Chromatography

Introduction

Chromatography (/ˌkroʊməˈtɒgrəfi/; from Greek χρῶμα *chroma* which means "color" and γράφειν *graphein* "to write") is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the *mobile phase*, which carries it through a structure holding another material called the *stationary phase*. The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for later use, and is thus a form of purification. Analytical chromatography is done normally with smaller amounts of material and is for establishing the presence or measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive

History

Thin layer chromatography is used to separate components of a plant extract, illustrating the experiment with plant pigments that gave chromatography its name

Chromatography was first employed in Russia by the Italian-born scientist Mikhail Tsvet in 1900. He continued to work with chromatography in the first decade of the 20th century, primarily for the separation of plant pigments such as chlorophyll, carotenes, and xanthophylls. Since these components have different colors (green, orange, and yellow, respectively) they gave the technique its name. New types of chromatography developed during the 1930s and 1940s made the technique useful for many separation processes.

Chromatography technique developed substantially as a result of the work of Archer John Porter Martin and Richard Laurence Millington Synge during the 1940s and 1950s, for which they won a Nobel prize. They established the principles and basic techniques of partition chromatography, and their work encouraged the rapid development of several chromatographic methods: paper chromatography, gas chromatography, and what would become known as high performance liquid chromatography. Since then, the technology has advanced rapidly. Researchers found that the main principles of Tsvet's chromatography could be applied in many different ways, resulting in the different varieties of chromatography described below. Advances are continually improving the technical performance of chromatography, allowing the separation of increasingly similar molecules.

Applications

Chromatography is a powerful separation tool that is used in all branches of science, and is often the only means of separating components from complex mixtures. The Russian botanist Mikhail Tswett coined the term chromatography in 1906. The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures.

A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties. Many types of chromatography have been developed. These include Column chromatography, High performance liquid chromatography (HPLC), Gas chromatography, Size exclusion chromatography, Ion exchange chromatography etc.

In this book contains more details about the applications of chromatography by various research findings. Each and every topics of this book have included lists of references at the end to provide students and researchers with starting points for independent chromatography explorations. I welcome comments, criticisms, and suggestions from students, faculty and researchers.

Types of Chromatographic

This article throws light upon the twelve types of chromatographic techniques used in biochemistry.

The twelve types are: (1) Column Chromatography (2) Paper Chromatography (3) Thin Layer Chromatography (4) Gas Chromatography (5) High Performance Liquid Chromatography (6) Fast Protein Liquid Chromatography (7) Supercritical Fluid Chromatography (8) Affinity Chromatography (9) Reversed Phase Chromatography (10) Two Dimensional Chromatography (11) Pyrolysis Gas Chromatography and (12) Counter Current Chromatography.

There are different kinds of chromatographic techniques and these are classified according to the shape of bed, physical state of mobile phase, separation mechanisms.

Apart from these there are certain modified forms of these chromatographic techniques involving different mechanisms and are hence categorized as modified or specialized chromatographic techniques.

Type # 1. Column Chromatography:

It is the preparative application of chromatography. It is used to obtain pure chemical compounds from a mixture of compounds on a scale from micrograms up to kilograms using large industrial columns. The classical preparative chromatography column is a glass tube with a diameter from 5 to 50 mm and a height of 50 cm to 1 m with a tap at the bottom.

Slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. Care must be taken to avoid air bubbles. A solution of the organic material is pipetted on top of the stationary phase.

This layer is usually topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the velocity of newly added eluent. Eluent is slowly passed through the column to advance the organic material. Often a spherical eluent reservoir or an eluent-filled and stoppered separating funnel is put on top of the column.

Fig. 9.1: A chemist using column chromatographic apparatus (*Courtesy:* Deptt. of Biochemical Engineering, IT, BHU)

The individual components are retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent. At the end of the column they elute one at a time. During the entire chromatography process the eluent is collected in a series of fractions.

The composition of the eluent flow can be monitored and each fraction is analyzed for dissolved compounds, e.g., by analytical chromatography, UV absorption, or fluorescence. Coloured compounds (or fluorescent compounds with the aid of an UV lamp) can be seen through the glass wall as moving bands.

The stationary phase or adsorbent in column chromatography is a solid. The most common stationary phase for column chromatography is —$C_{18}H_{37}$, followed by alumina. Cellulose powder has often been used in the past. Also possible are ion exchange chromatography,

reversed-phase chromatography (RP), affinity chromatography or expanded bed adsorption (EBA). The stationary phases are usually finely ground powders or gels and/or are micro porous for an increased surface; though in EBA a fluidized bed is used.

The mobile phase or eluent is either a pure solvent or a mixture of different solvents. It is chosen so that the retention factor value of the compound of interest is roughly around 0.75 in order to minimize the time and the amount of eluent to run the chromatography. The eluent has also been chosen so that the different compounds can be separated effectively. The eluent is optimized in small scale pretests, often using thin layer chromatography (TLC) with the same stationary phase.

A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation. A simple laboratory column runs by gravity flow. The flow rate of such a column can be increased by extending the fresh eluent filled column above the top of the stationary phase or decreased by the tap controls. Better flow rates can be achieved by using a pump or by using compressed gas (e.g., air, nitrogen, or argon) to push the solvent through the column (flash column chromatography).

Automated flash chromatography systems attempt to minimize human involvement in the purification process. Automated systems may include components normally found on HPLC systems (gradient pump, sample injection apparatus, UV detector) and a fraction collector to collect the eluent. The software controlling an automated system will coordinate the components and help the user to find the resulting purified material within the fraction collector. The software will also store results from the process for archival or later recall purposes.

Type # 2. Paper Chromatography:

It is an analytical technique for separating and identifying mixtures that are or can be coloured, especially pigments. This can also be used in secondary or primary schools in ink experiments. This method has been largely replaced by thin layer chromatography; however it is still a powerful teaching tool. Two-way paper chromatography, also called two-dimensional chromatography, involves using two solvents and rotating the paper 90° in between. This is useful for separating complex mixtures of similar compounds, for example, amino acids.

A small, ideally concentrated spot of solution that contains the sample is applied to a strip of chromatography paper about 1 cm from the base, usually using a capillary tube for maximum precision. This sample is absorbed onto the paper and may form interactions with it. Any substance that reacts or bonds with the paper cannot be measured using -Solvent front technique. The paper is then dipped into a suitable solvent, such as ethanol or water, taking care that the spot is above the surface of the solvent, and placed in a sealed container.

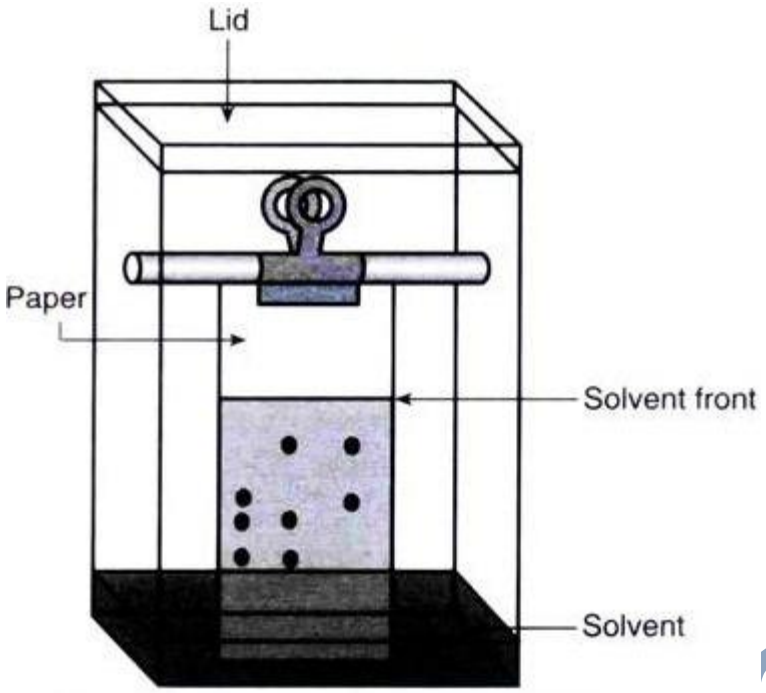


Fig. 9.2: Schematic diagram of paper chromatography

The solvent moves up the paper by capillary action, which occurs as a result of the attraction of the solvent molecules to the paper and to one another. As the solvent rises through the paper it meets and dissolves the sample mixture, which will then travel up the paper with the solvent. Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the fibers in the paper. Paper chromatography takes anywhere from several minutes to several hours.

