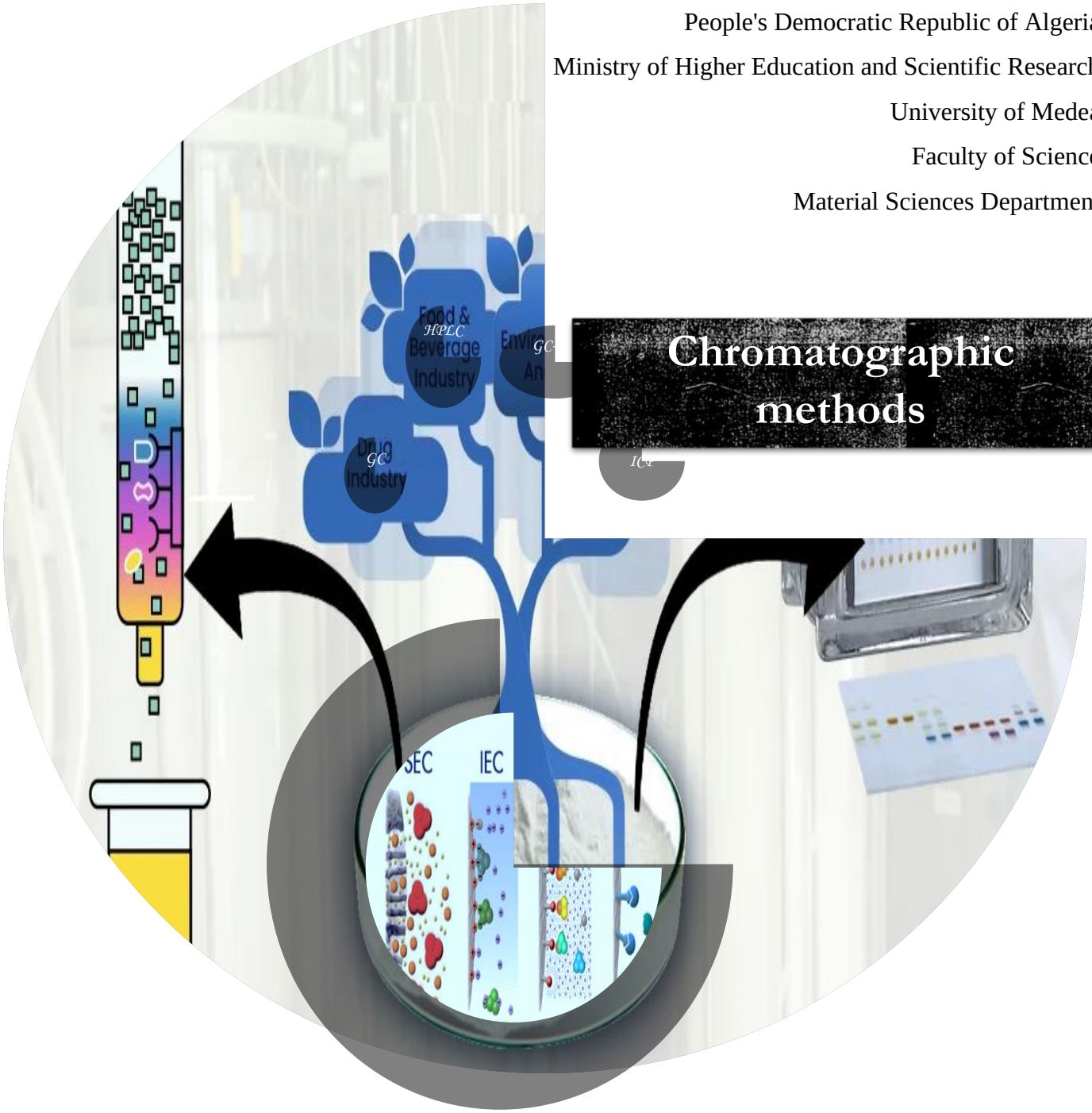
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Ministry of Higher Education and Scientific Research
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Faculty of Science
Material Sciences Department




# Chromatographic methods

According to the official pedagogical program of the 3rd year Licence,

Pharmaceutical Chemistry (Chemistry)

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Class A lecturer

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**Preamble**

This course is an approach to in-depth study of the instrumental separation methods, such as chromatography, most commonly used in chemistry and analytical research.

This course focuses on the principles, fundamentals and technical aspects of chromatographic separation methods, commonly used in the laboratory.

The principles and techniques contained in this textbook are aimed mainly at third-year Pharmaceutical Chemistry students, but may also be of interest to students of other chemistry specialties, and of Master's degrees in Biological Sciences and Process Engineering.

As a pedagogical course support, the present course material is organized into 8 chapters, as follows:

***The first chapter*** details the principles, definitions and fundamental laws of chromatography.

***The second chapter*** describes the theoretical and practical aspects of chromatographic methods such as partitioning, adsorption, ion exchange, steric exclusion and affinity.

***The third chapter*** focuses on conventional column chromatography, or gravity chromatography, where the various aspects and fundamentals of this type of chromatography are presented.

***The fourth chapter***, covers all the principles of surface chromatography such as thin layer chromatography "TLC" and paper chromatography "PC", as well as the various experimental aspects of the targeted methods.

***The fifth chapter*** provides an in-depth look at high-performance liquid chromatography "HPLC". Almost all the practical (equipment) and theoretical fundamentals of this method are highlighted.

***The sixth chapter*** focuses on the gas chromatography technique "GC" for gaseous or volatile compounds. All theoretical and experimental aspects are discussed.

***The seventh chapter*** deals with electrophoresis, highlighting both separation techniques.

***The eighth chapter*** looks at the current application of modern chromatography methods, such as HPLC and CPG, namely casting with mass spectroscopy and plasma induction.

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# Chapter I: Chromatography: Definitions and Fundamentals

## I. Introduction

Chromatography is an important analytical method that enables the separation, identification, purification and quantification of the components of a mixture for qualitative and quantitative analysis. Chromatography is one of the most common methods for achieving this type of separation. It is a useful form of separation science that uses intermolecular forces, to separate the components of a mixture according to their properties.

## II. Principle

According to IUPAC, chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction.
In this method, the components of a mixture are separated based on their differential interactions with two chemical or physical phases: a mobile phase and a stationary phase that is held in place by a supporting material.

This leads to a preferential distribution of the components between two immiscible phases in contact, (Figure I.1).

Based on this approach three components form the basis of the chromatography technique:
- ***Stationary phase*** (SP): This is either a solid with a high surface area or a liquid coated onto a solid support. It always stays still. The stationary phase is trapped in a column or fixed on a support. on SP the components of the mixture to be separated are interact selectively.
- ***Mobile phase*** (MP): This phase is always composed of liquid or a gaseous component. It moves in contact with the stationary phase without interaction.
- ***Separated molecules.***

The distribution coefficient (K) of solute A between the stationary phase and the mobile phase is defined by expression (I.1):

$$K = \frac{(C_A)_{SP}}{(C_A)_{MP}} \qquad (I.1)$$

**With:**

$(C_A)_{SP}$: Concentration of solute A in the stationary phase

$(C_A)MP$: Concentration of solute A in the mobile phase

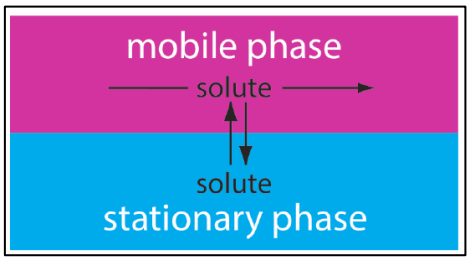



**Figure I.1.** Chromatographic separation principle

The separation is based on the differential entrainment of the constituents of the mixture. The latter travel through the stationary phase with times proportional to their intrinsic properties (size, structure, molecular weight) or their affinity with the stationary phase (polarity, etc.).

## III. Classification

Under the name of chromatography, we bring together a very large number of different techniques which can be classified into 3 categories according to (Figure I.2):

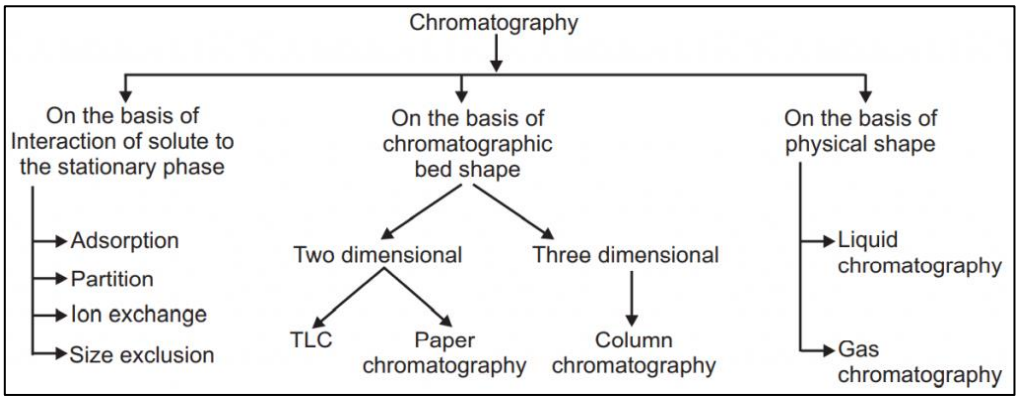



**Figure I.2.** Classification of chromatographic methods

**III.1. Based on the physical shape of phases** (mobile or stationary).

### *a) Stationary phase*

The stationary phase to be selected for a chromatographic process depends on the nature of the components to be separated and the type of chromatography. It may be:

***1)*** ***Solid:*** in column chromatography, a packed column is used as a stationary phase (gravity chromatography and high-performance chromatography or HPLC) or spread in a thin layer on a plate of glass, aluminium or a sheet of plastic material (thin layer chromatography TLC).
***2)*** ***Liquid:*** impregnating solid support by a carbon chain fixed on it (grafted phase) allows the separation of the components of the mixtures based on their adsorbent or partition properties.

***b) Mobile phase***

Is often forced through an immobile, immiscible stationary phase, carrying the analyte with it. It can be either a gas where the mobile phase is called vector gas or carrier gas. Or a liquid: it is also called eluent.

**III.2. Based on the interaction of solute to the stationary phase**

The phenomena which give rise to the separation depend on the nature of the stationary phases (SPs). There are five types of interaction existing between SP and solute molecules:

- Adsorption
- Partition
- Ion exchange
- Size exclusion
- Affinité
- Hydrophobic interaction.

**III.3. Based on bed shape**

Conditioning of the stationary phase:

***a) Two-dimensional or planar chromatography***

The SP is supported on a flat plate or the interstices of a paper and the mobile phase (MP) moves through the stationary phase by capillary action.

- Thin layer chromatography (TLC)
- Paper chromatography (PC)

***b) Three-dimensional***

Column chromatography: The SP is held in a narrow tube through which the MP is forced under pressure or by gravity

## IV. Basic chromatography terminology

***a) Elution:*** Percolation of a compound through a column

***b) Eluent:*** Solvent allowing the compound to be eluted

***c) Eluate:*** Solution collected at the bottom of the column

***d) Solute:*** Component of a solution eluted by the MP and which must be favourably retained on the SP

***e) Chromatogram:*** Recording of eluted peaks perceived by a detector

***f) Chromatograph:*** Instrument employed for chromatography.

## V. Nomenclature of chromatogram

The chromatogram is a two-dimensional diagram presented on a screen or on paper. It shows the evolution of a parameter which depends on the instantaneous concentration of the solute leaving the column as a function of time or volume, (Figures I.3 to I.5).

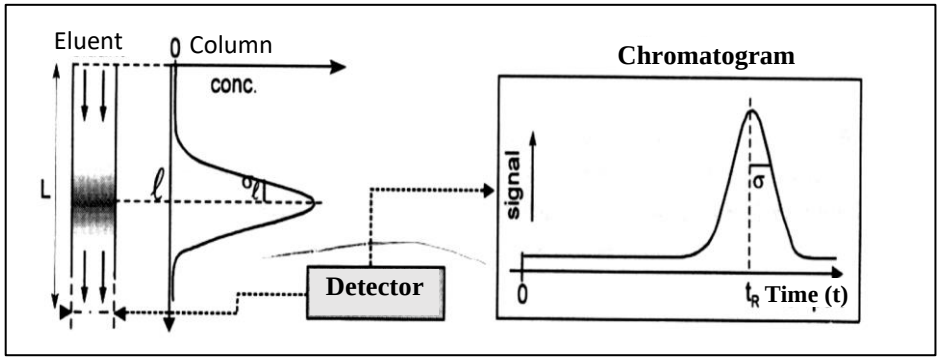



**Figure I.3.** Chromatographic pic.

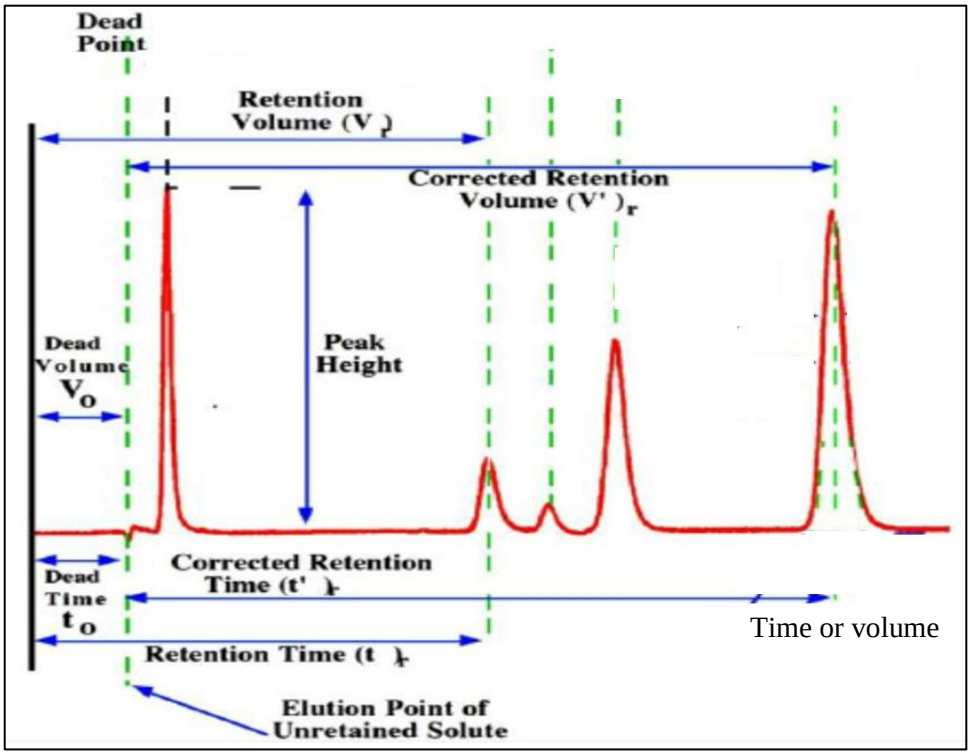



**Figure I.4.** Nomenclature of a chromatogram

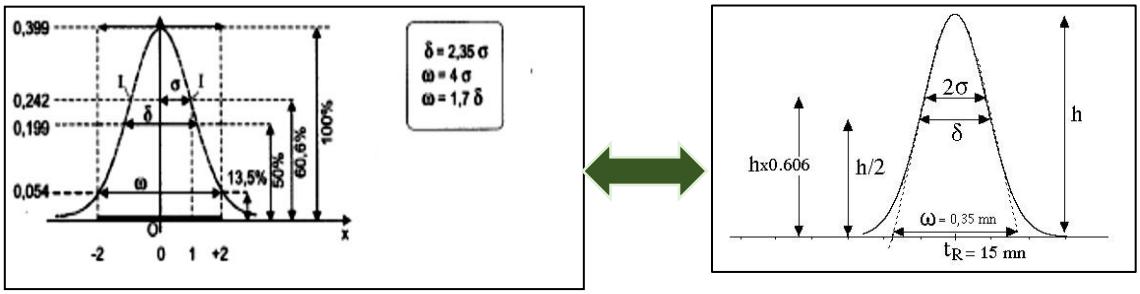



**Figure I.5.** Schematic representation of a Gaussian pic (ideal).

The chromatographic peak is characterised by several elements:

- **δ:** Width at half height (measured at 50% of the total height).
- **ω:** Width of the base of the peak measured at 13.5% of the total height, or by the tangents to the inflection points.
- **σ:** Variance of the peak
- **Peak area (s):** the surface between the peak and the baseline. It is proportional to the quantity of solute.
- **Peak height (h):** used during quantitative analyses.

## VI. Chromatographic parameters

### VI.1. Retention time ($t_R$)

A constituent is characterised by its retention time, the time elapsed between the moment of injection and that determined at the maximum of the peak corresponding to it on the chromatogram.

The retention time is independent of the quantity injected, the nature, and the abundance of other constituents in the mixture.

On the other hand, it depends on:

- Mass and nature of stationary phase in the column,
- Flow rate of the mobile phase,
- Dead volume of the chromatograph (injector, detector, pipes, etc.).

### VI.2. Dead time ($t_0$ or $t_M$)

An unretained constituent leaves the column at time $t_0$, called dead time or void time.

### VI.3. Dead volume ($V_0$ or $V_M$)

The volume of the MP in the column corresponds to the accessible interstitial volume. It can be calculated from the chromatogram of an unretained solute.

A relation between dead volume ($V_0$) and dead time ($t_0$) is expressed by the relationship (I.2):

$$V_0 = D.t_0 \qquad (I.2)$$

**With:**

D: Volume flow rate of mobile phase, ($mL.min^{-1}$).

### VI.4. Corrected or reduced retention time ($t'_R$)

It is obtained by the difference between the actual retention time and the dead time. It is linked only to the retention phenomenon itself. It is expressed by the relationship (I.3).

$$t'_R = t_R - t_0 \qquad (I.3)$$

### VI.5. Retention volume ($V_R$)

It represents the volume of the mobile phase necessary to make it migrate from one end of the column to the other. This volume corresponds on the chromatogram to the volume of the mobile phase which has elapsed between the moment of injection and that corresponding to the maximum of the peak. This volume is expressed by relationship (I.4).

$$V_R = t_R.D = t_R.u.s = t_R.u.s'.\varepsilon \quad (I.4)$$

**With:**

u: Linear elution velocity of the MP, ($cm.min^{-1}$)

s': Section of the column, ($cm^2$)

ε : Porosity of the SP, ($\varepsilon = V_0/V_T$)

### VI.6. Stationary phase volume (Vs)

It is calculated from the volume of the free column and the volume of the MP.

## VII. Chromatographic calculations

### VII.1. Capacity factor (k')

The total mass introduced into the column ($m_T$) is distributed into ($m_M$) in the mobile phase and $m_S$ in the stationary phase. These quantities depend on $m_T$ and the distribution coefficient (K). Their ratio is fixed and is called the retention factor or capacity factor (k'), (expressions (I.5) and (I.6)):

$$k' = \frac{m_S}{m_M} = \frac{C_S V_S}{C_M V_M} = K\frac{V_S}{V_M} \quad (I.5)$$

$$k' = \frac{t'_R}{t_0} = \frac{t_R - t_0}{t_0} \quad (I.6)$$

The k' factor is not a constant, it depends on the operating conditions such as temperature, and composition of the MP (it is independent of the flow rate and the length of the column). It is the most important parameter in chromatography to define the behaviour of a column.

- k'≤ 1: Elution too fast
- 1 < k'≤ 5 : Optimal elution
- 5 > k' : Elution too slow.

### VII.2. Relative retention, selectivity or separation factor (*α*)

Describes the ability of a chromatographic system to separate two compounds position of two adjacent peaks 1 and 2 located on a chromatogram, (expression (I.7), and Figure I.6). It corresponds to the ratio of the column retention factors for the two compounds.

$$\alpha = \frac{t'_{R2}}{t'_{R1}} = \frac{k'_2}{k'_1} \qquad (I.7)$$

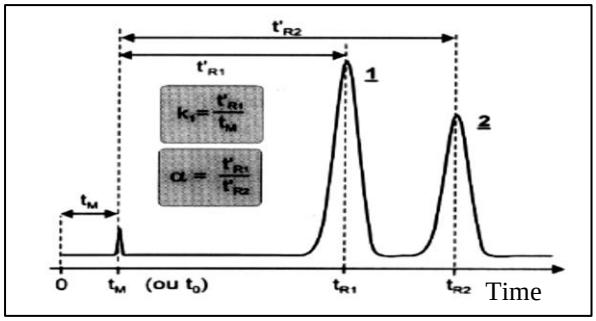



**Figure I.6.** Separation of two adjacent solutes.

**α:** *always greater than 1*. The selectivity factor of two analytes allows you to estimate how well the column can separate them. It defines whether the separation is chemically possible.

### VII.3. Theoretical plate number (theoretical column efficiency, $N_{th}$)

The width of the peak characterises the separation efficiency, it is the ability to give fine peaks. It is measured by the number of theoretical plates. As shown in Figure I.7, the molecules "undergo" a large number of simultaneous transfers from the MP to the SP and vice versa. The column having a length L is assumed to be unscrewed into $N_{th}$ small superimposed fictitious disks (theoretical plates) of the same height HETP, which means the height equivalent to a theoretical plate.

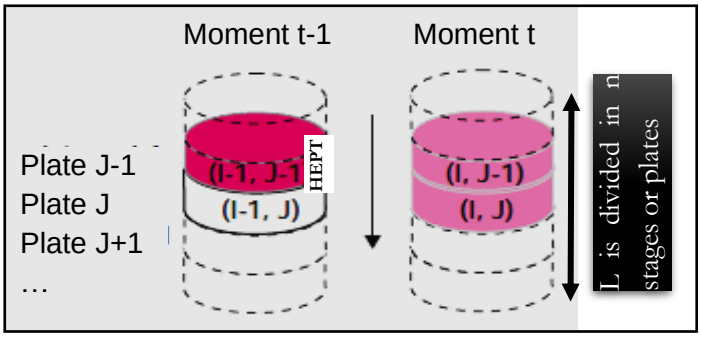



**Figure I.7.** Chromatography plate model

HETP and $N_{th}$ are expressed by the relations (I.8) to (I.11):

$$HETP = \frac{L}{N_{th}} = \frac{\sigma^2}{L} \quad \text{(IV.8)}$$

$$\sigma = L.\omega / 4t_R \quad \text{(IV.9)}$$

$$HETP = L\omega^2/16\, t_R^2 \quad \text{(IV.10)}$$

$$N_{th} = 16.\left(\frac{t_R}{w}\right)^2 = \left(\frac{t_R}{\sigma}\right)^2 \quad \text{(IV.11)}$$

**With**:

L: Length of the column

$N_{th}$: Number of theoretical plates: 100 - $10^6$

HETP: Height Equivalent to a Theoretical Plate: 1 cm - 10 µm

**Remarks:**

**1.** If N is large, the peaks become narrower and the efficiency increases

**2.** Efficiency N: ratio between the residence time of the solute in the column and the broadening of the peak (or standard deviation σ)

**3.** N can be optimised by playing on HEPT (reduction of the ø of the particles, optimisation of the ø of the column, L, and u (Van Deemter).

### VII.4. Effective plate number (Effective column efficiency, $N_{eff}$)

This number is used in High-Performance Liquid Chromatography (HPLC) because it characterises the actual time spent in the SP. It is given by expression (I.12):

$$N_{eff} = 16.\left(\frac{t'_R}{w}\right)^2 = \left(\frac{t'_r}{\sigma}\right)^2 \quad \text{(I.12)}$$

**VII.5. Resolution factor between two peaks (Rs)**

Resolution is a measure of the quality of a chromatographic separation. This depends both on the relative spacing of the peak peaks (thermodynamic factor expressed in relative retention time) and on the fineness of the peaks (kinetic factor expressed in column efficiency). It characterises the ability of a chromatographic system (column, solutes, solvents) to separate 2 solutes.

It is given by expression (I.13):

$$R_S = \frac{2[(t_R)_A - (t_R)_B]}{w_A + w_B} \quad \text{(I.13)}$$

If $w_A = W_B \Rightarrow$ The resolution is given by expressions (I.14) and (I.15):

$$R_S = \frac{\sqrt{N_{th}}}{4}.\left(\frac{\alpha - 1}{\alpha}\right).\left(\frac{k'_2}{1 + k'_2}\right) \quad \text{(I.14)}$$

$$R_S = \frac{\sqrt{N_{eff}}}{4}.\left(\frac{\alpha - 1}{\alpha}\right) \quad \text{(I.15)}$$

Where $k'_2$ is the retention factor of the slowest species.

- Rs <1 : Bad separation
- 1 ≤Rs ≤1.4 : Acceptable separation
- 1.4 <Rs ≤1.6 Optimal resolution
- Rs >1.6: Good resolution with extended analysis time.

The resolution, therefore, depends on two quantities (Figure I.8):

**(1)** The distance which separates the vertices of the two peaks

**(2)** The width of each of the peaks.

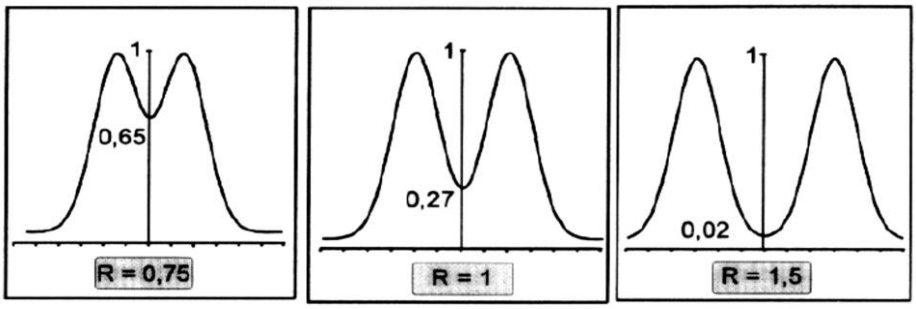



**Figure I.8.** Effect of the resolution on the separation of two adjacent pics of equal intensities.

To improve the resolution, it is necessary either to extend the length of the column to have a greater number of theoretical plates, or to change the operating conditions of the analysis (temperature, mobile phase, etc.).

While keeping k' between 1 and 5, it must therefore increase α by:

- Modification of the mobile phase (including pH changes)
- Change in temperature (CG, CL [ion exchange])
- Modification of the composition of the stationary phase

**VII.6. Kinetic model of chromatography**

This model was established by Jan Van Deemter, where he established a relationship between the HETP to the physical characteristics of the MP and the column (expression (I.16))

$$\mathrm{HETP} = \mathrm{A} + \frac{\mathrm{B}}{\mathrm{u}} + \mathrm{C}.\,\mathrm{u} \qquad \text{(I.16)}$$

This model comes from fluid mechanics, where the terms A, B and C mean:

**A**: Eddy-diffusion parameter, related to channelling through a non-ideal packing [m], depends on packing and particle diameter parameters, A= λ.dp, for a packed column: A ≠ 0 and for a capillary column (gas chromatography GC): A = 0.

**B**: Longitudinal diffusion term, B=2. τ.$D_G$

**With:**

τ: Obstruction or tortuosity factor

$D_M$: diffusion coefficient

**C**: Mass transfer term in each of the two phases.

As shown in Figure I.9, the Van Deemter curve shows the existence of an optimal MP flow rate for which the efficiency of the column is maximum (hence minimal HETP). The usefulness of this curve is important in HPLC and GC.

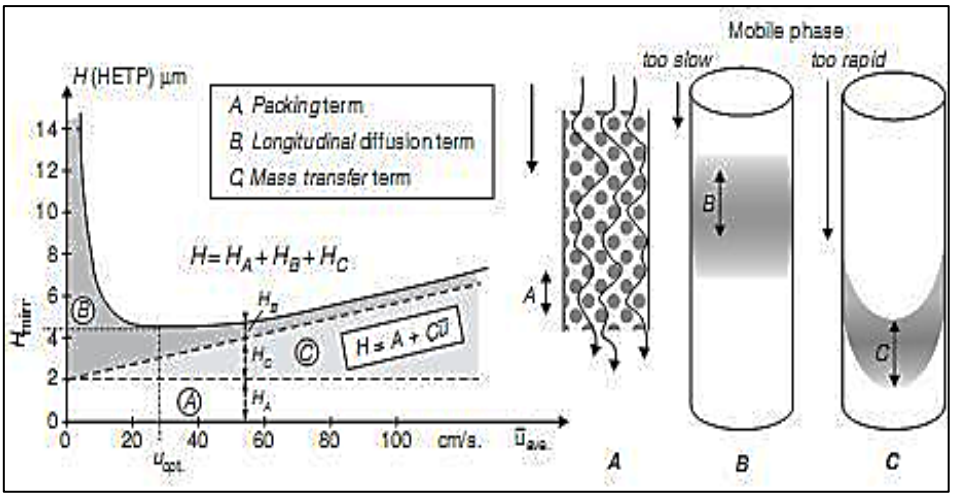



**Figure I.9.** Van Deemter's curve in gas chromatography with domains of parameters A, and C.

## VIII. Optimisation of chromatographic analysis

Resolution factor depends on √N, k and α; resolution and elution time are the two most important dependent variables to consider, see equation (I.17):

$$t_{R2} = \left(16 . \frac{R_S.H}{u}\right) . \left[\frac{\alpha}{\alpha-1}\right]^2 . \left[\frac{(1+k'_2)^3}{{k'_2}^2}\right] \qquad (I.17)$$

$R_S$ increases with k' but time is also taken into account (compromise: 1<k<10). To increase k': the temperature must be reduced in GC and modify MP composition in LC.

**Remark:** No need to optimise N and k' if α (separation factor) = 1. To increase α while maintaining 1<k<10: programming of temperature in GC, modifying the composition of the MP in LC, and modification of the SP, by adding a chemical species to interact with certain constituents.

The chromatographer is trapped in a triangle whose vertices correspond to resolution (Figure I.10), velocity, and capacity, three opposing parameters. Optimised analytical chromatography makes full use of the potential of the most effective parameter: selectivity. In this triangle, it is therefore located near the resolution top.

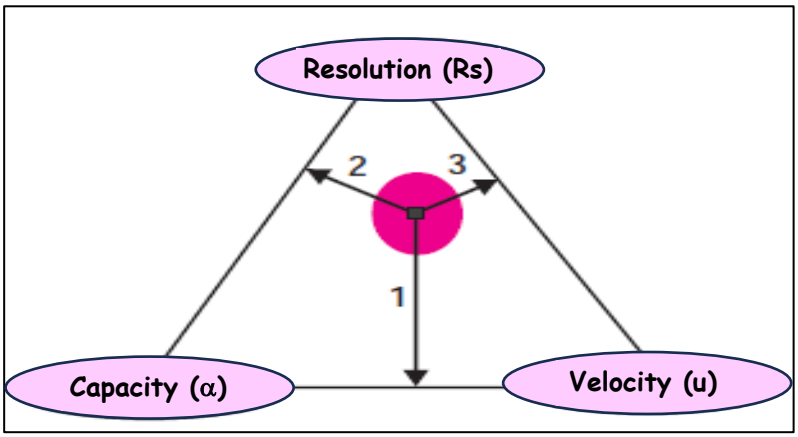



**Figure I.10.** Optimisation of a chromatographic analysis.

## IX. Pression loss in LC

The flow of the MP near the column wall and the particles of the SP generates friction (resistance to flow).

Maintaining the constant flow rate causes an increase in pressure and a decrease in the particle size of the SP. In the case of HPLC (high pressure), the pressure loss between the inlet and outlet of the column is expressed by Darcy's law (equation (I.18)):

$$\Delta P = \frac{\phi.\eta.L.u}{d_p^2} \qquad (I.18)$$

**With:**

$\phi$ : Flow resistance factor, [1]

$\eta$ : Viscosity (Pa.s)

L : Column length, (m)

u : Linear velocity of the MP, ($m.s^{-1}$)

dp : Mean particle diameter of the SP, (m).

# Chapter II: Classification of chromatographic methods

## I. Introduction

In light of the numerous applications of chromatography, this important class of physicochemical separation methods is considered to be the most potentially effective method for separating, identifying, and even characterising complex homogeneous and heterogeneous mixtures in fine detail. The properties of the solutes contained in the mixtures (chemical, biological, and others) are the source of this particularity. These solutes can be of very different polarity (polar, apolar), ionisation form (ionised or ionisable), and physical state (solid, liquid, or gas). Based on the nature of the molecules to be analysed, chromatographic methods are continually being developed.

## II. Classification

The chromatographic methods are classified according to several principles: the separation mechanisms, the physical nature of the stationary and mobile phases, and the technique used.

The factors involved in the partitioning of the molecules to be separated between the stationary and mobile phases are: solubility in a liquid solvent, size (shape), polarity, electric charge and the presence of atom groups forming specific sites. The different types of chromatography result from the focus on the effect of one of these factors, but the exclusivity of one mechanism is always present during a chromatographic separation.

The choice of chromatographic method depends on:

- ***The nature of the solute***: gas, volatile liquid, low volatility liquid, solid, macromolecule, organic species, polar, ionic, etc.
- ***Purpose of the analysis***: identification of the components of a mixture, whether or not it is necessary to "couple" chromatography with a spectroscopic method or with mass spectrometry (GC/MS or HPLC/MS), purity control, purification of products (preparative columns), continuous reaction monitoring to optimise parameters, dosages (quantification), etc.

