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Chromatographic Techniques

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Introduction:-

Chromatography, literally means ‘colour writing’ is a relatively new physical technique of separation, identification and purification of compounds of a mixture. Chromatography consist of a group of techniques that are used to separate the components of a mixture which are very closely related to each other. Chromatography is used in many areas of study particularly in chemistry, biology and medicine, pigments, dye, amino acids, vitamins, polymers etc. can be separated by using the technique of chromatography. Chromatography is used for the purification and separation of organic as well as inorganic substances. It is also found useful for the fractionation of complex mixture, separation of closely related compounds such as isomers and in the isolation of unstable substances.

IUPAC definition of chromatography:-

In 1993, the international commission of IUPAC (International Union for Pure and Applied Chemistry) defined Chromatography as a physical method of separation in which the components to be separated are, distributed between two phases, one of which is stationary phase while the other is mobile phase, moves in a definite directions.

History of Chromatography:-

Mikhail Tsvet: Inventore of Chromatography.

History of chromatography spans from the mid 19^{th} century to the 21^{st}. The technique was invented by Russian-Italian botanist Mikhail Tsvet. He used a liquid-adsorption column containing calcium carbonate to separate coloured plant pigments like xanthophylls (Yellow), carotenes (orange) and chlorophylls (green).

The method was described on 30th December 1901 at the 11th Congress of Naturalists and Doctors in Saint Petersburg.

Thin Layer Chromatography:-

Use of thin layer chromatography was first reported in 1938 by two Russian scientists, N.A. Izmailov and M.S Schreiber. They separated plant extracts on a slurried adsorption medium spread to a 2 mm thick layer by spotting an alcoholic plant extract in the center of the layer and observing rings as the solution spread. Analytical Chemists J. F. Meinhard and N. F. Hall improved the technique by the addition of binders to the sorbents in 1949. J.G. Kirchner and his colleagues at the U.S. Department of Agriculture found that silicic acid bound with amioca starch created a satisfactory layer for TLC.

He continued his work with sorbent layers on glass plates and developed TLC essentially as we know it today. Another major breakthrough came in the 1960s when convenient pre-coated plates become commercially available by Merck, Germany based on work by Egon Stahl. Attempt had been made for some time to couple TLC with this spectroscopic method. In 2013, pre-coated plates were finally introduced that were suitable for TLC-MS.

Paper chromatography:-

Paper chromatography was investigated by two British biochemists, Archer John Porter Martin and Richard Laurence Millington Synge. In 1941 Martin and Synge were trying to characterise a particular protin by determining the precise numbers of each amino acid present, however that the problem them had defeated a whole generation of biochemists.

Martin and Synge's development of paper chromatography successfully solved the problem of separating amino acids which are very similar to each other. It worked not only on amino acids but also on various other mixtures. The two scientists were awarded the 1952 Nobel Prize in chemistry for their work.

After Synge determined the structure of an antibiotic peptide called 'Gramicidin-S' Frederick Sanger used paper chromatography to figure out the structure of the insulin molecule. He determined the number of amino acids in it as well as the order in which they occurred.

The same paper chromatography technique was used by Melvin Calvin during the 1950s. Calvin discovered and identified at least ten different intermediates products in the process of photosynthesis.

Paper chromatography was also used by Australian- American biochemist. Erwin Chargaff, who modified the technique to study the components of the nucleic acid molecule. His research revealed four components, or nitrogenous bases, that occur in pairs. British biochemists James Dewey Watson and Francis Harry Campton Crick later used these results to work out the structure of DNA (deoxyribonucleic acid).

Ion Exchange chromatography:-

Ion exchange chromatography was originally introduced by two English researchers, agricultural Sir Thompson and chemists J T Way. They performed ion exchange methods to treat clays with the salts, resulting in the extraction of ammonia in addition to the release of calcium. Compounds known as zeolites were introduced to separate individual ions or electrically charged particles, in ion-exchange chromatography.

In the 1930s, synthetic resins were developed for complex ion-exchange processes. During World War II (1939-1945) ‘Life rafts’ were equipped with survival kits that contained resin for removing most salts from seawater. American chemist Frank Harold Spedding adapted this technique for the separation of rare-earth metals in 1947.

Gel permeation chromatography:-

Gel permeation chromatography technique is often used for the analysis of polymers. Size exclusion chromatography technique was first developed in 1955 by Lathe and Ruthven. Researcher J. C. Moore of the Dow chemical company investigated the technique of gel permeation chromatography in 1964.

The proprietary gel permeation column technology was licensed to Waters Corporation, who subsequently commercialized this technology in 1964.

Affinity chromatography:-

Affinity chromatography was first used in the isolation of enzymes in 1953 by Leonard Lerman, from Chicago university. He isolated enzyme tyrosinase on a column of cellulose with ethereally bound resorcinol residues. Immobilized leucyl-tRNA synthetase was used for the isolation of isoleucyl-tRNA, and also for the study of protein interactions with nucleic acid

Gas chromatography:-

Gas chromatography is one of the most popular chromatographic technique. German physical chemist Erika Cremer in 1947 together with Austrian graduate student Fritz Prior developed the theoretical foundations of GC and built the first liquid-gas chromatograph. Archer John Porter Martin, who was awarded the Nobel Prize for his work in developing liquid-liquid (1941) and paper (1944) chromatography, is therefore credited for the foundation of gas chromatography. The popularity of GC quickly rose after the development of the flame ionization detector.

