Chromatography

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 .

Based on this approach three components form the basis of he 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

Purpose of chromatography

- The purpose of chromatography is to separate a complex mixture into individual component exploiting the partition effect which distribute the molecules into the different phases. A distribution of a molecule between two phases A and B is given by a distribution coefficient, Kd. In most of the chromatography techniques, phase A is stationary phase or matrix and phase B is mobile phase or buffer.

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, esther, 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

Types of chromatographic techniques

The twelve types are:

(1) Column Chromatography

(2) Paper Chromatography

(3) Thin Layer Chromatography

(4) Gas Chromatography

(5) High Performance Liquid Chromatography

(6) Fast Protein Liquid Chromatography

(7) Supercritical Fluid Chromatography

(8) Affinity Chromatography

(9) Reversed Phase Chromatography

(10) Two Dimensional Chromatography

(11) Pyrolysis Gas Chromatography

(12) Counter Current Chromatography.

Different types of chromatography

Technique	Stationary phase	Mobile phase	Basis of separation
***Paper chromatography**	solid (cellulose)	liquid	polarity of molecules
***Thin layer chromatography (TLC)**	solid (silica or alumina)	liquid	polarity of molecules
***Liquid column chromatography**	solid (silica or alumina)	liquid	polarity of molecules
Size exclusion chromatography	solid (microporous beads of silica)	liquid	size of molecules
Ion-exchange chromatography	solid (cationic or anionic resin)	liquid	ionic charge of the molecules
Affinity chromatography	solid (agarose or porous glass beads on to which are immobilized molecules like enzymes and antibodies)	liquid	binding affinity of the analyte molecule to the molecule immobilized on the stationary phase
Gas chromatography	liquid or solid support	gas (inert gas like argon or helium)	boiling point of the molecules

Distribution Constant

The distribution coefficient is the ratio of the concentration of solute in the organic phase over the concentration of solute in the aqueous phase (the V-terms are the volume of the phases). This is essentially an equilibration process whereby we start with the solute in the aqueous phase and allow it to distribute into the organic phase.

$solute_{aq} = solute_{org}$

$D_c = [solute]org / [solute]aq = mol_{org}/V_{org} / mol_{aq}/V_{aq} = V_{aq}\ x mol_{aq} / mol_{aq} \times V_{org}$

The distribution coefficient represents the equilibrium constant for this process. If our goal is to extract a solute from the aqueous phase into the organic phase, there is one potential problem with using the distribution coefficient as a measure of how well you have accomplished this goal. The problem relates to the relative volumes of the phases. For example, suppose the volume of the organic phase was very small compared to the volume of the aqueous phase. (Imagine using 100 ml of organic solvent relative to a volume of water equal to that in an Olympic-sized swimming pool) You could have a very high concentration of the solute in the organic phase, but if we looked at the amount of solute in the organic phase relative to the amount still in the water, it might only be a small portion of the total solute in the system. Since we really want as much of the solute in the organic phase as possible, this system has not yet achieved that outcome.

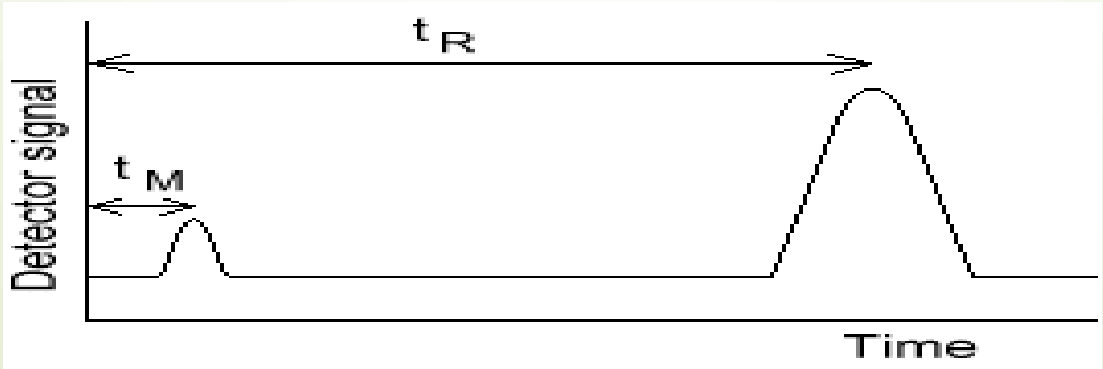
Distribution of analytes between phases

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases;

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

The equilibrium constant, K, is termed the partition coefficient; defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase.

