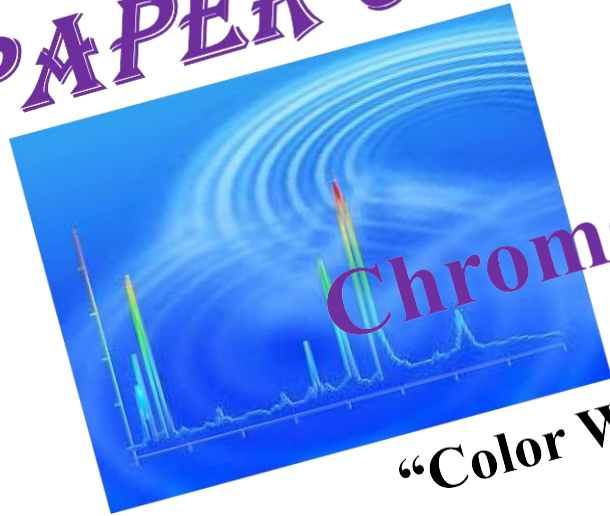


PAPER CHROMATOGRAPHY



Chroma-Graphein

“Color Writing” – Greek Word

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CONTENTS

- **Introduction**
- **Principle of Separation**
- **Modes of Paper Chromatography**
- **Experimental Details for Qualitative Analysis**
- **Experimental Details for Quantitative Analysis**
- **Applications**

INTRODUCTION

- ❖ The most dramatic advance in the history of chromatography took place in 1944.
- ❖ It was invented by two **British** biochemists, Archer John Porter **Martin** and Richard Laurence Millington **Synge**.
- ❖ In **1941** **Martin and Synge** began working together on proteins, which are made up of chains of amino acids.
- ❖ The duo was trying to characterize a particular protein by determining the precise numbers of each amino acid present.

- ❖ Amino acids are so similar to each other, however, that the problem of separating them had defeated a whole generation of biochemists.
- ❖ **Martin and Synge's development of paper chromatography** to solve this problem was an instant success.
- ❖ It worked not only on amino acids but also on various other mixtures. The **two scientists** were awarded the **1952 Nobel Prize** in **chemistry** for their work.
- ❖ Synge determined the structure of an **antibiotic** peptide called "**Gramicidin-S**."

- ❖ **Frederick Sanger** used paper chromatography to figure out the **structure of the insulin molecule**. He determined the number of amino acids in it as well as the order in which they occurred.
- ❖ **Calvin** discovered the **complex series of reactions** that enable **green plants to convert solar energy into the chemical energy stored** in food using paper chromatography in 1950s.

- ❖ It was also used by Austrian-American biochemist **Erwin Chargaff**, who modified the technique to **study the components of the nucleic acid molecule**. His research revealed four components, or nitrogenous bases, that occur in pairs.

- ❖ British biochemists **James Dewey Watson** and **Francis Harry Compton Crick** later used these results to work out the structure of **DNA (deoxyribonucleic acid)**.

There are **two types of paper chromatography**, they are:

PAPER ADSORPTION CHROMATOGRAPHY

Paper **impregnated** with silica or alumina acts as adsorbent (stationary phase) and solvent as mobile phase.

PAPER PARTITION CHROMATOGRAPHY

Moisture/ Water present in the **pores of cellulose fibers** present in filter paper acts as stationary phase and solvent used as mobile phase.

PRINCIPLE OF SEPARATION

- ❖ The **principle** involved is **partition chromatography** wherein the substances are distributed or partitioned between liquid phases. One phase is the **water**, which is held in the **pores of the filter paper** used; and other is the mobile phase which moves over the paper.
- ❖ The compounds in the mixture get **separated due to differences in their affinity** towards water (in stationary phase) and mobile phase solvents during the movement of mobile phase under the capillary action of pores in the paper.

Principle of separation Cont.,

- ❖ The principle can also be adsorption chromatography between solid and liquid phases, wherein the stationary phase is the solid surface of paper and the liquid phase is of mobile phase.
- ❖ But most of the applications of paper chromatography work on the principle of partition chromatography, i.e. partitioned between two liquid phases.

