Biyani's Think Tank

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Concept based notes

Instrumentation and Computer Course

[B.Sc.Biotect Part-I]

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Preface

I am glad to present this book, especially designed to serve the needs of the students. The book has been written keeping in mind the general weakness in understanding the fundamental concepts of the topics. The book is self-explanatory and adopts the “Teach Yourself” style. It is based on question-answer pattern. The language of book is quite easy and understandable based on scientific approach.

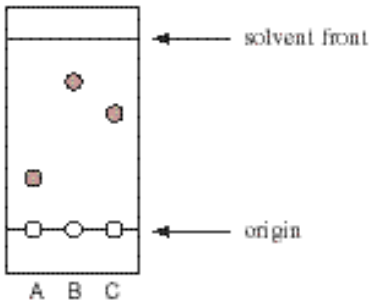
Any further improvement in the contents of the book by making corrections, omission and inclusion is keen to be achieved based on suggestions from the readers for which the author shall be obliged.

I acknowledge special thanks to Mr. Rajeev Biyani, *Chairman* & Dr. Sanjay Biyani, *Director* (*Acad.*) Biyani Group of Colleges, who are the backbones and main concept provider and also have been constant source of motivation throughout this Endeavour. They played an active role in coordinating the various stages of this Endeavour and spearheaded the publishing work.

I look forward to receiving valuable suggestions from professors of various educational institutions, other faculty members and students for improvement of the quality of the book. The reader may feel free to send in their comments and suggestions to the under mentioned address.

Author

Q 1. Following silica gel TLC plate of compounds A, B, and C developed in organic compounds (hexanes):



Answer the following questions:

a) Calculate the R_f values of compounds A, B, and C on the given silica gel TLC plate and hexanes as the solvent.

b) Arrange the compounds A, B, or C according to increasing order of their polarity.

c) What would you expect to happen to the R_f values if you used acetone instead of hexanes as the eluting solvent?

d) What will happen with the R_f values if eluted with hexanes using an alumina TLC plate?

Ans. a) Take a ruler and measure the distance between the origin and the solvent front: 2.75 cm. Then measure the distance between the origin and the center of spot A: 0.80 cm. Therefore the R_f for compound A is 0.80/2.75 = 0.29.

Similarly, compound B travels 2.20 cm, and therefore the R_f for compound B is 2.20/2.75 = 0.80. Compound C travels 1.70 cm, and therefore the R_f for compound C is 1.70/2.75 = 0.62.

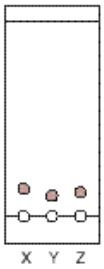
b) Compound A is the most polar because it does not travel as far as the other two compounds. Remember, polar compounds stick to the adsorbent more readily, and thus do not travel as far and have a lower value for R_f.

c) Acetone is a more polar solvent than is hexanes. If it were used to elute the same three compounds, each of the compounds would travel faster because the more polar eluting solvent is more proficient at eluting the compounds from the polar adsorbent. Since each compound travels faster, each

compound would have a larger R_f value if acetone were used to elute than when hexanes is used to elute the TLC plate.

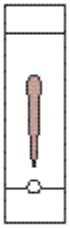
d) Alumina is more polar than is silica (see the first paragraph under "The Adsorbent" in the TLC section). Therefore, each of the compounds would travel slower on an alumina TLC plate than on a silica TLC plate, the R_f values for each of the compounds would be smaller.

Q. 2 **You are trying to determine a TLC solvent system which will separate the compounds X, Y, and Z. You ran the compounds on a TLC plate using hexanes/ethyl acetate 95:5 as the eluting solvent and obtained the chromatogram below. How could you change the solvent system to give better separation of these three compounds?**



Ans. In order to get the three compounds X, Y, and Z to separate, you need to get them to move further up the plate. To do this, you need to increase the polarity of the eluting solvent. Since the eluting solvent tried was hexanes/ethyl acetate 95:5, the first thing to try is a higher percentage of ethyl acetate in the eluting solvent, such as hexanes/ethyl acetate 85:15. If the three compounds still do not separate, try hexanes/ethyl acetate 50:50; continue increasing the percentage of ethyl acetate until separation is achieved. You could also try a more polar solvent such as acetone or methanol to replace the ethyl acetate.

Q. 3 **After a rather lengthy organic chemistry synthesis procedure, a student ran the product of the reaction on a TLC plate and obtained the result below. What might he/she have done wrong, if anything?**



Ans. A couple things can cause a TLC plate to streak as illustrated in the diagram of the plate shown. The plate might be overloaded, in other words, the solution used to spot the plate is too concentrated (to fix this, dilute the solution and try the TLC again). Or, there are simply too many components in the mixture to be separated by TLC.

Ref. orgchem.colorado.edu

Q. 4 A student spots an unknown sample on a TLC plate. After developing in hexanes/ethyl acetate 50:50, he/she saw a single spot with an R_f of 0.55. Does this indicate that the unknown material is a pure compound? What can be done to verify the purity of the sample?

Ans. The fact that you see only one spot on a TLC plate does not necessarily mean that the solution spotted contains only one component. This is because two compounds can have the same value of R_f in a particular eluting system. You must run the sample in a different eluting solvent and see if it again gives only one spot. This is a good indication that the sample is pure. However, you still should verify the purity of the sample by melting point, boiling point, and/or spectroscopic analysis.

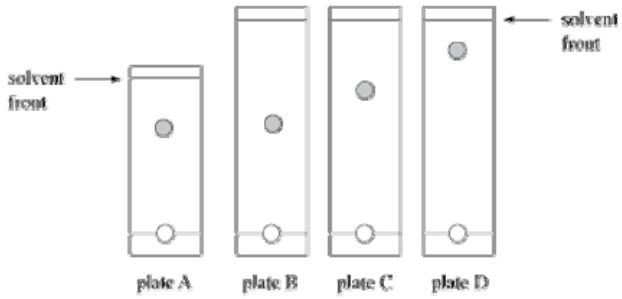
Ref. orgchem.colorado.edu

Q. 5 Consider a sample that is a mixture composed of biphenyl, benzoic acid, and benzyl alcohol. The sample is spotted on a TLC plate and developed in a hexanes/ethyl acetate solvent mixture. Predict the relative R_f values for the three compounds in the sample.

