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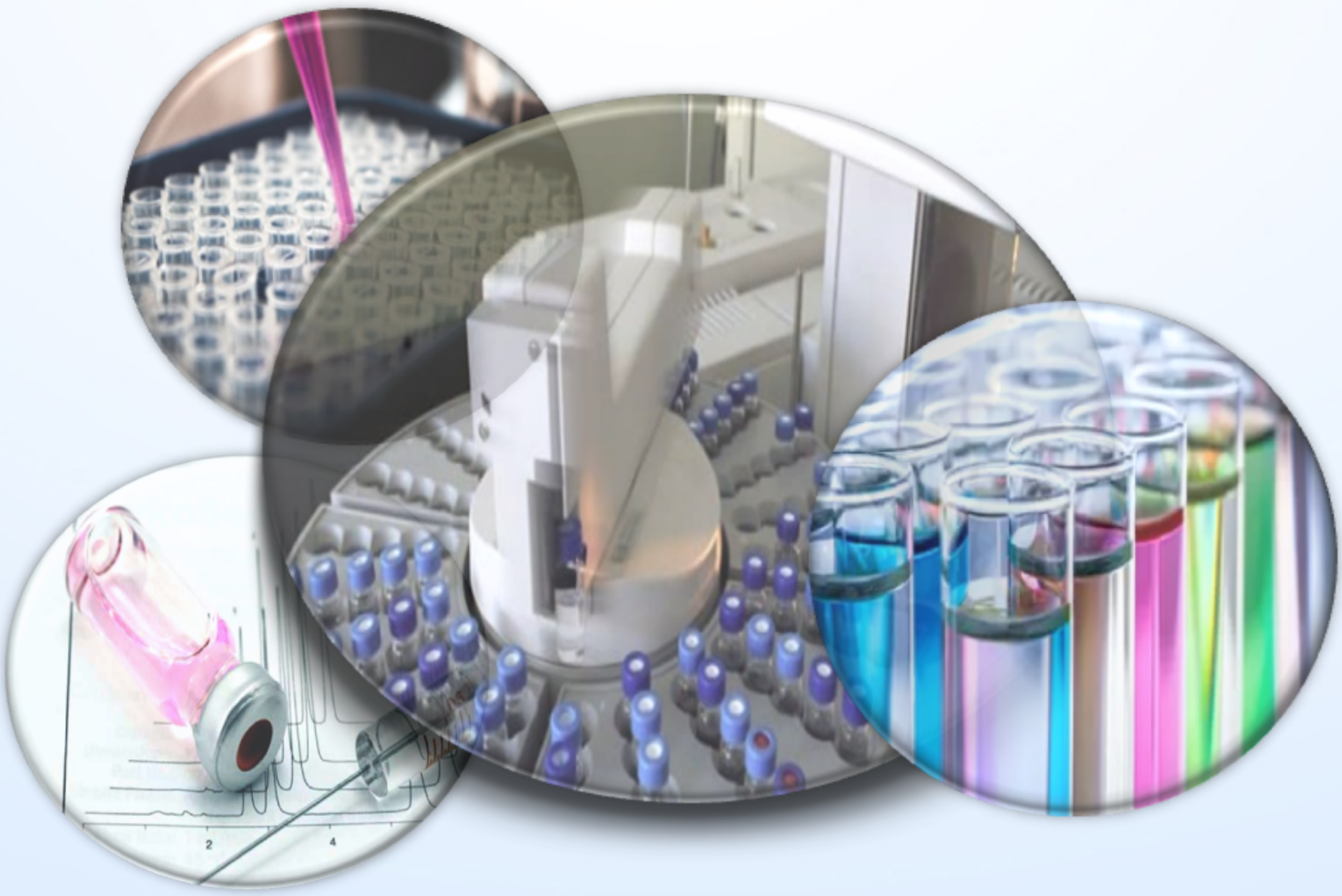
Université de Béjaïa

BIOLOGICAL ANALYSIS TECHNIQUES

COURSE

For 3rd year students in Microbiology and Microbial Biotechnology



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Preface

Biological analysis techniques refer to a diverse set of methods used to study and analyze biological systems, including cells, tissues, proteins, DNA, and other biomolecules. These techniques provide valuable information for understanding biological processes, diagnosing diseases, and developing new treatments.

The development and application of analytical techniques play a pivotal role in unraveling the complexities of living organisms.

This course is specifically designed for third-year Microbiology and Microbial Biotechnology students, providing an enlightening introduction to chromatographic and spectral methods, including UV/Vis spectroscopy and IR spectroscopy. Chromatographic techniques, are fundamental tools for separating and analyzing complex biological compounds. Understanding these techniques are essential for students aspiring to explore the molecular secrets of organisms.

On the other hand, spectral methods, especially UV/Vis spectroscopy and IR spectroscopy, provide insight into the structure of biological molecules. These techniques enable students to visualize molecular interactions and deepen their understanding of biological compounds.

This course has been carefully developed to provide students with a comprehensive educational resource. Each chapter aims to illuminate fundamental principles as well as practical applications. Students will be guided through analytical techniques, reinforcing their skills.

This work is intended for undergraduate students in Microbiology and Microbial Biotechnology as well as those pursuing a Master's degree in Fundamental and Applied Microbiology at the Faculty of Natural and Life Sciences. It can also be beneficially consulted by anyone who feels the need to update their knowledge in a constantly evolving field, which has become indispensable for the understanding of these analytical processes.

The course is presented in seven chapters. Chapter 1, titled introduction to chromatographic techniques, is dedicated to all techniques that can be implemented analytically or preparatively for the production of purified molecules. In Chapter 2, there is a detailed presentation of liquid chromatography, represented by planar chromatography

including paper and thin layer chromatography. Chapter 3 defines High-Performance Liquid Chromatography (HPLC), which represents an evolution of column chromatography to enhance its performance. This investigative method serves as the starting point for advancing knowledge in the field of separation. Chapter 4, will focus on Gas Chromatography, it will provide an understanding of the technique for separating and analyzing volatile compounds in a mixture.

Chapter 5 discusses another kind of technique : spectral methods. Spectroscopic methods refer to a set of analytical techniques that involve the interaction of electromagnetic radiation with matter to study and analyze its properties. These methods are widely used in various scientific disciplines, including chemistry, physics, biology, and environmental science. Spectroscopy provides valuable information about the composition, structure, and behavior of substances by measuring the way they absorb, emit, or scatter electromagnetic radiation. Chapters 6 and 7 will describe respectively, UV/Vis Spectroscopy and Infrared Spectroscopy.

Each chapter will be complemented by Applications.

List of Figures

CHAPTER 1 Introduction to Chromatographic Techniques

CHAPTER 2 Liquid Chromatography

CHAPTER 3 High Performance Liquid Chromatography

CHAPTER 4 Gas Chromatography

CHAPTER 5 Introduction to Spectral Methods

CHAPTER 6 UV/Visible Spectroscopy

CHAPTER 7 Infrared Spectroscopy

List of Tables

CHAPTER 3 High Performance Liquid Chromatography

Table of contents

Chapter 4. Gas Chromatography

Chapter 5. Introduction to Spectral Methods

Chapter 6. UV/Visible Spectroscopy

Chapter 7. Infrared Spectroscopy

Chapter 1

Introduction to chromatographic techniques

CHAPTER

1

Introduction to Chromatographic Techniques

1. What is chromatography ?

Chromatography is a laboratory technique for the separation of a mixture of color or pigments and chemicals. It is a powerful separation tool that is used in all branches of science, and is often the only means of separating components from complex mixtures. The Russian botanist Mikhail Tswett coined the term chromatography in 1906. The first analytical use of chromatography was described by James and Martin in1952, for the use of gas chromatography for the analysis of fatty acid mixture. This process involves dissolving the mixture in a liquid known as the **mobile phase**, which carries it through a structure holding another material called the **stationary phase**. The individual constituents of the mixture move at varying rates, leading to their distinct separation. The separation is based on differential partitioning between the nmobile and stationary phases. Minor variations in a compound's partition coefficient lead to divergent retention on the stationary phase, thereby influencing the separation process (Figure 1.1) (Coskun 2016). .

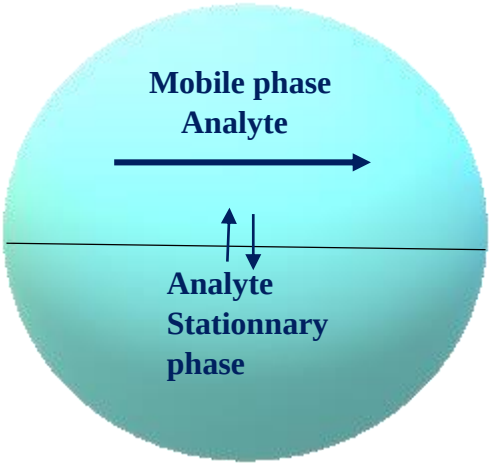


Figure 1.1. *The mobile phase passes through the stationary phase. When the sample is injected into the mobile phase, these components move with the mobile phase and distribute within the stationary phase. The solute that spends more time in the stationary phase takes longer to move through the system.*

2. History and development of chromatography

Chromatography can trace its origins to early attempts at separating and identifying compounds based on their visual properties.

Ancient pigment separation: The earliest recorded use of chromatography-like techniques can be found in ancient times when people separated pigments from natural materials to create dyes and inks. This process was often based on differences in solubility and color.

Mikhail Tswett and the birth of chromatography (1900s)

The modern history of chromatography began with the groundbreaking work of Mikhail Tswett, a Russian botanist. In 1901, Tswett sought to separate the pigments from plant leaves. He used a glass column filled with calcium carbonate as a stationary phase and passed a solution of plant pigments through it. Tsvet's work laid the foundation for liquid chromatography (Ettre 2003) (Box 1.1).

Tswett's work led to the term "chromatography," which he derived from the Greek words "chroma" (color) and "graphein" (to write), reflecting the initial use of the technique for separating plant pigments based on their colors.

Development of liquid chromatography

- **Column chromatography**: Tswett's work laid the foundation for column chromatography, which is still widely used today. Researchers further refined this technique, developing various stationary phases and mobile phases to separate a wide range of compounds.

- **Paper chromatography**: In the 1940s, Martin and Synge developed paper chromatography, which was a simpler and more accessible form of liquid chromatography. It found applications in fields like biochemistry (Box 1.2).
- **Birth of gas chromatography**: In the 1950s, Martin and Synge introduced gas-liquid chromatography. This technique used a gas as the mobile phase and a liquid stationary phase. It allowed for the separation of volatile compounds based on their interaction with te gas phase.

Advances in liquid chromatography

- **High-Performance Liquid Chromatography (HPLC)**: In the 1960s, HPLC was developed, which utilized high-pressure pumps and more advanced columns, allowing for greater separation efficiency and analysis of a broader range of compounds.

Modern chromatographic techniques

Chromatography has continued to evolve, with the development of specialized techniques and instrumentation, including:

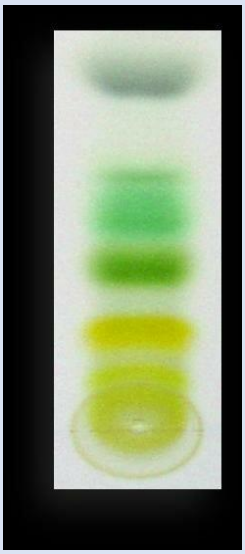
- **Ion Chromatography (IC)**: Introduced in the 1970s, IC is designed for the separation of ions and polar molecules.
- **Size Exclusion Chromatography (SEC)**: This technique, developed in the mid-20th century, separates molecules based on their size, making it valuable in biotechnology and materials science.
- **Affinity Chromatography**: Developed in the 1960s, it allows for highly specific separation by exploiting molecular interactions, such as antibody-antigen binding.
- **Advances in column technology**: Modern chromatographic columns, including those with different packing materials, have greatly improved resolution and speed.
- **Coupled techniques**: Chromatography has been combined with other analytical methods, such as mass spectrometry (LC-MS, GC-MS), further enhancing its capabilities for compounds identification.

In 1906 a Russian botanist Mikhail Tswett, an assistant at Warsaw University, presented a lecture at the meeting of the Biological Section of the Warsaw Society of Natural Sciences, titled “On a New Category of Adsorption Phenomena and Their Application to Biochemical Analysis.”

In this lecture, he discussed his wide-ranging investigations of leaf pigments performed during the previous couple of years. He made a $CaCO_3$ packed glass column as the stationary phase. $CaCO_3$ column is a solid adsorbent. Mobile phase used was petroleum ether.

After publishing his twin papers in 1906, Tswett continued to refine his chromatographic technique and conducted further research on various plant and animal pigments. He also introduced the chromatographic method to the German Botanical Society in Berlin, advocating its practical application.

In 1908, Tswett made the decision to consolidate all the accumulated knowledge into a book, which saw publication in 1910 by a Warsaw-based publisher. The book was titled "Chlorophylls in the Plant and Animal World." It also served as his thesis for attaining the Russian Doctor of Science degree.



Boxe 1.1. *Michel Tswett (1872-1919), Russian botanist and developer of the technique of chromatography (Ettre 2003).*

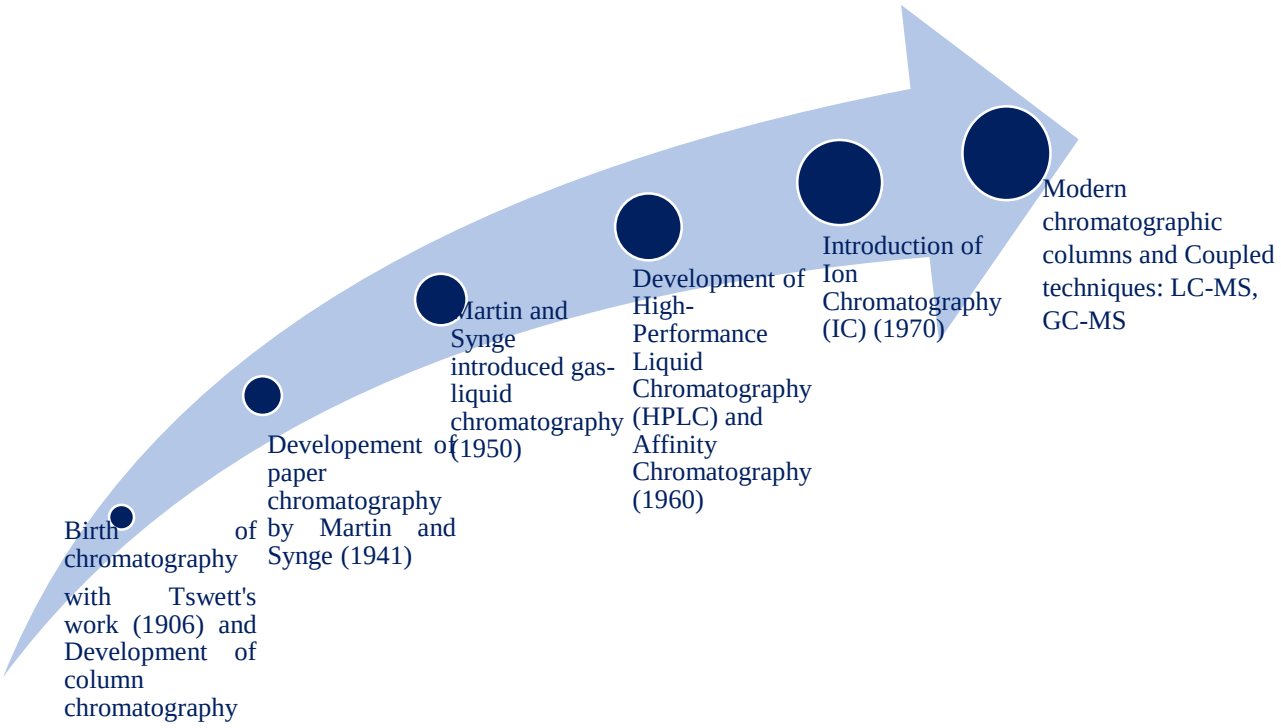


Figure 1.2. *Milestones in chromatography*

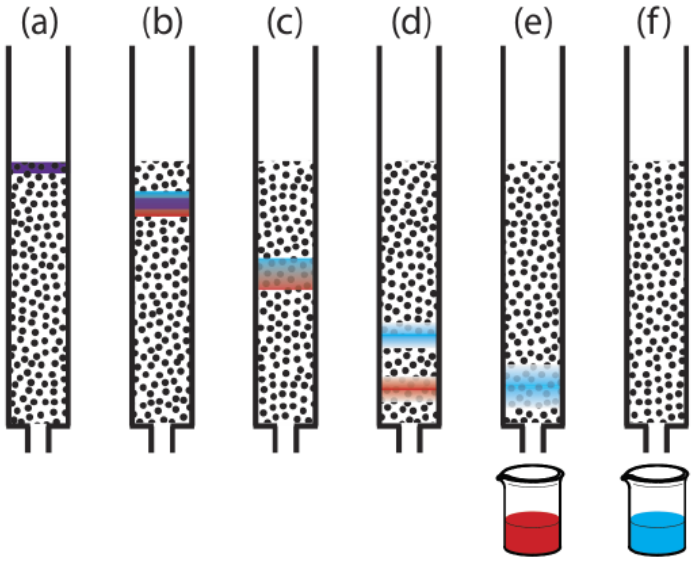


Figure 1. 3. *Evolution of a column chromatographic separation process for a two-component mixture.* ***(a)*** *the sample is initially applied on the stationary phase. As the mobile phase flows through the column, the sample divides into two solute bands* ***(b-d)****.* ***(e)*** *and* ***(f)*** *collect each solute as it elutes from the column (General Theory of Column Chromatography 2021).*

3. Importance of chromatography in various fields

The importance of chromatography lies in its ability to separate, identify, and quantify components within complex mixtures, making it an invaluable tool for quality control, research, and analysis in numerous industries and scientific disciplines. Its adaptability allows it to be tailored to specific analytical needs, from determining the purity of a drug to assessing the environmental impact of pollutants.

Pharmaceuticals

- *Drug development:* Chromatography is indispensable in the analysis of pharmaceutical compounds, ensuring the quality, purity, and efficacy of drugs during development.
- *Quality control*: It assists in quality control testing, to verify the consistency and purity of pharmaceutical products.

Environmental analysis

- *Pollutant detection*: Chromatography is used to detect and quantify environmental pollutants, helping in the monitoring and regulation of air, water, and soil quality.
- *Environmental research*: Researchers employ chromatography to study the fate and transport of chemicals in the environment.

Food industry

- *Food safety*: It ensures the safety and quality of food products by analyzing ingredients, additives, and contaminants.
- *Food flavor and aroma*: Chromatography helps in analyzing and developing flavors and aromas in the food industry.

Chemical industry

- *Process optimization*: Chromatography aids in optimizing chemical processes, enhancing product quality and purity.
- *New compound development*: It plays a vital role in developing and characterizing new chemical compounds.

Biotechnology

- *Biopharmaceutical production*: Chromatography is essential in the purification and analysis of proteins, nucleic acids, and other biomolecules used in biopharmaceuticals.
- *Bioprocess monitoring*: It's used to monitor and control bioprocesses for the production of biologics.

Forensic Science

- *Drug testing*: Chromatography is utilized for drug testing in forensic toxicology.

- *Criminal investigations*: It helps in analyzing physical evidence and identifying illicit substances.

Research and academia

- *Scientific research*: Chromatography is fundamental for scientific research, enabling the purification and analysis of compounds in various fields.
- *Teaching and training*: It's a common laboratory technique in academic institutions, teaching students essential analytical skills.

Clinical diagnostics

- *Clinical laboratories*: Clinical labs use chromatography for testing and diagnosing various medical conditions, including diabetes and metabolic disorders.

Material science

- *Polymer analysis*: Chromatography is used to analyze polymers, catalysts, and other materials, contributing to material development and characterization.
- *Nanomaterials*: It aids in characterizing nanoparticles and nanomaterials.

Petroleum industry

- *Crude oil analysis*: Chromatography helps in the analysis of crude oil and its products in the petroleum and petrochemical industries, aiding in refining processes.

4. Classification of chromatographic techniques

Chromatographic methods are classified into three ways:

4.1. Classification according to the physical nature of the phases

Depending on the nature of the mobile phase we distinguish:

- Liquid chromatography (LC)
- Gas chromatography (GC)
- Supercritical phase chromatography (SPC)

Depending on the nature of the stationary phase we distinguish:

- Liquid/solid chromatography (LSC)
- Liquid/liquid chromatography (LLC)
- Gas/solid chromatography (GSC)
- Gas/liquid chromatography (GLC)

4.2. Classification according to the technique involved

Depending on the technology involved, we distinguish:

- Column chromatography
- Surface chromatography (paper chromatography or thin layer chromatography)

4.3. Classification according to the retention mechanism

Chromatography is a powerful separation technique based on several fundamental principles. Understanding these principles is essential for successfully using chromatography in various applications.

Chromatographic separation principles

Chromatography relies on the differential partitioning of components within a mixture between two phases: the stationary phase and the mobile phase. This partitioning occurs due to the interactions between the components and the phases. Key separation principles include:

1. Partitioning

Partitioning is the distribution of components between the stationary and mobile phases.

Separation mechanism: Components that interact more strongly with the stationary phase spend more time within it, leading to differential retention and separation.

2. Adsorption

Adsorption is the adhesion of a component to the stationary phase surface.

Separation mechanism: Components with stronger adsorption to the stationary phase will be retained longer, leading to separation based on adsorption affinities.

3. Size exclusion

Exclusion refers to the exclusion of components from the stationary phase.

Separation mechanism: Components that are too large to enter the pores of the stationary phase will be excluded and elute faster, while smaller molecules enter the pores of the stationary phase and elute later, resulting in separation based on size.

4. Ion exchange

Ion exchange involves interactions between ions in the mobile phase and charged sites on the stationary phase.

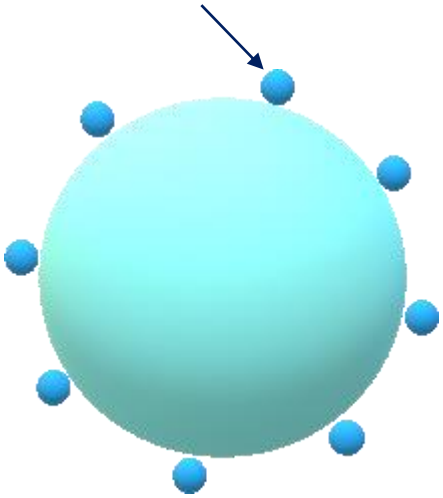
Separation mechanism: Components with different charge interactions will be retained differently, allowing for the separation of ions and charged molecules.

5. Affinity

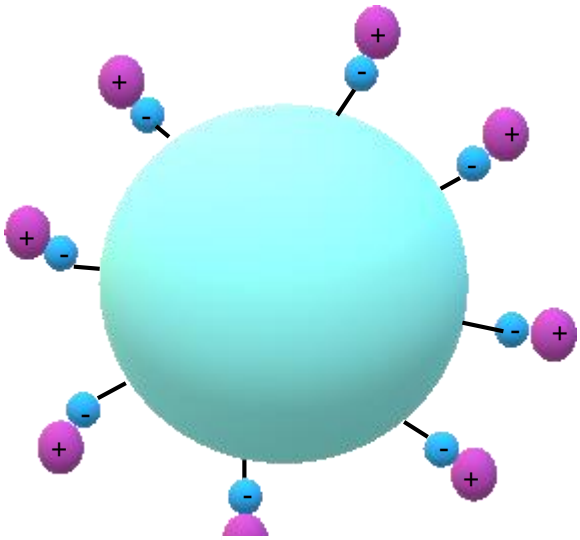
Affinity chromatography is based on highly specific interactions between a molecule of interest and a ligand attached to the stationary phase.

Separation mechanism: The molecule of interest binds selectively to the ligand, enabling purification or isolation (Figure 1. 4).

The analyte is adsorbed to the surface of the stationary phase



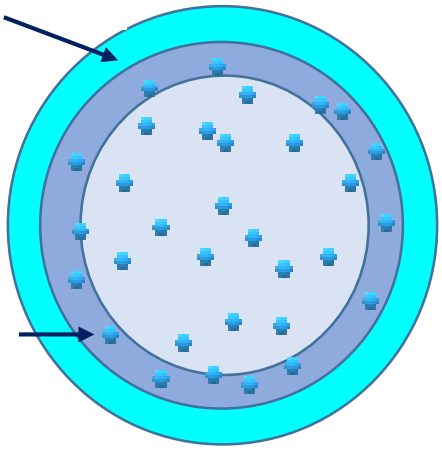
(a)



(b)

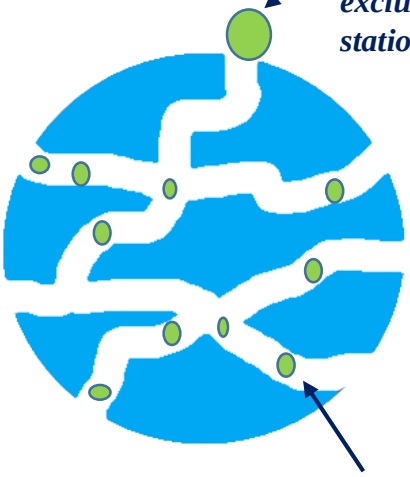
Cross-section of the open tubular column

Analyte dissolved in the liquid phase bound to the surface of the column



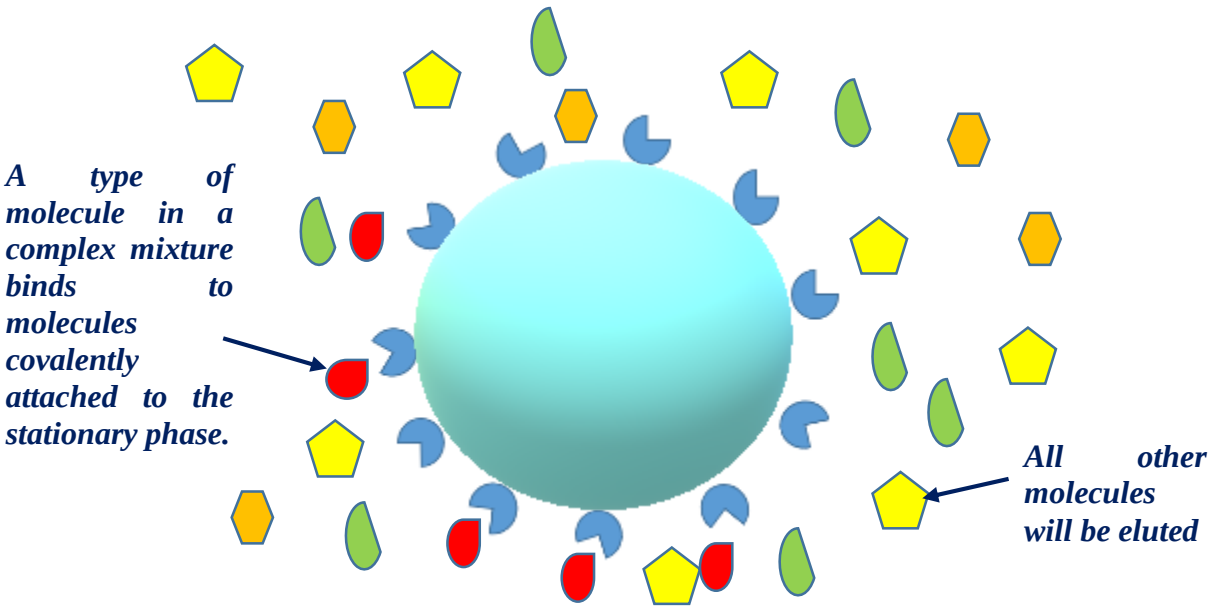
(c)

Large molecule excluded from the stationary phase



Small molecules penetrate the pores of the stationary phase

(d)



(e)

Figure 1. 4. *Five examples of interactions between an analyte and the stationary phase : (a) adsorption on a solide surface, (b) ions exchange, (c) liquid partioning, (d) exclusion, (e) affinity.*

5. Key terminology and concepts

To effectively navigate the world of chromatography, it's important to grasp key terminology and concepts:

5.1. Analyte

The substance or component of interest being separated and analyzed.

5.2. Stationary phase

The immobile material in which the analytes interact during separation.

5.3. Mobile phase

The fluid or gas that carries the analytes through the stationary phase.

5.4. Retention time (t_R)

The distance of the peak maxima from the injection point expressed in time units is called retention time (t_R). The time it takes for an analyte to move through the chromatographic system and elute from the column.

The retention time is characteristic of a species under specific analytical conditions and can be used for qualitative analysis. The area and height of a peak are proportional to the quantity of

the component. Retention time is independent of the injected sample quantity. It depends on the nature and velocity of the mobile phase.

5.5. Elution

The process of an analyte exiting the chromatographic column and becoming detectable.

5.6. Column efficiency

A measure of how well a column separates components; it depends on factors like column packing and particle size.

5.7. Chromatogramm

A chromatogram is a graphical or visual representation of the results obtained from a chromatographic separation. It is a critical output of chromatography, providing valuable information about the components in a mixture, their quantities, and their separation characteristics. Chromatograms are typically generated by detectors that record signals as analytes elute (exit) from the chromatographic column.

Chromatograms can be generated in various forms, depending on the chromatographic technique and the detector used. Common types of chromatograms include gas chromatograms (GC), liquid chromatograms (LC), and thin-layer chromatograms (TLC), each with its own specific format.

