Biophysical and Biochemical Techniques

Chromatography

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CONTENTS

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Chromatography

Separation, purification and identification of one or more biological compounds from a mixture of such compounds is the major problem faced by the biological chemists. One of the convenient techniques of separation is chromatography. The technique can be used both for preparative and analytical purposes including separation of biomolecules at micro and large scales and determination of purity of preparations and estimation of molecular weights. Chromatography is also used for identification and characterization of biological substances.

The selection of a particular chromatographic technique depends upon the nature of material to be isolated and often several chromatographic techniques may be used in sequence to achieve complete purification.

The basis of all form of chromatography is the partition or distribution coefficient (K_d) which describes the way in which a compound distributes between two phases, i.e.
K_d= concentration of the solute in phase A/ concentration of the solute in phase B

The two phases need not be liquid but a combination of liquid/liquid; liquid/gas; liquid/solid, gas/solid and so on. One of the phases acts as stationary and the other mobile phase.

Stationary and mobile phases are so selected that the compounds to be separated have different distribution coefficients. This may be achieved by:

(i) Partition equilibrium between stationary liquid phase (held on some inert solid support) and mobile liquid phase e.g. partition chromatography and counter current distribution.

(ii) An adsorption equilibrium between a solid adsorbent (stationary phase) and a mobile liquid or gas phase (adsorption chromatography).

(iii) An ion exchange equilibrium between a solid exchanger (stationary phase) and a mobile electrolyte phase (Ion-exchange chromatography).

(iv) An equilibrium between liquid phases inside and outside of porous structure or molecular sieve (exclusion, gel permeation or gel filtration chromatography).

(v) An equilibrium between a macromolecule and a micromolecule for which it has high biological affinity (affinity chromatography).

(vi) A variation of techniques (i) and (ii) where the mobile phase is a gas instead of liquid, is widely used for separation of less polar substances and known as gas liquid or gas solid chromatography.

Techniques

There are various techniques of developing a chromatogram. Chromatographic separation can be achieved on sheets of papers as such or modified, thin layers and columns, depending upon quantity of material to be separated, permitted time and degree of resolution desired.

Columns

Majority of separation is carried out in column wherein stationary phase is packed. Mostly glass columns are used. Minimum length to inner diameter should be 10:1. Stationary phase at the bottom of the column is supported by cotton wool, glass wool, sintered glass disc, nylon mesh,

etc. Below the column there should be minimum of dead space. Inner diameter of the column should be uniform. Some separations are required to be carried out at controlled temperature, such columns are jacketed so that water of desired temperature could be circulated.

Stationary phase

The chemical composition of the stationary phase depends upon type of chromatography to be performed. Most stationary phases are available in range of size and shape. Both the properties are important as they determine flow rate and resolution. The larger the particles, faster the flow rate with inferior resolution. In contrast, smaller particles offer greater surface area and thus improved resolution but the flow rate is affected. In practice a balance has to be settled between the two qualities. Particle size is expressed as mesh size which is the number of pores per square inch in a sieve, hence larger the mesh size, smaller the particle.

Column packing

This is the most critical step in achieving a successful separation in column chromatography. The column should be scrupulously cleaned. It is held vertically. The lower end is plugged with cotton, rayon, sintered glass or glass wool. A thin slurry of uniformly settling particles of stationary phase is prepared in a suitable buffer. Extreme care is taken to prevent entrapment of air bubbles which may later coalesce in the column and form channels. To do this air from buffer is removed by applying negative pressure and/or preparing the column at a temperature at which the experiment is to be performed. The slurry is placed in a container and connected to the column filled with the starting buffer. Slurry is then allowed to enter into the column and settle down uniformly under the influence of gravity. The slurry is gently and continuously stirred. Slurry is allowed to settle slightly higher in the column than required.

After the slurry has settled in the column, proper packing of slurry is affected by passing several bed volumes of starting buffer. When more compact packing is needed, a negative pressure is applied at the bottom of the column while packing and subsequent washing of the column. The stationary phase is now never allowed to dry and some buffer must remain over the top surface. While applying the sample, top surface of the matrix may get disturbed. To prevent this, it is often covered with filter paper disc cut to the size of inner diameter of column. Some solutes may get adsorbed on paper, thus rayon gauge, nylon mesh, glass wool or cotton wool packs may be used.

Application of sample

In most cases ionic composition and pH of the sample should be the same as that of starting or equilibrating buffer. This could be achieved either by preparing sample in the same buffer or dialyzing it against the starting buffer. For better resolution, the volume of sample should be as small as possible so that a narrow initial zone is formed. Thus the sample may be concentrated.

There are various methods of applying sample on to column:

(i) The liquid above the gel is drained off just below the gel and sample is carefully layered over it without disturbing the surface. Then it is washed to drain down the sample into gel with small volume of starting buffer. Fractions are collected right from now. The starting buffer is now layered above the matrix and column connected to eluent reservoir.

(ii) Density of the sample is increased by adding sucrose solution to make the concentration of sample 1% with respect to sucrose. This solution is carefully layered on the gel below the buffer. The method is useful if sucrose does not interfere in chromatographic process.

(iii) The sample may be injected below the solvent over the gel using syringe or peristaltic pump.

Column development

Column development means separation of components of sample by continuous passage of suitable eluent (mobile phase) through the column. Different methods are used for development of chromatogram depending upon type of chromatography being performed. The volume of mobile phase required to elute a given solute is known as it's elution volume (V_e).

During elution it is essential that eluent flow is maintained at a stable rate. This is very simply done by maintaining the pressure of the eluent constant by using a Mariotte flask or a pump.

Fraction collection and analysis

Column development and subsequent elution can be done by several methods. When a single solvent is needed for elution as in gel permeation chromatography, it is known as isocratic separation . In many cases, in order to increase the resolving power and elution, it is necessary to change the pH, ionic concentration or polarity of eluent continuously. This can be achieved by stepwise elution where solutions of increasing ionic concentration or solutions of different pH in fixed volumes are passed through the column one after the other. This method has the disadvantage that eluting power changes abruptly which may result in coelution of closely related solutes. For continuous smooth increase in eluting power gradient elution is carried out which gives better results. Depending upon the requirement, gradient elution is carried out in one of the following ways :

Convex exponential gradient

This gradient is produced by having a eluent reservoir A, attached to the mixing chamber B, which in turn is connected to the column. The two chambers are placed as shown in Fig. 1. Mixing chamber has the starting solvent. Chamber A has concentrated solution to increase the ionic strength of eluent (mobile phase). As the eluent flows from B to the column, eluent will flow from A to B at the same rate. The volume of liquid in B remains constant but the concentration of ion entering in the column increases in a fashion shown in Fig. 2. Concentration of eluent at any given instance is given by the equation $Cv = C_1 - (C_1 - C_2)\, e^{-\frac{v}{V_R}}$

where Cv is the concentration of eluent in the effluent from the apparatus after volume v has flown through it. C_1 is the concentration of the eluent in the reservoir A. C_2 is the concentration of eluent initially present in the mixing chamber B. V_R is the volume of liquid in B. If $C_2 = 0$, as is frequently the case, the equation simplifies to $Cv = C_1 (1 - e^{-v/V_R})$ which for the purpose of calculation may be rearranged as $2.303 \log_{10} C_1/C_1 - C_{v=} v/V_R$. Design of the apparatus to make this type of gradient is simple but resolution of solutes is inferior.

Concave gradient

It is of increasing importance. Apparatus for producing concave gradient is given in Fig. 3.

