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| Name of Unit | Basics of Phytochemistry |
| Subject /Course | Pharmacognosy and Photochemistry-II |
| Subject/Course ID | BP 504T |
| Class: B.Pharm. Semester | 5th |
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## Learning Outcome of Module 05

1. Modern methods of extraction

2. Application of latest techniques like spectroscopy, chromatography and Electrophoresis in isolation, purification and identification of crude drugs.

## Content Table

| Topic |
|---|
| • Conventional methods of extraction viz. maceration, percolation,infusion,digestion,decoction |
| • Soxhletextraction,Counter-current, sonication and modern methods like MAE, PLE,CLE. |
| • Application of Chromatographic techniques viz.TLC,HPTLC, PC, CC, GC, HPLC, Siz exclusion chromatographyfor isolation and purification of crude drugs. |
| • Application of latest techniques of spectroscopy like UV, Mass, IR,NMR for identification crude drugs. |
| • Application of Electrophoresis in separation and purification of crude drugs. |
| • Application of latest techniques like spectroscopy, chromatography and Electrophoresis isolation, purification and identification of crude drugs. |

## BASICS OF PHYTOCHEMISTRY

A plant is a biosynthetic laboratory not only for fulfilling food needs of humans and animals but also for production of muultitude of phytoconstituents i.e. primary and secondary metabolites having commercial and medicinal importance. Phytochemistry is concerned with the study of the chemicals produced by plants, particularly the secondary metabolites viz. alkaloids , glycosides, terpenoids, tannins, resins etc. which are used in medicine for their pharmacological properties. As has been discussed in chapter 1, they are not necessary for normal growth and development. They are synthesized for self-defense against pests, herbivores and environmental hazards. Phytochemistry involves:

1) Extraction of plant material by various methods viz. maceration, percolation, continuous hot percolation or with modern methods of extraction like microwave assisted extraction, pressurized liquid extraction etc.
2) Isolation and purification by various chromatographic methods, and
3) Identification (qualitatively and quantitatively) by performing qualitative tests and spectroscopic methods.

Phytochemistry also takes into account the biosynthetic pathways, functions and it's medicinal, industrial, and commercial applications.

### EXTRACTION

It is the method of removing active constituents from a solid or liquid by means of liquid solvent. It can be defined as the process of isolation of soluble material from an insoluble residue by treatment with solvent.

It is done for separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents.In this method the wanted components are dissolved by the use of selective solvents known as menstrum and undissolved part is a marc. After the extraction unwanted matter is removed by filtration.

Extraction is the process of efficiently dissolving and separating the desired constituents from the crude drug with the use of solvent/s. Plant constituents are usually contained inside the cells. Therefore, the solvent used for extraction must diffuse into the cell to dissolve the desired compounds.

The choice of solvent depends upon the charactristics of secondary metabolites like polarity, pH and thermal stability.

1. It should be highly selective for the compound to be extracted.
2. It should not react with the extracted compound or with other compounds in the plant material
3. Have a low price.

4. Be harmless to man and to the environment.

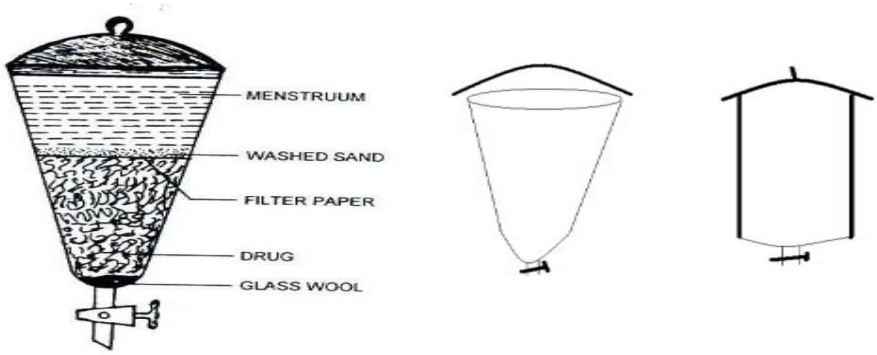
**Types**

**Solid extraction** i.e. extraction of solid from solid plant material using a solvent and

**Solvent extraction** It's partitioning with immiscible solvent after solid extraction .

**The conventional methods of extraction**

1. **Maceration (soaking)**The whole or coarsely powdered crude drug is placed in a stoppered container with the solvent.Allow it to stand at room temperature for a period of 7 days with frequent agitation until the soluble matter gets dissolved. The mixture then is strained, the marc (the damp solid material) is pressed. The combined liquids are clarified by filtration or decantation after standing.This method is best suitable for use in case of the thermolabile drugs.
   Modified maceration is used for unorganised drug like gums and resins The method can be modified to increase the yield.
2. **Percolation (pass through)** It is continuous flow of the solvent through the bed of crude drug material to get the extract. In this method a slow passage of menstrum under the influence of gravity pass through column of drug powder and during this movement it goes on extracting the drug molecules layerwise. In percolation the drug is exhaustively extracted by fresh menstrum.
   Percolation consists of three stages viz. imbibition i.e. moistening, maceration and percolation.Pack powered drug in percolator.Moisten the drug with sufficient quantity of menstrum.Add suffiecient mensrtum to saturate the drug. Allow to stand for 4 hrs. Add sufficient menstrum to form layer above drug,when liquid starts dropping, close the outlet.Allow to stand for 24 hrs(maceration) in closed vessel. Collect extract (percolate).Clarify further if necessary. Adjust final volume. Press marc and mix the liquid with extract Allow to percolate until 3/4th of final extract volume.



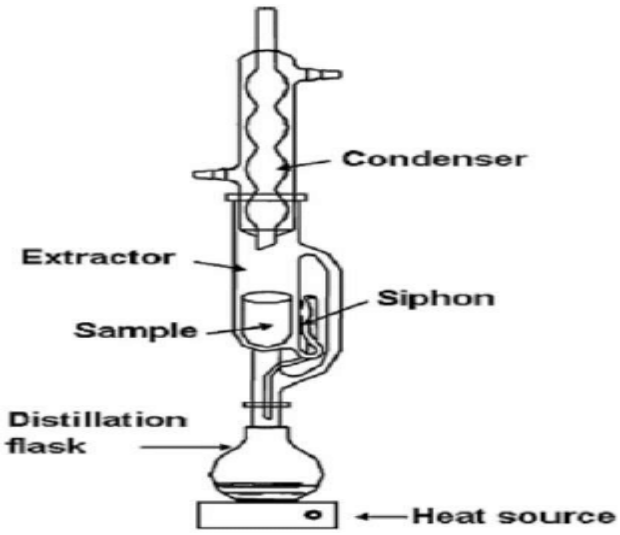

It is used for extraction of vitamins, volatile ingredients and soft ingredients in which the powdered drug is extracted with hot/cold water.