In some cases, paper chromatography does not separate pigments completely; this occurs when two substances appear to have the same values in a particular solvent. In these cases, two-way chromatography is used to separate the multiple-pigment spots. The chromatogram is turned by ninety degrees, and placed in a different solvent in the same way as before; some spots separate in the presence of more than one pigment.

As before, the value is calculated, and the two pigments are identified. The R_f value (retention factor) is the distance travelled by a particular component from the origin (where the sample was originally spotted) as a ratio to the distance travelled by the solvent front from the origin. Rf values for each substance will be unique, and can be used to identify components. A particular component will have the same R_f value if it is separated under identical conditions.

After development, the spots corresponding to different compounds may be located by their colour, ultraviolet light, ninhydrin (Triketohydrindane hydrate) or by treatment with iodine vapours. The final chromatogram can be compared with other known mixture chromatograms to identify sample mixture using the R_n value.

As in most other forms of chromatography, paper chromatography uses R_n values to help identify compounds. R_f values are calculated by dividing the distance the pigment travels up the paper by the distance the solvent travels (the solvent front). Because R_f values are standard for a given compound, known R_n values can be used to aid in the identification of an unknown substance in an experiment.

Type # 3. Thin Layer Chromatography:

Thin-layer chromatography (TLC) is a chromatographic technique that is useful for separating organic compounds. It involves a stationary phase consisting of a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose immobilized onto a flat, inert carrier sheet. A liquid phase consisting of the solution to be separated dissolved in an appropriate solvent is drawn through the plate via capillary action, separating the experimental solution.

When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are visualized with ultraviolet light or by placing the plate in iodine vapour. The different components in the mixture move up the plate at different rates due to differences in their portioning behavior between the mobile liquid phase and the stationary phase.

It can be used to determine the pigments a plant contains, to detect pesticides or insecticides in food, in forensics to analyze the dye composition of fibers, or to identify compounds present in a given substance, among other uses. It is a quick, generic method for organic reaction monitoring.

TLC plates are made by mixing the adsorbent, such as silica gel, with a small amount of inert binder like calcium sulphate (gypsum) and water. This mixture is spread as thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic, and the resultant plate is dried and activated by heating in an oven for thirty minutes at 110°C. The thickness of the adsorbent layer is typically around 0.1-0.25 mm for analytical purposes and around 1-2 mm for preparative TLC. Every type of chromatography contains a mobile phase and a stationary phase.

The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products.

A small spot of solution containing the sample is applied to a plate, about one centimetre from the base. The plate is then dipped into a suitable solvent, such as ethanol or water, and placed in a sealed container. The solvent moves up the plate by capillary action and meets the sample mixture, which is dissolved and is carried up the plate by the solvent.

Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the stationary phase. Results also vary depending on the solvent used. For example, if the solvent were a 90:10 mixture of hexane to ethyl acetate, then the solvent would be mostly non-polar.

This means that when analyzing the TLC, the non-polar parts will have moved further up the plate. The polar compounds, in contrast, will not have moved as much. The reverse is true when using a solvent that is more polar than non-polar (10:90 hexane to ethyl acetate). With these solvents, the polar compounds will move higher up the plate, while the non-polar compounds will not move as much.

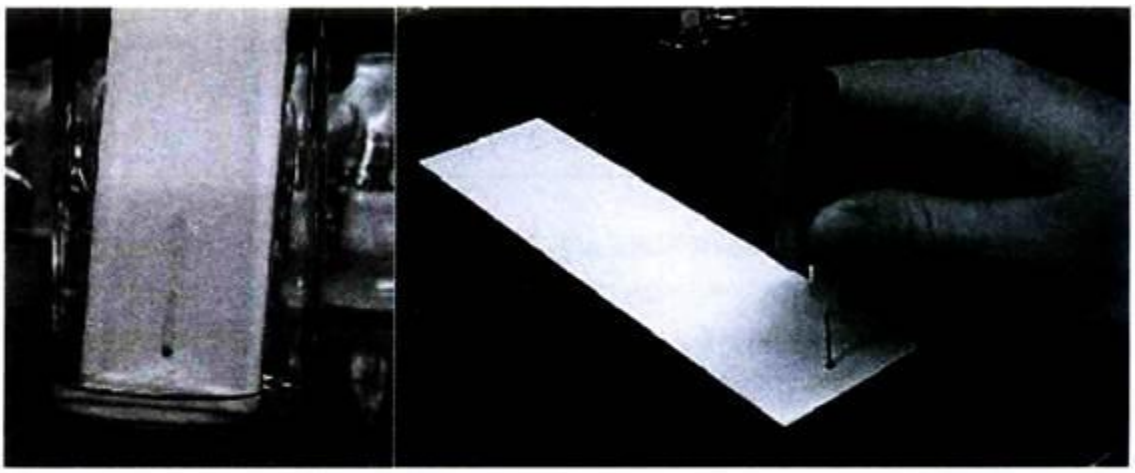


Fig. 9.3: Spotting of TLC plate with a sample and then running the TLC plate in the solvent (*Courtsey:* Dr D Dash, Deptt. of Biochemistry, IMS, BHU)

The appropriate solvent in context of thin layer chromatography will be one which differs from the stationary phase material in polarity. If polar solvent is used to dissolve the sample and spot is applied over polar stationary phase of TLC, the sample spot will grow radially due to capillary action, which is not advisable as one spot may mix with the other.

Hence, to restrict the radial growth of sample-spot, the solvent used for dissolving samples in order to apply them on plates should be as non-polar or semi-polar as possible when the stationary phase is polar, and vice versa.

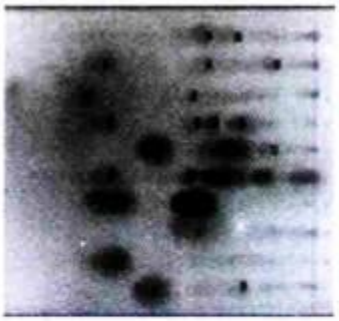


Fig. 9.4: Chromatogram of 10 essential oils coloured with vanillin reagent

As the chemicals being separated may be colourless, several methods exist to visualize the spots:

1. Often a small amount of a fluorescent compound, usually Manganese-activated Zinc Silicate, is added to the adsorbent that allows the visualization of spots under a black-light(UV_{254}). The adsorbent layer will thus fluoresce light green by itself, but spots of analyte quench this fluorescence.

2. Iodine vapours are a general unspecific colour reagent

3. Specific colour reagents exist into which the TLC plate is dipped or which are sprayed onto the plate

Once visible, the R_f value of each spot can be determined by dividing the distance travelled by the product by the total distance travelled by the solvent (the solvent front). These values depend on the solvent used, and the type of TLC plate, and are not physical constants.

Type # 4. Gas-Liquid Chromatography (GLC) or Simply Gas Chromatography (GC):

It is a type of chromatography in which the mobile phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen, and the stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside glass or metal tubing, called a column. The instrument used to perform gas chromatographic separations is called a gas chromatograph (also: aerograph, gas separator).

