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Higher School of Biological Sciences of Oran
Department of the Second Cycle

Educational Handout

Subject: Biochemical Analysis Techniques
Biochemical Analysis Techniques Course

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Level: 1st year of the second cycle
Option: **Biotechnology**
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Preamble

This comprehensive course material “Biochemistry Analysis Techniques” covers the fundamental principles and practical applications of key separation, chromatographic, electrophoretic, and spectroscopic techniques essential for modern biological and biochemical research.

The content is structured into four main chapters, each building upon the previous to provide a logical progression from basic separation methods to advanced analytical techniques:

- ✓ Chapter I introduces classical separation and fractionation techniques, including decantation, distillation, filtration, precipitation, centrifugation, and membrane-based methods, which are foundational for processing biological samples.
- ✓ Chapter II explores the wide range of chromatographic techniques, from planar methods (TLC, paper chromatography) to column techniques (HPLC, GC), as well as specialized modes such as size-exclusion, ion-exchange, and affinity chromatography, including IMAC for recombinant protein purification.
- ✓ Chapter III delves into electrophoretic techniques, covering both protein and nucleic acid separation methods, including PAGE, SDS-PAGE, agarose gel electrophoresis, capillary electrophoresis, isoelectric focusing, 2D electrophoresis, and blotting techniques (Southern, Northern, Western).
- ✓ Chapter IV provides an in-depth overview of spectroscopic methods, including atomic spectroscopy, UV-Visible spectroscopy, fluorescence spectroscopy, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry, with an emphasis on their applications in biomolecular analysis.

Target Public and Prerequisites

This subject is taught to first-year students specialized in Immunotechnology at the Higher School of Biological Sciences of Oran**.** It is also suitable for students in related fields such as biochemistry, molecular biology, biotechnology, and pharmaceutical sciences who require a solid foundation in analytical techniques for their research and professional careers. This course aims to equip students with the theoretical knowledge necessary to select, apply, and interpret results from a wide range of separation and analytical techniques, forming an essential toolkit for their future research in immunology, diagnostics, and biotechnology.

To successfully engage with this material, students should have:

- ✓ A solid understanding of general chemistry, including atomic structure, chemical bonding, and basic thermodynamics.
- ✓ Foundational knowledge of organic chemistry, particularly functional groups and their reactivity.
- ✓ Basic concepts of biochemistry, including the structure and function of proteins, nucleic acids, carbohydrates, and lipids.
- ✓ Familiarity with general physics, especially electricity, magnetism, and the properties of electromagnetic radiation.

Learning Objectives

After completing this course, students should be able to:

- ✓ Understand the fundamental principles of separation, chromatographic, electrophoretic, and spectroscopic techniques used in biological and biochemical analysis.
- ✓ Select appropriate separation methods (decantation, distillation, filtration, precipitation, centrifugation, membrane techniques) based on the physical and chemical properties of the target molecules.
- ✓ Analyze and interpret spectral data, chromatograms and electrophoretic profils.
- ✓ Evaluate the advantages and limitations of each technique and select the most appropriate method for a given analytical problem in immunology, biochemistry, or biotechnology.
- ✓ Demonstrate a solid theoretical foundation for future practical laboratory work involving these techniques, including sample preparation, instrument operation, and data analysis.

Disclaimer

This course material was developed with the assistance of artificial intelligence (AI) tools to enhance the academic style, clarity, and scientific rigor of the content. The AI was used to:

- ✓ Improve the structure and flow of the text
- ✓ Refine scientific explanations and terminology
- ✓ Format references according to academic standards
- ✓ Ensure consistency across chapters

All scientific concepts, experimental techniques, and applications presented in this document are based on established knowledge in the fields of biochemistry and analytical chemistry.

This disclaimer is provided in the spirit of transparency and academic integrity, acknowledging the role of AI as a collaborative tool.

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CHAPTER IV

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List of Abbreviations

A
A : Absorbance
AAS : Atomic Absorption Spectroscopy
AC : Affinity Chromatography
AEM : Anion Exchange Membrane
AES : Atomic Emission Spectroscopy
AP : Alkaline Phosphatase
APCI : Atmospheric Pressure Chemical Ionization
APD : Avalanche Photodiode
APPI : Atmospheric Pressure Photo Ionization
APS : Ammonium Persulfate

B
BME : β-Mercaptoethanol
BSA : Bovine Serum Albumin

C
CE : Capillary Electrophoresis
CEM : Cation Exchange Membrane
CGE : Capillary Gel Electrophoresis
CI : Chemical Ionization
CM : Carboxymethyl
CsCl : Cesium Chloride
CW : Continuous Wave
CZE : Capillary Zone Electrophoresis

D
DAD : Diode Array Detector
DEAE : Diethylaminoethyl
DNA : Deoxyribonucleic Acid
DTT : Dithiothreitol

E
ECD : Electron Capture Detector
ED : Electrodialysis
EEM : Excitation-Emission Matrix
EI : Electron Ionization
ELSD : Evaporative Light Scattering Detector
EMSA : Electrophoretic Mobility Shift Assay
EOF : Electroosmotic Flow
ESI : Electrospray Ionization
EtBr : Ethidium Bromide

F
FES : Flame Emission Spectrometry
FID : Flame Ionization Detector
FTIR : Fourier Transform Infrared Spectroscopy

G
GC : Gas Chromatography
GFC : Gel Filtration Chromatography
GFP : Green Fluorescent Protein
GLC : Gas-Liquid Chromatography
GPC : Gel Permeation Chromatography
GSC : Gas-Solid Chromatography

H
HA : Humic Acid
HD : Humification Degree
HPLC : High-Performance Liquid Chromatography
HRP : Horseradish Peroxidase

I
IC : Internal Conversion
ICP : Inductively Coupled Plasma
IDA : Iminodiacetic Acid
IEC : Ion Exchange Chromatography
IEF : Isoelectric Focusing
IFE : Inner Filter Effect
IMAC : Immobilized Metal Affinity Chromatography
IPG : Immobilized pH Gradient
IR : Infrared

J
J : Coupling Constant

K
K : Partition Coefficient
k' : Capacity Factor

L
LC : Liquid Chromatography
LED : Light-Emitting Diode
LIF : Laser-Induced Fluorescence
LIBS : Laser-Induced Breakdown Spectroscopy
LIDAR : Light Detection and Ranging

M
$M^{+\bullet}$: Molecular Ion
MALDI : Matrix-Assisted Laser Desorption/Ionization
MEKC : Micellar Electrokinetic Chromatography
MF : Microfiltration
MMI : Multimode Ionization
MPB : Microphytobenthos
MRI : Magnetic Resonance Imaging
MS : Mass Spectrometry
MW : Molecular Weight
MWCO : Molecular Weight Cut-Off

N
N : Number of Theoretical Plates
NF : Nanofiltration
NIR : Near-Infrared
NMR : Nuclear Magnetic Resonance
NTA : Nitrilotriacetic Acid

O
OM : Organic Matter

P
PAGE : Polyacrylamide Gel Electrophoresis
PAH : Polycyclic Aromatic Hydrocarbon
PARAFAC : Parallel Factor Analysis
PBS : Phosphate-Buffered Saline
PC : Paper Chromatography
pI : Isoelectric Point
PMT : Photomultiplier Tube
ppm : Parts Per Million
PTM : Post-Translational Modification

Q
Q : Quaternary Ammonium

R
RCF : Relative Centrifugal Force
RF : Radiofrequency
Rf : Retention Factor
RNA : Ribonucleic Acid
RO : Reverse Osmosis
RP-HPLC : Reverse Phase High-Performance Liquid Chromatography
RPM : Revolutions Per Minute
Rs : Resolution

S
S : Svedberg Unit
SDS : Sodium Dodecyl Sulfate
SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC : Size Exclusion Chromatography
SFS : Synchronous Fluorescence Spectroscopy
SNR : Signal-to-Noise Ratio
SOM : Soil Organic Matter
SP : Sulfopropyl

T
t_0 : Dead Time
t'R : Adjusted Retention Time
tR : Retention Time
TALIF : Two-Photon Absorption Laser-Induced Fluorescence
TBE : Tris-Borate-EDTA
TCD : Thermal Conductivity Detector
TCEP : Tris(2-carboxyethyl)phosphine
TCSPC : Time-Correlated Single Photon Counting
TEMED : N,N,N',N'-Tetramethylethylenediamine
TLC : Thin Layer Chromatography
TMS : Tetramethylsilane
TOF : Time-of-Flight
TSFS : Total Synchronous Fluorescence Spectroscopy

U
UF : Ultrafiltration
UV : Ultraviolet
UV-Vis : Ultraviolet-Visible

V
V_0 : Void Volume
Ve : Elution Volume
Vt : Total Volume

W
WB : Western Blot

X
Xe : Xenon

Chapter I: Separation and Fractionation Techniques

Table of Contents

Learning Objectives

After reading this chapter, you should be able to understand the fundamental principles of separation techniques used in biochemistry and cell biology. You will be able to distinguish between purification, concentration, and fractionation, and classify mixtures based on their physical properties. You will understand the mechanisms of decantation, distillation, filtration, precipitation, centrifugation, and membrane-based techniques, and know when to apply each method. Finally, you will be able to select the most appropriate technique for a given separation problem, such as desalting a protein solution, isolating cellular organelles, or concentrating a biological sample.

Lecture 01: Decantation, Distillation and Filtration

I. Introduction

Separation processes are fundamental techniques in both biochemical and chemical analysis, (Nichols & Geddes, 2021). These processes are critical across various industries, including medical, pharmaceutical, environmental, and chemical sectors, where high efficiency and selectivity are paramount (Khodakarami & Alagha, 2017). They encompass a set of mechanical and physico-chemical operations designed to transform a mixture of substances into two or more distinct product fractions (Belter, Cussler, & Hu, 1988). These processes are critical for achieving several key objectives such as:

- ✓ *Purification:* The removal of undesirable impurities from a target compound to increase its specificity and biological activity.
- ✓ *Concentration:* The increase in the abundance of a solute in a solution, typically by the removal of a portion of the solvent, to facilitate downstream analysis or reactions.
- ✓ *Fractionation:* The systematic subdivision of a complex mixture into multiple fractions.

The underlying principle of any separation technique is the exploitation of a difference in intrinsic physico-chemical properties between the target molecule and the rest of the matrix. These properties can include molecular size, density, solubility, volatility, charge, and affinity for a specific ligand. The efficacy of a separation is directly proportional to the magnitude of these differences (Scopes, 1994).

A primary consideration when selecting a separation method is the physical nature of the initial mixture. Mixtures are broadly classified as follows:

- ✓ *Heterogeneous Mixtures:* These are non-uniform mixtures where distinct phases or components are visible to the naked eye. Examples include liquid-solid suspensions (e.g., sand in water) or liquid-liquid emulsions (e.g., oil and water).
- ✓ *Homogeneous Mixtures:* These are uniform at the molecular or ionic level, presenting as a single phase, such as saline solution (salt in water) or a mixture of miscible liquids like ethanol and water.
- ✓ *Colloidal Mixtures:* A state where particles are dispersed in a continuous phase. These mixtures appear homogeneous to the naked eye but are actually heterogeneous at the microscopic scale (Eg: milk, blood..ect).

The selection of a separation strategy for liquid mixtures is based on three essential physico-chemical properties:

- ✓ *Solubility:* This refers to the maximum amount of a solute that can dissolve in a given amount of solvent at a specific temperature and pressure to form a stable homogeneous solution. Solubility is highly dependent on the chemical nature of the solute and solvent (e.g., polarity), as well as environmental conditions such as temperature, pH, and ionic strength
- ✓ *Miscibility:* This property describes the ability of two or more liquids to mix in all proportions, forming a single homogeneous liquid phase. For instance, water and ethanol are completely miscible. Conversely, immiscible liquids, such as chloroform and water, form separate layers upon standing, with their order determined by their relative densities.
- ✓ *Density:* Defined as mass per unit volume ($\rho = m/V$, typically in g/cm^3 or kg/m^3), density is a critical parameter in techniques relying on gravitational or centrifugal forces. In a mixture, components with higher density will sediment or sink below those with lower density.

II. Decantation

Decantation is a primary, mechanical separation technique that utilizes the force of gravity to separate phases of different densities within a heterogeneous mixture, where at least one phase is a liquid or a gas (McCabe, Smith, & Harriott, 2005).

I.1. Principle

The process relies on the natural settling of a denser phase under the influence of gravity. Given sufficient time, particles or an immiscible liquid phase with a higher density than the continuous phase will separate, allowing for physical removal of the clarified supernatant or the separated layer.

- **Solid-Liquid Separation:** This is applied to separate insoluble solids suspended in a liquid. A classic example is the purification of water by allowing suspended silt and sand particles to settle. In wastewater treatment plants, this principle is employed in primary settling tanks, where gravity separation removes a significant portion of organic and inorganic solids from the influent, forming primary sludge (Metcalf & Eddy, 2014).
- **Liquid-Liquid Separation:** For mixtures of immiscible liquids, such as oil and water, decantation occurs spontaneously. The liquid with the higher density forms the lower layer. An essential tool for this separation in a laboratory setting is the separatory funnel (Figure 01). The mixture is introduced into the funnel, allowed to separate into distinct layers, and the bottom

layer is then carefully drained out through the stopcock, effecting a clean separation. While decantation is a simple, low-cost technique, its major limitations are its dependence on a significant density difference.

Figure 01: Different shapes of separatory funnels. Adapted from Separatory Funnel, by Home Science Tools, (2017).

II. Distillation

Distillation is a separation process that exploits differences in the volatility of the components in a liquid mixture. The process involves the selective vaporization of the more volatile components followed by the condensation of the vapors, yielding a purified distillate (Perry & Green, 2007). It is a cornerstone technique for purifying solvents, separating liquid mixtures, and in industrial processes like petroleum refining.

II.1. Simple Distillation

Simple distillation is employed when the boiling points of the components in a mixture differ significantly, typically by more than 25-30°C.

II.1.1. Principle and Apparatus

A standard simple distillation setup represented in figure 02 consists of the following key components:

- ✓ *Heat Source:* A burner or hot plate is used to heat the liquid mixture
- ✓ *Distillation Flask (Pot):* A round-bottom flask containing the liquid mixture, heated by a controlled heat source.
- ✓ *Thermometer:* Positioned at the head of the flask to monitor the temperature of the vapor, which corresponds to the boiling point of the component currently being distilled.
- ✓ *Condenser:* A jacketed tube where vapor is cooled and liquefied. A coolant, usually water, circulates through the outer jacket.

- ✓ *Receiving Flask (Receiver):* Collects the condensed liquid, known as the distillate.
- ✓ *Cooling System:* Water is circulated through the condenser to maintain a constant temperature, ensuring efficient condensation

Upon heating, the component with the lowest boiling point (the most volatile) vaporizes first. These vapors travel into the condenser, where they are cooled and revert to the liquid state, and are collected. Since phase changes occur at a constant temperature under atmospheric pressure (or a set pressure), the mixture will maintain the boiling point of the most volatile liquid until it is completely transformed into vapor therefore the temperature observed at the thermometer remains relatively constant during the distillation of this primary component. The greater the difference in the boiling points of the components, the more efficient the distillation process However, simple distillation does not achieve a perfect separation; the initial distillate is enriched with the more volatile component, but some less volatile component is always co-distilled due to vapor pressure effects. A common example is the partial separation of a water-ethanol mixture.

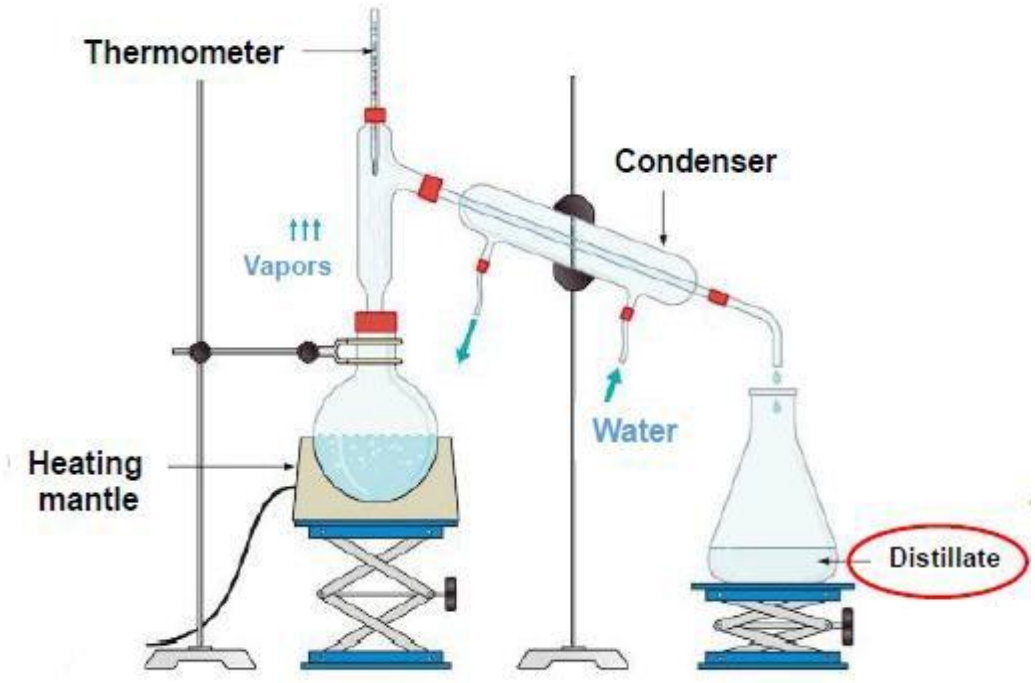


Figure 02: Main components of a simple distillation setup. Adapted from *Lab Procedure: Simple Distillation* by ChemTalk, (2022).

II.2. Simple Distillation at Reduced Pressure

II.2.1. Principle and Apparatus

This setup is indispensable for compounds that are thermolabile (decompose at their normal boiling point) or that have exceedingly high boiling points at atmospheric pressure. By connecting a vacuum pump to the system, the ambient pressure is reduced. A decrease in pressure lowers the boiling point of a liquid, allowing distillation to proceed at a much lower temperature, thereby preventing thermal degradation (Sittig, 1981). The apparatus of simple distillation at reduced pressure called a Rotavapor or Rotary evaporator is represented in figure 03 and is mainly composed of the following parts:

- ✓ *Rotary motor*: It is an electric-powered motor that plays an important role by rotating the evaporation flask at a continuous and constant speed. This results in an increased surface area for evaporation as well as even mixing, heating hence a stable and even evaporation.
- ✓ *Vacuum source*: This is the system responsible for creating a vacuum within the airtight set-up, thereby reducing the pressure within the system. The vacuum source can be simply a water aspirator consisting of a water immersed trap, or a complex mechanical vacuum pump consisting of a refrigerated trap.
- ✓ *Glass tube:* The glass tube has two functions, the first is to protect and support the sample during rotation while the second is to help in the creation of a vacuum.
- ✓ *Heating bath*: The mainly used heating bath is a hot water bath. The function of the heating pot is to heat the sample and help in temperature regulation.
- ✓ *Condenser:* It is mainly found in a double snake shape and the function is to condensate the evaporated sample as well as enhance convenient sample collection.
- ✓ *Receiving flask:* The function of the collecting flask is to receive the distilled sample.

