Module 5 Chromatographic Techniques

Lecture 28: Principles of Chromatography

Introduction: The molecules present in biological system or in synthetic chemistry are produced through a series of reactions involving intermediates. As discussed in previous lecture, at any moment of time biological organism has major fraction as desired product but has other compounds in minute quantities. The minor species present in a product is always referred as "impurities" and these compounds need to separate from desired product for biotechnology applications. **How two molecules can be separated from each other?** To answer this question we can take the example of three molecules given in Figure 28.1. These 3 molecules (benzene, phenol, aniline) are similar to each other but have distinct physical and chemical properties which can be used as a criteria to separate them. The physical and chemical properties which can be use to separate molecules are-

Physical Properties

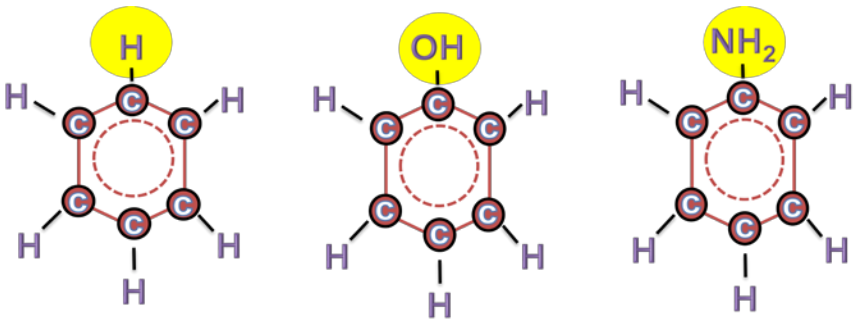
1. Molecular weight
2. Boiling point (in case both are liquid, as in this case)
3. Freezing point
4. Crystallization
5. Solubility
6. Density

Chemical Properties

1. Functional Group, for example, phenol has –OH where as aniline has NH2.

2. Reactivity towards other reagent to form complex

Now for example you have a mixture of compound 1 (benzene) and compound 3 (Aniline) and you would like to purify benzene rather than aniline. In this situation, you can take the physical and chemical properties of benzene into the account and isolate it from the mixture.



Name	Benzene	Phenol	Aniline
Molecular formula	C_6H_6	C_6H_6O	$C_6H_5NH_2$
Molar mass (g mol^{-1})	78.11	94.11	93.13
Density	0.8765 g cm^{-3}	1.07 g cm^{-3}	1.0217 g ml^{-1}
Melting point (°C)	5.5	40.5	-6.3
Boiling point (°C)	80.1	181.7	184.13

Figure 28.1: Chemical Structure and physical Properties of benzene, phenol and aniline.

Principle of Seperation: <u>How a physical or chemical property will allow to isolate a particular substance?</u> The mixture of compound 1 and 3 is shown in Figure 28.2 and assume if we are using boiling point as a criteria to isolate them. As we will heat the mixture there will two phase forms, one liquid phase and other is vapor phase. The molecules of compound 1 and 3 will distribute between these two phases and as the temp is near to boiling point of compound 1, more amount of 1 will be present in vapor phase than liquid phase. Where as more number of compound 3 will be in liquid phase. Eventually as this process will continue, at the end two molecules will get separated from each other. The distribution coefficient (Kd) to describe the distribution of compound 1 between two phase A and B is as follows:

$$\text{Kd} = \frac{\text{Concentration in Phase A}}{\text{Concentration in Phase B}}$$

Similarly one can also exploit other physical & chemical parameters as well. With each and every physical and chemical parameter the molecule present in the mixture will distribute as per their behavior in each parameter.

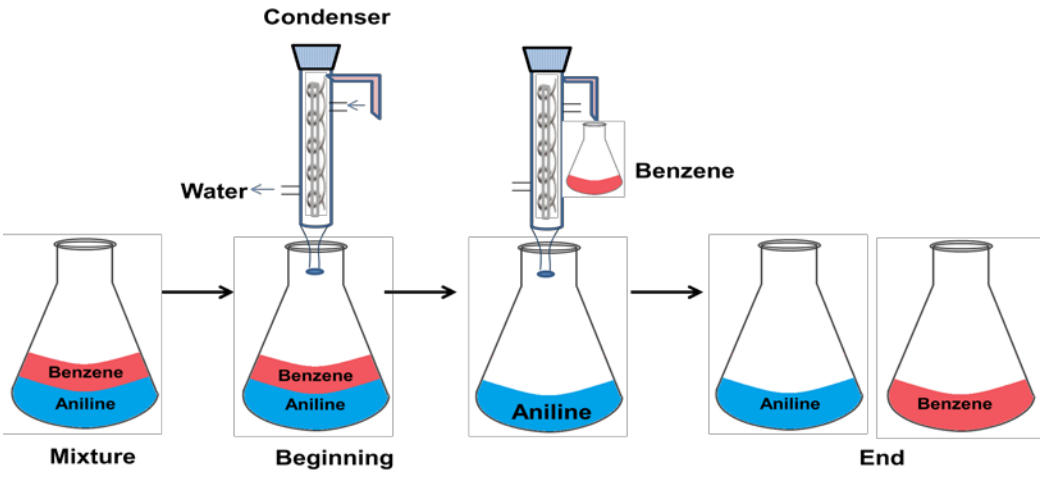


Figure 28.2: Distribution of molecules during distillation.

Chromatography: The purpose of chromatography is to separate a complex mixture into individual component exploiting the partition effect which distribute the molecules into the different phases. As discussed above, a distribution of a molecule between two phases A and B is given by a distribution coefficient, Kd. In most of the chromatography techniques, phase A is stationary phase or matrix and phase B is mobile phase or buffer.

Column Chromatography: In column chromatography, a stationary phase is filled into a cylindrical tube made up of glasss or steel. The mixture of analyte is loaded on the top and it runs from top to bottom. **How Kd is exploited in column chromatography ?** Assume two molecules, X and Y with a Kd value of 1 and 9 and they are traveling through a column with water as mobile phase as given in Figure 28.3. As they will travel, X and Y will partition between stationary phase and mobile phase. As there is a huge difference in Kd, Y will be associated with the matrix and remain on the top of the column where as X will move along the water. At the end of chromatography, X will come out first whereas Y will come out last.

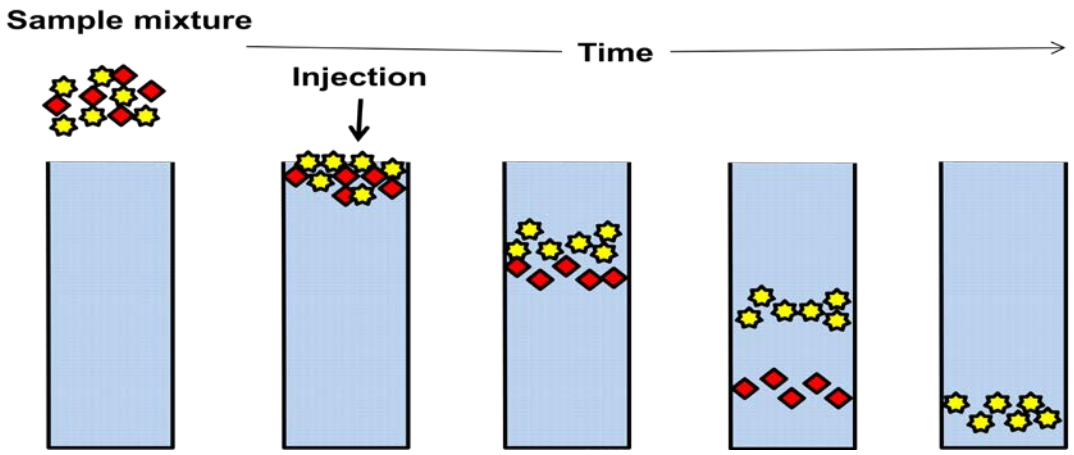


Figure 28.3: Seperation of two molecules on a column.

Chromatogram: The plot of elution volume along with the absorbance is known as chromatogram as given in Figure 28.4. The volume or time it takes for a analyte to come out from the column is known as retention volume or time. The chromatogram may have separate peaks (A and B) or peaks (C and D) with overlapping base, these peaks are called fused peaks.

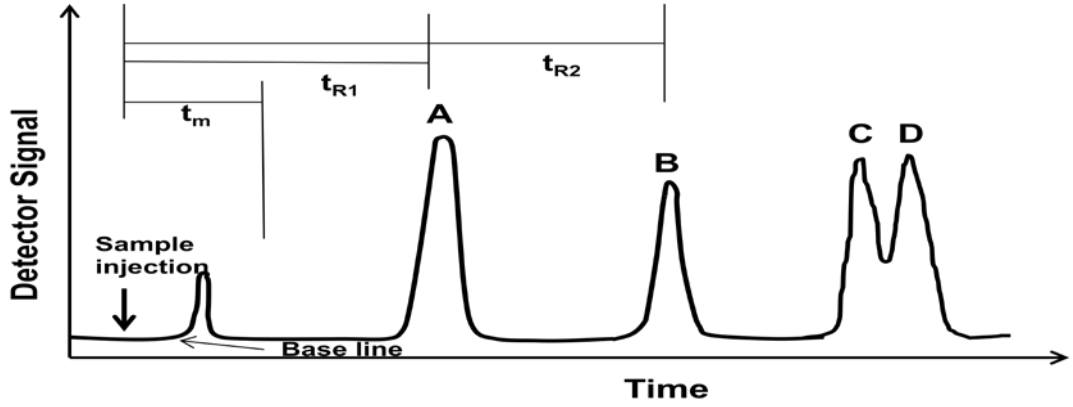


Figure 28.4: A typical chromatogram.

Resolution: The ability of a chromatography column to separate two analyte peak from another is known as resolution. It is defined as the ratio of difference in retention time between two peaks and average of base of peak width. It is given by

$$Rs = \frac{\Delta \mathrm{tR}}{\mathrm{Wav}}$$

When Rs=1, the separation of two peaks is 97.7% and a column with Rs more than 1.5 considered good. The number of distribution events govern the ability of a column to separate the two analytes. In another words, resolution is directly proportional to the number of distribution events. In column chromatography, each thin plain of column matrix participate in distribution of molecule. Assume height of a distribution plain is **H** and length of a column is **L**, hence number (**N**) of distribution plain in a column is given by,

$$N = \frac{\mathrm{L}}{\mathrm{H}}$$

$$\mathrm{N}=16\ (t_R/\mathrm{W})^2$$

$$\mathrm{N}=5.54\ (t_R/W_{1/2})^2$$

Hence, Number of distribution plain in a column is controlling two parameters:

(1) As number of distribution plain will go up, it will allow the analyte to travel for longer period of time, consequently it will increase the distance between two peaks.

(2) As number of distribution plain will go up, it will reduce the width of the base of peak, as a result the peaks will be more sharp. A representative example, how number of distribution plain affects the base of the peak is given in Figure 28.5. As the number is increasing, the peak width is decreasing. Hence, number of distribution is an indirect way to measure the column efficiency, higher N number is desirable for better separation.

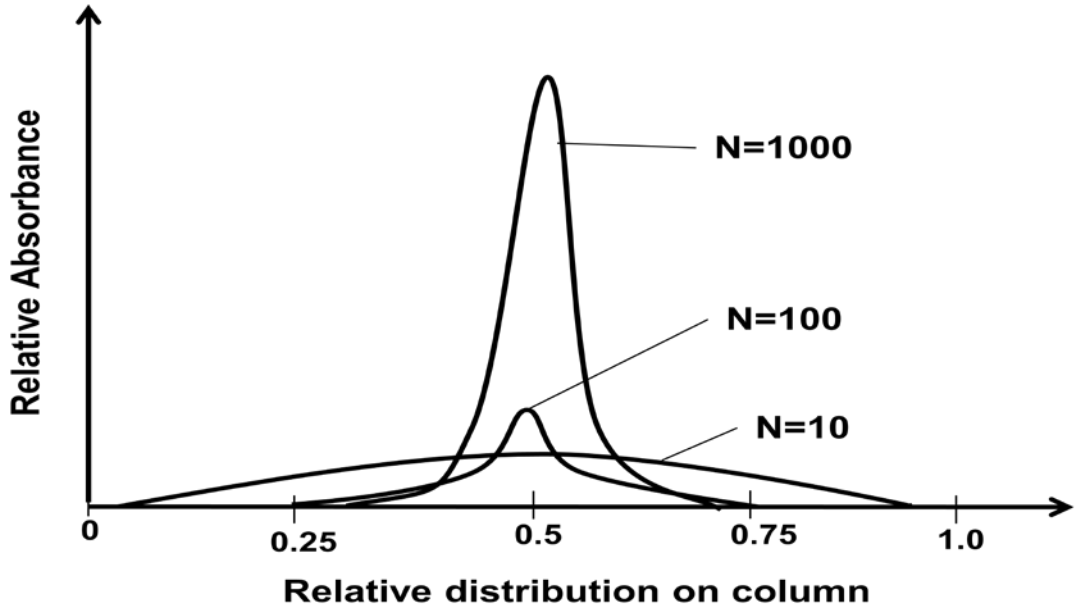


Figure 28.5: Relationship between number of distribution planes (N) and peak width.

Different components of chromatography system: The different components of a chromatography system are given in Figure 28.6. It has following components:

1. Reservoir: One or two reservoir for mobile phase (buffer).

2. Pump: One or two pump to flow the buffer from reservoir. Different types of pumps are used in chromatography system, mostly based on the pressure level required to perform chromatography. A pump is chosen as per the pressure required to run the mobile phase. Based on the pressure level, liquid chromatography can be classified into the following categories:

Low Pressure Liquid Chromatography: Pressure limit less than 5 Bar.

Medium Pressure Liquid Chromatography: Intermediate pressure limit (6-50 bar).

High Pressure Liquid Chromatography: Pressure limit more than 50-350 bar. A typical polysaccharide bead is not appropriate to withstand high pressure during HPLC. Hence, in HPLC silica based beads are recommended. Due to high pressure and smaller size of the silica beads gives higher number of theoretical plates. This gives HPLC superior resolving power to separate complex biological samples.

3. Mixer: A mixer is required to mix the buffer received from both pumps to form a linear or step gradient.

4. Column: A column made up of glass or steel.

5. Detector: The elution coming out from column goes to the online monitoring system to test the presence of the analyte based on different properties. There are different types of detectors are known in chromatography such as UV-Visible detector etc.

6. Fraction Collection- The eluent can be collected in different fractions by a fraction collector.

7. Recorder: The profile of eluent with respect to the measured property in a detector can be plotted in the recorder.

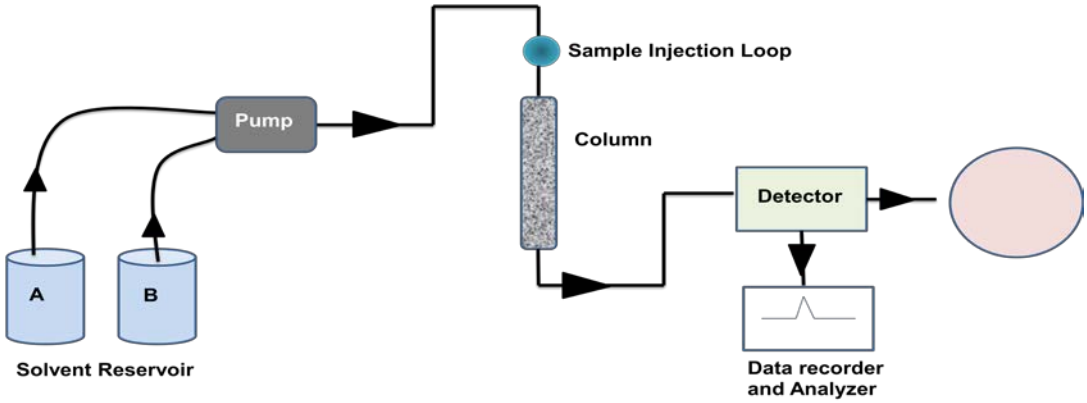


Figure 28.6: Different components of a chromatography system.

