High Performance Liquid Chromatography

What is HPLC ?

It is a separation technique that involves:

- *Injection of small volume of liquid sample*
- *Into a tube packed with a tiny particles* ***(stationary phase).***
- *Where the individual components of the sample are moved down the packed tube* ***(column)*** *with a liquid* ***(mobile phase)*** *forced through the column by* ***high pressure*** *delivered by the* ***pump.***

- *These component are separated from one another by column packing.*

- *The separated component are detected at the exit of the column by flow through* ***detector*** *that measure their amount.*

Theory :

Depending on the HPLC mode , there are different types of the adsorption forces :

- *Hydrophobic (non-specific) interaction in* ***reversed phase****.*
- *Dipole-dipole (polar) interaction in* ***normal phase****.*
- *Ionic interaction in the* ***ion exchange chromatography.***
- *separation of mixture by the molecular size of component in* ***Size exclusion chromatography****.*

Types of HPLC:

Classification based on the nature of the stationary phase and separation process:

a-Adsoption chromatography:

- *The stationary phase is an adsorbent (like silca gel).*
- *Separation based on repeated adsorption- adsorption steps.*

b- ion exchange chromatography:

- *The stationary bed has an ionically charged surface of opposite charged to the sample ions.*

- *The **stronger** the charge on the sample the stronger it will be attracted to the ionic surface the longer it will take to elute.*

C-Size exclusion chromatography:

- *The column filled with material having controlled pore sizes.*
- ***Larger molecules*** *are rapidly washed through the column.*
- ***smaller molecules*** *penetrate inside the porous of packing particles and elute later*.

Normal and reversed phase chromatography:

Normal phase :

- *The stationary bed is strongly polar in nature (e.g : silica gel).*
- *The mobile phase is non polar.*
- *Polar samples are retained on the polar surface of the column packing for longer , than less polar material.*

Reversed phase chromatography:

- *The stationary phase is (non polar)in nature .*
- *The mobile phase is a polar liquid.*
- *The more non polar the material is , the longer it will be retained.(water & methanol or acetonitrile).*

Instrumental HPLC system:

1-mobile phase reservoir.

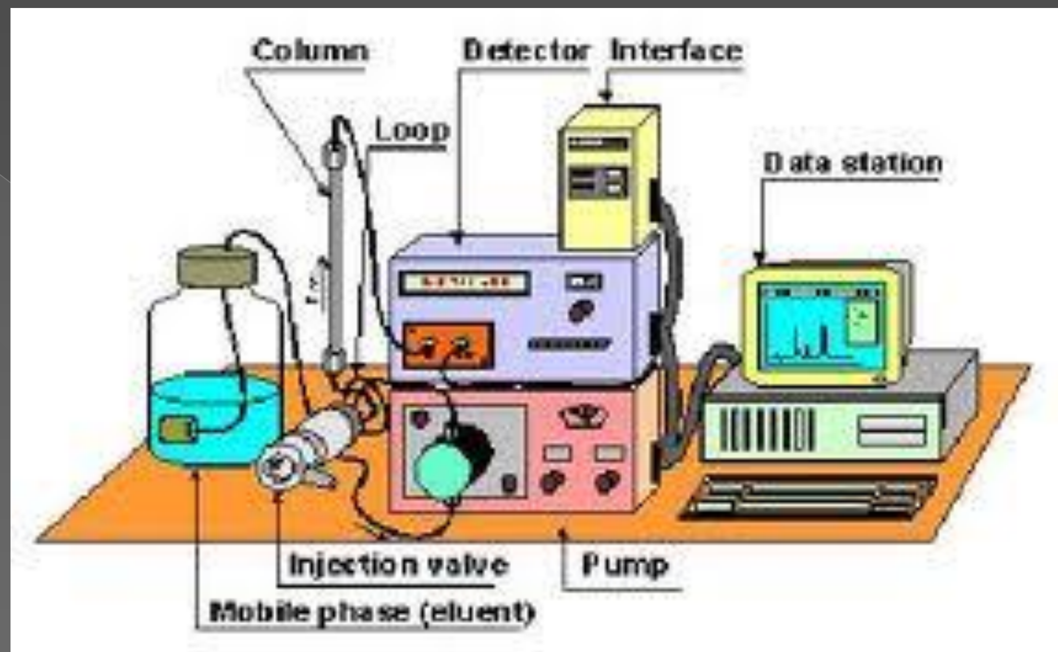
2-pumping.

3-injector.