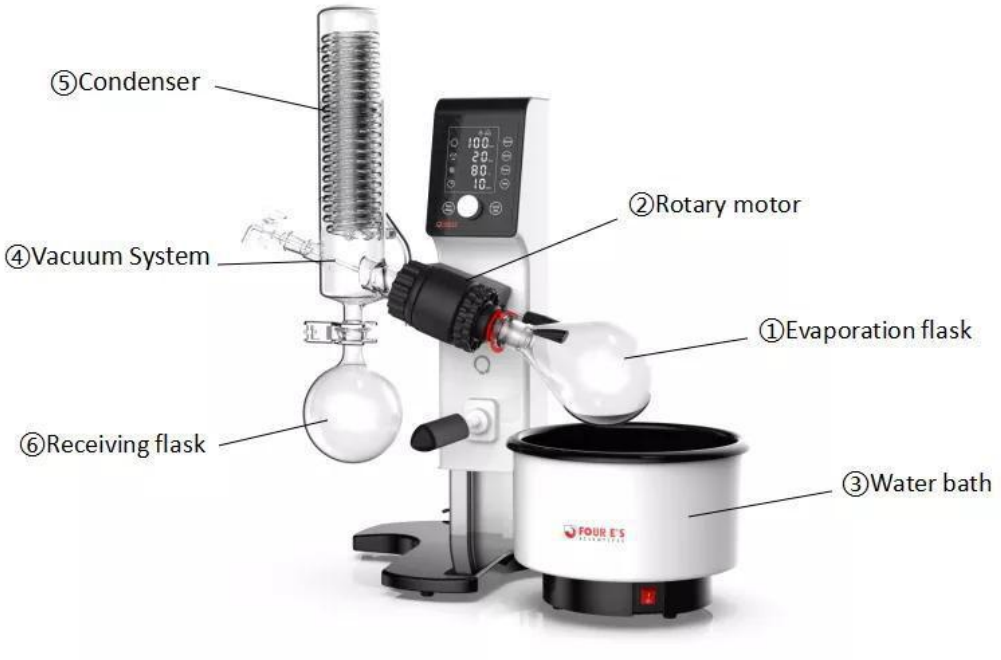


Figure 03: Main parts of a rotary evaporator. Adapted from Everything You Want to Know About Rotary Evaporator, by FOUR E's Scientific, (2023).

II.3. Fractional Distillation

Fractional distillation is the method of choice for separating liquid mixtures with components possessing similar, or closely-spaced, boiling points. It achieves multiple, sequential vaporization-condensation cycles within a single apparatus.

II.2.1. Principle and Apparatus

A fractional distillation setup is quite similar to a simple distillation setup, the critical addition to the setup is a fractionating column called Vigreux column (Figure 04) placed between the distillation flask and the condenser. This column contains usually internal structures (glass indentations) that provide a large surface area.

As the vapors from the boiling mixture rise through the column, they encounter a downward flow of condensed liquid (reflux). This contact facilitates continuous heat and mass transfer: the less volatile components in the vapor tend to condense, releasing heat that vaporizes the more volatile components from the descending liquid. This series of micro-distillations, or theoretical plates, results in a vapor phase increasingly enriched in the more volatile component at the top of the column. Consequently, a much sharper separation is achieved than with simple distillation. The different components, or "fractions," can be collected separately over distinct temperature ranges.

Figure 04: Vigreux column. Adapted from Labasics Colonne de Distillation Vigreux, 24/40 200 mm, by Labasics, (n.d)., Amazon.fr.

The most significant industrial application of fractional distillation is the refining of crude oil. In a massive fractionating tower, heated crude oil is separated into its primary fractions—such as refinery gases, gasoline, naphtha, kerosene, diesel, and lubricating oils—based on their respective boiling ranges. Each fraction is drawn off at different levels of the tower.

III. Filtration

Filtration is a fundamental physical separation process for isolating solids from a fluid (liquid or gas) by passing the mixture through a porous medium that retains the solid particles while allowing the fluid to pass through (Rushton, Ward, & Holdich, 2000). The efficiency of filtration depends on the particle size distribution, the porosity and chemical nature of the filter medium, and the driving force applied.

III.1. Gravity Filtration

This is the simplest form, relying solely on gravity to drive the fluid through the filter. A typical laboratory setup involves a funnel lined with filter paper. The retained solids form a residue, while the clarified fluid that passes through is termed the "filtrate." While straightforward, this method is often slow and is suitable only for small volumes or coarse precipitates (Figure 06).

III.2. Vacuum Filtration

To accelerate the filtration process, a pressure differential is created across the filter medium. This is typically achieved using a Büchner funnel, connected to a vacuum source (e.g., a water aspirator or a vacuum pump). The vacuum flask beneath the funnel collects the filtrate. The applied suction significantly increases the flow rate, making this method ideal for rapidly

separating fine crystalline products or precipitates from a mother liquor. The formation of a uniform filter cake is crucial for efficient operation (Figure 05).

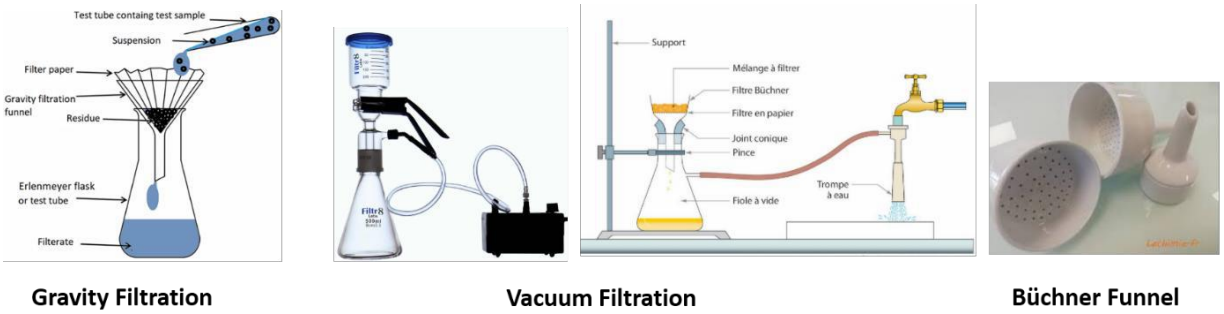


Figure 05: Gravity and vacuum filtration.

This figure was assembled by the author using components inspired by commercial filtration systems. Filtr8 Labs. (2022, February 20). Buchner funnel flask kit with pro pump.

Lecture 02: Precipitation, centrifugation and ultracentrifugation

I. Precipitation

Precipitation is a versatile technique that involves the formation of a solid phase (the precipitate) from a solution. It can be induced by various means to achieve either the removal of impurities or the selective isolation of a target molecule, such as a protein (Scopes, 1994).

I.1. Chemical Precipitation

This method relies on a chemical reaction in solution that generates an insoluble compound. It is frequently used in analytical chemistry for qualitative, and in industrial processes for metal recovery or water treatment. If the presence of a specific ion is suspected, a counter-ion that forms an insoluble salt with it is added. The appearance of a solid precipitate confirms the presence of the target ion, which can then be isolated by filtration or centrifugation.

The most common example is the addition of sodium hydroxide (NaOH) to a solution of copper sulfate ($CuSO_4$) resulting to the formation of a characteristic blue precipitate of copper(II) hydroxide, $Cu(OH)_2$. This reaction is a classic test for the presence of Cu^{2+} ions.

$$CuSO4(aq)+2NaOH(aq)\rightarrow Cu(OH)2(s)+Na2SO4(aq)$$

I.2. Protein Precipitation

Proteins solubility is highly dependent on their environment such as:

- ✓ Temperature
- ✓ pH
- ✓ Ionic concentration
- ✓ Nature of solvent
- ✓ The presence of biospecific agents that can interact with the proteins (eg: immunoprecipitation).

Manipulating these solution conditions can decrease protein-solvent interactions, leading to aggregation and precipitation. Protein precipitation can be total (precipitation of all proteins) which can be sometimes extreme and may lead to protein denaturation or differential (selective precipitation of a target protein based on subtle solubility differences) often described as soft and can preserve protein integrity.

I.2.1. Precipitation by Temperature Change

the effect of temperature on protein solubility is complex and can vary depending on the specific protein, its structure, and the solution conditions.

- *Kinetic Energy*

Higher Temperature: As temperature increases, the kinetic energy of molecules also increases. This can lead to more frequent and forceful interactions between water molecules and protein molecules, which can enhance solubility.

Lower Temperature: Conversely, at lower temperatures, molecular movement decreases, potentially leading to decreased solubility.

- *Protein Structure and Stability Denaturation*

High temperatures can lead to denaturation, where proteins lose their native structure. This can expose hydrophobic regions that may aggregate, thereby decreasing solubility

- *Hydration Shell Temperature Effects on Solvation*

The solvation of proteins depends on the ability of water to surround and stabilize protein molecules. Increased temperature can enhance the solvation dynamics, which may help in dissolving proteins. However, at extreme temperatures, the stability of the hydration shell may be compromised, affecting solubility.

I.2.2. Precipitation by pH Change (Isoelectric Precipitation)

Adjusting the pH of a protein solution to its pI is a highly effective method for precipitation. Each protein has a characteristic isoelectric point (pI), the pH at which the number of positive charges and negative charges is equal. At the pI, the absence of net electrostatic repulsion between protein molecules minimizes their solubility. Consequently, proteins tend to aggregate and precipitate out of solution. In fact, at pH values above or below the pI, the protein carries a net negative or positive charge, respectively, leading to electrostatic repulsion maintain them in a soluble state (Figure 01)

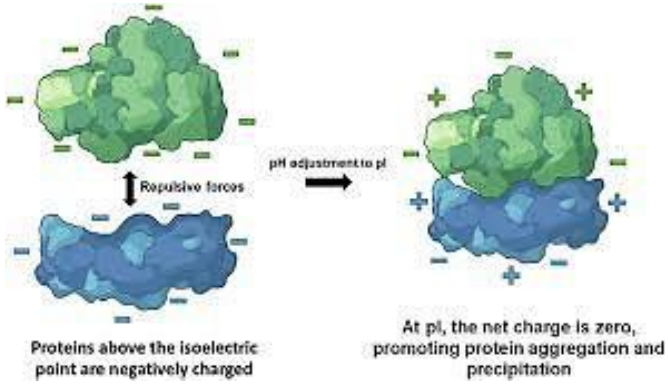
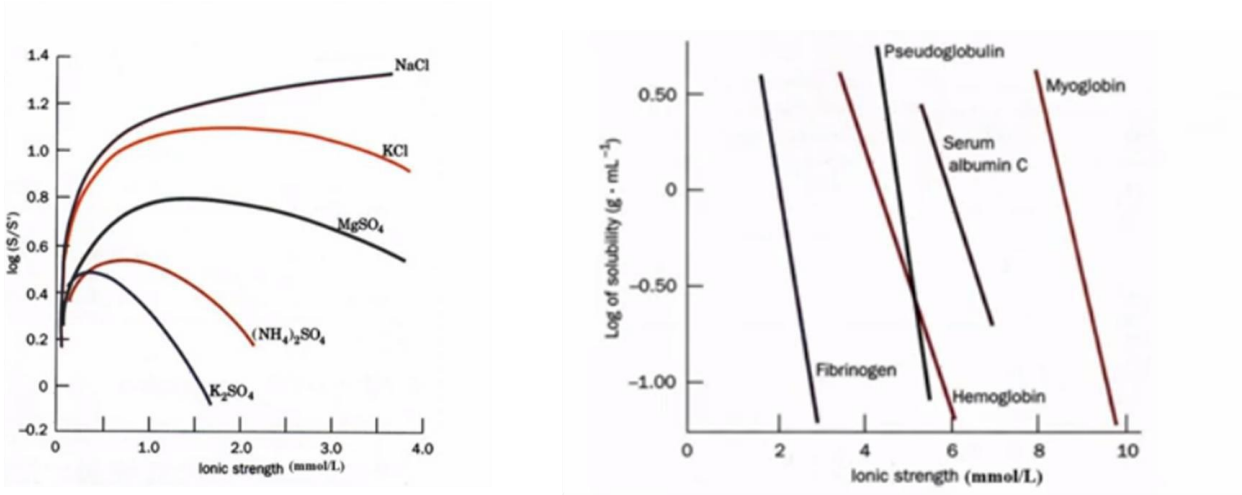


Figure 01: pH effect on protein solubility. Cruz-Solis et al., (2023)

I.2.3. Precipitation by Salting Out

This is one of the most common and powerful methods for fractionating proteins. It involves the addition of high concentrations of neutral salts, such as ammonium sulfate ($(NH_4)_2SO_4$). At high ionic strength, salt ions compete with the protein for hydration shells. Water molecules are "diverted" from the protein surface to solvate the added ions. This reduces the protein's solubility by decreasing the hydrophobic effect and, at very high concentrations, by shielding the protein's surface charges. As a result, protein-protein interactions become favored over protein-solvent interactions, leading to aggregation and precipitation (Cohn & Edsall, 1943).

As shown in the charts represented in figure 02, The solubility of proteins varies depending on the specific protein in question and the salts being considered.



Solubility of carboxyhemoglobin as a function of ionic strength and the nature of the ions.

Solubility of several proteins in ammonium sulfate solutions

Figure 02: Variability of Protein Solubility Depending on the Protein and Salt Type. Adapted from screenshots of the video Action des sels sur la solubilité d'une protéine, by S. Orsoni, (2021).

Different proteins precipitate at characteristic salt concentrations. As shown in the provided saturation table (table 01), ammonium sulfate can be added incrementally to a crude protein extract to precipitate different protein fractions sequentially. This technique, known as differential salting-out, is a classic first step in many protein purification protocols.

Table 01: Ammonium Sulfate Saturation Percentages for Protein Salting-Out

INITIAL CONCENTRATION OF AMMONIUM SULFATE (% saturation at 0 °C)	FINAL CONCENTRATION OF AMMONIUM SULFATE (% saturation at 0 °C)																
	%	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
		g solid ammonium sulfate to add to 100 mL of solution																
	0	10.6	13.4	16.4	19.4	22.6	25.8	29.1	32.6	36.1	39.8	43.6	47.6	51.6	55.9	60.3	63.0	69.7
	5	7.9	10.8	13.7	16.6	19.7	22.9	26.2	29.6	33.1	36.8	40.5	44.4	48.4	52.6	57.0	61.5	66.2
	10	5.3	8.1	10.9	13.9	16.9	20.0	23.3	26.6	30.1	33.7	37.4	41.2	45.2	49.3	53.6	58.1	62.7
	15	2.6	5.4	8.2	11.1	14.1	17.2	20.4	23.7	27.1	31.6	34.3	38.1	42.0	46.0	50.3	54.7	59.2
	20	0.0	2.7	5.5	8.3	11.3	14.3	17.5	20.7	24.1	27.6	31.2	34.9	38.7	42.7	46.9	51.2	55.7
	25		0.0	2.7	5.6	8.4	11.5	1.6	17.9	21.1	24.5	28.0	31.7	35.5	39.5	43.6	48.8	52.2
	30			0.0	2.8	5.6	8.6	11.7	14.8	18.1	21.4	24.9	28.5	32.3	36.2	40.2	44.5	48.8
	35				0.0	2.8	5.7	8.7	11.8	15.1	18.4	21.8	25.4	29.1	32.9	36.9	41.0	45.3
	40					0.0	2.9	5.8	8.9	12.0	15.3	18.2	21.2	25.8	29.6	33.5	37.6	41.8
	45						0.0	2.9	5.9	9.0	12.3	15.6	19.0	22.6	26.3	30.2	34.2	38.3
	50							0.0	3.0	6.0	9.2	12.5	15.9	19.4	23.0	26.8	30.8	34.8
	55								0.0	3.0	6.1	9.3	12.7	16.1	19.7	23.5	27.3	31.3
	60									0.0	3.1	6.2	9.5	12.9	16.4	20.1	23.9	27.9
	65										0.0	3.1	6.3	9.7	13.2	16.8	20.5	24.4
	70											0.0	3.2	6.5	9.9	13.4	17.1	20.9
	75												0.0	3.2	6.6	10.1	13.7	17.4
	80													0.0	3.3	6.7	10.3	13.9
	85														0.0	3.4	6.8	10.5
	90															0.0	3.4	7.0
	95																0.0	3.5
	100																	0.0

II. Centrifugation and Ultracentrifugation

Centrifugation is an accelerated form of sedimentation where the gravitational force is replaced by a powerful centrifugal force, enabling the rapid separation of particles based on differences in size, shape, and density (Rickwood, 1984). Ultracentrifugation refers to the use of very high speeds, capable of sedimenting even macromolecules and small organelles.

II.1. Fundamental Principle and Sedimentation Coefficient

A particle moving in a circular path is subjected to a centrifugal force, *Fc*, directed outward from the center of rotation. The effective force causing sedimentation is the difference between this centrifugal force and the buoyant force of the medium (Figure 03).

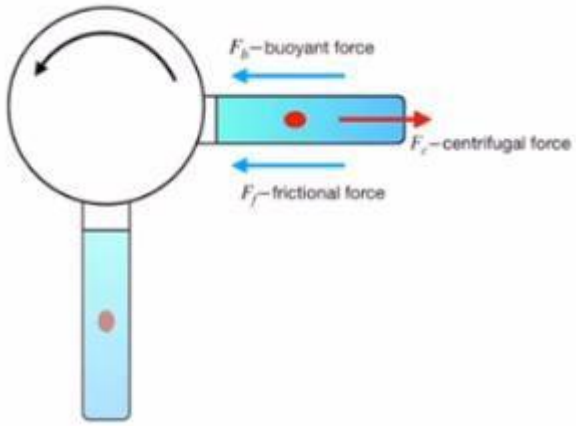


Figure 03: Schematic representation of the forces applied on a particle in centrifugation. Adapted from screenshot of Sedimentation during Centrifugation. Svedberg Equation. Sedimentation Coefficient, by Dr.AhMath Medicine, (2020).

The *sedimentation coefficient* is the ratio of the sedimentation velocity to the applied acceleration. During centrifugation, the sedimentation velocity of a particle depends on its mass, volume, and the density of the solvent (which determines the buoyant force), the acceleration to which it is subjected, as well as the frictional forces associated with its movement through the solution. These frictional forces depend on the size and shape of the particle. Thus, the sedimentation velocity involves numerous variables, and a sedimentation coefficient is measured, expressed in Svedberg units (S).

$$s = \frac{m\,(1-\bar{V}\rho)}{F}$$

Where:

m = mass of the particle

$\bar{V}$= partial specific volume (the increase in volume produced when 1 g of dry solute is added to the solvent) = 1/density of the particle

P = density of the solvent

F= Frictional force

II.2. Instrumentation and Parameters

Centrifuges are characterized by their maximum speed and the type of rotors used (fixed-angle, swinging-bucket, vertical), different types of centrifuges rotors are represented in figure 04. The separation power is expressed either in a Relative Centrifugal Force (RCF or g-force), which is a multiple of the Earth's gravitational acceleration or in Revolutions Per Minute **(**RPM), which is the measurement of the rotational speed.

To convert RPM to RCF, the following equation is used:

$$RCF = (RPM)^2 \times 1.118 \times 10^{-5} \times r.$$

Where r is the radius of the rotor

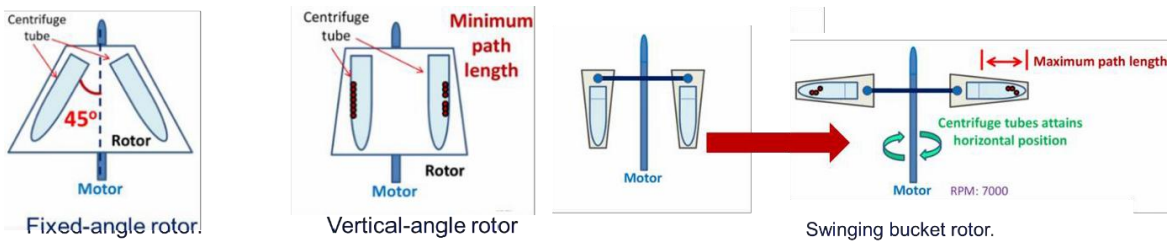


Figure 04: different types of rotors. Adapted from screenshots of the video Centrifuge Introduction, by Quick Biochemistry Basics, (2022).

I.3. Differential Ultracentrifugation

This is a widely used technique for the crude fractionation of cellular components (e.g., nuclei, mitochondria, microsomes, ribosomes). The sample is subjected to a series of centrifugation steps at incrementally higher RCF values and/or longer durations. At each step, the largest and densest particles that have not yet been sedimented will pellet at the bottom of the tube. The supernatant, now depleted of these particles, is then transferred to a new tube for the next, higher-speed centrifugation (Figure 05).