A classification according to the solute to be separated is shown in Figure II.1:

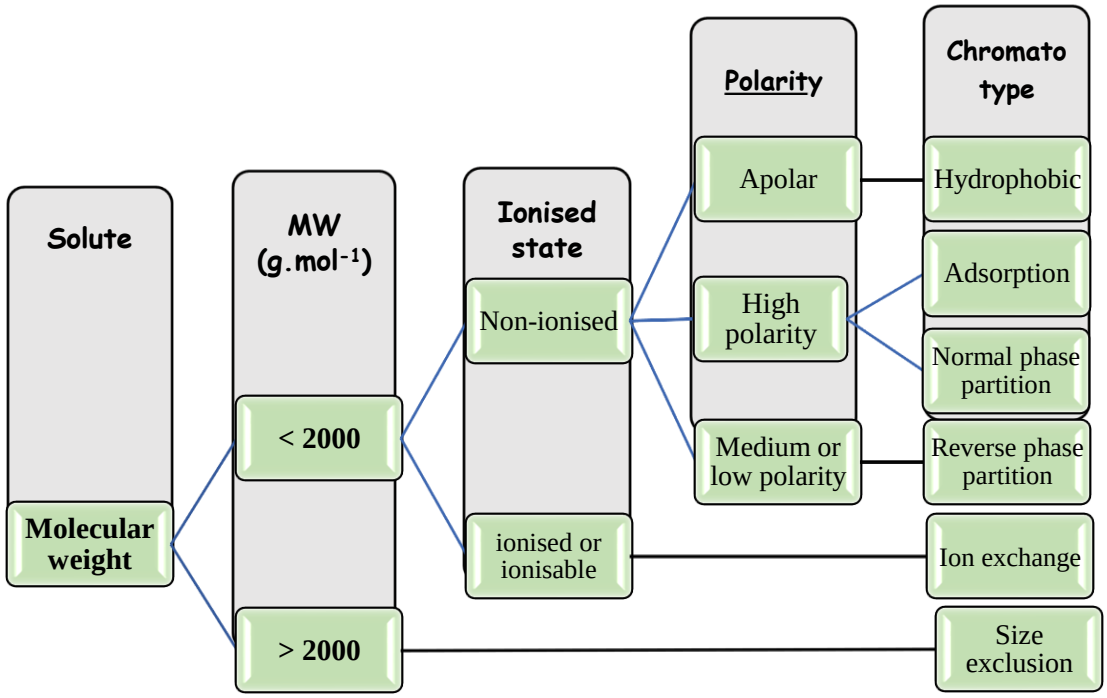



**Figure II.1.** Chromatographic methods classification.

## III. Liquid chromatography (LC)

In this type of chromatography, the mobile phase is a liquid and the stationary phase can be a solid or a liquid. A distinction is made between liquid/liquid chromatography and solid/liquid chromatography.

### III.1. Partition chromatography or liquid-liquid chromatography (LLC)

This technique is based on a partition or partitioning mechanism of a solute between the immiscible SP and MP solvents.

In this type, the distribution coefficient is known as the partition coefficient. It represents the difference in solubility of the solute between the two phases.

## III.2. Elements of LLC

### *a) Stationary phase*

It can be prepared:

***1) On a column***: Also known as 3D chromatography: where the SP, in the form of grains of a well-defined size, is filled with a support impregnated with a solvent or fixed by a covalent bond (grafted phase). This thick layer acts as a solvent for the solutes transported by the MP. The MP and solutes flow by gravity.

***2) On paper***: This is surface or 2D chromatography, the support is a cellulose membrane, which is introduced into a tank containing the fixed phase (often aqueous). The highly hydrophilic cellulose in the paper saturates with water, forming the SP.

On the top of the tank. The mobile phase migrates by capillary action, and the solutes are separated by partitioning between the two solvents.

#### *a) Grafting of the SP*

This is carried out by establishing covalent bonds through a silanisation reaction by forming siloxane (Si-O-Si) links on the silanol groups of the silica gel (the silica gel being the support). The silanisation reaction (reaction 1) is based on one reagent, generally monochlorodimethylsilane (monosilanisation reaction).

$$\text{->Si-OH} + \text{X->Si}(CH_3)_2\text{-R} \rightarrow \text{->Si-O-Si}(CH_3)_2\text{-R} + \text{HX} \qquad \text{(Reaction : 1)}$$

In this process, a liquid film is produced on the surface of the PS (silica gel) where the partition mechanism takes place between the SP and the MP (Figure II.2).

Depending on the nature of the R radicals, two types of PS can be obtained:

***1) Normal phase LC (NP):*** Where the SP is more polar than the MP, it is used for the separation of very polar organic compounds: diol, amine or nitrile functions.

**2) *Reverse phase LC (RP):*** Where the MP is more polar than the SP. The SP is grafted onto the adsorbent, and the organic molecules are apolar. It is used to separate apolar or slightly polar compounds.

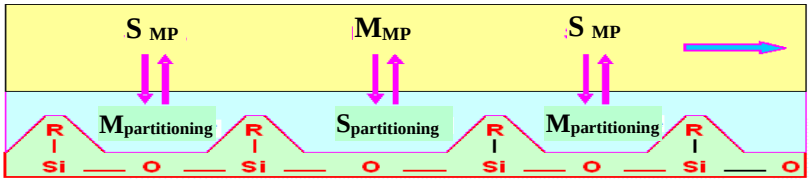
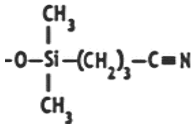
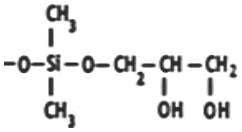
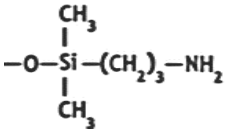
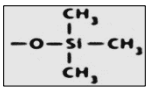
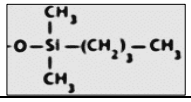
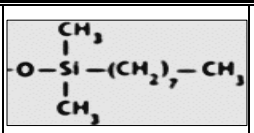
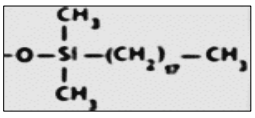
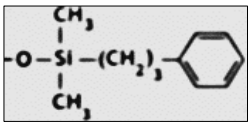



**Figure II.2.** Partitioning principle in the LLC

The major grafts used are summarised in Table 1.

**Table 1.** Grafts used in liquid-liquid chromatography

| ***Grafts of LLC in normal phase (NP)*** | | | | |
|---|---|---|---|---|
| **Structure** |  |  |  | |
| **Name** | Cyano3propyldimethyl silane | Dihydroxy2,3propyloxydimethyl silane | Amino3propyldimethyl silane | |
| ***Grafts of LLC in resverse phase (RP)*** | | | | |
| **Structure** | a)  b)  |  |  |  |
| **Name** | a) Trimethyl silane b)- Triméthylpropylsilane | Dimethylocty silane **(C8)** | Dimethylocta-decyl **(C18)** | Phenyl 3 propyldimethyl |

### *b) Mobile phase*

MP: depending on the nature of the SP, there are two types of MP, (Figure II.3):

***1) Case of normal phase***: The MP is a mixture of apolar solvents, lipophilic in nature: hexane, heptane, isoctane and polarity modifiers (low or polar): chloroform ($CHCl_3$), tetrahydrofuran (THF), ethanol, propyl ether (isopropyl) ($(CH_3)_2CHOCH(CH_3)_2$).

***2) Case of reverse phase:*** The MP is a polar or slightly polar solvent, such as water, ethanol, methanol, methyl cyanide (acetonitrile), THF, $CH_3Cl$, $CH_2Cl_2$.

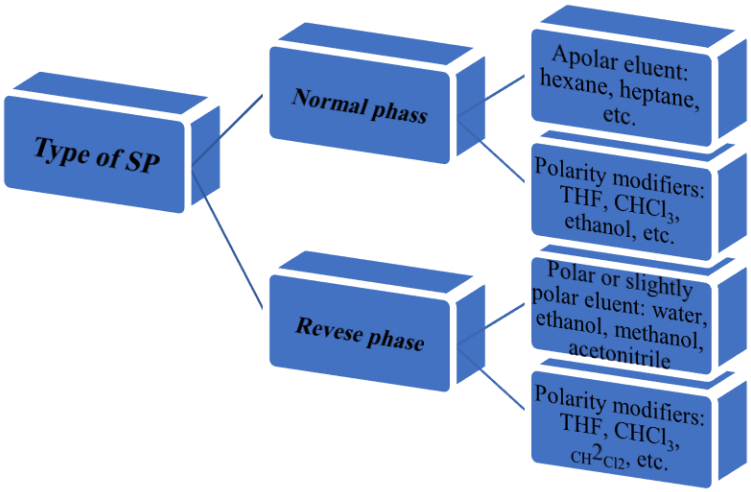



**Figure II.3.** Mobile phases in liquid-liquid chromatography.

The polarity of the MP ($\pi_{AB}$) depends on the composition of the solvents that compose it. This polarity is evaluated according to a relationship (II.1):

$$\pi_{AB} = \phi_A I_A + \phi_B I_B \qquad \text{(II.1)}$$

**With:**

$\phi_A$ and $\phi_B$: Volume fractions of solvents A and B respectively.

$Ip_A$ and $Ip_B$: Polarity indices of solvents A and B respectively.

The polar indices of the different solvents used in chromatography are given in Table 2.

Retention is related to the composition of the MP through the relationship (II.2):

$$\frac{k'_2}{k'_1} = 10^{(\pi_2 - \pi_1)/2} \qquad \text{(II.2)}$$

**With:**

$k'_1$ and $k'_2$: Retention factor in MP1 and MP2 respectively.

$\pi_1$ and $\pi_2$: Polarities of MP1 and MP2 respectively

**Table 2.** Polarity indices of solvents used in chromatography.

| **Solvent** | **(Ip)** | **Solvent** | **(Ip)** |
|---|---|---|---|
| Cyclopentane | 0.1 | n-Propyl Alcohol | 4.0 |
| Heptane | 0.1 | Tetrahydrofuran | 4.0 |
| Hexane | 0.1 | Chloroform | 4.1 |
| Iso-Octane | 0.1 | Ethanol | 4.3 |
| Petroleum Ether | 0.1 | Ethyl Acetate | 4.4 |
| Cyclohexane | 0.2 | 1,4-Dioxane | 4.8 |
| Toluene | 2.4 | Methanol | 5.1 |
| Ethyl Ether | 2.8 | Pyridine | 5.3 |
| Dichloromethane | 3.1 | Acetonitrile | 5.8 |
| Ethylene Dichloride | 3.5 | N,N-Dimethylformamide | 6.4 |
| Isopropyl Alcohol | 3.9 | Dimethyl Sulphoxide | 7.2 |
| n-Butyl Acetate | 4.0 | Water | 10.2 |

The relationship (II.2) is applied depending on the polarity of the SP, as indicated by relationships (II.3) and (II.4):

NP $\frac{k'_{i,B}}{k'_{i,A}} \approx 10^{\frac{P'_A - P'_B}{2}}$ $P'_B > P'_A$ (II.3)

RP $\frac{k'_{i,B}}{k'_{i,A}} \approx 10^{\frac{P'_B - P'_A}{2}}$ $P'_B > P'_A$ (II.4)

### III.3. Liquid-solid chromatography or adsorption chromatography

Adsorption chromatography is used to separate the constituents of a liquid (or gas) mixture by moving the solutes through a MP along a SP phase, which is a solid or liquid fixed to a support positioned in a column or on a plate. The retention mechanism on the SP is a surface phenomenon called "adsorption" which involves low-energy bonds (hydrogen bonds, Van-der-Waals, or secondary bonds).

SP is a finely divided solid to which molecules adhere through a dual physisorption and/or chemisorption effect. The physicochemical parameter here is the adsorption coefficient. In this case, the separation principle is based on the difference in polarity between the compounds to be separated.

### III.4. Elements of adsorption chromatography

#### *a) Stationary phase*

Is can be prepared :

***1) In a column:*** The SP is made up of spherical particles with a specific porosity and a diameter of up to 250 µm, with well-defined polarity. In this case, the chromatography is called three-dimensional.

***2) On a thin layer:*** The SP is made up of finely divided porous grains with a diameter of less than 5 µm. In this case, the chromatography is called two-dimensional.

The adsorbents used are mineral or organic:

- Inorganic (polar) adsorbents: silica ($SiO_2$), alumina ($Al_2O_3$, $nH_2O$), calcium carbonate ($CaCO_3$), magnesium carbonate ($MgCO_3$), magnesium oxide (MgO), etc. Silica gel exists in two forms: coated on glass beads or highly porous particles.
- Organic (low-polarity) adsorbents: cellulose, starch (and other bones), Kieselguhr (crushed diatom tests), etc.

The adsorbents (listed in ascending order of their interaction strength with polar compounds) and the functional groups used to modify the polarity of the adsorbents are listed in Figure II.4.

#### *b) Mobile phase*

The polarity of the MP must be different from that of the SP, and have the following properties:

- Insoluble in the stationary phase.
- Chemically inert to solutes and the stationary phase.
- High absorption power.

Eluents are ordered according to their polarity (in order of eluotropic strength) and according to the nature of the SP.

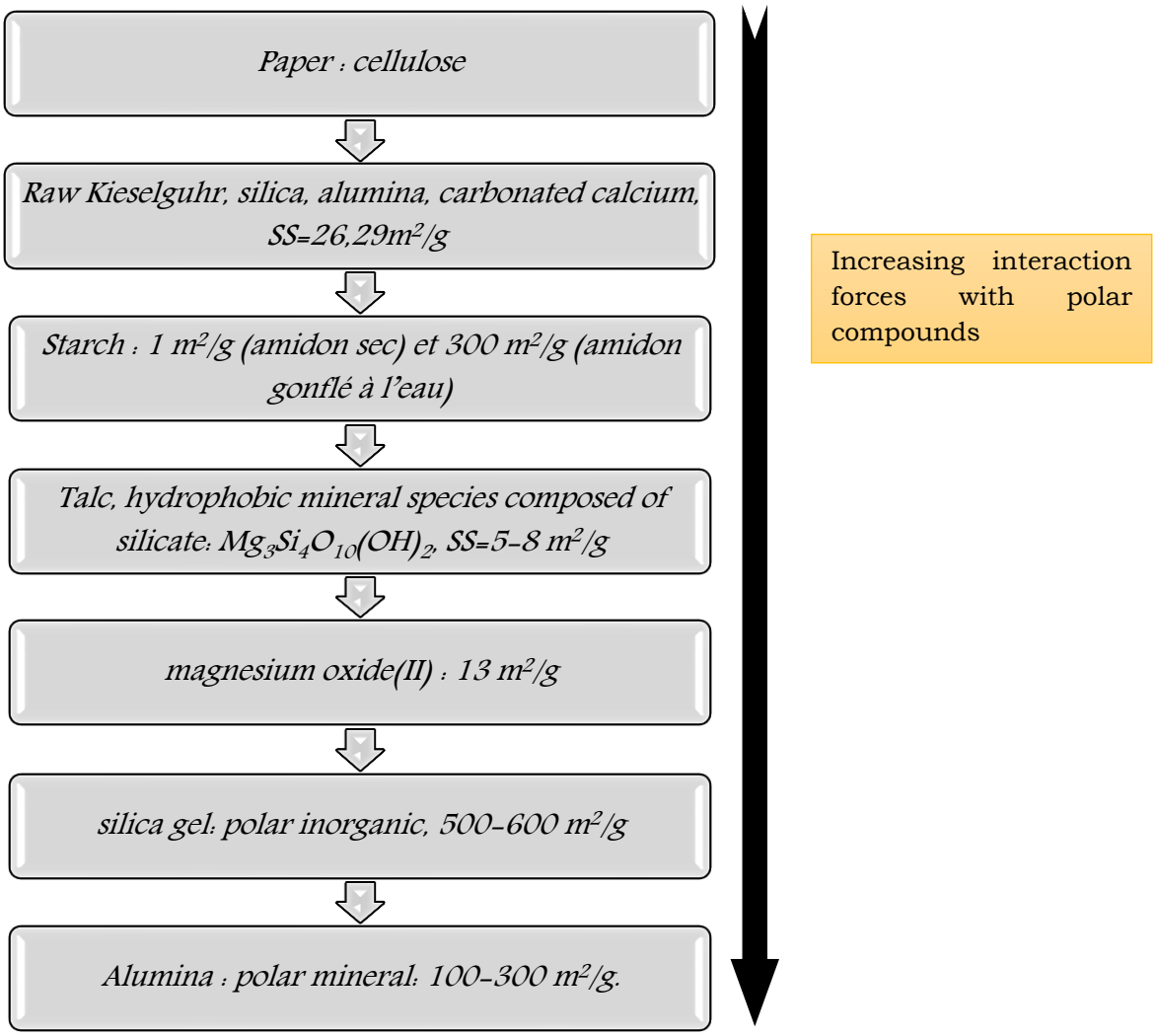



**Figure II.4.** Order of interaction of SP with polar compounds in adsorption chromatography.
*ss: specific surface*

## IV. Ion chromatography

Ion chromatography (IC), or ion-exchange chromatography (IEC), is based on electrostatic interactions between the solute and the SP, and is a form of chromatography that separates ionised, ionisable, or highly polar molecules. Separation is based on the ionic distribution coefficients between the two phases:

- MP: is an aqueous buffer solution

- SP: The most common is composed of spheres (of the order of a micron in diameter) of polymer, containing ionised or ionisable functional groups with opposite ions.

Therefore, positively charged ion-exchange matrices are called anion-exchange matrices and adsorb negatively charged molecules. While matrices bound with negatively charged groups are known as cation-exchange matrices, and adsorb positively charged molecules, (Figures II.5 and II.6)

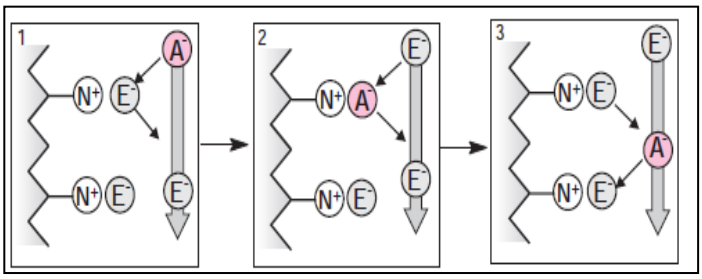



**Figure II.5.** IC retention principle.

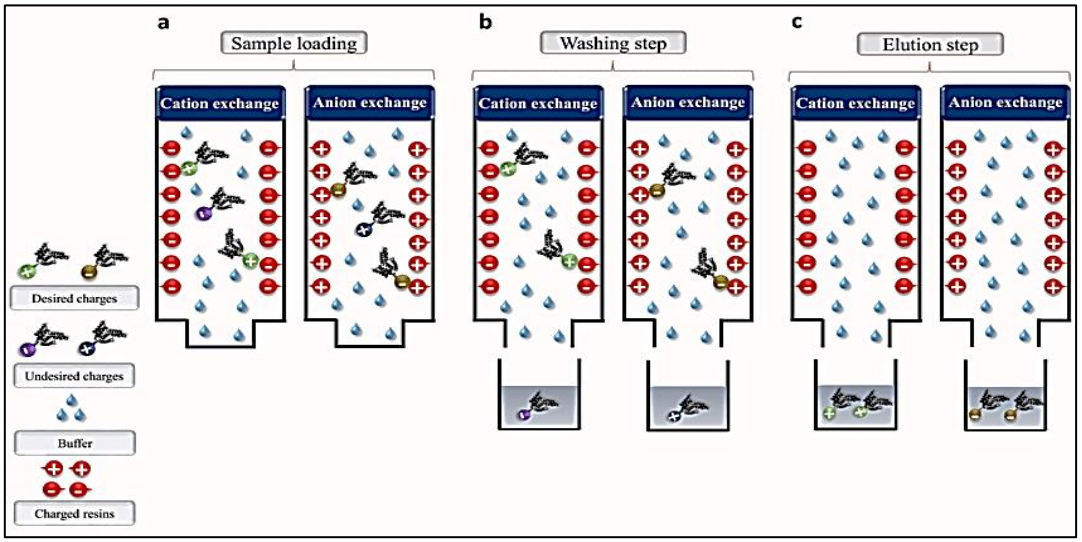



**Figure II.6.** Schematic illustration of ion-exchange chromatography process.

## IV.1. Elements of IC

### *a) Stationary phase*

Stationary phases for ion-exchange separations are characterised by the nature and strength of the acidic or basic functions on their surfaces and the types of ions that they attract and retain.

Cation exchange is used to retain and separate positively charged ions on a negative surface. Conversely, anion exchange is used to retain and separate negatively charged ions on a positive surface.

In ion-exchange chromatography, the stationary phase is a cross-linked polymer resin (as a support), with covalently attached ionic functional groups.

***1) Functional groups*** :

- **For anion exchange:** these groups can be strong, such as sulphonate ($-SO_3^-$) or phosphonate ($-PO_2^{-3}$), or weak, such as carboxylate ($-COO^-$).
- **For cation exchange:** the strong groups are quaternary ammonium: $-NR_3^+$, secondary ammonium: $-NRH_2^+$, or tertiary ammonium: $-NR_2H^+$, while the weak exchangers are primary ammonium: $-NH_3^+$ (weak exchangers).

The reactions governing cationic and anionic exchanges are given in reactions 2 and 3.

$$RSO_3^- H^+_{(SP)} + M^+_{(MP)} \rightarrow RSO_3^- M^+_{(SP)} + H^+_{(MP)} \quad \text{(Reaction : 2)}$$

$$RN(CH_3)_3^+ OH^-_{(SP)} + A^-_{(MP)} \rightarrow RN(CH_3)_3^+ A^-_{(SP)} + OH^-_{(MP)} \quad \text{(Reaction : 3)}$$

Separation is therefore based on the ionic distribution coefficients between the two phases. The expression of K for reaction 2 is given by relationship (II.5):

$$K = \frac{[RSO_3^- M^+(SP)].[H^+(MP)]}{[RSO_3^- H^+(SP)].[M^+(MP)]} \quad \text{(II.5)}$$

**With:**

$[RSO_3^- M^+_{(SP)}]$ : Concentration or activity of $M^+$ in the stationary phase, where relationship (II.6) is written:

$$\frac{[RSO_3^- M^+_{(SP)}]}{[M^+_{(MP)}]} = K\frac{[RSO_3^- H^+_{(SP)}]}{[H^+_{(MP)}]} \quad \text{(II.6)}$$

The concentration of protons in an aqueous solution is higher than that of $M^+$ ions. In addition, the concentration of exchange sites $[RSO_3^- H^+_{(SP)}]$ is much higher than the concentration of retained $M^+$. Therefore, the term: $\left[K\frac{[RSO_3^- H^+_{(SP)}]}{[H^+_{(MP)}]}\right]$ can be considered constant and K will be expressed by relation (II.7):

$$K = \frac{(C_A)_{SP}}{(C_A)_{MP}} = \frac{[RSO_3^- M^+_{(SP)}]}{[M^+_{(MP)}]} \qquad (II.7)$$

***2) Supports:*** there are different types:

- **Resins or gel**: this is a porous support made up of spherical particles, some microns in diameter, where the matrix is made up of a macromolecular network, often based on the styrene/divinyl benzene copolymer (DVB) on which the functional groups are grafted. Figure II.7 shows the typical and molecular structures of a polystyrene resin cross-linked by DVB with sulfonate groups
- **Porous silica particles:** (2-5 µm) Silica gel can be used as a support to attach alkyl phenyl chains, substituted by sulphonate or quaternary ammonium groups, via covalent bonds ([silica-R'-$NR_3$]$^+$OH), (Figure II.8).
- **Film resins:** These are polymers called Latex, prepared from a monomer that contains organic functions and deposited in the form of a continuous film of 1 to 2 µm on silica, glass, or polystyrene beads (impermeable support) with a diameter of between 5 and 50 µm.
- **Diethylaminoethyl cellulose (DEAE-C)***:* is a positively charged resin that enables the retention (at basic pH) and specific separation of negatively charged molecules. The retention force governing the association between the charged molecules and the matrix depends on the ionic strength and pH of the elution solution. It can be controlled by acting on these two components, leading to separation, Figure II.9.

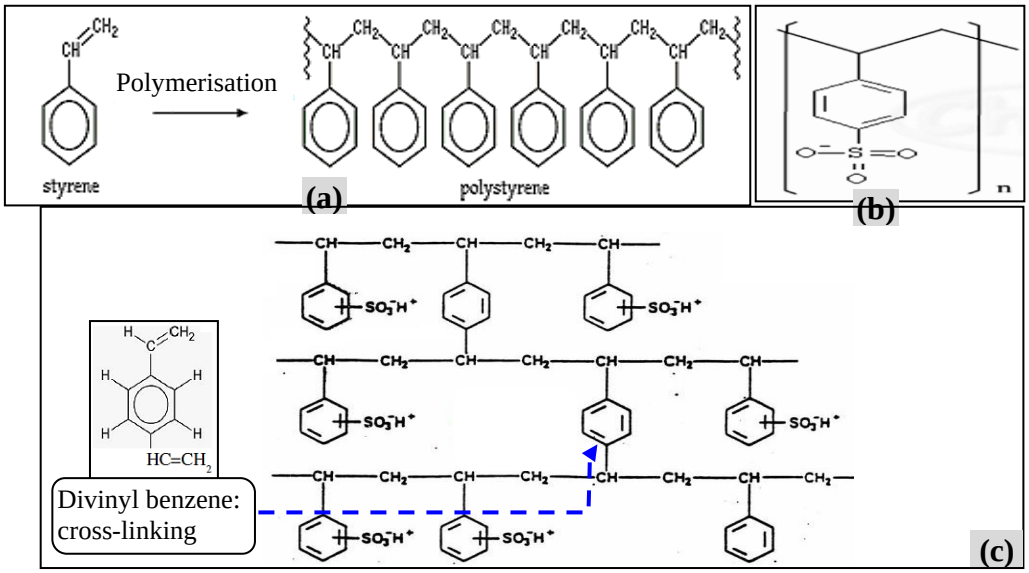



**Figure II.7.** Resin with sulphonate units, polymerisation of styrene: (a), electrophilic substitution of sulphonates: (b), linkage of DVB to polystyrene sulphonate chains: (c).

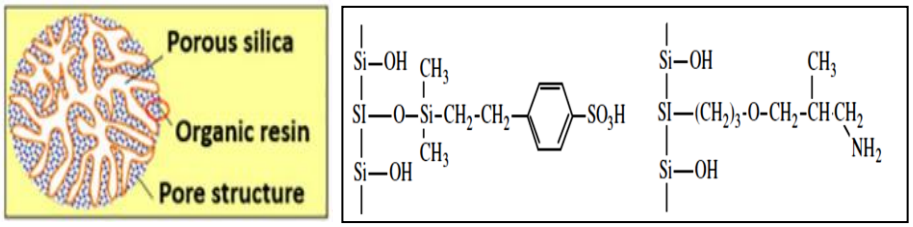



**Figure II.8.** Porous silica particle morphology: (a and sulphonate and quaternary ammonium groups grafted onto silanols.

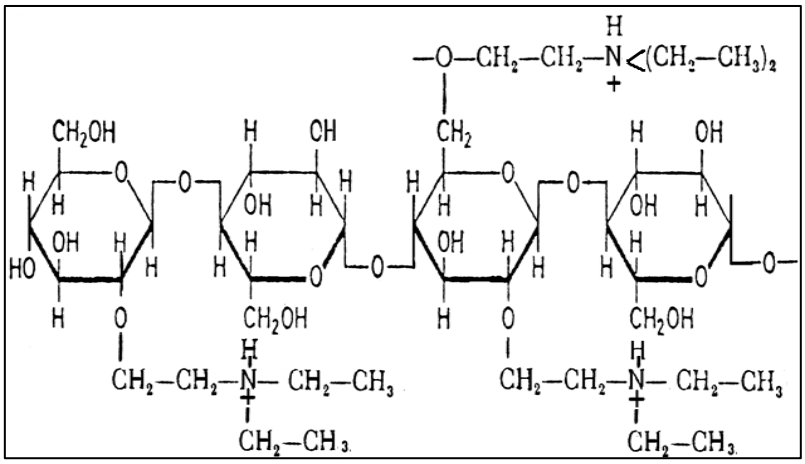


**Figure II.9.** Cellulose-based resin -DEAE.

Table 3 details the different functional groups of cationic and anionic SP.

**Table 3.** Nature of cationic and anionic SP in IC.

| **Ionisable function** | | **Ionisable function** | |
|---|---|---|---|
| **Strong alkali** | **Weak alkali** | **Strong acid** | **Weak acid** |
| Quaternary ammonium<br>---$NR_3^+$<br>or III: ---$NHR_2^+$<br>Example: Trimethylammonium<br>---$N(CH_3)_3^+$ | Protonated form of an amine I, II<br>Example: diethylaminoethyl (DEAE):<br>---$C_2H_4$-$NH^+(C_2H_5)_2$ | Examples: Sulphonate ---$SO_3^-$<br>Phosphonate ---$PO_3^{2-}$ | Example:<br>Carboxymethyl<br>---$OCH_2$-$CO_2^-$ |

***b) Mobile phase***

Most separations are carried out with an aqueous solution charged with saline or organic ions because of the solubilisation and ionisation properties of water. The cation exchange reaction is given by reaction (4):

$A^+_{stat} + B^+_{mob} \leftrightarrow B^+_{stat} + A^+_{mob}$ (Reaction : 4)

Elution is achieved by shifting the equilibria:

- Through the addition of an ion with a greater affinity for the resin
- Increasing the concentration of H+ or OH- ions
- By modifying the pH (in the case of weak exchangers), which affects the ionisation of both the exchangeable groups in the resin and those in the solutes.

**IV.2. Effect of parameters on ion exchange separation**

***a) pH***

This influences weak acids, such as amino acids, which are eluted according to their $pH_{isoelectric}$ (pHi). When the pH of the mobile phase is equal to pHi, the amino acid is in the form of an uncharged zwitterion and is therefore not retained.

***b) Type and concentration of counter-ion***

The order of increasing effectiveness of counter-ions for cation exchange resins is as follows:

- Monovalent cations: $Li^+ < H^+ < Na^+ < NH_4^+$
- Divalent cations : $Cd^{2+} < Mn^{2+} < Mg^{2+} < Zn^{2+} < Cu^{2+} < Ca^{2+}$
- Efficiency increases with load: $K^+ < Ca^{2+} < Al^{3+}$

***c) Temperature***

Increasing column temperature reduces solute retention. In addition, the viscosity of the mobile phase decreases, which increases the flow rate.

### IV.3. Applications

- *Water purification and softening:* consists of replacing the $Ca^{2+}$ and $Mg^{2+}$ cations (responsible for water hardness) with sodium cations.

- *Water demineralisation:* this consists of eliminating all the ions present in demineralised water.

- *Biochemistry:* separation of proteins and amino acids from a mixture.

## V. Size exclusion chromatography (SEC)

Size exclusion chromatography is a method where the separation of different compounds occurs according to their hydrodynamic volumes instead of their molecular weight measured by how efficiently they penetrate the pores of the stationary phase.

Size exclusion chromatography has two basic versions:

### *a) Gel permeation chromatography (GPC)*

When performed using organic solvents as MP. The main application field of GPC is polymer analysis.

### *b) Gel filtration chromatography*

When size exclusion chromatography is performed using aqueous solvents as MP. A typical example of gel filtration is the desalting of proteins.

SP is a porous polymer whose pores have dimensions chosen to the size of the species to be separated. A sort of molecular sieve is created. The larger molecules are excluded from the column, while the smaller ones diffuse into the pores until equilibrium is reached (Figure II.10).

In this case, the distribution coefficient is called the diffusion coefficient and is defined by the relationship (II.8):

$$K = \frac{(C_A)_{SP}}{(C_A)_{MP}} \qquad \text{(II.8)}$$

**With:**

$(C_A)_{SP}$: Concentration of (A) in the intragranular part

$(C_A)_{MP}$: Concentration of (A) in the extragranular part.

The diffusion coefficient is always between 0 (total exclusion) and 1 total diffusion: **$0 \leq K \leq 1$**

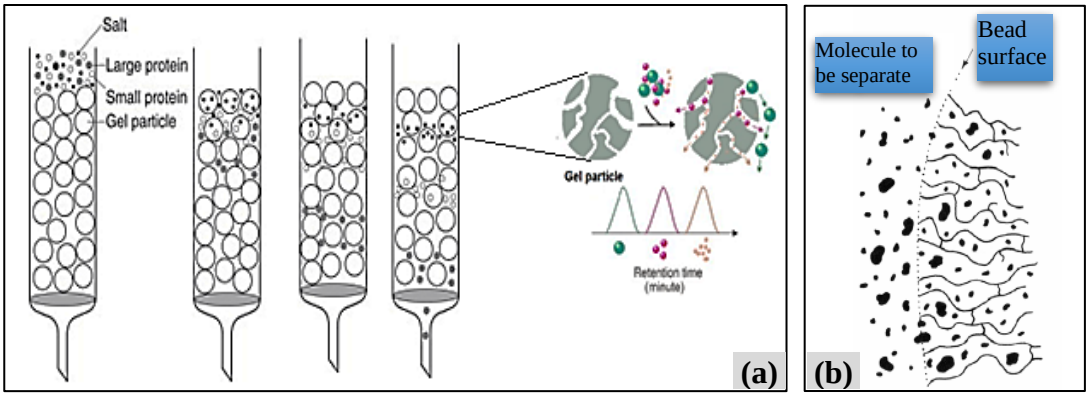



**Figure II.10.** SEC separation principle : (a and SP structure : (b.

**V.1. Elements of SEC**

***a) Stationary phase***

This is a gel (cross-linked organic or mineral polymer), sized into small, very regular porous particles (3<dp<400 µm and pore diameter between 4 and 200 nm) in the form of grains, beads or pearls. Gels are characterised by their chemical nature and their rigidity.

SP was characterised by some properties:

- It is a semi-permeable, porous bead with a defined range of pore sizes.
- Beads are crosslinked polymers
- Degree of crosslinking is controlled carefully to yield different pore sizes.
- Smaller pore sizes are used for rapid desalting of proteins or for protein purification.
- Intermediate pore sizes are used to separate relatively small proteins.
- Very large pore sizes are used for the purification of biological complexes.

