# Paper chromatography

It is a kind of partition chromatography . The paper used for chromatography are usually Whattman No.1 filter paper .since they are pure and have uniform fibers. The stationary phase in paper chromatography is made up of a combination of the paper's cellulose fibers and associated water molecules .the cellulose fibers are highly polar chains of covalently joined sugar molecules.Their many OH groups allow for extensive hydrogen bonding to free water molecules.Thus ,the paper chromatography stationary phase is a very polar matrix of cellulose and bound water.

- Stationary phase= cellulose paper
- Mobile phase=liquid solvent
- The solvent travels up the filter paper via capillary action and carries the the sample with it.

**Apparatus** : The apparatus required for paper chromatography consists of a support for paper , a solvent trough & an air – tight chamber in which the chromatogram is developed . The closed chamber is necessary to prevent the evaporation of the volatile solvents from the large exposed area of the paper. The size of the chamber vary according to the size of the paper used. The paper is cut to the size required for each separation , paper are used as such or they may be washed or treated according to the required technique, for example, by soaking them in paraffin or silicone oil in order to carry out 'reversed phase' chromatography for lipids. The tank or chamber or jar used are usually round tanks which has ground glass lid & it should be saturated with the solvent before introduction of the paper. The solvent should always have a depth of 1 Cm in the tank .

**Application of the sample spot :**

Usually a line is drown at a distance which is 1.5 Cm from the bottom of the paper , the line should be drawn with a pencil & any other colored pencil or ink should not be used otherwise it will interfere with the separation . The sample is then applied to this line with the aid of a hair drier & this is done for immediate evaporation of the sample giving a small circular spot on the base instead of letting the sample to evaporate by itself & so it will diffuse giving a big circular spots . The solvent front is also marked with a pencil at ¾ length of the plate. the first spot is applied at a distance of at least 1Cm from the edge of the paper . The sample should be completely dried before developing , the application is done with a capillary tube or micro pipette .

**Development :** Several developing techniques can be applied in paper chromatography:

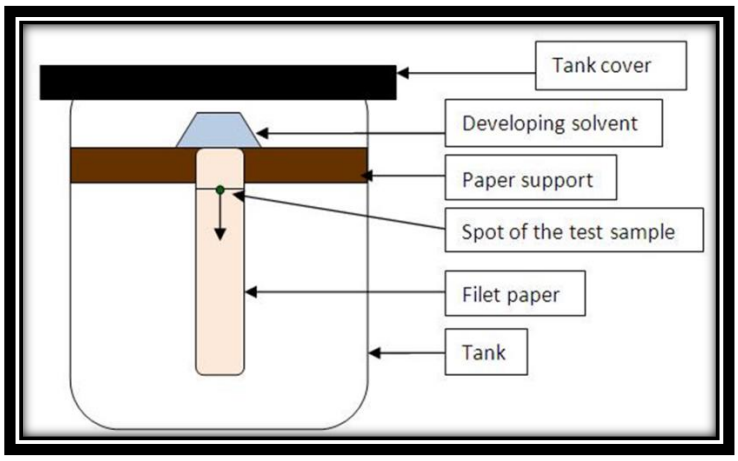
1-Ascending development :

This is the simplest & most popular type . The paper is suspended vertically with about 0.5-1 Cm above the surface of the solvent to prevent diffusion of the sample downward into the solvent reservoir. The solvent ascends through the paper by capillary action . The rate of ascent is slow & decrease with time because of gravity , however , the slow rate enhances the possibility of achieving partition equilibrium & often results in compact sharply defined spots .

