Chromatography

The "stuff" you do before you analyze a "complex" sample

Separation is needed before analysis

• Homogenous appearance, biological samples are complex mixture of different substances.
Example: (a) green plants mixture of different pigments. (b) Black ink is a mixture of different colored materials.

• Due to complexity of mixture & presence of interfering substances, it is difficult to analyze the target / desired components, even after using modern tools of biology.

• Thus removal of interference & separation of desired components is necessary.

• Some of these materials can be separated by the methods given in following table.

Methods of Separation

Physical & Mechanical: Hand picking, blowing, sieving, freezing & thawing, Using machine for the purpose.

Masking: Immobilization of interference as non-reactive complex

Mechanical Phase Separation:

(a) Precipitation & filtration: Difference in solubility of compounds formed.

(b) Distillation: Difference in volatility.

(c) Extraction: Difference in solubility in 2 immiscible solvents.

(d) Ion exchange: Difference in stability of reactants with ion-exchange resins.

Chromatography: Difference in rate of movement of solute through a stationary phase.

Electrophoresis: Difference in migration rate in an electrical field gradient.

What is chromatography?

Chromatography is a method of separation of organic & inorganic compounds so that they can be analyzed and studied.
Analysis of a compound enables to understand its role in life.
Chromatography means "color writing" .
The technique was invented by a Russian botanist in 1903 - M.S. Tswett.
He separated out pigments from green leaves using a glass column.
Chromatography is such an important technique: 2 Nobel prizes awarded.
Over 60% of chemical analysis is done with chromatography or its variation.
It is based on differential migration: solutes migrate & resolve on a stationary phase through a mobile phase. Solutes with a greater affinity to mobile phase will move faster than the solutes that have more affinity to the stationary phase.
As the solutes move through the stationary phase they separate.
This is called chromatographic development.

What is Chromatography?

Chromatography is the ability to **separate** molecules using **partitioning** characteristics of molecule to remain in a stationary phase versus a mobile phase. Once a molecule is separated from the mixture, it can be isolated and quantified.

Can chromatography identify components?

Not without the detector – chromatography is the process of separation!

Why is chromatography called chromatography?

First application by M. S. Tswett 1903

For the separation of plant pigments. Since the components had different colors the Greek *chromatos*, for *color*, was used to describe the process.

So, the detector was not needed?

IT WAS!!! YOU ALWAYS NEED A DETECTOR TO IDENTIFY chromatographically separated COMPONENTS.

In this case, the detector is an eye, Similarly, a nose can be used for a chromatography of fragrances.

Chromatography : Different Terms, Components & Phases

- Stationary phase: Or Chromatography surface on which target substances get separated or resolved.
- Mobile phase: Or solvent or carrier which flows over the stationary phase & carries target substances along with. Sometimes it is known as developer.
- Chromatograph: Or chromatogram is the system of result for observation & analysis when work is done
- Resolution: Differential separation of components.
- Detection: Methods that enable to observe or detect the separated components
- Types: Different types on the basis of stationary phase formate
- Basically 2 main types: (a) Planer – Paper, TLC (b) Column
- Mechanism (Principle): (a) Adsorption (b) Partition (c) Ion exchange (d) Exclusion (e) Affinity
- Mobile phase): (a) Liquid (2) Gas (3) GLC (4) HPLC
- Direction of flow: (a) Ascending (b) Descending
- Dimensions: (a) Single d (b) 2 – D, (c) Radial

The general principle.

- Use – to separate and identify components of mixtures.
- Several different types - paper, thin layer, gas-liquid.
- All use the principle of "partition"- affinity between two phases, to separate mixtures of substances.
- Stationary phase & mobile phase.
- Compounds with greatest affinity for mobile phase travel further.

Chromatography basics

- Mobile and Stationary phase
- Retention - Migration
- Bands or zones
- Equilibrium!

- Column vs. planar
- Liquid vs. gas vs. SF
- High vs. low resolution
- Partition
- Adsorption
- Ion exchange
- Size exclusion

All chromatography needs:

- support material – stationary phase
- solvent (or **carrier gas**) – mobile phase.

Chromatography Type	Stationary phase	Mobile phase
Paper	Paper	Organic or aqueous solvent.
Thin layer	Silica gel supported on plastic film	Organic or aqueous solvent.
G.L.C.	High boiling point liquid on inert solid support.	Inert gas e.g. nitrogen.