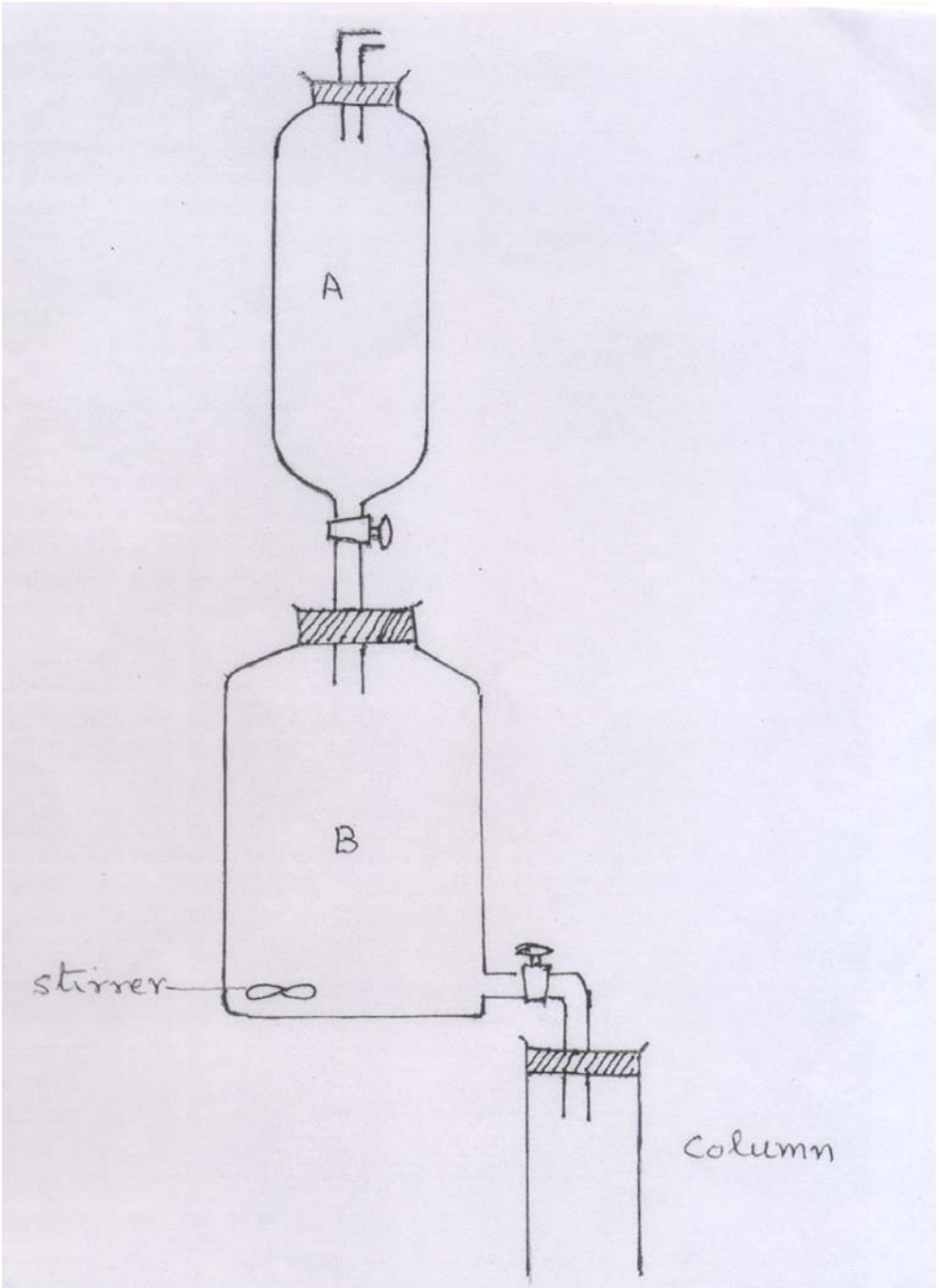


Fig. 1 : Arrangement for producing convex exponential gradient

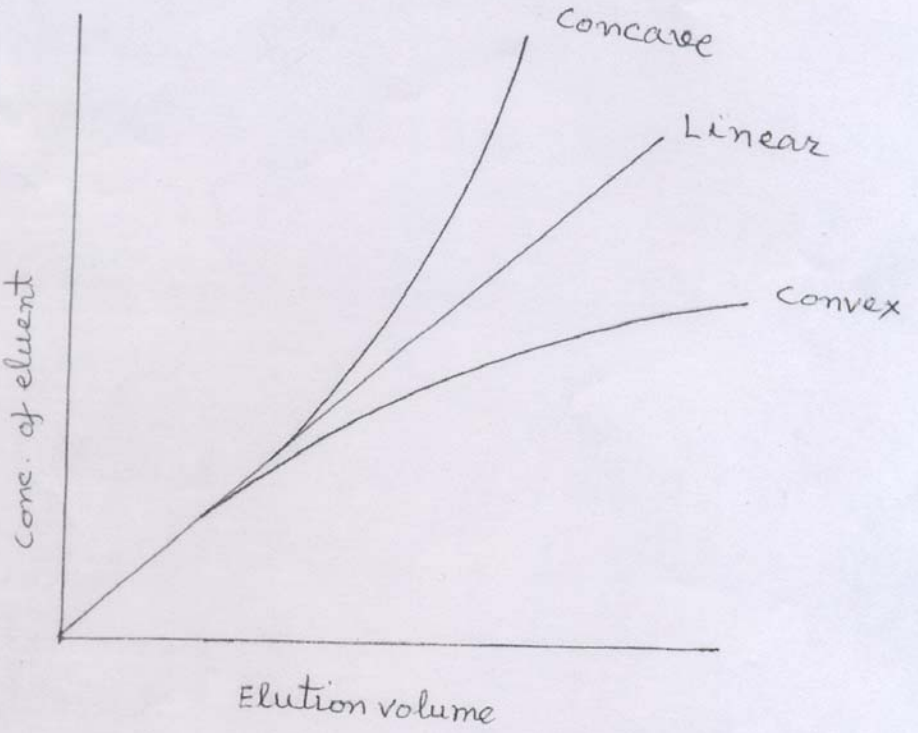


Fig. 2: Graph showing conc. of eluent through the column in concave, linear and convex gradient

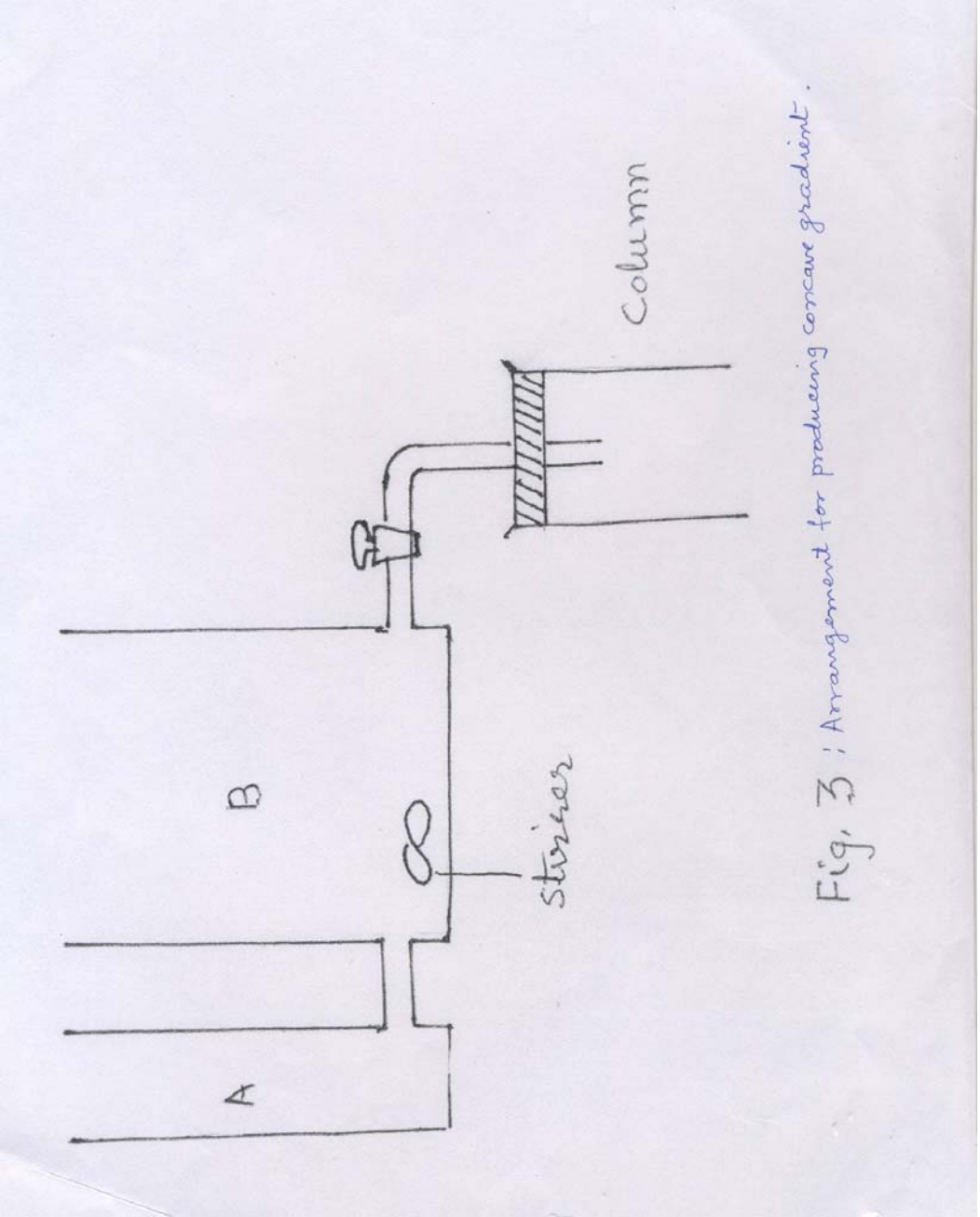


Fig. 3 : Arrangement for producing concave gradient.

