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A REVIEW ON COLUMNS USED IN CHROMATOGRAPHY

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Abstract

Column chromatography is used extensively in nucleic acid chemistry to purify or characterize products from chemical reactions. This appendix focuses on separations using silica gel or alumina. The procedures described include loading the column, assembling the apparatus, separating compounds and collecting fractions, and optimizing chromatography conditions. Column chromatography is simple and the most popular separation and purification technique. Column chromatography consists of a stationary solid phase that adsorbs and separates the compounds passing through it with the help of a liquid mobile phase. Various stationary phases, such as silica, alumina, calcium phosphate, calcium carbonate, starch, and magnesia, and different solvent compositions based on the nature of compounds to be separated and isolated, are used in column chromatography.

Keywords: Column, HPLC techniques, Separation, Absorption, Size exclusion.

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Introduction

Chromatography is physical method of separation of the mixture into its individual Components. It is used as analytical technique to get information about what is present in the mixture, how much the individual compound is in mixture. It is also used as a purification method to separate and collect the components of mixture. Chromatography is Greek word where chromates mean colour and graphy means writing. So, basically chromatography is colour writing process. The main advantage that differentiates chromatography from most other chemical and physical separation methods is that, two mutually immiscible phases brought into contact oneis stationary phase and other mobile. Equilibrium of solute or components of mixture between stationary phase and mobile phaseis repeatedly achieved Repeated interaction of species of sample in both phases may lead to gradual separation of sample into bands in the stationary phase. The separation of components of mixture is achieved on the simple fact that different component of mixture having different affinity towards mobile phase and stationary phase The least affianced component emerges first; the most strongly affinated compound (retained)elutes last [1]. The concept of chromatography is first time put forwarded in 1906 by Great Russian botanist, Michael T sweet, who has separated different plant pigments on solid support of calcium carbonate in a long tube. He observed that when methanolic extract of plant leaves are poured through the tube, various colour pigments are separated on calcium carbonate packed in tube. Each colour pigments occupy specific area in the column which is called as 'zone' and the coloured column is called as 'development of chromatogram.

Definition of Chromatography

Chromatography is defined as it is a physical method of separation into its individual components when the mixture is distributed between two phases one is fixed phase called stationary phase and other is movable phase called mobile phase OR Chromatography is a method of separation of components of mixture in which a sample is introduced into a mobile phase which is carried along with a column and solid support called stationary phase. Generally, the stationary phase is either solid or liquid and mobile phase is liquid or gas. Depending upon the stationary phase and mobile the chromatography is classified in different forms

Definition of Chromatography

Chromatography is a technique is used to affect the separation of two or more dissolved solids contained

within a solution in a very small quantites.In Greek ,the word chroma means colour and graphein is used to indicate writing. Initially, the technique is used for the separation of colours [2].

Types of HPLC techniques

Based on mode of chromatography

- ✓ Normal phase mode.
- ✓ Reverse phase mode.

Based on the principle of separation

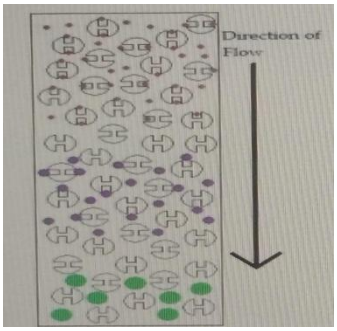
- ❖ Absorption chromatography
- ❖ Ion exchange chromatography
- ❖ Ion pair chromatography
- ❖ Size exclusion /gel permentation chromatography
- ❖ Affinity chromatography
- ❖ Chiral phase chromatography

Ion Exchange Chromatographic Columns

Ion exchange columns are used to separate ions and molecules that can be easily ionized. Separation of the ions depends on the ion's affinity for the stationary phase, which creates an ion exchange system. The electrostatic interactions between the analytes, moble phase, and the stationary phase, contribute to the separation of ions in the sample. Only positively or negatively charged complexes can interact with their respective cation or anion exchangers. Common packing materials for ion exchange columns are amines, sulfonic acid, diatomaceous earth, styrene-divinylbenzene, and cross-linked polystyrene resins. Some of the first ion exchangers used were inorganic and made from aluminosilicates (zeolites). Although aluminosilicates are not widely used as ion exchange resins used [3].

