Second Year blog on Chromatography: 11th May 2019

(This is a **revision** blog. It is merely a *summary* of some of the things that you need to know. Everything in this blog can be found in the relevant Chapters of the two books.)

If I had a mixture of different substances dissolved in Alcohol or in Water or in whatever, and I asked you how I could separate them, you would say to me "Dead easy: separation funnel or fractional distillation", and at that point I might reply "I'm so sorry, I forgot to tell you that I had just one single drop of the mixture" – and then you might go silent and scratch your head and start to think hard. Well, *that is where Chromatography comes in.* Despite the origins of the term in the Greek word for "colour", Chromatography has nothing whatsoever to do with colour[1] – instead, in essence, it is the science of the **separation** of mostly small volumes of mixtures of liquids and/or gases.

Chromatography is a very important process that enables the **SEPARATION** rather than the **identification** of a mixture of substances into their individual constituents to take place (very easily/quickly/and cheaply). The process is utilised extensively in forensic science where only very small samples are available, and also in industrial applications because Chromatography is inexpensive to carry out, and the results are obtained within a very short space of time. In order to separate the different substances that are being analysed, **all of them** depend on there being **two** mediums viz. a "**stationary phase**" and a **liquid** "**mobile phase**" for a liquid eluent, or a **gas** "**mobile phase**" for a gas eluent. (All the terms will be explained herein.)

There is one fundamental difference that needs to be understood in Chromatography viz. the difference between **Adsorption Separation** (where the term 'adsorption' is used in exactly the same way as we used it last year when we discussed the function of porous iron as the catalyst in the manufacture of Ammonia by the Haber process), and **Partition Separation**. (I am under the impression that in Chromatography separation by Adsorption is used more than is separation by Partition.)

Very often the identity of the substances under investigation are already known or are identified subsequently, and all that the process does is to separate them. The problem that is addressed in Chromatography is *how* to separate the different substances. The Royal Society of Chemistry (RSC) identifies the following different separation techniques in use today (all of which rely on the same underlying principles):

1) Paper Chromatography
2) Thin Layer Chromatography
3) Gas (also called Gas-Liquid) Chromatography
4) Liquid (or Liquid-Column) Chromatography
5) High Performance Liquid-Column Chromatography
6) Ion Exchange Chromatography
7) Gel Permeation or Size Exclusion Chromatography

There is a nice little table from the excellent Khan Academy on page 5.

[1] The science was given the name by Mikhail Tsweet following his pioneering work on the separation of plant pigments using starch packed into a vertically positioned glass burette as his stationary phase. Pigments by definition are coloured, therefore he called his technique "**Chroma**tography".

For UK 'A' Level purposes I am going to tackle this topic in two Sections
A The theoretical concepts behind the science
  A1 Definitions of the terms that are used.
  A2 An examination of how separation occurs in Chromatography, and
B A description of the seven different types of Chromatography that are cited above.

Section A) The theoretical concepts behind the science

A1 Definitions of the terms that are used

"Stickiness" is not a scientific term, but Chromatography separates things by differential 'stickiness' (in reality by differential **polarity** where the stickiness is created by van der Waals/London/Debye forces of attraction). If I made my mixture flow through the fibres inside a piece of paper[2] or over a piece of paper that was coated with a 'sticky' surface (i.e. the '**stationary phase**'), then the solute with greatest affinity for the fibres or the sticky surface would get stuck to the stationary phase first while the other substances would keep on flowing inside or over the paper in the '**mobile phase**' (or eluent), and gradually a separation of the different substances would occur as different substances got stuck to the paper at different points until eventually the substance that had the least affinity for the fibres or the sticky surface would get stuck and the game would then be over. Amazingly, that is **exactly** what happens in Chromatography.

