Chromatography is derived from Greek terms "chromo= colour & gram=bands." Hence as the name indicates, in chromatography there is the formation of colored bands. These bands are indicative of different components in the sample.

Chromatography' is an analytical technique commonly used for separating a mixture of chemical substances into its individual components, so that the individual components can be thoroughly analyzed. Chromatography is a separation technique that every organic chemist and biochemist is familiar with.

There are many types of chromatography e.g., liquid chromatography, gas chromatography, ion-exchange chromatography, affinity chromatography, but all of these employ the same basic principles.

Principle of paper chromatography:

Let's first familiarize ourselves with some terms that are commonly used in the context of chromatography:

Term	Definition
Mobile phase or carrier	solvent moving through the column
Stationary phase or adsorbent	substance that stays fixed inside the column
Eluent	fluid entering the column
Eluate	fluid exiting the column (that is collected in flasks)
Elution	the process of washing out a compound through a column using a suitable solvent
Analyte	mixture whose individual components have to be separated and analyzed

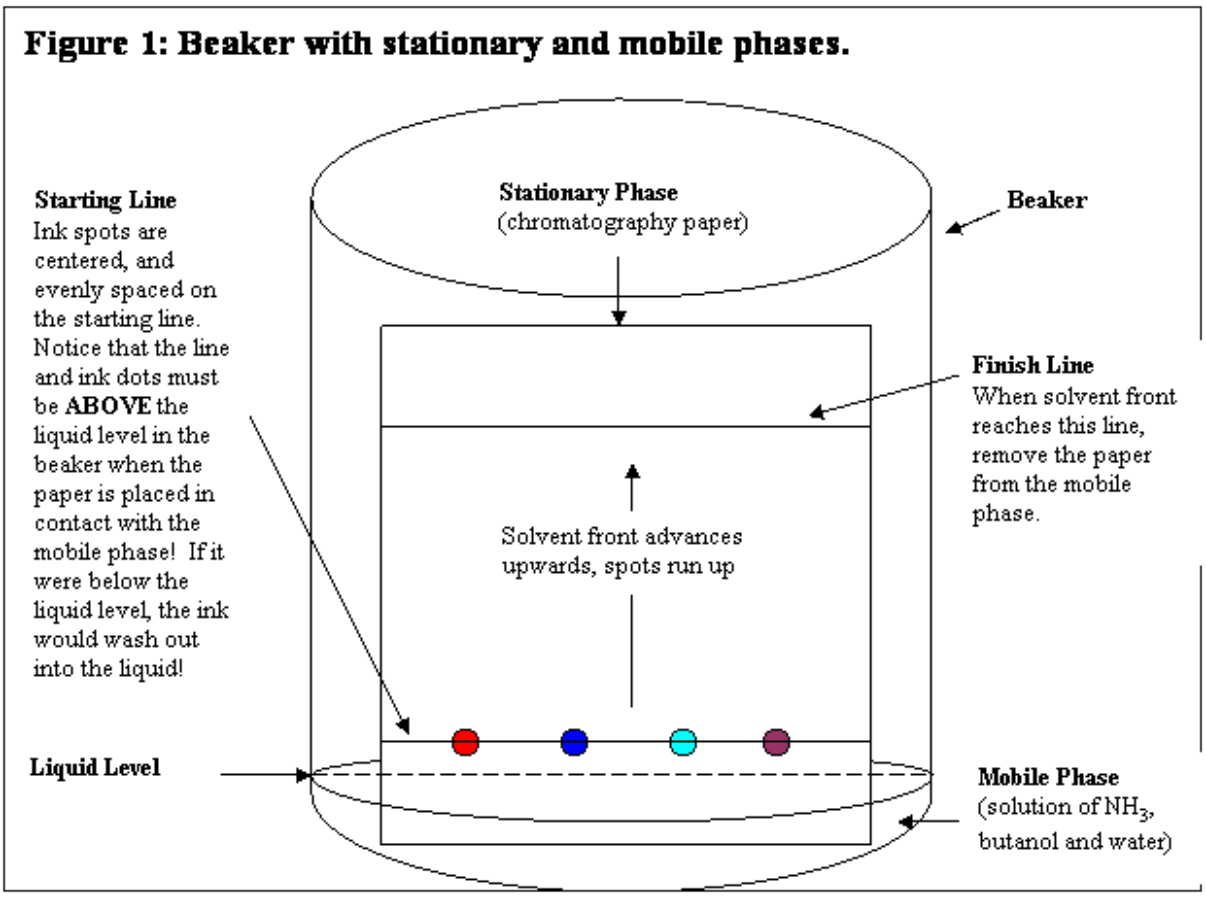
The principle involved is partition chromatography wherein the substances are distributed or partitioned between liquid phases. One phase is the water, which is held in the pores of the filter paper used; and other is the mobile phase which moves over the paper. The compounds in the mixture get separated due to differences in their affinity towards water (in stationary phase) and mobile phase solvents during the movement of mobile phase under the capillary action of pores in the paper.