Supercritical fluid chromatography:-

Supercritical fluid chromatography in earlier days was categorized as high pressure or dense chromatography (HPGC or DGC). Low boiling point hydrocarbons were used as supercritical mobile phase. By the late 1970s, carbon dioxide became the most preferred fluid because it has low critical temperature and pressure. It is also non toxic, non flammable and inexpensive.

Capillary electrophoresis:-

Capillary electrophoresis is a relatively new technique. The first system was developed in 1965 by Hjerten with the aim of separating proteins, nucleic acids and inorganic ions. However, the potential of the technique was further explored in 1980 by Jorgenson and Lukas, who published high resolution separations with a simple home-made system.

High Performance Chromatography:-

High Performance Chromatography was developed to overcome the ineffectiveness of GC because of the thermal instability of the solutes during biochemical analysis. Gas phase separation and analysis of very polar high molecular weight biopolymers was impossible by using GC. As a result, alternative methods were hypothesized which would soon result in the development of HPLC. Following on the seminal work of Martin and Synge in 1941, it was by Cal Giddings, Josef Hubber, and other in the 1960s predicted that liquid chromatography could be operated in the high-efficiency mode by reducing the particle diameter of packing material of the column below typical LC (and GC) level of 150 µm.

Paper chromatography:-

Principle: This is liquid - liquid partition type of chromatography. This old technique is used to analyze complex mixtures, such as ink, by separating the different chemicals it was made from.

Technique: The method involves placing a small spot of sample solution on to a strip of chromatography paper. The paper is placed into a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper it meets the sample mixture which starts to travel up the paper with the solvent. Different compounds in the sample mixture travel different distances according to how strongly they interact with the paper. Mixtures of different characteristics (size and solubility) travel at different speeds. This allows the calculation of an R_f, value and can be compared to standard compounds to aid in the identification of an unknown substance.

Thin layer chromatography:-

Principle: Thin layer chromatography (TLC) is a widely-employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate.

Technique : Different compounds in the sample mixture travel different distances according to how strongly they interact with the adsorbent. This allows the calculation of an R_f, value and can be compared to standard compounds to help in the identification of an unknown substance. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents.

Ion-exchange chromatography:-

Principle: Ion exchange chromatography (IEC) is a column chromatography based on charge. The stationary phase is usually an ion exchange resin that carries charged functional groups which interact with oppositely charged groups of the compound to be retained. It is used to separate charged compounds including amino acids, peptides, and proteins. Ion-exchange chromatography separates molecules based on their respective charged groups. Ion-exchange chromatography retains analyte molecules on the column based on coulombic (ionic) interactions. The ion exchange chromatography matrix consists of positively and negatively charged ions.

Essentially, molecules undergo electrostatic interactions with opposite charges on the stationary phase matrix.

Technique: The stationary phase consists of an immobile matrix that contains charged ionisable functional groups or ligands. The stationary phase surface displays ionic functional groups (R-X) that interact with analyte ions of opposite charge. To achieve electroneutrality these inert charges couple with exchangeable counter-ions in the solution. Ionisable molecules that are to be purified compete with these exchangeable counterions for binding to the immobilized charges on the stationary phase. These ionizable molecules are retained or eluted based on their charge.

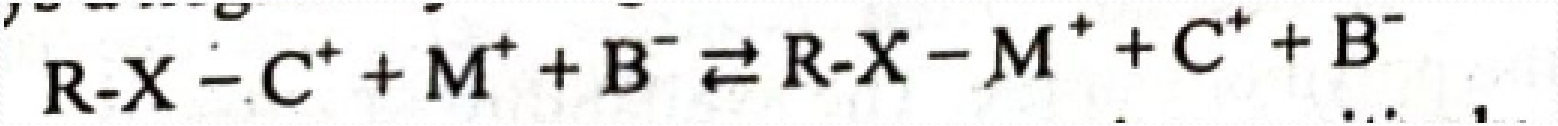
Initially, molecules that do not bind or bind weakly to the stationary phase are first to wash away. And molecule that bind strongly to stationary phase are eluted lastly. The concentration of the exchangeable counterions, which competes with the molecules for binding, can be increased or the pH can be changed.

This type of elution is called gradient elution. On the other hand, step elution can be used in which the concentration of counterions are varied in one step.

This type of chromatography is further subdivided into cation exchange chromatography and anion change chromatography.

Positively charged molecules bind to cation exchange resins while negatively charged molecules bind to anion exchange resins. The ionic compound consisting of the cationic species M^+ and the anionic species B^- can be retained by the stationary phase.

Cation exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group.



Anion exchange chromatography retains anions using positively charged functional group. In ion chromatography, the interaction of the solute ions and the stationary phase based on their charges determines which ions will bind and to what degree.

When the stationary phase features positive groups which attracts anions, it is called an anion exchanger, when there are negative groups on the stationary phase, cations are attracted and it is a cation exchanger. The attraction between ions and stationary phase also depends on the resin, organic particles used as ion exchangers.

Resins of ion exchange columns may include functional groups such as weak/strong acids and weak/strong bases. There are also special columns that have resin with amphoteric functional groups that can exchange both cations and anions. Some examples of functional groups of strong ion exchange resins are quaternary ammonium cation (Q), which is an anion exchanger, and sulfonic acid (S, $-SO_2OH$), which is a cation exchanger.

