

- **CHROMATOGRAPHY**

The term "chromatography" is derived from the original use of this method for separating yellow and green plant pigments. Chromatography has since evolved into a very general separation method for many types of mixtures. Examples of the application of chromatographic methods are (i) the purification of reaction mixtures in chemical synthesis, (ii) the purification of bio-molecules such as proteins for pharmaceutical research, (iii) the analysis of complex sample mixtures such as those obtained in forensics (body fluids, paints *etc*) and (iv) the analysis of environmental samples.

Key terms:

Absorption:	Interaction of solute molecules (or atoms or ions) with the surface of the stationary phase (note that it is different from absorption where the molecules fill the pores of a solid).
Column	In column chromatography, the stationary phase (e.g. silica or alumina) is a very fine powder contained within a tube standing vertically. The solvent (mobile phase) passes through the stationary phase due to gravity or with slight pressure applied. Column chromatography is most often used for purification of up to about 1g of compounds, but it is a slow and expensive process that requires skills and patience.
Eluent:	The mobile phase (usually for solvents), often mixture of solvents.
Elution:	The motion of the mobile phase through the stationary phase
Elution time:	The time taken for a solute to pass through the system. A solute with a short elution time travels through the stationary phase rapidly, <i>i.e.</i> it elutes fast.
Mobile phase:	The part of the chromatography system that is mobile. Commonly a solvent mixture (as in column chromatography or thin layer chromatography) or a gas stream (as in gas chromatography).
Normal phase:	"Unmodified" stationary phase where POLAR solutes interact strongly and run slowly
Reverse phase:	"Modified" stationary phase where POLAR solutes run fast <i>i.e.</i> reverse order

- Resolution:** Degree of separation of different solutes. In principle, resolution can be improved by using a longer stationary phase, finer stationary phase (e.g. column packing or TLC plate coating) or slower elution.
- Stationary phase:** The part of the chromatography system that is fixed in place. Most commonly a solid e.g. the packing in column chromatography or gas chromatography or the coating on a chromatographic plate.
- R_f value:** $\frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}}$
- R_f = retardation factor. The R_f value has to be between 0 and 1 (by definition), and it is typically reported to two decimal places.
- Thin layer** Stationary phase is a thin coating on a plate of metal, glass or plastic that once prepared is stood vertically in the solvent (mobile phase). The solvent climbs up through the plate due to capillary action. The plates most often used are about the size of a microscope slide (half a credit card) but range upto about 25 cm by 25 cm.

How does chromatography work ?

Chromatography is based on selective adsorption of compounds on a solid (or liquid) with high surface area (the *stationary phase*). As the solute mixture passes over and interacts with the stationary phase, the components are adsorbed and then released from the surface of the stationary phase at differing rates. This means that the solutes are continuously partitioned between the *stationary phase* and the *mobile phase*, either a gas or a solvent mixture. The stronger the interaction of the solute with stationary phase, the slower the solute will progress. The motion of the solute and solvent through the stationary phase is called *elution*. The mobile phase is often a mixture of solvents and changing the polarity of the solvent (more or less polar) typically changes how fast a compound elutes.

Perhaps the simplest type of chromatography is paper chromatography and this can be done at home using something like a coffee filter (stationary phase) and water as the solvent (mobile phase) to investigate the inks present in water soluble markers. In paper chromatography applications, the mixture (e.g. ink) is partitioned between the paper and a solvent that moves over the paper. If each of the component inks interacts with the molecules in the paper (which are polar) differently, then the components can be separated.

A chemist might use chromatography in different ways. In column chromatography and in thin layer chromatography (TLC) a solution of the mixture to be separated flows over a solid adsorbent. Separation occurs as molecules are adsorbed and desorbed while passing over the surface. In **gas chromatography** (GC, also called vapour phase chromatography), a mixture of volatile compounds is separated by passing

the vapour over an adsorbent packing in a long very thin tube heated in a thermostatically controlled oven.

