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Chromatographic Technique

Introduction

Chromatography, literally means "colour writing" is a relatively new physical technique of separation, identification and purification of components of a mixture. Chromatography consists 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, dyes, 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 mixtures, 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 direction.

History of Chromatography

Mikhail Tswett the Russian botanist who separated six pigments from a leaf pigment extraction.

The chromatography technique was found in the mid 1800s, by a German dye chemist known F.F Runge. He separated dyes on a filter paper and the separation took place due to two reasons, affinity of dye components for the filter paper and also different dye components had different molecular weights. Runge used water for the mobile phase and filter paper for stationary phase.

In 1906 a Russian botanist called Mikhail Tswett separated six pigments in a pigment extraction from leaves. He made a $CaCO_3$ packed glass column as the stationary phase. $CaCO_3$ column is a solid adsorbent. Mobile phase used was petroleum ether. At past chromatography was only used to separate colored compounds. The term chromatography is a Greek word, came as "Khroma" for colour and "graphian" for writing. Thus it is the analyzed results written in colour.

Mikhail Tsvet 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. The technique was first published in the Proceedings of Warsaw Society of Naturalists, section of biology in 1903. The term chromatography was coined by M. Tsvet in writing, in 1906, in his two research papers about chlorophyll, in a German botanical Journal. Later in 1907, he demonstrated his chromatography for the German Botanical Society.

New methods of column fractionation were developed in 1890s for separating the components of petroleum. Tsvet used a liquid-adsorption column containing calcium carbonate to separate coloured plant pigments. To isolate different types of chlorophyll, he trickled a mixture of dissolved pigments through a glass tube packed with calcium carbonate powder. As the solution washed downward each pigment stuck to the powder with a different degree of strength, creating a series of colored bands. Each band of color represented a different substance. Tsvet referred to the colored bands as a chromatogram. He also suggested that the technique (now called adsorption chromatography) could be used to separate colorless substances.

Thin Layer Chromatography

Use of a 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 strach 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 became commerically available by Merck, Germany based on work by Egon Stahl. Attempts had been made for some time to couple with this spectroscopic method. In 2013, pre-coated plates were finally introduced that were suitable for TLC-MS.

Paper chromatography

Paper chromatography was invented by two British biochemists, Archer John Porter Martin and Richard Laurence Millington synge. In 1941 Martin and Synge

were trying to characterise a particular protein 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 chromatographic technique was used by Melvin Calvin during the 1950s. Calvin discovered and identified at least ten different intermediate products in the process of photosynthesis.

Paper chromatography was also used by Austrian-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 Compton 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 chemist 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, 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 resins 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 exculsion 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 Lerman, who isolated tyrosinase on a column of cellulose with ethereally bound resorcinol residues. In subsequent year's affinity chromatography was employed only rarely, the reason obviously being the character of the insoluble supports that did not offer sufficient possibilities for complex formation between the product to be isolated and the attached affinant. Affinity chromatography is ideal for the study of interactions in biochemical processes. Immobilized leucyl-tRNA synthetase was used for the isolation of isoleucyl-tRNA, and also for the study of protein interactions with nucleic acid. The use of affinity chromatography for the determination of the inhibition constants of enzymes seems to have good prospects. On the basis of the elution volumes of the enzyme eluted from the column with immobilized inhibitor using various concentrations of soluble inhibitor—the inhibition constants can be determined both with bound inhibitors and with the soluble inhibitors employed.

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 deterctor.

Supercritical Fluid Chromatography

Supercritical fluid chromatography in earlier days was categorized as high pressure or dense gas chromatography (HPGC or DGC). Low boiling point 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 publidshed high resolution separations with a simple home-made system.

High Performance Chromatography

High Performance Chromatography was developed to overcome the ineffectiveness of Gas Chromatography 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 Huber, and others 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 the typical LC (and GC) level of 150 μm. The speed of mobile liquid phase can also be increased by applying high pressure. These predictions underwent extensive experimentation and refinement throughout the 60s into the 70s. Early development research began to improve LC particles, and the invention of Zipax, a superficially porous particle, was promising for HPLC technology.