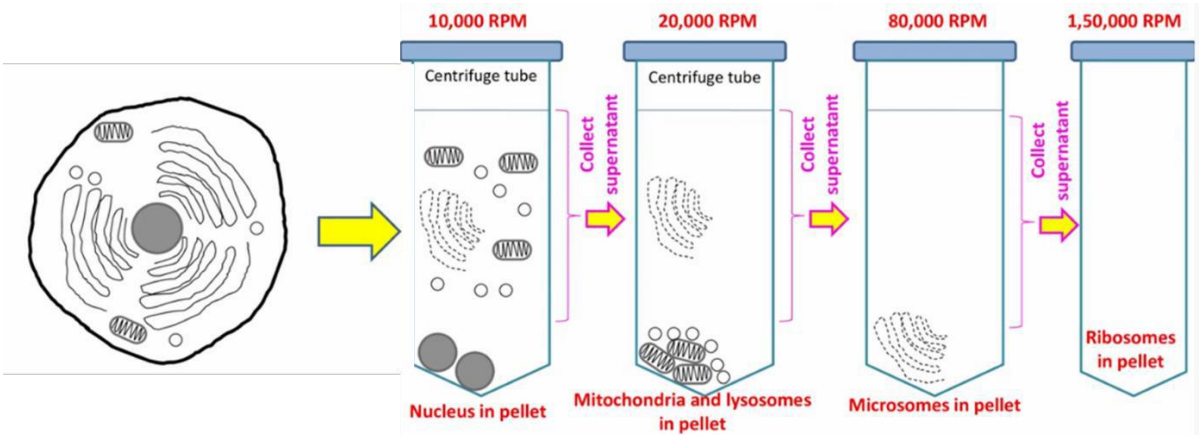


Figure 05: Graphic representation of the differential ultracentrifugation process. Adapted from screenshots of Differential Centrifuge, by Quick Biochemistry Basics, (2022).

I.4. Density Gradient ultracentrifugation

This technique exploits the fact that, in suspension, particles that are denser than the solvent sediment, while those that are less dense float. A high-speed ultracentrifuge is used to accelerate this process in order to separate biomolecules in a density gradient liquid, we distinguish two types of density gradient ultracentrifugation:

- **Rate-zonal density gradient ultracentrifugation**

This technique uses a discontinuous gradient which is often pre-formed. A small volume of sample is layered on top of a pre-formed density gradient (e.g., sucrose or glycerol). During centrifugation, particles sediment through the gradient as discrete zones based primarily on their sedimentation rate (a function of mass and shape). The run must be terminated before any particle reaches the bottom of the tube or their isopycnic equilibrium. Rate-zonal density gradient ultracentrifugation is ideal for separating particles of similar density but different sizes, such as ribosomes (30S and 50S subunits), nucleic acids, or subcellular organelles of similar density.

- **Isopycnic (Equilibrium) Ultracentrifugation**

This technique uses a continuous gradient which is often self-formed. The sample is mixed with, or layered on, a density gradient medium (e.g., CsCl, Cs_2SO_4, or Nycodenz). Under a high centrifugal force, particles migrate until they reach a position in the gradient where their buoyant density equals the density of the medium. At this isopycnic point, the net force is zero, and sedimentation ceases. The separation is based solely on buoyant density, independent of particle shape or size.

The most notorious example of this technique application is the seminal *Meselson-Stahl experiment (1958),* which demonstrated the semi-conservative replication of DNA, utilized isopycnic centrifugation in a CsCl gradient. *E. coli* grown in ^{15}N (heavy) and ^{14}N (light) media produced DNA of different densities, which formed distinct bands in the gradient, visually confirming the replication mechanism (Figure 06).

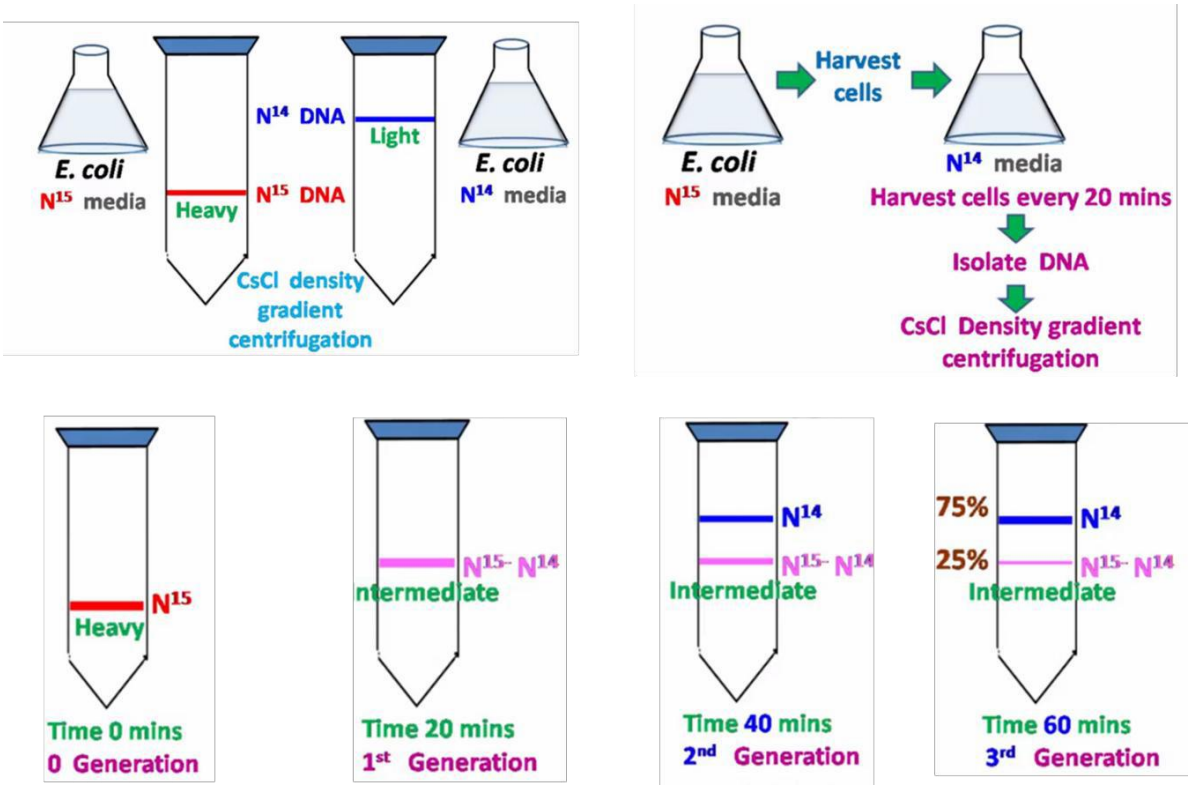


Figure 06: The Meselson and Stahl experiment 1958 (the semi-conservative replication of DNA). Adapted from screenshots of Meselson and Stahl Experiment, by Quick Biochemistry Basics, (2018)

Lecture 03: Concentration using membranes

I. Introduction

Membrane-based concentration techniques are powerful tools in bioseparations, offering selective transport based on size, charge, or solubility. They are typically efficient and can be performed under mild conditions, making them ideal for sensitive biological molecules (Zeman & Zydney, 2017).

II. Dialysis

Dialysis is a purification technique that separates molecules according to their size in a solution by virtue of their differential diffusion rates through a semi-permeable membrane (also known as a dialyzing membrane). The process is driven by the natural diffusion of solutes down their concentration gradient. A sample solution is placed inside a sealed dialysis bag or tubing, which is then immersed in a large volume of a well-stirred buffer (the dialysate). The membrane pores are sized to allow small molecules (e.g., salts, sugars, impurities) to pass through freely, while retaining larger macromolecules (e.g., proteins, nucleic acids). Over time, the small molecules diffuse out of the bag until their concentration inside and outside the bag reaches equilibrium (Figure 01). The efficiency of dialysis depends on the membrane pore size (characterized by its Molecular Weight Cut-Off, or MWCO), the surface area, the concentration gradient, temperature, and time.

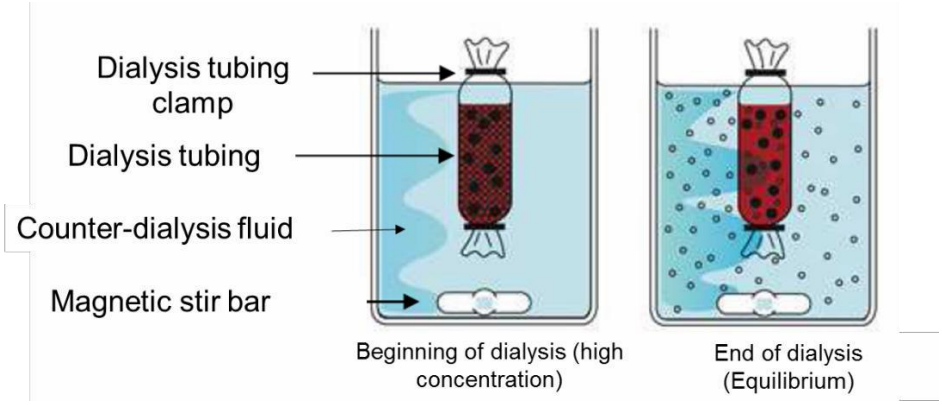


Figure 01: Principle of using a dialysis tubing. (Liu et al., 2016)

The main applications of this technique are:

- ✓ *Desalting:* Removal of salts or other small solutes from protein or DNA solutions.
- ✓ *Buffer Exchange:* Changing the solvent environment of a macromolecular solution to a different buffer system.
- ✓ *Concentration*: By placing the dialysis bag in a hygroscopic polymer (e.g., polyethylene glycol), water can be drawn out, concentrating the sample inside.

III. Electrodialysis

Electrodialysis (ED) is an advanced membrane process used specifically for the separation of ions from aqueous solutions under the influence of an applied electric field (Strathmann, 2011). Electrodialysis uses ion-exchange membranes arranged alternately: cation exchange membranes (CEM) and anion exchange membranes (AEM). Under the influence of an electric field, cations (positive ions) are attracted to the negative electrode, allowing them to pass through a cation exchange membrane (CEM) but preventing them from crossing an anion exchange membrane (AEM). Conversely, anions are attracted to the positive electrode, allowing them to pass through the AEM while they cannot cross the CEM. As a result, compartments that become depleted of ions (salts for seawater desalination) are referred to as "desalination or dilute compartments," while compartments that become enriched in salts are called "brine or concentrated compartments" (Figure 02).

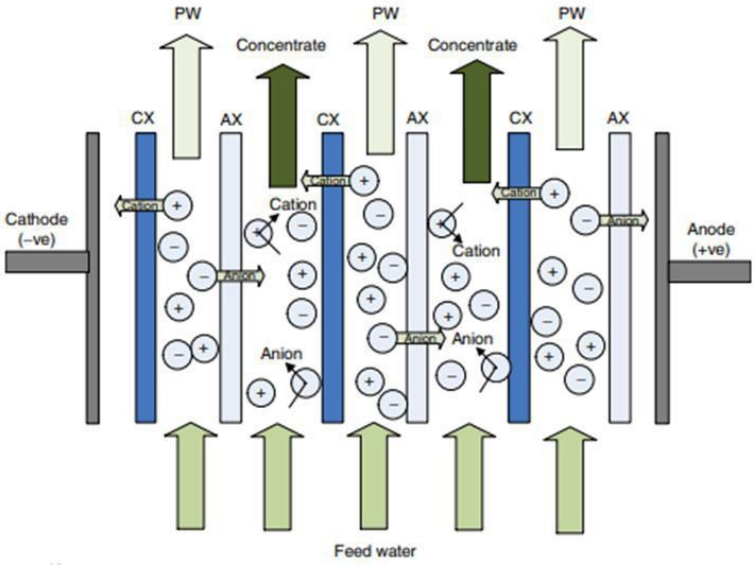


Figure 02: schematic representation of electrodialysis principle. CX: Cation exchange Membrane, AX: Anion exchange membrane, PW: Product water (Namboodiri & Rajagopalan, 2014)

Some of the main industrial applications of this technique:

- ✓ Desalination of brackish water and seawater.
- ✓ Demineralization of whey and other food products.
- ✓ Deacidification of fruit juices.

IV. smosis and Reverse Osmosis

Osmosis is the spontaneous net movement of solvent molecules (usually water) through a semi-permeable membrane from a region of lower solute concentration (hypotonic) to a region of higher solute concentration (hypertonic) (Figure 03).

Reverse Osmosis (RO) is a pressure-driven membrane process that reverses the natural direction of osmosis (Baker, 2012). By applying an external pressure to the concentrated feed solution that exceeds its osmotic pressure, the solvent is forced to flow against its concentration gradient, from the concentrated side to the dilute side. The semi-permeable RO membrane has a very dense structure with pores small enough to retain most ions and low-molecular-weight organic molecules (Figure 03).

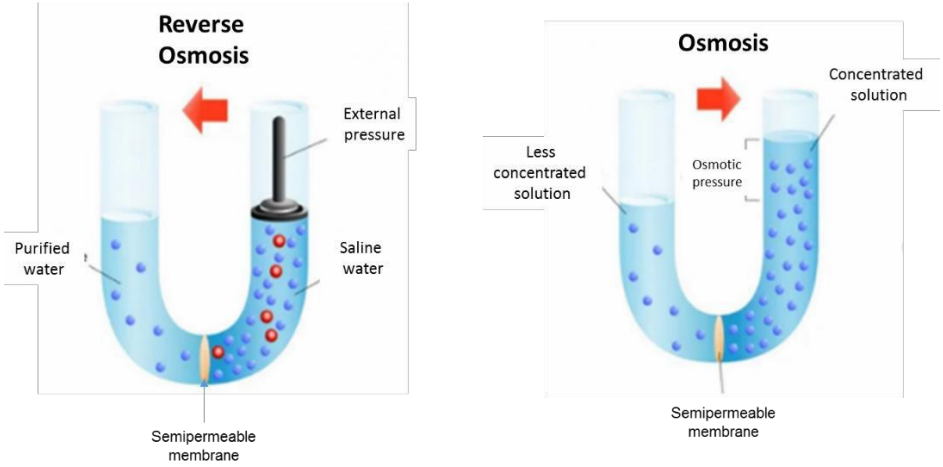


Figure 03: Schematic representation of osmosis and reverse osmosis. From Qu'est-ce que l'osmose inverse?, by Pure Aqua, Inc., (n.d).

The main industrial applications of RO are:

- ✓ Production of high-purity water for laboratories and pharmaceuticals.
- ✓ Potable water production from seawater or brackish water.

V. Concentration by Membrane Filtration

This encompasses a family of pressure-driven techniques differentiated by the size of the particles or molecules they retain.

- **Microfiltration (MF):** Uses membranes with pore sizes of 0.1–10 μm. It is primarily used to separate suspended particles, bacteria, and cells from a liquid.
- **Ultrafiltration (UF):** Uses membranes with pore sizes of approximately 1–100 nm (MWCO from 1 to 1000 kDa). It is used to concentrate and purify macromolecules like proteins, viruses, and colloids. It can also be used for buffer exchange via diafiltration, where fresh buffer is added to the retentate during filtration.

  A common laboratory application of UF is the use of centrifugal concentrators (spin columns). The sample is placed in a device containing an UF membrane and centrifuged. The solvent and small solutes pass through the membrane, while the macromolecules are retained and concentrated in the upper chamber.
- **Nanofiltration (NF):** Uses membranes with pore sizes around 0.5–2 nm. Capable of retaining small organic molecules and divalent ions (e.g., Mg^{2+}, SO_4^{2-}), while allowing monovalent ions to pass through.

Chapter Summary

Decantation: A simple gravity-based method for separating immiscible liquids or suspended solids from a liquid, relying on density differences.

Distillation: A thermal separation technique based on volatility differences.

Simple distillation is used for components with widely different boiling points.

Fractional distillation uses a column to achieve multiple vaporization-condensation cycles, enabling separation of components with close boiling points (e.g., petroleum refining).

Filtration: A physical separation using a porous medium.

Gravitational and vacuum filtration are used for coarse separations.

Microfiltration (MF), Ultrafiltration (UF), and Nanofiltration (NF) are pressure-driven membrane processes that separate based on particle/molecular size, with UF being a key method for concentrating proteins.

Protein precipitation can be achieved by altering temperature (denaturation), adjusting pH to the isoelectric point (pI), or adding high salt concentrations (salting out). Differential salting out with ammonium sulfate is a classic fractionation step

Centrifugation : An accelerated sedimentation technique.

Differential centrifugation separates cellular components by subjecting a homogenate to successive increases in centrifugal force.

Density gradient centrifugation offers higher resolution. **Rate-zonal** separates by sedimentation rate (size and shape), while **isopycnic** separates solely by buoyant density

Membrane Techniques :

Dialysis separates small molecules from large ones by diffusion through a semi-permeable membrane.

Electrodialysis uses an electric field and ion-exchange membranes to remove ions from a solution.

Osmosis is the natural flow of solvent across a membrane from low to high solute concentration, halted by **osmotic pressure (π)** .

Reverse osmosis (RO) applies external pressure greater than π to force solvent from a concentrated to a dilute side, producing pure water.

Review Questions

1. You have a mixture of ethanol (boiling point 78°C) and water (boiling point 100°C). Would you choose simple or fractional distillation to achieve a pure separation? Justify your answer.

2. A student wants to concentrate a dilute protein solution and exchange its buffer from phosphate-buffered saline (PBS) to a Tris-HCl buffer. Describe a procedure using ultrafiltration that would accomplish both goals.

3. Compare and contrast the principles of rate-zonal and isopycnic density gradient centrifugation. For each, provide a biological application.

4. The Meselson-Stahl experiment was a landmark in molecular biology. Explain how isopycnic centrifugation in a CsCl gradient allowed them to distinguish between semi-conservative, conservative, and dispersive models of DNA replication.

5. A solution contains a mixture of sodium chloride (NaCl) and a 50 kDa protein. You need to remove the salt. Which technique(s) would be most appropriate? Explain your choice.

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Chapter II: Chromatographic Techniques

Table of Contents

Learning Objectives

After reading this chapter, you should be able to understand the basic principle of chromatography based on the differential distribution of analytes between a mobile and a stationary phase. You will be able to classify chromatographic techniques by their separation mechanisms and formats, and explain key concepts such as polarity and partition coefficient. You will be able to interpret chromatograms using parameters like retention time and resolution. You will also be familiar with specialized techniques including size-exclusion, ion-exchange, and affinity chromatography, and most importantly you will know how to select the appropriate method based on the properties of the molecules to be separated.

Lecture 01: Introduction to Chromatographic Techniques

I. Introduction to Chromatography

Chromatography encompasses a diverse and powerful set of physico-chemical techniques used for the separation, identification, quantification, and purification of the components of complex mixtures. These methods are indispensable in modern analytical chemistry, biochemistry, and industrial processes, relying on the subtle differences in the physical and chemical properties of the molecules to be separated.

I.1. Historical Milestones

Chromatography was invented in 1906 by the Russian botanist Mikhail Tsvet, who sought to separate plant pigments. He notably observed the separation of the colored components of crude chlorophyll when its solution ascended along a filter paper. The different components indeed formed distinct color bands at different heights. However, it was not until the 1930s that chromatography began to be more widely used in various fields.

Key milestones in its subsequent development include:

1931 (Kuhn & Lederer): Successful application of liquid-solid column chromatography for the preparative separation of natural products.

1938 (Izmailov & Schraiber): Introduction of thin-layer chromatography (TLC).

1941 (Martin & Synge): Foundational work on liquid-liquid partition chromatography, proposing the concept of theoretical plates and foreshadowing gas-liquid chromatography. They were awarded the Nobel Prize in Chemistry in 1952 for this invention.

1952 (James & Martin): Practical development of gas-liquid chromatography (GC).

1955: Commercialization of the first gas chromatograph.

1965 (Horvath & Lipsky): Development of high-performance liquid chromatography (HPLC), utilizing small particle sizes and high pressures to achieve rapid and efficient separations.

I.2. General Principle

Chromatography separation process is based on the differential distribution of the components of a mixture between two immiscible phases:

A stationary phase, which can be a solid or a liquid immobilized on a solid support.

A mobile phase, which is either a liquid or a gas that percolates through or over the stationary phase.

The sample mixture is introduced into the system and carried by the mobile phase in a process called elution. As the mixture travels, its components interact differently with the stationary phase. Components that interact more strongly with the stationary phase will be retained longer, moving more slowly. Conversely, components that interact more readily with the mobile phase will be carried along more quickly. This differential migration results in the physical separation of the mixture's constituents over time and space.

I.3. Classification of Chromatographic Techniques

Chromatographic methods can be classified according to several criteria (Marouf, 2002):

- According to the nature of the Phases:

The mobile phase is a fluid → Liquid Chromatography (LC): mobile phase is a liquid
The mobile phase is a fluid → Gas Chromatography (GC): mobile phase is a gas.

