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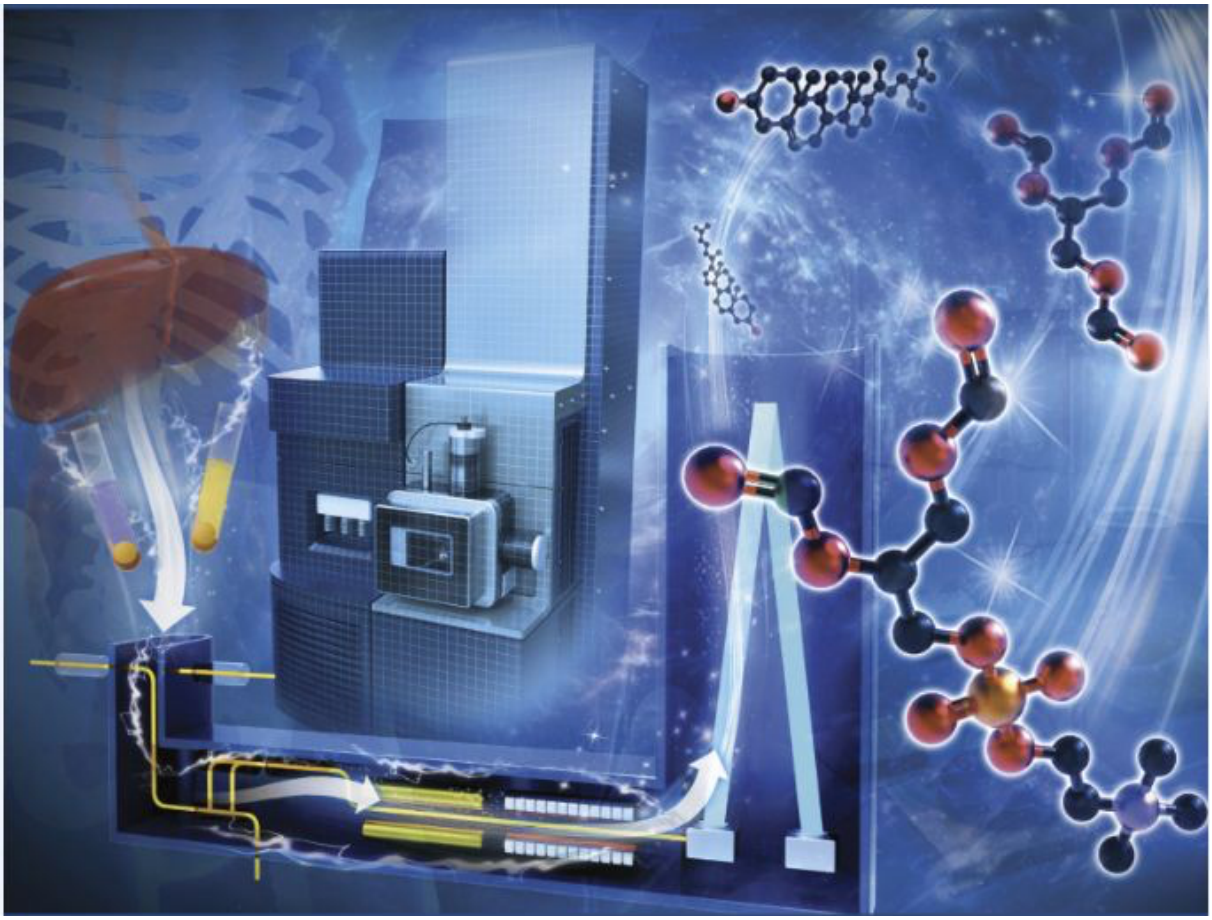
Physicochemical Analytical Methods



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PREFACE

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Analytical chemistry can be defined as the science that develops and applies physical and chemical methods for the identification, characterization, and quantification of substances. According to *Encyclopaedia Universalis*, "Analytical chemistry is the branch of chemistry whose aim is the identification, characterization, and quantification of chemical substances, as well as the development of the methods necessary for such analysis. It also seeks to understand the fundamental processes involved in analytical techniques in order to continuously improve them." This definition underlines the central role of analytical chemistry in ensuring quality control and advancing knowledge across diverse fields such as medicine, food science, pharmaceuticals, and environmental monitoring.

Practical examples of the importance of analytical chemistry abound. Every day, millions of blood samples are analyzed for oxygen and carbon dioxide levels to assist in diagnosis and treatment. The amounts of hydrocarbons, nitrogen oxides, and carbon monoxide in automotive exhaust gases are measured to evaluate the efficiency of emission control systems. Determining calcium ion concentrations in human serum contributes to the diagnosis of parathyroid diseases. During steel production, precise analysis of additives such as carbon, nickel, and chromium ensures the desired balance of hardness, ductility, and corrosion resistance. Even the concentration of odorants such as mercaptans in natural gas is continuously monitored to ensure public safety by enabling early detection of dangerous leaks.

Beyond industry and healthcare, quantitative chemical analysis plays a vital role in research across chemistry, biochemistry, biology, geology, physics, and related sciences. For example, measuring the levels of potassium, calcium, and sodium ions in intracellular fluids allows physiologists to investigate nerve impulse transmission and the mechanisms of muscle contraction and relaxation. These tasks, and countless others, illustrate the indispensable role of analytical chemistry in both applied and fundamental research.

This manuscript brings together essential knowledge on spectroscopic and chromatographic methods that are now routinely employed in modern chemical analysis. It is designed to provide students with a solid foundation in the principles and applications of these techniques, covering both qualitative and quantitative approaches as well as structural analysis. The content is structured to meet the academic needs of undergraduate, master's, and engineering students across a wide range of modules—*Physical Methods of Analysis, Physicochemical Analytical*

Methods, Analytical Chemistry, Analytical Techniques, and Physicochemical Methods and Techniques of Analysis.

The chapters are organized into two main parts. The first is devoted to spectroscopic methods, which explore the interactions between radiation and matter. These methods not only yield quantitative data but also provide crucial insights into the structure and dynamics of molecules, making them some of the most powerful tools available to the analytical chemist. The second part focuses on chromatographic methods, which separate complex mixtures into their individual components based on their interactions with stationary and mobile phases. Despite technological differences among various chromatographic approaches, they all share a common principle: identification through separation.

Throughout this manuscript, numerous examples and illustrations are provided to clarify theoretical developments and highlight practical applications. The objective is not only to familiarize students with fundamental principles but also to cultivate problem-solving skills that will allow them to design and interpret analytical experiments with confidence. Equally important, the course emphasizes laboratory practice, encouraging students to adopt the rigor and attention to detail required to produce reliable analytical results.

Written as clearly as possible, this teaching material is intended to support a wide audience of students in the sciences—whether they are refreshing previously studied concepts, preparing for advanced coursework, or aiming to apply analytical chemistry in professional or research settings. It is our hope that this manuscript, *Chromatographic and Spectroscopic Techniques: Principles and Applications in Chemical Analysis*, will serve as a valuable resource and learning companion for all those engaged in the fascinating and essential field of analytical chemistry.

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Part I
Spectroscopic Methods

Spectroscopic Methods

Spectroscopy, also known as spectrometry, is the experimental study of the spectrum of a physical phenomenon. Spectrometric techniques enable the analysis of the interaction between electromagnetic radiation and matter as a function of wavelength (λ) or frequency (ν) (Fig. 1). Historically, spectroscopy was defined as the study of visible light dispersed according to its wavelength, for instance, through a prism.

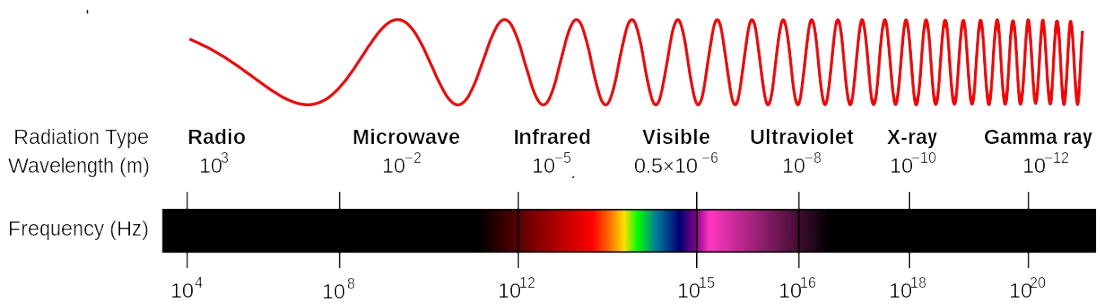


Figure 1. Main regions of the electromagnetic spectrum, shown by frequency, wavelength, and interaction with matter.

Figure 1 illustrates the classification of electromagnetic radiation, detailing the associated wavelengths and frequencies, each distinguished by their unique interactions with matter. From the longest to the shortest wavelengths, we observe: radio waves, microwaves, infrared (IR) radiation, visible light (VIS), ultraviolet (UV) radiation, X-rays, and gamma rays.
We categorize spectroscopic techniques into two main groups:

- **Techniques focused on the quantitative analysis of absorption or emission:**
  Examples include UV spectrophotometry, VIS spectrophotometry, atomic absorption, and flame emission photometry.
- **Techniques dedicated to the qualitative analysis of spectra:**
  Examples include IR spectroscopy, UV spectroscopy, and NMR.

Identification of Chemical Compounds:

1. **Classical Methods:**
   - Physical characterization (e.g., odor, color).
   - Characteristic qualitative tests (e.g., refractive index, solubility, boiling point, melting point).

- Characteristic chemical reactions (e.g., functional groups).

2. **Modern (Spectral) Methods:**
   - **Mass Spectrometry:** Primarily provides empirical formulas along with the identification of the constituent elements in a molecule.
   - **IR Spectroscopy:** Used to identify functional groups.
   - **UV Spectroscopy:** Assists in elucidating molecular structures and identifying certain functional groups.
   - **NMR:** Provides insights into the structural framework of molecules

Part I

Spectroscopic Methods

Chapter I

Molecular UV/VIS Absorption Spectroscopy

I. Molecular UV/VIS Absorption Spectroscopy

I.1. Overview

The measurement of electromagnetic radiation absorption by matter in the UV/Visible range provides limited structural information; however, it is highly valuable for quantitative analyses. The UV/VIS range spans from 190 to 800 nm and can be subdivided into two sections: ultraviolet (190 to 400 nm) and visible light (400 to 800 nm) (see Fig. 2).

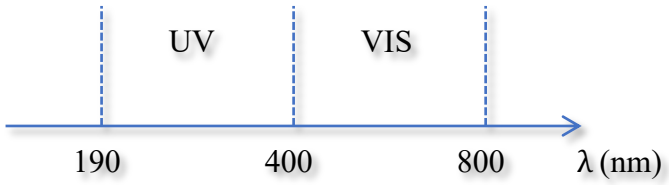


Figure 1. Spectrum of the UV-VIS range.

I.2. Principle

I.2.1. Beer-Lambert Law:

The measurement of the interaction between electromagnetic radiation and matter is expressed in terms of absorbance (A) or transmittance (T) of a chemical substance in solution, at a predefined wavelength (λ) (see Fig. 3). The more concentrated the sample, the greater its ability to absorb light, adhering to the proportionality limits defined by the Beer-Lambert Law.

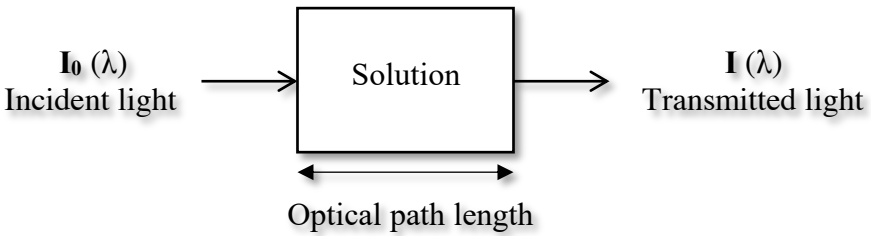


Figure 2. Diagram explaining the Beer-Lambert law.

This law was initially discovered by Pierre Bouguer in 1729, later refined by Johann Heinrich Lambert in 1760. Finally, August Beer introduced the concept of concentration in 1852 (Eq. 1).

$$I = I_0 . e^{-\varepsilon l c} \qquad \textbf{Eq.1}$$

Where:

- I_0: the incident light intensity.
- I: the transmitted light intensity.
- ε: the molar extinction coefficient in $L \cdot mol^{-1} \cdot cm^{-1}$ (dependent on the wavelength, solvent, substance under study, and temperature).

- l: the optical path length (or width of the cuvette in cm).
- c: the concentration of the solution in mol·L^{-1}.

Absorbance (A) is defined as the logarithm of the ratio of the incident intensity I_0 to the transmitted intensity III at a wavelength λ (Eq. 2):

$$A = \log\left(\frac{I}{I_0}\right) \qquad \textbf{Eq.2}$$

Transmittance (T) is defined as the ratio of the transmitted intensity I to the incident intensity I_0 at a wavelength λ (Eq. 3):

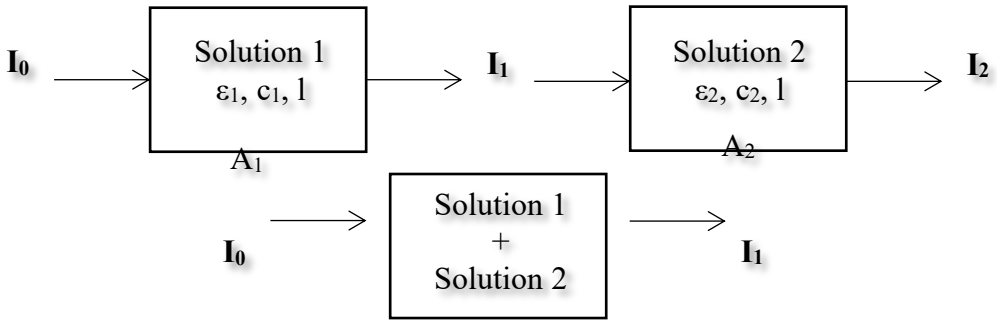
$$T = \frac{I}{I_0} \qquad \textbf{Eq. 3}$$

The most commonly used form is (Eq. 4):

$$A = \varepsilon l c \qquad \textbf{Eq.4}$$

- **Additivity of Absorbances**

The Beer-Lambert law is additive (see Fig. 4), meaning that if two solutions absorb light at the same wavelength, the absorbance of the mixture is equal to the sum of their individual absorbances.



$$A = A_1 + A_2 = (\varepsilon_1 c_1 + \varepsilon_2 c_2)\, l$$

Figure 3. Diagram explaining the additivity of the Beer-Lambert law.

- **Conditions for the Validity of the Beer-Lambert Law:**

1. **Monochromatic Light:** The light must have a constant wavelength (λ).
2. **Dilute Solutions:** The concentration must be less than or equal to 10^{-2} M.
3. **Clear Solutions:** The solutions must be transparent, free from fluorescence or suspensions.

I.2.2. Description of Electronic Energy Levels

Absorption occurs when radiation interacts with the sample. When a photon is absorbed, the energy of one or more valence electrons is increased, leading to electronic transitions among the molecular orbitals. These orbitals are designated as σ and π for bonding orbitals, σ* and π*

for antibonding orbitals, and n for non-bonding orbitals, which play a minimal role in chemical bonding. The energy levels of these molecular orbitals are illustrated in Figure 5 for organic compounds.

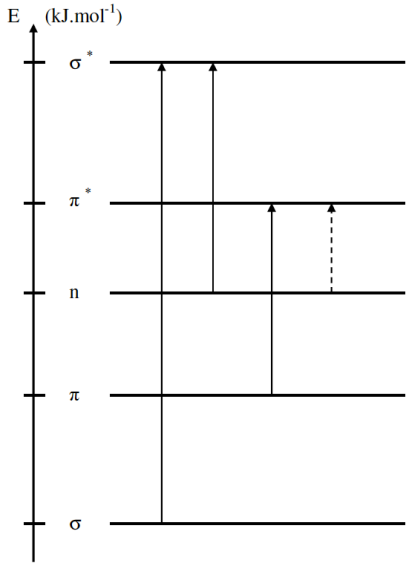


Figure 4. Energy levels of molecular orbitals involved in electronic transitions.

I.2.3. Selection Rules and Allowed Transitions

A UV-visible transition (190 to 800 nm) corresponds to the movement of an electron from a filled molecular orbital to an unoccupied excited molecular orbital. During this process, the matter absorbs a photon whose energy matches the energy difference between the ground state and the excited state. However, not all energetically possible transitions are allowed. Allowed transitions are those that result in a change in the electric dipole moment, as illustrated in Figure 5. In other words, the photon alters the symmetry of the occupied orbital of the electron before and after the transition, without affecting the electron's spin.

I.2.4. Different Types of Transitions and Chromophores

The functional groups responsible for a compound's UV/Visible absorbance are known as chromophores (C=C, C=O, C=N, C≡C, C≡N, etc.). When isolated, a specific chromophore consistently absorbs at the same wavelengths. However, when chromophores are in proximity to one another, they tend to absorb more strongly at higher wavelengths. The absorption wavelength depends on the nature of the orbitals involved.

- ***σ-σ* Transition****:* The high stability of σ bonds in organic compounds means that the transition of an electron from a bonding σ orbital to an antibonding σ* orbital requires

significant energy. The corresponding absorption band is intense and is located in the far UV, around 130 nm.

- ***n-π* Transition****:* This transition involves the movement of an electron from a non-bonding n orbital to an antibonding π* orbital. It occurs in molecules containing heteroatoms with lone pairs of electrons in an unsaturated system. The corresponding band is weak because the transition is forbidden; the most well-known example is the carbonyl band, which is found between 270 and 280 nm, and it has a low molar absorptivity coefficient.
- ***n-σ* Transition****:* This transition involves the transfer of an electron from the n lone pair of a heteroatom (such as O, N, S, Cl) to a σ* level, observed in alcohols, ethers, amines, and halogenated derivatives. It produces a band of medium intensity at the far edge of the near UV.
- ***π-π* Transition****:* This electronic transition in compounds with isolated double bonds leads to a strong absorption band around 165-200 nm.

In practice, only π→π* transitions will be observed, along with very weak n→π* transitions. Therefore, the information in an electronic spectrum primarily pertains to the unsaturations within the studied molecule; absorption can only occur in double bonds.

I.3. Instrumentation in UV/VIS

The spectrophotometer is one of the main instruments used in laboratories for quantitative analyses (see Fig. 6). It operates on the principles of light and its interaction with substances. The device is structured around three main components:

- **Light Source:** This may include a mercury vapor lamp, a xenon arc lamp, or a tungsten filament lamp.
- **Dispersive System (Monochromator):** This assembly of elements decomposes white light into radiation of different wavelengths, one of which is used to measure the analysis results.
- **Detector System:** This component detects the transmitted light and converts it into an electrical signal.

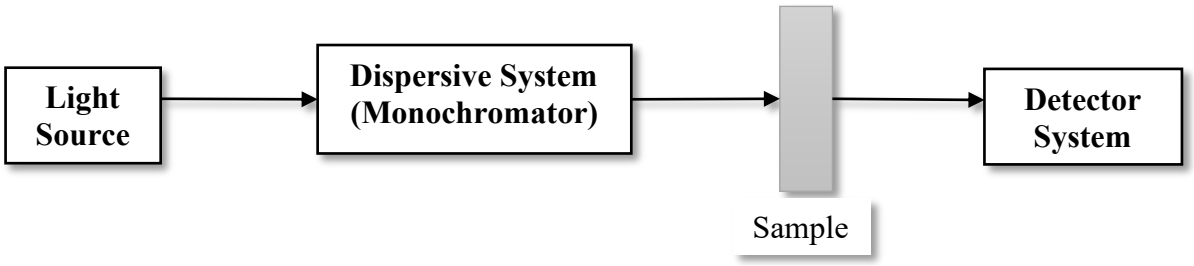


Figure 5. Simplified diagram of a UV/Visible spectrophotometer.

Note: It is essential to work with monochromatic light, as the molar extinction coefficient (ε) is dependent on the nature of the absorbing substance, temperature, and wavelength. To achieve optimal sensitivity, it is important to determine the wavelength at which the solution exhibits maximum absorption.

I.4. Different Configurations of UV/VIS Spectrometers

I.4.1. Single-Beam Spectrometers

In a single-beam spectrometer, a reference sample, either a blank containing only the solvent or a solution of the reagents used in the analysis (without the analyte), is placed sequentially in the optical path, followed by the solution prepared from the unknown concentration sample (see Fig. 7). The final measurement of absorbance (or transmittance) requires subtracting the absorbance of the blank from that of the sample at the same wavelength.

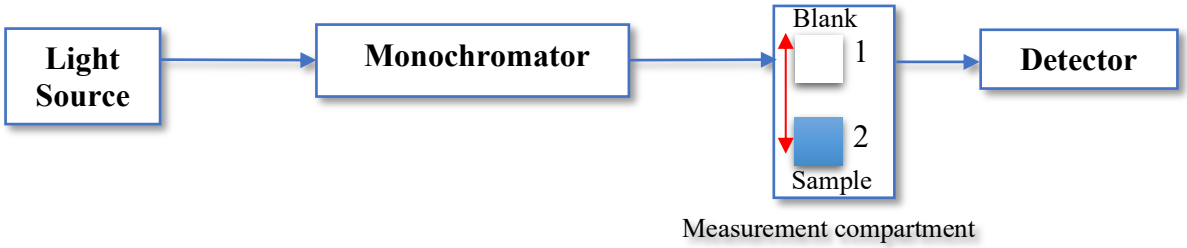


Figure 6. Simplified diagram of a single-beam spectrophotometer.

I.4.2. Double-Beam Spectrometers

The most advanced spectrophotometers in this field are still the double-beam devices, where one beam passes through the sample while the other serves as a reference path (see Fig. 8). Two rotating sector-shaped mirrors, synchronized with the stepwise movement of the diffraction grating, enable the detector to accurately compare the intensities transmitted through either path at the same wavelength.

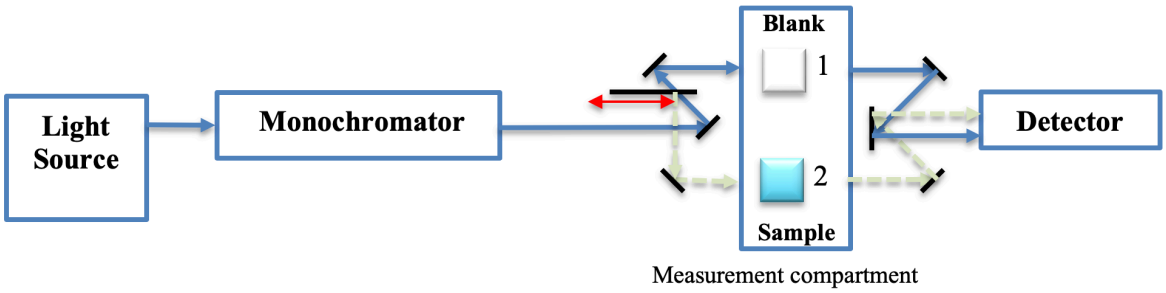


Figure 7. Simplified diagram of a double-beam spectrophotometer.

I.5. Presentation of an UV-Visible Spectrum

The UV-Visible spectrum is a graph representing the absorbance or transmittance of a substance as a function of the wavelength of light (typically between 190 and 800 nm). On the x-axis, we find the wavelength in nanometers (nm), while the y-axis represents either absorbance (dimensionless) or transmittance (in percentage).

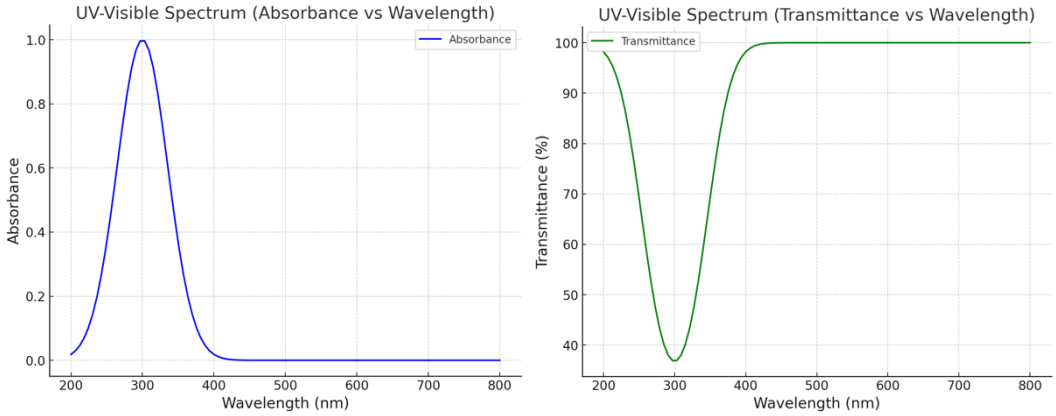


Figure 9. Representation of the UV/Visible spectrum.

