# Chromatography

# Importance

- Chromatography has application in every branch of the physical and biological sciences
  - 12 Nobel prizes were awarded between 1937 and 1972 alone for work in which chromatography played a vital role

# An Introduction to Chromatography

- What IS 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

**Chromatography is a physical method of separation in which the components to be separated are distributed between two phases**

**one of which is stationary (stationary phase) while the other (the mobile phase) moves through it in a definite direction.**

**The chromatographic process occurs due to differences in the distribution constant of the individual sample components.**

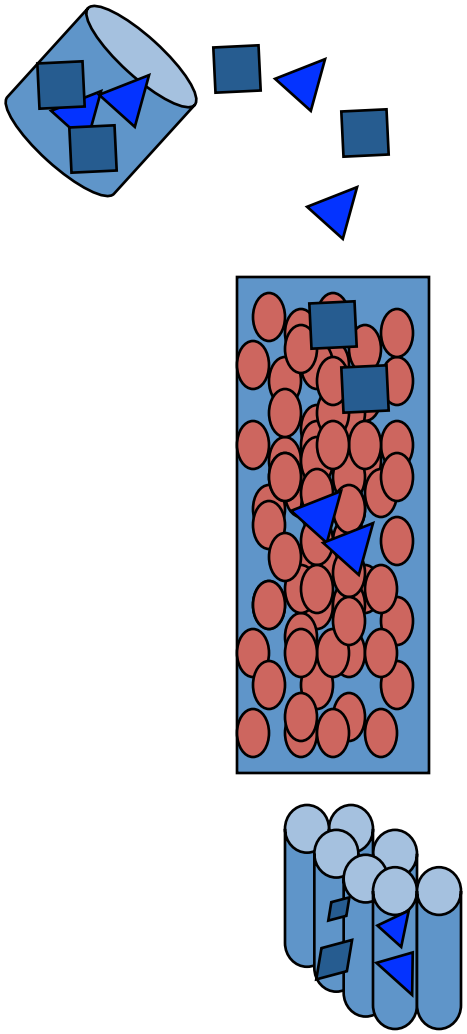
# Chromatography

**Is a technique used to separate and identify the components of a mixture.**

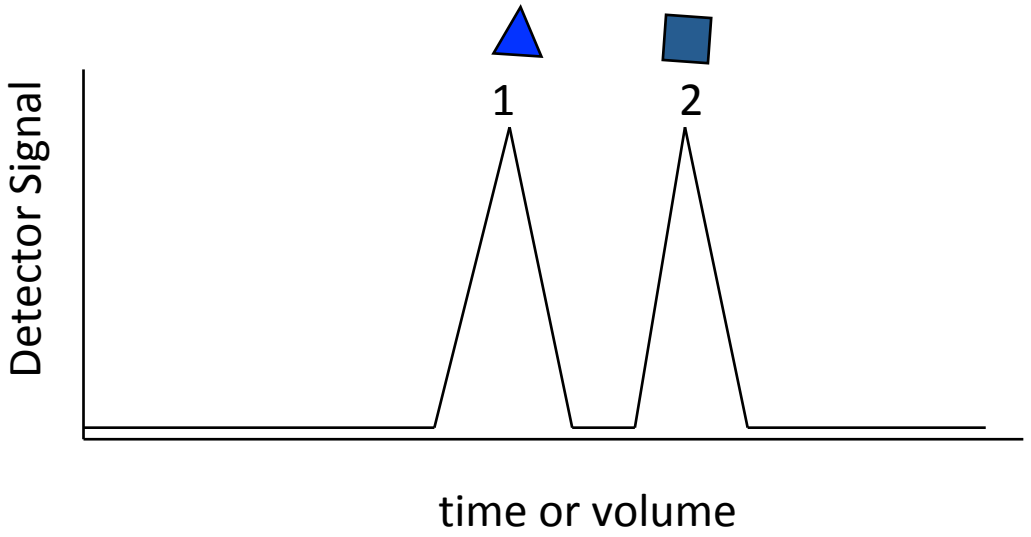
**Works by allowing the molecules present in the mixture to distribute themselves between a stationary and a mobile medium.**

**Molecules that spend most of their time in the mobile phase are carried along faster.**

# Chromatography



Chromatogram - Detector signal vs. retention time or volume



# Purpose of Chromatography

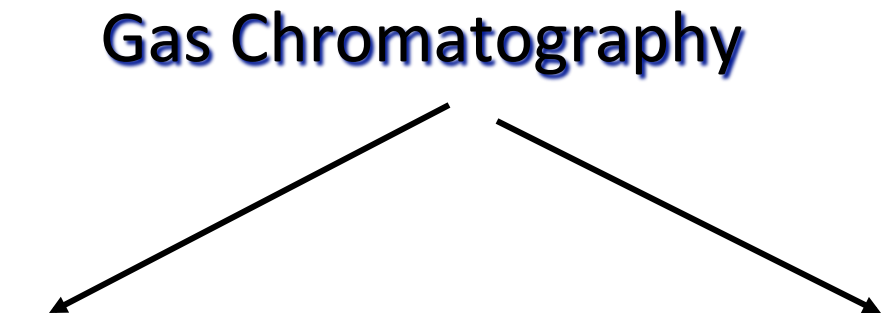
- **Analytical** - determine chemical composition of a sample

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

# Classification of Methods

- There are two classification schemes:
  - mobile phase
  - attractive forces

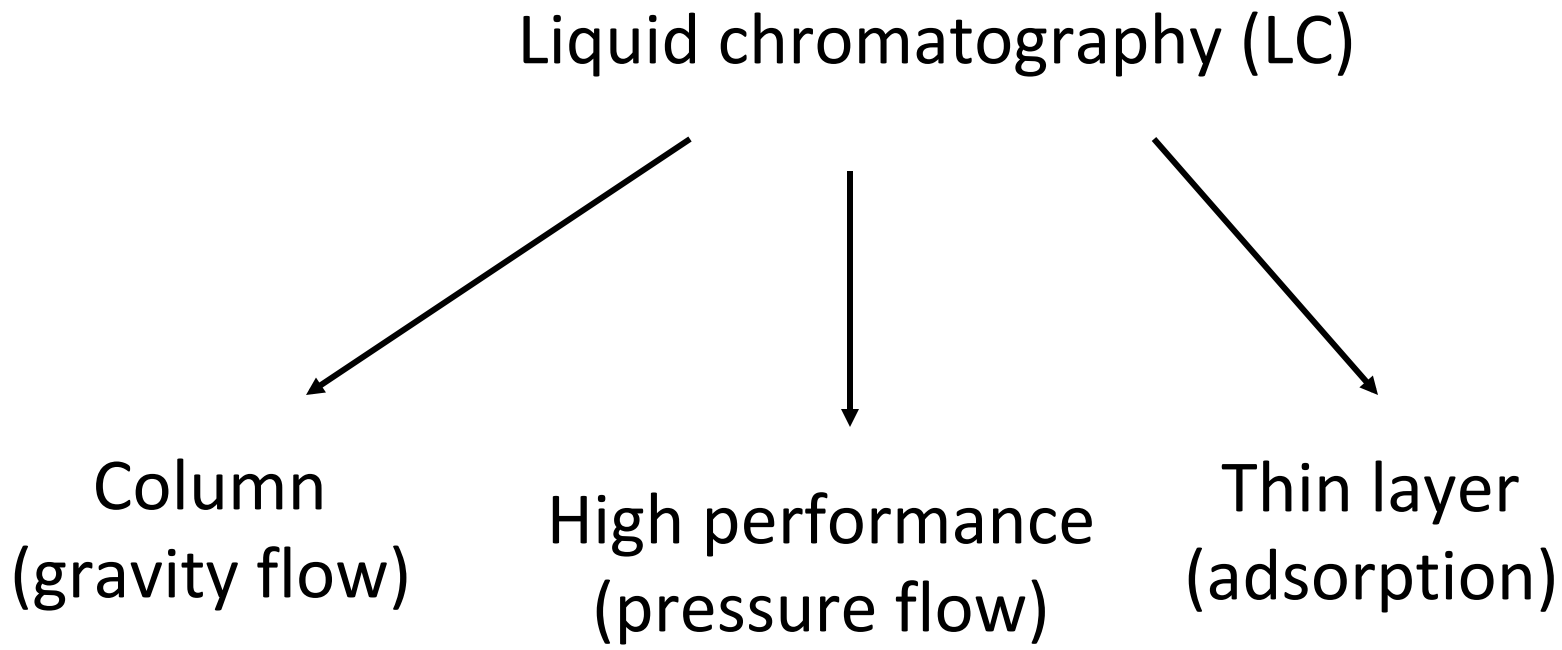
# Classification based on Mobile Phase




Pyrolysis GC -
heat solid materials
to 500 - 1000$^0$C
so they decompose
into gaseous products

Sample MUST be volatile at temperatures BELOW 350$^0$C

# Classification based on Mobile Phase



# Classification based on Attractive Forces

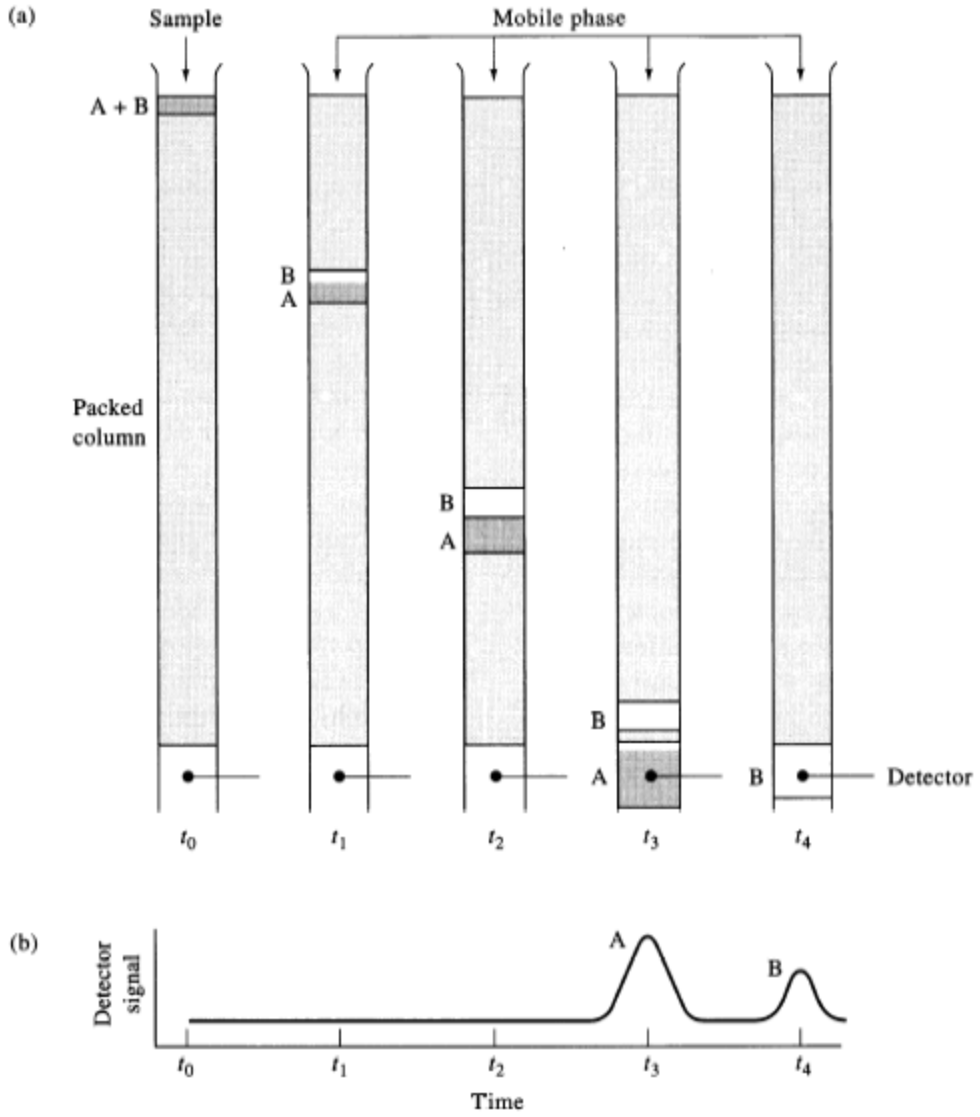
- Adsorption - for polar non-ionic compounds
- Ion Exchange - for ionic compounds
  - Anion - analyte is anion; bonded phase has positive charge
  - Cation – analyte is cation; bonded phase has negative charge
- Partition - based on the relative solubility of analyte in mobile and stationary phases
  - Normal – analyte is nonpolar organic; stationary phase MORE polar than the mobile phase
  - Reverse – analyte is polar organic; stationary phase LESS polar than the mobile phase
- Size Exclusion - stationary phase is a porous matrix; sieving