In this apparatus the mixing chamber has an extra outlet, in addition to one leading to the column. By regulating the entries of solvent in column and mixing chamber B, almost any type of gradient can be formed. The equation to calculate concentration of eluent at any given instance with the device is $C_v=C_1-(C_1-C_2)\ (1-v/V_{tot})^p$, where p= the ratio of the cross sectional areas of chambers A and B.

Linear gradient

Linear gradient is used most commonly and is very effective. The simplest way of producing linear gradient is to have two chambers, one containing eluent of final concentration A and the

other containing eluent of initial concentration B. The two containers are of exactly similar dimensions and are placed at leveled surface. The volume of liquid in the two chambers is equal and up to same level.

Concentration Cv of effluent after volume V has flown through the apparatus is given by $Cv = C_2 + (C_1 - C_2)\frac{V}{V_{tot}}$ where V_{tot} is the total volume of liquid in both the containers.

The advantages of gradient elution are as follows :

(1) Owing to a wide range of mobile phase properties which can be used in the course of single experiment, a correspondingly wide variety of solutes can be eluted from the column without using large effluent volumes.

(2) Gradient elution is suited for automatic operations.

(3) Multiple zoning of a single solute is entirely eliminated because there is no abrupt change in the properties of eluting power.

(4) Gradient elution causes sharpening of rear boundary of solute zones.

Disadvantages are (a) operational complexity and (b) loss of resolving power for difficult operations.

Fraction collection and analysis

Fractions of 2-5% of the bed volume are collected. As the separated compounds emerge from the column, it is necessary to detect them. This is best done by continuously monitoring the effluents by one of several ways. Monitoring for a given (particular) compound can be done by performing specific test in all the effluent fractions or for a group of compounds such as proteins or nucleic acids by monitoring the fractions at a wavelength characteristic to the group, change in refractive index, presence of radioactive label in the effluent or by performing specific chemical reactions.

A particular compound may be divided in several fractions and if the separation has been successful, number of these fractions will be less and separate from other compounds with intervening fractions that contain no or very little amount of any compound. Fraction can be collected manually or using automatic fraction collectors.

When the column effluent has been monitored to produce a chart recording then area of each peak is shown to be proportional to the one of the solute and hence can be used to quantitatively determine the total amount of a given component eluting from the column.

Flow rate

Smaller the flow rate, better would be separation, but smaller flow rate means larger time taken with attendant mixing of separated zones due to diffusion. Therefore, a compromise between the two is to be made.

Column efficiency

In practice, as the solute comes down the column, kinetic and flow phenomena cause the solute bands to broaden which may cause overlapping and intermixing of separated solute bands. In some cases asymmetrical displaying either fronting (extended front portion of the peak) or tailing may occur. Peak asymmetry has many causes including application of too much of sample, poor packing of the column, poor application of the sample, chromatography at high temperatures and solute-solute interactions etc. Band broadening can be avoided by applying smaller volume of sample or increasing the flow rate.

Thin Layer Chromatography

Principle

Partition, adsorption, ion-exchange, exclusion and high performance liquid chromatography, all can be carried out in thin layer mode. The technique is simple, quick and allows a large number of samples to be analyzed at one time. The technique can be used both for analytical and preparative purposes.

Thin layer preparation

A slurry of the holder of stationary phase usually suspended in water is applied to glass, plastic or foil plate uniformly either manually or by using a spreader. For analytical uses, thickness of slurry is usually 0.25 mm and for preparative purposes, up to 5 mm. Where stationary phase is to be used for adsorption chromatography, a binding agent such as $CaSO_4$ is incorporated into the slurry to facilitate adhesion of the adsorbent to the plate. With exception to permeation chromatography, after preparing the plates, they are heated to leave the coating of stationary phase. In case of adsorbent drying is carried out in an oven at 100-120^oC. This also serves to activate the adsorbent.

Sample application

Sample in small quantities is applied by micropipette and briefly dried in oven or with the help of hair dryer where applicable. Spot size should be as small as possible and therefore when a larger volume is to be loaded sample is applied in small volumes, dried and applied further. Sample is applied on plate at a position that it is not dipping in the solvent.

Plate development

The solvent is filled in glass chamber to a suitable height and filter paper is wrapped all around the inner wall so as to saturate the chamber with solvent vapours. The chamber is covered with air tight lid. It is then left for several hours for equilibration with solvent vapours. Unless this is done, the solvent runs irregularly on the plate with poor separation. The plate loaded with sample is then placed in the chamber. Chromatography is carried out at moderately low and constant temperature.

In order to increase the resolution, two dimensional chromatography is performed using two different solvent systems. The material to be separated is placed at one corner of the plate; run in one solvent, dried and then in another solvent where the plate is placed at 90^o of the previous run. TLC can also be linked to electrophoresis, where separation is carried out by TLC in one direction and unresolved solutes are separated by electrophoresis in other dimension.

Component detection

Spraying the plate with 50% H_2SO_4 or 25% H_2SO_4 in ethanol and heating will result in most compounds becoming charred and giving brown spots. Observation of plate in ultraviolet light will reveal UV absorbing and fluorescent solutes. Subjecting the plate to iodine vapor will reveal unsaturated compounds. Spraying of plate with specific colour reagents will stain certain compounds.

Identification of compounds

Although movement of compounds on TLC plates can be found out by R_f values, such values are not reliable in case of paper chromatography. Identification of components separated by TLC is more reliably done by comparing the movements with reference compounds chromatographed alongside the sample on the plate.

Quantitation

Quantitation may be achieved by use of radiochromatogram using radiolabelled compounds or generally by means of densitometry. There are several other methods for quantitation on plate such as measuring area of the spot and comparing it with that of the reference. The developed spots may be scrapped off, suspended in suitable solvent and assayed colorimetrically after removing the gel.

Paper Chromatography

Principle

Cellulose fibres of chromatography paper act as the supporting matrix to the stationary phase and separation occurs mainly by partition chromatography, but adsorption also plays significant role. This chromatography is done by either ascending or descending of solvent on the paper. Specially impregnated papers are available for specific separation. Papers of different running characteristic are available i.e. slow, medium and fast. In one dimensional chromatography, the paper should always be developed in its machined direction which is marked on the paper or packet.

Development

There are three techniques employed for developing paper chromatograms i.e. ascending, descending and horizontal. In all the cases chamber should be air tight and saturated with solvent vapours. Although ascending chromatography is preferred for its simplicity, in descending mode solvent flow is faster. In horizontal method solvent flow is no way affected by gravitation. Two dimensional chromatography on paper can be performed in a manner similar to that of TLC.

Component detection

The methods are same as described for TLC excepting H2SO4 can not be used as spraying agent. Identification of the spots can be made on the basis of their Rf values which is constant for the given solute under standard conditions. Sometimes other values such as Rg is used where the solvent has to be overrun for development. For example, chromatography of sugars takes a long time and the solvent is overrun in descending chromatography; the distance traveled by solvent front can not be measured and glucose or other sugar is taken as the reference.

Adsorption Chromatography

This technique is used for separation of substances differing in their charge. The mobile phase is a liquid whereas the stationary phase is a solid adsorbent that can adsorb charged substances on its surface. Degree of adsorption depends upon the (a) nature of mobile phase and (b) sign and magnitude of charge of the solutes on the adsorbent resulting in their separation. Adsorption chromatography can be done in column, thin layers or papers impregnated with adsorbent.

Adsorbents

Compounds such as silicic acid, silica gel, aluminum oxide, calcium carbonate, magnesium carbonate, magnesium oxide, cellulose and activated charcoal are commonly used adsorbents. The choice of an adsorbent and mobile phase depends upon the type of compound to be separated. Alumina C√ and hydroxyapatite (tricalcium phosphate) gels are widely used for separation of proteins, nucleic acids and viruses. These adsorbents also have some ion exchange properties which aid separation. One has to select out such an adsorbent where the adsorption of solutes is mild or solute does not get irreversibly adsorbed or undergoes denaturation. In general, adsorbents have tendency to adsorb water which may aid separation by partition chromatography.

