CHAPTER II:

Separation methods in chromatography

II.1 General:

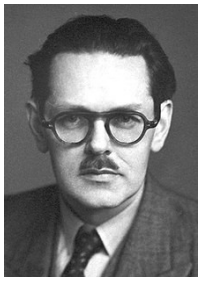
1- General introduction:

Chromatography is a fundamental analytical separation method, widely used in chemistry, pharmacy, and nanotechnology laboratories. This technique is based on the separation of the components of a mixture according to their affinity for a stationary phase and a mobile phase. It is crucial in high-precision analyses, such as the identification and quantification of chemical compounds in complex samples.

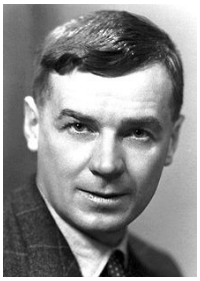
The concept of chromatography was developed in the early 20th century by Russian botanist **Mikhail Tswett** , who discovered that he could separate plant pigments by passing them through a chalk tube (a column) with a solvent.

Mikhail Semyonovich Tsvet , also transcribed as **Tswett** (in German, the most common spelling in botany) **Tswet** , **Zwet** or **Cvet**) (1872 - 1919) is a botanist Russian who invented adsorption chromatography . His name means *"color"* in Russian

This technique evolved and was refined by other scientists, including **Archer John Porter Martin** and **Richard Laurence Millington Synge** , who were awarded the Nobel Prize in Chemistry in 1952 for their work on partition chromatography.



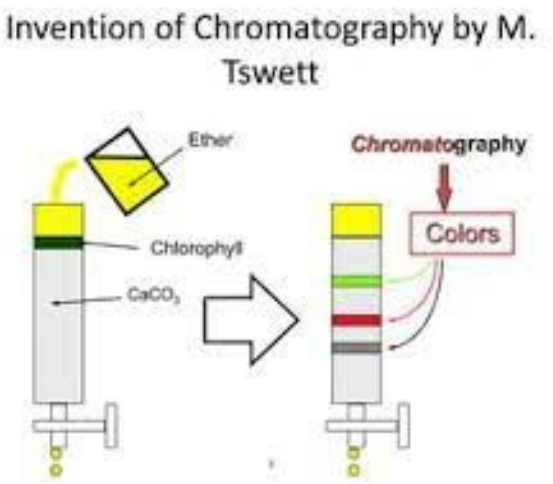
Archer John Porter Martin



Richard Laurence Millington Synge

Mikhail Tswett's experience:

Mikhail Tswett's experiment in 1903 was a pioneering experiment that laid the foundations for modern chromatography. Tswett, a Russian botanist, discovered the technique while studying plant pigments, including chlorophylls and carotenoids.

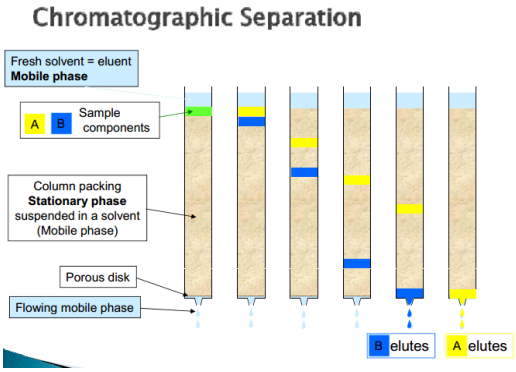


Purpose of the experiment: Tswett wanted to separate and identify the different pigments present in plant leaves. The analytical methods of the time did not allow the pigments to be isolated effectively.

- **Set up :**
  - He prepared an extract of plant pigments by dissolving leaves in a solvent.
  - Then he filled a vertical glass column with finely ground calcium carbonate, which he used as the stationary phase.
- **Procedure :**
  - Tswett poured his pigment extract into the column, then added a solvent that flowed through the column by gravity.
  - As the extract and solvent passed through the column, the different pigments separated into distinct bands.
- **Result :**
  - The pigments were separated into bands of different colors along the column, each band corresponding to a specific pigment.
  - The separation was done according to the affinities of the pigments with the stationary phase and the mobile phase.
- **Interpretation and term "chromatography":**
  - Tswett named this technique "chromatography", from the Greek "chroma" (color) and "graphein" (to write), because he observed colored "writings" on the column.
  - He thus discovered that different compounds can be separated by their affinity for a mobile phase (the solvent) and a stationary phase (the support in the column).

2- Principle of chromatography

Chromatography is a separation method, like extraction and distillation, but it is particularly suited to separating chemical compounds within a complex mixture. This technique relies on the interaction between a sample, a mobile phase (usually a liquid or gas), and a stationary phase (a solid or liquid attached to a surface). The sample dissolved in a solvent is injected into the system through an interface, then it passes through a column containing the stationary phase. The movement of the sample is facilitated by the flow of the mobile phase, such as nitrogen in gas chromatography.

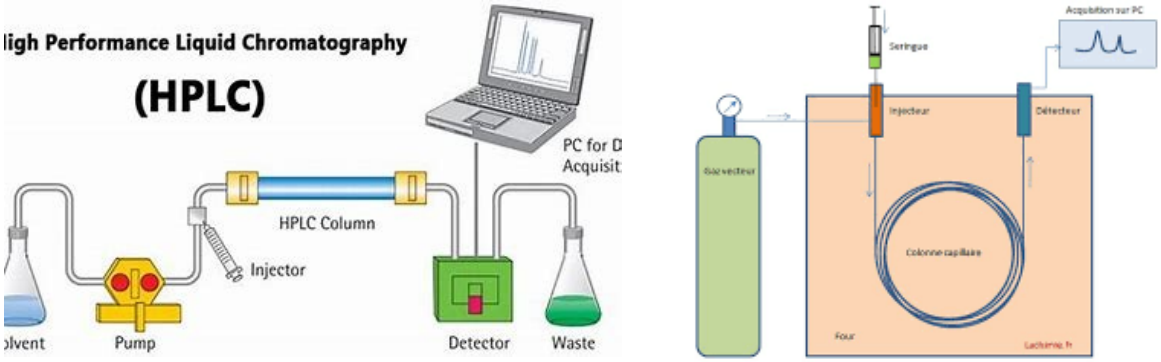


Principle of chromatography

- **General principle:** The basic principle consists of a separation of the sample constituents according to their respective affinities for the mobile phase and the stationary phase. Molecules with a greater affinity for the stationary phase will migrate more slowly, while those with a greater affinity for the mobile phase will migrate more quickly.
- **Injection and displacement:** The sample is introduced into the system by an injector. Under the effect of the mobile phase, it moves through the column. The nature of the gas or liquid used as the mobile phase depends on the type of chromatography and the type of sample to be analyzed.
- **Detection and analysis:** At the outlet of the column, the separated components are detected by different types of detectors (mass spectrometer, UV detector, etc.), which allows them to be identified and quantified.
- **Use for quantification and identification:** Chromatographic techniques allow the quantification of components present in a sample and the identification of substances based on their retention time and physical characteristics.

1- Structure of a Chromatographic System

A chromatographic system is composed of several essential elements that allow the **separation** , analysis **and** detection **of** compounds in a mixture.



HPLC CPG

Structure of a Chromatographic System

A typical chromatographic system consists of the following components:

a) **Mobile phase tank**

b) **Pump (or mobile phase drive system)**

c) **Injector (sample injection system)**

d) **Chromatographic column**

e) **Detector**

f) **Data acquisition system (computer and software)**

g) **Waste or fraction collector**

Explanation of each component

a) Mobile phase reservoir

- **Function** : Contains the **mobile phase** , which is the fluid used to transport analytes through the column.
- **Details** :
  - In **liquid chromatography** (HPLC), it can be a solvent or a mixture of organic solvents (water, acetonitrile, methanol).
  - In **gas chromatography** (GC), it is a carrier gas (helium, nitrogen, hydrogen).

b) Mobile phase pump or drive system

- **Function** : Ensures constant and controlled **flow** of the mobile phase through the system.
- **Details** :
  - In HPLC, the pump controls the flow rate (usually expressed in mL/min) and provides high pressure to push the mobile phase through the column.
  - In CPG, the carrier gas pressure is regulated for a constant flow rate.

c) Injector

- **Function** : Allows the sample to be analyzed to be **introduced** into the mobile phase.
- **Details** :
  - In HPLC, the sample is injected using an **injection loop** .
  - In GC, the sample is vaporized and injected into the carrier gas stream.
  - Injection must be rapid and reproducible to ensure good separation.

d) Chromatographic column

- **Function** : It is the heart of the chromatographic system where **separation** of analytes occurs.
- **Details** :
  - The column is filled with a **stationary phase** (liquid or solid) that interacts differently with each analyte, separating them based on their interactions.

