A glimpse of Thin layer Chromatography (TLC) and Column Chromatography

(Student reference)

Thin layer Chromatography (TLC)

Chromatography is a technique for separating two or more compounds or ions by the distribution between two phases in which one is moving and the other is stationary. These two phases can be consisted with solid-liquid, liquid-liquid or gas-liquid.

Thin-layer chromatography (TLC) is a solid-liquid form of chromatography in which the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. Different affinity of the analyte with the mobile and stationary phases achieves separation of complex mixtures of organic molecules by TLC.

Purpose and advantages of TLC

TLC is a quick, inexpensive, highly efficient and robust microanalytical (microscale) technique separation method which is associated with many advantages.

- determine the number of components in a mixture
- high sample throughput in a short time
- verify a substance's identity
- suitable for screening tests
- monitor the progress of the reaction
- rapid and cost-efficient optimisation of the separation due to easy change of mobile and stationary phase
- determine appropriate conditions for column chromatography
- analyze the fractions obtained from column chromatogrpahy
- ready-to-use layer acts as storage medium for data

Chromatographic adsorbents

Most Strongly Adsorbent	Alumina	Al_2O_3
	Charcoal	C
	Florisil	MgO/SiO_2 (anhydrous)
Least Strongly Adsorbent	Silica gel	SiO_2

Eluting solvents for chromatography

Least Eluting Power (alumina as adsorbent)	Petroleum ether (hexane; pentane)
	Cyclohexane
	Carbon tetrachloride
	Benzene
	Dichloromethane
	Chloroform
	Ether (anhydrous)
	Ethyl acetate (anhydrous)
	Acetone (anhydrous)
	Ethanol
	Methanol
	Water
	Pyridine
Greatest Eluting Power (alumina as adsorbent)	Organic acids

Stationary phase

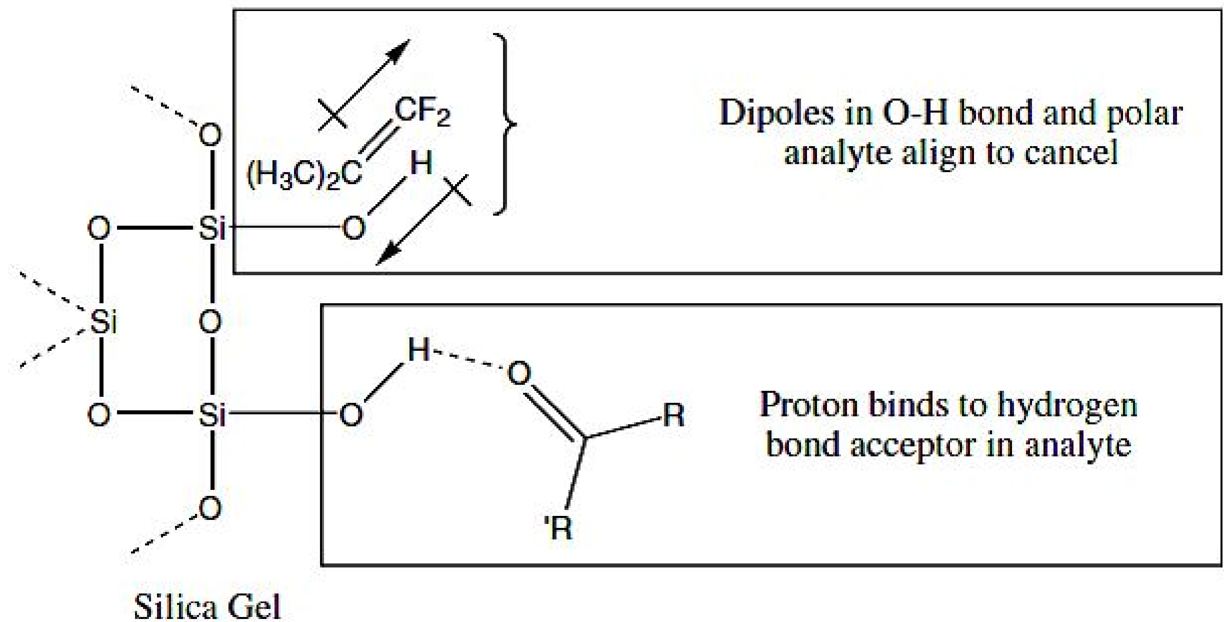
The common stationary phase is Silica gel with empirical formula SiO_2. At the surface of the silica gel, oxygens are usually bound to protons. The presence of hydroxyl groups makes the surface of silica gel highly polar. Consequently, polar functionality in the organic analyte interacts strongly with the surface of the gel particle while nonpolar organic analytes interact only weakly.

The mode of interaction of polar organic analyte molecules to the silica gel occurs in two ways:

1) hydrogen bonds
2) dipole-dipole or dipole induced dipole
3) Ion dipole
4) van der Waals forces

Thus, an analyte with multiple polar groups is in a position to interact with the surface of the stationary phase more strongly than an analyte with same polar functionality having no multidentate binding.

