

Scientific Practise

MCBG 2036

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Consultation Hours: Thursday- Friday: 13H30- 14H00

LEARNING OUTCOMES

Learners should be able to :

1. Define chromatography.
2. Explain classification of chromatography technique.
3. Explain type of phase in chromatography.
4. Describe principle, method & applications of chromatography.
5. Understanding related calculations.
6. Detection methods used in chromatography.

Chromatography

(from Greek :*chromatos* -- color ,
"graphein" -- to write)

History

Mikhail Tswett, Russian, 1872-1919

Botanist

In 1906 Tswett used the chromatography to separate plant pigments

He called the new technique chromatography because the result of the analysis was 'written in color' along the length of the adsorbent column



DEFINITION

CHROMATOGRAPHY

- ☞ The separation of a mixture by distribution of its components between a mobile and stationary phase over time
 - mobile phase = solvent
 - stationary phase = column packing material

Which means ...

Chromatography is the **physical separation** of a mixture into its **individual components**.

Mixtures & Compounds

Mixture – Two or more substances that are mixed together, but **not chemically combined**.

Examples of mixtures ...

Air – mixture of gases

Bowl of cereal – mixture of cereal and milk

Soda pop – mixture of soda syrup, water, and CO₂ gas

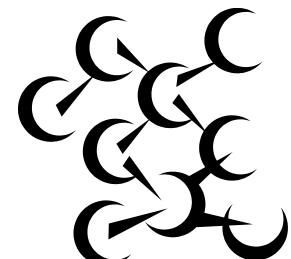
Fog –water suspended in air

Compounds – Two or more elements that are **chemically combined**.

Examples of compounds ...

Salt – Sodium and chlorine combined chemically

Water – Hydrogen and oxygen combined chemically



Solutions

Solutions are mixtures in which one substance is dissolved in another. **Solutions** have two parts: **solute and solvent**

The solute is the substance that **is dissolved**.

The solvent is the substance that **does the dissolving**.

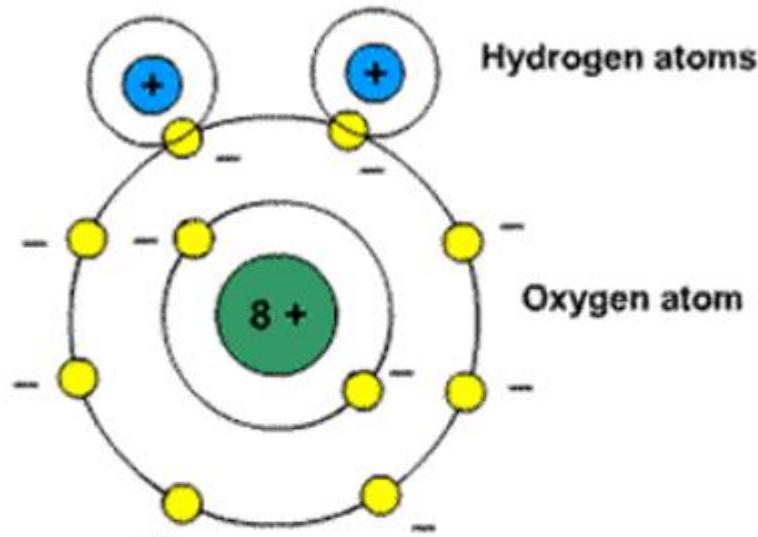
Solubility - A measure of how much of a given substance will dissolve in a liquid.

A substance that does not dissolve in water is called **insoluble**.

A substance that does dissolve in water is called **soluble**.

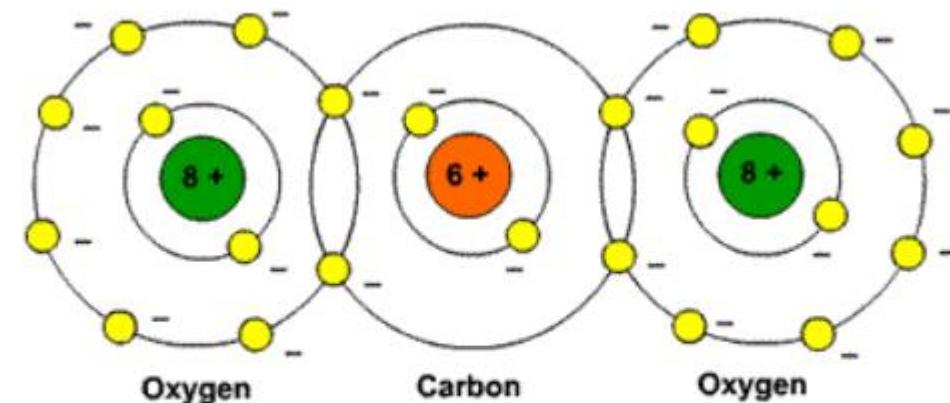
Polar vs Non-polar molecules

More positive charges



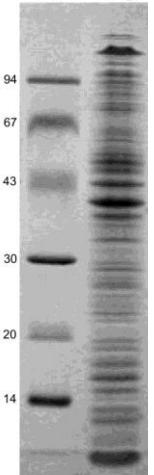
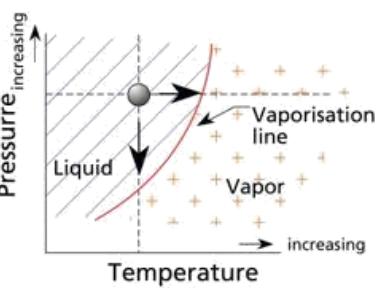
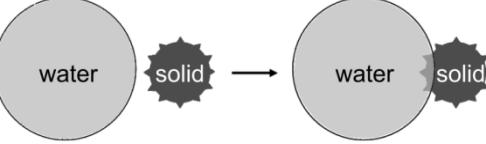
More negative charges

Water is a polar molecule with positive charges on one side and negative on the other

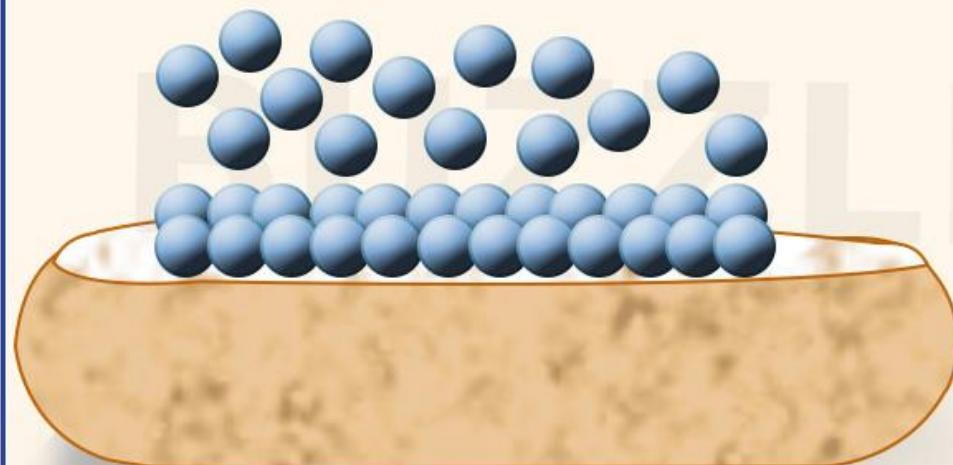


What is Chromatography?

- Separation of individual **components** of a **mixture** on basis of **differences** in **physical characteristics**

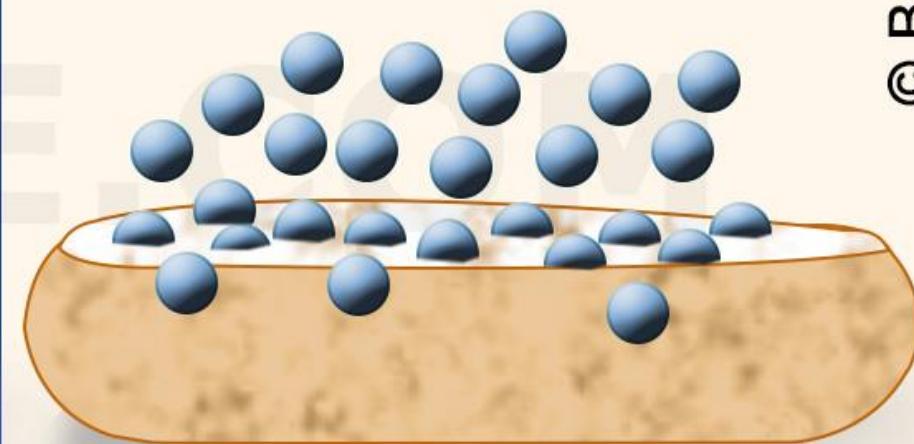
Molecular size	Shape	Charge	Volatility	Solubility	Adsorptivity
	 Linear				
	 Trigonal planar				
	 T-shaped				
					
				<p>Difference between Absorption and adsorption https://www.youtube.com/watch?v=djlzXvwIz5U</p>	
	 Trigonal bipyramidal				

ADSORPTION



Molecules adhere to the surface of the phase.

ABSORPTION



Molecules are drawn into the bulk of the phase.

Essential Components

- Stationary phase
- Chromatographic bed

Solid, gel or immobilised liquid



- Mobile phase
- Delivery system
- Detection system

Liquid or gas

Absorbance, fluorescence

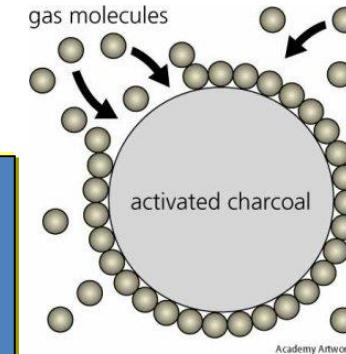
Separation based
on interaction
with stationary
phase



Chromatography procedure

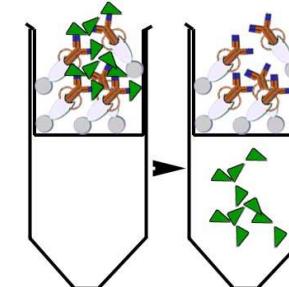
1) Adsorption

Select matrix with biophysical properties that result in adsorption of molecule of interest under certain conditions



2) Elution of non-adsorbed particles

Continuously apply buffer which promotes adsorption of molecule of interest and elution of unwanted/contaminating proteins



3) Selective elution

Gradient of changing conditions e.g. pH, [salt] or [ligand]

Purpose of Chromatography

- **Analytical** - determine chemical composition of a sample

uses small sample

e.g. environmental lab to look for pollutants- is to separate compounds in order to identify them

- **Preparative** - purify and collect one or more components of a sample

uses large sample

e.g. pharmaceutical industry- to remove impurities from a commercial product

Uses for Chromatography

Real-life examples of uses for chromatography:

- Pharmaceutical Company – determine amount of each chemical found in new product
- Hospital – detect blood or alcohol levels in a patient's blood stream
- Law Enforcement – to compare a sample found at a crime scene to samples from suspects
- Environmental Agency – determine the level of pollutants in the water supply
- Manufacturing Plant – to purify a chemical needed to make a product

Classification of Methods

There are two classification schemes:

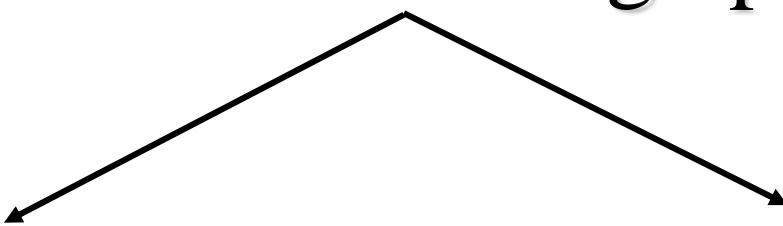
mobile phase

attractive forces

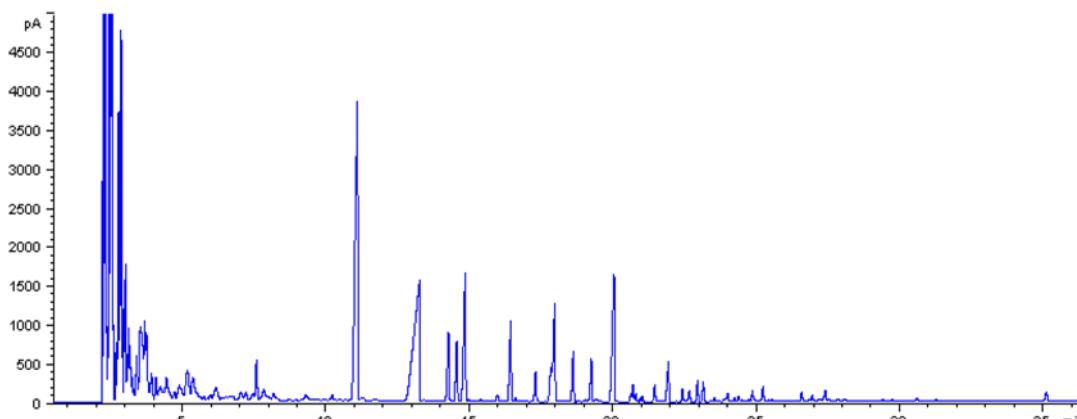
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graph TD; A[There are two classification schemes:]; A --> B[mobile phase]; A --> C[attractive forces]; B --> D["Gas (GC)  
Organic Solvent (LC)  
Water (LC)"]; C --> E["1. Adsorption  
2. Ion Exchange  
3. Partition  
4. Size exclusion  
5. Affinity"]
```

Classification based on Mobile Phase

Gas Chromatography

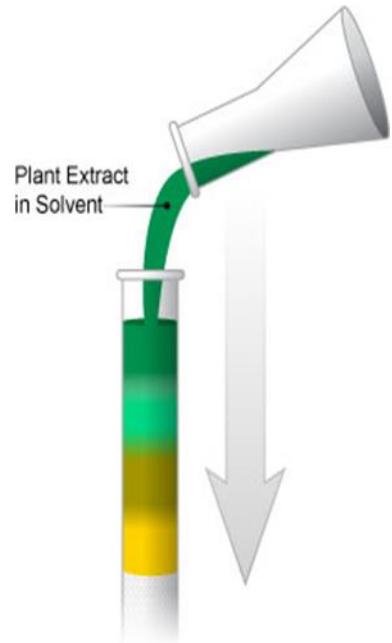


Gas - solid Gas - liquid



Sample MUST be volatile at
temperatures BELOW 250°C

Classification based on Mobile Phase

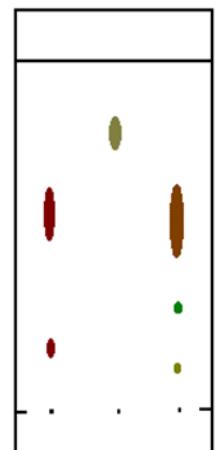


Liquid chromatography (LC)

Column
(gravity flow)

High performance
(pressure flow)

Thin layer
(adsorption)



Classification based on attractive forces

Techniques in chromatography are classified according to the method of solute separation

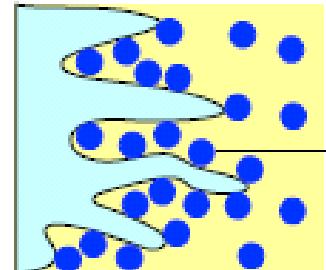
Adsorption chromatography

Partition chromatography

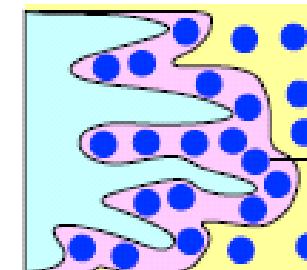
Affinity chromatography

Size-exclusion chromatography

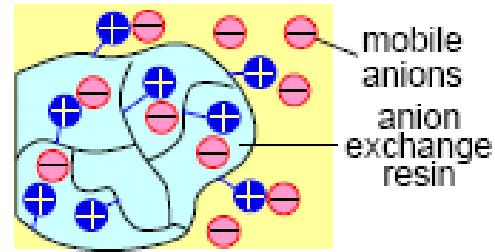
Ion-exchange chromatography



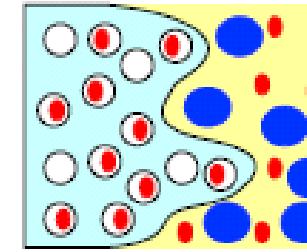
Adsorption Chromatography



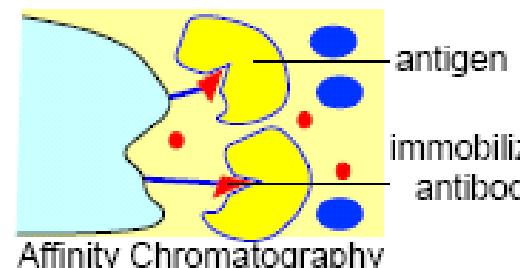
Partition Chromatography



Ion-Exchange Chromatography



Molecular Exclusion Chromatography
Gel Permeation Chromatography
Gel-Filtration Chromatography
Gel Chromatography



Affinity Chromatography

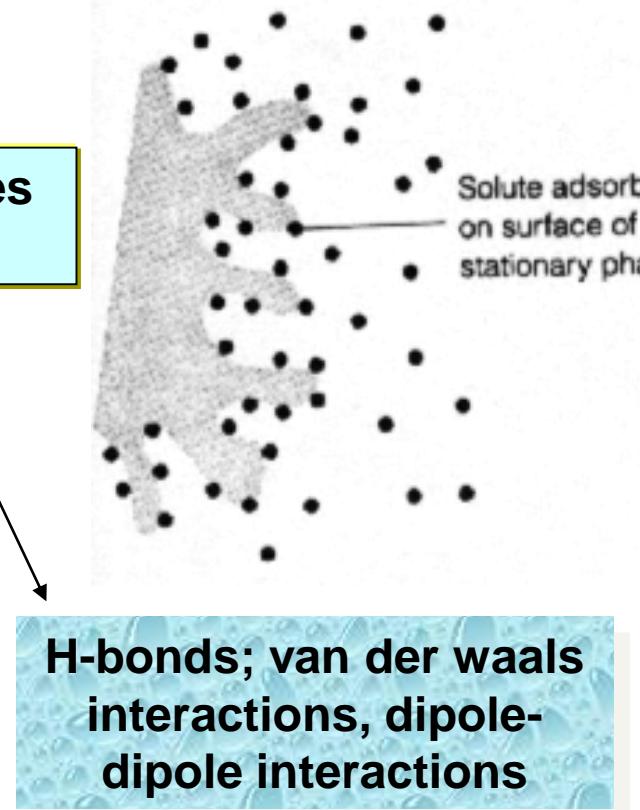
1. Adsorption Chromatography

Separates solutes based on their adsorption to solid particles.

- Mobile phase: Liquid or gas
- Stationary phase: Adsorbs molecules onto it's surface

Polystyrene-based: binds non-polar substances

Silica, aluminium-oxide or calcium phosphate: binds polar substances

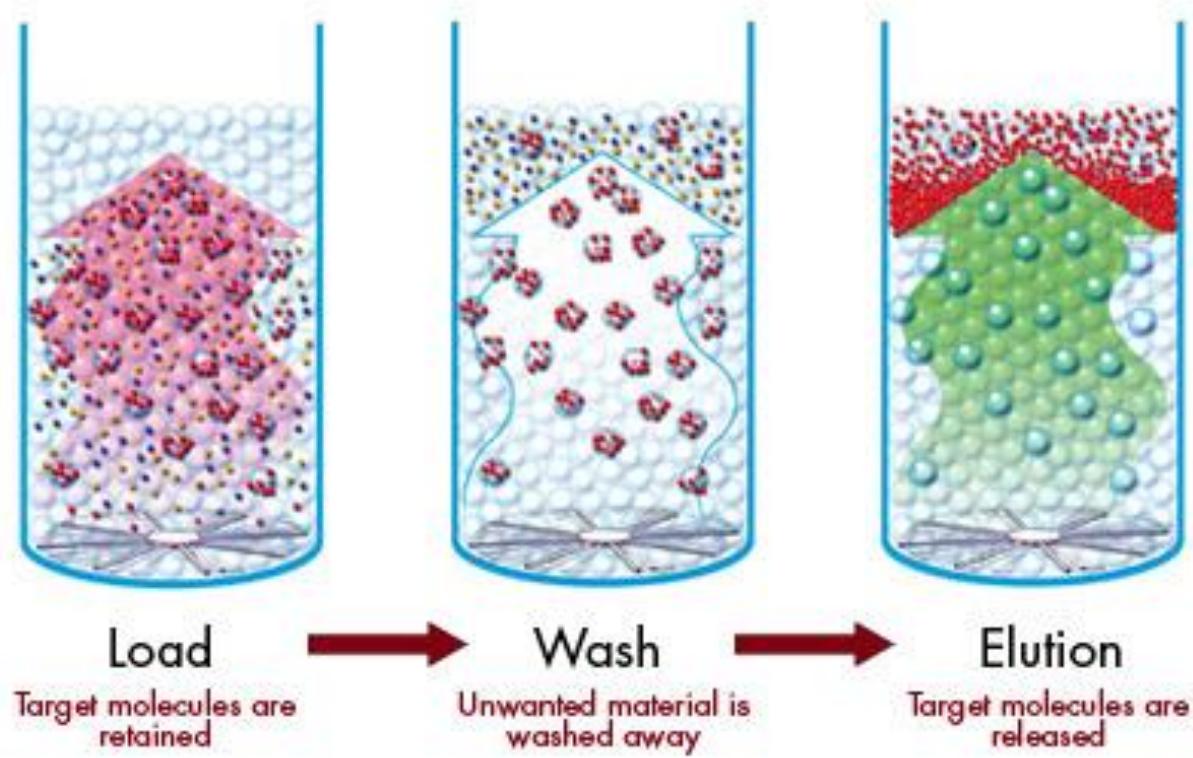


Polar molecules elute first

Non-polar molecules elute first

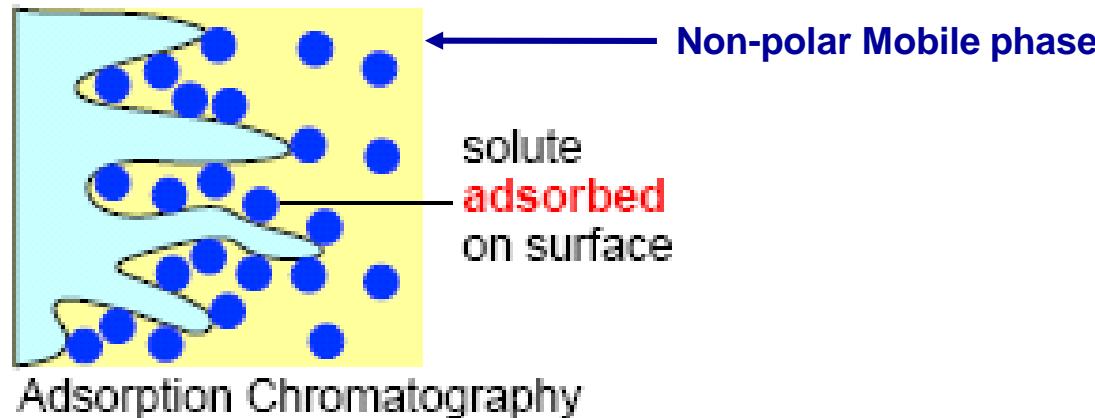
An example of a **dipole-dipole interaction** can be seen in hydrogen chloride (HCl): the positive end of a polar molecule will attract the negative end of the other molecule and influence its position. Polar molecules have a net attraction between them.

Adsorption Chromatography



Activate adsorbants by heating to 110 – 120 °C
Separates most materials

Adsorption Chromatography

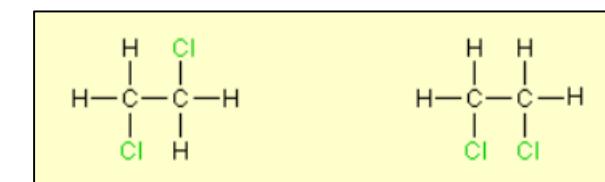


advantages:

- retain and separate some compounds that cannot be separated by other methods
- ② separation of geometrical isomers (cis-trans)

disadvantages:

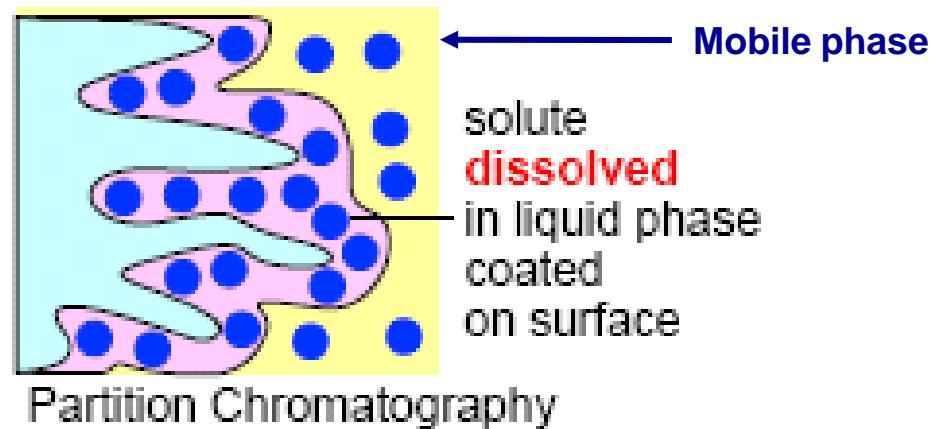
- very strong retention of some solutes
- may cause catalytic changes in solutes
- solid support may have a range of chemical and physical environments → non-symmetrical peaks and variable retention times



1,2-dichloroethene

2. Partition Chromatography

Separates solutes based on their partitioning between a **liquid mobile phase (Polar solvent (usually water) and a liquid stationary phase (Immiscible, non-polar organic solvent) coated on a solid support.**



Support Material – is usually silica, originally involved coating this support with some liquid stationary phase that was not readily soluble in the mobile phase

Separates peptides, proteins, oligosaccharides, vitamins

Two main types of partition chromatography based on the type of stationary phase:

- ② normal-phase liquid chromatography (Polar stationary phase)
- ② reversed-phase liquid chromatography (Non-polar stationary phase)

Comparison of RPLC & NPLC		
Type	Stationary phase	mobile phase
RPLC	Non-polar	Polar liquid
NPLC	polar	Non-polar liquid

3. Ion-Exchange Chromatography

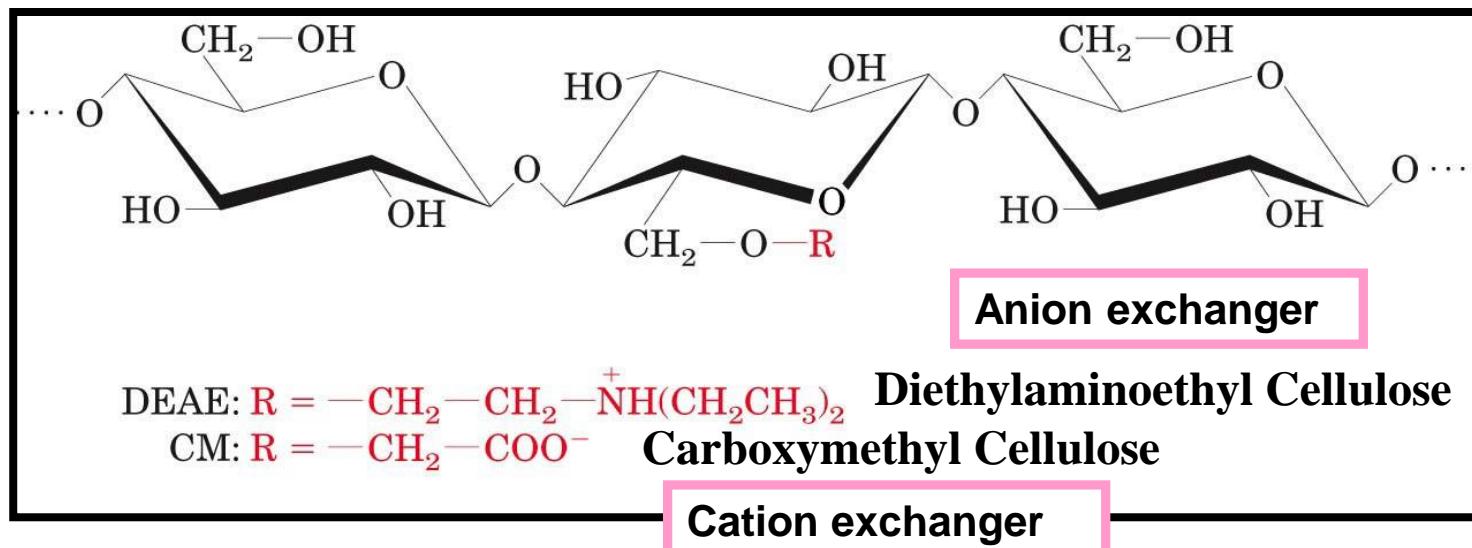
- ions separated on the basis of their tendency to displace counter ions adsorbed on stationary phase (Depends on charge, hydration, “solubility”...)

Stationary phase: **Either positively or negatively charged**



Common resins:

Anion exchanger



Ion-Exchange Chromatography (IEC)

