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**CHROMATOGRAPHY**

**INTRODUCTION:**

**Chromatography** (from [Greek](http://en.wikipedia.org/wiki/Greek_language) *chroma* "color" and *graphein* "to write") is the collective term for a set of [laboratory techniques](http://en.wikipedia.org/wiki/Laboratory_techniques) for the [separation of mixtures.](http://en.wikipedia.org/wiki/Separation_of_mixtures) It involves passing a mixture dissolved in a "mobile phase" through a *stationary phase*, which separates the [analyte](http://en.wikipedia.org/wiki/Analyte) to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's [partition coefficient](http://en.wikipedia.org/wiki/Partition_coefficient) result in differential retention on the stationary phase and thus **changing** the separation.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive

Chromatography involves a sample (or sample extract) being dissolved in a *mobile phase* (which may be a gas, a liquid or a supercritical fluid). The mobile phase is then forced through an immobile, immiscible *stationary phase*.Kaolin, alumina, silica, and activated charcoal have been used as adsorbing substances or stationary phases. The phases are chosen such that components of the sample have differing solubilities in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the stationary phase.

**TERMINOLOGIES USED**

1. The **analyte** is the substance to be separated during chromatography.
2. **Analytical chromatography** is used to determine the existence and possibly also theconcentration of analyte(s) in a [sample.](http://en.wikipedia.org/wiki/Sample_(material))
3. A **bonded phase** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.
4. A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.
5. A **chromatograph** is equipment that enables a sophisticated separation e.g. gas chromatographic or liquid chromatographic separation.
6. The **eluate** is the mobile phase leaving the column.
7. The **eluent** is the solvent that will carry the analyte.
8. An **immobilized phase** is a stationary phase which is immobilized on the support particles, or on the inner wall of the column tubing.
9. The **mobile phase** is the phase which moves in a definite direction. It may be a liquid (LC and CEC), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In the case of [HPLC](http://en.wikipedia.org/wiki/High-performance_liquid_chromatography) the mobile phase consists of a non-polar solvent(s) such as hexane in normal phase or polar solvents in reverse phase chromotagraphy and the sample being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
10. The **retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set conditions.
11. The **sample** is the matter analyzed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample.
12. The **solute** refers to the sample components in partition chromatography.
13. The **solvent** refers to any substance capable of solubilizing other substance, and especially the liquid mobile phase in LC.
14. The **stationary phase** is the substance which is fixed in place for the chromatography procedure. Examples include the [silica](http://en.wikipedia.org/wiki/Silica) layer in [thin layer chromatography](http://en.wikipedia.org/wiki/Chromatography#Thin_layer_chromatography)

**TYPES OF CHROMATOGRAPHY:**

Classification of chromatographic techniques tends to be confusing because it may be based on the type of stationary phase, the nature of the adsorptive force, the nature of the mobile phase, or the method by which the mobile phase is introduced.

1. **Adsorption chromatography:**

That in which the stationary phase is an adsorbent.. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

1. **Affinity chromatography**:

This is the most selective type of chromatography employed. It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase. For example, the immobilized molecule may be an antibody to some specific protein. When solute containing a mixture of proteins are passed by this molecule, only the specific protein is reacted to this antibody, binding it to the stationary phase. This protein is later extracted by changing the ionic strength or pH.

1. **Column chromatography:**

That in which the various solutes of a solution are allowed to travel down an absorptive column, the individual components being absorbed by the stationary phase.

1. **Gas chromatography:**

(GC) that in which an inert gas moves the vapours of the materials to be separated through a column of inert material.

1. **Gas-liquid chromatography:**

(GLC) gas chromatography in which the sorbent is a non-volatile liquid coated on a solid support.

1. **Gas-solid chromatography:**

(GSC) gas chromatography in which the sorbent is an inert porous solid.

1. **Gel-filtration chromatography** (**gel-permeation chromatography):**

That in which the stationary phase consists of gel-forming hydrophilic beads containing specifically sized pores that trap and delay molecules small enough to enter them.

1. **High-performance liquid chromatography** (**high-pressure liquid chromatography):** (HPLC) a type of automated chromatography in which the mobile phase is a liquid which is

forced under high pressure through a column packed with a sorbent.

1. **Ion exchange chromatography:**

That in which the stationary phase is an [ion exchange resin.](http://medical-dictionary.thefreedictionary.com/resin)

1. **Molecular exclusion chromatography** :

Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute. The liquid or gaseous phase passes through a porous gel which separates the molecules according to its size.

1. **Paper chromatography** :

That using a sheet of blotting paper, usually filter paper, for the adsorption column.

1. **Partition chromatography:**

A method using the partition of the solutes between two liquid phases (the original solvent and the film of solvent on the adsorption column).This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibriates between the mobile phase and the stationary liquid.

