Unit – 2 Chromatographic techniques

Chromatography 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 affect 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.

Chromatography was first employed in Russia by the Italian-born scientist Mikhail Tsvet in 1900. 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.[6] 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.

Chromatography terms

- **Analyte** is the substance to be separated during chromatography. It is also normally what is needed from the mixture.
- **Analytical chromatography** is used to determine the existence and possibly also the concentration of analyte(s) in a sample.
- **Bonded phase** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.

- **Chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.
- **Chromatograph** is equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.
- **Chromatography** is a physical method of separation that distributes components to separate between two phases, one stationary (stationary phase), the other (the mobile phase) moving in a definite direction.
- **Eluate** is the mobile phase leaving the column. This is also called effluent.
- **Eluent** is the solvent that carries the analyte.
- **Eluite** is the analyte, the eluted solute.
- **Immobilized phase** is a stationary phase that is immobilized on the support particles, or on the inner wall of the column tubing.
- **Mobile phase** is the phase that moves in a definite direction. It may be a liquid (LC and Capillary Electrochromatography (CEC)), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of HPLC the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or a polar solvent such as methanol in reverse phase chromatography and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Preparative chromatography** is used to purify sufficient quantities of a substance for further use, rather than analysis.
- **Retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.
- **Sample** is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.
- **Solute** refers to the sample components in partition chromatography.
- **Solvent** refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.

- **Stationary phase** is the substance fixed in place for the chromatography procedure. Examples include the silica layer in thin layer chromatography
- **Detector** refers to the instrument used for qualitative and quantitative detection of analytes after separation.

Types of chromatography techniques

1. Paper chromatography
2. Thin layer chromatography
3. Gel chromatography
4. Ion-exchange chromatography
5. Affinity chromatography
6. Hydrophobic interaction chromatography
7. Gas liquid chromatography
8. High pressure/ performance liquid chromatography (HPLC)

1. Paper chromatography

➢ Introduction and Principle

Paper chromatography is an analytical method used to separate colored chemicals or substances. This is useful for separating complex mixtures of compounds having similar polarity, for example, amino acids. The setup has three components. The mobile phase is a solution that travels up the stationary phase, due to capillary action. The mobile phase is generally an alcohol solvent mixture, while the stationary phase is a strip of chromatography paper, also called a chromatogram.

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of *chromatography paper*. The paper is placed in a container with 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. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

The stationary phase is the water trapped between the cellulose fibers of the paper. The mobile phase is a developing solution that travels up the stationary phase, carrying the samples with it. Components of the sample will

separate readily according to how strongly they adsorb onto the stationary phase versus how readily they dissolve in the mobile phase.

When a colored chemical sample is placed on a filter paper, the colors separate from the sample by placing one end of the paper in a solvent. The solvent diffuses up the paper, dissolving the various molecules in the sample according to the polarities of the molecules and the solvent. If the sample contains more than one color, that means it must have more than one kind of molecule. Because of the different chemical structures of each kind of molecule, the chances are very high that each molecule will have at least a slightly different polarity, giving each molecule a different solubility in the solvent. The unequal solubility causes the various color molecules to leave solution at different places as the solvent continues to move up the paper. The more soluble a molecule is, the higher it will migrate up the paper. If a chemical is very non-polar it will not dissolve at all in a very polar solvent

R_f values (Retention factor)

The retardation factor (R_f) may be defined as the ratio of the distance traveled by the substance to the distance traveled by the solvent.

Some compounds in a mixture travel almost as far as the solvent does; some stay much closer to the base line. The distance travelled relative to the solvent is a constant for a particular compound as long as you keep everything else constant - the type of paper and the exact composition of the solvent, for example.

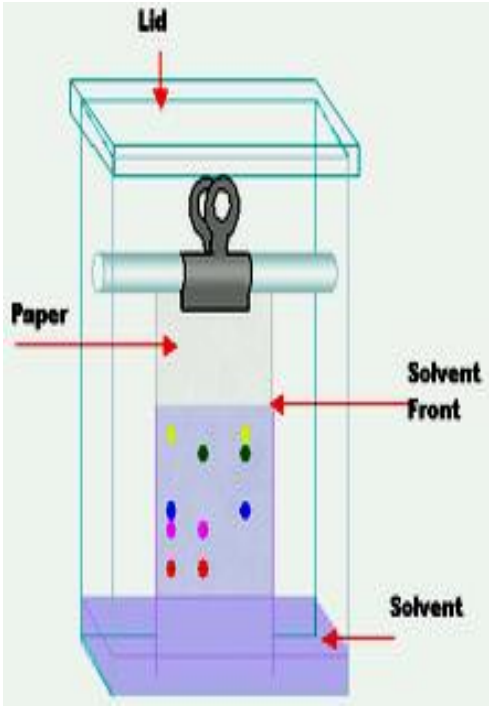
The distance travelled relative to the solvent is called the R_f value. The R_f value (retention factor) is the distance travelled by a particular component from the origin (where the sample was originally spotted) as a ratio to the distance travelled by the solvent front from the origin. For each compound it can be worked out using the formula:

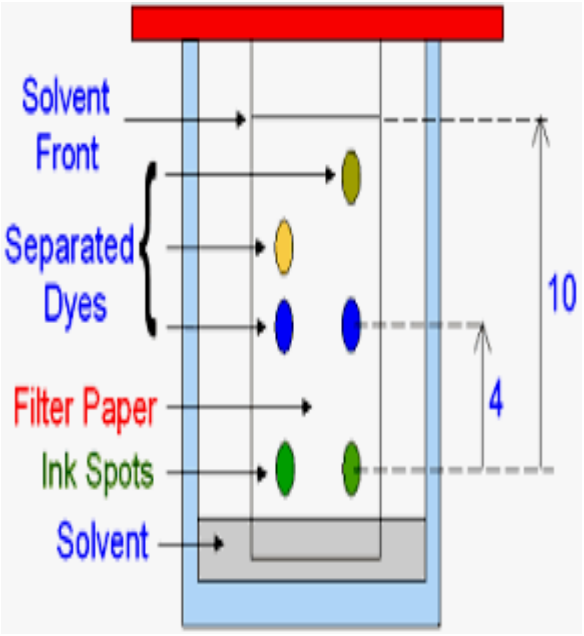
$$R_f = \frac{\text{distance travelled by compound}}{\text{distance travelled by solvent}}$$

Procedure

i. A small, ideally concentrated spot of solution that contains the sample is applied to a strip of chromatography paper about 1 to 3 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.

ii. 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.
iii. 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.
iv. 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.
v. After development, the spots corresponding to different compounds may be located by their colour, ultraviolet light, ninhydrin or by treatment with iodine vapours. The final chromatogram can be compared with other known mixture chromatograms to identify sample mixture using the R_f value.





Types of Paper Chromatography

a. Descending Paper Chromatography-In this type, development of the chromatogram is done by allowing the solvent to travel down the paper. Here, mobile phase is placed in solvent holder at the top. The spot is kept at the top and above solvent flow down the paper from above.

b. Ascending Paper Chromatography-Here the solvent travels up the chromatographic paper. Both Descending and Ascending Paper Chromatography are used for the separation of organic and inorganic substances.

c. Ascending-Descending Paper Chromatography-It is the hybrid of both of the above techniques. The upper part of Ascending Chromatography can be folded over a rod in order to allow the paper to become Descending after crossing the rod.

d. Radial Paper Chromatography-It is also called Circular Chromatography. Here a circular filter paper is taken and the sample is deposited at the center of the paper. After drying the spot, the filter paper is tied horizontally on a Petri dish containing solvent, so that the wick of the paper is dipped in the solvent. The solvent rises through the wick and the components are separated into concentric circles.

e. Two-Dimensional Paper Chromatography-In this technique a square or rectangular paper is used. Here the sample is applied to one of the corners and development is performed at right angle to the direction of the first run.

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- **Thin layer chromatography**

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. It may be performed on the analytical scale as a means of monitoring the progress of a reaction, or on the preparative scale to purify small amounts of a compound. TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity, and speed of separation.TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots.

