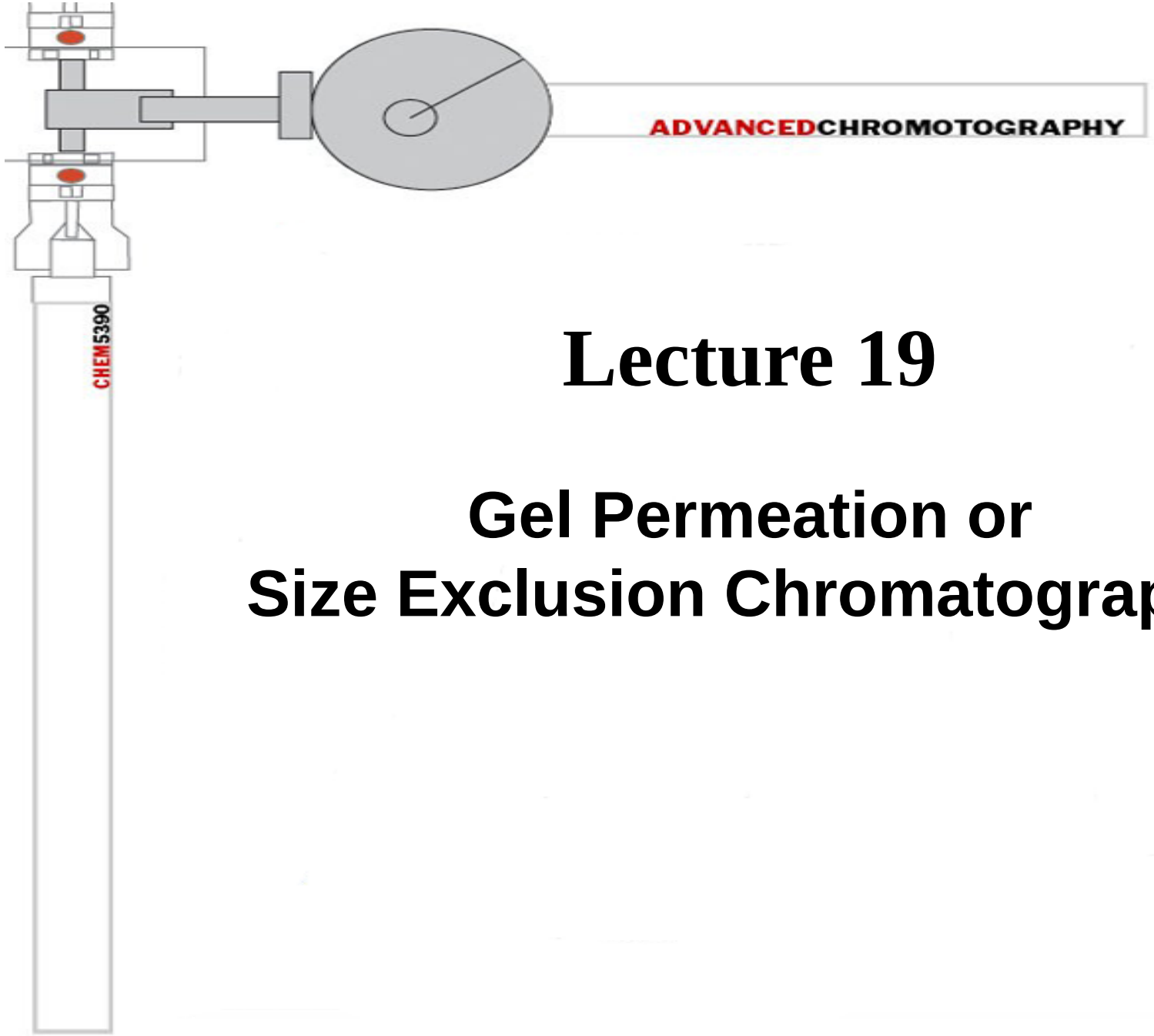
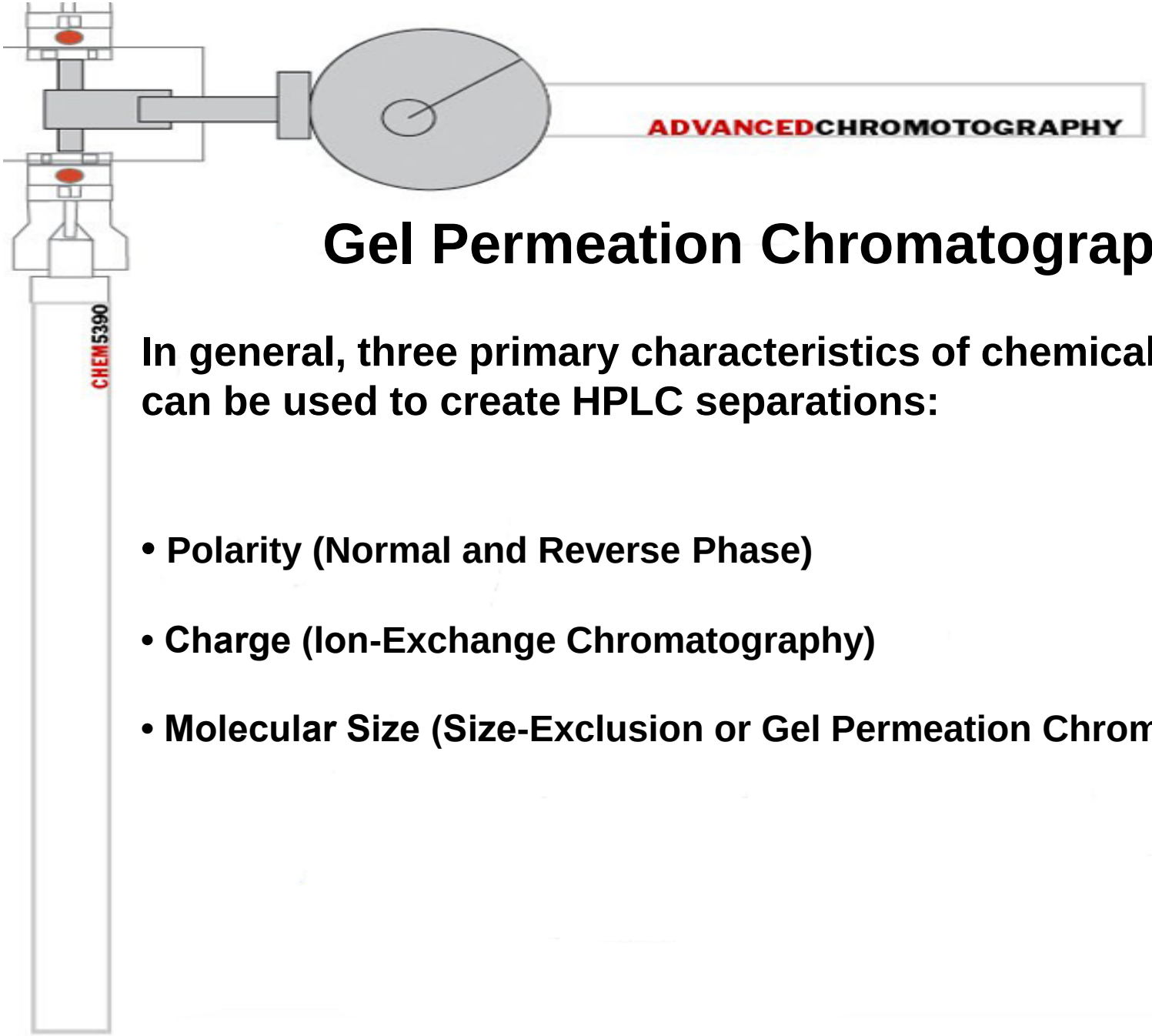


Lecture 19

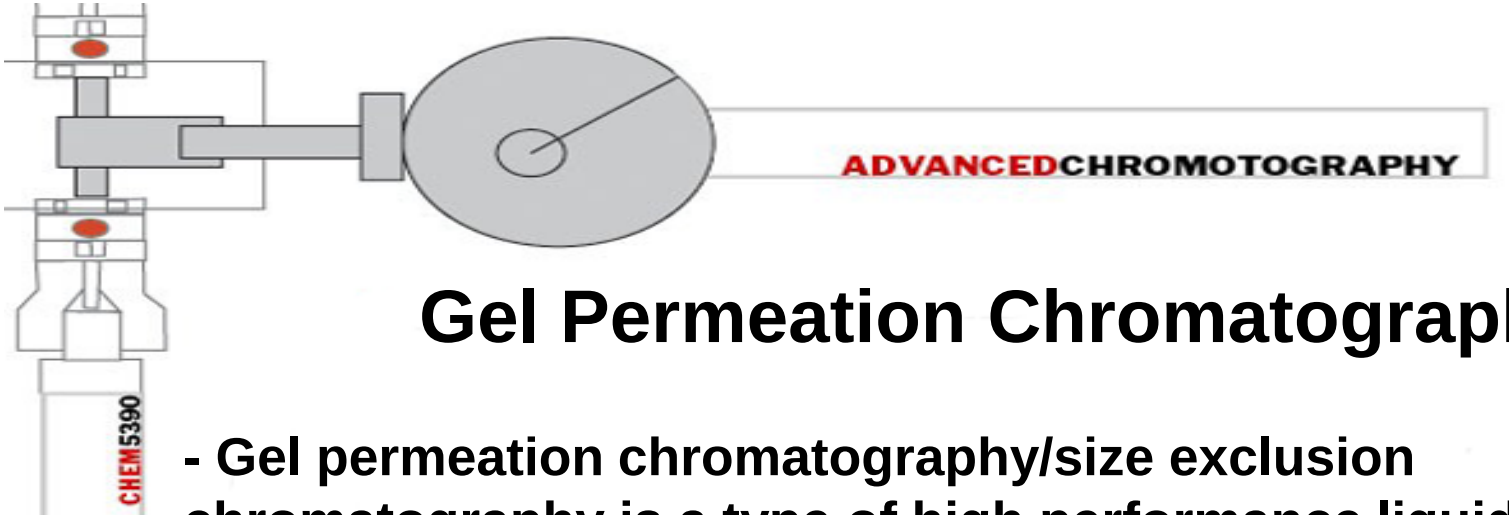
Gel Permeation or
Size Exclusion Chromatography



Gel Permeation Chromatography

In general, three primary characteristics of chemical compounds can be used to create HPLC separations:

- **Polarity (Normal and Reverse Phase)**
- **Charge (Ion-Exchange Chromatography)**
- **Molecular Size (Size-Exclusion or Gel Permeation Chromatography)**



Gel Permeation Chromatography

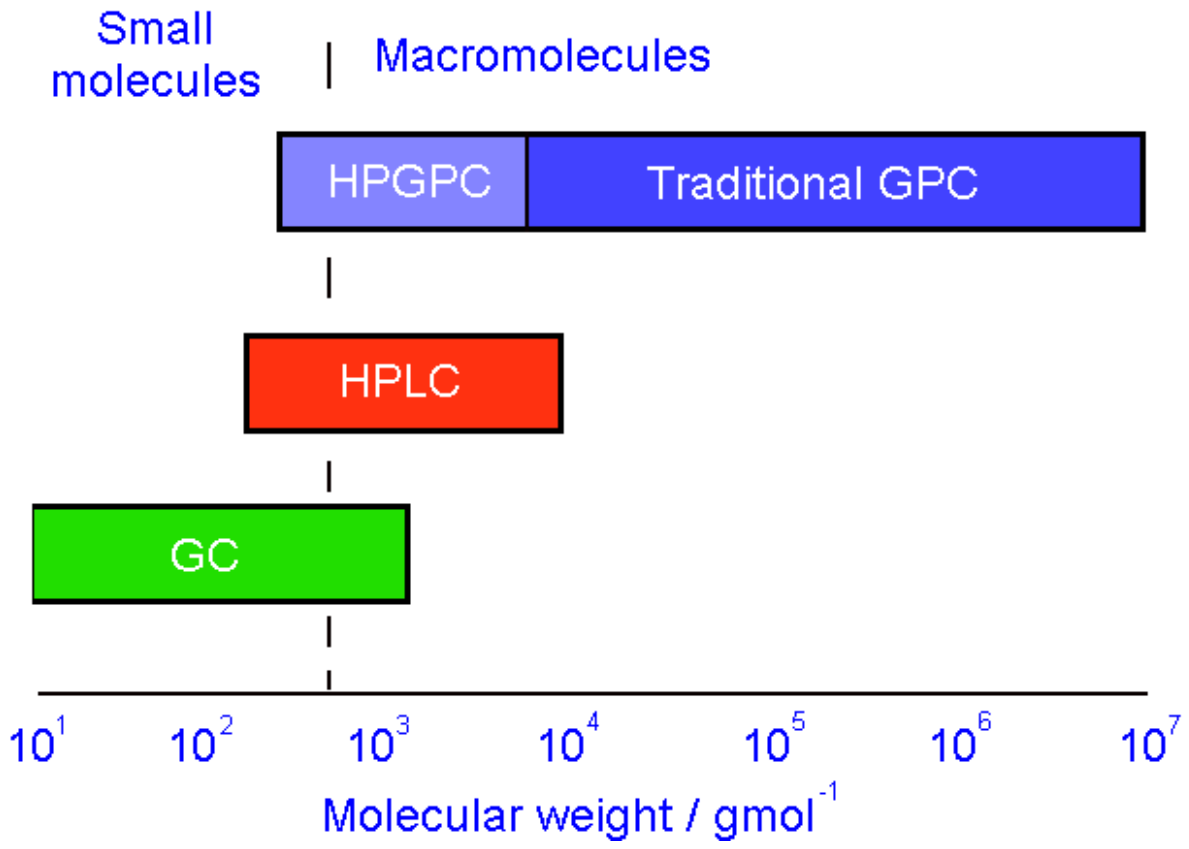
- Gel permeation chromatography/size exclusion chromatography is a type of high performance liquid chromatography (LC).

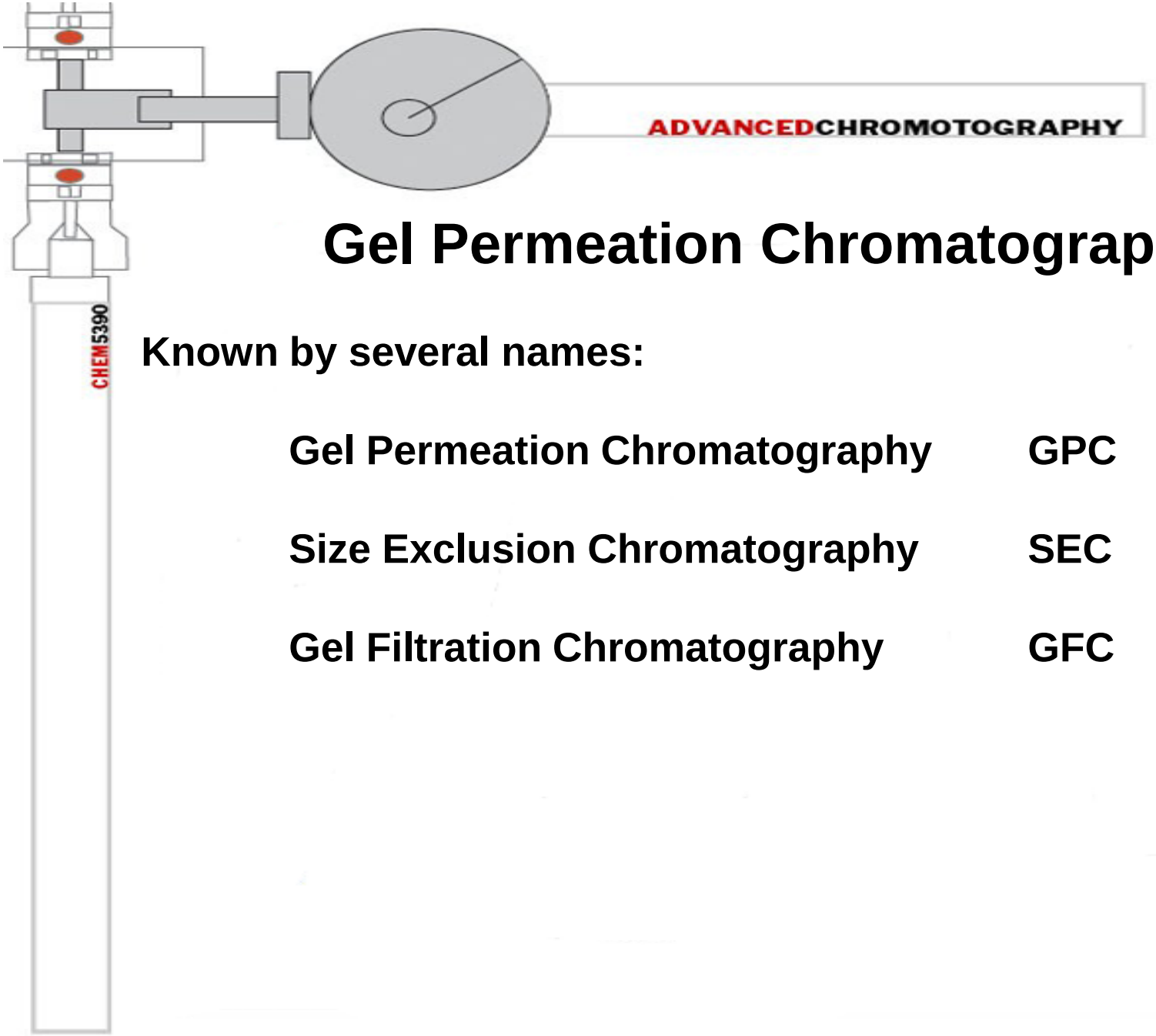
-GPC/SEC separates molecules on the basis of their size, hence ‘size exclusion’.

-The first GPC/SEC columns were packed with materials referred to as gels, hence ‘gel permeation’.

-GPC/SEC is used to determine the molecular weight distributions of polymers.

