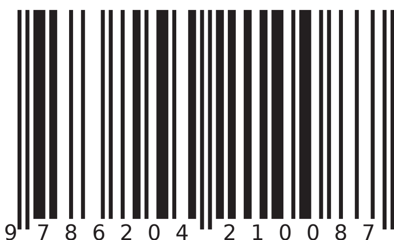
This book provides basic information on all the different types of chromatographic techniques under one cover. It is the most comprehensive resource available on the principles, theory techniques, applications and advantages of chromatographic techniques in various fields of science and technology. This book is consisting of six chapters. Chapter 1 deals with the basic concepts of chromatographic techniques. Chapter 2 deals with paper chromatography, its principle, nature of paper, developing solvents, components, retardation factors and factors affecting its types, experimental procedure, advantages, disadvantages and various applications. Chapter 3 gives details about gas chromatography. Chapter 4 consists of details about thin layer chromatography. Chapter 5 deals with column chromatography. Chapter 6 deals with discussion on high performance liquid chromatography. The details provided in this book will be very useful to students of UG and PG courses of Science and Technology.

FOR AUTHOR USE ONLY

Dasharath Chavhan
Santosh Agarkar

# Fundamental concepts of Chromatography

Dr. D. M. Chavhan, M.Sc. (Chem), NET-JRF, Ph.D. working as Assistant Professor at Indira Mahavidyalaya, Kalamb Dist. Yavatmal India. He has 12 years of Teaching experience and publishesd 2 books.
Dr. S.V. Agarkar, Ph.D.(Chemistry, Education and Civil Engineering) working as Principal at Ralegaon. He has 27 years' experience and published 7 books.



**Dasharath Chavhan**
**Santosh Agarkar**

# Fundamental concepts of Chromatography

FOR AUTHOR USE ONLY

FOR AUTHOR USE ONLY

Dasharath Chavhan
Santosh Agarkar

# Fundamental concepts of Chromatography

FOR AUTHOR USE ONLY

LAP LAMBERT Academic Publishing

**Imprint**
Any brand names and product names mentioned in this book are subject to trademark, brand or patent protection and are trademarks or registered trademarks of their respective holders. The use of brand names, product names, common names, trade names, product descriptions etc. even without a particular marking in this work is in no way to be construed to mean that such names may be regarded as unrestricted in respect of trademark and brand protection legislation and could thus be used by anyone.

Cover image: www.ingimage.com

Publisher:
LAP LAMBERT Academic Publishing
is a trademark of
Dodo Books Indian Ocean Ltd., member of the OmniScriptum S.R.L Publishing group
str. A.Russo 15, of. 61, Chisinau-2068, Republic of Moldova Europe
Printed at: see last page
**ISBN: 978-620-4-21008-7**

Copyright © Dasharath Chavhan, Santosh Agarkar
Copyright © 2021 Dodo Books Indian Ocean Ltd., member of the OmniScriptum S.R.L Publishing group

FOR AUTHOR USE ONLY

# Preface

"Fundamental concept of Chromatography" is designed to meet the scope and sequence requirement of students of undergraduate courses of Science, Engineering, Technology and Pharmaceuticals courses. The book provides an important opportunity for undergraduate students to learn the fundamental and core concept of chromatographic techniques and understand how those concepts are useful in various fields. The book is designed with main objective to enhance student learning and create interest in the subject.

Most of the universities and educational institutes have introduced chromatographic techniques as course for undergraduate level. This book has been written by keeping in new to cover all contents as per requirements of various courses. In this book basic concepts of chromatography, principles, components, procedures, advantages, disadvantages and applications of Paper chromatography, Gas chromatography, Thin Layer chromatography, Column chromatography and high performance Liquid chromatography are covered.

The author have consulted and referred several reputed books and other literature sources.

FOR AUTHOR USE ONLY

In framing, designing, organizing and preparing the script of this book we express our sense of gratitude to all authors, editors and sources which have been used directly or indirectly for the reading material.

The practical suggestions for the improvement of learning material provided in this book are most welcomed.

**Dr. D. M. Chavhan**

**Dr. S. V. Agarkar**

**FUNDAMENTAL CONCEPT OF CHROMATOGRAPHY**

**INDEX**


| Chapter | Particulars | | Page No |
|---|---|---|---|
| | **Basic Concepts of Chromatography** | | **5-10** |
| **1** | 1.1 | Introduction | 5 |
| | 1.2 | Definition of Chromatography | 6 |
| | 1.3 | Classification of Chromatography | 6 |
| | 1.4 | Principle of Chromatography | 7 |
| | 1.5 | Partition Chromatography | 7 |
| | 1.6 | Principle of Partition Chromatography | 8 |
| | 1.7 | Liquid-liquid chromatography (LLC) | 8 |
| | 1.8 | Adsorption Chromatography | 8 |
| | 1.9 | Principle of Adsorption Chromatography | 9 |
| | 1.10 | Summary | 9-10 |
| | 1.11 | References: | 10 |
| **Chapter** | **Paper Chromatography** | | **11-24** |
| 2 | 2.1 | Introduction | 11 |
| | 2.2 | Principle of paper Chromatography | 11-12 |
| | 2.3 | Nature of Paper Used | 12 |
| | 2.4 | Nature of Developing Solvents | 12-13 |
| | 2.5 | Components of Paper Chromatography | 13 |
| | 2.6 | Development of the Chromatogram | 13-14 |
| | 2.7 | Retardation Factor | 14-15 |
| | 2.8 | Factors Affecting Rf Value | 15 |
| | 2.9 | Types of Paper Chromatography | 15 |
| | 2.10 | Ascending Paper Chromatography | 15-16 |
| | 2.11 | Experimental Procedure of Ascending Paper Chromatography | 16-17 |
| | 2.12 | Descending Paper Chromatography | 17-18 |
| | 2.13 | Circular or Radial Paper Chromatography | 18-19 |
| | 2.14 | Two Dimensional Paper chromatography | 19 |
| | 2.15 | Ascending Descending hybrid Paper Chromatography | 20 |
| | 2.16 | Advantages of Paper Chromatography | 20-21 |


FOR AUTHOR USE ONLY

| | | | |
|---|---|---|---|
| | 2.17 | Disadvantages of paper Chromatography | 21 |
| | 2.18 | Applications of Paper Chromatography | 21-22 |
| | 2.19 | Paper Chromatography Experiment | 22-23 |
| | 2.20 | Summary | 23 |
| | 2.21 | References | 24 |
| **Chapter** | **Gas Chromatography** | | **25-33** |
| **3** | 3.1 | Introduction | 25-26 |
| | 3.2 | Principle of Gas Liquid Chromatography | 26 |
| | 3.3 | Retention Time (Rt) | 26 |
| | 3.4 | Retention volume (Rv) | 27 |
| | 3.5 | Procedure of Analysis of Mixtures | 27-29 |
| | 3.6 | Components of Gas Liquid Chromatography | 29 |
| | 3.7 | Carrier Gas or Mobile Phase | 29 |
| | 3.8 | Stationary Phase | 29-30 |
| | 3.9 | Column | 30 |
| | 3.10 | Detector | 30 |
| | 3.11 | Applications of Gas Liquid Chromatography | 30-31 |
| | 3.12 | Advantages Gas Liquid Chromatography | 31-32 |
| | 3.13 | Disadvantages of Gas-Liquid Chromatography | 32 |
| | 3.14 | Summary | 32-33 |
| | 3.15 | References | 33 |
| **Chapter** | **Thin Layer Chromatography** | | **34-39** |
| **4** | 4.1 | Introduction | 34-35 |
| | 4.2 | Principle of Thin Layer Chromatography | 35 |
| | 4.3 | Steps involved in Thin Layer Chromatography | 35-36 |
| | 4.4 | Components of Thin Layer Chromatography | 36-37 |
| | 4.5 | Applications of Thin Layer Chromatography | 37-38 |
| | 4.6 | Advantages of Thin Layer Chromatography | 38 |
| | 4.7 | Disadvantages of TLC (Limitations) | 38 |
| | 4.8 | Summary | 39 |
| | 4.9 | References | 39 |
| **Chapter** | **Column Chromatography** | | **40-46** |
| **5** | 5.1 | Introduction | 40 |
| | 5.2 | Principle of Column Chromatography | 41 |
| | 5.3 | Types of Preparation of column chromatography or | 41-42 |


FOR AUTHOR USE ONLY

| | | | |
|---|---|---|---|
| | | types of column packing | |
| | 5.4 | Components of Column Chromatography | 42 |
| | 5.5 | Experimental Procedure of Column Chromatography | 42-43 |
| | 5.6 | Types of Column Chromatography | 43-44 |
| | 5.7 | Factor's affecting separation in column Chromatography | 44 |
| | 5.8 | Applications of Column Chromatography | 44-45 |
| | 5.9 | Advantages of column Chromatography | 45 |
| | 5.10 | Disadvantages of Column Chromatography | 45 |
| | 5.11 | Summary | 45-46 |
| | 5.12 | References | 46 |
| **Chapter** | **High Performance Liquid Chromatography (HPLC)** | | **47-52** |
| **6** | 6.1 | Introduction | 47 |
| | 6.2 | Types of HPLC | 47-48 |
| | 6.3 | Components of HPLC | 49 |
| | 6.4 | Experimental Procedure of HPLC | 49 |
| | 6.5 | Applications of HPLC | 50 |
| | 6.6 | Advantages of HPLC | 50-51 |
| | 6.7 | Disadvantages of HPLC | 51 |
| | 6.8 | Summary | 51 |
| | 6.9 | References | 51-52 |


FOR AUTHOR USE ONLY

# Chapter 1
# Basic Concepts of Chromatography

## 1.1 Introduction:

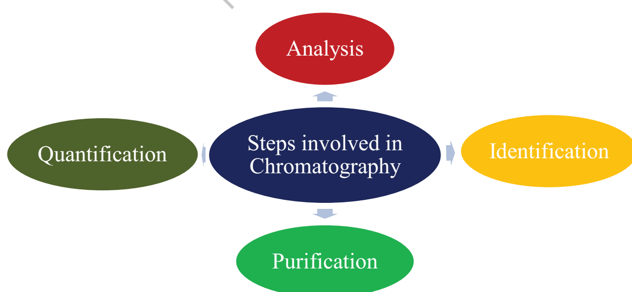
Chromatography is a very important technique in Analytical Chemistry from the point of view of separation and identification of various components presents in mixtures of a substance under analysis. The Chromatography can also be called as a Purification Technique. The technique is useful in organic synthesis for detection of number of products or isomers formed during preparation. Chromatography applied by scientist in separation of mixtures of organic and inorganic chemicals present in the substance. Many people used chromatography identifications of unknown substance in mixtures and also in forensic field for detection of crime. The chromatography technique was introduced by Russian Botanist M.S.Tswett in 1906. Tswett was the first scientist who introduced the concept of the term chromatography.

The word chromatography is a Greek word which split as

Chroma=Color and Graphien=to write.

The green pigment present in the plants contains different constituents. Tswett was the first used the technique of separation and isolation of different pigments present in green pigment chlorophyll. The dissolved plant pigments in petroleum ether and by using the calcium carbonate as column after running the pigment it resolved into different colored bands. Each color bands represent the different components present in the pigments. In any Chromatographic techniques mainly four Operations are involved.

FOR AUTHOR USE ONLY



## 1.2 Definition of Chromatography:

Chromatography can be defined as "It is a methods or process used in analytical chemistry for separation, identification and isolation of constituents present in the mixtures of substance into its individual components".