- Each is available with a variety of different ranges of pore size in the beads, permitting separation of macromolecules of different size

The SP can be made of particles of:

- Porous silica mineral, grafted or not, or coated with a hydrophilic polymer.
- Cross-linked organic polymers, the most commonly used in gel filtration chromatography include dextran (Sephadex™), polyacrylamide and dextran-polyacrylamide (Sephacryl™).

***Sephadex gels***, are composed of macroscopic beads synthetically derived from the polysaccharide dextran. Its organic chains are composed of glucose molecules linked by α-1 - 6 glucoside bonds which are cross-linked with epichlorohydrin to give a three-dimensional network having functional ionic groups attached by ether linkages to glucose units of the polysaccharide chains, (Figure II.11).

Available forms include anion and cation exchangers, as well as gel filtration resins, with varying degrees of porosity; bead sizes fall in discrete ranges between 20 and 300 μm.

Table 4 gives an overview of Sephadex gel varieties for a range of fractionation domains.

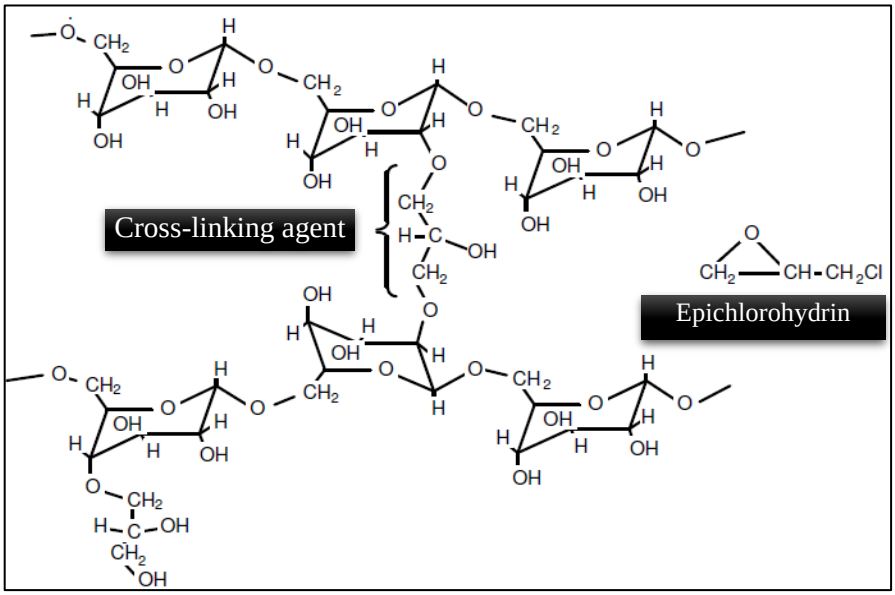



**Figure II.11.** Sephadex gel structure.

**Table 4.** Sephadex gels fractionation domains

| ***Sephadex gel*** | ***Fractionation range (MW, Da)*** |
|---|---|
| G-10 | 700 |
| G-15 | 1500 |
| G-25 | 1000-5000 |
| G-50 | 1500-30000 |
| G-75 | 3000-80000 |
| G-100 | 4000-150000 |
| G-150 | 5000-300000 |
| G-200 | 5000-600000 |

***b) Mobile phase***

The liquid used to dissolve the biomolecules to make the mobile phase is usually called a buffer.

Also:

- The mixture of biomolecules dissolved in the buffer is called the sample.
- The choice of MP to be used in any separation will depend on the type of separation to be achieved and the component to be separated.
- The MP must be compatible with the detector if used directly.
- The mobile phase, the trapped solvent and the gel should interact identically with the solute molecules.
- Aqueous buffers are sometimes required to stabilise pH, molecular composition, and molecular size.

Depending on the chemical nature of the SP, the MP may be:

- Water or buffer solutions for hydrophilic gels
- Organic solvent (Tetrahydrofuran, Chloroform, Dimethyl formamide, etc.) for hydrophobic gels.

### *c) Column*

The commercial columns used in SEC can have several types:

- analytical column- 7.5–8mm diameters.
- Preparative columns-22–25mm for.
- Usual column lengths-25, 30, 50, and 60 cm.
- Narrow bore columns- 2–3mm diameter, which save time and solve.

The choice of column type is therefore based on the following parameters:

- Shorter columns save time and solvent.
- Small particles (typically 5 mm) provide a better resolution.
- On the other hand, 5 mm (or even 3 mm) packings are more sensitive to contamination by samples containing impurities.
- Particles as large as 20 mm have been recommended for very high-molecular-weight polymers
- Small particle size packings can sometimes result in shear degradation of large polymer molecules because the space between particles is very narrow.
- Columns with different porosity or mixed-bed columns, provide a better separation.

### *d) Pump*

A highly constant flow rate has to be maintained during the entire chromatogram.

Also:

- A change of the flow rate of only 0.1% can cause an error in molar mass of up to 10%.
- Most pumps can only reproduce the flow rate to 0.2–0.3%.

In-line filters in the solvent reservoir may prevent particles from coming into the pump heads, which might damage the check valves or the pump seals.

The pump used in SEC can be a syringe pumps or reciprocating pump.

***e) Detectors***

Numerous types of detectors exist for SEC, including:

***1) Concentration-sensitive detectors:*** they can be:

- Bulk Property Detectors- Refractive Index (RI) Detector
- Solute Property Detectors- Ultraviolet (UV) Absorption Detector
- Evaporative Detectors- Evaporative Light Scattering Detector (ELSD)

***2) Molar mass sensitive detectors:*** The following sensors are used:

- Light Scattering Detectors
- Low Angle Light Scattering (LALS) Detectors
- Multiangle Light Scattering (MALS) detectors

## V.2. Applications

- *Desalting proteins from salts, sugars and amino acids:* application to remove lactose from milk, using a highly cross-linked gel.

- *Fractionation of a mixture of macromolecules.*

- *Identification of the quaternary structure of purified proteins*

***- Molecular weight determination****:*

The SEC is mainly used to measure the molecular weight of polymer compounds and macromolecules.

Regarding SEC, as molecules of various sizes flow through the column, molecules of small size flow slowly while penetrating deep into the pores in the packing material, while molecules of large size flow out of the column without penetrating the pores.
The calibration curve shows this relationship graphically. Figure II.12 shows the relationship between retention time (also called elution volume) and molecular weight for SEC. When molecules larger than a certain size are separated by size exclusion chromatography, the retention time is the same because the substance passes through the pores of the packing material. This is called the exclusion limit ($V_0$). Conversely, molecules below a certain size completely penetrate the pores and tend to elute almost in the same position. This is called the permeation limit .

In order to obtain an accurate molecular weight distribution of polymer materials, it is necessary to set analytical conditions such that the measured polymer materials are eluted between the exclusion limit and the penetration limit.

After constructing a calibration curve where Log (MW) is a linear function of retention volume (or retention time), the MW of the unknown macromolecules can be determined using a calibration curve, (Figure II.12).

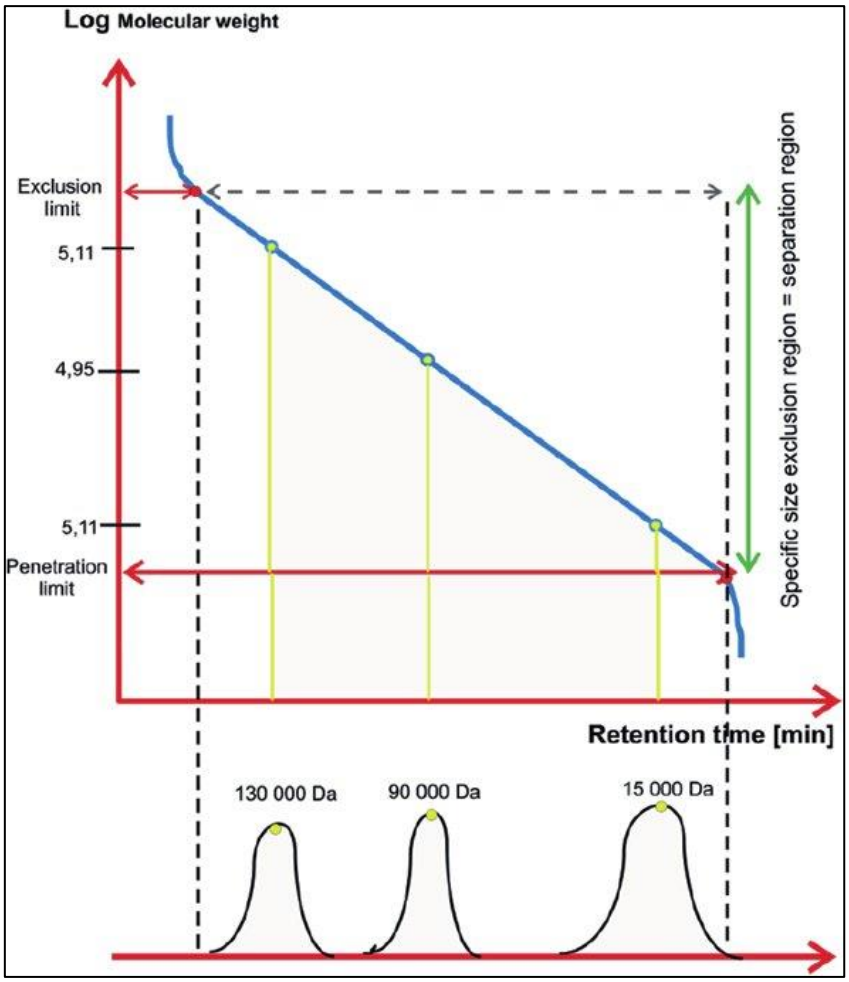



**Figure II.12.** Column calibration curve in size exclusion chromatography.

In SEC, the expressions (II.9) et (II.10) are used:

$$V_e = V_0 + KV_p \qquad \text{(II.9)}$$

$$V_t = V_0 + V_p \qquad \text{(II.10)}$$

**With:**

K: Diffusion coefficient

$V_0$: Interstitial volume or exclusion volume, (mL)

$V_e$: Elution volume, (mL)

$V_p$: Pore volume, (mL)

$V_t$ : Permeation volume, (mL)

## VI. Affinity chromatography (AC)

Affinity chromatography (AC) is a chromatographic technique based on biological-type interactions between a specific ligand (grafted onto a macromolecular matrix) and a specific substrate, which constitutes the molecule to be separated. AC is characterised by the purification of a component in a single step and is mainly applied to the purification of biomolecules.

### VI.1. Principle

A specific ligand for the molecule to be purified attaches to an insoluble matrix (SP) by binding reversibly with it.

As shown in Figure II.13, in the first stage (retention), the molecule of interest binds to the matrix via a specific ligand, and the other components are eluted. Then, in the second stage, it is detached from the matrix, from which the molecule is purified. Before separating components by AC, it is necessary to know the structure and specificity of the molecule to be purified to choose a suitable ligand, which must not bind to compounds other than the molecule.

Coupling: ligand-resin chromatography, which has no affinity for the molecule of interest, must be carried out without denaturing the ligand so that it retains its affinity for the molecule of interest.

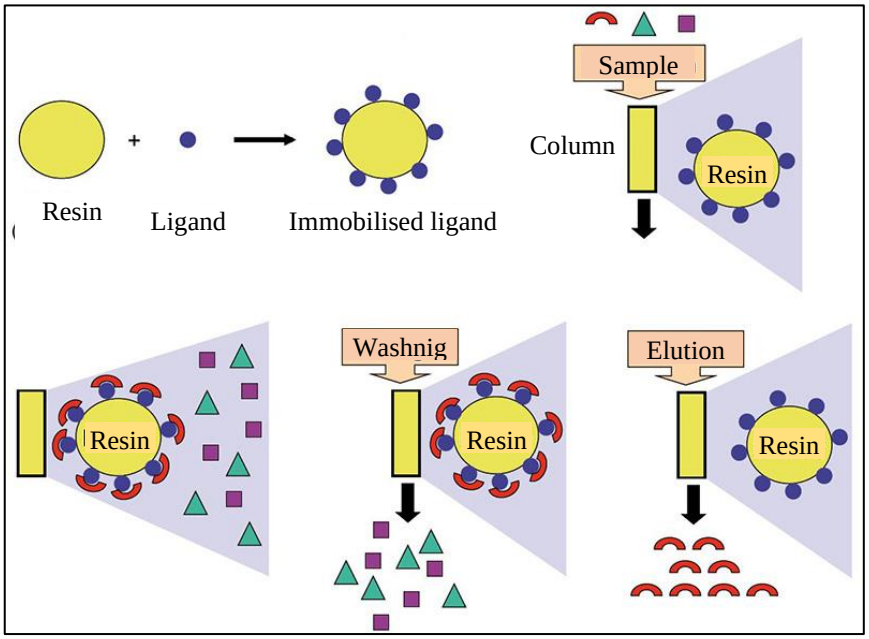



**Figure II.13.** Affinity chromatography principle.

## VI.2. Elements of AC

### *a) Stationary phase*

It consists of a chemically inert matrix or macromolecular support onto which a ligand (effector) is grafted, which has a specific biological affinity for a molecule in the sample to be analysed.

The supports used in AC must:

- be insoluble in water but wettable
- be porous
- be chemically and mechanically stable
- have a functional group that is reactive so that the fixing branches can be attached.

The matrices used are organic (osidic) or inorganic, such as cross-linked agarose, polyacrylamide gels, or silica.

Several derivatives of carboxymethylcellulose exist, such as aminohexyl M-cellulose, which has a long spacer branch, allowing a certain distance between the ligand and the support and

thus facilitating the complexation reaction. Succinylated aminohexyl CM and aminododecyl CM-cellulose are supports that allow the attachment of effectors with a reactive -$NH_2$ function.

Figure II.14 illustrates the activation and attachment of an amine-functional ligand to an agarose support.

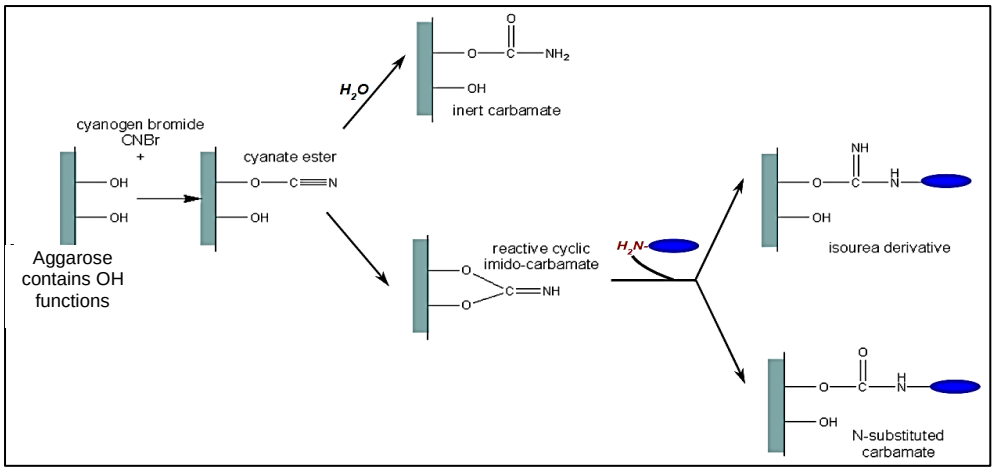



**Figure II.14.** Activation and cross-linking of a ligand in affinity chromatography

### *b) Effectors or ligands*

Ligands are substances that can form stable complexes with the molecules they bind. Ligands must contain a stable reactive group at a sufficient distance from the active site to reduce steric constraint and facilitate the bonding reaction.

The different types of effectors depend on the field of application:

- In enzymology, in the case of enzyme purification, substrate-like effectors must be chosen, such as reversible inhibitors, allosteric effectors, and coenzymes.
- In immunology, antibodies, haptens, and antigens are best suited.
- In biochemistry, hormones, peptides, and peptide analogs are used for receptor proteins.

### *c) Mobile phase*

Generally, it is a buffer solution that leads to the retention of a single biomolecule. The solution must not interact with the SP, but it must ensure that it is wetted.

There are two main methods for eluting the retained biomolecules, (Figure II.15):

***1) Elution by competition:*** A competitive agent is added to the MP at a sufficient (saturating) concentration to displace the retained biomolecule from the support. The competitor is then removed by an appropriate method, such as dialysis.

***2) Elution by modifying the physicochemical conditions of the medium:*** In this case, elution is achieved either by:

- Modification of pH
- Addition of detergents
- Ionic strength
- Addition of a hydrogen bond competitor.

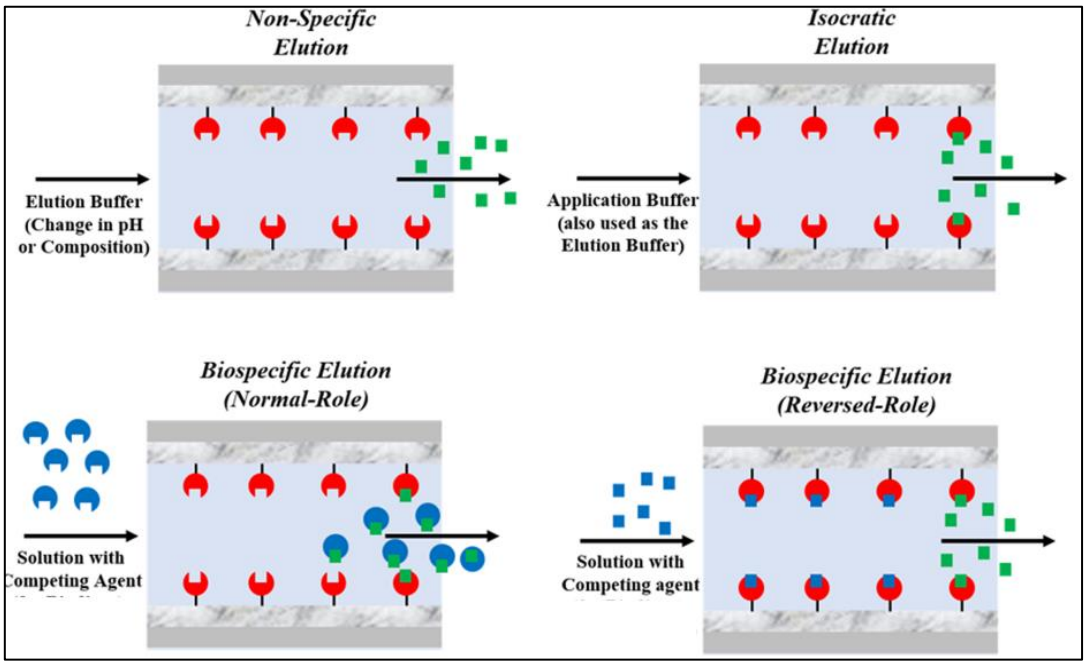



**Figure II.15.** Elution types in AC.

## VI.3. Applications

The AC mainly applies to:

- *Enzymology:* enzyme extraction and purification of enzyme extracts
- *Immunology*: purification of antibodies;

- *Biochemistry:* Isolation of proteins and nucleic acids. For the isolation of polyadenylated messenger RNA, chromatography on oligo(dT)-cellulose or poly(U)-Sepharose resins are used exclusively. However, at high ionic strength, the short polyadenine sequence at the 3' end of the mRNA can bind by simple base complementarity to oligo-deoxythymidine or poly-uridine sequences. Elution is then carried out at low ionic strength.

# Chapter III: Planar chromatography: Thin layer and paper chromatographies

## I. Introduction

Planar chromatography also named two-dimensional chromatography, includes paper chromatography (PC), thin layer chromatography (TLC), and planar electrophoresis. This is a liquid chromatography technique where the stationary phase is positioned on a flat or planar support. The nature of the mobile and stationary phases as well as the theory are the same as in column chromatography.

The two types PC and TLC use the same implementation and the same equipment.

## II. Thin layer chromatography (TLC)

### II.1. Objectives

TLC allows easy and rapid control of a separation. It is carried out for the following purposes:

***a) Analytical***

The separation of the constituents of a mixture is then the first step to determine the composition of this mixture:

- Check the purity of a product: If the analysis, carried out with various solvents and different adsorbents, reveals the presence of a single substance.
- Monitor the progress of a reaction: by indicating the number of components in a mixture.
- Check the effectiveness of liquid-liquid extraction.
- Identification of the eluent for chromatography in a silica gel column.

***b) Preparation***

Purification of small quantities of product (up to ~100 mg on a 20 cm* 20 cm plate). The strip containing the purified product is scraped, then the silica is extracted with a solvent.

## II.2. Interaction mechanism

TLC is based mainly on adsorption phenomena; the mobile phase, a mixture of volatile solvents, is then carried to the surface of the plate by capillarity action in an atmosphere saturated with solvent. When the plane on which the sample is deposited is placed in the chromatographic tank, the eluent rises through the stationary phase (SP).

But, depending on its density, its solubility in the mobile phase and the electrostatic forces holding the component on the SP, each solute in the sample moves at its own speed in the absorbent.

## II.3. Elements of TLC

The major elements of a chromatographic separation by TLC are:

### *a) Chromatographic chamber*

It is a container generally made of glass of variable shape, it is closed by a waterproof cover. Glass is used because it is not attacked by migration solvents. In the case of anthraquinone pigment chromatography, a transparent plastic tank is sufficient, because the solvents used are not corrosive.

During the development of the chromatogram, the tank must be saturated with solvent and remain closed and not moved.

### *b) Support*

These are glass plates, semi-rigid sheets of plastic, or aluminium or even on a sheet of paper, covered with a thin layer of stationary phase (modified or unmodified silica gel or another adsorbent), (Figure III.1).

### *c) Stationary phase (SP)*

It has the form of balanced granules which have a large specific surface area and characteristic functional groups. It is prepared in the form of a thin layer of thickness (100 – 250 μm) and fixed using a binder such as hydrated calcium sulphate, starch or an organic polymer, on a plastic support, metal or glass. A fluorescent pigment can also be added to the SP, which allows

detection of compounds by exposure under ultraviolet light. The mixture is placed on an inert support, left to dry then hot activated.

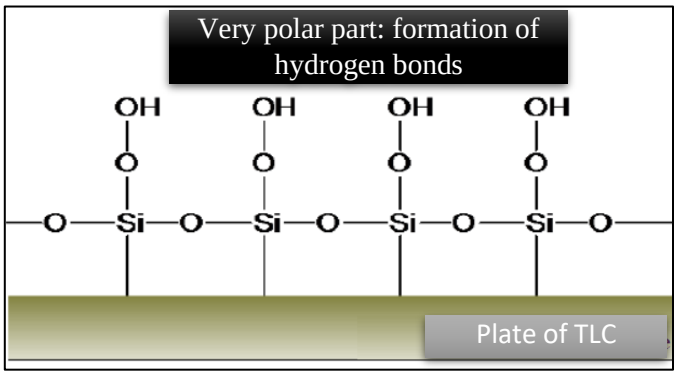



**Figure III.1.** Simplified illustration of an SP of silica gel.

***1)*** ***<u>Adsorbents type:</u>*** It is possible to classify the adsorbents used in chromatography according to their interaction strengths with polar compounds (Figure II.4).

Silica gel and alumina are the most commonly used adsorbents. In general, the more active an adsorbent is, the more strongly it retains polar compounds. It is however possible to treat an adsorbent to modify its adsorption capacities and its properties: The lower the water content of an adsorbent (which results in the presence of a greater number of adsorption sites for the solute), the more polar or active it is.

- **Silica gel ($Si(OH)_4$):** Often referred to as silica gel, it is a porous white powder with very fine particle sizes. It is in the form of microspheres of constant diameter which can vary from 2 to 5 µ (Figure III.2.a). The surface of the silica gel microspheres contains silanol groups which are essential to adsorption phenomena in particular because they allow the formation of hydrogen bonds and are responsible for the polarity and acidity of the silica gel, (Figure III. 2.b).
- ***Alumina ($Al_2O_3$):*** porous solid white powder of high surface area (between 100 to more than 300 $m^2.g^{-1}$), it is well suited for the separation of acidic solutes (phenols, carboxylic acids), (Figure III.3).

***2)*** ***<u>Interaction sites</u>*****:** they are of three types: free silanols, weakly hydrated, and bound free silanols (Hydrogen bound). Depending on the hydration state of the silica, the sites are to a

certain extent blocked. The interactions are electrostatic of the dipole-dipole type (Si-O-Si-O-H).

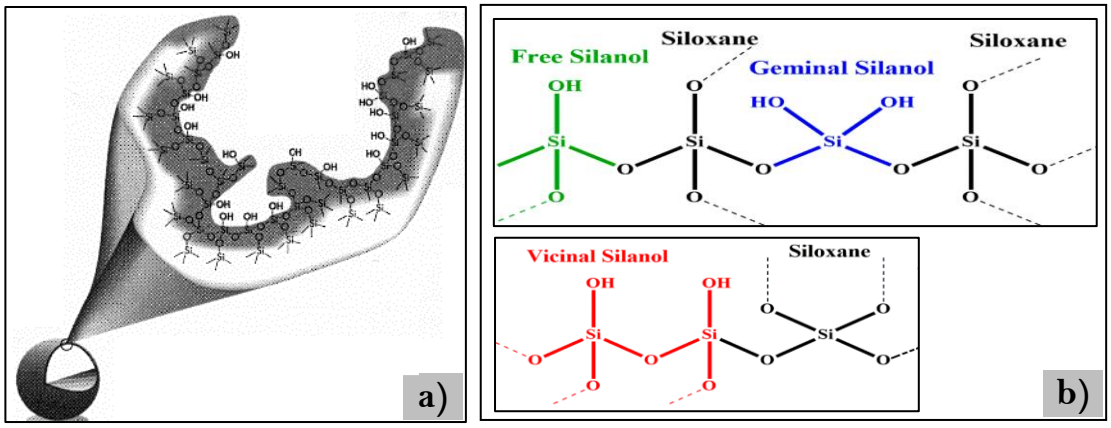



**Figure III.2.** Morphology of a silica gel grain pore: a) and surface of a silica gel grain: b).

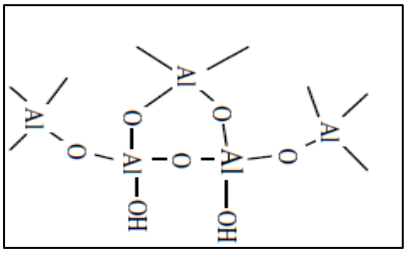


**Figure III.3.** Surface of an alumina grain.

***d) Sample (Analyte or solute)***

It can be polar, apolar, or in the form of an ion, its migration speed depends on the polarity of the phases. It is dissolved (2 to 5%) in a volatile solvent, which is not necessarily the same as the eluent: the most used are trichloromethane (chloroform), propanone, or dichloromethane. The solution is placed on the plate, approximately one (01) cm from the lower part. Polar molecules are always more adsorbed than non-polar molecules in the following order:

*Amides > amines > carboxylic acids > alcohols > ketones > esters > nitro compounds > ethers > aromatic hydrocarbons > unsaturated hydrocarbons > saturated hydrocarbons.*

***e) Mobile phase (Eluent)***

A pure solvent or a mixture, it migrates slowly along the plate carrying the components of the sample.

Two factors can affect the interaction between; the eluent and the solute (mixture of compounds to be separated):

***1) Solubility:*** It must be able to dissolve the solute in the eluent for migration to take place.

***2) Strength or eluting force ($\varepsilon_0$):*** It represents the ability of a solvent to desorb analyte molecules from the stationary phase. It is a function of the polarity of the solvent relative to the polarity of the SP. A list according to strength is referred to as an eluotropic series, (Table 5) and it represents the adsorption energy of an eluent molecule per unit area of the adsorbent. (In many tables the $\varepsilon_0$ values refer to alumina as an adsorbent which gives higher numbers.

• The solvents are classified according to the eluting force to facilitate their choice to control the capacity factor (k') of the analytes. The higher the eluting force, the more the retention coefficient (k') decreases.

• In chromatography on polar SP: the Eluting force increases as the polarity of the eluent increases.

• In reverse-phase chromatography: The eluting force increases as the polarity of the eluent decreases.

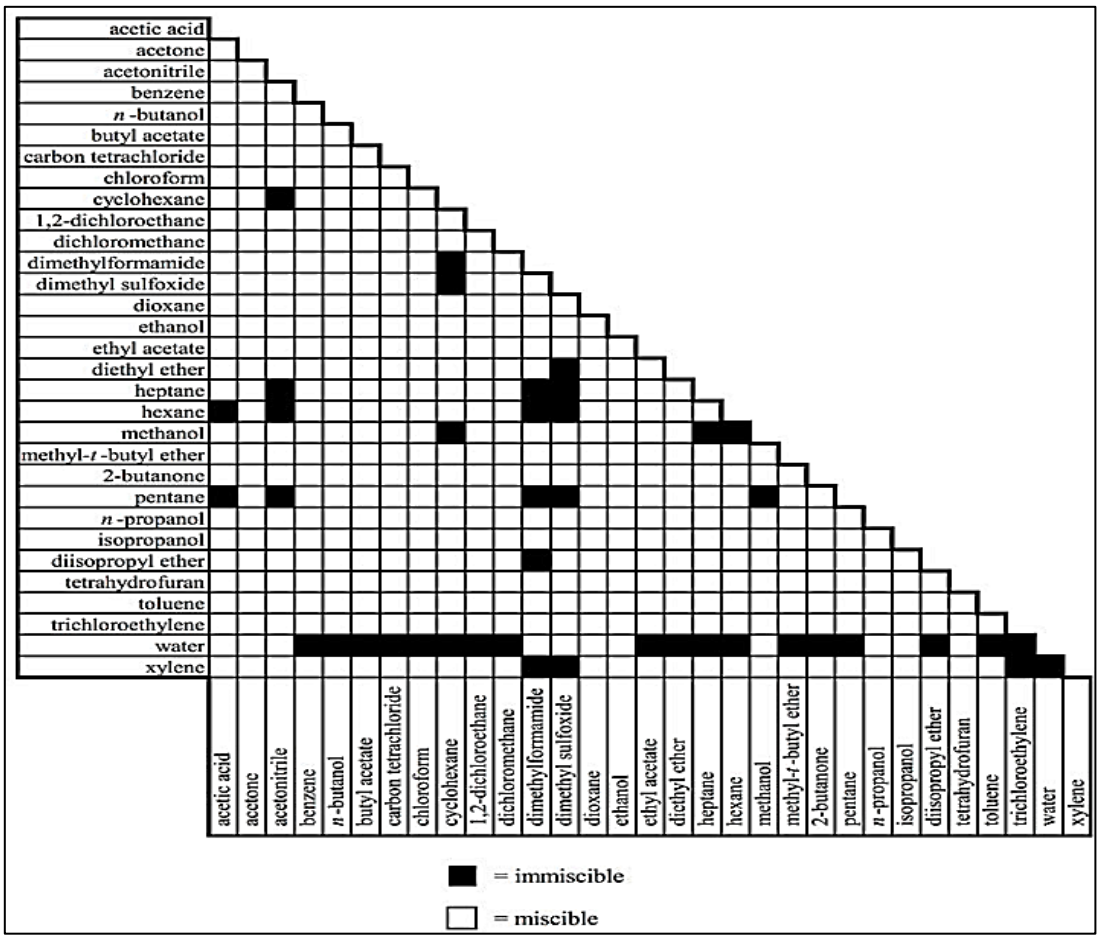
**Table 5.** Eluotropic forces **($\varepsilon_0$)** of solvents in alumina adsorption chromatography (for silica, multiply by 0.77).

| Solvent | Eluotropic force ($\varepsilon_0$) | Solvent | Eluotropic force ($\varepsilon_0$) | Solvent | Eluotropic force ($\varepsilon_0$) |
|---|---|---|---|---|---|
| Hexane | 0,01 | Ethyl ether | 0,36 | Ethyl acetate | 0,56 |
| Cyclohexane | 0,04 | Trichoromethane | 0,4 | Tetrahydrofuran | 0,57 |
| Methane tetrachloride | 0,18 | Methyl chloride | 0,42 | Methyl cyanide | 0,65 |
| Isopropyl ether | 0,28 | Acetone | 0,56 | Pyridine | 0,71 |
| Toluene | 0,29 | Dioxane | 0,56 | Ethanol | 0,66 |
| Benzene | 0,32 | Butanol | 0,56 | Methanol | 0,95 |

**Rremarks:**

1. Elution is started with slightly polar solvents and then continues with solvents of increasing polarity.
2. The mixture of solvents must meet the criteria: chemical inertness and miscibility, (Table 6).

**Table 6.** Solvent Miscibility.




## II.4. Carrying out a TLC

TLC takes place in three stages: tank preparation, plate preparation, and elution and development. Figure III.4 summarises the steps for carrying out thin-layer chromatography.

### *a) TLC Plate preparation*

Measure 1 cm from the bottom of the plate. Using a pencil, draw a line across the plate at the 0.5 cm mark. Under the line, mark lightly the name of the samples you will spot on the plate, or mark numbers for time points. Leave enough space between the samples so that they do not run together; about 4 samples on a 5 cm wide plate is advised.

### *b) Developing container Preparation*

In a tank with a lid, a volume of eluent (or a mixture of eluent) is introduced with an approximate height of 0.5 cm, then the lid will be closed while waiting a few minutes: this

allows the atmosphere to be saturated with vapours of eluent, to prevent it from evaporating from the plate during elution.

### *c) Spot the TLC plate*

This involves depositing the sample with a micropipette onto the baseline of the TLC plate.