Size Exclusion Chromatographic Columns

Size Exclusion Chromatographic columns separate molecules based upon their size, not molecular weight. A common packing material for these columns is molecular sieves. Zeolites are a common molecular sieve that is used. The molecular sieves have pores that small molecules can go into, but large molecules cannot. This allows the larger molecules to pass through the column faster than the smaller ones.[4]Other packing materials for size exclusion chromatographic columns are polysaccharides and other polymers, and silica. The pore sizefor size exclusion separations varies between 4 and 200 nm.



Chiral Columns

Chiral columns are used to separate enantiomers. Separation of chiral molecules is based upon steriochemistry. These columns have a stationary phase that selectively interacts with one enantiomer over the other. These types of columns are very useful for separating racemic mixtures. Some Stationary Phases Used to Separate Enantiomer is show in Table.

Table: This table shows some stationary phases that are used to separate enantiomers and the corresponding chromatographic methods that they are applied to.

Stationary phase Method(s) used
Metalchilates GC, LC
Amino acid derivatives GC, LC
Proteins LC
Helicol polymers LC
Cyclodextrin derivatives GC,LC

Principle of Choromatography

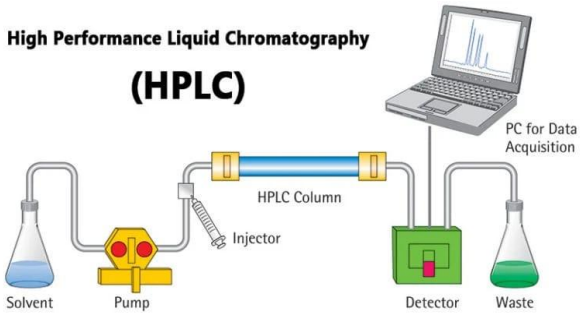
Chromatography is based on the principle where molecules in mixture applied on to the surface or in to the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase

Based on the approach three components from the basis of the chromatography technique:

- ➢ **Stationary phase**: this phase is always composed of a solid phase or a layer of a liquid absorbed on the surface a solid support.
- ➢ **Mobile phase**: this phase is always composed of liquid or a gaseous component.
- ➢ Separated molecules [5].

Instrumentation

The compounds separated in the column are detectd by a detector downstream of the coumn and each compound is identified and quantified.



Solvent

Solvents with low UV Absorbence, such as a methanol, acetonitrile, hexane, and Tetrahydrofurane (THF) are often used for the mobile phase in HPLC Because The UV detector is the most commonly used detector.

Pump

An HPLC Pump is also called as a "solvent delivery system."The purpose pump is to maintain a constant flow of mobile phase Through the HPLC System. This is accomplished regardless of the back pressure caused by the flow Resistance of the HPLC column.

Injector

A sampleInjector is a device used in conjugation with injecting samples in to high performance liquid chromatography or similar chromatography apparati.An HPLC injector allows the introduction of a precise sample volume on to the column [6].

Columns

In the **HPLC column**, the components of the sample separate based on their differing interactions with the column packing. If a species interacts more strongly with the stationary phase in the column, it will spend more time adsorbed to the column's adsorbent and will therefore have a greater retention time. Columns can be packed with solids such as silica or alumina; these columns are called **homogeneous columns**. If stationary phase in the column is a liquid, the column is deemed a **bonded column**. Bonded columns contain a liquid stationary phase bonded to a solid support, which's again usually silica or alumina. The value of the constant **C** described in the van Deemter equation is proportional, in HPLC, to the diameter of the particles that constitute the column's packing material

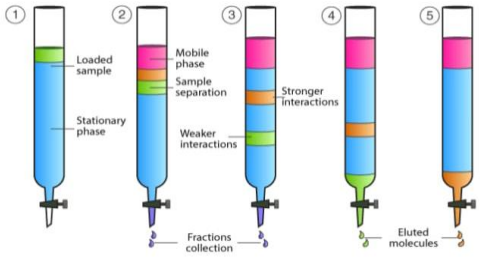
What Is Column Chromatography?

In chemistry, Column chromatography is a technique which is used to separate a single chemical compound from a mixture dissolved in a fluid. It separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allow them to get separated infractions. This technique can be used on small scale as well as large scale to purify materials that can be used in future experiments. This method is a type of adsorption chromatography techniques

Column Chromatography Principle

When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slow are eluted out last. The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as: Rf = the distance travelled by solute/ the distance travelled by solvent Rf is the retardation factor [7].

