

Principles of chromatography

'Chromatography' is an analytical technique commonly used for separating a mixture of chemical substances into its individual components, so that the individual components can be thoroughly analysed. There are many types of chromatography e.g., liquid chromatography, gas chromatography, ion-exchange chromatography, affinity chromatography, but all of these employ the same basic principles.

Chromatography is a separation technique that every organic chemist and biochemist is familiar with. I, myself, being an organic chemist, have routinely carried out chromatographic separations of a variety of mixture of compounds in the lab. In fact, I was leafing through my research slides and came across a pictorial representation of an actual chromatographic separation that I had carried out in the lab. I guess that picture would be a good starting point for this tutorial!

Let me first explain what I was trying to do here. I had two reactants 'A' and 'B'. I let them react with each other, under certain reaction conditions, to form a product 'C'. After the reaction was complete, I ended up with a reaction mixture that contained unreacted A, unreacted B and my desired product C. Now my task was to separate out A, B and C to isolate and analyze pure product C.

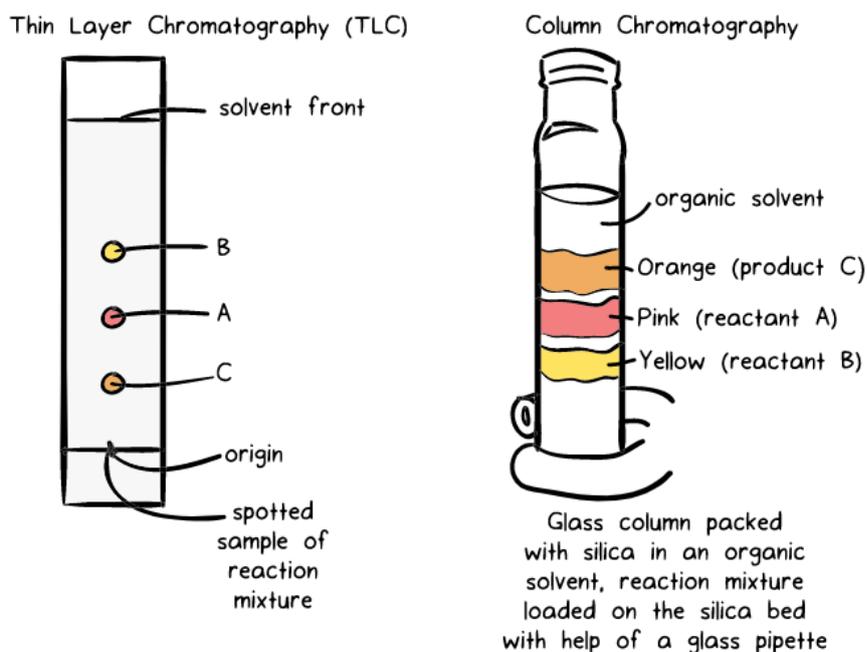


Illustration of thin layer chromatography (TLC) and glass column chromatography

First, as shown in the left hand side panel, I ran a *thin layer chromatography* (TLC) plate.

This is basically a rectangular piece of glass plate, coated with a thin layer of silica. I applied a spot of the reaction mixture just above the base of the plate (denoted with a solid line), and placed the plate in a jar that contained an appropriate organic solvent (in this case, 1:1 volume by volume mixture of hexane:ethyl acetate was used), with just enough volume to dip the lower edge of the plate. Gradually by capillary action, the solvent started rising up the silica plate, and as you can see the reaction mixture separated into 3 spots with distinct colors by the time the solvent had reached the solvent front mark.

Next, in order to actually perform the separation, I assembled a glass column (as shown on the right hand side of the picture). I took a glass column with a stopcock attached at the bottom, inserted a cotton plug at the bottom of the column and packed the column with a slurry of silica gel (prepared in an organic solvent). Once the column was packed, and the solvent volume above the bed reduced to less than 5 mm, I carefully poured the reaction mixture over the bed of silica from the top of the column, with the aid of a glass pipette. I opened the stopcock and let the solvent run slowly through the column. I constantly kept adding solvent from the top of the glass column. As you can see, the reaction mixture started separating into three distinct bands - yellow, pink and orange corresponding to unreacted B, unreacted A and the desired product C, respectively. I collected individual bands in separate flasks and was thus able to obtain pure C!