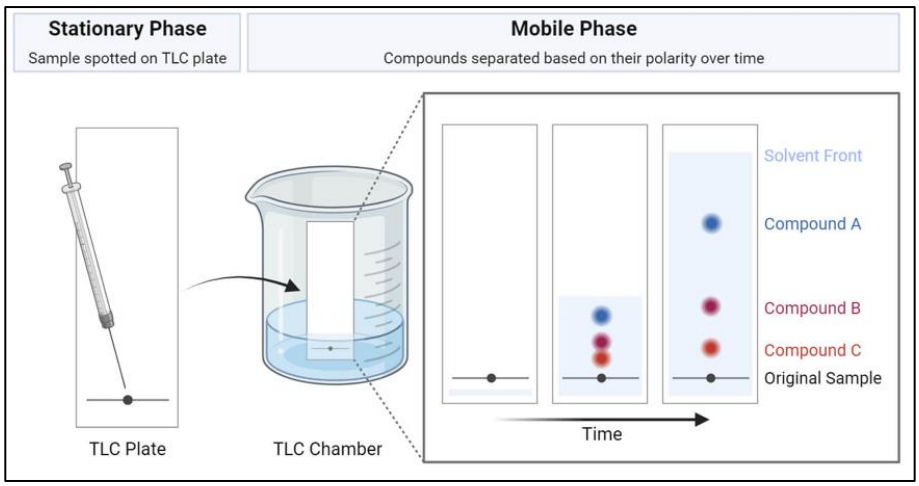



**Figure III.4.** Steps for a TLC, case of linear development.

### *d) Elution and development*

**1)** ***Linear development:*** the plate is placed in the eluent, immediately closing the lid. The eluent then rises slowly, by capillarity. Migration stops when the eluent reaches 2/3 or 3/4 of the height of the plate. The latter is removed where its level is marked immediately, in pencil: the position of the eluent front (before it dries). The plate can be dried to prevent any spreading of stains.

**2)** ***Radial development (circular chromatography):*** (Figure III.5.a): The compounds to be separated are deposited near the centre or at the circumference of a circular support and migration is carried out towards the end or the centre of the circle, as appropriate.

**3)** ***Two-dimensional development:*** It is possible to develop the plate in two directions, oriented at right angles (90°) to each other (Figure III.5.b). The sample to be separated is placed on the corner of a plate and development is carried out in an appropriate solvent. The plate is dried, rotated a quarter turn, and developed again in a second solvent of different selectivity to the first.

### *e) Visualisation/Revelation*

If the substances are coloured they are visually detected easily. Then for colourless substance physical and chemical methods are used.

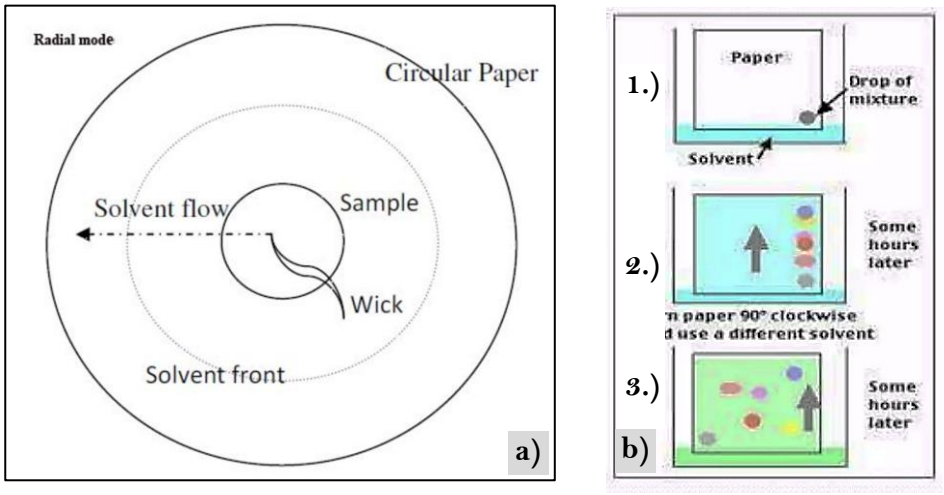



**Figure III.5.** Radial Development: a), Two-dimensional development: b).

***1) physical methods: Use of UV lamp:*** All substances with absorption in the region above 230 nm are studied on supports supplemented with fluorescent bodies by irradiation with short-wave UV light. If the plate is fluorescent, under a UV lamp, the entire plate appears green except where the spots are, which are circled with a pencil.

As a fluorescent agent, zinc sulphide, added to silica gel, makes the plate fluorescent when placed under a lamp. Some TLC plates contain a fluorescent indicator allowing resolution in UV, near UV (366nm) or far UV (254nm). The UV254 indicator is a zinc silicate activated with manganese, whose absorption maximum is 254nm. It exhibits green fluorescence. The UV366 indicator is also a mineral pigment with maximum absorption at 366nm. It exhibits blue fluorescence.

***2) Chemical method:*** It is based on the transformation of separated substances into coloured spots; products are often detected by their classic functional reactions:

- Amino acids by ninhydrin, which gives most of them a blue-violet colour:
- Organic acids by coloured indicators.
- Sugars using Molisch's reagent or 50% sulfuric acid (brown spots).

- Some reagents, such as iodine or permanganate, give non-specific colorations with most organic compounds.

**II.5. Advantages specific to TLC**

- Short development times (2 to 60 min)
- Small separation distances;
- Very good resolution;
- Very high sensitivity;
- Very small quantities of sample used.

**II.6. Chromatographic calculations**

***a) Retention factor or retardation factor, ($R_f$)***

Is characteristic of a product in a given eluent and for a given SP. It is given by expression (III.1).

$$R_f = \frac{\text{Distance travelled by the solute } (D_i)}{\text{Distance travelled by the MP } (D_{MP})} \quad \text{(III.1)}$$

The value of $R_f$ is therefore between 0 and 1. To obtain reproducible Rf, it is necessary to operate under identical conditions.

***b) Relationship between $R_f$ and k'***

In TLC the stationary phase is immobilised and migration takes place by capillarity. The migration speed of the MP is given by expression (III.2):

$$u_0 = \frac{d_{MP}}{t_M} \quad \text{(III.2)}$$

And that of the solute is given by the expression (III.3):

$$u_i = \frac{d_{MP}}{t_R} \quad \text{(III.3)}$$

And the expression of $R_f$ is given by relationship (III.4):

$$R_f = \frac{u_i}{u_0} = \frac{t_R}{t_M} \quad \text{(III.4)}$$

And as the retention time is expressed by the expression (III.5):

$$t_R = t_M\,(1+k') \quad \text{(III.5)}$$

Then the retention frontal relationship will have the expression (III.6):

$$R_f = \frac{1}{1+k'} \quad \text{(III.6)}$$

### *c) Efficiency in TLC*

The efficiency (N) of a TLC plate and the height equivalent to a theoretical plate (HETP) for a compound are given by relations (III.7) and (III.8), respectively:

$$N = 16 . \frac{d^2}{W^2} \quad \text{(III.7)}$$

$$HETP = \frac{d}{N} \quad \text{(III.8)}$$

The resolution is given by the expression (III.9):

$$Rs = 2. \frac{d_1 - d_2}{W_1 + W_2} \quad \text{(III.9)}$$

**With:**

d: Distance travelled (cm)
W: Spot diameter (cm).

## II.7. Applications

The success of TLC is mainly due to the ease of its implementation and the possibility of its use in the analytical domain rather than in the preparative domain.
There are several applications of TLC:

- Easy and rapid control of the purity of an organic compound
- Monitoring of the chemical reaction
- Chromatographic fractionation on the column and search for the best solvent, before starting a separation on the conventional column.
- Purification of small quantities of product (up to 100 mg).

The strip containing the purified product is scraped, then the silica is extracted with a solvent.

## III. Paper chromatography (PC)

This technique is similar to that of TLC but the principle is based on the phenomena of sharing (or partitioning) of a solute between solvents constituted respectively by immiscible SP and MP; it is therefore an LLC. This method is based on the difference in solubility of a solute between two phases.

The distribution coefficient is called the partition coefficient (relation (I.1)).

## III.1. Elements of the PC

### *a) Support*

The support is a cellulose membrane that is introduced into a tank containing the fixed phase (often aqueous). The cellulose in very hydrophilic paper becomes saturated with water, which constitutes SP. Ordinary filter paper can be used, but paper designed for this purpose must be used (low level of impurities and the physical characteristics are uniform). There are eight categories of Whatman paper, classified according to their thickness, the texture of their surface and the speed with which water diffuses through them. For example, the Whatman paper No. 1 paper is the most commonly used, but for high flow speeds, paper No. 4 is recommended. Paper No. 20 has a slow flow speed, but allows better separation, giving very dense, uniform spots.

### *b) Stationary phase*

The stationary phase consists of the water itself, absorbed by or chemically bonded to the cellulose in the paper. On the upper part of the tank, the mobile phase migrates by capillarity, the separation of the solutes is done by sharing between the two solvents.

### *c) Mobile phase*

The mobile phase is most often a mixture of organic solvent and water. Solvents such as acetic acid, ethanol, propanol, phenol or pyridine are the most frequently used solvents mixed with water to develop a chromatogram.

### *d) Sample*

PC is mainly used to separate very polar substances such as amino acids, sugars and polyfunctional compounds. Generally, compounds that are more soluble in water or those that easily form hydrogen bond associations are strongly retained by the stationary phase and therefore migrate slowly.

### III.2. Carrying out a PC

It is similar to TLC, but it takes some time (usually several hours). Once the operation is completed, the paper is removed from the tank, and the solvent is allowed to evaporate. The chromatogram obtained is used for comparison with other analyses carried out on known substances and taken under identical conditions, to identify the substances in the sample. Substances can be identified by calculating the $R_f$ value which can be compared to those found in the tables.

There are several methods of revelation:

1. For coloured substances, there is nothing special to do.
2. For fluorescent substances, identification is done under a UV lamp.

1. Iodine chamber method: where brown or amber is kept in a tank with few iodine crystals at the bottom.
2. For other substances, a developer which will react chemically with the products and whose result will be coloured is used.

### III.3. Disadvantages of PC

The biggest disadvantages of PC compared to CCM are:

- Much longer development time
- Generally, less good separation.

# Chapter IV : Conventional Column Liquid Chromatography

## I. Introduction

Column chromatography is one of the oldest physicochemical separation processes (like distillation, crystallisation, or fractional extraction of the constituents of a homogeneous liquid or gas mixture) among other techniques. chromatographic.

## II. Principle

This is a preparative method (qualitative and quantitative) which allows the constituents of a mixture to be separated and isolated. This technique is based mainly on adsorption, partitioning, ion exchange or steric exclusion phenomena and makes it possible to separate practically all possible mixtures. The stationary phase, in the form of small particles, fills a column of determined length and section. The mixture, in concentrated solution, is deposited at the top of the column. The separation of the constituents of the mixture results from the continuous flow of an eluent through the column by gravity, hence its name gravitational chromatography. In the conventional technique, the eluent is a single solvent whose polarity is modified so as to accelerate the movement of the constituents of the mixture.

## III. Some basic notions: (See chapter I)

**III.1.** Retention factor k'
**III.2.** Capacity factor ($\alpha$)
**III.3.** Efficiency of a column: Number of theoretical plates (N and HETP)
**III.4.** Resolution factor (Rs)

Resolution is the quality of separation of two neighbouring peaks. To numerically translate the more or less good separation between two compounds, we use the resolution factor which is calculated from the chromatogram, for two molecules.

## IV. Elements of liquid column chromatography

Conventional column chromatography requires the following constituents (Figure IV.1):

- Stationary or fixed phase
- Glass or steel column

- A mobile phase consisting of one or more eluents
- Various accessories such as: collector, MP tank.

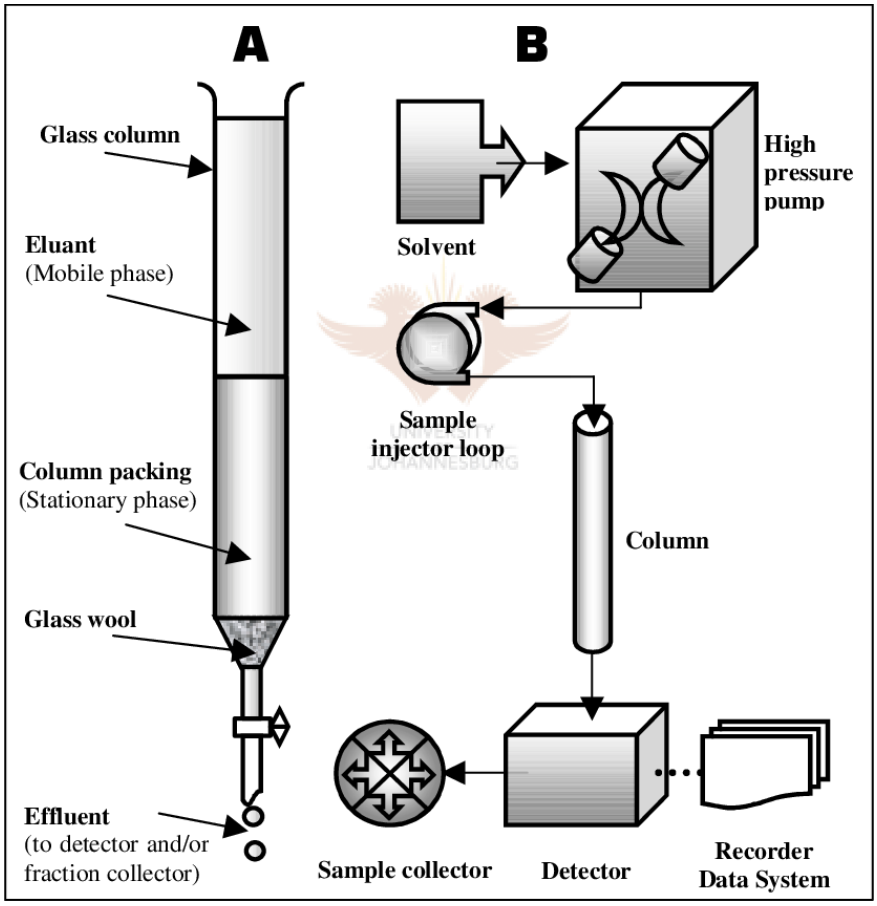



**Figure IV.1.** Description of liquid column chromatography.

## IV.1. Stationary phase

The type of SP depends on the phenomenon involved.

### *a) Case of adsorption chromatography*

The most used is alumina; however, it will be limited to stable organic compounds because, in its basic form, it can cause the dehydration of esters for example. Silica gel is also frequently used. In column chromatography, the particle size of the adsorbent must be greater than that of the adsorbents used in TLC. Their size is usually between 50 and 200 μm.

The amount of adsorbent depends on the difficulty of separation and the sample mass. We can consider that for each gram of sample, 30 to 50 g of adsorbent are required if the polarity of the components to be separated is very different and up to 200 g if separation is difficult.

***b) Case of partition chromatography***

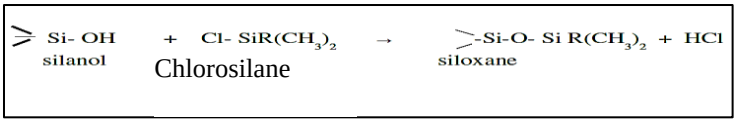
Two cases present:

***1) <u>Case of the normal phase</u>*:** The MP is not very polar, the substances are then eluted in the opposite direction to their polarity.

To modify the polarity of the SP, the main grafts are used: These are grafts comprising functions of a polar nature:

- Amine $NH_2$
- Nitrile: CN
- Diols: $CH_2 – CHOH – CH_2OH$

***<u>2) Case of the reversed phase</u>:*** The MP is polar, the substances are then eluted in the direction of their polarity. The crosslinking reaction is given by silanization expressed in reaction IV.1.




(IV.1)

The main grafts used in the reverse phase, are grafts comprising functions of an apolar nature, table 1.

The difference in electronegativity between C and H is small. $\chi$ (C) =2.5 and $\chi$ (H)=2.2. The longer the chain, the more the solute is retained and the more $t_R$ increases.

## IV.2. Mobile phase

The eluent (or all of the eluents) must be:

- Immiscible in the stationary phase.
- Pre-saturated with the stationary phase before entering the column.
- Compatible with the detectors used.

The polarity of the mobile phase has a great influence on the partition coefficient of solutes. Suitable retention times are then obtained by adding small quantities of a polar modifier to a given solvent.

***a) For LLC (partition)***

The nature (composition) of the MP depends on that of the SP (see III.1)

***b) In LSC (adsorption)***

The eluent generally used is a mixture of two solvents. Most often, at the start of the elution, we start with the least polar solvent which carries away the least polar constituents (the least retained by the adsorbent); the polarity of the eluent is then increased by gradual addition of the most polar solvent; the most polar constituents are thus elected. It is necessary to carry out tests on TLC with different eluents to have a good separation between the products. An $R_f$ close to 0.3 for the least polar product.

**IV.3. Sample**

***a) Case of LLC***

- For polar or very polar molecules: LC in normal phase
- For molecules with medium or low polarity: LC in reverse phase

***b) Case of SLC***

As in the case of TLC, polar molecules are always more adsorbed than non-polar molecules: amides > amines > carboxylic acids > alcohols > ketones > esters > nitro compounds > ethers > aromatic hydrocarbons > unsaturated hydrocarbons > saturated hydrocarbons.

**V. Factors influencing separation**

Four factors are involved: SP, MP and:

**V.1. Column size**

Columns specially designed for this use have at their base a sintered glass or porcelain plate which allows the free flow of the eluent while preventing the passage of the adsorbent. You can

also use a burette, at the bottom of which you place a pad of glass wool and sand (Figure IV.2). Long columns will give higher resolutions (Figure IV.3).



**Figure IV.2.** Conventional chromatographic column.

The quantity of adsorbent is such that it occupies a height equal to approximately 10 times the diameter of the column. It is also necessary to provide a space of approximately 10 cm above the adsorbent to place the solvent.

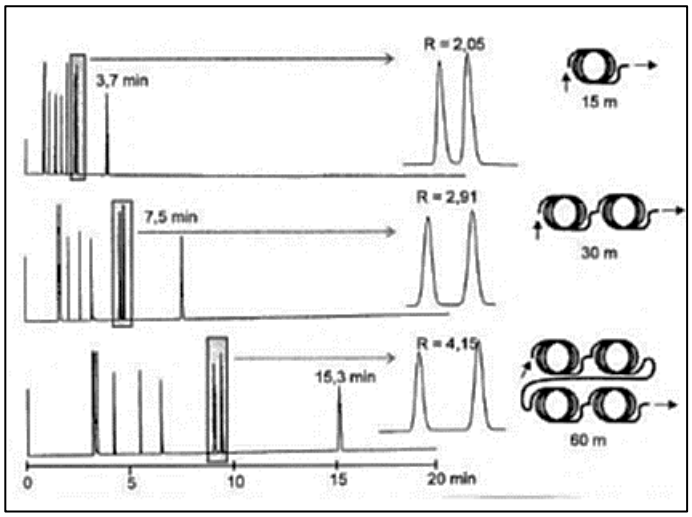



**Figure IV.3.** Effect of column length on separation quality.

### V.2. Elution velocity

It must be as constant as possible. It must be slow enough so that the solute is as close as possible to equilibrium between the liquid and adsorbed phases. It should not be too slow because the substances diffuse in the solvent and we obtain increasingly wider bands and poor separation.

## VI. Preparation of a chromatographic system in column adsorption chromatography

### VI.1. Packing the column

This is the most delicate operation because the packing must be as homogeneous as possible and free of air bubbles. The lower and upper surfaces of the adsorbent must be perfectly horizontal. The column being vertical, packing can be carried out using two methods.

***a) Wet packing***

A homogenised mixture of the adsorbent and the least polar solvents used for the development is prepared in a beaker by adding the adsorbent in small quantities to the solvent to obtain a slurry sufficiently fluid to flow easily.

Using a funnel, enough mixture is poured so that the adsorbent which is gradually deposited forms a layer of approximately 2 cm. The walls of the column are tapped to encourage compaction of the adsorbent. We then open the tap so that the solvent flows slowly and we continue to add the homogenised slurry in successive portions. When all the adsorbent is introduced, it is allowed to settle until the supernatant liquid is clear. During the operation, care must be taken to ensure that the level of solvent is always higher than that of the adsorbent. (See the link: https://www.youtube.com/watch?v=S-CDbA8tYKM).

***b) Dry packing***

The column is two-thirds filled with the less polar of the two solvents and the powdered adsorbent is added in successive portions to the column using a funnel; during the addition, we continuously tap on the walls to obtain maximum compaction. When the first portion forms a layer of approximately 2 cm, we open the tap to slowly let the solvent flow.

**VI.2. Deposit of products to be analysed**

They should form a narrow cylindrical area at the top of the column.
A liquid is deposited as is. A solid will be dissolved in the minimum of the less polar of the two solvents.
First, adjust the solvent level so that it is just above that of the adsorbent. Using a pipette, the sample is poured at the top of the column uniformly over the entire surface of the column without deforming it. If necessary, the liquid level of the column is adjusted again, as before: the sample is thus adsorbed uniformly at the top of the column. A sintered glass or a round of filter paper can be placed above the adsorbent to prevent resuspension of the adsorbent.

**VI.3. Elution**

The column can be fed by eluting it continuously using a dropping funnel or manually. In all cases, the surface of the adsorbent must never come into contact with air. The solvent is supplied

using a pouring funnel. The flow rate of the feed is set to be the same as the flow rate at the bottom of the column (typically 5 to 50 drops per minute). When the analysis of the fractions is completed, we bring together those that correspond to identical products, taking care to eliminate those that correspond to overlapping zones. The substances obtained in this way are generally of very high purity.

## VII. Properties

Column chromatography can be a preparatory method; it allows the separation of the constituents of a mixture and their isolation, from samples whose mass can reach several grams. It has the following properties:

- Large quantities of solvent are required for elution;
- The duration of the elution is generally very long;
- Detection of compounds requires constant attention.

# Chapitre V: High-performance liquid chromatography

## I. Introduction

The separation power of a chromatographic column increases with length and efficiency. Thus, the latter is linked to the nature and the surface presented by the SP, however, in the case of conventional chromatography, the MP passes through the SP by gravity or under low pressure (~0.1 MPa). To increase the flow rate, higher pressures (~10 MPa) were applied. The performances of the latter case in the form of optimisation of the technique were implemented, such as the reduction in particle size, regularity and compactness of filling, use of detectors, etc.

The analytical technique of High-Performance Liquid Chromatography (HPLC) can separate, identify, and quantify the compounds that are present in any sample that can dissolve in liquid. HPLC is the most accurate analytical method widely used for the quantitative as well as qualitative.

All the chromatographic methods already seen are commonly involved in HPLC.

## II. Principle

The liquid mixture to be separated is introduced into the MP which passes through a very well developed and finely designed SP (diameter of 1.8 to 10µm) for porous support or a porous monolithic material, under high pressure ensured by a pumping system to minimise loss of charge created, which thus ensures a regular and continuous flow of the MP. This chromatography must be accompanied by a detection system. The principle of separation is based on the interaction of the solute with the stationary phase, which depends on its affinity with the SP. Depending on the nature of the stationary phase, a variety of chromatographic modes have been developed, on the basis of the mechanisms of retention and operation, which consider as the more common classification, (Figure V.1).

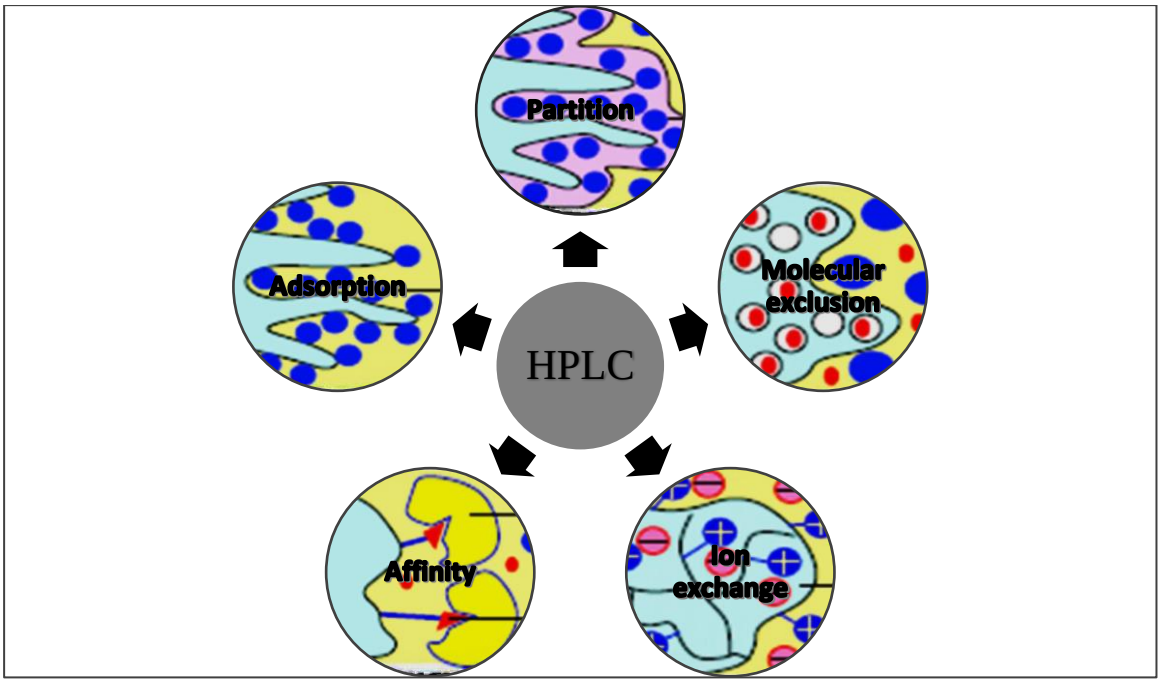



**Figure V.1.** Major mode of HPLC separation.

### III. HPLC instrument

An HPLC installation includes various specialised modules (Figure V.2): pumping system, injector, column, and detector.

### III.1. Solvent delivery system (pump)

Pumping systems are designed to deliver either single or multiple (binary, tertiary, and even quaternary) eluents. These depend on application which extends the capabilities of the technique. Every HPLC installation has at least one pump to force the passage of the MP through the column. These pumps are designed to maintain a non-pulsating and stable flow rate (0.1 and 10 mL.min$^{-1}$) while minimising the pressure loss constraint (Chapter I, expressions: (V.1) and (V.2)).

$$\Delta P = \frac{\eta . L . u}{K_0} \qquad (V.1)$$

$$K_0 = \frac{d_p^2}{180} . \frac{\varepsilon^3}{(1-\varepsilon)^2} \qquad (V.2)$$

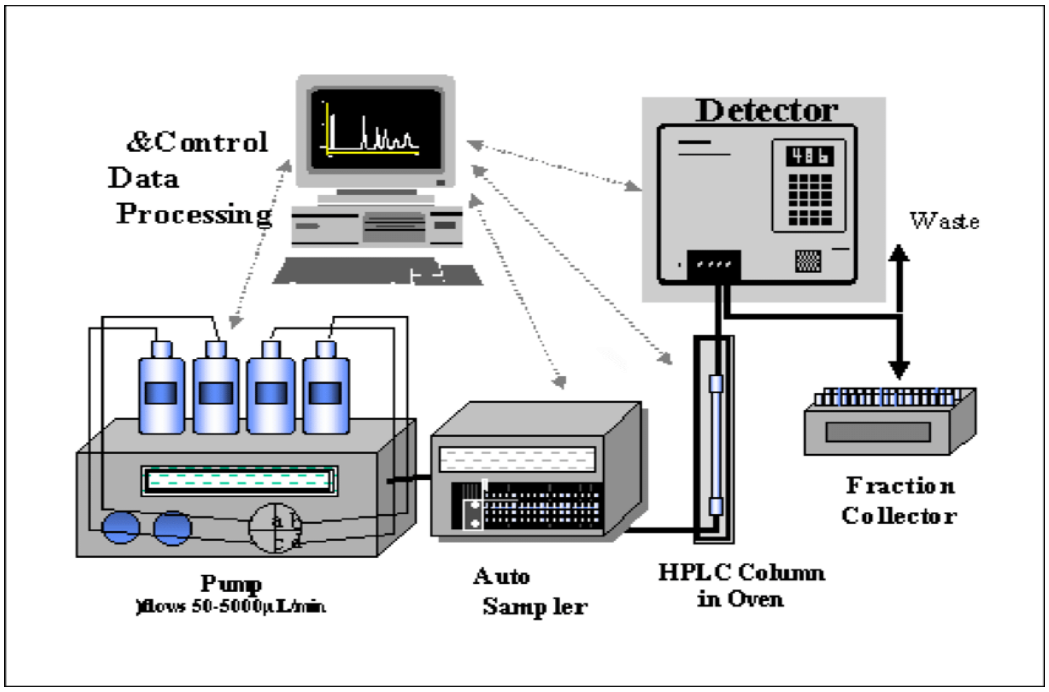



**Figure V.2.** Simplified schematic representation of an HPLC system.

**With:**

η : Dynamic viscosity, (Pa.s)

L : Column length, (m)

u : Linear velocity of the MP, ($m.s^{-1}$)

dp : Mean particle diameters, (m)

$K_0$ : Permeability constant ($cm^2$)

ε : Interstitial porosity (void fraction).

Therefore, depending on the MP power supply mode, there are two modes of chromatography:

***a) Isocratic mode***

The composition of the MP is kept constant during the separation.

***b) Gradient mode***

The composition of the mobile phase may need to be changed as the substances migrate through the column when complex sample mixtures are involved. Changes must be accompanied by an increase in mobile phase elution strength so that peaks which would otherwise be eluted late or not at all are accelerated.

### III.2. Sample injectors

Sample introduction is one of the critical aspects of HPLC. Even the best column produces a poor separation result if injection is not carried out carefully. In theory, an infinitely small volume of sample mixture should be placed in the centre of the column head, care being taken to prevent any air from entering at the same time.

There are various options for sample injection:

- by using syringe and septum injector;
- by using a loop valve;
- by using an automated injection system (autosampler).

High pressure systems require a special type of sample injection since injections with standard syringes are not possible. A multi-way high pressure (four-, six-, and eight-port) valves, manual or motorised in the case of automatic injectors, are used.

Figure V.3 shows how a loop valve operates. These valves are known as six-port valves as they have six connections.

- Loading position (a): filling of the loop where the syringe is inserted at position N° 4
- Injection position (b): the valve is turned manually (rotating 60° of a lever) or electronically so that the loop is connected to MP and the sample is thereby injected onto the column.
- Most modern injectors are autosamplers, which allow programmed injections (sequence) of different volumes of samples that withdraw from the vials in the autosampler tray.

### III.3. Column

Most HPLC columns are made of stainless steel, glass, polymers, or fused silica (Figure V.4), which is resistant to the usual HPLC pressure and also relatively inert to chemical corrosion (chloride ions and lithium ions at low pH being important exceptions). The inside of the column should have no rough surfaces, grooves, or microporous structures.

In most analyses, a post-column (or guard column) is used with the analytical column (Figure V.5):

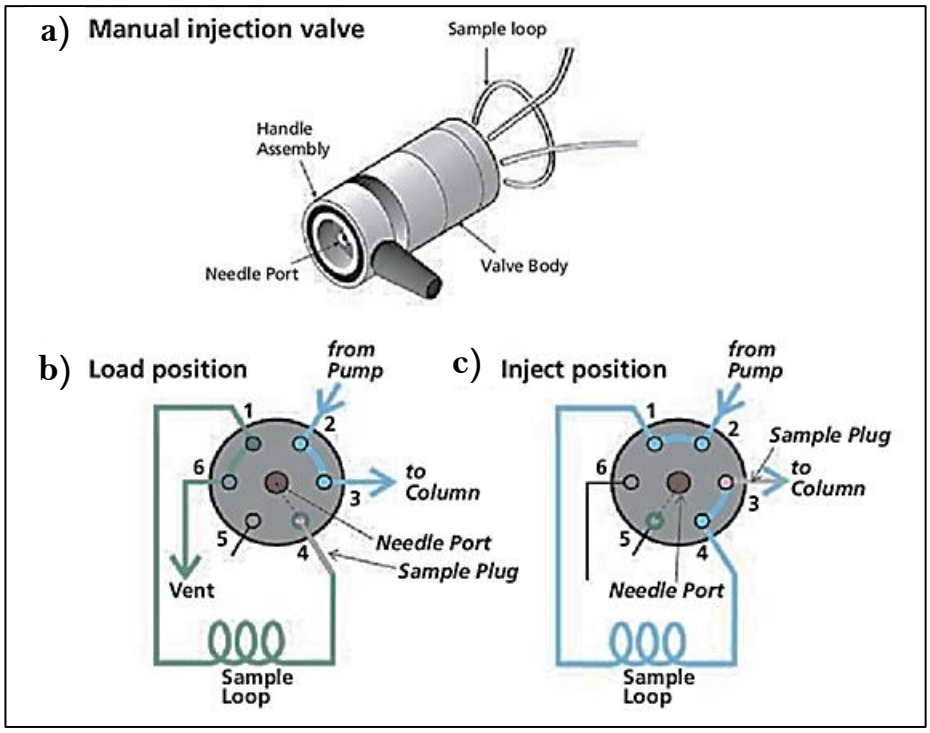



**Figure V.3.** Schematic diagram of the manual injection valve: a), schematic of the injector in load position: b) and in inject position: c).