The principle can also be adsorption chromatography between solid and liquid phases, wherein the stationary phase is the solid surface of paper and the liquid phase is of mobile phase. But most of the applications of paper chromatography work on the principle of partition chromatography, i.e. partitioned between to liquid phases.

Paper chromatography:

This is probably the first, and the simplest, type of chromatography that people meet. A drop of a solution of a mixture of dyes or inks is placed on a piece of chromatography paper and allowed to dry. The mixture separates as the solvent front advances past the mixture. Filter paper and blotting paper are frequently substituted for chromatography paper if precision is not required. Separation is most efficient if the atmosphere is saturated in the solvent vapour (Fig below).

Figure 1: Beaker with stationary and mobile phases.



Some simple materials that can be separated by using this method are inks from fountain and fibre-tipped pens, food colourings and dyes. The components can be regenerated by dissolving them out of the cut up paper.

The efficiency of the separation can be optimised by trying different solvents, and this remains the way that the best solvents for industrial separations are discovered (some experience and knowledge of different solvent systems is advantageous).

The experimental method involves:

Selection of suitable type of development:

This depends on the complexity of the mixture, solvent, paper, etc. But in general ascending type or radial type chromatography is used as they are easy to perform, handle, less time-consuming and also give chromatogram faster.

Selection of suitable filter paper:

Filter paper is selected based on pore size, the quality of the sample to be separated, and also mode of development.

Preparation of sample:

Preparation of sample involves dissolution of sample in suitable solvent used in making mobile phase. The *solvent used should be inert with the sample* under analysis.

Spotting of sample on the paper:

Samples are to be spotted at proper position on the paper preferably using a capillary tube.

Development of chromatogram:

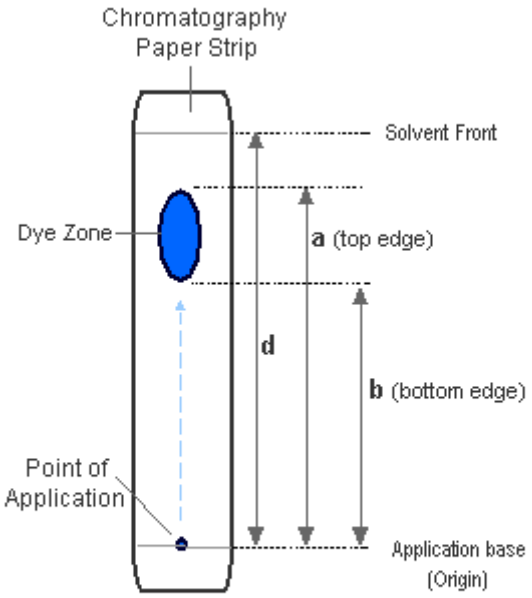
Sample spotted paper is subjected to development by immersing it in the mobile phase. The mobile phase moves over the sample on the paper under the capillary action of paper.

Drying of the paper and detection of the compounds:

Once the development of chromatogram is over, the paper is held carefully at the borders so as to avoid touching the sample spots and dried using an air drier. Sometimes the detecting solution is sprayed in the developed paper and dried to identify the sample chromatogram spots.

Paper chromatography works by the partition of solutes between water in the paper fibres (stationary phase) and the solvent (mobile phase). Common solvents that are used include pentane, propanone and ethanol. Mixtures of solvents are also used, including aqueous solutions, and solvent systems with a range of polarities can be made. A mixture useful for separating the dyes is a 3:1:1 mixture (by volume) of butan-1-ol: ethanol: 0.880 ammonia solution.