Column Chromatography



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Column Chromatography Procedure

Before starting with the Column Chromatography Experiment let us understand the different phases involved. Mobile phase – This phase is made up of solvents and it performs the following functions:

1. It acts as a solvent – sample mixture can be introduced in the column.
2. It acts as a developing agent – helps in the separation of components in the sample to form bands.
3. It acts as an eluting agent – the components that are separated during the experiment are removed from the column
4. Some examples of solvents used as mobile phase based on their polarity are – ethanol, acetone, water, acetic acid, pyridine, etc. Stationary phase – It is a solid material which should have good adsorption property and meet the conditions given below:
   - ✓ Shape and size of particle: Particles should have uniform shape and size in the range of 60 – 200µ in diameter.
   - ✓ Stability and inertness of particles: high mechanical stability and chemically inert. Also, no reaction with acids or bases or any other solvents used during the experiment.
   - ✓ It should be colourless, inexpensive and readily available.
   - ✓ Should allow free flow of mobile phase
   - ✓ It should be suitable for the separation of mixtures of various compounds.

Column Chromatography (Traditional column chromatography)

Traditional column chromatography is characterized by addition of mobile phase under atmospheric pressure and the stationary phase is packed in a glass column. ¬Following slides contain the stepwise procedure of experimental techniques of Traditional coloumn chromatography [8]. Packing & operating the column

- ➢ Packing the selection of the method of packing depends mainly on the density of the solid. Techniques used are the wet, dry & slurry methods In all cases avoid inclusion of air bubbles
- ➢ Sample Application Apply evenly & in a concentrated solution to the top of the column which is protected from disturbance (e.g. add glass wool or filter paper)

FACTOR	EFFECT
Particle size of solid stationary phase decrease of size improve separation(but very sma	
(or of support) need high pressure	ll particles
Solvent flourate &low flourate gives better resolution	uniform
Continuity of flow continuous flow distrubs resolution	dis
Condition of deactivation of adsorbent decrease separation	absorbent

Conceration of solutes of high concentration move slowly	substance

Number of Theoretical Plates (N) H = Theoretical Plate Height L = Length of the Column. N = L / H as HETP decreases efficiency of the column increases

Types of Column Chromatography

- Strength of adsorption of polar groups (solutes) on polar support is in the following order
- -C=C- < O-CH3 < -COOR <>C = O < -CHO < -NH2 < - OH < -COOH ¬
- Olefins < Ethers < Esters < Lactones < Aldehydes < Amines < Phenols < Acids

Applications in separation of natural products

- Alumina: sterols, dyestuffs, vitamins, esters, alkaloids & inorganic compounds: Not used for compounds containing phenolic or carboxylic groups
- Silica gel: sterols & amino acids. ¬ Carbon: peptides, carbohydrates & amino acids.
- Calcium carbonate: carotenoids &xanthophylls

A. Partition Column Chromatography

- In this type, the packing consists of a theoretically inert support material coated with a film of the liquid stationary phase.
- The division into adsorption & partition is only of theoretical significance as in partition chromatography the adsorption effects of the support can be felt.

Selection of the solid support

The support material should: ¬adsorb & retain the mobile stationary phase.

- expose as large surface as possible to the mobile phase
- be mechanically stable.
- be easy to pack.
- not retard the solvent flow

Examples of solid supports

Silica gel, diatomaceous earth (as kieselguhr, celite etc.) & cellulose.

Selection of the mobile phase

- The liquid stationary & mobile phases should have a considerable difference between their solvent strength parameters
- Pure water > Methanol > Ethanol > Propanol > Acetone > Ethyl acetate> Ether > Chloroform > Dichloromethane >Benzene > Toluene > Carbon tetrachloride tetrachloride > Cyclohexane Cyclohexane > Hexane > Pentane Pentane.
- e.g. if the stationary phase is water, pentane would be the eluent of choice.
- The mobile phase is usually saturated with the stationary phase to overcome "stripping" (washing of the stationary phase from the colum [9].

Preparation of the Column

The column mostly consists of a glass tube packed with a suitable stationary phase. Glass wool/cotton wool or an asbestos pad is placed at the bottom of the column before packing the stationary phase. After packing, a paper disc kept on the top, so that the stationary layer is not disturbed during the introduction of sample or mobile phase.