The stationary (fixed) phase → A solid with adsorptive properties
The stationary (fixed) phase → A solid impregnated with a liquid

- According to the Physical Format of the Stationary Phase:

Planar Chromatography: The stationary phase is supported on a flat plane. → Paper Chromatography (PC)
Planar Chromatography: The stationary phase is supported on a flat plane. → Thin-Layer Chromatography (TLC)

Column Chromatography: The stationary Phase is packed into a cylindrical column → At high pressure (e.g: HPLC)
Column Chromatography: The stationary Phase is packed into a cylindrical column → At low pressure (e.g. traditional LC)

➢ According to the factor determining the separation

- An inert solid is impregnated with a liquid that can be hydrophilic (normal partition) or hydrophobic (reverse phase partition).
- A solid bearing ionizable groups, e.g., ion-exchange chromatography.
- A solid with calibrated porosity, e.g., size-exclusion chromatography
- A solid bearing groups with specific characteristics, e.g., affinity chromatography.

II. Main Chromatographic Modes

II.1. Partition Chromatography

In partition chromatography, the stationary phase is a liquid immobilized on an inert solid support. The mobile phase can be either a liquid (liquid-liquid partition chromatography) or a gas (gas-liquid chromatography, GLC) (Martin & Synge, 1941).

Separation is achieved based on the differential solubility (partitioning) of the analytes between the two immiscible liquid phases. Analytes that are more soluble in the stationary liquid phase will partition into it more frequently and be retained longer, while those more soluble in the mobile phase will travel faster. The relative polarity of the analyte and the two phases governs this distribution.

II.1.1. Fundamental Concepts

II.1.1.1. Concept of Molecular Polarity

Polarity is a fundamental molecular property that governs interactions in chromatography. It describes the distribution of electric charge within a molecule, leading to the formation of partial positive (δ^+) and negative (δ^-) regions (Atkins & de Paula, 2010).

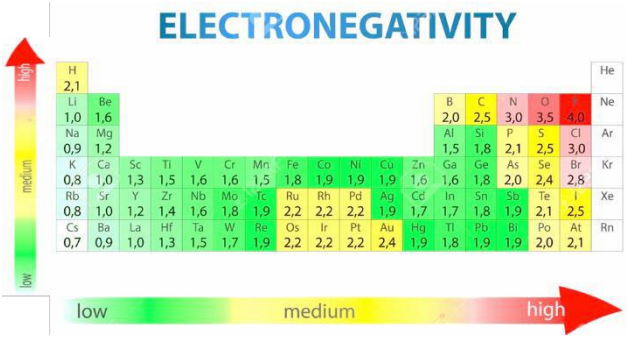
Polarity arises from two main factors:

- Electronegativity defined as the ability of an atom to attract shared electrons during the formation of a chemical bond.
- Molecular Geometry.

How to determine if a molecule is polar? (The answer to the following questions will indicate if a molecule is polar or not)

Q1. Does it contain a polar bond?

✓ A bond is polarized if the difference in electronegativity between the two atoms is greater than 0,45 (See periodic table of elements)



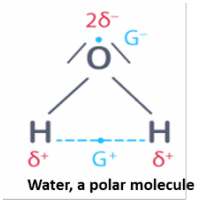
NB. When the difference in electronegativity is very large (> 1.7), the partial charges become formal, and the bond loses its covalent character, tending towards an ionic bond.

Q2. Where is the center of mass_located?

- ✓ Nonpolar molecule: the center of positive charges and the center of negative charges coincide; the distribution of charges is symmetrical within the molecule.
- ✓ Polar molecule: the two centers do not coincide; there are two distinct poles within the molecule

NB. Even if a molecule contains polar bonds, it can be nonpolar if its three-dimensional structure is symmetric, causing the bond dipoles to cancel each other out.

Examples:



Water, a polar molecule

Water (H_2O): A highly polar molecule due to the high electronegativity of oxygen and the bent geometry of the molecule, resulting in a net dipole moment.

Carbon Dioxide (CO_2): A nonpolar molecule. Although the C=O bonds are polar, the linear geometry of the molecule causes the two bond dipoles to point in opposite directions and cancel each other perfectly.

Carbon dioxide, a nonpolar molecule

In chromatography, polarity dictates the strength and type of intermolecular forces (e.g., hydrogen bonding, dipole-dipole interactions, van der Waals forces) between the solute, the mobile phase, and the stationary phase. The principle "like dissolves like" generally applies: polar compounds interact strongly with polar stationary phases and are retained longer in normal-phase systems, while nonpolar compounds interact preferentially with nonpolar phases.

II.1.1.2. Partition Coefficient (*K*)

The partition coefficient, denoted as *K* (P), is a parameter that measures the distribution of a substance between two immiscible phases, typically an organic phase and an aqueous phase, in a chemical system. it describes the equilibrium distribution of an analyte between the stationary phase and the mobile phase (Harris, 2010).

It is defined as the ratio of the solute's concentration in the stationary phase (Cs) to its concentration in the mobile phase (Cm):

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

A compound with a high *K* has a greater affinity for the stationary phase and will spend more time there, resulting in a longer retention time in the column. Conversely, a compound with a low K prefers the mobile phase and will elute quickly.

II.2. Adsorption Chromatography

This is the oldest form of chromatography, classically performed in columns or on TLC plates. Separation is based on the repeated adsorption and desorption of solute molecules onto the surface of a solid stationary phase (the adsorbent) as they are carried by a liquid or gaseous mobile phase (the eluent) (Geiss, 1987).

The process relies on competitive interactions between the solute molecules and the solvent molecules for active sites on the adsorbent surface. The most polar solutes interact most strongly with the polar active sites and are, therefore, retained the longest. Table 01 represents the commonly used stationary phases (Adsorbents) and mobile phases (Eluents).

Table 01: Commonly Used Stationary Phases (Adsorbents) and Mobile Phases (Eluents) (Marouf , 2002).

Stationary Phase (Polar)	Mobile Phase (Eluent) - Increasing Polarity
Silica gel (SiO_2)*	Hexane, Heptane
Alumina (Al_2O_3)	Cyclohexane,
Magnesium silicate	Carbon tetrachloride
Activated carbon	Benzene, Toluene
Calcium carbonate	Diethyl ether, Chloroform
	Dichloromethane, Ethyl acetate
	Acetone, 1-Butanol, Pyridine
	n-Propanol, Ethanol, Methanol
	Water

*Silica gel is the most widely used adsorbent due to its high capacity, well-defined pore structure, and relatively low cost.

Lecture 02: Planar Chromatography

I. Paper Chromatography (PC)

Paper chromatography is a simple and inexpensive form of planar chromatography where the stationary phase is a sheet of high-quality filter paper (Sherma & Fried, 2003). The paper is composed of cellulose, a hydrophilic polymer of glucose. It acts as a support for a stationary liquid phase, which is essentially the water molecules adsorbed from the air and from the manufacturing process onto the cellulose fibers. Thus, the true stationary phase is a water-cellulose complex. The mobile phase is a solvent immiscible with the stationary phase.

Procedure:

Sample Application: A small spot of the sample solution is applied near one edge of the paper.

- Development: The edge of the paper is immersed in a shallow pool of the mobile phase (a solvent or solvent mixture immiscible with water). The solvent ascends the paper by capillary action.
- Separation: As the solvent passes over the sample spot, the components partition between the mobile phase and the stationary aqueous phase. Components with different partition coefficients move at different rates, separating into distinct spots.
- Visualization: Colored substances (e.g., chlorophyll pigments) are visible directly. Colorless substances (e.g., amino acids) must be visualized by spraying the dried paper with a revealing reagent, such as ninhydrin, which forms a purple complex with amino acids.

PC exists in three main modes: ascending (most common), descending, and circular (radial).

II. Thin Layer Chromatography (TLC)

TLC is a more versatile and widely used planar technique than paper chromatography. The stationary phase is a thin layer of adsorbent (typically 0.1-0.25 mm thick), such as silica gel, alumina, or cellulose, coated onto a flat, inert support (glass, aluminum, or plastic plate) (Touchstone, 1992).

In TLC:

The mobile phase is a liquid immiscible with the stationary phase, chosen according to the polarity of the substances to be separated.

The stationary phase is most commonly an anhydrous silica gel (Adsorption chromatography) with a fluorescent product, or a hydrated silica gel (Partition chromatography) (less common).

II.1. Key Steps in TLC

Key steps of TLC are represented in figure 01

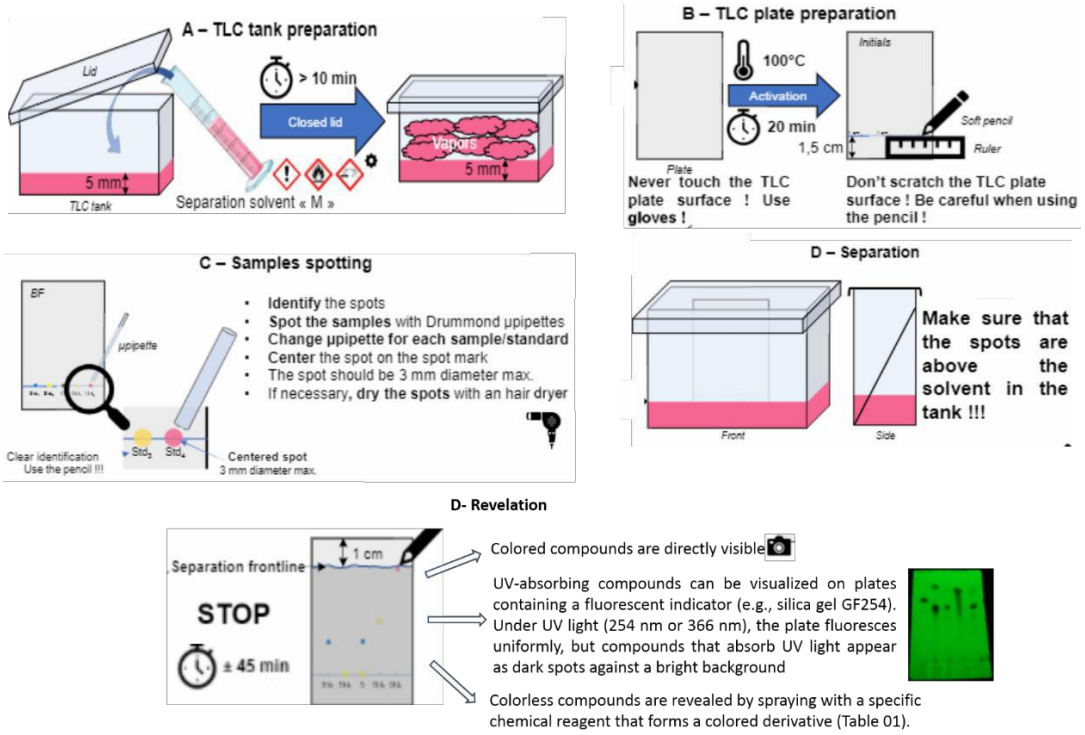


Figure 01: Main steps of TLC workflow. Adapted from Faurie, (2020).

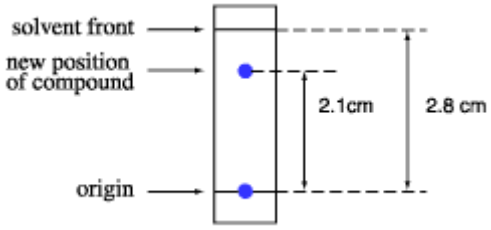
Table 01: Chromatographic Revealing Agents and Their Targeted Substances (Marouf , 2002).

Revealing agents	**Substances revealed**
Copper acetate	Prostaglandins
Magnesium acetate	Aldehydes and ketones
Phosphomolybdic acid	Antioxidants
Aniline-diphenylamine	Reducing sugars
Aluminum chloride	Flavonoids
Iron chloride	Phenols and phenolic acids
Iodine	Unsaturated fatty acids, steroids
Isatin	Proline and hydroxyproline
Ninhydrin	Amino acids and amines
Antimony pentachloride	Vitamins A, D, essential oils, terpenes, steroids
Dragendorff's reagent	Organic bases, alkaloids
Vanillin	Terpenes
Bromocresol green	Organic acids and bases

III. Retention Factor (Rf)

The position of a compound on a developed TLC or paper chromatogram is characterized by its retention factor (Rf) , a dimensionless quantity defined as (Fried & Sherma, 1999):

$$Rf = \frac{\text{Distance traveled by the substance (cm).}}{\text{Distance traveled by the solvent in the same time (cm).}}$$



Rf values range from 0 (compound remains at the origin, strongly retained) to 1 (compound migrates with the solvent front, not retained). A larger Rf indicates a greater affinity for the mobile phase and lower retention. Rf values are used for tentative identification by comparing them with those of known standards run on the same plate under identical conditions.

IV. uantification and Recovery of Compounds

Semi-quantitative Analysis: By visually comparing the size and intensity of a sample spot with a series of spots from known concentrations of a standard, an approximate concentration can be estimated.

Quantitative Analysis & Recovery: For preparative purposes, the spot corresponding to the desired compound can be scraped from the plate. The compound is then eluted (extracted) from the adsorbent using a suitable solvent, filtered to remove the adsorbent, and recovered for further analysis.

V. Two-Dimensional Planar Chromatography

In a two-dimensional linear development, one substance mixture is applied in a plate corner and first developed linearly. After drying, the plate is rotated by 90 and developed again linearly in a second solvent with chemical properties different to the first solvent (Hölzl & Dörmann, 2021) (Figure 02).

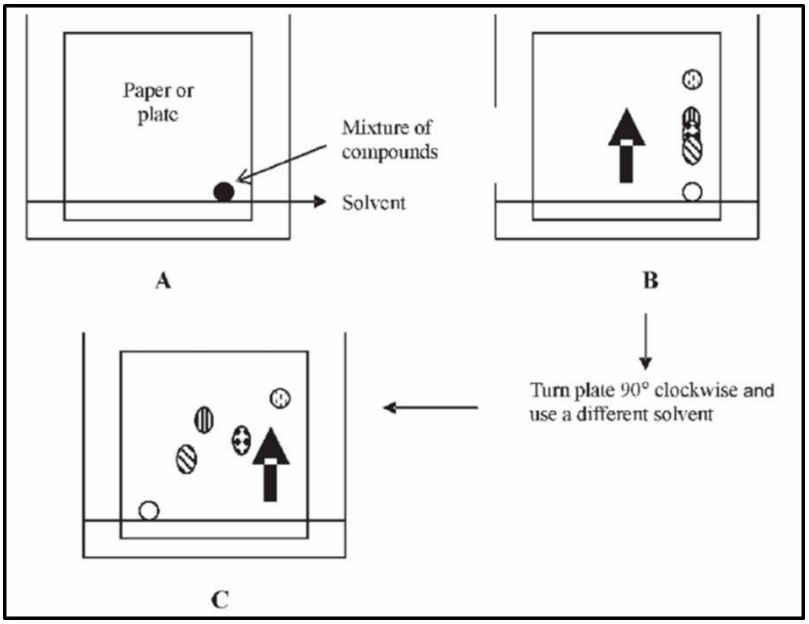


Figure 02: Schematic representation of a 2D planar chromatography (Debenedetti, 2009).

Lecture 03: Column Chromatography, HPLC and GC

I. Column Chromatography

I.1. Basic Principle

In its simplest form, column chromatography involves packing a vertical glass column with a stationary phase (e.g., silica gel). The sample is applied to the top of the column, and the mobile phase (eluent) is allowed to flow through the column continuously. As the sample moves down the column, its components separate based on their differential affinities for the stationary and mobile phases (Figure 01). Fractions of the eluent are collected at the bottom, and the separated components are recovered by evaporating the solvent.

Preparative TLC is often used as a scouting experiment to determine the optimal solvent system and its proportions for a successful column separation (Figure 01).

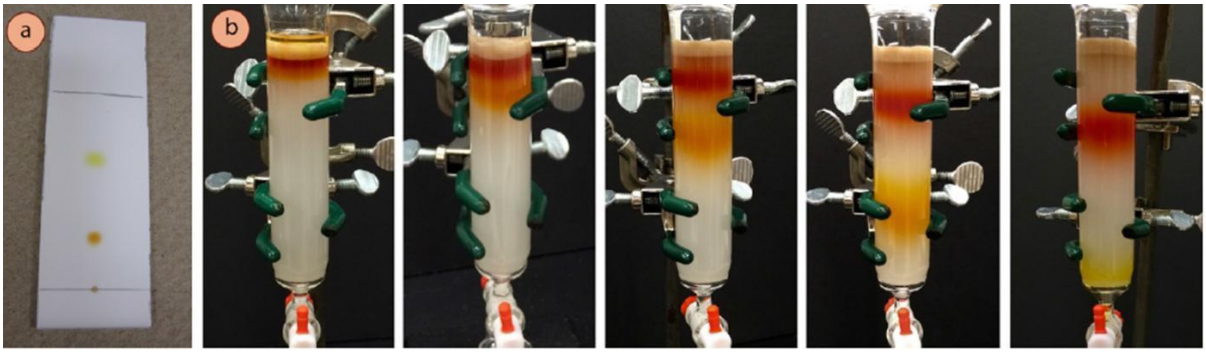


Figure 01: Photography of preparative TLC (a) preceding a column chromatography (b). Adapted from Nichols (2017), Organic Chemistry Lab Techniques, LibreTexts Chemistry

II. High Performance Liquid Chromatography (HPLC)

HPLC is a sophisticated form of column chromatography that represents a significant advancement over traditional LC (Snyder, Kirkland, & Dolan, 2010). The term "High Performance" refers to the use of stationary phases with very small, uniformly sized particles (typically 3-10 µm). This results in a vastly increased surface area for interaction and reduced band broadening, leading to dramatically improved resolution, speed, and sensitivity. However, the small particle size creates a high back-pressure, requiring the use of high-pressure pumps to force the mobile phase through the column (hence the alternative name, High Pressure Liquid Chromatography).

II.1. Advantages of HPLC

- ✓ High Resolution: Ability to separate closely related compounds.
- ✓ High Speed: Analysis times are often a few minutes.
- ✓ High Sensitivity: Detectors can quantify compounds at very low concentrations.
- ✓ Reproducibility: Precise control over parameters yields highly reproducible results.
- ✓ Versatility: Applicable to a wide range of compounds, from small organic molecules to large biomolecules.

II.2. Analytical vs Preparative HPLC

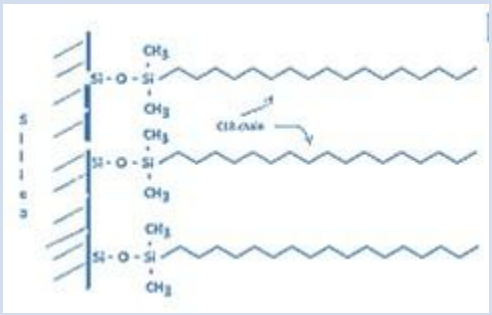
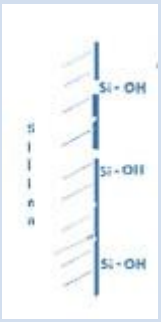
- ✓ Analytical HPLC: The goal is qualitative and quantitative analysis. A small amount of sample is injected, and the separated components are sent to a detector and then to waste.
- ✓ Preparative HPLC: The goal is to isolate and purify a specific compound for subsequent use. Larger sample amounts are injected, and the separated components are collected individually using a fraction collector.

II.3. Normal Phase vs Reverse Phase HPLC:

The two most common separation modes in HPLC are based on the relative polarities of the stationary and mobile phases. Table 01 summarizes the differences between normal phase HPLC and reverse phase HPLC.

Table 01: Normal phase Vs Reverse phase HPLC

REVERSE PHASE HPLC	NORMAL PHASE HPLC
Separation Mode: Reverse phase chromatography is the most commonly used HPLC technique. It is used to separate nonpolar molecules in solution.	**Separation Mode**: Neutral solutes are separated based on polarity. More polar solutes are retained longer on the stationary phase.
Phases: **Stationary Phase**: Nonpolar. **Mobile Phase**: Polar.	**Phases**: **Stationary Phase**: polar **Mobile Phase**: Nonpolar
Columns: Two types of stationary phases are used: **Silica bonded** with alkyl chains (C18, C8, phenyl, cyanopropyl groups). **Resin/polymers** for extreme pH conditions or more hydrophobic environments.	**Columns**: Primarily silica or alumina, both polar due to surface hydroxyl groups (-OH). Silica is preferred for its availability, performance, and low cost, while alumina is used for basic compounds like amines.