Proteins bound to ion exchange resins are bound via non-covalent ionic (salt-bridge) interactions.

Proteins and IEC

Binding dependent on net charge

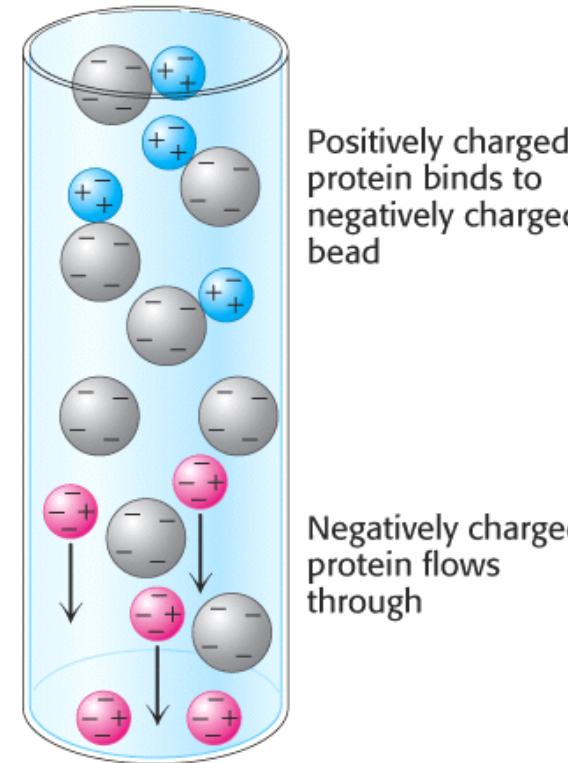
Affinity is based on charge

Select resin & buffer pH so that sample is tightly bound to column & all “contaminants”/unwanted material is eluted

Elution:

**Change pH or
Increase salt concentration
(stepwise or continuous gradient)**

Elution from weakly bound to strongly bound

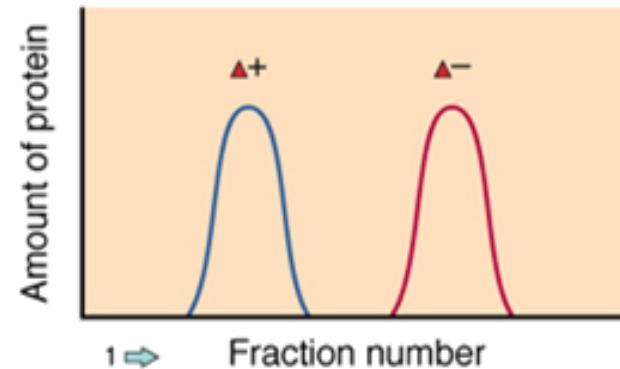
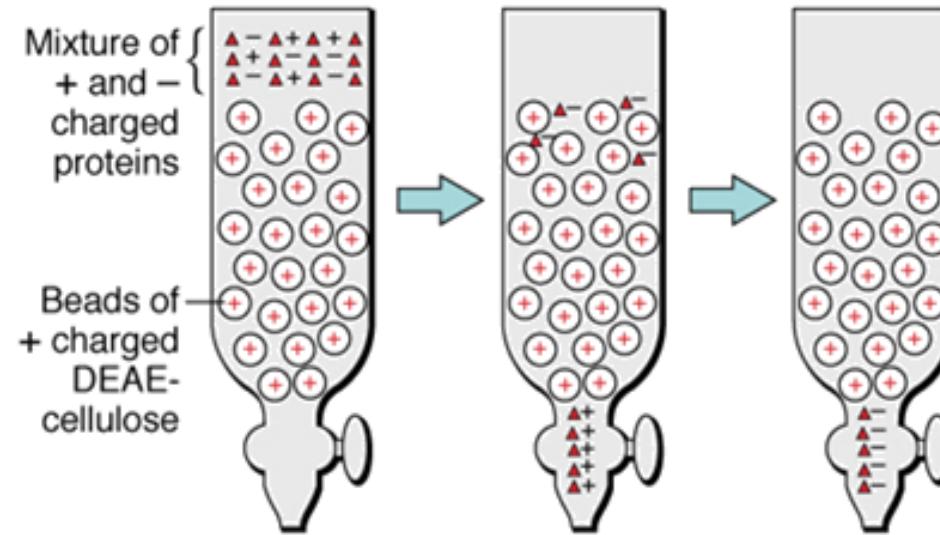


Ion-Exchange Chromatography

“short” and “fat” columns preferable

to maximise linear flow rate

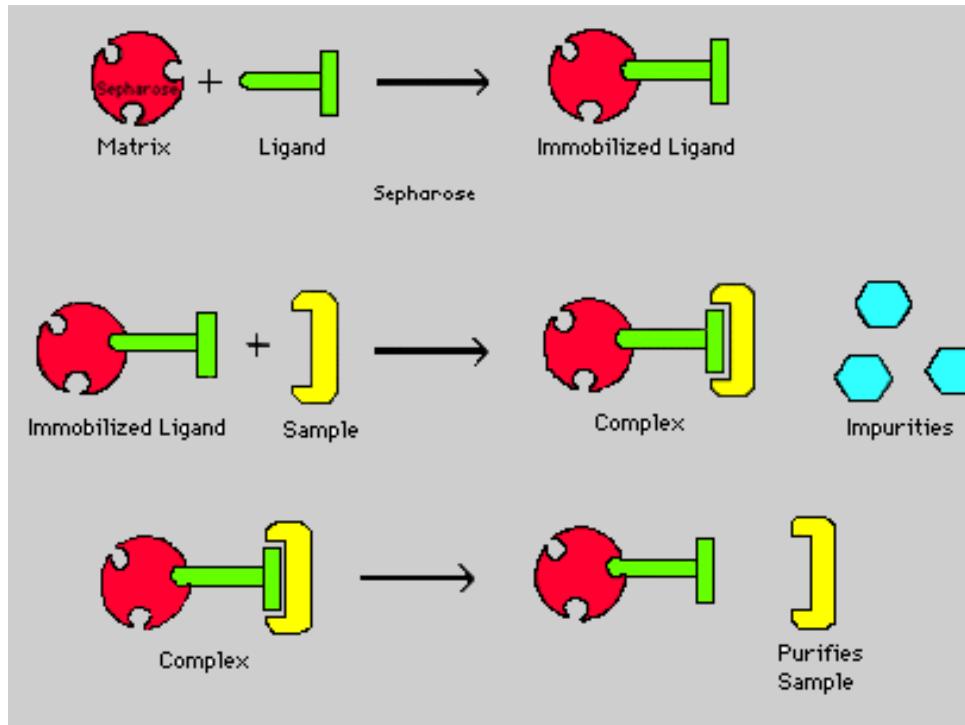
Separates large range of charged biomolecules



e.g. proteins and nucleotides

4. Affinity Chromatography (AC)

Separates based on the use of immobilized biological molecules (and related compounds) as the stationary phase



Based on the selective, reversible interactions that characterize most biological systems

- binding of an enzyme with its substrate or a hormone with its receptor
- immobilize one of a pair of interacting molecules onto a solid support
- immobilized molecule on column is referred to as the *affinity ligand*

Two Main Types of Affinity Ligands Used in AC:

High-specificity ligands – which bind to only one or a few very closely related molecules

Affinity Ligand	Retained molecules
Antibodies	Antigens
Antigens	Antibodies
Inhibitors/Substrates	Enzymes
Nucleic Acids	Complimentary Nucleic acids

General or group specific ligands – which bind to a family or class of related molecules

Affinity Ligand	Retained Compounds
Lectins	Glycoproteins, carbohydrates, membrane proteins
Triazine dyes	NADH- or NADPH Dependent Enzymes
Phenylboronic acid	Cis-Diol Containing Compounds
Protein A/Protein G	Antibodies
Metal Chelates	Metal-Binding Proteins & Peptides

Note: the affinity ligand does not necessarily have to be of biological origin

Affinity Chromatography

Used to purify:

Enzymes

Antibodies

Transport proteins

Receptor proteins

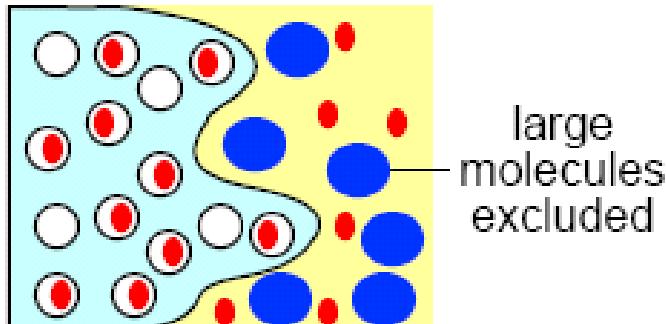
Glycoproteins, lipoproteins & membrane receptors

mRNA (+ proteins that bind mRNA)

Recombinant-tagged proteins

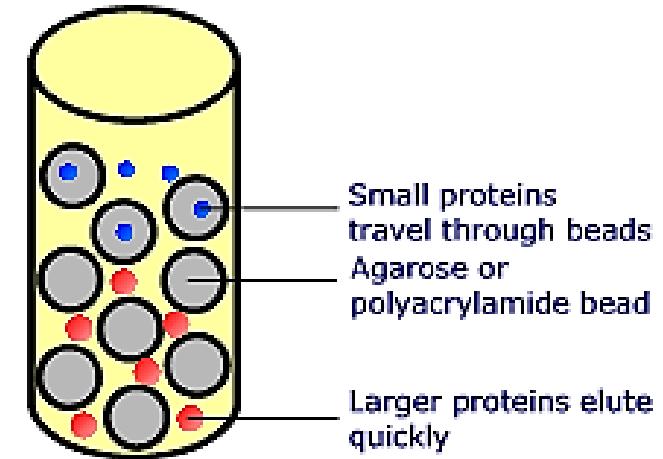
5. Size Exclusion Chromatography (SEC)

separates molecules according to **differences in their size**



Molecular Exclusion Chromatography

Separation is a result of
“trapping” of molecules in
the pores of the packing
material



SEC is based on the use of a support material that has a certain range of pore sizes-

- Very large molecules can't get into the pores – unretained
- Very small molecules get hung up in to pores for a long time - most retained – longest retention time

Gel Filtration / Size Exclusion

Preferably used to separate **globular** molecules

e.g. enzymes, antibodies & other globular proteins

Resins:

Cross linked dextrans

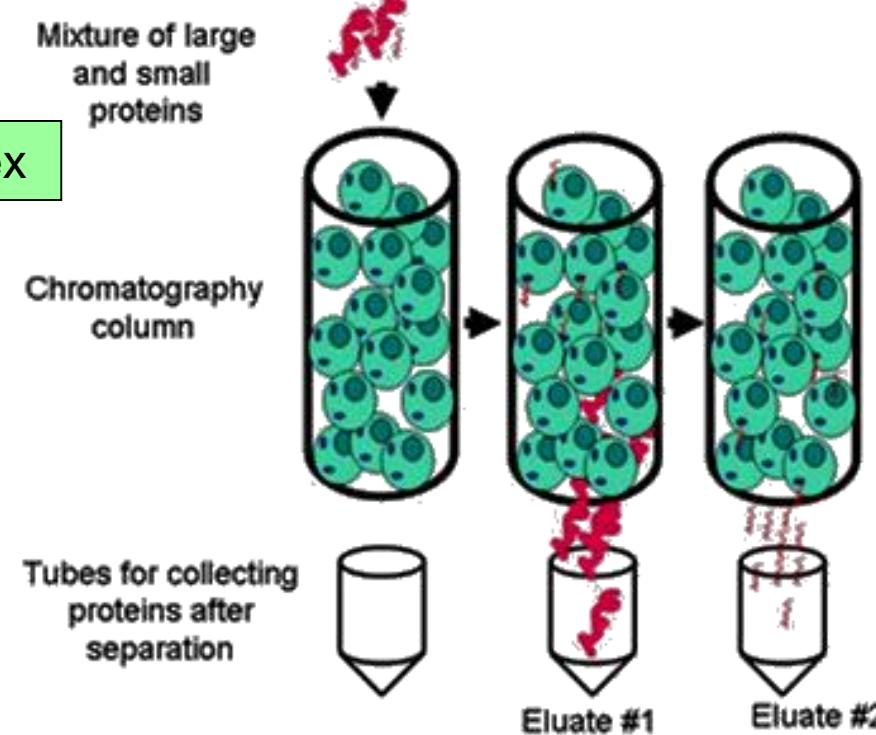
e.g. Sephadex

Polyacrylamide

e.g. SephAcryl

Agarose

e.g. Sepharose

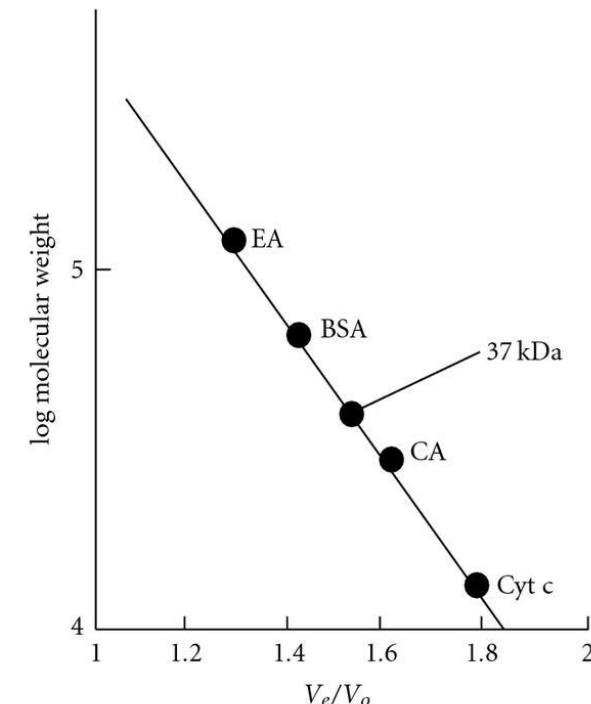
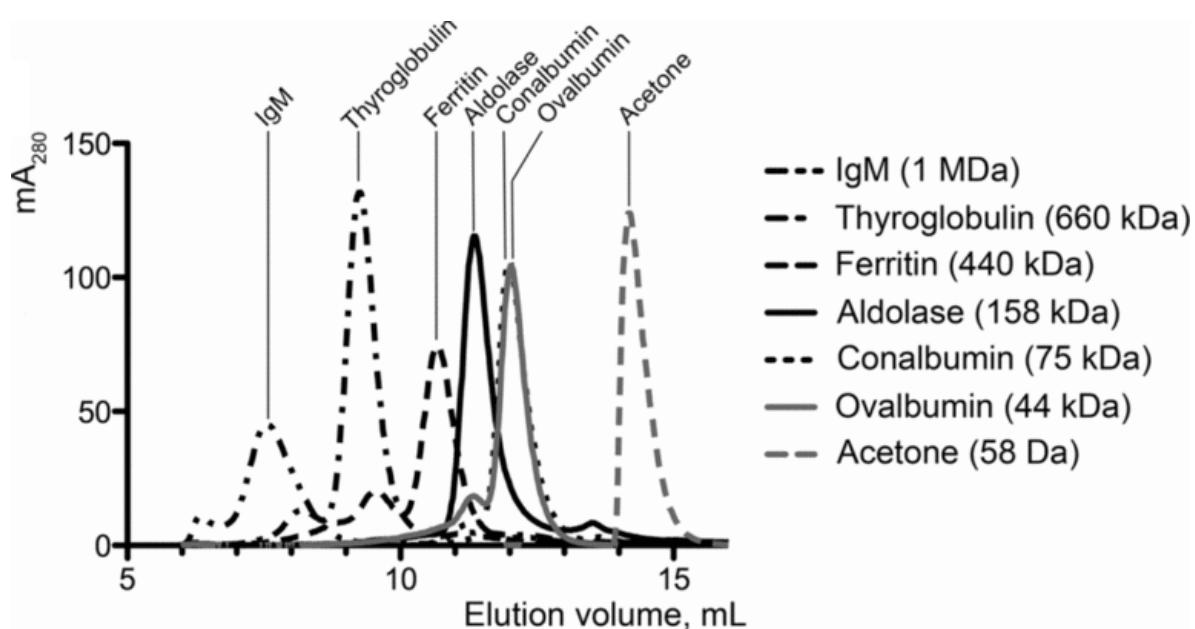


SEC does not have a “weak” or “strong” mobile phase since retention is based only on size/shape of the analyte and the pore distribution of the support.

- *gel filtration chromatography*: if an aqueous mobile phase is used
- *gel permeation chromatography*: if an organic mobile phase is used
(usually tetrahydrofuran- polar solvent)

Common applications of SEC:

- Separation of Biological Molecules (e.g., proteins from peptides)
- Separation/analysis of organic polymers
- molecular-weight determination



Summary of Different types of Liquid Chromatography

Mode or type	Stationary phase	Mobile phase	Mechanism
Adsorption	Solid that attracts the solutes	Liquid or gas	Solutes move at different rates according to the forces of attraction to the stationary phase.
Partition	Thin film of liquid formed on the surface of a solid inert support	Liquid or gas	Solutes equilibrate between the 2 phases according to their partition coefficients