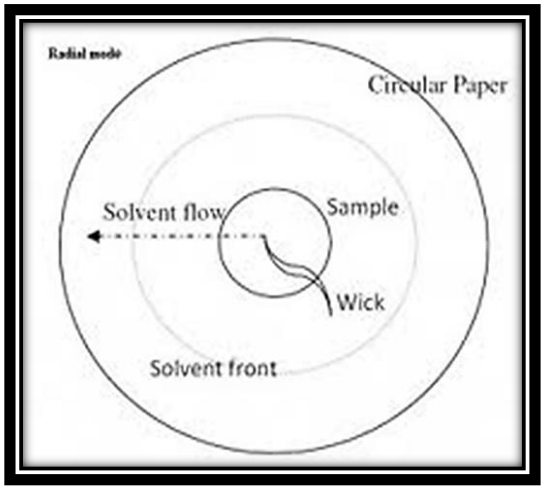
2-Descending development :

This is done with a solvent trough at the top of the tank or chamber , the direction of flow is downward . To prevent rapid siphoning , the paper is folded into a U-shape with a short initial rise from the solvent trough . The method is much faster than the ascending development , longer pieces of

paper may be used & large amount of solvents can be used if necessary for slow moving spots .



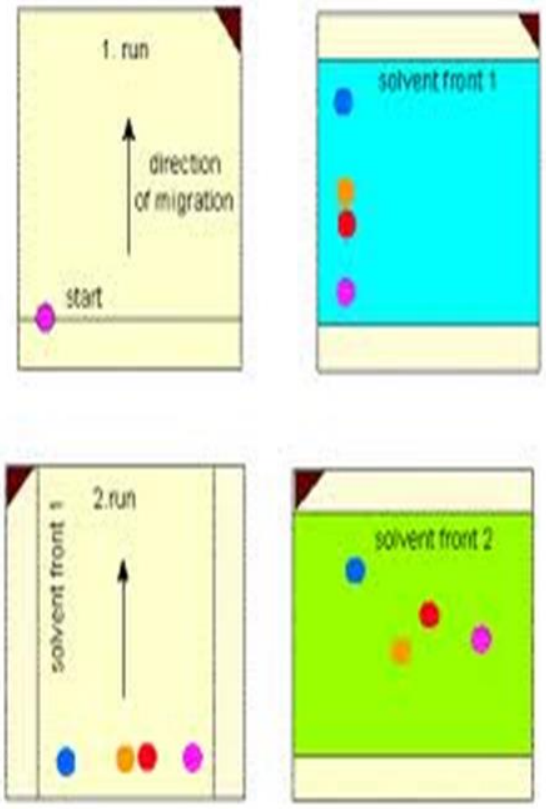

3-Radial or horizontal development : In this method the sample is spotted around the center of the horizontally placed filter paper with a wick in the center to supply the solvent to the center of the paper from a supply trough below . The solvent move outward separating the components of the sample along radial paths forming circles of increasing diameter . A covered Petri dish is an adequate container.




4-Two dimensional development :

In all previously described methods of chromatography the eluting solvent could flow in one direction only across the paper . On a plane

surface such as a piece of paper , it is possible to carry out a sequential development in two directions . The sample is applied as a spot close to a corner . This is developed in the normal fashion by ascending or descending procedure until the fastest moving spot approaches the end of the paper . Then the paper is removed & after evaporating the solvent it is turned 90 degree & developed a second time with another solvent having different eluting properties  In this manner , samples which could be only partially separated with either solvent alone may be completely separated by the combination of solvents.



5-Multiple development :

It is a simple ascending development that is repeated several times between each two development the paper should be removed & dried then introduced again into the jar . This method enhance the separation by increasing the distance between two successive spots .

**Detection of the separated spots :**

If the spots are not colored the spots are detected either under UV lamp , or sprayed with a spray reagents .

Spots of separated antibiotics can be detected by applying the chromatogram on a seeded plate ( seeded with living microorganism ) & noticing the inhibition zones after 24 hours .

**Retention factor**(Rf.):is the ratio of distance traveled by substance to the distance traveled by the solvent.Rf.value is always between 0 and 1and has no unit.The Rf value of unknown compounds is compared with the Rf value table of unknown compounds for identification.

$$Rf = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

Rf (retardation factor) depends on the following parameters:

- Solvent system
- Absorbent (grian size, water content,thickness)
- Amount of material spotted
- Temperature
- Ideal Rf between 0.2 and 0.8.
- The functional groups play a big role in the migration of a solute . Non polar groups: CH3_ , CH3O_ , Ph_ , CH3CH2_ ,----

  Polar groups : -CO2H , -OH, -NH2, -SO3H, PO3H2 --

Preparative paper chromatography :

For large scale separations thick sheets of chromatography filter paper are available(Whattman no.3) of 3mm thickness which the separated components are easily removed by cutting the paper to the separated bands then each separated band is cut to small pieces to ensure the complete removal of sample when shaken with the solvent.