The chrmatogramm is used in both qualitative and quantitative analysis.

- *Qualitative analysis*: allows the identification of compounds by the position of the peak.
- *Quantitative analysis*: evaluate the concentration or mass of a compound using the peak area

5.8. Peak

A peak in the chromatogram represents an individual component of the sample. It is characterized by its retention time, peak width, and peak area.

5.9. Dead time

Dead time (t_M) or hold up time is the time taken by a compound that is not retained by the stationary phase of the column to travel from the inlet to the outlet of the column (or the time taken by the mobile phase to pass through the column).

5.10. Reduced retention time

Reduced retention time (t_R)' represents the time the solute spends in the stationary phase.

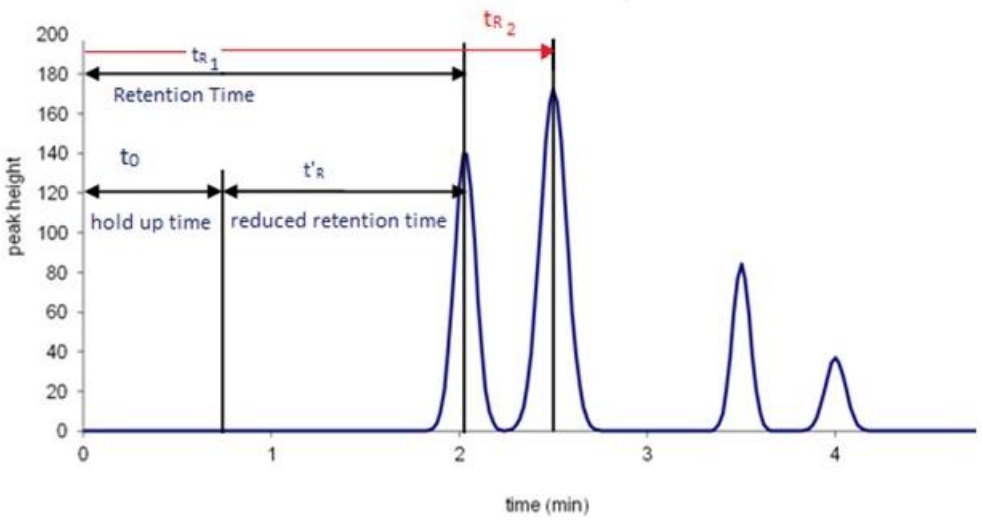


Figure 1. 5. *Chromatogram showing the retention time t_R, dead time of the column t_M, as well as the reduced retention time $t_{R'}$*

5.11. Distribution coefficient (K)

In liquid chromatography, separations are based on the difference in distribution of species between two immiscible phases, one stationary (solid particles impregnated or not with a liquid), the other mobile (liquid). For a given chromatographic system, the distribution coefficient K (or coefficient of sharing) is defined by :

$$\mathbf{K} = \frac{Cs}{Cm}$$

5.12. Retention factor (k') (Capacity factor)

When a compound is introduced into the column, its total mass mT is distributed into two quantities mM in the mobile phase and mS in the stationary phase. These quantities remain constant during its migration in the column. They depend on mT and K. Their ratio is fixed and is called retention factor:

$$\mathbf{k'} = \frac{ms}{mM} = \frac{Cs\,Vs}{CM\,VM} = \mathbf{K}\,\frac{Vs}{VM}$$ **K : Distribution coefficient**

With Vs: volume of the stationary phase which is calculated by the difference between the total volume of the column and the volume of the mobile phase (V_M).

k' is not a constant but varies with the operating conditions (temperature, composition of the mobile phase, etc.).

It is the most important parameter in chromatography to define the behavior of a column.

In order to prevent prolonging the analysis time, it is advisable to maintain k' values at moderate, avoiding excessively hight values.

k' can be determined directly from the chromatogram:

$$\mathbf{k'} = \frac{VR - VM}{VM} = \frac{VR'}{VM} = \frac{tR - tM}{tM}$$

5.13. Selectivity (α)

The ability of the chromatographic system to discriminate different analytes is called selectivity (α). Selectivity is determined as the ratio of the retention factors of two analytes, or the ratio of the reduced retention times.

$$\alpha = \frac{k2}{K1} = \frac{tR2 - tM}{tR1 - tM}$$

5.14. Retention volume

Given the constant flow rate (D) of the mobile phase, we define the retention volume (V_R).

$$\mathbf{V_R = D.t_R}$$

The volume of the mobile phase in the column, also known as the "dead volume," can be calculated from the chromatogram, provided that a solute not retained by the stationary phase is introduced. This parameter is expressed as:

$$\mathbf{V_M = D.\ t_M}$$

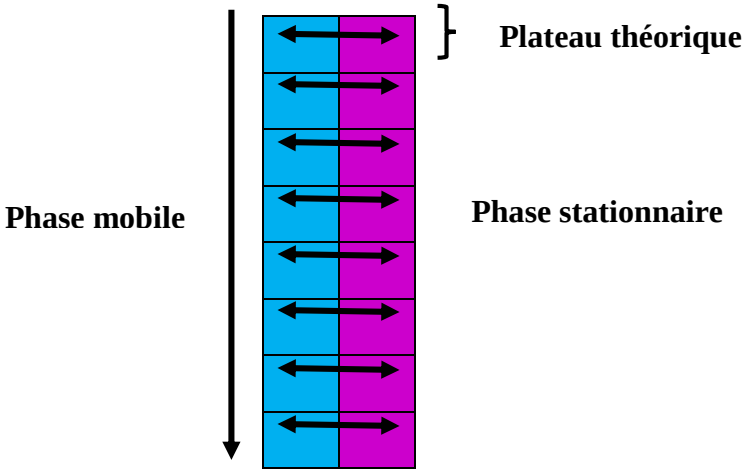
The volume of the stationary phase, designated as V_S, is calculated by subtracting the volume of the mobile phase from the total internal volume of the empty column.

5.15. Column efficiency (N)

The efficiency of a chromatographic column is measured, for each compound, by the number of theoretical plates (N) in the column and the height equivalent to a theoretical plate (H).

This theory originated from the quest for a static model to describe the operation of a chromatographic column, similar to that of a distillation column. Instead of considering the actual, continuous movement of the mobile phase, it is assumed to progress in discrete steps,

coming into equilibrium with the stationary phase between transfers. This allows the column to be conceptually divided into a certain number of zones in which equilibria are achieved, and these zones are referred to as theoretical plates.



The elution peaks can be compared to Gaussian curves. The geometric characteristics of the Gaussian curve (Fig. 6) allow to calculate, for a given solute, N from the chromatogram.

To express the efficiency of a column with a length L and N theoretical plates, the height H equivalent to a theoretical plate is defined.

To compare columns of different lengths, we define the height equivalent to a theoretical plate.

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

H is called the Height Equivalent to a Theoretical Plate (HETP).
In HPLC, the HETP ranges from 0.001 to 1 mm.

The efficiency of chromatographic columns increases if the number of theoretical plates increases or if the height equivalent to a theoretical plate decreases at constant length.
When you double the length of the column or the speed of the mobile phase, the retention time is multiplied

$$\mathbf{N= 16 \left(\frac{tR}{\omega}\right)^2}$$

ω: Peak width at the base

$$N = 5.54 \left(\frac{tR}{\delta}\right)^2$$

δ : Peak width at half height

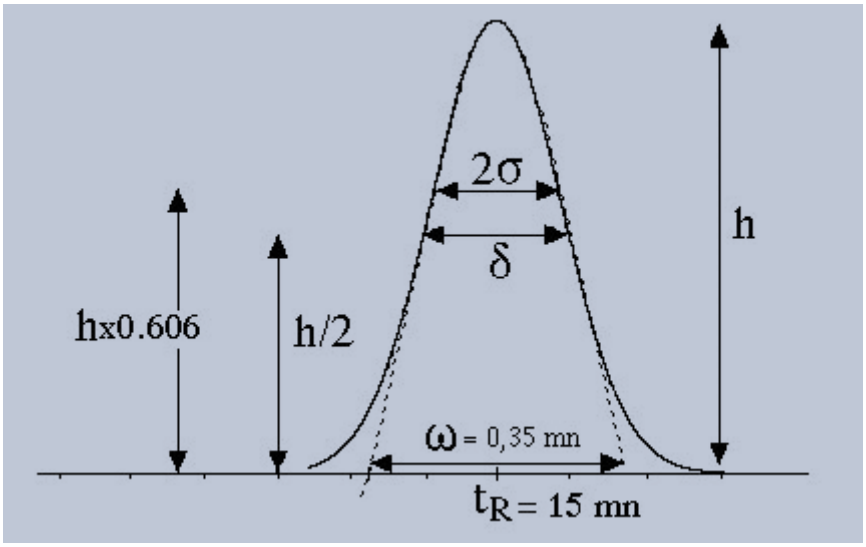


Figure 1. 6. *Gauss curve profile*

5.16 Resolution (R)

The resolution between two chromatographic peaks, R_{AB}, is a quantitative measure of their separation, and is defined as

$$R = 2 \left(\frac{tR2 - tR1}{\omega 1 + \omega 2}\right)$$

As shown in Figure 4, the separation of two chromatographic peaks improves with an increase in R.

- Two peaks are well resolved, if R ≥ 1.5
- For values of R larger than 1, the separation of the peaks is not better, but the separation time becomes unnecessarily long.
- Resolution is a measure of the quality of a separation and to optimize it, it is important to link the factors of selectivity and capacity to the resolution

Assuming ω1=ω2, by combining the expressions, we obtain the Purnell relationship:

$$R = \frac{1}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'_2}{(k'_2 + 1)} \cdot \sqrt{N_2}$$

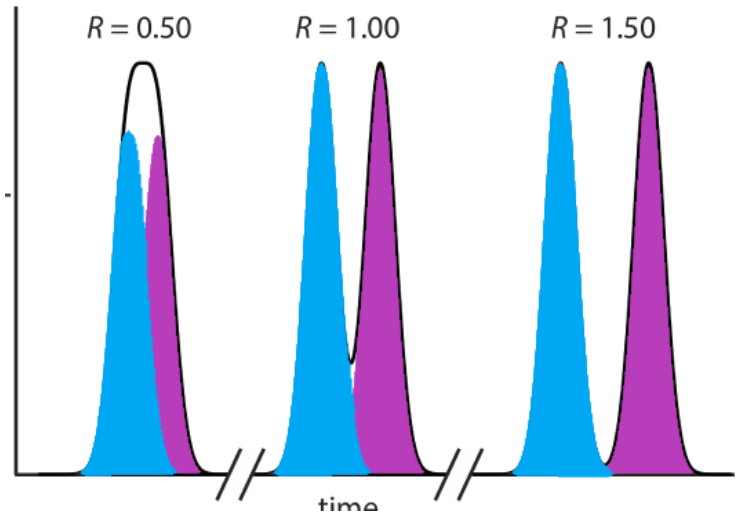


Figure 1. 7. *Relationship between resolution and the separation of two component mixture.*

6. Optimization of a chromatographic analysis

Optimization of a chromatographic analysis is a crucial aspect of analytical chemistry, particularly in high-performance liquid chromatography (HPLC) and gas chromatography (GC). The goal of optimization is to achieve the best separation and detection of analytes in a sample while minimizing analysis time and solvent usage.

Choice of column and stationary phase: The selection of the appropriate column and stationary phase is fundamental. Different columns and phases have varying selectivities, which can significantly impact separation. Consider factors such as analyte properties, matrix complexity, and required resolution. Mobile phase composition: The composition of the mobile phase, including solvent type and gradient conditions, is essential for achieving desired separation. Experimentation with different mobile phase compositions can help optimize results so that the selectivity factor is not too close to 1 and the capacity factors are between 1 and 10.

Applications

Application 1

In a chromatographic analysis of low molecular weight acids, butyric acid elutes with a retention time of 7.63 min. The column's void time is 0.31 min. Calculate the retention factor for butyric acid.

Application 2

In a chromatographic analysis of lemon oil, a peak for limonene has a retention time of 8.36 min with a baseline width of 0.96 min. γ-Terpinene elutes at 9.54 min with a baseline width of 0.64 min. What is the resolution between the two peaks?

Application 3

A chromatographic analysis for the chlorinated pesticide Dieldrin gives a peak with a retention time of 8.68 min and a baseline width of 0.29 min. Calculate the number of theoretical plates? Given that the column is 2.0 m long, what is the height of a theoretical plate in mm?

Application 4

Figure 1.8 is the chromatogram for a two-component mixture.

1. Determine the retention factor for each solute assuming the sample was injected at time $t = 0$.

2. For each solute in the chromatogram, calculate the number of theoretical plates and the average height of a theoretical plate. The column is 0.5 m long.

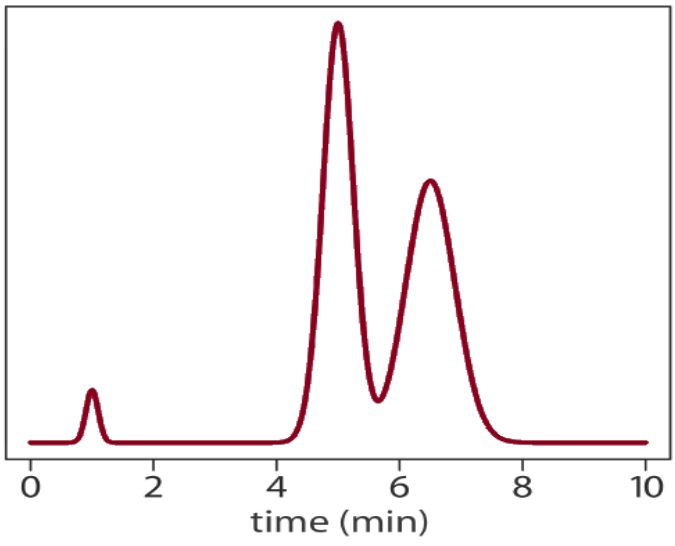


Figure 1. 8.

Chapter 2

Liquid Chromatography

CHAPTER

2

Liquid Chromatography

I. Planar chromatography

Paper and thin layer chromatography

Paper chromatography and thin-layer chromatography (TLC) are two common chromatographic techniques used for separation and analysis. These methods are particularly useful for separating and identifying compounds in mixtures. The resulting chromatogram is usually qualitative, it can only be used to identify substances.

1. Paper chromatography

The discovery of paper chromatography is attributed to the work of Synge and Martin in 1941 (Martin and Synge, 1941).

Paper chromatography is a chromatographic technique that employs paper sheets or strips as the stationary phase. In this method, a solution is passed through the paper, allowing the separation of dissolved chemical substances based on their distinct migration rates across the paper sheets. It is known for its cost-effectiveness and the ability to separate compounds with high precision. This analytical tool is particularly advantageous as it requires only minimal quantities of materials.

2. Theory and principles

The principle of paper chromatography is based on the differential affinities of compounds in a mixture for two phases: the stationary phase (the paper) and the mobile phase (the solvent).

Paper chromatography is a form of liquid chromatography where the basic principle involved can be either partition chromatography or adsorption chromatography. The compounds in the mixture get separated due to differences in their affinity towards water (in the stationary phase) and mobile phase solvents during the movement of the mobile phase. This mobile phase moves under the capillary action of pores in the paper.

The principle can also be adsorption chromatography between solid and liquid phases, wherein the stationary phase is the solid surface of the paper, and the liquid phase is the mobile phase. But most of the applications of paper chromatography work on the principle of partition chromatography (i.e., partitioned between two liquid phases).

3. Uses and applications of paper chromatography

Paper chromatography is a versatile and widely applicable technique that provides valuable information about the composition and characteristics of various substances. Its simplicity and low cost make it accessible for a wide range of applications in research, industry, and education (Figure 2.1). Here are some common uses and applications of paper chromatography:

- ***Separation of amino acids***: paper chromatography is frequently used to separate and analyze amino acids in biological and biochemical research. It is valuable for determining the composition of proteins and the presence of specific amino acids in a mixture.
- ***Separation of plant pigments***: in botany and plant biology, paper chromatography is used to separate and identify the pigments found in plant leaves, such as chlorophyll, carotenoids, and xanthophylls.
- ***Food analysis***: paper chromatography is employed in the food industry to analyze and detect various food components, such as food dyes, flavor compounds, and additives. It can help ensure the quality and safety of food products.
- ***Pharmaceuticals***: the technique is used for the analysis of pharmaceutical compounds, including the separation and identification of active ingredients and impurities in drugs and pharmaceutical formulations.
- ***Clinical chemistry***: paper chromatography is used in clinical laboratories for the separation and identification of various compounds in body fluids, such as urine and blood. It can be utilized in tests for amino acid disorders, metabolic diseases, and drug analysis in clinical settings.

- ***Environmental monitoring***: paper chromatography can be applied to analyze environmental samples to identify pollutants, such as pesticides, herbicides, and other contaminants in water and soil.
- ***Forensic science***: it has applications in forensic laboratories for analyzing drugs, poisons, and ink samples. It can help in identifying substances related to criminal investigations.
- ***Chemistry education***: paper chromatography is commonly used in educational settings to teach students about separation techniques, including the principles of chromatography and how to analyze mixtures.
- ***Art conservation***: in art conservation and restoration, paper chromatography can be used to analyze the composition of pigments in artworks and historical artifacts.
- ***Quality control***: various industries, including the chemical and manufacturing sectors, use paper chromatography as a quality control tool to assess the purity of chemical compounds and manufactured products.

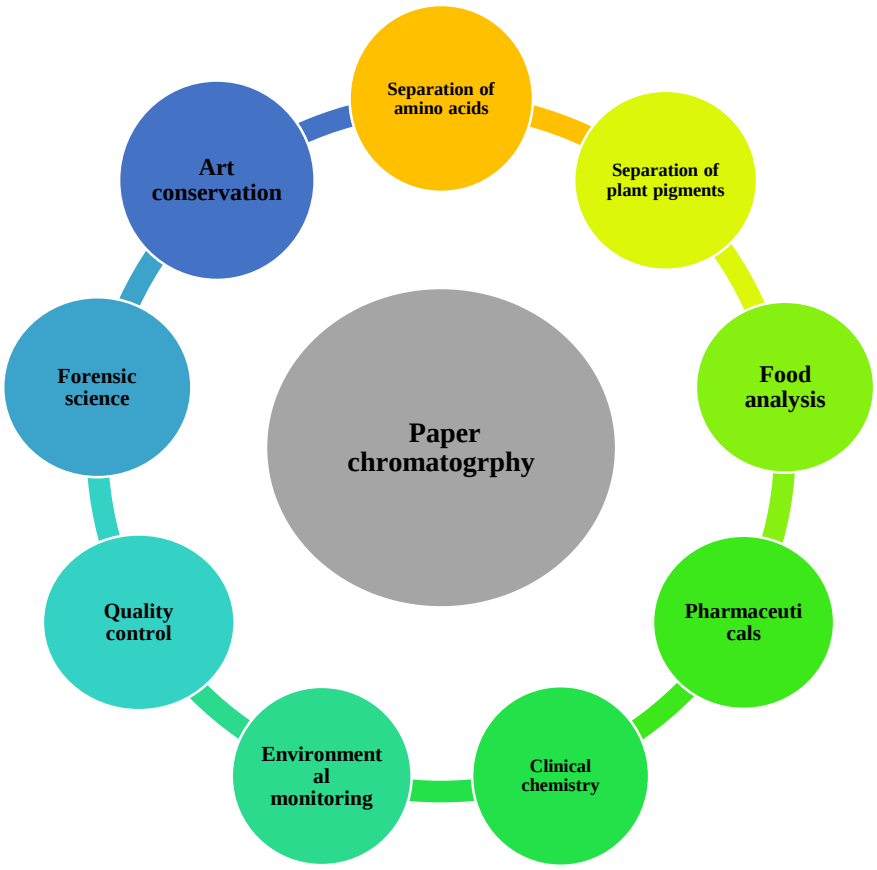


Figure 2.1. *Applications of paper chromatography*

4. Ascending paper chromatography

Ascending paper chromatography is particularly useful when working with samples that have limited solubility in the solvent or when separation is more efficient with the solvent moving upward. It is commonly employed in educational settings and in laboratories for the separation and analysis of a wide range of compounds, including amino acids, pigments, and various organic and inorganic substances.

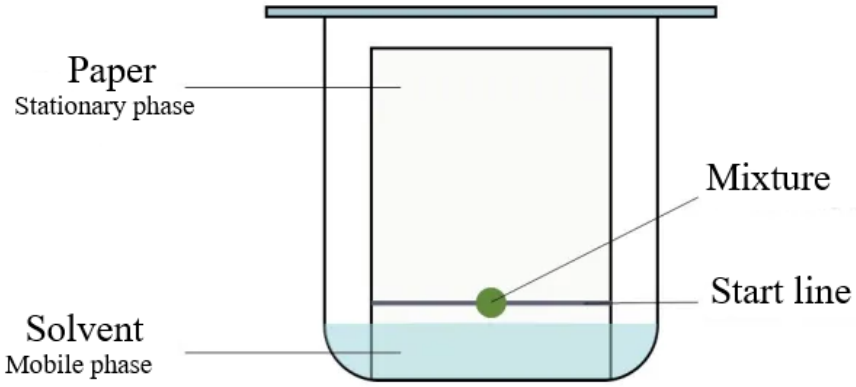
The key steps in ascending paper chromatography are as follows:

Preparation of the stationary phase

Begin by cutting a strip of chromatography paper, which is typically a special type of paper with high porosity that allows for efficient capillary action. The paper strip should be marked at the bottom with a pencil line where the sample will be applied.

Sample application

Apply a small spot or line of the mixture you want to separate on the marked line at the bottom of the paper strip. This is typically done using a capillary tube, micropipette, or similar applicator.

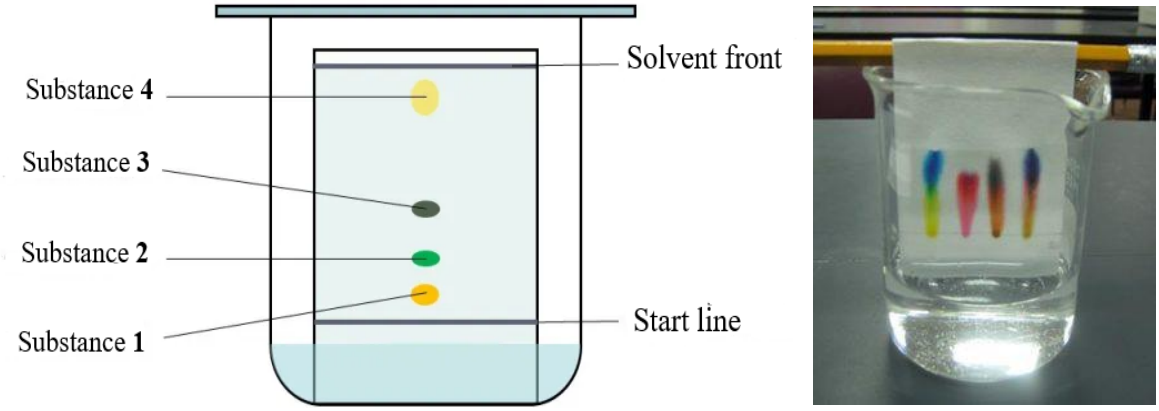


Absorbent paper on which the mixture has been dotted before the separation process

Development

In ascending paper chromatography, the bottom of the paper strip is immersed in a container with a solvent, which serves as the mobile phase. The solvent should only come into contact with the bottom of the paper strip, not the sample itself. Capillary action causes the solvent to move up the paper, carrying the sample components with it. As the solvent migrates upward, it

interacts with the components of the mixture, causing them to separate based on their affinity for the paper and the solvent.



A chromatogram with separated spots

Visualization

The visualization step in paper chromatography is crucial for identifying and observing the separated components of the mixture on the chromatogram. Once the solvent has traveled up the paper to the desired height or when sufficient separation is achieved, the paper strip is removed from the container.

The separated components can be visualized using various techniques, such as UV light, chemical reagents, or by observing color changes or fluorescence, depending on the nature of the compounds. Here are some common visualization techniques used in paper chromatography:

UV light: Some compounds exhibit natural fluorescence when exposed to ultraviolet (UV) light. In such cases, you can visualize the separated spots or bands by placing the chromatogram under a UV lamp. The compounds that fluoresce will appear as bright spots against a dark background.

Iodine vapor: Iodine vapor is commonly used for visualizing non-fluorescent or weakly fluorescent compounds. Place the chromatogram in a closed container with a source of iodine, such as iodine crystals, and allow the vapor to interact with the paper. The compounds will react with iodine, resulting in dark spots or bands.

Chemical reagents: Specific chemical reagents can be sprayed or applied to the chromatogram to react with the separated compounds, producing color changes or visible spots. For example,

ninhydrin can be used to detect amino acids, and Dragendorff's reagent is used to detect alkaloids.

***Analysis*:** The retention factor (R_f) values can be calculated to describe the relative distances traveled by the components from the starting line. This can help in comparing and identifying the compounds.

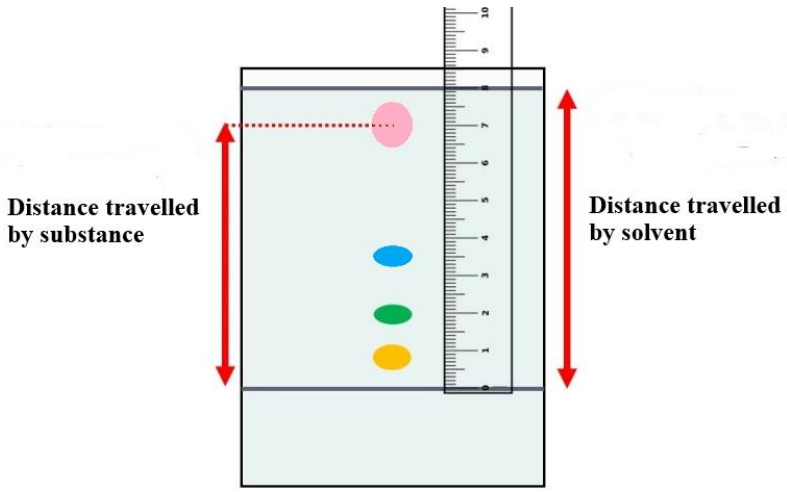
How to calculate R_f value in chromatography?

The substances can be identified by calculating the ratio of the distance they migrated compared to the distance travelled by the solvent. This measurement is known as the Rf (retention factor) value. The formula for calculating it is:

$$\mathbf{R_f} = \frac{\textit{Distance travelled by a substance}}{\textit{Distance travelled by the solvent}}$$

Here are a few important points to keep in mind when calculating R_f values:

- Rf values are specific to the particular chromatographic conditions, including the type of paper, solvent, and environmental factors. Therefore, they are useful for relative comparisons within the same experiment but may not be directly comparable between different experiments.
- Rf values are commonly used for compound identification by comparing them to known Rf values for standard compounds under the same chromatographic conditions.
- In cases where you have multiple compounds on the chromatogram, each compound will have its own unique Rf value, allowing for easy identification and comparison.



2. Thin Layer Chromatography (TLC)

Thin layer chromatography, or TLC, is a chromatographic separation technique that employs a thin layer of adsorbent material, such as silica gel or alumina, coated onto a flat, solid support (often glass, plastic, or aluminum). This thin layer acts as the stationary phase on which the separation takes place.

1. Theory and principles

TLC operates on the same fundamental principles as other chromatographic methods, which involve the distribution of compounds between a stationary and a mobile phase. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast (Thin Layer Chromatography, 2022).

2. Thin layer chromatography procedure

The diagram below illustrates the complete thin-layer chromatography process.

Sample application

A small amount of the mixture to be separated is applied as a spot or line near the bottom of the TLC plate. This is often done using a capillary tube or micropipette.

Development

The bottom of the TLC plate is placed in a container with a solvent, which acts as the mobile phase.

TLC development is usually performed in chambers. Depending on the application and goals, there are various chambers available, differing in materials and chromatographic results. For instance, chambers for ascending chromatography are made of glass and have glass or stainless steel lids, while those for horizontal TLC are made of PTFE and have glass lids.

As the solvent moves up the plate through capillary action, it carries the sample components with it. Compounds within the mixture will interact differently with the stationary phase (the adsorbent) and the mobile phase, leading to separation based on their affinities.

Visualization

After the solvent has traveled to a desired height or when the separation is complete, the TLC plate is removed from the container. The separated components can be visualized using various techniques, including UV light, chemical reagents, or by observing color changes or fluorescence.

R_f value calculation

The Rf (retention factor) value is calculated to describe the relative distances traveled by the components from the starting line.

The Rf value is calculated using the formula:

$$\mathbf{R_f} = \frac{\boldsymbol{Distance\ travelled\ by\ a\ substance}}{\boldsymbol{Distance\ travelled\ by\ the\ solvent}}$$

3. Plates (Stationary phase)

TLC plates can be prepared in the lab, but are most commonly purchased. Silica gel and alumina are among the most common stationary phases, but others are available as well. Many plates incorporate a compound which fluoresces under short-wave UV (254 nm). The backing of TLC plates is often composed of glass, aluminum, or plastic. Glass plates are chemically inert and best withstand reactive stains and heat, but are brittle and can be difficult to cut. Aluminum and plastic plates can be cut with scissors, but aluminum may not withstand strongly acidic or oxidizing stains, and plastic does not withstand the high heat required to develop many stains. Aluminum and plastic plates are also flexible, which may result in flaking of the stationary phase. Never under any circumstances touch the face of a TLC plate with your fingers as contamination from skin oils or residues on gloves can obscure results.