Gel Permeation Chromatography

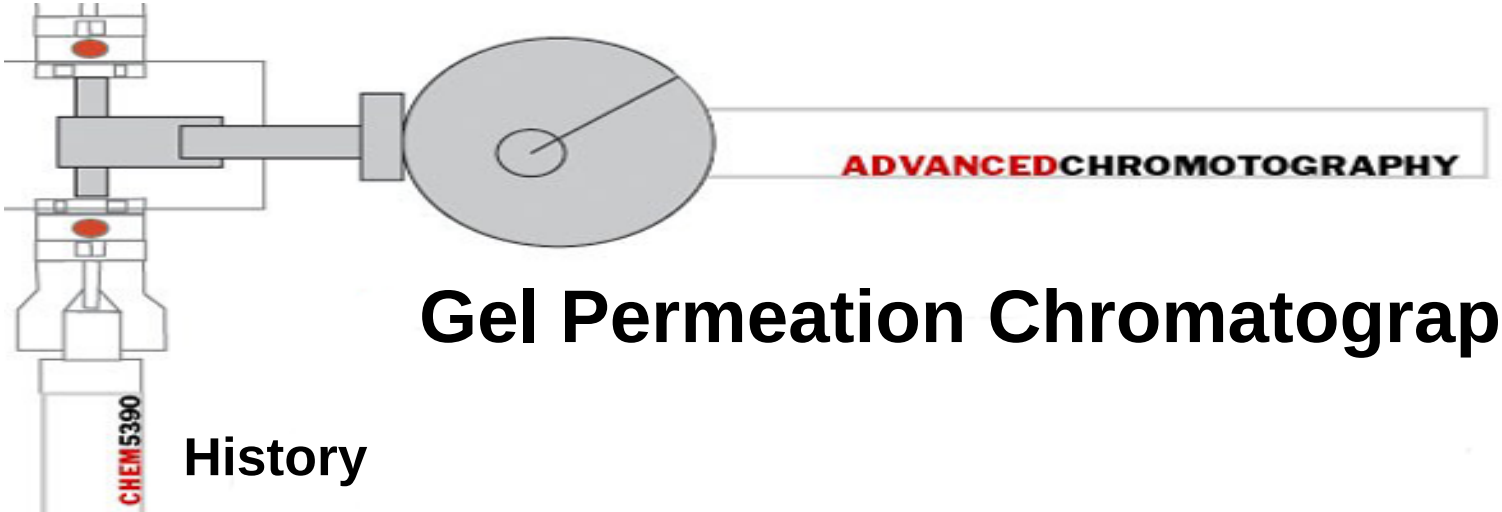




Gel Permeation Chromatography

Known by several names:

Gel Permeation Chromatography	**GPC**
Size Exclusion Chromatography	**SEC**
Gel Filtration Chromatography	**GFC**

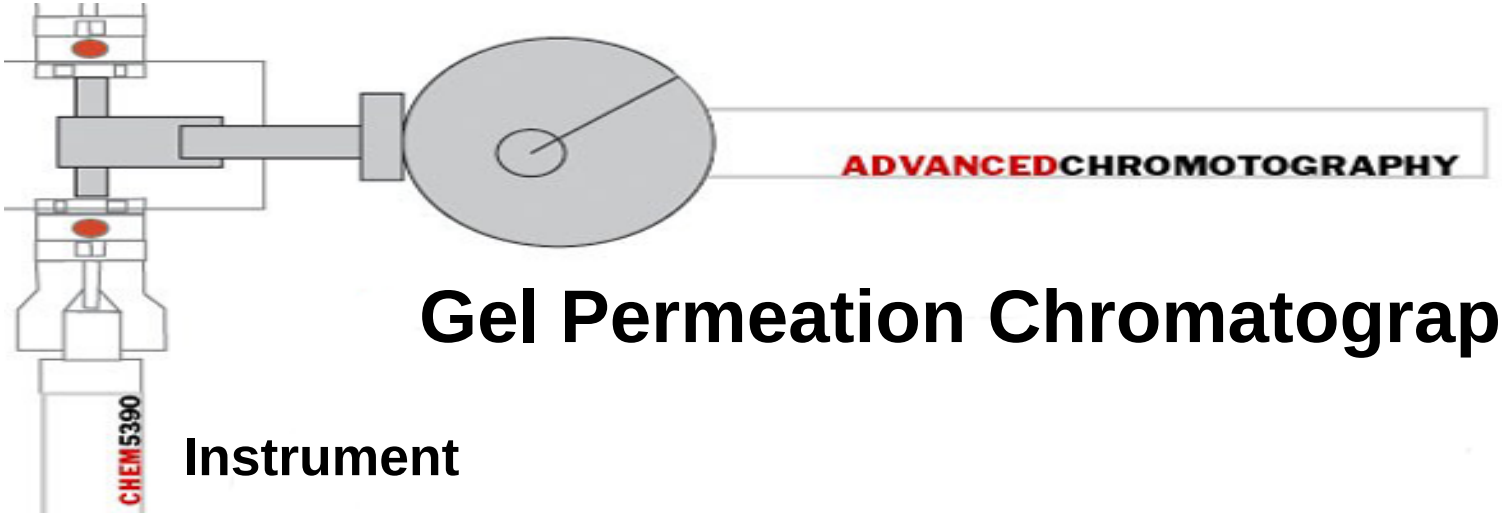


Gel Permeation Chromatography

History

In the 1950s, Porath and Flodin discovered that biomolecules could be separated based on their size, rather than on their charge or polarity, by passing, or filtering, them through a controlled-porosity, hydrophilic dextran polymer.

Introduced in 1963, the first commercial HPLC instruments were designed for GPC applications.



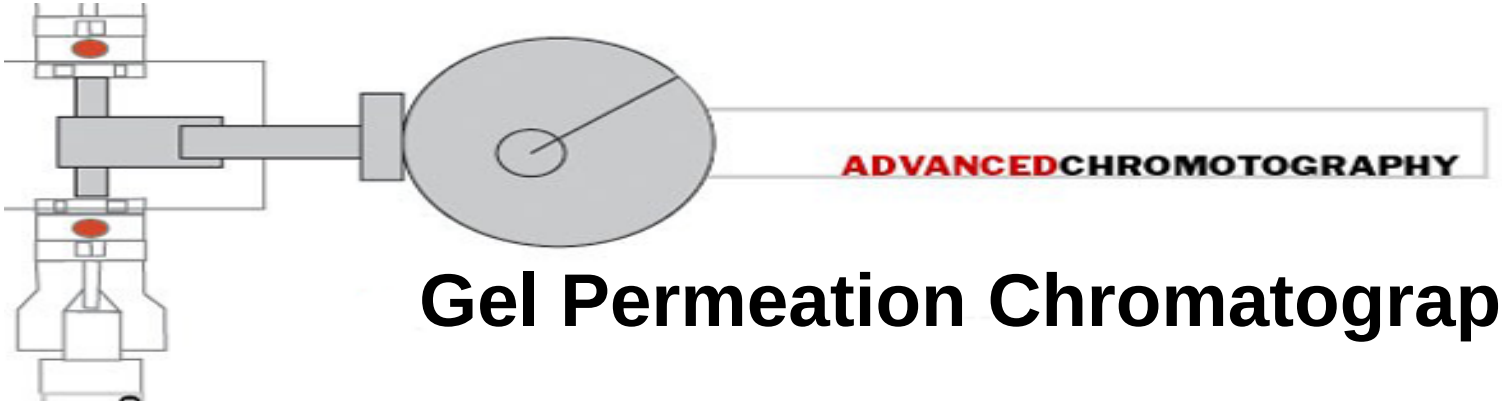
Gel Permeation Chromatography

Instrument

A GPC/SEC instrument consists of:

- **mobile phase container**
- **pump**
- **injection port**
- **column/stationary phase (sometimes in an oven)**
- **one or more detectors**

So similar instrumentation as an HPLC.



Gel Permeation Chromatography

Instrument

Column/stationary phase (sometimes in an oven)

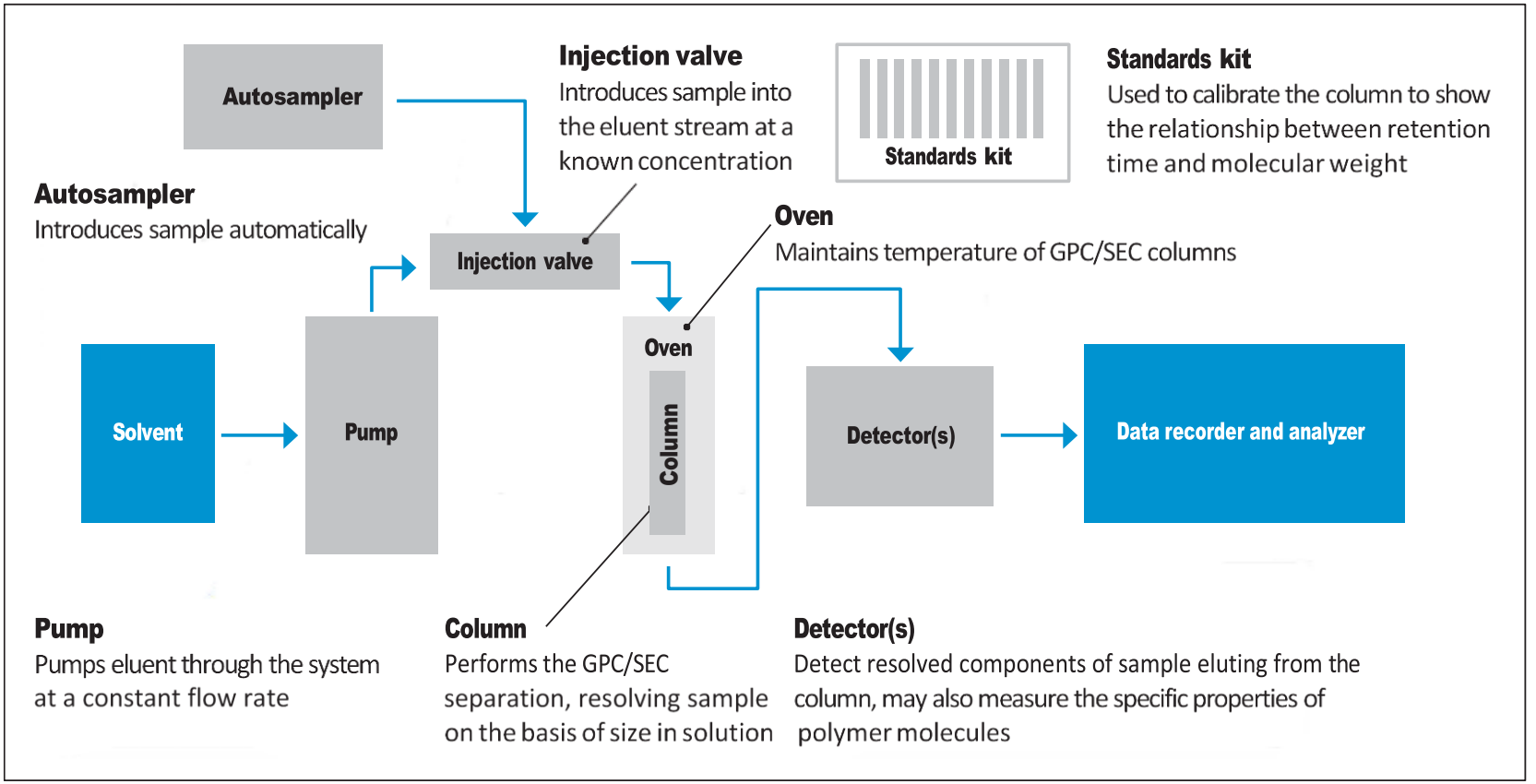
Sometimes the column temperature must be higher than room temp and controlled using a column oven typically up to 220 ^{o}C:

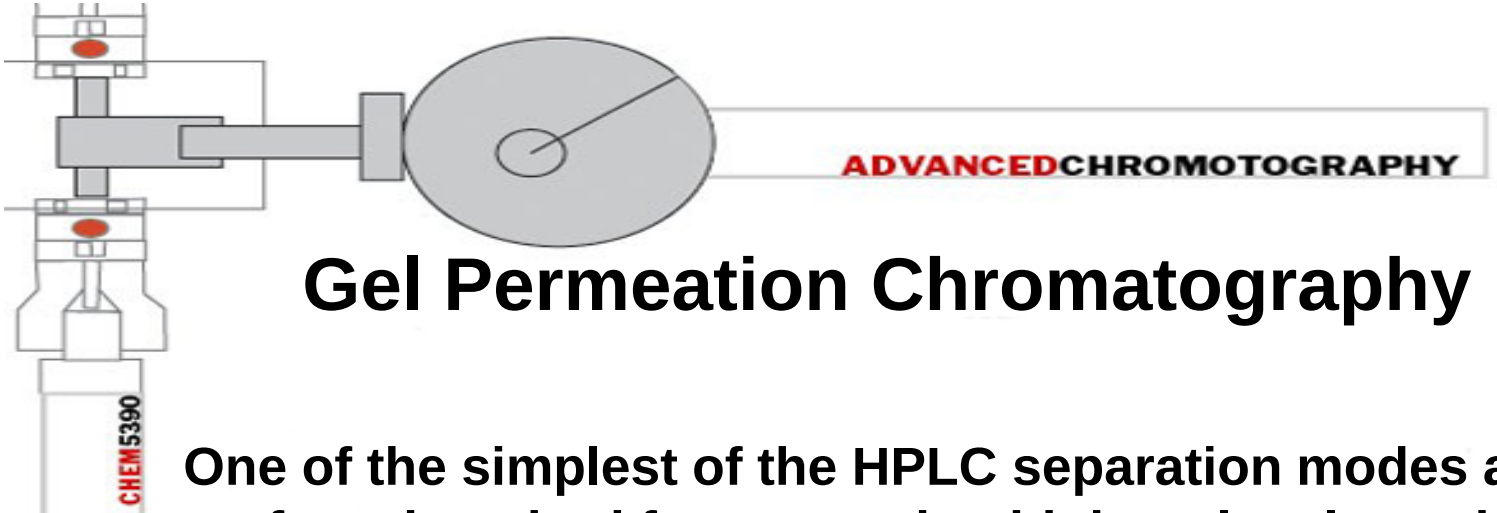
- Higher temperatures, up to 220 °C, are necessary for some solvents that have much higher viscosities, such as trichlorobenzene or chloronaphthalene, than more usual organic solvents like tetrahydrofuran, chloroform or toluene. Operating the instrument at high temperatures reduces viscosity and hence column back pressure, with a corresponding increase in efficiency.

- Some samples are also insoluble at lower temperatures; in these cases high temperature is required to maintain the solubility of the sample during analysis.

Gel Permeation Chromatography

Instrument



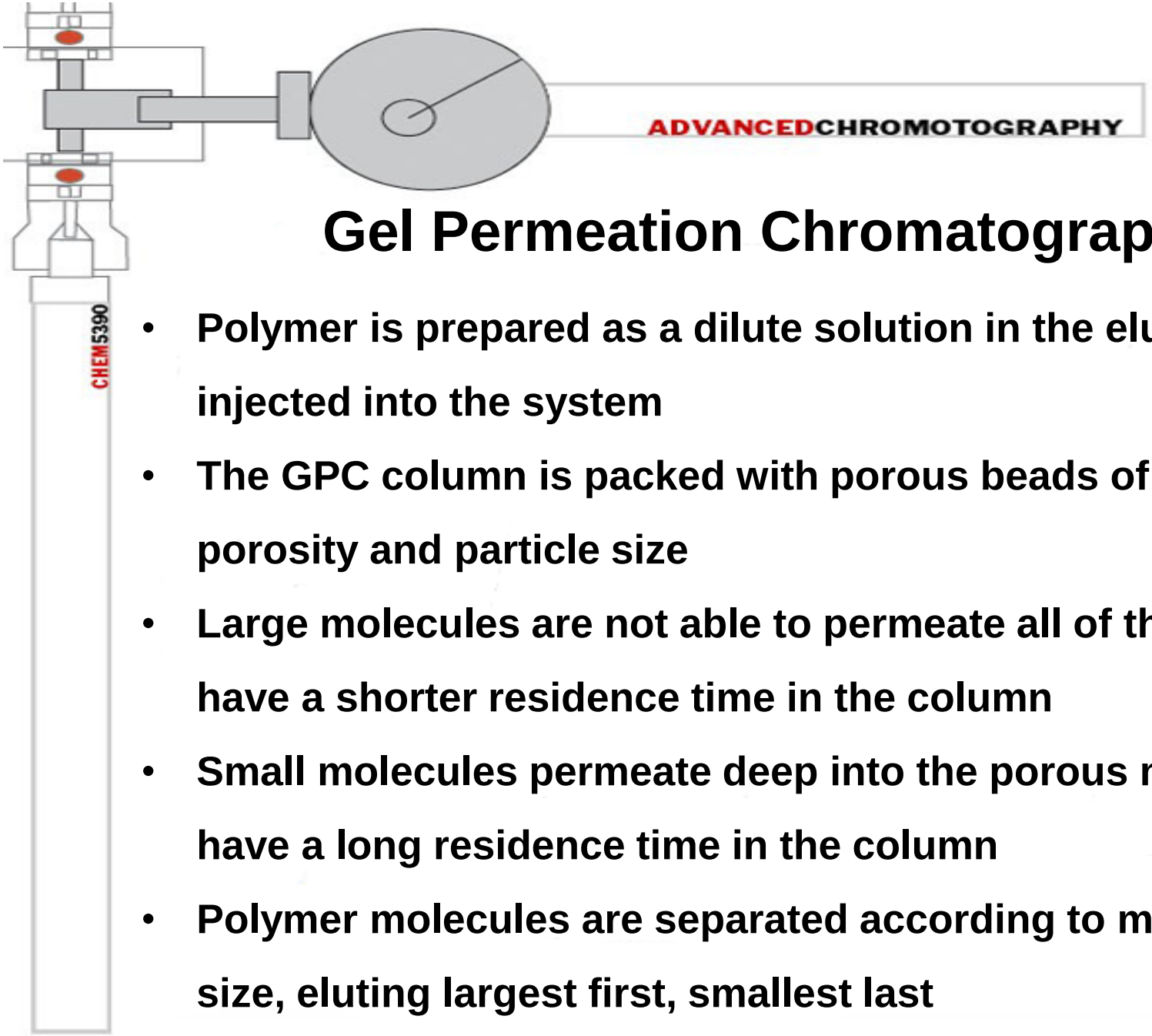


Gel Permeation Chromatography (GPC)

One of the simplest of the HPLC separation modes and is the preferred method for separating high molecular weight (>2000-3000) neutral solutes.

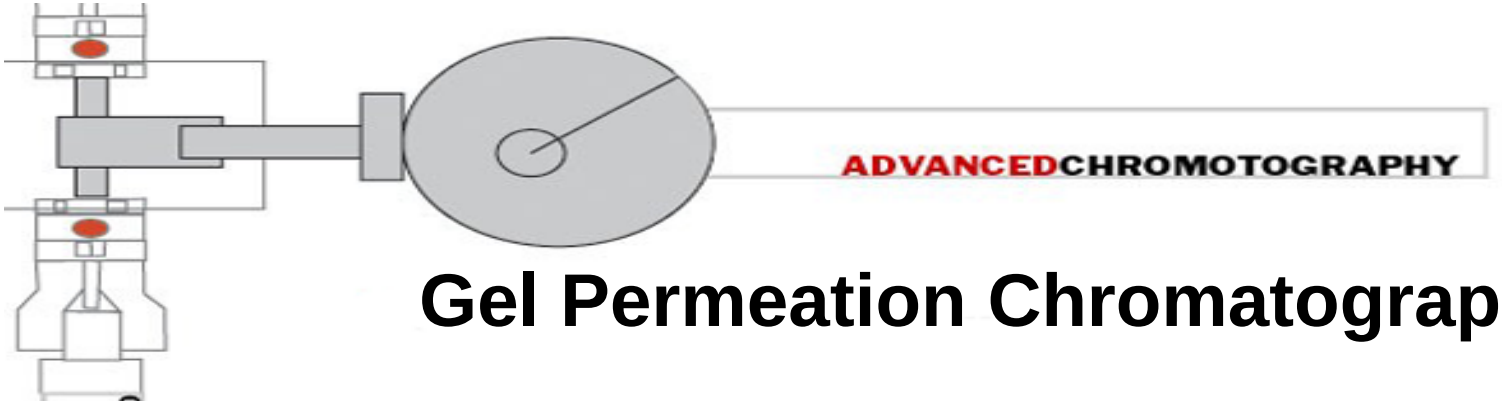
Uses size exclusion for separation. The stationary phase contains solvent filled pores of certain sizes that allow smaller molecules to enter while excluding larger molecules.

Technique is mostly used to characterize the MW distribution of polymer materials or separation of high MW weight proteins.



