Chromatography

اعداد

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Thin Layer Chromatography.

Thin layer chromatography, or TLC, is a method for analyzing mixtures by separating the compounds in the mixture. TLC can be used to help determine the number of components in a mixture, the identity of compounds, and the purity of a compound. By observing the appearance of a product or the disappearance of a reactant, it can also be used to monitor the progress of a reaction. TLC is a sensitive technique - microgram (0.000001 g) quantities can be analyzed by TLC - and it takes little time for an analysis (about 5-10 minutes). TLC consists of three steps - spotting, development, and visualization. Photographs of each step are shown on the course website. First the sample to be analyzed is dissolved in a volatile (easily evaporated) solvent to produce a very dilute (about 1%) solution. Spotting consists of using a micro pipet to transfer a small amount of this dilute solution to one end

of a TLC plate, in this case a thin layer of powdered silica gel that has been coated onto a plastic sheet. The spotting solvent quickly evaporates and leaves behind a small spot of the material. Development consists of placing the bottom of the TLC plate into a shallow pool of a development solvent, which then travels up the plate by capillary action. As the solvent travels up the plate, it moves over the original spot. A competition is set up between the silica gel plate and the development solvent for the spotted material. The very polar silica gel tries to hold the spot in its original place and the solvent tries to move the spot along with it as it travels up the plate. The outcome depends upon a balance among three polarities - that of the plate, the development solvent and the spot material. If the development solvent is polar enough, the spot will move some distance from its original location. Different components in the original spot, having different polarities, will move different distances from the original spot location and show up as separate spots. When the solvent has traveled almost to the top of the plate, the

plate is removed, the solvent front marked with a pencil, and the solvent allowed to evaporate. Visualization of colored compounds is simple – the spots can be directly observed after development. Because most compounds are colorless however, a visualization method is needed. The silica gel on the TLC plate is impregnated with a fluorescent material that glows under ultraviolet (UV) light. A spot will interfere with the fluorescence and appear as a dark spot on a glowing background. While under the UV light, the spots can be outlined with a pencil to mark their locations. A second method of visualization is accomplished by placing the plate into iodine vapors for a few minutes. Most organic compounds will form a dark-colored complex with iodine. It is good practice to use at least two visualization techniques in case a compound does not show up with one particular method. The Rf value is used to quantify the movement of the materials along the plate. Rf is equal to the distance traveled by the substance divided by the distance traveled by the solvent. Its value is always between zero and one. A TLC analysis

might be summarized something like, "Using a silica gel plate and ethyl acetate as the development solvent, unknown mixture X showed three spots having Rf's of 0.12, 0.25, and 0.87". Comparing these Rf's with the Rf's of known compounds might enable a tentative identification to be made. Note that observing three spots means only that there are at least three components in the mixture. Some components may have such similar polarities that they appear under one spot after development. 2 Rf = Y/X (always ≤ 1) If a development solvent of too high a polarity is used, all components in the mixture will move along with the solvent and no separation will be observed (Rf's will be too large). If the solvent is of too low a polarity the components will not move enough, and again separation will not occur (Rf's will be too small). In practice, different solvents or mixtures of solvents are tried until a good separation is observed. Typically an effective solvent is one that gives Rf's in the range of 0.3 - 0.7. Note that the spotting solvent is simply used as a vehicle to transfer the material to be analyzed to the TLC plate. Once the transfer

is made the spotting solvent evaporates. It has no effect on the separation. It is the development solvent that effects the separation. What's going on at the molecular level during development? There are three components in TLC: (1) the TLC plate (stationary phase), the development solvent (mobile phase), and the sample to be analyzed (solute). In our experiment the TLC plate consists of a thin plastic sheet covered with a thin layer of silica gel, a portion of the structure of which is shown below. Si O OH O Si OH O O Si OH O O Si O O Si Si O Silica gel consists of a three-dimensional network of thousands of alternating silicon and oxygen bonds, with O-H groups on the outside surface. Silica gel is simply very finely ground very pure sand. It should be noted that silica gel is highly polar and is capable of hydrogen bonding. Consider the side-on view of the development of a TLC plate below. As the solvent travels up the plate, over the spot, an equilibrium is set up, as development solvent competes with the TLC plate for the solute. The silica gel binds to the solute and the development solvent tries to dissolve it away, carrying the

solute(s) along as the solvent travels up the plate. 3 A balance of intermolecular forces determines the position of equilibrium and thus the ability of the solvent to move the solute up the plate. In other words, would the spot prefer to be stuck on the plate or would it prefer to move along with solvent. The balance depends upon (1) the polarity of the TLC plate (constant and high), (2) the polarity of the development solvent (can be varied by using different solvents), and (3) the polarity of the compounds in the spot (this varies depending upon what compounds are in the spot). For example, if a sample consists of two components, one more polar than the other, the more polar will tend to stick more tightly to the plate and the less polar will tend to move along more freely with the solvent. Using a more polar development solvent would cause both to move along further. If the approximate structures of the solutes are known, it is possible to make an educated guess as to what solvent or mixture of solvents to use. In practice though, for a given mixture of compounds to be analyzed, a solvent or

mixture of solvents is chosen by trial and error to give the best separation. (A caveat: the polarity argument is helpful in understanding the principles of TLC. Because most compounds have some polarity the argument works well. For compounds having very low polarity however, a lower-polarity solvent may be more effective in moving the solute up the plate.) To illustrate a TLC experiment, consider the following example of the analysis of a two-component mixture. 4 The polarity of molecules, solutes and solvents alike, is ordered as follows, from least to most polar: Alkanes (least polar), alkyl halides, alkenes, aromatic hydrocarbons, ethers, esters, ketones, aldehydes, amines, alcohols, and carboxylic acids (most polar). Note however that many molecules contain multiple functional groups and that the overall polarity would be determined by all of the groups. Experimental Procedure. (rev 1/08) This experiment consists of two parts: (1) analyzing two different analgesics to determine the number of compounds in each and the identity of those compounds and (2) studying the effect of solvent polarity on Rf. Look

over the photos showing TLC on the course website. (1.) TLC analysis of analgesics. Spotting. The powdered side of the silica gel plate, not the shiny, plastic side is the side that is spotted. Lightly draw a pencil line about 1 cm from the end of a plate. Use a pencil, not a pen, and be careful to not scratch the surface of the plate too deeply. The plates are of such a size that only three spots can be run on each. Use a fresh pipet for each spot. Make each spot as small as possible (less than about 1 mm diameter). After applying the spots check them under the UV lamp to see that they are of a reasonable size. The UV light must be held close to the plate to see the spots (CAUTION: do not look directly into the UV light.) If the spot is much too large, prepare a new plate. If a spot is too small, add more solution. Analyze two unknown analgesics in the following way: two of the three known compounds (references) plus the first analgesic to be analyzed (unknown 1) will be spotted on one plate, the same two knowns plus the second analgesic (unknown 2) will be spotted on a second plate, and both analgesic 1 and analgesic 2 along with the

third known compound will be spotted on a third plate (see Figure below). (If the plates were 5 wider, you could analyze each unknown on just one plate.) To obtain a sample of a known solution, bring a micropipet to the sample solution at the side bench, dip it into the solution to fill it, then take it back to your workspace for spotting (Use only micropipets for obtaining samples; do not use Pasteur pipets to take larger amounts of samples – that could lead to contamination). Try to minimize traffic at the side bench and take care to not contaminate the solutions with a micropipet that has already been used. The purpose of the experiment is to determine which of the known compounds are contained in the analgesics that you chose to analyze. Solutions of the known compounds will already be made. You must make 1% solutions of the unknown analgesics. To do this, take a small part of a crushed tablet and add enough ethanol to make an approximately 1% solution (very approximate - do not weigh samples). About 1 mg in several drops of solvent will produce an approximately 1% solution. The analgesic contains

insoluble binders so not all of it will dissolve. Development. The development chamber is a small screw-capped jar with a 5.5 cm filter paper placed into it to ensure that the atmosphere is saturated with vapor. Each run is relatively brief so please use only one ethyl acetate development chamber. Otherwise there may not be enough for everyone. Place about 2 mL of ethyl acetate into the chamber (2 mL is a full squeeze of the bulb using a Pasteur pipet). Place the TLC plate into the jar with the spotted side at the bottom. Keep the cap on the jar at all times except when placing the plate into or taking the plate out of the jar. Watch out for the following: the solvent must be below the level of the spots or the spotted material will dissolve in the development solvent; the plate must stand vertically in the tank and the silica gel must not touch the filter paper; the solvent must not be allowed to run all of the way to the top of the plate; the correct amount of material must be spotted (checking under UV light before development may help - if spots are too large, another plate can be made - if the spots are too

small, more material can be spotted). The development takes little time so if a plate comes out poorly, another can easily be done. After the solvent has almost reached the top of the plate, the plate is removed and the position of the solvent front marked before the solvent evaporates. The plate is then allowed to dry in the hood. Always allow the developing solvent to run almost completely to the top of the plate. This will use the entire plate and allow for the best possible separation. Visualization. This is done by two methods. First, by short wavelenth UV light. (CAUTION: do not look directly into the light.) The light must be held close to the plate to see the the spots. Some spots may be very faint. The observed spots should be outlined with a pencil. The plate is then placed into a jar containing iodine crystals for a few minutes. Keep the iodine jar capped and in the fume hood. The spots may appear different from those shown with the UV light. They should also be circled. A comparison of the two visualization techniques can be made and may help in 6 identifying the unknown spots. The iodine method must be done after the UV method

because the compound may react with the iodine, possibly changing the results. Calculate the Rf values for the known compounds and for all components of the analgesic. From your results, identify which components are present in a particular analgesic. Some of the components may have very similar Rf's under these conditions. However, their spots may look different upon treatment with iodine and this may allow a distinction to be made. Note that caffeine is present in small amounts and may not show up well. Purposely overloading a spot may help to show a component present in small amounts. You may choose from the following analgesics: Anacin, Extra Strength Excedrin, Extra Strength Tylenol, and CVS Super Strength Pain Reliever. You may also analyze your personally-preferred legal analgesic if you wish. Just bring a tablet to lab. (2.) Solvent Effect on Rf Values. Three known compounds, anthracene, benzil, and triphenylmethanol, will be developed in two different solvents. Prepare two identical plates, spotted with each of those compounds, and develop one in ethyl acetate and the other in a

mixture of 95% hexane and 5% t-butyl methyl ether (you may use a second chamber for this solvent mixture). Calculate Rf values, and in the post-lab write-up, discuss the effect of solvent on Rf, keeping in mind the structures and polarities of the compounds and solvents. O O OH Anthracene Benzil Triphenylmethanol SAFETY: Carefully note the warning about glass slivers above. Do not stare into the UV lamp. BEFORE YOU LEAVE THE LAB: turn off any electrical equipment that you may have used, put away your equipment and lock your drawer, clean up your work areas, close the fume hood sash completely, and ask your TA for her or his signature. In general, please try to keep the lab in as good condition as you found it. If you see caps off of bottles, replace the caps. If you see spilled chemicals, clean them up or at least report it to your TA. WASTE: Never dispose of glass waste in the regular trash. The custodian could become injured. Dispose of all glass waste in the cardboard "Glass Only" boxes. Used capillary pipets may be placed into those boxes also or into the dishes placed around the lab for that purpose. Dispose of

all solutions and developing solvents in the ORGANIC LIQUID WASTE container in the hood. When finished, pour as much of the solvent as possible into the waste container and leave the screwcapped jars open in the hood so they will dry. Leave the filter paper in the jars. 7 P

PAPER CHROMATOGRAPHY

This page is an introduction to paper chromatography - including two way chromatography.

Carrying out paper chromatography

Background

Chromatography is used to separate mixtures of substances into their components. All forms of chromatography work on the same principle.

They all have a *stationary phase* (a solid, or a liquid

supported on a solid) and a *mobile phase* (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates. We'll look at the reasons for this further down the page.

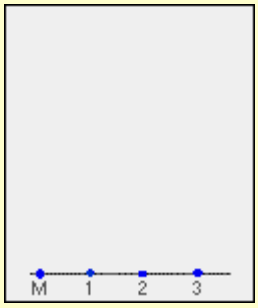
In paper chromatography, the stationary phase is a very uniform absorbent paper. The mobile phase is a suitable liquid solvent or mixture of solvents.

Producing a paper chromatogram

You probably used paper chromatography as one of the first things you ever did in chemistry to separate out mixtures of coloured dyes - for example, the dyes which make up a particular ink. That's an easy example to take, so let's start from there.

Suppose you have three blue pens and you want to find out which one was used to write a message. Samples of each ink are spotted on to a pencil line drawn on a sheet of chromatography paper. Some of the ink from the

message is dissolved in the minimum possible amount of a suitable solvent, and that is also spotted onto the same line. In the diagram, the pens are labelled 1, 2 and 3, and the message ink as M.

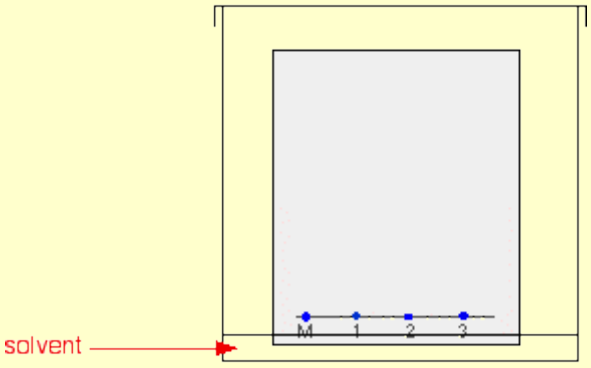


Note: The chromatography paper will in fact be pure white - not pale grey. I'm forced to show it as off-white because of the way I construct the diagrams. Anything I draw as pure white allows the background colour of the page to show through.