4. Solvent (Mobile phase)

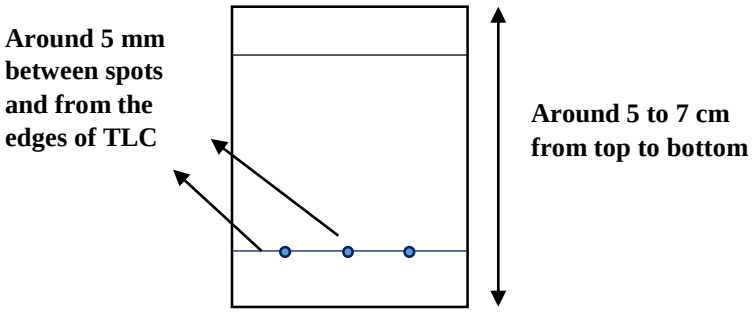
The selection of an appropriate solvent is the most critical factor in TLC, and finding the optimal solvent may involve some trial and error. Similar to the consideration of plate selection, it is important to take into account the chemical properties of the analytes.

A commonly used initial solvent is hexane and ethyl acetate 1:1. Adjusting the ratio can significantly influence the Rf values. Rf values range from 0 to 1, where 0 indicates very low solvent polarity, and 1 indicates very high solvent polarity.

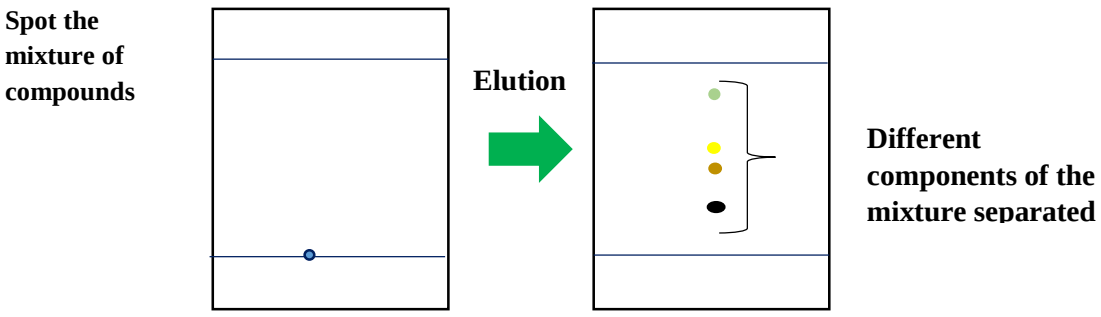
In your experimental setup, it is undesirable for your values to be exactly 0 or 1 because the components you are separating likely have different polarities. If the value is 0, you should increase the solvent polarity as the sample is not moving and adhering to the stationary phase.

Conversely, if the value is 1, you should decrease the solvent polarity because the compound was unable to separate.

Acids, bases, and strongly polar compounds often produce streaks rather than spots in neutral solvents. Streaks make it difficult to calculate an R_f and may occlude other spots. Adding a few percent of acetic or formic acid to the solvent can correct streaking with acids. Similarly for bases, adding a few percent triethylamine can improve results. For polar compounds adding a few percent methanol can also improve results (Thin Layer Chromatography, 2022).



Typical TLC size and arrangment

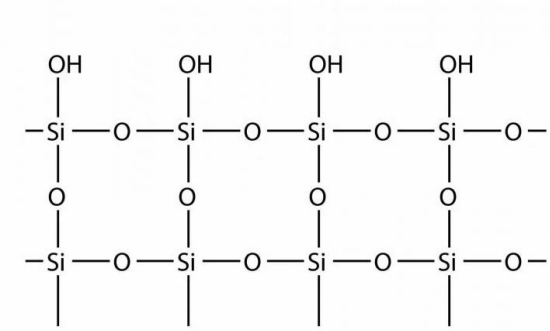


Spot the TLC mixtures at the corresponding mark in the line above the bottom of the plate.

What's going on at the molecular level during development?

TLC plate consists of a thin plastic sheet covered with a thin layer of silica gel, a portion of the structure of which is shown below.

Silica gel consists of a three-dimensional network of thousands of alternating silicon and oxygen bonds, with O-H groups on the outside surface. Silica gel is highly polar and is capable of hydrogen bonding.



As the solvent ascends the plate, passing over the spot, an equilibrium is established, where the development solvent competes with the TLC plate for the solute. The silica gel adheres to the solute, while the development solvent attempts to dissolve it, transporting the solute(s) as the solvent moves upward on the plate.

A balance of intermolecular forces determines the position of equilibrium and thus the ability of the solvent to move the solute up the plate.

If a sample consists of two components, one more polar than the other, the more polar will tend to stick more tightly to the plate and the less polar will tend to move along more freely with the solvent.

5. Applications of TLC

TLC has a wide range of applications (Figure 2.2), including:

Purity assessment: It is commonly used to determine the purity of a compound by comparing the number of spots or the intensity of spots in a sample to those in a reference substance.

***Compound identification*:** TLC can be used for the preliminary identification of compounds by comparing the Rf values of the sample components to known reference substances.

Reaction monitoring: Chemists use TLC to monitor the progress of chemical reactions, ensuring that reactants have been converted into products or to identify the appearance of intermediates.

Forensic analysis: In forensic science, TLC is used to analyze substances found at crime scenes, identify drugs and toxins, and match substances to reference samples.

Pharmaceutical analysis: TLC is employed in the pharmaceutical industry for quality control, assessing the purity of drug formulations, and identifying impurities.

Botanical analysis: TLC is used to identify the presence of various compounds in plant extracts, including alkaloids, flavonoids, and terpenes.

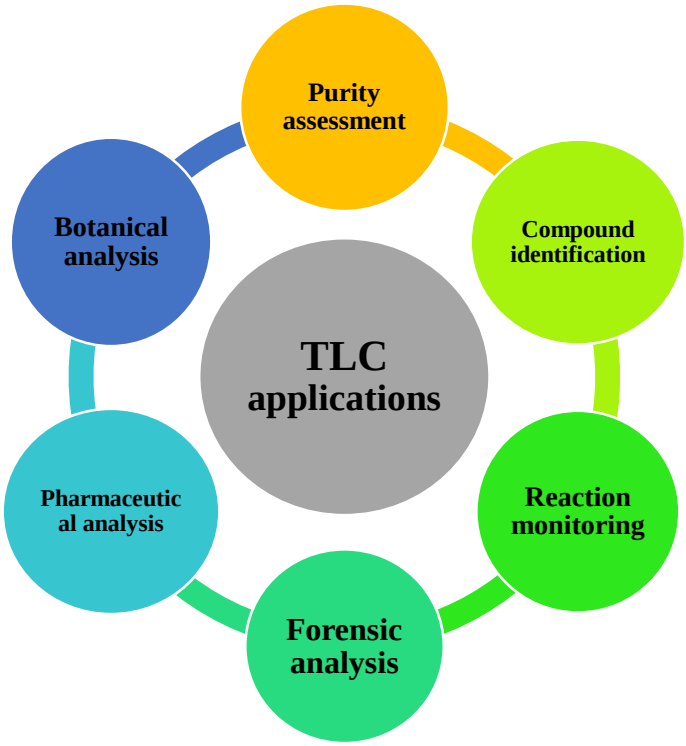


Figure 2.2. *Applications of thin layer chromatography (TLC)*

6. Advantages of TLC

TLC is a cost-effective and user-friendly technique that requires minimal materials, including a chamber, watch glass, capillary, plate, solvent, pencil, and UV-light. Once the optimal solvent is identified, it can be transferred to other techniques like High-Performance Liquid Chromatography (HPLC). Multiple compounds can be separated on a single TLC plate as long as each compound is compatible with the chosen mobile phase. The solvents for the TLC plate are easily interchangeable, allowing the use of different solvents for varied results. Purity assessment is straightforward with UV-light, and the identification of most compounds can be achieved by comparing Rf values to literature values. Chromatography conditions can be easily adjusted to enhance the optimization for the resolution of a specific component (Touchstone, 1993).

3. Reversed phase Thin Layer Chromatography (RP-TLC)

Reversed-Phase Thin-Layer Chromatography (RP-TLC) is a chromatographic technique that separates and analyzes compounds based on their hydrophobicity or lipophilicity. Unlike traditional normal-phase TLC, where the stationary phase is polar and the mobile phase is non-polar, RP-TLC involves a reversed setup. In RP-TLC, the stationary phase is non-polar, consisting of a hydrophobic layer, while the mobile phase is polar.

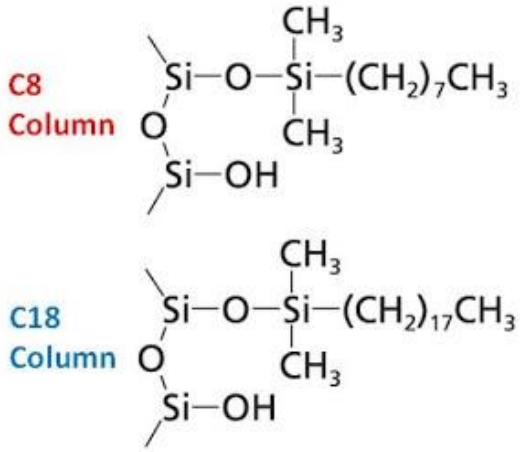
1. Theory and principles

Stationary phase

- The stationary phase in RP-TLC is a non-polar material, such as a silica gel modified with hydrophobic groups like C18 or C8 (octadecyl or octyl chains).
- The hydrophobic interactions between the non-polar stationary phase and analytes drive the separation.

Mobile phase

- The mobile phase in RP-TLC is polar, often composed of a mixture of water and an organic solvent (e.g., methanol or acetonitrile).
- The composition of the mobile phase influences the elution of analytes.



2. Steps in RP-TLC

Sample application

Samples are applied as small spots near the base of the TLC plate, and then developed in a closed chamber with the mobile phase.

Development

The mobile phase migrates up the TLC plate through capillary action, carrying the sample components with it. Hydrophobic analytes interact more with the non-polar stationary phase and move more slowly, resulting in separation.

Visualization

After development, the plate is usually dried, and the separated compounds are visualized using techniques such as UV light or specific staining reagents.

4. Analytical and preparative Thin-Layer Chromatography (TLC)

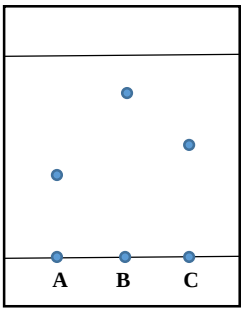
Analytical Thin-Layer Chromatography is a chromatographic technique primarily used for qualitative and quantitative analysis of complex mixtures. It involves the separation of components based on their relative affinities for the stationary and mobile phases.

Preparative Thin-Layer Chromatography is an extension of analytical TLC and is specifically designed for the isolation and purification of larger quantities of compounds. It is widely used in the isolation of pure compounds from complex mixtures.

Applications

Application 1

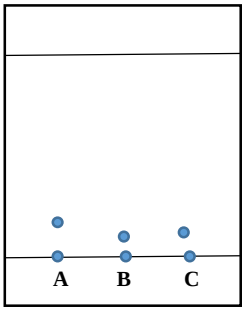
Consider the following silica gel TLC plate of compounds A, B, and C developed in hexane.



1. Determine the R_f values of compounds A, B, and C run on a silica gel TLC plate using hexanes as the solvent.
2. Which compound, A, B, or C, is the most polar?
3. What would you expect to happen to the R_f values if you used acetone instead of hexane as the eluting solvent?

Application 2

You are trying to determine a TLC solvent system which will separate the compounds A, B, and C. You ran the compounds on a TLC plate using hexanes/ethyl acetate 95:5 as the eluting solvent and obtained the chromatogram below. How could you change the solvent system to give better separation of these three compounds?



Application 3

When water is included in the composition of the mobile phase, are we working in reverse phase or normal phase?

II. Column chromatography

1. Principles

Column chromatography is a widely employed technique for the separation and purification of compounds. It is based on the differential interaction of compounds with a stationary phase and a mobile phase as they move through a vertical column.

This method is employed in various scientific disciplines, from organic chemistry to biochemistry, for the purification and isolation of compounds.

Stationary phase

The stationary phase is a solid material packed into a column, often silica gel or alumina. The choice of stationary phase depends on the nature of the compounds being separated.

- *Silica gel (SiO2)*

It is presented in a white powder, particularly used to separate organic compounds that don't have sufficient stability to be separated on alumina. The particle size of an adsorbent used in column chromatography is larger than that of an adsorbent used in TLC (80 to 200 µm instead of 70 µm).

- *Alumina (Al_2O_3)*

. This adsorbent in acidic, basic, or neutral forms, can only be used with stable organic compounds (acidic alumina causes dehydration of tertiary alcohols, while basic alumina leads to the hydrolysis of esters). Alumina is a polar adsorbent; the most polar component will be strongly retained, and it is the least polar component that will be eluted first.

Mobile phase

The mobile phase is a liquid solvent or a mixture of solvents that flows through the column. The sample mixture is introduced onto the column, and as the mobile phase percolates through the stationary phase.

The commonly used eluent is a mixture of two solvents. At the beginning of elution, the process starts with the less polar solvent, which carries the least polar components. Subsequently, the eluent's polarity is increased by gradually adding the more polar solvent, thus eluting the more polar components. It is necessary to conduct tests on TLC with different eluents to achieve a good separation between products.

2. Process of column chromatography

Sample loading

The sample is carefully loaded onto the top of the column. It can be dissolved in the mobile phase or applied as a dry powder.

Elution and separation

The mobile phase is allowed to flow through the column, carrying the sample components with it. As the components interact differently with the stationary phase, they separate along the column.

Fraction collection

Fractions are collected at regular intervals as the elution progresses. Each fraction represents a different component of the mixture.

Analysis

The collected fractions are analyzed using various techniques such as spectroscopy or chromatography to identify and quantify the separated compounds (Figure 2.3) (General Theory of Column Chromatography 2021).

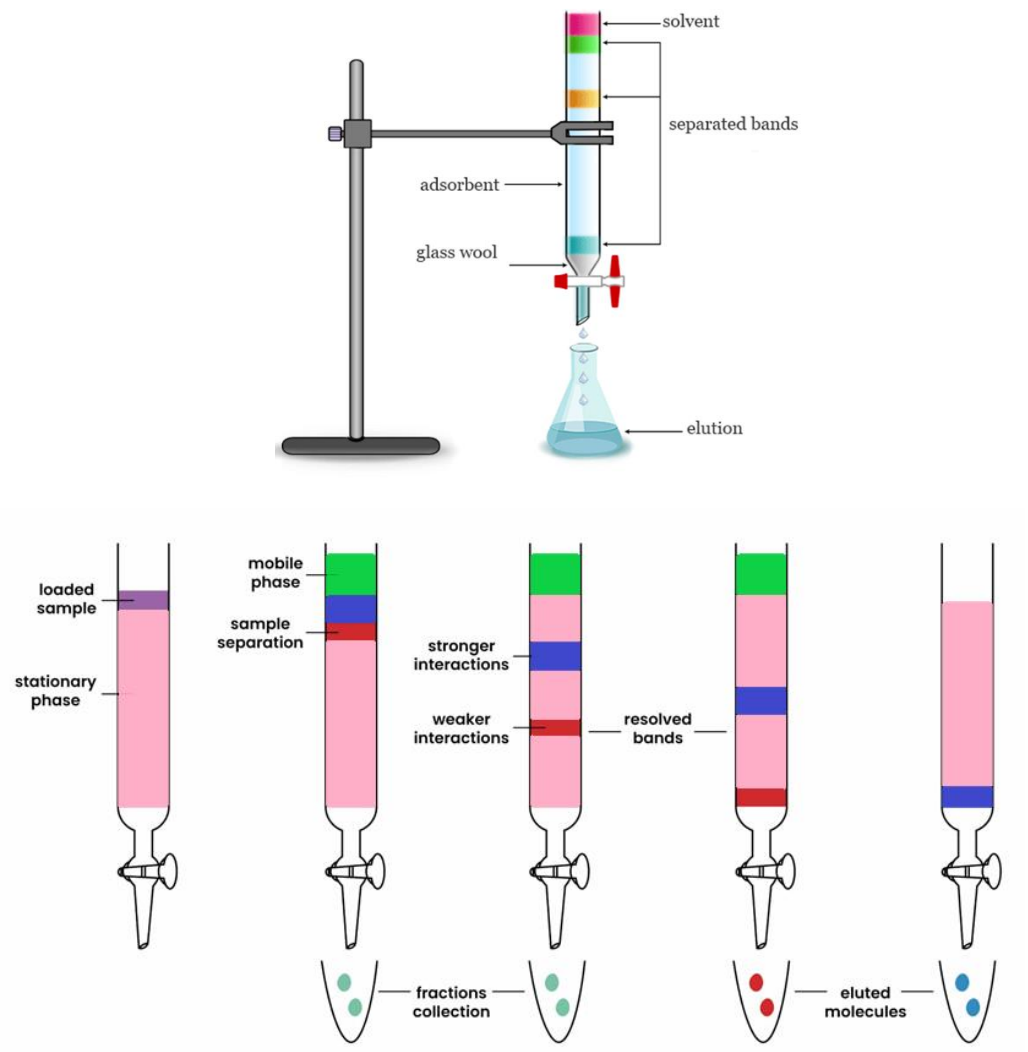


Figure 2.3. *Different steps of column chromatography process*

Chapter 3

High Performance Liquid Chromatography

CHAPTER

3

High Performance Liquid Chromatography

I. Size Exclusion Chromatography (SEC)

1. Definition

Size exclusion chromatography, also known as size exclusion chromatography (SEC) or gel filtration chromatography, is a powerful and widely used separation technique based on the size and molecular weight of analytes. This method is particularly effective for biomolecules, polymers, and other macromolecules.

2. Principles of exclusion

The stationary phase in exclusion chromatography is a porous gel matrix, often composed of cross-linked polymers. The gel consists of beads with specific pore sizes, allowing molecules to enter or be excluded based on their size.

Larger molecules are excluded from the pores and, therefore, have a shorter path through the column, resulting in faster elution.

Smaller molecules enter the pores and take a longer path, resulting in slower elution.

The mobile phase is a liquid buffer that permeates the gel matrix. Analytes of different sizes interact differently with the gel, leading to their separation.

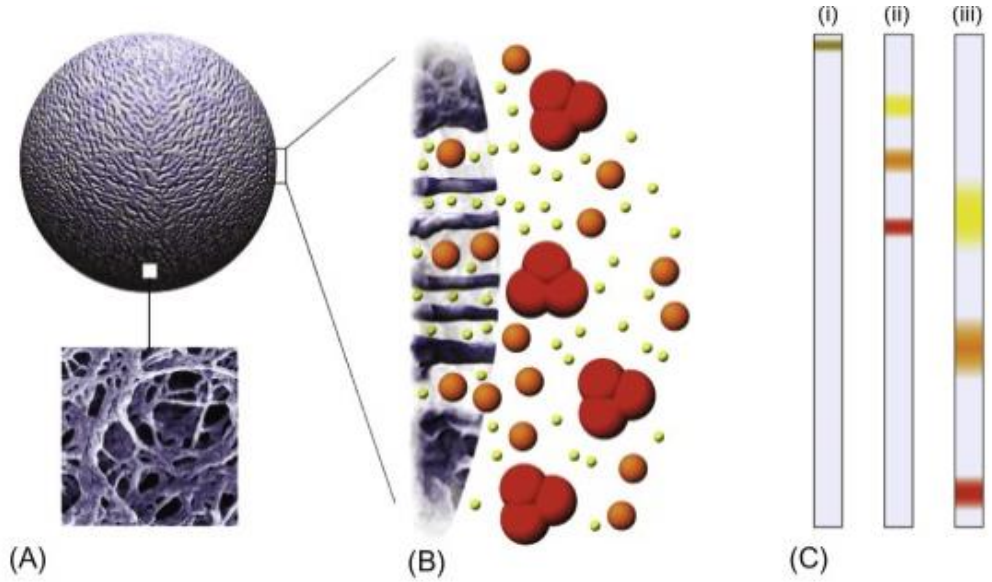


Figure 3.1. *(A) Schematic picture of a chromatography resin bead with an inserted electron microscopic enlargement. (B) Schematic drawing of sample molecules diffusing into or being excluded from the bead pores. (C) Graphical description of separation: (I) sample applied on the column; (II) the smallest molecule (yellow) is more delayed than the largest molecule (red); (III) the largest molecule is eluted first from the column (Hall 2018).*

3. Process of size exclusion chromatography

Sample loading

The sample containing a mixture of molecules is applied to the top of the column filled with the porous gel.

Elution

The mobile phase is allowed to flow through the column, carrying the sample with it. Larger molecules are excluded, while smaller ones enter the pores.

Elution profile

As the mobile phase progresses, molecules elute at different times, creating an elution profile. Larger molecules elute earlier, and smaller ones later.

Fraction collection

Fractions are collected at different elution times, and each fraction represents a group of molecules with similar sizes.

The results of SEC experiments are commonly presented in the form of an elution profile or chromatogram, depicting the changes in the concentration of eluted sample components. For

proteins, this is often represented by UV absorbance at 280nm, showcasing their elution from the column based on their apparent size (Figure 3.2 and 3.3).

The total volume of a column CV, can be divided in different partial volumes.

- Molecules that are too large to enter pores in the matrix are eluted together in the void volume, V_0, as they pass directly through the column at the same speed as the flow of eluent.
- Molecules with partial access to the pores of the matrix, elute from the column in order of decreasing size, that is, the smaller the molecule, the larger the accessible pore volume and the later the elution.
- Small molecules that have full access to the intraparticle pores, move through the column, but do not separate from each other. These molecules elute at the total liquid volume $CV-Vs=Vt$ such as Vs : the volume of the solid matrix.
- The part of the column volume which is accessible for each component is expressed in terms of the component elution volume, Ve, which is measured/calculated from the chromatogram.
- Ve is an intrinsic property of a sample component specific to a given SEC resin and sample within a column of fixed dimensions. Therefore, it cannot be directly applied for scale-up purposes as it varies with the total volume of the packed bed and the quality of column packing. Hence, for scale-up or comparative assessments, the elution of a component is more accurately characterized by a distribution coefficient, Kd. Kd remains unaffected by column dimensions, facilitating comparisons and predictions between columns of different sizes when utilizing the same resin and sample. The distribution coefficient, Kd, for a specific sample component is defined as the fraction of the total intraparticle pore volume, Vi, that is accessible to that particular component. The calculation of Kd is as follows:

$$K_d = \frac{Ve - V0}{Vt - V0} = \frac{Ve - V0}{Vi}$$

where Ve is the component elution volume, V0 is the column void volume, Vt is the total liquid volume, and Vi is the intra-particle pore volume.

Since in practice, Vt, and therefore Vi, can be difficult to estimate due to weak interactions between small probes and groups on the surface of pores in the beads of the stationary phase, it is more convenient to employ the term $(CV-V0)$ in calculations of the distribution coefficient. The distribution coefficient thus achieved is designated K_{av} (Hall, 2018).

$$K_{av} = \frac{Ve - V0}{CV - V0}$$

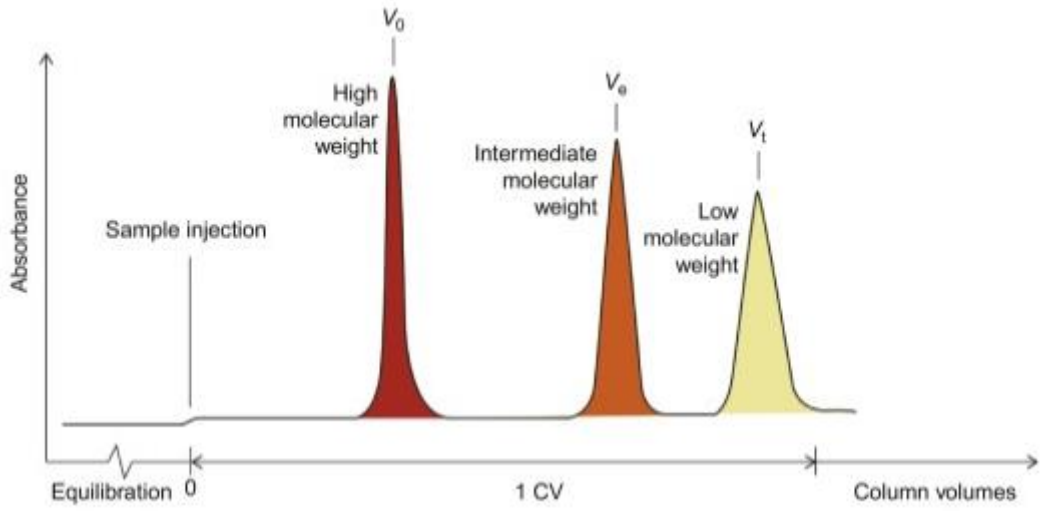


Figure 3.2. *Hypothetical chromatogram (Hall 2018)*

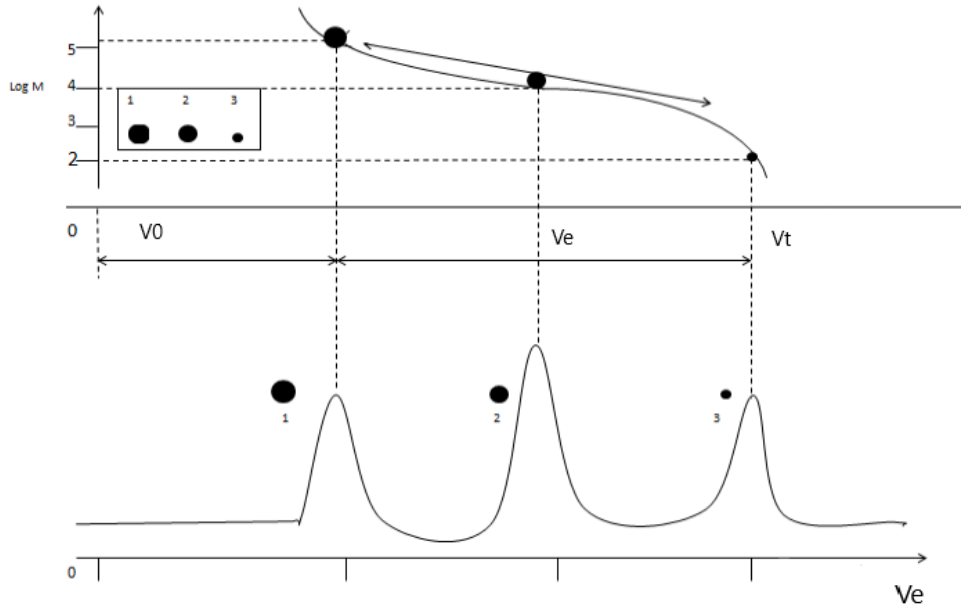


Figure 3.3. *Log (M) curve against the elution volume*

4. Applications of size exclusion chromatography

Applications of size exclusion chromatography (SEC) can be categorized into two primary types based on the purpose and complexity of the separation:

1. *Buffer exchange/group separation*

This application is suitable when there is a substantial difference in sizes between the target molecule and other molecules that need to be either removed or exchanged. The goal is often to isolate or purify a specific target from a mixture. It is preferable to use a concentrated sample in this scenario to enhance productivity. The sample volume is typically limited to approximately 15%–30% of the column volume (CV) in buffer exchange/group separation.

Example group separation -virus

The example in Figure shows a group separation of cell culture-derived influenza virus from impurities (Hall, 2018). The virus particles are much larger than the impurities, which makes group separation possible. The porosity of the resin will allow salts and the bulk of the impurities to eter the pores, while the large virus particles are excluded.

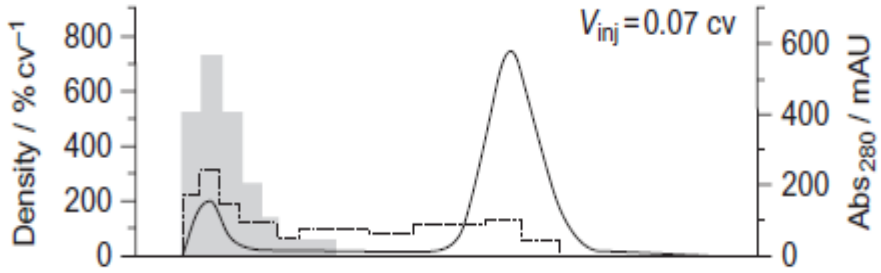


Figure 3.4. *Loading study of an agarose based SEC resin using concentrated influenza virus preparation. Column was loaded with 7% of the CV. Eluates were analyzed for hemagglutinin (HA) activity (shaded) and total protein (dotted line) and UV absorbance at 280nm (solid line) (Hall, 2018).*

***2.** Fractionation*

It is employed when molecules of similar sizes need to be separated. This application is more challenging as it requires the differentiation of molecules with close size ranges. Similar to buffer exchange/group separation, a concentrated sample is preferred to increase productivity. However, in fractionation, the sample volume is even more restricted, typically ranging from about 2%–6% of the column volume (CV) (Hall, 2018).

In both cases, SEC is a versatile chromatographic technique used for size-based separations. It is applied in various fields, including biochemistry, biotechnology, pharmaceuticals, and environmental sciences. The choice between buffer exchange/group separation and fractionation depends on the specific objectives of the separation and the

characteristics of the molecules involved. The concentration of the sample plays a crucial role in optimizing the efficiency and productivity of the SEC process.