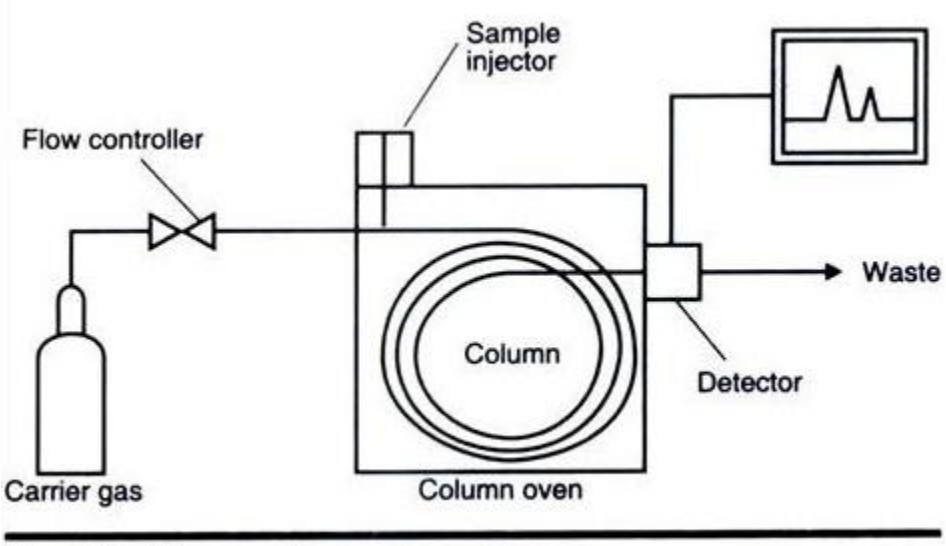


Fig. 9.5: Diagram of a gas chromatograph

A gas chromatograph is a chemical analysis instrument for separating chemicals in a complex sample. 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, and the temperature.

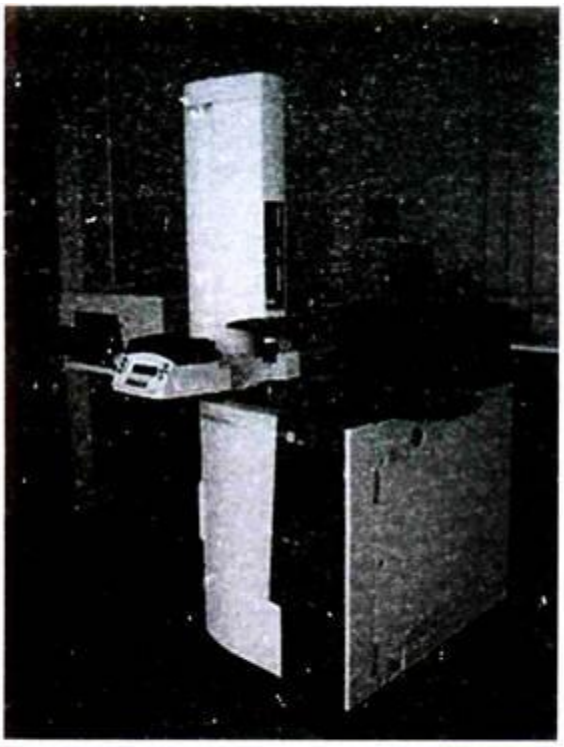


Fig. 9.6: A gas chromatograph with an headspace sampler (*Courtesy*: Deptt. of Pharmaceutics, IT, BHU)

In a GC analysis, a known volume of gaseous or liquid analyte is injected into the "entrance" (head) of the column, usually using a micro syringe (or, solid phase micro-extraction fibers, or a gas source switching system). 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 molecule and on the stationary phase materials. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column and reach the end 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.

Auto Samplers:

The auto sampler provides the means to introduce automatically a sample into the inlets. Manual insertion of the sample is possible but very rare nowadays. Automatic insertion provides better reproducibility and time-optimization. Different kinds of auto samplers exist. Auto samplers can be classified in relation to sample capacity (auto-injectors VS. auto samplers, where auto-injectors can work a small number of samples), to robotic technologies (XYZ robot VS rotating/SCARA-robot – the most common), or to analysis:

i. Liquid

ii. Static head-space by syringe technology

iii. Dynamic head-space by transfer-line technology

iv. Solid phase micro extraction (SPME).

Inlets:

The column inlet (or injector) provides the means to introduce a sample into a continuous flow of carrier gas. The inlet is a piece of hardware attached to the column head.

Common inlet types are:

1. S/SL (Split/Split less) injector:

A sample is introduced into a heated small chamber via a syringe through a septum; the heat facilitates volatilization of the sample and sample matrix. The carrier gas then either sweeps the entirety (split less mode) or a portion (split mode) of the sample into the column. In split mode, a part of the sample/carrier gas mixture in the injection chamber is exhausted through the split vent.

2. On-column inlet:

The sample is here introduced in its entirety without heat.

3. PTV injector:

Temperature-programmed sample introduction was first described by Vogt in 1979. Originally Vogt developed the technique as a method for the introduction of large sample volumes (up to 250 µl) in capillary GC. Vogt introduced the sample into the liner at a controlled injection rate. The temperature of the liner was chosen slightly below the boiling point of the solvent.

The low-boiling solvent was continuously evaporated and vented through the split line. Based on this technique, Poy developed the Programmed Temperature Vaporizing Injector PTV. By introducing the sample at a low initial liner temperature many of the disadvantages of the classic hot injection techniques could be circumvented.

4. Gas source inlet or gas switching valve:

Gaseous samples in collection bottles are connected to what is most commonly a six-port switching valve. The carrier gas flow is not interrupted while a sample can be expanded into a previously evacuated sample loop. Upon switching, the contents of the sample loop are inserted into the carrier gas stream.

5. P/T (Purge-and-Trap) system:

An inert gas is bubbled through an aqueous sample causing insoluble volatile chemicals to be purged from the matrix. The volatiles are 'trapped' on an absorbent column (known as a trap or concentrator) at ambient temperature. The trap is then heated and the volatiles are directed into the carrier gas stream. Samples requiring pre-concentration or purification can be introduced via such a system, usually hooked up to the S/SL port.

6. SPME (solid phase micro extraction) offers a convenient, low-cost alternative to P/T systems with the versatility of a syringe and simple use of the S/SL port.

Columns:

Two types of columns are used in GC:

1. Packed columns are 1.5-10 m in length and have an internal diameter of 2-4 mm. The tubing is usually made of stainless steel or glass and contains a packing of finely divided, inert, solid support material (e.g., diatomaceous earth) that is coated with a liquid or solid stationary phase. The nature of the coating material determines what type of materials will be most strongly adsorbed. Thus numerous columns are available that are designed to separate specific types of compounds.

2. Capillary columns have a very small internal diameter, on the order of a few tenths of millimeters, and lengths between 25-60 metres are common. The inner column walls are coated with the active materials (WCOT columns), some columns are quasi-solid filled with many parallel microspores (PLOT columns). Most capillary columns are made of fused-silica with a polyimide outer coating. These columns are flexible, so a very long column can be wound into a small coil.

3. New developments are sought where stationary phase incompatibilities lead to geometric solutions of parallel columns within one column.

Among these new developments are:

(i) Internally heated micro FAST columns, where two columns, an internal heating wire and a temperature sensor are combined within a common column sheath (micro FAST);

(ii) Micro packed columns (1/16″ OD) are column-in-column packed columns where the outer column space has a packing different from the inner column space, thus providing the separation behaviour of two columns in one. They can be easily fit to inlets and detectors of a capillary column instrument.

