

# Paper Chromatography

Paper chromatography is the method in which the analysis of an unknown substance is mainly done by the flow of solvents on specially designed filter paper. One of the two solvents is immiscible or partially miscible in the other solvent. The solvent rises up by the capillary action and by adsorption on the paper; the separation is effected by the differential migration of the mixture of substances. This occurs due to differences in partition coefficients.

## 1.1 Principle

The principle is a type of partition chromatography in which the substances are distributed between two liquids, i.e., one is the stationary liquid (usually water) which is held in the fibres of the paper and called the stationary phase; the other is the moving liquid or developing solvent and called the moving phase. The components of the mixture to be separated migrate at different rates and appear as spots at different points on the paper.

The principle can also be adsorption chromatography between solid and liquid phases, where in 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 two liquid phases.

## 1.2 Technique

The technique of paper chromatography is very simple. A drop of the solution containing the mixture of substances is placed near one end of the filter paper. The spot is then allowed to dry. The portion of the paper with the spot is then placed in a suitable solvent. The solvent passes the spot and carries the substances along with it while moving up the filter paper. Different substances move with different rates depending on the size, nature of the substances, solvent, diffusion coefficient and many other factors. The finished paper is known as paper chromatogram.

### 1.3 Types of Paper Chromatography

Based on the way the development of chromatogram on the paper is done in the procedures, we have broadly 5 types of chromatography.

1. **Ascending chromatography:** When the development of the paper is done by allowing the solvent to travel up the paper, it is known as ascending technique. The solvent reservoir is at the bottom of beaker. The paper tip with sample spots just dips into the solvent at bottom such that spots remain well above the solvent.

2. **Descending chromatography:** When the development of the paper is done by allowing the solvent to travel down the paper, it is known as descending technique. The solvent reservoir is at the top. The movement of solvent is assisted by gravity besides capillary action.

3. **Ascending- descending chromatography:** It is the hybrid of the above two techniques. In this technique, the upper part of the ascending chromatography can be folded over a glass rod allowing the ascending development to change over into the descending after crossing the glass rod.

4. **Radial paper chromatography:** Here the solvent travels from center (mid point) towards periphery of circular chromatography paper. The entire system is kept in a covered petridish for development of chromatogram.

The wick at the center of paper dips into mobile phase in a petridish by which the solvent drains on to the paper and moves the sample radically to form the sample spots of different compounds as concentric rings.

5. **Two-dimensional chromatography:** Here the chromatogram development occurs in two directions at right angles. In this mode the samples are spotted to one corner of rectangular paper and allowed for first development. Then the paper is again immersed in mobile phase at right angle to previous development for second chromatogram.

Paper chromatography can be divided into two classes.