- The equivalent to my piece of paper is called the "**substrate**".
- The sticky film on my piece of paper is called the "**stationary phase**" (and very often it is just water – water being a polar substance).
- The medium in which the different substances are dissolved is called the "**solvent**" or the "**mobile phase**". The solvent can also be called the "**eluent**".
- The different dissolved substances are called the "**solutes**" or the "**analytes**" (and strictly speaking the solvent is called the "**eluent**", but the mixture of the different substances dissolved in the solvent can also loosely be called the "**eluent**". Basically, the eluent is the liquid or the gas that is flowing through the system).

Let us start by defining some terms.

Solute : is a substance that is dissolved by another substance (the latter is called a solvent). In chromatography a solute can also be called an "**analyte**" and a mixture of solutes dissolved in the solvent can also be referred to as "the analyte".

Solvent : a substance that dissolves another substance is called a solvent. The amount of a solute that can be dissolved in a given volume of solvent will vary from solvent to solvent. This is a function of the solubility of a given solute in a given solvent (cf. page 4).

[2] Paper is made from "cellulose" (a condensation polymer of glucose). In a piece of Chromatography paper the 'stickiness' would be due to the highly polar '–OH' species in the molecules of the cellulose in the paper.

Eluent : in Chromatography, **the *mobile* solvent** (either liquid or gas), either with or without the solutes dissolved in it, is called the "eluent". To "elute" is a term that means to be carried along or carried out of the differentiating mechanism by the solvent. (The term comes from Latin: where "*eluere*" is the infinitive form of the verb "to wash".)

Immiscible : is a term that means that two substances (usually liquids) cannot be mixed (and if shaken up together and left for a period of time will separate out into two separate liquids).

State : there are four common states for matter viz. solid (s), liquid (l), gas (g), and "aqueous" (aq) which means dissolved in water – but it is possible to have something dissolved in something else and then it is common practice to indicate this as "(soln)" for solution, meaning that the solute has been dissolved in the solvent. There are other phases such as plasma, but the above are the common ones.
Phase : phases are represented by the normal states, other than for the fact that if two states are immiscible, then two identical states will be in different **phases**. For example, oil and water are both liquids and are immiscible, and when oil and water are put in the same container, the oil will lie on top of the water (*they are in the same state but in different phases*). Equally, ice and water are different states of H_2O, but ice will float on top of water – here *they are in different states and in different phases*.

In Chromatography, there are always two phases
- a **mobile phase** which is capable of moving around within the system, and
- a **stationary phase** which is in a fixed location within the system.

Retardation factor/Retention factor, R_f = $\dfrac{\text{distance travelled by the substance}}{\text{distance travelled by the solvent front}}$

Locator : If the separated solutes are colourless, then a variety of different 'locators' can be used to pinpoint the location of the differentiated solutes: spraying with Ninhydrin for amino acids / suspension in a container of iodine vapour / uv light / Geiger counters for radioactive substances / and so on.

There are two more terms ("**Adsorption**" and **Partition**") that we need to talk about and then we can talk about the different types of Chromatography.

A2) <u>An examination of how separation occurs in Chromatography</u>

A2a) <u>Separation by Adsorption</u>

Adsorption: SiO_2 and Al_2O_3 gels, and crushed/powdered $CaCO_3$ all operate through Adsorption separation via Hydrogen bonding[3]**.** It is the Oxygen in the above that makes them

[3] Oxygen is the second most polar element that there is. Only three elements can form Hydrogen bonds: F/O/and N.

into polar substances. *(Oxygen is the second most electro-negative element in the Periodic Table.)* The gels "adsorb" by virtue of the hydrogen bonding that occurs at the surface of the gel.

When we talked about the action of the solid porous iron catalyst in the manufacture of Ammonia by the Haber process (Chapter 2, Rates of Reaction, last year), we talked about how the two gases (Nitrogen and Hydrogen) were **adsorbed** onto the surface of the porous iron for one brief moment of time and that this broke the bonds in the N–N and H–H molecules and enabled them to form the three new N–H bonds that were required for Ammonia. Well, that is precisely the **adsorption** that we are talking about here.