The paper is suspended in a container with a shallow

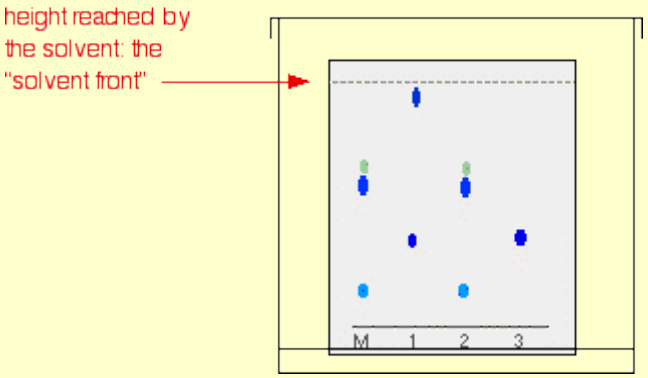
layer of a suitable solvent or mixture of solvents in it. It is important that the solvent level is below the line with the spots on it. The next diagram doesn't show details of how the paper is suspended because there are too many possible ways of doing it and it clutters the diagram. Sometimes the paper is just coiled into a loose cylinder and fastened with paper clips top and bottom. The cylinder then just stands in the bottom of the container.

The reason for covering the container is to make sure that the atmosphere in the beaker is saturated with solvent vapour. Saturating the atmosphere in the beaker with vapour stops the solvent from evaporating as it rises up the paper.



As the solvent slowly travels up the paper, the different components of the ink mixtures travel at different rates and the mixtures are separated into different coloured spots.

The diagram shows what the plate might look like after the solvent has moved almost to the top.



It is fairly easy to see from the final chromatogram that the pen that wrote the message contained the same dyes as pen 2. You can also see that pen 1 contains a mixture of two different blue dyes - one of which *might* be the

same as the single dye in pen 3.

R_f values

Some compounds in a mixture travel almost as far as the solvent does; some stay much closer to the base line. The distance travelled relative to the solvent is a constant for a particular compound as long as you keep everything else constant - the type of paper and the exact composition of the solvent, for example.

The distance travelled relative to the solvent is called the R_f value. For each compound it can be worked out using the formula:

$$R_f = \frac{\text{distance travelled by compound}}{\text{distance travelled by solvent}}$$

For example, if one component of a mixture travelled 9.6 cm from the base line while the solvent had travelled 12.0

cm, then the R_f value for that component is:

$$R_f = \frac{9.6}{12.0} = 0.80$$

In the example we looked at with the various pens, it wasn't necessary to measure R_f values because you are making a direct comparison just by looking at the chromatogram.

You are making the assumption that if you have two spots in the final chromatogram which are the same colour and have travelled the same distance up the paper, they are most likely the same compound. It isn't necessarily true of course - you could have two similarly coloured compounds with very similar R_f values. We'll look at how you can get around that problem further down the page.

What if the substances you are interested in are

colourless?

In some cases, it may be possible to make the spots visible by reacting them with something which produces a coloured product. A good example of this is in chromatograms produced from amino acid mixtures.

Suppose you had a mixture of amino acids and wanted to find out which particular amino acids the mixture contained. For simplicity we'll assume that you know the mixture can only possibly contain five of the common amino acids.

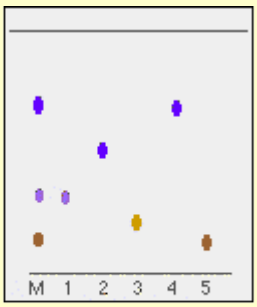
A small drop of a solution of the mixture is placed on the base line of the paper, and similar small spots of the known amino acids are placed alongside it. The paper is then stood in a suitable solvent and left to develop as before. In the diagram, the mixture is M, and the known amino acids are labelled 1 to 5.

The position of the solvent front is marked in pencil and the chromatogram is allowed to dry and is then sprayed with a solution of *ninhydrin*. Ninhydrin reacts with amino

acids to give coloured compounds, mainly brown or purple.

The left-hand diagram shows the paper after the solvent front has almost reached the top. The spots are still invisible. The second diagram shows what it might look like after spraying with ninhydrin.

before spraying with ninhydrin



after spraying with ninhydrin

There is no need to measure the R_f values because you can easily compare the spots in the mixture with those of the known amino acids - both from their positions and their colours.

In this example, the mixture contains the amino acids

labelled as 1, 4 and 5.

And what if the mixture contained amino acids other than the ones we have used for comparison? There would be spots in the mixture which didn't match those from the known amino acids. You would have to re-run the experiment using other amino acids for comparison.

Two way paper chromatography

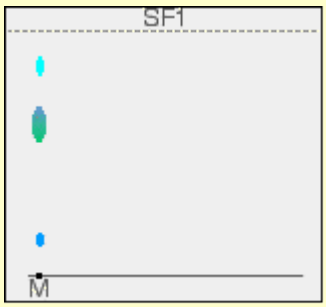
Two way paper chromatography gets around the problem of separating out substances which have very similar R_f values.

I'm going to go back to talking about coloured compounds because it is much easier to see what is happening. You can perfectly well do this with colourless compounds - but you have to use quite a lot of imagination in the explanation of what is going on!

This time a chromatogram is made starting from a single spot of mixture placed towards one end of the base line.

It is stood in a solvent as before and left until the solvent front gets close to the top of the paper.

In the diagram, the position of the solvent front is marked in pencil before the paper dries out. This is labelled as SF1 - the solvent front for the first solvent. We shall be using two different solvents.



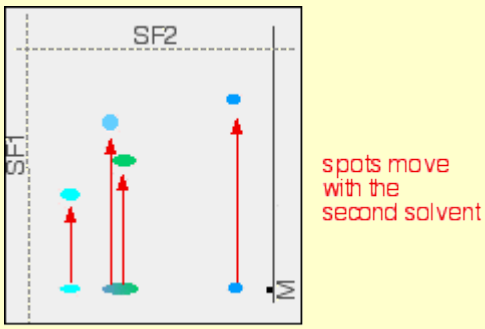
If you look closely, you may be able to see that the large central spot in the chromatogram is partly blue and partly green. Two dyes in the mixture have almost the same R_f values. They could equally well, of course, both have been the same colour - in which case you couldn't tell whether there was one or more dye present in that

spot.

What you do now is to wait for the paper to dry out completely, and then rotate it through 90°, and develop the chromatogram again in a different solvent.

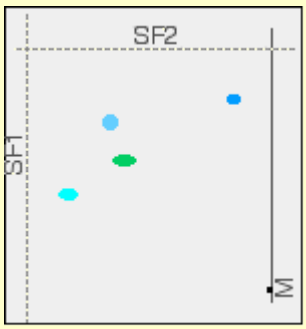
It is very unlikely that the two confusing spots will have the same R_f values in the second solvent as well as the first, and so the spots will move by a different amount.

The next diagram shows what might happen to the various spots on the original chromatogram. The position of the second solvent front is also marked.



You wouldn't, of course, see these spots in both their original and final positions - they have moved! The final

chromatogram would look like this:



Two way chromatography has completely separated out the mixture into four distinct spots.

If you want to identify the spots in the mixture, you obviously can't do it with comparison substances on the same chromatogram as we looked at earlier with the pens or amino acids examples. You would end up with a meaningless mess of spots.

You can, though, work out the R_f values for each of the spots in both solvents, and then compare these with values that you have measured for known compounds under exactly the same conditions.

How does paper chromatography work?

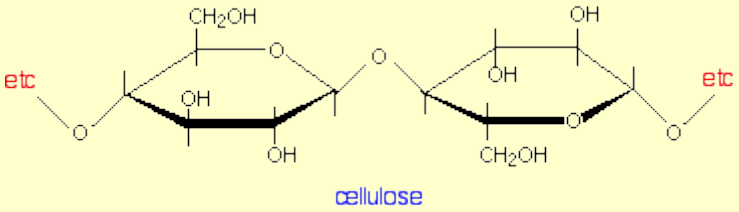
Although paper chromatography is simple to do, it is quite difficult to explain compared with thin layer chromatography. The explanation depends to some extent on what sort of solvent you are using, and many sources gloss over the problem completely. If you haven't already done so, it would be helpful if you could read the explanation for how thin layer chromatography works (link below). That will save me a lot of repetition, and I can concentrate on the problems.

Note: You will find the explanation for how thin layer chromatography works by following this link.

Use the BACK button on your browser to return quickly to this page when yhou have read it.

The essential structure of paper

Paper is made of cellulose fibres, and cellulose is a polymer of the simple sugar, glucose.



The key point about cellulose is that the polymer chains have -OH groups sticking out all around them. To that extent, it presents the same sort of surface as silica gel or alumina in thin layer chromatography.

It would be tempting to try to explain paper chromatography in terms of the way that different compounds are adsorbed to different extents on to the paper surface. In other words, it would be nice to be able to use the same explanation for both thin layer and paper

chromatography. Unfortunately, it is more complicated than that!

The complication arises because the cellulose fibres attract water vapour from the atmosphere as well as any water that was present when the paper was made. You can therefore think of paper as being cellulose fibres with a very thin layer of water molecules bound to the surface.

It is the interaction with this water which is the most important effect during paper chromatography.

Paper chromatography using a non-polar solvent

Suppose you use a non-polar solvent such as hexane to develop your chromatogram.

Non-polar molecules in the mixture that you are trying to separate will have little attraction for the water molecules attached to the cellulose, and so will spend most of their time dissolved in the moving solvent. Molecules like this will therefore travel a long way up the

paper carried by the solvent. They will have relatively high R_f values.

On the other hand, polar molecules *will* have a high attraction for the water molecules and much less for the non-polar solvent. They will therefore tend to dissolve in the thin layer of water around the cellulose fibres much more than in the moving solvent.

Because they spend more time dissolved in the stationary phase and less time in the mobile phase, they aren't going to travel very fast up the paper.

The tendency for a compound to divide its time between two immiscible solvents (solvents such as hexane and water which won't mix) is known as *partition*. Paper chromatography using a non-polar solvent is therefore a type of *partition chromatography*.

Paper chromatography using a water and other polar solvents

A moment's thought will tell you that partition can't be the explanation if you are using water as the solvent for your mixture. If you have water as the mobile phase and the water bound on to the cellulose as the stationary phase, there can't be any meaningful difference between the amount of time a substance spends in solution in either of them. All substances should be equally soluble (or equally insoluble) in both.

And yet the first chromatograms that you made were probably of inks using water as your solvent.

If water works as the mobile phase as well being the stationary phase, there has to be some quite different mechanism at work - and that must be equally true for other polar solvents like the alcohols, for example. Partition only happens between solvents which don't mix with each other. Polar solvents like the small alcohols do mix with water.

In researching this topic, I haven't found any easy explanation for what happens in these cases. Most sources ignore the problem altogether and just quote the

partition explanation without making any allowance for the type of solvent you are using. Other sources quote mechanisms which have so many strands to them that they are far too complicated for this introductory level. I'm therefore not taking this any further - you shouldn't need to worry about this at UK A level, or its various equivalents.

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Column Chromatography-Principle, Types, Applications

Written By Adeel Abbas

Column chromatography is a widely used technique of chromatography in chemistry to separate and purify a mixture of chemical compounds. Mostly it is used in natural product isolation.

I also used this technique during my research on Boerhavia Procumbens. The goal of this technique is to isolate each component in a mixture based on its physical and chemical properties. In this article, we will take a closer look at what column chromatography is, its principle, procedure, and applications.

What is Column Chromatography?

Column chromatography is a separation technique in which a column is used. The column is filled with a solid stationary phase which is silica. it is tightly packed for better separation. A liquid mobile phase to separate a mixture of chemical compounds is passed through the column. The mobile phase dissolves compounds and flows out of the column.

Column Chromatography Principle

The principle of column chromatography is based on the fact that different components in a mixture have different affinities for the stationary and mobile phases. When the mixture is passed through the column, the components will separate based on their different interactions with the stationary and mobile phases. This allows each component to be isolated and collected separately.

Common mobile and stationary phase used in column chromatography

Mobile Phase	Stationary Phase
Hexane	**Silica gel**

Ethanol	**Sephadex LH-20**
Acetonitrile	**C18**
Methanol	**Florisil**
Water	**Reverse-phase silica**
Toluene	**Alumina**
Chloroform	**Diol**
Acetone	**Ion exchange resins**
Isopropanol	**Cellulose**
Petroleum ether	**Polymeric resins**

Table showing different mobile and stationary phases used in column chromatography

Column Chromatography Diagram

A column chromatography setup typically consists of a column, a pump, a detector, and a collection system, and holding clamps. A

detector is used in advance columns. At basic level it is done manually by performing thing layer chromatography and checking it under uv lamp.

The column is filled with the stationary phase and the mixture is introduced at the top of the column. The mobile phase is then pumped through the column, carrying the mixture along with it. As the mixture passes through the column, the components will separate based on their different interactions with the stationary and mobile phases. The separated components are then collected at the bottom of the column.

