CHROMATOGRAPHY

THIN LAYER CHROMATOGRAPHY

TLC is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate).

A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analyzed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action.

As the solvent moves past the spot that was applied, an equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized. If the compounds are colored, visualization is straightforward. Usually the compounds are not colored, so a UV lamp is used to visualize the plates. (The plate itself contains a fluorescent dye which glows everywhere *except* where an organic compound is on the plate.)

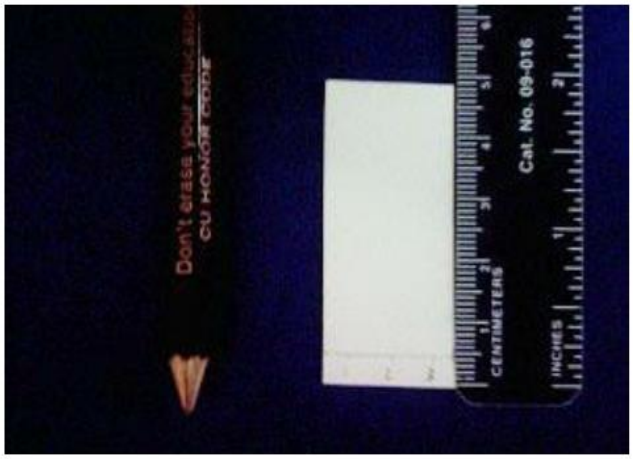
How To Run a TLC Plate

Step 1: Prepare the developing container

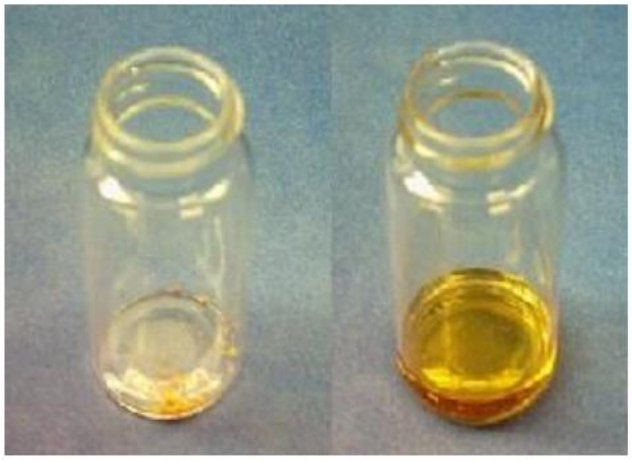
The developing container for TLC can be a specially designed chamber, a jar with a lid, or a beaker with a watch glass on the top (the latter is used in the undergrad labs at CU). Pour solvent into the chamber to a depth of just less than 0.5 cm. To aid in the saturation of the TLC chamber with solvent vapors, you can line part of the inside of the beaker with filter paper. Cover the beaker with a watch glass, swirl it gently, and allow it to stand while you prepare your TLC plate.

Step 2: Prepare the TLC plate

TLC plates used in the organic chem teaching labs are purchased as 5 cm x 20 cm sheets. Each large sheet is cut horizontally into plates which are 5 cm tall by various widths; the more samples you plan to run on a plate, the wider it needs to be. Shown in the photo to the left is a box of TLC plates, a large un-cut TLC sheet, and a small TLC plate which has been cut to a convenient size. Handle the plates carefully so that you do not disturb the coating of adsorbent or get them dirty.



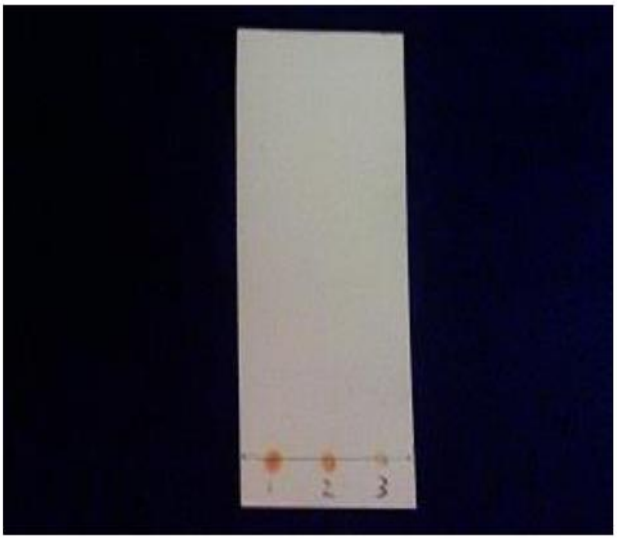
Measure 0.5 cm from the bottom of the plate. Using a pencil, draw a line across the plate at the 0.5 cm mark. This is the*origin*: the line on which you will spot the plate. Take care not to press so hard with the pencil that you disturb the adsorbent. Under the line, mark lightly the name of the samples you will spot on the plate, or mark numbers for time points. Leave enough space between the samples so that they do not run together; about 4 samples on a 5 cm wide plate is advised.



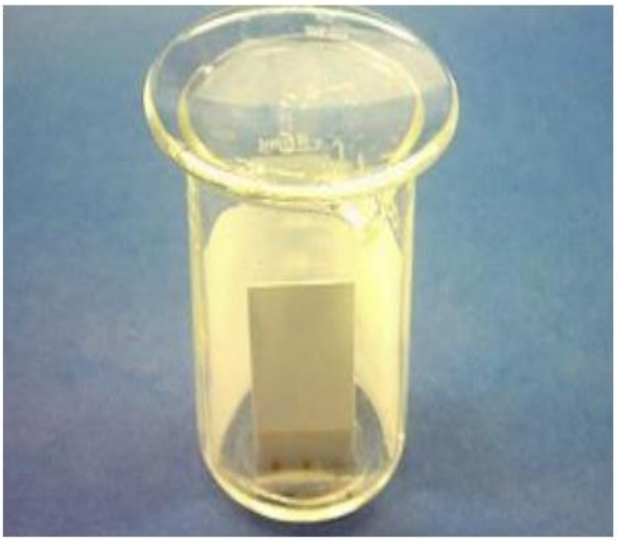
Step 3: Spot the TLC plate

If the sample is not already in solution, dissolve about 1 mg in 1 mL of a volatile solvent such as hexanes, ethyl acetate, or methylene chloride. As a rule of thumb, a concentration of 1% usually works well for TLC analysis. If the sample is too concentrated, it will run as a smear or streak (see troubleshooting section below); if it is not concentrated enough, you will see nothing on the plate. Sometimes you will need to use trial and error to get well-sized, easy to read spots.

Obtain a a microcapillary. In the organic teaching labs, we use 10µL microcaps - they are easier to handle than the smaller ones used in research labs. Dip the microcap into the solution and then **gently** touch the end of it onto the proper location on the TLC plate. Don't allow the spot to become too large - if necessary, you can touch it to the plate, lift it off and blow on the spot. If you repeat these steps, the wet area on the plate will stay small.