This technique is also applied to the fractionation of mixtures of macromolecules and the determination of protein molecular weight. There is a linear relationship between the elution volume and the logarithm of the molecular weight. In the latter case, the column needs to be first calibrated with proteins of known molecular weight, plotting the log(M) curve against the elution volume, and then performing a graphical determination.

II. Ion Exchange Chromatography (IEC)

1. Definition

Ion exchange chromatography (IEC) is a liquid chromatography used for the separation and purification of proteins, peptides, nucleic acids, and other charged biomolecules based on their net charge. This chromatographic method exploits the electrostatic interactions between charged biomolecules and charged groups on the stationary phase.

2. Principle

Ion exchange chromatography employs a stationary phase consisting of ion exchange resins, which are typically made of cross-linked polymers with charged functional groups. The two main types of ion exchange resins are :

- *Anion exchange resin*: It is composed of positively charged functional groups, such as quaternary ammonium groups. These resins attract and bind negatively charged biomolecules (anions) from the sample.
- *Cation exchange resin:* It has negatively charged functional groups, such as sulfonic acid groups. They attract and bind positively charged biomolecules (cations) from the sample.

Thus, charged molecules can adsorb reversibly on the ion exchanger and then be desorbed from the resin, altering the ionic composition of the solvent (Figure 3.5).

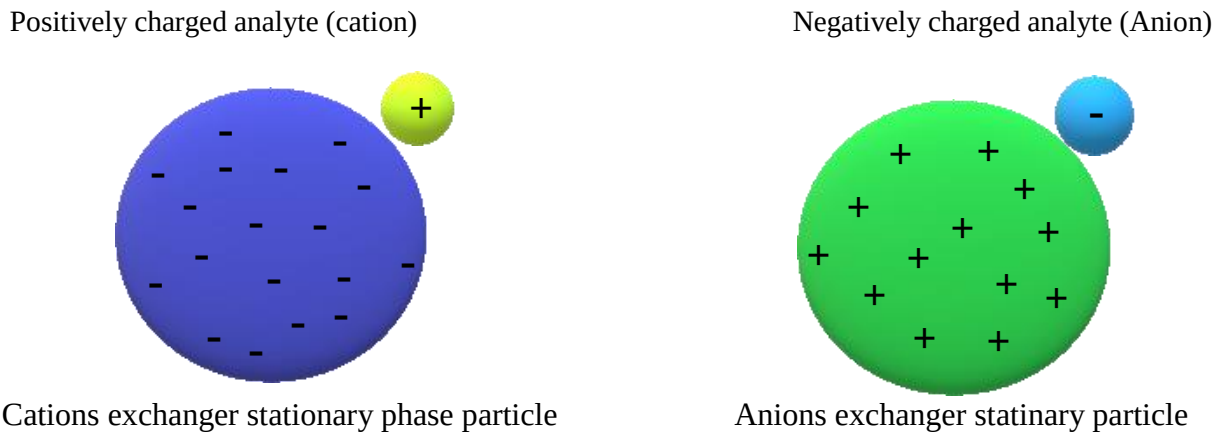


Figure 3.5. *Types of ion exchangers*

3. Stationary phase

The stationary phase consists of a cross-linked polymer resin, typically divinylbenzene cross-linked polystyrene, featuring covalently attached ionic functional groups. The

counterions associated with these fixed charges are mobile and can be replaced by ions that compete more favorably for the exchange sites.

Ion-exchange resins are categorized into four groups: strong acid cation exchangers, weak acid cation exchangers, strong base anion exchangers, and weak base anion exchangers (Table).

Table 3.1. *Examples of common ion-exchange resins*

Type	Functional group	Examples	pH range
Strong acid cation exchanger	Sulfonic acid	$-SO_3^-$ $-CH_2CH_2SO^{3-}$	0-14
Weak acid cation exchanger	Carboxylic acid	$-COO^-$ $-CH_2COO^-$	2-9
Strong base anion exchanger	Quaternary amine	$-CH_2N(CH_3)_3^+$ $-CH_2CH_2N(CH_2CH_3)_3^+$	0-14
Weak base anion exchanger	Amine	$-NH_4^+$ $-CH_2CH_2NH(CH_2CH_3)_3^+$	2-9

An ion-exchange resin's selectivity is somewhat dependent on whether it includes strong or weak exchange sites and on the extent of cross-linking. The latter is particularly important as it controls the resin's permeability, and, therefore, the accessibility of exchange sites.

Note that highly charged cations bind more strongly than cations of lower charge, and that for cations of similar charge, those with a smaller hydrated radius, or that are more polarizable, bind more strongly.

Anions of higher charge and of smaller hydrated radius bind more strongly than anions with a lower charge and a larger hydrated radius.

4. Mobile phase

The mobile phase in IEC usually is an aqueous buffer, the pH and ionic composition of which determines a solute's retention time. Gradient elutions are possible in which the mobile phase's ionic strength or pH is changed with time.

5. Procedure of ion exchange chromatography

Equilibration

As a first step, the stationary phase is washed with the start buffer (initial buffer composition) until the baseline is stabilized and eluent pH remains constant. This step ensures

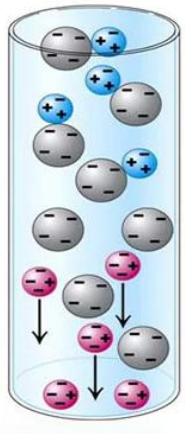
that the ionizable groups on the column are available to interact with the charged analyte molecules.

Sample loading

The sample, which may contain a mixture of charged biomolecules, is loaded onto the column. The choice of resin (anion or cation exchange) depends on the charge of the target biomolecules.

Ion exchange process

During the chromatographic process, ions in the sample undergo exchange with ions on the resin. Positively charged biomolecules (cations) bind to anion exchange resins, while negatively charged biomoleculs (anions) bind to cation exchange resins.



Technology in Science

Elution

Elution is achieved by changing the ionic strength or pH of the mobile phase. This disrupts the electrostatic interactions between the biomolecules and the resin, causing them to be released from the column.

Detection

Detection in ion exchange chromatography involves monitoring the elution of sample components based on their ionic interactions with the stationary phase. Several detection methods can be employed to observe and quantify the separated ions.

Conductivity detection

This is a widely used detection method for ion exchange chromatography. It measures changes in the electrical conductivity of the eluent as ions pass through the detector. The conductivity increases as ions are eluted, providing information about the presence and concentration of ions.

UV-Visible absorbance

UV-Visible absorbance detection is suitable for compounds that absorb light in the UV or visible spectrum. Some ions or compounds may have characteristic absorbance at specific wavelengths, allowing their detection during elution.

Column regeneration

Finally, the column capacity is restored for the next run by washing out any molecules bound on the column. To achieve this, a high ionic strength buffer is allowed to flow through the column until the baseline and pH of the eluent stabilize. The column is then conditioned with the starting buffer prior to the next run.

6. Applications of ion exchange chromatography

Ion exchange chromatography is widely used in various fields for separating and purifying charged molecules based on their ionic interactions with a charged stationary phase. Here are some applications:

- Ion exchange chromatography is commonly employed for purifying proteins. Proteins carry a net charge, and their interactions with the charged resin can be controlled by adjusting the pH and ionic strength of the mobile phase.
- Ion exchange chromatography is used for separating and analyzing amino acids.
- Ion exchange chromatography is applied in water treatment processes for removing ions, such as hardness ions (calcium and magnesium) or heavy metal ions, from water.
- In the pharmaceutical industry, ion exchange chromatography is used for analyzing and purifying drugs, pharmaceutical intermediates, and related compounds.
- It plays a crucial role in the purification of biotechnological products, including enzymes, nucleic acids, and other biomolecules.
- This technique is used to analyze and purify food and beverage products, especially for determining the levels of specific ions or contaminants.
- Ion exchange chromatography is utilized for monitoring and analyzing environmental samples, including soil, water, and air, to assess the presence of ions and pollutants.

III. Affinity chromatography

1. Definition

Affinity chromatography is a powerful chromatographic technique used for the separation and purification of biomolecules based on their specific interactions with a ligand or a stationary phase. The principle behind affinity chromatography relies on the selective binding between the target molecule (analyte) and a ligand immobilized on a solid support. This method is particularly effective for purifying proteins, enzymes, antibodies, and other biomolecules with high specificity.

2. Principles of affinity

The principles of affinity chromatography are based on the specific interactions between a target analyte (molecule to be separated or purified) and a ligand (molecule immobilized on the chromatographic support).

The stationary phase comprises a supporting medium onto which the substrate (ligand) is covalently attached, ensuring that the reactive groups necessary for binding the target molecule are accessible.

As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.

After eluting the other substances, the bound target molecules can be released using methods such as introducing a competing ligand in the mobile phase or altering the conditions of pH, ionic strength, or polarity.

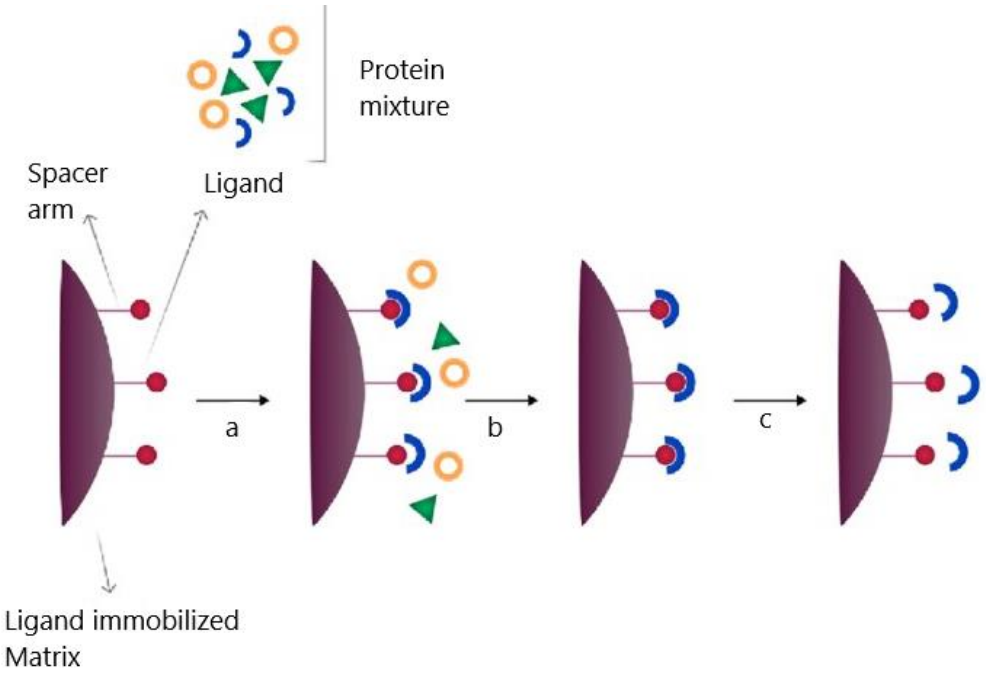


Figure 3.6. *Schematic diagram of affinity chromatography: (a) loading, (b) capture of a target molecule, and (c) elution of the target molecule.*

Examples of ligands that are used in affinity chromatography are given in Table.

Table 3.2. *Affinity ligands and their target compounds (Hage, 2013)*

Type of affinity ligand	Typical targets
Biological agents	
Lectins	Glycoproteins, cells
Carbohydrates	Lectins and other carbohydrate-binding proteins
Nucleic acids	Exonucleases, endonucleases, and polymerases
Cofactors, substrates, and inhibitors	Enzymes
Protein A and protein G	Antibodies
Hormones and drugs	Receptors
Antibodies	Antigens
Nonbiological ligands	
Metal-ion chelates	Proteins and peptides that bind metal ions
Synthetic dyes	Enzymes and various nucleotide-binding proteins

3. Components of affinity chromatography

Stationary phase

It is a solid support or matrix that contains an immobilized ligand. Table shows various materials that have been employed for this purpose. The immobilized ligand is the key element that imparts specificity to the chromatographic separation. The ligand is chosen based on its high affinity for the target molecule that needs to be isolated or purified.

The choice of the ligand immobilized on the stationary phase depends on the specific affinity between the ligand and the target molecule. Common ligands include antibodies, antigens, enzymes, receptors, or other molecules with a high binding specificity for the target. The interaction between the immobilized ligand and the target molecule allows for selective retention and subsequent elution of the target from the column.

Table 3.3. *Materials used as supports in affinity chromatography (Hage, 2013).*

Support material	**Approximate usable pH range**
Carbohydrate-based supports	
Agarose	2–14
Cellulose	1–14
Dextran	2–14
Synthetic organic polymers	
N-Acryloyl-2-amino-2-hydroxymethyl-1,3-propane diol	1–11
Hydroxyethylmethacrylate polymer	2–12
Oxirane-acrylic polymer	0–12
Polyacrylamide	3–10
Polytetrafluoroethylene	0–14
Poly(vinyl alcohol)	1–14
Styrene–divinylbenzene polymer	1–13
Inorganic supports	
Glass	2–8
Silica	2–8

This material should be stable under the flow rate, pressure, and solvent conditions to be employed in the analysis or purification of samples. In addition, the support should be readily available and simple to use in method development (Hage, 2013).

Mobile phase

The mobile phase in affinity chromatography is a liquid that flows over or through the stationary phase, facilitating the separation of components based on their specific interactions with the immobilized ligands.

The mobile phase typically consists of a buffer solution or other aqueous solutions, and its composition can be modified based on the specific requirements of the affinity chromatography experiment. A change in the mobile phase (pH, ionic strength or addition of a competitor) allows the substances of interest to be eluted.

4. Steps of affinity chromatogarphy

The steps of affinity chromatography typically include

Column preparation

Pack a chromatography column with a stationary phase such as sepharose, agarose, cellulose etc. that contains the immobilized ligand specific to the target molecule.

The ligand could be an antibody, antigen, enzyme, or any other molecule with high affinity for the target.

Equilibration

The chromatography column is initially equilibrated with a buffer solution to create a stable environment for interactions between the immobilized ligand and the sample.

Sample loading

The sample containing the mixture of substances is applied to the column. Target molecules with an affinity for the immobilized ligand will selectively bind to it, while non-target molecules are washed away.

Washing

The column is washed with the mobile phase to remove any unbound or weakly bound substances, reducing nonspecific interactions.

Elution

Target molecules are selectively eluted from the column. This is often achieved by introducing a competing ligand or altering the conditions (e.g., pH, ionic strength) to disrupt the binding between the target and the immobilized ligand.

Regeneration

The column is regenerated to remove any remaining bound material and to prepare it for subsequent chromatographic runs. This may involve washing with a regeneration solution or re-equilibrating with the initial buffer.

5. Advantages and desadvantages of affinity chromatography

- Affinity chromatography offers high specificity, targeting the isolation of a particular molecule based on its affinity for the immobilized ligand.Target molecules can be obtained in a highly pure state.
- The technique can achieve high purity in the isolated sample due to the specificity of the ligand-target interactions.
- Affinity chromatography is a relatively gentle method that preserves the biological activity of the isolated molecule.
- It can be applied to complex mixtures, allowing for the purification of a specific target from a complex biological sample.
- The method is reproducible, providing consistent results when properly controlled and optimized.
- Affinity columns may have limited capacity for binding, which can be a challenge when dealing with large-scale purifications.

- Immobilized ligands can be expensive to produce, impacting the cost of the chromatography process.
- Achieving specificity depends on the availability of a suitable ligand, and not all molecules have well-defined affinity ligands.
- The method may have slower flow rates compared to other chromatographic techniques, impacting the overall process time.

6. Applications of affinity chromatography

Affinity chromatography plays a crucial role in various fields, contributing to the isolation and study of biomolecules with high specificity and efficiency.

- Affinity chromatography is widely used for purifying proteins by exploiting the specific interactions between proteins and immobilized ligands.
- Enzymes with specific binding properties can be purified using affinity chromatography, allowing for high yields and purity.
- Monoclonal and polyclonal antibodies can be efficiently purified using affinity chromatography, taking advantage of their affinity for specific ligands.
- Recombinant proteins expressed in host cells can be isolated and purified based on their tags or specific binding domains.
- Affinity chromatography is employed for isolating DNA or RNA fragments by using ligands that specifically interact with nucleic acid sequences.
- Researchers use affinity chromatography to study cell surface receptors by isolating and purifying them based on their interactions with ligands.
- Affinity chromatography is a valuable tool in drug development for isolating and studying drug targets or specific binding molecules.
- Affinity chromatography is employed to investigate biomolecular interactions, including ligand-receptor binding studies.
- Affinity chromatography facilitates the study of protein-protein interactions by isolating interacting partners.

IV. Partition Chromatography (PC)

1. Definition

Partition chromatography is a chromatographic technique based on the differential partitioning of analytes between a stationary liquid phase and a mobile gas or liquid phase. The principle behind this method lies in the distribution or partitioning of solutes between these phases, leading to their separation.

2. Partition chromatography principle

The separation is based on the different affinities of analytes for the stationary and mobile phases. The more a component partitions into the stationary phase, the longer it stays in that phase and the slower it moves through the system. An equilibrium is established which depends on the relative solubility of the solute in the two solvents and therefore on the partition coefficient.

In each phase, the concentration is given by the partition coefficient in chromatography which is represented as $\mathbf{K = C_S/C_M}$

C_S stands for concentration of solute in the stationary phase

C_M stands for concentration of solute in the mobile phase

In a situation when the solute is equally distributed amongst the two phases, then K = 1.

Stationary phase

Partition chromatography can be subdivided into liquid-liquid chromatography and grafted liquid-phase chromatography; the difference lies in how the stationary phase is linked to the support particles. In liquid-liquid partition chromatography, the liquid is physically adsorbed onto the surface of the support, while it is chemically bonded in grafted phase chromatography.

According to the polarity of the stationary and mobile phases, we can distinguish.

Grafted polar phases (normal phase)

The presence of specific functions on the grafting (-CN, $-NH_2$, etc.) can provide interesting selectivity. In normal-phase chromatography, the least polar component is eluted first; increasing the polarity of the mobile phase reduces its elution time.

Non-polar grafted phases (reversed phase)

They are the most commonly used supports, mainly with an alkyl graft (octadecylsilane or -ODS or -C18). Within these systems, water, as the most polar solvent, exhibits the least eluting capacity. The addition of an organic solvent, such as methanol or acetonitrile, enhances the

elution strength of the mobile phase. Consequently, the least polar component is eluted initially, and augmenting the polarity of the mobile phase results in an extended elution time.

Mobile phase

The eluent must be immiscible with the stationary phase. The solutes' partition coefficient is notably affected by the polarity of the mobile phase, usually consisting of a mixture of water and organic solvent in different ratios.

3. Applications of partition chromatography

Partition chromatography is a versatile technique with applications in various fields. Some notable applications include:

Partition chromatography is used for the separation of organic compounds, it is widely employed in the pharmaceutical industry for the isolation and purification of drugs and drug intermediates, for the analysis of environmental samples, such as detecting pollutants and contaminants in water and soil, in biochemistry, for the separation of biomolecules like proteins, amino acids, and nucleic acids. It finds applications in the analysis and quality control of food and beverages, separating and identifying various components and in the petroleum industry, partition chromatography is used to separate and analyze complex mixtures of hydrocarbons.

V. Hydrophobic Interaction Chromatography (HIC)

1. Definition

Hydrophobic Interaction Chromatography (HIC) is a chromatographic technique that separates biomolecules based on their hydrophobicity or, more precisely, their ability to interact with hydrophobic surfaces. Unlike other chromatographic methods that rely on charge differences (like ion exchange chromatography) or size (like size exclusion chromatography), HIC exploits the hydrophobic regions present in biomolecules.

Hydrophobic Interaction Chromatography is a valuable approach for protein purification as it preserves the biological activity of proteins while providing a less denaturing environment. HIC facilitates the separation and purification of proteins based on their hydrophobicity, utilizing their hydrophobic characteristics. This makes it a valuable tool in the field of bioseparations and protein purification.

2. Principles

The principle of HIC is based on interactions between the hydrophobic regions of protein molecules in the sample and a hydrophobic stationary phase or matrix. This principle enables the separation and purification of proteins based on their hydrophobicity.

In HIC, protein molecules from the sample are introduced into a column containing a high-salt buffer. The presence of salt in the buffer plays a crucial role in promoting the binding of proteins to the hydrophobic stationary phase. Salt reduces the solvation of sample molecules, exposing their hydrophobic regions. As a result, the hydrophobic regions of proteins interact with the hydrophobic environment, leading to their adsorption on the stationary phase.

The amount of salt required to promote binding is inversely proportional to the hydrophobicity of protein molecules. Proteins with higher hydrophobicity will have more exposed hydrophobic regions, requiring less salt for binding. Conversely, proteins with low hydrophobicity will need a higher salt concentration to facilitate their interaction with the hydrophobic environment. This property allows the separation of proteins based on their relative hydrophobicity.

To elute the bounded proteins from the HIC column, a decreasing salt gradient is typically employed. By gradually reducing the salt concentration, the strength of hydrophobic interactions between proteins and the stationary phase is progressively weakened. Consequently, proteins are released from the column in order of increasing hydrophobicity.

3. Procedure

The hydrophobic interaction chromatography protocol involves several key steps to effectively separate and purify proteins based on their hydrophobicity. Here is a general overview of the HIC protocol.

Column preparation

The hydrophobic ligands are attached to the support matrix to create the stationary phase.

The column is equilibrated with a buffer containing a high concentration of salt.

Sample application

The protein sample, dissolved in a moderately high-salt buffer, is applied to the column.

Washing

Unbound proteins are washed away with a high-salt buffer.

Elution

A salt gradient is applied to decrease salt concentration gradually, eluting proteins based on their hydrophobicity.

4. Advantages of Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography offers several advantages, making it a valuable technique in the field of bioseparations. Here are some key advantages of HIC

Mild conditions : HIC operates under relatively mild conditions, preserving the structure and activity of biomolecules.

Selective separation : HIC allows for the selective separation of proteins based on their hydrophobicity. This is particularly useful when dealing with proteins that have similar charges or sizes.

High resolution: HIC provides high resolution in separating proteins, especially those that form aggregates.

Versatility: HIC is versatile and applicable to a wide range of biomolecules, including proteins, peptides, and nucleic acids. It is commonly used in the purification of biotherapeutics and recombinant proteins.

Robustness: HIC resins are often robust and stable under various experimental conditions, allowing for reliable and reproducible separations.

Cost-effective: HIC is often considered cost-effective in terms of the equipment required and the simplicity of the process. The absence of expensive affinity ligands or specialized equipment contributes to its cost efficiency.

5. Limitations of HIC

Hydrophobic Interaction Chromatography is a powerful technique, but it also has some limitations.

HIC is generally less specific than techniques like affinity chromatography. It relies on the overall hydrophobicity of proteins, and therefore, different proteins with similar hydrophobicity may co-elute.

HIC is sensitive to changes in salt concentration. Small changes in salt concentration can significantly impact the retention of proteins. Optimization of salt conditions is crucial for successful separations.

Between runs, HIC columns often require re-equilibration with high concentrations of salt, which can be time-consuming.

Extremely hydrophobic proteins may interact too strongly with the stationary phase, making elution difficult. Extremely hydrophilic proteins may not interact strongly enough with the hydrophobic ligands, leading to poor retention. The conditions required for HIC, including the use of high salt concentrations, may not be suitable for samples sensitive to these conditions.

Applications

Application 1

The retention times (tr) are calculated during Sephadex chromatography for the specified proteins, each with a known molecular mass (MM). The column operates at a flow rate of 5 ml/min.

Protéines	MM (Da)	tr (min)
Aldolase	145000	10.4
Lactate déshydrogénase	135000	11.4
Phosphatase alcaline	80000	18.4
Ovalbumine	45000	26.2
lactoglobuline	37100	28.6

1. Explain the principle of this technique.
2. Determine the corresponding elution volumes (Ve). Plot the logarithm of MM against Ve; what do you observe?
3. For glucokinase with Tr=21, determine its molecular mass using the previous graph.

Application 2

The height equivalent to a theoretical plate (HETP) for a gel filtration column is found to be 0.02 cm. Calculate the number of theoretical plates (N) for the column, given its length is 30 cm.

Application 3

We aim to determine the molecular weight (MW) of a protein P through exclusion chromatography. The gel exclusion limit is between 40,000 and 400,000 MW. The calibration of the gel is performed using various substances, and their molecular weights (expressed in daltons) and elution volumes Ve (ml) are indicated in the following table:

Proteines	MW (Da)	Ve (mL)
Dextran	2000000	45
Fibrinogène	340000	60
Catalase	230000	75
Lactoglobuline	19000	132

1. Determine the dead volume (V_0) of the column.

2. Plot the logarithm of MM against Ve. What do you observe ?

3. The protein P exhibits an elution volume Ve=113 ml. Determine its MW.

Application 4

Carboxymethylcellulose is an ion exchanger whose exchange capacity depends on the pH of the mobile phase. The functional exchange group -CH2-COOH has a pKa of 4.76.

1. Calculate the ionization rate of the exchanger when using an ionic mobile phase with a pH of 2.5 and a pH of 7.0.

2. Indicate in a table the charge of the exchanger at these two pH value.

3. Indicate the charge of proteins ovalbumine (pHi=4.6), cytochrome C (pHi=10.65) et lysozyme (pHi=11).

What are the proteins retained by the ion exchanger at pH 2.5 et 7.0 ?

Application 5

In an anion exchange chromatography experiment, a protein of interest has a pI of 6.0. The mobile phase contains a salt gradient. Discuss how varying the salt concentration in the mobile phase affects the separation and elution of the protein.

Application 6

Explain the role of the ligand in affinity chromatography. How does the specific interaction between the ligand and the target molecule contribute to the separation process?

Application 7

A solute is distributed between a stationary phase and a mobile phase. The concentration of the solute in the stationary phase is 0.05 mol/L, and in the mobile phase, it is 0.2 mol/L. Calculate the partition coefficient (K).

Chapter 4
Gas Chromatography

CHAPTER

4

Gas Chromatography

1. Definition

Gas Chromatography (GC) is a powerful analytical technique used for the separation and analysis of volatile and semi-volatile compounds in a mixture. It utilizes a gaseous mobile phase to carry the sample through a stationary phase, separating the components based on their interaction with the stationary phase.

2. Principle

The principle of Gas Chromatography is based on the separation of components in a mixture due to differences in their distribution between a stationary phase and a mobile phase. The heart of the GC system is a long, coiled column typically made of stainless steel or glass. The column is coated with a thin layer of a stationary phase, which can be a liquid (Gas-Liquid Chromatography, GLC) or a solid (Gas-Solid Chromatography, GSC). The column is enclosed in a temperature-controlled oven.

The sample, in the form of a vapor or gas, is introduced into the chromatograph through an injector. The injector vaporizes the sample and introduces it into the column. A carrier gas, commonly helium, nitrogen, or hydrogen, flows through the column. The carrier gas serves as the mobile phase that carries the sample through the column (Figures 4.1 and 4.2).

As the sample travels through the column, different components interact with the stationary phase based on their chemical properties. In Gas-Liquid Chromatography (GLC), separation occurs due to differences in vapor pressure, solubility, and boiling points of the components.

In Gas-Solid Chromatography (GSC), separation is based on adsorption and desorption processes on the solid stationary phase.

As components exit the column, they reach a detector. The detector responds to the presence of analytes and generates electrical signals. Common detectors include Flame Ionization Detector (FID), Thermal Conductivity Detector (TCD), and Mass Spectrometry (MS).

The signals from the detector are recorded and plotted as a chromatogram. The chromatogram is a graph that shows the separation of components over time. Peaks in the chromatogram represent individual components (Figure 4.3).