Mode of interaction with analyte



Factors influencing the mode of interactions with silica gel

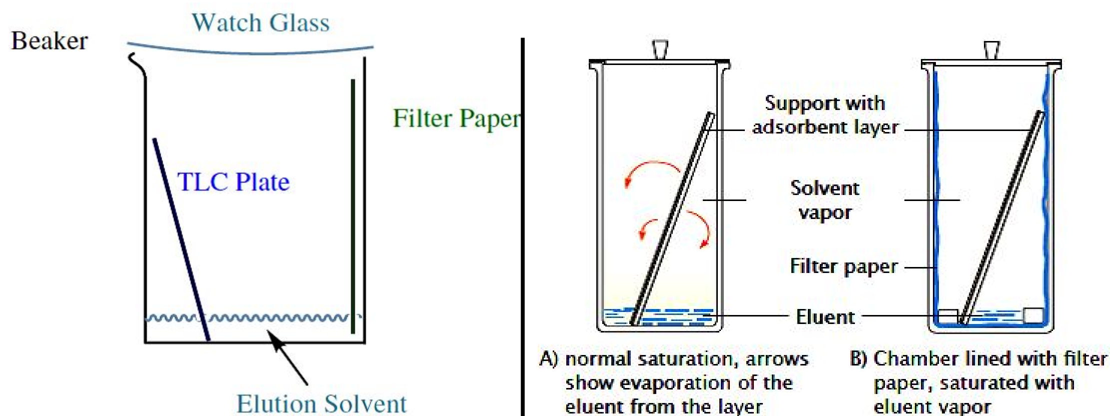
The total strength of the interaction with silica gel is a sum of these two components.

1) Polarity of organic analyte (electronic effect)
2) the shape of the organic analyte (steric effect)

Mobile phase

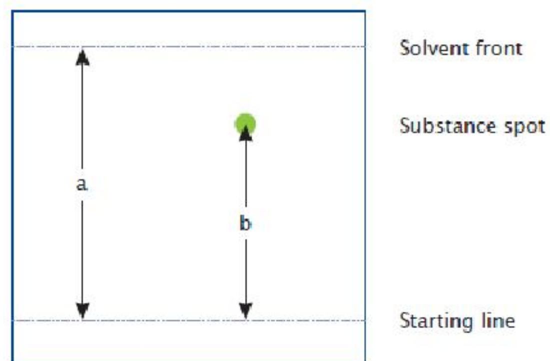
To conduct TLC experiment, the selection of elution solvent is crucial. Hexane and ethyl acetate are used in combination as an elution solvent for most organic analyte molecules. For example, hexane: ethyl acetate = 20:1, 10:1, 7:1, 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4 etc. are commonly employed. Nevertheless, ethy acetate-dichloromethane, methanol-dichloromethane, methanol-water are also used.

