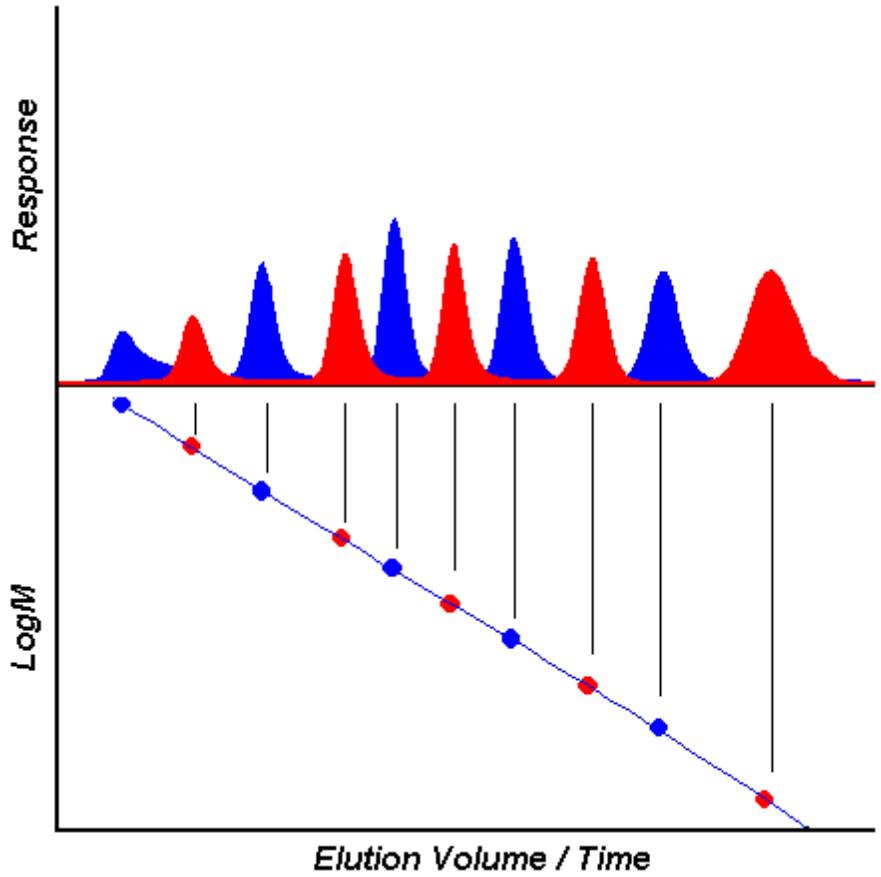




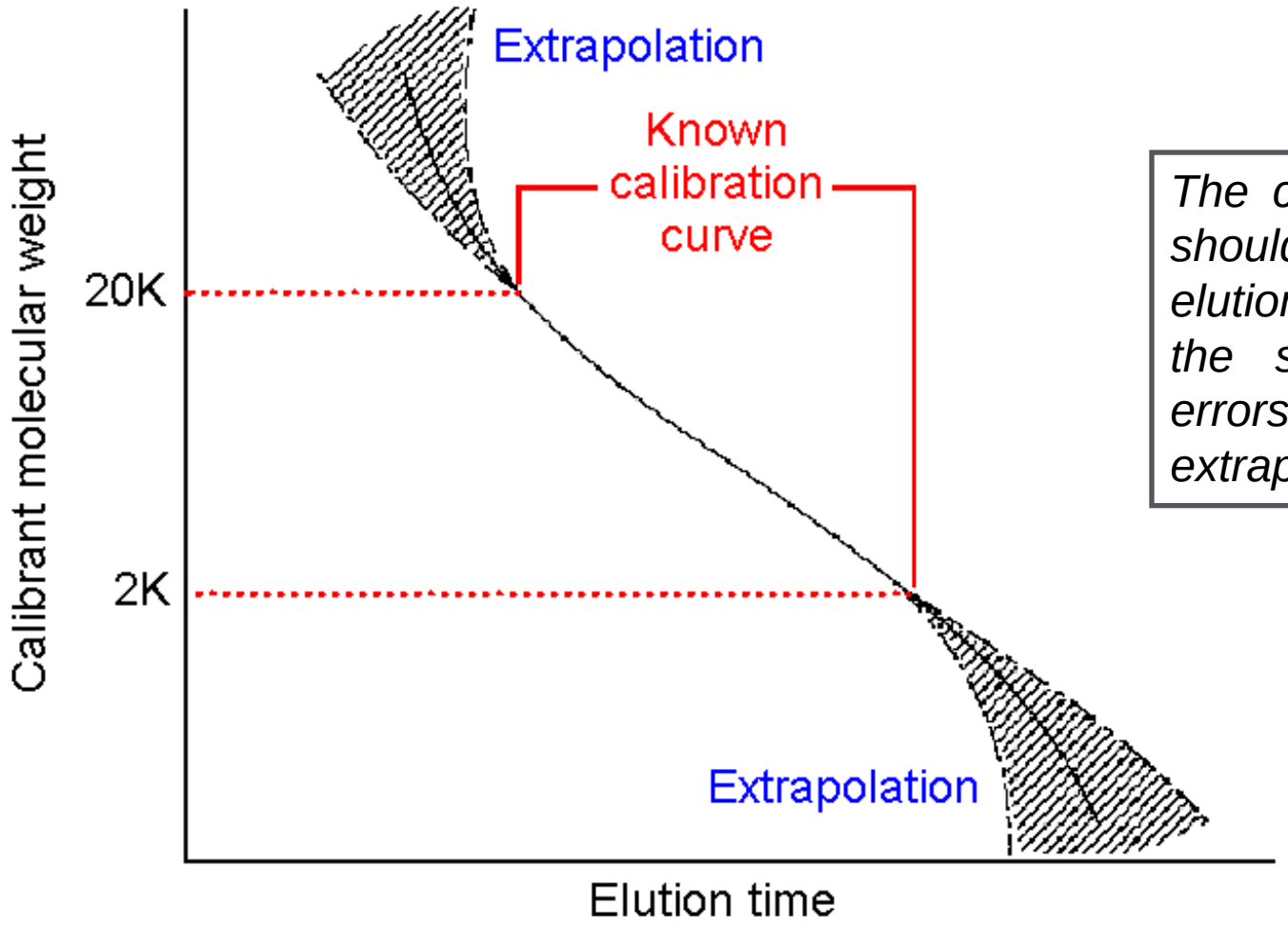
Calibration of GPC/SEC Columns

Conventional GPC:

- Chromatograph a series of well characterized, narrow polydispersity polymer standards
- Plot peak retention time (RT) versus peak log molecular weight (logM)
- Fit the data using a mathematical function (e.g. polynomial order 1,2,3, etc)
- The calibration curve will be characteristic of the GPC column set used

