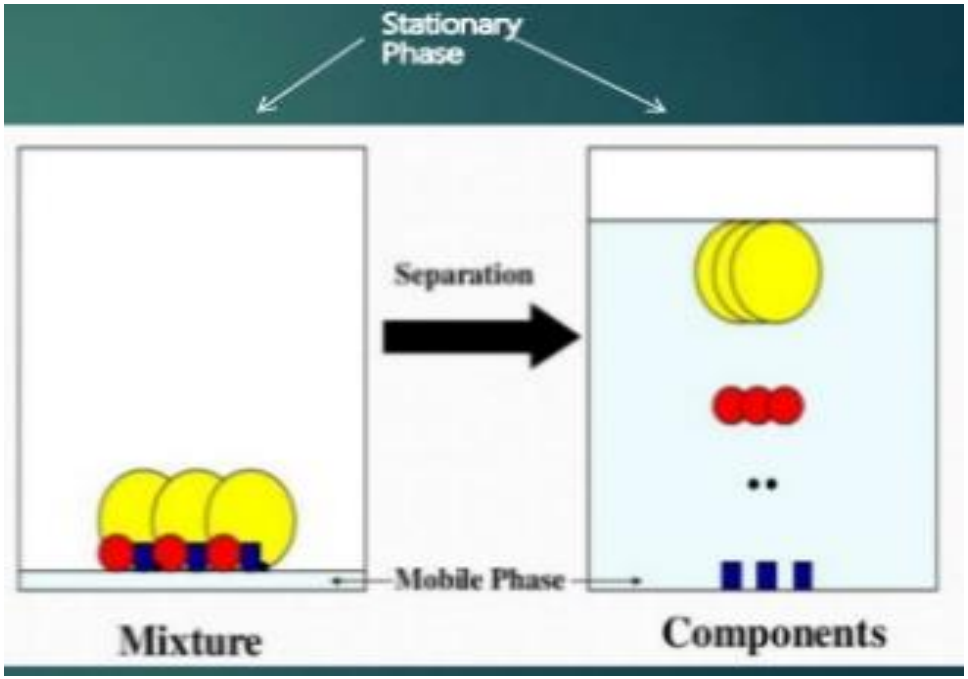
Chromatography

- Chromatography is a physical process where the components (solutes) of a sample mixture are separated as a result of their differential distribution between stationary and mobile phases. Greek chroma meaning ‘color’ and graphein meaning ‘writing’ Stationary Phase
- Tswet, Russian botanist (referred to as Father of chromatography) is credited for the development of chromatography.



PRINCIPLES OF CHROMATOGRAPHY

Distribution coefficients The basis of all forms of chromatography is the distribution or partition coefficient

(Kd), which describes the way in which a compound (the analyte) distributes between two immiscible phases. For two such phases A and B, the value for this coefficient is a

constant at a given temperature and is given by the expression:

$$\frac{\text{concentration in phase A}}{\text{concentration in phase B}} = K_d$$

The term effective distribution coefficient is defined as the total amount, as distinct from the concentration, of analyte present in one phase divided by the total amount present in the other phase.

Column chromatography

- Column chromatography is described as the useful technique in which the substances to be isolated are presented onto the highest point of a column loaded with an adsorbent (stationary phase), go through the column at various rates that rely upon the affinity of every substance for the adsorbent and the solvent or solvent mixture, and are typically gathered in solution as they pass from the column at various time.
- The two most common examples of stationary phases for column chromatography are silica gel and alumina while organic solvents are regarded as the most common.