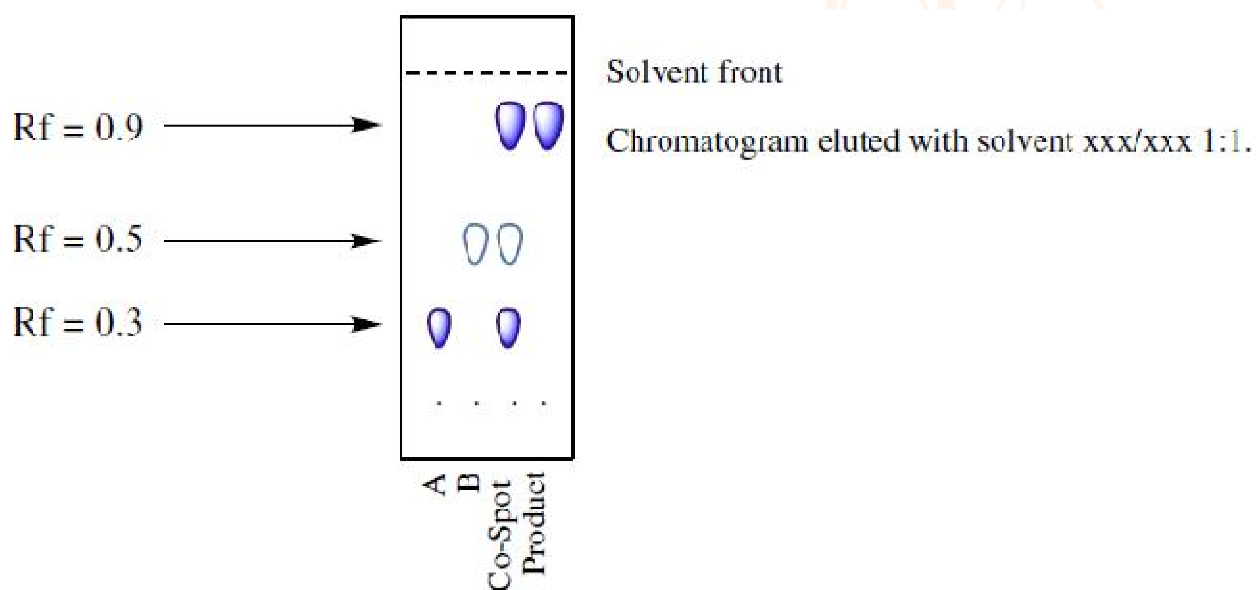
Procedure



10 mL of the elution solvent is place in a 100 mL beaker covered with a watch glass. A piece of filter paper, torn into a square, is inserted along the inside wall of the beaker to ensure the atmosphere in the elution chamber saturated with solvent vapour. The bottom of the filter paper touches the solvent. A line is drawn on the TLC plate about 5 mm from the bottom followed by the cross line in three places with short pencil lines. Three intersections are the locations to place the sample. A solution of the sample (About 1 mg) is prepared in the least polar solvent in which it is soluble. Very small volume of sample solution is spotted onto the pencil marked TLC plate using a micro capillary that already dipped into the sample solution. The spots on the TLC plate should not be more than 3 mm in diameter. The spotting solvent is evaporated for a few seconds. TLC plate is then inserted into the elution chamber with the sample spots at the bottom such that the sample spots should be above the level of the elution solvent. The sample is allowed to elute up to a point where the solvent front is about 5 mm from the top of the TLC plate. To visualize the spots, TLC plate is exposed under UV light, iodine or a series of chemical stains depending on the nature of the analytes. The polarity of the solvent is adjusted to avoid the retention factor (R_f) of the analyte is too large or too small. The R_f values is the ratio of the distance travelled by the compound to the distance travelled by the solvent. Thus, R_f is calculated by dividing the distance traversed by the analyte by the distance traversed by the solvent. It is definite for a particular solvent system. It varies when solvent system changes. R_f should be greater than 0.1 to less than 0.9. It is recommended to use a solvent that gives R_f of the analyte 0.3. R_f value is always less than unity.

$$R_f \text{ value} = \frac{\text{distance traveled by substance}}{\text{distance traveled by solvent front}}$$





The R_f for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant:

- solvent system
- adsorbent
- thickness of the adsorbent
- amount of material spotted
- temperature
- pH of the medium
- nature of other contaminant in the mixture

The R_f for a compound/ solute is highly dependent on the nature and the ration of the solvent system (mobile phase) applied. It is purely a relative quantity which

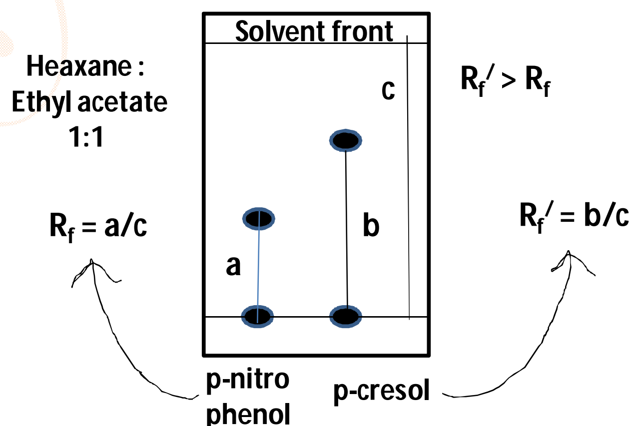
relies on the experimental factors. The higher an R_f of a compound, the larger the distance it travels on the TLC plate. *When comparing two different compounds run under identical chromatography conditions, the compound with the larger R_f is less polar because it interacts less strongly with the polar adsorbent on the TLC plate.* Looking at the structures of the compounds in a mixture, it is predicted that a compound with low polarity will have a larger R_f value than a polar compound run on the same plate. It does not necessarily means that two compounds having similar R_f value would have likewise compound; rather they may be enantiomers (in some cases diastereomers) difficult to be isolated under conventional TLC technique.

Q. *An unknown sample spotted on a TLC plate is eluted with hexane. Only one spot with R_f value 0.1 is observed. Does this indicate that the unknown is a pure compound? If so, how could the purity be verified.*

Ans: The spot at R_f 0.1 or less in non polar solvent like hexane should never be considered as pure compound unless it is being checked in relatively polar solvent. Because, the compound may mix with other polar analyte which is in the base line and is unable to elute in hexane. Thus, hexane and ethylacetate solvent mixture can be a good option so that the compound will have $R_f > 0.5$ with single spot.

Q. *Illustrate by comparing between 4-nitrophenol and p-cresol in 1:1 hexane and EtOAc solvent system?*

Ans: In 1:1 hexane: Ethyl acetate, the retention factor of (R_f) of 4-nitrophenol will be less than p-cresol since the for is more polar owing to larger dipole moment by hydroxyl and nitro groups.