Eluents

Choice of eluent depends upon the polarity of compounds to be separated. Elution of small molecular weight substances can be carried by polar organic solvents. Commonly used low polarity solvents are hexane, heptane, toluene, diethyl ether, chloroform, etc. Intermediate polarity solvents are dichloromethane and pyridine. High polarity solvents include propane-1-ol, butane-1-ol, methanol, ethanol and water. For labile macromolecules these solvents are not suited as they may denature them. Common method of elution of these substances is by changing pH of the eluent or increasing its ionic strength in steps or using a gradient.

Partition Chromatography

Principle

Basic requirements for partition chromatography are: (i) There are two phases, one is the stationary phase and the other mobile phase. (ii) Both the phases are liquid; in some cases (e.g. GLC) the mobile phase may be as gas. (iii) The two phases are immiscible in to each other. (iv) The solutes to be separated should be soluble in both the phases.

Relative solubility of the solutes in the two phases decides the migration rates of solutes in the column. While traveling through the column, solutes are partitioned or distributed between the two phases. Since different solutes have different relative solubility in the two solvents i.e. have different distribution or partition coefficients and therefore move at different rates in the column. A solute with high affinity for mobile phase will travel in the column along with it and thus have faster mobility. A solute with high affinity for stationary phase will have slowest mobility. Solutes with intermediate solubility in two phases will likewise move at different rates resulting in their separation.

In normal phase chromatography, the stationary phase is usually water, immobilized on a chromatographically inert solid matrix such as cellulose, silicic acid, starch, etc. For paper

chromatography, paper itself and for TLC silicic acid or silica gel is commonly used but here adsorption also plays significant role in separation process. Mobile phase is generally an organic solvent.

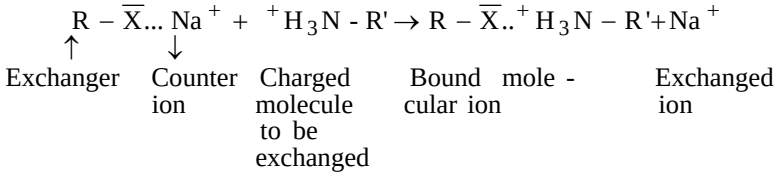
In reverse phase partition chromatography, the stationary phase is a non-polar compound such as liquid paraffin supported by suitable matrix. The phase is prepared by treating the supporting matrix with a solution of stationary phase in a suitable non-polar volatile solvent. The solvent is subsequently removed by evaporation.

Ion Exchange Chromatography

Principle

This technique is used for the separation of charged solutes such as proteins and amino acids. The separation is based on the differences in the sign and magnitude of charge on the solutes undergoing separation. Ion exchangers are either highly insoluble acids or bases. Acids have positively charged exchangeable groups and are thus called cation exchangers whereas bases have negatively charged exchangeable groups and are called anion exchangers.

When a solute mixture containing solutes having different magnitude of positive charge is passed through a bed of cation exchanger, positively charged exchangeable groups are exchanged for counter ion on the exchanger.



Anion exchanger for the separation of anionic solutes.

$$R - N...Cl^- + {}^-OOCR' \rightarrow R - N...OOCR' + Cl^-$$

Thus binding and subsequent retention of solutes in a column depends upon, provided other conditions remain constant, the number of charged groups a solute possesses. A substance without charge will move through the column unrestricted and emerge from the column first. Through cation exchanger, a solute having minimum positive charge will move down with less restriction as compared to one having more positive charge because it is held less firmly by the exchanger as compared to that having more charges. Charge difference on the solute molecules, therefore, determine their migration rate through the column and resulting in their separation. The actual ion exchange mechanism is thought to be composed of five distinct steps : (i) Diffusion of the ion to exchanger surface. This occurs very quickly in homogeneous solutions. (ii) Diffusion of the ion through matrix structure of the exchanger to the exchange site. This is dependent upon degree of cross-linking of the exchanger and number of exchangeable groups per unit surface area. This controls the rate of whole ion exchange process. (iii) Exchange of ions at exchange site. The more highly is charged molecule to be exchanged, the tighter it binds to the exchanger and less readily displaced by other ions. (iv) Diffusion of the exchanged ion through the exchanger to the surface. (v) Selective desorption by the eluent and diffusion of the molecule into external solution. Selective desorption of the molecule is achieved by changes in

pH (decreasing the pH for anionic and increasing the pH for cationic exchangers), or increase in the ionic concentration of eluent or by affinity elution for a specific solute. Elution by the above first two methods can be achieved by stepwise change or gradient method. Labile biomacromolecules often may undergo inactivation or denaturation by pH changes. Thus elution by increasing ionic concentration of salt is preferred. Relative abundance of ion in the eluent cause desorption of the exchanged solutes resulting in their elution.

Material

For successful separation of small molecular weight ions, such as amino acids, polymerized styrene derivatives with suitable acid or basic groups have been abundantly used. Styrene is copolymerized to give linear polystyrene. During polymerization divinyl benzene is added to form cross linked porous polystyrene beads. By varying the ratio of styrene to divinyl benzene, resins with varying degree of cross-linking and thus pore size can be obtained. Resins of low degree of cross linking are permeable to high molecular weight compounds and the *vice-versa*. These resins are highly hydrophilic and swell in aqueous solutions. Swelling characteristics must be taken into account when a column is prepared.

Sulphonation of the resin introduces $-SO_3H$ group which is highly ionized and acts as a strong cation exchanger. Anion exchanger is produced by reacting cross linked polystyrene with chloromethyl ether and then reacting chlorogroups with tertiary amines.

Determination of Exchange Capacity of an Exchanger

Cation exchanger

Known quality of dried exchanger is suspended in excess of NaCl solution and mixed for sometime for ion exchange to occur. Suspension is then titrated with standard alkali. From the volume of alkali consumed exchange capacity is determined in terms of mg of Na^+ consumed/gm of exchanger.

Anion exchanger

Known amount of dried exchanger is shaken for sometime with excess of NaCl. Then titrated with standard NaCl using methyl orange as indicator. Exchange capacity is expressed as mg. Cl^- taken up/gm of exchanger.

Separation of solutes in ion exchange column depend upon a number of factors viz.

(a) Nature of exchangeable ions :

(i) if a solute is polyvalent, it is preferably exchanged and thus moves slowly in the column than di or monovalent solutes,

(ii) larger the size of solute more preferably it is exchanged and thus retained in the column. Moreover, movement of proteins is further hindered due to its adsorption on the exchanger, which sometimes may be irreversible.

(b) Nature of exchange resins:

(i) exchange capacity depends upon the number of functional groups present per unit weight of the resin,

(ii) degree of cross-linking also decides the extent of exchange. Higher the degree of cross linking results in lowering of the exchange capacity.

(c) Medium in which exchange takes place :

(i) degree of exchange depends upon the total concentration of solutes present, (ii) exchange of a particular ion depends upon the nature and concentration of other ions present and

(iii) it also depends upon the pH and ionic concentration of equilibrating and eluting solvent.

Separation of Proteins by Ion Exchange Chromatography (An example)

For separation of proteins and nucleic acids polystyrene ion exchangers are entirely unsuited as these are labile macromolecules and (i) because of larger molecular weight proteins can not be exchanged by entering into resin beads and exchange, is therefore, incomplete because they are exchanged only at the surface of the resin beads; (ii) binding between the exchanger and the proteins is so firm that this itself and subsequent drastic conditions for elution may cause their inactivation or denaturation; (iii) the time taken for separation of proteins by these exchangers is too long.

For separation of macromolecular ions, porous exchanger with mild exchanger groups are needed where binding of solutes and their elution can be carried out under milder conditions. Such exchangers were introduced by Peterson and Sober who used cellulose powder and later agarose or acrylamide matrices were also introduced, wherein suitable exchangeable groups are introduced to produce anion or cation exchangers. Most commonly used cellulose exchangers are, carboxy methyl (CM)-cellulose (cation exchanger), diethyl aminoethyl (DEAE)-cellulose (anion exchanger), phosphocellulose, oxy-cellulose, cellulose succinate half ester, ECTEOLA cellulose (chloroethanol triethyl cellulose).

