ISBN: 978-93-90847-49-5

3. Chromatographic Study of Biological Active Compound

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3.1 Introduction:

Education is lifelong process. We learn different things from different situations formally and It is the analytical method of separating different mixtures of the component from the stationary and mobile phase. In the chromatography technique, the different mixtures of a component will appear as different spots on the **adsorbent**. The sample moves upward through the **capillary action** of the mobile phase. This technique was first introduced in **1903** by the scientist M.S. Tswett. Therefore, chromatography is merely the separation technique that separates the different components of a molecule by making the use of two distinct phases, namely a stationary and mobile phase. To get the process started, the mixture is dissolved in a substance called the **mobile phase,** which carries it through a second substance called the **stationary phase**. The different components of the mixture travel through the stationary phase at different speeds, causing them to separate from one another. The nature of the specific mobile and stationary phases determines which substances travel more quickly or slowly, and is how they are separated.

These different travel times are termed **retention time**. Chromatography gets its name from a technique first used in the late 19th century to separate pigments in a complex mixture. The largest molecules of the mixture will travel more slowly while the smallest ones race ahead, causing the stationary phase to develop discrete bands of colour corresponding to each component of the mixture. This gives the technique the name “chromatography” or “writing colour.” By altering the mobile phase, the stationary phase, and/or the factor determining speed of travel, a wide variety of chromatographic methods have been created, each serving a different purpose and ideal for different mixtures. Some of the most common forms of chromatography are as follows.

In **gas chromatography,** the mixture of interest is vaporized and carried through a stationary phase (usually a metal or glass separation column) with an inert gas, usually nitrogen or helium. Larger molecules in the mixture take longer to pass through the column and reach the detector at the far end.

In **liquid chromatography,** the mixture of interest is dissolved in a liquid and passed through a solid stationary phase, which is often made of a silica material. Several varieties of liquid chromatography exist, depending on the relative polarities of the mobile and stationary phases (**normal-phase** versus **reverse-phase**) and whether the mobile phase is pressurized (**high-performance**).

In **thin-layer chromatography (TLC)**, the stationary phase is a thin layer of solid material, usually silica-based, and the mobile phase is a liquid in which the mixture of interest is dissolved. Thin-layer chromatography comes with the advantage of photographing well, making its output easy to digitize.

Ion exchange chromatography separates the components of a mixture based on their charge, in addition to or instead of their size. In essence, positively (cations) or negatively (anions) charged ions are separated using different stationary phases and different pH mobile phases.

Chromatography can be used as an analytical tool, feeding its output into a detector that reads the contents of the mixture. It can also be used as a purification tool, separating the components of a mixture for use in other experiments or procedures. Typically, analytical chromatography uses a much smaller quantity of material than chromatography meant to purify a mixture or extract specific components from it.

For example, **solid-phase extraction** is a kind of liquid chromatography in which different mobile phases are used in sequence to separate out different components of a mixture trapped in a solid phase.

Chromatography as a purification technique has major roles in petrochemical and other organic chemistry laboratories, where it can be one of the more cost-effective ways to remove impurities from organic solutions, particularly if the components of the mixture are heat-sensitive.

3.2 Thin Layer Chromatography (TLC):

Principle

The separation depends on the relative affinity of compounds towards stationary and the mobile phase. The compounds under the influence of the mobile phase (driven by capillary action) are travelled over the surface of the stationary phase.

During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, the separation of components in the mixture is achieved. Once separation occurs, the individual components are visualized as spots at different level of travel on the plate. Their nature or character is identified using suitable detection techniques.

3.2.1 Requirements:

TLC plates, preferably ready-made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in fine particle size.

TLC chamber, this is used for the development of the TLC plate. The chamber maintains a stable environment inside for proper development of spots. It also prevents the evaporation of solvents and keeps the process dust-free.

Mobile Phase, this comprises of a solvent or solvent mixture. The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.