*Components:*

mobile phase: a solvent that flows through the supporting medium

stationary phase: a layer or coating on the supporting medium that interacts with the analytes

supporting medium: a solid surface on which the stationary phase is bound or coated




The analytes interacting most strongly with the stationary phase will take longer to pass through the system than those with weaker interactions.

These interactions are usually chemical in nature, but in some cases physical interactions can also be used.

*Types of Chromatography:* - chromatography can be classified based on the type of mobile phase, stationary phase and support material

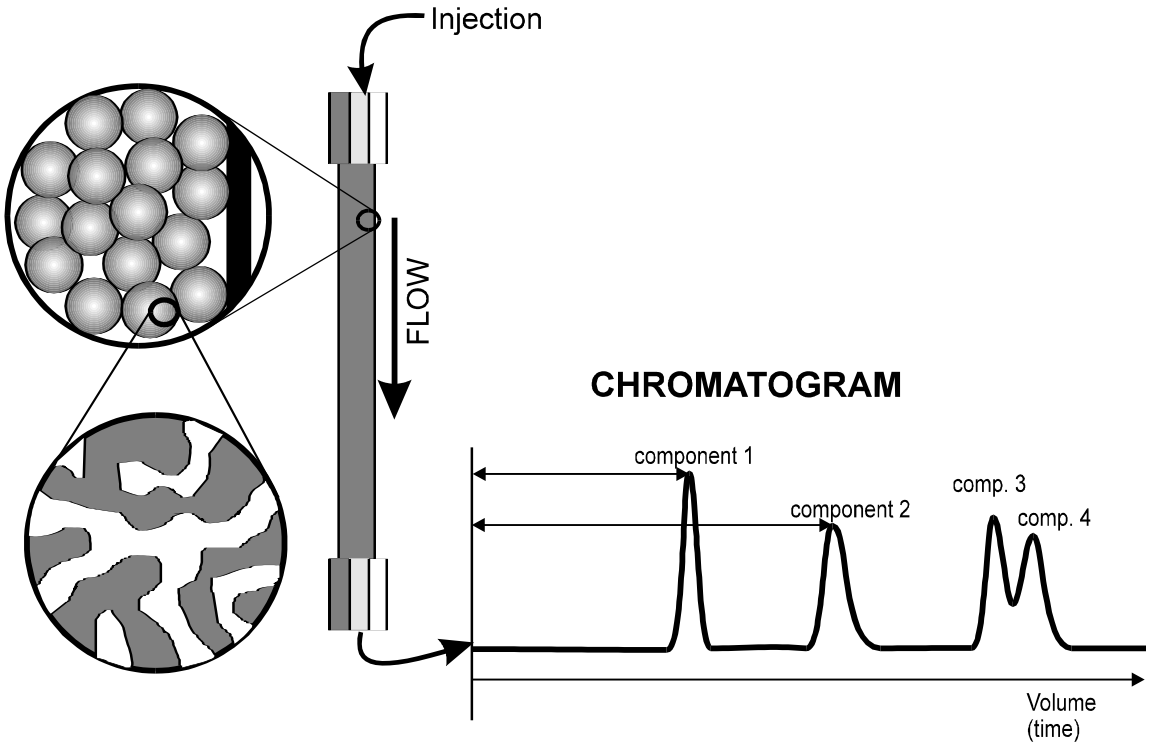
**TABLE 26-1 Classification of Column Chromatographic Methods**

| 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<br>Size exclusion | Ion-exchange resin<br>Liquid in interstices of a polymeric solid | Ion exchange<br>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 |

# *Types of Chromatography*

## Liquid Chromatography

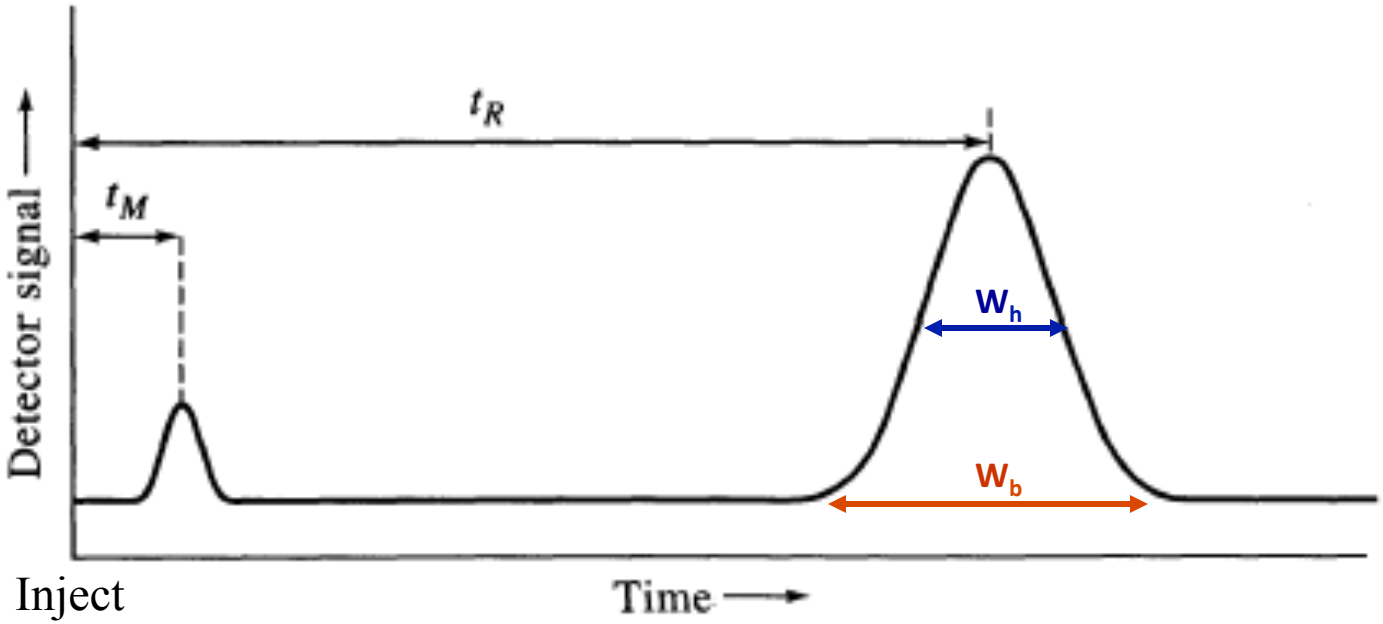
| Name of LC Method | Type of Stationary Phase |
|---|---|
| Adsorption chromatography | solid, underivatized support |
| Partition chromatography | liquid-coated or derivatized support |
| Ion-exchange chromatography | support containing fixed charges |
| Size exclusion chromatography | porous support |
| Affinity chromatography | support with immobilized ligand |



## *Theory of Chromatography*

Typical response obtained by chromatography (i.e., a chromatogram):

chromatogram - concentration versus elution time



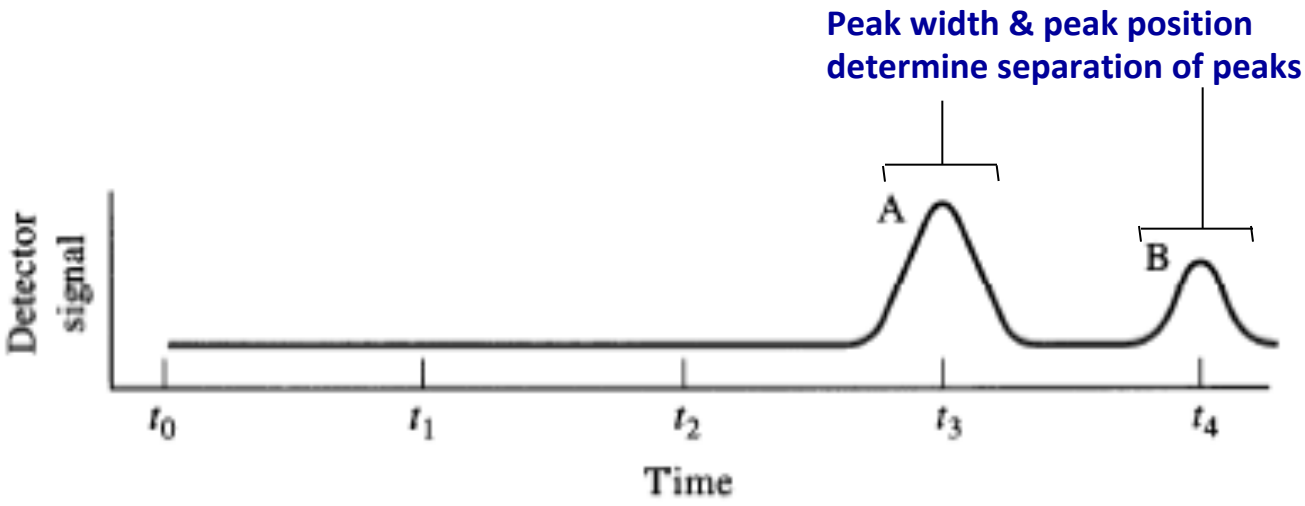

Where:

$t_R$ = retention time
$t_M$ = void time
$W_b$ = baseline width of the peak in time units
$W_h$ = half-height width of the peak in time units

*Note*: The separation of solutes in chromatography depends on two factors:

(a) a difference in the retention of solutes (i.e., a difference in their time or volume of elution

(b) a sufficiently narrow width of the solute peaks (i.e, good efficiency for the separation system)




A similar plot can be made in terms of elution volume instead of elution time. If volumes are used, the volume of the mobile phase that it takes to elute a peak off of the column is referred to as the *retention volume* ($V_R$) and the amount of mobile phase that it takes to elute a non-retained component is referred to as the *void volume* ($V_M$).