MODES OF PAPER CHROMATOGRAPHY

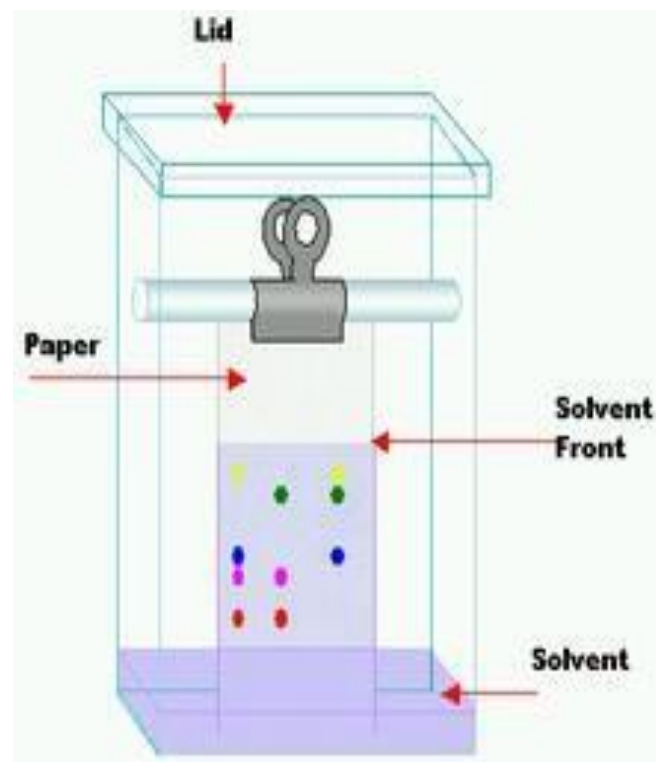
Based on the way the development of chromatogram on paper is done in procedures, we have, broadly, five modes of chromatography.

- 1. Ascending**
- 2. Descending**
- 3. Ascending-Descending**
- 4. Circular/ Radial**
- 5. Two-Dimensional**

1. Ascending Technique

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

- ❖ The chromatogram of this technique ascends slowly due to the **mobile phase movement** in a **upward direction**.
- ❖ The solvent is kept at the bottom of the filter paper or stationary phase with the end of the filter paper dipped in.
- ❖ The component mixture spot is kept well above the solvent level and is not allowed to touch the spot.



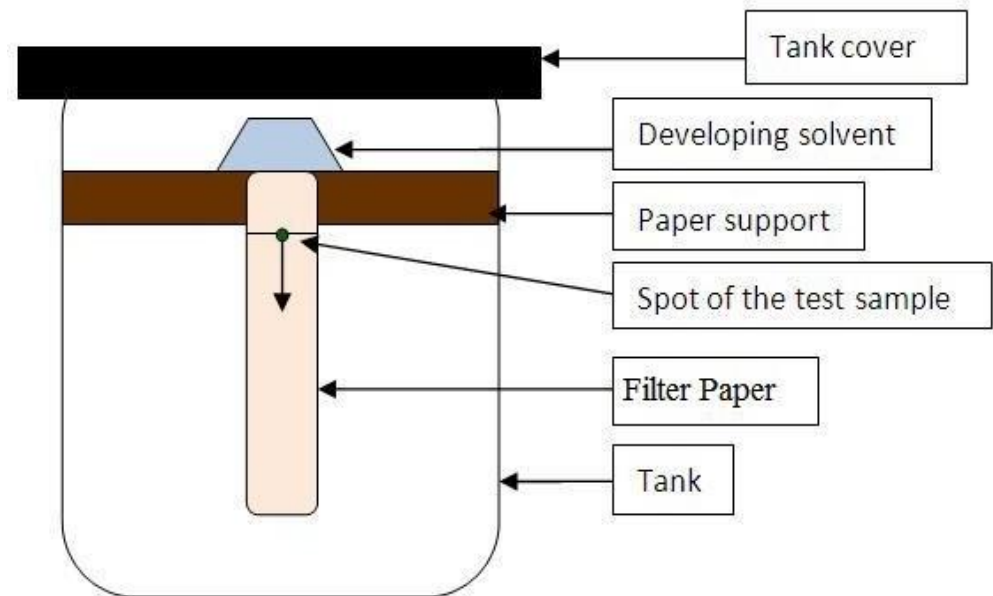
2. Descending Technique

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

The mobile phase in this type is kept at the top of the chromatogram and the components of the mixture separates out **downward** due to **gravity and capillary** action of the filter paper.