1. **Thin-layer chromatography:**

(TLC) chromatography through a thin layer of inert material, such as cellulose



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|  | **ADSORPTION CHROMATOGRAPHY** | **PARTITION CHROMATOGRAPHY** |  |
|  |  |  |  |
|  | In this, mobile phase is adsorbed on the surface | In this, two phase system of immiscible |  |
|  | of stationary phase. Stationary phase in an | solvents are used. The stationary phase is the |  |
|  | adsorption column system is an inorganic or | solvent coating the particles of the support |  |
|  | organic powdered material which has the | solid |  |
|  | ability to adsorb compounds from solution. |  |  |
|  | Common adsorbents used are alumina, silica |  |  |
|  | gel, calcium carbonate, microcrystalline |  |  |
|  | cellulose, starch and finely divided sucrose. |  |  |
|  | **RATE OF SEPARATION** | |  |
|  |  |  |  |
|  | Degree of adsorption depends upon chemical | Rate of separation depends upon solubility |  |
|  | nature and surface area of adsorbent and | of solute. If solute is more soluble in S.P. |  |
|  | affinity of solute between two phases. | there is decrease separation. If solute more |  |
|  | Increase in affinity with S.P. results in less | soluble in M.P. there is increase separation. |  |
|  | separation, Increase in affinity with M.P. results |  |  |
|  | in more separation |  |  |
|  | **EXAMPLES** | |  |
|  |  |  |  |
|  | Column chromatography is the typical example | Paper chromatography is the typical example |  |
|  | in which solid S.P. is placed in tubular column, | of partition chromatography in which S.P. is |  |
|  | M.P. is allowed to flow through S.P. and adsorb. | a layer of water adsorbed on the sheet of |  |
|  | Other example is Thin layer chromatography | paper. |  |
|  |  |  |  |
|  | **USES** | |  |
|  |  |  |  |
|  | It is used to separate unwanted from wanted | It is particularly well suited to the |  |
|  | components in a sample, e.g. separation of | fractionation of biological mixtures, e.g. |  |
|  | hydrocarbons and terpenes from oxygenated | separation of |  |
|  | components of an essential oil. | flavonoids from polyphenolic extract |  |
|  | It is also used to determine the purity of the |  |  |
|  | compounds or employed to remove substances |  |  |
|  | which could interfere with subsequent assay |  |  |
|  | method |  |  |
|  |  |  |  |

**THEORIES OF CHROMATOGRAPHY:**

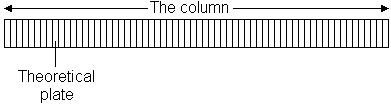
* 1. Plate theory
  2. Rate theory
  3. Band broad theory

1. **Plate theory**

This theory is compared with fractional distillation and totally imaginary theory.

*“More the number of plates or fraction, more separation occur.”*

The plate model supposes that the chromatographic column is contains a large number of separate layers, called *theoretical plates*. Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.



**It is important to remember that the plates do not really exist**; They are a figment of theimagination that helps us understand the processes at work in the column. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, *N* (the more plates the better), or by stating the plate height; the *Height Equivalent to* *a Theoretical Plate* (the smaller the better).

1. **Rate theory**

It is the realistic theory of chromatography.

It depends on the time taken by the mobile phase to flow and results in formation of different chromatographic bands.

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If time of flow increase, Rate of separation decrease, less bands are formed and vice versa

1. **Band broad theory**

Mechanism of action of this theory are:

1. **Eddy diffusion**

The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause broadening of the solute band, because different paths are of different lengths.

1. **Longitudinal diffusion**

The concentration of analyte is less at the edges of the band than at the center. Analyte diffuses out from the center to the edges. This causes band broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion.

1. **Resistance to mass transfer**

The analyte takes a certain amount of time to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase. The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

**PLANE CHROMATOGRAPHY**

Plane chromatography is a form of liquid chromatography in which stationary phase is held in/on a plane surface than in a column.

Planar material used consist of glass, aluminium foil, inert plastic sheet covered with thin layer of column adsorbent.

There are two common types:

1. Paper chromatography
2. Thin layer chromatography

**Paper Chromatography**

1. Paper chromatography is one method for testing the purity of compounds and identifying substances. Paper chromatography is a useful technique because it is relatively quick and requires small quantities of material.
2. Separations in paper chromatography involve the same principles as those in thin layer chromatography. In paper chromatography, like thin layer chromatography, substances are distributed between a stationary phase and a mobile phase. The stationary phase is usually a piece of high quality filter paper. The mobile phase is a developing solution that travels up the stationary phase, carrying the samples with it. Components of the sample will separate on the



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stationary phase according to how strongly they adsorb to the stationary phase versus how much they dissolve in the mobile phase.

**TYPES OF PAPER CHROMATOGRAPHY**

There are two types of development methods:

* + 1. One-dimensional Separation
    2. Two-dimensional Separation

1. **One-dimensional Separation** These are of two types:
   * + 1. Ascending Paper Chromatography
       2. Descending Paper Chromatography
   1. **Ascending Paper Chromatography**

In this solvent is in a pool at the bottom of the vessel in which paper is supported. It rises up the paper by capillary action

* 1. **Descending Paper Chromatography**

In this solvent is in trough from which paper is hung. Solvent flows down the paper by a combination of capillary and gravity action

1. **Two-dimensional Separation**

These are usually carried out by ascending technique and are developed on square paper. Single sample is applied near one corner of the paper and the chromatogram developed as usual. It is then removed from the tank, dried, turned through 90o and replaced in the same a second one and redeveloped.