	
Mobile Phase: Polar solvent (usually water) combined with a less polar organic solvent (acetonitrile, methanol). Factors affecting separation include solvent type, proportion, and pH.	**Mobile Phase**: Nonpolar hydrocarbons (hexane, heptane, octane) with a small amount of a more polar solvent added (e.g., 2-propanol).
Mechanism: non-polar or less polar molecules in the sample interact with the hydrophobic stationary phase. These molecules are retained longer on the stationary phase. More polar molecules interact more strongly with the polar mobile phase, these polar molecules are eluted more quickly because they prefer to remain dissolved in the mobile phase rather than adsorbing onto the stationary phase.	**Mechanism:** Polar compounds functional groups interact with the stationary phase's hydroxyl groups and are retained longer than nonpolar compounds.
Applications: Widely used for separating low MW (<2500) Pharmaceutical industry: Separation of vitamins, steroids, β-blockers. Food and beverage industry: Analysis of sweeteners, food additives, carbohydrates. Chemical industry: Analysis of polymer additives. Environmental monitoring: Analysis of pesticides and herbicides. Clinical analysis: Determination of catecholamines.	**Applications:** Used to analyze samples soluble in nonpolar solvents, particularly effective for separating isomeric and multifunctional compounds. Commonly used for separating vitamins, pesticides, and hydrocarbons.
Advantages for Polar Molecules: Used for separating polar molecules (amino acids, peptides, proteins) that are soluble in polar solvents or strongly retained in normal phase chromatography	/

NB. Other separation mode can be used in HPLC technique depending on the stationary phase packed in the column such as size exclusion HPLC (SEC-HPLC) or Ion exchange HPLC (IEX-HPLC).

II.4. Instrumentation

A basic HPLC system represented in figure 02 consists mainly of the components listed below.

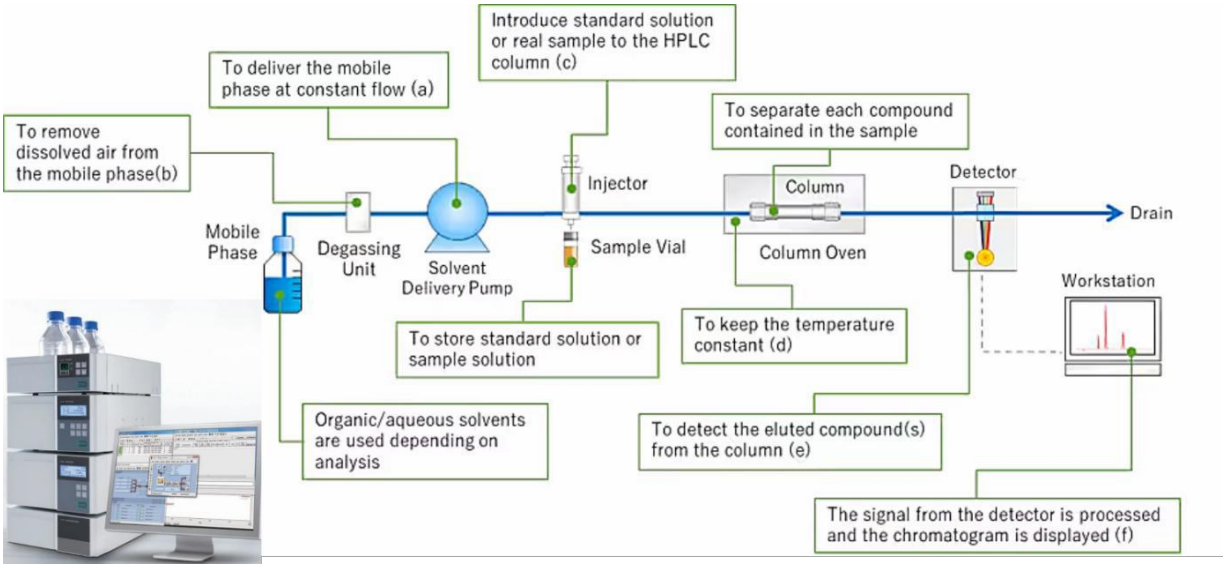


Figure 02: HPLC flow diagram and the role of each component. Adapted from What is HPLC?, by Shimadzu Corporation, n.d

➢ Mobile Phase Reservoirs: It is usually composed of several glass bottles, ranging from 200 to 1000 ml, with special caps (Figure 03).

The mobile phase must be degassed (to remove dissolved gases) and filtered to eliminate any suspended particles. This is necessary because all the tubes in the apparatus have an extremely small diameter and are at risk of clogging.

Figure 03: Mobile Phase Reservoirs. Adapted from Le réservoir de la phase mobile, by Labster Theory, n.d.

It should be noted that there are two types of elution:

Isocratic mode: The composition of the mobile phase remains constant throughout the duration of the experiment.

Gradient elution: The composition of the mobile phase changes over time.

- Pump: Pump in HPLC play a major role; it facilitates the circulation of the mobile phase throughout the system and control its composition through the operating modes mentioned earlier. The flow rate and maximum pressure vary depending on the model of the pump used. The most commonly used pumps in HPLC are designed to maintain a non-pulsed and stable flow rate, even when the composition of the mobile phase varies.
- Injector: Introduces the sample into the flowing mobile phase. Modern systems use an autosampler with an injection valve and a sample loop for precise and reproducible injections.
- Column: The heart of the system, where separation occurs. The column is typically presented as a tube, most often made of steel, with varying lengths and diameters depending on the model (Figure 04 a). "Standard" columns have an internal diameter (ID) of approximately 4.5 mm and a length of 10 cm (column lengths can go up to 30 cm), but they are increasingly being replaced by columns with smaller diameters, ranging from 0.1 to 1 mm in diameter. The stationary phase is held in place by two porous discs located at its ends. The column is often preceded by a guard column, which is short (0.4 to 1 cm) and filled with the same stationary phase, serving to retain certain impurities. This helps extend the lifespan of the main column while preserving its performance. It is still recommended to pass samples through a filter with a pore size of less than 0.5 mm before analysis (Figure 04 b). The column can be either at ambient temperature or temperature-controlled.

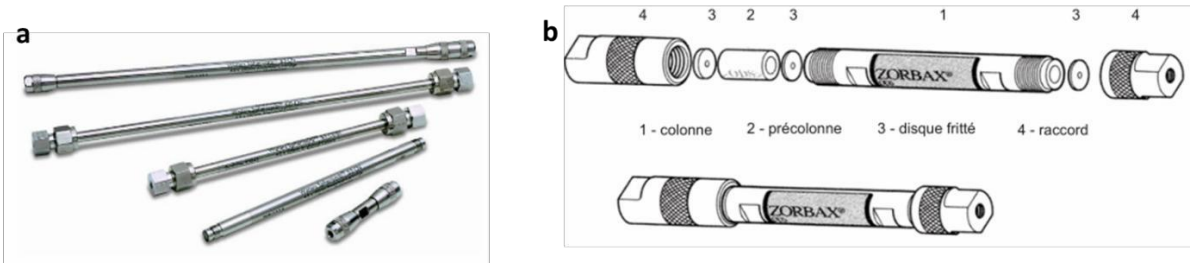


Figure 04: Stainless steel HPLC columns (a) and schematic representation of a column components (b). Adapted from Chromatographie Liquide Haute Performance (HPLC), by Analytical Toxicology, n.d.

- Detector: Continuously monitors the eluent from the column and generates an electrical signal proportional to the concentration of the analytes. Figure 05 provides an overview of HPLC detector types and their relative usage rates.
- Data System: A computer that records the detector signal, processes the data, and controls the instrument.

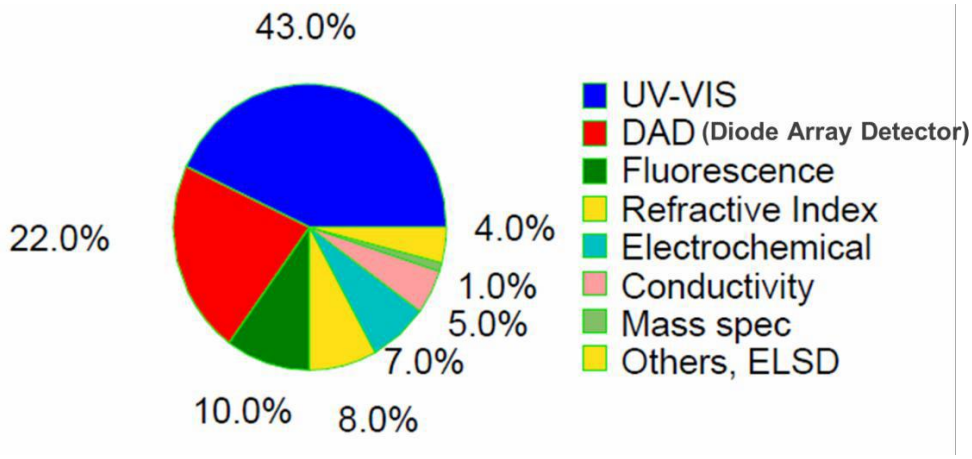


Figure 05: An overview of HPLC detector types and their relative usage rates. Adapted from Sandford (2020)

III. Gas Chromatography (GC)

GC is a powerful analytical technique for separating and analyzing compounds that can be vaporized without decomposition (McNair & Miller, 2009). It is used in various fields, such as perfumery, the petroleum industry, biology, fine chemistry, and the plastics industry.

III.1. Principle

The principle of GC is based on the interaction of analytes with a mobile phase (carrier gas) and a stationary phase contained within a column. The sample is vaporized and carried through the column by the inert carrier gas.

Depending on the nature of the stationary phase, we distinguish two main types:

- ✓ Gas-Solid Chromatography (GSC): The stationary phase is a solid adsorbent (e.g., silica gel, molecular sieves). Separation is based on adsorption.
- ✓ Gas-Liquid Chromatography (GLC): The stationary phase is a non-volatile liquid coated on the inside of the column or on an inert solid support. Separation is based on partition, and this is the vastly more common form of GC.

Every molecule introduced into this chromatographic system alternates between the stationary phase and the mobile phase and is subjected to a set of opposing forces:

- ➢ A retention force exerted by the stationary phase causes the molecule to be "held" by these forces, either through solubilization in the liquid (in partition chromatography) or through physical adsorption (in adsorption chromatography).

In partition chromatography, this retention force depends on two factors: the volatility of the molecule and its affinity for the liquid phase. Volatility, which is related to the boiling point, represents the molecule's tendency to escape into the gas phase. Affinity represents the strength of intermolecular interactions (such as van der Waals forces, dipole-dipole interactions, or hydrogen bonding) between the solute and the stationary phase. This affinity is governed by polarity: a polar molecule will be strongly retained by a polar stationary phase, while a non-polar molecule will prefer a non-polar stationary phase and therefore be less retained. This is why the choice of column polarity is essential: on a non-polar column, molecules tend to elute in order of their boiling points, whereas on a polar column, the elution order can be completely different due to specific chemical interactions.

In adsorption chromatography, the retention force depends on the molecule's ability to adhere to a solid surface. This involves not only but also molecular geometry and the ability to enter pores, which can create a molecular sieving effect. This mode is often used for permanent gases or very volatile compounds.

- A driving force is exerted by the mobile phase (the carrier gas) towards the exit of the column, constantly pushing the molecules forward regardless of their chemical nature.

The more a molecule introduced into the system is retained by the stationary phase, the slower it migrates towards the exit, resulting in a longer retention time. For example, in partition chromatography, a polar molecule injected into a polar column will spend more time dissolved in the liquid phase due to strong intermolecular interactions, hence it will be retained longer. In adsorption chromatography, a molecule that adsorbs strongly to the solid surface will also take more time to desorb and move forward.

The less a molecule introduced into the system is retained by the stationary phase, the faster it will exit the column, resulting in a shorter retention time. Conversely, a non-polar molecule on a polar column will spend most of its time in the mobile gas phase and will elute quickly, even if its boiling point is relatively high.

Ultimately, separation occurs because different molecules have different balances of volatility and polarity (Partition chromatography) or different adsorption strengths (Adsorption chromatography), causing them to spend different amounts of time in the stationary phase and therefore travel through the column at different speeds.

III.2. Instrumentation

A basic GC system represented in figure 06 consists mainly of the components listed below.

- Carrier Gas Supply: A high-pressure cylinder provides the inert carrier gas (mobile phase), typically helium, nitrogen, or hydrogen. The choice of gas depends on the detector and the desired efficiency (Table 02).

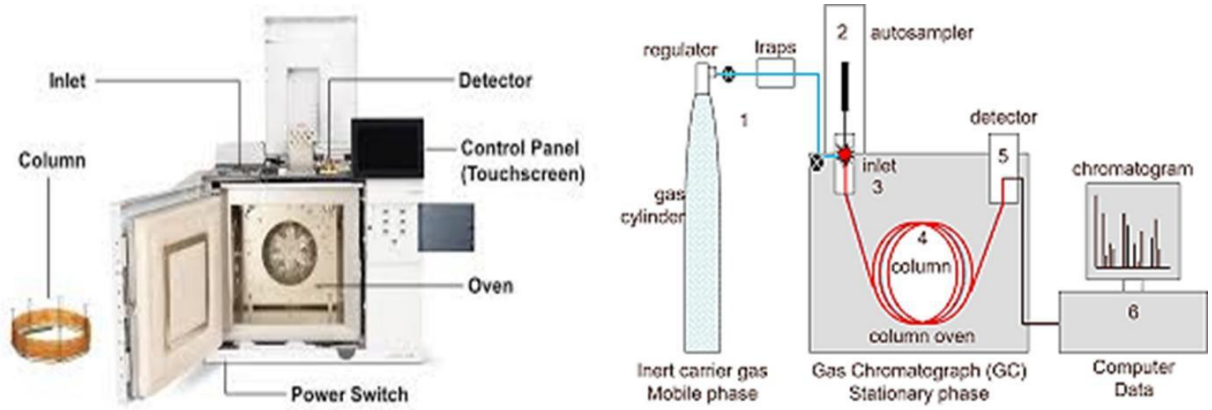


Figure 06: GC system diagram. Adapted from Agilent Technologies (n.d.), Gas Chromatography Fundamentals, and Technology Networks (2022)

Table 2: Common Carrier Gases in GC

Gas	Efficiency	Speed	Cost	Safety
Helium	High	Fast	High	Safe
Hydrogen	Very High	Fast	Moderate	Flammable
Nitrogen	Moderate	Slow	Low	Safe

- Flow Controller (Regulator): Maintains a constant and precise flow rate of the carrier gas.
- Injection system: whether manual injection or an autosampler is used, the needle is introduced through a self-sealing septum into an inlet containing a glass liner, which is typically packed with deactivated glass wool to trap non-volatile residues and protect the column.
- Columns: located in a thermostatted oven (an hermetically sealed chamber), we distinguish two main types (Figure 07).:
  - ✓ Packed Columns: made of glass or stainless steel (2-4 mm ID, 1-5 m long), filled with an inert solid support coated with the stationary phase. They have lower resolution.
  - ✓ Capillary Columns (Open Tubular Columns): These are long, narrow tubes made of fused silica (0.1-0.5 mm ID, 10-100 m long, to fit within the device, the column is coiled into spirals with a diameter of 10 to 30 cm). The stationary phase is coated as a thin film

(0.1-5 µm) on the inner wall. They are of common use nowadays and offer extremely high resolution.

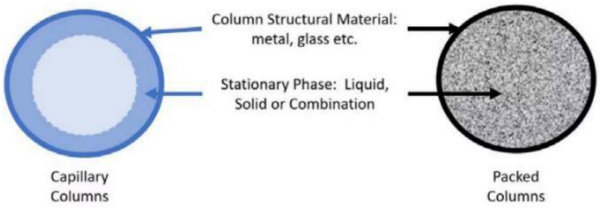


Figure 07: Capillary and packed column structure (Atkins, 2022).

- Detector: Positioned at the column exit, it continuously measures the amount of each analyte as it elutes. Common detectors include: Flame Ionization Detector (FID) for organic compounds, Thermal Conductivity Detector (TCD), Electron Capture Detector (ECD) for compounds containing electronegative atoms, such as halogens (e.g., pesticides, PCBs) and last but not least Mass Spectrometer (MS): The most powerful detector, providing both quantitative and qualitative (structural) information. *GC-MS is the gold standard for identifying unknown compounds.*
- Data System: Records and processes the detector signal, producing a chromatogram and controlling the instrument.

III.3. Effect of temperature on analytes elution

The oven temperature is precisely controlled and is evenly distributed by a fan. It can be held constant (isothermal) or increased during the run (temperature programming). The higher the temperature of the oven (and thus of the column), the faster the analytes move through the column; however, they interact less with the stationary phase, resulting in less separation of the analytes. Conversely, the lower the oven temperature, the better the separation of the analytes, but the longer the analysis takes. Therefore, the choice of temperature is a compromise between the duration of the analysis and the desired level of separation. Figure 08 shows 3 charts representing how the set of temperature can affect the time and quality of separation.

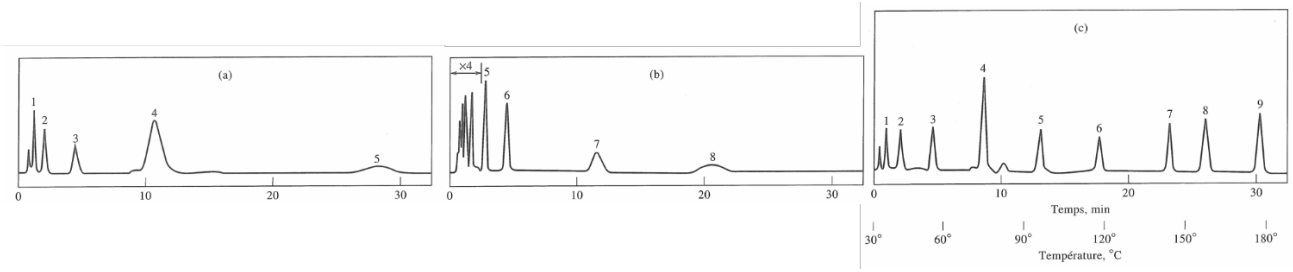


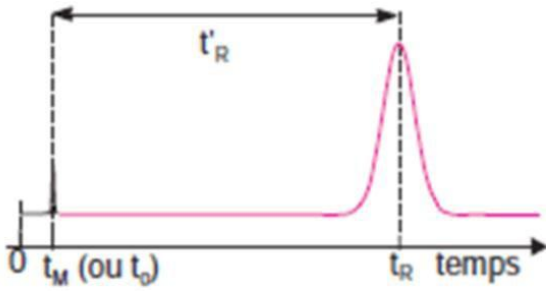
Figure 08: chromatograms of the same sample obtained under different conditions: (a) using an isothermal method at 45 °C; (b) using an isothermal method at 145 °C; (c) using a method with a temperature gradient from 30 °C to 180 °C over 30 minutes (Cai & Stearns, 2023).

IV. Chromatographic Parameters and Data Analysis

A **chromatogram** is a graphical representation of a chromatography experiment. It displays the detector response as a function of time (or sometimes volume) as different components in a mixture pass through the detector.

Retention Time, tR: Retention time is the total time a compound takes to travel through the chromatographic column from the point of injection to the detector. It represents the time at which a specific analyte elutes from the column.

Dead Time, t0: Dead time is the time taken by an unretained compound (one that is not retained by the stationary phase) to pass through the chromatographic system and reach the detector. It represents the time for the mobile phase to flow through the column without any interaction with the stationary phase.



Reduced (adjusted) Retention Time, t′R: Reduced retention time is the actual time an analyte spends interacting with the stationary phase. It is the retention time corrected by subtracting the dead time.

Several parameters are used to describe and optimize the separation:

Capacity Factor or Retention factor (k'): A measure of how well a compound is retained by the column. Optimal k' values are typically between 1 and 10.

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

Selectivity Factor (α): A measure of the relative retention of two compounds, indicating their separation efficiency. k′2 and k′1 are the capacity factors of the two analytes (with k′2 for the more retained compound).

