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A review on paper chromatography

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Abstract
Chromatography is indeed a widely-used technique for separating and analyzing mixtures, and paper chromatography stands out as a simple yet effective method, particularly for the separation of colored compounds. The technique relies on the principle of partition and differential affinity of compounds toward the stationary (paper) and mobile phases (usually a solvent). Compounds move at different rates based on their solubility in the solvent and their attraction to the paper, resulting in separation.While paper chromatography has been utilized for decades, especially in regions like the United States, it is not restricted to organic compounds. It can also be applied for inorganic analysis. Future research in this field can focus on advancements such as improving the sensitivity of paper chromatography, exploring new types of paper with unique properties (e.g., porosity, thickness, or surface chemistry), and integrating gel permeation processes for separating macromolecules based on size. Modern developments may also include coupling with advanced detection techniques for enhanced accuracy and quantification. Paper chromatography is a simple and widely used technique for separating and analyzing mixtures of substances, especially when dealing with small amounts of materials. It works on the principle of differential solubility and capillary action, where the components of a mixture move at different rates on a strip of chromatography paper when exposed to a solvent.In the process, a small sample of the mixture is placed near the bottom of the paper. The lower edge of the paper is then immersed in a solvent (like water or alcohol), while the sample spot stays above the solvent level. As the solvent ascends through the paper by capillary action, it dissolves the components of the mixture, which travel up the paper at different speeds depending on their affinity for the paper (stationary phase) versus the solvent (mobile phase). Substances that are more soluble in the solvent will travel farther, while less soluble substances remain closer to the origin. The separation of components results in distinct spots on the paper. These can be analyzed by measuring the distance each component has traveled compared to the solvent front, yielding the retention factor (Rf value), used for identification purposes. Paper chromatography is commonly used in fields like biochemistry for separating amino acids, identifying pigments in plants, and analyzing inks or dyes in forensic science due to its efficiency, low cost, and ease of use.

Keywords: Chromatography, history & principle of chromatography, classification of chromatography, paper chromatography, development techniques involved in paper chromatography, preparation of stationary phase & mobile phase

Introduction

Chromatography

- It is a physical method of preparation in which the components are separated or distributed between two phases. These are stationary phase and mobile phase.
- This method is highly efficient in components separation, and to purify the compounds and for determination of purity of the compounds.[1]
- It covers a brief description of major chromatography types and applications, general set up configurations for different chromatographic systems, common media used in chromatography including the basic chemistry behind them as well guidance on selecting appropriate media targeting standard protein purification methods.[2]

History

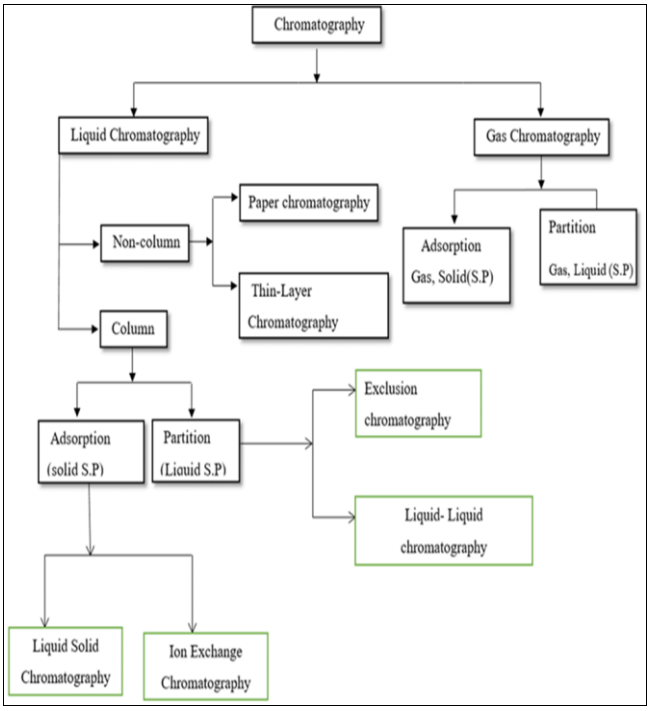
- In the year 1903, Chromatography was invented by a Russian Botanist “Mikhail Tsvet”. He is known as the “Father of Chromatography”.
- The term “chromatography is derived from Greek; chroma means color and graphein means to write. Chromatography involves separation of solutes between two phases: stationary and mobile.[3]

Mikhail Tsvet

Principle

Chromatography relies on the separation of solutes between two phases, stationary phase and mobile phase.[4]
Ex:- a combination of red ink and blue ink can be separated by chromatography process.