Some other conventional extraction methods are :

3. **Infusion** Fresh infusions can be prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.
4. **Digestion** This is a form of maceration in which gentle heat is used during the process of extraction. The moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased.
5. **Decoction** In this process, the crude drug is boiled in a specified volume of water for a definite time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat-stable constituents. The starting ratio of crude drug to water is fixed, e.g. 1:4 or 1:16; the volume is then brought down to one-fourth its original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

**Sohxlet extraction (Continuous Hot Percolation)** It's the most preferred method of extraction. Same quantity of solvent is made to circulate through extractor of drug by evaporation and subsequent condensation. Its suitable for extraction of thermostable constituents.

In this method, the finely ground crude drug is placed in a "thimble" made of fine filter paper, which is placed in chamber extractor of the Soxhlet apparatus. The extracting solvent in flask RB is heated, and its vapors condense in condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in extracter rises to the top of siphon tube, the liquid contents of extractor siphon into flask. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated.




**Advantages**

The traditional extracting methods require a large amount of solvent, and they are time-consuming and inefficient.

The advantage of Soxhlet extraction is that it can be extracted multiple times. Compared with the general extraction methods, it has the advantages of small solvent dosage, high efficiency and complete extraction.

**Counter-current extraction (CCE)** In counter-current extraction pulverized raw material is used to produce a fine slurry. In this process, the plant material generally in the form of a fine slurry, is moved in one direction (within a cylindrical extractor) where it comes in contact with extraction solvent. The further movements of material, produces more concentrated extract. Complete extraction is thus possible when the quantities of solvent and material and their flow rates are optimized. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end.

**This extraction process has significant advantages:**

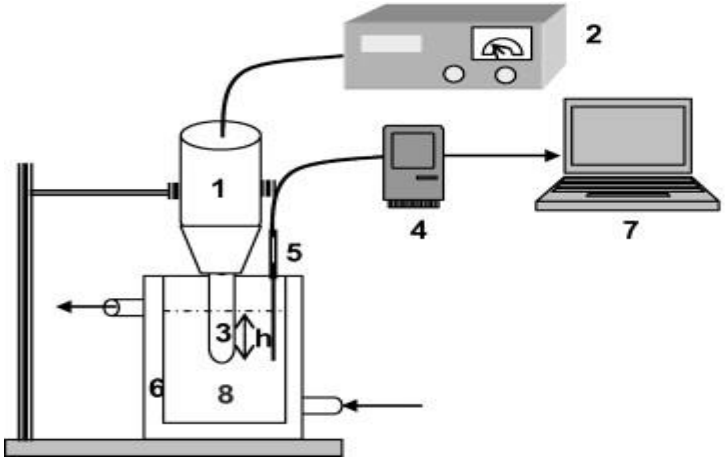
i) A unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, percolation.

ii) CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques.

iii) As the pulverization of the drug is done under wet conditions, the heat generated during comminution is neutralized by water. This again spares the thermolabile constituents from exposure to heat.

iv) The extraction procedure has been rated to be more efficient and effective than continuous hot extraction.

**Sonication (Ultrasound Extraction)** The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, its large-scale application is limited due to the higher costs.




(1) Ultrasound transducer
(2) Ultrasonic generator
(3) Ultrasound probe
(4) Data logger
(5) Temperature probe
(6) Jacketed beaker
(7) Computer
(8) Sample and solvent
(h) Depth of probe in to the sample (2.5 cm)

One disadvantage of the procedure is the occasional deleterious effect of ultrasound energy on the active constituents of medicinal plants through formation of free radicals and resulting undesirable changes in the drug molecules.

**Some common extraction methods of Volatile oils**

1. **By Hydrodistillation-** It includes water distillation, water and steam distillation & steamdistillation used for extraction of volatile oil from herbal drugs. The fresh crude drug is subjected to hydrodistillation for volatile oil isolation. The apparatus used is Clevenger apparatus.
2. **Enfluerage method-** It is used for extraction of delicate perfumes. For this fresh flowerpetals are mechanically spread on fatty material layer. It was allowed to imbibe and the exhausted petals are replaced with fresh petals. The process is continued till the fatty material layer is saturated with volatile oil which is further extracted with lipid solvents.
3. **Ecuelle method-** it is used for extraction of citrus oils. In this the oil cells are rupturedmechanically using pointed projections by twisting raw material over them in clockwise direction either mechanically or manually.
4. **By using liquid Carbondioxide-** CO2 is liquefied under pressure and it act as solvent forextraction of essential oils. It reverses back to gaseous nature when pressure is reduced and leaving no any residue of solvent

**Modem Methods of Extraction**

1. **Microwave assisted extraction**The prefix ‘micro’ indicates that these waves are shorter than of radiowaves. Microwaves (frequency 300 MHz- 300 GHz) are nonioinizing electromagnetic waves. The two types of oscillating perpendicular fields that generate

microwaves are the electric field and magneticfield.The ionic conduction and dipole rotation are responsible for heating of substances. When the microwaves interact with polar solvents, heating of substance is caused due to any one of the above mentioned phenomena, individually or simultaneously.

The electrophoretic migration of ions under the influence of the changing electric field is called Ionic conduction.If the solution offers a resistance to this migration of ions, a friction is generated and the solution is heated.

The realignment of the dipoles of the molecule with the rapidly changing electric field is called Dipole rotation. At a frequency of 2450 MHz the process of heating occurs.

In this method high temperature is produced by microwaves evaporate the moisure in cell, dehydrates cellulose causing rupturing of cellwallthereby facilitates quick extraction with solvent. It's a rapid extraction method, completing extraction within 5-10 min.

**Principle**

The target for heating in dried plant material is the minute microscopic traces of moisture that occurs in plant cells. The heating up of this moisture inside the plant cell due to microwave effect, results in evaporation and generates tremendous pressure on the cell wall. The cell wall is pushed from inside due to the pressure and the cell wall ruptures. Thus the exudation of active constituents from the ruptured cells occurs, hence increasing the yield of phytoconstituents.The yields from plant matrices can also be enhanced by using selective solvent. Higher yields can be obtained also by increasing the temperature, which facilitates faster penetration of solvent into the cell walls.The free water molecules present in the gland and vascular systems of plant matrices are heated and this causes a localized heating and expansion of their walls, thus resulting into the flow of constituents outside the cells.

In order to prevent the degradation of thermo-labile components the sample matrix is immersed in a microwave transparent solvent like hexane.