Ion Exchange	Solid resin that carries fixed ions	Liquid containing electrolytes	Solute ions of charge opposite to the fixed ions are attracted to the resin by electrostatic forces & replace the mobile counterions.
Molecular Exclusion	Porous gel with no attractive action on solute molecules	Liquid	Molecules separate according to their size: 1. Smaller molecules enter the pores of the gel, and need a larger volume of eluent. 2. Larger molecules pass through the column at a faster rate.
Affinity	Solid on which specific molecules are immobilized	Liquid or gas	Special kind of solute molecules interact with those immobilized on the stationary phase

Most commonly used types of Chromatography

- Thin-Layer Chromatography – separates dried liquid samples with a liquid solvent (mobile phase) and a glass plate covered with a thin layer of alumina or silica gel (stationary phase)
- Liquid Chromatography – separates liquid samples with a liquid solvent (mobile phase) and a column composed of solid beads (stationary phase)

Thin layer chromatography (TLC)

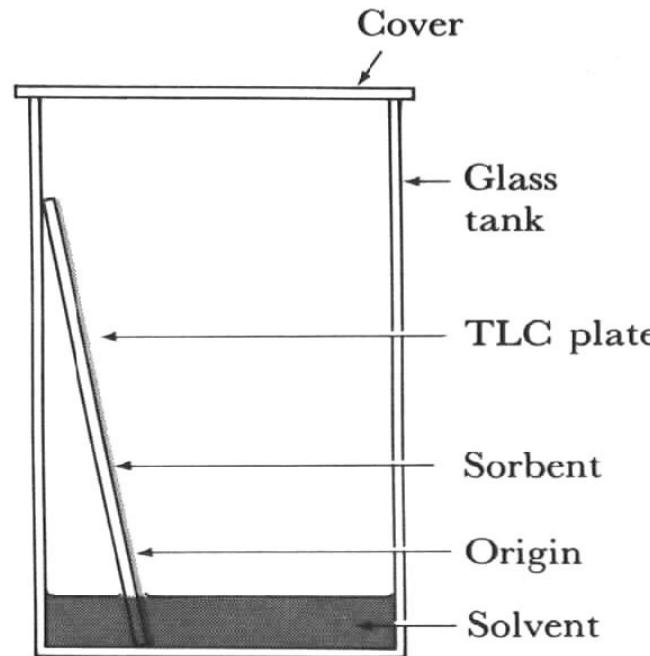
- is a method for **identifying** substances and **testing** the purity of compounds.
- TLC is a useful technique because it is relatively **quick** and **requires small quantities** of material.

Example of
Adsorption
chromatography

PRINCIPLE

- With capillary action the solvent will move up to the plate.
- Every separated component will move to certain distance.

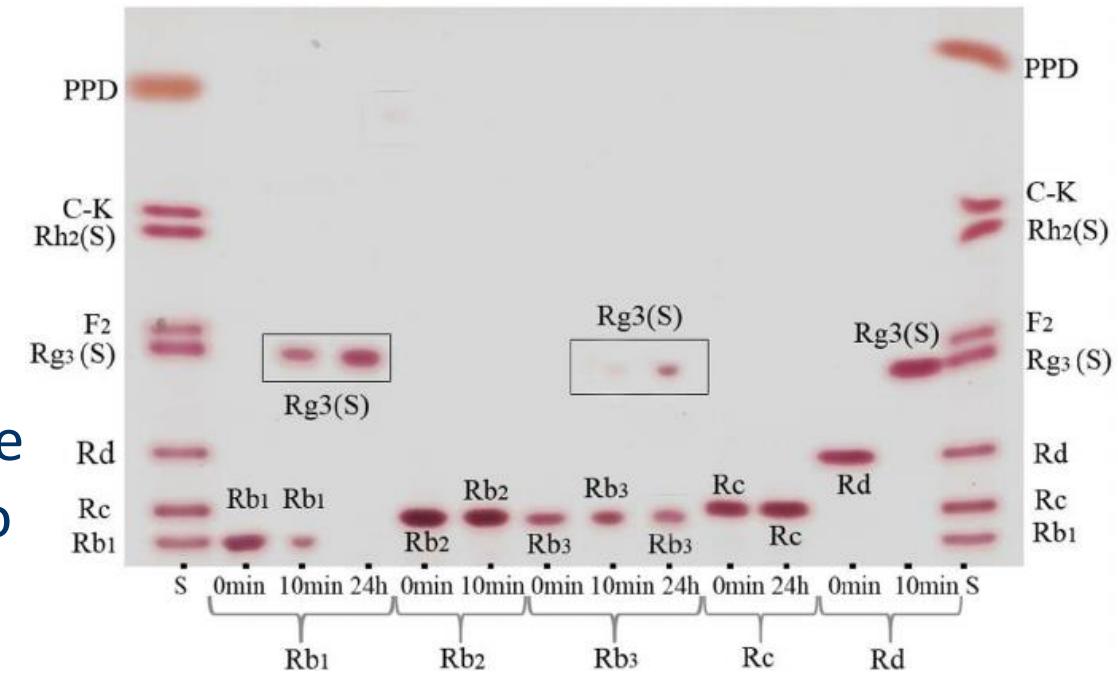
Based on adsorption or partition chromatography



Thin Layer Chromatography (TLC)

To a jar with a tight-fitting lid add enough of the appropriate developing liquid so that it is 0.5 to 1 cm deep in the bottom of the jar.

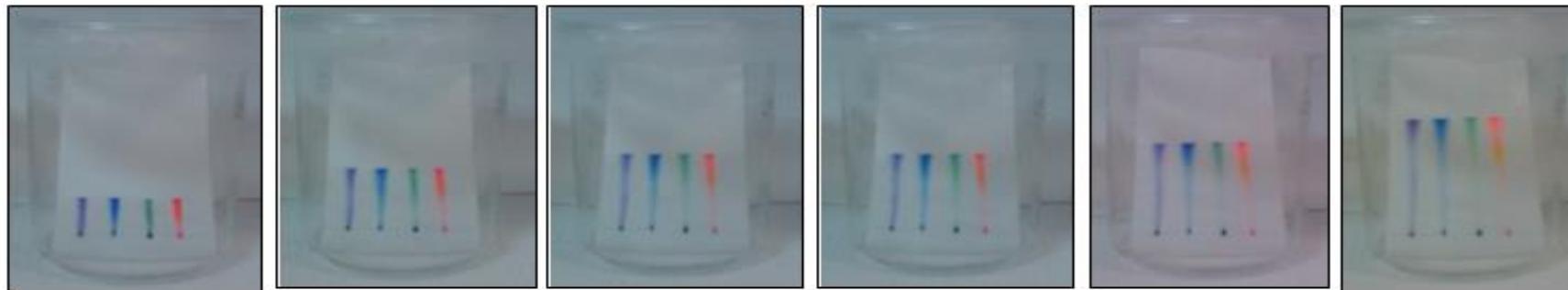
Close the jar tightly, and let it stand for about 30 minutes so that the atmosphere in the jar becomes saturated with solvent.



Most common solvents used to separate organic compounds are mixture of hexane (non-polar) and ethyl acetate (polar)

Developing the Chromatograms

50% Isopropanol



0 min

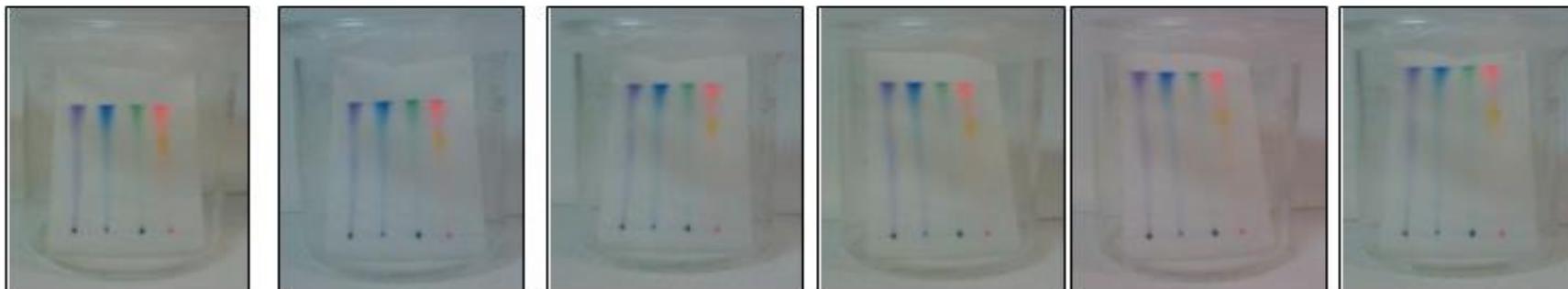
4 min

9 min

14 min

20 min

25 min



30 min

35 min

40 min

45 min

50 min

55 min

Identifying the Spots (visualization)



If the spots can be seen, outline them with a pencil.

If no spots are obvious, the most common visualization technique is to **hold the plate under a UV lamp**.

Many organic compounds can be seen using this technique.

Visualizing Agents

Alkaloids: Dragendorff's reagent

Cardiac glycosides: Antimony trichloride

Sugar: Aniline phthalate

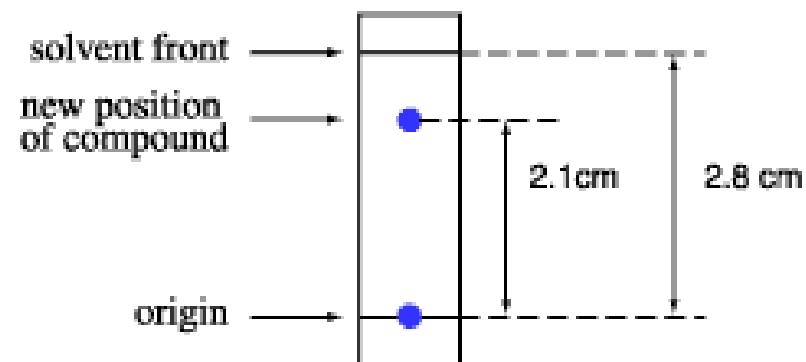
Amino acids: Ninhydrin (A deep blue colour is produced)

Interpreting the Data

The retention factor, or R_f , is defined as the distance traveled by the compound divided by the distance traveled by the solvent

$$R_f = \frac{\text{distance moved by substance}}{\text{distance moved by solvent front}}$$

For example, if a compound travels 2.1 cm and the solvent front travels 2.8 cm, the R_f is 0.75:



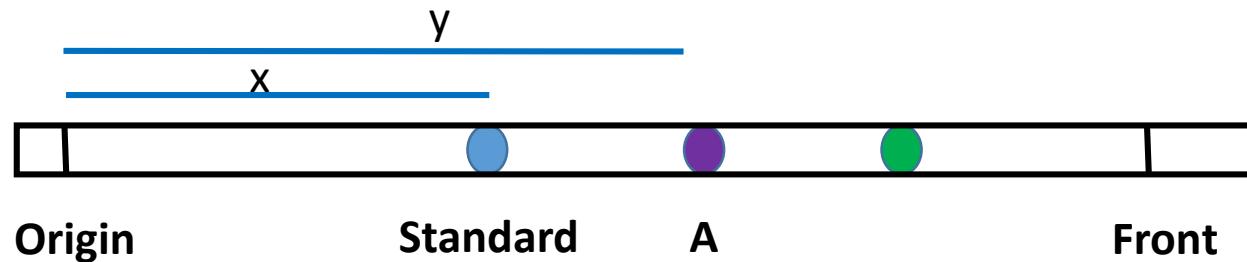
For substances that are very soluble in the liquid R_f will be close to 1

For substances that are rather insoluble in the liquid R_f will be close to 0

$$R_f = \frac{2.1}{2.8} = 0.75$$

The movement with respect to a standard of known mobility is expressed as Rx

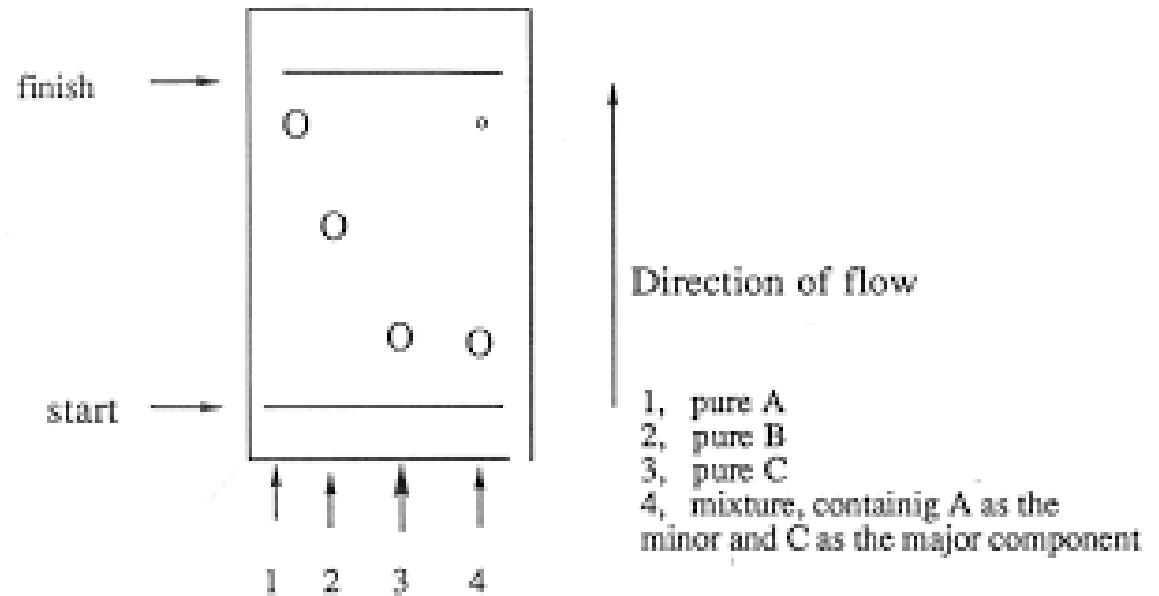
$$Rx = \frac{\text{Distance moved by test substance}}{\text{Distance moved by standard}}$$



$$Rx = \frac{Y}{X}$$

In addition, the **purity** of a sample may be estimated from the chromatogram.

An impure sample will often develop as two or more spots, while a pure sample will show only one spot



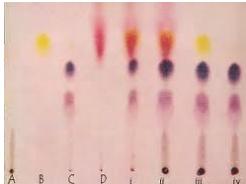
Summary of TLC

- ✓ A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina).
- ✓ A small amount of the mixture to be analyzed is spotted near the bottom of this plate.
- ✓ The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid.
- ✓ This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action.
