Exercise 4: Chromatography

1.- INTRODUCTION

1.1.- Size-exclusion chromatography

Chromatographic methods are employed to separate the components in a mixture. This separation might have analytical purposes, i.e., to figure out the components comprising a mixture; or be preparative, to obtain separately some of the components in a mixture in larger quantities for further study or subsequent use.

Within biological macromolecules, proteins have a greater diversity of sizes and structures. They have been studied for several decades using a large variety of techniques and methodological approximations. In the majority of these cases, a preliminary isolation or purification step is required.

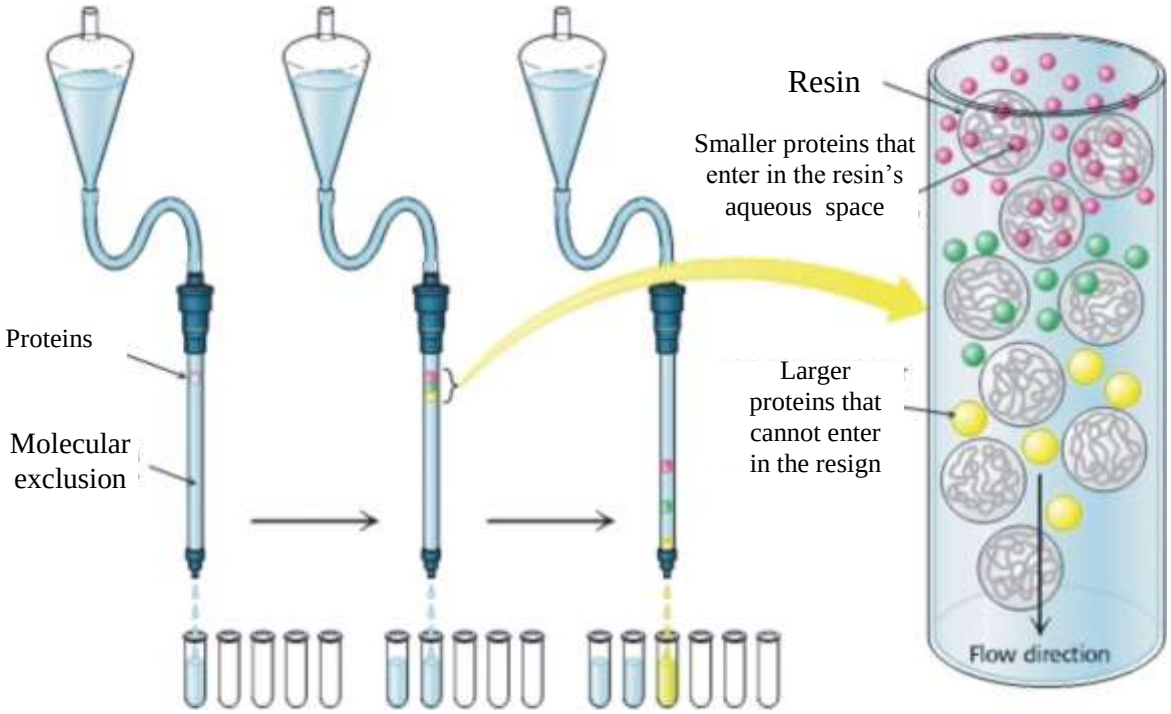
Several chromatographic techniques exist for protein purification, but in all of them, there is a **mobile phase** consisting in a fluid that drags the proteins through a **stationary phase** of solid consistency. The proteins interact with the stationary phase in a distinct manner, in which they pass through at different speeds, and this causes them to separate.

Size-exclusion chromatography, or **gel-filtration chromatography**, is one of the most utilized chromatographic techniques. This allows for molecular separation by molecular size. Component separation is attained by making the mixture pass through a resin in a glass column. Once the sample has completely penetrated the resin, it is dragged by an eluent, which is normally the same buffer or solution within which the resin is equilibrated in the column. Different components in the mixture descend through the column at different speeds, separating themselves. The liquid exiting or eluting from the column is collected in small fractions in different tubes, and a total or partial component separation can be carried out from this.

The explanation for the mixture components descending the column at different speeds is due to, on the one hand, the different sizes these components have. On the other, this is due to the resin structure. The resins used are polymers insoluble in water having different degrees of porosity; they form microscopic spherical particles that constitute a true molecular framework. The degree of porosity is such that some of the smaller molecules are able to penetrate the resin while larger ones cannot, and so they freely descend within the resin interstices. However, the small molecules able to penetrate the resin pores due to their small size are hindered, remaining within the labyrinth or porous framework comprising the resin interior for a longer time. This delay will be successively greater as the molecules become smaller.