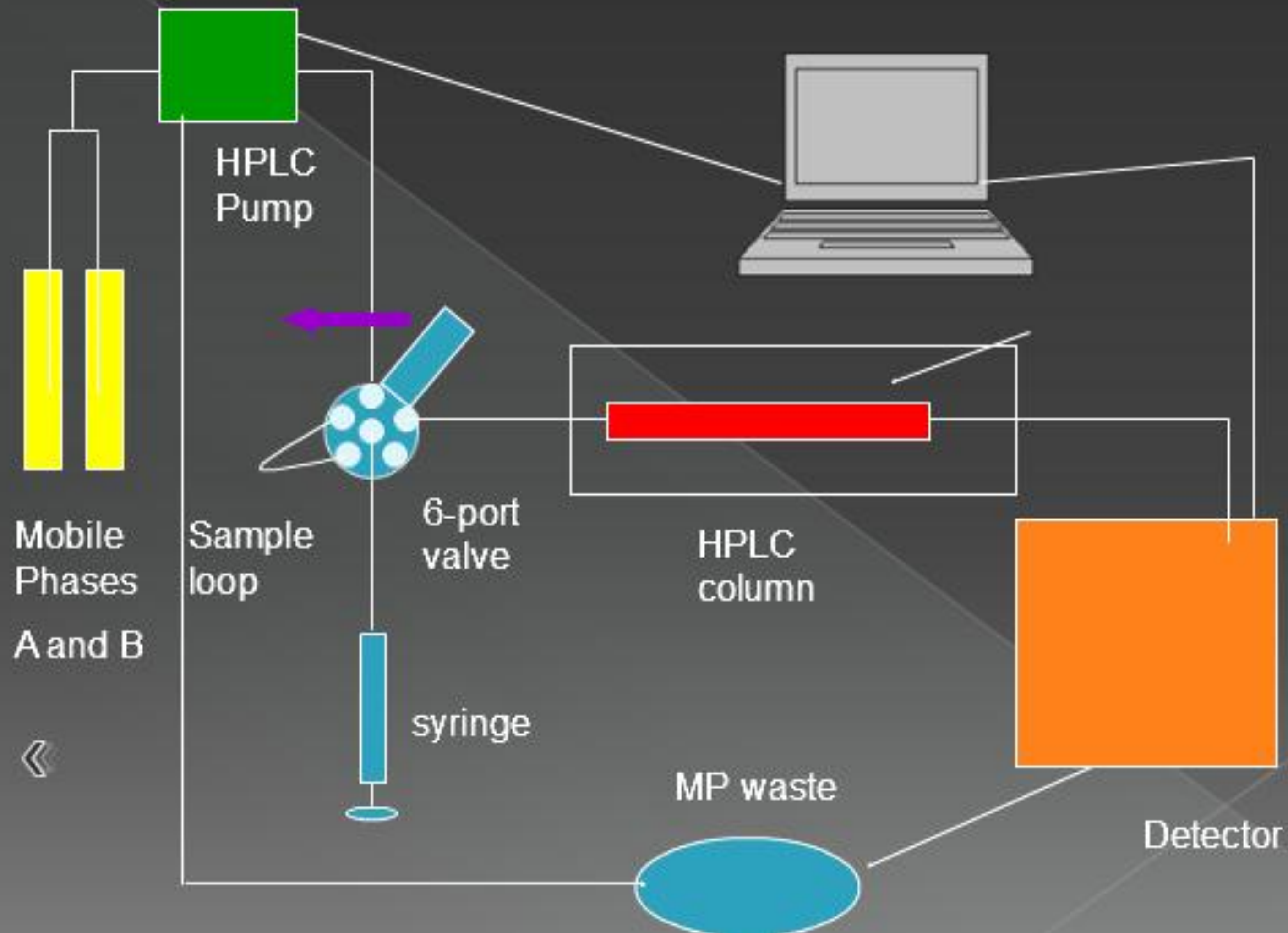
4-column.

5-detector.

6-Data system.



HPLC-UV:-



mobile phase reservoir:

- *Individual reservoirs store the mobile phase components until they are mixed and used.*

- *May also manually prepare the mobile phase mixture and store in a single reservoir*

Mobile phase degassing:

Dissolved gases in the mobile phase may block flow through the system.

Degassing by:

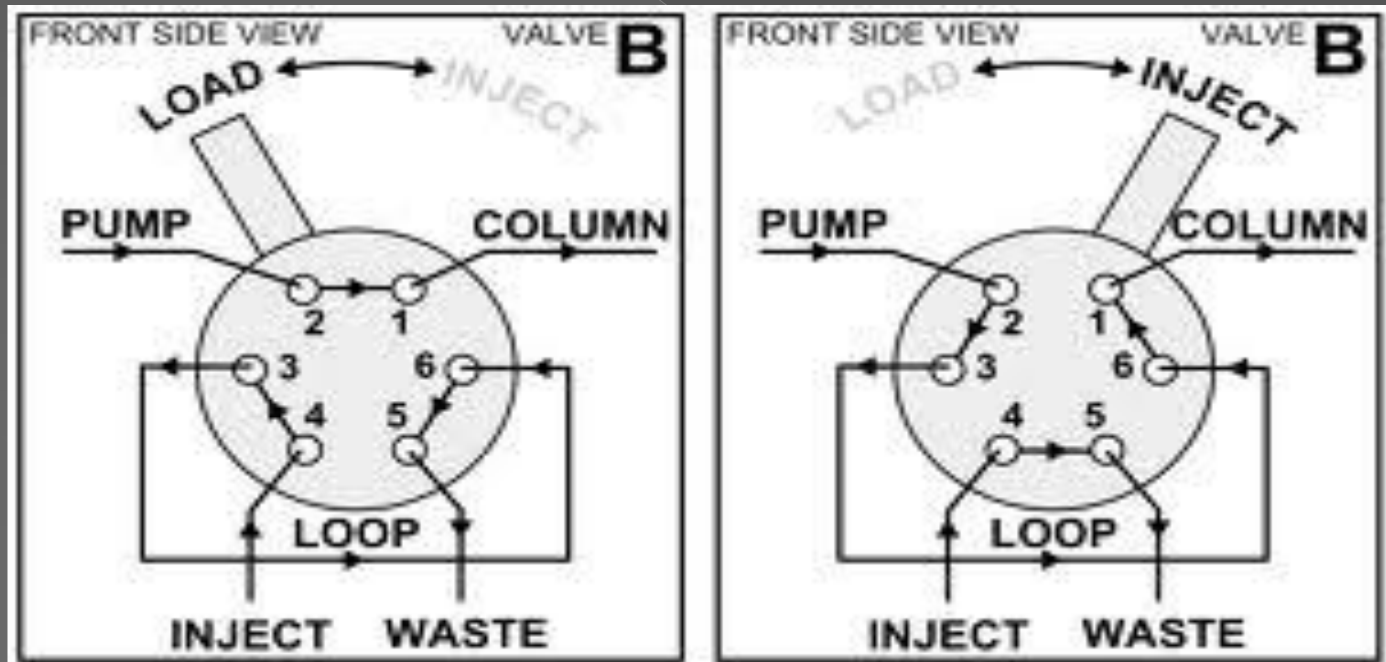
- *Inert insoluble gass. (e.g: helium)*
- *Filter mobile phase under vaccum.*

Pump:

- *High pressure pump is needed to force solvent through packed stationary phase beds.*
- *Very Stable flow rate only essential in SEC.*
- *Flow rate range: from 0.01 to 10 ml/min.*
- *Pressure range: from 1 to 5,000 psi.*

Injector::

- *A sample is injected into the flow path at continuous pressure. for analysis. Using manual injector or an auto-sampler.*
- *Each type is equipped with* ***six-port valves****.*



Column:

Guard column: *Protects the analytical column*

- *Particles*
- *Interferences*
- *Prolongs the life of the analytical column*

Analytical column: *Performs the separation.*

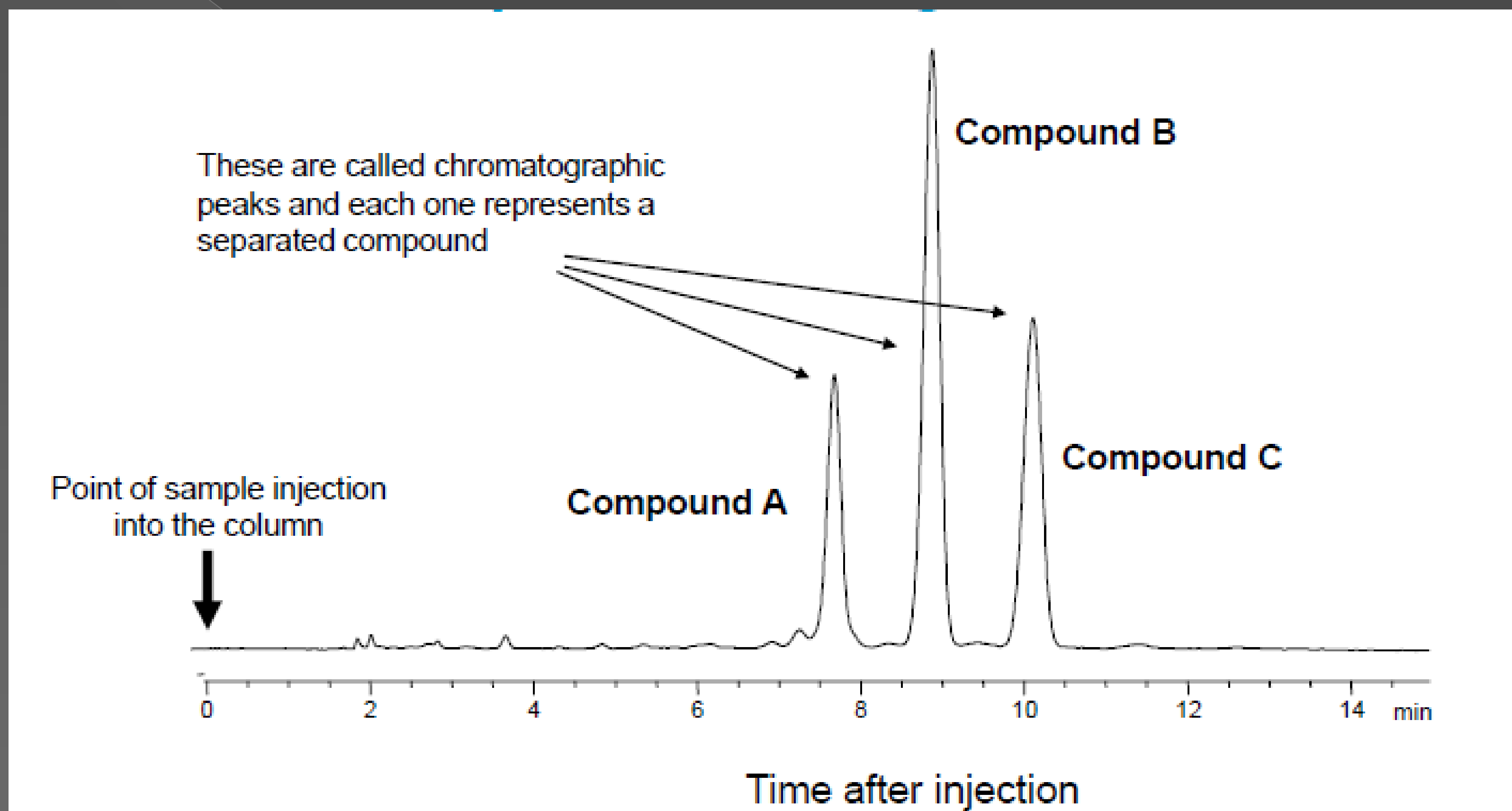
Detector:

- *UV spectrophotometer*
- *Mass spectrometer*
- *Refractive index*
- *Electrochemical*
- *Conductivity*
- *Fluoresence*

Application:

- *Qualitative/quantitative analysis of nucleic acid , amino acid and protein in physiological sample.*
- *Measuring level of active drug and degradation product in pharmaceutics.*
- *Measuring level of hazardous compound.*
- *Monitoring environmental sample.*
- *Purifying compounds from mixture.*

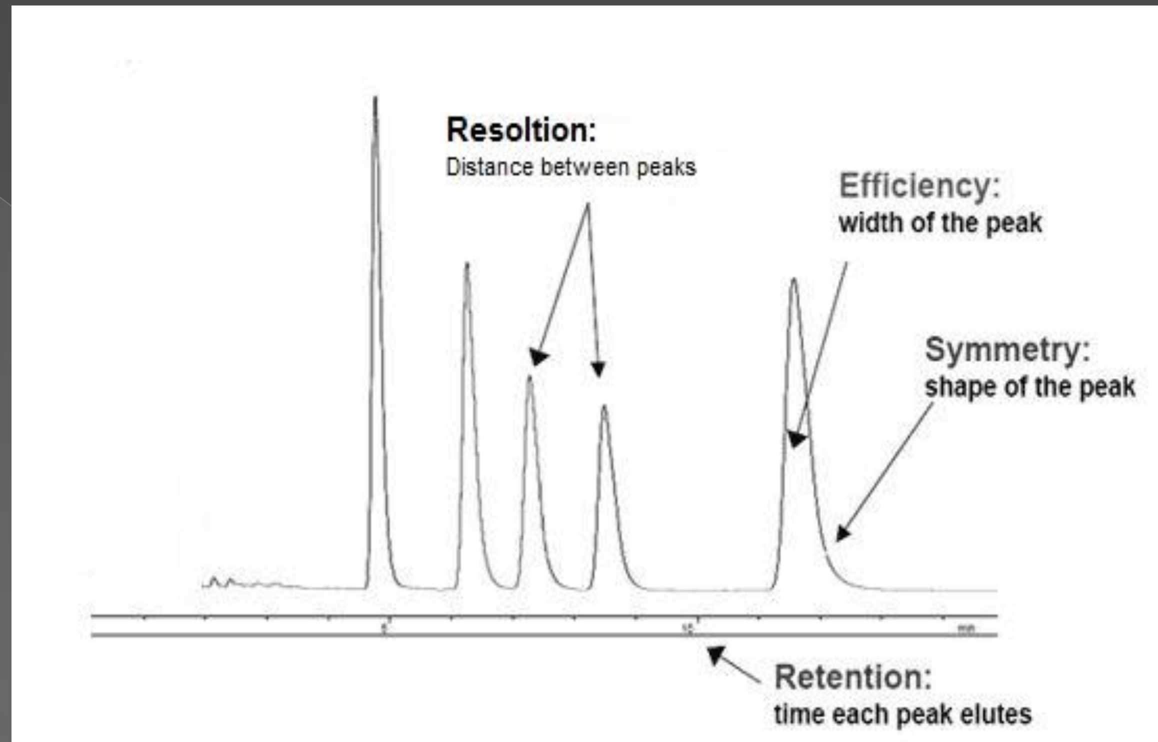
HPLC chromatogram:



This is the chromatogram resulting from the injection of a small volume of liquid extracted from a vitamin E capsule that was dissolved in an organic solvent. Modern HPLC separations usually require 10- to 30-minutes each.

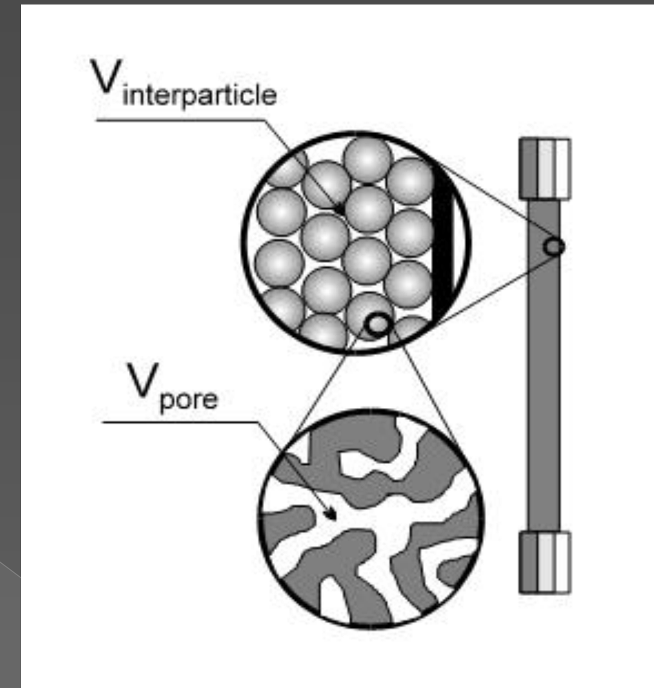
HPLC parameters:

- *Dead volume*
- *Retention volume(V_R)*
- *Retention time (t_R)*
- *Void time (t_0)*
- *Capacity factor (k)*
- *Selectivity (α)*
- *Efficiency*
- *Resolution (R)*
- *Peak symmetry factor (s)*



Dead volume (V_0):

is the total volume of the mobile phase in the chromatographic column.



$$V_0 = V_{column} - V_{partical} + V_{pores}$$

$$V_0 = 0.65\ (\pi\ D^2\ L\ /\ 4\ )$$

where dead volume equal 65% of empty column volume

Retention Volume (V_r):

Total volume of mobile phase (in ml) require to elute certain substance.

$$V_r = t_r \times F$$

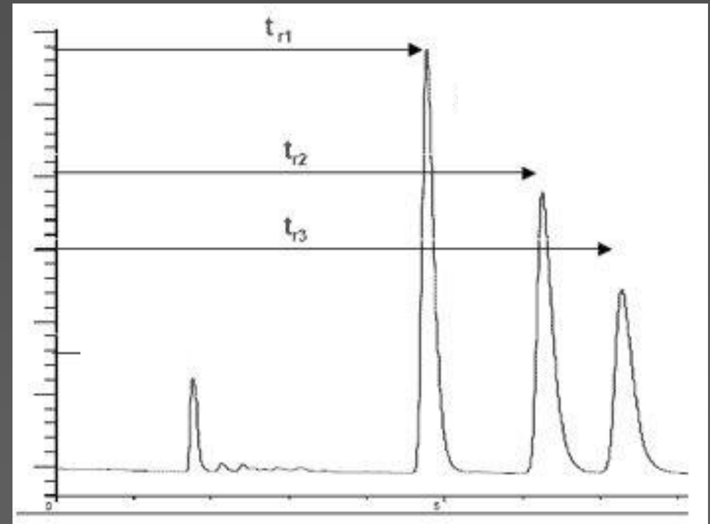
Where:

F (Flow rate) :

volume of mobile phase per unit time passing through the column. usually reported as ml / min.