Solute Retention:

A solute's retention time or retention volume in chromatography is directly related to the strength of the solute's interaction with the mobile and stationary phases.

Retention on a given column pertain to the particulars of that system:

- size of the column
- flow rate of the mobile phase

$$\text{average migration rate} \quad \bar{v} = \frac{L}{t_R} \qquad \text{column length}$$

*Capacity factor (k')*: more universal measure of retention, determined from $t_R$ or $V_R$.

$$k' = (t_R - t_M)/t_M$$

or

$$k' = (V_R - V_M)/V_M$$

capacity factor is useful for comparing results obtained on different systems since it is independent on column length and flow-rate.

The value of the capacity factor is useful in understanding the retention mechanisms for a solute, since the fundamental definition of k' is:

$$k' = \frac{\text{moles A}_{\text{stationary phase}}}{\text{moles A}_{\text{mobile phase}}}$$

*k' is directly related to the strength of the interaction between a solute with the stationary and mobile phases.*

Moles $A_{\text{stationary phase}}$ and moles $A_{\text{mobile phase}}$ represents the amount of solute present in each phase at equilibrium.

Equilibrium is achieved or approached at the center of a chromatographic peak.

When k' is **#** 1.0, separation is poor
When k' is > 30, separation is slow
When k' is = 2-10, separation is optimum

A simple example relating k' to the interactions of a solute in a column is illustrated for partition chromatography:

$$\text{A (mobile phase)} \underset{}{\overset{K_D}{\rightleftharpoons}} \text{A (stationary phase)}$$

where: $K_D$ = equilibrium constant for the distribution of A between the mobile phase and stationary phase

Assuming local equilibrium at the center of the chromatographic peak:

$$k' = \frac{[A]_{\text{stationary phase}}\,\text{Volume}_{\text{stationary phase}}}{[A]_{\text{mobile phase}}\,\text{Volume}_{\text{mobile phase}}}$$

$$k' = K_D\,\frac{\text{Volume}_{\text{stationary phase}}}{\text{Volume}_{\text{mobile phase}}}$$

*As $K_D$ increases, interaction of the solute with the stationary phase becomes more favorable and the solute's retention (k') increases*

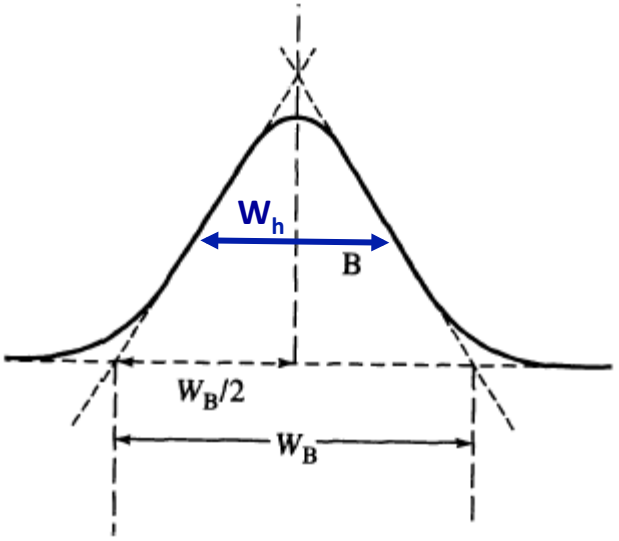
Efficiency:

Efficiency is related <u>experimentally</u> to a solute's peak width.

- an efficient system will produce narrow peaks
- narrow peaks → smaller difference in interactions in order to separate two solutes

Efficiency is related <u>theoretically</u> to the various kinetic processes that are involved in solute retention and transport in the column

- determine the width or standard deviation ($\sigma$) of peaks




Estimate $\sigma$ from peak widths, assuming Gaussian shaped peak:

$$W_b = 4\sigma$$

$$W_h = 2.354\sigma$$

*Dependent on the amount of time that a solute spends in the column (k' or $t_R$)*

*Number of theoretical plates (N)*: compare efficiencies of a system for solutes that have different retention times

$$N = (t_R/\sigma)^2$$

or for a Gaussian shaped peak

$$N = 16\,(t_R/W_b)^2$$

$$N = 5.54\,(t_R/W_h)^2$$

The larger the value of N is for a column, the better the column will be able to separate two compounds.

- the better the ability to resolve solutes that have small differences in retention
- N is independent of solute retention
- N is dependent on the length of the column

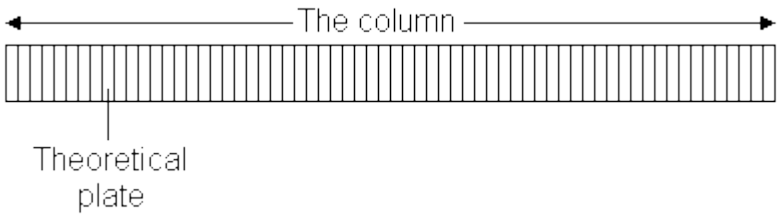


*Plate height or height equivalent of a theoretical plate (H or HETP)*: compare efficiencies of columns with different lengths:

$$H = L/N$$

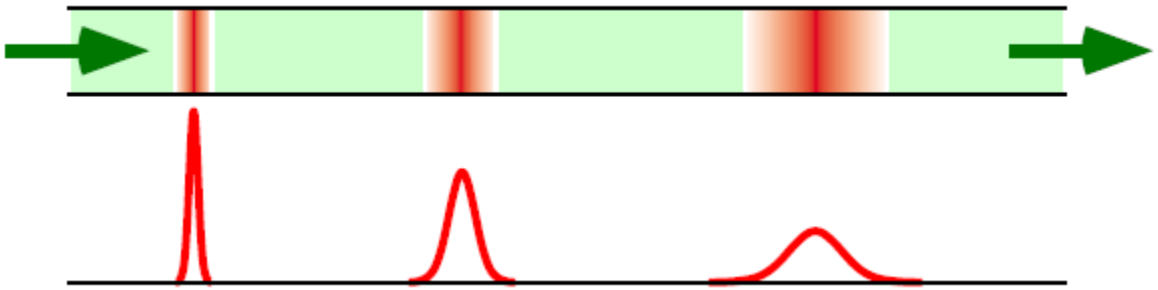
where: L = column length
N = number of theoretical plates for the column

*Note*: H simply gives the length of the column that corresponds to one theoretical plate

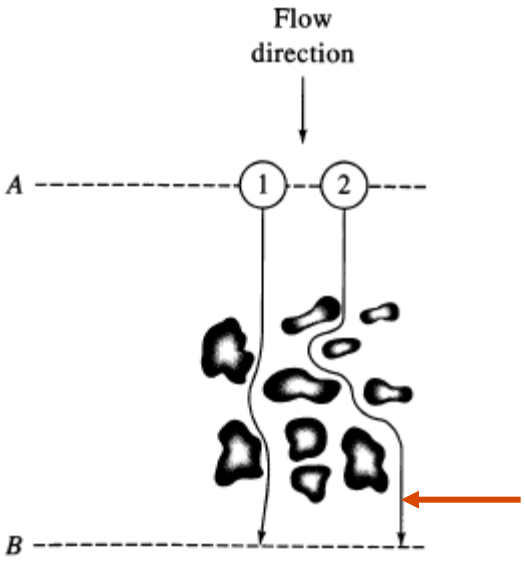
H can be also used to relate various chromatographic parameters (e.g., flow rate, particle size, etc.) to the kinetic processes that give rise to peak broadening:

*Why Do Bands Spread?*

a. Eddy diffusion
b. Mobile phase mass transfer
c. Stagnant mobile phase mass transfer
d. Stationary phase mass transfer
e. Longitudinal diffusion

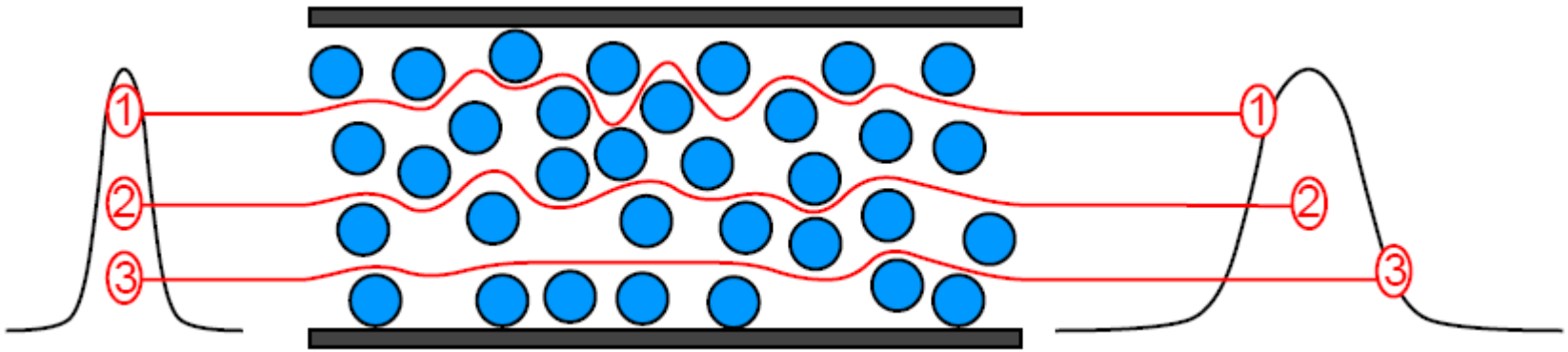


a.) *Eddy diffusion* – a process that leads to peak (band) broadening due to the presence of multiple flow paths through a packed column.

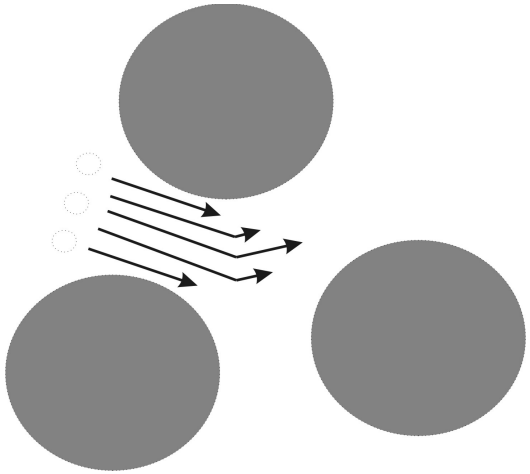



As solute molecules travel through the column, some arrive at the end sooner then others simply due to the different path traveled around the support particles in the column that result in different travel distances.

Longer path arrives at end of column after (1).



*b.) Mobile phase mass transfer* – a process of peak broadening caused by the presence of different flow profile within channels or between particles of the support in the column.