Applications:-

(1) Ion exchange chromatography is commonly used to purify any kind of charged molecule including large proteins, small nucleotides, and amino acids.

(2) Today ion chromatography is important for investigating aqueous systems, such as drinking water. It is a popular method for analyzing anionic elements or complexes that helps to solve environmentally relevant problems.

Gel Permeation Chromatography:-

Principal: Gel permeation chromatography (GPC) is a type of size exclusion chromatography (SEC), that separates analytes on the basis of size.

GPC separates the analytes based on the size or hydrodynamic volume (radius of gyration). This differs from other separation techniques which depend upon chemical or physical interactions to separate analytes. Separation occurs via the use of porous beads packed in a column. The smaller analytes can enter the pores more easily and therefore spend more time in these pores, increasing their retention time. These smaller molecule spend more time in the column and therefore will elute last. Conversely, larger analytes spend little if any time in the pores and are eluted quickly. All columns have a range of molecular weights that can be separated.

Technique Instrumentation:-

Gel permeation chromatography is conducted almost exclusively in chromatography columns. The experimental design is not much different from other techniques of liquid chromatography. Samples are dissolved in an appropriate organic solvent and after filtering the solution it is injected onto a column. The separation of multicomponent component mixture takes place in the column. The constant supply of fresh eluent to the column is accomplished by the use of a pump. Since most analytes are not visible to the naked eye a detector is needed. Often multiple detectors are used to gain additional information about the polymer sample. The availability of a detector makes the fractionation convenient and accurate.

Gels : Gels are used as stationary phase for GPC. The pore size of a gel must be carefully controlled in order to be able to apply the gel to a given separation. Other desirable properties of the gel forming agent are the absence of ionizing groups and, in a given solvent, low affinity for the substances to be separated. Commercial gels like PLgel Sephadex, Bio-Gel (cross-linked polyacrylamide), agarose gel and Styragel are often used based on different separation requirements.

Column: The column used for GPC is filled with a microporous packing material. The column is filled with the gel.

Eluent: The eluent (mobile phase) should be a good solvent for the polymer, should permit high detector response from the polymer and should wet the packing surface. The most common eluents in for polymers that dissolve at room temperature GPC are tetrahydrofuran (THF), o-dichlorobenzene and trichlorobenzene at 130-150^{o}C for crystalline polyalkynes and m-cresol and o-chlorophenol at 90^{o}C for crystalline conditions polymers such as polyamides and polyesters.

Pump:

There are two types of pumps available for uniform delivery of relatively small liquid volumes for GPC piston or peristaltic pumps.

Detector: In GPC, the concentration by weight of polymer in the eluting solvent may be monitored continuously with a detector. There are many detector types available and they can be divided into two main categories. The first is concentration sensitive detectors which includes UV absorption, differential refractometer (DRI) or refractive index (RI) detectors infrared (IR) absorption and density detectors.

The second category is molecular weight sensitive detectors, which include low angle light scattering detectors (LALLS) and multi angle light scattering (MALLS) detectors.

Applications of GPC:-

The technique is often used for the analysis of polymers. When characterizing polymers, it is important to consider the dispersity (D) as well the molecular weight.

Polymers can be characterized by a variety of definitions for molecular weight including the number average molecular weight (M_n), the weight average molecular weight (M_w) the size average molecular weight (M_z), or the viscosity molecular weight (M_v). GPC allows for the determination of D as well as M_v, and based on other data, the M_n, M_w, and M_z can be determined.

Affinity Chromatography:-

Principal: Affinity chromatography is a method of separating biochemical mixture based on a highly specific interaction between antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid. It is a type of chromatographic laboratory technique used for purifying biological molecules within a mixture by exploiting molecular properties Protein could be eluted by ligand solution.

Biological macromolecules, such as enzymes and other proteins, interact with other molecules with high specificity through several different types of bonds and interaction. Such interactions include hydrogen bonding, ionic interaction, disulfide bridges, hydrophobic interaction, and more.

Technique: The high selectivity of affinity chromatography is caused by allowing the desired molecule to interact with the stationary phase and be bound within the column in order to be separated from the undesired material which will not interact and elute first.

The molecules no longer needed are first washed away with a buffer while the desired proteins are let go in the presence of the eluting solvent (of higher salt concentration). This process creates a competitive interaction between the desired protein and the immobilized stationary molecules, which eventually lets now highly purified proteins be released.

Applications of Affinity chromatography:-

(1) Affinity chromatography can be used to purify and concentrate a substance from a mixture into a buffering solution, reduce the amount of unwanted substances in a mixture, identify the biological compounds binding to a particular substance, purify and concentrate an enzyme solution.

(2) Affinity chromatography is used for the determination of the inhibition constants of enzymes.

(3) Affinity chromatography is ideal for the study of interactions in biochemical processes.

Gas Chromatography:-

Gas chromatography (GC) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition.

Principal: In gas chromatography, the mobile phase (or "moving phase") is a carrier gas usually an inert gas such as helium or an unreactive gas such as nitrogen.

Helium remains the most commonly used carrier gas in about 90% of instruments although hydrogen is preferred for improved separations. The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The instrument used to perform gas chromatography is called a gas chromatograph (or "aerograph", "gas separator").

Technique: The gaseous compounds being analyzed interact with the walls of the column which is coated with a stationary phase. This causes each compound to elute at a differ time, known as the retention time of the compound. The comparison of retention times is what gives GC its analytical usefulness.

