CHROMATOGRAPHY

INTRODUCTION TO CHROMATOGRAPHY

At the beginning of the twentieth century, the Russian botanist Mikhail Tswett invented and named chromatography.

He separated plant pigments by passing solution mixtures through a glass column packed with fine particles of calcium carbonate.

The separation of those pigments appeared as colored bands on the column.

Tswett named his separation method for the two Greek words "chroma" and "graphein," which mean "color" and "to write," respectively (Skoog et al., 1998).

In the past six decades, chromatography has been extensively applied to all branches of science. The 1952 Nobel Prize in chemistry was awarded to A. J. P. Martin and R. L. M. Synge for their contributions to chromatographic separations, which tremendously impacted chemistry-related SCIences.

More impressively between 1937 and 1972, a total of 12 Nobel Prizes were based on working which chromatography was a key tool.

In all chromatographic separations, the sample is carried by the mobile phase, which may be a gas, a liquid, or a supercritical fluid.

The mobile phase is then percoated through an immiscible stationary phase that is fixed on a solid substrate.

When the sample passes through the stationary phase, species are retained to varying degrees as a result of the physicochemical interaction between the sample species and the stationary phase. The separation of species appears in the form of bands or zones resulting from various retentions. Chemical information can thus be analyzed qualitatively and/or quantitatively on the basis of these separated zones.

Based on the physical means by which the stationary phase and mobile phase are brought into contact, chromatography can be classified as planar or column (Giddings, 1991).

In planar chromatography the stationary phase is supported on a flat plate or a piece of paper, while the mobile phase is usually driven by capillary force, gravity, or an electric field.

In a few cases, the mobile phase is forced under pressure, for example, in overpressure planar chromatography.

When a tube holds the stationary phase, the chromatographic method is referred to as column chromatography.

In *column* {:hromatography, the mobile phase is driven by pressure, gravity, or an electric field. Because of its astonishing separation power, column chromatography has become the most frequently practiced means of analytical separation.

Three types of mobile phases are used in column chromatography: liquids, gases, and supercritical fluids.

Among these three types, liquids are the most frequently used.

Therefore, LC is the predominant technique used in modem analytical separations.

Early LC was operated in glass columns, and the mobile phase was driven by gravity.

To ensure a reasonable flow rate (F), the column was packed with large particles in the 150 to *200* urn range.

Such packing yielded poor results with long separation times, often several hours.

Beginning in the late 1960s, small particles were packed in a steel tube, which was subjected to high pressure.

Such a system dramatically improved the separation power of column chromatography; in the early years, "HPLC" stood for "high pressure liquid chromatography".

Three to ten micrometers particle diameter (dp) are commonly used as stationary phases in HPLC.

Separation can thus be done in a high performance mode, which means high resolution and short analysis time.

Therefore, these newer procedures are termed "high-performance liquid chromatography" to distinguish them from the earliest methods.

Basic Chromatographic terminology

- **Chromatogram:** Graph showing detector response as a function of a time.
- **Flow rate:** How much mobile phase passed *I* minute (mIlmin).
- **Linear velocity:** Distance passed by mobile phase per I min in the column (em/min).
- **Bed volume (Vt)** is the total volume inside the column.
- **Void volume (VO)** is the volume of solution not trapped in the beads.
- **Internal volume (Vi)** is the volume of solution trapped in the beads.
- **Volume of the gel matrix (Vg):** Vt = VO + Vi + V g.
- **Elution volume (Ve)** is the volume necessary to elute a substance from the column.

2. GENERAL PRINCIPLES OF CHROMATOGRAPHY

The partition principle: Partition chromatography

When a solute is aIIowed to equilibrate itself between two equal volumes of two immiscible liquids, the ratio of the concentration of the solute in the two phases at equilibrium at a given temperature is caIIed the partition coefficient.

A mixture of substances with different partition coefficient can be quantitatively separated by a technique known as countercurrent distribution, first developed by L.c. Craig, in which many repetitive partition steps take place.

Partition chromatography is logical extension of the countercurrent partition principle for achieving chromatographic separation of mixtures.

The technique, originaIIy developed by A.J.P Martin and R.L.M synge has since been applied to an enormous number of separations.

The separation is achieved in a huge number of partition steps which takes place on microscopic granules of hydrated insoluble inert substances, such as starch or silica gel packed in a column or layered on a plate, or the granules of ceIIulose in paper.

The granules, through inert, are hydrophilic and as such are surrounded by a layer of tightly bound water.

This tightly bound water, since it is immobilized, serves as the stationary phase.

On the surface of this stationary phase flows, the mobile phase of an immiscible solvent containing the mixture to be separated.

