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## Separation techniques: Chromatography

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### Abstract

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. Proteins can be purified based on characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase. Four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion. Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography. Column chromatography is one of the most common methods of protein purification.

**Keywords:** *Chromatography, column chromatography, protein purification*

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights [1, 2]. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster [3].

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a “solid” phase or “a layer of a liquid adsorbed on the surface a solid support”.
- Mobile phase: This phase is always composed of “liquid” or a “gaseous component.”
- Separated molecules

The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (ie. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, ester, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses [4].

Stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable, and non-volatile samples [5].

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achieve a satisfactory separation within a suitable time interval. Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography [6].

### **Types of chromatography**

- Column chromatography
- Ion-exchange chromatography
- Gel-permeation (molecular sieve) chromatography
- Affinity chromatography
- Paper chromatography
- Thin-layer chromatography
- Gas chromatography
- Dye-ligand chromatography
- Hydrophobic interaction chromatography
- Pseudoaffinity chromatography
- High-pressure liquid chromatography (HPLC)

### **Column chromatography**

Since proteins have different characteristic features as size, shape, net charge, stationary phase used, and binding capacity, each one of these characteristic components can be purified using chromatographic methods. Among these methods, most frequently column chromatography is applied. This technique is used for the purification of biomolecules. On a column (stationary phase) firstly the sample to be separated, then wash buffer (mobile phase) are applied ([Figure 1](#)). Their flow through inside column material placed on a fiberglass support is ensured. The samples are accumulated at the bottom of the device in a time-, and volume-dependent manner [[7](#)].

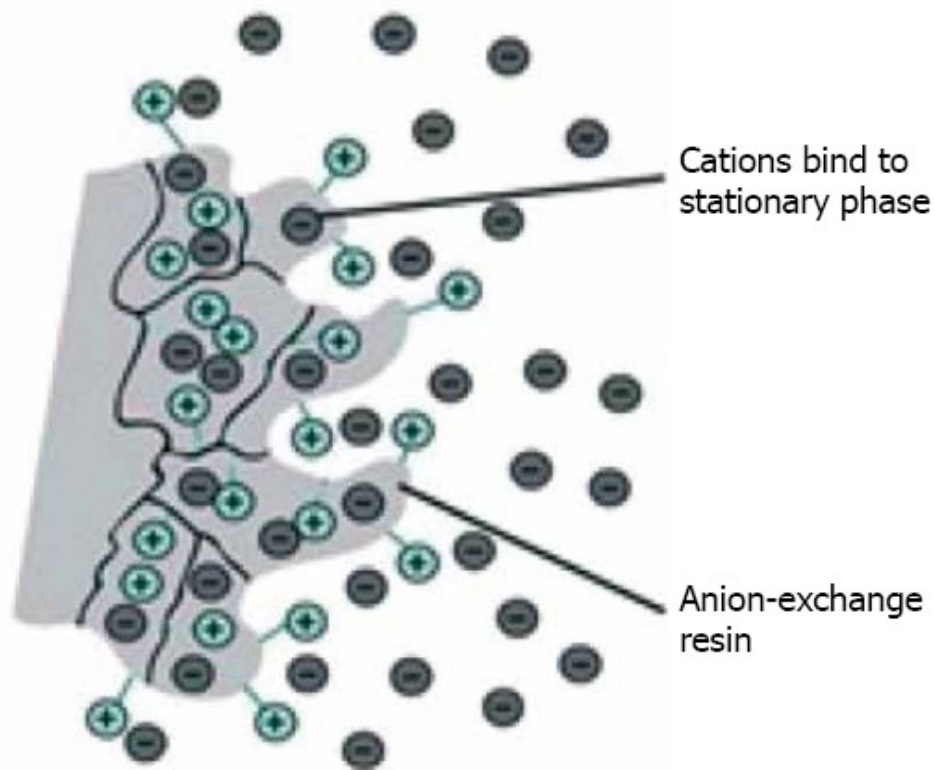


[FIGURE 1](#)

Column chromatography.

### **Ion- exchange chromatography**

Ion- exchange chromatography is based on electrostatic interactions between charged protein groups, and solid support material (matrix). Matrix has an ion load opposite to that of the protein to be separated, and the affinity of the protein to the column is achieved with ionic ties. Proteins are separated from the column either by changing pH, concentration of ion salts or ionic strength of the buffer solution [[8](#)]. Positively charged ion- exchange matrices are called anion-exchange matrices, and adsorb negatively charged proteins. While matrices bound with negatively charged groups are known as cation-exchange matrices, and adsorb positively charged proteins ([Figure 2](#)) [[9](#)].