Gas chromatography is in principle similar to column chromatography (as well as other forms of chromatography, such as HPLC, TLC), but has several notable differences. First, the process of separating the compounds in a mixture is carried out between a liquid stationary phase and a gas mobile phase, whereas in column chromatography the stationary phase is a solid and the mobile phase is a liquid. Hence the full name of the procedure is "Gas-liquid chromatography", referring to the mobile and stationary phases, respectively. The column through which the gas phase passes is located in an oven where the temperature of the gas can be controlled, whereas column chromatography has no such temperature control. Finally, the concentration of a compound in the gas phase is solely a function of the vapour pressure of the gas.

Gas chromatography is also sometimes known as vapor-phase chromatography (VPC), or gas-liquid partition chromatography (GLPC). These alternative names, as well as their respective abbreviations, are frequently used in scientific literature. Strictly speaking GLPC is the most correct terminology, and is thus preferred by many authors.

Instrumentation : A gas chromatograph uses a flow-through narrow tube known as the column, through which different chemical constituents of a sample pass in a gas stream (carrier gas, mobile phase) at different rates depending on their various chemical and physical properties and their interaction with a specific column filling, called the stationary phase.

As the chemicals exit the end of the column, they are detected and identified electronically. The function of the stationary phase in the column is to separate different components, causing each one to exit the column at a different time (retention time), other parameters that can be used to alter the order or time of retention are the carrier gas flow rate, column length and the temperature.

In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a microsyringe. As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the adsorption of analyte molecules either onto the column walls or onto packing materials in the column.

The rate at which the molecules progress along the column depends on the strength of adsorption which in turn depends on the type of molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of analyte mixture are separated as they progress along the column and reach the end of column at different times (retention time). A detector is used to monitor the outlet stream from the column; thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are identified (qualitatively) by the order which they emerge (elute) from the column and by the retention time of the analyte in the column.

Various detectors such as Thermal Conductivity Detector (TCD), Flame ionization Detector (FID), Electron Capture Detector (ECD) Vacuum ultraviolet (VUV) Mass Spectrometer (MS), Infrared Detector (IRD), Photo-lonization Detector (PID) are used for quantitative and qualitative analysis.

Applications of Gas Chromatography:-

(1) In general, substances that vaporize below 300 °C (and therefore are stable up to that temperature) can be measured quantitatively. 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.

(2) Professionals working with GC analyze 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, GC is very accurate if used properly and can measure pico-moles of a substance in a 1ml liquid sample, or parts-per-billion concentrations in gaseous samples.

(3) GC analyse hydrocarbons (C2-C40+). In a typical experiment, a packed column is used to separate the light gases, which are then detected with a TCD. The hydrocarbons are separated using a capillary column and detected with a FID. 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.

Supercritical-Fluid Chromatography (SFC)-(Introduction):-

Supercritical-fluid chromatography is a combination (hybrid) of gas and liquid chromatography. Hence, it is associated with some of the best features of each. Though it is GLC and HPLC for certain types of applications.

Some important properties of supercritical fluids:-

A Supercritical fluid is formed whenever a substance is heated above its critical temperature. At this temperature, a substance can no longer be condensed into its liquid state the application of pressure.

For example CO_2, becomes a supercritical fluid at above 31°C. In this supercritical fluid state, the molecules of CO_2 behave freely of one another simply as they do in a gas.

Carbon dioxide is the preferred SFC mobile phase at present for the following reasons:

i) Low critical temperature and relatively low critical pressure.

ii) It can be used with Flame Ionisation Detector FID, the sensitivity of detection of which is only slightly decreased by CO_2.

iii) It is relatively inert, although strongly basic compounds would undergo chemical reactions. (e.g. formation of carbamates with amines).

Critical solution temperatures for fluids used in chromatography differ from 30°C to above 200°C.

Lower critical temperatures are useful due to several reasons hence lot of attention has been focused on supercritical fluids such as CO_2 (31°C), C_2H_6 (32°C) and N_2O (37°C, nitrous oxide). It is clear from these figures that these temperatures and pressures are well within the operating conditions of ordinary HPLC.

Instrumentation and Operating variables:-

Instrumentation pattern for both SFC and HPLC is same.

However, in SFC instruments additional provision is made for controlling and measuring the column pressure.

The Effect of Pressure:- The density of a supercritical-fluid increases speedily with increase in pressure, For example, it has been observed that the elution time for hexadecane decrease from 25 to 5 min. as the pressure of CO_2 is raised from 70 to 90 atm.

Columns:- Both packed and open tubular columns are used in SFC. Packed columns are fabricated from hard glass or metal-stainless steel, Cu or Al tubes, length 2 to 4m and inside diameters of 2 to 4 mm. with porous organic polymer, open tubular columns are preferred. Polysiloxanes are used as column coating material, because these are chemically bonded to the inner silica wall of the capillary tubing.

Mobile Phases:- Carbon dioxide is the most widely used mobile phase for SFC. It is a superior solvent for a variety of organic molecules. It transmits in UV region and it is odourless, non-toxic, readily available and comparatively cheaper. Its critical temperature and pressure are low (31°C and 73 atm). Hence it permits a wide selection of temperature. Other mobile phases employed in SFC include ethane, pentane, dichlorodifluoromethane diethyl ether and tetrahydrofuran (THF).

Detectors:- The important advantage of SFC technique is that one can use sensitive and universal detectors which are used in GLC. For example Flame lonization Detector (FID) can be employed in SFC.