**Microwave Oven Design:** The microwave extraction assembly comprises of four major components

**Magnetron** is responsible for generation of microwaves

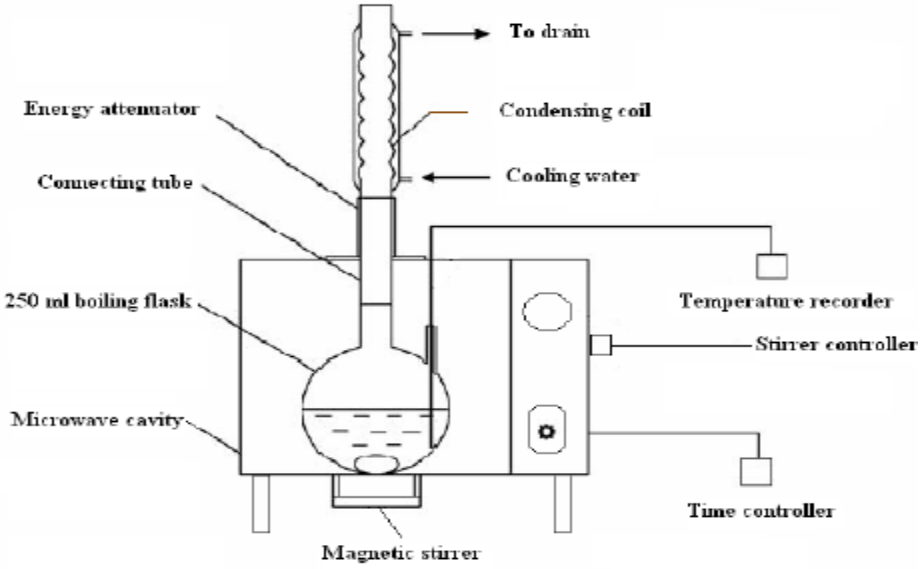
**Wave guide** is used to direct the propagation of microwave from the source to the microwave cavity.

**Applicator**, where the sample holder along with the sample is placed.

In case of multi- mode systems the applicator is a **closed cavity inside** which a random dispersion of microwaves is brought about.

**Circulator** which regulates the movement of microwaves only in the forward direction.

**Open systems** Open vessels can be operated at atmospheric pressure. The major advantage of the instrument is the ability to process large samples without the requirement of a cooling process. The equipment can be obtained at a low cost and a complete automation with Open-vessel operation can be done.




In a ***closed vessel system*** higher temperatures can be reached due to the increased pressure inside the vessel that raises the boiling point of the solvents used thus increasing both speed and efficiency of extraction. There is considerably no loss of volatile substances in a closed system vessel and very less volume of solvent is required. There is no need of addition of solvent/s repeatedly and hence risk of air-borne contamination is lowered.

## Advantages

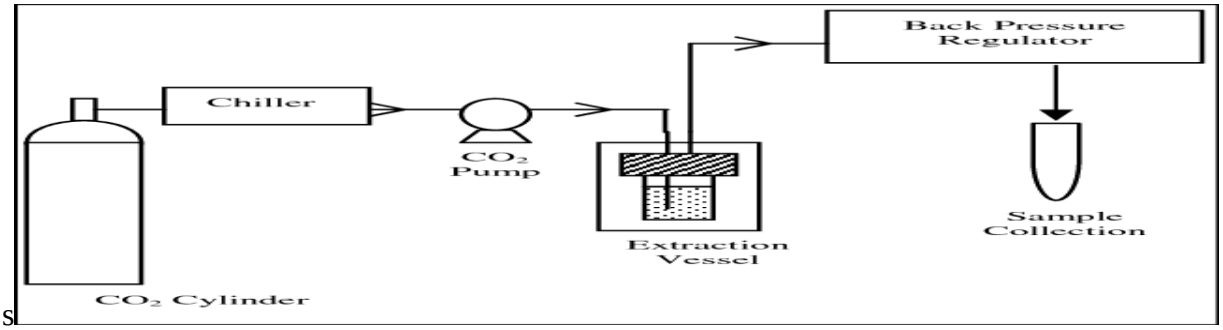
1. It's a rapid extraction method, completing extraction within 5-10 min.
2. The process can be automated .
3. Improved extraction yields .
4. Low solvent consumption and
5. The method is suitable for thermostable phytoconstituents.

## Applications

1. Extraction of caffeine from the green tea leaves using 50% ethanol as solvent in just 4 minutes.
2. Extraction of Ginsenosides from the Ginseng root using ethanol as solvent in just 15 minutes.
3. Extraction of Anthraquinones from the Morindacitrifolia root using ethanol as solvent in just 15 minutes.

2. **Supercritical Fluid extraction (SCF extraction)** At critical point, substance exists in vapour-liquid equilibrium state. SCF offer liquid like density, gas like viscocity, gas like compressibilty and high diffusivity than liquid. Carbon dioxide ($CO_2$) is the most commonly used supercritical fluid, sometimes modified by co- solvents. Extraction conditions for supercritical carbon dioxide are above the critical temperature of 31 °C and critical pressure of 74 bar. Addition of modifiers may slightly alter this.

   The system must contain a pump for the $CO_2$, a pressure cell to contain the sample, a means of maintaining pressure in the system and a collecting vessel. The liquid is pumped to a heating zone, where it is heated to supercritical conditions. It then passes into the extraction vessel, where it rapidly diffuses into the solid matrix and dissolves the material to be extracted. The dissolved material is swept from the extraction cell into a separator at lower pressure, and the extracted material settles out. The $CO_2$ can then be cooled, re-compressed and recycled, or discharged to atmosphere.

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   SFE used for obtaining residual solvent free extracts. It'scan be used as a sample preparation step for analytical purposes, or on a larger scale to either strip unwanted material from a product (e.g. decaffeination), refining cooking oils or collect a desired product (e.g. essential oils) or spices.

   **Advantages**

   Selective, fast and efficient method.

   **Disadvantages**

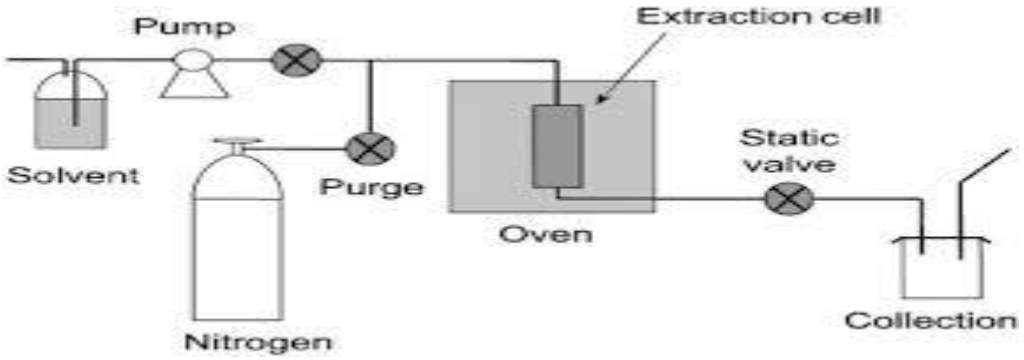
   High cost operation.

   Due to non polar nature of $CO_2$, the additional use of modifiers becomes necessity.

3. **Pressurized Liquid extraction(PLE)** Pressurized liquid extraction (PLE; also referred to as pressurized solvent extraction, accelerated solvent extraction, high-pressure solvent extraction, high-pressure, high-temperature solvent extraction, pressurized hot solvent extraction and subcritical solvent extraction) is one of the most promising techniques in bioactive compound extraction. The process uses high temperature and pressure. This technique is a solid–liquid

extraction process performed at high temperatures (50–200 °C) with high pressures for short time periods (5–10 min) in solid and semisolid matrices**.** The extraction taking less time and requiring less solvent, and possibly also giving better analyte recovery. The elevated temperature is employed to increase extraction efficiency of the analyte of interest. The elevated pressure (100-200 bars) is used to keep the solvent in a liquid state as the temperature is increased above its boiling point and also increases permeability of solvent. *The temperatue and pressure is below supercritical point. The $CO_2$ is not used as solvent.* It requires minutes to complete extraction process. It's a solid liquid extraction technique which has been developed as an alternative to current extraction methods such as Soxhlet, maceration, percolation or reflux, offering advantages with respect to solvent consumption, extraction yields, extraction time and reproducibility.