Or

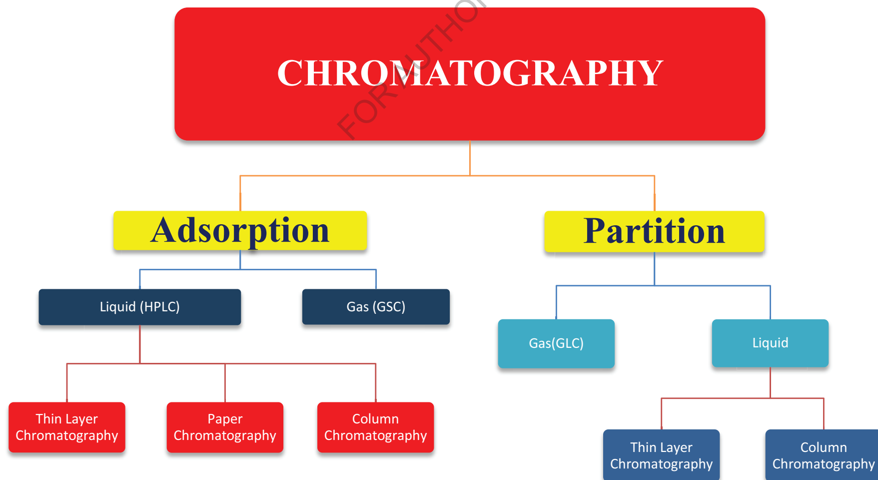
Chromatography can also be defined in as "the process of distribution of components present into the mixtures on the basis of its affinity with two different phases involved during process out of which one is a stationary phase and other is a mobile phase"

In terms of IUPAC the Chromatography can be defined as "it is a kind of physical method of analysis in which the components to be separated are distributed between two phases, one of which is stationary while the other phase is mobile phase moves in a definite direction along with the component"

In any Chromatographic technique, there are two phases are involved the first phase that support the movement of running solvent is a stationary phase and the other phase that moves along with the component it is mobile phase. Stationary phase may be solid or liquid while mobile phase may be liquid or gas.

FOR AUTHOR USE ONLY

## 1.3 Classification of Chromatography Technique:



## 1.4 Principle of Chromatography:

Chromatographic technique is based on the different rate of migration of components in mixtures. Some components move faster with mobile phase against the stationary phase. There are different types of chromatography technique but in any types there are two phases are involved one is stationary phase and other is mobile phase. The stationary phase is the support for mobile phase to move with the components and it may be solid or liquid supported on suitable rigid material. The mobile phase as the name indicates it moves over the stationary phase by dissolving them the components in mixture. In any chromatography methods the mobile phase always in liquid or gaseous state. The stationary phase as the name indicates it remains stationary and doesn't move like the mobile phase. While the stationary phase move by taking with it the solutes presents in the mixture of sample under analysis. Components which is more soluble or more affinity for the mobile phase will remained intact and spend more time in this phase than the components that greater affinity with the stationary phase adhered to that phase and move slower. Due to this different affinity with different phases the components or solutes separate. Because of these two opposite tendencies of solute separation will occur and the phenomenon is known as differential migration. The migration rate of solute is depends on the following two factors.

1. A Factor to move the solute forward
2. A factor tending to retard the solute

The first factor or the force that is responsible for the forward movement of the solute is called propelling force the moving solvent or mobile phase is responsible for the forward movement of solute. The factors that appose for the forward movement of solute due to adsorption phenomenon of solute on stationary phase is known as retardation factor or retarding force.

FOR AUTHOR USE ONLY

## 1.5 Partition Chromatography:

Partition chromatography is very simple methods of separation and identification of mixtures of samples. It is based on very simple principle of distribution of components on the basis of their solubility with respective phases. The components itself distributed into mobile phase and stationary phase and separation of mixtures take place. It was discovered in 1940 by Synge and Archer Martin. In this method of separation the two phases involved is either liquid in nature or one is liquid and other is gas out which one is lipophilic and the other is hydrophilic in nature. The components on the basis of its affinity distributed either in lipophilic or hydrophilic part of solvents.

### 1.6 Principle of Partition Chromatography:

The mobile phase covered to the stationary phase and both of these are immiscible with each other. The components present in the mixtures are different affinity with the two phases. In partition chromatography, the components which have more affinity with the mobile phases move with it and separated first and the other components which have affinity with stationary phase move slowly in this way the components participated in the respective phases. After distribution of components in two phases they migrate in forward direction against the mobile phases and the components are separated on the basis of their different partition coefficient. In partition chromatography, the stationary phase is liquid in nature and mobile phase is either gas or liquid. On the basis of mobile phase involved partition chromatography is of following two types.

1. Liquid-liquid chromatography.
2. Gas-liquid chromatography .

### 1.7 Liquid-liquid chromatography (LLC):

As the name indicates, in this type of chromatography the both the phases involved are liquid in nature. The stationary phase is supported on a special kind of blotting paper. The mobile phase which is immiscible with another liquid stationary phase move in forward direction by taking the components of mixture. The solutes due to their difference in migration rate, migrates differently and separation takes place. After complete running of mobile phase the spots are stained to make the chromatogram visible.

FOR AUTHOR USE ONLY

### 1.8 Adsorption Chromatography

In this technique of chromatography, components are separated by very week interaction force between components and stationary phase or mobile phase. The components which can create interaction with stationary phase may spend more of its time than those components which remain sticky with mobile phase. The components that adsorbed strongly with mobile phase separate first. In this technique, out of the two phases involved the stationary phase is solid in nature and mobile phase is either liquid or in gaseous state. The main difference between adsorption chromatography and Partition chromatography is that in adsorption chromatography the mixtures separated by the principle of adsorption and the stationary phase is in solid state while in partition chromatography the mixtures separated by principle of partition or distribution and the stationary phase is liquid in state.

## 1.9 Principle of Adsorption Chromatography:

In this technique, the solute to be separated creates the weak interaction with stationary phase the interaction may be Vander waal interaction, hydrogen bonding and electrostatic interaction etc. In adsorption chromatography, the stationary phase is solid supported on specific material known as column and mobile phase run through the stationary phase by taking the components of mixtures. The components that strongly adsorbed on the stationary phase remain stick while those components have greater affinity with mobile phase pass easily with mobile phase and separated first. The stationary phase or adsorbents used commonly during preparation of column are silica gel, Silica gel H, silica gel G, silica gel N, silica gel S, hydrated gel silica, alumina, cellulose microcrystalline etc.

In the adsorption chromatography, Adsorbent is the stationary phase the different forces develop during separation help to remove solutes from the adsorbent so that they can move with the mobile phase. The forces develop by the solute with the mobile phase help to remove solutes from the adsorbent so that they can move with the mobile phase. In adsorption chromatography, the mobile phase involved is liquid, it is called LSC (Liquid-Solid Chromatography). When a gas is used as a mobile phase, it is called GSC (Gas-Solid Chromatography).

FOR AUTHOR USE ONLY

Adsorption Chromatography is of two types

1. Liquid Chromatography or HPLC (high Performance Liquid Chromatography)
2. Gas Chromatography or GLC (Gas Liquid Chromatography)

There are various type of Chromatography

1. Paper Chromatography
2. Thin layer Chromatography
3. Colum Chromatography
4. Gas Chromatography
5. High Performance Liquid Chromatography (HPLC)

We have discussed all above types of chromatography one by one through various chapter.

## 1.10 Summary:

1. Chromatography is a very important technique in Analytical Chemistry from the point of view of separation and identification of various components presents in mixtures of a substance.
2. The word chromatography is a Greek word which split as

   Chroma=Color and Graphien=to write.

3. The chromatography technique was introduced by Russian Botanist M.S.Tswett in 1906.
4. Component which is more soluble or more affinity for the mobile phase will remained intact and spend more time in this phase than the Components that greater affinity with the stationary phase move slower.
5. The factors that opposes for the forward movement of solute due to adsorption phenomenon of solute on stationary phase is known as retardation factor or retarding force.

## 1.11 References:

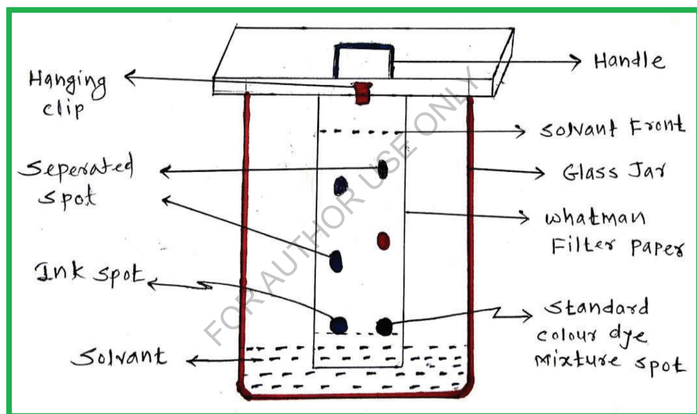

1. Analytical Chemistry-G. D. Christion, P. K. Dasgupta and K. A. Schug , 2013 7th edition, willey publication
2. A Textbook of Analytical Chemistry-Dr. B. Zaware, Dr. S. Kuchekar, Dr. H. Aher, Dr. M. Sangle and Dr. A. Deshpande,2010 Vision Publication, Pune
3. Chromatography-Concepts and Contrasts- J. M. Miller, 2009 2nd edition, willey publication
4. The Essence of Chromatography-C. F. Poole.2002 1st edition Elsevier Publication
5. Chromatographic Methods-A. Braithwaite and F.J. Smith,1999 5th edition Springer Netherlands
6. Analytical Chromatography-Dr. G.R.Chatwal,2017 4th edition Himalaya Publishing House, Mumbai
7. Chromatography-Principles and Instrumentation- M. F. Vitha, 2016 willey publication


FOR AUTHOR USE ONLY

# Chapter 2
# Paper Chromatography

## 2.1 Introduction:

The paper chromatography was introduced by consden, Gorden and martin in 1944. The technique of paper chromatography is useful in separating amino acids anions and RNA. The technique in the finger printing, separating and testing histamines, antibiotics, etc. Paper chromatography is a very simple technique in which the components of mixtures to be separated are dissolved in a special type of solvents and the solvents run along a special type of filter paper. This technique of paper chromatography is applied for both quantitative and qualitative analysis of mixtures. The technique is very simple because it requires simple apparatus and the sample required for analysis is very less. Coloured separated spots or components can be easily identified and separated by this method



FOR AUTHOR USE ONLY

**Fig.2.1 Paper Chromatography**

## 2.2 Principles of Paper Chromatography:

In paper chromatography, the stationary phase and mobile phases both are liquid in nature. While the stationary phase is supported on cellulose fibers of filter paper where the capillary action is used to pull the solvents up through the paper and separate the solutes.

In paper chromatography, the components of mixture to be separated are distributed between the stationary phase and mobile phase. So there is liquid-liquid partition takes place and mobile phase moving along a special kind of filter paper run the component with it and the paper act as small cell containing water in it and can hold

up to 20 percentage of water. The holded water on cellulose paper acts as stationary phase. If the components or solute present in mixture is more soluble in moving solvent or mobile phase than water then it will move faster. On the other hand the components or solute is more soluble in water or stationary phase will move slowly and remain behind. Due to such distribution of components or solute in two different phases the separation of mixture occurs.

## 2.3 Nature of Paper Used:

The polymeric papers containing hydroxyl functional group bonded to the polymer chains are generally preferred. In paper chromatographic technique, the Whatman filter paper no.1 is generally used. The filter papers with short fiber cellulose filter paper with high purity are preferentially used. The filter papers available are different grade such as slow, medium and fast depending on the rate of flow

Following are the criteria for using the filter paper in paper chromatography

1. The paper used should have maximum degree and clarity of separation.
2. The spot generated after running of the moving phase should not diffuse in the paper.
3. The paper should not be reactive toward the developing solvents.
4. Should not be soluble in moving solvent or developing solvents.
5. Should give the clear spot of components.
6. Paper should not affect the speed of components.
7. The solvent front rate of movement should be excellent.

FOR AUTHOR USE ONLY

## 2.4 Nature of Developing Solvents:

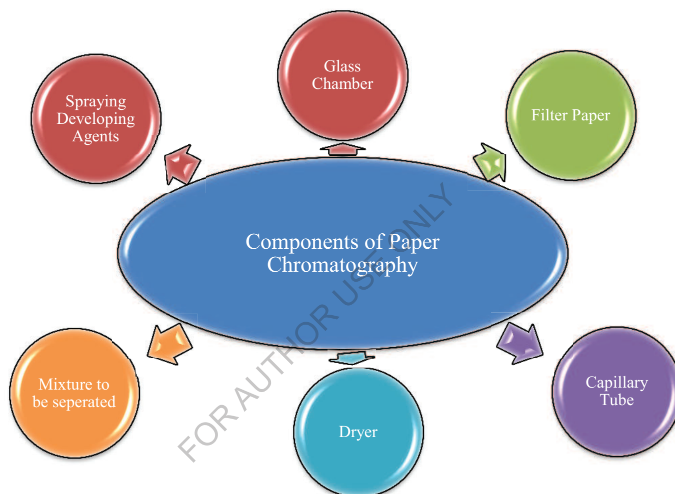
In paper chromatography, the solvent plays important role because it forms the mobile phase and move over the paper by dissolving them the solute. The pure solvents or undiluted solvent with water or mixture of miscible solvents are prefer. In paper chromatography, the mixture of n-butanol, acetic acid, ether, acetone and water is generally used. The solvent or mixtures of solvent move across filter paper are called developing solvents or developing agents. The polar solvents dissolved the polar solute and vice versa. Therefore for the separation of polar components the polar solvents are chosen and for non-polar components non polar solvents generally used.