Advantages of SFC:- SFC technique is applicable to a class of compounds that cannot be easily analyzed by GLC or HPLC. The resolving power is approximately five times that of HPLC. SFC can analyze non-volatile, polar or adsorptive solutes without derivatization. It can also analyze thermally labile compounds and solutes of very high molecular weight. Supercritical fluids have low viscosities hence give faster analysis. SFC can be used with a wide range of sensitive detectors. Another advantage of SFC is that supercritical columns are much easier to interface with mass spectrometers that liquid chromatographic columns.

Applications of SFC:- SFC has been applied to a wide variety of samples including natural products, drugs, foods, pesticides, herbicides, surfactants, polymers, polymer additives etc.

High Performance Liquid Chromatography:-

Principal: High-performance liquid chromatography (HPLC, formerly referred to as high pressure liquid chromatography) is a technique in analytical chemistry used to separate identify, and quantify each component in a mixture.

Technique: HPLC relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

HPLC is distinguished from traditional "low pressure" liquid chromatography because pressures are significantly higher (50-350 bar), while ordinary liquid chromatography typically relies on the force of gravity to pass the mobile phase through the column. Due to the small sample amount separated in analytical HPLC, typical column dimensions are 2.1-4.6 mm diameter, and 30-250 mm length. Also HPLC columns are made with smaller adsorbent particles (2-50 µm in average particle size). This gives HPLC superior resolving power when separating mixtures, which makes it a popular chromatographic technique.

Instrumentation:-

A typical HPLC unit has the following basic components namely solvent reservoir system, pump system, sample injection system, column, detector and recorder.

Autosampler:-

Large numbers of samples can be automatically injected onto an HPLC system, by the use of HPLC autosamplers. In addition, HPLC autosamplers have an injection volume and technique which is exactly the same for each injection, consequently they provide a high degree of injection volume precision.

Columns:-

The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.

Analytical scale columns (4.6 mm) have been the most common type of columns, through smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.

Narrow-bore columns (1-2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry. Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle size:-

Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 µm beads being the most common. Smaller particles generally provide more surface area and better separation, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

This means that changing to particles that are half as big, kipping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

Pore size:-

Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.

Pump pressure:-

Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible volumetric flow rate. Pressure may reach as high as 60 MPa (6000 lbf/in^2), or about 600 atmospheres.

Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (< 2μm). These ultra high performance liquid chromatography systems or UHPLCs can work at up to 120 MPa (17,405 lbf/in^2), or about 1200 atmospheres. The term "UPLC" is a trademark of the Waters Corporation, but is sometimes used to refer to the more general technique of UHPLC.

Detectors:-

HPLC detectors fall into two main categories: universal or selective. Universal detectors typically measure a bulk property (e.g, refractive index) by measuring a difference of a physical property between the mobile phase and mobile phase with solute while selective detectors measure a solute property (e.g, UV-Vis absorbance) by simply responding to the physical or chemical property of the solute.

HPLC most commonly uses a UV-Vis absorbance detector, however, a wide range of other chromatography detectors can be used. A universal detector that complements UV-Vis absorbance detection is the charged aerosol detector (CAD). In certain cases, it is possible to use multiple detectors, for example LCMS normally combines UV-Vis with a mass spectrometer.

Applications:-

1) Manufacturing: HPLC has many applications in both laboratory and clinical science. It is a common technique used in pharmaceutical development, as it is a dependable way to obtain and ensure product purity.

2) Legal: This technique is also used for detection of illicit drugs in urine. The most common method of drug detection is an immunoassay. This technique has been used to detect a variety of agents like doping agents, drug metabolites, glucuronide conjugate, amphetamines, opioids, cocaine, BZDs, ketamine, LSD, cannabis, and pesticides. Performing HPLC in conjunction with Mass spectrometry reduces the absolute need for standardizing HPLC experimental runs.

3) Research: Similar assays can be performed for research purposes, detecting concentrations of potential clinical candidates like anti-fungal and asthma drugs. It is used as a method to confirm results of synthesis reactions, as purity is essential in this type research.

4) Medical: Medical use of HPLC can include drug analysis, but falls more closely under the category of nutrient analysis. While urine is the most common medium for analyzing drug concentrations, blood serum is the sample collected for most medical analyses with HPLC. It is used for diagnosing vitamin D deficiencies in children.

Capillary Electrophoresis:-

CE instrumentation is relatively simple. It consist of two platinum electrodes (anode and cathode) connected to a high voltage power supply and a fused silica capillary tube, whose ends are immersed into a reservoir containing buffer solution. For detection the capillary tube must have an optical window, simply made by removing the polymer coating. This window is aligned with the detector, which is often a UV.

A buffer solution can be modified by adding substances, which leads to alternative mechanisms of retention and therefore to different modes of capillary electrophoresis. The modes or methods that can be mentioned are: Capillary Zone Electrophoresis (CZE), Micellar Electrokinetic Chromatography (MEKC), Capillary Gel Electrophoresis (CGE), Capillary Isoelectric Focusing (CIEF), and Capillary Isotachophoresis (CITP).

Applications of Capillary Electrophoresis:-

The technique used in different research areas such as biotechnology, pharmacy and Medicine.

1) CZE has been employed in bioscience in order to separate peptides and proteins and also for the separation of organic acids and inorganic ions.

2) MEKC is used for the separation of charged solutes as in CZE, but also neutral ones; some examples are amino acids, vitamins, pharmaceutical products, nucleotides and explosives.

