Swayam Course - Analytical Techniques

Week: 3, Module 8 - Planar Chromatography

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1. Learning outcomes: After completing this chapter, the student shall be able to:

b. Describe the principle and types of planar chromatography

c. List the various methodological aspects of paper and thin layer chromatography

d. Describe the set up and process of planar chromatography

e. List different applications of planar chromatography

2. **Introduction:**

a. **History and Overview:** Pliny the Elder, in Ancient Rome, described the use of papyrus impregnated with gall apple extracts for identification of chemical substances. Modern chromatography owes its origins to F.F. Runge, a manager of a chemical factory in Germany in the mid-nineteenth century. Runge used various kinds of paper available, to examine the compounds being produced in his factory, to differentiate between color dyes and used his observations on the same as a tool to monitor and control the dye producing process. He inferred that paper was a good medium for this analysis because of the capillary properties of paper fibres. Around the same time, German chemists Schonbein and his student Goppelsröder were also studying filter paper and its properties with respect to the movement of different chemical substances. However, the elucidation of the principle and establishment of paper chromatography as a widespread technique in the current era was the result of the work of Liesegang, and especially, Archer Martin[1], who also went on to develop the technique of gas-liquid chromatography with AT James. The technique of Thin layer chromatography (TLC) owes its pioneering efforts to the work of Russian Scientists, Izmailov and Schraiber in the late 1930s, based on principles described by Tsvet. They used a thin slurry for separating a plant alcoholic extract, and then observed the concentric rings under a UV lamp. Kirchner and Stahl at the US department of Agriculture were instrumental in promoting the widespread use of this technique, through their experiments on

[1] Martin's work on partition chromatography along with that of his colleague, Richard Synge won them the Nobel Prize in Chemistry in 1952

developing various kinds of materials as sorbent layers for glass plates, binders for improving the thin layer coating, spreaders etc. leading to improvement in the separation performance and reproducibility.

b. ***Introduction***- In nature, things are rarely pure, in fact, it is the inherent "*impurity*" of natural substances that confers unique and often interesting properties to these substances. However, in order to understand, manipulate and use substances in novel ways, pure forms are required so that their organization and properties may be understood. Chromatographic techniques are used widely across scientific disciplines and in industry for multiple applications, and have become an important tool for the separation, detection, identification and quantitation of chemical species both in biology and in industry. The general chromatographic principle involves the separation of components (i.e. analytes of interest) of a mixture (of widely varying constitution e.g. peptides, lipids, pigments etc.) based on their relative solubility in, and hence movement through "phases" i.e. mobile and stationary, used in the technique. Chromatography is broadly classified into **planar** and **column** chromatography, based on how the stationary phase is arranged i.e. in "column chromatography, the stationary phase is packed into a narrow column and the mobile phase is passed through the column using gravity or by applying pressure; in "planar chromatography", the stationary phase is a solid particle or a thin, liquid film coated on either a solid particulate packing material or on an inert matrix like glass or plastic. Capillary action allows the mobile phase to move over the stationary phase.

Chromatography is further divided into **gas** and **liquid** based on the nature of the mobile phase. When the mobile phase is liquid then the technique is referred to as "liquid chromatography" and when the mobile phase is gaseous then as "gas chromatography" (GC).

3. **Principle of Planar chromatography**

Planar chromatography can be divided into two types: i). Thin layer chromatography i.e. TLC and ii). Paper Chromatography. The separation involves both "partition" and "adsorption" chromatography principles.

3.1 Thin layer chromatography

The basic principle of TLC is the same as that of other chromatographic techniques, i.e. differential affinities of analytes for stationary and mobile phases. In TLC, the components of a mixture are separated on a thin stationary phase supported by an inert backing. TLC may be used for preparative or analytical purposes. It is generally useful for qualitative analysis and small-scale preparations. TLC is a simple, relatively inexpensive, highly sensitive (often in micrograms), and a fast separation technique. The early forms of TLC were considered quantitative or semi-quantitative but the improvement in the coating layers, instrumentation and increased automation facilitated by computer-controlled devices, has led to modern thin-layer chromatography being able to provide accurate and precise quantitative results based on *in situ* measurements and a record of the separation in the form of a chromatogram. The use of chromatographic layers of high separation efficiency, employment of state-of-the-art instrumentation throughout the procedure, with precise sample application, standardized reproducible chromatogram development and software controlled evaluation have led to a format of TLC described as High Performance TLC (HPTLC).