Following are the criteria of using the good developing solvents.

1. The composition of mixture of developing solvents should not be change with time.
2. The viscosity of solvent should be less as it does not affect the running across filter paper.
3. The toxicity of solvent should be less.

4. It must be neutral toward the components to be separated.
5. The solvent must give the clear and circular spot.
6. There will be no tailing during the development of spot.
7. After the development of chromatogram it should not be interfere with detection of components.
8. The solvents must be easy to handle during process.
9. The solvents must be easily available.
10. Rf value should be lie between 0.05 to 0.85.

## 2.5 Components of Paper Chromatography:




FOR AUTHOR USE ONLY

## 2.6 Development of the Chromatogram:

In this chromatographic technique the chromatogram development involves following four stages

1. Sample Preparation
2. Sample Application
3. Developing of the Chromatogram
4. Visualization of separated spots

In sample preparation, stage solid sample cannot be applied directly, these are to be dissolved in proper solvent and the resulted liquid sample should be spotted on the paper. In case of liquid samples to be separated, these can be directly spotted on the paper and separated

In second stage, the liquid sample or sample dissolved in proper solventis applied on the paper by using capillary tube or any other technique. This is applied on the baseline ie nearer solvent line in a very fine and neat manner. Then spot is allowed to dry and then the paper should be immersed in the glass chamber with solvent with the marked spot just above the solvent level.

This stage involves the development of chromatogram. In this the solvent start to move from the level and takes spotted mixture spot components differently along with it. After paper should be taken out of jar and solvent front should be properly marked which is required to calculate the Rf value. Allow the paper to dry along with separated components in separate chamber before visualization of separated spot of components

Finally, separated spot can be visualized by various techniques when separated components are colorless. Some spraying reagents are required to develop them into colored spots.

## 2.7 Retardation Factor:

Rf is represented as Retardation Factor or Retention Factor and in paper chromatographic techniques the separated components are identified from their Rf Value. Rf is the characteristics of any substance and it ranges in between 0 to 1. Rf can be defined as the ratio of distance traveled by solute from base line to the distance travel by solvent from base line. The term Rf or Retardation factor is unit less quantity and there is no unit for Rf Value.

FOR AUTHOR USE ONLY

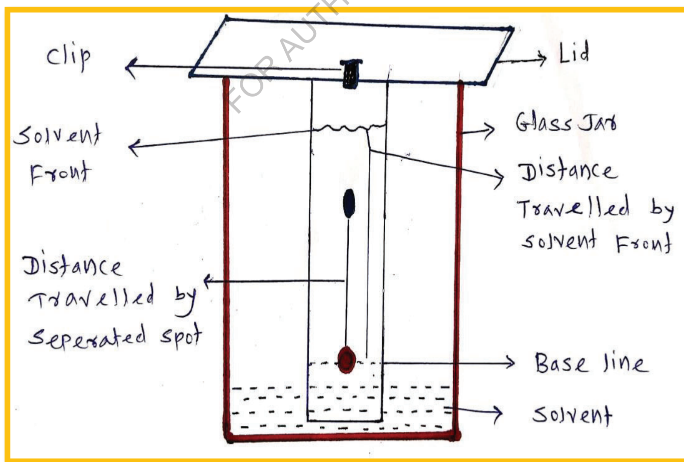



**Fig :2.2 Calculation of Rf Value from chromatogram**

$$Rf = \frac{\text{Distance travelled by the solute from base line}}{\text{Distance travelled by the solvent from base line}}$$

The distances travelled by the components are measure from the spot given before running. If there is the development of tail of spot then the center of tail of spot of that component to be considered.

## 2.8 Factors Affecting Rf Value:

As it is mentioned above that the particular component have their fix Rf value but still there are some factors which deviate the original Rf value of components the factors that affect the Rf Value are listed below.

1. The Rf value depends on concentration of developing solvents and its purity.
2. The quality of paper used for developing chromatogram also affect the Rf Value.
3. The amount of sample spotted can also affect the Rf Value.
4. Rf Value also Depends on the Temperature of medium.
5. Rf also depend on the pH of the mobile phase.
6. If the composition of mixture of developing solvents change the Rf Value also changes.
7. Variation in The thickness of the stationary phase also affect Rf Value.
8. Rf also changes as the Polarity of components change.

## 2.9 Types of Paper Chromatography:

Paper chromatography on the basis of direction of flow of the components it is classified into four different types.

1. Ascending Paper Chromatography
2. Descending Paper Chromatography
3. Circular or Radial Paper Chromatography
4. Two Dimensional Paper Chromatography

## 2.10 Ascending Paper Chromatography

This is the commonly applied technique of paper chromatography. As the name suggest the developing solvents or components move against the gravity in upward direction

FOR AUTHOR USE ONLY

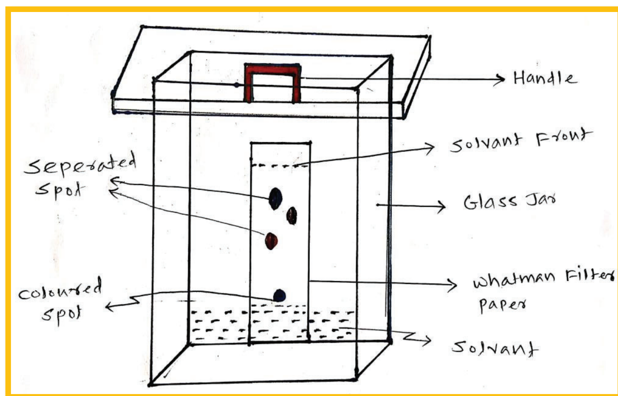



**Fig:2.3 Ascending Paper Chromatography**

## 2.11 Experimental Procedure of Ascending Paper Chromatography

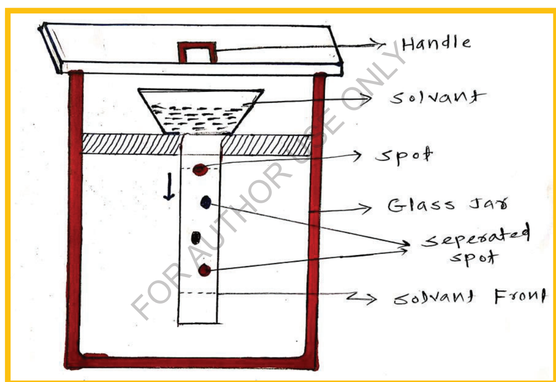
1. A strip of whatman filter paper no. 1 with height of 25-30 cm taken depending upon the tank or apparatus used for running the chromatogram.
2. Draw the horizontal baseline about 1 cm above from the bottom of paper, the baseline should be drawn in such a way that the spot of mixtures or components does not dipped in the running solvents.
3. The sample to be separated should take in a suitable solvent.
4. Select the center of horizontal baseline and apply the spot of mixture to be separated by using small capillary tube.
5. To get the sharp separation point, while applying the spot care should be taken that the spot must not be too large.
6. After spot applied by using the electrical dryer, dry the applied spot and follow the same procedure twice or thrice.
7. A suitable running solvent as per the nature of components are taken in to tank or chromatographic chamber and covered the tank with so that the tank is saturated with vapors of developing solvents.
8. Now the strips of filter paper with applied sample spot are dipped into running solvent without dipping the horizontal line into the solvents.
9. As the solvent rises up due to capillary action the separation of components presents in the mixture started.
10. After the sufficient rise in running solvent the stop and taken out the filter paper
11. Dry the filter paper with dryer.

FOR AUTHOR USE ONLY

12. If the components present in the mixtures are coloured then spot can be visible itself it does not required the spraying agents or locating agents.
13. If the components are colorless the spray the rubianic acid which is the locating solution for clear visible of spots.
14. After locating the center of spot the different components of mixture marked with pencil.
15. After marking measure the distance travelled by solute front and solvent front and calculate the Rf Value.

## 2.12 Descending Paper Chromatography:

The setup of this chromatographic technique is complicated but in this chromatographic technique the development of chromatogram occurs the faster than ascending paper chromatography, because the running solvent moves towards the gravity. The running solvents placed at the top and horizontal line and spot are present at the top of the paper as shown in the figure below.



FOR AUTHOR USE ONLY

**Fig :2.4 Descending Paper Chromatography**

The filter paper before carrying out running of developing solvent it is saturated first with stationary phase and hung in to a paper support with immersing into the developing solvents chamber present in the upper region of the chromatographic tank. In descending paper chromatography, the separation of the mixtures occurs in the same manner as in ascending paper chromatography, the solvents move down towards the gravity pull by capillary action. After the sufficient run up of the developing solvent the paper taken out of the tank and mark or locate the spot with locating agent. Rf value can be calculated in the same manner as in Ascending Paper Chromatography.

The technique of descending paper chromatography is applied for those mixtures or components which are too viscous and required more time to separate. For such a component the descending paper chromatography is more beneficial.

## 2.13 Circular or Radial Paper Chromatography:

As the name indicate, in this paper chromatography the circular filter paper used for development of chromatogram. The developing solvents move radially and develops the spot therefore the name circular or radial paper Chromatography. In this technique the circular filter paper is taking and paper is cut from the edge of paper to the center in such a way that tongue or wick is formed. The spots are given with the fine capillary at the center of circular filter paper. The wick or tongue is dipped in the Petridis containing the developing solvent in such a way that the developing solvent run through the wick. Cover the paper with another petridish. Then leave the petridish assembly undisturbed to allow the development of chromatogram. When the solvent front has almost reached three-fourth of the chromatogram, remove it and immediately mark the solvent front with a pencil. Spray the chromatogram with spraying reagent (ninhydrin reagent) to visualize the separated components. Calculate Rf value of each components as described in earlier method.

FOR AUTHOR USE ONLY

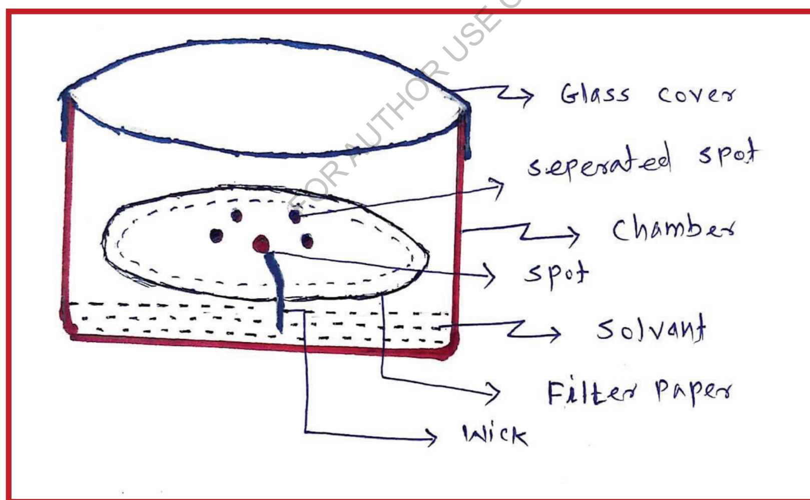



**Fig:2.5 Circular or Radial Paper Chromatography**

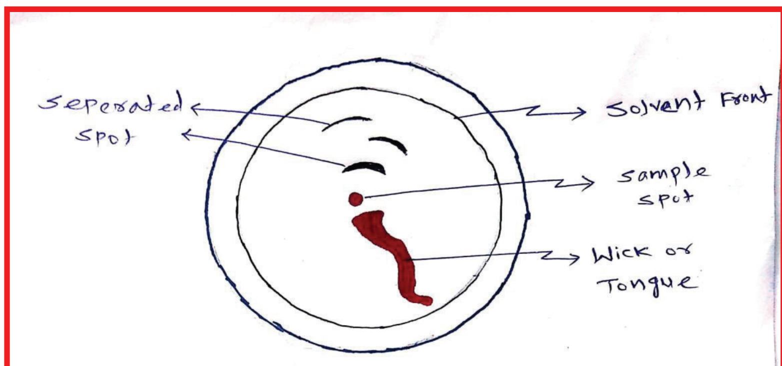



**Fig:2.6 Circular Filter paper or developed radial chromatography**

## 2.14 Two Dimensional Paper chromatography:

In this paper chromatographic technique, the paper are taken as same in case of ascending paper chromatography. The spots are given on horizontal line at corner of paper. After that the paper dipped in developing solvent keep in mind the spots doesn't dipped in solvent. The solvents run in upward direction against the gravity after few hours the filter paper turn $90^0$ clockwise and the chromatographic chamber or tank is filled with another type of solvent. Run the developing solvent in as in ascending paper chromatography. If there is no clear development of spot occur after some hours then go into further turning of $90^0$ clockwise and use another solvent and again run. The same procedure follow until the satisfactory separation of mixture is carried out.this is time consuming technique but still gives the accurate separation of samples.