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

Resolution: Resolution measures how well two peaks are separated. Good resolution ensures accurate quantification, especially for closely eluting compounds. tR1 and tR2 are the retention times of the two peaks w1 and w2 are the peak widths at the base. A resolution of 1.5 or greater indicates baseline separation.

$$R_s = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2}$$

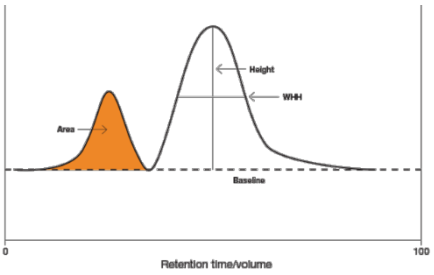
Column Efficiency (Number of Theoretical Plates, N): Efficiency indicates how well the column separates compounds. tR is the retention time and w is the width of the peak at the base

$$N = 16 \times \left(\frac{t_R}{w}\right)^2$$

V. Qualitative and Quantitative Analysis

Qualitative Analysis: Identification is achieved by comparing the retention time of a peak in the sample with that of a known standard analyzed under identical conditions.

The **peak area** in chromatography refers to the area under a chromatographic peak in a chromatogram. It is directly proportional to the amount or concentration of the analyte present in the sample.



Key Points about Peak Area:

- Proportional to Concentration: The peak area is used to quantify the amount of analyte in the sample. A larger area indicates a higher concentration of the substance.
- Units: The peak area doesn't have specific units but is reported in terms of detector response, often as arbitrary units. It is calculated using numerical integration methods.

Quantitative Analysis: The concentration of an analyte is determined from its peak area. Quantification of a compound using a single standard with the following formula:

$$C_{sam} = \frac{C_{std} \times A_{sam}}{A_{std}}$$

Or using a calibration curve is constructed by plotting the peak areas of several standard solutions of known concentration against their concentrations. The concentration of the analyte in the sample is then determined by comparing its peak area to this curve.

Internal standard concept

A known amount of a compound not present in the sample (the internal standard) is added to both the calibration standards and the sample. The ratio of the analyte's peak area to the internal standard's peak area is used for quantification, correcting for any variations in injection volume or sample preparation. The following ratio remains constant.

$$\frac{\text{Tr sample}}{\text{Tr internal standard}}$$

Lecture 04: Size Exclusion Chromatography, Ion Exchange Chromatography and Affinity Chromatography

I. Size Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC), also called gel filtration chromatography (GFC), or gel permeation chromatography (GPC), it is a molecular sieving chromatography where the main factor for separation is the size of the molecules. The separation of molecules of different sizes is achieved on a stationary phase made up of a granular gel, allowing for calibrated pores between these granules. The gel granules themselves are perforated by a large number of calibrated pores (Figure 01).

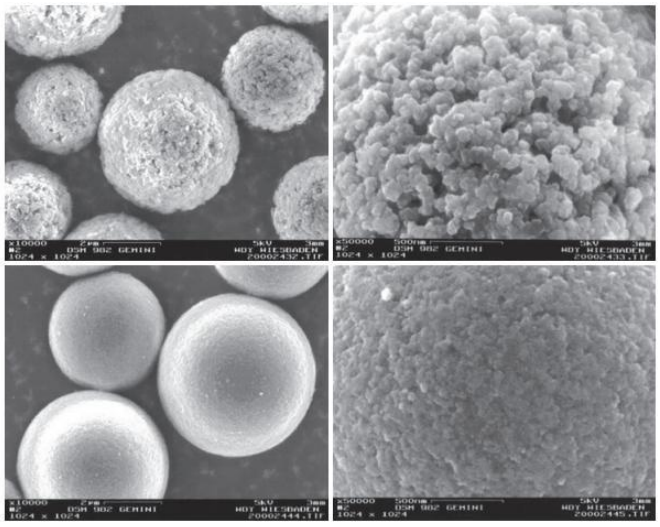


Figure 01: Electron micrographs at different magnifications of SEC gels with various pore sizes (Hoshino et al., 2015)

A large number of solid supports can constitute the stationary phase in size exclusion chromatography (SEC), with the main differences being their nature and porosities. These supports can be made of organic, natural polymers (such as polysaccharides) or synthetic materials. The fractionation range of a given gel identifies the molecular weight (MW) interval between the smallest molecules that can completely penetrate the gel and the largest molecules that are completely excluded (Table 01).

Table 01: Gels commonly used in size exclusion chromatography and their fractionation ranges (Marouf, 2002).

Gel (Commercial name)	Fractionation range (MM)
	Dextrans
Sephadex G-10	> 700
Sephadex G-15	>1500
Sephadex G-25	1000 - 5000
Sephadex G-50	1500 - 30 000
Sephadex G-75	3000 - 70 000
Sephadex G-100	4000 - 150 000
Sephadex G-200	5000 – 800 000
	Polyacrylamide
Biogel P-2	100 – 1800
Biogel P-10	1500 – 20 000
Biogel P-100	5000 – 100 000
Biogel P-150	15 000 – 150 000
Biogel P-300	60 000 – 400 000
	Agarose
Sepharose CL-6B	10^4- 4. 10^6
Sepharose CL-4B	6. 10^4 - 2. 10^7
Sepharose CL-2B	7. 10^4 - 4. 10^7
Biogel A-0,5M	10^4 - 5. 10^7
Biogel A-5M	10^4 - 5. 10^7
Biogel A-50M	10^5 - 5. 10^7

I.1. Principle and Elution Order

As the sample solution flows through the column, molecules that are larger than the largest pores cannot enter the particles and are excluded. They travel only through the interstitial space between the particles and elute first. Molecules that are small enough to enter the pores will diffuse in and out of the stationary phase. Smaller molecules can access more of the pore volume and are, therefore, retarded more, eluting later (Figure 02). The separation is purely based on size and shape, with no chemical interaction between the analyte and the stationary phase. The mobile phases are typically low-salinity aqueous phases (e.g., 0.9% NaCl) or those with a slight buffering capacity (e.g., 0.05 M PBS, pH 7.0).

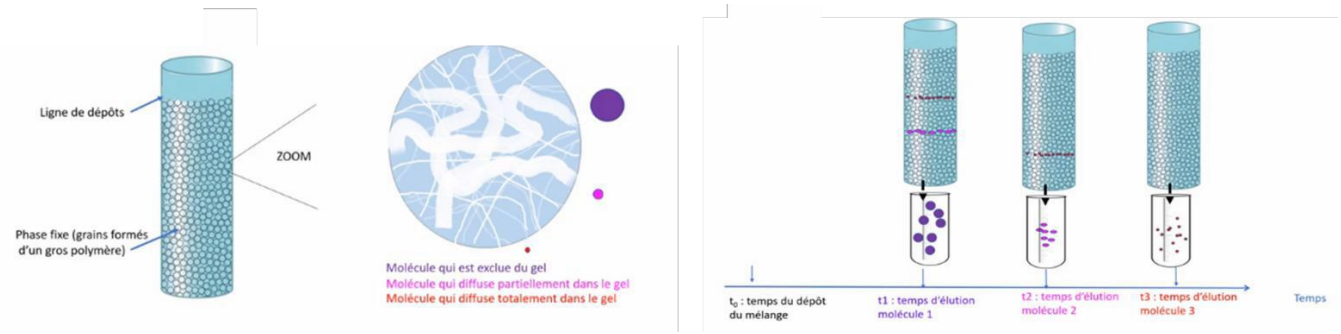
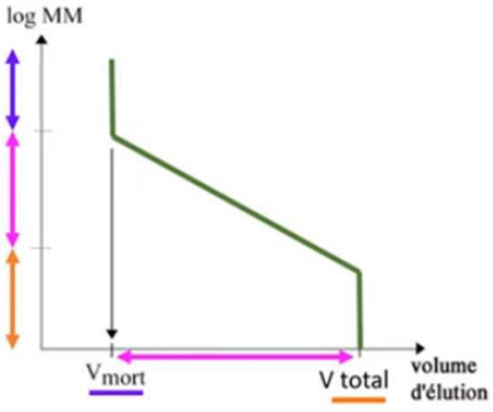


Figure 02: Elution order of molecules with different sizes. Adapted from screenshots of La chromatographie par gel filtration, by S. Orsoni, (2020).

I.2. Results Expression

The results are typically expressed in the form of a graph showing the variation of solute concentration as a function of elution volume, representing the logarithm of the molecular weight (MW) as a function of elution volume: log MW = f(elution volume), or the logarithm of the molecular weight as a function of K AV , the partition coefficient between the liquid phase and the stationary phase: log MW = f(K AV) (AV: Accessible Volume)



The partition coefficient K_AV quantifies the degree to which a molecule of a particular size can access the pore volume within the stationary phase of the chromatography column.

I.2.1. Volumes and the Partition Coefficient

Volumes

The elution of a solute is characterized by its elution volume. The elution volume (Ve) is the volume of the 'eluting' mobile phase required to cause the substance to exit the column.

Note: The Ve depends on other volumes involved in size exclusion chromatography (SEC), which are closely related to the distribution coefficient.

$$Vt = V0 + Vi$$

Vt: Total column volume

Vd (or V0): Dead volume, void volume, or exclusion volume; it represents the elution volume of a substance that is completely excluded from the gel.

VI: Volume inside the gel grains.

- Consider two molecules with respective diameters X and Y that are greater than the diameter of the largest pore in the gel, with Y>X; these two molecules are *completely excluded*, and their elution volumes are equal to the outer volume of the gel grains. In this case **Ve= V0**.
- Consider two molecules with respective diameters X and Y that are smaller than the diameter of the smallest pore in the gel, with Y>X; these two molecules *completely diffuse* into the gel, and their elution volumes are equal to the total volume. In this case, **$V_e = V_t = V_0 + V_i$.**
- Consider two molecules with respective diameters X and Y that are between the diameter of the smallest pore and that of the largest pore in the gel, with Y>X; these two molecules partially diffuse into the gel, and their elution volumes for each molecule represent the volume of the mobile phase between V_0 and V_t. In this case, Ve for Y is not equal but greater than Ve for X. When there is partial diffusion of molecules in a gel (i.e., within its fractionation range), we can say that the larger the size of a molecule, the smaller its elution volume

Partition Coefficient

A solute is distributed between the solution outside the gel granules (V_0) and the solution inside the gel granules (V_i) according to a partition coefficient (K).

If K = 0, the solute is totally excluded.

If 0 < K < 1, the solute is partially included. The inclusion rate increases with K.

If K = 1, theoretical value corresponding to total inclusion of a compound in the gel.

The general expression that relates the elution volume of a solute to its distribution coefficient is as follows:

$$Ve = V0 + K.\ Vi$$

Knowing that Vi = Vt- V0, we can thus write:

$$K=Ve-V0\ /\ Vt-V0$$

We can therefore say that the partition coefficient K better characterizes the elution of a solute.

1.3. Applications

Desalting and Buffer Exchange: Rapidly separating high molecular weight proteins from low molecular weight salts and buffers.

Molecular Weight Determination: Estimating the molecular weight of native or denatured proteins and polymers.

Fractionation of Complex Mixtures: Separating proteins, nucleic acids, polysaccharides, and other macromolecules based on size.

II. Ion Exchange Chromatography (IEC)

IEC separates molecules based on differences in their surface charge (Himmelhoch, 1971). It is widely used for proteins, peptides, nucleic acids, and other charged biomolecules.

II.1. Principle

The stationary phase is an insoluble macromolecular support onto which positively (+) or negatively (-) charged groups are covalently attached. These groups are capable of non-covalently binding ions of opposite charges (counter ions) (Figure 03).

There are two types of ion exchange chromatography → **Cation exchange chromatography**

There are two types of ion exchange chromatography → **Anion exchange chromatography**

In ion exchange chromatography, ion exchangers are classified as either strong or weak, for both anions and cations.

Strong Cation Exchanger: e.g., R-sulfonated $R–SO_3^-$ groups. maintain a negative charge regardless of the working pH

Weak Cation Exchanger: e.g., Carboxymethyl (CM) groups. their ionization dependent on the pH.

Strong Anion Exchanger: e.g., Quaternary ammonium (Q) groups. Their charge is independent of pH below pH 11-12.

Weak Anion Exchanger: e.g., Diethylaminoethyl (DEAE) groups. Their charge decreases above pH 9.

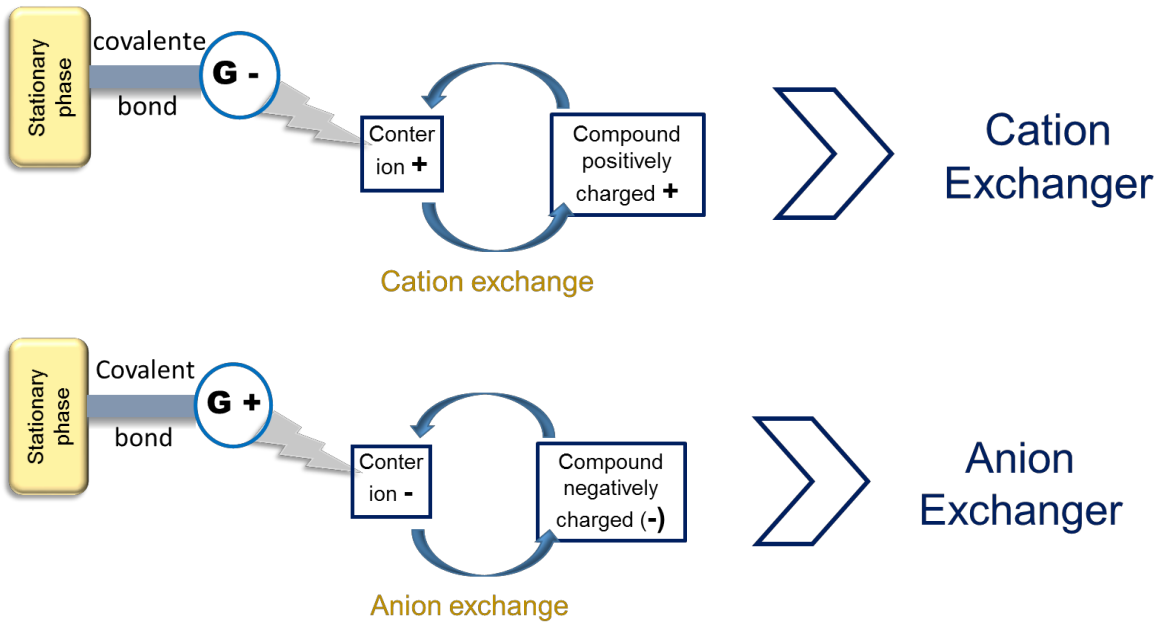


Figure 03: Schematic representation of cation and anion exchanger.

II.2. Steps in Ion Exchange Chromatography

II.2.1. Column Preparation (Equilibration)

The column is equilibrated with a starting buffer at a specific pH and low ionic strength that promotes binding of the target molecule(s). The pH is chosen so that the target protein has the opposite net charge to the exchanger (i.e., for a cation exchanger, the buffer pH must be below the protein's pI to give it a net positive charge; for an anion exchanger, the pH must be above the protein's pI).

II.2.2. Sample Loading & Binding (Fixation)

The sample, dissolved in the starting buffer, is applied to the column. The target protein and other proteins with the appropriate charge will bind, displacing the counter-ions. Unbound materials (with the same charge as the exchanger, or neutral) flow through the column.

II.2.3. Elution

Bound proteins are selectively released (eluted) from the column by altering the buffer conditions to disrupt the electrostatic interactions. This is typically done in two ways:

- ✓ Increasing Ionic Strength (Salt Gradient): Adding increasing concentrations of a salt (e.g., NaCl) introduces competing ions (Na^+, Cl^-). These ions shield the protein's charge and compete for binding sites on the exchanger. Proteins with lower net charge elute at lower salt concentrations, while those with higher net charge elute at higher salt concentrations.
- ✓ Changing pH (pH Gradient): Altering the pH changes the net charge of the protein. As the pH approaches the protein's pI, its net charge approaches zero, weakening its binding to the exchanger and causing elution.

III. Affinity Chromatography (AC)

Affinity chromatography is the most specific and powerful separation technique, exploiting the unique biological properties of molecules (Hage, 1999). It relies on the highly specific and reversible interaction between a target molecule and a complementary molecule called a ligand.

III.1. Principle

The ligand, which has a specific affinity for the molecule of interest (e.g., an enzyme's substrate, an antigen's antibody), is covalently attached to an insoluble, inert matrix (the stationary phase). When a complex mixture is passed through the column, the target molecule binds specifically to the immobilized ligand, while all other components (with no affinity for the ligand) flow through unretained. The bound target molecule is then eluted by disrupting the specific interaction.

III.2. Matrix, Ligand, and Spacer Arms

Matrix: The ideal matrix is inert, chemically stable, has good flow properties, and is easily derivatized. Common matrices include agarose (e.g., Sepharose), polyacrylamide, and silica.

Ligand: The molecule that specifically binds the target. It can be a small molecule (e.g., a cofactor) or a large biomolecule (e.g., an antibody). It must be coupled to the matrix in a way that retains its biological activity.

Spacer Arm: If the ligand is small, or if the target molecule is large, a "spacer arm" (a short hydrocarbon chain, 2-12 atoms long) may be introduced between the matrix and the ligand. This moves the ligand away from the matrix surface, reducing steric hindrance and making it more accessible for binding, hence a better ligation (Figure 04).

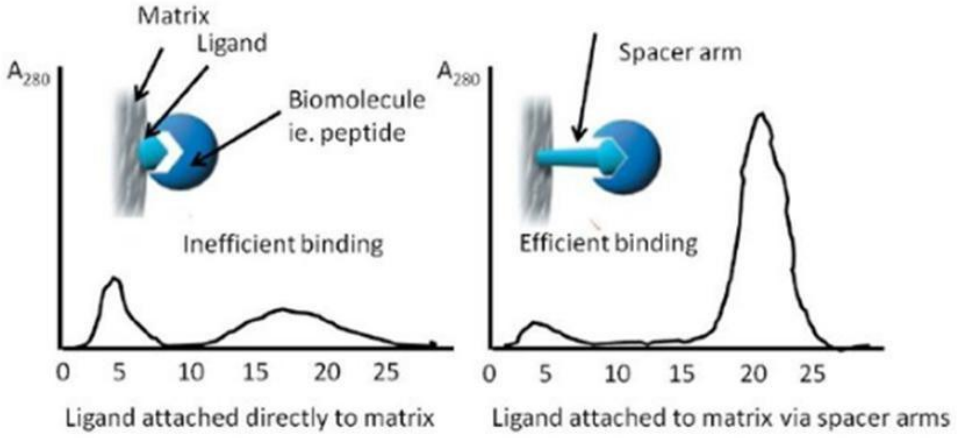


Figure 04: Chromatogram showing better ligation and elution when spacer arms are introduced between the ligand and matrix. (Magdeldin & Moser, 2012)

III.3. Elution Methods:

Elution is achieved by breaking the specific ligand-target interaction without irreversibly damaging the target.

- Biospecific Elution (Competition): A high concentration of a free ligand (competitive) or the target molecule itself is passed through the column. The free ligand competes with the immobilized ligand for binding to the target, displacing it (Figure 05).

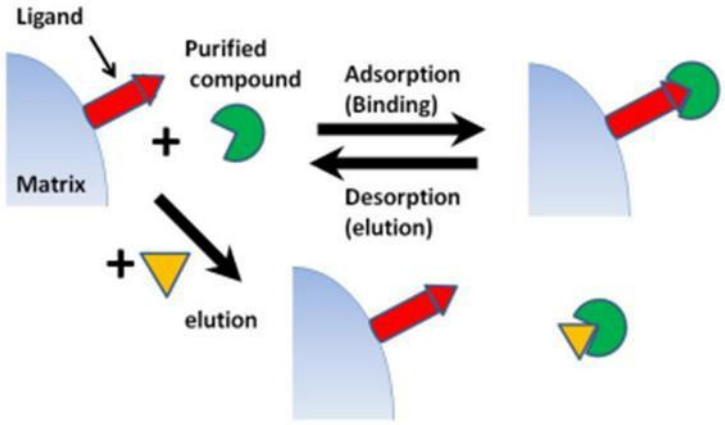


Figure 05: Competitive elution of the target by adding a competitive free ligand (triangle). (Magdeldin & Moser, 2012).

- Non-Specific Elution (Physicochemical Change): Changing the buffer conditions (e.g., lowering pH, increasing ionic strength, adding chaotropic agents, changing polarity) disrupts the electrostatic, hydrophobic, or hydrogen bonding interactions that stabilize the complex.

After elution, the column must be regenerated by washing with appropriate buffers to remove any remaining bound material and re-equilibrate it for the next run.