**Radial Separation**

In this method sample is applied at the centre of the horizontally placed disc of paper/ at the circumference of a small circle whose centre is that of a large paper disc. A wick is situated so that it supplies solvent to the centre from a supply through and the components move outwards along radial paths forming circle of increasing diameter

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| **ONE DIMENSIONAL** | **TWO DIMENSIONAL** |
|  |  |
| One way component separation | Two way component separation |
|  |  |
| Separation occur in one dimension | Separation occur in two dimensions |
|  |  |
| Single direction | 2 solvents are used, moved in 2 directions |
|  |  |
| Single angle | Two angles (90o) |
|  |  |
| Single run | Two runs |
|  |  |
| Single component | Different components |
|  |  |
| Single solvent used | Double solvents |
|  |  |
| Single process | Complex process |
|  |  |

**Stationary phase**

1. Stationary phase is a paper.
2. Whatmann paper which is a glass fiber paper, does not affected by the reagents is usually used.
3. Some **modified cellulose paper** are also used with different components as some cellulose paper with high carboxylic groups is used for the separation of amines, cations and amino acids.
4. **Cellulose ester paper** is used for most of organic substances
5. **ORIENTATION** of the fibre in paper paper also affect the movement of mobile phase overstationary phase
6. Paper usually used consist of highly purified cellulose. Polymeric cellulose structure contain several thousand Anhydro-glucose units linked through oxygen bridges. Theoretically there are three hydroxyl groups on each glucose unit, but during manufacture many of these will have been partially oxidized to aldehyde, ketone or carboxyl group.. these variations are emphasized in practice by presence of manufacturing impurities including Adsorbent salts and mineral matter held on hydroxyl group. Water & polar solvents swell the paper by the formation of hydrogen bond so, acid wash it and dry before use.

**Mobile phase**

1. Surface of paper binds the solute strongly, so mobile phase should be more polar.
2. In most cases CHCl3 hydrophilic, Benzene  polar Cyclohexane  hydrophobic.
3. This combination is used for unknown components.

**PROCEDURE**

1. **Preparing the Mobile phase**

As experimental required, e.g. for one dimensional

n-butanol : water : acetic acid

12 : 5 : 3

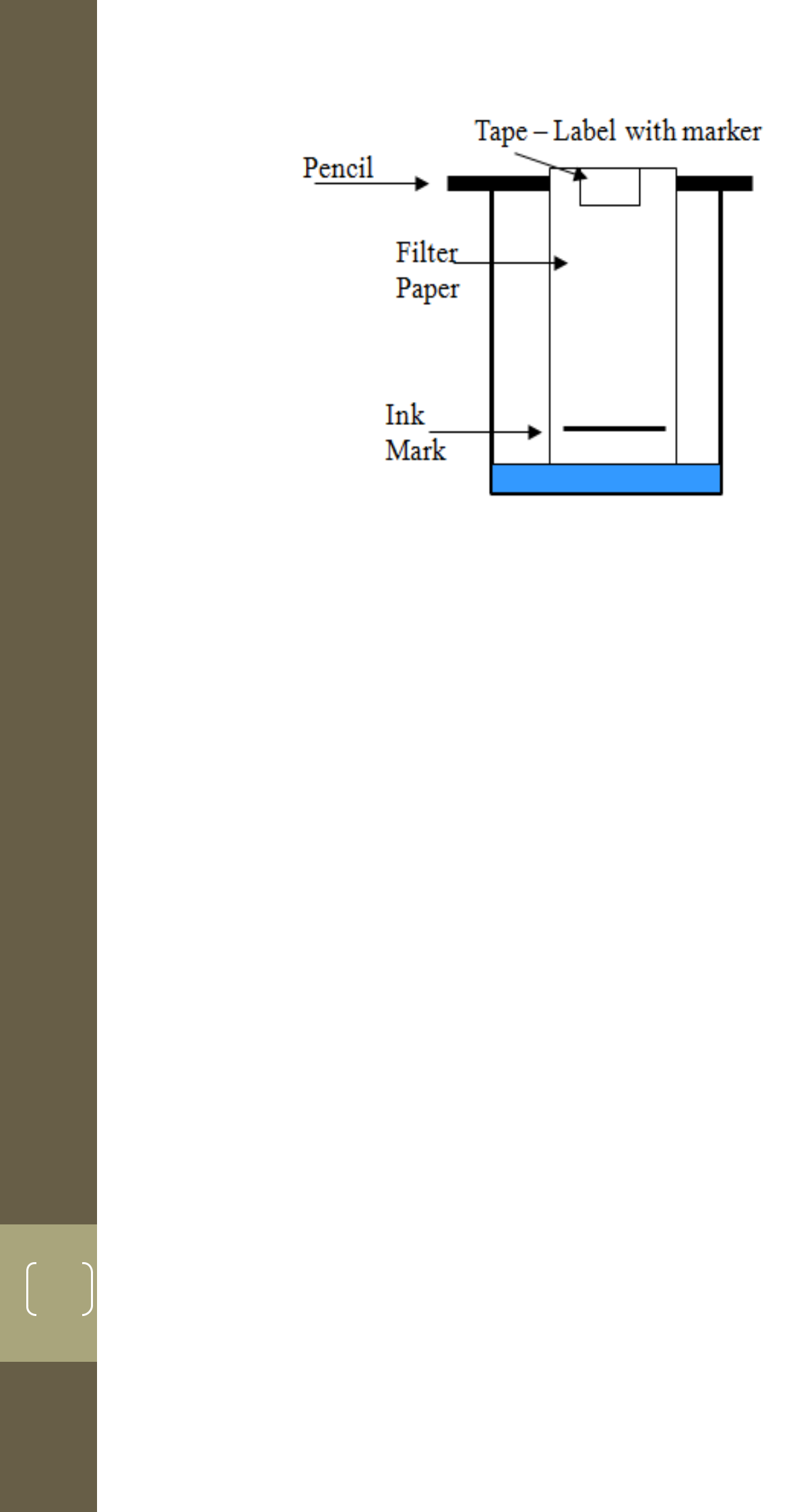
1. **Preparing the Chamber**
   1. Choose a developing chamber that can be sealed well. The chamber should be large enough to hold the paper that is to be developed**.**
   2. The chamber should be clean and dry before use.
   3. Add the mobile phase to the chamber so that it is about 2 cm deep. Seal the chamber tightly and let the chamber stand overnight if possible.
2. **Preparing the Stationary Phase**
   1. Cut a square piece of high-quality filter paper to fit into your development chamber.
   2. With a pencil, draw a straight line about 3 cm from the bottom edge of the paper**.**
3. **Spotting the Samples**
   1. First, each sample should be dissolved in an appropriate volatile.
   2. Conc. Of sample should be 0.1--1000µg depending upon the sensitivity of detection method and purpose of analysis
   3. In case **of ascending chromatography** the line to which sample is applied should be 3— 5cm from base.
   4. Sample applied with microlitre pipette, sample size should be 3—8mm in diameter.
   5. Different samples are applied with distance of 2—3cm or1—5cm.
   6. All spots on the chromatogram should be 2 to 2.5 cm away from the edges of the paper and from each other.
4. **Developing the Chromatograms**
5. After preparing the chamber and spotting the samples, the paper is ready for development. Be careful to handle the paper only by its edges, and try to leave the development chamber uncovered for as little time as possible.
6. Initially, the chromatogram should be suspended in the chamber without touching the solvent. To suspend the chromatogram, to the top of the paper and thread a piece of string throught the paper clip. Then tape the string to the outside of the chamber to hold the chromatogram in place. The paper should hang in the development chamber overnight, if possible.
7. After the chromatogram has hung in the chamber, immerse the paper's bottom edge into the developing solvent.
8. Allow the chromatogram to dry in a well-ventilated area.