The design of the extractor is such that it is capable enough to withstand high pressures, helps the extraction temperature to be raised above the boiling point of the solvent used. The high pressure helps in maintaining the solvent in a liquid state at a high temperature. Under such conditions, the solvent has properties favoring the extraction process, such as high diffusion coefficients, low viscosity and high solvent strength. This assists to attain a good dissolution processes and favors desorption of analytes from the cellular matrix. Additionally, pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. An increased temperature augments diffusivity of the solvent resulting in an increased extraction rate.




The sample is placed in an extraction vesselas shown in figure made of stainless steel. Following addition of the solvent, the cell is pressurized, heated to the desired temperature, and the sample is extracted statically for a specific period of time. Next, the extract is removed from the cell and the cell is flushed with fresh solvent. The cycle can be repeated. When the extraction is complete, compressed nitrogen moves all of the solvent from the cell to the vial for analysis. The extract is filtered prior to being collected in the receiver, thus eliminating the need for a separate filtration step.

PLE has been used successfully for the extraction of analytes from medicinal plants, food, environmental samples, etc.It can be used to flavonoids from flowers and polyphenols from fruits. However this method is not suitable for thermolabilephytoconstituents.

**Phytonics Extraction**

The Phytonics process involves the use of a novel non-toxic solvent based on hydrofluorocarbon - 134a, having a boiling point of - 25° C and a vapor pressure of 5.6 bar at ambient temperature and technology to optimize its remarkable properties in the extraction of plant materials.

The products mostly extracted by this process are high quality natural fragrant essential oils, flavors and biological extracts which can be directly used without further physical or chemical treatment.

**Advantages**:

i) Unlike other processes that employ high temperatures, the phytonics process is cool and gentle and its products are never damaged by exposure to temperatures.

ii) No vacuum stripping is needed which, in other processes, leads to the loss of precious volatiles.

iii) The technique is highly selective, offering a choice of operating conditions and hence a choice of end products.

iv) It is less threatening to the environment.

v) It requires a minimum amount of electrical energy.

**Disadvantages:**

i) By most standards this is a poor solvent as the solvent does not mix with mineral oils or triglycerides.

ii) The solvent does not dissolve plant wastes.

**Applications:**

1. The phytonics process can be used for extraction of the production of antibiotics, in the herbal drug industry, in the food, essential oil and flavor industries, and in the production of other pharmacologically active products.
2. In particular, it is used in the production of top quality pharmaceutical - grade extracts, pharmacologically active intermediates, antibiotic extracts and phytopharmaceuticals.
3. The technique is also used in refining crude products obtained from other extraction processes.

# CHROMATOGRAPHY

It is a laboratory technique for the separation of a mixture of phytoconstituents. 'Chromatography'is derived from two Greek words, chroma "color and graphein "to write. Chromatography was first employed in Russia by the Italian born scientist Mikhail Tsvet in 1900. In this technique 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 separation is based on differential partitioning between the mobile and stationary phases.

**Principle**: Chromatography usually consists of mobile phase and stationary phase. The mobile phase is mixture of substances to be separated plus a liquid or a gas.The stationary phase is a porous solid matrix through which the mobile phase along with sample percolates. The interaction between the mobile phase and the stationary phase results in the separation of the compounds from the mixture depending upon its partition coefficient between mobile phase and stationary phase. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase. Due to this various constituents of the mixture travel at different speeds along with mobile phase, causing them to separate.

The 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.

**Classification of chromatography**

**I. On the basis of interaction of solute to the stationary phase**

1. Adsorption Chromatography
2. Partition Chromatography
3. Ion Exchange Chromatography
4. Size Exclusion Chromatography

**II. On the basis of physical state of mobile phase**

1. Liquid Chromatography
2. Gas Chromatography
3. Super Critical Fluid Chromatography

**Thin layer chromatography (TLC)** It's is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different

adsorbents. The mobile phase runs upward along the plate due to capillary action and the components get separated depending upon their polarity towards stationary phase.

**Principle**: It is based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed. The components with more affinity towards stationary phase travels slower andcomponents with less affinity towards stationary phase travels faster.

Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character is identified by means of suitable detection techniques.

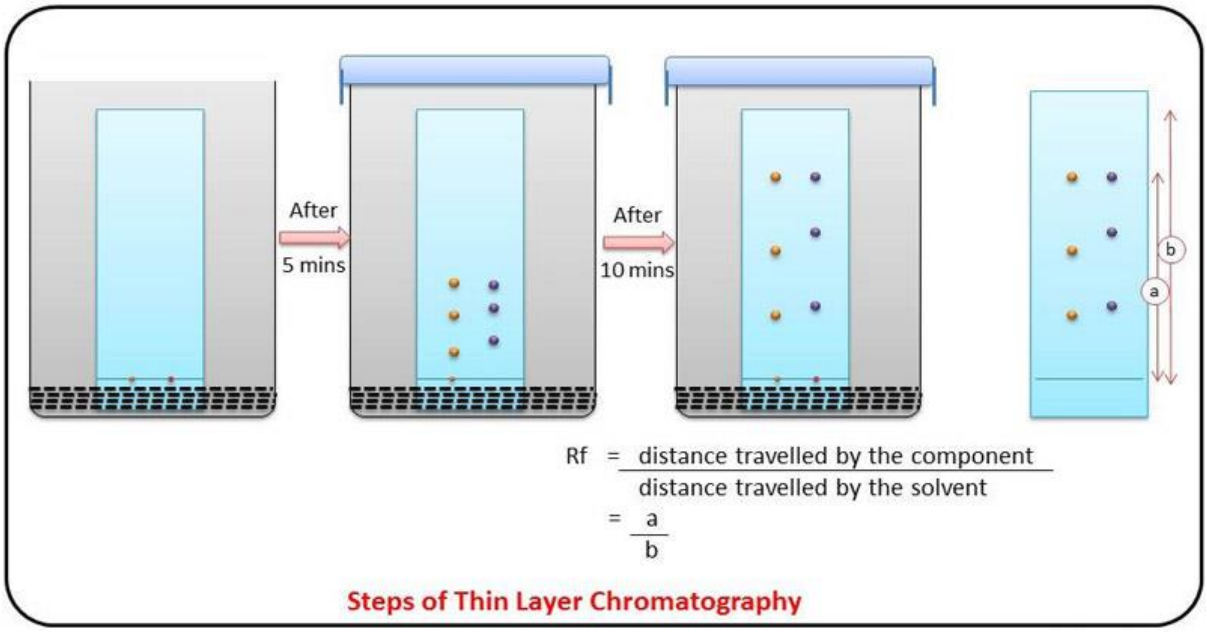
**TLC system components consists of:**

1. **TLC plates, preferably ready made with a stationary phase**: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
2. **TLC chamber-** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
3. **Mobile phase-** This comprises of a solvent or solvent mixture. The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.