Retention Factor (R_f)

After a separation is complete, individual compounds appear as spots separated vertically. Each spot has a retention factor (Rf) which is equal to the distance migrated over the total distance covered by the solvent.

$$R_f = \frac{\text{distance travelled by component}}{\text{distance travelled by solvent}}$$

The Rf value can be used to identify compounds due to their uniqueness to each compound. When comparing two different compounds under the same conditions, the compound with the larger Rf value is less polar because it does not stick to the stationary phase as long as the polar compound, which would have a lower Rf value.

Rf values and reproducibility can be affected by a number of different factors such as layer thickness, moisture on the TLC plate, vessel saturation, temperature, depth of mobile phase, nature of the TLC plate, sample size, and solvent parameters. These effects normally cause an increase in Rf values. However, in the case of layer thickness, the Rf value would decrease because the mobile phase moves slower up the plate.

Apparatus

Plates

As stated earlier, TLC plates (also known as chromatoplates) can be prepared in the lab, but are most commonly purchased. Silica gel and alumina are among the most common stationary phases, but others are available as well. Many plates incorporate a compound which fluoresces under short-wave UV (254 nm). The backing of TLC plates is often composed of glass, aluminum, or plastic. Glass plates are chemically inert and best withstand reactive stains and heat, but are brittle and can be difficult to cut. Aluminum and plastic plates can be cut with scissors, but aluminum may not withstand strongly acidic or oxidizing stains, and plastic does not withstand the high heat required to develop many stains. Aluminum and plastic plates are also flexible, which may result in flaking of the stationary phase.

Gel / Stationary phase

The properties of your sample should be considered when selecting the stationary phase. Silica gel can be exclusively used for amino acids and

hydrocarbons. It is also important to note that silica gel is acidic. Therefore, silica gel offers poor separation of basic samples and can cause a deterioration of acid-labile molecules. This would be true for alumina plates in acidic solutions as well. It is important to note that there are differences between silica gel and alumina. Alumina is basic and it will not separate sample sizes as large as silica gel would at a given layer thickness. Also, alumina is more chemically reactive than silica gel and as a result, would require more care of compounds and compound classes. This care would avoid decomposition and rearrangement of the sample.

Table: Stationary phase and mode of separation

Stationary Phase	**Chromatographic Mechanism**	**Typical Application**
Silica Gel	adsorption	steroids, amino acids, alcohols, hydrocarbons, lipids, aflaxtoxin, bile, acids, vitamins, alkaloids
Silica Gel RP	reversed phase	fatty acids, vitamins, steroids, hormones, carotenoids
Cellulose, kieselguhr	partition	carbohydrates, sugars, alcohols, amino acids, carboxylic acids, fatty acids
Aluminum oxide	adsorption	amines, alcohols, steroids, lipids, aflatoxins, bile acids, vitamins, alkaloids
PEI cellulose	ion exchange	nucleic acids, nucleotides, nucelosides, purines, pyrimidines
Magnesium silicate	adsorption	steroids, pesticides, lipids, alkaloids

Solvent system / Mobile phase

Proper solvent selection is perhaps the most important aspect of TLC, and determining the best solvent may require a degree of trial and error. As with plate selection, keep in mind the chemical properties of the analytes. A common starting solvent is 1:1 hexane:ethyl acetate. Varying the ratio can have a pronounced effect of Rf.

Rf values range from 0 to 1 with 0 indicating that the solvent polarity is very low and 1 indicating that the solvent polarity is very high.

If you know that one component of a mixture is insoluble in a given solvent, but another component is freely soluble in it, it often gives good separations. How fast the compounds travel up the plate depends on two things:

- If the compound is soluble in the solvent, it will travel further up the TLC plate
- How well the compound likes the stationary phase. If the compound likes the stationary phase, it will stick to it, which will cause it to not move very far on the chromatogram.

Acids, bases, and strongly polar compounds often produce streaks rather than spots in neutral solvents. Streaks make it difficult to calculate an Rf and may occlude other spots. Adding a few percent of acetic or formic acid to the solvent can correct streaking with acids. Similarly for bases, adding a few percent triethylamine can improve results. For polar compounds adding a few percent methanol can also improve results.

Common Solvents, grouped in order of increasing polarity are Fluoroalkanes, Cyclohexane, *n*- Hexane, 1-Chlorobutane, Carbon tetrachloride, *i*- Propyl ether, Toluene, Diethyl ether, Tetrahydrofuran, Chloroform, Ethanol, Ethyl acetate, Dioxane, Methanol, Acetonitrile, Nitromethane, Ethylene glycol, Water.

Useful Solvent Mixtures

- A solvent which can be used for separating mixtures of strongly polar compounds is ethyl acetate : butanol : acetic acid : water, 80:10:5:5.
- To separate strongly basic components, make a mixture of 10% NH_4OH in methanol, and then make a 1 to 10% mixture of this in dichlormethane.
- Mixtures of 10% methanol or less can be useful for separating polar compounds.

Pipettes

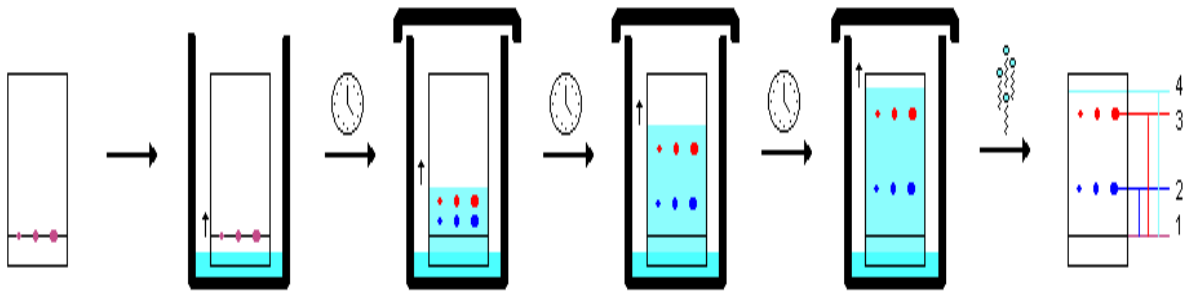
- Spots are applied to the plate using very thin glass pipettes. The capillary should be thin enough to apply a neat spot, but not so thin as to prevent the uptake of an adequate quantity of analyte. Here is a popular method of producing TLC pipettes.

Developing chamber or vessel

This can be as simple as a wide-mouth jar, but more specialized pieces of glassware to accommodate large plates are available. The chamber should contain enough solvent to just cover the bottom. It should also contain a piece of filter paper, or other absorbent material to saturate the atmosphere with solvent vapors. Finally, it should have a lid or other covering to minimize evaporation.

Procedure

1. Cut the plate to the correct size and using a pencil (never ever use a pen), gently draw a straight line across the plate approximately 1 cm from the bottom. Do not use excessive forces when writing on a TLC plate as this will remove the stationary phase. It is important to use a pencil rather than a pen because inks commonly travel up the plate with the solvent. An example of how black ink separates is shown in the section labeled "examples".
2. Using TLC pipettes, apply spots of analyte to the line. Make sure enough sample is spotted on the plate. This can be done by using the short-wave UV. A purple spot should be seen. If the spot is not visible, more sample needs to be applied to the plate. If a standard of the target compound is available, it is good practice to produce a co-spot by spotting the standard onto a spot of the unknown mixture. This ensures the identity of the target compound.
3. Place the plate into the chamber as evenly as possible and lean it against the side. Never allow the bulk solvent to rise above the line you drew. Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end. Never allow the solvent to migrate all the way to the end of the plate.
4. Remove the plate and immediately draw a pencil line across the solvent front.
5. Use a short-wave UV light and circle the components shown with a pencil.