I.6. Interpretation of an UV-Visible spectrum

To interpret an UV-Visible Spectrum we must:

- **Identify absorption peaks**: The wavelengths where peaks appear depend on the nature of the electronic transitions. Conjugated molecules, chromophores such as double bonds or carbonyl groups, show specific absorptions.
- **Analyze the electronic structure**: The $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions provide clues about the presence of double bonds, conjugated systems, or functional groups.
- **Use absorbance for quantitative analysis**: Through the Beer-Lambert law, absorbance can be related to the concentration of the studied substance, allowing for quantitative analysis.

Example: **Acetone (CH_3COCH_3)**

Acetone, a molecule containing a carbonyl group (C=O), exhibits specific absorption peaks in the UV region.

- **UV-Visible Spectrum of acetone**:

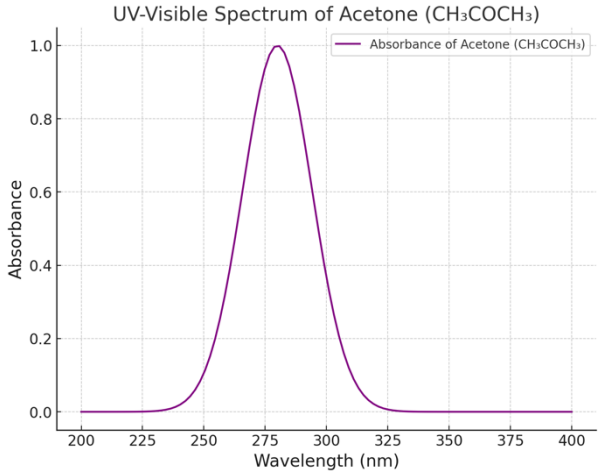


Figure 10. UV/Visible spectrum of acetone.

The UV spectrum of acetone shows a main absorption peak at **λ = 280 nm**, corresponding to the n → π* transition, where a non-bonding electron (n) from the oxygen of the carbonyl group is excited to an antibonding π* orbital.

Interpretation:

The peak at 280 nm is typical of carbonyl compounds. The n → π* transition is characteristic of C=O groups, as the non-bonding electron on oxygen is excited to an antibonding orbital. This transition is less energetic than the π → π* transition, hence its higher wavelength. Generally, compounds with carbonyl groups show absorptions in this region.

Part I

Spectroscopic Methods

Chapter II

Infrared (IR) Spectroscopy

II. Infrared (IR) Spectroscopy

II.1. Overview

Infrared radiation is the portion of the electromagnetic spectrum that ranges from 780 to 1,000,000 nm (see Fig. 1), lying between the visible light and radio waves. This range corresponds to energy transitions associated with the vibrational states of atoms or the rotational states of molecules. Infrared radiation is divided into three regions (Table 1):

Table 1. Ranges of the three infrared regions.

Region	λ Range (nm)	δ Range (cm^{-1})
Near Infrared (NIR)	780 – 2,500 nm	4,000 – 13,000 cm^{-1}
Mid Infrared (MIR)	2,500 – 25,000 nm	400 – 4,000 cm^{-1}
Far Infrared (FIR)	25,000 – 1,000,000 nm	10 – 400 cm^{-1}

Note: $\delta = 1/\lambda$, where λ is the wavelength and δ is the wavenumber.

The most commonly used IR region is the one associated with atomic vibrations, located between 400 and 4,000 cm^{-1}, which corresponds to the MIR region (Table 1).

II.2. Principle

In a chemical bond, the atoms on either side of the bond vibrate relative to one another at a frequency specific to that bond. The frequency of vibration, estimated between the ground state and the first excited state, is calculated using the harmonic oscillator model, in which the two atoms are considered to be connected by a spring with a stiffness constant k (see Fig. 11), analogous to the bond strength.

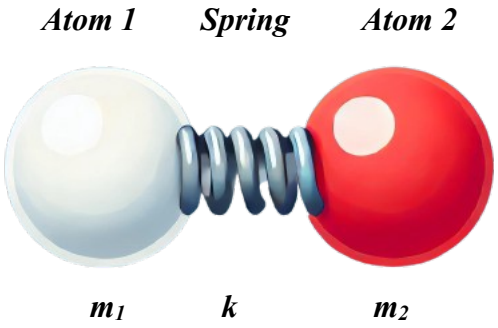


Figure 8. Molecular vibration model atoms connected by a spring.

The frequency specific to the bond is calculated according to the equation (Eq. 6):

$$\nu = \frac{1}{2\pi} \times \sqrt{\frac{k}{\mu}} \qquad \textbf{Éq. 6}$$

With:

- **k**: the stiffness constant And **μ**: the reduced mass.

μ is the reduced mass of the model, calculated according to equation 2 :

$$\mu = \frac{m_1 \times m_2}{m_1 + m_2} \qquad \textbf{Éq. 7}$$

However, the classical mechanics model is not sufficient, as it does not allow for the introduction of the quantization of energy levels, which is necessary to interpret the discontinuity of the spectra observed in the infrared region, where the energy levels correspond to different vibrational states of the molecule. It is the quantum mechanics model that allows for the quantization of energy, as described in Eq. 8.

$$E = \left(V + \frac{1}{2}\right) \times h \times \nu = \left(V + \frac{1}{2}\right) \times hc \times \bar{\nu} \qquad \textbf{Éq. 8}$$

V being the vibrational quantum number (a non-negative integer) and h the Planck constant. According to this expression of energy, we observe that the quantized energy levels are equidistant and that this energy is never zero.

When a bond is irradiated with an electromagnetic wave of the same frequency as its vibrational frequency, the energy of the diatomic system increases, which results in a decrease in emitted radiation and an increase in the amplitude of vibration of the bond. This is the phenomenon of absorption. However, the calculated atomic distance and the experimentally observed one are not perfectly identical, as the harmonic oscillator model is an approximate model. To obtain a more accurate value, the anharmonic oscillator model must be used, which explains the absorption bands observed in the near-infrared region.

According to the anharmonic oscillator model, it is assumed that the curve representing the potential energy as a function of the distance r between two atoms is no longer superimposable on a parabola but is described by the Morse curve. The possible eigenvalues (solutions to the Schrödinger equation) lead to the expressions for energy (Eqs. 9, 10, and 11).

$$E = \left(V + \frac{1}{2}\right) \times h \times \nu_a - \left(V + \frac{1}{2}\right)^2 \times h \times \nu_a \times \chi_e \qquad \textbf{Éq. 9}$$

$$E = \left(V + \frac{1}{2}\right) \times h \times \overline{\nu_a} - \left(V + \frac{1}{2}\right)^2 \times h \times \overline{\nu_a} \times \chi_e \qquad \textbf{Éq. 10}$$

$$\Delta E = h\nu_a = hc\overline{\nu_a} \qquad \textbf{Éq. 11}$$

With $\boldsymbol{\nu_a}$ as the oscillation frequency, $\boldsymbol{\overline{\nu_a}}$ va as the reduced wavenumber, and $\boldsymbol{\chi_e}$ as the anharmonicity constant (its value is small and always positive). As a result, the energy levels are no longer equidistant, and if V increases, the distance separating two consecutive levels decreases; it even tends toward zero as one approaches the dissociation energy.

Figure 12 shows the energy of the bond as a function of atomic distance, as well as the energy calculated according to the harmonic and anharmonic oscillator models.

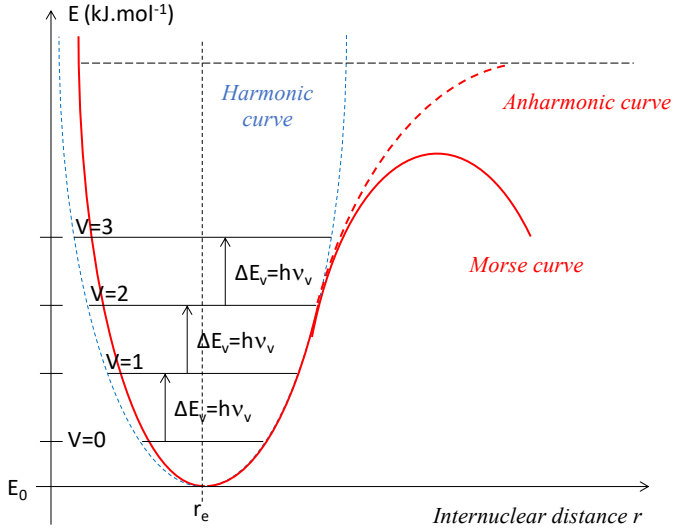


Figure 9. Energy curve as a function of displacement.

A very important consequence of anharmonicity is that a given chemical bond generally vibrates at multiple frequencies, which correspond to the fundamental vibration and harmonic vibrations of lower amplitudes.

These frequencies can be determined from (Eq. 12, 13, and 14).

Fundamental vibration:	$\nu_1 = \nu_0(1 - \chi_e)$	**Éq. 12**
First harmonic vibration:	$\nu_2 = 2\nu_0(1 - 3\chi_e)$	**Éq. 13**
Second harmonic vibration:	$\nu_3 = 3\nu_0(1 - 4\chi_e)$	**Éq. 14**

Where $\boldsymbol{\nu_0}$ is the fundamental frequency, depending on the nature of the bond. Since $\boldsymbol{\chi_e}$ is small, the frequencies are respectively close to $\boldsymbol{\nu_0}$, $\boldsymbol{2\nu_0}$ and $\boldsymbol{3\nu_0}$. Thus, these harmonics, appearing at 2 or 3 times the fundamental vibration frequency, see their absorption decrease as the number of harmonics increases. However, as the number of harmonics increases, the overlap of bands intensifies (Fig. 13).

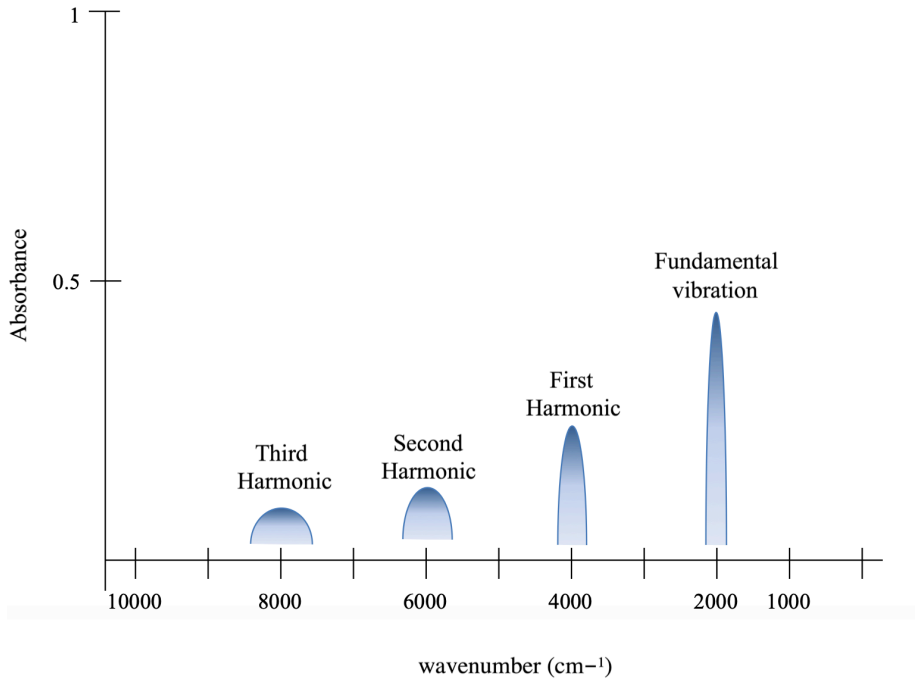


Figure 10. Positioning of the different harmonics relative to the fundamental vibration.

MIR (Mid-Infrared) spectroscopy is based on the absorption of radiation in a spectral range varying from 400 to 4000 cm^{-1}, where every organic compound has its own unique fingerprint. Several functional groups of organic compounds will exhibit characteristic vibrations. Consequently, MIR spectroscopy is very useful for qualitative analysis. Quantitative analysis is also possible since the intensity of absorption is proportional to the concentration of the absorbing species.

MIR absorption bands are mainly due to the fundamental vibrations of molecules. The mid-infrared spectrum can be roughly divided into three regions:

- From 1500 to 4000 cm^{-1} are found the absorption bands of stretching vibrations ν of major bonds (O-H, N-H, C-H, C=O, C=C),
- From 1000 to 1500 cm^{-1} are the deformation bands δ (C-H) as well as some stretching vibrations (C-O, P=O),
- For wavenumbers below 1000 cm^{-1}, complex vibrations ("breathing" of cyclic structures) and ethylenic or aromatic systems are found (Fig. 14).

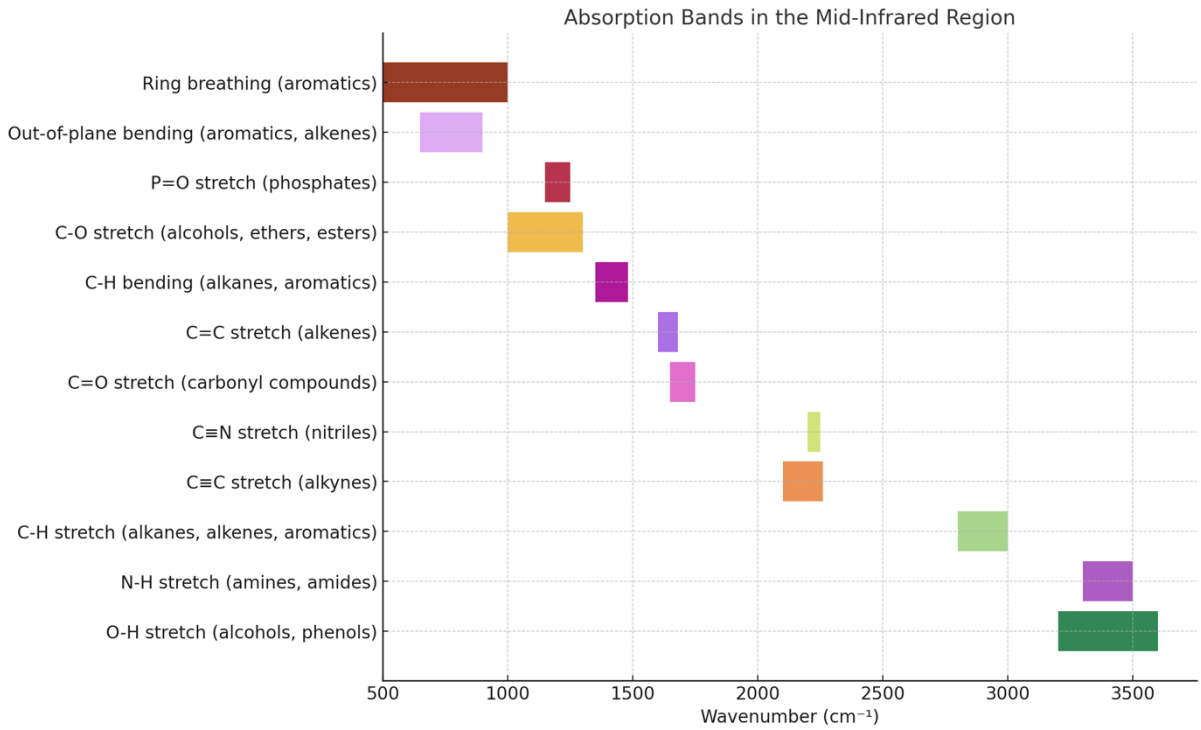


Figure 14. Absorption bands in the Mid-Infrared region.

II.3. Factors Influencing Vibration Frequencies

- **Bond Multiplicity**:

When the number of bonds increases, the stiffness constant k increases.

Example:

$$C = C \xrightarrow{k \text{ increases}} C \equiv C$$

$$\nu \approx 1650\ cm^{-1} \xrightarrow{\nu \text{ increases}} \nu \approx 2200\ cm^{-1}$$

- **Increase in Mass**:

If the mass of one of the atoms increases, the frequency ν decreases.

Example:

$$C — H \xrightarrow{k \text{ increases}} C — O$$

$$\nu \approx 2900\ cm^{-1} \xrightarrow{\nu \text{ decreases}} \nu \approx 1200\ cm^{-1}$$

- **Inductive and Mesomeric Effects**:
  - An electronegative group (O=C–Cl) increases the frequency of C=O.
  - The mesomeric effect (O=C–C=C–) weakens the C=O bond and causes a decrease in the frequency.
- **Hydrogen Bonding**:

Intermolecular association via hydrogen bonding involves multiple molecules, and this reversible formation is favored by higher concentration.

Example:

$$3\,R-O-H \quad \rightleftharpoons \quad R-O-H\cdots R-O-H\cdots R-O-H$$

$$\nu_{OH} \approx 3600\ cm^{-1} \quad \rightarrow \quad \nu_{OH} \approx 3450\ cm^{-1}$$

ν decreases

II.4. Instrumentation

Spectra are collected using a spectrometer, typically a double-beam spectrometer, which operates on the same principle as UV-VIS spectrometry. A spectrum can be defined as the measurement of the absorption or transmission of electromagnetic radiation as a function of wavelength (or wavenumber).

Regardless of the type of spectrometer used, two key elements are essential:

- A source of Electromagnetic Radiation (EM),
- One or more detectors.

II.4.1. Light Source

The radiation emitted by the source must be, on the one hand, powerful enough to enable good signal detection, and on the other hand, stable during the measurement acquisition time in order to obtain reproducible absorbance measurements.

For MIR spectrometers, the radiation sources come from solids heated to high temperatures that emit radiation. Among the most common IR sources in MIR, we have the "globar" (glowing-bar), which consists of silicon carbide heated to about 1500°C by the Joule effect (Fig. 15). It dissipates a large amount of heat, which requires cooling. The spectral range extends from 5000 cm^{-1} to 50 cm^{-1}.

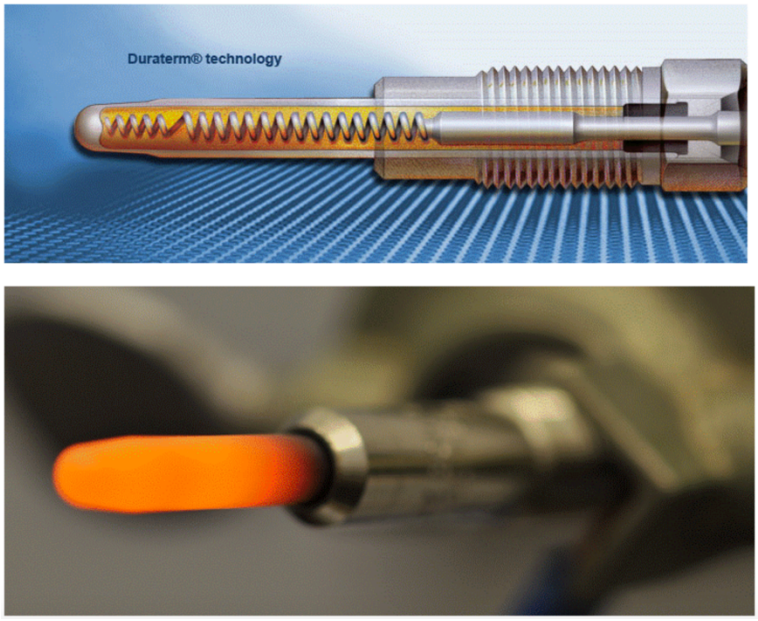


Figure 11. "Globar" light source made of silicon carbide.

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II.4.2. Detectors

Today, various types of detectors can be utilized in infrared spectroscopy, with the most common being thermal detectors and semiconductor detectors. These detectors have been developed to enhance the speed of data acquisition.

In the mid-infrared region, for applications requiring high sensitivity, a sensor made from a Mercury-Cadmium-Telluride (MCT) alloy is often employed, which is cooled with liquid nitrogen at -196 °C (see Fig. 16). Alternatively, Indium-Antimonide (In/Sb) deposited on an inert substrate may also be used.

Figure 12. MCT detector.

II.5. Presentation of an IR Spectrum

An IR spectrum is a graphical representation of infrared light absorption by a molecule as a function of wavenumber (typically in cm^{-1}). On the x-axis, we have the wavenumber, ranging from 4000 cm^{-1} to 400 cm^{-1}, and on the y-axis, we have either transmittance (in percentage) or absorbance (without units). Each peak in the IR spectrum corresponds to a specific vibrational mode of the molecule, such as stretching or bending vibrations of bonds between atoms.

II.6. Interpretation of an IR spectrum

To interpret an IR Spectrum we must :

- **Identify Functional Groups**: Each peak corresponds to a specific vibrational mode of a functional group. The position and intensity of these peaks allow us to deduce which functional groups are present in the molecule.
- **Analyze Absorbance or Transmittance**: Strong peaks indicate strong absorption of IR radiation, which corresponds to specific bond vibrations in the molecule.
- **Compare to Reference Spectra**: For complex molecules, it is often useful to compare the spectrum to reference spectra to confirm the identity of the compound.

Example **: Ethanol (CH_3CH_2OH)**

The IR spectrum of ethanol shows several characteristic peaks:

- **O-H stretching**: A broad and strong peak appears around **3200–3600 cm^{-1}**, corresponding to the hydrogen-bonded hydroxyl (O-H) group.
- **C-H stretching**: Peaks around **2800–3000 cm^{-1}** correspond to the C-H bonds in the alkyl (CH_2 and CH_3) groups.
- **C-O stretching**: A peak around **1050–1150 cm^{-1}** corresponds to the C-O bond stretching vibration in the alcohol.

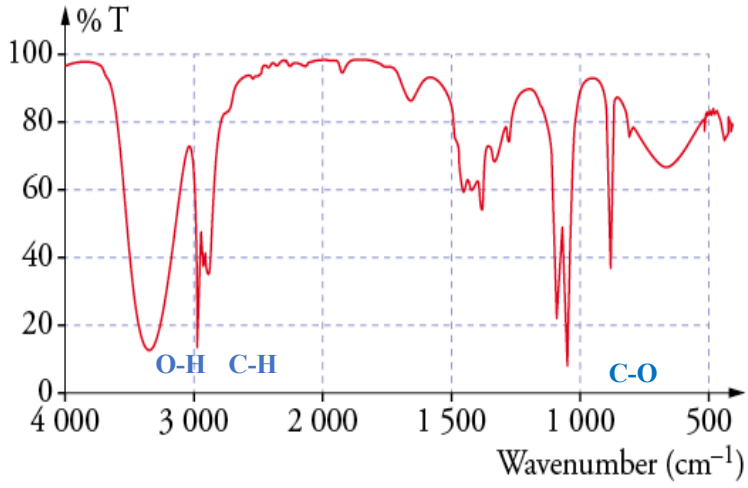


Figure 13. Ethanol IR spectrum.

Interpretation:

The broad O-H peak is a clear indicator of an alcohol group, while the peaks in the C-H and C-O regions provide additional confirmation of ethanol's structure. The presence of these peaks allows us to confirm the functional groups present in the ethanol molecule.

Part I

Spectroscopic Methods

Chapter III

Mass Spectrometry (MS)

III. Mass Spectrometry (MS)

Mass spectrometry (MS) is a powerful analytical technique used to determine the molecular mass, elemental composition, and structure of molecules by measuring the mass-to-charge ratio (m/z) of ions.

III.1. Principle

The process begins with ionization, where neutral molecules are converted into charged particles by different ionization methods, such as electron impact (EI) in which a high-energy electron beam collides with the molecule (M), producing a radical cation ($M^{+\bullet}$) and often generating characteristic fragments that reveal structural details. Once formed, the ions are focused and directed to the mass analyzer, where they are separated according to their m/z values by electric or magnetic fields. The separated ions are then detected, and their relative abundances are recorded in a mass spectrum. This spectrum acts as a molecular fingerprint, enabling both the identification and quantification of analytes through comparison with reference spectral libraries.

III.2. Theoretical Concepts

III.2.1 Mass-to-Charge Ratio (m/z)

The central parameter in mass spectrometry is the mass-to-charge ratio (m/z):

$$m/z = \frac{mass\ of\ the\ ion\ (m)}{charge\ (z)} \quad \textbf{Éq. 15}$$

For most ions, z = 1 :

In mass spectrometry, for most ions, the charge number z is equal to 1, meaning the ion carries a single elementary charge. As a result, the mass-to-charge ratio m/z is approximately equal to the mass of the ion. This is why the m/z value observed in a mass spectrum is often considered to reflect the ion's mass directly when the charge is +1.

