PAPER CHROMATOGRAPHY
AND
COLUMN CHROMATOGRAPHY

1. Chromatography:
1.1 Introduction:

Chromatography (Greek: Khromatos-colour and graphos-writing), a relatively new separation technique, was discovered by Dr. Michael Tswett (1906) in Warsaw.

He was successful in doing the separation of chlorophyll, xanthophylls and several other colored substances by percolating vegetable extracts through a column of calcium carbonate. The calcium carbonate column acted as an adsorbent and the different substances got adsorbed to different extent and this gives rise to colored bands at different positions. Tswett termed this system of colored bands as the chromatogram and the method as chromatography.

- In 1855, Friedrich Runge obtained a colored chromatogram by impregnating a filter paper with ferric sulphate, drying and adding a drop of potassium ferrocyanide solution.
- In 1865, Schoenbein observed that when a strip of filter paper was dipped into an aqueous solution of salt, water raised up the filter paper by capillary action carrying the salt with it.
- Kuhn’s remarkable work of separating carotene and resolving it into α and β isomers caused an awakening of the importance of adsorption in analytical field.
- In 1941, Martin and Synge who introduced partition column chromatography.
- In 1952, Martin and James were awarded Nobel Prize for developing gas chromatography.

1.2 Definition:

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases, based on the differences in the rate at which the components of a mixture move through a porous medium under the influence of some solvent or gas.

1.3 Classification of Chromatographic methods:

In all the chromatographic techniques, difference in affinity involves the process of either adsorption or partition. In adsorption, the binding of a compound, to the surface of solid phase takes place where as in partition the relative solubility of a compound in two phase’s results, in the partition of a compound between two phases.
Chromatography

1. Adsorption chromatography competition between solid and
   - Gas: gas-solid Chromatography (GSC).
   - Liquid: Liquid Chromatography (LLC).
     - Adsorption column Chromatography (ACC).
     - Thin layer Chromatography (TLC).

2. Partition chromatography competition between liquid and
   - Gas: gas-liquid Chromatography (GLC).
   - Liquid: Liquid-liquid Chromatography (LLC).
     - Partition column Chromatography
     - Paper Chromatography (PC).
     - Thin layer Chromatography (TLC).
     - Reversed phase partition Chromatography (RPC).

1.4 TECHNIQUES:

1. **Paper chromatography**: It is a technique in which mixtures of substances are separated by means of partition between a moving solvent and a stationary liquid which is held on a suitable solid support.

2. **Adsorption chromatography**: It is a technique in which small differences in the adsorption behavior of substances between a moving solvent (liquid or gas) and a stationary solid phase are utilized to achieve the separation.

3. **Ion exchange chromatography**: This method is based upon differences in the exchange potential between various ions exchange resin packed in a column.

   In cation exchange, cations with high valency are strongly retained while monovalent ions are easily removed. In anion exchange methods, anions with a high charge are strongly retained in relation to ones having a low charge.

4. **Exclusion chromatography**: The technique is based fundamentally upon exploitation of the size or molecular geometry of the components.

5. **Electro chromatography**: In electro chromatographic methods columns or plates are used. Separation based on the difference in mobility of different ions when an external DC potential is applied.
2. PAPER CHROMATOGRAPHY

2.1 Introduction:

The principle involved is partition chromatography where in the substances are distributed or partitioned between to liquid phases. One phase is the water which is held in pores of filter paper used and other phase is that of 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 Paper chromatography:

The principle can also be adsorption chromatography between solid and liquid phases, where in 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 to liquid phases.

Types of principle of Paper chromatography:

1. Paper partition chromatography: In PPC technique, the substances in a mixture move in two non mixing solvents and create partition between them.

2. Paper adsorption chromatography: In PAC the filter paper is impregnated with adsorbent such as alumina or silica and a single solvent is allowed to flow over the unknown components. Some of the components may be adsorbed more strongly than the others facilitating separation of the components.

2.2 Theory of Paper chromatography:

In paper chromatography two types of forces operate when a drop of solution is applied on the filter paper and treated with a solvent.

- Propelling force: it tries to drag the substance in the direction of the flow of solvent. This depends upon;
  1. The rate of the solvent flow and
  2. The solubility of the substances in the solvent.

- Retarding force (R<sub>f</sub>): retarding force tries to drag the substance behind towards its point of application. This retardation depends on the adsorption and partition.

Retardation factor, R<sub>f</sub> value: it is expressed in terms of retention volume (VR) or retention time (t<sub>R</sub>). The relative rate of the movement of solvent and solute is expressed by a term R<sub>f</sub> value. It is defined as the ratio of the distance travelled by the compound at its point of max concentration to the distance travelled by the solvent. R<sub>f</sub> value has no units.
Distance traveled by component from application point

$$R_f = \frac{\text{Distance traveled by component from application point}}{\text{Distance traveled by solvent from application point}}$$

**Rx value:** is the ratio of the distance travelled by the substance to the distance travelled by a chemically similar standard substance.