Column Chromatography Procedure

The procedure for column chromatography can vary depending on the type of column and the specific requirements of the separation. However, the basic steps of a column chromatography procedure are as follows:

1. **Prepare the stationary phase and fill the column with it.**
2. **Load the mixture to be separated at the top of the column.**
3. **Start the flow of the mobile phase through the column.**
4. **Monitor the progress of the separation with a detector.**

5. **Collect the separated components at the bottom of the column.**
6. **Perform elution to wash the components from the column.**

Column Chromatography Experiment

Column chromatography experiments are a key part of many chemistry research projects and are used to separate and purify different components in a mixture. The following is a step-by-step guide to performing a column chromatography experiment:

1: Choose the appropriate column chromatography technique

There are several different techniques of column chromatography to choose from, including ion exchange chromatography, gel filtration chromatography, reverse phase chromatography, affinity chromatography, and hydrophobic interaction chromatography. The choice of technique will depend on the properties of the mixture and the desired outcome of the separation.

2: Prepare the stationary phase

The stationary phase is the material in the column that will retain the components of the mixture. It is important to choose the appropriate stationary phase for the separation and to prepare it according to the manufacturer's instructions.

3: Load the mixture

The mixture to be separated should be carefully loaded into the column. It is important to take care not to damage the stationary phase or to introduce any contaminants into the column.

4: Start the flow of the mobile phase

The mobile phase is the liquid that will flow through the column and carry the mixture along with it. It is important to choose the

appropriate mobile phase for the separation and to set the flow rate correctly.

5: Monitor the progress of the separation

The progress of the separation can be monitored using a detector, such as a UV-Vis spectrophotometer. The detector measures the amount of each component in the mixture as it passes through the column.

6: Collect the separated components

The separated components will be collected at the bottom of the column. It is important to collect the components in a way that preserves their purity and to store them correctly for further analysis.

7: Perform elution

Elution is the process of washing the separated components from the column. This is done by changing the conditions of the mobile phase, such as the solvent or the flow rate, to release the retained components from the stationary phase. The eluted components are then collected for further analysis or use.

In conclusion, a column chromatography experiment is a carefully controlled process that requires careful attention to detail in order to obtain accurate and reproducible results. By following the steps outlined above, you can perform a successful column chromatography experiment and obtain high-quality separated components for your research.

Types of Column Chromatography

There are several types of column chromatography, each with its own unique characteristics and applications. Some of the most commonly used types of column chromatography include:

- **Ion Exchange Column Chromatography**
- **Gel Filtration Chromatography**
- **Reverse Phase Chromatography**
- **Affinity Chromatography**
- **Hydrophobic Interaction Chromatography**

Column Chromatography Applications

Column chromatography is a powerful tool for separating and purifying different components in a mixture. The following are some of the most common applications of column chromatography:

1. **Biotechnology: Column chromatography is used extensively in the biotechnology industry to purify proteins and other biological molecules for use in research and as therapeutic drugs.**
2. **Pharmaceuticals: Column chromatography is used in the pharmaceutical industry to purify active ingredients and impurities from drug products. This is important for ensuring the quality and safety of drugs.**
3. **Environmental analysis: Column chromatography is used in environmental analysis to separate and identify contaminants in water, air, and soil samples.**
4. **Food and beverage analysis: Column chromatography is used in the food and beverage industry to analyze food ingredients, such as vitamins and minerals, and to monitor the quality of food and beverage products.**
5. **Analytical chemistry: Column chromatography is a common technique in analytical chemistry for separating and purifying different components in a sample for further analysis. This can be used in fields such as drug discovery, forensic science, and metabolic profiling.**
6. **Process chromatography: Column chromatography is also used in process chromatography to purify large quantities of a**

product, such as a food ingredient or a pharmaceutical, for commercial use.

Basics & Fundamentals Gas Chromatography Basics & Fundamentals: Gas Chromatography Table of Contents Introduction 3 1. GC Structure and Fundamentals 4 1-1. Basic instrumentation of GC 4 1-2. Applications of GC 5 2. Sample Injection Method 6 2-1. Split Injection Method 6 2-2. Splitless Injection Method 7 2-3. Direct Injection Method 7 2-4. Cold Injection Method 8 3. Separation Columns 9 4. Carrier Gas 10 4-1. Choice of Carrier Gas 10 4-2. Flow

Control Modes 10 5. Detectors 12 5-1. Thermal Conductivity Detector (TCD) 13 5-2. Flame Ionization Detector (FID) 13 5-3. Barrier Discharge Ionization Detector (BID) 14 5-4. Mass Spectrometer (MS) 14 5-5. Electron Capture Detector (ECD) 14 5-6. Flame Photometric Detector (FPD) 15 5-7. Flame Thermionic Detector (FTD) 15 5-8. Sulfur Chemiluminescence Detector (SCD) 16 6. Sample Introduction 16 7. Sample Pre-treatment Device 17 7-1. Static Headspace (SHS) 17 7-2. Dynamic Headspace (DHS) 17 7-3. Thermal Desorption (TD) 19 7-4. Pyrolysis Analysis (Py) 19 8. Summary 20 Shimadzu 2 Basics & Fundamentals: Gas Chromatography Introduction Gas chromatography (GC) is an analytical methodology, which was devised by Nobel Laureate, Martin, et al. in 1952. More than 60 years after the award, GC systems are widely commercialized and used in various industries, capable of both of quantitation and qualifcation. GC is applicable for many types of analysis in the markets such as residual solvent analysis in pharmaceuticals, residual pesticides analysis in food safety, trace level analysis for environmental as well as petrochemical and fne chemical industry. This article describes the basics and fundamentals of GC with tips on the instrumental operations in laboratory use. There may be other more advanced applications which require Shimadzu 3 GC customization (aka Process GC or

System GC), which will not be covered in this primer. Jackie Product Specialist (GC & GCMS) Shimadzu (Asia Pacifc) Pte Ltd. Basics & Fundamentals: Gas Chromatography 1. GC Structure and Fundamentals Gas chromatograph is an analytical instrument used to analyze the different components in a sample. An analytical method using a gas chromatograph is called gas chromatography (GC). Table 1: Terminologies and Defnitions of Gas Chromatography Term Defnition Chromatography Method for Separation Chromatograph Instrument for Chromatography Chromatogram Data of Chromatography 1-1. Basic instrumentation of GC As shown in Figure 1, the GC consists of a fow control section, a sample injection port, a column, a column oven, and a detector in which is connected to a data processor. Carrier gases such as helium (He), nitrogen (N2) or hydrogen (H2) are preferred to be supplied at a constant fow rate to the sample to the injection port. A separation tube called a column is connected between the sample injection port and the detector, all three parts are maintained at an appropriate temperature. The sample injected into the sample injection port instantaneously vaporizes and fows into the column with the carrier gas. In the column, a liquid stationary phase (for example, silicone polymers) is chemically bonded or coated, the vaporized sample is repeatedly dissolved and vaporized in the liquid stationary phase and

travels downstream with the carrier gas. Since the process of dissolving and vaporizing the sample in the stationary phase depends on the physicochemical properties such as the boiling point and the nature of the column, the time of dissolving in the liquid phase and the time of vaporizing will be different for each compound. Therefore, even when mixed components are injected, the time for the components to arrive at the column exit is different, and separation can be detected. The column exit connects to a detector and when substances other than the carrier gas are eluted from the column, the detector converts them into electrical signals which are amplifed and sent to a data processor. By analyzing the electric signal of the detector on the data processor, the GC will be able to identify the sample and determine its quantity. Under certain conditions, the time to reach the detector (retention time) is the same after injection of the compound. By injecting the standard sample and the unknown sample, and comparing the retention times, quantitation can be done by comparing the sizes of their peaks. Flow controller Injection port Column oven Detector Data processor Column Gas cylinder ELECTRIC SIGNAL Figure 1: Typical GC Confguration Shimadzu 4 Basics & Fundamentals: Gas Chromatography Shimadzu Basics & Fundamentals: Gas Chromatography 1-2. Applications of GC Shimadzu GC Application

Data Sheet Table 2: Some Industrial Applications of Gas Chromatography https://www.shimadzu.com/an/gc-datasheet.html Industry Type of Analysis Pharmaceutical Residual solvent analysis Food and beverages Component analysis, food safety analysis, halal analysis of alcohol Environmental Air, water, soil Petrochemicals Simulated distillation, component analysis Chemicals Material, polymer, additive, gas purity analysis, gas emission in automotives Energy and gas Artifcial photosynthesis research Shimadzu 5 Basics & Fundamentals: Gas Chromatography 2. Sample Injection Methods There are many different injection methods: split injection, splitless injection, direct injection, on-column injection. 2-1. Split Injection Method Split injection is the most popular and versatile method in capillary GC analysis. Split injection can be applied to many types of analysis, and whilst it may be less sensitive, the resolution of the chromatogram is not affected. Figure 2 shows the fow diagram of split injection. The sample is introduced into the inlet and is vaporized, moving downstream. Large portions of the vaporized sample have to be exhausted before the sample is separated in the column and detected. The split line has a role to protect the column from any sample overload and increase the moving velocity of the component in the injector port. The split ratio indicates "the ratio between the column fow rate and the split fow rate" and indicates the

approximate branching ratio of the injected sample. When the split ratio is high (for example 1:400), the rate of the injected sample being introduced into the 3mL/min 0mL/min 2mL/min 5mL/min Septum purge Capillary column Split resistance Vent Trap Carrier gas 150kPa 2mL/min 3mL/min 49mL/min 2mL/min 53mL/min Septum purge Capillary column Split resistance Vent Trap Carrier gas 150kPa 50mL/min 1~2 minutes Sample injection port Sample needle Sample gas Sample injection port Sample needle Sample gas Figure 3: Schematic of a Splitless Injector Port 3mL/min 49mL/min 53mL/MIN Septum purge Split resistance Trap Carrier gas 100kPa Sample gas 50mL/min Split vent Sample injection port Sample needle Figure 2: Schematics of a Split Injector Port column decreases as well as the sensitivity. On the other hand, when the split ratio is low (for example 1:30), the rate of introduction into the column increases as the sensitivity increases. The commonly used split ratio is 1:50 to 1:100 in the column with the inner diameter of 0.25 mm to 0.32 mm but considering the required sensitivity and the load amount on the column, the analysis is performed using the optimum split ratio. Glass inserts used in the split method are flled with fllers such as glass wool which have been deactivated. Quantity values and reproducibility may differ if wool quality, quantity, and the packing position are different. Since most manufacturers provide the specifc

requirements for wool and position, it is better to adhere Shimadzu 6 Basics & Fundamentals: Gas Chromatography to this. The split method is a relatively simple sample injection method. Higher quality data can be obtained by understanding and analyzing features well. 2-2. Splitless Injection Method The inlet for splitless injection can be shared with split injection. The splitless injection method is suitable for higher boiling temperature compounds and it is used to measure lower concentrations in environmental and residual pesticides feld. Figure 3 shows the conceptual diagram of the splitless injection method. It is similar to the split injection fow diagram, because when the sampling time is set to zero, the splitless injector essentially operates like a split injector. The sampling time is the amount of time set to direct samples into the column, before the split vent is open. The operation during sample injection and the fow of carrier gas will be described with reference to Figure 3. Before injecting the sample, keep the column initial temperature at 50 to 60 °C and close the split line as shown on the left side of Figure 3. When the sample is injected into the inlet, it will be vaporized. The vaporized sample gradually moves to the column due to the carrier gas. At this time, the component with relatively high boiling point is concentrated in a narrow band at the tip end portion of the column because the column initial temperature is low. It takes 1 to 2 minutes for most of the

vaporized sample to be introduced into the column, and this is the typical set amount of sampling time in a splitless injection. Thereafter, as shown in the right diagram of Figure 3, the split line is opened, the solvent and the sample at the inlet and the column are removed, the temperature is raised, and the concentrated component is separated at the tip end portion of the column to detect. with a low analyte concentration. There are some caveats but it is widely used in environmental and residual pesticide analysis as a method that can easily achieve high sensitivity. 2-3. Direct Injection Method The majority of the sample is introduced into the capillary column. The inner diameter of the capillary column is 0.45 mm or more, which is called wide bore. It is recommended that the column length be 25 m or more. Short columns of 15 m or less are often diffcult to use due to low set pressure. Figure 4 shows an example of a structural view of an injection port dedicated to the wide bore column. In order to suppress sample retention in the glass insert and the wide bore column connection part, a part of the carrier gas is supplied as a purge gas in the column connection part, or a taper is formed in the lower part of the insert so that the wide bore column closely adheres to the bottom part of the insert. When using a septum purge type total volume injection port, it can be used with a carrier gas fow rate near the optimum fow rate (about 5 mL /min of He) for a wide bore

column. Septum Carrier purge gas Sample gas Glass insert Heater Wide bore column • Possible to set suitable ˜ow rate for separation • Reduce ghost peak from septum and improve separation with solvent peak 9mL/min 3mL/min The splitless method is an analytical method that is Figure 4: Schematic of Direct Injection Port (with only septum suitable for relatively high boiling point compounds purge fow) Shimadzu 7 Basics & Fundamentals: Gas Chromatography The direct injection method is an excellent analytical technique which enables simple capillary analysis by simple inlet port structure. 2-4. Cold Injection Method a. Cold on-column caps Injection (Cold OCI) Inlet temperature is kept below the boiling point of the sample solvent and the tip of the microsyringe is inserted directly into the tip of the capillary column for sample injection. Thereafter, by raising the temperature of the inlet and column, the sample is gradually vaporized directly inside the capillary column. The tip of the capillary column corresponds to the vaporization chamber. Cold OCI is suitable for analyzing compounds that are unstable to heat (easy to decompose). Due to there being no compositional change of samples, it is an analysis method with high accuracy for measures such as area value reproducibility. Samples with low concentrations (less than about 200 ppm per component) are also suitable. 3mL/min 0.25-0.53mm l.D. Cooling air Septum purge Carrier gas Sample gas