3.2.2 Methodology:

The stationary phase (**Table 3.1**) is applied onto the plate uniformly and then allowed to dry and stabilize. At present, ready-made plates are preferred.

With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots. Then, samples solutions are applied on the spots marked on the line in equal distances.

The mobile phase is poured into the TLC chamber to a levelled few centimetres above the chamber bottom.

Now, the plate prepared with sample spotting is placed in the TLC chamber in such a way that the side of the plate with the sample line is facing to the mobile phase. Then the chamber is closed with a lid.

The plate is then immersed with mobile phase in such level that the sample spots are well above the level of mobile phase (i.e. not immersed in the solvent) for development.

Allow sufficient time to move mobile phase at sufficient up the plate (**Figure 3.1 and 3.2**). Then remove the plates and allow them to dry.

The sample spots can now be seen in a suitable UV light chamber or any other methods as recommended for the said sample.

3.3 Retention Factor:

Retardation factor is an important parameter in thin layer determinations. It defines the distance travelled by the mixture component from application or starting point relative to the distance travelled by the solvent front over a given time period. Rf lies between 0 and 1.0.

Ideally two spots should not overlap with each other. Rf is influenced by impurities in the paper or solvent mixture, temperature and degree of saturation of the vapour inside the development chamber.

After a separation is complete, individual compounds appear as spots separated vertically. Each spot has a retention factor (R_f) which is equal to relative distance migrated over the total distance covered by the solvent. The *Rf* formula is

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

The *Rf* value can be used to identify compounds due to their uniqueness to each compound. When comparing two different compounds under the same conditions, the compound with the larger *Rf* value is less polar because it does not stick to the stationary phase as long as the polar compound, which would have a lower *Rf* value. *Rf* values and reproducibility can be affected by a number of different factors such as layer thickness, moisture on the TLC plate, vessel saturation, temperature, depth of mobile phase, nature of the TLC plate, sample size, and solvent parameters.

These effects normally cause an increase in *Rf* values.

However, in the case of layer thickness, the *Rf* value would decrease because the mobile phase moves slower up the plate.

Table 3.1: Stationary phase applied in different compounds

Stationary Phase	Chromatographic Mechanism	Typical Application
Silica Gel	adsorption	steroids, amino acids, alcohols, hydrocarbons, lipids, aflaxtoxin, bile, acids, vitamins, alkaloids
Silica Gel RP	reversed phase	fatty acids, vitamins, steroids, hormones, carotenoids
Cellulose, kieselguhr	partition	carbohydrates, sugars, alcohols, amino acids, carboxylic acids, fatty acids
Aluminum oxide	adsorption	amines, alcohols, steroids, lipids, aflatoxins, bile acids, vitamins, alkaloids
PEI cellulose	ion exchange	nucleic acids, nucleotides, nucelosides, purines, pyrimidines
Magnesium silicate	adsorption	steroids, pesticides, lipids, alkaloids