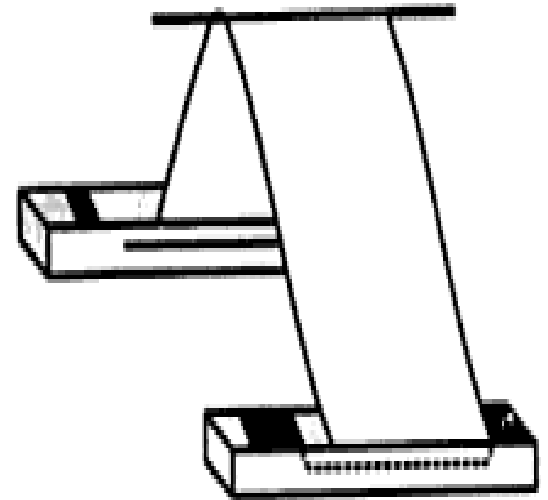
ADVANTAGE:

Development is faster



3. Ascending-Descending Technique

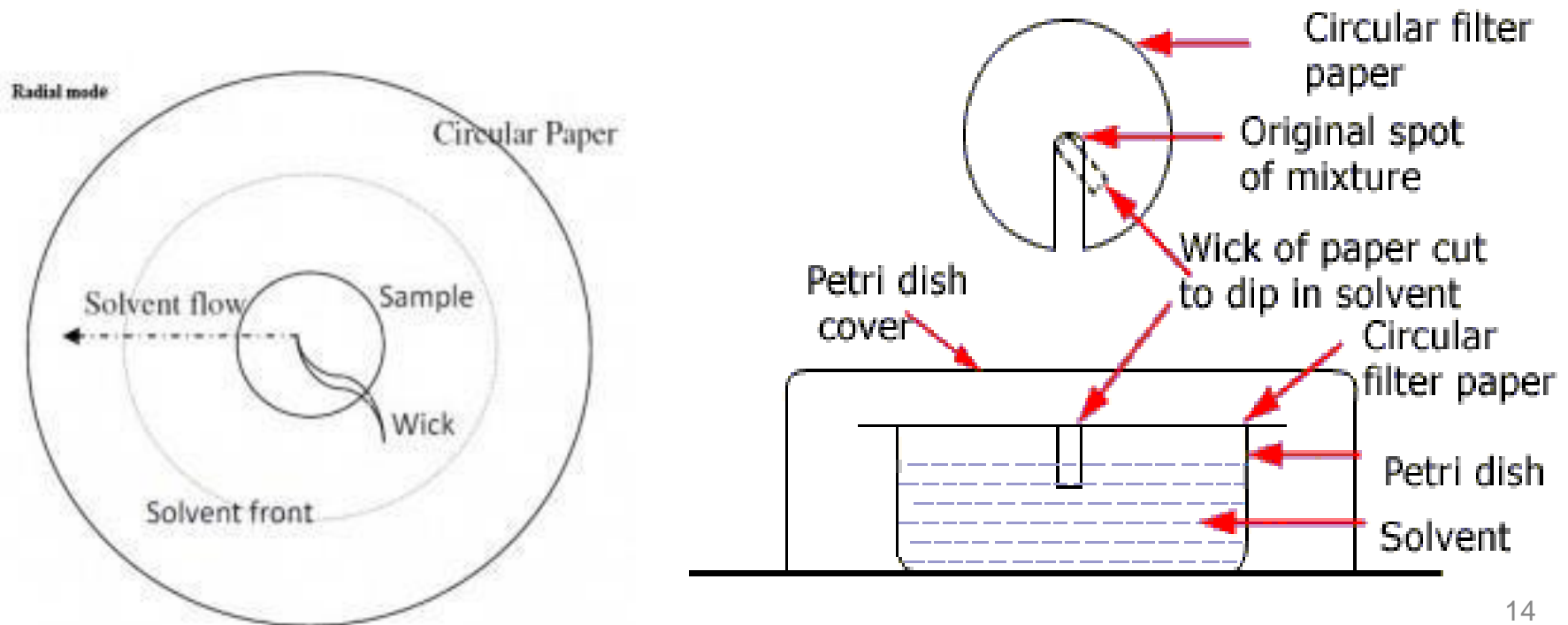
- ❑ A **hybrid** of above two techniques is called ascending-descending chromatography.
- ❑ Only length of separation increased, first ascending takes place followed by descending.
- ❑ Overall shows a **bi-directional movement** of the mixture components.