The solute molecules are subjected to microscopic partition processes between the immobilized water layer and the flowing solvent.

Since the process takes place on the sulface of each granule the number of partition steps is so great that the substances move along the column or surface at a differeut rate as the mobile phase **flows through it.**

The principle of partition is *exploited* in gas/liquid chromatography (GLC) technique also. Separations depend upon the partition of the solute molecules between a liquid supported on a **suitable solid, and the gas flowing through the system.**

In true partition chromatography, the only factor which influences the movement of a compound **as the solvent travels along the stationary of that compound in the two phases.**

Substances more soluble in the mobile phase will migrate greater distances as compared with the **substances more soluble in the stationary phase.**

Other compounds of intennediate solubility between the two phases will migrate to intennediate **distances depending upon their partition coefficient.**

Adsorption chromatography:

Substances differ in their adsotption-desotption behavior between a moviug solvent (a liquid or a **gas) and a stationary solid phase.**

This behavior of a substance can be exploited to achieve its separation.

Adsorption is a surface phenomeuon which signifies a higher concentration at an interface as **compared to that present in the surroundings medium.**

Adsotption should not be confused with absotption, which signifies the penetration of one **substance into the body of another.**

For the PUlpOse of chromatography, the term adsotption has limited meaning; it usually denotes interactions involving hydrogen bonding and weaker electrostatic forces of the substances with **the adsorbent.**

The solute molecule which interacts more with the adsorbent, which is *also* the stationary phase, **is retarded more while less interacting solute molecules are retarded less.**

In this way a separation of sample components is achieved.

3. LON-EXCHANGE CHROMATOGRAPHY

This procedure was first developed by W. Cohn and maybe defined as the reversible exchange of ions in solution with ions electrostatically bound to some sort insoluble support mediwn.

The ion exchanger consists of an inert support medium coupled covalently to positive (anion **exchanger) or negative (cation exchanger) functional groups.**

To these covalently bound functional groups are bound, through electrostatic attraction, **oppositely charged ions which will be exchanged with like charged ions in the sample.**

Thus if anion exchange chromatography is perfonned, negativeiy charged sample components will interact more with the stationary phase and will be exchanged for like charged ions already **bound to the matrix.**

These sample ions will be retarded whereas other uncharged or positively charged ions will not **be retarded to the same degree and will be eluted out fast.**

The situation will be exactly reversed for cation exchange

chromatography. Molecular size: Gel-filtration chromatography

This technique exploits the molecular size as the basis of separation.

The support medium, a gel, consists of porous beads where pore size is strictly controlled. Macromolecules smaller than the pores get entrapped in the pores (and move siowly), while those bigger than the pores travel unhindered through the column (and elute out faster than the **smaller molecules).**

Thus the main interaction between the *solute* and the stationary phase is with respect to the size **and this is ultimately the basis of separation also.**

This technique is also used to determine relative molecular weight of an given macromolecule. Affinity chromatography:

The technique utiiizes the specificity of an enzyme for its substrate (also receptor for its

agonist, antibody for antigen) or substrate analogue for enzyme's (other proteins with biological **specificity) separation.**

A substrate analogue is coupled to the gel matrix and the cellular suspension is allowed to percolate through.

The enzyme which is specific for the substrate analogue binds to the gel becoming immobile while all other components move down and out. The technique has high resolution power.

Table 1: The various types of Chromatography Technique

Technique	Stationary Phase	Mobile phase
1. column or adsorption chromatography	**Solid**	**Liquid**
chromatography	**Liquid**	**Liquid**
2. partition chromatography	**Liquid**	**Liquid**
3. paper chromatography	**Liquid or**	**Liquid**
4. thin layer cbromatography (TLC)	**solid**	**Gas**
5. Gas-liquid chromatography (GLC)	**Liquid**	**Gas**
6. Gas-solid chromatography (GSC)	**Solid**	**Liquid**
7. Ion exchange chromatography	**Solid**	

PAPER CHROMATOGRAPHY:

In paper chromatography, the mobile phase is a solvent, and the stationary phase is water held in the fibers of chromatography paper.

Solution of the mixture to be separated is spotted onto a strip of chromatography paper (or filter paper) with a dropper.

The solvent moves through the paper due to capillary action and dissolves the mixture spot. The components of the sample start to move along the paper at the same rate as the solvent. Components of the mixture with a stronger attraction to the paper (stationary phase) than to the solvent will move more slowly than the components with a strong attraction to the solvent (mobile phase).

The difference in the rates with which the components travel along the paper, over time, leads to their separation.