If you were to think of Adsorption in Chromatography as "stickiness", then you would not go far wrong. For any given sticky substance, some things will stick to it well while other substances might not find it sticky at all – **and it is this difference in stickiness that would determine how far the substances move over the surface of the sticky substance**. (NB In Chromatography, the 'stickiness' is mostly caused by polarity which can lead to Hydrogen bonding and to other sorts of binding mechanisms.)

If the substances to be separated (i.e. the solutes in Chromatography) were being carried along by a mobile phase over the 'sticky' surface of a stationary phase, then the differing propensities to a**d**sorb to the surface of the 'stationary phase' is what would separate the substances that are being separated in Chromatography.
At 'A' Level you would be expected to be only vaguely aware of the fact that the propensity to a**d**sorb to the stationary phase is related to many different factors including surface area/temperature/polarity/ surface tension/etc – but **the most important of these is polarity**. The substances that are being separated would have different polarities, and the stationary phase would also be polar. (In a piece of chromatography paper this would be due to the highly polar '–OH' species in the molecules of the cellulose in the paper. Paper is made from "cellulose", a condensation polymer of glucose. In a piece of Chromatography paper the 'stickiness' would be due to the highly polar '–OH' species in the molecules of the cellulose in the paper, and the resultant Hydrogen bonding that this causes.)

A2b) Separation by Solubility / Partition Separation

It is not only the ability of the stationary phase to *adsorb* that will affect the separation of the different substances in the mobile phase. The **solubility** of the different substances in the mobile phase will also be a factor. A substance that is more soluble than another substance in the mobile phase will get carried farther than will a less soluble substance.

If a liquid is used on the surface of the stationary phase, then it is almost certain to be Partition Separation. For example, paper will have molecules of Water in the interstices of the fibres, and therefore Paper Chromatography works by Partition Separation.

If a solute (the substance that is being dissolved by the solvent) is capable of being dissolved in two different solvents that are immiscible, then differing amounts of the solute will dissolve in a given volume of each of the two solvents. If two immiscible solvents are put into a test-tube and

a small amount of a solute is added to the test-tube, and the test-tube is shaken and left for a period of time, then the two solvents will separate out with the denser one being under the lighter one. There will be more of the solute dissolved in one solvent than in the other. That is the **partition** to which I am referring here. **It is all about differential solubility.**

Partition Separation involves the reaching of a position of dynamic equilibrium in a reversible reaction. The reaction that is involved here is a *solubility* reaction in which the solubility of a solute that is dissolved in a solvent called the mobile phase differs from the solubility of that same solute in a second solvent called the stationary phase.

The concentrations of the solute/the analyte in the mobile and the stationary phases is given by the reversible reaction

$$\text{Solute in the Mobile phase} \rightleftharpoons \text{Solute in the Stationary phase}$$

therefore there must be an Equilibrium Constant (called the **Partition Coefficient**) where

$$\text{Partition Coefficient} = \frac{\text{Concentration of the solute in the stationary phase}}{\text{Concentration of the solute in the mobile phase}}$$

In Partition Chromatography the stationary phase is usually a layer of a polar liquid (such as water) that is held in place by a solid surface (such as paper) and the mobile phase is a liquid that is immiscible with the stationary phase – and the mobile phase travels over the surface of the stationary phase.

Let us now talk about the techniques that are involved in the different types of Chromatography (and then the technical terms will become much more clear to you).
To help you keep in mind where we are going, the table below is from the excellent Khan Academy website.

Technique	**Stationary phase**	**Mobile phase**	**Basis of separation**	**Notes**
***Paper chromatography**	solid (cellulose)	liquid	polarity of molecules	Compound spotted directly onto cellulose paper
***Thin layer chromatography (TLC)**	solid (silica or alumina)	liquid	polarity of molecules	Glass coated with a thin layer of silica on which is spotted the compound
***Liquid column chromatography**	solid (silica or alumina)	liquid	polarity of molecules	Glass column 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, while large molecules flow through the gaps between the beads and have very small retention times. Larger molecules

				therefore come out first. In this type of chromatography there isn't any interaction, physical or chemical, between the analyte and the stationary phase.
Ion-exchange chromatography	solid (cationic or anionic resin)	liquid	ionic charge of the molecules	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.
Affinity chromatography	solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and antibodies)	liquid	binding affinity of the analyte molecule to the molecule immobilized on the stationary phase	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 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.