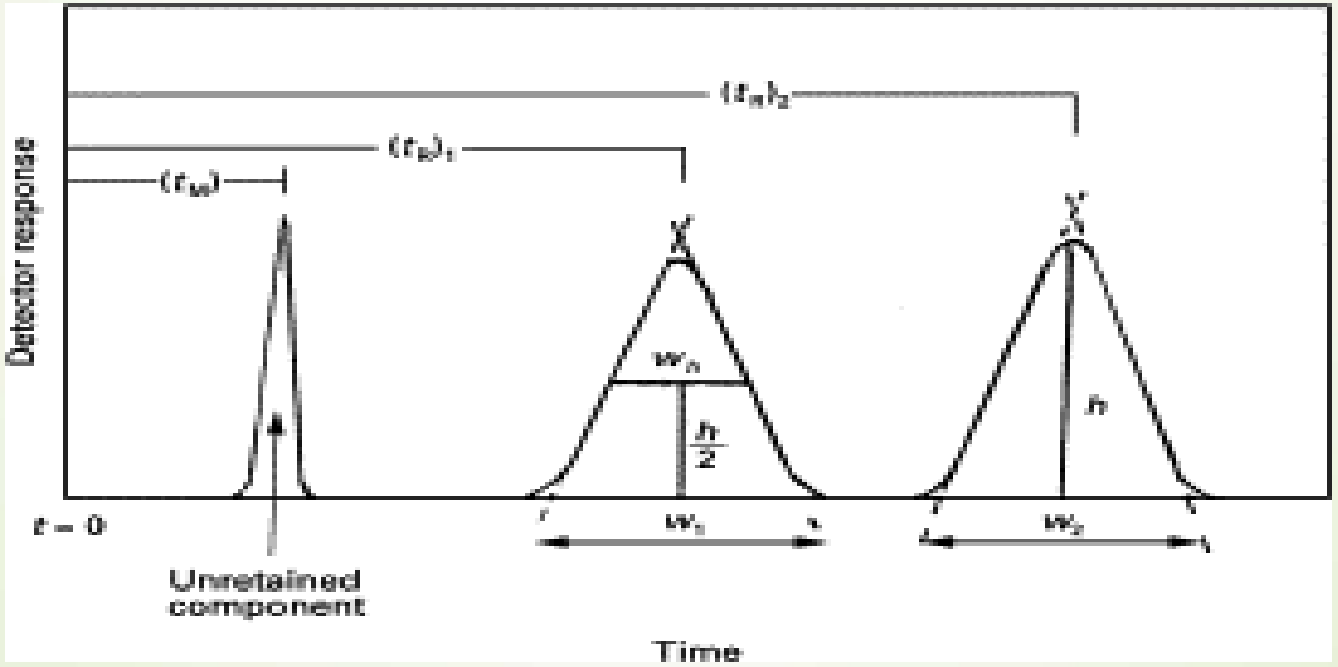
The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the retention time (tR). Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called t_M..



Retention Factor

- Retention parameters are measured in terms of chart distances or times, mobile phase volumes or retention factors (k) (previously called capacity factors, k′). With a constant recorder speed, chart distances are directly proportional to times. Likewise if the flow rate is constant, the volumes are proportional to times, e.g. t_R (time) is analagous to V_R (volume). In GC with a compressible carrier gas, V_M, V_R and V'_R represent volumes under column outlet pressure. If F_c is used in their calculation these correspond to volumes at column temperature.