Principles of chromatography

Let's first familiarize ourselves with some terms that are commonly used in the context of chromatography:

Illustration of column chromatography with labeled terms

Term	Definition
<i>Mobile phase or carrier</i>	solvent moving through the column
<i>Stationary phase or adsorbent</i>	substance that stays fixed inside the column
<i>Eluent</i>	fluid entering the column
<i>Eluate</i>	fluid exiting the column (that is collected in flasks)

Term	Definition
<i>Elution</i>	the process of washing out a compound through a column using a suitable solvent
<i>Analyte</i>	mixture whose individual components have to be separated and analyzed

Now let's try to understand the principle of chromatography. Let us draw a pictorial representation of a column chromatographic separation set up.

Illustration of a column chromatographic separation

As depicted above, the analyte is loaded over the silica bed (packed in the column) and allowed to adhere to the silica. Here, silica acts as the stationary phase. Solvent (mobile phase) is then made to flow through the silica bed (under gravity or pressure). The different components of the analyte exhibit varying degrees of adhesion to the silica (see later), and as a result they travel at different speeds through the stationary phase as the solvent flows through it, indicated by the separation of the different bands. *The components that adhere more strongly to the stationary phase travel more slowly compared to those with a weaker adhesion.* Analytical chromatography can be used to purify compounds ranging from milligram to gram scale.

Before we move on, let's conduct a simple experiment to exemplify the power of a chromatographic separation.

1. Take a few leaves and crush them in a mortar.
2. Spot a drop of the leaf extract on a strip of chromatographic paper ~ 0.5 cm above the edge of the paper. Chromatographic paper is made of cellulose and is quite polar in nature.
3. Place the strip of paper in a jar that contains a small volume of propanone (acetone). There should be just enough propanone that the edge of the paper dips in it comfortably. Place a lid on the jar to avoid any evaporation of the solvent.
4. Let the solvent rise up the paper by capillary action. Remove the paper strip from the jar once the solvent has reached the 'solvent front' level. 5) What do you think you will notice?

Illustration of thin layer chromatographic (TLC) separation experiment involving crushed leaves

The various components of the leaf pigment separate out! Could you have ever imagined that a leaf pigment was made up of so many compounds?

Principle of separation of different components: *Differential affinities (strength of adhesion) of the various components of the analyte towards the stationary and mobile phase results in the differential separation of the components. Affinity, in turn, is dictated by two properties of the molecule: 'Adsorption' and 'Solubility'.*

We can define adsorption as the property of how well a component of the mixture sticks to the stationary phase, while solubility is the property of how well a component of the mixture dissolves in the mobile phase.

- Higher the adsorption to the stationary phase, the slower the molecule will move through the column.
- Higher the solubility in the mobile phase, the faster the molecule will move through the column.

So, the interplay between the above two factors determines the differential rates at which the different components of the analyte will move through the column. Adsorption and solubility of a molecule can be manipulated by choosing the appropriate stationary phase and mobile phase.

Now, the question arises why do different compounds possess different affinities towards the stationary and mobile phases? *"Polarity" of the compounds dictates their affinities towards the stationary and mobile phases.* Let's understand this through an example.

Suppose we have a mixture of two molecules A and B, where 'A' is a protein and 'B' is a lipid. Our column is packed with silica, which is polar in nature; our mobile phase is hexane, which is non-polar in nature. What do you think will happen when we load this mixture of A and B onto this column?

'A', being polar in nature, will adsorb on to the polar stationary phase (silica). 'B' being non-polar in nature, will readily dissolve in the non-polar mobile phase (hexane) without adhering to silica, and will thus elute out of the column with hexane. Once B is eluted out, the mobile phase will be changed to something polar like acetonitrile. By doing so we will now force A

to detach from the silica and dissolve in the polar solvent, acetonitrile, and get eluted out of the column with acetonitrile. This is illustrated in the diagram below.

Illustration of column chromatography with hexane eluent and lipid (nonpolar) eluate;
column chromatography with acetonitrile eluent and protein (polar) eluate

Different types of chromatography

Throughout this article we are dealing with what we refer to as **normal-phase** chromatography, implying that our stationary phase is polar (hydrophilic) in nature and our mobile phase is non-polar (hydrophobic) in nature. For special applications, scientists sometimes employ **reverse-phase** chromatographic techniques where the scenario is reversed i.e. the stationary phase is non-polar while the mobile phase is polar.