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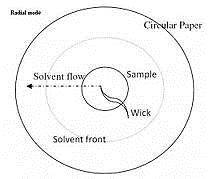


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1. **Development methods**
   1. **Radial separation:**

In this we use pteri dish or a jar. In pteri dish we use a wick and in jar we use a thread.

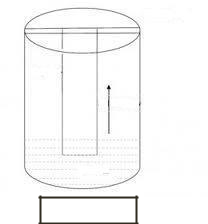


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Radial mode

1. **Ascending development:**

In this capillary action is involved.



Paper 

Solvent flow

Solvent

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Ascending type

1. **Descending development:**

Capillary action and gravity is involved So , separation is faster.

Wire



 Cork

Spot Chamber jar

PaperDescending chamber

1. **Identifying the Spots**
   1. If the spots can be seen, outline them with a pencil.
   2. If the spots are not obvious, the most common visualization technique is to hold the paper under an ultraviolet lamp. (Caution: Do not look directly into the lamp!) Many organic compounds can be seen using this technique. Locating agents for different components e.g. ninhydrin for amino acids. Outline the spots with a pencil.
   3. Several methods are used to locate the compound and make them visible.
      1. **Chemical method**

Colourless compounds on the paper are converted to coloured products by treating with coloured reagent.

Solution of reagent can be applied by dipping the paper into the solution or by spraying the solution onto paper.

* + - 1. **Dipping technique**

Take shallow tray in which chromatogram is dipped without touching the slides and then dry it, e.g. acetone (better because evaporate rapidly)

* + - 1. **Spraying technique**

A solution of reagent is sprayed over the surface of chromatogram by means of an atomiser or by special chromatogram spray bottle, e.g. Ninhydrine

* + 1. **Physical methods**
       1. **UV Detection**

Compounds which are invisible on chromatogram in ordinary light can readily be detected under an UV-lamp. Wavelength of light emitted and colour is observed which is characteristics of the compounds and are useful for the purpose of identification (wavelength = 254nm)

* + - 1. **Radioactivity**

Widespread use of nuclear power has made available a number of labelled or radioactive compounds which can be used to detect components on chromatograms by means of special counter.

1. **Interpreting the Data**
   1. The Rf value for each spot should be calculated.
   2. Rf stands for "ratio of fronts" and is characteristic for any given compound.
   3. Hence, known Rf values can be compared to those of unknown substances to aid in their identifications**.**

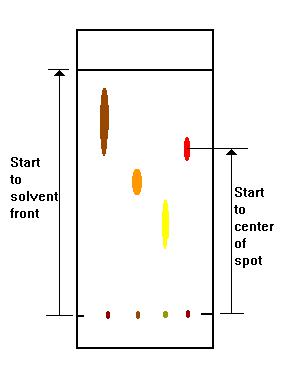


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(**Note**: Rf values often depend on the temperature, solvent, and type of paper used in the experiment; the most effective way to identify a compound is to spot known substances next to unknown substances on the same chromatogram.)