Ans. Biphenyl is an unsaturated hydrocarbon, and therefore is the least polar and will have the largest R f value of the three compounds. The benzoic acid - an acid - would be the most polar and therefore will have the smallest Rf value. Benzyl alcohol is between these two compounds in polarity and will have an Rf value between the two. (See the chart in the TLC section under "Interactions of the compound and the adsorbent".)

Ref. orgchem.colorado.edu

Q. 6 Plate A, below, shows the TLC chromatogram of a compound run in hexanes. The same compound was then spotted on a large TLC plate and again run in hexanes. Which TLC plate, B, C, or D, correctly represents how far the compound would run on the longer plate?



compound will move with the same Rf value no matter what length the TLC plate. So, determine first the Rf value on plate A, and then see which of the longer plates gives the same Rf value for the compound. Take a ruler and measure the distance between the origin and the solvent front on plate A; you should measure about 2.5 cm. Then measure the distance to the center of the spot; you should measure about 1.75 cm. Determine the Rf by dividing 1.75 by 2.5; you should get 0.7 for this Rf value. Now look at plates B, C, and D. On plate C, if you measure how far the spot moves (2.3 cm) and divide this value by the solvent front (3.4 cm), you get about 0.7 for the Rf. Thus, the answer is plate C.

Ref. orgchem.colorado.edu

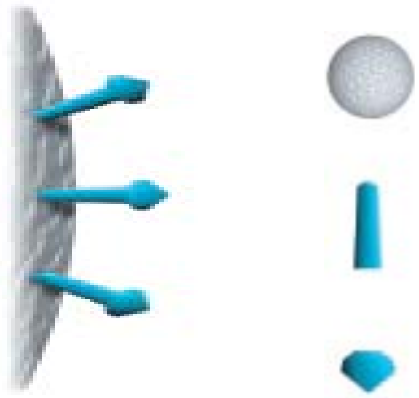
Q.7 Describe affinity chromatography in brief.

A technique exhibiting great selectivity, affinity chromatography, was first described by Pedro Cuatrecasas and his coworkers in 1968. In these separations, a biomolecule such as an enzyme binds to a substrate attached to the solid phase while other components are eluted. The retained molecule can subsequently be eluted by changing the chemical conditions of the separation.

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix.

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatographic matrix. The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. Purification can be in the order of several thousand-fold and recoveries of active material are generally very high.

Common terms in affinity chromatography



Matrix: for ligand attachment. Matrix should be chemically and physically inert.

Spacer arm: used to improve binding between ligand and target molecule by overcoming any effects of

steric hindrance.

Ligand: molecule that binds reversibly to a specific target molecule or group of target molecules.

Binding: buffer conditions are optimized to ensure that the target molecules interact effectively with the ligand and are retained by the affinity medium as all other molecules wash through the column.

Elution: buffer conditions are changed to reverse (weaken) the interaction between the target molecules and the ligand so that the target molecules can be eluted from the column.

Wash: buffer conditions that wash unbound substances from the column without eluting the target molecules or that re-equilibrate the column back to the starting conditions (in most cases the binding buffer is used as a wash buffer).

Ligand coupling: covalent attachment of a ligand to a suitable pre-activated matrix to create an affinity medium.

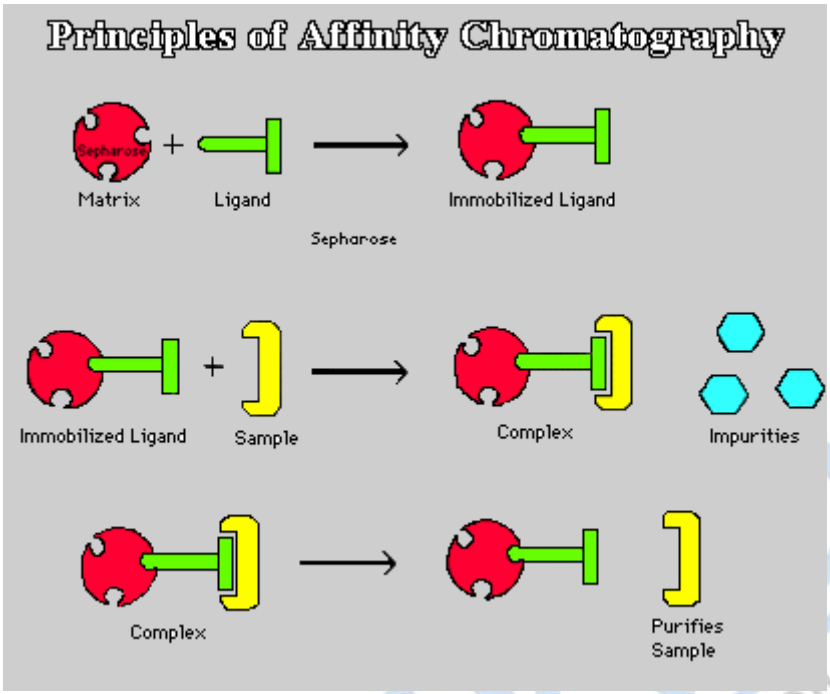
Pre-activated matrices: matrices which have been chemically modified to facilitate the coupling of specific types of ligand.

The technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest. With high selectivity, hence high resolution, and high capacity for the protein(s) of interest, purification levels in the order of several thousand-fold with high recovery of active material are achievable. Target protein(s) is collected in a purified, concentrated form.

Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. In a single step, affinity purification can offer immense time-saving over less selective multistep procedures. The concentrating effect enables large volumes to be processed. Target molecules can be purified from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances. For an even higher degree of purity, or when there is no suitable ligand for affinity purification, an efficient multi-step process must be developed using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP). When applying this strategy affinity chromatography offers an ideal capture or intermediate step in any purification protocol and can be used whenever a suitable ligand is available for the protein of interest.

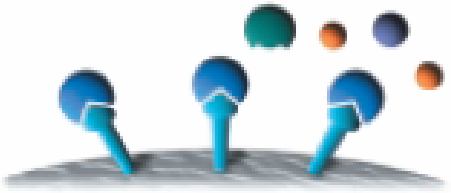
Successful affinity purification requires a biospecific ligand that can be covalently attached to a chromatographic matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in affinity chromatography, are listed below:

- Enzyme substrate analogue, inhibitor, cofactor.
- Antibody antigen, virus, cell.
- Lectin polysaccharide, glycoprotein, cell surface receptor, cell.
- Nucleic acid complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein.
- Hormone, vitamin receptor, carrier protein.
- Glutathione glutathione-S-transferase or GST fusion proteins.
- Metal ions Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces.

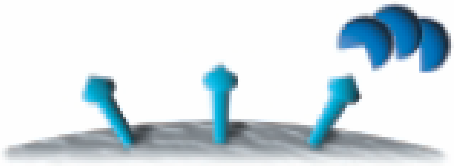


Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose™ 6 Fast Flow can remove serine proteases, such as thrombin and Factor Xa. Figure 2 shows the key stages in an affinity purification.

1. Affinity medium is equilibrated in binding buffer.

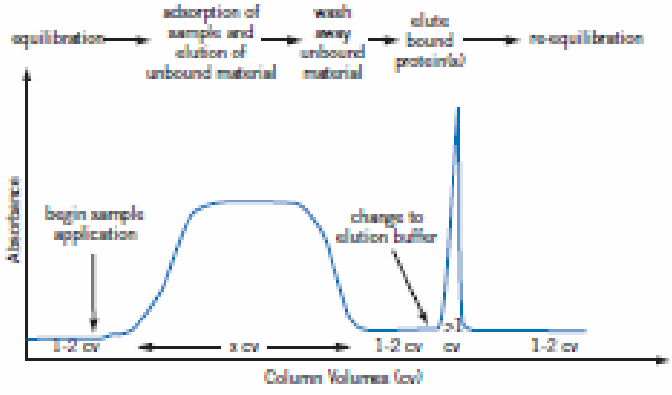


2. Sample is applied under conditions that favour specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.



3. Target protein is recovered by changing conditions to favour elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.

4. Affinity medium is re-equilibrated with binding buffer.



Q.8 What are the main types of chromatography?

Ans.

- Ion Exchange Chromatography (IEC)
  separates biomolecules based on the their net surface charge
- Ion Chromatography (IC)
  more general form of IEC allows separation of ions and polar molecules based on the charge properties of the molecules
- Affinity Chromatography (AC)
  is the purification of a biomolecule with respect to the specific binding of that biomolecule due to the chemical structure
- Gas Chromatography (GC)
  is a technique used to separate organic molecules that are volatile
- Gas-Liquid Chromatography (GLC)
  another name for GC

Q.9 Write a note on ion exchange chromatography.

Ion-Exchange Chromatography

- Usually employed with HPLC
- Ions are charged molecules

  - cation positively charged ion

  - anion negatively charged ion
- These ions do not separate smoothly under the traditional methods of the liquid and mobile phases of chromatography
- Requires alteration methods of either the mobile phase or stationary phase are required

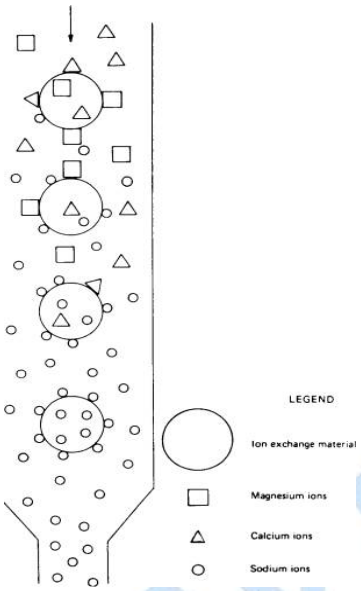
- mobile phase suppresses the ionic nature of the analyte

- stationary phase incorporate ions of the opposite charge to attract and retain analyte

How do you get those columns to work

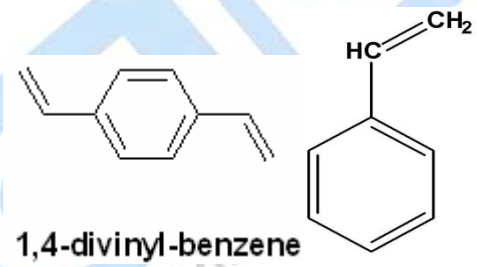
- There is a glass column coated with a resin polymer
- The resin is either positively charged (an acid) or negatively charged (a base)

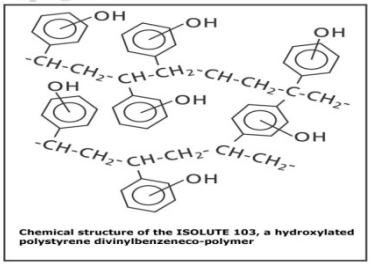
- An analyte will have ions opposite of the resins charge eluting off the ion of interest



Resin

- Common resins are copolymerized styrenes
- vinylic and aromatic functional groups styrene derivative and divinylbenzene