### 2.3 TYPES OF PAPER CHROMATOGRAPHY:

**A) Descending paper chromatography:**

When the development of the paper is done by allowing the solvent to travel down the paper, it is known as descending technique. The apparatus consists of well sealed glass tank of suitable size and shape which is provided with a trough for the mobile phase in the upper portion. The paper with the same sample spotted is inserted with the upper end in the trough containing the mobile phase, the jar itself having been equilibrated with the mobile phase prior to elution.

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

![Fig1: Descending paper chromatography](image)

**B) Ascending paper chromatography:**

When the development of the paper is done by allowing the solvent to travel up the paper it is known as ascending technique. The mobile phase is placed in a suitable container at the bottom of the chamber. The sample is applied a few centimeters from the bottom edge of the paper suspended from a hook. Alternatively the paper may be rolled into a cylinder, held together by staple, string or plastic clips.
C) **Ascending-Descending chromatography:**
It is the hybrid of the above two techniques. The upper part of the ascending chromatography can be folded over a glass rod allowing the ascending development to change over into the descending after crossing the glass rod.

D) **Radial paper chromatography:**
This is also known as circular paper chromatography. This makes use of radial development. A circular filter paper is employed. Then the various materials to be analyzed are placed at the center. After drying the spot the paper is fixed horizontally on the petridish possessing the solvent so that the tongue or the wick of the paper dips into the solvent. Cover the paper by means of petridish cover. The solvent rises through the tongue or the wick. When the solvent front has moved through a sufficient large distance, the components get separated in the form of concentric circular zones.
E) **Two-dimensional chromatography:**

The square or rectangular paper is used. The sample is applied to one of the corners. The second development is performed at right angle to the direction of the first run. This type of chromatography can be carried out with identical solvent system in both the directions or by two solvent systems.

This technique can be understood by spotting the sample at lower corner of the rectangular sheet of the filter paper so that the spot is situated above the surface of the developing solvent in the trough. The dried paper is kept at its edge in the solvent and developed by either ascending or descending technique. When the solvent reaches the opposite edge of the paper, it is removed from the tank and dried. The system is changed to second liquid. The filter paper is now rotated through $90^\circ$ so that the edge having the series of spots is now at the bottom, just above the solvent trough and the chromatogram is run as before. The chromatogram is now having spots of the solute scattered all over the paper.
2.4 TECHNIQUE OF PAPER CHROMATOGRAPHY:

1) Choice of the proper chromatographic technique:
First select the mode of paper chromatographic technique, i.e., ascending, descending, ascending-descending, and radial or two dimensional techniques. The choice of the technique depends on the nature substance to be separated.

2) Choice of the filter paper:
The filter paper plays an important role in the success of paper chromatography. The choice of paper depends on as follows:
- Whether the paper is being used for quantitative or qualitative analysis.
- Whether it is used for analytical or preparative chromatography.
- Whether the substances used are hydrophilic or lipophilic neutral or charged species.
Various types of Whatmann chromatography papers are available upon type of separation. The chemical composition of Whatmann filter is:
- α -cellulose 99%,
- β –cellulose 0.3-1%
- Pentosans 0.5-0.8%
- Ash 0.07-0.08%
- Ether soluble substances 0.01-0.1%

### Characteristics of Whatmann filter paper

<table>
<thead>
<tr>
<th>S.No</th>
<th>Rate of flow</th>
<th>Fast</th>
<th>Medium</th>
<th>Slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Thin paper</td>
<td>No.4</td>
<td>No.7</td>
<td>No.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No.54</td>
<td>No.1</td>
<td>No.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No.540</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Thick paper</td>
<td>No.31</td>
<td>No.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No.17</td>
<td>No.3MM</td>
<td>-</td>
</tr>
</tbody>
</table>

### Modified papers for paper chromatography

<table>
<thead>
<tr>
<th>S.No</th>
<th>Type</th>
<th>Typical uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Carboxyl papers</td>
<td>Cationic separation of protonated amines and amino acids.</td>
</tr>
<tr>
<td>2</td>
<td>Acetylated papers</td>
<td>Reversed phase chromatography of lipophilic substances like steroids, insecticides and pigments and also metal cations.</td>
</tr>
<tr>
<td>3</td>
<td>Kieselguhrpapers, Aluminapapers, Zirconiapapers, Silica papers</td>
<td>Separation of low polarity substances such as amines, fatty acids, steroids triglycerides, vitamins and pesticides.</td>
</tr>
<tr>
<td>4</td>
<td>Ion exchange papers</td>
<td>Ion exchange paper chromatography.</td>
</tr>
</tbody>
</table>

### Proper developing solvent:

The choice of this depends upon the simple fact that Rf values should be different for different constituents present in a mixture. Many combinations of solvent systems can be used provided they are not completely miscible with one another. The following are usual criteria for a good solvent system.