- In **HPLC** , columns are often made of stainless steel and packed with modified silica particles.
- In **GC** , columns are often capillary tubes covered with a liquid film of stationary phase.

e) Detector

- **Function** : Measures the **concentration** of analytes after their separation in the column.
- **Details** :
  - The detector sends an electrical signal proportional to the amount of analyte detected.
  - Different detectors are used depending on the analytes (UV-Vis, FID, MS, etc.).
  - The generated signal is transformed into a chromatogram.

f) Data acquisition system (computer and software)

- **Function** : Collects and **analyzes** signals from the detector.
- **Details** :
  - It allows you to visualize the **chromatogram** , identify peaks and quantify analytes.
  - The software allows statistical analysis and comparison of results.

(g) Waste or fraction collector

- **Function** : Collects effluent after detection or allows fractions to be recovered for further analysis.
- **Details** :
  - In HPLC, the liquid is collected in a waste tank.
  - In CPG, gases usually exit through an exhaust.

■ **General Operating Process**

a) **Mobile Phase Preparation** : The mobile phase is prepared in the reservoir and the pump is activated to initiate flow through the system.

b) **Sample injection** : The sample is introduced into the mobile phase by the injector.

c) **Separation in the column** : The components of the sample interact with the stationary phase of the column, causing them to separate according to their physicochemical properties.

d) **Detection** : Separated analytes are detected based on their specific properties.

e) **Data acquisition and analysis** : The detector signal is recorded, generating a chromatogram allowing the identification and quantification of analytes.

f) Detection result: The chromatogram

g) The **chromatogram** is the graphical output of the chromatographic analysis.

A. It shows the **signal intensity** (on the ordinate) as a function of **time** (on the abscissa).
B. Each **peak** corresponds to a different analyte.

C. **Retention time** is used for identification, and **peak area** for quantification.

2- Classification of chromatographic methods

Chromatography can be classified according to the type of sample, mobile phase, stationary phase and partition coefficient between phases:

(a) Classification according to the nature of the phases

- **Gas-Solid Chromatography (GSC)** : Solid stationary phase and gas mobile phase.
- **Gas-Liquid Chromatography (GLC)** : Liquid stationary phase immobilized on a solid support, with a gaseous mobile phase.
- **Liquid-Solid Chromatography (LSC)** : Solid stationary phase, and liquid mobile phase.
- **Liquid-Liquid Chromatography (LLC)** : Liquid stationary phase immobilized on a support, and liquid mobile phase.
- **Supercritical Chromatography** : Uses a supercritical mobile phase, usually CO_2 .

(b) Classification according to the separation phenomenon

- **Adsorption chromatography** : Separation is based on the adsorption of components onto the solid stationary phase.
- **Partition chromatography** : Based on the difference in affinity of components between two liquid phases.
- **Ion exchange chromatography** : Sample ions interact with charged sites in the stationary phase.
- **Gel permeation chromatography (or size exclusion)** : Molecules are separated according to their size, the stationary phase is a porous gel.
- **Affinity chromatography** : Exploits the specific affinity between a target molecule and an immobilized ligand.

(c) Classification according to the processes used

- **Column chromatography** : The sample passes through a column filled with stationary phase.
- **Thin layer chromatography (TLC)** : The stationary phase is a thin film on a plate and the sample migrates over it.
- **Paper chromatography** : Uses paper as the stationary phase and the solvent migrates by capillary action.
- **High-performance liquid chromatography (HPLC)** : A column method using high pressures to improve separation.
- **Gas chromatography (GC)** : The sample is vaporized and transported through a column by a carrier gas.

(d) Classification according to the parameters involved in the separation

- **Temperature** : Mainly used in gas chromatography (GC) where high temperatures allow evaporation and separation of volatile components.
- **Pressure** : Particularly relevant in HPLC to improve separation speed and resolution.

- **Polarizability and polarity** : Affect the interactions between the mobile phase, stationary phase, and analytes.
- **pH** : In ion exchange chromatography, it influences the charge of analytes and exchange resins.
- **Molecular size** : Essential in size exclusion chromatography (gel permeation) where molecules are separated by size.

1. ***Liquid chromatography (LC):*** *Uses a liquid mobile phase to separate compounds that are soluble in the mobile phase. It is often used to analyze thermally unstable or nonvolatile compounds.*
2. ***Gas chromatography (GC):*** *Uses a gas (e.g., nitrogen or helium) as a mobile phase to separate volatile and thermally stable compounds.*
3. ***Ion chromatography:*** *Designed for ionic or polar compounds; it uses stationary phases capable of retaining ions of opposite charges to those in the sample.*
4. ***Reversed-phase chromatography (RP-LC):*** *Uses a polar mobile phase (such as water) and a non-polar stationary phase, allowing polar compounds to be separated in the mobile phase.*
5. ***Ion pair chromatography:*** *Suitable for polar and ionizable compounds, with the addition of counterions in the mobile phase to improve separation.*
6. ***Supercritical chromatography:*** *Uses supercritical CO_2 as □ □ □ □ mobile phase, allowing rapid separation for semi-volatile compounds.*

3- How to choose the chromatographic technique?

The selection of the method depends on the physicochemical properties of the sample:

- **Nature of the sample** : For example, volatile compounds will be suitable for GC, while non-volatile or thermally unstable compounds will be suitable for LC.
- **Polarity** : Polarity influences the choice of stationary and mobile phase.
- **Solubility** : Depending on the solubility of the sample in the mobile phase.

Each technique has advantages depending on the type of analysis, and the final choice often depends on the needs for quantification, identification and the nature of the sample.

For chromatography, the sample should ideally be **homogeneous** to ensure efficient and reproducible separation of components. If a sample is heterogeneous, it must be prepared by dissolution, filtration or homogenization to prevent particles from disturbing the separation process in the column.

Table for the choice of chromatographic method

Selection criteria	Liquid chromatography (LC)	Gas chromatography (GC)	Ion chromatography	Reversed phase chromatography (RP-LC)	Ion pair chromatography	Supercritical chromatography (SFC)
Physical state of the sample	Solid or liquid	Volatile or easily vaporizable	Solid or liquid, ionic	Solid or liquid	Solid or liquid	Solid or liquid
Volatility	Not required	Required	Not required	Not required	Not required	Semi-volatile compounds
Stability	Thermosensitiv	Thermally	Compounds	Heat sensitive	Thermosensitiv	Moderate

Selection criteria	Liquid chromatography (LC)	Gas chromatography (GC)	Ion chromatography	Reversed phase chromatography (RP-LC)	Ion pair chromatography	Supercritical chromatography (SFC)
temperature	e compounds	stable compounds	sensitive to high temperatures	compounds	e compounds	
Polarity	Polar to non-polar	Non-polar to slightly polar	Ionic	Polar to slightly polar	Ionic	Semi-polar
Mobile phase type	Liquid	Gas (nitrogen, helium)	Liquid (electrolyte)	Liquid (polar, often water)	Liquid (with counterion)	Supercritical gas (CO_2)
Stationary phase type	Solid or liquid	Solid	Solid	Non-polar solid	Solid	Solid or liquid
Type of analysis	Quantification, identification	Quantification, identification	Quantification of ions	Quantification of polar compounds	Quantification of ionizable compounds	Rapid identification and separation
Main applications	Pharmaceuticals, biomolecules	Volatile organic compounds	Anions, cations, polar compounds	Pharmaceuticals, polar compounds	Organic, polar products	Organic compounds and polymers

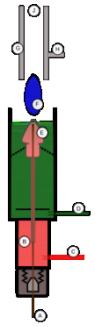
Additional Considerations

- **Sample preparation** : The sample should be prepared so that it is compatible with the mobile phase and the stationary phase.
- **Analytical objectives** : The precision and sensitivity required for the analysis influence the choice of method. For example, for rapid qualitative analysis, GC is ideal for volatile compounds. For temperature-sensitive compounds, LC is preferable.

Interest and advantages of chromatography

- **High precision** : Enables high precision separation and identification of components in complex mixtures.
- **Speed** : Certain techniques, such as CPG and HPLC, allow rapid analyses, essential in pharmaceutical production.
- **Flexibility** : The diversity of techniques allows it to be adapted to the chemical and physical properties of the samples.
- **Possibility of studying a tiny quantity, sample of the order of nono-gram** .

6 - Detector:



6.1 Definition: Chromatographic detector

A **chromatographic detector** is an instrument placed at the outlet of the column that measures **the** concentration of solutes present in the column effluent. It converts this measurement into an **electrical signal** proportional to the concentration of the solute, thus producing a **chromatogram** .

Principle of detection:

The principle of detection is based on measuring a physical or **chemical property** of the solute as it passes through the detector. These properties may include:

- **Light absorption** (UV-visible)
- **Conductivity**
- **Refractometry**
- **Ionization** or **mass variation** .

Variations in these properties are converted into **signals** by the detector, which are then recorded as a **chromatogram** .

