Lecture 2: Protein separation techniques: General concepts of Chromatography

Key words: Purification; Chromatography; Separation techniques

In order to analyse different components of a mixture, their separation is necessary. A successful separation needs different separation techniques. There are several physical and chemical methods to separate the different components of a simple mixture but for a complex mixture as, mixture of amino acids or proteins, we need more advanced techniques. Chromatography is the result of advancement of separation and purification techniques. The word Chromatography comes from Greek words chroma *chroma* "color" and *graphein* "to write". Literally meaning of Chromatography is "color writing" as initially color of separated component was used for identification. This separation technique credited to a Russian botanist Dr. Michel Tswett (1903). He separated the green plant pigment and showed different components of chlorophyll in form of different colour bands on a $CaCO_3$ column through adsorption. Likewise Richard Kuhn resolves α and β isomers of carotene and proved the importance of adsorption in analytical field (**The Nobel Prize in Chemistry 1938)**.

Column Chromatography

This is the most commonly used mode of chromatography. It can be defined as a separation process involving the uniform percolation of a liquid through a column packed with finely divided material. The stationary phase attached to a matrix (inert insoluble support) is packed in a glass or metal column and mobile phase is passed through it either by gravitational flow or with help of a pump. The selected stationary phase retards the movement of certain components of mobile phase which leads to effective separation of different components. This retardation in movement may be either due to direct interaction of solute component with stationary phase or indirect adsorption of solute component on the surface of stationary phase.

A typical column chromatographic system uses liquid mobile phase, consist of a column, a mobile phase reservoir and delivery system (pump), a detector for identification of separated

analytes as they emerge in the effluent from the column, a recorder to maintain the record of analytes in combination with detector and a fraction collector to collect each analyte separately (Fig.1).

The pictorial representation of detector response as a function of elution time or volume is known as chromatogram which consists of a series of peaks, representing the individual analytes. After application on the column the time require to elute by an analyte is known as retention time (t_R) of that analyte. The retention time of any analyte has two components. The first one is time taken by that analyte to cross the column through free spaces between matrixes. This volume is referred as void volume (V_0) and the time taken is called dead time (t_M). The value of dead time (t_M) will be same for all analytes and can be measured by the analyte which does not show any interaction with stationary phase. The second component is the time the analyte retained by the stationary phase and known as adjusted retention time (t_R'). This is the characteristic of analyte and can be given by;

$$\mathbf{t_R' = t_R - t_M}$$

The additional time taken by an analyte to elute from the column relative to an excluded analyte that does not interact with the stationary phase is known as capacity factor (k').

Thus, **Capacity factor (k') = $t_R - t_M / t_M = t_R' / t_M$** (capacity factor has no units)

Column efficiency and resolution: The efficiency of a chromatographic column is a measure of the capacity of the column to restrain peak dispersion and thus, provide high resolution. The higher the efficiency, the more the peak dispersion is restrained, and the better the column. The column efficiency will vary with the retention of the peak. In capillary columns, the efficiency generally falls as the retention increases and for a packed column the efficiency generally increases with retention.

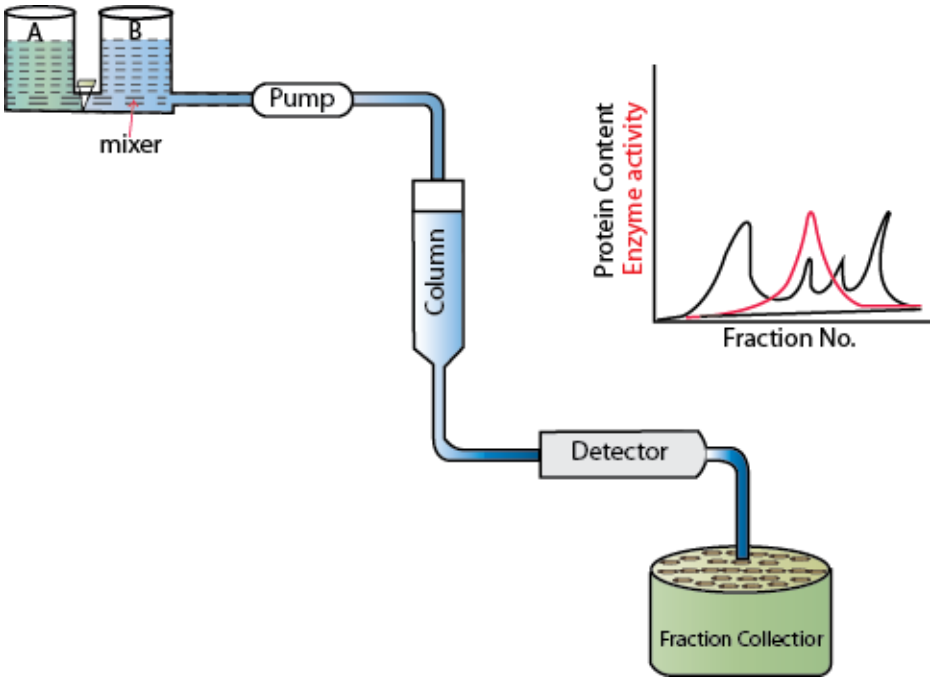

Figure 1: A pictorial representation of column chromatography

The expression for calculating the column efficiency can be derived from the plate theory. Column efficiency (N) is measured in theoretical plates (from the plate theory) and is taken as 16 times the square of the ratio of the retention time (the time between the injection point and the peak maximum) to the peak width at the points of inflection.

$$N = 16\frac{t_{R^2}}{w^2}$$

The height equivalent to the theoretical plate (HETP) or the variance per unit Length of a column is calculated as the ratio of the column length to the column efficiency (number of plates). If the length of the column is L, then the HETP (height equivalent to the theoretical plate) is;

HETP = L / N

Note: The plate model supposes that the chromatographic column contains a large number of separate layers, called theoretical plates. Separate equilibrations of the sample between the

stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next. It is important to remember that the plates do not really exist; they are a figment of the imagination that helps us understand the processes at work in the column. They also serve as a way of measuring column efficiency, either by stating the number of theoretical plates in a column, *N* (the more plates the better), or by stating the plate height; (the height equivalent to a theoretical plate) (Smaller the HEPT, better the column efficiency).