# Thin layer chromatography (TLC)

Planar liquid chromatography (PLC) involves the separation of mixtures on thin layers of adsorbents that are usually coated on glass, plastic, or aluminum sheets. The most common form of planar liquid chromatography is thin-layer chromatography (TLC); & this particular technique is the easiest , cheapest , & most widely used method for the isolation of natural products. TLC is a kind of adsorption chromatography . The operations performed in TLC are essentially the same as in paper chromatography but instead of a piece of paper , a thin layer of finely divided adsorbent supported on a glass plate is used.

Separation by TLC is effected by the application of the mixture or extracts as a spot or thin line onto a sorbent that has been applied on a backing plate. The plate is then placed into a tank with sufficient suitable solvent .The solvent then migrates up the plate through the sorbent by capillary action, a process known as development. As a consequence of development , compounds of a mixture will be separated according to their relative polarities. Polarity is related to the type & number of functional groups present on a molecule capable of hydrogen-bonding.

Advantages of TLC over paper chromatography

1.small quantities are easily detected by TLC.

2.The spots are more compact & clear in TLC than paper chromatography.

3. We can use drastic reagent in TLC ex: concentrated $H_2SO_4$ in TLC but we cannot use it on paper chromatography.

## Analytical TLC

1- **Preparation of the layer** : The glass plate is usually flat & smooth , &different sizes & shapes of glass plates are used . Microscopic slides are cheap , readily available & adequate for small scale work. In any event the glass surface must be cleaned with a detergent & / or on organic solvent to remove any grease.

-The thickness of the layer will determine the capacity of the system . Layers of 0.1-0.25 mm is satisfactory for analytical purpose. The primary concern is to obtain a layer of uniform thickness . Most thin layers are produced by spreading a film of an aqueous slurry of the adsorbent over the entire surface . The slurry must be neither too thick nor too thin , or it will not spread properly . Silica gel when mixed with about twice its weight of water makes a very satisfactory slurry.

-The slurry after preparation should be spreaded on the plates & there are several types of spreaders used commercially , but the well-known manufacturers who have developed simple method for TLC are the Desaga & quickfit .

In the Desaga method the plates are placed on a templet or tray which have flat surface & it should be placed on flat table , after fitting the plates on the templet , a spreader is used which is usually placed at the end of the templet & it has a rotating chamber through which the adsorbent is placed & there is a gauge to set the thickness of the adsorbent layer to be spreaded .

-The slurry is poured into the spreader & slowly & steadily moved along the row of plates to give a thin film of adsorbent on the glass plate . The plates are left on the templet until they are air dried & then they are activated . Activation is the removal of water molecules from

the adsorbent by placing the plates , after holding them in a rack, in the oven & heating it at 110 C for 30-60 minute . The activation process will change the type of chromatography from partition to adsorption type , because the water molecules which are found in the slurry will act as a stationary phase if not removed.

-The plates are left to cool & the edges are cleaned then it is kept in the desicator till use . Desicator is a well clossed chamber which contains a substance which has the ability to absorb moisture ex : silica gel blue .

2- **Preparation of the tank** (jar) : This include the following steps :

-introduction of the solvent.

-lining of the tank .

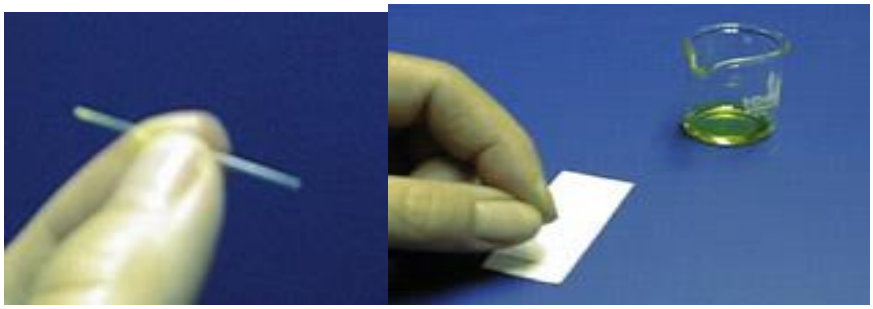
-saturation of the tank.