The temperature-dependence of molecular adsorption and of the rate of progression along the column necessitates a careful control of the column temperature to within a few tenths of a degree for precise work. Reducing the temperature produces the greatest level of separation, but can result in very long elution times. For some cases, temperature is ramped either continuously or in steps to provide the desired separation. This is referred to as a temperature program. Electronic pressure control can also be used to modify flow rate during the analysis, aiding in faster run times while keeping acceptable levels of separation.

The choice of carrier gas (mobile phase) is important, with hydrogen being the most efficient and providing the best separation. However, helium has a larger range of flow rates that are comparable to hydrogen in efficiency, with the added advantage that helium is non-flammable, and works with a greater number of detectors. Therefore, helium is the most common carrier gas used.

Detectors:

A number of detectors are used in gas chromatography. The most common are the flame ionization detector (FID) and the thermal conductivity detector (TCD). Both are sensitive to a wide range of components, and both work over a wide range of concentrations.

While TCDs are essentially universal and can be used to detect any component other than the carrier gas (as long as their thermal conductivities are different than that of the carrier gas, at detector temperature), FIDs are sensitive primarily to hydrocarbons, and are more sensitive to them than TCD.

However, an FID cannot detect water. Both detectors are also quite robust. Since TCD is non-destructive, it can be operated in series before an FID (destructive), thus providing complementary detection of the same eluents. Other detectors are sensitive only to specific types of substances, or work well only in narrower ranges of concentrations.

They include:

i. Discharge ionization detector (DID)

ii. Electron capture detector (ECD)

iii. Flame photometric detector (FPD)

iv. Hall electrolytic conductivity detector (E1CD)

v. Helium ionization detector (HID)

vi. Nitrogen phosphorus detector (NPD)

vii. Mass selective detector (MSD)

viii. Photo-ionization detector (PID)

ix. Pulsed discharge ionization detector (PDD)

Some gas chromatographs are connected to a mass spectrometer which acts as the detector. The combination is known as GC-MS. Some GC-MS are connected to an Nuclear Magnetic Resonance Spectrometer which acts as a back-up detector. This combination is known as GC-MS- NMR. Some GC-MS-NMR are connected to an infrared spectra which acts as a back-up detector. This combination is known as GC-MS-NMR-IR. It must, however, be stressed that this is very rare as most analysis needed can be concluded via purely GC-MS.

Methods:

The method is the collection of conditions in which the GC operates for a given analysis. Method development is the process of determining what conditions are adequate and/or ideal for the analysis required. Conditions which can be varied to accommodate a required analysis include inlet temperature, detector temperature, column temperature and temperature program, carrier gas and carrier gas flow rates, the column's stationary phase, diameter and length, inlet type and flow rates, sample size and injection technique.

Depending on the detector(s) (see below) installed on the GC, there may be a number of detector conditions that can also be varied. Some GCs also include valves which can change the route of sample and carrier flow, and the timing of the turning of these valves can be important to method development.

Carrier Gas Selection and Flow Rates:

Typical carrier gases include helium, nitrogen, argon, hydrogen and air. Which gas to use is usually determined by the detector being used, for example, a DID requires helium as the carrier gas. When analyzing gas samples, however, the carrier is sometimes selected based on the sample's matrix, for example, when analyzing a mixture in argon, an argon carrier is preferred, because the argon in the sample does not show upon the chromatogram. Safety and availability can also influence carrier selection, for example, hydrogen is flammable, and high-purity helium can be difficult to obtain in some areas of the world.

The purity of the carrier gas is also frequently determined by the detector, though the level of sensitivity needed can also play a significant role. Typically, purities of 99.995% or higher are used. Trade names for typical purities include "Zero Grade", "Ultra-High Purity (UHP) Grade", "4.5 Grade" and "5.0 Grade".

The carrier gas flow rate affects the analysis in the same way that temperature does (see above). The higher the flow rates the faster the analysis, but the lower the separation between analytes. Selecting the flow rate is, therefore, the same compromise between the level of separation and length of analysis as selecting the column temperature.

With GCs made before 1990s, carrier flow rate was controlled indirectly by controlling the carrier inlet pressure, or "column head pressure". The actual flow rate was measured at the outlet of the column or the detector with an electronic flow meter, or a bubble flow meter, and could be an involved, time consuming, and frustrating process. The pressure setting was not able to be varied during the run, and thus the flow was essentially constant during the analysis.

Many modern GCs, however, electronically measure the flow rate, and electronically control the carrier gas pressure to set the flow rate. Consequently, carrier pressures and flow rates can be adjusted during the run, creating pressure/flow programs similar to temperature programs.

Inlet Types and Flow Rates:

The choice of inlet type and injection technique depends on if the sample is in liquid, gas, adsorbed, or solid form, and on whether a solvent matrix is present that has to be vaporized. Dissolved samples can be introduced directly onto the column via a COC injector, if the conditions are well known; if a solvent matrix has to be vaporized and partially removed, a S/SL injector is used (most common injection technique); gaseous samples (e.g., air cylinders) are usually injected using a gas switching valve system; adsorbed samples (e.g., on adsorbent tubes) are introduced using either an external (on-line or off-line) desorption apparatus such as a purge-and-trap system, or are desorbed in the S/SL injector (SPME applications).

Sample size and injection technique:

The real chromatographic analysis starts with the introduction of the sample onto the column. The development of capillary gas chromatography resulted in many practical problems with the injection technique. The technique of on-column injection, often used with packed columns, is usually not possible with capillary columns.

The injection system, in the capillary gas chromatograph, should fulfill the following two requirements:

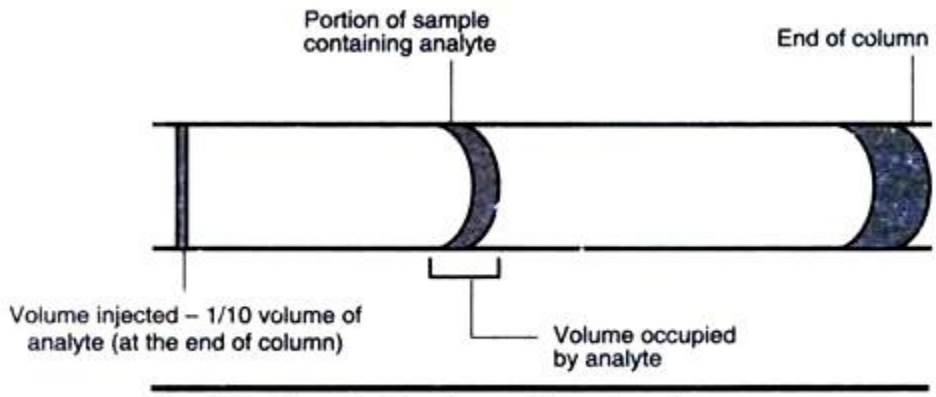


Fig. 9.7: The rule of ten in gas chromatography

1. The amount injected should not overload the column.

2. The width of the injected plug should be small compared to the spreading due to the chromatographic process. Failure to comply with this requirement will reduce the separation capability of the column. As a general rule, the volume injected, V_{inj}, and the volume of the detecor cell, V_{det}, should be about 1/10 of the volume occupied by the portion of sample containing the molecules of interest (analytes) when they exit the column.

Some general requirements, which a good injection technique should fulfill, are:

(i) It should be possible to obtain the column's optimum separation efficiency.

(ii) It should allow accurate and reproducible injections of small amounts of representative samples.

(iii) It should induce no change in sample composition. It should not exhibit discrimination based on differences in boiling point, polarity, concentration or thermal/catalytic stability.

(iv) It should be applicable for trace analysis as well as for undiluted samples.

