وزارة التعليم العالي والبحث العلمي
Badji Mokhtar-Annaba University
Faculty of Science
Department of Chemistry

Chromatographic Analysis Methods

Course Handout for Use by Undergraduate's and Master's Students in Organic, Pharmaceutical, and Analytical Chemistry.

Courses, Tutorials and Practical Work

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This manual on chromatographic separation methods is intended for undergraduate students (L3) in analytical and pharmaceutical chemistry, as well as for master's students (M1) in analytical and organic chemistry. It aligns with the new LMD curriculum and is divided into six chapters:

- *The first chapter provides a bibliographical overview of the general principles of chromatography.*
- *The second chapter introduces the technique of thin-layer chromatography (TLC), paper chromatography, and conventional column chromatography.*
- *The third chapter is devoted to gas chromatography (GC).*
- *In the fourth and fifth chapters, we present the key concepts of high-performance liquid chromatography (HPLC) and ion chromatography (IC).*
- *The final chapter focuses on the fundamental concepts of electrophoresis.*

It is important to note that this manual serves as a supplementary resource and, under no circumstances, can replace the student's attendance in class.

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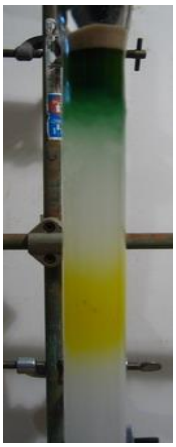
Chapter I

Chromatography - Basic Concepts.

I- Introduction :

Chromatography is a method of analysis and separation of chemical substances widely used in all sectors of industry and scientific laboratories. The success of this analytical method is attributed to its exceptional performance: qualitative and quantitative analyses of the most complex mixtures can be achieved with better resolution and in shorter times than with most other analytical methods.

II- Definition:



The method of analyzing colored pigments based on the difference in affinity for an adsorbent was proposed in 1903 by Mr. *Tswett* and called "chromatography" from "chromos" meaning color and "graphein" meaning to write. Although the method was applied to non-colored substances as well, the name "chromatography" continues to be used to this day.

III- Principle of Chromatography :

Chromatographic methods are based on the phenomenon of separating the substances to be analyzed between two immiscible phases: one of these phases is a stationary phase (ϕ_s), fixed and immobile on a support, while the other is a mobile phase (ϕ_m), either a gas or a liquid. The relationship between the two phases is determined by the following equation:

$$\beta = \frac{V_m}{V_s}$$

Under the antagonistic influence of retention forces (solute/ϕs) and driving forces (solute/ϕm), the various components of a sample (referred to as the solute) can be collected separately at the end of migration. Each compound is characterized by a Nernst distribution coefficient K (also known as the partition coefficient), defined as follows: (Please provide the specific definition you'd like to include for a more detailed translation.)

$$K = \frac{C_s}{C_m} = \frac{\text{The concentration of the solute in the stationary phase}}{\text{The concentration of the solute in the mobile phase.}}$$

- ♣ K depends on temperature and three interaction forces: ϕ_s/solute, ϕ_m/solute, and ϕ_m/ϕ_s.

Chromatographic separations involve techniques based on the physicochemical properties of molecules:

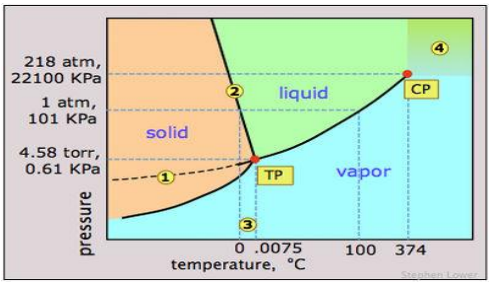
- ♣ Solubility: The tendency of a molecule to dissolve in a liquid.
- ♣ Volatility: The tendency of a molecule to vaporize.
- ♣ Adsorption: The tendency of a molecule to bind to a finely divided solid.

IV- Classification of Chromatographic Methods:

Chromatographic methods encompass a wide range of techniques that can be classified based on the physical nature of the phases, the phenomenon involved, and the operating procedure.

IV-1- Classification According to the Physical Nature of Phases:

The mobile phase is a fluid, which can be a liquid, a gas, or a supercritical fluid, while the stationary phase can be either a solid or a liquid.
The following distinctions are made:



- ♣ Liquid-Solid Chromatography (LSC).
- ♣ Liquid-Liquid Chromatography (LLC).
- ♣ Gas-Solid Chromatography (GSC).
- ♣ Gas-Liquid Chromatography (GLC).
- ♣ Supercritical Fluid Chromatography (SFC); supercritical fluids have properties intermediate between those of liquids and gases, for example, CO2 at 50°C and 150 bars.

IV-2- Classification According to the Phenomenon Involved:

This classification is based on the nature of the stationary phase and its interaction with the molecules to be separated. The distinctions made include:

- ♣ Adsorption or Affinity Chromatography (when the stationary phase is a solid); this corresponds to a case where the adsorption properties of the stationary phase are specific to a compound(s).

- ♣ Ion Exchange Chromatography, where the stationary phase carries acidic or basic functional groups, designed to separate ionized compounds.

- ♣ Partition Chromatography, when the stationary phase is a non-miscible liquid with the mobile phase.

- ♣ Exclusion Chromatography, where the porous stationary phase acts like a sieve and separates compounds based on their size; also known as gel permeation chromatography.

IV-3- Classification According to the Operating Procedure:

Based on the conditioning of the stationary phase, the distinctions made are:

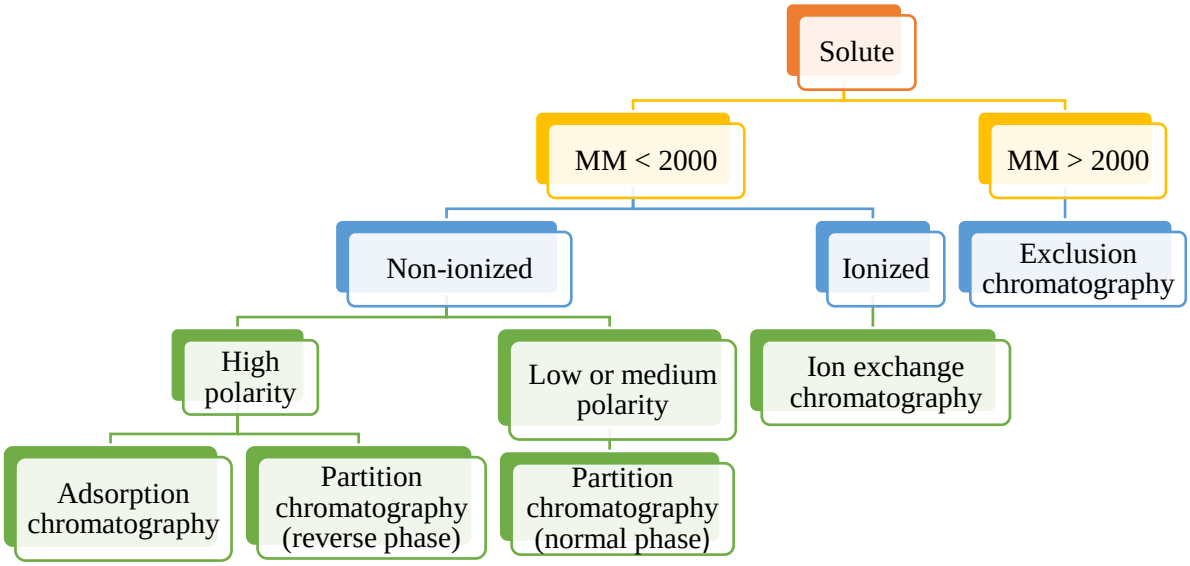
- ♣ Column Chromatography or Elution Chromatography: Substances are carried out of the stationary phase.

- ♣ Thin-Layer Chromatography (paper) or Development Chromatography: The components of the sample remain on the stationary phase.

V- Choosing Chromatographic Methods:

The choice between different chromatographic techniques depends on two essential factors:

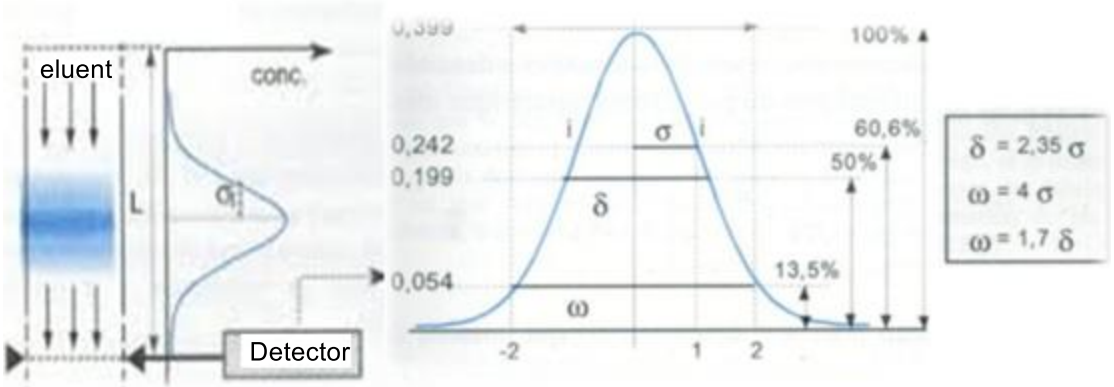
- ♣ The physical nature of the solute: gas, liquid, solid, macromolecule, organic species (polar or non-polar), ionic.
- ♣ The purpose of the analysis: identification, purity control, product purification, monitoring a reaction, quantification, etc.

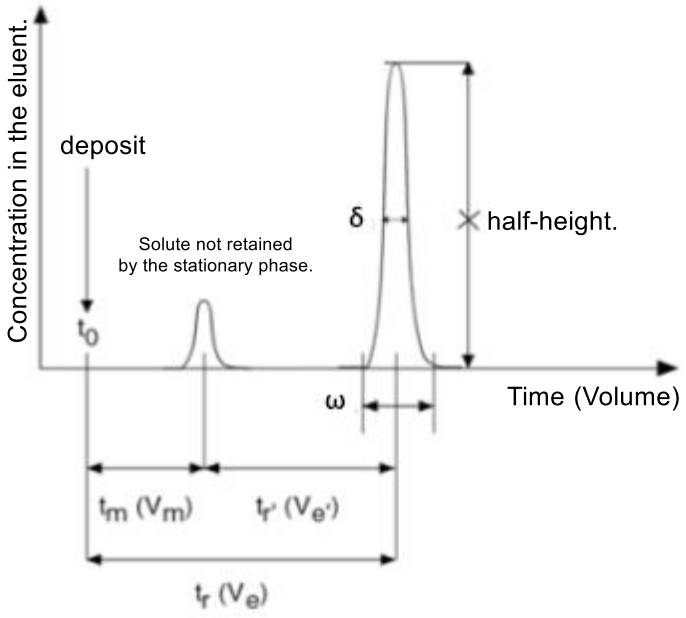


VI- Chromatographic Parameters:

VI-1- The Chromatogram:

All chromatographic instruments include a column containing the stationary phase and a detector downstream to detect changes in the composition of the mobile phase during elution. Each separation corresponds to a recording called a "Chromatogram," which is ideally a Gaussian curve as a function of time. In chromatography, δ denotes the width at half-height ($\delta=2.35\sigma$), and σ^2 represents the variance of the peak. The peak width, ω, is measured at 13.5% of the height, and $\omega=4\sigma$.





With:

(**t_0**): Injection start
(**V_m**): dead volume.
(**t_m**): dead time.
(**V_e**): Elution volume (or retention volume, Vr) of a compound.
(**t_r**): Elution time (t_e) of a compound.
(**V_e**) = Flow x time.
(**V_e'**): Reduced elution volume ($V_e=V_e'+V_m$).
(t_r'): Reduced retention time ($t_r=t_r'+t_m$).
(**ω**): Peak width at the base.
(**δ**): Peak width at half-height.

VI-2- Retention Time and Displacement Velocity:

Retention time (t_r) is the time it takes for a solute to reach the detector, where its average linear displacement velocity is equal to:

$$\bar{v} = \frac{L}{t_r} \ldots\ldots (1)$$

With L as the length of the column. The average linear velocity of mobile phase molecules is given by:

$$\mu = \frac{L}{t_m} \ldots\ldots (2)$$

For given analysis conditions, retention time (t_r) is a characteristic value; it is independent of the injected sample quantity but depends on the nature and velocity of the mobile phase.

To establish a relationship between the displacement velocity of a solute and its distribution coefficient, its velocity is expressed as a function of the displacement velocity of the mobile phase, which is:

$$\bar{v} = \mu * a \ldots\ldots (3)$$

Where: **a** is the fraction of time spent by the solute in the mobile phase; it is equal to the quantity of solute present in the mobile phase (Q_S (ϕ_m)) divided by the total quantity of solute in the column (Q_S (t)):

$$\bar{v} = \mu * \frac{Q_s(\Phi_m)}{Q_S(t)} = \mu * \frac{C_m * V_m}{C_m * V_m + C_S * V_S} = \mu * \frac{1}{1+\frac{C_S * V_S}{C_m * V_m}}$$

$$\bar{v} = \mu * \frac{1}{1+K*\left(\frac{V_S}{V_m}\right)} \ldots\ldots (4)$$

VI-3- Capacity Factor:

The capacity factor (k_A) describes the rate at which solute A progresses through the column. It is defined in terms of the quantity of A using the following equation:

$$k_A = \frac{C_S * V_S}{C_m * V_m} = \frac{K_A * V_S}{V_m} \ldots\ldots (5)$$

Using equations (1), (2), and (4), the capacity factor (kA) can be expressed as a function of the retention time:

$$\bar{v} = \mu * \frac{1}{1+k_A} \Leftrightarrow \frac{L}{t_r} = \frac{L}{t_m} * \frac{1}{1+k_A} \Leftrightarrow k_A = \frac{t_r - t_m}{t_m} \ldots\ldots (6)$$

It is said that the separation is optimal if the values of k_A for various solutes fall between 1 and 5. In gas chromatography, k_A values are optimized by adjusting:

- ♣ Temperature.
- ♣ Column packing.

In liquid chromatography, this factor is optimized by adjusting:

- ♣ The composition of the stationary phase.
- ♣ The composition of the mobile phase.

VI-4- The selectivity factor between two solutes A and B:

The selectivity factor (α) corresponds to the ratio of distribution factors (K), capacity factors (k), or even reduced retention times (tr'). It indicates the column's ability to separate two compounds, α is always > 1.

$$\alpha = \frac{K_A}{K_B} = \frac{k_A}{k_B} = \frac{(t_r)_B - t_m}{(t_r)_A - t_m} \ldots\ldots (7)$$

VI-5- Column Efficiency:

In their theoretical studies, chemists ***Martin*** and ***Synge*** treat the chromatographic column as if it were made up of a series of plates similar to those in fractional distillation of petroleum. The efficiency of a chromatographic column is evaluated based on:

- ♣ The equivalent height of a theoretical plate (***H***).
- ♣ The number of theoretical plates (***N***).

These two quantities are related by the following equation:

$$N = \frac{L}{H} \ldots\ldots (8)$$

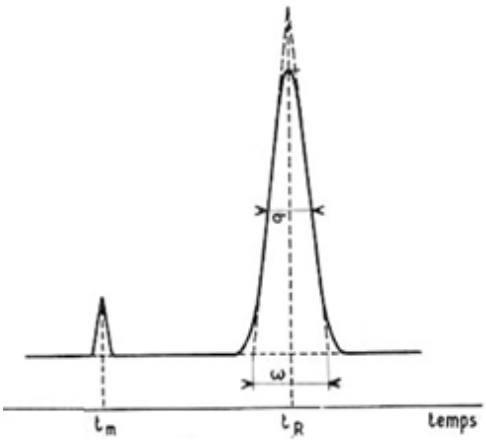
Where L is the length of the column. It can be observed that the greater the number of theoretical plates, the better the efficiency of a column. The following figure illustrates how (***N***) and (***H***) can be determined from a chromatogram:

$$H = \frac{\sigma^2}{L} \ldots (9)$$

$$H = \frac{L * \omega^2}{16 t_r^2} \ldots\ldots (10)$$

$$N = 16 \left(\frac{t_r}{\omega}\right)^2 \ldots\ldots (11)$$

$$N = 5{,}54 \left(\frac{t_r}{\delta}\right)^2 \ldots\ldots (12)$$



The following table summarizes the key parameters that affect the efficiency of a column:

Parameter	**Symbol**	**Units**
Linear velocity of the mobile phase	μ	$cm.s^{-1}$
Diffusion coefficient of the mobile phase	D_M	$cm^2.s^{-1}$
Diffusion coefficient of the stationary phase	D_S	$cm^2.s^{-1}$
Capacity factor	k	-
Particle diameter of the support	d_p	µm
Thickness of the liquid layer on the stationary phase	d_f	µm

VII- Effect of Elution Velocity on Column Efficiency, Van Deemter Equation:

The efficiency of a column is directly related to the number of theoretical plates. To obtain the maximum number of theoretical plates (***N***) in a column, one must define conditions where the plate height (***H***) is minimized (see equation (8)).

Therefore, it is necessary to study the different mechanisms of solute peak broadening during its progression within a chromatographic column. Solute peak broadening can be considered to have three origins:

- ♣ Dispersion of molecules due to longitudinal diffusion.
- ♣ The existence of "multiple paths" caused by the packing material.
- ♣ Resistance to mass transfer in each of the two phases.