When a mixture is chromatographed, the time taken for a component to be eluted from the column, the (total) retention time (t_R), is measured from the moment of injection to the appearance of the peak maximum. This, together with the width of the peak measured at the baseline (w) or at half peak height (w_h), and the elution of an 'unretained peak', are important parameters in chromatography. These are illustrated in Figure, which represents the separation of a two-component mixture.



- The retention volume (V_M) of an unretained peak (where $V_M=F\times t_M$) is also called the gas hold-up volume or dead volume, and is equal to the volume (both inter- and intra-particle) available to the mobile phase in the column. The corrected gas hold-up volume (V_{Mo}) is corrected for gas compressibility where $V_{Mo}=V_{Mj}$.
- Injection techniques in GC, where the sample is held at the head of the column before it starts moving through the column, have necessitated the introduction of additional terms. These are the peak time/volume (t_R, V_R), where the time/volume is measured from the start of elution rather than time of injection, and the adjusted retention time/volume (t_R'/V_R'), which is the total elution time/volume minus the gas hold-up time/volume

R^j

The corrected retention time/volume ($t_R°/V_R°$) is the total retention time/volume corrected for carrier gas compressibility:

$t_R°=t_Rj=V_Rj/F_c=V_R°/F_c;V_R°=V$

The net retention time/volume (t_N, V_N) is the adjusted retention time/volume corrected for carrier gas compressibility:

tN=VR′j/Fc=VN/Fc;VN=VR′j

The specific retention volume at column temperature normalizes the retention for the amount of stationary phase on the column (Ws):

$V_g^{\theta}=V_N/Ws$

Normalizing the specific retention volume to 0°C (273.15 K) gives rise to the specific retention volume at 0°C (Vg):

$V_g=V_g^{\theta}\times 273.15k/Tc=V_N/Ws\times 273.15k/Tc$

where Tc is the column temperature.

The unretained peak is given by a substance that has no affinity for the stationary phase and therefore passes through the column at the same speed as the mobile phase. A substance that shows affinity for the stationary phase moves through the column more slowly than the mobile phase and is said to be retained. The ratio of the two velocities is known as the retardation factor (R):

R=rate of movement of retained peak/rate of movement of mobile phase

A retained component spends time in both the mobile phase (t_M) and the stationary phase t_S), and retention time t_R is given by:

$t_R=t_M+t_S$

The time spent in the stationary phase is dependent on the distribution coefficient (Kc) such that $t_S=KcV_S$. If C_S and C_M are the concentrations of a component in the stationary phase and mobile phase, respectively, then the distribution constant is given by:

$Kc=C_S/C_M$

The time spent in the stationary phase is dependent on the distribution coefficient (Kc) such that $t_S=K_cV_S$. If C_S and C_M are the concentrations of a component in the stationary phase and mobile phase, respectively, then the distribution constant is given by:

$K_c=C_S/C_M$

The rate of movement of a component through the column is inversely proportional to the distribution constant, i.e. a substance with a high concentration in the stationary phase (a high distribution coefficient) moves slowly through the column. Components of a mixture are, therefore, separated only if their distribution coefficients differ. Using volumes rather than times we can write:

$V_R=V_M+K_C V_s$ or $V_R'=K_CV_s$

which is the fundamental equation for chromatography, neglecting the effects of nonlinearity of the sorption isotherm and band broadening.

In adsorption chromatography the stationary phase volume is replaced by the surface area (A_S) of the stationary phase, and the distribution coefficient is replaced by the adsorption coefficient (K_A). In GC both V_R and V_M have to be corrected for gas compressibility.

An alternative expression (the retention factor, k) for the distribution of a sample component is in terms of the relative number of moles (n) of a component in the stationary and mobile phases, such that:

$k=nS/nM=Kc(VS/VM)$

The ratio V_S/V_M is the phase ratio. Early literature will refer to the retention factor as the capacity ratio (k').