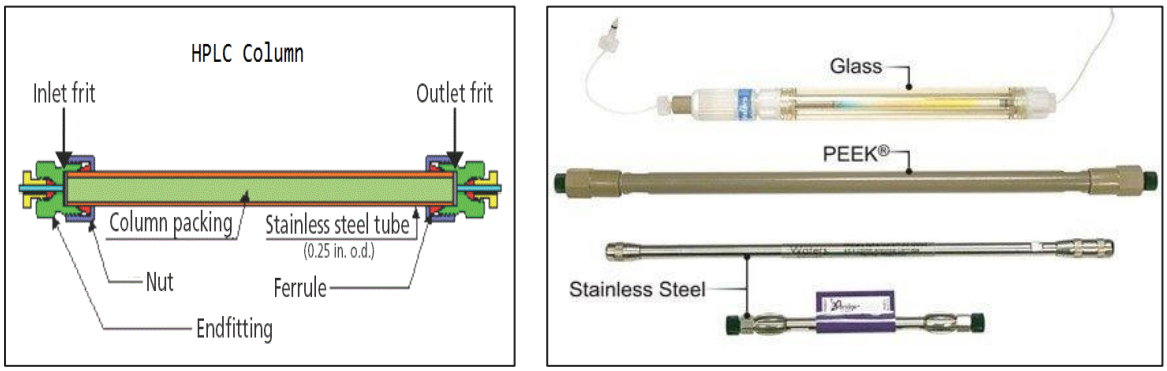



**Figure V.4.** Types of HPLC column.

### *a) Guard Column*

These columns are miniature versions of the analytical (separation) column and they are used as a protection between the injector and column. They are packed with the same or a chemically similar stationary phase as the main column and prevent impurities such as strongly retained compounds from contaminating it. When chosen and mounted properly a guard column does not affect the separation performance. they are replaced at regular time intervals or after a certain number of injections to ensure a maximum lifetime of the separation column.

### *b) Analytical column*

Modern column technology for HPLC has produced columns having various dimensions, with a trend toward smaller internal volumes. However, these columns have typical internal diameters that range from 2 to 5 mm and lengths ranging from 5 to 30 cm (Table 7). The narrow bore analytical columns are used for improved performance and MS applications and the capillary columns are used for MS detectors (from 0.075 -0.1 mm in diameter). For instance, packed capillary columns can be used that have inner diameters of 0.1 to 0.5 mm and lengths of 20 to 200 cm. Open tubular capillary columns can be constructed by placing a thin film or coating of the stationary phase onto the inner wall of a fused silica tube. However, the separation columns must be perfectly filled, with no empty spaces, as the presence of a few empty areas in a column will affect the theoretical height of the tray and reduce the resolution.

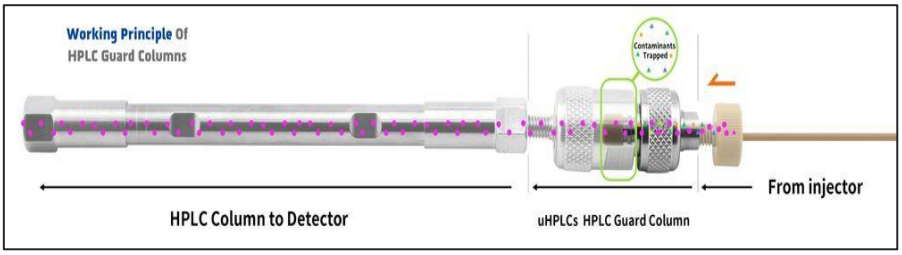



**Figure V.5.** HPLC with guard column.

**Table 7.** Typical column sizes used in analytical HPLC columns

| Type of column | Typical inner diameter (ID) and length | Typical flow rate range |
|---|---|---|
| Conventional packed column | 4-5 mm<br>5-30 cm | 1-3 mL.min$^{-1}$ |
| Narrow-bore column | 2-3 mm<br>5-15 cm | 0.2-0.6 mL.min$^{-1}$ |
| Microbore column | 1-2 mm<br>10-100 cm | 0.05-0.2 mL.min$^{-1}$ |
| Packed capillary | 0.1-0.5 mm<br>20-200 cm | 0.1-20 µL.min$^{-1}$ |
| Open tubular column | 0.01-0.075 mm<br>1-100 cm | 0.05-2 µL.min$^{-1}$ |

For these columns (with the exception of the conventional column), the flow rate is low, requiring special pumps and detectors. These narrow columns not only considerably reduce the amount of MP, but also improve resolution by reducing diffusion inside the column. They are also more sensitive and used in HPLC-MS coupling.

### III.4. Stationary phases

The particle size distribution should be as narrow as possible (with a ratio of diameters of the smallest and the largest particles of 1:1.5 or 1:2, as already mentioned). This is because the smallest particles determine the column permeability and the largest particles fix the plate number. Small particles produce a high flow resistance and large particles are responsible for a high degree of band broadening. For this reason, a particle size analysis accompanies many products.

Many types of particles and support formats have been developed for HPLC, (Figure V.6): Porous particles, non-porous particles of small diameter, porous layer beads, perfusive particles, and monolithic materials and grafted phases.

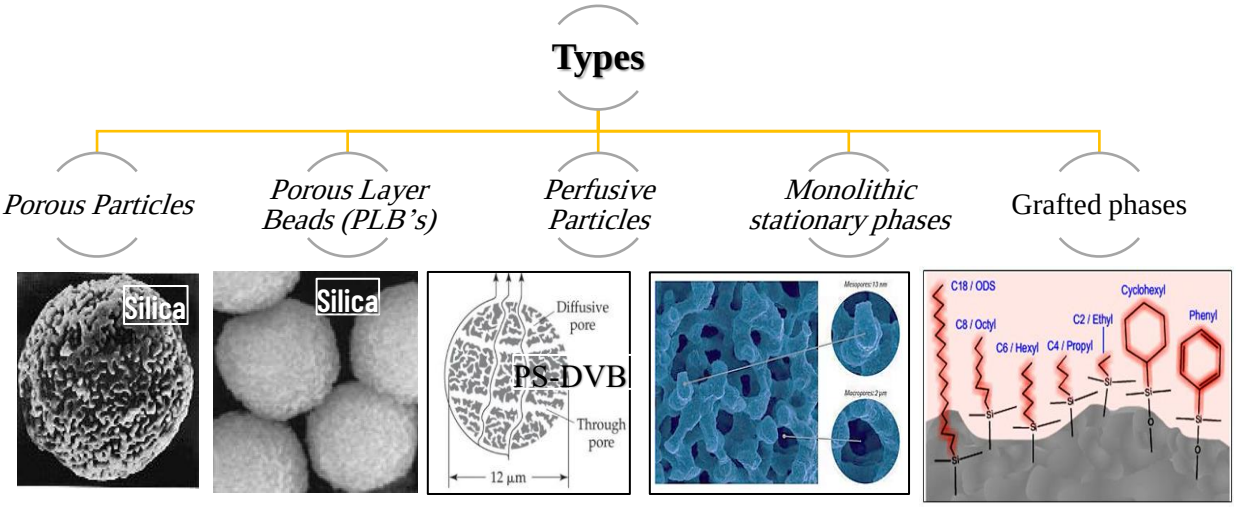



**Figure V.6.** Main supports and materials used in HPLC.

#### *a) Porous Particles*

This is the usual type of HPLC stationary phase. Their internal structure is fully porous with size materials varying from 3, 3.5, 5 or 10 µm. As a rule of thumb, their performance, i.e. the plate number per unit length, doubles each time from 10 to 5 and 3 mm, whereas the pressure drop increases each time by a factor of four. Within the pores, the mobile phase (and the analytes) does not flow but moves only by diffusion.

### *b) Non-porous, Small-diameter Particles*

To maintain a certain sample capacity (specific surface) a small particle should be used with a diameter of 1–2 μm. The capacity is about 50 times lower than compared to conventional porous phases and the pressure drop is high. The retention volume of the peaks is low because the pore volume is missing; the fraction of liquid within the column (the column porosity ε) is only ca. 0.4 compared to 0.8 of a column with porous packing. Therefore, extra-column volumes and time constants need to be kept low. Chromatography of biopolymers seems to be more advantageous with these stationary phases concerning denaturation and recovery.

### *c) Porous Layer Beads (PLB's)*

These are large particles with a diameter in the 30 mm range which allows to pack them dry. They consist of a non-porous core (e.g. from glass) which is covered with a 1–3 mm thick layer of a chromatographically active material. PLB's are rarely used but can be found in guard columns or as a repair material for deteriorated columns with collapsed packing.

### *d) Perfusive Particles*

They consist of highly crosslinked styrene-divinylbenzene (PS-DVB, Figure V.7) with two types of pores: very large through pores with a diameter of 600–800 nm and narrow diffusion pores of 80–150 nm. The active stationary phase (e.g. a reversed phase, ion exchanger, or affinity phase) fully covers the external and internal surface of the particles. The through-pores are wide enough to allow the mobile phase to flow through, whereas it stagnates in the diffusion pores. The analytes are rapidly transported in and out by the flowing eluent and diffusion paths in the narrow pores are short, therefore separation is fast and the Van Deemter curve is very favourable. It is not necessary to prepare really small particles: the typical diameter of perfusive particles is 20 mm. Their flow resistance is very low.

### *e) Monolithic stationary phases*

It is possible to synthesise stationary phases which consist of one single piece of porous material such as organic polymers or silica. For these phases, the chromatographic bed is not a packing of particles but a porous rod that totally fills the cylindrical volume of the column.

These columns have a bi-porous structure of larger macropores (2.0 µm), which permits high flow rates with low back-pressure and smaller mesopores (13.0 nm); they also provide a high surface area for enough efficiency. The unique features and good advantages of monolithic silica HPLC columns over packed microparticulate columns are the ability to independently control the macro- and mesopore diameters as well as the silica skeleton diameter Such materials have a porosity of more than 0.8, their separation performance is similar to packed beds, and their Van Deemter curve is very favourable.

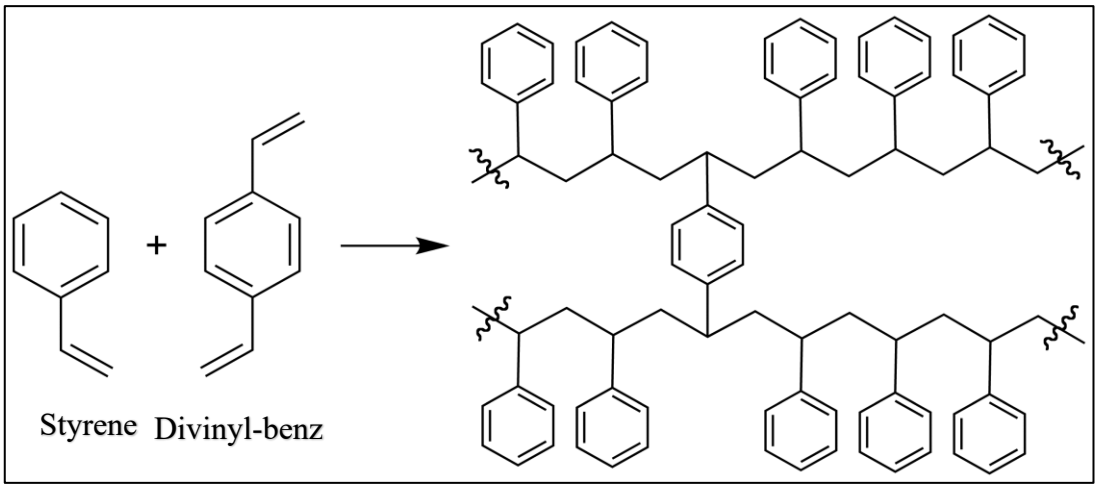



**Figure V.7.** Styrene–divinylbenzene support.

***f) Grafted phases***

***1) Silica*:** Its description has been discussed in Chapter III (TLC part). Silica gel, a highly polar material, exists in the form of spherical particles, sometimes porous, with a diameter of between 2 and 5 µm. This ensures that the column is packed compactly and homogeneously, allowing the mobile phase to circulate evenly without forming preferential pathways in the column. Recently, a new monolithic type has appeared, packed with a porous silica gel that unites to form a single entity (Figure V.6). This material is more permeable to solvents than traditional bead-type stationary phases. The characteristics of silica gel depend on several parameters: internal structure, particle size, porosity (pore size and distribution), specific surface area, crush resistance, and polarity. Silica gels used in HPLC contain around five silanol groups per $\mu m^2$. They can be non-porous or porous (pore size around 10 nm). The volume of the MP varies from 30 to 70% of the total volume of the column.

***2) Chemically modified silica*:** Silica carries OH groups (silanol groups) on its surface and may be chemically modified at these points to give stationary phases with specific properties. These

grafted phases, whose polarity can be precisely adjusted, are used in almost all separations. These modifications to the gel surface led to two types of phases (Figure V.8):

- ***Monomeric phases*** (10-15 µm thick). These are obtained by reacting a monochlorosilane with the silanol functions on the surface. The most commonly used inverse SP are C8 (or RP8) and C18 (or RP18).
- ***Polymeric phases*** (25 µm or more in thickness). This time a di- or trichlorosilane is used.

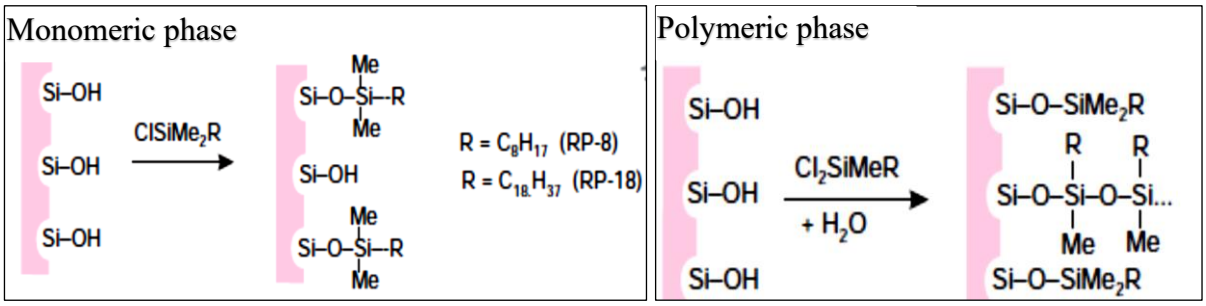



**Figure V.8**. Synthesis of organosilanes grafted at the silica gel interface.

### III.5. Mobile phases

The MPs used are those described for the types of chromatography we have already seen. They may consist of a single solvent or a mixture of several solvents. The MP used must be recognised by:

- Compatibility with the detector.
- Miscibility between solvents (Table 6).
- Solubility of the solutes.
- Low viscosity (η), is given in mPa.s at 20 °C. Solvents with a higher viscosity than water with η = 1.00 Pa.s are less suited for HPLC because the pressure drop will be high.
- Chemical inertness.
- Sufficient eluent force to prevent solute desorption from the SP (see Table 5, case of adsorption chromatography).
- UV cutoff: This is the wavelength at which the absorbance of the pure solvent is 1.0, measured in a 1 cm cell with air as the reference (10% transmittance).

## III.6. Detectors

The detector should be able to recognise when a substance zone is eluted from the column. Therefore, it has to monitor the change in MP composition in some way, convert this into an electric signal, and then convey the latter to the recorder or display where it is shown as a deviation from the baseline.

The ideal detector should:

1. either be equally sensitive to all eluted peaks or record only those of interest
2. not be affected by changes in temperature or mobile phase composition (as happens, for example, in gradient elution)
3. be able to monitor small amounts of compound (trace analysis)
4. not contribute to band broadening, hence the cell volumes should be small
5. react quickly to pick up correctly narrow peaks that pass rapidly through the cell
6. be easy to manipulate, robust, and cheap.

### *a) Detectors exigences*

Hence, detectors are considered in terms of the following characteristics.

***1) Concentration or Mass-Sensitivity:*** Concentration-sensitive detectors produce a signal, S, which is proportional to the concentration, c, of the sample in the eluate:

Mass-sensitive detectors produce a signal which is proportional to the mass flux, i.e. to the number, n, of sample molecules or ions per unit time, $\Delta t$, in the eluate: $S \propto n/\Delta t (g.s^{-1})$

The type of detector used is determined by switching off the pump at the peak maximum, the signal (recorder amplitude) retains its actual value in concentration-sensitive detectors but drops to its initial level (baseline) in mass-sensitive detectors.

***2) Selectivity:*** The detectors may be near universal detectors that will respond to any organic chemical structure to specialty detectors that only respond to a few analytes. Both have their advantages. Universal detectors provide economic use for a large diversity of chemicals but specific analyte detection can be hampered if two or more analytes elute from the analytical column at the same retention time. Specialty detectors provide near-interference free detection of only a few analytes.

***3) Noise:*** Increased amplification of the detector signal shows that the baseline is irregular even when no peak is eluted. High-frequency (short-term) noise may be due to inadequate grounding of the detector and/or recorder or also to amplification electronics. However, a minimum noise

level persists despite good grounding and the use of the most modern equipment. High-frequency noise oscillates with slower, low-frequency (long-term) noise

***4) Detection Limit:*** The smallest detectable signal cannot be less than double the height of the largest noise peak. If the amount injected is even less, then the signal ceases to be distinguishable from noise. For qualitative analysis the signal-to-noise ratio should not be lower than 3–5; for quantitative analysis, it must be higher than 10.

***5) Linear Range:*** The ideal detector gives a signal with an area proportional to the amount injected, irrespective of whether it is large or small. This linearity is not infinite but should embrace as wide a range as possible.

***6) Time Constant:*** The time constant (t), is a measure of how quickly a detector can record a peak. As the detector works together with the data acquisition, the time constant of this combination is an important factor. The time constant can be defined as the minimum time required by a system to reach 63% of its full-scale value (98% is also often specified). It should not be greater than 0.3 s for detecting very narrow HPLC peaks, i.e. those that are rapidly eluted at a volume of less than 100 ml, and should not exceed 0.1 s for high-speed HPLC.

***7) Cell Volume:*** The volume of the detector cell should have a negligible influence on peak broadening and should, therefore, amount to less than 10% of the elution volume of the narrowest (first) peak. A cell volume of 8 ml is standard. Too small a cell volume impairs the detection limit (as a certain amount of product is needed to produce any signal at all). The cell must have no dead corners which may prevent the peak being fully removed by subsequent eluent. Most of the original pressure has gone from the eluate by the time it leaves the column.

### *b) Detector types*

The most common detection methods are based on the optical properties of compounds: absorption, fluorescence, and refractive index. Common detectors, along with their use and approximate detection limits are given in Table 8.

***1) Refractometric detector:*** This comprises a differential refractometer whose purpose is to continuously measure the difference in refractive index between the mobile phase and the column effluent. This detector is considered to be almost universal, but it has the disadvantage of being less sensitive than the UV detector, and of being too sensitive to temperature variations.

**Table 8.** Common Commercially Available HPLC detectors, applications, and detection limits.

| Detector | Application (s) | Detection limits |
|---|---|---|
| UV-Visible Absorbance | For Compounds that absorb in the UV or visible range | pg quantities |
| Fluorescence | For Compounds with fluorophores (especially polyaromatic hydrocarbons | fg quantities |
| Refactive Index (RI) | For alcohol, sugar, saccharides, fatty acid and polymer analysis with refractive indices different from the solvent | ng quantities |
| Electrochemical | For analysing a wide range of compounds | High pg quantities |
| Conductivity fo IC | Mainly for inorganic ions | ng |
| Evaporative light scattering (ELS) | For a wide variety of compounds that lack UV/Visible chromophores and no volatile compounds | µg quantities |
| Fourier Transfer infrared | For compounds with vibrational functional groups | µg quantities |
| Mass spectrometry | A universal detector | µg to pg quantities depending on the type of mass spectrometer |

***2) <u>Absorbance Detectors:</u>*** Many organic compounds with aromatic groups, amide bonds, carboxylic acids, and many other groups can have substantial absorption of light. In addition, some ions, inorganic compounds, and metal complexes can be detected by their absorption of light in the UV or visible range.

Three types of absorbance detectors can be used in liquid chromatography (Figure V.9).

### III.7. Recorder

The signal transmitted by the detector is recorded in the form of spectra (chromatogram) and the integrator calculates the various parameters.

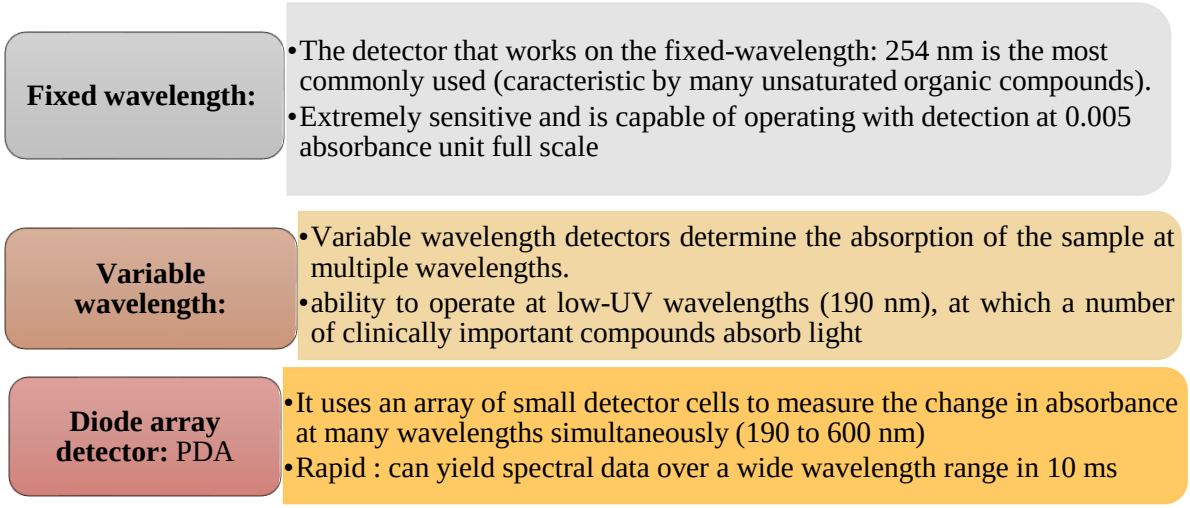



**Figure V.9.** Absorbances detectors used in HPLC.

# Chapter VI: Gas chromatography

## I. Introduction

The separation on the gas chromatography (GC) column is carried out on compounds that must be in the gaseous state or capable of being volatilised by raising the temperature. This technique is limited to the study of thermostable and volatile molecular compounds and the separation requires sample quantities of the order of mg to µg. GC constitutes the most powerful and finest method for separating, identifying, and quantifying gaseous or volatilisable bodies. It thus allows the analysis of possibly very complex mixtures whose constituents can differ considerably in their nature and volatility. Currently, GC devices are coupled with a mass spectrometer for the identification of compounds as they are separated.

Depending on the nature of the SP, two chromatography types exist:

- Gas adsorption chromatography (gas-solid chromatography GSC): if the SP is solid
- Partition gas chromatography (gas-liquid chromatography GLC): if the SP is liquid.

## II. Principle

The method is based on the affinity of the solutes in a mixture concerning the stationary phase (adsorbent solid GSC, liquid film GLC). The mobile phase (inert gas) has no affinity for solutes.

Temperature is the only important modification parameter. Furthermore, the viscosity and velocity of the gas in the column influence the dispersion of the compounds in the stationary phase and the diffusion in the mobile phase (Van Deemter equation) and consequently on the efficiency parameter N and the sensitivity of detection.

Same as for the LC:

- 

- 

## III. Equipment

The GC equipment mainly consists of three compartments: injection system, column and detection system. The MP is a gas, that drives the injected and vaporised sample through a column containing the SP. The solutes are carried at unequal speeds and the outlet of the column there is a detector connected to a recorder (Figure VI.1).

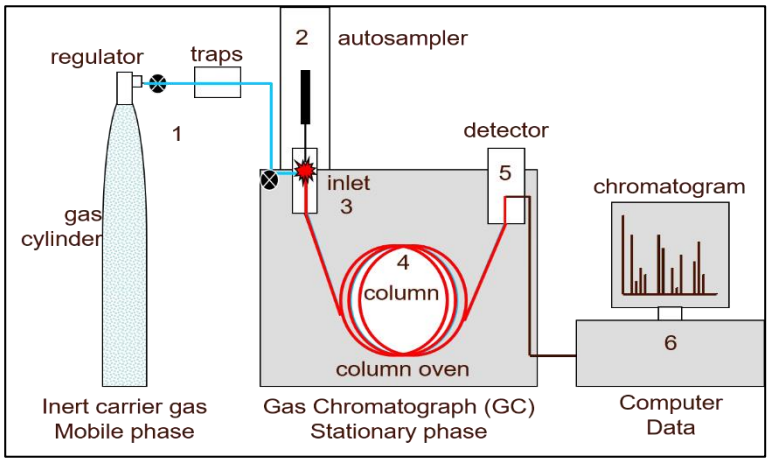



**Figure VI.1.** A gas chromatography apparatus.

### III.1. Carrier gas source

Elution is ensured by a gas, called vector or carrier gas, the most used is helium, and the others are hydrogen, nitrogen, or argon. It must be inert, very pure, dried out and above all contain neither oxygen nor water. Helium (He) is the most used gas, along with argon, nitrogen and hydrogen (the choice of gas depends on the detector used, Catharometer: $H_2$, He; Electron capture: $N_2$ or $H_2$; flame ionisation: $N_2$, $H_2$, He; masse spectroscope: $N_2$, $H_2$, He). It comes from a cylinder under pressure and its flow rate is adjusted by a pressure regulator or pressure gauge, (Figure VI.2). The pressure at the head of the column is a few tens to a few hundred kPa and is stabilised so that the flow rate remains constant. In general, the gas flow rate is 25 to 100 $mL.min^{-1}$ for packed columns and 0.5 to 5 $mL.min^{-1}$ for capillary columns.

### III.2. Injection system: inlet or autosampler

This is an important step in the analysis. Introducing samples too slowly often causes peak broadening and reduces resolution. The characteristics of the injectors as well as the injection

modes differ depending on the types of columns to which they are connected. The injector temperature should be set to cause vaporisation of all analytes in the sample: it is generally maintained at 20 to 50°C above the boiling temperature of the least volatile analyte, (it is greater than that of the column).



**Figure VI.2.** Carrier gas flow regulator in a GC.

Depending on the condition of the sample, the injection is made:

- Using a micro-syringe for liquids and solutions.
- Using a loop valve for gas mixtures.

The choice of injector is dictated by the type of column used (packed or capillary) and by the nature of the products to be separated.

***a) For packed columns***

Injection is carried out hot with direct vaporisation in the body of the injector. Most chromatographs use thermostatically controlled injection chambers. The carrier gas, previously heated, passes through the injection chamber and the sample is injected using a micro-syringe through a septum and is vaporised and mixed with the carrier gas carrying it towards the column. This type of injection has the disadvantage of promoting the decomposition of fragile substances because the temperature of the injector is significantly higher than that of the column, (Figure VI.3.a).

***b) For capillary columns***

Injectors operate in two modes: with or without division (split or splitless: SSL, Figures VI.3.b and VI.3.c) because these columns require low flow and small sample volumes.

***1) In split mode:*** A large portion of the injected sample, vaporised and mixed with the carrier gas, is removed from the injector through a leaking valve. This leak valve separates the gas stream into two parts, the smallest of which is the only one to enter the column. A device regulates the leak rate (generally between 50 and 100 mL.min$^{-1}$), and the division factor varies between 1/20$^{th}$ and 1/500$^{th}$. The disadvantage of this injection method is the selective evaporation of the most volatile compounds, with modification of the composition of the injected sample.

***2) In splitless mode*:** This injection mode is reserved for trace analysis. The sample is injected with the leak valve closed for 0.5 to 1 minute so that the compounds vaporise at the same time as the solvent concentrates on the first centimetres of the column. Opening the leak valve then eliminates from the injector the less volatile compounds that would harm the analysis.

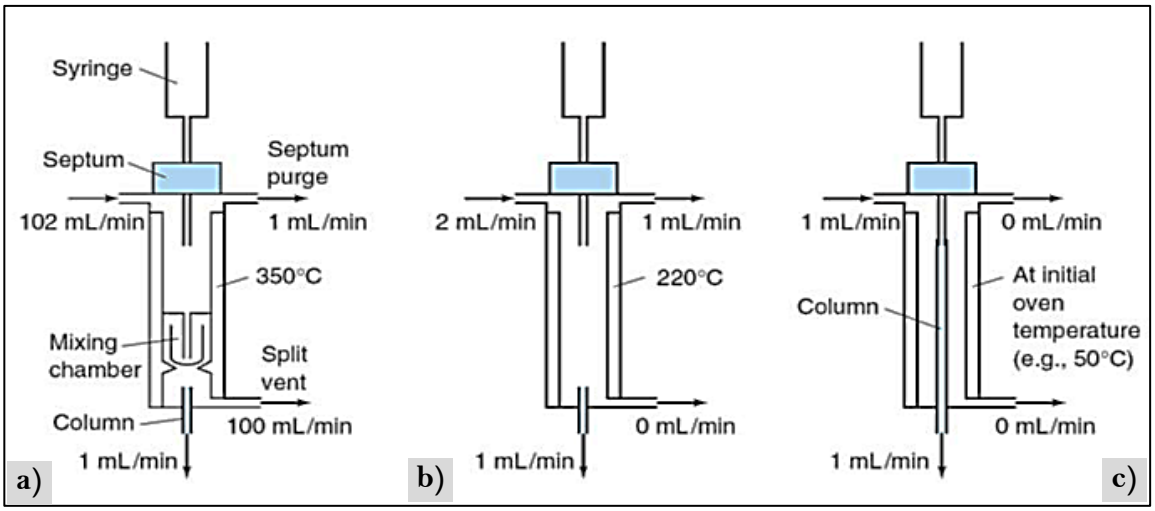



**Figure VI.3.** Injections port operation for a) split, b) splitless, and c) on-column injection into an open paked column.

**Remarks:** The SSL inlet can also operate in splitless mode, where significantly more sample vapor flows onto the column. This mode yields increased sensitivity for trace-level analysis at the cost of increased peak broadening and thermal degradation of unstable analytes due to longer residence time of the sample in the inlet.

Other common gas chromatography inlets include:

- Programmable temperature vaporiser (PTV), which is similar to SSL but enables rapid ramping of the inlet temperature during the injection.
- Cool on-column (COC), where the needle enters and injects the entire sample directly onto the head of the column at a lower temperature to further protect against possible thermal degradation and facilitate trace-level analysis.
- Multimode Inlet (MMI) that combines the flexibility of a standard split/splitless inlet with the capabilities of a PTV inlet.

**III.3. Column oven**

It is a thermostatically controlled enclosure that allows the temperature of the column to be controlled down to a few tenths of a degree. The column oven can operate in two ways:

***a) At constant temperature***

The temperature of the column is kept constant throughout the separation. The optimum column temperature for isothermal operation is approximately at the midpoint of the boiling range of the sample. However, this mode works best if the boiling point range of the sample is narrow.

***b) With programming temperature***

This method is well-suited to separate a mixture with a wide boiling point range.
In this case, the column temperature is either continuously or in stages as the separation progresses. The analysis starts at a low temperature to separate the low-boiling compound and increases during separation to separate the less volatile compounds. Rates of 5 to 7°C/minute are typical for temperature programming separations.

**III.4. Columns**

There are two types:

***a) Packed columns***

These columns, with a diameter of 2 to 15 mm and 1 to 4 m long, are made from a steel tube glass or other metal. They support a carrier gas flow rate ranging from 10 to 40 $mL.min^{-1}$. They contain, continuously, a bed of porous and inert granules. These are spherical grains with a

specific surface area of 2 to 8 $m^2.g^{-1}$ from 0.5 to 300 µm in diameter. The SP is immobilised by impregnation or by chemical reaction with the porous support. (Figure VI.4).

SP materials are marketed under the name:

• *Chromosorb*®: These are fossil silicates (diatomaceous earth) whose skeleton is chemically comparable to amorphous silica.

• *Spherosorb*®: It is a synthetic material made up of small silica beads. The presence of numerous silanol groups gives all these supports a reactivity comparable to that of silica gel.

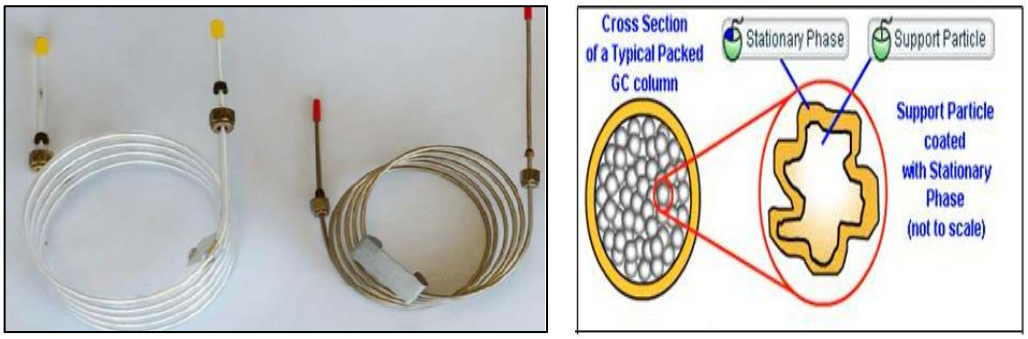



**Figure VI.4.** Packed column for GC.

***b) Open tubular columns, or capillary columns***

The internal diameter of these columns ranges from 0.1 to 0.35 mm ($d_{SP}$ = 0.1-5.5 µm) and the length from 15 to 100 m spirally wound. These columns are empty tubes inside which the SP is deposited on the internal wall in the form of a regular film. They are of types, (Figure VI.5):

***1) WCOT (Wall Coated Open Tubular):*** When the stationary phase is a thin layer of a liquid film coated along the column wall.

***2) PLOT (Porous Layer Opent Tubular):*** When the stationary phase is a porous solid layer (adsorption chromatography).

***3) SCOT (Support Coated Open Tubular):*** When the stationary phase is coated with a thin layer (about 30%µm thick) which is treated with the liquid stationary phase.

The walls of capillary columns are drawn from purified silica containing minimal metal oxides. These columns are much thinner than glass columns, with diameters as small as 0.1 mm and lengths as long as 100 m.