Different forms of chromatography:

Partition Chromatography: In this form of chromatography, an analyte distribute themselves into two phases, liquid stationary and mobile phase. The major advantage of this chromatography is that it is simple, low cost and has broad specificity. It is further divided into liquid-liquid chromatography and bonded-phase liquid chromatography. The example of this chromatography is cellulose, starch or silica matrix.

Adsorption Chromatography: In this form of chromatography, matrix molecule has ability to hold the analyte on their surface through a mutual interaction due to different types of forces such as hydrogen bonding, electrostatic interaction, vander waal etc. The example are ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography etc.

Lecture 29: Ion-Exchange Chromatography-I

Ion exchange chromatography: Ion-exchange chromatography is a versatile, high resolution chromatography techniques to purify the protein from a complex mixture. In addition, this chromatography has a high loading capacity to handle large sample volume and the chromatography operation is very simple.

Principle: This chromatography distributes the analyte molecule as per charge and their affinity towards the oppositively charged matrix. The analytes bound to the matrix are exchanged with a competitive counter ion to elute. The interaction between matrix and analyte is determined by net charge, ionic strength and pH of the buffer. For example, when a mixture of positively charged analyte (M, M^{+}, M^{-1}, M^{-2}) loaded onto a positively charged matrix, the neutral or positively charged analyte will not bind to the matrix where as negatively charged analyte will bind as per their relative charge and needed higher concentration of counter ion to elute from matrix (Figure 29.1).

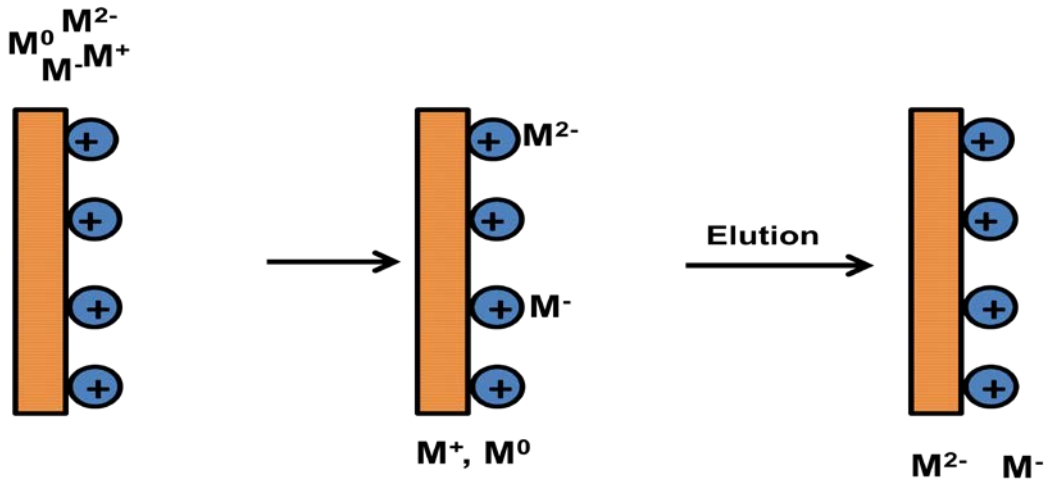


Figure 29.1: Affinity of analytes (M, M^{+}, M^{-1}, M^{-2}) towards positively charged matrix.

The matrix used in ion-exchange chromatography is present in the ionized form with reversibly bound ion to the matrix. The ion present on matrix participitate in the reversible exchange process with analyte. Hence, there are two types of ion-exchange chromatography:

1. Cation exchange chromatography- In cation exchange chromatography, matrix has a negatively charged functional group with a affinity towards positively charged molecules. The positively charged analyte replaces the reversible bound cation and binds to the matrix (Figure 29.2). In the presence of a strong cation (such as Na^+) in the mobile phase, the matrix bound positively charged analyte is replaced with the elution of analyte. The popular cation exchangers used are given in Table 29.1.

2. Anion Exchange chromatography- In anion exchange chromatography, matrix has a positively charged functional group with a affinity towards negatively charged molecules. The negatively charged analyte replaces the reversible bound anion and binds to the matrix (Figure 29.2, B). In the presence of a strong anion (such as Cl^-) in the mobile phase, the matrix bound negatively charged analyte is replaced with the elution of analyte. The popular anion exchangers used are given in Table 29.1.

Table 30.1: List of selected Ion-exchange matrix

S.No	Name	Functional Group	Type of Ion-exchanger
1	Carboxyl methyl (CM)	-OCH2COOH	Cation Exchanger
2	Sulphopropyl (SP)	-OCH2CH2CH2SO3H	Cation Exchanger
3	Sulphonate (S)	-OCH2SO3H	Cation Exchanger
4	Diethylaminoethyl (DEAE)	-OCH2CH2NH (C2H5)2	Anion Exchanger
5	Quaternary aminomethyl (Q)	-OCH2N(CH3)3	Anion Exchanger

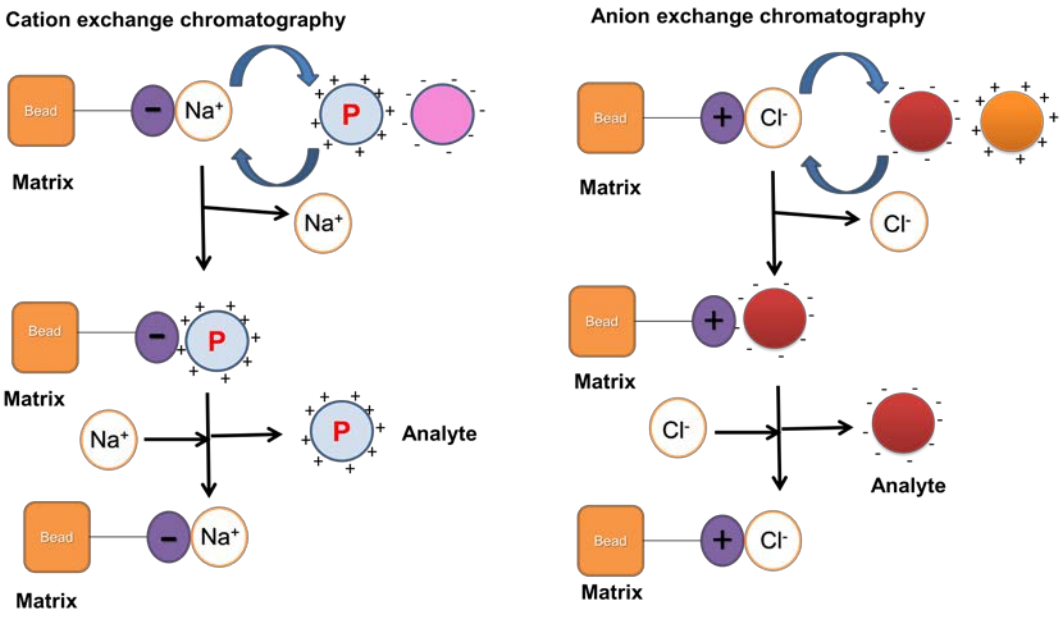


Figure 29.2: Cation and Anion exchange chromatography.

Isoelectric point and charge on a protein: Protein is a polymer made up of amino acids with ionizable side chain. At a particular pH, these amino acid side chain ionizes differentially to give a net charge (positive/negative) to the protein. The pH at which the net charge on a protein is zero is called as Isoelectric point (pI). The protein will have a net positive charge below the pI where as it has net negative charge above the pI value (Figure 29.3).

Choice of a Ion-exchange column matrix-Before starting the isolation and purification of a substance, a choice for a suitable ion-exchange chromatography is important. There are multiple parameter which can be consider for choosing the right column matrix.

1. pI value and Net charge- The information of a pI will be allow you to calculate the net charge at a particular pH on a protein. As discussed above, a cation exchange chromatography can be use below the pI where as an anion exchange chromatography can be use above the pI value.

2. Structural stability-3-D structure of a protein is maintained by electrostatic and vander waal interaction between charged amino acid, Π-Π interaction between hydrophobic side chain of amino acids. As a result, protein structure is stable in a narrow range around its pI and a large deviation from it may affect its 3-D structure.

3. Enzymatic activity-Similar to structural stability, enzymes are active in a narrow range of pH and this range should be consider for choosing an ion-exchange chromatography.

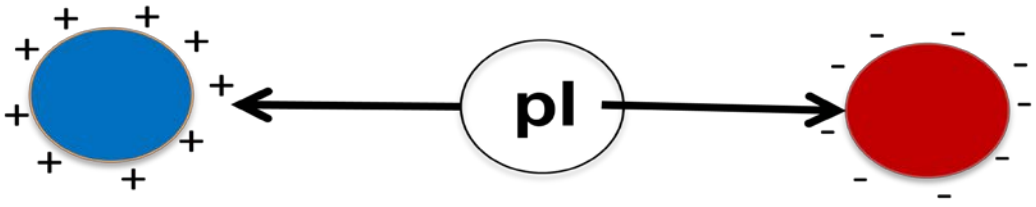


Figure 29.3: Change of charge with respect to the pI.

Operation of the technique-Several parameters needs to be consider to perform ion-exchange chromatography (Figure 29.4).

1. Column material and stationary phase-Column material should be chemically inert to avoid destruction of biological sample. It should allow free low of liquid with minimum clogging. It should be capable to withstand the back pressure and it should not compress or expand during the operation.

2. Mobile Phase-The ionic strength and pH are the crucial parameters to influence the property of the mobile phase.

3. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

4. Elution- There are many ways to elute a analyte from the ion-exchange column. (1) Isocratic elution (2) Step-wise gradient (3) Continuous gradient either by salt or pH (4) affinity elution (5) displacement chromatography

5. Column Regeneration- After the elution of analyte, ion-exchange chromatography column require a regeneration step to use next time. column is washed with a salt solution with a ionic strength of 2M to remove all non-specifically bound analytes and also to make all functional group in a iononized form to bind fresh analyte.

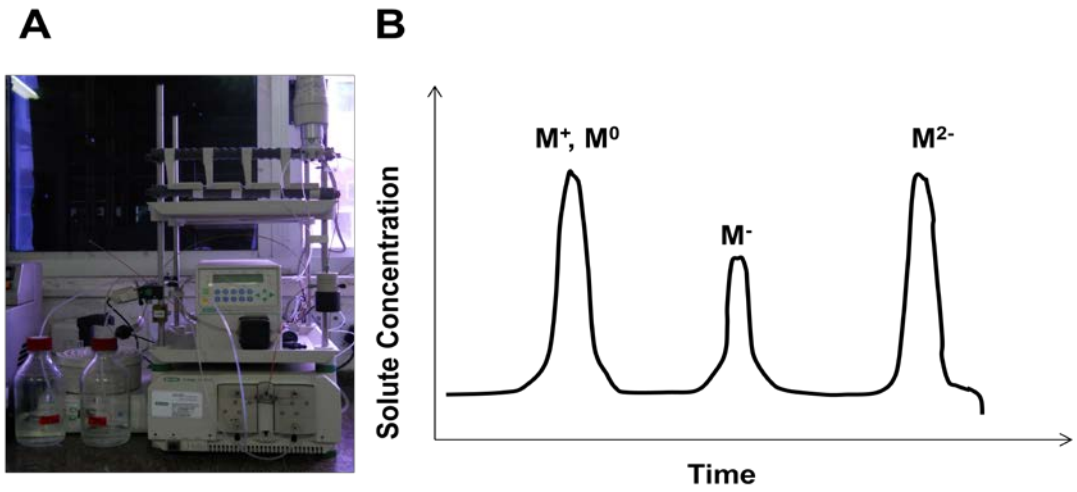


Figure 29.4 : Operation of the Ion-exchange Chromatography. (A) Chromatography system to perform gradient elution of analytes to give an (B) elution profile.

Lecture 30: Ion-Exchange Chromatography-II

Applications of Ion-exchange chromatography

1. Protein Purification-In the previous lecture we have already discussed how protein can be purified using ion-exchange chromatography.

2. Protein-DNA interaction-Ion-exchange column is used as a tool to study interaction between DNA and a particular protein. DNA is negatively charged and has strong affinity towards anion exchange chromatography. A schematic figure to depict the steps involved in DNA-protein interaction is given in (Figure 30.1). In this approach, anion exchange matrix is incubated with the DNA and allowed it to bind tightly. Excesss DNA is washed from the column. Now the pure protein is passed through the DNA bound beads, followed by washing with the buffer to remove unbounded proteins. Now the DNA is eluted from the matrix either by adding high salt concentration or with denaturating condition. Now the fractions are tested for the presence of DNA and protein. Eluted protein is analyzed in the SDS-PAGE and DNA is in agarose. As a control, protein is also added to the matrix without DNA to rule out the possibility of protein binding directly to the matrix. If protein will have a affinity towards DNA, they both will comes out from the column at the same time and should give similar pattern in the elution profile. It could be possible that high salt may break interaction between DNA and protein, in such situation protein will comes out first followed by DNA. Besides this ion-exchange chromatography approach still be able to answer whether the DNA-protein are interacting with each other or not.

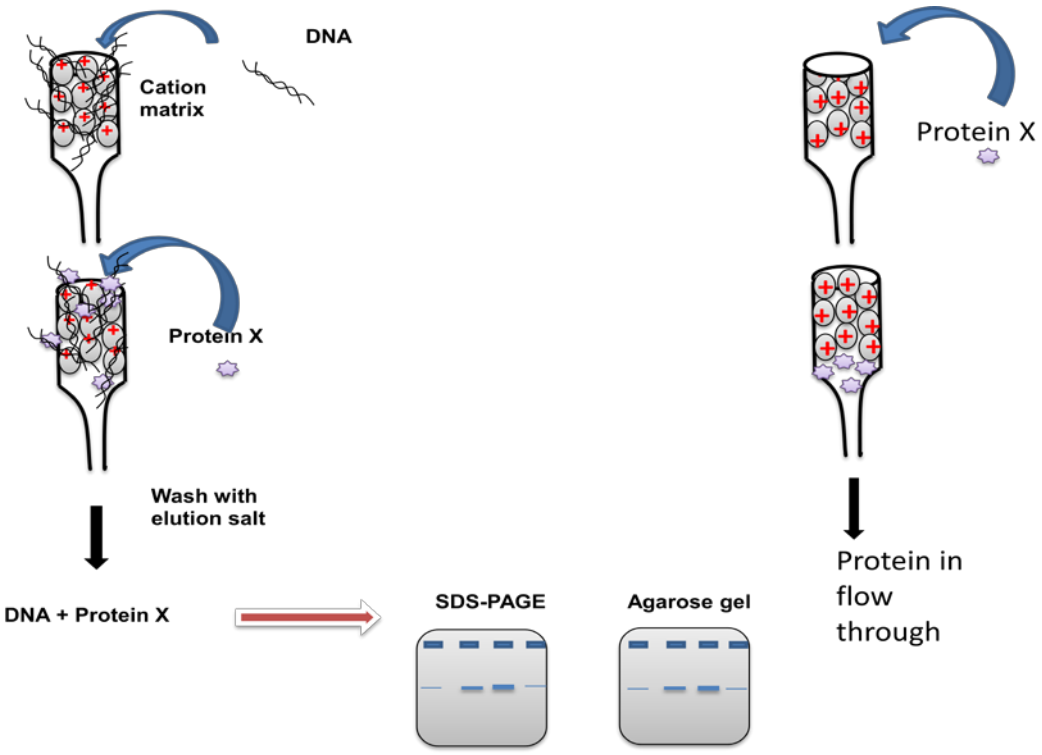


Figure 30.1: DNA-Protein interaction.