FOR AUTHOR USE ONLY

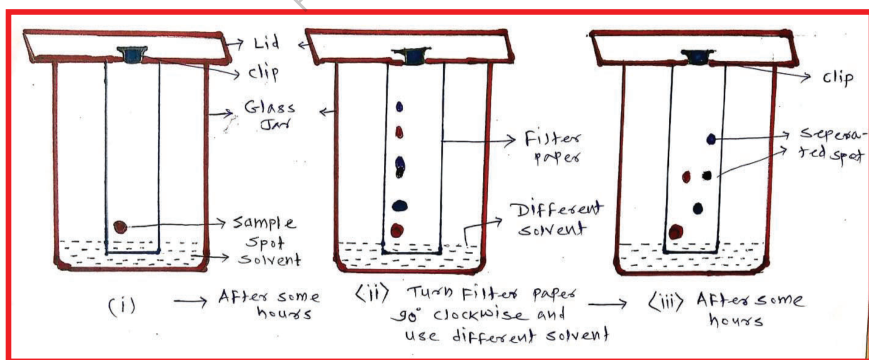



**Fig : 2.7 Two Dimensional Paper chromatography**

## 2.15 Ascending Descending hybrid Paper Chromatography:

In this type of paper chromatography, the solvent selected first travels upward on the paper strip which is folded over a rod at the top of jar and then it continues with its travel downwards after crossing the rod. This hybrid technique facilitate longer development period for better resolution of various mixtures selected for separation.

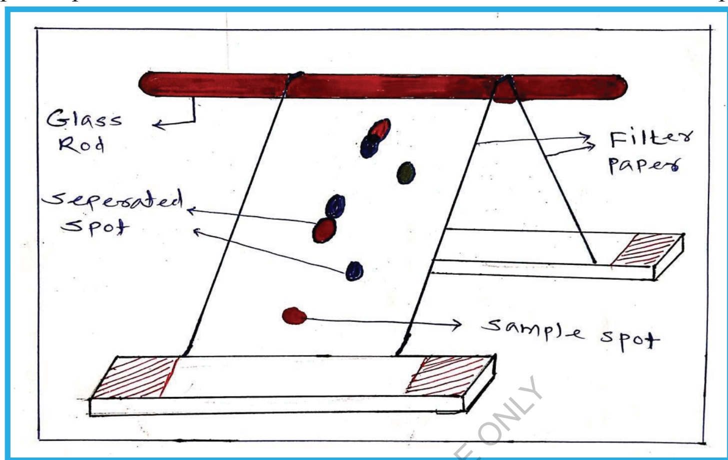



Fig:2.8 Ascending Descending hybrid Paper Chromatography

FOR AUTHOR USE ONLY

## 2.16 Advantages of Paper Chromatography:

1. It is very simple technique of separation.
2. The apparatus required are very simple and easily available.
3. The material or samples required are very less.
4. In calculation of Rf value of separated components or spots does not involves any sophisticated software or technique.
5. The technique is low cost.
6. For performing the experiments minimal training is required.
7. By this technique one can analyzed organic as well as inorganic samples.
8. It is cheaper method of analysis.
9. The space required by the equipment's of this chromatography is less as compared to other technique.
10. The method is cost effective.
11. The analysis of samples carry out within a short period of time.
12. No much maintenance and repairing is essential, because the assembly does not have any moving parts or delicate spare parts.

13. handling the setup of paper chromatography does not required much more skill and easy to handle.

## 2.17 Disadvantages of paper Chromatography:

1. Paper chromatography fails to separate the large quantity of samples.
2. Like the other chromatographic technique the data cannot saved for longer time.
3. In paper chromatography only qualitative analysis will be carry out instead of quantitative analysis.
4. The complex mixtures analysis cannot be carry out by this technique.
5. It fails to analyses the sample which is volatile in nature.
6. As compared to other technique it does not give the accurate analysis of samples.

## 2.18 Application of Paper Chromatography:

1. It is useful in analysis of food coloures in synthetic drinks and beverages.
2. It is useful in analysis of following class of compounds.
    - Alkaloids
    - Amino acids
    - Various Organic Acids
    - Proteins and peptides
    - Polysaccharides
    - Natural and Artificial Pigments
    - Inorganic Cations etc.
3. The technique is useful for analysis of mixture of sugars and mixtures of amino acids.
4. The technique is used for separation and identification of metals.
5. The technique is useful for detection of adulterants in various products.
6. It is useful in separation and analysis of pharmaceutical samples and drugs.
7. It is used for qualitative analysis of organic, inorganic and Biochemical samples.
8. It is used in the identification of contaminants in various drinks.
9. It is used in the identification of contaminants in various Foods.
10. It is used in analysis of cosmetic products.
11. The technique is useful in to determine dopes and drugs in humans and animals.
12. It is useful in analysis of food coloures in synthetic drinks and beverages.
13. In Pharmaceutical for getting information and development of new drug. molecule, Reaction completion and progress of manufacturing or production of drugs.

FOR AUTHOR USE ONLY

14. It is commonly used in monitoring of progress of reactions in industries ad research work.
15. It is commonly employed for the isolation and purification of components of various mixtures.

## 2.19 Paper Chromatography Experiment:

**Aim:** to separate the pigments present in the leaves by paper chromatographic technique

**Materials/Apparatus Requiered:** 1) Glass Jar 2) Rod or Rubber Cork 3) Test Tubes 4) Mortar and Pestle 5) Capillary Tube 6) Pencil 7) Scale 8) Dropper 9) Dryer 10) Filter Paper 12) Leaves

**Chemicals Required**:1) Distilled Water 2) Methanol or Acetone 3) Petroleum Ether 4) Developing Reagent

**Theory:** Paper Chromatography is one of the widely used techniques for separation of pigments present in plants leaves extracts. In this experiment, the water molecule present in pores of filter paper selected as stationary phase ad solvent such as acetone Toluene or mixture of solvents or Methanol- Water mixture etc as moving phase. The rate of movement of each component or substance can be represent by relative font or retardation factor ie. Rf Value Rf Value can be represented by following Formula

$$Rf = \frac{\text{Distance Traveled by the substance/ compound/Components from Reference line (cm)}}{\text{Distance Travelled by the solvent front from the reference line (cm)}}$$

Rf Value of different component/Compound are different though the solvent used are same

It is very easy to locate the coloured spot separate and in case of colorless spot some developing reagent required.

FOR AUTHOR USE ONLY

**Procedure:**

1. Select plant leaves of extraction of plant pigments.
2. Wash the leaves properly before taking extract.
3. Grind the leaves in mortar and pistle then transfer this extract or paste in test tube or small beaker or conical flask.
4. Then add small amount of solvent Methanol or Acetone in the leaves extracts or paste.
5. Cover the test tube or small beaker or conical flask containing extracts or paste and shake well.
6. Collect the filtrate in another test tube and cork it.
7. Select the Whatman filter paper of approximate size(4cmx17cm).

8. Draw a line at a distance of 3 cm from one end of filter paper by a pencil as shown in figure.
9. Take a capillary tube and dip in the leaves paste.
10. Put a spot A on filter paper above a base line as shown in fig and allow to dry.
11. Then hang or attached whatman filter paper strip having leaves paste spot A in the glass jar containing the mixture of petroleum ether and chloroform (19ml:1ml) keep the solvent just below the reference line marked on the jar.
12. Allow the mobile phase rise up 2/3 of the length of the filter paper.
13. Then remove the filter paper from the glass jar and mark the solvent front.
14. Now outline the separated component spot by pencil and allow the filter paper to dry.
15. Measure the distance travelled by solvent front and the center of different separated spot with respect to the reference line as shown in fig which is required for the calculation of Rf value using formula.
16. Now observe and identify the number of pigments present the leave extract.
17. Record your observation in the observation table and finely record your result of separated pigments leaves extracts and Rf Value.

FOR AUTHOR USE ONLY

## 2.20 Summary:

1. Paper chromatography, is a very simple technique in which the components of mixtures to be separated are dissolved in a special type of solvents and the solvents run along a special type of filter paper.
2. In paper chromatography, the stationary phase and mobile phases both are liquid in nature.
3. While the stationary phase is supported on cellulose fibers of filter paper where the capillary action is used to pull the solvents up through the paper and separate the solutes.
4. In paper chromatography, the components of mixture to be separated are distributed between the stationary phase and mobile phase.
5. In paper chromatographic technique, the Whatman filter paper no.1 is generally used.
6. Separated spot can be visualized by various techniques when separated components are colorless. Some spraying reagents are required to develop them into colored spots.
7. The apparatus required are very simple and easily available.
8. Paper chromatography is useful in separating amino acids anions and RNA. The technique in the fingerprinting, separating and testing histamines, antibiotics.

## 2.21 References:


1. Introduction to Modern Liquid Chromatography-L. R. Snyder, J. J. Kirkland and J.W. Dolan, 2009 3rd edition wiley Publication USA
2. Instrumentental Methods of Chemical Analysis-H.Kour,2010 Pragati Prakashan Meerut UP India
3. Analytical Chemistry-A.L.Gupta,2018 Pragati Prakashan Meerut UP India
4. Liquid Chromatography Fundamental and Instrumentation-S. Fanali, P. Haddad, C. Poole, P.Schoenmakers and D. Lioyd,2017 2nd edition Elsevier Publication
5. Chromatography Applied to Quality Testing-E.E.Pfeiffer,1984 kindle edition Bio-dynamic Literature USA
6. Chromatography Theory-J.C. Raymonds and P.W.Scott,2002 1st edition CRC Press USA
7. Handbook of Chromatography-Prof. A.P.Nikalje and Dr. Dileep Bhosle, 2015 Nirali Publication Pune, India


FOR AUTHOR USE ONLY

# Chapter 3
# Gas Chromatography

## 3.1 Introduction:

Gas Chromatography is one of the most important chromatography techniques in this, the mobile phases involved are gas. The stationary phase in this chromatography may be liquid or solid. But the detector used in both the technique is same. Depending on the basis of stationary phase involved gas chromatography are of two different types

1. Gas-Solid Chromatography
2. Gas-Liquid Chromatography

The stationary phase in gas solid chromatography is solid and it is coated on column used during detection of sample hence the name Gas-Solid Chromatography and in Gas-Liquid chromatography the stationary phase is liquid and mobile phase is gas.

Although the mobile phase involved in both technique is same but the separation of mixture takes place by two different principles. In Gas-Solid Chromatography, the separation of mixtures takes place by the principle of adsorption of different components present in mixture of sample. While in Gas-Liquid Chromatography, the mixture separated by partition principle as the component itself distributed between mobile gaseous phase and stationary liquid phase.

FOR AUTHOR USE ONLY

Charcoal and molecular sieves are the materials that are generally used as stationary phase In Gas-Solid Chromatography. Due to limited stationary phase available for Gas-Solid Chromatography, this technique less commonly used for separation. The technique is also limited to those substances which have low solubility in stationary phase charcoal and Molecular sieves. Due to above limitations of Gas-Solid Chromatography, the commonly used gas chromatography is Gas-Liquid Chromatography which discussed in details as follow.