Experimental Details

1. Selection of exchanger

For chromatography of ampholytes such as proteins, both anionic and cationic exchangers can be used. In selecting an exchanger one has to keep in mind the following characteristics into consideration:

(a) exchanger gives maximum resolution and the protein intended to be purified is obtained in purest possible form and

(b) conditions employed in chromatography and elution such as pH, ionic concentration, etc. do not interfere in purification process or effect the proteins to be separated in any way.

2. Preparation of sample

Ionic concentration of sample should be same as that of starting (equilibrating) buffer. The sample must, therefore, be dialyzed against the starting buffer before chromatography. To have narrow starting zone the sample volume must be as small as possible and therefore the sample must be concentrated. Total volume of sample should be 2-5% of the volume of the column.

3. Preparation of exchanger

Before packing into the column, the exchanger should be washed thoroughly with water to remove water soluble impurities. During this washing finer particles are removed by decantation and uniformly settling cake of the exchanger is used. The exchanger is then equilibrated against

starting buffer by repeated washing with buffer and then leaving for 5-10 hr in it. In general, for separation of 0.5 mg protein 10 mg exchanger is sufficient.
The exchangers can be used several times after regeneration. Regeneration is carried out as follows :
Anion exchanger is washed several times with 0.5 *N* NaOH, washed with water to remove OH ions, left in 0.5 *N* HCl for 30 min. and then washed with water to remove Cl^-. Exchanger is then equilibrated with starting buffer as usual. The cation exchanger is regenerated in the reverse order.

4. Selection of buffer system
Starting buffer should be of low ionic strength; usually 0.1 *M*. Exchange occurs best at slightly alkaline pH in an anionic exchanger and at an acidic pH in case of cationic exchanger.

5. Preparation of column
Preparation of column is carried out preferably at the temperature at which chromatography is to be performed to prevent formation of channels in the column due to bursting and coalesce of air bubbles.

A glass tube with uniform inner diameter (it may be jacketed to circulate water to maintain desired temperature) having diameter to length ratio of 1:10 to 1:20 is clamped vertically. The lower end is plugged with glass wool to prevent escape of exchanger. Column is filled up to $2/3^{rd}$ height with starting buffer. A thin slurry of the exchanger is prepared in the starting buffer and connected to the column. The slurry is continuously mixed. When stopper of the column is opened, the slurry enters the column and settles uniformly. After the desired quantity of exchanger has settled, the column is washed with several bed volumes of starting buffer to ensure uniform and compact packing of the column. The top surface is now never allowed to dry and there must be some solution above the exchanger. To prevent disturbance of the top surface during application of sample, it may be covered with a filter paper disc cut to size of inner diameter of the tube or with glass or cotton wool.

6. Application of sample
The buffer is drained down just below the exchanger and sample is loaded carefully without disturbing the top surface, on the column and washed down with small volume of starting buffer. Fractions are collected just after applying the sample.

7. Development of chromatogram
Chromatogram is developed by passing the eluent through the column. Elution can be carried out by lowering the pH of eluent for anion exchanger or raising it for cation exchanger. Elution by changing pH may often lead to structural alterations, inactivation or even denaturation of proteins and is thus generally not suited. Elution is better carried out by increasing ionic strength of eluent which may be accomplished by stepwise elution where equal volume of eluent of increasing ionic strength is passed through the column one after the other. This technique suffers from the major disadvantage that two nearly identically exchangeable solutes coelute from the column in a single band thus causing remixing of the separated zones. Best way of carrying elution is by applying salt gradient the details of which have been already discussed.

Affinity elution: This is a highly specific type of elution wherein a given protein is selectively eluted from the column by passing a solution of a ligand (substrate, substrate analogue, activator or inhibitor or any other substance specific to the enzyme or protein) specific for the protein under study. In this type of elution such experimental conditions are selected at which unwanted proteins are loosely bound with the exchanger and are thus readily eluted by passing suitable eluent. The protein of interest is retained in the column, which is subsequently eluted by the specific ligand.

8. Collection of resolved fractions and their analysis

In ion exchange chromatography fairly faster flow rates can be afforded. Flow rate of 1 ml per minute is suitable. The fractions should be collected in smallest possible volume. Ideal volume of fraction collected depends upon the volume of sample loaded. Fractions are collected manually or by using automatic fraction collector.

If all the proteins are to be purified then each fraction is analyzed for presence of proteins by performing some sensitive protein test or measuring absorbance of samples at 280 nm and a graph between fraction number or elution volume and absorbance or protein concentration is plotted. A number of peaks are obtained, each corresponding to at least one protein. Fractions under each peak may be pooled and rechromatographed for further purification. Area under each peak corresponds to concentration of the protein. If interested in the purification of a particular protein, in each fraction, test specific for that protein is performed to locate that protein.

Countercurrent Distribution Chromatography (CCD)

This separation process is based upon distribution of solutes between two immiscible phases. These phases may be mixtures of solvents, buffers, salts and various complexing agents. The only difference in this technique from partition chromatography is that in counter current distribution there is no solid supporting matrix. Separation of compounds is, nevertheless, based on the difference in distribution coefficients of solutes between the two phases.

The apparatus generally used is Craig's counter current distribution apparatus. It consists of 30-1000 interconnected vessels (called train), each of which retains a fixed volume of stationary phase. The solute mixture is introduced into the first vessel of train and equilibrated with immiscible and less dense mobile phase by repeated rocking of the vessel through 90^{o}. After equilibration is complete (1-2 min) the mobile phase is transferred to next vessel as a result of complete tipping of the first vessel. When this returns to its original position, fresh upper phase is automatically introduced. The whole process is repeated so that the mobile phase is transferred progressively along the series of lower phases. The solutes are transferred at a rate determined by their partition coefficients and the relative volumes of the two solvents in each vessel. Each solute eventually accumulates in specific group of vessels. The resolution being determined by the total number of transfers and the difference in partition coefficient of solutes.

Recently several new versions of counter current distribution have been developed. Helix or torroidal CCD uses a helically wound tube located circumferentially on a centrifuge rotor. The centrifugal field holds one of the phases stationary in the coil so that when a second phase is

pumped through the coil, mixing occurs without displacement of the stationary phase. Introduced sample, therefore, undergoes a series of partitions as that solutes more soluble in the stationary phase elute more slowly than those soluble in the pumped mobile phase.

CCD is the only form of chromatography which has been successfully used for cell organelle fractionation. It has also been used for cell fractionation and membrane receptor isolation.

Gas Liquid Chromatography (GLC)

This technique, which is based on partitioning of compounds between a liquid and gas phase, is widely used method for qualitative and quantitative analyses of a large number of compounds since it has high sensitivity, reproducibility and speed of separation. It is useful for separation of solutes of low polarity. A stationary phase of liquid material such as silicone grease is supported on an inert granular solid. This material is packed into narrow glass or steel coiled column which is of 2-4 mm inner diameter and 2-3 meter long through which an inert carrier gas (the mobile phase) such as nitrogen, argon or helium is passed. The column is maintained at an elevated temperature which volatilizes the compounds to be separated. The basis of separation is difference in the partition coefficient of volatilized solutes between the liquid and gas phases as the compounds are carried through the column by carrier gas. As the compounds leave the column they pass through recorder. GLC may also be performed using capillary columns of 0.03-1.0 mm diameter and 100 m. in length.

There are two types of capillary columns: (a) wall coated open tubular (WCOT) and (b) support coated upon tubular columns (SCOT). In WCOT columns the stationary phase is coated directly onto the wall of capillary tubing. As there is only small amount of stationary phase present, very small amount of sample can be chromatographed but as the length of these columns is very long, very high efficiencies are obtained. In SCOT columns support material is bonded to the wall of column and stationary phase is coated to the support, thus the capacity of SCOT columns is very high. Sample can be directly loaded on such columns. SCOT columns are simpler in use, their efficiency being less than WCOT columns but better than conventional GLC columns.

Solid support

Since this is used to provide supporting system for stationary phase, the support should be inert to the sample. Most commonly used support is celite (diatomaceous silica). Celite has free-OH groups that may interact with sample. To prevent this, celite is usually silanized with hexamethyl disilazane. Glass column and glass wool plug at the bottom of the column are also silanized. The support particles must have even size and for majority of applications the size is 60-80, 80-100 or 100-120 mesh.

Stationary phase

The stationary phase must be non-volatile and thermally stable at the temperature of experiment. Often the stationary phases are high boiling point organic compounds and are coated on the support to provide 1-25% loading. Such phases are of two types: (a) selective where separation occurs by utilization of different chemical characteristics of the compound and (b) non-selective where separation is achieved by difference in boiling points of the sample components.