**The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are more commonly used.**

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom.
4. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect).
5. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
6. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent) for development.
7. Sufficient time is given for the development of spots.
8. The plates are then removed and allowed to dry.

9. The sample spots are then seen in a suitable UV light chamber, or any other methods as recommended for the given sample.




Some common techniques for visualizing the results of a TLC plate include

1. UV light
2. Iodine chamber

Retention Factor ($R_f$) Value: The behaviour of a compound on a TLC is usually described in terms of its relative mobility or Rf value.

$R_f$ = the distance travelled by solute/ the distance travelled by the solvent.

It's a simple, quick,cheap, qualitative and purity checkinglaboratory tool.

However it cannot be used for quantitative purposes.

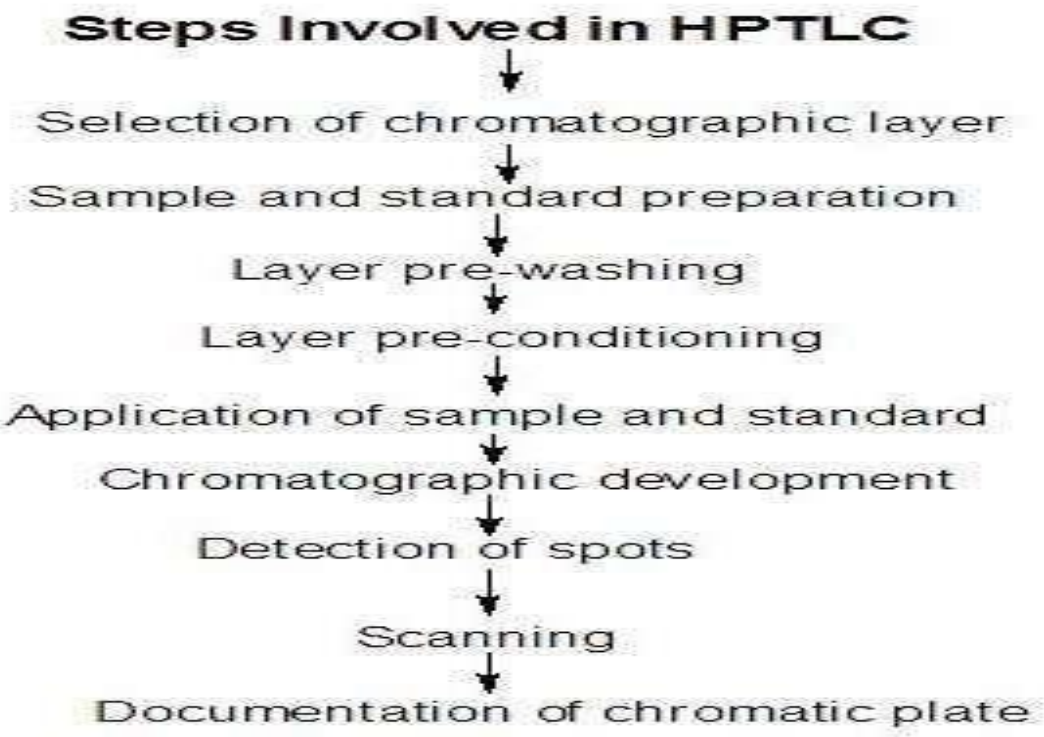
**Applications**

TLC is used for separation and identification of alkaloids, glycosides, carbohydrates, protein and peptides, terpenoids, fats and fatty acids etc.

**High-performance thin-layer chromatography (HPTLC)**

High-performance thin-layer chromatography is an enhanced form of thin-layer chromatography. A number of enhancements can be made to the basic method of thin-layer chromatography to automate the different steps, to increase the resolution achieved, and to allow more accurate quantitative measurements.

The plates used in HPTLC contain silica gel particles with a very small size and the packing density of the gel on the plate is high. The surface of the plate is smooth and gives efficient separation. The analysis is faster than the TLC technique. The sensitivity of this method is also high. In HPTLC, several different samples can be separated in the same plate without any sample preparation step.




HPTLC has a special instrument named HPTLC scanner which can scan developed TLC plates and measure the optical density of separated spot and convert it into a graph called chromatogram. These chromatograms are utilized for quantification of specific secondary metabolites.

HPTLC is suitable for both qualitative and quantitative analysis of herbal extracts and formulation; the data can be stored digitally and being offline technique sample application, scanning and data analysis can be done individually.

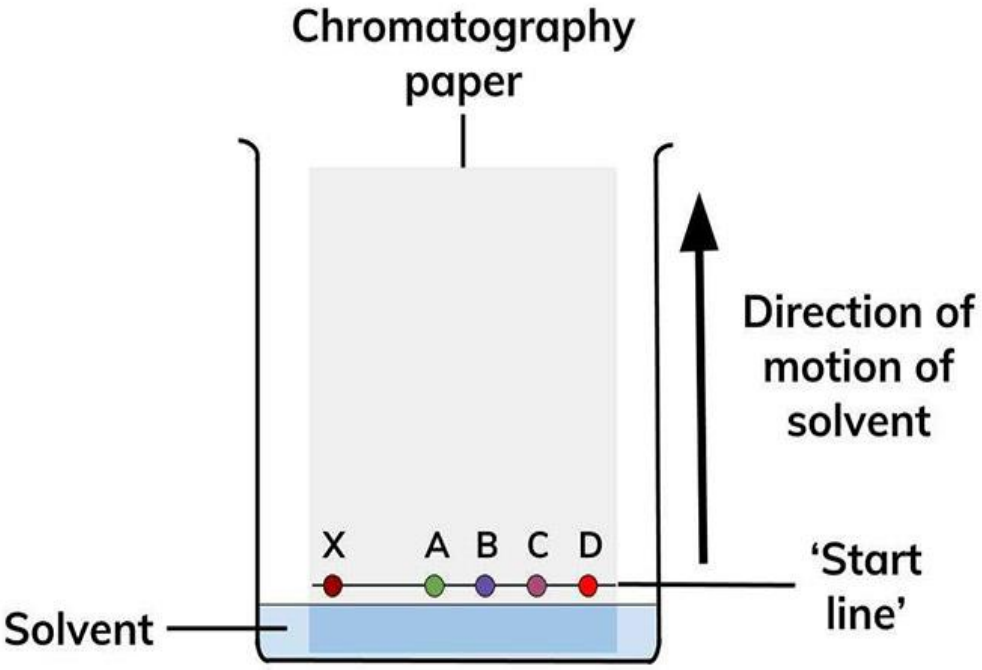
However mobile phase composition cannot be altered till operation is complete.

HPTLC is also useful stability testing of finished product and in finger printing profile of herbal drugs.

**Paper chromatography** **(**PC)It's 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 jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent. 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.