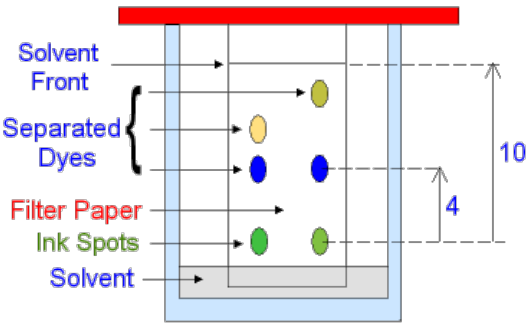
**Falls under the category of 'Liquid Chromatography'.*

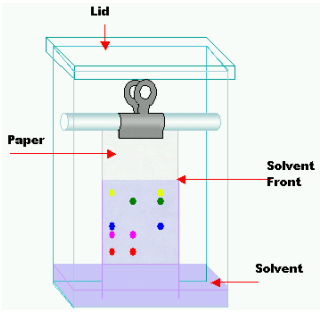
B) The different separation techniques in Chromatography

B1) <u>Paper Chromatography</u>

- Paper Chromatography is an example of *Partition* Chromatography where the stationary phase is the molecules of water that are trapped in the paper, and the mobile phase is the solvent (immiscible with water) that travels up the paper by virtue of capillary action.[4]

- The web contains a large number of very simple demonstrations of Chromatography. The two below are one from gcsescience.com and one from Wikipedia





- The procedure in Paper Chromatography is to put a spot of the mixture of the solutes onto a piece of chromatographic paper (about 2 cm from the bottom of the paper), and then suspend the paper in a small amount of solvent in a lidded jar (with the paper just touching the solvent). *For reference purposes spots of known chemicals can be put at exactly the same horizontal level as the mixture of solutes under investigation.*

- When the mobile phase solvent reaches almost the top of the paper the experiment must be stopped and measurements taken. Capillary action will have carried the mobile phase solvent and the different solutes therein up the paper. It is the different distances that have been travelled in relation to the distance travelled by the mobile phase solvent that enable the identification of the different solutes (cf. below).

- **Retardation factor/Retention factor R_f** = $\frac{\text{distance travelled by the substance}}{\text{distance travelled by the solvent front}}$ and published data on Retention/Retardation factors are available.

- In reality, the identification of a solute requires the researcher to have some prior knowledge of what the solutes are.

- Paper Chromatography is a moderately unsophisticated form of analysis and, in order to obtain better results, the procedure can be modified as follows

[4] In a narrow tube, forces such as surface tension cause a liquid to travel up the tube against the force of gravity.

Paper or cardboard —

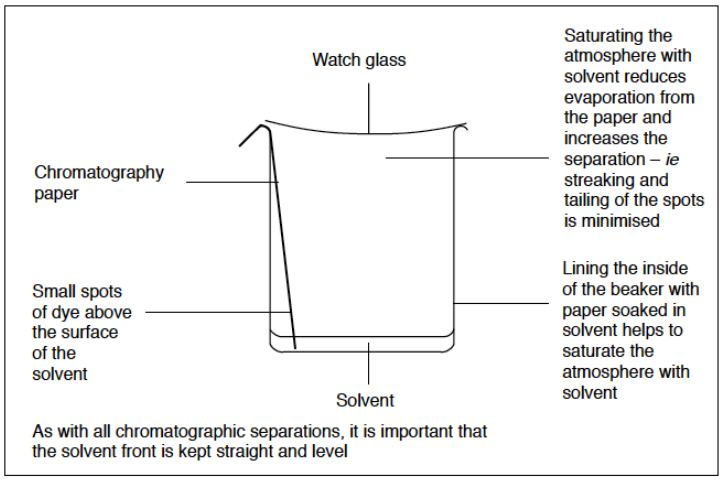


Figure 5 Paper chromatography

Source: RSC

- **Multiple pass separation**: If the solutes have not separated sufficiently in one pass then the slide can be turned upside down and replaced in the same mobile solvent (or even a different mobile solvent) for a second pass (or a much longer slide can be used).