Commonly used stationary phases include polyethylene glycols, methyl phenyl and methyl vinyl silicone gums, esters of adipic, succinic and phthallic acids and squalene. The column is dry packed under a slight positive gaseous pressure and after packing, is conditioned for 24-48 hr by heating to near upper working temperature and passing the carrier gas at normal flow rates. During conditioning the column should not be connected to detector to prevent it's fouling.

Preparation and application of sample

Compounds having -OH, -NH_2, -COOH groups are generally retained in the columns if they are applied directly for excessive period of time resulting in poor resolution and peak tailing. This problem is overcome by derivatization of polar groups.

The sample for chromatography is dissolved in suitable solvent such as ether, heptane or methanol. The sample is then injected into the column through a septum in the injection port attached to the top of the column using a microsyringe. The injection zone is maintained at a slightly elevated temperature to ensure rapid and complete volatilization of the sample.

Separation conditions

Nitrogen, helium and argon are the most commonly used carrier gases. They are passed through the column at the rate of 40-80 cm^3/min. In isothermal analysis, a constant temperature is employed. In the separation of compounds of widely different polarity or molecular weights, it may be advantageous to gradually increase the temperature. This is referred to as temperature programming.

Detection systems

Most widely used detector in GLC is flame ionization detector (FID). It responds to almost all ionic compounds and can detect as low as 1 ng. A mixture of H_2 and air is introduced into detector to give a flame, the jet of which forms one electrode, while the other electrode is brass or platinum wire mounted near the tip of the flame. When sample components emerge from the column they are ionized in the flame, resulting in a signal being passed to the recorder. The nitrogen phosphorus detector, also known as the thermionic detector is similar in design as FID, but has sodium salt fused on to the electrode system or a burner tip embedded in a ceramic tube containing a sodium salt or a rubidium chloride tip. NP detector has excellent selectivity towards nitrogen and phosphorus containing compounds.

Electron capture detector responds to substances that capture electrons, particularly halogen containing compounds. This detector is, therefore, used in the detection of polyhalogenated compounds such as DDT, aldrin and similar pesticides.

Applications

GLC is mainly used for the separation of volatile non-polar compounds. Compounds are characterized by their retention time or preferably by their relative retention time to a standard reference compound.

High Performance (Pressure) Liquid Chromatography (HPLC)

Principle

Resolving power of a column increases with column length and number of theoretical plates per unit length. As the number of theoretical plates is related to the surface area of the stationary phase, it follows that the smaller the particle size, the better the resolution. But decrease of particle size is associated with greater resistance to eluent flow. In all the chromatographic processes; the eluent travels either under the influence of gravity or low pressure pumping. This results in low flow rates and gives greater time for band broadening by diffusion. Use of faster flow rates is not possible because it creates a back pressure which can damage matrix structure of the stationary phase thereby reducing eluent flow and impairing resolution.

Now a days special very small particle size stationary phases are available which can withstand high pressures and pumping systems which give high and reliable flow rates. These developments, which have occurred in adsorption, partition, ion exchange, exclusion and affinity chromatography have resulted in faster and better resolution. HPLC has, therefore, come out as the most popular, powerful and versatile form of chromatography.

Column

HPLC columns are generally made up of stainless steel and are capable of withstanding pressure upto 8,000 PSI. Straight columns of 20-50 cm in length and 1 to 4 mm in diameter are generally used. Porous plugs of stainless steel or teflon are used at the ends of the column to retain packing material.

Column packing

Prepared column with all necessary details are commercially available. Some may prefer to pack their own column. Several methods are available for packing column and a method depends upon the nature of packing material and dimensions of the particles to be packed. The particle size of the packing material should be uniform and the packed column should not have cracks or channels. Hard gels should be packed as densely as possible but without fracturing the particles. The most common technique is the high pressure slurry method. A suspension of packing is made in a solvent of equal density to the packing material. The slurry is then rapidly pumped at high pressure in the column with a porous plug at the outlet. The column is then washed with starting solvent to equilibrate it. The gel must be allowed to swell in the solvent to be used during chromatography before packing. Soft gels can not be packed under pressure and are packed under gravity in the usual fashion.

Mobile phase

The choice of mobile phase depends upon the type of chromatography to be performed. Isocratic separation can be done by a single solvent or two or more solvents mixed in a definite proportion. Gradient elution is also performed. All solvents to be used in the HPLC should be highly pure, since traces of impurity may affect the column and interfere in the detection system. The solvents must also be degassed before use as gassing tends to occur in most pumps.

Pumping system

There is high resistance to flow of solvents due to narrow columns packed with small particles. High pressures are required to achieve satisfactory flow rate. The pump should be able to

develop an output of at least 5,000 PSI and there must be no pulses of flow through the system. There must be a flow delivery of at least 10 cm^3/min for normal analyses and upto 30 cm^3/min for preparative analysis. All materials in the pump should be chemically resistant to all solvents.

Detector systems

The detector system should be very sensitive because a very small sample is analyzed. Most commonly used detector is variable wavelength UV-VIS spectrophotometer, a fluorimeter, a refractive index monitor or an electrochemical detector. Now a days some HPLC include mass spectrometer as the detector.

Practical procedure

For achieving successful separation, correct application of sample is essential. The initial bands of sample should be as narrow as possible. The sample is introduced through a microsyringe which can withstand high pressure or sample is injected through a septum in the injection port either directly on the column packing or on small plug of inert material just above the column packing. This can be done while the system is under pressure or the pump may be turned off before injection and after loading the sample the pressure recovered by switching the pump on.

Repeated application of impure samples may cause columns to loose their resolving power. To prevent this, a short column is introduced between the injector and the main column, the packing of which can be replaced as and when needed.

Applications

HPLC has proved to be most applicable technique of separation and identification because of its speed of separation, sensitivity and resolving power. Almost all types of biological molecules have been separated by HPLC. Reverse phase partition HPLC is particularly useful for polar substances such as peptides, vitamins, polyphenols and steroids. The technique is particularly useful in clinical and pharmacological work. HPLC has been most successfully used for the separation of proteins and oligopeptides. Instrument used for separation of proteins has given rise to the technique of fast protein liquid chromatography (FPLC).

Chromatofocussing

It is a technique of ion exchange chromatography and is in principle same as isoelectric focussing. The technique is particularly suitable for protein separation. A linear pH gradient is generated in the column by exploiting buffering action of the exchanger and using amphoteric buffers which have even buffering action over a wide range of pH. Proteins elute from such gradients in order of their isoionic points. Focussing effect occurs which result in sample concentration, band sharpening and very high resolution.

Affinity Chromatography

The technique employs highly specific biological interaction between solute to be separated and its ligand for purpose of purification of the former. Enzymes are highly specific for their substrates, inhibitors, activators or substrate analogues, which specifically but non-covalently bind to the enzyme for expression of their affect. This property has been utilized for purification of a given enzyme from other enzymes and proteins. Thus theoretically the technique is capable

of giving absolute purification, even from complex mixture, in a single step. The technique was originally developed for purification of enzymes, but has since been extended to nucleotides, nucleic acids, immunoglobulins, membrane receptors and even whole cells and cell fragments.

The technique requires that the material to be isolated, is capable of selectively and reversibly binding to the specific ligand which is covalently attached to chromatographically inert matrix.

Under appropriate experimental conditions, when a complex mixture, containing specific compound to be purified, is added to the insolubilized ligand contained in a column, only that compound would be bound to the matrix containing ligand, and thus its movement be retarded through the column. All other compounds can be washed away. The compound under study is then recovered by displacement from the ligand by either passing higher concentration of the substrate, changing the pH or ionic strength of the eluent.

The method requires a detailed knowledge of structure and biological properties of the compound to be purified, so that ideal separation conditions could be carefully planned. The conditions chosen should be such that are optimal for ligand-enzyme binding.

Matrix

An ideal matrix for affinity chromatography must possess the following characteristics : It must have sufficient and suitable chemical groups to which the ligand may covalently be added and it must be stable under the conditions of attachment. It must be stable during the binding of the macromolecule and subsequent elution. The matrix should have minimum interaction with other macromolecules to minimize non-specific adsorption i.e. it must be chromatographically inert to other macromolecules. It should exhibit good flow properties. In practice, uniform, spherical and rigid particles are used. The most common ones are cross linked dextrans, agarose, polyacrylamide, etc.