- Creates better stability due to crosslinking of the benzene rings
- Creates a swelling within the polymer affecting the porosity while taking in the mobile phase liquid
- Aromatic substitution reaction makes these polymers ideal for charged functional groups

Cation Exchange Resins :

- The functional group in cation exchange resins are usually acids
- Sulfonic acids $-SO_3H$ (strong acid resin) are added to the resin by sulfonation reactions

  $Res\text{-}(SO_3H) + M^+ \rightarrow Res\text{-}(SO_3M) + H^+$
- Carboxylic acid –COOH (weak acid resin)

  $Res\text{-}COOH + M^+ \rightarrow Res\text{-}COOM + H^+$
- With both the strong and the weak acid exchange sites an acidic Hydrogen is attached to a functional group chemically bound to the resin
- Cation exchange is good for removing metal ions from an aqueous solution

Anion Exchange Resins :

- The functional groups added to the resin is similar to cation resins but are basic instead of acidic
- Quaternary ammonium a strong base -- $CH_2N(CH_3)_3{+}OH^-$
  - $CH_2N(CH_3)_3{+}OH^- + B^- \rightarrow Res\text{-}CH_2N(CH_3)_3{}^+Cl^- + OH^-$
- Polyalky amine a weak base -- $NH(\text{-}R)_2{+}OH^-$

  $NH(\text{-}R)_2{+}OH^- + B^- \rightarrow Res\text{-}NH(\text{-}R)_2{}^+B^- + OH^-$
- The rate of ion exchange is controlled by the law of mass action. At equal concentration the greater affinity molecule will control the cation exchange resins in the acid form.
- However if a much higher concentration of strong acid passed through the greater affinity molecule, such as sodium, will form the resin, reversing equilibrium and convert the resin back to an acidic form.
- Generally it is possible to return either ion exchange resin column to a desired starting form by passing a large excess of the desired ion at very high concentration through the resin

  Application of Ion exchange chromatography
- In cell and molecular biology, ion exchange chromatography is used to separate different proteins out of an eluant.
- areas of research such as the environment, industry, commercial products of organic molecules without UV-vis absorption

- The analysis of ionic analytes by separation on ion exchange stationary phases with eluent suppression of excess eluent ions
- ex) when cations are being exchanged to effect a separation, variable concentrations of HCl are used as an eluent passing through the analytical anion column without being retained forming largely undissociated species such as water, carbonic acid and bicarbonate ions

Q.9 What do you know about electrophoresis?

Ans. Electrophoresis may be the main technique for molecular separation in today's cell biology laboratory. Because it is such a powerful technique, and yet reasonably easy and inexpensive, it has become commonplace. In spite of the many physical arrangments for the apparatus, and regardless of the medium through which molecules are allowed to migrate, all electrophoretic separations depend upon the charge distribution of the molecules being separated.

Electrophoresis can be one dimensional (i.e. one plane of separation) or two dimensional. One dimensional electrophoresis is used for most routine protein and nucleic acid separations. Two dimensional separation of proteins is used for finger printing , and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell (greater than 1,500).

The support medium for electrophoresis can be formed into a gel within a tube or it can be layered into flat sheets. The tubes are used for easy one dimensional separations (nearly anyone can make their own apparatus from inexpensive materials found in any lab), while the sheets have a larger surface area and are better for two- dimensional separations. Following figure shows a typical slab electrophoresis unit.

When the detergent SDS (sodium dodecyl sulfate) is used with proteins, all of the proteins become negatively charged by their attachment to the SDS anions. When separated on a polyacrylamide gel, the procedure is abbreviated as SDS--PAGE (for Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis). The technique has become a standard means for molecular weight determination.

Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N,N1-methylene- bis-acrylamide (Bis, for short). Bis is a cross-linking agent for the gels. The polymerization is initiated by the addition of ammonium persulfate along with either β-dimethyl amino-propionitrile (DMAP) or N,N,N1,N1,- tetramethylethylenediamine (TEMED). The gels are neutral, hydrophillic, three-dimensional networks of long hydrocarbons crosslinked by methylene groups.

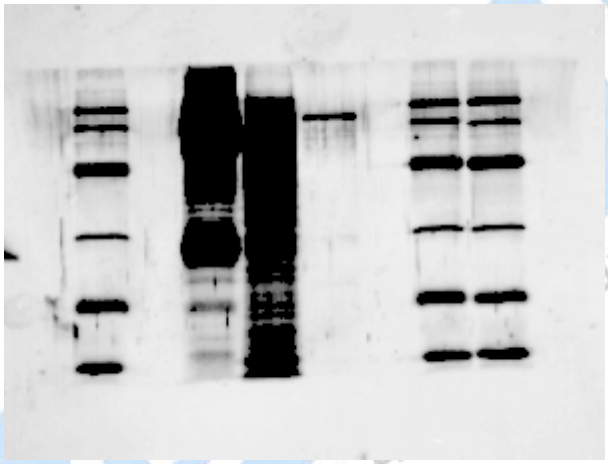
The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The pore size of a gel is determined by two factors, the total amount of acrylamide present (designated as %T) and the amount of cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases. With cross- linking, 5%C gives the smallest pore size. Any increase or decrease in %C increases the pore size. Gels are designated as percent solutions and will have two necessary parameters. The total acrylamide is given as a % (w/v) of the acrylamide plus the bis-acrylamide. Thus, a 7 1/2 %T would indicate that there is a total of 7.5 gms of acrylamide and bis per 100 ml of gel. A gel designated as 7.5%T:5%C would have a total of 7.5% (w/v) acrylamide + bis, and the bis would be 5% of the total (with pure acrylamide composing the remaining 2.5%).