4. Circular/ Radial Technique

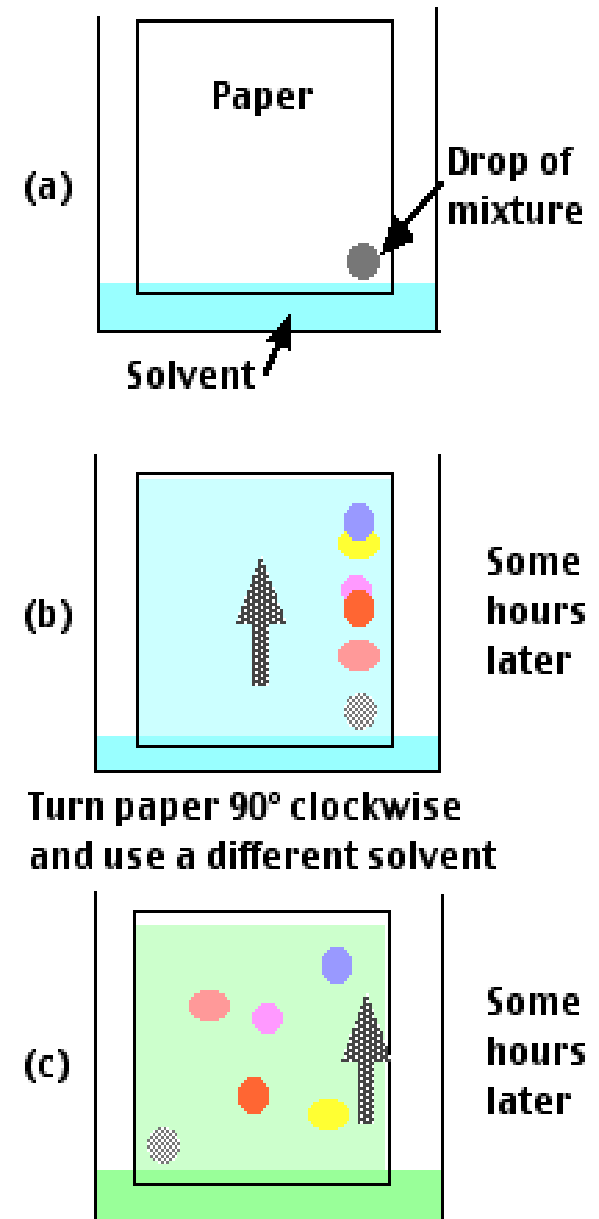
In this type of paper chromatography the **solvent moves** from the **centre towards the peripheral regions** of the filter paper. The radiating mixture component is allowed to spread till all the components have separated out. For precaution the entire system is covered with the help of a Petri dish.

The centre of the paper is allowed to be dipped into the solvent and the coloured components radiates out in concentric circles.



5. Two-Dimensional Technique

- ❖ The chromatogram in this type develops at **right angle** to each other and the filter paper is dipped at right angle once the first chromatogram is complete.
- ❖ The second chromatogram then develops at right angle to the first one.



Experimental details for qualitative analysis


1. **Choice of Development Technique**
2. **Choice of Stationary Phase**
3. **Proper Developing Solvent System**
4. **Preparation of Sample**
5. **Application of Sample (Spotting)**
6. **Drying the Chromatogram**
7. **Detecting or Visualizing**
8. **Calculation of R_f Value**

1. Choice of Development Technique

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

- ❖ **Ascending**
- ❖ **Descending**
- ❖ **Ascending-Descending**
- ❖ **Circular/ Radial**
- ❖ **Two-Dimensional**

2. Choice of Stationary Phase

- ❖ Chromatography makes use of **paper** which **acts** as a **stationary phase**.
- ❖ Paper essentially consists of **cellulose fibers** which are **polymers** having **-OH functional groups** sticking out of the polymer chains.

- ❖ These groups lead to **retention and separation** of surface adsorbed molecules.
- ❖ In practice the **separating molecules** **equilibrate** between the layer of **adsorbed water** and **the mobile phase solvent**.

2. Choice of Stationary Phase Cont.,

The **choice** of paper is **dependent** on the **type of problem under investigation**. The prime factors, that govern the choice, are as follows:

- ❖ Whether the paper is being used for **quantitative or qualitative** analysis;
- ❖ Whether it is used for **analytical or preparative** chromatography; or
- ❖ Whether the substances used are **hydropilic or lipophilic, neutral or charged** species.