Classification of chromatography



Introduction of paper chromatography

Definition

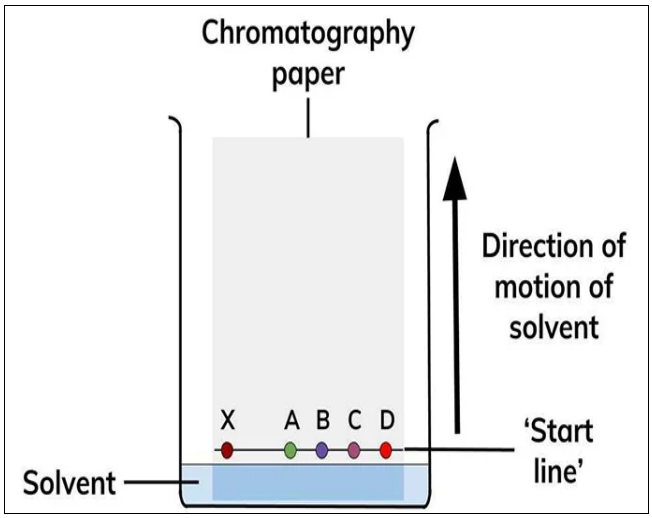
- This is a method where, passing of solvents on the filter paper already designed by known substance and this in return will be used to analyze unknown compounds.[5]
- German scientist "Christian Friedrich Schonbein" first produced it in 1865.

Christian Friedrich Schonbein

- Paper chromatography is the form of planar chromatography and employs a flat relatively thin layer of paper as the stationary support.[6]

Principle

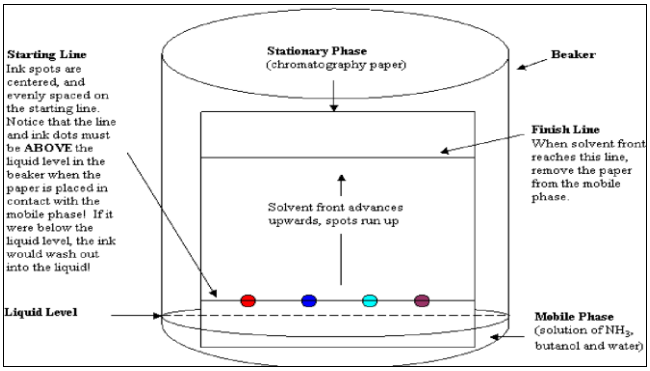
- The separation mechanism is essentially partition, not adsorption. As we already aware, separation is achieved serially utilizing equilibrium of sample between stationary phase and mobile gel.[7]
- Cellulose layers in filter paper to allow wetting, so that moisture or water is used as stationary phase.
- Instead of water, organic solvents can be used as stationary phase with mobile phase using the form parameters such as organic solvent based or else buffer.
- In this method, 1 drop of the test solution is added as spot on a filter paper slightly away from edge and allowed to dry. The paper is stored in a closed chamber and the former edge of filter land thereby submerged at developing solvent.
- When the liquid moves in upward in filter paper through its capillary axis and it meets to spot of test solution (mixture two or more substances) than various components are moved by solvent system at different speed.



Theory

- The travel of sample on paper is related to the type and quantity of stationary phase but also number of mobile phases in that region as well partition coefficient.
- Rf value is the ratio of distance travelled by solute and distance travelled by solvent front.
- They provide a not even sort of identification, which will translate into less reference substances tested in a given experiment and simplify the experimental work.