Q. Give the relative order of R_f of isomeric hydroxyl benzaldehyde.

Hint Ans: The order of Rf values: Salisaldehyde > meta hydroxyl benzaldehyde > para-hydroxy benzaldehyde.

Due to intramolecular hydrogen bonding, salisaldehyde exhibits less interaction with silica and thus it gives high R_f value.

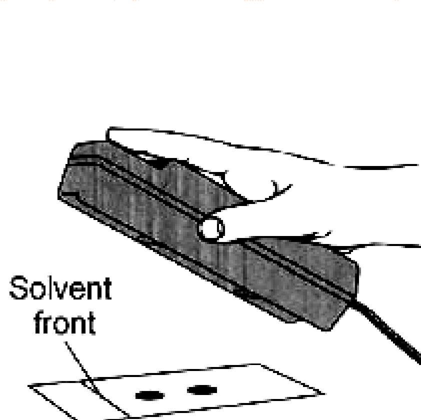
Q. How does steric effect influence the TLC? Explain with example.

Hint Ans: The steric crowding in the molecule reduces the absorption of the molecule to silica or alumina plate.

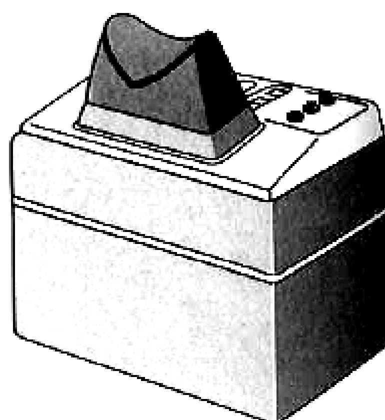
Troubleshooting TLC

- The compound runs as a streak rather than a spot
- The sample runs as a smear or a upward crescent
- The sample runs as a downward crescent
- The plate solvent front runs crookedly: Crooked plates make it harder to measure Rf values accurately.
- Many random spots are seen on the plate
- Visualization of a blur of blue spots on the plate as it develops
- No spots are seen on the plate

Visualization of TLC spot by UV light



(a) Handheld UV lamp


(b) UV lamp with dark box

Those analytes which have chromophoric group(s) can easily absorb the UV light and illuminate the spot as blue spot. Both handled UV lamp and UV lamp with dark box are commonly used to observe the spot.

For alumina

Fluoroalkanes
Pentane
Isooctane
Petroleum ether (light)
Hexane
Cyclohexane
Cyclopentane
Carbon tetrachloride
Carbon disulfide
Xylene
Di-i-propyl ether
Toluene
1-Chloropropane
Chlorobenzene
Benzene
Ethyl bromide
Diethyl ether
Diethyl sulfide
Chloroform
Methylene chloride
Tetrahydrofuran
1,2-Dichloroethane
Ethyl methyl ketone
1-Nitropropane
(Acetone)
1,4-Dioxane
Ethyl acetate
Methyl acetate
1-Pentanol
Dimethyl sulfoxide
Aniline
Diethylamine
Nitromethane
Acetonitrile
Pyridine
Butyl cellosolve
2-Propanol
1-Propanol
Ethanol
Methanol
Ethylene glycol
Acetic acid

For silica

Cyclohexane
Heptane
Pentane
Carbon tetrachloride
Carbon disulfide
Chlorobenzene
Ethylbenzene
Toluene
Benzene
2-Chloropropane
Chloroform
Nitrobenzene
Di-i-propyl ether
Diethyl ether
Ethyl acetate
2-Butanol
Ethanol
Water
Acetone
Acetic acid
Methanol
Pyruvic acid

For magnesium silicate

Pentane
Carbon tetrachloride
Benzene
Chloroform
Methylene chloride
Diethyl ether

It should be noted that to obtain the best results in any of these quantitative TLC methods, the spots being used should have ***R,*** values between 0.3 and 0.7; spots with R_f values <0.3 tend to be too concentrated whereas those with R_f values >0.7 are too diffuse.

Column Chromatography

Principle and Theory

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids when carrying out small-scale experiments. The separation can be liquid/solid (adsorption) or liquid/liquid (partition) in column chromatography. The stationary phase, a solid adsorbent, is usually placed in a vertical glass column and the mobile phase, is added from the top and let flow down through the column by either gravity or external pressure (Figure 1). The principle of column chromatography is based on differential adsorption of substance by the adsorbent.

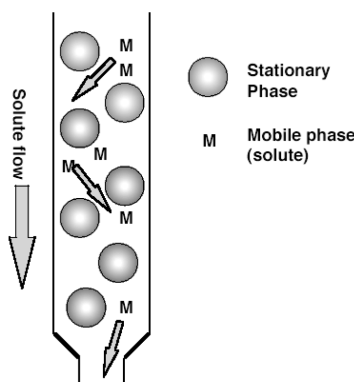


Figure 1. Column chromatography involves a mobile phase flowing over a stationary phase.