9mL/min Heater Empty, inactive treated 0.53mm l.D. column, without stationary phase (retention gap) Upper part of 0.53mm l.D. column or Figure 5: Schematic of a Cold On-Column Injection However, since the sample is injected directly into the column, the column is liable to be contaminated. 3mL/min 0.25-0.53mm l.D. purge gas 9mL/min air Septum Carrier Sample gas Cooling Heater Split resistance variable Figure 6: Schematic of a Programmed Temperature Vaporizer b. PTV Injection System (Programmable Temperature Vaporizer) At the point of sample injection, the injection port is set to a low temperature, typically below the boiling point of the solvent. Thereafter the PVT is rapidly heated to vaporize the entire sample, along with the solvent. This method is suitable for analyzing compounds that are unstable to heat (easy to decompose) with little change in composition due to the warming of the remaining components at the tip of the syringe needle. Unlike Cold On-Column Injection analysis, glass inserts can be used in a PTV. Additionally, both split and splitless modes can be applied onto the PTV. As a result, PTV can cope with high and low concentration samples. With the right setting, PTV can prevent nonvolatile contaminants from entering the analytical column altogether. Shimadzu 8 Basics & Fundamentals: Gas Chromatography 3. Separation Columns There are two main types of separation column

used in GC available on the market: capillary and packed columns. A capillary column typically has 0.1-0.53 mm internal diameter and 10-100 m length with very high resolution. The material is fused silica and the inside wall is chemically bonded with the liquid phase. The outside is coated by polyimide resin to increase the Table 3: Polarity and Liquid Phase of Capillary Columns Polarity Example of Column Typical Liquid Phase Non-polar Squalane SH-Rtx-1 100% Dimethylpolysiloxane Low-polar SH-Rtx-5 SH-Rtx-1301, 624 5% Diphenyl 95% Dimethylpolysiloxane 6% Cyanopropylphenyl 94% Dimethylpolysiloxane Mid-polar SH-Rtx-1701 4% Cyanopropylphenyl 86% Dimethylpolysiloxane SH-Rtx-17 50% Phenyl 50% Methylpolysiloxane SH-Rtx-200 Trifuoropropylmethylpolysiloxane High-polar SH-Rtx-Wax Polyethyleneglycol BPX-90 90% Bis-cyanopropyl 10% Cyanopropylphenylpolysiloxane intensity. The column with 0.25 mm internal diameter and 30 m length is frequently used. The GC user chooses the column based on certain attributes such as; the target compounds, the number of components and the instrument confguration. A smaller diameter and longer column is suitable if higher resolution is needed, while a larger diameter column can be used if higher resolution is unnecessary. Inner Stationary phase diameter (types and ÿlm Column of column thickness) length Carrier

gas Oven (types and linear temperature velocity) Figure 7: Factors Which Affect Separation in a GC Column Shimadzu 9 Basics & Fundamentals: Gas Chromatography 4. Carrier Gas 4-1. Choice of Carrier Gas The carrier gas should be an inert gas, carrying the sample but not interacting with the target compounds. Such examples are He, N2 , H2 , and Ar, of which, He and N2 are the most commonly used. In capillary columns, He is preferred due to its ability to maintain the separating resolution at high linear velocity (the speed at which sample travels through the column). Table 4: Advantages and Disadvantages of Carrier Gases Carrier Gas Advantages Disadvantages • Safe Helium • Relatively wide optimum linear velocity • Expensive range Nitrogen • Cheap • Safe • Optimum linear velocity range is narrow and slow • Long analysis time Carrier gas always fows into the detector, therefore it is necessary to use one with a high purity (99.995 % or higher). Carrier gases with high purity can suppress baseline noise. Measures and Proposals to Reduce Helium Gas Consumption are found in the link below: https://www.shimadzu.com/an/gc/eco/gas.html 4-2. Flow Control Modes When GC was frst developed, the primary mechanism to direct the fow within the system was by controlling the pressure. For convenience, the initial pressure (sometimes known as head pressure) is fxed and all the resultant methods created are based on constant

pressure mode. There is, however, a major faw: peak broadening. There are three different fow control modes that are commonly employed. We can look directly into the associated units to compare the three control modes: · constant pressure mode: Pa (pressure) · constant fow mode: cm^3/s (volume per second) · constant linear velocity mode: cm/s (distance per second) In constant pressure mode, the head pressure is fxed. As the sample travels further and further from the injector port, it experiences less force, and hence it slows down. This results in peak broadening as the retention time increases. Such an impact is more signifcant in capillary columns, in which the column length is much longer than in packed columns. Over time, GC methods have shifted from this mode in favor of signifcant improvements. Next, we look into the very similar constant fow and linear velocity modes. They both overcome the weakness of constant pressure mode, correcting the volume passing a column or distance travelled down to the "per second" accuracy level. However, columns expand at a higher temperature (typical in GC oven programming) and the difference between these two modes become signifcant. In an ideal situation, when a tube has a smaller diameter, the same volume occupies a greater length. For the same volume to pass points 1 and 2 in a given time (Figure 8), the speed must be greater at point 2. Shimadzu 10 Basics & Fundamentals: Gas

Chromatography Figure 8: Fluid Flows Through a Narrower Column Faster: The same volume of fuid occupies a different cross-sectional areas (A) at points 1 and 2, where the column size is different. This causes a different fow rate, and thus a different linear velocity (v). A1 A2 2 V2 V1 1 The process is exactly reversible; if the fuid fows in the opposite direction, its speed will decrease when the tube widens. This "speed" is what we know as the linear velocity. During analysis, with the heating program of the GC oven, capillary columns expands gradually. As a result, if the constant fow mode is used, linear velocity will slow down progressively, affecting the separation effciency. While not illustrated in any fgure of this article, there is another situation where the constant linear velocity mode comes in handy. When the tail pressure is lower (i.e. vacuum), the fow will be faster than it would be with a higher tail pressure (i.e. atmospheric pressure). As a result, the retention time between a GC with an atmospheric detector (e.g. a fame ion detector (FID)) and a GC with a detector in a vacuum state (e.g. a mass spectrometer (MS)) cannot be matched interchangeably. The issue can be resolved, however, if the constant linear velocity mode is employed; in this mode, carrier gas fows through the column regardless of the detector pressure. In GC instrumentation, it is easier to control the pressure (you only need a regulator to do that). To control the fow, you then

need to implement some correction factors. A feedback mechanism is required to adjust the pressure regularly so that the volume passing through a column is maintained. This automatic pressure control mechanism is called “electronic pneumatic control”. To control the linear velocity, an additional correction factor for the cross-sectional area is needed. The electronics need a more sophisticated calculation algorithm. Within one instrument, using constant linear velocity mode can maintain the optimal resolution effciency of chromatographic separation. Across multiple instruments, constant linear velocity ensures the reproducibility of results. This is true even for reproducibility between vacuum detectors and ambient pressure detectors. In other words, constant linear velocity mode can be extremely benefcial for its method transferability, e.g. between R&D and QC departments of an organization. Shimadzu 11 Basics & Fundamentals: Gas Chromatography 5. Detectors Table 5: Features of GC Detectors. This table serve as a rough indication, it may be different depending on the compound chemical structure and analytical condition Detector Example of Detectable Compound Example of Minimum Detectable Amount* Universal Detector Thermal Conductivity Detector TCD All compounds except for carrier gas 10 ppm (10 ng) Flame Ionization Detector FID Organic compounds 0.1 ppm (0.1 ng) Barrier Discharge Ionization Detector

BID All compounds except for He and Ne 0.07 ppm (0.07 ng) Mass Spectrometer MS Ionized molecule 10 ppm (10 ng) in Scan mode 0.5 ppm (0.5 ng) in SIM mode 10 ppb (10 pg) in MRM mode Selective Highsensitivity Detector Electron Capture Detector ECD Organic Halogen compounds Organic mercury compounds 0.01 ppb (0.01 pg) Flame Photometric Detector FPD Sulfur compounds Organic phosphorus compounds Organic tin compounds 10 ppb (10 pg) Flame Thermionic Detector FTD (NPD) Organic phosphorus compounds Organic nitrogen compounds 0.1 ppb (0.1 pg) 1 ppb (1 pg) Sulfur Chemiluminescence Detector SCD Sulfur compounds 1 ppb (1 pg) Table 6. The Compounds in the TCD and the Unique Conductivity Constant Compounds Thermal Conductivity Constant (10-6 cal/s·cm·˚C) He 408 H2 547 (Very High) N2 73 Ar 52 O2 76 H O2 60 Ethane 77 Methanol 52 Acetone 40 Chloroform 24 Shimadzu 12 Basics & Fundamentals: Gas Chromatography Reference side column TO outlet DATA PROCESSOR A B C D Figure 9: Schematic of a Thermal Conductivity Detector 5-1. Thermal Conductivity Detector (TCD) TCD detects using difference of thermal conductivity of between sample and carrier gas. Generally, He is used as carrier gas. However, He has a high thermal conductivity constant number. When the target compound is He and H2 , N2 or Ar is used as carrier gas. All compounds have each unique thermal conductivity constant

as shown below. When thermal conductivity of target compound is larger than the carrier gas, peak is detected as a negative value. TCD can detect most compounds except for carrier gas but its sensitivity is not as high as compared Collector Hydrogen ˜ame Quartz nozzle Air Hydrogen (+ makeup gas) TO DATA PROCESSOR Column outlet HIGH VOLTAGE Data processor Figure 10: Schematic of Flame Ionization Detector (FID) Analysis side column outlet The voltage or a direct current is applied between A and B. While only carrier gas is ˜owing at constant ˜ow, each ÿlament is kept at constant temperature and shows constant voltage between C and D. Components are eluted from an analysis side column. The temperature of ÿlament rises up (since the thermal conductivity is smaller than that of carrier gas, resistance value changes). Voltage between C and D changes. to other detectors, such as the FID or BID. Among the other GC detectors, it has almost the lowest sensitivity. Its main application is analysis of permanent gases it can analyze the compounds that are undetectable using FID, such as water, formaldehyde, and formic acid. 5-2. Flame Ionization Detector (FID) In FID, the components are burned and ionized; it is used for almost all organic compounds, which have C-H or C-N structure. There are several exceptions such as CO, CO2 and CS2 which are non-organic compounds that cannot be detected. Carbonyl group and C=O carbon atom of carboxyl group are also

not detectable. FID can Electric current °ows when ions are collected by collector Organic compounds burn in hydrogen ˜ame and several ppm of those become the following ions: CH CHO+ + e− (O) OXIDATION CN NO+ + CO + e− 2(O) OXIDATION Shimadzu 13 Basics & Fundamentals: Gas Chromatography detect most hydrocarbons which have a C-H bond in its structure, except for HCHO and HCOOH. CHCOOH, and acetaldehyde (CH3 CHO) which are from the same group as HCHO and HCOOH respectively, and have one more carbon. They are detectable because a carbon of C-H exists in addition to C=O. FID is mainly used to analyze organic compounds and overall, it is a stable and highly sensitive detector, used in various felds. 5-3. Barrier Discharge Ionization Detector (BID) The BID is a highly sensitive device that creates ionization from a He-based, dielectric barrier discharge plasma. A 17.7 eV plasma is generated by applying a high voltage to a quartz dielectric chamber, in the presence of helium at a relatively low temperature. Compounds that elute from the GC column are ionized by this He plasma energy and then detected by the collection electrode to be processed as peaks. BID is able to detect most of compounds except for He and Ne. The BID is more sensitive than both TCD and FID with the ability to analyze down to sub-ppm levels of water and inorganic gases. Exhaust Collector 63Ni radiation source TO DATA

PROCESSOR Column outlet 10mCi Makeup gas Data processor N2 used as carrier gas (makeup gas) is ionized by the beta ray emitted from 63Ni. N2 N2 + + e− ß RAY Electric current °ows when electrons are captured by collector (background current) If electrophilic compounds, such as PCB, enter then PCB + e− PCB − Since PCB is highly electrophilic, it will absorb the background electrons Ion current decreases Peak detected 5-4. Mass Spectrometer (MS) The GC-MS is superior in qualitative and quantitative capability and, with greatly improved performance and operability, it is rapidly spreading in the market. The composition and principle of the GC-MS will be explained in a separate document. 5-5. Electron Capture Detector (ECD) Radioactive isotopes are equipped (63Ni for Shimadzu product) in ECD. It is highly selective for electrophilic compounds which become negative ions after obtaining electrons such as organic halogens, organic metal compounds, and diketones. The sensitivity depends on the type of halogen, its number and its structure. ECD's main application is environmental analysis, such as residual pesticides, residual PCB, Chlorine VOC in drain water, organic mercury in environmental feld, etc. Figure 11: Schematic of an Electron Capture Detector (ECD) Shimadzu 14 Basics & Fundamentals: Gas Chromatography Air Hydrogen (+ makeup gas) TO DATA PROCESSOR Column outlet Quartz tube Filter*