Chromatographic methods have high "resolving power", *i.e.* they are capable of sharp separations of closely related compounds, particularly when very small samples are used. Both GC and column chromatography can be carried out with instruments that detect extremely small amounts of compounds in the gas or liquid stream, as it leaves the chromatographic column. In GC, the detector responds to the thermal conductivity of the gas stream or the ionisation of the gas as it passes through a flame. This gas stream can be introduced straight into a mass spectrometer (MS) to give the very important technique of GCMS which facilitates the separation and identification of samples within a mixture. In liquid (column) chromatography instruments, the detector senses changes in the refractive index or uv-visible absorption of the solution. Signals from the detector corresponding to each component in the mixture and proportional to the amount of the compound are recorded automatically on a chart. These instruments thus provide powerful methods for quantitative analysis.

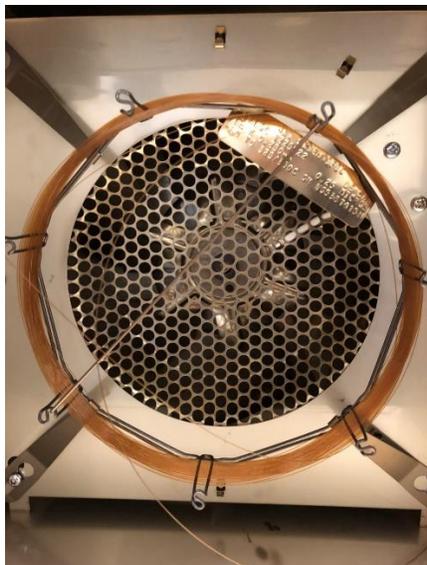
Analysis of a Mixture by Gas Chromatography

Gas chromatography (GC) is carried out using an instrument containing a long (*e.g.* 30 m) but very thin (*e.g.* 0.25 mm) tube filled with an inert support (usually silica) as the stationary phase and a stream of helium gas as the mobile phase. The coiled tube, the "column", is heated in a thermostatically controlled oven.



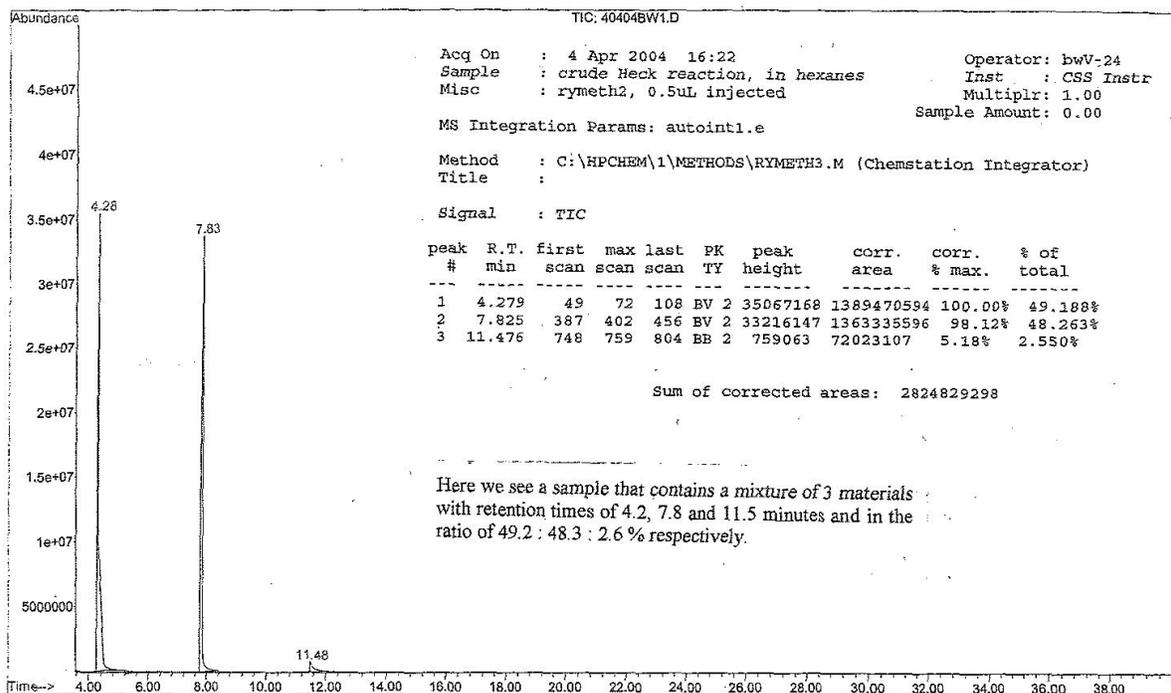
A GC in EEEL with the oven door open to show the coiled column within

A dilute solution of the liquid sample is typically prepared in a volatile solvent such as diethyl ether. A small amount of this sample solution (maybe 1 μL) is then injected onto the column and is carried forward by the helium carrier gas. The oven temperature is typically gradually increased up to about 270°C over a



A close up of the coiled column in the oven of a GC.

