PAPER AND THIN LAYER CHROMATOGRAPHY (TLC)

Lab#4

BCH 333

Chromatography:

- It is a collective term for a set of laboratory techniques that enables the ***separation, identification, and purification*** of the components of a mixture.

- ***The general principle for all chromatography techniques:***

➢ Separation of molecules ➔ by distribution between a stationary phase and a mobile phase.

➔ *The stationary phase* can be solid, gel, or liquid. Also called matrix, resin, or beads.

➔ *The mobile phase* is the solvent, and it is usually a liquid, but may also be a gas.

(Usually one phase is hydrophilic and the other is lipophilic).

➢ The compounds to be separated are considered <u>solutes</u>.

➢ The separation of materials is based on differential partitioning [retardation] between the mobile and stationary phases.

Types of Chromatography:

- Paper chromatography.

- Thin-layer chromatography.

- Gel filtration (molecular sieve) chromatography.

- Ion-exchange chromatography.

- Affinity chromatography.

- Gas chromatography.

- Dye-ligand chromatography.

- Hydrophobic interaction chromatography.

- Pseudoaffinity chromatography.

- High-pressure liquid chromatography (HPLC).

Partition Chromatography:

- Is the distribution of a solute between two liquid phases (mobile and stationary phase).

- This may involve direct extraction using two liquids, or it may use a liquid immobilized on a solid support as in the case of paper and thin layer chromatography.

➔ Originally, partition chromatography involved coating the support with a liquid stationary phase that was immiscible with the mobile phase.
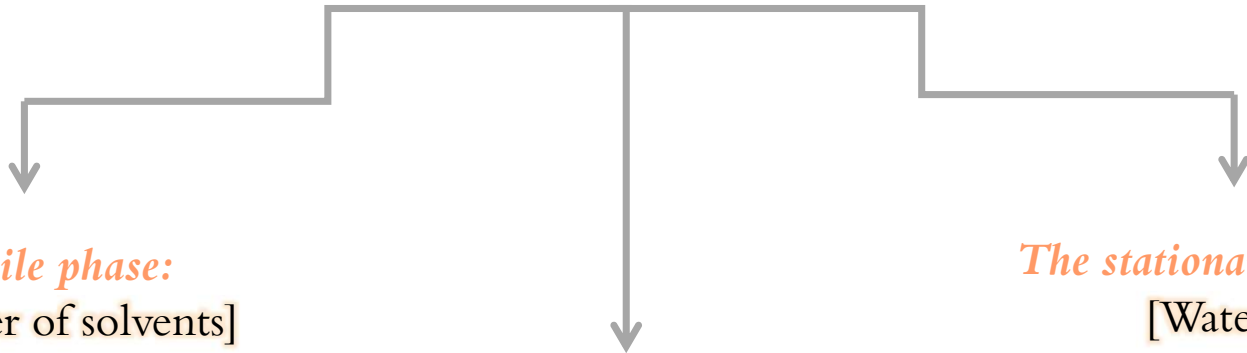
1. Paper Chromatography (PC):

- Paper chromatography is one of the chromatography types, procedures which runs on a piece of **specialized paper.**

- It is a "liquid–liquid" chromatography.

- Separation of solutes depend on the *polarity.*

- Requires small quantities of material.

- Rapid.

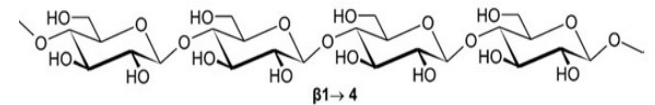# 1. Paper Chromatography (PC) cont':

The system is composed of

The mobile phase:
[shallow layer of solvents]
Is a developing solution that travels up the stationary phase, carrying the samples with it.

Non-Polar mixture "Liquid"

Separated molecules

The stationary phase:
[Water]
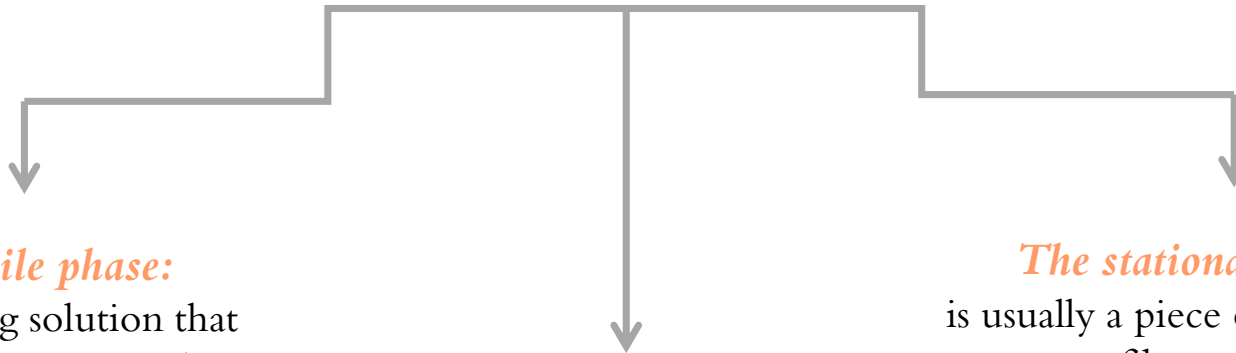Layer of cellulose highly saturated with water (the water trapped between the cellulose fibres of the paper).