3. Softening of water-Ground water has several metals such as Ca^{2+}, Mg^{2+} and other cationic metals. Due to presence of the metal, hard water creates problem in industrial settings. Ion-exchange chromatography is used to remove the metals present in the water through an exchange of matrix bound Na^{+} (Figure 30.2). Calcium or magnesium present in the hard water has more affinity towards the matrix and it replaces with matrix bound sodium ions. The schematic presentation of water softening is given in Figure 30.3. A cation exchanger matrix with bound sodium is packed in the column and the hard water containing calcium, magnesium is passed

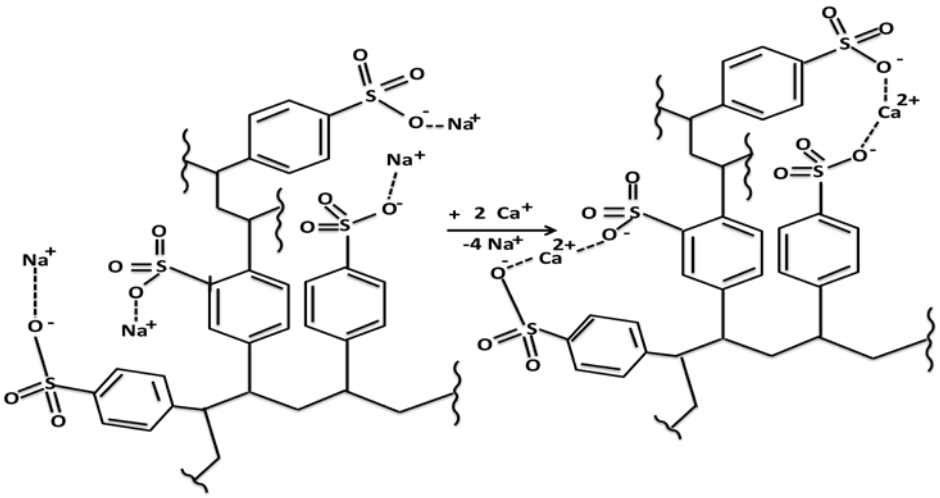


Figure 30.2: Mechanism of metal exchange during water softening.

through the column. In this process, calcium present in the solution preferentially migrate from the solution to the matrix where as sodium ion present on the matrix migrate into the solution. The matrix can be use for softening of the water until it has bound sodium ions. Once sodium ions are exhausted, matrix can be regenerated by flowing a solution of sodium chloride or sodium hydroxide. The calcium/magnesium bound to the matrix comes out in the solution and can be dumped into the sewage.

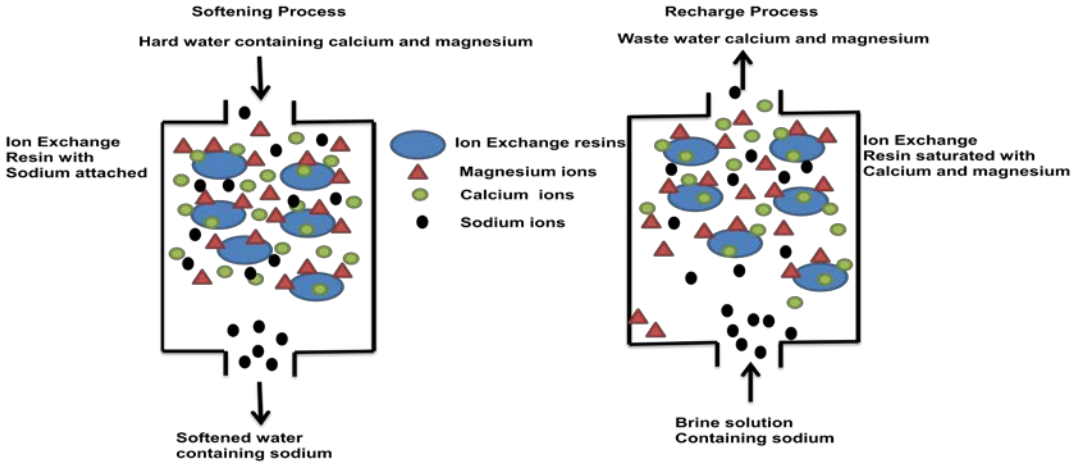


Figure 30.3: Softening of water by a cation exchanger matrix column. [NEEDS RECONSTRUCTION].

4. Protein kinase assay- Protein kinase are class of enzyme responsible for transfer of phosphate group on the substrate molecule. In the protein kinase assay, a radioactive substrate (preferable a radioactivity on carbon) was incubated with the enzyme protein kinase, $MgCl_2$ and non-radioactive ATP. A negative control is also been included where enzyme protein kinase is absent from the assay mixture. Reaction mixture from negative control and experimental will be loaded on two separate cation exchange chromatography columns to bind unphosphorylated substrate from the reaction mixture where as phosphorylated radioactive substrate is present in the flow through. The radioactive count of the flow through was measured using a liquid scintillation reagent (Figure 30.4).

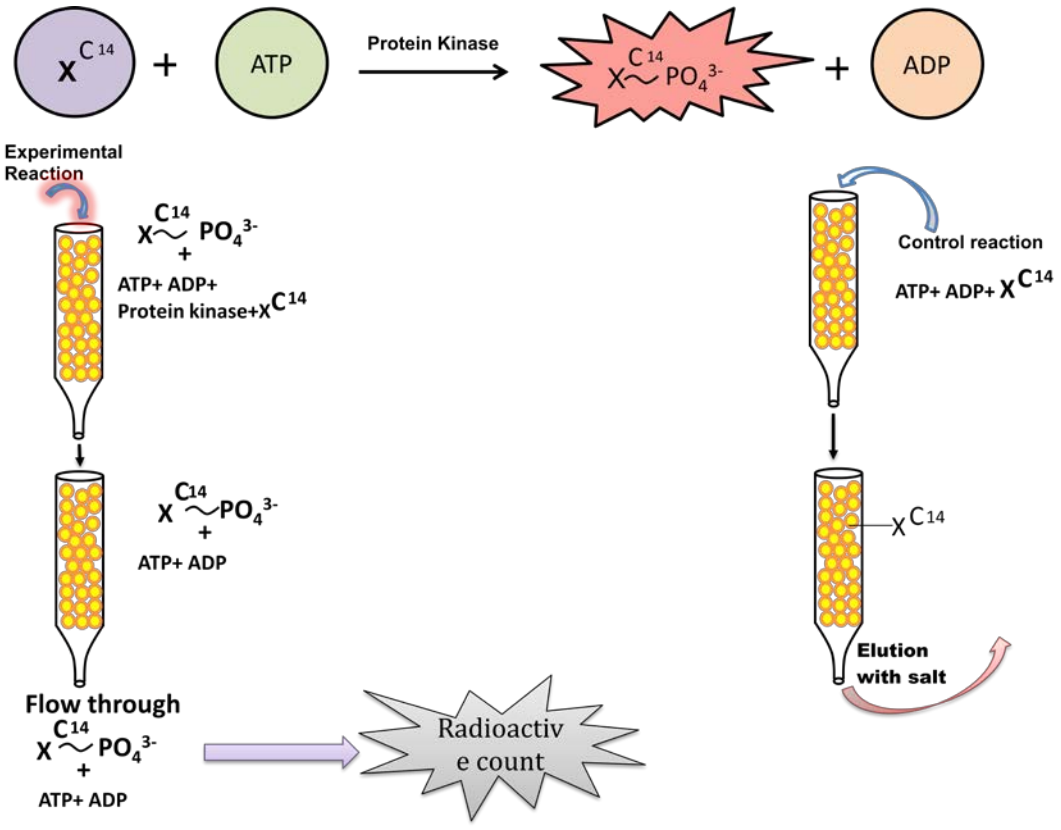


Figure 30.4: Protein kinase assay using ion-exchange chromatography.

5. Purification of rare earth metals from nuclear waste- Ion-exchange matrix is used to isolate and purify rare earth metals such as uranium or plutonium. The first process to isolate uranium in large quantities was developed by frank spedding. Ion-exchange beads are also found suitable to recover uranium from the water coming out of the nuclear power plant. Uranium binds to the matrix through the ion-exchange process. The uranium bound bead is sent to the processing unit where uranium is isolated from the beads to form **'yellow cake'** and stored in drum for further processing. The ion-exchange beads can be reused in the ion-exchange facility.

6. Concentrating a sample-A ion-exchange bead can be used to bind the analyte from a diluted solution and then sample can be eluted in smaller volume to increase the concentration.

Lecture 31: Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography: Hydrophobic interaction chromatography exploits the ability of a strong interaction between hydrophobic group attached to the matrix and hydrophobic patches present on an analyte such as protein. Protein is made-up of amino acids with acidic, basic, polar and non-polar (aliphatic or aromatic) side chain. Protein is synthesized from ribosome as a linear chain and afterwards it gets folded into a 3-D conformation mostly guided by the environment of side chain and the outer medium. Local environment in a cell is aqeous and it favors the folding of protein to keep the polar or charged amino acids on the surface and non-polar side chain within the inner core (Figure 31.1). Most of the hydrophobic amino acids are shielded from the outer polar environment where as polar amino acid present on the surface has bound water molecule to form a hydration shell.

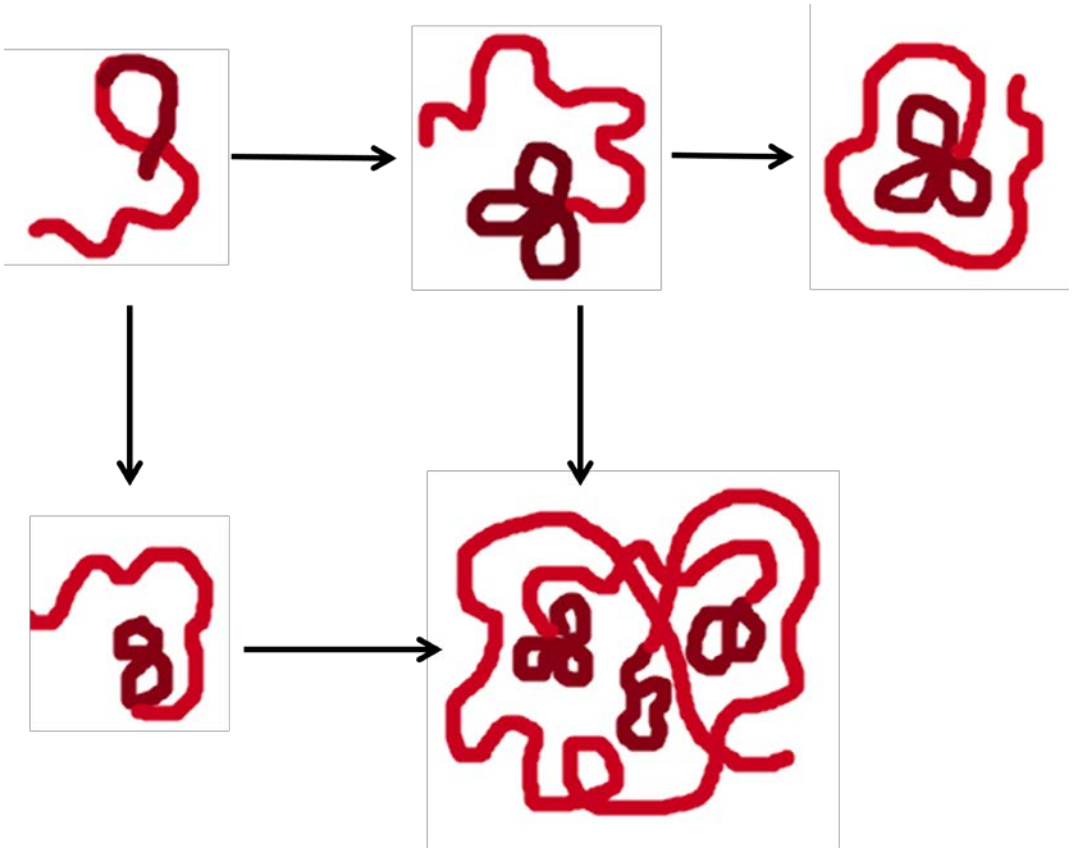


Figure 31.1: Folding of Protein in an aqueous environment. Following a series of folding stages, protein adopts a 3-D conformation with hydrophobic patches present in the core.

Addition of low amount of salt to the protein solution results in the displacement of bonded water molecule with an increase in protein solubility (Figure 31.2). This effect is called as **"salting-in"**. In the presence of more amount of salt, water molecule shielding protein side chains are displaced completely with an exposure of hydrophobic patches on protein surface to induce protein precipitation or decrease in protein solubility. This effect is called as **"salting-out"**. The phenomenon of salting out is modulated so that addition of salt induces exposure of hydrophobic patches on protein but does not cause precipitation or aggregation. The exposure of hydrophobic patches facilitates the binding of protein to the non-polar ligand attached to the matrix. When the concentration of salt is decreased, the exposed hydrophobic patches on protein reduces the affinity towards matrix and as a result it get eluted (Figure 31.3).

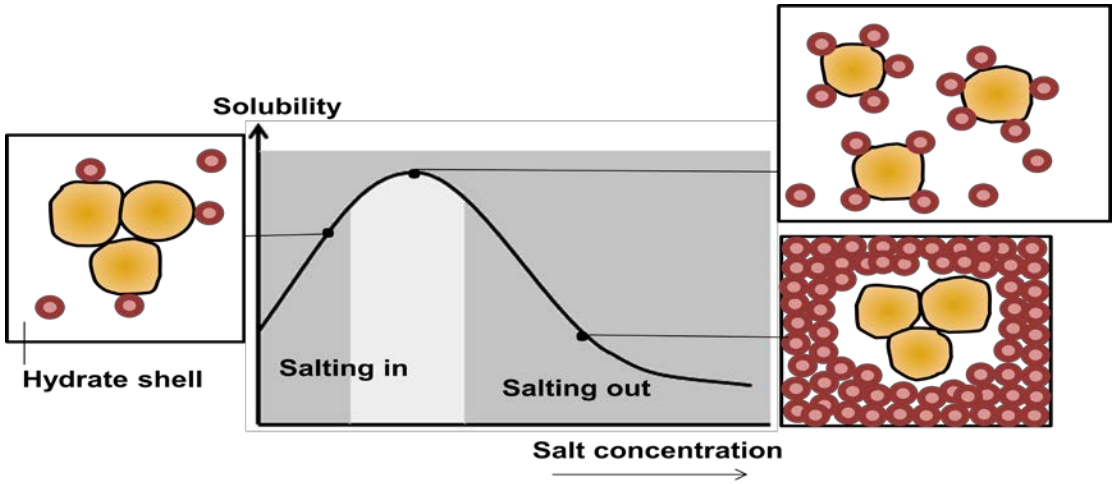


Figure 31.2: Effect of salt on protein, salting in and salting out effect.