B2) Thin Layer Chromatography (TLC)

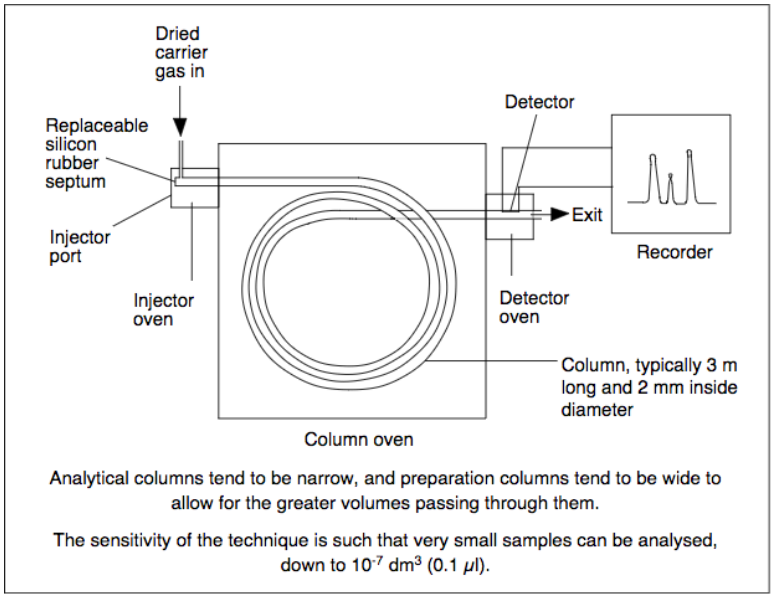
- Thin Layer Chromatography uses a rigid surface of an inert substance such as a piece of glass. The glass is coated with a polar substance such as Silica (SiO_2), or Alumina (Al_2O_3), in gel form, and it is the Silica or the Alumina that forms the stationary phase.

- Chemistry LibreTexts makes the following point "TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity, and speed of separation. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. (If used for the purpose of identification) the goal of TLC is to obtain well defined, well separated spots.". *At 'A' Level you do **not** need the detail below. I show it merely to illustrate the types/uses of TLC.*

Table 1: Stationary phase and mode of separation

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

B3) Gas (or Gas-Liquid) Chromatography



Source: RSC

- As with all other sorts of Chromatography, there must be

a) A **substrate** for the stationary phase. The substrate is a long thin heated coiled metal capillary tube called a "column", the **inner** surface of which is coated with the **stationary phase** (0.25 μm film coating the inside surface of a 0.32 mm capillary tube), and the **mobile phase** (which is also called the "carrier gas") is a flow of inert gas such as Helium (used in 90% of GC machines) or Argon or Nitrogen. The inert gas is the mobile phase or eluent that carries the solutes along the tube.

b) A **stationary phase**, consisting of a paste or gel or a liquid polymer with a high boiling point. The stationary phase that coats the inner surface of the tube can even consist of a solid provided that it adheres to the inner surface of the substrate metal tube. (If the stationary phase is a solid, then the separation will take place by **ad**sorption.) The greater is the interaction between the solute and the stationary phase, the **longer** will the solute take

to travel through the tube. If the stationary phase is polar and the solute is also polar then there will be an attraction between the two. The greater the polarity, the greater the attraction and the longer will the retention time be. The stationary phase must have a high boiling point (higher than the temperature of the oven otherwise the stationary phase would boil and become a gas and be carried out of the tube with the inert gas and thus it would no longer be the stationary phase that was coating the inner surface of the tube).

c) A **mobile phase or eluent** which is an inert gas and which will **not** interact with either the stationary phase or the solutes. The solutes will thus be gases in a gaseous mobile phase or eluent.