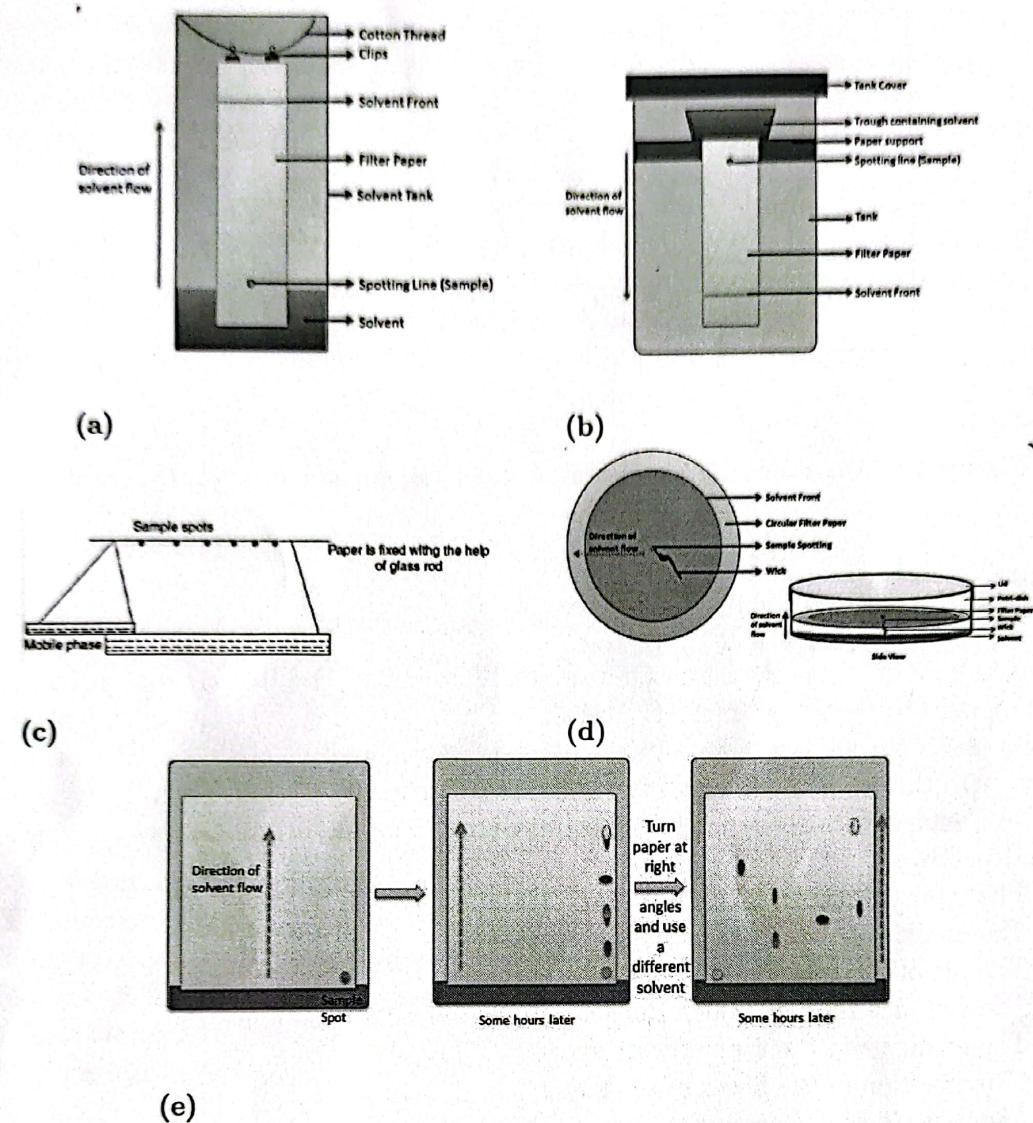
1. **Paper partition chromatography:** The standard method of analysis in which paper is used as an inter support with one solvent as mobile phase and the other solvent as immobile phase is called paper partition chromatography.

2. **Paper adsorption chromatography:** In some case paper is impregnated with an adsorbent like alumina or silica. The paper so modified, is used as the adsorbent and the single solvent is allowed to flow over the unknown components. This process is known as paper adsorption chromatography.

### 1.4 Migration parameter

The positions of migrated spots on the chromatograms are indicated by different terms such  $R_f$ ,  $R_x$ ,  $R_m$  and  $R_c$ . These parameters are also qualitative and quantitative parameters, characteristic of a substance.

$R_f$ :  $R_f$  is defined as the ratio of the distance travelled by the compound at its point of maximum concentration to the distance travelled by the solvent. Both the distances are measured from the point of application of the sample.  $R_f$  value has no



**Figure 1.1:** Types of paper chromatography. (a) ascending chromatography; (b) descending chromatography; (c) ascending-descending chromatography; (d) radial paper chromatography; (e) two-dimensional paper chromatography.