This simplification holds because the mass spectrometer measures the ratio of the ion's mass to its charge, and since the charge is usually one elementary charge (z=1), the m/z essentially represents the ion's mass.

III.2.2 Ionization and Fragmentation

Ionization is the process by which neutral molecules are converted into positively or negatively charged ions. The choice of ionization technique depends on the physicochemical properties of the sample, such as volatility, thermal stability, and molecular size (e.g., biological macromolecules).

- **Example of Ionization:** In **electron impact (EI) ionization**, a high-energy electron collides with a molecule (M), removing an electron and producing a radical cation ($M^{+\bullet}$):

$$\mathbf{M + e^- \longrightarrow M^{+\bullet} + 2e^-}$$

For methane (CH_4), EI generates a molecular ion at **m/z 16 ($CH_4^{+\bullet}$)**.

Fragmentation is the process by which an ionized molecule breaks into smaller charged and neutral fragments during mass spectrometry. This occurs because the molecular ion ($M^{+\bullet}$) is often unstable after ionization. The resulting fragment ions provide valuable structural information, since the pattern of bond cleavage depends on the molecular structure.

- **Molecular ion ($M^{+\bullet}$):** The intact ionized molecule, usually the highest m/z value.
- **Fragment ions:** Produced from bond cleavage, appearing at lower m/z values.
- **Base peak:** The most intense fragment ion, set to 100% relative intensity.

Example of Fragmentation: In ethanol (C_2H_6O), the molecular ion at **m/z 46** may fragment into ions at **m/z 31 (CH_2OH^+)**, **m/z 29 ($C_2H_5^+$)**, and **m/z 15 (CH_3^+)**.

III.2.3. Isotopes and Isotopic Patterns

Atoms of the same element may exist in different forms, called isotopes, which differ in the number of neutrons but have the same number of protons. In mass spectrometry, isotopes produce characteristic peak patterns in the spectrum, since ions containing different isotopes have slightly different masses.

- Isotopic peaks appear at M+1, M+2, etc**.**, next to the molecular ion ($M^{+\bullet}$).
- Their intensities depend on the natural abundance of isotopes.
- The analysis of isotopic patterns helps in identifying elements such as chlorine, bromine, or sulfur in a molecule.

Table 2. Common isotopic patterns

Element	Isotopes	Typical Pattern
Cl	^{35}Cl (75%), ^{37}Cl (25%)	Two peaks: M and M+2 in ~3:1 ratio
Br	^{79}Br (50%), ^{81}Br (50%)	Two peaks: M and M+2 in ~1:1 ratio
S	^{32}S (95%), ^{34}S (4%)	Small M+2 peak
C	^{12}C (98.9%), ^{13}C (1.1% per carbon)	M+1 peak increases with number of carbons
N	^{14}N (99.6%), ^{15}N (0.4%)	Small M+1 peak

Example

- A compound containing **one chlorine atom** will show two molecular ion peaks:
    - M (with ^{35}Cl)
    - M+2 (with ^{37}Cl)

    → Intensity ratio ~3:1.
- A compound with **two chlorine atoms** will show three peaks:
    - M, M+2, M+4 → Ratio ~9:6:1.
- A compound containing one bromine atom will show two peaks at M and M+2 of equal intensity**.**

III.2.3. Resolution and Accuracy

In mass spectrometry, the ability to distinguish between ions with similar m/z values and the precision of mass measurement are critical for reliable results. Two key concepts are involved:

a. Resolution (R)

- **Definition:** The ability of a mass spectrometer to separate two adjacent peaks at close m/z values.
- **Formula:**

$$R = \frac{m}{\Delta m} \quad \textbf{Éq. 16}$$

Where:

- m = mass of the ion
- Δm = difference in m/z between two resolvable peaks

Low resolution → Peaks may overlap, making it difficult to identify compounds.
High resolution (HRMS) → Allows differentiation between molecules with nearly identical masses.

b. Mass Accuracy

- **Definition:** The closeness of the measured mass to the true (theoretical) mass.
- **Expressed in parts per million (ppm):**

$$Error\ (ppm) = \frac{Measured\ mass - Theoretical\ mass}{Theoretical\ mass} \times 10^6 \quad \textbf{Éq. 17}$$

High mass accuracy is essential for determining the molecular formula of unknown compounds.

c. Comparison of Resolution Levels

Table 3. Comparison of Resolution Levels, Accuracy, and Applications of Different Types of Mass Spectrometers

Type of MS	Resolution (R)	Accuracy	Applications
Quadrupole MS	~1,000	± 0.1 Da	Routine analysis, quantification
TOF (Time-of-Flight)	10,000–20,000	± 0.01 Da	Proteomics, biomolecules
Orbitrap	>100,000	± 1–2 ppm	Structural elucidation, metabolomics
FT-ICR MS	>1,000,000	< 1 ppm	Ultra-high precision, research-grade

Example

Two molecules: C_3H_6O (acetone, exact mass = 58.0419 Da) and $C_2H_6N_2$ (methylhydrazine, exact mass = 58.0531 Da)**.**

- At **low resolution**, both appear as a single peak at m/z ≈ 58.
- At **high resolution**, they can be clearly separated, allowing correct identification.

III.3. Instrumentation

A mass spectrometer consists of three fundamental components that work together to produce a mass spectrum:

1. **Ion Source** – Converts neutral molecules into ions.
2. **Mass Analyzer** – Separates ions according to their mass-to-charge ratio (m/z).
3. **Detector** – Records the number of ions at each m/z and produces the spectrum.

All mass spectrometers, regardless of their design, share these three essential elements, but the technique used for ionization and the type of analyzer strongly influence performance and applications.

III.3.1 Ion Source

The ion source is the part of the spectrometer where ions are generated. Different ionization methods are available, and the choice depends on the sample's properties (volatile, polar, thermally stable, or large biomolecule).

Table 4. Ionization Methods in Mass Spectrometry

Ionization Method	Principle	Applications
Electron Impact (EI)	High-energy electrons knock out electrons from molecules	Small volatile molecules, gases
Chemical Ionization (CI)	Ion-molecule reactions with reagent gas	Molecular weight determination
Electrospray Ionization (ESI)	Charged droplets evaporate, leaving multiply charged ions	Proteins, peptides, biomolecules
MALDI	Laser desorption with matrix	Large biomolecules (proteins, polymers)

III.3.2 Mass Analyzer

The analyzer separates ions based on their m/z. Different analyzers offer trade-offs between resolution, speed, and mass range.

Table 5. Ionization Methods in Mass Spectrometry

Analyzer	Principle	Advantages	Limitations
Quadrupole	Uses oscillating electric fields to allow only selected m/z ions to pass	Simple, robust, good for quantitative analysis	Limited resolution
Time-of-Flight (TOF)	Ions separated by velocity (lighter ions reach detector faster)	High mass range, fast analysis	Requires pulsed ionization
Ion Trap	Ions confined in an oscillating electric field	Enables MS/MS experiments, compact	Limited dynamic range
Orbitrap	Ions oscillate around a central electrode; frequency measured to determine m/z	Very high resolution and accuracy	Expensive
FT-ICR (Fourier Transform Ion Cyclotron Resonance)	Ions trapped in a magnetic field; cyclotron frequency analyzed	Ultra-high resolution (<1 ppm accuracy)	Very expensive, requires strong magnets

III. 3.3 Detector

The detector records the separated ions and converts them into an electrical signal, which is processed into a spectrum.

Table 6. Mass Spectrometer Detectors

Detector	Principle of Operation	Advantages	Limitations
Electron Multiplier (EM)	Ions strike a surface, releasing secondary electrons that are multiplied to create an amplified signal	Very high sensitivity, fast response, widely used	Limited dynamic range, can degrade with time
Faraday Cup	Ions directly hit a metal cup, generating a measurable current proportional to ion abundance	Simple, robust, stable over time	Low sensitivity, unsuitable for very low ion intensities
Microchannel Plate (MCP)	Ions strike microscopic channels, generating cascades of electrons that produce an amplified signal	High sensitivity, fast detection, useful for imaging MS	More expensive, limited lifespan
Photomultiplier	Ions first hit a phosphor screen → photons → converted into amplified electrical signal	High gain, useful in specialized applications	Rarely used in modern MS, bulky
Array Detectors (e.g., CCD)	Multiple channels detect ions simultaneously	Enables parallel detection, imaging	More complex, less common

III.4. Mass Spectrum Presentation and Interpretation

A mass spectrum is the graphical output of a mass spectrometer, showing how ions are distributed according to their mass-to-charge ratio (m/z). It provides both qualitative and quantitative information about the sample.

III.4.2 Key Features

- ***Molecular Ion ($M^{+\bullet}$):***
  - Represents the intact ionized molecule.
  - Provides the molecular weight.
  - May be weak or absent if the molecule fragments easily.

- **Fragment Ions:**
  - Produced by bond cleavage after ionization.
  - Appear at lower m/z values than $M^{+}\bullet$.
  - Provide structural information.
- **Base Peak:**
  - The most intense peak in the spectrum.
  - Defined as 100% relative intensity.
  - Does not always correspond to the molecular ion.
- **Isotopic Peaks (M+1, M+2, …):**
  - Due to naturally occurring isotopes (e.g., ^{13}C, ^{37}Cl, ^{81}Br).
  - Useful for identifying heteroatoms.

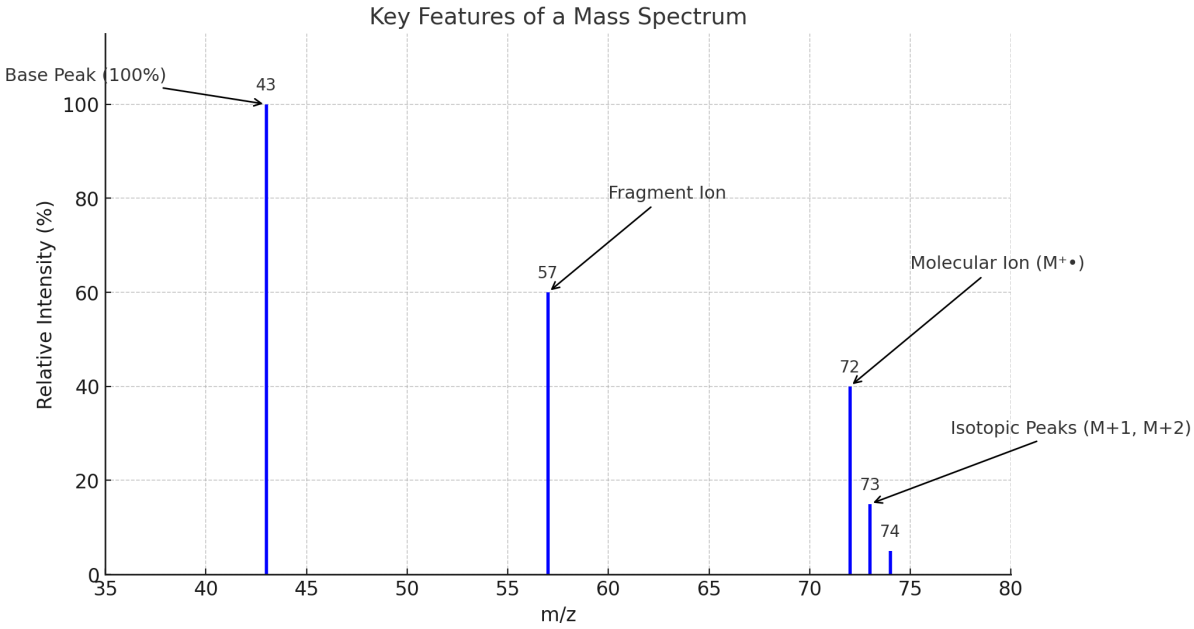


Figure 18. Key features of a mass spectrum

Examples

- ***Methane (CH_4)***

Ionization:

$$CH_4 + e^- \longrightarrow CH_4^{+\bullet} + 2e^-$$

- The molecular ion ($M^{+}\bullet$) appears at m/z = 16.
- Because methane is very small, the molecular ion is unstable and fragments easily.

Fragmentation:

- m/z = 15 → CH_3^+ (major fragment)
- m/z = 14 → CH_2^+ (smaller fragment)
- m/z = 12 → C^+ (minor fragment)

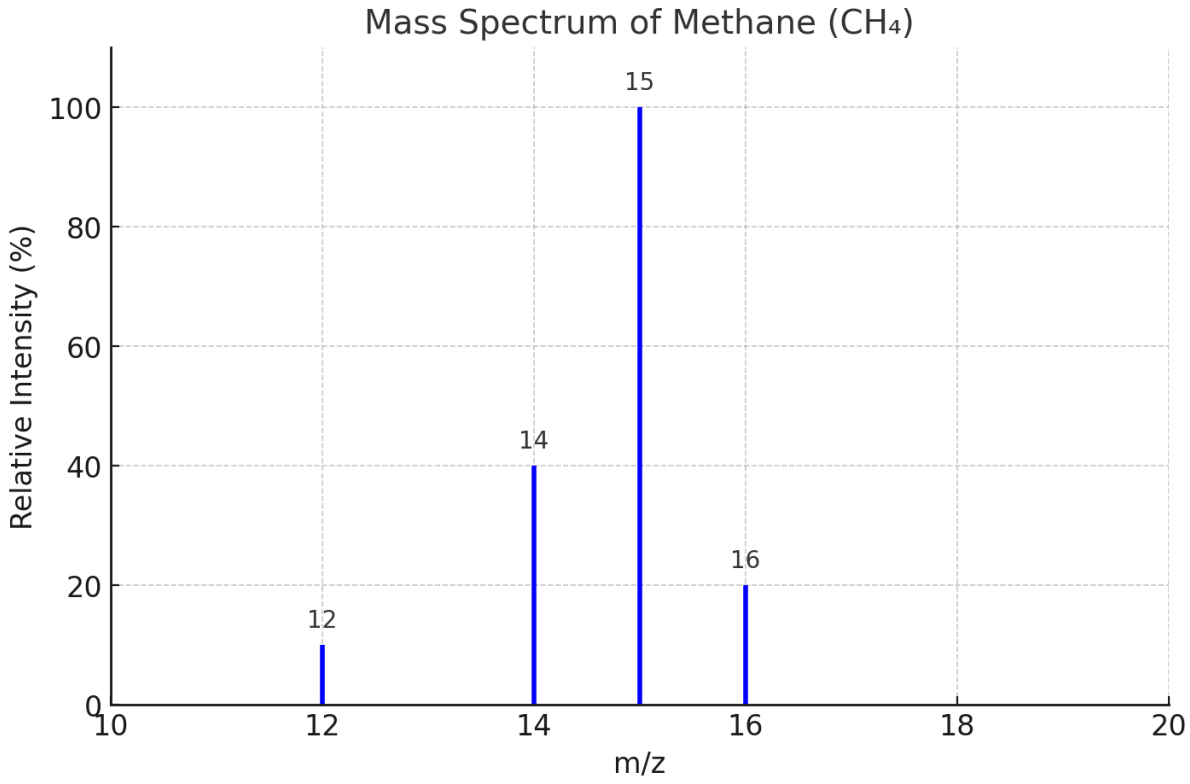


Figure 19. Mass spectrum of methane.

Interpretation:

- The spectrum shows a weak molecular ion at m/z 16, a base peak at m/z 15 (CH_3^+), and smaller peaks at m/z 14 and 12.
- This fragmentation pattern is typical of very small hydrocarbons.

Example 2

- **Ethanol (C_2H_5OH)**

Ionization:

$$C_2H_5OH + e^- \longrightarrow C_2H_5OH^{+\bullet} + 2e^-$$

The molecular ion ($M^{+\bullet}$) appears at m/z = 46.

Fragmentation:

- m/z = 31 → CH_2OH^+ (base peak)
- m/z = 29 → $C_2H_5^+$
- m/z = 15 → CH_3^+

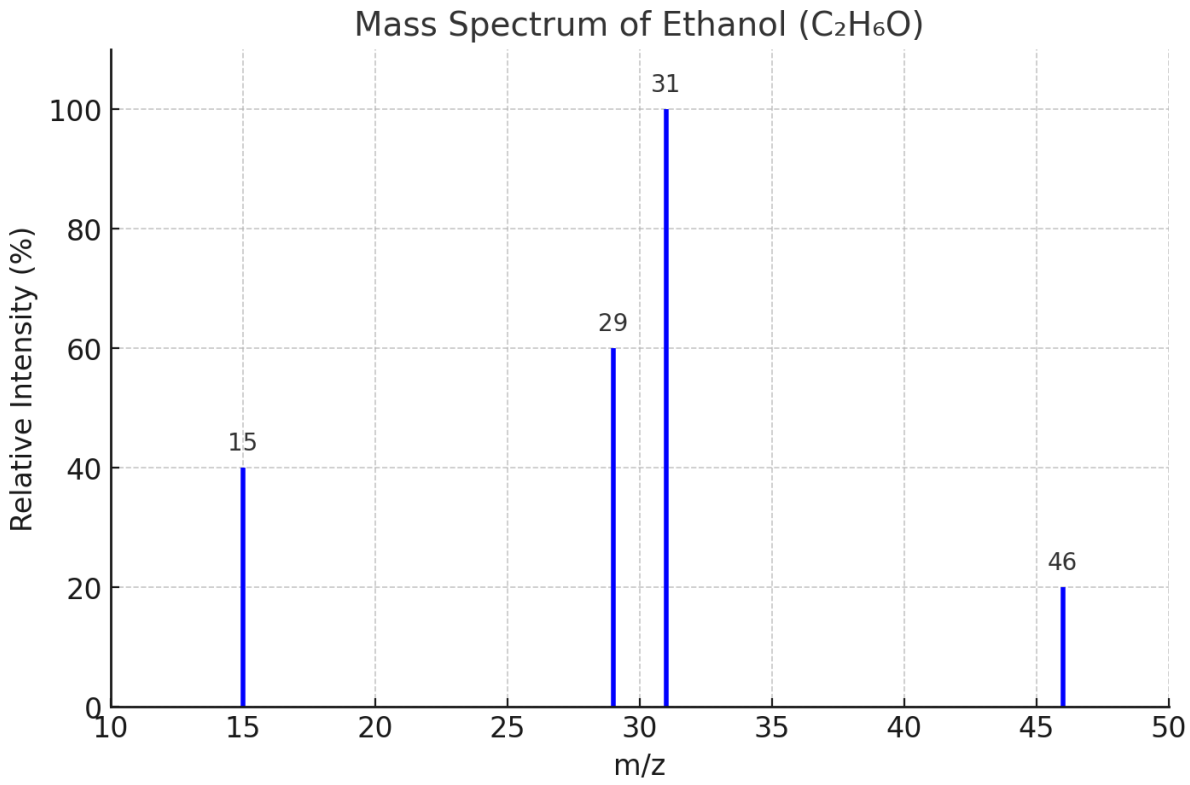


Figure 20. Mass spectrum of methane.

Interpretation:

- The spectrum shows a molecular ion at m/z 46, with fragmentation peaks at 31, 29, and 15.
- The base peak at m/z 31 corresponds to the most stable fragment (CH_2OH^+).
- This fragmentation pattern is characteristic of alcohols.

Part I

Spectroscopic Methods

Chapter IV

Nuclear Magnetic Resonance (NMR)

IV. Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is a relatively recent technique compared to classical spectroscopies. It is currently considered the most powerful and versatile method for the structural analysis of organic molecules and certain types of inorganic materials. NMR provides information from the interaction between atomic nuclei in a sample when subjected to a strong, constant magnetic field generated by a superconducting magnet.

NMR therefore holds particular importance in organic chemistry and biochemistry. It enables the study of compounds both in solution and in the solid state. While it can be applied to quantitative analysis as well as structural elucidation, its true strength lies in the latter, where it has become an indispensable tool.

This technique investigates molecular absorption in the radiofrequency range of the electromagnetic spectrum, corresponding to wavelengths between 10^{-1} and 10^{4} m, or frequencies between 10^{5} and 10^{10} Hz (Fig. 21).

IV.1. Principle of NMR

Nuclear Magnetic Resonance (NMR) is based on the measurement of the absorption of radiofrequency (RF) radiation by atomic nuclei placed in a strong external magnetic field ($\vec{B}_0$).

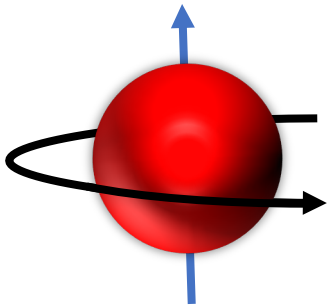


Figure 21. Simplified model of an atomic nucleus.

As shown in Figure 21, the nucleus of an atom can be approximated as a small spherical particle spinning around an axis. This spinning motion is associated with an angular momentum, known as spin, which is represented by a vector I attached to the nucleus.

The nuclear spin behaves like a tiny magnetic needle, capable of pointing in different directions. Its associated magnetic moment (μ) originates from the nucleus itself, not from the surrounding electrons. Nuclei are therefore characterized by two intrinsic properties: the spin quantum number (S) and the magnetic moment (μ), which are related by:

$$\boldsymbol{\mu = \gamma S} \qquad \textbf{Eq.18}$$

The value of the spin quantum number **S** determines the number N of possible orientations of the nucleus in a magnetic field:

$$N = 2S + 1 \qquad \textbf{Eq.19}$$

- If both the number of protons (p) and neutrons (n) are even → S = 0 (no NMR signal, e.g., ^{12}C, ^{16}O).
- If both p and n are odd → S is an integer (e.g., ^{2}H, ^{14}N with S = 1).
- If (p + n) is odd → S is a half-integer (e.g., ^{1}H, ^{13}C, ^{31}P with S = ½).

For NMR spectroscopy, we are mainly interested in nuclei with **S = ½**, since these are the most widely used in practice (^{1}H NMR, ^{13}C NMR, ^{31}P NMR).

When **S = ½**, there are two possible spin states and therefore two possible orientations of the magnetic moment (μ) in an external field (Figure 22):

Figure 22. Simplified diagram representing the two possible spin states for a half-integer nucleus.

- **+½ (α state):** aligned with B_0, lower energy, stable.
- **–½ (β state):** opposed to B_0, higher energy, less stable.

When a strong external magnetic field B_0 is applied (Figure 23):

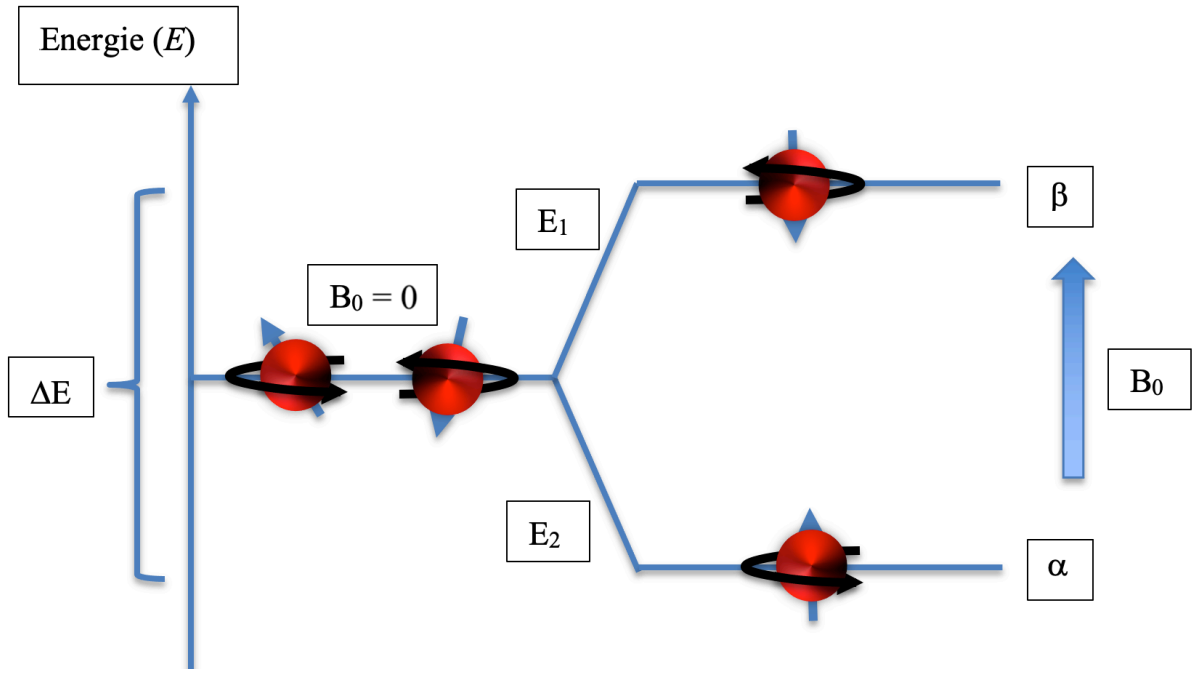


Figure 23. Orientations of nuclei exposed to an external magnetic field (B_0).