- The Rf values of sample should be lie between 0.05 and 0.85 in the system.
- The difference between the Rf value of any two components must be 0.05 the min value necessary in order to separate any two components.
- The distribution ratios of the components in the solvent system should be independent of concentration so as to get circular spots.
- The solvents should not undergo chemical reaction with any of the components of the sample mixture.
- The solvent should not interfere with the detection of the spots on the developed chromatogram.
- The composition of the solvent system should not alter with time.
Mayri (1st M.Pharm, Pharmaceutical analysis, NRI College of pharmacy, A.P, India)

<table>
<thead>
<tr>
<th>S.No</th>
<th>solvent</th>
<th>t20</th>
<th>t25</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n-hexane</td>
<td>1.89</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Cyclo hexane</td>
<td>2.02</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Carbon tetra chloride</td>
<td>2.24</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Benzene</td>
<td>2.29</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Toluene</td>
<td>2.44</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Trichloroethylene</td>
<td>3.40</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Diethyl ether</td>
<td>4.34</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Chloroform</td>
<td>4.91</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Water</td>
<td>80.40</td>
<td>78.50</td>
</tr>
</tbody>
</table>

The solvent systems have been formed to be most suited for separating almost all mixtures. They are listed below in the order of decreasing polarity of the stationary phase.

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>Isopropanol: ammonia water(9:1:2)</td>
</tr>
<tr>
<td>Water</td>
<td>n-butanol : acetic acid: water(4:1:5)</td>
</tr>
<tr>
<td>Water</td>
<td>Phenol saturated with water</td>
</tr>
<tr>
<td>Liquid paraffin</td>
<td>Dimethyl formamide: methanol: :water(10:10:1)</td>
</tr>
</tbody>
</table>

Multiple developments are the improvement in the spot resolution can be attained by multiple development technique. The paper spotted with the sample is first developed using one solvent system as the mobile phase. As soon as the solvent front reaches the edge of the paper, the development gets stopped and the chromatogram dried the paper is then developed in a second solvent system.

4) Preparation of samples: This technique provides an easy way to separate the components of a mixture.

- A drop of mixture is placed in one corner of a square of absorbent paper.
- One edge of the paper is immersed in a solvent.
- The solvent migrates up the sheet by capillary attraction.
- As it does so, the substances in the drop are carried along at different rates.
- Each compound migrates at a rate that reflects solubility in the solvent.
- After a second run at right angles to the first (often using a different solvent), the various substances will be spread out at distinct spots across the sheet, forming a chromatogram.
- The identity of each spot can be determined by comparing its position with the position occupied by known substances under the same conditions.
- In many cases, a fragment of the paper can be cut away from the sheet and chemical analysis run on the tiny amount of substance in it.

It is not possible to give any standard procedure for preparation of samples. The sample volume of 10-20 microns having as many microgram of the substance is ideal quantity to be sported.

5) Spotting: The horizontal line is drawn on the filter paper by lead pencil. This line is known as origin line; on this cross marks are made at least 2cm away from each other.
6) **Drying the chromatogram:** The wet chromatograms after development are dried in special drying cabinets which are being heated electrically with temperature controls.

7) **Detection:**
In detection two methods are available:
- a) Non specific methods.
- b) Specific methods.

a) **Non specific methods:**
- **Iodine chamber:** In a beaker between two cotton beds iodine bolls are taken, the chamber is filled with iodine vapors then chromatogram is placed in it after some time we can observe brown spots.
- **U.V chamber:** Fluorescent compounds are observed light if short wavelength and emitted light is characteristic of the compound and imparts a color to it.

b) **Specific methods:** Some reagents are specific for some specific compounds.
- Ninhydrin reagent for amino acids.
- Dragendorff’s reagent for alkaloids.
- Ferric chloride for phenols.
- 3, 5dinitro benzoic acid for cardiac glycosides.
- 2, 4dinitro phenyl hydrazine for aldehydes and ketones.

The detection methods can also be classified into two types:
- a) **Destructive methods**
  Eg:
  - Ninhydrin reagent for amino acids.
  - Dragendorff’s reagent for alkaloids.
  - Ferric chloride for phenols.
  - 3, 5dinitro benzoic acid for cardiac glycosides.
  - 2, 4dinitro phenyl hydrazine for aldehydes and ketones.
- b) **Non Destructive methods**
  Eg:
  - **Iodine chamber:** In a beaker between two cotton beds iodine bolls are taken, the chamber is filled with iodine vapours then chromatogram is placed in it after some time we can observe brown spots.
  - **U.V chamber:** Fluorescent compounds are observed light if short wavelength and emitted light is characteristic of the compound and imparts a color to it.
2.5 APPLICATIONS:

Uses and applications of paper chromatography
1. Paper chromatography is specially used for separation of mixture having polar and non polar compounds.
2. for separation of antibiotics, alkaloids, glycosides, amino acids.
3. It is used to determine organic compounds, biochemicals in urine etc.
4. In Pharma sector for determination of hormones, drugs.
5. Sometimes used for evaluation of inorganic compounds like salts and complexes.
6. Useful for the identification of impurities and foreign substances in drugs and plant extracts.
7. Identification of Inks by Paper Chromatography:

A. This lab will be done as collaboration between both lab pairs at a bench. One lab pair will use 2 parts isopropanol to 1 part water as a solvent (labeled 2:1 IPA). One lab pair will use 1 part isopropanol to 2 parts water as a solvent (labeled 1:2 IPA).