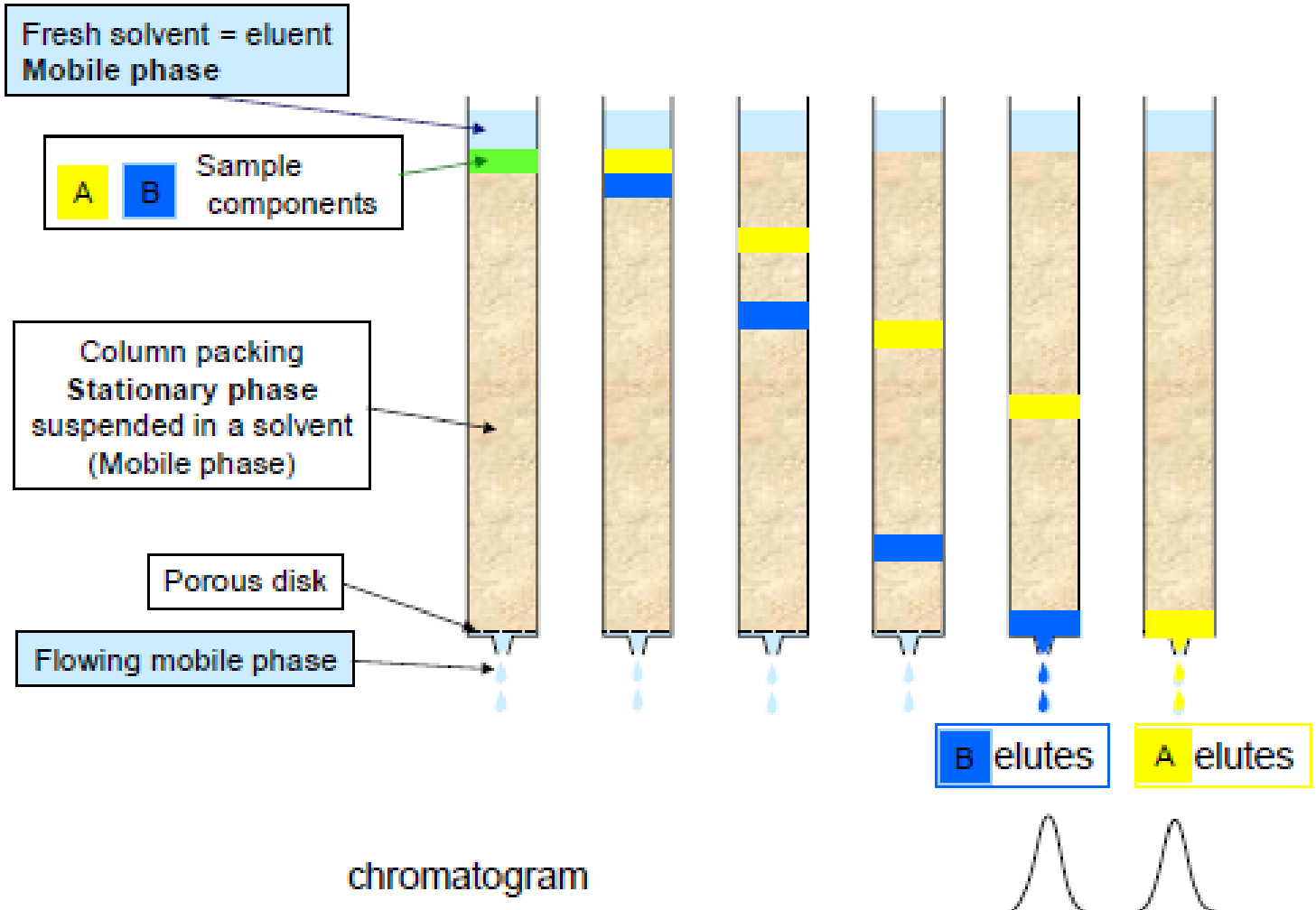
Chromatography

A. Column Chromatograpgy
B. Planar Chromatograpgy – Paper chromatography / TLC

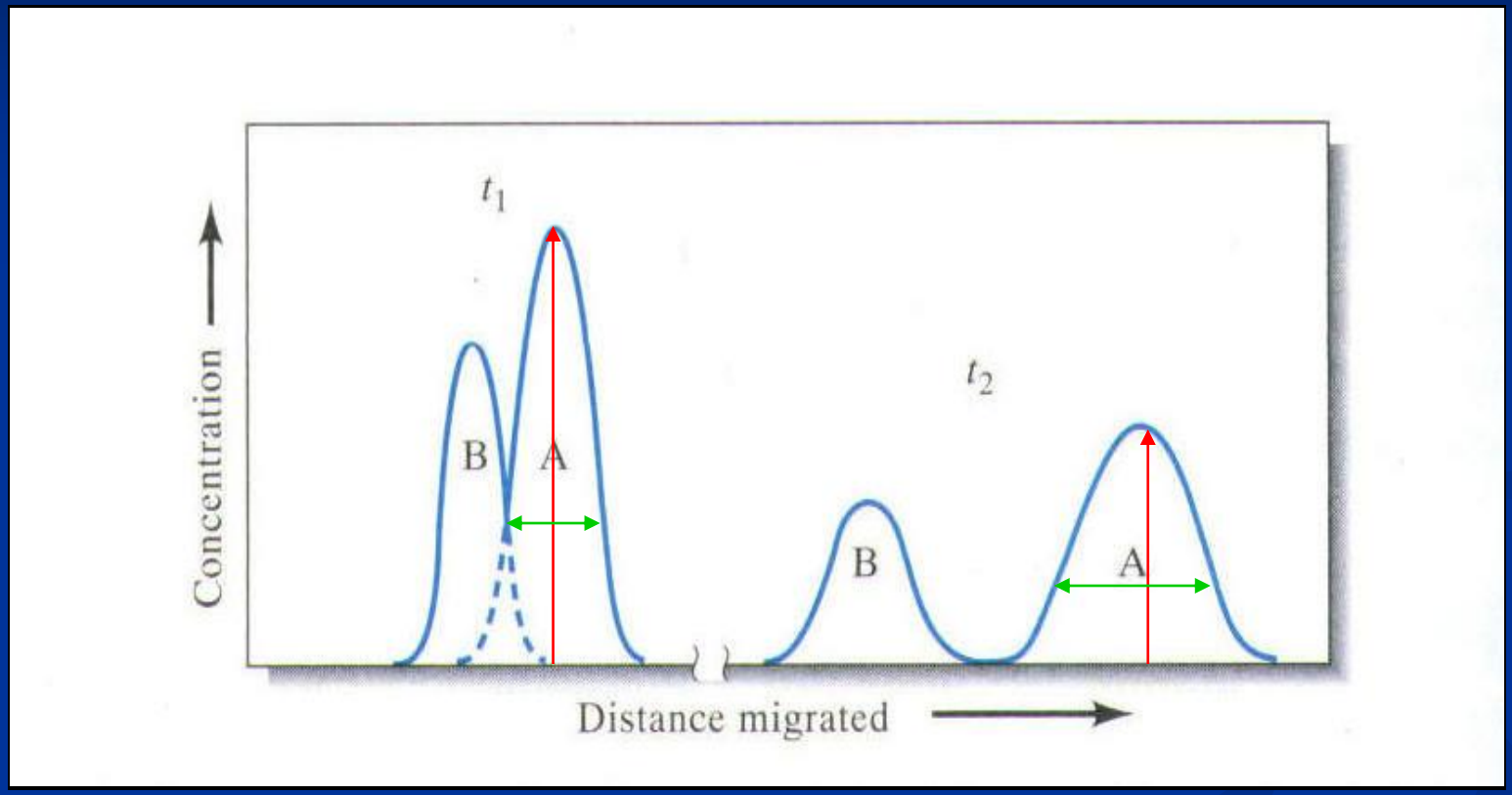
General Classification	Specific Method	Stationary Phase	Type of Equilibrium
Liquid chromatography (LC) (mobile phase: liquid)	Liquid-liquid, or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Liquid-solid, or adsorption	Solid	Adsorption
	Ion exchange Size exclusion	Ion-exchange resin Liquid in interstices of a polymeric solid	Ion exchange Partition/sieving
Gas chromatography (GC) (mobile phase: gas)	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Column Chromatography