- Most nuclei occupy the **α state** (low energy, aligned with B_0).
- A smaller number of nuclei occupy the **β state** (high energy, opposed to B_0).

IV.2. Steps of NMR Phenomenon

a) **Application of B_0:** Nuclei align with or against the field, creating an energy gap between the α and β states.

b) **Resonance:** When an RF pulse of the correct frequency is applied (perpendicular magnetic field B_1), nuclei absorb the energy and transition from the α to the β state. This absorption is the essence of NMR.

c) **Relaxation:** After the RF pulse stops, nuclei return to equilibrium, releasing energy as a weak RF signal. This signal is detected by a receiving coil and processed by a computer to produce the NMR spectrum.

IV.3. The NMR Spectrum

An NMR spectrum is defined by several key parameters that characterize each nucleus. These parameters provide information about the proton, carbon, or phosphorus atom being observed and its local chemical environment.

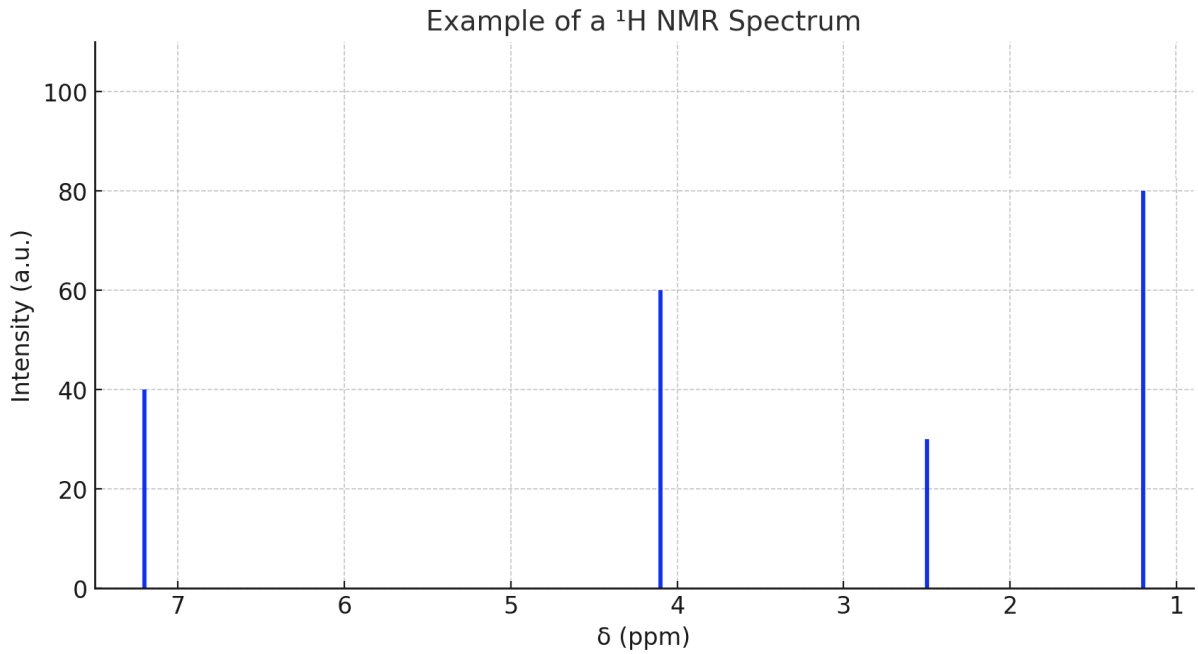


Figure 24. Example of ^{1}H NMR spectrum

As illustrated in Figure 24, an example of a ^{1}H NMR spectrum is shown:

- The x-axis (abscissa) represents the chemical shift (δ), expressed in parts per million (ppm), and sometimes in Hertz (Hz). The chemical shift indicates the electronic environment surrounding a nucleus.
- The y-axis (ordinate) represents the signal intensity (I), which is proportional to the number of equivalent nuclei contributing to the signal.

IV.4. Theoretical Concept

IV.4.1. Chemical Shift

When a nucleus is placed in an external magnetic field (B_0), the magnetic forces reaching the nucleus must pass through the surrounding electron cloud, which partially shields the nucleus from the external field (Figure 25). This effect is called shielding.

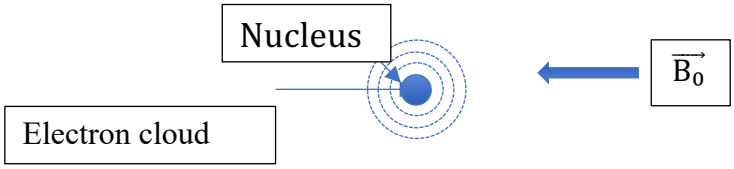


Figure 14. Representation of a nucleus exposed to a B field

To quantitatively evaluate the shielding experienced by protons, a reference compound is needed. The standard reference is tetramethylsilane (TMS, $Si(CH_3)_4$), which is added in small amounts (1–2%) to the sample. TMS offers several advantages:

- All 12 protons are chemically equivalent, giving rise to a single sharp signal.
- Its signal is intense and requires only a very small quantity of TMS.
- Its resonance occurs at a higher field than most other protons, so its absorption peak is well separated and appears on the far right of the spectrum.
- By convention, the TMS peak is set to 0 ppm, which defines the origin of the chemical shift scale.

For any given proton, the difference between its resonance frequency and that of TMS defines its chemical shift (δ). To avoid unit inconsistencies, δ is expressed as a dimensionless value in parts per million (ppm):

$$\delta = \frac{\nu - \nu_{ref}}{\nu_{ref}} \times 10^6 \qquad \textbf{Eq.20}$$

Where:

- ν = resonance frequency of the observed proton
- ν_{ref} = resonance frequency of TMS protons

Since these frequencies are very large (MHz range), the δ scale in ppm allows easy comparison of spectra recorded at different magnetic field strengths.

IV.4.2. Effect of Electronegativity

The chemical shift increases with the electronegativity of atoms attached to the proton. Electronegative atoms withdraw electron density, reducing shielding and shifting the resonance downfield (to the left, higher δ values).

Table 7. Electronegativity and Corresponding Chemical Shifts

	CH_3F	CH_3OH	CH_3Cl	CH_3Br	CH_3I	TMS
Electronegativity	4,0	3,5	3,1	2,8	2,5	1,0
Chemical Shift (ppm)	4,26	3,40	3,05	2,68	2,16	0,00

IV.4.3. Shielding and Deshielding

When a molecule is subjected to an external magnetic field (B_0):

- The field induces circulation of electrons around protons, generating an induced magnetic field**.**
- If the induced field opposes B_0 → the nucleus is shielded, requiring a stronger B_0 to achieve resonance. Shielded protons resonate at lower δ values (upfield, right side of the spectrum).

- If the induced field reinforces B_0 → the nucleus is deshielded, resonating at higher δ values (downfield, left side of the spectrum).

Thus, the position of absorption peaks in an NMR spectrum directly reflects the electronic environment of nuclei (Figure 26).

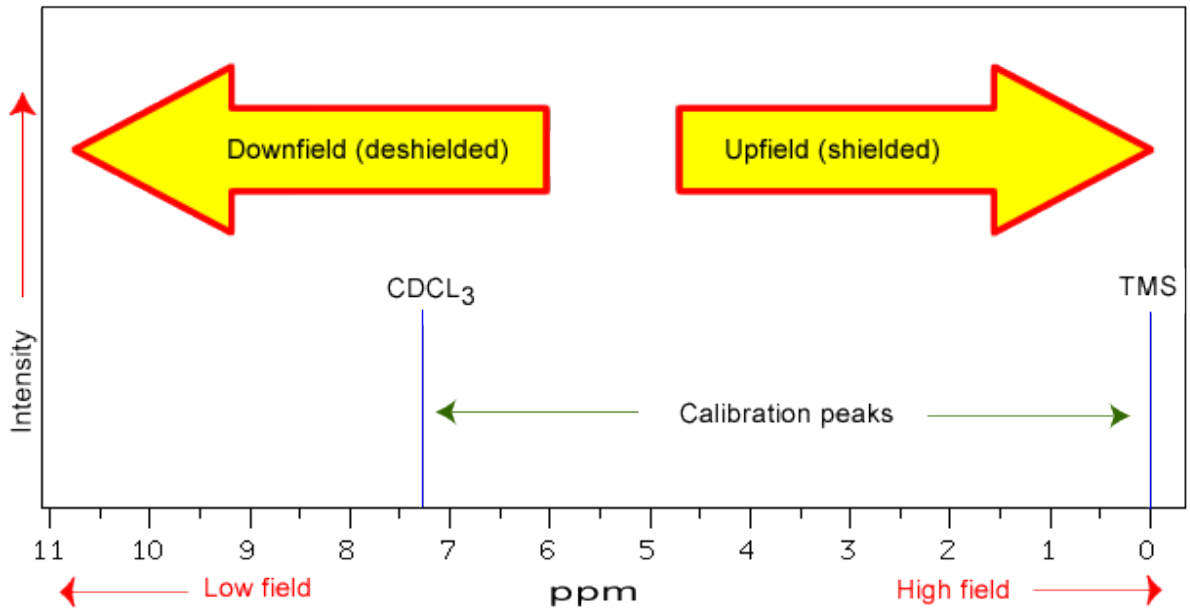


Figure 26. Nuclear shielding and deshielding as a function of the external magnetic field strength (B_0)

The intensity of shielding or deshielding therefore depends on the chemical environment of the proton, and consequently on the molecular structure of the compound. For a given proton, the main influence is the functional group to which it is attached. Thus, the chemical shift (δ) provides valuable information about the nature of the functional group. Figures 27 and 28 illustrate the typical chemical shift ranges of protons as a function of δ.

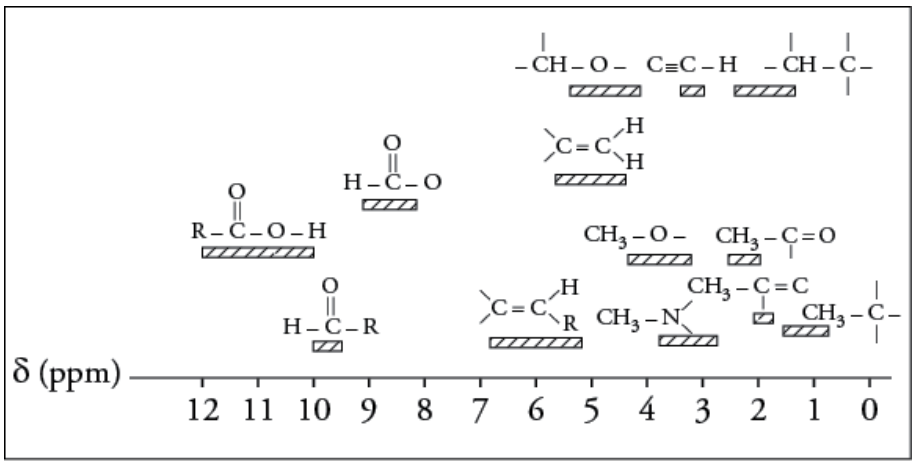


Figure 27. Chemical shifts of protons as a function of δ.

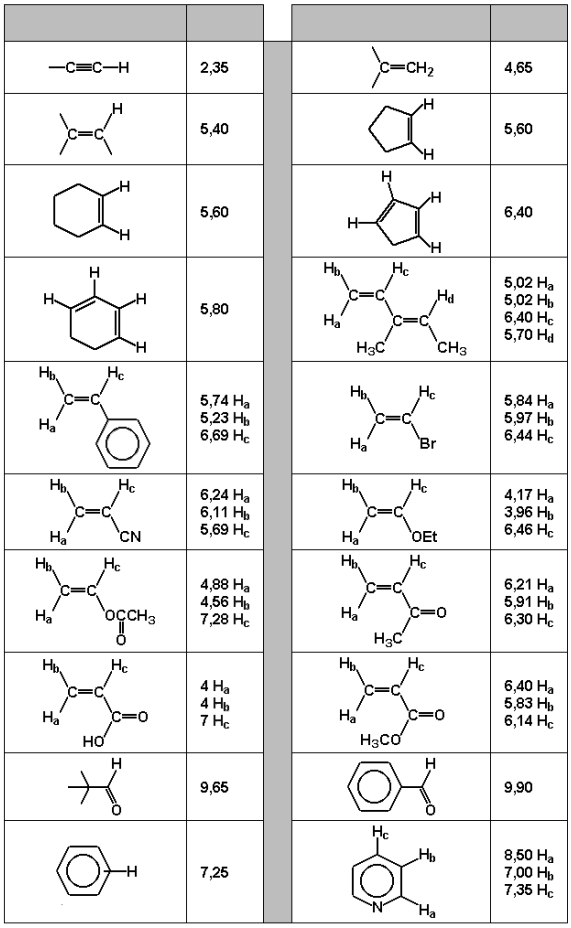


Figure 28. Chemical shifts of selected protons as a function of δ.

IV.4.4. Equivalent Protons (Isochronous Protons)

When protons are located in the same chemical environment, they resonate at the same frequency (ν). Such protons are called equivalent protons, and they share the same chemical shift (δ).

Examples:

1. Each family of equivalent protons is shown in parentheses:
   - **C(H₄):** one type of proton
   - **(H₃)C–C(H₂)–C(H₃):** two types of protons
   - **(H₃)C–C(H)O:** two types of protons
   - **(H₃)C–C(H₂)–CO–C(H₂)–C(H₃):** two types of protons
   - **(H₃)C–CO–C(H₂)–C(H₃):** three types of protons
   - **(H₃)C–C(H₂)–C(H)O(H)–C(H₂)–C(H₃):** four types of protons
2. The NMR spectrum of benzene :

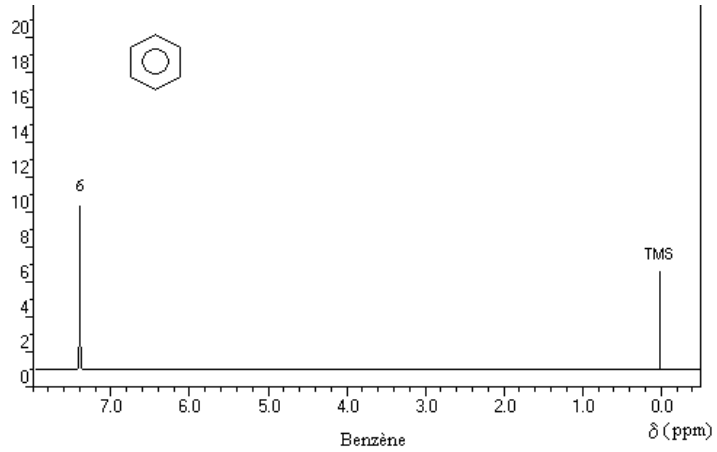


Figure 29. NMR spectrum of benzene

In benzene (C_6H_6), all six protons are chemically equivalent due to the symmetry of the aromatic ring. As a result, the ^1H NMR spectrum shows a single sharp signal at around $\delta \approx 7.26$ ppm (in $CDCl_3$), characteristic of aromatic protons.

IV.4.5. Signal Areas (Integration Curves)

The integration mode in NMR spectra allows determination of the relative number of equivalent protons. In a given molecule:

- The greater the number of hydrogens (protons) in the same chemical environment, the larger the corresponding signal.
- Similarly, the higher the concentration of the compound, the more intense the signal.

The spectrometer converts the area under each peak into a stepwise integration curve. The total height of this curve (hT) is proportional to the total number of protons in the molecule. To interpret the spectrum, one must compare the relative step heights (h) of different signals.

Examples

1. **2,2-Dimethylpropanal:**

The relative heights of the three integration steps correspond to a ratio of **1 : 2 : 9**, which reflects 1H, 2H, and 9H. This matches the 12 total protons present in the molecule.

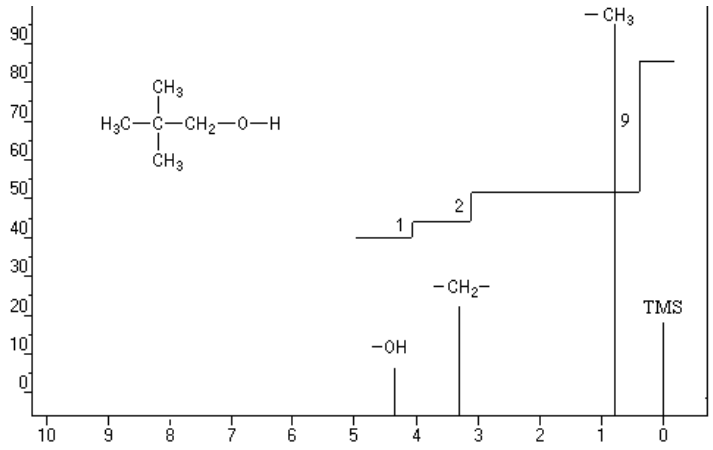


Figure 30. NMR spectrum of 2,2-Dimethylpropanal

2. **Tert-butyl acetate :**

The relative heights of the two integration steps correspond to 3H and 9H, consistent with the 12 total protons in the compound.

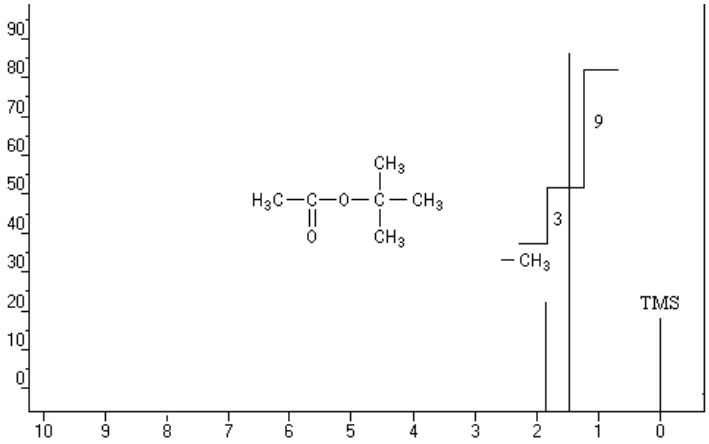


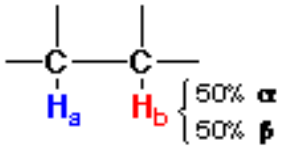
Figure 31. NMR spectrum of Tert-butyl acetate

IV.4.6. Spin–Spin Coupling

When NMR spectra are recorded with sufficient resolution, some signals are observed to split into multiple peaks. This signal splitting arises from interactions between neighboring nuclei, either through space (direct coupling) or via bonding electrons (indirect coupling). This phenomenon is called spin–spin coupling.

- ***Coupling Between Two Neighboring Protons (H_a and H_b)***

Consider two protons, $\mathbf{H_a}$ and $\mathbf{H_b}$, bonded to two adjacent carbon atoms.



- In an external magnetic field (B_0), the spin of H_b can adopt two orientations: α (parallel) or β (antiparallel), each with a probability of 50%.
- As a result, H_b behaves like a tiny magnet that can either reinforce (+ε) or oppose (–ε) the external field.
- Consequently, H_a experiences two slightly different effective magnetic fields: B_0 + ε or B_0 – ε (Figure 32).

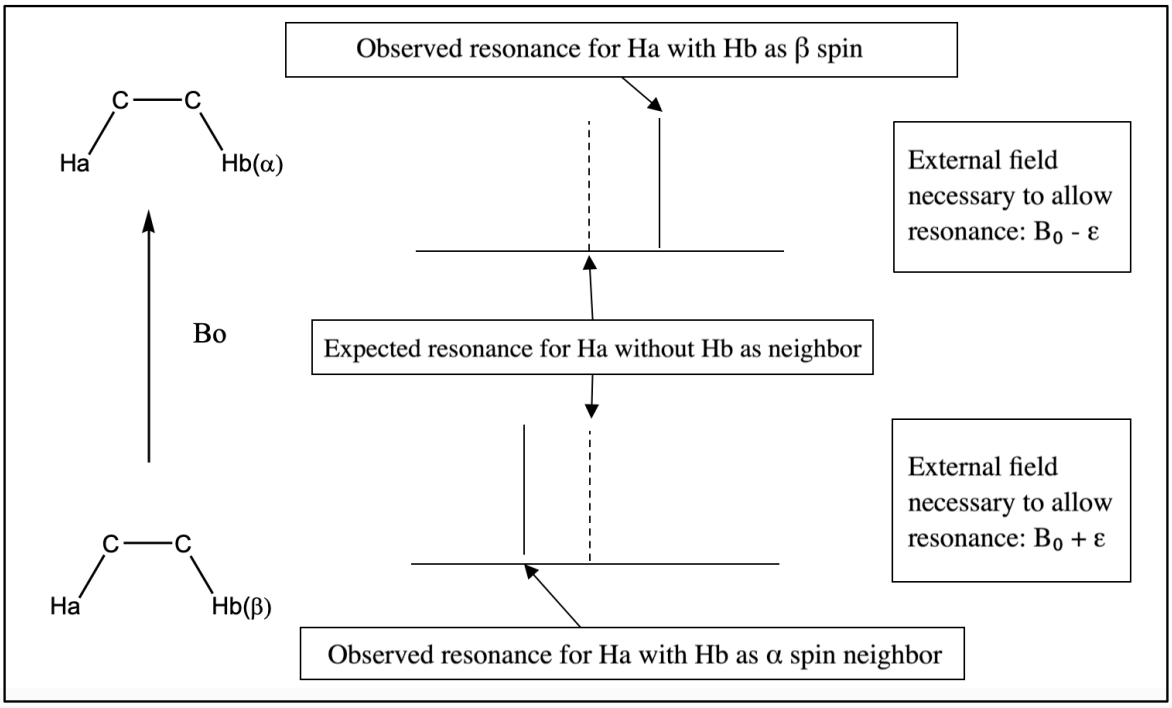


Figure 32. Explanatory Diagram of Spin-Spin Coupling

This causes the signal of H_a to split into two peaks (a doublet), centered around the chemical shift of H_a if it were isolated. The distance between the two peaks corresponds to the coupling constant, noted J_{ab}, and is

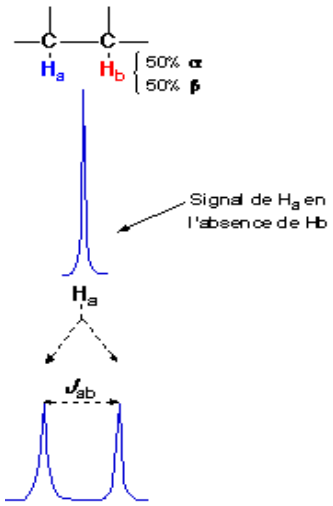


Figure 33. Spin–spin coupling between H_a and H_b.

This causes the signal of Ha to split into two peaks (a doublet), centered around the chemical shift of Ha if it were isolated. The distance between the two peaks corresponds to the coupling constant, noted J_{ab}, and is expressed in Hertz (Hz) (Figure 33).

- ***(N+1) Rule***

If Ha has n equivalent neighboring protons (H_b), its signal will be split into (n + 1) peaks:

- 0 neighbor → singlet (1 peak)
- 1 neighbor → doublet (2 peaks)
- 2 neighbors → triplet (3 peaks)
- 3 neighbors → quartet (4 peaks)

This is known as the multiplicity rule (Figure 34).