For each resin type, there are two limit values to keep in mind according to the degree of porosity: One is a maximum value referencing the molecular weight beginning with the molecules excluded from the resin; the other is a minimum value, below which all molecules are completely included in the resin. Different types of resins exist for gel-filtration chromatography, and Sephadex is one of the most used, of which several types exist in response to the fractioning range, i.e., the molecular weight limits among which the resin is capable of discriminating. For example, Sephadex G-50 possesses a fractioning range for globular proteins from 1500-30000 Da. This means that this resin could resolve a mixture of 2 proteins of 8000 and 20000 Da, but would be of no use in separating 2 proteins of 35.000 and 50.000 Da. In each case, one resin or another will be chosen, depending upon the size of the proteins in the mixture.

Representative diagram of size-exclusion chromatography



The results from size-exclusion chromatography are expressed in an **elution diagram,** where the appearance of the solute is represented in the form of concentration, absorbance, etc., in function of the volume of the eluent that passes through the column. The **elution volume** (Ve) for a substance corresponds with the volume of eluent that has passed through the column, beginning with the application of a sample until the appearance of the maximum concentration of such substance.

It is important to consider two parameters in chromatography:

1.- The **void volume** (Vo) is the volume remaining in the resin exterior. In other words, this is the volume of eluent of the molecules excluded by the resin. To determine this, a colored and high-molecular weight substance, such as blue dextran (MW=2,000,000), is usually employed. It is enough to measure the volume eluted through the column from its application until the appearance of the characteristic blue color of the dextran.

2.- The other important parameter is the volume of the **stationary phase** (Vi), i.e., the volume of eluent in the resin interior where the smallest molecules can access. To calculate this, we resort to the same system as before; in this case, using a colored substance with a very low molecular weight, which could be a salt like potassium dichromate, vitamin B12, etc.

To normalize the chromatography results instead of expressing the elution volume of a component, the **distribution coefficient** (Kd) can be expressed, which would represent the fraction of the stationary phase accessible to the diffusion of the solute.

$$Kd = \frac{Ve - Vo}{Vi}$$

It is not possible to precisely determine *Vi* in some cases. In this case, its value can be approximated knowing the total bed volume (Vt), whose determination can be done well mathematically, or either by measuring the equivalent volume of water with a test tube. Here, *Vt-Vo* would produce the volume the resin would occupy in the stationary phase of its interior as well as the volume of the fibers constituting its matrix.

In this case, the normalized coefficient used would be *Kav*:

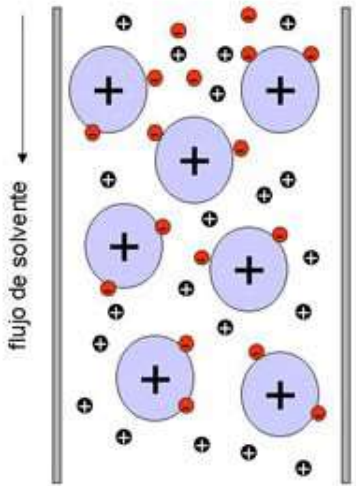
$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Kd and *Kav* both define the chromatographic behavior of the solute independently of the bed length or degree of packing. Other ways to normalize the results exist, for example, *Ve/Vt* or *Ve/Vo*.

1.2.- Ion exchange chromatography

Ion exchange chromatography is based on the acid-base properties of proteins. **One protein will be positively charged if the pH is less than its pI (isoelectric point), while it will be negatively charged if the pH is greater than its pI**. Therefore, in the first case, it will be bound to a negatively charged resin (carboxymethyl cellulose, CMC); while in the second case, it will do so with a positively charged resin (diethylaminoethyl cellulose, DEAE-C).

FUNDAMENTALS OF ION EXCHANGE CHROMATOGRAPHY



Negatively charged particles bond to the positively charged solid matrix and retained.

Positively charged particles are repulsed by the positively charged solid matrix and eluted.

The elution of the negatively charged particles is attained by changing the solvent's pH until it equals its isoelectric point or its net charge reverses.

Once the protein bonds to the resins, they can be eluted one of two ways:

1. **Varying the pH** of the medium until it reaches the pI.
2. **By ion gradient**, adding the corresponding counterion (Na^+ in the case of cationic exchange, and Cl^- for anionic exchange).

As hemoglobin's pI is approximately 6.8, at a pH of 8, the protein will have a net negative charge, and at a pH of 4, it will be positive. In this exercise, we will check its behavior in an anion exchanger at pH 4 and pH 8.

2.- OBJECTIVE

To learn and interpret the separation principals of size-exclusion and ion exchange chromatography.