2. Choice of Stationary Phase Cont.,

Various types of *Whatmann* papers are available.

	Rate of Flow		
	Fast	Medium	Slow
Thin Paper	No. 4	No. 7	No. 2
	No. 54	No. 1	No. 20
	No. 540		
Thick Paper	No. 31	No. 3	
	No. 17	No. 3 MM	

2. Choice of Stationary Phase Cont.,

- ❖ Whatmann filter papers commonly used for chromatographic purposes have a content of **99% of α -cellulose**. The rest is mineral content.
- ❖ For the **efficient separation of polar substances**, the **exchange capacity** of the paper is increased by increasing the carboxyl content (1.4%) by partial oxidation.
- ❖ It is possible to **increase the capillarity of the paper** by partial hydrolysis which is achieved by soaking the filter paper for 24 h in 7% hydrochloric acid and washing successfully with water and ethanol.

2. Choice of Stationary Phase Cont.,

- ❖ Cellulose paper is also used as a support for various adsorbents like alumina, silica, zirconium oxide etc. which get precipitated in the pores of the filter paper to produce a thin sheet of the adsorbent with the flexibility of the paper but having adsorbent characteristics of the precipitate.
- ❖ Chelating agents such as 8-hydroxyquinoline, dimethyl glyoxime and several other compounds impart papers with special characteristics.
- ❖ Papers have also been impregnated with powdered or liquid ion exchangers to produce ion exchange papers.

2. Choice of Stationary Phase Cont.,

Modified Papers for Paper Chromatography

Type	Mobile Phase
Carboxyl papers	Cationic separation of protonated amines and amino acids
Acetylated Papers	RP chromatography of lipophilic substances like steroids, insecticides and pigments and also metal cations.
Kieselguhr papers, Alumina papers, Zirconia papers, Silica papers	Separation of low polarity substances such as amines, fatty acids, steroids, triglycerides, vitamins and pesticides
Ion Exchange Papers	Ion Exchange paper chromatography

3. Proper Developing Solvent System

Criteria for selection of a working solvent system:

- ❖ Solvents should **not** be **toxic or carcinogenic**. In such cases **adequate handling care** should be exercised.
- ❖ The solvent mixture **composition should not change** with **time**.
- ❖ Solvent constituents **should not chemically react** with any of the sample constituents.
- ❖ Solvents **should not interfere** with the **detection of spots** on the developed paper.

- ❖ Differences in R_f values of any two components **should be at least 0.05** for differentiation between two closely spaced spots.
- ❖ The R_f values of the sample **should lie between 0.05 and 0.85** in the system.

Suitable solvent systems for paper chromatography

Water as a stationary Phase

- Isopropanol: ammonia: water [9:1:2]
- N-butanol: glacial acetic acid: water [4:1:5]
- Phenol saturated with water

SP (dimethyl ether):- Cyclohexane

SP (kerosene) :- 70% isopropanol

Commonly used solvent combinations:

❖ Strongly polar compounds:

- ethyl acetate: n-butanol: acetic acid: water - 80:10:5:5

❖ Polar compounds:

- 10% methanol or less in dichloromethane

❖ Strongly basic compounds:

- 10% ammonium hydroxide in methanol and make 1-10% mixture of this in dichloromethane

