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|---|---|
| Name of Unit: | Introduction to chromatography: Adsorption and partition column chromatography, Thin layer chromatography, Paper Chromatography, Electrophoresis |
| Course/Subject Name: | Instrumental Methods of Analysis |
| Course/Subject Code: | BP701T |
| Class: B. Pharm. Semester | VII |
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## Learning Outcome of Module-III

| LO | Learning Outcome (LO) | Course Outcome Code |
|---|---|---|
| LO1 | To understand the chromatographic separation and analysis of drugs. | BP701.1, BP701.4, BP701.6 |
| LO2 | To understand the chromatographic separation using coloumn chromatography | BP701.1, BP701.4, BP701.6 |
| LO3 | To understand the chromatographic separation using TLC and Paper chromatography | BP701.1, BP701.4, BP701.6 |
| LO4 | To understand the separation and analysis of drugs using Electrophoresis. | BP701.1, BP701.4, BP701.6 |

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## Column Chromatography

Chromatography is a technique used for the separation and analysis of complex mixtures. The separation phenomenon was first reported by a Russian botanist Mikhail Tswett in 1906. He separated different colored constituents of plant extract by passing them through a column of finely divided calcium carbonate, alumina and sucrose. Tswett observed that various pigments such as xanthophylls and chlorophylls appeared as colored bands on the column and he coined the term the chromatography for this technique (Greek word *chroma* means 'column' and *graphein* means 'to write' ). Many advances have been made in this field in the last century, notably by Martin and Synge who introduced the concept of gas-liquid chromatography in 1941. They were awarded Noble Prize for their contribution to chromatographic science. Broadly, chromatographic techniques can be divided into planar and column chromatography.

As the name suggests, planar technique incorporates the use of a flat, planar, two-dimensional surface (where only length and breadth are taken as area) on which chromatograms are formed. This surface is coated with the stationary phase. Paper Chromatography, Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) are different types of planar techniques. Column Chromatography has stationary phase held in a column or a narrow tube. Most common types are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC).

## Principle of Column Chromatography

Column chromatography separations involve stationary phase being held in a narrow tube (column). The mobile phase or eluent, along with the mixture of components (solutes) is forced through the tube having stationary phase, under pressure. This process is termed as elution. Eluate is fluid emerging from the end of the column. Columns are either packed or open tubular. Packed column is filled with small particles of stationary phase whereas open tubular column is usually a narrow capillary tube with stationary phase coated on the inside wall.

## Classification of Column Chromatography

Chromatography is a dynamic process and all its types involve two immiscible phases- one is stationary phase (which stays inside the column or on a planar surface) and the other mobile phase (which moves through the column or over planar surface). Mobile phase can be liquid or gas and helps in redistributing the components of a mixture between the two phases, thus aiding

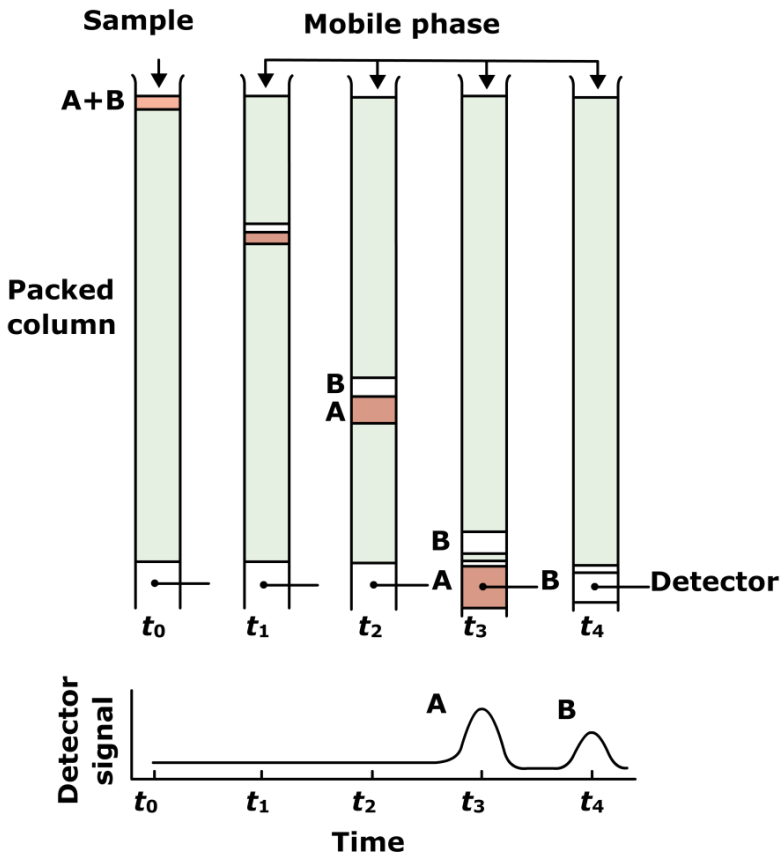
in their separation. The stationary phase can be solid, liquid, liquid adsorbed on solid or organic species bonded to solid surface. Solid forms include non-exchange resins.

Following table classifies the various types of chromatographic techniques based on the type of mobile phase used- liquid, gas or supercritical fluid. (Supercritical fluid mobile phase method is relatively new and is gaining popularity) Liquid chromatography can be further divided into five categories depending upon the stationary phase used and the mechanism of redistribution followed to attain equilibrium. Components of the mixture follow different mechanisms of interaction or redistribution namely, adsorption, partition, ion exchange or size exclusion.

Likewise, gas chromatography can be further sub-divided into three types. It is to be noted that liquid chromatography can be carried out in column as well as on planar surface but gas chromatography and supercritical chromatography can only be performed in a column.

## Process of Elution

The process of elution and separation of various components by column chromatography can be depicted by the following diagram.



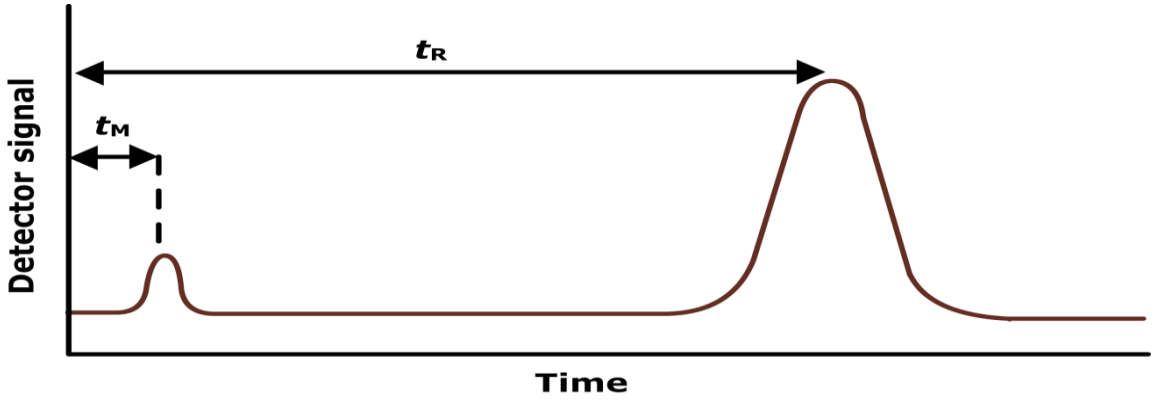



***Fig.1: Showing process of elution and separation of various components by column chromatography***

A solution having components A and B in the mobile phase is introduced at the head of the column. Elution process begins when addition of fresh solvent (mobile phase or eluent) which is applied to the top of the column. The mixture of A and B in washed down into the column and these components distribute themselves between the mobile phase and stationary phase. The average time spent by a particular component in either stationary or mobile phase depends upon its affinity for that phase.

In the diagram, component B is retained on the stationary phase for longer period than component A. These difference in their rates of movement (A moving faster than B) along the length of the column causes the components of the mixture to separate into bands or zones. Complete isolation of the separated components is achieved by passing sufficient mobile phase through the column and collecting (or detecting) them at different times (timest3 & t4).

**The Chromatogram**

Various solutes emerging from the column are observed with "Detectors". The detectors may be specific for the type of solute detected and are described in later modules. A chromatogram is a graph showing the detector response plotted against elution time. Fig.1 is a chromatogram of two components. Such a plot is useful for carrying out both qualitative and quantitative analysis. The positions of the peaks on the time axis are used for qualitative analysis whereas the peak areas reveal the quantitative aspect of the components.

***Fig.2: Chromatogram depicting detector response against elution time***

Before proceeding ahead, we should be aware of certain terms used in column chromatography.

**Retention Time**

Retention time is the time taken by a solute to reach the detector after the mixture is injected onto the column. It is represented by $t_R$.

Adjusted retention time, $t'_R$ is given by the expression

$$t'_R = t_R - t_M$$

Where, $t_M$ is the time taken by unretained species or mobile phase to reach the detector. It is also referred to as **Dead Time**.

So, the adjusted retention time for a solute is to move through the column beyond the time required by mobile phase to travel through the length of the column.

