Swayam Course - Analytical Techniques

Week: 3, Module 7 - Gel Filtration: Principles, Methodology& Applications

Content Writer - Dr. Savita Yadav, professor, Department of Biophysics,All India Institute of Medical sciences, New Delhi.

1. Learning outcomes

- Basic terminology
- Principle of gel filtration chromatography
- Theory of gel filtration chromatography
- Selection of media
- Column packing and process of gel filtration chromatography
- Factors affecting resolution
- Applications

2. Introduction

Chromatography is a laboratory process for the segregation of a mixture. The mixture to be separated is applied to a chromatography column after dissolving in a fluid (liquid or gas) which is called the mobile phase. This mobile phase moves the mixture through the stationary phase, which separates it into its constituents. This separation occurs as the various constituents of the mixture move at different speeds through the stationary phase. In gel filtration chromatography, the reason for this difference in speeds is the difference in size of the molecules of various constituents.

Gel filtration chromatography is used to separate proteins, peptides and oligo-nucleotides on the basis of their size only. It is also known as size exclusion chromatography. The sample, which is a mixture needing separation is applied to the mobile phase and moves through a column of porous beads. Molecules of different sizes diffuse into these pores to a greater or lesser extent. Small molecules enter pores easily and thus, elute slowly. Whereas, large molecules cannot enter many pores and move directly without entering-leaving-re-entering them thus, eluting rapidly.

Gel filtration chromatography may be preparative or analytical. Preparative chromatography is basically a purification process while analytical chromatography is demonstrating the presence or evaluating the relative percentages of analytes in a mixture. Gel filtration chromatography is widely applied for molecular size analysis, separation of components of a mixture, as well as for desalting and buffer exchange.

Nearly 50 years after Tsvett discovered column liquid chromatography, gel filtration chromatography was recognized, accidently, as a new method for molecular separation. In the early 1950's, some researchers noted that neutral small molecules and oligomers were eluted on the basis of decreasing molecular weight from a column of cross-linked polystyrene ion-exchange resins. Deuel and Lindquist also reported similar results for the elution of low-molecular weight molecules using cross-linked galactomannan and starch granules. Then, in 1955–1956, Lathe and Ruthven used columns packed with starch granules to study the elution profiles of low and high molecular weight polysaccharides and proteins. Surprisingly, they observed that high molecular weight molecules eluted faster than low molecular weight molecules. They performed the first biopolymer size separation process. Lathe and Ruthven called the process "gel filtration". In 1959, two Biochemists- Per Flodin and Jerker Porath- synthesized a series of cross-linked dextran resins with different pore sizes for size separation of peptides and oligosaccharides. Molecular weight separation of non-polar molecules was first performed by Brewer who used swollen rubber granules for the separation of hydrocarbons. In 1962, John Moore developed cross-linked polystyrene

resins of known porosities and particle sizes for the Gel Permeation Chromatography (Gel Filtration Chromatography was known as Gel Permeation Chromatography at that time) of synthetic polymers. This was revolutionary as now the molecular weight distribution of polymers, which could be dissolved in toluene (the mobile phase used), could be obtained in hours which was much faster compared to weeks or months with traditional methods.

3. Terminology

Some basic terms and components should be understood before proceeding.

- Analyte: It is the mixture which needs to be separated. It can also be used to denote the fraction or part of interest in the mixture.
- Stationary/immobilized/solid phase: It is bonded to support particles or to the column's wall and hence immobilized. It can be a solid, gel, liquid or a solid/liquid mixture.
- Mobile phase: It moves in a definite direction over or through the stationary phase after the mixture to be resolved has been applied over it. It may be a liquid or a gas. The mobile phase flows through the column where the analyte interacts with the stationary phase and gets resolved.
- Eluate/effluent: It is the mobile phase exiting the column.
- Eluent: It is the solvent that moves the analyte.
- Detector: It is the device used for detection of analytes when they come out of the column after resolution.
- Chromatogram: It is the visible product, in the form of a graph, of the chromatograph.