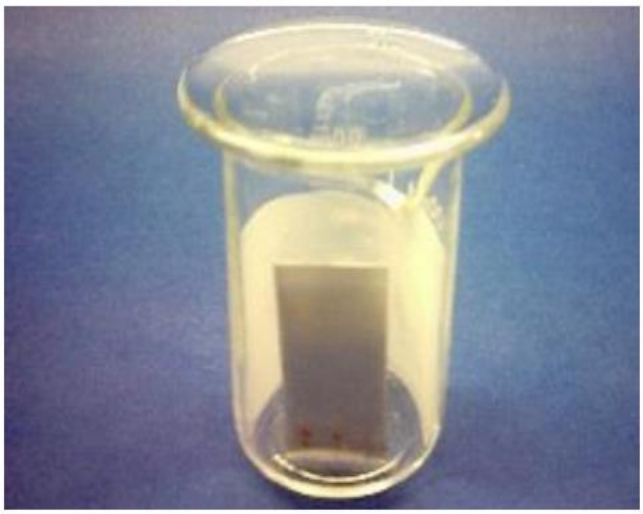


This example plate has been spotted with three different quantities of the same solution and is ready to develop. If you are unsure of how much sample to spot, you can always spot multiple quantities and see which looks best.

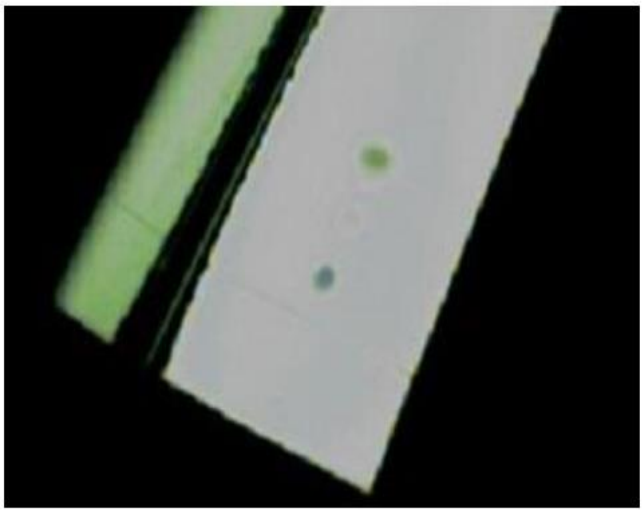


Step 4: Develop the plate

Place the prepared TLC plate in the developing beaker, cover the beaker with the watch glass, and leave it undisturbed on your bench top. The solvent will rise up the TLC plate by capillary action. Make sure the solvent does not cover the spot.

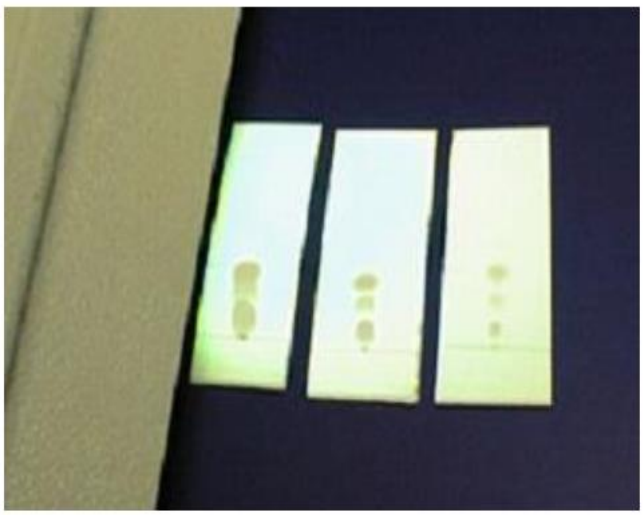


Allow the plate to develop until the solvent is about half a centimeter below the top of the plate. Remove the plate from the beaker and immediately mark the solvent front with a pencil. Allow the plate to dry.

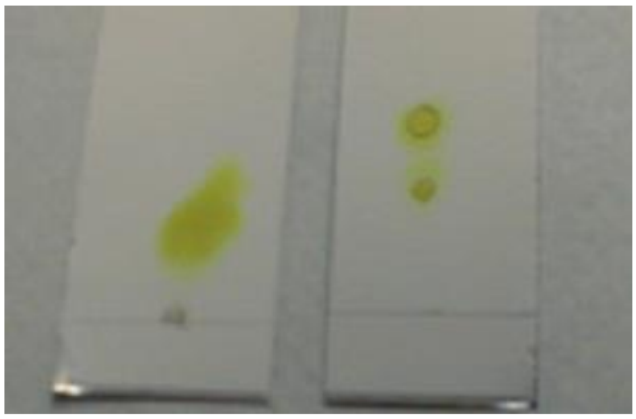


Step 5: Visualize the spots

If there are any colored spots, circle them lightly with a pencil. Most samples are not colored and need to be visualized with a UV lamp. Hold a UV lamp over the plate and circle any spots you see. Beware! UV light is damaging both to your eyes and to your skin! Make sure you are wearing your goggles and do not look directly into the lamp. Protect your skin by wearing gloves.



If the TLC plate runs samples which are too concentrated, the spots will be streaked and/or run together. If this happens, you will have to start over with a more dilute sample to spot and run on a TLC plate.



Here's what overloaded plates look like compared to well-spotted plates. The plate on the left has a large yellow smear; this smear contains the same two compounds which are nicely resolved on the plate next to it.

TLC Solvents Choice

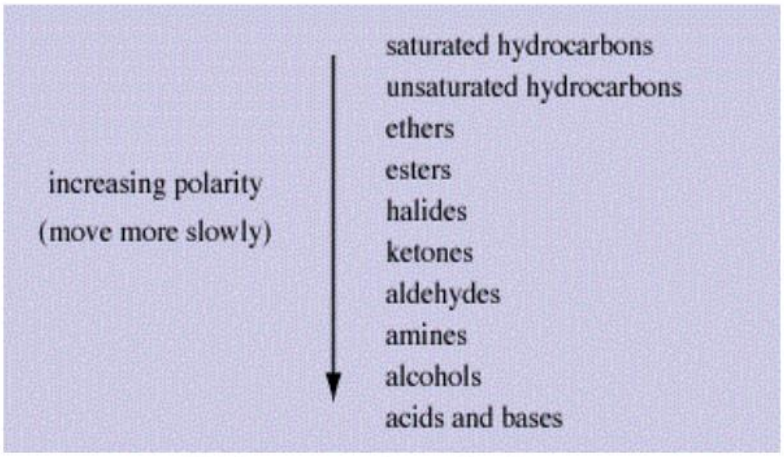
When you need to determine the best solvent or mixture of solvents (a "solvent system") to develop a TLC plate or chromatography column loaded with an unknown mixture, vary the polarity of the solvent in several trial runs: a process of trial and error. Carefully observe and record the results of the chromatography in each solvent system. You will find that as you increase the polarity of the solvent system, all the components of the mixture move faster (and vice versa with lowering the polarity). The ideal solvent system is simply the system that gives the best separation.

TLC elution patterns usually carry over to column chromatography elution patterns. Since TLC is a much faster procedure than column chromatography, TLC is often used to determine the best solvent system for column chromatography. For instance, in determining the solvent system for a flash chromatography procedure, the ideal system is the one that moves the desired component of the mixture to a TLC R_f of 0.25-0.35 and will separate this component from its nearest neighbor by difference in TLC R_f values of at least 0.20. Therefore a mixture is analyzed by TLC to determine the ideal solvent(s) for a flash chromatography procedure.