Gas liquid chromatography is one of the modern computer assisted method of separation of mixture of sample proposed first by the Scientists in 1941 by Archer J. P. Martin and Richard L. M. Synge. This is the important technique of separation which performs both qualitative quantitative analysis of mixture of sample. Gas liquid chromatography is the type of partition chromatography in this technique, the mobile phase involve the gaseous state and stationary phase is high boiling point liquid coated as film on solid support column. In this technique of separation, the mixture after vaporization distributed on the basis of its solubility in stationary liquid phase and mobile gaseous phase. The mobile phase used in this technique are inert gases like helium, argon, nitrogen and sometimes hydrogen can also be used as mobile phase. The sample or mixtures to be separated are first vaporized under high temperature under

controlled condition in the column. The components after vaporization distributed between mobile gaseous phase and stationary phase liquid supported on solid column. The rate of separation of components of mixture depends on how much the components have affinity with both the phases.

## 3.2 Principle of Gas-Liquid Chromatography:

The separation of components in Gas Liquid Chromatography is based on the phenomenon of Partition of components in to two phases. The separation of mixture occurs by the phenomenon of partition of the components in to the two immiscible phases like in the paper chromatography. In this technique, the component distributed itself in two liquid phases where both phases are liquid in nature. The sample injected in the high temperature injector gets vaporized and the carrier gas helium moves the sample into the column. After that vapor phase of the mixture distributed itself between two different phases. The component of a mixture which is more soluble in liquid phase spend more time in column with stationary phase and those component which is less soluble in liquid stationary phase is move with the carrier gas and fall on the detector where the detector detect the nature or type of component present in the mixture of sample. Due to the difference in solubility of components of mixture into different phase the separation of mixture takes place. This distribution of components of a mixture into two different immiscible states is due to solubility of components into the particular state is known as Partition coefficient can be given by equation as follow.

$$\textbf{Partition coefficient (Kc)} = \frac{\textbf{Concentration of components in liquid phase}}{\textbf{Concentration of components in gas phase}}$$

FOR AUTHOR USE ONLY

In gas liquid chromatographic technique, the two terms involved on the basis of affinity of a particular components present in the sample with the particular phase.

(i) Retention Time and (ii) Retention volume.

## 3.3 Retention Time (Rt):

The components which have more affinity with the stationary phase delayed there time to fall on the detector during analysis. The time which is required by the components of mixture to fall on the detector to generate peak in a gas chromatographic column is known as Retention time. The peak generated by detector is the characteristics and unique peak of particular components present in the sample and area under the peak give the information about the quantity of particular components in the given sample. The characterization of mixture in unknown sample is done through retention time by comparing with those of reference compounds. Hence the gas liquid Chromatographic technique is important from the point of view of Qualitative and Quantitative analysis of

samples. The relative proportion of various components in a mixture is determined by calculating their peak areas and then calculating the percentage of peaks are out of the total area of various peaks obtained.

## 3.4 Retention volume (Rv):

Retention volume can be defined as the definite amount of volume of carrier gas required to carry a component maximum through the column to the detector is known as Retention volume. And can be given by the equation as

$$Rv = Rt\ Fc$$

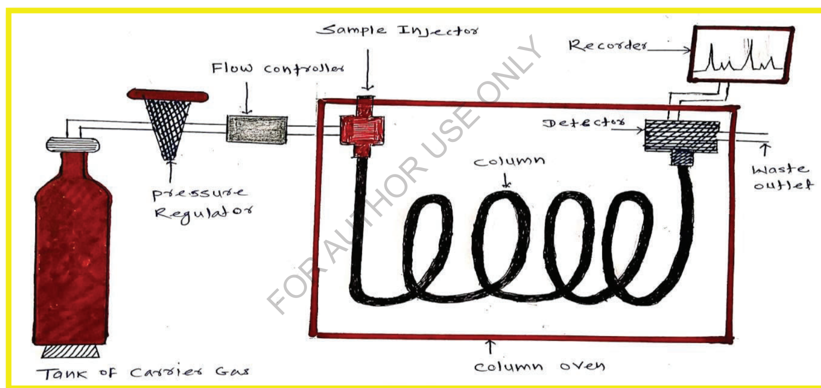
Where Fc is the volume flow rate of the gas at outlet.

Rv= **Retention volume**

Rt= **Retention Time**

## 3.5 Procedure for Analysis of Mixtures:

The schematic diagram of gas liquid chromatography is represented in the following figure.



FOR AUTHOR USE ONLY

**Fig:3.1 Gas-Liquid Chromatograph**

**The stepwise procedure is given below.**

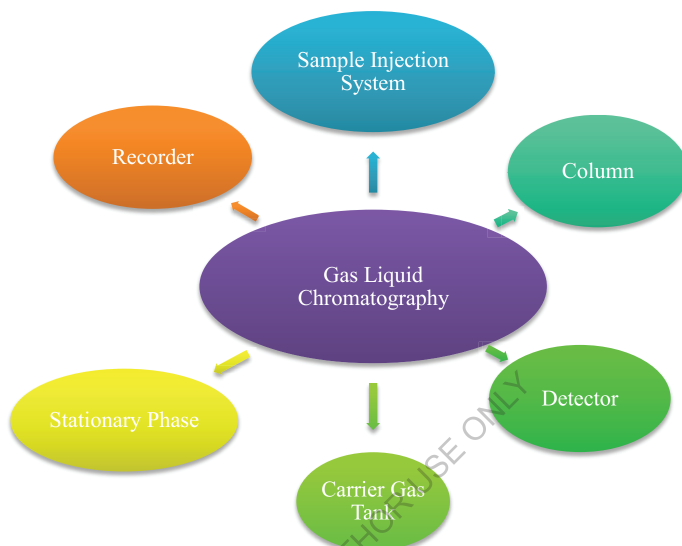
1. The equipment used in GLC technique is known as gas chromatograph.
2. The sample or mixture to be analyzed is injected into the sample injection system. Depending on the nature of sample the method of injections are different.
3. If the sample is liquid in nature, it can be injected a small amount of it by syringes through a septum made up of silicon.

4. If the sample is solid in nature, then it is first sealed into a small glass tube and smoothly placed into the sample injection system where the pressure of carrier gas burst the sealed tube and solid sample itself mix with the carrier gas stream.
5. The injector are fitted into a hot oven whose temperature are maintain by temperature controller, the temperature of injector system maintain as such that the sample boiled and converted into vapors.
6. The inert carrier gas caries the sample into the column after vaporization of sample.
7. The column is made up of sophisticated glass or stainless steel and it is about 2 to 4 meter long and internal diameter is about 2 to 4 mm it is fitted with coiled in oven in such a way that the temperature of oven can be controlled by controller.
8. The temperature of oven and column are different. Oven temperature is greater than column and in column it varies from 50 to $300^0$C and in injector oven temperature varies from 5 to $400^0$C.
9. The carrier inert gas flows from the carrier gas tank into the column continuously and then with regulated pressure passed itself to the detector.
10. The pressure of carrier gas regulated by flow regulator gauge.
11. Vaporized sample distributed itself into the mobile phase carrier gas and coated stationary liquid phase as per the principle of Partition and separation of mixtures takes place.
12. The component which is more soluble into the carrier gas mobile phase reaches first to the detector and chromatogram record the peaks of particular components and the generation of data begin into the data recording system.
13. The components or substance which less soluble in more soluble in stationary liquid phase required more times to fall on the detector due to its more retention time and it analyses later in gas chromatography.
14. Due to temperature regulator, by varying the temperature of column all the components are passed into the detector.
15. To obtain the clear separation peaks, it is necessary to vary the temperature of column regularly.
16. If the temperature of column maintain constant then the peaks obtained in recorder chromatogram merge together and it became hard to identify the peaks of a particular components
17. With increasing the temperature, the components which hold by stationary phase can also easily passed with carrier inert gas to the detector.

FOR AUTHOR USE ONLY

18. The recorder itself adjusts the peaks in Chromatogram depending on the nature of components whether components comes first or later to the detector.

## 3.6 Components of Gas Liquid Chromatography:




FOR AUTHOR USE ONLY

## 3.7 Carrier Gas or Mobile Phase:

In Gas Chromatography, the selection of the moving phase or carrier gas is based on fact that it is unreactive or neutral with the stationary phase as well as to the sample under analysis. For the purpose of carrier gas neutral or inert dried gas are generally selected. Helium is the most commonly used carrier gas in Gas-Liquid Chromatography. The Nitrogen, Argon, Hydrogen or mixtures of Nitrogen with hydrogen can also use as mobile phase.

## 3.8 Stationary Phase:

In Gas Chromatography, while choosing the stationary phase the two things are considered one is polarity of substance and temperature tolerance during operating process. The two types of solvents as Polar and Non Polar are generally in used. as per the concept of like dissolved like the polar solvents always dissolved the polar components of sample and vice versa. The non-polar solvents are generally hydrocarbons such as methyl silicons, sqaline and Apiezen greases. The polar solvents

contains the functional group hydroxyl, cyno and carbonyl groups to bind with components and retain with for more time the solute. The examples of polar solvent used in Gas-Liquid Chromatography are Polyethylene glycol and Polyester.

## 3.9 Column:

In Gas Liquid Chromatography, the column used during detection of sample is made up of either stateless steel or the glass material. The column length ranges of about 1 to 4 m long and 3 to 4 mm diameter. Depending on the nature of sample the packed column or open tubular column are employed. The packed column is shorter length but provide more space for mobile phase and separation of mixture became easy. Due to small internal diameter in capillary column is 0.25 to 0.50 mm. the packed column are generally used in gas liquid chromatography.

## 3.10 Detector:

To detect the components present in the sample Detector is the most important part of gas liquid chromatogram as it performs two function one to detects and second to sends the signal to recorder. If the gas chromatogram is connected to Mass spectrometer, then it becomes very important to select the detector which does not destroy the sample. There are various types of detector to use some of them listed below

1. Photo Ionization Detector
2. Gas Density Detector
3. Flame Ionization Detector
4. Thermal Conductivity Detector
5. Beta-Ray Ionization Detector
6. Glow Discharge Detector
7. Flame Temperature Detector
8. Dielectric Constant Detector

FOR AUTHOR USE ONLY

The commonly used detector in Gas-Liquid Chromatography is the flame ionization detector but it is not in general practice when the gas chromatography coupled with the Mass Spectrophotometer as it destroys whole sample in its flame of high temperature.

## 3.11 Applications of Gas Liquid Chromatography:

The gas liquid chromatography is the modern computer assisted technique of separation and identification of mixtures of sample from diverse field’s few of them are as follow.

1. The technique is useful to analyze the pesticidal residue from the foods and food products.

2. The technique is applicable in Pharmaceutical industry for identification of different chemicals in particular drugs.
3. Drugs residue from urine of patient.
4. Drug residue from Blood samples.
5. Some of the substance secreted by human body are volatile in nature such secreted substance can be identified by GLC technique which helps to find out diseases in human body.
6. GLC is useful in the determination of quality of air both qualitative and quantitative.
7. The technique is also useful in determining the various pollutants from soil and water samples.
8. The technique is useful in analysis of agricultural products.
9. GLC is useful in the determination of sample in agricultural industry both qualitative and quantitative.
10. GLC is also used in cosmetic industry for detection of chemicals in cosmetic industry.
11. The technique is also useful to researcher and manufacturer to identify and separate the different products formed during the progress of reaction.
12. In forensic department for analysis of quantity of alcohols present in the blood.
13. Toxic chemicals or poison present in the blood samples of patient also be analyzed by GC.
14. The GLC can also be used for separation of amino acids present in urine sample of patients.
15. It is useful in analysis and separation of carbohydrates and proteins.
16. The GLC can also apply in petrochemical industry for separation and identification of different hydrocarbons.
17. It is useful in analysis and separation of various vitamins from food sample and biological products.
18. The technique is useful in analysis and identification of food adulterants.
19. It is useful in separation and identification of different isomers of organic compounds.
20. It is useful in beverage industry to check the impurity present the various brands.