The choice of HIC gel-The different commercially available HIC matrix are given in Table 31.1. Choosing a suitable HIC matrix is essential to achieve best result. The strength of the binding of analyte on a HIC column is governed by the length of the aliphatic linear ligand. Matrix with aromatic ring containing ligand makes additional Π-Π interaction and they will bind analyte more strongly than same number of carbon aliphatic ligand. In addition, presence of Π-Π interaction gives selectivity as well, such as ring containing aromatic ligand, phenylalanine. At last, ligand density plays a vital role in the strength of binding of an analyte to the matrix. Hence, these points should be consider to choose a suitable matrix for purification.

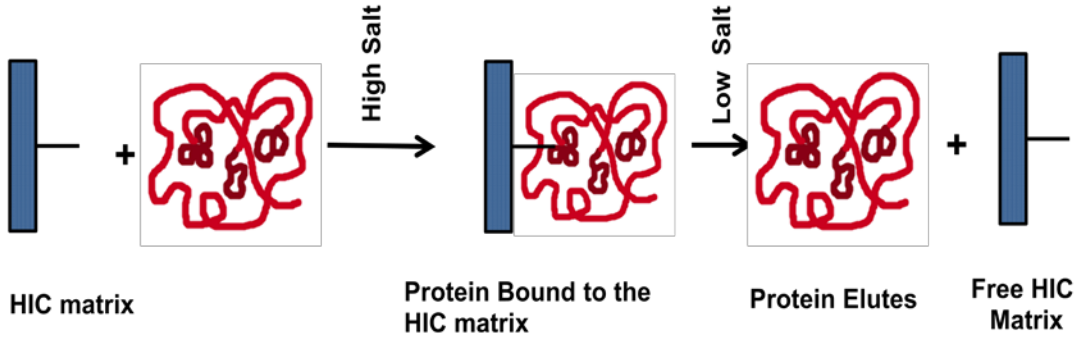


Figure 31.3: Principle of the hydrophobic interaction chromatography.

Table 31.1: Selected list of popular HIC column matrix.

S.NO	Column Material	Functional Group
1	Butyl-S-Sepharose	-Butyl
2	Phenyl Sepharose (Low Sub)	-Phenyl, low density
3	Phenyl Sepharose (High Sub)	-Phenyl, high density
4	Capto phenyl sepharose	-Phenyl
5	Octyl Sepharose	-Octyl

Operation of the technique-Several parameters needs to be consider to perform hydrophobic interaction chromatography.

1. Equlibration-HIC column material packed in a column and equilibrate with a buffer containing 0.5-1.5M ammonium sulphate (mobile phase). The salt must be below the concentration where it has salting-out effect.

2. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

3. Elution- There are many ways to elute a analyte from the hydrophobic interaction column. (1) decreasing salt concentration, (2) changing the polarity of the mobile phase such as alchol, (3) By a detergent to displace the bound protein.

4. Column Regeneration- After the elution of analyte, HIC column require a regeneration step to use next time. column is washed with 6M urea or guanidine hydrochloride to remove all non-specifically bound protein. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 4^0C in the presence of 20% alchol containing 0.05% sodium azide.

Lecture 32 Gel Filtration Chromatography

Introduction: This chromatography distributes the protein or analyte, based on their size by passing through a porous beads. The first report in 1955 described performing a chromatography column with swollen gel of maize starch to separate the protein based on their size. '**Porath and Floidin**'coined the term "gel filtration" for this chromatography technique separating the analytes based on molecular sizes. Since then the chromatography technique evolved in terms of developed of different sizes beads to separate protein of narrow range, as well as performing the technique in aqueous and non-aqueous mobile phase. The beads used in gel filtration chromatography is made up of cross linked material (such as dextran in sephadex) to form a 3-D mesh. These 3-D mesh swell in the mobile phase to develop pores of different sizes (Figure 32.1). The extent of cross linking controls the pores size within the gel beads.

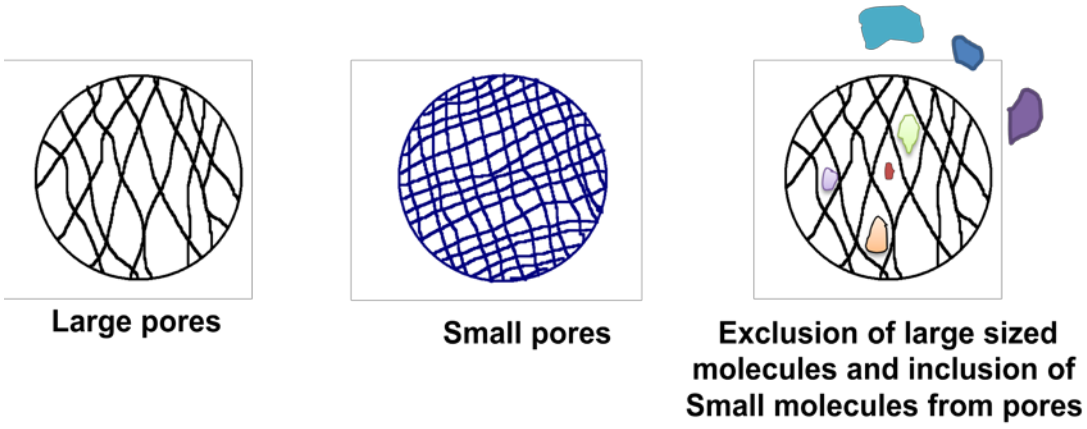


Figure 32.1: Gel Filtration Matrix has Beads with different pore sizes.

Principle: The principle of the chromatography technique is illustrated in Figure 32.2. The column is packed with the beads containing pores to allow entry of molecules based on their sizes. Smallest size in the inner part of pore followed by gradual increasing size and largest molecule excluded from entering into the gel. The separation between molecules occur due to the time they travel to come out from the pores. When the mobile phase pass through the column, it takes protein along with it. The small molecules present in the inner part of the gel takes longer flow of liquid (or time) and travel longer path to come out where as larger molecules travel less distance to come out. As a result, the large molecule and small molecule get separated from each other. A schematic gel filtration chromatogram is given in Figure 32.3.

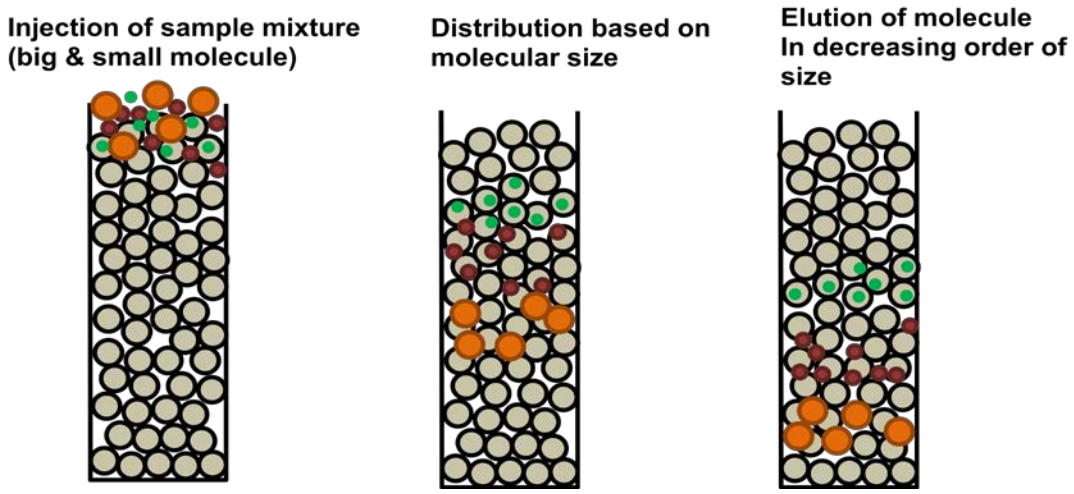


Figure 32.2: Principle of Gel Filtration Chromatography.

Suppose the total column volume of a gel is V_t and then it is given by-

$$\mathbf{V_t=V_g+V_i+V_o}\text{……Eq (32.1)}$$

V_g is the volume of gel matrix, V_i is the pore volume and V_o is the void volume. The volume of mobile phase flow to elute a column from a column is known as elution volume (V_e). The elution volum is related to the void volume and the distribution coefficient Kd as given below

$$\mathbf{V_e=V_o+KdV_i}\text{……Eq (32.2)}$$

$$\mathbf{Kd} = \frac{Ve-Vo}{Vi}\text{……..……Eq (32.3)}$$

Kd is the ratio of inner volume available for an analyte and it is independent to the column geometry or length. As per relationship given in Eq 32.3, three different type of analytes are possible:

1. Analyte with **Kd=0**, or Ve=Vo, these analytes will be completely excluded from the column.

2. Analyte with **Kd=1** or Ve=Vo+Vi, these analytes will be completely in the pore of the column.

3. Analyte with **Kd>1**, in this situation analyte will adsorb to the column matrix.

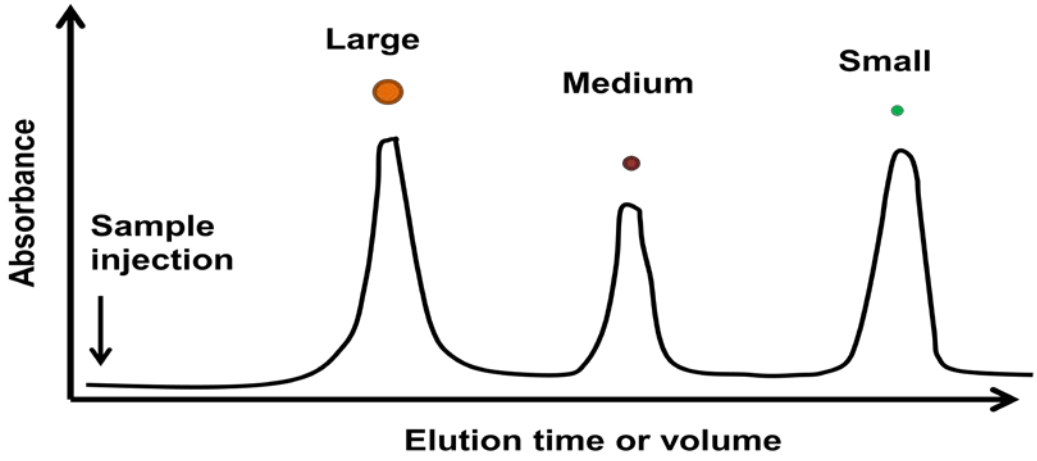


Figure 32.3: A typical Gel Filtration Chromatogram.

Choice of matrix for gel filtration chromatography-The choice of the column depends on the range of molecular weight and the pressure limit of the operating equipment. A list of popular gel filtration column matrix with the fractionation range are given in Table 32.1.

Operation of the chromatography-

1. Column packing-The column material is allowed to swell in the mobile phase. It is poured into the glass tube and allow the beads to settle without trapping air bubble within the column. Once the matrix is settled to give a column, it can be tested for presence of air channel and well packing by flowing a analyte with Kd=1, it is expected that the elution volume (Ve) in this case should be V_o+V_i.

2. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

3. Elution- In gel filtration column, no gradient of salt is used to elute the sample from the column. The flow of mobile phase is used to elute the molecules from the column.

4. Column Regeneration- After the analysis of analyte, gel filtration column is washed with the salt containing mobile phase to remove all non-specifically adsorb protein to the matrix. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 4^0C in the presence of 20% alchol containing 0.05% sodium azide.

Table 32.1: List of popular gel filtration matrix

S.No	Name of the matrix	Fractionation Range (Daltons)
1	Sephadex G10	Upto 700
2	Sephadex G25	1000-5000
3	Sephadex G50	1500-30,000
4	Sephadex G100	4000-150,000
5	Sephadex G200	5000-600,000
6	Sepharose 4B	60,000-20,000,000
7	Sepharose 6B	10,000-4,000,000

Determination of native molecular weight of a protein using gel filtration chromatography

The molecular weight and size of a protein is related to the shape of the molecule and the relationship between molecular weight (M) and radius of gyration (R_g)is as follows-

$$Rg \propto M^a \quad \text{……Eq 32.4}$$

here “a” is a constant and it depends on shape of the molecule, a=1 for Rod, a=0.5 for coils and a=0.33 for spherical molecules.

The set of known molecular weight standard protein can be run on a gel filitration column and elution volume can be calculated from the chromatogram (Figure 32.4). A separate run with the analyte will give elution volume for unknown sample. Using following formula, Kd value for all standard protein and the test analyte can be calculated.

$$\mathbf{Kd} = \frac{Ve - Vo}{Vi}$$

A plot of Kd versus log mol wt is given in Figure 32.4, B and it will allow us to calculate the molecular weight of the unknown analyte.

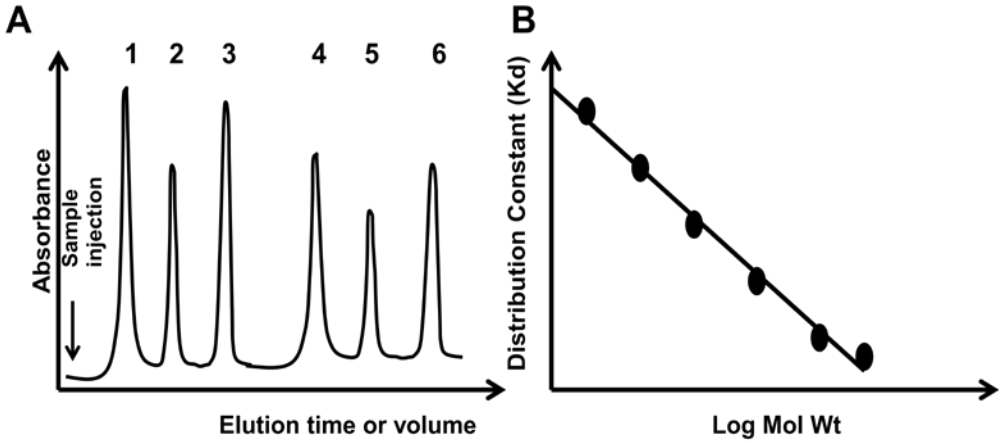


Figure 32.3: Determination of molecular weight by gel filtration chromatography. (A) Gel filtration chromatogram with the standard proteins (1-6), (B) Relationship between distribution constant (Kd) and Log Molecular weight.

Lecture 33 Gel Filtration Chromatography-II

Oligomeric status of the protein-Native molecular weight determination by gel filtration in conjugation with the SDS-PAGE can be used to determine the oligomeric status of the protein.

$$\text{Oligomeric Status} = \frac{\text{Molecular weight (Gel Filtration)}}{\text{Molecular weight (SDS-PAGE)}}$$

Studying protein folding-Protein is madeup of the different types of amio acid residues linked by the peptide bond. As soon as peptide chain comes out from the ribosome, it folds into the 3-D conformation directed by the amino acid sequence, external environment and other factors. Protein structure has multilevel organization; Primary structure (sequence of protein), secondary (α-helix, β-sheet and turn), tertiary and quaternary structure. When protein is incubated with the increasing concentration of denaturating agents (such as urea), it unfolds the native structure into the unfolded extended conformation following multiple stages. The different protein conformation forms during unfolding pathway has distinct hydrodynamic surface area and it can be use to follow protein folding-unfolding stages with the gel filtration chromatography. The details of the experimental setup is given in the Figure 33.1. Protein is incubated with different concentration of urea (0-8M) for 8-10hrs at 37^0C. A gel filtration column is equiliberated with the buffer containing urea (same as in incubation mixture) and the incubation mixture is analyzed. As the concentration of denaturating agent is increasing, protein will unfold with an increase in hydrodynamic surface area. As a result, protein peak shifts towards left. At highest concentration of denaturant, protein unfolds completely and mostly appear in void volume.