Retention time (t_r) :

- *Time from injection point to maximum detector response for corresponding compound.*



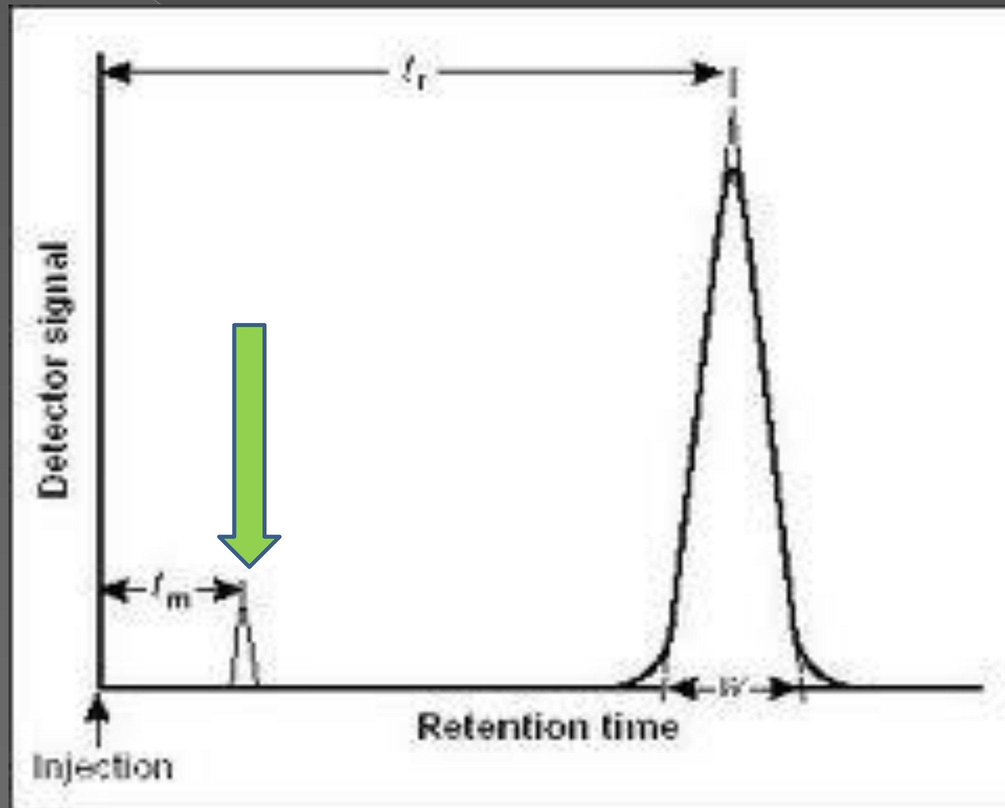
- *Qualitative analysis.*

Parameter affecting retention time :

- *temperature of column.*
- *column length.*
- *packing material.*
- *flow rate & type of mobile phase.*

Void Time (t_m) or (t_0) :

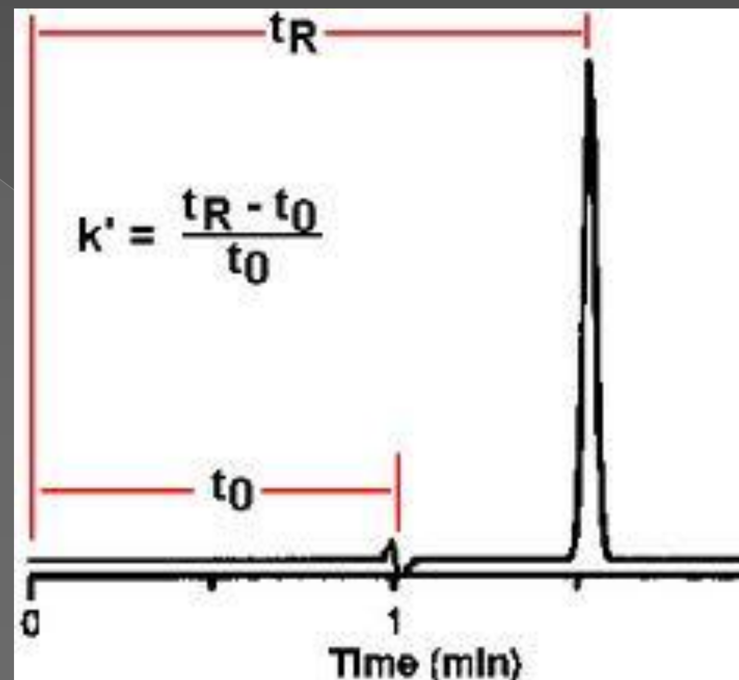
The time of mobile phase required to elute ***non-retained*** *components.*



Capacity factor (K):