## 3.12 Advantages Gas Liquid Chromatography:

1. GLC required in very few quantity of sample for analysis.
2. The time required for analysis is very short.

FOR AUTHOR USE ONLY

3. By GLC the sample can be analyzed qualitatively and quantitatively this is the main advantage of this chromatography.
4. If TLC is attached to Mass spectroscopy then it also gives the information of molecular weight of different components present in the mixture.
5. The technique very much sensitive to various samples.
6. The technique provides the accurate information of sample.
7. The compounds with lower boiling point can also be analyzed.
8. The gas chromatography can analyzed all states of compounds such as solid, liquid and gaseous state of mixture can be analyses.
9. The process of analysis is very simple and operation of technique is very easy.
10. The system is automated.
11. It can analyze heavy organic complex compounds.
12. The power consumption is very low for operation of Equipment.
13. It does not give any peaks of stationary phase and mobile phase in chromatograph as they are not detected.
14. The method is very much selective.

## 3.13 Disadvantages of Gas-Liquid Chromatography:

1. By this technique only volatile sample can analyzed this is the main limitation.
2. The semi-volatile components of mixture cannot be detected.
3. The technique is not suitable to the compounds which are temperature sensitive.
4. To operate the instrument the training of person is necessary.
5. It does not provide the information of behavior of components in mixture.
6. Maintenance of equipment is costly, as sensor are moisture sensitive.
7. The sensor is not fully specific about the sample.
8. Frequent calibration of equipment is required.
9. Regular variation of temperature at detector and strong electrical fields can affects the instrumental signal.

FOR AUTHOR USE ONLY

## 3.14 Summary:

1. In this stationary phase used in chromatography can be liquid or solid substance.
2. In the Gas-Solid chromatography, when the substance coated on the column is solid which acts as stationary phase hence the name Gas-Solid Chromatography.
3. In Gas chromatography the mobile phase is gas and stationary phase use is liquid in nature it is Gas-Liquid chromatography.
4. In any type of gas chromatography techniques, the detector used are same.
5. Gas liquid chromatography is the type of partition chromatography in which unlike paper chromatography the mobile phase involve the gaseous state and

6. Stationary phase is high boiling point liquid coated as film on solid support column.
7. The gas liquid Chromatographic technique is important from the point of view of Qualitative and Quantitative analysis of samples.
8. The mobile phase used in this technique is inert gas such as helium, argon or Nitrogen in pure forms.

## 3.15 References:


1. Basic Gas Chromatography-H. M. Mcnair, J.M. Miller and N. H. Snow,2019 3rd Edition Wiley Publications USA
2. Gas Chromatography-C.Poole,2012 1st edition Elsevier publication Netherland
3. Gas Chromatography Analysis, Methods and Practices-V.Warren, 2017 Nova Science Publishers USA
4. Columns for Gas Chromatography performance and Selection-E.F.Barry and R.L.Grob,2007 viley Publication USA
5. Modern Analytical Chemistry-A.K. Gupta, 2015 Pragati Prakashan Meerut UP India
6. An Introduction to Chromatography-H.Kaur,2010 Pragati Prakashan Meerut UP India


FOR AUTHOR USE ONLY

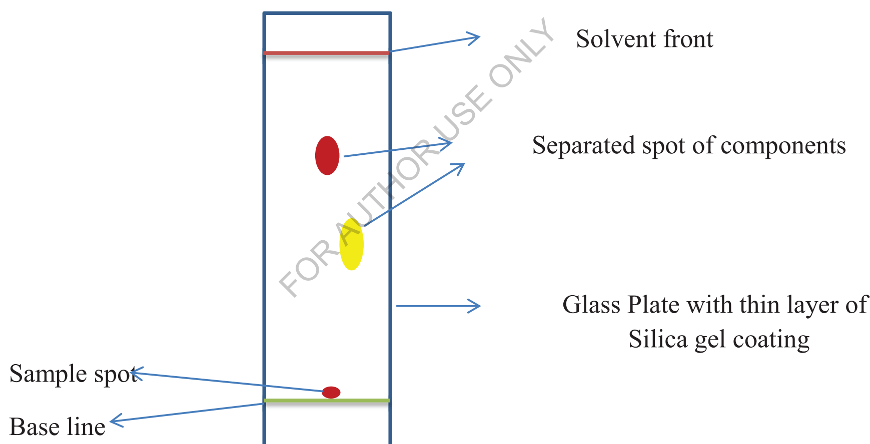
# Chapter 4
# Thin Layer Chromatography

## 4.1 Introduction:

It is a solid-liquid based method in which a solid phase acts as stationary phase and a liquid phase acts as moving phase. It is commonly applied method of identification by researchers and pharmacists to check out the progress of reactions. It is the type of adsorption chromatography in which the solid layer is used as stationary phase and liquid or gas can be used as mobile phase. Depending on the type of mobile phase used thin layer chromatography is of two types

1. In TLC, if the mobile phase involved is liquid then it is known as liquid-liquid Chromatography
2. In TLC, if the mobile phase is Gas then it is known as Gas Solid Chromatography.

In this type of Chromatography, generally solid phase of silica gel or alumina is used.



FOR AUTHOR USE ONLY

**Fig: 4.1 thin layer silica gel on glass plate with separated spots**

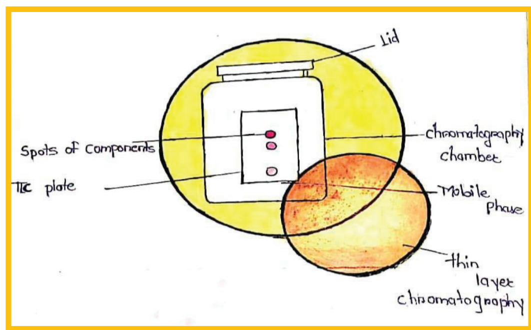



**Fig: 4.2 thin layer chromatography chamber**

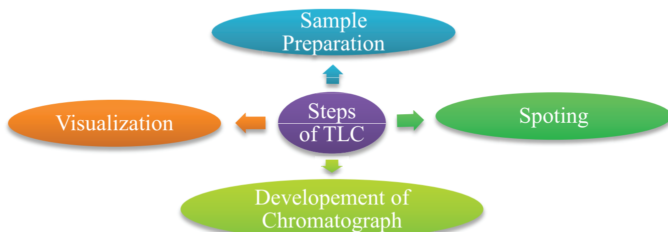
## 4.2 Principle of thin layer chromatography:

In thin Layer Chromatography, the components of sample separated on the basis present in the sample are separated on the basis how more the particular component adheres on to the solid stationary phase. The components which remained more stick to the stationary phase will separate last and the component which have remain less stick to thin layer of plate or stationary phase separate first. As the components which adsorbed less on the thin layer solid stationary phase, it became easy for liquid mobile phase to carry along with it the component by this principle the separation of different components present in mixture are separated. After the sufficient distance travelled by components of mixture develops as spot on the TLC plate. If the developed spots are coloured then it can be identified by naked eyes, but if the spots are colorless then it can be identified after development of color of spot after the application of suitable methods.

FOR AUTHOR USE ONLY

## 4.3 Steps involved in Thin Layer Chromatography:

In thin layer chromatography, following four steps are involved




In the first step, the sample to be analyzed is dissolved in such solvent which is easy to evaporate and to produce very dilute solution. While in second step, spotting by a

micropipette for putting a small amount of the dilute solution of sample to one end of thin glass plate. The spotting is allowed to dry and evaporate solvent leaves behind small spot of the sample selected.

In third step, development of spotted sample is done by keeping the thin layered plate into glass container consist of developing solvent and the sample travel up along with solvent. The moving of spotted sample varies with solvent and spotted sample. The solvent moves almost to the top of the plate and spot moves as per the its propertis on to the plate. The solvent front should be marked by pencil after removing the plate from the jar with solvent. Then, solvent is allowed to evaporate

In final step, the separated spot of sample is visualized. It is very simple to observe the colour of components, but very difficult to observe in case of colourless components. In this some techniques are adopted such as impregnation of fluorescent agents which glow under UV light or use of Iodine is also employed.

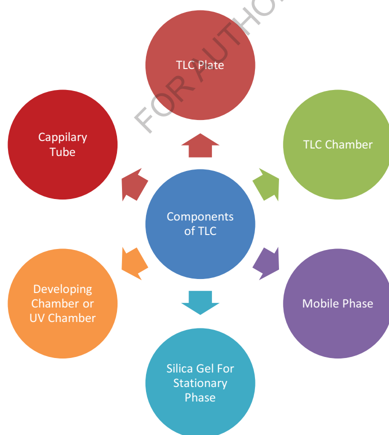
Rf Value:

It is calculated by using following formula

$$\mathbf{Rf} = \frac{\textbf{Distance Traveled by the sample spot}}{\textbf{Distance Traveled by the Solvent}}$$

## 4.4 Components of Thin Layer Chromatography:

FOR AUTHOR USE ONLY




In Thin layer chromatographic technique, the TLC plates are easily prepared in laboratory by applying the thin slurry of silica gel in suitable solvents or on the foil of alumina. The plates are generally made up of glass or it may be foil of alumina or the sheet of plastics can also be used as TLC plate. The solvent generally used for

preparation of slurry is the Chloroform. The various adsorbent material are also available and it includes hydrated gel silica, cellulose microcrystalline, alumina or modified silica gel such as silica gel G, Silica gel H, silica gel N, silica gel S can be used. The running solvent or mobile phase used in TLC may be single pure solvents or it may be a mixture of two solvents. The solvent mixture for mobile phase includes pure n-Hexane and Pure Ethyl acetate. The proportion of mixture of running solvent or mobile phase may be 30:70 or the proportion may vary depending on the polarity of sample to be analyzed. Instead of Ethyl Acetate the other solvents includes Ethanol, Methanol, Acetone or Chloroform. For application of spot of sample the sample dissolved first in a specific solvent. The solvent used for dissolving the sample must be volatile in nature as after the application of spot it can easily evaporated leaving behind only the spot of sample of analysis. For calculation of Rf Value and location of colourless spot the TLC plate put in the chamber containing pure Iodine, as iodine is most reactive towards organic compounds develops coloured complex due to which the naked eyes can easily identify the spot of components on developed TLC plate.

## 4.5 Applications of Thin Layer Chromatography:

1. It is very important tool in determining the number of components present in given mixture.
2. It also helps researcher & Pharmacists to monitor the progress of various chemical reactions.
3. To determine the identity of component present in mixture this is under investigation or analysis.
4. It is employed for purification of various samples.
5. Is useful in isolation of various biochemical metabolites, blood Plasma, Serum, Urine etc.
6. Separation of multicomponent pharmaceutical formulations.
7. It is useful in food Industry.
8. It is useful in cosmetic Industry.
9. It is useful in preservative and sweetening agent manufacturing units.
10. It is highly employed in phytochemical investigations.
11. In plant biotechnology for different clones of the same gene can also be compared to see check any variation between the clones
12. It is useful in determining the Pesticides & Insecticides.
13. It is useful in detecting by products in synthetic processes related to research and manufacturing processes.
14. It is also useful in the study of alkaloids, Steroids, Vitamins etc.

FOR AUTHOR USE ONLY

15. The TLC used to identify and separate the natural products like alkaloids, essential oils, glycoside and different types of waxes.
16. TLC also has important application in medicine for qualitative analysis of drugs such as hypnotics, analgesics, antihistamines, steroids, sedatives and local anesthetics.

## 4.6 Advantages of Thin Layer Chromatography:

1. It is very important method and can be applied to various organic and inorganic compounds for their identification and separation
2. By using this technique identification & separation can be done quickly.
3. The cost of maintenance & repairing is very low.
4. It does not require any software and delicate costly instrument.
5. Acidic and other strong chemical agents can be used as spraying agent on thin layer of Chromatoplate without any change.
6. In this technique the diffusion is minimum.
7. This technique requires small amount compounds mixture or substances to be analyzed.
8. The plate can be operated at somewhat high temperature compared to paper Chromatography.
9. It is very sensitive method as compared to some other methods.
10. The non-volatile compounds or substances are separated by this technique.