3) CGE is usually used in biological science for the separation of macromolecules such as proteins and nucleic acids.

4) CIEF is the mode used to separate peptides and proteins based on their pI (isoelectric points).

5) CITP is useful for analyzing cations and anions simultaneously.

Classification of Chromatographic methods:-

Chromatographic separations may be classified in three ways based on.

Physical state of the mobile phase and stationary phase:-

The mobile phase is usually a liquid or a gas, and the stationary phase, when present, is a solid or a liquid film coated on a solid surface. Chromatographic techniques are often named by listing the type of

mobile phase, followed by the type of stationary phase. Thus, in gas-liquid chromatography the mobile phase is a gas and the stationary phase is a liquid. If only one phase is indicated, as in gas chromatography, it is assumed to be the mobile phase.

Method of contact between the mobile phase and stationary phase:-

Two common approaches are used to bring the mobile phase and stationary phase into contact.

In column chromatography, the stationary phase is placed in a narrow column through which the mobile phase moves under the influence of gravity or pressure. The stationary phase is either a solid or a thin, liquid film coating on a solid particulate packing material or the column's walls. In paper chromatography the stationary phase coats a flat glass, metal, or plastic plate and is placed in a developing chamber. A reservoir containing the mobile phase is placed in contact with the stationary phase, and the mobile phase moves by capillary action.

Chemical or physical mechanism responsible for separating the sample’s constituents:-

The mechanism by which solutes separate provides a third means for characterizing a separation.

In adsorption chromatography, solutes separate based on their ability adsorb to a solid stationary phase. In partition chromatography, a thin liquid film coating a solid support serves as the stationary phase. Separation is based on a difference in the equilibrium partitioning of solutes between the liquid stationary phase and the mobile phase. Stationary phases consisting of a solid support with covalently attached anions (e.g. $–SO^{3-}$) or cationic (e.g. $-N(CH_3)^{3-}$) functional groups are used in ion-exchange chromatography. Ionic solutes are attracted to the stationary phase by electrostatic forces. Porous gels are used as stationary phases in size-exclusion chromatography, in which separation is due to differences in the size of the solutes.

Large solutes are unable to penetrate into the porous stationary phase and so quickly pass through the column. Smaller solutes enter into the porous stationary phase, increasing the time spent on the column. Not all separation methods require a stationary phase. In an electrophoretic separation, for example, charged solutes migrate under the influence of an applied potential field. Differences in the mobility of the ions account for their separation.

Paper Chromatography:-

Paper chromatography may be defined as the technique in which the analysis of an unknown substance is carried out mainly by the flow of solvent on specially designed filter paper.

Origin and overview of Technique:-

The technique paper chromatography may be regarded as the extension of partition chromatography. The technique paper chromatography is a simple form of chromatographic method. It is a valuable analytical technique to the organic and biochemist. The technique was induced by Schonbein. It is known as Capillary analysis. It was than further develop by Consden, Garden, Martin and Syngle (1964). Cellulose filter paper as it is hydrophilic in nature, hence it is often used as the stationary phase in paper chromatography. The technique is often regarded as liquid-liquid chromatography.

Types of papers:-

Special paper are usually used for this technique. These papers should be highly purified. The paper should possess porosity and thickness. The papers used for this technique contain sufficient adsorbed water. The other liquids such as silicone or paraffin oil are used for this purpose. Sometimes special papers that contain an adsorbent or an ion-exchange resin are also used.

The choice of filter paper plays an important role in this type of chromatography. This choice of paper is dependent on the type of analysis under investigation. Different types of Whatman chromatographic papers are available in the market.

The choice of Whatman chromatographic paper depends upon the type of separation.

Sample preparation:-

There is no any standard procedure for preparation of samples. This problem is due to the several factors of the given samples especially the presence of other systems like fats, protein, salts etc. Generally, the sample, volume of 10-20 μ is the ideal quantity to be spotted.

Principle:-

It is a kind of partition chromatography. Here the stationary phase is water and mobile phase is a mixture of one or more organic solvents and water. Filter paper is used as a solid support to the stationary liquid phase. The cellulose fibers of the filter paper are hydrophilic. A thin coat of water adsorbed on the cellulose fiber acts as a stationary liquid phase. A filter paper is used in the form of a rectangular sheet. The mobile phase moves over the stationary phase by capillary action.

It is a type of liquid-liquid partition chromatography in which the separation is based on the differences in the distribution (solubility) of the solutes in the two liquid phases. That is the solutes to be separated are distributed between the two liquids, according to their partition coefficients.

The two liquids are immiscible. The solutes which are more soluble in the stationary phase move slowly, while those more soluble in mobile phase move fast. Thus, different solutes move at different rates and appear as spots at different places on the paper.

Steps in Paper Chromatography:-

Following are the important steps in paper chromatography

1. Application of the sample on the paper.

2. Saturation of the tank

3. Development of the chromatogram.

4. Location of the spots and

5. Measurement of R_f, values and identification of the solutes.

1) Application of the sample on the paper:-

A Whatman filter paper of suitable size (15 to 30 cm. in length and 10 to 15 cm. in width) is used. A thin pencil line is drawn at a distance of two centimeter from the bottom of the paper. A small quantity of the mixture is dissolved in a minimum quantity of a volatile solvent. The sample solution is spotted on this line with a micropipette or capillary tubing as shown in Fig. 4.1. The spot must be made as small as possible for maximum separation and minimum tailing (spread up).