Gel Permeation Chromatography

- Polymer is prepared as a dilute solution in the eluent and injected into the system
- The GPC column is packed with porous beads of controlled porosity and particle size
- 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
- Polymer molecules are separated according to molecular size, eluting largest first, smallest last



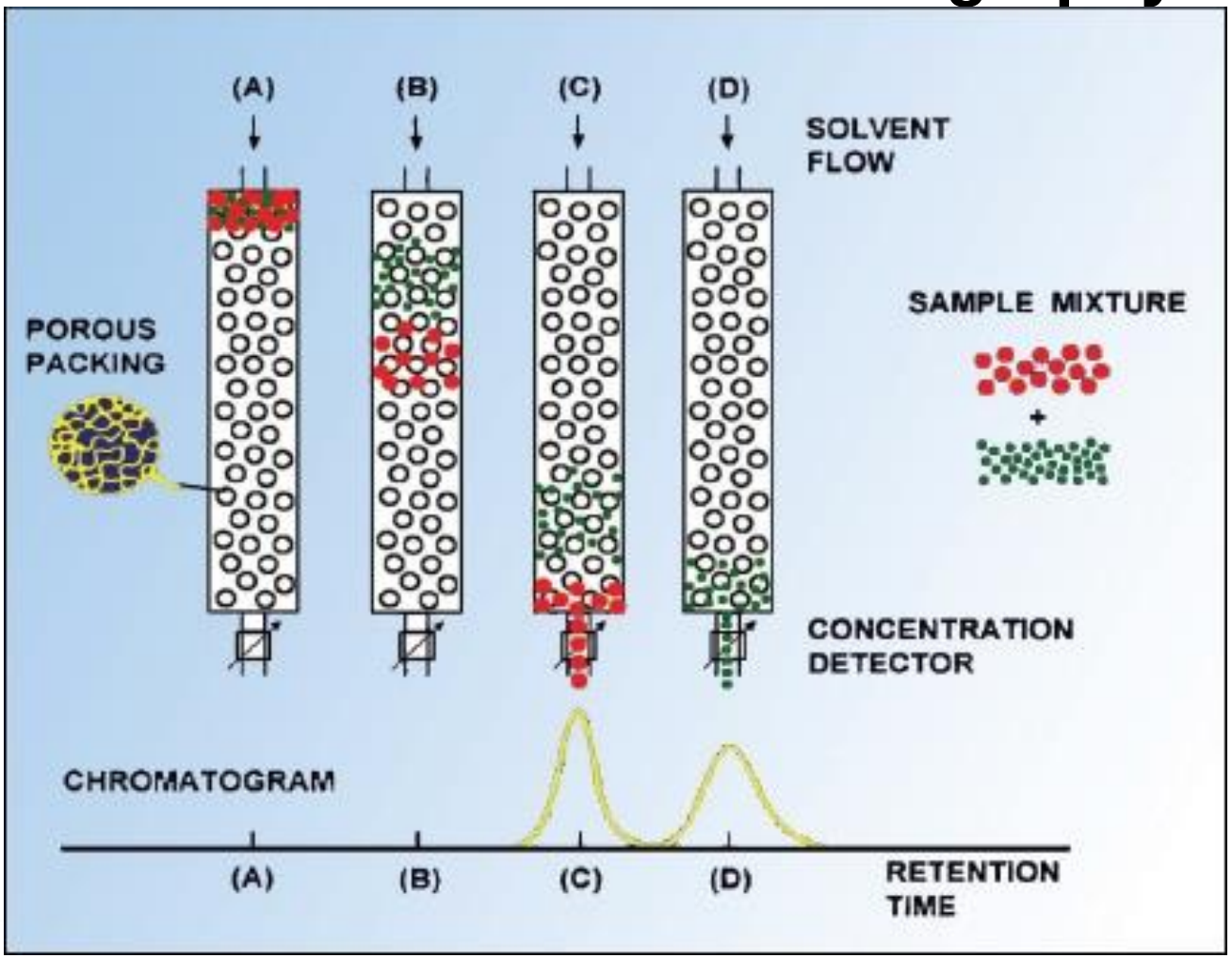
Gel Permeation Chromatography

Sample Preparation is very important.

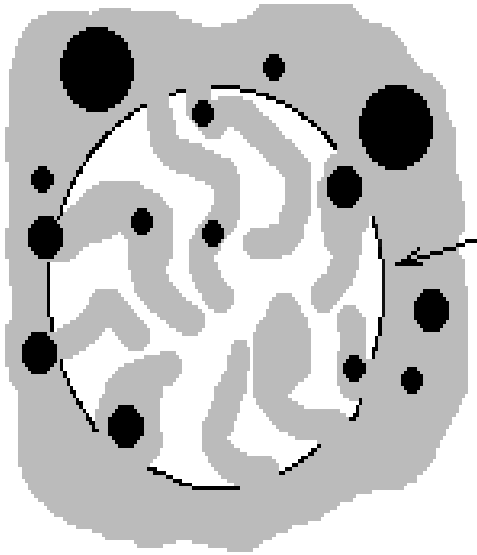
- **To prepare a sample for analysis it is first dissolved in an appropriate solvent, such as tetrahydrofuran (THF) for organic GPC or water based buffers for aqueous SEC.**

- **Since the separation obtained depends on the size of the sample molecules, it is important that they are allowed to swell and then fully dissolve in the solvent before being put through the chromatograph, which may take up to 12-24 hours.**

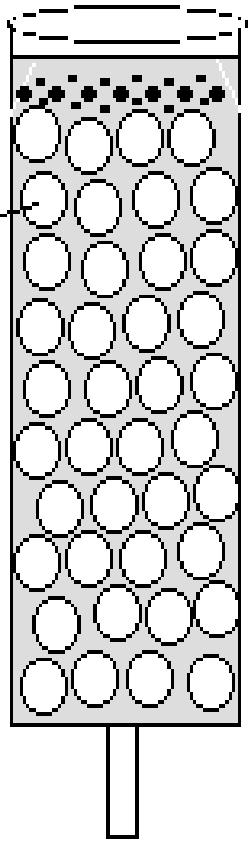
Gel Permeation Chromatography

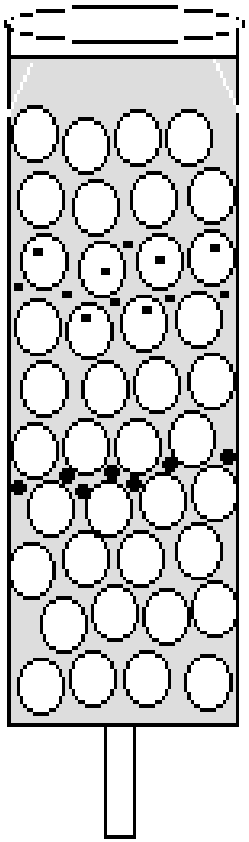


Gel Permeation Chromatography



Gel beads have pores in them of a defined size range which allows smaller molecules to enter but excludes molecules larger than the pore diameters.

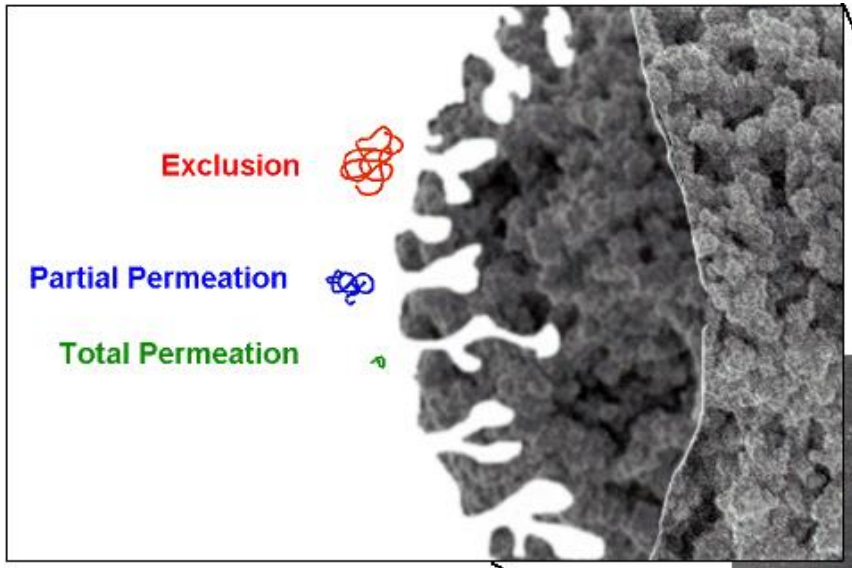


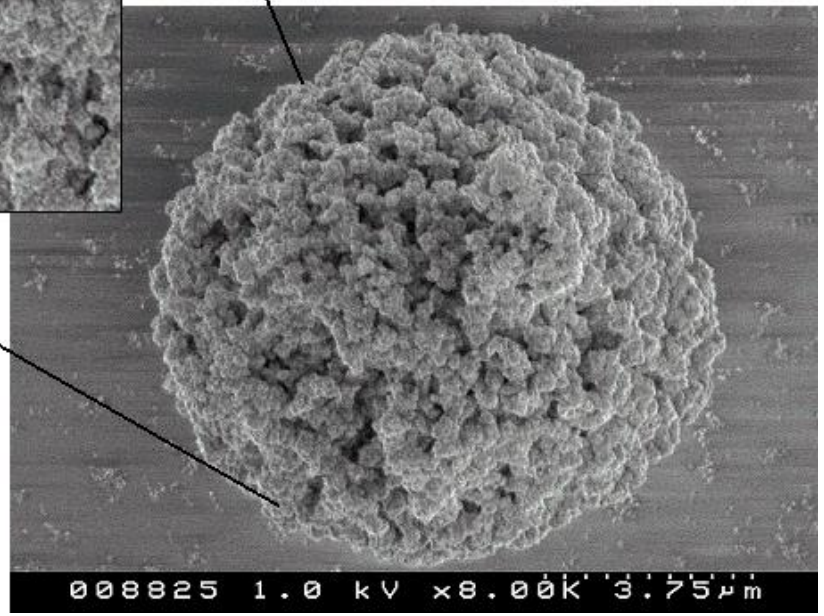


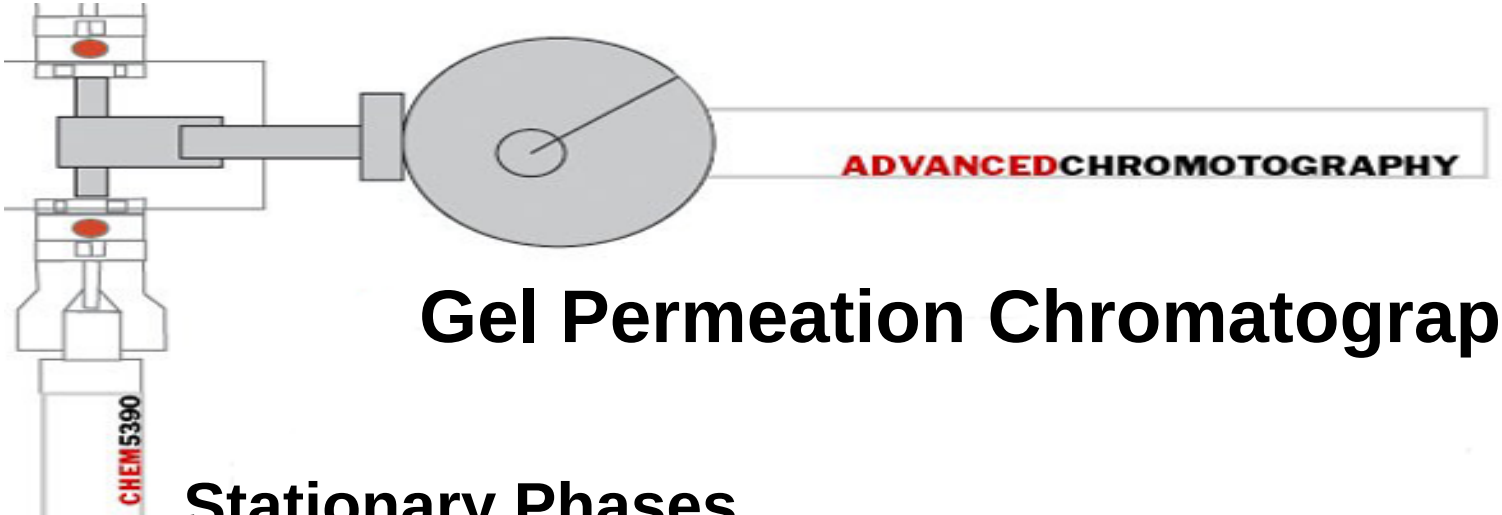
Molecules smaller than gel bead pores

Molecules larger than gel bead pores

Gel Permeation Chromatography





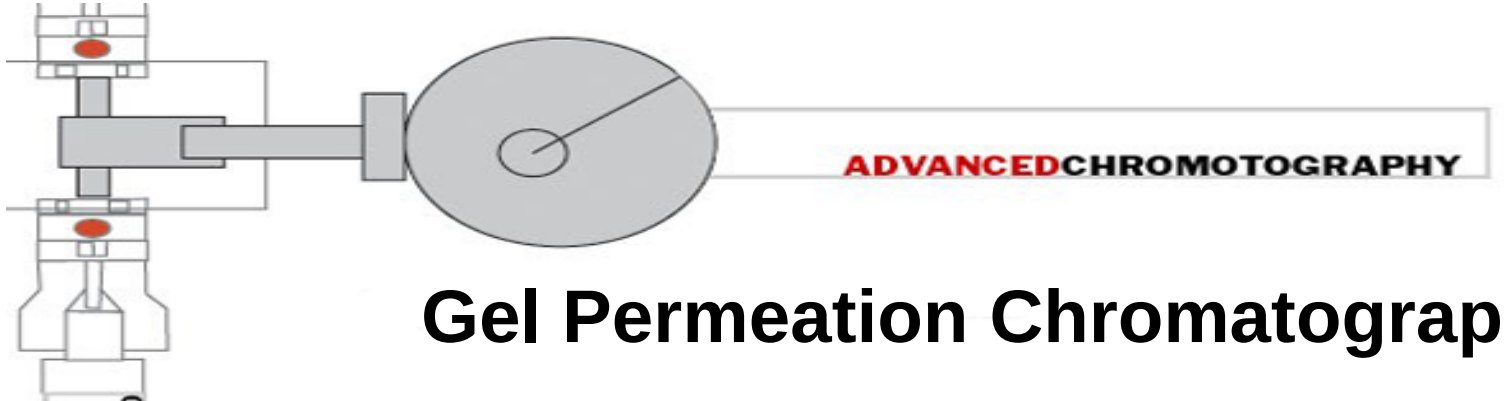


Gel Permeation Chromatography

Stationary Phases

GPC/SEC can be performed in a wide range of solvents. From non-polar organics to aqueous applications.

The composition of the mobile phase is generally not used to control retention or selectivity. The stationary phase is used almost exclusively to vary these parameters.



Gel Permeation Chromatography

Stationary Phases

GPC/SEC uses columns packed with very small, round, porous particles to separate molecules contained in the solvent that is passed through them.

The particles in the columns are made from polymers that have been cross-linked to make them insoluble, or inorganic materials, such as spherical silicas.

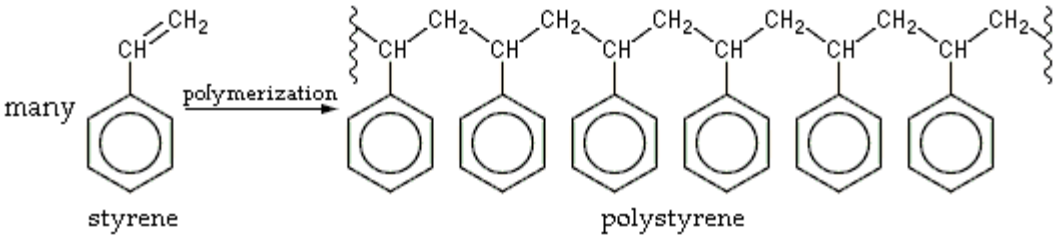
Modern columns are packed with 7-10 um particles and have separation efficiencies of 10-15,000 plates per column.

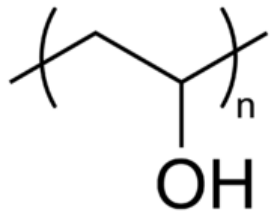
Gel Permeation Chromatography

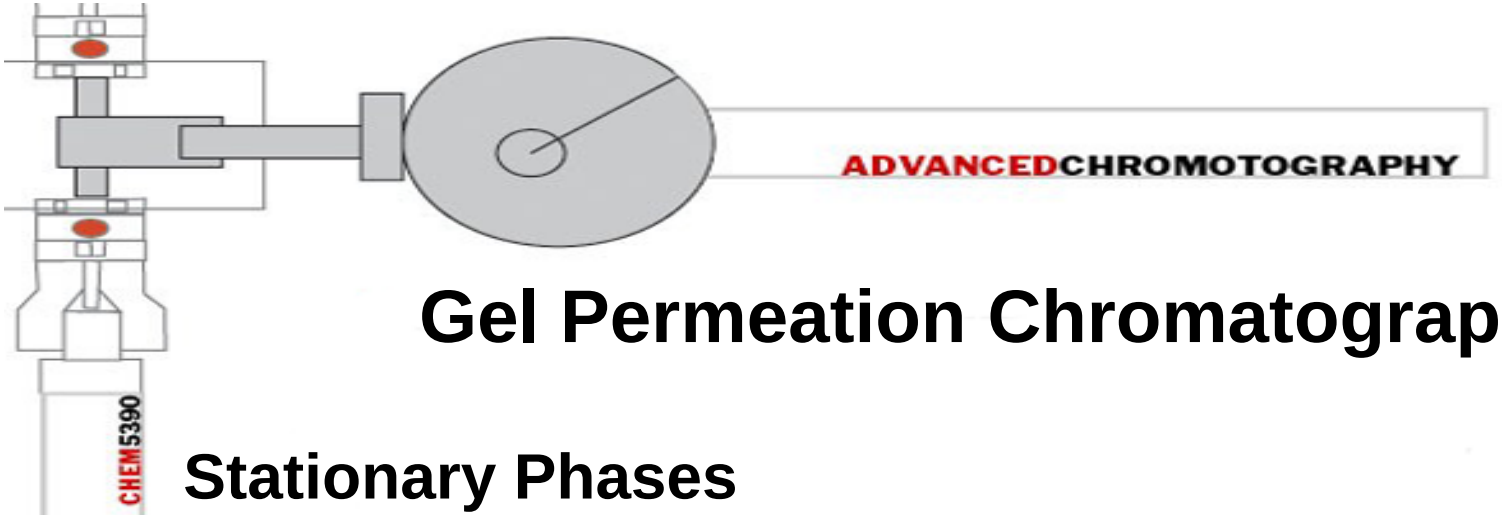
Stationary Phases

Divided into two classes:

- **cross-linked, rigid polystyrene divinylbenzene gels (Shodex)**
- **polydextrans, polyvinylalcohol, hydroxylated methacylate, and silica gels**







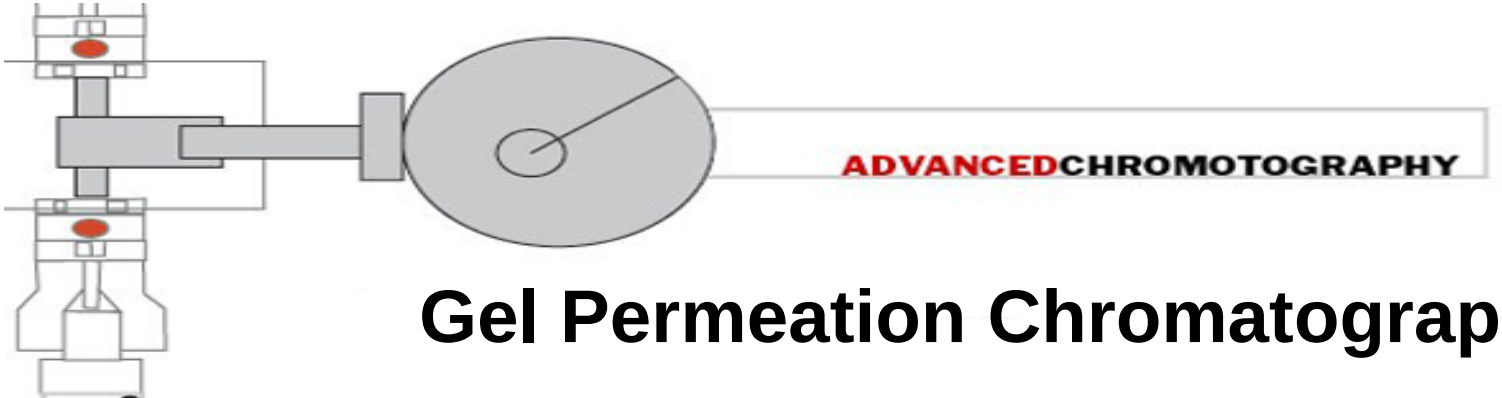
Gel Permeation Chromatography

Stationary Phases

<u>Soft gel</u> e.g.- dextran(Sephadex), Polyacrylamide gels
Separation of proteins.

