Chapter VII:

Separation methods in chromatography

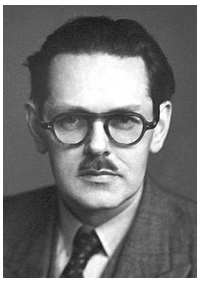
VII General introduction:

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.

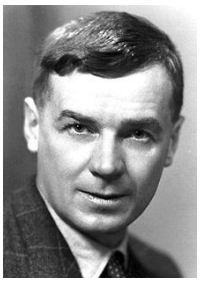
The concept of chromatography was developed in the early 20th century by Russian botanist **Mikhail Tswett** , who discovered that he could separate plant pigments by passing them through a chalk tube (a column) with a solvent.

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

VII.1.1 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.

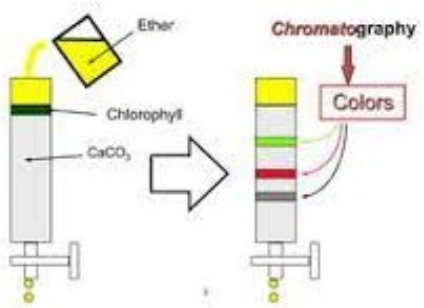


Fig. VII.1: Mikhail Tswett's experience

- **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).

VII.1.2 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.

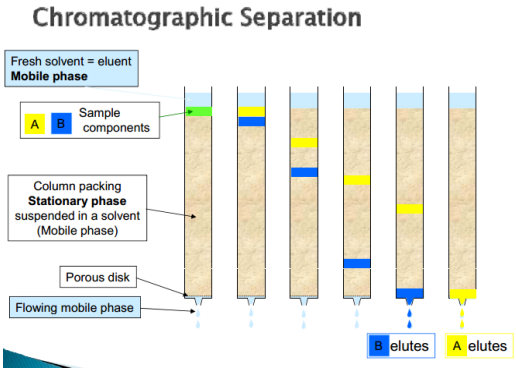
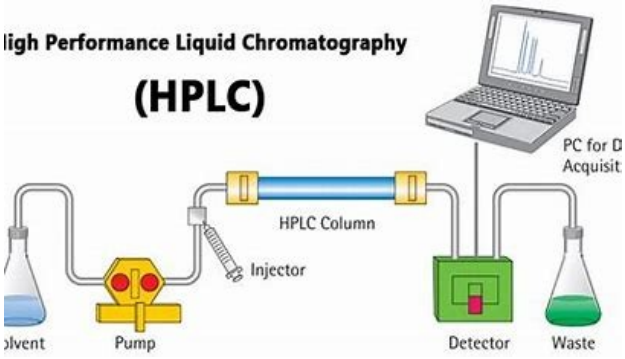


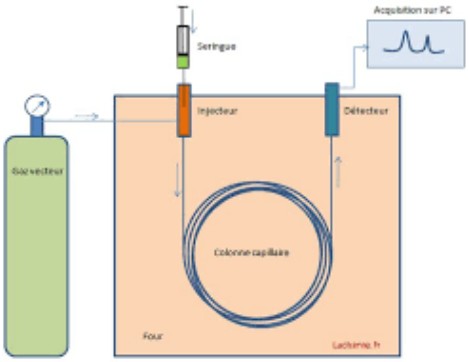
Fig.VII.2: Principle of chromatography

- **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.

VII.1.3 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

Fig. VII.3: Structure of a Chromatographic System[1]

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 : Contains the **mobile phase** , which is the fluid used to transport analytes through the column.

- 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: Ensures constant and controlled **flow** of the mobile phase through the system.

- 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: Allows the sample to be analyzed to be **introduced** into the mobile phase.

- In HPLC, the sample is injected using an **injection loop**
- In CPG, the sample is vaporized and injected into the carrier gas stream.
- Injection must be rapid and reproducible to ensure good separation.

d) Chromatographic column: It is the heart of the chromatographic system where **separation** of analytes occurs.

- 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: Measures the **concentration** of analytes after their separation in the column.

[1] https://www.analyticaltoxicology.com/chromatographie-phase-gazeuse-cpg/

https://www.analyticaltoxicology.com/methodes-separatives/

- 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): Collects and **analyzes** signals from the detector.

- 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: Collects effluent after detection or allows fractions to be recovered for further analysis.

- 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.

VII.1.4 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:

1. **Gas-Solid Chromatography (GSC)** : Solid stationary phase and gas mobile phase.
2. **Gas-Liquid Chromatography (GLC)** : Liquid stationary phase immobilized on a solid support, with a gaseous mobile phase.
3. **Liquid-Solid Chromatography (LSC)** : Solid stationary phase, and liquid mobile phase.

4. **Liquid-Liquid Chromatography (LLC)** : Liquid stationary phase immobilized on a support, and liquid mobile phase.
5. **Supercritical Chromatography** : Uses a supercritical mobile phase, usually CO_2 .

(b) Classification according to the separation phenomenon:

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

(c) Classification according to the processes used:

1. **Column chromatography** : The sample passes through a column filled with stationary phase.
2. **Thin layer chromatography (TLC)** : The stationary phase is a thin film on a plate and the sample migrates over it.
3. **Paper chromatography** : Uses paper as the stationary phase and the solvent migrates by capillary action.
4. **High-performance liquid chromatography (HPLC)** : A column method using high pressures to improve separation.
5. **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:

1. **Temperature** : Mainly used in gas chromatography (GC) where high temperatures allow evaporation and separation of volatile components.
2. **Pressure** : Particularly relevant in HPLC to improve separation speed and resolution.
3. **Polarizability and polarity** : Affect the interactions between the mobile phase, stationary phase, and analytes.
4. **pH** : In ion exchange chromatography, it influences the charge of analytes and exchange resins.
5. **Molecular size** : Essential in size exclusion chromatography (gel permeation) where molecules are separated by size.

VII.1.5 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 VII.1: 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 temperature	Thermosensitive compounds	Thermally stable compounds	Compounds sensitive to high temperatures	Heat sensitive compounds	Thermosensitive compounds	Moderate
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 applicati	Pharmaceuticals,	Volatile organic	Anions, cations,	Pharmaceuticals, polar	Organic, polar	Organic compounds

Selection criteria	Liquid chromatography (LC)	Gas chromatography (GC)	Ion chromatography	Reversed phase chromatography (RP-LC)	Ion pair chromatography	Supercritical chromatography (SFC)
ons	biomolecules	compounds	polar compounds	compounds	products	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.