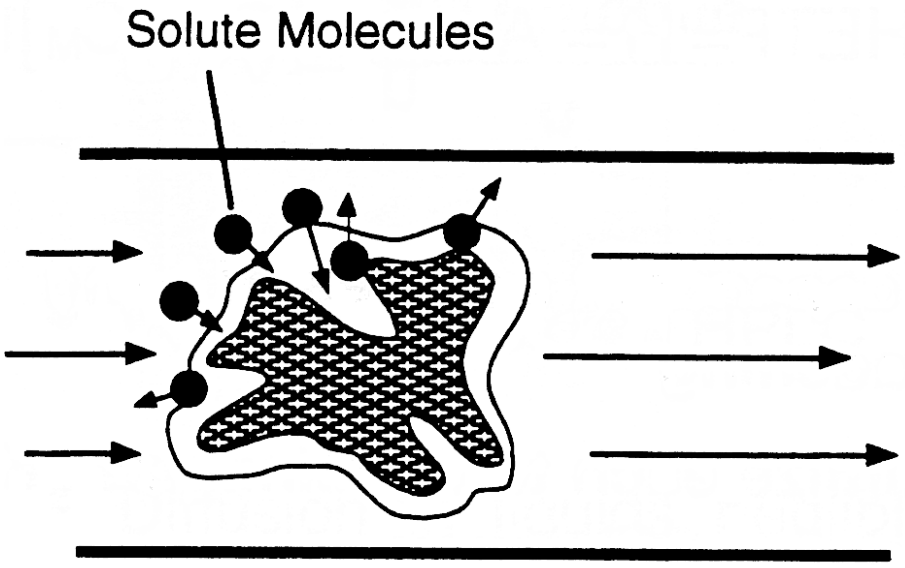


A solute in the center of the channel moves more quickly than solute at the edges, it will tend to reach the end of the channel first leading to band-broadening

The degree of band-broadening due to eddy diffusion and mobile phase mass transfer depends mainly on:

1) the size of the packing material
2) the diffusion rate of the solute

*c.) Stagnant mobile phase mass transfer* – band-broadening due to differences in the rate of diffusion of the solute molecules between the mobile phase outside the pores of the support (*flowing mobile phase*) to the mobile phase within the pores of the support (*stagnant mobile phase*).

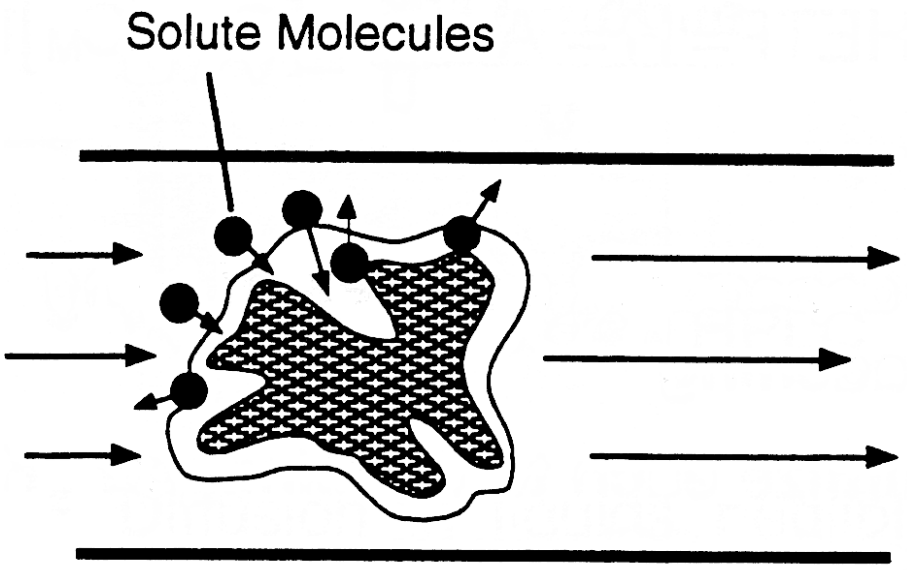



Since a solute does not travel down the column when it is in the stagnant mobile phase, it spends a longer time in the column than solute that remains in the flowing mobile phase.

The degree of band-broadening due to stagnant mobile phase mass transfer depends on:

1) the size, shape and pore structure of the packing material
2) the diffusion and retention of the solute
3) the flow-rate of the solute through the column

*d.) Stationary phase mass transfer* – band-broadening due to the movement of solute between the stagnant phase and the stationary phase.

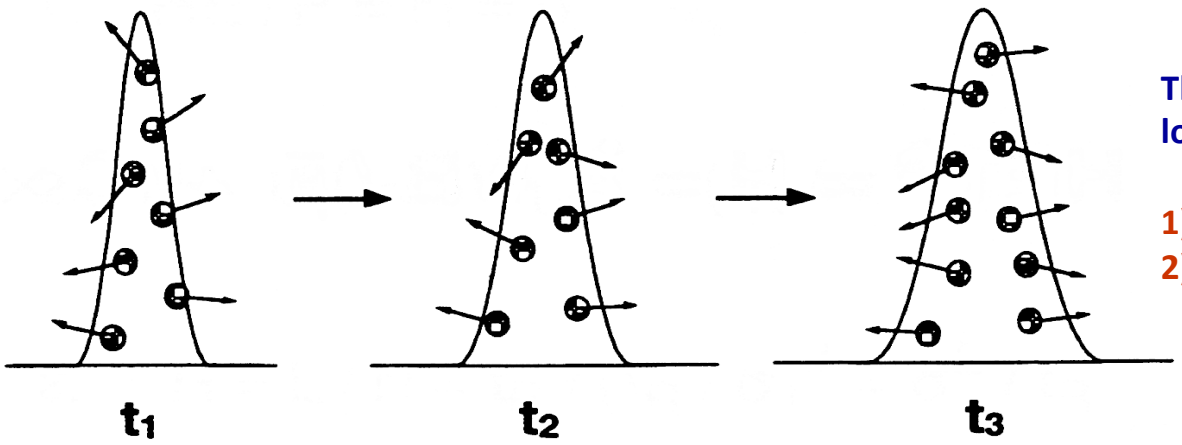



Since different solute molecules spend different lengths of time in the stationary phase, they also spend different amounts of time on the column, giving rise to band-broadening.

The degree of band-broadening due to stationary phase mass transfer depends on:

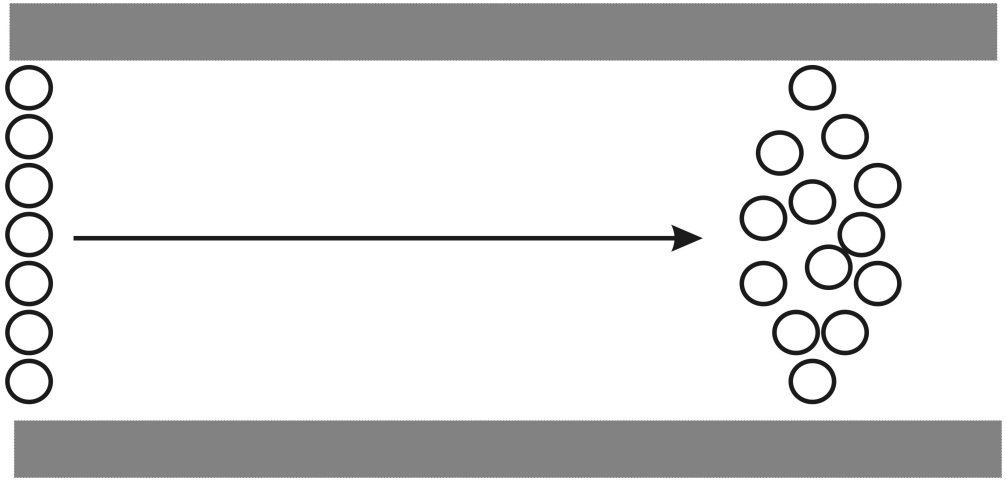
1) the retention and diffusion of the solute
2) the flow-rate of the solute through the column
3) the kinetics of interaction between the solute and the stationary phase

*e.) Longitudinal diffusion* – band-broadening due to the diffusion of the solute along the length of the column in the flowing mobile phase.




**The degree of band-broadening due to longitudinal diffusion depends on:**

**1) the diffusion of the solute**
**2) the flow-rate of the solute through the column**



Measures of Solute Separation:

separation factor ($\alpha$) – parameter used to describe how well two solutes are separated by a chromatographic system:

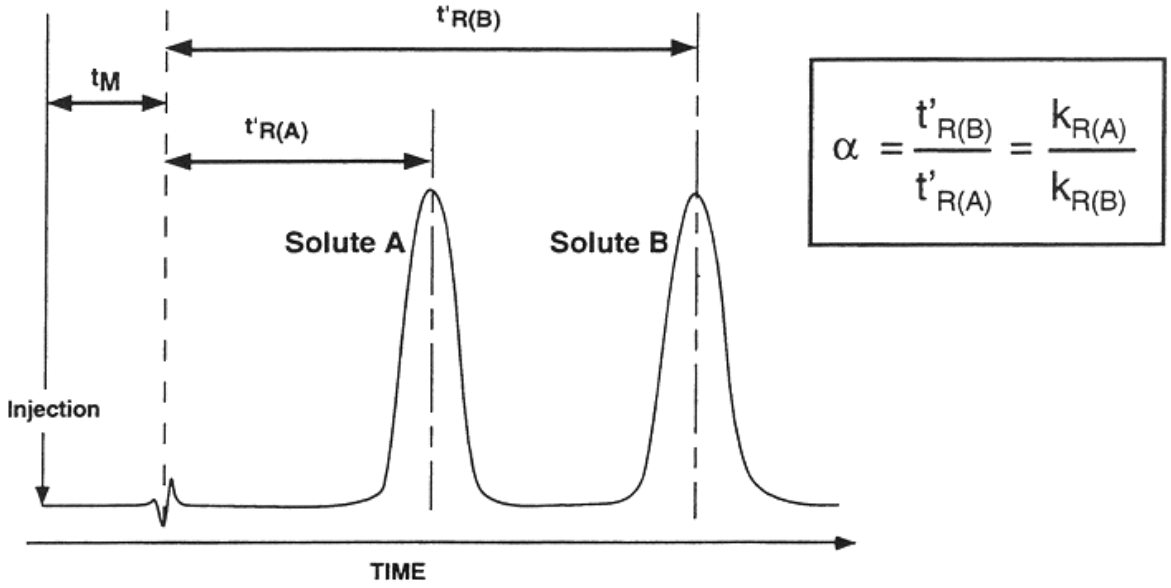
$$\alpha = k'_2/k'_1 \qquad k' = (t_R - t_M)/t_M$$

where:

$k'_1$ = the capacity factor of the first solute

$k'_2$ = the capacity factor of the second solute, with $k'_2 \geq k'_1$

A value of $\alpha \geq 1.1$ is usually indicative of a good separation




*Does not consider the effect of column efficiency or peak widths, only retention.*