**Partition Coefficient**

The solute components partition themselves in the mobile phase and stationary phase as they move through the column. If the column is run slow enough, the concentration of solute in the stationary phase and mobile phase reaches equilibrium. The equilibrium constant or partition coefficient, K, for this reaction is defined as

$$K = C_S / C_M$$

Where, $C_S$ is the molar concentration of the solute in the stationary phase and $C_M$ is the molar concentration in the mobile phase. Most of the chromatographic separations are performed under linear condition i.e. $C_S$ is directly proportional to $C_M$. Thus, for solute X, it may be written

$$X_{mobile} \leftrightarrow X_{stationary}$$

**Capacity Factor**

It is also called retention factor and is used to describe the rate of migration of the solute on column. The capacity factor $k'$ is defined as

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

This equation implies that longer a solute is retained by the column, greater is the capacity factor. If this factor is in the range of 20 to 30, elution time becomes too long, but if it is less than 1, the solute passes out without interacting with stationary phase. Ideal value lies between 1

to 5.

**Selectivity Factor or Relative Relation, $\alpha$**

This term describes the extent of separation between two components. Greater the value of selectivity factor or relative retention, $\alpha$, greater is the separation between closely spaced components in the chromatogram.

It is expressed as:

$$\alpha = k'B / k'A$$

Where, k'B is the capacity factor for more strongly retained solute B and k'A is the capacity factor for solute A, which is eluted faster. It can be rearranged to give:

$$\alpha = (tR)B - tM / (tR)A - tM$$

Both capacity factor and selectivity factor can be easily calculated from the chromatogram and are used to monitor the performance of the column.

**Thin Layer Chromatography (TLC)**

Thin layer chromatography (TLC) is a quick, sensitive, and inexpensive technique used to determine the number of components in a mixture, verify the identity and purity of a compound, monitor the progress of a reaction, determine the solvent composition for preparative separations, and analyze the fractions obtained from column chromatography. This unit is mainly aimed at novice experimenters, describing in detail the strategies and principal steps for performing a TLC experiment, with illustrations of the relevant instruments, as well as approaches for obtaining and understanding results

**OVERVIEW AND PRINCIPLES**

The first widespread application of partition chromatography on a planar surface was paper chromatography, introduced in the 1940s. However, paper chromatography was gradually replaced by thin-layer chromatography (TLC), which has become one of the most routinely used chromatography techniques (Ettre and Kalasz, 2001). TLC is also ´ a liquid-solid adsorption technique where the mobile phase ascends the thin layer of stationary phase coated onto a backing support plate resembles column chromatography (UNIT 6.2), where the solvents

(eluents) flow down through the column's adsorbent. However, unlike column chromatography, TLC is a quick, sensitive, and inexpensive technique that only requires a few micrograms of sample for one successful analysis. TLC is commonly used to (1) determine the number of components in a mixture; (2) verify the identity and purity of a compound; (3) monitor the progress of a reaction; (4) determine the solvent composition for preparative separations; and (5) analyze the fractions obtained from column chromatography. Like all forms of chromatography, TLC involves a dynamic and rapid equilibrium of molecules between the two phases (mobile phase and stationary phase). However, TLC differs from all other chromatographic techniques in the fact that a gas phase is present, which can influence the results of separation significantly. Between the components of the mobile phase and its vapor, an equilibrium will be established gradually (also called chamber saturation). The part of the stationary layer that is already wetted with mobile phase also contributes to the formation of the equilibrium (Fig. 6.3.1). During development, molecules are continuously moving back and forth between the free and adsorbed states (Fig. 6.3.2A). A balance of intermolecular forces determines the position of equilibrium and thus the ability of the solvent to move the solute up the plate (also see Strategic Planning for details). This balance depends on (1) the polarity of the TLC coating material, (2) the polarity of the development solvent, and (3) the polarity of the sample molecule(s). For example, with a sample consisting of two compounds A and B as illustrated in Fig 6.3.2B, if the molecules A spend more time in the mobile phase, they will be carried through the stationary phase more rapidly and move further in a certain time. While molecules B are adsorbed to the stationary phase more than A, B molecules spend less time in the mobile phase and therefore move through the stationary phase more slowly, and do not move as far in the same amount of time. The consequence is that A is gradually separated from B as the mobile phase flows (ascends).

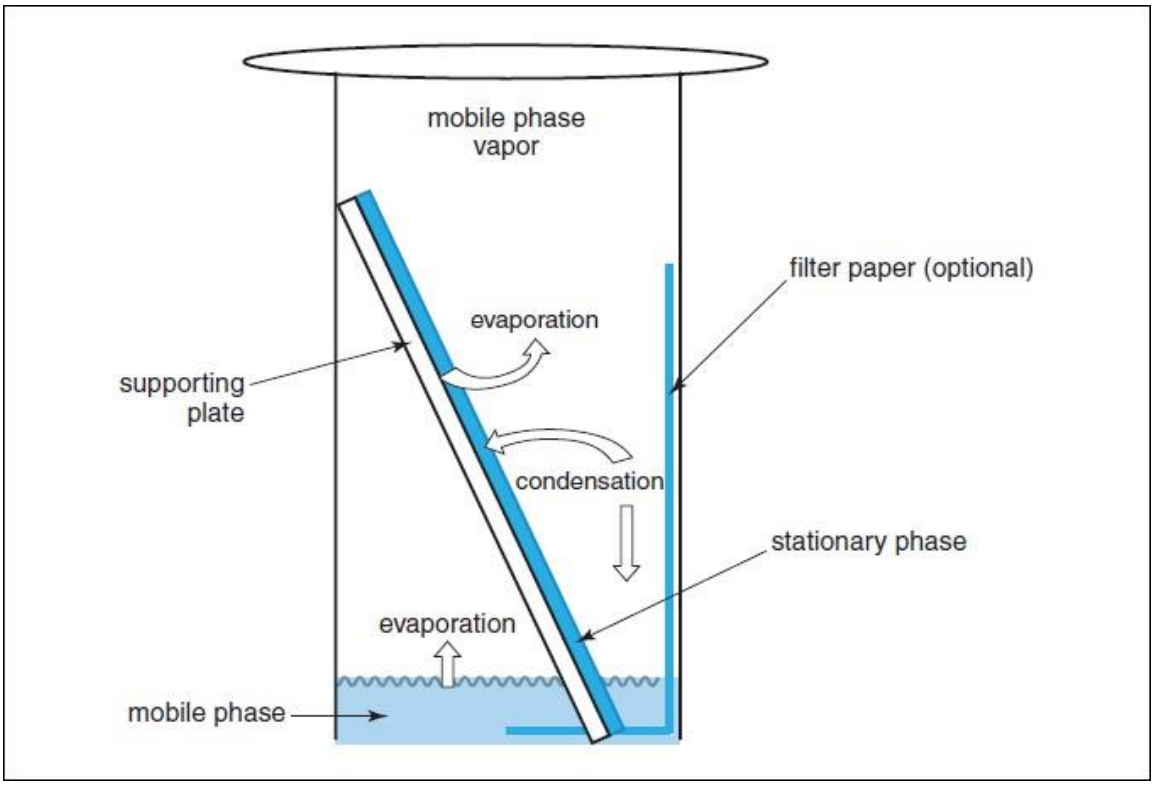
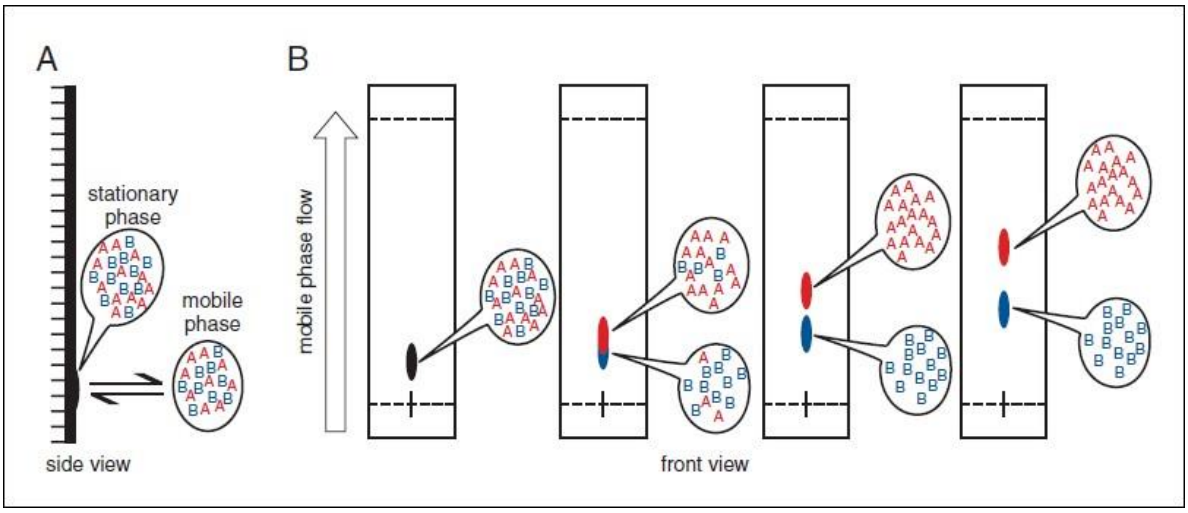