Beginners often do not know where to start: What solvents should they pull off the shelf to use to elute a TLC plate? Because of toxicity, cost, and flammability concerns, the common solvents are hexanes (or petroleum ethers/ligroin) and ethyl acetate (an ester). Diethyl ether can be used, but it is very flammable and volatile. Alcohols (methanol, ethanol) can be used. Acetic acid (a carboxylic acid) can be used, usually as a small percentage component of the system, since it is corrosive, non-volatile, very polar, and has irritating vapors. Acetone (a ketone) can be used. Methylene chloride or and chloroform (halogenated hydrocarbons) are good solvents, but are toxic and should be avoided whenever possible. If two solvents are equal in performance and toxicity, the more volatile solvent is preferred in chromatography because it will be easier to remove from the desired compound after isolation from a column chromatography procedure.

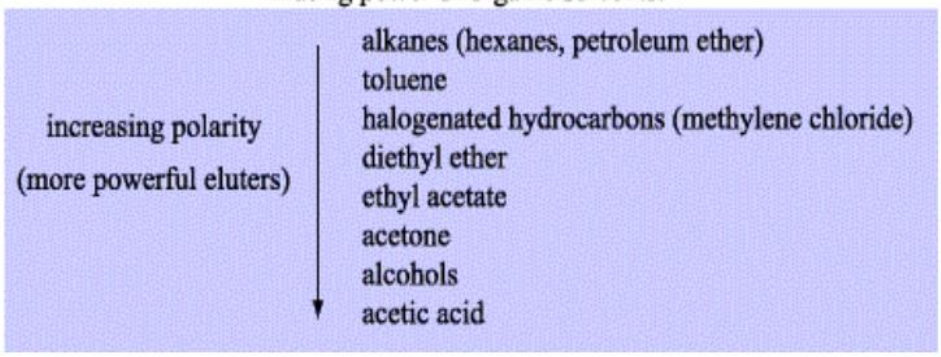
Ask the lab instructor what solvents are available and advisable. Then, mix a non-polar solvent (hexanes, a mixture of 6-carbon alkanes) with a polar solvent (ethyl acetate or acetone) in varying percent combinations to make solvent systems of greater and lesser polarity. The charts below should help you in your solvent selection. You can also download this pdf chart of elution

The expected elution order of organic classes.



order.

Eluting power of organic solvents.



Interactions Between the Compound and the Adsorbent

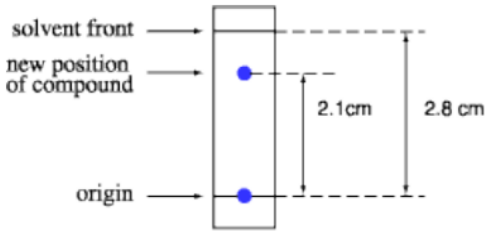
The strength with which an organic compound binds to an adsorbent depends on the strength of the following types of interactions: ion-dipole, dipole-dipole, hydrogen bonding, dipole induced dipole, and van der Waals forces. With silica gel, the dominant interactive forces between the adsorbent and the materials to be separated are of the dipole-dipole type. Highly polar molecules interact fairly strongly with the polar SiOH groups at the surface of these adsorbents, and will tend to stick or adsorb onto the fine particles of the adsorbent while weakly polar molecules are held less tightly. Weakly polar molecules generally tend to move through the adsorbent more rapidly than the polar species. Roughly, the compounds follow the elution order given above.

The R_f value

The retention factor, or R_f, is defined as the distance traveled by the compound divided by the distance traveled by the solvent.

$$R_f = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

For example, if a compound travels 2.1 cm and the solvent front travels 2.8 cm, the R_f is 0.75:



$$R_f = \frac{2.1}{2.8} = 0.75$$

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

Since these factors are difficult to keep constant from experiment to experiment, relative R_f values are generally considered. "Relative R_f" means that the values are reported relative to a standard, or it means that you compare the R_f values of compounds run on the same plate at the same time.

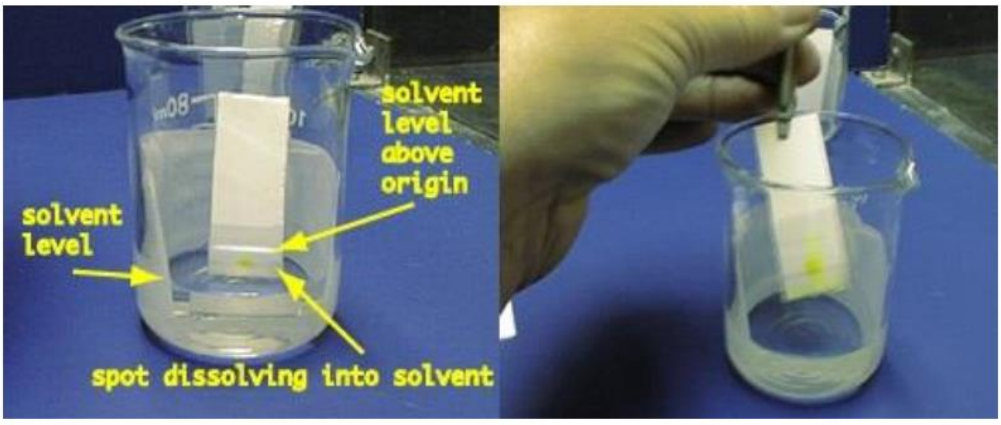
The larger an R_f of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger R_f is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. Conversely, if you know the structures of the compounds in a mixture, you can predict that a compound of low polarity will have a larger R_f value than a polar compound run on the same plate.

The R_f can provide corroborative evidence as to the identity of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top of each other) with the compound in question. If two substances have the same R_f value, they are likely (but not necessarily) the same compound. If they have different R_f values, they are definitely different compounds. Note that this identity check must be performed on a single plate, because it is difficult to duplicate all the factors which influence R_f exactly from experiment to experiment.

Troubleshooting TLC

All of the above (including the procedure page) might sound like TLC is quite an easy procedure. But what about the first time you run a TLC, and see spots everywhere and blurred, streaked spots? As with any technique, with practice you get better. Examples of common problems encountered in TLC:

- **The compound runs as a streak rather than a spot:** The sample was overloaded. Run the TLC again after diluting your sample. Or, your sample might just contain many components, creating many spots which run together and appear as a streak. Perhaps, the experiment did not go as well as expected.
- **The sample runs as a smear or a upward crescent:** Compounds which possess strongly acidic or basic groups (amines or carboxylic acids) sometimes show up on a TLC plate with this behavior. Add a few drops of ammonium hydroxide (amines) or acetic acid (carboxylic acids) to the eluting solvent to obtain clearer plates.
- **The sample runs as a downward crescent:** Likely, the adsorbent was disturbed during the spotting, causing the crescent shape.
- **The plate solvent front runs crookedly:** Either the adsorbent has flaked off the sides of the plate or the sides of the plate are touching the sides of the container (or the paper used to saturate the container) as the plate develops. Crooked plates make it harder to measure R_f values accurately.
- **Many random spots are seen on the plate:** Make sure that you do not accidentally drop any organic compound on the plate. If get a TLC plate and leave it laying on your workbench as you do the experiment, you might drop or splash an organic compound on the plate.
- **You see a blur of blue spots on the plate as it develops:** Perhaps you used an ink pen instead of a pencil to mark the origin?
- **No spots are seen on the plate:** You might not have spotted enough compound, perhaps because the solution of the compound is too dilute. Try concentrating the solution, or spot it several times in one place, allowing the solvent to dry between applications. Some compounds do not show up under UV light; try another method of visualizing the plate (such as staining or exposing to iodine vapor). Or, perhaps you do not have any compound because your experiment did not go as well as planned. If the solvent level in the developing jar is deeper than the origin (spotting line) of the TLC plate, the solvent will dissolve the compounds into the solvent reservoir instead of allowing them to move up the plate by capillary action. Thus, you will not see spots after the plate is developed. These photos show how the yellow compound is running into the solvent when lifted from the developing jar.