Particular mixtures will have chromatographic patterns that are consistent and reproducible as long as the paper, solvent, and time are constant.

This makes paper chromatography a qualitative method for identifying some of the components in a mixture.

Principle: This technique is a type of partition Chromatography in which the substance are distributed between two liquids i.e. one is stationary liquid which is held in the fibers of the paper and called stationary phase and other is the moving liquid or *developing solvent* and called the moving phase.

The components of the mixture to be separated migrate at different rates and appear as sports at different point on the paper.

In this technique a drop of the test *solution* is applied as a small spot on a filter paper and the post is dried.

The paper is kept in close chamber and the edge of paper is dipped into a solvent called developing solvent.

As the filter paper gets the liquid through its capillary axis and when it reacbes the spot of the test *solution,* the various substances are moved by *solvent* system at various appends. When reacbed or travelled to a suitable length the paper is dried and spot are visualised by suitable reagents called visualizing reagents.

The movement of substance relative to the *solvent* is expressed in terms of RF values i.e. migration parameters.

4. MIGRATION PARAMETERS

The positions of migrated spots on the Chromatogram are indicated by terms such as RF, Rx, and RM.

R: The R is related to the migrations of solute front from solvent front.

RF = distance travelled by the *solute* from the origin line distance travelled by the *solvent* from the origin *line.*

R is a function of partition coefficient. It is constant for a given substance provided the conditions of the Chromatographic system are kept constant, with respect to temperature, type of paper, duration and direction of development, amount of liquid in the reservoir, humidity etc. i.e. quality of paper I this case

- Nature of mixture.
- Temperature and,
- The size of the vessel in which the operation is carried.

Rx: **In** some cases the solvent front runs off the end of filter paper, the movement of substance is expresses as Rx.

Rx = Distance travelled by the substance from the origin line/distance travelled by the standard substance from the origin line.

RM: The term RM is additive and is composed of the partial RM values of the individual functional groups of atoms in molecule.

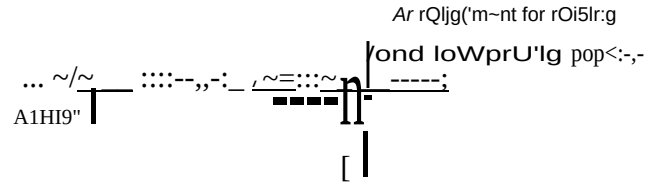
RM = Log [1/ RF -1]

TYPES OF PAPER CHROMATOGRAPHY:

Ascending Chromatography:

When the development of the paper is done by allowing the solvent to travel up the paper it is known ascending technique.

In the ascending mode of development the paper is suspended so that the lower edge is below the



An alternative to suspending the paper was to form a self-supporting cylinder from the paper.

As with TLC, mnltiple developments, with either the same or different solvent was used to Improve resolution, although the time taken for each development must have made this especis

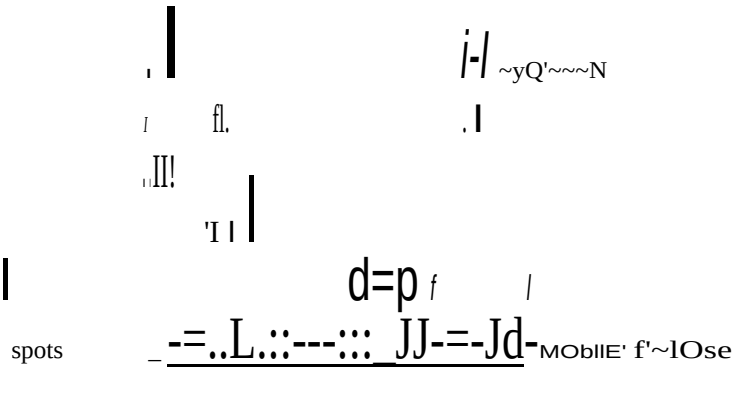


Fig.I: **Ascending Chromatography**

ascending and descending technique have been employed for separation but descending technique is preferred if the RF values of various constitutions are almost same.

Descending Chromatography:

When the development of the paper IS done by allowing the solvent to travel down the paper it is known descending technique.

The advantage of descending technique is that the development can be continued indefinitely even though the solvent runs off at the other end of paper.

The descending method of chromatogram development was that originally proposed by Martin and his co-workers.

In descending paper chromatography the upper end of the paper is immersed in a solvent contained in a suspended trough so that the Sow, initiated as in the ascending mode by capillary action, is

sustained by gravity and will continue so long as there is solvent to feed it.

This had the useful consequence that a sheet of any (practical) length could be used.