Chromatographic Separation



Chromatography: Peak separations

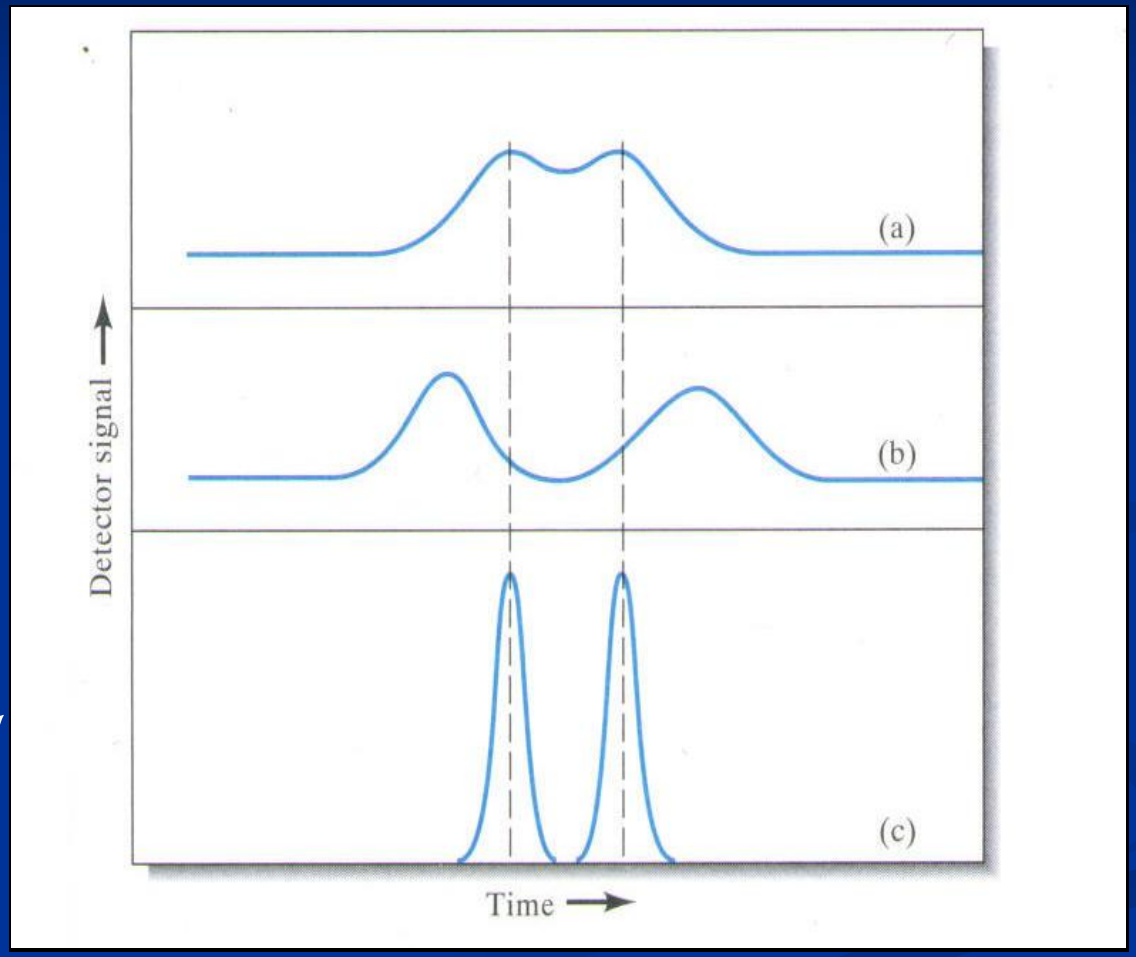


Chromatography: Peak Resolution

Poor resolution

More separation

Less band spread



Quantitative Analysis

- Peak areas
- Peak height
- Calibration and standards
- Internal Standard method

Summary

- Relate to column chromatography
- Retention times
- Velocities of mobile and component
- Height equivalent of theoretical plates
- Peak or zone broadening
- Resolution