3.- MATERIALS

1. Sephadex G-75 size-exclusion column (fractionation range 3000 - 100000 Da).
2. DEAE-C column.
3. Blue dextran solution (MW: 2000000 Da, 5 mg/ml)
4. Potassium dichromate solution (PM: 294.2 Da, 10 mg/ml).
5. Hemoglobin solution (5 mg/ml).
6. Buffer A: Tris HCl 50 mM pH 8.
7. Buffer B: Tris HCl 50 mM pH 8 + 0.5 M NaCl.
8. Buffer C: Na acetate 50 mM pH 4.
9. Buffer D: Na acetate 50 mM pH 4 + 0.5 M NaCl.
10. Pasteur pipette, micropipettes, graduated pipettes, timer, eppendorf tubes, permanent marker, rack, glass cuvette and spectrophotometer.

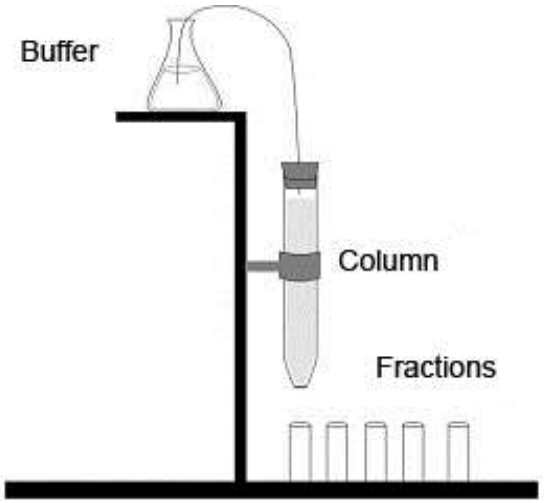
4.- METHODS

4.1.- First day task: column preparation.

On the **first day** of this exercise, the supports used for the size-exclusion and ion exchange chromatography that were previously swollen in the adequate buffer will be packed and the flow will be regulated at 1 ml/min in both cases. On the **second day**, the application and elution of the samples will proceed. Furthermore, detection of the chromatographed samples will be conducted and the elution profile obtained.

4.1.1.- Column preparation for size-exclusion chromatography: pack the resin to a height of 35 cm. Pass 2 volumes of buffer to balance, and regulate its flow until reaching approximately 1,5 ml/min. Use buffer A for the size exclusion.

4.1.2.- Column preparation for ion exchange chromatography: pack the DEAE-C column to a height of 4 cm, pass 2 volumes (aproximately15 ml) of buffer to balance and regulate its flow until reaching approximately 1,5 ml/min. Use either buffer A or C as indicated by the instructor.



4.2.- Second day task.

4.2.1.- Task to carry out employing the size-exclusion column:

1. Number 45 eppendorf tubes (from 1 to 45).
2. Extract the liquid from the column that exceeds the resin surface with a Pasteur pipette, being careful not to disturb the chromatographic layer.
3. Apply 0.2 ml of the problem mixture that contains blue dextran, potassium dichromate and the problem proteins.
4. Place the first eppendorf tube beneath the opening of the column and allow the liquid to flow by opening its valve. When the colored sample has penetrated into the resin, add buffer A to the column with a Pasteur pipette. To perform this step, it is recommended to add the buffer upon the column walls, and not add the buffer directly upon the resin.
5. Once there is a 4 cm buffer height above the resin, connect the column to the Erlenmeyer flask again.
6. Collect 2 ml fractions in the numbered tubes in a successive and orderly fashion. Observe the presence of colored substances in the tubes. Record the volume where the blue dextran (Vo) and the potassium dichromate (Vi) elute.
7. Measure the absorbance at 412 nm.
8. Make the chromatographic profile, i.e., represent the absorbance versus the volume in a graph.
9. **Take 20 µl of the fraction containing greater absorbance and keep at 4ºC**.

4.2.2.- Task to carry out with the ion exchange column:

1. Number 30 eppendorf tubes (from 1 to 30).
2. Deposit 0.2 ml of the hemoglobin solution in the DEAE-C column balanced with buffers A or C.
3. **Place the first eppendorf tube beneath the opening of the column and allow the liquid to flow by opening its valve. When the colored sample has penetrated the resin, add buffers A or C to the column with a Pasteur pipette in accordance with the pH in which the column is balanced. To perform this step, it is recommended to add the buffer upon the column walls, and not add the buffer directly upon the resin**.