Representation of paper chromatography



Types of paper chromatography

Paper chromatography can be categorized into two types:

1. Paper adsorption chromatography
2. Paper partition chromatography

1. Paper adsorption chromatography

- This is a form of chromatography where paper serves as the stationary phase. It separates mixtures based on the differences in adsorption between the paper and the sample components.
- In this method, the paper is treated with silica or alumina which acts as the adsorbent (stationary phase) while a solvent is used as the mobile phase.[8]

- It is used for identifying compounds separating components and for purposes.

2. Paper partition chromatography

- This chromatography technique is a method that aids in separating and identifying components through their varying solubility in a stationary phase and a mobile phase.[9]
- In this process, the moisture trapped within the cellulose fiber pores of filter paper serves as the stationary phase while the solvent acts as the mobile phase.

Types of instrumentation in paper chromatography

1. Paper as stationary phase
2. Mobile phase
3. Sample application on paper
4. Chromatographic chambers
5. Development of chromatogram
6. Drying
7. Quantitative analysis and Qualitative analysis
8. Estimation

1. Paper as stationary phase

- Whatmann filter papers of various grades like No.1, 2, 3etc; are used. The commonly used are Whatman's no.4 and the general α-cellulose of the papers was 98-99% whereas β-cellulose fraction was just around 1%.
- These papers which are used for paper chromatography procedure may varies in size, shape, porosity and thickness.
- Acid or base washed filter paper, glass fiber type papers or other modified sheets can also be employed in this method.
- Hydrophilic papers- Methanol, formamide, glycol and glycerol are used for modification of the papers.
- Hydrophobic papers- acetylation of OH groups make them hydrophobic; thus, they may be used for reverse phase chromatography.

2. Mobile phase

- Pure solvents, buffers or mixture of solvents can be used as mobile phase. The mobile phase can either be hydrophilic or hydrophobic in nature. Some of the examples of[10]

1. Hydrophilic mobile phases

- 2-propanol: Ammonia: Water (9:1:2)
- Methanol: H2O (4:1 or 3:1)

2. Hydrophobic mobile phases

- Kerosene-Isopropyl-alcohol 70%
- Dimethyl ether: Cyclohexane

- Some of the frequently used solvents are polar in nature; nevertheless, its selection is based upon the compound whether it can be natural product or synthetic one.[11]

3. Application of sample

- The sample to be injected is dissolved in the solvent of mobile phase and injection of the sample can be done with capillary by micropipette.
- The filter paper after the sample is applied on it, dried by using air drying or gentle flow of warm air.[12]

4. Developing or chromatographic chambers

- These chambers differ in the material used in which, they are made up of, they are plastic, glass or stainless steel.
- Glass tanks are used the most and comes in different sizes, which depend on the paper size and type of development required.

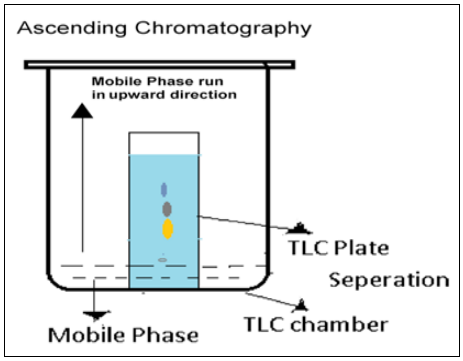
Development technique

- In this, the sample loaded paper is carefully dipped into solvent and left until travelling front of solvent reaches near to edge of paper. Development practices can differ.
- Five types of development techniques have deployed in here. They are:

a. Ascending development
b. Descending chromatography
c. Development: Ascending - descending
d. Circular development
e. Two-dimensional development

A. Ascending development

- In this type, the spots are kept on the bottom of paper and placed in mobile phase which is solvent.



- This type of development, the solvent flows against gravity.