Column chromatography isolates desired compounds from a mixture in such a way that the mixture is applied from the top of the column. The columns are usually glass or plastic with sinter frits to hold the packing. The liquid solvent (eluent) is passed through the column by gravity or by the application of air pressure. The eluent, instead of rising by capillary action up a thin layer, flows down through the column filled with the adsorbent. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Stationary phases are almost always adsorbents. Adsorbent is a substance that causes passing molecules or ions to adhere to the surface of its particles. The mobile phase is a solvent that flows past the stationary phase, dissolving the molecules of the compounds to be separated some of the time.

Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as solvent drips from the bottom of the column.

Many compounds are not visible to the eye when dissolved in a solvent or adsorbed on a adsorbent. Visualization processes make these substances visible. The used techniques for this purpose include UV lights that cause fluorescence or phosphorescence and chemical reactions that give colored compounds.

Purpose, benefits and advantages

Column chromatography is advantageous over most other chromatographic techniques because

i) it can be used in both *analytical* and *preparative* applications.
ii) it can be used to determine the number of components of a mixture and as well as the separation and purification of those components.
iii) those compounds which are not separated by fractional distillation or by fractional crystallization are also able to separate.
iv) separation of a by-product from desired product in a reaction
v) isomeric compounds or compounds having close proximity of molecular weight or polarity can be separated.

Choice of adsorbent

i) silica,
ii) alumina,
iii) calcium carbonate,
iv) calcium phosphate,
v) magnesia,
vi) starch

Silica gel (SiO_2) and alumina (Al_2O_3) are two adsorbents commonly used by organic chemists for column chromatography. These adsorbents are sold in different mesh sizes: "silica gel 60", "silica gel 230-400" etc. This number refers

to the mesh of the sieve used to size the silica, specifically, the number of holes in the mesh or sieve through which the crude silica particle mixture is passed in the manufacturing process. If there are more holes per unit area, those holes are smaller, thus only smaller silica particles are allowed to pass the sieve. The larger the mesh size, the smaller the adsorbent particles are.

Adsorbent particle size affects the way the solvent flows through the column. Smaller particles (higher mesh values) are used for flash chromatography; larger particles (lower mesh values) are used for gravity chromatography.

Alumina is quite sensitive to the amount of water which is bound to it; the higher its water content, the less polar sites it has to bind organic compounds, and thus the less "sticky" it is. This stickiness or activity is designated as I, II, or III with I being the most active. Alumina comes in three forms: acidic, neutral, and basic. The neutral form of activity II or III, 150 mesh, is most commonly employed.

Choice of solvent

Selection of solvent is based on the nature of both the solvent and the adsorbent. The polarity of the solvent, which is passed through the column, affects the relative rates at which compounds move through the column. Polar solvents can more effectively compete with the polar molecules of a mixture for the polar sites on the adsorbent surface and will also better solve the polar constituents. Consequently, a highly polar solvent will move even the highly polar molecules rapidly through the column. If a solvent is too polar, movement becomes too rapid, and little or no separation of the components of a mixture will result. On the other hand, if a solvent is not polar enough, no compounds will elute from the column. Proper choice of an eluting solvent is thus crucial for a successful application of column chromatography as a separation technique since compounds interact with the silica or alumina largely due to polar interactions.

In nutshell, the rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent. If the activity of the adsorbent is very high and polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is

rapid but gives only a poor separation, i.e., the components separated are not 100% pure.

Elution Chromatography

The adsorbent is made into slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through the column. Elution involves transporting a species through a column by continuous addition of fresh mobile phase. Introduction of additional mobile phase (the *eluent*) forces the solvent containing a part of the sample down the column where further partition between the mobile phase and fresh portions of the stationary phase occurs (time t_1). Simultaneously, partitioning between the fresh solvent and the stationary phase takes place at the site of the original sample. Continued additions of solvent carry solute molecules move down the column in a continuous series of transfers between the mobile and the stationary phases. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The component with greater adsorption power will be adsorbed at the top and the other will be adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as *elution*. That is, the process of dissolving out of the components from the adsorbent is called elution and the solvent is called is called eluent. The weakly adsorbed component will be eluted more rapidly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions gives the pure components.

Chromatograms

If a detector that responds to the presence of analyte (dye) is placed at the end of the column and its signal is plotted as a function of time (volume of added mobile phase), a series of peaks is obtained. Such a plot, called a chromatogram, is useful for both qualitative and quantitative analysis.

Critical view

Intermolecular forces, which vary in strength according to their type, make organic molecules to bind to the stationary phase. The stronger the intermolecular force, the stronger the binding to the stationary phase, therefore the longer the compound takes to go through the column.

Intra-molecular hydrogen bonding is present in ortho- nitro phenol. This is due to the polar nature of the O-H bonds which can result in the formation of hydrogen bonds within the same molecule. But in para-nitro phenol, inter molecular hydrogen bonding (between H and O atoms of two different para-nitro phenol molecules) is possible. As result of inter molecular hydrogen bonding para-nitro phenol undergo association that increases the molecular weight, whereby decreasing volatility.