3.2. **Paper chromatography**: This involves exploiting the principle of differential partitioning or adsorption over the solid support i.e. the stationary phase which is a specific kind of paper. The method is mostly used to separate coloured chemicals or substances e.g. inks, mixture of small molecules e.g. amino acids. Paper chromatography was initially developed by Martin and Synge and Consden, Gordon, and Martin in the 1940s.

4. The planar chromatographic process: overall the process of planar chromatography involves:

a. spotting/application of the sample mixture to be separated,

b. separation and

c. derivatization and/or development (for visualizing the separated products).

4.1 Thin Layer chromatography: In TLC, the sample application is done by means of a thin glass pipettes[2] onto plates (also called chromatoplates) made of glass, metal, or plastic, coated with a thin layer of solid adsorbent material such as silica gel (silicon dioxide)/alumina (aluminium oxide) placed in a small volume of development solvent. Sample application is a critical step since the amount of sample applied, state of analyte in solvent before initiating separation

Each of the plate materials has its own advantages and disadvantages. Glass is chemically inert and good when working with reactive stains or for experiments that involve high temperatures, but it is brittle and can be difficult to cut. Aluminium is not good if strongly acidic or oxidizing stains are being used. Plastic is poorly heat resistant and both these can be cut easily. However, the flexibility of aluminium and plastic can cause flaking of the stationary phase. Nowadays, high-performance TLC(HPTLC) can be performed as the particle sizes of stationary phases such as silica coating can be as small as a few microns and modifications of classical silica phases to materials such as monolithic formats and specialized compound formulations allow for improved separation.

The "Ascending" form of TLC is the most frequently used separation technique using a customary trough chamber which has been equilibrated with the mobile phase in which the TLC plate is kept. The sample mixture to be resolved, is dissolved in a volatile solvent which will evaporate quickly after application onto the separating matrix. The separation of the components depends on the balance of the polarities - of the plate, the development solvent and the spot material. The components of the applied spot will move different distances from the original spot location based on their differing polarities and hence varying interactions with the components of the chromatographic system, and will appear as separate spots. The TLC plate is removed once the solvent has almost reached the top, dried, and the separated components of the mixture are visualized.

TLC plate stationary phases: The selection of stationary phase depends on the properties of the molecules being separated and analysed. Usually, stationary phases are polar, and while mobile phases vary in polarity, they are less polar than the stationary phase. This combination is called "normal phase chromatography". If the conditions are reversed i.e. the mobile phase is *more* polar than the stationary phase, the process is called "reverse phase chromatography".

The TLC plate coating can serve two purposes in the separation process. The surface of the coated material can made to contain chemical groups that actually interact directly with the solutes themselves and, thus, determines the extent of their retention and the selectivity of the phase system in which case the material acts as the stationary phase proper. This function is amply demonstrated in the use of silica gel. The material can alternatively merely act a support for other substances carrying the interactive groups that will establish the degree of retention and selectivity but makes minimal to solute retention. Cellulose fiber is such an example. In practical situations, stationary phases and supports may have some overlapping properties.

Silica gel is still the most commonly used stationary phase in TLC. Amino acids and hydrocarbons can be separated using silica gel. The separation on silica gels depends on the relative amounts of different silanol groups which affects its pH, making it more or less acidic. Hence, separation of basic samples on silica gel is poor; there can be a deterioration of acid-labile molecules. The usual range of the silica gel particles used for TLC is typically described in terms of mesh size, typically 230-400, corresponding to a

[2] Traditionally, these thin glass pipettes can be made in the laboratory by heating pasteur pipettes and pulling them to make a glass tube with narrow waist. This is split into two at the waist, and the narrow tipped pipettes are used for sample application onto a TLC plate.

particle size of 40-63 microns. Both normal and reverse phase TLC is possible on silica gels. Alumina gel is more reactive than silica and can lead to deterioration of samples if not handled with care. Alumina is prepared by heating aluminum hydroxide precipitated from aluminum chloride solution to moderate temperatures. Alumina gel can be in acidic, basic or neutral. The acidic form is used for separation of carboxylic acids and amino acids, basic for amines and neutral for non-basic and non-acidic compounds. Acetylated sugars, steroids and essential oils are separated by magnesium silicate. Modifications can be made to the stationary phase and TLC can be used for specialized applications such as Silver ion-thin-layer chromatography (Ag-TLC) which is the method of choice to check the purity, to identify fatty acid methyl esters (FAME) or triacylglycerol (TAG) component(s).