6.2 **The different types of detectors in chromatography:**

There are several types of detectors used depending on the type of chromatography and the properties of the solutes. Detectors can be classified into two main categories: **universal detectors** and **specific detectors** .

a) Detectors for Liquid Chromatography (HPLC):

1. **UV-Visible (UV-Vis) Detector:**
   - **Principle** : Measures the absorption of UV or visible light by solutes. Light-absorbing compounds generate a signal proportional to their concentration.
   - **Advantages** : Sensitive and widely used for aromatic or conjugated compounds.
   - **Disadvantages** : Less effective for compounds that do not absorb UV light.
2. **Differential Refractometry Detector (RID):**
   - **Principle** : Measures the variations in the refractive index between the pure mobile phase and the mobile phase containing the solute.

- **Advantages** : Universal detector, works for compounds that do not absorb UV light.
- **Disadvantages** : Less sensitive than UV-Vis detector.

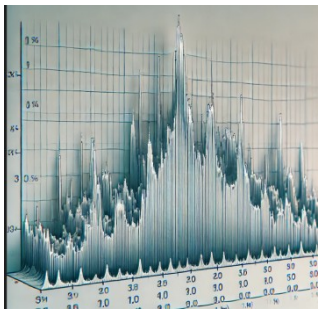
3. **Fluorescence Detector:**
   - **Principle** : Measures the emission of light by solutes after excitation by a light source.
   - **Advantages** : Very sensitive and selective for fluorescent compounds.
   - **Disadvantages** : Limited to fluorescent compounds or compounds labeled with fluorescent agents.
4. **Electrochemical Conductivity Detector:**
   - **Principle** : Measures the electrical conductivity of the solution in the presence of ionic compounds.
   - **Advantages** : Sensitive to ionic compounds.
   - **Disadvantages** : Requires a conductive mobile phase.

b) Detectors for Gas Chromatography (GC):

1. **Flame Ionization Detector (FID):**
   - **Principle** : Burns organic solutes in a hydrogen flame, generating ions that are detected as an electric current.
   - **Advantages** : Sensitive to organic compounds. Universal detector for hydrocarbons.
   - **Disadvantages** : Does not detect inorganic or non-ionizable compounds.
2. **Electron Capture Detector (ECD):**
   - **Principle** : Measures the capture of electrons by electronegative compounds (often halogens).
   - **Advantages** : Very sensitive to pesticides, PCBs, and other halogenated compounds.
   - **Disadvantages** : Selective for certain compounds.
3. **Mass Detector (MS):**
   - **Principle** : Ionizes solutes and separates them according to their mass/charge ratio (m/z).
   - **Advantages** : Very sensitive and allows precise identification of compounds.
   - **Disadvantages** : Complex and expensive.

Conclusion: Detection results: The chromatogram A **chromatogram** is the graph produced by the detector. It represents the **signal** as a function of **time** .

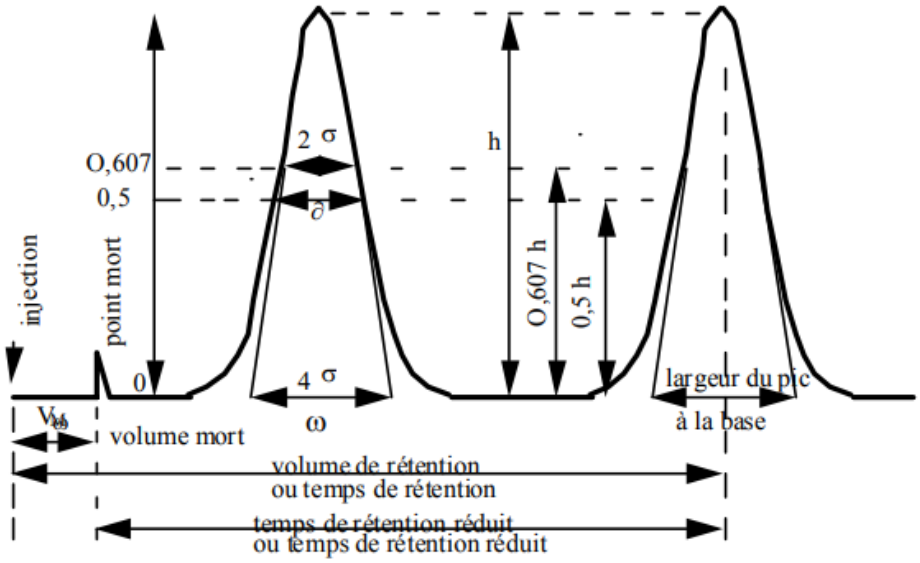
6.1 Chromatogram



A chromatogram is the graphical result of a chromatographic analysis, allowing to visualize the separation of the components of a mixture according to their retention time in the column. Here is a detailed explanation of its elements and how to interpret it.

(a) Definition of a Chromatogram

A chromatogram is a graphical representation where the intensity of the detected signal (usually by a UV, fluorescence, or mass detector) is plotted against time . It allows one to observe the separation of compounds in a mixture, with each compound appearing as a distinct peak.



Chromatogram: Variations and Coordinate Axis

In a chromatogram, the y-axis **represents** the intensity of the detected signal, which is often related to the concentration or amount of a compound in the sample. The x-axis **represents** time, and each peak on the chromatogram corresponds to a specific time of detection, that is, the retention time of the compound.

- **Signal variation over time** : The chromatogram shows how the detected signal varies over time, where each peak appears at the time a specific component is detected.

- **Interpretation of results** : By comparing the retention times obtained with the known retention times for standard compounds, each peak can be identified.

Meaning of a Straight Line (No Peaks)

- A straight line on a chromatogram (absence of peaks) means that no compounds were detected during that period. This may indicate a phase where there are no components retained by the stationary phase or a period where the components are either very low in concentration or undetectable by the detector used.
- A straight line may also appear before sample injection, during the equilibration or signal stabilization period.

Identification of Compounds

To determine the identity of a compound:

- ✓ **Retention time** : The first indicator is the retention time of the peak. By comparing it to known standards, it is possible to make a first identification.
- ✓ **Analysis of spectrum or other characteristics** : In some techniques (such as chromatography coupled with mass spectrometry), the spectrum of each peak can be analyzed to provide a more precise identification.
- ✓ **Peak intensity** : The intensity and shape of the peak can also give clues to the nature and concentration of the compound.

In conclusion, the chromatogram is a powerful method to analyze and quantify the components of a mixture, each peak corresponding to a distinct compound and its retention time helping to identify the compounds in comparison with standards.

❖ **Retention time (t_R):** The **retention time** is the time elapsed between the injection of the sample into the chromatographic column and the appearance of the peak corresponding to the detected solute. It represents the time required for the solute to migrate through the column under the influence of the mobile phase: **Interpretation** : A high retention time indicates that the solute has interacted for a longer time with the stationary phase, while a short retention time indicates a weak interaction.

❖ **Retention volume (V_R):** The **retention volume** is the volume of mobile phase required to elute (pull out) a specific solute from the column. It is calculated by multiplying the retention time by the volumetric flow rate of the mobile phase (F):

$$V_R = t_R \times F$$

$$V_R = t_R \times v \times s$$

- (v): linear velocity of the mobile phase
- (s): reduced section of the column
- . $s = s' \times \varepsilon$
- (s'): section of the column

$$\varepsilon = V_M / V_T$$

- **ε: porosity**

$$V_M = F \times t_0$$

❖ **V_M : volume of the mobile phase in the column**

- the spaces not retained by the stationary phase appear in the effluent after the time (t_0) corresponding to the flow of the interstitial volume of the column or volume of mobile phase (V_M) contained in the column.

The retention volume (V_R) is directly related to the distribution coefficient K by the relation:

$$V_R = V_M + K.V_S$$

(V_S): volume of the stationary phase (or mass or specific surface area according to the units of K); this relationship only applies in the case of linear elution, i.e. when K varies linearly with the concentration of the compound in each phase.

❖ **Definition of dead time (t_0):**

- ***Dead time*** *is also called* ***interstitial volume passage time*** *or* ***mobile phase flow time*** .
- ***Unretained molecules*** *are those that have* ***no*** *or negligible interaction with the stationary phase. They therefore move only with the mobile phase.*

(c) **Interstitial volume (Dead volume or V_M):**

Example :

If you perform a chromatography and observe a very early peak at a time $t_0 = 2min$, this means that the mobile phase took 2 minutes to pass through the column. Solutes that have interactions with the stationary phase will appear after this time, for example at $t_R = 5$, $t_R = 7min$, etc.

In summary, t0 gives you an idea of the ***velocity*** *of the mobile phase and serves as a reference* ***to*** *estimate the actual retention time of solutes interacting with the stationary phase.*

ω : width of the peak at the base, distance between the points of intersection of the tangents at the inflection point with the baseline

δ : peak width at mid-height

❖ **Reduced retention time ($t_{R'}$)**

The **reduced retention time** is defined as the solute retention time minus the dead time (t_M):

$$t_{R'} = t_R - t_M$$

- **Interpretation** : It represents the time during which the solute actually interacts with the stationary phase, excluding the time that the mobile phase takes to pass through the column.