IV. mmobilized Metal Affinity Chromatography (IMAC)

IV.1. Principle

IMAC is a popular and versatile affinity technique, particularly for purifying recombinant proteins (Porath et al., 1975). It is based on the affinity of certain amino acid side chains (especially histidine and cysteine) for immobilized transition metal ions.

In this technique a chelating agent (e.g., iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA)) is covalently attached to the matrix. This chelator is then charged with a metal ion, most commonly Ni^{2+}, but also Co^{2+}, Zn^{2+}, or Cu^{2+}(Figure 06)

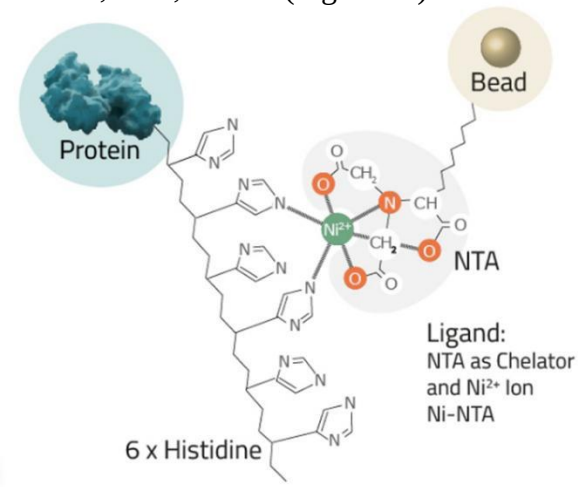


Figure 06: A schematic representation of Nickel affinity chromatography principle. From The His-Tag: Fundamentals And Principles, by Cube Biotech, n.d.

A common use of this technique is the purification of recombinant proteins, due to the presence on this laters of Histamine-tag.

His-tag Technology: The gene encoding the target protein is engineered to include a sequence coding for a short tag of polyhistidine residues, typically 6 (a hexahistidine tag or His-tag), at either the N- or C-terminus. These histidine residues have a high affinity for the immobilized Ni^{2+} ions (Figure 07).

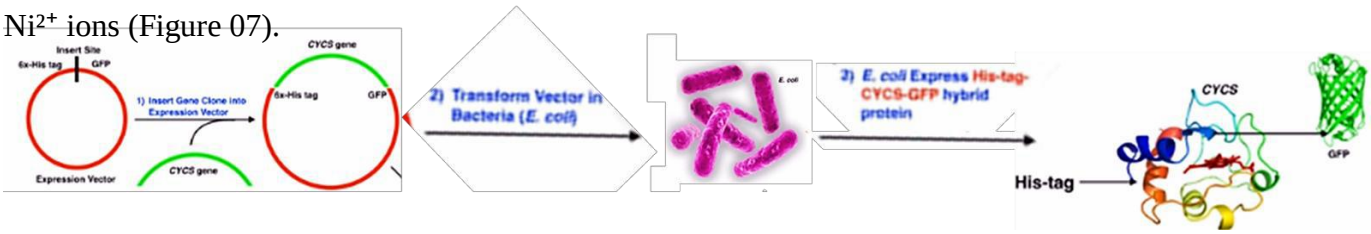


Figure 07: Cloning and Expression of a His-CYCS-GFP Fusion Protein in E. coli (CYCS: Cytochrome C, Somatic , GFP: green fluorescent protein). Adapted from screenshots of Biotechniques | Basics of Making His-Tags & Nickel Affinity Chromatography, by Catalyst University, 2019.

IV.2. Elution

When a crude cell lysate containing the His-tagged protein is passed through the Ni^{2+}-NTA column, the His-tagged protein binds specifically, while most other proteins flow through. After washing away unbound contaminants and other proteins with small concentration of imidazole, the bound protein is eluted by either:

Imidazole competition: Imidazole, which has a structure similar to the histidine side chain, is added to the elution buffer. At high concentration, it competes with the His-tag for binding to the Ni^{2+} ions, displacing the protein (Figure 08).

Lowering pH: Protonation of the histidine residues at low pH (e.g., pH 4-5) reduces their affinity for the metal ions, causing elution.

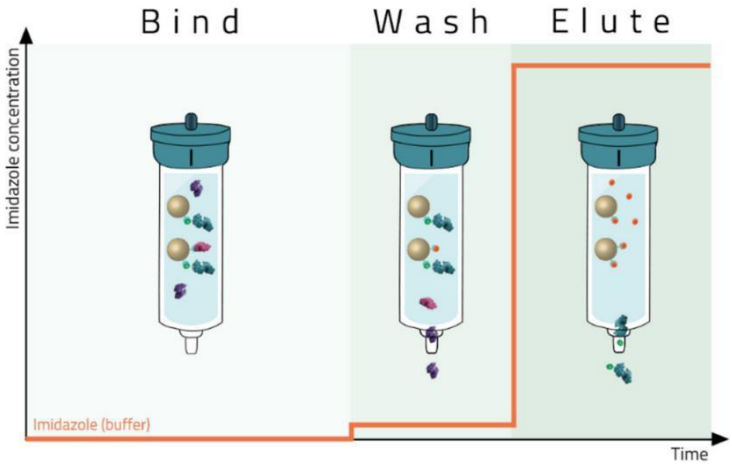


Figure 08: His-tag protein elution using imidazole competition. From The His-Tag: Fundamentals And Principles, by Cube Biotech, n.d.

Finally, IMAC is considered a very powerful tool for protein purification, in figure 09 an SDS-PAGE profil shows an example of a result of purification of Cysc using IMAC.

Figure 09: Example of results after purification using IMAC. Adapted from screenshots of Biotechniques | Basics of Making His-Tags & Nickel Affinity Chromatography, by Catalyst University, 2019.

Chapter Summary

Chromatography is a separation technique based on the differential distribution of analytes between a mobile phase (liquid or gas) and a stationary phase (solid or liquid immobilized on a solid support).

Adsorption Chromatography: Uses a solid stationary phase (e.g., silica, alumina). Separation is based on differential adsorption. The most polar compounds are retained longest.

Partition Chromatography: Uses a liquid stationary phase immobilized on a solid. Separation is based on differential solubility between two liquid phases.

Paper Chromatography (PC): Stationary phase is water bound to cellulose.

Thin-Layer Chromatography (TLC): Stationary phase (e.g., silica) is coated on a plate. More versatile than PC.

Retention Factor (Rf): Rf = distance traveled by compound / distance traveled by solvent front. Used for tentative identification.

HPLC (High-Performance Liquid Chromatography): Uses high pressure to pump mobile phase through columns packed with small particles. Offers high speed, resolution, and sensitivity. Key modes are normal-phase (polar stationary phase, nonpolar mobile phase) and reverse-phase (nonpolar stationary phase, polar mobile phase).

GC (Gas Chromatography): For volatile compounds. Mobile phase is an inert carrier gas (He, N_2, H_2). Separation occurs in a heated column coated with a liquid stationary phase. Temperature programming is used to optimize separation of mixtures with a wide boiling range.

Chromatographic Parameters: Key parameters include retention time (tR), dead time (t0), capacity factor (k'), selectivity factor (α), resolution (Rs), and number of theoretical plates (N). These are used to evaluate and optimize separations.

Size-Exclusion Chromatography (SEC): Separates molecules by size. Largest molecules elute first, smallest last. Used for molecular weight determination and desalting.

Ion-Exchange Chromatography (IEC): Separates charged molecules (e.g., proteins) using a stationary phase with charged groups. Binding is controlled by pH and ionic strength. Elution is achieved by increasing salt concentration or changing pH.

Affinity Chromatography (AC): The most specific technique. Uses a ligand immobilized on a matrix to bind a target molecule (e.g., enzyme-substrate, antibody-antigen) with high specificity.

IMAC (Immobilized Metal Affinity Chromatography): A type of affinity chromatography using metal ions (e.g., Ni^{2+}) to purify His-tagged recombinant proteins. Elution is achieved by imidazole competition or pH change.

Review Questions

1. Explain the fundamental principle of chromatography. What are the roles of the mobile phase and the stationary phase in the separation process?
2. A student runs a TLC plate and obtains an Rf value of 0.2 for compound A and 0.8 for compound B. Which compound is more polar? Justify your answer based on the principles of adsorption chromatography.
3. Compare and contrast normal-phase HPLC and reverse-phase HPLC in terms of stationary phase polarity, mobile phase polarity, and elution order of polar vs. nonpolar compounds.
4. In gas chromatography (GC), what is the purpose of temperature programming? Describe a scenario where an isothermal method would be insufficient.
5. Why do larger molecules elute first in SEC?
6. In ion-exchange chromatography (IEC), how does the choice of buffer pH affect the binding of a protein to a cation exchange resin? Use the concept of isoelectric point (pI) in your explanation.
7. Explain how a His-tagged protein is purified using immobilized metal affinity chromatography (IMAC). What is the role of imidazole in the elution step?

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Chapter III : Electrophoretic Techniques

Table of Contents

Learning Objectives

After reading this chapter, you should be able to understand the principle of electrophoresis and the factors affecting the migration of charged molecules in an electric field. You will be able to explain the role of gels as molecular sieves and distinguish between different types of zone electrophoresis, including native PAGE, SDS-PAGE, and agarose gel electrophoresis. You will understand specialized techniques such as isoelectric focusing (IEF), two-dimensional electrophoresis (2D), and capillary electrophoresis (CE). Finally, you will be familiar with membrane transfer techniques (Southern, Northern, and Western blot) and be able to choose the appropriate electrophoretic method for analyzing proteins or nucleic acids.

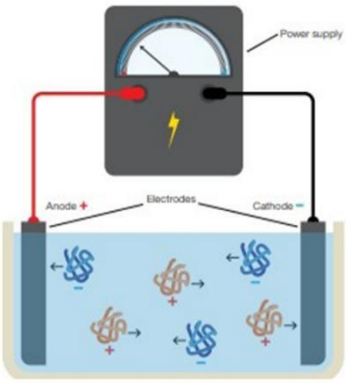
Lecture 01: Different types of electrophoresis (I)
Zone Electrophoresis: SDS-PAGE

1. Introduction

Electrophoresis is a fundamental analytical technique widely used in molecular biology, biochemistry, and clinical diagnostics for the separation and characterization of charged molecules, primarily proteins and nucleic acids (Westermeier, 2016). The method relies on the differential migration of charged particles in a liquid medium under the influence of an electric field.

1.1. General Principle

A sample containing a mixture of molecules is placed in a conductive buffer solution or on a supportive medium (e.g., a gel) that is in contact with buffer reservoirs containing electrodes. When an electric current is applied, molecules with a net negative charge migrate toward the positively charged electrode (anode), while molecules with a net positive charge migrate toward the negatively charged electrode (cathode). The rate of migration, or velocity, of a molecule depends on its intrinsic properties and the experimental conditions.



Movement of proteins during electrophoresis

1.2. Electrophoretic Mobility

The electrophoretic mobility (μ) of an ion is defined as its steady-state velocity (v) per unit electric field strength (E) (Andrews, 1986). For a spherical particle, this mobility can be described by balancing the electrical force driving the molecule with the frictional force resisting its movement through the medium (Figure 01).

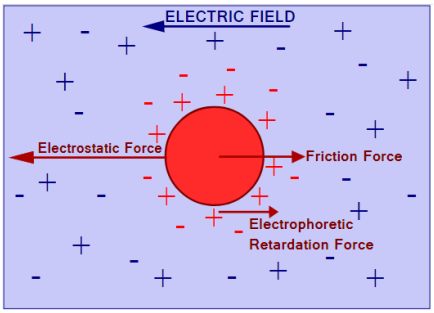


Figure 01: Forces affecting the electrophoretic mobility.
(https://upload.wikimedia.org/wikipedia/commons/a/ab/Electrophoresis.svg)

Various factors can affect electrophoretic mobility:

- ✓ The charge, size, and shape of the molecule
- ✓ The intensity of the electric field
- ✓ Frictional force

The mobility of an ionic constituent is the ratio between its velocity v in the direction of the current and the electric field E that drives the migration.

$$\mu = \frac{v}{E} \qquad m^2 \cdot V^{-1} \cdot s^{-1}$$

The velocity of a molecule is therefore proportional to its charge and the intensity of the electric field, and inversely proportional to its radius and the viscosity of the fluid.

$$v_{EP} = \frac{q.E}{6.\pi.\eta.r}$$

where:

q is the net charge of the molecule,

E is the electric field strength,

η (eta) is the viscosity coefficient of the buffer,

r is the hydrodynamic radius (Stokes' radius) of the molecule.

This equation highlights the key factors influencing electrophoretic velocity:

Velocity is directly proportional to the molecule's net charge (q). A higher charge results in a stronger electrical force.

Velocity is directly proportional to the electric field strength (E). A higher voltage leads to faster migration.

Velocity is inversely proportional to the molecule's size (r) and the buffer viscosity (η). Larger molecules and more viscous media experience greater frictional resistance, slowing them down.

II. Zone Electrophoresis

In zone electrophoresis, the sample is applied as a narrow zone or band at a starting point on or within a support medium. Upon application of the electric field, each component of the mixture migrates from this starting zone at its characteristic rate, theoretically separating into distinct, pure zones or bands (Gordon, 1969).

II.1. Gel Electrophoresis

Gel electrophoresis is a form of zone electrophoresis where the support medium is a gel. The gel acts as a molecular sieve, adding a size-based separation mechanism to the charge-based

separation of free electrophoresis. As molecules migrate through the gel matrix, their movement is hindered by the gel's pores. Smaller molecules navigate the pores more easily and migrate faster, while larger molecules are retarded. This sieving effect is crucial for resolving molecules of similar charge but different sizes.

II.1.1. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels are the most commonly used medium for separating proteins in the molecular weight range of 5–250 kDa. For larger proteins, protein complexes, or nucleic acids (>300 kDa), agarose gels, which have larger pores, are preferred.

PAGE is typically performed with the gel cast vertically between two glass plates. A comb is inserted into the unpolymerized gel solution at the top to create sample wells. The gel assembly is then placed between two buffer chambers (upper and lower), and an electric current is passed through the system via electrodes immersed in the buffer, forcing the migration of charged molecules into and through the gel (Figure 02).

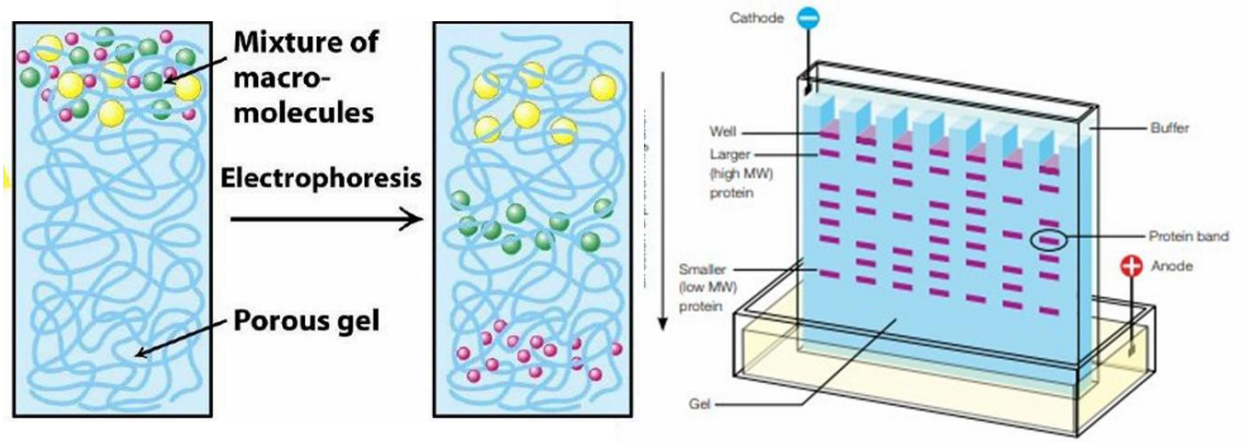


Figure 02: Diagram of protein separation by polyacrylamide gel electrophoresis. Adapted from Bio-Rad Laboratories (n.d.) p.10 and Semmame. Techniques d'analyse de laboratoire (n.d) p.5.

II.1.2. Buffer Systems

Two main types of buffer systems are used in gel electrophoresis

- **Continuous Buffer System:** The same buffer, at a constant pH, is used in the gel, and the electrode reservoirs. This system is simple but can lead to sample diffusion and band broadening, as proteins do not enter the gel simultaneously. It is more commonly used for nucleic acid electrophoresis.
- **Discontinuous Buffer System:** This system, developed by Ornstein and Davis, uses different buffers in the gel and the electrode solutions, along with a gel composed of two distinct sections: a large-pore stacking gel on top and a small-pore resolving (or separating) gel below.

Stacking Gel: The sample is loaded into wells in this gel. The buffer chemistry and large pores cause all proteins to migrate rapidly and become concentrated, or "stacked," into an extremely thin, sharp band before they enter the resolving gel. This is achieved by using a leading ion ., Cl^-) and a trailing ion (e.g., glycine) in the buffers, which create a local voltage gradient that concentrates the proteins.

Resolving Gel: Once the stacked protein band enters the small-pore resolving gel, the trailing ion overtakes the proteins, eliminating the stacking effect. Here, proteins are separated primarily based on their size due to the molecular sieving effect of the gel matrix.

The discontinuous system dramatically improves resolution by ensuring all proteins start their size-based separation from the same narrow starting point (Figure 03).

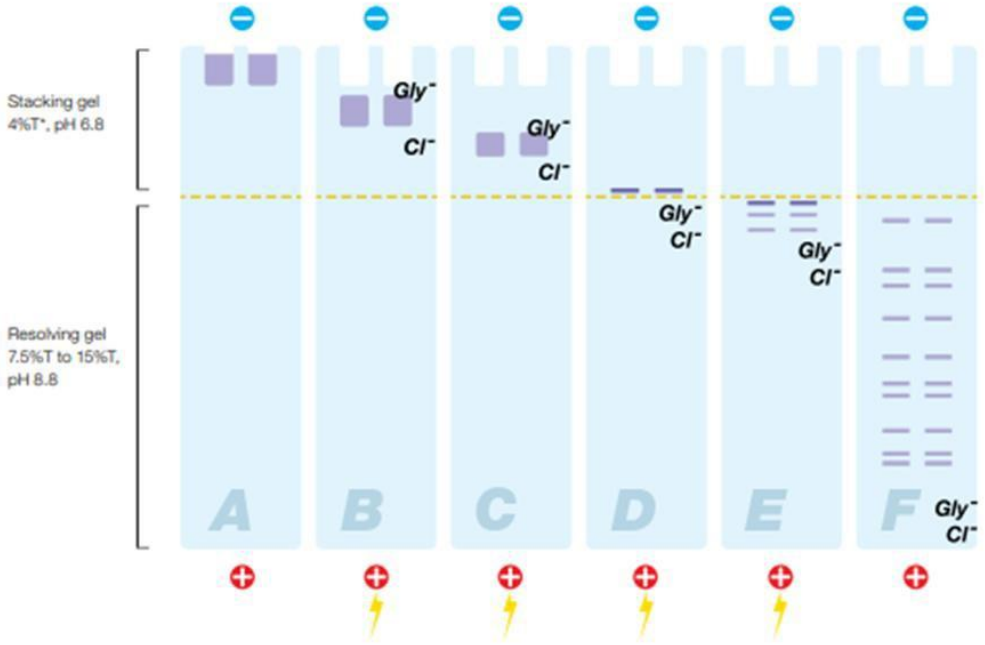


Figure 03: Migration of proteins and buffer ions in a discontinuous PAGE under denaturing conditions. Adapted from Bio-Rad Laboratories (n.d.) p.11.

II.1.3. Discontinuous Native PAGE

In native PAGE, proteins are prepared and electrophoresed under non-denaturing and non-reducing conditions. The sample buffer lacks denaturing agents like SDS and reducing agents like β-mercaptoethanol. This preserves the protein's native conformation, secondary structures, subunit interactions, and biological activity. While useful for studying functional protein complexes, native PAGE has significant drawbacks:

Mobility is complex: A protein's migration is governed by a complex combination of its size, shape, and intrinsic net charge.

Unpredictable interactions: Proteins may migrate as complexes, making interpretation difficult.
Charge-dependent migration: Proteins can migrate toward either electrode based on their native charge, which complicates comparisons.

Consequently, native PAGE is generally unsuitable for determining the molecular weight of unknown proteins.