<u>Semi-rigid</u> gel e.g.- bio beads
Separation of non-polar polymers in non-polar solvents.

<u>Highly rigid</u> gels and glasses
Separation of polar systems.



Gel Permeation Chromatography

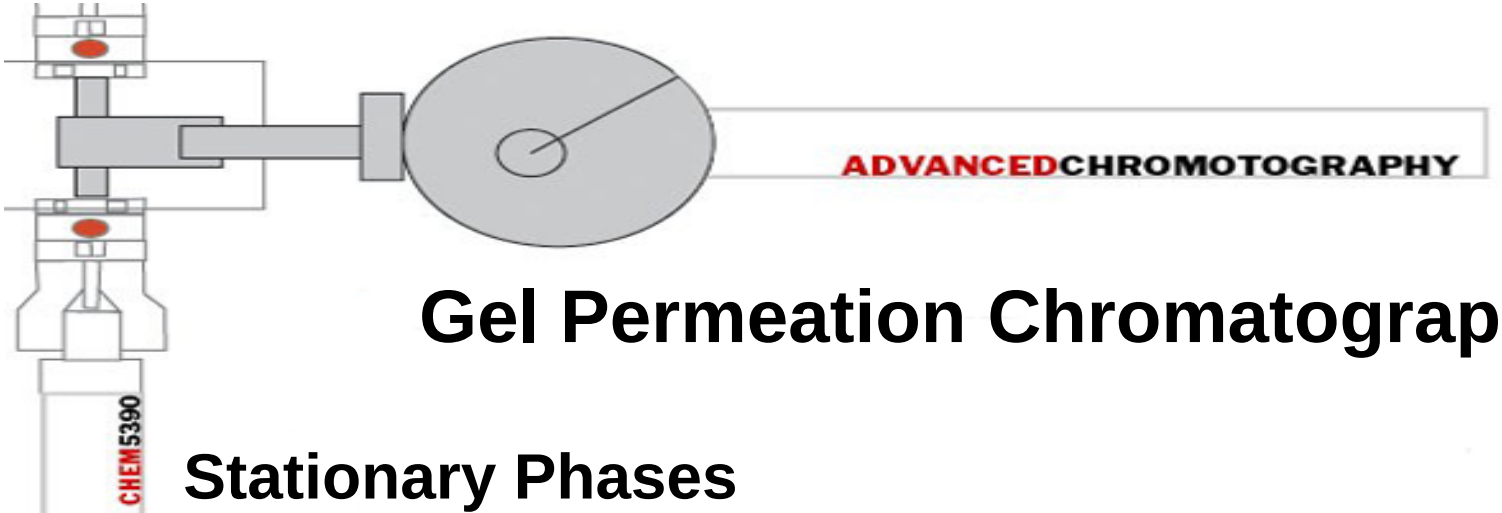
Stationary Phases

Dextran

A homopolysaccharide of glucose residues.

Prepared with various degrees of cross-linking to control pore size.
Bought as dry beads, the beads swell when water is added.
The trade name is sephadex.

Mainly used for separation of small peptides and globular proteins with small to average molecular mass



Gel Permeation Chromatography

Stationary Phases

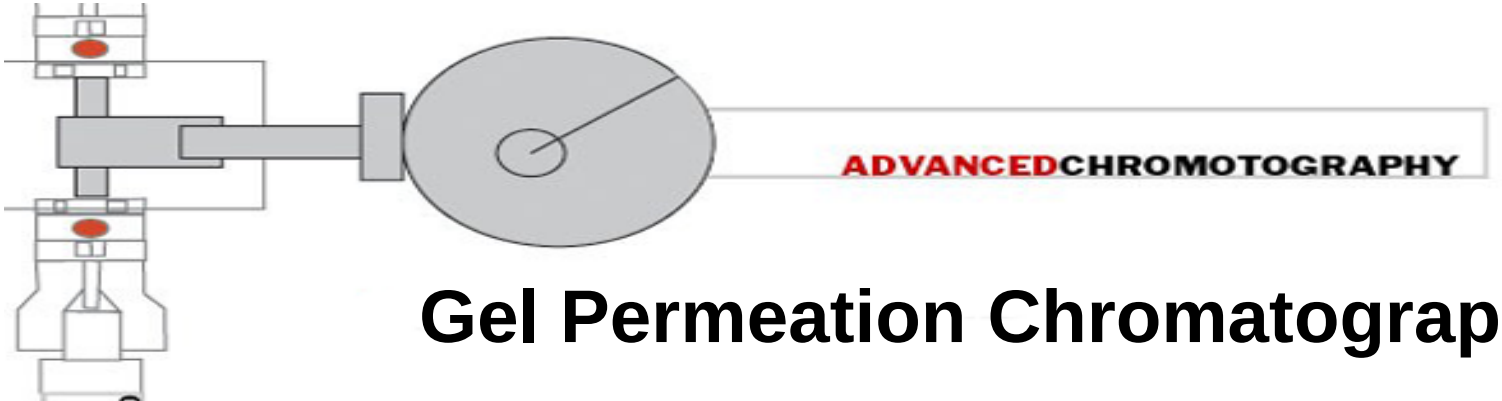
Polyacrylamide

These gels are prepared by cross linking acrylamide with N,N-methylene bis acrylamide.

The pore size is determined by the degree of cross-linking.

The separation properties of polyacrylamide gels are mainly the same as those of dextrans.

They are sold as bio-gel P. They are available in wide range of pore sizes.



Gel Permeation Chromatography

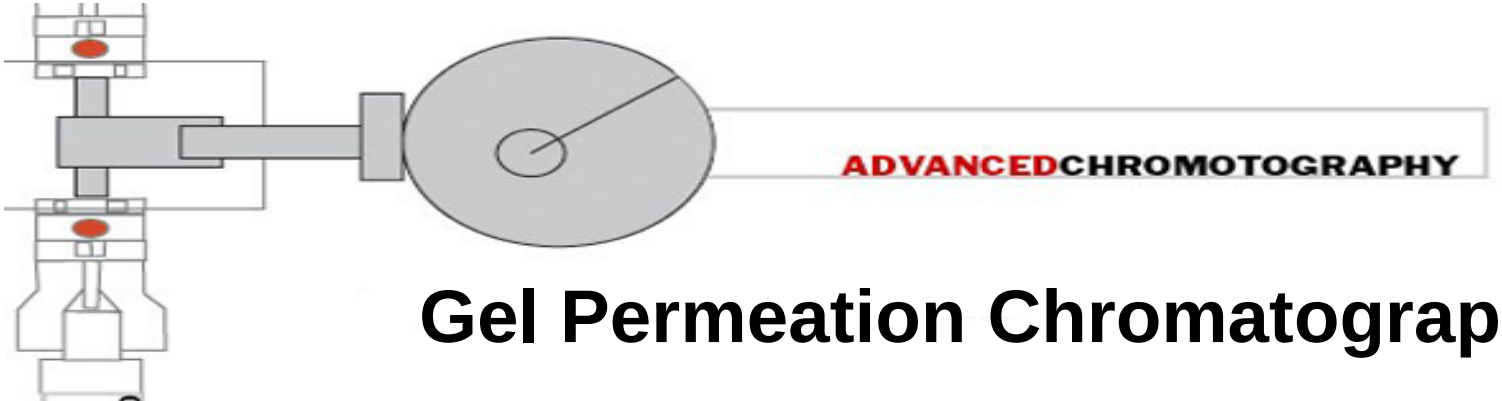
Stationary Phases

Agarose

Linear polymers of D-galactose and 3,6 anhydro-1-galactose.

Forms a gel that's held together with H bonds. It's dissolved in boiling water and forms a gel when cooled.
The concentration of the material in the gel determines the pore size.
The pores of agarose gel are much larger than those of sephadex or bio-gel p.

Useful for analysis or separation of large globular proteins or long linear molecules such as DNA



Gel Permeation Chromatography

Stationary Phases

Further classified according to pore size:

The pore size of the column is matched to the expected range of solute sizes so that they show selective permeation on the gel.

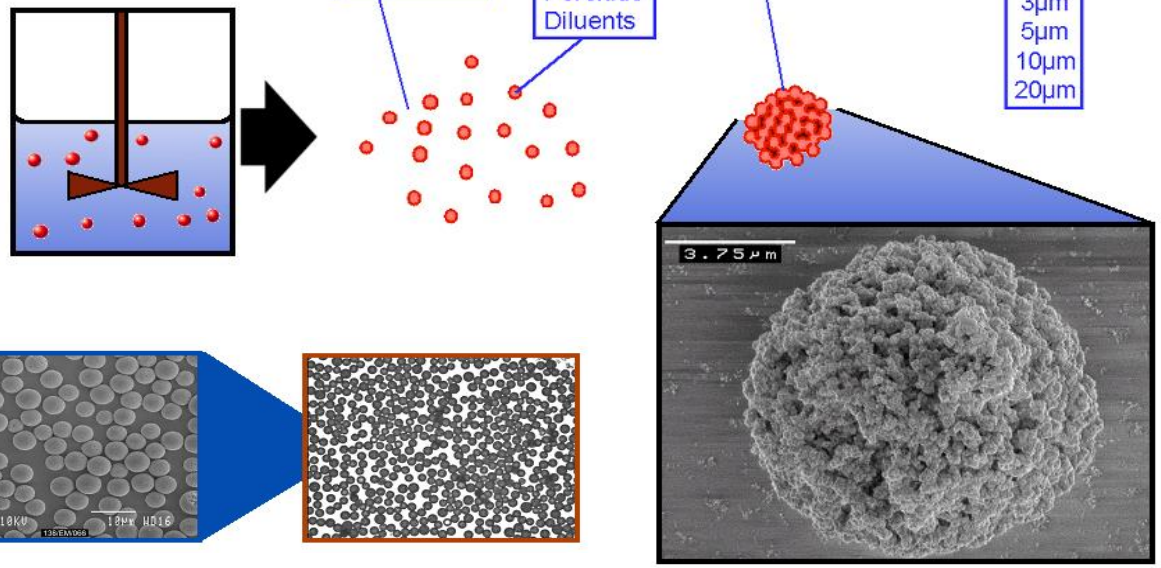
Historically, the pore size of a GLC column is defined as the extended chain length (Å) of a molecule of polystyrene which is just large enough to be excluded totally from all the pores.

This value is referred to as the exclusion, or pullulan, limit of the column.

Gel Permeation Chromatography

Synthesis of Stationary Phase

High cross-link content gives a rigid, low swelling product with a well-defined pore structure



Gel Permeation Chromatography

Synthesis of Stationary Phase

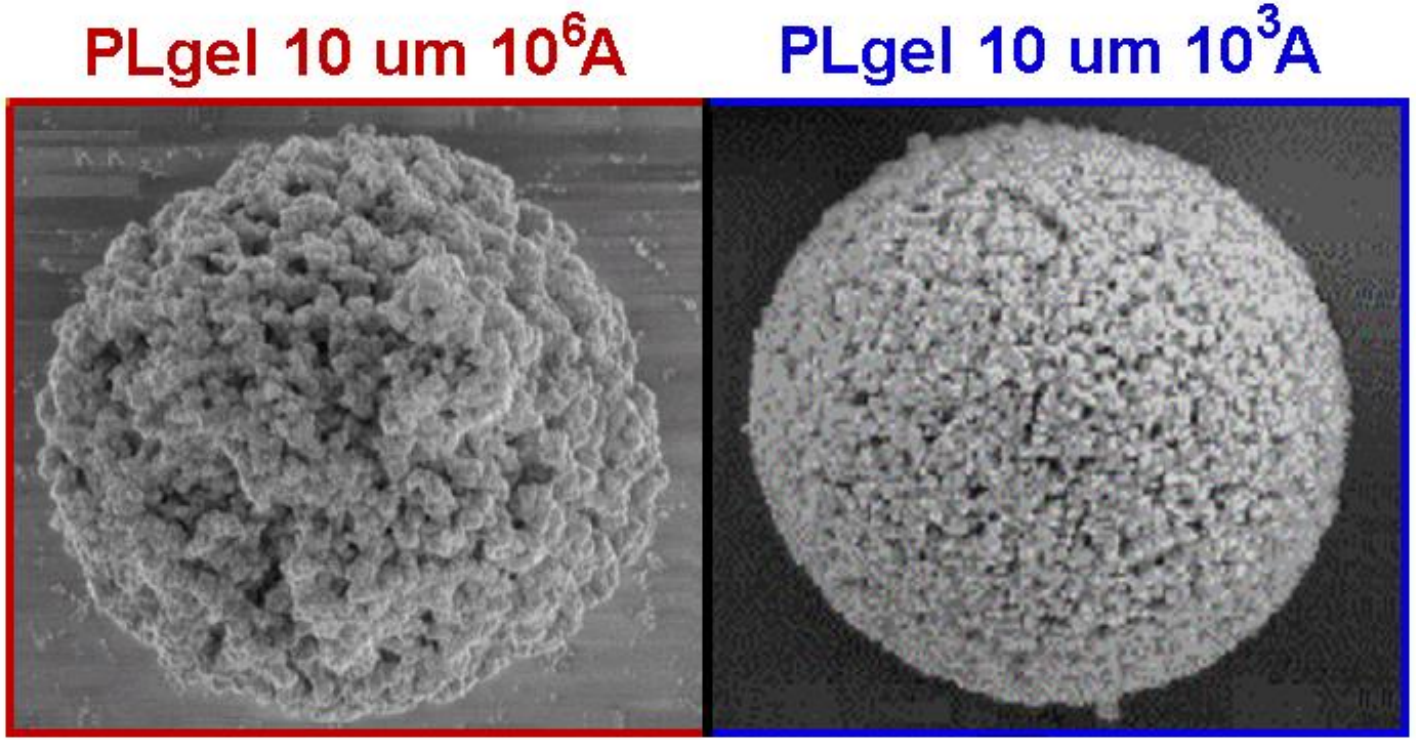
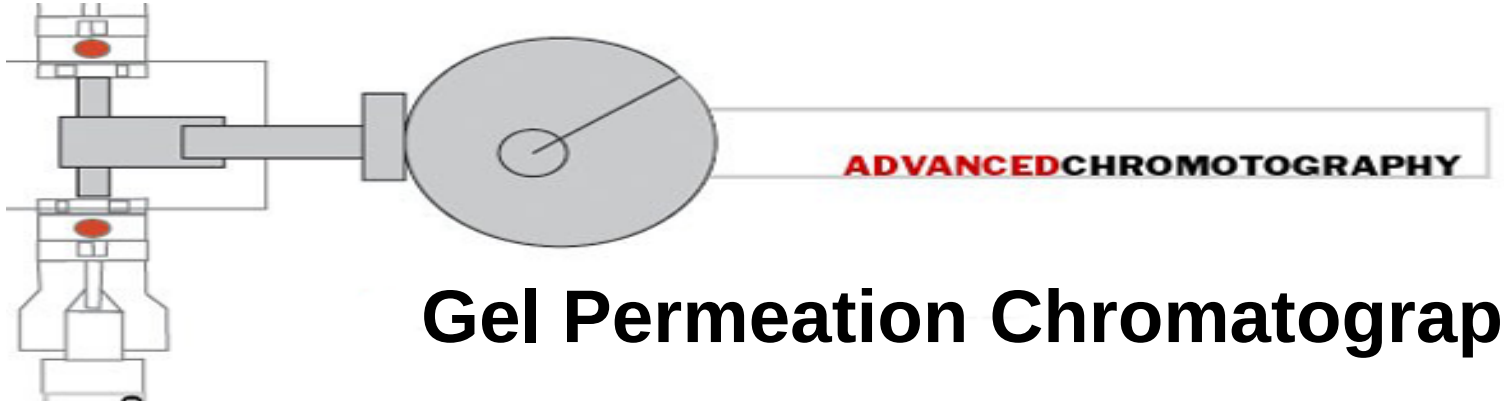


Table 6.9 Characteristics of non-aqueous (upper) and aqueous (lower) GPC column ranges available commercially from Waters.

Column range and individual designations	Packing material	Column range exclusion limits (lowest to highest)	Solvent(s)
Nonaqueous columns			
Ultrastyragel 100, 500, 10^3, 10^4, 10^5, 10^6, linear	PS-DVB	500 up to 10^7	THF, toluene
μStyragel 100, 500, 10^3, 10^4, 10^5, 10^6, linear	PS-DVB	500 up to 10^7	Toluene
μStyragel HT 10^3, 10^4, 10^5, 10^6, linear	PS-DVB	30 000 up to 10^7	MEK
Styragel 60, 100, 200, 500, 10^3, 10^4, 10^5, 10^6, 10^7	PS-DVB	500 up to 2×10^7	Toluene
Shodex K series 801, 802, 802.5, 803, 804, 804L, 805, 806, 805, 806, 807, 80-linear	PS-DVB	1.5×10^3 up to 2×10^8	THF, DMF, chloroform
Shodex HFIP series 803, 804, 805, 806, 807, 80-linear	PS-DVB	7×10^4 up to 2×10^8	HFIP
Shodex AT series 803, 804, 805, 806, 807, 80-linear	PS-DVB	7×10^4 up to 2×10^8	Toluene
Aqueous columns			
Shodex OHpak B series 803, 804, 805, 806	Methacrylate	10^5 up to 2×10^7	Water
Shodex OHpak Q series 803, 804, 805, 806	Polyvinylalcohol	10^5 up to 2×10^7	Water
Ultrahydrogel 120, 250, 500, 1000, 2000, linear, DP	Methacrylate	5×10^3 up to 7×10^6	Water
Protein-Pak 60, 125, 300SW	Silica gel	2×10^4 up to 3×10^5	Propanol
Shodex Protein KW 802.5, 803, 804	Diol-bonded silica gel	5×10^4 up to 6×10^5	Water

PS-DVB, polystyrene divinylbenzene; THF, tetrahydrofuran; MEK, methylethyl ketone; DMF, dimethylformamide; HFIP, hexafluoroisopropanone.

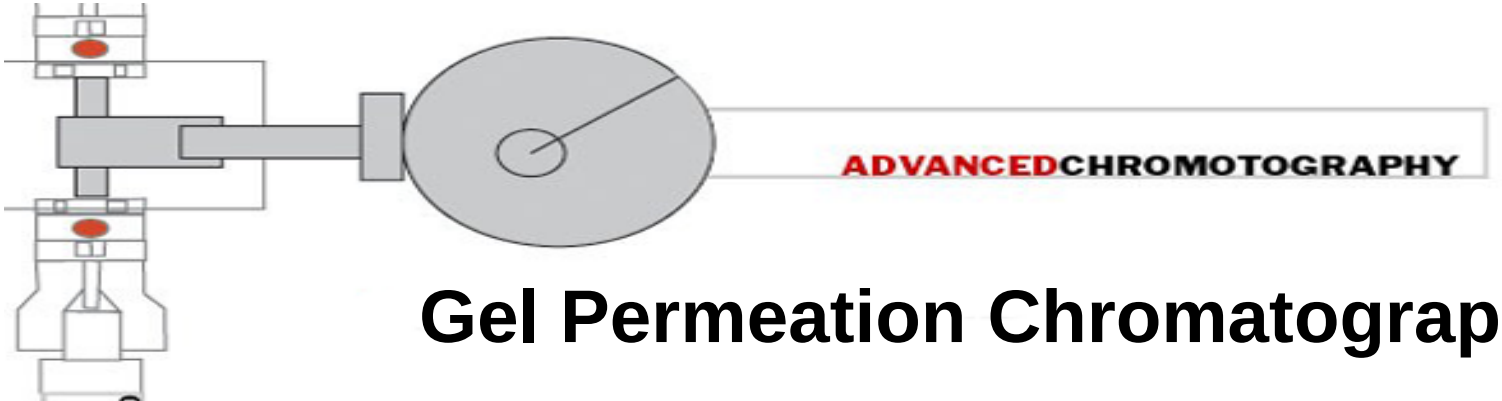


Gel Permeation Chromatography

Mobile Phases

GPC/SEC can be performed in a wide range of solvents. From non-polar organics to aqueous applications.