- The coiled metal capillary tube is heated so as to prevent the solutes and the mobile phase from condensing and becoming liquids and thus stop being gases. The solutes may temporarily become liquids, but ultimately they must become gases to be carried out of the capillary column. **This is GAS Chromatography.** If the solutes were to be liquid, then they would no longer be carried along in the stream of the inert gas eluent. However, if the temperature of the oven is higher than the boiling point of the stationary phase, then the stationary phase would boil off and be carried out of the capillary tube by the eluent gas.
- It can be seen therefore that the temperature of the oven is quite critical. In any case, if the temperature of the oven were too high, then the eluent and solutes would pass through the column too quickly and there would be an inadequate separation of the solutes.

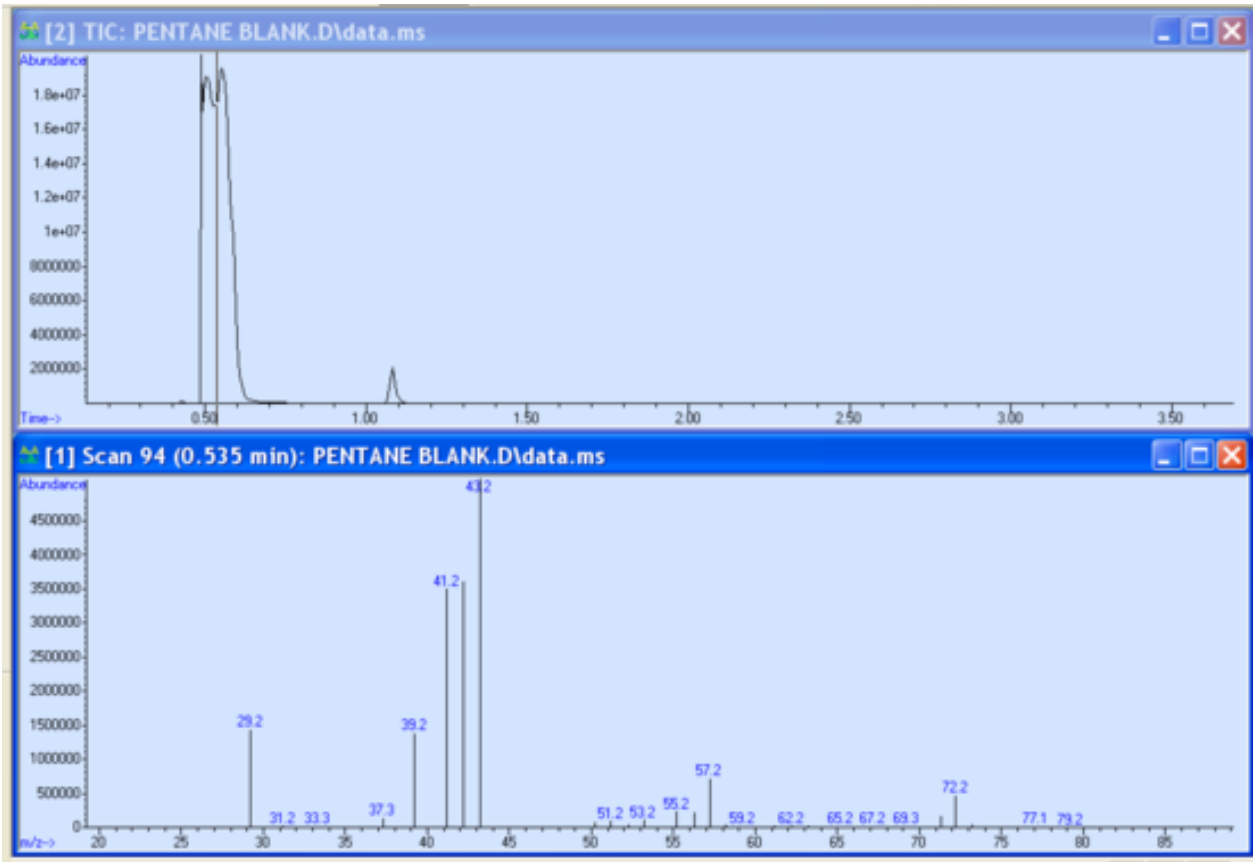
- The strength of the interaction between the solutes and the stationary phase determines the speed at which the solutes pass through the tube (and so does the speed at which the eluent passes through the column).

