6Th Semester Theory

DSE4T : Research Methodology and Design
Tools techniques for research in Physiology and Health Science :

COLUMN CHROMATOGRAPHY :

➢ What Is Column Chromatography?

In chemistry, Column chromatography is a technique which is used to separate a single chemical compound from a mixture dissolved in a fluid. It separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allow them to get separated in fractions. This technique can be used on small scale as well as large scale to purify materials that can be used in future experiments. This method is a type of adsorption chromatography technique.

➢ Column Chromatography Principle

When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slow are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

R_f = the distance travelled by solute/ the distance travelled by solvent

R_f is the retardation factor.

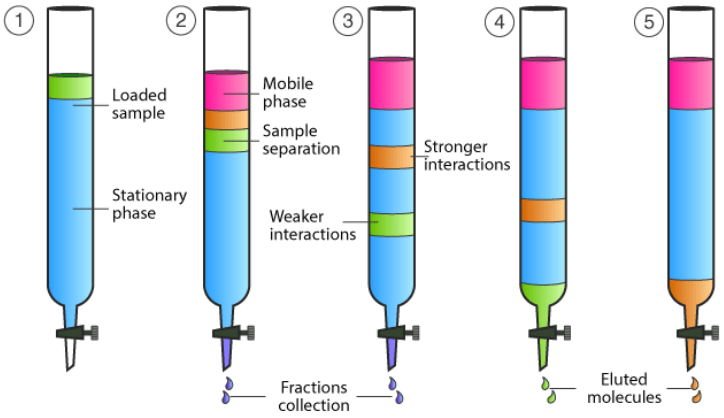


Figure : Column Chromatography

➢ Column Chromatography Procedure

Before starting with the Column Chromatography Experiment let us understand the different phases involved.

Mobile phase – This phase is made up of solvents and it performs the following functions:

1. It acts as a solvent – sample mixture can be introduced in the column.
2. It acts as a developing agent – helps in the separation of components in the sample to form bands.
3. It acts as an eluting agent – the components that are separated during the experiment are removed from the column
4. Some examples of solvents used as mobile phase based on their polarity are – ethanol, acetone, water, acetic acid, pyridine, etc.

Stationary phase – It is a solid material which should have good adsorption property and meet the conditions given below:

1. Shape and size of particle: Particles should have uniform shape and size in the range of 60 – 200μ in diameter.
2. Stability and inertness of particles: high mechanical stability and chemically inert. Also, no reaction with acids or bases or any other solvents used during the experiment.
3. It should be colourless, inexpensive and readily available.
4. Should allow free flow of mobile phase
5. It should be suitable for the separation of mixtures of various compounds.

➢ Column Chromatography Experiment

- The stationary phase is made wet with the help of solvent as the upper level of the mobile phase and the stationary phase should match. The mobile phase or eluent is either solvent or mixture of solvents. In the first step the compound mixture that needs to be separated, is added from the top of the column without disturbing the top level. The tap is turned on and the adsorption process on the surface of silica begins.
- Without disturbing the stationary phase solvent mixture is added slowly by touching the sides of the glass column. The solvent is added throughout the experiment as per the requirement.
- The tap is turned on to initiate the movement of compounds in the mixture. The movement is based on the polarity of molecules in the sample. The non-polar components move at a greater speed when compared to the polar components.
- For example, a compound mixture consists of three different compounds viz red, blue, green then their order based on polarity will be as follows blue>red>green
- As the polarity of the green compound is less, it will move first. When it arrives at the end of the column it is collected in a clean test tube. After this,

the red compound is collected and at last blue compound is collected. All these are collected in separate test tubes.

➢ Column Chromatography Applications

- Column Chromatography is used to isolate active ingredients.
- It is very helpful in Separating compound mixtures.
- It is used to determine drug estimation from drug formulations
- It is used to remove impurities.
- Used to isolation metabolites from biological fluids.

➢ Types of Column Chromatography:

1. Adsorption column chromatography – Adsorption chromatography is a technique of separation, in which the components of the mixture are adsorbed on the surface of the adsorbent.

2. Partition column chromatography – The stationary phase, as well as mobile phase, are liquid in partition chromatography.