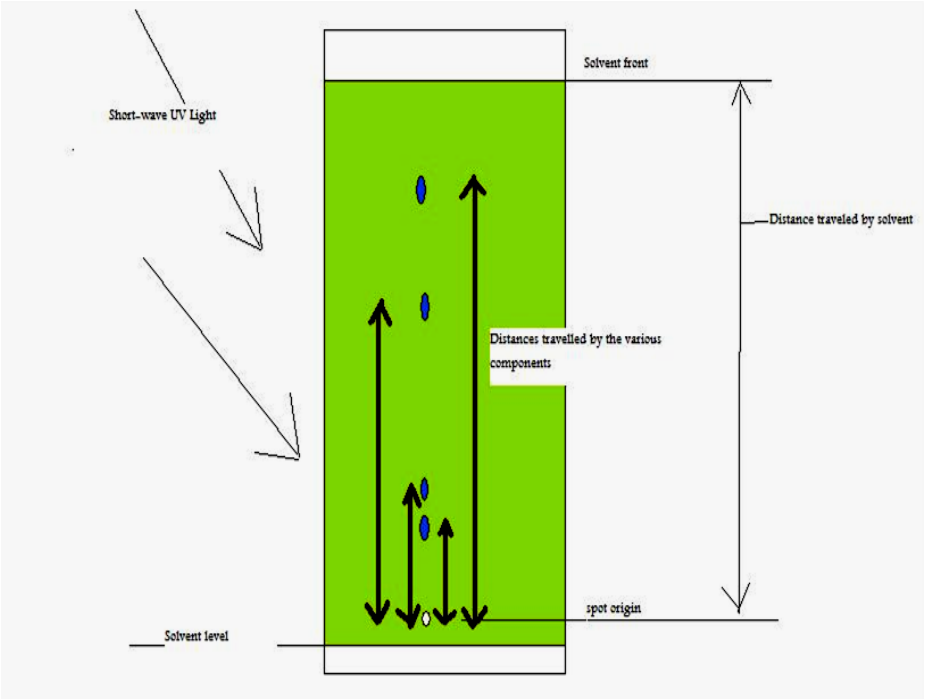
Visualizing

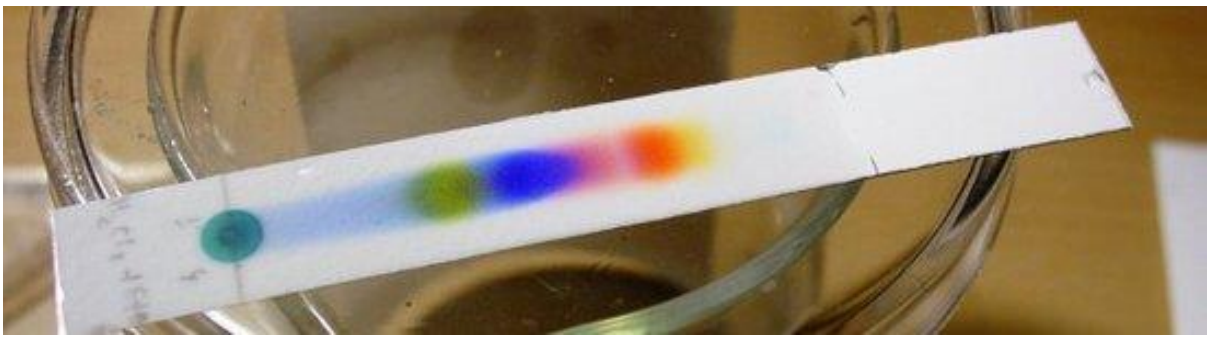
If fluorescent plates are used, a number of compounds can be seen by illuminating the plate with short-wave UV. Quenching causes dark spots on the surface of the plate. These dark patches should be circled with a pencil. For compounds which are not UV active, a number of chemical stains can be used. These can be very general, or they can be specific for a particular molecule or functional group.

Iodine is among the most common stains. Plates are placed in a jar containing iodine crystals, or covered in silica gel with iodine dispersed throughout, for approximately one minute. Most organic compounds will be temporarily stained brown. Some popular general use stains are Permanganate, ceric ammonium molybdate (CAM), and p-anisaldehyde. These can be kept in jars which plates are dipped into, or in spray bottles.

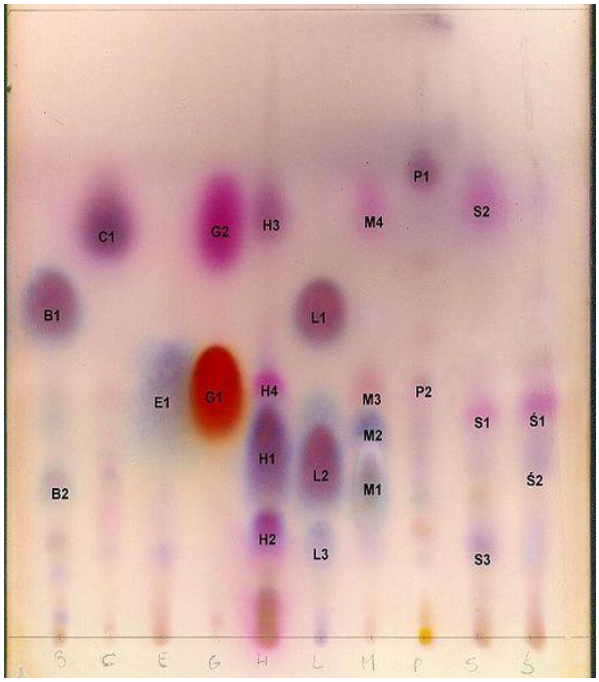
To develop a plate with permanganate, spray or dip the plate and heat it with a heat-gun. Hold the plate face up 10 to 20 cm above the heat gun until the bulk water evaporates. Then move the plate to 5 to 10 cm above the heat gun and heat it until white/yellow/brown spots appear. Overheating will turn the entire plate brown, obscuring the spots. If glass plates are used it is often easier to see spots through the backing because it is harder to overheat.

CAM and p-anisaldehyde stained plates are developed similarly. Overheating CAM stained plates turns everything blue.





Chromatogram of TLC plate



Chromatogram of 10 essential oils

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❖ Gel-filtration chromatography

the technique is known as **Size-exclusion chromatography** (**SEC**) / **molecular sieve chromatography** / **gel permeation chromatography** is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight.

It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. It is used when an organic solvent is used as a mobile phase. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution (Mw) results for polymers.

The advantages of this method include good separation of large molecules from the small molecules with a minimal volume of eluate and that various solutions can be applied without interfering with the filtration process, all while preserving the biological activity of the particles to separate. The technique is generally combined with others that further separate molecules by other characteristics, such as acidity, basicity, charge and affinity for certain compounds. With size exclusion chromatography, there are short and well-defined separation times and narrow bands, which lead to good sensitivity. There is also no sample loss because solutes do not interact with the stationary phase.

Discovery

The technique was invented by Grant Henry Lathe and Colin R Ruthven, working at Queen Charlotte's Hospital, London. While Lathe and Ruthven used starch gels as the matrix, Jerker Porath and Per Flodin later introduced dextran gels;[9] other gels with size fractionation properties include agarose and polyacrylamide

Theory and method

SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping smaller molecules in the pores of the adsorbent materials adsorption ("stationary phases"). This process is usually performed with 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 faster the elution. The larger molecules simply pass by the pores because those molecules are too large to enter the pores. Larger molecules therefore flow through the

column more quickly than smaller molecules, that is, the smaller the molecule, the longer the retention time.

One requirement for SEC is that the analyte does not interact with the surface of the stationary phases, with differences in elution time between analytes ideally being based solely on the solute volume the analytes can enter, rather than chemical or electrostatic interactions with the stationary phases. Thus, a small molecule that can penetrate every region of the stationary phase pore system can enter a total volume equal to the sum of the entire pore volume and the interparticle volume. This small molecule elutes late (after the molecule has penetrated all of the pore- and interparticle volume—approximately 80% of the column volume). At the other extreme, a very large molecule that cannot penetrate any the smaller pores can enter only the interparticle volume (~35% of the column volume) and elutes earlier when this volume of mobile phase has passed through the column. The underlying principle of SEC is that particles of different sizes elute (filter) through a stationary phase at different rates. This results 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.

Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of the column 'working' range and is where molecules are too large to get trapped in the stationary phase. The lower end of the range is defined by the permeation limit, which defines the molecular weight of a molecule that is small enough to penetrate all pores of the stationary phase. All molecules below this molecular mass are so small that they elute as a single band.

The filtered solution that is collected at the end is known as the **eluate**. The **void volume** includes any particles too large to enter the medium, and the solvent volume is known as the **column volume**.

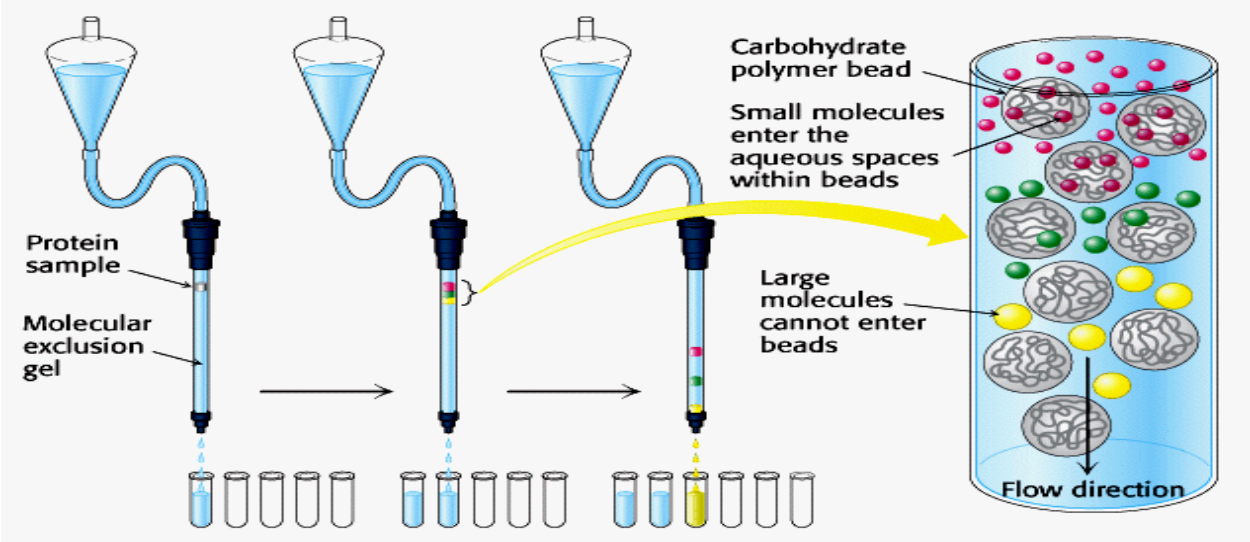
The basic components of the gel filtration experiment are the matrix, chromatography column and the elution buffer. The matrix is the material in the column that is actually the separation medium. It is the stationary phase of the chromatography.

The column is a tube with a frit and elution spout fitted at the bottom. The frit is a membrane or porous disk that supports and retains the matrix in the column but allows water and dissolved solutes to pass. The elution buffer is the mobile phase of the chromatography and flows through the matrix and out of the column. The column, with the matrix and applied sample, is “developed” by the elution buffer. This means that the molecules in the sample are carried by the flow of buffer into the matrix where they are gradually separated. The

separated zones of molecules then flow out of the column where they are collected for analysis Filling the chromatography column with matrix is referred to as “packing”. The packed matrix is called the “bed” and the volume it occupies is termed the “bed volume”.

The gel filtration matrix consists of microscopic beads that contain pores and internal channels. The larger the molecule, the more difficult it is for it to pass through the pores and penetrate the beads. Larger molecules tend to flow around and in between the beads. The total volume of buffer between the beads is the “void volume”. Smaller molecules tend to spend more time in the maze of channels and pores in the bed. Consequently, the larger, higher molecular weight molecules are eluted from the column before smaller molecules. Larger molecules take the faster, more direct path that involves less time in the beads (Figure)

Commonly used media for gel filtration chromatography are based on agarose or polyacrylamide beads, dextrose for gravity or low-pressure systems, and polymeric resins for medium-pressure systems.



Analysis

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 are 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. Common spectroscopy detection techniques are refractive index (RI) 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.

Gel Filtration Chromatography Applications

- Fractionation of molecules and complexes within a predetermined size range
- Size analysis and determination
- Removal of large proteins and complexes
- Buffer exchange
- Desalting
- Removal of small molecules such as nucleotides, primers, dyes, and contaminants
- Assessment of sample purity
- Separation of bound from unbound radioisotopes

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❖ Ion-exchange chromatography

W. Cohn first developed this procedure. **Ion exchange chromatography** (or **ion chromatography**) is a process that allows the separation of **ions** and polar molecules based on their affinity to the **ion exchanger**. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids

Ion-exchange chromatography (IEC) is part of ion chromatography which is an important analytical technique for the separation and determination of ionic compounds, together with ion-partition/interaction and ion-exclusion chromatography. Ion chromatography separation is based on ionic (or electrostatic) interactions between ionic and polar analytes, ions present in the eluent and ionic functional groups fixed to the chromatographic support.

Two distinct mechanisms as follows; ion exchange due to competitive ionic binding (attraction) and ion exclusion due to repulsion between similarly charged analyte ions and the ions fixed on the chromatographic support, play a role in the separation in ion chromatography.

Ion exchange mechanism

Ion-exchange chromatography which is designed specifically for the separation of differently charged or ionizable compounds comprises from mobile and stationary phases similar to other forms of column based liquid chromatography techniques.

Mobil phases consist an aqueous buffer system into which the mixture to be resolved. The stationary phase usually made from inert organic matrix chemically derivative with ionizable functional groups (fixed ions) which carry displaceable oppositely charged ion. Ions which exist in a state of equilibrium between the mobile phase and stationary phases giving rise to two possible formats, anion and cation exchange are referred to as counter ion (Figure).

Exchangeable matrix counter ions may include protons (H^+), hydroxide groups (OH^-), single charged mono atomic ions (Na^+, K^+, Cl^-), double charged mono atomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}) as well as organic bases (NR_2H^+) and acids (COO^-). Cations are separated on cation-exchange resin column and anions on an anion exchange resin column. Separation based on the binding of analytes to positively or negatively charged groups which are fixed on a stationary phase and which are in equilibrium with free counter ions in the mobile phase according to differences in their net surface charge

Ion exchange chromatography involves separation of ionic and polar analytes using chromatographic supports derivatized with ionic functional groups that have charges opposite that of the analyte ions. The analyte ions and similarly charged ions of the eluent compete to bind to the oppositely charged ionic functional group on the surface of the stationary phase. Assuming that the exchanging ions (analytes and ions in the mobile phase) are cations.