The number of theoretical plates that a real column possesses can be found by examining a chromatographic peak after elution;

$$\mathbf{N = 5.55\ t_R^2/w_{1/2}^2}$$ (where $w_{1/2}$ is the peak width at half-height)

Resolution of chromatographic column is the ability to resolve one analyte peak to other. Resolution can be defined as the ratio of the difference in retention time between the two peaks to the mean of their base widths (w_{av}).

$$\mathbf{R_S = 2(t_{RA} - t_{RB})/w_A + w_B = dt_R/w_{av}}$$

On the basis of the pressure generated inside the column the liquid column chromatography can be further subdivided as -

(a) Low pressure liquid chromatography (LPLC) - < 5 Bar.

(b) Medium pressure liquid chromatography (MPLC) – 6 to 50 Bar.

(c) High pressure liquid chromatography (HPLC) - > 50 Bar.

In low pressure chromatography, stationary phase are mainly polysaccharides which are mechanically weak. Therefore, even if such particles are produced with small diameters, they would not be sufficiently strong to withstand the high pressure required for high resolution chromatography. Thus, in HPLC silica based particles (5-10μm) are used. Smaller size of particle (large surface area) reduces size of theoretical chromatographic plat (thus number of

plate in a given length is more). Higher number of chromatographic plate results in better resolution .

Types of column chromatography: On the basis of type of interaction between the stationary and the mobile phases the column chromatography can be divided into following types.

1. Adsorption chromatography
2. Partition chromatography
3. Ion-Exchange chromatography
4. Molecular Exclusion (Gel Filtration) chromatography
5. Affinity chromatography

Adsorption chromatography

Adsorption is a surface phenomenon in which molecules get attached to column particle. The molecule is called adsorbate while column particle is adsorbent. Molecules can be separated based on their adsorption properties. Many inert materials can be used as adsorbents. e.g. alumina, charcoal, calcium carbonate.

Partition chromatography

Chromatography is a non-destructive separation technique for successful separation and purification of individual components of a complex mixture which can not be separated by usual methods due to their similar physical and chemical properties. The basic principal of any chromatography is, how a compound get distributed in between two immiscible phases, a stationary phase and a mobile phase. Those components have higher affinity for the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase which leads the separation of different components of mixture.

An analyte remains in equilibrium between the two phases;

$$A_{mobile} \rightleftharpoons A_{stationary}$$

The equilibrium constant, K_d is termed the partition coefficient or distribution coefficient, defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase. It explains about the distribution of a compound in between two phases. Suppose there are two phases A & B (immiscible to each other) and these are present together. When a compound X is mixed, it distributes itself in these two phases and the concentration of compound in phase A is X_A and in phase B is X_B. The distribution coefficient of X can be expressed as,

$$K_d = X_A/X_B \qquad (K_d \text{ is a constant at a particular temperature})$$

Factors Affecting the Magnitude of the Distribution Coefficient (K_d):

The magnitude of (K_d) is determined by the relative affinity of the solute for the two phases. Those solutes interacting more strongly with the stationary phase will exhibit a high distribution coefficient and will be retained longer in the chromatographic system. Molecular interaction results from intermolecular forces of which there are three basic types. (a) Dispersion forces (b) Polar forces (c) Ionic forces

If the compound X has same affinity for both the phase, it gets distributed equally in both the phases but compound Y has differential affinity for two phases and accordingly gets distributed in stationary and mobile phase.

References.

- David Sheehan., Physical Biochemistry (Wiley) ISBN: 978-0-470-85602-4
- Raymond P. W. Scott., *Principles and Practice of Chromatography,* (Chrom-Ed Book Series). http://faculty.ksu.edu.sa/Dr.almajed/Books/practical%20HPLC.pdf

Quiz-1

Q. What is the basis of primary classification of chromatography?

Ans. Primary classification of chromatography is based on the phenomenon involving the process of either partition or adsorption.

Q. What is partition or distribution coefficient?

Ans. Partition or distribution coefficient is the molar ratio of concentrations of analyte in stationary and mobile phase.

Partition or distribution coefficient $\mathbf{K_d = X_A/}X_B$

Where X_A = Molar concentration of X in phase A.

X_B = Molar concentration of X in phase B.

Q. What are three molecular forces which affects the partition or distribution coefficient?

Ans.(a) Dispersion forces (b) Polar forces (c) Ionic forces

Q. What are the different types of column chromatography?

Ans. Different types of column chromatographies are as fallows

Adsorption chromatography, Partition chromatography, Ion-Exchange chromatography, Molecular Exclusion (Gel Filtration) chromatography, Affinity chromatography.

Q. What is capacity factor and what is the unit of capacity factor?

Ans. The additional time taken by an analyte to elute from the column relative to an excluded analyte that does not interact with the stationary phase is known as capacity factor (k'). It has no unit.

Capacity factor (k') = $t_R - t_M / t_M = t_R' / t_M$