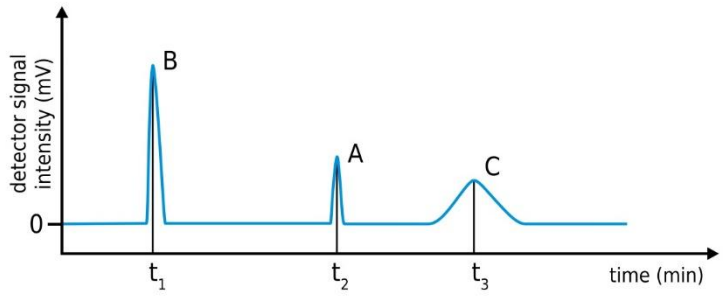

Figure: Chromatogram

4. Principle

Gel Filtration Chromatography achieves separation of molecules on the basis of their molecular size and shape. It is normally done in a column. This is a hollow tube packed with beads having pores of different sizes. It uses the molecular sieve properties of various porous resins. These pores may be troughs on the surface of beads or passages formed between them. They make-up the solid or stationary phase. As the mobile phase passes down the chromatographic column, particles or molecules of the mixture enter into the pores. Large molecules or particles will be unable to enter a majority of these pores. They directly pass by these pores as they are too big to enter them. Whereas, small molecules are able to enter a majority of these pores. They enter into a pore, exit it and then enter another pore and get distributed between the mobile phase inside and outside the beads and therefore pass through the column at a slower rate. Therefore, the larger the size of the particle, the faster it travels down the column as its path is straighter. While smaller the size of a particle, the slower it travels down the column as its path is more zig-zag. In other words, a small molecule can pass through every volume of the stationary phase pore system and therefore can penetrate a volume equal to the total pore and interparticle volume. This small molecule passes through the entire pore and interparticle volume (~ 80% of the column volume) and hence exits the column late. On the other hand, a very large molecule cannot pass through small pores and can enter only the interparticle volume (~35% of the column volume) and therefore exits the column earlier when this much volume of the mobile phase has flown down the column. This results in the resolution of a mixture based on the size of the molecules.
An important consideration is that the all the particles of the mixture should be applied concurrently or almost concurrently. Also, these molecules should not have any interactions with the stationary phase. Its

passage should ideally be based only on the pores it can or cannot enter depending on its size and not on any chemical or electrostatic interactions with the stationary phase.

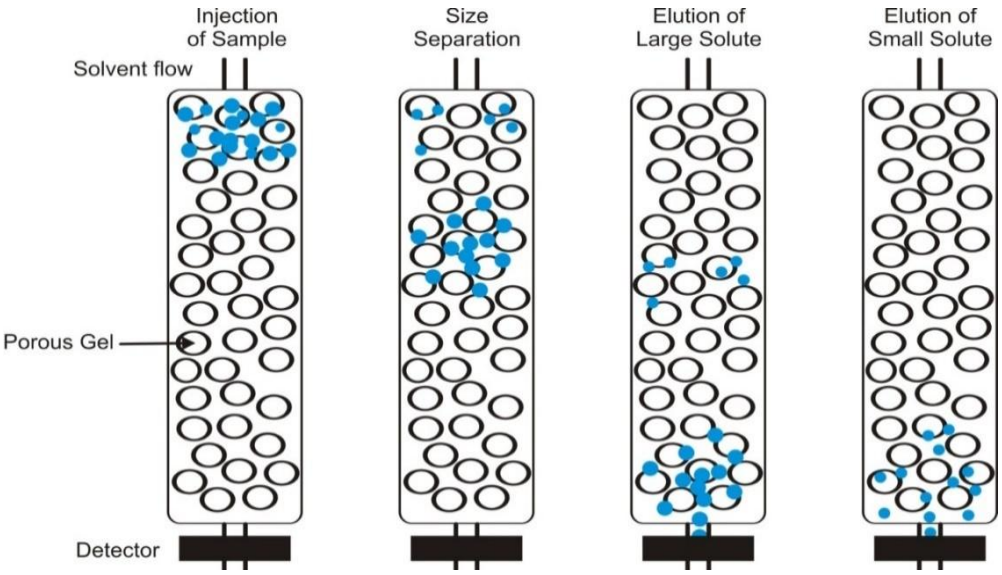

Figure: Principle of Gel filtration chromatography

5. Theory

Distribution/partition coefficient (K_d)