*Figure 1 Schematic representation of ascending development chamber for conventional TLC*




*(side-on view).*

**Figure 2 (A) Mixture of A and B adsorbed on the stationary phase and free in mobile phase and (B) schematicrepresentations of the principle of separation.**

## TLC Plates

### *Supports for stationary phases (glass, aluminum, and plastic)*

Glass has been found to be a very robust support. It is rigid and transparent, and has high chemical resistance and good heat stability. The glass backing is economical (reusable). However, glass plates are relatively heavy and thick. They cannot be easily cut to desired size (see steps for handling and cutting TLC plates in the Basic Protocol, below). Because glass backing is fragile and highly susceptible to breakage, there is also a potential safety issue. Aluminum foil is preferable to all other materials for TLC plates. Compared with glass plates, foil plates are thin, lightweight, and easy to handle. They can easily be cut to desired dimensions with scissors and can be stored in a laboratory notebook. Moreover, aluminum plates have strong adsorbent layer adherence and are good for use with eluents containing a high concentration of water. However, they are not as chemically resistant as glass to reagents that contain strong acids, concentrated ammonia, or iodine (i.e., they do not tolerate long treatments in an iodine chamber). Plastic—polyethylene terephthalate (PET) film— plates are becoming less frequently used. Their advantages (thin, lightweight, easy to handle, can be easily cut, etc.) are similar to aluminum-foil plates, but their flexibility (adsorbent layer may be more susceptible to cracking) and considerably inferior heat stability are very marked disadvantages.

## Adsorbent layers and stationary phases

The standard silica coating (silica 60 with a mean pore diameter of 60 A° ) is the most commonly used adsorbent in TLC, although for some very sensitive substances less active adsorbents such as aluminum oxide are preferred to prevent sample decomposition. Moreover, in the early days, the use of cellulose, polyamide, and Florisil (magnesium silicate) as adsorbent agents was also described. For selection of an adsorbent, one considers the properties of the compounds to be separated: first, the solubility of the sample compounds (hydrophilic or hydrophobic); then, whether the compounds can chemically react with the adsorbent or the eluent. Based on these considerations it is recommended that:

1. for lipophilic substances: silica, aluminum oxide, acetylated cellulose, polyamide should be used;
2. for hydrophilic substances: cellulose, cellulose ion exchangers, polyamide, and reversed-

phase silica should be used.

Several different types of TLC stationary phases are listed according to polarity in Figure 3. Figure 4 shows affinity of common functional groups for silica gel (approximate). Assuming that a polar adsorbent (silica gel) is used, the more polar compounds will be eluted more

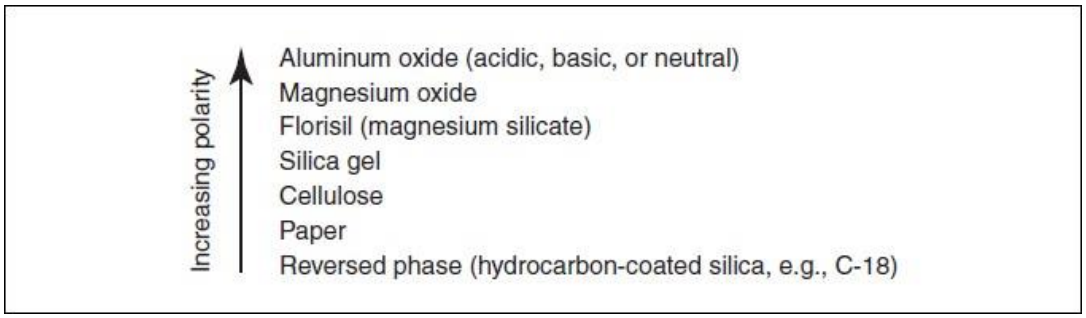



***Figure 3 TLC stationary phase polarities***.

slowly and the more nonpolar compounds will be eluted more rapidly. The charts depicted in these figures are very useful to help predict the order of elution; however, the functional groups should always be viewed and considered within the context of a whole molecule. Clear answers come from real experiments!

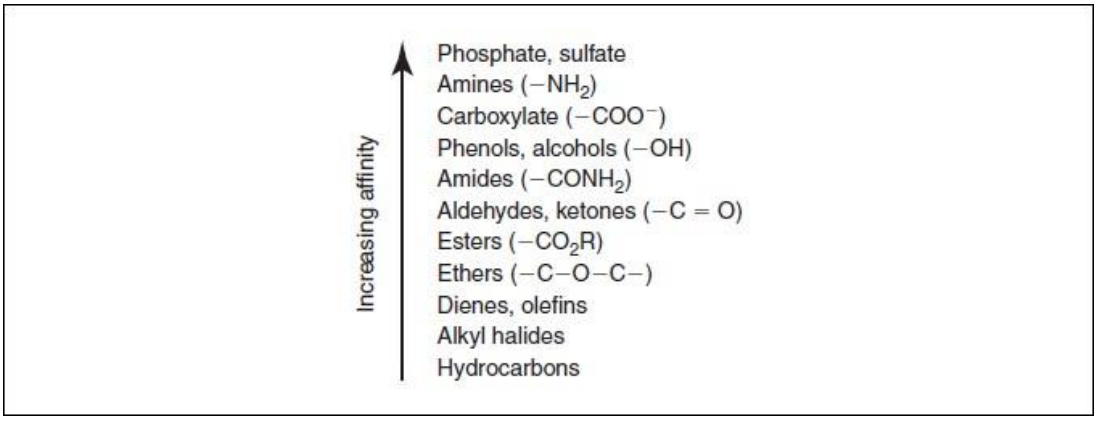



***Figure 4 Affinity of common functional groups for silica gel (approximate).***

## Solvent System (Mobile Phase)

Finding a suitable solvent system is usually the most difficult part of TLC experiments, and solvent system is the factor with the greatest influence on TLC. Only in a few cases does the solvent consist of only one component, and mixtures of up to five components are commonly used. No matter how many components are present, the prepared solvent system must be a homogenous system with no sign of cloudiness. Three criteria are usually considered for

choosing a solvent system: solubility, affinity, and resolution. The first step in solvent selection is to determine the solubility of the sample. The desired mobile phase will be able to provide the greatest solubility while balancing the sample affinity for the solvent and the stationary phase to achieve separation. Resolution is improved by optimizing the affinity between sample, solvent, and stationary phase.Most TLC solvent systems contain a polar solvent and a chromatographically less polar solvent. Figure 5 lists some common mobile phase solvents according to their polarities and elution power with silica 60 as the stationary phase (Halpaap's eluotropic series, Halpaap and Ripphahn, 1976; Hahn-Deinstrop, 2006). With these solvents, there are some common combinations for organic molecules with silica gel as the stationary phase (see Troubleshooting for combinations of three solvents for very polar compounds):

Hexane (or petroleum ether)/ethyl acetate Dichloromethane (or chloroform)/methanol Pentane/ether

Petroleum ether/acetone Hexane/dichloromethane Dichloromethane/ethyl acetate Ethyl acetate/methanol Toluene/acetonitrile

Water/methanol (for C18-reversed phase silica) Water/acetonitrile (for C18-reversed phase silica).

The easiest way to find a starting point for development is to look up a reference for chromatography conditions of compounds with similar structure. Meanwhile, consider the affinity for the type of compound (Fig. 6.3.4), as well as the solvent strength (Fig. 6.3.5), to make adjustments. If the mobile phase has not been previous reported or determined, start with a less polar combination such as hexane/ethyl acetate and observe the separation. If the components do not move very far, try adding a greater volume or a higher ratio/percentage of the polar solvent. Always compare the separation to the previous plate. If the spots stay at the starting line of the plate, add more of the polar solvent or switch to a more polar combination such as dichloromethane/methanol. If they run with the solvent front (or $R$f > 0.8), then add more nonpolar solvent or switch to an even less polar combination such as pentane/ether. It is common to try three to six solvent systems for the first round of method development. As a general guide, a substitution in the more polar solvent often results in a change in resolution, while a change in the less polar solvent results primarily in a change in $R$f of the sample