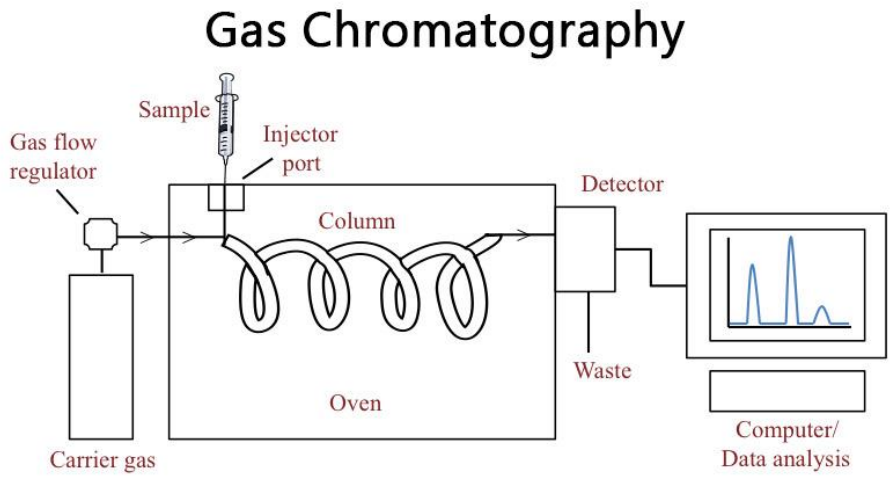
3. Gel column chromatography – In this method of chromatography, the separation takes place through a column packed with gel. The stationary phase is a solvent held in the gap of a solvent.

4. Ion exchange column chromatography – A chromatography technique in which the stationary phase is always ion exchange resin.

GAS CHROMATOGRAPHY :

- Gas chromatography differs from other forms of **chromatography** in that the mobile phase is a gas and the components are separated as vapors.
- It is thus used to separate and detect small molecular weight compounds in the gas phase.
- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas, typically helium because of its low molecular weight and being chemically inert.
- The pressure is applied and the mobile phase moves the analyte through the column. The separation is accomplished using a column coated with a stationary phase.
- The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase.
- Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer **retention time (Rt)** than samples that have a higher affinity for the mobile phase.

- Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation.
- Ideal peaks are Gaussian distributions and symmetrical, because of the random nature of the analyte interactions with the column.
  - ❖ The separation is hence accomplished by partitioning the sample between the gas and a thin layer of a nonvolatile liquid held on a solid support.
  - ❖ A sample containing the solutes is injected into a heated block where it is immediately vaporized and swept as a plug of vapor by the carrier gas stream into the column inlet.
  - ❖ The solutes are adsorbed by the stationary phase and then desorbed by a fresh carrier gas.
  - ❖ The process is repeated in each plate as the sample is moved toward the outlet.
  - ❖ Each solute will travel at its own rate through the column.
  - ❖ Their bands will separate into distinct zones depending on the partition coefficients, and band spreading.
  - ❖ The solutes are eluted one after another in the increasing order of their kd, and enter into a detector attached to the exit end of the column.
  - ❖ Here they register a series of signals resulting from concentration changes and rates of elution on the recorder as a plot of time versus the composition of carrier gas stream.
  - ❖ The appearance time, height, width, and area of these peaks can be measured to yield quantitative data.



Gas chromatography is mainly composed of the following parts:

1. **Carrier gas in a high-pressure cylinder with attendant pressure regulators and flow meters**
   - Helium, N_2, H, Argon are used as carrier gases.

- Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapors.
- N_2 is preferable when a large consumption of carrier gas is employed.
- Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml/min), capillary restrictors, and a pressure gauge (1-4 atm).
- Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.
- The operating efficiency of the gas chromatograph is directly dependant on the maintenance of constant gas flow.

2. **Sample injection system**
   - Liquid samples are injected by a microsyringe with a needle inserted through a self-scaling, silicon-rubber septum into a heated metal block by a resistance heater.
   - Gaseous samples are injected by a gas-tight syringe or through a by-pass loop and valves.
   - Typical sample volumes range from 0.1 to 0.2 ml.
3. **The separation column**
   - The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.
   - Copper is useful up to 250^0
   - Swege lock fittings make column insertion easy.
   - Several sizes of columns are used depending upon the requirements.
4. **Liquid phases**
   - An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.
   - No single phase will serve for all separation problems at all temperatures.