principle

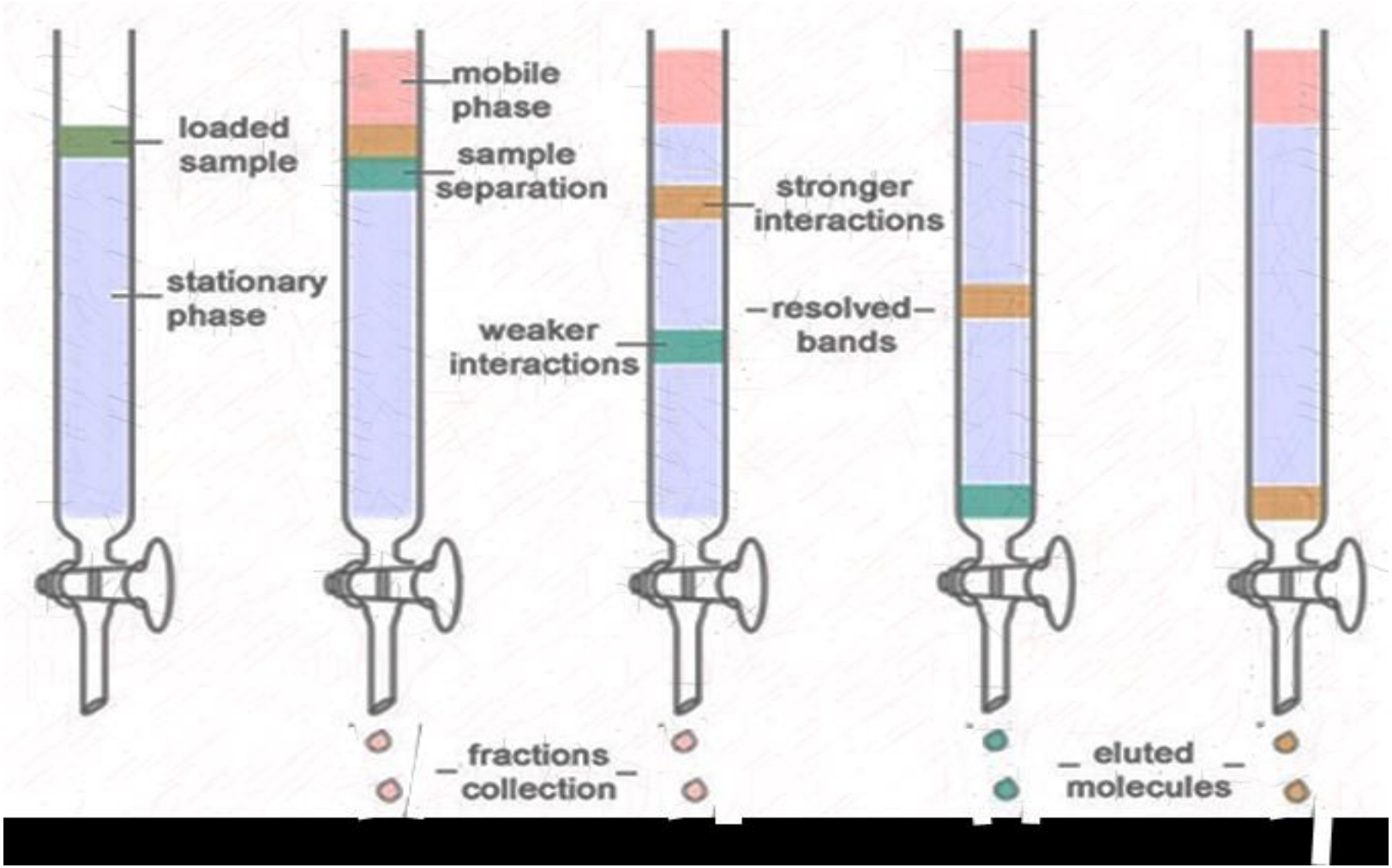
- The main principle involved in column chromatography is the adsorption of the solutes of the solution with the help of a stationary phase and afterward separates the mixture into independent components.
- At the point when the mobile phase together with the mixture that requires to be isolated is brought in from the top of the column, the movement of the individual components of the mixture is at various rates on mobile phases.

- The components with lower adsorption and affinity to the stationary phase head out quicker when contrasted with the greater adsorption and affinity with the stationary phase. The components that move rapidly are taken out first through the components that move slowly are eluted out last.

The adsorption of solute molecules to the column happens reversibly. The pace of the movement of the components is communicated as:

Rf = the distance traveled by solute/ the distance traveled by the solvent

Where Rf is called retardation factor


loaded sample
stationary phase
mobile phase
sample separation
weaker interactions
stronger interactions
resolved bands
fractions collection
eluted molecules

Column chromatography

Basic column chromatographic components

- **A stationary phase:** Chosen to be appropriate for the analytes to be separated.
- **A column**: In liquid chromatography these are generally 25-50 cm long and 4mm internal diameter and made of stainless steel whereas in gas chromatography they are 1-3m long and 2-4mm internal diameter and made of either glass or stainless steel. They may be either of the conventional type filled with the stationary phase, or of the microbore type in which the stationary phase is coated directly on the inside wall of the column.
- **A mobile phase and delivery system:** Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.
- **An injector system**: To deliver test samples to the top of the column in a reproducible

- **A detector and chart recorder:** To give a continuous record of the presence of the analytes in the eluate as it emerges from the column. Detection is usually based on the measurement of a physical parameter such as visible or ultraviolet absorption or fluorescence. A peak on the chart recorder represents each separated analyte.
- **A fraction collector:** For collecting the separated analytes for further biochemical studies.

Analyte development and elution-

- **zonal development -**analytes in the sample are separated on the basis of their distribution coefficients between the stationary and mobile phases.
- **displacement or affinity development-** sample are separated on the basis of their affinity for the stationary phase.

Column chromatography Procedure

preparation of the column

- Mostly the column is comprised of a glass tube with an appropriate stationary phase The bottom end of the column is packed with a glass wool/cotton wool or an asbestos pad after which the stationary phase is packed.
- After packing the column, a paper disc is placed on the top to avoid the disturbance of the stationary phase during the introduction of the sample or mobile phase.
- The disturbance in the stationary phase (adsorbent layer) leads to the irregular bands of separation.
- Two types of preparing the column, known as packing techniques namely
- **Dry packing technique** – The amount of absorbent needed is added as a fine dry powder in the column and the solvent flows freely through the column until equilibrium is achieved.
- **Wet packing technique** – The slurry of adsorbent is prepared along with the mobile phase and is poured into the column.
- It is regarded as the ideal technique for packaging.

Introduction of the sample

- The sample (a mixture of components) is dissolved in the minimum amount of the mobile phase.
- At one instant, the sample is introduced into the column and on the top portion of the column, it is absorbed.
- Through the elution process, the individual sample can be isolated from this zone.