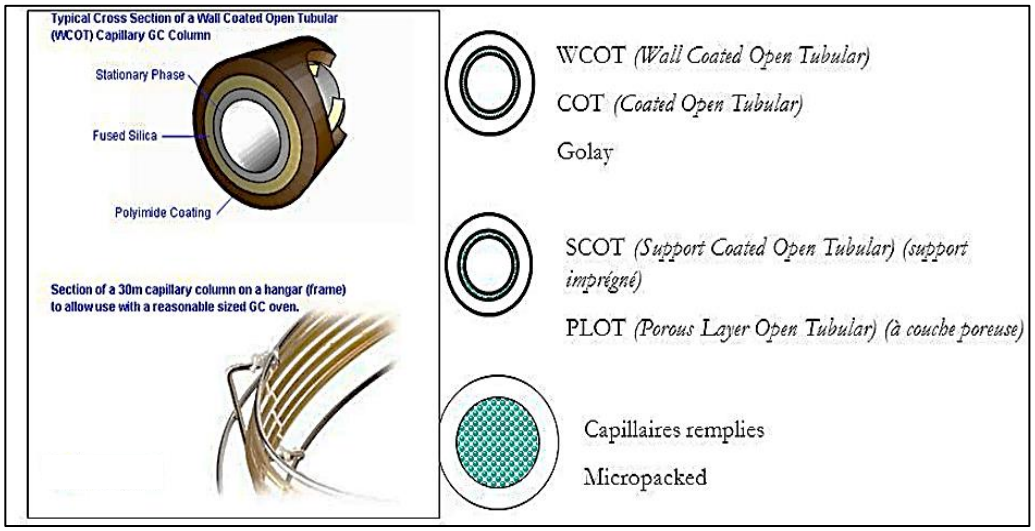



**Figure VI.5**. Capillary GC columns.

To protect the column, a polyimide coating is applied to the outside of the tubing and bent into coils to fit inside the thermos-statted oven of the gas chromatography unit. The columns thus obtained are very flexible and can be wound into a spiral of around ten centimetres. The internal wall of the silica undergoes various treatments which depend on the technique of fixing the stationary phase which will consist of a uniform film of liquid a few tenths of a micrometre thick.

They have the following properties:

- Greater column efficiencies, it is possible to achieve up to $4.10^5$ theoretical plates with a 100 m WCOT column, yet the world record for the largest number of theoretical plates is over 2 million plates for 1.3 km section of column.
- Higher speed, (shorter analysis time).
- No packed particles in the column (very low loss in pressure).
- Cost, fragility (breakage, oxidation), low capacity.
- Variable polarity.

## III.5. Stationary phases

Stationary phases must meet a certain number of requirements:

1. They must have high selectivity to interact selectively with different solutes
2. They must cover a wide temperature range as possible (-60 to 400 °C).
3. They must have a low vapor pressure as possible
4. They must be thermally stable
5. They must be chemically inert
6. They must have low viscosity under working conditions
7. They must wet the sample by adhering effectively so that they do not entrain with the MP.

Although separations are made at high temperatures, the most commonly used SP are high molecular weight liquid polymers.

Impregnation techniques are very simple for packed columns, more than 100 SPs of different types have been proposed in the literature.

For capillary columns, the most used SP are:

***a) For GLC***

The liquid is fixed by impregnation or by grafting onto solid particles or onto an inert support which can also be the wall of the column. The current phases correspond to two main types of compounds:

***1) Polysiloxanes or silicones:*** They are characterised by high thermal stability and the possibility of chemically modifying the basic structure to obtain phases of different polarities and selectivity. There are approximately 20 different compositions of alkyl or aryl chains (methyl or phenyl) into, which other functional groups (cyanopropyl, trifluoropropyl), can be incorporated (Figure VI.6).

***2) Polyethylene glycols (PEG):*** These SP are very useful for the separation of polar compounds by the formation of hydrogen bonds. They are prepared by the polymerisation reaction of ethylene oxide. Like polysiloxanes, PEGs can be subject to structural modifications (Figure VI.7).

The retention characteristic of this SP is the content of OH groups, while the molecular weight is responsible for thermal stability. These polar polymers (M = 1500 to 20,000 g.mol$^{-1}$ for Carbowax 20M) can be used for deposition, impregnation or as bonded phases (40 < T < 240-260 °C, depending on the diameter of the column and the thickness of the film). The disadvantage of these SPs is their ease of oxidation.

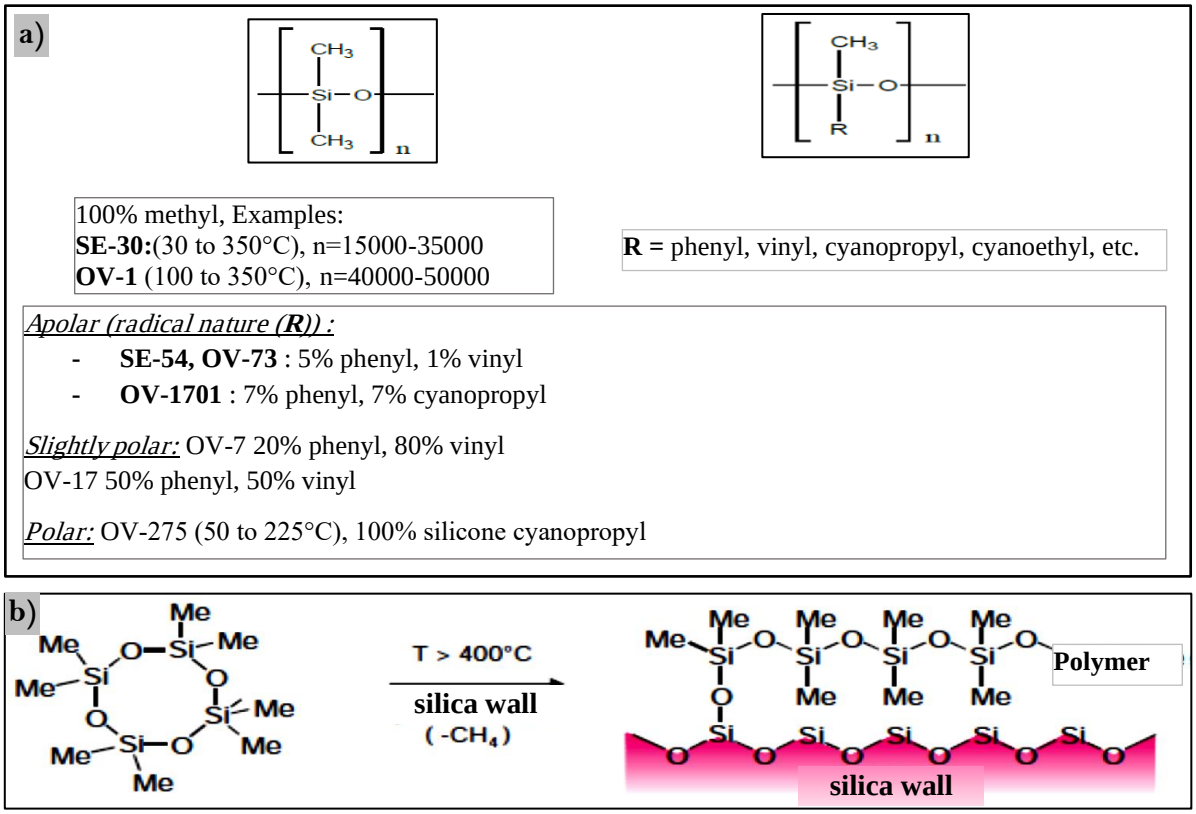



**Figure VI.6.** Structure of polysiloxanes (silicones): a). Example of formation of a grafted phase: b). Non-polar SP SE-30: polydimethylsiloxane film grafted onto the internal surface of the fused silica capillary tube: c)

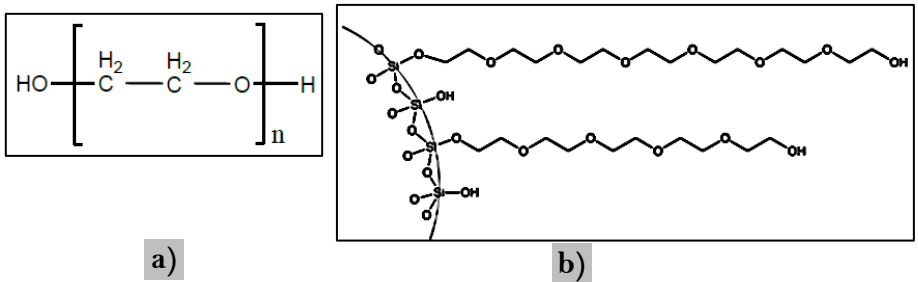



**Figure VI.7.** Structure of polyethylene glycols (PEG): a) and Carbowax type SP: b).

***b) For GSC***

The GSC is less used than the GLC, this is due to some factors:

1. Incomplete elution of certain solutes and appearance of asymmetric peaks, due to the dependence of the retention volumes on the quantity of sample (non-linear adsorption isotherms.
2. Many adsorbents exhibit catalysis properties at column operating temperatures.
3. Excessively long retention time due to the high specific surface areas of the adsorbents.

In the case of porous layer capillary columns (PLOT), the main SP used are adsorbents: carbon graphite, silica gel, or alumina, deposited in the form of very fine particles on the internal wall of the capillary columns.

They are used to separate gaseous or very volatile compounds. Graphite phase columns have been developed for the separation of $N_2$, CO, $CO_2$, and very light hydrocarbons.

**III.6. Samples**

Sensitivity limits vary depending on the device, they are in the nanogram or even picogram range. Sample processing varies depending on the substances analysed:
- When the solutes are directly volatilisable, the substances are solubilised in a very volatile solvent and then chromatographed.
- When the solutes are not volatile at the temperature of the chromatograph or are decomposed at this temperature, they must be transformed into stable volatile derivatives: the amino acids are thus esterified by butanol, the fatty acids esterified by methanol, the oses reduced to alditols, then acetylated, etc.

**III.7. Detectors**

The solutes modify a chemical or physical property of the carrier gas and these variations are transformed by the detector into electrical signals which are amplified and transcribed in the form of a graph. An ideal detector should have the following characteristics:

- Good sensitivity.
- Good stability and reproducibility.

- Linear response over several decades of concentrations.
- Operating temperature range between ambient temperature and at least 400°C.
- Fast response time and independent of flow speed.
- High reliability and ease of use.
- Uniform or selective response for different solutes.
- Preservation of sample integrity.

***a) Terminologies***

***1) Detection limit (DL):*** it is defined by the quantity of solute required to have a signal-to-noise ratio at least equal to 3.

***2) Limit of quantification (LQ):*** it expresses the quantity of solute required to have a signal-to-noise ratio at least equal to 10.

***3) Linearity domain (LD):*** it is a domain in which the response of the detector is directly proportional to the concentration.

***4) Selectivity:*** it is the property of the detector which must not respond in the same way to all species, it characterises the ability to analyse specific molecules.

***5) Sensitivity:*** a detector can respond to small variations in concentration or quantities.

A variety of detectors can be used in GC systems (Table 9). These include universal detectors that can detect a broad range of analytes and more selective devices that may detect only specific groups of analytes.

***b) Types***

***1) Flame ionisation detector (FID):*** During the operation of a FID, the carrier gas that is leaving the column is mixed with hydrogen, and the eluting compounds are burned by a flame that is surrounded by air and an oxygen-rich environment. Approximately one organic molecule in $10^3$ results in the production of a gas-phase ion. These ions are detected by a collector electrode that is positioned above the flame. The magnitude of the current that is generated by these ions is related to the mass of carbon that was delivered to the detector. This signal can then be used for both the detection and quantification of organic compounds that are eluting from the column, (Figure VI.8).

**Table 9** Somes detectors used in GC.

| Type of detector | Principle of operation | Selectivity | Approximate of the detection limit |
|---|---|---|---|
| Flame ionisation detector (FID) | Production of gas phase ions from combustion of organic compounds | General : Organic compounds | $10^{-12}$ g |
| Electron capture detector (ECD) | Capture of electrons by chemicals with electronegative groups | Chemicals with electronegative groups | $10^{-15}$-$10^{-13}$ g |
| Mass spectrometry (MS) | Production of gas phase ions, followed by separation/ analysis of these ions based on their mass-to-charge ratios | Universal: Full-scan mode Selective: Selected ion monitoring mode (SIM) | $10^{-10}$-$10^{-9}$ g Full-scan mode $10^{-12}$ - $10^{-11}$ g SIM mode |
| Thermal conductivity detector (TCD) | Measurement of change in thermal conductivity of carrier gas as compounds elute from the column | Universal | $10^{-9}$ g |
| Photoionisation detector | Measurement of gas phase ions that are produced due to chemical ionisation with light | General: Organic compounds | $10^{-12}$ - $10^{-11}$ g |
| Infrared (IR) spectroscopy | Absorption of IR light | IR-absorbing compounds | $10^{-9}$ g |
| Flame photometric detector (FPD) | Phosphorus- and sulfur-containing compounds emit light when burned in a flame; emitted light is detected | Phosphorus and sulfurcontaining compounds | $10^{-12}$ g phosphorus $10^{-11}$ g sulfur |

The different of flame characteristics ionisation detectors are detailed in table 10.

***2) Thermal Conductivity Detector (TCD) or katharometer:*** The katharometer is a simple and non-destructive device, with a universal response, but it is not very sensitive. A TCD is a general detector that can monitor both inorganic and organic compounds.

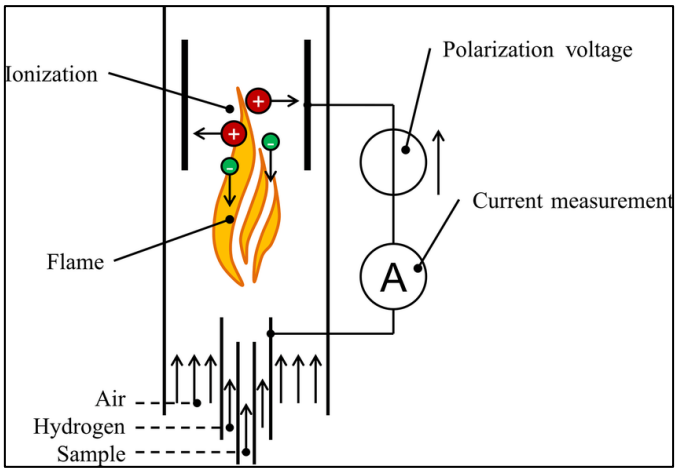



**Figure IV.8.** Flame ionisation detector.

**Table 10.** FID Detector characteristics.

| Property | Evaluation |
|---|---|
| Linearity | $10^6$ |
| Stability | Excellent |
| Temperature limite | 400 °C |
| Carrier gas | $N_2$, He, $H_2$ |
| Simplicity, reliability, versatility, and ease of operation | Affected |

It detects and measures inorganic and organic compounds based on their ability to change how the carrier gas/ analyte mixture will conduct heat away from a hot wire filament (a property referred to as thermal conductivity). The carrier gas used with a TCD is often helium or hydrogen, which have the greatest differences in their thermal conductivities from most organic or inorganic compounds. However, nitrogen or other carrier gases also can be used with a TCD.

***3) Mass spectrometers***: Mass spectrometers are also used as detectors for GC. This combination is known as gas chromatography/mass spectrometry (GC/MS). GC/MS is a powerful method for identifying analytes and quantifying them as they elute from a GC column. Ionisation methods that are often used in GC/MS include electron impact ionisation and

chemical ionisation. Some mass analysers that are commonly used in GC/MS are quadrupole mass analysers and ion traps, although other types of mass analysers can be used as well.

## IV. Effect of mobile phase speed on column efficiency

The viscosity and speed of the gas in the column influence the dispersion of the compounds in the SP and on the diffusion in the mobile phase (Van Deemter equation), therefore on the efficiency parameter N and the sensitivity of detection (Figure. IV.9).

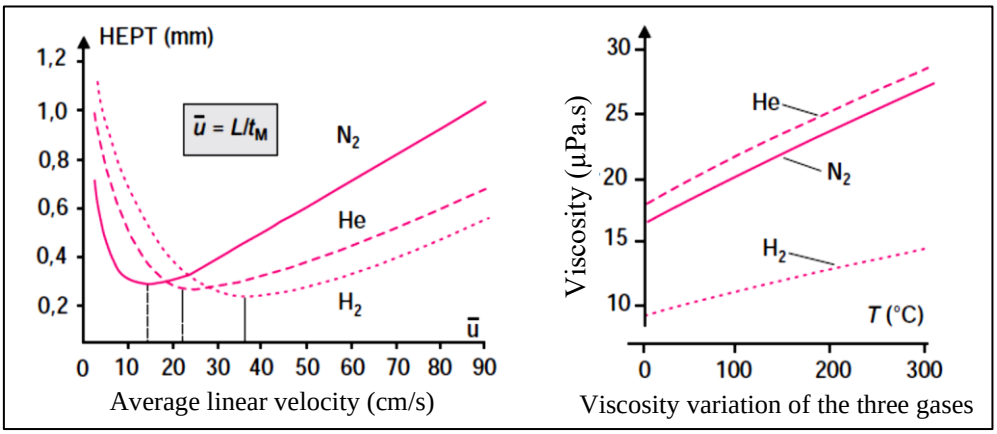



**Figure VI.9.** Influence of carrier gas type and flow rate on column efficiency

In GC, the A, B and C terms appearing in Van Deemter's law ((expression I.16) are defined as follows:

### IV.1. A term, [m]

Is related to the size of the particles, their dimensional distribution, and the regularity of the packing (parameter λ), (Expression (VI.1)), which are at the origin of preferential paths which can lead to imperfect exchanges between the two phases. (This is the turbulent diffusion factor, considered unimportant in liquid chromatography and absent by for capillary columns).

$$A = 2.\ \lambda \cdot dp \qquad (VI.1)$$

Because of this factor, many HPLC column manufacturers have concentrated their efforts in producing packed columns with smaller particle sizes, and more uniform shape and size distribution throughout the column. However, one consequence of reducing the particle size is that the pressure drop across the column per unit length is also increased. hence high performance liquid chromatography has also been referred to as high pressure liquid chromatography.

### IV.2. B term, [$m^2.s^{-1}$]

It relates to the diffusion of the molecules in a longitudinal direction and describes the band broadening processes due to the random motion of the solute molecules in the mobile phase as they travel through the column, (expression (VI.2)).

$$B = 2.\tau.D_G \qquad (VI.2)$$

**With:**

$D_G$: Diffusion coefficient of the analyte in the MP, ($m^2.s^{-1}$)

$\tau$ : Tortuosity factor, to be considered especially if the MP is a gas (main cause of peak broadening).

The effect of the B term is flow-dependent and also dependent on the nature of the mobile phase and the analyte under study. At lower flow rates the effect of longitudinal diffusion is more pronounced, resulting in a greater band broadening of the peak. This effect has a greater significance in gas chromatography since the diffusion coefficients in liquid chromatography are an order of magnitude smaller than in gas chromatography. Since this term is dominant at low flow rates it is generally not considered a limitation within liquid chromatography, as most analysts would like shorter analysis times.

B is inversely proportional to the average linear velocity ($\bar{u}$), (less diffusion when the analyte stays less long in the column).

### IV.3. C term

Is relates to the mass transfer of the analyte through the mobile phase and to the stationary phase, and any subsequent diffusion on the surface. Under laminar flow in an open tubular system, flow at the sides of the tube is slower due to greater frictional forces, resulting in a parabolic flow profile and hence band broadening. Unless there is a dynamic equilibrium between the radial movement of fluid and the movement of fluid longitudinally through the column, a parabolic flow profile for an open tubular system, given in Figure VI.10, will exist. Thus, fluid at the surface of the open tubular system will be effectively stationary, with the velocity of the fluid increasing as the central flow path is approached, due to the drag effect of the many parallel fluid paths. Since diffusion is a slow process, increasing the fluid flow will

exaggerate any discrepancies in the flow profile. This results, in a chromatographic environment, in peak tailing at higher laminar flow rates. Thus, although the components will elute quicker at higher flow rates, because of the hydrodynamics, peak tailing will result.

These typical Van Deemter curves (Figure IV.9) show that hydrogen is, among the three gases studied, allows the fastest separations, with equal performance, while giving more flexibility with regard to the flow rate, which is very useful in temperature programming mode.

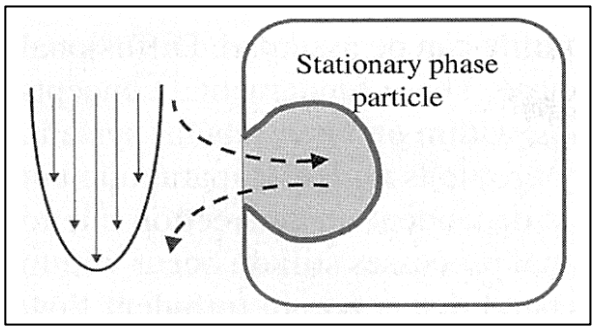



**Figure VI.10.** Schematic representation of the C term in the Van Deemter equation.

## V. Applications

There are numerous applications of GC in all fields such as perfumery, enology, environmental research, the oil industry, biology, the food industry, fine chemicals, and the plastics industry. The development of high-speed or multidimensional gas chromatography makes this technique even more attractive. It's very high sensitivity makes it possible to detect quantities of the order of picograms for certain compounds.

# Chapter VII: Electrophoresis

## I. Introduction

Electrophoresis is the most useful separation technique in molecular biology and biochemistry for the analysis, purification, and characterisation of complex charged biological molecules such as proteins and nucleic acids. It is a very powerful and easy-to-use technique for separating macromolecules of interest.

The term "electrophoresis" is composed of:

- The prefix "electro" for electricity, from the Greek "èlektron", meaning yellow amber.
- The root: "phoresis" from the Greek phoros, meaning to carry from one side to the other.

Electrophoresis is defined as a method of separation and analysis (which can be qualitative or quantitative), generally preparative, based on the differential displacement of electrically charged particles under the influence of an electric field. The particle may be simple ions, complex macromolecules and colloids or particulate matter- either living cells such as bacteria or inert material such as oil emulsion, droplet and clay.

## II. Principle

If a charged particle is subjected to a potential gradient, the foreign phase migrates through the continuous medium towards the cathode or anode depending on the sign of the charge on the particles. This phenomenon is called electrophoresis. Migration can also occur in a non-uniform alternating current, provided that the particles are polarisable, and its transport phenomenon is called dielectrophoresis. Some techniques result in complete separation of the component into distinct zones; this is known as zone electrophoresis.

Electrophoretic migration depends on the following parameters:

### II.1. Electrophoretic mobility, (μ)

It characterises the speed of migration and depends on the charge and shape of the particle. By applying an electric field E to a particle that has an electric charge q, the particle is subjected to a force F that causes it to move towards the electrode of the opposite sign (expression (VII.1)):

$$F = q.E \qquad \text{(VII.1)}$$

During migration, the particle is subjected to a frictional force, $F_f$, (expression (VII.2):

$$F_f = \mathbf{6\pi}.\eta.\mathbf{r}.\mathbf{v} \qquad \text{(VII.2)}$$

**With** :

$\eta$ : Fluid viscosity, (Pa.s)

v : Particle migration velocity, ($m.s^{-1}$)

r : Particle radius, (m)

At equilibrium (Figure VII.1), where the rate of migration is constant, we write relationships (VII.3) and (VII.4):

$$Q.E = 6.\pi.\eta.r.v \qquad \text{(VII.3)}$$

Hence:

$$v = \frac{Q.E}{6.\pi.\eta.r} \qquad \text{(VII.4)}$$

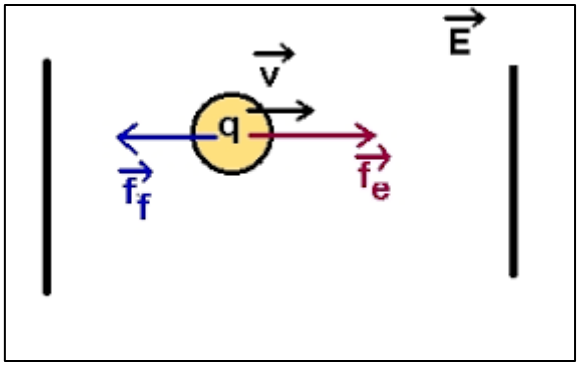



**Figure VII.1.** Movement of the charged particle in an external field.

Electrophoretic mobility is defined by the ratio of expression (VII.5):

$$\mu = \frac{v}{E} \qquad \text{(VII.5)}$$

Hence, electrophoretic mobility ($\mu$) is directly proportional to the charge and inversely proportional to the viscosity of the medium, size and shape of the molecule. In the case of relative mobility, it is directly related to the charge/radius of the molecule. For a globular protein, the radius (r) of the molecule is related to the molecular mass of the macromolecule.

The relative mobility, ($\mu$') is as follows (relation (VII.6)):

$$\mu' = \frac{Charge}{\text{Masse}} \qquad \text{(VII.6)}$$

And it's given by relation (VII.7)

$$\mu = \frac{q}{6.\pi.\eta.r} \qquad \text{(VII.7)}$$

Electrophoretic mobility is independent of the electric field and depends on the properties of the particle to be separated.

The charge q is a function of the isoelectric pH of the particle and the pH of the solvent. The difference between these pH values defines the intensity and sign of the charge q:

- When : pH > pHi : q < 0 : (anion) - Anode migration
- When : pH < pHi : q > 0 : (cation) - Cathode migration
- When : pH = pHi : q = 0 - No migration

**II.2. Electric Field E**

It is expressed by relationship (VII.8)

$$E = v/\mu \qquad \text{(VII.8)}$$

**II.3 Liquid current**

***a) Electroendosmosis current***

The support can have a net overall charge, which causes particles to accelerate or decelerate towards either the anode or the cathode.

***b) Evaporation current***

During the passage of the electric current, the substrate heats up, causing the water in the liquid phase to evaporate and a liquid current to be established at each end towards the centre of the strip. To limit this phenomenon, the tank is closed and cooled.

**II.4. Migration time**

It affects the migration distance according to relationship (VII.8):

$$d = v.\ t \qquad \text{(VII.8)}$$

**With:**

d: Distance covered, (cm)

v: Migration velocity, ($cm.s^{-1}$)

t: Current flow time, (s)

In the case of electrophoresis on a support, the previous formula is not applicable, as the molecules follow a non-linear path through the microchannels of the support.

### II.5. Factors associated with the nature of the base material

The nature of the support affects particle migration through adsorption and the texture of the support.

## III. Types of electrophoresis

### III.1. Zone electrophoresis

#### *a) On a thin film*

The most commonly used supports are in solid form: paper, cellulose acetate, or silica. Applied for the separation of amino acids, peptides, proteins, and nucleotides.

#### *b) On gel*

Gels take the place of porous supports. Gels are characterised by their hydrophilic, semi-colloidal properties and their insolubility in water, which gives them a wide range of uses (resolution). The gels used are starch, agarose, and polyacrylamide (Figure VII.2).

***1) Polyacrylamide gel electrophoresis (PAGE)*** : The monomer (acrylamide: ($CH_2$=CHCO-$NH_2$) is polymerised with a cross-linking agent (N,N' -methylenebisacrylamide : $CH_2$(NH-CO-CH=$CH_2$)$_2$)) to form polymeric network.

These gels are characterised by a percentage of acrylamide that varies between 3 and 30% (giving different pore diameters), which allows molecules with a molecular weight of $10^6$ to $10^4$ to be separated (Table 11). The gel has a low absorption capacity and does not absorb UV light.

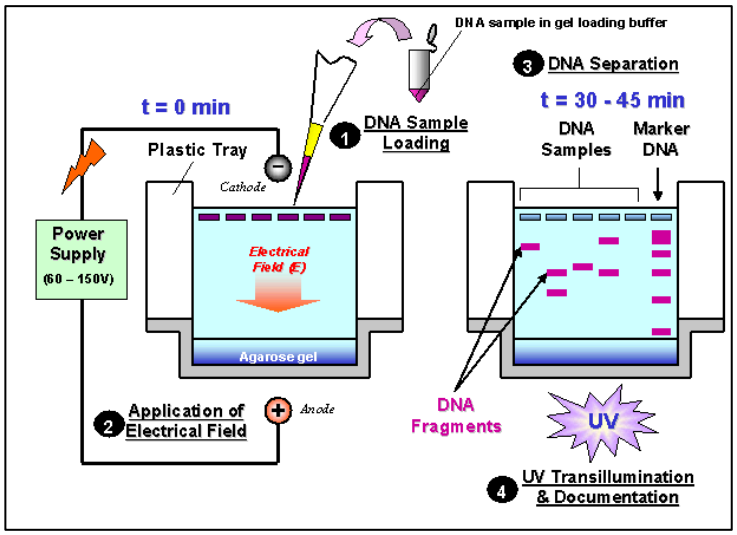



**Figure VII.2.** Gel electrophoresis.

**Table 11.** Polyacrylamide content as a function of the MW

| Polyacrylamide (%) | Separation range (kDa) |
|---|---|
| **7.5** | 40-400 |
| **10** | 20-300 |
| **12** | 15-200 |
| **15** | 6-90 |

The method is summarised as follows (Figure VII.3):

Samples are placed in pre-formed wells at the top of the gel and the buffer is the same in the top and bottom wells (basic pH determined so that all proteins have the same charge -), a direct current of 100 to 200 volts flows through the gel during the migration time.

- The proteins will migrate towards the anode (+) according to their charge/mass ratio.
- After migration, the gel is removed and the protein bands are visualised by revelation.

***2) SDS-PAGE:*** Commonly used for separation and determination of protein molecular weights.

SDS: Sodium dodecyl sulphate or sodium lauryl sulphate is an anionic surfactant (Figure VII.4) that binds strongly to proteins and denatures them: SDS forms a micelle whose charge is $\propto$ to the length of the peptide chain, the strong overall negative charge provided by SDS masks the intrinsic charge of proteins, so the charge/mass ratio will be the same for all proteins of the same mass and the electrophoretic separation of the gel with SDS will depend solely on the gel-filtration phenomenon through the pores of the gel.

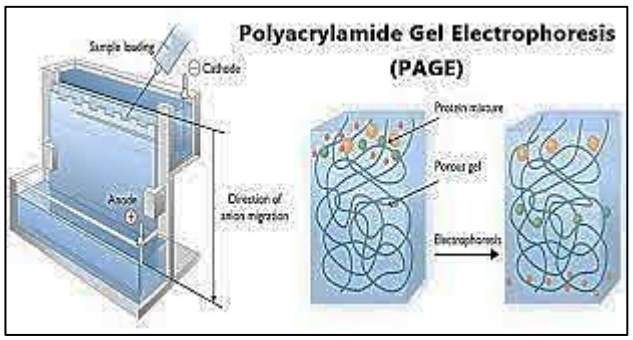



**Figure VII.3.** Polyacrylamide Gel Electrophoresis

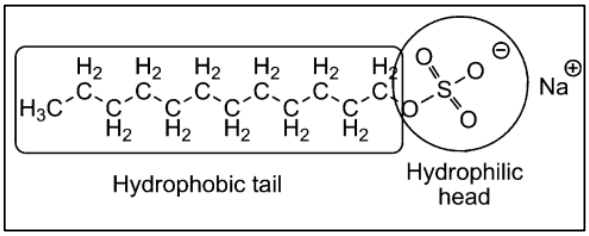



**Figure VII.4.** A molecular structure of sodium dodecyl sulphate.

Procedure of SDS-PAGE is summarised as following steps (Figure VII.5):

- Gel preparation
- Sample preparation and protein denaturation
- Electrophoresis: injection, analysis and detection

Depending on their size, each protein moves differently through the gel matrix; short proteins move more easily fit through the pores in the gel, while larger ones move with more difficulty. After the separation is over, the gel is gently taken out and transferred to the staining box and treated with the staining dye (Coomassie Brilliant Blue R-250). Excess stains are removed by de-staining using an acetic acid solution. The bands appear to be blue-stained, which are then analysed according to the needs of the experiment.

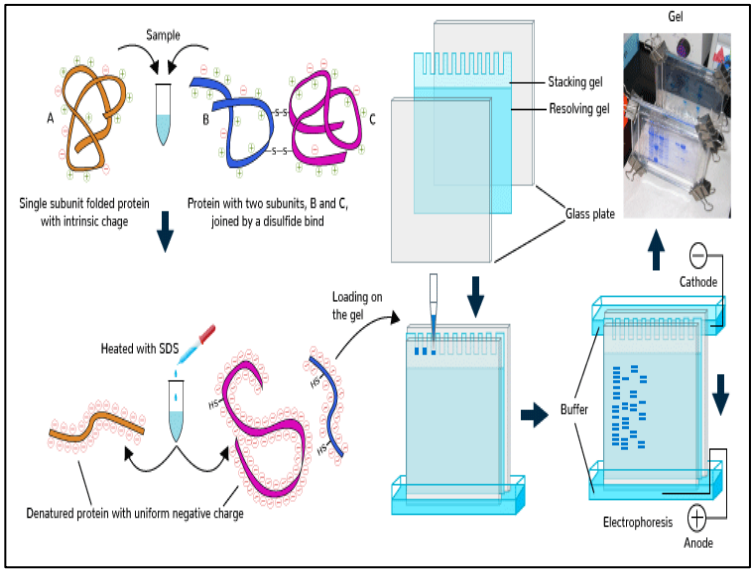



**Figure VII.5.** Gel electrophoresis SDS/PAGE process.

Calculation of molecular weight of the unknown protein sample:

- Resolve the protein sample on the SDS-PAGE along with the molecular weight markers.
- Calculate the relative mobility $R_f$ using the following formula (VII.9):

$$R_f = \frac{\text{Migration of protein from thhe lane}}{\text{Migration of tracking dye}} \qquad \text{(VII.9)}$$

It has been shown that a linear relationship can be obtained between relative electrophoretic mobility and the logarithm of molecular weight (Figure VII.6).