Since a sample molecule only migrates through the column when it is in the mobile phase, the retardation factor (R) may be written:

R=amount of solute in the mobile phase amount of solute in mobile +stationary phases

or:

$R=n_M/(n_M+n_S)=1/(1+k)$

Substituting the retention factor into the equation:

$V_R=V_M+K_CV_S$

gives:

$V_R=V_M(1+k)$

or using retention times:

$t_R=t_M(1+k)$

and on rearrangement:

$k=t_R-t_M/t_M$

This last expression is widely used as a simple way of expressing retention from values easily measured from the chromatogram, and without the need to measure flow rates. Since:

$t_M=L/u—M$

we can write:

$t_R=L_u—(1+k)$

Effect of Temperature

- If the column temperature is increased, the chromatographic separation process becomes faster. A rule of thumb for reversed-phase isocratic separation predicts a retention time decrease of 1–2% for each 1 °C column temperature increase. In that case, retention factors of sample compounds change, sample compounds may co-elute, or their selectivity can be inverted by a small variation of the column temperature .Consequently, a proper column temperature control is essential for separations with marginal resolution of the critical peak pair or if retention times are used for the identification of individual sample compounds.

Partition isotherm

An isotherm is a graphical representation of the interaction of an adsorbent and a solute in solution (gas or liquid) at a specified temperature. The isotherm is a graphical representation of the partition coefficient or distribution constant K:

$$K=C_S/C_G$$

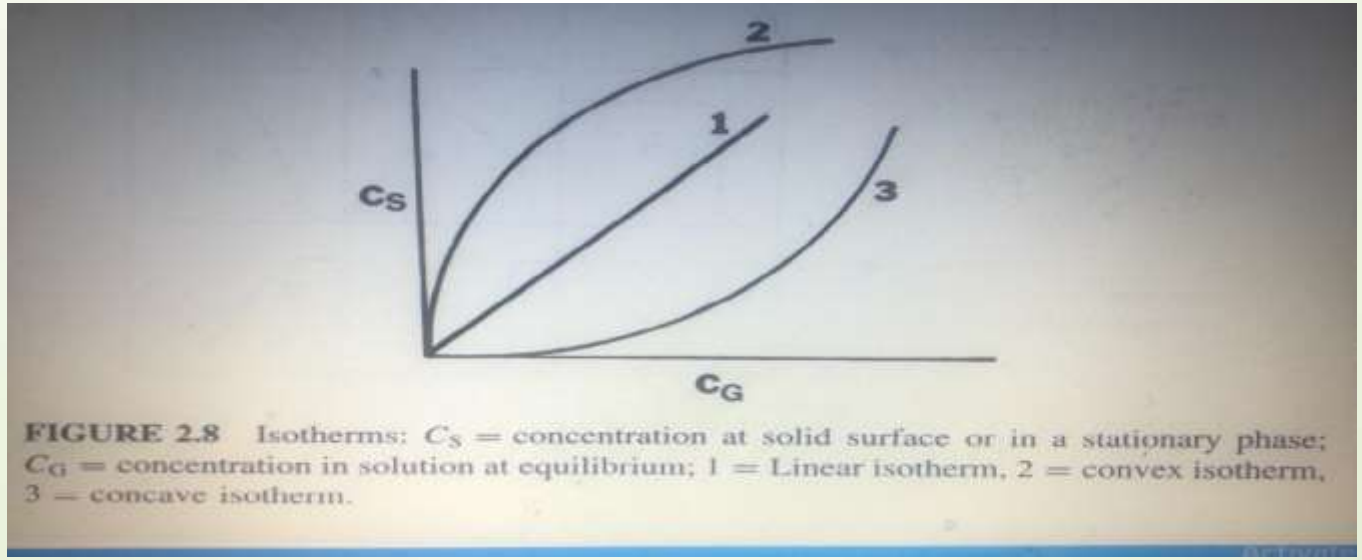


FIGURE 2.8 Isotherms: C_S = concentration at solid surface or in a stationary phase; C_G = concentration in solution at equilibrium; 1 = Linear isotherm, 2 = convex isotherm, 3 = concave isotherm.

and on rearrangement:

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we can write:

$t_R=L/u—(1+k)$

Hence the retention time is directly proportional to the column length and inversely proportional to the linear flow rate of the mobile phase.