Elution technique

- Through this technique, the individual components are separated completely from the column.The process of elution can be carried out by employing two techniques:
- ***Isocratic elution technique*** – Throughout the procedure, a solvent of the same polarity or same solvent composition is utilized.

Example: Use of chloroform alone

- ***Gradient elution technique*** – Throughout the separation procedure, solvents of gradually increased polarity or increased elution strength are utilized.

Example: Benzene → Chloroform → Ethyl acetate → Chloroform

Detection of Components

- In case the mixture separated in a column chromatography procedure are colored compounds, then monitoring the separation progress is simple.
- In case the compounds undergoing separation are colorless, then small fractions of the eluent are sequentially collected in tubes that are labeled. Thorugh TLC, the composition of each fraction is determined.

Types of Column chromatography

- Adsorption column chromatography
- Partition column chromatography.
- Gel column chromatography
- Ion exchange column chromatography Gas Chromatography (GC)
- High-Performance Liquid Chromatography (HPLC)

Column chromatography uses

- Column chromatography is one of the versatile methods for purifying and separating both solids and liquids.

Major applications:

- To isolate active constituents
- To separate compound mixtures
- To remove impurities or carry purification process
- To isolate metabolites from biological fluids
- To estimate drugs in drug formulations or crude extracts

CHROMATOGRAPHIC PERFORMANCE PARAMETER

Retention time

The retention time tR for each analyte has two components. The first is the time it takes the analyte molecules to pass through the free spaces between the particles of the matrix coated with the stationary phase. This time is referred to as the dead time, tM.

The volume of the free space is referred to as the column void volume, V0. The value of tM will be the same for all analytes and can be measured by using an analyte that does not interact with the stationary phase but simply spends all of the elution time in the mobile phase travelling through the void volume.

The second component is the time the stationary phase retains the analyte, referred to as the adjusted retention time, t'R. This time is characteristic of the analyte and is the difference between the observed retention time and the dead time.

$$t'_R = t_R - t_M$$

Retention factor

- One of the most important parameters in chromatography is the retention factor, k (previously called capacity factor and represented by the symbol k0). It is simply the additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase and which, by definition, has a k value of 0.

$$k = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M}$$

$$k = \frac{t'_R}{t_M} = \frac{M_S}{M_M} = K_d \times \frac{V_S}{V_M}$$

- where MS is the mass of analyte in the stationary phase, MM is the mass of analyte in the mobile phase, VS is the volume of stationary phase and VM is the volume of mobile phase. The ratio VS/VM is referred to as the volumetric phase ratio, b. Hence:

$$k = K_d \beta$$

Retention factors are important because they are independent of the physical dimensions of the column and the rate of flow of mobile phase through it. They can therefore be used to compare the behaviour of an analyte in different chromatographic systems .

selectivity is expressed by the selectivity or separation factor, a, which can also be viewed as simply the relative retention ratio for the two analytes

$$\alpha = \frac{k_{\mathrm{A}}}{k_{\mathrm{B}}} = \frac{K_{\mathrm{d_A}}}{K_{\mathrm{d_B}}} = \frac{t'_{\mathrm{R_A}}}{t'_{\mathrm{R_B}}}$$

The selectivity factor is influenced by the chemical nature of the stationary and mobile phases. Some chromatographic mechanisms are inherently highly selective.

Plate height and resolution

- **Plate height**

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for an analyte to completely equilibrate between the two phases. Each zone is called a theoretical plate (of which there are N in total in the column). The length of column containing one theoretical plate is referred to as the plate height, H.

$$H = \frac{\sigma^2}{x}$$

$$N = \frac{L}{H} = \frac{Lx}{\sigma^2}$$

The number of theoretical plates in the whole column of length L is equal to L divided by the plate height

Peak broadening

A number of processes oppose the formation of a narrow analyte peak thereby increasing the plate height:

Application of the sample to the column: It takes a finite time to apply the analyte mixture to the column, so that the part of the sample applied first will already be moving along the column by the time the final part is applied. The part of the sample applied first will elute at the front of the peak.

Longitudinal diffusion: Fick's law of diffusion states that an analyte will diffuse from a region of high concentration to one of low concentration at a rate determined by the concentration gradient between the two regions and the diffusion coefficient (P) of the analyte. Thus the analyte within a narrow band will tend to diffuse outwards from the centre of the band, resulting in band broadening.

Multiple pathways: The random packing of the particles in the column results in the availability of many routes between the particles for both mobile phase and analytes. These pathways will vary slightly in length and hence elution time. The smaller the particle size the less serious is this problem and in open tubular columns the phenomenon is totally absent, which is one of the reasons why they give shorter elution times and better resolution than packed columns

Asymmetric peaks

In some chromatographic separations, the ideal Gaussian-shaped peaks are not obtained, but rather asymmetrical peaks are produced.