Proteins with molecular weights ranging from 10,000 to 1,000,000 may be separated with 7 1/2% acrylamide gels, while proteins with higher molecular weights require lower acrylamide gel concentrations. Conversely, gels up to 30% have been used to separate small polypeptides. The higher the gel concentration, the smaller the pore size of the gel and the better it will be able to separate smaller molecules. The percent gel to use depends on the molecular weight of the protein to be separated. Use 5% gels for proteins ranging from 60,000 to 200,000 daltons, 10% gels for a range of 16,000 to 70,000 daltons and 15% gels for a range of 12,000 to 45,000 daltons.

Cationic vs anionic systems *:*

In electrophoresis, proteins are separated on the basis of charge, and the charge of a protein can be either + or -- , depending upon the pH of the buffer. In normal operation, a column of gel is partitioned into three sections, known as the Separating or Running Gel, the Stacking Gel and the Sample Gel. The sample gel may be eliminated and the sample introduced via a dense non-convective medium such as sucrose. Electrodes are attached to the ends of the column and an electric current passed through the partitioned gels. If the electrodes are arranged in such a way that the upper bath is -- (cathode), while the lower bath is + (anode), and -- anions are allowed to flow toward the anode, the system is known as an anionic system. Flow in the opposite direction, with + cations flowing to the cathode is a cationic system.

Tube vs Slab Systems :



Electrophoretic separations of proteins

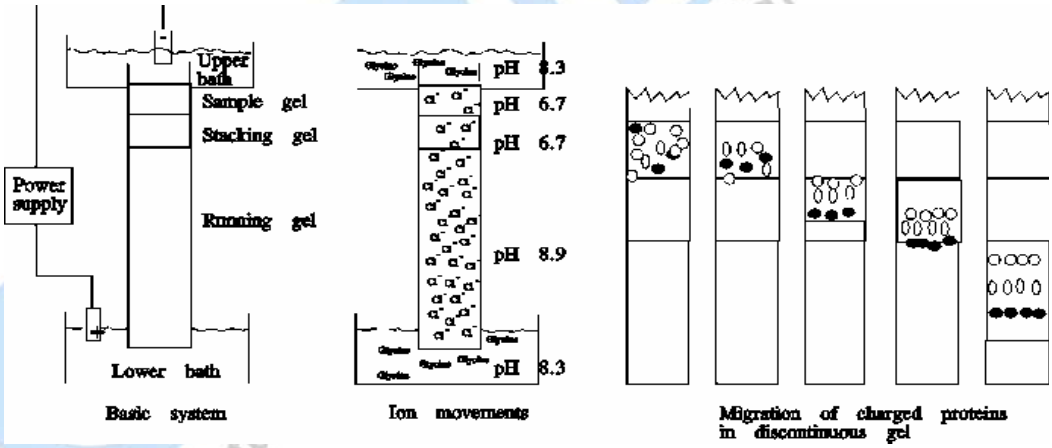
Two basic approaches have been used in the design of electrophoresis protocols. One, column electrophoresis, uses tubular gels formed in glass tubes, while the other, slab gel electrophoresis, uses flat gels formed between two plates of glass. Tube gels have an advantage in that the movement of molecules through the gels is less prone to lateral movement and thus there is a slightly improved resolution of the bands, particularly for proteins. It is also more economical, since it is relatively easy to construct homemade systems from materials on hand. However, slab gels have the advantage of allowing for two dimensional analysis, and of running multiple samples simultaneously in the same gel.

Slab gels are designed with multiple lanes set up such that samples run in parallel. The size and number of the lanes can be varied and, since the samples run in the same medium, there is less likelihood of sample variation due to minor changes in the gel structure. Slab gels are unquestionably the the technique of choice for any blot analyses and for autoradiographic analysis. Consequently, for laboratories performing routine nucleic acid analyses, and those employing antigenic controls, slab gels have become standard. The availability of reasonably priced commercial slab gel units has increased the use of slab gel systems, and the use of tube gels is becoming rare.

The theory and operation of slab gel electrophoresis is identical to tube gel electrophoresis. Which system is used depends more on the experience of the investigator than on any other factor, and the availability of equipment.

Above figure presents a typical protein separation pattern.

Continuous vs discontinuous gel systems :



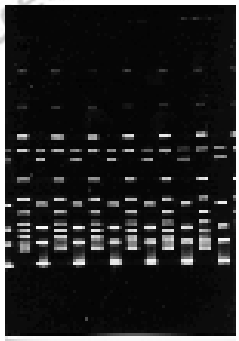
Schematic diagram of electrophoresis

The original use of gels as separating media involved using a single gel with a uniform pH throughout. Molecules were separated on the basis of their mobility through a single gel matrix. This system has only occasional use in today's laboratory. It has been replaced with discontinous, multiple gel systems. In multiple gel systems, a separating gel is augmented with a stacking gel and an optional sample gel. These gels can have different concentrations of the same support media, or may be completely different agents. The key difference is how the molecules separate when they enter the separating gel. The proteins in the

sample gel will concentrate into a small zone in the stacking gel before entering the separating gel. The zone within the stacking gel can range in thickness from a few microns to a full millimeter. As the proteins are stacked in concentrated bands, they continue to migrate into the separating gel in concentrated narrow bands. The bands then are separated from each other on a discontinuous (i.e. disc) pH gel.

Once the protein bands enter the separating gel, separation of the bands is enhanced by ions passing through the gel column in pairs. Each ioin in the pair has the same charge polarity as the protein (usually negative), but differ in charge magnitude. One ion will have a much greater charge magnitude than the proteins, while the other has a lesser charge magnitude than the proteins. The ion having a greater charge will move faster and is thus the leading ion, while the ion with the lesser charge will be the trailing ion. When an anionic system is employed, the Cl^{-} and glycinate (glycine as its acid derivative) ions are derived from the reservoir buffer (Tris-Glycine). The leading ion is usually Cl^{-} glycinate is the trailing ion. A schematic of this anionic system is shown in above figure Chloride ions enter the separating gel first and rapidly move down the gel, followed by the proteins and then the glycinate ions. The glycinate ions overtake the proteins and ultimately establish a uniform linear voltage gradient within the gel. The proteins then sort themselves within this gradient according to their charge and size.