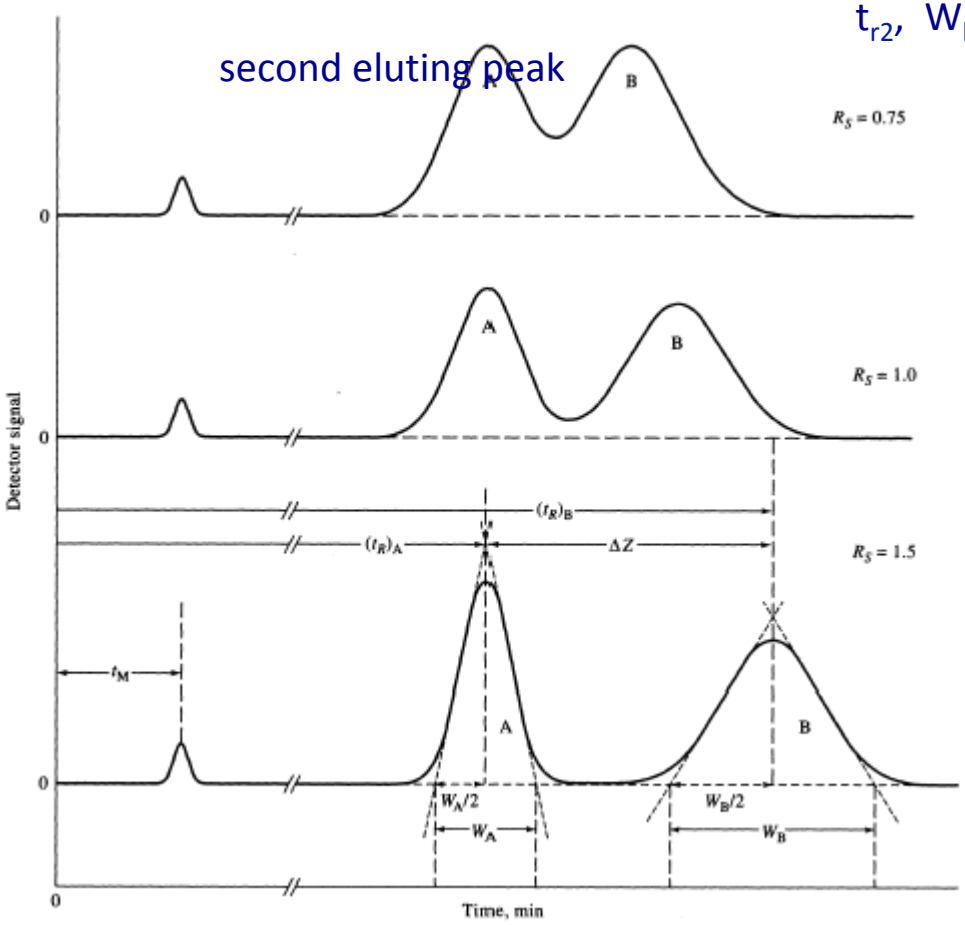
resolution ($R_s$) – resolution between two peaks is a second measure of how well two peaks are separated:

$$R_S = \frac{t_{r2} - t_{r1}}{(W_{b2} + W_{b1})/2}$$

where:

$t_{r1}$, $W_{b1}$ = retention time and baseline width for the first eluting peak

$t_{r2}$, $W_{b2}$ = retention time and baseline width for the second eluting peak




$R_s$ is preferred over $\alpha$ since both retention ($t_r$) and column efficiency ($W_b$) are considered in defining peak separation.

$R_s$ **$** 1.5 represents *baseline resolution*, or complete separation of two neighboring solutes → ideal case.

$R_s$ **$** 1.0 considered adequate for most separations.

**Classification of chromatography according to mobile phase:**

**1- Liquid chromatography: mobile phase is a liquid. (LLC, LSC).**

**2- Gas chromatography : mobile phase is a gas. (GSC, GLC).**

**Classification according to the packing of the stationary phase:**

**1- Thin layer chromatography (TLC): the stationary phase is a thin layer supported on glass, plastic or aluminium plates.**

**2- Paper chromatography (PC): the stationary phase is a thin film of liquid supported on an inert support.**

**3- Column chromatography (CC): stationary phase is packed in a glass column.**

## Classification according to the force of separation:

1- Adsorption chromatography.

2- Partition chromatography.

3- Ion exchange chromatography.

4- Gel filtration chromatography.

5- Affinity chromatography.

# 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.**

**Separations in TLC involve distributing a mixture of two or more substances between a stationary phase and a mobile phase.**

**The stationary phase:**

**is a thin layer of adsorbent (usually silica gel or alumina) coated on a plate.**

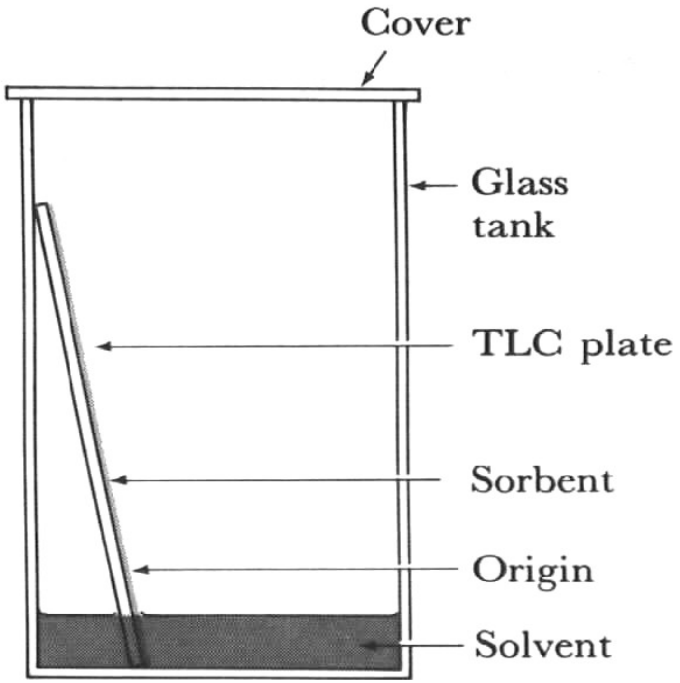
**The mobile phase:**

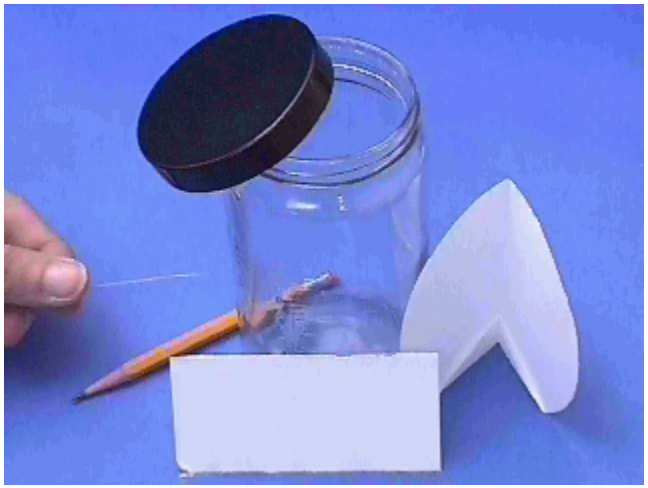
**is a developing liquid which travels up the stationary phase, carrying the samples with it.**

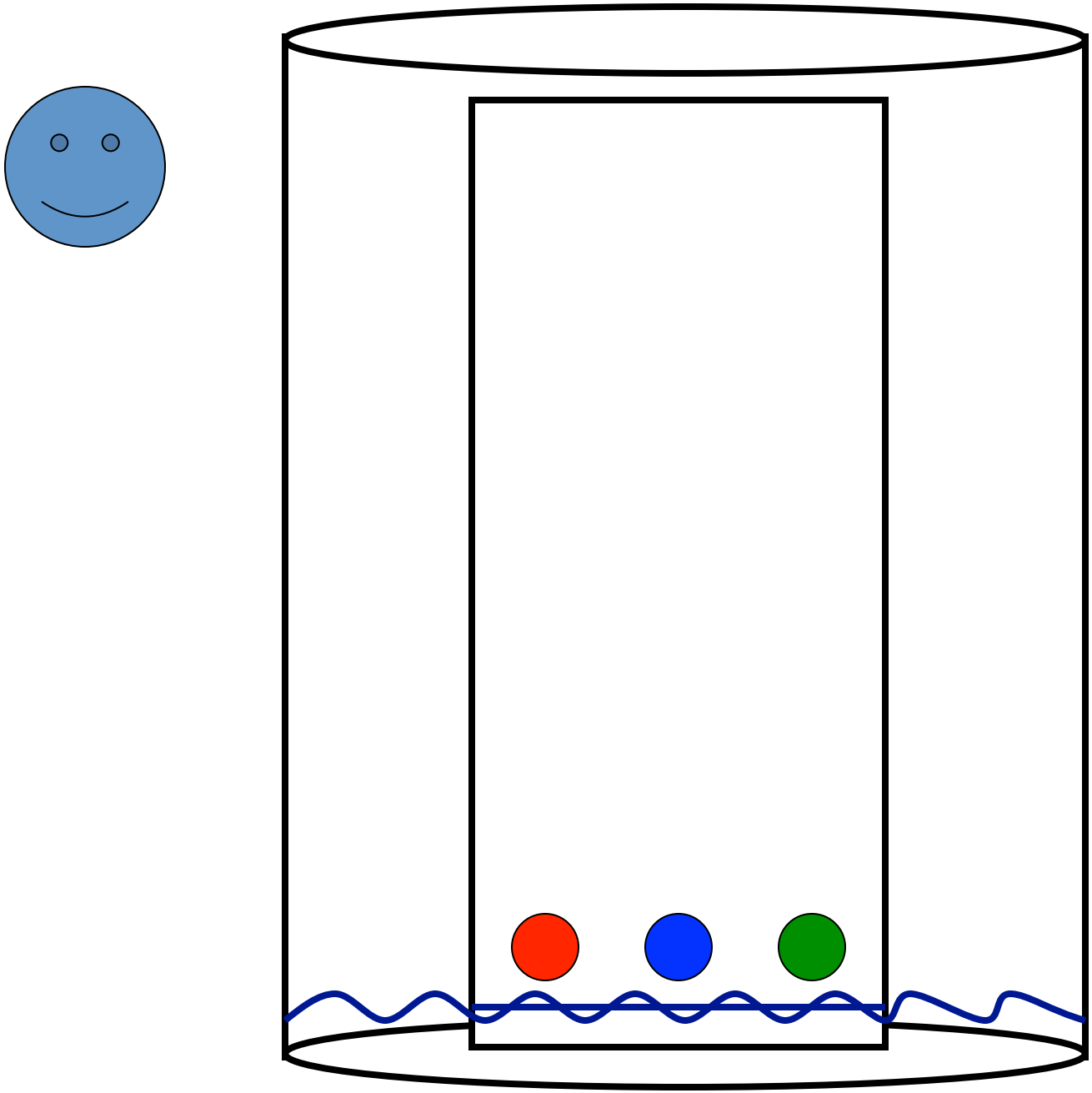
**Components of the samples will separate on the stationary phase according to**

**how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase.**

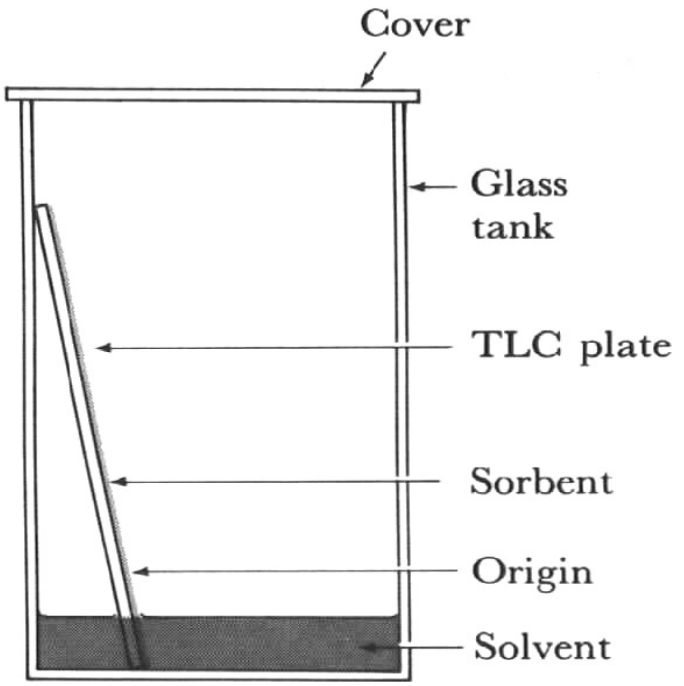
# Thin Layer Chromatography (TLC)

