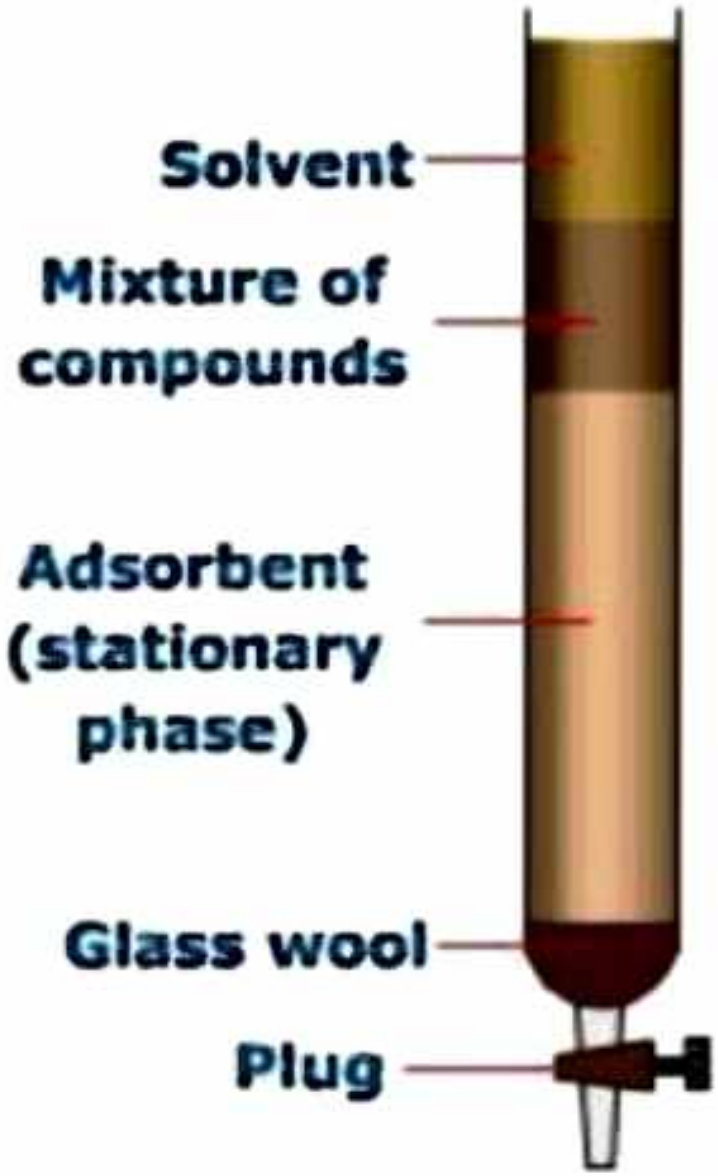
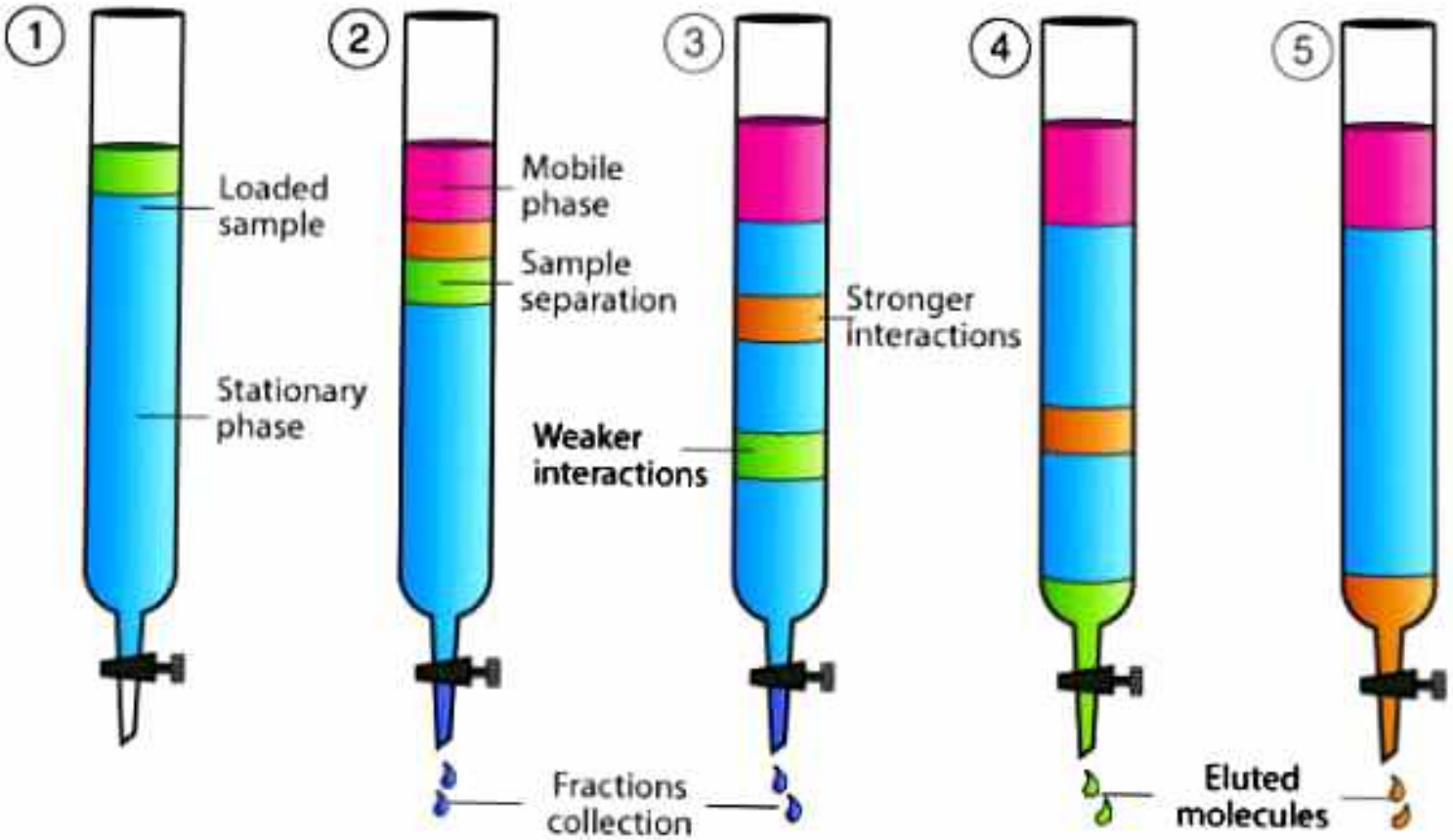
COLUMN CHROMATOGRAPHY