❖ **Reduced retention volume ($V_{R'}$)**

The **reduced retention volume** is calculated in the same way as the reduced retention time, taking into account the dead volume (V_M):

$$V_{R'} = V_R - V_M$$

- **Interpretation** : It corresponds to the volume of mobile phase that the solute has traveled through while interacting with the stationary phase.

❖ **Dead point (Dead time or Dead time (t_M))**

The **dead point** or **dead time** is the time required for a molecule not retained (or poorly retained) by the stationary phase to pass through the column. This time is taken as a reference to calculate the reduced retention time:

- **Interpretation** : It represents the time taken for an unretained compound (not interacting with the stationary phase) to travel through the column, and gives an idea of the speed of the mobile phase.

❖ **Dead volume (V_M)**

The dead volume corresponds to the volume of mobile phase required to pass through the column without interaction with the stationary phase.

$$V_M = F \times t_M$$

- **F** : volumetric flow rate of the mobile phase (mL/min).
- t_M : time out (in minutes).

If we know the column section and the mobile phase velocity, we can express the dead volume by :

$$V_M = V \times s \times \epsilon$$

- **S** : cross-section of the column, with r the radius of the column.
- **V: linear velocity of the mobile phase**
- **ϵ** : porosity of the column, which represents the fraction of the volume occupied by the mobile phase.

❖ **Reduced section of the column**

The reduced column section refers to the column surface area taken into account after reduction by the presence of stationary phase particles.

(b) **Partition coefficient (K)**

The **partition coefficient** (*Distribution coefficient*) is the ratio between the concentration of the solute (analyte) in the stationary phase (Cs) and in the mobile phase (Cm):

$$K = \frac{C_S}{C_m}$$

- **Interpretation** : A high partition coefficient means that the solute has a strong affinity for the stationary phase, thus increasing the retention time.

Chromatogram Analysis: Identification and Quantification of Analytes

a) Identification:

- **Retention time** :
  - **Retention time** is the time required for an analyte to reach the detector after injection.
  - Each compound has a specific retention time, which depends on its interactions with the stationary phase and the mobile phase.
  - By comparing the tRt_R tR of a peak with that of a known **standard substance** , the analyte can be identified.
- **Comparison with standards** :
  - Standard solutions (reference samples) are injected and their retention times are compared with those of the analytes in the sample to identify them.

b) Quantification:

To quantify the concentration of analytes, two methods are mainly used:

- **Peak height method** : The peak height (h) is used to estimate the concentration if the relationship is linear.
- **Peak area method** : The **peak area** (A) is calculated as the peak integral (area under the curve).

7- **Columns/stationary phase/** Number of theoretical plates

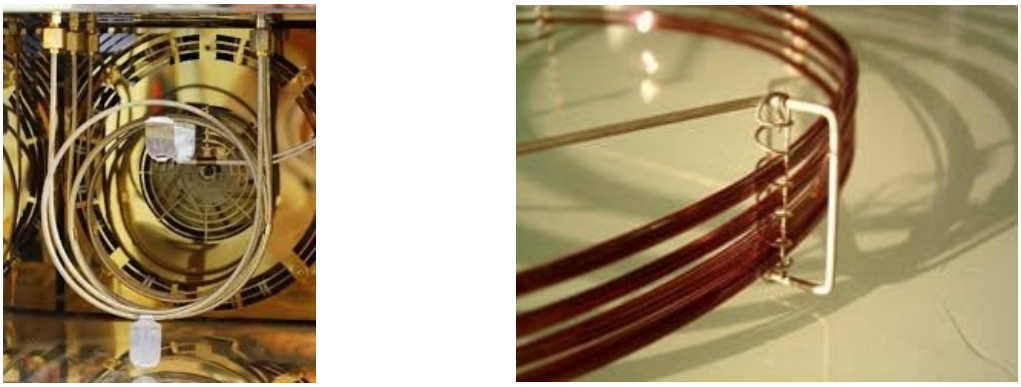


Fig: Chromatography columns

7.1 **. Chromatographic column**

A chromatographic column is a cylindrical tube containing the **stationary phase** , through which the **mobile phase** transports the analytes. The column is essential for the separation of compounds in a mixture.

a) Column Types

- **Packed columns** :
  - Used in liquid chromatography (HPLC) and gas chromatography (GC).
  - **stationary phase** particles or support material coated with stationary phase.
  - Typical internal diameter: 2-4 mm, length: 10-30 cm for HPLC.
- **Capillary columns (open tubular columns)** :
  - Mainly used in gas chromatography (GC).
  - Capillary tube with a thin layer of **stationary phase** on the inner wall.
  - Inner diameter: 0.1-0.5mm, length: 10-100m.

b) Column settings

- **Column length** (L): Affects the number of theoretical plates and the resolution of the separation.
- **Internal Diameter** : The smaller the diameter, the better the resolution, but this may result in increased pressure.
- **Material Type** : Stainless steel, glass, or polymers, depending on analytical needs.

7.2 Stationary Phase

The **stationary phase** is the component of the column with which the analytes interact. It can be solid or liquid (supported by a solid) and determines the separation mechanism.

a) Types of Stationary Phases

- **Polar stationary phase** :
  - Capable of retaining polar compounds.

- Example: Silica (SiO_2), cyano (CN).

- **Nonpolar stationary phase** :
  - Capable of retaining non-polar compounds.
  - Example: C18 (octadecylsilane, ODS), C8.
- **Chiral stationary phase** :
  - Used to separate enantiomers.
  - Composed of materials that interact differently with each enantiomer.

b) Choice of Stationary Phase

- Depends on the nature of the analytes and the purpose of the separation.
- A nonpolar stationary phase is chosen for nonpolar compounds, and a polar phase for polar compounds.

7.3 Number of Theoretical Plates (N)

The **number of theoretical plates** is a measure of the efficiency of the chromatographic column. It reflects the degree of separation that the column can achieve.

a) Definition

- The number of theoretical plates (N) indicates the number of equilibrium zones in a column where the analyte distributes between the mobile phase and the stationary phase.
- The higher the N , the more efficient the separation and the narrower the chromatographic peaks.

b) Calculation formula

The number of theoretical plates is calculated by:

$$N = 16\left(\frac{t_R}{\varpi}\right)^2 = 5.54\left(\frac{t_R}{\delta}\right)^2$$

c) Equivalent Height of a Theoretical Plateau (HEPT or H)

- HEPT **or H is another measure** of column efficiency, given by:

$$HEPT = \frac{L}{N}$$

  - **L** : Length of the column.
  - The smaller the value of H , the better the efficiency of the column.

d) Factors influencing N **and** H

- **Particle size** : Smaller particles increase N .
- **Column length** : A longer column increases N , but may require higher pressure.
- **Mobile phase flow rate** : An optimal flow rate maximizes N .

e) Applications of the Number of Theoretical Plateaus

- **Performance evaluation** : N is used to compare the efficiency of the columns.

- **Separation optimization** : Column parameters can be adjusted to maximize N .
- **Chromatographic peak quality : A high** NN N value corresponds to narrower and better defined peaks.

■ **Application Example: Separation of Aniline and Toluene**

Suppose we are analyzing a mixture of aniline and toluene. The chromatogram shows two distinct peaks, with:

- **Retention time** for aniline t_R =5.2 min.
- **Retention time** for toluene t_R 7.8 min.
- **Half-height width** of aniline $\omega_{1/2}$ = 0.12 min.
- **Half-height width** of toluene $\omega_{1/2}$ = 0.15 min.
- By applying the formula N

- **Conclusion**
- Understanding column parameters, stationary phase, and number of theoretical plates is crucial to optimizing a chromatographic method. Column efficiency can be adjusted by changing parameters such as particle size, column length, and mobile phase flow rate to achieve optimal separations and accurate analytical results.

f) **Symmetry Factor**

■ **Definition**

The symmetry factor (or asymmetry factor) is a measure of the shape of a chromatographic peak. Ideally, a chromatographic peak should be symmetrical and Gaussian-shaped, but in practice, peaks can be asymmetrical. The symmetry factor (As) is used to quantify this deviation from ideal symmetry.

■ **Formula**

The symmetry factor is given by:

$$A_S = \frac{b}{a}$$

- **a** : Distance from peak start to left inflection point (measured at 10% of peak height).
- **b** : Distance from the end of the peak to the right inflection point (measured at 10% of the peak height).

■ **Interpretation**

- **As = 1** : The peak is perfectly symmetrical.
- **As > 1** : The peak is spread to the right (peak tail or "tailing").
- **As<1** : The peak is spread to the left (peak front or "fronting").

■ **Importance**

- **Optimization** : A symmetry factor close to 1 is desirable for good separation and accurate quantification of analytes.

- **Diagnosis** : An asymmetric peak may indicate chromatographic column problems, such as sample overload or unwanted interactions with the stationary phase.

■ **Examples of use**

In HPLC and GC chromatography, the symmetry factor is used to assess peak quality. High symmetry factors (e.g. As > 2) may require optimization of chromatographic conditions or column cleaning.