Agarose Gels



Agarose separation of cDNA

While acrylamide gels have become the standard for protein analysis, they are less suitable for extremely high molecular weight nucleic acids (above 200,000 daltons). In order to properly separate these large molecules, the acrylamide concentration needs to be reduced to a level where it remains liquid.

The gels can be formed, however, by the addition of agarose, a naturally linear polysaccharide, to the low concentration of acrylamide. With the addition of agarose, acrylamide concentrations of 0.5% can be used and molecular weights of up to 3.5 x 10^6 daltons can be separated. This is particularly useful for the separation of large sequences of DNA. Consequently, agarose-acrylamide gels are used extensively in today's genetic laboratories for the determination of gene maps. This chapter will concentrate on the separation of proteins, but above figure demonstrates the separation of DNA fragments on an agarose gel.

Q. 10 Write a note on centrifugation.

Ans. Contents.

I. Introduction
II. Increasing the effect of gravity: the centrifuge
III. Types of centrifugal separation
IV. Rotor categories
V. Selection of centrifuge tubes
VI. Common centrifuge vocabulary and formulas
VII. Reference and suggested readings

I. Introduction :

Centrifugation is one of the most important and widely applied research techniques in biochemistry, cellular and molecular biology, and in medicine. Current research and clinical applications rely on isolation of cells, subcellular organelles, and macromolecules, often in high yields.

A centrifuge uses centrifugal force (g-force) to isolate suspended particles from their surrounding medium on either a batch or a continuous-flow basis. Applications for centrifugation are many and may include sedimentation of cells and viruses, separation of subcellular organelles, and isolation of macromolecules such as DNA, RNA, proteins, or lipids.

II. Increasing the effect of gravity: the centrifuge:

Many particles or cells in a liquid suspension, given time, will eventually settle at the bottom of a container due to gravity (1 x g). However, the length of time required for such separations is impractical. Other particles, extremely small in size, will not separate at all in solution, unless subjected to high centrifugal force.

When a suspension is rotated at a certain speed or revolutions per minute (RPM), centrifugal force causes the particles to move radially away from the axis of rotation. The force on the particles (compared to gravity) is called Relative Centrifugal Force (RCF). For example, an RCF of 500 x g indicates that the centrifugal force applied is 500 times greater than Earthâ€™s gravitational force. Table 1 illustrates common centrifuge classes and their applications.

Table 1. Classes of centrifuges and their applications.

	Centrifuge Classes		
	Lowspeed	**High-speed**	**Ultra/micro-ultra**
Maximum Speed (rpm $x10^3$)	10	28	100/150
Maximum RCF ($x10^3$)	7	100	800/900
Pelleting applications			
Bacteria	Yes	Yes	(Yes)
Animal and plant cells	Yes	Yes	(Yes)
Nuclei	Yes	Yes	(Yes)
Precipitates	Some	Most	(Yes)
Membrane fractions	Some	Some	Yes
Ribosomes/Polysomes	-	-	Yes
Macromolecules	-	-	Yes
Viruses	-	Most	Yes

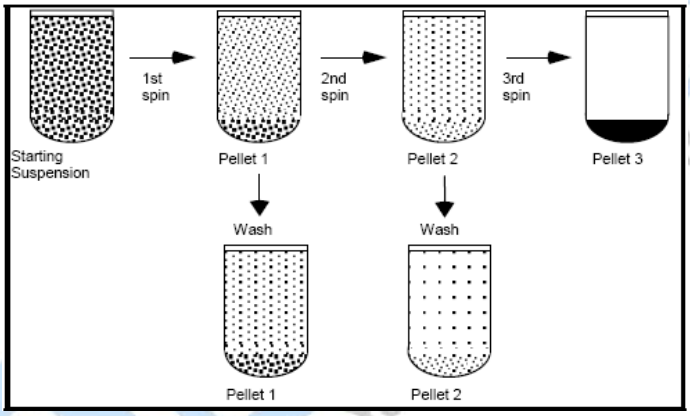
( ) = can be done but not usually used for this purpose.

III. Types of Centrifugal Separations.

1. **Differential centrifugation.** Separation is achieved primarily based on the size of the particles in differential centrifugation. This type of separation is commonly used in simple pelleting and in obtaining partially-pure preparation of subcellular organelles and macromolecules. For the study of subcellular organelles, tissue or cells are first disrupted to release their internal contents. This crude disrupted cell mixture is referred to as a homogenate. During centrifugation of a cell homogenate, larger particles sediment faster than smaller ones and this provides the basis for obtaining crude organelle fractions by differential centrifugation. A cell homogenate can be centrifuged at a series of progressively higher g-forces and times to generate pellets of partially-purified organelles.

When a cell homogenate is centrifuged at 1000 x g for 10 minutes, unbroken cells and heavy nuclei pellet to the bottom of the tube. The supernatant can be further centrifuged at 10,000 x g for 20 minutes to pellet subcellular organelles of intermediate velocities such as mitochondria, lysosomes, and microbodies. Some of these sedimenting organelles can obtained in partial purity and are typically contaminated with other particles. Repeated washing of the pellets by resuspending in isotonic solvents and re-pelleting may result in removal of contaminants that are smaller in size (Figure 1). Obtaining partially-purified organelles by differential centrifugation serves as the preliminary step for further purification using other types of centrifugal separation (density gradient separation).

Figure 1. Differential Centrifugation



2. Density gradient centrifugation. Density gradient centrifugation is the preferred method to purify subcellular organelles and macromolecules. Density gradients can be generated by placing layer after layer of gradient media (Table 2) such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous or continuous mode. The cell fraction to be separated is placed on top of the layer and centrifuged. Density gradient separation can be classified into two categories. 2a. Rate-zonal (size) separation. 2b. Isopycnic (density) separation.