**Retention factor** :R*f* = zero, - Solute remains in the stationary phase and thus it is immobile. R*f* = 1 - Solute has no affinity for the stationary phase and travels with the solvent front.

The technique and applications are similar to TLC, however drastic spraying reagents cannot be used with PC

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**Column chromatography (**CC)

It's a technique which is used to separate a single chemical compound from a mixture dissolved in a fluid. It separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allow them to get separated in fractions. This technique can be used on a small scale as well as large scale to purify materials that can be used in future experiments.

**Principle**

When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slowly are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

$R_f$ = the distance travelled by solute/ the distance travelled by the solvent

$R_f$ is the retardation factor

Column chromatography involves the following:

1. Adsorption/retention of substance on stationary phase
2. Separation of adsorbed substance using mobile phase.
3. Recovery of individual components by continuous flow of mobile phase. Stationary phase: silica gel, alumina.

**Preparation of the Column**

- The column mostly consists of a glass tube packed with a suitable stationary phase.
- A glass wool/cotton wool or an asbestos pad is placed at the botton of the column before packing the stationary phase.
- After packing, a paper disc kept on the top, so that the stationary layer is not disturbed during the introduction of sample or mobile phase.

There are two types of preparing the column, they are:

**1. Dry packing / dry filling**

In this the required quantity of adsorbent is poured as fine dry powder in the column and the solvent is allowed to flow through the column till equilibrium is reached.

**2. Wet packing / wet filling**

In this, the slurry of adsorbent with the mobile phase is prepared and is poured into the column. It is considered as the ideal technique for packing.

The various steps include:

1. **Introduction of the Sample**

   The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase.The entire sample is introduced into the column at once and get adsorbed on the top portion of the column.

2. **Elution** By elution technique, the individual components are separated out from the column.

   It can be achieved by two techniques:

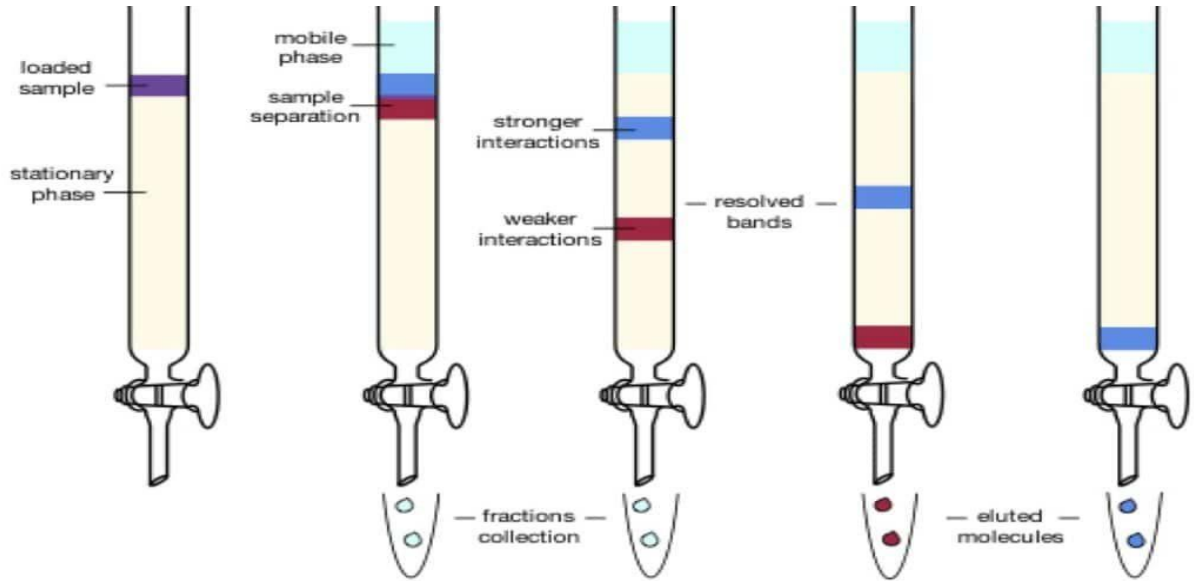
   **Isocratic elution technique:** Same solvent composition or solvent of same polarity is used throughout the process of separation.e.g. Use of chloroform alone.

   **Gradient elution technique:** Solvents of gradually increasing polarity orincreasing elution strength are used during the process of separation.e.g. initially benzene, then chloroform, then ethyl acetate then chloroform

3. **Detection of Components**

   If the compounds separated in a column chromatography procedure are colored, the progress of the separation can simply be monitored visually and If the compounds to be isolated from column chromatography are colorless then, small fractions of the eluent are collected sequentially in labelled tubes and the composition of each fraction is analyzed by TLC.

   This method needs large quantities of plant material and solvents. The isolated phytoconstituents need further purification.



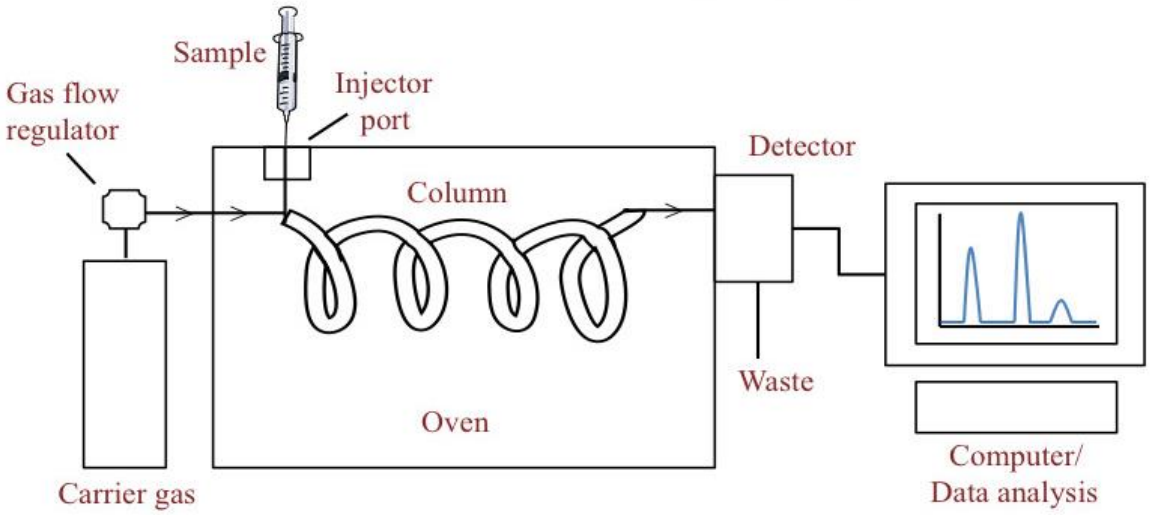

**Gas chromatography (GC)** It's also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. It is the method of choice for the separation of volatile substances or the volatile derivatives of certain non-volatile substances. Stationary phase is an inert solid material (kieselgurh/ firebrick) impregnated with a non-volatile liquid (silicon/PEG). This is packed in narrow column and maintained at high temperature around 2000C.