Photomultiplier Data processor *Only the light of speciÿc wavelength can pass the ÿlter: •°For S (blue) — 394nm •°For P (yellow) — 526nm •°For Y (orange) — 610nm Combustion of S compounds, P compounds, and Sn compounds emit the light of respectively inherent wavelength. By letting it pass in a ÿlter, only the light of inherent wavelength reaches a photomultiplier. The intensity of light is ampliÿed and changed into electric signal by the photomultiplier. Figure 12: Schematic of a Flame Photometric Detector 5-6. Flame Photometric Detector (FPD) FPD is a highly selective and sensitive detector, especially for phosphorus (P), sulfur (S), and tin (Sn) compounds. It detects light unique to P, S and Sn in the hydrogen fame through interference flter. FPD is so stable and sensitive that it is easy to use alongside selective detectors. It has been used in food analysis, — to detect phosphorus pesticides, sulfur odors and food favors — and in environmental analysis to detect organic tin compounds in sea products. 5-7. Flame Thermionic Detector (FTD) A fame thermionic detector is a highly selective and sensitive detector for organic nitrogen compounds, inorganic and organic phosphorus compounds. It is also known as a nitrogen phosphorus detector (NPD) because it can detect nitrogen and phosphorus compounds. Its principle and basic structure is the same as FPD. Inorganic nitrogen compounds cannot be detected using FTD, so it cannot be applied to

ammonium analysis. Its selectivity to phosphorus compounds is lower than FPD. The main applications of FTD include the analysis of drugs, nitrogen pesticides, and phosphorus pesticides, etc. HIGH VOLTAGE Air Hydrogen (+ makeup gas) POWER CONTROLLER Column outlet Collector Quartz nozzle Rb2 SO4 bead TO DATA PROCESSOR Data processor When the platinum coil to which rubidium sulfate adhered to is heated by electric current, plasma-like atmosphere is generated into the surrounding of Rb2 SO4 bead. In this atmosphere, Rb* (rubidium radical) is generated, and −CN and −PO2 (generates by the oxidation of organic phosphorus compounds) react as follows with Rb*, and become ions. CN + Rb* CN− + Rb+ PO2 + Rb* PO2 − + Rb+ Electric current °ows when ions are collected by collector Figure 13: Schematic of a Flame Thermionic Detector Shimadzu 15 Basics & Fundamentals: Gas Chromatography In FTD, organic nitrogen compounds and organic phosphorus compounds are decomposed and ionized in the vicinity of the collector surface of superheated rubidium salt and detected. It is highly sensitive, but this rubidium collector is a consumable. If not restored or replaced, the overall detector's sensitivity tends to drop over time. 5-8. Sulfur Chemiluminescence Detector (SCD) The sulfur-containing compound (RS) is burned at about 800 °C in a hydrogen-flled atmosphere, to generate sulfur monoxide (SO). SO

then reacts with ozone from the ozone generator to obtain an excited state of sulfur dioxide (SO2),and detects chemiluminescence when it returns to the ground state. A series of reactions is carried out under reduced pressure of 5 Torr. The emitted light (hν) passes through the optical flter and is detected by the photomultiplier tube (PMT). R-S + O → SO + H-R + H2 O SO + O3 → SO2 * + O2 SO2 *→ SO2 + hν (300~400nm) 6. Sample Introduction SCD has high selectivity and sensitivity with a wider dynamic range as compared with FPD for the same sulfur compound. Since the response is hardly infuenced by the structure of the compound, it is possible in principle to quantify all the sulfur compounds with one standard substance. There is no unavoidable quenching phenomenon in FPD. SCD is widely used for analysis of petroleum products and foods. Redox cell Chemical luminescence PMT (Reaction cell) reaction chamber (Photomultiplier tube) EXHAUST PUMP Column TO DATA PROCESSOR O2 OXIDATION REDUCTION OZONATION LUMINESCENCE Ozone generator H2 O3 Figure 14: Schematic of a Sulfur Chemiluminescene Detector (SCD) The target samples to be analyzed by GC are mostly gas or liquid. In the case of manual injection, the gas sample is sampled by a gas tight syringe and injected into the device. For liquid samples, the sample is injected with a microsyringe exclusively for liquid. Auto injectors capable of automatically

injecting samples into liquid samples are widely used. The injection volume often used for analysis is 0.2 to 1 mL for gas and 1 to 2 μL for liquid. Improvement in sensitivity can be expected if the injection amount is large, but separation often becomes worse due to excessive loading. Hence great care is needed as it will cause problems unless an appropriate amount is injected. Shimadzu 16 Basics & Fundamentals: Gas Chromatography 7. Sample Pre-treatment Devices Various types of sample pre-treatment devices are used with GC and GC/MS. The major sample pretreatment system and methods are described in this chapter. However, you may refer to the complete guide of choosing the right GC pre-treatment device in a separated document. 7-1. Static Headspace (SHS) The sample is frst sealed and heated. The gas in the space (headspace) above the sample matrix, within sealed container is introduced into the GC. Components with relatively high volatility can be detected with high sensitivity. Samples are comprised of liquids and solids, but often are liquid samples. In the case of a liquid sample, when kept at a constant temperature for a certain period of time, the compound concentration of the headspace gas is in an equilibrium state. Therefore, measuring the headspace gas makes it possible to quantify the liquid sample. After sealing the sample, it should be kept at an appropriate temperature and time, so that gas can be collected and

introduced to the GC. A headspace sampler that automatically performs temperature, heat retention time, and gas sampling is also used to automate and improve the accuracy of analysis (Figure 15). 7-2. Dynamic Headspace (DHS) Purge-and-trap is a device used for high sensitivity measurement of low concentration volatile organic compounds (VOC) in water that operates under the principle of dynamic headspace. Gas is passed through sample water, and VOC is driven out together with gas. This gas is collected and desorbed by a trap tube to be analyzed. There are several kinds of adsorbents for trap pipes, and they are selected by target components. There are generally four steps in the preprocessing process (Figure 16). 1. Purge trap An inert gas such as He or N2 is passed through the water sample to purge the volatile VOC, and the evacuated VOC is then trapped in the trap tube (trap). 2. Dry purge (water removal) At the time of the purge trap, moisture is also trapped in the trap tube, so there is concern about its infuence Equilibration CG: concentration in gas phase CM: concentration in matrix (e.g. sample solution) Autosampler Manual Injection Vial Gas tight syringe • Possible to set suitable ˜ow rate for separation • Reduce ghost peak from septum and improve separation with solvent peak k=CM / CG K: PARTITION RATIO Silicon rubber or butyl rubber septum Aluminum cap with hole PTFE Figure 15: Principle of Static

Headspace Shimadzu 17 Basics & Fundamentals: Gas Chromatography 2 / Dry purge (removal of water) Carrier gas Trap tube WATER Purge PURGE 1 / Purge trap Carrier gas Trap tube Trap tube (heating) Carrier gas 3 / Sample introduction Trap tube (heating) Carrier gas 4 / Conditioning Figure 16: Principle of Dynamic Headspace on the measurement. Only the inert gas is allowed to fow into the trap pipe after the purge trap to remove moisture as much as possible. It is necessary to perform dry purging to the extent that the component to be measured does not desorb. This method is unsuitable when the holding power of the component to be measured to the adsorbent is low and the difference in holding power with water is small. 3. Sample introduction Heat the trap tube to desorb the VOC and introduce the sample into GC and GC/MS. When heating and desorbing the trap tube, the direction of the carrier gas is made to fow opposite to that during the time of trapping. This could improve the desorption effciency. 4. Conditioning While fowing inert gas, heat and purge any remaining organic matter in the trap tube. Collecting tube (heating) Carrier gas Pump Integrating ˜ow meter Collecting tube (heating) Carrier gas Cold trap (cooling) Trap tube (heating) COLLECTING TUBE (REMOVE AFTER COOLING) Carrier gas 1 / Collection (off-line) 2 / Cold trap (on-line) 3 / Sample introduction (on-line) 4 /

Conditioning (off-line) Collecting tube (room-temp) Figure 17: Principle of Thermal Desorption Shimadzu 18 Basics & Fundamentals: Gas Chromatography 7-3. Thermal Desorption (TD) In order to measure low concentration volatile organic compounds (VOC) in the environment and indoor atmospheric air, a certain amount of gas has to be passed through a collection tube flled with an adsorbent, the VOC is to be concentrated and then heated. It is a method of desorbing and analyzing components. The process consists of four steps of collection, cold trap, sample introduction, and conditioning (Figure 17). 1. Collection A pump is connected to the collecting pipe, and the sample gas is passed, so that the VOC in the atmosphere is collected. The cumulative fow meter is connected to the rear stage of the pump in order to fnd the collected volume. 2. Cold trap The VOC desorbed from the collection tube needs to be re-concentrated before it is introduced the GC and GCMS via the cold trap. The trap flled with adsorbent is cooled to below room temperature and the collection time is heated with the VOC being desorbed as to be able to recapture it with the trap. 3. Sample introduction The trap is heated to desorb the VOC and introduce it to GC and GC-MS. 4. Conditioning By heating the collecting tube, it is possible to reuse it by expelling the remaining organic matter. It is desirable to heat the tube just before collection as much as possible.

The collecting tube has to be capped and not exposed to the outside air as to prevent contamination of organic matter. 7-4. Pyrolysis Analysis (Py) When a polymer sample is heated to about 400 to 900 °C, the decomposed gas is generated. It is a method used to obtain information on macromolecular monomers, dimers, trimers and compounds derived from polymer structures by introducing decomposition gas into GC and GC/MS for analysis. In order to obtain the information on the decomposed gas with good reproducibility, it is required to heat the sample instantaneously at a temperature as accurate as possible. Typical methods for instantaneous heating include flament type, induction (Curie point) heating furnace type and heating furnace type (free fall). In addition, an inert material or surface treatment that does not cause any catalytic reaction is required for the container (cup) with a minimum sample amount (about 100 to 500 µg) (Figure 18). Carrier gas Polymer sample 100~500 ˜g Sample cup Inert quartz tube To pyrolysis GC Figure 18: Principle of Pyrolysis Analysis (Py) 400~900°C Shimadzu 19 Basics & Fundamentals: Gas Chromatography 8. Summary GC is useful and universal analytical instrument. By choosing injection method, column, detector and sample pretreatment method, it can be applied for wide variety of application. Understanding the system features and appropriate

sample treatment can result to a more effcient process. To analyze actual samples, clean-up process such as dissolution and extraction may be needed. To maintain higher sensitivity, it is important to ensure the instrument condition is optimized.

High-Pressure Liquid Chromatography (HPLC), Principle, Instruments, and Applications

- **Bimal Raut**

- October 8, 2021
- Separation

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High-Pressure Liquid Chromatography (HPLC)

Definition: High-Pressure Liquid Chromatography (HPLC) is a separation technique in which a liquid or properly dissolved solid sample is passed through a column at high pressure. It is also

referred to as High-Performance Liquid Chromatography and is used to separate, identify or quantify each component in a mixture.

Principle of HPLC (High-Performance liquid Chromatography)

The basic principle of HPLC is that it separates a sample into its constituent parts based on the relative affinities of distinct molecules for the mobile phase and the stationary phase used in the separation.

The distribution of the analyte between a mobile phase (eluent) and a stationary phase (packing material of the column) is the basis for HPLC separation. The molecules are retarded while passing through the stationary phase, depending on the chemical structure of the analyte. The duration a sample spends "on-column" is determined by the unique intermolecular interactions between its molecules and the packing material. As a consequence, the constituents of a sample get eluted at different times, and hence the separation is achieved.

High Performance Liquid Chromatography Instrumentation

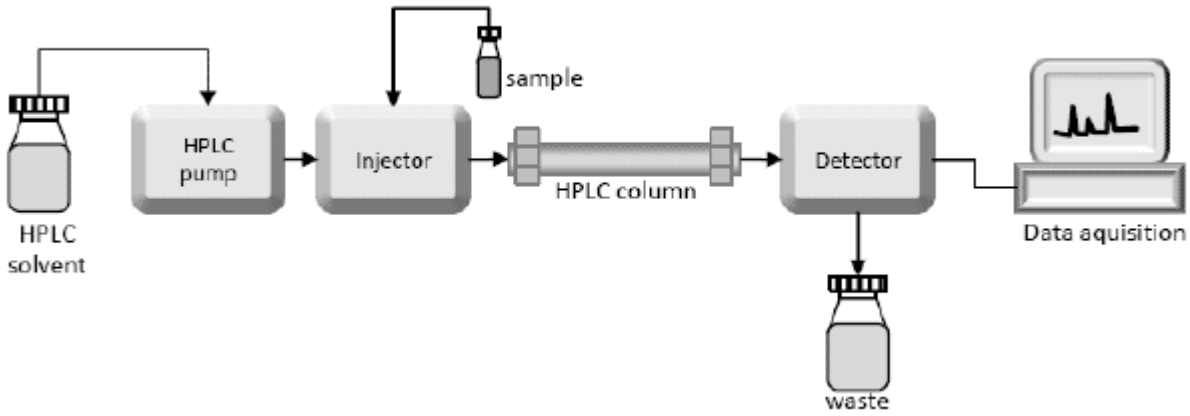

Image Source: https://www.researchgate.net/figure/Schematic-diagram-of-the-High-Performance-Liquid-Chromatography-HPLC-system_fig2_236146377

1. **Solvent reservoir: Solvent reservoir is also known as mobile phase reservoir. The high viscous solvent is discouraged to use as it takes much more time to travel through column, and high pressure is required for the viscous solvent. These leads to peak broadening, and hence better not to use such sovent. The choice of solvent depends on the nature of sample and the sensitivity of the detector.**
2. **Pump: The pump's function is to propel a liquid (known as the mobile phase) through the liquid chromatograph at a set flow rate, which is measured in milliliters per minute (mL/min). A pump can deliver a constant mobile phase composition (isocratic) or a rising mobile phase composition (gradient) during the chromatographic experiment.**

3. **Injector: The injector is used to insert the liquid sample into the mobile phase's flow stream. Sample quantities range from 5 to 20 microliters (L).**
4. **HPLC Column: It is known as the heart of the chromatograph. The column length generally varies from 5 cm to 30 cm,and its diameter ranges from 2-50 mm. Mostly, stainless steel is used as materials for the construction of the tubing, while Silica and alumina particle is used as packing materials. The mobile phase is aspirated from the solvent resorvoir and forced through the system's column and detector by a pump.**
5. **Detector: The detector detects individual molecules leaving the column and delivers an output to a recorder or computer, resulting in a liquid chromatogram.**
6. **Computer: It takes the signal from the detector and makes use of it to decide the time of elution (retention time) of the sample components (qualitative analysis) and the quantity of pattern (quantitative analysis).**

Application of High Performance Liquid Chromatography

High-Performance Liquid Chromatography is used for both qualitative and quantitative analysis. Some of the major applications of HPLC are listed below:

- **Pharmacetical applications for the analysis of drugs**
- **Purification of water**
- **Ligand-exchange chromatography**
- **Used for analyzing air and water pollutants**
- **Used for analyzing complex mixtures in chemistry and biochemistry research**
- **Used in quality control to ensure the quality of raw materrials**
- **Ion-exchange Chromatography of proteins**
- **Separation and analysis of non-volatile or thermally unstable compounds**

Advantages of High Performance Liquid Chromatography

- **High resolution**
- **Quick analysis**
- **Separation of volatile and non-volatile components**

Disadvantages of HPLC:

- **High cost**
- **Relatively difficult to operate**

High Performance Liquid Chromatography (HPLC) : Principle, Types, Instrumentation and Applications

By[**Editorial Team**]**March 9, 2022**

High-performance liquid chromatography, abbreviated as HPLC, is a chromatographic technique of great versatility and analytic power used in many aspects of drug manufacturing and research.