Solvents used in chromatagraphy should always be pure & dry . The amount of solvent used is calculated to give a solvent height in the tank to about 1Cm.

The tank after introduction of the solvent should be lined with filter paper , this lining will ensure saturation of the tank , the tank is closed tightly to ensure saturation.

Saturation of the tank is of great importance in separation because the migration of the component is greatly affected by the migration of the solvent system which in turn will evaporate from the plate if the tank is not saturated with the vapors of the solvent . The tank should be placed after preparation on a flat surface away from air current or heat or sun light because they affect saturation & hence the migration of the solute .


2- **Application of the sample** : Before application of the sample several parameters should be adjusted ex:



-The base line (the line on which the sample will be spotted ) should be located & it is about 1.5-2 cm above the lower edge of the plate & a mark is made with a fine needle to indicate its position . The base line level should be higher than the level of the solvent in the tank otherwise the sample will dissolve in the solvent before migration.

-The solvent front ( the level or height to which the solvent will move or travel ) should also be adjusted .

-About 0.5 cm is removed from both sides of the plate , this is necessary to prevent any adsorbent from the sides of the plate from absorbing solvent from lining of the tank & also for easiness of handling the plates .

-The sample is applied on the plate by the aid of a capillary or micro pipet , the first spot is applied about 1.5-2 Cm from the edge of the plate & about 1.5 Cm between two successive spots . After each application the spot should be air dried so that to prevent diffusion of the spot .

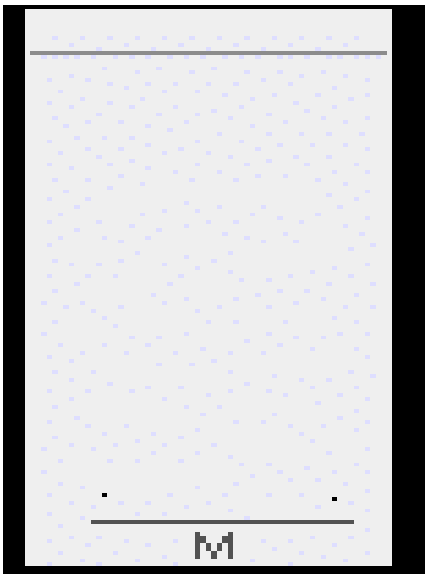
-The concentration of the sample is very important to have a good separation because overloading the TLC plate will lead to streaking & if insufficient amount of the sample is applied then the trace impurities may not be observed . After application & drying the spots , the plate is introduced in the tank and the tank is covered & the solvent is allowed to move or develop until it reaches the solvent front , then the plate is removed from the tank or jar & allowed to dry .

3- **Detection of spots** : There are several methods can be used to detect the separated spots ex :

❖ **Visualizing Colorless Compounds**

What if the compounds being separated are colorless? How are the spots visualized?

■ Two ways to get around this problem:

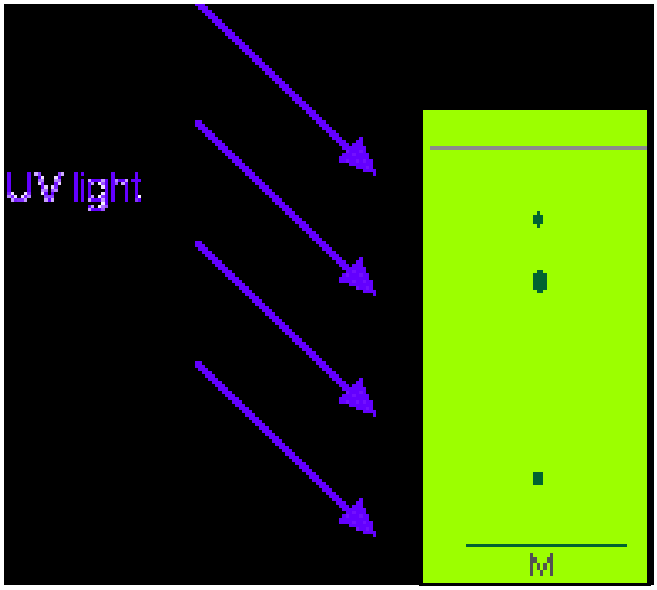


A) Use fluorescence

B) Use chemical methods

Using Fluorescence to Visualize Spots

- Substance which can fluoresce under UV light is added to stationary phase.
  - So, when the TLC plate is exposed to UV light, the entire plate will glow.
  - On the final chromatogram, the glow will be masked at positions were spots are located.
  - Examples: zinc sulfide




❖ Visualizing Spots Chemically

- In some cases it may be possible to visualize the spots by reacting them with something that produces a colored product.
- Iodine Crystals:

- The dried chromatogram is placed into a closed container containing iodine crystals.
- The iodine vapor either:
- Reacts with the spots
- Sticks more to the spots than it does to the rest of the chromatogram

- **Ninhydrin:**
  - The dried chromatogram is sprayed with a ninhydrin solution.
  - Reacts with amino acids to produce a colored product.
  - mainly brown or purple
- **Rhodamine B:**
  - Visualization of lipids
- **Aniline phthalate:**
  - Visualization of carbohydrates

.

❖ **Spraying the plate with concentrated H2SO4** which upon warming will chars the sample components leaving black spots .

Different spraying reagents are used to detect different compounds ex:

Dragendorff spray reagent used for detection of alkaloids.

Vanillin in H2SO4 spray reagent used for detection of terpens .

Antimony trichloride spray reagent used for detection of steroids .

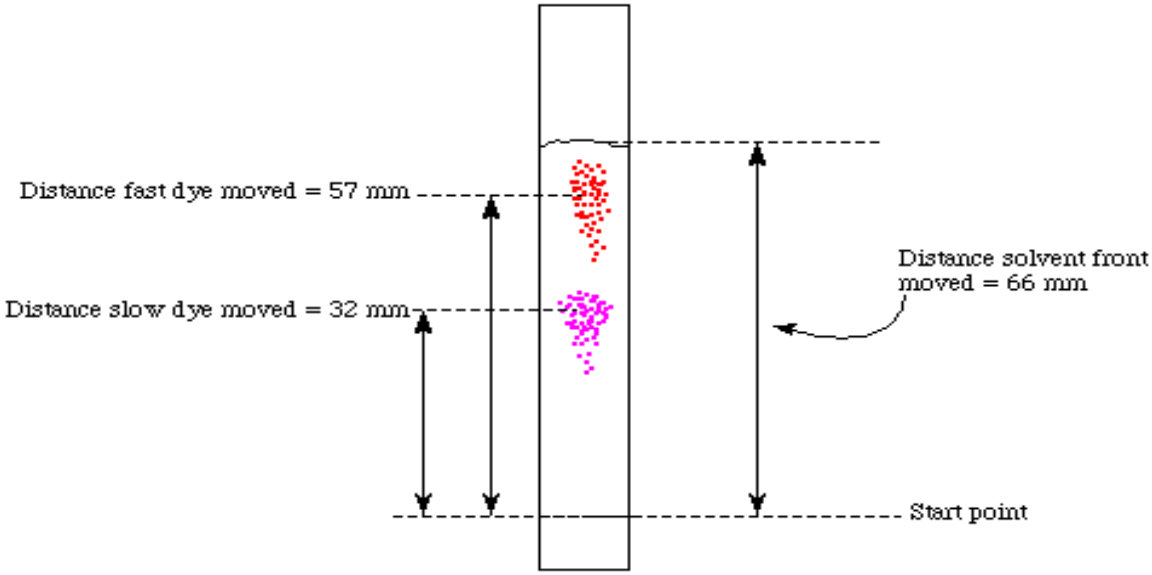
Coloured compounds are easily detected by their colour .

5- **Measurments of chromatographic data** :

Several parameters should be measured to identify the separated spots . One of the important methods used to identify the separated components is the use of standard material that could be spotted between the spots of the unknown components .