- In Gas Chromatography, identification is achieved by the time taken to exit the tube (much like a time-of-flight mass spectrometer), and the process can become very sophisticated with drying/cleansing/etc functions – but in essence the above is all that you need to know.

- You may remember that I told you (when we were talking about Mass Spectroscopy) that it is becoming quite common for the separated gases that exit a Gas Chromatographic separator to be injected straight into a Mass Spectrometer and thus conduct a Mass Spectrometric analysis in series with the separation.

- Modern analytical techniques are thus becoming very sophisticated indeed.

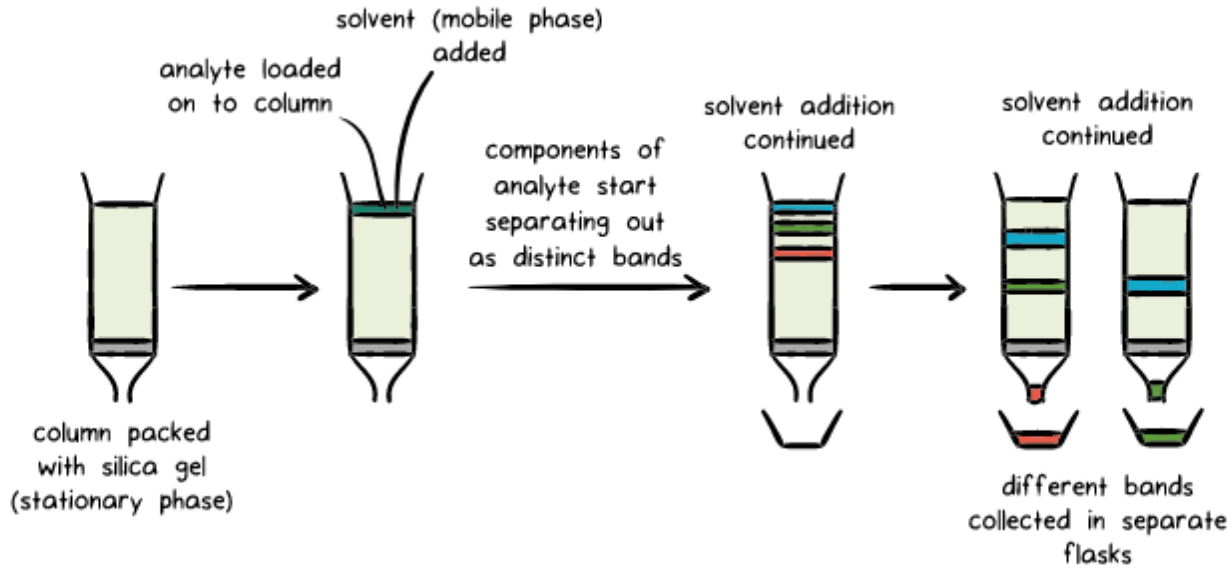
- The following is the spectrograph of an alkane from a Gas Chromatograph in conjunction with a Mass Spectrometer.



Source: Chemistry LibreTexts

B4) **<u>Liquid (or Liquid Column) Chromatography</u>**
(Very good for separating amino acids)

The most common form of this technique is that of a stationary glass column as shown below and, when the column is packed with a gel, separation takes place by adsorption.