1. In addition, the purity of a sample may be estimated from the chromatogram. An impure sample will often develop as two or more spots, while a [**pure sample will show only one**](http://www.uwplatt.edu/chemep/chem/chemscape/labdocs/catofp/chromato/paper/purity.htm) [**spot.**](http://www.uwplatt.edu/chemep/chem/chemscape/labdocs/catofp/chromato/paper/purity.htm)

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Reagent** |  |  | **Colour of Spots** |  |  | **Components Detected** |  |
|  |  |  |  |  |  |
|  | |  |  |  |  |  |  |  |
|  | **Ninhydrin** |  |  | Pink/purple |  |  | Amino acid & Amines |  |
|  | |  |  |  |  |  |  |  |
| **Iodine Vapours** | |  |  | Brown |  |  | General organic & | |
|  |  |  |  |  |  |  | Unsaturated compounds | |
|  | **Antimony Chloride** |  |  | Various |  |  | Steroids, Acyclic, |  |
|  |  |  |  |  |  |  | Vitamins, Carotenoids |  |
| **Bromophenol Blue** | |  |  | Yellow |  |  | Carboxylic acids | |
|  |  |  |  |  |  |  |  |  |

**APPLICATIONS**

1. It is used in the separation of amino acids and peptides in the investigation of protein structure.
2. It is used for routine examination of urine and other body fluids for amino acids and sugars.
3. For separation of purine bases and nucleotides in the examination of nucleic acids.
4. For separation of steroids.
5. For analysis of polymers.
6. 6. In investigation of phenolic materials in plant extract.
   1. For the separation of alkaloids.
   2. For the detection metals in soils and geological specimens.
   3. In the separation of radioisotopically labelled compounds.

**ADVANTAGES**

Major advantage of the paper chromatography is the sensitivity with which compound can be located after separation.

**DISADVANTAGES**

This technique cannot be used in the separation of volatile substances such as hydrocarbons and volatile fatty acids

**Thin Layer Chromatography**

**Definition:**

Thin layer chromatography is a method of analysis in which a stationary phase, a finely divided solid, is spread as thin layer on a rigid supporting plate; and the mobile phase, a liquid, is allowed to migrate across the surface of the plate.

**Principle:**

An analyte migrates up or across the layer of stationary phase under the influence of the mobile phase which moves through the stationary phase by capillary action and the distance moved by the analyte is determined by its relative affinity or solubility for stationary phase and mobile phase.

**THEORY OF THIN LAYER CHROMATOGRAPHY**

1. In the thin layer chromatography, a solid phase ,the adsorbant, is coated on a solid support as a thin layer (about 0.25 mm thick).
2. In many cases, a small amount of a binder such as plaster of Paris is mixed with the absorbent to facilitate the coating. Many different solid supports are employed, including thin sheets of glass, plastic, and aluminum.
3. The mixture (A plus B) to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom.
4. A solvent, or mixture of solvents, called the **eluant**, is allowed to flow up the plate by capillary action. At all times, the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. Any one molecule will spend part of the time sitting still on the adsorbent with the remainder moving up the plate with the solvent.
5. A substance that is strongly adsorbed (say, A) will have a greater fraction of its molecules adsorbed at any one time, and thus any one molecule of A will spend more time sitting still and less time moving.
6. In contrast, a weakly adsorbed substance (B) will have a smaller fraction of its molecules adsorbed at any one time, and hence any one molecule of B will spend less time sitting and more time moving.
7. Thus, the more weakly a substance is adsorbed, the farther up the plate it will move. The more strongly a substance is adsorbed, the closer it will stay near the origin.
8. Several factors determine the efficiency of a chromatographic separation.
9. The adsorbent should show a maximum of selectivity toward the substances being separated so that the differences in rate of elution will be large.
10. For the separation of any given mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing.
11. Table 1 lists a number of adsorbents in order of adsorptive power.

**CHROMATOGRAPHIC ADSORBENTS**.

1. The order in the table is approximate, since it depends upon the substance being adsorbed, and the solvent used for elution.