components

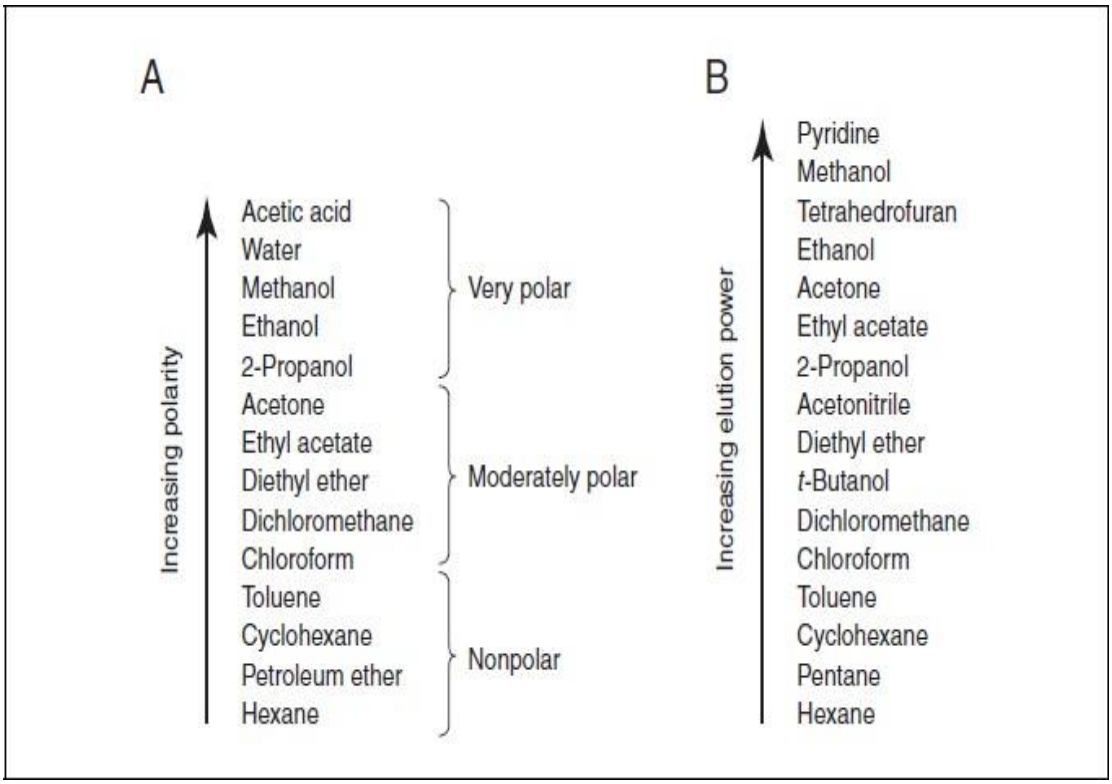



***Figure 5 (A) Common mobile phase solvents listed by increasing polarity (adapted from Grace catalog for TLC accessories: and (B) elution power with silica gel as the stationary phase***

## SAFETY CONSIDERATIONS

1. Take extra caution when breaking scored glass TLC plates. The resulting sharp edges may cause cuts to the hands.
2. Inhaling silica gel (dust form particularly) is highly dangerous and may cause severe lung irritation. Long-term exposure may cause the lung disease silicosis. A safety mask is recommended when handling silica-gel TLC plates.
3. Many organic solvents used for developing TLC are flammable or combustible, and inhalation of their vapors is to be avoided. Some organic solvents are potentially carcinogenic, such as benzene (proven group 1 carcinogenic; should be replaced with toluene), chloroform, and dichloromethane.
4. Many reagents used in TLC staining are toxic and must be handled with care. If heating (with a hot plate or a heat gun) is required for staining, make sure all steps are carried out in a fume hood with care to avoid inhalation of any toxic or irritant smoke or vapor. Personal

protections (disposal gloves, safety goggles, and masks) are required.

## PROTOCOL

### Basic Protocol: Principal Steps of TLC and Required Instrumentation

In this protocol, preparation of the TLC plates, preparation of the spotting capillary, spotting of the sample, development of theTLCplate, and visualization of the components are described. Necessary TLC accessories are illustrated in the associated figures with instructions for their use included in the protocol steps.

### Materials

Organic sample solution: the sample for TLC can be dissolved in any compatible solvent because the solvent used to dissolve the sample will be completely dried out after the sample is spotted on the TLC (avoid high-boiling-point solvents which would make it difficult to dry the sample after spotting and cause the TLC look like a spread; see Troubleshooting) Developing solvents (CH2Cl2, hexanes, ethyl acetate, methanol, etc.)

Iodine (I2)

TLC staining reagents

TLC plates (aluminum, glass, or plastic; e.g., EMD Millipore, SORBTECH, Sigma-Aldrich)

Guillotine paper trimmer

Diamond-tipped glass cutter

Glass Pasteur pipets for capillary spotters

TLC chamber or small wide-mouth flat-bottom glass jar/bottle with a lidHeat gun (optional)

UV lamp

Iodine chamber: a screw-top glass jar with a well-fitting lid can be used as the vaporization chamber

Filter paper

Hot plate or TLC plate heater

### *Handling and cutting TLC plates*

*For an aluminum-foil TLC plate:* Place the aluminum foil TLC plate on a paper trimmer board with the coating side down (Fig 6) and use the guillotine to cut the aluminum-foil plates into desired sizes. Gently wipe the cut edges with a spatula to remove loose layer material.

***Sample application (spotting the TLC plate)***

The most common technique for TLC sample application is to use a glass capillary spotter.

1. The spotting capillaries are extremely small and easy to make from glass Pasteur pipets (steps2 to 4, illustrated in Fig. 8).
2. Heat the middle part of the Pasteur pipet over a Bunsen burner blue flame. *Hold both ends carefully, as they will get hot! Use heat-proof gloves if necessary!*
3. When the middle part is hot enough, it will become pliable; pull both ends apart quickly andsmoothly. *This will draw the middle part into a thin string-like tube.*
4. After cooling for a few minutes, gently break the middle string-like part into 2- in. sections to provide good TLC capillary spotters. *The end should be as flat as possible, as a jagged edge will lead to unsymmetrical spots or no spots on the TLC plate.*
5. Fill the prepared capillary spotter by quickly dipping it into the organic sample solution. *With aqueous solutions, filling will be much slower—an Eppendorf pipettor with 10-µl tipis thus recommended for applying aqueous sample solutions.*

**1.** Place the capillary spotter at the starting line (labeled in pencil) on the coated side of the plate vertically and carefully, to allow capillary action to draw the solution onto the plate. If more than one sample is running at the same time, make sure to properly label the plate with a pencil (Fig. 6.3.9) and use a different capillary for each sample to avoid contamination. Blow with cold or hot air to facilitate solvent evaporation of **Thin Layer** the applied samples. *To keep spots small and compact, it is better to apply a sample in several portions with intermediate drying (to avoid diffusing), instead of holding the capillary against the plate for a long time. For beginners, it is best to practice spotting a few times on a spare TLC strip. In this way, you will discover the capillary emptying time and have better control over the application pressure to be used with capillaries (avoiding damage to the layer).*