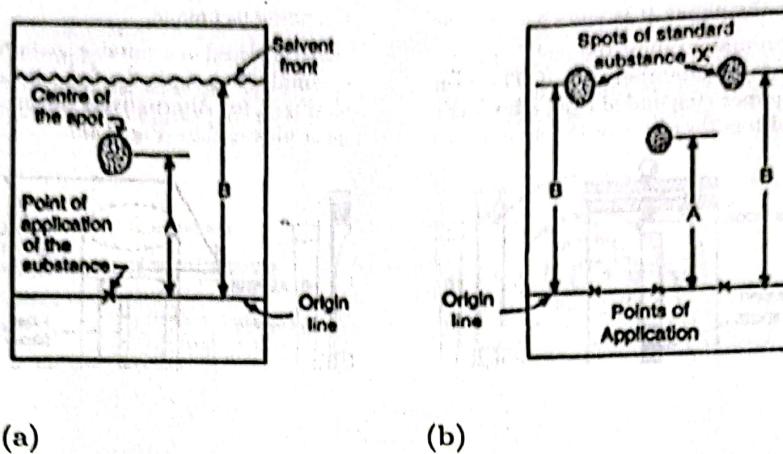


Figure 1.2: Diagrammatic representation of migration parameter. (a)  $R_f$ ; (b)  $R_x$ .

unit.

$$R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}}$$

It is to be noted that  $R_f$  value is always less than unity. The  $R_f$  values of different compounds are entirely different. Just as the half potential, melting point, boiling point and other physical constants are different for different compounds. The values vary with the solvents used e.g., two solvents will give two values. Thus  $R_f$  value is always quoted with reference to the solvent used. The important factors on which  $R_f$  depends are:

1. The temperature.
2. The quality of the paper used.
3. The pH of the solution.
4. The distance through which the solvent has moved on the paper.
5. The quality and the type of water used.
6. The direction of the fibres of the paper.
7. The method of development.
8. The distance through which the spot travels.
9. The concentration of the separated substances.
10. The method of drying, locating the colourless substances and many other operations involved in the method.
11. The impurities present on the surface of the paper.
12. Irreversible adsorption on paper.
13. Chemical reactions between substances being partitioned and the paper and ion exchange with acid groups of cellulose.

When the solvent moves over the spots, there are two types of forces which are involved. They are-

1. Propelling forces: This assists in the propagation of the substances in the direction of the flow of the solvent.
2. Retarding forces: This type of force tries to drag the substances behind towards their point of application. The  $R_f$  value or the distance through which the substances move on the paper under the influence of the solvent, is due to the resultant

of these two types of forces.

$R_x$ : In some cases, the solvent front runs off the end of filter paper, the movement of a substance in such cases is expressed as  $R_x$  but not  $R_f$ :

$$R_f = \frac{\text{Distance travelled by the substance from the origin line}}{\text{Distance travelled by the standard substance } x \text{ from the origin line}}$$

Pictorial representation of  $R_f$  and  $R_x$  have been made in the figure.

$R_m$ : The  $R_f$  values of chemically related compounds are very close. The influence of individual functional groups was presumed to be added to a rough approximation. According to Bate-Smith and Westall,  $R_m$  is defined as follows:

$$R_m = \log \frac{1}{R_f - 1}$$

The term  $R_m$  is additive and is composed of the partial  $R_m$  value of the individual functional groups or other grouping of atoms in the molecules.

## 1.5 Method

By this time numerous suggestions have been made regarding the way in which these experiments should be done. There are many essentialities which should be taken into account while doing experiments on paper chromatography on sheets or strips of paper. The following operations, which are most important, are involved.

1. Choice of filter paper
2. Preparation of the paper
3. Preparation of sample
4. Application of sample to the paper
5. Choice of solvents
6. Development of chromatograms
7. Drying the chromatogram after development
8. Development of spots
9. Quantitative estimation

### 1.5.1 Choice of filter paper

The basic material of the paper used in paper chromatography is  $\alpha$ -cellulose of high purity which is present in 98.99%. Besides, there are other substances which are also present, like  $\beta$ -cellulose ether soluble matter, ammonia, nitrogen like compounds and mineral matters. Cellulose chemically consists of long chains of glucose molecules and has the normal properties of a polyhydroxy alcohol. There are also present aldehydic groups with normal reducing properties of a polyhydroxy alcohol. Whatman filter paper has been extensively used in chromatographic work. The chemical composition is as

$\alpha$ -cellulose	98.99%
$\beta$ -cellulose	0.3-1.0%
pentosans	0.4-0.8%
ash	0.07-0.01%
Ether soluble matter	0.015-0.1%

Since there are various grades and types of paper the most suitable is the one which satisfies the following conditions.