- **Fronting-** In cases where there is a gradual rise at the front of the peak and a sharp fall after the peak . The most common cause of fronting is overloading the column so that reducing the amount of mixture applied to the column often resolves the problem.
- **Tailing**-In cases where the rise in the peak is normal but the tail is protracted. The probable explanation for tailing is the retention of analyte by a few active sites on the stationary phase, commonly on the inert support matrix. Such sites strongly adsorb molecules of the analyte and only slowly release them. This problem can be overcome by chemically removing the sites, frequently hydroxyl groups, by treating the matrix with a silanising reagent such as hexamethyldisilazine. This process is sometimes referred to as **capping.**

Resolution

The success of a chromatographic separation is judged by the ability of the system to resolve one analyte peak from another. Resolution (RS) is defined as the ratio of the difference in retention time (tR) between the two peaks (tRA and tRB) to the mean (wav) of their base widths (wA and wB).

$$R_S = \frac{\Delta t_R}{w_{av}} = \frac{2(t_{R_A} - t_{R_B})}{w_A + w_B}$$

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_{av}}\right)$$

Resolution is influenced by column efficiency, selectivity factors and retention factorswhere k2 is the retention factor for the longest retained peak and kav is the mean retention factor for the two analytes

High Pressure liquid chromatography(HPLC)

Principle-

The number of theoretical plates (N) in the column and hence plate height (H). The value of N increases with column length but there are practical limits to the length of a column owing to the problem of peak broadening

- the selectivity of the column, a; and
- the retentivity of the column as determined by the retention factor, k.

- As the number of theoretical plates in the column is related to the surface area of the stationary phase, it follows that the smaller the particle size of the stationary phase, the greater the value of N, i.e. N is inversely proportional to particle size.
- Unfortunately, the smaller the particle size, the greater is the resistance to the flow of the mobile phase for a given flow rate. This resistance creates a backpressure in the column that is directly proportional to both the flow rate and the column length and inversely proportional to the square of the particle size
- The back-pressure may be sufficient to cause the structure of the matrix to collapse, thereby actually further reducing eluent flow and impairing resolution. This problem has been solved by the development of small particle size stationary phases.

Columns

Conventional columns used for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of up to 50MPa.

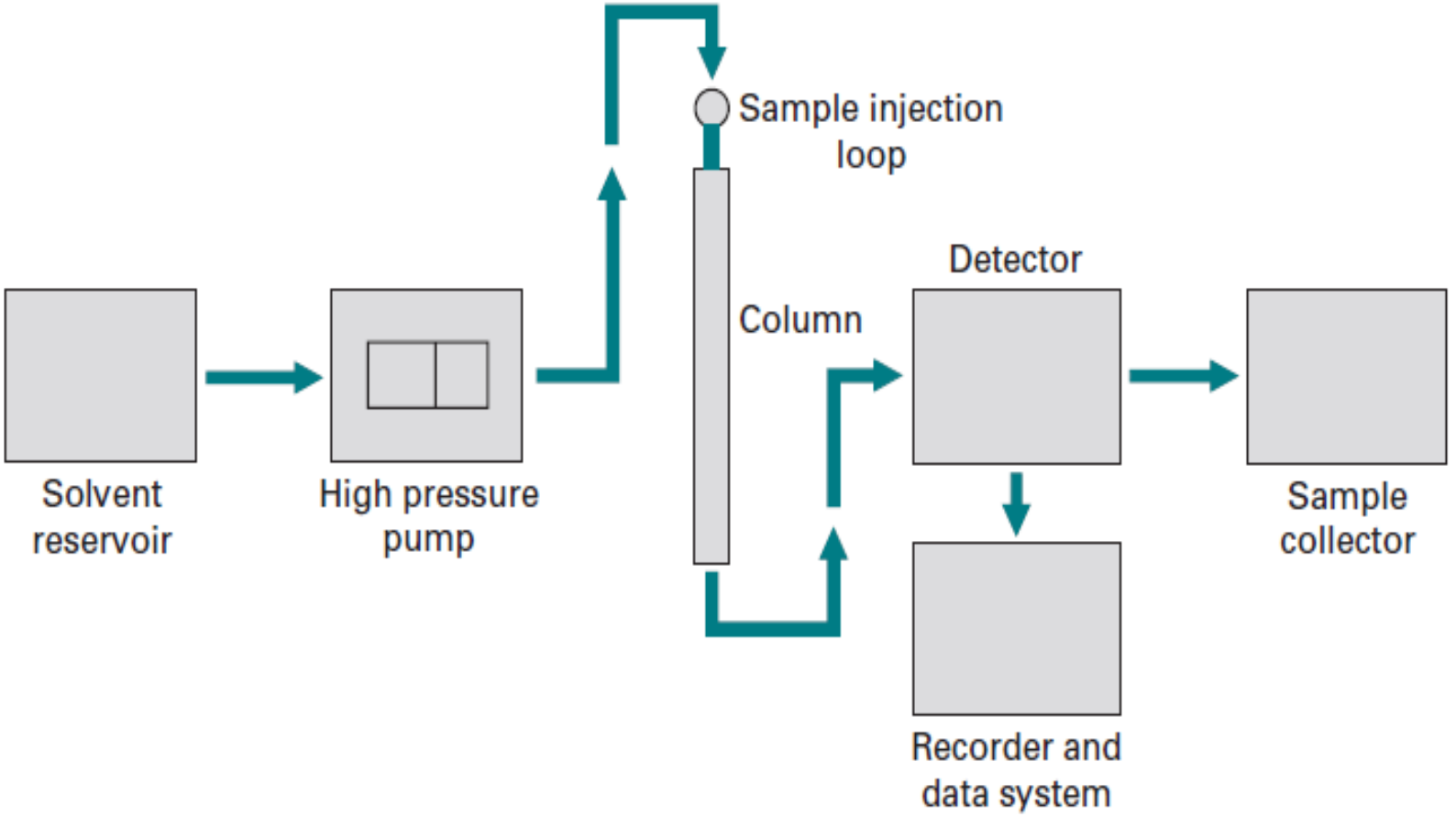
Microbore or open tubular columns have an internal diameter of 1–2mm and are generally 25-50 cm long. They can sustain flow rates of 5-20mm3 per min. Microbore columns have three important advantages over conventional columns:

- reduced eluent consumption due to the slower flow rates;
- ideal for interfacing with a mass spectrometer due to the reduced flow rate; and
- increased sensitivity due to the higher concentration of analytes that can be used.

Matrices and stationary phases

Made of chemically modified silica or styrene/divinylbenzene copolymers. The two forms are:

- **Microporous supports:** In which micropores ramify through the particles that are generally 5-10 mm in diameter.
- **Bonded phases:** In which the stationary phase is chemically bonded onto an inert support such as silica.


Sample injection loop
Detector
Column
Solvent reservoir
High pressure pump
Sample collector
Recorder and data system

Application of sample

The most common method of sample introduction is by use of a loop injector This consists of a metal loop, of fixed small volume, that can be filled with the sample. The eluent from the pump is then channelled through the loop by means of a valve switching system and the sample flushed onto the column via the loop outlet without interruption of the flow of eluent to the column.

Mobile phases

The choice of mobile phase to be used in any separation depends on the type of separation to be achieved***. Isocratic elution*** may be made with a single pump, using a single eluent or two or more eluents premixed in fixed proportions. ***Gradient elution*** generally uses separate pumps to deliver two eluents in proportions predetermined by a gradient programmer. It is also essential that all eluents be degassed before use otherwise gassing (the presence of air bubbles in the eluent) tends to occur in most pumps. Degassing of the eluent may be carried out in several ways – by warming, by stirring vigorously with a magnetic stirrer, by applying a vacuum, by ultrasonication, and by bubbling helium gas through the eluent reservoir.

Pumps

Pumping systems for delivery of the eluent are one of the most important features of HPLC systems.

Constant displacement pumps maintain a constant flow rate through the column irrespective of changing conditions within the column. The ***reciprocating pump*** is the most commonly used form of constant displacement pump. Such pumps produce small pulses of flow and pulse dampeners are usually incorporated into the system to minimise this pulsing effect.

Detectors

Variable wavelength detectors: These are based upon ultraviolet–visible spectrophotometry. These types of detector are capable of measuring absorbances down to 190nm and can give full-scale deflection (AUFS) for as little as 0.001 absorbance units.

Scanning wavelength detectors: These have the facility to record the complete absorption spectrum of each analyte, thus aiding identification.

Fluorescence detectors: These are extremely valuable for HPLC because of their greater sensitivity (10–12 g cm–3) than UV detectors but they have a slightly reduced linear range (104).

NMR spectrometer detectors: These give structural information about the analyte that is complementary to that obtained via HPLC–MS.

Evaporative light-scattering detectors (ELSD): These rely on the vaporisation of the eluate, evaporation of the eluent and the quantification of the analyte by light scattering.

- The sensitivity of ultraviolet absorption, fluorescence and electrochemical detectors can often be increased significantly by the process of derivatisation, whereby the analyte is converted pre- or post-column to a chemical derivative.

ADVANTAGES

- It is simple, rapid , reproducible.
- High sensitivity.
- High performance.
- Rapid process and hence time saving.
- It is having a high resolution and separation capacity.
- Accuracy and Precision.
- Stationary phase was chemically innert.
- Wide varities of stationary phase.
- Mobile phase was chemically innert.
- Less requirement of mobile phase in developing chamber.
- Early recovery of separated component.
- Easy visualization of separated components.
- It is having Good reproducibility and repeatability.
- It is analytical technique is important for validation of product, quality control studies of product.
- It is important for qualitative and quantitative analysis.
- It is used for both analytical and preparative purpose.

Gas Liquid chromatography

Principle

- It exploits differences in the partition coefficients between a stationary liquid phase and a mobile gas phase of the volatilised analytes as they are carried through the column by the mobile gas phase.
- Its use is therefore confined to analytes that are volatile but thermally stable. The partition coefficients are inversely proportional to the volatility of the analytes so that the most volatile elute first.
- The temperature of the column is raised to 50-300 oC to facilitate analyte volatilisation. The stationary phase consists of a high-boiling-point liquid material such as silicone grease or wax that is either coated onto the internal wall of the column or supported on an inert granular solid and packed into the column
- Gas chromatography is widely used for the qualitative and quantitative analysis of a large number of low-polarity compounds because it has high sensitivity, reproducibility and speed of resolution. Analytically, it is a very powerful technique when coupled to mass spectrometry.