10 to 20 minute period. In general, the lower the boiling point of the liquid, the quicker it will be carried through the column (so it has a short retention time). When the sample exits the column, the liquid is detected by the detector and the amount of liquid is measured, this information is usually plotted as a "trace" with x-axis as time and the y-axis the response of the detector as the abundance (*i.e.* the amount detected). Therefore, the area of the peak is directly proportional to the amount of material it represents. A typical GC trace is shown below. This sample contained three compounds. Based on the peak areas we see a 49 : 48 : 3 ratio (approximately). The minor (3%) impurity (retention time 11.48 minutes) is a high boiling contaminant.

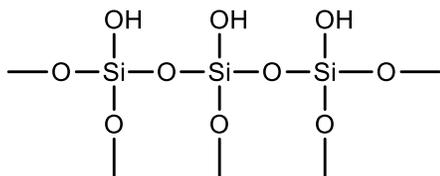


The typical results of a simple GC analysis

Analysis of a Mixture by Thin Layer Chromatography

Thin layer chromatography (often just referred to as TLC) can be used to determine the identity and purity of compounds as well as possibly determining the number of compounds in a mixture. It can also be used to monitor the progress of a reaction by observing the appearance of the product or the disappearance of a reactant as a reaction progresses. A synthetic chemist might use this to help make decisions about a synthesis they are carrying out (not that unlike a baker checking on cake in the oven to determine if it is done). TLC is very sensitive (μg scale) and often reasonably quick (5-10 mins.).

TLC is carried out on glass plates or strips of plastic or metal coated on one side with a thin layer of adsorbent. The adsorbent contains a small amount of gypsum (CaSO_4) which acts as a binder to give an adherent coating. For routine work, small TLC plates can be prepared by dipping microscope slides in a slurry of the adsorbent in chloroform. More uniform plates are obtained by mixing the adsorbent with an equal weight of water, spreading the mixture on a glass plate and allowing it to set dry. Precoated TLC plates are commercially available with various adsorbents in very uniform layers. The most common adsorbent is silica gel.



Silica gel is a 3D network of silicon oxygen bonds with polar $-\text{OH}$ groups on the outside surface. The image on the right shows a scoop of silica for column chromatography)

(https://en.wikipedia.org/wiki/Column_chromatography#/media/File:Feinpulvriges_Silicat.jpg)

There are three stages to running a TLC plate:

1. Plate preparation
2. Development
3. Visualisation

A series of photographs are provided at the end of this document to show the steps in running a TLC plate.

To "load" the plate, very small samples of the sample mixture in some volatile solvent (e.g. diethyl ether or chloroform) are applied as spots near one end and the volatile application solvent is allowed to evaporate. The plate is then placed, sample end down, in a closed vessel containing a shallow pool of

the "developing solvent". The solvent rises on the plate by capillary action, passing over the sample and causing the compounds to move at varying rates depending on their relative affinities for the stationary adsorbent and the solvent. When the solvent front has risen to just below the top of the plate, the plate is removed, the solvent front marked and the solvent is allowed to evaporate. The zones or spots containing the various components of the mixture are then detected at various points along the plate. If the compounds are colourless, they are made visible by treating the plate with a reagent, such as iodine vapour, that causes colour to develop.

Identification by TLC

The main uses of TLC are for quick observation of the number of compounds present in a sample, and for qualitative detection of a given compound in a mixture. For the latter purpose, a sample of the compound in question is placed in one position at the bottom of the plate and a sample of the mixture is placed in an adjacent position. With a plate 3 to 4 cm wide, it is possible to place samples in as many as five or six "lanes". If identical compounds are present in two or more lanes, they should appear at the same height after the plate has been developed with solvent. The position of the spot relative to the solvent front, called the R_f value (distance travelled by spot / distance travelled by solvent), depends on the thickness of the coating, the amount of sample, the temperature, the solvent mixture and may vary from one plate to the next. Comparison of two samples on the same plate is therefore essential.