Selection and attachment of ligand

The chemical nature of ligand is determined by prior knowledge of the biological specificity of the compound to be purified. In practice it is frequently possible to select a ligand which displays absolute specificity to the macromolecule to be purified. Alternatively, a ligand may be selected which displays group specificity. It is essential that the ligand possesses a suitable chemical group which is not involved in reversible binding of the ligand to macromolecule and is used to attach the ligand to the matrix. The most common groups are – NH_2, -COOH, - SH and – OH. To prevent the attachment of the ligand to the matrix interfering with its ability to bind macromolecule, it is generally advantageous to introduce 6-10 carbon chain spacer arm between the ligand and the matrix. In some cases, the chemical nature of this spacer is critical for separation. Some spacers are purely hydrophobic, most commonly consisting of methylene groups, others are hydrophilic, possessing carbonyl or imido-groups.

The most common method of attachment of the ligand to matrix involves treatment of matrix with cyanogen bromide (CNBr) at pH 11. The reaction conditions and the relative proportion of the reagent will determine the number of ligand molecules which can be attached to the matrix particle. The spacer arm is then covalently linked to the matrix. Example of spacer arms include 1,6-diaminohexane, 6-amino-hexanoic acid and 1,4-bis(2,3-epoxy propoxy) butane. They must

possess a second functional group to which the ligand may be attached by conventional organosynthetic procedures which frequently involve succinic anhydride and a water soluble carbodiimide.

Practical procedure

First the supporting matrix, usually agarose, is activated by treating with –CNBr. To the decanted agarose, equal volume of water is added at a well ventilated place. Finely divided –CNBr (50-300 mg/ml packed agarose) is added at once to the stirred solution and pH is immediately raised to 11 with NaOH. The pH is maintained by constant manual titration of the mixture. The temperature is maintained at 20^{o}C by adding pieces of ice as needed. The reaction is complete within 8-10 min, as indicated by cessation of base uptake. A large amount of solid is then rapidly added to the suspension, which is quickly transferred to Buchner funnel and washed under suction with cold buffer. The buffer should be the same to be used in coupling stages and the volume of wash should be 10-15 times that of the packed agarose. The compound to be coupled is taken in cold buffer whose volume is same as that of packed agarose and added to the agarose still in the Buchner funnel and mixed immediately within 90 sec. The treated agarose is transferred to a beaker and stirred at 4^{o} for 16-20 hr very gently. The substituted agarose is washed with large volumes of water and then with buffer until there is no free ligand in the solution.

Chromatography

The procedure for affinity chromatography is similar to that used in other forms of chromatography. The ligand treated matrix is packed into the column. A buffer is used which will encourage the desired macromolecule to be strongly bound to ligand of the matrix. The buffer must also contain any cofactors necessary for macromolecule-ligand interaction. Once the sample has been applied and the macromolecule bound, the column is washed with more buffer to remove non-specifically bound contaminants. The purified compound is finally removed by specific or non-specific elution. Non-specific elution may be achieved by a change in either pH or ionic strength. pH shift elution results from a change in the ionization of groups in the ligand and/or the macromolecule which are critical to ligand-macromolecule binding. A change in ionic strength also causes elution due to disruption of ligand macromolecule interaction.
1 *M* NaCl is frequently used for this purpose. Elution may also be accomplished by affinity elution.

Applications

A wide range of enzymes and other proteins including receptor proteins and immunoglobulins are purified by this technique. The technique is limited in application only on the availability of immobilized specific ligand for the protein under purification.

Messenger RNA is routinely isolated by selective hybridization on Poly(u) Sepharose 4B and the viral RNA on the corresponding Poly (A). Immobilized single stranded DNA can be used to isolate complementary RNA and DNA. Immobilized nucleotides are useful for isolation of proteins involved in nucleic acid metabolism. Affinity chromatography is used to concentrate the dilute samples and to separate native and denatured macromolecules.

Metal chelate or immobilized metal affinity chromatography is another extension of affinity chromatography. It enables proteins of similar molecular weight and isoelectric pH to be separated on the basis of their differential binding to metal ions which have been immobilized by chelation. Binding of metal ions is pH dependent. The sample is applied at neutral pH and is eluted by lowering the pH and ionic strength or by including EDTA in the eluent. The two most commonly used ligands for chelating metal ions are iminodiacetic acid and Tris (carboxy methyl)ethylene diamine. The binding of metal atoms to the protein involves mostly the histidine residues. Proteins purified by this technique include fibrinogen, super oxide dismutase and non histone nuclear proteins.

Covalent chromatography

It differs from other forms of chromatography in that it involves covalent bond formation between the bound ligand and the compound to be separated (most commonly proteins). The most common form involves formation of disulfide bond between thiol groups in the compound and ligand. Commercially available ligands include thiopropyl sepharose and thiol sepharose. Elution is carried by dithiothreitol or cysteine. The technique is best suited for proteins that have many free-SH groups.

Specific elution with substrate

Purification of enzymes and certain proteins can be achieved by selective elution of the enzyme with its substrate, activator or reversible inhibitor which, because being specific to the enzyme, selectively binds the enzyme and elutes leaving other proteins in the column.

Ion exchange column using suitable anionic or cationic matrix is prepared as usual. It is then loaded with crude preparation to the extent to saturate the column with respect to the enzyme being purified. Selective elution of the enzyme is carried out by passing substrate, specific activator or reversible inhibitor of the enzyme. Since the enzyme forms specific non-covalent bond with ligand, it leaves the exchanger and is eluted out leaving behind other proteins.

Hydrophobic Interaction Chromatography (HIC) and Reverse Phase Chromatography (RPC)

Macrmomolecules, particularly proteins have surface exposed hydrophobic regions whose size varies from protein to protein and may be used for their separation. This form of chromatography uses hydrophilic gel based matrix which has been partially substituted on the surface by non polar alkyl (e.g. methyl or octyl) or aryl (e.g phenyl) groups. Similar materials are used in reverse phase chromatography but the degree of substitution in latter case is much more than hydrophobic interaction matrices. The solvent used for elution in HIC is low ionic strength buffer whereas in RPC an organic solvent is used.

The binding of proteins to an HIC matrix depends on several factors such as type of the matrix, nature of functional groups and degree of substitution, nature of protein, specially the character and size of surface exposed hydrophobic regions and the composition of buffer used. Proteins with large surface exposed hydrophobic regions will bind the matrix more strongly than those with smaller regions. The hydrophobic effect is strongly dependent on concentration and nature

of dissolved salts in the eluent consequently binding and subsequent elution can be carried by controlling the buffer conditions. Binding is tighter at higher salt concentrations and elution may be carried out by decreasing concentration of salt. Typically sample binding is carried out in a 20 m*M* phosphate buffer, pH 7.0 in presence of 1 *M* ammonium sulfate and elution is carried by decreasing the ammonium sulfate gradient from 1 *M* to 0. Desorption may be facilitated by incorporation of ethylene glycol, glycerol or non ionic detergent Triton X-100. Since hydrophobic effect is highly dependent on temperature, lowering the temperature may also effect desorption. Hydrophobic matrices often cause irreversible denaturation of proteins, thus limiting utility of the technique. HIC matrices have high capacity for binding proteins (e.g. 40 mg albumin can be bound to 1.0 gm gel at 1.5 *M* $(NH_4)_2SO_4$. The salt composition of the sample should be same as equilibrating buffer. Elution volume should not be more than 10 times the volume of the column.

Gel Filtration, Molecular Sieve, Exclusion, Permeation Chromatography

Principle

Separation of molecules on the basis of their molecular size and shape uses molecular sieve properties of a variety of porous materials. These polymeric organic compounds possess a three dimensional network of pores which confer gel properties upon them. Gels of various organic substances such as cross linked dextran, agarose and polyacrylamide are available. These are of uniform pore sizes. The pore size can be varied by controlling the degree of cross linking during their synthesis, thus by selecting the gel of suitable pore size molecules of any size can be fractionated.

The pores are funnel shaped and therefore a molecule larger than the size of the pore will not be able to enter into the pore and will move along with the solvent and come out of the column first. Molecules of decreasing order of size will be eluted in increasing order because smaller the size deeper they will be able to enter into the pores and thus their movement through the column will be more restricted and they will come out of the column with passage of larger volume of eluent.

Gel of a given pore size has a fractionation range. Molecules larger than the pore size of the gel will not separate and will come out of the column together with the pass through. Similarly molecules smaller than a given size will not be fractionated and retained in the gel pores together. The lowest molecular weight substance would be eluted at the end of passing one bed volume of eluent through the column after that there will not be separation of smaller molecular weight substances.