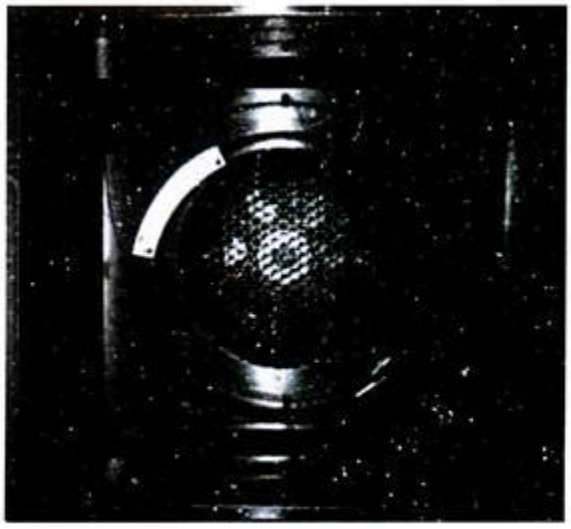


Fig. 9.8: A gas chromatography oven, open to show a capillary column

Column Selection:

The column in a GC is contained in an oven, the temperature of which is precisely controlled electronically. The rate at which a sample passes through the column is directly proportional to the temperature of the column. The higher the column temperature, the faster the sample moves through the column. However, the faster a sample moves through the column, the less it interacts with the stationary phase, and the less the analytes are separated. In general, the column temperature is selected to compromise between the length of the analysis and the level of separation.

A method which holds the column at the same temperature for the entire analysis is called "isothermal". Most methods, however, increase the column temperature during the analysis, the initial temperature, rate of temperature increase (the temperature "ramp") and final temperature is called the "temperature program". A temperature program allows analytes that elute early in the analysis to separate adequately, while shortening the time it takes for late-eluting analytes to pass through the column.

Data Reduction and Analysis:

Qualitative analysis:

Generally chromatographic data is presented as a graph of detector response (y-axis) against retention time (x-axis). This provides a spectrum of peaks for a sample representing the analytes present in a sample eluting from the column at different times. Retention time can be used to identify analytes if the method conditions are constant.

Also, the pattern of peaks will be constant for a sample under constant conditions and can identify complex mixtures of analytes. In most modern applications, however, the GC is

connected to a mass spectrometer or similar detector that is capable of identifying the analytes represented by the peaks.

Quantitive analysis:

The area under a peak is proportional to the amount of analyte present. By calculating the area of the peak using the mathematical function of integration, the concentration of an analyte in the original sample can be determined. Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the response factor of an analyte.

The response factor is the expected ratio of an analyte to an internal standard and is calculated by finding the response of a known amount of analyte and a constant amount of internal standard (a chemical added to the sample at a constant concentration, with a distinct retention time to the analyte). In most modern GC-MS systems, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.

Limitations Associated with Gas Chromatography:

In fact, a GC analysis takes much more time; sometimes a single sample must be run more than an hour according to the chosen program; and even more time is needed to "heat out" the column; so it is free from the first sample and can be used for the next. Equally, several runs are needed to confirm the results of a study—a GC analysis of a single sample may simply yield a result per chance.

Also, GC does not positively identify most samples; and not all substances in a sample will necessarily be detected. All a GC truly tells is at which relative time a component eluted from the column and that the detector was sensitive to it.

To make results meaningful, analysts need to know which components at which concentrations are to be expected; and even then a small amount of a substance can hide itself behind a substance having both a higher concentration and the same relative elution time. Last but not least it is often needed to check the results of the sample against a GC analysis of a reference sample containing only the suspected substance. A GC-MS can remove much of this ambiguity, since the mass spectrometer will identify the component's molecular weight. But this still takes time and skill to do properly.

Similarly, most GC analyses are not push-button operations. According to the substances one expects to find the operating program must be carefully chosen. A push-button operation can exist for running similar samples repeatedly, such as in a chemical production environment or for comparing 20 samples from the same experiment to calculate the mean content of the same substance. However, for the kind of investigative work this is clearly not the case.

Type # 5. High Performance Liquid Chromatography:

High-performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry. It is also sometimes referred to as high-pressure liquid chromatography. HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analyzed (analyte) and the chromatography column.

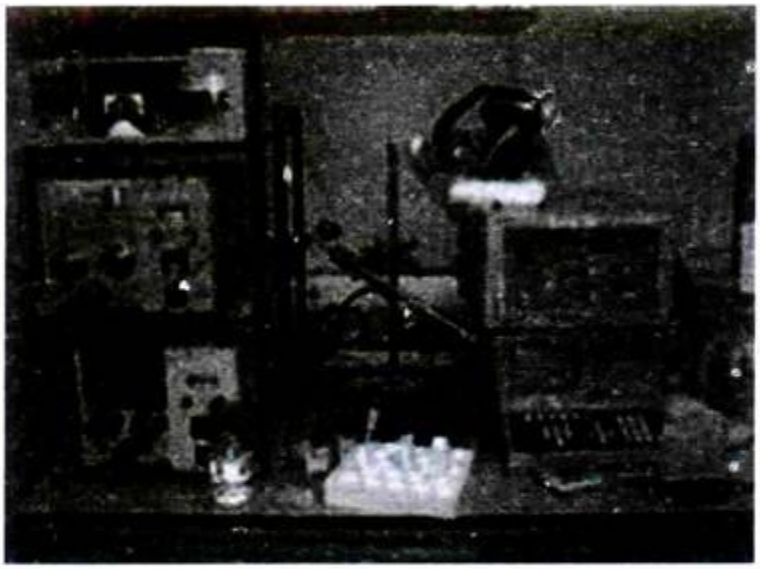


Fig. 9.9: A HPLC. From left to right: A pumping device generating a gradient of two different solvents, a steel enforced column and an apparatus for measuring the absorbance. (*Courtesy:* Deptt. of Phamacology, IMS, BHU)

In isocratic HPLC, the analyte is forced through a column of the stationary phase (usually a tube packed with small round particles with a certain surface chemistry) by pumping a liquid (mobile phase) at high pressure through the column. The sample to be analyzed is introduced in a small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column.

The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram.

Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in

the separation of the analyte components, or compounds such as Trifluoroacetic acid which acts as an ion pairing agent.

A further refinement to HPLC has been to vary the mobile phase composition during the analysis; this is known as gradient elution. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes, depending on how hydrophobic the analyte is.

The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, the more hydrophobic components will elute (come off the column) under conditions of relatively high methanol; whereas the more hydrophilic compounds will elute under conditions of relatively low methanol.

The choice of solvents, additives and gradient, depends on the nature of the stationary phase and the analyte. Often a series of tests are performed on the analyte and a number of generic runs may be processed in order to find the optimum HPLC method for the analyte — the method which gives the best separation of peaks.

Types of HPLC:

1. Normal phase chromatography:

Normal phase HPLC (NP-HPLC) was the first kind of HPLC chemistry used, and separates analytes based on polarity. This method uses a polar stationary phase and a non-polar mobile phase, and is used when the analyte of interest is fairly polar in nature. The polar analyte associates with and is retained by the polar stationary phase.

Adsorption strengths increase with increase in analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on steric factors, and structural isomers are often resolved from one another.

Use of more polar solvents in the mobile phase will decrease the retention time of the analytes while more hydrophobic solvents tend to increase retention times. Particularly polar solvents in a mixture tend to deactivate the column by occupying the stationary phase surface. This is somewhat particular to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

NP-HPLC had fallen out of favour in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently it has become useful again with the development of HILIC bonded phases which utilize a partition mechanism which provides reproducibility.

2. Reversed phase chromatography:

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and a moderately polar mobile phase. One common stationary phase is a silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is, therefore, longer for molecules which are more non-polar in nature, allowing polar molecules to elute more readily.

Retention time is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic solvent. Reversed phase chromatography is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification.

RP-HPLC operates on the principle of hydrophobic interactions which result from repulsive forces between a relatively polar solvent, the relatively non-polar analyte, and the non-polar stationary phase. The driving force in the binding of the analyte to the stationary phase is the decrease in the area of the non-polar segment of the analyte molecule exposed to the solvent.