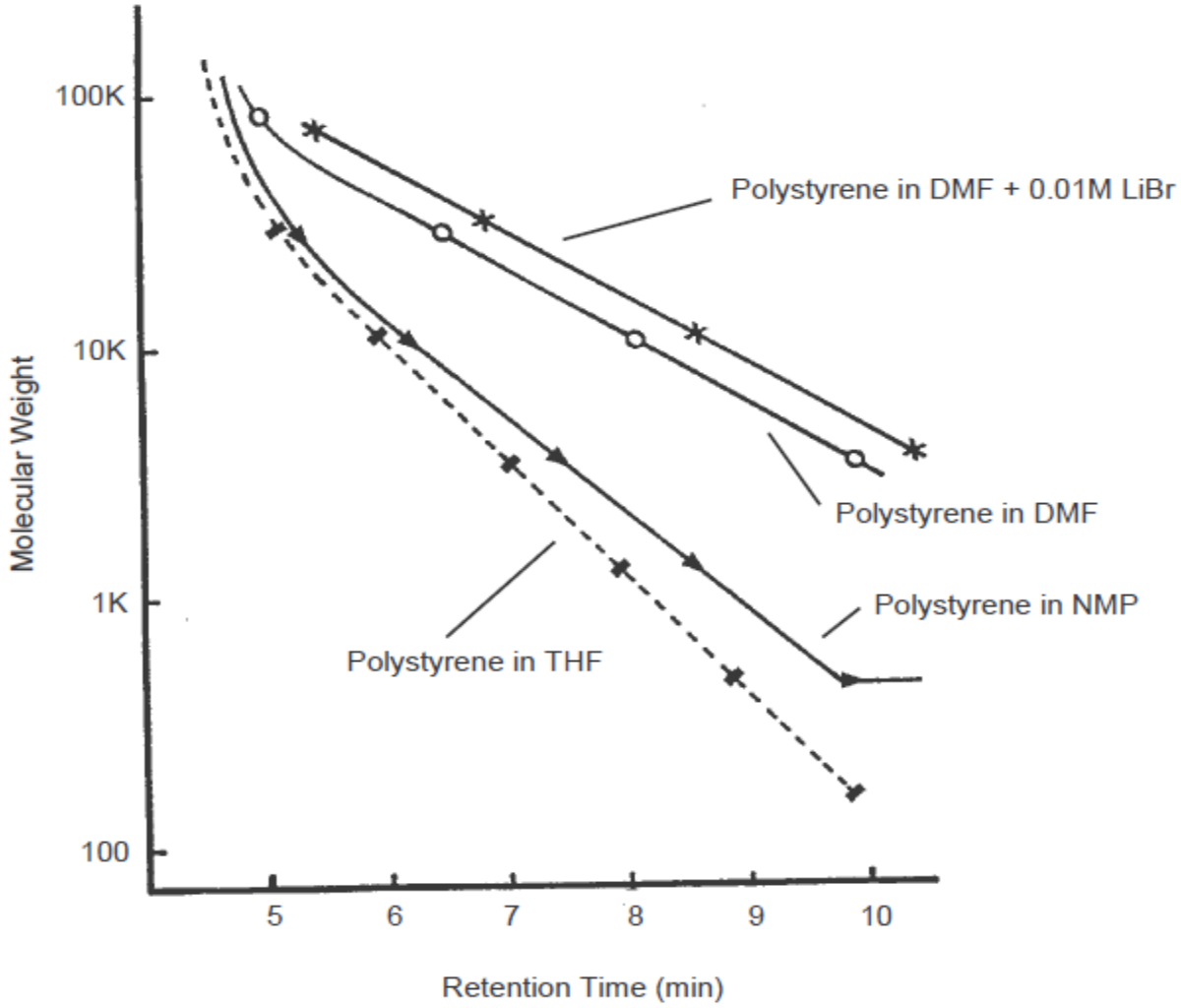


Errors Due to Limited Calibration Region



The column calibration should cover the full elution time region of the sample to avoid errors due to extrapolation

Importance of Choosing CORRECT Calibration Standards



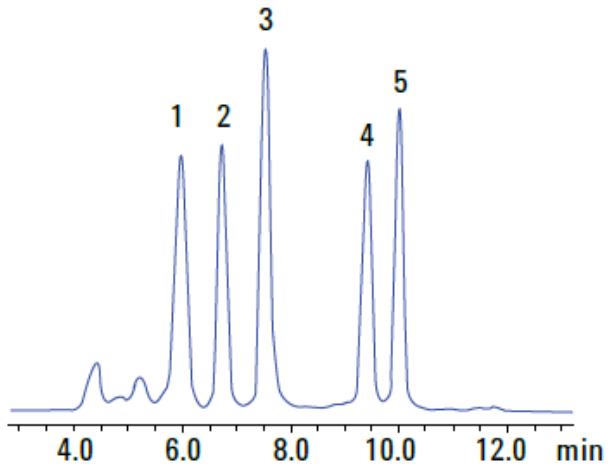
Calibration Standards – selecting the standard

Solvent Type	GPC/SEC Standards Type
Organic ex THF, toluene chloroform, MeCl	• Polystyrene (PS) • Polymethylmethacrylate (PM)
Mixed or Polar Organic ex DMF, DMAc, DMSO, NMP	• Polymethylmethacrylate (PM) • Polyethylene glycol/oxide (PEG/PEO)
Aqueous ex water, buffer	• Polyethylene glycol/oxide (PEG/PEO) • Polysaccharide (SAC) • Polyacrylic acid (PAA)

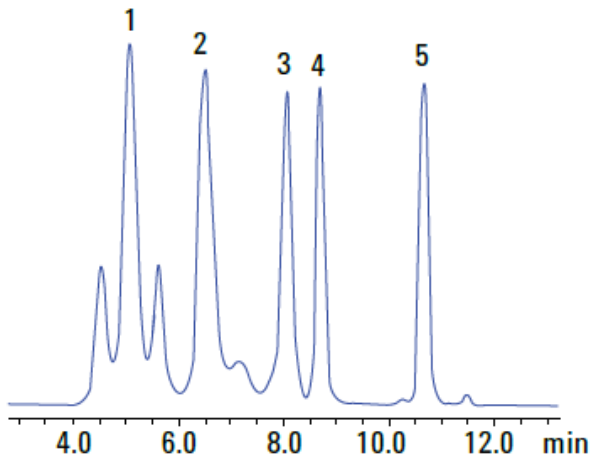
What TYPE of kits best suits my needs?