(g) Capacity Factor (or Retention Factor) in Chromatography

The capacity factor, also called retention factor (symbolized by **k'**), is a fundamental measurement used in chromatography to describe the behavior of an analyte during separation. It indicates how much time a molecule spends in the stationary phase relative to the mobile phase. It is an essential parameter for evaluating the efficiency of the separation.

■ Definition: The capacity factor **k'** is defined as the ratio of the time a solute spends in the stationary phase to the time it spends in the mobile phase. It is also interpreted as the number of times a molecule interacts with the stationary phase before being eluted: The formula for the capacity factor is given by:

$$k' = \frac{t_R - t_0}{t_0}$$

- t_R : Solute retention time (time taken for the solute to be eluted from the column).
- t_0 : Dead time or retention time of the unretained compound (this is the time required for the mobile phase to pass through the column without interacting with the stationary phase).

■ Principle

- If **k′ = 0** , the compound is not retained and flows directly with the mobile phase.
- If **k′** is high, it means that the solute interacts strongly with the stationary phase and spends more time in it, thus increasing the retention time.
- In general, an optimal value of **k′** is between **1 and 10** to ensure good separation without excessively extending the analysis time.

■ Example

Let's take a simple example to illustrate the calculation of the capacity factor:

Suppose that during a chromatographic analysis we have the following data:

- **(solute retention time)** : 10 minutes
- **(time out)** : 2 minutes

The capacity factor **k'** = 4: This means that the solute spends 4 times more time in the stationary phase than in the mobile phase.

■ Importance of Capacity Factor

- **Efficiency assessment** : Too low a **k'** indicates poor retention, which can lead to peak overlap. Too high a **k'** lengthens the analysis time unnecessarily.
- **Separation optimization** : The capacity factor helps to adjust the chromatographic conditions (type of stationary phase, choice of mobile phase) to obtain efficient separation of compounds.

- **Method comparison** : Using the capacity factor, different columns and mobile phases can be compared to assess which offers better retention and separation.

■ **Relationship with other chromatographic parameters**

The capacity factor is often used in conjunction with other parameters such as the **selectivity factor** (α) and the **number of theoretical plates** (N) to describe and optimize the separation process.

- **Selectivity factor (α)** : Defined by the ratio of the capacity factors of two compounds, it allows the resolution between two peaks to be evaluated.

$$\alpha = \frac{k'_2}{k'_1} \quad \left[k'_2 \triangleright k'_1\right]$$

■ Application examples

- **High-performance liquid chromatography (HPLC)** : By adjusting the polarity of the mobile phase, the capacity factor of the analytes can be changed to improve separation.
- **Gas chromatography (GC)** : By changing the column temperature, the capacity factor of the analytes can be influenced.

■ Advantages and disadvantages

- **Benefits** :
  - Allows rapid estimation of analyte retention.
  - Helps to optimize chromatographic conditions.
- **Disadvantages** :
  - a **k'** lengthens the analysis time and can impair the separation efficiency.
  - a **k'** can result in poor separation of compounds.

■ **Conclusion**

The capacity factor is an essential tool in chromatography to evaluate and optimize separation conditions. By playing on experimental variables such as the nature of the stationary phase or the composition of the mobile phase, the capacity factor can be adjusted to obtain optimal separations and faster and more precise analyses.

(h) Selectivity factor (α) in chromatography

■ Definition

The selectivity factor, denoted **α** , is a key parameter in chromatography that measures the ability of a chromatographic column to distinguish two different compounds. It indicates the difference in retention between two analytes on the same column and allows their separation to be evaluated.

■ Formula

The selectivity factor is defined by the ratio of the capacity factors (k′) of two distinct analytes:

$$\alpha = \frac{k'_2}{k'_1}$$

- k'_2 : Capacity factor of the most retained compound ($k'_2 \triangleright k'_1$).
- k'_1 : Capacity factor of the least retained compound.

The selectivity factor is always greater than 1 ($\alpha > 1$

■ Principle

The selectivity factor is used to quantify the difference in retention between two solutes. The higher the selectivity factor, the greater the difference in retention, which results in a better separation of the two compounds.

- **($\alpha \approx$ 1)** : The analytes are weakly separated or not separated at all (overlapping peaks).
- **(** $\alpha >$ 1) : Indicates good separation of analytes.
- **Very high α** : Indicates a sharper separation, but if α is too large it may result in a longer analysis time for the most retained compound.

■ Calculation Example

Suppose we have two compounds, A and B, analyzed by chromatography with the following parameters:

- Retention time of compound A (t_{R1}): 5 minutes
- Retention time of compound B (t_{R2}): 10 minutes
- Timeout (t_0): 2 minutes

Let's first calculate the capacity factors:

$$k'_2 = \left(\frac{10-2}{2}\right) = 4 \quad \text{and} \quad k'_1 = \left(\frac{5-2}{2}\right) = 1.5 \quad \text{So}: \alpha = 2.67$$

Interpretation : A selectivity factor of 2.67 means that compound B is significantly more retained than compound A, indicating good separation.

■ **Importance of Selectivity Factor**

- **Separation Optimization** : The selectivity factor is used to adjust experimental conditions to improve the separation of analytes.
- **Stationary Phase Selection** : Selectivity depends on the nature of the stationary phase. By changing the column, better separation of analytes can be achieved by increasing α\alpha α .
- **Comparison of Analytes** : The selectivity factor allows to compare the retention of several compounds on the same column.

■ **Improved Selectivity**

- **Modification of the mobile phase** : By changing the polarity or composition of the mobile phase, one can influence the retention of analytes and increase the selectivity factor.
- **Changing the stationary phase** : Using a stationary phase with different chemical interactions can increase the retention difference between two analytes.
- **Temperature** : In gas chromatography (GC), adjusting temperature can affect retention times and improve selectivity.

Practical Example

Let's assume an HPLC analysis where we want to separate two isomers: ibuprofen and naproxen.

- Using a C18 column with an aqueous acetonitrile mobile phase (50:50), we observe that the retention times are very close, and $\alpha \approx 1.05$.
- By adjusting the mobile phase composition to 60% acetonitrile, the retention time of naproxen increases more than that of ibuprofen, and α increases to 1.2.
- This improvement in selectivity (α) allows better separation of peaks on the chromatogram.

Relationship with Resolution (Rs)

The selectivity factor is a key component of the resolution formula (Rs), which measures the separation between two chromatographic peaks:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_2'}{1 + k_2'}$$

- **N** : Number of theoretical plates
- **α** : Selectivity factor
- $\mathbf{k_2'}$: Capacity factor of the most retained compound

Benefits :

- Allows to evaluate and optimize the separation of analytes.
- Used to compare the efficiency of different columns and experimental conditions.

Limitations :

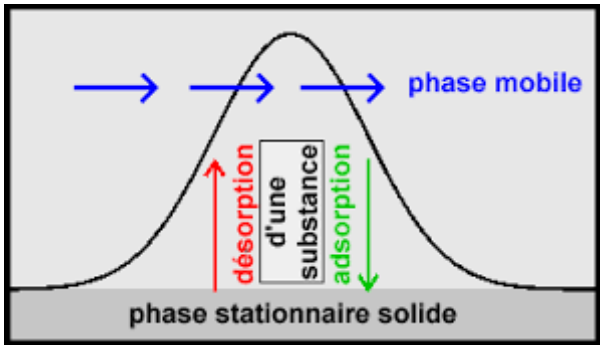
- An α close to 1 makes separation difficult and may require significant adjustments to chromatographic conditions.
- If α is very high, the analysis time may be unnecessarily prolonged for some analytes.

Conclusion

The selectivity factor (α) is an essential parameter to evaluate and optimize the separation of analytes in chromatography. It allows to understand and adjust the interactions between analytes and the stationary phase, directly influencing the quality of the separation and the resolution of chromatographic peaks. Adjusting the mobile phase, choosing the stationary phase and modifying experimental conditions are key methods to optimize selectivity and thus obtain accurate and reliable analyses.

7.2 Mobile phase:

In chromatography, the eluent, or mobile phase, is the liquid or gas that flows through the chromatographic column and carries the components of the sample to be analyzed. Its main function is to carry these molecules along the stationary phase, which is fixed inside the column.



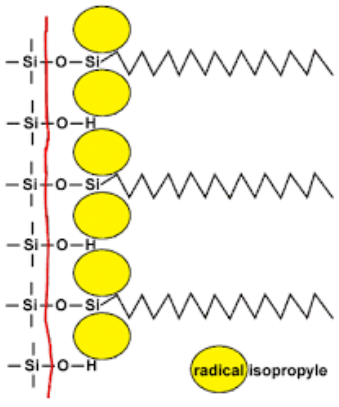
- **Interaction with stationary phase:** In the column, the sample is separated according to its interaction with the stationary phase and the mobile phase. Components that interact weakly with the stationary phase are carried away more quickly by the mobile phase and therefore have a shorter retention time, while those that interact more strongly remain in the column longer.
- **Polarity of the eluent:** In liquid chromatography, for example, the polarity of the eluent is crucial, as it can influence the separation of components. For example, in normal-phase chromatography, a less polar eluent promotes the retention of polar compounds on the polar stationary phase. In contrast, in reversed-phase chromatography, a more polar mobile phase more easily carries away polar compounds.
- **Separation speed:** The speed of movement of the mobile phase also influences the resolution of the separation. A fast mobile phase reduces the analysis time, but may result in less separation of components (less well resolved peaks).