This hydrophobic effect is dominated by the decrease in free energy from entropy associated with the minimization of the ordered molecule-polar solvent interface. The hydrophobic effect is decreased by adding more non-polar solvent into the mobile phase. This shifts the partition coefficient such that the analyte spends some portion of time moving down the column in the mobile phase, eventually eluting from the column.

The characteristics of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a longer alkyl chain length results in a longer retention time because it increases the molecule's hydrophobicity.

Very large molecules, however, can result in incomplete interaction between the large analyte surface and the alkyl chain. Retention time increases with hydrophobic surface area which is roughly inversely proportional to solute size. Branched chain compounds elute more rapidly than their corresponding isomers because the overall surface area is decreased.

Apart from mobile phase hydrophobicity, other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a linear increase in the surface tension of aqueous solutions, and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tends to increase the retention time.

Another important component is pH since this can change the hydrophobicity of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. An organic acid such as formic acid or most commonly trifluoro-acetic acid is often added to the mobile phase.

These serve multiple purposes: they control pH, neutralize the charge on any residual exposed silica on the stationary phase and act as ion pairing agents to neutralize charge on the analyte. The effect varies depending on use but generally improves the chromatography.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reverse phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica backbone. They can be used with aqueous acid but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment.

The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be retained. A good test for the metal content of a column is to inject a sample which is a mixture of 2, 2′- and 4, 4′- bipyridine. Because the 2,2′- bipyridine can chelate the metal it is normal that when a metal ion is present on the surface of the silica the shape of the peak for the 2,2′-bipyridine will be distorted, tailing will be seen on this distorted peak.

3. Size exclusion chromatography:

Size exclusion chromatography (SEC) is a chromatographic method in which particles are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. When an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography.

The name gel permeation chromatography is used when an organic solvent is used as a mobile phase. The main application of gel filtration chromatography is the fractionation of proteins and other water-soluble polymers, while gel permeation chromatography is used to analyze the molecular weight distribution of organic-soluble polymers. Either technique should not be confused with gel electrophoresis, where an electric field is used to “pull” or “push” molecules through the gel depending on their electrical charges.

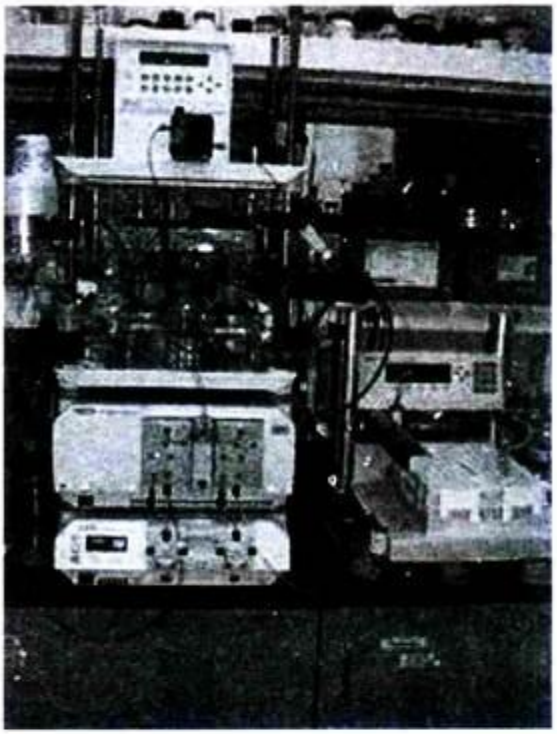


Fig. 9.10: Equipment for running size exclusion chromatography. The buffer is pumped through the column (right) by a computer controlled device.

SEC is a widely used technique for the purification and analysis of synthetic and biological polymers, such as proteins, polysaccharides and nucleic acids. Biologists and biochemists typically use a gel medium—usually polyacrylamide, dextran or agarose—and filter under low pressure. Polymer chemists typically use either a silica or cross-linked polystyrene medium under a higher pressure. These media are known as the stationary phase.

The advantage of this method is that the various solutions can be applied without interfering with the filtration process, while preserving the biological activity of the particles to be separated. The technique is generally combined with others that further separate molecules by other characteristics, such as acidity, basicity, charge, and affinity for certain compounds.

The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This result in the separation of a solution of particles based on size, provided that all the particles are loaded simultaneously or near simultaneously, particles of the same size should elute together.

This is usually achieved with an apparatus called a column, which consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes. These pores may be depressions on the surface or channels through the bead. As the

solution travels down the column some particles enter into the pores. Larger particles cannot enter into as many pores. The larger the particles, the less overall volume to traverse over the length of the column, and the faster the elution.

The filtered solution that is collected at the end is known as the eluent. The void volume consists of any particles too large to enter the medium, and the solvent volume is known as the column volume. In real life situations, particles in solution do not have a constant, fixed size, resulting in the probability that a particle which would otherwise be hampered by a pore may pass right by it. Also, the stationary phase particles are not ideally defined; both particles and pores may vary in size.

Elution curves, therefore, resemble Gaussian distributions. The stationary phase may also interact in undesirable ways with a particle and influence retention times, though great care is taken by column manufacturers to use stationary phases which are inert and minimize this issue.

Like other forms of chromatography, increasing the column length will tighten the resolution, and increasing the column diameter increases the capacity of the column. Proper column packing is important to maximize resolution: an over packed column can collapse the pores in the beads, resulting in a loss of resolution. An under packed column can reduce the relative surface area of the stationary phase accessible to smaller species, resulting in those species spending less time trapped in pores.

In simple manual columns the eluent is collected in constant volumes, known as fractions. The more similar the particles are in size, the more likely they will be in the same fraction and not detected separately. More advanced columns overcome this problem by constantly monitoring the eluent.

The collected fractions are often examined by spectroscopic techniques to determine the concentration of the particles eluted. Three common spectroscopy detection techniques are refractive index (RI), evaporative light scattering (ELS), and ultraviolet (UV). When eluting spectroscopically similar species (such as during biological purification) other techniques may be necessary to identify the contents of each fraction.

The elution volume decreases roughly linearly with the logarithm of the molecular hydrodynamic volume (often assumed to be proportional to molecular weight). Columns are often calibrated using 4-5 standard samples (e.g., folded proteins of known molecular weight) to determine the void volume and the slope of the logarithmic dependence. This calibration may need to be repeated under different solution conditions.

Type # 6. Fast Protein Liquid Chromatography:

Fast Protein Liquid Chromatography, usually referred to as FPLC, is a form of column chroma-tography used to separate or purify proteins from complex mixtures. It is very commonly used in biochemistry and enzymology. Columns used with an FPLC can separate macromolecules based on size, charge distribution, hydrophobicity, or bio-recognition (as with affinity chromatography).

Typical columns used for protein purification include:

i. Ion exchange chromatography, cation or anion — separates proteins based on surface- charges

ii. Gel filtration chromatography, also known as Gel permeation chromatography or desalting — separates proteins based on size.

iii. Reversed phase or hydrophobic interaction — separates based on hydrophobicity which depends heavily on surface area.

iv. Affinity chromatography — columns which purify based on ligand affinity, such as a His-tagged protein uses a nickel column.

Type # 7. Supercritical Fluid Chromatography (SFC):

It is a robust and easy-to-use form of normal phase chromatography ideally suited to the analysis and purification of low to moderate molecular weight, thermally labile molecules. It is especially suited to the separation of chiral compounds. Similar to high performance liquid chromatography (HPLC), SFC typically utilizes carbon dioxide as the mobile phase; therefore, the entire chromatographic flow path must be pressurized.