As sample is rapidly heated and vaporized at the injection port, the sample is transported through the column by a mobile phase consisting of an inert gas (Ar/He/N). The sample components are separated based on partioncoefficeint between mobile phase and staionary phase. The higher a component's affinity for the stationary phase, the slower it comes off the column. The components are then detected and represented as peaks on a chromatogram. It is commonly used for quantitative estimation of lipids, drugs and vitamins.

Gas chromatography is mainly composed of the following parts:

1. **Carrier gas** in a high-pressure cylinder with attendant pressure regulators and flow meters e.g. Helium, $N_2$, H, Argon are used as carrier gases.
2. **Sample injection system** Liquid samples are injected by a microsyringe with a needle inserted through a self-scaling, silicon-rubber septum into a heated metal block by a resistance heater.Typical sample volumes range from 0.1 to 0.2 ml.
3. **The separation column** The heart of the gas chromatography is the column which is made of metals (stainless steel )bent in U shape or coiled into an open spiral or a flat pancake shape.
4. **Liquid phases** No single phase will serve for all separation problems at all temperatures.
5. **Detector** These are either concentration-dependent or mass dependant.Detectors sense the arrival of the separated components and provide a signal.

6. **Recorder** The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.




**Step 1: Sample Injection and Vapourization**

1. A small amount of liquid sample to be analyzed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
3. The injection of the sample is considered to be a "point" in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporize.
5. The vaporized components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

**Step 2: Separation in the Column**

- Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will, therefore, have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.

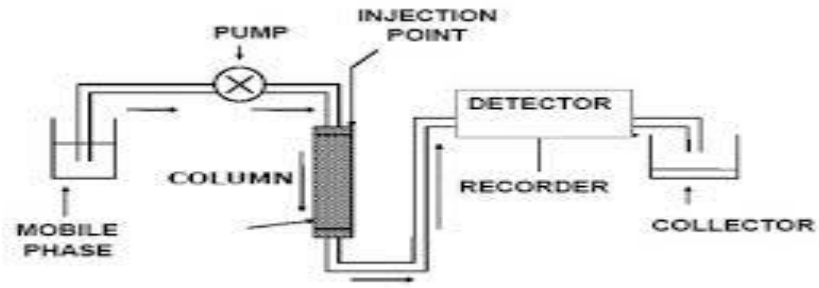
**Step 3: Detecting and Recording Results**

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last.

**High Pressure Liquid Chromatography** It's also called as high performance liquid chromatography. It relies on pumps to pass a pressurized (5000-1000 psi) liquid solvent ie mobile phase containing the sample mixture through a column filled with a solid adsorbent material. The stationary phase-immobilized thin layer liquid on micro glass or plastic beads is tightly packed on narrow column. The mobile phase- solvent system is passed under high pressure through column. The eluents can be detected by detectors. It can be applied in the form of adsorption, ion exchange, partition or molecular sieve chromatography. Due to rapidity in detection,its is used for detection of amino acids, peptides, carbohydrates, proteins, lipids, nucleic acids, vitamins, hormones, drugs, etc.

**Principle**

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.




## Instrumentation

**The Pump** High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.

**Injector** An injector is placed next to the pump.The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

**Columns** The recent columns are often prepared in a stainless steel housing, instead of glass columns.The packing material generally used is silica or polymer gels.

**Detectors** Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.

**Recorder** There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

## Types

1. **Normal phase:** Column packing is polar (e.g silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cis-trans isomers, and chiral compounds.
2. **Reverse phase:** The column packing is non-polar (e.g C18), the mobile phase is water+ miscible solvent (e.g methanol). It can be used for polar, non-polar, ionizable and ionic samples.
3. Ion exchange:Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.

## Advantages

1. Speed
2. Efficiency
3. Accuracy

4. Versatile and extremely precise when it comes to identifying and quantifying chemical components.

**Disadvantages**

1. Cost: Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics.
2. Complexity of operation
3. Volatile substances are better separated by gas chromatography.

**Size-exclusion chromatography (SEC)** It's also known as gel permeation chromatography (GPC) or gel filtration chromatography and separates molecules according to their size, shape & molecular weight. It is also referred to as molecular sieving or molecular exclusion chromatography. The chromatography column is packed with fine, porous beads which are composed of dextran polymers (Sephadex), agarose (Sepharose), or polyacrylamide. The pore sizes of these beads are used to estimate the dimensions of macromolecules. Smaller molecules are able to enter the pores of the media and, therefore, molecules are trapped and removed from the flow of the mobile phase. The molecules that are larger than the average pore size of the packing are excluded and thus suffer essentially no retention, the first to be eluted. This is how the molecules are separated. It is also useful for determining the tertiary structure and quaternary structure of purified proteins, especially since it can be carried out under native solution conditions.

**Some other methods of purification of isolated compounds include :**

Sublimation, fractional crystallization, fractional distillation, fractional liberation, etc. Chemical derivatization can also be employed based on groups or moieties present in the compound and chemical reactions.

In fractional crystallization the compound is mixed with a solvent, heated, and then gradually cooled so that, as each of its constituent components crystallizes, it can be removed in its pure form from the solution. Many natural products are crystalline in nature even in mixture, process such as concentration, slow evaporation, refrigeration are used for crystallization.

Fractional distillation is a process by which components in a chemical mixture are separated into different parts (called fractions) according to their different boiling points. This method is used for the separation of the components from volatile mixtures. It's largely used in the separation of hydrocarbons from oxygenated volatile oil e.g. citral, eucalyptol.

# SPECTROSCOPY

Spectroscopy is concerned with the interaction between matter and electromagnetic radiation. It can involve any interaction between light and matter, including absorption, emission, scattering, etc. When a beam of electromagnetic radiation passes through a sample, the photons interact with the sample. They may be absorbed, reflected, refracted, etc. The absorbed radiation affects the electrons and chemical bonds in a sample. In some cases, the absorbed radiation leads to the emission of lower- energy photons. Spectroscopy looks at how the incident radiation affects the sample. Emitted and absorbed spectra can be used to gain information about the material. Depending upon the wavelength or frequency or energy different types of electromagnetic radiations are named as

1) Visible radiation
2) UV radiation
3) Infrared radiation
4) Radio rays
5) X-rays
6) Γ-rays
7) Microwaves

## UV spectroscopy

**Principle** It is absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet (200-400nm) and the full, adjacent visible spectral regions (400-800 nm). The compounds which are colorless absorb radiation in UV range whereas the colord ones absorb light in visible range. Absorption of the ultra-violet radiations results in the excitation of the electrons from the ground state to higher energy state. The absorption of energy is characteristic of a particular atom. It's useful both for qualitative and quantitative determinations.

The absorption pattern (curve with wavelength on X axis vs. absorbance on Y-axis) is unique for every colored compound. The wavelength at which maximum absorption of radiation takes place is known as $\lambda_{max}$, which is constant for a compound. This technique can be used for qualitative determination.

The quantitative determination can be obtained by preparing calibration or standard curve between concentration on X-axis and absorbance on Y-axis. So from absorbance, the unknown concentration can be determined.