The calculation of the variance corresponding to each of these factors was first demonstrated by Van Deemter according to the following relationship:

$$H = A + \frac{B}{\mu} + C * \mu \ldots\ldots (13)$$

With:

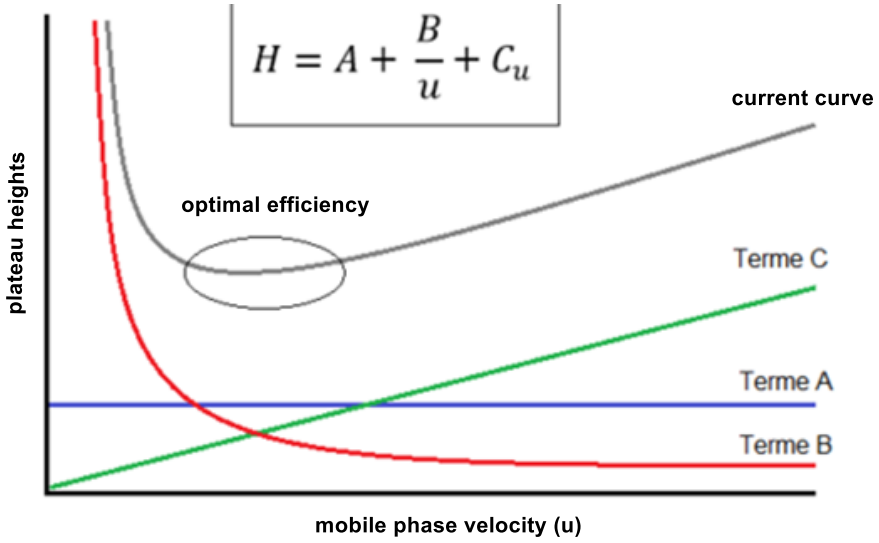
A: Turbulent diffusion coefficient or packing term: It refers to the preferential path taken by the solute due to the particle size and distribution of the stationary phase. A is related to the particle diameter of the stationary phase and the regularity of the packing.

B: Longitudinal diffusion coefficient: It refers to the solute's migration from more concentrated regions to less concentrated regions. The diffusion coefficient is more significant in gases than in liquids.

C: Mass transfer coefficient of the solute between the two phases.

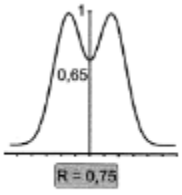
μ : The average velocity of the mobile phase (cm/s).

In conclusion, the most efficient columns are those that are consistently well-packed with the smallest possible particle diameter, along with optimal mobile phase flow rates.

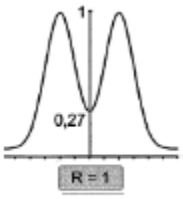


VIII- Column Resolution:

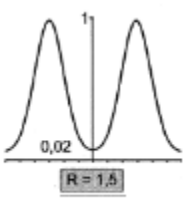
Column resolution (R) reflects the quality of separation between two solutes. The following figure illustrates chromatograms of species A and B on three columns with different resolution values:



C-1



C-2



C-3

Resolution (R) depends on the distance separating the peaks' summits and their base widths (ω) according to the following relationship:

$$R = 2 * \frac{t_r(B) - t_r(A)}{\omega_B + \omega_A} \ldots\ldots (14)$$

To highlight the main factors that influence the resolution factor, the following approximate expressions are often used:

$$R = \frac{\sqrt{N}}{4} * \left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_B}{1 + k_B}\right) \ldots\ldots (15) \Rightarrow N = 16R^2 \left(\frac{\alpha}{\alpha - 1}\right)^2 \left(\frac{1 + k_B}{k_B}\right)^2 \ldots\ldots (16)$$

The retention time (t_r (B)) can be expressed in terms of resolution (R) and the velocity of the mobile phase (μ):

$$t_r(B) = \frac{16R^2 H}{\mu} * \left(\frac{\alpha}{\alpha - 1}\right)^2 \left(\frac{1 + k_B}{k_B}\right)^3 \ldots\ldots (17)$$

According to the previous expressions, resolution depends on:

- ♣ Column efficiency (N).
- ♣ Selectivity (α).
- ♣ Capacity factor (k) or the retention times of the two species A and B. Therefore, to optimize a chromatographic analysis and obtain well-separated peaks, these three factors should be optimized first. a. Optimization of column efficiency: To achieve the maximum number of theoretical plates in a column (N), the conditions where the plate height (H) is minimized must be defined (see equation 8). The methods to minimize (H) include reducing:

The diameter of the stationary phase particles.

- ♣ The support diameter.
- ♣ Temperature (for gas chromatography (GC)).
- ♣ The thickness of the liquid film (for liquid chromatography (LC)).

The flow rate of the mobile phase can also be optimized:

VIII-1- Optimization of the capacity factor:

Significant improvement in separation can be achieved by modifying the capacity factor (*k*). Resolution (*R*) increases with (*k*) in the following two cases:

- ♣ Decreasing the temperature in the case of gas chromatography.
- ♣ Modifying the composition of the mobile phase in the case of liquid chromatography.

VIII-2- Optimization of selectivity:

There is no need to optimize N and k if the selectivity factor α is equal to 1. Several options are available to increase α while keeping $1 < k < 10$:

- ♣ Modify the mobile phase.
- ♣ Adjust the column temperature.
- ♣ Modify the composition of the stationary phase.

IX- Applications of Chromatography:

Chromatographic techniques are used for qualitative and quantitative analysis, as well as preparative applications, for the identification, quantification, and purification of various substances.

IX-1- Qualitative Analysis:

The number of peaks obtained on a chromatogram corresponds to the number of components present in the sample. Each molecule present in the sample is characterized by a retention time, which is reproducible to within 1% if all the relevant factors affecting it are carefully chosen.

IX-2- Quantitative Analysis:

The area under the peaks is proportional to the quantity of the injected substance. Each component of the mixture generates a signal (peak) with a larger area when its

concentration in the mixture is higher. The presence of only one peak in the chromatogram indicates that the product is pure; or the different components of the mixture are not separated on the stationary phase used.

IX-3- Preparative Applications:

In organic synthesis, chromatography is used to purify products obtained at the end of a reaction before identifying them using various spectroscopic methods (IR, NMR, X-ray, etc.).

Series 1

Exercise 1:

In an Erlenmeyer flask, 6 ml of silica gel and 40 ml of a solvent containing 100 mg of a compound considered non-volatile in solution are mixed. After thorough mixing, the mixture is allowed to settle, and 10 ml of the solvent is collected and evaporated. The residue weighs 12 mg.

1. Calculate the adsorption coefficient K (Nernst coefficient) of this compound in this experiment.

Exercise 2:

1. Calculate the separation factor between two compounds with retention volumes of 6 and 7 ml, respectively. The dead volume of the column used is 1 ml.

2. Demonstrate that this factor is equal to the ratio of the distribution coefficients K_2/K_1 of these compounds.

Exercise 3:

The resolution factor *R* for two solutes 1 and 2, whose elution peaks are adjacent, is expressed by equation (1):

$$R = 2 * \frac{t_{r(2)} - t_{r(1)}}{\omega_2 + \omega_1} \ldots . (1)$$

1. Show that, if we assume that the two adjacent peaks have the same width at the base ($\omega_1 = \omega_2$), expression (2) is equivalent to (1).

$$R = \frac{\sqrt{N}}{4} * \left(\frac{\alpha - 1}{\alpha}\right) * \left(\frac{k_2}{1 + k_2}\right) \ldots . . (2)$$

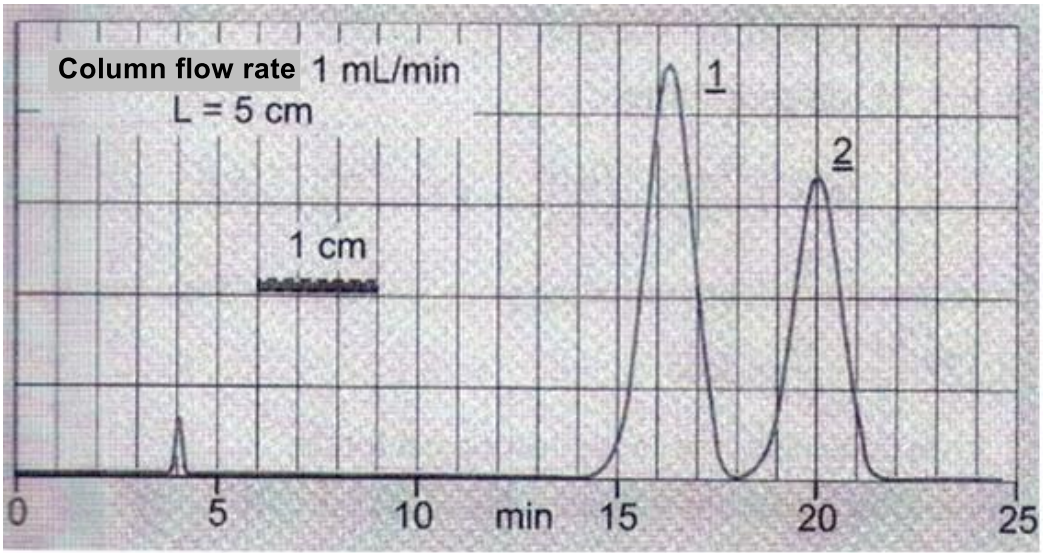
2. The following formula allows you to calculate the number of theoretical plates *N* as a function of α for a given resolution *R*. Find this expression:

$$N = 16R^2 \frac{\alpha^2}{(1-\alpha)^2} \ldots . . (3)$$

Exercise 4:

From the following chromatogram, calculate the following for compounds 1 and 2 at the origin of their respective peaks:

1. The dead volume of the column and the retention volumes.
2. The retention factors and the selectivity factor.
3. The height of a theoretical plate for compound 2 and the resolution factor between compounds 1 and 2.
4. Write the relationship that connects α to the distribution factors K_1 and K_2 for 1 and 2.



Tp n° 1	*Thin Layer Chromatography (TLC)*

Keywords: TLC, developer, retention factor (R_f), eluent, polarity.

Purpose of the experiment :

1. Determination of the purity of a solution.
2. Determination of the best mobile phase (eluent).
3. Use of universal and specific developers for the identification of the composition of three unknown solutions.

Products	Solvents / Solutions	Materials
3,3-dimethylbutan-2-ol *N*, *N*-dimethylpyridin-4-amine 1-(diethoxymethyl) -3-nitrobenzene 3,3-dimethylbutan-2-one Phenylmethanamine 3-nitrobenzaldehyde Aniline	Pentane Ethyl acetate Potassium permanganate solution Ninhydrin solution Three solutions of unknown composition	Tanks Test tubes Beakers Pasteur pipettes Capillaries Funnels

Thin layer chromatography (TLC) is an analytical technique used to visualize the contents of a mixture and thus determine the purity of a product. The results obtained can then be used in column chromatography of the mixture, using the same stationary phase and mobile phase.

Procedure :

Step 1 :

1. Cut three plates with dimensions close to 1.5 x 8 cm.

2. Using a capillary, make three deposits of a first solution (Sol-1) of unknown composition. These deposits of the diluted solution are made on a line drawn with a pencil 1 cm from the bottom edge of the plate.

3. Prepare three eluents with different polarities: 100% pentane, 100% ethyl acetate, and 80% pentane / 20% ethyl acetate.

4. Place the plates in tanks containing the eluent (1 to 2 mm high), with the deposits above the eluent. The eluent should rise vertically by capillarity; remove the plates when the solvent front level is 1 cm from the top.

5. Place the three plates under a UV lamp at 254 nm.

Step 2:

After selecting the best eluent, repeat the same instructions with solutions Sol-2 and Sol-3 using ninhydrin and $KMnO_4$ solutions as chemical developers.

Questions:

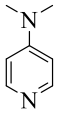
Q-1: What is the best mobile phase among the three eluents studied? Why?

Q-2: Knowing that the KMnO4 solution is a specific developer for alcohols, and ninhydrin is a developer for amines and amino acids, identify the composition of the three solutions Sol-1, Sol-2, and Sol-3.

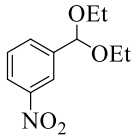
Q-3: Classify the different components of each solution in increasing order of polarity.

Data: Topological formula of the products used in this lab experiment.

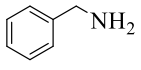
3,3-dimethylbutan-2-ol



N,*N*-dimethylpyridin-4-amine

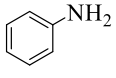


1-(diethoxymethyl)-3-nitrobenzene

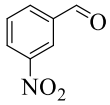


phenylmethanamine

3,3-dimethylbutan-2-one



aniline



3-nitrobenzaldehyde

Chapter II

TL, paper and conventional column chromatography.

I- Definition of thin layer chromatography:

Components in the sample are isolated using thin-layer chromatography (TLC), a technique in which a thin layer of stationary phase, typically made of silica gel, is applied to a flat rectangular surface like glass, plastic, or aluminum plates measuring 100-200 micrometers in thickness. To secure the stationary phase on the support and ensure the particles stick together, an organic binder is added during the plate's manufacturing process.

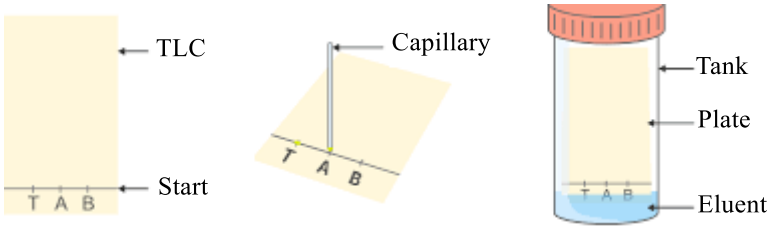
Thin-layer chromatography (TLC) is a complementary technique to HPLC; it offers several advantages:

1- It is faster and more cost-effective than HPLC.
2- It accommodates complex matrices.
3- It allows for two-dimensional analyses.
4- It makes it possible to run multiple analyses simultaneously.

II- Separation Steps :

II-1- Sample deposition:

We start by depositing a small volume of the diluted sample, near the bottom edge of the plate, in the form of a spot measuring 1 to 3 mm in diameter. This deposition can be done either manually or automatically, using a capillary with a flat tip.

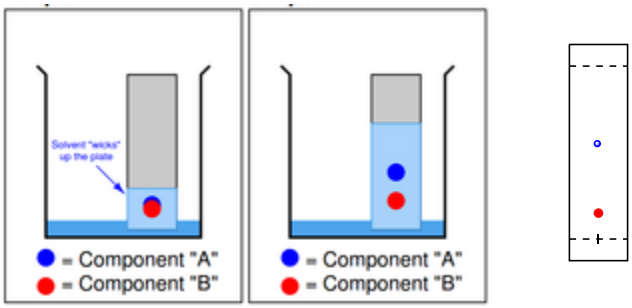


The prepared plate is then placed into a special chamber equipped with a cover, at the bottom of which is a small amount of the mobile phase, serving as the eluent. The spot containing the sample should be positioned above the immersion level.

II-2- Thin-Layer Chromatography development:

The mobile phase migrates through the dry stationary phase by capillarity, carrying the components to be separated at different rates. The migration time, which can span several minutes, depends on various parameters.

When the solvent front has traveled a distance considered sufficient (a few centimeters), the plate is removed from the chamber, the endpoint reached by the mobile phase is marked, and the latter is evaporated.



II-3- Thin-Layer Chromatography visualization:

The localization of compounds after migration is done on the plate using various visualizing agents, which can be spectroscopic or chemical:

a. Using a mercury vapor UV lamp (λ= 254 nm), any compound that absorbs at this wavelength (such as aromatic compounds) appears as a dark (or colored) spot against a green-illuminated background (due to the presence of zinc salts on the stationary phase).

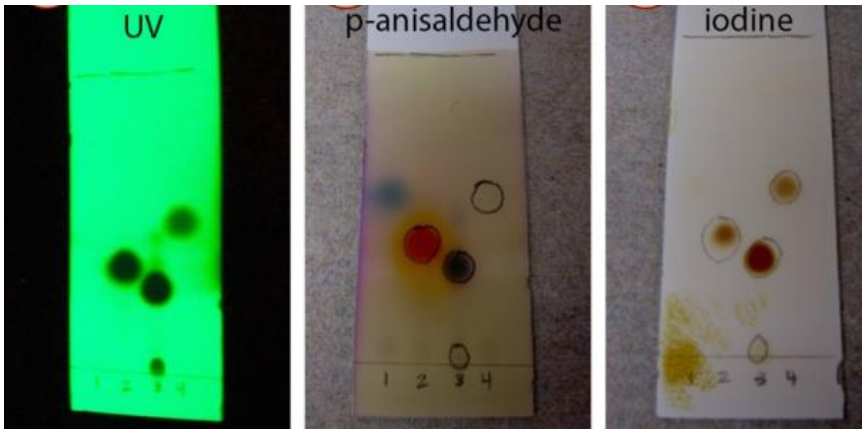
b. Alternatively, chemical visualizers can be used. This method involves charring the compounds by heating the plate after subjecting it to a spray of sulfuric acid, or visualizing by immersion in general reagents (phosphomolybdic acid, vanillin, iodine, $KMnO_4$), or specific ones (e.g., alcoholic ninhydrin for amino acids).

Here is a summary of various visualization techniques and the functional groups with which they typically react:

Chemical developer	Visualization	Preparation	Chemical Stains
Iodine	Strongly reacts with aromatics	An "iodine chamber" can be created by adding a few iodine crystals to a TLC chamber, or by adding a few iodine crystals to a chamber containing a portion of powdered silica or alumina	
***p*-Anisaldehyde**	aldehydes, ketones, and alcohols	135mL absolute ethanol, 5mL concentrated H_2SO_4, 1.5mL glacial acetic acid, and 3.7mL *p*-anisaldehyde.	
Vanillin	aldehydes, ketones, and alcohols	250mL ethanol, 15g vanillin, and 2.5mL concentrated H_2SO_4	
Permanganate	alkenes, alkynes, or oxidizable groups (aldehydes, alcohols)	1.5g $KMnO_4$, 10g K_2CO_3, 1.25mL10%NaOH(aq) and 200mL water	
Phosphomolybdic Acid	alcohols, phenols, alkenes, and many carbonyl compounds	5g phosphomolybdic acid in 500mL ethanol.	