- a. The paper with maximum degree and clarity of the separation.
- b. The paper with negligible diffuseness of the spots.
- c. The paper with excellent rate of movement of solvent front.

The choice of the paper depends on the thickness flow rate, purity and net strength. If the paper does not have the desired properties as mentioned above, they can be made so just by the modification method.

Paper can be modified for chromatographic work either by impregnating with other substances or by changing the chemical structure of the cellulose. The impregnation of the paper can be done with aqueous buffer solutions, which affects the separation in many ways. The stabilization of pH enhances or suppresses the ionization of acids or base.

### **1.5.2 Preparation of the paper**

After having selected the quality of the paper which has to be used the next question that comes in is about the shape and size of the paper. This is more particularly important in descending technique. Shape of the paper facilitates in giving better resolution of less polar compounds. Shape and size depend on the type of the work involved. Though paper of shapes like rectangular and square have been used by many workers but the most common is rectangular shaped paper.

In some, very precise measurements, it is always advisable to wash the paper wash the paper before use in order to remove reducing substances. After washing, the paper is used at once otherwise some of the compounds may be again formed by oxidation or break down of cellulose.

### **1.5.3 Preparation of sample**

In the preparation of the samples for chromatographic work, the important question is to get and choose the proper solvent for making solution. The complexity arises in cases of qualitative or quantitative type of work involved therewith. For solids, a weighed amount is dissolved in a volatile solvent and by careful means the minimum volume of the concentrated solution is applied on the paper. This is done simply to avoid diffusion through paper which ultimately results in larger zones. Extracts from plant and animal tissues are taken out with the help of some solvent which is then directly applied on the paper.

### **1.5.4 Application of sample to the paper**

The amount of sample to be applied is again important factor. The factors which govern the amount of sample to be applied are, (1) The capacity of the solvent, (2) Optimum concentration required for the quantitative determination, (3) The time required for development. With these considerations in mind, final decision is made regarding the amount of the sample to be applied.

The samples may be applied either as spots or bands. A line is drawn with the help

of a pencil at a suitable distance from one end of the paper. A number of points are marked on the line at equal distances. A drop of each sample is then put on these points with the help of capillary tubing, microsyringe or platinum loop. It is to be seen that spots occupy small areas which should at the most be of 5 mm in diameter. The volume of the liquid may vary from 1 – 2 $\mu$ L or more as the case may be.

For more practical purposes platinum loop is preferred because it can be used again and again by carefully washing and heating strongly in a flame after each application. In order that the spots may not diffuse, stream of hot nitrogen or air is blown at the place after putting the spot. This keeps the spots intact at its position by evaporating the volatile solvents. After doing so, the paper becomes ready for development.

### 1.5.5 Choice of solvents

There are a number of solvents which can be used in paper chromatography. The final selection depends entirely on the nature of the substances to be separated. There are two ways to solve this problem. The first is the knowledge which one could get from literatures and the second is by trial and error method.

The most commonly used is the polar solvent which is adsorbed on the paper. A less polar solvent, which is called the mobile phase, advances ahead by capillary action and separates the applied substances. Water miscible solvents such as furan propanol and cellosolve have been excessively used. In most cases a mixture of n-butanol acetic acid and water has been used. But in place of n-butanol, some tertiary alcohol is preferred because of the tendency of the former to esterify with organic acids. The selection of solvents should be such so that when the compounds are mixed together, two definite layers result. When using solvents it is essential that they are used in the specified ratios and are well shaken before use.