- ✓ As the solvent moves past the spot that was applied, an equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution.

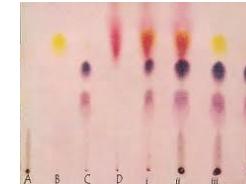
In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others.

When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized.

If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp is used to visualize the plates.



Paper Chromatography



Example of
partition
chromatography

Stationary phase:

Cellulose

Mobile phase:

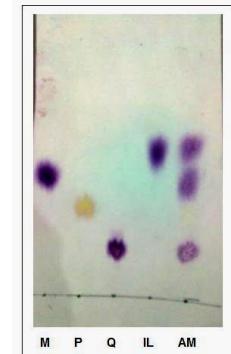
Organic solvent

∴ Non-polar substances will move faster than polar substances

Staining of amino acids:

Ninhydrin

Paper Chromatography vs. TLC

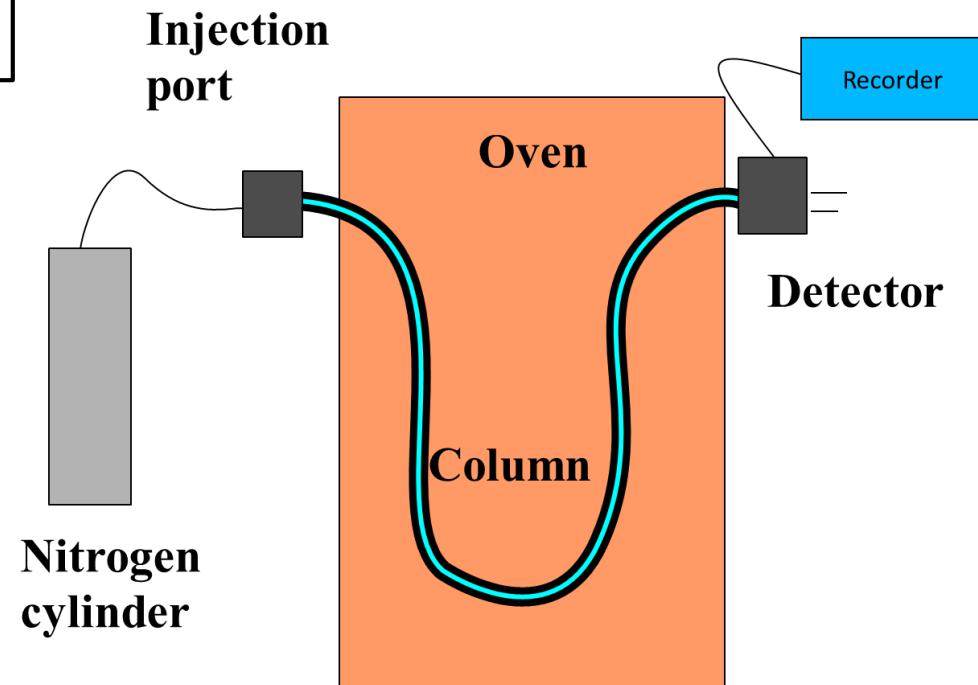


Gas Liquid Chromatography

used to separate volatile organic compounds

Here the mobile phase is an **unreactive gas** (eg Nitrogen) flowing through a tube.

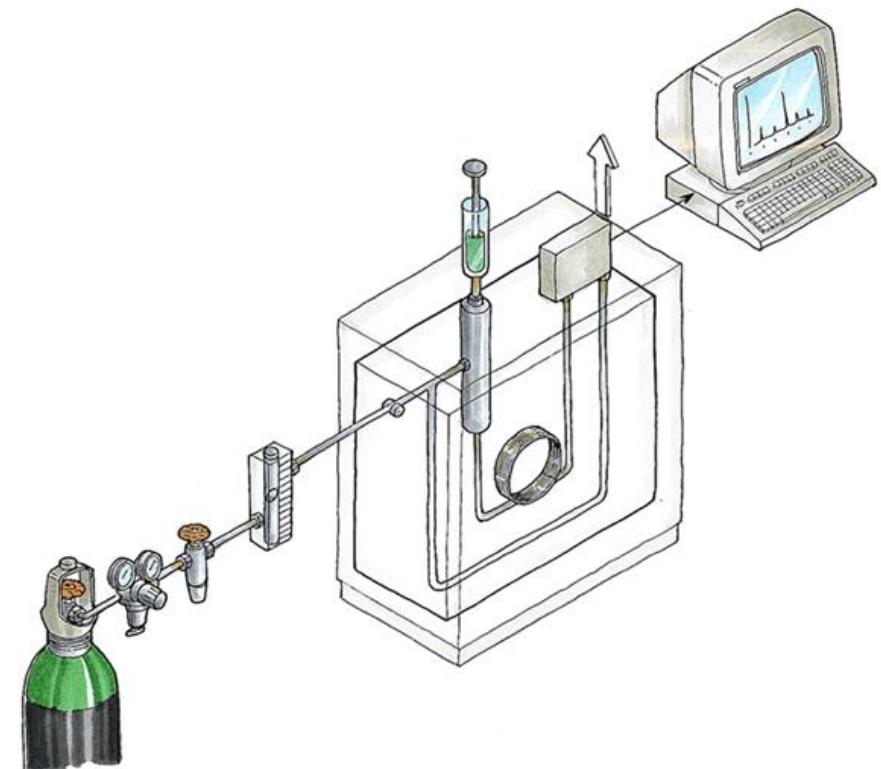
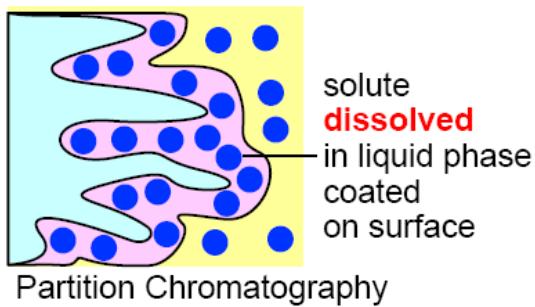
And the stationary phase is an **involatile liquid** held on particles of a solid support.



About 1 μ L of liquid is injected into one end of the column. As each component reaches the other end it is detected and registered on a chart recorder.

Principle

The organic compounds are separated due to differences in their **partitioning behavior** between the mobile gas phase and the stationary phase in the column.



Gas Chromatography

- ❖ Good for **volatile** samples (up to about 250 °C)
- ❖ 0.1-1.0 microliter of liquid or 1-10 ml vapor
- ❖ Can detect <1 ppm with certain detectors
- ❖ Can be easily automated for injection and data analysis

Definition- One **ppm** is equivalent to 1 milligram of something **per** liter of water (mg/l) or 1 milligram of something **per** kilogram soil (mg/kg)

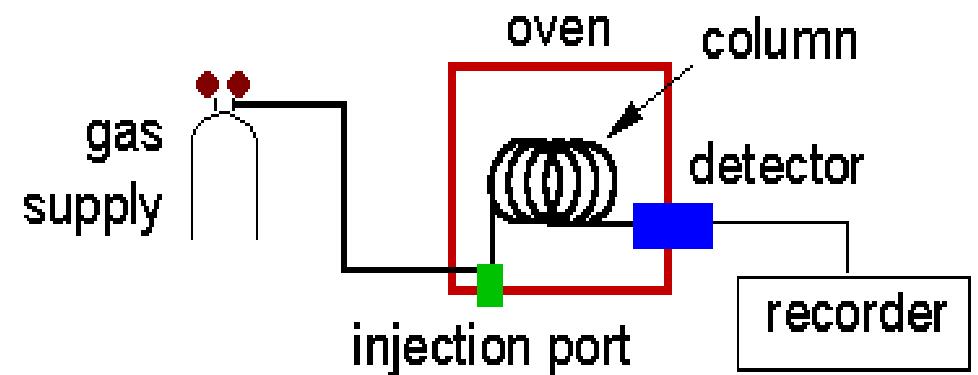
Components of a Gas Chromatograph

Gas Supply: (usually N₂ or He)

Sample Injector: (syringe / septum)

Column: 1/8" or 1/4" x 6-50' tubing packed with
small uniform size (.5-1.0 mm), inert support
coated with thin film of nonvolatile liquid

Detector: TC - thermal conductivity
FID - flame ionization detector



✓ Since the partitioning behavior is dependent on temperature, the separation column is usually contained in a thermostat-controlled oven.

✓ Separating components with a wide range of boiling points is accomplished by starting at a low oven temperature and increasing the temperature over time to elute the high-boiling point components.

Polyunsaturated Fatty Acid Methyl Esters

1. C14:0

2. C16:0

3. C16:1w7

4. C18:0

5. C18:1w9

6. C18:1w7

7. C18:2w6

8. C18:3w3

9. C20:1w9

10. C20:2w6

11. C20:3w6

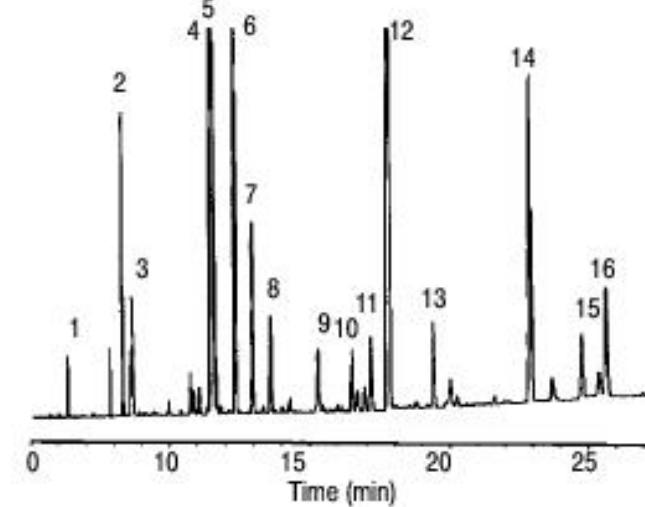
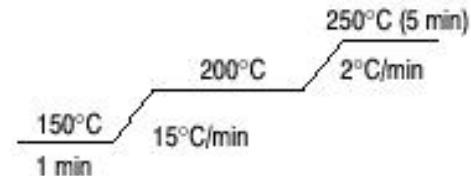
12. C20:4w6

13. C20:5w3

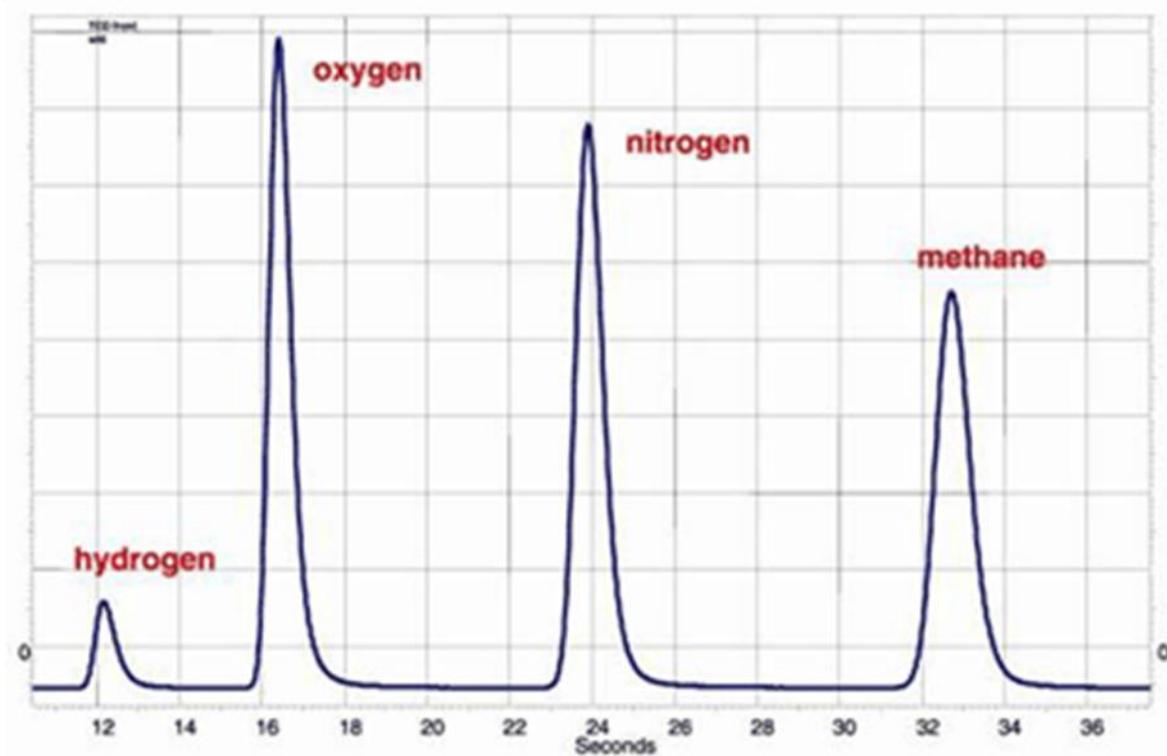
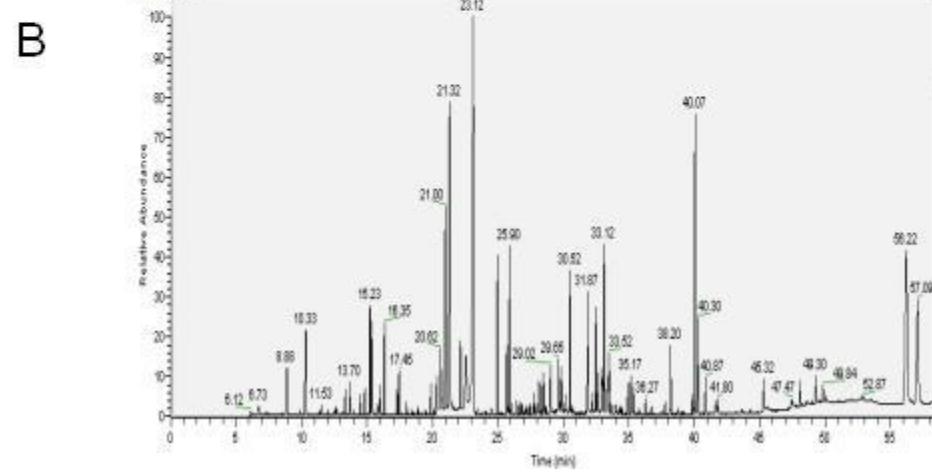
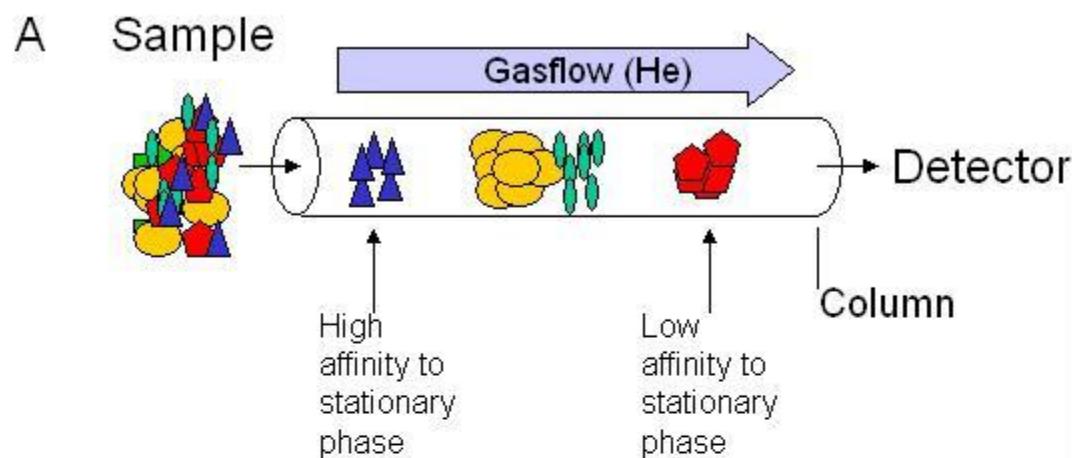
14. C22:4w6

15. C22:5w3

16. C22:6w3

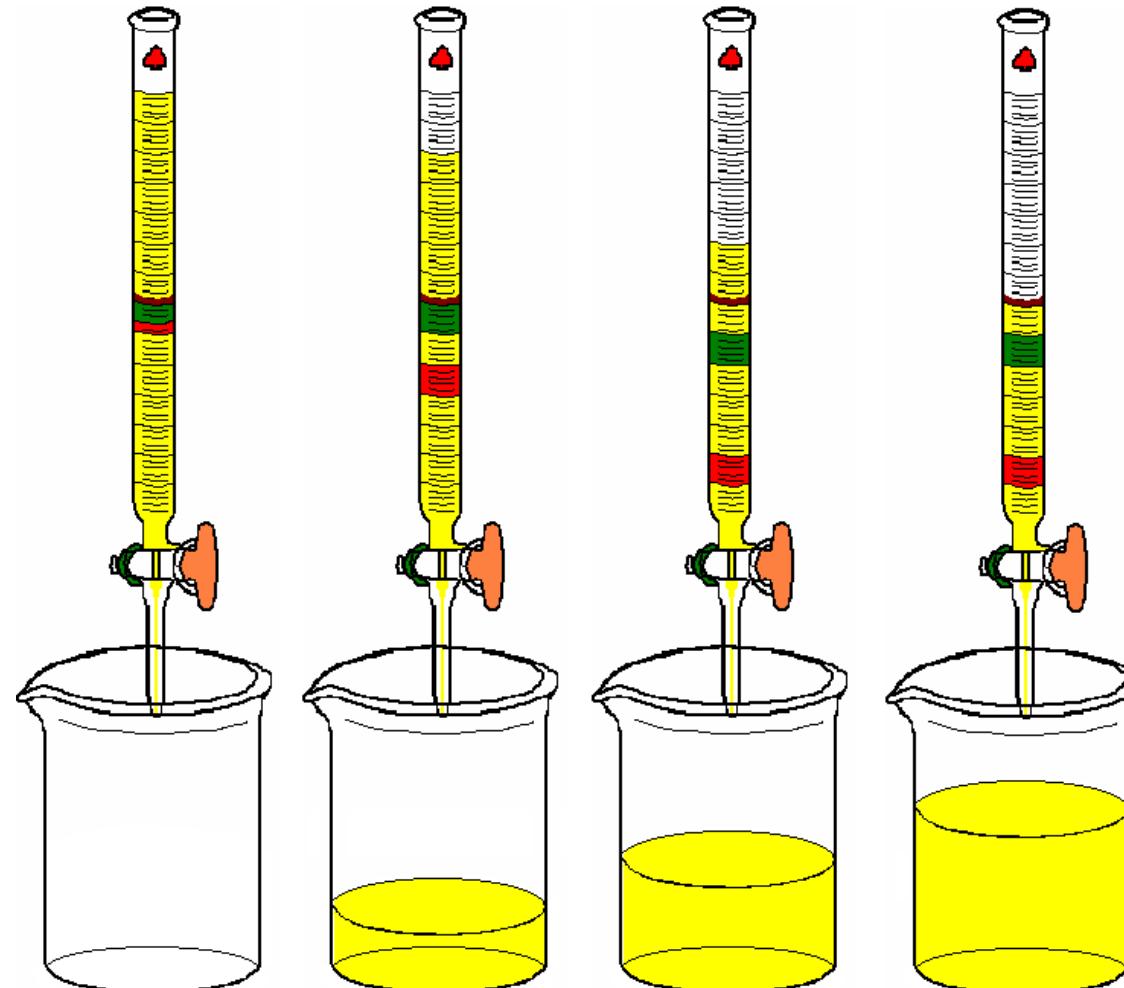


Sample: 50 g/l in methylene chloride
Carrier: Helium, 30 cm/sec, 10.7 psi (150°C), 1.5 ml/min constant flow
Column: HP-INNOWax (Cross-Linked PEG), 30 m x 0.32 mm x 0.5 µm
(Part No. 19091N-213)
Injection: Split (60:1), 0.5 µl, inlet 220°C
Oven: Temperature program listed above
Detector: FID 275°C

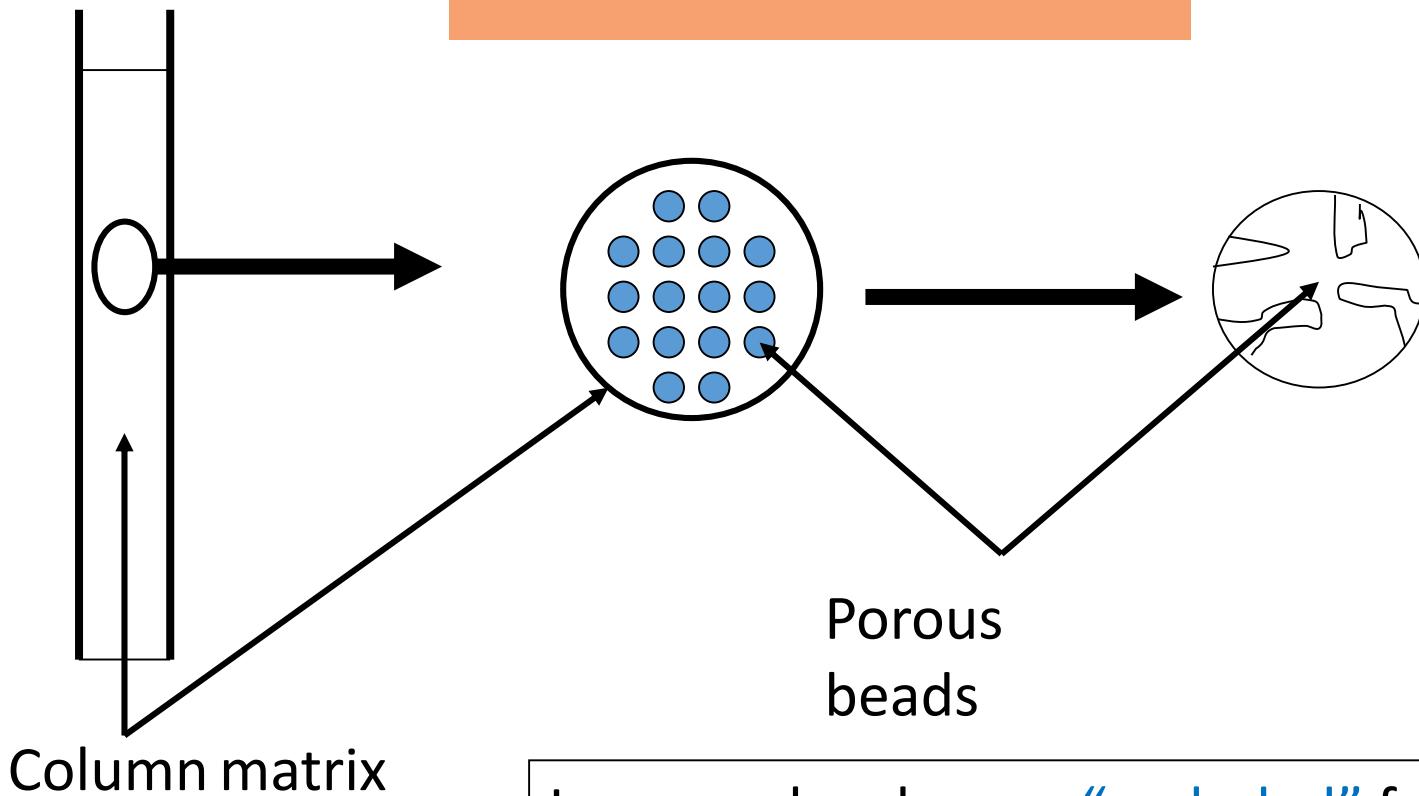


Column Chromatography

Stationary phase
is held in a narrow
tube through
which the mobile
phase is forced
under pressure or
under the effect of
gravity



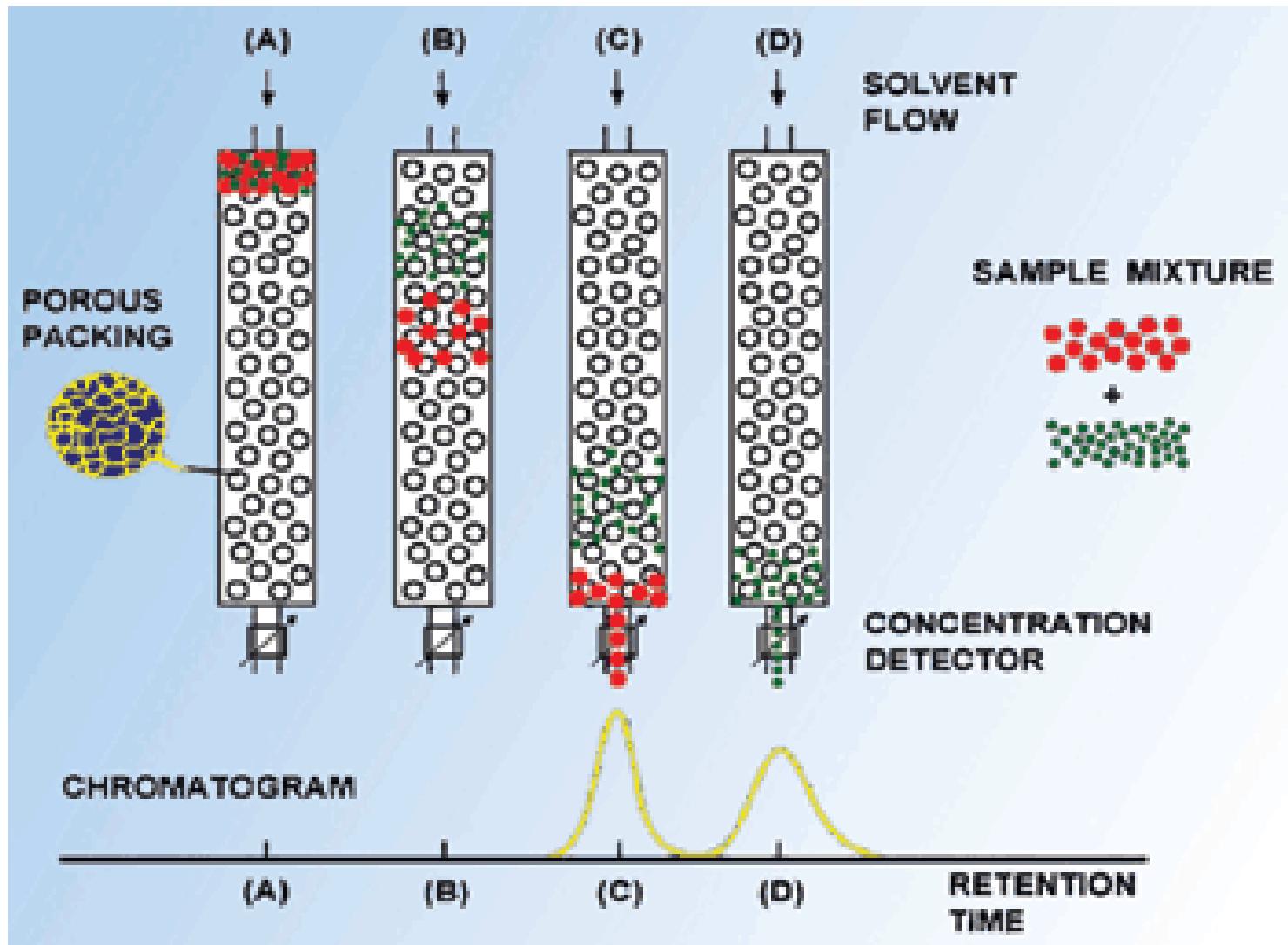
Theory



Large molecules are “excluded” from the pores and travel through the column fastest

Small molecules are “included” – can diffuse into the pores and elute later

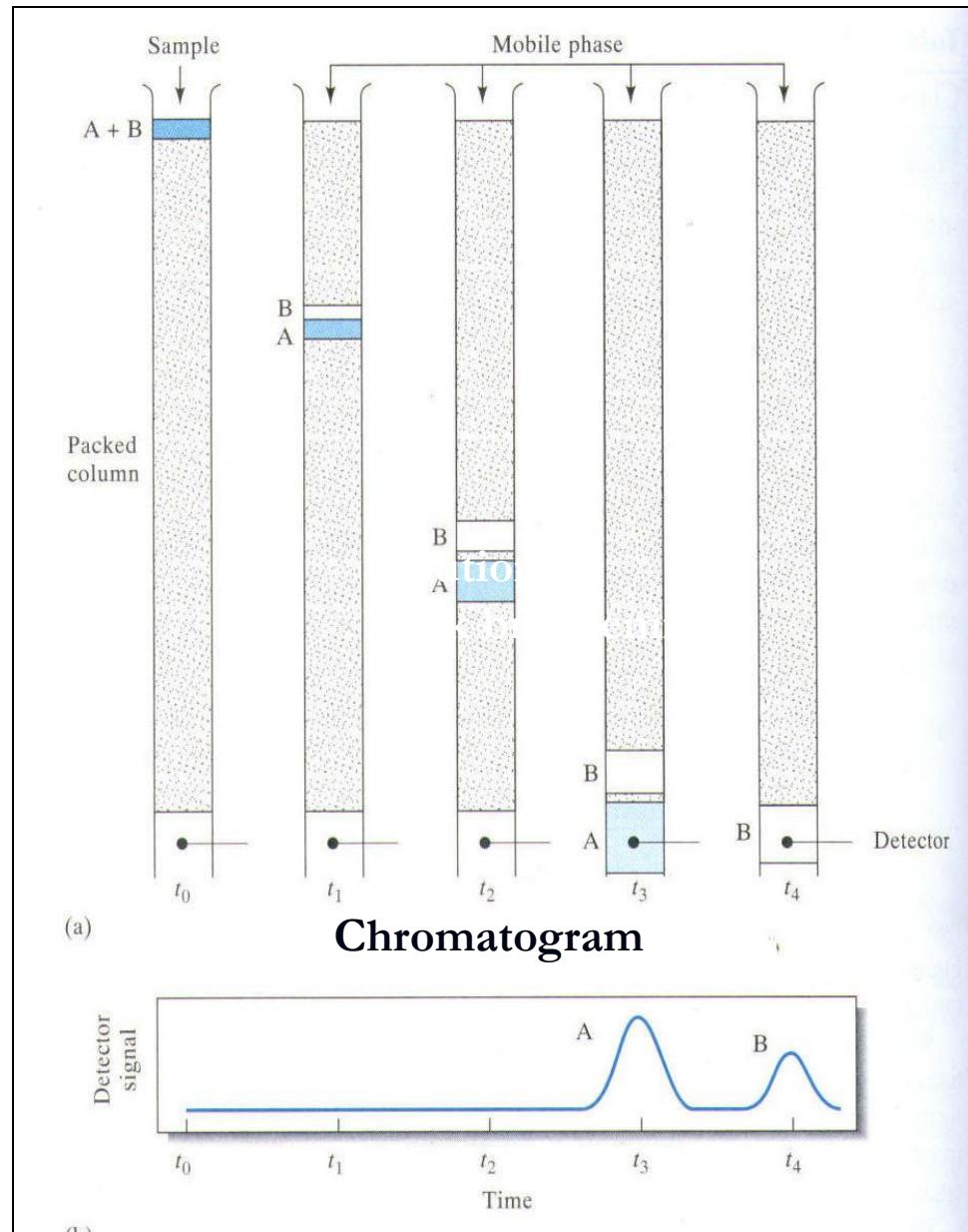
Theory



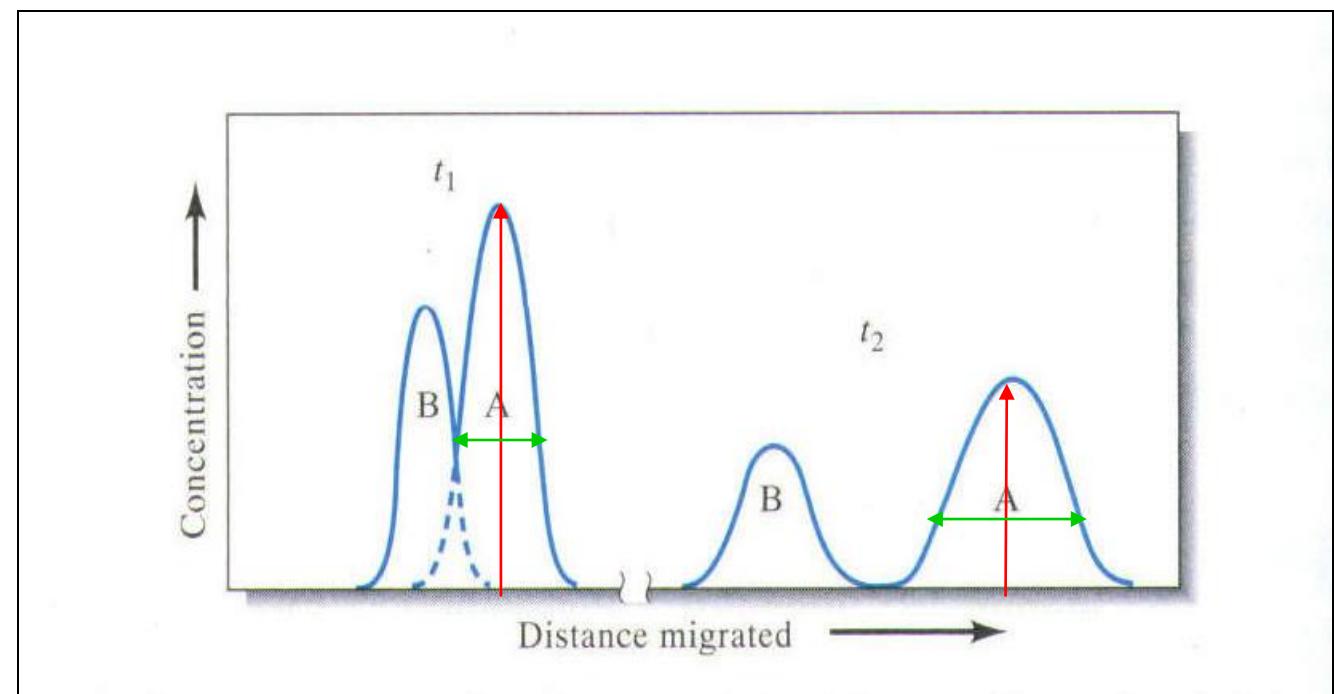
Running the column

- Sample size / Fraction size
 - 0.5 – 5% of total bed volume (V_t).
 - Concentration limited by viscosity (The *viscosity* of a fluid is a measure of its resistance to gradual deformation by shear stress or tensile stress.)
- Running time
 - Determined by “trial and error”
 - Slow rates allow efficient partitioning into pores and thus increase resolution