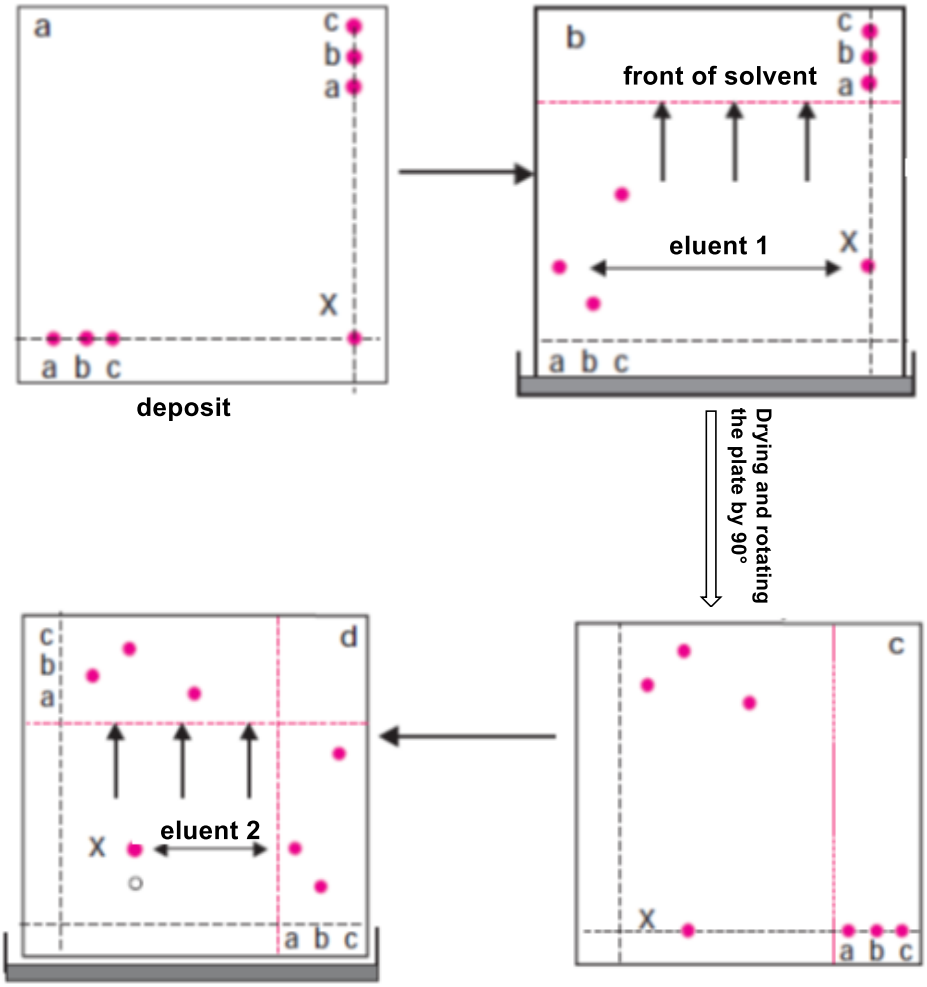
Iron (III)Chloride	phenols	1% $FeCl_3$ in water and CH_3OH (50%)	
Bromocresol Green	acidic compounds	100mL absolute ethanol, 0.04g bromocresol green, and 0.10MNaOH(aq) drop wise until solution turns from yellow to blue	

Visualization techniques are often customized to selectively detect specific functional groups. For example, in the following figure, various compounds are visualized using UV, *p*-anisaldehyde stain, and iodine. The compound in lane 1 of all the plates was only detectable with *p*-anisaldehyde stain, appearing as a blue spot, and not with UV or I_2. The compound in lane 4 of all the plates did not react with p-anisaldehyde stain but could be visualized using UV and I_2. The impurity located at the baseline of lane 3 exhibited strong UV activity but was barely visible with the other staining methods.



III-Two-Dimensional Thin-Layer Chromatography (2D-TLC):

Using a square-shaped plate allows for two-dimensional chromatography by performing two successive elutions with two different eluents. This experiment helps determine if a "spot" on the TLC plate actually contains two distinct compounds.



IV- Separation and Retention Parameters:

Each compound is defined by its R_f (short for "retention factor"), which corresponds to its relative migration compared to the solvent:

$$R_f = \frac{\text{distance traveled by the solute}}{\text{distance traveled by the solvent}} = \frac{x}{x_0}$$

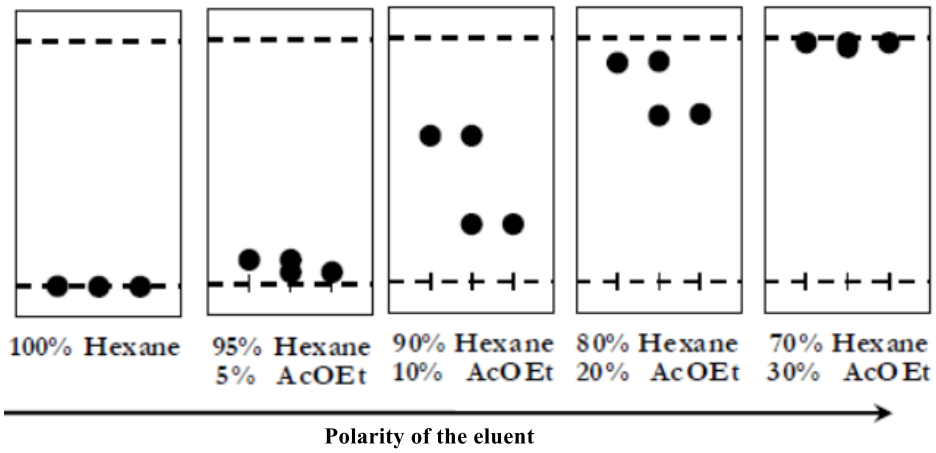
We define the efficiency *N* and the theoretical plate height *H* for a compound with a migration distance of x and a spot diameter ω using the following relationships:

$$N = \frac{16x^2}{\omega^2}, \quad H = \frac{x}{N}$$

To calculate the retention factor *k* of a compound or the selectivity or resolution R between two compounds, it is common practice to correlate the migration distances on the plate with the migration times read from a chromatogram:

$$k = \frac{1}{R_f} - 1, \quad R = 2\frac{x_2 - x_1}{\omega_2 + \omega_1}$$

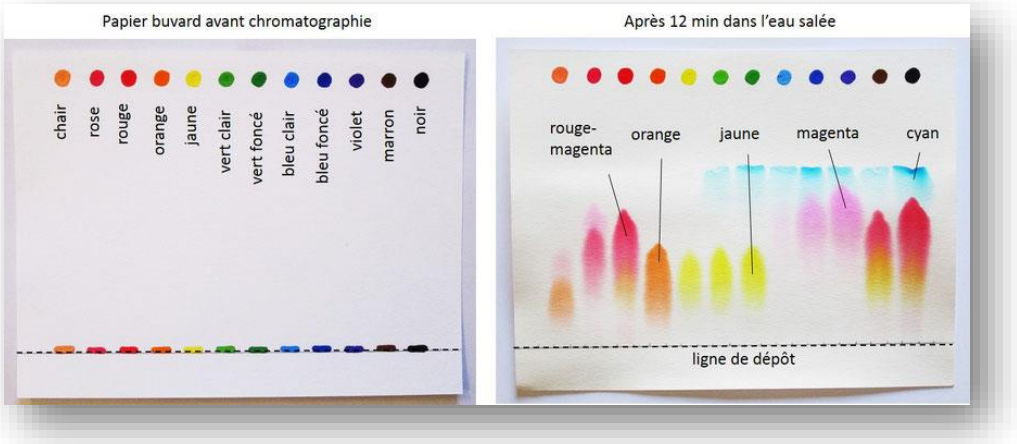
The choice of the mobile phase is crucial in achieving satisfactory resolution between two compounds. When performing a separation of two products by thin-layer chromatography, one seeks an eluent that provides the largest possible ΔR_f, with an R_f value for the least polar product around 0,3.



V- Paper Chromatography:

Paper chromatography is a planar chromatography technique that shares the same steps and separation principles as thin-layer chromatography. The difference between the two techniques lies in the nature of the stationary phase.

In paper chromatography, separation is typically achieved using specialized paper. In 1943, *Porter Martin* and *Richard Synge* conducted the initial separation of plant extracts through paper chromatography to identify the constituents within the extracts.



IV- Column Chromatography:

This solid-liquid chromatography technique enables separations of solute quantities ranging from 500 mg to several grams, depending on the column diameter and the amount of stationary phase used.

a. Initially, the column is filled with the stationary phase (silica or alumina) impregnated with a solvent. The liquid level in the column is lowered to the top of the stationary phase.

b. The sample to be analyzed is dissolved in a minimal amount of solvent (the mobile phase), and it is applied at the top of the column for elution. The solute components are carried downward at different rates.

The polarity of the eluent is an important factor. If the eluent is polar enough, the solute components are carried along; if it is nonpolar, the nonpolar components (less retained) will elute first, and the polar products will only migrate by the addition of a polar solvent (mixtures).

Polarity is the property of molecules having separated positive and negative centers, and this arrangement results from the nature of the atoms involved and their configuration. In chromatography, the definition is broadened to include properties such as hydrogen bonding and the phenomenon of polarization.

Oxygenated organic compounds, such as alcohols, ketones, esters, etc., have lower dipole moments and form hydrogen bonds less readily than water (a highly polar solvent). The relative polarity of solvents is characterized by their dielectric constant.

Less polar

1- Light hydrocarbons.
2- Cyclohexane.
3- Tetrachloromethane.
4- Trichloroethylene.
5- Toluene.
6- Benzene.
7- Dichloromethane.
8- Chloroforme.
9- Ethylic ether.
10- Ethyle acétate.
11- Acetone.
12- *N*-Propanol.
13- Ethanol.
14- Methanol.

More polar

15- Eau.

Series 2

Exercise 1:

A mixture of two compounds, A and B, results in two spots with the following characteristics (migration distance x and spot diameter ω):

* x_A = 27 mm et ω_A = 2 mm * x_B = 33 mm et ω_B = 2,5 mm

The migration of the solvent front in this experiment is 60 mm.

a. Calculate R_f, N, and H for each of the compounds.
b. Calculate the resolution factor between the two compounds, A and B.
c. Establish the relationship between the selectivity factor and the R_f of the two compounds. Calculate its numerical value.

Exercise 2:

1. The analysis by thin-layer chromatography (silica, eluent: hexane/ether 80/20) of 1.5g of a nonpolar organic compound gives us two spots: A with R_f=0.8 and B with R_f=0.2.

a. What can be concluded from the result of this TLC?
b. Which purification method should be used to purify this compound?
c. What chromatographic method will allow for the qualitative and quantitative analysis of this compound?

2. Arrange the following solvents in ascending order of polarity: Toluene, Propanol, Petroleum Ether, Chloroform.

Exercise 3:

The following figure represents the result of scanning a normal-phase TLC plate (mobile phase: hexane/acetone 80:20). The three compounds have structures A, B, and C.

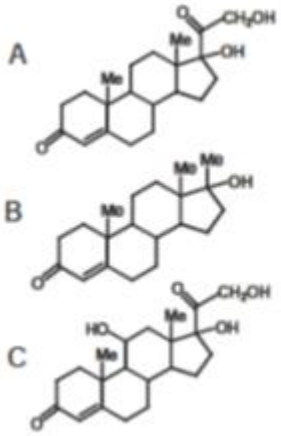
a. Assign each peak in the scan to the corresponding compound.

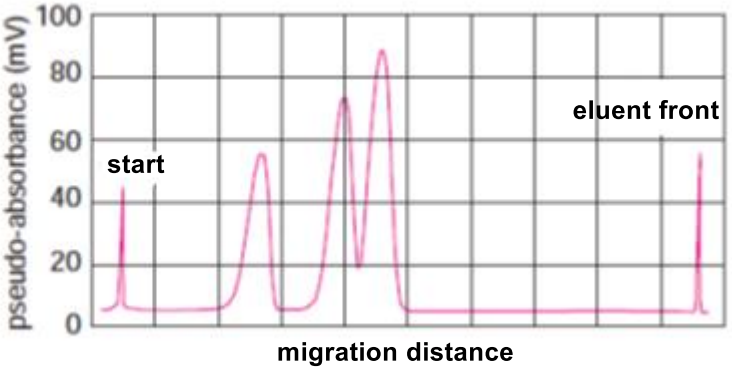
b. What would be the elution order of compounds A, B, and C on a column containing the same type of stationary phase and the same mobile phase?

c. What would be the elution order of compounds A, B, and C on a column containing an RP-18 type stationary phase with a mobile phase of acetonitrile/methanol (8:2)?

d. Calculate the R_f of the compound that migrates the fastest on the plate.

e. For the same compound, calculate, from the reproduced scan, the efficiency of the TLC plate and the corresponding HETP (height equivalent to a theoretical plate).





Exercise 4:

The substances A and B have respective retention times of 16,40 and 17,63 minutes on a 30 cm column. A non-retained species passes through the column in 1,30 minutes. The widths (at the base) of the A and B peaks are 1,11 and 1,21, respectively. Calculate:

a. The column resolution.

b. The average number of theoretical plates in the column.

c. The height equivalent to a theoretical plate.

d. The column length required to achieve a resolution of 1,5.

Tp n° 2	*Column Chromatography*

Keywords: TLC, Acetalization, Kaolin, Eluent, Polarity.

Purpose of the Experiment :

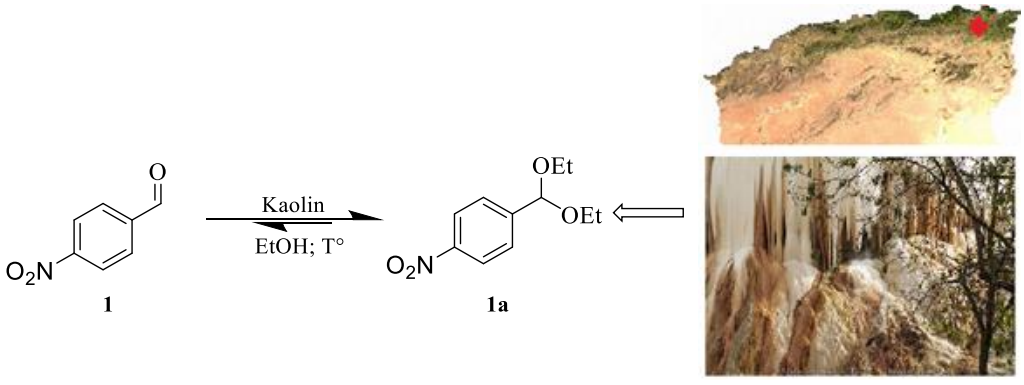
1. Determination of the amount of acetal formed.
2. Deduction of the extent of the acetalization reaction.
3. Characterization of the formed acetal through infrared spectroscopy.

Products	Solvents / Solutions	Materials
4-nitrobenzaldehyde. Kaolin.	Petroleum ether Ethyl acetate Ethanol.	Tanks, columns Test tubes, Beakers Pasteur pipettes, Capillaries Funnels, Test tubes TLC

Introduction :

Acetals are the most commonly used protective groups for carbonylated compounds, they are an intermediary's key for the synthesis of many target molecules as the preparation of perfumes, cosmetics, food additives, drugs and polymers.[1] The synthesis of acetals can be achieved using various catalysts.

In this manipulation, we are interested in the valorization of local kaolin clay for the synthesis of acyclic acetals under green chemistry conditions.[2]



[1] (a) M. Zhang, S. Lu, G. Li, L. Hong. *Catalysts*, 10, **2020**, 392. (b) A. K. Ghosh**,** M. R. Belcher., *J. Org. Chem.* **2020**, 85, 10399–10412.
[2] M. Boukachabia, H. Bendjeffal, L. Aribi-Zouioueche, O. Riant, Y. Bouhedja, *ChemistrySelect*. **2022**, 22, e202104610

Procedure :

Step n° 1:

Natural kaolin (100 mg) was added to a solution of 4-nitrobenzaldehyde **1** (1 mmol) in ethanol (4 ml), the reaction mixture was heated at 40°C and stirred for 24 h. the mixture was filtered and the solvent was removed in vacuo.

Step n° 2:

After evaporation of solvent, the product was purified by flash chromatography on silica gel (80/20 petroleum ether / ethyl acetate), according to the following steps:

- **a.** Preparation of silica gel by introducing an appropriate amount of SiO_2 into an Erlenmeyer flask containing the mobile phase (80/20 petroleum ether / ethyl acetate).
- **b.** Introduce the silica gel into a glass column and let it rest for several minutes.
- **c.** Number several test tubes and cut three TLC plates of similar dimensions.
- **d.** After 30 minutes of resting, introduce the reaction mixture onto the surface of the column using a pasteur pipette.
- **e.** Fill the test tubes and, using a capillary, make spots from each tube on a TLC plate.

Questions:

1. Find the quantity of the substance (n) of acetal formed **1a**.
2. Calculate the yield of the acetalization reaction.
3. Identify on the infrared spectrum, the characteristic bands of the formed acetal **1a**.

Chapter III

Gas Chromatography (GC)

I- Introduction:

Gas Chromatography (GC) is a widely used technique in both industrial and scientific research fields, and its development is primarily attributed to several advantages:

- **a.** To its extreme sensitivity,
- **b.** Its versatility,
- **c.** Automation possibilities,
- **d.** Quick development of new analyses.

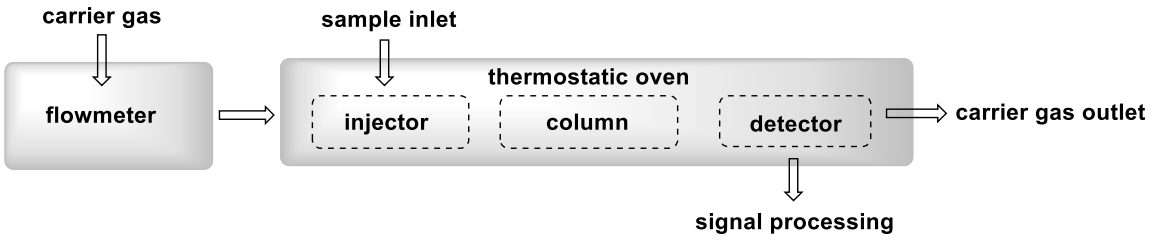
However, the use of GC is limited to compounds that must be in the gaseous state; the analysis of liquids or solids requires the ability to transform them into vapor through heating. This limitation is a major drawback of this technique, as it restricts its application to the study of thermally stable and sufficiently volatile molecular compounds. Two types of GC are distinguished based on the stationary phase:

1- Gas-liquid chromatography: The stationary phase is a liquid immobilized on a solid support by impregnation or grafting.

2- Gas-solid chromatography: The stationary phase is a porous solid, primarily used for the analysis of mixtures of gases or low-boiling-point liquids.