It separates or identifies mixtures of substances into their components based on their molecular structure and composition.

The other name for high-performance liquid chromatography is high-pressure liquid chromatography.

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Introduction

High-performance liquid chromatography (HPLC) is the most widely used separation technique. It can be very sensitive, specific, and precise.

- **It is a particular form of column chromatography used in biochemistry and analysis to separate, identify, and quantify the active compounds in a mixture.**
- **In HPLC, a column holds packing material (stationary phase), a pump moves the mobile phase(s) through the column, and a detector shows the retention times of the molecules.**
- **Retention time is variable and mainly depends on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.**
- **A small volume of sample to be analyzed is introduced to the mobile phase stream and is retarded by specific chemical or physical interactions with the stationary phase.**
- **The amount of retardation mainly depends on the nature of the analyte and the composition of both stationary and mobile phases.**
- **The most common solvents used in high-performance liquid chromatography (HPLC) are methanol and acetonitrile.**

Brief history

Michael Tswett (1872-1920) is credited as the father of chromatography due to his demonstration of liquid chromatography. In 1903, he separated the green-leaf pigments into bands of colors. After that, in 1937-38, thin-layer chromatography (TLC) was used. The next significant advancement was the use of paper chromatography in the mid-1940s.

Thin-layer chromatography (TLC) advanced slowly during the next few years, but Egon Stahl made significant development in 1956. Egon Stahl standardized the preparation of the sorbents used to make the plates. High-pressure liquid chromatography (HPLC) was later developed in the 1970s.

The term high-performance liquid chromatography (HPLC) was introduced in the 1970s to distinguish the modern high-performance technique from classical low-pressure column chromatography, developed in the 1930s.

HPLC Principle

High-performance liquid chromatography (HPLC) involves the injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 microns (µm) in diameter called the stationary phase) where individual components of the sample are moved down

the packed tube with a liquid (mobile phase) forced through the column by high pressure delivered through a pump.

The column packing is used to separate the components from one another. It involves various chemical and/or physical interactions between their molecules and the packing particles.

The separated components are then detected at the exit of the column by a detector that measures their amount. Output from this detector is called a "liquid chromatogram."

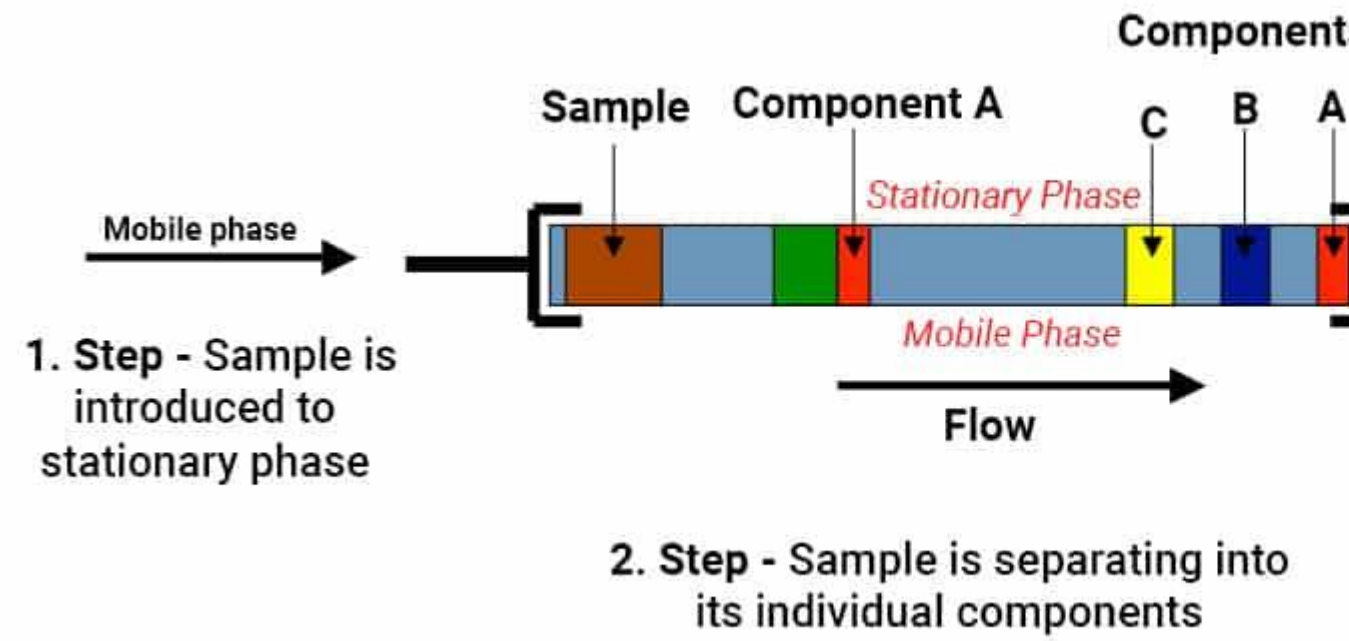


Figure: 1 Describes the basic principle of HPLC. Step 1. The sample is introduced (mobile phase). Step 2. The sample is separated into its components(stationary phase). Step 3. The sample is separated into its components (mobile phase).

Advantages over low-pressure column liquid chromatography

There are many advantages of High-performance liquid chromatography (HPLC) over traditional low-pressure column liquid chromatography.

- **Greater sensitivity (various detectors can be employed)**
- **Improved resolution**
- **Speed**
- **Easy sample recovery (less eluent volume to remove)**
- **A wide variety of stationary phases**

Branches of HPLC

Chromatography is divided into gas, liquid, and supercritical fluid techniques. Gas chromatography is further divided into gas-liquid and gas-solid techniques.

Liquid chromatography is divided into a relatively large collection of techniques like thin layer chromatography. Pressurized liquid chromatography can be divided into ion exchange, exclusion, partition, and liquid-solid chromatography.

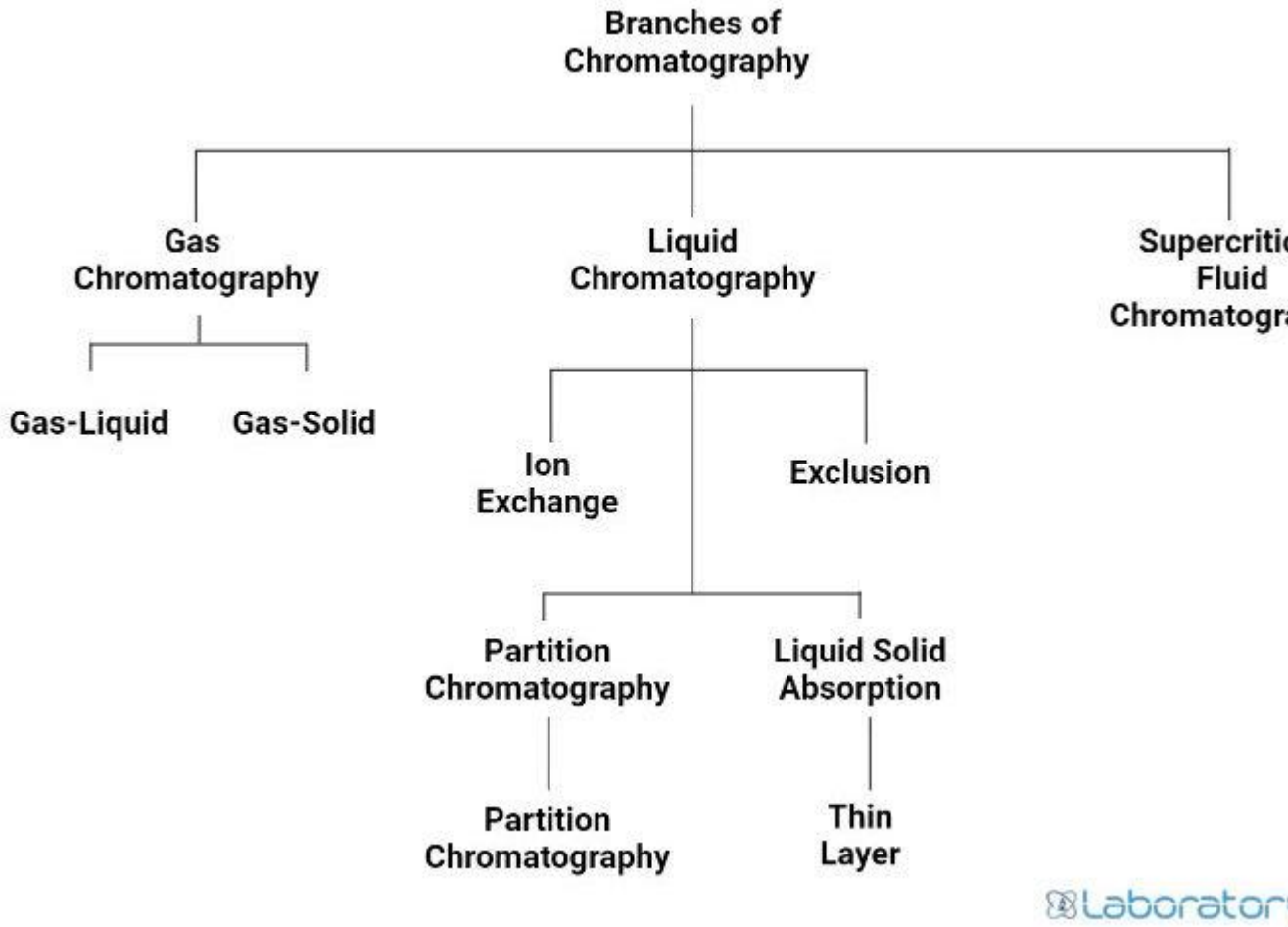


Figure 2. Describes the Branches of Chromatography.

Types of HPLC

The following variants of HPLC depend upon the phase system (stationary) in the process.