Ion exchange materials used in TLC include the *inorganic ion exchangers,* the *ion exchange resins,* the *ion exchange celluloses* and the *ion exchange bonded phases*. The inorganic ion exchange materials are largely the zeolites (the aluminosilicates), apatite and hydroxy apatite. However, the phosphates, molybdates, tungstates and vanadates of some tetravalent metals have also shown utility as ion exchange separations. The most popular ion exchange substrates used in TLC are the ion exchange resins and the ion exchange celluloses.

In addition to the primary material of the stationary phase that is used for separation, the stability of the coated plate is achieved by the use of a *binder*. A commonly used binder is gypsum; organic polymers (particularly the polymethacrylates) have also been employed as binders but, partially dehydrated gypsum is still used in the production of over 80% of all thin layer chromatography plates.

Solvents: The selection of solvent system is critical for good separation. Most commonly, a mixture of solvents is necessary for optimum separation. Hexane is a commonly used non-polar solvent. Others include petroleum ether, carbon tetrachloride, benzene, toluene, ethanol, acetone, acetic acid, chloroform, and methanol, the last being the most polar. Often, the selection of solvents is based on the classification by Snyder who classified solvents into eight groups for normal phase chromatography based on their properties as proton acceptors, proton donors and their dipole-dipole interactions. Optimization of solvents is performed by selecting solvents from this list and running preliminary experiments on the TLC plates. Further refining and optimization of mobile phase is done based on models such as "PRISMA" and correlations between mobile-phase composition and resolution for saturated TLC systems, can be described by mathematical functions. The characteristics of an ideal solvent system include- adequate purity, stability, low viscosity, linear partition isotherm, moderate vapor pressure and minimal toxicity.[3]

Competing processes occurring in the developing chamber in which TLC is being run affect the outcome of the TLC. An equilibrium is obtained between the components of the developing solvent and their vapor. This equilibrium is called chamber saturation. Depending on the vapor pressure of the individual components the composition of the gas phase can differ significantly from that of the developing solvent. While still dry, the stationary phase adsorbs molecules from the gas phase. During this process of adsorptive saturation, an equilibrium is achieved in which the polar components will be withdrawn from the gas phase and loaded onto the surface of the stationary phase. At the same time, the part of the layer which is already wetted with mobile phase interacts with the gas phase. Thereby especially the less polar components of the liquid are released into in the gas phase. The latter process is governed primarily by adsorption forces. During migration, the components of the mobile phase can be separated by the stationary phase under certain conditions, causing the formation of secondary fronts.

To illustrate influence of analyte-solvent interactions in separation, consider that a less polar compound will move higher than one that is more polar. Essentially the solvent molecules will compete with the

[3] TLC and HPTLC differ from all other chromatographic techniques in the fact that in addition to stationary and mobile phases, a gas phase is present which can significantly influence the result of the separation.

molecules from the sample for sites on the stationary phase. Therefore, a less polar solvent will not compete as much with the polar stationary phase, allowing the molecule to stay bound to the stationary phase. This will result in a slower elution rate. Higher polarity solvents will compete more, leading to faster movement of the unbound solutes in the mobile phase. The polarity of commonly used molecules can be summarized as Alkanes (least polar), alkyl halides, alkenes, aromatic hydrocarbons, ethers, esters, ketones, aldehydes, amines, alcohols, and carboxylic acids (most polar). There could be multiple functional groups on molecules and so the final polarity will be the outcome of the combined properties of all of them.

Detection: Detection relies on the properties of the analyte i.e. natural color such as for plants pigments and food colors; natural fluorescence as for polycyclic aromatic hydrocarbons, riboflavin, quinine, aflatoxins etc. In order to detect separated analytes, plates can be visualized in visible or UV light (short wave or long wave) or, if they have fluorescence properties, through fluorescence excitation. Very few of the compounds being analyzed have natural color in the visible range however, and thus most compounds require an alternative detection strategy.