Non-Polar – Parafin, squalane, silicone greases, apiezon L, silicone gum rubber. These materials separate the components in order of their boiling points.
Intermediate Polarity – These materials contain a polar or polarizable group on a long non-polar skeleton which can dissolve both polar and non-polar solutes. For example. diethyl hexyl phthalate is used for the separation of high boiling alcohols.
Polar – Carbowaxes – Liquid phases with a large proportion of polar groups. Separation of polar and non-polar substances.
Hydrogen bonding – Polar liquid phases with high hydrogen bonding e.g. Glycol.
Specific purpose phases – Relying on a chemical reaction with solute to achieve separations. e.g AgNO3 in glycol separates unsaturated hydrocarbons.

5. **Supports**
   - The structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.

- The support should be inert but capable of immobilizing a large volume of liquid phase as a thin film over its surface.
- The surface area should be large to ensure the rapid attainment of equilibrium between stationary and mobile phases.
- Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.
- Diatomaceous earth, kieselguhr treated with Na 2CO 3 for 900^0 C causes the particle fusion into coarser aggregates.
- Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases.
- Porous polymer beads differing in the degree of cross-linking of styrene with alkyl-vinyl benzene are also used which are stable up to 250^0

6. **Detector**
   - Detectors sense the arrival of the separated components and provide a signal.
   - These are either concentration-dependent or mass dependant.
   - The detector should be close to the column exit and the correct temperature to prevent decomposition.
7. **Recorder**
   - The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.
   - An integrator may be a good addition.

Procedure of Gas Chroatography :

Step 1: Sample Injection and Vapourization

1. A small amount of liquid sample to be analyzed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
3. The injection of the sample is considered to be a “point” in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
5. The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

Step 2: Separation in the Column

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will,

therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.

- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.
- If we consider a 2 component mixture in which component A is more polar than component B then:
    1. component A will have a **longer retention time** in a polar column than component B
    2. component A will have a **shorter retention time** in a non-polar column than component B

Step 3: Detecting and Recording Results

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.

Applications :

- GC analysis is used to calculate the content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water.
- Gas chromatography is used in the analysis of:
  (a) air-borne pollutants
  (b) performance-enhancing drugs in athlete's urine samples
  (c) oil spills
  (d) essential oils in perfume preparation
- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Gas Chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence.

Advantages :

- The use of longer columns and higher velocity of carrier gas permits the fast separation in a matter of a few minutes.

- Higher working temperatures up to 5000C and the possibility of converting any material into a volatile component make gas chromatography one of the most versatile techniques.
- GC is popular for environmental monitoring and industrial applications because it is very reliable and can be run nearly continuously.
- GC is typically used in applications where small, volatile molecules are detected and with non-aqueous solutions.
- GC is favored for non-polar molecules.

Limitations :

- Compound to be analyzed should be stable under GC operation conditions.
- They should have a vapor pressure significantly greater than zero.
- Typically, the compounds analyzed are less than 1,000 Da, because it is difficult to vaporize larger compounds.
- The samples are also required to be salt-free; they should not contain ions.
- Very minute amounts of a substance can be measured, but it is often required that the sample must be measured in comparison to a sample containing the pure, suspected substance known as a reference standard.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY :

Affinities for Mobile and Stationary Phases

All chromatographic separations, including HPLC operate under the same basic principle; every compound interacts with other chemical species in a characteristic manner. Chromatography separates a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

Distribution Constant

All chemical reactions have a characteristic equilibrium constant. For the reaction