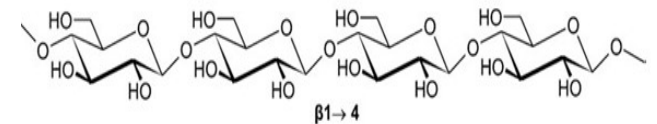**Polar substance "Liquid"**

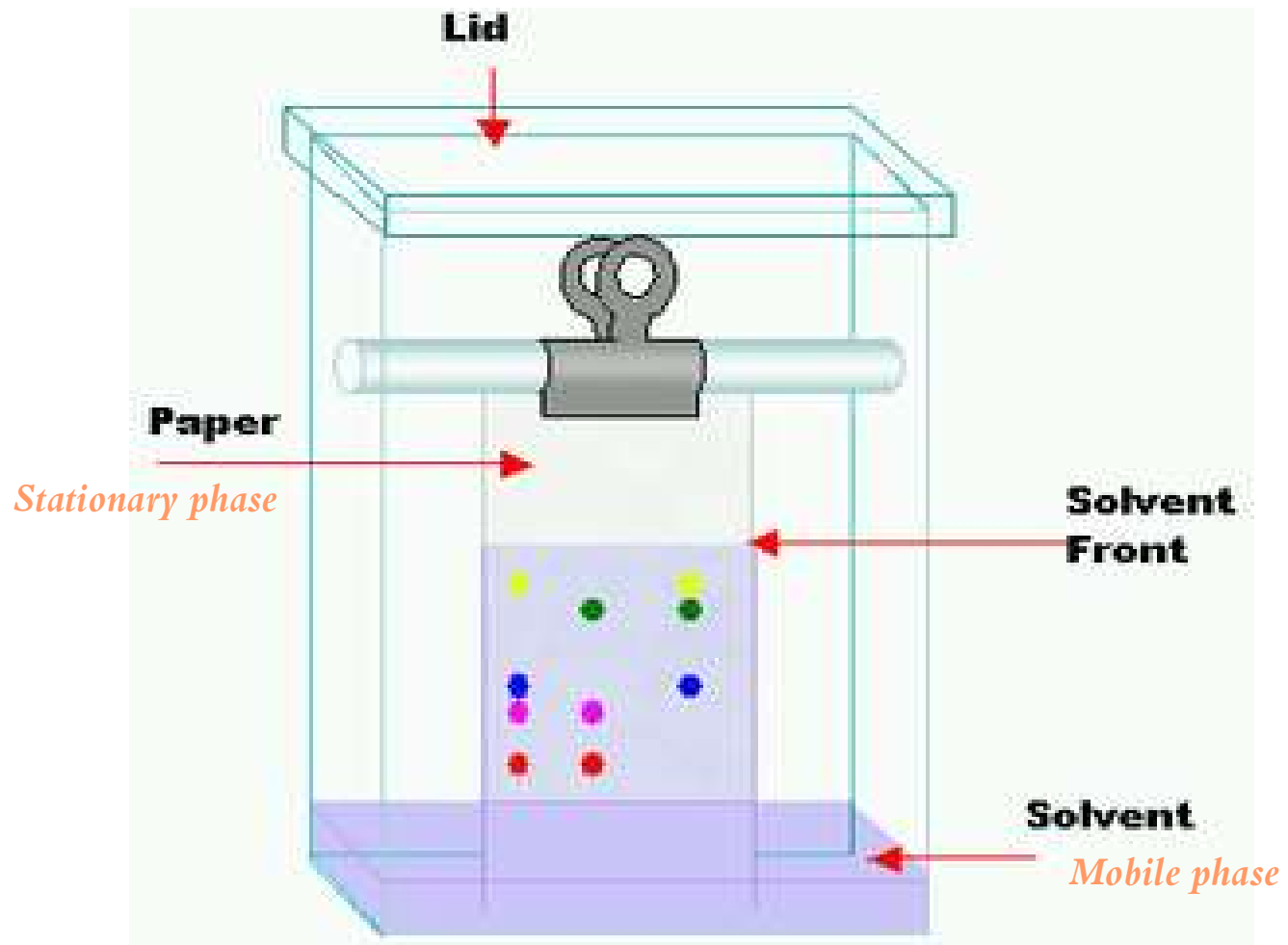# 1. Paper Chromatography (PC) cont':

The system is composed of

The mobile phase:
is a developing solution that travels up the stationary phase, carrying the samples with it. [shallow layer of solvents]. **Non-Polar mixture**
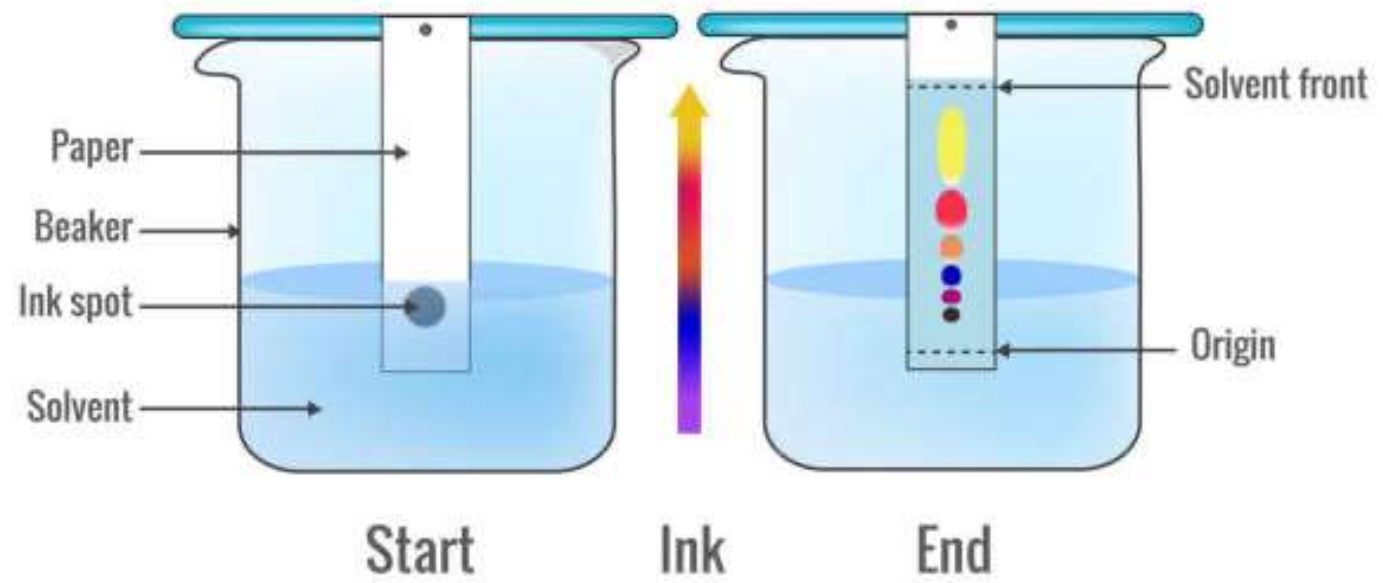
Separated molecules