Visualizing the separated compounds involves various techniques like using fluorescence material impregnated into the silica gel which is then visualized by illuminating the plate with short-wave UV. UV is the most common non-destructive visualization method for TLC plates is ultraviolet (UV) light. A UV lamp can be used to shine either short-wave or long-wave ultraviolet light on a TLC plate. Analytes not amenable to these approaches for detection and characterization, would require derivatization, often post-chromatographic to facilitate chemical reactions on the TLC plate. Derivatization (pre-or post-separation) is employed when analyte detection is not feasible just with visible/UV detection so that they can be visualized with suitable detection reagents to form colored, fluorescent, or UV-absorbing compounds. Some non-specific reactions used are iodine adsorption and the charring technique (spraying with sulphuric acid and heat treatment). Universally used charring reagents include sulfuric acid and perchloric acids. Post-derivatization procedures usually result in destruction of sample.

Iodine is a universal detection reagent used especially when unknown compounds are expected, since it reacts with almost all organic compounds. A range of stains are available depending upon the types of compounds and the conditions under which they are being separated. Some stains/derivatization reagents can be used for a wide range of chemical compounds and others are more specific, e.g. ferric chloride for phenols, ninhydrin for amino acids, bromocresol green for functional groups whose pKa is approximately 5.0 and lower, potassium dichromate for tricyclic antidepressants) that will allow visualization of the molecules. cerium molybdate[4], phosphomolybdic acid, potassium permanganate, vanillin, p-anisaldehyde and morin chloride (fluorescent) as general, multipurpose stains.

Chemicals[5] used for post-separation development are either sprayed onto the plate or the plate is dipped in a chamber containing a solution of the chemical/chemical vapor[6]. Dipping usually results in more uniform application. The method to be used for visualization is also dependent on the purpose of the separation e.g. fluorescent detection is acceptable for preparative separation but analytical separation requires higher sensitivity and hence other detection modalities. Some methods like UV light detection and iodine vapor staining are non-destructive methods of development.

[4] This stain can stain non-specific compounds, so to ensure accurate results when employing this stain, the TLC plate needs to be heated vigorously. Thus, this stain is not recommended for volatile samples.

[5] Some reagents are hazardous-ninhydrin and iodine are toxic, sulfuric acid is corrosive. Other solvents used commonly earlier like benzene are being supplanted by toluene since it is much less toxic.

[6] Plates should be well dried prior to visualization. Drying, if being done using the heating method, should be done at temperatures that will prevent any heat induced decomposition.

Currently, *in situ* scanning of TLC plates employing optical instrumentation is extensively used and is now considered essential for both the *accurate identification* of the spot position and the *precise quantitative estimation* of its content. This procedure measures the light scattered, reflected or generated by fluorescence from the spot and compares it electronically with light from a part of the plate where no sample has passed (*e.g*. the channel between the spots).

While there is fairly wide range of sensitivity depending on the analyte and detection method, absorbance typically has sensitivity values in nanogram range while fluorescence methods have higher sensitivities, in picograms.

Starting from manual preparation of plates to the availability of pre-coated TLC plates in the 1960s, the range of stationary phases available for coating has expanded immensely, that are compatible with a range of detection methodologies. In 2013, pre-coated plates that were suitable for TLC-MS have also been introduced. These plates have enhanced sensitivity and a very low background signal compared to standard HPTLC plates, yet offer the same separation efficiency and selectivity.

The classical form of TLC is a simple and relatively inexpensive technique, but the restriction on the length of the stationary phase that can be coated onto the plates, limits the range of separation that can be achieved. The open system format of TLC also makes the results susceptible to being affected by conditions like humidity and temperature. Finally, the limit of detection is higher than other chromatographic techniques, e.g. those that can be achieved by various forms of column chromatography. However, it is a good preparative technique for preliminary analysis or fractionation.

Major recent innovations that have led to improvement in the utility of TLC as an accurate and quantitative technique include newer stationary phases with increased range of separation, automated multiple development, interfaces with spectroscopic instruments and emerging techniques for video densitometry.