The composition of the mobile phase is generally not used to control retention or selectivity. The stationary phase is used almost exclusively to vary these parameters.

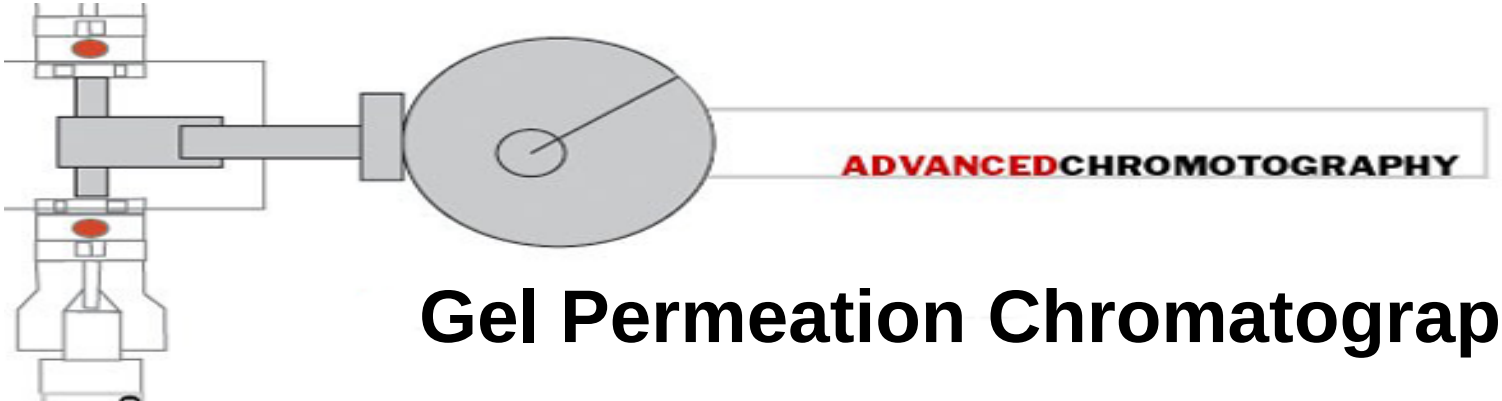


Gel Permeation Chromatography

Mobile Phases

Since mobile phase is not normally used to control retention and selectivity, the primary consideration when selecting a mobile phase is sample solubility.

Also needs to have a low viscosity and compatible with the detection method.



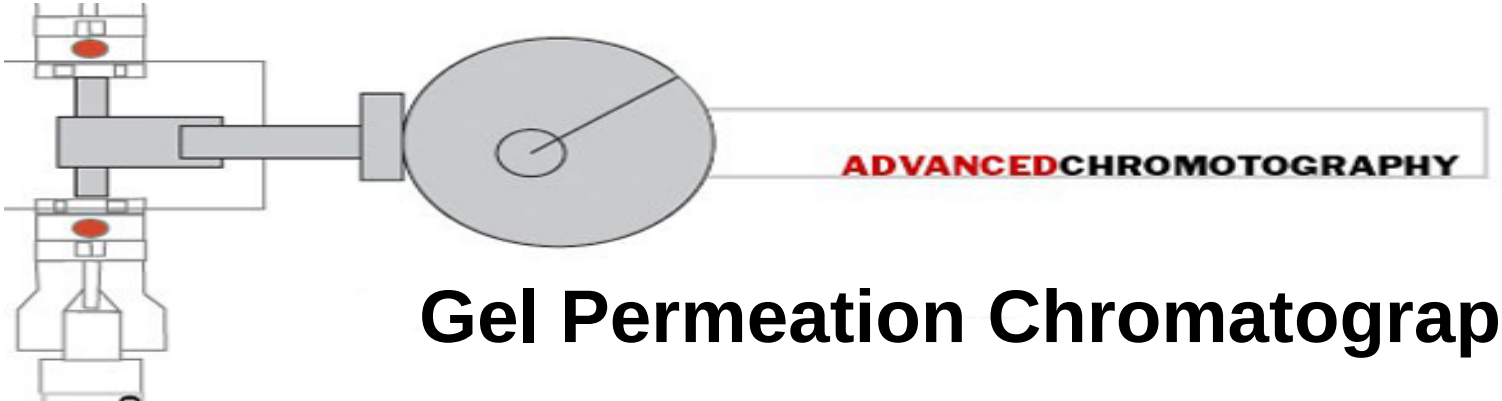
Gel Permeation Chromatography

Mobile Phases

Polar solvents, i.e., methanol, ethanol, etc… should not be used with polystyrene packings which can permanently damage the column.

Solvents that are compatible are toluene, chloroform, tetrahydrafuran, etc…

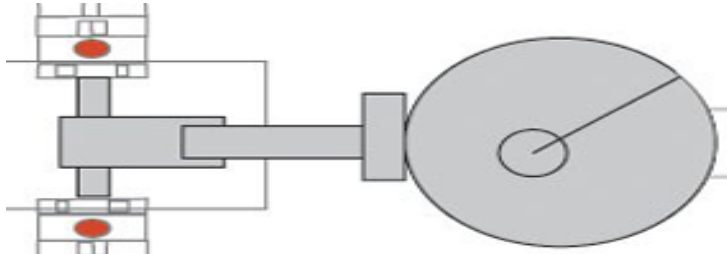
Of course these solvents with silica gel and other aqueous compatible packings will work with these types of columns.



Gel Permeation Chromatography

Mobile Phases

The most common eluents for polymers that dissolve at room temperature are Tetrahydrofuran, Chloroform, and Dimethyl formamide.



Gel Permeation Chromatography

Table 6.10 Properties of solvents commonly used for polymer GPC.

Solvent	u.v. cut-off (nm)	Viscosity at 20°C (c*P*)	Refractive index at 20°C
Dimethyl formamide*	295	0.924	1.4294
Ethylene chloride	225	0.84	1.4444
Dioxane	220	1.439	1.4221
Trichloroethane	225	1.20	1.4791
Cyclohexane*	220	0.98	1.4262
Carbon tetrachloride	265	0.969	1.4630
Toluene	285	0.59	1.4969
Trifluoroethanol*	190	1.996	1.2910
Benzene	280	0.652	1.5011
Hexafluoroisopropanol*	190	1.021	1.2752
Chloroform	245	0.58	1.4457
Hexane*	210	0.326	1.3749
Xylene	290	0.81	1.4972
Tetrahydrofuran	220	0.55	1.4072
Methylene chloride	220	0.44	1.4237

*Care must be taken when using these solvents with 100–500 Å pore size columns.
Data from Snyder [4].

Gel Permeation Chromatography

Detection Techniques

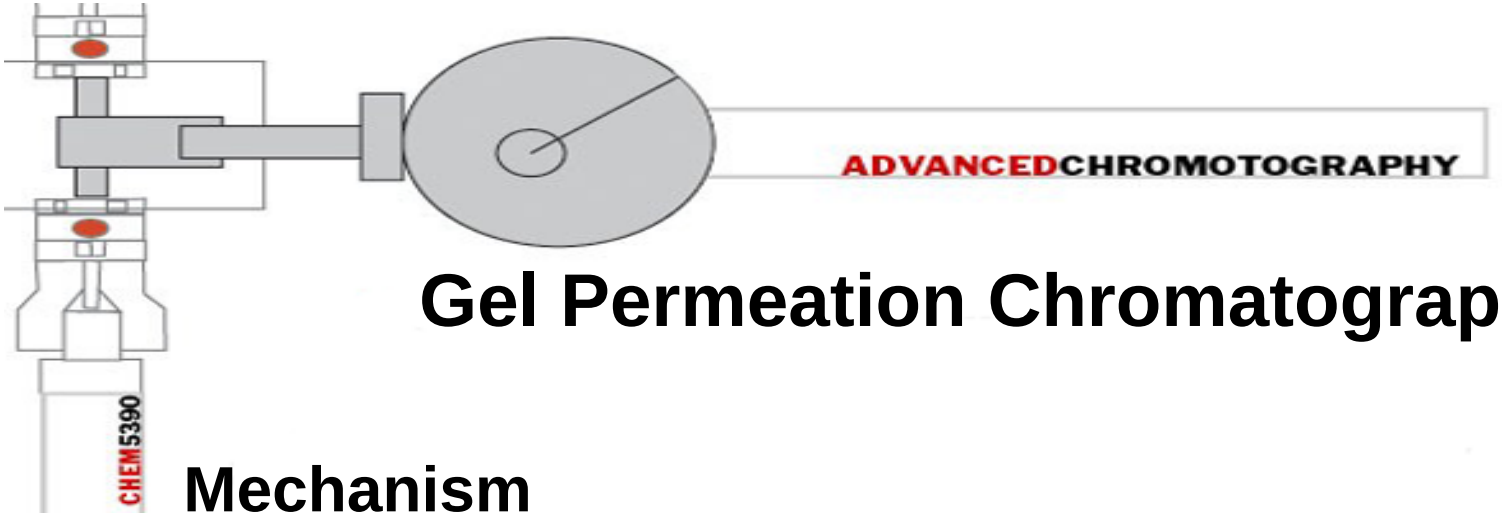
Concentration detectors

- Differential refractometer (RI)
- Ultraviolet absorbance (UV)
- Evaporative light scattering or mass detector (ELS, EMD)
- Infra-red (IR)

Molecular weight sensitive detectors

- Viscometry
- Light scattering

Most common detection techniques are refractive index (RI) and ultraviolet (UV).

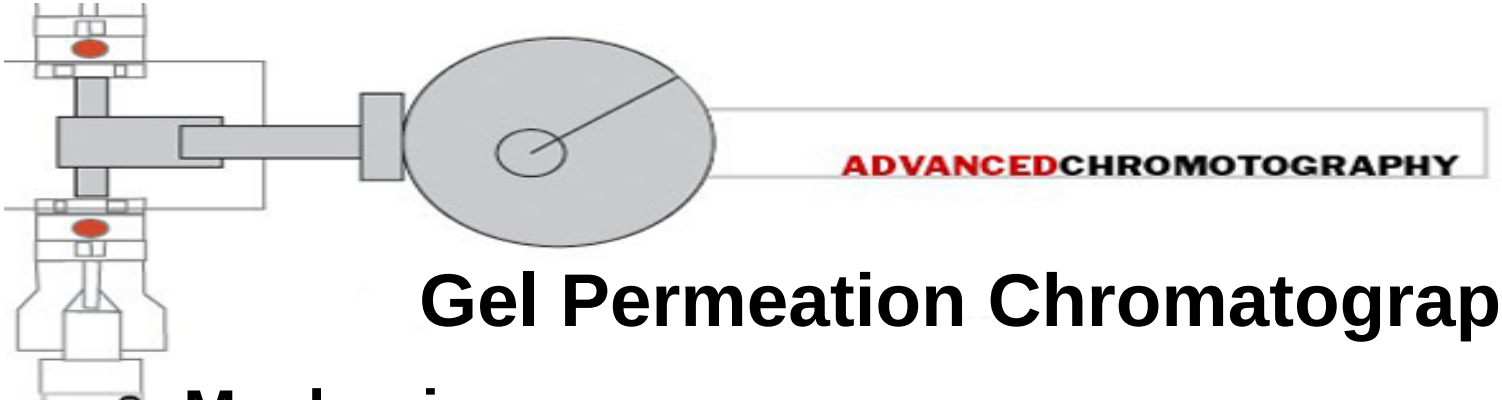


Gel Permeation Chromatography

Mechanism

Mechanism is straightforward and molecules are separated according to their effective size in solution.

In this mode the solute molecules do not interact with the stationary phase by adsorption or hydrophobic interaction.



Gel Permeation Chromatography

Mechanism

If there are any interactions, the following problems can occur:

- **polymer - polymer (aggregation) – earlier elution, artificially increased molecular weight**
- **polymer - column packing (adsorption) - delayed elution, artificially decreased molecular weight**
- **eluent-column packing (repulsion) – earlier elution, artificially increased molecular weight**
- **polymer - eluent (aggregation) - earlier elution, artificially increased molecular weight**

Gel Permeation Chromatography (GPC)

GPC can determine several important parameters

These include: number average molecular weight, weight average molecular weight, Z weight average molecular weight, and molecular weight distribution.

These values are important, since they affect many of the characteristic physical properties of a polymer.
Some of these properties include: Tensile strength, Brittleness, Impact strength, Adhesive strength, Cure time, Melt viscosity, Hardness

Gel Permeation Chromatography (GPC)

In polymers, molecular weight occurs as a distribution.

To accurately assess the molecular weight distribution of a polymer, have to count how many particles there are at every weight in the distribution, and from these counts, calculate an average that describes the whole sample.

Gel Permeation Chromatography (GPC)

In polymers, molecular weight occurs as a distribution.

The typical position of molecular weight average.

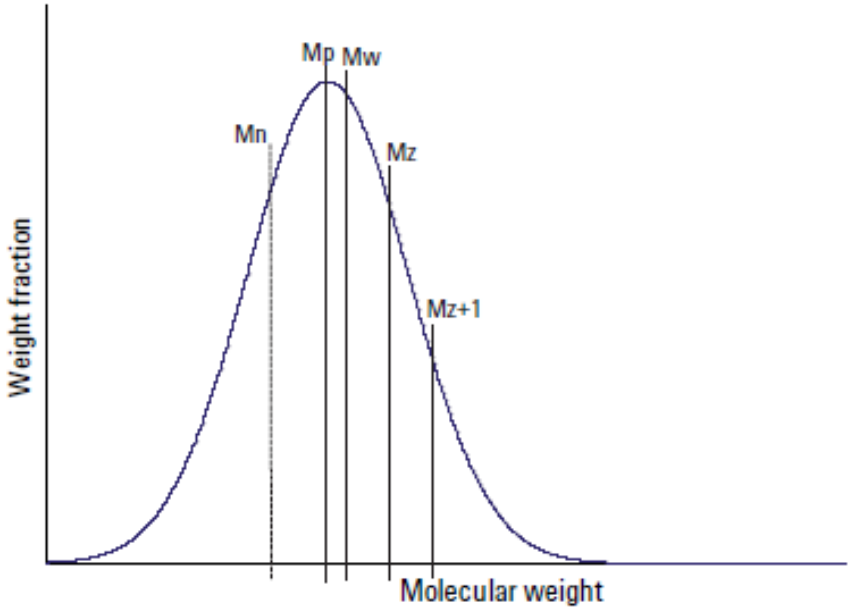


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*

Gel Permeation Chromatography (GPC)

The commonly calculated average is called the number average molecular weight, abbreviated to Mn.
Looking at the distribution in the figure – the Mn value marks the value at which there are equal numbers of molecules on each side, at higher and lower molecular weight. The value of Mn influences the thermodynamic properties of the molecule.

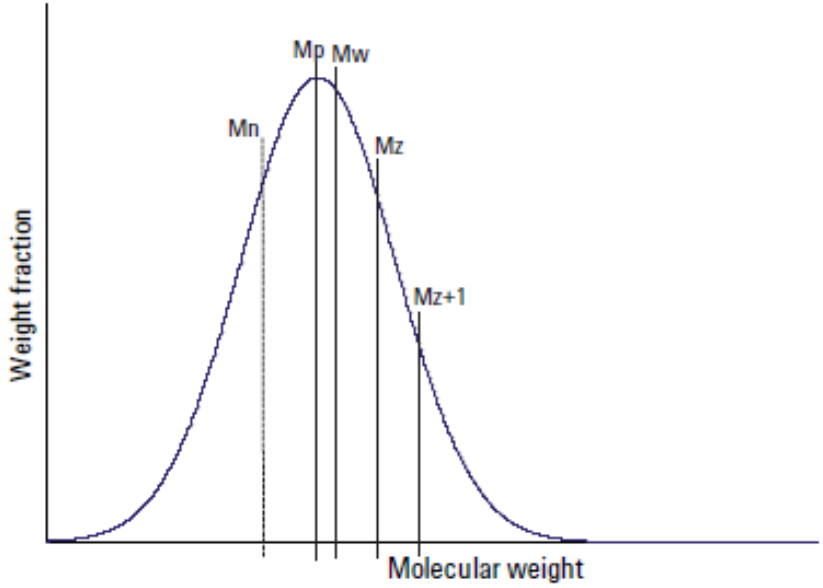


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*

Gel Permeation Chromatography (GPC)

Other ways of describing molecular weight average include weight average molecular weight (Mw).
Mw is defined as the value at which there are equal masses of molecules on each side, at higher and lower molecular weight.

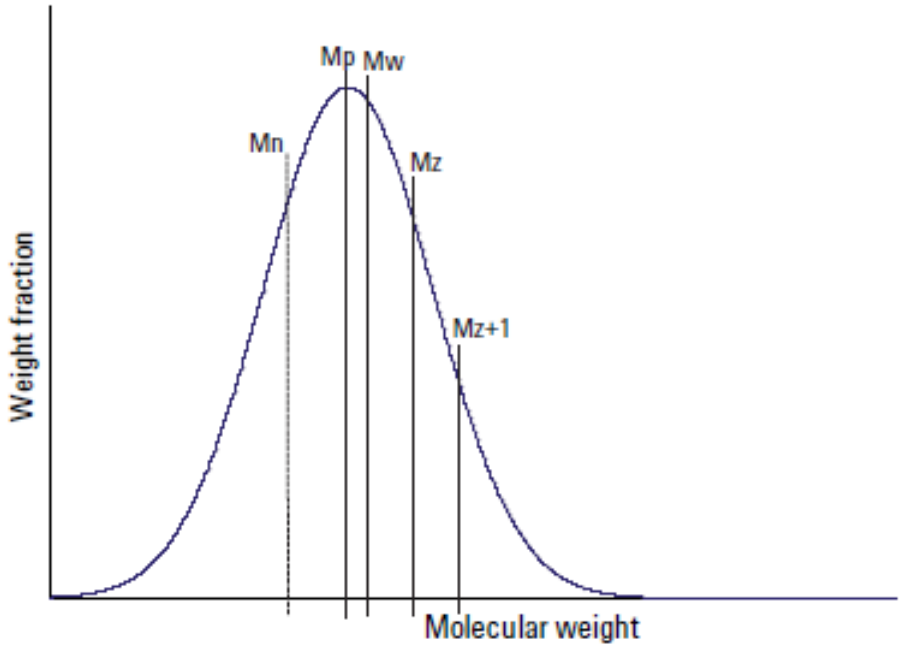


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*

Gel Permeation Chromatography (GPC)

The Mw value is always greater than the Mn value unless the polymer is completely monodisperse.
Mw is large-molecule sensitive and influences the bulk properties and toughness of the polymer, and is the most often quoted molecular weight average.

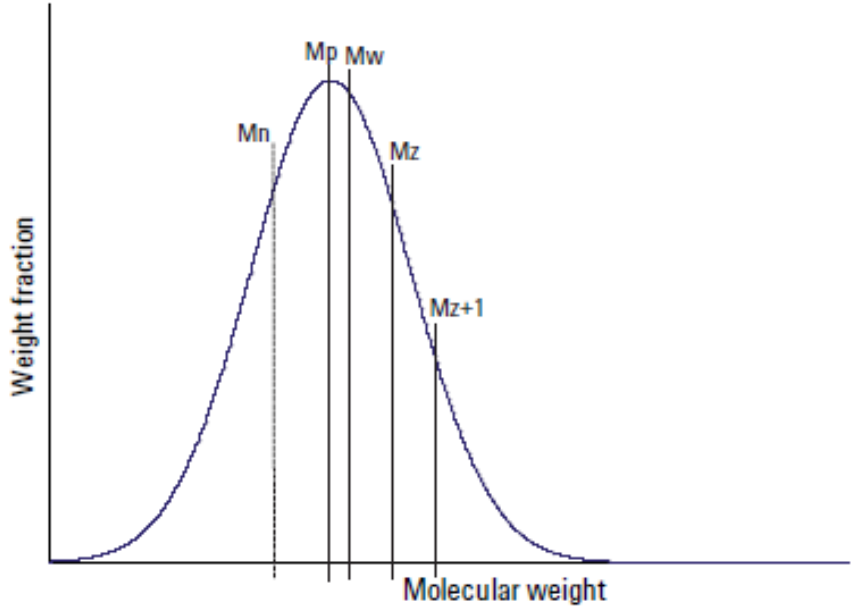


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*

Gel Permeation Chromatography (GPC)

The ratio of Mw to Mn is used to calculate the polydispersity index (PDI) of a polymer, which provides an indication of the material's range of molecular mass.
The broader the molecular weight distribution, the larger the PDI.