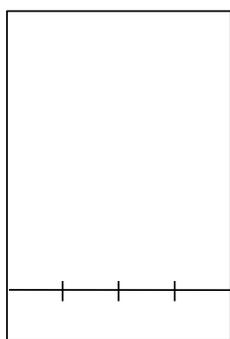
In addition to the position of the known sample and that of a spot in the mixture, the appearance and / or colour of the compound on the developed plate may greatly strengthen the identification if it is distinctive after visualisation. If there is a reagent specific for the compound of interest, this is sprayed in a fine mist over the surface. A general reagent such as iodine will cause brown or black spots with practically all compounds; these may be slightly different shades, but the colour is usually not very characteristic. Another method for visualising spots is illumination of the plate with an ultraviolet lamp. Many substances, particularly aromatic compounds, will show a bright fluorescence, which may have a characteristic colour. A further possibility is use of an adsorbent layer that contains a trace of fluorescent dye. Compounds that are fluorescent still show up as bright spots on a light background; any others appear as a dark spot since they quench the fluorescence of the background dye.

Preparing to run TLC

Capillaries or TLC applicators for applying the samples to the chromatographic plate can be prepared by heating and drawing out a soft glass pipette or melting point or capillary tubes. Soften a 1 cm section in the centre of the glass tube by heating in a low Bunsen burner flame, then remove the tube from the flame and draw it out to a thread-like thickness. Allow the glass to cool then carefully break the glass in the thin portion gives you the capillaries.



The **developing jar** can be prepared from a beaker or jar with watch glass as a lid. The developing jar is usually lined with a piece of filter paper (cut to shape and size) which helps create a solvent saturated environment. The solvent system is added to the developing jar which is then carefully turned at an angle to saturate the filter paper. The solvent depth should be less than 5mm (typically 2-5 mm).

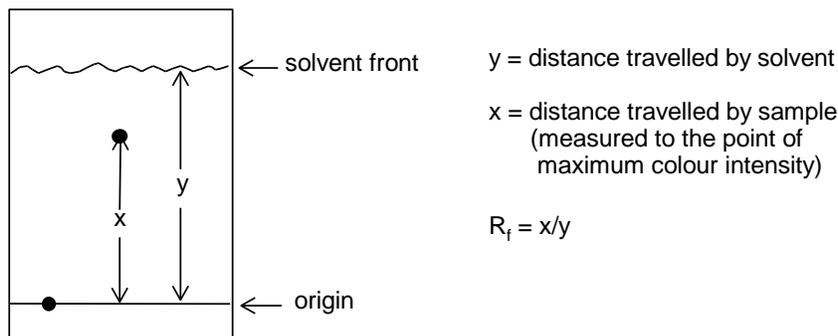


TLC plates or sheets should be handled by the edges, and you should avoid touching the coated surface otherwise fingerprints will ruin the experiment. Samples of the test solutions should be spotted on the coated side about 1 cm from one end of the sheet, and about 0.5 cm apart, with the outer two spots about 0.5 cm from the edge of the sheet. It is common practice to draw a light pencil line about 1cm up from the bottom edge of the plate (this line defines the origin) and then divide this line at about 5mm intervals to create the lanes.

Samples of unknowns are typically spotted in the middle lanes of the plate. It is important to avoid applying too large an amount of sample. The spot after application should be about 1 to 2 mm in diameter. *It is a good idea first to practice applying spots to a small scrap of the sheet. You can check to see how well you are doing by examining the spots under the UV lamp. If the spots are too pale at this stage, then you will need more applications to the plate, or you should concentrate your samples.*

To apply the samples, touch the end of a capillary tube to the solution and then touch this gently to the plate at the proper place. When the samples have been applied, place the sheet, spotted end (origin) down, in a developing jar containing a pool of solvent no more than 5 mm deep. On no account must spots dip below the surface of the solvent pool when the sheet is placed in the developing jar. The solvent system used depends on the nature of the samples. Many experimental procedures will define the solvent system used, but at times a chemist will need to develop their own system. It is important to handle the plates without touching the front surface of the silica covered plate or fingerprints will result and the plate may be spoiled. Use clean forceps for transferring the plates in and out of the developing and visualising jars. Once the plate is in place, cap the jar securely and develop the chromatogram until the solvent has risen to within 1 or 2 cm of the top of the sheet. When the solvent front is close to the top of the sheet remove the sheet from the jar, and mark the solvent front with a small scratch (using a

spatula or forceps) or a pencil mark. Recap the developing jar and out the TLC sheet to one side and it allow it to dry horizontally. In some cases the spots on the plate will be visible to the naked eye. In other cases the plate will need to be visualised using other methods such as a UV lamp or with a stain such as iodine. It is a good idea to draw circles around the spots with a pencil as a permanent record of the visualisation. The R_f value (retardation factor) for a spot can now be calculated and recorded along with the colour (if any) of the spot.