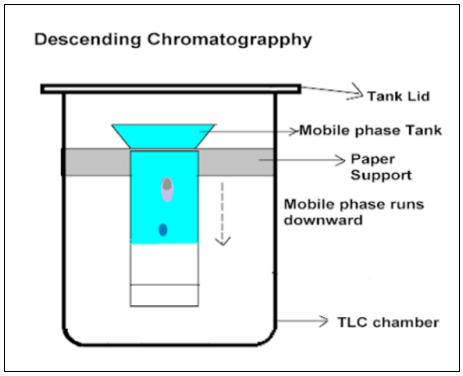
Advantages

- In this type, development is faster
- Setup for the process is simplified.

Ascending development diagram

B. Descending development

- Silver ion chromatography:- This kind of technique is mainly performed in the special chamber and where solvent present at top. The point is maintained on the paper as a top, though solvent pass down the card.

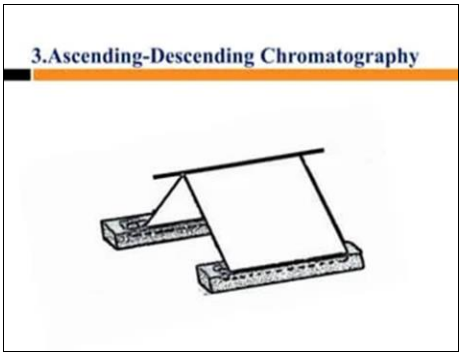


- So, in this way the solvent moves from up to down so that type of chromatography is called as descending chromatography.

Advantages
- Mostly suited for larger paper sizes
- This technique is easy to handle.

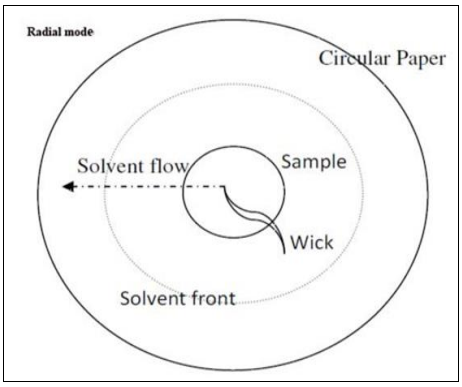
Descending development diagram

C. Ascending – descending development
- This is ascending and descending using two step process. The length of the paper increased as well as the length of separation.
- This tends from up development to down trends. First up rise afterwards downtrend.



Ascending- descending development diagram

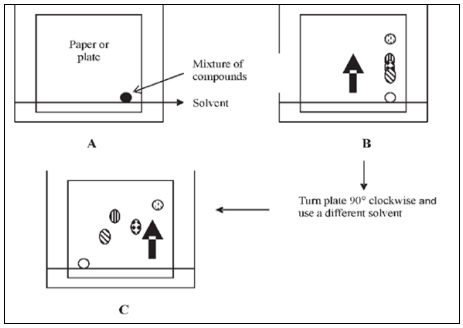
D. Circular development
- Here, the spot is placed at midpoint of paper where solvent will travel through same wick present in sampling machine that spread throughout.
- Thus, the individual spots after it, are arranged in wave like appearances like those of a target. The perforations to form the quadrants can be done radially.



Circular development diagram

Advantages
- Faster development type
- Smaller paper sizes are suitable for this type.

E. Two-dimensional development
- This process is almost similar to 2-Dimensional TLC (Thin layer chromatography).
- The paper is first developed in one direction and after development, same piece of paper used for 2nd development which allows better separation as more complex are separated to individual spots.
- In this Two Seperated chromatographic system are connected in series transferring the effluent from the first system to the second column. Typically the second column has a different separation mechanim, so that bands which were poorly resolved in the first column can be completely separated in the second column.
- Alternately, the two columns may run at different temperatures. During the second stage,the separation has to occur faster than in the first stage, because there is still one detector; thus, the development occurs smarter, utilizing smaller paper, and the development of the plane surface has the option of sequential development in two directions with two different solvents.