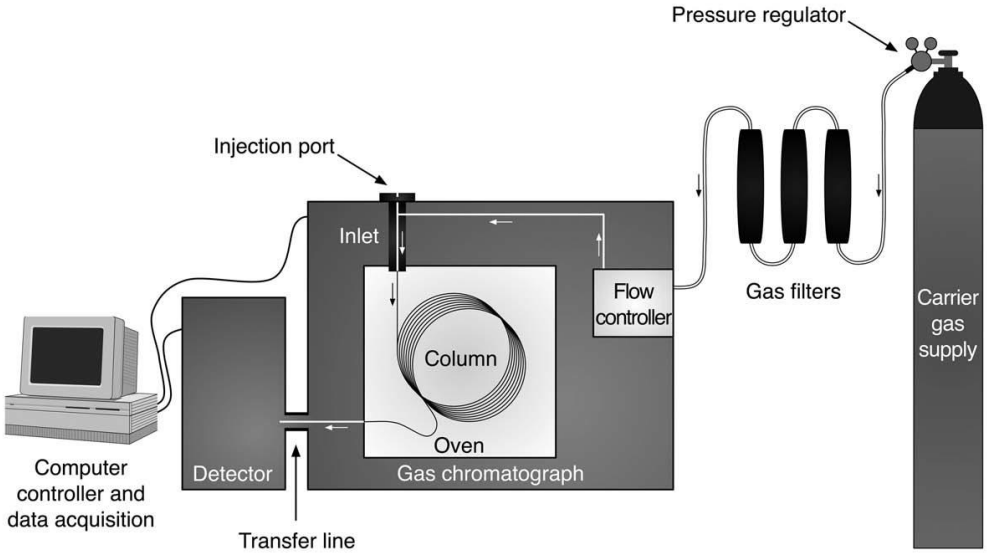


***Figure 4.1**. A gas chromatograph and its main components (Stauffer, 2008).*

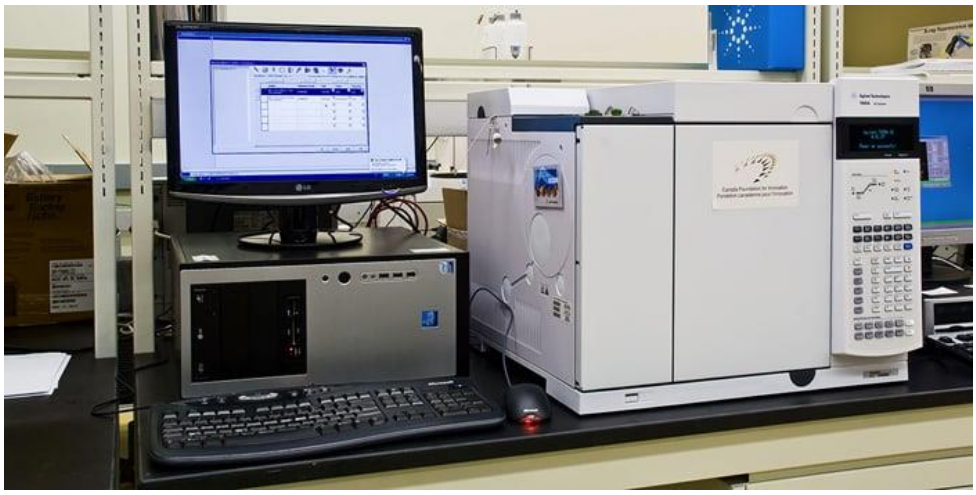


Figure 4.2. Gas chromatography instrument

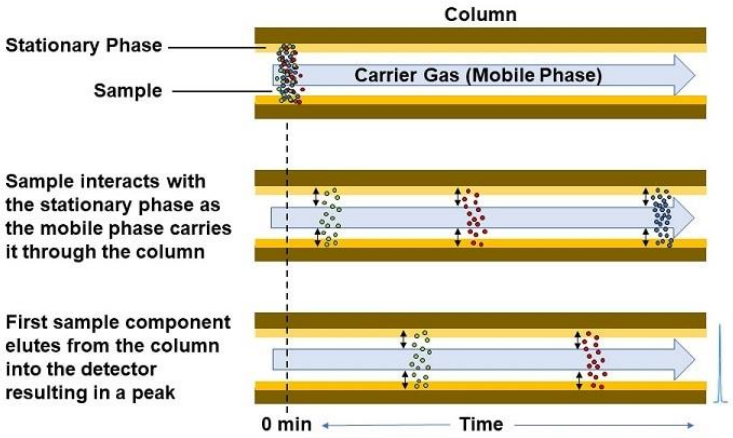


Figure 4.3. *Principle of gas chromatography*

3. Parts of Gas Chromatography

Gas chromatography is mainly composed of the following parts:

3. 1.Carrier gas

The carrier gas, serving as the mobile phase in gas chromatography, remains inert and exhibits no interaction with the analytes. This distinguishes gas chromatography from other chromatographic systems, where the mobile phase's chemical properties often influence separation. The selection of a carrier gas is typically determined by the type of detector employed and, when multiple gases are suitable, by factors such as efficiency and availability.

The most commonly used carrier gases for capillary gas chromatography are helium (He), hydrogen (H2), and nitrogen (N2). The efficiency of a chromatographic system with a given carrier gas varies with the fl ow rate (in ml/min), also expressed in terms of average linear velocity (in cm/sec) (Stauffer et al., 2008).

3.2. Sample injection system

Liquid samples are injected using a microsyringe equipped with a needle, which is inserted through a self-sealing silicon rubber septum into a heated metal block powered by a resistance heater. Gaseous samples can be introduced either using a gas-tight syringe or through a bypass loop and valves. Typical sample volumes range from 0.1 to 0.2 ml.

3.3. Separation column

Originally, gas chromatography used exclusively packed columns—columns that were fi lled with an inert solid support that was coated with the liquid stationary phase (Stauffer et al., 2008). In 1958, Golay introduced the open-tubular (OT) column, commonly known as a

capillary column, which is widely employed in most gas chromatographs due to its unmatched resolution (Ettre, 2005).

A packed column can achieve a maximum of approximately 3,000 plates per meter, corresponding to an HETP of 300 μm. Since these columns are usually not longer than 5 meters, they can reach approximately 15,000 plates. A capillary column may possess an HETP slightly below 100 μm, representing a moderate improvement over packed columns. Nevertheless, owing to their typically longer length, ranging from 20 to 60 meters, they can demonstrate between 150,000 to 500,000 plates (Jennings, 2000). This clearly illustrates the efficiency improvement achieved with a capillary column in comparison to a packed column.

The selection of a column is entirely dependent on the nature of the samples under analysis. Four key variables govern column selection: the chemical nature of the stationary phase, its thickness, column length, and diameter. It is important to clearly defi ne what types of compounds are analyzed and what the goal of the analysis is

Columns typically have dimensions ranging from 15 to 60 meters in length and from 0.20 to 0.53 mm in internal diameter. The stationary phase usually possesses a thickness ranging from 0.1 to 1 μm. When the column dimensions are known, the internal volume can be quickly calculated (as the thickness of the stationary phase is negligible, the internal diameter is used to determine the cross-sectional area). Knowing the column volume is valuable for verifying the flow rate of a system.

Figure 4.4. illustrates a cross-section of a chromatographic column. Capillary columns are constructed from a fused silica open cylinder with an external polyimide coating. The stationary phase is chemically bonded to the inner wall of the cylinder.

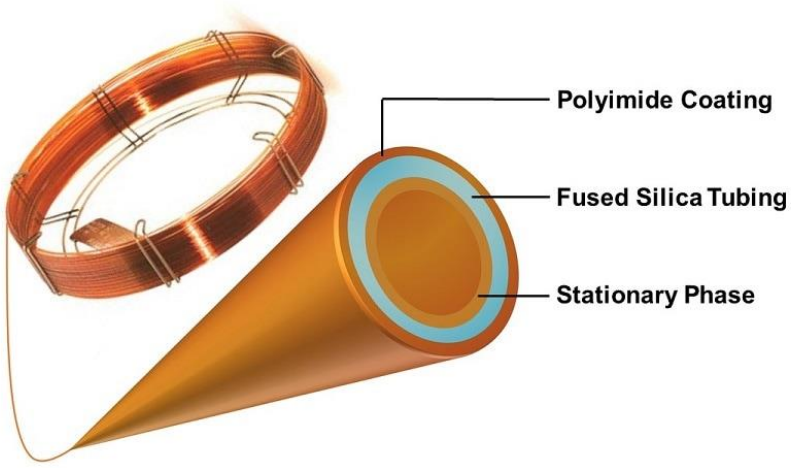


Figure 4.4. *Cross-section of a chromatographic column*

3.4. Supports

The efficiency of the support and the extent of separation are contingent on the structure and surface characteristics of the support materials. The support must be inert while having the ability to immobilize a substantial volume of liquid phase in the form of a thin film across its surface. The surface area should be extensive to facilitate the rapid achievement of equilibrium between the stationary and mobile phases. Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.

3.5. Detector

The role of a GC detector is to generate a detectable electronic signal in response to the compounds eluting from the column. There are various detectors designed for GC applications.

4. Procedure of Gas chromatography

Step 1: Sample injection and vapourization

Step 2: Separation in the column

Components in the mixture are separated based on their abilities to adsorb on or bind to, the stationary phase.

The component with the highest adsorption affinity to the stationary phase will spend the longest time in the column, resulting in the longest retention time (Rt). Consequently, it will be the last to exit the gas chromatograph. A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will, therefore, have the shortest retention time (Rt). It will emerge from the gas chromatograph first.

Step 3: Detecting and recording results

Variations in the retention times of individual components in the mixture lead to their sequential arrival at the detector. The initial detection occurs for the component with the shortest retention time, while the component with the longest retention time is detected last.

The detector transmits a signal to the recorder, generating a peak. The initial detection corresponds to the first-recorded component, while the component detected last is recorded at the end.

5. Applications of GC

Gas chromatography (GC) is a widely used analytical technique that separates and analyzes volatile compounds in a gas phase. It has numerous applications across various fields due to its high sensitivity, precision, and efficiency. Some common applications of gas chromatography include:

Environmental analysis

Detection of pollutants in air, water, and soil.

Monitoring of pesticide residues in food and water.

Pharmaceuticals

Quality control of pharmaceutical products.

Analysis of drug formulations.

Food and beverage industry

Detection of flavor compounds in food products.

Analysis of food additives and contaminants.

Petrochemical industry

Determination of hydrocarbons in petroleum products.

Monitoring of impurities in fuels.

Biotechnology

Monitoring fermentation processes.

Analysis of volatile compounds in biological samples.

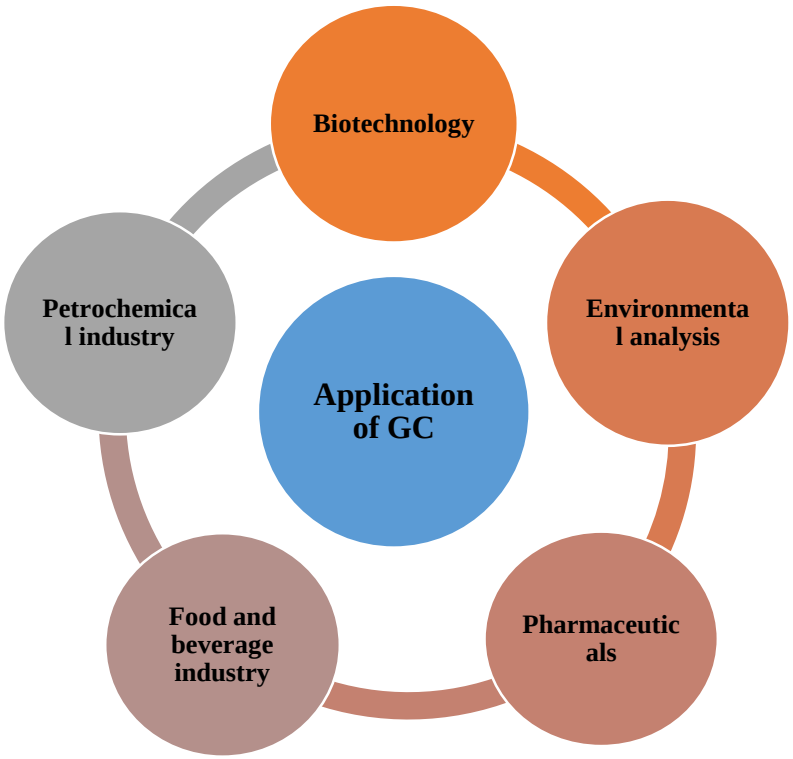


Figure 4.5. *Applications of gas chromatography.*

Chapter 5
Introduction to Spectral Methods

CHAPTER

5

Introduction to Spectral Methods

1. Definition

Spectral methods refer to a set of techniques used to analyze and study the interaction of electromagnetic radiation with matter. These methods involve the measurement and interpretation of spectra, which are the distribution of radiation intensity as a function of wavelength or frequency.

They are widely employed in various scientific fields, including chemistry, physics, biology, materials science, and environmental science.

Spectral methods provide valuable information about the composition, structure, and properties of substances, allowing researchers to identify and characterize molecules, study molecular interactions, determine chemical and physical properties, and investigate energy transitions within systems. These methods encompass different techniques such as UV/Visible spectroscopy, infrared spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry, each offering unique insights into the behavior and properties of matter at the molecular level.

- One of the most commonly used spectral methods is UV/Visible spectroscopy, which involves the measurement of the absorption or emission of ultraviolet and visible light by molecules. This technique is widely employed in chemistry, biochemistry, and

materials science to analyze the electronic structure of compounds, determine their concentration, and investigate chemical reactions.

- Infrared spectroscopy is another important spectral method that focuses on the interaction of infrared radiation with molecules. By measuring the absorption or transmission of infrared light, this technique provides information about molecular vibrations and functional groups present in a compound. It is extensively used in organic chemistry, pharmaceutical analysis, and environmental science.
- Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique that exploits the magnetic properties of atomic nuclei to study molecular structure, dynamics, and interactions. NMR spectroscopy is widely used in chemistry, biochemistry, and medicine for elucidating the structures of organic compounds, characterizing proteins and nucleic acids, and studying metabolic processes in living organisms.
- Mass spectrometry is another important spectral method that involves the ionization, separation, and detection of ions based on their mass-to-charge ratio. This technique provides information about the molecular weight, structure, and fragmentation patterns of compounds. Mass spectrometry finds applications in a wide range of fields, including proteomics, metabolomics, environmental analysis, and forensic science.

The significance of spectral methods lies in their ability to provide detailed and precise information about the molecular and structural characteristics of substances. These methods enable scientists to identify unknown compounds, quantify their concentrations, study molecular interactions, investigate reaction mechanisms, and explore the physical and chemical properties of materials. Spectral methods also play a crucial role in quality control, drug discovery, environmental monitoring, and forensic analysis.

Overall, spectral methods are indispensable tools in scientific research and analysis, providing valuable insights into the microscopic world and contributing to advancements in various disciplines.

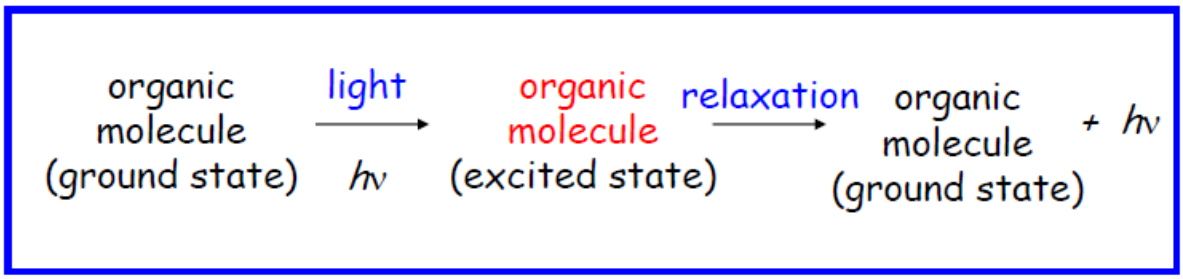
What is Spectroscopy ?

- Study the properties of matter through its interactions with radiation at different frequencies of the electromagnetic spectrum.

  Latin : « spectrum »- ghost or spirit

  Greek : to see

- With light you don't look directly at the molecule (matter), but at its "ghost"



2. Historical background and development of the spectroscopy

The foundation of spectroscopy dates back to the early 17th century when scientists began to experiment with light and its interactions with prisms and lenses.

Sir Isaac Newton's experiments with prisms demonstrated the dispersion of white light into a spectrum of colors, leading to the understanding of the rainbow phenomenon (Figures 5.1, 5.2).

In the early 19th century, Joseph von Fraunhofer observed dark absorption lines in the solar spectrum, now known as Fraunhofer lines. These lines corresponded to the absorption of specific wavelengths by chemical elements in the Sun's atmosphere, providing early insights into the concept of absorption spectroscopy.

In the mid-19th century, the study of spectroscopy began to advance rapidly with the development of more precise instruments.

In 1859, Gustav Kirchhoff and Robert Bunsen introduced the first spectroscope, which allowed for the observation and analysis of atomic emission spectra (Figure 5.3).

The study of absorption and emission spectra of elements laid the groundwork for further advancements in spectroscopy.

Figure 5.1. *Newton's prism experiments demonstrated that different colors were all components of white light.*

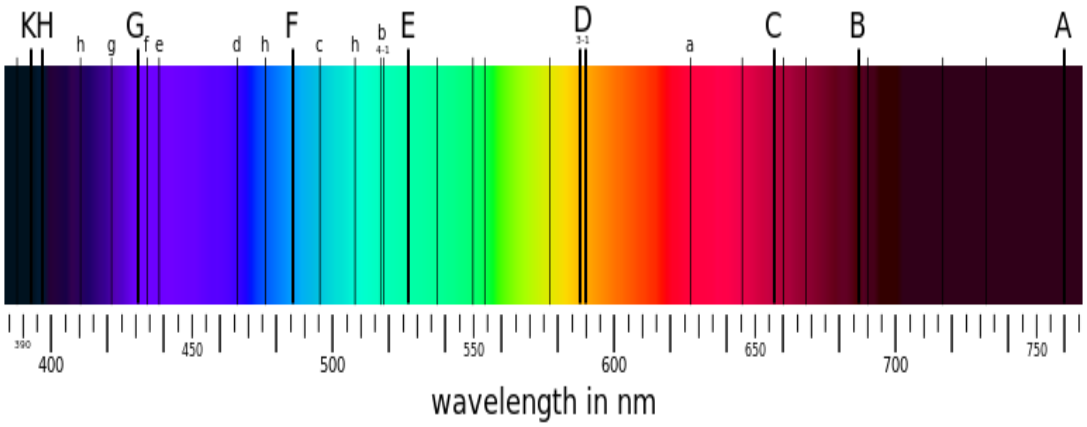


Figure 5.2. *Fraunhofer lines*

Those little black lines are among the most important discoveries in optics. They are absorption lines, and Fraunhofer mapped 570 of them. Each element on the periodic table absorbs and scatters a different set of wavelengths.

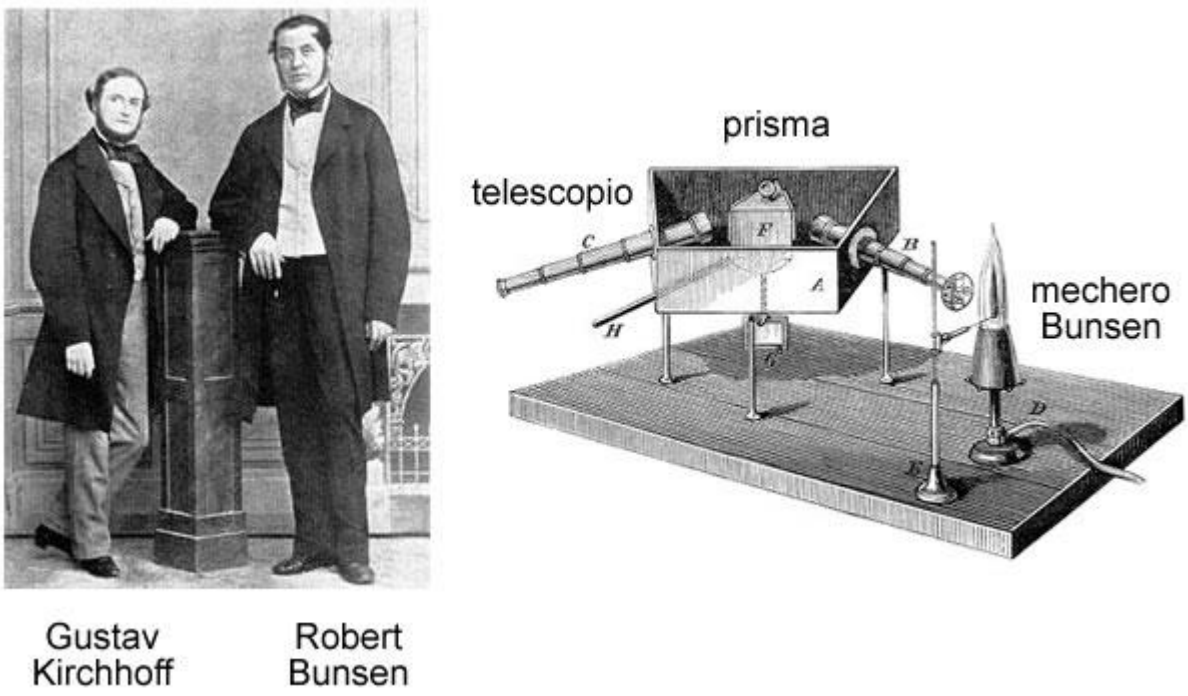


Figure 5.3. *The Bunsen-Kirchhoff spectroscope*

3. Electromagnetic spectrum

The electromagnetic spectrum refers to the entire range of electromagnetic radiation, which includes various forms of energy that travel through space in the form of waves. These waves differ in their wavelengths, frequencies, and energies and they are classified into different regions based on these properties.

- The electromagnetic spectrum is typically divided into the following regions (**Figure 5.4**):
- Radio Waves: Radio waves have the longest wavelengths and lowest frequencies in the electromagnetic spectrum. They are used for communication, broadcasting, and radar systems.
- Microwaves: Microwaves have shorter wavelengths and higher frequencies than radio waves. They are commonly used in microwave ovens, telecommunications, and satellite communications.
- Infrared (IR) Radiation: Infrared radiation has longer wavelengths and lower frequencies than visible light. It is associated with the heat emitted by objects and is used in applications such as thermal imaging, remote sensing, and infrared spectroscopy.
- Visible Light: Visible light is the portion of the electromagnetic spectrum that is visible to the human eye. It consists of different colors, ranging from violet (shortest wavelength) to red (longest wavelength). Visible light is responsible for our sense of vision and is used in various lighting applications.
- Ultraviolet (UV) Radiation: Ultraviolet radiation has shorter wavelengths and higher frequencies than visible light. It is known for its ability to cause sunburn and is used in applications such as sterilization, fluorescent lighting, and UV spectroscopy.
- X-Rays: X-rays have shorter wavelengths and higher frequencies than UV radiation. They possess high energy and can penetrate materials, making them useful in medical imaging, security scanning, and industrial testing.
- Gamma Rays: Gamma rays have the shortest wavelengths and highest frequencies in the electromagnetic spectrum. They are produced by nuclear reactions and radioactive decay. Gamma rays are used in radiation therapy, sterilization, and the study of nuclear processes.
- Each region of the electromagnetic spectrum has unique properties and applications. Scientists and engineers utilize these different regions for a wide range of purposes, from communication and imaging to medical diagnostics and scientific research. Understanding the electromagnetic spectrum and its various regions is crucial for exploring and harnessing the power of electromagnetic radiation (Figure 5.4).

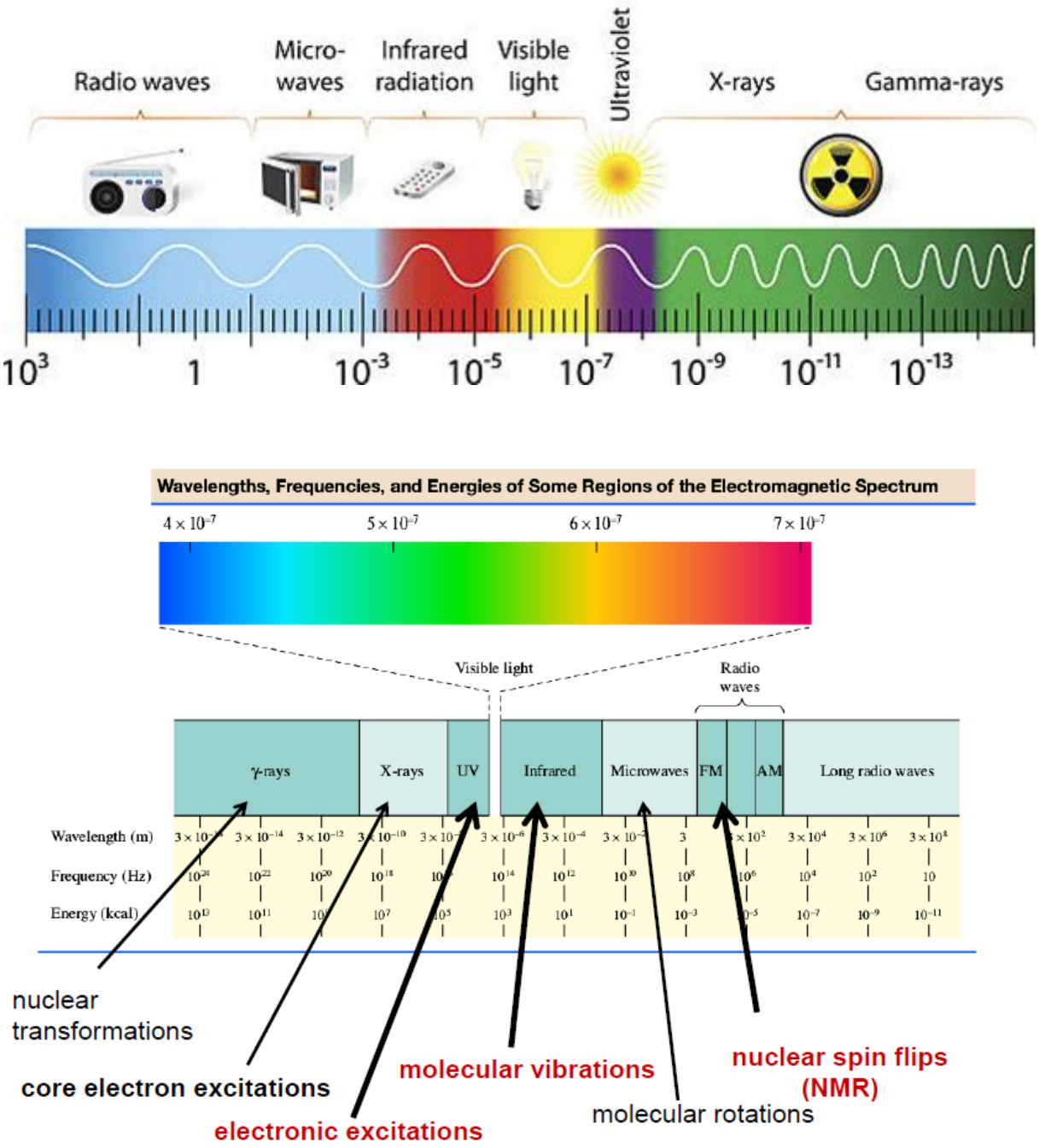


Figure 5.4. *Electromagnetic spectrum*

4. Light-Matter interaction and basic principles

Electromagnetic radiation is a form of energy that travels in waves and exhibits both wave-like and particle-like properties. It consists of photons, which are discrete packets of energy. The electromagnetic spectrum encompasses a wide range of wavelengths, from gamma rays (shortest wavelength) to radio waves (longest wavelength).

Photons are fundamental particles of light and carry energy proportional to their wavelength. The energy of a photon is inversely proportional to its wavelength, meaning that higher energy photons have shorter wavelengths. This relationship is described by Planck's equation: **E = h * ν,** where E is the energy of a photon, h is Planck's constant, and ν is the frequency of the photon.

When light interacts with matter (atoms, molecules, or materials), several processes can occur (Figure 5.5).

Absorption: In absorption, photons are absorbed by the material, promoting electrons from lower energy levels (ground state) to higher energy levels (excited states). The energy difference between these levels corresponds to the energy of the absorbed photon.

Transmission: In transmission, light passes through the material without being absorbed. The material is said to be transparent at specific wavelengths.

Reflection: In reflection, light bounces off the surface of the material without being absorbed. This process gives objects their color or appearance.

Scattering: Scattering occurs when light interacts with small particles or irregularities in the material, causing the light to change direction without being absorbed. Scattering is responsible for phenomena such as blue sky and the white appearance of clouds.

Light-matter interaction is fundamental to many natural processes and technological applications. In UV/Vis spectroscopy, the absorption of light by molecules provides valuable information about their electronic structure and properties. Understanding how light interacts with matter is crucial in various scientific fields, including chemistry, physics, biology, and material science.

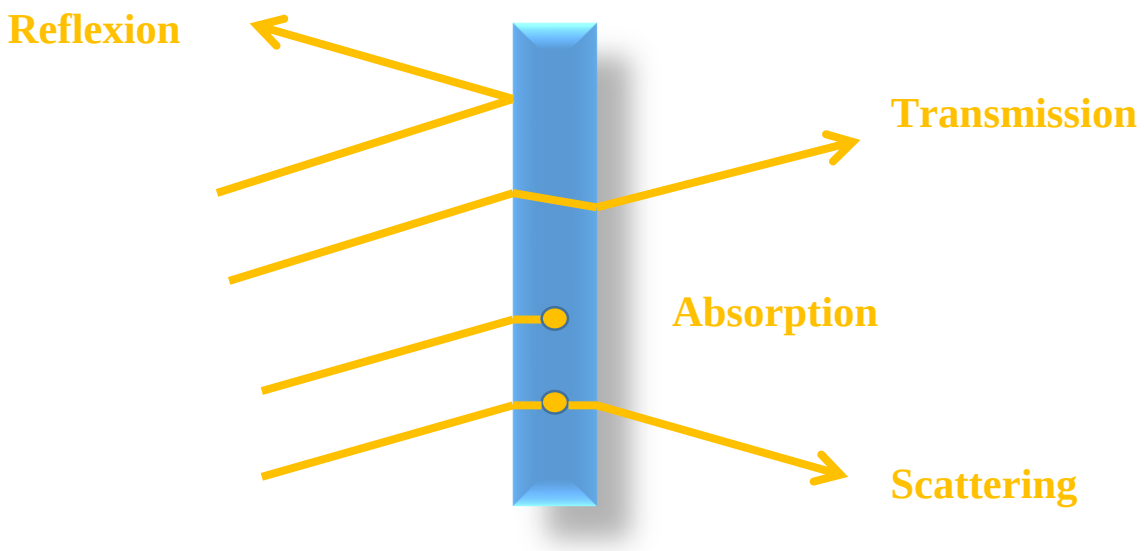


Figure 5. 5. *Light-matter interaction*

5. Importance and applications

The importance of spectral methods lies in their ability to provide valuable information about the composition, structure, and properties of various substances. These methods involve the measurement and analysis of the interaction between electromagnetic radiation and matter, allowing scientists to gain insights into the molecular and atomic characteristics of materials. Spectral methods find extensive applications in various fields (Figure 5.6), including:

- ***Chemistry***: Spectral methods such as UV/Visible spectroscopy, infrared spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy are widely used for compound identification, quantification, and structural analysis in chemical research.
- ***Material science***: Spectral techniques such as X-ray diffraction (XRD) and Raman spectroscopy help in the characterization of materials, including crystalline structures, molecular bonding, and surface analysis.
- ***Environmental science***: Spectral methods play a crucial role in monitoring and analyzing environmental pollutants, studying water quality, air pollution, and soil composition. Techniques like atomic absorption spectroscopy (AAS) and inductively coupled plasma-mass spectrometry (ICP-MS) are employed for elemental analysis.
- ***Pharmaceutical science***: Spectral methods are extensively utilized in drug discovery, development, and quality control. Techniques such as nuclear magnetic resonance (NMR), mass spectrometry (MS), and infrared (IR) spectroscopy help in compound identification, purity assessment, and stability studies.
- ***Biomedical research***: Spectral methods are used in medical diagnostics, biomolecular analysis, and imaging techniques. Infrared spectroscopy, fluorescence spectroscopy, and Raman spectroscopy are employed for disease diagnosis, protein analysis, and cellular imaging.
- ***Food science***: Spectral methods are valuable in analyzing the composition, quality, and safety of food products. Techniques like Fourier transform infrared (FTIR) spectroscopy and near-infrared spectroscopy (NIRS) help in detecting contaminants, determining nutritional content, and evaluating food authenticity.