Studying protein-ligand interaction-Gel filtration chromatography separates the molecules based on their size. Ligand binding to the protein induces conformational changes, result into the change in size or shape (Figure 33.2). In addition, ligand is small in size where as protein-ligand complex is big and may appear at a distinct place in the column. In step 1, a gel filtration column is equiliberated with the buffer and elution profile of ligand is recorded. Now column is equliberated with the buffer containing ligand molecule. As the concentration of ligand is increased, protein binds ligand and form a larger complex with an increase in hydrodynamic surface area. As a result, protein peak shifts towards left. As the concentration of ligand will

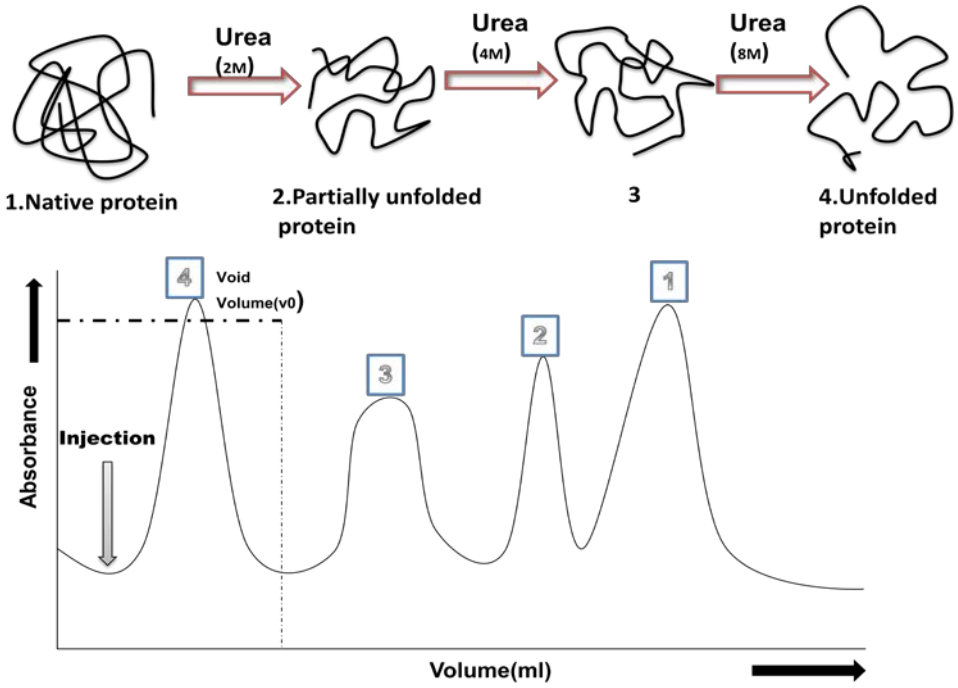


Figure 33.1: Studying Protein folding stages by gel filtration chromatography.

increase with a fixed amount of the protein, free ligand will appear in the chromatogram. The protein amount and the concentration at which free ligand appeared, and the elution data can be use to calculate the stoichiometric ratio of ligand/protein and the equilibrium constant.

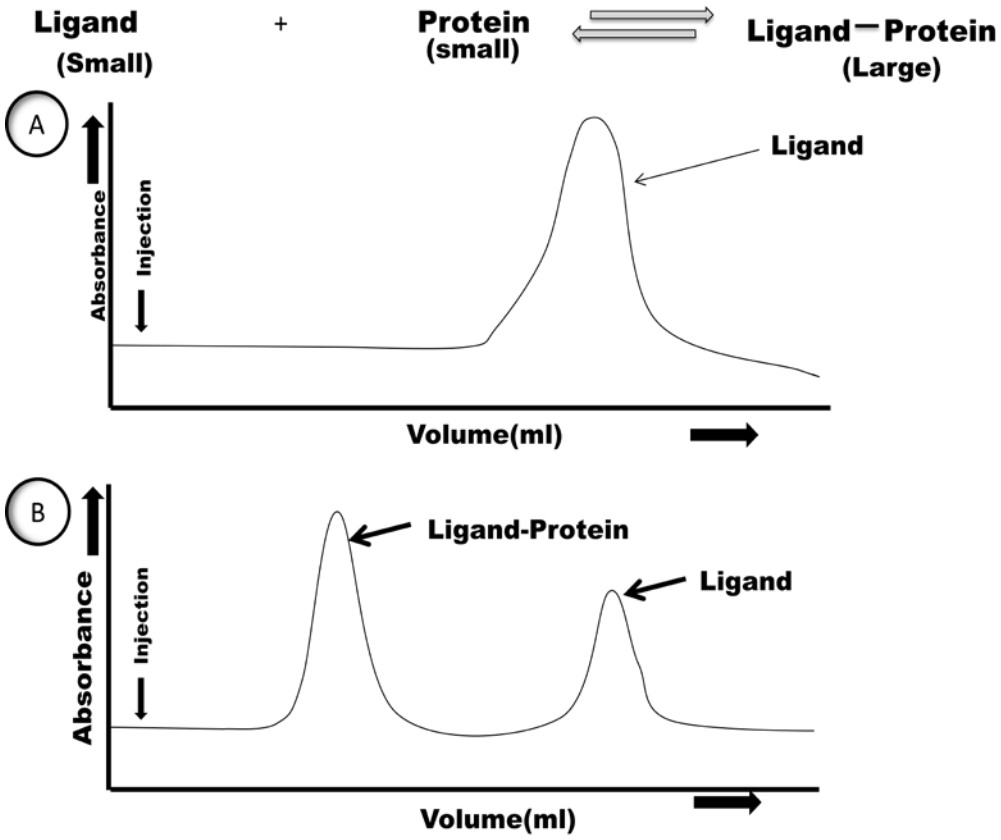


Figure 33.2: Studying Protein-ligand interaction by gel filtration chromatography.

Desalting- Desalting or removal of the small molecule from the protein is important for activity assay and other down stream processes. A gel filtration column is equilibrated with the buffer or water and then the sample for desalting is loaded. After the run the protein and salt are eluted separaetely as peak (Figure 33.3).

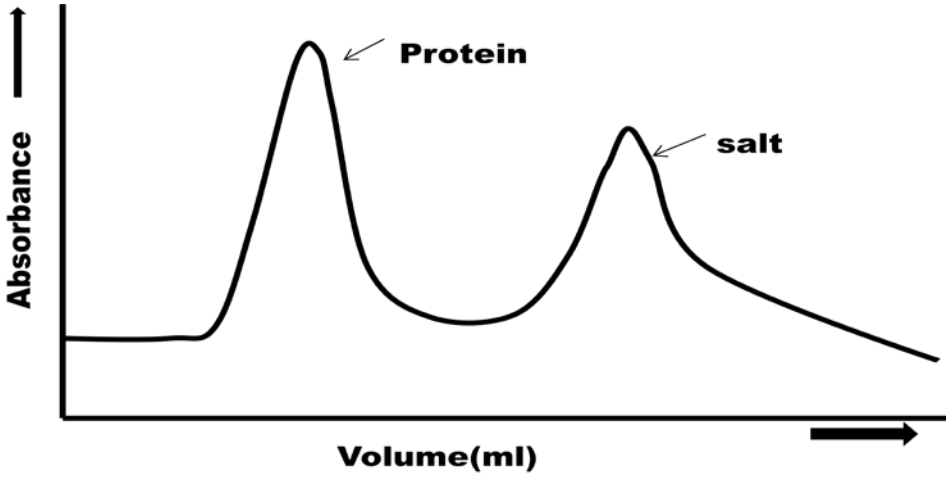


Figure 33.3: Desalting of a sample by gel filtration chromatography.

Lecture 34 Affinity Chromatography-I

Introduction: The chromatography techniques we discussed so far were exploiting different types of interactions between the matrix and the group present on the analyte but these chromatography techniques are not specific towards a particular analyte per se. The generalized chromatography approaches needs higher sample volume to isolate the molecule of interest. In the current lecture we will discuss another chromatography technique where a chromatography matrix is specific for a particular molecule or group of protein.

Principle: The affinity chromatography works on the principle of mutual recognition forces between a ligand and receptor. The major determinants, responsible to provide specificity are shape complementarity, electrostatic, hydrogen bonding, vander waal interaction between the groups present on the ligand-receptor pair (Figure 34.1). A mutual interaction between a ligand (L) and receptor (R) forms ligand-receptor complex (RL) with a dissociation constant Kd, which is expressed as follows-

$$\mathbf{R + L \leftrightharpoons RL}$$

$$\mathbf{Kd = \frac{[R][L]}{[RL]}} \text{.....33.1}$$

Dissociation constant is specific to the receptor-ligand pair and number of interaction between them. when a crude mixture is passed through an affinity column, the receptor present on the matrix reacts with the ligand present on different molecules. The mutual collision between receptor on matrix and ligands from different molecule test the affinity between them and consequently the best choice bind to the receptor where as all other molecules do not bind and appear in flow through. A wash step removes remaining weakly bound molecules on matrix. Subsquently, a counter ligand is used to elute the bound molecule through a competition between the matrix bound molecule and counter ligand (Figure 34.2).

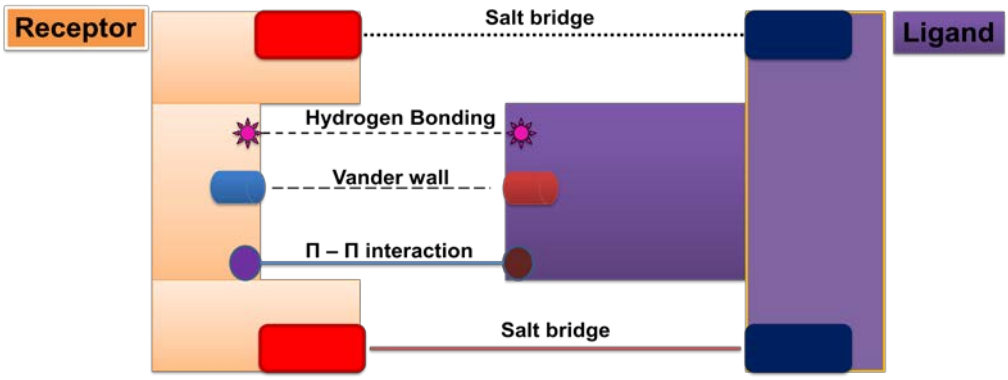


Figure 34.1: Interactions playing crucial role in providing specificity.

Advantages of Affinity chromatography-

1. Specificity: Affinity chromatography is specific to the analyte in comparison to other purification technique which are utilizing molecular size, charge, hydrophobic patches or isoelectric point etc.

2. Purification Yield: Compared to other purification method, affinity purification gives very high level of purification fold with high yield. In a typical affinity purification more than 90% recovery is possible.

3. Reproducible: Affinity purification is reproducible and gives consistent results from one purification to other as long as it is independent to the presence of contaminating species.

4. Easy to perform: Affinity purification is very robust and it depends on force governing ligand-receptor complex formation. Compared to other techniques, no

column packing, no special purification system and sample preparation required for affinity purification.

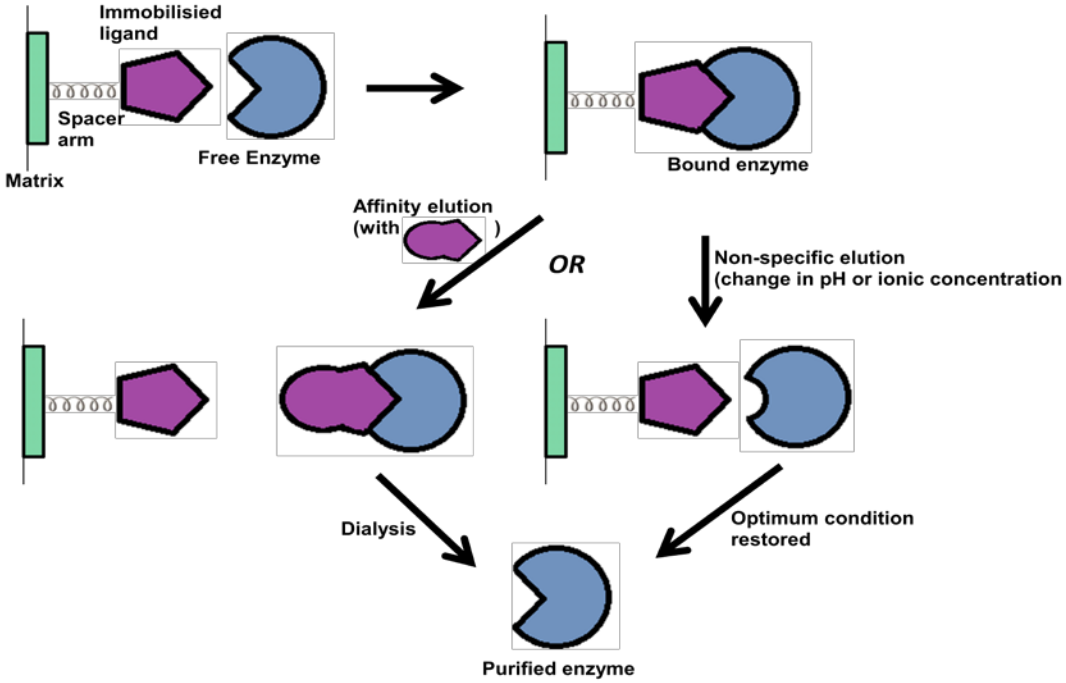


Figure 34.2: Principle of affinity chromatography.

Different types of affinity chromatography: Affinity chromatography is further divided into the different types based on the nature of receptor present on matrix to binds tag present on the analyte molecule. Different types of affinity chromatography are-

Bio-affinity chromatography- In this type of affinity chromatography, biomolecules are used as receptor present on matrix and it exploit the biological affinity phenomenon such as antibody-antigen. In addition, enzyme-substrate or enzyme-inhibitor is also belong to this class. Ex. GST-Glutathione.

Pseudo-affinity chromatography-In this affinity chromatography, a non-biological molecule is used as receptor on matrix to exploit the separation and purification of biomolecules. There are two specific example to this class-

A. Dye-affinity chromatography-In this method, matrix is coupled to the reactive dye and the matrix bound dye has specificity towards a particular enzyme. For ex. Cibacron Blue F3G-A dye coupled to the dextran matrix has strong affinity towards dehydrogenases.

B. Metal-affinity chromatography-In this method, transition metals such as Fe^{2+}, Ni^{2+} or Zn^{2+} is coupled to the matrix and the matrix bound metal form multidentate complex with protein containing poly-his tag (6x His). The affinity of protein for matrix bound metal is different and these differences are been exploited in metal affinity chromatography to purify the protein.