The important parameters measured are :

Rf value = **distance moved by the solute/distance moved by the solvent**. Rf value could be one or less than one but it cannot be more than one because the sample components cannot move above the solvent front .




If a standard (reference) material is used then another parameter is measured which is Rx value .

Rx value = **distance moved by the sample/ distance moved by the reference**

If Rx value =1 then the sample & the reference could be the same specially if these sample & reference give Rx value =1 in different solvent systems .

HRf value = Rf value x 100

Finally the developed plate is traced on tracing paper to be kept as a document. This tracing paper is called chromatogram on which all the materials & parameters used in the development of the plate is written ex:

adsorbent used

solvent system

sample used

reference material used

spray reagent

- etc..

**Factors affecting migration of solute in TLC**

These factors include the followings :

1- Nature of adsorbent –

-strong or active adsorbent

- medium activity
- weak activity

2- **Nature of the solvent** – following the eluting power of the solvent

3- **Nature of the solute** – depending on the molecular weight , functional groups, configuration .

Nature of adsorbent :

The adsorbing power of a material depends on the chemical nature of the surface, the particle size , & by its moisture content .

The particle size determines the surface area & this determines the active sites , the more fine powder give larger surface area , so the adsorbent become stronger by decreasing the particle size . In general chromatographic adsorbents are used at a standard particle size. The adsorbents used for TLC are of fine particle size than the adsorbents

for column chromatography . Examples of common adsorbents in a decreasing order of adsorption power:

Alumina

Silica gel

Magnesia

Calcium carbonate

Sucrose

Starch

Powdered cellulose

It is usually preferable to use an adsorbent with a binder like gypsum which is added to help in setting the adsorbent at a shorter time than the material without a binder for this reason adsorbent having a binder should be immediately spread once the slurry is prepared otherwise it hard to spread.

The adsorbents are classified into polar , medium polar , non- polar . On a non- polar ( weak ) adsorbent the interaction between the solute & the adsorbent depend on the physical forces (Vander Waals forces). In the case of polar adsorbent the dipole-dipole interaction & hydrogen bonding between the active site on the solid surface & the solute will act & it helps in retention . The polar adsorbent are very strong adsorbent & much stronger than the medium polar or the non-polar adsorbent .

The strength of particular adsorbent may be changed by the particle size & its moisture content . The particle size determines the surface area of the powder & the number of active sites because solid surfaces increase with increasing the surface area , for this reason the more fine a powder, the more is the surface area. Polar adsorbent like silica gel contains free moisture which is held together by hydrogen bonding . By heating, part or sometimes all the moisture may be removed , for this reason the nature of adsorbent may be changed

after removal of water from it & also the type of chromatography from partition to adsorption .

In partition the adsorbent contains from 10-20% of free moisture while in the adsorption the adsorbent contains 4-6% of free moisture for this reason it is necessary to activate TLC plates after spreading the slurry to ensure the removal of water & to change the adsorbent to an active adsorbent & hence the system will be changed from partition to adsorption .

Nature of the solvent :

Solvents may be graded into a series according to their power of elution , the more polar a solvent is the greater is the eluting power of that solvent for this reason adsorption of a solute on an adsorbent will be very high starting from a very non polar solvent up the list of polarity this is because the solute is only slightly soluble in the solvent , we call this as the solute- solvent interaction is weak & therefore the solute- adsorbent interaction is higher & for this reason the solute will remain on the base line.

The weak migration if a solute is due to the weak solute-solvent interaction . In an open column chromatography ex : TLC when polar solvent is added to the system it will push the solute up the plate depending upon the nature of the solute & therefore Rf value of the solute will be increased .

For open column chromatography like TLC a major solvent is always chosed in which the solute is slightly soluble , this solvent may be of moderate or non-polar type , then another solvent is added to increase or change the Rf value of the solute according to the nature of the solute to be separated ex : if strychnine alkaloid is separated on TLC , silica gel is used as adsorbent , abase solvent is of ethyl acetate is choosed . The plate is developed in this solvent & the Rf value is recorded . Ethyl acetate being a moderatly polar solvent , will not

migrate strychnine alkaloid because the interaction between strychnine & silica gel (adsorbent) is higher than the interaction between ethylacetate & strychnine for this reason a more polar solvent like methanol is added to increase the polarity of the solvent & hence increase the Rf value of strychnine.