 - Slow rates increase diffusion of sample on column thus increasing peak width and reducing resolution.
 - Protein about $5\text{mL cm}^{-2}\text{ h}^{-1}$

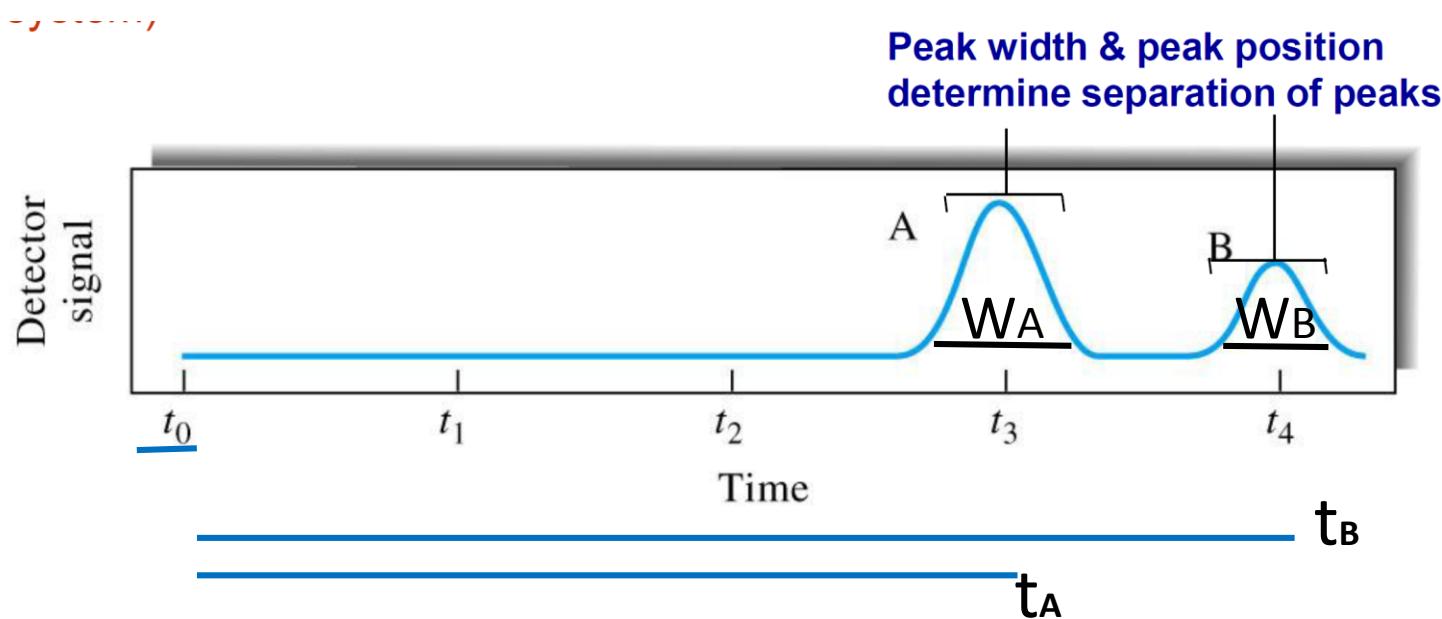
Column Chromatography



Peak separations



Separation Efficiency



Selectivity

$$\text{Selectivity } (\alpha) = \frac{t_B - t_0}{t_A - t_0}$$

Ability of a column to distinguish between two similar substances

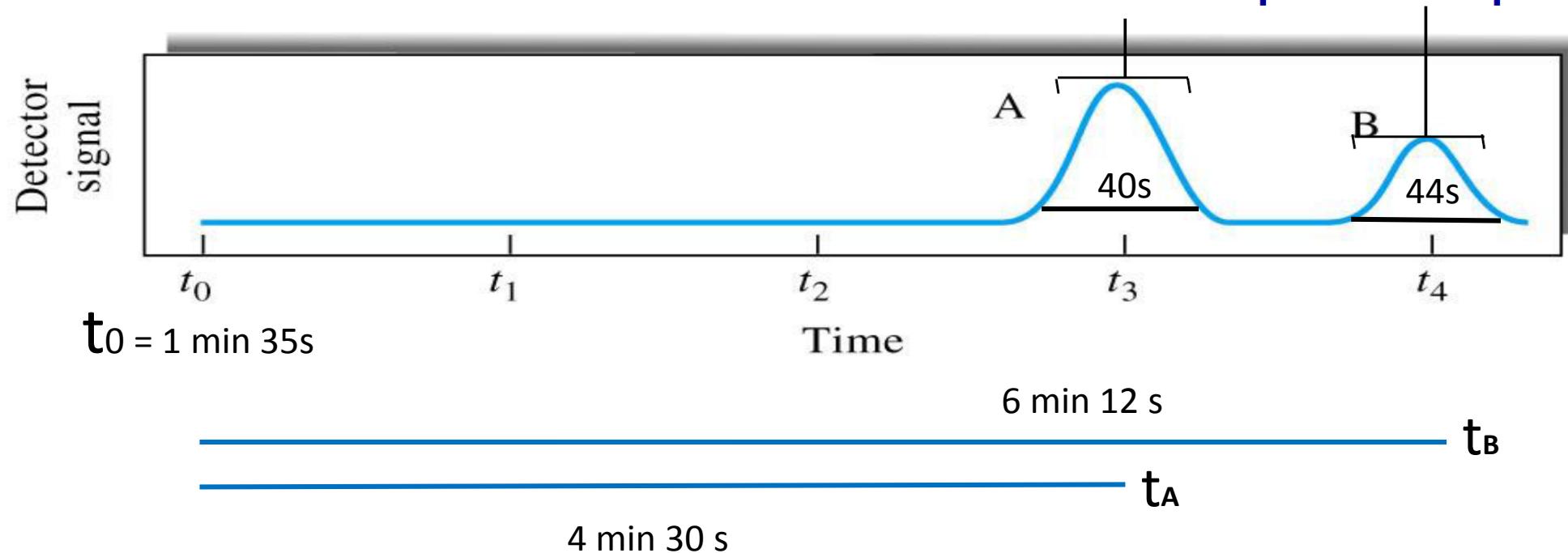
Resolution

$$R = \frac{2(t_A - t_B)}{W_A + W_B}$$

R values of 1 or more are satisfactory for symmetrical peaks

QUESTION

Peak width & peak position
determine separation of peaks

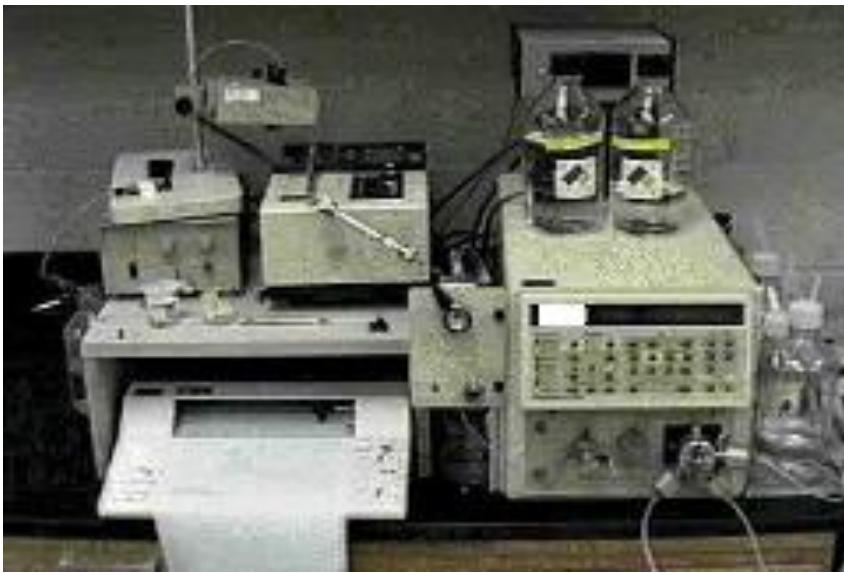


Calculate Selectivity (1.58) and resolution (2.43)

Example calculation

Types of Column Systems

- Low Performance Liquid Chromatography (CC)
- High Performance Liquid Chromatography (HPLC)



Low-performance liquid chromatography

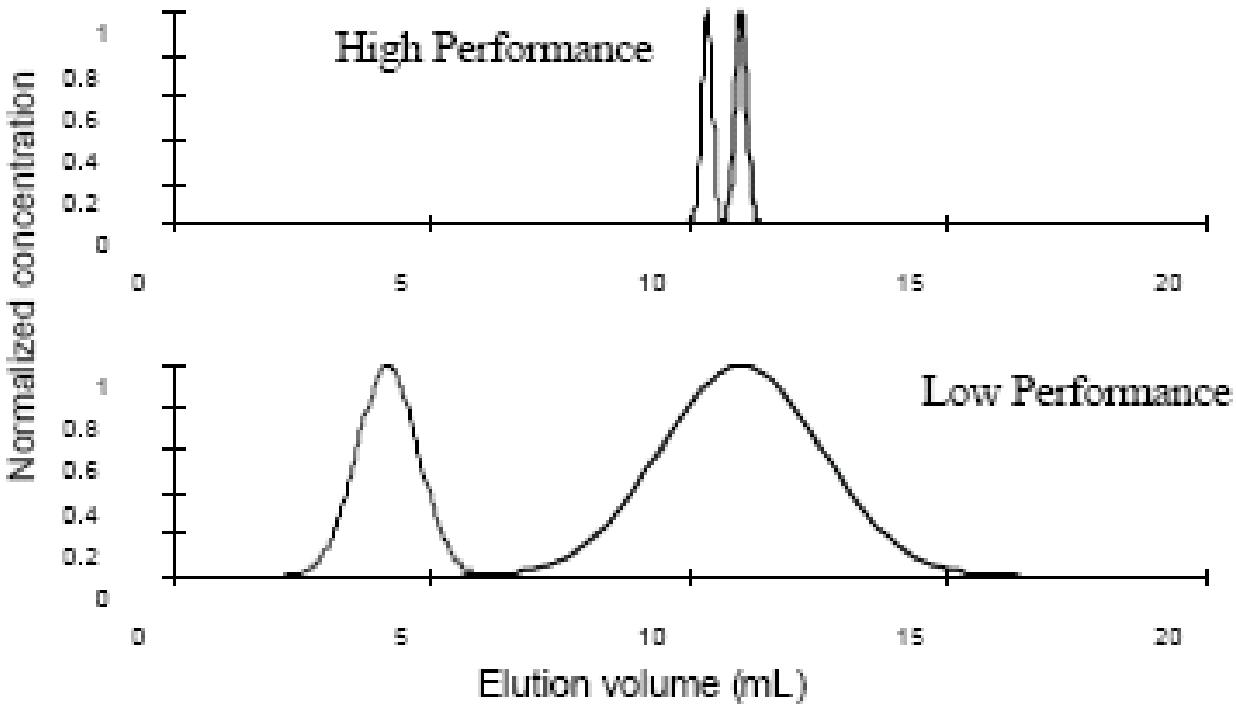
(e.g. column chromatography)

Advantages:

- simple system requirements
- low cost
- popular in sample purification
- used in the removal of interferences from samples
- used in some analytical applications

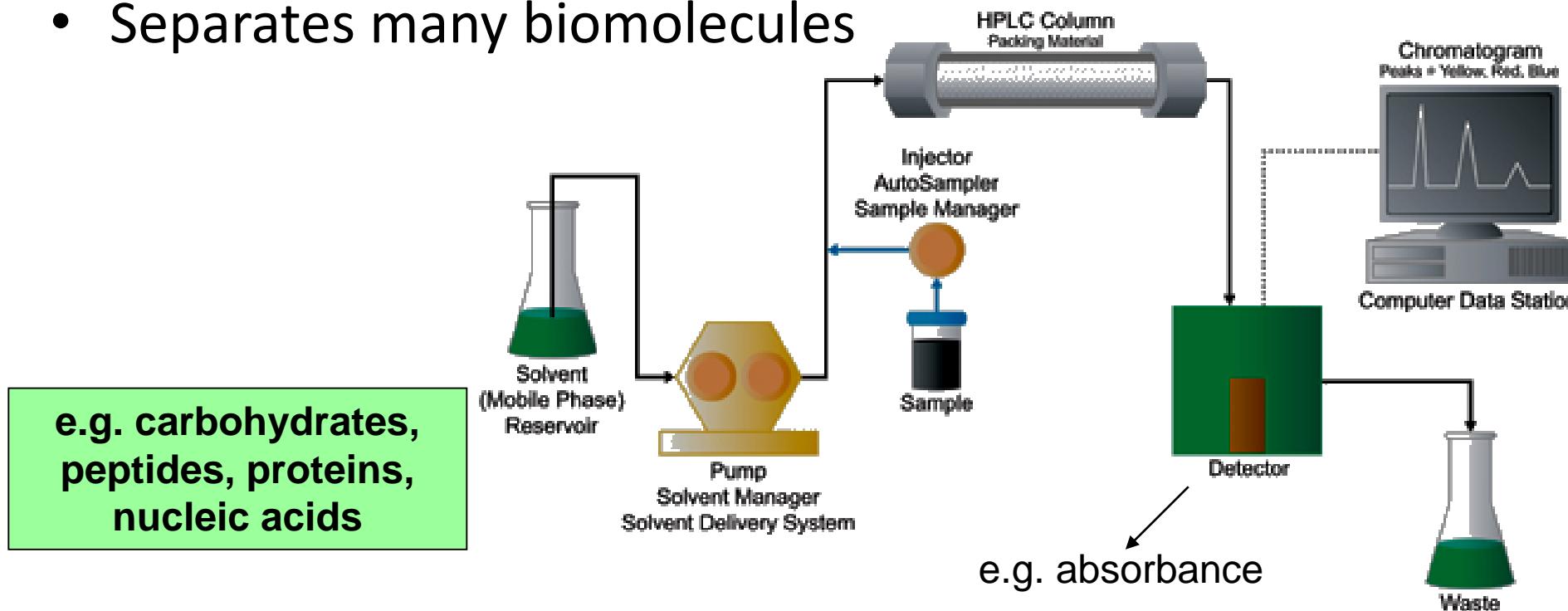
Disadvantages:

low efficiency, long analysis times and poor limits of detection



High Performance Liquid Chromatography (HPLC)

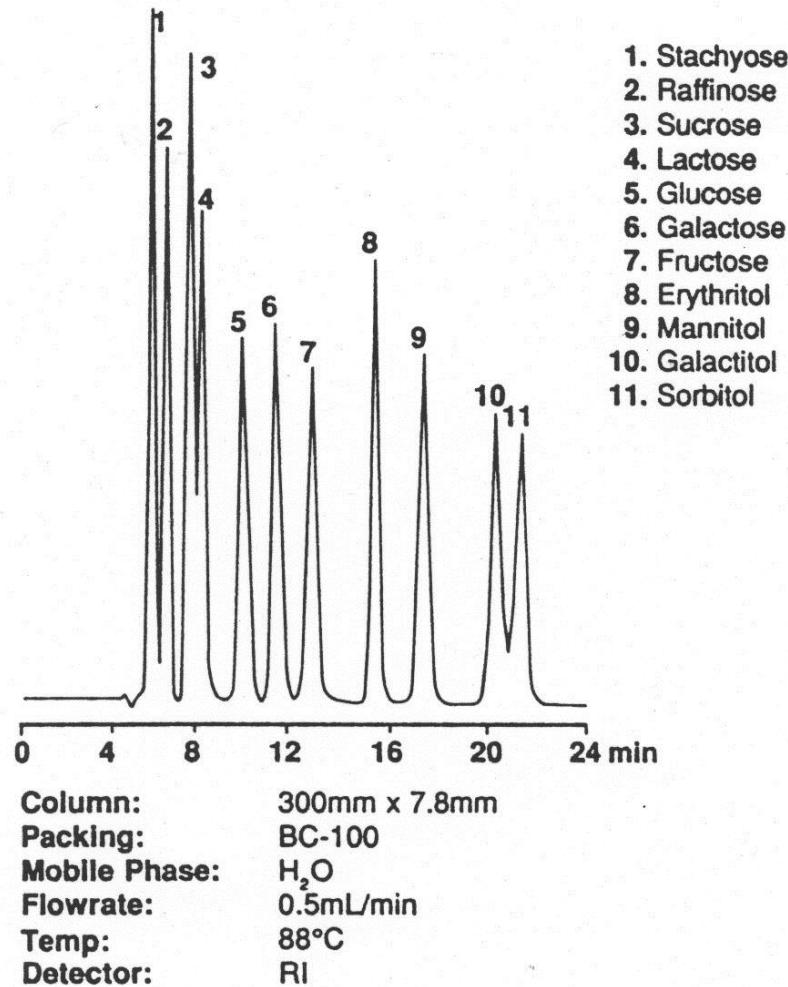
- Stationary phase small **incompressible** beads
- Mobile phase pumped at **HIGH PRESSURE**
- Rapid separation & sharper resolution
- Separates many biomolecules



High-performance Liquid Chromatography (HPLC)

- LC methods that use small, uniform, rigid support material
 - particles < 40 µm in diameter
 - usually 3-10 µm in practice
- good system efficiencies
- such systems have the following characteristics:
 - narrow peaks
 - low limits of detection
 - short separation times
 - columns can only tolerate high operating pressures and faster flow-rates

Standard Mixture of Sugars and Alcohols



High-performance liquid chromatography

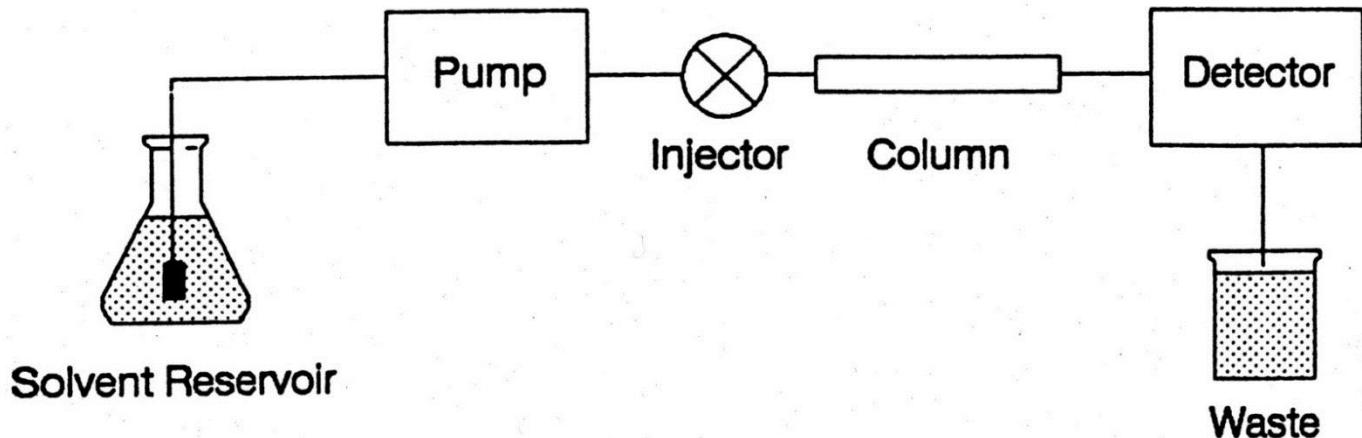
advantages:

- fast analysis time
- ease of automation
- good limits of detection
- preferred choice for analytical applications
- popular for purification work

e.g. carbohydrates,
peptides, proteins,
nucleic acids

disadvantages:

- greater expense
- lower sample capacities

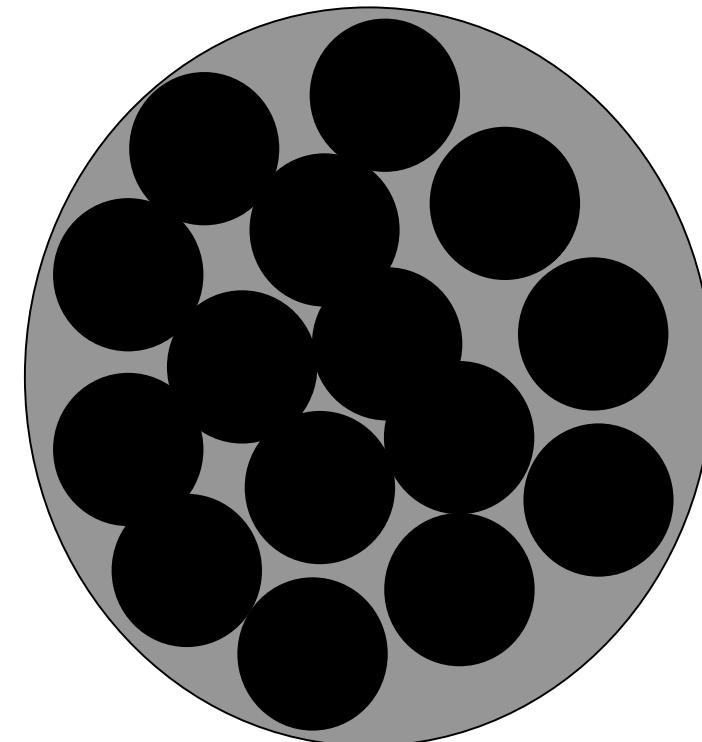


Gel filtration chromatography

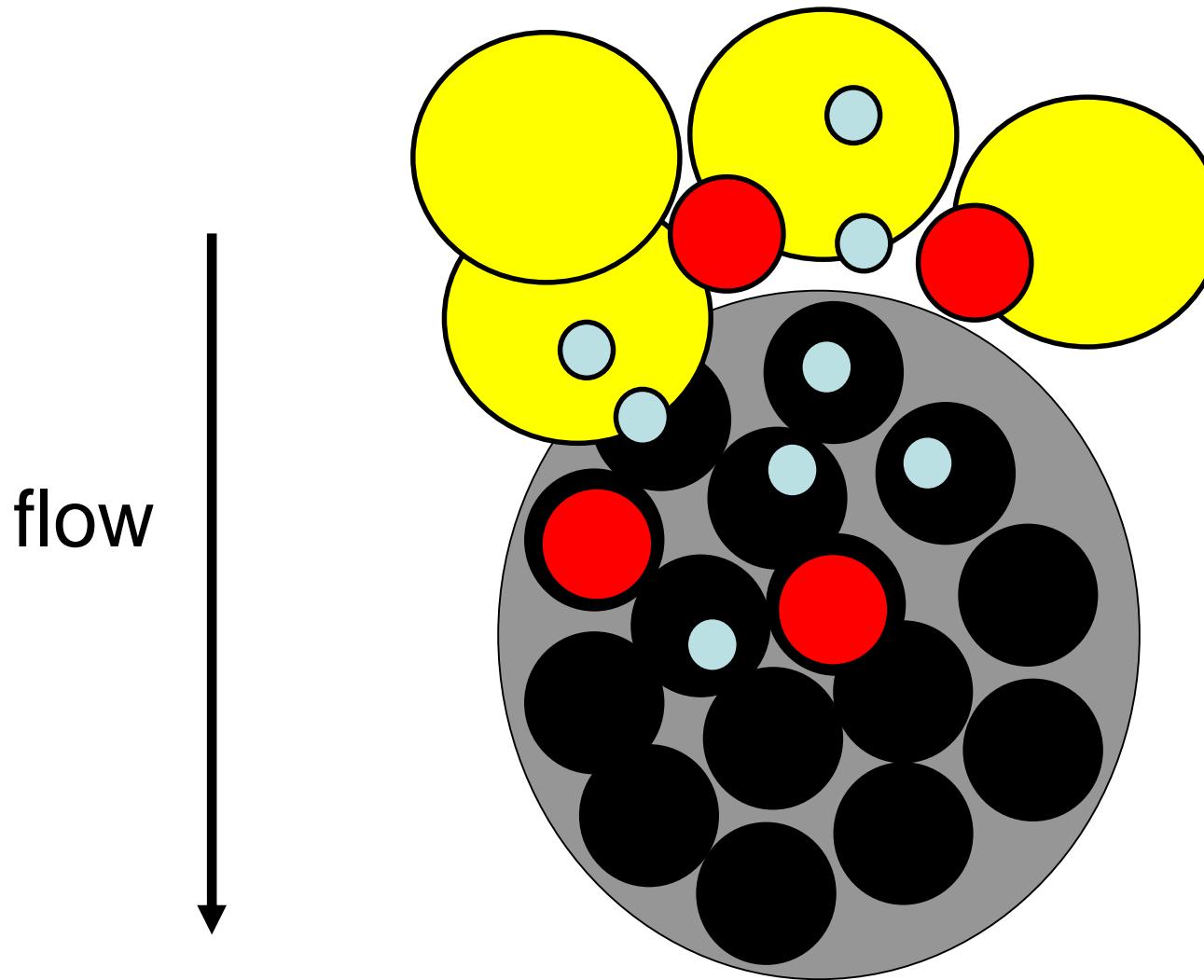
- Also called **size exclusion chromatography** or **molecular sieve chromatography**.

How does it work? If we assume proteins are spherical...

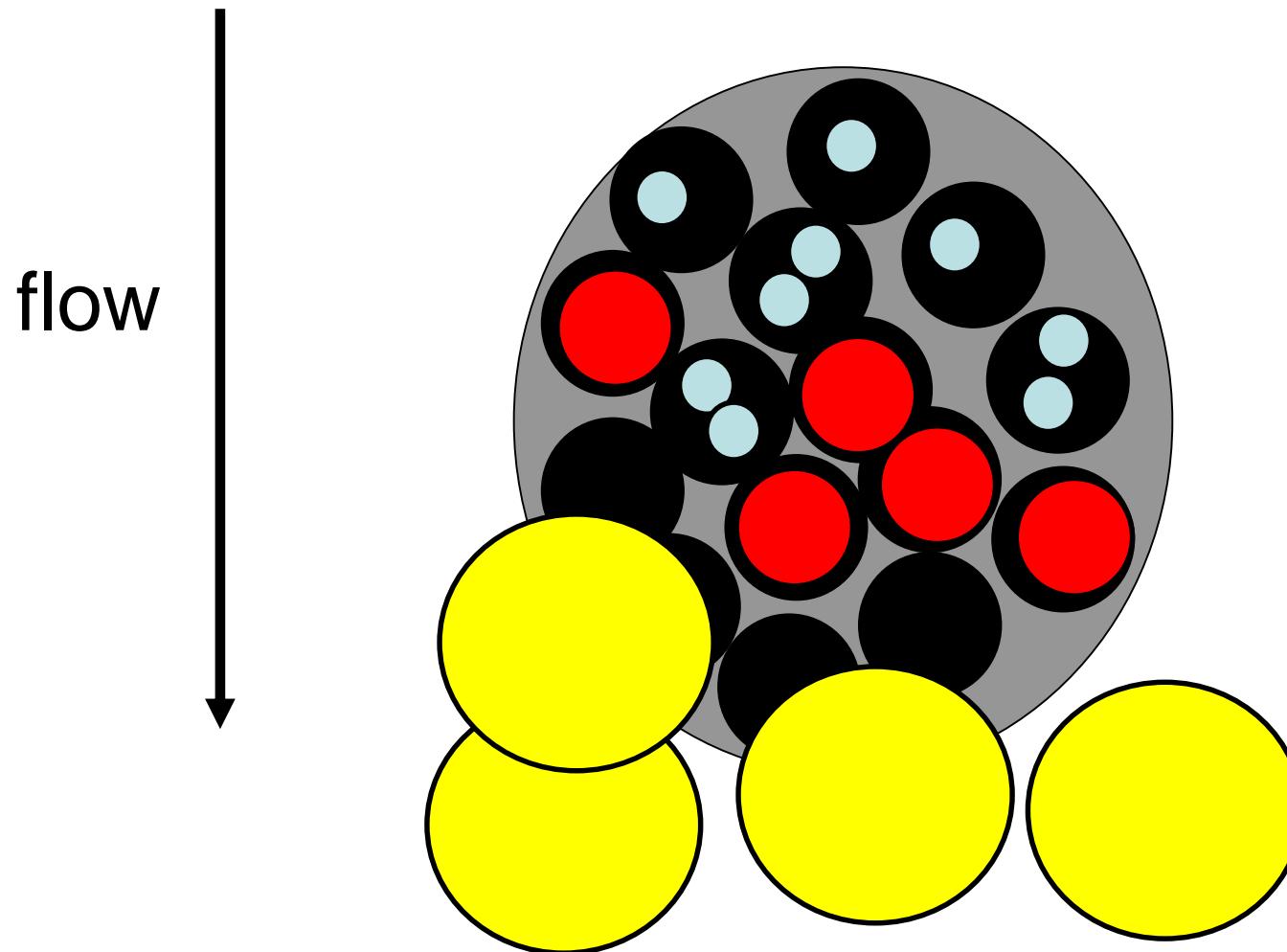
<u>size</u>	<u>Molecular mass (daltons)</u>
●	10,000
●	30,000
●	100,000



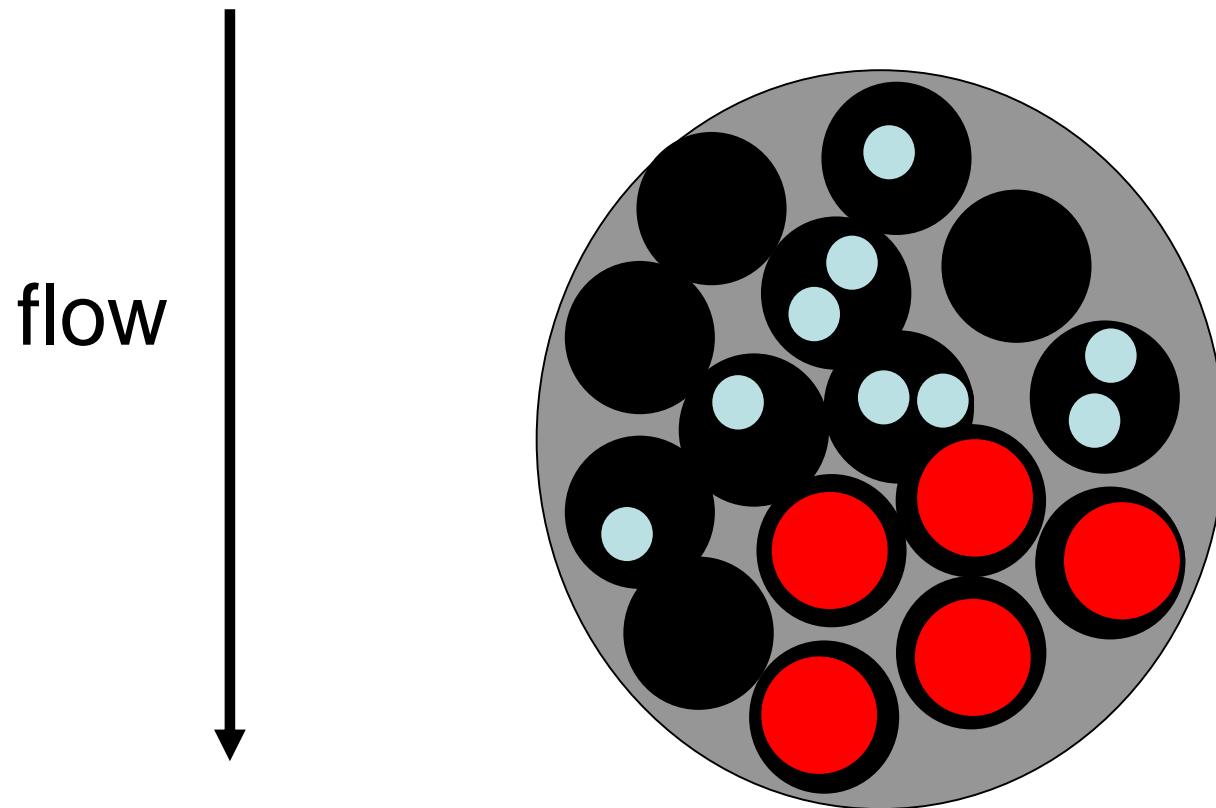
Gel filtration chromatography



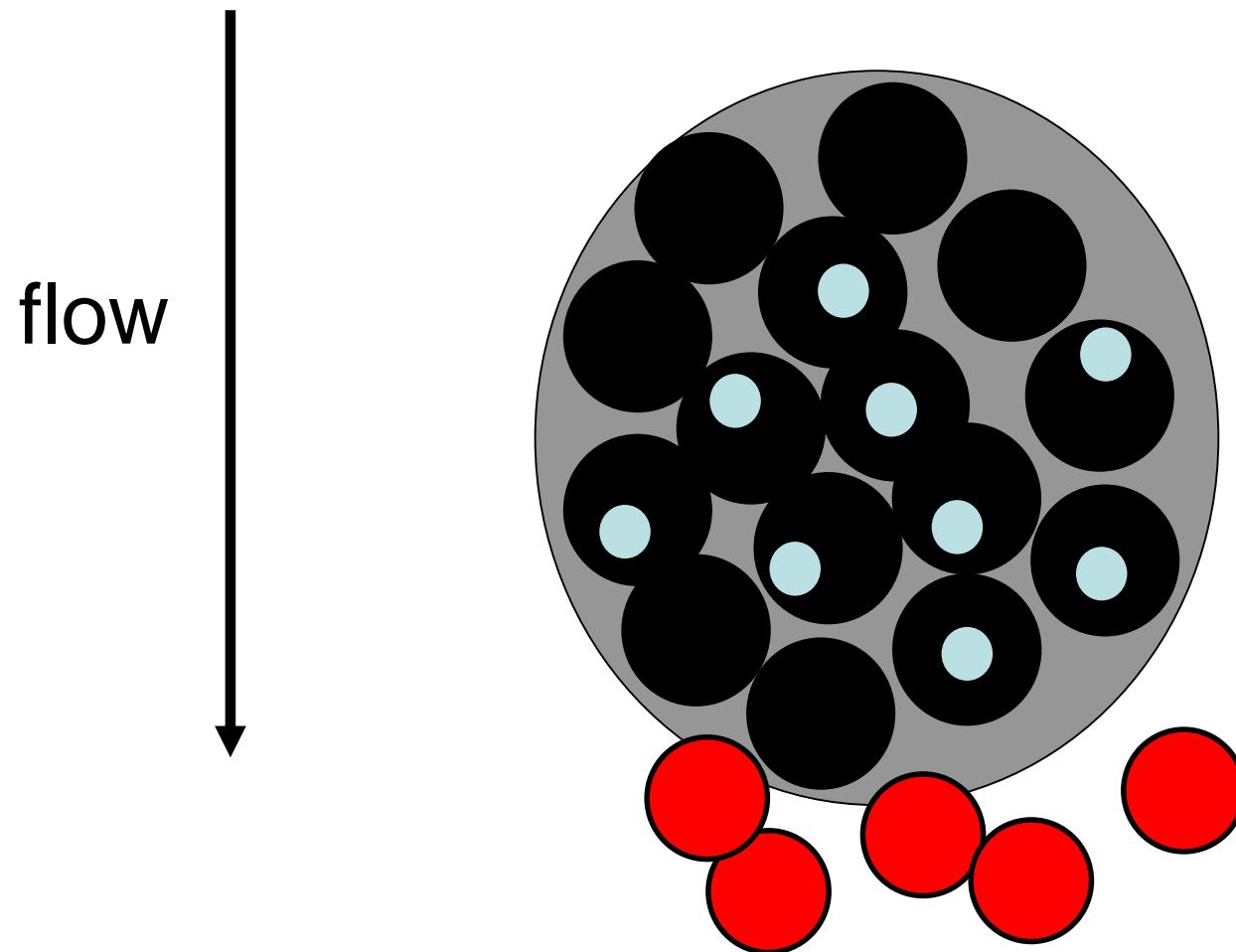
Gel filtration chromatography



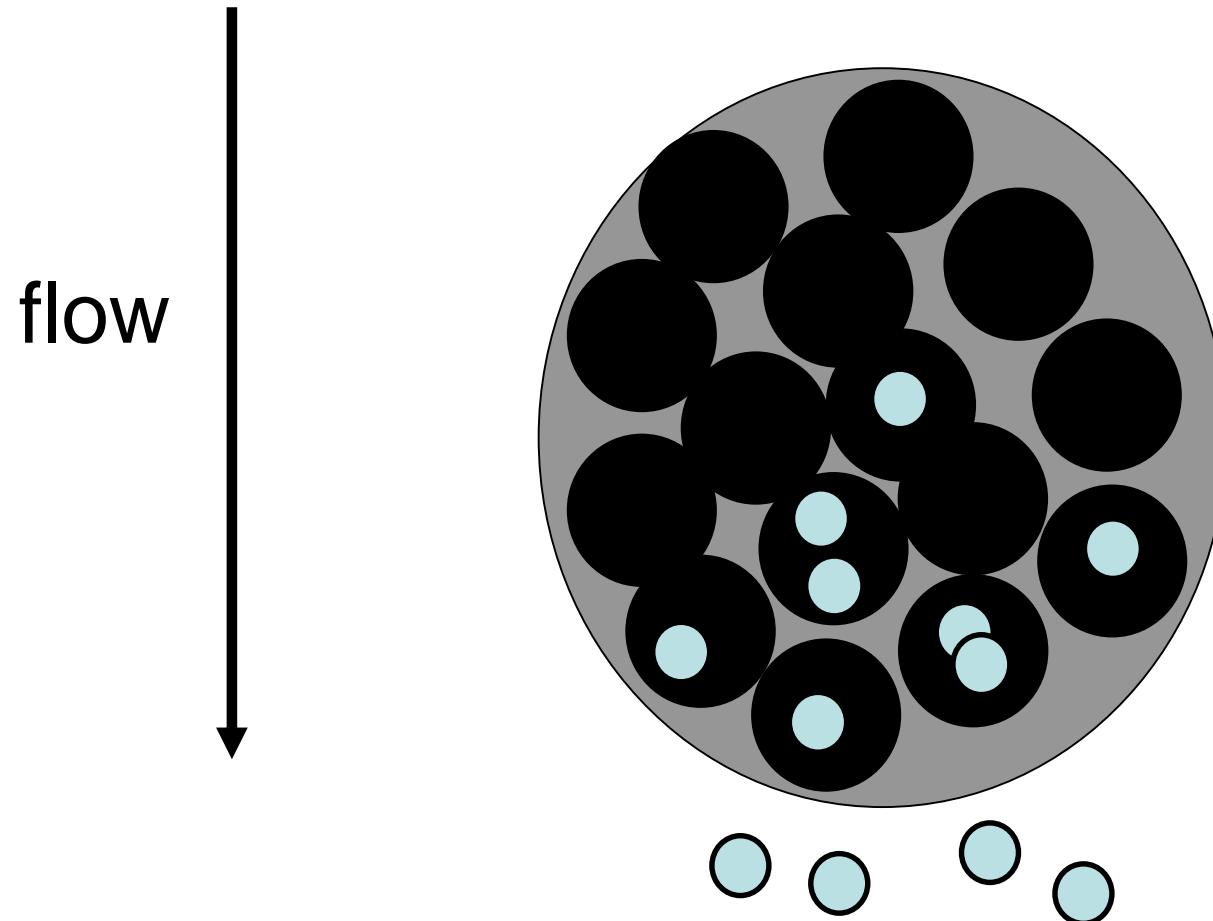
Gel filtration chromatography



Gel filtration chromatography



Gel filtration chromatography



Design of Column

- Column size
 - Analytical or preparative
- Solvent
 - Inert matrix most solvents OK
- Matrix
 - Most important consideration
 - Many different types
 - Material
 - Pore size

Some Commonly Used Gel Filtration Materials

Name ^a	Type	Fractionation Range (kD)
Sephadex G-10	Dextran	0.05–0.7
Sephadex G-25	Dextran	1–5
Sephadex G-50	Dextran	1–30
Sephadex G-100	Dextran	4–150
Sephadex G-200	Dextran	5–600
Bio-Gel P-2	Polyacrylamide	0.1–1.8
Bio-Gel P-6	Polyacrylamide	1–6
Bio-Gel P-10	Polyacrylamide	1.5–20
Bio-Gel P-30	Polyacrylamide	2.4–40
Bio-Gel P-100	Polyacrylamide	5–100
Bio-Gel P-300	Polyacrylamide	60–400
Sepharose 6B	Agarose	10–4,000
Sepharose 4B	Agarose	60–20,000
Sepharose 2B	Agarose	70–40,000
Bio-Gel A-5	Agarose	10–5000
Bio-Gel A-50	Agarose	100–50,000
Bio-Gel A-150	Agarose	1000–150,000

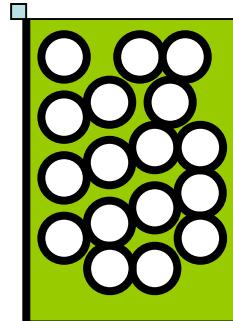
^aSephadex and Sepharose gels are products of Amersham Pharmacia Biotech; Bio-Gel gels are manufactured by BioRad Laboratories.