II- Description of the GC Apparatus:

A GC apparatus consists of the following compartments: injector, column, detector, and a thermostatically controlled oven that allows the column to reach a specific temperature. The mobile phase that carries the sample through the column is a gas, referred to as the carrier gas. Precisely controlled flow rates ensure high reproducibility of retention times.



The analysis begins when a very small quantity of the sample, either in liquid or gaseous form, is introduced into the injector. The injector serves the dual purpose of vaporizing the sample and introducing it into the gas flow at the head of the column. The column is typically a coiled tube of small diameter, ranging from 1 to over 100 meters in length. This column is placed within a temperature-regulated enclosure. The gaseous phase that has passed through the column is directed to a detector before being released into the open air.

III- Carrier gas:

One of the three following gases is used as the mobile phase: **helium**, **nitrogen**, or **hydrogen**. These gases are sourced either from a pressurized cylinder or a generator (water electrolysis for H_2 and air separation for N_2).

The carrier gas must be free from traces of hydrocarbons, water vapor, and oxygen, as these act as harmful impurities for certain polar stationary phases and reduce the sensitivity of the detectors.

IV- Introduction of the Sample:

A very small quantity of the sample in solution (0.5 ml) is introduced into the apparatus using a micro syringe, of which there are numerous models adapted to various injectors and columns.

To better control the reproducibility of results, an automatic injector can be adapted, allowing for the automation of syringe movements. When combined with a sample holder, it becomes possible to program the cyclic sequence of sample withdrawal, its rapid introduction into the injector, and syringe rinsing.

V- Injectors and Types of Injectors:

The injector is the gateway for the sample into the chromatograph. It has two additional functions:

a. Vaporizing the sample
b. Carrying it to the head of the column mixed with the carrier gas.

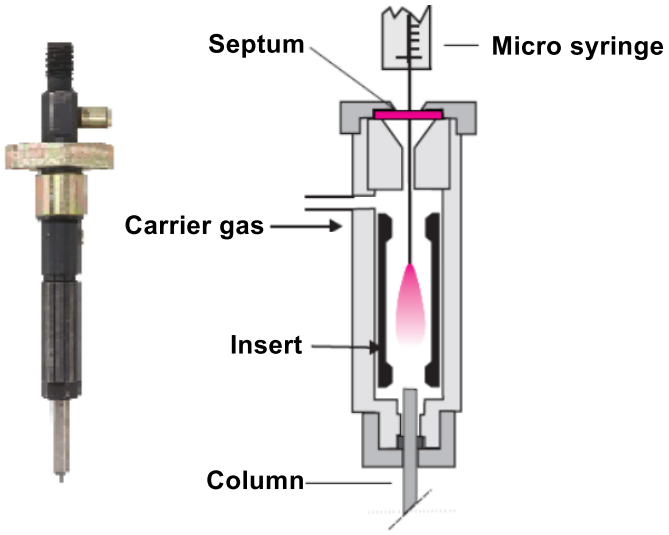
The characteristics of injectors, as well as the injection modes, vary depending on the types of columns they are connected to. Three types of injectors are distinguished:

V-1- Direct injection injector :

It consists of a metal tube lined with a glass liner (referred to as an insert) that is swept by the carrier gas and heated to the average boiling temperature of the compounds to be chromatographed.

The needle of the micro syringe containing the sample passes through one of the sealed ends of the injector, which is covered by a septum. The other end is connected to the also heated column. The entire introduced sample, immediately vaporized, travels into the column in a matter of seconds.

This method is suitable for packed columns and large capillary columns when the carrier gas flow rate reaches at least 8 ml/min.

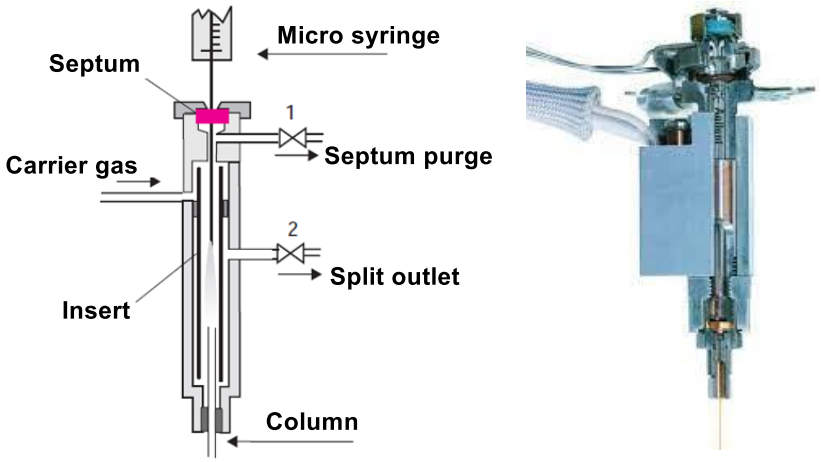


V-2- Split or splitless injector:

For capillary columns with low sample capacity, the smallest volumes that can be drawn with a micro syringe can saturate the column. In such cases, injectors can operate in two modes: with or without splitting (commonly referred to as split or splitless).

A stream of carrier gas enters the vaporization chamber at a high flow rate, where it mixes with the injected sample. A split valve, typically set between 50 and 100 ml/min, divides this flow into two fractions, with the larger one being eliminated from the injector body.

This type of injector can also operate in splitless mode. In this introduction mode, intended for very dilute solution samples, the contents of the micro syringe are injected slowly, with valve 2 kept closed for 0.5 to 1 minute. This allows the compounds vaporized with the solvent to concentrate in the initial centimeters of the column.



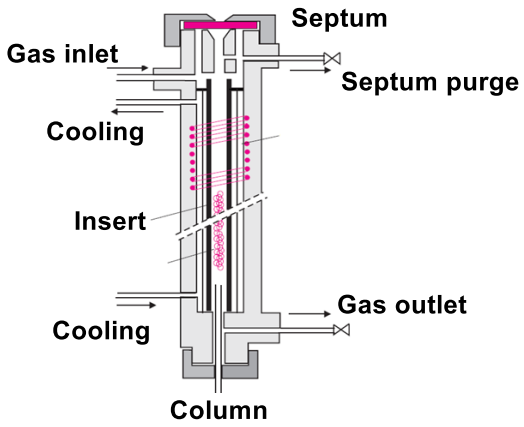
V-3- Programmable temperature injector:

This injector, also known as PTV (Programmed Temperature Vaporizer), is designed similarly to the split/splitless injector. However, the injection chamber's temperature can be programmed, ranging from 20 to over 300°C. Its three main operating modes are:

a. **In the cold split injection**, the sample is introduced into the cold vaporization chamber. Since the sample is not instantly vaporized, the solvent and different compounds enter the column in order of their boiling points.

b. **The cold splitless injection mode** is used for trace analysis.

c. In the **solvent elimination injection mode**, the sample is introduced into the cold injector. After injection, the split valve is opened to remove all the solvent. Then, the injector is heated to allow the transfer of heavy compounds into the column.



VI- Thermostatic Chamber:

The chromatograph includes a chamber that allows heating the column to over 400°C. It must have low thermal inertia to enable controlled and rapid temperature increase (with a ramp of up to 100°C/min) and excellent stabilization.

By adding a cryogenic valve supplied with liquid N_2 or CO_2, the chamber can be regulated at low temperatures.

VII- Columns and Types of Columns:

There are two types of columns: packed columns (or packed columns) and capillary columns. For packed columns, the stationary phase is immobilized by impregnation or by chemical reaction with the porous support. For capillary columns, a thin layer of the stationary phase is either deposited or grafted onto the inner surface of the column.

VII-1- Packed Columns:

These columns, with diameters of 3.18 or 6.35 mm and lengths ranging from 1 to 3 m, are made from steel or glass tubes with their inner walls treated to prevent potential catalytic effects on the sample. They can handle a carrier gas flow rate ranging from 10 to 40 ml/min. Packed columns are not suitable for trace analysis.

VII-2- Capillary Columns:

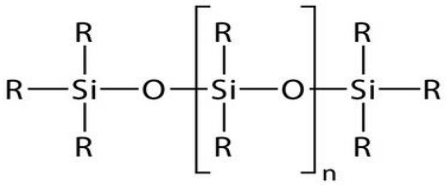
These columns are typically made of high-purity fused silica, with internal diameters ranging from 100 to 530 µm and lengths of up to 100 m. They have an outer brown polyimide coating, a thermally stable polymer (T_{max} = 370°C), to make them less fragile and allow them to be coiled using a suitable metal support.

VIII- Stationary Phases:

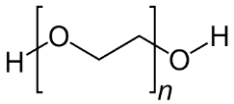
Four types of stationary phases are distinguished:

1. **Polysiloxane Type Stationary Phase:** Polysiloxanes (also known as silicone gum) consist of the repetition of a basic pattern that includes two carbon chains per silicon atom. By combining monomers in various proportions, it is possible to adjust the

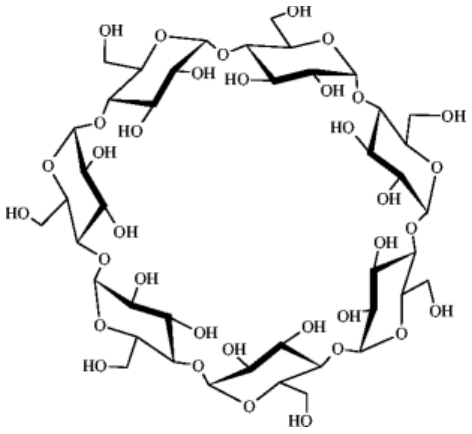
properties of the stationary phases, such as polarity and the range of stability. Due to their wide temperature range, these are the most commonly used phases for capillary columns.



2- **Polyethylene Glycols (PEG) Type Stationary Phase:** The most well-known representatives of this family are Carbowax, which are polar polymers (1,500 < M < 20,000) that can be used in the deposited, impregnated, or grafted mode.



3- **Chiral Stationary Phase:** These are typically polysiloxane phases containing between 10 and 20% by mass of *β*-cyclodextrin molecules (a chiral polysaccharide) incorporated into the base polymer. This type of column is used when examining the optical purity of analytes. If an organic compound, for example, has an asymmetric carbon, the *R* and *S* enantiomers will not have exactly the same affinity for the cyclodextrin-loaded stationary phase, resulting in different retention times. Therefore, a chemically pure compound in the form of a racemate will produce two equal peaks, each corresponding to a single enantiomer.



4- **Solid Stationary Phase:** These phases are composed of various adsorbent materials: deactivated silica or alumina with mineral salts, 5 Å molecular sieves, glass, porous polymers, and graphitic carbon.

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

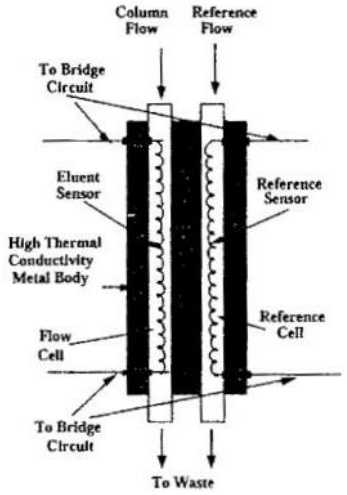
IX- Main Detectors:

Some detectors are universal, meaning they are sensitive to almost all eluted compounds, while others are much more sensitive to a particular type of molecule. All detectors provide a response that depends on the molar or mass concentration of the solute in the carrier gas. There are five main types of detectors:

IX-1- Thermal Conductivity Detector (TCD):

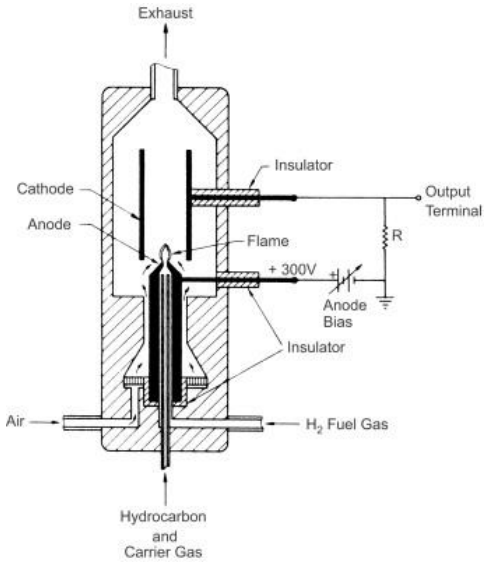
Is a universal but relatively insensitive device. It is based on a continuous comparison between the heat flow carried by the pure carrier gas and the heat flow carried by the carrier gas loaded with solute molecules, using two thermistors placed in parallel.

The thermal conductivity detector (TCD) has the advantage of not destroying the analyzed substances. However, its main drawback is its low sensitivity.

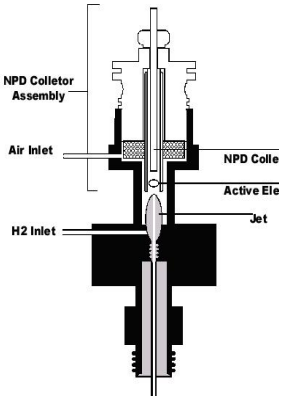


IX-2- Flame Ionization Detector (FID):

Considered almost universal for organic compounds, the gas stream from the column enters the flame of a small burner fed by a mixture of hydrogen and air. This detector destroys the sample, and the combustion produces ions and charged particles, leading to the flow of an extremely low ionic current (10^{-12} A) between two electrodes. The tip of the burner serves as the polarization electrode, while the second electrode, in the form of a ring, surrounds the flame. The signal is amplified by an electrometer into a measurable voltage. For organic compounds, the signal intensity is sensitive to the mass flow rate of the sample.



IX-3- Nitrogen-Phosphorus Detector (NPD):

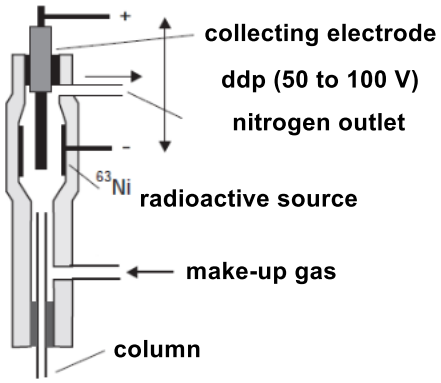


This detector is highly sensitive to compounds containing nitrogen (N) or phosphorus (P). It consists of a small cylinder made of ceramic doped with an alkali salt (e.g., rubidium sulfate) to which an electric voltage is applied to sustain a small plasma (800°C) fueled by the combustion of an air/hydrogen mixture.

Unlike the FID, the flame is smaller. Compounds containing N or P produce decomposition fragments that are transformed into negative ions. These ions are collected on a collector electrode.

IX-4- Electron Capture Detector (ECD):

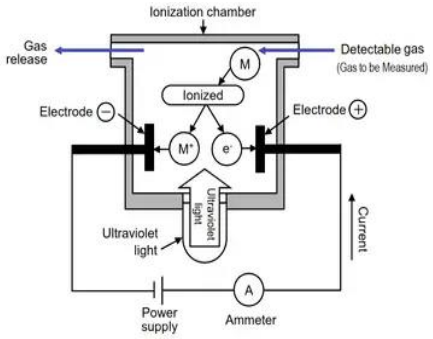
This detector is considered selective because it is much more sensitive to halogenated derivatives. A flow of nitrogen, ionized by a stream of electrons generated by a low-energy radioactive source, circulates between two electrodes. If solute molecules containing a halogen (F, Cl, or Br) pass through the region between the two electrodes, they capture some of the electrons to form heavy negative ions, which are less mobile.



The intensity collected follows a decreasing exponential law of the type $\mathbf{I = I_0 \exp[-kc]}$. The presence of a radioactive source in this detector subjects it to special regulations. It is often used in the analysis of chlorinated pesticides.

IX-5- Photoionization Detector (PID):

The photoionization detector is quite selective and suitable for hydrocarbons as well as derivatives containing sulfur (S) or phosphorus (P). The principle of this detector involves exposing the eluted compound to a UV lamp emitting highly energetic photons (from 8.4 to 11.8 eV). Photoionization occurs when the energy of the photon is greater than the compound's first ionization energy.



Collecting the electrons released by an electrode connected to an electrometer allows for concentration measurements. This is a detector that can operate at temperatures exceeding

400 °C and is non-destructive. Ionization is reversible and only affects a small fraction of the molecules of each compound.

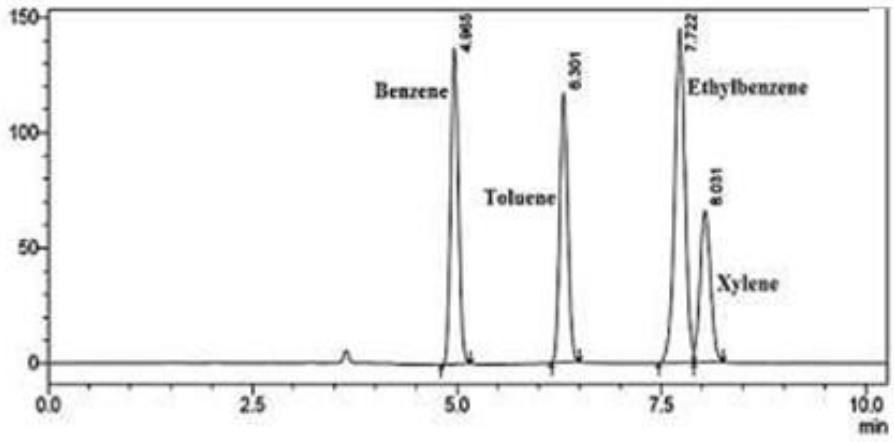
The following table summarizes the various types of detectors based on the nature of the products to be analyzed:

Detector type	Selectivity	Detectability
Thermal Conductivity Detector (TCD)	Universal	1 ng
Flame Ionization Detector (FID)	Organic products	100 pg
Electron Capture Detector (ECD)	Halogenated and organometallic products	50 pg
Nitrogen-Phosphorus Detector (NPD)	Nitrogenous or phosphorous products	10 pg
Photoionization Detector (PID)	Oxygenated and sulfur products	2 pg

X- Qualitative Analysis/Quantitative Analysis :

X-1- Qualitative Analysis:

The number of peaks obtained on a chromatogram corresponds to the number of components present in the sample. Each molecule in the sample is characterized by a retention time, which is reproducible to 1% if all the factors on which it depends are carefully selected.