4. Maintain 4 cm of buffer above the resin. Once there are 4 cm of buffer above the resin, connect the column to the Erlenmeyer flask again and allow the buffer to run through.
5. Once 15 ml of buffer has run through close the valve.
6. Elute the compound(s) retained in the matrices with buffer B or D according to the column's pH. To perform this, open the valve and allow the buffer to run through until no liquid above the resin is observed. Then add buffer B or D with a Pasteur pipette.
7. Collect 2 ml fractions in the numbered tubes in a successive and orderly fashion. Observe the presence of colored substances in the tubes and measure the absorbance at 412 nm.
8. Make the chromatographic profile. Indicate the wash and the elution.
9. **Take 20 µl of the fraction containing greater Absorbance and keep at 4ºC**

5.- EVALUATION

5.1.- Each student have to prepare a report with the results obtained in sections 4.1. and 4.2. This report must include the graph with the chromatographic profile that the students have made in **epigraphs 4.2.1.8.** and **4.2.2.8.**

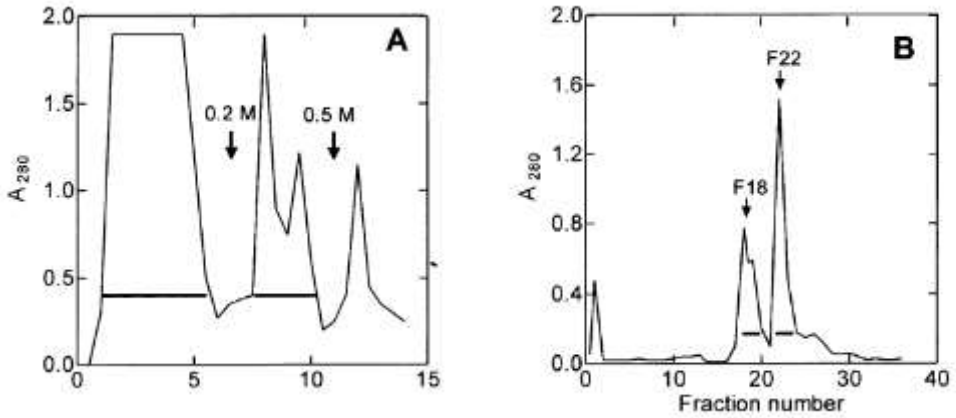
5.2.- The report has to include the answers to the following questions:

Size-exclusion chromatography:

1. How many colored substances appear in the column during the chromatography development?
2. What is the chromatography Vt?
3. What are they and what are the values of Vo and Vi?
4. Why is the Kav calculation made instead of directly employing the elution volume?
5. In a Sephadex G-50 size-exclusion (separation range 1500-30000), could the ovalbumin have been taken as a reference to confect the column calibration curve? Justify the response.
6. What is the value of Kd and Kav for the hemoglobin?
7. What is the elution order for the following proteins in a Sephadex G-50 column: catalase (222 kDa), chymotrypsin (21.6 kDa), concanavalin B (42.5 kDa), lipase (6.7 kDa) and myoglobin (PM 17 kDa)?

Ion exchange chromatography:

1. What happens when buffer B or D is added in the column?
2. Make a table indicating how "retained" or "not retained" is the hemoglobin fraction in function of pH for the wash and elution of each column.
3. In what way can the elution of the proteins retained in the matrix improve? Base the response.
4. In what order would the following proteins in a CMC ion exchange column elute as the salt gradient was increased to pH 7: hemoglobin (pI 6.8), lysozyme (pI 11), pepsin (pI 1) and ribonuclease A (pI 9.6)?
5. Answer the questions:



Purification of antifungal proteins from sunflower flowers. **A**, Cation exchange chromatography of a heat-resistant protein fraction. The arrows indicate the elution with 0.2 and 0.5 M NaCl. **B**, The fraction eluted with 0.2 M NaCl from **A** was further purified by gel filtration on Superose 12 FPLC. Bars indicate fractions with antifungal activity.

a. In A, Which is the charge of the eluted fraction?
b. Which fraction has more charge, the fraction eluted with 0.2 M or 0.5 M NaCl?
c. In B, How could you determine the purity of F18? Justify your answer.
d. Which fraction has higher molecular weight, F18 or F22?

6.- HANDING IN WORK

Each student must make:

A Word file with the results responding to that asked for in epigraph **5.EVALUATION**.

To name in a systematic manner, you must follow the following example:
I am student *Emilio Moreno Serrano*, and I am turning in the following files:
4_MorenoSerrano_Emilio.doc. This file will be turned in TOGETHER AS A SINGLE PACKAGE via the HOMEWORK system that **the student has personal access to** on the course webpage.

EMAILED DOCUMENTS WILL NOT BE ACCEPTED.