B. Obtain a strip of chromatography paper about 2.5 cm wide by 10 cm tall. Along one of the shorter sides, draw a horizontal line in pencil about 1.5 cm from the edge of the strip. This will be your “baseline”, the starting line where the samples will be spotted. Graphite will not be carried up the chromatography paper. This baseline will be used later in the calculations of R_f values.

C. Using the black felt tip pens, apply generous dots of ink on the baseline according to the diagram in the technique section.

D. With a pencil, label each dot with the identity of the pen from which the ink came.

E. Obtain a paper strip from your instructor that is marked with one of the three pens. This is the unknown ink sample that you must identify.

F. Once your two strips are prepared, set up a developing chamber using a 400 ml beaker. Pour some of the developing solvent in the beaker, using only enough solvent to cover the bottom of the beaker (about 5-10 ml). Remember, one lab pair will use 1:2 IPA and the other lab pair will use 2:1 IPA.

G. Place the paper strips into the developing chamber so that the edge near the ink spots is submerged in the solvent. The ink spot must be above the solvent level. You should avoid allowing the paper strips to touch each other or the sides of the beaker. (Taping the top of the strips to the outside of the beaker may be helpful.) Once you have inserted the paper, cover the chamber with a watchglass and make sure the solvent is progressing up the paper.

H. When the solvent has risen almost to the top of the paper strip, remove the strip from the chamber and immediately mark the level to which the solvent has risen and circle each spot present on the strip with a pencil. (The solvent front and the spots will continue to move for several minutes after removing the strip from the beaker). Measure the distance each spot traveled and calculate R_f values for each spot. Use the circles you drew around each spot with your pencil and not the actual spot.

J. Turn your chromatogram in as part of your report.
8. Separation of Food Colorings
A. Remove the piston and insert the long end of the C18 cartridge snugly into the syringe tip.
B. Add 5 ml of IPA, insert the piston, and slowly flush at a flow rate not exceeding 5-10 ml per minute. It is important to not flush liquid through the cartridge more rapidly than 10 ml per minute. Repeat this step with another 5 ml portion of IPA.
C. Flush with two 5 ml portions of distilled water.
D. Add about 1 ml of the food coloring mixture to the syringe, insert the piston, and push the mixture onto the cartridge. As you push the sample through the cartridge, immediately begin collecting the drops in a test tube. The first color comes off quickly. Once the 1ml of food coloring mixture is pushed onto the cartridge, add water to the syringe and push it through the cartridge until all the yellow is gone. Collect the yellow liquid. The process of pushing solvent(s) through a cartridge is called elution. In this case, water elutes the yellow color. The remaining colors in the mixture must be removed by eluting with a water/IPA mixture. Prepare a 1% IPA solution by placing 5mL of water into the syringe and adding 1 drop of IPA. Mix well by shaking the syringe. Push this solution through the cartridge and collect all the colors that come off. If coloring still remains on the cartridge, prepare a 2% IPA solution by adding 2 drops of IPA to 5ml of water, mix, and elute this solution through the cartridge. Continue to increase the percentage of IPA (3% - 3 drops IPA + 5ml water, 4% - 4 drops IPA + 5 ml water, etc.) until all or most of the coloring has been removed from the cartridge. A 5% IPA solution may be the most concentrated solution needed to remove all of the colors from the cartridge. Any coloring residue remaining on the cartridge should be removed using a 1:1 IPA: water mixture. The residue may need to be collected. Save the separated colored solutions for the next part.
E. See what would happen if several of the separated colored solutions were mixed. Try to make a green solution from the colors you separated.
F. Make sure the cartridge is clean before returning it.

An Example

The figure (courtesy of Dr. James A. Bassham) shows autoradiograms of the type that were essential in working out the dark reactions of photosynthesis. The dark spots show the radioactive compounds produced

- After 10 secs (top) and

- 2 minutes (bottom) of photosynthesis by the green algae Scenedesmus. The alga was supplied with carbon dioxide labeled with $^{14}$C, a radioactive isotope of carbon.
- At 10 seconds, most of the radioactivity is found in 3-phosphoglyceric acid ("P-Glyceric").
- At 2 minutes, phosphorylated 6-carbon sugars (glucose and fructose) have been synthesized as well as a number of amino acids.
3. COLUMN CHROMATOGRAPHY

3.1 Introduction:

Column chromatography is the actual or the basic type of chromatography procedure which was developed during early stages of chromatography discovery. From it the principle of chromatography is described and these are still followed in the most advanced modes of chromatography. Other types of chromatography methods were developed with column chromatography as a module and with slight variations.