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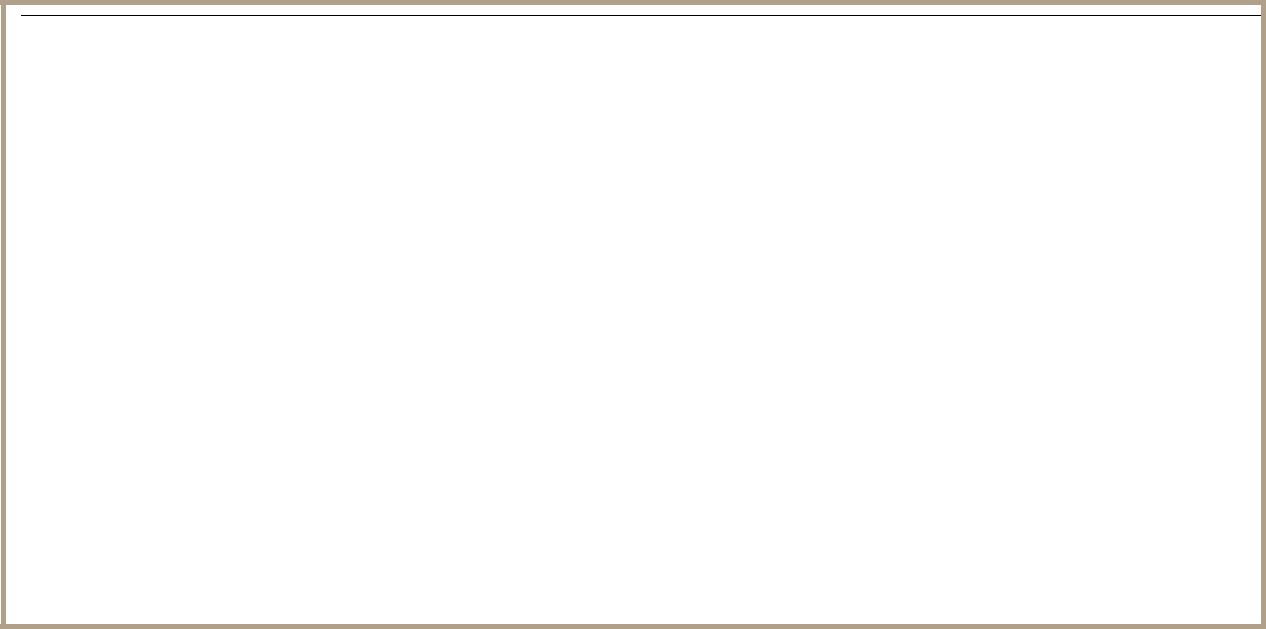


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| *Most Strongly Adsorbent* | Alumina | Al2O3 |
|  | Charcoal | C |
|  | Florisil | MgO/SiO2(anhydrous) |
| *Least Strongly Adsorbent* | Silica gel | SiO2 |

**PROPERTIES OF ELUTING SOLVENTS**

1. The eluting solvent should also show a maximum of selectivity in its ability to dissolve or desorb the substances being separated. The fact that one substance is relatively soluble in a solvent can result in its being eluted faster than another substance. However, a more important property of the solvent is its ability to be itself adsorbed on the adsorbent. If the solvent is more strongly adsorbed than the substances being separated, it can take their place on the adsorbent and all the substances will flow together.
2. If the solvent is less strongly adsorbed than any of the components of the mixture, its contribution to different rates of elution will be only through its difference in solvent power toward them. If, however, it is more, strongly adsorbed than some components of the mixture and less strongly than others, it will greatly speed the elution of those substances that it can replace on the absorbent, without speeding the elution of the others.
3. Lists a number of common solvents in approximate order of increasing adsorbability, and hence in order of increasing eluting power. The order is only approximate since it depends upon the nature of the adsorbent



**Eluting solvents for chromatography**

*Least Eluting Power (alumina as adsorbent)* Petroleum ether (hexane; pentane)

Cyclohexane

Carbon tetrachloride

Benzene

DichIoromethane

Chloroform

Ether (anhydrous)

Ethyl acetate (anhydrous)

Acetone (anhydrous)

Ethanol

Methanol

Water

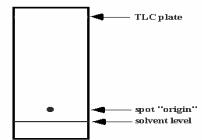
Pyridine

*Greatest Eluting Power* Organic acids

*(alumina as adsorbent)*

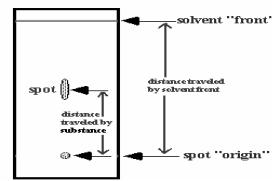
**TECHNIQUE OF THIN LAYER CHROMATOGRAPHY**

1. The sample is applied to the layer of adsorbent, near one edge, as a small spot of a solution.
2.  After the solvent has evaporated, the adsorbent-coated sheet is propped more or less vertically in a closed container, with the edge to which the spot was applied down.
   1. The spot on the thin layer plate must be positioned above the level of the solvent in the container. If it is below the level of the solvent, the spot will be washed off the plate into the developing solvent.
3. The solvent, which is in the bottom of the container, creeps up the layer of adsorbent, passes over the spot, and, as it continues up, effects a separation of the materials in the spot ("develops" the chromatogram).
4. When the solvent front has nearly reached nearly the top of the adsorbent, the thin layer plate is removed from the container.



***Position of the spot on a thin layer plate***

1. Since the amount of adsorbent involved is relatively small, and the ratio of adsorbent to sample must be high, the amount of sample must be very small, usually much less than a milligram.
2. For this reason, thin-layer chromatography (TLC) is usually used as an analytical technique rather than a preparative method.
3. With thicker layers (about 2 mm) and large plates with a number of spots or a stripe of sample, it can be used as a preparative method.
4. The separated substances are recovered by scraping the adsorbent off the plate (or cutting out the spots if the supporting material can be cut) and extracting the substance from the adsorbent.