In addition, the solvent could be allowed to run off the end of the paper, thus extending the chromatographic run if needed to improve resolution, or enabling compounds to be eluted from the paper and collected for further experiments.

The results obtained for a particular sample/solvent system combination run in either ascending or descending mode were usually similar; however, the latter was generally faster.

2.3.3. Ascending - Descending Chromatography:

It is a hybrid of two techniques. The upper part of ascending chromatography can be folded over a glass rod allowing the descending development to change over into the descending after crossing the glass rod.

Circularl Radial Paper Chromatography:

This is also known as circular paper chromatography. In this technique a circular filter paper is employed, various materials to be analyzed are placed in a centre.

After drying the spot the filter paper IS fixed horizontally on pet dish possessing solvent, so that tongue of paper dips in solvent when

solvent front has moved through a sufficient large distance the components gets separated. Forming concentric circular spots.

Two-dimensional Separations on Paper

Where separations were not achieved in a single development, it was often possible to achieve the desired result using a second solvent system of different composition and development in a second dimension at 903 to the original direction of chromatography.

Two-dimensional paper chromatography was described by Consden, Gordon and Martin for the separation of20 amino acids, but was subsequently widely employed.

An additional possibility was the use of paper chromatography in one direction with paper electrophoresis (both high and low voltage) in the second.

Indeed, there are numerous examples in the literature of either chromatography followed by electrophoresis or electrophoresis followed by chromatography.

Table: 2 : **Solvent Listed In Order**

of Increasing Polarity
Solvent
n-hexane
Cyclohexane
Carbon tetrachloride
Benzene
Toluene

Procedure for paper chromatography:

• Choice of the proper chromatographic technique:

The choice of technique is depends upon the nature of substances to be separated.

• Choice of filter paper:

../' Whether the paper is being used for quantitative or qualitative analysis.

../' Whether it is used for analytical or preparative chromatography.

../' Whether substance used are hydrophilic or lipophilic neutral or charged species. • Proper developing solvent:

Trichloroethylene
Diethyl ether
Chloroform
Ethyl acetate
n-butanol
n-propanol
Acetone
Ethanol
Methanol
Water

The choice of this depends upon the simple fact that RF values should be different for different constituents present in mixture. A solvent or mixture of solvent, which gives a RF 0.2 _ 0.8 for sample,

should be selected. The solvent are listed in order of increasing polarity in table 2

RfValues:

The distance a substance travels related to the distance the solvent travels is called the Rf value. The Rf value can be calculated by measuring the distance of the substance from its starting point in millimeters, as well as the distance the solvent travelled from its starting pointing millimeters, then dividing the substance distance by the solvent distance.

$$R_f = \frac{\text{distance the solute (componen) moved} \quad D}{\text{distance the solvent front moved} \quad L}$$

The equation is: It does not matter if the solvent moves 10 mm or 100 mm, the Rf value of a substance will remain the same. For example the average Rf value for a dye called "methylene blue" is 0.50.S0, that means if the substance moved 5 mrn, the solvent moved 10 mm. It also means if the substance moved 50 mm, the solvent moved 100 mm.

Applications:

Paper chromatography is widely used for qualitative analysis of inorganic, organic and biochemical interests. It is also useful is analysis of mixture of amino acid and mixture of sugars. Given the importance of paper chromatography in its heyday, a list of its applications covers all types of analytes, including proteins, peptides, amino acids, poly-, oligo-, di- and monosaccharides, natural products, sterols, steroids, bile acids, pigment, dyes and inorganic species.

5. SUMMARY

Chromatography is a method of analysis by which components of a mixture are separated by redistribution of molecules of mixture between two or more phases. It is used for purification, separation and preparation purposes. Phases in chromatography are Stationary phase and Mobile phase. One of the methods of classification of chromatographic techniques is based on state of matter of stationary and mobile phases. Another method of classification is based on principles. General classification contains different well known methods. Different principles of chromatography are adsorption, partition, ion exchange, exclusion. Diagnostically important method of chromatography is paper chromatography, which is used for separation of serum amino acids. Other methods are also clinically useful. Paper Chromatography is a method used to separate mixtures into their different parts. Paper chromatography has been most commonly used to separate pigments, dyes and inks. To do paper chromatography you need paper with a lot of cellulose fiber (fiber found in wood) and chromatography solution, which is usually made from a

mixture of water and alcohol. Paper chromatography works because inks usually contain several different colors. Ink manufacturers mix different amounts of primary colors like blue, red and yellow to make many other colors. During paper chromatography, we can separate basic colored inks that contain different ingredients because some inks are more attracted to the paper and some are more attracted to the alcohol or water.