2) Saturation of the tank:-

The atmosphere in the tank (chamber) must be saturated with the mobile liquid. The solvent is placed at the bottom of the tank. The tank is closed. The air saturated with solvent vapour prevents the evaporation of the solvent from the surface of the paper as it moves up. The liquid is called as developing solvent.

3) Development of the chromatogram:-

The paper is placed in the chamber with its end dipping in the developing solvent as shown in Fig. 4.2. There are two way of development.

(i) Ascending development: The developing solvent moves in the upward direction on the paper by the capillary action as shown in Fig. 4.3.

(ii) Descending development: The developing solvent moves down by the capillary as well as by the pull of gravity as shown in Fig. 4.4.

The process of separation of components of the mixture in the form of band or spots of pure substances at different places on the chromatogram is know as development. Time required for development may be one hour or more. The amount of development depends upon the nature of the solutes in the mixture to be separated. Many samples can be spotted along the bottom and developed simultaneously, if wide paper is used.

The main advantage of paper chromatography is that greater separation power can be obtained by using Two-dimensional Paper Chromatography. This is the method of developing the paper in two dimensions (directions). In this method, a large square pieces of paper is used. A single spot of the mixture is applied at a bottom corner of the paper. The paper is first developed in one direction in one solvent as usual. The paper is then turned 90^{o} and then developed in a second solvent system as shown in Fig. 4.5. Thus, if two or more solutes are not completely separated with the first solvent, it may be possible to separate them with a second, solvent. After development of the chromatogram, that is, when the solvent has travelled required distance or the required time, the paper is removed from the chamber and the position of the solvent front is marked with the help of a pencil. The paper is then dried by a fan or hair dryer or I. R. lamp.