Column chromatography is basically a type of adsorption chromatography techniques. Here the separation of components depends upon the extent of adsorption to stationary phase. Here the stationary phase is a solid material packed in a vertical column made of glass or metal.

![Table 26-1: Classification of Column Chromatographic Methods](source)

<table>
<thead>
<tr>
<th>General Classification</th>
<th>Specific Method</th>
<th>Stationary Phase</th>
<th>Type of Equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gas chromatography (GC)</td>
<td>a. Gas-liquid chromatography (GLC)</td>
<td>Liquid adsorbed or bonded to a solid surface</td>
<td>Partition between gas and liquid</td>
</tr>
<tr>
<td></td>
<td>b. Gas-solid</td>
<td>Solid</td>
<td>Adsorption</td>
</tr>
<tr>
<td>2. Liquid chromatography (LC)</td>
<td>a. Liquid-liquid, or partition</td>
<td>Liquid adsorbed or bonded to a solid surface</td>
<td>Partition between immiscible liquids</td>
</tr>
<tr>
<td></td>
<td>b. Liquid-solid, or adsorption</td>
<td>Solid</td>
<td>Adsorption</td>
</tr>
<tr>
<td></td>
<td>c. Ion exchange</td>
<td>Ion-exchange resin</td>
<td>Ion exchange</td>
</tr>
<tr>
<td></td>
<td>d. Size exclusion</td>
<td>Liquid in interstices of a polymeric solid</td>
<td>Partition/sieving</td>
</tr>
<tr>
<td></td>
<td>e. Affinity</td>
<td>Group specific liquid bonded to a solid surface</td>
<td>Partition between surface liquid and mobile liquid</td>
</tr>
<tr>
<td>3. Supercritical fluid chromatography (SFC; mobile phase: supercritical fluid)</td>
<td></td>
<td>Organic species bonded to a solid surface</td>
<td>Partition between supercritical fluid and bonded surface</td>
</tr>
</tbody>
</table>

3.2 Principle:

When a mixture of mobile phase and sample to be separated are introduced from top of the column, the individual components of mixture move with different rates. Those with lower affinity and adsorption to stationary phase move faster and eluted out first while those with greater adsorption affinity move or travel slower and get eluted out last.

The solute molecules adsorb to the column in a reversible manner. The rate of the movement of the components is given as follows:
Rf = Rate of movement of a component / Rate of movement of mobile phase. i.e., it is the ratio of distance moved by solute to the distance moved by solvent.

3.4 Theory:

Stationary phase

Column chromatography proceeds by a series of steps.

The stationary phase or adsorbent in column chromatography is a solid. The most common stationary phase for column chromatography is silica gel, followed by alumina. Cellulose powder has often been used in the past. Also possible are ion exchange chromatography, reversed-phase chromatography (RP), affinity chromatography or expanded bed adsorption (EBA). The stationary phases are usually finely ground powders or gels and/or are microporous for an increased surface; though in EBA a fluidized bed is used. There is an important ratio between the stationary phase weight and the dry weight of the analyte mixture that can be applied onto the column. For silica column chromatography, this ratio lies within 20:1 to 100:1, depending on how close to each other the analyte components are being eluted.

Mobile phase (eluent)

The mobile phase or eluent is either a pure solvent or a mixture of different solvents. It is chosen so that the retention factor value of the compound of interest is roughly around 0.2 - 0.3 in order to minimize the time and the amount of eluent to run the chromatography. The eluent has also been chosen so that the different compounds can be separated effectively. The eluent is optimized in small scale pretests, often using thin layer chromatography (TLC) with the same stationary phase.

There is an optimum flow rate for each particular separation. A faster flow rate of the eluent minimizes the time required to run a column and thereby minimizes diffusion, resulting in a better separation. However, the maximum flow rate is limited because a finite time is required for analyte to equilibrate between stationary phase and mobile phase, see Van Deemter's equation. A simple laboratory column runs by gravity flow. The flow rate of such a column can be increased by extending the fresh eluent filled column above the top of the stationary phase or decreased by the tap controls. Faster flow rates can be achieved by using a pump or by using compressed gas (e.g. air, nitrogen, or argon) to push the solvent through the column (flash column chromatography).
particle size of the stationary phase is generally finer in flash column chromatography than in gravity column chromatography. For example, one of the most widely used silica gel grades in the former technique is mesh 230 – 400 (40 – 63 µm), while the latter technique typically requires mesh 70 – 230 (63 – 200 µm) silica gel.

A spreadsheet that assists in the successful development of flash columns has been developed. The spreadsheet estimates the retention volume and band volume of analytes, the fraction numbers expected to contain each analyte, and the resolution between adjacent peaks. This information allows users to select optimal parameters for preparative-scale separations before the flash column itself is attempted.