In addition, SFC metering pumps require that the pump head be kept cold in order to maintain the carbon dioxide in a supercritical state, where it can be effectively metered at some specified flow rate. The chemist sets mobile phase flow rate, composition, and column temperature. In addition, SFC provides an additional control parameter, pressure, which the chemist similarly sets through the keyboard. From an operational standpoint, SFC is as simple and robust as HPLC.

Any molecule that will dissolve in methanol or a less polar solvent is an ideal candidate for SFC. SFC can even separate polar solutes. Many strong bases that are difficult to separate by other techniques separate rapidly and efficiently with good peak shapes.

Type # 8. Affinity Chromatography:

It is a chromatographic method of separating biochemical mixtures, based on a highly specific biologic interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography with the ability to design a stationary phase that reversibly binds to a known subset of molecules.

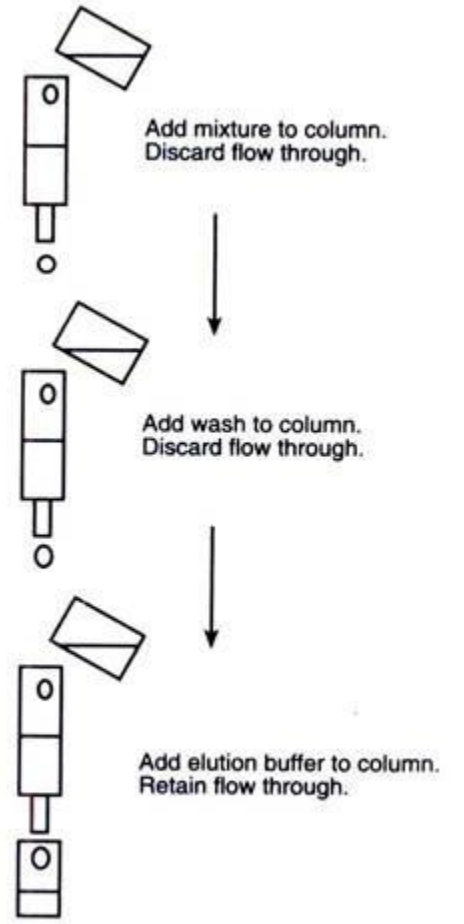


Fig. 9.12: Column chromatography

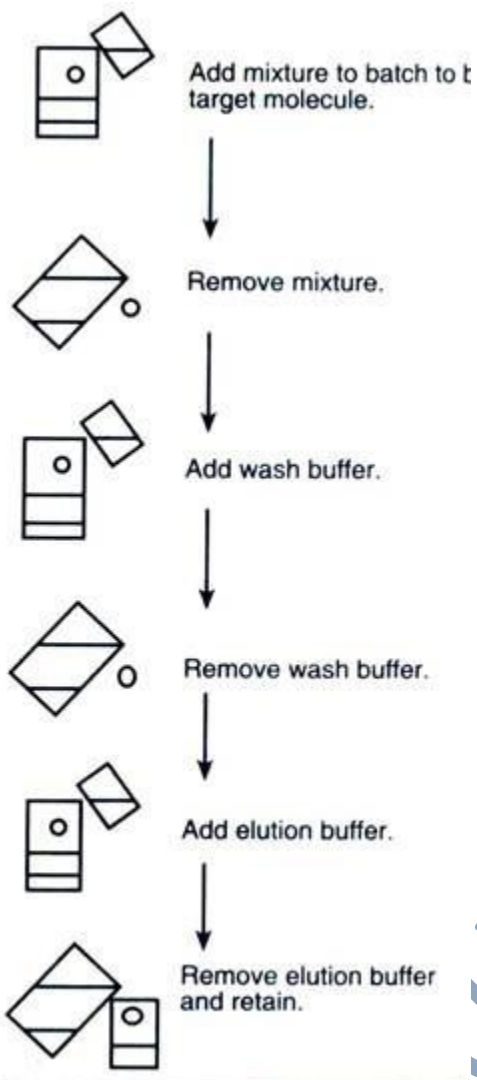


Fig. 9.13: Batch chromatography

Affinity chromatography can be used to:

i. Purify and concentrate a molecule from a mixture into a buffering solution.

ii. Reduce the amount of a molecule in a mixture.

iii. Discern what biological compounds bind to a particular molecule, such as drugs.

Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum. The molecule of interest will have a well known and defined property which can be exploited during the affinity purification process. The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium.

The other molecules in solution will not become trapped as they do not possess this property. The solid medium can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution.

Binding to the solid phase may be achieved by column chromatography, whereby the solid medium is packed onto a chromatography column, the initial mixture run through the column to allow binding, a wash buffer run through the column and the elution buffer subsequently applied to the column and collected. These steps are usually done at ambient pressure (as opposed to HPLC or FPLC).

Alternatively binding may be achieved using a batch treatment, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase (by centrifugation, for example), removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the eluate. Sometimes a hybrid method is employed, the binding is done by the batch method, then the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

A third method, expanded bed adsorption, which combines the advantages of the two methods mentioned above, has also been developed. The solid phase particles are placed in a column where liquid phase is pumped in from the bottom and exits at the top. The gravity of the particles ensures that the solid phase does not exit the column with the liquid phase.

Type # 9. Reversed-Phase Chromatography:

Reverse-phase chromatography (RPC) includes any chromatographic method that uses a non-polar stationary phase. All of the mathematical and experimental considerations used in other chromatographic methods apply (i.e., separation resolution proportional to the column length and inversely proportional to the column width).

Reversed-phase column chromatography is widely used in the pharmaceutical, chemical, and biochemical industry for separating molecules of small molecular weight. In more recent years RPC has been used to separate larger molecules. Any inert non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography. Common examples include larger hydrocarbons, diphenyl, and divinylbenzene.

Typically mobile phases are made of water or a polar organic compound such as acetonitrile or the lighter alcohols. Buffer solutions are used to control the protonation of functional groups. For example, a buffer of high pH will encourage elution of alcohols because they are charged in basic solutions and, therefore, prefer to be in the mobile phase. For the same reason, very acidic solutions encourage the elution of nitrogen containing molecules. Using multiple buffers allows for selective elution of a wide variety of chemicals.

Type # 10. Two-Dimensional Chromatography:

By using an additional physicochemical (Chemical classification) criterion for separation of the mixture of analytes (sample), the resolution and quality of chromatographic separation can be

increased. As a result, higher specificity concerning the separational capability of the chromatographic technique is obtained, allowing separation and preparation or analysis of compounds indistinguishable by one-dimensional chromatography.

In Gas-Phase Chromatography, two-dimensional separation is achieved by coupling a second, short column to the first long column. Coupling is achieved by different techniques, for example, shock-freezing the elutes in order of elution from the first column at fixed time-intervals, and then reheating them in order of elution, releasing them into the second column. The time of traversal through the second column needs to be shorter than the time remaining until the next sample is reheated to prevent compound build-up and to fully exploit the separational capability.

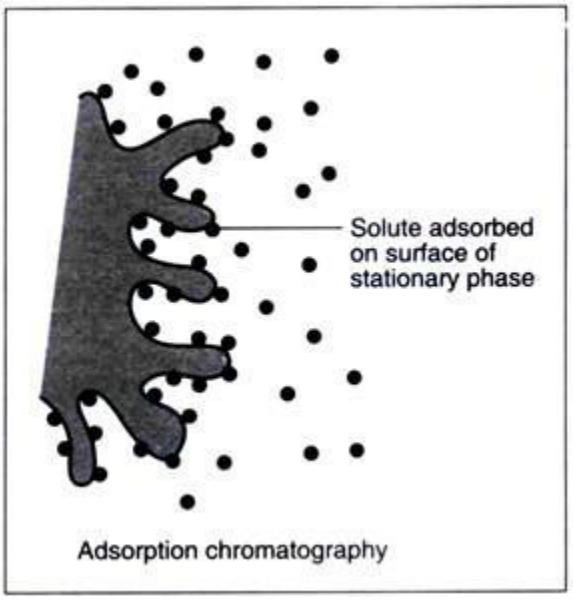
Type # 11. Pyrolysis Gas Chromatography:

Pyrolysis-chromatography is a potent analytical tool able to thermally crack (fragment) essentially non-volatile molecules into fragments suitable for chromatographic analysis. The technique enables a reproducible and characteristic "fingerprint" to be generated of a non-volatile sample. The technique can be applied to such varied tasks as bacterial strain differentiation and forensic characterisation of paints, polymers and fibre cross-matching.