It is the ratio of concentration of a compound (analyte) in 2 immiscible phases, X and Y, at a given temperature. It is the basis of all forms of chromatography. It explains the way in which the analyte distributes between X and Y.

$$K_d = \frac{concentration\ of\ analyte\ in\ X}{concentration\ of\ analyte\ in\ Y}$$

Effective distribution coefficient

It is the ratio of the total amount of a compound (analyte) in 2 immiscible phases X and Y, at a given temperature.

$$= \frac{amount\ in\ X}{amount\ in\ Y}$$

$$= \frac{concentration\ of\ analyte\ in\ X * volume\ of\ X}{concentration\ of\ analyte\ in\ Y *\ volume\ of\ Y}$$

$$= K_d * \frac{volume\ of\ X}{volume\ of\ Y}$$

$$= K_d *\ Volume\ ratio$$

During the chromatography, the analytes repeatedly move to and fro between two phases and their different K_d's lead to their separation.

Bed volume (V_t)
The total column volume is referred as bed volume (V_t).

Void volume (V_0)
The space between the gel particles is called void volume (V_0). It is the free space between the particles of the matrix coated with the stationary phase. In well packed columns, the void volume is about 1/3rd of the bed volume. The large molecules pass through the void volume. While the small molecules which gain full access to the complete mobile phase, both in this free space and that in the pores, will elute after the one bed volume.
$V_0 = V_t$ - Volume occupied by gel matrix (V_{gel}) – Volume of Stationary phase (V_s)

Elution Volume (V_e)
The volume of the buffer that elutes from the column before a particular peak in the elution profile appears is called elution volume (V_e).

It is tough to estimate the concentration of an analyte in the 2 phases. Hence, K_d needs to be defined in terms of simply quantifiable variables, V_e and V_o. The analyte spends some time in the stationary phase and elutes at volume V_e. The volume V_e - V_0 is the 'extra' volume that it passes through due to its small size. This 'extra' volume is a part of the total pore volume (V_i). Now, K_d can be defined as

$$K_d = \frac{V_e - V_0}{V_i}$$

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

Retention time (t_R)
Retention time for an analyte is the time period between sample loading and detection of that analyte by the detector (time between sample loading and analyte peak). It has 2 parts.

- Dead time
- Adjusted retention time

Dead time (t_M)
It is the time taken (t_M) by the analyte to pass through the void volume (V_0). This is constant for all analytes and can be calculated by passing an analyte which has large size and therefore doesn't interact with the stationary phase by entering any pore. This large molecule passes through the mobile phase in the void volume.

Adjusted retention time (t'_R)
Adjusted retention time for an analyte is the time it spends in the stationary phase. It is specific for a specific analyte and column. It is equal to an analyte's retention time (tR) minus the elution time of an unretained peak (tm) i.e.

$$t'_R = t_R - t_M$$

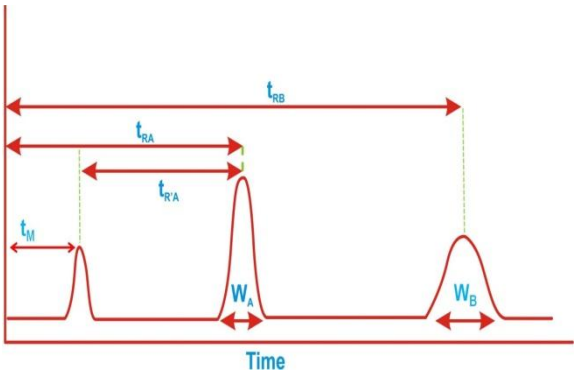

Figure: Representative chromatogram

Retention/capacity factor (k)

It is the extra time that an analyte takes to elute from the column compared to an excluded analyte that does not enter any pore which, by definition, has k = 0. It is a crucial criterion in chromatography.

$$k = \frac{t_R - t_M}{t_M}$$

$$k = \frac{t'_R}{t_M}$$

k is dimensionless and has no units. Its value, ideally, should be between 1 and 10.