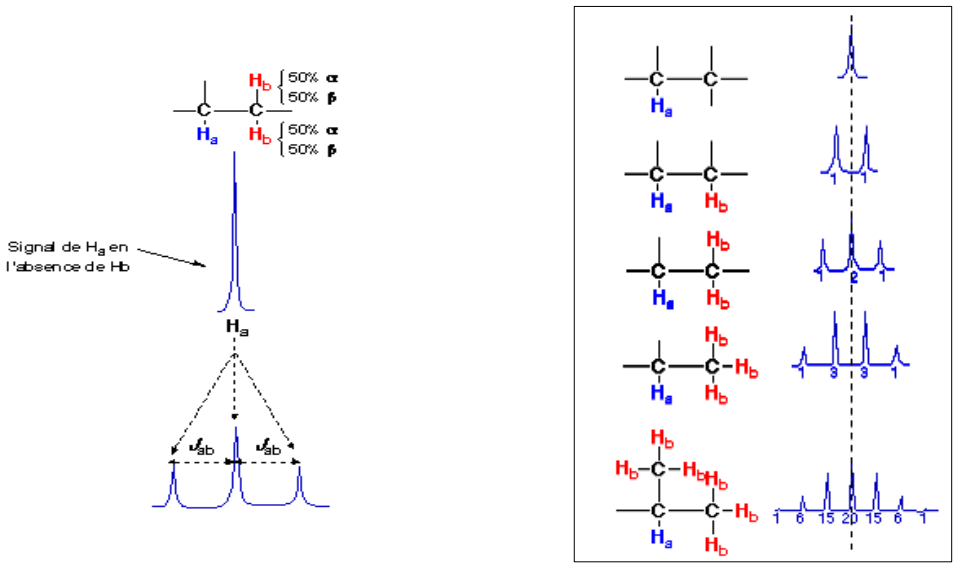


Figure 15. Multiplicity rule

The relative intensities of the peaks within a multiplet follow the coefficients of Pascal's triangle (Figure 35):

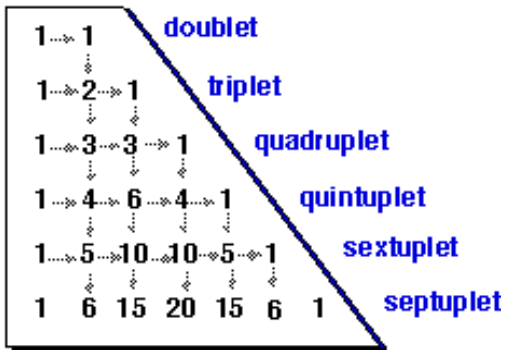


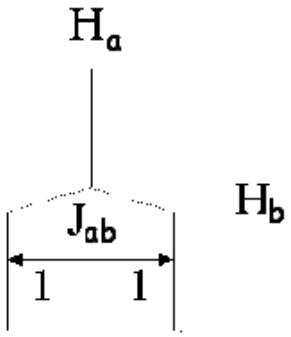
Figure 35. Pascal's triangle

- Doublet → 1:1
- Triplet → 1:2:1
- Quartet → 1:3:3:1

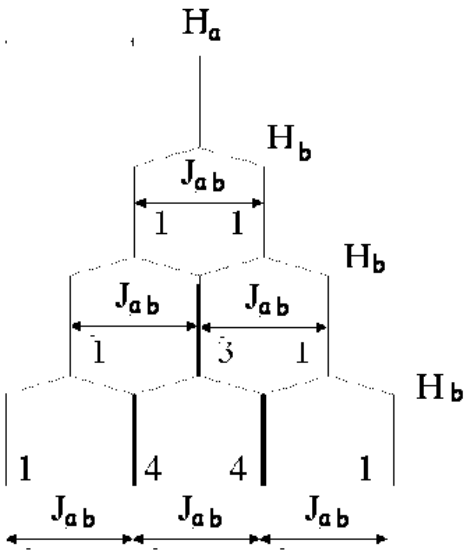
Such splitting patterns are extremely useful because they provide information about the number of neighboring protons and thus the connectivity of atoms within the molecule.

It is possible to represent proton couplings using a branching diagram (tree diagram) that gathers all the possible spin orientations of neighboring protons. Such a representation provides, for first-order spectra, a good estimation of both the position of peaks and their relative intensities.

- **Case 1:** Proton Ha coupled with one neighboring proton H_b → results in a doublet (two peaks of equal intensity, 1:1).



- **Case 2:** Proton H_a coupled with three equivalent protons H_b → results in a quartet (four peaks with relative intensities 1:3:3:1).



IV.5. Instrumentation

An NMR spectrometer consists of three main components: a superconducting magnet, a radiofrequency (RF) transmitter, and an RF receiver. In modern instruments, the transmitter and receiver are often combined into a single RF coil that can both emit and detect the signal (Figures 36).

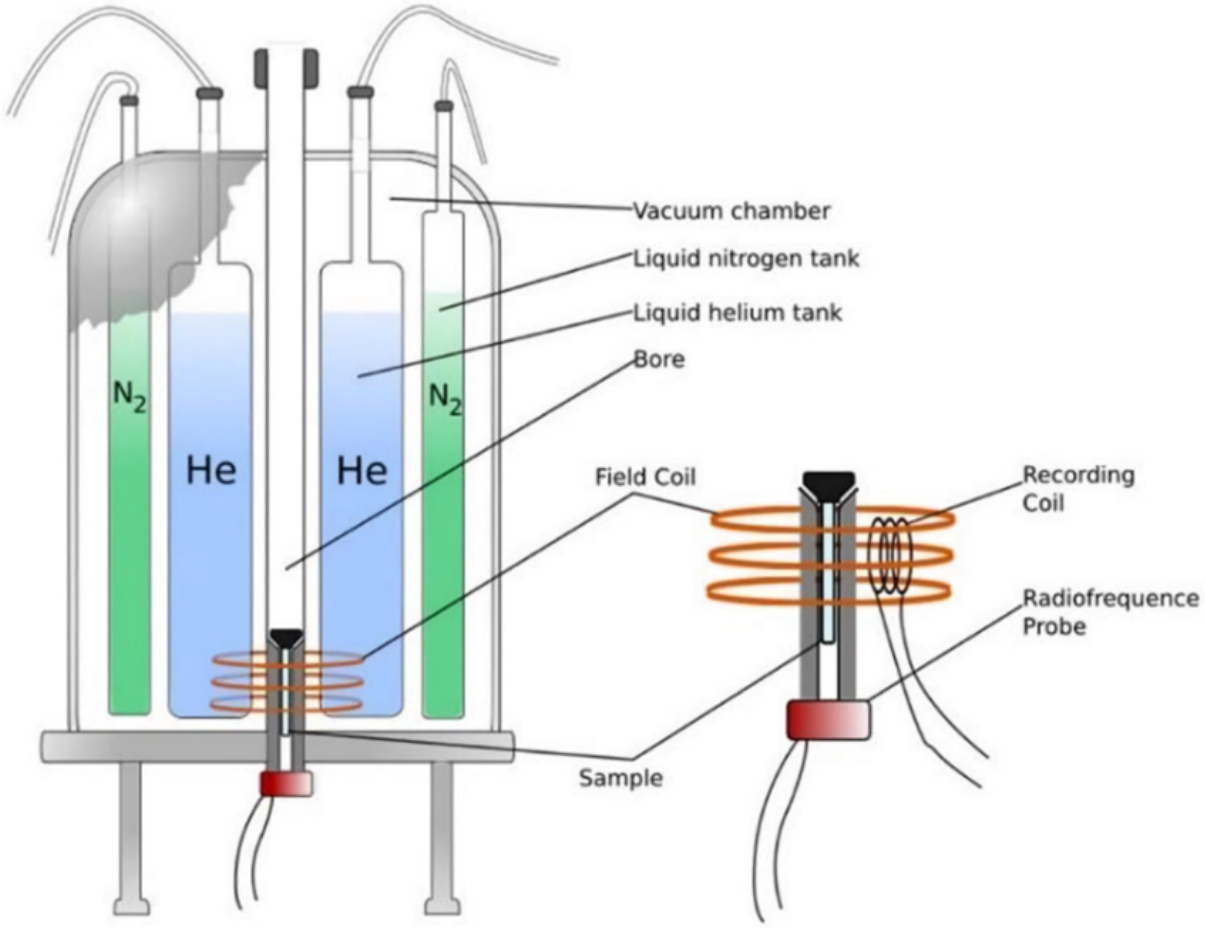


Figure 36. Diagram of an NMR Spectrometer

a) **Superconducting Magnet**

The sensitivity of an NMR experiment increases with the strength of the magnetic field B_0; therefore, the magnet must be as strong as possible. To maintain superconductivity, the magnet is immersed in liquid helium and enclosed in a Dewar (similar to a Thermos flask) for thermal insulation. To minimize helium evaporation, the system is surrounded by an additional outer Dewar filled with liquid nitrogen. The evaporation of helium and nitrogen is monitored, and liquid nitrogen is refilled approximately once per week.

b) **Radiofrequency Generator**

The RF transmitter consists of a coil powered by an alternating current. This coil generates the oscillating magnetic field B_1, oriented perpendicular to B_0, at a specific radiofrequency needed to induce resonance in the nuclei.

c) **Radiofrequency Receiver**

The same RF coil can also act as a receiver, detecting the weak signal emitted by nuclei as they return to equilibrium after excitation. This signal is then processed and transformed into the NMR spectrum.

- ***Sample Preparation***

A small volume of the solution (~0.5 mL) is placed in a narrow glass or nylon tube (5 mm diameter) and positioned perpendicular to the magnetic field B_0, inside the RF coil. To improve the apparent homogeneity of the magnetic field, the tube is spun at a rate of 30–50 revolutions per second during data acquisition.

IV.4. Example with interpretation

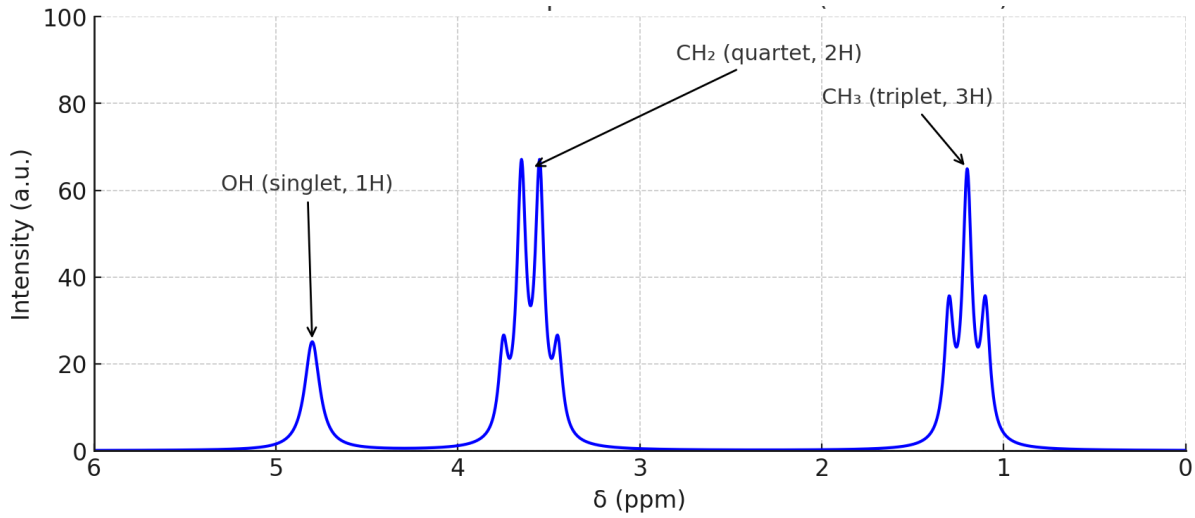


Figure 37. ^{1}H NMR Spectrum of Ethanol (CH_3–CH_2–OH)

Observed spectrum (in $CDCl_3$ solvent)

1. **CH_3 group (methyl):**
   - Appears at δ ≈ 1.2 ppm
   - Splits into a triplet (3H) because of coupling with the two neighboring protons of CH_2
   - Integration: corresponds to 3 protons
2. **CH_2 group (methylene):**
   - Appears at δ ≈ 3.6 ppm
   - Splits into a quartet (2H) due to coupling with the three neighboring protons of CH_3
   - Integration: corresponds to 2 protons
3. **OH group (hydroxyl proton):**
   - Appears at δ ≈ 4.5–5 ppm (variable, broad signal)

- Usually appears as a singlet, because proton exchange makes spin–spin coupling negligible
- Integration: corresponds to 1 proton

Interpretation

- The integration ratio of 3:2:1 confirms the presence of CH_3, CH_2, and OH groups.
- The multiplicities (triplet for CH_3, quartet for CH_2) are consistent with the (N+1) rule and show that the CH_3 and CH_2 groups are neighbors.
- The broad OH singlet is characteristic of alcohols.

Part I

Spectroscopic Methods

Comparative Overview (Spectroscopic Methods)

V. Comparative Overview (Spectroscopic Methods)

Spectroscopy and spectrometry are complementary analytical techniques that provide a wide range of molecular information.

While mass spectrometry (MS) focuses on determining molecular weight, isotopic composition, and fragmentation-based structural details, other spectroscopic methods offer different insights. Infrared (IR) spectroscopy reveals functional groups through vibrational transitions, ultraviolet–visible (UV–Vis) spectroscopy provides information on electronic transitions and chromophores, and nuclear magnetic resonance (NMR) gives access to atomic connectivity, stereochemistry, and molecular dynamics.

The following table summarizes the main principles, information provided, advantages, and limitations of these four commonly used analytical techniques.

Table 8. Comparative Overview of Common Spectroscopic.

Technique	*Principle*	*Information Provided*	*Advantages*	*Limitations*
Infrared (IR) Spectroscopy	Absorption of IR radiation causes vibrational transitions in molecular bonds.	Functional groups, bond types, molecular fingerprint.	Rapid, non-destructive, good for qualitative identification.	Limited to functional group identification, requires pure samples.
Ultraviolet–Visible (UV–Vis) Spectroscopy	Absorption of UV–Vis light promotes electrons to higher energy levels.	Electronic structure, conjugation, chromophores.	Simple, fast, suitable for concentration measurements and conjugated systems.	Less structural detail, mainly useful for conjugated molecules.
Nuclear Magnetic Resonance (NMR)	Absorption of radiofrequency radiation by nuclei in a magnetic field.	Molecular structure, atomic connectivity, stereochemistry, dynamics.	Detailed structural information, quantitative, non-destructive.	Requires relatively large amounts of sample, expensive instrumentation.
Mass Spectrometry (MS)	Ionization of molecules followed by separation of ions according to mass-to-charge ratio (m/z).	Molecular weight, elemental composition, fragmentation patterns, isotopic distribution.	High sensitivity, precise molecular mass, structural elucidation possible.	Requires ionizable samples, fragmentation may complicate interpretation.

Part II
Chromatographic Methods

Chromatographic Methods

Chromatography is a physicochemical separation technique in which the components of a homogeneous mixture (liquid or gaseous) are separated through the use of two immiscible phases: a mobile phase and a stationary phase.

- The mobile phase (which can be a gas, a liquid, or a supercritical fluid) carries the analytes through the stationary phase.
- The stationary phase (such as silica, polymers, or chemically modified silica) interacts differently with each component of the mixture.
- The migration speed of solutes depends on their chemical and physical properties as well as on their interactions with both phases.

Chromatographic methods can be classified according to:

1. The nature of the phases involved (gas–solid, liquid–solid, liquid–liquid, etc.), or
2. The separation mechanisms at play (adsorption, partition, ion exchange, size exclusion, affinity, etc.).

In the following chapters, we will focus on the four most widely used chromatographic techniques:

- Thin Layer Chromatography (TLC)
- Column Chromatography
- Gas Chromatography (GC)
- High-Performance Liquid Chromatography (HPLC)

Part II

Chromatographic Methods

Chapter I

Thin Layer Chromatography (TLC)

I. Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is a simple qualitative separation technique and one of the most accessible chromatographic methods. It is based on the different affinities of the components in a mixture for two phases:

- A stationary phase (usually a thin layer of silica gel, alumina, or cellulose deposited on a plate).
- A mobile phase (a solvent or mixture of solvents) that migrates by capillary action through the stationary phase.

Each substance interacts differently with the two phases:

- Compounds with a stronger affinity for the stationary phase move more slowly.
- Compounds with a stronger affinity for the mobile phase travel further.

This difference in migration creates a separation of spots on the TLC plate, allowing rapid and inexpensive analysis.

I.1. Principle

The originality of Thin Layer Chromatography (TLC) lies in the fact that the stationary phase is deposited as a thin, strongly adhesive film (0.1–2.5 mm thick) on a solid (glass) or flexible (aluminum, plastic) support, forming the so-called TLC plates.

A small quantity of the mixture to be separated is applied as a spot near the bottom of the stationary phase. The plate is then placed in contact with the mobile phase (also called the developing solvent or eluent).

The mobile phase migrates upward by capillary action through the stationary phase, carrying the components of the mixture with it. This process, known as elution, is responsible for separating the mixture's constituents.

- Each compound migrates at its own characteristic rate, depending on its relative affinity for the stationary phase and the mobile phase.
- Molecules that interact more strongly with the stationary phase move more slowly, while those with a greater affinity for the mobile phase travel further.

This difference in migration results in the separation of compounds and the formation of distinct spots on the TLC plate.

I.2. Equipment Used

I.2.1. TLC Plates

TLC plates consist of a thin layer of stationary phase (usually silica gel) spread uniformly over a solid support made of glass, metal, or plastic.

Plates are available in different dimensions, most commonly:

- 5 × 5 cm
- 5 × 20 cm
- 10 × 20 cm
- 20 × 20 cm

These plates are often pre-coated and ready to use.

Figure 38. Thin Layer Chromatography plates

I.2.2. Development Chamber (Migration Tank)

The development chamber is a sealed glass container designed to hold both the mobile phase and the TLC plate during development. It is fitted with a lid to maintain a saturated atmosphere with solvent vapor and water, ensuring proper migration of the solvent front (Figure 39).

Figure 39. TLC Separation Tank.

⚠ ***Note :***

- The mobile phase should be placed in the chamber some time before starting the chromatographic run, in order **to** saturate the atmosphere inside the chamber.
- When highly volatile solvents are used, saturation can be improved by lining the inner walls of the chamber with filter paper soaked in the mobile phase.

I.2.3. Mobile Phase (Developing Solvent)

The mobile phase in TLC is usually an organic solvent or a mixture of solvents. Its composition directly determines the polarity of the eluent, which strongly influences the migration of compounds.

- Silica gel, the most commonly used stationary phase in TLC, is polar because of the presence of hydroxyl groups on its surface.

As a result:

- Polar compounds interact strongly with the stationary phase and migrate slowly.
- Non-polar compounds interact weakly and migrate faster with the mobile phase.

To optimize separation, the polarity of the mobile phase can be adjusted using single solvents or solvent mixtures.

Additives such as acids (e.g., acetic acid) or bases (e.g., ammonia, amines) may also be used to minimize streaking caused by ionizable compounds.

To illustrate this concept, Table 9 summarizes common solvents used in TLC, their polarity, and typical applications.

Table 9. Examples of Solvents and Their Polarity in TLC

Solvent	Polarity	Typical Use in TLC
Hexane	Non-polar	Good for separating non-polar compounds (lipids, hydrocarbons)
Toluene	Low polarity	Slightly stronger eluent than hexane
Dichloromethane	Medium polarity	Useful for moderately polar compounds
Ethyl acetate	Polar	Common solvent for organic compounds (esters, aromatics)
Methanol	Very polar	Strong eluent, often mixed with less polar solvents
Acetic acid	Polar protic	Used as an additive to prevent tailing of acidic compounds
Ammonia / Amines	Polar basic	Used as additives to prevent tailing of basic compounds

I.3. Operating procedure

I.3.1. Sample Application

- The reference samples (known solutions) and the mixture to be separated are spotted separately on the TLC plate, about 1–3 cm from one edge (the baseline). Reference spots allow direct comparison between known compounds and the mixture under analysis.
- The sample is applied using a micropipette or a capillary tube, followed by drying to ensure small, concentrated spots.
- The plate is then placed in the development chamber containing the mobile phase (Figure 40).

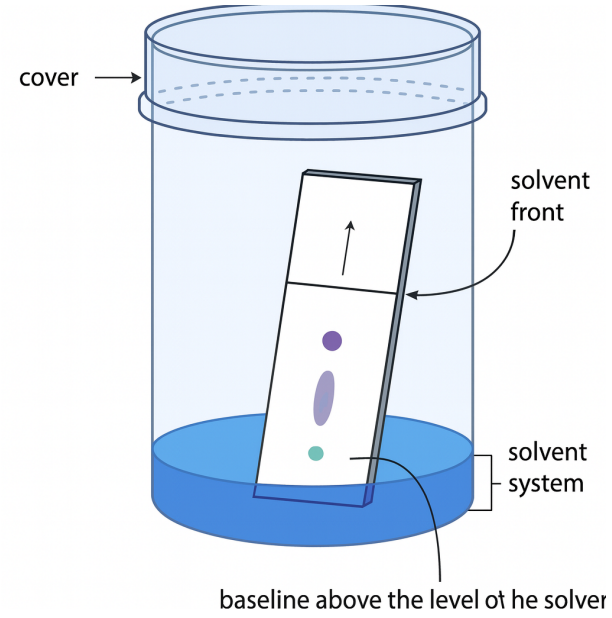


Figure 40. TLC plate introduced into the development chamber.

I.3.2. Development (Migration)

- During this process, the sample is carried upward by the mobile phase, moving through the stationary phase.
- The solvent migrates by capillary action, transporting analytes at different rates depending on their affinities for the mobile and stationary phases.
- Migration is usually stopped once the solvent front has traveled 10–15 cm. The plate is then removed, and the solvent front is marked (this mark can also be pre-drawn as a reference for stopping the elution).
- The duration of development depends on the solvent system and may range from 30 minutes to 3 hours.

I.3.3. Visualization (Detection of Spots)

After development, the plates are removed from the chamber and dried to eliminate residual solvent. The analytes must then be visualized, since many are colorless.

- ***Colored compounds***: directly visible on the TLC plate.
- ***Colorless compounds***: require visualization techniques, including:

a) Fluorescence under UV light :

- At **254 nm (short-wave UV)**: compounds appear as dark spots on a fluorescent green background, since commercial plates are impregnated with a fluorescent indicator.

- At **366 nm (long-wave UV)**: fluorescent compounds appear as bright colored spots on a dark background.

Figure 41. Visualization of TLC plates under UV light.

In general, spots are outlined with a pencil to mark their position. The area can also be scraped off, and the collected powder dissolved in a suitable solvent (in which the stationary phase is insoluble) for further analysis.

b) Color-forming reagents (Chemical staining):

- This technique involves spraying the plate with a visualizing reagent, followed by heating at around 100 °C, to convert colorless compounds into colored spots.
- General reagents: concentrated sulfuric acid**,** phosphoric acid.
- Strong oxidizing reagents: potassium dichromate**,** potassium permanganate.
- Specific reagents: used for particular classes of compounds, such as alkaloids, steroids, phenols, sugars, amino acids, etc.

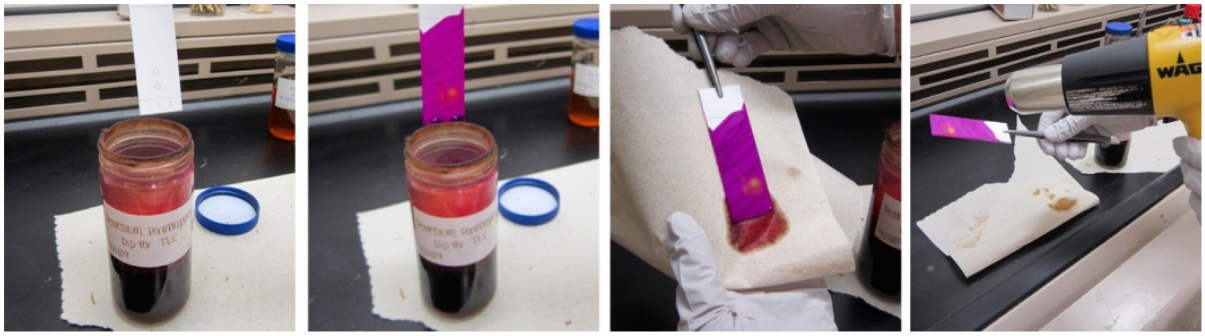


Figure 42. TLC plate visualization by chemical staining with potassium permanganate ($KMnO_4$).

c) Exposure to iodine vapors:

- The plate is placed for a few minutes in a sealed chamber containing solid iodine crystals.
- Iodine vapors adsorb onto most organic compounds, making them appear as dark brown spots.

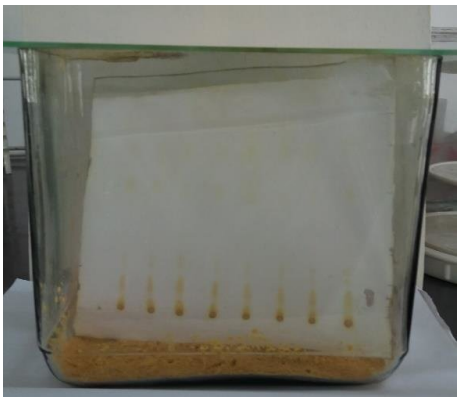


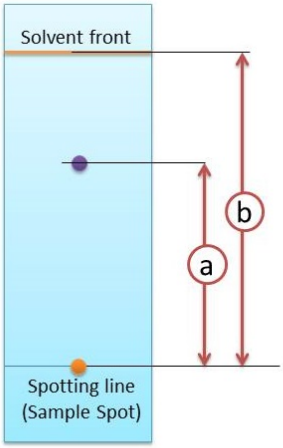
Figure 43. Visualization of a TLC plate after exposure to iodine vapors in a sealed chamber..