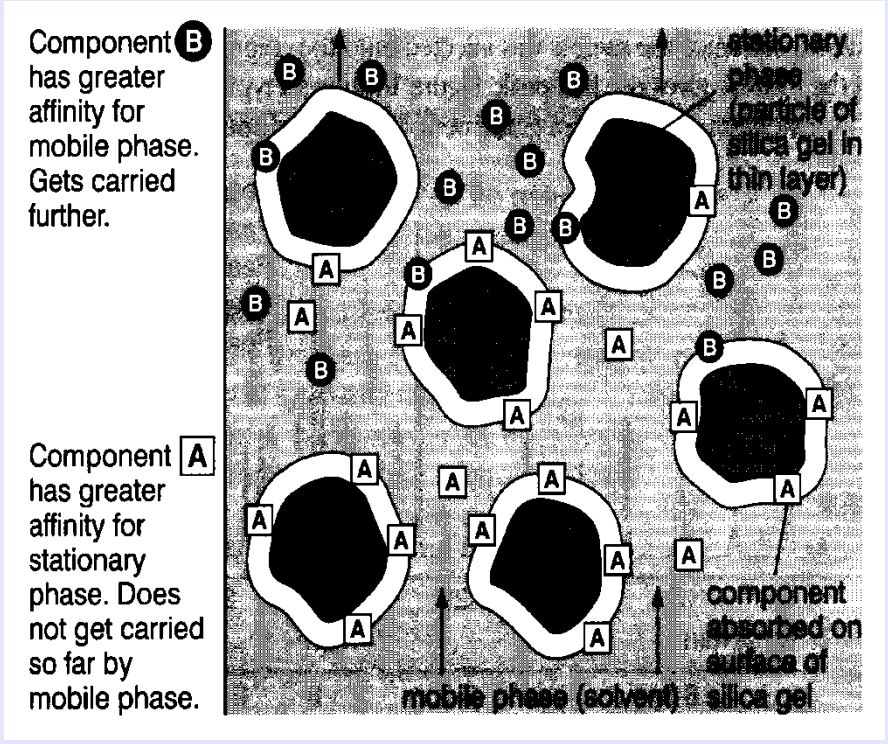
What happens in practice.

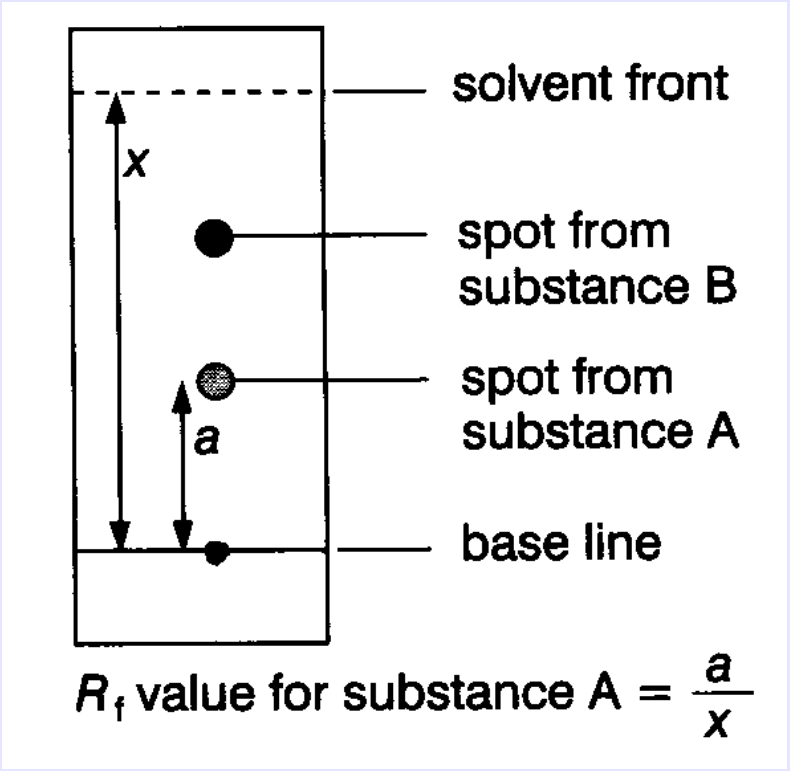
- Compounds that have **high affinity for mobile phase emerge first, (most volatile).**
- Chromatogram charts recorder response against time.
- Each component - separate peak.
- **Retention time** - characteristic of the compound under given conditions.

Factors affecting retention time:

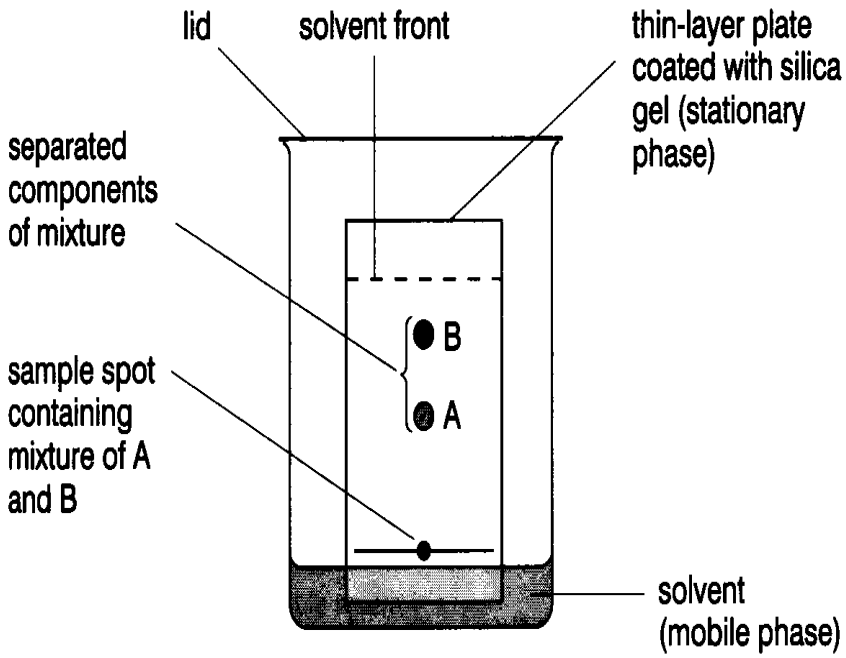
- *length of column*
- ***packing material***
- ***type of carrier gas***
- *flow rate of carrier gas*
- ***temperature of column.***

Separation and identification.