Advantages
- Easy identification of the compounds
- Separation of complex mixtures.[13]

Paper preparation used in paper chromatography

The process of preparing paper used for the separation of complex mixtures and onward identification is as:-

1. Cutting of the paper
2. Washing
3. Drying
4. Drawing the baseline
5. Application of the sample
6. Drying of the sample
7. Development

1. **Cutting of the paper: -**Cut the paper into your wish size used for the separation process of complex mixtures by using paper cutter or scissors.

2. **Washing: -**After cutting the paper, wash the selected paper with water or suitable solvent to remove the impurities present in paper that might alter the results.

3. **Drying: -** Dry the washed paper by air drying or by using gentle stream of warm air.

4. **Drawing the baseline: -**By using a fine tip pen or pencil, draw a straight line across the paper around 1-2 cm from the edge of the paper. This line is known as baseline.
   - After drawing the baseline, mark the sample application point after that.

5. **Application of the sample: -** The sample to be applied is dissolved in mobile phase or solvent before applying and application of the sample on the paper is done by using capillary tube or micropipette.

6. **Drying: -** Allow the paper to dry after the application of the sample by using air drying

7. Development: - The paper is then ready for the development in chromatography tank.

Techniques of paper chromatography

The different operations related to paper chromatography are:

1. Filter paper used
2. Solution preparation
3. Sample being applied to paper
4. Choice of the solvent
5. Development of chromatogram
6. Drying
7. Retention factor of compound on paper against travelled from starting line
8. The quantitative estimation

1. Filter paper used

The type of filter paper varies with the

a. Thickness
b. Flow rate
c. Purity
d. Net power of the paper

- Whatmann filter papers are used most in paper chromatography.
- Whatmann filter paper have the chemical compositions as:

a. α-cellulose 99%
b. β-cellulose 0.3-1%
c. pentosans 0.5-0.8%
d. Ash 0.01-0.07%
e. Ether soluble substances 0.01-0.1%

- A suitable paper should meet the following criteria;

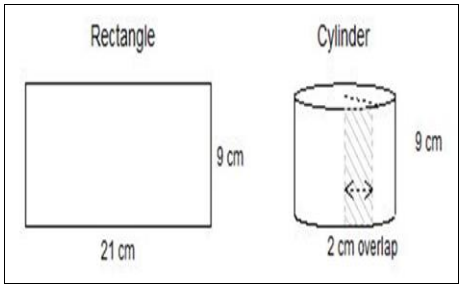
a. Good solvent front migration
b. Almost no spreading from the spots
c. The separation is defined concisely.

Modification of the paper

For specific chromatographic work, the chemical composition of the paper can be modified by dipping the filter paper in aqueous solution of other substances or by changing the chemical composition of the cellulose present in the filter paper.

Preparation of the paper

- The rectangular filter paper is most commonly used, though square shaped filter papers may be required depending on the instrument.
- Average dimensions are 15 to 30cm long and width can range 1 between several cms depending on the can go up to more than m if directed work type.[14]



2. Solution preparation

- Preference should be given to the solvent used to create solution. They are applied as pure solutions on the paper, solid substances. However, are always dissolved in a small amount of an appropriate solvent.
- Solubilizing the biological tissues using their suitable solvents and then extracting.
- Protein and salts content is high in biological extracts which disturbs partitioning step. High levels of proteins and salts may be present in the biological extract, impacting upon the success rate of early binding.
- Alcohols are used for precipitating proteins and salts can be removed by ion exchange resin and electrolysis.
- Concentrated solutions are often placed on the filter paper (as not to diffuse through it)

3. Sample being applied to paper

The parameters that will dictate the volume of the sample inlcude: -

a. Capacity of the solvent
b. Optimum concentration
c. Time-consuming in development

- A pencil line around 5cm from one end is drawn once the kind of paper and quality have been selected.
- The line is flooded with gliadin, and then a pencil marked number of crosses (depends upon how many samples are to be analysed)
- A capillary pipette of narrow hole is used to spot a drop of concentrated solutions per each sample on the marked positions at appropriate places using platinum loops for the sample placement.