Thus, the mobile phase is essential for the movement of analytes in the column and directly affects the separation efficiency and the quality of analytical results.

7.3 Stationary Phase:

Definition :

The stationary phase is a key component in chromatography techniques. It is the solid or liquid material immobilized in a column or on a surface where the different compounds of the sample interact. Its role is to allow the separation of the different analytes according to their interactions with it, and with the mobile phase (the solvent or gas that passes through the stationary phase)



Principle:

The principle of the stationary phase is based on the differential interaction of analytes (molecules present in the sample) with the stationary phase. When a sample passes through the stationary phase with the help of the mobile phase, molecules that interact strongly with the stationary phase move more slowly, while those that interact less pass more quickly. This leads to a separation of the components according to their affinity with the stationary phase.

Types of Stationary Phase:

There are several types of stationary phases used depending on the type of chromatography used:

- **Solid Stationary Phase (Adsorption Chromatography):**
  - Used mainly in adsorption chromatography, it consists of adsorbent solids such as silica gel, alumina, or activated carbon.
  - Analytes temporarily attach to the surface of stationary phase particles, causing them to separate.
- **Liquid Stationary Phase (Partition Chromatography):**
  - In partition chromatography, the stationary phase is a layer of liquid immobilized on a solid support.
  - Examples: Octadecylsilane (C18) used in high performance liquid chromatography (HPLC) is a common liquid stationary phase.
- **Polymer Stationary Phase:**
  - Used mainly in ion exchange chromatography.
  - Composed of charged polymer resins that interact with ions based on their opposite charges.
- **Gas Stationary Phase (Gas Chromatography - GC):**
  - The stationary phase is an immobilized liquid or polymer coating the interior of the capillary column.
  - The analytes dissolve in this liquid before redistributing into the mobile phase.

Stationary Phase Forms:

- **Columns Filled:**
  - Packed columns contain stationary phase particles in powder or gel form.
  - Used in liquid chromatography.
- **Capillary Columns:**
  - In capillary columns (especially for GC), a thin layer of stationary phase is coated on the inner walls of a capillary tube.

Interactions with the Stationary Phase:

Analyte-stationary phase interactions depend on several physical and chemical phenomena:

- **Adsorption:** Interaction of analytes with the surface of a solid stationary phase.
  - Examples: Van der Waals interactions, London forces.

- **Partition:** Distribution of analytes between the liquid stationary phase and the mobile phase.
  - The molecules temporarily dissolve in the stationary phase before returning to the mobile phase.
- **Ion Exchange:** Charged analytes exchange their ions with ions of the opposite charged stationary phase.
  - Used for ionic compounds, amino acids, proteins.
- **Steric Exclusion:** The separation is done according to the size of the molecules.
  - Small molecules enter the pores of the stationary phase and are retained longer.

Laws Governing Interactions:

- **Nernst's Law (Partition):**
  - Describes the distribution of an analyte between two phases (stationary and mobile).
- **Adsorption isotherms (Langmuir, Freundlich):**
  - Describe how an analyte adsorbs to the surface of a solid stationary phase.

Choice of Stationary Phase:

- The choice of stationary phase depends on several factors:
- **Nature of Analytes:** Polarity, size, charge of analytes.
  - Examples:
    - Polar analytes often use polar stationary phases such as silica.
    - Non-polar analytes use non-polar phases such as C18.
- **Type of Chromatography:**
  - For adsorption chromatography, solids such as silica are common.
  - For reversed phase chromatography (HPLC), phases like C8 or C18 are chosen.
- **Compatibility with Mobile Phase:**
  - The stationary phase must be stable and insoluble in the mobile phase to avoid contamination and deterioration.

Distribution of the Stationary Phase in the Column:

- **On the Surface:**
  - In liquid chromatography, the liquid stationary phase is immobilized on a solid surface such as silica beads.
- **Inside the Pores:**
  - For size exclusion chromatography, the stationary phase has pores into which small molecules can enter.

Practical Examples:

- **Reversed Phase Chromatography (HPLC):**
  - Stationary phase: Octadecylsilane (C18)

- Mobile phase: Water/methanol
- Application: Separation of non-polar organic compounds such as essential oils.

Ion Exchange Chromatography:

- Stationary phase: Sulfonate resin for cation exchange.
- Application: Analysis of amino acids.

7.4 Sorption isotherm

Sorption isotherms are mathematical models that describe how a solute (or analyte) interacts with an adsorbent surface (the stationary phase) at a constant temperature. These isotherms show the relationship between the amount of solute adsorbed per unit mass of adsorbent and the concentration of the solute in the mobile phase.

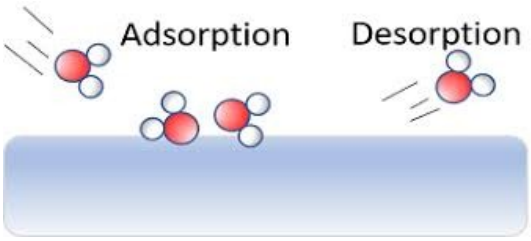


Fig: Sorption phenomenon

Sorption isotherms are particularly important in chromatography and materials analysis because they help to understand adsorption behavior and optimize separation processes.

Types of Sorption Isotherms:

There are several types of adsorption isotherms that are commonly used to model the interaction between the adsorbent and the adsorbate:

1. Langmuir **isotherm :**

- **Principle:**
  - The Langmuir isotherm assumes that adsorption occurs on a homogeneous surface with a finite number of adsorption sites, where each site is equivalent.
  - Once a molecule is adsorbed to a site, there is no further interaction with neighboring molecules, and each site can accommodate only one molecule.

Equation:

$$q_e = \frac{K_L.C_e.q_{max}}{1+K_L.C_e}$$

- q_e : Quantity adsorbed at equilibrium (mg/g).
- q_{max} : Maximum adsorption capacity (mg/g).
- K_L : Langmuir constant (L/mg).
- C_e : Concentration of solute in mobile phase at equilibrium (mg/L).

Applications:

- Used for systems where adsorption reaches saturation, for example in adsorption chromatography for the purification of organic compounds.

(a) **Freundlich isotherm:**

- **Principle:**
  - The Freundlich isotherm is an empirical model that assumes a heterogeneous surface with adsorption sites of different energies. This model allows for multilayer adsorption.
  - It is often used to describe systems where adsorption is nonlinear and applies to both high and low concentrations.
- **Equation:**

$$q_e = K_F . C_e^{1/n}$$

- K_F : Adsorption capacity (mg/g)(L/mg).
- $1/n$: Heterogeneity index (unitless).

  Yes $(1/n) \prec 1$, adsorption is favorable.

- **Applications:**
  - Used for heterogeneous systems, for example in the separation of complex aromatic compounds.

(b) **BET (Brunauer, Emmett, Teller) isotherm:**

- **Principle:**
  - The BET isotherm extends the Langmuir model for multilayer adsorptions. It considers that the molecules adsorbed on the surface form several layers and that the adsorption in the successive layers follows a geometric distribution.
- **Equation:**

$$q_e = \frac{q_{max} . C_e . K_{BET}}{(1 - C_e)[1 + (K_{BET} - 1) . C_e]}$$

K_{BET} : BET constant.

- **Applications:**
  - Used in the analysis of surfaces of solids, for example to determine the specific surface area of catalysts.

(c) **Temkin isotherm:**

- **Principle:**
  - The Temkin isotherm takes into account the interactions between the adsorbent and the adsorbate. It assumes that the heat of adsorption decreases linearly due to the interactions.
- **Equation:**

$$q_e = \frac{RT}{b} \ln(K_T C_e)$$

- b : Temkin constant.
- K_T : Adsorption equilibrium constant.

■ **Applications:**

- Used for systems where molecular interactions are significant, for example in the separation of volatile chemicals.

■ **Practical Applications of Sorption Isotherms in Chromatography:**

Adsorption isotherms help to understand how analytes distribute between the mobile phase and the stationary phase in chromatography. This helps to predict retention, elution, and separation efficiency.

- **Optimization of chromatographic conditions:** Knowing the isotherm, it is possible to adjust the mobile phase to obtain optimal separation.
- **Stationary phase capacity analysis:** Isotherms provide information on the maximum adsorption capacity of the stationary phase.
- **Chromatographic column design:** Isotherms allow selection of the type and amount of stationary phase for efficient separation.