Suppose,

Time for which an analyte is present in stationary phase (t_S) = x

Time for which an analyte is present in mobile phase (t_M) = x

For this analyte,

$t_R = t_S + t_M = 2x = 2(t_S) = 2(t_M)$

$$k = \frac{t_R - t_M}{t_M}$$

$$k = \frac{2t_M - t_M}{t_M}$$

$$k = 1$$

Similarly, if the analyte spends x in the mobile phase and 8x in the stationary phase, t_R = 9x and k = 8.

Hence, it is seen that k is related to K_d as follows.

$$k = \frac{t'_R}{t_M}$$

$$k = \frac{M_S}{M_M}$$

M_S = mass of analyte in the stationary phase
M_M = mass of analyte in the mobile phase

$$k = \frac{concentration\ of\ analyte\ in\ stationary\ phase * volume\ of\ stationary\ phase}{concentration\ of\ analyte\ in\ mobile\ phase * volume\ of\ mobile\ phase}$$

$$k = \frac{concentration\ of\ analyte\ in\ stationary\ phase}{concentration\ of\ analyte\ in\ mobile\ phase} * \frac{volume\ of\ stationary\ phase}{volume\ of\ mobile\ phase}$$

$$k = K_d * \frac{V_S}{V_M}$$

V_S = volume of the stationary phase
V_M = volume of the mobile phase

$$k = K_d * \beta$$

β = Volumetric phase ratio = V_S/V_M

Volumetric phase ratio (β)
It is the ratio of the volume of stationary phase to the volume of mobile phase.

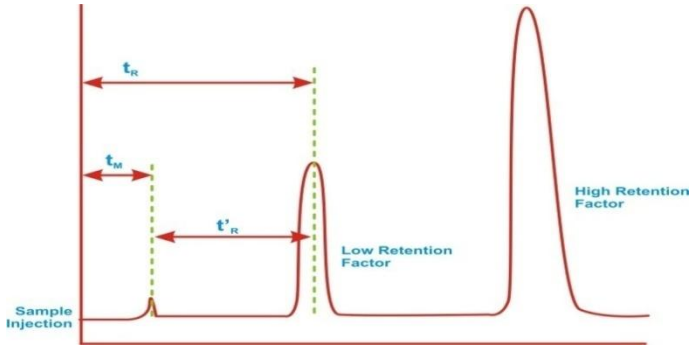

Figure: Representative chromatogram

6. Gel filtration media

The commonly used media for gel filtration chromatography are dextran (Sephadex™), polyacrylamide (Bio-Gel P™), dextran-polyacrylamide (Sephacryl™) and agarose (Sepharose™ and BioGel A™). Every media has a span of molecular weights that it can resolve. At the upper limit come the molecules having molecular weight so large that they cannot penetrate the pores at all while at the lower limit come the molecules having molecular weight so small that they can penetrate all the pores. The media are available in a large range of pore sizes for separation of macromolecules of different sizes.
A gel with a smaller range of pore sizes gives higher resolution, while a gel with a wider range gives lower resolution. However, the benefit of using wider range gels is that they can be used when molecular weights of the constituents of the analyte are unknown as they have the ability to resolve a higher range of sizes. Some widely used media are listed in the table below.

S. No.	Type of media	Commercial name	Molecular weight range (kDa)
1.	Dextran	Sephadex G-50	1.5-30
		Sephadex G-75	3-80
		Sephadex G-100	4-150
		Sephadex G-200	5-600
2.	Polyacrylamide	Bio-Gel P-10	1.5-20
		Bio-Gel P-30	2.5-40
		Bio-Gel P-100	5-100
		Bio-Gel P-150	15-150
		BioGel P-200	30-200
3.	Dextran-polyacrylamide gels	Sephacryl S-200	5-250
		Sephacryl S-300	10-1500
		Sephacryl S-400	20-8000
4.	Agarose	Sepharose 6B	10-4000
		Sepharose 4B	60-20,000
		Bio-Gel A-0.5	10-500
		Bio-Gel A-1.5	10-1500
		Bio-Gel A-5	10-5000

Table: Different commercially available media for gel filtration chromatography.