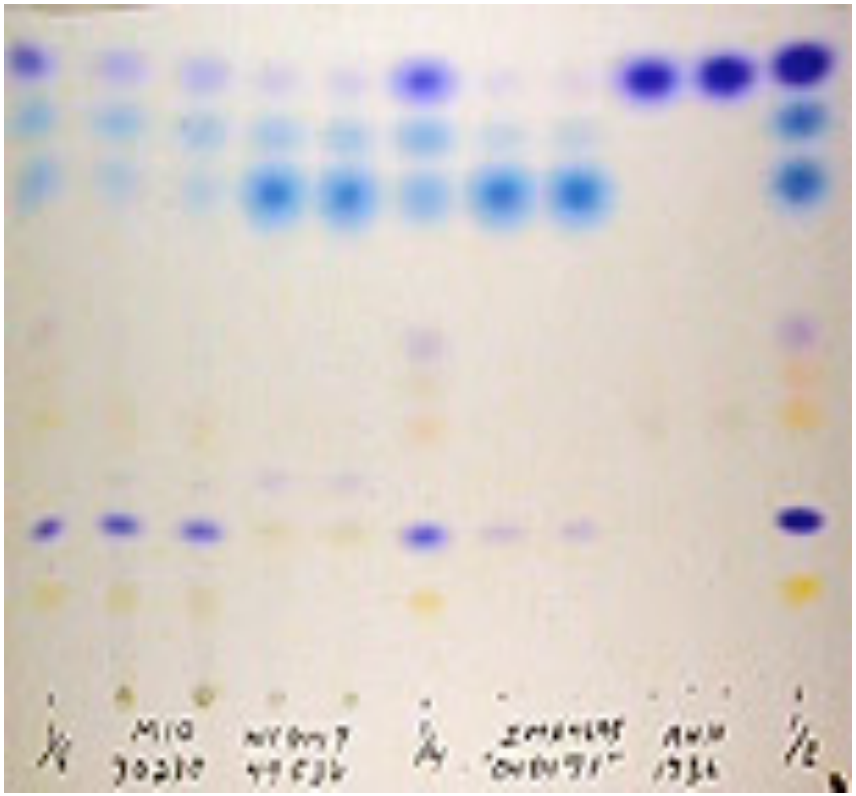




# TLC



# Preparing the Chamber

**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.**

# Preparing the Plates for Development

**With a pencil, etch two small notches into the adsorbent about 2 cm from the bottom of the plate.**

**The notches should be on the edges of the plate, and each notch should be the same distance up from the bottom of the plate.**

**The notches must be farther from the bottom of the plate than the depth of the solvent in the jar.**

**Using a drawn-out capillary tube, spot the samples on the plate so that they line up with the notches you etched.**

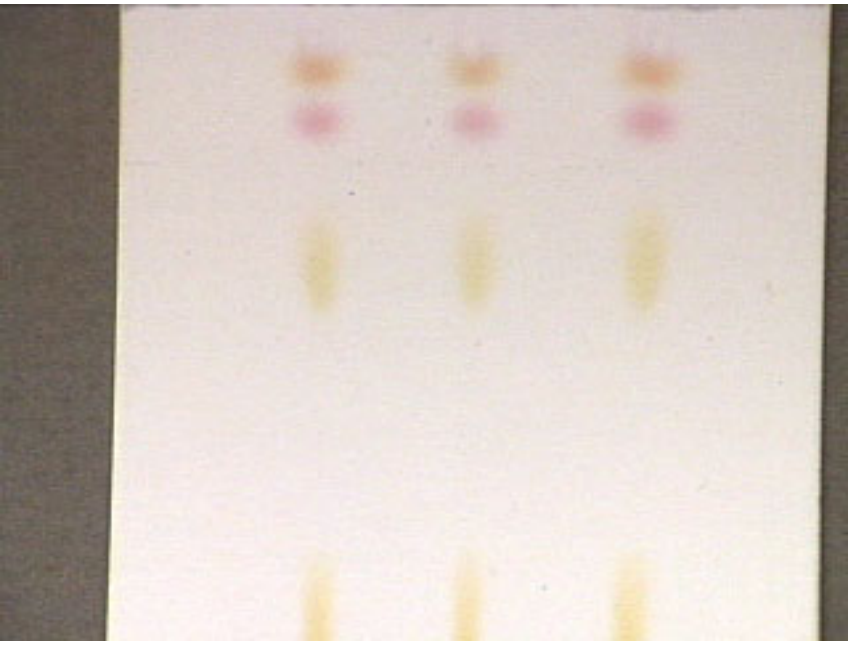
# Developing the Plates

**After preparing the development chamber and spotting the samples, the plates are ready for development.**

**Be careful to handle the plates only by their edges, and try to leave the development chamber uncovered for as little time as possible.**

**When the plates are removed from the chamber, quickly trace the solvent front (the highest solvent level on the plate) with a pencil.**

# 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, and many commercially made plates often contain a substance which aids in the visualization of compounds.**

# Visualizing Agents

Alkaloids:  Dragendorff’s reagent

Cardiac glycosides: Antimony trichloride

Sugar: Aniline phthalate

Amino acids: Ninhydrin

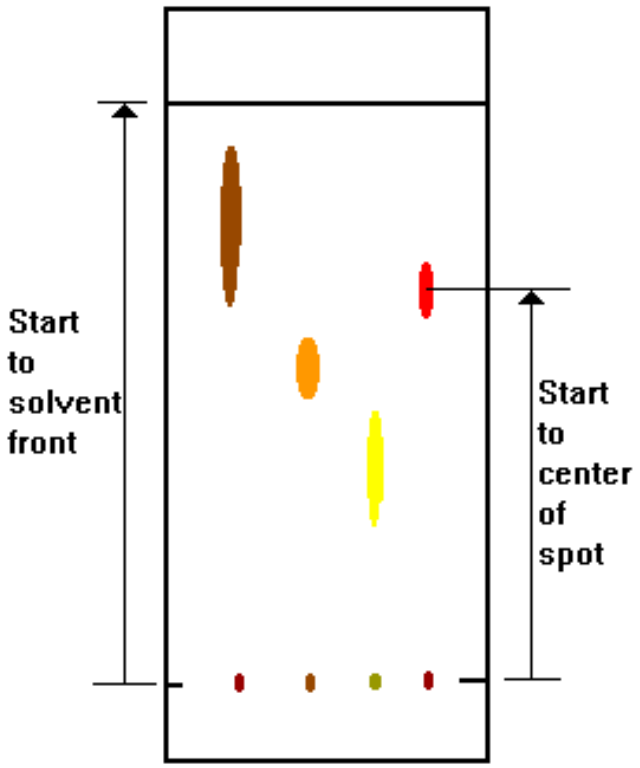
# Interpreting the Data

**The $R_f$ (retention factor) value for each spot should be calculated.**

**It is characteristic for any given compound on the same stationary phase using the same mobile phase for development of the plates.**

**Hence, known $R_f$ values can be compared to those of unknown substances to aid in their identifications.**

$$R_f = \frac{\text{Distance from start to center of substance spot}}{\text{Distance from start to solvent front}}$$



**(Note: $R_f$ values often depend on the temperature and the solvent used in the TLC experiment.**

**the most effective way to identify a compound is to spot known substances – authentic - next to unknown substances on the same plate.)**

**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

**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.**

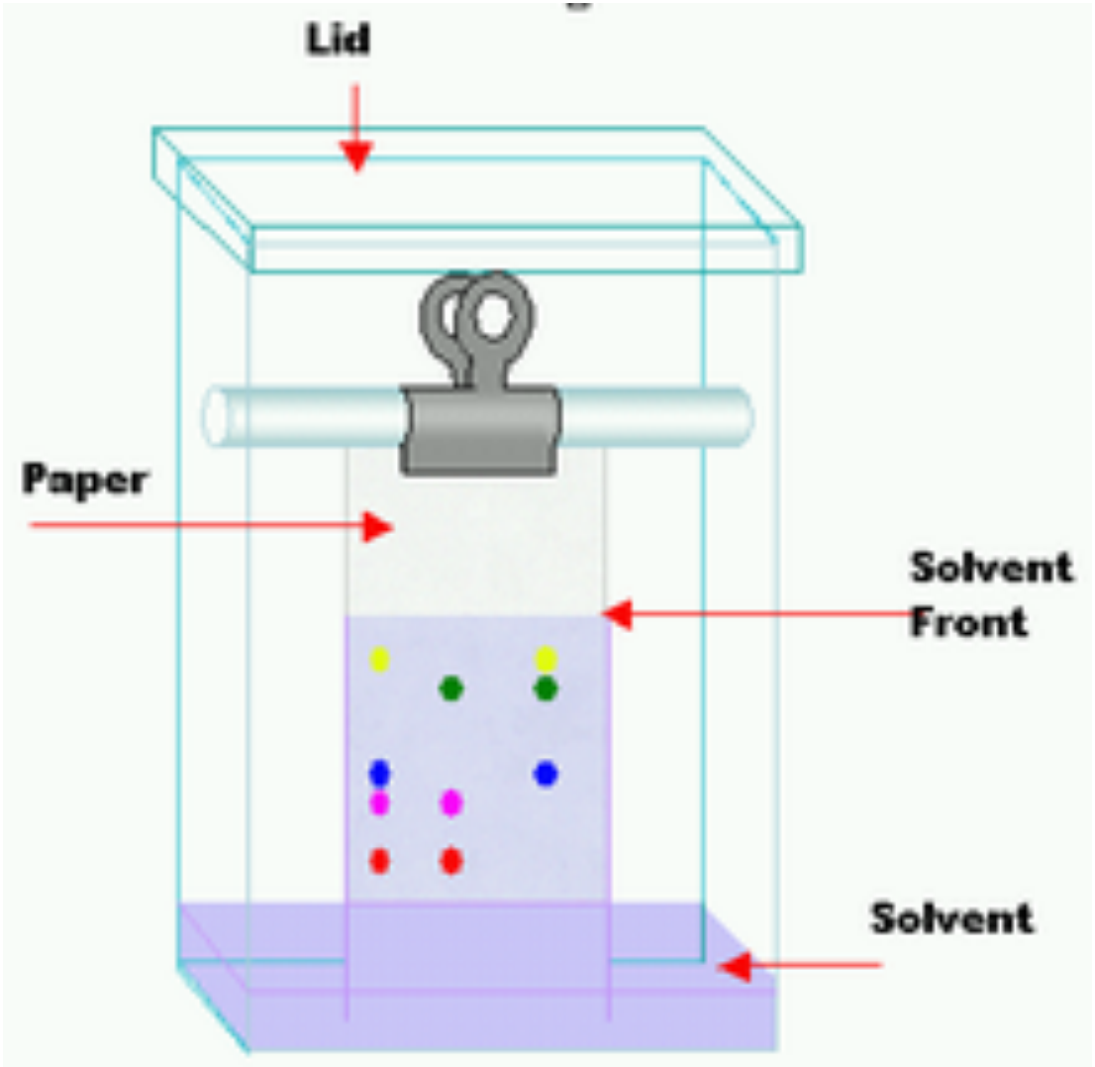
# Paper Chromatography

**A method of partition chromatography using filter paper strips as carrier or inert support.**

**The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases.**

**One is usually water adsorbed on cellulose fibres in the paper (stationary phase).**

**The second is the organic solvent flows past the sample on the paper (stationary phase).**


Lid
Paper
Solvent
Front
Solvent

**Partition occurs between the mobile phase and the stationary aqueous phase bound by the cellulose.**

**The isolation depends on partition coefficient of the solute.**

$$K = \frac{c(stationary)}{c(mobile)}$$

# General Procedure

1- Choice of paper and solvent to be used.