COLUMN CHROMATOGRAPHY

Principals

Ion Exchange Chromatography relies on charge-charge interactions between the proteins in your sample and the charges immobilized on the resin of your choice. Ion exchange chromatography can be subdivided into cation exchange chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, in which the binding ions are negative, and the immobilized functional group is positive. Once the solutes are bound, the column is washed to equilibrate it in your starting buffer, which should be of low ionic strength, then the bound molecules are eluted off using a gradient of a second buffer which steadily increases the ionic strength of the eluent solution. Alternatively, the pH of the eluent buffer can be modified as to give your protein or the matrix a charge at which they will not interact and your molecule of interest elutes from the resin. If you know the pH you want to run at and need to decide what type of ion exchange to use paste your protein sequence into the **titration curve generator**. If it is negatively charged at the pH you wish, use an anion exchanger; if it is positive, use a cation exchanger. Of course this means that your protein will be binding under the conditions you choose. In many cases it may be more advantageous to actually select conditions at which your protein will flow through while the contaminants will bind. This mode of binding is often referred to as "flow through mode". This is a particularly good mode to use in the case of anion exchange. Here one could use this type of mode to bind up endotoxins or other highly negatively charged substances well at the same time relatively simply flowing your protein through the matrix.

Considerations

Anion Exchange Chromatography (AEC)

The surface charge of the solutes (proteins, nucleic acids, endotoxin) which bind will be net negative, thus to get binding of a specific protein one should be above the pI of that protein. Commonly used anion exchange resins are Q-resin, a Quaternary amine; and DEAE resin, DiEthylAminoEthane (see figure below). AEC is often used as a primary chromatography

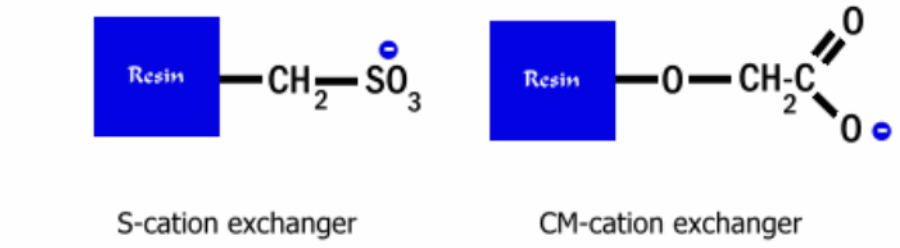
Q-anion exchanger DEAE-anion exchanger

step due to its high capacity, (Matrices can bind from 10 to 100 mg of protein per ml) and ability to bind up and separate fragmented nucleic acids and lipopolysaccharides from the initial slurry. Typically, AEC is performed using buffers at pH's between 7 and 10 and running a gradient from a solution containing just this buffer to a solution containing this

buffer with 1M NaCl. The salt in the solution competes for binding to the immobilized matrix and releases the protein from its bound state at a given concentration. Proteins separate because the amount of salt needed to compete varies with the external charge of the protein. Uses of AEC include initial clean up of a crude slurry, separation of proteins from each other, concentrating a protein, and the removal of negatively charged endotoxin from protein preparations.

Cation Exchange Chromatography (CEC)

The surface charge of the solutes (proteins, nucleic acids, endotoxin) which bind will be net positive, thus to get binding of a specific protein one should be below the pI of that protein. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions (see figure below).



S-cation exchanger CM-cation exchanger

CEC is less commonly used compared to AEC, largely due to the fact that often proteins do not stick to this resin at physiological pHs and one is reluctant to titrate a protein through its isoelectric point to get it to adhere to the resin. Nonetheless, it is as powerful as AEC for initial separations with equivalently high capacity. Typically, CEC is performed using buffers at pH's between 4 and 7 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1M NaCl. Uses of CEC include initial clean up of a crude slurry, separation of proteins from each other, concentrating a protein, and as a common first purification step for proteins expressed under acidic conditions such as in *P. pastoris*.

Buffers and Buffer Systems

1. Buffers for anion exchange chromatography*

Molecule	**pKa**	**dpKa/degree C.**	**Counter ion**
N-methyl piperazine	4.75	-0.015	chloride
piperazine	5.68	-0.015	chloride or formate
L-histidine	5.96		chloride
bis-Tris	6.46	-0.017	chloride

bis-Tris propane	6.80		chloride
triethanolamine	7.76	-0.020	chloride or acetate
Tris	8.06	-0.028	chloride
N-methyl-diethanolamine	8.52	-0.028	chloride
diethanolamine	8.88	-0.025	chloride
1,3-diaminopropane	8.64	-0.031	chloride
ethanolamine	9.50	-0.029	chloride
piperazine	9.73	-0.026	chloride
1,3-diaminopropane	10.47	-0.026	chloride
piperidine	11.12	-0.031	chloride
phosphate	12.33	-0.026	chloride

***These values were taken from the Pharmacia biotech "Ion exchange chromatography, principles and methods" guidebook.**

2. Buffers for cation exchange chromatography:*

Molecule	**pKa**	**dpKa/degree C.**	**Counter ion**
Maleic acid	2.00		sodium
Malonic acid	2.88		sodium
citric acid	3.13	-0.0024	sodium
lactic acid	3.81		sodium
formic acid	3.75	0.0002	sodium or lithium
butaneandioic acid	4.21	-0.0018	sodium
acetic acid	4.76	0.0002	sodium or lithium
malonic acid	5.68		sodium or lithium
phosphate	7.20	-0.0028	sodium
HEPES	7.55	-0.0140	sodium or lithium

BICINE	8.35 -0.0180	sodium

*These values were taken from the Pharmacia biotech "Ion exchange chromatography, principles and methods" guidebook.

Buffer system 1
Buffer A = 20 mM Tris, pH=8.0
Buffer B = 20 mM Tris, 1 M NaCl, pH=8.0
Equilibrate column in buffer A, cycle once through buffer B. Bind protein dialyzed against buffer A. Elute with a linear gradient of A to 100% B.

Buffer system 2 (Common CEC buffer system)
Buffer A = 30 mM sodium acetate, pH=4.5
Buffer B = 30 mM sodium acetate, 1 M NaCl, pH=4.5
Treat as above.