Migration Rates of Solutes

The Partition Coefficient

An analyte is in equilibrium between the two phases;

$$A_{mobile} \leftrightarrow A_{stationary} \qquad K = C_S / C_M$$

where the equilibrium constant K is called the partition coefficient.

C_S: molar concentration of analyte in stationary phase

C_M: molar concentration of analyte in mobile phase

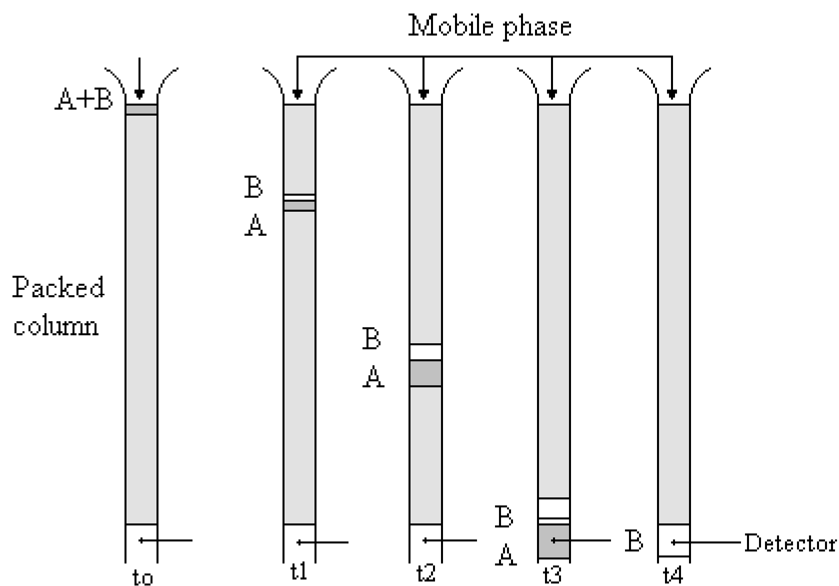


Figure 2. Diagram showing the separation of a mixture of components A and B by column chromatography

Retention Time

The time it takes after sample injection for the analyte peak to reach the detector is called retention time and its symbol is t_R.

$v = L/t_R$ $u = L/t_M$

where v: average linear rate of analyte migration

L: length of column packing

u: average linear rate of movement of molecules of mobile phase

t_M: time required for an ave. molecule of mobile phase to pass through the column, dead time

Relation between retention time & partition coefficients

The migration rate as a fraction of mobile phase velocity

$v = u$ * (moles of analyte in mobile phase / total moles of analyte)

$$v = u[(C_M V_M /(C_M V_M + C_S V_S))] = u[1/(1+(C_S V_S / C_M V_M))] = u[1/(1+(KV_S / V_M))]$$

The rate of Solute Migration

The capacity or retention factor, k'_A, is used to describe the migration rates of analytes on columns for a species A.

$k'_A = K_A V_S / V_M$ then $v = u/(1+k'_A)$

So, the following equation may be used to derive k'_A from a chromatogram,

$L/t_R = L/(t_M * (1+k'_A))$

Selectivity Factors

The selectivity factor, α, of a column for the two species A and B is defined as, $\alpha = K_B / K_A$

where K_B: the partition ratio for the more strongly retained species B

K_A: the partition ratio for the less strongly held or more rapidly eluted species A.

By this definition, α is always greater than unity.

Variables that affect Column Efficiency

- Linear velocity of mobile phase
- Diffusion coefficient of mobile phase
- Diffusion coefficient of stationary phase
- Capacity factor
- Diameter of packing particle

EXPERIMENTAL PROCEDURE

Materials

- Methylene blue (MW = 373.90 g/mol)
- Methyl orange (MW = 327.34 g/mol)
- Ethyl alcohol
- Water
- Alumina 90

Apparatus

- Column
- Stopper
- Glass wool
- Fraction collecting tubes
- UV-visible spectrophotometer

Procedure

1. Pack a conventional size chromatography column with activated alumina. You will need to use a glass wool plug in the bottom. The column has a clamp to stop the flow of the solvent. Mix 10 ml ethanol and 10 g alumina to obtain slurry. Fill this slurry to the column and wait until alumina settles down to 4-5 cm height.
2. Always keep some ethanol above the top of the alumina. Newer allow the alumina to be dry. Allow the ethanol to come to about 1 mm at the top of the column and stopper the bottom of the column with a hose clamp.
3. You are given a mixture prepared with 0.6 ml methylene blue and 0.4 ml methyl orange which have the same concentration, 0.25 mg/ml. Introduce gently 1 ml dye solution onto the top of the column.
4. Carefully add about 10 ml of ethanol to the column and allow it to drip through. Collect uncolored eluent in the waste, but as soon as the colored compound begins to emerge, collect this in a beaker. Record the time to reach first dye drop.You might need to add an additional few ml of ethanol to the column to prevent it from becoming dry.
5. Record the rate of movement of colored rings through the packing.