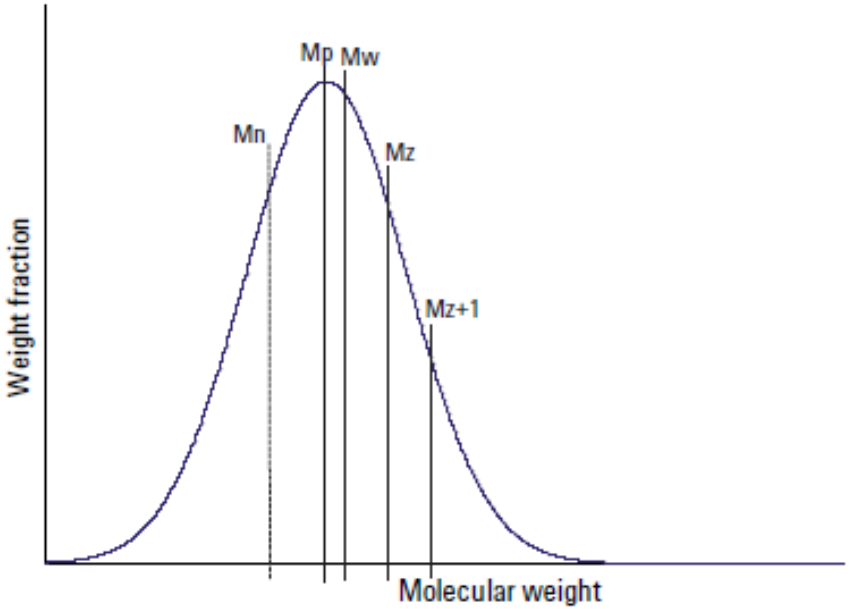


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*

Gel Permeation Chromatography (GPC)

Other molecular weight averages take into account the higher molecular weight components of the sample, such as z-average molecular weight (Mz) and Mz+1.
Mz is sensitive to even larger molecules and influences viscoelasticity and melt flow behavior.

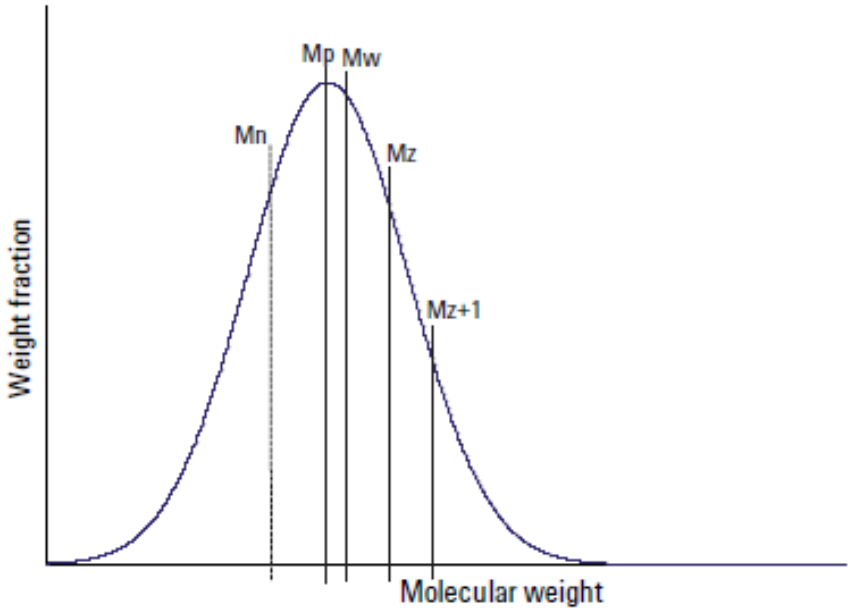
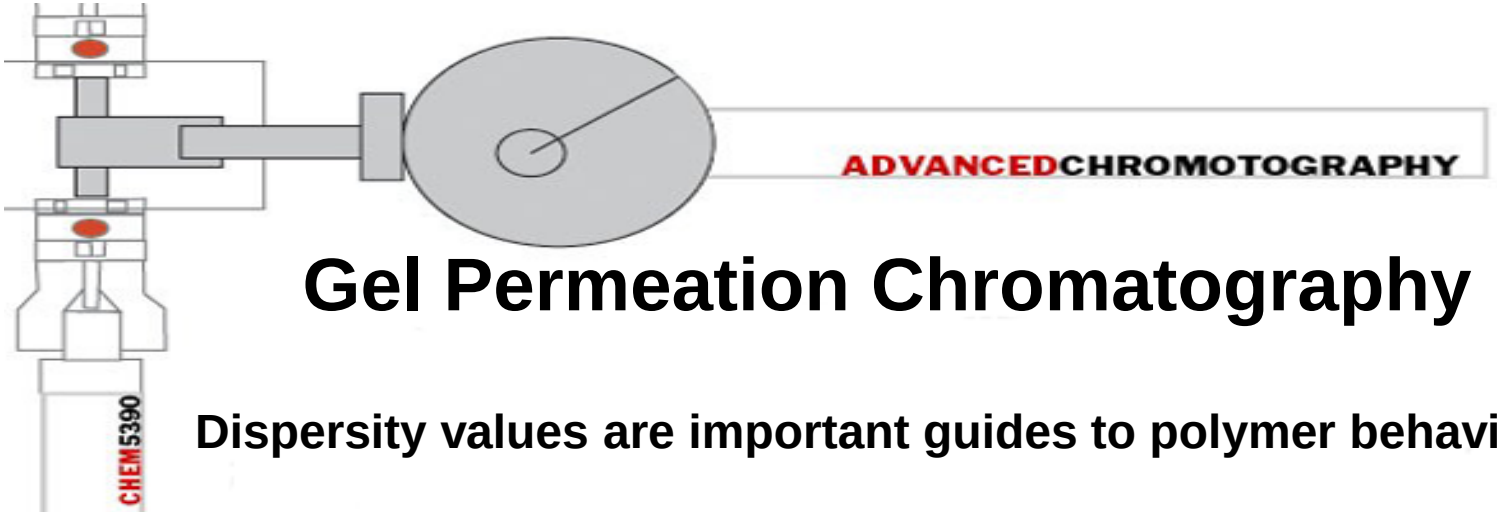


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*



Gel Permeation Chromatography (GPC)

Dispersity values are important guides to polymer behavior:

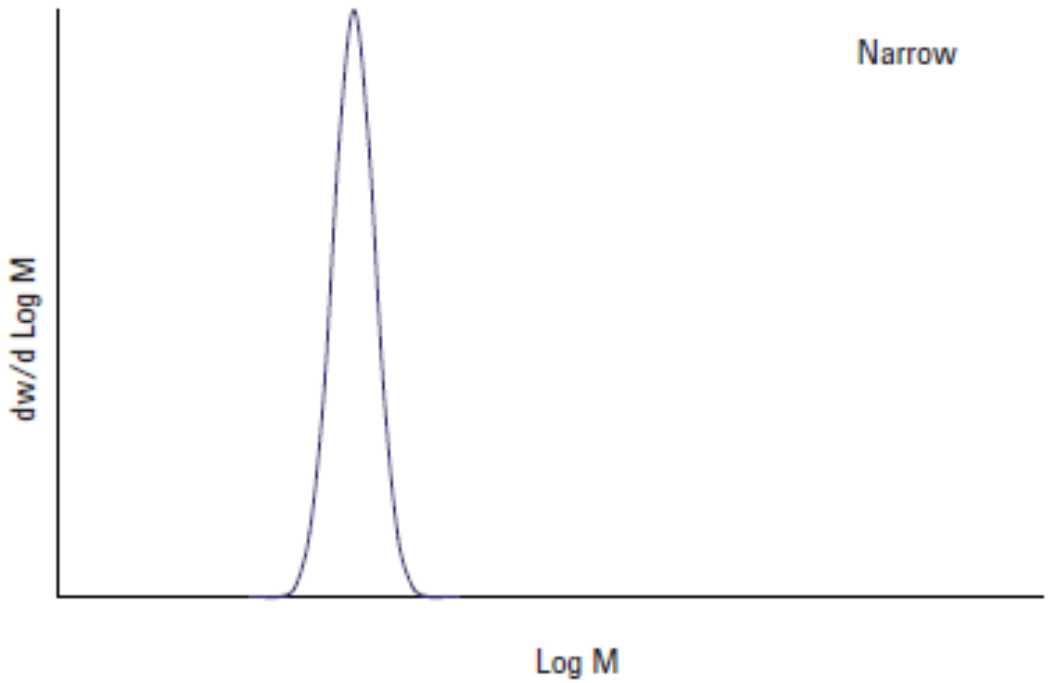
- Monodisperse polymers consist of molecules all with the same molecular weight where the values of Mn, Mw and Mz are identical.
In practical terms, the only monodisperse polymers are those found in nature, such as proteins and nucleic acids.

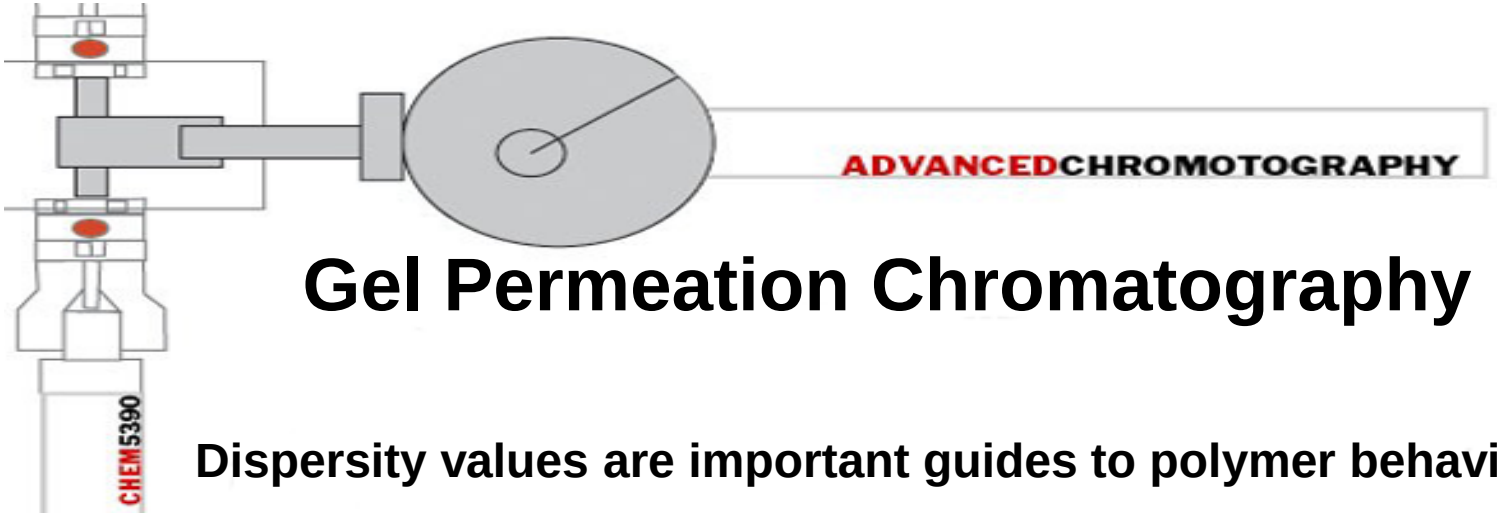
- Narrow distribution polymers are synthetic compounds where all the chains are close in molecular weight as possible.
The Mw/Mn polydispersity ratio is less than 1.2,
Examples include polymers used as standards for calibrations.

Gel Permeation Chromatography (GPC)

Dispersity values are important guides to polymer behavior:

- Narrow distribution polymers





Gel Permeation Chromatography (GPC)

Dispersity values are important guides to polymer behavior:

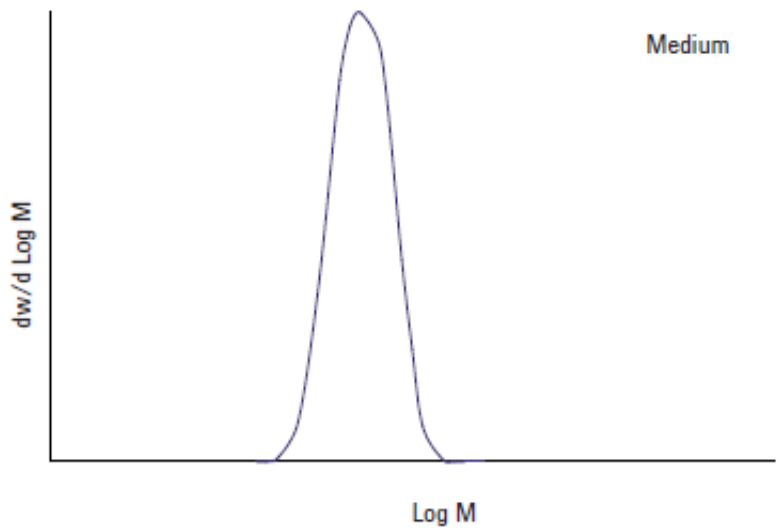
- The most common synthetic polymers have a medium width distribution, with an Mw/Mn ratio between 1.2 and 3, because many of the synthetic procedures used tend statistically towards these values.

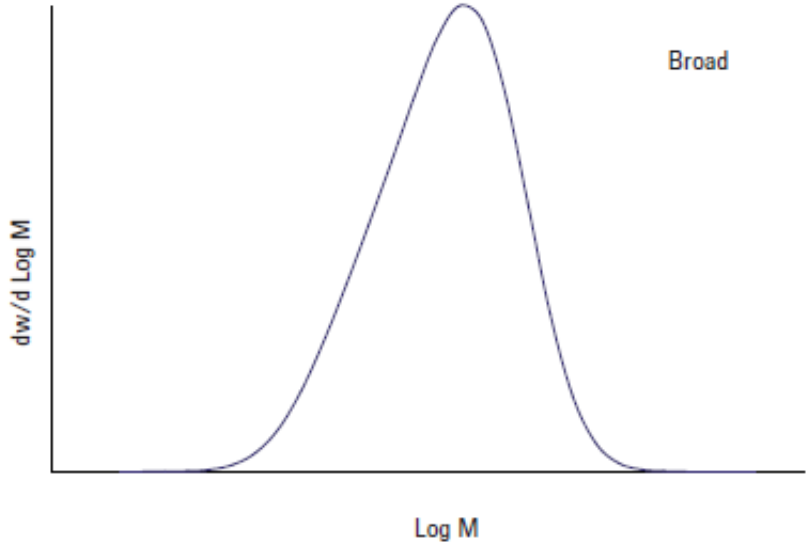
- Broad distribution polymers may also be synthetic, or natural such as polysaccharide and starch carbohydrates.
These distributions are rarely normal in shape; for example, they may have a long tail towards the lower molecular weight. The presence of the low molecular weight compounds shifts Mn more than Mw and Mz.

Gel Permeation Chromatography (GPC)

Dispersity values are important guides to polymer behavior:

- Medium width distribution polymers
- Broad distribution polymers

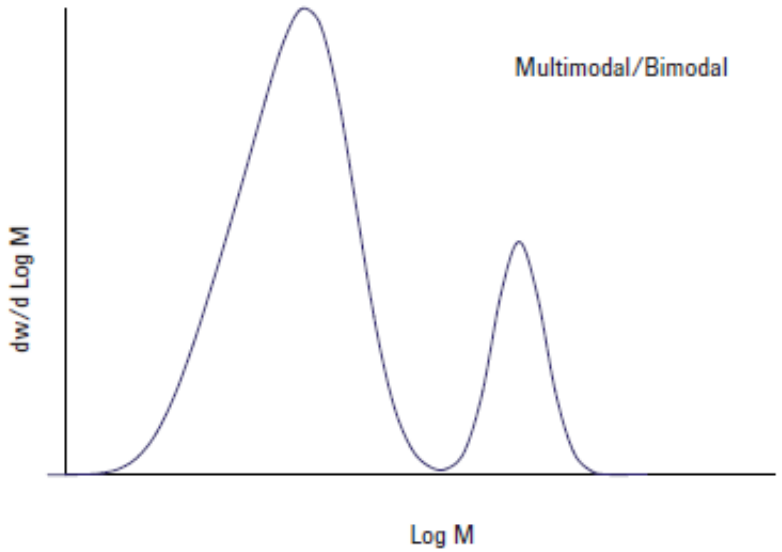




Gel Permeation Chromatography (GPC)

Dispersity values are important guides to polymer behavior:

Sometimes synthetic polymers have multimodal distributions.
If two or more polymers of differing molecular weight are present, then the two peaks can overlap, resulting in a bimodal distribution



Gel Permeation Chromatography (GPC)

For standards - need to be of very high quality and with extremely narrow molecular weight distributions so that the position of the top of the peak, the peak molecular weight (Mp) can be assigned with confidence. It is the Mp value used to set up the calibration.