- Chromatography is the term used to describe a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.
- There are a number of different kinds of chromatography, which differ in the mobile and the stationary phase used.
- Column Chromatography was developed by the American chemist D.T Day in 1900, M.S. Tswett, the Polish botanist, in 1906 used adsorption columns in his investigations of

plant pigments.


1
2
3
4
5
Loaded sample
Stationary phase
Mobile phase
Sample separation
Stronger interactions
Weaker interactions
Fractions collection
Eluted molecules

Liquid Phase Column Chromatography

Liquid phase column chromatographic methods are those in which a packed column is employed with a mobile liquid phase for achieving separations. The solid column itself may be the stationary phase as in *adsorption* or *ion exchange chromatography* or the packing may act as the support for a liquid stationary phase as in liquid-liquid chromatography. A more recent development in liquid-liqud chromatography is *Countercurrent Chromatography* which entirely eliminates the use of a solid matrix support. Another form of chromatography where the stationary phase is a porous gel and the separation is according to the size of the molecule, is *Gel (Exclusion) Chromatography*. All these come under the category of Liquid Phase Column Chromatography. Chromatography using gels modified to develop highly specific biochemical reactions for separations is called *Affinity Chromatography* (also called *Bioaffinity Chromatography*). Other modifications of this technique are, *Metal-Chelate Affinity Chromatography, Ligand Exchange Chromatography* , and *Dye-Ligand Affinity Chromatogrraphy.* Ion exchange Chromatography is described in the chapter dealing with ion exchange separations. A newer technique which makes use of all the above principles permits very rapid separations is *High Performance Liquid Chromatography* (HPLC).

10.1 Adsorption Chromatography

Adsorption chromatography is based on the specific interaction of the solute with the surface of the finely divided adsorbent. In a multicomponent system, the competition between the solute and the solvent molecules for the adsorption sites establishes a dynamic process. The solute and the solvent molecules continuously come in contact with the adsorption sites, reside there and then leave to reenter the mobile phase. The entire process is governed by the strength of the adsorption forces between the adsorbent and the adsorbates. These can be classified as

1. van der Waals forces (London dispersion forces)
2. Polar forces
3. Ionic forces, and
4. Chemisorption

van der Waals forces (London dispersion forces) are intermolecular forces which hold neutral molecules together in the liquid or solid state. Since these forces are purely physical in character, they do not lead to the formation of any chemical bonds. The process arising from these forces is called physical adsorption. Since their energies are low, there is a rapid establishment of equilibria leading to good chromatographic separations. Dispersion forces account for all the adsorption energy in the case of adsorption of nonpolar solutes onto nonpolar adsorbents. They arise from charge fluctuations rather than permanent electric charges in the molecules. Examples of purely dispersive interactions are the molecular forces that exist between the hydrocarbon molecules. For example, n-heptane is not a gas due to the collective effect of all dispersive interactions resulting in the molecules held together in the forms of a liquid. These solutes can be held together selectively by nonpolar adsorbents (e.g., hydrocarbons on graphite) or by hydrocarbon-type materials such as reverse-bonded phase (to be described later). To enable the dispersive selectivity to be exercised by the stationary phase, the mobile phase must be polar or significantly less dispersive (e.g., ethanol-water and acetonitrile-water mixtures).

Polar forces, which include hydrogen bonding forces, arise from permanent or induced dipoles and not from net charges as in the case of ionic forces. Some examples of polar substances with permanent dipoles are: alcohols, ketones, aldehydes and aromatic hydrocarbons (e.g., benzene). When a molecule carrying a permanent dipole approaches a polarizable molecule, electrical interactions occur. For this reason, the stationary phase must be polar for retaining a polar solute. Examples are, silica (acidic) and alumina (acidic and basic).

Ionic interactions result from the permanent electrical charges that exist on the molecules in the form of ions. Examples are, organic acids and salts. Such interactions are exploited in ion exchange chromatography described in an earlier chapter.

Chemisorption occurs when chemical bonds are formed between the solutes and the active sites on the surface of the adsorbents which have not been deactivated. For example, silica surfaces may contain residual acidic sites which strongly chemisorb acids. Similarly, alumina contains basic sites which strongly chemisorb acids. This can lead to strongly tailing elution bands resulting in incomplete resolution and sample recovery.