This what we mean by mixing of solvents i.e use solvent system ex : for the separation of strychnine the solvent system has been choosed which consist of ethyl acetate – methanol 98:2 .

Nature of solute :

The functional groups play a big role in the migration of a solute . The presence of polar groups will lead to low migration . Also the configuration & conformation ex : cis & trans have effect on the migration because they will determine the way of attachment of the functional groups to the adsorbent.

Non polar groups: $CH_3$_ , $CH_3O$_ , Ph_ , $CH_3CH_2$_ ,----

Polar groups : -$CO_2H$ , -OH, -$NH_2$, -$SO_3H$, $PO_3H_2$ ----

The relationship between the molecular weight & adsorption has a rule which states that within a similar series the adsorption increase with an increase in the molecular weight & this what is called Traubes rule . As the adsorption increase the Rf value will decrease.

Natural products may be "tracked" by running analytical TLC of fractions from other separation processes , such as column chromatography , HPLC. More than one solvent system should always be used for TLC separation , as even apparently "pure" spots may consist of several compounds with identical Rf values .

The similarity of different extracts from the same species can also be assessed in this way & the discision to combine non polar & polar extracts can be made on the basis of identical or similar TLC chromatogram.

**Preparative TLC**

This technique is used for qualitative as well as quantitative analysis . The size of the plates used here large ex : 20x20 or 20x40 Cm . In this technique usually a thick plates (preparative plates) are prepared with a thickness varying from 0.5-2mm .

For preparation of such plates the same techniques discussed before in the analytical TLC are applied , the only difference is that the amount of the adsorbent in preparative TLC usually double the quantity of that used in the preparation of the slurry in analytical TLC .

The preparative plates are much thicker than analytical plates & so it should be dried at room temperature over night before activation in the oven to prevent cracking of the adsorbent layer .

The sample is dissolved in the minimum amount of the solvent & then applied on to the plates at a distance of 1.5 Cm from the bottom of the plate in the form of a continuous line with the aid of a capillary tube or automatic syringe & left to dry , then developed in the appropriate solvent .

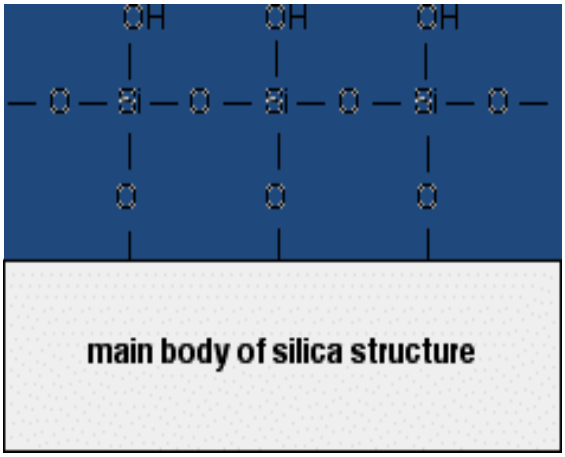
After development , the plates are left to dry then it is observed under UV light to locate the separated bands . If the components are not florescent then one side of the plate is cut with a needle (the adsorbent is cut) & then the rest of the plate is covered with another glass plate & the exposed part is sprayed with a spray reagent to indicate the location of the separated bands .

Then the glass plate is removed & the different components which are separated in the form of bands are removed or scrapped & the scrapped adsorbent is placed in a glass container (flask) & a solvent is added to each flask then it is shaken with the solvent to ensure the complete removal of the component from the adsorbent (elution) &

then filtered & taken to dryness & estimated qualitatively & quantitatively.

# How does TLC work??

- The surface of the silica (or alumina) gel has free hydroxyl groups which makes it very polar.
- The primary interactionsbetween the silica gel and the molecules of the sample is H-bonding.




- The silica gel may act as:
- H-bond donor

OR

- H-bond acceptor
- This bonding between the sample molecules and the silica gel is also known as adsorption.