Source: The Khan Academy (https://www.khanacademy.org/test-prep/mcat/chemical-processes/separations-purifications/a/principles-of-chromatography)

NB It is even better to have a tap at the bottom of the tube so that the flow of the mobile phase solvent can be controlled. Ceramic wool (or sintered glass) is put at the bottom of the column to stop the gel from being washed out of the column.

- A glass tube can be packed with chalk (calcium carbonate, $CaCO_3$), silica (SiO_2), or Alumina (Al_2O_3), and the tube filled with the mobile phase solvent and **there must be no air bubbles that could interfere with the flow of the mobile phase solvent**. The analyte containing the solutes is placed on top of the chalk/silica/alumina and the tap at the bottom of the column should be opened to allow the mobile phase solvent to start flowing out of the column. As the mobile phase solvent flows down through the silica/alumina, it will take the analyte down with it and the column should be continuously topped-up with fresh solvent. As the analyte flows down through the column, the solutes will start to separate through adsorption and the solutes will eventually exit the bottom of the column at different time intervals. Separation will thus have been effected.

B5) High Performance Liquid Chromatography (HPLC)

- HPLC is just a more sophisticated form of Liquid Column Chromatography where the eluent is forced through the column under pressure and where the packing material in the column (grains of Silica) is of fine particle size thus providing a much larger surface area.

- SiO_2 is polar and a non-polar eluent is used, but if the packing material in the column is non-polar, then the eluent must be polar and the process is called Reverse Phase HPLC.

- If a more sophisticated instrument is needed, then the process can be developed even further by using it in conjunction with a Spectroscopic machine (such as Mass/IR/UV/etc).

B6) Ion Exchange (IEX) Chromatography

- At 'A' Level, the normal context in which I have seen questions on this subject is with regard to water softeners where one set of ions (the ones that result in the hardness of water) are exchanged for another set (those that do not cause hardness in water). The mobile phase thus exchanges an unwanted set of ions for a desirable set of ions.

- Positively charged Calcium and Magnesium cations in Water make Water 'hard' and affect the working of soap/'fur' up electric kettles, washing machines and central heating pipes/ disrupt the moving parts of central-heating boilers/leave limescale spots on crockery/ make the washing of clothes less efficient/etc.

- In a Water-softener, negatively charged resin beads attract the positively charged Calcium, Magnesium and Sodium cations in Water, and then when Brine is flushed through the softener, the cations are flushed out of the Water-Softener.

- Water softening is a very crude/primitive form of IEX Chromatography. Extraordinarily sophisticated machines exist in Biochemistry, and these make use of the fact that different biological substances have different isoelectric points.

- There are two types of IEX viz. (a) cation-exchange and (b) anion-exchange.
  a) Cation-exchange chromatography is used when the molecule of interest is positively charged and the stationary phase is negatively charged and positively charged molecules are loaded to be attracted to it.
  b) Anion-exchange chromatography is when the stationary phase is positively charged and negatively charged molecules are loaded to be attracted to it. It is often used in protein purification, and quality control.

B7) Size Exclusion or Gel Permeation Chromatography

- This is a form of Chromatography where separation is achieved by the size of the molecules; and, contrary to expectations, **the physically LARGEST species move the fastest**. Small particles get trapped in the 'beads' more easily and hence spend a longer time in the separation process. It is used in polymer separation.

- If you go into Dentistry when you leave school, you will find that fillers for holes in teeth used to be made from a mercury amalgam, but for aesthetic purposes composite polymer-filler matrices are increasingly used because they can be made to have the same appearance as natural teeth (whereas amalgam fillers are noticeably different from ordinary teeth). If something is going to remain in your mouth for 10-15 years then it needs to be pure and non-toxic, and unfortunately the monomer (BPA) from which the resin polymer matrix (Bis-GMA) is made is moderately toxic whereas the polymer is not. For Biomedical purposes it is therefore important that non-toxic substances are separated efficiently from toxic ones.

I think that that is all that you need to know for 'A' Level purposes.