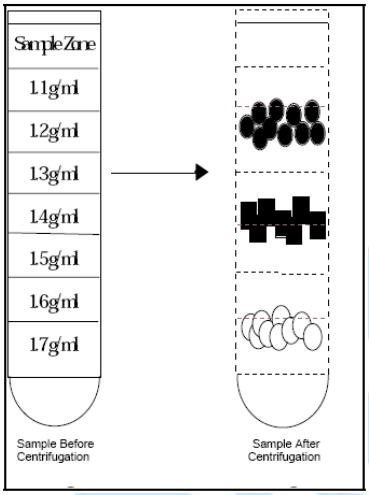
2a. Rate zonal (size) separation

Rate-zonal separation takes advantage of particle size and mass instead of particle density for sedimentation. Figure 2 illustrates a rate-zonal separation process and the criteria for successful rate-zonal separation. Examples of common applications include separation of cellular organelles such as

endosomes or separation of proteins, such as antibodies. For instance, Antibody classes all have very similar densities, but different masses. Thus, separation based on mass will separate the different classes, whereas separation based on density will not be able to resolve these antibody classes.

Certain types of rotors are more applicable for this type of separation than others. Please See rotor categories (below) and Table 2.

Figure 2. RATE-ZONAL (SIZE) SEPARATION



Criteria for successful rate-zonal centrifugation:

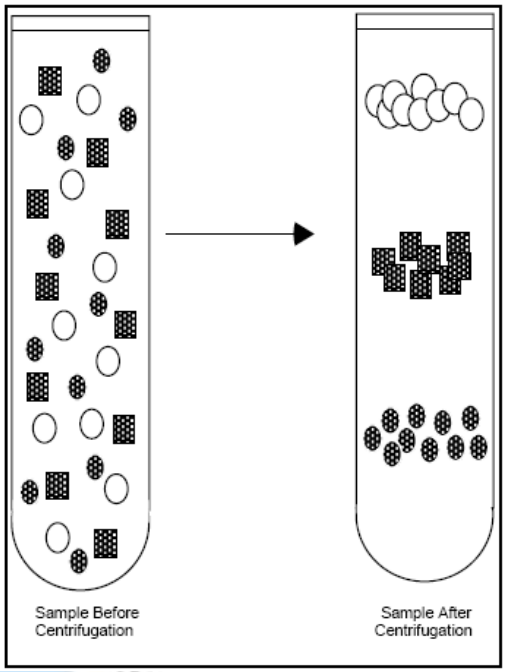
- Density of the sample solution must be less than that of the lowest density portion of the gradient.
- Density of the sample particle must be greater than that of the highest density portion of the gradient.
- The pathlength of the gradient must be sufficient for the separation to occur.
- Time is important. If you perform too long runs, particles may all pellet at the bottom of the tube.

2b. Isopycnic separation :

In this type of separation, a particle of a particular density will sink during centrifugation until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle. Once this quasi-

equilibrium is reached, the length of centrifugation does not have any influence on the migration of the particle. A common example for this method is separation of nucleic acids in a CsCl gradient. Figure 3 illustrates the isopycnic separation and criteria for successful separation. A variety of gradient media can be used for isopycnic separations and their biological applications are listed in Table 2.

Figure 3. ISOPYCNIC (DENSITY) SEPARATION



Criteria for successful isopycnic separation :

- Density of the sample particle must fall within the limits of the gradient densities.
- Any gradient length is acceptable.
- The run time must be sufficient for the particles to band at their isopycnic point. Excessive run times have no adverse effect.

Table 2. Applications of density gradient media for isopycnic separations.

Gradient media	Cells	Viruses	Organelles	Nucleoproteins	Macro-molecules
Sugars (e.g sucrose)	+	+ + +	+ + +	+	-
Polysaccharides (e.g Ficoll)	+ +	+ +	+ +	-	-
Colloidal silica (e.g Percoll)	+ + +	+	+ + +	-	-
Iodinated media (e.g Nycodenz)	+ + + +	+ +	+ + + +	+ + +	+
Alkali metal salts (e.g. CsCl)	-	+ +	-	+ +	+ + + +

+ + + + excellent, + + + good, + + good for some applications, + limited use, - unsatisfactory

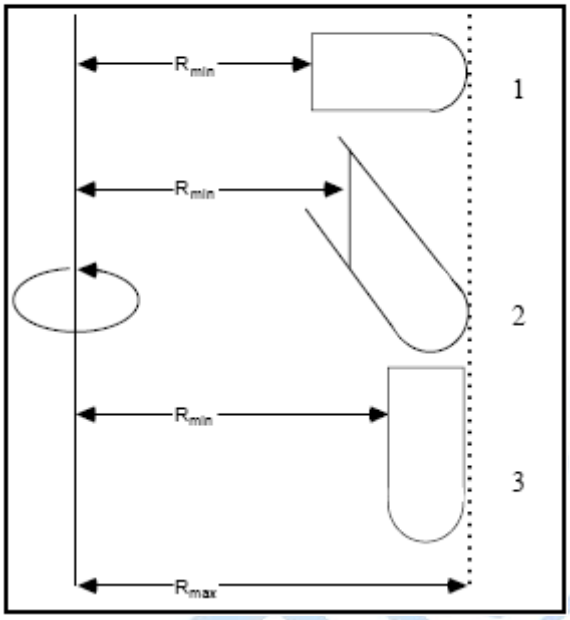
Source: D. Rickwood, T.C. Ford, J. Steensgard (1994) *Centrifugation essential data,* John Wiley & Sons Ltd. U.K.