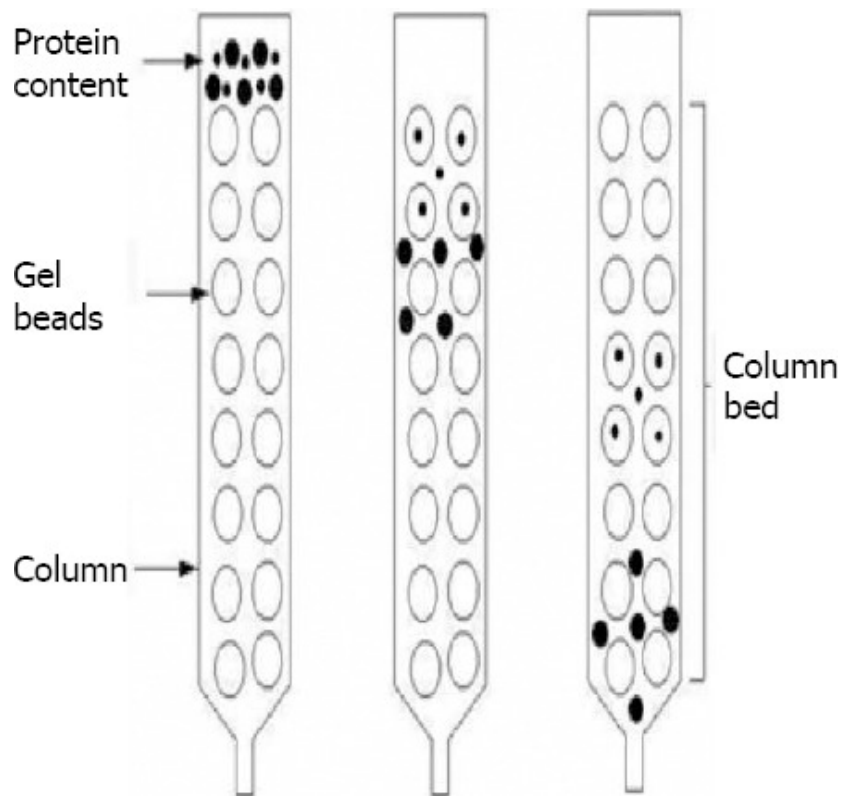


**FIGURE 2**

Ion- exchange chromatography.

### **Gel- permeation (molecular sieve) chromatography**

The basic principle of this method is to use dextran containing materials to separate macromolecules based on their differences in molecular sizes. This procedure is basically used to determine molecular weights of proteins, and to decrease salt concentrations of protein solutions [10]. In a gel- permeation column stationary phase consists of inert molecules with small pores. The solution containing molecules of different dimensions are passed continuously with a constant flow rate through the column. Molecules larger than pores can not permeate into gel particles, and they are retained between particles within a restricted area. Larger molecules pass through spaces between porous particles, and move rapidly through inside the column. Molecules smaller than the pores are diffused into pores, and as molecules get smaller, they leave the column with proportionally longer retention times (Figure 3) [11]. Sephadex G type is the most frequently used column material. Besides, dextran, agarose, polyacrylamide are also used as column materials [12].



**FIGURE 3**

Gel-permeation (molecular sieve) chromatography.

### Affinity chromatography

This chromatography technique is used for the purification of enzymes, hormones, antibodies, nucleic acids, and specific proteins [13]. A ligand which can make a complex with specific protein (dextran, polyacrylamide, cellulose etc) binds the filling material of the column. The specific protein which makes a complex with the ligand is attached to the solid support (matrix), and retained in the column, while free proteins leave the column. Then the bound protein leaves the column by means of changing its ionic strength through alteration of pH or addition of a salt solution (Figure 4) [14].



**FIGURE 4**

Affinity chromatography.

### Paper chromatography

In paper chromatography support material consists of a layer of cellulose highly saturated with water. In this method a thick filter paper comprised the support, and water drops settled in its pores made up the stationary “liquid phase.” Mobile phase consists of an appropriate fluid placed in a developing tank. Paper chromatography is a “liquid-liquid” chromatography [15].