FOR AUTHOR USE ONLY

## 4.7 Disadvantages of TLC (Limitations):

1. In this method, only soluble components of the mixture to be analyzed are possible to separate.
2. It is only applicable for qualitative analysis of the mixture of components.
3. Generally TLC used is on automatic in nature, it is manually operated by researchers and person involved in analytical work.
4. In this technique, the results obtained during one analysis are difficult to produce for the same sample.
5. It is operated in open system and therefore the result can be affected by temperature & humidity factor.
6. The stationary phase in TLC is not remaining longer.
7. The quantitative analysis of sample cannot be done by Thin Layer Chromatography it is limited for qualitative analysis only.

## 4.8 Summary:

1. It is a solid-liquid based method in which a solid phase acts as stationary phase and a liquid phase acts as moving phase.
2. In TLC, if the mobile phase involved is liquid then it is known as liquid-liquid Chromatography
3. In TLC, if the mobile phase is Gas then it is known as Gas Solid Chromatography.
4. Samples are separated on the basis how more the particular component adheres on to the solid stationary phase.
5. If the developed spots are coloured, then it can be identified by naked eyes, but if the spots are colorless, then it can be identified after development of color of spot after the application of suitable methods.

## 4.9 References:

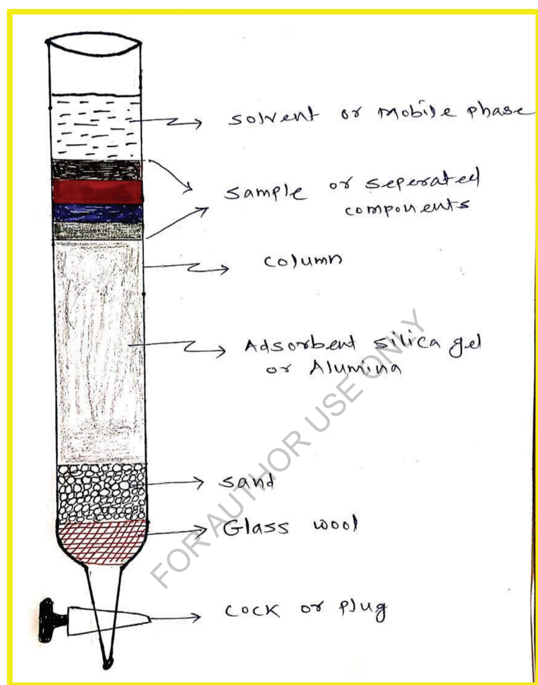

1. A Handbook of Thin Layer Chromatography- J. Sherma ad B. Fried,2003 CRC Press USA
2. Instrumental Thin Layer Chromatography-C. Poole,2014 1st edition Elsevier publication Netherland
3. Practical Thin Layer Chromatography-B.Fried and J. Sherma,1996 CRC Press USA
4. Thin Layer Chromatography- E. Stahl,1965 1st edition Springer-Verlag Berlin Heidelberg
5. Handbook of Analytical Instruments-R.S. Khandpur,2006 2nd edition McGraw Hill education
6. Quantitative Thin layer Chromatography a practical Survey-B. Spangenberg, C. Poole and C. Weins,2011 1st edition Springer-Verlag Berlin Heidelberg
7. Practice of Thin Layer Chromatography-J.C. Touchstone,1992 3rd edition wiley publication USA
8. Thin layer Chromatography-R. Hamilton and S. Hamilton,2008 wiley india Pvt Ltd


FOR AUTHOR USE ONLY

# Chapter 5
# Column Chromatography:

## 5.1 Introduction:

This is one of the simple types of Chromatographic techniques which is employed for purifying compounds or isolate a single chemical compound from a mixture to be analyzed.



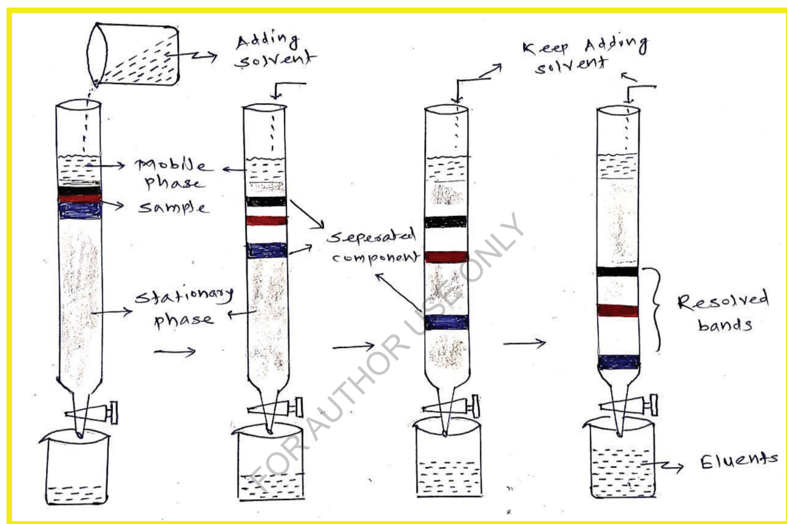
FOR AUTHOR USE ONLY

**Fig: 5.1Column Chromatography**

It consists of two phase: one mobile phase and another stationary phase. The column is filled or prepared by mixing the adsorbent silica gel with suitable solvent which is mobile phase. The solvent used is moved along mixture of components through the glass column as per their polarity property.

## 5.2 Principle of Column Chromatography:

The column chromatography principle is based on adsorption of the solutes of solution through a stationary phase and separates the mixtures into individual compounds or components. This works on affinity between the mobile phase and stationary phase involved in column chromatography. The components which have more affinity towards stationary phase remained adsorbed and separate letter than those components which have less affinity towards stationary phase. The components which adsorbed less on the stationary phase can traveled down easily towards gravity with mobile phase and separate first.



FOR AUTHOR USE ONLY

**Fig: 5.2 separation of mixture via different steps**

## 5.3 Types of Preparation of column chromatography or types of column packing:

Types on the basis of procedure to prepare the column by packing adsorbent silica or alumina

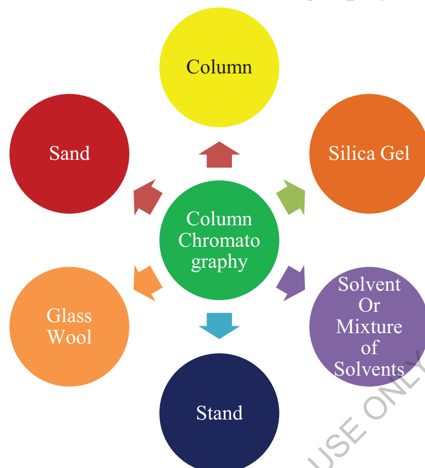
### 5.3.1 Dry Method

In this method, the column is filled with dry powdered adsorbent such as silica or alumina. Then the mobile phase solvent is poured from the top of glass column till the complete silica gel wet and properly settled in the column

### 5.3.2 Wet Method:

In this method slurry of adsorbent silica and selected solvent is prepared And then it is poured into glass column. It is suggested to use more solvent so as to allow silica to settle properly.

## 5.4 Components of Column Chromatography:




FOR AUTHOR USE ONLY

## 5.5 Experimental Procedure of Column Chromatography:

1. Before starting the experiment select the proper solvent with proper polarity as per the sample to be separated as mobile phase by performing the TLC of sample.
2. After selecting the solvent either single or mixtures prepare the slurry of silica gel in beaker or conical flask.
3. Place the glass wool at the bottom of column to pack properly the stationary phase.
4. Place the some amount of sand over the glass wool or cotton wool.
5. Clamp the required size column in vertical position to the stand.
6. Fill up the column with slurry of silica gel by pouring the from upper opening end of the column
7. Add more amount of solvent or mixture of solvent in to the beaker to collect all the silica gel slurry in to the column.
8. Open the cock gently; the solvent move downward and the stationary phase packed in the column uniformly.
9. Prepare the sample for analysis by dissolving the sample in the solvent in which the slurry of silica gel is prepared.

10. Attached the funnel at the upper opening of the column
11. Pour the sample through the funnel in to the column containing the slurry of silica gel.
12. Open the cock and allow the solvent to pass through the layer of sand.
13. Add the solvent or mixture of solvent from the funnel without disturbing the stationary phase and allow standing.
14. After some time depending on the polarity there is separation of colour band or layers occur.
15. The less polar component move down first due to gravity by opening the cock of column collect the fraction in clean beaker or test tube.
16. Collect all the components by repeated addition of solvents from the funnel to collect each component in separate beaker or test tube.
17. After collecting each of the components evaporate the solvent by rotary evaporator to get the pure components.

## 5.6 Types of Column Chromatography:

There are five basic type of column chromatography are commonly used

1. Adsorption Chromatography
2. Partition Chromatography
3. Ion Exchange Chromatography
4. Affinity Chromatography
5. Exclusion Chromatography

FOR AUTHOR USE ONLY

In case of adsorption method, on solute may be adsorbed selectively absorbed on adsorbent which is stationary phase from a mixture of component. The separation of the components will depend on the differences in their degree of adsorption and solubility in the selected solvent.

In case of partition chromatography there is partitioning of components between a liquid stationary phase and a liquid mobile phase. The liquid which is stationary phase can be held on any solid support.

In case of ion exchange, the attraction between oppositely charged particles takes places. The net charge carried by them depends on their Pka and on the pH of the solution. In this method cation and anion exchangers are used

In case of affinity chromatography the specific Biological interaction of the compounds to be separated with special molecule which is attached on the stationary phase. These special molecules are called as ligands.

The exclusion chromatography involves the separation of molecules on the basis of their molecular size and shape. Also on the molecular sieve properties, in case of porous solid material.

## 5.7 Factor's affecting separation in column Chromatography:

The efficiency of column chromatography is mainly governed by separation process. Some of the important factors affecting separation are discussed below.

1. Nature of column and its dimension: the efficiency of the column improves with increase in dimensions i.e. Length:Width ratio. The ideal dimension are in the 10:1 to 100:1
2. Temperature of the column play important role and generally operated at room temperature only. Because as temperature of column increases adsorption reduces and elution speed cease.
3. The poor quality of adsorbent decreases the efficiency of column
4. The particle size of adsorbent used should be small to improve the adsorption process in the column
5. The packing of adsorbent by dry or wet process should be uniform and even, otherwise it affects efficiency
6. The solvent purity and quality also affects the efficiency of column
7. The efficiency of separation enhances when high pressure on the column and low pressure under the column.

FOR AUTHOR USE ONLY

## 5.8 Application of Column Chromatography:

It is versatile technique commonly used in various field such as academic, research, Pharmaceuticals and analysis of various substances required in many fields for the welfare of human being. Some of the important applications are discussed below.

1. It is used in the separation of the mixtures of compounds
2. It is useful in phytochemical study such as alkaloids, glycosides, plant extracts etc.
3. It is employed in the purification of components, mixtures, compounds etc.
4. It is helpful in estimation of the drugs in the pharmaceutical formulations
5. Metabolites can be separated from biological fluids by this method.
6. It is helpful for removing the impurity present in desired product
7. The method is usefule to separate the side products formed during synthesis of various products
8. It is helpful in isolation of isomers.
9. It is used in pharmaceutical for purification of biomolecules such as protiens and hormones

10. It is useful in extraction of pesticides residue from different food materials
11. It is useful in isolation and identification of inorganic ions.
12. It is usefule in isolation and purification of vitamins.
13. It is useful for determination of phytomenadione in tablets and injections.
14. Useful in purification of compounds isolated from plants.
15. Preparation of sample for further analysis.
16. The method useful for extraction of lanthanides and actinides elements.
17. The technique is useful in homogenization of substance containing different color impurity

## 5.9 Advantages of column Chromatography:

1. It is low cost analytical method.
2. It is very simple to handle and operate
3. All types of simple and complex mixtures of compounds or components can be separated
4. Any amount of mixture can be separated
5. It has a broad range of mobile phase
6. In this method analytes can be separated and reused.
7. It is employed for removal of impurities
8. There is wider choice of mobile phase.
9. It is flexible process.
10. In this method both solid and liquid sample can be analyzed
11. The process can be applied at laboratory scale to commercial or large scale.
12. The separated analytes can be reused in this method

FOR AUTHOR USE ONLY

## 5.10 Disadvantages of Column Chromatography:

1. It is time consuming technique to separate the components of mixture.
2. It requires more quantity of solvents.
3. Automation of this method is more tedious and costly.
4. The method required technical skill.
5. Air entered into the stationary phase and mobile phase then crack appear in adsorbent layer
6. The complete attention required till the experiment end.
7. The separation power of this method is low

## 5.11 Summary:

1. This is one of the simple types of Chromatographic techniques
2. It consists of two phase: one mobile phase and another stationary phase
3. The mobile phase is single solvent or mixture of solvents

4. Stationary phase used in this technique is silica gel or alumina
5. The separation of mixture takes place on the basis of principle of adsorption
6. The little quantity of sample can be analyzed by this method
7. The technique has diverse application in various fields.