Adsorbents

Adsorbents for adsorption chromatography are finely divided porous solids. The most common adsorbents are silica and alumina, whose lattices are terminated at the lattice surface with polar hydroxyl groups which provide the means of surface interactions. The role of the adsorbent is determined by several factors such as surface area, chemical composition and the geometrical arrangements of the atoms or groups. The stronger adsorbents naturally possess more polar structure. Table X.1 contains a list of adsorbents used in adsorption chromatography.

TABLE X.1

ADSORBENTS USED IN ADSORPTION CHROMATOGRAPHY
(in the order of increasing strength)

Adsorbent	*Nature of active sites*
Sucrose	Neutral
Starch	Neutral
Kieselgurh	Neutral
Carbon	Neutral and acidic
Silica	Acidic
Magnesium silicate (Florisil)	Acidic
Alumina	Acidic and basic
Fuller's earth	Acidic
Magnesia	Basic

Other materials used as adsorbents include, calcium sulphate, molecular sieves, organo clays and polyamides.

The selectivities are a function of the type of adsorbent and the solute. Polar adsorbents selectively adsorb unsaturated, aromatic and polar molecules. A polar adsorbent can be acidic, basic or neutral depending on the pH of the surface. Examples of acidic adsorbents are silica and Florisil. These chemisorb bases. Similarly basic adsorbents (e.g., magnesia) chemisorb acids. While chemisorption is an effective concentration method, it will not enable separations because of the difficulties of desorption. Thus acids are best separated on acidic adsorbents and bases on basic adsorbents.

Silica (or silica gel) is a commonly used adsorbent. It has the general formula $SiO_2 . xH_2O$. There is a wide variation in its properties such as surface area, pH, and pore size distribution depending on the method of its preparation and subsequent thermal treatment. To obtain reproducible results it is necessary to use a standardized sample.

Another commonly used polar adsorbent is alumina. It is preferred for the separation of mixtures of aromatic hydrocarbons. It contains a number of strongly basic sites and therefore shows a preferential adsorption for acidic samples. While strong acids with $pK_a < 5$ are chemisorbed, weaker acids can be separated in the order of their pK_a values

using basic eluants. It cannot however be used for some separations because the solutes undergo chemical reactions at the reactive sites. Some examples are: saponifications of esters and anhydrides, condensation reactions with aldehydes and ketones, isomerization and polymerization of olefins, oxidation and complex formation.

Table X.2 gives details of separations that can be achieved with some solid adsorbents.

TABLE X.2

ADSORBENTS FOR CHROMATOGRAPHIC SEPARATIONS

Adsorbent	*Applications*
Sucrose	Chlorophyll, xanthophylls
Starch	Enzymes
Aluminium silicate	Sterols
Calcium carbonate	Carotenoids, xanthophylls
Magnesium silicate	Sterols, esters, glycerides, alkaloids
Carbon	Peptides, carbohydrates, amino acids.
Silica gel	Sterols, amino acids
Alumina, Magnesia	Sterols, dyestuffs, vitamins, esters, alkaloids, inorganic compds.

The properties of silica gel and other polar adsorbents can be modified by incorporating a complexing agent into the adsorbent. For example, silica gel impregnated with silver nitrate gives a more satisfactory separation of olefins from saturated hydrocarbons.

Rigid nonporous supports such as glass beads covered by thin layers of adsorbent, on which liquid stationary phases can be deposited, are used for HPLC separations. These will be described in the next chapter.

Eluants

The choice of the eluant (mobile phase) in adsorption chromatography is based on the fact that the more strongly the mobile phase interacts with the adsorbent, the quicker the solute will elute from the column. Thus, the major factor determining the retention of the solute is the relative polarities of the mobile phase and the solutes. A polarity scale, called the *eluotropic series*, for the solvents is established by arranging the order of their strength of adsorption on an adsorbent. A solvent with higher polarity will displace one lower in the polarity scale. Table X.3 contains such a scale developed for alumina. The order is similar for silica and other polar adsorbents. To obtain an eluant of intermediate strengths, mixtures of solvents are used. Selection of the correct mobile phase is generally a matter of trial and error. In practice, the best way of choosing the correct eluant is by carrying out a trial TLC run on microscopic slides coated with the same adsorbent. Other factors

that have to be taken into consideration in choosing the appropriate eluant are: volatility, method of detection of the solute, viscosity, solubility of the solute and effect on the adsorbent.

TABLE X. 3

ELUOTROPIC SCALE FOR ALUMINA

Solvent	*Eluant strength* ε^{o}
n-Pentane	0.00
Iso-pentane	0.01
Cyclohexane	0.04
Cyclopentane	0.05
Carbon disulphide	0.15
Carbon tetrachloride	0.18
Xylene	0.26
Toluene	0.29
Benzene	0.32
Ethyl ether	0.38
Chloroform	0.40
Methylene chloride	0.42
Tetrahydrofuran	0.45
Ethylene dichloride	0.49
Methylethyl ketone	0.51
Acetone	0.56
Dioxane	0.56
Ethyl acetate	0.58
Methyl acetate	0.60
Amyl alcohol	0.61
Aniline	0.62
Nitromethane	0.64
Acetonitrile	0.65
Pyridine	0.71
Isopropanol	0.82
Ethanol	0.88
Methanol	0.95
Water	>1.0
Ethylene glycol	1.11
Acetic acid	large