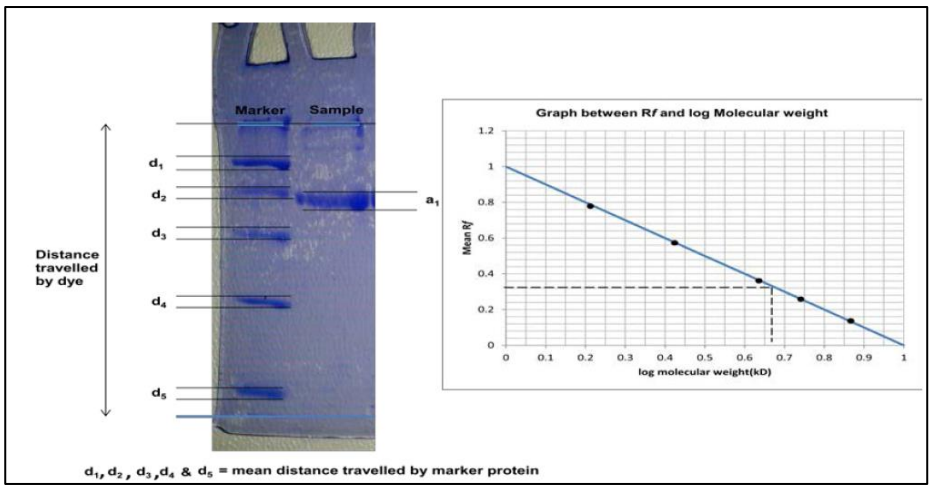



**Figure VII.6.** Molecular weight Estimation using gel electrophoresis SDS/PAEG.

### III.2 Isoelectric focusing electrophoresis

Amphoteric biological molecules are separated by a pH gradient where the anodic region has a lower pH than the cathodic region. When subjected to an electric field and below their isoelectric point, the analytes are positively charged and migrate towards the cathode where they encounter a medium which, at a given pH, behaves like a zwitterion: migration stops and bands form for the different pHi.

Under the effect of a high voltage (~1kV), the stationary bands formed by the analytes focus for a period of several tens of hours (Figure VII.7).

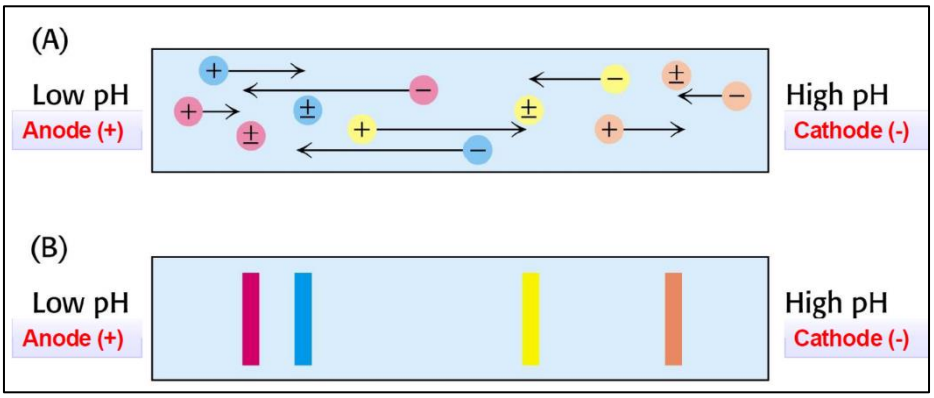



**Figure VII.7.** Isoelectric focusing electrophoresis.

## IV. Applications

- DNA analysis
- Explosives chemical and residue analysis
- Protein detection
- Analysis of drug abuse:
- Test for antibodies
- Vaccine testing

# Chapter VIII : Coupling chromatographic methods

## I. Introduction

Today, chromatography covers a range of different techniques, all based on the principle of the selective retention of the constituents of a mixture on a fixed medium (capillary or packed column, plate, etc.), transported towards the outlet by a mobile fluid (gaseous, liquid, supercritical). This fluid can be set in motion either by the action of a pressure difference, or by the action of an electric field, or by a combination of the two.

Modern instrumentation is used for increasingly complex analyses, due to the large number of constituents present and the extremely small quantities to be detected. However, these analyses must meet the requirements of rapidity, sensitivity and reproducibility, and must therefore be automated in order to acquire more or less accurate results for a large number of samples.

The most widely applied coupling methods are those involving liquid and gas chromatography with mass spectroscopy. These couplings generally have the particularity of responding to a number of criteria, including the limit of detection (up to pico-metres) and the complexity of the analysts in terms of number and chemical nature.

## II. Coupling architecture of Chromatography-Mass spectrometry

Chromatography-mass spectrometry coupling is designed to create an analytical methodology that allows the collection in a series of several sub-units through which the samples to be separated and analysed pass in succession.

Combining the chromatographic part with the mass spectrometer part poses a number of specific problems that do not arise when each of the two parts is used separately. The solution is to find a balance that allows each unit to operate under optimum conditions. For a long time, the design and operation of the interface involved adding a third compartment. This notion of a separate interface is not clear in modern integrated devices.

Two types of coupling can be used:

- On-line coupling is applied when the operator does not intervene at the interface between the chromatograph and the mass spectrometer source.

- Off-line coupling is used when there is no practical way of making a direct transition. The operator, or a robot, must be involved to carry out a manipulation. This is the case for chromatographic methods on planar supports, or mass spectrometry methods using desorption of a surface or solid matrix (MALDI: Matrix-Assisted Laser Desorption Ionisation).

## III. Coupling chromatographic methods

These methods are numerous and various, and some specialists use the terminology ‘separative sciences’ rather than chromatography.

These methods include:

- Gas chromatography (GC)
- Liquid chromatography (LC)
- Supercritical phase chromatography (SPC)
- Planar (or thin layer) chromatography
- Steric exclusion chromatography (SEC)
- Capillary electrophoresis (CE)
- Micellar electrokinetic chromatography (MEKC)
- Capillary isotachophoresis (CITP).

In recent decades, all these separative methods have been coupled to mass spectrometry, and some of the most viable devices have been commercialised, while others remain laboratory equipment. Currently, the field of separative sciences includes sample preparation techniques that make it possible to take a selective sample from a medium, or to transfer a well-defined part of the samples to another piece of equipment:

- Direct injection into a stream (DIF)
- Solid phase microextraction (SPE and SPME)
- Selective permeation through membranes (MI).

These sample preparation methods may or may not precede a chromatographic separation stage (GC or LC) coupled to a mass spectrometer. When the separation stage is absent or very simplified, the result is rapid methods capable of processing large numbers of samples, or even ensuring real-time detection of a physicochemical process. These methods can be used for a wide range of applications.

In this case, the addition of a second specific chromatographic detector, either in series between the end of the column and the mass spectrometer or in parallel.

## IV. HPLC-ICP-MS coupling

High-Performance Liquid Chromatography Inductively Coupled Plasma-Mass Spectrometry is one of the most widely used techniques for analysing the chemical forms of trace elements. It is one of the most widely used techniques for analysing the chemical forms of trace elements.

ICP-MS is a high-performance elemental detector that can achieve very low concentrations with good specificity. This detector, coupled online with HPLC, is widely used in speciation analyses for several reasons:

1. It is a specific elemental detector, so the presence of other elements does not a priori interfere with the analysis of species of the element in question
2. Its high sensitivity over a wide range of elements often eliminates the need for post-column pre-concentration or sample pre-treatment
3. Baseline stability is good compared to other detectors and no negative signal can occur, making signal processing easier
4. Its multi-element capability provides more complete information on the content of different chemical elements in complex samples
5. Simultaneous analysis of several isotopes of an element enables the use of quantification methods with a high metrological standard, such as the isotope dilution method
6. Sample introduction rate is compatible with liquid chromatography, making coupling easy to implement. Pour toutes ces raisons et malgré son coût élevé en investissement et fonctionnement, l'ICP-MS est le détecteur de choix après séparation des espèces chimiques par HPLC.

### IV.1. Principle

Inductively Coupled Mass Spectrometry (ICP-MS) is an analytical technique that atomises, excites and ionises metallic and non-metallic species in liquid samples and even isotopes of the same element in very low concentrations (down to one part per billion ($10^{-15}$)), using a high-

frequency inductively coupled plasma. The role of the mass spectrometer is to separate the different ions present and specifically select the analyte and send it to the detector.

## IV.2. Definitions

### *a) Atomisation*

Involves creating small atomic and polyatomic ions.

### *b) Plasma*

Which is often considered to be a fourth state of matter, is defined as a high-temperature (7000 K) gas with a high density of ions and free electrons that is electrically neutral. The induced-current plasma is maintained by energy supplied in the form of an electric field by a radio-frequency generator.

## IV.3. Design of the HPLC-ICP-MS coupling system

The coupling is installed by connecting the outlet of the chromatographic column to the inlet of the nebuliser (Figure VIII.1). This operation is made possible by the compatibility between the output flow rate of the chromatographic column and the input flow rate of a conventional pneumatic nebuliser (~ 1 $mL.min^{-1}$). In addition, in the chromatographic module, the separation mechanisms can be: ion exchange, reverse phase chromatography with or without ion pairing and steric exclusion, allowing the analysis of very different chemical compounds such as redox species, organometallic compounds, biomolecules and xenobiotic metabolites. The main limitation of this type of coupling is the lack of compatibility between MP of the HPLC part and ICP-MS. High levels of salts or organic solvents can lead to disturbances in the nebuliser, plasma and/or cones.

### *a) Source*

The most commonly used plasma is argon plasma since this gas has the following characteristics:

- It does not recombine with chemical species in a stable manner
- It has a very high ionisation potential (15.6 eV), which allows most ions to be excited.

- As a rare gas, it is difficult to ionise and can reach very high plasma temperatures (around 10 000 K).
- It has the advantage of being one of the most abundant gases on Earth.

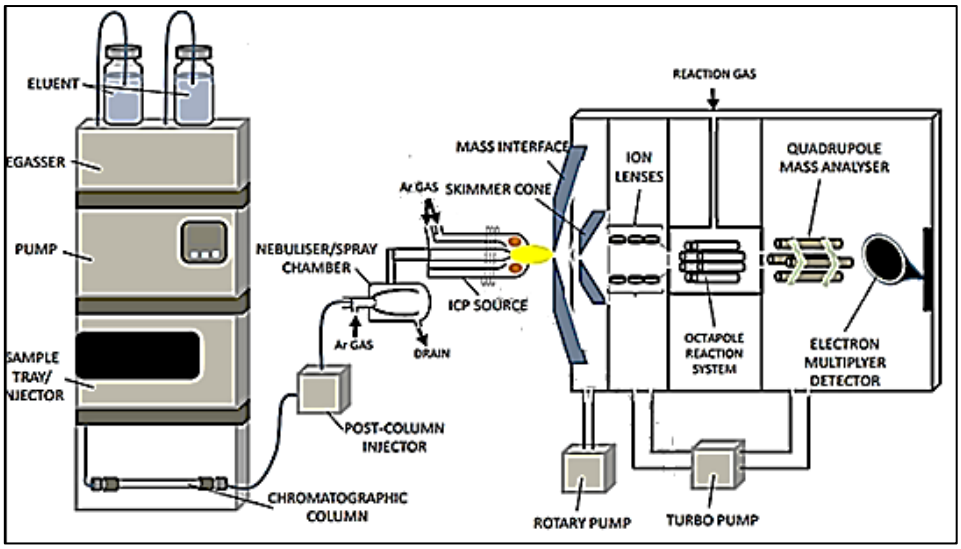



**Figure VIII.1**. General diagram of a HPLC-ICP-MS coupling.

Helium could be used, but has the disadvantage of being expensive. An argon plasma (Figure VIII.2) is made up of argon atoms, argon Ar+ ions and electrons. The disadvantage of argon is that it has poor thermal conductivity, so it is necessary to optimise the parameters to ensure very similar conditions between measurements of different samples, particularly in terms of temperature.

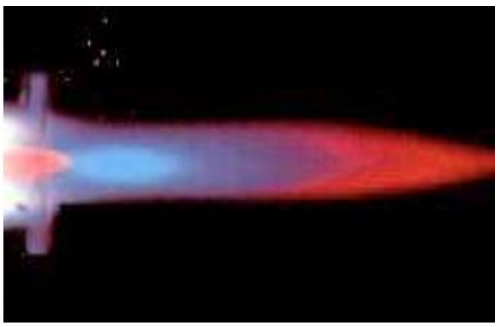


**Figure VIII.2.** Argon plasma.

***b) Interfaces : Sample input system***

Coupling HPLC with ICP spectrometry is a relatively simple process. This is due to the compatibility between the flow rates of the mobile phase used in HPLC and the flow rate of the sample liquid generally used in ICP analysis.

There are different instrumental systems used as interfaces between LC and ICP-MS.

***1) Micronebulisers/spray chambers***

It consists of a nebuliser and a spray chamber. The nebuliser transforms the sample, which is in liquid form at the column outlet, into a dense aerosol made up of very fine droplets (1-10 µm). The spray chamber limits the solvent load in the plasma and therefore makes the aerosol homogeneous, by limiting the size of the droplets and reducing the speed of the aerosol (Figure VIII.3). Several interfaces are available: pneumatic nebulisers, microconcentric nebulisers (MCN), ultrasonic nebulisers and direct injection nebulisers. The latter is used specifically for the LC-ICP-MS interface and is placed inside the ICP torch. Nebulisation efficiency can reach 100% and flow rates are (30-150) µl.min$^{-1}$ with very high efficiency.

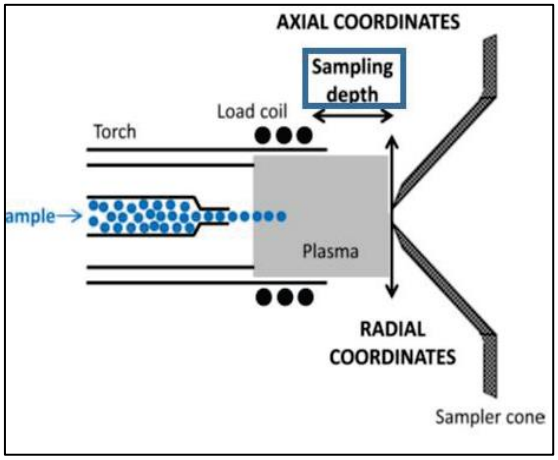



**Figure VIII.3.** Shematic of micronebulisers/spray chambers HPLC-ICP-MS.

***2) Direct injection nebulisers (DIN)***

The DIN is located some millimetres below the plasma base, which promotes the use of micro-bore HPLC columns because all the column effluent is introduced into the plasma. Therefore, they offer a 100% analyte transport efficiency, low dead volume, improved precision, minimum

peak broadening and enhanced sensitivity. The DIN operates at liquid flow rates from 30 to 120 $\mu L.min^{-1}$, it has an extremely low dead volume (< 2 µL) and the sample wash-out is fast, leading to minimal memory effects.

There are two types of spray chamber: cyclonic and vertical rotation (Figure VIII.4). The fine droplets and carrier gas are directed towards the torch from the top of the chamber, while the large droplets are directed downwards and drained in liquid form. Only droplets smaller than 10 µm in diameter pass through the plasma. This means that only 1% of the solution aspirated passes into the plasma.

***c) Atomisation and excitation system***

The excitation process represents all the phenomena occurring in the plasma. The nebulisate obtained after the nebulisation of the sample is subjected to various processes (desolvation, vaporisation, atomisation and possibly ionisation) before the excitation of the atoms or ions in the sample (Figure VIII.5).

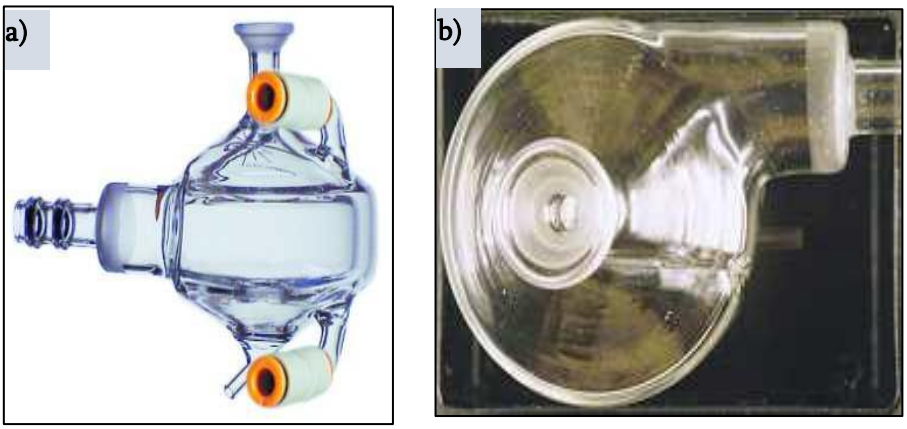



**Figure VIII.4**. Spray chamber, cyclone: a) and vertical rotation: b).

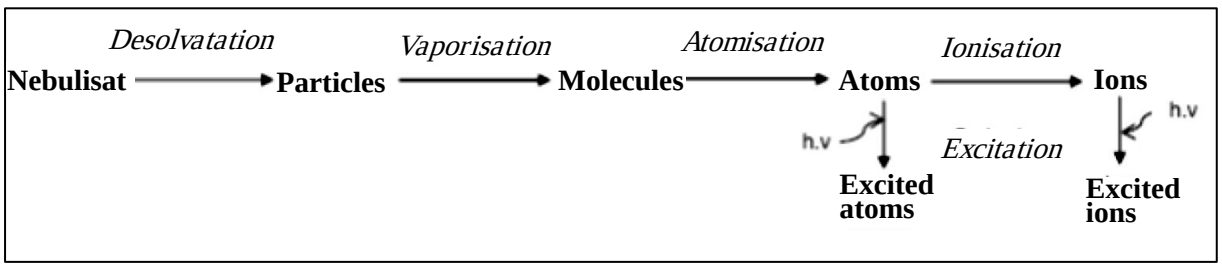



**Figure VIII.5.** Diagram showing the different processes involved in atomisation and excitation.

During atomisation and ionisation, when a particle collides with an atom, three processes can occur (Reactions (5) to (7)):

- Excitation : $Ar + M \rightarrow Ar + M^*$ ou $Ar^+ + M \rightarrow Ar^+ + M^*$ (5)

- Ionisation : $Ar + M \rightarrow Ar + M^{+}* + e^-$ ou $Ar^+ + M \rightarrow Ar^+ + M^{+}* + e^-$ (6)

- Charge transfer: $Ar^+ + M \rightarrow Ar + M^+$ (7)

Excitation and ionisation occur at high collision speeds, in the hottest part of the plasma, while charge transfer takes place at low speeds, in the least hot part of the plasma.

***d) Mass spectrometer detector***

For mass spectrometry coupling, the plasma ions are extracted through a series of cones towards the spectrometer, generally a quadrupole analyser. The quadrupole operates in a vacuum at ambient temperature. An interface consisting of two successive cones is required. The ions are separated according to their mass-to-charge ratio (m/z) and a detector receives a signal proportional to the ion concentration

## V. Applications of HPLC-ICP-MS

The technique (HPLC-ICP-MS) has a very wide range of applications:

- Pharmaceutical industry
- Elemental analysis
- Metal speciation
- Quantification of proteins and biomolecules

## VI. Gas chromatography-mass spectroscopy coupling

This coupling, abbreviated GC-MS: Gas chromatography-Mass Spectrometry, is an analysis technique that combines the performance of separating compounds via gas chromatography, and mass spectrometry, as a means of detection and identification of compounds according to their mass-to-charge ratio. This technique makes it possible to precisely identify and/or quantify numerous substances present in very small quantities, or even traces.

### VI.1. Principle

It is based on the injection, evaporation, separation, then detection of the different components of a mixture which can be as complex as possible. Detection is carried out by mass spectroscope which currently tends to supplant all other detection systems because it is the only one to provide structural information on the compounds separated by chromatography. Therefore, the same components and principle of a capillary column gas chromatogram and those of a mass spectroscope are applicable.

The difference in chemical properties between different molecules in a sample separates them as the sample moves along the column. Molecules are retained differently in the column before being eluted, allowing the downstream mass spectrometer to capture, ionise, accelerate, deflect and detect the ionised molecules separately. The mass spectrometer then destroys each molecule into ionised fragments and detects these fragments based on their mass-to-charge ratio. These two components used together allow the identification of a substance to a much finer degree than each unit used separately.

### VI.2. Conception

A GC-MS is made up of two main compartments: a gas chromatograph and a mass spectrometer (Figure VIII.6). The gas chromatograph uses a capillary column which depends on the dimensions of the column (length, diameter, film thickness) as well as the properties of the phase (for example: 5% polyphenyl siloxane).

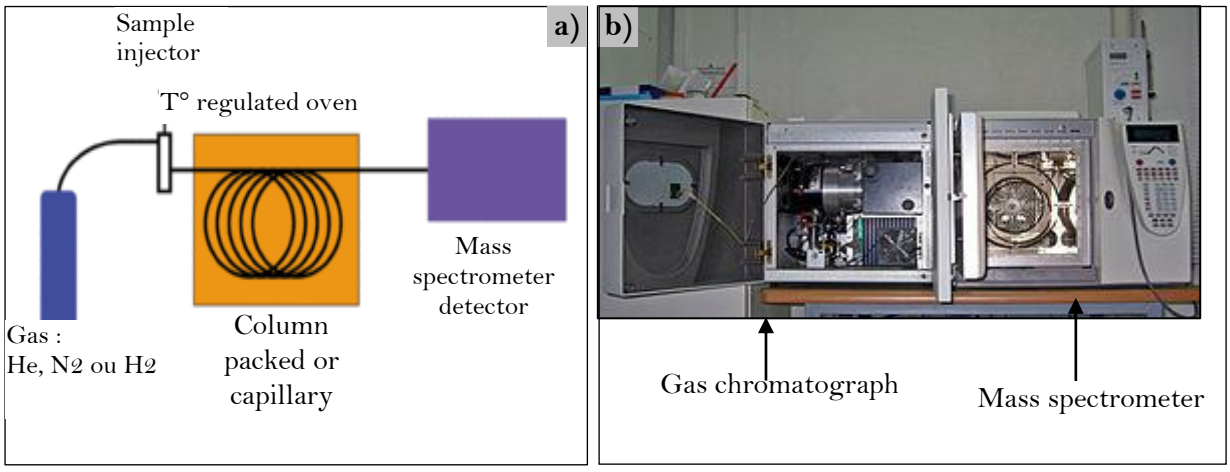



**Figure VIII.6.** General diagram of a GC-MS: a) and Apparatus of a GC-MS: b).

### *a) Gas chromatography*

Is described previously. The role of the chromatograph is to separate the constituents of a mixture. Gas chromatography is reserved for the analysis of relatively volatile and thermally stable compounds.

### *b) Mass spectrometry*

There are many types of mass spectrometers, all of which have three elements: a source, an analyser, and a detector (Figure VIII.7). Only mass spectrometers equipped with type: quadrupole analysers are presented, which represent the vast majority of analysers used in GC-MS coupling, due to their:

- Very high scanning speed.
- Reasonable cost.
- Ease of maintenance.
- Good functioning of the filament and the electron multiplier which are extremely weakened by too high pressure.

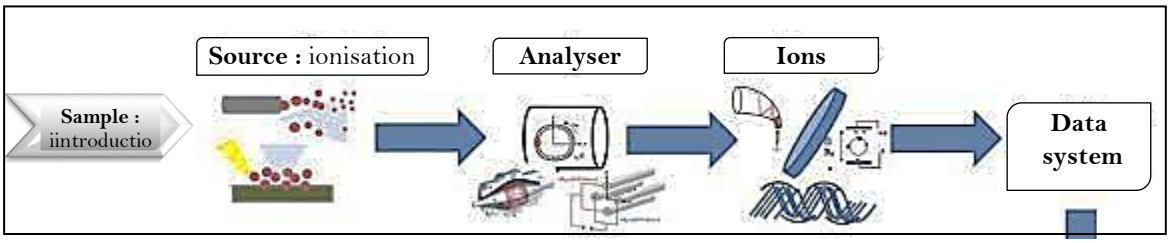



**Figure VIII.7.** Mass spectrometer elements.

***1) Sample introduction: inlet:*** In coupling with a gas chromatograph, where the eluted compounds arrive at the spectrometer in the gaseous state, the compounds are introduced into a tank which communicates with the ionisation chamber. The sample has a mass ranging from a few nanograms to a few milligrams. The sources used are of the type: electronic impact "EI" or chemical ionisation "CI". Their use is reserved for the analysis of gaseous or easily volatilisable compounds (for boiling point not exceeding 400°C). The source is maintained at an elevated temperature (typically between 100 and 250°C) to prevent analyte condensation.

***1.1. Chemical ionisation (CI):*** It consists of ionising a reactive gas of low molecular mass (methane, ethane, ammonia, isobutane) by electronic impact in the ionisation chamber then protoning the sample molecule by the reactive gas with ammonia, reactions (8) to (10):

$$NH_3 + e^- \rightarrow NH_3^{+\bullet} + 2\ e^- \quad (8)$$

$$NH_3^{+\bullet} + NH_3 \rightarrow NH_4^+ + NH_2^- \quad (9)$$

$$M + NH_4^+ \rightarrow MH^+ + NH_3 \quad (10)$$

***m/z* =18** ***m/z* =M+1**

This mode of ionisation involves a high abundance of pseudomolecular ion ($MH^+$) and a low probability of fragmentation which results in an absence of structural information of the molecule (M).

***1.2. Electron impact ionisation (EI) :*** The molecule is bombarded by high energy electrons (70 ev) which eject a weakly bound electron from the molecule (reaction (11), Figure VIII.8).

$$M + e^- \rightarrow M^{+\bullet} + 2\ e^- \quad (11)$$

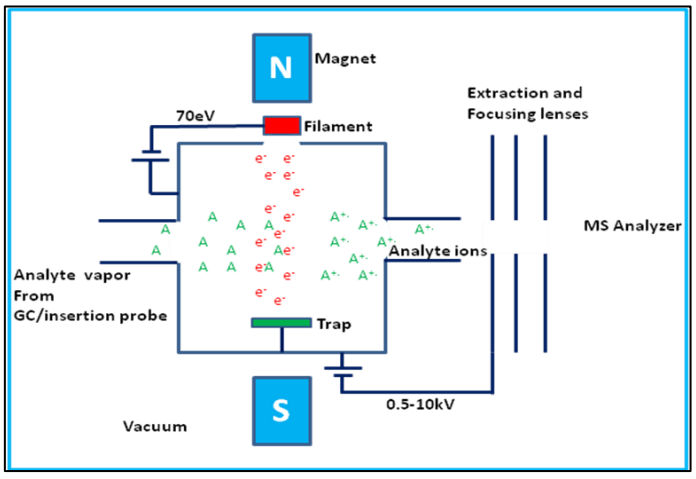



**Figure VIII.8.** Schematic diagram of EI source.

The excess ionisation energy lets the molecular ion undergo partial fragmentation, producing fragmentation ions, radicals or molecules. The ions formed continue to fragment and so on.

The study of these daughter ions makes it possible to elucidate the structure of the molecule (M).

***2) Ionisation chamber and ion acceleration:*** In the ionisation chamber, a plasma of ions is formed, the lightest of which is $H^+$ and the heaviest is $M^{+\bullet}$(EI). In order to avoid collisions between positive and negative ions, an electric potential is applied to the exit of the ionisation chamber. Thus, during fragmentation, the neutral fragments formed are eliminated by a pumping system. At the exit of the ionisation chamber, all ions with the same charge have the same kinetic energy. These ions, of different masses and different speeds, are accelerated by means of several plates brought to increasing negative potentials (case of positively charged ions), the total voltage U being between 2 and 10 kV (kinetic energy = $z \cdot e \cdot U$) and the vacuum must be high ($P < 10^{-4}$ Pa).

***3) Mass analyser:*** The analyser is used to separate the ions formed according to their mass (m) to charge (z) ratio (m/z). There are several types of mass analysers:

- Quadrupole Mass Analyser
- Time of Flight Mass Analyser
- Magnetic Sector Mass Analyser
- Electrostatic Sector Mass Analyser
- Quadrupole Ion Trap Mass Analysers
- Ion Cyclotron Resonance

In a GC-MS coupling context, the ions generally have a unitary charge (z = 1), the ions being too small to be charged several times (we consider that in the gas phase an ion can carry a charge for approximately 1000 units of mass). The analysers are characterised by four parameters: resolution, scanning speed, mass/charge scanned ratio range, and transmission. The resolution reflects the precision in measuring the m/z ratios of the ions. That of a so-called "quadrupole" analyser is linked to its scanning speed in m/z: the faster the scanning, the worse the resolution.

### *1.3. Quadrupole Mass Analyser*

As shown in Figure VIII.10, a quadrupole is systematically associated with a system for extracting and focusing the ions produced in the source. It is most often a hexapole or an electrostatic lens (electrical analogue of an optical lens). The quadrupole is made up of four

parallel metal electrodes electrically connected two by two, of ideally hyperbolic section. These electrodes are cylindrical and 12 to 20 cm a long depending on the model. The application of a U + Vcos(ωt) type potential creates a quadrupolar field between the electrodes. Two electrodes symmetrical to the central axis of the quadrupole are brought to a potential of type U + Vcos(ωt), the two others to the same potential but of opposite sign (- U – Vcos(ωt)). U and Vcos(ωt) are, respectively, the direct voltage and alternating voltage components of the radio frequency potential. V and ω are, respectively, the amplitude and the pulsation of the alternating voltage. In practice, the quadrupole functions like an ion filter: the values of U and V are varied simultaneously so that the ions produced by the source are stable in turn. At a given time t, only ions with a given m/z ratio are detected. The other ions will hit the electrodes or the internal walls of the spectrometer; they discharge and are driven by the pumping system.

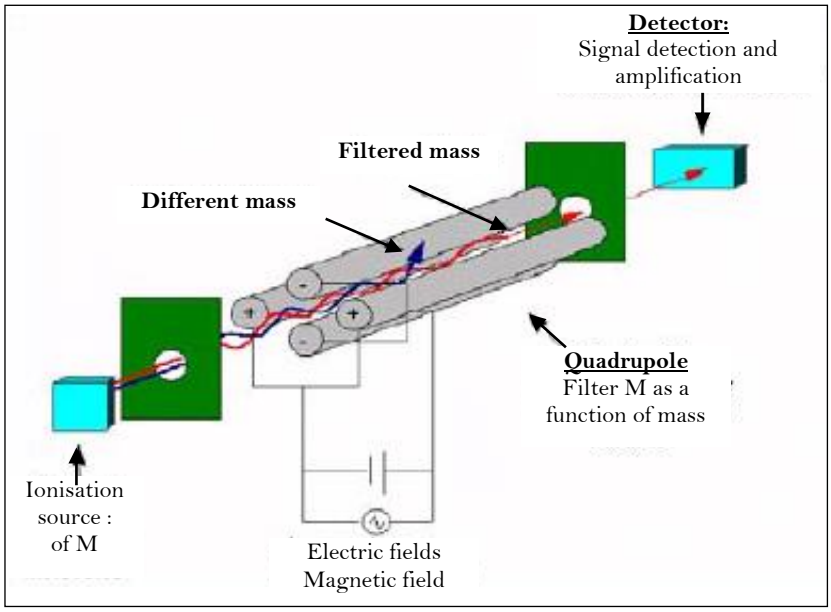



**Figure VIII.10.** Mass spectroscope with a quadrupole type detector.

***4) Detectors:*** The role of the detector is twofold: detect the ions proportionally to their number and amplify

the corresponding current (of the order of $10^{-12}$ A) to make it detectable by the system electronics. Most GC-MS devices are equipped with Chaneltron type detectors. A "chaneltron" is shaped like a curved funnel or cornucopia. The interior is coated with a lead/lead oxide alloy which has the property of emitting electrons under the effect of an impact. A potential difference of 1000 to 3000 V is applied between the inlet and outlet of the funnel. When an ion hits the internal wall, there is emission of electrons which, accelerated by the potential difference,

bounce back on either side of the wall. Each shock releases new electrons which are themselves accelerated; we speak of an "electronic cascade". A chaneltron provides a gain of around $10^5$.

***5) Chromatograph/mass spectrometer interfaces:*** The use of coupled capillary columns (GC/MS) has become widespread and the appropriate interfaces are part of the basic configuration. Three types of interfaces for capillary columns are generally used, (Figure VIII.11):

***5.1. The direct interface***: all of the effluent leaving the column enters the ionisation source. Good sensitivity is obtained but the maximum internal diameter allowed for the column depends on the pumping flow rate.

***5.2. The open-split interface:*** the effluent leaving the chromatographic column is diluted with an additional sweeping gas; part of the gas mixture enters the ionisation chamber through a capillary tube which acts as a restrictor, the rest of the mixture is sucked up by the pump.

***5.3. The jet separator:*** most of the carrier gas is eliminated; This results in a relative enrichment of the effluent in molecules to be analysed. The simplest and most common interface is the direct interface. The open-split interface and jet separator are usually used for megabore columns. The interface is independent of the ionisation chamber, in which the molecules are ionised by electronic ionisation (EI), positive chemical ionisation (CI+) or negative chemical ionisation (CI -).

## VII. Applications of GC-MS coupling

Nowadays, GC-MS coupling is an essential technology in modern analytical chemistry laboratories. The main applications include:

- Environmental monitoring and analysis (soil, water, and air pollutants, water quality, etc.)
- Geochemistry-age determination, soil and rock composition, oil and gas surveying
- Chemical and petrochemical industry quality control
- Identify structures of biomolecules, such as carbohydrates, nucleic acids, sequence biopolymers such as proteins and oligosaccharides
- Determination of molecular mass of peptides proteins and oligonucleotides.
- Monitoring gases in patients' breath during surgery.
- Identification of drug abuse and metabolites of drugs of abuse in blood, urine, and saliva.