The general considerations for selecting gel filtration chromatography media may include fractionation range, operating pressure & temperature, flow rate, sample viscosity, pH range and tolerance for detergents, salts, etc.

7. Column Dimensions

The column dimensions are very important. For gel filtration chromatography, resolution increases with the column length. The column diameter increases the capacity of the column as bed volume increases. General recommendation for gel filtration column is more in length but less in diameter, in comparison to other types of chromatography.

Figure: Chromatography column

8. Packing of a column

Packing a column can be divided into 3 parts - swelling the gel, pouring the gel into the column and equilibration of the column.

Swelling the gel

- Add appropriate amount of filtered ddH_2O to the gel matrix depending on the swelling capacity of the beads as per the manufacturer's recommendations.
- Once the gel has swollen and settled down in the beaker, decant the ddH_2O on the top.
- Add appropriate buffer to the gel, mix gently (do not use magnetic stirrer for mixing) and wait for it to settle down.
- Decant and resuspend the gel in an equal volume of the buffer and degas the gel in order to remove any trapped air.
- Rapid swelling of the gel can be achieved by heating the slurry at 90^{o}C for 5 hours. Swelling at room temperature is considerably slower, and takes approximately 72 hrs.
- All buffers and the gel should be degassed to avoid air bubbles. Air bubbles cause uneven packing of the column and give rise to poor resolution.
- All buffers should be filtered before use.

Pouring the gel into the column

- Mount the glass column vertically on a metal stand and adjust the bottom adapter.
- Add some buffer to the empty column and allow it to pass through outlet tube so that any trapped air is removed.
- Block the outlet tubing and pour the gel slurry to fill the column up to the required height.
- Gel slurry should be poured with the help of a glass rod in such a way that the glass rod touches the inner wall of the column.
- This avoids the introduction of any air bubble.
- A gel reservoir should be used for smooth and continuous pouring of the gel.
- Allow it to settle down and decant any excess buffer or gel.
- Put the top adapter.

Equilibration of the column

- Attach the top tubing to the peristaltic pump and buffer reservoir.
- Keep the bottom tubing to waste.
- The operating pressure should be according to the manufacturer's recommendations for the gel matrix.
- Choose the buffer as per purification needs and equilibrate the column with 2-3 bed volumes of this buffer.

9. Process of gel filtration

Buffer selection

- Any buffer in which the analyte to be separated is stable can be used as pH, ionic strength and composition do not significantly affect resolution.
- Low strength salt can be added to the buffer to avoid any interaction with the gel particles or if there is an ionic interaction between the molecules to be separated.
- The buffer in which the sample is dissolved (sample buffer) does not have to be the same buffer as the column, but prior to running, the column should be preferably equilibrated with the sample buffer.
- Extreme pH conditions and ionic strengths should be avoided because that may affect the gel as well as the separation.

- All buffers should be filtered through a 0.22µm membrane before use.
- Some commonly used buffers are Tris-HCl and phosphate.

Sample preparation & loading

- The sample should not be diluted as it may cause band broadening.
- However, it should not be so concentrated that it precipitates.
- Ideally, sample volume equal to 1% of the bed volume should be loaded onto the column.
- The sample should be filtered prior to loading and be free of any debris.
- To load the sample manually, remove the upper adapter and remove excess buffer on the top of the gel without disturbing the gel surface.
- Then, gently pipette in the sufficient amount of sample and allow the sample to pass through the surface.
- Care should be taken that the surface of the gel does not dry.
- Finally, add a little buffer to the column and set the upper adapter.
- The above processes can also be done by automated sample injector.

Flow rate

- Ideally the flow rate should be slow, in the range 6-12mL/hr.
- A flow rate slower than this may cause diffusion of the band.
- A flow rate faster than this may cause poor resolution.

Elution

- A fraction collector is attached to the system to collect the fractions of the elution.
- The sample is eluted isocratically using a single buffer system.
- The length of tubing connected to fraction collector should be small and small fractions of size 1mL or less should be collected to avoid mixing of peaks.
- The detector connected to the fraction collector analyses the separation.
- The total elution should be equal to or more than one bed volume.