The 1970s brought about many developments in hardware and instrumentation. Researchers began using pumps and injectors to make a rudimentary design of an HPLC system. Gas amplifer pumps were ideal because they operated at constant pressure and did not require leak free seals or checks valves for steady flow and good quantitation. Hardware milestones were made at Dupont IPD (Industrial Polymers Division) such as replacing the septum injector with a loop injection value.

Paper Chromatography

Paper chromatography definition explains that is an inexpensive and powerful analytical technique, which requires a piece of paper or strips serving as an adsorbent in the stationary phase across which a particular solution is allowed to pass.

For the separation of dissolved chemical substances and lipid samples (in particular), paper chromatography is found to be very trustable. This analytical tool employs very few quantities of material.

Principle of Paper Chromatography

Paper chromatography is a form of liquid chromatography where the basic principle involved can be either partition chromatography or adsorption chromatography.

In paper chromatography separation of component is distributed between phases of liquid. Here, one phase of liquid is water that is held amidst the pores of filter paper and the other liquid is the mobile phase that travels along with the filter paper. Separation of the mixture is the result that is obtained from the differences in the affinities towards the water and mobile phase when travelling under capillary action between the pores of the filter paper.

Though in a majority of paper chromatography applications, the principle is based on partition chromatography but sometimes, adsorption chromatography can take place where the stationary phase is the solid surface of the paper and the mobile phase is the liquid phase.

Technique

The method involves placing a small spot of sample solution onto 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 Rf value and compared to standard compounds to aid in the identification of an unknown substance.

Thin Layer Chromatography

Principle

Thin layer chromatography is a kind of chromatography used to separate and isolate mixtures that are non-volatile in nature. Just like other chromatography processes, this one consists of a mobile phase and a stationary phase.

The latter one here is a thin layer of absorbent material, such as aluminium oxide, silica gel, or cellulose. This layer is applied to plastic, glass, or aluminium foil sheets called an inert substrate. The mobile phase in the TLC procedure is a solvent or a mixture of it.

If you want to learn more about the thin layer chromatography procedure, you have landed at the right place. Here we will be discussing its principle, process, and applications in different industries.

Thin Layer Chromatography Principle

The separation principle of the TLC procedure is based on the given compound's relative affinity towards the mobile and the stationary phase. The process begins here by moving the mobile phase over the stationary phase's surface. During this movement, the higher affinity compounds gain less speed as compared to the lower affinity compounds. This results in their separation.

Once the procedure gets completed, different spots can be found on the stationary surface at distinct levels, reflecting various elements of the mixture. Basically, the compounds that are more attracted towards the stationary phase secure their position at lower levels while others move towards the higher levels of the surface. So their spots can be seen accordingly.

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 Rf 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 choice between different adsorbents.

Ion -Exchange Chromatography

Ion-exchange chromatography (IEC) is an important analytical technique that is frequently utilized for the separation and determination of ions and polar molecules on the basis of their affinity towards the ion exchanger.

Here, Stationary phase = A resin; Mobile phase = An eluent

Ion exchange chromatography along with the ion partition/interaction and ion-exclusion chromatography is an important component of ion chromatography.

Ion exchange chromatography is applicable for various charged molecules such as large proteins, amino acids, and small nucleotides.

Principle

Ion exchange chromatography 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 grops (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 thar are to be purified complete 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, moleucles that do not bind or bind weakly to the stationary phase are first to wash away. Altered conditions are needed for the elution of the molecules that bind to the stationary phase. The concentration of the exchangeable counterions, which competes with the molecules for binding, can be increased or the pH can be changed.