Type of Chromatography	Applications in the Real World	Why and What is it
Liquid Chromatography	testing water samples to look for pollution	Used to analyze metal ions and organic compounds in solutions. It uses liquids which may incorporate hydrophilic, insoluble molecules.
Gas Chromatography	detecting bombs in airports, identifying and quantifying such drugs as alcohol, being used in forensics to compare fibres found on a victim	Used to analyze volatile gases. Helium is used to move the gaseous mixture through a column of absorbent material.
Thin-Layer Chromatography	detecting pesticide or insecticide residues in food, also used in forensics to analyze the dye composition of fibres	Uses an absorbent material on flat glass plates. This is a simple and rapid method to check the purity of the organic compound.
Paper Chromatography	separating amino acids and anions, RNA fingerprinting, separating and testing histamines, antibiotics	The most common type of chromatography. The paper is the stationary phase. This uses capillary action to pull the solutes up through the paper and separate the solutes.

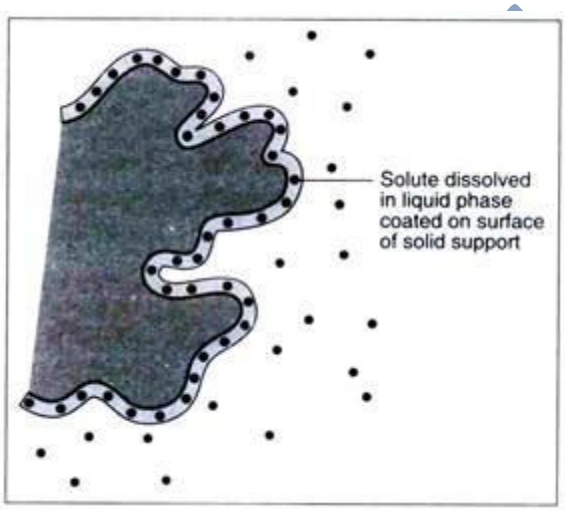
Adsorption Chromatography:

Adsorption chromatography is probably one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phases accounts for the separation of different solutes.



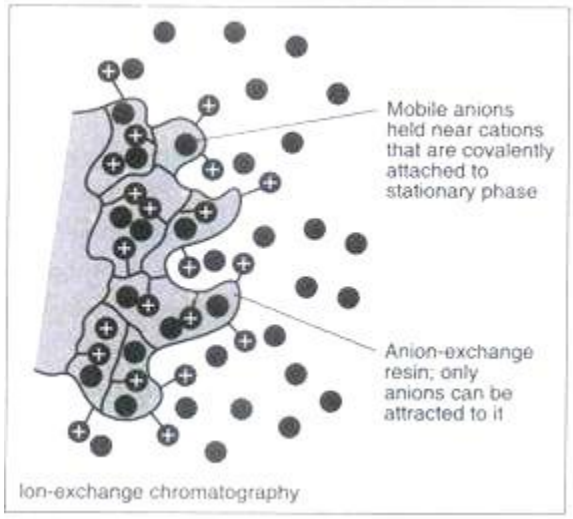
Partition Chromatography:

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid.



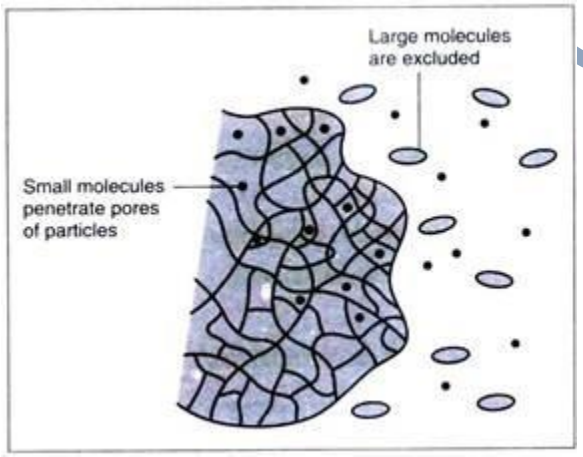
Ion Exchange Chromatography:

In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it. Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.



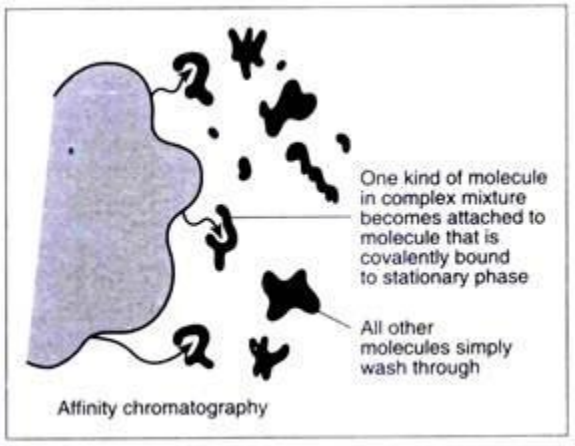
Molecular Exclusion Chromatography:

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size. The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through the column at a faster rate than the smaller ones.



Affinity Chromatography:

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins are passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.



Type # 12. Counter-Current Chromatography:

CCC or Partition Chromatography is a category of liquid-liquid chromatography techniques. Chromatography in general is used to separate components of a mixture based on their differing affinities for mobile and stationary phases of a column. The components can then be analyzed separately by various sorts of detectors which may or may not be integrated into an apparatus. In liquid-liquid chromatography, both the mobile and stationary phases are liquid.

In contrast, standard column chromatography uses a solid stationary phase and a liquid mobile phase, while gas chromatography uses a liquid stationary phase on a solid support and a gaseous mobile phase. By eliminating solid supports, permanent adsorption of the analyte onto the column is avoided, and a near 100% recovery of the analyte can be achieved.

The instrument is also easily switched between various modes of operation simply by changing solvents. With liquid-liquid chromatography, researchers are not limited by the composition of the columns commercially available for their instrument. Nearly any pair of immiscible solutions can be used in liquid-liquid chromatography, and most instruments can be operated in standard or reverse-phase modes. Solvent costs are also generally cheaper than that HPLC, and the cost of purchasing and disposing of solid adsorbents is completely eliminated.

Another advantage is that experiments conducted in the lab can easily be scaled to industrial volumes. When GC or HPLC is done with large volumes, resolution is lost due to issues with surface-to-volume ratios and flow dynamics; this is avoided when both phases are liquid.

CCC can be thought of as occurring in three stages: mixing, settling, and separation (although they often occur continuously). Mixing of the phases is necessary so that the interface between them has a large area, and the analyte can move between the phases according to its partition coefficient.

A partition coefficient is a ratio of the amount of analyte found in each of the solvents at equilibrium and is related to the analyses' affinity for one over the other. The mobile phase is mixing with them settling from the stationary phase throughout the column.

The degree of stationary phase retention (inversely proportional to the amount of stationary phase loss or “bleed” in the course of a separation) is a crucial parameter. Higher quality instruments have greater stationary phase retention. The settling time is a property of the solvent system and the sample matrix, both of which greatly influence stationary phase retention.

Reference

- **www.google.com**
- **www.wikipedia.com**
- **www.studymafia.org**