4. Choice of the solvent

- The solvents usually used are polar in nature but it is depending on what type of substance to be separated from.
- Water miscible solvents like furan, propanol etc. have been used extensively. In view of the above fact, adsorption is an intrinsic part of separation process and can be used as suitable simple mobile phase.
- If pure solvents do not give proper separation, then a mixture of solvents having suitable polarity can be used.
- Such solvents must be acids, bases or complexing agents. A solution consisting of n-butanol, acetic acid and H20 was generally used.
- However, n-butanol should be replaced with a tertiary alcohol due to its formation of esters by organic acids.

5. Development of chromatogram

The chromatogram is developed in a chemical chamber constructed of various materials like glass, stainless steel or plastic.

6. **Drying: -** After the development of the chromatogram, it is dried in oven at 100 - 105°c until colored spots are detected.

7. **Retention factor of compound on paper against travelled drom starting line**

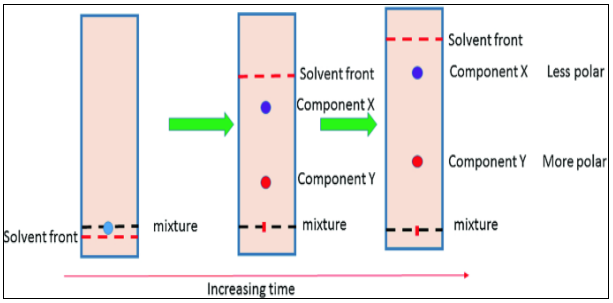
- since retention of components separated by chromatography vary from each other. Rf value can be used approximately to locate the position on a plate where component is situated.
- The Rf value is determined by dividing the distance travelled of a component to be chromatographed and that of pure solvent.

8. **Estimation: -** The estimation of the sample is done by using detecting or spraying agents and estimation is done by comparing the obtained Rf values with standard values.

Procedure for seperation of amino acids

- Amino acids have different types of amino acids and can be isolated by paper chromatography. The solution is spotted on paper, eluted then detected by using ninhydrin reagent which gives rise to purple or brown color.
- Rf values gives the qualitative identification. Quantitative identification is done by measuring the intensity of color with those standards. The experimental means used are given as follows.
- Prepare 0.1g/100ml each of valine, threonine, glycine, crystine, isoleucine (these are all amino acids).
- Take 8" x 10" sheet of Whatmann filter paper No.1 and make six equally spaced small circle with a pencil 0.5" from the bottom. Label the paper at top with the names of amino acids and the sixth as unknown.
- Place four small drops of amino acid on one circle and dry it carefully over between each drop application. Similarly repeat the process for other amino acids.
- After this roll the paper sheet in a cylindrical form (the spots should lie at the bottom) and put it into a beaker containing 5% ethyl alcohol.
- In a way that the paper should not touch the sides of beaker. The top and bottom of the paper is stapled. Allow the eluent agent to rise about 6".

- This process may take 3 to 4 hours. At this moment, remove the paper from the paper, and mark the solvent front position immediately with pencil. The chromatogram is dried and then sprayed with 0.2% ninhydrin reagent solution in water saturated with butyl alcohol.
- The chromatogram is then dried in an oven at about 100 to 105°c until colored spots appear.
- Find the Rf value of each amino acids and the spots for the unknown and thus find out quantitatively the composition of the unknown.



Sources of errors

The sources of errors in paper chromatography are enumerated as follows:

- **Spot application error**

However, when it is applied onto the paper in spots, should be careful to place as low a volume of each concentrated solution possible. Since even if nothing of that stuff has been diluted yet (none moved anywhere from its original position), it can cause error.

Development

- it happens when the paper in the tank is incorrectly adjusted, this issue can happen if the papers are rested horizontally.
- Hence, hold it vertically. Otherwise, the error should be due to not equilibrating of developing solvent and atmosphere in the tank.

- **Detection: -**The spraying and dipping methods affect the final result considerably.

- **Quantitative spot detection methods: -** There are various quantitative ways to estimate on paper a spot.