2- Desalting of sample.

3- Application of the sample.

4- Equilibration of paper.

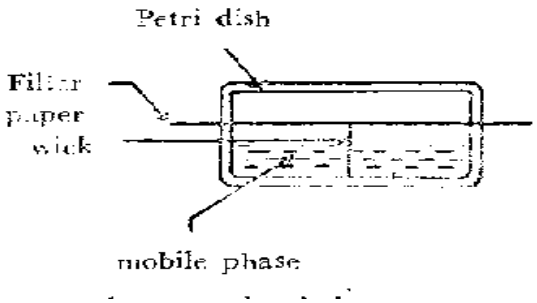
5- Development.

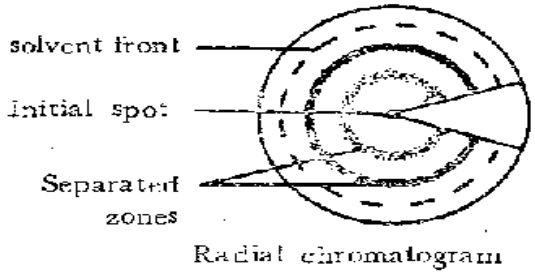
6- Detection.

7- Identification of substances.

# Techniques of development with various flow directions

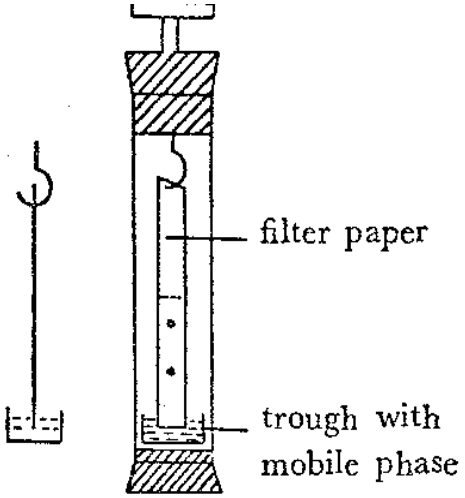
## Radial development



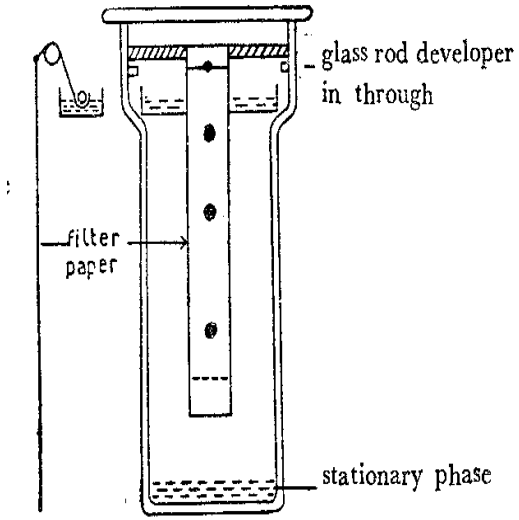




Radial chromatogram

## Ascending development




Cylinder for ascending chromatography




Descending development

## Descending development

# Columnar Chromatography (CC)

**This includes chromatographic methods in which:**

**The stationary phase is packed into a column.**

**The mobile phase is a moving liquid or gas.**

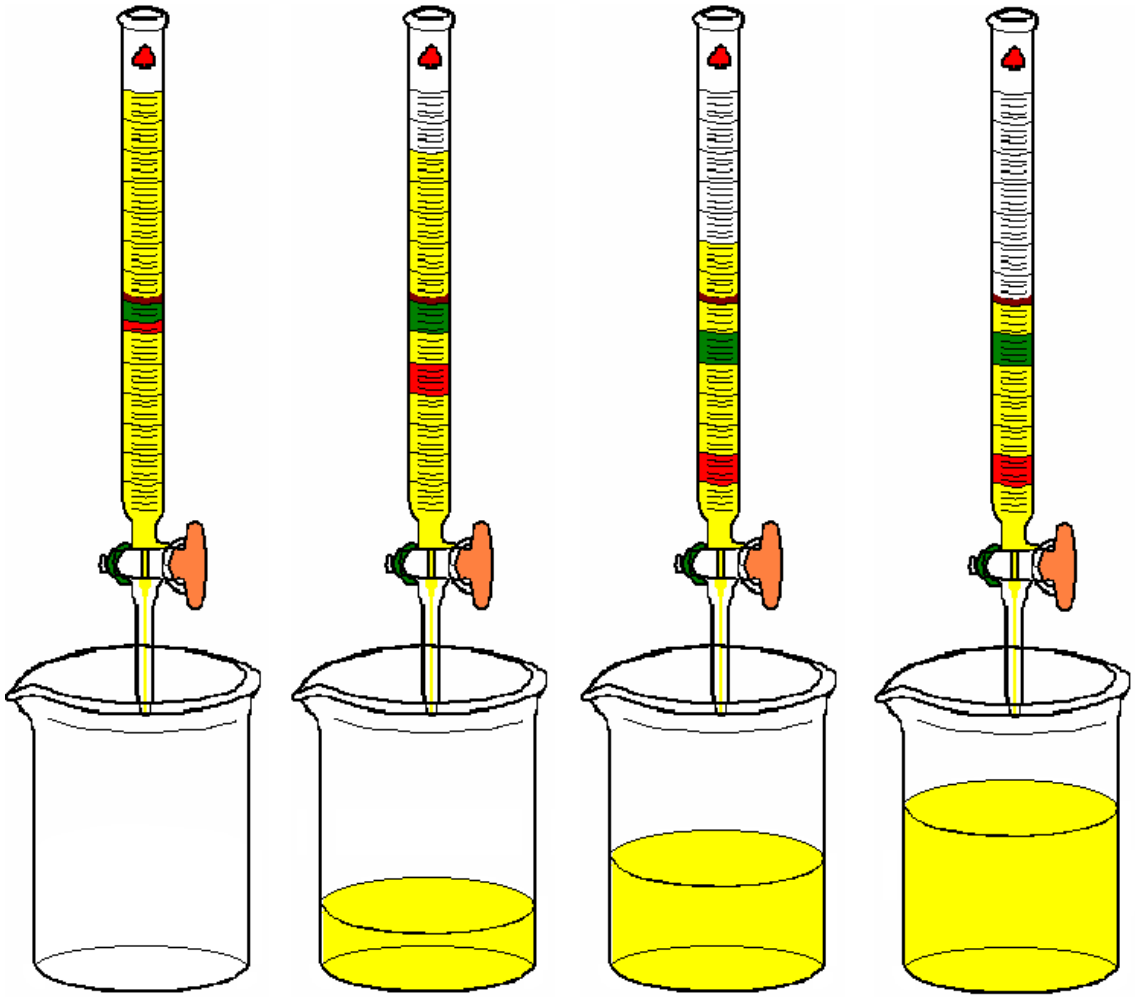
**According to the mechanism of separation of solutes, five major types of CC are ditinguished. Usually, one mechanism predominates but does not exclude the others**

# Different Types of chromatography

| Mode or type | Stationary phase | Mobile phase | Mechanism |
|---|---|---|---|
| Adsorption Chromatography | Solid that attracts the solutes | Liquid or gas | Solutes move at different rates according to the forces of attraction to the stationary phase. |
| Partition Chromatography | 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 Chromatography | Solid resin that carries fixed ions & mobile couterions of opposite charge attached by covalent bonds | 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 Chromatography | Porous gel with no attractive action on solute molecules | Liquid | Molecules separate according to their size:<br>1.Smaller molecules enter the pores of the gel, and need a larger volume of eluent.<br>2.Larger molecules pass through the column at a faster rate. |
| Affinity Chromatography | Solid on which specific molecules | Liquid or gas | Special kind of solute molecules interact with those immobilized on |

# Column Chromatography

- 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



| 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.** |

# Open Column Chromatography
# (Traditional column chromatography)

Traditional column chromatography is characterized by addition of **mobile phase** under **atmospheric pressure** and the **stationary phase** is **packed in a glass column**.

# Packing & operating the column
## 1- Packing

The selection of the method of packing **depends** mainly **on the density of the solid**.Techniques used are the **wet**, **dry** & **slurry** methods.

In all cases **avoid inclusion of air bubbles**

# 2- Sample Application

Apply **evenly & in a concentrated solution** to the top of the column which is protected from disturbance (e.g. add glass wool or filter paper).

# 1.Elution techniques

| Technique | Procedure |
|---|---|
| **Isocratic elution** | **Addition of solvent mixture of fixed composition during the whole process.** |
| **Gradient elution** | **<u>Continuous or linear elution</u>: in which there is continuous change in the composition of the mobile phase over a period of time (e.g. polarity, pH or ionic strength).** |
| | **<u>Step wise or fractional elution</u>: in which the change is not continuous i.e. a sudden change in the composition of the mobile phase is followed by a period where the mobile phase is held constant.** |

# 4- Detection

**On-column detection** for **colored** or **fluorescent** compounds directly after developing the chromatogram.

**Monitoring of eluted fractions** (PC or TLC).

**Using special detectors** connected to the column such as refractive index, UV detectors, etc…

# Factors affecting solutes separation in CC
# ( Factors affecting column efficiency)

| Factor | Effect |
|---|---|
| Particle size of solid stationary phase (or of support) | Decrease of size improves separation (but very small particles need high pressure). |
| Column dimensions | Efficiency increases as ratio length / width increases. |
| Uniformity of packing | Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing). |
| Column temperature | Increase in column temperature results in speed of elution but does not improve separation (tailing). |
| Eluting solvent | Solvents should be of low viscosity (to give efficient resolution) & h igh volatility (to get rapid recovery of the substances). |
| Solvent flow rate | Uniform & low flow rate gives better resolution. |
| Continuity of flow | Discontinuous flow disturbs resolution |
| Condition of adsorbent | Deactivation of adsorbent decreases separation. |
| Concentration of solutes | Substances of high concentration move slowly. |

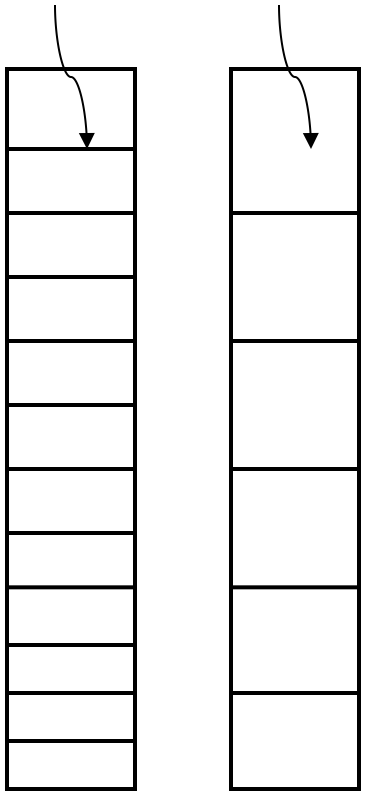
# Number of Theoretical Plates (N)

**H = Theoretical Plate Height**

**L = Length of the Column.**

$$N = L / H$$

**As HETP decreases efficiency of the column increases.**



# Adsorption Column Chromatography

**Adsorbents:**

The most common are **Alumina** & **Silica gel** in which the **interactions** with solute molecules is **due to OH groups present on their surface**.