Buffer system 3 (AEC for proteins which are very insoluble or have a very high pI)
Buffer A = 30 mM Ethanolamine, 8M urea, pH=10.0
Buffer B = 30 mM Ethanolamine, 8M urea, 1 M NaCl, pH=10.0
Treat as above

GEL FILTRATION CHROMATOGRAPHY

Gel filtration chromatography, a type of size exclusion chromatography, can be used to either fractionate molecules and complexes in a sample into fractions with a particular size range, to remove all molecules larger than a particular size from the sample, or a combination of both operations. Gel filtration chromatography can be used to separate compounds such as small molecules, proteins, protein complexes, polysaccharides, and nucleic acids when in aqueous solution. When an organic solvent is used as the mobile phase, the process is instead referred to as gel permeation chromatography.

Gel filtration chromatography can also be used for:

- Fractionation of molecules and complexes within a predetermined size range
- Size analysis and determination
- Removal of large proteins and complexes
- Buffer exchange
- Desalting
- Removal of small molecules such as nucleotides, primers, dyes, and contaminants
- Assessment of sample purity
- Separation of bound from unbound radioisotopes

Gel filtration chromatography media for all of the above uses are available in prepacked gravity flow columns, spin columns, low-pressure and medium-pressure chromatography columns, and bottled resins.

Gel Filtration Chromatography Mechanism

In a gel filtration chromatography column, the stationary phase is composed of a porous matrix, and the mobile phase is the buffer that flows in between the matrix beads. The beads have a defined pore size range, known as the fractionation range. Molecules and complexes that are too large to enter the pores stay in the mobile phase and move through the column with the flow of the buffer. Smaller molecules and complexes that are able to move into the pores enter the

stationary phase and move through the gel filtration column by a longer path through pores of the

beads.

Any molecule or complex that is above the fractionation range for a particular gel filtration chromatography column will move through the column faster than any molecule that can enter the stationary phase. Therefore, any constituent in the sample that is above the fractionation range will elute first (in the void volume) before anything that is in the fractionation range. The minimum size that will remain in the mobile phase and not enter the stationary phase is known as the exclusion limit. Bio-Rad offers gel filtration chromatography media and columns with exclusion limits ranging over three orders of magnitude, from 100 daltons to 100,000 daltons (100 kDa).

Molecules and complexes that can enter the stationary phase will be fractionated according to their sizes. Smaller molecules will migrate deep into the pores and will be retarded more than larger molecules that do not so easily enter the pores, and are thus eluted from the column more quickly. This difference in pore migration leads to fractionation of components by size with the largest eluting first.

In gel filtration chromatography columns designed for desalting, buffer exchange, and the removal of small molecules such as nucleotides, the salts and small compounds readily enter the pores, are retarded, and migrate more slowly through the column than the larger proteins or nucleic acids. Therefore, the components of interest in the sample are eluted in advance of salts, nucleotides, etc. DNA cleanup kits using this mechanism often contain gel filtration spin columns.

Resolution, here defined as the sharpness of the boundaries between size fractions, is determined by bead size and a number of other factors. Smaller bead size generally yields higher resolution in a gel filtration chromatography column. Compact molecules diffuse through the stationary phase faster than linear molecules. Size exclusion, fractionation range, and elution rate are

affected by buffer composition, ionic strength, and pH. For the fractionation of complex mixtures of proteins, elution times and size exclusion limits may need to be determined empirically.

Gel Filtration Chromatography Media

An important criterion for gel filtration chromatography media is that media is inert and that nothing in the sample or any buffer binds to the media. Another consideration is the type of gel filtration column being used and whether it is used in a pressurized chromatography system or gravity flow or spin columns. If a pressurized chromatography system is being used, both the column and the media must be able to tolerate the pressure and flow rates used.

Commonly used media for gel filtration chromatography are based on agarose or polyacrylamide beads, dextrose for gravity or low-pressure systems, and polymeric resins for medium-pressure systems. The choice of media depends on the properties of the components to be separated and other experimental factors. The following are general considerations when determining the choice of gel filtration chromatography media:

- Fractionation range
- Size exclusion limit
- Operating pressure
- Flow rate
- Sample viscosity
- pH range
- Autoclavability
- Tolerance for water-miscible organic solvents; some samples may be more soluble in a water-organic mix
- Tolerance for detergents, chaotropic agents, formamide, etc.
- Operating temperature

The types of samples, choice of media, and the chromatography system setup will determine which parameters are the most important for a given purification application.

AFFINITY CHROMATOGRPHY

Affinity chromotography, also known as bioselective adsorption, is a protein purification technique developed thirty years ago. The fundamental principal composing the method is the isolation of a particular protein by chromatography on a bioligand that has been immobilized on an inert matrix. It relies on the specificity of a protein binding site for a particular ligand. For this precise coupling to take place a number of variable conditions must be addressed first.

Sample Before any attention can be paid to the actual technique, a protein sample is needed**.** Once the desired protein's tissue location has been determined, cells comprising this tissue must be isolated. Once done, a crude cellular extract is performed and all endogenous substrates are removed. The sample is now ready tfor the column.

Matrix A sound matrix is an essential part of affinity chromatography. A matrix, in its use here, is a substance, usually in bead form, to which a specific ligand is covalently bound. In order for the matrix to be affective it must have certain characteristics: 1) it must be insoluble in solvents and buffers employed in the process; 2) it must be chemically and mechanically stable; 3) it must be easily coupled to a ligand or spacer arm onto which the ligand can be attached; 4) it must exhibit good flow properties and have a relativley large surface area for attachment. It is common for matrices to be made out of agarose, glass, cellulose, or a duel composition polyacrylimide based compound. (Scouten 20)

Solvents The primary buffer in affinity chromatography is the one in which the matrix resides. This buffer should not degrade the matrix in any way. The buffer should also have a negliable effect on the sample. The ideal buffer minimizes nonspecific interactions while maximizing the specific interaction between the sample and the ligand.

The other major solvent to consider in affinity chromatography is the elution buffer. The purpose of the elution buffer is to wash away unbound proteins initially and at higher concentration release the desired protein from the ligand. Salt solutions of various concentrations as well as buffers containing specifc analogs for the sample can be used. It is important that the elution buffer work quickly and to not change the function or activity of the desired protein.

Spacer arms and coupling methods Since the success of affinity chromatography resides in its ability to bind an active site to its corresponding ligand, if the protein binding region cannot join with the immobilized ligand the technique is effectively useless. Spacer arms, though not always necessary, can improve binding probability. Spacer arms distance the ligand from the matrix reducing steric hinderance which can occur when the ligand is bound directly to the bead. Spacer arms should neither chemically or structually affect the sample or the ligand. (Scouten 38)

When coupling the spacer arm or ligand to the matrix, multipoint or single point attachment may be used. Single point attachment offers high ligand flexabilty and easier ligand access to the sample's active site. Though single point coupling does provide better site recognition, it is not nearly as strong as a multipoint attachment. Multipoint coupling is stronger than single point attachment and will thus show less degradation. Unfortunately it can impede binding between the ligand and the sample.