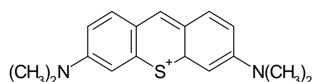
6. When the first dye is either completely or nearly emerged from the column, add 10 ml of water carefully to the top of the column. Once again, collect clear eluent in the waste. The second dye should be collected in a separate beaker.
7. Note the volume of each of the two dye solutions. If they are too dark, they may need to be diluted. To do this, take 1 ml of the solution and carefully dilute it to 5 or 10 ml with the eluent in a clean tube.
8. Using UV Spectrophotometer, find the concentrations of the dye solutions. Plotting absorbance versus concentration of standard solutions, draw the calibration lines. The calibration lines for methylene blue and methyl orange should be drawn at 650 nm and 450 nm respectively.

Flash Chromatography

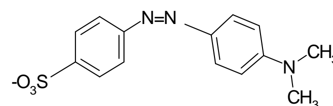
Column chromatography is often very time consuming process. Allowing the solvent to elute through the column one drop at a time takes patience. One method to speed up the process is to use Flash Chromatography. This method uses a pressure of about 10 psi of air or nitrogen to force the mobile phase through the column. Because the rate of the mobile phase is increased, in general, this method gives a poorer separation. However, by using a finer grade of alumina or silica, flash chromatography can be used to increase the speed without lowering the quality of the separation.

Questions

1. Which eluent is more polar?
2. Which dye is more polar? Is this reflected in their structures given below?

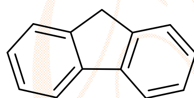


Methylene blue (A)

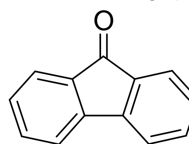


Methyl Orange (B)

3. Calculate average linear rate of movement of the molecules of the mobile phase.
4. Calculate average linear rate of analyte migration.
5. Calculate moles of analyte in mobile phase.
6. Calculate the partition coefficient and capacity factor for each analyte.
7. Calculate the selectivity factor (for the first system only).
8. Suggest a better method to control the rate of flow of mobile phase through the column.
9. How can this system be automated?
10. Predict the order of elution of fluorene and fluorenone from an alumina chromatography column. Explain in terms of compound structure (shown below) and polarity and its interaction with the alumina stationary phase.

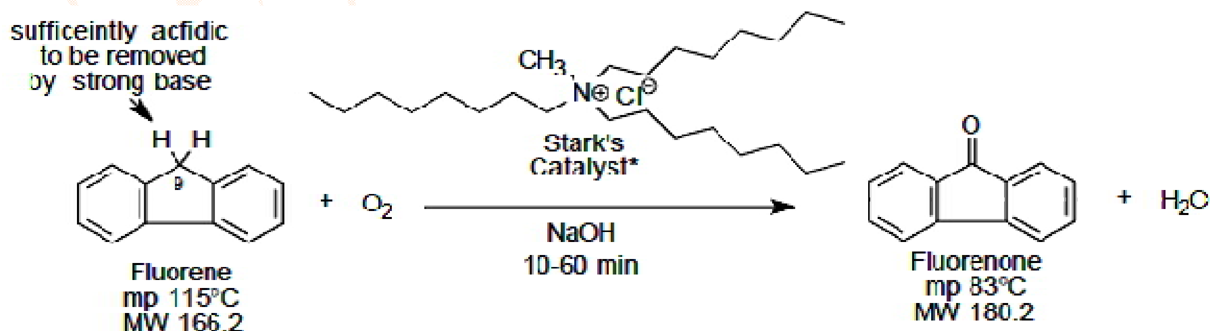


Fluorene



Fluorenone

11. Consider the following reaction and predict the order of elution of fluorene and fluorenone from an alumina chromatography column.