- *Use to describe the migration rate of solutes on columns.*

$$K = (t_R - t_0) / t_0$$

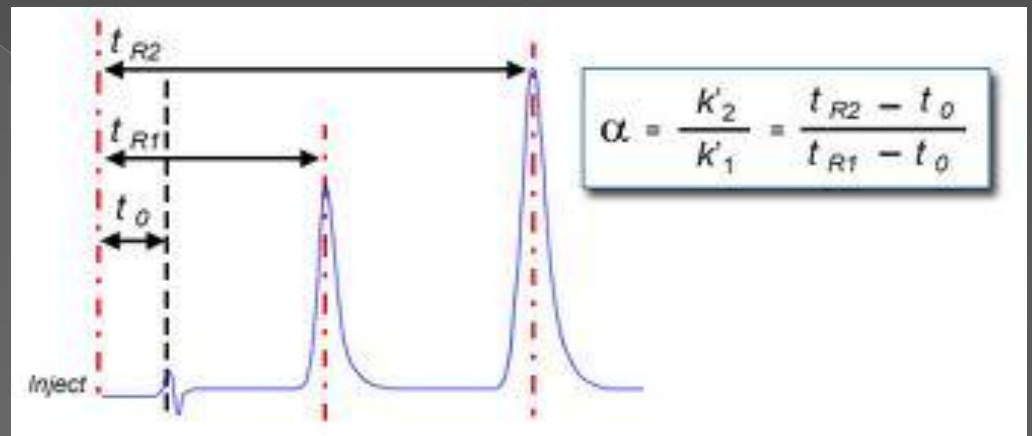


- *Recommended to be (2 – 10).*

Selectivity (α):

it describes the separation of band centres

$$\alpha = K_2 / K_1$$

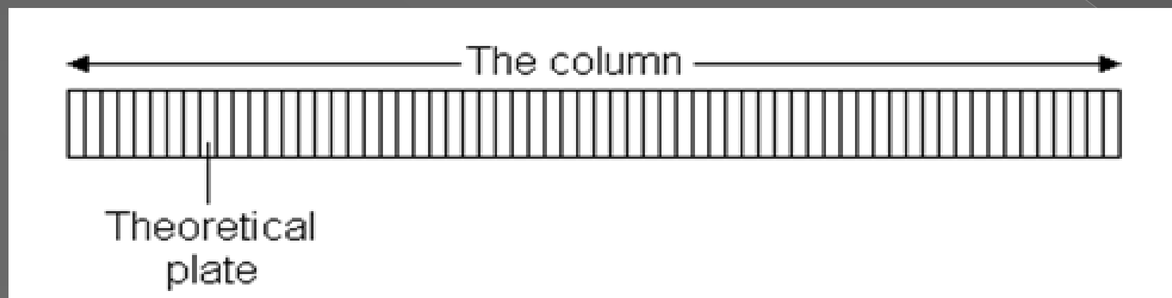


$$\alpha = t_{r_2} - t_0 / t_{r_1} - t_0$$

Efficiency:

It is important to remember that the plates do not really exist.

- *They are a figment of the imagination that helps us to understand processes at work in the column.*
- *They also serve as a way of measuring column* ***efficiency.***



- *Narrow peaks have high efficiency.*

- *Units of efficiency are "theoretical plates" N (the more plates the better),*

$$N = 16\,(t_R / w)^2$$

Parameters affecting efficiency:

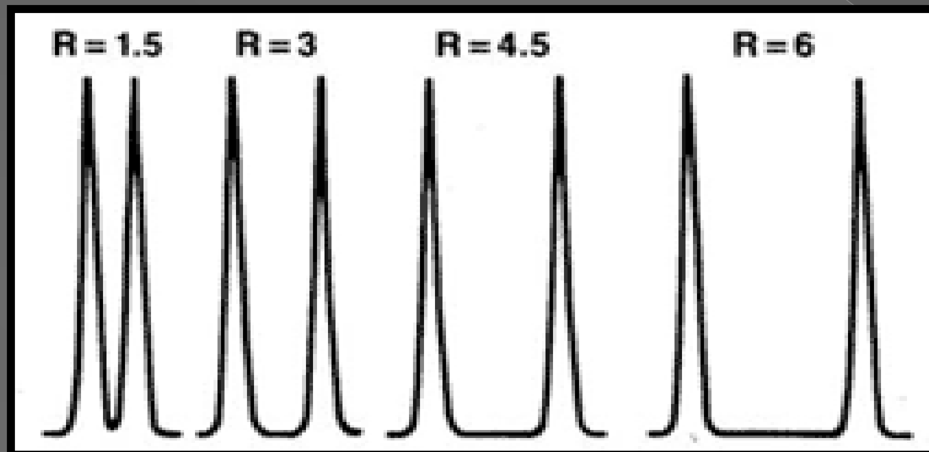
- *Flow rate*
- *Column length*
- *Particle diameter*
- *Particle size distribution*