The order of the eluotropic series for nonpolar adsorbents such as graphitized carbon is reversed because nonpolar molecules are preferentially adsorbed on this adsorbent. Thus the series is

water < methanol < ethanol < acetone < propanol < ethyl

ether < butanol < ethyl acetate < n-hexane < benzene

A number of macroporous polymeric adsorbents of varying porosities are now commercially available under the trade names Bio-Bead

SM (Bio-Rad), Amberlite-XAD (Rohm and Haas) and XUS (Dowex). The polystyrene-DVB nonpolar adsorbents are particularly useful for the adsorption of nonpolar substances or surfactants from aqueous solutions. They can be used for the removal of detergents, emulsifiers and wetting agents as well separating water-soluble steroids, phenols, drugs and pesticides. The polar acrylic polymer beads can adsorb polar compounds from nonpolar solvents or nonpolar compounds from polar solvents. Depending on the solvent, they can adsorb either the polar or nonpolar portion of molecules having regions of both types. Examples are: adsorption of fatty acids from water or toluene, proteins from aqueous solutions and phenols and coloured organics. Other applications include, purification of Vitamin B_{12}, antibiotics such as Oxytetracycline and Oleandomycin, removal of pesticides and metals in trace concentrations from aqueous solutions. The adsorbents can be regenerated by eluting the adsorbed molecules.

10.2 Liquid-liquid Chromatography

In liquid-liquid chromatography, the solid adsorbent is replaced by a support material coated with a stationary liquid phase which should be insoluble or at the most, sparingly miscible with the mobile phase. In principle, it is much like liquid-liquid extraction. Resolution of the components in the mixture occurs due to their differences in their distribution ratios. While large samples can be handled in liquid-liquid extractions, liquid-liquid chromatography is capable of faster and more difficult separations.

The ideal partition system is one in which the stationary liquid phase is in equilibrium with the mobile phase, i.e., the amount and distribution of the stationary liquid in the system remains constant. But this condition is rarely achieved due to several effects such as the mutual solubility of the two contacting phases and the effect of changing temperature on solubility. One practical approach to realize this condition is through the saturation of the mobile phase by shaking it for 24 hours with excess of the stationary phase at the column temperature and using it after the suspended droplets of the stationary phase are settled.

Solid Supports

In LLC, the stationary liquid phase (normally water) is supported on a material which is usually of a high surface area with small particle size (1-200 microns) and inert. The support materials most commonly used are, silica gel, alumina, diatomaceous earths (e.g., Celite, Kieselgurh), porous polymers and cellulose. Since it is not possible to achieve complete screening of the support surface, adsorption and other factors also play some role in the separations. Silica gel which is used in the form of fine white powder, can hold about 30% water which can act as the stationary phase. Table X.4 gives details of some LLC systems and their

applications. In addition, new support materials are developed for faster and better separations. These will be described in the next chapter.

TABLE X.4

SUPPORT MATERIALS AND THEIR APPLICATIONS IN LIQUID-LIQUID CHROMATOGRAPHY

Support	*Stationary phase*	*Mobile phase*	*Applications*
Celite	Water	$CHCl_3$ or CCl_4	C_1-C_4 alcohols
Silica gel	Water	$CHCl_3$/butanol	C_2-C_8 fatty acids, acetylated amino acids
Silica gel	water	iso-Octane	phenols
Cellulose	Water	Acetone HCl	inorganic ions
Starch	Water	Methanol/Butanol-$CHCl_3$ Propanol or Butanol-HCl	phenols amino acids

Mobile Phase

At the present level of understanding of the properties of solutions, the choice of a mobile phase to achieve the resolution of components of a mixture is mostly empirical. In addition to activity coefficients, the factors that guide the choice of a suitable mobile phase are: ability to achieve the desired selectivity, stability and volatility of the phase, viscosity and convenience of use. Polarity is a rough guide to selectivity which a solvent is likely to show for a series of different compound types. Other parameters which come into consideration are, solubility parameter and critical solution temperature.

In the traditional LLC, polar supports (e.g., silica and alumina) are used to hold the polar compound as the stationary phase. The polarity of the silica surface arises from the silanol groups (Si-OH), the greater their number, the greater the polarity. At 170°C, the polarity of the surface is maximum. Heating above 200°C leads to the conversion of the silanol groups into siloxane groups (Si-O-Si) resulting in the decrease of polarity of the surface.

Substituting a hydrocarbon group (e.g., $C_{18}H_{39}$) on the surface forms a bonded nonpolar phase (Fig. X.1). Nonpolar solutes which are held by such nonpolar phases can be eluted by aqueous mobile phases, a reverse of the traditional LLC. This is called *Reversed Phase Chromatography* (RPC), a term which covers stationary phases that are nonpolar in nature. Such phases retain solutes predominantly on the basis of dispersion interactions. These will be described in detail in the next chapter.

The macroporous polymeric adsorbents Biobead SM, Amberlite - XAD, and XUS, mentioned earlier, are also suitable supports for RPC.