A change in pH affects the charge on the particular molecules and therefore, alters binding. The molecules then start eluting out based on the changes in their charges from the adjustments. Further such adjustments can be used to release the protein of interest. Additionally, concentration of counterions can be gradually varied to separate ionized molecules. 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 exchange 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.

$$R\text{-}X\text{-}C^+ + M^+ + B^- \rightleftharpoons R\text{-}X\text{-}M^+ + C^+ + B^-$$

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 (often termed 'beads') of ion exchange columns may include functional groups such as weak/strong acids and weak/strong bases. There are also special columns that have resins 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. These types of exchangers can maintain their charge density over a pH range of 0-14. Examples of functional groups of weak ion exchange resins include diethylaminoethyl (DEAE, $-C_2H_4N(CH_2H_5)_2$, which is an anion exchanger, and carboxymethyl (CM, $-CH_2$-COOH), which is a cation exchanger. These two types of exchangers can maintain the charge density of their columns over a pH range of 5-9.

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

Principle

Gel permeation chromatography (GPC) is a type of size exclusion chromatography (SEC), that separates analytes on the basis of size, typically in organic solvents. The technique is often used for the analysis of polymers. As a technique, SEC was first developed in 1955 by Lathe and Ruthven.

GPC separates the analytes based on the size or hydrodynamic volume (radius of gyration). This differs from other separation technoques 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 molecules 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 solvents and after filtering the solution it is injected onto a column. The separation of multicomponent 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. Commerical 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 130-135 ℃ for crystalline polyalkynes amd m-cresol and o-chlorophenol at 90 ℃ for crystalline condensation 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. The resulting chromatogram is therefore a weight distribution of the polymer as a function of retention volume. The most sensitive detector is the differential UV photometer and the most common detector is the differential refractometer (DRI). When characterizing copolymer, it is necessary to have two detectors in series. For accurate determinations of copolymer composition atleast two of those detectors should be concentration detectors. The determination of most copolymer compositions is done using UV and RI detectors, although other combinations can be used.

Applications of GPC

The technique is often used for the analysis of polymers. When characterizing polymers, it is important to consider the dispersity (*Đ*) as well the moecular 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 *Đ* as well as M_v and based on other data, the M_n, M_w, and M_z can be determined.

Affinity Chromatography

Affinity chromatography is a method of separating a biomolecule from a mixture, based on a highly specific macromolecular binding interaction between the biomolecule and another substance. The specific type of binding interaction depends on the biomolecule of interest; antigen and antibody, enzyme and substrate, receptor and ligand, or protein and nucleic acid binding interactions are frequently exploited for isolation of various biomolecules. Affinity chromatography is ueful for its high selectivity and resolution of separation compared to other chromatographic methods.

Principle

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 comnpounds binding to a particular substance, purify and concentrate an enzymen 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 is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition.

Chromatography is a technique that separates components in a mixture by the difference in partitioning behavior between mobile and stationary phases. Gas chromatography (GC) is one of the popular chromatography techniques to separate volatile compounds or substances. The mobile phase is a gas such as helium, and the stationary phase is a high-boiling liquid that is adsorbed on a solid. Because of its

simplicity, high sensitivity, and the ability to effectively separate mixtures, gas chromatography has become one of the most important tools in chemistry.

Principle

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 different time, known as the retention time of the compound. The comparsion of retention times gives GC its analytical usefulness.

Gas chromatography is in principle similar to column 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 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 Vapour-phase chromatography (VPC) or gas - liquid partition chromatography (GLPC).

Instrumentation

A gas chromatography 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.

Gas chromatography 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 the 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 the molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analytic mixture are separated as they progress along the column and reach the end of of the 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 in 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, Electronic Capture Detector (ECD), Vacuum Ultraviolet (VUV), Mass Spectrophotometer (MS) Infrared Detector (IRD), Photo Ionization Detector (PID) are used for quantitative and qualitative analysis.

Application of Gas Chromatography

1. In general substance that vaporize below 300℃ (and therefore are stable up to that temperature) can be measured quantitatively. The sample 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.Professional workings with GC analysze 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 1 mL liquid sample or parts per billion concentrations in a gaseous samples.