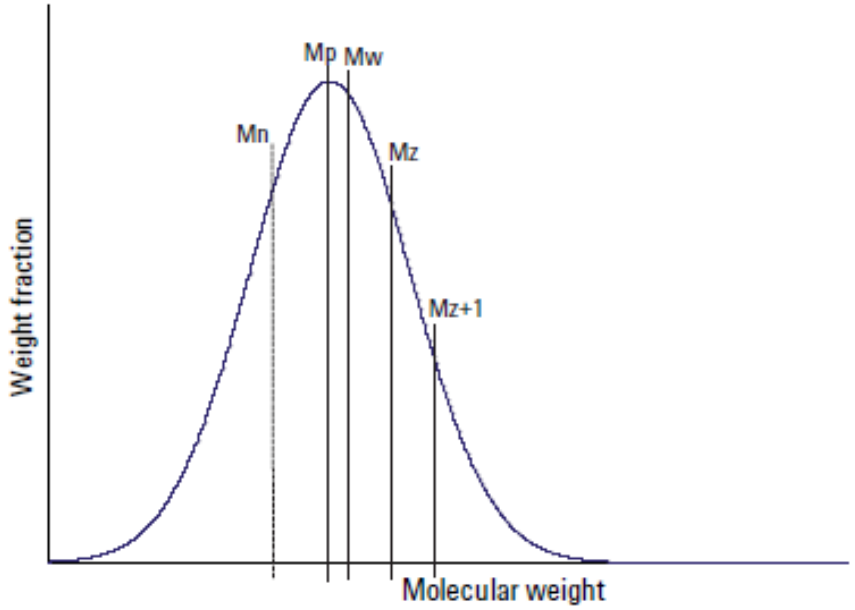


Figure 3. *The average molecular weights of a mono-modal polymer – in this case the distribution is nearly symmetrical*

Gel Permeation Chromatography

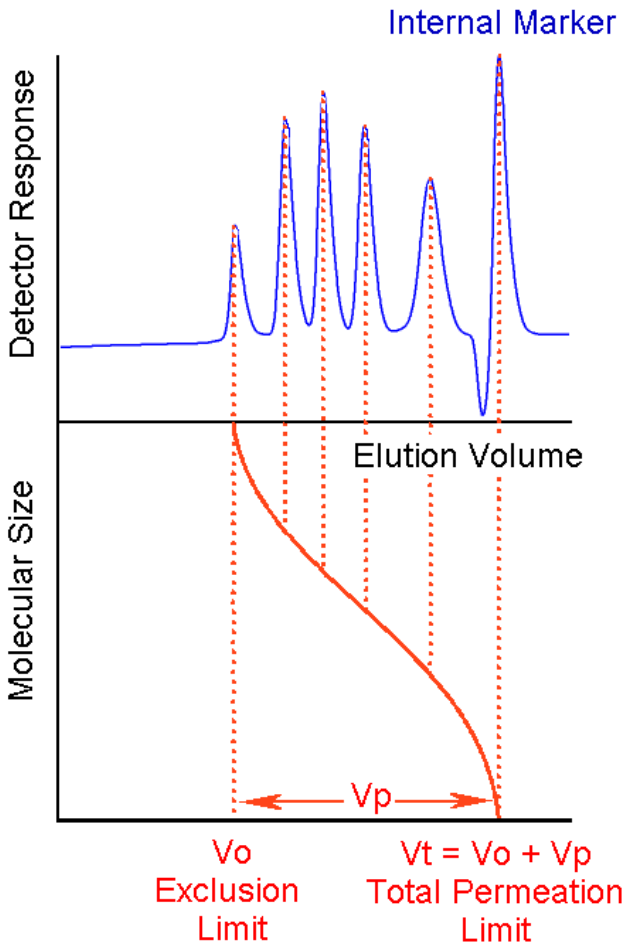
Elution Profile

Exclusion volume (Vo) –
Upper MW limit
(also known as void volume)

Total permeation volume (Vt) –
Lower MW limit

Pore volume (Vp) –
Working resolving range of MW

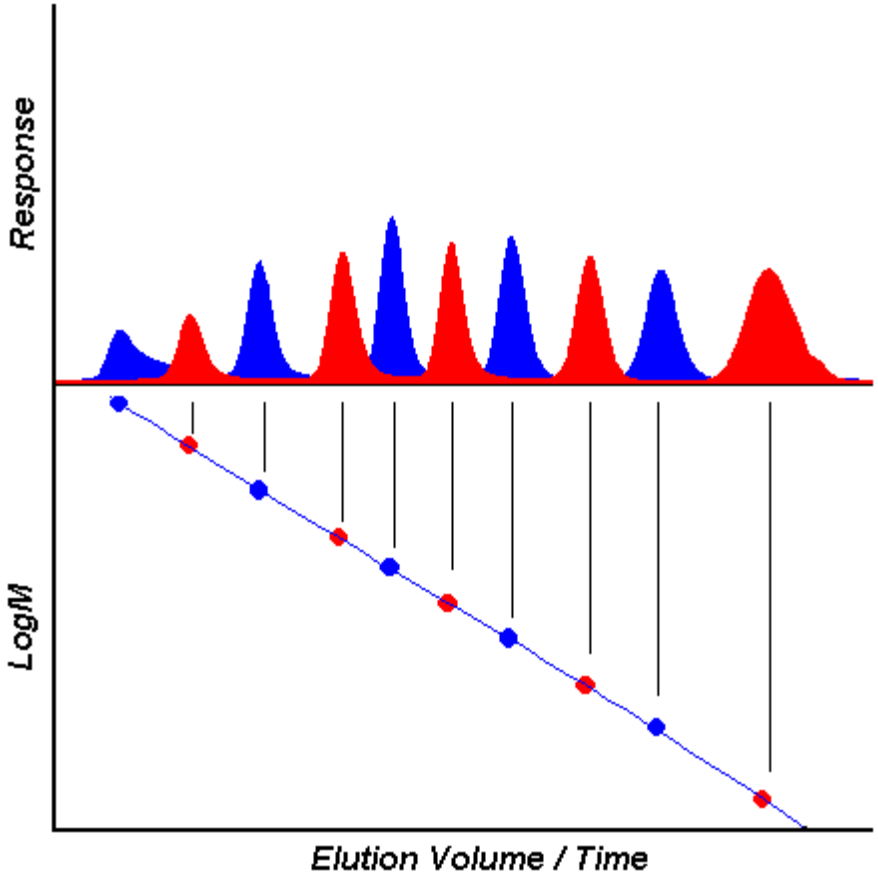
$$Vp = Vt - Vo$$



Gel Permeation Chromatography

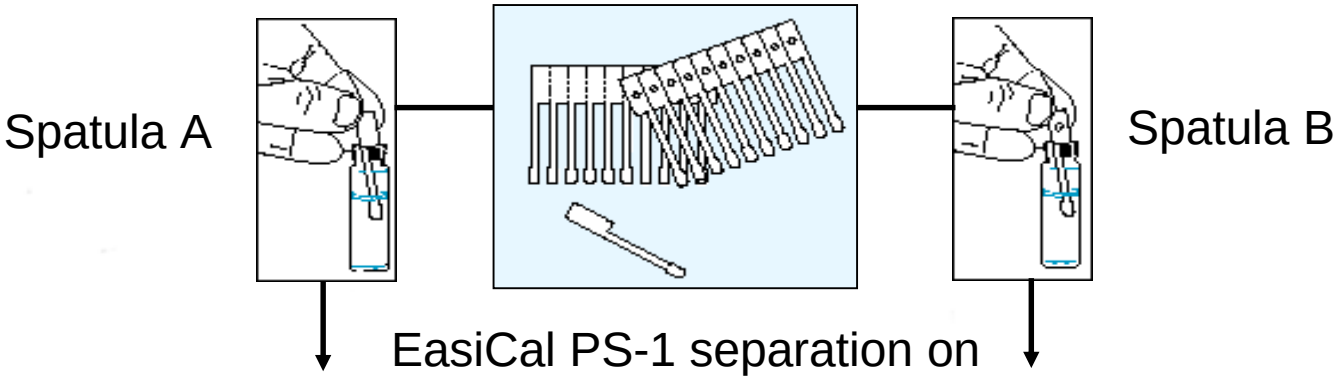
Calibration

Injections of multiple narrow standards reduces the time taken to calibrate the system.

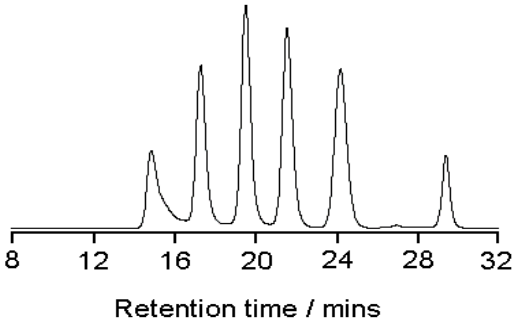


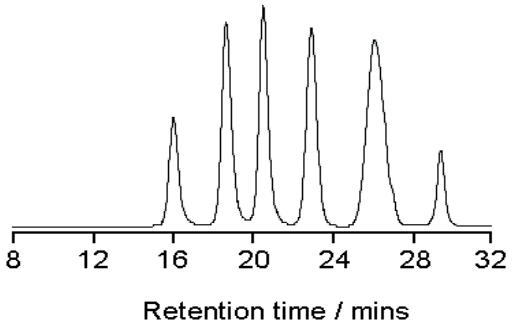
Gel Permeation Chromatography

Calibration - EasiCal Pre-pared stds (example: Varian)



EasiCal PS-1 separation on
3 x PLgel 10µm MIXED-B





Gel Permeation Chromatography

Calibration - EasiCal Pre-pared example

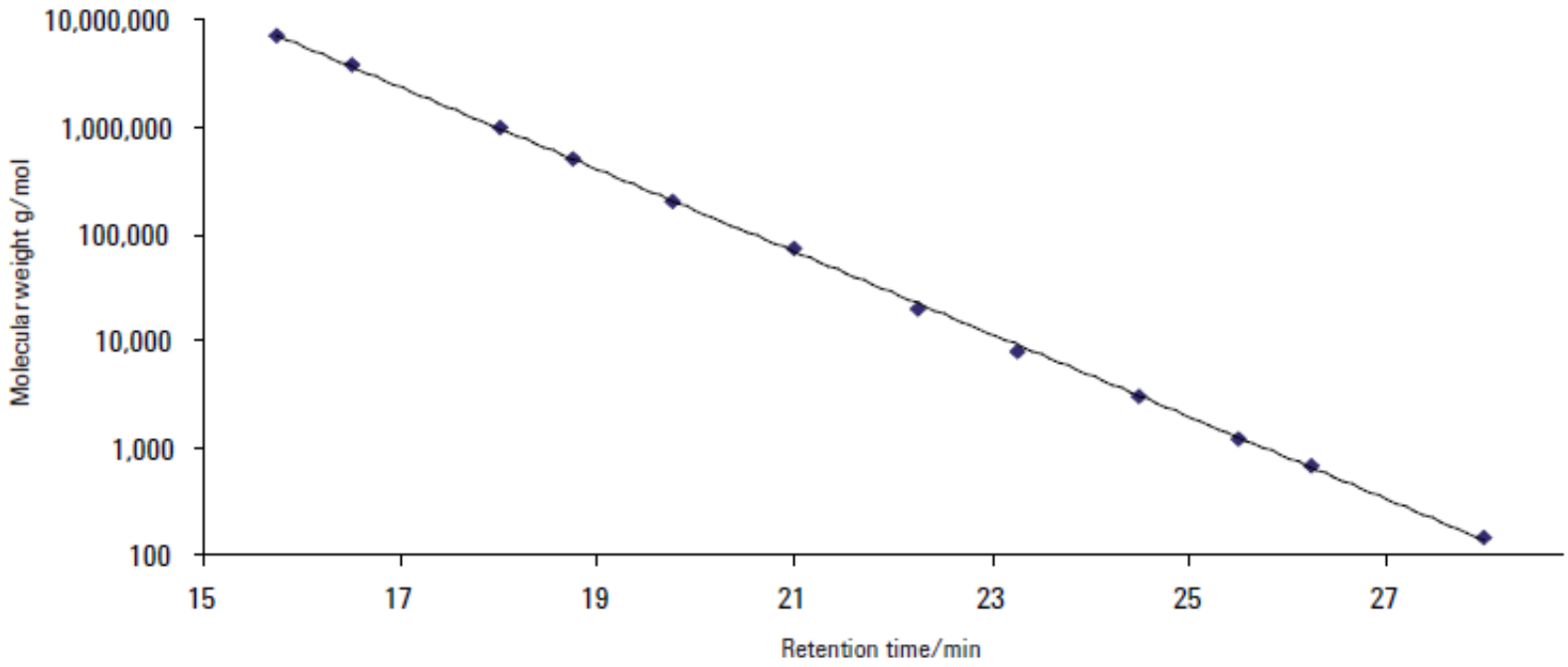
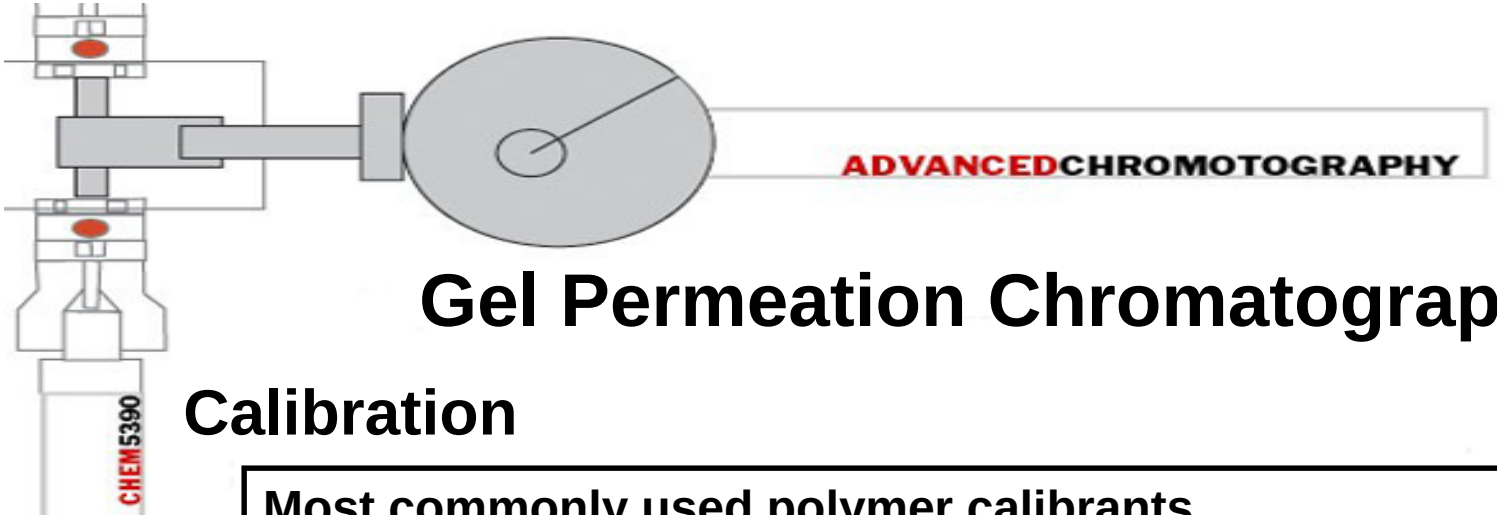


Figure 2. *A calibration graph used to determine the molecular weight of a polymer from its retention time*



Gel Permeation Chromatography

Calibration

Most commonly used polymer calibrants

Polystyrene - THF, toluene, chloroform, TCB

Polymethyl methacrylate - MEK, ethyl acetate, acetone, DMF

Polyethylene oxide/glycol - aqueous eluents, DMF, DMSO

Mn - number average molecular weight
Mw - weight average molecular weight
Mv- viscosity average molecular weight
Mp - peak molecular weight
Mw/Mn - polydispersity by GPC

Must be extremely well characterized

Gel Permeation Chromatography

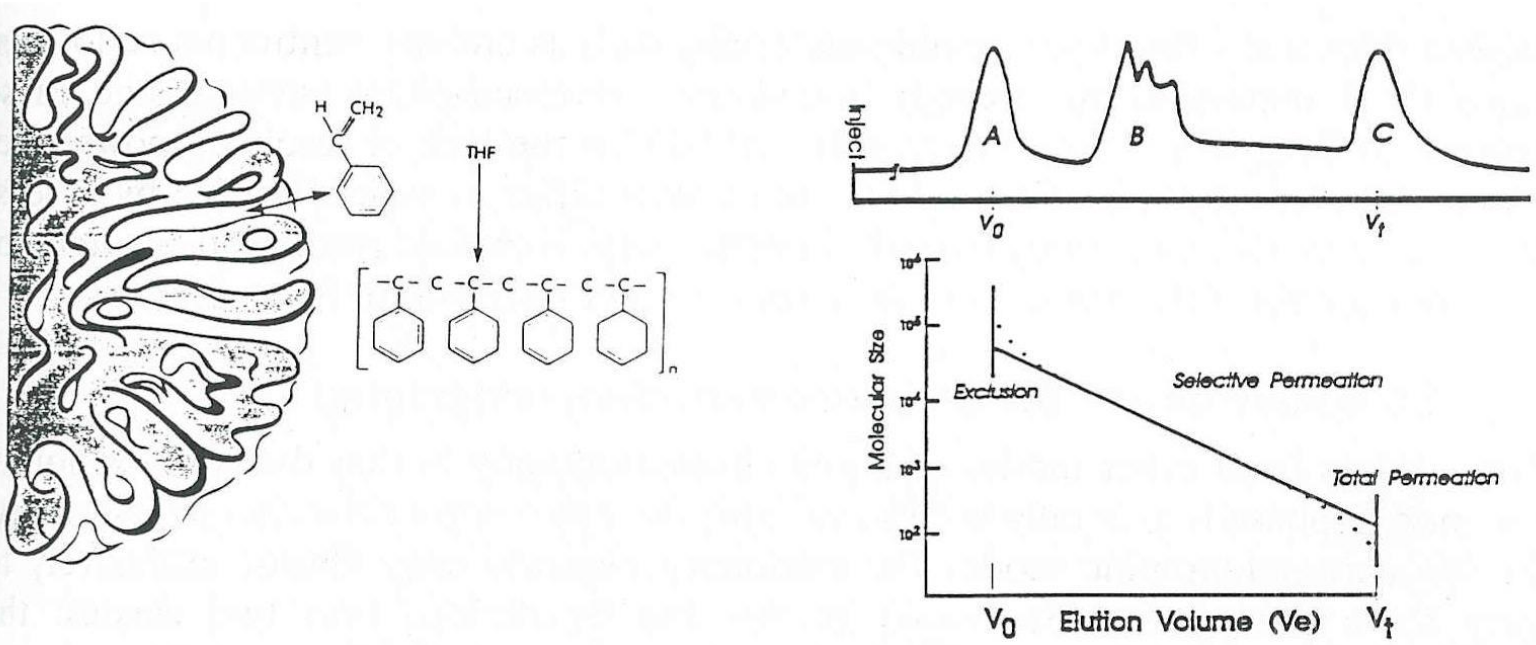


Fig. 6.22. Schematic illustration of the permeation process. In the diagram on the left, the smaller molecule is able to diffuse into solvent within the pores, while the larger solute is excluded. In the diagram on the right, V_o and V_t represent the totally excluded and included volumes, respectively. Reprinted from *Developing HPLC Separations, Book One* (1991), Millipore, Milford, with permission.