UV spectroscopy obeys the Beer-Lambert law, which states that when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of

radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution. The expression of Beer-Lambert law is

$A = \log (I_0/I) = Ecl$

Where, A is absorbance, $I_0$ is intensity of light incident upon sample cell, I is intensity of light leaving sample cell, C is molar concentration of solute, L is length of sample cell (cm.) and E is molar absorptivity

Chromophore is defined as any isolated covalently bonded group that shows a characteristic absorption in the ultraviolet or visible region (200-800 nm). The chromophores can be divided into two groups-

a) Chromophores which contain p electrons and which undergo pie to pie* transitions. e.g: Ethylenes and acetylenes

b) Chromophores which contain both p and nonbonding electrons. They undergo two types of transitions; pie to pie* and nonbonding to pie*. e.g: Carbonyl, nitriles,azo compounds, nitro compounds.

Auxochromes- An auxochrome can be defined as any group which does not itself act as a chromophore but whose presence brings about a shift of the absorption band towards the longer wavelength of the spectrum. e.g: –OH,-OR,-NH2,-NHR, -SH etc.

**IR spectroscopy (Infrared Spectroscopy)** It deals with the infrared region of the electromagnetic spectrum, i.e. light having a longer wavelength and a lower frequency than visible light(wave number 400/cm to 4000/cm). The IR spectroscopy concept can generally be analyzed in three ways by measuring reflection, emission, and absorption. The major use of infrared spectroscopy is to determine the functional groups of molecules, relevant to both organic and inorganic chemistry.

**Principle** The absorption of IR is restricted to compounds with small energy differences in the possible vibrational and rotational states. For a molecule to absorb IR, the vibrations or rotations within a molecule must cause a net change in the dipole moment of the molecule. The alternating electrical field of the radiation (remember that electromagnetic radiation consists of an oscillating electrical field and an oscillating magnetic field, perpendicular to each other) interacts with fluctuations in the dipole moment of the molecule. If the frequency of the radiation matches the vibrational frequency of the molecule (natural frequency of amolecule) then radiation will be absorbed, with the generation of peak(IR spectra).

In IR, the region below 1500 cm-1 is rich in many absorption bands and the region is known as fingerprint region. Here the number of bending vibrations are usually more than the number of stretching vibrations. In this region, small difference in the structure and constitution of a

molecule results significant changes in the absorption bands. Many compounds show unique absorption bands in this region and which is very useful for the identification of the compound.

IR spectroscopy is useful for identification functional groups in a compound, identification of compounds and detecting impurities by comparing with IR spectrum of a standard.

**Mass spectrometry** It' a powerful analytical technique used to determine molecular weight of a compound, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules.

**Principle** In MS the results are presented in the form of a graph known as mass spectrum.The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. A mass spectrum is the plot of relative abundance (intensity) of ions against their mass/charge ratio. Here no electromagnetic radiation is used for excitation to occur.

The instrument used carry out mass spectroscopy is called as Mass spectrometer. The basic aspect of organic mass spectrometry consist of bombarding the vapour of an organic compound with a beam of energetic electron accelarated from a filament to an energy of 70 eV to form positively charged ions (molecular ions). The additional energy of the elecrons is dissipated in breaking the bonds in the molecular ion, which undergoes fragmentation to yield several neutral or positively charged species. The ions are then separated according to their mass to charge ration by virtue of magnet and are thus detected by a detector.

**Nuclear Magnetic Resonance (NMR)** It'sis the most powerful tool available for organic structure determination.

**Principle** The spinning charged nucleus generates a magnetic field. When placed in an external field, spinning protons act like bar magnets. The magnetic fields of the spinning nuclei will align either with the external field, or against the field. *A photon with the right amount of energy can be absorbed and cause the spinning proton to flip. Energy difference is proportional to the magnetic fieldstrength. This energy is provided through radio waves.It is used to study a wide variety of nuclei like 1H, 13C, 15N, 19F, 31P etc.A nucleus with an odd atomic number or an odd mass number has a nuclear spin***.**

Depending on their chemical environment, protons in a molecule are shielded by different amounts. The number of signals shows how many different kinds of protons are present. The location of the signals shows how shielded or deshielded the proton is. The intensity of the signal shows the number of protons of that type. Signal splitting shows the number of protons on adjacent atoms. Trimethylsilane (TMS) is added to the sample. Since silicon is less

electronegative than carbon, TMS protons are highly shielded. Signal defined as zero.Organic protons absorb downfield (to the left) of the TMS signal.

**Electrophoresis**

Electrophoresis is the movement of charged particles or molecules in a medium under the influence of an applied electric field. It's a comprehensive term that refers to the migration of charged particle of any size in liquid medium under the influence of an electric field. Depending on kind of charge the molecule carry, they move towards either to cathode or to anode.It's a cheaper separation technique as compared to others. The electrophoresis technique is extensively used for the analysis of RNA, DNA and proteins.

An ampholyte become positively charged in acidic condition and migrate to cathode, in alkaline condition they become negatively charge and migrate to anode. Its commonly used for isolation of amino acids and proteins.

The rate of migration of an ion in electrical field depend on factors,

1. Net charge of molecule
2. Size and shape of particle
3. Strength of electrical field
4. Properties of supporting medium and
5. Temperature of operation

**Types of Electrophoresis**

**FreeElectrophoresis i.e.** method performed without any support media.

**Zone Electrophoresis** The separation is carried out by using support media like paper or gel. The components are separated as zones hence the named so. It's also known as electromatography.

**Capillary Electrophoresis** Here narrow bore tubes are used to effect separation of the samples. It's a simple,quick, easyand more efficient method and requires less sample.

**Gel Electrophoresis** Here gels like agarose or sodium dodecyl polyacrylamide is used as a support media. The technique is most suitable for separation of proteins.It can be used for analytical purposes and as preparative technique for purification of molecules before before subjecting to other analytical techniques.

## IMPORTANT QUESTIONS

### 2 MARKS

1. Write principle/applications ofSoxhlet extraction/ Counter-current/ Sonication/ MAE/ PLE/ CLE.
2. Write principle/applications of TLC/ HPTLC/ PC/ CC/ GC/ HPLC/ Size exclusion chromatography
3. Write principle/applications of UV/ Mass/ IR/ NMR/ Electrophoresis.

### 5 MARKS

1. Write a note on Soxhlet extraction/ Counter-current/ Sonication/ MAE/ PLE/ CLE.
2. Write a note on Electrophoresis.
3. Discuss briefly TLC/ HPTLC/ PC/ CC/ GC/ HPLC/ Size exclusion chromatography
4. Discuss briefly UV/ Mass/ IR/ NMR/ Electrophoresis.

### 10 MARKS

1. Modern methods of extractionused in phytochemistry.
2. Discuss Chromatographic techniques used in phytochemistry.
3. Discuss spectroscopic techniques used in phytochemistry.
4. Discuss application of latest techniques of spectroscopy/ chromatography in isolation, purification and identification of crude drugs.