The retention time depends on the following factors: flow rate of the carrier gas, nature of the stationary phase, column dimensions and temperature.

$$t'_r = t_m * \frac{K}{\beta} \quad ou \quad t_r = t_m * \left(1 + \frac{K}{\beta}\right) \ldots\ldots (1)$$

K, varies with temperature according to the following equation:

$$Ln\ K = -\frac{\Delta G^0}{RT} \ldots\ldots (2)$$

If K >> 1, then ΔG^0 is negative. By combining this expression (2) with the equation for reduced retention time (1), we find the relationship between reduced retention time and temperature (3):

$$Lnt'_r = -\frac{\Delta G^0}{RT} - Ln\beta + Lnt_m \ldots\ldots (3)$$

With: $\Delta G^0 = \Delta H^0 + T\Delta S^0$ One can deduce: $Lnt'_r = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} - Ln\beta + Lnt_m \ldots (4)$

Or: $Lnt'_r = \frac{A}{T} + B \ldots (5)$

The dead time, t_m can be assumed to be independent of temperature for a given column and pressure. For two temperatures: $T_2 > T_1$, with their corresponding retention factors: k_2 and k_1, formula (4) becomes:

$$Ln\frac{t'_{r1}}{t'_{r2}} = -\frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) = -\frac{\Delta H(T_2 - T_1)}{RT_1T_2} \ldots\ldots (6)$$

a. <u>Kovats index:</u>

The Kovats index is a reference tool for compound identification. If a solute is eluted between two alkanes (n) and (n+1), its Kovats index Ix is given by the following formula:

$$I_x = 100n + 100\frac{Logt'_r(x) - Logt'_r(n)}{Logt'_r(n+1) - Logt'_r(n)} \ldots\ldots (7)$$

Many chemicals are listed in Adams tables with their Kovats indices[1]:

Compounds	Kovats retention index[a]	Peak area %
Sabinene	975	1.9
Myrcene	990	0.9
Limonene	1029	0.2
(*E*)-β-Ocimene	1050	0.5
γ-Terpinene	1059	0.3
cis-Linalool oxide	1072	1.0
trans-Linalool oxide	1086	0.5
Linalool	1096	4.6
Citronellal	1153	59.9
Isopulegol	1159	0.2
Terpinen-4-ol	1177	0.6
Citronellol	1225	20.7
Nerol	1229	0.3
Citronellyl acetate	1352	4.5
Neryl acetate	1361	0.1
Geranyl acetate	1381	0.5
(*E*)-Caryophyllene	1419	0.9
α-Humulene	1454	0.2
Bicyclogermacrene	1500	0.2
δ-Cadinene	1523	0.3
Hedycaryol	1548	0.2
(*E*)-Nerolidol	1563	0.8
Caryophyllene oxide	1583	0.2

GC-MS, gas chromatography mass spectrometry.
[a]Kovats retention index was identified by Adams and Davies (1990).[31,32]

b. <u>McReynolds constant:</u>

The McReynolds constant is used to characterize the polarity of a stationary phase. It is defined as the difference between the Kovats index of a compound on the phase under study and its index on a reference phase called squalane:

$$\Delta I = I_{phase} - I_{squalane} \dots\dots (8)$$

[1] (a) V. I. Babushok, P. J. Linstrom, I. G. Zenkevich. *J. Phys. Chem. Ref.* Data, Vol. 40, No. 4, **2011**, (b) M. Lucero, R. Estell, M. Tellez, E. Fredrickson, *Phytochem. Anal*. **2009**, 20, 378–384.

X-2- Quantitative Analysis:

Theory shows that the area under the peaks is proportional to the amount of injected product. In other words, each component of the mixture produces a signal (peak) with a larger area when the concentration of that component in the mixture is higher.

The presence of a single peak on the chromatogram proves that the product is pure, or that the different components of the mixture are not separated on the stationary phase used.

The stationary phases differ in their polarity. Polar phases will adsorb polar substances more strongly, while on non-polar phases, the components of the mixture will elute in order of their respective increasing boiling points.

Series 3

Exercise 1:

Here is a table of the values of the capacity factor (*k*) for four refinery gases studied at three different temperatures on the same capillary column (LC = 30 m, ID = 250 mm) with an SE-30 stationary phase. The chromatograph is equipped with a cryogenic accessory.

		Column temperature (°C)		
Compound	**Bp (°C)**	**-35**	**25**	**40**
Ethylene	-104	0,249	0,102	0,083
Ethane	-89	0,408	0,148	0,117
Propene	-47	1,899	0,432	0,324
Propane	-42	2,123	0,481	0,352

a. Based on the elution order, can we say if the SE-30 phase is polar or not?

b. Calculate the selectivity factor for the propene-propane pair at the three indicated temperatures.

c. Why does k decrease as the temperature increases for the same compound?

d. What is the number of theoretical plates in the column for propane at 40 °C, knowing that at this temperature, the resolution factor for the propene-propane pair is 2? Calculate the corresponding HETP.

Exercise 2:

In a series of GC (Gas Chromatography) analyses, we aim to determine the influence of column length on certain parameters of the chromatogram. All experiments are conducted under the same temperature and gas flow rate conditions.

L (m)	$a = \sqrt{L}$	t_r (min)	t_r/L	R	R/a
15		3,7		2,05	
30		7,5		2,91	
60		15,3		4,15	

a. Complete the table.

b. What simple relationship can be considered between retention time and column length?

c. What simple relationship can be considered between resolution and the square root of the column length?

d. The two peaks used to calculate R have retention times of 8.3 minutes and 9.7 minutes with a column length of 60 meters. Calculate the width at half-height of these peaks (assuming that both peaks have nearly the same width at half-height) and the column efficiency for the solute with a retention time of 8.3 minutes.

Exercise 3:

Multiple injections of the same compound mixture are performed isothermally at different temperatures on the same column:

- At 120°C, the compound X of interest elutes at 15.2 minutes with a peak width (tm) of 0.2 minutes.
- At 150°C, the same compound elutes at 10.1 minutes with a tm of 0.17 minutes. Based on the interpretation of several chromatograms, it appears that the most suitable working temperature to separate the compounds of interest is between 132°C.

At what retention time (tr) should the compound X elute if tm is 0.19 minutes?

Exercise 4:

The chromatogram of nicotine, the component responsible for the toxicity of tobacco, was obtained by GC under the following conditions: packed column: 10% Carbowax 20% KOH on 80/100 Chromosorb (183 cm) x 2 mm ID, mobile phase flow rate: 20 ml/min, injection volume: 1 microliter. Oven temperature: 200°C, retention time of nicotine = 3.20 min. Oven temperature: 180°C, retention time of nicotine = 6.60 min.

a. Calculate the Linear velocity (in cm/s) of the mobile phase in the column.

b. Deduce the dead time of this analysis.

c. Calculate the retention time of nicotine at 160°C.

d. Can nicotine be eluted in 1 minute, knowing that the column's maximum operating temperature is 250°C?

e. The nicotine peak has a width at half-height of 10 seconds. Calculate N (number of theoretical plates) and H (height equivalent to a theoretical plate).

Tp n° 3	*Gas Chromatography (GC)*

Keywords: Terpene, Acylation, Kaolin, GC.

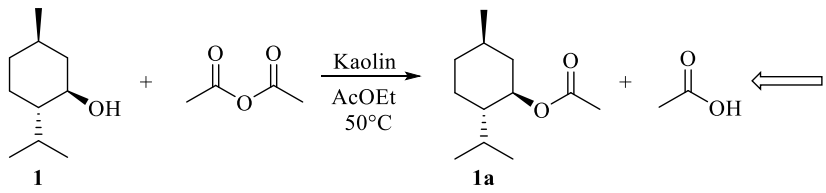
Purpose of the Experiment :

1. Calibration of the GC.
2. Study the kinetics and calculate the activation energy of an acylation reaction.
3. Calculate the enthalpy, entropy, and Gibbs energy of this reaction.

Products	Solvents / Solutions	Materials
Menthol. Kaolin. Acetic anhydride. Ethyl acetate.	Petroleum ether. Ethyl acetate.	Tanks, Pasteur pipettes, Capillaries TLC, GC.

Introduction :

Terpene esters are highly desirable molecules for their organoleptic and phytotherapeutic properties; Acetates of: Menthyl, genaryl and linalyl are specific components of several essential oils[1]. A fairly large number of methods have been developed to synthesize these types of compounds. In a recent work, we successfully used a local kaolin originating from Hammam Debagh-Guelma in northeastern Algeria for the chemoselective synthesis of acyclic acetals under green chemistry conditions[2]



[1] Zeferino, R. C. F., Piaia, V. A. A., Orso, V. T., Pinheiro, V. M., Zanetti M., Colpani, G. L., Padoin, N., Soares, C., Fiori M. A., Riella H. G. *Chem. Eng. Res. Des.* **2021**, (*168*), 156–168.
[2] M. Boukachabia, H. Bendjeffal, M. M. Khelassi, O. Riant. *Flavour Fragr. J.*, **2023**, 1-11

Procedure:

Step n° 1:

Natural kaolin (100 mg) was added to a mixture of alcohol (1 mmol of *l*-menthol) and acetic anhydride (1.5 mmol) diluted with ethyl acetate (3 ml). The reaction mixture was stirred for 1h at 50°C.

Step n° 2:

1. To determine the separation conditions of *l*-menthol **1** and its corresponding acetate **1a**, reference injections are performed on the GC at different temperatures.
2. In order to determine the calibration factor, three solutions **1/1a** of different concentrations are successively injected into the GC under the previously found separation conditions.
3. Note the areas of the peaks: alcohol **1** / acetate **1a**, and use the following formulas to calculate the calibration factor:
4. Take samples from the acylation reaction every 10 minutes and calculate the yield using the previous formulas.
5. Re-run the acylation reaction at temperatures of 30°C, 40°C, and 50°C to calculate the thermodynamic parameters.

Questions:

1. Calculate the calibration factor.
2. Find the reaction order and calculate the activation energy for the acylation of *l*-menthol.
3. Calculate the enthalpy, entropy, and Gibbs energy of this reaction.
4. Determine the nature of the stationary phase.

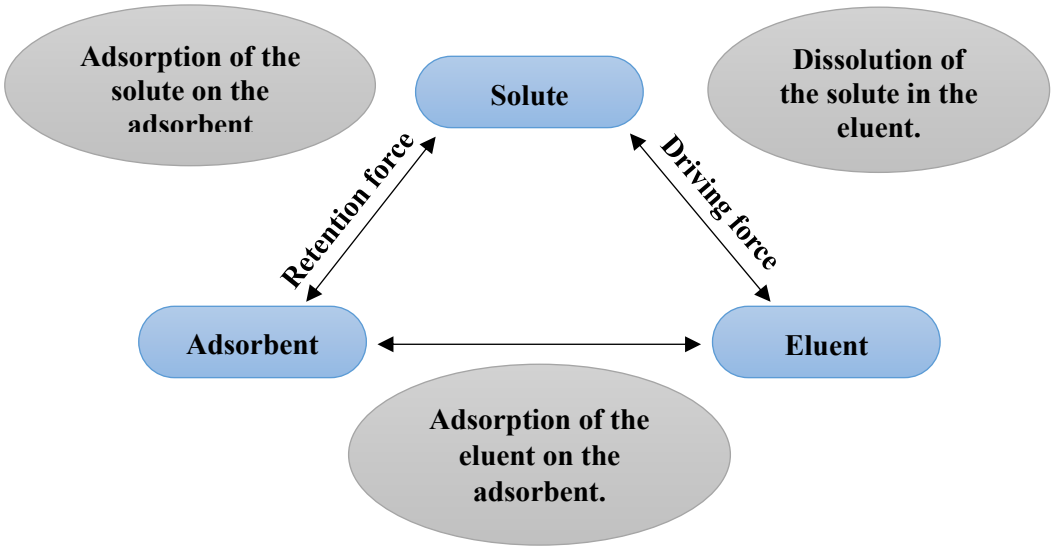
Chapter IV

High-Performance Liquid Chromatography (HPLC)

I- Introduction:

High-Performance Liquid Chromatography (HPLC), is an instrumental technique that allows for the separation of components in a non-volatile, thermosensitive, polar, and high molecular weight mixture for the purpose of identification and quantification.

Its success is attributed to the precise control over compound selectivity by choosing the appropriate column and mobile phase composition, which involves exploiting solute interactions with the mobile phase and stationary phase.



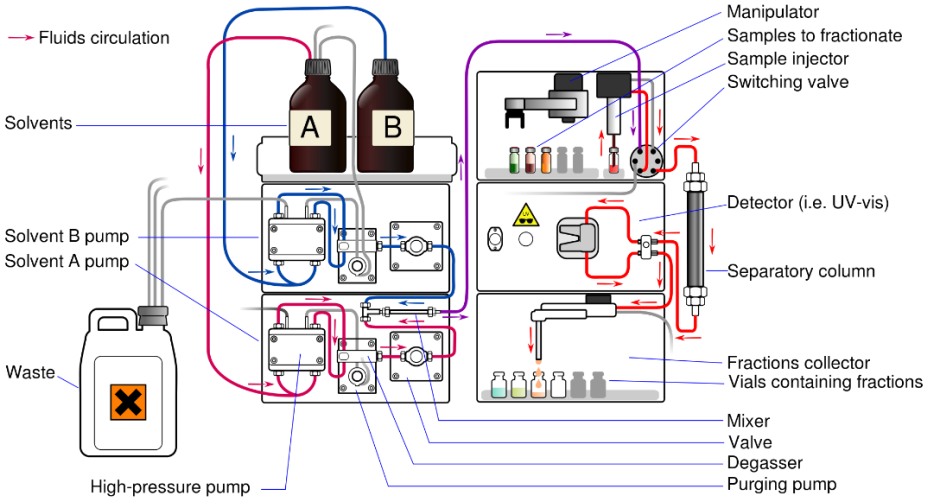
What sets HPLC apart is its utilization of solute/mobile phase/stationary phase exchange mechanisms based on adsorption or partition coefficients.

II- General Design of an HPLC Apparatus:

An HPLC setup consists of various specialized modules, which are housed in separate enclosures or integrated within the same chassis.

An HPLC apparatus typically includes the following modules: a reservoir containing the mobile phase, a degasser, a pumping system, an injector, a column, a detector, through which a liquid carries the components of a mixture to be separated, and a data acquisition

system. These modules are interconnected via very narrow internal diameter (0.1 mm) pipelines to ensure the circulation of the mobile phase. These pipelines can be made of stainless steel or solvent-resistant polymers, even under high pressures.



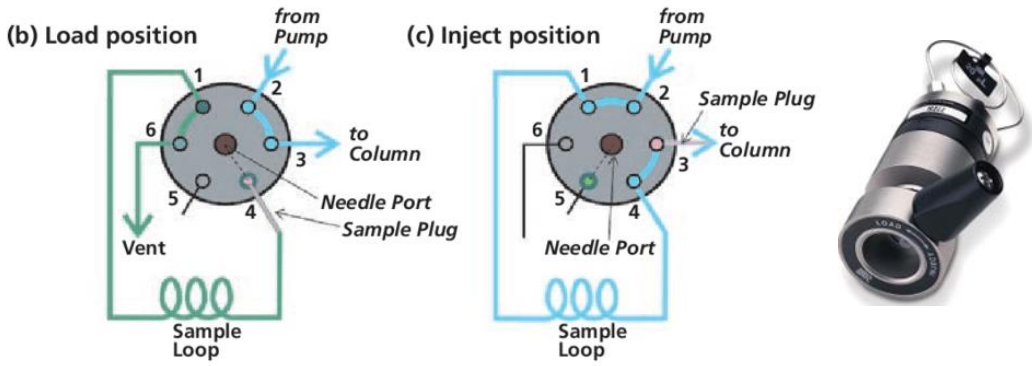
II-1- Pumps:

Every HPLC setup includes at least one pump to propel the mobile phase through the highly compact column. This results in a substantial pressure at the injector level, which can reach up to 20,000 kPa (200 bars) depending on the flow rate imposed on the mobile phase, its viscosity, and the nature of the stationary phase.

II-2- Injectors:

The injection of a precise volume of the sample at the column's head must occur rapidly to minimize disruption to the flow of the mobile phase, which needs to remain stable from the column to the detector. The sample injection is carried out using a valve:

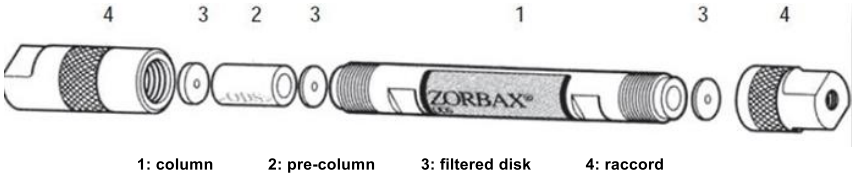
1. First, the sample is introduced into a loop of known volume (loading position).
2. After rotating the valve lever 60° to reverse the flow direction within the loop (injection position), the mobile phase carries the sample to the column's head.



II-3- Columns:

The column is typically a stainless-steel tube with varying lengths and diameters depending on the specific model. The column is often preceded by a pre-column, called a guard column, which is short (0.4 to 1 cm) and filled with the same stationary phase, used to retain certain impurities.

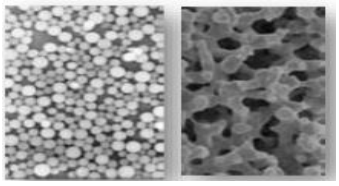
These columns have the advantage of faster analysis, lower solvent consumption, and improved analysis resolution.