Apparatus and experimental procedure

- a column housed in an oven that can be temperature programmed;
- a sample inlet point;
- a carrier gas supply and control; and
- a detector, amplifier and data recorder system

Columns

Packed conventional columns:

- These consist of a coiled glass or stainless steel column 1-3m long and 2-4mm internal diameter. They are packed with stationary phase coated on an inert silica support. Commonly used stationary phases include the polyethylene glycols .
- The most commonly used support is Celite (diatomaceous silica), which because of the problem of support–sample interaction is often treated so that the hydroxyl groups that occur in the Celite are modified. This is normally achieved by silanisation of the support with such compounds as hexamethyldisilazane.

Capillary (open tubular) columns:

These are made of high-quality fused quartz and are 10-100m long and 0.1-1.0mm internal diameter.

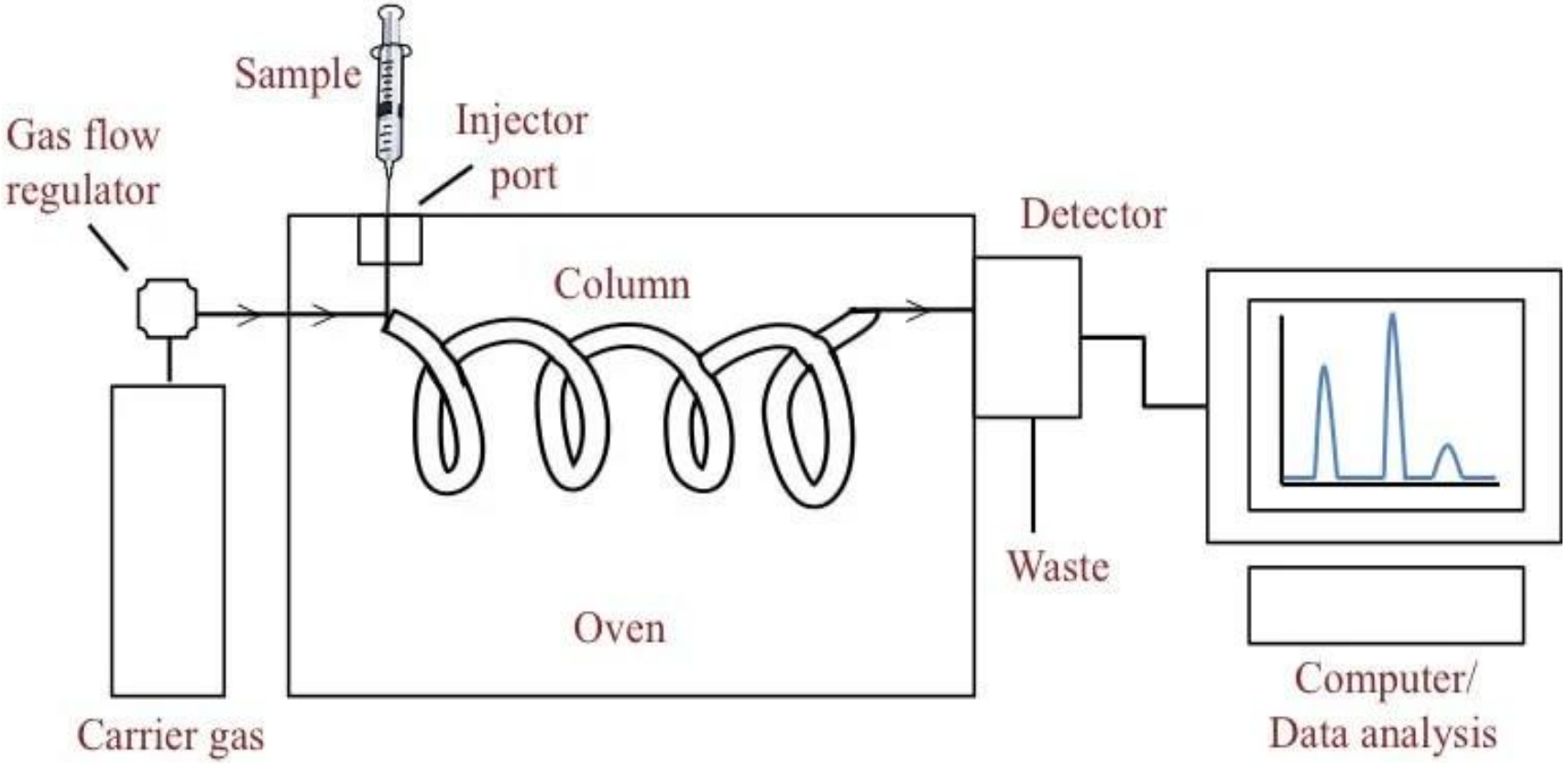
wall-coated open tubular (WCOT) In WCOT columns the stationary phase is thinly coated (0.15 mm) directly onto the walls of the capillary .

Support-coated open tubular in SCOT columns the support matrix is bonded to the walls of the capillary column and the stationary phase coated onto the support.

Commonly used stationary phases include polyethylene glycol (CP wax and DB wax, very polar) and methyl and phenyl-polysiloxanes.

The operating temperature for all types of column must be compatible with the stationary phase chosen for use. Too high a temperature results in excessive column bleed owing to the phase being volatilised off, contaminating the detector and giving an unstable recorder baseline.

- **Isothermal analysis:** Here a constant temperature is employed.
- **Temperature programming:** The temperature is gradually increased to facilitate the separation of compounds.