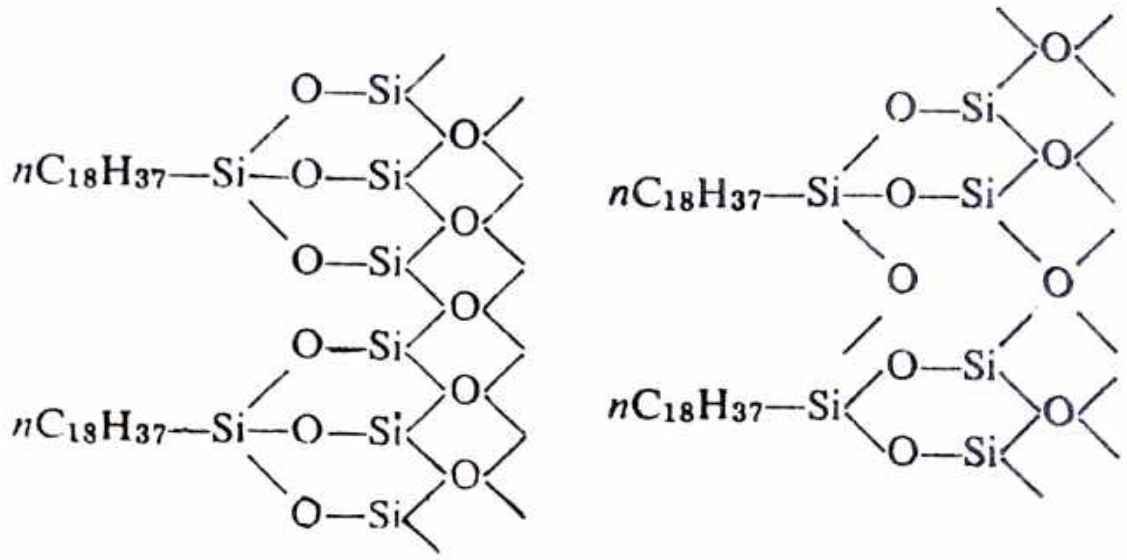


Fig. X.l: Bonded nonpolar Silica phases

Advantages and limitations of Adsorption and Liquid-Liquid (Partition) Chromatography

Adsorption Chromatography

- Adsorption is experimentally easier than partition.
- Adsorption provides a more uniform, reproducibel behaviour since only a solid and mobile eluting phase are involved.
- Adsorption is very sensitive to steric differences for similar molecules and is therefore applicable to the separations of such mixtures of molecules.
- The polarity of the mobile phase can be varied widely since miscibility with a stationary phase is not involved.
- Adsorption chromatography is suitable for large amounts of samples.
- Adsorption chromatography is preferred for the separation of mixtures whose components differ widely in polarity or structure.

Liquid-Liquid Partition Chromatography

- Liquid-liquid partition chromatography provides much larger resolving power than adsorption chromatography even though it is more difficult to reproduce the amount of stationary liquid phase and the interaction between the stationary and mobile liquid phases.
- Partition, in general, is more suitable to low concentrations of mixtures (In some cases however these limitations can be overcome).
- Since partition is dependent on solubility in two liquids, small differences in molecular weight will influence partitioning. Therefore partitioning is preferred for the separation of homologous series.

- The tendency is for the partition coefficient to be independent of concentration over a greater range than for adsoption coefficient (Partition provides a linear isotherm over a wider concentration range).
- There appears to be a more rational relationship between structure and substituent influences in partition in comparison to adsorption.

Adsorption and partition are based on polarity differences. Since adsorption increases with an increase in polarity, it becomes difficult to elute very polar molecules from the adsorbent. For this reason, adsorption is used for non-polar or less polar molecules while partition is used for polar molecules.

Some Solvent Systems for Liquid-Liquid Chromatography

Stationary Phase	Mobile Phase
Normal Partition	
Water	Alcohols (n-and iso-butanol)
Water + acid & Water + alkali	Hydrocarbons (benzene, toluene, hexane & cyclohexane)
Water + buffers	Chloroform
Aq. Alcohols (MeOH, EtOH)	Ethylacetate
Alcohols (MeOH, EtOH)	Ethylene glycol monomethyl ether
Formamide	Methylethyl ketone
Glycols (Ethylene glycol, Propylene glycol, Glycerol)	Pyridine
Reverse Phase Partition	
n-Butanol	Water
Octanol	Water + acid
Chloroform	Water + alkali
Chlorosilane & Silicone	Water + buffers
Mineral oil	Aq. Alcohols (MeOH & EtOH)
Paraffin	Alcohols (MeOH & EtOH), Formamide, Glycols (Ethylene glycol, Propylene glycol, Glycerol)

10.4 Gel (Exclusion) Chromatography

Selectivity based on steric factors between the adsorbate and the adsorbent is utilised in achieving specific separations through gel chromatography. The technique is variously known as *gel filtration, molecular sieve filtration,* and *exclusion chromatography*. All these names essentially refer to the same process.

Gel chromatography columns contain porous gel particles with different diameters and porosities presenting a gradation of size barriers ranging from 10 to 75 microns to the solute molecules. A solute injected into such a column will diffuse into the gel pores that have diameters greater than the effective diameter of the solute. As the effective diameter of the solute gets larger, the number of pores that it can fit into and its ability to diffuse into the pores decreases. Thus, if a solute is of such a diameter that it cannot diffuse into any of the pores, it is defined as totally excluded and consequently will not be retained. It will elute into the void volume of the column and then out of the column. Solutes with smaller molecules which enter the gel beads to varying extents, depending on their size and shape, are retarded during their passage through the column bed to differing degrees and are resolved. Thus, the solute molecules are eluted in a predictable way in the order of decreasing molecular size (Fig. X.4).