**Figure *:****TLC plate showing distances travelled by the spot and the solvent after solvent front**nearly reached the top of the adsorbent.*

1. Because the distance travelled by a substance relative to the distance travelled by the solvent front depends upon the molecular structure of the substance, TLC can be used to identify substances as well as to separate them. The relationship between the distance traveled by the

solvent front and the substance is usually expressed as the **Rf** **value**:

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1. The Rf values are strongly dependent upon the nature of the adsorbent and solvent.
2. Therefore, experimental Rf values and literature values do not often agree very well.



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1. In order to determine whether an unknown substance is the same as a substance of known structure, it is necessary to run the two substances side by side in the same chromatogram, preferably at the same concentration.

**Stationary Phase**

As stationary phase, a special finely ground matrix (silica gel, alumina, or similar material) is coated on a glass plate, a metal or a plastic film as a thin layer (~0.25 mm). In addition a binder like gypsum is mixed into the stationary phase to make it stick better to the slide. In many cases, a fluorescent powder is mixed into the stationary phase to simplify the visualization later on (e.g. bright green when you expose it to 254 nm UV light).

**Mobile phase**

Criteria for Choosing a Preparative Solvent includes

* 1. Solubility
  2. Affinity
  3. Resolution

1. **Solubility**

Many solvent systems provide the minimal solubility for the sample, but to elute a sample from a column the mobile phase must have a greater solubility for the sample, as the sample concentration is usually very high. When possible, it is best to dissolve the sample in the mobile phase. The first step in solvent selection determination of the solubility of the sample. The desired mobile phase would provide the greatest solubility, while providing affinity for the sample on the stationary phase.

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**Solvent Solubility Screening Table**

1. Water
2. Methanol
3. Ethanol
4. Acetone
5. Diethyl Ether
6. Ethyl Acetate
7. Dichloromethane
8. Toluene
9. Chloroform
10. Cyclohexane
11. Petroleum Ether
12. Hexane



1. **Affinity**

To achieve a separation, the sample must have a relatively equal affinity for the solvent and the packing material. If the sample has a higher affinity for the stationary phase than the solvent, the sample will remain at the origin (Rf value will be too low).

1. **Resolution**

Resolution is improved by optimizing the affinity between sample, solvent, and support. The optimum solvent for separating two or more compounds will maximize the difference in the compounds. Most TLC and preparative mobile phase systems contain a polar solvent and a chromatographically dissimilar less-polar solvent. As a guide for method development, a

substitution in the polar solvent often results in a change in resolution, while a change in the

1. less-polar solvent results primarily in a change in Rf of the sample components. The table below shows some common tendencies of various functional groups to adsorb onto the silica.

**Affinity of Functional Groups for Silica Gel**

-NH2 Amine



-COOH Carboxylic Acid



-COH Alcohol

-CONH2 Amide

-C=O Carbonyl

-C=-CO2R Ester

-C-O-C Ether

-C1 Halocarbons

-CC- Hydrocarbons

**PROCEDURE**

1. **Preparation Of Plates**

Different sized plates are available

* 1. 5×20 cm
  2. 10×20cm
  3. 20×20cm

They are usually made up of glass but plastic stainless steel or aluminium backing are also used

1. **Preparation of slurry**

**Stationary phase** should consist of small particles of uniform size so as to provide a large areafor interaction and a small void volume (to reduce band broadening). The particles are mixed with water or organic solvent to form a slurry. A suitable binding agent added and fluorescent indicator (internal indicator) such as zinc silicate may be included to aid in detection of solute after development

After this slurry is applied on the plates by one of the following methods

1. **Method to apply the slurry:**

Main objective is to produce and absolutely even layer with lumps or gaps which adhere evenly and securely to the sport

* 1. **Spreading Method:**

It is used to spread the slurry on the rigid plate by using an applicator e.g. by roller or glass rod.

* 1. **Dipping Method:**

Used for microscopic slides for analysis for some components. We make slurry and dip glass slide in it, then withdraw and dry it.

* 1. **Tapping Method:**

Plastic tape is applied on both sides of glass plates and in the center thin layer of slurry is formed by using roller or glass rod.

1. **Sample application**

After the plates have been dried and conditioned, if necessary in a controlled humidity chamber, the samples, which may range from a few µg to mg dissolved in 10 to1000µL of a volatile solvent, a spotted usually with a capillary tube or a microliter syringe. Samples may be applied as spot or as thin streaks, but it is essential that all of solvents be evaporated between repeated applications and the area of sample application be kept as small as possible, because the bands will broaden as they travel up the plate.

1. **Detection methods**

Once the chromatogram has developed the solute spots must be made visible in order to determine their Rf value if the substance are highly coloured there is no difficult in visual detection.

**In case of colourless substance:**

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In case of colourless substance most organic compounds don’t absorb visible light and ultraviolet used to examine the separated compounds

**Fluorescence quenching:**

It is a particularly used technique for detection of compound that absorb at 254 nm .

Usually iodine vapours (iodine ions) is used as quencher, which forms charge transfer with the invisible components decreasing their fluorescence and making U.V light absorption possible and making them visible.