After the completion of each and every run, the column should be washed thoroughly to remove any analytes left in the gel. Ideally, it should be done using 1-2 bed volumes of buffer. For long term storage, antimicrobial agents should be added to the buffer.

10. Calibration of the column

For accurate determination of the molecular weights of separated molecules, a gel filtration column needs to be calibrated with known standard molecular weights. For this purpose, a calibration curve is drawn by plotting the logarithm of the molecular weights of standard proteins against their respective K_d. The calibration standards should be selected in such a way that the relationship between molecular weights of standards and molecular weights of the analyte is the same.

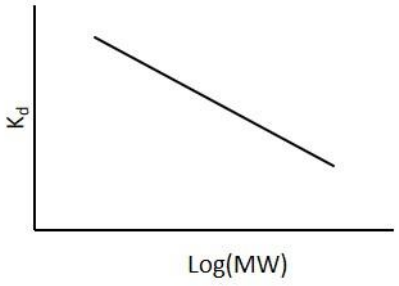

Figure: Plot of Log (MW) against K_d

11. Factors affecting resolution in gel filtration chromatography

There are numerous factors which affect the separation quality in gel filtration. The important factors include-

1) Column dimensions
2) Selection of media
3) Composition of buffer
4) Sample volume and concentration
5) Sample loading
6) Flow rate
7) Length of tubing
8) Fraction size

12. Applications

1) Molecular weight determination
   Gel filtration chromatography is widely applied for determination of the molecular weight of proteins. The molecular weight of a given protein is estimated by comparing its elution volume with that of known protein standards. The standard curve is constructed by plotting logarithm of the molecular weights of standard proteins against their respective K_d. This standard curve is then used to determine the molecular weight of unknown proteins.

2) Desalting
   Salt molecules and buffer components are several times smaller in comparison to macromolecules. For this reason, gel filtration chromatography using a resin with smaller size-exclusion limits can be used for desalting and buffer exchange applications. Desalting is the process of salt removal from a protein sample, while in buffer exchange a more appropriate buffer is used to replace a less appropriate one. The separation limits for resins are generally up to 10 kDa. Resins with large exclusion limits are not suitable for buffer exchange and desalting application.

3) Separation of macromolecules
   Gel filtration chromatography is commonly applied in research laboratories for the separation of proteins and peptides. It is also used for detection and separation of oligomers.

4) Purification
   Gel filtration chromatography can be used to purify viruses, enzymes, hormones, antibodies, nucleic acids etc. by using appropriate gels. is also applied for fractionation of crude samples into low and high molecular weight protein groups.

5) Concentration of samples
   Solutions can be concentrated by introducing dry Sephadex G-10 which soaks up water and small molecules while large molecules remain in solution. The gel can be separated by centrifugation thereby enriching the solution without effecting other properties like pH, ionic strength etc.

13. Precautions

- Selection of appropriate media is very essential. It should be selected according to the molecular weight of the molecule to be separated.
- The column length should be enough, approximately 4ft, to provide better resolution.
- Flow rate should be appropriate. It should not be very slow such that it allows diffusion of sample or very fast such that it results in poor resolution.
- Sample should not be precipitated before loading.

14. Explanatory example

Suppose we have a mixture of equal quantities of three proteins - albumin, pepsin and lysozyme which needs to be resolved. The average molecular weights of these proteins are 66 kDa, 35 kDa and 15 kDa respectively. We choose pre-made Sephacryl S-100 (cross-linked copolymer of allyl dextran and N,N'-methylene bisacrylamide) column bed volume = 320 mL (GE Life Sciences) which has a resolving range of 1-100 kDa. As the molecular weights of our proteins are within this range, this resin can be used. After column preparation according to the method described in the text above and as per manufacturers recommendations, the sample is loaded into the column and fractions collected.
The following chromatogram is obtained.