3. GC analyse hydrocarbons (C-2- 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 crime- scence evidences.

Supercritical-Fluid Chromatography (SFC)

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 both GLC and HPLC for certain types of applications.

some important properties 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 through the application of pressure. For example carbon dioxide becomes a supercritical fluid temperatures above 31℃. In this supercritical fluid state, the molecules of carbon dioxide behave freely of one another simply as they do in a gas.

Carbon dioxide is the preferred supercritical fluid chromatography mobile phase at present for the following reasons:

1. Low critical temperature and relative kilo critical pressure.
2. It can be used with Flame Ionization Detector (FID) the sensitivity of detection of which is only slightly decreased by carbon dioxide.
3. It is relative inert although strongly basic compounds would undergo chemical reactions (example formation of carbamates with amines).

Critical solution temperature for fluids used in chromatography differ from 30 ℃ to about 200 ℃. Lower critical temperatures are useful due to several reasons hence lot of attention has been focused on supercritical fluids such as Carbon dioxide (31 ℃) ethane (32 ℃) and nitrous oxide (N_2O) (37 ℃). It is clear from these figures that these temperatures and pressures are well within the 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 in 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 decreases from 25 to 5 minute as the pressure of carbon dioxide is raised from 70 to 90 atmosphere.

Columns: Both packed and open tubular columns are used in SFC. Packed columns are fabricated from hard glass or metal stainless steel, copper or aluminium tubes length 2 to 3 metre 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 they 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 ℃ and 73 atmosphere). Hence it permits a wide selection of temperature. Other mobile phases employed in SFC include ethane, pentane, dichlorodifluoromethane, diethyl Ether and tetrahydrofuran.

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

Advantages of SFC : SFC technique is applicable to a class of compounds that cannot be easily analysed by GLC or HPLC. The resolving power is approximately five times that of HPLC. SFC can analyse non-volatile polar or adsorptive solutes without derivatization. It can also analyse 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 spectrophotometers that liquid chromatography 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 absorbent 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 operational pressures are slightly 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 2.1 - 4.6 mm diameter and 30- 250 mm length. Also HPLC column are made with smaller adsorbent particles (2 - 50 μm in the average particle size). This gives HPLC superior resolving power when separating mixtures, which makes it a popular chromatography technique.

Instrumentation

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

Autosamplers

Large number 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 of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity gradient elution. It also determine 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. Lower 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) the most common type of columns, through the smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of sample and often use a UV visible absorbance detector

Narrow-bore columns (1-2mm) are used for applications when more sensitivity is desired either with special UV visible 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 beds begin the most common. Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of of the particle diameter squared.

This means that changing to particles that are half as a big, keeping 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 large 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 do not easily 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 (< 2 μm). These ultra high performance liquid chromatography systems or UPLCs 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 illict 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 conjugates , amphyetamines, 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, as purity is essential in this type of research.

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

Capillary Electrophoresis

Principle

Capillary Electrophoresis (CE) is an analytic separation technique which allows ion and charged molecule separation based on mobility differences in short periods of time with high efficiency and low solvent consumption. The name of the technique is due to the site where the separation takes place: a capillary , which is a tube with inner diameters ranging from 10 to 100 micrometers.

Technique and Instrumentation

CE instrumentation is relatively simple. It consists 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 point).
5. CITP is useful for analyzing cations and anions simultaneously.

Classification Chromatographic methods

Chromatographic separations may be classified in three ways based on

a) 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.

b) 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.

c) Chemical or physical mechanism responsible for separating the sample's constituents

The mechanism by which solutes separate provides a thrid means for characterizing a separation. In absorption chromatography, solutes separate based on their ability to 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 anionic (e.g. $-SO_3^{3-}$) 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 onto 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 an biochemist. The technique was introduced by Schonbein. It is known as 'Capillary analysis'. It was than further developed y 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 papers 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. Usually, coarser and faster paper are used when substances to be separated have sufficiently R_f values.