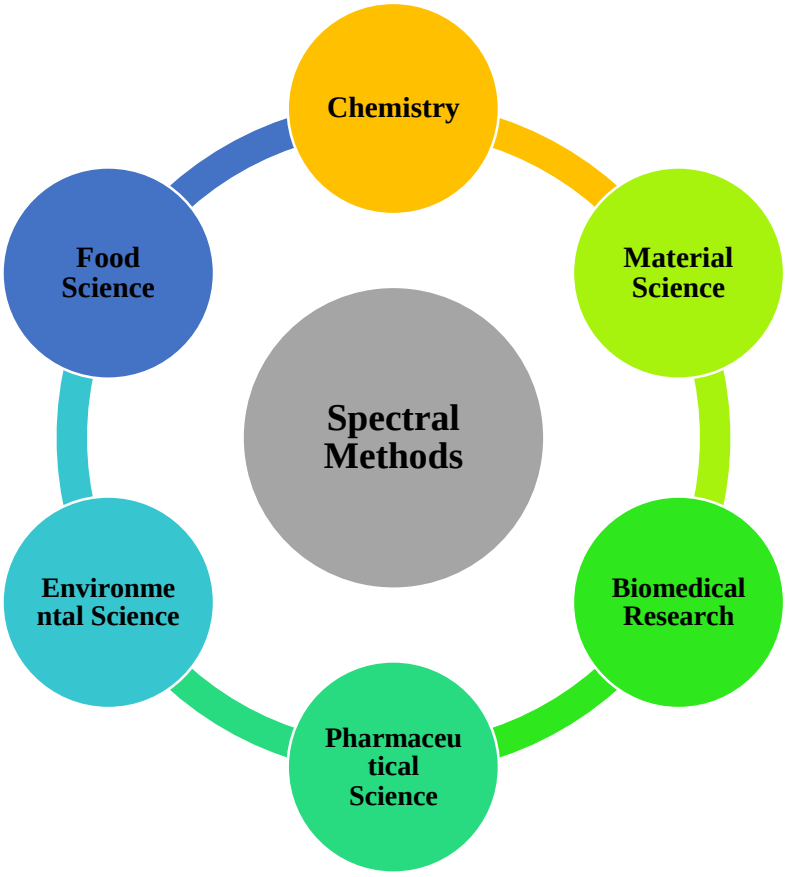


Figure 5.6. *Applications of spectral methods*

Chapter 6

UV/Visible Spectroscopy

CHAPTER

6

UV/Visible Spectroscopy

1. Definition

UV/Visible spectroscopy is a widely used analytical technique that involves the measurement of the interaction between ultraviolet (UV) and visible (Vis) light with matter. It provides valuable information about the electronic structure and properties of molecules, making it a powerful tool in various scientific fields.

UV/Visible spectroscopy relies on the principle that molecules can absorb specific wavelengths of UV or visible light, leading to transitions of electrons between different energy levels. By analyzing the pattern and intensity of light absorption, we can gain insights into the chemical composition, concentration, and structural characteristics of substances.

This technique finds extensive applications in chemistry, biochemistry, pharmaceuticals, environmental sciences, materials science, and many other areas. It allows researchers to study the properties of organic and inorganic compounds, quantify their concentrations, and monitor chemical reactions in real-time.

UV/Visible spectroscopy is particularly useful in determining the presence and concentration of chromophores, which are groups of atoms responsible for light absorption. The technique is non-destructive, requires minimal sample preparation, and provides rapid results, making it highly efficient for routine analysis in laboratories.

In the upcoming sections of this lecture, we will explore the principles of UV/Visible spectroscopy, discuss the instrumentation and experimental setup, delve into quantitative

analysis using the Beer-Lambert Law, examine the interpretation of spectra, and explore various applications of this technique (Kafle 2000).

2. Historical background

The development of spectroscopy techniques continued into the late 19th and early 20th centuries, with the focus shifting to the ultraviolet and visible light regions.

Notably, Sir William Crookes and Johann Balmer made significant contributions to the understanding of the hydrogen spectrum, leading to the formulation of Balmer's formula.

The early 20th century witnessed the emergence of quantum mechanics, which revolutionized the understanding of light-matter interactions.

Pioneering works by Niels Bohr, Werner Heisenberg, and Erwin Schrödinger provided the theoretical framework for explaining electronic transitions in atoms and molecules.

The first practical UV/Vis spectrophotometers were developed in the 1930s, allowing scientists to measure and quantify the absorption of light by various substances.

These early instruments employed prisms and later, diffraction gratings, to disperse light into its component wavelengths.

The mid-20th century saw significant advancements in UV/Vis spectroscopy instrumentation, including the use of photomultiplier tubes and solid-state detectors for improved sensitivity and accuracy.

Introduction of double-beam spectrophotometers enabled simultaneous measurements of sample and reference, minimizing baseline drift.

In recent decades, UV/Vis spectroscopy has been combined with other techniques, such as chromatography and mass spectrometry, to provide more comprehensive analytical information. The development of fiber-optic and microspectroscopy techniques expanded the applications of UV/Vis spectroscopy to non-traditional sample formats and microscale analyses.

3. Theoretical foundations of UV/Vis spectroscopy

3.1 Spectral range

The UV-visible range extends approximately from 800 to 10 nm.

In a molecule, electronic transitions take place in the ultraviolet region (approximately 400-10 nm) and the visible region (800-400 nm).

- **Visible:** 800 nm (red) - 400 nm (indigo)
- **Near-UV**: 400 nm - 200 nm
- **Far-UV**: 200 nm - 10 nm

UV range used in analysis: 190 – 400 nm. Therefore, the far-UV is not accessible for measurements under these conditions.

Visible range used in analysis: 400 – 800 nm.

3.2 Principle

An electronic transition corresponds to the movement of an electron from an occupied molecular orbital in the ground state to an unoccupied excited molecular orbital, through the absorption of a photon whose energy matches the energy difference between the ground state and the excited state (Kafle 2000) (Figure 6.1).

Four types of electronic transitions are possible:

$\sigma \longrightarrow \sigma^*$

$n \longrightarrow \sigma^*$

$n \longrightarrow \pi^*$

$\pi \longrightarrow \pi^*$

Each transition is characterized both by its wavelength λ_{max} and by its molar absorption coefficient: ε_{max} at that wavelength.

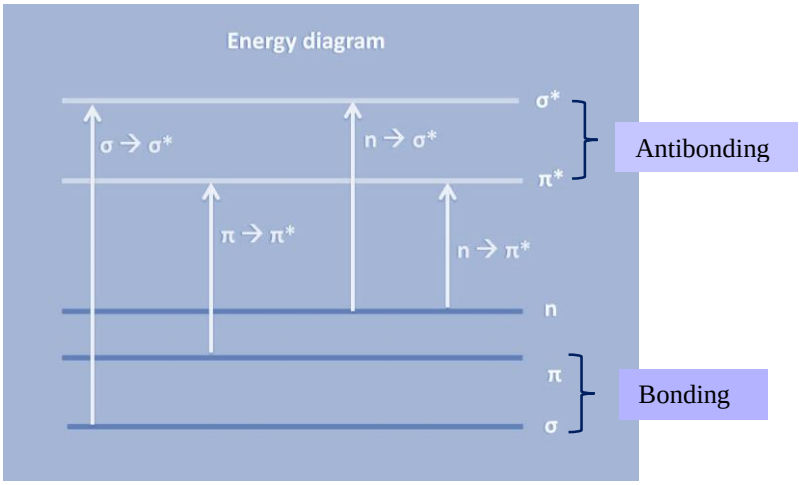


Figure 6.1. *Electronic transitions and types of electrons*

3.3 Different types of transitions

Electronic transitions correspond to the movement of electrons from filled bonding and non-bonding molecular orbitals to vacant anti-bonding molecular orbitals. The absorption wavelength depends on the nature of the involved orbitals. The following diagram illustrates this for σ, π, and n-type orbitals (Figure 6.1)

The absorption of a photon in the UV-visible range can often be attributed to electrons belonging to small groups of atoms called chromophores (C=C, C=O, C=N, C≡C, C≡N…). The absorption wavelength depends on the nature of the involved orbitals.

Transition σ ⟶ σ*

The strong stability of σ bonds in organic compounds means that the transition of an electron from a bonding σ to an anti-bonding σ* requires a significant amount of energy. The corresponding absorption band is intense and located in the far-UV, around 130 nm.

Transition n ⟶ π*

This transition results from the movement of an electron from a non-bonding n to an anti-bonding π*

This type of transition occurs in molecules containing an heteroatom with unshared electron pairs that belong to an unsaturated system.

These transitions originate from functional groups such as (C=O; N=O; N=N; etc). The molar absorption coefficient is generally low (ε < 100).

Transition n ⟶ σ*

The transfer of an electron from the lone pair n of an heteroatom (O, N, S, Cl...) to a σ* level is observed for alcohols, ethers, amines, as well as halogenated derivatives. This transition results in a moderately intense band that lies at the extreme limit of the near-UV range. It can be detected by standard instruments if λ≥ 200 nm.

Transition π ⟶ π*

These transitions are found in compounds that possess double bonds. They involve the movement of an electron from a π molecular orbital to a π* molecular orbital. These transitions are strong, with an absorption coefficient ranging from 1000 to 10000 $L \cdot mol^{-1} \cdot cm^{-1}$.

4. Absorption and emission spectra

4.1 What does an absorption spectrum looks like ?

When light passes through a sample, certain wavelengths are absorbed by the sample's molecules, causing electronic transitions. The energy levels of the molecules dictate which wavelengths they can absorb. In the UV-Vis range, these transitions often involve movement of electrons between different molecular energy levels. The resulting absorption spectrum can be used to identify the presence of specific compounds in a sample and to quantify their concentrations.

An absorption spectrum in UV-Vis spectroscopy shows the wavelengths of light absorbed by a sample as it transitions from a lower energy state to a higher energy state. It

illustrates which specific wavelengths of light are absorbed by a substance. The absorption spectrum is usually plotted with absorbance on the vertical axis and the wavelength of light on the horizontal axis (Figure 6.2).

Amoxicillin is a famous antibiotic that gives an identification peak at approximately 265nm on the wavelength scale.

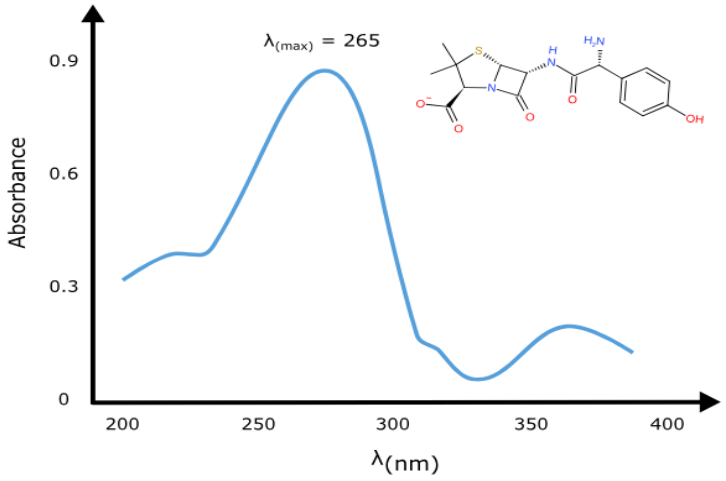


Figure 6.2. *UV-Vis spectrum of amoxicillin*

UV-Vis spectroscopy is additionally employed in the quantitative analysis of analytes. According to the Beer-Lambert law, the absorbance at a specific light wavelength demonstrates direct proportionality to the concentration.

4.2 Emission spectrum

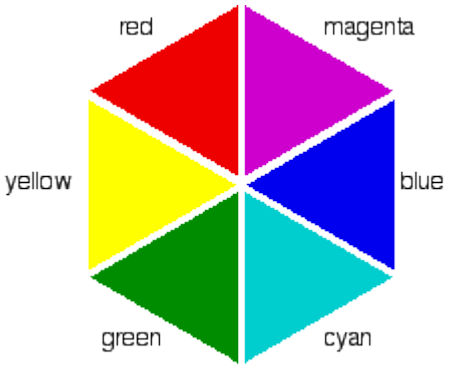
An emission spectrum is a graphical representation of the wavelengths or colors of light emitted by a substance when it undergoes electronic transitions from higher energy states to lower energy states. It's the counterpart to an absorption spectrum, where instead of absorbing light, the substance emits light at specific wavelengths.

Each element or molecule has a unique emission spectrum, which can provide information about its atomic or molecular structure. In the case of atoms, the emission spectrum consists of discrete lines, while molecules may have more complex emission patterns due to vibrational and rotational transitions in addition to electronic transitions.

Complementary colors

Colors directly opposite each other on the color wheel are said to be complementary colors. Blue and yellow are complementary colors; red and cyan are complementary; and so are green and magenta. Mixing together two complementary colors of light will give you white light (Figure 6.3).

If a particular color is absorbed from white light, what your eye detects by mixing up all the other wavelengths of light is its complementary color. In the beta-carotene case, the situation is more confused because you are absorbing such a range of wavelengths. However, if you think of the peak absorption running from the blue into the cyan, it would be reasonable to think of the color you would see as being opposite that where yellow runs into red - in other words, orange.



Violet: 400 - 420 nm
Indigo: 420 - 440 nm
Blue: 440 - 490 nm
Green: 490 - 570 nm
Yellow: 570 - 585 nm
Orange: 585 - 620 nm
Red: 620 - 780 nm

Figure 6.3. *Color complementation in UV-Vis spectroscopy*

5. Shifting of absorption band and change in intensity

In structural analysis, the values of λ_{max} and ε_{max} are used to identify the atom groups responsible for absorption within the molecule, known as chromophores.

Chromophore Group: An unsaturated covalent group responsible for absorption.

Examples include C=C, C=O, C=N, C≡C, C≡N, and more.

Bathochromic shift: The shift of absorption bands towards longer wavelengths is called the bathochromic effect.

Hypsochromic shift: The shift of absorption bands towards shorter wavelengths is called the hypsochromic effect.

Hyperchromic effect: The increase in absorption intensity is called the hyperchromic effect.

Hypochromic effect: The decrease in absorption intensity is called the hypochromic effect.

These terms are commonly used to describe how certain factors, such as chemical modifications or environmental changes, can influence the absorption characteristics of molecules (Figure 6.4).

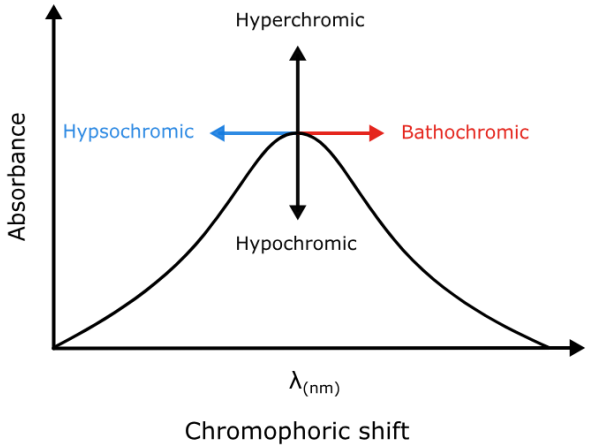


Figure 6.4. *Changes in absorption spectra*

6. Factors affecting change in the absorption and wavelength

These are the factors that result in changes in absorption and wavelength:

1. Effect of conjugation

As per Molecular Orbital Theory (MOT), an escalation in the count of pi electrons leads to an augmented delocalization. This expanded delocalization brings about the stabilization of the sample molecules, consequently reaching a lower energy state. This energy reduction prompts a shift in the wavelength towards a lengthier wavelength, commonly referred to as a redshift.

For instance, 1,2-butadiene demonstrates maximum absorption at 210nm, while 1,3-butadiene exhibits it at 217nm due to the involvement of conjugated double bonds. This wavelength shift becomes more pronounced, extending to 260nm for 1,3,5-hexatriene, attributed to the presence of conjugation at two distinct positions (Figure 6.5).

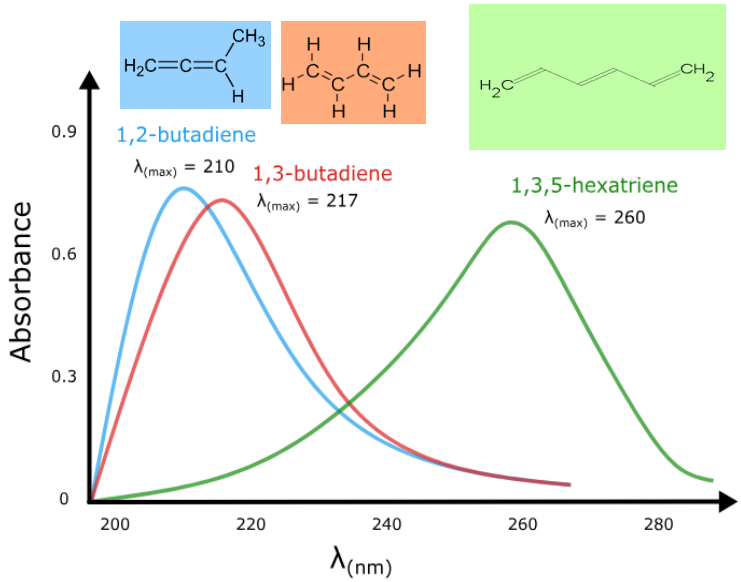


Figure 6.5. *Effect of conjugation*

2. Effect of additive characteristics

When a molecule contains two or more chromophores separated by more than one single bond the total absorption is equal to the sum of absorption characteristics of each chromophore.

For example, ethylene and 1,5 hexadiene absorb the same wavelength. But the amount of radiation absorbed for similar concentrations is almost doubled for 1,5 hexadiene than ethylene.

3. Effect of the aromatic ring

The aromatic ring especially when two or more rings in conjugation (polycyclic compounds) absorbs a higher wavelength in the visible region, it alters the spectrum of absorption.

For example

Naphthalene (C_6H_6) absorbs at 268nm, anthracene absorbs at 311nm, tetracene absorbs at 476nm (Figure 6.6).

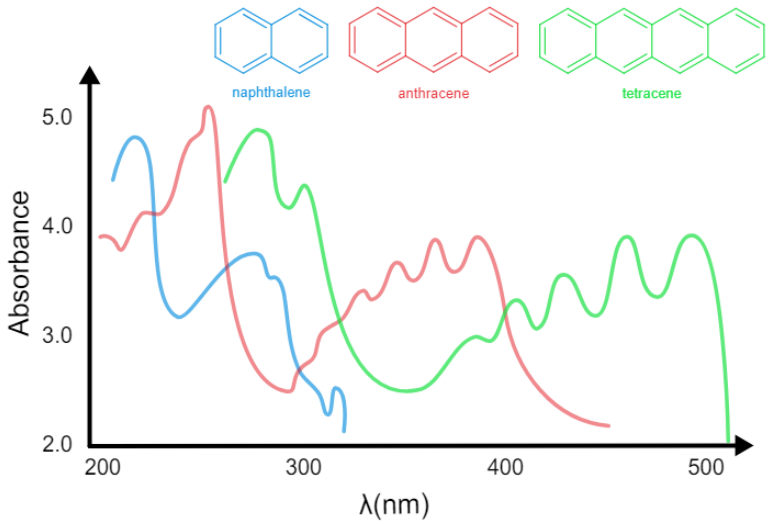


Figure 6.6. *UV-Vis spectrum of polycyclic organic compounds*

4. Effect of substitution of auxochrome

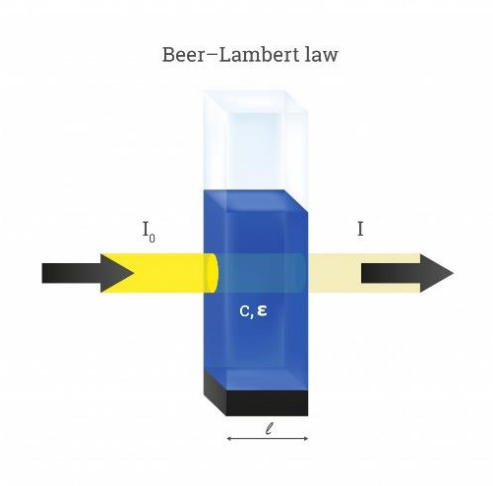
Benzene is a less effective chromophore, the substitution of a polar group to it causes an increase in λ_{max} in the visible region and hence ε_{max} value increase.

For example

Auxochrome	compound	λ_{max}	ε_{max}
	Benzene	256	250
-NH_2	Aniline	280	200
-Cl	Chlorobenzene	265	360

7. Law of light absorption - Beer-Lambert Law

The Beer-Lambert Law, also known as Beer's Law, is a fundamental principle in spectroscopy that relates the absorption of light by a substance to its concentration and the path length through which the light passes. This law is essential for quantifying the relationship between the amount of light absorbed by a sample and the properties of that sample.



Mathematically, the Beer-Lambert Law is expressed as:

$$A = \varepsilon * c * l$$

Where:

A is the absorbance of the sample.

ε (epsilon) is the molar absorptivity or molar extinction coefficient, which represents how strongly the substance absorbs light at a specific wavelength.

c is the concentration of the substance in the sample.

l is the path length through which the light travels through the sample.

The law is often written in logarithmic form as:

$$A = \log(I_0/I) = \varepsilon * c * l$$

Where I_0 is the intensity of the incident light and I is the intensity of the transmitted light.

Overall, the Beer-Lambert Law is a foundational concept that has applications in many fields, including chemistry, biochemistry, environmental science, and more, where the absorption of light is used to quantify the amount of a specific substance in a sample**.**

The law is a fundamental basis for quantitative analysis, allowing scientists to determine concentrations of unknown samples based on their absorbance measurements and a calibration curve.

8. Limitations of lambert beer law

- The light source used must be monochromatic.
- This is not suitable for concentrated solutions i.e. It can only be applicable to dilute solutions.
- With an increase in dilution, the dissociation of weak acids occurs. The weak acids reach equilibrium with their conjugate base. The acid (HA) and conjugate base (A–) cannot have the same absorbance. Hence this law is not completely applicable to weak acidic solutions.

Additivity property

Additivity, in the context of the Beer-Lambert Law and spectroscopy, refers to the principle that the total absorption of light by a mixture of absorbing substances is the sum of the individual absorptions contributed by each component. This principle is particularly relevant when multiple substances with distinct absorbing characteristics are present in a solution.

In other words, if you have a solution containing multiple absorbing species, you can determine the overall absorption at a certain wavelength by adding up the absorptions that each individual component would produce at that wavelength.

This concept is useful in situations where different components in a solution contribute to the overall absorption spectrum. It allows you to analyze complex mixtures and determine the contributions of individual components to the total absorption.

9. Determination of solution concentration through calibration

Using the Beer-Lambert law, it's possible to determine the concentration of a species by measuring its absorbance.

- Determining the wavelength corresponding to the maximum absorption, λmax.

- A series of solutions is prepared at various concentrations, C_i, and the absorbance, A_i, of each of these solutions is measured at λ_{max}.
- The calibration curve $A_i=f(C_i)$ is plotted (Figure 6.7).
- The absorbance, A of our solution of unknown concentration is measured at λmax. Using the curve, we can read the concentration, C of our solution from the absorbance A.

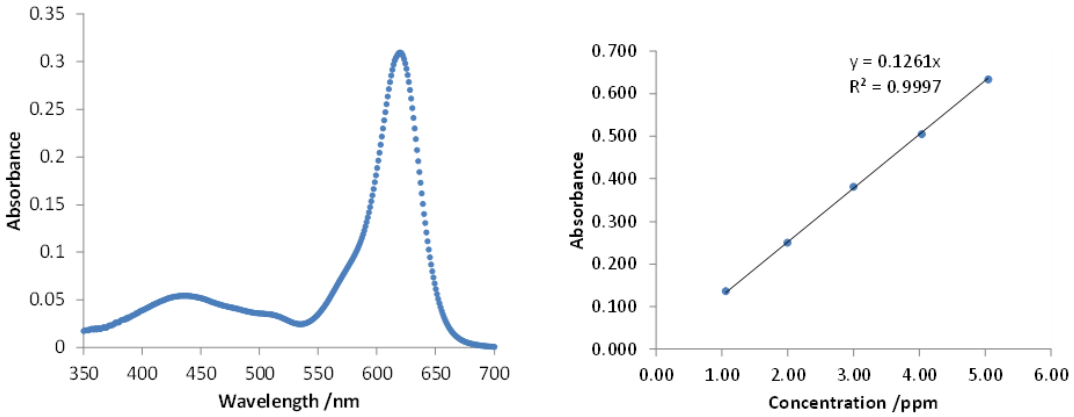


Figure 6.7. *A UV-Vis spectrum of a metabolite extracted from a sample is shown on the left graph. A calibration curve shown on the right graph was developed from standard diluted solutions of the metabolite.*

Reproducible results are essential to ensure precise, high quality measurements. Standard deviation, relative standard deviation, or the coefficient of variation help to determine how precise the system and measurements are. A low deviation or variation indicates a higher level of precision and reliability.

10. Instruments and experimental methods

Description of UV/Vis spectrophotometer

There are two types of UV-Vis spectrophotometers

- Single beam UV-Vis spectrometer
- Double beam UV-Vis spectrometer

10.1 Single beam UV-Visible spectrophotometer

Single beam UV-Vis spectrophotometer has a single beam as the name indicates. The incident light coming from the source is passed through a monochromator then that incident monochromatic light moves through a slit. Then it passes through the sample solution. Where some of the incident light is absorbed by the sample while other is transmitted. That transmitted light is detected by the detector. The detected light is then amplified, recorded,

and displayed on a suitable readout device (Figure 6.8). Spectrum is plotted and the λ_{max} is located.

Single beam UV-Vis spectrophotometer comprises of:

- Light source
- Wavelength selector
- Sample container/cuvette
- Detector
- Digital meter/Recorder

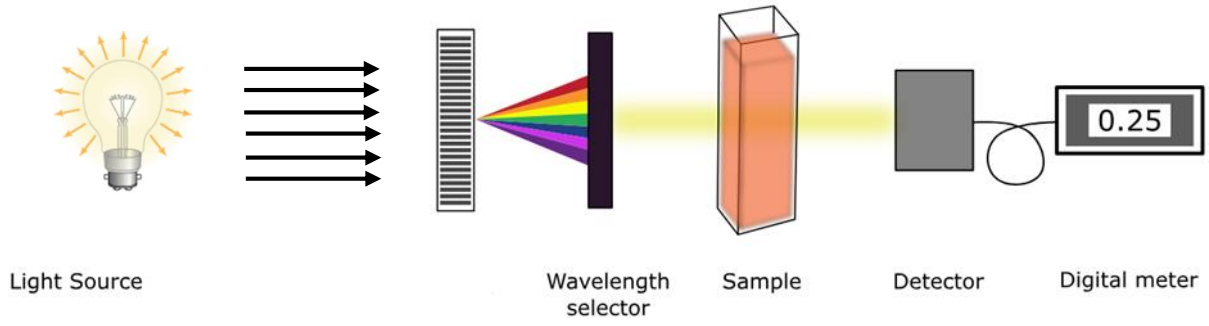


Figure 6.8. *A simplified schematic of the main components in a single beam UV-Visible spectrophotometer*

Light sources

- Deuterium lamps provide UV light, while tungsten or halogen lamps offer visible light.
- Xenon arc lamps cover a broader range, making them suitable for UV-Vis measurements.

Monochromator

- Selects specific wavelengths by dispersing light into its component colors using prisms or diffraction gratings.
- Allows for the adjustment of wavelength and bandwidth to optimize measurements.