**APPLICATIONS**

1. Used for qualitative analysis by measuring Rf values and for quantitative analysis by using scraping and spectrodensitometric method.
2. For detection of narcotive and stimulant drugs
3. Identification and isolation of active chemical from plants and crude extracts
4. Determination of glycerol in tobacco
5. Determination of VitaminB1 in pharmaceutical products
6. Determination of essential oils in herbal drugs.
7. Determinations of plant pigments
8. Detection of pesticides or insecticides in food.
9. Analysing dye composition of fibres in forensics.
10. Monitoring chemical reactions and for qualitative analysis of reaction products

**IMPORTANCE OF TLC**

1. Pharmaceutics and drugs

Identification, purity, testing and determination of concentration of active ingredients and preservatives in drugs and drug preparations process control in synthetic manufacturing process.

1. Clinical chemistry, forensic chemistry & biochemistry

Determination of active ingredients and their metabolites in biological matrices, diagnosis of metabolic disorders, e.g. phenylketonuria

1. Cosmetology

Dye, raw materials and end products, preservatives, surfactants, fatty acids constituents of perfume.

1. Food analysis

Determination of pesticides and fungicides in drinking water and food. Residues in vegetables, salads and meat. Vitamins in soft drinks.

1. Environmental analysis

Ground water analysis, determination of pollutants from abandoned ornaments in soil and surface water.

**ADVANTAGES**

Preferred over the paper chromatography due to following:

1. Faster runs
2. Better separation
3. Choice between different stationary phases
4. Simplicity
5. Speedy method
6. No expensive equipment used

**COLUMN CHROMATOGRAPHY**

It is a separation technique in which solid phase (adsorbent) is held in a vertical tube. Mixture to be separated is placed to the top of column and mobile phase is forced through the column under the influence of pressure or gravity.

**TYPES OF COLUMN CHROMATOGRAPHY**

Column chromatography is divided into two categories, depending on how the solvent flows down the column.

* 1. Gravity Column Chromatography
  2. Flash Chromatography

1. **Gravity Column Chromatography**

If solvent is allowed to flow down by gravity or percolation, it is called Gravity Column Chromatography.

1. **Flash Chromatography**

If solvent is forced down the column by positive air pressure through the pumps, it is called Flash Chromatography.

**COLUMN**

The classical preparative chromatography column, is a glass tube with a diameter from 5 mm to 50 mm and a height of 50 cm to 1 m with a tap and some kind of a filter (a glass frit or glass wool plug – to prevent the loss of the stationary phase) at the bottom.

**ADSORBENT (Stationary Phase)**

Adsorbent in column chromatography must be solid porous to finely divided powder. Most commonly used adsorbent is Silica gel, followed by Alumina Cellulose Powder has often been used.

Adsorbent particle size affects how the solvent flows through the column.

1. **Smaller particle**

Smaller particles are used for flash chromatography, e.g. 230-400 mesh.

1. **Larger particle**

Larger particles are used for gravity chromatography, e.g. 70-230 silica gel

**ELUENT (Mobile phase)**

1. Eluent is either a pure solvent (organic) or mixture of different solvents, e.g. Hexene, Ethyl acetate, ethanol and water.
2. The polarity of the solvent, which is passed through the column, affects the relative rates at which compounds move through the column.
3. If the solvent is too polar, movement becomes too rapid, and little or no separation of the components of the mixture will result.
4. If solvent is polar enough, no compounds will elute from the column.
5. Often a series of increasingly polar system are used to elute a column. A non-polar solvent is first used to elute a less polar compound.
6. Once the less polar compound is passed off the column, a more polar solvent is added to the column to elute the more polar compound.

**PACKING OF COLUMN**

1. First step is slurry preparation, in which adsorbent is mixed with the solvent which is miscible with the eluent.
2. Then fix cotton plug in the glass column.
3. Pour sand on it until a layer is formed on its surface.
4. Place slurry on this layer an d then fix filter paper on the slurry.
5. Again place sand over filter paper to prevent shock absorber.



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1. Now add mobile phase from top.
2. Open stopper until all materials are evenly packed or compressed.

**SAMPLE APPARATUS**

Sample to be analysed is dissolved in a very small amount of solvent or in concentrated solution and added to the top of the column.

**ELUTION**

1. As mobile phase is entered from the top, pinch clamp is opened and the solvent is allowed to drain just to the top of the column.
2. A couple small portions of the eluting solvent are added and allowed to drain in until mixture is a little ways absorb into the adsorbent and then column is filled to the top with eluting solvent.
3. Column is now ready to run continue adding at the top with eluting solvent, and collecting fractions at the bottom until the compounds elute.
4. The compound having more affinity with stationary phase, retain in the column.
5. If applicable, change the eluting solvent to a more polar solvent during the eluting process.
6. Never let the solvent level drop below the top level of the adsorbent . the process is discontinued when the compounds desired are off the column.

**ANALYSIS**

1. If the compounds separated in a column chromatography procedure are coloured progress of the separation can simply be monitored visually.
2. More commonly, compounds to be isolated from the column chromatography are colourless.

In this case, after specific period of time, small fractions of the eluent are collected sequentially in labelled tubes and the composition of each fraction is analysed by Thin Layer Chromatography.

After running the sample over TLC, identify and observe in day light or UV light. In every test tube separate component is obtained

**ADVANTAGES**

1. Column Chromatography is meant for preparative and analytical purpose.
2. Pure single component can be separated
3. Special attention and special technique is required.

**DISADVANTAGES**

Main disadvantages of this technique is that it is more time consuming process.

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