I.4. Analysis of the Plate

I.4.1. Qualitative Analysis

The identification of an unknown substance can be achieved by comparing its migration distance with those of reference compounds deposited on the same TLC plate. This migration distance is expressed as the *Rf* value (Retention factor or Front ratio):

$$Rf = \frac{\textbf{Distance traveled by the spot } (a)}{\textbf{Distance traveled by the solvant } (b)}$$



- Each spot correspons to a specific constituent.
- A compound is identified by comparing its *Rf* value with that of a standard.
- Under identical operating conditions, the same substance will migrate the same distance, giving the same *Rf* value.

I.4.2. Quantitative Analysis

Since the **spot area** is proportional to the **logarithm of the mass** of the eluted compound, it is theoretically possible to estimate concentration by:

- Applying precisely known volumes of both the sample solution and standard solutions on the same plate.
- Comparing the spot intensities or areas.

Alternatively, the portion of the plate containing the analyte can be scraped off, dissolved in a suitable solvent, and analyzed by colorimetric or spectrophotometric methods.

⚠ ***Note :*** Quantitative analysis by TLC is generally unreliable. This technique is mainly used for qualitative identification or at best for semi-quantitative estimations.

Part II

Chromatographic Methods

Chapter II

Column

Chromatography (CC)

II. Column Chromatography (CC)

Column Chromatography (CC) is a separation method widely used in organic chemistry to isolate and purify the constituents of a mixture. The technique employs a stationary phase (usually silica gel, SiO_2) packed into a vertical glass column, and a mobile phase (solvent or mixture of solvents) that flows through it, carrying analytes at different speeds depending on their affinities.

This technique was first developed in 1906 by the Russian botanist Mikhail Tswett, who demonstrated that plant pigments could be separated by passing their solution in petroleum ether through a column filled with calcium carbonate.

II.1. Principle

The principle of column chromatography is similar to that of Thin Layer Chromatography (TLC).

- Separation depends on the relative affinity of compounds for the stationary phase (adsorbent) and the mobile phase (eluent).
- Unlike TLC, where the stationary phase is spread on a flat plate and solvent rises by capillary action, in column chromatography the stationary phase is packed inside a vertical column, and elution occurs by gravity (or pressure in some techniques).
- Compounds with stronger interactions with silica gel (polar compounds) are retained longer, while less polar compounds elute faster.

II.2. Procedure

II.2.1. Column Packing

The quality of separation depends heavily on the homogeneity of the stationary phase. Two main packing methods exist:

- **Dry packing**:
  - The column is partially filled with eluent.
  - The adsorbent (silica) is poured carefully as a dry powder using a funnel.
  - The column is tapped continuously to ensure even distribution and prevent air bubbles.
- **Wet packing**:
  - A slurry of adsorbent and eluent is prepared in a beaker.
  - The slurry is poured into the column with a funnel.

- This technique generally provides better homogeneity and is preferred for precise separations.

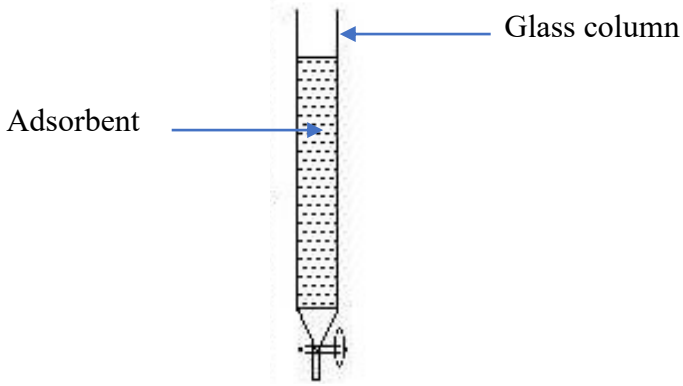


Figure 44. Step 1 – Column packing.

II.2.2. Sample Loading and Elution

- Before loading, the solvent level must be aligned with the top of the stationary phase.
- The mixture to be separated is then deposited gently along the column wall using a Pasteur pipette, ensuring an even distribution.
- Elution is initiated by adding solvent (eluent) dropwise, maintaining a constant flow with the help of a Pasteur pipette, wash bottle, or separatory funnel.

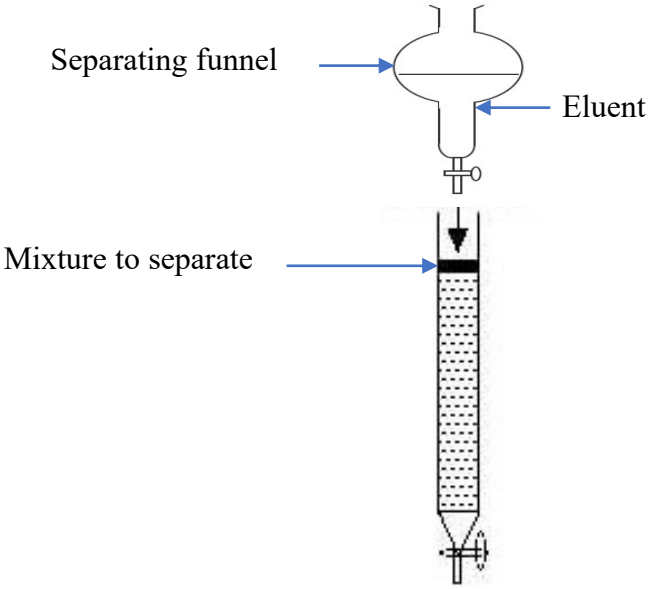


Figure 45. Step 2 – Sample loading and elution.

⚠ **Important:** The column should never be allowed to run dry, as this can create cracks in the adsorbent and destroy the separation.

II.2.3. Fraction Collection

- The eluate is collected in numbered test tubes (fractions).
- Each fraction can be checked for purity using TLC, GC, or HPLC.
- Fractions containing identical compounds are pooled together.

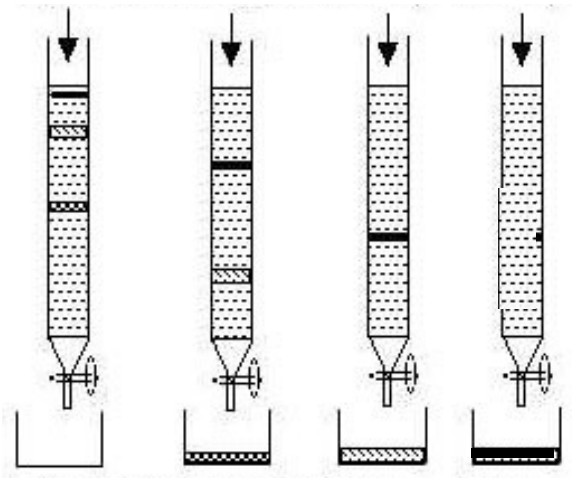


Figure 46. Step 3 – Fraction collection.

II.3. Parameters Affecting Separation

II.3.1. Amount of Adsorbent

- A typical ratio is 50 g of adsorbent per 1 g of sample, though up to 200 g may be required for difficult separations.

II.3.2 Eluent (Mobile Phase)

- Usually a mixture of polar and non-polar solvents.
- Separation often begins with a weak eluent (low polarity).
- Once non-polar compounds are eluted, the polarity of the eluent can be gradually increased to elute more polar compounds.
- This process is called gradient elution.

II.3.3 Column Dimensions

- The length and diameter of the column are chosen based on the amount of sample and the desired purity.

- In laboratories, typical separations range from 10 mg to 20 g of sample; in industry, up to 1 kg can be processed.
- Special columns are equipped with a sintered glass frit at the base, allowing solvent flow while retaining the adsorbent.

II.3.4. Eluent Flow Rate

- The eluent should flow at a slow, constant rate to ensure good resolution.
- Too fast → poor separation.
- Too slow → unnecessarily long separation times.

II.4. Types of Column Chromatography

II.4.1. Gravity Chromatography

- Uses silica particles of 70–200 µm.
- Eluent flows naturally by gravity, drop by drop.
- This is the classical method described above.

II.4.2. Flash Chromatography

- Uses smaller silica particles **(**35–70 µm**)**.
- Eluent is pushed through the column using compressed air or nitrogen, which accelerates separation.
- Provides better resolution and is widely used in modern organic laboratories.

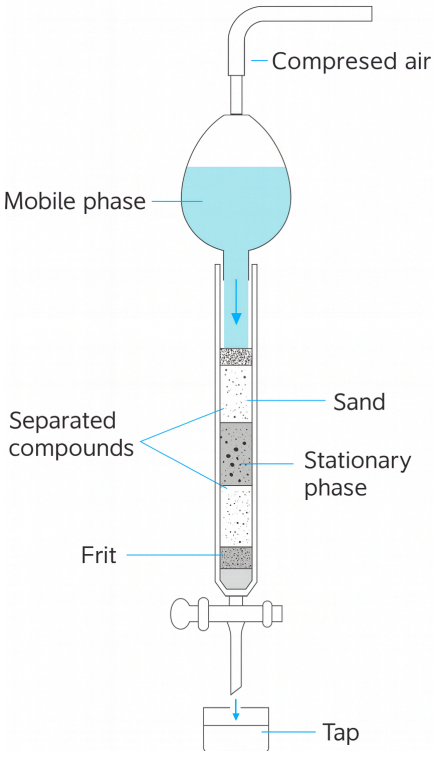


Figure 47. Flash Chromatography

Part II

Chromatographic Methods

Chapter III

Gas Chromatography (GC)

III. Gas Chromatography (GC)

Gas Chromatography (GC) is a powerful separation technique developed by Archer John Porter Martin and Richard Laurence Millington Synge, who were awarded the 1952 Nobel Prize in Chemistry for their pioneering work on partition chromatography. GC is primarily used to separate and analyze volatile and thermally stable organic molecules in complex mixtures.

III.1. Principle

In GC, a very small volume of sample (typically 0.1–1 μL) is injected into a heated injection chamber (injector) where it is instantly vaporized.

- The vaporized sample is transported by an inert carrier gas (mobile phase, often helium, nitrogen, or hydrogen) through a column containing the stationary phase (solid or liquid immobilized on a support).
- Molecules interact differently with the stationary phase depending on their polarity, volatility, and molecular size.
- Compounds that interact weakly with the stationary phase move quickly, while those with stronger interactions are retained longer.
- This leads to their separation, and each compound exits the column after a characteristic retention time (t_R).

⚡ **Key definitions:**

- **Retention time (tR):** Time taken for a compound to travel through the column and be detected.
- **Dead time (tM):** Time required for a non-retained compound to travel through the column.
- **Chromatogram:** Plot showing detector response vs. time, where each peak corresponds to a compound.

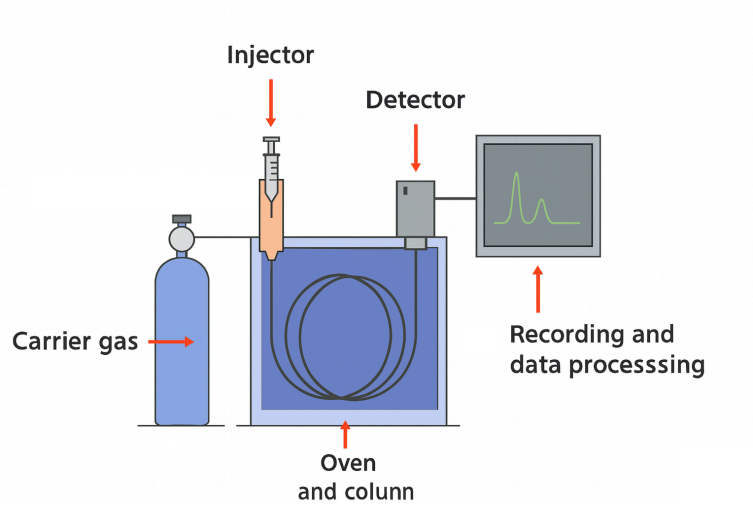


Figure 48. Gas Chromatograph setup.

III.2. Instrumentation

A gas chromatograph consists of three main elements:

- **Injector** (introduction and vaporization of the sample),
- **Column** (separation of analytes),
- **Detector** (detection and quantification of separated analytes).

a) The Injector

Several types of injectors are available on the market, each designed to accommodate different types of samples and chromatographic systems. Among these, the most widely used is the Split/Splitless injector, which was also employed in the context of this study. The injector is a critical component of the gas chromatograph, as it is responsible for the rapid vaporization of the injected liquid sample and its efficient transfer into the column by means of the carrier gas. Structurally, the injector is composed of several key parts: an inlet for the carrier gas, a sealed system for sample introduction through a rubber septum (which can be pierced by a syringe needle), a vent that allows the division of the flow when operating in split mode, a vaporization chamber (commonly referred to as the liner or insert), and an outlet for connection to the chromatographic column. All of these elements are enclosed in a thermostated metallic block, which ensures that the injector is maintained at a high and constant temperature suitable for the complete vaporization of the analyte (Fig. 49).

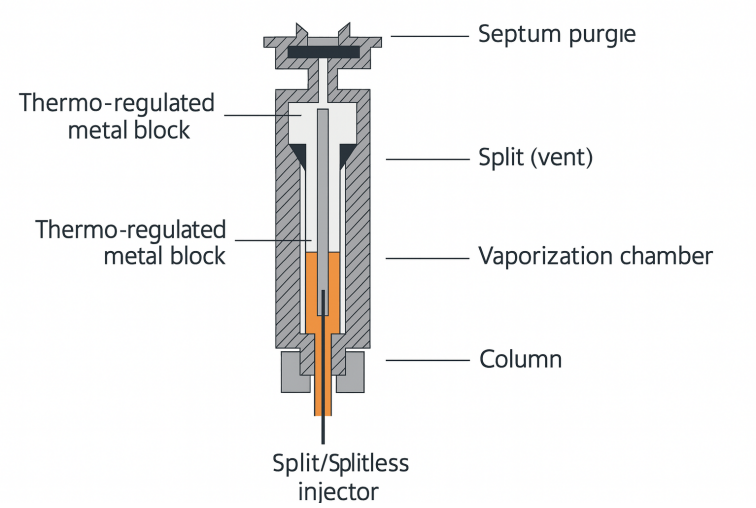


Figure 49. Split/Splitless injector schematic.

Once injected through the septum, the sample is immediately vaporized inside the liner. The vaporized analyte is then swept along by the carrier gas and directed either partially or completely into the chromatographic column, depending on the selected operating mode. Two main injection modes can be distinguished:

- **Split mode (for concentrated samples):** In this configuration, the split vent is left open. As a result, the majority of the vaporized sample is diverted out of the injector through the vent, while only a small, controlled fraction enters the column. This prevents column overloading and ensures sharp, well-defined chromatographic peaks.
- **Splitless mode (for dilute samples):** In this configuration, the split vent is closed, and the entirety of the vaporized sample is introduced into the column. This mode maximizes sensitivity and allows the detection of trace analytes, but it requires precise control of the injection volume and conditions to avoid band broadening.

In practice, the choice between split and splitless injection depends on the concentration of the analyte and the sensitivity required for the analysis. Advanced GC systems may also integrate programmable temperature-controlled injectors (PTV), which allow temperature ramping during injection to further optimize vaporization and minimize sample degradation.

b) The Column

A wide variety of chromatographic columns are commercially available, and the choice of column depends primarily on the type of molecules to be separated. Today, however, the most commonly used are capillary columns, which have become the standard in modern gas chromatography due to their superior separation efficiency.

Capillary columns are narrow, tubular open columns with an internal diameter typically below 1 mm (semi-capillary columns have diameters ranging between 0.5 and 1 mm). The inner wall of the tube is coated with the stationary phase, which may be a thin film of a liquid or polymer. The carrier gas (the mobile phase) flows through the hollow center of the column, transporting the analytes while their interactions with the stationary phase control their retention times (Figure 50).

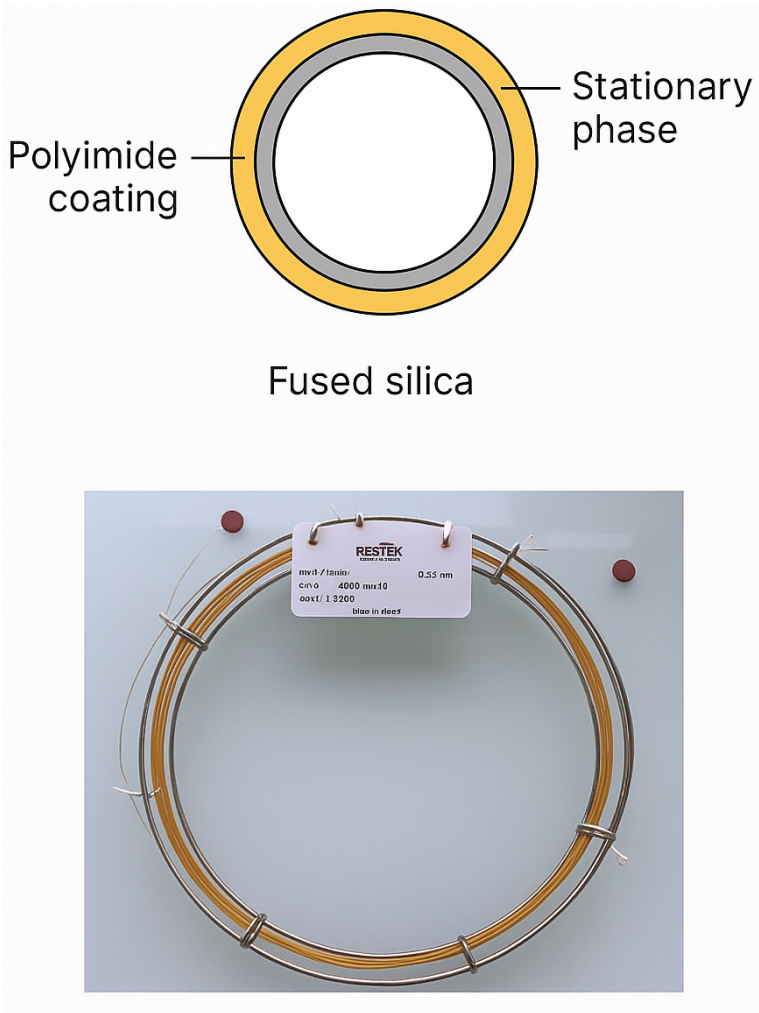


Figure 50. Capillary column (scheme and photo).

Depending on the chemical nature of the stationary phase, columns can be tailored for specific applications:

- **Polar columns**, designed to retain polar molecules through dipole-dipole interactions or hydrogen bonding.
- **Nonpolar (apolar) columns**, used to separate hydrocarbons or nonpolar analytes, where separation is mainly based on volatility.

- **Chiral columns**, which contain a chiral stationary phase capable of separating enantiomers, making them essential in pharmaceutical and biochemical analyses.
- **Achiral columns**, suitable for general-purpose separations where chirality is not relevant.

The diversity of stationary phases available allows chromatographers to optimize selectivity, resolution, and analysis time for a wide range of compounds, from simple hydrocarbons to complex mixtures such as essential oils, pesticides, or pharmaceutical ingredients.

c) Detectors

Just like injectors, gas chromatographs can be equipped with different types of detectors depending on the type of analytes to be studied. Among the many options available, the Flame Ionization Detector (FID) and the Mass Spectrometry Detector (MSD, in GC–MS coupling) are by far the most widely used for organic compound analysis.

➢ The **Flame Ionization Detector (FID)** is considered a semi-universal detector because it can detect the majority of organic substances with high sensitivity. The detector consists of several key components: a column outlet, a hydrogen supply (fuel for the flame), an air supply (oxygen as oxidizer), a combustion chamber, a collector electrode, and an ignition source (Figure 51)

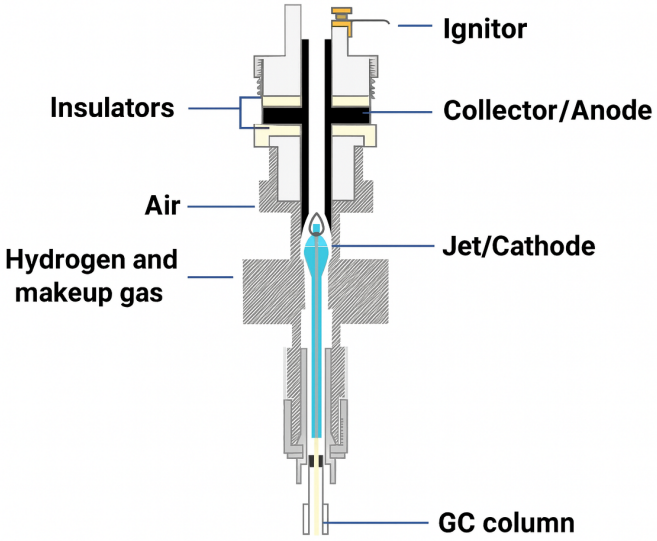


Figure 51. Flame Ionization Detector (FID).

In operation, a potential difference of about 300 volts is maintained between the burner nozzle and the collector electrode. As analytes exit the chromatographic column, they enter the flame where they are pyrolyzed and ionized. The resulting ions generate an electric current between the electrodes, which is then amplified by an electrometer and recorded by the data acquisition

system. The intensity of the signal is directly proportional to the number of carbon atoms entering the detector, which means that the peak area in the chromatogram is proportional to the concentration of the analyte. FID is particularly appreciated for its wide linear dynamic range, sensitivity (down to 10^{-12} g), and robustness.

- The **Mass Spectrometry Detector (MSD)**, used in combination with GC (GC–MS), provides structural information about analytes, making it one of the most powerful tools in modern analytical chemistry. A mass spectrometer is generally composed of three essential parts: the ion source, the mass analyzer, and the detector. In GC–MS, the most common mode of ionization is electron impact (EI). In this process, molecules (M) are bombarded with high-energy electrons, which leads to the ejection of an electron from the molecule and the formation of a radical cation (M^{+}•). This molecular ion may then fragment into smaller ions, producing a unique mass spectrum that serves as a molecular fingerprint. For example, in the study of essential oils, EI ionization is particularly useful since it allows both the determination of molecular weights and structural information through characteristic fragmentation patterns.

Once the ions are formed in the source, they are accelerated and guided into the mass analyzer (in this study, a quadrupole analyzer was used). The analyzer separates the ions based on their mass-to-charge ratio (m/z). Finally, the separated ions are detected, and the resulting signal is converted into a mass spectrum. By comparing these spectra with commercial or in-house spectral libraries, unknown compounds can be identified with high confidence.

In addition to mass spectrometry, other approaches exist to aid in the identification of unknown compounds in GC. One common method is the comparison of retention times (t_R) with those of known standards, since each compound under given chromatographic conditions exhibits a characteristic retention time. Another complementary approach is the calculation of retention indices, introduced by James and Martin. These indices, such as the Kováts index (IK) or retention indices on polar (IRp) and nonpolar (IRa) columns, are determined using a homologous series of n-alkanes as reference compounds. Retention indices provide greater reliability than raw retention times alone because they are less sensitive to variations in operating conditions or column aging. By comparing experimental retention indices with literature values, analysts can achieve a more accurate identification of unknown compounds.

Together, the use of retention times, retention indices, and mass spectra makes GC–MS one of the most versatile and reliable analytical techniques for both qualitative and quantitative studies of volatile organic compounds.

Part II

Chromatographic Methods

Chapter IV

High Performance Liquid Chromatography (HPLC)

IV. High Performance Liquid Chromatography (HPLC)

HPLC is both an analytical and preparative technique in which the mobile phase is a liquid, unlike Gas Chromatography (GC) where the mobile phase is a gas. It was developed around 1967 by Huker and Hulsman.

Unlike GC, which is limited to volatile and thermally stable compounds, HPLC can separate almost any type of mixture, regardless of volatility. This makes it one of the most versatile separation methods in chemistry, biochemistry, pharmaceuticals, and environmental sciences.

IV.1. Principle

- The mixture to be analyzed is injected into the mobile phase using an injection valve placed between the pump and the column.
- The **pump** drives the mobile phase through the column at high pressure to overcome resistance due to the fine particles of the stationary phase.
- Inside the column, molecules are separated based on their differential affinity toward the mobile phase (eluent) and the stationary phase (packing material).
- As they elute, compounds are detected by an appropriate detector, producing peaks on a chromatogram.
- The set of peaks obtained represents the chromatogram, where retention times and peak areas provide both qualitative (identification) and quantitative (concentration) information.