The stationary phase:
is usually a piece of high-quality filter paper. [cellulose]. **Polar substance**

Figure 1. *Ascending Paper Chromatography*

Figure 1.Ascending Paper Chromatography

1. Principle of Paper Chromatography (PC):

- *The Separation is depending on:*

➢ Components of the sample will separate on the stationary phase according to <u>how strongly they adsorb to the stationary phase versus how much they dissolve in the mobile phase</u>. (how?)
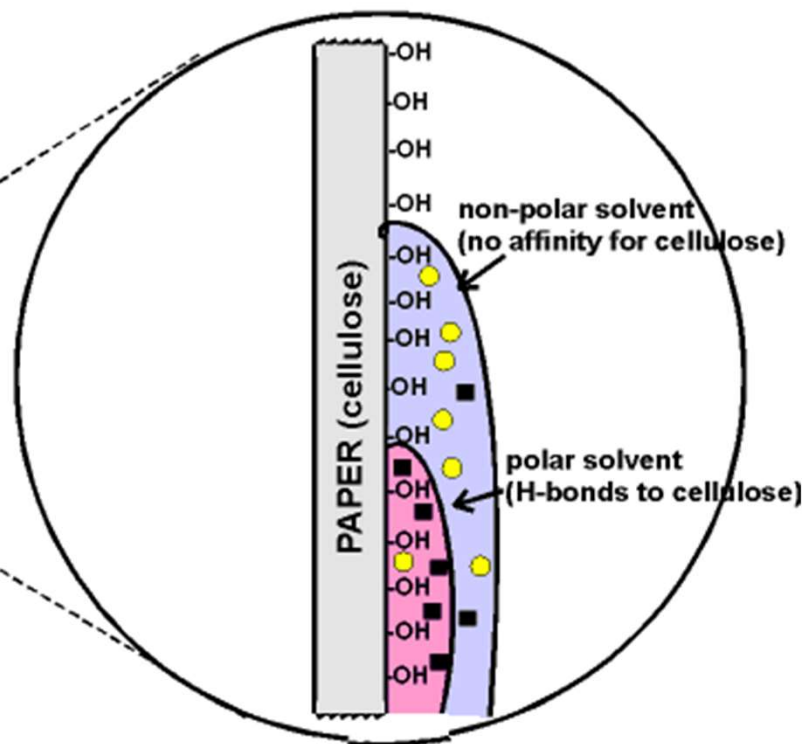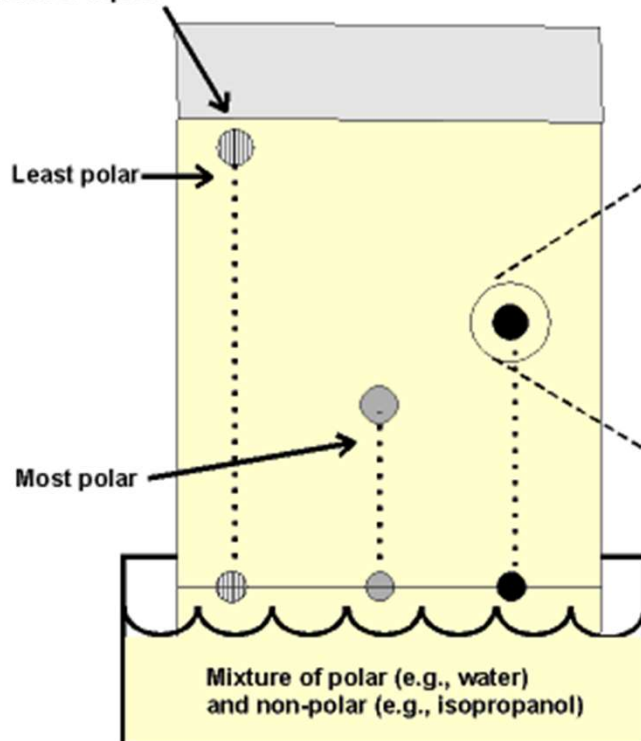
- *Explanation:*

➢ This paper is consisting of a layer of cellulose highly saturated with <u>water</u> [a polar substance, stationary phase], as the solvent travel up [non-polar, mobile phase], the compounds within the mixture travel farther if they are non-polar.

➢ So, *more polar* substances bond with the hydrated cellulose paper more quickly, and therefore do not travel as far.

➡ *As a result, different compounds in the sample mixture travel at different rates due to:*

- Differences in their *solubility* in the solvent [mobile phase].

- Differences in their *attraction* to the fibres in the cellulose paper [stationary phase].

Paper chromatography

R_f = ratio of the mobility (distance traveled) of the compound to the mobility of the front of liquid.

Least polar

Most polar

Mixture of polar (e.g., water) and non-polar (e.g., isopropanol)

PAPER (cellulose)

-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH
-OH

non-polar solvent (no affinity for cellulose)

polar solvent (H-bonds to cellulose)

In the mixture:

■ more *polar* molecule (spends most of its time in the polar solvent)