Thin Layer Chromatography - TLC

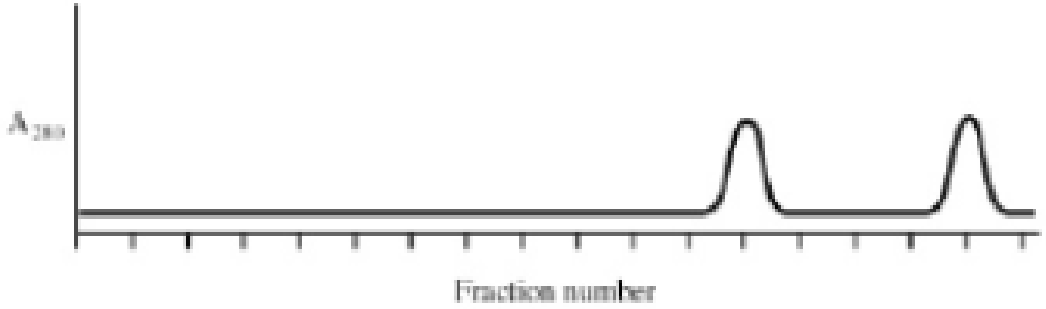


- Series of spots forms
- Compare samples in mixture with known substances.
- Measure R_f values.
- Coloured compounds & colourless compounds.

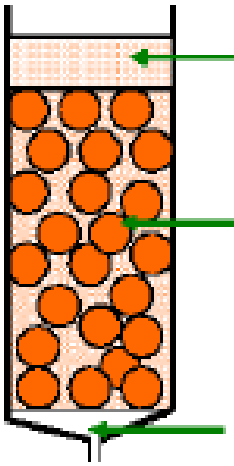
Gel-filtration chromatography: proteins passed over a column filled with a hydrated porous beads made of a carbohydrate or polyacrylamide polymer [large molecules exit (elute) first]

Ion-exchange chromatography: separation of proteins over a column filled with charged polymer beads (bead +charge = anion-exchange; bead -charge = cation exchange). Positively charged proteins bind to beads of negative charge & vice versa. Bound proteins are eluted with salt. Least charged proteins will elute first.

Affinity chromatography: proteins are passed through a column of beads containing a covalently bound high affinity group for the protein of interest. Bound protein is eluted by free high affinity group.



Size Exclusion (gel filtration)



Wait for gel to settle before equilibrating with elution buffer. Make sure gel does not run dry.

Step 2

Prepare gel slurry and pour it gently into column avoid introducing air bubbles

Step 1

Fill the column with elution buffer and ensure that air spaces are eliminated from the column

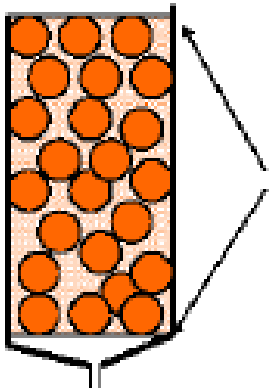
Sephadex G-50 1-30 kD
Sephadex G-100 4-150 kD
Sephadex G-200 5-600 kD
Bio-Gel P-10 1.5-20 kD
Bio-Gel P-30 2.4-40 kD
Bio-Gel P-100 5-100 kD
Bio-Gel P-300 60-400 kD

Sephadex is a trademark of Pharmacia.
Bio-Gel is a trademark of Bio-Rad.

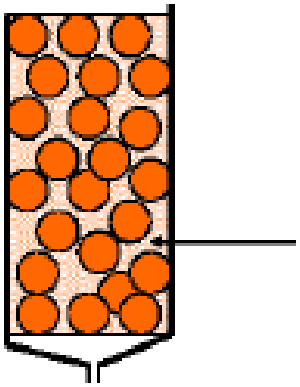
Biochemists refer to a protein's size in terms of its molecular weight, in kDa (a kilodalton, kD or kDa, is 1000 times the molecular mass of hydrogen)
Each amino acid residue counts for about 110 daltons, that is, about 0.11 kDa.

Size Exclusion (gel filtration)

Vt or total column volume
Refers to total volume occupied by the gel in the column, and not the size of the column



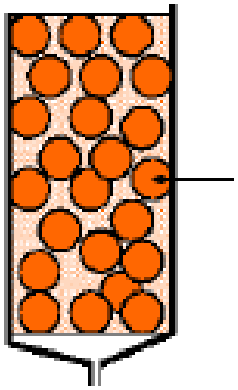
Vt = Total column volume



Vo or void volume

Void volume = space outside the granules

Rule: Vo = 1/3 column volume



Vt – Vo = volume occupied by gel space, including gel matrix (Vgel)