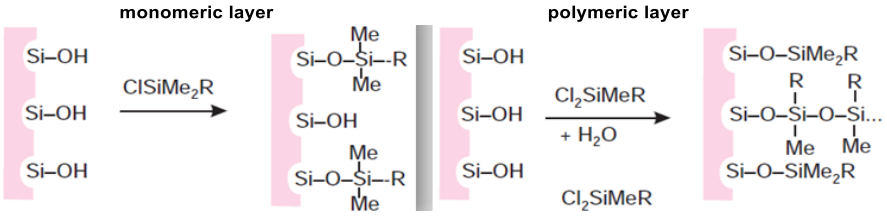
II-4- Stationary Phases:

The quest for effective chromatographic resolution has led to the development of stationary phases with various natures and structures. Several types of stationary phases are distinguished:

Silica-based Stationary Phase: The properties of silica gels depend on various parameters, including internal structure, porosity (size and distribution of pores), specific surface area, crush resistance, and polarity.

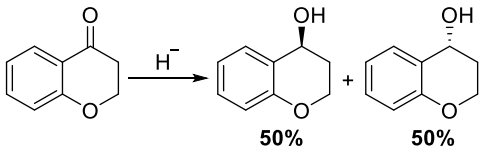


Silica Grafted Stationary Phase: Grafted phases are the foundation of reverse-phase polarity partition chromatography, which is used in nearly all separations. Surface modifications of the gel result in two types of phases: monomeric phases (10–15 µm thick) and polymeric phases (25 µm or thicker).



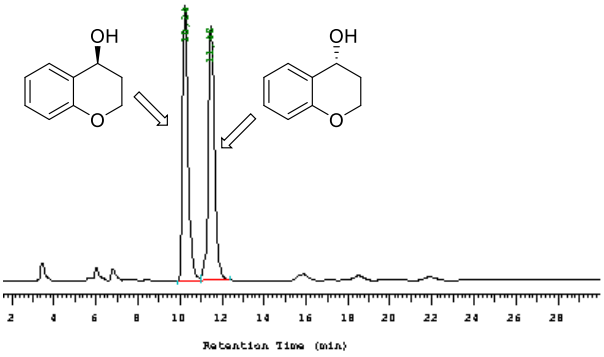
III- Chiral Chromatography:

An organic compound with a structural formula that reveals the presence of a chiral center typically leads to a mixture of the two possible enantiomers, *R* and *S*.

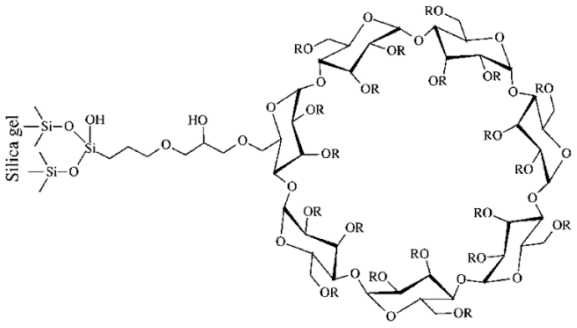


When this compound is chromatographed on a column with a chiral stationary phase, meaning it possesses identical chiral centers corresponding to only one enantiomer (*R* or *S*), two peaks are observed on the chromatogram. These peaks result from reversible

interactions between the enantiomers of the compound and the enantiomer of the stationary phase.

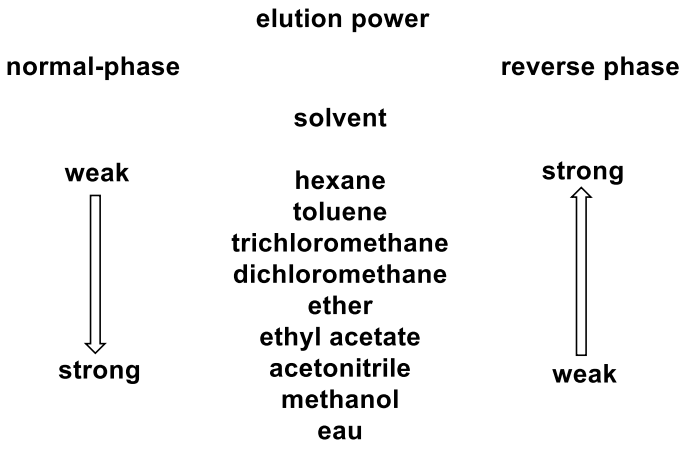


The stationary phases are optically active resins or silica gels grafted with cyclodextrins (cyclic assemblies of 5 to 7 glucose molecules) via an "arm" containing multiple carbon atoms.

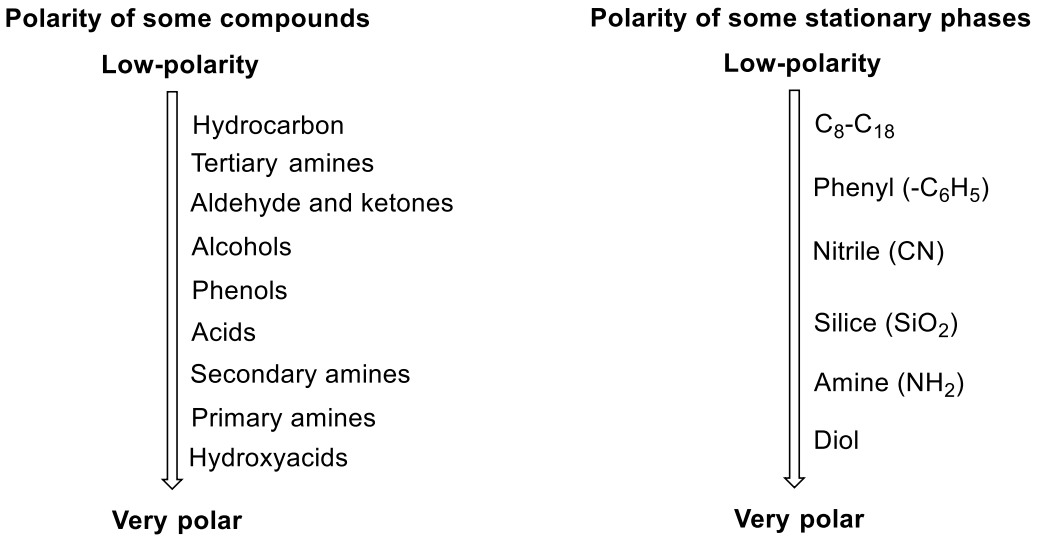


IV- Mobile Phases:

Following a general principle, a polar stationary phase is paired with a nonpolar or weakly polar mobile phase. In this case, the chromatography is referred to as "normal phase." Conversely, when the opposite occurs, it is called "reverse phase" chromatography.



With an apolar stationary phase (such as a C_8 and C_{18} paraffinic layer), the elution order is reversed compared to that of normal phase chromatography (polar stationary phase). Therefore, with a polar eluent, a polar compound migrates faster than a nonpolar one. Under these conditions, hydrocarbons are strongly retained.



V- Major Detectors:

In chromatography, a detector must possess several qualities: provide a response proportional to the instantaneous concentration for each detected compound, be sensitive, have minimal background noise, and be stable over time. The most common detection

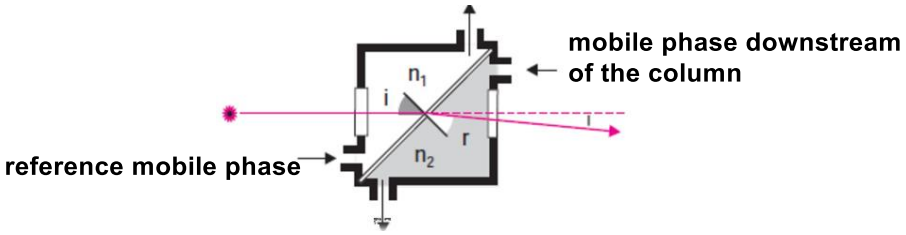
methods are based on the optical properties of compounds: absorption, fluorescence, and refractive index.

V-1- Spectrophotometric Detectors: Detection is based on Lambert-Beer's law (A = εlc): the absorbance A of the mobile phase is measured at the column outlet, at the wavelength λ, or several wavelengths in the UV or visible range.

UV detection is a selective detection method used for compounds containing chromophoric groups.

V-2- Spectrofluorimetric Detector: Fluorescent compounds re-emit a greater or lesser fraction of the radiation from the source to which they are exposed in the form of light emissions. The intensity of fluorescence is proportional to the substance's concentration. Fluorescence detectors are suitable for naturally fluorescent compounds.

V-3- Refractometric Detector: This type of detector includes a differential refractometer designed to continuously measure the difference in refractive index between the mobile phase and the column effluent. The optimal sensitivity corresponds to a maximum difference between the refractive indices of the effluents and the mobile phase.



Series 4

Exercise 1:

1. What is the elution order of the following acids in HPLC with a column having a C_{18} stationary phase and a mobile phase of 200 mM formate buffer at pH 9?

a. Linoleic acid: $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$.

b. Arachidic acid: $CH_3(CH_2)_{18}COOH$.

c. Oleic acid: $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$.

2. Indicate next to each of these chromatographic techniques the term that represents the primary mode of attachment to the phase:

1- Reverse phase	**a-** Hydrophobicity.
2- Gel permeation	**b-** Hydrophilicity.
3- Ion-exchange chromatography	**c-** Molecular weight.
4- Normal phase	**d-** Ionization.

Exercise 2:

In HPLC, the separation of two compounds, A and B, is studied using an RP-18 column. The mobile phase is a binary mixture of water and acetonitrile. It is assumed that there is a linear relationship between the logarithm of the capacity factor and the percentage of acetonitrile in the binary water/acetonitrile mixture used. From two chromatograms obtained, one with a mobile phase consisting of a 70/30 v/v water/acetonitrile mixture and the other with a 30/70 v/v water/acetonitrile mixture, the equations of two straight lines are given as follows:

a. For compound A: $\log kA = -6.075 \times 10^{-3}$ (% MeCN) + 1.3283

b. For compound B: $\log kB = -0.0107$ (% MeCN) + 1.5235

1- Find the composition of the binary phase that would result in a selectivity factor of 1.

2- It is assumed that for each compound, the peak width at half-height is the same, and the column efficiency is not affected by the composition of the mobile phase. Is the resolution between the two peaks better for the mobile phase containing 70% water or 30% water? Demonstrate the practical importance of the previous choice.

Exercise 3:

A separation of two amphetamines is performed on an HPLC column (C_{18} stationary phase: 15cm x 4.6mm) filled with 5µm diameter particles, using acetonitrile as the mobile phase and at a temperature of 30°C. A dead time of 1.07 minutes is measured. Under these conditions, the following separation of the two amphetamines is obtained:

- **a.** Amphetamine A: retention time of 2.4 minutes; peak width at half-height δA is 5s.
- **b.** Methamphetamine M: retention time of 2.85 minutes; peak width at half-height δM is 6s.

1. Calculate the separation factor.
2. Evaluate the number of theoretical plates of the column.
3. Calculate the resolution factor.
4. Calculate the difference in free energy of dissolution Δ(ΔG°) between these two compounds (R = 8.31 J. $K^{-1}\cdot mol^{-1}$.

Exercise 4:

Current HPLC instruments can use columns with an internal diameter of 300 µm, for which the recommended optimum flow rate is 4 $\mu L.min^{-1}$.

1. Show, through a simple calculation, that this flow rate practically leads to the same linear velocity of the mobile phase as for a column of the same type but with a standard diameter of 4.6 mm, for which the recommended flow rate is 1 $mL.min^{-1}$.

2. The separation of a sample containing 16 PAHs (Polycyclic Aromatic Hydrocarbons) was performed on an RP-18 column (LC = 25 cm, d = 300 µm). The flow rate of the mobile phase (acetonitrile/water) is 4 $\mu L.min^{-1}$. One of these PAHs has a retention time

of 48 minutes. Calculate the retention volume of this compound. What can be concluded?

3. Two columns filled with the same stationary phase are available, one with an internal diameter d of 4.6 mm, and the other with 300 µm. The columns have the same lengths and packing ratios (V_S/V_M). It is decided to use them successively with the same chromatograph and under identical conditions, with the recommended flow rates mentioned above for each of them. The same amount of the same compound is injected in both experiments.

a. When switching from one column to the other, is the retention (or elution) volume of the compound modified?

b. If the detector's sensitivity has not been changed between the two experiments, will the intensity of the corresponding peak be different?"

Tp n° 4	*High-Performance Liquid Chromatography (HPLC)*

Keywords: Kinetic resolution, Acylation, Enzyme, HPLC.

Purpose of the Experiment :

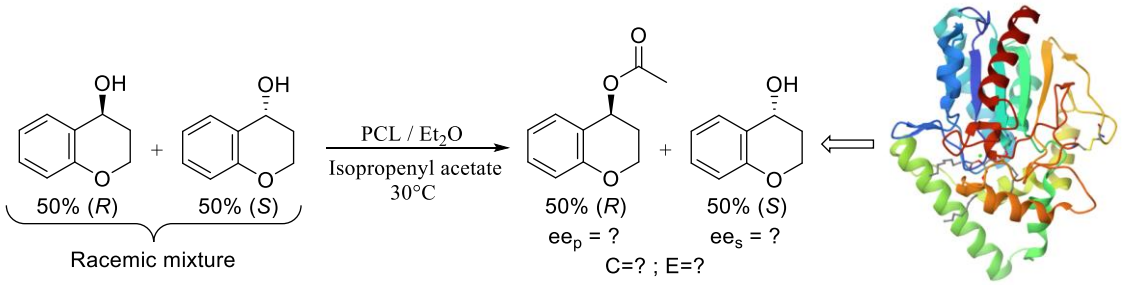
1. Acquiring proficiency in using a chiral stationary phase.
2. Measuring the enantiomeric excess of an optically pure alcohol.

Products	Solvents / Solutions	Materials
Chroman-4-ol. Lipase. Isopropenyl acetate. Diethyl ether.	Petroleum ether. Ethyl acetate.	Tanks, columns, Test tubes, Beakers, Pasteur pipettes, Capillaries, TLC, HPLC.

Introduction :

Chiral alcohols serve as crucial intermediates in the synthesis of numerous target molecules, particularly in the preparation of biologically active compounds. The significance of this compound family on an industrial scale has led to the development of several synthesis protocols. Kinetic resolution reactions through enzymatic acylation are the most commonly employed for the resolution of racemates.[1]

In this reaction, we conducted a kinetic resolution of a heterocyclic alcohol, chroman-4-ol, in the presence of *Pseudomonas Cepacia Lipase* (PCL) as a biocatalyst.



[1] (a) A. Zaks, D.R. Dodds, *Drug Discovery Today*. *Review*., **1997**, Vol. 2. N°. 12, 513-531. (b) M Merabet-Khelassi, L Aribi-Zouioueche, O Riant. *Tetrahedron: Asymmetry* 19 (**2008**), 2378-2384, (c) N Braïa, M Merabet-Khelassi, M Toffano, L Aribi-Zouioueche. Biocatalysis and Biotransformation 41 (**2023**), 261-269. (d) N Melaïs, M Boukachabia, L Aribi-Zouioueche, O Riant, Bioprocess Biosyst Eng, (**2015**) 1579-1588.

Procedure:

Step n° 1:

In a Schlenk tube equipped with a magnetic stir bar, 1 mmol of racemic alcohol and 3 mmol of isopropenyl acetate (IA) are dissolved in 5 ml of diethyl ether, followed by the addition of the appropriate amount of enzyme. The mixture is then subjected to magnetic stirring at 40°C. The resulting solution, which contains a mixture of residual alcohol and formed acetate, is filtered over Celite under reduced pressure, and the ether is evaporated.

Step n° 2:

The components of the mixture are separated using silica gel column chromatography with an eluent typically composed of petroleum ether and ethyl acetate (80/20).

Step n° 3:

Turn on the HPLC and allow it to stabilize for a few minutes under the separation conditions determined later, then inject the two samples.

Questions:

1. Calculate the enantiomeric excess of the formed acetate and the residual alcohol.
2. Calculate the conversion rate using the following formula:

$$\mathrm{C} = \frac{ee_s}{ee_s + ee_p}$$

3. Calculate the selectivity factor using the following formula:

$$\mathrm{E} = \mathrm{Ln}\frac{[(1-C)(1-ee_s)]}{[(1-C)(1+ee_s)]}$$

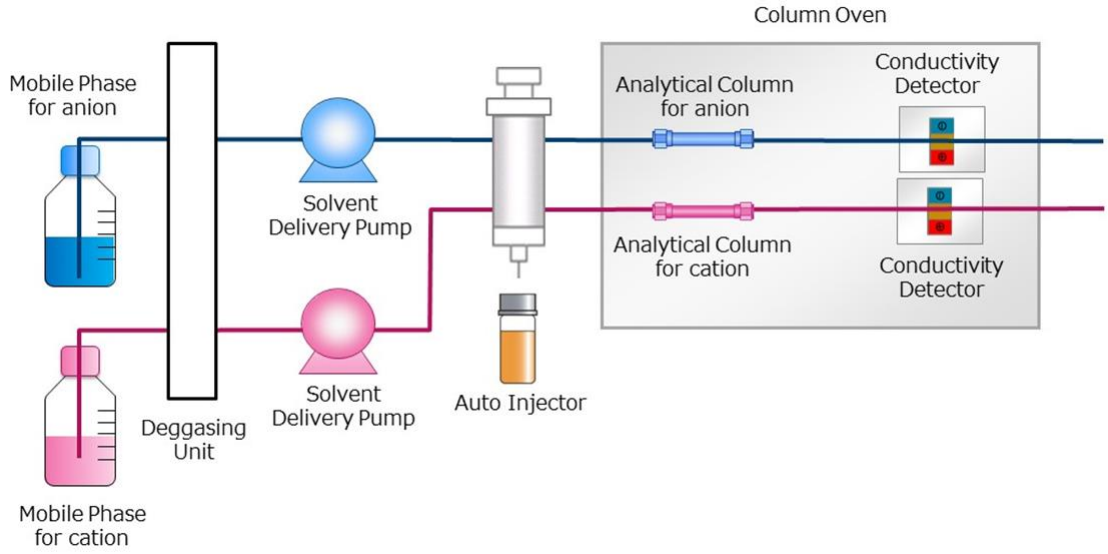
Chapter V

Ion Chromatography.