- EasiVial – pre-prepared for fast and easy, accurate concentration, 12-point column calibration for organic and aqueous solvents
- EasiCal – easy 3-step process for accurate 10-point calibration, for organic solvents
- Calibration kits and individual standards – Polystyrene, PMMA, PEG/PEO, PAA, Polysaccharide

AdvanceBio SEC Protein Standards:



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



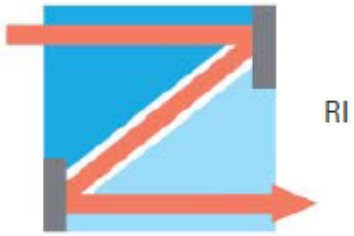
AdvanceBio SEC 300Å Protein Standard separation on AdvanceBio SEC 300Å column

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

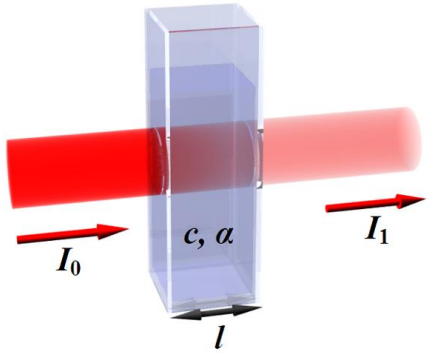
AdvanceBio SEC 300Å Protein Standard (p/n 5190-9417, 1.5 mL vial)	
Analyte	**MW**
1. Thyroglobulin	670,000
2. γ-globulin	150,000
3. Ovalbumin	45,000
4. Myoglobin	17,000
5. Angiotensin II	1,000

Detection
Concentration Detectors

- Most common detectors for GPC/SEC are *concentration* detectors:



RID



UV/DAD

ELSD

- These provide information on the amount of polymer eluting from the column at any given time

Advanced Detection
Molecular Weight Sensitive Detectors

- Viscometer detector
  - Response proportional to the intrinsic viscosity (IV) of the polymer
  - Generate accurate molecular weight for polymers using the *Universal Calibration* principle
  - Determination of branching
  - Conformation & size of polymer

- Dual angle light scattering (LS) detector
  - Response directly proportional to molecular weight (Mw) of the polymer – 'Absolute Molecular Weight'
  - Scattered light measured at more than one angle permits determination of Radius of gyration (Rg)
  - Determination of branching
  - No column calibration required

Expanding Conventional GPC/SEC

add Viscometer and Light Scattering Detectors

Advanced detectors give a greater understanding of the analyte as well as overcoming the limitations of Conventional GPC.

GPC/SEC Technique	Molecular Weight	Molecular Size	Information
Conventional (RI or UV)	Relative to standards used for calibration	No	Molecular weight distribution, concentration
Viscometry	More accurate from Universal Calibration	Yes, hydrodynamic radius (**Rh).**	Conformation, branching. Works with copolymers
Light Scattering	Absolute determination	Yes, Radius of Gyration (Rg) directly.	Conformation, branching.
Triple	Absolute determination	Yes, Rg and Rh, directly.	The ultimate configuration for comprehensive polymer characterisation

Summary

Sample	What is the type of sample -> polymer or biomolecule
Solvent	Is my sample organic soluble, aqueous soluble & is the solvent appropriate for use with my columns, calibrants, and detection
GPC/SEC Column Selection	Columns must be appropriate for both the type of sample & for the solvent being used. Choose correct pore size for MW range needed. Consider KEY Requirements for the method: reproducibility, resolution, speed
Calibrants	Choose calibrants appropriate for solvent, sample, & column. Calibrants range should cover the full MW range for the column or column set being used.
Detectors	RI, UV, ELSD; concentration detectors & most commonly used for conventional GPC/SEC Advanced detectors: LS, Viscometry & Triple detection for maximum sample MW & size/conformation information

THANK YOU FOR ATTENDING

ANY QUESTIONS??

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

*available 8am – 5pm EST – PST in US and Canada

- gc-column-support@agilent.com
- lc-column-support@agilent.com
- spp-support@agilent.com
- spectro-supplies-support@agilent.com

Community @ Agilent.com -> Resources for Columns & Consumables

Customers can visit here for
Agilent Website pages for best collection of 'links' for Columns, Supplies, and Standards:

https://community.agilent.com/docs/DOC-1952-collection-of-consumables-resources

Agilent GPC/SEC Product webpages:

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

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