The solvent absorbed by a swollen gel is available to a solute to an extent which is dependent on porosity of the gel particle and size of the solute molecules. Thus distribution of a solute in a column of a swollen gel is determined only by the total volume of solvent, both inside and outside the gel particles which is available to it.

For a given type of gel, the distribution coefficient, Kd, of a particular solute between the inner and outer solvent is function of its molecular size. If the solute is large and completely excluded from the solvent within the gel, Kd = 0, whereas if a solute is sufficiently small to gain

complete accessibility to the inner solvent, Kd = 1. Due to variation in pore size of a given gel, there is solvent which will be available and some which will not be available to the solutes of intermediate size, hence Kd values vary from 0-1. It is the complete variation of Kd between these limits which makes possible separation of solutes within narrow molecular size range on a given gel. The elution volume, Ve, of a given solute depends on the void volume Vo, the distribution coefficient and the volume within the gel matrix Vi.

Thus $Ve = Vo + Kd\ Vi$.

The inner volume Vi can be calculated from the known dry weight of the gel and the swelling volume Wr since Vi = a Wr.

For substances of two different molecular weights and Kd values, the difference in their effluent volumes Vs is given by $V_s = V_e' - V_e'' = (V_o + K_d'V_i) - (V_o + K_d''V_i)$ or $V_s = (K_d' - K_d'')\ V_i$.

Thus for complete separation of two substances, sample volume should not be larger then Vs and thus should, in practice, be as small as possible.

In practice the above parameters can be experimentally determined after packing of the column:

a) Calculate out total volume of the packed column using eqn. $V_{tot} = \pi r^2 h$
b) Calculate out void volume by passing colour dye of very high molecular weight, such as blue dextran or India ink which does not enter into gel beads, and noting the elution volume of the dye. This treatment will also show the irregularity in column packing such as air channels or un uniform packing etc.

Gel filtration chromatography can also be carried (specially for molecular weight determinations) on thin layer mode. Gel beads are spread on glass plates. They adhere to the plate without fixative and form stationary phase. Interstitial fluid forms the mobile phase. The plate is placed in an air tight chamber inclined 20^o to the horizontal plane and both the ends connected to the solvent reservoirs by paper wicks. The plate is equilibrated in the chamber for a minimum of 12 hr. The main function is to normalize the ratio between mobile and stationary phase volumes. The sample is applied as a spot or a band and the plate developed for suitable period of time and spots visualized by appropriate method. The main advantage of TLGFC is that a number of samples can be chromatographed under identical conditions at the same time. In addition, very small amounts of samples can be used; thus it is ideal for clinical purposes.

Materials

Commonly used gels are cross linked dextran (Sephadex), agarose (Sepharose, BioGel A, Sagavac), polyacrylamide (Biogel P), polyacryloyl morphine (Enzocryl Gel) and polystyrenes (Bio beads S).

Gels of varying pore sizes can be made by controlling the degree of cross linking and are useful over different molecular size ranges. Each type of gel is characterized by its water regain i.e. the amount of water taken up in completely swollen gel granules by 1 gm of gel.

Dextran gels allow separation of spherical molecules such as globular proteins of dimensions corresponding to molecular weights upto 800,000 Daltons, the agarose gels may be used to separate molecules and particles upto molecular weight of several million Daltons. The agarose

gels have, therefore, been used for the study of viruses, nucleic acids and polysaccharides. Polyacrylamide gels are prepared by polymerization of acrylamide and cross linking by addition of N'N'-methylene (bis)-acrylamide. By varying the ratio of acrylamide to bisacryl amide, gels of various pore sizes can be prepared. They have characteristics very similar to dextran and agarose gels. They have a molecular weight exclusion limit ranging from 1800-400,000 Daltons. Moreover, many gels are available in range of sizes, superfine, fine, medium and coarse. Coarser the bead, better the flow rate but poorer the resolution. Thus fine and superfine gels are used for analytical and coarse gels for preparative purposes.

Applications

Purification

The main application of gel filtration chromatography is the purification of labile biological macromolecules. Viruses, proteins, enzymes, nucleic acids, hormones, antibodies, polysaccharides all have been purified by this technique.

Experimental Details

Selection of suitable gel

If one is interested in purification of a particular protein and there is an idea of its molecular weight then the gel of suitable fractionation range can be selected easily. If all the components of a crude preparation are to be separated, then selection of gel is carried out by trial and error starting from the gel of maximum fractionation range downwards.

Packing of the column

Depending on the recommended swelling time, gels of desired pour size is allowed to swell preferably at the temperature of experiment. During swelling and subsequent washing of the gel, finer particles are decanted off and the slurry of uniformly settling particles is used to pack the column. Though, chromatography can be done in pure water, addition of low concentration of salt such as NaCl in the slurry or eluent is advised to minimize interionic attractions between the solute molecule.

For gel filtration chromatography relatively larger columns are employed to increase fractionation surface area. Usually the ratio of column length to diameter is kept about 1:10 to 1:20.

The column of uniform inner diameter is held vertically and filled with water upto $2/3^{rd}$ of its length with buffer or salt solution. The uniformly settling slurry of the gel is poured in the column and allowed to settle under the gravity. At no stage entrapment of air bubbles should be allowed in the column. As these, with change in temperature, may coalesce and form channels in the column. To prevent this, the column packing should be done at the temperature of the experiment and buffer solution be degassed. After the desired amount of gel has been loaded into the column, it is washed with 1-2 bed volumes of starting buffer to properly compress it. Once the column has been prepared, it is never allowed to run dry. To prevent disturbance of the top surface a nylon sheet may be layered over it.

Checking of the column packing

Since the resolution of the solutes greatly depends upon the uniformity of the packed gel, before use, the packing of the column is checked by passing coloured substances of very high molecular weight, such as India ink or blue dextan. These substances do not enter into the gel pores. As they pass through the column, packing deformity and channeling becomes visible. Moreover, by following the elution volume of these substance, void volume of packed column can also be determined.

Application of sample

Usually sample solution is not needed to be specially treated for loading onto the column. Excepting that it should be sufficiently concentrated so that a smaller volume is loaded on the column. Buffer above the gel top is removed and the sample is applied carefully so as not to disturb the surface of the gel. Sample solution is allowed to enter into gel by opening the stopper of the column. Fractions are collected from now. As the sample solution has entered the gel, gel surface is washed with small volume of buffer and the column is connected to the eluent reservoir which has a salt solution or a buffer.

Collection of fraction and analysis

Once the sample is loaded, eluent is continuously passed. Theoretically, by passing one bed volume of eluent the smallest fractionable solute of the mixture is eluted out. For better resolution, the flow rate should be low as 2-10 ml/hr and also the volume of eluent fraction collected should be small (2-5 ml each). Each fraction is then analyzed for the presence of solute by specific reaction or absorption at the characteristic wavelength. A graph between elution volume and absorbance is plotted. A number of peaks of eluted solutes are obtained. Each peak represents at least one solute. Fractions under each peak may be pooled, concentrated and rechromatographed for further resolution.

Molecular Weight Determination by Gel Filtration Chromatography

As discussed earlier, molecular weight of macromolecules can be determined on thin layers of gel filtration media. Spot of unknown sample, along with proteins of known molecular weight are applied and chromatography performed for predetermined time. Spots are visualized and distances traveled by spot of each protein are measured. With the help of distances traveled by marker proteins, a graph between molecular weight and distance traveled is plotted. By interpolating the distance traveled by the unknown protein in the graph, its molecular weight is determined. This method is quick and reliable. Alternatively, molecular weight of the unknown macromolecule can also be determined using appropriate gel filtration media packed in a suitable column. Markers, along with unknown are loaded in the column and elution volume of each eluting protein is noted. A calibration curve between molecular weight and elution volume is constructed with the markers. Elution volume of unknown is interpolated into this curve to find out its molecular weight (Fig.4).

Fig 4. Molecular Weight determination by gel filtration chromatography. Graph is made by plotting elution volume of proteins of known molecular weight. Molecular weight of unknown protein is determined by interpolating its mobility in to the graph.

36

Suggested Readings

1. Separation Methods in Biochemistry by C.J.O.R. Morris and P. Morris.
2. Practical Biochemistry and Techniques by K. Wilson and J. Walker.
3. Biochemical Methods-A concise guide for students and researchers by A. Pingoud, C. Urbanke, J. Hoggett and A. Jeltsch.
4. Laboratory techniques in biochemistry and molecular biology by T.S. Work and E. Work.
5. Methods in enzymology Vol. 182 Guide to protein purification M.P. Deutscher, ed.