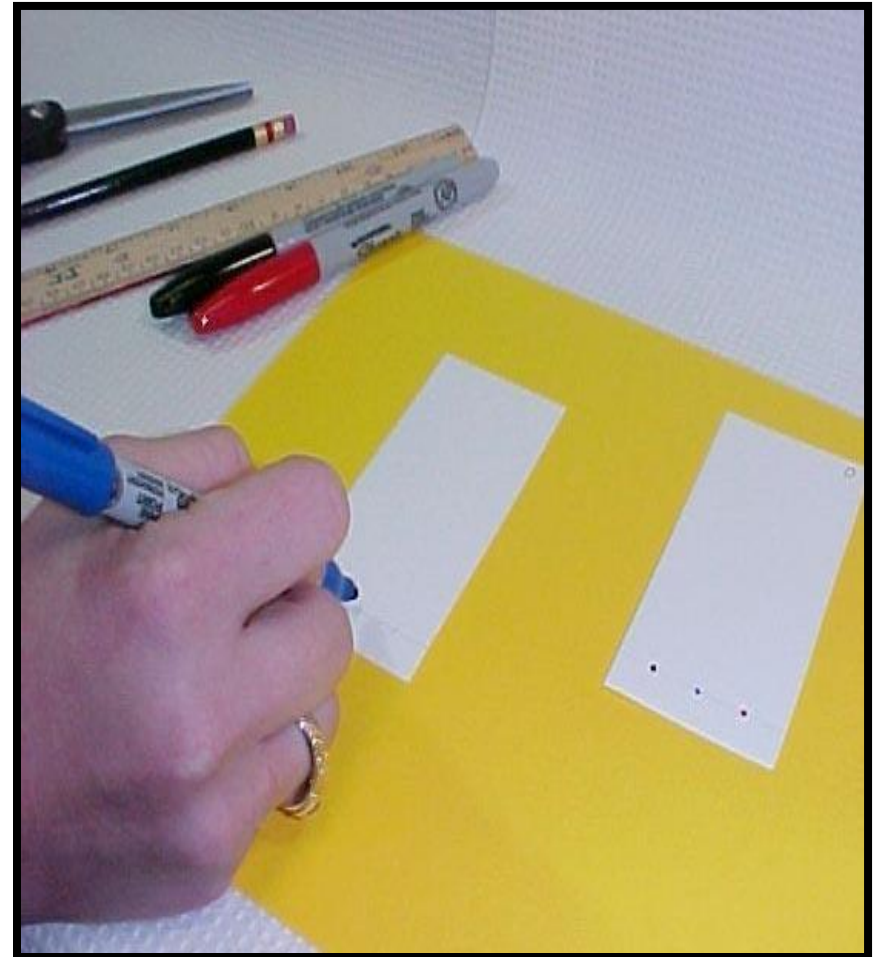
4. Preparation of Sample

- ❖ Pure solutions can be applied directly on the paper but solids are always dissolved in small quantity of a suitable solvent.
- ❖ Biological tissues are treated with suitable solvents and their extracts obtained.
- ❖ The sample volume of 10-20 μL having as many μg of the substances is that ideal quantity to be spotted.

5. Application of Sample (Spotting)

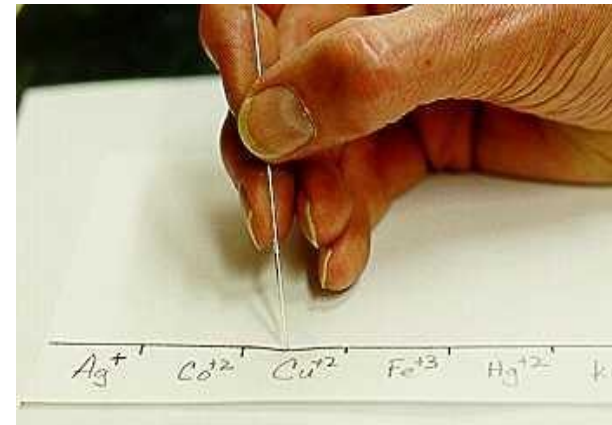
Preparation of Paper

- ❖ Cut the paper into desired shape and size depending upon work to be carried out.
- ❖ The starting line is marked on the paper with an ordinary pencil 2 cm from the bottom edge.
- ❖ On the starting line marks are made 2 cm apart from each other.



Spotting:

- ❖ The sample to be applied is dissolved in the mobile phase and applied as a **small spot** on the origin line, **using capillary tube or micropipette**.
- ❖ **Very low concentration** is used to avoid larger zone.
- ❖ The sample is **applied as a neat spot on a horizontal line** drawn with a pencil close to one edge.
- ❖ The spot is dried on the filter paper and is placed in developing chamber.



Developing:

- ❖ Allow the spot to dry and then **immerse the paper** in the developing chamber as per the selected technique with the marked spot above the solvent level.
- ❖ The solvent begins to move and draws the sample components differentially along with it.
- ❖ At the end of the development take out the paper and mark the solvent front with another line.



6. Drying of Chromatogram

They are dried by cold or hot air depending on volatility of solvents. A simple hair dryer is a convenient device to dry chromatograms.



7. Detecting or Visualizing

- ❑ Coloured spots are easily observed on developed chromatograms.
- ❑ However, different approaches need to be adopted when colourless components are to be observed.
- ❑ It is convenient to classify such methods as specific or non-specific.