Development chromatogram

- In terms of operation, in development chromatography the mobile phase flow is stopped before solutes reach the end of the bed of stationary phase. The mobile phase is called the developer, and the movement of the liquid along the bed is referred to as development. With glass columns of diameter in the centimetre range and large samples (cubic-centimetre range), the bed is extruded from the column, the solute zones carved out, and solutes recovered by solvent extraction. Although this is easily done with coloured solutes, colourless solutes require some manner of detection, such as ultraviolet light absorption or fluorescence or the streaking of the column with a reagent that reacts with the solute to form a coloured product.

Are three types of chromatographic development

- Displacement development
- Frontal analysis.
- Elution development
- Elution development is the only development technique employed in both GC and LC

Displacement development

-Displacement development is only effective with a solid stationary phase where the solutes are adsorbed on its surface.

-The components array themselves along the distribution system in order of their decreasing adsorption strength.

-The sample components are usually held on the stationary phase so strongly that they are eluted very slowly or even not at all.

The solute must be displaced by a substance more strongly held than any of the solutes - (called displacer which is the mobile phase).

-In displacement development the solutes are never actually separated from one another.

-The solutes leave the system sequentially and somewhat mixed with its neighbor.

Frontal Analysis

- It can be effectively employed in a column chromatography. The sample is fed continuously onto the column as a dilute solution in the mobile phase. - Frontal analysis can only separate part of the first component in a relatively pure state, each subsequent component being mixed with those previously eluted.
- For a three components mixture, containing solutes (A), (B)and (C) as a dilute solution is fed continuously onto a column:
- The first component to elute, (A), will have less affinity to the stationary phase. Then the second solute, (B), will elute but it will be mixed with the first solute. Finally, the third solute (C),will elute in conjunction with (A) and (B). It is clear that only solute (A) is eluted in a pure form.
- Thus, frontal analysis is not suitable for most practical analytical applications.

Elution Development

- Elution development is best described as a series of adsorption-extraction processes which are continuous from the time the sample is injected.

- -As the elution proceeds, the moving phase will continuously displace the concentration profile of the solute in the mobile phase forward, relative to that in the stationary phase.

Types of elution techniques

- Simple elution: the column is eluted with the same solvent all the time. This is suitable when the components have similar affinities for the stationary phase and are therefore eluted rapidly, one after another.

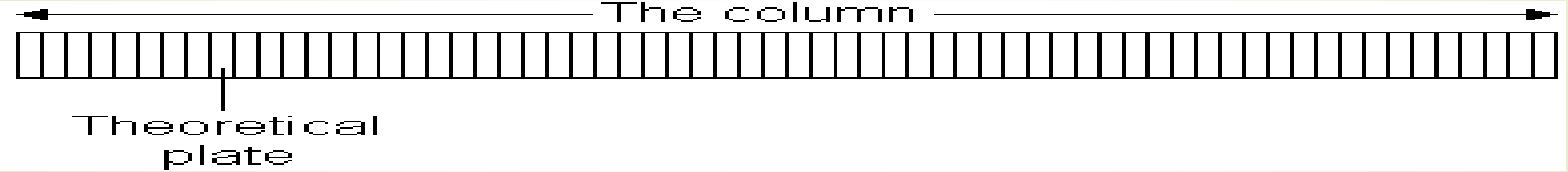
- -Stepwise elution: the eluent (solvent) is changed after a predetermined period of time. The eluents are chosen to have increasing eluting power, that is, increasing affinity to the remaining components, and there for releasing them from the stationary phase

Gradient elution

- The composition of the eluting solvent is changed gradually to achieve separation of widely varying affinities for the stationary phase. The ratio of two solvents is gradually changed to increase slowly the eluting power of the mobile phase. Thus enhancing the resolution (narrows the zones and reduces tailing). The solvent composition gradient may be a concentration, pH, polarity or ionic strength gradient.

Theoretical Plate

The plate model supposes that the chromatographic column is 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* (the smaller the better). If the length of the column is L, then the HETP is

HETP = L / N

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

$$N = \frac{5.55\ t_R^{\ 2}}{w_{1/2}^{\ 2}}$$

where $w_{1/2}$ is the peak width at half-height.