**Automated Systems**

![Automated Ion Chromatography System](image)

An automated ion chromatography system.

Column chromatography is an extremely time consuming stage in any lab and can quickly become the bottleneck for any process lab. Therefore, several manufacturers like Teledyne Isco, have developed automated flash chromatography systems (typically referred to as LPLC, low pressure liquid chromatography, around 350–525 kPa/51–76.1 psi) that minimize human involvement in the purification process. Automated systems will include components normally found on more expensive high performance liquid chromatography (HPLC) systems such as a gradient pump, sample injection ports, a UV detector and a fraction collector to collect the eluent. Typically these automated systems can separate samples from a few milligrams up to an industrial many kilogram scale and offer a much cheaper and quicker solution to doing multiple injections on prep-HPLC systems.

The resolution (or the ability to separate a mixture) on an LPLC system will always be lower compared to HPLC, as the packing material in an HPLC column can be much smaller, typically only 5 micrometre thus increasing stationary phase surface area, increasing surface interactions and giving better separation. However, the use of this small packing media causes the high back pressure and is why it is termed high pressure liquid chromatography. The LPLC columns are typically packed with silica of around 50 micrometres, thus reducing back pressure and resolution, but it also removes the need for expensive high pressure pumps. Manufacturers are now starting to move into higher pressure flash chromatography systems and have termed these as medium pressure liquid chromatography (MPLC) systems which operate above 1 MPa (150 psi).
Typical set up for manual column chromatography.

The software controlling an automated system will coordinate the components, allow a user to only collect the fractions that contain their target compound (assuming they are detectable on the system's detector) and help the user to find the resulting purified material within the fraction collector. The software will also save the resulting chromatograph from the process for archival and/or later recall purposes.

A representative example of column chromatography as part of an undergraduate laboratory exercise is the separation of three components (out of 28) in the oil of spearmint: carvone, limonene and dehydrocarveol. A micro scale setup consisting of a Pasteur pipette as column with silica gel stationary phase can suffice. The starting eluent is hexane and solvent polarity is increased during the process by adding ethyl acetate.
Column Chromatogram Resolution Calculation

Powdery silicate for column chromatography

Typically, column chromatography is set up with peristaltic pumps, flowing buffers and the solution sample through the top of the column. The solutions and buffers pass through the column where a fraction collector at the end of the column setup collects the eluted samples. Prior to the fraction collection, the samples that are eluted from the column pass through a detector such as a spectrophotometer or mass spectrometer so that the concentration of the separated samples in the sample solution mixture can be determined.

For example, if you were to separate two different proteins with different binding capacities to the column from a solution sample, a good type of detector would be a spectrophotometer using a wavelength of 280 nm. The higher the concentration of protein that passes through the eluted solution through the column, the higher the absorbance of that wavelength.

Because the column chromatography has a constant flow of eluted solution passing through the detector at varying concentrations, the detector must plot the concentration of the eluted sample over a course of time. This plot of sample concentration versus time is called a chromatogram.

The ultimate goal of chromatography is to separate different components from a solution mixture. The resolution expresses the extent of separation between the components from the mixture. The higher the resolution of the chromatogram, the better the extent of separation of the samples the column gives. This data is a good way of determining the column’s separation properties of that particular sample. The resolution can be calculated from the chromatogram.

The separate curves in the diagram represent different sample elution concentration profiles over time based on their affinity to the column resin. To calculate resolution, the retention time and curve width are required.

**Retention Time:** The time from the start of signal detection by the detector to the peak height of the elution concentration profile of each different sample.

**Curve Width:** The width of the concentration profile curve of the different samples in the chromatogram in units of time.
A simplified method of calculating chromatogram resolution is to use the plate model. The plate model assumes that the column can be divided into a certain number of sections, or plates and the mass balance can be calculated for each individual plate. This approach approximates a typical chromatogram curve as a Gaussian distribution curve. By doing this, the curve width is estimated as 4 times the standard deviation of the curve, 4σ. The retention time is the time from the start of signal detection to the time of the peak height of the Gaussian curve.

From the variables in the figure above, the resolution, plate number, and plate height of the column plate model can be calculated using the equations:

**Resolution (Rₛ)**

\[
Rₛ = \frac{2(t_{RB} - t_{RA})}{w_B + w_A}
\]

Where:

- \( t_{RB} \) = retention time of solute B
- \( t_{RA} \) = retention time of solute A
- \( w_B \) = Gaussian curve width of solute B
- \( w_A \) = Gaussian curve width of solute A

**Plate Number (N):**

\[
N = \frac{(t_R)^2}{(w/4)^2}
\]

**Plate Height (H):**

\[
H = \frac{L}{N}
\]

Where \( L \) is the length of the column.

**Column Adsorption Equilibrium**

For an adsorption column, the column resin (the stationary phase) is composed of microbeads. Even smaller particles such as proteins, carbohydrates, metal ions, or other chemical compounds are conjugated onto the microbeads. Each binding particle that is attached to the microbead can be assumed to bind in a 1:1 ratio with the solute sample sent through the column that needs to be purified or separated.