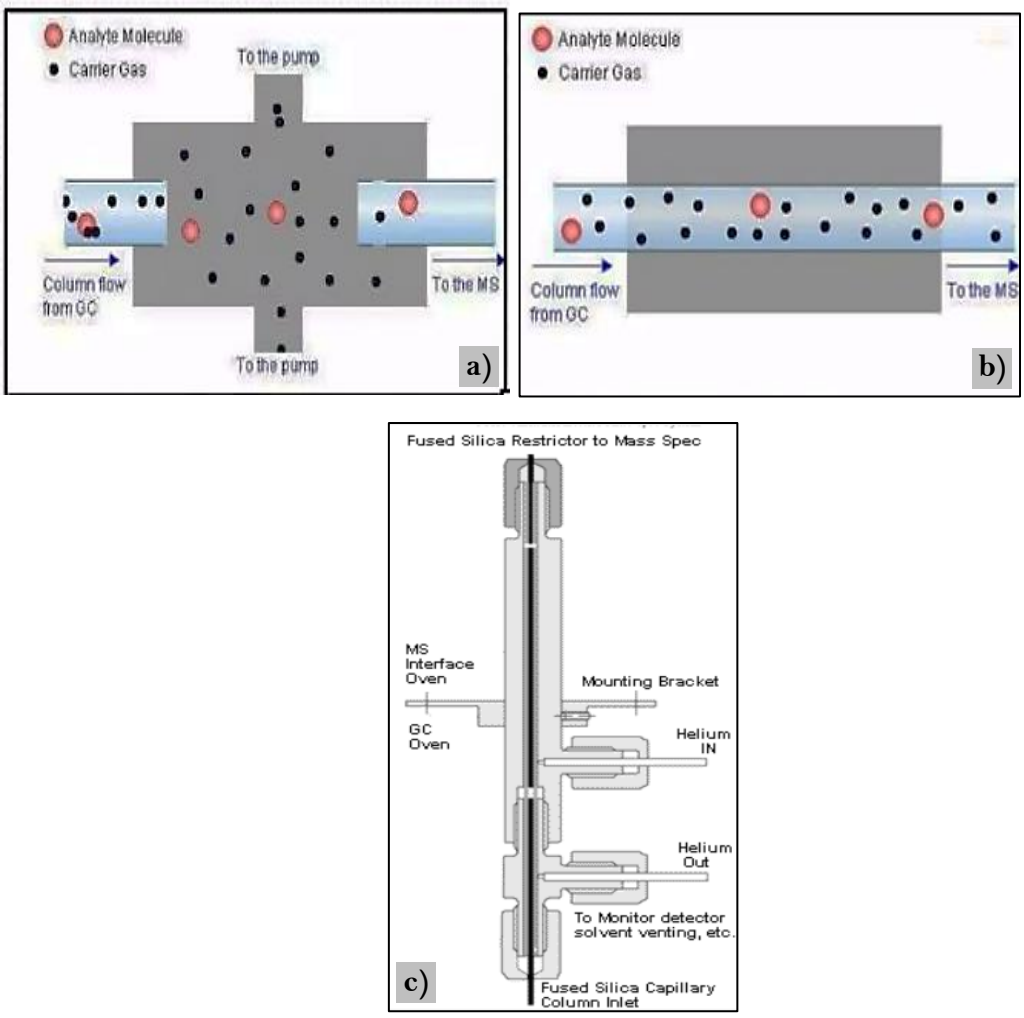



**Figure VIII.11.** GC-MS interface, jet separator: a), capillary direction: b) and open-split.

# Exercises Chapter 1: Chromatography: Fundamental and Calculations

## ***Exercise 1:***

6 mL of silica gel and 40 mL of a solvent containing 100 mg of a non-volatile compound A in solution are mixed in an Erlenmeyer flask. After shaking the mixture well, 10 mL of the solution is decanted and the solvent evaporated. The residue weighs 12 mg. Calculate the adsorption coefficient of compound A.

*Response:*

$K_A = 7.22$

## ***Exercise N°2:***

Calculate the linear flow velocity of a fluid (in cm/min), flowing at a rate of 1.0 $mL.min^{-1}$, through a tubular column with an internal diameter of 2 mm and porosity equal to 0.5.

*Response:*

$D=u.s.\varepsilon \Rightarrow u=D/s.\varepsilon$

u= 31.84 cm/min.

## ***Exercise N°3:***

Van Deemter's equation is applied for a gas chromatographic column (d=10 mm and L=2 m), this equation is expressed by:

HEPT=A+ B/u+C.u

Where: A = $2.10^{-3}$ cm, B = 0.1 $cm^2. s^{-1}$, C = $1.6.10^{-3}$ s

1. Calculate the optimum MP flow rate, if the porosity ε = 0.5.
2. What would then be the minimum height equivalent to a theoretical column plateau?
3. What is the number of theoretical plates?

*Response:*

1. $u_{opt} = \sqrt{\frac{B}{C}} = 7.905 \frac{cm}{s}$

D = u.s.ε = 3.103 ml/s

2. $HEPT_{min}$ =0.0273 cm
3. N=HEPT/L; N = 7326.

# Exercises Chapter 2: Chromatographic methods

## ***Exercise N°1:***

A cation exchange resin used to separate amino acids is composed of polystyrene beads containing sulphonate groups ($SO_3^-$) bonded covalently to phenyl rings. Two types of effect are used to separate amino acids: electrostatic interactions with the ($SO_3^-$) group and hydrophobic interactions with the apolar phenyl ring.

Since the pH of the column is kept at pH 7.0 using an appropriate buffer, which order of elution will be predicted for the following pairs of amino acids: Asp/Arg, Lys/Met, Gly/Leu, Ser/Phe, and Lys/His?

*Response:*

Before knowing the order of elution of the AA pairs, it is necessary to know the pka or pHi of the AAs.

- The Met/Lys pair: Met (zero charge) elutes first before Lys (+ charge);
- The Gly/Leu pair: Gly (zero charge, less hydrophobic) elutes first before Leu (also zero charge, more hydrophobic);
- Ser/Phe: Ser (zero charge, less hydrophobic) elutes first before Phe (zero charge, more hydrophobic);
- His/Lys: His (partial charge +) elutes first before Lys (total charge +).

## ***Exercise N°2:***

1/- An enzyme A (with a MW of 24 kDa and an isoelectric point (pHi) of 5.5) is contaminated by two other proteins, one with a similar MW and a pHi of 7.0, and the other with a MW of 100 kDa and a pHi of 5.4. Suggest a general purification protocol for enzyme A.

2/- Diethyl aminoethyl cellulose (DEAE-cellulose) is an anion exchange support obtained by substituting cellulose with diethyl aminoethyl groups:

1) What is the proportion of positively charged DEAE radicals at the following pH values: 2; 7; 9.4; 12 (the pKa of the tertiary amine of the DEAE group is 9.4). Using the Henderson-Hasselbalch equation: pH = pKa+log[base]/acid].

2) Among the following proteins: Serum albumin (pHi = 4.9), Urease (pHi = 5), Chymotrypsinogen (pHi = 9.5), which proteins are retained by DEAE-cellulose at pH 7? (Protein-DEAE-cellulose interactions are electrostatic only).

*Response*

1/- The protocol involves two chromatographic methods:

1) SEC: to separate the P2 protein: recovery of a mixture E with P1

2) IC uses a cation exchange resin adjusted to pH 7: to separate P1 first, then E last, after lowering the pH to 5.5.

2/-

1) **<u>At pH=2</u>**: pH-pka = -7.4 $\Rightarrow \frac{[NR2]}{[NR2H^+]} = 3.98.10^{-8}$ since $[NR_2]+[NR_2HH^+]$ = 100 %, we deduce that: $[NR_2HH^+]$ = 100 % and $[NR_2]$ = 0%.

**<u>At pH=7:</u>** $[NR_2HH^+]$ = 99.6 % and $[NR_2]$ = 0.4%.

**<u>At pH=9.4:</u>** $[NR_2HH^+]$ = 50 % and $[NR_2]$ = 50%.

**<u>At pH=12:</u>** $[NR_2HH^+]$ = 0.25 % and $[NR_2]$ = 99.75%.

2) At pH 7 the resin is positively charged, so serum albumin is retained, urease is retained but chymotrypsinogen is eluted.

***<u>Exercise N°3:</u>***

An unpurified enzyme solution was purified in two stages:

- <u>First stage of purification</u>: on an anion exchange resin. Various proteins were eluted by gradually decreasing the pH from 8 to 5. Only one fraction had enzyme activity: fraction F2. It is eluted at pH 6.

1) What can be said about the pHi of the enzyme?

- <u>Second stage of purification:</u> using an exclusion chromatography resin. The active fraction F2 is deposited on a column (Sephadex G100 previously calibrated with 4 proteins) and eluted with a pH 7 buffer. The elution volumes are shown in the Figure 1. Only one fraction has enzymatic activity (fraction F3).

2) What property of proteins is exclusion chromatography based on?

3) Why are the chromatography steps followed by a measurement at 280 nm?

4) Using the semi-log grid below, give an estimate of the molecular mass of this enzyme.

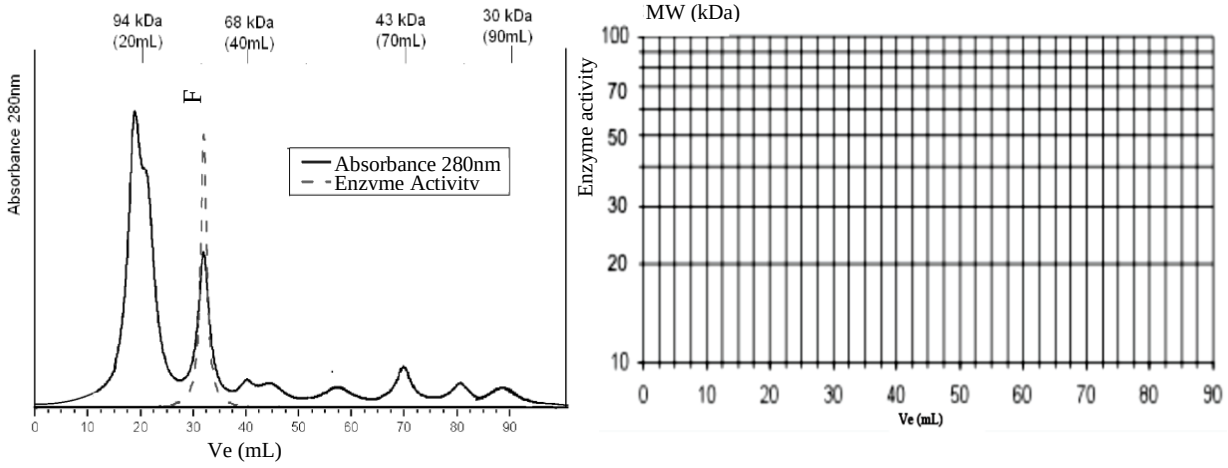



**Figure 1.** Chromatogram of five protein fractions

*Response:*

1) The pHi of the enzyme is 6.
2) SEC is based on the elution of macromolecules according to molecular weight, ranging from those with the highest MW to those with the lowest MW. In this chromatographic method, the SP permeation interval is crucial, as it determines the elution and order of macromolecules.
3) Almost all proteins absorb at 280 nm, due to their aromatic AA constituents such as tryptophan and tyrosine.
4) Using the given semi-log grid, plot the variation in MW, Next, reading the elution volume for the protein fraction containing the enzyme, its elution volume is 31.9 ml, then injecting it onto the plotted graph: so the MW is 77 kDa.

## Exercises Chapter 3: Planar Chromatography: TLC and PC

### ***Exercice N°1:***

Thin-layer chromatography of three compounds

A, B, and C using hexane as eluent gave the chromatogram shown in Figure 2 :

1) Determine the $R_f$ of these three compounds

2) Classify the compounds in order of increasing polarity.

3) How would the elution order of the three compounds be evaluated if hexane were replaced by acetone?

4) If silica gel is replaced by alumina while retaining the hexane, how do the three $R_f$'s compare?

**Data:** Eluotropic forces with Alumina: ($\varepsilon_{0\ hexane}$) =0.01 and ($\varepsilon_{0\ Acetone}$) =0.56

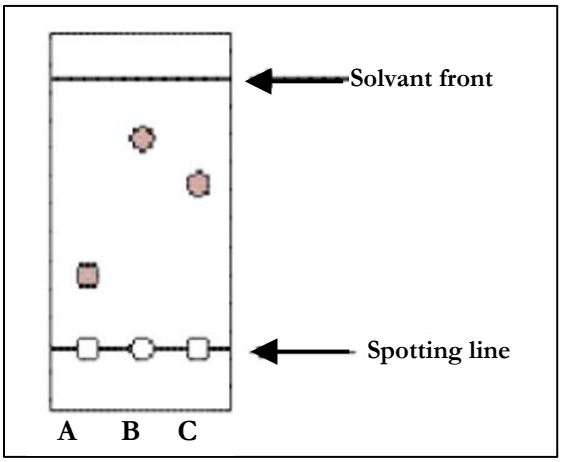



**Figure 2.** Migration of compounds A, B and C by TLC

*Response:*

1) $R_f = \frac{\text{Distance travelled by the solute } (D_i)}{\text{Distance travelled by the MP } (D_{MP})}$

$R_A$=0.28
$R_B$=0.7
$R_C$=0.6

2) ***B<C<A***

3) Acetone has a higher eluotropic force than hexane, so if we continue elution with acetone, we'll have: compound A will be entrained more quickly, followed by C and finally B.

4) For compound A, (polar), it will be more retained on alumina than on silica gel, so the $R_f$ will be multiplied *0.77

For compound b: the least polar, it will have even less affinity for alumina, so its Rf will be divided by 0.77.

For compound C, its Rf is less than 0.5, so it has an apolar character; hence, its Rf will be divided by 0.77.

## ***Exercise N°2 :***

To separate compounds X, Y, and Z on a silica gel plate, tests were carried out to determine the appropriate MP for achieving satisfactory separation. A 95:5 (v/v) hexane/ethyl acetate mixture was used as the MP1. The migration results for these analytes are shown in the chromatogram in Figure 3. Propose a protocol to improve separation.
**Data:** Ip (Ethyl acetate) = 4.4 and Ip (Hexane) = 0.1

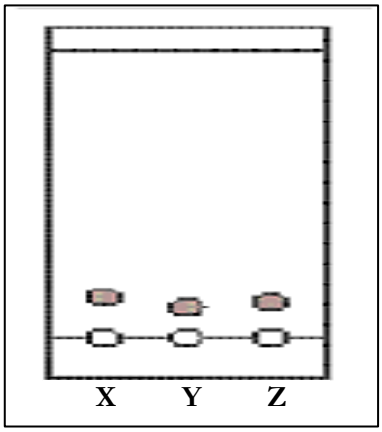



**Figure 3.** Migration results of compounds X, Y and Z by MP1

*Response:*

The protocol is as follows: start the elution with : 95 :5 (hexane/ethyl acetate), giving the same positions as the current chromatogram. Then increase the proportion of ethyl acetate to 38.14% (61.86% hexane) to migrate compound y (to d=1.4 cm). Next, the proportion of ethyl acetate is increased to 58.4% (41.6% hexane) to migrate compound x (up to d = 2.45cm).

## ***Exercise N°3 :***

A sample contains two compounds A and B, separated by TLC on silica gel. After migration, the revelation results in two spots. The migration of the solvent front in this experiment is 6 cm. Calculate:

1) Calculate $R_f$, N and H for each compound.

2) Calculate the resolution factor between the two compounds A and B.

**Data:** $x_A$= 3.1 mm and $w_A$= 1.5 mm, $x_B$= 2.6 mm and $w_B$= 2.2 mm.

*Response :*

1)

$R_f = dx/d_M$ ; $R_{f\,A} = 3.1/6$ ; $\mathbf{R_f = 0.516}$

$R_{f\,B} = 2.6/6$; $\mathbf{R_f = 0.433}$

$N_A = 16 . \frac{x_A^2}{W^2}$ ; $N_A = 68 \Rightarrow H_A = \frac{x_A}{N_A}$ ; $\mathbf{H_A = 0.0455\ cm}$

$N_B = 16 . \frac{x_B^2}{W^2}$ ; $N_A = 22 \Rightarrow H_B = \frac{x_B}{N_B}$ ; **$H_B = 0.118$ cm**

2) $Rs = 2. \frac{d_1 - d_2}{W_A + W_B}$ ; $Rs = 2. \frac{3.1-2.6}{1.5+2.2} \Rightarrow$ **Rs = 0.135**

## Exercises Chapter 4: Column Chromatography

### ***Exercise N°1:***

Analyses are to be carried out on a 5 cm long column with an internal diameter of 4.6 mm, packed with silica particles 5 µm in diameter.

1) Calculate the dead volume of the column (estimated porosity 70%).

We wish to precondition (equilibrate) the column with a 50:50 water/methanol mobile phase. The flow rate of the mobile phase is adjusted to 1 mL/min. Knowing that a volume of mobile phase equal to 5 times the column volume is required to equilibrate the column.

2) Calculate the time required to equilibrate the column.

*Response:*

1) $V_0$=1.12 ml.
2) t= 5.6 min

### ***Exercise N°2:***

Two substances A and B have retention times of 16.40 min and 17.63 min, respectively on a column of length L=30 cm. The retention time of a non-retained solute is 1.30 min. Peak widths at the base are 1.11 and 1.21 min, respectively.

1) Evaluate the separation quality of compounds A and B.

2) Propose a method(s) to improve the separation quality of these two compounds.

3) In the laboratory, you have a new column of length L', all the data are the same as above: Calculate the length L', to have a resolution of 1.5.

4) Calculate the HETP and efficiency required to achieve a resolution of 1.5 with length L. Compare and comment on these results with those of column L'.

*Response:*

1) Rs=1.06
2) to improve separation:
   - Increase column length
   - increase the number of interaction sites
   - Optimize flow rate
3) L'=58.42 cm
4) To have the same resolution, then N is identical: Nb2 = 6802 so HETP= 0.0044, in which case we can say that :

-either increase the number of interaction sites to increase the N
- or optimize the rate

## Exercises Chapter 5: HPLC

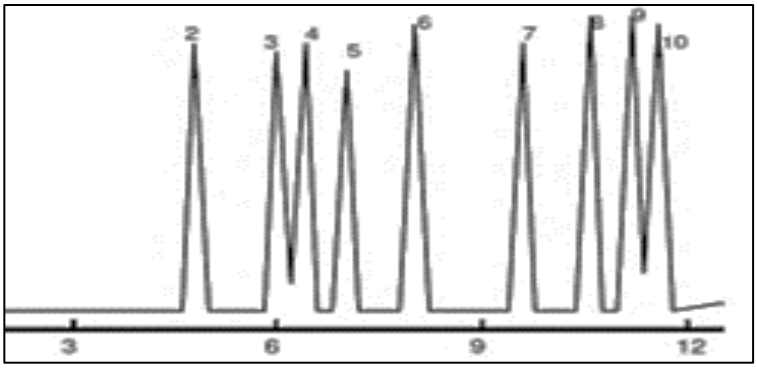
### ***Exercise N°1:***

The separation of fatty acids is carried out on a column (L= 15 cm and $d_i$=4.6 mm), which the SP consists of spherical particles of octyl silica 4 µm in diameter.
- The mobile phase has the composition shown in Table 1.
- The flow rate of the PM is 1 ml/min. The mixture injected is 10 µL of a 0.1 mM solution of fatty acids in the mobile phase. The diagram of a standard chromatogram is given in the figure 4 (time in min):

1) What type of chromatography is used?
2) What is the role of phosphoric acid in the mobile phase?
3) The interstitial porosity is 0.5. What is the capacity factor of these compounds?
4) How would you reduce the analysis time? (Ip= 4 and 5.8 for THF and acetonitrile respectively).

**Table 1.** MP composition

| $CH_3$-CN |  | $H_3PO_4$ à 1 % |
|---|---|---|
| V/V = 50 | 22 | 28 |




| | | | |
|---|---|---|---|
| 2 : acide linolénique | (C18:3,cis) | 7: acide palmitique | (C16:0) |
| 3: acide palmitoléique | (C16:1,cis) | 8: acide oléique | (C18:1,cis) |
| 4: acide trans-héxadécénoïque | (C16:1,trans) | 9: acide élaïdique | (C18:1,trans) |
| 5: acide linoléique | (C18:2,cis) | 10: acide stéarique | (C18:0) |
| 6: acide trans-linoléique | (C18:2,trans) | | |

**Figure 4.** Chromatogram of the nine (09) fatty acids

*Response :*

1) Partition chromatography
2) To maintain the hydrophobic character of fatty acids
3) $V_M$=1.25 ml

| Fatty acid | k' | Fatty acid | k' |
|---|---|---|---|
| linolenic acid | 2.86 | Palmitoleic acid | 3.8 |
| trans-hexa acid | 4.08 | Cis-linoic acid | 4.55 |
| trans-linoleic acid | 5.4 | palmitic acid | 6.62 |
| Oleic acid | 7.38 | elaidiqic acid | 7.94 |
| stearic acid | 8.22 | | |

4) The following is required: increase the acetonitrile content or increase the flow rate

## ***Exercise N°2 :***

Benzene derivatives were analysed using a high-performance chromatograph equipped with a C8 column (porosity = 0.5, dp = 5 µm, length 15 cm and internal diameter=3.9 mm); the MP consisted of water and isopropanol (17.5% isopropanol) at a flow rate of 1 $mL.min^{-1}$. These derivatives are nitrobenzene (NB), 1,3- dinitrobenzene (DNB), 1,3,5-trinitrobenzene (TNB), 3-nitrotoluene (NT), 2,4-dinitrotoluene (DNT) and 2,4,6-trinitrotoluene (TNT). The chromatogram obtained is shown in Figure 5.

**1)** Explain the retention mechanism and interpret the order of elution of these various compounds. What type of detector is best suited to this system?

**2)** Calculate the dead time of the column.

**3)** Calculate the capacity factors for nitro derivatives of benzene. Give comments.

**4)** How can the analysis time be reduced? Give its value in the case where k'(NT) = 10

**5)** Calculate the pressure drop if the viscosity of the MP is 1 cps.

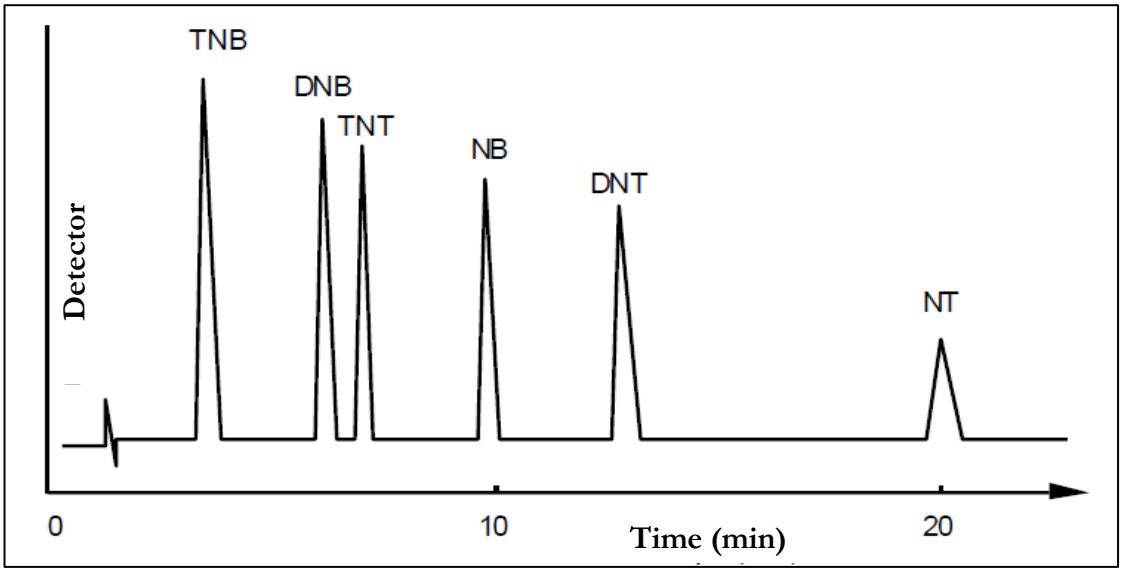



**Figure 5.** Chromatogram of the benzene derivatives

*Response:*

1) This is a reverse-phase LLC. Retention is based on hydrophobic interactions between the hydrophobic part of each molecule and the C8 hydrocarbon chain.
The higher the number of $NO_2$s, the greater the polarity of the compound and the shorter its retention time. Due to the presence of the methyl group, toluene derivatives have a higher hydrophobicity than benzene derivatives; their retention times, all other conditions being equal, are therefore higher.

2) $V_M = t_0 . D = \varepsilon . V_T$ Where :

$$t_0 = \frac{\varepsilon . V_T}{D} = \frac{0,5 . 15 . (0,39)^2 . \pi}{4 . 1} = 0,9 \text{ min}$$

3) According to the chromatogram, the analysis time is long (>21 min), and toluene derivatives (DNT and NT) are eluted too late (very high retention and low elution): k'>10.

4) By increasing the PM flow rate or changing the PM composition (by decreasing its polarity). et $\phi_{isopropanol}$ = **0.2788** (27.88%) et $\phi_{water}$ **= 0.7212** (27.12%).

| Molecule | $t_R$ (min) | k' |
|---|---|---|
| TNB | 3.25 | 2.61 |
| DNB | 5.15 | 4.72 |
| TNT | 6.1 | 5.77 |
| NB | 10 | 10.11 |
| DNT | 13.1 | 13.55 |
| NT | 20 | 21.22 |

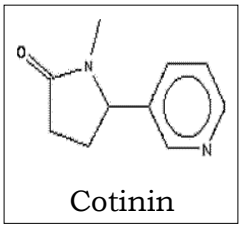
$t_{analysis}$ = 10.3 min

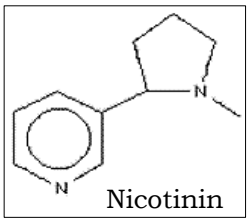
5) $\Delta P = 59.35.10^5$ Pa = 59.35 bars

## Exercises Chapter 6: GC

### ***Exercise N°1 :***

Using a GC separation of a mixture: cotinine, nicotinine, a C8 grafted silica SP column and a MP consisting of a mixture of $N_2$ and He gas 50:50 (v/v) are used, the structures of the solute molecules being represented below:


Cotinin


Nicotinin

1) Suggest an elution order for the solutes.

2) Since: tm= 1min; $k'_{Nicotinine}$= 3.6 and $\alpha$ = 1.2,

Calculate the retention times.

3) If the linear velocity of the PM $\bar{u}$=1 cm.min$^{-1}$

and $\omega_1 = \omega_2$ =0.4cm, calculate the theoretical plate number (N) for the retained compound and the resolution of the column (Rs).

*Response:*

1) Cotinin first, nicotinin second
2) tr(cotinine) = 3 min, tr(nicotin)=3.6min
3) $N_{cotinin}$ = 900 ; $N_{Nicotinin}$ = 1296

Rs = 1.5

### ***Exercise N°2:***

Table 2 shows the capacity factor values k' (in italics) for four refinery gases, studied at three different temperatures on the same capillary column (LC = 30 m, ID = 250 mm) with a SE-30 stationary phase. The chromatograph is fitted with a cryogenic accessory.

1) According to the elution order, is the SE-30 phase polar or not?
2) Calculate the selectivity factor for the propene-propane pair at the three temperatures
3) Why does k' decrease with increasing temperature for the same compound?

4) What is the theoretical number of plates in the column for propane at 40°C, given that at this temperature the resolution factor for the propene-propane pair is equal to 2?

5) Calculate the corresponding HETP.

**Table 2.** Values of k' at different column temperatures

| Compound | Boiling temperature (°C) | k'(-35 (°C)) | k' (25 (°C)) | k' '40 (°C)) |
|---|---|---|---|---|
| Ethylene | -104 | 0.249 | 0.102 | 0.0833 |
| Ethane | -89 | 0.408 | 0.148 | 0.117 |
| Propene | -47 | 1.899 | 0.432 | 0.324 |
| Propane | -42 | 2.123 | 0.481 | 0.352 |

*Response :*

1) SE-30 is an apolar SP.

2) $\alpha$ = k'2/k'1 : $\alpha$ = 1,12 (at : −35 ◦C), $\alpha$= 1, 11 (at : 25 ◦C) and $\alpha$ = 1,09 (at: 40 ◦C)

3) For the same column, depending on the relationship: $\text{Ln(t'r)} = \frac{A}{T(K)} + B$

when Tpe ↑ retention time ↓.

*4)* $N = 16.\left[Rs\frac{\alpha}{\alpha-1}.\frac{k'2+1}{k'2}\right]^2 = 16.\left[2.\frac{1.09}{0.09}.\frac{0.352+1}{0.352}\right]^2$

*N = 138 489*

5) $H_{\text{min theo}}$ = L/N = 0, 216 mm.

## ***Exercise N°3 :***

Heptane and ethylbenzene were separated by GC on a column filled with SP type OV17 (silicone: 50% phenyl, 50% vinyl, L = 2m, T= 60°C and u(He)opt = 20 cm/s). The analysis resulted in k' values of 4 and 4.4.

1) What is the nature of the PS used (polar or apolar)?

2) Which compound is the most retained?

3) Calculate the efficiency N of the most retained compound to obtain an Rs = 1.5 and calculate its retention time.

4) Above the optimum helium velocity, Van Deemter's equation shows that the HETP follows the relationship:

$$\text{HETP} = \left(\frac{2}{3}\right).\left(\frac{\text{HETP}_{\text{opt}}}{u_{\text{opt}}}\right).u$$

What are the resolution and retention times if the helium velocity is 60 $cm.s^{-1}$?

5) What is the length of the column (OV17) required to maintain an Rs= 1.5. Calculate the corresponding retention times. What does this mean?

*Response:*

1) The PS used is liquid and apolar.
2) Ethyl benzene.
3) N = 6561 theoretical plates

$t_m$ = L/u = 200/20 ; **$t_m$ = 10 s** and tr = $t_m(1+k')$ ; **$t_r$ = 54 s**

**4) Rs=1.07 and tr = 16.65 s**

5) **tr = 35.42 s.**

**In conclusion:** for a high helium flow rate, you need to use a column that is twice as long, in which case retention (or analysis) time will be reduced by more than 35%.

## Exercises Chapter 7: Electrophoresis

### ***Exercise N°1:***

1) Predict the migration of the following proteins in an electric field:
   - Chymotrypsinogen at pH 6.0, 9.5 and 12.0
   - Haemoglobin at pH 4.5 and 8.0
   - Lysozyme at pH 7.0
   - Pepsin at pH 7.0
2) What is the most favourable pH for separating the following mixtures:
   - Serum albumin and myoglobin
   - Urease, γ1 globulin and haemoglobin?

**Table 3.** Different protein pHi

| **Protein** | **pHi** |
|---|---|
| Pepsine | 1.0 |
| Ovalbumine | 4.6 |
| Sérum-albumine | 4.9 |
| Urease | 5.0 |
| **γ** 1 globulin | 6.6 |
| Hemoglobin | 6.8 |
| Myoglobin | 7.0 |
| Chymotrypsinogen | 9.5 |
| Lysozym | 11.0 |

*Response :*

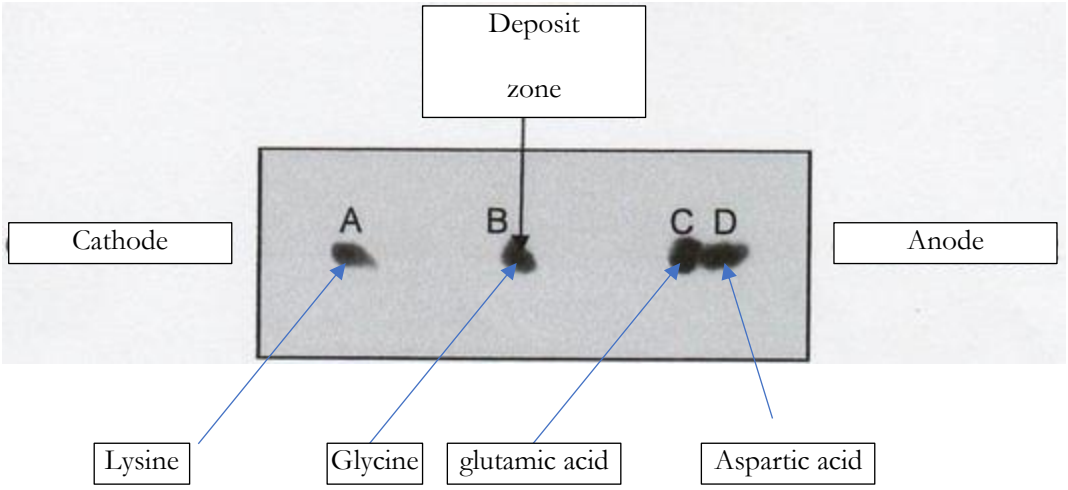
1)
- Chymotrypsinogen at pH 6.0, : to cathode ; at 9.5: immobile and at 12.0: to anode
- Hemoglobin at pH 4.5 to cathode and at 8.0 to anode
- Lysozyme at pH 7.0 to cathode
- Pepsin at pH 7.0 : to cathode

2) - Serum albumin and myoglobin : pH 6
- Urease, γ1 globulin and hemoglobin : pH:6.6

***Exercise N°2:***

Predict the migration of amino acids in a mixture using electrophoresis: glycine, leucine, aspartic acid, and glutamic acid.

*Response*:



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