○ more *non-polar* molecule (spends most of its time in non-polar solvent)
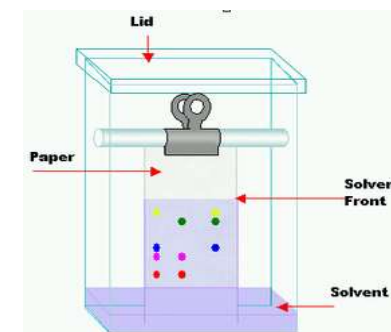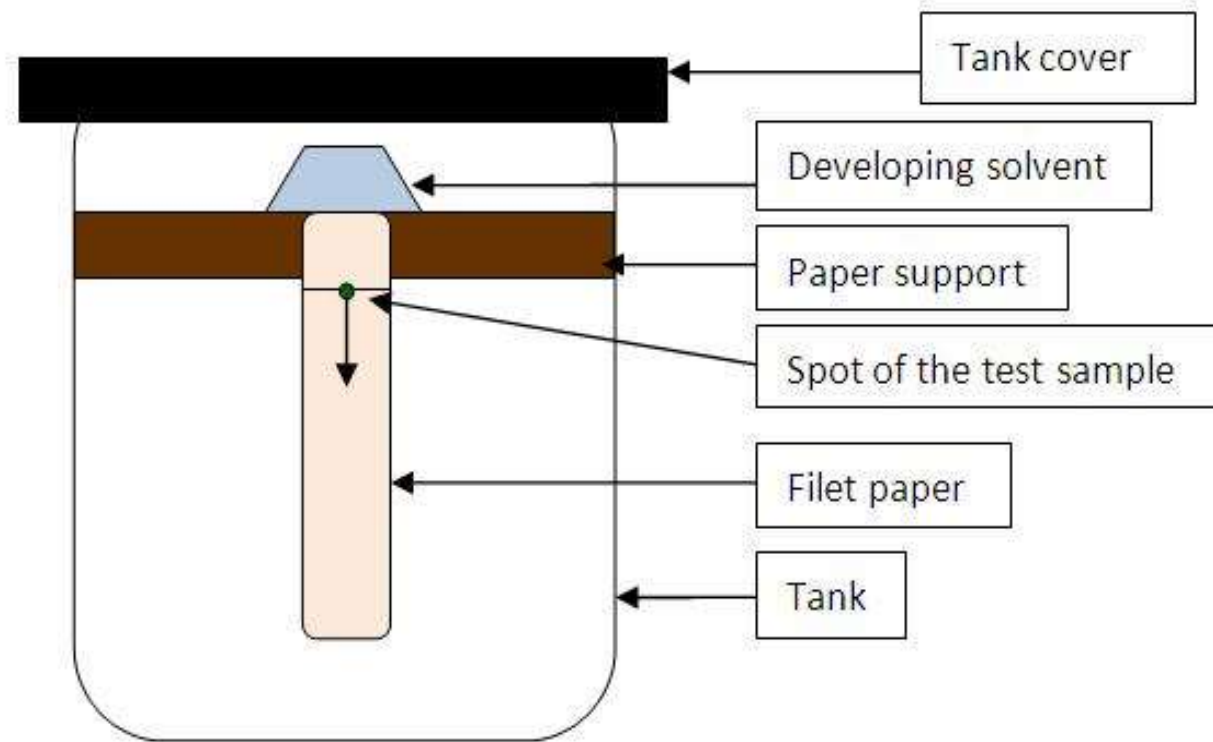
Paper Chromatography Modes

Descending:

- In this method, the solvent is kept in a trough at the top of the chamber and is allowed to *flow down* the paper.
- The liquid moves down by capillary action as well as by the gravitational force.
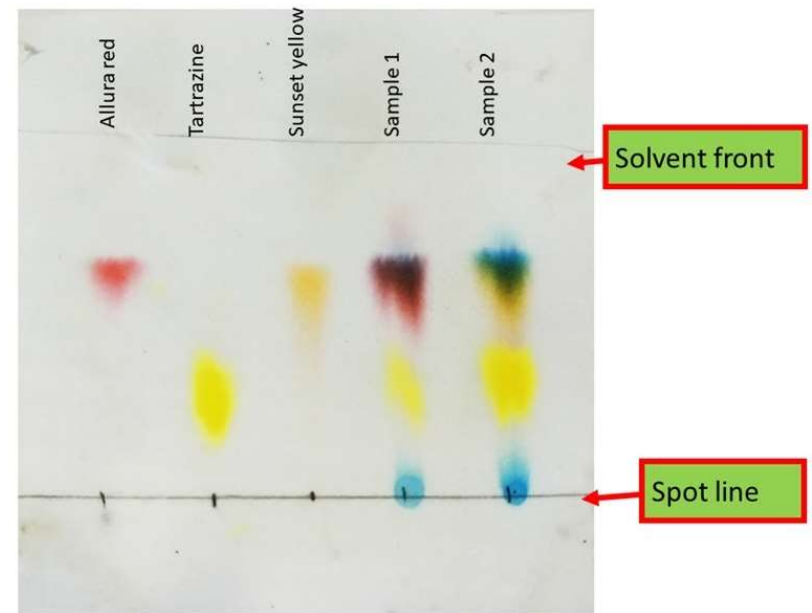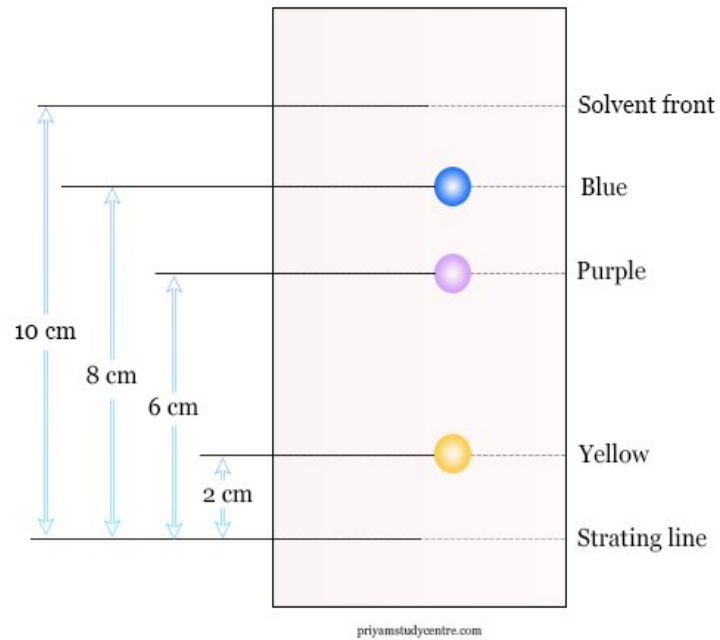- In this case, the flow is <u>more rapid</u> as compared to the ascending method.
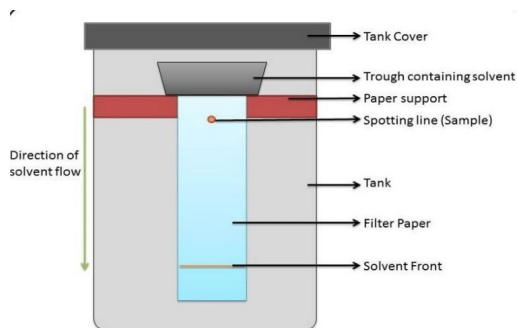
Ascending:

- In this method, the solvent moves *upward* against gravitational force.
- The only force that cause the motion of solvent and the compounds is capillary force, so the speed of the process is <u>slow.</u>

Figure 1. Descending *Paper Chromatography*

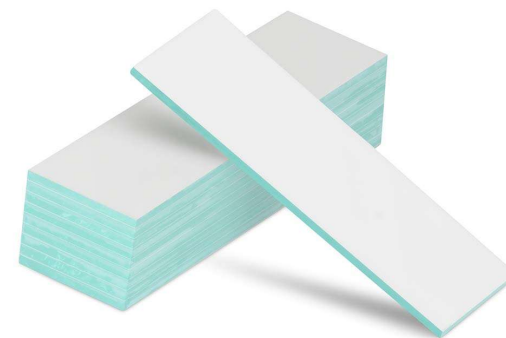# 1. Result of Paper Chromatography (PC):

Chromatogram

## 2. Thin layer Chromatography (TLC):

- Procedures which runs on a ***solid adsorbent substance coated on glass plates.***

- Rapid, the separation can be completed in less than one hour.

- It is a "solid–liquid adsorption" chromatography. ?

- Separation depend on the ***polarity.***

- Requires small quantities of material.

- Widely used.

2. Thin Layer Chromatography (TLC) cont':

The system is composed of
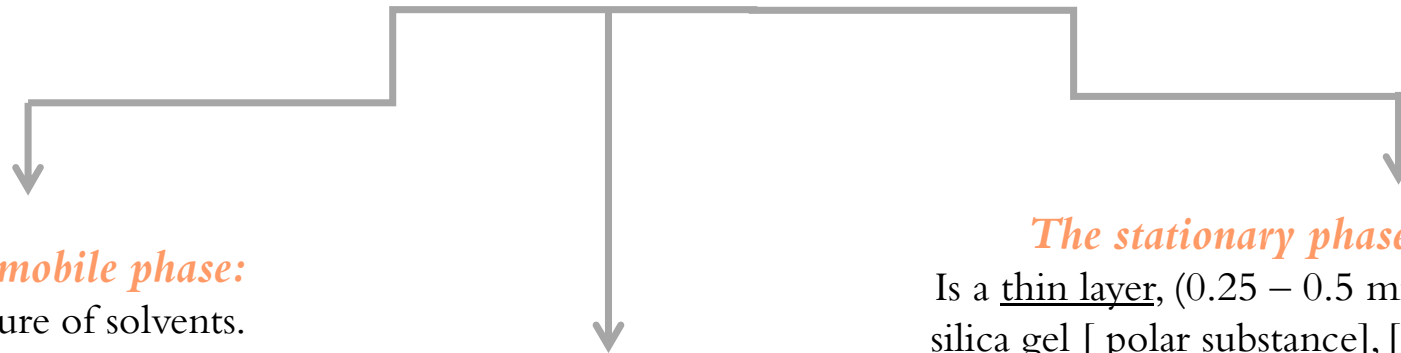
The mobile phase:
Mixture of solvents.
Non–Polar mixture

Separated molecules
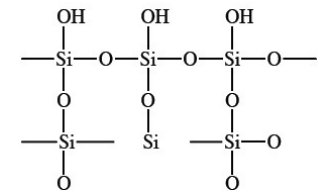
The stationary phase (adsorbent):
Is a <u>thin layer</u>, (0.25 − 0.5 mm) of ***adsorbent*** like silica gel [polar substance], [aluminium oxide or magnesium silicate] spread uniformly over the surface of a flat, inert surface of the glass plastic plate.

The stationary phase+ support medium ➔ should be inert.

Polar substance

2. Principle of Thin Layer Chromatography (TLC):

- Partition of a solute between a moving solvent phase and a stationary aqueous phase.

- The solute moves in the direction of a solvent flow at a ***rate determined by the solubility*** of the ***solute*** in the moving phase.

➔ Thus a compound with high mobility [less polarity] is more attracted to the moving solvent [mobile phase] than to the stationary phase.

- Separation depend on the polarity.
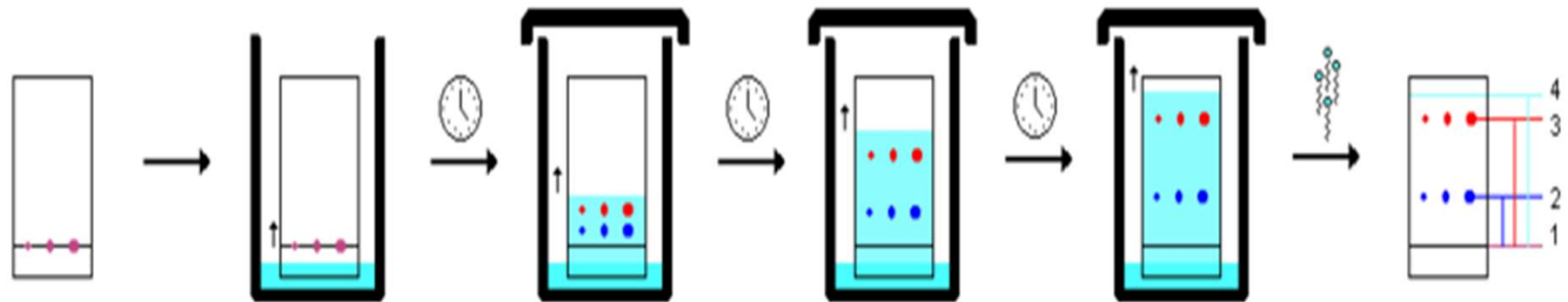
Figure 1. Traveling of the solvent in TLC via capillary action.