Binding between the target molecule to be separated and the binding molecule on the column beads can be modeled using a simple equilibrium reaction \( K_{eq} = [CS]/([C][S]) \) where \( K_{eq} \) is the equilibrium constant, \([C]\) and \([S]\) are the concentrations of the target molecule and the binding molecule on the column resin, respectively, \([CS]\) is the concentration of the complex of the target molecule bound to the column resin.

Using this as a basis, three different isotherms can be used to describe the binding dynamics of a column chromatography: linear, Langmuir, and Freundlich.

The linear isotherm occurs when the solute concentration needed to be purified is very small relative to the binding molecule. Thus, the equilibrium can be defined as:
[CS] = K_{eq}[C].

For industrial scale uses, the total binding molecules on the column resin beads must be factored in because unoccupied sites must be taken into account. The Langmuir isotherm and Freundlich isotherm are useful in describing this equilibrium. Langmuir Isotherm: 

$$[CS] = (K_{eq}S_{tot}[C])/(1 + K_{eq}[C]),$$

where $S_{tot}$ is the total binding molecules on the beads.

**Freundlich Isotherm:**

$$[CS] = K_{eq}[C]^{1/n}$$

The Freundlich isotherm is used when the column can bind to many different samples in the solution that needs to be purified. Because the many different samples have different binding constants to the beads, there are many different $K_{eq}$’s. Therefore, the Langmuir isotherm is not a good model for binding in this case.

### 3.5 Procedure Requirements:

1. **Adsorption column:**

   **Column Characteristics:** The material of the column is mostly good quality neutral glass since it should not be effected by solvents, acids or alkalis. An ordinary burette can also be used as column for separation. The column dimensions are important for effective column separation. The length: diameter ratio ranges from 10: 1 to 30: 1. For more efficiency the length: diameter ratio can be 100: 1. The length of the column depends upon:
   - Affinity of compounds towards the adsorbent used
   - Number of compounds to be separated.
   - Type of adsorbent used.
   - Quantity of sample.

**Packing of the column**

- **Wet packing:** This is the ideal technique. The required quantity of the adsorbent is mixed with the mobile phase solvent in the beaker and poured into the column. The stationary phase settles uniformly in the column and there is no entrapment of air bubbles. There will not be any crack in the column of adsorbent. The bands eluted from the column will be uniform and ideal for separation.

- **Dry packing:** In this technique, the required quantity of adsorbent is packed in the column in dry form and the solvent allowed to flow through the column till equilibrium is reached. The demerit with this technique is that air bubbles are entrapped between the solvent and the stationary phase and the column may not be uniformly packed. Cracks appear in the adsorbent present in the column. Hence uniformity in flow characteristics and clear bands of the separated component cannot be obtained.
Adsorbents:

An adsorbent used in column chromatography should meet the following criteria:

- **The particle size and geometry**: the particle should have uniform size distribution and have spherical shape. Particle size 60 to 200µ.
- Should have high mechanical stability.
- Should be inert and should not react with solute or other components.
- Insoluble in the solvent or mobile phase used.
- It should be colorless to facilitate observation of zones and recovery of components.
- It should allow free flow of mobile phase.
- It should be useful for separating for wide variety of compounds.
- Above all it should be freely available, inexpensive, etc……

Selection of stationary phase:

The success of chromatography depends upon the proper selection of stationary phase. The selection of stationary phase in column chromatography depends on the following:

- **Removal of impurities**: when a small quantity impurity is present and there is difference in affinity when compare to the major component, a weak adsorbent is sufficient.
- **Number of components to be separated**: when few components are to be separated, weak adsorbent is used. When more components are to be separated, a strong adsorbent is selected
- **Affinity differences between components**: when components of similar affinities, a strong adsorbent will be effective. When there are more differences in affinities, a weak adsorbent is selected.
- **Length of the column used**: when a shorter is used, strong adsorbent has to be used. When a longer column is used a weak adsorbent can be used.
- **Quantity of adsorbent used**: 20 or 30 times the weight of the adsorbent is used for effective separation. Adsorbate: adsorbent ratio 1: 20 or 1: 30.

2. Solvents:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Solvent</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>n-hexane</td>
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<tr>
<td>2.</td>
<td>Cyclo hexane</td>
</tr>
<tr>
<td>3.</td>
<td>Carbon tetra chloride</td>
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<tr>
<td>4.</td>
<td>Benzene</td>
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<tr>
<td>5.</td>
<td>Toluene</td>
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<tr>
<td>6.</td>
<td>Trichloro ethylene</td>
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7. Diethyl ether
8. Chloroform
9. Water

3. Elution technique:
   - **Isocratic elution**: only one type eluent is passed throughout the separation process. Eg: (1:1) petroleum ether : benzene
   - **Gradient elution**: more than one eluent is taken in the increasing order of their polarity or solvent strength or eluent strength. Eg: benzene < chloroform < methanol < formic acid.