IV. Rotor categories

Rotors can be broadly classified into three common categories namely swinging-bucket rotors, fixed-angle rotors, and vertical rotors (Figure 4, Table 3). Note that each type of rotor has strengths and limitations depending on the type of separation.

1. swinging-bucket
2. fixed-angle
3. vertical

Figure 4. Rotor Types



Other rotors include continuous flow and elutriation rotors.

Table 3. Types of rotors and their applications.

Type of rotor	Pelleting	Rate-zonal Sedimentation	Isopycnic
Fixed-angle	Excellent	Limited	Variable*
Swinging-Bucket	Inefficient	Good	Good**
Vertical	NS	Good	Excellent
Zonal	NS	Excellent	Good

NS = not suitable
*Good for macromolecules, poor for cells, and organelles
**Good for cells and organelles, caution needed if used with CsCl

In swinging bucket rotors, the sample tubes are loaded into individual buckets that hang vertically while the rotor is at rest. When the rotor begins to rotate the buckets swing out to a horizontal position (Figure 4). This rotor is particularly useful when samples are to be resolved in density gradients. The longer pathlength permits better separation of individual particle types from a mixture. However, this rotor is relatively inefficient for pelleting. Also, care must be taken

to avoid "point loads" caused by spinning CsCl or other dense gradient materials that can precipitate.

In fixed-angle rotors, the sample tubes are held fixed at the angle of the rotor cavity. When the rotor begins to rotate, the solution in the tubes reorients (Figure 4). This rotor type is most commonly used for pelleting applications. Examples include pelleting bacteria, yeast, and other mammalian cells. It is also useful for isopycnic separations of macromolecules such as nucleic acids.

In vertical rotors, sample tubes are held in vertical position during rotation. This type of rotor is not suitable for pelleting applications but is most efficient for isopycnic (density) separations due to the short pathlength. Applications include plasmid DNA, RNA, and lipoprotein isolations.

V. Selection of Centrifuge Tubes.

Table 4 and Table 5 illustrate properties of centrifuge tubes and the proper rotors in which they should be used.

Selection of the appropriate centrifuge tube:

- Prevents sample leakage or loss
- Ensures chemical compatibility
- Allows easy sample recovery

Major factor in selection of a tube (plastic) material:

- Clarity
- Chemical resistance
- Sealing mechanism (if needed)

Table 4 - Chemical Compatibility of Popular Tube Materials

Tube Plastic type	Clarity	Chemical Resistance*
Polypropylene (PP)	Opaque	Good
Polyallomer (PA)	Opaque	Good
Polycarbonate (PC)	Clear	Poor
Polyethylene terephtalate (PET)	Clear	Poor

* For more information, please check our chemical resistance chart available on this website.

- check product guide pages or tube packaging for notes on recommended sample volume and maximum speed.
- always run thin-walled, sealed tubes full in a fixed angle or vertical rotor. Examples:
  - open top tube with multiple sealing assembly
  - Re-seal tubes
  - Ultracrimp® and Clearcrimp® tubes
- autoclave tubes only if absolutely necessary and only at 121°C for 15 min.
- avoid cleaning plastic tubes in automated dishwashers or glassware washers, which may produce excessively hot temperatures.
- we recommend that you clean tubes with a mild laboratory detergent in warm water, rinse, and thin air dry.
- tube must be carefully matched with rotor type to prevent sample loss and/or failure as illustrated in Table 5 below.

To prolong tube life and avoid breakage or collapse:

Table 5 – Tube Type and Rotor Compatibility

	Rotor Type		
Tube type	**Fixed-angle**	**Swinging-bucket**	**Vertical**
Thin wall open top	No	Yes	No
Thick wall open top	Yes	Yes	No
Thin wall sealed	Yes	Some tube types	Yes
Oak ridge	Yes	No	No

VI. Common Centrifugation Vocabulary and Formulas.

- **Pellet:** hard-packed concentration of particles in a tube or rotor after centrifugation.
- **Supernatant:** The clarified liquid above the pellet.
- **Adapter:** A device used to fit smaller tubes or centrifugal devices in the rotor cavities.
- **RPM:** Revolutions Per Minute (Speed).

- **R_{max}:** Maximum radius from the axis of rotation in centimeters.
- **R_{min}:** Minimum radius from the axis of rotation in centimeters.
- **RCF:** Relative centrifugal Force. RCF = 11.17 x Rmax $(RPM/1000)^2$
- **K-factor:** Pelleting efficiency of a rotor. Smaller the K-factor, better the pelleting efficiency.

$$K = \frac{2.53 \times 10^{11} \, Ln(R_{max}/R_{min})}{(RPM)^2}$$

- **S-value:** the sedimentation coefficient is a number that gives information about the molecular weight and shape of the particle. S-value is expressed in Svedberg units. The larger the S-value, the faster the particle separates.

  For more information about sedimentation coefficients, please refer to the section on references and suggested readings in this article.

- **Pelleting time:** time taken to pellet a given particle. T = K/S where T= pellet time in hours. K = K-factor of the rotor, and S = sedimentation coefficient.
- **Rotor conversion formula:** If the time to pellet a sample in your "old" rotor is known, one could determine the time it would take for the same sample to pellet in a "new" rotor. The formula for this determination is as follows:

$$\frac{T_1}{K_1} = \frac{T_2}{K_2} \longrightarrow T_1 = T_2\left(\frac{K_1}{K_2}\right)$$

Where:
T1 = Time to pellet in the "new" rotor
T2 = Time to pellet in the "old" rotor
K1 = K-factor of the "new" rotor
K2 = K-factor of the "old" rotor

Example of a rotor conversion:

Old Rotor (Beckman® JA-10) New Rotor (Sorvall® SLC-1500)

T2 = 20 min; K2 = 3610 T1 = (?) min; K1 = 1676

Old Pelleting Time = 20 min New Pelleting Time = 9.2 min

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