TLC developed plate

Solvent front

More affinity with mobile phase

Intermediate affinities

More affinity with stationary phase

Base Line
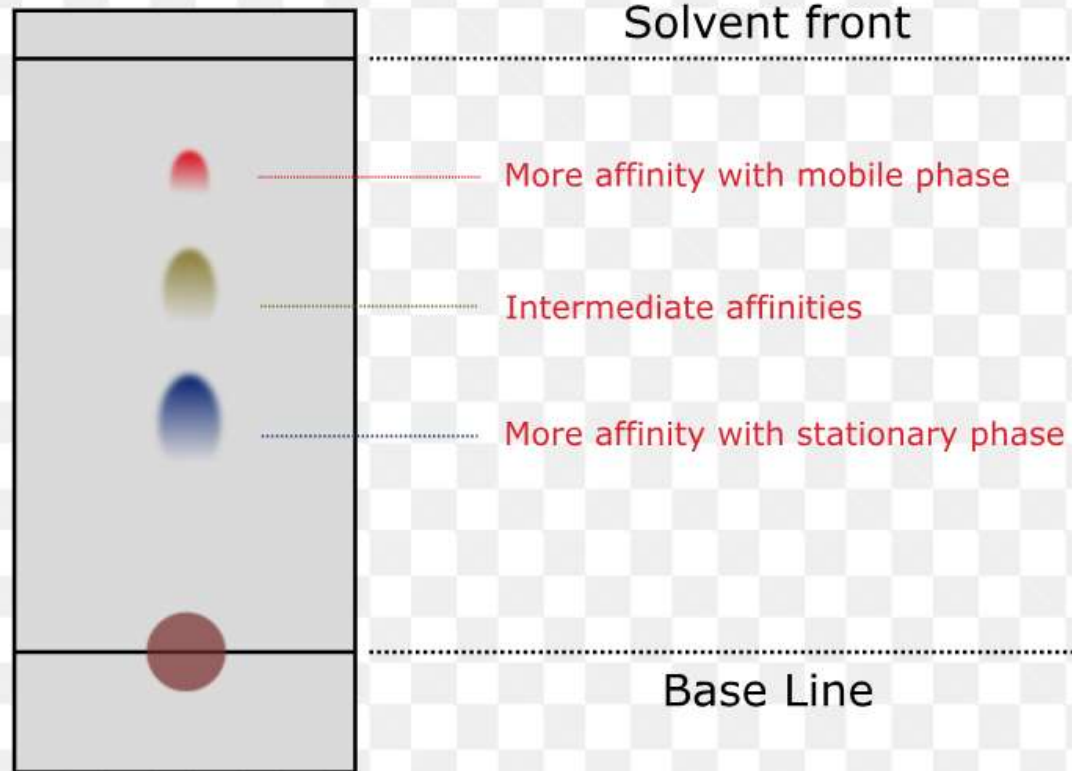
2. Result of Thin layer Chromatography (TLC):

Chromatogram

Factors affect the resolution of separation:
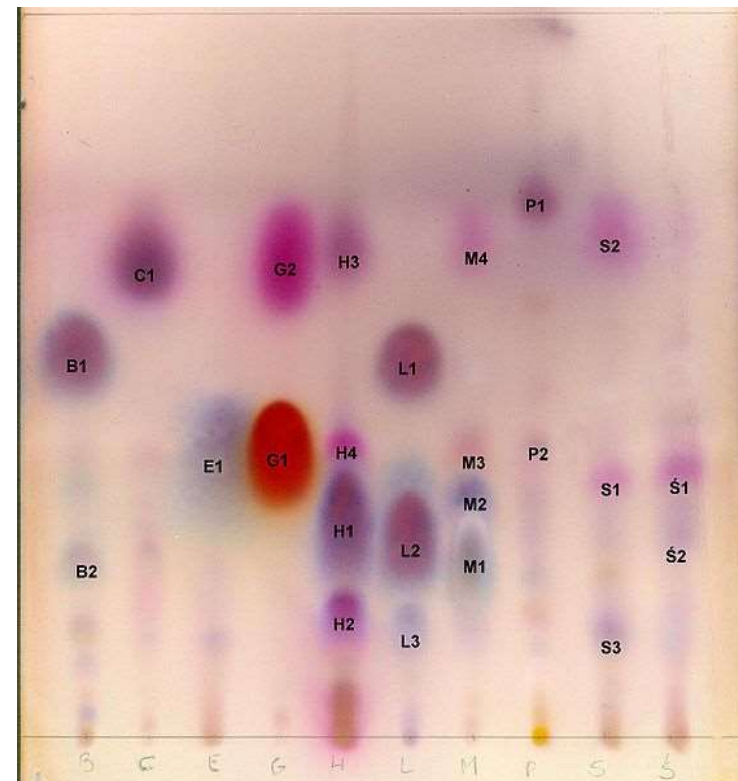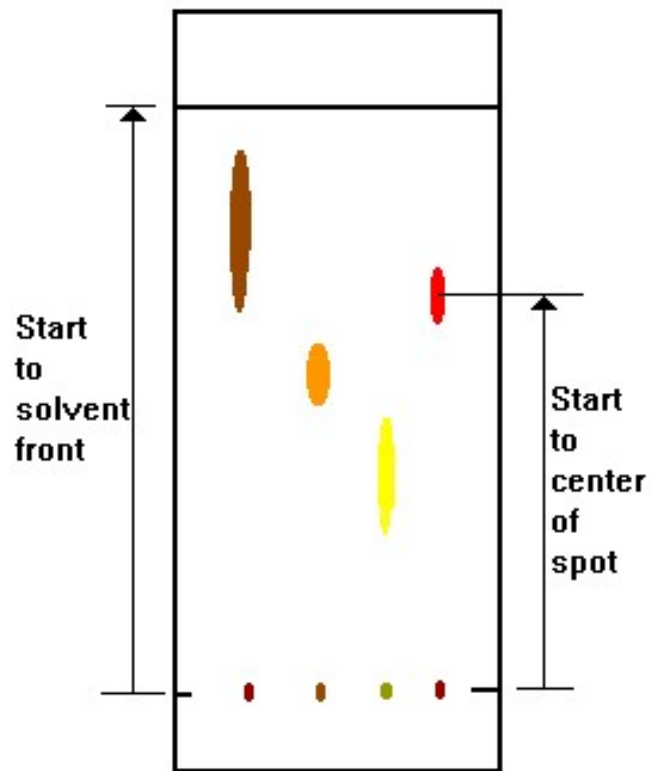
1. *Ion exchange effect:* Any ionized impurities in the support medium will tend to bind or attract oppositely charged ions and will therefore reduce the mobility of these solutes [resulting in bad resolution].