4.2 Advantages of TLC:

i. Several samples can be analyzed at the same time; two-dimensional separations can be also be achieved by rotating plates by 90° after drying the TLC plate after the first dimensional development, and running it with a second mobile phase.

ii. Components at extreme positions can also be detected (at site of application; end of plate)

iii. Extensive clean-up of samples is not necessary and enrichment of solutes to be detected can be done

iv. Detection approaches can be combined to allow multimode detection, such as detection of visible spots, which can be followed by detection under UV light; detection by specific color reagents and detection by radioactivity. This possibility is especially important at screening biologically active components, where each component of the raw extract is scouted.

v. TLC separations can be combined with highly sophisticated methods, such as mass spectrometry (e.g., direct MS of spots, MALDI TOF MS).

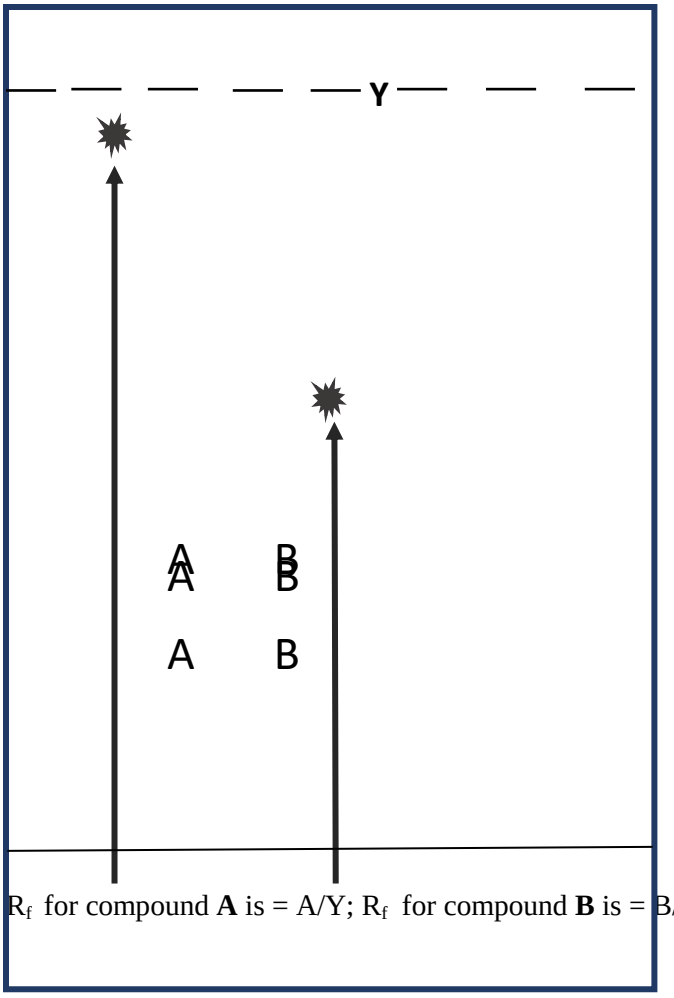
5. Paper chromatography: The paper chromatographic process involves placing the stationary phase (a paper of uniform consistency that is usually filter paper, chromatography paper or sometimes, even a paper towel[7]) in a container with the solvent/mixture of solvents so that the base the paper is dipped in the solvent. This allows the solvent to rise through the stationary phase carrying the sample mix with it. Once the sample mix is applied close to the base of the paper (above the level of the solvent in which the paper is dipped), the relative mobility of the different solutes will allow their separation. The container with the solvent is kept covered to allow the atmosphere inside the container to equilibrate thus preventing evaporation and facilitating a uniform separation as well.

The method is usually carried out in a single dimension but double-way or two-dimensional paper chromatography is also used in which the paper being developed is rotated by 90 degrees during the process and dipped again in a different solvent. The addition of the second dimension improves the resolution of the spots and leads to better separation. Further, apart from the solvent traveling up the stationary phase as in the classical "ascending" paper chromatography, there are formats like "descending" and radial" paper chromatography also. In the descending techniques, the solvent moves downwards under gravity. Radial development is particularly used for separation of compounds in the lower RF range.
If the components of the mixture are colorless, they are visualized using appropriate detection reagents, e.g. amino acids are visualized after separation using a chemical ninhydrin that reacts with almost all amino acids to form a "blue-violet" compound.

Interpretation: Once the compounds are separated and visualized, their individual identities are established by, calculating their **"R_f"** value and comparing it with known standards/compounds. The **"R_f"** or retardation (retention) factor, is equal to the **distance traveled by the substance** divided by the **distance traveled by the solvent**. Its value is always between **zero and one**[8]. For any given chromatographic system, an **R_f** value is characteristic for a compound. The figure below displays the basic outcomes of a simplified planar chromatographic process.