Stationary phase (Vs) = Vt-Vo-Vgel
This is difficult to measure

Vs also labeled as Vi

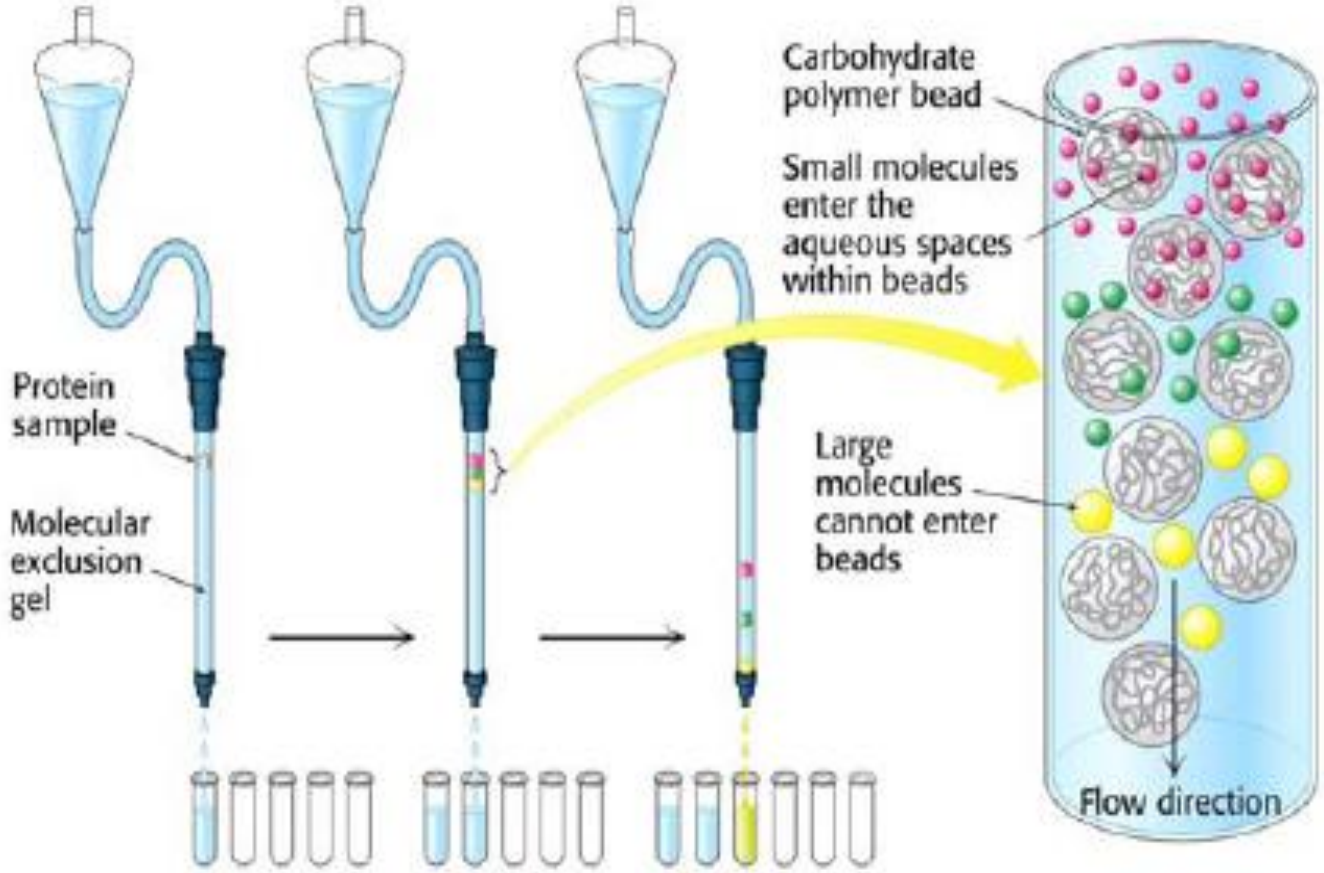
$$K_d = \frac{V_e - V_o}{V_s}$$

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Ve = elution volume of solute
Vo = void volume of column
Vs = volume of stationary phase (= Vi)
Vi = Vt-Vo-Vgel matrix.

For convenience, expression Kav is used

Gel-Filtration Chromatography – Separation based on size



Ion Exchange Chromatography

Two common examples of ion exchangers are:

Anion exchanger:

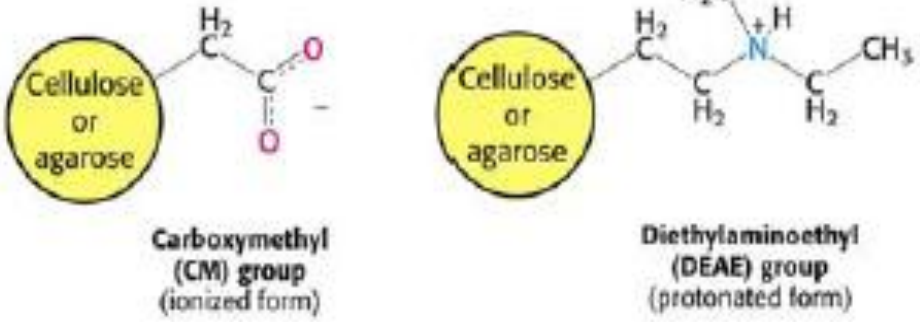
Inert Matrix—CH_2—CH_2—$NH(CH_2CH_3)^{2+}$ diethylaminoethyl (DEAE) group

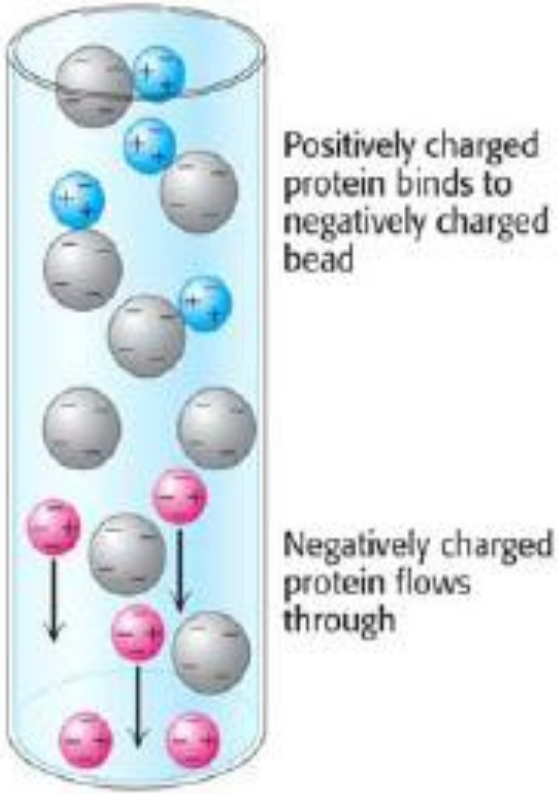
Cation exchanger:

Inert Matrix —CH_2—COO^- carboxymethyl (CM) group

The inert (uncharged) matrix is

most commonly cellulose or agarose





❖ The surface of a protein has both positive and negative charges, and therefore can bind to both cation and anion exchangers.