Complex mixtures of anions or cations can usually be separated and quantitative amounts of each ion measured in a relatively short time by ion exchange chromatography. In classical ion-exchange chromatography separations have been performed in the open-column mode. Column which is loosely packed with stationary phase as small particles made of 1-2 cm diameter glass. The mobile phase or eluent contains the competing ion and is passed continuously into the column and percolates through it under gravity. Sample

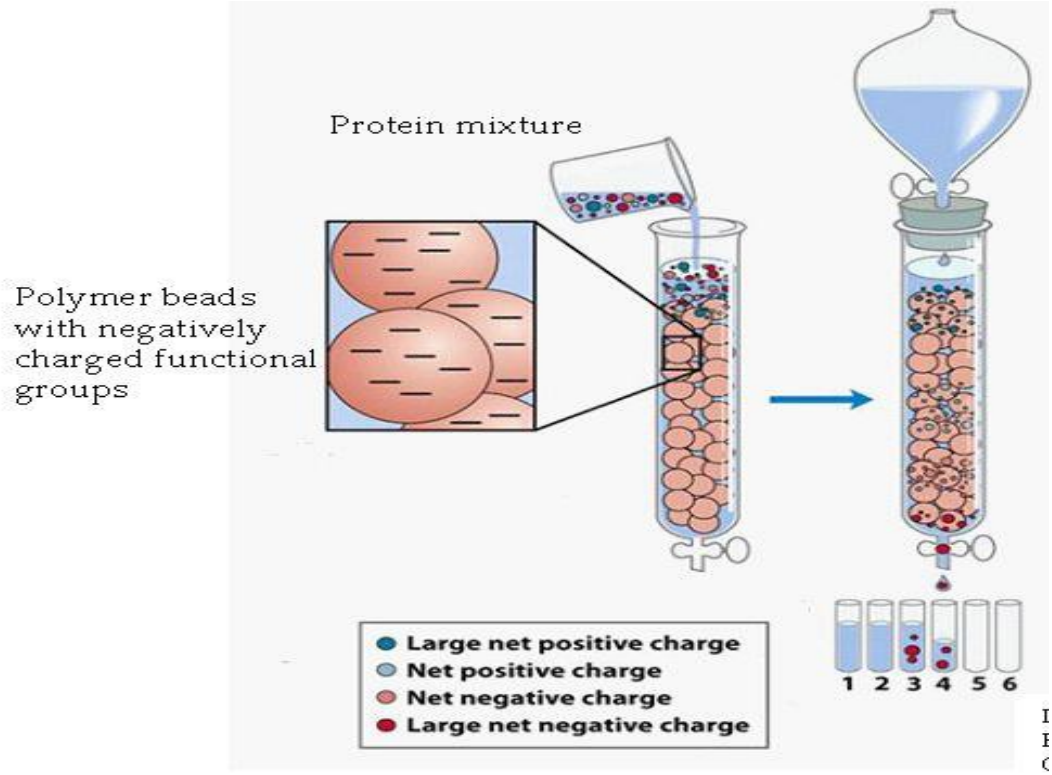
mixture is applied to the top of the column and allowed to pass into the bed of ion- exchange material.

Eluent flow is then resumed and fractions of eluent are collected at regular intervals from the column outlet. Open column ion-exchange chromatography is very slow due to low eluent flow-rates. Increasing flow rate may result in deteriorated separation efficiency (Figure). In modern ion-exchange chromatography the usage of high efficiency ion exchange materials combined with flow-through detection have overcome of these challenges.

Separations are performed on the column which is filled with ion-exchanger as particles in uniform size. The particles of ion-exchange material are generally very much smaller than those used for classical open column ion-exchange chromatography. However ion-exchange resins used in modern chromatography have lower capacity than older resins. The eluent must be pumped through the column due to the small particle size of stationary phase. The sample mixture is applied into eluent by the injection port. Finally the separated ions are detected with a flow-through detection instrument

In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

- **Cationic exchangers** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called “Acidic ion exchange” materials, because their negative charges result from the ionization of acidic group
- **Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called “Basic ion exchange” materials.



Stationary phase

In ion-exchange chromatography, numerous stationary phases are available from different manufacturers, which vary significantly in a number of chemical and physical properties. Stationary phases comprised of two structural elements; the charged groups which are involved in the exchange process and the matrix on which the charged groups are fixed.

General structure (fibrous or beaded form), particle size and variation, pore structures and dimensions, surface chemistry (hydrophilic or hydrophobic), swelling characteristics of matrix are important factors which effect chromatographic resolution. Porosity of ion exchange beads can be categorized as non-porous, microporous and macroporous.

Cellulose, Dextran, Agarose, Polyacrylamide, Acrylate-copolymer, Polystyrene-divinilybenzene, Coated polystyrene-divinilybenzene, Silica, Coated Silica are used as stationary phase.

Mobile phase (Eluent)

In ion exchange chromatography generally eluents which consist of an aqueous solution of a suitable salt or mixtures of salts with a small percentage

of an organic solvent are used in which most of the ionic compounds are dissolved better than in others in.

Sodium chloride is probably the most widely used and mild eluent for protein separation due to has no important effect on protein structure.

The salt mixture can itself be a buffer or a separate buffer can be added to the eluent if required. The competing ion which has the function of eluting sample components through the column within reasonable time is the essential component of eluting sample.

Commonly used eluent additives which have been successfully used in ion exchange chromatography can be given as follow;

- EDTA; Ethylenediamine tetraacetic acid
- Polyols; Glycerol, glucose, and saccharose
- Detergents;
- Urea and guanidinium chloride
- Lipids
- Organic solvents

Buffer

In ion exchange chromatography, pH value is an important parameter for separation and can be controlled and adjusted carefully by means of buffer substance

A number of buffers are suitable for ion-exchange chromatography. A number of important factors influences the selection of mobile phase including buffer charge, buffer strength and buffer pH.

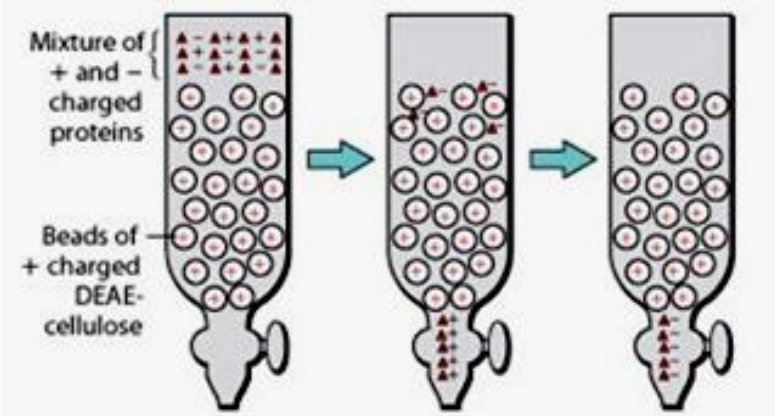
Generally, applications of ıon exchange chromatography are performed under slightly acidic or alkali conditions, pH range 6.0-8.5 but there are also more acidic and more alkali buffers.

Buffers	**PH range**
Ammonium acetate	4 to 6
Ammonium formate	3 to 5
Pyridinium formate	3 to 6
Pyridinium acetate	4 to 6
Ammonium carbonate	8 to 10

Detection

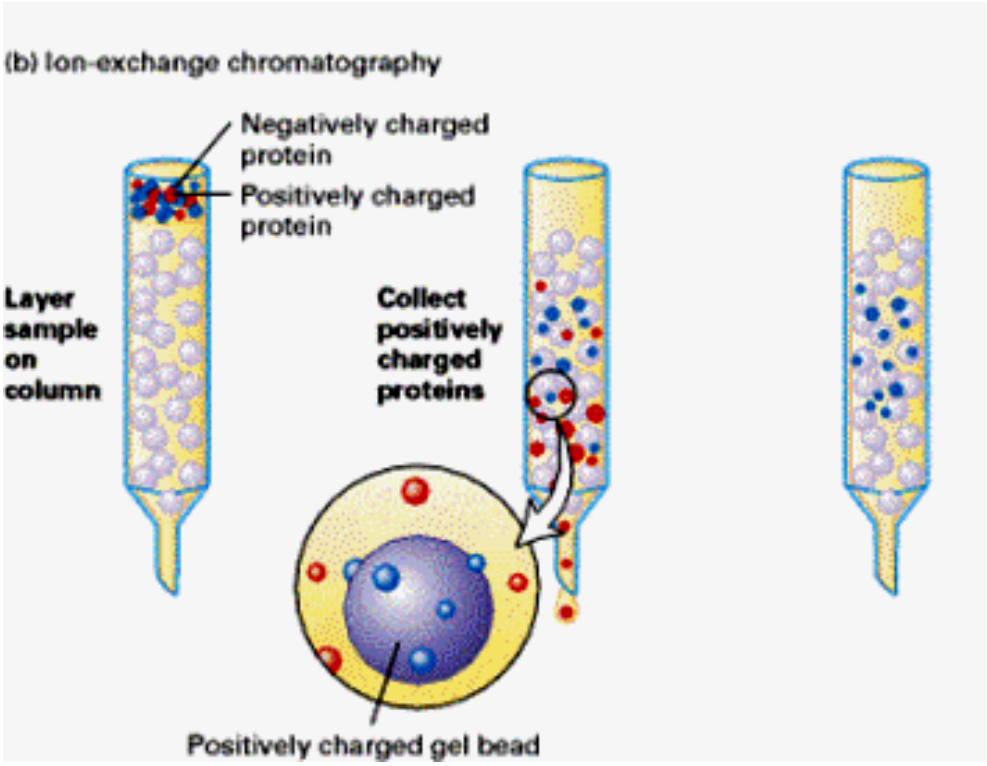
Conductivity detector is the most common and useful detector in ion exchange chromatography. However UV and other detectors can also be useful

For example anions that absorb in the UV spectral region can be detected spectrophotometrically. In this case, an eluent anion is selected that does not absorb UV.