HPLC can therefore be used for:

- **Analytical chromatography** → identification and quantification.
- **Preparative chromatography** → purification of target compounds.

V.2. Instrumentation

As with Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC) consists of four main components:

- **Pump** (to deliver the mobile phase under high pressure),
- **Injector** (sample introduction system),
- **Column** (where separation occurs),
- **Detector** (to visualize and quantify analytes).

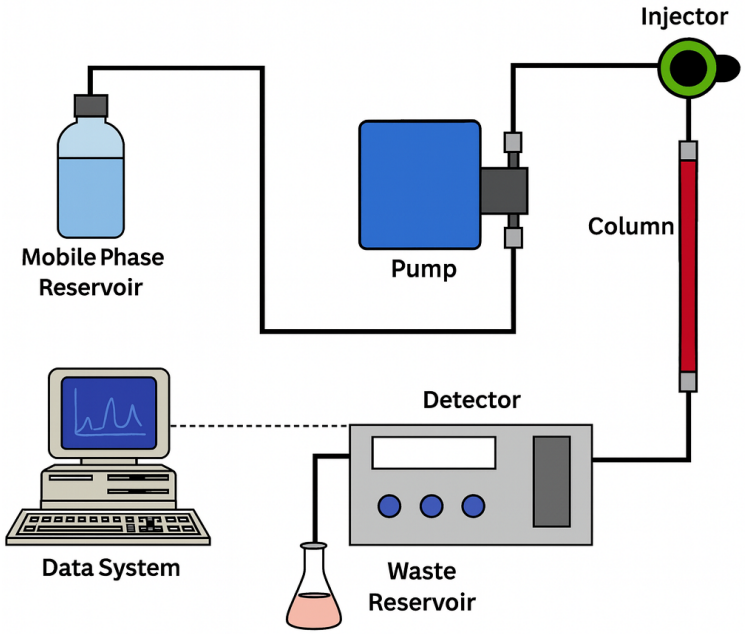


Figure 52. General scheme of an HPLC system.

IV.2.1. Pump

The pump in High-Performance Liquid Chromatography (HPLC) plays a critical role: it drives the mobile phase through the column at high pressure and with a constant, stable flow rate. Because HPLC uses solvents of relatively high viscosity and stationary phases composed of very fine particles, a significant pressure drop (backpressure) occurs between the top and bottom of the column. This pressure drop can reach 50–100 bars under standard conditions. To overcome this, HPLC pumps are designed to deliver much higher pressures: the most commonly used systems operate up to 420 bars, while advanced instruments can reach 600 bars or more.

The pumps most frequently used are reciprocating piston pumps, known for their ability to generate constant flow rates under high pressure. Depending on the analytical need, several configurations are available:

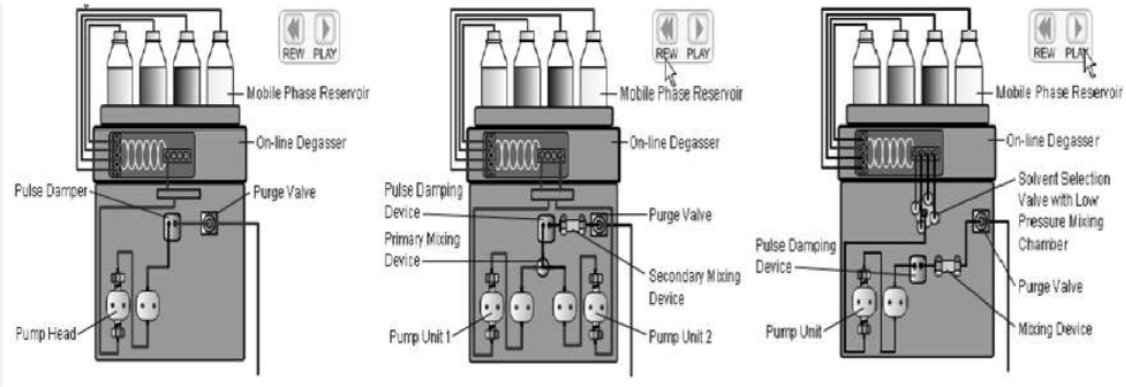


Figure 53. Diagrams of isocratic (A), binary (B) and quaternary (C) pumps.

- **Isocratic pumps**: These deliver a single solvent throughout the entire analysis. Equipped with a single pump head, they are the simplest and most stable, but limited to systems where one fixed mobile phase is sufficient.
- **Binary pumps**: These use two separate pump heads, allowing the use of two solvents. They can operate in either isocratic mode or gradient mode. In gradient mode, the relative proportion of each solvent changes during the run, which is particularly useful for separating complex mixtures with components of very different polarities. Binary pumps are highly precise and reproducible, which makes them the standard in most modern HPLC systems.
- **Quaternary pumps**: These have one pump head but can handle up to four solvents simultaneously. Mixing occurs in a low-pressure chamber before the pump head, enabling both isocratic and gradient elution. While quaternary pumps are more versatile and cost-effective, their gradients are generally less precise and less reproducible than those obtained with binary pumps.

The internal volume of the pump head determines the displacement volume per piston stroke. When combined with the piston's reciprocating frequency (number of strokes per minute), this defines the mobile phase flow rate delivered to the column (Fig. 54).

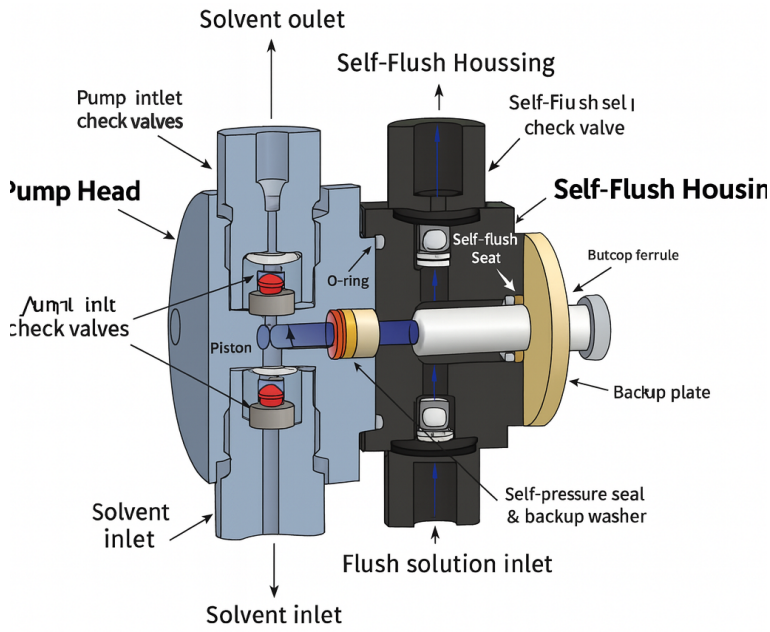


Figure 54. HPLC piston pump head.

This precise flow control is crucial because even small variations in flow rate can significantly affect retention times, peak shape, and overall chromatographic performance.

V.2.2. Injection systems

Injection systems in HPLC are designed to introduce precise and reproducible amounts of sample into the chromatographic column, even under very high pressures that can reach up to 600 bar. Because the pressure inside the pump–column circuit is extremely high, it is not possible to inject the sample directly with a syringe, as this would cause abrupt pressure fluctuations and damage the system. Instead, HPLC systems use an indirect injection method involving a sample loop.

The loop is a small, closed volume (ranging from a few microliters to several hundred microliters) into which the sample is first introduced at atmospheric pressure. Once filled, the loop can be switched into the high-pressure mobile phase flow using a specially designed valve system. In this way, the content of the loop is carried into the column by the mobile phase in a smooth and reproducible manner. This method minimizes pressure shocks, avoids irregular injections, and guarantees that the exact loop volume is always delivered to the column, regardless of the operator's technique.

Nearly all modern HPLC systems are equipped with loop injection valves, which are considered the standard because of their accuracy and repeatability. The most widely used type is the Rheodyne injection valve (named after the well-known manufacturer). This valve allows the user to load the loop with the sample using a syringe through the injection port, then switch the

valve handle to the "inject" position, placing the loop in line with the mobile phase. The sample is then swept into the column in a controlled and reproducible way.

The reliability of loop valves, particularly Rheodyne systems, makes them indispensable for analytical HPLC. They allow reproducibility of injection volumes, which is critical when comparing chromatographic peaks quantitatively. Figure 55 shows an example of a Rheodyne loop injection valve, highlighting its main components such as the syringe port, the loop, and the switching handle.

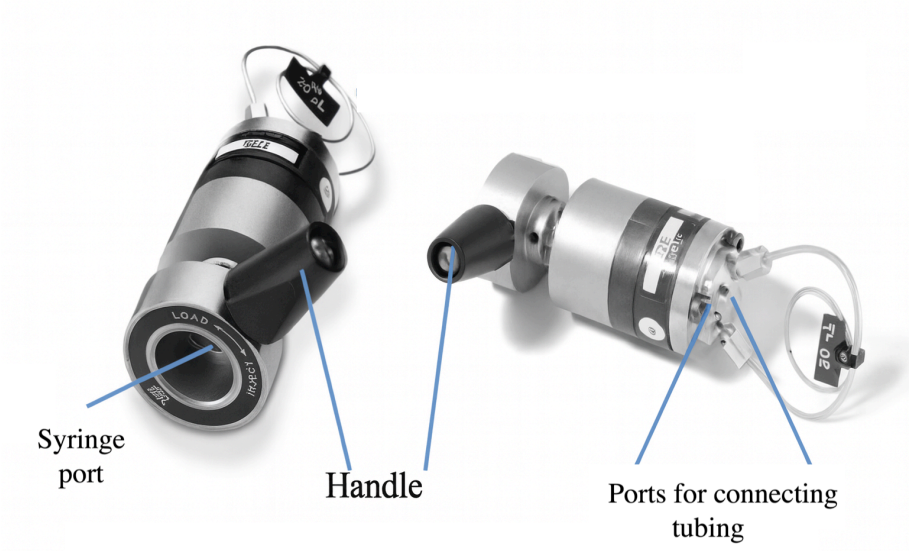


Figure 55. Rheodyne injection valve.

IV.2.3. Column

The columns used in High-Performance Liquid Chromatography (HPLC) are defined both by their geometry and by the nature of the stationary phases they contain. They are typically made of stainless steel to withstand the high operating pressures of HPLC. The internal diameter of analytical columns generally ranges from 2 to 8 mm, with lengths between 10 and 30 cm. Thanks to their robust construction, these columns can tolerate pressures up to several hundred bars without deformation or leakage.

Commercially, a wide variety of HPLC columns are available, and the choice of column depends largely on the type of molecules to be separated and the goals of the analysis. Most HPLC columns are packed columns, meaning they are filled with porous stationary-phase particles (often silica-based, sometimes modified chemically with bonded phases such as C18, C8, phenyl, cyano, or polar functional groups). These particles provide a very high surface area, allowing efficient interactions with analytes and thus improving separation resolution. Column

efficiency is closely related to particle size: smaller particles (e.g., 3–5 µm in traditional HPLC or <2 µm in UHPLC) offer higher efficiency but also require higher operating pressures.

HPLC columns are available in different chemistries to cover a wide range of applications. Reversed-phase columns (such as C18-bonded silica) are the most widely used and are suitable for separating non-volatile and moderately polar compounds. Normal-phase columns, which use polar stationary phases, are useful for the separation of polar compounds. Specialized columns also exist, such as chiral columns for enantiomeric separations, ion-exchange columns for charged molecules, and size-exclusion (gel permeation) columns for the separation of macromolecules based on molecular size.

In practice, HPLC columns are often preceded by a guard column (or pre-column), which is a short cartridge containing the same stationary phase as the main column. The guard column acts as a protective barrier, preventing contaminants, strongly retained compounds, or particulate matter from reaching and fouling the analytical column. This significantly prolongs the lifetime of the main column and reduces the risk of premature deterioration of the stationary phase (Fig. 56).

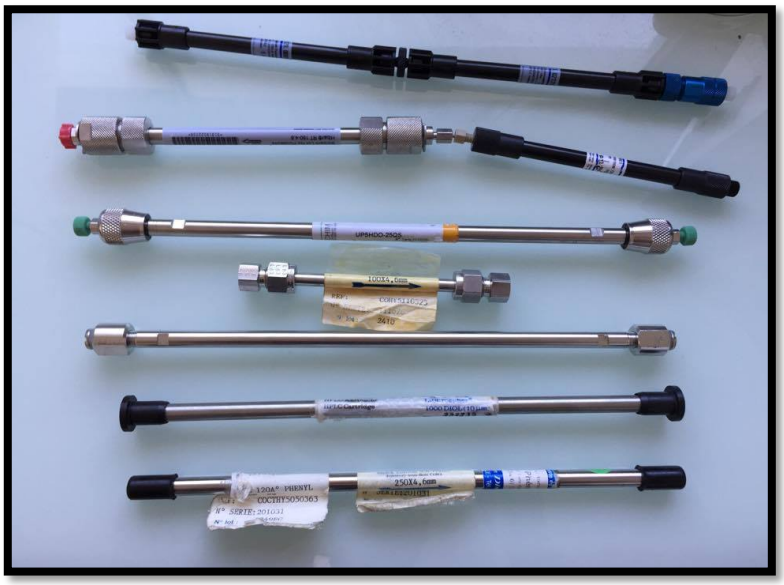


Figure 56. HPLC columns.

IV.2.4. Detectors

Detection systems in HPLC play a central role, as they continuously monitor the presence of analytes as they elute from the column. Several types of detectors are available, each with specific advantages depending on the chemical properties of the analytes and the application of

interest. The most commonly used detectors include UV–visible spectrophotometers, diode array detectors (DAD), refractometers, fluorescence detectors, polarimeters, and, more recently, mass spectrometers (MS) coupled to HPLC.

1. Spectrophotometric Detectors (UV–Vis and DAD):

The most widely used detectors in HPLC are spectrophotometric detectors, which measure the absorption of light by analytes at specific wavelengths. Classical UV–visible detectors operate at a single wavelength, while diode array detectors (DAD) can simultaneously measure absorbance across multiple wavelengths or even across an entire spectrum. In a DAD system (Fig. 57), a polychromatic UV light source passes through the flow cell containing the eluate from the column. The transmitted light is then dispersed by a concave grating, and the absorbance is recorded by an array of photodiodes. This allows simultaneous monitoring at multiple wavelengths, identification of compounds based on spectral patterns, and detection of co-eluting species. The sensitivity and spectral resolution depend on the number and size of the diodes.

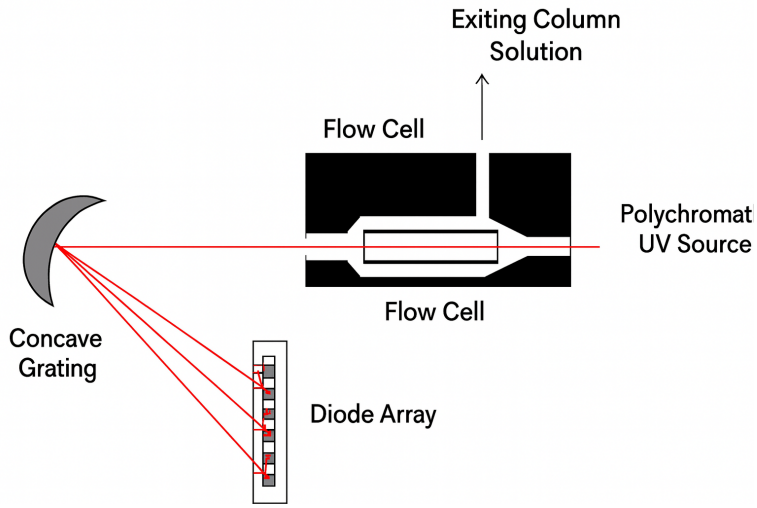


Figure 57. DAD detector diagram.

Importantly, the detector's response is generally proportional to the concentration of the analyte. Thus, the area of a chromatographic peak is directly proportional to the amount of compound present. Quantification is typically performed by external calibration using known standards, or by internal calibration, where a reference compound (not present in the sample) is added at a fixed concentration to improve precision and correct for injection variability.

2. Refractive Index Detector (RID):

The refractometer measures the difference in refractive index between the pure mobile phase (used as a reference) and the eluate containing analytes. While simple and universal (since all

compounds have a refractive index), its sensitivity is relatively poor compared to spectrophotometric detection. RID is highly sensitive to temperature fluctuations and flow rate variations, which can lead to signal instability. Additionally, it is unsuitable for gradient elution, since the refractive index of the solvent mixture changes during the run. Consequently, refractometers are mainly used in size-exclusion chromatography (SEC) or when compounds lack chromophores and cannot be detected by UV.

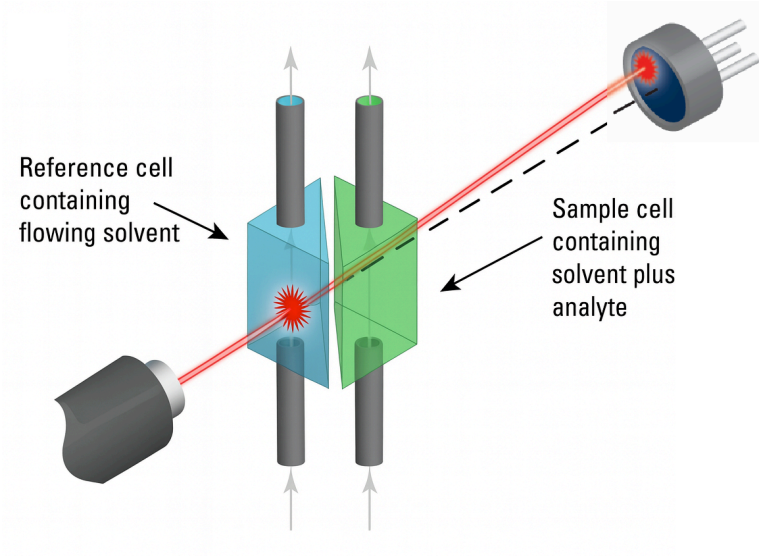


Figure 58. Refractive index detector diagram.

3. Fluorescence Detector:

Fluorescence detection is extremely sensitive and selective, but only applicable to compounds that naturally fluoresce under UV excitation (e.g., aromatic compounds, polycyclic structures). The eluate is irradiated with light at a specific excitation wavelength, and the emitted fluorescence at a longer wavelength is measured. This technique offers sensitivities several orders of magnitude higher than UV detection. Its application range can be extended by chemically derivatizing non-fluorescent compounds into fluorescent derivatives either before injection or post-column. Modern fluorescence detectors often employ lasers as excitation sources, offering high stability and monochromaticity.

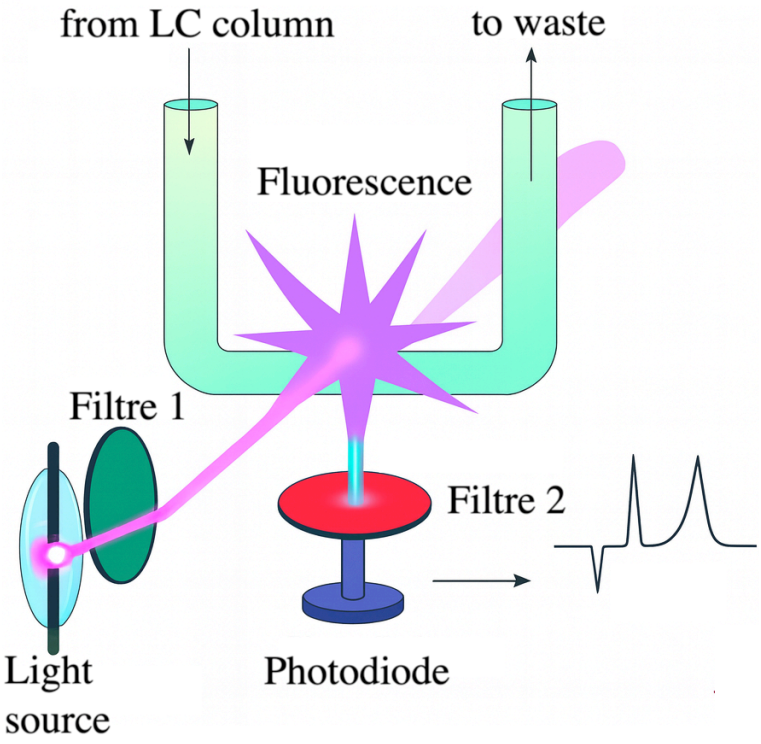


Figure 59. Fluorescence detector diagram.

4. Polarimetric Detector (Polarimeter):

Polarimetric detection is based on measuring the rotation of polarized light as it passes through a sample containing optically active (chiral) compounds. This method is mainly used in chiral HPLC, where enantiomers must be separated and quantified. Although less sensitive than UV or fluorescence detection, it provides unique information about the stereochemistry of analytes, which is crucial in pharmaceutical and biochemical studies.

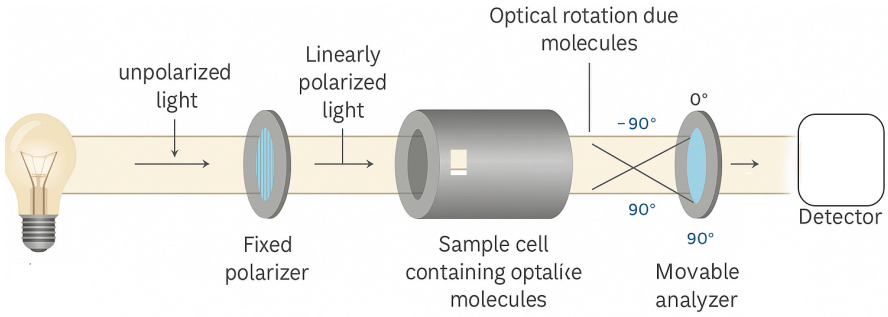


Figure 60. Polarimetric detector diagram.

5. Mass Spectrometric Detection (HPLC–MS):

While not part of the original text, it is worth highlighting the increasing importance of HPLC–MS coupling. In this setup, molecules eluting from the column are ionized (commonly by electrospray ionization, ESI) and then analyzed based on their mass-to-charge ratio (m/z). This provides structural information and high sensitivity, making it the gold standard in fields such as drug analysis, metabolomics, proteomics, and environmental chemistry.

Part II

Chromatographic Methods

Chapter V

Chromatography Theory

V. Chromatography Theory

V.1. Characteristics of a Chromatogram

When a compound elutes from the chromatographic column, it produces a chromatographic peak, generally Gaussian in shape. Each peak can be described by several fundamental parameters that are essential for both qualitative and quantitative analysis (Figure 61).

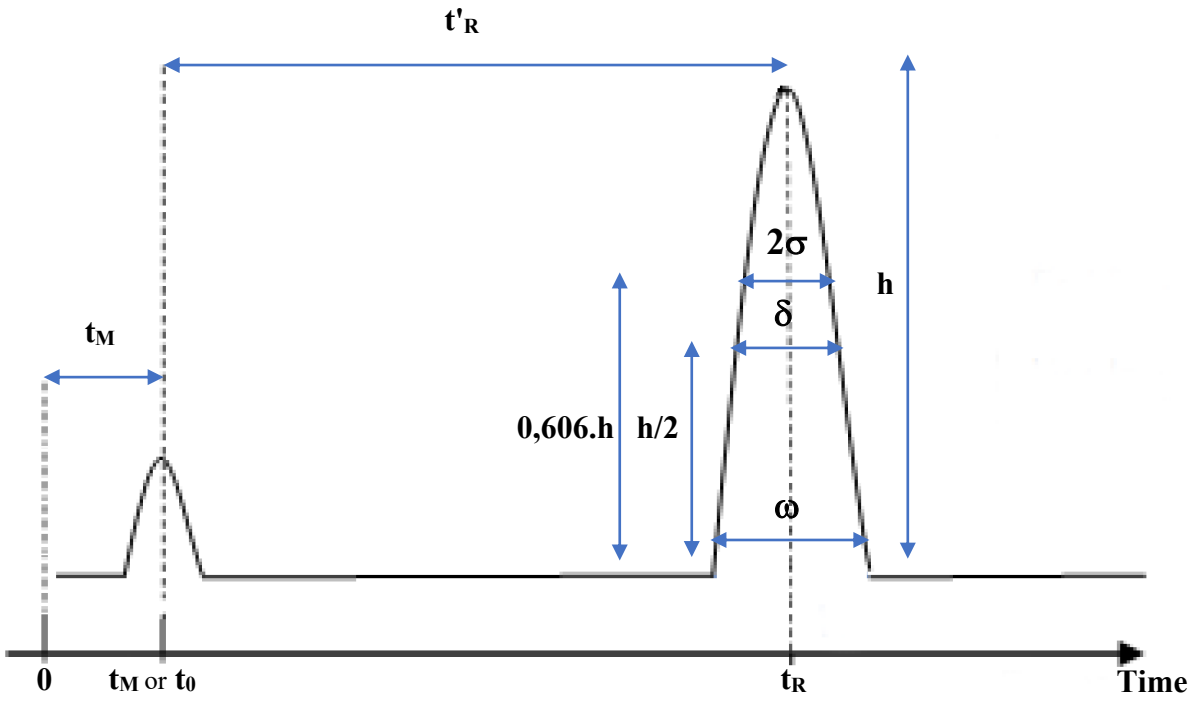


Figure 61. **Profile of chromatographic peaks.**

V.1.1. Dead Time (t_0 or t_M)

The dead time corresponds to the time required for an unretained compound to travel through the column from injection to detection. It represents the minimum time any molecule needs to pass through the column and provides the baseline reference for all retention measurements.