Gel Permeation Chromatography

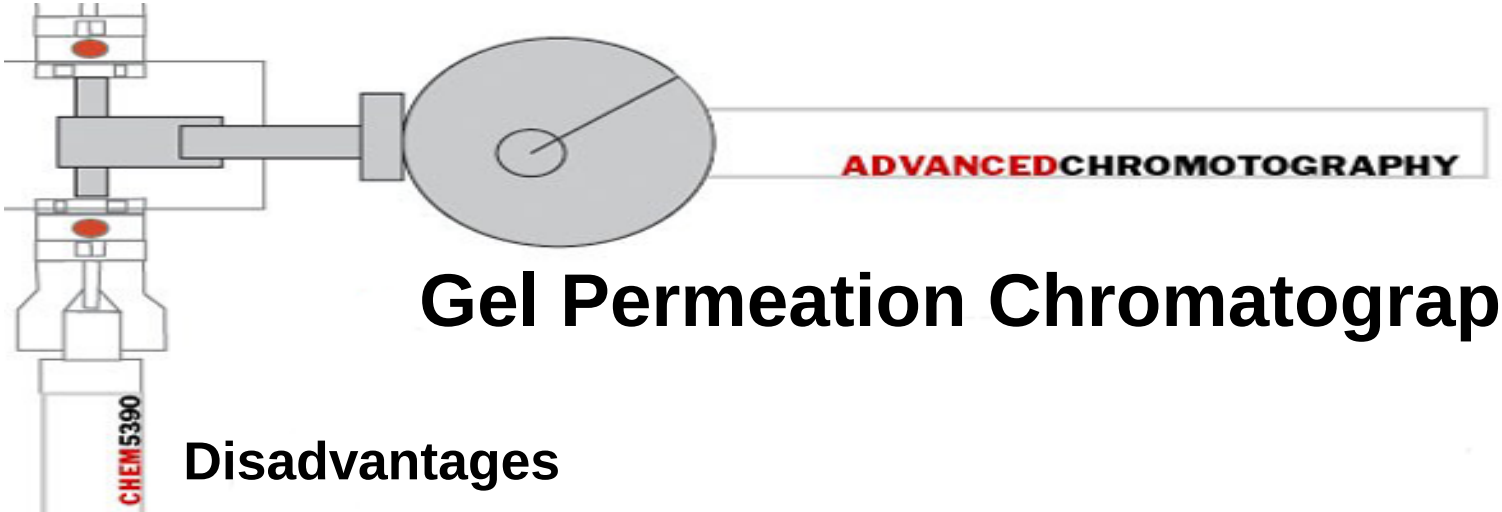
Advantages

- **Since solutes are eluted within a small retention volume, peaks are narrow**
- **Inexpensive detectors can be used, like RI**
- **Retention is very predictable, so retention times can be used to predict the MW or size of an unknown molecule**

Gel Permeation Chromatography

Advantages

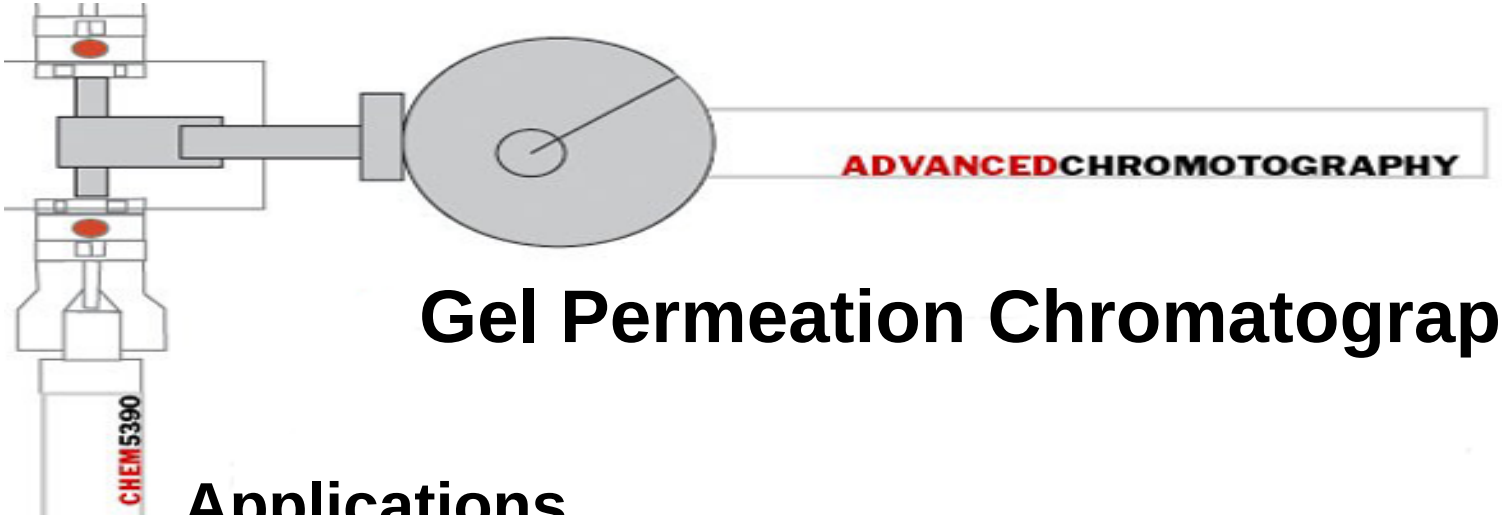
- **Simplicity – can use an isocratic elution**
- **GPC is a good alternative to RPLC for samples that contain high levels of triglycerides and other hydrophobic materials, i.e. creams, ointments, or lotions. These tend to bind strongly on reverse phase surfaces.**



Gel Permeation Chromatography

Disadvantages

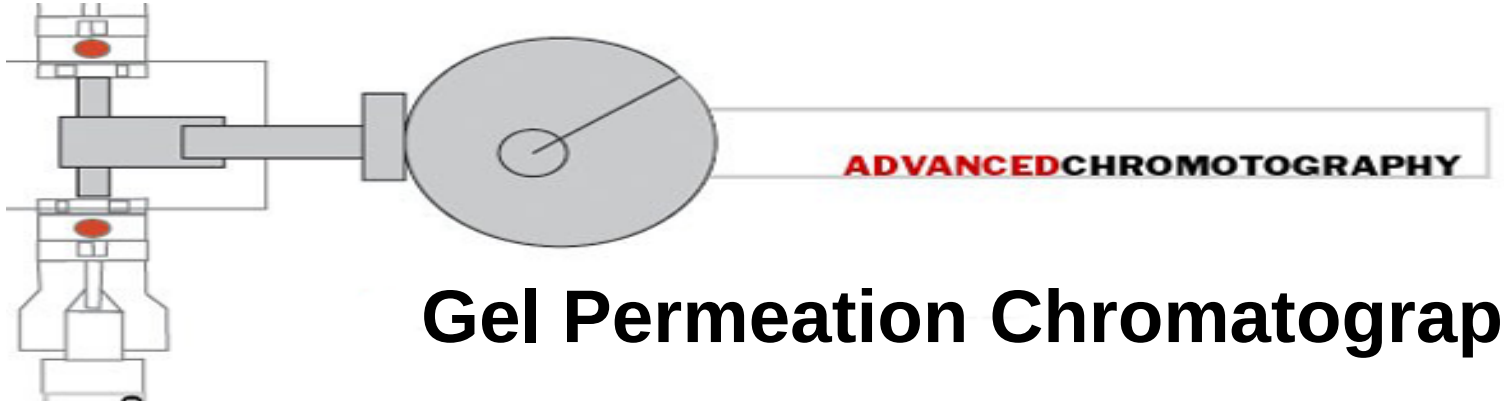
- **Lack resolving power because of limited peak capacity, molecules must differ in molecular size by 10% in order to be resolved by GPC.**



Gel Permeation Chromatography

Applications

- to characterize polymers and mixtures into discrete fractions, such as polymer, oligomer, monomer and any non-polymeric additives.
- to separate biomolecules (i.e. proteins fractionation)
- has been used to purify certain RNA and viruses



Gel Permeation Chromatography

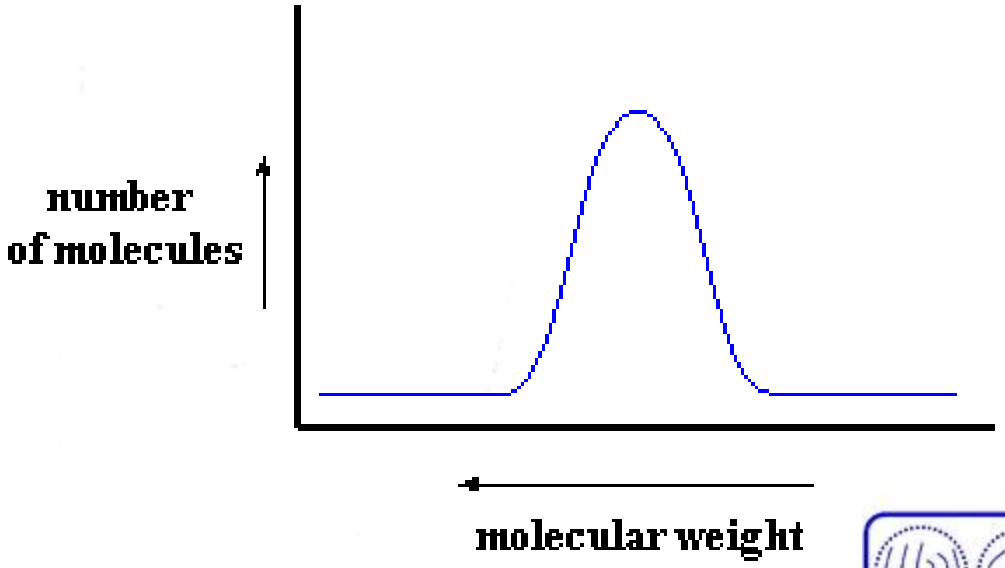
Applications

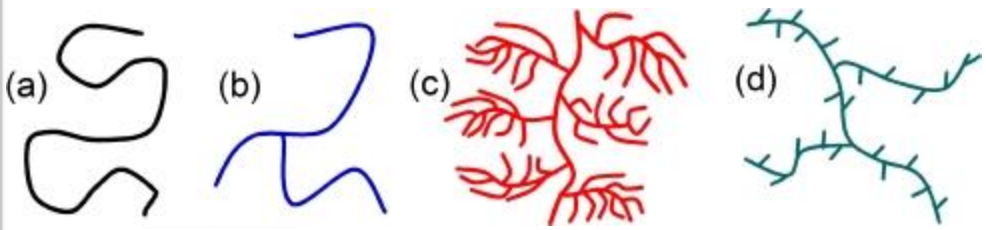
Characterization of polymers

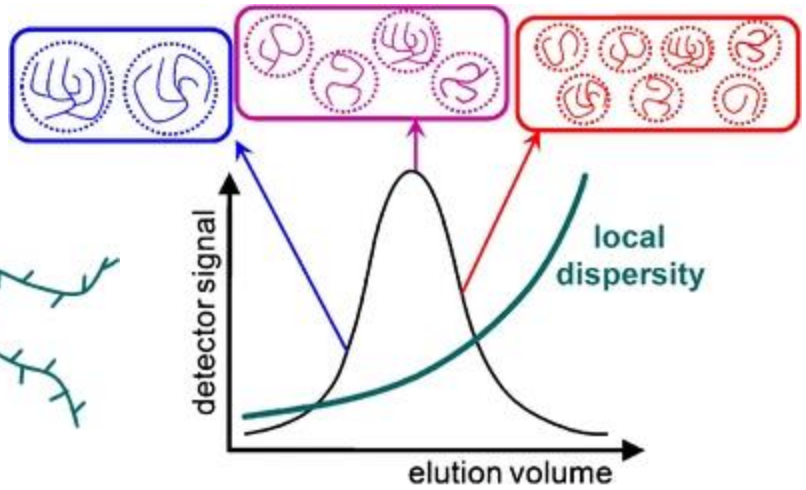
Molecular weight distribution determines a polymer's properties, i.e., tensile strength, brittleness, hardness, melt viscosity.

When dealing with a polymer, it is not a single molecular weight, but a distribution of molecular weights, an average molecular weight.

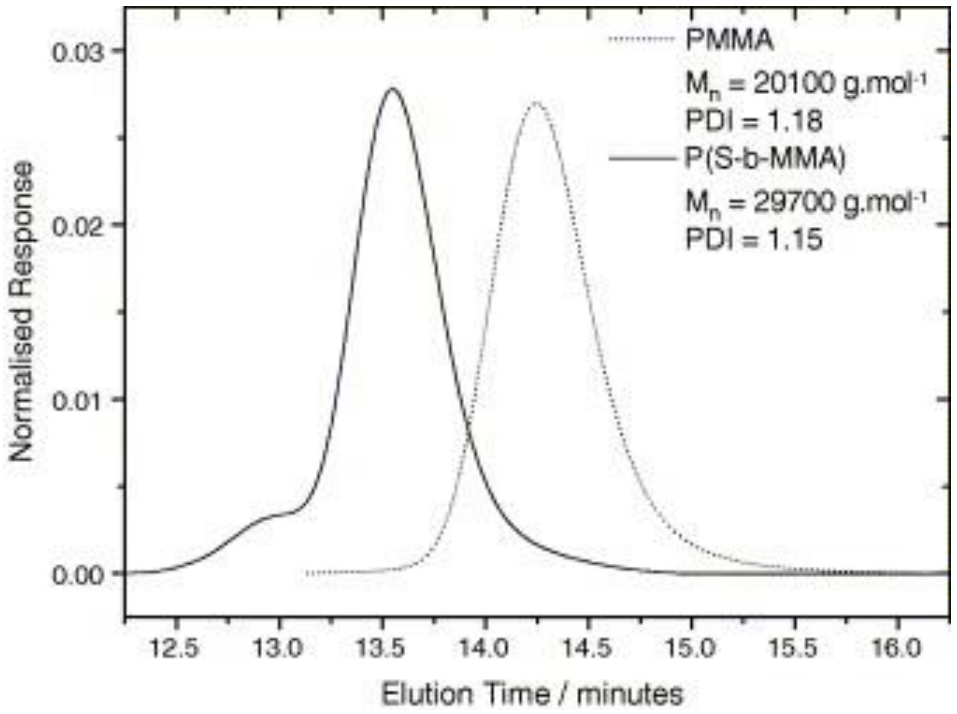
Gel Permeation Chromatography

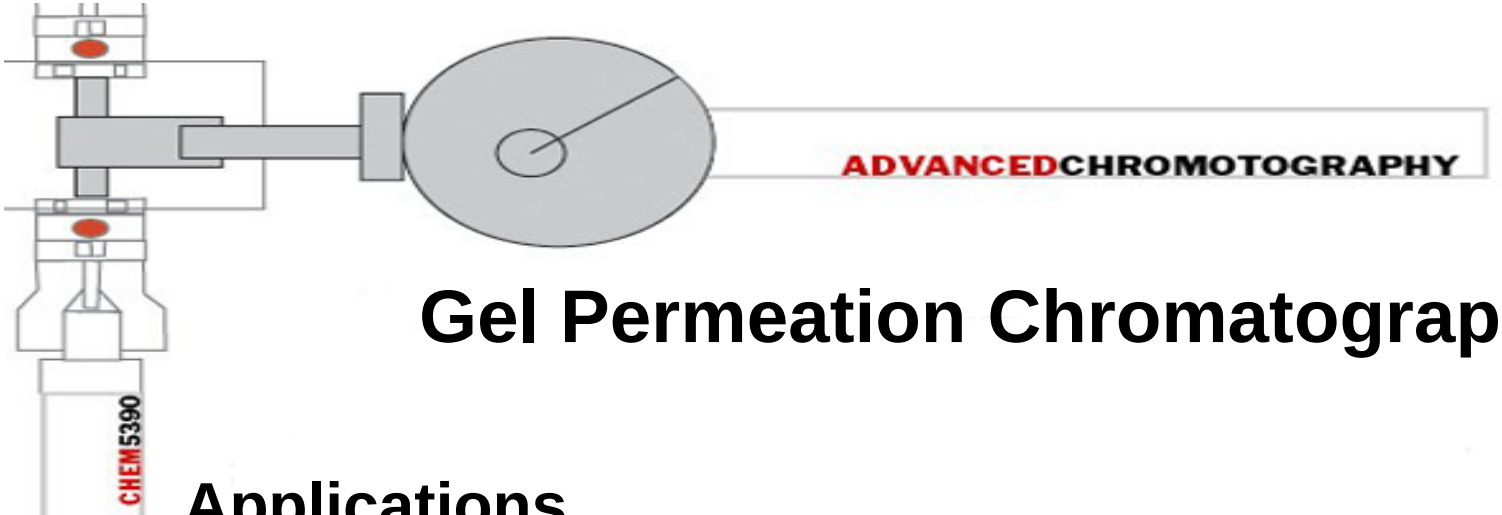






Gel Permeation Chromatography





Gel Permeation Chromatography

Applications

Characterization of proteins

Used to separate, purify, and characterize.

Gel Permeation Chromatography

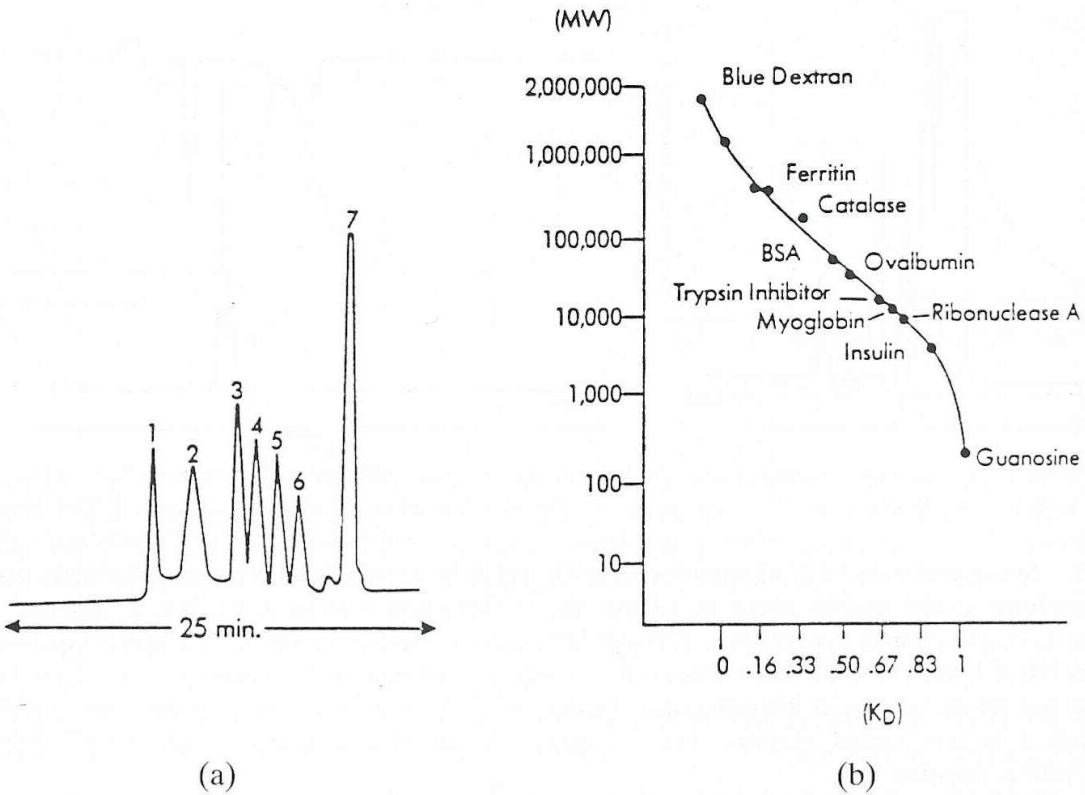
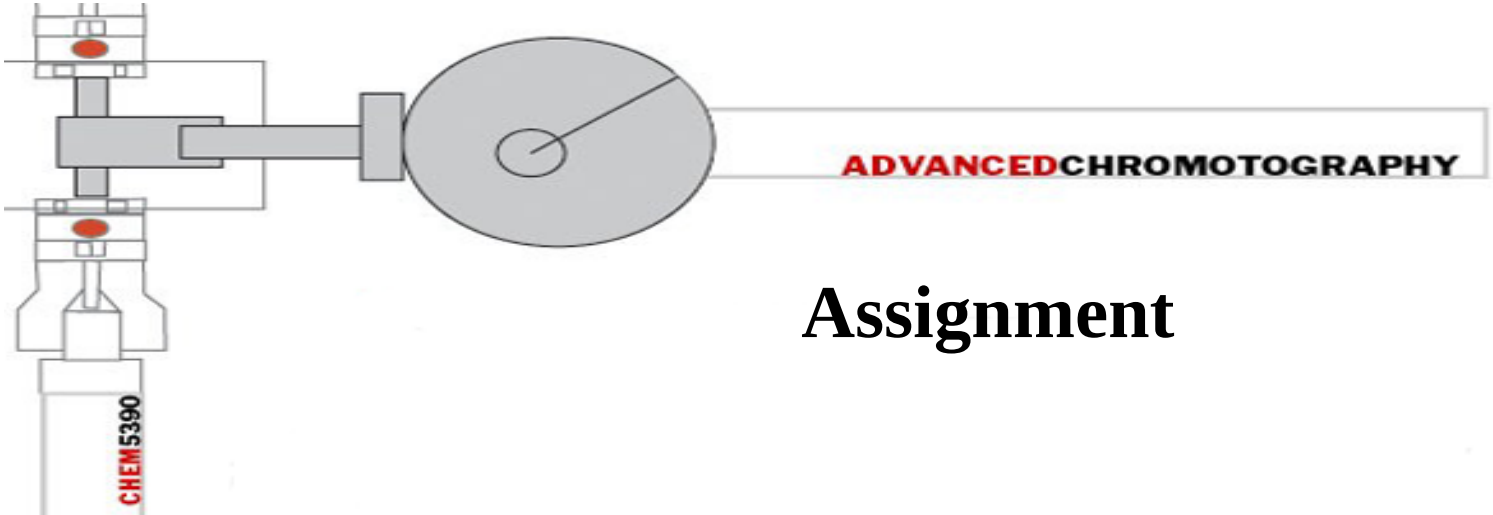


Fig. 6.26. Gel filtration chromatography of proteins. Waters Protein-Pak 300 SW columns (×2) were used with 0.1 M potassium phosphate monobasic as the mobile phase at 1.0 ml min^{-1}. Detection was by direct u.v. at 280 nm. Solute identities: 1, blue dextran; 2, ferritin; 3, bovine serum albumin; 4, ovalbumin; 5, trypsin inhibitor; 6, ribonuclease A; 7, guanosine. Reprinted from *Waters Sourcebook of Chromatography* (1992), Millipore, Milford, with permission.



Assignment

- Research papers Due Tuesday December 5th