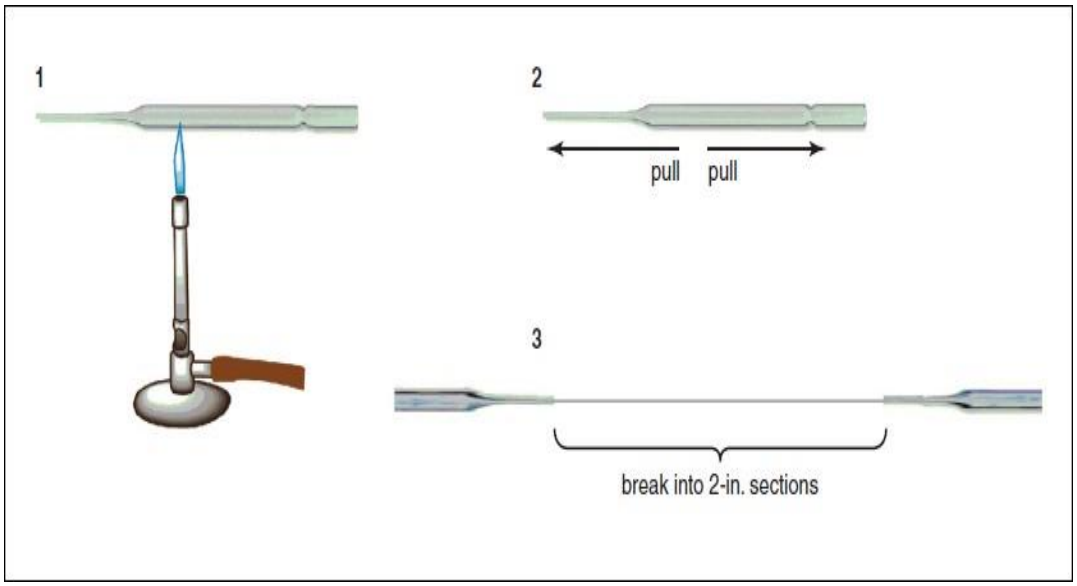



***Figure 8 Making glass capillary spotter from Pasteur pipets*.**

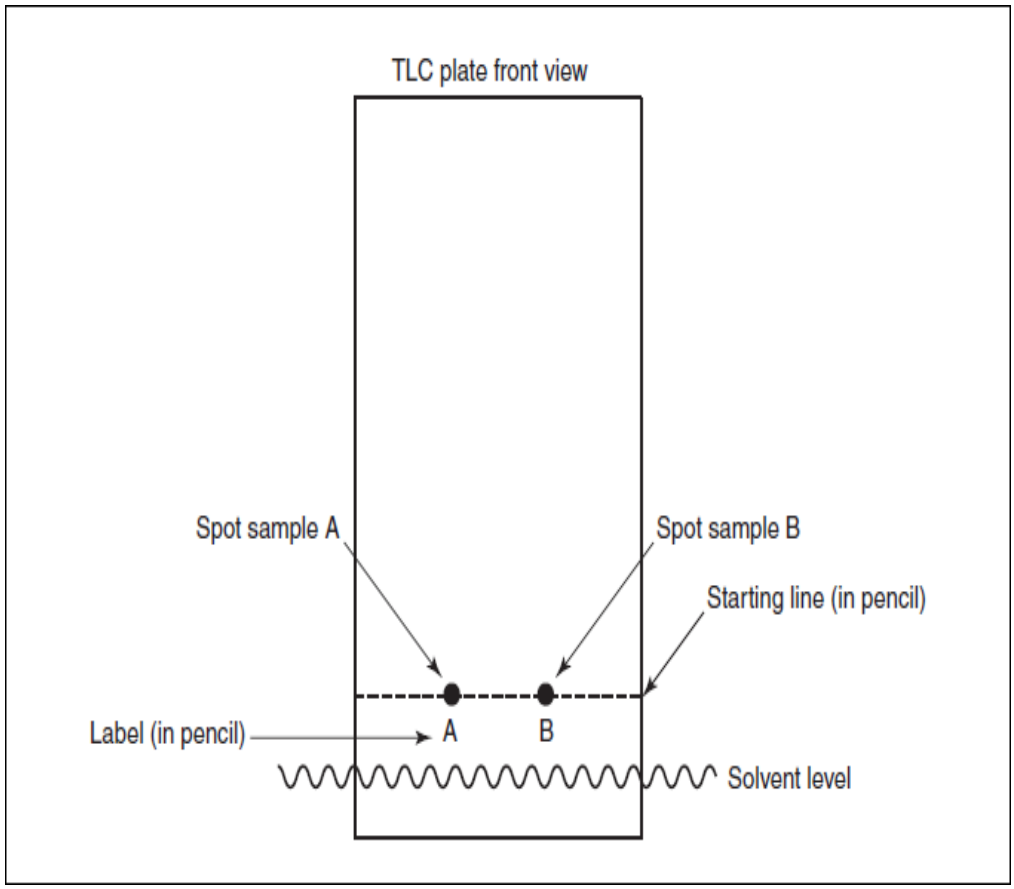



***Figure 9 Spotting TLC plate*.**


***Figure 10 Flat bottom TLC chamber.***

***Development of TLC plates***

In most cases, ascending TLC is applied in a TLC chamber as for development once with a single solvent system (single development). Commercial TLC chambers of different sizes are readily available (Fig. 6.3.10), but usually a small wide-mouth flatbottom glass jar/bottle with a lid large enough to fit the TLC plate works just as well.

7. First, fill the chamber with development solvent to a depth no greater than 0.5 cm.

8. Use tweezers to place the TLC plate in the prepared development chamber with its back layer leaning against the chamber's inside wall, and immediately cover the chamber with the lid (Fig. 6.3.1).

*Make sure that the starting line is above the solvent level (Fig. 6.3.9). Saturation of the chamber atmosphere with solvent vapor is important for TLC. In most cases, lining the chamber with a piece of filter paper (wetted with the eluent) will promote the saturation and may improve the separation and reproducibility (Fig. 6.3.1). Thus, to maintain the atmosphere in the developing chamber, it must not be opened during the development. Observe the solvent front through the side wall while keeping the chamber closed!*

9. Development starts once the TLC plate is immersed; when the solvent front has reached an appropriate level (usually within 0.7 cm of the top of the plate), quickly remove the lid, take out the plate with tweezers, and mark the solvent front with a pencil.

*Proper marking of the solvent front will be of benefit in the analysis of results (see*

*Understanding Results).*

*10.* Allow the plate to dry in a fume hood or with a heat gun (heat gently) before proceeding to the visualization step. *If certain separation cannot be achieved by a single development, multiple developments (with or without change of solvent system) may be performed: the TLC is developed two or more times with intermediate drying (also see Troubleshooting).*

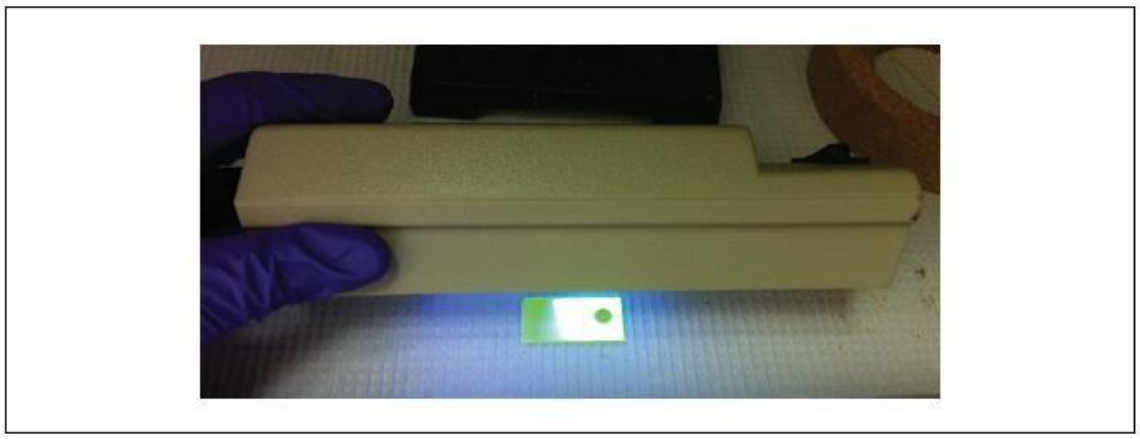


***Figure 11 Visualization under a UV lamp***.

***Visualization***

***Nondestructive visualization***

**11a.** ***For nondestructive visualization with the naked eye:*** Simply put the plate under a UV lamp (Fig. 11), and the compounds become visible to the naked eye. Lightly circle the spots with a pencil, so that you will have a permanent record of their location for later qualitative assignment.

*Only in a few cases is the sample a dye (colored) that can be seen with naked eye. Much more often, substance visualization can be achieved under UV light, since many substances have UV absorption. TLC plates normally contain a fluorescent indicator that makes the TLC plate glow green under UV light of wavelength 254 nm (less frequently at 365 nm with a mercury lamp). Substances absorbing UV light in the respective region of avelength will quench the green fluorescence, yielding dark purple or bluish spots on the plate (Fig. 6.3.11).*

**11b.** ***For nondestructive visualization using iodine****:* Use tweezers to insert the TLC plate into the prepared iodine chamber (described in Table 6.3.1) and remove it after it develops a light brown color over the entire plate. Cover the iodine-treated TLC plates with a clean glass plate,

since the color stainwill eventually fade.

*You may also circle the observed spots with a pencil for documentation. Iodine sublimes and will absorb to organic molecules. This method is therefore nonspecific, but usually does not cause decomposition. The organic spots on the plate will turn brown and can be easily identified. Dwell times for plates in a vaporization chamber can range from a few minutes for detection up to 20 hr for purity tests. It should be noted that, with long dwell times in iodine vapor, aluminum TLC plates may react with iodine and become impossible to evaluate.*

***For stepwise dipping technique***

11d. Hold the edge of the developed plate (dried) with tweezers, quickly immerse the whole plate into the staining solution, and immediately take out the plate. Close the dipping chamber with a lid immediately.

*As an alternative derivatization technique, brief dipping of the TLC plates in staining reagent solutions is being used increasingly for many reasons: (1) the application of the reagent solution to the TLC layer is more homogeneous and uniform; (2) this technique gives improved reproducibility; (3) the consumption of reagent is low; (4) complex spraying equipment is not needed; (5) contamination of the workplace with reagent is minimized.*

*Dipping chambers/jars vary in size and shape, and dipping solutions are generally less concentrated than praying solutions. Under certain circumstances, the dwell time in the dipping chamber should be determined experimentally.*

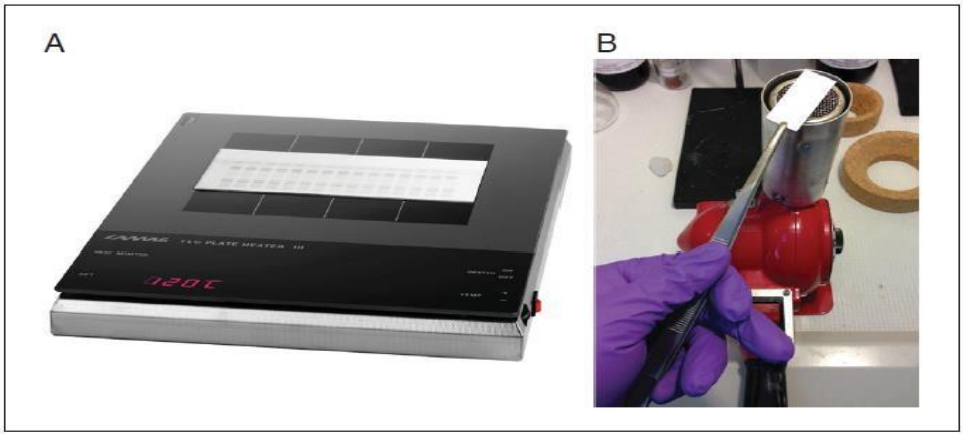



***Figure 13 (A) TLC Plate Heater. (B) Heating TLC plate with a heat gun.***

12d. After the dipping operation (especially before heating), carefully wipe the back of the plate with a paper towel that has been wetted with the staining solution. *The dipping chamber should always be closed with a lid after dipping to prevent evaporation of solvent.*