**More polar molecules** are **adsorbed more strongly** & thus, will **elute more slowly**

Strength of adsorption of polar groups (solutes) on polar support is in the following order:

**-C=C- < O-CH3 < -COOR < >C = O < -CHO < -NH2 < -OH < -COOH**

**Olefins < Ethers < Esters < Lactones < Aldehydes < Amines < Phenols < Acids.**

# Applications in separation of natural products

**Alumina**: sterols, dyestuffs, vitamins, esters, alkaloids & inorganic compounds.

Not used for compounds containing phenolic or carboxylic groups

**Silica gel:** sterols & amino acids.

**Carbon:** peptides, carbohydrates & amino acids.

**Calcium carbonate:** carotenoids & xanthophylls.

# Partition Column Chromatography

In this type, the **packing consists of a theoretically inert support material coated with a film of the liquid stationary phase**.

The **division into adsorption & partition** is only of **theoretical** significance as **in partition chromatography the adsorption effects of the support can be felt.**

# Selection of the solid support

The support material should:
adsorb & retain the mobile stationary phase.
expose as large surface as possible to the mobile phase
be mechanically stable.
be easy to pack.
not retard the solvent flow

**Examples of solid supports**:
Silica gel, diatomaceous earth (as kieselguhr, celite etc.) & cellulose.

# Selection of the mobile phase

The **liquid stationary & mobile phases** should have a considerable difference between their solvent strength parameters.

**Pure water > Methanol > Ethanol > Propanol > Acetone > Ethyl acetate> Ether > Chloroform > Dichloromethane >Benzene > Toluene > Carbon tetrachloride > Cyclohexane > Hexane > Pentane.**

e.g. if the **stationary phase** is **water**, **pentane** would be the **eluent** of choice.

The **mobile phase** is usually **saturated** with the **stationary phase** to **overcome** "**stripping**" (washing of the stationary phase from the column by the mobile phase).

# Gel Permeation Chromatography (GPC)

This type is also known as:

**Size Exclusion Chromatography (SEC)**

**Molecular Exclusion Chromatography (MEC)**

**Molecular Sieve Chromatography (MSC)**

**Gel Filtration Chromatography (GFC)**

**Gel Chromatography**.

# Stationary phase

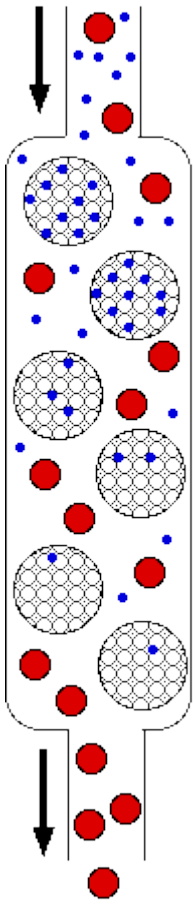
**Porous polymeric matrix:** formed of spongy particles, with pores completely filled with the liquid mobile phase **(gel)**.

The gels (polymers) consist of **open**, **three-dimensional networks** formed by cross-linking of long polymeric chains.

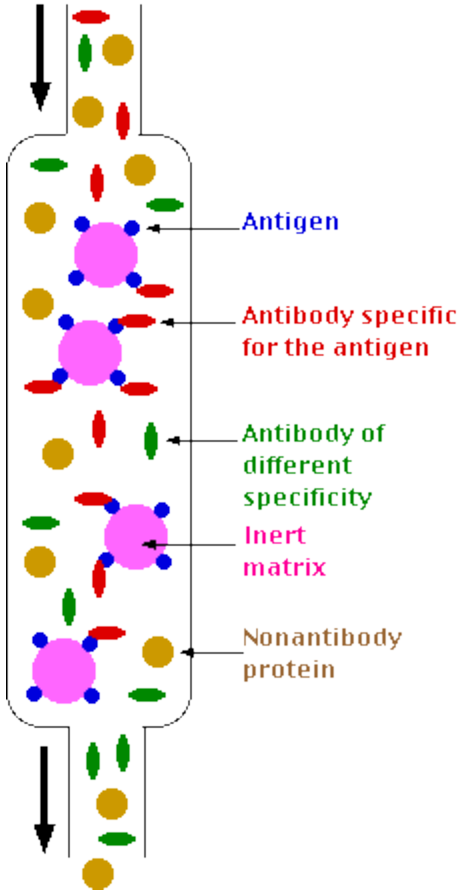
The **pore size** varies with the degree of cross-linking.

The **diameter of the pores is critical as** separation is based on that **molecules above certain size are totally excluded from the pores because they can not enter the gel**.

The **interior of the pores is reached**, partially or wholly, **by smaller molecules**.



# Affinity Chromatography



**Mobile phase**

Mobile phase is a liquid as **water** or **dilute acohol**

**Separation mechanism**

Based on difference between the solutes molecular weights.

Molecules will distribute themselves outside & inside the pores according to their size.

**Larger** are excluded, **medium sized** enter half-way & **smallest** permeate all the way.

The **retention volume Vo** of a substance is inversely proportional to the molecular weight (M. Wt) of the solute.

$$Vo \simeq 1 / \text{M.wt}$$

*Vo* = retention volume

M.wt = Molecular Weight

# Applications of GPC to natural products

**Determination of M. wt. of peptides, proteins & polysaccharides.**

**Desalting of colloids e.g. desalting of albumin prepared with 2% $(NH_4)_2SO_4$.**

**Separation of mixture of mono- & polysaccharides.**

**Separation of amino acids from peptides & proteins.**

**Separation of proteins of different molecular weights.**

**Separation of mucopolysaccharides & soluble RNA.**

**Separation of myoglobin & haemoglobin.**

**Separation of alkaloids & purification of enzymes.**

# Ion Exchange Chromatography

**Principle**

Process by which **ions** of an **electrolyte solution** are brought into contact with an **ion exchange resin**.

The **ion exchange resin** is an insoluble polymer consisting of a **"matrix"** (Lattice or framework) that **carries fixed charges** (not exchangeable) and mobile **active ions "counter ions"** which are **loosely attached to the matrix.**

In **water,** the **counter-ions move more or less freely in the framework & can be replaced by ions of the same sign present in the surrounding solution**.

The **"matrix"** (framework) **of a "cation exchanger"** is considered as a crystalline non-ionized "**polyanion**" & the **matrix of an "anion exchanger"** as a non-ionized "**polycation**".

# Cation Exchangers

**Active ions (counter ions) are cations**.

The **polar groups attached to the matrix are acidic** (sulphonic acids, carboxylic acids, phenols, phosphoric acids) e.g. a cation exchanger in the free carboxylic acid form:

**$X\text{-}COO^- H^+$**

**X = Frame work (matrix)**

**$-COO^-$ = Fixed charge (anionic),**

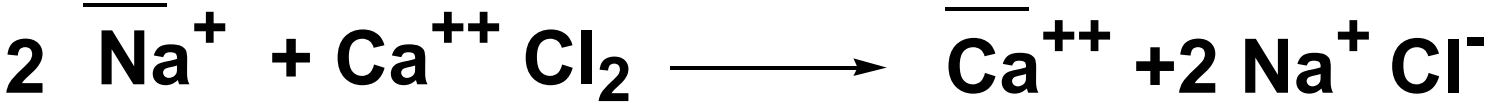
**Non-exchangeable**

**$H^+$ = Counter ion (cation), Exchangeable**

They are usually (but not always) supplied in the $Na^+$ form: **X-COO$^-$Na+**

**or $\overline{Na}^+$, Where — = Matrix**

**e.g. exchange with $CaCl_2$ aqueous solution**



# Anion Exchangers

**Active ions (counter ions) are anions**.

The polar groups attached to the matrix are tertiary or quaternary ammonium groups (basic).

e.g. Anion exchanger in the quaternary ammonium form:

**X. $NR3^{+}OH^{-}$**

**X = Framework (matrix)**

**$-NR3^{+}$ = Fixed charge (cationic)**

**Non exchangeable**

**$-OH^{-}$ = counter ion (anion), Exchangeable**

They are supplied as the chloride rather than the hydroxide as the chloride form is a more stable. Represented as: **X. $NR3^{+}Cl^{-}$**

**or $\overline{Cl}^{-}$ (where, —— is the matrix)**

**e.g. exchange with $Na_2SO_4$ solution**

$$2\,\overline{Cl}^{-} + Na_2^{+2}\,SO_4^{-2} \longrightarrow SO_4^{-2} + 2\,Na^{+}Cl^{-}$$

# Regeneration of the resin

**Ion exchange process is generally reversible e.g in the following:**


**The cation exchanger could be exhausted after exchanging all $Na^+$ for $Ca^{++}$, the exchanger could be regenerated (loaded again with $Na^+$) by contacting it with excess $Na^+$ ions e.g. a solution of NaCl.**

# Types of Exchangers

**Ion Exchangers**

These are either **cation** or **anion exchangers** of either **organic** or **inorganic** nature.

**A- Inorganic ion exchangers**

Common clays, soils, minerals e.g. zeolites used for "softening water".

**Disadvantage:** low ion-exchange capacity.

**Advantages:**

Great resistance to high temperatures.

High volume capacity.

Great selectivity towards simple inorganic ions.

# B- Organic exchangers

**They may be natural or synthetic.**

**Preparation of organic synthetic ion exchangers** :

Polycondensation of **phenols**, **sulpho-** & **carboxy-derivatives with formaldehyde** → **cationic** exchangers.

Polycondensation of **aromatic amines with formaldehyde** → **anionic** exchangers.

These techniques yield products **linear** in structure & relatively **soluble in water** which are now replaced by resin materials based on **styrene divinyl benzene copolymers** and **polyacrylate**.

# Applications of Ion Exchange Chromatography

**1- Water softening:**

Removal of Ca2+, Mg2+ & other multivalent ions causing hardness of water by filtration through a layer of strong cation resin.

**2-Water demineralization:**

Removal of cations & anions dissolved in water. Usually carried by the two step technique in which two columns of strongly acid cation exchanger in [H+] form & strongly basic anion exchanger in [OH-] form are used in sequence**.**

**3- Neutralization:**

Cationic exchanger in [H+] can be used to neutralize alkali hydroxide & anionic exchanger in [OH+] form to neutralize the acidity.

**4- Separation of electrolytes from non-electrolytes.**

**5- Separation of carbohydrates & their derivatives:**

**Uronic acids** separated on anion exchanger.

**Sugars** converted into ionized form by using borate& separated on strong anion exchanger.

**Hexosamines** separated on strong cation exchanger.