Types of preparing the column

There are two types of preparing the column, they are:

1. Dry packing / dry filling

In this the required quantity of adsorbent is poured as fine dry powder in the column and the solvent is allowed to flow through the column till equilibrium is reached.

2. Wet packing / wet filling

In this, the slurry of adsorbent with the mobile phase is prepared and is poured into the column. It is considered as the ideal technique for packing.

B. Introduction of the Sample

The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase.The entire sample is introduced into the column at once and get adsorbed on the top portion of the column.From this zone, individual sample can be separated by a process of elution.

C. Elution

By elution technique, the individual components are separated out from the column.It can be achieved by two techniques: Isocratic elution technique: Same solvent composition or solvent of same polarity is used throughout the process of separation [10].

Eg. Use of chloroform alone.

Gradient elution technique: Solvents of gradually ↑ polarity or ↑ elution strength are used during the process of separation.

E.g. initially benzene, then chloroform, then ethyl acetate then

- chloroform

D. Detection of Components

If the compounds separated in a column chromatography procedure are colored, the progress of the separation can simply be monitored visually. If the compounds to be isolated from column chromatography are colorless. In this case, small fractions of the eluent are collected sequentially in labelled tubes and the composition of each fraction is analyzed by TLC.

Column chromatography is a commonly used purification technique in labs of organic chemistry. Done right it can simply and quickly isolate desired compounds from a mixture. But like many aspects of practical chemistry, the quick and efficient setting up and running of a column is something that can take time to master. Here are some instructions to help you set up the column. 1. Introduction In a typical column (Fig. 1), the stationary phase, a solid adsorbent normally silica gel (SiO2), is placed in a vertical glass column.

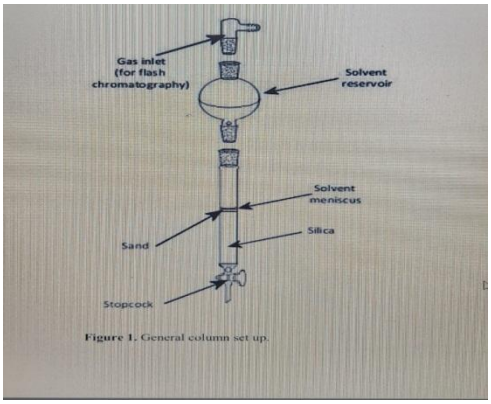


Figure 1. General column set up.

The ball of cotton or glass wool should be large enough to plug the bottom of the column, but not so large and densely packed that it restricts solvent flow (Fig. 3). A piece the size of the tip of your little finger should be suitable for most columns.

1. Position the cotton or glass wool ball securely in the narrowest part of the column using a long glass rod or other suitable device.

2. Clamp the column securely and close the tap or stopcock (Fig. 2, 2). 3. Add a layer of sand until it reaches the main body of the column (approx. 2 cm, Fig.

3). This will give the stationary phase an even base and prevent concentration and streaking of the bands as they come off the column and are collected

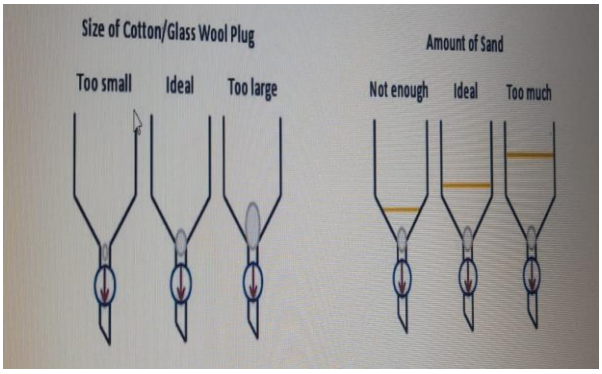
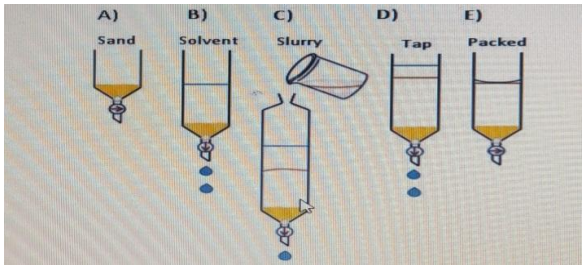


Figure 3. Guidelines for the correct size of cotton or glass wool and sand for non-fritted columns