Sample
Injector port
Gas flow regulator
Detector
Column
Waste
Oven
Computer/ Data analysis
Carrier gas

Application of sample

- The majority of non- and low-polar compounds are directly amenable to GC, but other compounds possessing such polar groups as -OH, -NH2 and -COOH are generally retained on the column for excessive periods of time if they are applied directly.Poor resolution and peak tailing usually accompany this excessive retention. This problem can be overcome by derivatisation of the polar groups.
- This increases the volatility and effective distribution coefficients of the compounds.Methylation, silanisation and perfluoracylation are common derivatisation methods for fatty acids, carbohydrates and amino acids.
- The test sample is dissolved in a suitable solvent such as acetone, heptane or methanol. Chlorinated organic solvents are generally avoided as they contaminate the detector.
- For packed and SCOT columns the sample is injected onto the column using a microsyringe through a septum in the injection port attached to the top of the column.

Mobile phase

- The mobile phase consists of an inert gas such as nitrogen for packed columns or helium or argon for capillary columns. The gas from a cylinder is pre-purified by passing through a variety of molecular sieves to remove oxygen, hydrocarbons and water vapour.
- A gas-flow controller is used to ensure a constant flow irrespective of the back-pressure and temperature of the column.

Detectors-

Flame ionisation detector (FID): This responds to almost all organic compounds. An upper temperature limit of 400 C. A mixture of hydrogen and air is introduced into the detector to give a flame, the jet of which forms one electrode, whilst the other electrode is a brass or platinum wire mounted near the tip of the flame When the sample analytes emerge from the column they are ionised in the flame, resulting in an increased signal being passed to the recorder. The carrier gas passing through the column and the detector gives a small background signal, which can be offset electronically to give a stable baseline.

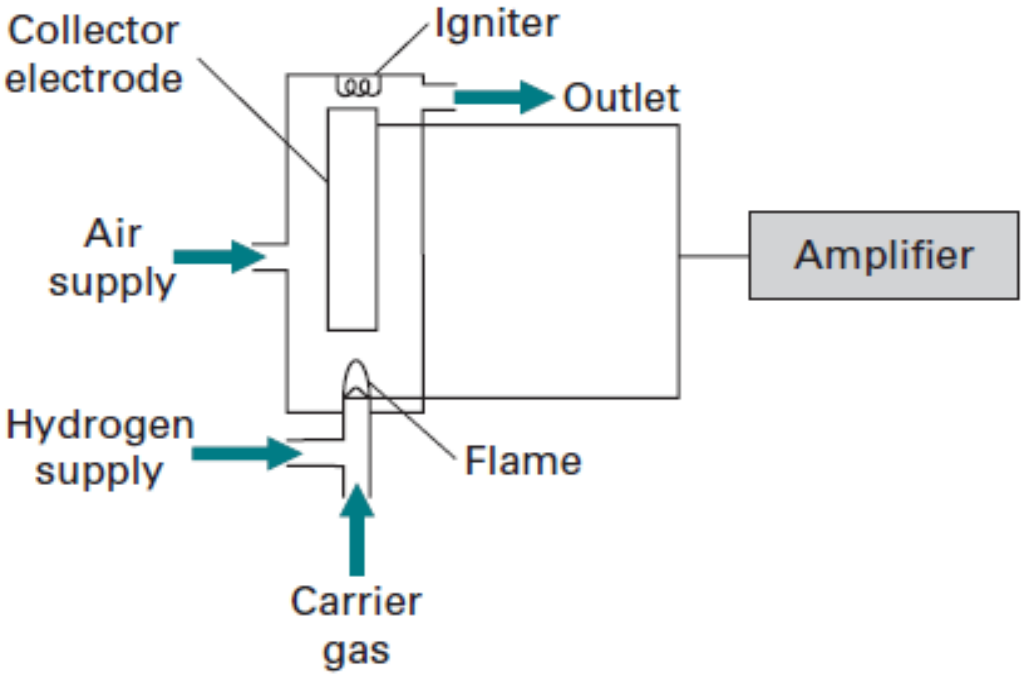


Fig. 11.13 GC flame ionisation detector. The tip of the flame forms the anode and the collector electrode the cathode.

$$\text{analyte} + H_2 + O_2 \longrightarrow \text{combustion products} + H_2O + \text{ions} + \text{radicals} + \text{electrons}$$

$$\sum(\text{ions})^- + \sum(\text{electrons})^- \longrightarrow \text{current}$$

Nitrogen–phosphorus detector (NPD) (also called a thermionic detector): This is similar in design to an FID but has a crystal of a sodium salt fused onto the electrode system, or a burner tip embedded in a ceramic tube containing a sodium salt or a rubidium chloride tip. The NPD has excellent selectivity towards nitrogen- and phosphorus-containing analytes . It is widely used in organophosphorus pesticide residue analysis.

Electron capture detector (ECD): This responds only to analytes that capture electrons, particularly halogen-containing compounds. This detector is widely used in the analysis of polychlorinated compounds, such as the pesticides DDT, dieldrin and aldrin.

Flame photometric detector: This exploits the fact the P- and S-containing analytes emit light when they are burned in a FID-type detector. This light is detected and quantified.

Mass spectrometer detector: This is a universal detector that gives a mass spectrum ofthe analyte and therefore gives both structural and quantitative data.