■ **Practical Example:**

Suppose we want to separate a mixture of two compounds A and B using liquid chromatography on a column packed with silica gel (solid adsorbent). The Langmuir isotherm can be used to model the retention of the compounds. If compound A has a stronger interaction with silica, its retention factor will be higher and it will elute more slowly than compound B. By adjusting the polarity of the mobile phase, we can modulate these interactions to achieve an efficient separation.

■ **Conclusion :**

Sorption isotherms are essential tools for understanding and predicting interactions between analytes and the stationary phase in chromatography. Langmuir, Freundlich, BET, and Temkin models provide mathematical frameworks to describe these interactions and thus optimize the analytical separation process.

7.5 Intermolecular interaction:

In chromatography, intermolecular interactions play a key role in the separation process. These interactions manifest themselves in different forms, such as electrostatic forces and Van der Waals forces. Here is a more detailed explanation of the main intermolecular forces and their role in chromatography.

(a) Electrostatic Forces:

■ Definition: Electrostatic forces are forces of attraction or repulsion between two charged particles. They result from the interaction between positive and negative charges.

■ Principle:

- In chromatography, electrostatic forces are manifested mainly in ion exchange stationary phases.
- Ions present in the mobile phase interact with charged sites in the stationary phase.
- The stronger the ionic interaction, the higher the retention of the analyte.

■ Example :

In cation exchange chromatography, cations present in the solution interact with sulfonate groups ($-SO_3^-$) attached to the stationary phase.

(b) Van der Waals forces:

Van der Waals forces are a group of several types of weak but cumulative intermolecular interactions between neutral molecules. They fall into three main categories: **London dispersion forces** , **Debye induction forces** , and **Keesom orientation forces** .

b. 1. London Dispersion Forces:

Definition: London forces are attractive forces that arise between nonpolar molecules due to the formation of instantaneous and temporary dipoles.

Principle:

- These forces are due to a temporary fluctuation in the distribution of electrons in a molecule, creating an instantaneous dipole.
- They increase with the size and polarization of the molecules.

Example: In reversed-phase chromatography (C18 HPLC), nonpolar compounds such as hydrocarbons interact with the nonpolar stationary phase via London dispersion forces, increasing their retention.

b.2 . Debye Induction Forces:

Definition : Debye forces (or induction forces) are forces of attraction between a polar molecule (having a permanent dipole) and a non-polar molecule.

Principle:

- The polar molecule induces a temporary dipole in the apolar molecule by attraction of electrons.
- These forces are influenced by the polarity of the polar molecule and the polarization capacity of the nonpolar molecule.

Example: When analyzing mixtures with polar solvents (such as ethanol), the solvent molecule induces a dipole in the analyte, increasing Debye interactions and retention.

b.3. Keesom Orientation Forces:

Definition: Keesom forces (or dipole-dipole interactions) are attractive forces between two polar molecules having permanent dipoles.

Principle:

- The permanent dipoles of two molecules align in such a way as to minimize energy, creating an attraction between the opposite poles.
- These forces are temperature dependent: they decrease as the temperature increases.

Example: In a polar stationary phase (such as silica), polar analytes such as ketones or alcohols are retained by dipole-dipole interactions with silanol groups (Si-OH).

(c) Hydrogen Bonds:

Definition: Hydrogen bonding is a strong interaction between a hydrogen atom bonded to an electronegative atom (such as O, N, or F) and another electronegative atom possessing a lone pair of electrons.

Principle:

- Hydrogen bonding strongly influences the retention of analytes in chromatography.
- The more hydrogen bonding opportunities there are, the more analyte is retained in the column.

Example: In normal phase chromatography (HPLC), an analyte containing hydroxyl groups (e.g., glucose) will form hydrogen bonds with the stationary phase, increasing its retention.

(d) Hydrophobic Interactions:

Definition: Hydrophobic interactions occur when nonpolar molecules group together to avoid contact with an aqueous phase.

Principle:

- These interactions play an important role in reversed-phase chromatography (HPLC), where nonpolar analytes are retained by the hydrophobic stationary phase (such as C18).
- The more apolar the analyte, the stronger the hydrophobic interaction and the higher the retention.

Example: In the analysis of aromatic hydrocarbons, these show strong retention in a C18 column due to hydrophobic interactions.

⌗ **Choice of Stationary Phase:**

The choice of stationary phase depends on the intermolecular interactions desired for the separation:

- **Nonpolar phases** (C18, C8) for nonpolar analytes (hydrocarbons).
- **Polar phases** (silica, cyano) for polar analytes (alcohols, amines).
- **Ion exchange phases** for ionized analytes (amino acids, proteins).

⌗ **Distribution in the Column:**

The distribution of analytes between the mobile phase and the stationary phase depends on:

- **The affinity** of the analyte for the stationary phase (based on intermolecular interactions).
- **The flow rate** of the mobile phase.
- **The type of stationary phase** used (film-covered surface, solid particles).

Analytes continuously adsorb and desorb to the surface of the stationary phase, allowing separation as they progress through the column.

■ **Conclusion :**

Intermolecular interactions are critical to separation mechanisms in chromatography. Understanding these forces allows for the selection of the appropriate stationary phase and optimization of mobile phase conditions for efficient separation of analytes, while improving resolution and efficiency of the analysis.

This includes an in-depth understanding of London, Debye and Keesom forces, as well as hydrophobic interactions and hydrogen bonding, which influence the retention and selectivity of analytes in a chromatographic analysis.

7.6 Introduction to kinetic theory in chromatography

Kinetic theory in chromatography explains how solutes separate based on different factors that influence the separation efficiency. The efficiency is measured in terms of the number of theoretical plates. The **Van Deemter, Knox, and Giddings equations** are mathematical models used to describe the effect of these factors on the equivalent height of a theoretical plate (HETP).

(a) **Van Deemter equation**

- **Formula** :

$$H = A + B/u + C.u$$

$$B = 2\gamma D_M$$

- Or :
  - H : Equivalent height of a theoretical plate (HETP)
  - A : Eddy term (turbulent diffusion)
  - B : Longitudinal diffusion term
  - C : Mass transfer term
  - u : Linear velocity of the mobile phase: *reduced speed*
  - γ ***tortuosity*** factor > 1
  - D_M : Diffusion coefficient of the solute in the mobile phase
- **Explanation :**
  - **Term A (Eddy Diffusion):** Related to the multiple paths that molecules can take through the stationary phase particles.
  - **B/u term (Longitudinal diffusion):** More significant at low mobile phase speeds. Molecules diffuse along the column, which broadens the peaks.
  - **Term C · u (Mass transfer):** More important at high speeds. Mass transfer is the resistance to the movement of solutes between the mobile and stationary phases.

⌗ **Application:** This equation helps determine the optimum mobile phase velocity that minimizes H and maximizes separation efficiency.

(b) Knox equation

- **Formula:**

$$H = A' + B'.u^{0.5} + C'.u$$

- Where A', B' and C' are specific constants related to the nature of the column and the solutes.
- **Application:** The Knox equation is useful for modern high performance LC columns where mass transfer is a limiting factor at high speed.

(c) Giddings equation

Formula:

$$H = A + B/u + C_1.u + C_2.u^2$$

- Where C1 and C2 represent different mass transfer terms in the mobile and stationary phase.

- **Explanation :**
  - Adds an extra term ($C_2 \cdot u^2$) to represent the extra dispersion at very high speeds.
  - Takes into account the complex effects of mass transfer and molecular interactions under extreme conditions.
- **Application:** Useful for describing the behavior of solutes in columns using modern stationary phases or high viscosity mobile phases.
- **Application of kinetic theory in the analysis of chromatograms**

- **Reading a chromatogram:** Using these equations, we can estimate the height of the peaks, their width at half-height, and evaluate the quality of separation.
  - HH H value indicates an efficient column with narrow peaks.
  - High efficiency translates into a high number of theoretical plateaus.
- **Example:** When analyzing a complex mixture, optimizing the mobile phase velocity using the Van Deemter equation can reduce analysis time while maintaining good resolution.

7.7 Pressure loss in a column: (DARCY's law)

Pressure drop in a chromatographic column is a decrease in pressure across the column as the mobile phase flows through the stationary phase. This pressure drop is essential to understand because it directly influences flow rate, analysis time, and separation efficiency.

Introduction to pressure loss

In the context of chromatography, pressure drop refers to the pressure difference between the inlet and outlet of the column. This pressure drop is due

to the resistance of the flow of the mobile phase through the stationary phase particles.

Principle of pressure loss (Darcy's law)

Darcy's law is used to describe fluid flow in a porous medium, and it is applied to chromatographic columns to model pressure drop.