Gel chromatography is a simple and reliable method for separating molecules according to size. Its versatility makes it

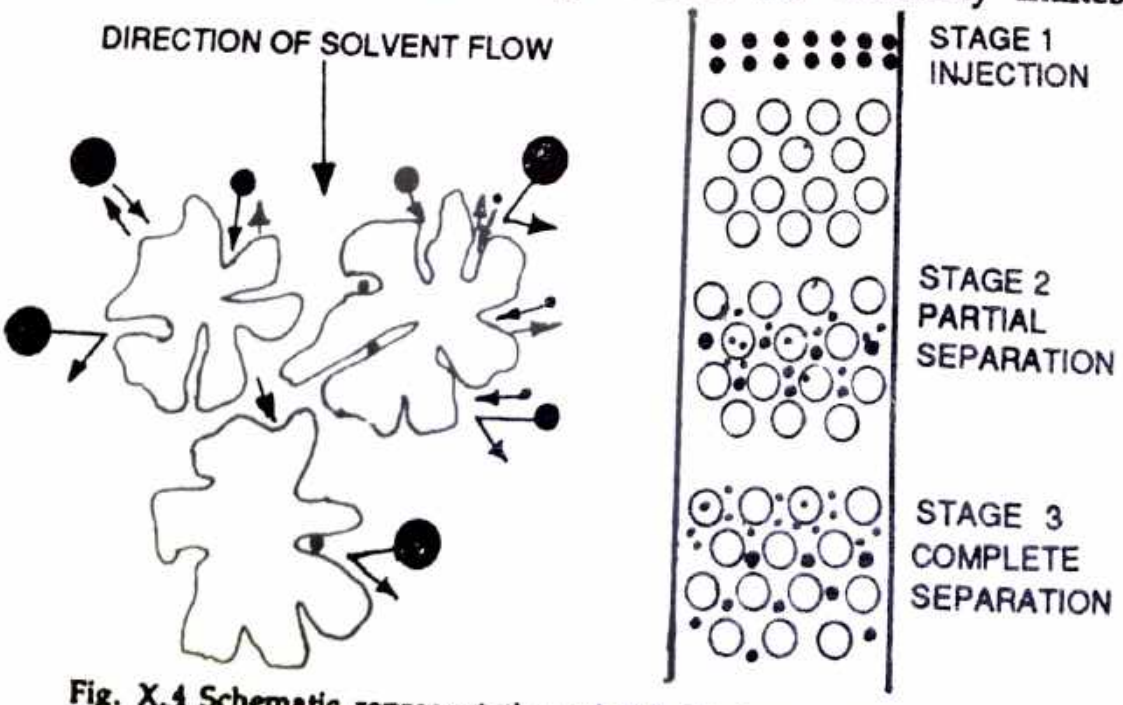


Fig. X.4 Schematic representation of Gel (Exclusion) Chromatography

generally applicable to the purification of all classes of biological substances, including giant macromolecules not readily fractionated by other techniques. Good separations and high activity yields are easily obtained.

Column Materials

A suitable material is one whose fractionation range covers the molecular sizes to be fractionated. The technique is essentially employed for the separation of biological molecules which range in size from a few hundred to many million in molecular weight. Four forms of gel materials are commercially available. They are.

(i) bead-formed cross-linked dextran gels of different porosities.

(ii) bead-formed agarose gels with different agarose concentrations in the gel beads,

(iii) porous polyacrylamide beads prepared by the copolymerization of acrylamide and N-N'-methylene-bis-acrylamide, and

(iv) porous polystyrene beads prepared by the copolymerization of styrene and divinylbenzene.

Table X.5 contains details of some commercially available gels and their useful separation ranges.

Gels belonging to the dextran, agarose and acrylamide groups are soft gels while those belonging to the polystyrene group are semirigid. In addition, rigid gels based on silica are available. Soft gels are useful for separating water-soluble substances in the molecular weight range 100 to 2.5×10^7. They typically have a large range of pore sizes and are particularly useful for the separation of

TABLE X.5

GEL CHROMATOGRAPHY MEDIA AND THEIR CHARACTERISTICS

Type	*Useful fractionation range (Molecular weight)*	
DEXTRAN GROUP		
Sephadex, G-10 to G-200	$700 - 2 \times 10^5$	for dextrans
	$700 - 6 \times 10^5$	for peptides
AGAROSE GROUPS		
Sepharose CL-6B/4B/2B	$1 \times 10^4 - 2 \times 10^7$	for dextrans
	$1 \times 10^4 - 4 \times 10^7$	for peptides
Bio-Gel A 0.5m to 150m	$1 \times 10^4 - 1.5 \times 10^8$	for proteins, peptides, and complex carbohydrates
ACRYLAMIDE GROUP		
Sephacryl S-200 to S-1000	$5 \times 10^3 - 8 \times 10^6$	for peptides
	$1 \times 10^3 - 10^8$	for dextrans
Bio-Gel P-2 to P-300	$100 - 4 \times 10^5$	
POLYSTYRENE GROUP		
Ultrastyragel 100A° – 10^6A°	$50 - 10^7$	for polymers
Bio-Beads SX1 and SX2	$400 - 14 \times 10^4$	for removal of pesticides and PCB residues

[Sephadex, Sepharose and Sephacryl gels are supplied by Pharmacia-Inc. Bio-Gel gels are supplied by Bio-Rad-Ultrastyragel gels are supplied by Waters]

water-soluble polymers and characterization of proteins and enzymes. Semirigid gels based on polystyrene have high efficiencies and wide pore ranges. They are most useful for fractionation of synthetic polymers. They are compatible with most organic solvents. Water cannot be used because it does not wet the surface. The rigid gels have certain advantages like wide choice of aqueous and organic solvents, and adaptability for high flow-rate. The major disadvantages are: instability to alkaline solution with pH > 7.5 and adsorption effects.