Alcohols, acids, ketones, esters, phenols, amines or other bases, aromatic or aliphatic hydrocarbons, constituting single phase solvent systems are also used. As far as possible minimum number of solvents are used. This facilitates in making the chamber saturated with the solvent which prevents evaporation from the paper during the development.

### 1.5.6 Development of chromatograms

There are a number of apparatus designed to carry on paper chromatography. The following things are taken into consideration:

- a. There is sufficient amount of solvent at the bottom of the reservoir.
- b. There is proper arrangement so that the paper is freely suspended.
- c. It is particularly to be noted that large temperature variations are avoided to the maximum.

The reservoir is made from a variety of materials. The material should be insoluble in the solvent and that purpose glass, polythene, stainless steel and many other materials have been successfully used. The paper may be held in position by clamps.

The choice of the method of development depends upon the class of compounds to be investigated. It is in the successful experiment that the type of development must be selected before and this governs the choice of the solvent system, its amount, clipping arrangement and also the method of application. The following are the types of developments.

a. **Ascending development:** This is the simplest of all the methods. It consists of dipping the lower end of the paper into the solvent and then allowing it to rise up the paper by capillary action. This method is preferable for quick analysis of a larger number of substances. Proper care is taken to ensure that the lower end of the paper containing spots is so placed so that it is above the solvent depth. The two ends of the paper are not allowed to touch with each other and kept at a distance apart by clipping or by any other method.

b. **Descending development:** This form of chromatography is used very frequently. 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 pull of the gravity. In this case the flow is more rapid as compared to ascending method. Because of this rapid speed the chromatogram is completed in the comparatively shorter time.

The apparatus required in this case is more sophisticated. The developing solvent is placed in a trough at the top which is usually made up of an inert material. The paper is then suspended in the solvent and the lid is placed at the top. By following this method it is rather impossible to carry on two dimensional development. This is because of the paper must be cut to fit into the trough.

Compound with low  $R_f$  values are not completely separated by ascending method but this case can be done by following descending technique.

c. **Radial or disk development:** This type of development is very rarely used and that too in some very special cases. In this method, a circular piece of paper is taken which has a wick cut parallel to the radius, from edge to centre. The sample is deposited at the centre of the paper and at the upper end of the wick. This paper is then laid on the edge of a circular disk with the wick dipping into the solvent at the bottom of the dish. The liquid ascends the wick and flow radially through the paper. While moving it carries the compounds with it. As the solvent moves ahead, the radial becomes slower and takes more time for the solvent to reach the last circle.

### 1.5.7 Drying the chromatogram after development

After the development by any of the methods as described earlier, the paper is removed from the reservoir and the position of the solvent front is marked with the help of the pencil at the two edges of the paper. The chromatogram is then dried by keeping in an oven or over a hot plate for few minutes. This is done so that the solvents are completely dried from the paper. The drying is best done by means of a fan or hair dryer.

### 1.5.8 Development of spots

After drying of the developed chromatogram, it is very essential to locate the separated substances. If the substances are coloured, no difficulty arises but the problem stands with the colorless substances. There are several methods which have been described to locate the spots, all of which can be grouped into (1) physical and (2) chemical methods.

#### 1.5.8.1 Physical methods

There are a number of methods which are used to locate the substances. Physical methods have got an edge over chemical methods in the sense that the substances on the paper are not converted into other compounds. Thus they can be removed and studied.

In such cases where the compounds are invisible in ordinary light, UV lamp can be used to locate their positions. The other method is the use of radioactivity in which a number of labeled compounds are available which can be detected by means of Geiger Muller counter.

#### 1.5.8.2 Chemical methods

In those cases where a physical method cannot be used, the help is taken of the chemical methods. The colourless compounds are converted into coloured compound by reaction with some chemical reagents. The chemicals so used are known technically as locating reagents. The locating reagents can be gas, liquid or solid but mostly liquid and solid are used for this purpose. The  $H_2S$  gas is mostly used in detecting metallic ions of II and IV group elements. Detection by colour reactions is the normal methods used for colourless substances that cannot be located by any other methods. The substances can be located with an indicator and other reagents but the beauty of the method is that it will not interfere with other estimation methods.