Non-specific methods (Physical):

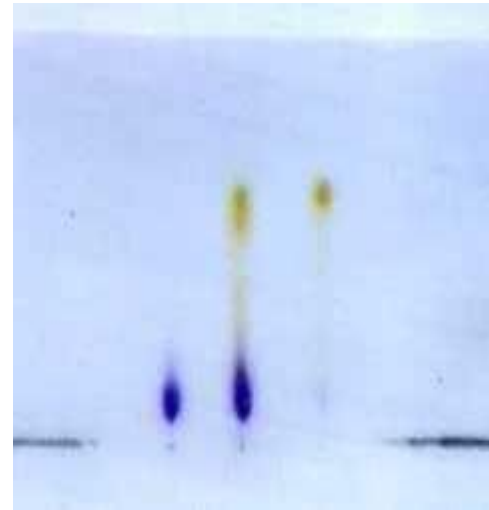
❑ **Iodine chamber:** The developed plate is suspended in a closed jar containing a few crystals of iodine for about a minute. In presence of iodine vapour **most organic compounds** appear as **brown spots**.

❑ **UV viewing cabinet:**

- Majority of colourless compounds can be viewed under illumination with UV light in a UV viewing cabinet.
- Commonly the cabinets are equipped with a long wavelength (**366 nm**) and short wavelength (**254 nm**) light sources.

Specific methods (Chemical methods)

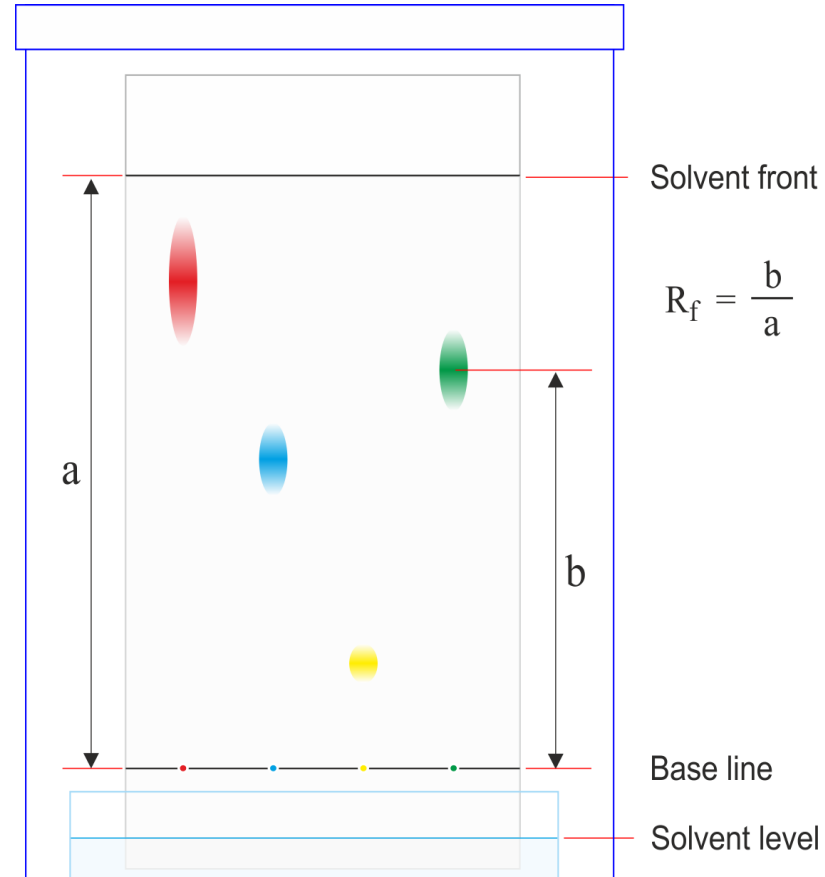
- Ferric chloride
- Ninhydrin in acetone
- Dragendroff's reagents
- 3,5-dinitro benzoic acid
- Molisch reagent
- Phenolic comp. & tannins
- Amino acids
- Alkaloids
- Cardiac glycosides
- Carbohydrates



8. Calculation of R_f VALUE

R_f VALUE (Retardation Factor)

The R_f defines the movement of the substance relative to the solvent front in a given chromatographic system.