13. If heating is necessary, perform this on a hotplate (or a TLC plate heater, Fig. 13A), or with a heatgun (Fig. 13B).

*Make sure to remove the TLC plate from the heat source once the spots are visible and before the background color obscures the spots. When glass plates are used, the spots can sometimes be seen more clearly from the glass side of the plate.*

### *Retention factor (Rf)*

The analysis of TLC depends on the purpose. For most qualitative determinations, localization of substances is sufficient. This can be easily achieved by parallel runs with reference compounds (e.g., authentic sample, starting material, compound with similar structure, etc.). A practical example of a TLC analysis for monitoring the progress of a reaction is illustrated in Figure 6.3.14 (adapted from Zhao et al., 2010). The starting material, *N*-acetylglucosamine (Lane A), and the product, *N*-acetylglucosamine-1-phosphate authentic sample (Lane B), were used as controls by parallel runs with the reaction mixture (Lane C). The disappearance of the starting material spot and generation of the new product spot in the reaction mixture clearly indicate complete consumption of the starting material and completion of the reaction. An important parameter often used for qualitative analysis of TLC is the *R*f value (retention factor, Fig. 6.3.15). If two spots travel the same distance or have the same *R*f value, then it might be concluded that the two components are the same molecule. However, identical *R*f values do not necessarily mean identical compounds. For *R*f value comparisons to be valid and reproducible *R*f values to be obtained, TLC plates must be run under the same exact conditions with respect to chamber saturation, composition of solvents, temperature, etc. As shown in Figure 6.3.14, the authentic sample of *N*-acetylglucosamine-1-phosphate (Lane B) and *N*-acetylglucosamine-1-phosphate product from the reactionmixture (Lane C) have the same *R*f value, providing evidence that the desired product was obtained.

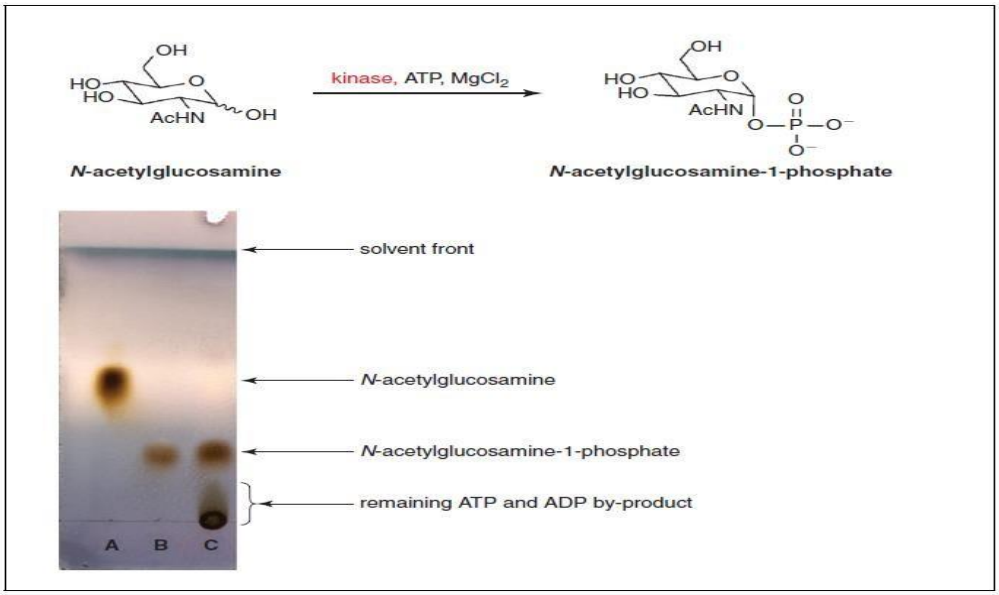



**Figure 14** Use of TLC to monitor the progress of an enzymatic synthesis reaction The developing solvent used was 2:1:1 (v/v/v) n-butanol:acetic acid:water for highly polar carbohydrates. The developed plate was stained with p-anisaldehyde stain. Lane: A, N-acetylglucosamine; B, N-acetylglucosamine-1-phosphate authentic sample; C, reaction mixture of N- acetylglucosamine and ATP using a kinase. The N-acetylglucosamine and N-acetylglucosamine-1-phosphate show dark/light brown on the TLC plates. The authentic sample of N-acetylglucosamine-1-phosphate and the N- acetylglucosamine-1-phosphate product have the same Rf value.

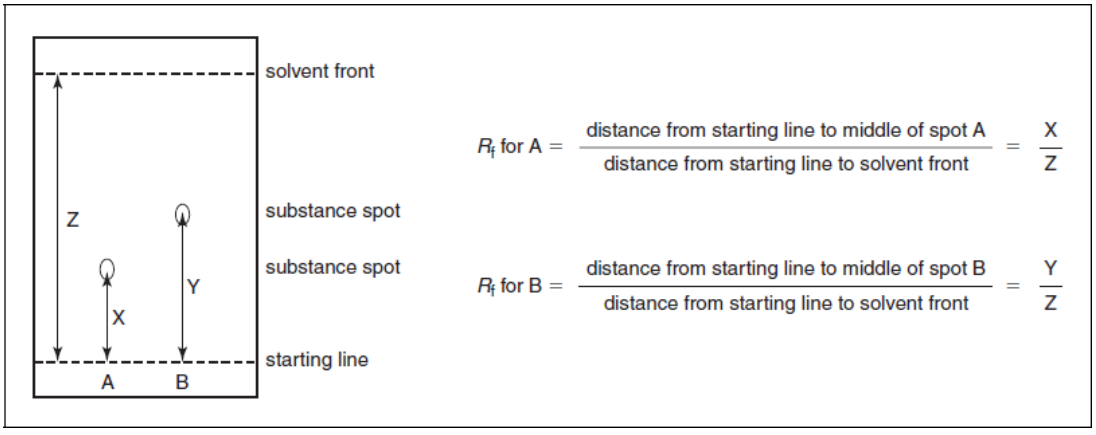



**Figure 15** Calculation of Rf value. This figure shows how Rf can be measured and calculated for each spot observed on a TLC plate: the ratio of distance start–substance zone to distance

start–solvent front. The Rf values are between 0 and 1. However, the optimum separation of compounds by TLC is usually achieved when Rf values are between 0.3 to 0.5. For scale-up to preparative separations, the TLC solvent system's polarity must be decreased to lower the Rf between 0.15 and 0.3.

## Paper chromatography

Chromatography is a group of techniques in which a mixture is separated into individual components due to differential distribution of the solutes between 'stationary phase' and the 'mobile phase'. Chromatographic techniques are invaluable tools of analysis as the components of mixture are first separated, facilitating the analysis of complex mixtures.

The history of chromatography dates back to early 1900's, when a Russian scientist, Mikhail Tswett successfully separated a mixture of plant pigments using calcium carbonate and alumina packed in a glass column. In his experiment, he made a solvent extract of homogenized plant leaves and applied this extract on to the calcium carbonate column. The sample was allowed to travel through the column along with the solvent under the gravity. The components of the plant pigment mixture separated into different colored bands in the column. Tswett coined the name chromatography from the Greek words *chroma* meaning color and *graph*, meaning writing. Two British researchers, Martin and Synge, years later amended upon Tswett's chromatographic technique and devised a technique known as paper chromatography. They were able to use this technique to separate the amino acids in a protein hydrolyzate and were awarded with the Nobel Prize in 1952 for their work.

Over the last century, the technique of chromatography has provided significant contribution in the field of molecular characterization in addition to the purification process. Quantitative chromatographic analysis is invaluable in pharmaceutical industry for research as well as for quality control. Similarly, biotechnology industries widely use chromatographic techniques and in many cases alternative methods are not available. As an example, chromatographic techniques have been applied for separation of stereoisomers that are very similar in structure and properties. The development of bio- therapeutics would have been impossible without

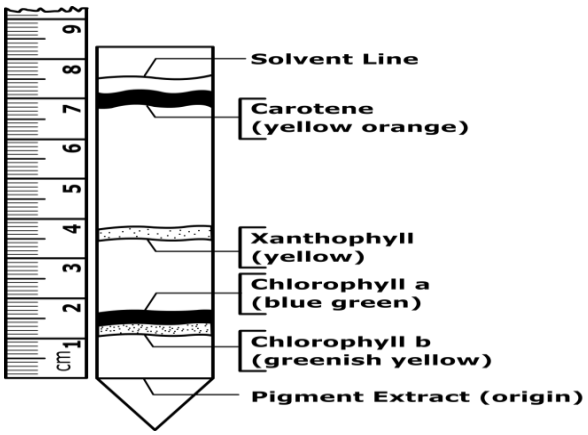
chromatography-based purification strategies. The reasons for popularity of these techniques lie in their unique sensitivity, flexibility and scalability. Moreover, chromatographic separations are relatively fast and simple and afford considerable ease of operation compared with the other instrumental techniques.