As each solute distributes itself (equilibrates) between the stationary and the mobile phase, the distance a solute moves is always the same fraction of the distance moved by the solvent. This fraction is variously called the retardation factor or the retention ratio, and is given the symbol R or Rf: Retention ratio = distance moved by solute distance moved by solvent = = Rf



RF top = RF(a) = (a) / d
RF bottom = RF(b) = (b) / d
RF center = RF(c) = [(a+b)/2] / d

It is possible that two solutes have the same Rf values using one solvent, but different values using another solvent (e.g. this occurs with some amino acids). This means that if a multi component system is not efficiently separated by one solvent the chromatogram can be dried, turned through 900, and run again using a second solvent.

There are five types of paper chromatography based mainly on the development of chromatogram.

Radial paper chromatography:

In this type of paper chromatography the solvent moves from the centre towards the peripheral regions of the filter paper. The radiating mixture component is allowed to spread till all the components have separated out. For precaution the entire system is covered with the help of a Petri dish.

The centre of the paper is allowed to be dipped into the solvent and the coloured components radiates out in concentric circles.

Ascending paper chromatography:

The chromatogram of this paper chromatography ascends slowly due to the mobile phase movement in a upward direction. The solvent is kept at the bottom of the filter paper or stationary phase with the end of the filter paper dipped in.

The component mixture spot is kept well above the solvent level and is not allowed to touch the spot.

Descending paper chromatography:

The mobile phase in this type is kept at the top of the chromatogram and the components of the mixture separate out downward due to gravity and capillary action of the filter paper.

Two dimensional paper chromatography:

The chromatogram in this type of paper chromatography develops at right angle to each other and the filter paper is dipped at right angle once the first chromatogram is complete.

The second chromatogram then develops at right angle to the first one.

Ascending and descending paper chromatography:

The chromatogram in this type first develops in upward direction and then is reversed and allowed to move again in reverse direction which overall shows a bi-directional movement of the mixture components.

Advantages of Paper Chromatography:

- ✓ This type of chromatography helps us identifying the affinity of molecules especially when a mixture of polar and non-polar components is taken for separation.
- ✓ The separation of various types of amino acids is carried out using this method.
- ✓ Paper chromatography very essential to segregate and determine the molecules present in urine sample.
- ✓ The forensic science and pharmaceutical industries make use of this method to determine the level of hormones and drug levels in samples.
- ✓ The paper chromatography is very useful in evaluating the various salts and metal complexes present in sample compounds.

Thin layer chromatography (TLC):

Thin layer chromatography is similar to paper chromatography, but the stationary phase is a thin layer of a solid such as alumina or silica supported on an inert base such as glass, aluminium foil or insoluble plastic. The mixture is ‘spotted’ at the bottom of the TLC plate and allowed to dry. The plate is placed in a closed vessel containing solvent (the mobile phase) so that the liquid level is below the spot.

TLC is a type of planar chromatography. It is routinely used by researchers in the field of phyto-chemicals, biochemistry, and so forth, to identify the components in a compound mixture, like alkaloids, phospholipids, and amino acids. It is a semi quantitative method consisting of analysis. High performance thin layer chromatography (HPTLC) is the more sophisticated or more precise quantitative version.

TLC has advantages over paper chromatography in that its results are more reproducible, and that separations are very efficient because of the much smaller particle size of the stationary phase.

The solvent ascends the plate by capillary action, the liquid filling the spaces between the solid particles. This technique is usually done in a closed vessel to ensure that the atmosphere is saturated with solvent vapour and that evaporation from the plate is minimised before the run is complete. The plate is removed when the solvent front approaches the top of the plate and the position of the solvent front recorded before it is dried (this allows the Rf value to be calculated).

TLC has applications in industry in determining the progress of a reaction by studying the components present; and in separating reaction intermediates. In the latter case a line of the reaction mixture is 'painted' across the TLC plate instead of a single spot, and the line of product after separation is cut out of the plate and dissolved in an appropriate solvent.

Principle:

Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation.