After the ligand has been bound to the matrix it is important as a final step to block all unreacted groups of the matrix. This provides a higher degree of certainty that all binding will be between the sample and the ligand. (Deutscher 363)

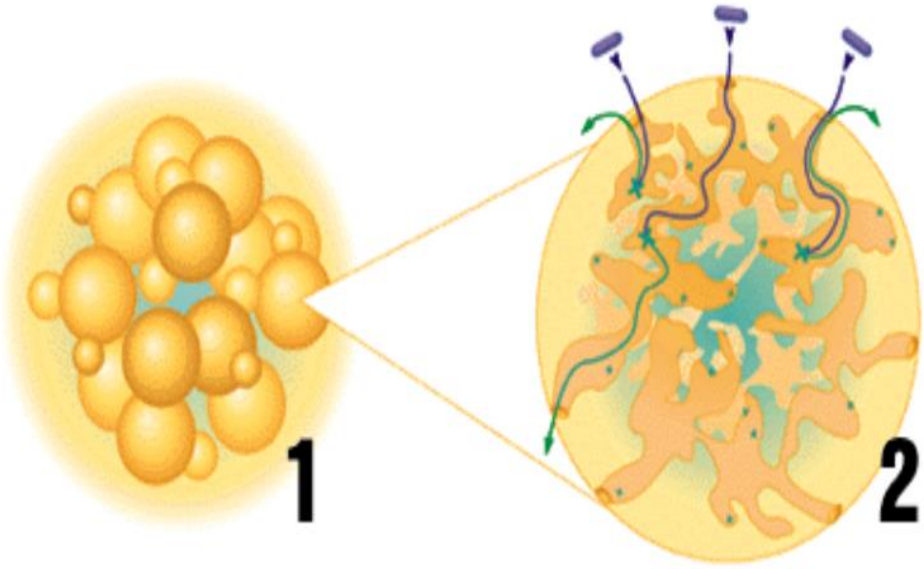


Fig1. This is a depiction of a matrix bead showing ligand and protein binding. The X's represent a ligand and the pill-shaped images represent a protein. The image comes from the Bio-Rad homepage.

Pouring the column Once the ligand has been immobilized, the column packed, and the sample prepared the sample can finally be poured. Since ligand binding is based on hydrogen bonding, site/ligand motifs and other noncovalent interaction, a certain degree of care must be used to make binding as opportune as possible. Variations in flow rate of the sample, and of the wash and elution buffers can exhibit monumental effects on the success of affinity chromatography. If the sample is poured too quickly proper binding may not take place. If, when pouring the wash, the flow rate is too high the bound protein may release as well. And finally, though the elution may be at a higher flow rate, if it exceeds the rate used to pack the original matrix the entire complex may come apart. (Deutscher 365)

Just as impotant as flow rate is the concentration and acidity of the buffers used. As mentioned above, buffers should in no way chemically or structually interfere with ligand binding. In the elution phase it is necessary for buffer to seperate the ligand from the sample, but prior to that buffers should remain inocuous because of the sensitive ligand/sample interaction.

Fig 2. This is a depiction of a column used in affinity chromatography. The image comes from the Bio-Rad hompage.

What do now After elution, a protein, specific to the ligand, has been isolated. To determine protein purity and activity SDS-PAGE or immunoelectrophoresis can be used.

Applications Affinity chromatography has a large range of protein purfying applications. For example, the control of transcription factors to activate DNA transcription makes them a extremely important to understanding gene expression. Using a a specific DNA sequence as a ligand, these proteins can be isolated via affinity chromatography. Another major group is enzymes. Enzymes can be isolated by a host a different ligands fit for bioselective adsoprtion. For example, adenosine monophosphate (AMP) can be immobilized and used to bind those proteins exhibiting an affinity for AMP, ADP, or ATP. Extra cellular and other receptor proteins can also be purified by affinity chromatography. For example, determination of whether a particular hormone has recpetor in a specific tissue group can be accomplished by using the hormone in question as the immobilized ligand. These examples are only a small sample of the vast applications of affinity chromatography. (Ngo 4-9)

Affinity chromotography is a powerful technique in that it allows protein purifiaction in a relatively short amount of time with a high yield. It simplifies the isolation process by using the preexisting ligand binding relationships already in an organism.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - HPLC

Introduction

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster.

It also allows you to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

The other major improvement over column chromatography concerns the detection methods which can be used. These methods are highly automated and extremely sensitive.

The column and the solvent

Confusingly, there are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase.

Normal phase HPLC

This is essentially just the same as you will already have read about in thin layer chromatography or column chromatography. Although it is described as "normal", it isn't the most commonly used form of HPLC.

The column is filled with tiny silica particles, and the solvent is non-polar - hexane, for example. A typical column has an internal diameter of 4.6 mm (and may be less than that), and a length of 150 to 250 mm.

Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to

make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol.

In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column. There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent.

Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules, for example. They therefore spend less time in solution in the solvent and this will slow them down on their way through the column.

That means that now it is the polar molecules that will travel through the column more quickly.

Reversed phase HPLC is the most commonly used form of HPLC.

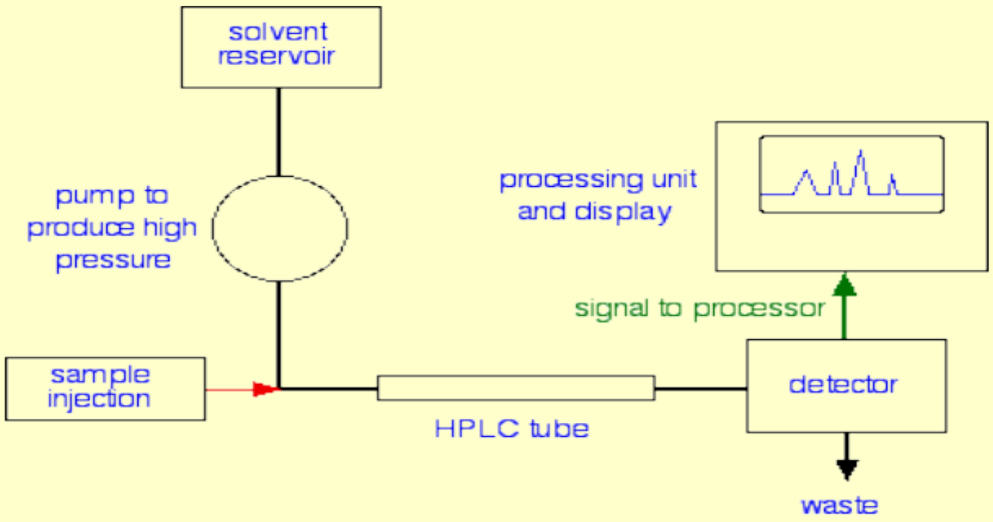
Note: I have been a bit careful about how I have described the attractions of the non-polar molecules to the surface of the stationary phase. In particular, I have avoided the use of the word "adsorpion". Adsorption is when a molecule sticks to the surface of a solid. Especially if you had small molecules in your mixture, some could get in between the long C_{18} chains to give what is essentially a solution.

You could therefore say that non-polar molecules were more soluble in the hydrocarbon on the surface of the silica than they are in the polar solvent - and so spend more time in this alternative "solvent". Where a solute divides itself between two different solvents because it is more soluble in one than the other, we call it ***partition***.

So is this adsorption or partition? You could argue it both ways! Be prepared to find it described as either.

Looking at the whole process

A flow scheme for HPLC



Injection of the sample

Injection of the sample is entirely automated, and you wouldn't be expected to know how this is done at this introductory level. Because of the pressures involved, it is *not* the same as in gas chromatography (if you have already studied that).