Covalent chromatography- This is a different type of chromatography technique where binding of analyte to the matrix is not reversible as it involves the formation a covalent bond between functional group present on matrix and analyte. Thiol group (-SH) present on neighbouring residues of protein forms disulphide bond after oxidation and under reducing environment, disulphide reversible broken back to free thiol group. The matrix in covalent chromatography has immobilized thio group which forms covalent linkage with the free thiol group containing protein present in the mixture (Figure 34.3). After a washing step to remove non-specifically bound protein, a mobile phase containing compound with reducing thio group is passed to elute the bound protein. The thio group containing compound present in mobile phase breaks the disulphide bond between protein and matrix thio group to release the protein in the mobile phase (Figure 34.3).

Choice of matrix for Affinity chromatography- Different popular affinity matrix used for protein purification is given in Table 34.1. The choice of matrix solely depends on the affinity tag present on the recombinant protein produced after genetic engineering.

Table 34.1: Matrix containing receptor for ligand present on protein.

S.No.	Receptor	Affinity towards protein ligand
1	5'AMP	NAD^+-dependent dehydrogenase
2	2'5'-ADP	$NADP^+$-dependent dehydrogenase
3	Avidin	Biotin-containing enzymes
4	Protein A and Protein G	Immunoglobulin
5	Concanavalin A	Glycoprotein
6	Poly-A	Poly U mRNA
7	Lysine	rRNA
8	Cibacron Blue F3GA	NAD+ Containing dehydrogenase
9	Lectin	Glycoprotein
10	Heparin	DNA binding site

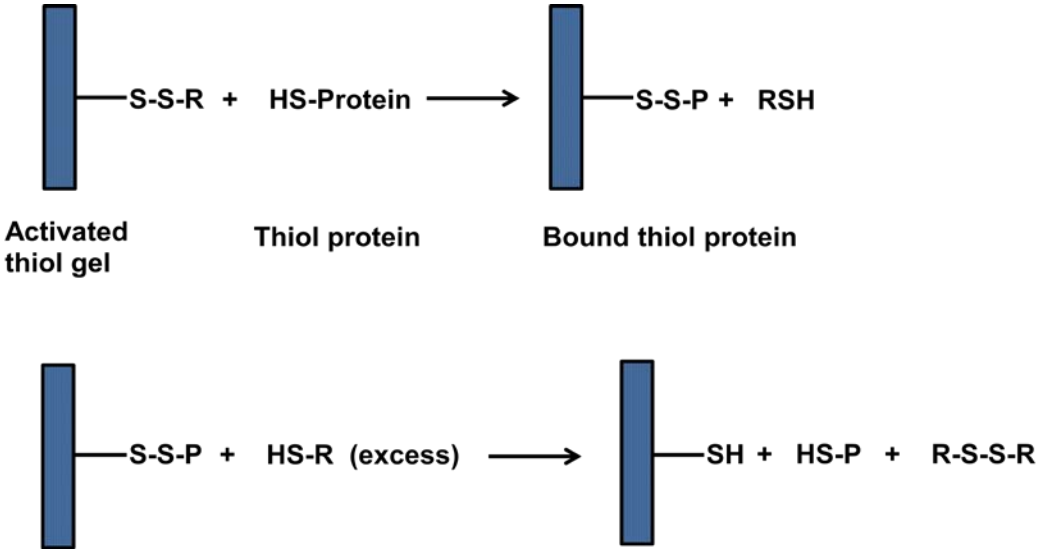


Figure 34.3: Principle of covalent chromatography.

Lecture 35 Affinity Chromatography-II

Generation of Receptor-The receptor molecule present on the matrix can be produced either by genetic engineering, isolation from the crude extract or in the case of antibody, it is produced in the mouse/rabbit model and purify. The generation of receptor molecule is beyond the discussion in the current lecture and interested student are advised to follow it from other relevant course.

Coupling of the Receptor-Once the receptor molecule is available, it can be couple to the matrix by following steps. (1) Matrix activation (2) covalent coupling utilizing reactive group on ligand. (3) deactivation of the remaining active group on matrix.

CNBr mediated receptor coupling-CNBr mediated coupling is more suitable for protein/peptide to the polysaccharide matrix such as agarose or dextran. CNBr reacts with polysaccharide at pH 11-12 to form reactive cynate ester with matrix or less reactive cyclic imidocarbonate group. Under alkaline condition these cynogen ester reacts with the amine group on receptor to form isourea derivative (Figure 35.1). The amount of cynate ester is more with agarose whereas imidacarbonate is more formed with dextran as a matrix. The protein or peptide ligand with free amino group is added to the activated matrix to couple the receptor for affinity purification.

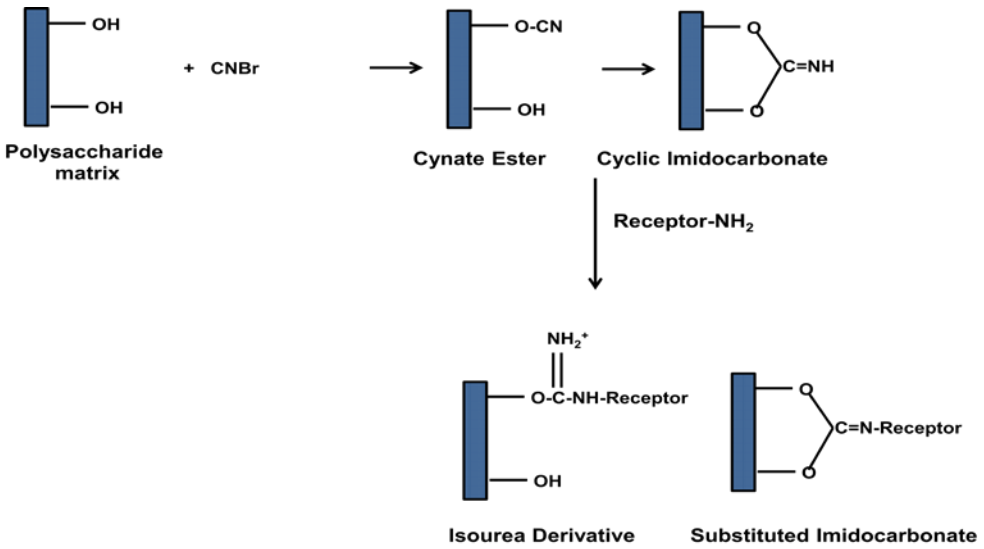


Figure 35.1: CNBr mediated coupling of receptor to the matrix.

Epichlorohydrin mediated receptor coupling- Epichlorohydrin activates the polysaccharide matrix by adding oxirane group with a 3 carbon alchol group (propanol) spacer arm (Figure 35.2). Activated matrix reacts with the receptors containing primary amine or thiol group. Receptor are couple to the matrix by a thioester or a secondary amine linkage. It can be able to couple hydroxyl group containing receptor molecule as well as by a ether linkage.

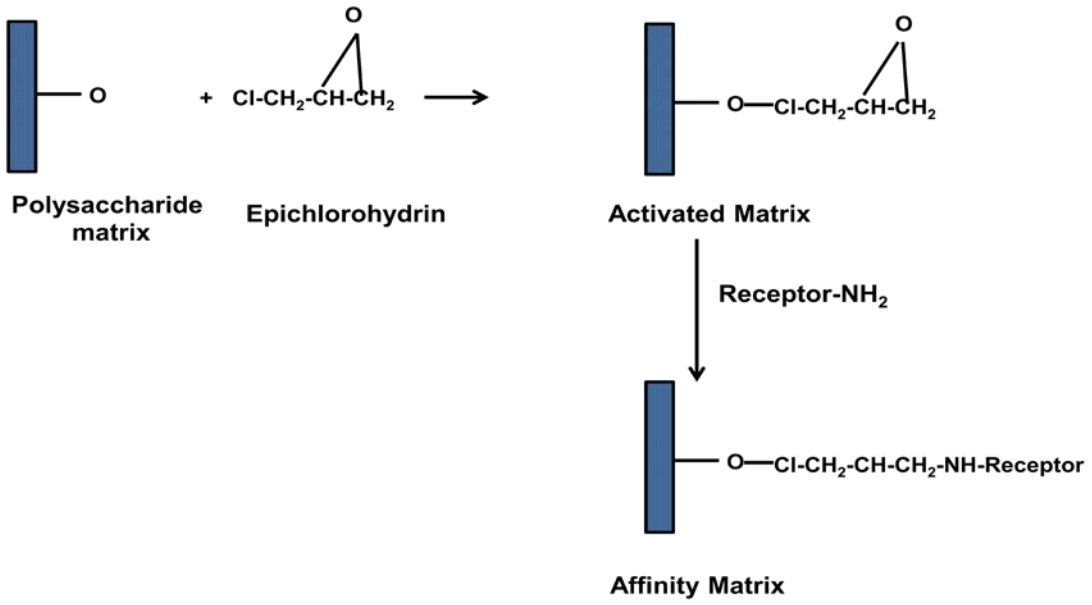


Figure 35.2: Epichlorohydrin mediated coupling of receptor to the matrix.

Carbodiimides mediated receptor coupling-Carboiimides reacts with the matrix containing carboxyl group to form isourea ester. The activated matrix is then allowed to react with the receptor molecule containing carboxyl or free amino group. Receptor are couple to the matrix by a secondary amine linkage.

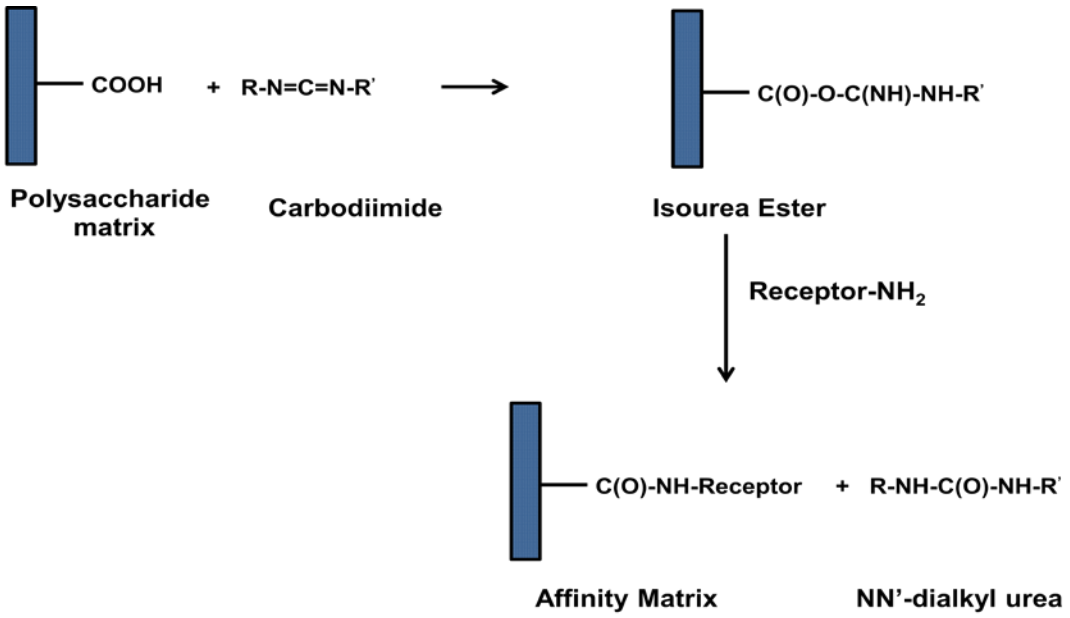


Figure 35.3: Carbodiimides mediated coupling of receptor to the matrix.

Operation of the Affinity chromatography-Different steps in affinity chromatography is given in Figure 34.4.

1. Equlibration-Affinity column material packed in a column and equilibrate with a buffer containing high salt (0.5M NaCl) to reduce the non-specific interaction of protein with the analyte.

2. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.

3. Elution- There are many ways to elute a analyte from the affinity column. (1) increasing concentration of counter ligand, (2) changing the pH polarity of the mobile phase, (3) By a detergent or chaotrophic salt to partially denature the receptor to reduce the affinity for bound ligand.

4. Column Regeneration- After the elution of analyte, affinity column requires a regeneration step to use next time. column is washed with 6M urea or guanidine hydrochloride to remove all non-specifically bound protein. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 4^0C in the presence of 20% alchol containing 0.05% sodium azide.

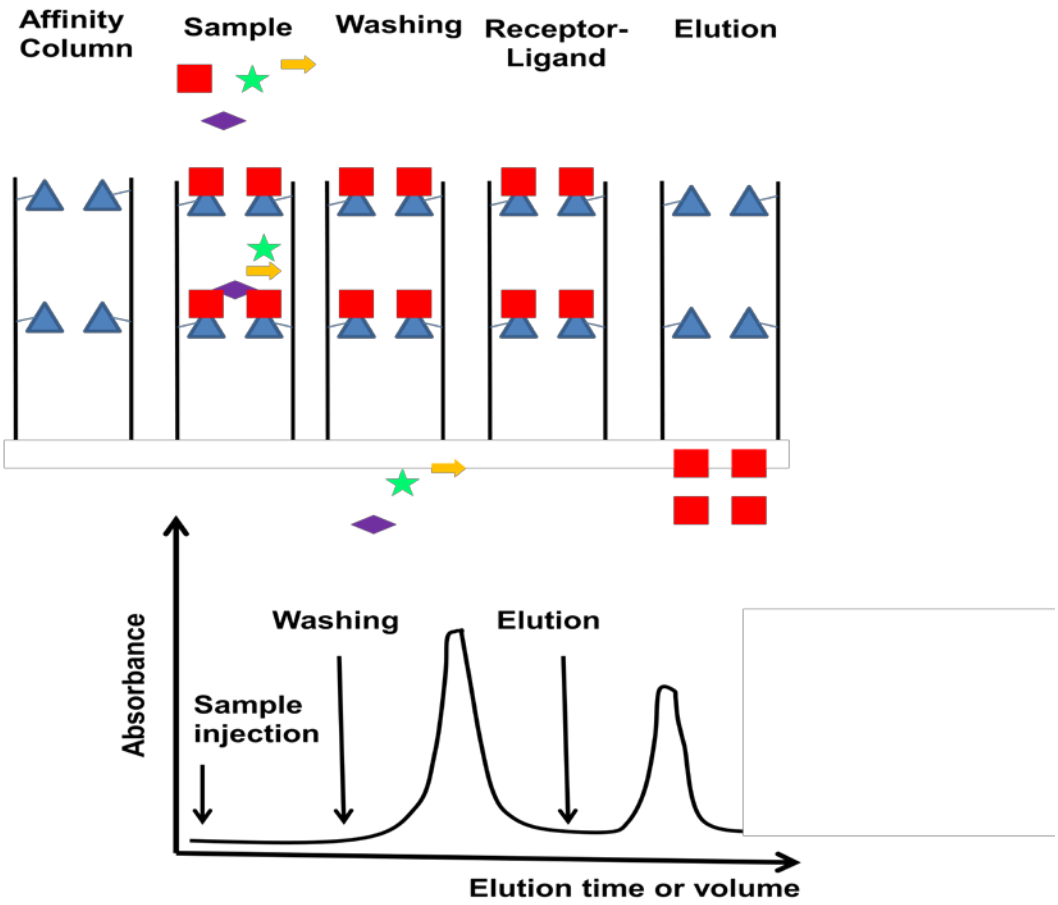


Figure 35.4: Performing Affinity chromatography

Lecture 36 Affinity Chromatography-III

Applications of Affinity Chromatography

1. Purification of biomolecules

GST Based Purification- Glutathione S-transferase (GST) utilizes glutathione as a substrate to catalyze conjugation reactions for xenobiotic detoxification purposes. The recombinant fusion protein contains GST as a tag is purified with glutathione coupled matrix (Figure 36.1). GST fusion protein is produced by the recombining protein of interest with the GST coding sequence present in the expression vector (either before or after coding sequence of protein of interest). It is transformed, over-expressed and the bacterial lysate containing fusion protein is purified, using affinity column. The sample is loaded on the column previously equiliberated with the buffer containing high salt (0.5M NaCl). Unbound protein is washed with the equilibration buffer and then the fusion proein is eluted with different concentration of glutathione dissolved in the equilibration buffer. Purified fusion can be treated with the thrombin to remove the GST tag from the protein of interest. The mixture containing free GST tag and the protein can be purified using the affinity column again as tag will bind to the matrix but protein will come out in the unbound fraction.