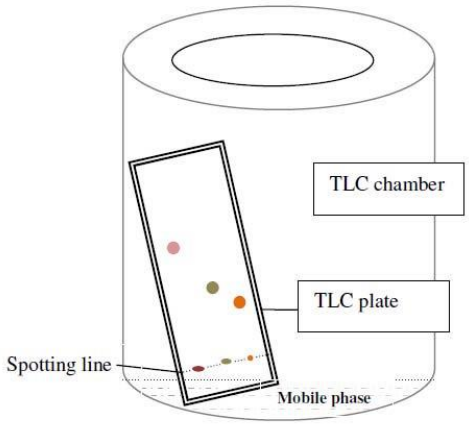
1. The separation depends on the relative affinity of compounds towards stationary and the mobile phase.
2. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved.
3. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character is identified by means of suitable detection techniques.

System Components:

TLC system components consists of

1. **TLC plates,** preferably readymade with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
2. **TLC chamber.** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.

3. **Mobile phase.** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.
4. **A filter paper.** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.



Procedure:

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are preferred.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect this way).
4. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
5. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent — as shown in the picture) for development.
6. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber, or any other methods as recommended for the said sample.

Advantages:

- ✓ It is a simple process with a short development time.
- ✓ It helps with the visualization of separated compound spots easily.
- ✓ The method helps to identify the individual compounds.
- ✓ It helps in isolating of most of the compounds.
- ✓ The separation process is faster and the selectivity for compounds is higher (even small differences in chemistry is enough for clear separation).
- ✓ The purity standards of the given sample can be assessed easily.
- ✓ It is a cheaper chromatographic technique.

Applications:

1. To check the purity of given samples.
2. Identification of compounds like acids, alcohols, proteins, alkaloids, amines, antibiotics, and more.
3. To evaluate the reaction process by assessment of intermediates, reaction course, and so forth.
4. To purify samples, i.e. for the purification process.
5. To keep a check on the performance of other separation processes.

Being a semi quantitative technique, TLC is used more for rapid qualitative measurements than for quantitative purposes. But due its rapidity of results, easy handling and inexpensive procedure, it finds its application as one of the most widely used chromatography techniques.

Different types of chromatography:

Throughout this article we are dealing with what we refer to as **normal-phase** chromatography, implying that our stationary phase is polar (hydrophilic) in nature and our mobile phase is non-polar (hydrophobic) in nature. For special applications, scientists sometimes employ **reverse-phase** chromatographic techniques where the scenario is reversed i.e. the stationary phase is non-polar while the mobile phase is polar.

There are several types of chromatography, each differing in the kind of stationary and mobile phase they use. The underlying principle though remains the same: *differential affinities of the various components of the analyte towards the stationary and mobile phases results in the differential separation of the components*. Again, the mode of interaction of the various components with the stationary and mobile phases may change depending on the chromatographic technique used. The commonly used chromatographic techniques are tabulated below.

Technique	Stationary phase	Mobile phase	Basis of separation	Notes
Paper chromatography	solid (cellulose)	liquid	polarity of molecules	compound spotted directly on a cellulose paper
Thin layer chromatography (TLC)	solid (silica or alumina)	liquid	polarity of molecules	glass is coated with thin layer of silica on which is spotted the compound
Liquid column chromatography	solid (silica or alumina)	liquid	polarity of molecules	glass column is packed with slurry of silica
Size exclusion chromatography	solid (micro porous beads of silica)	liquid	size of molecules	Small molecules get trapped in the pores of the stationary phase, while large molecules flow through the gaps between the beads and have very small retention times. So larger molecules come out first. In this type of chromatography there isn't any interaction, physical or chemical, between the analyte and the stationary phase.
Ion-exchange chromatography	solid (cationic or anionic resin)	liquid	ionic charge of the molecules	Molecules possessing the opposite charge as the resin will bind tightly to the resin, and molecules having the same charge as the resin will flow through the column and elute out first.
Affinity chromatography	solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and antibodies)	liquid	binding affinity of the analyte molecule to the molecule immobilized on the stationary phase	If the molecule is a substrate for the enzyme, it will bind tightly to the enzyme and the unbound analytes will pass through in the mobile phase, and elute out of the column, leaving the substrate bound to the enzyme, which can then be detached from the stationary phase and eluted out of the column with an appropriate solvent.
Gas chromatography	liquid or solid support	gas (inert gas like argon or helium)	boiling point of the molecules	Samples are volatilized and the molecule with lowest boiling point comes out of the column first. The molecule with the highest boiling point comes out of the column last.