Sample preparation

There is no any standard procedure for preparation of samples. This problem is due to the several factors of the given samples esspecially the presence of other systems like fats, proteins, 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 fibres of the filter paper are hydrophilic. A thin coat of water adsorbed on the cellulose fibre 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 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 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. 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

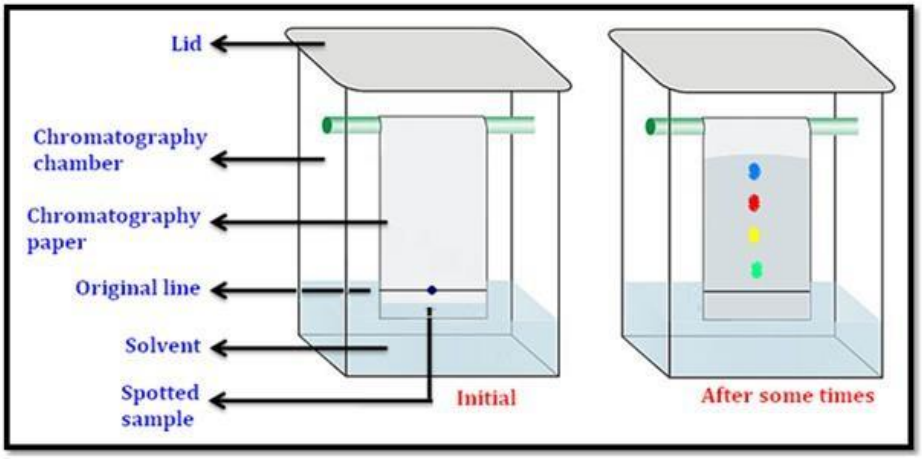
The paper is placed in the chamber with its end dipping in the developing solvent. There are two ways of development.

i. Ascending development : The developing solvent moves in the upward direction on the paper by the capillary action.

ii. Descending development : The developing solvent moves down by the capillary action as well as the pull of gravity

The process of separation of components of the mixture in the form of bands or spots of pure substances at different places on the chromatogram is known 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 advantages 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 piece of paper is used. A single spot of the mixture is applied at a bottom corrner of the paper. The paper is first developed in one direction in one solvent as usual. The paper is then turned 90° and then developed in a second solvent system. 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 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.



4. Location of the spots

i. 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 is drawn around the spots for permanent identification.

ii. Chemical method

The colourless components are converted into coloured components by reaction with chemical reagents. A chemical can be 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 chemical reagents 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 the short form of retardation factor. R_f is defined as the ratio of the distances 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. Rf valur 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 Rs, a retardation factor with respect to a standard.

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

In sugars, the movements are compared with that of the glucose and the values are expressed as R_g 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.

Application 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 often 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.

Thin Layer Chromatography (TLC)

Introduction

Thin-layer chromatography resembles column and paper chromatography. In TLC partition occurs on a thin layer of finely divided adsorbent. This adsorbent is supported on a glass plate or on a other supporting medium. Hence it is known as thin-layer chromatography. The adsorbents like Al2O3, MgO or SiO_2 are used as the substrate instead of paper. A thin layer plate may be prepared by spreading an aqueous slurry of the adsorbent over the surface of a glass plate. The plate is then allowed to stand until the layer has set up for many purposes. Some-times the plate may be heated in an oven for several hours.

This technique was first invented by M. T. Sweet in 1906. The thin-layer chromatography technique was then developed by Izamailov and Shraiber in 1938. The other scientists namely, Meinhard end Hall (1949), Kirchner, Miller and Keller (1951), Mottirer (1952) and Reitsema (1954) also helped to this technique.

Chromatography is the most single analytical technique. It may be defined as a method of separating a mixture components into individual components through equilibrium distribution between two phases.

Principle

It is a kind of adsorption chromatography. The chromatography using thin layer of an adsorbent spread over a glass plate, aluminium plate or plastic plate is called thin layer chromatography. It is similar to paper chromatography except that the stationary phase is a thin layer of finely divided adsorbent on a plate. The solids used in column chromatography can be used as a stationary phase hence thin layer chromatography is called open column chromatography.