[7] Paper is made up of cellulose fibres with a very thin layer of water molecules bound to the surface. The interaction with this water is the critical for the separation effect during paper chromatography.

[8] An R_f value of 0 indicates that the solvent polarity is very low and 1 indicates that the solvent polarity is very high.



SOLVENT FRONT

Application point

R_f for compound **A** is = A/Y; R_f for compound **B** is = B/Y

5. **Applications of planar chromatography** Planar chromatography has many applications, both quantitative and qualitative. These include separation and analysis for:

   a. Drug residues: steroids, hormones, antibiotics

   b. Bioactive components' analysis: alkaloids, nucleotides, purines

   c. Forensic medicine: alcohols, hydrocarbons, pesticides residues.

   d. Consumer goods quality control

   e. Food analysis: vitamins, antioxidants, fatty acids

6. **Factors affecting chromatographic separation in planar chromatography:** In general, the R_f is affected by layer thickness, moisture on the TLC plate, vessel saturation, temperature, depth of mobile phase, nature of the TLC plate, sample size, and solvent parameters all of which generally cause an increase in $\mathbf{R_f}$.

   • **Development solution**: the polarity and volatility of solvent mixtures will determine the quality of separation. A solvent used for separating highly polar mixtures is ethyl acetate: butanol: acetic acid: water, 80:10:5:5. For strongly basic components, a mixture of 10% NH_4OH in methanol, made to 1 to 10% mixture in dichlormethane is often used. Adjusting the pH of the development solution is a strategy used to optimize the separation and obtain clearly demarcated spots.
   Strongly acidic or basic compounds can cause **streaking** instead of forming neat spots. Adding acetic or formic acid to the solvent for acids and triethylamine for bases can improve results.

   - **Type of visualization protocols used**: the sensitivity of each method differs. Overstaining can lead to non-specificity e.g. overheating ceric ammonium molybdate stained plates can cause the whole plate to turn blue.

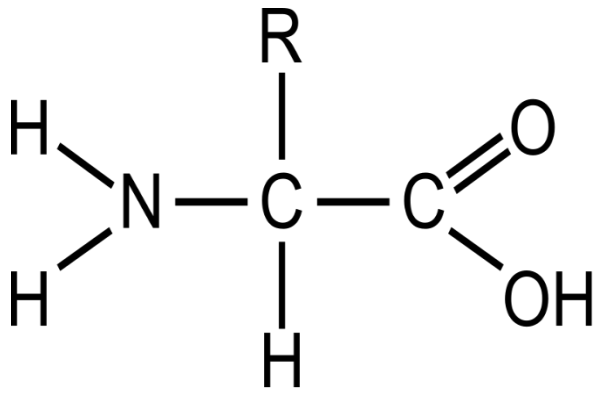
   - **Humidity and temperature**.

   - **Spotting size**: The applied sample should be within 1-2 mm or very large spots may be formed with overlapping component separation.

   - **Uneven solvent front movement**: this may be due to insufficient solvent, improper placement of the stationary phase/plates, uneven plates.

7. **A. Illustrative example showing the separation of amino acids in a mixture by paper chromatography:**

   Amino acids mixtures are separated and visualized by paper chromatography. The different amino acids will have unique $\mathbf{R_f}$ values as, each amino acid has an amino group, -NH2, and a carboxylic acid group, -COOH. The other two valencies of the central carbon are occupied by a hydrogen and a side chain, "R". The 20 different amino acids that are constituents of proteins in humans, and those of most other living things, differ in the identity of the side chain R. The presence of these functional groups affects the charge on the respective amino acids in solutions of different pH and hence the solubility of the amino acid. This allows a differential mobility and separation depending on the solvent used for separation.



Procedure: On the filter paper that will be used for separation, draw a faint pencil line about 1 to 2 cm from one of the long edges and parallel to that edge. This will be the bottom of the chromatogram. Mark off four/five equally spaced points along this line. (They should be separated by about 2 cm). Your samples will be applied to these spots. Keep the paper in a glass jar containing the solvent such that the lower edge of the paper is in contact with the solvent. Apply the sample to the marked positions just above the surface of the solvent using glass capillary tubes. As the solvent moves vertically up the paper by capillary action, separation of the amino acids will take place.