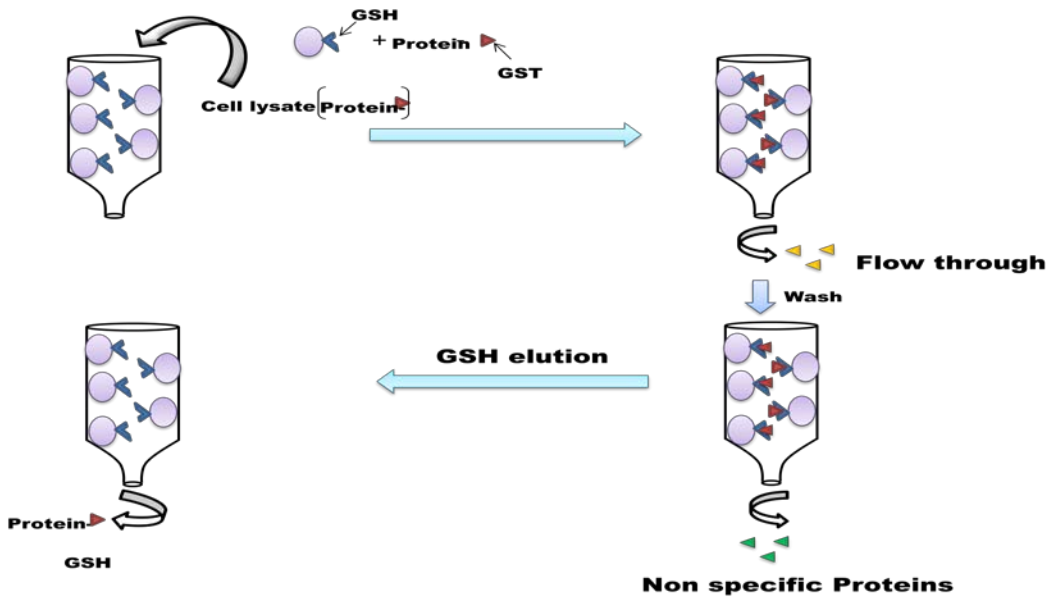


Figure 36.1: GST purification

2. Protein-Protein interaction-Protein-protein interaction can be studied through multiple techniques or approaches. Affinity column also can be used as a tool to study or isolate interacting partner of a particular protein. A schematic figure to depict the steps involved in the studying protein-protein interaction is given in (Figure 36.2). In this approach, matrix is incubated with the pure protein-1 and then washed to ensure tight binding. All other sites on the bead is blocked with an non-specific protein such as BSA or an unrelated cell lysate. Now cell lysate or the pure protein-2 is passed to the protein-1 containing beads, followed by washing with the buffer to remove unbounded proteins. Now the protein-1 is eluted from the matrix either by adding high concentration of ligand, or with denaturating condition. Now the eluted protein is analyzed in the SDS-PAGE or SDS-PAGE followed by the western blotting to detect protein 1 or protein 2. As a control, cell lysate or protein-2 is also added to the matrix without protein-1 to rule out the possibility of protein-2 binding directly to the matrix.

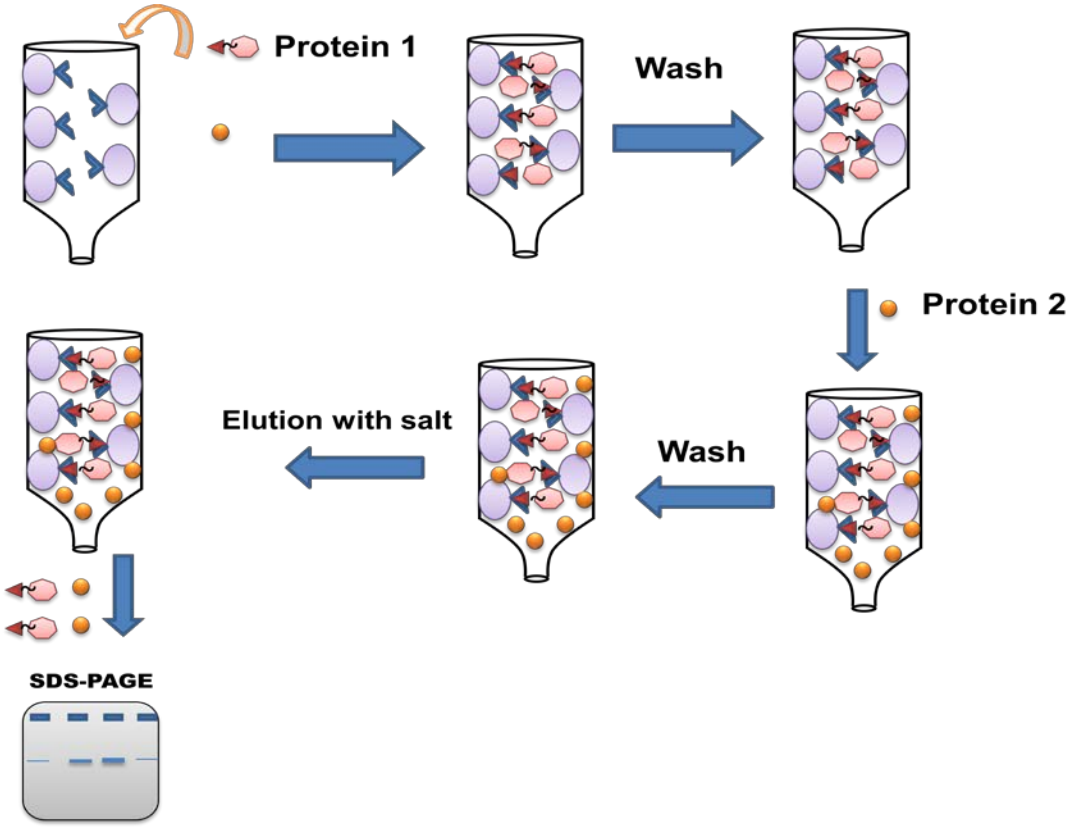


Figure 36.2: Protein-protein interaction studies with an affinity chromatography.

3. Enzymatic Assay-Affinity chromatography can be use to perform enzymatic assay such as protease assay (Figure 36.3). In this assay, peptides with different amino acid sequence bound with the terminal residue to the affinity bead are incubated with the protease for an optimal time. Enzyme acts on the attached peptide and relases the free portion into the supernatant. The supernatant is recovered from the reaction mixture and can be analyzed in a MALDI-tof to deduce the amino acid sequence from the molecular weight. Analysis of a set of reaction may allow to predict the protease recognition and cutting site.

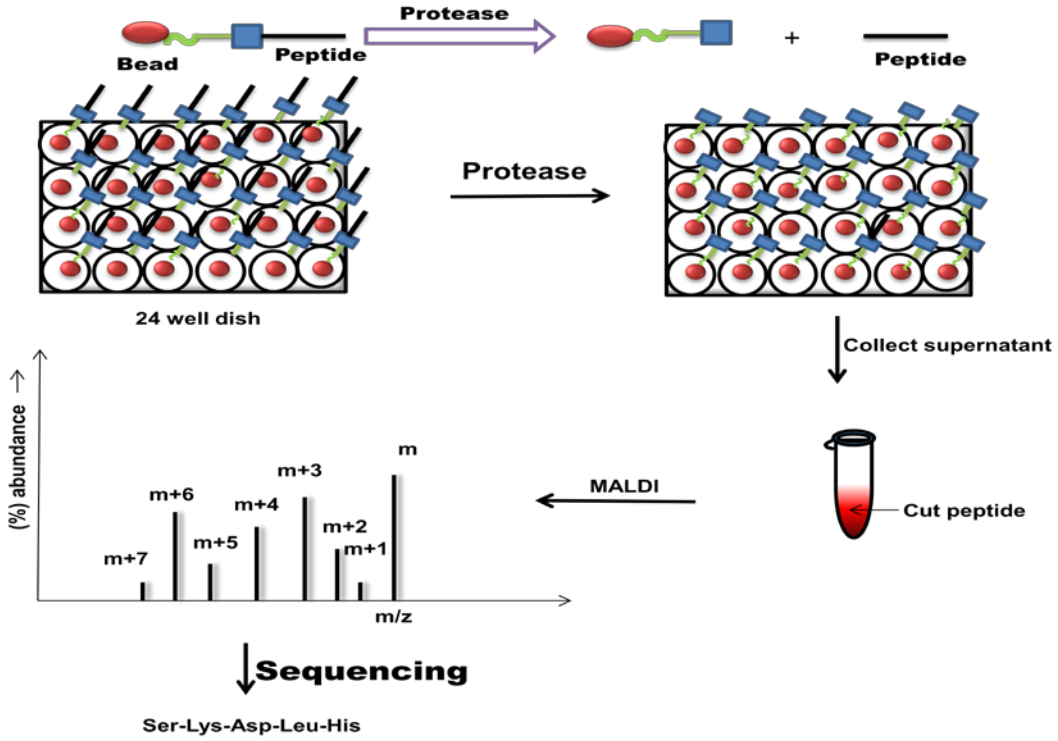


Figure 36.3: Protease assay by an affinity chromatography.

4. Clinical diagnosis-Receptor present on the matrix provides a unique tool to isolate, detect and characterize biomolecules from the crude mixture. For example, matrix containing boronic acid is used to separate and quantify glycosylated hemoglobin from diabetic patients blood. Ribonucleoside in patient urine can be identify by an affinity matrix containing boronic acid followed by the reverse phase chromatography.

5. Immuno-purification- The avidin-biotin system is used to capture and isolate cytokines from immune cells (Figure 36.4). Biotinylation of antibodies allows immobilization of antibodies in the correct orientation on the streptomycin coated glass beads. Lymphocyte lysate is passed to the column packed with the glass beads containing antibodies binds cytokines. The cytokines are eluted by flowing buffer of decreasing pH or by chaotrophic ions. The antibodies remain bound to the column due to strong affinity between avidin-biotin which is resistant to these chemical treatment.

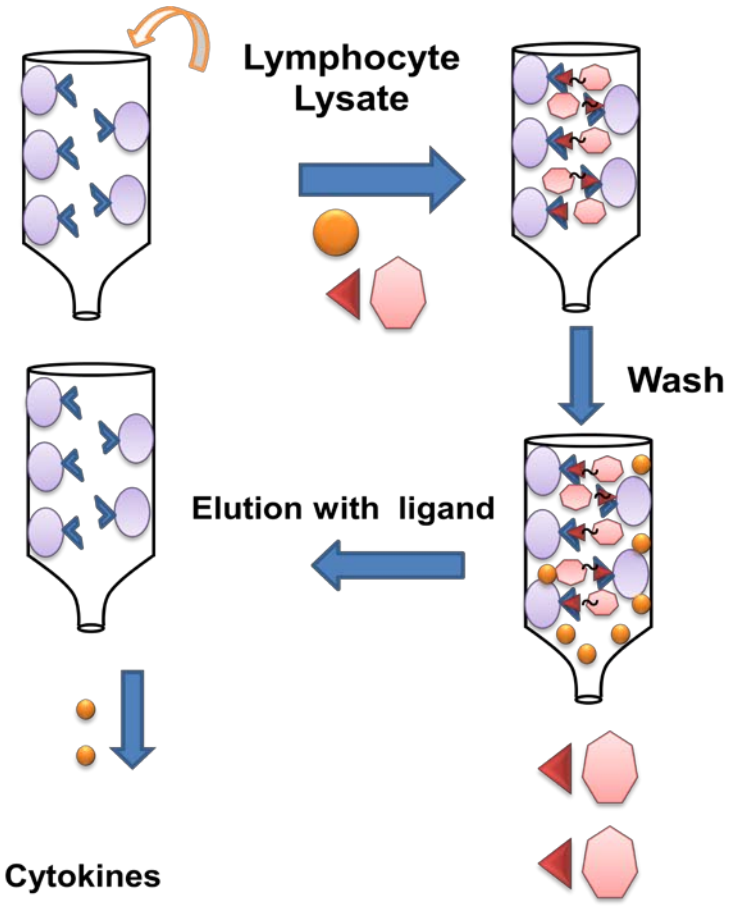


Figure 36.4: Immuno-purification with an affinity column.

Lecture 37 Thin Layer Chromatography

The thin layer chromatography technique is an analytical chromatography to separate and analyze complex biological or non-biological samples into their constituents. It is most popular for monitoring the progress of a chemical reaction or estimation of a substance in a mixture. It is also one of the popular technique for testing the purity of a sample. In this method, the silica or alumina as a stationery phase is coated on to a glass or aluminium foil as thin layer and then a sample is allowed to run in the presence of a mobile phase (solvent). In comparison to other chromatography techniques, the mobile phase runs from bottom to top by diffusion (in most of the chromatography techniques, mobile phase runs from top to bottom by gravity or pump). As sample runs along with the mobile phase, it get distributed into the solvent phase and stationery phase. The interaction of sample with the stationery phase retard the movement of the molecule where as mobile phase implies an effective force onto the sample. Supose the force caused by mobile phase is $\mathbf{F_m}$ and the retardation force by stationery phase is $\mathbf{F_s}$, then effective force on the molecule will be $\mathbf{(F_m-F_s)}$ through which it will move (Figure 37.1). The molecule immobilizes on the silica gel (where, $F_m=F_s$) and the position will be controlled by multiple factors.