Stationary Phase for TLC (Outline of the method)

The thin layer of a finely divided powder (particle size 5 to 50 mμ) coated on a glass plate acts as a stationary phase. It can be an adsorbent, an ion exchamger, a molecular sieve or act as a support for a liquid film. The most commonly used stationary phases are adsorbents like silica gel, alumina and powdered cellulose. The solid adsorbent adheres to the plate due to the addition of the binding agent like calcium sulphate or plaster of paris. This glass plate having a thin uniform layer of the finely divided adsorbent on it is called as a chromatoplate.

Preparation of the chromatoplate

The plate having a thin uniform layer of the finely divided solid on it is called as a chromatoplate. The plate may be of glass or aluminium plate.

The chromatoplate is prepared as follows

An aqueous slurry of the powder is prepared. A suitable binder such as plaster of paris, gypsum (calcium sulphate) or polyvinyl alcohol is added to the slurry, to hold the solid on the plate. The commercially available applicator is used to spread the slurry on the plate as a thin uniform film. The thickness of the film is in the range of 0.1 to 0.3 mm. The plate is dried to evaporate the solvent and heated in an oven at 110℃ to activate the adsorbent. Readymade of weakly polar compounds. But silica gel is used for polar compounds such as amino acids.

Types of thin layer chromatography

Thin layer chromatography can be divided into four types on the basis of the nature of the substance in thin layer.

1. Adsorption thin layer chromatography

2. Liquid-liquid partition thin layer chromatography

3. Ion exchange TLC and

4. Size exclusion TLC

1. Adsorption Thin Layer Chromatography

The most commonly used stationary phases are adsorbents. Silica gel, alumina and powdered cellulose are the most commonly used adsorbents.

i. **Silica-gel** : Silica gel particles contain hydroxyl groups on their surface which form hydrogen bond-with polar molecules. The silica gel chromatoplate is activated by heating at 110℃ in an oven for many hours to remove adsorbed water. Adsorbed water prevents the adsorption of the polar molecules and hence decreases the efficiency of the separation.

ii. **Alumina** : It also contains hydroxyl groups or oxygen atoms on its surface. Generally alumina is used for the separation of weekly polar compounds while silica-gel used for the separation of polar compounds such as amino acids and sugars.

iii. Other adsorbents such as activated charcoal or magnesium silicate or calcium silicate are used in TLC.

2. Liquid-liquid Partition Thin Layer Chromatography

In this type , the stationary and mobile phases are liquids. In this case the adsorbed or residual water of the slurry acts as the stationary liquid phase. Hence the chromatoplate is not activated by heating. The thin film of water supported on materials such as silica gel or diatomaceous earth acts as stationary liquid phase. This is similar to column chromatography and hence this type of TLC is also called as open column chromatography.

3. Ion-Exchange Thin Layer Chromatography

In this type, ion-exchange resins are used for preparing thin layer plates. The particle size of the resin is in the range of 40 to 80 mμ. For example

a. Strong acid cation exchanger in hydrogen or sodium form is Dowex-50W; $R_zSO_3^-H^+$ or $R_zSO_3^-Na^+$

b. Strong base anion exchanger in chloride form is Dowex 1; $R_zNR_3^+Cl^-$

A layer of 0.2 to 0.3 mm thickness of the resin is obtained by spreading a slurry of six parts of resin and one part of cellulose powder.

4. Size Exclusion Thin Layer Chromatography

Superfine sephadex is used for preparing chromatoplates. The gel is first soaked in water for about four days to complete the swelling and then spread on the plate. The plates are not dried but kept wet. As compared to other thin layers the capillary action of these molecular sieves is very slow. Hence the development time is about 8 to 10 hours while for other types of stationary phases it is only 25 minutes.

Mobile Phases for Thin Layer Chromatography

The mobile phase in TLC is liquid. A single solvent or a mixture of two or three solvents should be used. The mobile liquid phase is also called a developing solvent. Usually, mixed solvents give better separation of complex mixture. The selection of the developing solvent in TLC is nearly similar to that in column chromatography. The developing solvent must be pure and should not contain traces of water or other impurities. Otherwise irreproducible chromatograms are obtained.