2. *Temperature:* Since temperature can <u>affect the solubility</u> of a solute in a given solvent temperature is also an important factor and often a chromatography laboratory has a fixed temperature for optimum results [increased temperature ➔ increase solubility].

3. *Composition of the solvent:* Since some compounds are more soluble in one solvent than in the other the mixture of solvents used affect separation of the compounds.

4. Concentration and volume of the sample.

Sample Analysis

Sample Analysis:

1. *Visualization:*

- Amino acids ➔ Specific color reagents are sprayed onto the plate or the paper [ninhydrin].

- Sugars ➔ Spraying the plate or the paper with [aniline diphenylamine].

★The paper or plate remaining after the experiment is known as the Chromatogram.

Sample Analysis:

2. *Expression of the results:*

➢ Relative flow [R_f]:

- Also known as relative mobility.
- Used to express the performance of a solute in a given <u>solvent system/stationary medium.</u>
- **<u>Rf value may be defined as:</u>** the ratio of the distance moved by a compound to that moved by the solvent.
- Rf value is <u>constant</u> for ➔ a particular compound, solvent system and stationary phase.
- Its value is always between zero and one "remember it is a ratio value".

$$Rf = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

Relative flow [R_f] value:

$$R_f = \frac{\text{Distance of migration of solute}}{\text{Distance moved by solvent}}$$

➢ If R_f value of a solute is *closer to zero* ➜ The solute has more attraction to <u>stationary</u> phase.

➢ If R_f value of a solute is *closer to 1* ➜ Then the solute has more affinity for the <u>mobile</u> phase and travels further.

• The final chromatogram can be compared with other <u>known</u> mixture chromatograms to identify sample mixes, *using the R_f value in an experiment.*

Example:

Solvent Front
12 cm

8.3 cm - - - - - - - - - - - ⬤
2

2.9 cm - - - - - - - - - ⬤
1

Adsorbent Layer
(Si Coated Side)

Origin - - - - - - ○ -
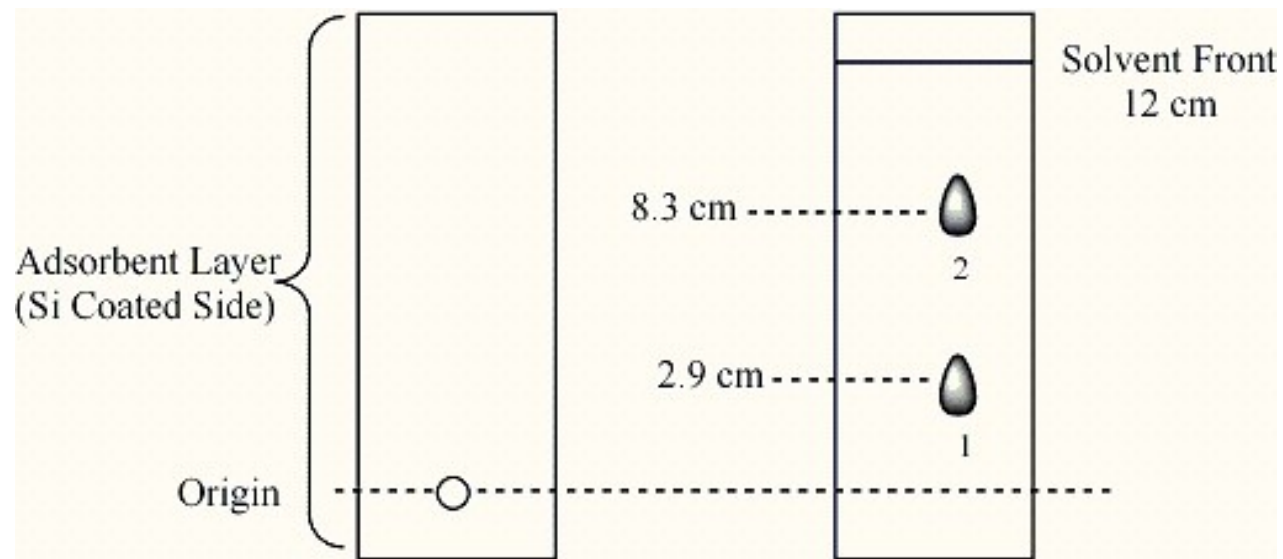
R_f = Distance Traveled by Spot / Distance Traveled by Solvent

$$R_f(1) = 2.9 \text{ cm}/12 \text{ cm} = 0.24$$

$$R_f(2) = 8.3 \text{ cm}/12 \text{ cm} = 0.69$$

Note: R_f Values Are Always Less Than 1

Comparison between PC and TLC:

	TLC	*Paper chromatography.*
Separation	It has better separations than paper chromatography	Separate the sample.
Stationary phase	Wide choice between different adsorbents (stationary phase).	Hydrated Cellulose "water"
Principle	Based on adsorption	Based on partition.
Resolution	It has better resolution.	Low resolution.
Quantification	Allow for quantification.	Allow for quantification.
Zonal spread	Compact zonal spread (concentrated for quantification analysis in need).	Expanded zonal spread (not concentrated for quantification analysis in need).