Chromatographic separation involves a stationary and mobile phase. The analyte mixture interacts with the stationary phase. Depending upon different types of interactive forces of components of the mixture with the stationary and the mobile phases, some molecules are detained in the column more than the others. The difference in the migration rate of the components of the mixture brings about the separation as the mobile phase moves and flows out of the system. The mobile phase can be either liquid or gas. The stationary phase can be solid or liquid.

## Chromatogram

A chromatogram is the visual output of the chromatograph. Different patterns or peaks are obtained on the chromatogram, during an optimal separation, wherein the different peaks correspond to different components present in the separated mixture.

In all of the different forms of chromatography, a rapid dynamic equilibrium of molecules exists between the two phases- the ‘stationary phase’ and the ‘mobile phase’. For example, consider a mixture of molecules A and B dissolved in a mobile phase and flowing over a stationary phase. The molecules of compound A and B exist either as completely dissolved in the mobile phase or may be bound to the stationary phase.



The equilibrium of the molecules between the mobile and stationary phases depends upon:

1. Polarity and size of the molecule
2. Polarity of the stationary phase
3. Polarity of the solvent.

Depending upon the above three factors the components of the mixture, partition differently between the mobile and the stationary phase. For example, consider separation of a mixture containing compounds A and B. Compound B has a greater affinity towards the stationary phase as compared to the compound A. When the mixture flows over the stationary phase, the molecules of compound A spend more time in the mobile phase, and move farther in a given amount of time. Whereas, since compound B has greater affinity towards the stationary phase, the molecules of compound B move at a slower pace through the stationary phase particles. As a result of this, the components of the mixture can be gradually separated. A suitable solvent mixture can be selected as a mobile phase to achieve the desired separation.

**Paper Chromatography**

In paper chromatography, substances are distributed between the paper as a stationary phase and a suitable mobile phase. The chromatography paper has highly uniform texture and thickness. It is made from cotton cellulose and has low content of additives. For the separation of the mixture, a discreet spot is applied on the paper. The components of the mixture partition between the two phases: aqueous phase consisting of water adsorbed in the pores of the chromatography paper (stationary phase) and solvent (mobile phase) that moves over the paper due to capillary action. The compounds in the mixture get separated due to differences in their affinity towards the two phases. An additional factor could be adsorption of the components of the mixture by the filter paper. Paper chromatography is a useful technique for separation and analysis of molecules because it is quick and requires small quantities of material.

The equipment for paper chromatography consists of a vapor tight glass chamber with inlets for addition of solvents in addition to supports for solvent troughs and chromatography paper. In Paper Chromatography, a volatile solvent is used to carry the components of the sample along the paper. This is called the developing solvent. The choice of solvent depends on the chemical nature of the materials to be analyzed and needs to be optimized for every type of mixture.

Usually solvents such as ethanol, methanol, isopropanol, acetone, diethyl ether, chloroform, methylene chloride, formamide, acetic acid, ammonia, cyclohexane, benzene can be used alone or as mixture as the mobile phase. The general procedure in paper chromatography is as follows:

**Preparation of chromatographic chamber**

The procedure for paper chromatography starts with equilibration of chamber with the solvent vapors. The pre-saturation of the chromatography chamber is carried out well in advance before the development. The saturation of the chamber with the solvent may be facilitated by lining the chamber with filter papers wetted with the developing solvent.

**Preparation of paper**

The paper may be used as such, where water adsorbed in the fibers acts as the stationary phase. Alternatively, paper may be impregnated with a polar solvent other than water, such as buffer, salt solution, methanol or glycol. Paper may also be modified with silica, diatomaceous earth, and alumina. Such type of modification papers would illustrate adsorbing capacity of the respective modifiers.

Paper may be made hydrophobic by impregnating with silicone or other non-polar solvents. Paper may also be acetylated so that it becomes more hydrophobic and retains a similar hydrophobic component.

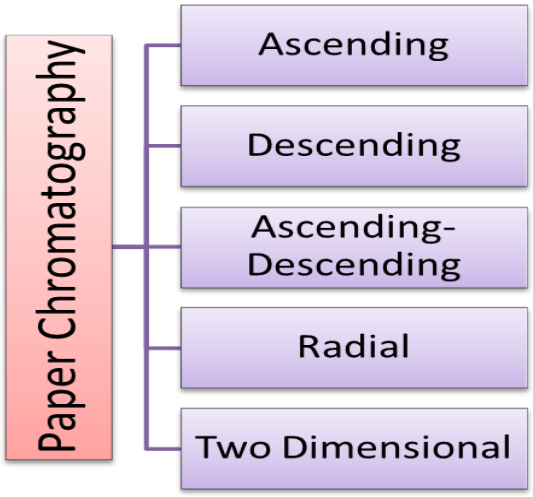
**Application of samples**

The mixture to be analyzed is dissolved in a suitable solvent. A fine line is drawn on the filter paper so that it is few centimeters away from the level of developing solvent. The dissolved samples are applied to the spots (diameter: 6-10mm) that lie at least 3 cm apart on the line drawn on the chromatography paper. The sample application is usually performed using micropipettes. The sample application may be repeated after solvent evaporates from the previous spot, to concentrate the sample.

**Development of the chromatogram**

After preparing the chamber and spotting the samples, the paper is ready for development.

Depending upon the type of development, paper chromatography can be classified as:




**Ascending Paper Chromatography-** Here the developing solvent is poured at the bottom of chromatography chamber. The solvent travels in upward direction due to capillary action.

**Descending Paper Chromatography-** Here the solvent is allowed to travel down the paper. The mobile phase is kept in a trough located at the top of the chromatography chamber and it is allowed to descend over the chromatography paper.

**Ascending-Descending Paper Chromatography-** It is the hybrid of both the above technique. The upper portion of the ascending chromatographic sheet is folded on a rod while allowing the descent of paper after crossing the rod.

**Radial Paper Chromatography-** It is also called as Circular Chromatography. Here a circular filter paper is taken and the sample is applied at the center of the paper. After drying the spot the filter paper is tied horizontally on a shallow chamber containing solvent. A wick of the paper is dipped inside the solvent. The solvent rises through the wick and the components get separated in the form of concentric circular zone.

**Two-Dimensional Paper Chromatography-** In this technique, a square or rectangular paper is used. Here the sample is applied at one corner and development is performed at right angle to the direction of first run, using a different solvent. This facilitates better separation of the components of the mixture.

## Applications of Paper Chromatography

1. Paper chromatography has been used for separation of mixtures having polar and non-polar compounds such as amino acids, hormones, drugs and other bio- chemicals.
2. It has also been used for the evaluation of inorganic compounds.
3. Although paper chromatography has become very popular due to its simplicity, it suffers from various limitations such as long development time, poor separation, low accuracy in quantitative determinations and low reproducibility.

## Electrophoresis

Electrophoresis may be defined as the migration of colloidal particles through a solution under the influence of an electrical field. Electrophoresis basically is the movement of distributed particles corresponding to a fluid under the influence of electric field. Electrophoresis is mostly known as electro - kinetic phenomena. The technique of electrophoresis was discovered by Reuss in 1809 when he experimented that soil particles dispersed in water migrate under effect of an applied electric field. Electrophoresis takes place because particles dispersed in a fluid nearly at all times carry an electric surface charge. The charged molecule migrates to their oppositely charged electrodes but that electric field is removed before it reaches there completely. Passage of charged particle in an electric field provides differential motion to the sample on the basis of charge and consequently resolve them. An electric field exerts electrostatic Coulomb force on the particles through these charges. It has been witnessed that the colloidal particles are transfered to either the positive or negative electrode, whenever a potential difference is applied between the two electrodes in a colloidal solution. Since, Electrophoresis is based on the differential movement of electrically charged particles in an electric field. Fundamentally, the technique is appropriate only to ionic or ionogenic materials, i.e., substances transformable to ionic species. Electrophoresis is recurrently used in forensic science, molecular biology and medicine.

## Principle and Methodology

All types of electrophoresis are directed by the single set of general principles illustrated byEquation:

$$\text{Mobility of a Molecule} = \frac{\text{(Applied Voltage) (Net charge on the Molecule)}}{\text{Friction of the Molecule}}$$

The mobility or rate of migration, of a molecule increases with increased applied voltage and increased net charge on the molecule. On the other hand, the mobility of a molecule decreases with increased molecular friction, or resistance to movement through the viscous medium, caused by molecular size and shape. Overall actual movement of the molecules increases with increased time, since movement is defined as the rate of migration.