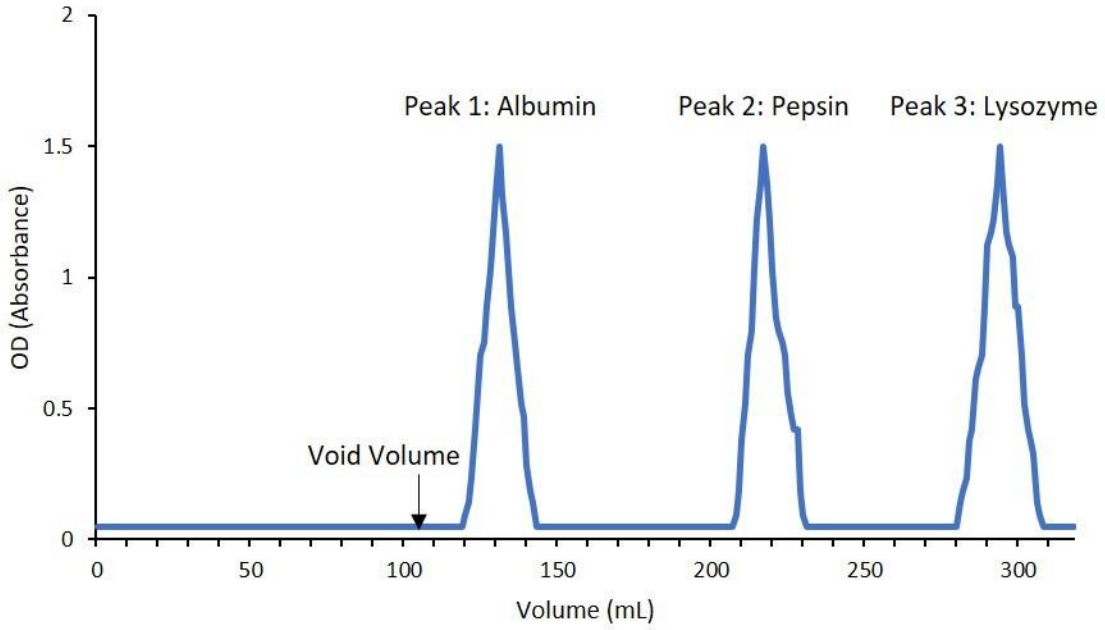

Figure: Chromatogram showing Gel Filtration Chromatography - Albumin, Pepsin, Lysozyme

As albumin is the heaviest and largest molecule in our mixture, it will travel the fastest. It can access the least number of pores in the column and hence its elution volume is the shortest. Therefore, it exits the column earliest (1).
As lysozyme is the lightest and smallest molecule in our mixture, it will travel the slowest. It can access the maximum number of pores in the column and hence its elution volume is the largest. Therefore, it exits the column in the end (3).
As pepsin is intermediate compared to albumin and lysozyme, it will exit after albumin and before lysozyme (2).

15. Comparison of three commonly used chromatographic techniques

S. No.	Gel filtration chromatography	Ion exchange chromatography	Affinity chromatography
1.	It is based on separating molecules based on their molecular size and weight alone.	It is based on separating molecules pased on their charge which can be changed by adjusting the pH in relation to their pI.	It is based on specific interactions between the molecule of interest and a specifically designed ligand.
2.	The molecular weight/size of the molecules of interest should be different.	The pI of the molecules of interest should be different.	Specific ligands of the molecules of interest should be available.
3.	It can also be used to determine the molecular weight of the components of a mixture.	It can also be used for protein purification.	It can also be used to study protein-protein interactions.
4.	It can be used for purification,	It is used in water	It is used to deplete

	concentration and desalting.	treatment and other applications like pharmaceutical research food industry, chemical industry etc.	high abundant proteins like albumin from a sample. It can also be used to enrichment specific proteins or protein groups and in the study of protein-protein interactions or the purification of specific antibodies and proteins.
5.	Upon elution, purified protein will be diluted and will have to be concentrated for further experimentation.	Upon elution, purified protein will always be in salt, which will have to be removed for further experimentation.	Upon elution, protein will be bound tightly to the ligand and may require severe conditions for separation which can cause denaturisation.

16. Summary

- Gel filtration chromatography is used to separate macromolecules on the basis of their size.
- Wide range of gel filtration media is available to choose from as per one's requirements.
- Factors that affect separation and resolution may include column dimensions, media, flow rate, etc.
- Gel filtration is commonly applied for molecular weight determination, purification of proteins and peptide, desalting and buffer exchange.