When looking at a developed TLC plate it is good idea to review the “quality” of the analysis or note if there are any problems with the plate that means it might need to be rerun. A poor quality plate would mean that it is difficult to obtain the desired information from the plate. Some common problems are described below:

Problem

Samples does not show up

Possible causes

Not enough sample, different visualisation method required

Streaking

This could be due to the nature of the sample, due to sample overload (too much applied to the plate) or a poor choice of solvent mixture.

Diffuse / blurred spots

The origin line was below the solvent level or the plate was developed for too long so the solvent front reached the top of the plate.

Sample “lanes” are not straight / parallel

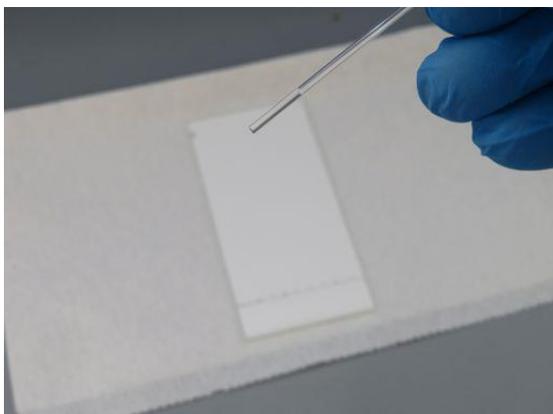
A problem with the way the solvent ran due to the plate being crooked in the developing jar, touching the filter paper in the developing jar or the plate was chipped.



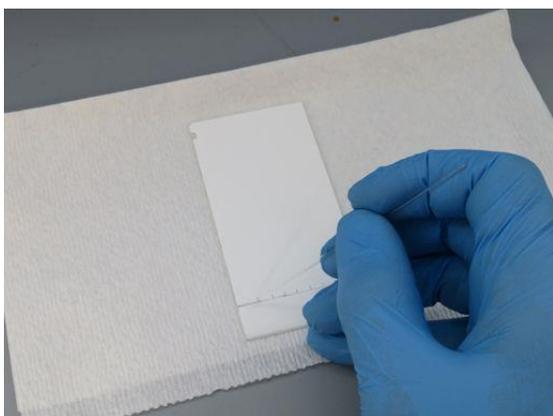
Preparing a sample to use on TLC by dissolving a small amount of the sample in a small vial



Adding the volatile solvent to dissolve the sample



A glass capillary tube is used as a micro pipette



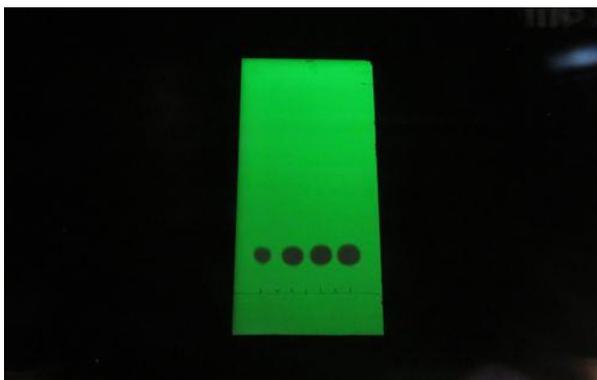
Spotting the sample onto the TLC plate with a microcapillary



Handling the TLC plate with forceps in order to place it in the developing tank.



With the lid on, the TLC plate is developed by allowing the solvent to run up the plate. Note that the solvent level is below the pencil line on the plate that marks the origin (i.e. the line where the samples were applied).



After development, the TLC plate is removed and visualised. Here we see it under a UV lamp. In this example, all the spots were the same sample, and they only moved a short distance.