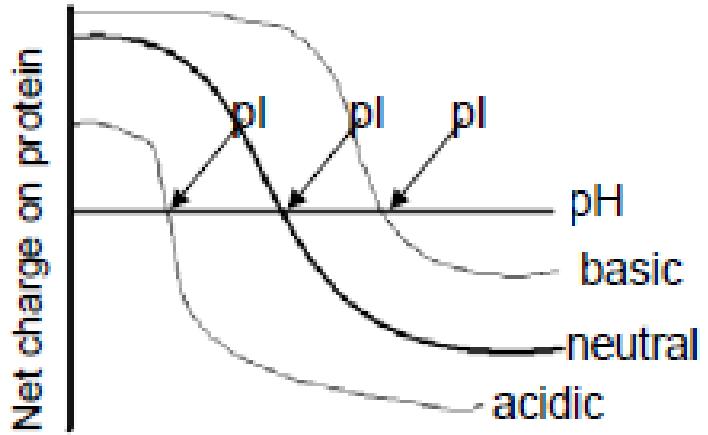
❖ The binding affinity of a protein depends on:

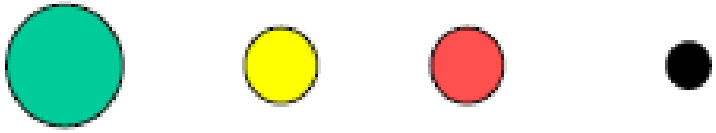
a. the concentration of salt ions in the mobile phase that compete with the protein for binding to the ion exchanger.

b. the pH of the mobile phase, which influences the ionization (and therefore the charge) properties of the protein.

❖ A protein can be eluted from the matrix by applying a buffer at higher salt concentration (or different pH) that reduces the protein's affinity for the matrix.

pH vs pI





60 Kd
pI 4.2

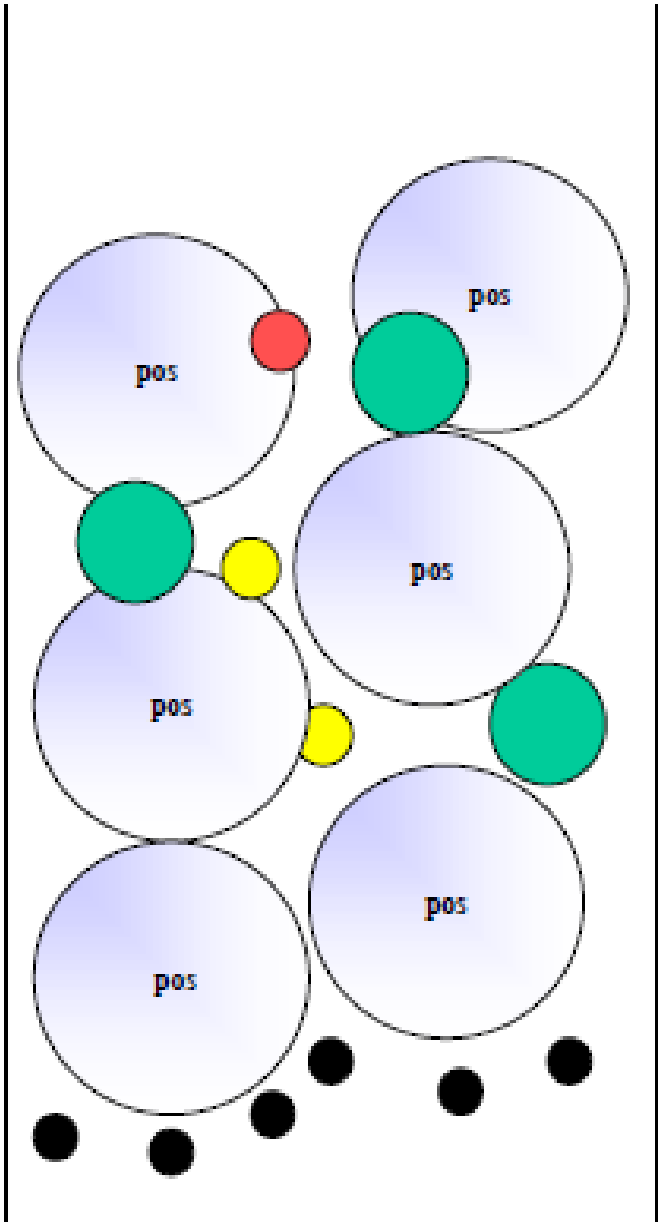
20 Kd
pI 5.4

20 Kd
pI 6.0

5 Kd
pI 8.5

- Ion-exchange column chromatography separates proteins on the basis of charge.

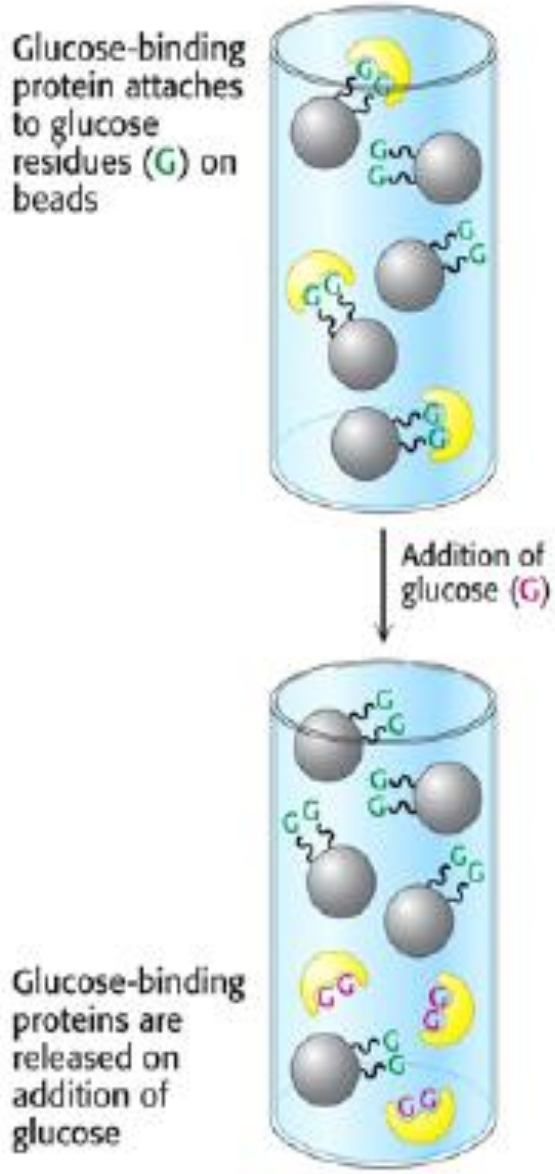
- pH 7.2
- positively charged column



Affinity Chromatography

Small molecules are attached to beads and complex protein mixtures are applied.