1. Normal Phase HPLC

- **They are also known as normal-phase or absorption chromatography. This method separates analytes based on polarity.**
- **It has a polar stationary phase and a non-polar mobile phase.**

- **Therefore, the stationary phase is usually silica, and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures.**
- **The technique is used for water-sensitive compounds, geometric isomers, cis-trans isomers, class separations, and chiral compounds.**

2. Reverse Phase HPLC

- **The stationary phase is nonpolar (hydrophobic), while the mobile phase is an aqueous, moderate polar.**
- **It works on the principle of hydrophobic interactions; hence the more nonpolar the material is, the longer it will be retained.**
- **This technique is used for non-polar, polar, ionizable, and ionic molecules.**

3. Size-exclusion HPLC

- **It is also known as gel permeation chromatography or gel filtration chromatography.**
- **The column is filled with a material having precisely controlled pore sizes, and the particles are separated according to their molecular size.**

- **Larger molecules are rapidly washed through the column; smaller molecules penetrate the porous packing particles and elute later.**
- **Size-exclusion chromatography is also helpful in determining the tertiary and quaternary structure of proteins and amino acids.**
- **It is also used for the determination of the molecular weight of polysaccharides.**

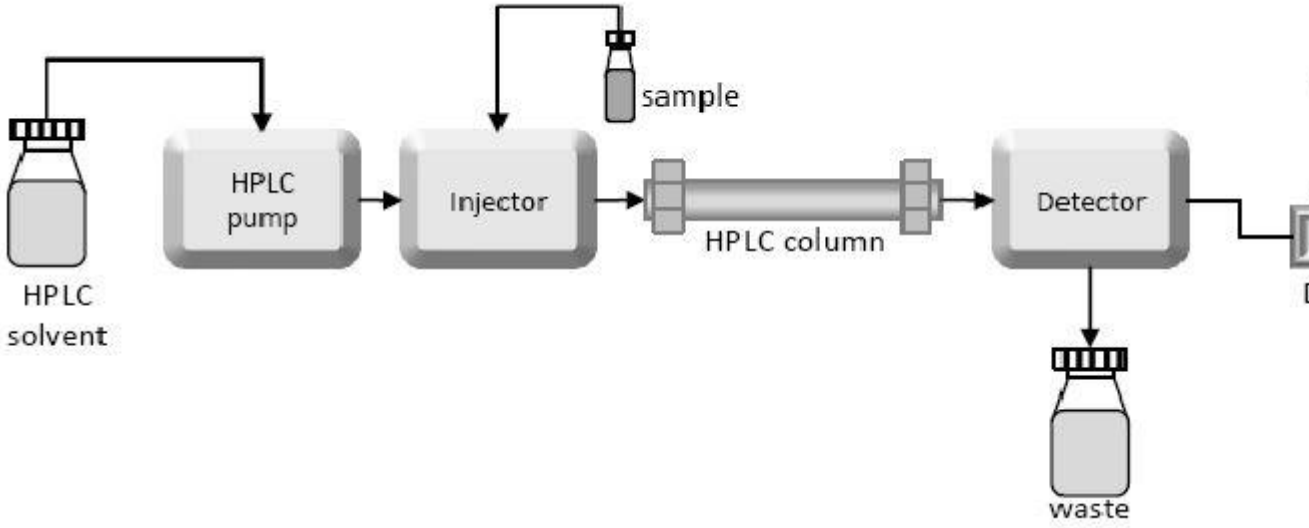
4. Ion-Exchange HPLC

- **In this type of chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase.**
- **Same charged ions are excluded.**
- **This technique is used in purifying water, Ligand and Ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc.**

5. Bio-affinity HPLC

- **In this type of chromatography, separation is based on the reversible interaction of proteins with ligands.**

Instrumentation of HPLC



The schematic diagram the above figure shows that the basic HPLC system consists of a pump, injector, column, detector, and integrator or acquisition and display system.

The heart of the system is the column where separation occurs.

1. Solvent Reservoir

- **Mobile phase contents are contained in a glass reservoir.**
- **The mobile phase, or solvent, in HPLC, is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.**

2. Pump

- **A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detecter.**
- **Depending on several factors, including column dimensions, the particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.**

3. Sample Injector

- **The injector can be a single injection or an automated injection system.**
- **An injector for an HPLC system should provide an injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).**

4. Columns

- **Columns are usually made of polished stainless steel, are between 50 and 300 mm long, and have an internal diameter between 2 and 5 mm.**
- **They are commonly filled with a stationary phase with a particle size of 3–10 µm.**

- **Columns with internal diameters of less than 2 mm are often called microbore columns.**
- **Ideally, the temperature of the mobile phase and the column should be kept constant during an analysis.**

5. Detector

- **The HPLC detector, located at the end of the column, detects the analytes as they elute from the chromatographic column.**
- **Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.**

6. Data Collection Devices

- **Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and their ability to process, store and reprocess chromatographic data.**
- **The computer integrates the detector's response to each component and places it into a chromatograph that is easy to read and interpret.**

Applications of HPLC

The information that HPLC can obtain includes resolution, identification, and quantification of a compound. It also aids in

chemical separation and purification. The other applications of HPLC include

Pharmaceutical Applications

1. **To control drug stability.**
2. **Tablet dissolution study of pharmaceutical dosages form.**
3. **Pharmaceutical quality control.**

Environmental Applications

1. **Detection of phenolic compounds in drinking water.**
2. **Bio-monitoring of pollutants.**

Applications in Forensics

1. **Quantification of drugs in biological samples.**
2. **Identification of steroids in blood, urine, etc.**
3. **Forensic analysis of textile dyes.**
4. **Determination of cocaine and other drugs of abuse in blood, urine, etc.**

Food and Flavour

1. **Measurement of Quality of soft drinks and water.**
2. **Sugar analysis in fruit juices.**

3. **Analysis of polycyclic compounds in vegetables.**
4. **Preservative analysis.**

Applications in Clinical Tests

1. **Urine analysis, antibiotics analysis in blood.**
2. **Analysis of bilirubin, biliverdin in hepatic disorders.**
3. **Detection of endogenous Neuropeptides in the extracellular fluid of the brain etc.**

Limitations

The limitation of using high-performance liquid chromatography (HPLC) is the following.

- **HPLC is much more costly requires a large number of expensive organics.**
- **HPLC may have low sensitivity for certain compounds, and some cannot even be detected as they are irreversibly adsorbed.**
- **Complexity**
- **Volatile substances are much better to be separated by gas chromatography.**

Frequently asked Questions

Q 1. What is liquid chromatography?

Liquid chromatography is one of the three main branches of chromatography. It involves a small volume of liquid sample placement into a tube packed with porous particles. The individual components of the sample are transported along the column by a liquid moved with gravity. The sample components are separated and then collected at the exit of this column.

Q 2. What is the principle of HPLC?

The principle of HPLC is based on analyte distribution between the mobile and stationary phases. It is crucial to remember that the sample's different constituents elute at various times before the sample ingredients' separation is achieved. The intermolecular interactions between sample and packaging materials molecules determine their time on-column.

Q 3. What are the types of HPLC?

There are four primary types of HPLC

1. Normal phase HPLC (effective method for separating phospholipid classes)

2. Reverse phase HPLC (the most common method used to separate

compounds that have hydrophobic moieties)

3. Size-exclusion HPLC/molecular sieve chromatography (Used in large molecules/macromolecular complexes such as industrial polymers and proteins)

4. Ion-exchange HPLC (separates ions and polar molecules according to their ion exchanger.

Q 4. What are the four types of chromatography?

The four types of chromatography are

1. Liquid chromatography (test for pollution in water samples like lakes and rivers)

2. Gas chromatography (detect bombs and valuable in forensic investigations)

3. Thin-layer chromatography (used to check the purity of organic compounds such as the presence of insecticide or pesticide in foods)

4. Paper chromatography (uses a strip of paper in the stationary phase).

Q5. Why is high pressure needed in HPLC?

HPLC uses a moderate to high pressure to achieve the desired flow rate of the solvent through the chromatographic column as small particles have more excellent resistance to flow.

Q 6. What is the difference between isocratic and gradient?

Your application can be run in different ways – isocratic and gradient.

Isocratic is when the mobile phase mixture is consistent over the total testing time.

With a gradient, the compounding of the eluent mixture is changed during measurement, which significantly affects analyte retention. It can accelerate or decelerate the separation process.

Q 7. What solvent is used in HPLC?

Different solvents are used in HPLC, such as aqueous solvent (water) and organic solvent (methanol, acetonitrile, and propanol). To improve the chromatographic peak shape, acids such as acetic acid, formic acid, and trifluoroacetic acid can be used.

Q 8. What is the difference between UV and PDA detectors?

The PDA and UV are both absorbance detectors, which provide sensitivity for light-absorbing compounds. The UV detector is most commonly used for HPLC analysis.

The UV absorbance differs on the wavelength used, so it is essential to choose the right wavelength based on the type of analyte. On the other hand, the PDA detector adds a third dimension wavelength,

which is a more convenient way of finding out the wavelength without repeating the analysis.

Q 9. What are the advantages of HPLC?

The advantages of HPLC are as follows:

1. It can test both raw materials and finished products.

2. It can reverse engineer formulations.

3. It helps solve product failure problems.

4. It can detect contaminants and other impurities.

5. It can perform competitor product analysis.

6. It can determine product stability and shelf life.

7. The testing can be done even with just a small sample size.

8. It enables you to modify the testing depending on the needed quantification level.

9. The results it produced are reliable.

10. It helps develop better products.

11. It lets you gain a better understanding of the competitor's products.

Q 10. What is Rf value?

In chromatography, the RF value pertains to the distance a particular component traveled divided by the distance traveled by

the solvent front. In other words, it is the characteristic of the component which is helpful in the identification of the components.

Q 11. What are the two main types of chromatography?

There are different types of chromatography, but the two primary types are liquid chromatography and gas chromatography.

Q 12. What is retention time?

It is when a specific analyte comes out of the end of the column.

PAPER CHROMATOGRAPHY

This page is an introduction to paper chromatography - including two way chromatography.

Carrying out paper chromatography

Background

Chromatography is used to separate mixtures of substances into their components. All forms of chromatography work on the same principle.

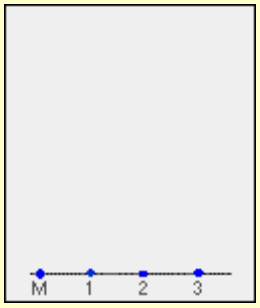
They all have a ***stationary phase*** (a solid, or a liquid supported on a solid) and a ***mobile phase*** (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Different components travel at different rates. We'll look at the reasons for this further down the page.

In paper chromatography, the stationary phase is a very uniform absorbent paper. The mobile phase is a suitable liquid solvent or mixture of solvents.

Producing a paper chromatogram

You probably used paper chromatography as one of the first things you ever did in chemistry to separate out mixtures of coloured dyes - for example, the dyes which make up a particular ink. That's an easy example to take, so let's start from there.

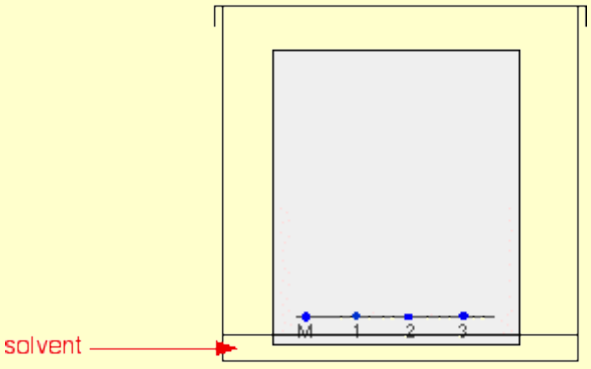
Suppose you have three blue pens and you want to find out which one was used to write a message. Samples of each ink are spotted on to a pencil line drawn on a sheet of chromatography paper. Some of the ink from the message is dissolved in the minimum possible amount of a suitable solvent, and that is also spotted onto the same line. In the diagram, the pens are labelled 1, 2 and 3, and the message ink as M.



Note: The chromatography paper will in fact be pure white - not pale grey. I'm forced to show it as off-white because of the way I construct the diagrams. Anything I draw as pure white allows the background colour of the page to show through.

The paper is suspended in a container with a shallow layer of a suitable solvent or mixture of solvents in it. It is important that the solvent level is below the line with the spots on it. The next diagram doesn't show details of how the paper is suspended because there are too many possible ways of doing it and it clutters the diagram. Sometimes the paper is just coiled into a loose cylinder and fastened with paper clips top and bottom. The cylinder then just stands in the bottom of the container.

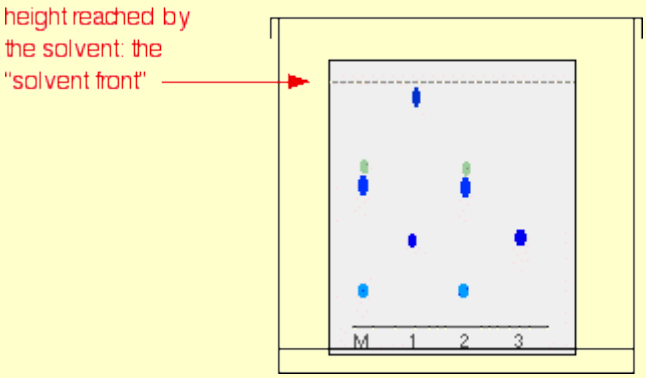
The reason for covering the container is to make sure that the atmosphere in the beaker is saturated with solvent vapour. Saturating the atmosphere in the beaker with vapour stops the solvent from evaporating as it rises up the paper.



As the solvent slowly travels up the paper, the different components of the ink mixtures travel at different rates and the

mixtures are separated into different coloured spots.

The diagram shows what the plate might look like after the solvent has moved almost to the top.



It is fairly easy to see from the final chromatogram that the pen that wrote the message contained the same dyes as pen 2. You can also see that pen 1 contains a mixture of two different blue dyes - one of which *might* be the same as the single dye in pen 3.

R_f values

Some compounds in a mixture travel almost as far as the solvent does; some stay much closer to the base line. The distance travelled relative to the solvent is a constant for a particular compound as long as you keep everything else constant - the type of paper and the exact composition of the solvent, for example.

The distance travelled relative to the solvent is called the R_f value. For each compound it can be worked out using the formula:

$$R_f = \frac{\text{distance travelled by compound}}{\text{distance travelled by solvent}}$$

For example, if one component of a mixture travelled 9.6 cm from the base line while the solvent had travelled 12.0 cm, then

the R_f value for that component is:

$$R_f = \frac{9.6}{12.0} = 0.80$$

In the example we looked at with the various pens, it wasn't necessary to measure R_f values because you are making a direct comparison just by looking at the chromatogram.

You are making the assumption that if you have two spots in the final chromatogram which are the same colour and have travelled the same distance up the paper, they are most likely the same compound. It isn't necessarily true of course - you could have two similarly coloured compounds with very similar R_f values. We'll look at how you can get around that problem further down the page.

What if the substances you are interested in are colourless?

In some cases, it may be possible to make the spots visible by reacting them with something which produces a coloured product. A good example of this is in chromatograms produced from amino acid mixtures.