The locating reagents are applied by

1. Spraying the solution on the paper and or
2. Dipping the paper into the reagent solution.

**Spraying method:** The solution of the locating reagent is sprayed on the paper uniformly with the help of a glass atomizer. Chromatograms are hanged on suitable racks and fixed in the position with the help of stainless steel clips. The atomizer held in the position normally at a distance of 12-15 inches from the paper and is moved slowly from top to bottom in a left to right direction. It is to be noted that much of the spray is not done on the paper. This will result in the diffuseness of the spots.

**Dipping method:** In this method it is essential to choose a solvent in which the substances are insoluble. Dipping is done in a tray of smaller size. The volatile solvents are most suitable because they are rapidly evaporated from the chromatogram. Ninhydrin (0.2-0.3%) solution is used for amino acids detection, heating the chromatogram at 105 °C for 5-6 minutes is extremely essential. Ninhydrin is a triketohydrindene hydrate which reacts with amino group of amino acids. As a result

of this reaction the ninhydrin is reduced and the amino acid is deaminated and decarboxylated. The product so formed has a characteristic purple colour which has an absorption of maximum at 540 nm.

### 1.5.9 Quantitative estimation

In most of the chromatographic work the simple measurement of  $R_f$  values are done either by the comparison with the reference substances or with the standard values. Thus the identification of the substance is done. But in order that paper chromatography may form the basis of quantitative tool, it is a must that the amount of material in each spot be measured. For this purpose, variety of methods, depending on the properties of the compounds has been used. All the available methods can be divided into two main groups:

- Direct measurement of the amount of the substances.
- Prior elution methods.

The choice of the methods depends upon the factors like the chemical and physical properties of the compound, composition and capacity of the system and also on the degree of required accuracy.

- Direct measurement methods:

i. **Comparison of visible spots:** This is one of the simplest methods known so far. The method involves comparison of the size and intensity of colour, by fluorescence of UV absorbance of the spot on the chromatogram, with the known amounts of the desired compounds. Thus the unknown compound is identified.

ii. **Densitometry:** This method is used with the chromatograms of coloured compounds. A thin strip is cut from the chromatogram, placed between two sheets of glass and then into densitometer. A plot is made of transmission or absorbance against the distance from the origin. The peak heights or areas under the peaks are measured and then compared with similar measurements made standards treated in an identical manner. This method gives determinations accurate within 5% in most cases.

iii. **Fluorimetry:** Direct fluorometric measurements are made by the technique as described above. The essential condition is that the compound to be determined must be naturally fluorescent or else must be convertible into fluorescent derivatives. The measurements are then made on a densitometer or similar light source and filters.

iv. **Radiotracer method:** The radioactive elements are used to locate and determine the quantity of material on the chromatogram. The compound on the chromatogram is identified by subjecting to neutron bombardment. The activity and location are measured by passing the paper either in a gas flow counting chamber or a thin window Geiger Muller tube. The results so obtained are compared with standards under similar conditions.

v. **Miscellaneous methods:** Several other methods like polarographic and conductometric methods have been used to measure the amount of material in the spot on the chromatogram.

b. **Elution method:** The spots from the developed chromatogram are eluted. The

bands are cut off from the paper and then shaken with the appropriate solvent in the flask. The elutes as obtained from the chromatogram may be diluted or concentrated as the case may be and then analyzed by any technique that does not react with the components.