Practical procedure:

- Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.
- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers. In next step using corresponding buffer separates the tightly bound particles.
- Then these particles are analyzed spectroscopically.



Applications of Ion Exchange Chromatography:

This technique has been used for the

- analyses of anions and cations, including metal ions, mono- and oligosaccharides, alditols and other polyhydroxy compounds,
- aminoglycosides (antibiotics),
- amino acids and peptides,
- organic acids,
- amines, alcohols, phenols, thiols,
- nucleotides and nucleosides and other polar molecules.
- analysis of raw materials, bulk active ingredients, counter ions, impurities, and degradation products, excipients, diluents and at different stages of the production process
- analysis of production equipment cleaning solutions, waste streams, container compatibility and other applications

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❖ Affinity chromatography

Affinity chromatography is a method of separating biochemical mixtures based on a highly specific interaction between antigen and antibody, enzyme and substrate, or receptor and ligand.

It is a type of chromatographic laboratory technique used for purifying biological molecules within a mixture by exploiting molecular properties. 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 including hydrogen bonding, ionic interaction, disulfide bridges, hydrophobic interaction, and more.

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 the now highly purified proteins be released.

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
- Discern what biological compounds bind to a particular substance
- Purify and concentrate an enzyme solution.

- **Principle**

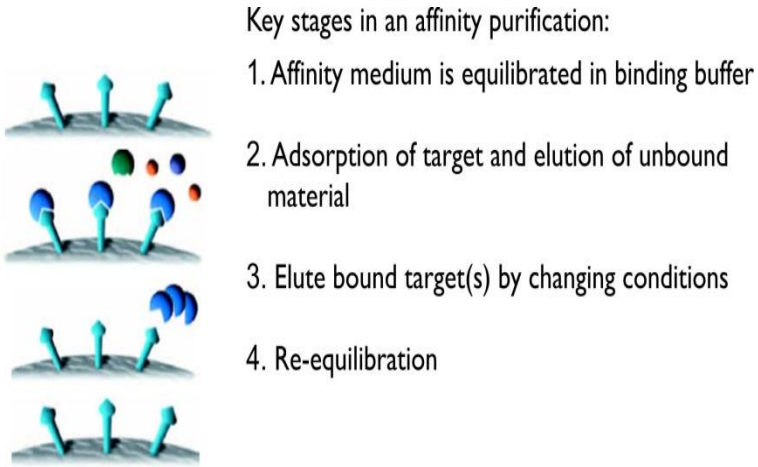
The stationary phase is typically a gel matrix, often of agarose; a linear sugar molecule derived from algae. Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum. The molecule of interest will have a well known and defined property, and 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 the mobile phase will not become trapped as they do not possess this property. The stationary phase can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as dialysis. Possibly the most common use of affinity chromatography is for the purification of recombinant proteins.

These matrices include interaction between:

Molecule	**Ligand**
Antigen	Antibody
Enzyme	Substrate
Receptor	Ligand
Nucleic Acid Binding Protein	Nucleic Acid
Polysaccharide, glycoprotein	Lectin

The phases of affinity chromatography are shown in Figure



Procedure

1. *Sample preparation*

The sample must be a clear solution free from solid particles. This can be achieved by centrifugation or filtration. Protein solutions should be centrifuged at at least 10000 g. Cell lysates should be centrifuged at 40-50000 g. A 0.45-µm pore size filter can be used for filtration.

One must also consider how the solubility and stability of the sample or the desired protein can be influenced by the pH, the salt concentration, or the presence of any organic solvent. The factors affecting the interactions between the desired target protein and the matrix-bound ligand (pH, salt concentration, temperature) should also be determined. The composition of the initial binding buffer must be adjusted accordingly.

Sample components interfering with the target protein and/or the ligand (e.g. metabolites in cell lysates) should be removed before loading onto the column.

2. *Equilibration with a buffer facilitating the specific interaction*

The chromatographic column is washed with 3-4 column volumes of the starting (binding) buffer. The sample must also be equilibrated with this starting binding buffer (if necessary, via solvent exchange or dialysis).

3. *Binding of the molecule of interest and wash-out of the unbound material*

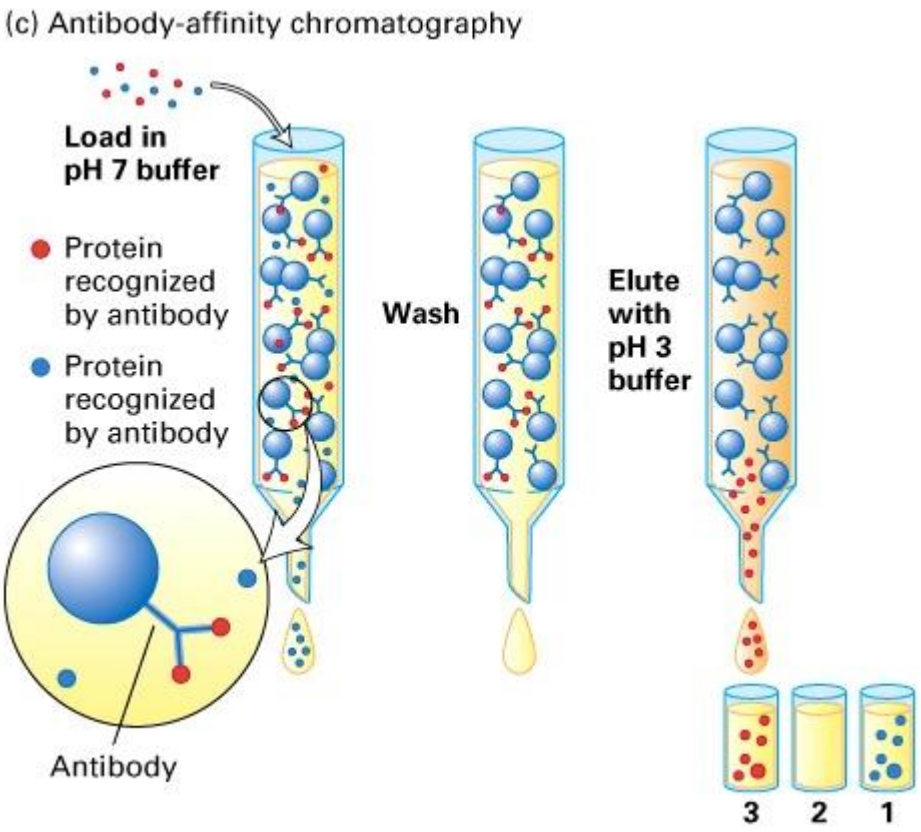
During sample loading, consider the strength of the interaction. In case of high-affinity samples, a high flow rate may be applied. In case of a weak interaction and/or a slow equilibration process, reduce the rate of sample loading. After sample application, the column should be further washed with binding buffer until all unbound components are removed.

4. *Elution of the molecules of interest by changing the composition of the mobile phase*

Elution via pH and/or ionic strength changes: One possible and simple means of elution is achieved through decreasing the interaction strength between the ligand and the target protein. Changes in the pH will change the ionisation state of charged groups of the ligand and/or the target protein, thereby changing the strength of the interaction. Similarly, increasing the ionic strength (usually by raising the NaCl concentration) will generally reduce the interaction strength. In either case, the solubility and stability of the target protein should be considered.