Development of the Chromatogram

The technique of the development in TLC is nearly similar to that for paper chromatography. The mixture to be separated is applied to the plate in the form of a very small spot. A fine glass capillary is used for the application. The capillary is not allowed to touch the adsorbent, otherwise, a hole will result due to detachment of the adsorbent. The spots are applied near one end of the plate. Sample spot should be 2 to 5 mm in diameter. Typically, sample sizes range from 10 to 100 μg per spot. The position of the spot is marked and the plate is placed in a closed glass tank containing a little mobile phase at the bottom. The spot is at the lower end and should not dip into the solvent. The tank is saturated with the vapours of the solvent. In this vertical position, the mobile phase moves slowly up the plate under the influence of capillary action. When the moving phase has almost reached the top of the plate, the plate is removed. The position of the solvent front is marked and the plate is dried by hair dryer. The development is generally more rapid taking 10-50 minutes less than the paper chromatography. More, separations are more clear cut than in paper chromatography.

Detection of the Spots

It is easy to locate the positions of the coloured components but difficult to locate the colourless components on the chromatoplate. TLC separates many of the colourless components and their positions are located with the help of

locating agents. The positions of the components having the property to fluoresce can be located by observing the developed plate under ultra violet lamp.

Usually aromatic organic compounds fluoresce in ultraviolet lamp. Non-fluorescent compounds can be detected by using the thin layer plate containing fluorescent indicator in the Powdered adsorbent.

Chemical Methods of Detection

A. **Use of iodine** :

The developed plate is exposed to iodine vapours. Iodine vapours interact with the sample components to produce coloured compounds. This method is mainly used for the detection of the organic compound. It is non specific.

B. **Use of sulphuric acid** :

A solution of concentrated sulphuric acid (4 mL) in methanol (100 mL) can be sprayed on the plate for the detection of organic compounds. After treatment with sulphuric acid solution the plate is heated in an oven to about 200℃ until organic compound is seen as dark charred spots.

C. Other chemical reagents :

Many other chemical reagents are used for the detection of colourless components. For example, ninhydrin reagent is used for the detection of amino acids, the solution of of acid- base indicator is used for the detection of acids and bases. Usually the solutions the chemical reagents are spread on the plate. The detection of spots is sometimes easier than with paper chromatography.

Qualitative Detection of Components

R_f Values of the substances

The results of the chromatographic separation are express in terms of R_f values. R_f value is the short form of retardation factor. R_f is defined as the ratio of the distance travelled by the given substance (component) from the origin to the distance travelled by the moving solvent.

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

In other words, R_f values express the relative rates of movements of the substance and solvent. Under constant experimental conditions in a given chromatography system the ratio of movement of any substance relative to the movement of the developing solvent is constant the constant is called the R_f value

Applications of thin layer chromatography

1. TLC is used to detect and analyse mixtures of terpenes, oils, vitamins, chlorophylls, steroids.
2. Clinically, TLC is used to identify sugar in urine.
3. In forensic science, TLC is used to detect poisons, metal ions, drugs, tranquilizers in body fluids.
4. TLC is used in routine synthetic chemistry to check the preparation and purity of an organic compound.

Superiority of Thin Layer Chromatography over Paper Chromatography

Although the technique used in TLC is very similar to that in paper chromatography, TLC is considered to be superior to paper chromatography.

TLC has following advantages

1. It gives very sharp and clear-cut separation.
2. it can be used in organic chemistry, inorganic chemistry, biochemistry and Pharmaceutical laboratories.
3. It is faster than paper chromatography since it requires short development time (only twenty minutes).
4. It can be used for separating and identifying the components of the mixture on very small scale and very quickly.
5. It gives more reproducible Rf values.
6. It can be used for adsorption, partition ion- exchange etc. types of chromatography because of the wide choice of the stationary phase on the plate.