Resolution (R) :

- *is a quantitative measure of the degree of separation between two chromatographic peaks, A and B , and is defined as:*

$$R = 2\,(t_{r\,B} - t_{r\,A}) \,/\, (w_B + w_A)$$

- *Recommended to be* ***1.5***



Peak symmetry:

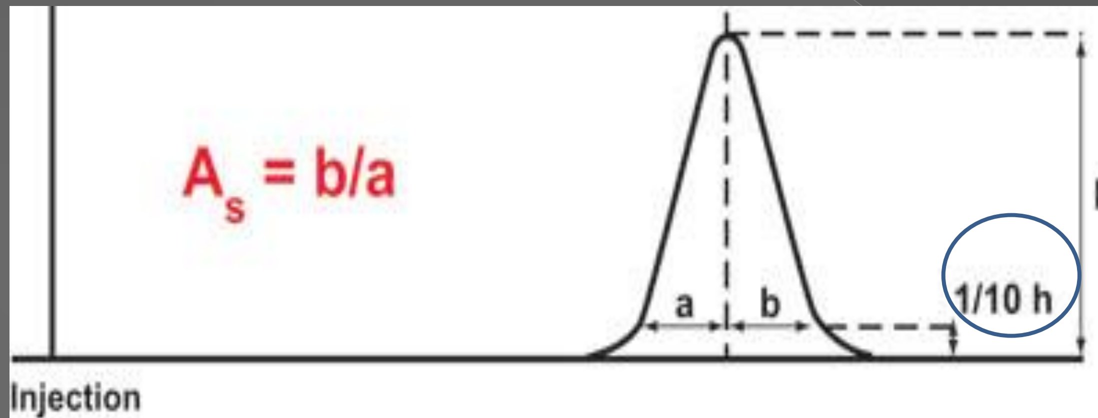
- *The symmetry of a peak is judged by the values of two half peak widths , a and b .*
- *When a = b, a peak is called **symmetric**, which is desired.*
- *Unsymmetrical peaks are often described as :*

"tailing" or "fronting".

Calculation of peak Asymmetry Factor:

Where:

A_s *= peak asymmetry factor*
b *= distance from the point at peak midpoint to the trailing edge (measured at* **10%** *of peak height)*
a *= distance from the leading edge of peak to the midpoint (measured at* **10%** *of peak height)*



Calculation of Tailing Factor :

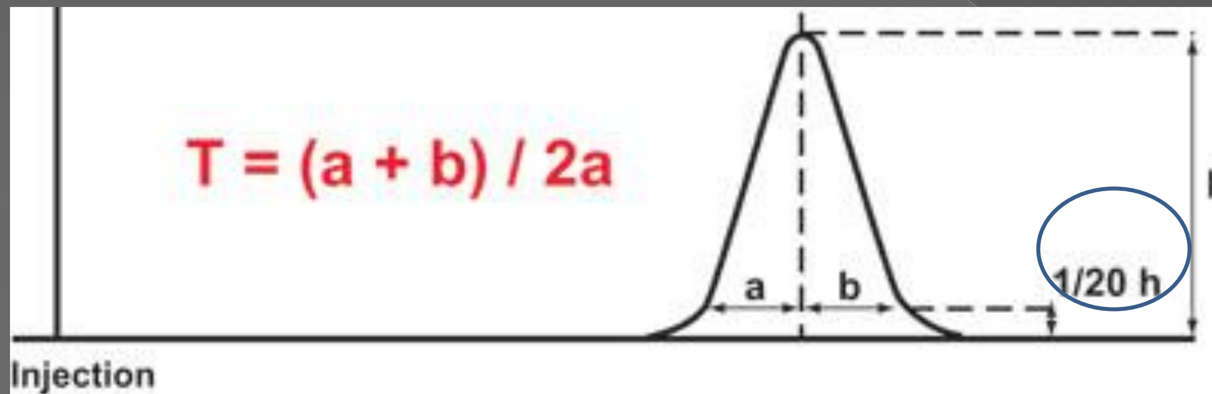
$T_f > 1$ ***Tailing*** , $T_f < 1$ ***fronting***

Where:

T *= tailing factor (measured at* ***5%*** *of peak height)*

b *= distance from the point at peak midpoint to the trailing edge*

a *= distance from the leading edge of the peak to the midpoint*



For ideal separation:

The attributions of an ideal separation are as follows:

- *Should meet baseline resolution of the compounds of interest.*
- *Each desired peak is narrow and symmetrical.*
- *Has no wasted dead time between peaks.*
- *Takes a minimal amount of time to run.*
- *The result is reproducible.*