As can be seen from this equation, columns behave as if they have different numbers of plates for different solutes in a mixture.

Rate Theory of Chromatography

Based on a random walk mechanism for the migration of molecules through a column • Takes into account: – mechanism of band broadening – effect of rate of elution on band shape – availability of different paths for different solute molecules to follow – diffusion of solute along length

$H = H_L + H_S + H_M + H_{SM}$

H = height equivalent to theoretical plate (as in Plate Theory)

H_L = contribution due to longitudinal diffusion

H_S = stationary phase mass transfer contribution

H_M = diffusion associated with mobile phase effects

H_{SM} = diffusion into or mass transfer across a stagnant layer of mobile phase (neglect)

$H = B/\mu + C\mu + A$

van Deemter Equation A, B & C are coefficients, μ = velocity)

Resolution (RS)

- we can define an equation for the resolution (RS) of two compounds. This will be a measure of how much two compounds in a chromatogram are separated from each other.
- $Rs=2(t_2 - t_1) /(W_1 + W_2)$.....1
- The terms t_2 and t_1 refer to the retention time of the two compounds, and W_1 and W_2 to the width of each peak at baseline.

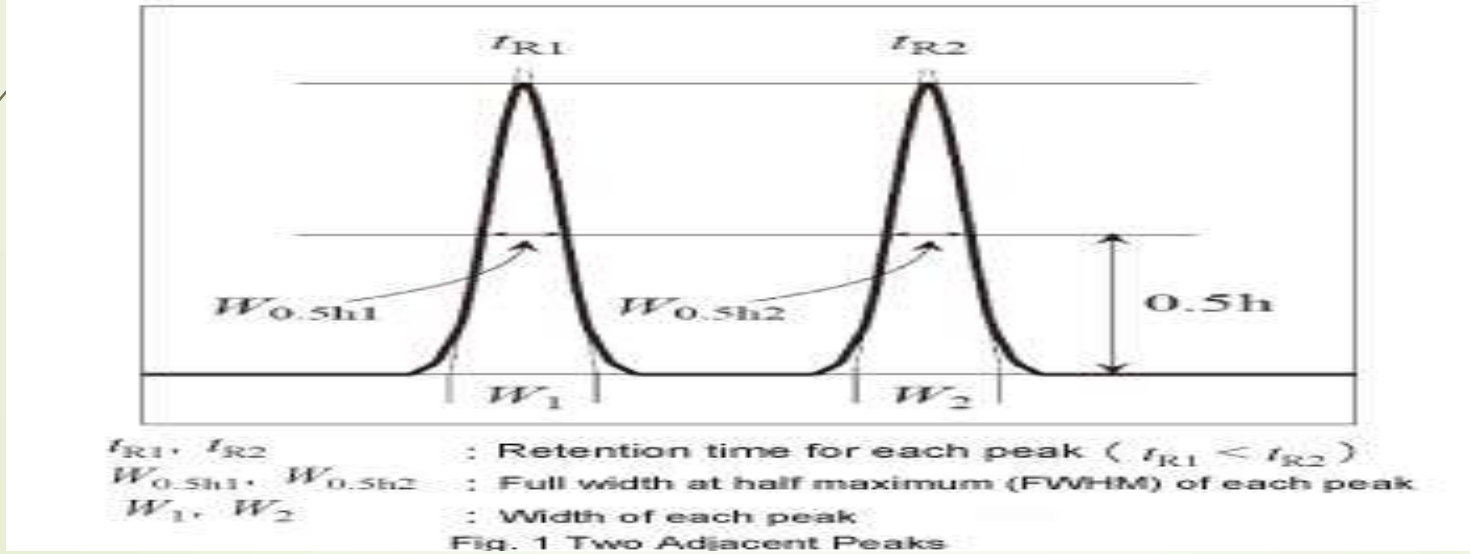


Fig. 1 Two Adjacent Peaks

quation (1) indicates that the resolution is the difference between peak retention times divided by the average peak width.