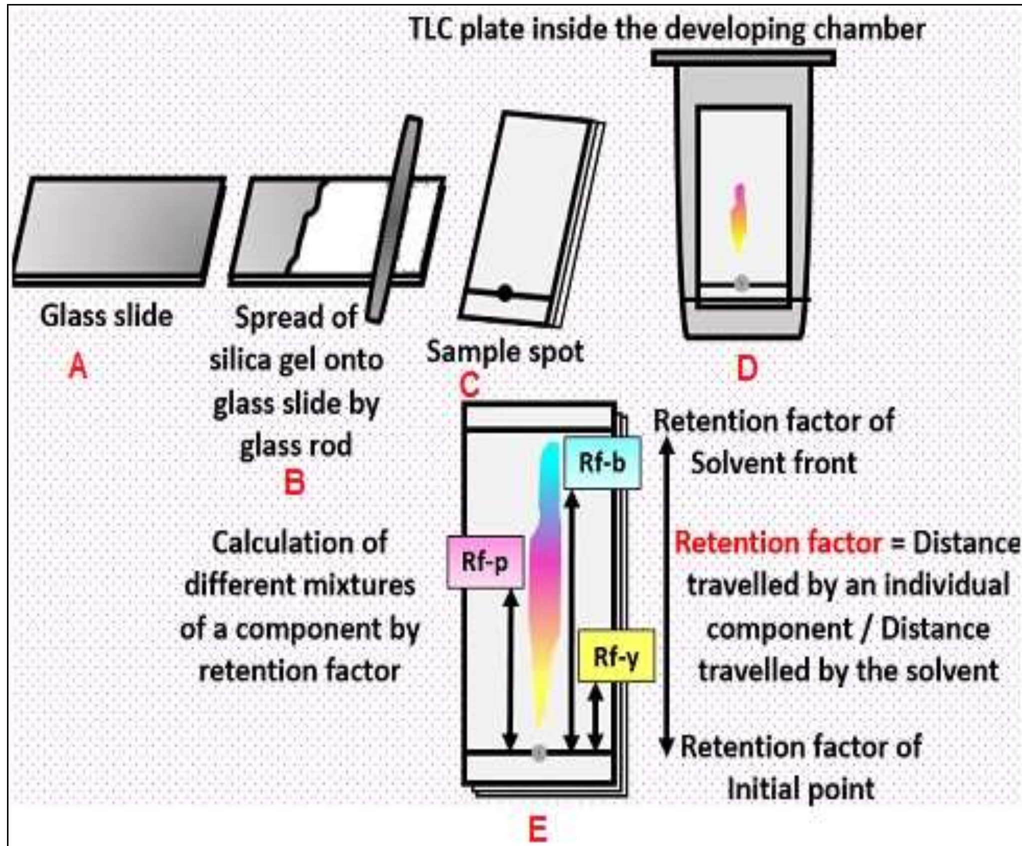

Figure 3.1: process of Thin Layer Chromatography (A-E)