V.1.2. Retention Time (t_R)

The retention time is the elapsed time between the injection of the sample and the apex of the analyte peak. It is a unique characteristic for each compound under constant chromatographic conditions and is frequently used for qualitative identification of analytes.

V.1.3. Adjusted Retention Time (t'_R)

The adjusted retention time is the difference between the retention time and the dead time

($\mathbf{t'_R = t_R - t_M}$). This parameter reflects the actual interaction of the analyte with the stationary phase and eliminates the contribution of the mobile phase transit.

V.1.4. Peak Width (ω)

The peak width is measured at 13.5% of the peak height. It provides insight into column efficiency: narrower peaks generally indicate better separation performance and reduced band broadening.

V.1.5. Peak Width at Half-Height (δ)

The half-width is measured at 50% of the peak height. It is another measure of peak sharpness and is often used in calculations of column efficiency (number of theoretical plates).

V.1.6. Standard Deviation of the Peak (σ)

The standard deviation corresponds to the half-width measured at 60.6% of the peak height. It is a statistical measure of peak dispersion and forms the basis of many chromatographic equations related to column efficiency.

V.1.7. Retention Volume (V_R)

The retention volume, also called the elution volume, is the volume of mobile phase required to elute the analyte. It is calculated as $\mathbf{V_R = t_R \times D}$, where D is the flow rate of the mobile phase. On a chromatogram, it corresponds to the volume of eluent that has passed through the column until the apex of the peak.

V.1.8. Dead Volume (V_M)

The dead volume is the volume of mobile phase required to pass through the column in the dead time (t_M). It represents the volume of the mobile phase occupying the column and can be calculated as $\mathbf{V_M = t_M \times D}$.

V.1.9. Adjusted Retention Volume (V'_R)

The adjusted retention volume is defined as $\mathbf{V'_R = V_R - V_M}$. It eliminates the contribution of the dead volume and provides a more accurate reflection of analyte retention due to interactions with the stationary phase.

V.1.10. Linear Velocity of the Mobile Phase (u)

The average linear velocity of the mobile phase is calculated as $\mathbf{u = L / t_M}$, where L is the column length. It characterizes the rate at which the mobile phase travels through the column.

V.1.11. Linear Velocity of the Solute (v)

The solute's average linear velocity is defined as $\mathbf{v = L / t_R}$. It measures how fast the analyte travels through the column, accounting for its retention behavior.

V.1.12. Distribution Coefficient (K)

The distribution coefficient expresses the equilibrium of analyte partitioning between the stationary phase and the mobile phase:

$$\mathbf{K = C_S / C_M}$$

where C_S is the concentration of the analyte in the stationary phase and C_M is its concentration in the mobile phase. This thermodynamic parameter fundamentally governs chromatographic separation.

V.1.13. Capacity Factor (k′)

The capacity factor, also called the retention factor, expresses the relative time a compound spends in the stationary phase compared to the mobile phase. It can be calculated using time or volume parameters:

$$\mathbf{k' = (t_R - t_M) / t_M = t'_R / t_M}$$

or

$$\mathbf{k' = (V_R - V_M) / V_M = V'_R / V_M}$$

Values of k′ between 1 and 10 are generally considered optimal, as they provide good retention and separation without excessively long analysis times.

V.1.14. Separation Factor (α)

The separation factor, also called the selectivity factor (α), describes the relative position of two adjacent peaks (1 and 2) on a chromatogram. It is mathematically expressed as:

$$\alpha = \frac{k'_2}{k'_1} = \frac{t'_{R2}}{t'_{R1}} = \frac{V'_{R2}}{V'_{R1}}$$

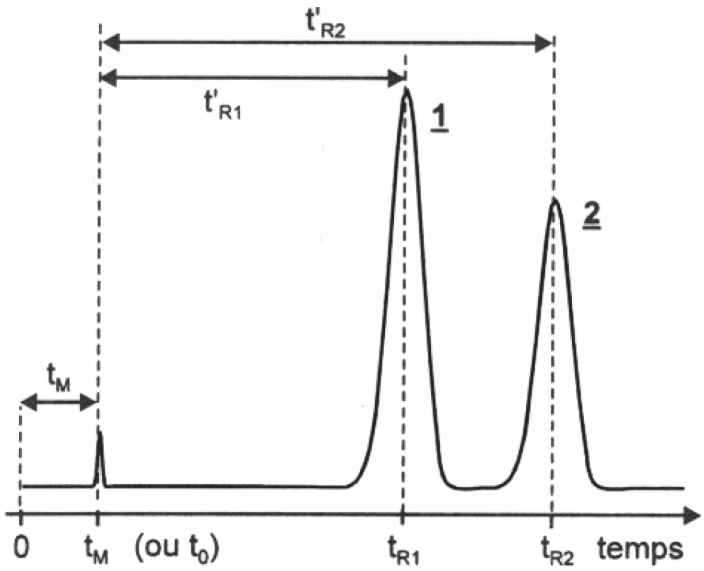


Figure 62. Separation of two adjacent peaks.

where k′, t'_R, and V'_R represent the retention factor, adjusted retention time, and adjusted retention volume, respectively.

A higher α indicates that the two compounds have different interactions with the stationary phase, but this factor alone does not guarantee a complete baseline separation. In practice, **$\alpha > 1$** is necessary for separation, but additional factors such as peak width and resolution must be considered to assess whether the separation is truly effective.

V.1.15. Resolution (R_S)

Resolution (R_S) is a key parameter that quantifies the ability of a chromatographic system to separate two analytes in a mixture. It is defined by the following equation:

$$R_s = 2\,\frac{t_{R2} - t_{R1}}{\omega_1 + \omega_2}$$

where t_{R1} and t_{R2} are the retention times of the two analytes, and ω_1 and ω_2 are the peak widths at baseline.

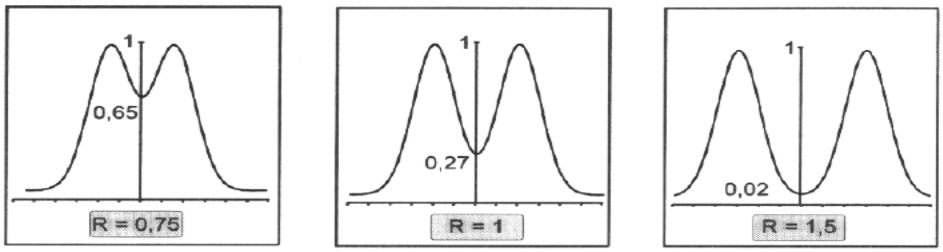


Figure 63. **Improved resolution of two adjacent peaks.**

Resolution provides a numerical measure of how well two peaks are separated:

- **$R_S < 0.75$** → insufficient separation (peaks strongly overlap).
- **$R_S \approx 1$** → partial separation with noticeable peak overlap.
- **$R_S \geq 1.5$** → good baseline separation, considered acceptable in analytical practice.

Improving R_S can be achieved by optimizing column efficiency, selectivity (α), or analysis conditions such as temperature, flow rate, or mobile-phase composition.

V.1.16. Column Efficiency

Column efficiency reflects the ability of a chromatographic column to produce sharp and narrow peaks for the separated compounds. The sharper the peak, the more efficient the column. Efficiency is expressed using two key parameters: the number of theoretical plates (N) and the height equivalent to a theoretical plate (HETP, or H).

a) Number of Theoretical Plates (N)

The number of theoretical plates (N) represents the number of successive equilibrium stages that a solute undergoes during its migration through the column. It is calculated using the following relationships:

$$N = 16\frac{t_R^2}{\omega^2} = 5{,}54\frac{t_R^2}{\delta^2}$$

where:

- t_R = retention time of the analyte,
- ω = peak width at the baseline (measured at 13.5% of peak height),
- δ = peak width at half height (measured at 50% of peak height).

The retention time and the width of the peak must be expressed in the same unit (time, distance, or volume if flow rate is constant).

A higher N indicates a narrower peak and, therefore, better column performance. The number of theoretical plates is characteristic of a given chromatographic system (column + stationary phase + mobile phase + analytes).

b) Height Equivalent to a Theoretical Plate (HETP)

To standardize efficiency relative to column length, the height equivalent to a theoretical plate (H) is defined. It is calculated as:

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

where L is the length of the column.

A lower H value corresponds to higher efficiency, as it means more equilibrium stages (N) are achieved over a shorter length of column. In practice, the H provides a useful way to compare the efficiency of columns of different lengths and packing materials.

V.2. Qualitative and Quantitative Analysis in Chromatography

Chromatography is widely used not only for the separation of compounds but also for their identification (qualitative analysis) and quantification (quantitative analysis). In fact, reliable quantitative analysis is only possible after correct identification of the peaks on a chromatogram.

V.2.1. Qualitative Analysis

V.2.1.1. Identification by Retention Parameters

In principle, compounds can be identified using their retention times (t_R). Under strictly controlled conditions, each compound has a characteristic retention time, which can be compared with that of a reference standard (an authentic sample analyzed under identical chromatographic conditions).

However, retention times are influenced by multiple experimental factors, including:

- The nature of the stationary and mobile phases,
- Column dimensions (length and diameter),
- Flow rate of the mobile phase (HPLC) or carrier gas (GC),
- Elution gradient in HPLC,
- Temperature (in GC) and pressure (in HPLC).

Because retention time depends on so many variables, strict control of conditions is required to ensure reproducibility and reliability of identification.

V.2.1.2. Identification by Retention Indices (Kováts Index and Retention Index)

To overcome the variability of retention times, retention indices were introduced.

In 1958, Ervan Kováts proposed a system to compare analytes with a homologous series of n-alkanes. The Kováts Index (KI) assigns a numerical value to each compound based on its position relative to two alkanes (n and n+1) that elute just before and after the analyte, under isothermal conditions:

$$KI_X = 100 \times \left(n + \frac{\log t'_{R(X)} - \log t'_{R(n)}}{\log t'_{R(n+1)} - \log t'_{R(n)}} \right)$$

where t_R' are the reduced retention times.

The calculated KI can then be compared with published reference values for identification.

Later, the Retention Index (RI) was developed, which uses the same principle but does not require isothermal conditions. Instead, it can be applied under temperature programming:

$$RI_X = 100 \times \left(n + \frac{t_{R(X)} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}} \right)$$

Retention indices are extremely useful because they provide standardized values that are less sensitive to experimental variations, making them a robust tool for compound identification.

V.2.2. Quantitative Analysis

Chromatographic quantification is fundamentally comparative. The concentration of an analyte is determined by relating the area of its peak (Ai) to the amount of compound injected, using a proportionality factor known as the response factor (Ki):

$$m_i = K_i . A_i$$

where:

- m_i = mass of compound i injected,
- A_i = area of the chromatographic peak,
- Ki = response factor (instrument- and compound-dependent).

Since:

$$m_i = Ci \cdot V$$

(where Ci = concentration of analyte, and V = injected volume), we obtain:

$$C_i = m_i / V \quad ===> \quad m_i = C_i . V$$

Thus, quantification requires accurate measurement of peak area and determination of the response factor.

V.2.2.1. Methods of Quantification

Quantitative chromatography relies on different calibration strategies to determine the concentration of analytes in a mixture. Each method has its own advantages, limitations, and applications depending on the sample type, detector, and precision required. The most common approaches are outlined below.

a) External Standard Calibration (Comparison Injections)

This is the simplest and most widely used quantification method. It is based on comparing chromatograms of a reference solution with known concentration and a test solution with unknown concentration, under identical chromatographic conditions to ensure the response factor Ki remains constant.

- **Reference Chromatogram:** A reproducible injection volume V of a standard solution with known concentration C_{ref} is introduced. The area of the peak (A_{ref}) is measured.
- **Sample Chromatogram:** The same injection volume of the unknown sample is introduced, giving a peak area (A_{sample}).

The relationship is:

$$M_{sample} = C_{sample} .V = K. A_{sample}$$

$$m_{ref} = C_{ref} . V = K. A_{ref}$$

Since volumes are equal:

$$C_{sample} = C_{ref.} A_{sample} / A_{ref}$$

❖ **Calibration curve:**

While a single-point calibration is possible, better accuracy is achieved by injecting multiple concentrations of the standard and plotting peak area vs. concentration (Fig. 64). The calibration curve should be linear, and unknown concentrations are obtained by interpolation.

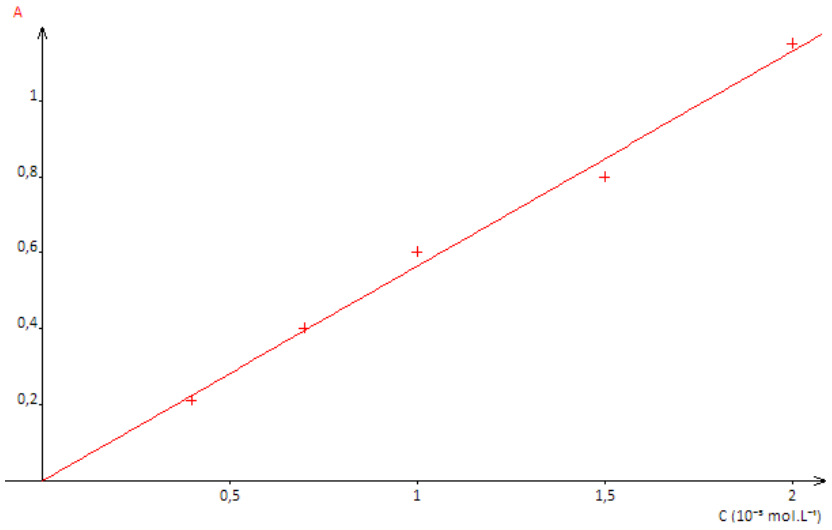


Figure 64. Calibration line (area-concentration proportionality).

Advantages:

- Simple and direct.
- Suitable for both gases and liquids.
- No need to add foreign compounds to the sample.

Limitations:

- Requires highly reproducible injection volumes (often ensured with an autosampler).
- Reference and sample concentrations must be in the same order of magnitude.
- Sensitive to instrumental drift since no internal correction is made.

b) Internal Standard Method

The internal standard (IS) method corrects for injection variability and instrumental fluctuations by adding a known quantity of a reference compound (the IS) to both calibration standards and the unknown sample.

- **Reference Chromatogram:** A solution containing known concentrations of analytes and IS is injected. From the peak areas A_1,A_2,A_E and concentrations C_1,C_2,C_E, response factors relative to the IS are calculated:

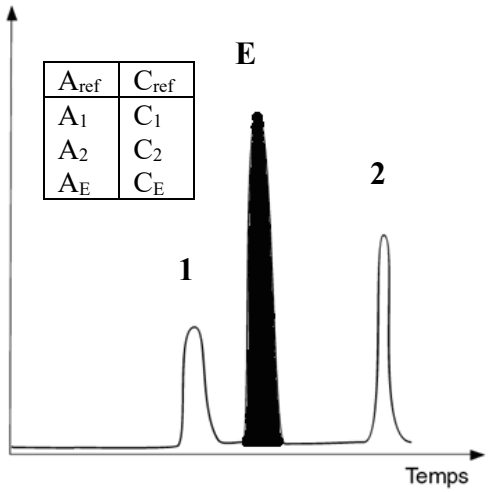


Figure 65. Calibration chromatogram (internal calibration).

$$\frac{K_1}{E} = \frac{K_1}{K_E} = \frac{C_1 A_E}{C_E A_1}$$

$$\frac{K_2}{E} = \frac{K_2}{K_E} = \frac{C_2 A_E}{C_E A_2}$$

- **Sample Chromatogram:** The same IS is added to the unknown sample, and peak areas A_1',A_2',A_E'are measured. The concentrations of analytes are then obtained using the known $K_{i/E}$.

Criteria for choosing an internal standard:

- Must be chemically and physically similar to the analytes.
- Inert with respect to sample components and the stationary/mobile phases.
- Not naturally present in the sample.
- Elution peak must be well resolved.
- Retention time close to that of analytes.
- Response factor should be similar in magnitude to the analytes.

Advantages:

- Corrects for errors in injection volume.
- High reproducibility and accuracy.

- Eliminates need to determine absolute response factors.

Limitations:

- Requires careful selection of a suitable internal standard.

c. Delayed Calibration

This method is similar to internal calibration, but the internal standard is injected separately rather than mixed with the sample. It is placed on the chromatogram so its peak does not interfere with analyte peaks. Sometimes one of the sample components itself may serve as the calibration reference.

d. Internal Normalization

In this method, it is assumed that all sample components elute completely and are detected. The relative proportions of peak areas are then used for quantification.

For three compounds (1, 2, and 3):

$$m_1 = K_1 . A_1$$

$$m_2 = K_2 . A_2$$

$$m_3 = K_3 . A_3$$

$$\frac{m_i}{m_1 + \quad m_2 + \quad m_3} = \frac{K_1 A_1}{K_1 A_1 + K_2 A_2 + K_3 A_3}$$

$$X_1\% \text{ compound } (1) = \frac{K_1 A_1}{K_1 A_1 + \ K_2 A_2 + K_3 A_3} 100$$

If all response factors are equal ($K_1=K_2=\cdots=K_n$), this simplifies to:

$$X_i \ \% = \frac{A_i}{\Sigma \, A_i} . 100$$

Advantages:

- Simple and requires no addition of external/internal standards.

Limitations:

- Semi-quantitative only.
- Requires full resolution and detection of all compounds.
- Response factors must be known or assumed to be equal.

e. Standard Addition Method (Spiking Method)

This method is mainly used for trace analysis or in complex matrices where matrix effects influence quantification. The analyte it self is used as the standard:

- Increasing amounts of analyte are added (spiked) to the sample.
- Only the analyte peak area increases proportionally, while matrix peaks remain constant (Fig. 66).

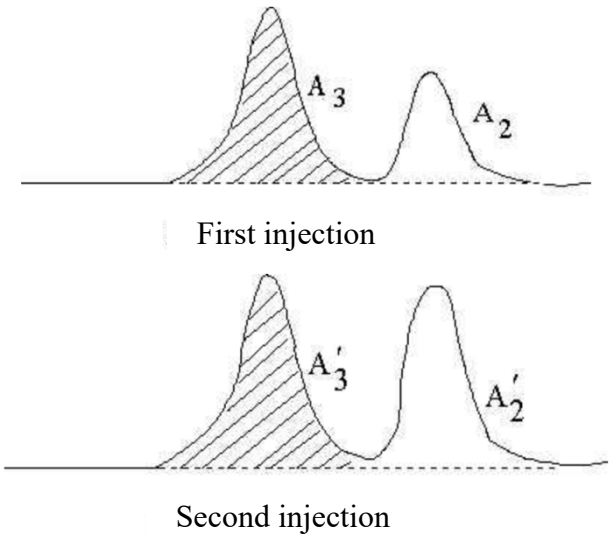


Figure 66. **Method of measured additions.**

For the analyte peak:

$$m_i = K_i . A_i \text{ (first injection, unspiked sample)}$$

$$m_i' = K_i . A_i' \text{ (after addition of standard)}$$

$$m_i (ad) = K_i (A_i' - A_i) \text{ (mass from added standard)}$$

$$m_i = \frac{m_i(ad) \quad A_i}{(A'_i \quad - \quad A_i)}$$

- A calibration curve of peak area vs. added concentration is plotted.
- Extrapolation to zero response (y = 0) gives the original concentration in the sample (Fig. 67).

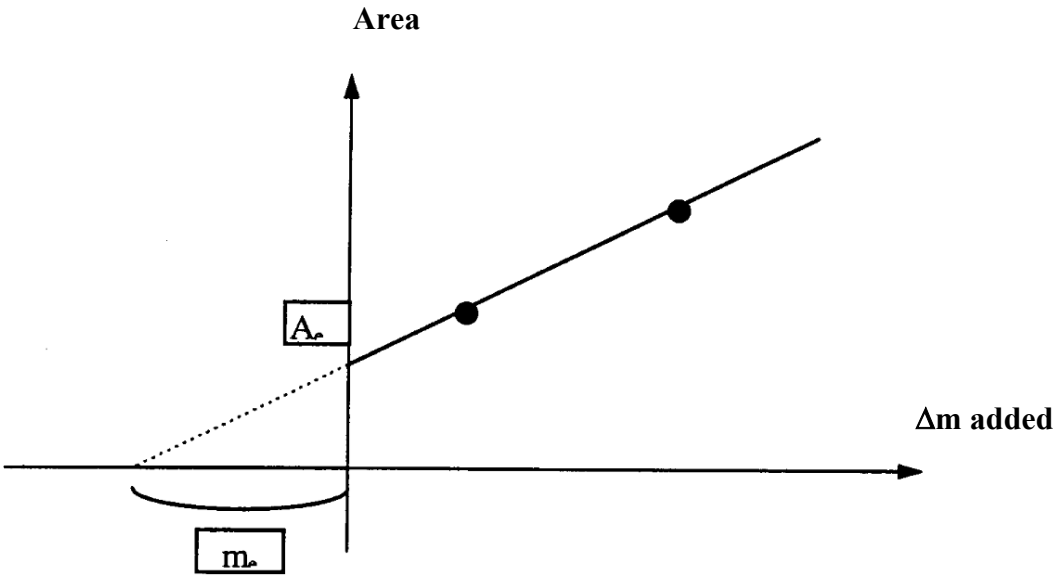


Figure 67. Method of measured additions (graphical method)

Advantages:

- Corrects for matrix effects.
- Very useful in complex samples and for trace analysis.

Limitations:

- Requires highly reproducible injections.
- More labor-intensive than external calibration.

Part II
Chromatographic Methods

Comparative Overview (Chromatographic Methods)

VI. Comparative Overview (Chromatographic Methods)

Chromatography comprises a family of separation techniques widely applied in chemistry, biology, pharmaceuticals, and environmental sciences. Although all chromatographic methods rely on the distribution of analytes between a mobile phase and a stationary phase, they differ in the nature of these phases, the type of analytes they can separate, and their performance in terms of resolution, speed, and sensitivity. Thin-layer chromatography (TLC) and column chromatography are often used for simple, qualitative, or preparative separations, while gas chromatography (GC) and high-performance liquid chromatography (HPLC) provide higher resolution and quantitative precision.

The following table summarizes the main principles, information provided, advantages, and limitations of these four commonly used chromatographic techniques.

Table 10. Comparative Overview of Common Chromatographic Methods (TLC, CC, GC, HPLC).

Technique	*Principle*	*Information Provided*	*Advantages*	*Limitations*
Thin-Layer Chromatography (TLC)	Separation based on migration of analytes on a thin stationary layer using capillary action of a solvent.	Qualitative identification, Rf values, mixture composition.	Simple, low-cost, rapid, requires very small sample amounts.	Limited quantitative precision, lower resolution, mainly qualitative.
Column Chromatography (CC)	Separation of analytes through a packed column of stationary phase with gravity- or pressure-driven solvent.	Purification of compounds, preparative separations.	Versatile, inexpensive, scalable for preparative purposes.	Time-consuming, lower resolution compared to HPLC/GC, requires larger sample.
Gas Chromatography (GC)	Separation of volatile compounds based on partitioning between gas mobile phase and liquid/solid stationary phase.	Identification, quantification, purity of volatile and thermally stable compounds.	High resolution, sensitive, can be coupled to MS for structural analysis.	Limited to volatile/thermally stable compounds, requires derivatization for some analytes.
High-Performance Liquid Chromatography (HPLC)	Separation of analytes based on their interaction with a stationary phase under high-pressure liquid flow.	Identification, quantification, separation of complex mixtures.	High resolution, quantitative accuracy, applicable to non-volatile compounds.	Expensive instrumentation, requires high-purity solvents, maintenance-intensive.

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