Application of TLC and PC:

1. Identifying unknown compounds present in a given substance.

2. Qualitative and semi quantification tests.

3. Separation of compounds in a sample.

4. Identify the purity of the sample.

5. Diagnosis.

Using of TLC and PC in diagnosis:

➢ *Phenylketonuria:*

- Non-functional <u>phenylalanine hydroxylase enzyme.</u>
- This enzyme is necessary to metabolize the amino acid phenylalanine (Phe) to the amino acid tyrosine.
- Phenylalanine accumulates and is converted into phenylpyruvate (also known as phenylketone), which is detected in the urine.

➢ *Cystinuria:*

- Cystinuria is an inborn error of <u>amino acid transport </u>that results in the defective reabsorption by the kidneys of the amino acid called cystine. The name means "cystine in the urine."
- When the kidneys do not reabsorbed cystine, this compound builds up in the urine.

Practical part

OBJECTIVES:

- Using paper chromatography and TLC to diagnose two samples of urine with phenylketonuria and cystinuria.

METHOD:

- *You are provided with silica gel Thin Layer Chromatography [TLC] plate and paper chromatography[PC]:*

1. Draw a sample starting line (base-line) about 2.5–3 cm from the bottom of the TLC plate and PC, divide it uniformly then apply one small drop (5 μl) of each standard (Phe & Cys) and sample (1 &2), allow to dry.
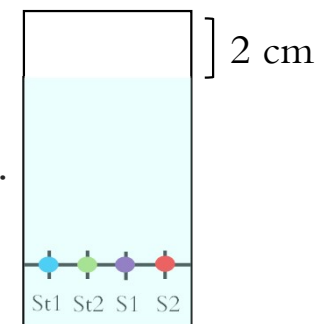
Base-line

2.5 cm

Standard 1 ●
Standard 2 ●
Sample 1 ●
Sample 2 ●
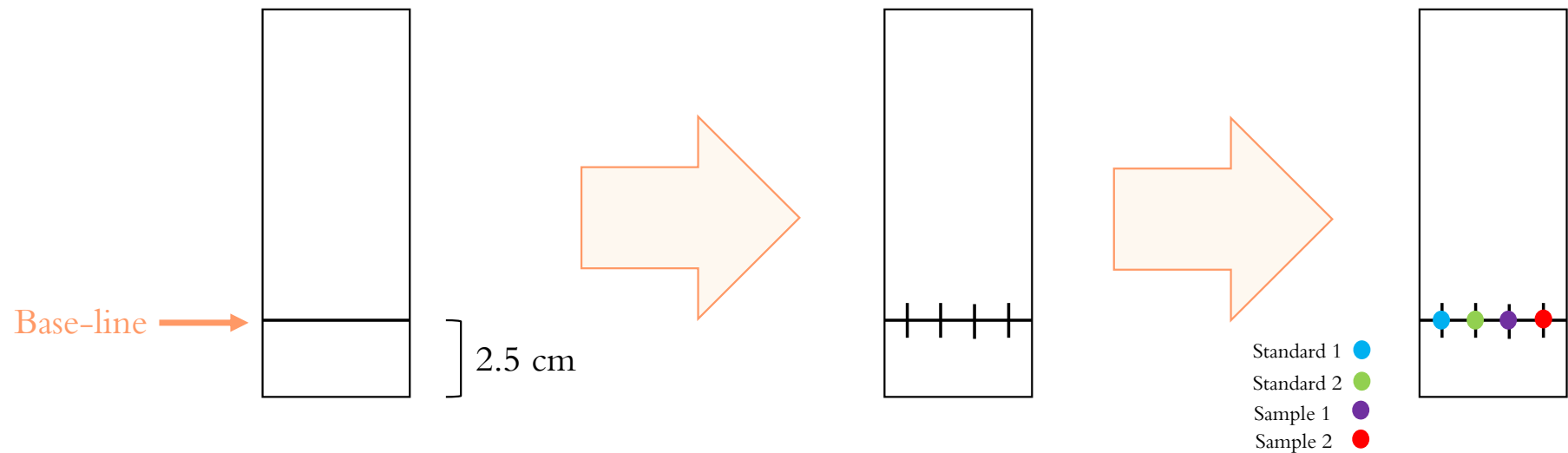
METHOD:

2. Label your TLC plate and PC, then place them in the solvent chamber. Do not forget to cover the solvent chamber.

Standard 1 ●
Standard 2 ●
Sample 1 ●
Sample 2 ●

St1 St2 S1 S2

] 2 cm

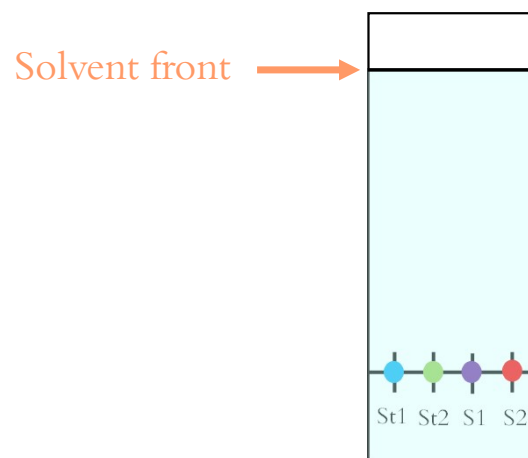3. Leave them for 45 min (or until the level of the solvent is 2 cm from the top).

St1 St2 S1 S2

METHOD:

4. Remove the TLC plate and PC paper from the solvent chamber draw a line to mark the solvent front.
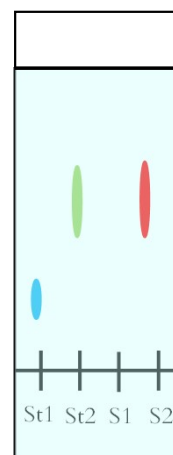
Solvent front

St1 St2 S1 S2

METHOD:

5. Spray the TLC plate and PC paper with ninhydrin (wear gloves and be very careful ninhydrin is carcinogenic).

6. Put the TLC plate and PC paper in the oven at 70 ℃ , until the color develops.

Color develops ➡️

RESULTS:

- *For TLC and PC record the following:*

1. Distance migration by solvent front = _____ cm.
2. Fill the table:

Sample	Distance (unit)	R_f
Phe standard		
Cys standard		
Sample 1		
Sample 2		