Competitive elution: For competitive elution, materials are applied that react with the target protein or the ligand, competing for the pre-existing interaction. For instance, His-Tag fusion proteins can be readily displaced from the metal chelate matrix by imidazole buffer

In all cases, the flow rate of the buffer should be reduced during elution, thereby avoiding excessive dilution of the target protein.



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❖ **Hydrophobic Interaction Chromatography (HIC)**

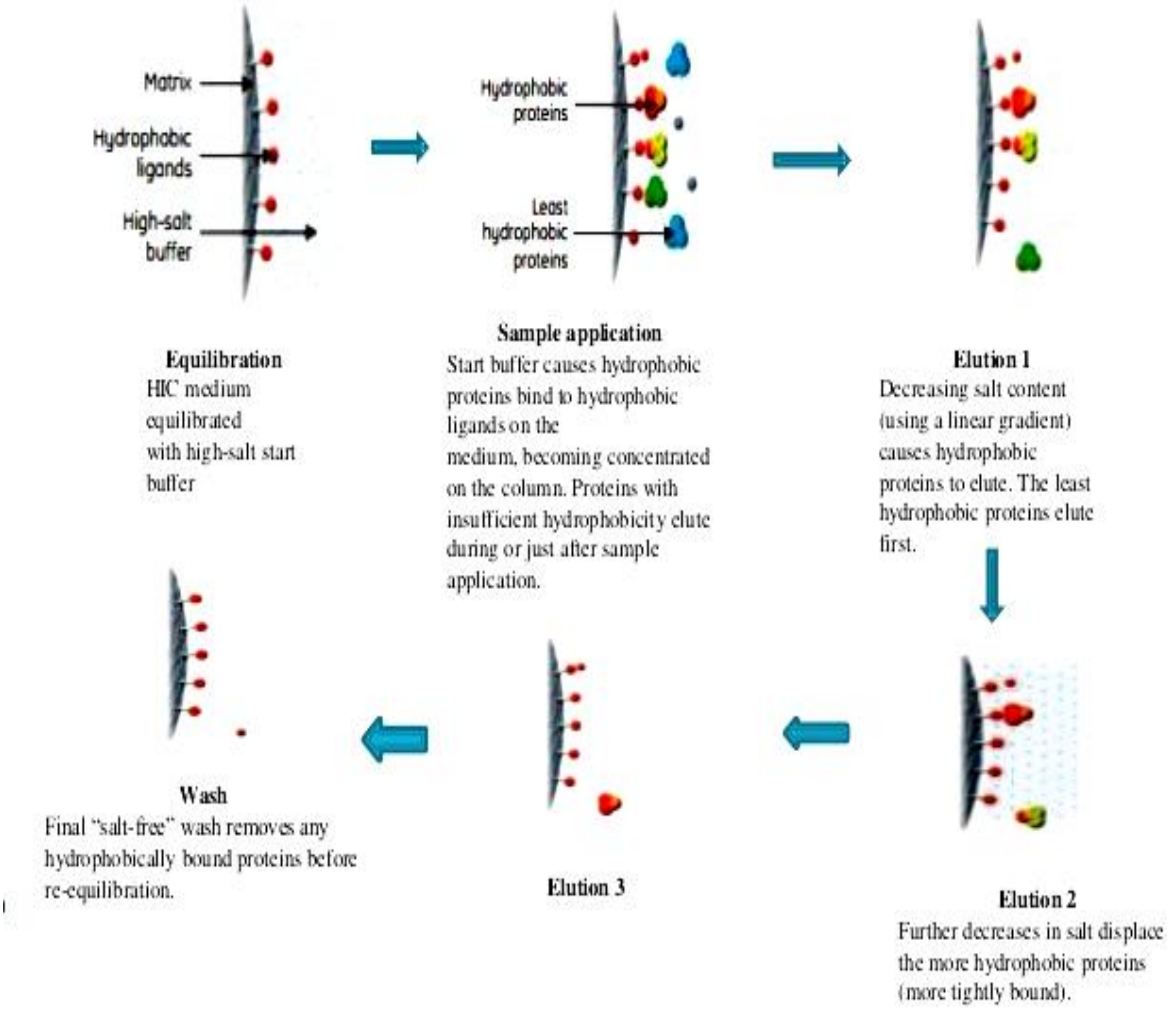
Hydrophobic Interaction Chromatography is a separation technique that uses the properties of hydrophobicity to separate proteins from one another. In this type of chromatography, hydrophobic groups such as phenyl, octyl, or butyl, are attached to the stationary column. Proteins that pass through the column that have hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column.

HIC separations are often designed using the opposite conditions of those used in ion exchange chromatography. In this separation, a buffer with a high ionic strength, usually ammonium sulfate, is initially applied to the column. The salt in the buffer reduces the solvation of sample solutes thus as solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium.

The more hydrophobic the molecule, the less salt needed to promote binding. To elute the proteins, the salt concentration is gradually decreased, eluting bound species in order of increasing hydrophobicity. Additionally, elution can also be achieved through the use of mild organic modifiers or detergent.

The stationary phase is designed to form hydrophobic interactions with other molecules. These interactions are too weak in water. However, addition of salts to the buffer result in hydrophobic interactions. The following is a list of salts that increase hydrophobic interactions in the order of their ability to enhance interactions:

1. Na_2SO_4
2. K_2SO_4
3. $(NH_4)_2SO_4$
4. NaCl
5. NH_4Cl
6. NaBr
7. NaSCN



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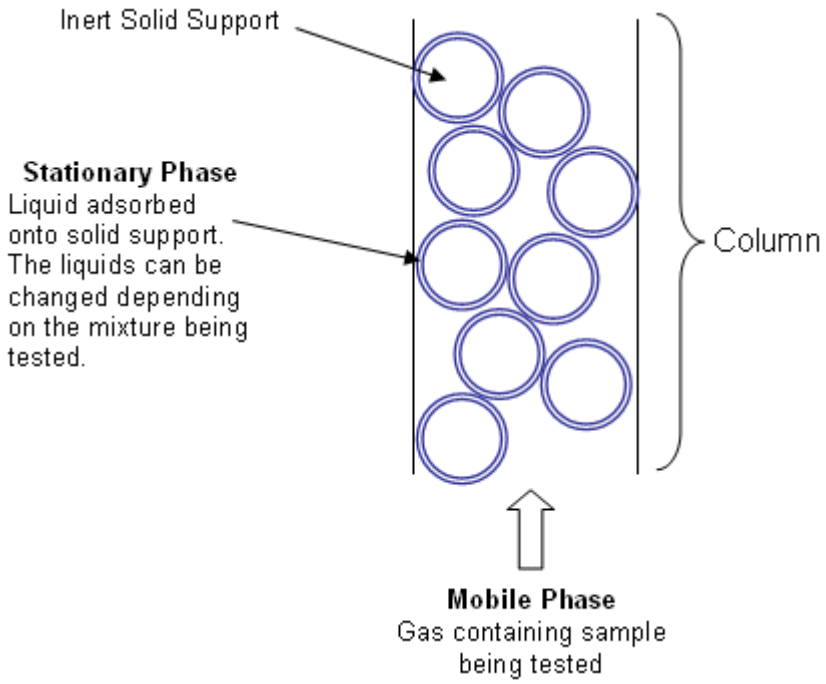
Gas-liquid chromatography