## 5.12 References:


1. Dynamics of Chromatography-Principles and Theory-J. C. Giddings,1965 1st edition CRC Press USA
2. Ion Chromatography Application-R.E. Smith,1987 CRC Press USA
3. Advances in Chromatography-N.Grinberg and P.W. Carr,2017 1st edition CRC Press USA
4. Analytical Chemistry A Chemist and Laboratory Technicians Toolkits-B. H. Ham and A. M. Ham,2015 Wiley Publication
5. Analytical Chemistry for Techicians-K Kenkel,2014 4th edition CRC Press USA
6. Microscale organic laboratory : with multistep and multiscale syntheses. D. W. Mayo, R. M. Pike and D. C. Forbes, 2013 6th edition J. Wiley & Sons Hoboken
7. Purification of laboratory chemicals. W. L. F. Armarego and C. L. L Chai, 2009 6th edition Butterworth-Heinemann Amsterdam, Boston


FOR AUTHOR USE ONLY

# Chapter 6
# High Performance Liquid Chromatography (HPLC)

## 6.1 Introduction:

It is one of the versatile analytical techniques used to separate, identify or quantify components present in a mixture. In this method, the components in mixture separation are done on the basic principle of column chromatography. The main difference between the column chromatography and HPLC is that, in column chromatography the mobile phase is moved towards the gravity by itself under the atmospheric pressure or no any external pressure applied for separation. But due to time consuming process of column chromatography and from the point of view of accuracy of separation, the technique is automated and as the name indicates the solvents are allowed to flow under the applied external high pressure in column. The process of identification of components and also the quantification is done by spectroscopic studies. It is improved version of column liquid chromatography and it is automated in nature.

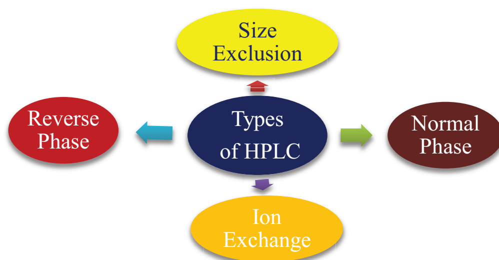
The technique of HPLC also can be called as modern methods of separation and identification of mixture. It is fast method as compared to low pressure column chromatography used conventionally. In conventional method the solvents are allowed to flow as per the gravity. It is time consuming and operator can continuously observe the separation of layer of components. In case of HPLC, the solvents are allowed to move fast by applying the pressure by pump. In this case the data automatically recorded by computer within a very short period of time. The data obtained can be identifyied after working procedure at any time. The HPLC instruments are automated; therefore the identification or separation of samples became easy by using the HPLC instruments.

There are two phases involved such as stationary phase and mobile phase similar to other chromatographic techniques. The stationary phase employed is a granular porous material, while mobile phase is a solvents or mixture of solvents which is forced at high pressure through the separation column present in the assembly.

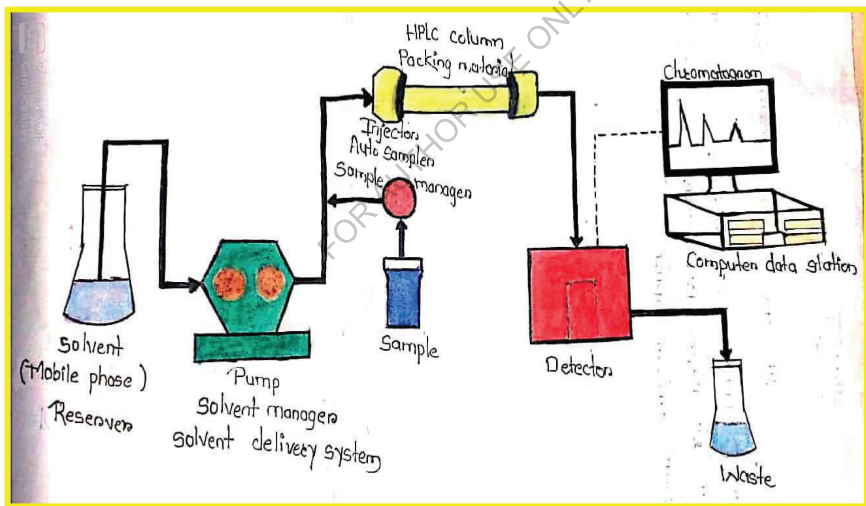
FOR AUTHOR USE ONLY

## 6.2 Types of HPLC:

This technique commonly used to get accurate results. There are four types of HPLC on the basis of column packing and diffusion of molecules



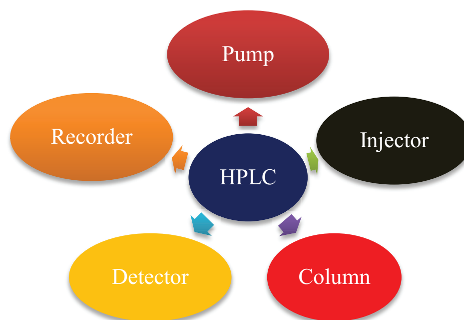

The difference between normal phase and reverse phase is only the polarity of phases used in fist case for the stationary phase the column is packed by using polar material silica whereas non-polar solvent as mobile phase. In case of reverse phase, as the name reverse non-polar material is used as stationary phase and it is packed in the column such as C18 while mobile phase is water and miscible solvent which is polar in nature. In ion exchange technique, column packing is done by using ionic group and the buffer is used as mobile phase. In size exclusion process, a molecule diffuses into pores of porous medium and they are separated as per their relative size to the pore size.




FOR AUTHOR USE ONLY

**Fig: 6.1 High Performance Liquid Chromatography**

## 6.3 Components of HPLC:




## 6.4 Experimental Procedure of HPLC:

1. Before starting of experiment keep the equipment turn on for fifteen minutes & be sure that the pump column and detector are in turn on position
2. Turn on the recorder equipment such computer and printer.
3. As you start the computer and HPLC equipment's, read the message display on the window as per the manual and follow the procedure for each component of HPLC instruments.
4. Set up the pump flow rate as per the instruction shown on display and select the proper solvent for mobile phase.
5. Dissolved the mixture or sample into suitable solvent.
6. Fill up the syringe for injection of sample into injector.
7. After the injection of sample into injector, pressure of flow rate of sample must adjust by pressure knob.
8. As the time passes, the sample pass through the column & components are separated as per the principle of adsorption.
9. The components reach on the detector; it detects the particular kind of components and passes the information to recorder system.
10. The computer start writing the peaks for each components against the retention time
11. The software itself integrates and calculates the area of each components peak.
12. Take the print out of the data recorded and identify the components present in mixture of sample under analysis.

FOR AUTHOR USE ONLY

## 6.5 Applications of HPLC:

1. It is very versatile, accurate and widely accepted chromatographic technique & therefore used in chemistry, Biochemistry, Biological Sciences, Biotechnology, Pharmaceutical Sciences etc.
2. It is useful in analysis of various pollutants in environmental pollution control studies.
3. It is employed in isolation of variety of valuable products.
4. It is used in quality control section of industry for checking purity and quality of products generated in industry.
5. Coenzymes, nucleic acid Separation and purification can be done by this technique.
6. It is employed for the analysis of various drugs.
7. This technique is useful in water purification.
8. This technique also employed in field of clinical separation
9. It is applied for molecular weight determination.
10. HPLC is useful in food technology and consumer product development.
11. It is useful in identification of bile acid metabolites from the sample of human
12. The technique is useful in forensic department for determination of toxic chemicals in samples
13. The technique is also useful in determination of chemical structure.
14. It is useful in the synthetic process for identification of intermediate and its quantity.
15. It is useful in polymer, paints and chemical industry.
16. Separation and identification of natural products can be done by HPLC.
17. It is useful for determination of quantity of banned drug from urine and blood samples
18. It is useful in determination of phenolic compounds from the water samples

FOR AUTHOR USE ONLY

## 6.6 Advantages of HPLC:

1. It is fast and absolute method to identify specific chemical components.
2. This method employed the pump to force liquid phase to solid adsorbent phase instead of working on gravity.
3. This technique is reproducible and highly accurate.
4. It is automated chromatographic method
5. It can be run by a person on minimal short training.
6. In identifying and quantifying chemical components, it is versatile and precise technique

7. This technique has good efficiency.
8. By this technique sample can be analyzed both qualitatively and quantitatively.
9. In this technique the column used can be reusable without any process.
10. The multiple component analysis can be done by HPLC.
11. Complex mixture can analyzed within short period of time by this technique.

### 6.7 Disadvantages of HPLC:

1. It is costly method than other chromatographic methods because of requirement of large quantity of solvents.
2. Due to irreversible adsorption some compounds cannot be detected by this method.
3. Solution makes difficulty in determining exactly which part of the sample is eluted in case components having nearly same time.
4. Some components of sample bind with the beads shortly in this technique; it is makes trouble to realize them.
5. It has low sensitivity for certain compounds.
6. It is very hard to operate for the new beginner.
7. It is less efficient than the gas chromatography.
8. The instruments required specific detector which is not easily available.

FOR AUTHOR USE ONLY

### 6.8 Summary:

1. In this method, the separation of components in mixture is done on the basic principle of column chromatography.
2. The process of identification and quantification of components is done by spectroscopic studies.
3. There are two phases involved such as stationary phase and mobile phase similar to other chromatographic techniques.
4. This method employed the pump to force liquid phase to solid adsorbent phase instead of working on gravity.
5. It has low sensitivity for certain compounds.

## 6.9 References:

1. Practical HPLC Methodology and Applications-B.A. Bidlingmeyer,1993 Wiley Publication, Hoboken USA
2. High Performance Liquid Chromatography Fundamentals Principles and Practice-W. J. Lough and I. W. Wainer,1995 Springer Publication New York City
3. Textbook of Pharmaceutical Analysis- Dr. S. R. Sankar,2010 4th edition RX Publication, Aurangabad, Maharashtra, India

4. Instrumental Methods of Chemical Analysis-G.R.Chatwal and S.K.Anand, 2014 Himalaya Publishing House, Mumbai India
5. Introduction to Chemical Analysis-R.D. Broun, 1982 McGraw Hill USA
6. Modern HPLC for Practicing Scientists- M.W.Dong, 2006 Wiley–Blackwell, USA
7. A Practical Handbook of Preparative HPLC-D.A.Wellings, 2011 1st edition Elsevier science publication, Amsterdam Netherlands
8. HPLC Columns : Theory, Technology, and Practice-U.D.Neue, 1997 1st edition Wiley-VCH Publication USA
9. Practical High-Performance Liquid Chromatography-V.R.Meyer,2010 5th edition John Wiley & Sons, Ltd USA
10. HPLC in Pharmaceutical Industry-G.W.Fong and S.K. Lam,1991 1st edition CRC Press USA

FOR AUTHOR USE ONLY

FOR AUTHOR USE ONLY

FOR AUTHOR USE ONLY

More Books!

yes
I want morebooks!

Buy your books fast and straightforward online - at one of world's fastest growing online book stores! Environmentally sound due to Print-on-Demand technologies.

Buy your books online at
**www.morebooks.shop**

Kaufen Sie Ihre Bücher schnell und unkompliziert online – auf einer der am schnellsten wachsenden Buchhandelsplattformen weltweit! Dank Print-On-Demand umwelt- und ressourcenschonend produzi ert.

Bücher schneller online kaufen
**www.morebooks.shop**

KS OmniScriptum Publishing
Brivibas gatve 197
LV-1039 Riga, Latvia
Telefax: +371 686 204 55

info@omniscriptum.com
www.omniscriptum.com

OMNIScriptum

FOR AUTHOR USE ONLY

FOR AUTHOR USE ONLY