Applications

The most important application of gel chromatography is the separation and characterization of molecules of different molecular weights. Very often it is possible to separate molecules of similar molecular weights by a proper selection of the appropriate gel and column length. Another important application is the separation of large molecules of biological origin from inorganic and ionizable species. This is known as *desalting*. For example, using a column of Sephadex gel, haemoglobin can be separated from sodium chloride. Fractionation of mixtures of biopolymers is the most widespread application of gel chromatography. The method can also be used for preparative purposes.

10.5 Affinity Chromatography

Affinity chromatography is based on unique interactions between biomolecules. It involves the use of a bioselective stationary phase placed in contact with the material to be processed. Because of its rather selective interaction, sometimes called *lock and key mechanism*, the method is more selective than other liquid chromatographic systems. For example, enzymes bind coenzymes and inhibitors, antibodies bind haptens and protein A, and a single strand DNA binds its complement.

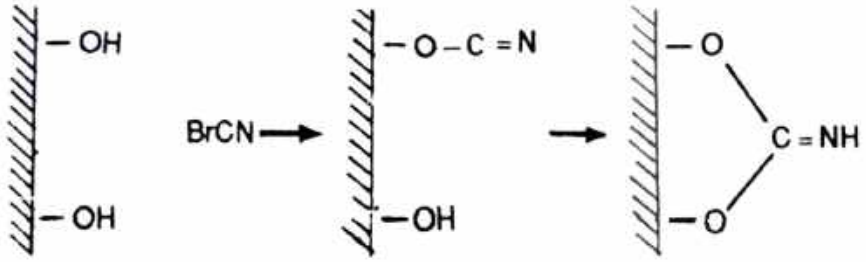
In affinity chromatography, a ligand is covalently bonded to a solid matrix which is packed into a chromatographic column. A mixture of components is then applied to the column. The unbound contaminants which have no affinity for the ligand are washed through the column, leaving the desired component (e.g., protein, peptide or a DNA fragment) bound to the matrix. Elution is accomplished by changing the pH, applying salt solution or organic solvents or by eluting with a molecule which competes for the bound ligand.

The correct choice of the coupling matrix is governed by the type of the groups available in the ligand molecule for coupling and by the nature of the binding reactions with the substance to be purified. The most commonly used matrix is agarose. Other materials are, cross-linked agarose, polyacrylamide gels and dextrans. The selection of the method of attachment of the affinity ligand depends upon which type of

affinity ligand can be used for linkage to the matrix without affecting its binding sites. Other considerations are:

1. The attachment should not introduce non-specific sorbing groups into the specific adsorbent, and
2. The linkage of the matrix and the reactive group should be stable during adsorption, desorption and regeneration.

One commonly used method of activation of the gel matrix involves the reaction with cyanogen bromide.



LABILE INTERMEDIATE IMIDOCARBONATE

A free amino group in a protein molecule will react under mild conditions with the imidocarbonate matrix.

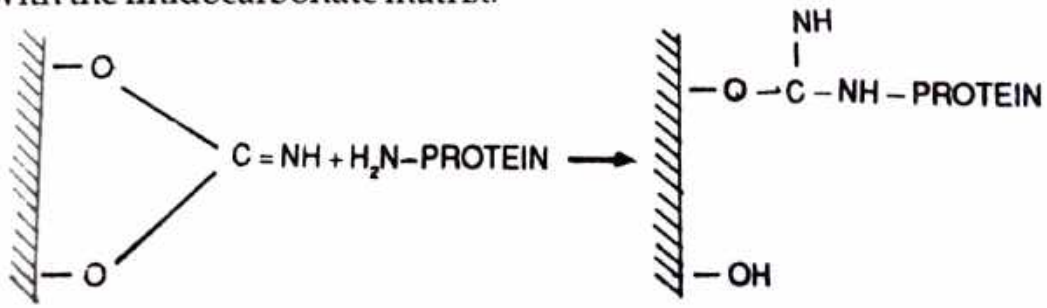


Fig. X. 5 outlines the separation of a biomolecule by affinity chromatography.