$$A_{aq} + B_s \rightleftharpoons A_{bs} \quad (1)$$

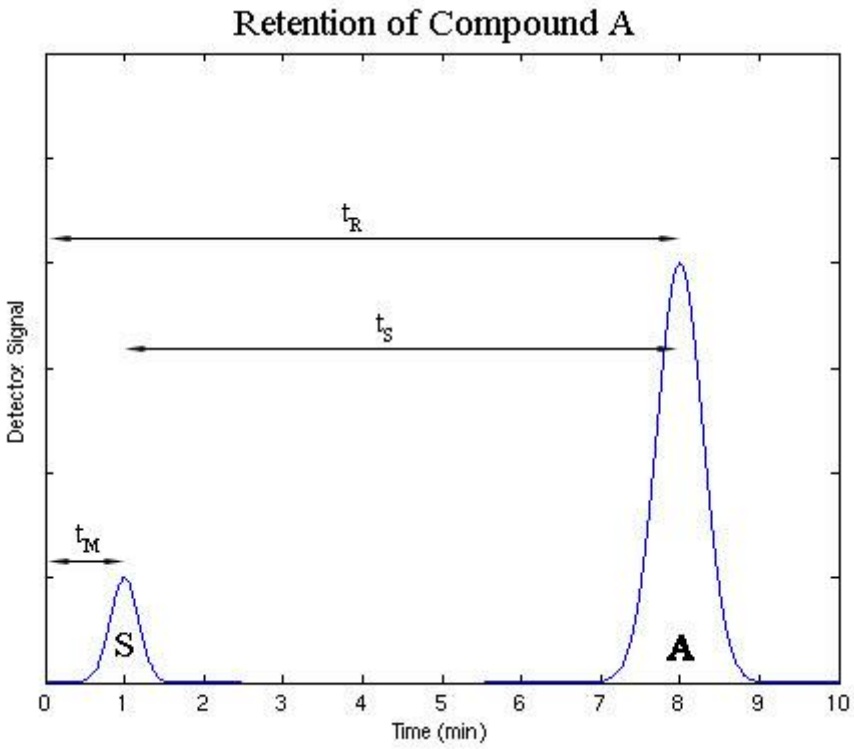
There is a chemical equilibrium constant $\mathbf{K_{eq}}$ that dictates what percentage of compound **A** will be in solution and what percentage will be bound to the stationary compound **B**. During a chromatographic separation, there is similar relationship between compound **A** and the solvent, or **mobile phase**, **C**. This will yield an overall equilibrium equation which dictates the quantity of **A** that will be associated with the stationary phase and the quantity of **A** that will be associated with the mobile phase.

$$A_{mobile} \rightleftharpoons A_{stationary} \quad (2)$$

The equilibrium between the mobile phase and stationary phase is given by the constant $\mathbf{K_c}$.

$$Kc=(aA)S/(aA)M \approx cS/cM (3)$$

Where $\mathbf{K_c}$, the **distribution constant**, is the ratio of the activity of compound **A** in the stationary phase and activity of compound **A** in the mobile phase. In most separations, which contain low concentrations of the species to be separated, the activity of **A** in each is approximately equal to the concentration of **A** in that state. The distribution constant indicates the amount of time that compound **A** spends adsorbed to the stationary phase as the opposed to the amount of time **A** spends solvated by the mobile phase. This relationship determines the amount of time it will take for compound **A** to travel the length of the column. The more time **A** spends adsorbed to the stationary phase, the more time compound **A** will take to travel the length of the column. The amount of time between the injection of a sample and its elution from the column is known as the **retention time**; it is given the symbol $\mathbf{t_R}$.



HPLC as a solution to efficiency problems

While all of these basic principles hold true for all chromatographic separations, HPLC was developed as method to solve some of the shortcomings of standard liquid chromatography. Classic liquid chromatography has several severe limitations as a separation method. When the solvent is driven by gravity, the separation is very slow, and if the solvent is driven by vacuum, in a standard packed column, the plate height increases and the effect of the vacuum is negated. The limiting factor in liquid chromatography was originally the size of the column packing, once columns could be packed with particles as small as 3 µm, faster separations could be performed in smaller, narrower, columns. High pressure was required to force the mobile phase and sample through these new columns, and previously unneeded apparatus was required to maintain reproducibility of results in this new instruments. The use of high

pressures in a narrow column allowed for a more effective separation to be achieved in much less time than was required for previous forms of liquid chromatography.

Apparatus

Specialized apparatus is required for an HPLC separation because of the high pressures and low tolerances under which the separation occurs. If the results are to be reproducible, then the conditions of the separation must also be reproducible. Thus HPLC equipment must be of high quality; it is therefore expensive.

Solvent

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. As the solvent is passed through a very narrow bore column, any contaminants could at worst plug the column, or at the very least add variability to the retention times during repeated different trials. Therefore HPLC solvent must be kept free of dissolved gases, which could come out of solution mid-separation, and particulates.