I- Introduction:

Ion chromatography (IC) is an instrumental technique suitable for the separation of mineral ions and polar organic molecules. The mobile phase consists of an ionic aqueous medium, and the stationary phase is a solid that acts as an ion exchanger. Detection methods in this technique are based on the conductance of solutions. Compounds separable by ion chromatography include:

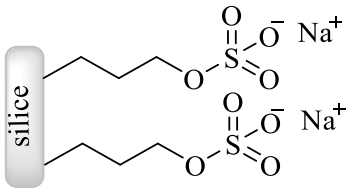
a. Mono- or polysaccharides.
b. Nucleosides and nucleotides.
c. Carboxylic acids.
d. Various organic or mineral anions and cations (transition metals, rare earth elements).



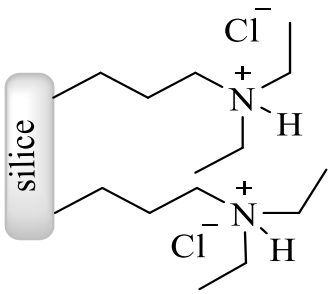
II- Principle:

Ion chromatography (IC) utilizes stationary phases containing ionic sites to create dipolar interactions with the solute to be separated. The greater the charge carried by a solute, the more it is retained by the stationary phase. The separation of sample compounds relies on ion exchange phenomena, and two situations are distinguished:

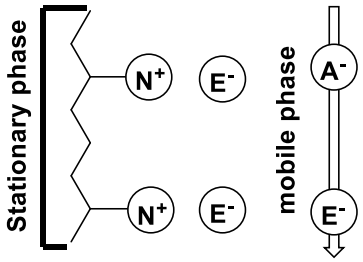
1. To separate cationic species (M^+ type), a cationic column is chosen, with the stationary phase consisting of a polymer grafted with groups capable of cation exchange ($-SO_3H$).



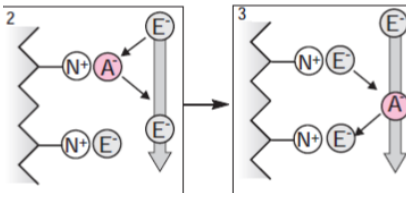
2. To separate anionic species (A^- type), an anionic column is chosen, which is derived from a polymer containing ammonium groups.



For an anionic column containing ammonium groups in equilibrium with a mobile phase rich in bicarbonate anions, all the cationic sites on the stationary phase are paired with ions from the mobile phase.



When an anion A^-, introduced by the sample, is carried by the eluent, a series of reversible equilibria occurs between the two phases according to the following equation:



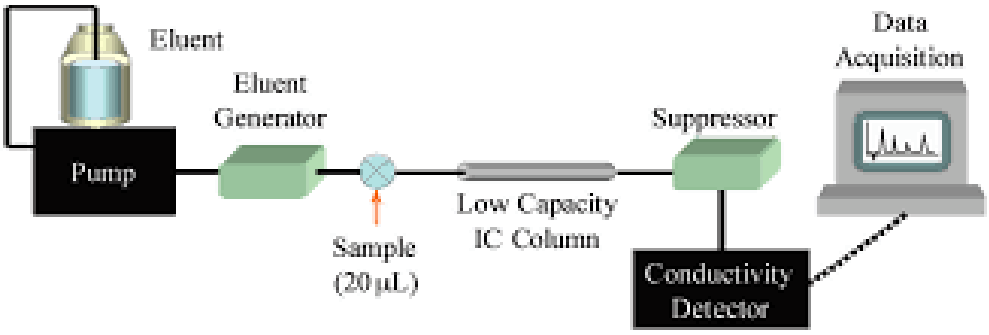
$$A^-_{PM} + [HCO_3]^-_{PS} \underset{2}{\overset{1}{\rightleftharpoons}} A^-_{PS} + [HCO_3]^-_{PM}$$

$$K = \frac{[A^-_{PS}][(HCO^-_3)_{PM}]}{[A^-_{PM}][(HCO^-_3)_{PS}]}$$

The direction 1 corresponds to the fixation of the anion A^- on the stationary phase, and direction 2 corresponds to its return to the mobile phase. If the sample contains two ions, X and Y, and if $K_y > K_x$, Y is more retained by the column than X.

III- General Design of an IC Apparatus:

The devices are made up of modules similar to those encountered in HPLC (High-Performance Liquid Chromatography). The parts in contact with the mobile phase must be made of inert materials to prevent its reactivity.



A suppressor: a device placed between the column and the detector, which serves to eliminate eluent ions through an acid-base reaction.

IV- Stationary Phase:

The stationary phases must meet the following requirements:

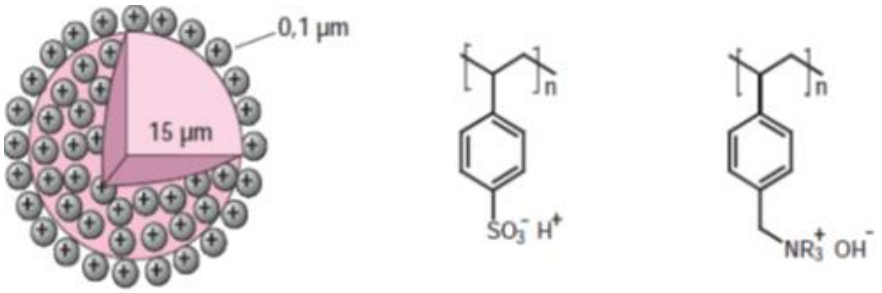
- **a.** Narrow particle size distribution.
- **b.** High specific surface area.
- **c.** Mechanical strength.

d. Resistance to acidic or alkaline pH.

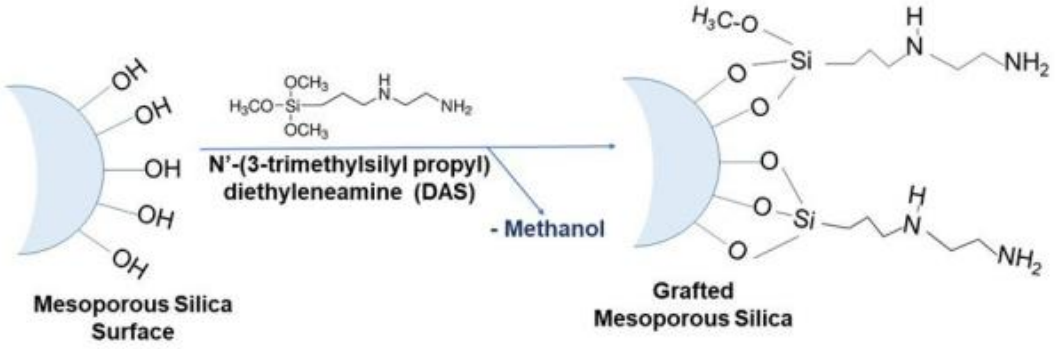
e. Rapid ion transfer.

Three types of stationary phases are distinguished:

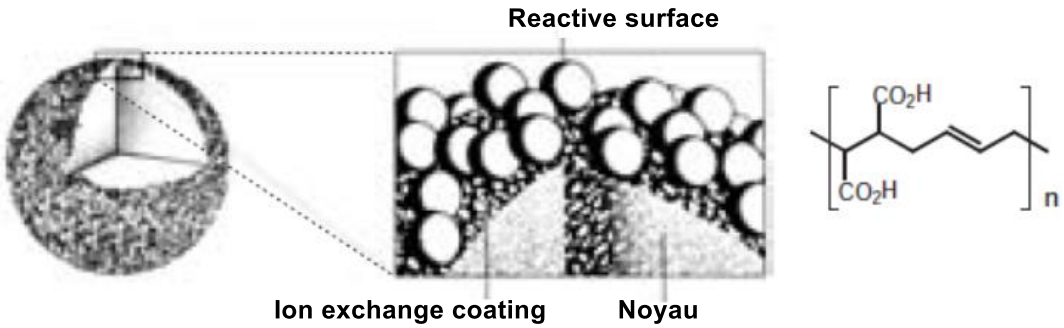
1. **Synthetic copolymers**: Obtained by copolymerizing styrene/divinylbenzene to create cross-linked phases that are resistant to compression in spherical form. Subsequently, grafting of -SO_3H or tertiary amine groups is performed according to the type of column (anionic or cationic).



2. **Grafted Silicas:** Silica gel can be used as a support to anchor, through covalent bonds, alkyl phenyl chains carrying sulfonated or quaternary ammonium groups.



3. **Pellicular Resins:** A polymer called latex, prepared from an organic functional monomer, is deposited in the form of tiny spheres to create a continuous film on a support made of silica, glass, or polystyrene.



V- Mobile Phase:

The mobile phase refers to the liquid or solvent used in liquid chromatography to carry the sample through the chromatographic column. It typically consists of aqueous solutions containing various salts or organic compounds, along with a small amount of methanol or acetone to aid in the dissolution of certain samples.

Depending on whether the column is cationic or anionic, the ions in the mobile phase can be provided by either mineral or organic acids (such as perchloric, benzoic, or phthalic acids) or by bases (such as KOH, NaOH, Na_2CO_3).

VI- Conductivity Detectors:

In this technique, detection is based on the sample's ability to conduct electrical current. Using a conductometer, the conductance of the mobile phase between two microelectrodes is measured at the outlet of the chromatographic column.

Series 5

Exercise 1:

Which of the following two mobile phases will elute an anion from an ion exchange polymeric column more quickly?

a. M oxalate.
b. 0,1 M oxalate.

Exercise 2:

To separate a mixture of proteins, a column containing a carboxymethyl cellulose-based phase is used. The internal diameter (DI) of the column is 0,75 cm, and its length is 20 cm. The pH of the mobile phase is adjusted to 4,8. The flow rate of the mobile phase is set at 1 mL/min. The dead volume is 3 mL It is observed that three peaks appear corresponding to elution volumes V_1, V_2, and V_3 of 12, 18, and 34 mL

a. Is it an anion-exchange or cation-exchange phase?

b. Why does increasing the pH of the mobile phase significantly change the retention times of these three compounds? Predict whether these times will be increased or decreased.

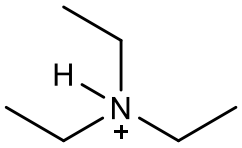
Exercise 3:

We want to separate 3 amino acids: L-glutamic acid, L-leucine, and L-lysine using chromatography on a polystyrene resin substituted with sulfonate groups ($-SO_3-$). The isoelectric pH values of L-glutamic acid, L-leucine, and L-lysine are, respectively: 3.22, 5,98; and 9,74 at 25 °C. These amino acids are deposited on the column at pH 2, and then elution is carried out by gradually adjusting the pH to 7.

a. Which amino acids are eluted and in what order? (We will consider that the amino acid-resin interactions are solely electrostatic in nature).

Exercise 4:

Diethylaminoethyl cellulose (DEAE-cellulose) is an anion-exchange support obtained by substituting cellulose with diethylaminoethyl groups:



1. What is the proportion of positively charged DEAE radicals at the following pH levels: 2, 7, 9.4, 12? (Assuming the pKa of the tertiary amine group of the DEAE group is 9,4).

2. Among the following proteins: Serum Albumin (pI=4,9), Urease (pI=5), Chymotrypsinogen (pI = 9,5), which ones, at pH 7, are retained by DEAE-cellulose? (Considering that the protein-DEAE-cellulose interactions are solely electrostatic in nature).

Tp n° 5	*Ionic Chromatography (IC)*

Keywords: Spring water, Dosage, Ion, IC.

Purpose of the Experiment :

1. Calibrate the device by analyzing water samples containing increasing amounts of ions.
2. Determine the ion concentration in tap water.
3. Check the technical sheet on the label of the spring water bottle (*Ifri*).

Products	Solvents / Solutions	Materials
NaCl, KCl, $CaCl_2$ and $MgCl_2$	Nitric acid 1mM. Spring water (*Ifri*).	Ionic Chromatograph.

Introduction:

We will prepare solutions containing four cations: Na^+, K^+, Ca^{2+}, Mg^{2+}, but at increasing concentrations, varying from one sample to another. These solutions will be prepared from concentrated cation solutions.

The blank, used for calibration and observing the solvent peak, will be prepared with distilled water, theoretically free of ions.

Procedure :

1. 0.5092 g of NaCl, 0.1691 g of KCl, 0.1840 g of $CaCl_2$, and 0.2140 g of $MgCl_2$ are weighed, transferred, and brought to a volume of 1L with deionized water. Then,

20 ml of this solution is transferred into a 100 ml volumetric flask and topped up to 100 ml with H_2O.

2. These solutions are used to create a calibration curve as follows: 5; 10; 15; 20; and 25 ml are transferred into 100 ml volumetric flasks and topped up with H_2O (solutions A, B, C, D, E).

3. On the other hand, 5 ml of tap water and spring water (*Ifri*) are brought to a volume of 250 ml. Then, 10 ml of these solutions are diluted 10 times before being analyzed by ion chromatography (solution X).

Cation	Standard solution	Concentration (mol / l)	Cation area (%)
Na^+	A		
	B		
	C		
	D		
	E		
K^+	A		
	B		
	C		
	D		
	E		
Ca^{2+}	A		
	B		
	C		
	D		
	E		
Mg^{2+}	A		
	B		
	C		
	D		
	E		
Na^+, K^+, Ca^{2+}, Mg^{2+}	X tap water	$[Na^+]$	
		$[K^+]$	
		$[Ca^{2+}]$	
		$[Mg^{2+}]$	
Na^+, K^+, Ca^{2+}, Mg^{2+}	X (*Ifri*)	$[Na^+]$	
		$[K^+]$	
		$[Ca^{2+}]$	
		$[Mg^{2+}]$	

Questions:

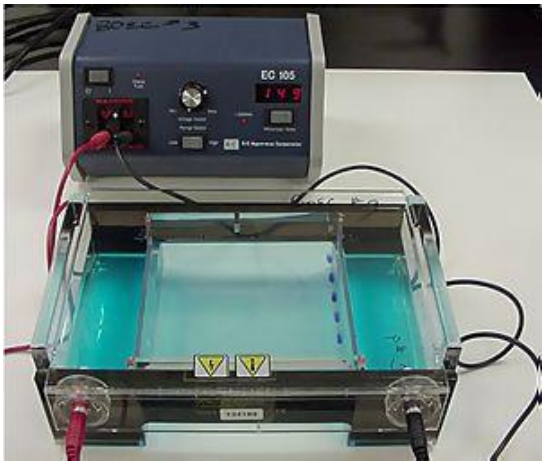
1. Calculate the mass concentrations of the standard solutions.
2. Establish the calibration curves.
3. What is the molar concentration of each cation in tap water and spring water (*Ifri*)?
4. What can be concluded?

Chapter VI

Electrophoresis

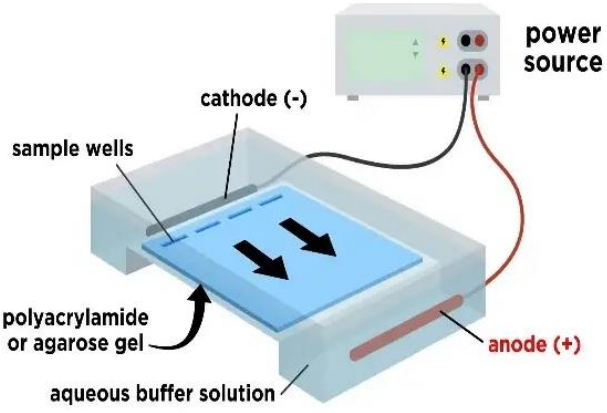
I- Introduction:

Electrophoresis is based on the migration in an electric field and in contact with a suitable support of species present in the sample in solution, whether they carry an electric charge or not. This method, also known as high-performance capillary electrophoresis (HPCE), allows for the separation of both biomolecules and low molecular weight compounds.



II- Principle:

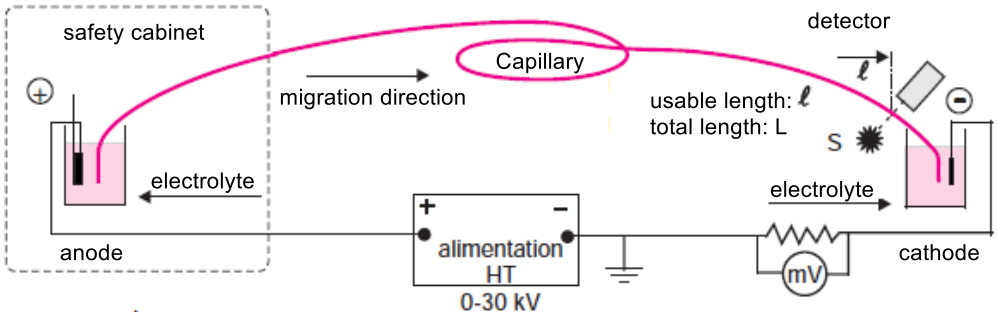
A plastic strip covered with a porous substance impregnated with an electrolyte is used. Its ends dip into two independent reservoirs also containing this electrolyte and are connected to the electrodes of a direct current generator.



The sample is deposited as a small transverse line on the strip. The hydrated species present migrate at very different rates towards one or the other end of the strip. Each compound is differentiated by its mobility.

III- Capillary Electrophoresis:

In capillary electrophoresis, the flat support of the traditional technique is replaced by an open-ended silica glass capillary with a very small diameter (15 to 150 µm). This capillary, with a length L ranging from 20 to 80 cm, is filled with a buffer electrolyte.



A detector is placed at a distance from the upstream end of the capillary near the cathodic compartment. The signal obtained forms the basis for obtaining the electropherogram, which provides information about the composition of the sample.

To limit the heating of the capillary, it is advisable to place it in a temperature-controlled enclosure.

IV- Electrophoretic Mobility and Electroosmotic Flow:

In electrophoresis, the components of a mixture separate over time due to two main factors known as electrophoretic mobility and electroosmotic flow.

IV-1- Electrophoretic Mobility:

Any compound carrying an electric charge moves through the electrolyte at a velocity (v_{EP}) that depends on the experimental conditions and its own electrophoretic mobility (μ_{EP}). This parameter is defined based on the electrophoretic migration velocity of the compound and the electric field (E):

$$\mu_{EP} = \frac{v_{EP}}{E} = v_{EP} \cdot \frac{L}{V}$$

Where **L** represents the total length of the capillary, and **V** represents the applied potential difference (ddp) at its ends. Electrophoretic mobility is assigned a sign (+ or -) depending on the

cationic or anionic nature of the species; $\boldsymbol{\mu_{EP}}$ ($cm^2 \cdot V^{-1} \cdot s^{-1}$) is zero for a globally neutral species, and $\boldsymbol{v_{EP}}$ is the velocity of the compound in an electric field (E).