## 1.6 Sources of error

There are the possibilities of loss and error involved which are enumerated as follows:

- a. **Application:** While applying the spots or bands on the paper with the help of platinum loop or capillary tube, care must be taken to see that the same diameter exists for all the spots. Even distributions of the spot will cause them to develop in an unevenly manner.
- b. **Development:** This error is mainly due to the improper adjustment of the paper in the tank. The paper should be held vertically; otherwise it will lead to erroneous results. The other possibility of error may be due to lack of equilibrium between the developing solvent and the atmosphere in the tank. Loss may also be due to irreversible adsorption of the compound by the paper and due to diffusion as well. The plot of recoveries versus time of development is made and from that, the chromatograms are developed for the fixed time and the recovery is corrected for the loss occurring during that time.
- c. **Detection:** The additional error may also be due to the detection method. The spraying or the dipping method affects the final result considerably.
- d. **Elution:** The error also arises because of incomplete elution of the compound from the paper. This is corrected by choosing the solvent of a more polar in character.

## 1.7 Uses and applications

Paper chromatography is specially used for separation of mixture having polar and non polar compounds.

For separation of amino acids.

It is used to determine organic compounds, biochemicals in urine etc.

In pharmaceutical sector for determination of hormones, drugs.

Sometimes used for evaluation of inorganic compounds like salts and complexes.

## 1.8 Experimental method

The experimental method involves

- a) **Selection of suitable type of development:** This depends on complexity of the mixture, solvent, paper etc. But in general ascending type or radial type of chromatography are used as they are easy to perform, handle, less time consuming and also give chromatogram faster.
- b) **Selection of suitable filter paper:** Filter paper is selected based on pore size,

quality of the sample to be separated and also mode of development.

c) **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.

d) **Spotting of sample on the paper:** Samples are to be spotted at proper position on the paper using preferably a capillary tube.

e) **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.

f) **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.

## 1.9 General preparation

In the experiments on paper chromatography, decision must be made beforehand regarding the facts like,

1. The colour of the solution.
2. If colourless, then what locating' reagents or which physical methods have to be applied.
3. The solvents to be used which again depends on the nature of the substance in question.
4. Whether ascending or descending method has to be used.

After having solved the above problems, preliminary preparations are made in the stepwise manner.

1. **Paper:** Normally a rectangular sheet of Whatman filter paper No.1 is used. As the case may be, the size of the paper is cut accordingly. On the paper, a line is drawn at a distance of about 2 cm. from one end. On this line are put dots equal in numbers of the test solutions at equal distances, leaving 2 cm. from each end.

2. **Solvent:** Suitable solvent (about 25 mL) is kept in a jar which is then covered with the lid to avoid evaporation.

3. **Sample application:** In the laboratory work capillaries are used to put the samples on the paper. Fresh capillary tubes are dipped in each test solution and are put on the dots of the paper. Every drop has to be dried before a second drop is put on the paper. For drying purpose, a hot plate or better a hair dryer is used. Below every spot, the specifications are made with the help of the pencil.

4. **Operation:** The two edges of the paper are brought together and held at their positions with the help of stainless steel clips or scotch tape so that they do not touch with each other. By doing so they are curled into a cylindrical form.

The paper is then dipped into the tank with the spotted end in the lower position. While dipping into the solvent, It has to be noted that the spotted line remains a little above the level of the solvent and also the sides of the tank are not touched by the paper.

Soon after the paper is dipped, the solvent begins to flow and moves over the spots. As it flows over the spot, separation of the components starts, which may be completed in a couple of hours time. When the solvent has reached a few cm below the top height, the experiment is stopped and the paper is unfolded and solvent front is marked with the help of a pencil.

With the coloured substances there is no difficulty in noting the position of each substance but the situation is complicated with the colourless substances.

**5. Drying the chromatogram:** After unfolding the paper, it is essential to dry the chromatogram which is done with the help of hair dryer and electric fan.

**6. Location of the different, substances:** For colourless substances, the location' is done by dipping or spraying techniques which have already been described. After treatment, the spots may become coloured at once or after the chromatogram is heated to 100 – 105 °C in an oven for 5 to 6 minutes.

**7. Calculation of  $R_f$  value:** The distance of the solvent front is noted from the origin: This is what is known as the distance of the compound moved from origin. By the knowledge of these two,  $R_f$  values of each individuals are calculated.

## Problems