Retention time

The time taken for a particular compound to travel through the column to the detector is known as its ***retention time***. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. For a particular compound, the retention time will vary depending on:

- the pressure used (because that affects the flow rate of the solvent)
- the nature of the stationary phase (not only what material it is made of, but also particle size)
- the exact composition of the solvent
- the temperature of the column

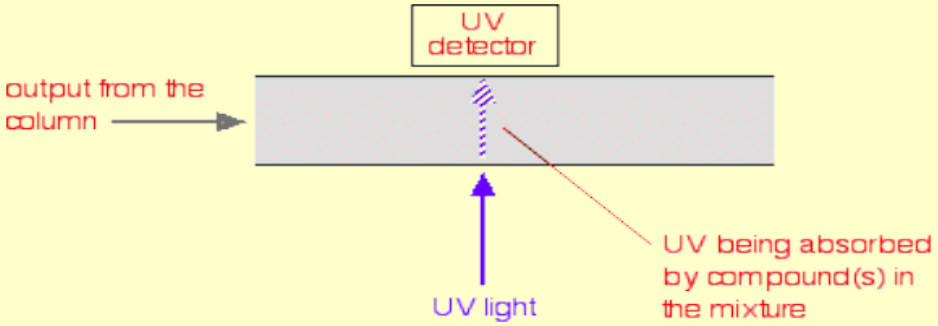
That means that conditions have to be carefully controlled if you are using retention times as a way of identifying compounds.

The detector

There are several ways of detecting when a substance has passed through the column. A common method which is easy to explain uses ultra-violet absorption.

Many organic compounds absorb UV light of various wavelengths. If you have a beam of UV light shining through the stream of liquid coming out of the column, and a UV detector on the opposite side of the stream, you can get a direct reading of how much of the light is absorbed.

The amount of light absorbed will depend on the amount of a particular compound that is passing through the beam at the time.



You might wonder why the solvents used don't absorb UV light. They do! But different compounds absorb most strongly in different parts of the UV spectrum.

Methanol, for example, absorbs at wavelengths below 205 nm, and water below 190 nm. If you were using a methanol-water mixture as the solvent, you would therefore have to use a wavelength greater than 205 nm to avoid false readings from the solvent.

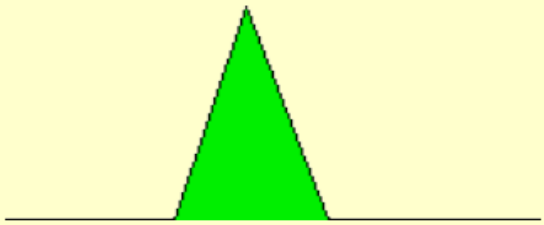
Interpreting the output from the detector

The output will be recorded as a series of peaks - each one representing a compound in the mixture passing through the detector and absorbing UV light. As long as you were careful to control the conditions on the column, you could use the retention times to help to identify the compounds present - provided, of course, that you (or somebody else) had already measured them for pure samples of the various compounds under those identical conditions.

But you can also use the peaks as a way of measuring the quantities of the compounds present. Let's suppose that you are interested in a particular compound, X.

If you injected a solution containing a known amount of pure X into the machine, not only could you record its retention time, but you could also relate the amount of X to the peak that was formed.

The area under the peak is proportional to the amount of X which has passed the detector, and this area can be calculated automatically by the computer linked to the display. The area it would measure is shown in green in the (very simplified) diagram.



If the solution of X was less concentrated, the area under the peak would be less - although the retention time will still be the same. For example:

This means that it is possible to calibrate the machine so that it can be used to find how much of a substance is present - even in very small quantities.

Be careful, though! If you had two different substances in the mixture (X and Y) could you say anything about their relative amounts? *Not if you were using UV absorption as your detection method.*

In the diagram, the area under the peak for Y is less than that for X. That may be because there is less Y than X, but it could equally well be because Y absorbs UV light at the wavelength you are using less than X does. There might be large quantities of Y present, but if it only absorbed weakly, it would only give a small peak.

Note: If you want lots more detail about HPLC you could explore the site operated by Waters Corporation - a supplier of HPLC equipment.

You will also find a useful industry training video which talks through the whole process by following this link.

Linking to other sites is always a little bit hazardous because sites change. If you find that either of these links don't work, please contact me via the address on the About this sitepage.

Coupling HPLC to a mass spectrometer

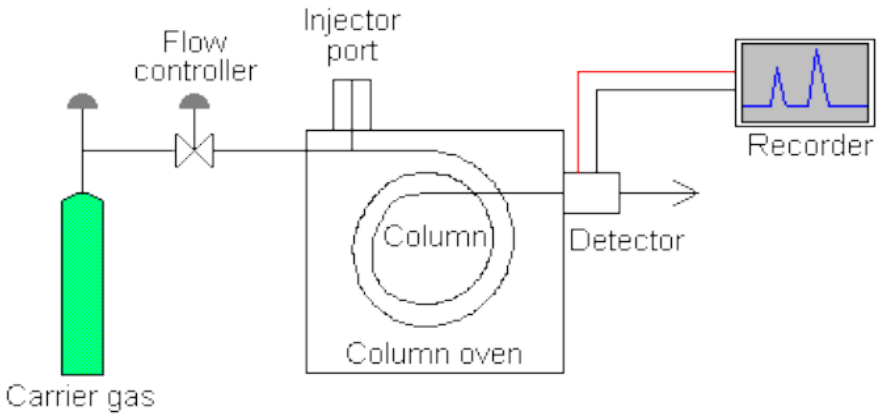
This is where it gets really clever! When the detector is showing a peak, some of what is passing through the detector at that time can be diverted to a mass spectrometer. There it will give a fragmentation pattern which can be compared against a computer database of known patterns. That means that the identity of a huge range of compounds can be found without having to know their retention times.

GAS CHROMATOGRAPHY

Introduction

Gas chromatography - specifically gas-liquid chromatography - involves a sample being vapourised and injected onto the head of the chromatographic column. The sample is transported through the column by the flow of inert, gaseous mobile phase. The column itself contains a liquid stationary phase which is adsorbed onto the surface of an inert solid.