*** Composition of solvent:**

Butanol (12)

Water (5)

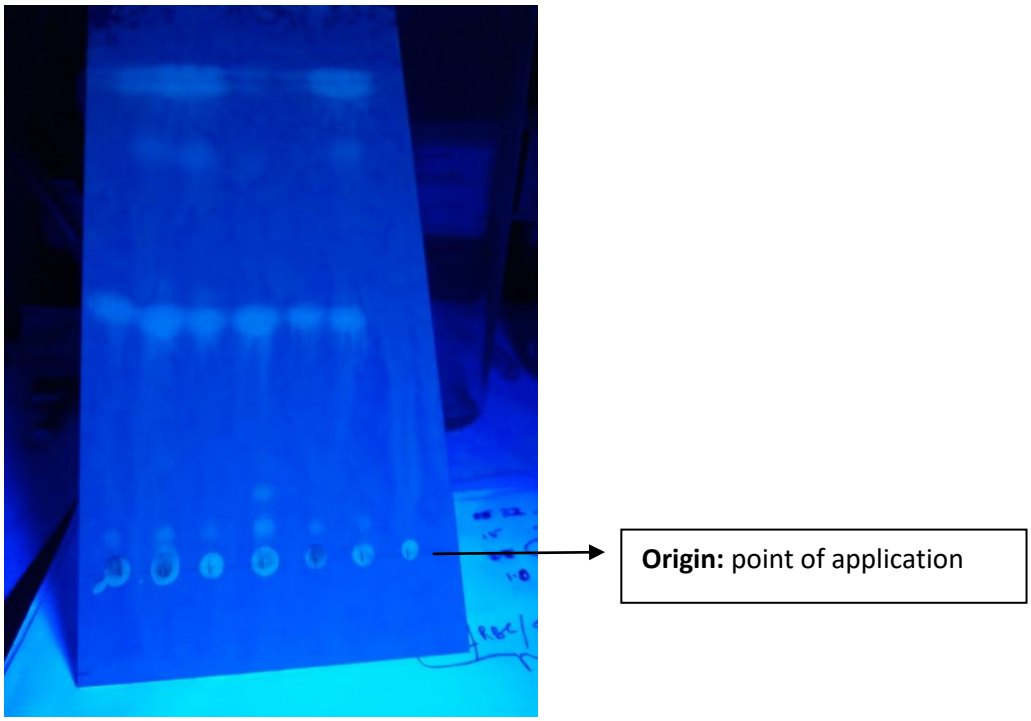
Acetic acid (3)

Component Detection: Spraying of the paper with ninhydrin will stain amino acids. The identification of a given amino acid may be on the basis of its Retardation (Retention) fraction (R_f) value, which is defined as:

$$R_f = \frac{The\ distance\ moved\ by\ solute\ front}{The\ distance\ moved\ by\ solvent\ front}$$

This value is constant for a particular compound under standard conditions. Quantification of the amino acid may be carried out after eluting it with a suitable solvent.

7.B. Illustration of a typical TLC run for separation of lipid components:



The picture shows spot visualization of serum lipid components under Ultraviolet light after separation and staining with Primulin.Each of the lanes contain samples applied at the origin and separated on a silica gel coated glass plate. These are then compared to a standard run for identification of the separated spot.

***Picture credit**: Dr.Ruby Gupta, Public Health Foundation of India,Gurgaon, India.*

Summary:

Planar chromatography is a type of chromatographic process in which the stationary phase is a solid particle or a thin, liquid film coated on either a solid particulate packing material or on glass or plastic which is thus called Thin-layer chromatography (TLC); when the stationary phase is paper, the sub-type is called paper chromatography. Some common stationary phases used in TLC are silica gel, magnesium silicate and silver–ion impregnated matrices for special applications like lipid analysis. The resolution in planar chromatography depends on factors like layer thickness, moisture on the TLC plate, vessel saturation, temperature, depth of mobile phase, nature of the TLC plate, sample size, and solvent parameters. The application of planar chromatography can be both preparative and analytical and some of these include analysis of drug residues, bioactive components like alkaloids, alcohols, hydrocarbons, pesticides residues, and nutrients like vitamins, antioxidants and fatty acids.