Darcy equation:

$$\Delta P = \frac{\eta L v}{K^0} \quad \text{and} \quad K^0 = \frac{d_p^2}{180} \times \frac{\varepsilon^3}{(1-\varepsilon)^2}$$

- ΔP : Pressure loss or pressure difference (Pa)
- η : Viscosity of the mobile phase (Pa.s)
- L : Length of the column (m)
- v : Linear velocity of the mobile phase (m/s)
- k^0 : Permeability of the stationary phase (m^2)
- dp : Diameter of stationary phase particles (m)

- ***Viscosity (η)*** *: A measure of the resistance of the mobile phase to flow. Higher viscosity results in greater pressure drop.*
- ***Column length (L)*** *: The longer the column, the higher the pressure loss.*
- ***Linear velocity (v)*** *: The higher the velocity of the mobile phase, the greater the pressure drop.*
- ***Permeability (k)*** *: Measure of the ability of the stationary phase to allow the mobile phase to pass through. Low permeability (due to fine and dense particles) results in high pressure drop.*
- ***Particle diameter (dp)*** *: Smaller particle diameters increase contact area and flow resistance, which increases pressure loss.*

Relationship between pressure drop and separation efficiency

The pressure drop directly influences the flow rate and the maximum pressure that a column can support. If the pressure becomes too high:

- There may be deformation or rupture of the spine.
- The optimal flow rate of the mobile phase cannot be maintained, which affects the efficiency and resolution of chromatographic peaks.

Flow optimization:

- **Choice of stationary phase** : Using larger particles or stationary phases with greater permeability can reduce pressure drop.

- **Mobile phase speed** : It is crucial to adjust the speed to avoid excessive pressure increase while maintaining good separation efficiency.

Practical application of Darcy's law in chromatography

In HPLC chromatography, pressure drop is a critical factor when choosing analysis parameters. For example:

- For rapid separation with high flow rate, short columns with larger particles are used to minimize pressure drop.
- In analyses requiring high resolution, finer stationary phase particles are used, which increases the pressure drop. In this case, the systems must be equipped with pumps capable of handling high pressures.

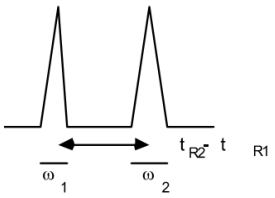
7.8 Optimizing the conditions of an analysis:

The ideal is to obtain a high resolution in very short time:

- It is possible to consider increasing the length of the column (the analysis speed will increase as well as the pressure drop)
- The particle diameter can be reduced (HEPT decreases, but the pressure loss increases)
- can be increased (HEPT increases the pressure too).

The resolution Rs between two peaks is defined by the relation:

$$R = 2 \times \left(\frac{t_{R1} - t_{R2}}{\varpi_1 + \varpi_2} \right)$$



Resolution optimization:

It is necessary to increase Rs

$$\begin{cases} R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_2}{1 + k'_2} \\ R_S = \frac{\sqrt{N_{ef}}}{4} \times \frac{\alpha - 1}{\alpha} \end{cases} \quad \text{with } N_{ef} = 16 \left(\frac{t_R - t_0}{\varpi} \right)^2$$

N_{ef} *number of effective trays*

The peak capacity of a column is defined by:

$$n = 1 + \sqrt{N/16} \ln \left(\frac{t_0}{t_\alpha} \right)$$

$(t_\alpha ; t_0)$Retention time of the first and last peak of the chromatogram, according to HERMAN (1985): Shown for n = 60, there is a 90% probability of separating 9 compounds.

7.8 Optimization of Chromatographic Analysis Conditions

Optimization of analytical conditions in chromatography is essential to obtain efficient separations, rapid analyses and reproducible results. This step aims to adjust various chromatographic parameters in order to improve resolution, reduce analysis time and ensure optimal sensitivity.

- **Objectives of optimization:**
  - **Improve Resolution (R)** : Effectively separate closely related compounds.
  - **Reduce analysis time (tR)** : Speed up the analytical process.
  - **Achieve maximum reproducibility** : Ensure consistent results across multiple analyses.
  - **Increase sensitivity** : Ensure accurate detection of low concentrations.
- **Parameters influencing optimization:**

Mobile phase:

- **Composition of the mobile phase:**
  - In liquid chromatography (HPLC), vary the water/organic solvent ratio (acetonitrile, methanol, etc.) to adjust the polarity.
  - In gas chromatography (GC), change the type of carrier gas (helium, nitrogen, hydrogen) or its flow rate.
- **pH and buffer concentration:** In liquid chromatography, pH influences the loading of analytes and the stationary phase. It must be adjusted to avoid degradation of sensitive compounds.

Stationary phase:

- **Nature of the stationary phase:** Adapt the type of stationary phase (polar, apolar, chiral, etc.) according to the polarity or chirality of the analytes.
- **Column dimensions:** A small diameter column and small particle size (in HPLC) increases efficiency, but also increases pressure.

Mobile phase flow rate: In liquid or gas chromatography, adjusting the flow rate reduces retention times and improves resolution. However, too high a flow rate can reduce efficiency (effect on the number of theoretical plates).

Temperature :

- In gas chromatography, high temperature reduces retention times, but may affect the stability of thermolabile analytes.

- In liquid chromatography, increasing the temperature can decrease the viscosity of the mobile phase, facilitating flow.

⌗ **Column length:** A longer column increases resolution, but at the cost of longer analysis time.

⌗**Injection volume:** Too large an injection volume can saturate the column and reduce separation efficiency.

8. Injectors/injection

8.1 Definition: An injector is an essential component of the HPLC system that allows the sample to be introduced into the mobile phase flow before it enters the column. It ensures precise and reproducible injection.

8.2 Principle:

The injector introduces a controlled amount of sample into the moving mobile phase. This is done without disturbing the flow or pressure of the system. The sample is typically mixed with the mobile phase before passing into the column.

8.3 Different Types of Injectors:

- ✓ **Depending on the sample type:**
  - Liquid samples: conventional injectors.
  - Gas samples: specific injectors (in gas chromatography - GC).
- ✓ **Depending on the type of chromatography:**
  - **Classic HPLC:** manual and automatic injectors.
  - **UHPLC chromatography:** high precision injectors suitable for high pressures.
- ✓ **Common types of injectors:**
  - **Loop Injector:** Allows reproducible injection of a fixed volume.
  - **Automatic Injectors (Autosampler):** Automate injection for multiple samples, reducing human error.
  - **Inline injectors:** Integrated into the mobile phase flow for continuous samples.

8.4 Injection Methodology:

- Prepare a syringe or use an automatic sampler.
- Introduce the sample into the injection loop or directly into the system depending on the injector type.

- Activate the system so that the sample is carried by the mobile phase towards the column.

8.5 How to Inject the Sample:

✓ **Amount of sample injected:**

- Depends on column capacity and sample concentration.
- Typically between 1 and 100 µL for standard HPLC columns.

✓ **Tips for an optimal injection:**

- Use a clean syringe without air bubbles.
- Ensure that the sample is homogeneous.
- Inject slowly to avoid disturbances in the system.

8.5 Sample Preparation:

✓ **Filtration:** Use 0.22 µm or 0.45 µm filters to remove particles that could clog the column.

✓ **Dissolution:** Dissolve the sample in a solvent compatible with the mobile phase.

✓ **Balancing:** Check that the concentration is suitable to avoid saturations or interferences.

8.6 Sample Standards:

✓ **Purity:** Samples must be free of contaminants that could affect results.

✓ **Compatibility:** The dissolution solvent must be compatible with the mobile phase to avoid precipitation or incompatible mixtures.

✓ **Concentration:** Adjusted according to analytical needs and detector detection limits.

8.7 Standards and Their Preparation:

➢ **Definition:** Standards are reference substances used for instrument calibration.

➢ **Preparation :**

- Accurately weigh a known quantity of standard.
- Dissolve in a suitable solvent to obtain an exact concentration.

➢ **Standards:**

- Store standards under appropriate conditions to prevent degradation.
- Prepare standard solutions from the standard to calibrate the system.

9. Mobile Phase

9.1 Definition: The mobile phase is the moving liquid in the HPLC system. It transports the analytes through the column and plays a crucial role in their separation.

9.2 Different Types of Mobile Phases:

✓ **According to polarity:**

- **Polar:** Water, weak acids, buffers (used in normal phase).
- **Nonpolar:** Organic solvents such as methanol or acetonitrile (used in reversed phase).
- **Mixed:** Mixture of water and organic solvents to modulate polarity.

✓ **Depending on the type of samples:**

- Polar samples: non-polar mobile phases.
- Nonpolar samples: polar mobile phases.
- Biomolecules: Specific buffers to maintain the stability of molecules.

9.3 How to Adjust the Flow Rate of the Mobile Phase:

- Use a pump to adjust the flow rate.
- The flow rate is expressed in mL/min and must be adapted to the column used (generally 0.5 to 2 mL/min).
- Equilibrate the column with the mobile phase before sample injection.

9.4 Pumps **:**

✓ **Role of Pumps:**

- Maintain a constant and precise flow rate of mobile phase.
- Withstand high pressures up to 600 bar.

✓ **Types of Pumps:**

- **Isocratic pumps:** Use a single mobile phase composition.
- **Gradient pumps:** Allow the composition of the mobile phase to be varied during analysis (used for complex mixtures).