### Thin-layer chromatography

Thin-layer chromatography is a “solid-liquid adsorption” chromatography. In this method stationary phase is a solid adsorbent substance coated on glass plates. As adsorbent material all solid substances used. in column chromatography (alumina, silica gel, cellulose) can be utilized. In this method, the mobile phase travels upward through the stationary phase. The solvent travels up the thin plate soaked with the solvent by means of capillary action. During this procedure, it also drives the mixture priorly dropped on the lower parts of the plate with a pipette upwards with different flow rates. Thus the separation of analytes is achieved. This upward travelling rate depends on the polarity of the material, solid phase, and of the solvent [16].

In cases where molecules of the sample are colorless, fluorescence, radioactivity or a specific chemical substance can be used to produce a visible coloured reactive product so as to identify their positions on the chromatogram. Formation of a visible colour can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the the distances travelled by the molecule and the solvent. This measurement value is called relative mobility, and expressed with a symbol  $R_f$ .  $R_f$  value is used for qualitative description of the molecules [17].

### Gas chromatography

In this method stationary phase is a column which is placed in the device, and contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. Gas chromatography is a “gas-liquid” chromatography. Its carrier phase consists of gases as He or N<sub>2</sub>. Mobile phase which is an inert gas is passed through a column under high pressure. The sample to be analyzed is vaporized, and enters into a gaseous mobile phase phase. The components contained in the sample are dispersed between mobile phase, and stationary phase on the solid support. Gas chromatography is a simple, multifaceted, highly sensitive, and rapidly applied technique for the extremely excellent separation of very minute molecules. It is used in the separation of very little amounts of analytes [18].

### **Dye- ligand chromatography**

Development of this technique was based on the demonstration of the ability of many enzymes to bind purine nucleotides for Cibacron Blue F3GA dye [19]. The planar ring structure with negatively charged groups is analogous to the structure of NAD. This analogy has been evidenced by demonstration of the binding of Cibacron Blue F3GA dye to adenine, ribose binding sites of NAD. The dye behaves as an analogue of ADP-ribose. The binding capacity of this type adsorbents is 10–20-fold stronger than that of the affinity of other adsorbents. Under appropriate pH conditions, elution with high-ionic strength solutions, and using ion-exchange property of adsorbent, the adsorbed proteins are separated from the column [20, 21].

### **Hydrophobic interaction chromatography (HIC)**

In this method the adsorbents prepared as column material for the ligand binding in affinity chromatography are used. HIC technique is based on hydrophobic interactions between side chains bound to chromatography matrix [22, 23].

### **Pseudoaffinity chromatography**

Some compounds as anthraquinone dyes, and azo-dyes can be used as ligands because of their affinity especially for dehydrogenases, kinases, transferases, and reductases. The mostly known type of this kind of chromatography is immobilized metal affinity chromatography (IMAC) [24].

### **High-pressure liquid chromatography (HPLC)**

Using this chromatography technique it is possible to perform structural, and functional analysis, and purification of many molecules within a short time. This technique yields perfect results in the separation, and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and other biologically active molecules. In HPLC, mobile phase passes through columns under 10–400 atmospheric pressure, and with a high (0.1–5 cm/sec) flow rate. In this technique, use of small particles, and application of high pressure on the rate of solvent flow increases separation power, of HPLC and the analysis is completed within a short time.

Essential components of a HPLC device are solvent depot, high- pressure pump, commercially prepared column, detector, and recorder. Duration of separation is controlled with the aid of a computerized system, and material is accrued [25].

### **Application areas of chromatography in medicine**

Chromatography technique is a valuable tool for biochemists, besides it can be applied easily during studies performed in clinical laboratories. For instance, paper chromatography is used to determine some types of sugar, and amino acids in bodily fluids which are associated with hereditary metabolic disorders. Gas chromatography is used in laboratories to measure steroids, barbiturates, and lipids. Chromatographic technique is also used in the separation of vitamins, and proteins.



## Conclusion

Initially chromatographic techniques were used to separate substances based on their color as was the case with herbal pigments. With time its application area was extended considerably. Nowadays, chromatography is accepted as an extremely sensitive, and effective separation method. Column chromatography is one of the useful separation, and determination methods. Column chromatography is a protein purification method realized especially based on one of the characteristic features of proteins. Besides, these methods are used to control purity of a protein. HPLC technique which has many superior features including especially its higher sensitivity, rapid turnover rate, its use as a quantitative method, can purify amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, drugs, antibiotics, and steroids.

## Footnotes

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