There are several types of chromatography, each differing in the kind of stationary and mobile phase they use. The underlying principle though remains the same: *differential affinities of the various components of the analyte towards the stationary and mobile phases results in the differential separation of the components*. Again, the mode of interaction of the various components with the stationary and mobile phases may change depending on the chromatographic technique used. The commonly used chromatographic techniques are tabulated below.

Technique	Stationary phase	Mobile phase	Basis of separation	Notes
*Paper chromatography	solid (cellulose)	liquid	polarity of molecules	compound spotted directly on a cellulose paper
*Thin layer chromatography (TLC)	solid (silica or alumina)	liquid	polarity of molecules	glass is coated with thin layer of silica on which is spotted the compound
*Liquid column chromatography	solid (silica or alumina)	liquid	polarity of molecules	glass column is packed with slurry of silica
Size exclusion chromatography	solid (microporous beads of silica)	liquid	size of molecules	small molecules get trapped in the pores of the stationary phase,

				<p>while large molecules flow through the gaps between the beads and have very small retention times. So larger molecules come out first. In this type of chromatography there isn't any interaction, physical or chemical, between the analyte and the stationary phase.</p>
<p>Ion-exchange chromatography</p>	<p>solid (cationic or anionic resin)</p>	<p>liquid</p>	<p>ionic charge of the molecules</p>	<p>molecules possessing the opposite charge as the resin will bind tightly to the resin, and molecules having the same charge as the resin will flow through the column and elute out first.</p>
<p>Affinity chromatography</p>	<p>solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and antibodies)</p>	<p>liquid</p>	<p>binding affinity of the analyte molecule to the molecule immobilized on the stationary phase</p>	<p>if the molecule is a substrate for the enzyme, it will bind tightly to the enzyme and the unbound analytes will pass through in the mobile phase, and elute out of the column, leaving the substrate bound to the enzyme, which can then be detached from the stationary phase and</p>

				eluted out of the column with an appropriate solvent.
Gas chromatography	liquid or solid support	gas (inert gas like argon or helium)	boiling point of the molecules	samples are volatilized and the molecule with lowest boiling point comes out of the column first. The molecule with the highest boiling point comes out of the column last.

**Fall under the category of 'Liquid Chromatography'*

Thin layer chromatography (TLC): Retention factors (R_f)

Just to refresh our memories, normal phase thin layer chromatography is performed on a piece of glass plate that is coated with a thin layer of silica. Here, silica acts as the *stationary phase* and the solvent in which the plate is dipped and that runs up the plate by capillary action is the *mobile phase*. The stationary phase i.e. silica is very polar in nature, while the solvent is less polar compared to silica.

The polar components of the analyte will adhere to the silica tightly and thus travel slowly up the plate, while the less polar or non-polar components will not adhere that strongly to the silica and travel up the plate relatively fast with the solvent. Now let's again go back to the very first picture, discussed in this tutorial.

Illustration of TLC plate showing upward travel of solvent components

As shown above, the three components A, B and C of the reaction mixture travelled different distances, as the solvent moved up the TLC plate. Measured from the origin (where we spotted the reaction mixture): component C travelled 1 cm, component A travelled 2 cms and component B travelled 3 cms. The solvent travelled 5 cms (distance from origin to solvent front).

Rule of thumb:

- The component that travels the least distance on the TLC plate is the most polar, since it binds to the silica most tightly.

- The component that travels the maximum distance is the least polar; it binds to the silica least tightly and is most soluble in the non-polar solvent (mobile phase), and hence moves up the plate with the solvent.

So just by looking at a TLC plate, you can tell which component is more polar and which component is less polar. There is also a quantitative parameter, termed as *retention factor* (R_f) that can be calculated for every individual component and this value is very commonly used in the ‘world of chemical syntheses’. This value is invariably reported in manuscripts so that people who replicate the synthesis of a compound can verify that they too are getting the same R_f values for the same compounds.

Retention factor is defined as the distance travelled by the individual component divided by the total distance travelled by the solvent. ‘Lower the R_f value, more polar the component.’

Component	Distance travelled by the component (cm)	Distance travelled by the solvent (cm)	Retention factor (R_f) of the component
C	1	5	$R_{fC} = \frac{1}{5} = 0.2$
A	2	5	$R_{fA} = \frac{2}{5} = 0.4$
B	3	5	$R_{fB} = \frac{3}{5} = 0.6$

Based on the R_f values (as calculated above), component C is the most polar and component B is the least polar.