1. Nature or functional group present on the molecule or analyte.

2. Nature or composition of the mobile phase

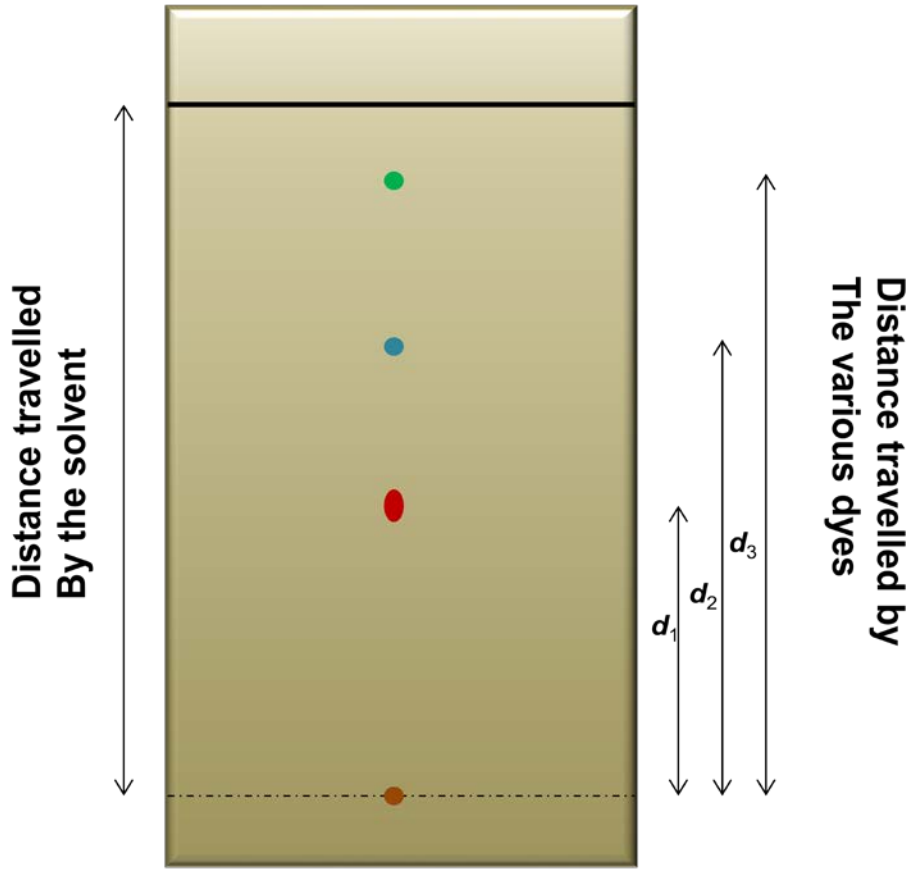
3. Thickness of the stationery phase.

4. Functional group present on stationery phase.

If the distance travelled by a molecule on TLC plate is $\mathbf{D_m}$ where as the distance travelled by the solvent is $\mathbf{D_s}$, then the retardation factor (R_f) of molecule is given by:

$$\mathbf{Rf} = \frac{\mathbf{Distance\ travelled\ by\ substance\ (Dm)}}{\mathbf{Distance\ travelled\ by\ solvent\ (Ds)}}$$

R_f value is characterstic to the molecule as long as the solvent system and TLC plate remains unchanged. It can be used to identify the substance in a crude mixture.



$$R_f = \frac{\text{Distance travelled By the solute } (D_1)}{\text{Distance travelled By the solvent } (D_s)}$$

Figure 37.1: Principle of thin layer chromatography.

Operation of the technique-Several steps are required to perform a thin layer chromatography to analyze a complex samples. These preparatory and operational steps are as follows:

Thin Layer Chromatography Chamber- Thin layer chromatographjy chamber (rectangular or cylindrical) is made up of transparent non-reactive material, mostly glass (Figure 37.2). It is covered from top with a thick glass sheet and the joints are sealed with a high vacuum grease to avoid loss of solvent vapor. All three sides of the chamber is covered with a whatman filter paper to uniformly equilibrate the chamber. A solvent system is filled in the chamber and it is allowed to humidify the chamber with the solvent vapor. It is important for uniform running of solvent front during TLC.

Preparation of TLC plate- A silica slurry is prepared in water and spread on the glass or alumina sheet as a thin layer and allowed to dry. It is baked at 110^0C for 1hr in a hot air oven and then the plate is ready for TLC. The layer is thin (~ 0.1-0.25 mm) for analytical application and thick (0.4-2.1 mm) for preparative or bio-assay purposes.

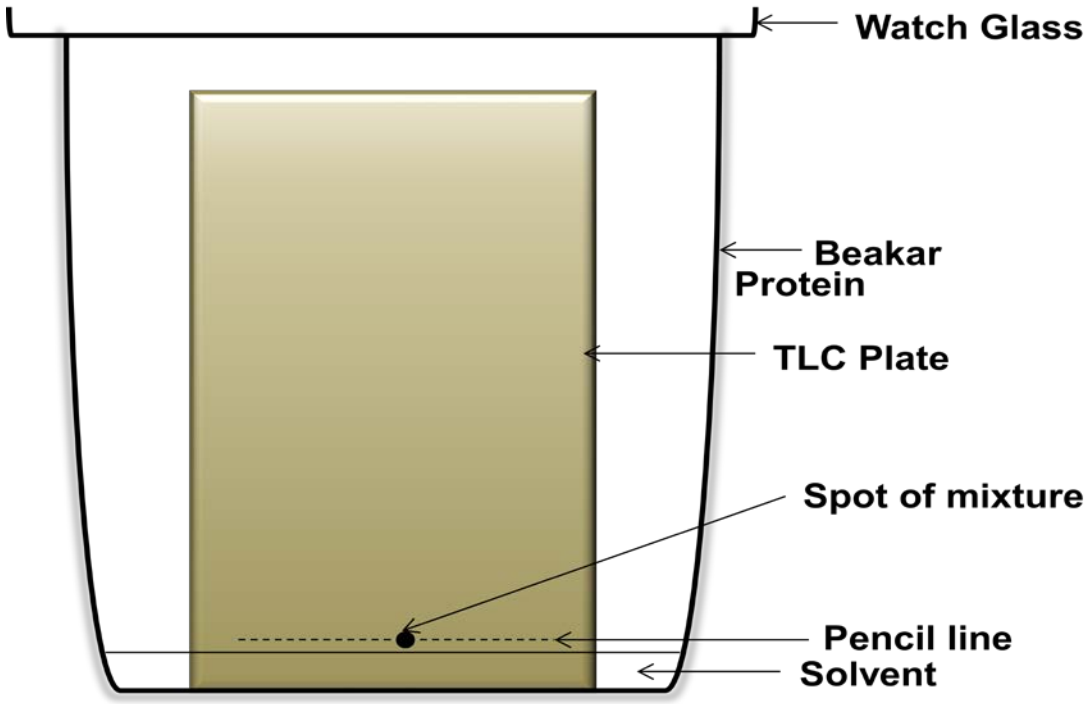


Figure 37.2: Thin Layer chromatography chamber

Spoting: The events involved in spotting is given in Figure 37.3. A line is draw with a pencil little away from the bottom. Sample is taken into the capillary tube or in a pipette. Capillary is touched onto the silica plate and sample is allowed to dispense. It is important that depending on the thickness of the layer, a suitable volume should be taken to apply. Spot is allowed to dry in air or a hair dryer can be used instead.

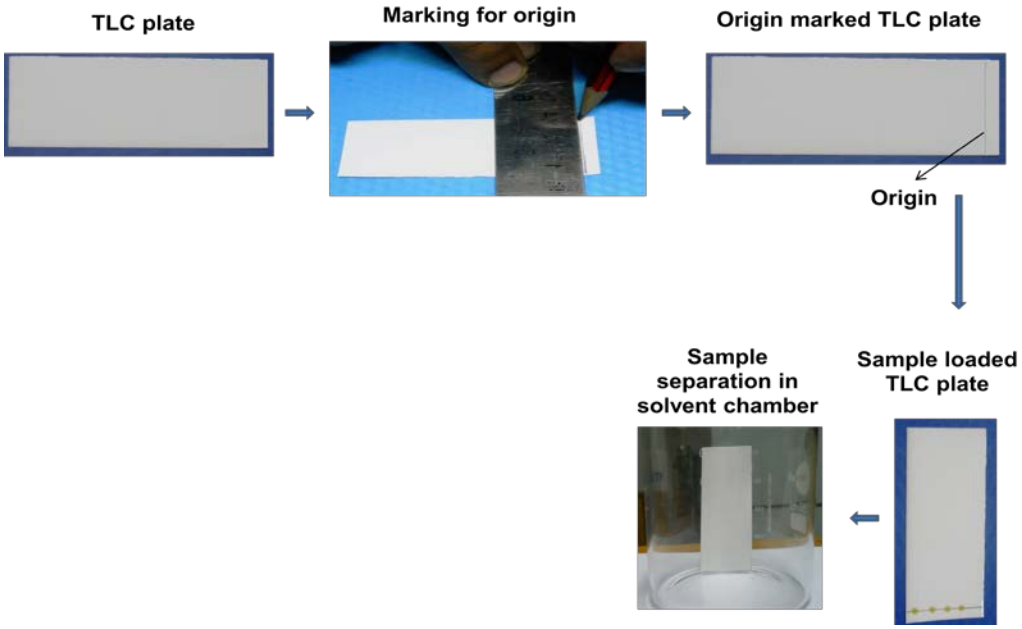


Figure 37.3: Events in spotting during thin layer chromatography.

Running of the TLC: Once the spot is dried, it is placed in the TLC chamber in such a way that spot should not be below the solvent level. Solvent front is allowed to move until the end of the plate.

Analysis of the chromatography plate- The plate is taken out from the chamber and air dried. If the compound is colored, it forms spot and for these substances there is no additional staining required. There are two methods of developing a chromatogram-

Staining procedure- In the staining procedure, TLC plate is sprayed with the staining reagent to stain the functional group present in the compound. Forx. Ninhydrin is used to stain amino acids.

Non-staining procedure- In non-staining procedure spot can be identify by following methods-

1. Autoradiography- A TLC plate can be placed along with the X-ray film for 48-72 hrs (exposure time depends on type and concentration of radioactivity) and then X-ray film is processed.

2. Fluorescence- Several heterocyclic compounds give fluorescence in UV due to presence of conjugate double bond system. TLC plate can be visualized in an UV-chamber (Figure 37.4) to identify the spots on TLC plate.

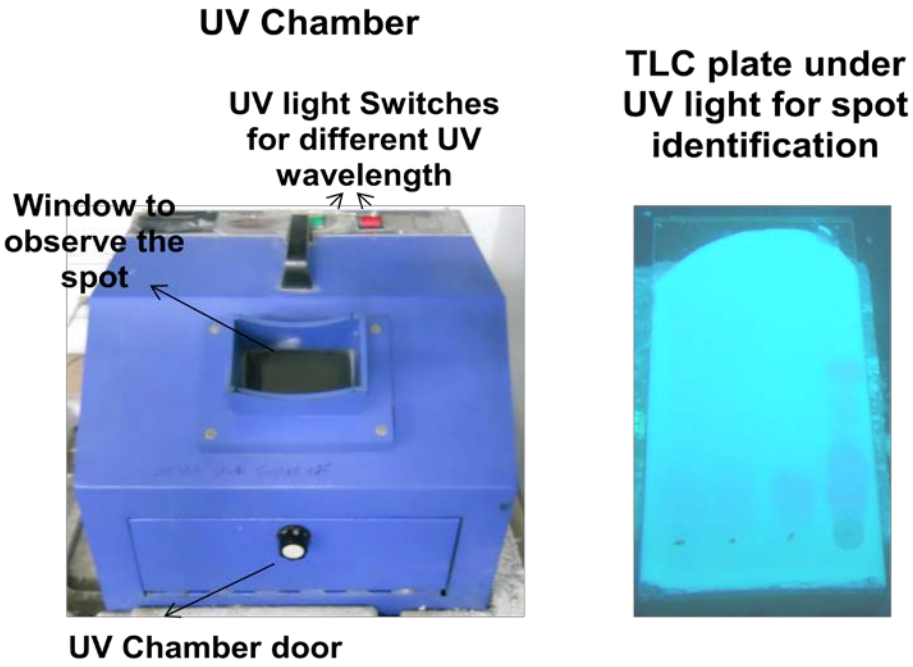


Figure 37.4: UV-Chamber and UV illuminated TLC plate.

Technical troubles with thin layer chromatography-

1. Tailing effect-In general sample forms round circular spot on the TLC plate. It is due to the uniform movement of the solvent front through out the plate. But in few cases instead of forming a spot, a compound forms a spot with long trail or rocket shape spot (Figure 37.5). it is due to few reasons as given below:

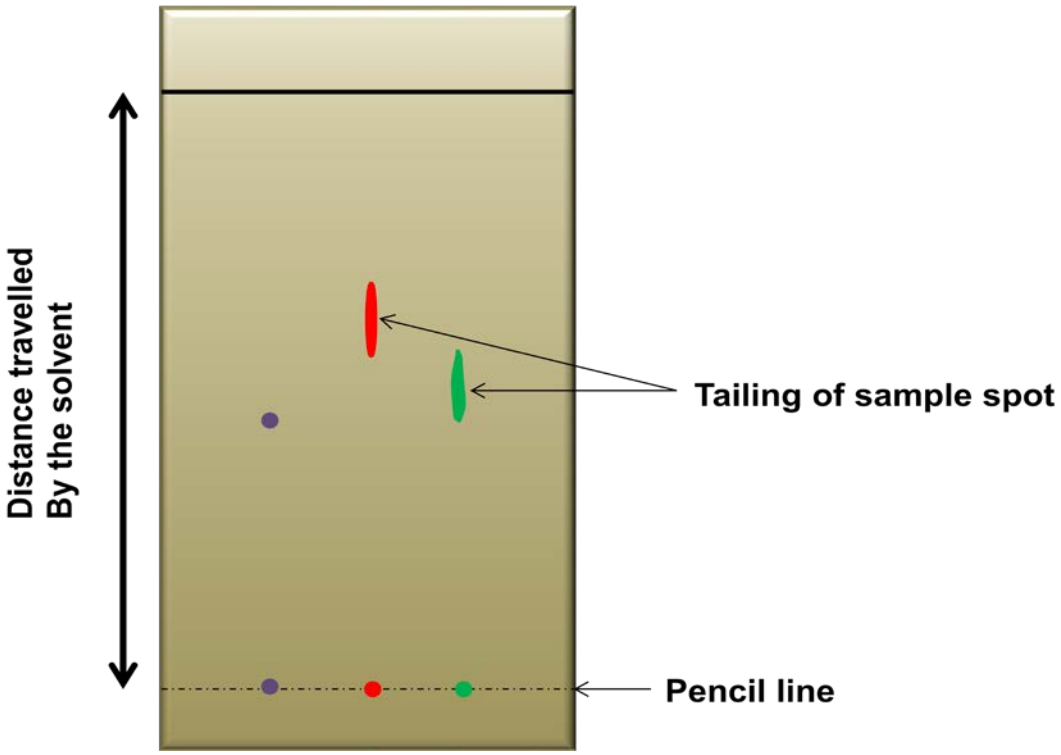


Figure 37.5: Tailing effect in thin layer chromatography.

A. Over-loading- if the sample is loaded much more than the loading capacity of the TLC plate, it appears as spot with trail or rocket shape spot. A diluted sample can be tested to avoid this.

B. Fluctuation in temp or opening of chamber- If there will be fluntuation in temperature or solvent saturation in the chamber (due to opening of the chamber during running), it disturb the flow of solvent front and consequently cause spot with trail. it can be avoided by maintaining a uniform temperature and the opening of the chamber should be minimized especially during running.

2. No movement of sample- In few cases, a sample doesn't move from the spot after the run is completed. These problems are common with high molecular weight substances such as protein or chemicals with large number of functional group. In this case, a change in polarity or pH of solvent system can be explored to bring the compound into the solvent front so that it run on silica plate to get resolved.

3. Movement is too fast-In few cases, the movement of a compound is too fast and does not give time to interact with the matrix to resolve into individual compounds. In this case, a change in polarity of solvent system can be explored to retard the running of the sample.

Applications of Thin layer Chromatography

1. Composition analysis of biomolecules/synthetic preparation
2. Quality testing of compound.
3. Identification of impurities in a sample
4. Progress of chemical reaction
5. Estimation of biomolecules
6. Bio-assay