Have a look at this schematic diagram of a gas chromatograph:



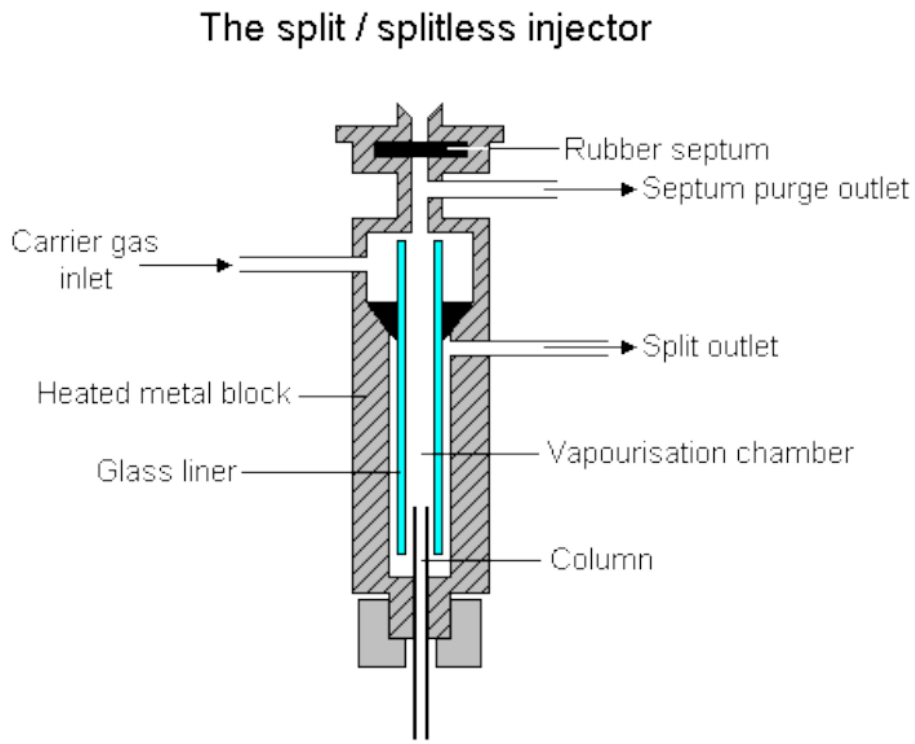
Instrumental components

Carrier gas

The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependant upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities.

Sample injection port

For optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapour - slow injection of large samples causes band broadening and loss of resolution. The most common injection method is where a microsyringe is used to inject sample through a rubber septum into a flash vapouriser port at the head of the column. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters. Capillary columns, on the other hand, need much less sample, typically around 10^{-3} mL. For capillary GC, split/splitless injection is used. Have a look at this diagram of a split/splitless injector;



The injector can be used in one of two modes; split or splitless. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum. The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vapourises to form a mixture of carrier gas, vapourised solvent and vapourised solutes. A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.

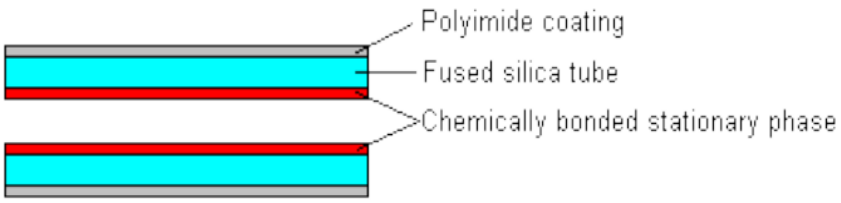
Columns

There are two general types of column, *packed* and *capillary* (also known as *open tubular*). Packed columns contain a finely divided, inert, solid support material (commonly based on *diatomaceous earth*) coated with liquid stationary phase. Most packed columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm.

Capillary columns have an internal diameter of a few tenths of a millimeter. They can be one of two types; *wall-coated open tubular* (WCOT) or *support-coated open tubular* (SCOT). Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase. In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the stationary phase has been adsorbed. SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns.

In 1979, a new type of WCOT column was devised - the *Fused Silica Open Tubular* (FSOT) column;

Cross section of a Fused Silica Open Tubular Column



These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating. These columns are flexible and can be wound into coils. They have the advantages of physical strength, flexibility and low reactivity.

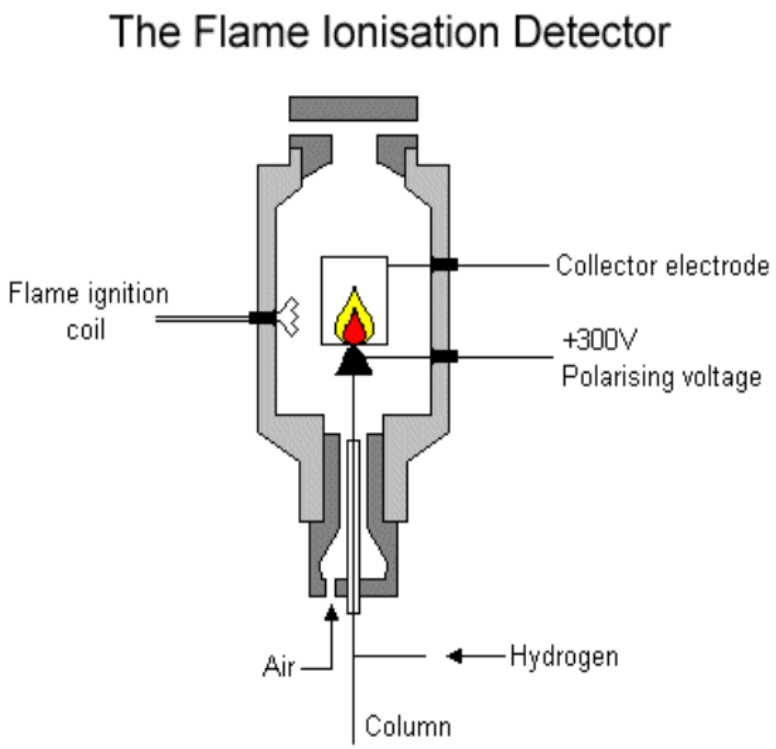
Column temperature

For precise work, column temperature must be controlled to within tenths of a degree. The optimum column temperature is dependant upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes. Minimal temperatures give good resolution, but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful. The column temperature is increased (either continuously or in steps) as separation proceeds.

Detectors

There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A *non-selective* detector responds to all compounds except the carrier gas, a*selective detector* responds to a range of compounds with a common physical or chemical property and a *specific detector* responds to a single chemical compound. Detectors can also be grouped into*concentration dependant detectors* and *mass flow dependant detectors*. The signal from a concentration dependant detector is related to the concentration of solute in the detector, and does not usually destroy the sample Dilution of with make-up gas will lower the detectors response. Mass flow dependant detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependant detector is unaffected by make-up gas. Have a look at this tabular summary of common GC detectors:

Detector	Type	Support gases	Selectivity	Detectability	Dynamic range
Flame ionization (FID)	Mass flow	Hydrogen and air	Most organic cpds.	100 pg	10^7
Thermal conductivity (TCD)	Concentration	Reference	Universal	1 ng	10^7
Electron capture (ECD)	Concentration	Make-up	Halides, nitrates, nitriles, peroxides, anhydrides, organometallics	50 fg	10^5
Nitrogen-phosphorus	Mass flow	Hydrogen and air	Nitrogen, phosphorus	10 pg	10^6
Flame photometric (FPD)	Mass flow	Hydrogen and air possibly oxygen	Sulphur, phosphorus, tin, boron, arsenic, germanium, selenium, chromium	100 pg	10^3
Photo-ionization (PID)	Concentration	Make-up	Aliphatics, aromatics, ketones, esters, aldehydes, amines, heterocyclics, organosulphurs, some organometallics	2 pg	10^7
Hall electrolytic conductivity	Mass flow	Hydrogen, oxygen	Halide, nitrogen, nitrosamine, sulphur		



The effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons which can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. FIDs are mass sensitive rather than concentration sensitive; this gives the advantage that changes in mobile phase flow rate do not affect the detector's response. The FID is a useful general detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise. It is also robust and easy to use, but unfortunately, it destroys the sample.