Sample compartment

Whichever wavelength selector is used in the spectrophotometer, the light then passes through a sample. For all analyses, measuring a reference sample, often referred to as the "blank sample", such as a cuvette filled with a similar solvent used to prepare the sample, is imperative.

When examining bacterial cultures, the sterile culture media would be used as the reference. The reference sample signal is then later used automatically by the instrument to help obtain the true absorbance values of the analytes.

Sample containers or cuvettes may be made up of quartz, borosilicate and plastic

- Only quartz is transparent in both UV and visible regions (200-700nm range).
- Glass and plastic are suitable for the visible region only.
- Glass is not suitable for the UV region because it absorbs UV radiation i.e. it is not transparent in the UV region.
- Plastic cells are not used for organic solvents.

The most common cuvette size is 1 cm, although it can vary from 0.1-10 cm.

Cuvette-free systems are also available that enable the analysis of very small sample volumes, for example in DNA or RNA analyses.

Detectors

After the light has passed through the sample, a detector is used to convert the light into a readable electronic signal. Generally, detectors are based on photoelectric coatings or semiconductors.

10.2 Double beam UV-Visible spectrophotometer

Double-beam spectrophotometers have two beams: one passes through the sample compartment, and the other through the reference compartment. In this case, there is no need to perform a blank measurement, as the subtraction is automatically handled by the calculation software (Figure 6.9).

The incident light splits and is directed towards both the reference and sample cuvette. The refracted or transmitted beam is detected by the detectors. A double beam UV-Vis spectrophotometer needs two detectors that detect electron ratio to measure absorbance in a test sample. It also requires a stabilized voltage supply.

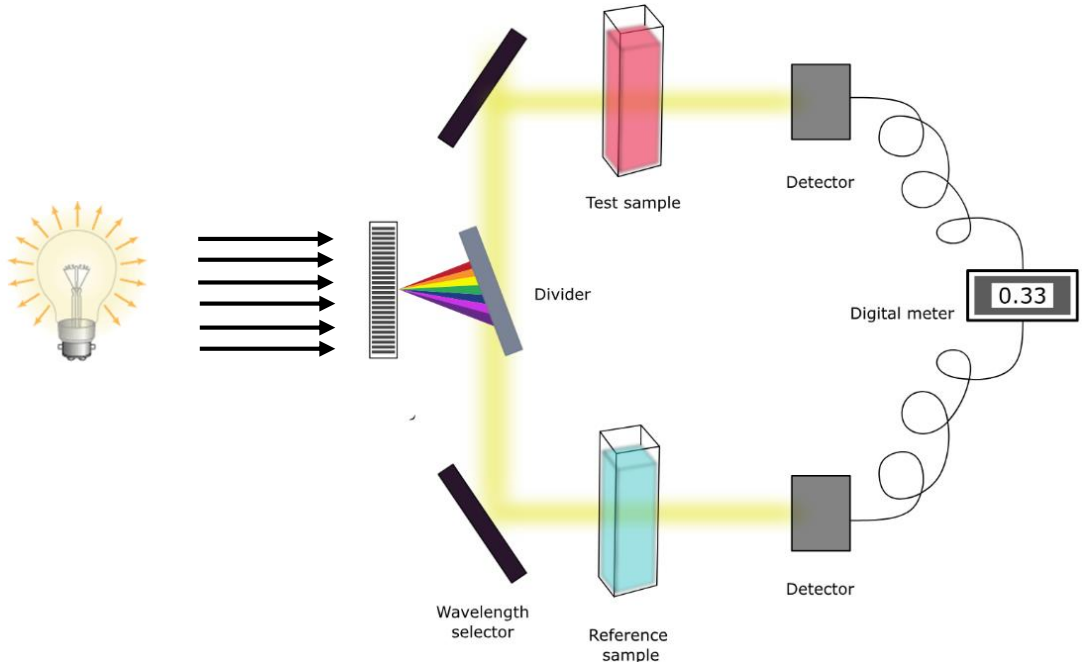


Figure 6.9. *A simplified schematic of the main components in a double beam UV-Visible spectrophotometer*

Experimental methods

Preparation of Samples

Samples should be appropriately diluted to ensure measurements fall within the linear range of the Beer-Lambert Law. Solutions should be clear and free from particulates that might scatter light.

Blank measurement

A blank reference containing the solvent is measured to account for solvent absorbance. Subtracting this reference from sample measurements corrects for solvent contributions.

Data acquisition

Absorbance or transmittance values are recorded across the desired wavelength range. Data can be collected in continuous or discrete modes, depending on the instrument and experiment.

Data analysis

Quantitative analysis involves constructing calibration curves using standards of known concentrations. Software tools assist in curve fitting, determining concentrations, and identifying unknowns.

11. Importance and applications

UV/Visible spectroscopy holds significant importance in the field of analytical chemistry and has a wide scope of applications. Here are some key points highlighting its importance and areas of application.

- ***Quantitative analysis***

UV/Visible spectroscopy allows for the quantitative determination of substances in various samples. By measuring the absorbance of light at specific wavelengths, we can establish concentration relationships using the Beer-Lambert Law. This makes it an invaluable tool for determining the concentration of compounds in solution, such as pharmaceuticals, environmental pollutants, and biological samples.

- ***Structural analysis***

The absorption spectra obtained from UV/Visible spectroscopy provide insights into the structural characteristics of molecules. It helps identify functional groups, electronic transitions, and conjugated systems within compounds. This information is crucial for understanding the chemical properties, reactivity, and behavior of substances.

- ***Chemical kinetics and reaction monitoring***

UV/Visible spectroscopy enables real-time monitoring of chemical reactions. By continuously measuring the absorbance of reactants and products at specific wavelengths, it allows researchers to study reaction rates, identify intermediate species, and investigate reaction mechanisms. This is particularly useful in areas such as enzymology, catalysis, and organic synthesis.

- ***Pharmaceutical analysis***

UV/Visible spectroscopy plays a vital role in pharmaceutical analysis. It is used for quality control, determination of drug concentrations in formulations, stability studies, and identification of impurities. The technique is widely employed in the pharmaceutical industry to ensure the safety, efficacy, and consistency of drug products.

- ***Environmental analysis***

UV/Visible spectroscopy is applied in environmental analysis for the detection and quantification of pollutants. It helps identify harmful compounds in air, water, and soil samples, such as heavy metals, pesticides, and organic pollutants. Monitoring these substances is crucial for assessing environmental impact, ensuring regulatory compliance, and safeguarding public health.

- ***Biochemical and biological studies***

UV/Visible spectroscopy is extensively used in biochemical and biological research. It aids in the characterization of biomolecules such as proteins, nucleic acids, and carbohydrates. By examining their absorption spectra, researchers can study protein folding, DNA-protein interactions, enzyme kinetics, and ligand binding. It also assists in determining the purity and concentration of biomolecular samples.

- ***Food and beverage analysis***

UV/Visible spectroscopy finds application in the food and beverage industry. It helps in assessing the quality, authenticity, and safety of food products. The technique is used for determining nutrient content, identifying additives, detecting contaminants, and evaluating the degree of food spoilage (Figure 6.10).

The scope of UV/Visible spectroscopy is non-destructive nature, simplicity, and versatility make it is an indispensable tool in various scientific disciplines. Whether in academic research, industrial laboratories, or quality control settings, UV/Visible spectroscopy offers valuable insights into the chemical and biological properties of substances, contributing to advancements in numerous fields.

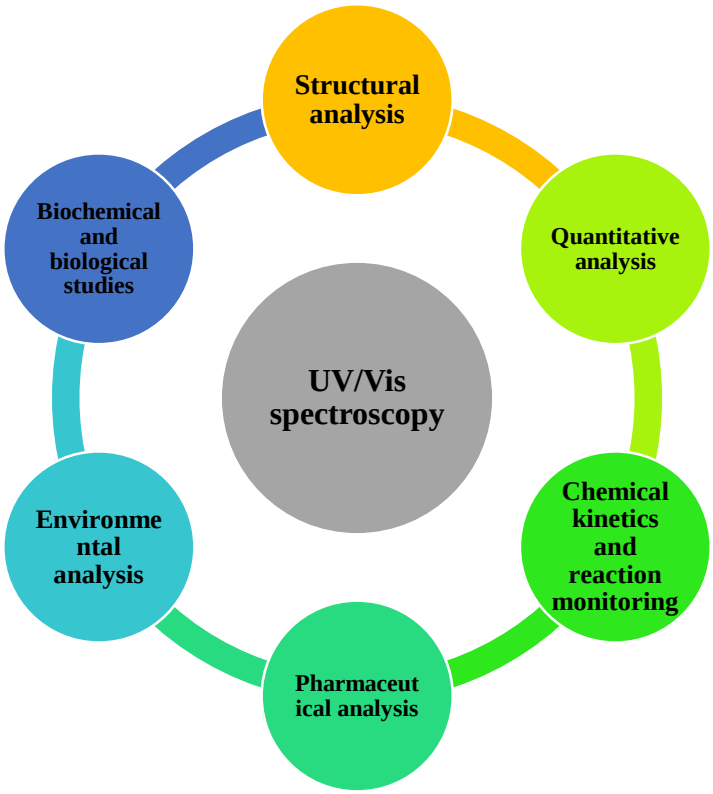


Figure 6.10. *Different fields of UV/Vis spectroscopy applications*

Applications

Application 1

Paints and varnishes are used outside buildings to protect them against the effects of solar radiation which can accelerate their degradation (photolysis and photochemical reactions). What should be the concentration expressed in g/L of a UV "M" additive such that 90% of the radiation is absorbed by a 0.3 mm thick coating?

Data: Molar mass of M = 500 g/mol, $\varepsilon = 15{,}000\ M^{-1}.cm^{-1}$ for $\lambda max = 350$ nm.

Application 2

We have a stock solution of copper sulfate at 1 mol/L. Various dilutions are made, and their absorbance is measured at the wavelength of 655 nm, which corresponds to the maximum of the A = f(λ) curve for a copper sulfate solution. The cuvette's width is 1 cm. The obtained table is as follows:

C(mol/L)	0.20	0.10	0.050	0.020	0.010	0.0050
A	0.601	0.302	0.151	0.060	0.031	0.016

1. Why was this wavelength chosen for work? In what range is it located?

2. Is the Beer-Lambert law verified?

3. Determine the molar absorbance coefficient under these conditions.

4. What is the concentration of a copper sulfate solution with an absorbance of A=0.2?

Application 3

We are studying the absorption of light by a solution containing two compounds, A and B. Their molar masses are M_A = 200 g/mol and M_B = 400 g/mol, respectively. In the solution, the concentration of compound A is 0.05 g/L, and that of B is 0.06 g/L. The molar extinction coefficients are as follows :

For A: $\varepsilon_1 = 500\ M^{-1}\cdot cm^{-1}$, $\varepsilon_2 = 1000$ m=$M^{-1}\cdot cm^{-1}$

For B: $\varepsilon_1 = 2000\ M^{-1}\cdot cm^{-1}$, $\varepsilon_2 = 3000\ M^{-1}\cdot cm^{-1}$.

The cuvette thickness is 1 cm.

What will be the absorbance of the solution at two wavelengths: 500 nm and 450 nm?

Application 4

An aqueous solution of potassium permanganate ($C = 1.28.10^{-4}M$) has a transmittance of 0.5 at 525 nm, if a cell with an optical path of 10 mm is used.

1. Calculate the molar absorption coefficient of permanganate for this wavelength.

2. If we double the concentration, calculate the absorbance and transmittance of the new solution.

Chapter 7

Infrared Spectroscopy

CHAPTER

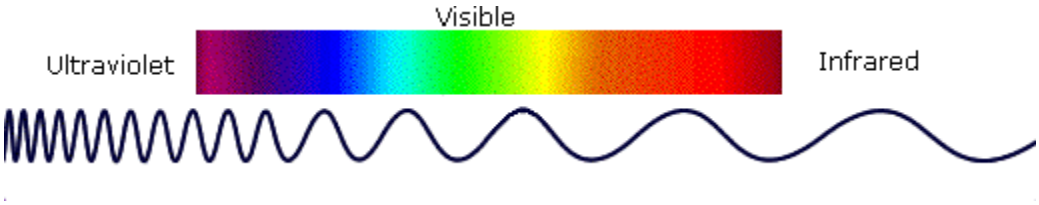
7

Infrared Spectroscopy

1. Definition

Infrared spectroscopy deals with the infrared region of the electromagnetic spectrum, i.e. light having a longer wavelength and a lower frequency than visible light.

It is a scientific technique that involves the study of molecular vibrations by measuring the absorption and transmission of infrared radiation through a sample. On the immediate high energy side of the visible spectrum lies the ultraviolet, and on the low energy side is the infrared. The portion of the infrared region most useful for analysis of organic compounds is not immediately adjacent to the visible spectrum, but is that having a wavelength range from 2,500 to 16,000 nm, with a corresponding frequency range from $1.9*10^{13}$ to $1.2*10^{14}$ Hz.



It provides valuable information about the types of chemical bonds and functional groups present in a molecule, helping to identify and characterize compounds in various fields such as chemistry, biology, material science, and more.

2. Historical background

The groundwork for infrared spectroscopy was laid by early 19th-century scientists like Sir William Herschel, who discovered the existence of infrared radiation beyond the visible spectrum in 1800 (Figure 7.1).

However, the full potential of infrared radiation and its applications in spectroscopy were not realized until later.

The development of the first practical infrared spectrometer is attributed to Samuel Ruben and Horace Isbell at the National Carbon Company (NCC) in the early 1940s.

They designed a dispersive infrared spectrometer, which was used to study the absorption of infrared radiation by various organic compounds.

In 1945, Hermann Arthur Jahn and Edward Teller published a groundbreaking paper on molecular vibrations and the theory of infrared absorption.

The 1950s saw advancements in instrumentation, with the introduction of Fourier Transform Infrared (FTIR) spectroscopy by Peter Fellgett and Alan Walsh in the late 1950s. This FTIR technique revolutionized infrared spectroscopy by offering faster data collection and greater sensitivity compared to dispersive methods.

Figure 7.1. *Sir William Herschel (1738-1822), Royal Astronomer of England, already famous for his discovery of the planet Uranus, was searching for an optical filter material to reduce the brightness of the Sun's image in telescopes during solar observations*

3. Theoretical foundations of infrared spectroscopy

3.1 Spectral range

The infrared spectrum is typically divided into several regions based on wavelength.

Near-Infrared (NIR)

Wavelength range: Approximately **700 nm to 2500 nm**.

This region is closest to the visible spectrum and is often used in applications such as remote sensing, fiber optics, and imaging.

Mid-Infrared (MIR)

Wavelength Range: Approximately **2500 nm to 25,000 nm (2.5 µm to 25 µm)**.

The MIR region is most commonly used in traditional infrared spectroscopy and is highly informative for identifying molecular functional groups and chemical bonds.

Far-Infrared (FIR) region

Wavelength Range: Approximately **25,000 nm to 1 mm (25 µm to 1000 µm)**.

This region has applications in astronomy, material science, and the study of molecular rotations.

3.2 Principle

The principle of infrared spectroscopy is based on the interaction between molecules and infrared radiation. Molecules consist of atoms connected by chemical bonds, and these bonds can vibrate in different ways. When a molecule absorbs infrared radiation, it undergoes vibrational transitions, leading to changes in its energy state. This interaction between molecular vibrations and infrared radiation forms the foundation of IR spectroscopy.

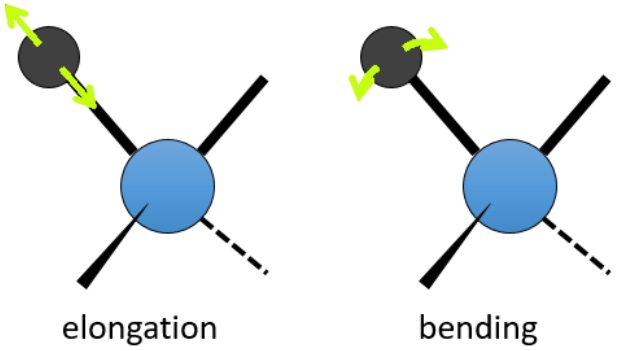
IR Spectroscopy detects frequencies of infrared light that are absorbed by a molecule. Molecules tend to absorb these specific frequencies of light since they correspond to the frequency of the vibration of bonds in the molecule.

Different types of chemical bonds and functional groups have characteristic vibrational frequencies. As a result, when a molecule absorbs infrared radiation, it does so at specific frequencies that correspond to its vibrational modes. These absorbed frequencies are recorded as peaks in an infrared spectrum (Bec et al., 2020).

3.3 Molecular vibrations

Molecules are not static entities; their atoms are constantly in motion due to thermal energy. This motion includes vibrations of the atoms around their equilibrium positions. Bonds can stretch (increase or decrease in bond length) or bend (change in bond angle) in different ways.

There are two main modes of vibration: the **elongation** and the **bending**.



In the elongation mode, the atoms vibrate around their mass centre with a frequency that is characteristic of the pair of atoms.

The bending is a vibration out of the axis of the liaison. It leads to a variation of the angles between liaisons.

Each mode shows a specific vibration with a given wavelength. The wavelengths of the elongation modes are very close to each other and the bending mode has a smaller wavenumber (Bec et al., 2020) (Figure 7.2).

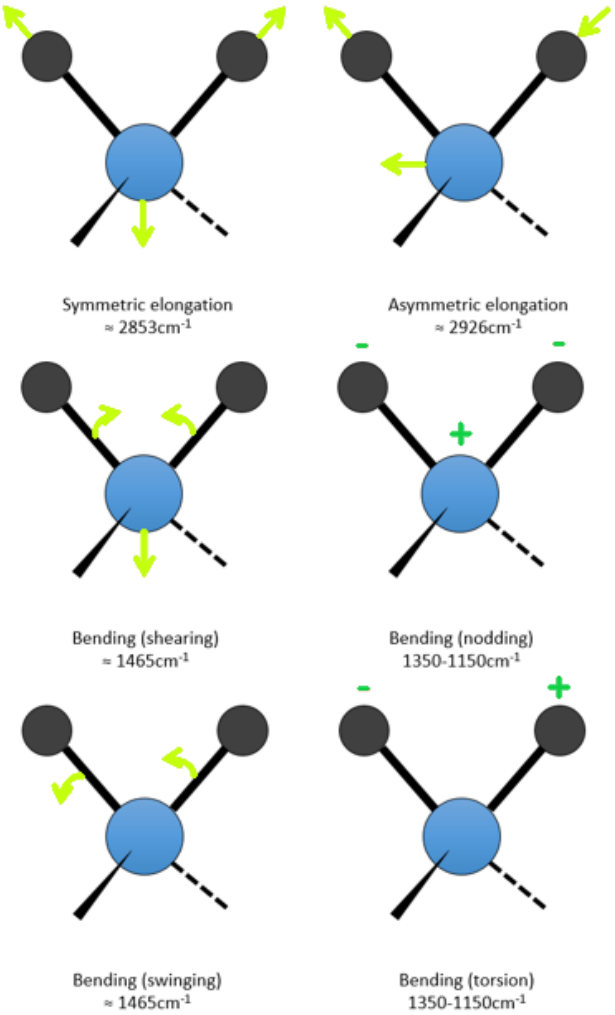


Figure 7.2. *Modes of molecular vibration*

Some bonds can stretch in-plane (symmetric stretching) or out-of-plane (asymmetric elongation).

Bending vibrations can be either in-plane (as; shearing, swinging) or out-of-plane (as; nodding, torsion) bending vibrations.

Stretching Vibrations

Generally, stretching vibrations require more energy and show absorption bands in the higher wavenumber/frequency region.

4. Infrared spectrum

4.1 What does an absorption spectrum looks like ?

The output of an IR spectroscopy experiment is a graph called an infrared spectrum. The spectrum displays the intensity of absorbed radiation (transmittance) on the y-axis and the

frequency of the absorbed radiation (measured in wavenumbers, cm^{-1}) on the x-axis. Peaks on the spectrum correspond to specific vibrational modes and provide information about the types of bonds and functional groups in the molecule.

The spectrum is to be read from top to bottom with bands dropping low in the spectrum (Figure 7.3).

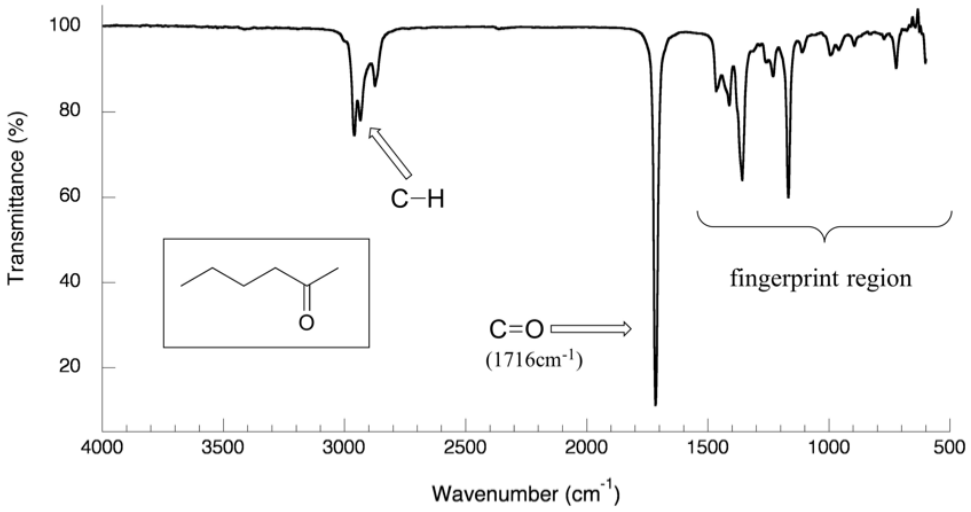


Figure 7.3. *IR spectrum of 2-hexanone*

4.2 Analysis of a spectrum

A precise interpretation of an IR spectrum is challenging. The interactions between different frequencies, the lack of specific vibration patterns, and the varying width of certain bands contribute to the complexity of identifying the precise characteristics of the bands. Nonetheless, each unique IR spectrum functions akin to a distinctive fingerprint for a particular compound. When one spectrum aligns with the spectrum of a recognized molecule (using the same solvent, concentration, and experimental conditions), the task is accomplished.

Most of the bands that indicate what functional group is present are found in the region from 4000 cm^{-1} to 1300 cm^{-1}. Their bands can be identified and used to determine the functional group of an unknown compound.

The spectrum, usually between 4000 and 400cm^{-1} can be split in three important regions to analyse:

Between 4000 and 1300cm^{-1}, called region of the functional groups

It contains stretching bands corresponding to specific functional groups, such as: O-H, C=O, N-H, and C-H. Valence vibrations of hydrogen atoms (X-H) are intense and are found in the

range from 3700 to 2700 cm^{-1}. O-H and N-H vibrations appear between 3700 and 3100 cm^{-1}, while those of Csp-H and Csp2-H are situated between 3300 and 3000 cm^{-1}, and the C-H vibrations of alkanes between 2980 and 2840 cm^{-1}. A characteristic vibration of Carbonyl-H appears as a doublet between 2830 and 2695 cm^{-1}.

A limited number of groups absorb in the region of triple bonds: 2700 – 1850 cm-1. In this region, vibration peaks of X-H, with X = S, P, are also located. However, all organic compounds containing a carbonyl group C=O exhibit a distinct and intense characteristic absorption between 1870 and 1540 cm-1. Absorption peaks of C=C and C=N valence vibrations are situated in the range of 1690 – 1600 cm-1. The 1650 – 1450 cm-1 region is significant concerning aromatic derivatives.

1300- 900cm^{-1} called fingerprint region

In this region, numerous deformation bands and some stretching bands are located. However, analyzing this region yields a characteristic spectrum for each substance. The in-plane deformation vibrations are as follows:

- Symmetric CH_3 deformation (1380 - 1370 cm^{-1}) and asymmetric deformation (1470-1430 cm^{-1}).
- In-plane CH_2 deformation: between 1485 and 1445 cm^{-1},
- Angular (Car-H) deformation in-plane: situated in the range of 1300 to 1000 cm^{-1},
- (O—H) deformation appears between 1440 and 1330 cm^{-1},
- Deformation of the Carbonyl-H bond: located between 1440 and 1320 cm^{-1}. Characteristic valence vibrations in this region involve the C-O bond in various organic functions and C-N bond for alcohols. The range extends from 1260 to 1000 cm^{-1}. For acids, the absorption band appears between 1300 and 1200 cm^{-1}. Thus, for esters, two bands appear: one between 1210-1260 cm^{-1}, and the other coupled with the C–O bond: O-C-C, dependent on the alcohol.

Under 900 cm^{-1} aromatic region

This is a crucial region for determining ethylenic and aromatic structures, thanks to the out-of-plane deformation bands of C-H bonds and the deformation bands of the ring (Figure 7.4).

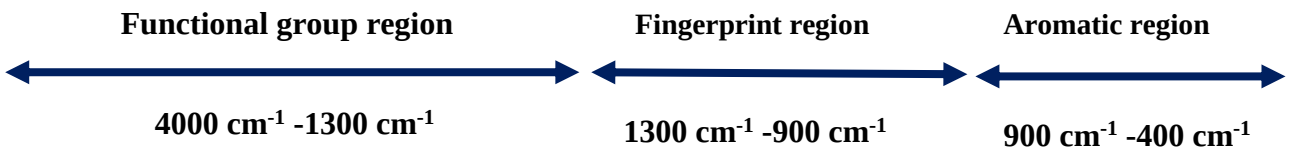


Figure 7.4. *Regions of the infrared spectrum*

The vibration frequency values of certain functions are grouped in the following Figure 7.5.

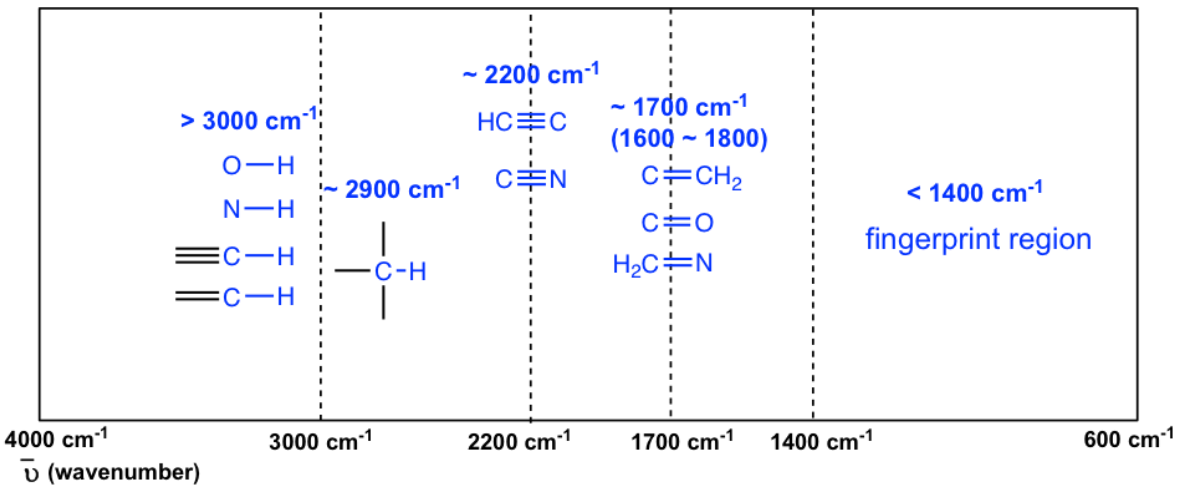


Figure 7.5. *Approximate IR absorption range*

The absorption bands in IR spectra have different intensities that can usually be referred to as **strong** (s), **medium** (m), **weak** (w), broad and sharp (Figure 7.6). The intensity of an absorption band depends on the polarity of the bond, and a bond with higher polarity will show a more intense absorption band. The intensity also depends on the number of bonds responsible for the absorption, and an absorption band with more bonds involved has a higher intensity.

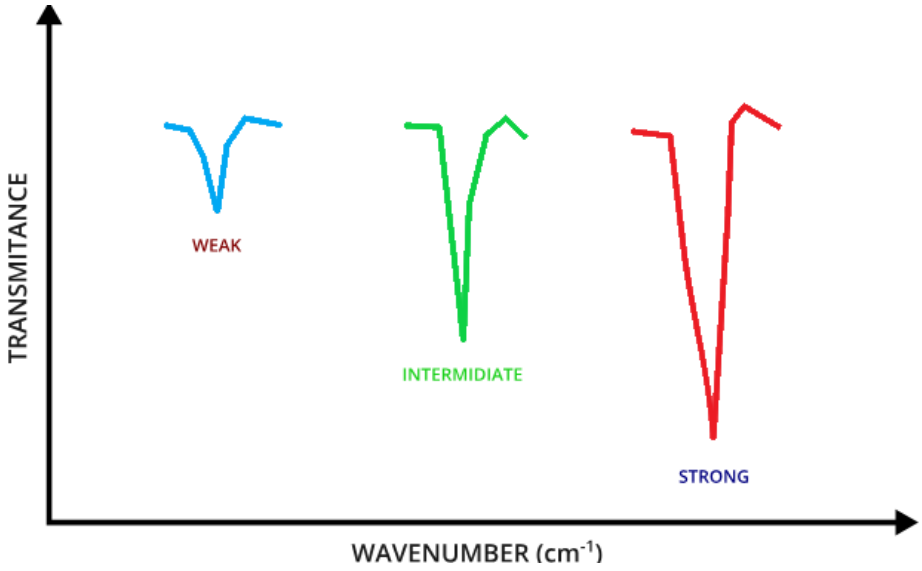


Figure 7.6. *Types of bands in IR spectrum*

The polar **O-H** bond, commonly found in alcohols and carboxylic acids, typically exhibits pronounced and wide absorption bands that are easily recognizable. The broad nature of the absorption band arises due to hydrogen bonding interactions among OH groups between molecules. The O-H bond in alcohol groups typically demonstrates absorption within the range of 3200–3600 cm-1, whereas the O-H bond in carboxylic acid groups is typically observed at approximately 2500–3300 cm-1.