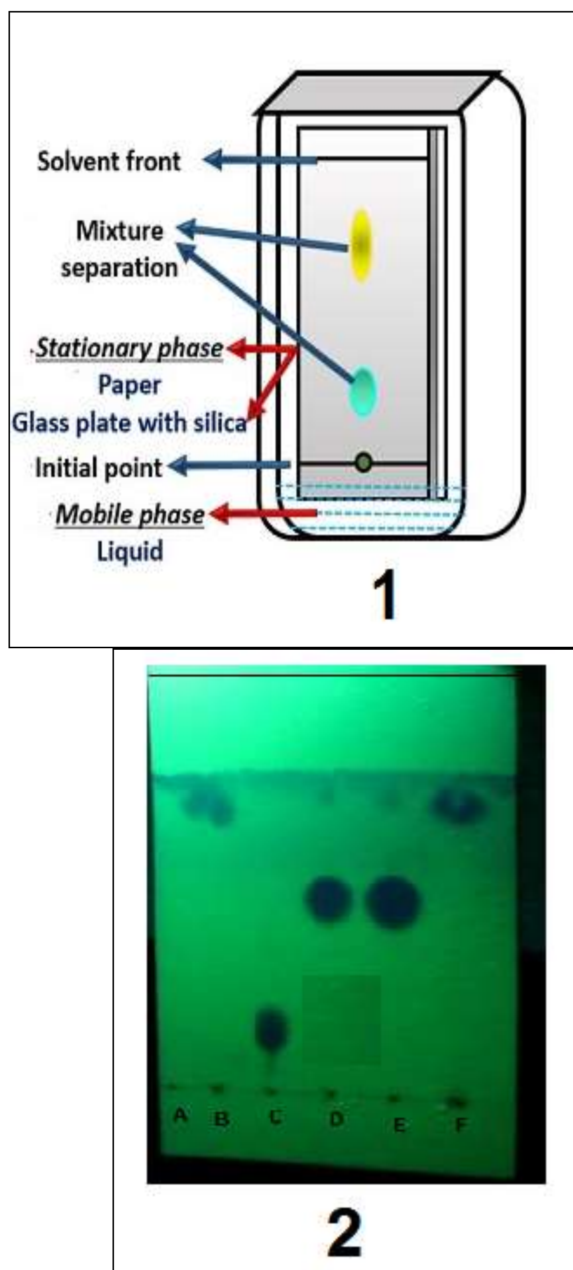

Figure 3.2: Thin Layer Chromatography (TLC). 1- TLC assay; 2- TLC plate

Table 3.2: Determination of Retention factor (*Rf* value) in Thin Layer Chromatography

Compound	Distance travelled by compound/sample	Distance travelled by solvent	Retention factor (*Rf* value)
A	35	42	0.83
B	32	42	0.76

C	7	42	0.166
D	24	42	0.57
E	25	42	0.59
F	32	42	0.76

The Technique for Evaluation of Spots with Colour Development:

- Natural colour observation as in case of coloured compounds
- Complexation reactions with colour producing reagents
- Spraying for inducing fluorescence
- The measurement of radioactivity
- **Other commonly used reagents are** Ninhydrin (for Amino acids), 2,4 – dinitrophenyl hydrazine (for Aldehydes and Ketones), Chloroplatinic acids (for Alkaloids), Bromothymol blue (for Lipids), etc.

3.4 TLC Bio Autography:

Direct bioassay on chromatographic plate make the TLC a powerful screening tool for rapid identification of bioactive components in crude plant extracts. To search for bioactive compounds in plant extracts with a targeted activity, TLC is hyphenated with an appropriate bioassay enabling direct in vitro biological study of the components that have been previously separated on the plate.

Post chromatographic derivatization by dipping or spraying can be done with either a universal micro chemical derivatization using for example anisaldehyde/sulphuric acid for detection of phenols, sugars, steroids, and terpenes or with selective micro chemical derivatization.

The diphenylpicrylhydrazyl (DPPH*) free radical can be used to detect antioxidants or free radical scavengers while fast blue salt B is a stain that can used to detect various enzymes.

Other reagents can be used to detect specific functional groups of phytochemical interest like natural products reagent (2-aminoethyl diphenylborinate) for flavonoids or Brady’s reagent (2, 4-dinitrophenylhydrazine) for aldehydes and ketones.

The bioautographic procedure for screening for antimicrobial activity involves direct localization of antifungal activity on a TLC chromatogram. The “agar diffusion” technique traditionally used, involves transfer of the antifungal compound from the chromatographic plate to an inoculated agar plate by diffusion, where the zones of inhibition are then visualized.

The bioautography technique (Dewanjee *et al.* 2015) is a simplified version of this method where a suspension of a fungal spores (fungi produce pigmented spores may be used for visualization as model organism for antifungal bio-assay) in a suitable broth is directly applied to the TLC plate by dipping the plate into the broth.

The plate is than incubated in a humid atmosphere to allow growth of the fungus and zones of inhibition are visualized after derivatization (**Figure 3.3**). Tetrazolium dyes, for example methylthiazolyltetrazlium bromide can be used to detect fungal dehydrogenase activity for

non-pigmented fungi. Yellow tetrazolium salts are reduced with fungal dehydrogenases into the intensely coloured bluish/violet formazans while antifungal compounds (inhibition zones) will appear as colourless spots against a coloured background.

Figure 3.3: The TLC bioautography for direct screening of antifungal compound from crud plant extract. C indicates control; A- indicates bioactivity.

3.5 Reference:

1. Dewanjee S, Gangopadhyay M, Bhattacharya N, Khanra R, Dua TK. Bioautography and its scope in the field of natural product chemistry. J. Pharnmaceu. Analysis. 5:75-84, 2015.