The number of theoretical plates ***N*** in a capillary electrophoresis column is given by the relationship:

$$N = \frac{\mu_{EP} .V.\ell}{2D.L}$$

D: the diffusion coefficient of the solute (cm^2/s).

VI-2- Electroosmotic Flow:

The second factor that controls the migration of solutes is the flow of the electrolyte known as electroosmotic flow, characterized by its electroosmotic mobility, μ_{EOS}; defined by the following relationship:

$$\mu_{EOS} = \frac{v_{EOS}}{E} = v_{EOS} \cdot \frac{L}{V}$$

A capillary made of silica glass has numerous silanol (Si–OH) groups on its surface that ionize into silanate (Si–O−) groups if the electrolyte's pH is above 3.

These fixed anionic sites attract cations present in the solution and arrange them in two layers, one of which is adhered to the wall, and the other is less mobile. Between these two layers, a potential difference arises, the value of which depends on the electrolyte concentration and pH. The electric field causes the migration of cations toward the cathode. Since these ions are solvated by water molecules, an electrolyte flow appears that moves in the same direction.

To calculate μ_{EOS}, you need to determine v_{EOS}. This corresponds to the flow velocity in the electrolyte of species with no net charge. You can access it based on the migration time (t_{mn}) it takes for a neutral marker, used as a tracer, to travel the effective distance of the capillary (ℓ).

$$v_{EOS} = \frac{\ell}{t_{mn}}$$

- The effective migration distance ℓ is shorter by about ten centimeters than the total length (L) of the capillary.

- You choose a nonpolar organic molecule as the marker, which remains nonpolar at the pH of the electrolyte used and is easily detectable by absorption in the near UV (e.g., acetone or benzyl alcohol).

Note:

In general, a negative surface causes electroosmotic flow directed towards the cathode. However, if we add a surfactant, such as a tetra alkyl ammonium, to reverse the wall's polarity, the electroosmotic flow will move towards the anode.

Based on the preceding information, each ion has an apparent migration velocity (v_{app}) that depends on both electrophoretic velocity and electroosmotic flow velocity.

$$\nu_{app} = \nu_{EP} + \nu_{EOS}$$

v_{app} can be easily calculated from the electropherogram using ℓ, the effective length of the capillary between the injection and detection points, and tm, the migration time. v_{app} is given by the following relationship:

$$\nu_{app} = \frac{\ell}{t_m}$$

The apparent electrophoretic mobility ($\boldsymbol{\mu_{app}}$) is defined by the following relationship:

$$\mu_{app} = \frac{\nu_{app}}{E} = \nu_{app} \cdot \frac{L}{V}$$

Therefore, we have: $$\mu_{app} = \frac{\ell}{t_m} \cdot \frac{L}{V}$$

By combining the electroosmotic flow of the electrolyte ($\boldsymbol{\mu_{EOS}}$) and the apparent mobility ($\boldsymbol{\mu_{app}}$), it is possible to calculate the electrophoretic mobility ($\boldsymbol{\mu_{EP}}$) using the following relationship:

$$\mu_{EP} = \mu_{app} - \mu_{EOS} \quad \text{either} \quad \mu_{EP} = \frac{L \,.\, \ell}{V}\left(\frac{1}{t_m} - \frac{1}{t_{mn}}\right)$$

V- Instrumentation:

To introduce a micro volume of the sample, which should not exceed 1% of the effective length of the capillary (ℓ), two methods are used:

a. **Hydrostatic injection:** This involves immersing the end of the capillary in the sample solution and inducing suction at the other end.

b. **Electromigration injection:** This involves immersing the end of the capillary in the sample for a certain time while applying a potential (50 mV/cm) to it, and choosing the polarity with respect to the other end.

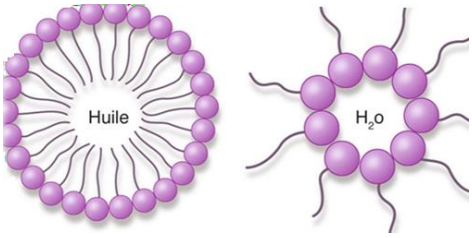
Detection Modes: To determine various solutes, several detection modes are available:

- ✓ UV/VIS detection.
- ✓ Fluorescence detection.
- ✓ Electrochemical detection.
- ✓ Mass spectrometry detection.

IV- Electrophoretic Techniques:

1. **Capillary Zone Electrophoresis:** This is the most common electrophoresis method. The capillary is filled with an electrolyte, which can be a buffer solution that, depending on the application, is either acidic (phosphate or citrate) or basic (borate), or an ampholyte (a molecule with both acidic and basic functions).

2. **Capillary Micellar Electrokinetic Chromatography (CMEKC):** In this variant, a cationic or anionic compound, such as sodium dodecyl sulfate, is added to the mobile phase to form micelles with ionic properties, making them charged. These microdroplets, which are immiscible with the solution, trap neutral compounds to varying degrees through hydrophilic/hydrophobic affinity.



1- **Capillary Gel Electrophoresis:** This is the adaptation of electrophoresis using polyacrylamide or agarose gels. The capillary is filled with an electrolyte containing a gel. The gel acts as a filtration medium, slowing down larger molecules and minimizing convection or diffusion phenomena. This method is particularly useful for separating less fragile molecules, such as oligonucleotides.

2- **Isoelectric Focusing Electrophoresis:** Also known as isoelectric focusing in electrophoresis, this technique involves creating a linear pH gradient in a treated-wall capillary containing an ampholyte. The capillary is immersed in H_3PO_4 at the anode and NaOH at the cathode. Each compound migrates and focuses at the pH that matches its isoelectric point (pI). Subsequently, under the influence of hydrostatic pressure and while maintaining the electric field, the separated species are moved towards the detector. The high resolutions achieved with this method allow for the separation of peptides with pI values that differ by only 0,02 pH units.

Series 6

Exercise 1:

An electrophoresis setup in solution consists of a capillary with a total length L = 32 cm and an effective length ℓ = 24.5 cm. The applied voltage is 30 kV. Under the experimental conditions, the migration time (t_{mn}) of a neutral marker is 3 minutes.

a. Calculate the electrophoretic mobility (μ_{EP}) of a compound with an apparent migration time of 2.5 minutes.

b. Under these conditions, calculate the diffusion coefficients of this compound, assuming that the calculated efficiency is N = 80,000.

Exercise 2:

In CE, when the capillary contains acrylamide gel in addition to the electrolyte, it is observed that the migration velocity is slowed down due to a mechanical filtration effect by the gel. This phenomenon becomes more significant as the molecule size increases. It is considered that the following relationship is verified:

$$\text{Log } M = a.v + b$$

Where a and b are constants, and M represents the molecular mass (Da) of a migrating molecule with velocity v.

In an experiment aimed at using this relationship to calculate the molecular mass of an unknown protein, two calibration standards are used:

- ✓ Ovalbumin (M = 45,000 Da) with a migration velocity of 1.5 cm/min
- ✓ Myoglobin (M = 17,200 Da) with a velocity of 5.5 cm/min.

In the same experiment, the unknown protein has a migration velocity of 3.25 cm/min. Calculate its mass in daltons.

Exercise 3:

On a capillary electrophoresis setup consisting of an untreated silica glass capillary with a "negative wall," L = 1 m in total length and ℓ = 90 cm in effective length (up to the detector). The applied potential difference across the capillary is 20 kV. The detector is located toward the cathodic end of the capillary. The electrolyte is a buffer solution with a pH of 5. A compound present in the sample has a migration time t_m=10min.

a. Can we deduce whether the net charge carried by this compound is positive or negative?

b. Calculate the apparent electrophoretic mobility μ_{app} of this compound.

c. Knowing that a neutral marker has a migration time t_m of 5 min, deduce the value of the electroosmotic flow μ_{EOS}.

d. Calculate the electrophoretic mobility μ_{EP} of the compound. Deduce the sign of its net charge.

e. What would happen if a capillary with a treated wall to make it neutral were used?

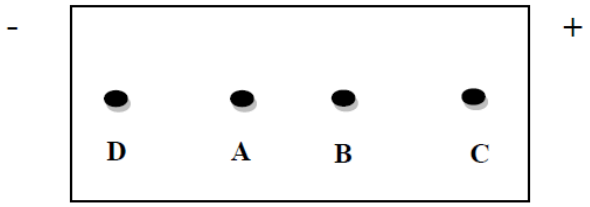
f. Assuming that the compound's isoelectric point (pI) is 4, what will be the sign of its net charge if the pH of the electrolyte is lowered to 3?

g. Calculate N for the studied compound, knowing that $D=2\times10^{-5}$ $cm^2.s^{-1}$.

h. Knowing the relationship between efficiency N and diffusion D, explain why small molecules generally lead to less effective separations in capillary electrophoresis than large molecules and why separations are better when the capillary diameter is narrower.

Exercise 4:

On donne un mélange de quatre protéines A, B, C, D. L'électrophorèse réalisée sur ce mélange à pH =7 a donné le résultat suivant :



Le calcul du pHi de ces quatre protéines a donné les valeurs suivantes : $pH_i = 5.1$, $pH_i = 6.2$, $pH_i = 8$, $pH_i = 8.5$.

a. Indiquer le pHi correspondant à chaque protéine.

b. Ce mélange est déposé au sommet d'une colonne de résine échangeuse d'anions éluée par un tampon de pH 7. Parmi les quatre protéines données, quelles sont celles qui s'accrochent la plus fortement à la résine.

c. Comment doit-on faire varier le pH afin de faciliter la libération de ces protéines ? Justifier votre réponse.

d. Indiquer l'ordre d'élution des protéines. Justifier votre réponse.

Tp n° 6	*Electrophoresis*

Keywords: Electrophoresis, Peptide synthesis, Agarose.

Purpose of the Experiment :

1. Develop a basic understanding of the theory of electrophoresis.
2. Gain hands-on experience with the separation processes of various molecules through horizontal gel electrophoresis.

Products	Solvents / Solutions	Materials
Boc-*L*-proline. *L*-valine. *L*-leucine. Agarose. DCC.	Dichloromethane. Buffer. Distilled water.	Tanks, Transfer micropipettes, Horizontal electrophoresis apparatus, DC power supply, Hot plate, Beakers, Pasteur pipettes.

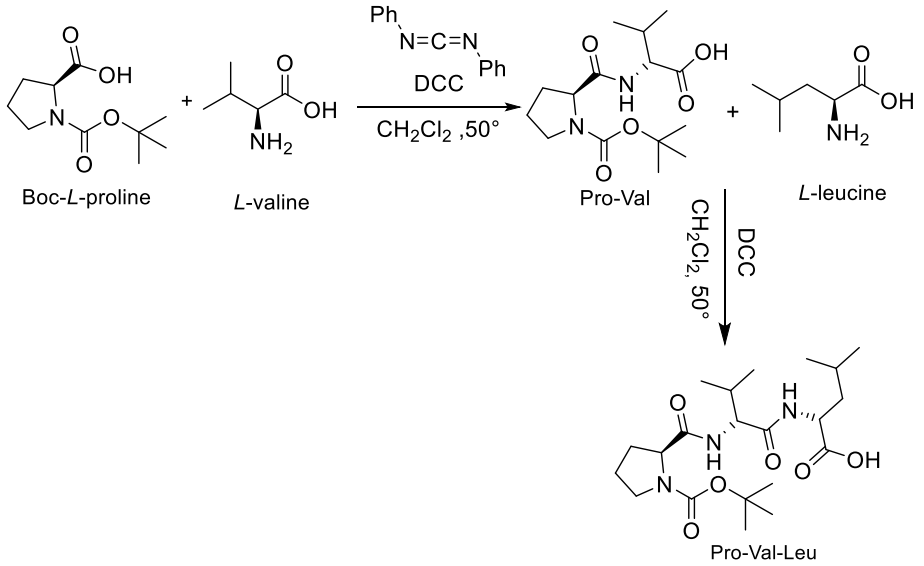
Introduction:

Peptide synthesis is the chemical process used to construct peptides, which are chains of amino acids linked by peptide bonds. These peptides can be small proteins or fragments of larger proteins. Peptide synthesis is a crucial step in the production of biological compounds with various applications, ranging from scientific research to drug design.

The creation of peptides is essential in drug development. Many drugs, such as hormones and antibodies, are based on peptides. The ability to synthesize specific peptides enables the design of more precise and effective medications[1].

We synthesized a pseudopeptide composed of three amino acids in this reaction: Boc-*L*-proline, *L*-valine, and *L*-leucine:

[1] (a) Arno F. Spatola, in Methods in *Neurosciences*, 1993, (b) Marije Marsman, ... Jacques Neefjes, in *Advances in Cancer Research*, 2005, (c) Markus Fischer, ... Silke Leimkühler, in *Comprehensive Natural Products II*, 2010, (d) Z (S) Han, ... C.H. Senanayake, in *Comprehensive Chirality*, 2012, (e) Berin Karaman, ... Wolfgang Sippl, in *Epi-Informatics*, 2016.



Procedure:

Step n° 1:

Initially, we synthesized a proline-valine peptide bond according to the following protocol:

In a 250 ml flask, 3 mmol of Boc-*L*-proline and 3.3 mmol of dicyclohexylcarbodiimide (DCC) are introduced into 40 ml of dichloromethane at 50°C. After 30 minutes of stirring, a solution of *L*-valine (3 mmol) is added to the previous solution. After 5 hours of stirring, a solution of *L*-leucine (3 mmol) is added dropwise to the reaction mixture, and the progress of the reaction is monitored by horizontal gel electrophoresis.

Step n° 2:

Agarose gel electrophoresis is used to separate biomolecules (DNA, RNA, proteins, peptides) based on their charge and size. The preparation of a 0.8% agarose gel is carried out according to the following steps:

1. Verse 3 g of agarose powder into a 500 ml flask containing 380 ml of distilled water 7,5 ml of buffer. Gently swirl to remove any lumps.
2. Heat the agarose solution using a hotplate until the solution is homogeneous.
3. Pour each volume of cooled agarose solution into the casting mold. Use 30 ml for 7x7 cm molds, 50 ml for 7x10 cm molds, and 60 ml for 7x14 cm molds.
4. Allow the gel to finish solidifying to complete the electrophoresis setup.

Step n° 3:

1. Place the gel in the electrophoresis chamber and cover it with the electrophoresis buffer. The gel must be fully submerged.
2. Prepare the samples by mixing them with components that provide increased density, such as glycerol or sucrose.
3. Deposit the samples (Proline, valine, leucine and the reaction mixture) using a micropipette or transfer pipette into wells created in the gel using a template during casting.
4. Connect the plugs to the power source and run the electrophoresis.
5. Once electrophoresis is completed, remove the gel and the casting mold from the electrophoresis chamber and visualize the agarose gel.

Questions:

1- Interpret the obtained results.

- For more information, you can consult the following website: https://www.youtube.com/watch?v=GUXKQBknYQo

References

1. F. Rouessac, A. Rouessac. *Méthodes et techniques instrumentales modernes*, 6e edition, Dunod, Paris, **2004**, ISBN : 2100484257.

2. S. R Crouch, F. J. Holler, D. A. Skoog, D. M. West. *Chimie analytique*, 4e Edition, DeBoeck, **2023**, ISBN: 9782807337299.

3. A. Rouessac, F. Rouessac, *Techniques instrumentales d'analyse chimique en 23 fiches*. Dunod, **2011**. ISBN : 978-2-10-055614-4.

4. K. Lukasz, W-H. Monika, S. Joseph. *Thin Layer Chromatography in Drug Analysis*. CRC PRESS, **2020**.

5. G. Eli, G. Nelu. *Advances in Chromatography*. CRC PRESS, **2019**.

6. M. Zhang, S. Lu, G. Li, L. Hong. *Catalysts*, 10, **2020**, 392.

7. A. K. Ghosh**,** M. R. Belcher., *J. Org. Chem.* **2020**, 85, 10399–10412.

8. M. Boukachabia, H. Bendjeffal, L. Aribi-Zouioueche, O. Riant, Y. Bouhedja, *ChemistrySelect*. **2022**, 22, e202104610

9. Zeferino, R. C. F., Piaia, V. A. A., Orso, V. T., Pinheiro, V. M., Zanetti M., Colpani, G. L., Padoin, N., Soares, C., Fiori M. A., Riella H. G. *Chem. Eng. Res. Des.* **2021**, (*168*), 156–168.

10. M. Boukachabia, H. Bendjeffal, M. M. Khelassi, O. Riant. *Flavour Fragr. J.*, **2023**, 1.

11. A. Zaks, D.R. Dodds, *Drug Discovery Today*. *Review*., **1997**, Vol. 2. N°. 12, 513-531.

12. M Merabet-Khelassi, L Aribi-Zouioueche, O Riant. *Tetrahedron: Asymmetry* 19 (**2008**), 2378-2384,

13. N Braïa, M Merabet-Khelassi, M Toffano, L Aribi-Zouioueche. Biocatalysis and Biotransformation 41 (**2023**), 261-269.

14. N Melaïs, M Boukachabia, L Aribi-Zouioueche, O Riant, Bioprocess Biosyst Eng, (**2015**) 1579-1588.

15. Arno F. Spatola, in Methods in *Neurosciences*, **1993**.

16. Marije Marsman, Jacques Neefjes, in *Advances in Cancer Research*, **2005**.

17. Markus Fischer, Silke Leimkühler, in *Comprehensive Natural Products II*, **2010**.

18. Z (S) Han, C.H. Senanayake, in *Comprehensive Chirality*, **2012**.

19. Berin Karaman, Wolfgang Sippl, in *Epi-Informatics*, **2016**.

20. F. Esther and T. Cserhati; *Chromatography in Food Science and Technology*, **2020**, ISBN: 1000160114.

21. L-C. Mapatac; *Chromatography: Concepts, Methods and Applications*, **2022**, ISBN:1680957228.

22. B. Andry and A. Dispas; *Chromatography: Principles and Instrumentation*, **2020**, ISBN: 1642242721.

23. Mark F. Vitha; *Chromatography Principles and Instrumentation*, **2017**, ISBN: 2016011576.