Procedure:

The column chromatography requires a vertical column (preferably glass column) with a knob at the bottom end. This is preferably a burette shaped cylindrical column without graduations or readings.

Stationary phase or adsorbent, mostly fine sand will do or that recommended for the experiment. The particles of stationary phase should be of uniform size and shape without contamination.

Mobile phase preferably solvents of chromatography grade either a single solvent or a mixture of solvents as required for the separation.

Cotton wool or asbestos pad to plug the exit of column at the bottom and there by hold the column of stationary phase and let only escape of solvent and sample.

The stationary phase material is suitably moistened with mobile phase and packed sufficiently in the column with a cotton or asbestos pad at the bottom. The extract material or sample to be separated is placed on the top of packed stationary phase with a second cotton or asbestos pad in between.

The mobile phase is poured into the column over the sample. A collecting beaker is placed at the bottom of column near the end to collect the elute.

Detectors:

**Chromatography detector** is a device used in gas chromatography (GC) or liquid chromatography (LC) to visualize components of the mixture being eluted off the chromatography column. There are two general types of detectors: destructive and non-destructive. The destructive detectors perform continuous transformation of the column effluent (burning, evaporation or mixing with reagents) with subsequent measurement of some physical property of the resulting material (plasma, aerosol or reaction mixture). The non-destructive detectors are directly measuring some
property of the column effluent (for example UV absorption) and thus affords for the further analyte recovery.

**Destructive detectors:**

- **Flame ionization detector**: The column effluent is injected into hydrogen flame and the flame conductivity is measured. Only used in GC.
- **Aerosol-based detector [NQAD]**: The column effluent is nebulized, the mobile phase evaporated, a water shell condensed on it and then it is detected. Very sensitive detection for reverse phase applications.
- **Flame photometric detector, (FPD)**: Column eluate is burned in hydrogen flame. The light of the flame is filtered through an optical filter. Allows the detection of sulfur and phosphorus.
- **Atomic-emission detector (AED)**: The column effluent is injected into plasma and the plasma spectrum is recorded. This detector allows for immediate determination of the elements present in the analyte.
- **MS detector**: The column effluent is continuously injected into mass spectrometer. This detector allows for immediate determination of the molecular weight of analyte and/or fragmentation pattern thus providing a significant amount of information about analyte.
- **Evaporative light scattering detector (ELD)**: The column effluent is continuously evaporated and the light scattering of the resulting aerosol is measured. Only used in LC.

**Non-destructive detectors:**

- **UV detectors**: fixed or variable wavelength, which includes diode array detector (DAD or PDA). The UV absorption of the effluent is continuously measured at single or multiple wavelengths. These are by far most popular detectors for LC.[3]
- **Thermal conductivity detector, (TCD)**: Measures the thermal conductivity of the effluent. Only used in GC.
- **Fluorescence detector**: Irradiates the effluent with a light of set wavelength and measure the fluorescence of the effluent detector. At a single or multiple wavelength. Used only in LC
- **Electron Capture Detector, (ECD)**: The most sensitive detector known. Allows for the detection of organic molecules containing halogen, nitro groups etc.
- **Conductivity monitor**: Continuously measures the conductivity of the effluent. Used only in LC when conductive eluents (water or alcohols) are used.
- **Photoionization detector, (PID)**: Measures the increase in conductivity achieved by ionizing the effluent gas with UV radiation.
- **Refractive index detector (RI or RID)**: Continuously measures the refractive index of the effluent. Used only in LC. The lowest sensitivity of all detectors. Useful when nothing else works and at high analyte concentrations.
- **Radio flow detector**: Measures radioactivity of the effluent. This detector can be continuously measures the optical angle of rotation of the eluant. Used only in LC when chiral compounds are being analyzed.
3.6 Precautions:

1. Keep the column in a clean and dust free place.

2. Do not disturb the column till the separation is complete.

3. Avoid gaps within the stationary phase packing.

3.7 Applications:

- Column chromatography is best suited to separate active principle from plant materials.
- In separation of compounds after organic synthesis to obtain desired molecule.
- To separate or purify natural compound mixtures like alkaloids, glycosides.
- Impurities along with important constituents can be separated.
- Useful for isolation of active constituents of plants.
- Isolation of metabolites from important components.
- Used for determination of phytomenadione in tablets and injections.
• Used for determination of primary and secondary glycosides in digitalis leaves.
• Determination of flucinolone, acetonide or betamethasone in formulations.
• Used for the separation diastereomers, geometric isomers from tautomer’s, recemates.
• Used for separation of inorganic ions like copper ion, cobalt ion, nickel ion.
References:

1. Instrumental methods of chemical analysis by Gurdeep R.Chatwal & Sham K. Anand.
2. Instrumental methods of chemical analysis by Kaur.