Column

In the **HPLC column**, the components of the sample separate based on their differing interactions with the column packing. If a species interacts more strongly with the stationary phase in the column, it will spend more time adsorbed to the column's adsorbent and will therefore have a greater retention time. Columns can be packed with solids such as silica or alumina; these columns are called **homogeneous columns**. If stationary phase in the column is a liquid, the column is deemed a **bonded column**. Bonded columns contain a liquid stationary phase bonded to a sold support, which is again usually silica or alumina. The value of the constant **C** described in the van Deemter equation is proportional, in HPLC, to the diameter of the particles that constitute the column's packing material.

Pump

The **HPLC pump** drives the solvent and sample through the column. To reduce variation in the elution, the pump must maintain a constant, pulse free, flow rate; this is achieved with **multi-piston pumps**. The presence of two pistons allows the flow rate to be controlled by one piston as the other recharges. A **syringe pump** can be used for even greater control of flow rate; however, the syringe pump is unable to produce as much pressure as a piston pump, so it cannot be used in all HPLC applications.

Detector

The **HPLC detector**, located at the end of the column, must register the presence of various components of the sample, but must not detect the solvent. For that reason there is no universal detector that works for all separations. A common HPLC detector is a **UV absorption detector**, as most medium to large molecules absorb UV radiation. Detectors that measure fluorescence and refractive index are also used for special applications. A relatively new development is the combination of an HPLC separation with an **NMR detector**. This

allows the pure components of the sample to be identified and quantified by nuclear magnetic resonance after having been separated by HPLC, in one integrated process.

Technique

Normal Phase vs. Reverse Phase

If the stationary phase is more polar than the mobile phase, the separation is deemed **normal phase**. If the stationary phase is less polar than the mobile phase, the separation is **reverse phase**. In reverse phase HPLC the retention time of a compound increases with decreasing polarity of the particular species. The key to an effective and efficient separation is to determine the appropriate ratio between polar and non-polar components in the mobile phase. The goal is for all the compounds to elute in as short a time as possible, while still allowing for the resolution of individual peaks. Typical columns for normal phase separation are packed with alumina or silica. Alkyl, aliphatic or phenyl bonded phases are typically used for reverse phase separation.

Gradient Elution vs. Isocratic Elution

If the composition of the mobile phase remains constant throughout the HPLC separation, the separation is deemed an **isocratic elution**. Often the only way to elute all of the compounds in the sample in a reasonable amount of time, while still maintaining peak resolution, is to change the ratio of polar to non-polar compounds in the mobile phase during the sample run. Known as **gradient chromatography**, this is the technique of choice when a sample contains components of a wide range of polarities. For a **reverse phase gradient**, the solvent starts out relatively polar and slowly becomes more non-polar. The gradient elution offers the most complete separation of the peaks, without taking an inordinate amount of time. A sample containing compounds of a wide range of polarities can be separated by a gradient elution in a shorter time period without a loss of resolution in the earlier peaks or excessive broadening of later peaks. However, gradient elution requires more complex and expensive equipment and it is more difficult to maintain a constant flow rate while there are constant changes in mobile phase composition. Gradient elution, especially at high speeds, brings out the limitations of lower quality experimental apparatus, making the results obtained less reproducible in equipment already prone to variation. If the flow rate or mobile phase composition fluctuates, the results will not be reproducible.

Applications

HPLC can be used in both qualitative and quantitative applications, that is for both compound identification and quantification. Normal phase HPLC is only rarely used now, almost all HPLC separation can be performed in reverse phase. Reverse phase HPLC (RPLC) is ineffective in for only a few separation types; it cannot separate inorganic ions (they can be separated by ion exchange chromatography). It cannot separate polysaccharides (they are too hydrophilic for any solid phase adsorption to occur), nor polynucleotides (they adsorb irreversibly to the reverse phase packing). Lastly, incredibly hydrophobic compounds cannot be separated effectively by RPLC (there is little selectivity). Aside from these few exceptions, RPLC is used for the separation of almost all other compound varieties. RPLC can be used to effectively separate similar simple and aromatic hydrocarbons, even those that differ only by a single methylene group. RPLC effectively separates simple amines, sugars,

lipids, and even pharmaceutically active compounds. RPLC is also used in the separation of amino acids, peptides, and proteins. Finally RPLC is used to separate molecules of biological origin. The determination of caffeine content in coffee products is routinely done by RPLC in commercial applications in order to guarantee purity and quality of ground coffee. HPLC is a useful addition to an analytical arsenal, especially for the separation of a sample before further analysis.