Table I shows some properties of different types of Sephadexes.

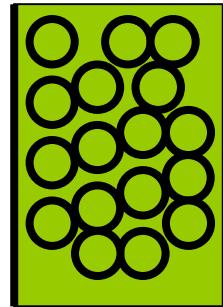
Sephadex type	Water absorbtion [ml/g dry mass]	Molecular weight fractionation range [daltons]
G - 10	1.0	to 700
G - 15	1.5	to 1 500
G - 25	2.5	1 000 – 5 000
G - 50	5.0	1 500 – 30 000
G - 75	7.5	3 000 – 70 000
G - 100	10.0	4 000 – 150 000
G - 150	15.0	5 000 – 400 000
G - 200	20.0	5 000 – 800 000

Blue Dextran is a high-molecular-weight glucose polymer (original mol wt $2 \times 10(6)$ g/mol) containing covalently bonded Reactive **Blue 2** dye (approximately mmol/g **dextran**). This **blue** dye is known for its high binding affinity to a wide variety of proteins, with a particularly high affinity for serum albumin.

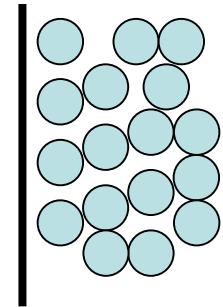
Column Parameters



V_o = void volume



V_t = total volume



V_i = volume of solvent held in the pores. This is normally approximated to
 $V_t - V_o$ = volume of beads

V_o = Elution volume of a large “totally excluded” molecule such as blue dextran

V_t = Physical volume of column

Gel filtration chromatography

- The molecular mass of the smallest molecule unable to penetrate the pores of the gel is the **exclusion limit**.
- The exclusion limit is a function of molecular shape, since elongated molecules are less likely to penetrate a gel pore than other shapes.
- Behavior of the molecule on the gel can be quantitatively characterized.

Total bed volume of the column

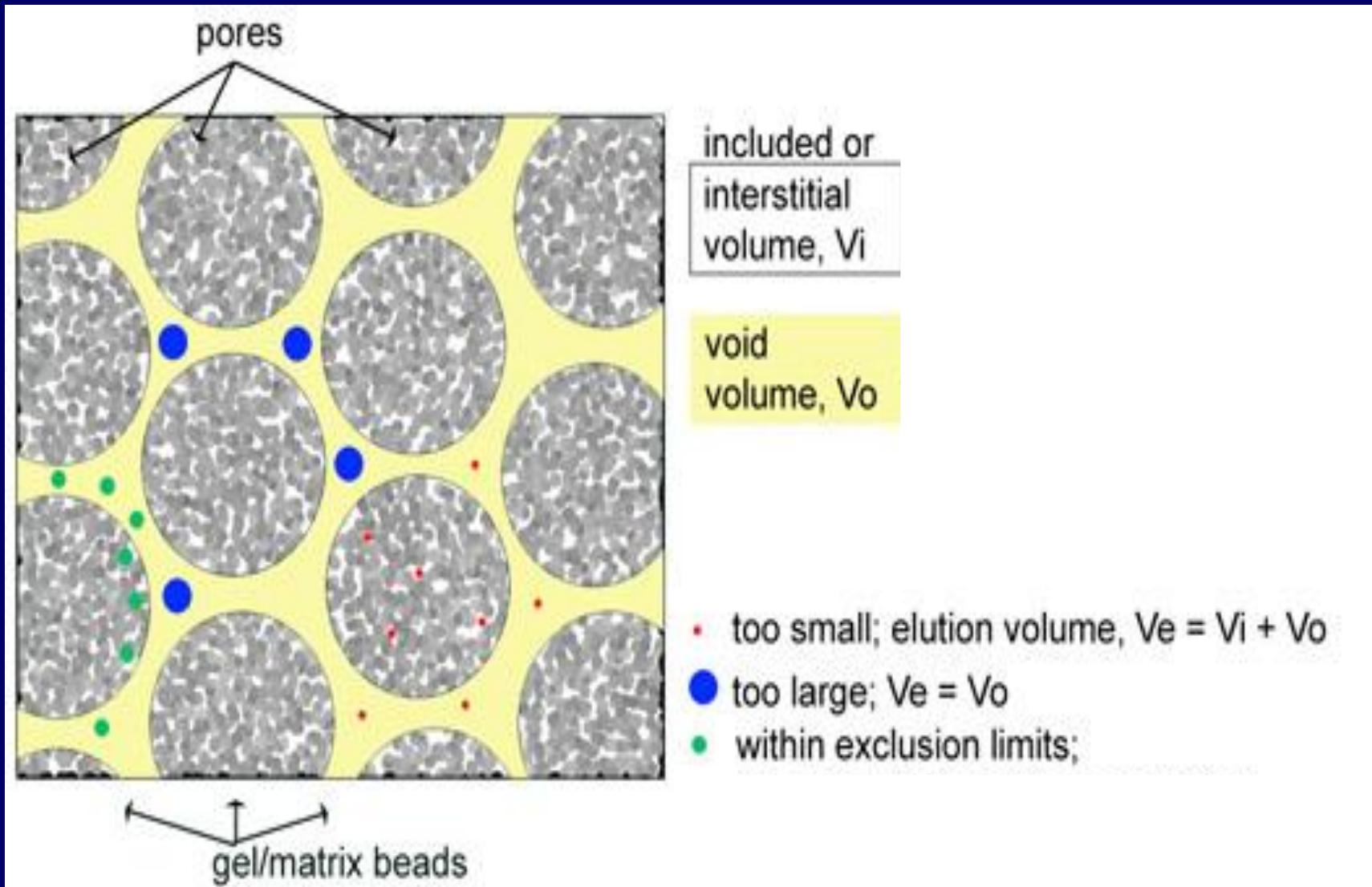
$$V_t = V_i + V_0$$

V_i = internal volume occupied by gel beads

V_0 = volume of solvent space surrounding gel; Typically 35%

Gel filtration chromatography

- **Elution volume (V_e)** is the volume of a solvent required to elute a given solute from the column after it has first contacted the gel.
- **Relative elution volume (V_e/V_0)** is the behavior of a particular solute on a given gel that is independent of the size of the column.
- This effectually means that molecules with molecular masses ranging below the exclusion limit of a gel will elute from a gel in the order of their molecular masses with the largest eluting first.



Partition coefficient (Kd)

- **Kd:** is the partition coefficient for solute, (the extent to which the molecules can penetrate the pores in stationary phase)
- its value range between 0 and 1

$$Kd = \frac{V_e - V_o}{V_i}$$

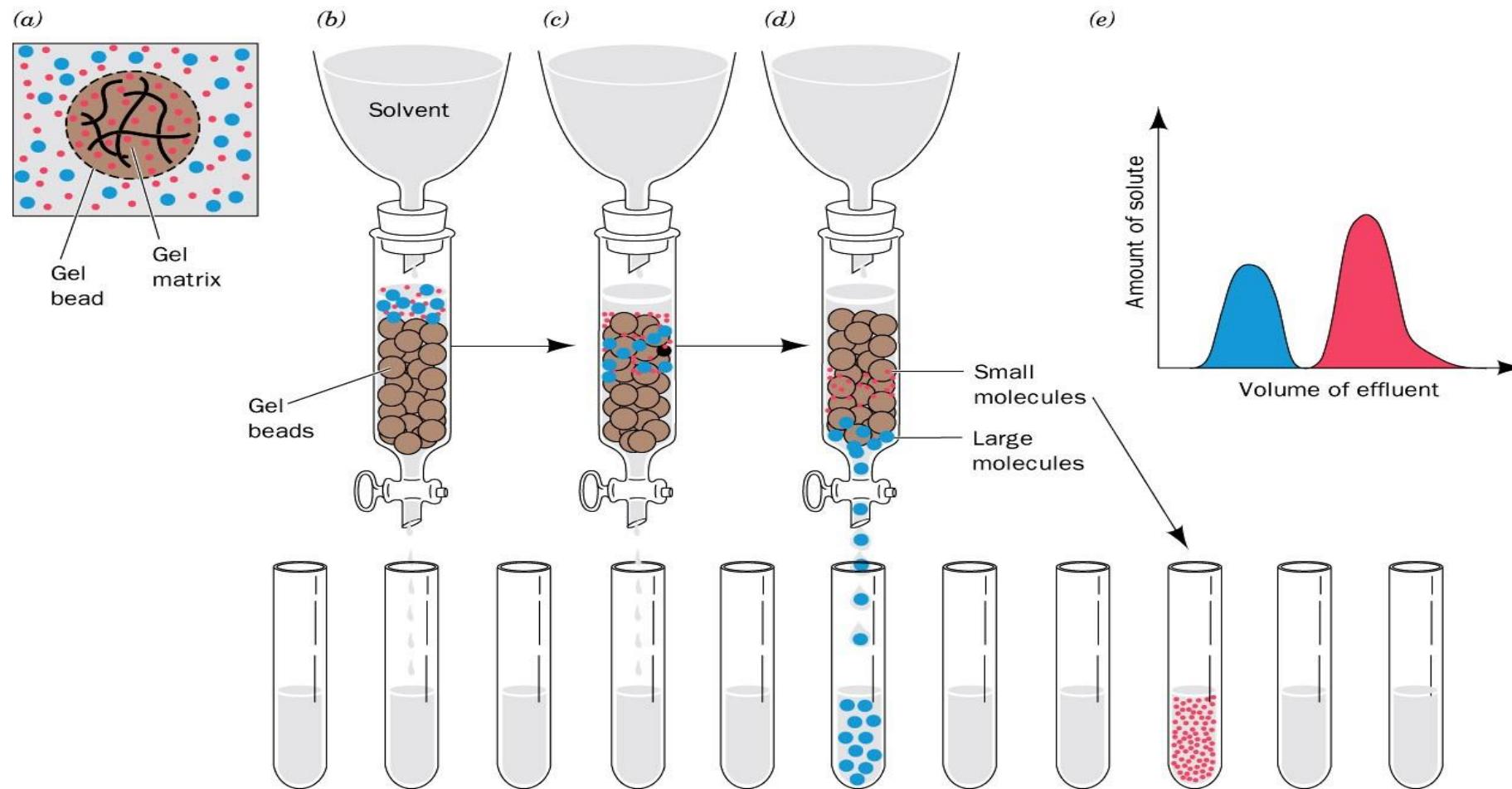
- it is difficult to measure V_i precisely, the equation modified to determine available part of the resin (Kav).

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

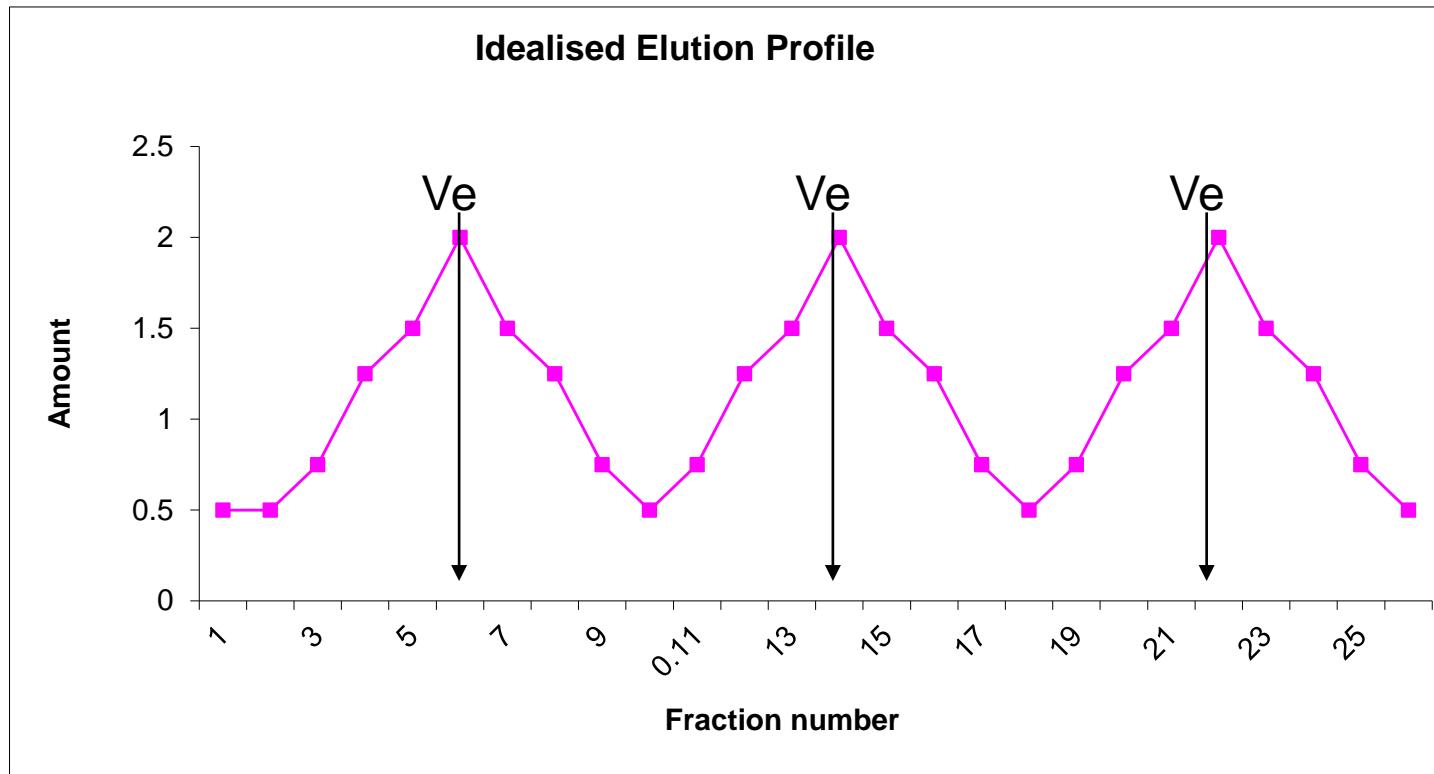
- Sample components (solutes) are easily separated if their (Kav) value different from each other.

Kav = proportion of pores available to the molecule.

Gel filtration chromatography



Elution Profile

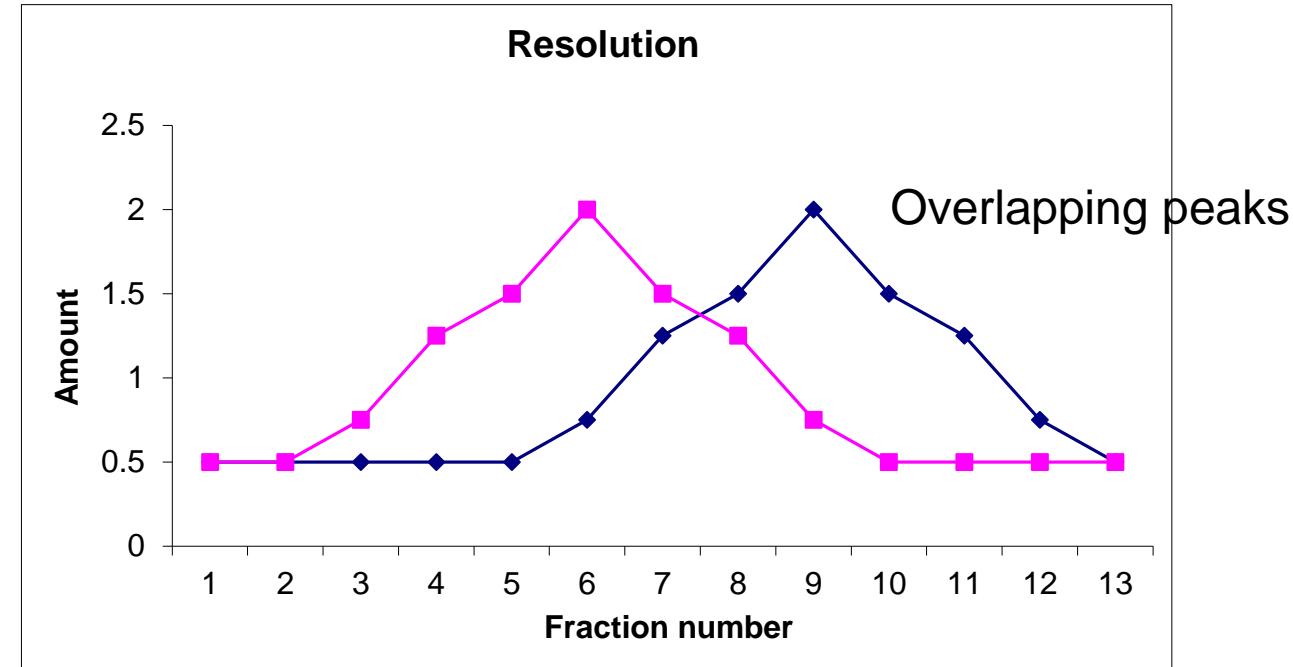


V_e = Elution volume (volume of solvent between injection and elution). Dictated by proportion of porous matrix available to molecules (K_d).

Bubbles in the column would affect the resolution

Resolution

Soak column properly
Maintain flow while running the column



Resolution proportional to square root of column length. Also affected by rate at which column is run.

Calculation of V_e

For a molecule that can partially enter the pores:

$$V_e = V_o + K_d (V_i)$$

$$\text{or } V_e = V_o + K_{av} (V_t - V_o)$$

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

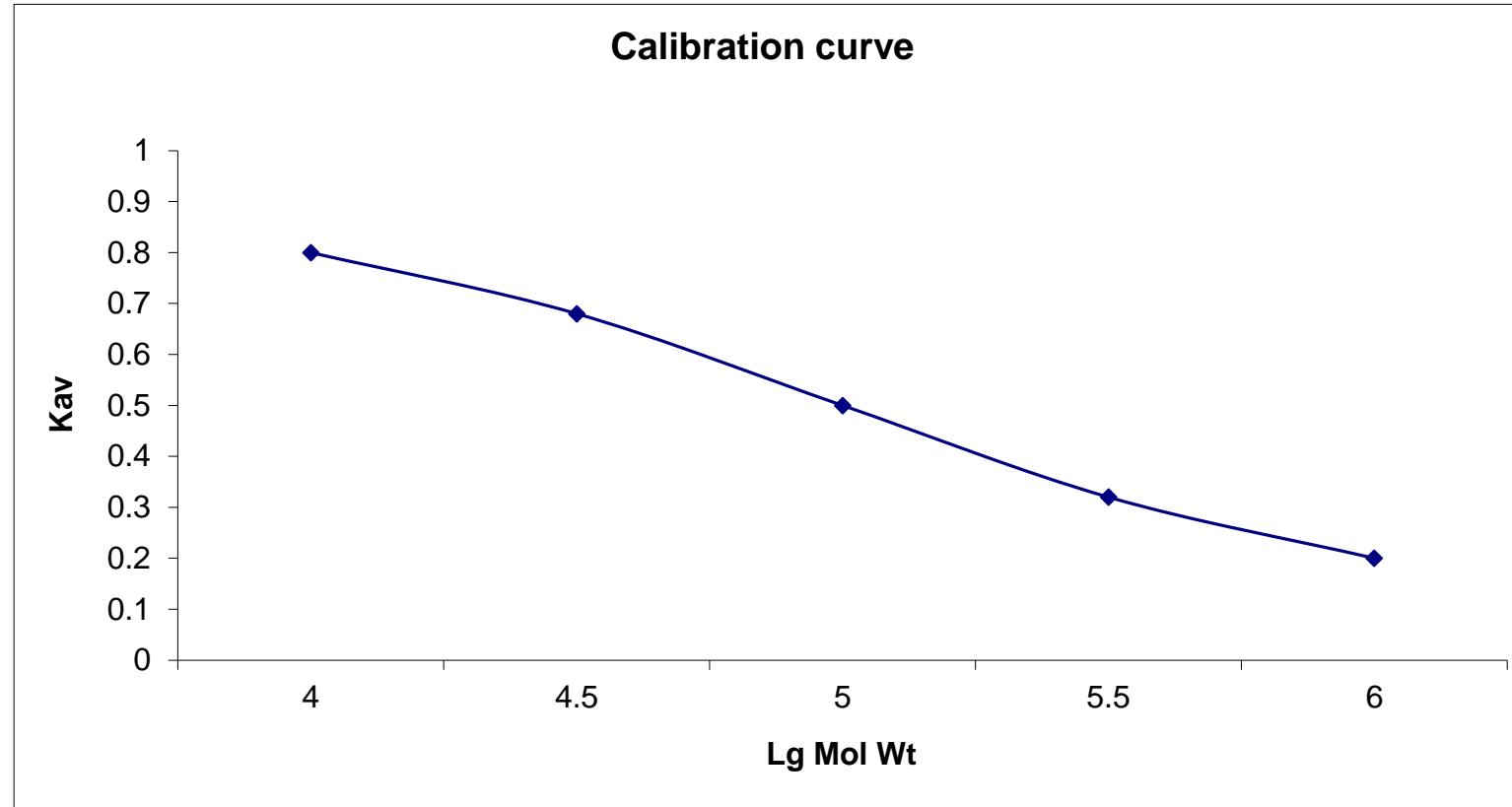
K_{av} = proportion of pores available to the molecule.

Totally “excluded” $K_{av} = 0$ and $V_e = V_o$

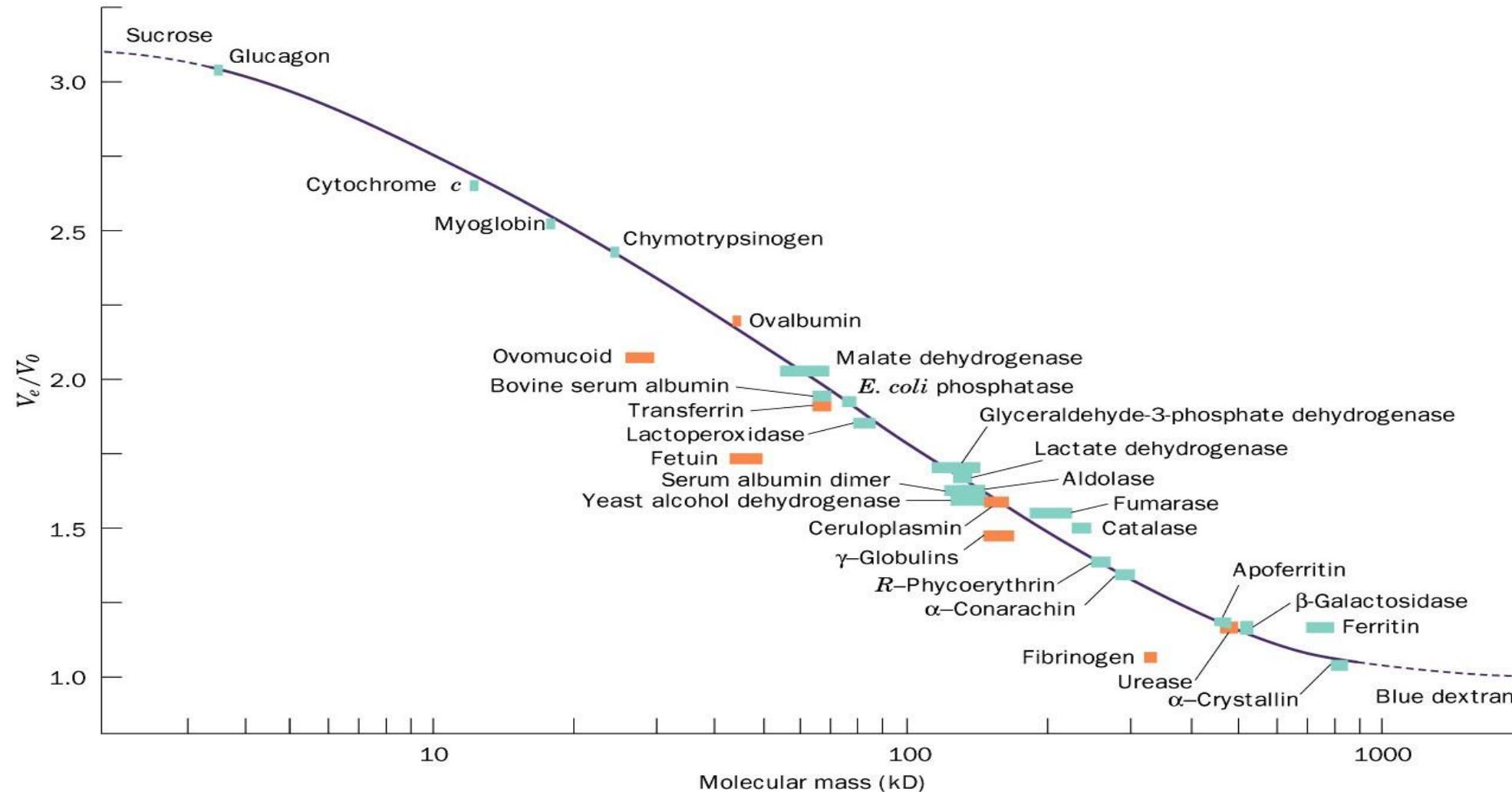
Totally “included” $K_{av} = 1$ and $V_e = V_t$

Determination of Molecular Weight

- Calibrate column with known standards
- Plot K_av against lg Mol Wt



Molecular mass determination by gel filtration chromatography.



Optimising chromatographic separations

Complete separation required

Minimise possibility of overlapping peaks

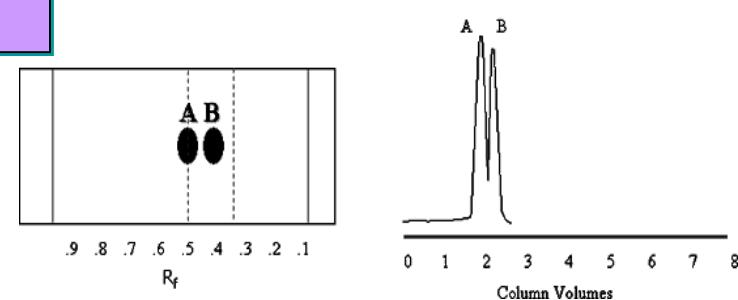
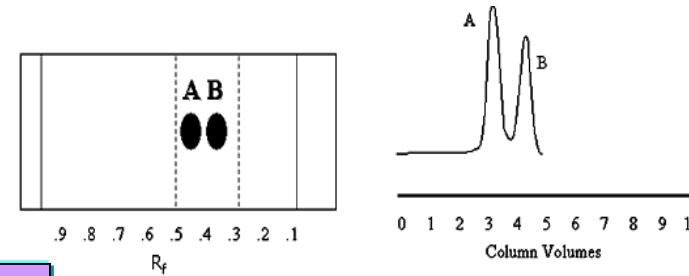
Resolution depends on:

Selectivity of column

Large distance between two peaks

Band broadening properties

Peaks as narrow as possible



Optimising chromatographic separations

Resolution influenced by:

Amount of each substance

Large differences in amounts may be problematic

Separation flow-rate

Faster for smaller molecules to prevent diffusion

Ensure correct packing

Chromatography: Detection & Analysis

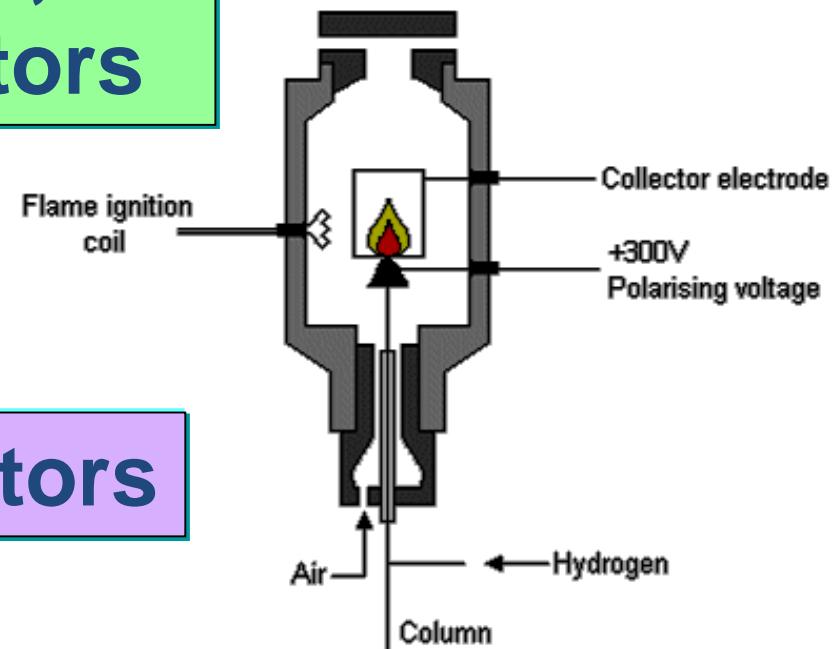
Require **HIGH** sensitivity & **MINIMAL** noise

Liquid chromatography detectors

**UV/Vis, fluorescence,
electrochemical detectors**

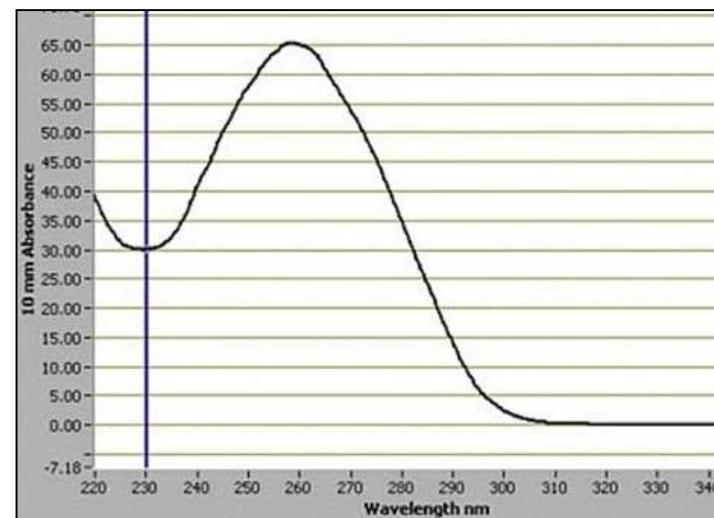
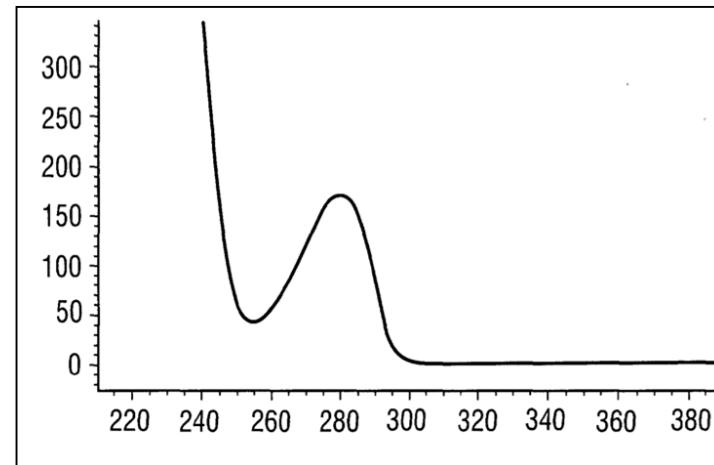
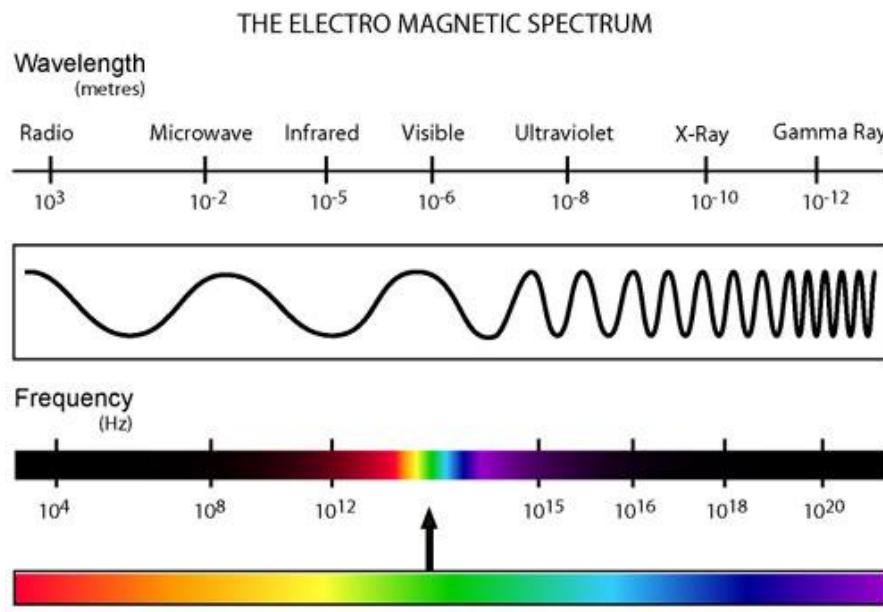
Gas chromatography detector

Flame ionisation detectors



UV/Vis

- Versatile, sensitive & stable
- Fixed vs. variable wavelength detector
- Proteins **280 nm**
- Nucleic acids **260 nm**
- Buffer may create noise





Fluorescence



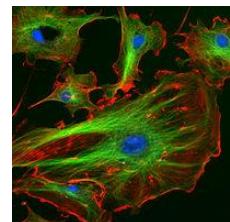
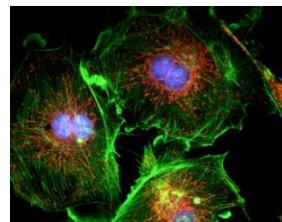
Natural fluorescence

Amino acids, vitamins, nucleic acids, porphyrins

Principle of Fluorescence

Fluorophore is excited by light of certain wavelength & emits light of longer wavelength as it returns to ground state

Protein fluorophores



Excitation

Emission

Tryptophan: 295; 330 - 360 nm

Tyrosine: 280; 310 nm

LC Detectors

Refractive Index Detector

UV/Vis Absorbance Detector

Fluorescence Detector

Conductivity Detector

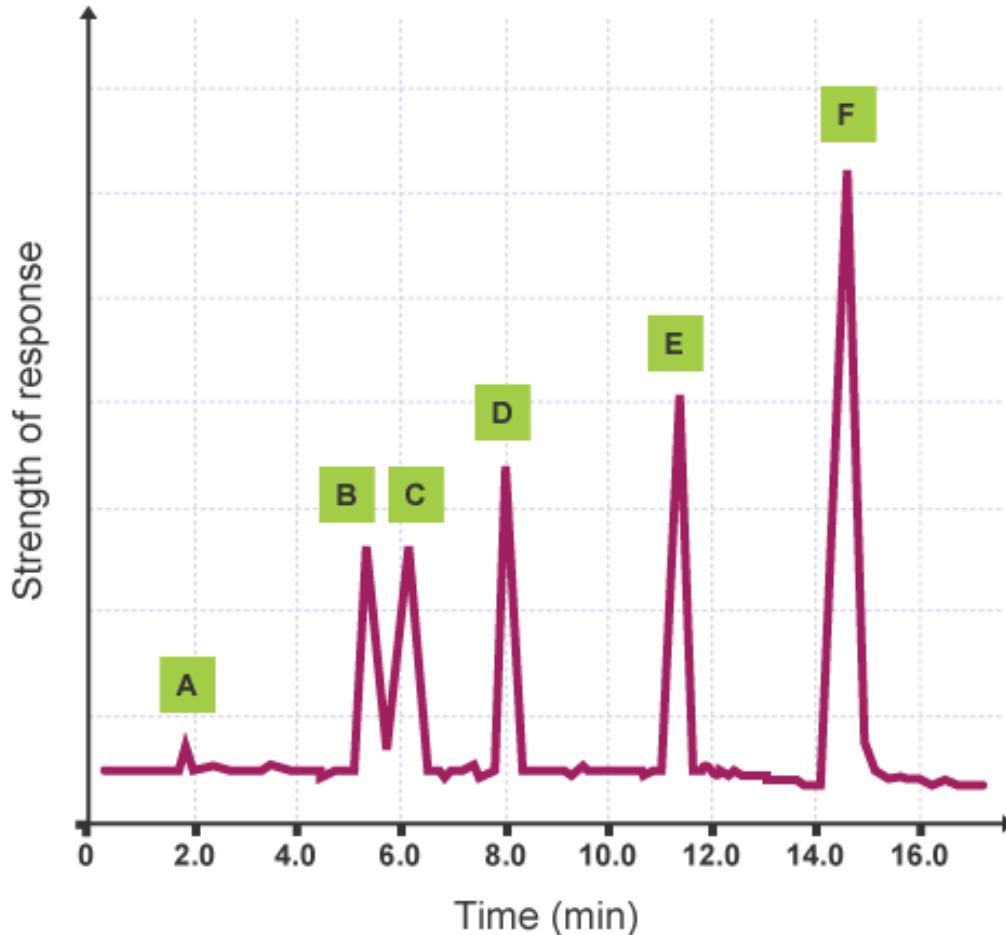
Electrochemical Detector

Detector	Selectivity	Sensitivity	Notes
Refractive Index	Poor	Poor	Any component that differs in refractive index from the eluate can be detected, despite its low sensitivity. Cannot be used to perform gradient analysis.
UV/Vis	Moderate	Good	A wide variety of substances can be detected that absorb light from 190 to 900 nm. Sensitivity depends strongly on the component.
Fluorescence	Good	Excellent	Components emitting fluorescence can be detected selectively with high sensitivity. This is often used for pre-column and post-column derivatization.
Conductivity	Moderate	Good	Ionized components are detected. This detector is used mainly for ion chromatography.
Electrochemical	Good	Excellent	Electric currents are detected that are generated by electric oxidation-reduction reactions. Electrically active components are detected with high sensitivity.

As in GC, the choice of detector will depend on the analyte and how the method is being used (*i.e.*, analytical or preparative scale)

Term	Definition
Solvent	Mobile liquid phase with no affinity to the stationary phase (i.e. inert towards it) & no effect on solutes.
Developer	Any liquid with more affinity to the stationary phase than the solvent but less than solutes and just capable to move them through the column.
Effluent	Any liquid that passes out of the column.
Eluent	Any liquid that has lesser affinity to the stationary phase than solutes but is capable to move them out of the column.
Eluate	Fraction of eluent containing a required specific substance.
Retention volume (V_R)	(or retardation volume): Volume of mobile phase that passes out of the column, before elution of a specific substance.

Understanding Chromatogram



- Substance A was present in the **smallest quantity** (it has the **smallest peak**)
- Substance A had the **shortest retention time**
- Substances B and C were present in **equal amounts**
- Substance F had the **longest retention time**
- Substance F was present in the **greatest quantity** (it has the **largest peak**)
- Substance F had the **greatest affinity** for the stationary phase

Quantitative analysis

- Determine response of the detector to test and reference substances- response factor (r)

$$r = \frac{\text{peak area (or height) of test substance}}{\text{peak area (height) of reference substance}}$$

Quantify the amount of test substance (Qt)

$$Qt = \frac{\text{peak area (or height) of test substance}}{\text{peak area (height) of reference substance}} \times (Q_r/r)$$

Q_r = known amount of the reference compound