3. Filling the Column



Procedure

- ✓ Fill the column about one third with solvent (10% EtOAc/Hexanes, Fig. 3, step B).
- ✓ In a beaker, measure out the required amount of In a separate flask or beaker, measure solvent approximately one and a half times the volume of silica.
- ✓ In a separate flask or beaker, measure solvent approximately one and a half times the volume of silica
- ✓ Add the silica to the solvent, a little at a time, while swirling. Use a Pasteur pipette or glass rod to mix the slurry.
- ✓ Pour or pipette some of the slurry into the column. Allow the solvent to drain to prevent overflowing (Fig. 3, step C).
- ✓ Tap the column gently to encourage bubbles to rise and the silica to settle (Fig. 3, step D).
- ✓ Continue to transfer the slurry to the column until all the silica gel is added.
- ✓ Rinse the inside of the column by pipetting solvent down the inside edge.
- ✓ Drain the solvent until the solvent level is just even with the surface of the stationary phase (Fig. 3, step E).
- ✓ Add a 3 mm layer of sand on top of the silica gel to prevent it from moving during solvent addition.[11]

4. Emptying

The Column Once you have your products isolated, all that remains is to empty and clean the column ready for next time. To speed up the process, elute all of the solvent using compressed air and allow air to flow through the column for approximately 15 min. This will give dry, free-flowing silica that is easy to pour into the silica waste container. Alternatively, elute all the solvent and secure the column upside down over a large beaker and allow to dry overnight in a fume hood. Cleaning the column by rinsing with water and acetone is usually sufficient.

Column Chromatogram Resolution Calculation

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.

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 at 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: [12.13]

Resolution (Rs)

Rs = 2(tRB – t RA)/(wB + wA)

Where:

t RB = retention time of solute B

t RA = retention time of solute A

wB = Gaussian curve width of solute B

wA = Gaussian curve width of solute A

Plate Number (N):

N = (tR) 2 /(w/4)2

Plate Height (H)

H = L/N

Where L is the length of the column [7].

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 [17, 15].

Detectors

HPLC analysis requires a detector to monitor the compounds themselves in order to quantify and identify the compounds separated in the column. In other words, detector plays a role of "eyes "for HPLC analysis.

Advantages of Column Chromatogrphy

- Any type of mixture can be separated.
- Any quantity of mixture can be separated.
- Wider choice of mobile phase.
- Automation is possible.
- Column chromatography is advantageous over most other chromatographic techniques because it can be used to separate and purify sub stational quantities of components of mixture.
- Fast and economic methods for the synthesis laboratory.
- Ideal for the separation of compounds up to gram quantities.
- No expensive equipment required.
- Ideal way transfer result from TLC to CLC .[16,17]
- Automated changes between normal phase and reverse phase chromatography.

Disadvantages

- Time consuming
- More amount of mobile phase is required.
- Automation makes the techniques more complicated & Expensive.
- In this method the late length is limited and hence separation takes place only up to certain length.[18]
- The separation takes place in an open system.
- Time required is large.
- it can't be used as a preparative method.
- Heating or corrosive reagents can't be used for detection.
- Large quantity of sample can't be applied on paper chromatography in quantitative analysis paper chromatography is not effective [19].

Applications

- Separation of mixture of compounds
- Removal of impurities or purification process [20].
- Isolation of active constituents
- Isolation of metabolites from biological fluids
- Estimation of drug in formulation and crude drug extract
- In the separation of the mixture in to the pure individual components
- Removal of impurities and in the purification of compounds [21].

Conclusion

It can be concluded that column chromatography is an effective way of separating compounds of a mixture .the crude pigment was sent throughout the coumn however three distantly separated layer where collected (yellow ,green-blue and blue).each coloured layer represented a different pigment Column chromatography is one of the most useful method for the separation and purification of liquids and solids. In this experiment it was used to successfully separate and purify a mixture of two organ metallic compounds. The begin the experiment a glass column was packed with wool and sand to create layers along with an ether solvent. Silica gel was then added to the column and another of sand and ether solvent was added. Once the column was packed and ready a 0.1g sample ferrocene: acetile ferrocin was mixed with ethyl acetate and added into the coloumn along with sample of 1:6 ero acpet ether the first component (yellow band.) was eluted using 1:EtO Ac:pet either making sure the colomn never became dry and a flask was available to collect the eluting fluid once the first component was collected.

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