Bound proteins can be eluted with the small molecule or with denaturing reagents (urea, guanidine, etc.)



Hydrophobic Interaction Chromatography

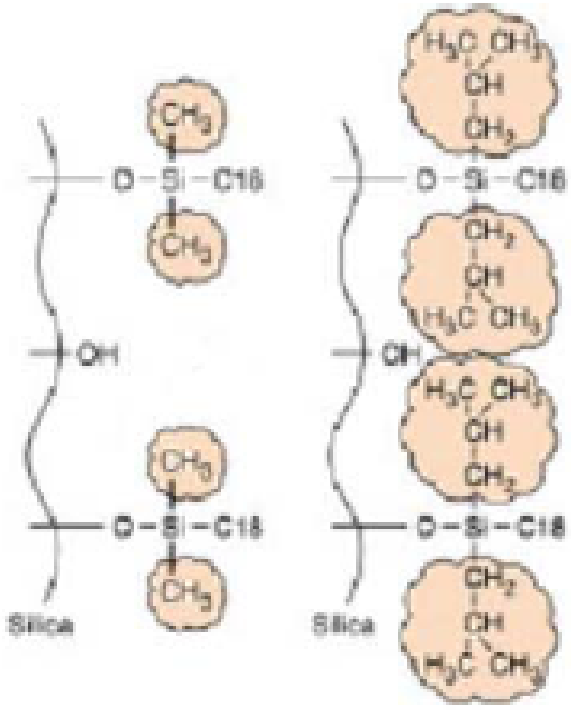
Stationary phase: Non-polar (octyl or phenyl) groups attached to an inert matrix

Exposed hydrophobic regions on proteins will bind to similar groups on the resin

Possible elution strategies:

1. Decreasing salt concentration (since higher salt augments hydrophobic interactions)

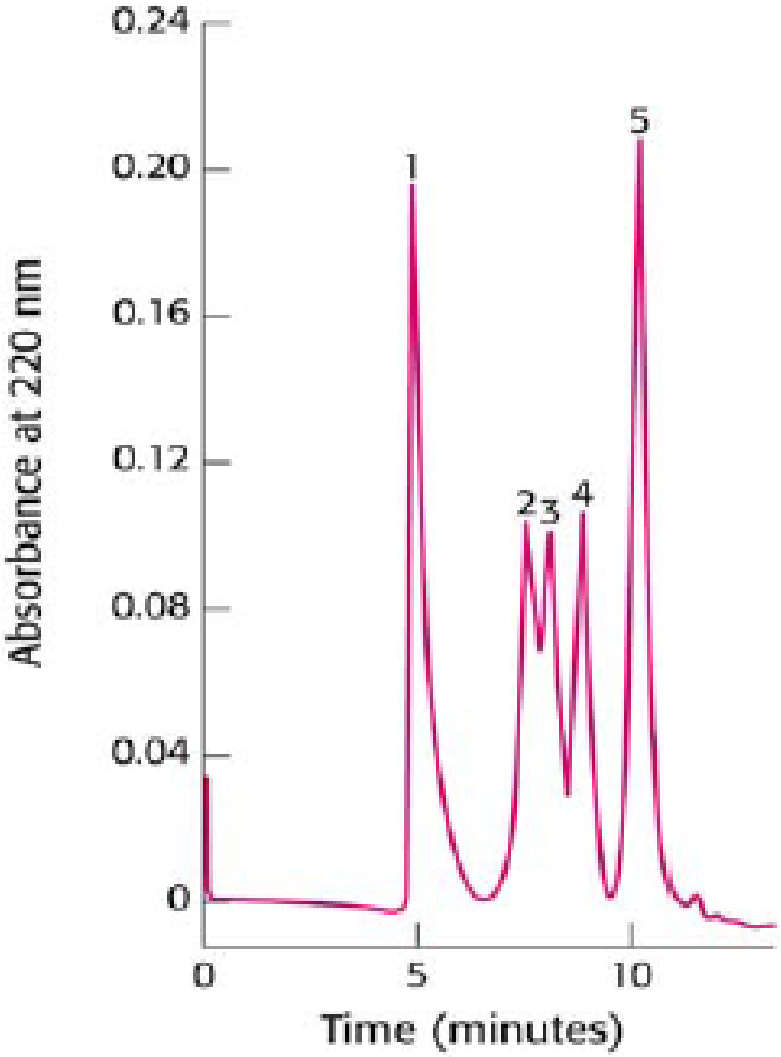
2. Increasing concentrations organic solvents



High Pressure Liquid Chromatography (HPLC)

- **sample is vaporized and injected;**
- **moves through a column containing stationary phase under high pressure;**
- **separates mixture into compounds according to their affinity for the stationary phase**

High Pressure Liquid Chromatography



High pressure limits diffusion and increases interactions with chromatography media

HPLC gives very high resolution of protein components

- HPLC Columns:
  - Stainless steel
  - 10-30 cm long
  - 4-10 mm internal diameter
  - 1-10 mm particle size - 40,000-60,000 plates/m