The polarity of the **N-H** bond (in amine and amide) is weaker than the OH bond, so the absorption band of N-H is not as intense or as broad as O-H, and the position is in the 3300–3500 cm^{-1} region.

The **C-H** bond stretching of all hydrocarbons occurs in the range of 2800–3300 cm-1, and the exact location can be used to distinguish between alkane, alkene and alkyne. Specifically:

- ≡C-H (sp C-H) bond of terminal alkyne gives absorption at about 3300 cm^{-1}
- =C-H (sp^2 C-H) bond of alkene gives absorption at about 3000-3100 cm^{-1}
- -C-H (sp^3 C-H) bond of alkane gives absorption at about ~2900 cm^{-1} (see the example of the IR spectrum of 2-hexanone in Figure 7.3 **;** the C-H absorption band at about 2900 cm^{-1}).
- A special note should be made for the C-H bond stretching of an aldehyde group that shows two absorption bands: one at ~2800 cm^{-1} and the other at ~ 2700 cm^{-1}. It is therefore relatively easy to identify the aldehyde group (together with the C=O stretching at about 1700 cm^{-1}) since essentially no other absorptions occur at these wavenumbers (see the example of the IR spectrum of butanal in Figure 7.7.

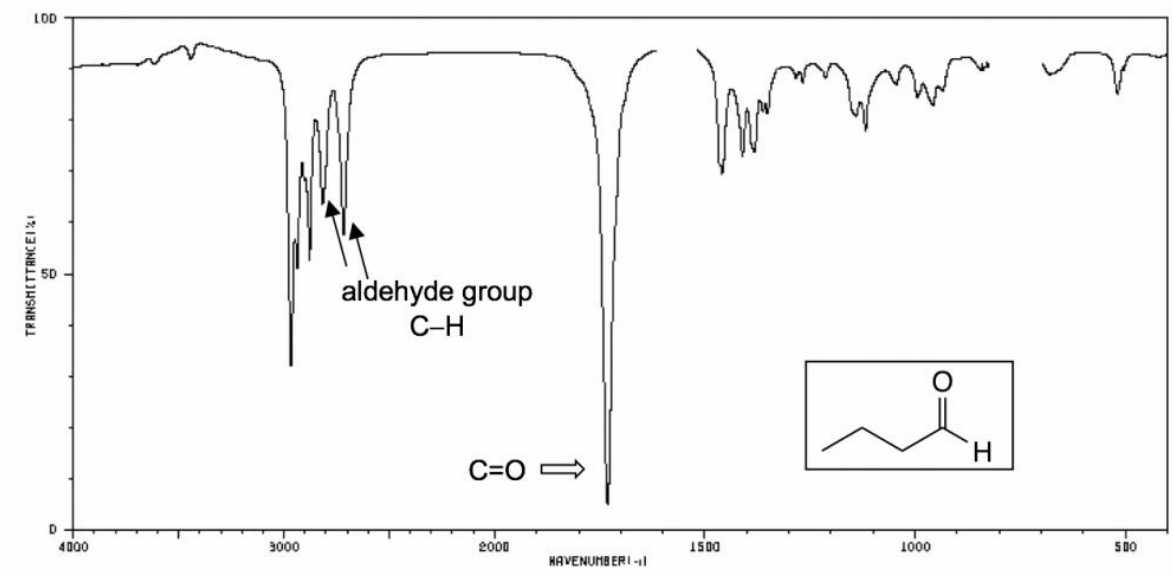


Figure 7.7. *IR spectrum of butanal*

5. IR spectrum interpretation practice

It's crucial to note that the objective isn't usually to identify every absorption band within an IR spectrum. Instead, our focus is on identifying the distinctive absorption band to confirm whether a specific functional group is present or absent.

In most cases, an IR spectrum doesn't offer sufficient data for a comprehensive determination of a molecule's complete structure. Complementary instrumental techniques, like NMR, need to be employed in tandem, as NMR provides more targeted insights into molecular structures and is a more potent analytical tool.

IR spectrum of 1-hexanol

There are sp3 C-H stretching bands of alkane at about 2800–3000 cm^{-1} as expected. There is a very broad peak centered at about 3400 cm^{-1} which is the characteristic band of the O-H stretching mode of alcohols (Figure 7.8). .

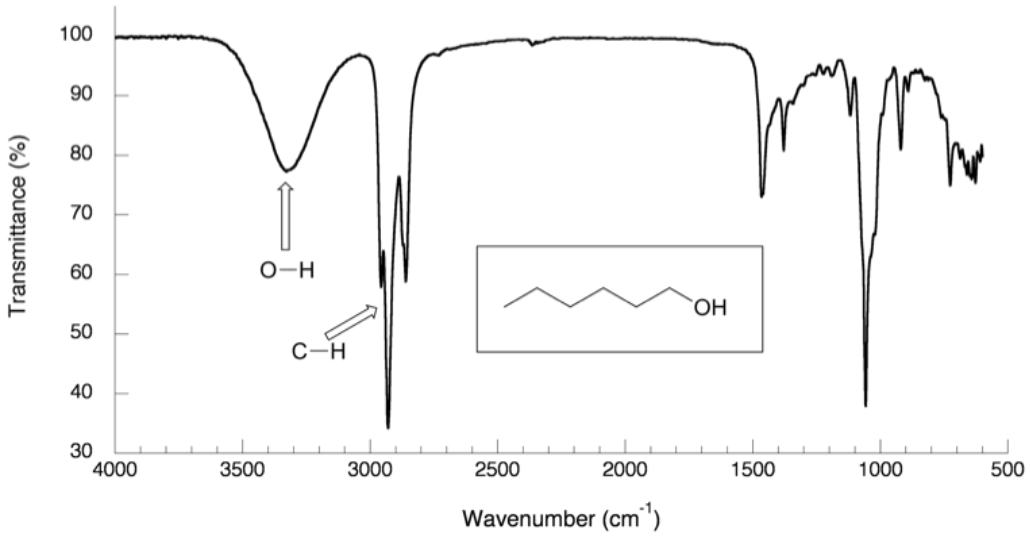


Figure 7.8. *IR spectrum of 1-hexanol*

IR Spectrum of 1-octene

It shows two bands that are characteristic of alkenes: the one at 1642 cm^{-1} is due to stretching of the carbon-carbon double bond, and the one at 3079 cm^{-1} is due to stretching of the σ bond between the sp^2-hybridized alkene carbons and their attached hydrogens (Figure 7.9).

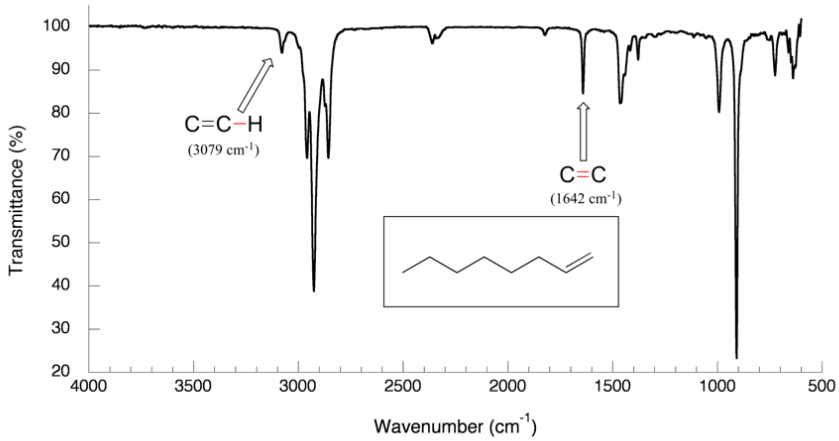


Figure 7.9. *IR Spectrum of 1-octene*

6. Samples in infrared spectroscopy

The samples used in IR spectroscopy can be either in the solid, liquid, or gaseous state.

- **Solid samples** are commonly analyzed in infrared spectroscopy. These samples can be in the form of powders, films, crystals, or solids pressed into pellets. The method of sample preparation depends on the nature of the solid and the instrument being used. Attenuated Total Reflectance (ATR) is a technique commonly employed for solid samples, as it allows for direct analysis of samples without extensive preparation.
- **Liquid samples** can be analyzed using transmission or ATR techniques. For transmission, the liquid is often placed between two transparent windows to allow the infrared light to pass through.
- Since the concentration of **gaseous samples** can be in parts per million, the sample cell must have a relatively long pathlength, i.e. light must travel for a relatively long distance in the sample cell.

7. Instrumentation

The components of an IR spectrometer consist of an infrared radiation source, a radiation separation system (monochromator), and a signal detector.

1. IR radiation sources

IR instruments require a source of radiant energy which emits IR radiation which must be steady, intense enough for detection, and extend over the desired wavelength.

Various sources of IR radiations are as follows.

1. Nernst glower
2. Incandescent lamp
3. Mercury arc
4. Tungsten lamp
5. Glober source
6. Nichrome wire

2. Sample cells and sampling of substances

IR spectroscopy has been used for the characterization of solid, liquid, or gas samples.

- Solid : Various techniques are used for preparing solid samples such as pressed pellet technique, solid run in solution, solid films, mull technique, etc.
- Liquid – Samples can be held using a liquid sample cell made of alkali halides. Aqueous solvents cannot be used as they will dissolve alkali halides. Only organic solvents like chloroform can be used.
- Gas– Sampling of gas is similar to the sampling of liquids.

3. Monochromators

Various types of monochromators are prism, gratings and filters.

- Prisms are made of Potassium bromide, Sodium chloride or Caesium iodide.
- Filters are made up of Lithium Fluoride and Diffraction gratings are made up of alkali halides.

4. Detectors

Detectors are used to measure the intensity of unabsorbed infrared radiation.

Detectors like thermocouples, Bolometers, thermisters, Golay cell, and pyro-electric detectors are used.

5. Recorders

Recorders are used to record the IR spectrum.

In an infrared spectrophotometer, a beam of IR radiation passes through the sample, and some radiation is absorbed by the sample, while the remaining radiation goes through it. Another beam of IR radiation passes through the cell with blank (no sample, no absorption) and all the light goes through it. The detector in the instrument records and compares the radiation transmitted through the sample with that transmitted in the absence of the sample.

Any frequencies absorbed by the sample will be apparent by the difference. The computer plots the result as a graph showing transmittance vs frequency.

The sample is prepared by pelleting the sample with potassium bromide (KBr). Then, 1 mg of the sample is mixed thoroughly with 100mg of anhydrous KBr powder. Now, the mixture is pressed to produce a disk shape pallet. This disc is placed in the IR beam for determination.

The instruments used for analysis based on IR spectroscopy are of two types,

- Dispersive IR spectrophotometer
- FT-IR spectrophotometer

Dispersive IR spectrophotometer

A dispersive IR spectrophotometer is the instrument used to analyze samples by analyzing the IR radiation absorbed by the sample. It uses a prism or grating to separate the different wavelengths of light and detect the intensity of the light at each wavelength using a detector. However, this technique is laborious and hence rarely used (Kalf 2020) (Figure 7.10).

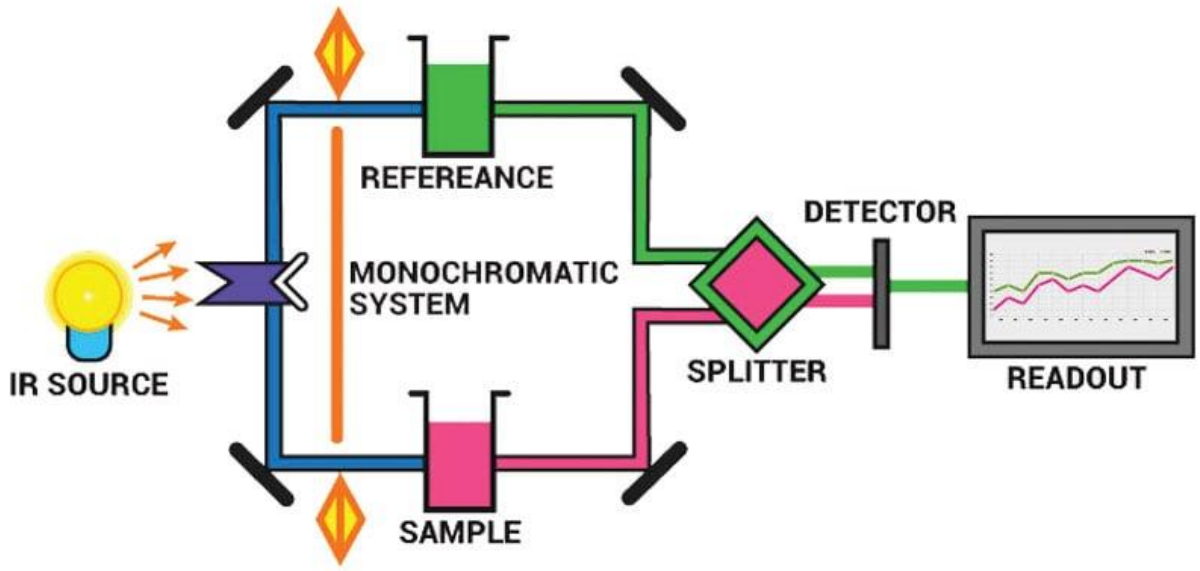


Figure 7.10. *Scheem of a dispersive IR spectrometer*

Fourier transform infrared spectrophotometer

Fourier transform infrared spectrophotometer (FTIR) is an analytical instrument that works on the principle of IR spectroscopy. Fourier transform is an algorithm designed to transform the data obtained from the analysis in the spectral form and plot absorbance against wave number.

It gives rapid results, such that 60 seconds are enough to complete one analysis. The main components of the instrument are described below:

FTIR spectrometer contains a single-beam optical assembly. The interferometer is the essential component – often of the Michelson type – located between the source and the sample (Figure 7.11).

The principle of Fourier-transform infrared spectroscopy is based on the use of interferometry to gather information about molecular interactions in the infrared range. Instead of directly analyzing the frequencies of light waves, as in traditional dispersive spectrometers, the Fourier transform technique measures light intensity as a function of wavelength while utilizing interference properties.

The process begins by splitting the beam of light from the polychromatic source. The beam is divided into two: one passes through a fixed mirror, and the other through a movable mirror. These two beams are then reflected and recombined after passing through a sample. The difference in optical path between the two beams creates an interferogram, a sequence of interference fringes, which is recorded by the detector.

The Fourier transform is then applied to this interferogram, translating spatial information into a frequency spectrum. This spectrum represents the frequency components of molecular interactions in the sample. By comparing the obtained spectrum with reference spectra, functional groups and bonds present in the sample can be identified (Kalf 2020).

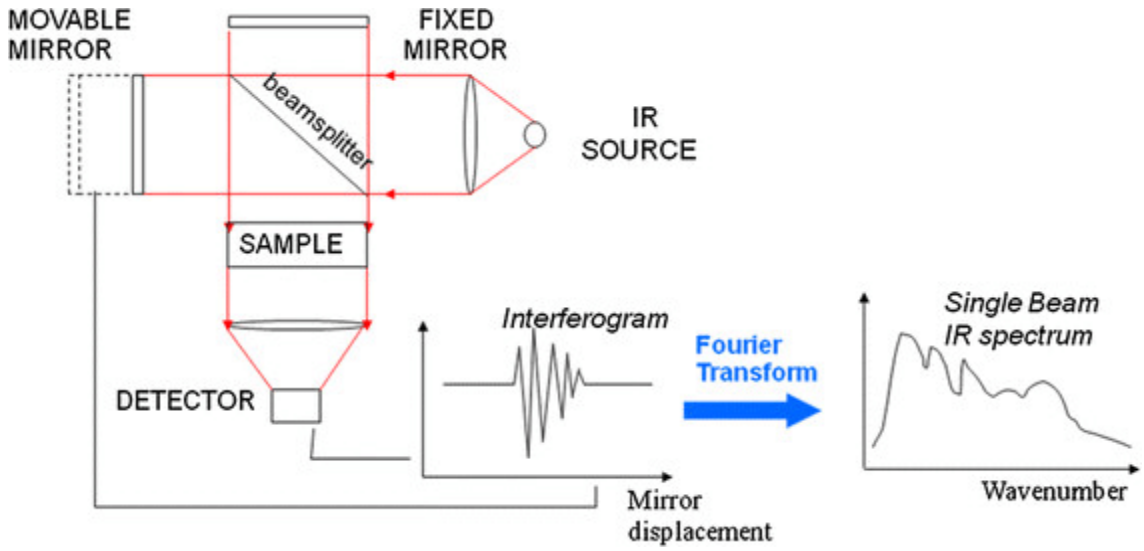


***Figure* 7.11.** *Scheem of a Fourier transform infrared spectrophotometer*

Fourier-transform infrared spectroscopy offers several advantages, including higher sensitivity and faster data acquisition compared to traditional dispersive methods. Here are some of the key advantages:

Speed: FTIR spectrophotometers are significantly faster than dispersive spectrophotometers. They can collect a complete spectrum over a wide range of wavelengths in a matter of seconds, whereas dispersive instruments may take longer to scan the same range.

High sensitivity: FTIR spectrophotometers are highly sensitive, capable of detecting even small changes in sample composition. This sensitivity is especially useful for identifying trace compounds and analyzing samples with low concentrations.

Simplicity: The interferometric design of FTIR spectrophotometers simplifies the optical layout, reducing the need for moving parts and complex mechanisms. This results in greater reliability and ease of maintenance.

Multiplex advantage: FTIR spectrophotometers use interferometry to collect all wavelengths simultaneously, whereas dispersive spectrophotometers collect wavelengths sequentially. This "multiplex advantage" allows FTIR instruments to gather data over a wide spectral range with every measurement.

Versatility: FTIR spectroscopy can analyze a wide range of sample types, including liquids, solids, gases, and even complex mixtures. It's used in various fields, such as chemistry, pharmaceuticals, material science, forensic analysis, and environmental monitoring.

Sample flexibility: FTIR spectrophotometers allow for various sampling techniques, including transmission, reflection, and attenuated total reflectance (ATR). This flexibility accommodates different types of samples and reduces the need for extensive sample preparation.

Resolution: FTIR instruments can achieve high spectral resolution, which means they can distinguish fine details and closely spaced peaks in a spectrum. This is particularly useful for identifying complex molecular structures.

Data quality: FTIR spectra are less affected by stray light and other artifacts, leading to cleaner and more accurate spectral data. This improved data quality enhances the accuracy of qualitative and quantitative analyses.

Interpretation: FTIR spectra are often easier to interpret due to their characteristic absorption bands associated with specific functional groups. This simplifies compound identification and analysis.

Real-time monitoring: FTIR can be used for real-time monitoring of chemical reactions and processes, making it valuable in reaction kinetics studies and process control applications.

8. What's the difference betwen FTIR and ATR ?

ATR stands for Attenuated Total Reflectance. It is a technique which involves the interaction of infrared light with a sample at the surface. It uses total internal reflection to create an evanescent wave that penetrates the sample slightly. The attenuated light that emerges from the sample is analyzed to determine its absorbance (Figure 7.12).

In ATR, the infrared light interacts with the outer layer of the sample, typically within a few micrometers of the surface. This makes it suitable for analyzing solid samples, liquids, and even sticky or viscous materials without extensive sample preparation.

ATR is particularly useful for samples that are difficult to handle or that have limited transparency, such as powders, polymers, films, and coatings.

Among these advantages: minimal sample preparation, non-destructive, and can be used with a wide variety of sample types. It provides information about surface properties, composition, and chemical structure near the surface.

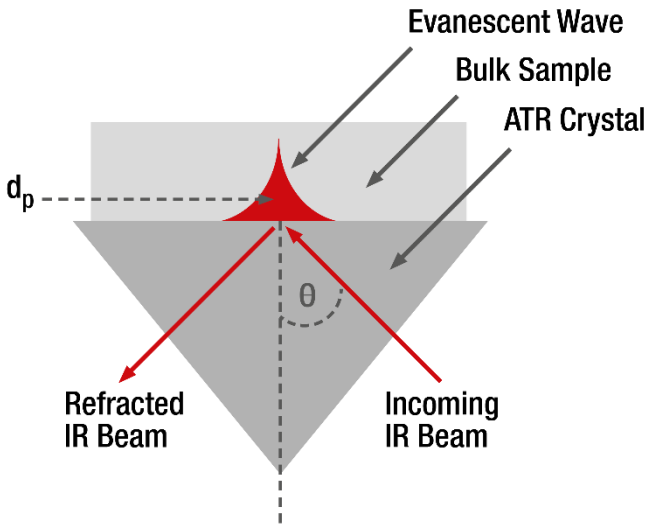


***Figure 7.12**. Evanescent wave resulting from total internal reflection*

Many different ATR accessories are available for FTIR spectrometers. They can be divided into ATR cells with a single reflection (one bounce) and cells with multiple reflections (multiple bounce). Depending on the application and the measured samples, different materials are used as the ATR crystal. Typical materials include zinc selenide (ZnSe), germanium (Ge), and diamond.

9. Applications of IR spectroscopy

IR spectroscopy is a widely used analytical technique in a variety of fields. It provides valuable information about the chemical composition, structure, and bonding of a wide range of compounds. Here are some of the applications of IR spectroscopy:

1. ***Identifying functional groups*:** IR spectroscopy is commonly used to identify functional groups in organic and inorganic compounds. Different functional groups absorb infrared radiation at specific wavelengths, resulting in characteristic peaks in the spectrum.
2. ***Organic chemistry*:** In organic chemistry, IR spectroscopy helps in identifying the presence of various functional groups such as carbonyl, hydroxyl, amino, and alkene groups. It's used for compound identification, purity determination, and reaction monitoring.
3. ***Quality control*:** IR spectroscopy is employed in quality control processes across industries, such as pharmaceuticals and food. It ensures the consistency and integrity of products by identifying impurities, contaminants, and structural changes.
4. ***Polymer characterization*:** IR spectroscopy is crucial for characterizing polymers and plastics. It can determine polymer composition, identify additives, and analyze copolymers. It's used in assessing polymer degradation and understanding polymer structure.
5. ***Forensic analysis*:** IR spectroscopy is used in forensic science to analyze trace evidence, identify unknown substances, and examine questioned documents. It helps in criminal investigations and court cases.
6. ***Environmental monitoring*:** IR spectroscopy is used to monitor air and water pollution. It can identify pollutants, analyze gas emissions, and assess the quality of water sources.
7. ***Pharmaceutical analysis*:** In pharmaceutical research and development, IR spectroscopy is used to analyze drug formulations, monitor drug stability, and study drug interactions with excipients.
8. ***Biomolecular analysis*:** IR spectroscopy plays a role in studying biomolecules like proteins, nucleic acids, and lipids. It provides insights into protein secondary structure, protein-ligand interactions, and conformational changes.
9. ***Art and archaeology*:** IR spectroscopy helps analyze pigments, dyes, and materials in art and archaeological objects. It aids in conservation efforts and understanding historical artifacts.
10. ***Mineral identification*:** Geologists use IR spectroscopy to identify minerals in rocks and minerals. Different minerals have distinct infrared absorption patterns that can be used for identification.

11. ***Food industry*:** IR spectroscopy is used to analyze food composition, quality, and authenticity. It can identify additives, determine fat and protein content, and detect adulteration.
12. ***Fuel analysis*:** IR spectroscopy is applied to analyze fuels, such as gasoline and diesel, to determine their composition, quality, and the presence of contaminants.
13. ***Process monitoring*:** In industrial processes, IR spectroscopy is used for real-time monitoring of chemical reactions and processes. It helps ensure product quality and optimize production efficiency.

These are just a few examples of the wide-ranging applications of IR spectroscopy. Its non-destructive nature, ability to analyze a variety of sample types, and its role in providing valuable chemical information make it an indispensable tool in various scientific and industrial fields.

Applications

Application 1

The IR spectrum of a compound with the formula C_3H_6O does not show bands around 3500 cm^{-1} and 1720 cm^{-1}. Which structures can be eliminated?

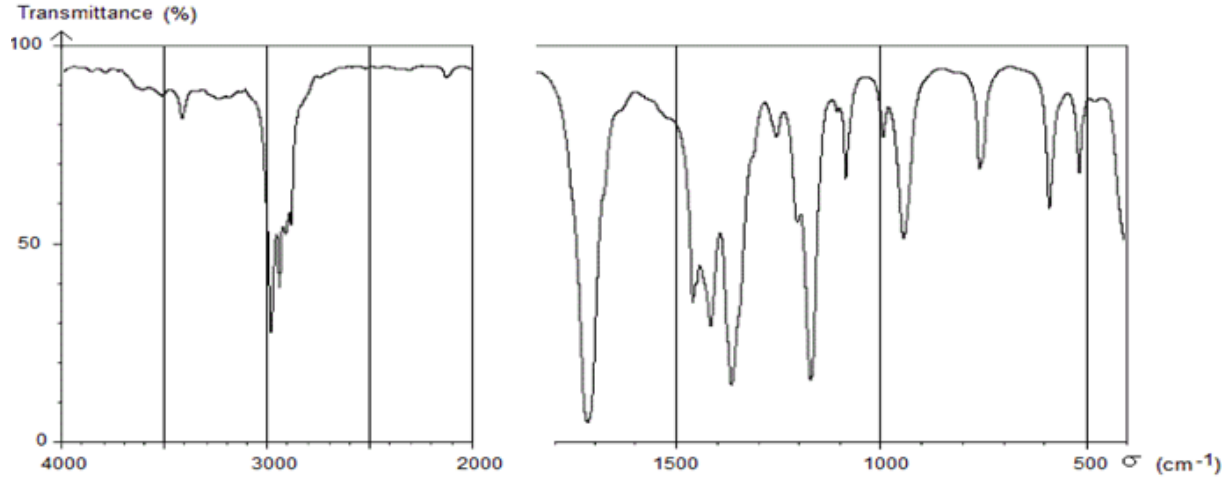
Suggest a possible structure and explain how its accuracy can be ensured.

Application 2

An organic molecule has the chemical formula C_4H_8O.

1. Give the possible semi-structural formulas of this molecule having a single characteristic group.

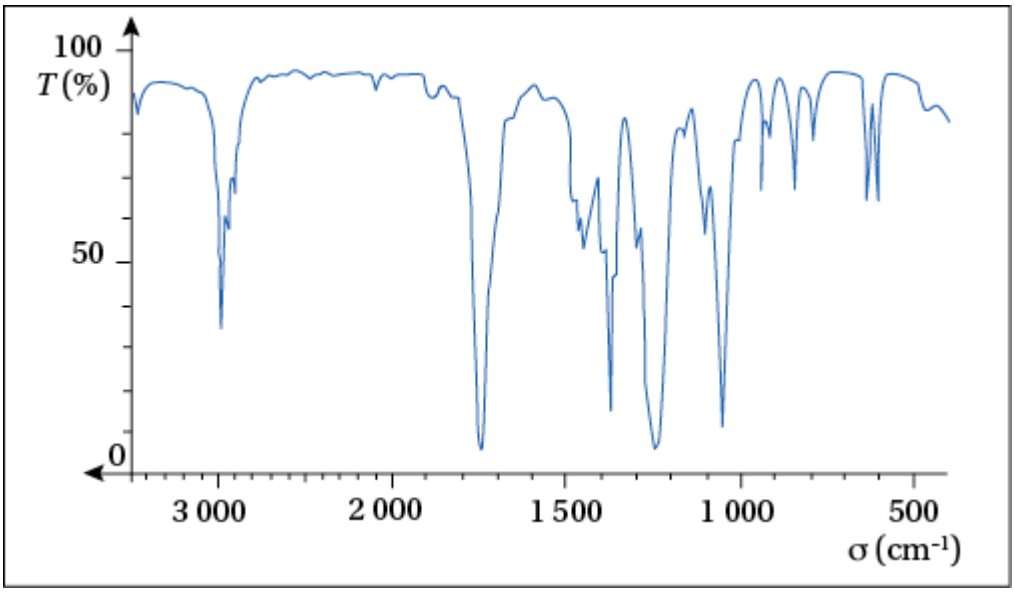
2. Determine the characteristic group by looking at the infrared spectrum of this molecule.



Bond	O - H free	O - H with hydrogen bridge	N-H	C_{tri} - H	C_{tetra} – H longitudinal vibration	C=O	C = C	C_{tetra} - H vibration angle $\widehat{HCH}$	C-O	C_{tetra}-C_{tetra}
Number of waves (cm^{-1})	3580 - 3650	3200-3400	3100 - 3500	3000 - 3100	2800 - 3000	1650 - 1750	1625 - 1685	1415 - 1470	1050 - 1450	1000 - 1250

Application 3

1. Below is the infrared spectrum of an unknown compound. What can you deduce from this spectrum? Can the exact function of the compound be determined?



2. From a complementary analysis, we determine the crude formula of the unknown compound $C_4H_8O_2$. Is this formula compatible with the previous analysis?

3. Then give the possible semi-structural formulas of the three isomers of $C_4H_8O_2$.

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