- **Measurement of spot length: -c**an be calculated as the logarithm of drug concentration in a spot is directly proportional to that for its geometrical dimension.

$$\text{Log (L)} = \propto \text{Log [C]}$$

- **Planimetric measurement: -**Area of the spot size is measured using a planimeter.

- **Methods of counting squares: -** A sheet of millimeter-ruled graph paper is placed over the spot (or a tracing of the spot) and all or some part falls within counted number square, last being equals to area.

- **Weighing the excised spot: -**After development, the spot was dried and is weighed which compared with blank paper chromatogram of an equivalent area.
- **Method of aspect contrast: -**considering the fact that depth from color and also area dimensions tend to be proportional to level involving medication, very difficult features in a ambiguous attentiveness can find obtaining around same column chromatogram group of dilutions together with traditional as well as ambiguous answers.

- Unknown concentrations that are equal to the standard appear as a matching spot of area, density of color.

- All uses should be of equal volume, since the spots are all made to approximately the same size by adding different volumes of solution.

Elution

- The incorrect choice of solvent for elution results in an insufficiently complete elution path with even greater error in paper chromatography.
- Compounds to be eluted should not attach with solvent, so the polarity of the solvent is more.
- It always has slightly higher polarity than stationary phase and would be completable miscible in nature to solubilize compound for different sorbents.
- One can cut the spot, place it in a small amount of solvent, put that into suspension and remove with drawn up and rinsed aliquot (using fresh solution) then finally bring to volume.
- Between two glass slides in a petri dish of water where the item with substance needs to be squirted saline.
- The water ascends through the capillary action between these slides, until it falls over edge of paper into a small beaker collection.
- Slides must be placed in a beaker and insert that into some larger outer vessel or will not have enough supply of liquid, it evaporated easily.

Precautions in paper chromatography

- The minimum volume amount of the concentrated sample solution is necessary to ensure that no diffusion occurs through the chromatographic paper.
- Solvent for elution should be properly selected.
- To get reproducibility, the stable solvent should be selected.
- Vapor- solvent equilibrium should be properly established.
- Natural spots should be avoided because they disturb the cellulose water which results in separation of water on to the paper.

Applications of paper chromatography

1. Identification of drugs, impurities and related compounds carried out by paper chromatography.
2. Polymers analyzed.
3. Detection and measuring the metals in soil and geological samples (mineral soils).
4. Phenolic materials in plant extracts.
5. To detect the contaminants in foods and drinks.
6. Separation of barbiturates, alkaloids, antibiotics, hormones and carbamyl phosphates.
7. Identification of decomposition of compounds.
8. Metabolites of drugs in blood, urine etc; for pharmacokinetic analysis.
9. For fermentation and maturation study
10. Since it is possible to obtain directly the solid-phase in dry form, separation of amino acids and peptides are commonly used for protein structure analysis.
11. Urine and other body fluids examination for the indoles, amino acids of sugars.
12. Separation of steroids.
13. Chromatography of purine nucleotides and bases in the analysis of nucleic acids.
14. Steroids separation.

Advantages

1. The equipment is very simple and easily available.
2. It has high efficiency of separation.
3. Separation can be possible on macro, micro and semi microscale.
4. Closely related homologues, isotopes, isomers and very labile and reactive substance can be separated readily.
5. Simple and rapid.

Disadvantages

1. Paper chromatography is not applicable in separation of volatile compounds like hydrocarbons and volatile fatty acids.
2. Longer development time required as compared to TLC (thin layer chromatography)
3. Spots are not always sharply defined.[15]
4. Accuracy in quantitative analysis is only fair.

Conclusion

- So, as a conclusion we can say that paper chromatography is used for valuable analytical technique which credit being simplest method for separation of very complex mixtures, identifying the compounds and purification purpose.
- Paper chromatography is low cost, simple, rapid and have various applications in chemistry, biology and pharmaceutical research.
- Compared to other techniques, paper chromatography provides unique combination of flexibility, easy usage and provides informative data or output.
- Therefore, it is concluded that the separation and identification of the compounds is done by detecting the colors on the filter paper and by comparing the Rf values of the unknown compounds with standard Rf values.

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