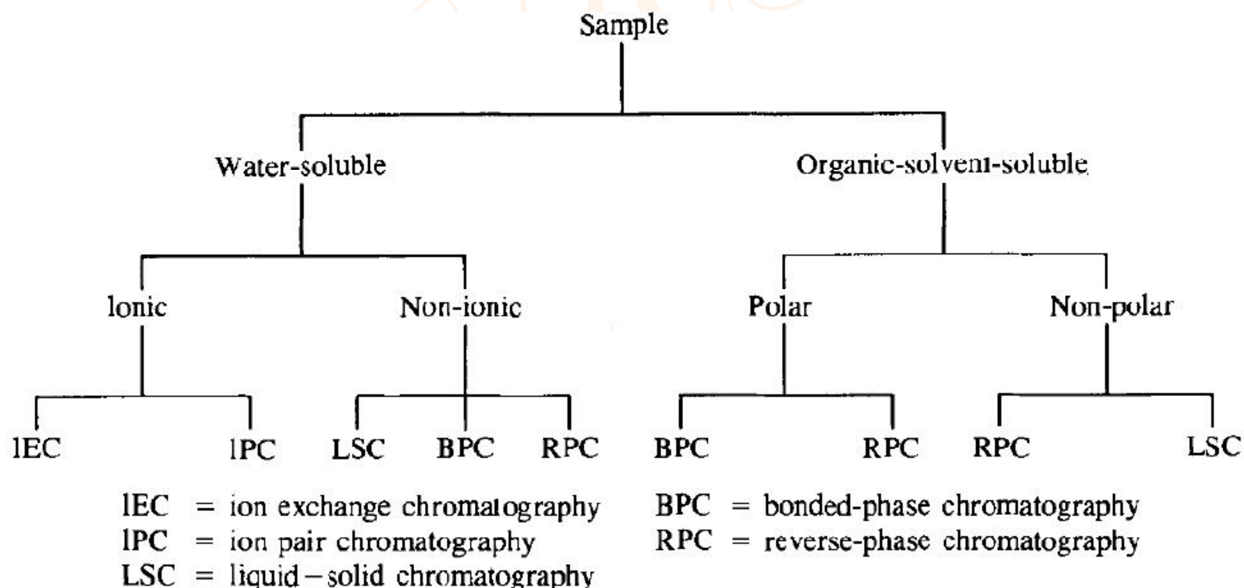
Point to be noted

Typical stationary and mobile phases for normal and reverse phase chromatography

Stationary phases	Mobile phases
Normal	
β, β′-Oxydipropionitrile Carbowax (400, 600, 750, etc.) Glycols (ethylene, diethylene) Cyanoethylsilicone	Saturated hydrocarbons, e.g. hexane, heptane; aromatic solvents, e.g. benzene, xylene; saturated hydrocarbons mixed with up to 10 per cent dioxan, methanol, ethanol, chloroform, methylene chloride (dichloromethane)
Reverse-phase	
Squalane Zipax-HCP Cyanoethylsilicone	Water and alcohol water mixtures; acetonitrile and acetonitrile–water mixtures

Choice of mode of separation

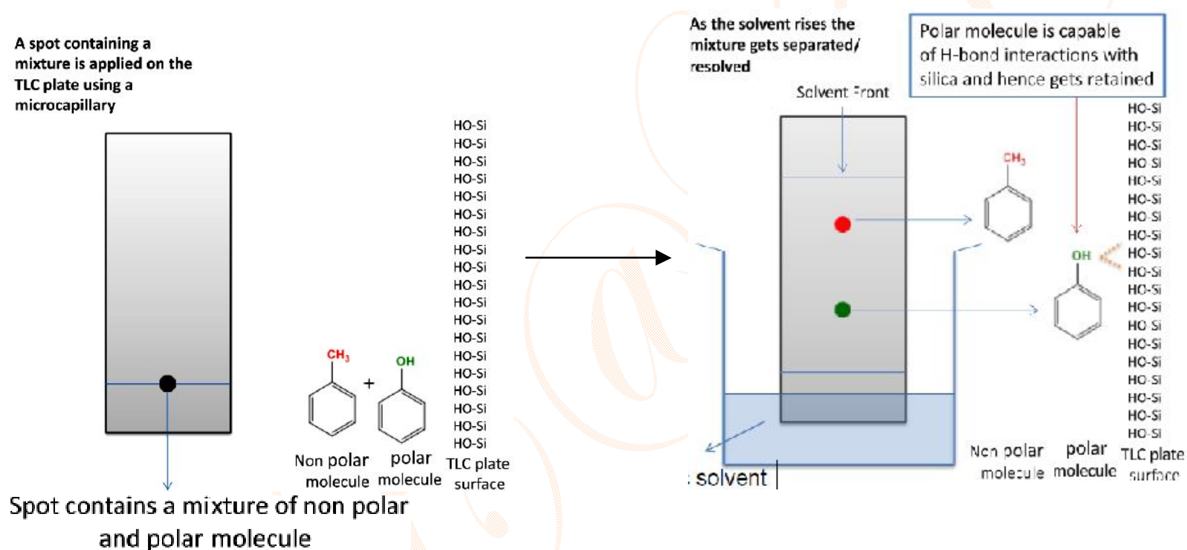
The diagram provides a general guide to the selection of a chromatographic method for separation of compounds of molecular weight <2000; for samples of higher molecular weight (>2000) the method of choice would be size-exclusion or -gel-permeation chromatography.



Q. Distinguish between TLC and paper chromatography.

TLC	Paper Chromatography
Wide choice between different Adsorbents [stationary phase].	cellulose
It has better resolution and to allow for quantitation.	Low resolution but also allow for quantitation
compact zonal spread [concentrated for quantitation analysis in need].	Expanded zonal spread[not concentrated for quantitation analysis in need].

Q. Illustrate the TLC of toluene and phenol.



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2. Fried, B., Sharma, J. Thin-layer chromatography, fourth edition, revised and expanded, Marcel Dekker inc., New York - Basel, 1999, 499.