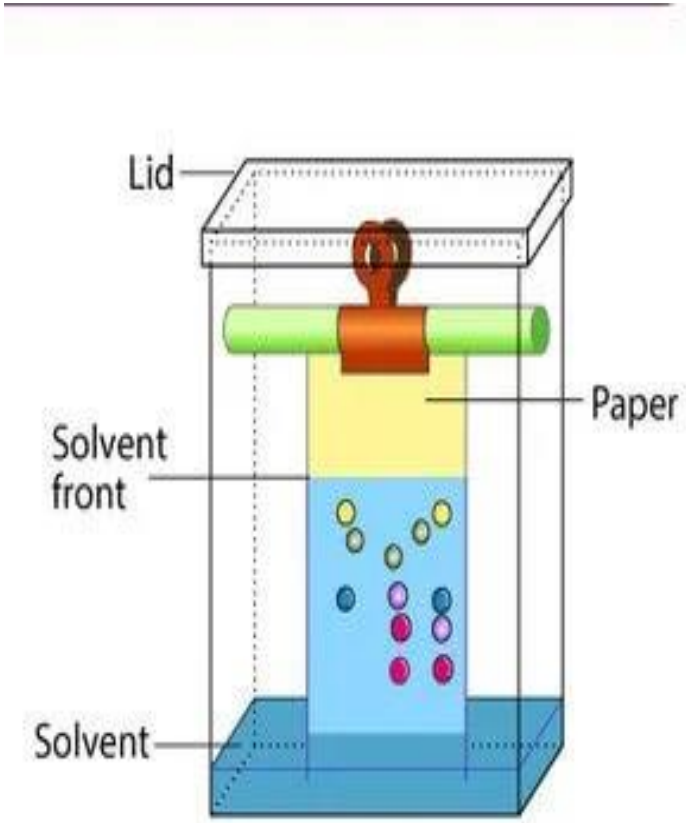
APPLICATION

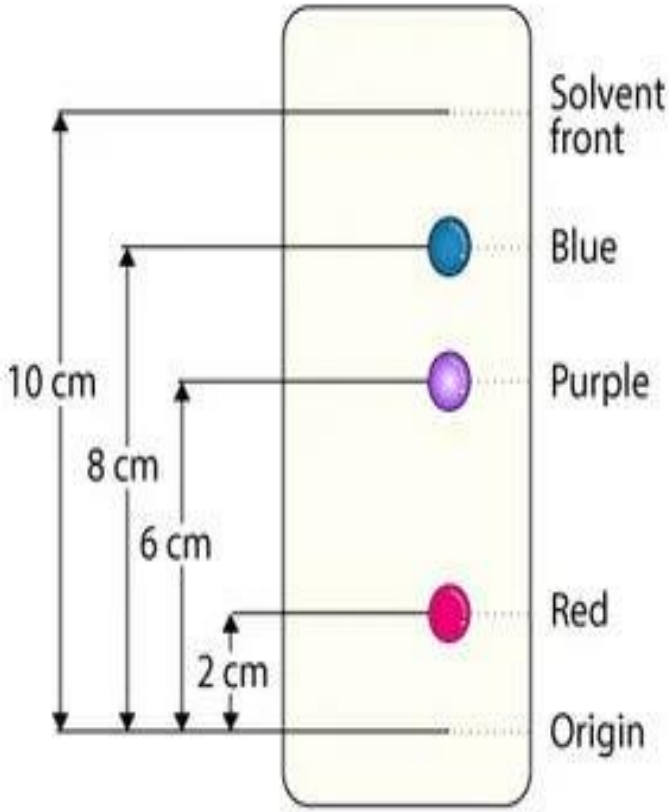
- Quantitative & Qualitative analysis of low polarity compounds
- Analytical chemistry, Biochemistry, Petrochemical, Environmental monitoring
- Measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples
- Measuring toxic substances in soil, air or water.

Paper Chromatography

Principle- Based on two principles

- The first is the paper adsorption chromatography that is based on the varying degree of interaction between the molecules and the stationary phase.
- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
- The second type of paper chromatography is the paper partition chromatography. It is based on the principle that the moisture on the cellulose paper acts as a stationary phase for the molecules moving with the mobile phase.
- The separation of the molecules is thus based on how strongly they adsorb onto the stationary phase.
- An additional concept of ‘retention factor’ is applied during the separation of molecules in the paper chromatography.


Lid
Paper
Solvent front
Solvent


Solvent front
Blue
Purple
Red
Origin
10 cm
8 cm
6 cm
2 cm

Steps of Paper chromatography

- The stationary phase is selected as a fine quality cellulosic paper.
- Different combinations of organic and inorganic solvents are taken as the mobile phase.
- About 2-200 µl of the sample solution is injected at the baseline of the paper, and it is allowed to air dry.
- The sample loaded paper is then carefully dipped into the mobile phase not more than the height of 1 cm.
- After the mobile phase reaches near the edge of the paper, the paper is taken out.
- The retention factor is calculated, and the separated components are detected by different techniques.

Uses of Paper chromatography

- Paper chromatography is performed to detect the purity of various pharmaceutical products.
- It can also be employed to detect contamination in various samples, like food and beverages.
- This method can also be used for the separation of impurities from various industrial products