Suppose you had a mixture of amino acids and wanted to find out which particular amino acids the mixture contained. For simplicity we'll assume that you know the mixture can only possibly contain five of the common amino acids.

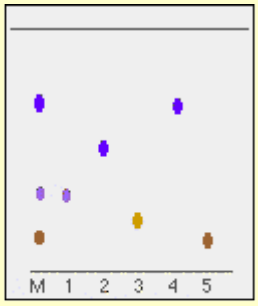
A small drop of a solution of the mixture is placed on the base line of the paper, and similar small spots of the known amino acids are placed alongside it. The paper is then stood in a suitable solvent and left to develop as before. In the diagram, the mixture is M, and the known amino acids are labelled 1 to 5.

The position of the solvent front is marked in pencil and the chromatogram is allowed to dry and is then sprayed with a solution of ***ninhydrin***. Ninhydrin reacts with amino acids to give coloured compounds, mainly brown or purple.

The left-hand diagram shows the paper after the solvent front has almost reached the top. The spots are still invisible. The second diagram shows what it might look like after spraying with

ninhydrin.

before spraying with ninhydrin



after spraying with ninhydrin

There is no need to measure the R_f values because you can easily compare the spots in the mixture with those of the known amino acids - both from their positions and their colours.

In this example, the mixture contains the amino acids labelled as 1, 4 and 5.

And what if the mixture contained amino acids other than the ones we have used for comparison? There would be spots in the mixture which didn't match those from the known amino acids. You would have to re-run the experiment using other amino acids for comparison.

Two way paper chromatography

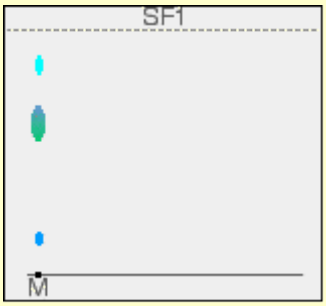
Two way paper chromatography gets around the problem of separating out substances which have very similar R_f values.

I'm going to go back to talking about coloured compounds because it is much easier to see what is happening. You can perfectly well do this with colourless compounds - but you have to use quite a lot of imagination in the explanation of what is going on!

This time a chromatogram is made starting from a single spot of mixture placed towards one end of the base line. It is stood in a solvent as before and left until the solvent front gets close to the top of the paper.

In the diagram, the position of the solvent front is marked in pencil before the paper dries out. This is labelled as SF1 - the

solvent front for the first solvent. We shall be using two different solvents.

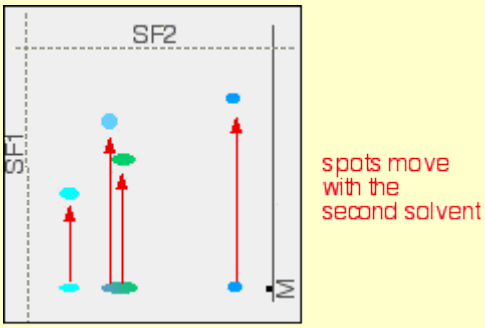


If you look closely, you may be able to see that the large central spot in the chromatogram is partly blue and partly green. Two dyes in the mixture have almost the same R_f values. They could equally well, of course, both have been the same colour - in which case you couldn't tell whether there was one or more dye present in that spot.

What you do now is to wait for the paper to dry out completely, and then rotate it through 90°, and develop the chromatogram again in a different solvent.

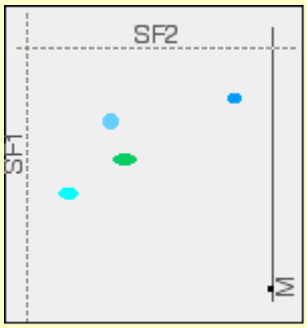
It is very unlikely that the two confusing spots will have the same R_f values in the second solvent as well as the first, and so the spots will move by a different amount.

The next diagram shows what might happen to the various spots on the original chromatogram. The position of the second solvent front is also marked.



You wouldn't, of course, see these spots in both their original

and final positions - they have moved! The final chromatogram would look like this:



Two way chromatography has completely separated out the mixture into four distinct spots.

If you want to identify the spots in the mixture, you obviously can't do it with comparison substances on the same chromatogram as we looked at earlier with the pens or amino acids examples. You would end up with a meaningless mess of spots.

You can, though, work out the R_f values for each of the spots in both solvents, and then compare these with values that you have measured for known compounds under exactly the same conditions.

How does paper chromatography work?

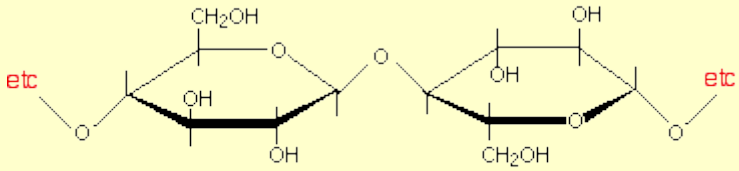
Although paper chromatography is simple to do, it is quite difficult to explain compared with thin layer chromatography. The explanation depends to some extent on what sort of solvent you are using, and many sources gloss over the problem completely. If you haven't already done so, it would be helpful if you could read the explanation for how thin layer chromatography works (link below). That will save me a lot of repetition, and I can concentrate on the problems.

Note: You will find the explanation for how thin layer chromatography works by following this link.

Use the BACK button on your browser to return quickly to this page when yhou have read it.

The essential structure of paper

Paper is made of cellulose fibres, and cellulose is a polymer of the simple sugar, glucose.



cellulose

The key point about cellulose is that the polymer chains have -OH groups sticking out all around them. To that extent, it presents the same sort of surface as silica gel or alumina in thin layer chromatography.

It would be tempting to try to explain paper chromatography in terms of the way that different compounds are adsorbed to different extents on to the paper surface. In other words, it would be nice to be able to use the same explanation for both thin layer and paper chromatography. Unfortunately, it is more complicated than that!

The complication arises because the cellulose fibres attract water vapour from the atmosphere as well as any water that was present when the paper was made. You can therefore think of paper as being cellulose fibres with a very thin layer of water molecules bound to the surface.

It is the interaction with this water which is the most important effect during paper chromatography.

Paper chromatography using a non-polar solvent

Suppose you use a non-polar solvent such as hexane to develop your chromatogram.

Non-polar molecules in the mixture that you are trying to separate will have little attraction for the water molecules attached to the cellulose, and so will spend most of their time

dissolved in the moving solvent. Molecules like this will therefore travel a long way up the paper carried by the solvent. They will have relatively high R_f values.

On the other hand, polar molecules *will* have a high attraction for the water molecules and much less for the non-polar solvent. They will therefore tend to dissolve in the thin layer of water around the cellulose fibres much more than in the moving solvent.

Because they spend more time dissolved in the stationary phase and less time in the mobile phase, they aren't going to travel very fast up the paper.

The tendency for a compound to divide its time between two immiscible solvents (solvents such as hexane and water which won't mix) is known as ***partition***. Paper chromatography using a non-polar solvent is therefore a type of ***partition chromatography***.

Paper chromatography using a water and other polar solvents

A moment's thought will tell you that partition can't be the explanation if you are using water as the solvent for your mixture. If you have water as the mobile phase and the water bound on to the cellulose as the stationary phase, there can't be any meaningful difference between the amount of time a substance spends in solution in either of them. All substances should be equally soluble (or equally insoluble) in both.

And yet the first chromatograms that you made were probably of inks using water as your solvent.

If water works as the mobile phase as well being the stationary phase, there has to be some quite different mechanism at work - and that must be equally true for other polar solvents like the alcohols, for example. Partition only happens between solvents which don't mix with each other. Polar solvents like the small alcohols do mix with water.

In researching this topic, I haven't found any easy explanation for what happens in these cases. Most sources ignore the problem altogether and just quote the partition explanation without making any allowance for the type of solvent you are using. Other sources quote mechanisms which have so many strands to them that they are far too complicated for this

introductory level. I'm therefore not taking this any further - you shouldn't need to worry about this at UK A level, or its various equivalents.

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Column Chromatography-Principle, Types, Applications

Written By Adeel Abbas

Column chromatography is a widely used technique of chromatography in chemistry to separate and purify a mixture of chemical compounds. Mostly it is used in natural product isolation.

I also used this technique during my research on Boerhavia Procumbens. The goal of this technique is to isolate each component in a mixture based on its physical and chemical properties. In this article, we will take a closer look at what column chromatography is, its principle, procedure, and applications.

What is Column Chromatography?

Column chromatography is a separation technique in which a column is used. The column is filled with a solid stationary phase which is silica. it is tightly packed for better separation. A liquid mobile phase to separate a mixture of chemical compounds is passed through the column. The mobile phase dissolves compounds and flows out of the column.

Column Chromatography Principle

The principle of column chromatography is based on the fact that different components in a mixture have different affinities for the stationary and mobile phases. When the mixture is passed through the column, the components will separate based on their different interactions with the stationary and mobile phases. This allows each component to be isolated and collected separately.

Common mobile and stationary phase used in column chromatography

Mobile Phase	Stationary Phase
Hexane	Silica gel
Ethanol	Sephadex LH-20
Acetonitrile	C18
Methanol	Florisil
Water	Reverse-phase silica
Toluene	Alumina
Chloroform	Diol
Acetone	Ion exchange resins
Isopropanol	Cellulose
Petroleum ether	Polymeric resins

Table showing different mobile and stationary phases used in column chromatography

Column Chromatography Diagram

A column chromatography setup typically consists of a column, a pump, a detector, and a collection system, and holding clamps. A detector is used in advance columns. At basic level it is done manually by performing thing layer chromatography and checking it under uv lamp.

The column is filled with the stationary phase and the mixture is introduced at the top of the column. The mobile phase is then pumped through the column, carrying the mixture along with it. As the mixture passes through the column, the components will separate based on their different interactions with the stationary and mobile phases. The separated components are then collected at the bottom of the column.

Column Chromatography Procedure

The procedure for column chromatography can vary depending on the type of column and the specific requirements of the separation. However, the basic steps of a column chromatography procedure are as follows:

1. Prepare the stationary phase and fill the column with it.
2. Load the mixture to be separated at the top of the column.
3. Start the flow of the mobile phase through the column.
4. Monitor the progress of the separation with a detector.
5. Collect the separated components at the bottom of the column.
6. Perform elution to wash the components from the column.

Column Chromatography Experiment

Column chromatography experiments are a key part of many chemistry research projects and are used to separate and purify different components in a mixture. The following is a step-by-step guide to performing a column chromatography experiment:

1: Choose the appropriate column chromatography technique

There are several different techniques of column chromatography to choose from, including ion exchange chromatography, gel filtration chromatography, reverse phase chromatography, affinity chromatography, and hydrophobic interaction chromatography. The choice of technique will depend on the properties of the mixture and the desired outcome of the separation.

2: Prepare the stationary phase

The stationary phase is the material in the column that will retain the components of the mixture. It is important to choose the appropriate stationary phase for the separation and to prepare it according to the manufacturer's instructions.

3: Load the mixture

The mixture to be separated should be carefully loaded into the column. It is important to take care not to damage the stationary phase or to introduce any contaminants into the column.

4: Start the flow of the mobile phase

The mobile phase is the liquid that will flow through the column and carry the mixture along with it. It is important to choose the appropriate mobile phase for the separation and to set the flow rate correctly.

5: Monitor the progress of the separation

The progress of the separation can be monitored using a detector, such as a UV-Vis spectrophotometer. The detector measures the amount of each component in the mixture as it passes through the column.

6: Collect the separated components

The separated components will be collected at the bottom of the column. It is important to collect the components in a way that preserves their purity and to store them correctly for further analysis.

7: Perform elution

Elution is the process of washing the separated components from the column. This is done by changing the conditions of the mobile phase, such as the solvent or the flow rate, to release the retained components from the stationary phase. The eluted components are then collected for further analysis or use.

In conclusion, a column chromatography experiment is a carefully controlled process that requires careful attention to detail in order to obtain accurate and reproducible results. By following the steps outlined above, you can perform a successful column chromatography experiment and obtain high-quality separated components for your research.

Types of Column Chromatography

There are several types of column chromatography, each with its own unique characteristics and applications. Some of the most commonly used types of column chromatography include:

- Ion Exchange Column Chromatography

- Gel Filtration Chromatography
- Reverse Phase Chromatography
- Affinity Chromatography
- Hydrophobic Interaction Chromatography

Column Chromatography Applications

Column chromatography is a powerful tool for separating and purifying different components in a mixture. The following are some of the most common applications of column chromatography:

1. **Biotechnology:** Column chromatography is used extensively in the biotechnology industry to purify proteins and other biological molecules for use in research and as therapeutic drugs.
2. **Pharmaceuticals:** Column chromatography is used in the pharmaceutical industry to purify active ingredients and impurities from drug products. This is important for ensuring the quality and safety of drugs.
3. **Environmental analysis:** Column chromatography is used in environmental analysis to separate and identify contaminants in water, air, and soil samples.
4. **Food and beverage analysis:** Column chromatography is used in the food and beverage industry to analyze food ingredients, such as vitamins and minerals, and to monitor the quality of food and beverage products.
5. **Analytical chemistry:** Column chromatography is a common technique in analytical chemistry for separating and purifying different components in a sample for further analysis. This can be used in fields such as drug discovery, forensic science, and metabolic profiling.
6. **Process chromatography:** Column chromatography is also used in process chromatography to purify large quantities of a product, such as a food ingredient or a pharmaceutical, for commercial use.