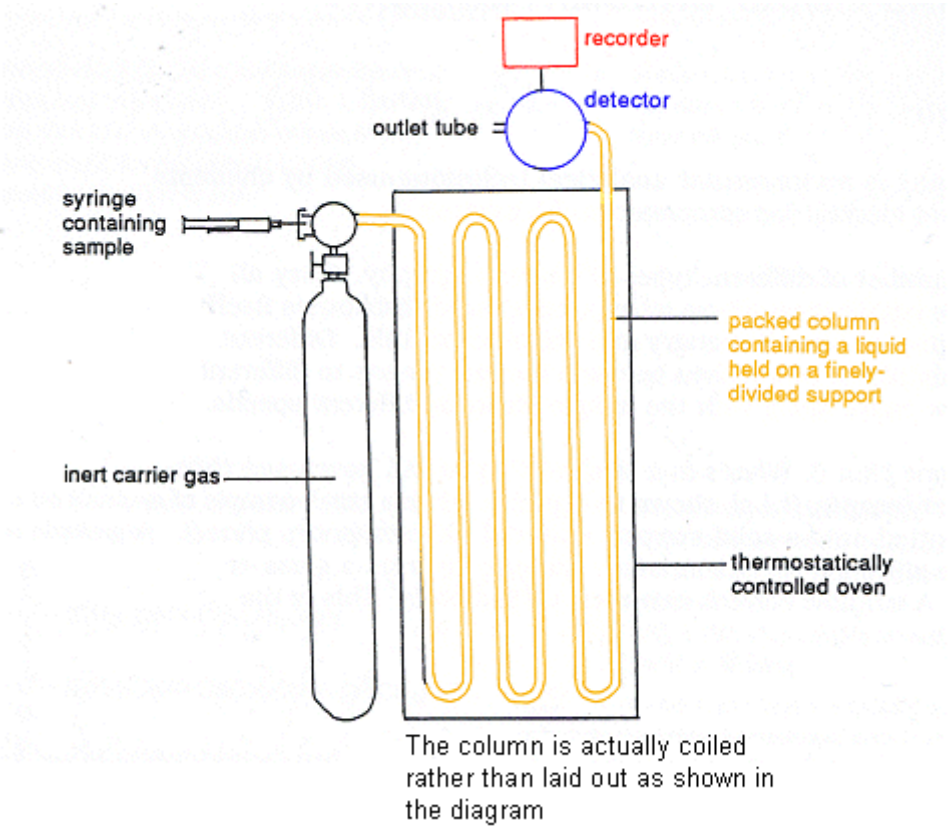
In gas-liquid chromatography, mobile phase is an unreactive gas, such as nitrogen (the carrier gas), and the stationary phase comprises of a small amount of liquid held on a finely-divided inert solid support. The solid support is in the form of a powder which is packed into a long, thin tube (the column). The column is coiled inside an oven.



The Instrument

- The sample to be reacted is injected into the gas stream just before it enters the column.
- The components of the mixture are then carried through the column in a stream of gas.
- Each compound distributes itself between the phases to different extents and therefore emerges from the column at a different time.
- Some of the compounds dissolve in the stationary solvents more readily than others; these travel through the column slower and so emerge last.
- The most volatile compounds usually emerge first.
- A detector on the outlet tube monitors compounds emerging from the column.
- Signals from the detector are plotted out by a recorder as a **chromatogram**
- The chromatogram shows the recorder response against the time which has elapsed since the sample was injected into the column.

- Each component of the mixture gives rise to a peak on the chromatogram



- The time a compound is held on a column under given conditions is characteristic of each compound and is referred to as its **retention time**; this can be affected by many factors, such as:
  - The length and packing of the column.
  - The nature and flow rate of the carrier gas.
  - The temperature of the column.
- The retention time can be used to identify the different compounds.
- The instrument is calibrated with known compounds, so that the conditions are kept constant throughout the analysis.
- The area under each peak represents the amount of each compound present.
- Gas-liquid chromatography is very sensitive and can be used to detect small quantities of substances; it is often used in forensic tests.
- In more sophisticated instruments, the outlet tube is connected to a mass spectrometer, so that each compound can be detected directly.

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❖ High-performance liquid chromatography (HPLC)

Introduction

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. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Principle

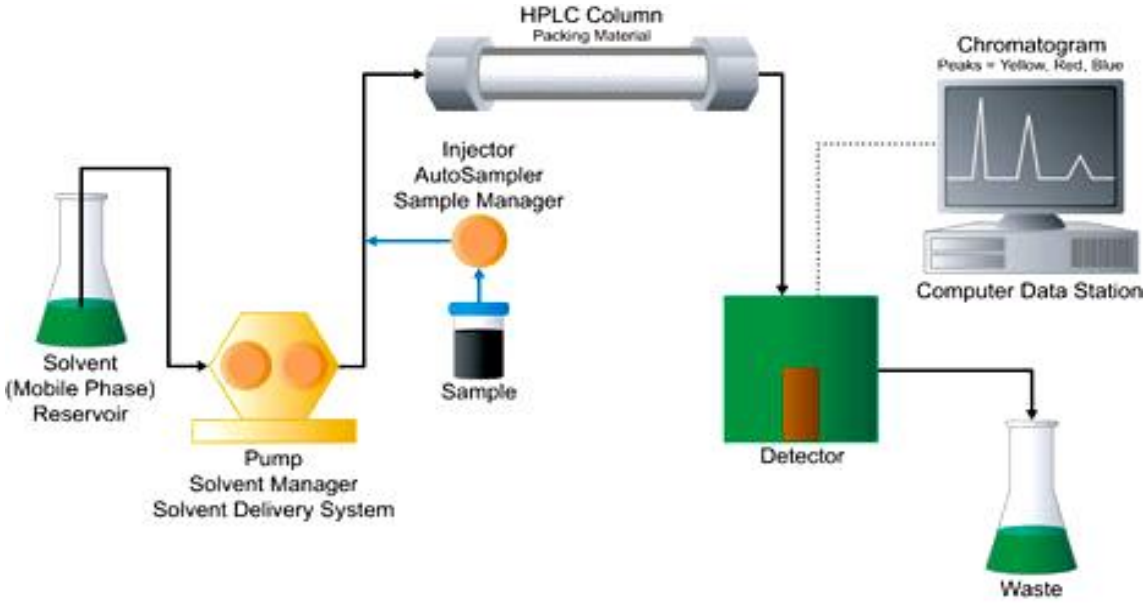
HPLC can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (*e.g.* silica, polymers, etc.), 2–50 μm in size.

The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (*e.g.* water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.

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

Instrumentation / Procedure

1. HPLC instrument typically includes a degasser, sampler, pumps, and a detector.
2. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase).
3. The pumps deliver the desired flow and composition of the mobile phase through the column.
4. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components.
5. A digital microprocessor and user software control the HPLC instrument and provide data analysis.
6. Some models of mechanical pumps in a HPLC instrument can mix multiple solvents together in ratios changing in time, generating a composition gradient in the mobile phase.
7. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or based on mass spectrometry. Most HPLC instruments also have a column oven that allows for adjusting the temperature at which the separation is performed.



The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analyte.

Stationary pahse / columns

Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface. The use of smaller particle size packing materials requires the use of higher operational pressure and typically improves chromatographic resolution (*i.e.* the degree of separation between consecutive analytes emerging from the column). Sorbent particles may be hydrophobic or polar in nature.

Mobile phase

Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water-free mobile phases. The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components.

The composition of the mobile phase may be kept constant or varied during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are very different in their affinity for the stationary phase. In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (=short retention times).

The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary phase (*e.g.* hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phases analytes partition between the two during the separation process taking place in the column.

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components. Often a series of trial runs is performed with the sample in order to find the HPLC method which gives adequate separation

- **Applications of HPLC**

1. HPLC has many applications in both laboratory and clinical science.
2. It is a common technique used in pharmaceutical development, as it is a dependable way to obtain and ensure product purity.
3. HPLC can produce extremely high quality (pure) products, it is not always the primary method used in the production of bulk drug materials.
4. HPLC has been used for manufacturing (*e.g.* during the production process of pharmaceutical and biological products).
5. Legal (*e.g.* detecting performance enhancement drugs in urine).
6. Research (*e.g.* separating the components of a complex biological sample, or of similar synthetic chemicals from each other).
7. Medical (*e.g.* detecting vitamin D levels in blood serum) purposes.

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