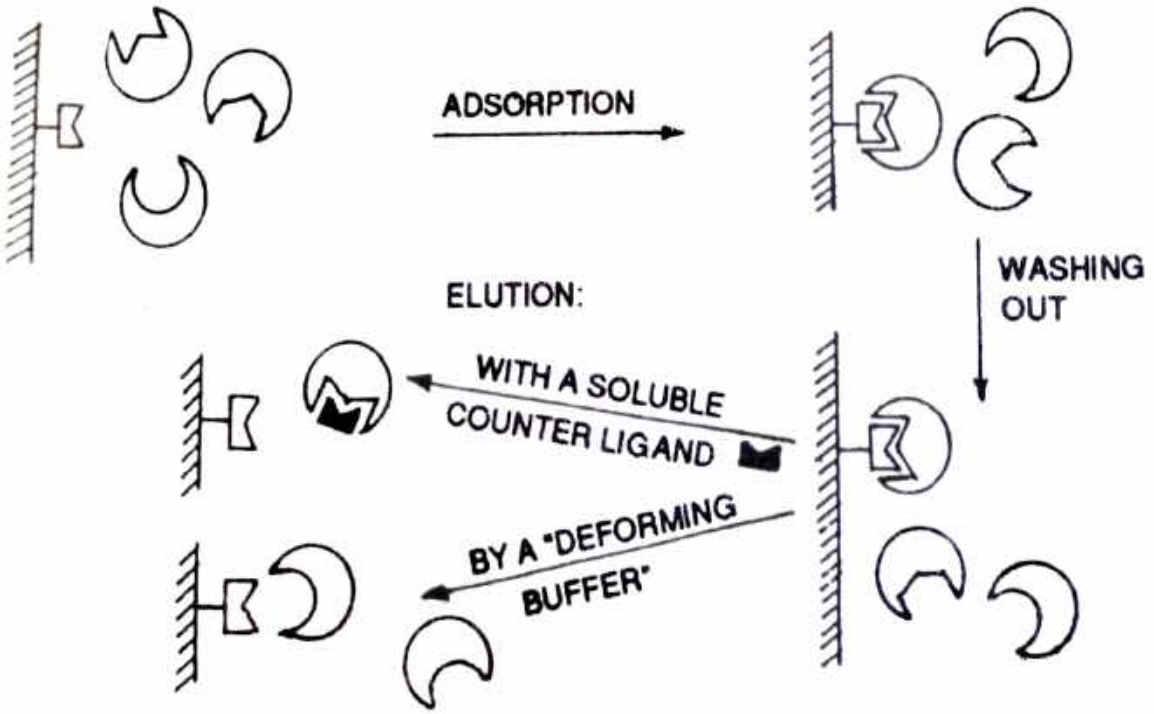


Fig. X.5 Bioaffinity chromatography separation (schematic)

It frequently happens that a ligand is too close to the matrix to interact freely with another biomolecule, invariably a macromolecule. To get over this steric problem, a *spacer arm* (e.g., hydrocarbon chains $-(CH_2)_n-$) is incorporated in the matrix to allow the ligand greater flexibility for interaction with the molecules in the samples (Fig. X.6).

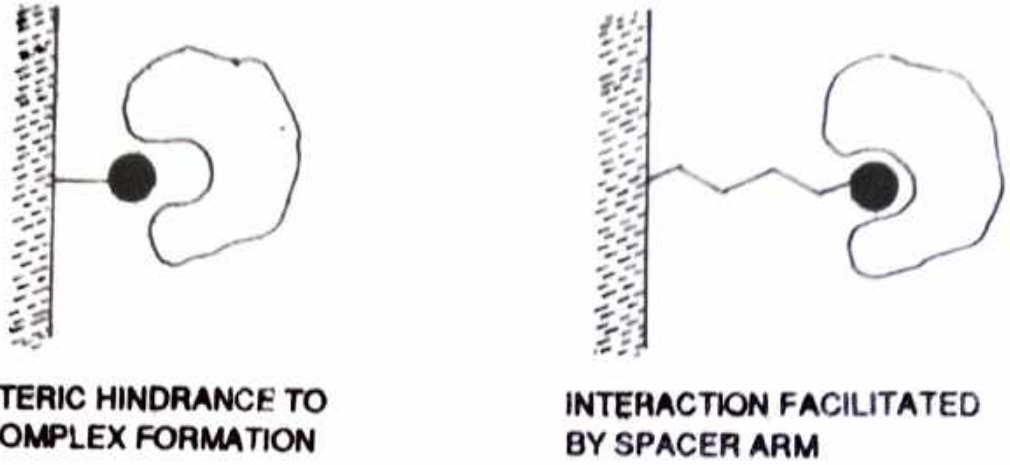


Fig. X.6 Function of spacer arm in bioaffinity chromatography separation

A wide range of affinity media are commercially available for specific requirements from Pharmacia (Sepharose CL) and Bio-Rad (Affi-Gel). Table X.6 contains a list of affinity chromatographic media. Suitable ligands are immobilized on the activated matrix for purification/binding of biomolecules.

Affinity chromatography finds extensive use in biosciences involving separation of antigens, enzymes, proteins, viruses and hormones. An outstanding application is the isolation of genes.

A variant of affinity chromatography is *Dye-Ligand Affinity Chromatography*, in which certain synthetic dyes with triazine structure are used as immobilized ligands. These dyes have affinity for a wide variety of enzymes and other proteins. Using this method, nearly 200 of them have been separated in a pure form.

10.6 Metal Chelate Affinity Chromatography

Metal Chelate Affinity Chromatography (MCAC), is also called *Immobilized Metal Ion Adsorption Chromatography* (IMAC). In this method, the gel is first charged with metal ions such as Cu^{2+} or Zn^{2+}, to form a chelate (Fig. X.7). Proteins and other biomolecules, depending on the presence of surface groups (e.g., histidine) which have affinity for the chelated metal ions, will bind to the gel. The binding strength is mainly affected by the matrix of the metal ion and pH of the buffers. The adsorbed proteins are eluted either by competitive elution, lowering of pH or by the use of chelating agents that have greater affinity for the metal ion. When a stronger chelating agent is used as the eluant, the technique is called *Ligand Exchange Affinity Chromatography*.

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