In a free buffer solution, in the absence of molecular sieving, the velocity **v** of a particle is proportional to the field strength **E**, multiplied by its electrophoretic mobility **μ**:

$$v = \mu E$$

The velocity **(v)** and Field strength **(E)** both are vectors, while the mobility **(μ)** is scalar, being positive for cations and negative for anions.

The rate at which migration takes place is dependent upon the strength of field, size and shape of molecules, net charge, ionic strength, viscosity and temperature of medium in which molecules are moving. As an analytical technique, it is rapid, simple and very sensitive. . Beside a separation technique it is also utilised diagnostically to study the properties of a single charged species. It provides the foundation for a number of analytical techniques used for separating molecules by charge, size, or binding affinity. The charged molecules under the stimulus of electric field travel in the direction of oppositely charged electrodes. Those molecules which are positively charged moves towards the cathode and negatively charged molecules moves towards the anode. The charge on the molecules and potential applied through the electrodes is responsible for this movement. The specimen under examination is sited at one end of the electrodes. The molecules starts moving to respective electrodes as soon as the electricity is applied. But the movement is influenced by molecular weight of the molecule. Consequently when a mixture is placed on the electrophoresis paper or gel, different bands are observed along the paper after the process. This is a result of differential rate of migration by molecules based on their weight.

## Classification of Electrophoretic Techniques

Electrophoresis is commonly categorised on the basis of the existence or absence of a solid supporting medium or matrix by means of which the charged molecules travel in the electrophoretic system. Solution electrophoresis systems utilises aqueous buffers in the lack of presence of a solid support medium. Such systems can suffer from sample mixing caused by diffusion of the charged molecules, with consequential loss of resolution during sample application, separation, and removal phases. Therefore, solution electrophoresis systems must employ specific resources for stabilizing the aqueous solutions in the electrophoresis cell.

***Generally, Electrophoresis is classified on the basis of two criteria:***

1. Initial component distribution
2. Boundary permeability

***Electrophoretic techniques can be classified into four main types:***

1. Zone electrophoresis (ZE)
2. Moving-boundary electrophoresis (MBE)
3. Isotachophoresis (ITP)
4. Isoelectric focusing (IEF)

**Common Mediums used in Electrophoresis**

**Cellulose Acetate:** Cellulose acetate is a cellulose derivative in which each hexose ring of the polysaccharide chain has two hydroxyl groups esterified to acetate (in general, in the C-3 and C-6 positions). Cellulose acetate is quite prevalent in clinical chemistry, as it offers a convenient support that can be equilibrated with buffer in a few seconds and yields good separations of proteins from biological fluids. Fully automatic systems have been manufactured around, so that this system has become the principal model of combined electrophoresis and robotics. Cellulose acetate allows migration of even large serum proteins. However, the use of cellulose acetate is limited to clinical electrophoresis and is not much in trend in basic research for high-resolution runs, despite the fact that focusing and two-dimensional techniques on cellulose acetate membranes have been described.

**Agarose Gels:** Agarose is a polysaccharide taken out from seaweed. Agarose is a purified linear galactan hydrocolloid, extracted from agar or recuperated directly from agar-bearing marine algae, like the Rhodophyta. Agarose gels are extremely easy to prepare and also nontoxic. Agarose gels have an extensive range of separation, but comparatively low resolving power.

**Polyacrylamide Gels:** The most resourceful of all matrices are the polyacrylamide gels. Their popularity are because of several fundamental properties:

- Optical clarity, including ultraviolet (280 nm) transparency
- Electrical neutrality, due to the absence of charged groups: and
- Availability in a wide range of pore sizes.

Polyacrylamide is a cross-linked polymer of acrylamide. Polyacrylamide gels are considerably more exasperating to formulate than agarose gels. These gels have comparatively small range of separation but have very high resolving power. For characterization and separation of mixtures of proteins, these gels are used. Acrylamide is a

known potent neurotoxin.

## Types of Electrophoresis

### Affinity electrophoresis

The procedures consist of the charge shift electrophoresis, mobility shift electrophoresis and affinity capillary electrophoresis. The techniques are established on changes in the electrophoretic pattern of molecules through bio- specific interaction or complex formation. The interaction or binding of a molecule, charged or uncharged, usually changes the electrophoretic properties of a molecule. Membrane proteins may be recognised by an alteration in mobility prompted by a charged detergent. Nucleic acids or its fragments may be categorised by their affinity to other molecules. The techniques has been used for assessment of binding constants, as for example in lectin affinity electrophoresis or categorization of molecules with particular characteristics like glycan content or ligand binding. Affinity electrophoresis may be used as an alternative quantification of the protein.

### Capillary electrophoresis

Capillary electrophoresis (CE), can be applied to separate ionic species by their charge and frictional forces and the hydrodynamic radius. In standard electrophoresis, electrically charged analytes migrate in a conductive liquid medium under the impact of an electric field. The technique of capillary electrophoresis (CE) was considered, in 1960, to isolate species onthe basis of their size to charge ratio inside the small capillary filled with an electrolyte.

### Pulsed field gel electrophoresis

Pulsed field gel electrophoresis or PFGE is a system utilised for isolation of large Deoxyribo Nucleic Acid (DNA) molecules by application of an electric field to a gel matrix that intermittently changes its course. Nevertheless, with periodic changing of field direction, the different lengths of DNA react to the change at differing rates. Specifically, larger pieces of DNA will be slower to rearrange their charge when field direction is changed, while smaller pieces will be faster. Over the course of time with the constant changing of directions, each band will begin to separate gradually even at very large lengths. Thus separation of very largeDNA pieces using PFGE is made possible.

### Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

One of the conventional means of examining proteins by electrophoresis is by using Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis. SDS is a detergent which coats the linear protein sequence with a layer of SDS molecules and denatures it by binding to the hydrophobic regions of the protein. Due to its negative charge it becomes the dominant charge of the complex. The binding of number of SDS molecules is directly proportional to the size of the protein. Therefore, there should not be any change in charge to mas ratio with size. In solution, principally all different sized proteins covered with SDS would run at about the same mobility. However, the proteins do not run through water. Instead they run through an inert polymer known as polyacrylamide.

## Native Gels

It is also possible to run protein gels without the SDS. These are known as Native Gels in that one does not deliberately denature the protein. Here, the native charge on the protein, divided by its mass, governs the movement of protein ni terms of speed and direction.

## Electrofocusing Gels

A different variant of gel electrophoresis is to decant a gel that purposefully has a pH gradient from one end to the other. As the protein travels through this pH gradient, its different ionizable groups either pick up or lose protons. Ultimately, it will find a pH where its charge is zero and it will get focused at that point.

## DNA Agarose Gels

It is a simple method of separating fairly large fragments of DNA from one another by size is to use an agarose gel. DNA does not need a detergent, since it already has a large under of negative phosphate groups evenly spaced. Thus, as with SDS-PAGE, the charge to mass ratio is constant. Also like SDS-PAGE, the separation results from the matrix itself. The range of size sensitivity can be varied by changing the density of the agarose. DNA denaturing polyacrylamide gels (often called as Sequencing Gels). These gels usually contain additional denaturing compounds such as Urea. Two pieces of DNA that differ in size by 1 base can be distinguished from each other this way.

## Long answer type Questions (10 Marks)

1. What is electrophoresis? Describe paper electrophoresis technique.
2. Explain the principle and techniques involved in Paper Electrophoresis.
3. Explain the principle and techniques involved in capillary electrophoresis.
4. Explain the separation techniques involved in column chromatography
5. Discuss different methods of preparation and elution techniques of column chromatography.
6. Explain the experimental methodology involved in preparing TLC plates. Add a note on detection methods in TLC?
7. Explain different qualitative and quantitative analytical methods in TLC.

## Short answer type questions (5Marks)

1. Describe the practical steps involved in paper electrophoresis..
2. Add a note on gel electrophoresis.
3. Briefly explain the operational techniques of column chromatography.
4. Explain the concept of plate theory and rate theory for increasing the efficiency of column in chromatography.
5. Explain the practical steps involved in TLC for separation of components.
6. Explain the various methods of preparation of TLC plates
7. Write the advantages of TLC over paper chromatography.
8. What is two dimensional paper chromatography?

## Very Short answer type questions (2 Marks)

1. What is two dimensional paper chromatography?
2. Classify detecting reagents in paper chromatography with suitable examples.
3. Give the principle of paper chromatography.
4. Define Rf.
5. List of adsorbent used in TLC.

6. How will you grade TLC plate (G,H).
7. Give the principle of TLC.
8. What are the elution techniques in column chromatography.
9. Define Column efficiency.
10. Full form of HETP and factors affecting HETP
11. What is Van Deemeter equation
12. What is electrophoresis?
13. Give the principle of gel electrophoresis
14. Give the principle of paper electrophoresis.