R_f value is calculated as : $\frac{\text{Distance travelled by the solute front}}{\text{Distance travelled by the solvent front}}$

Factors affecting R_f VALUE

- ❖ The temperature
- ❖ The purity of the solvents used
- ❖ The quality of the paper, adsorbents & impurities present in the adsorbents
- ❖ Chamber saturation techniques, method of drying & development
- ❖ The distance travelled by the solute & solvent
- ❖ Chemical reaction between the substances being partitioned.
- ❖ pH of the solution

R_x VALUE

- ❖ In many cases it has been observed that the solvent front is run off the end of the paper.
- ❖ It is the ratio of distance travelled by the sample and the distance travelled by the standard.
- ❖ R_x value is always closer to 1.

Experimental details for Quantitative Analysis

For quantitative analysis, the preliminary separation is done in the same way as in qualitative analysis. Then, the assay can be performed either after **extraction** from the paper or *in situ* on the paper.

1. **Estimation after Extraction from the paper**
2. *In situ* methods

1. Estimation after Extraction from the paper

In this technique, the spots are cut into portions and eluted with solvents. This solution can be analyzed by any techniques of analysis like gravimetric estimation, spectrophotometry, Colorimetry, Polarography, Flame photometry, etc.

In the assessment of the merits of any procedure, the following information is required:

- ✓ The nature of the substance to be assayed.
- ✓ The scientific equipment available and its sensitivity.
- ✓ The time available and
- ✓ The alternative method, available, if any, and their relative accuracies

2. *In situ* methods

- ❖ **By Visual Assessment:** The simplest procedure is to see the spot by the human eye. However, it is not very accurate.
- ❖ **By Measurement of areas:**
 - ✓ if the outline of the **spots or zones are well defined**, the size of the spot (length or areas) may serve for determining the quantity of the substances.
 - ✓ Then a **linear relationship** is obtained between the spot area and amount of the substance present.
 - ✓ The random errors in the procedure are generally high as a results of the **variations of the spot shape during separation**.
 - ✓ Other drawbacks: **Volume of applied sample and speed of application**

2. *In situ* methods cont.,

- ❖ **By Densitometer:** It is a method whereby the intensity of the colour of a substance is measured directly on the chromatogram.
- ❖ **By Potentiometer:** Changes in the potential of a metal electrode in contact with the filter paper is also utilised with quadrant electrometer or electronic voltmeters.
- ❖ **Fluorimetry:** The compound to be determined by fluorimetry must be fluorescent or convertible into fluorescent derivatives.
- ❖ **Radiotracer Method:** The compound containing radioactive element is labeled and treated with locating reagent. Using Geiger Muller counter.

SOURCES of ERROR

❖ Error during application of the spots:

- **Apply minimum volume** of the concentrated solution in order to avoid diffusion through the paper which leads to poor separation
- **Spots should be approximately of the same diameter.**

❖ Development:

- **Improper adjustment** of the paper in the tank leads to this error so the paper should be held vertically.
- **Do chamber saturation**

❖ Detection:

- The **spraying methods** affect the final result

APPLICATIONS

Innumerable applications have been reported in analysis of different classes of compounds such as:

- Amino acids and organic acids
- Alkaloids
- Polysaccharides
- Proteins and peptides
- Natural and artificial pigments
- Inorganic cations
- Plant extracts

Applications cont.,

Typical applications in key areas are briefly outlined here:

1. Reaction monitoring:

In a chemical reaction over a period of time the concentration of reactants decreases whereas the concentration of products increases.

Developing the chromatogram over different time intervals by spotting the reactants can give a fair idea on the progress of reaction.

Traditionally the technique was used for qualitative monitoring but availability of densitometers made quantitative estimations possible.

Applications cont.,

2. Isolation & Purification:

Paper chromatography has been put to use as a purification and isolation technique for components of mixtures. The separated components on the paper are cut, dissolved in suitable solvents and their absorption characterised at specific wavelengths using spectrophotometric methods.

3. Foods & Beverages:

Paper chromatography has been used for analysis of food colours in synthetic drinks and beverages, ice creams, jams & jellies, sweets, etc.

Applications cont.,

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Applications cont.,

4. Forensics:

The major applications are for identification and comparison against reference standards for drugs and their metabolites in viscera, explosive residues from blast sites, inks used in forgery of documents and paint pigments investigations in hit and run road accident cases.

5. Pharmaceuticals:

It offers a cost-effective alternative to monitoring the active ingredients in drug forms administered in bioanalytical studies. Its main contribution is when the quantity of sample available is in minute amounts.