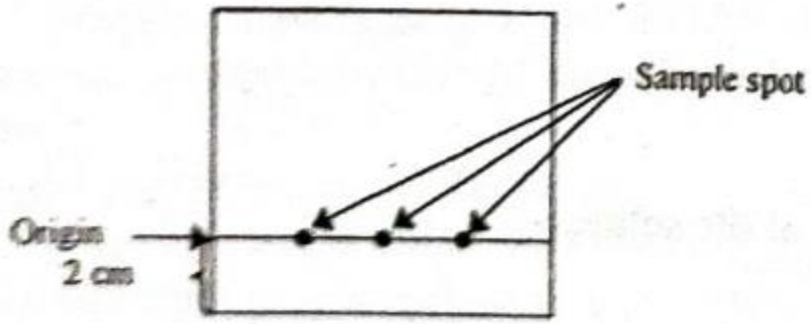


Fig. 4.1 : Spotting of the sample solution

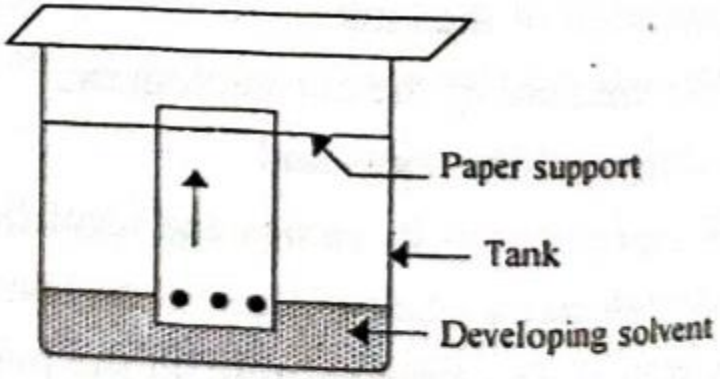


Fig. 4.2 : Paper chromatographic set up

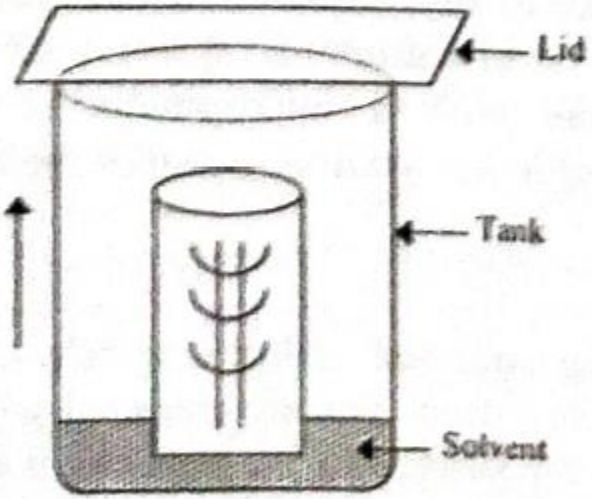


Fig. 4.3 : Ascending development

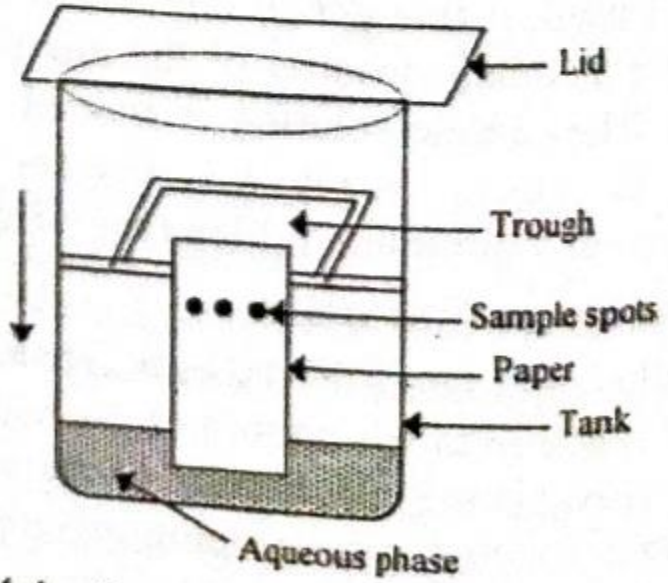


Fig. 4.4 : Descending development

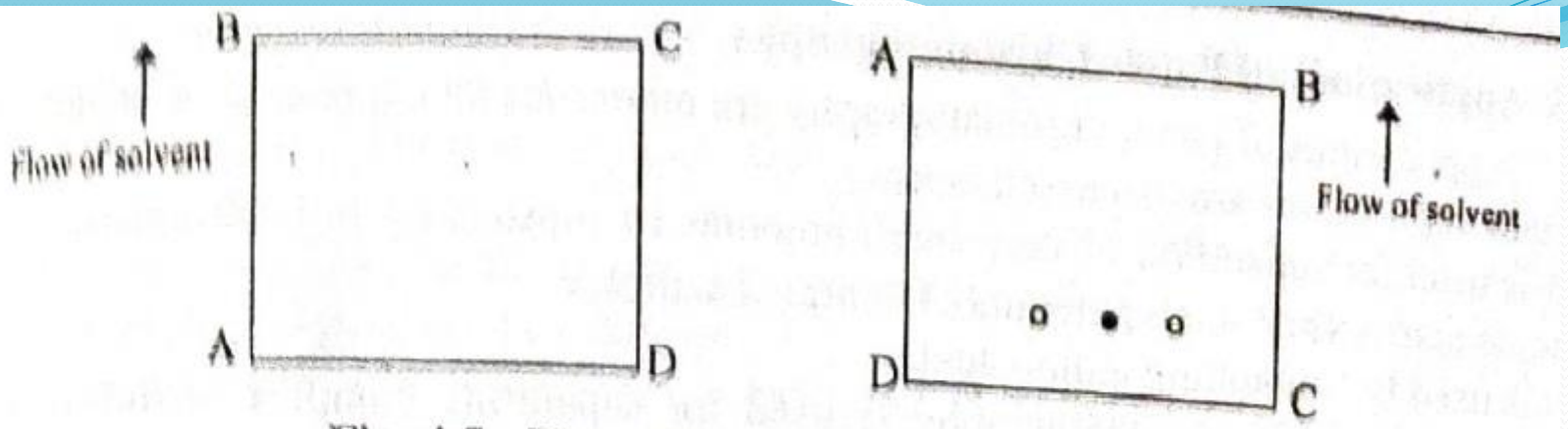


Fig. 4.5 : Two dimensional chromatography

4) Location of the spots:-

Physical method:-

The paper is observed under the ultraviolet lamp to locate the positions of the coloured components. The fluorescent compounds show fluorescence in ultraviolet light and hence their position is detected. A pencil line is drawn around the spots for permanent identification.

Chemical method:-

The colourless components are converted into coloured components by reaction with chemical reagents. A chemical can be in the state of gas, liquid or solid but mostly liquids and solids are used for this purpose. Solids like K_2CrO_4, and liquids like water, methyl, ethyl and t-butyl alcohols are used. The identification of metallic ions of II group elements is carried out by using H_2S gas. Exposure to iodine vapours produces a colour with colourless components but usually iodine is not used in paper chromatography. The suitable chemicals are sprayed on the paper. For example, the amino acids are located by spraying the paper with a solution of ninhydrin, which is converted to a blue or purple colour.

5) Identification of the components:-

The results of chromatography separation are expressed in terms of R_f values. R_f is de short form of retardation factor. R_f is defined as the ratio of the distance travelled by the given substance from the origin to the distance travelled by the moving solvent.

$$R_f = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent}}$$

R_f value is a characteristic property of a substance like melting point and boiling point. R_f value is used for the qualitative detection of the separated component. Under constant experimental conditions, the R_f values are reproducible.

In some cases, it is observed that the solvent front goes beyond the end of the paper. In such cases, it is more convenient to express the movement of any substance just by comparing with the movement of some standard substances. This is represented by R_s, a retardation factor with respect to a standard.

$$R_S = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the standard substance}}$$

In sugars, the movements are compared with that of glucose and the values are expressed as R_S values. It should be noted that R_f, value is always less than one. In case of compounds, which are highly soluble in the mobile phase, the R_f values are nearly equal to 1.

Applications of Paper Chromatography:-

Applications of paper chromatography are numerous and appear to be endless. Some important applications are discussed below.

1) It is used for separation of very small amounts of substances. In biochemistry we either come across very small amounts of complex samples.

2) It is used for separating amino acids.

3) Two dimensional chromatography is used for separating complex mixtures, such as protein hydrolysates

4) It can be used for the separation of organic as well as inorganic substances.

5) Modified cellulose paper can be used for various techniques of chromatography. The paper can be modified by changing the chemical structure of the cellulose. For example paper can be impregnated with alumina, silica-gel, ion-exchange etc and these can be used for the separation of various substances.

6) It has also been used in the analysis of mixture of sugars. In this case spots are visualised by spraying with aniline hydrogen phthalate


Thank you!