II.1.4. SDS-PAGE (Denaturing Conditions)

To overcome the limitations of native PAGE, Laemmli (1970) introduced sodium dodecyl sulfate (SDS) into the discontinuous buffer system, creating the most widely used protein electrophoresis technique: **SDS-PAGE**.

- **Sodium Dodecyl Sulfate (SDS)**

SDS is an anionic detergent with a long hydrophobic tail and a negatively charged sulfate head group. Its functions are twofold (Figure: 04):

Denaturation: SDS binds non-covalently to proteins along their polypeptide backbone, disrupting hydrogen bonds and hydrophobic interactions, thereby unfolding the protein into a linear rod-like shape. The binding ratio is approximately constant, about 1.4 g of SDS per gram of protein (or roughly one SDS molecule per two amino acid residues).

Charge Equalization: The bound SDS coats the protein with a large net negative charge. This overwhelming negative charge effectively masks the protein's intrinsic charge, creating a complex with a uniform charge-to-mass ratio.

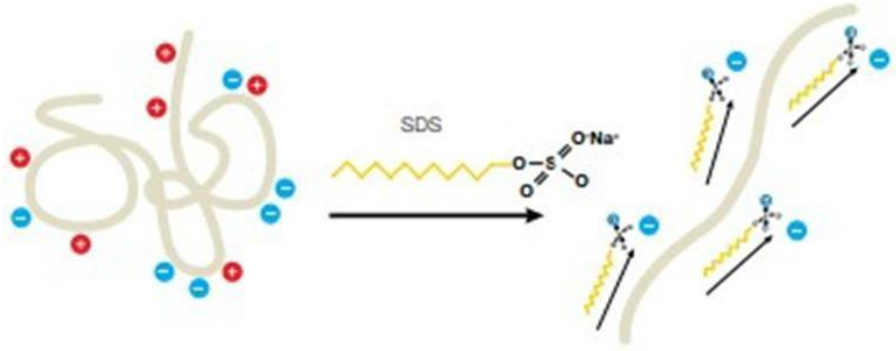


Figure 04: Effect of SDS on the conformation and charge of proteins. Adapted from Bio-Rad Laboratories (n.d.) p.11.

As a result, when an electric field is applied, all SDS-protein complexes migrate toward the anode. The electrophoretic mobility in the gel is then determined almost exclusively by the molecular sieving effect. Smaller proteins navigate the gel pores more easily and migrate faster, while larger proteins are retarded. Thus, proteins are separated based on their molecular weight alone.

- **Reduction of Disulfide Bonds**

Many proteins have tertiary and quaternary structures stabilized by disulfide bonds (S-S bonds) between cysteine residues. SDS alone does not break these covalent bonds. To achieve complete denaturation and ensure that proteins are separated as their individual polypeptide chains, samples are treated with a reducing agent such as: β-mercaptoethanol (BME), (DTT, also known as Cleland's reagent) or TCEP (Tris(2-carboxyethyl)phosphine). These agents reduce disulfide bonds to free sulfhydryl groups (-SH) (Figure 05).

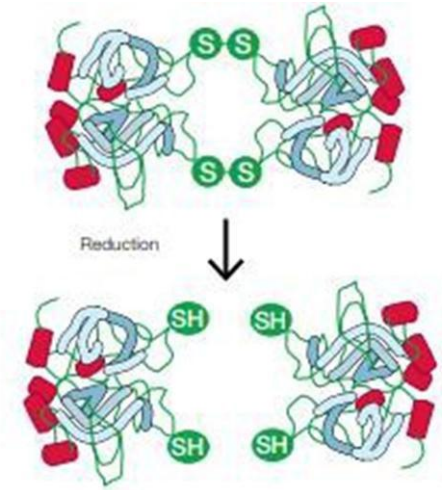


Figure 05: Reduction of proteins by DTT. Adapted from Bio-Rad Laboratories (n.d.) p.20.

- **Polyacrylamide Gel Chemistry and Properties**

Polyacrylamide gels are formed by the co-polymerization of two compounds (Chrambach & Rodbard, 1971): Acrylamide (the monomer) and N,N'-methylenebisacrylamide (the cross-linker) (Figure 06). Polymerization is a free radical reaction, typically initiated by ammonium persulfate (APS) and stabilized by TEMED (N,N,N',N'-tetramethylethylenediamine) , which acts as a catalyst by accelerating the decomposition of APS to form free radicals.

Polyacrylamide gel presents many advantages:

- Stable
- Chemically inert
- Hydrophilic
- Optically transparent
- It has a low affinity for specific protein stains (e.g., Coomassie Blue).

However, one of the main inconvenient for its use is its high toxicity.

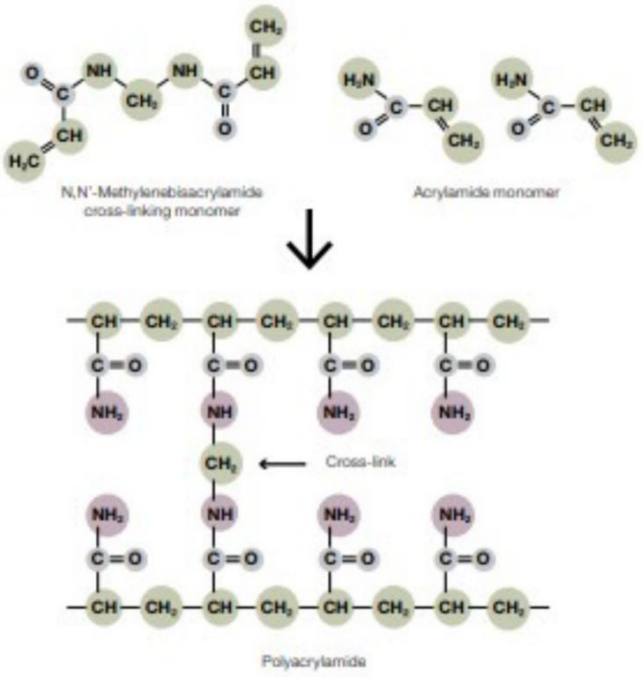


Figure 06: Polymerization of acrylamide and bisacrylamide. Adapted from Bio-Rad Laboratories (n.d.) p.27.

The physical properties of the gel are defined by two parameters:

Total monomer concentration (%T): The total weight of acrylamide and bis-acrylamide per 100 mL of solution and consists generally of solutions in the range of 30 to 40% with different ratios of acrylamide and bis-acrylamide (e.g., 38:2).

Cross-linking concentration (%C): The percentage of the crosslinking agent (C%).

$$\%T = \frac{\text{g acrylamide} + \text{g cross-linker}}{\text{Total volume, ml}} \times 100$$

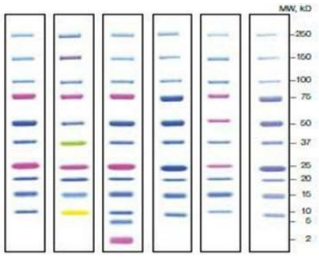
$$\%C = \frac{\text{g cross-linker}}{\text{g acrylamide} + \text{g cross-linker}} \times 100$$

By varying %T and %C, the pore size of the gel can be precisely controlled. A higher %T results in a denser gel matrix with smaller pores, better suited for separating lower molecular weight proteins.

- **Visualization and Molecular Weight Determination**

Following electrophoresis, proteins are invisible and must be stained. The most common stain is Coomassie Brilliant Blue. For higher sensitivity, silver staining or fluorescent dyes can be used.

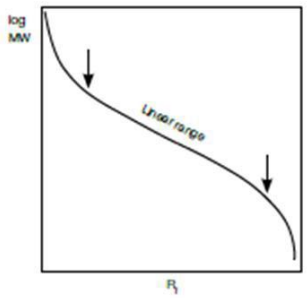
To determine the molecular weight of an unknown protein:
A molecular weight marker (ladder) containing proteins of known sizes is run alongside the samples.
After staining, the distance each protein has migrated from the top of the resolving gel is measured.
The relative mobility (Rf) is calculated for each band in the marker and the sample:



Example of size markers

$$R_f = (\text{distance to band})/(\text{distance to dye front})$$

A calibration curve is constructed by plotting the logarithm of the molecular weight (log MW) of the marker proteins against their Rf values. This typically yields a linear relationship within a certain range. The molecular weight of the unknown protein is estimated by interpolating its Rf value on the calibration curve.



Example of Rf ploting

Lecture 02: Different types of electrophoresis (II)
Zone Electrophoresis: Agarose Gel Electrophoresis- Capillary Zone Electrophoresis

I. Agarose Gel Electrophoresis

Agarose gel electrophoresis is the standard method for separating, identifying, and purifying nucleic acids (DNA and RNA) (Sambrook & Russell, 2001). It is valued for its ease of use, speed, and sensitivity, allowing visualization of nanogram quantities of nucleic acids.Nucleic acids have a constant negative charge-to-mass ratio due to their phosphate backbone. Therefore, under an electric field, they all migrate toward the anode. Their separation is primarily achieved through the molecular sieving effect of the agarose matrix, with smaller fragments migrating faster than larger ones.

I.1. Structure and Properties of Agarose

Agarose is a natural product that forms an inert and non-toxic matrix. It is a linear polymer with agarobiose as its fundamental unit, which is a saccharide dimer composed of D-galactose and 3,6-anhydro-L-galactopyranose linked by α-(1→3) and β-(1→4) glycosidic bonds. It is a white powder that dissolves in boiling water. The agarose solution remains liquid as long as the temperature is above 40-45°C. When the temperature drops below 40°C, the solution solidifies into a stable gel that does not melt as long as the temperature stays below 100°C.

The cross-linking of a gel depends on its agarose concentration: the pore size becomes smaller as the agarose concentration in the gel increases. Concentrations typically range from 0,4% to 2% (weight/volume). Low-percentage gels have larger pores and are used to resolve very large DNA fragments, while high-percentage gels have smaller pores and are better for separating small fragments. The cohesion between the polysaccharide chains is maintained mainly by weak hydrogen bonds. Agarose gels are therefore fragile and must be handled with care.

I.2. Buffers for Agarose Gel Electrophoresis

The gel and the electrophoresis running buffer must be the same to maintain constant pH and ionic strength. The two most common buffers are :

TAE (Tris-Acetate-EDTA): Offers good resolution for large DNA fragments and is preferred when recovering DNA from gels for downstream applications (e.g., cloning, ligation), as boric acid in TBE can inhibit some enzymes.

TBE (Tris-Borate-EDTA): Has a higher buffering capacity than TAE, making it suitable for longer runs and higher voltages. It provides better resolution for small fragments.

I.3. Sample Preparation and Visualization

In order to monitor the migration of DNA samples, they are mixed with a loading solution before being placed in the wells. This solution contains:

- ✓ A densifying agent (glycerol, Ficoll, or sucrose) to help the DNA sink to the bottom of the wells.
- ✓ Mobility markers (bromophenol blue and xylene cyanol).
- ✓ Possibly a denaturing agent to stop enzymatic reactions or restriction enzyme digestion

The migration speed is not affected between 4°C and 30°C, so gel migration can be performed at room temperature, ideally using a low voltage of 5 to 10 V/cm of gel.

For visualization, an ultraviolet light is used (due to the presence of intercalating agents such as ethidium bromide (EtBr) or SYBR Green).

The size of an unknown DNA fragment can be estimated by comparing its migration distance to that of fragments in a DNA ladder (size marker) run on the same gel. A standard curve is plotted as log(bp) vs. migration distance.

A schematic representation of agarose gel electrophoresis workflow is illustrated in figure 01.

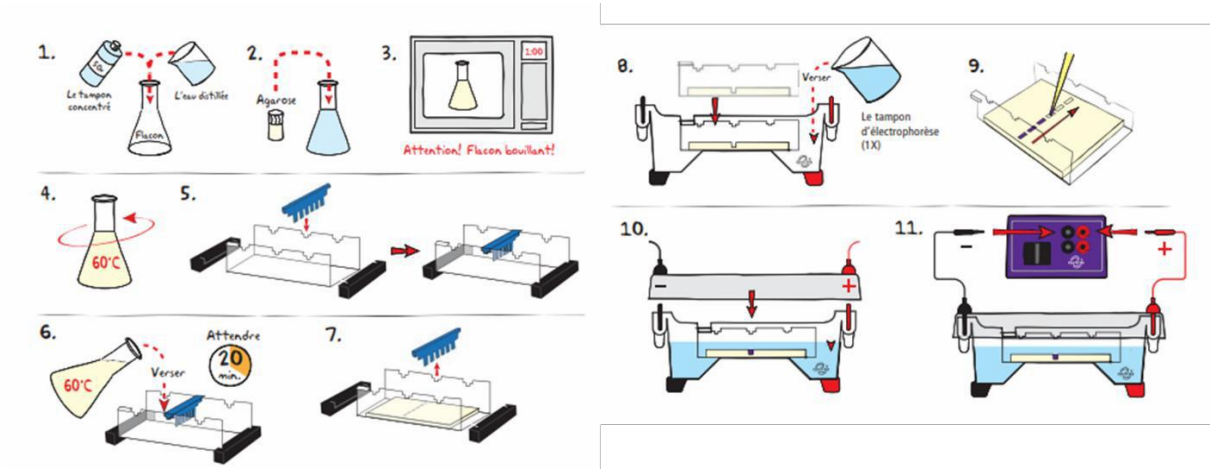


Figure 01: Schematic representation of agarose gel electrophoresis workflow. Adapted from Semmame. Techniques d'analyse de laboratoire (n.d) p.9.

II. Electrophoretic Mobility Shift Assay (EMSA)

EMSA, also called a gel shift assay, is a powerful technique to study protein-nucleic acid interactions (Garner & Revzin, 1981; Fried & Crothers, 1981).

The assay relies on the observation that a protein-DNA or protein-RNA complex is larger and has a different charge than the free nucleic acid fragment. When such a binding reaction is run on a non-denaturing polyacrylamide or agarose gel, the protein-nucleic acid complex migrates more slowly than the unbound nucleic acid, resulting in a "shifted" band at a higher position in the gel. This technique is widely used to:

- ✓ Detect and characterize DNA-binding proteins (e.g., transcription factors).
- ✓ Study the specificity and kinetics of protein-nucleic acid interactions.
- ✓ Map protein-binding sites on DNA.
- ✓ Screen for compounds that disrupt these interactions.

III. Capillary Zone Electrophoresis (CZE)

Capillary electrophoresis (CE) encompasses a family of related techniques that perform electrophoretic separations in narrow-bore capillaries (typically 15–150 µm internal diameter) (Jorgenson & Lukacs, 1981). CZE is a technique that allows the separation of a large number of molecules by the migration of charged species in an electric field using a capillary tube as a migration support.

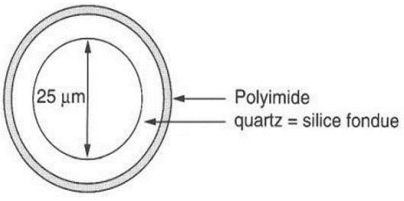
Example of CE apparatus

The main advantage of this technique is that the capillary tube enables better dissipation of the heat produced by the electric current. Consequently, higher voltages can be used (20 to 30 kV). As a result, CE offers high resolution, high sensitivity, and shorter separation times. CE also offers other advantages such as the easy conditioning of the separation capillary, the low consumption of samples (in the order of nanoliters) and separation buffers, and compatibility with many detectors.

III.1. Instrumentation

The equipment required for this type of electrophoresis consists of two reservoirs filled with buffer, into which the ends of capillary tubes are immersed. These capillaries have an internal diameter ranging from 15 to 150 µm and a length of 0.2 to 1 m. The electrodes for the electric current are also immersed in these buffers (Figure 02).

The capillary used in CE is typically made of fused silica and coated with a layer of polyimide* to ensure flexibility and protection.



* Colored polymers containing imide groups (secondary amides) in their main chain

A window of detection

The detector is positioned near the cathode and is connected to a data analyzer. Most commercial systems use UV-Visible spectroscopy for detection. This detection is performed directly through the capillary at a window created by removing the external polyimide layer. Other types of detection are also used, such as conductometry (for inorganic species), amperometry, fluorimetry, and mass spectrometry for organic species.

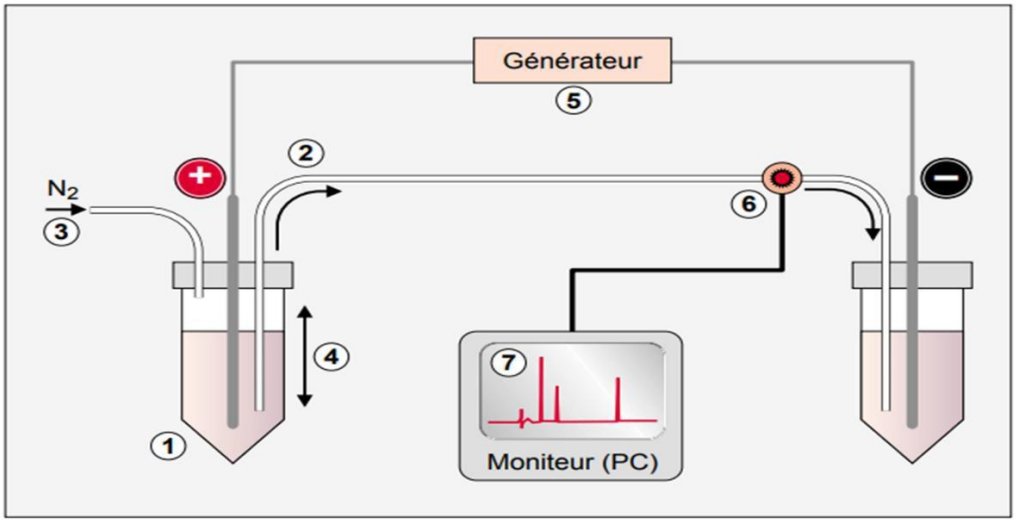


Figure 02: Schematic diagram of a capillary zone electrophoresis (CZE) apparatus (Feuilloley et al., 1999).

A few nanoliters of sample (1) are injected into the capillary (2) using nitrogen pressure (3). The sample tube is then replaced with a tube containing the electrophoresis buffer (4). The generator (5) is immediately activated to apply an electric field of approximately 500 volts/cm between the two ends of the capillary. A detector (6) records the passage of the separated molecules during electrophoresis and transmits this information to a computer (7), where a graph (electropherogram) is generated, showing the variation in optical density or fluorescence as a function of time**.**

III.2. Principle: Electrophoretic Mobility and Electroosmotic Flow

The movement of compounds in CE occurs from the anode (+) to the cathode (-).

The separation of compounds in CE results from two transport mechanisms:

- Electrophoretic Mobility
- Electroosmotic Flow

Electrophoretic Mobility: Resulting from the movement of a charged species when subjected to an electric field, the acquired electrophoretic velocity is proportional to the electric field and to the electrophoretic mobility of the ion. The latter is proportional to the charge and the electric field and inversely proportional to the frictional forces. When subjected to an electric field, ions

move at a characteristic constant speed that depends on their size and charge: an ion will be more mobile the higher its charge and the smaller its size (or ionic radius).

Electroosmotic Flow (EOF): The electroosmotic flow, on the other hand, is a phenomenon specific to the silica capillary, corresponding to the flow of a liquid filling a capillary (whose internal wall has a surface charge) when subjected to an electric field. In the case of a silica capillary, the silanol groups are very acidic and easily form Si-O- groups when the pH is above 2, which gives the capillary an internal negative charge. When the capillary is filled with an electrophoretic buffer, the cations in the buffer adsorb onto the internal wall forming an electrical double layer consisting of a compact layer of adsorbed cations and a diffuse layer where cations remain in excess (Figure 03). When a voltage is applied to the capillary, the solution is driven toward the cathode by the creation of a flow, carrying with it all species in the fluid, whether they are charged or not.

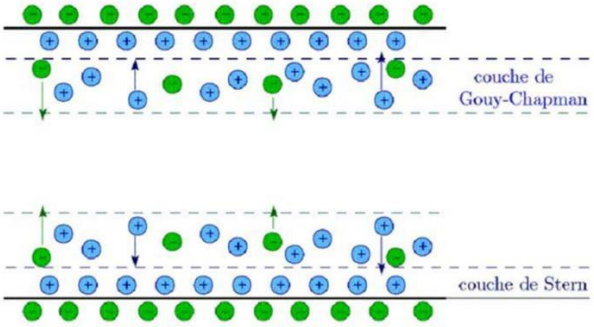
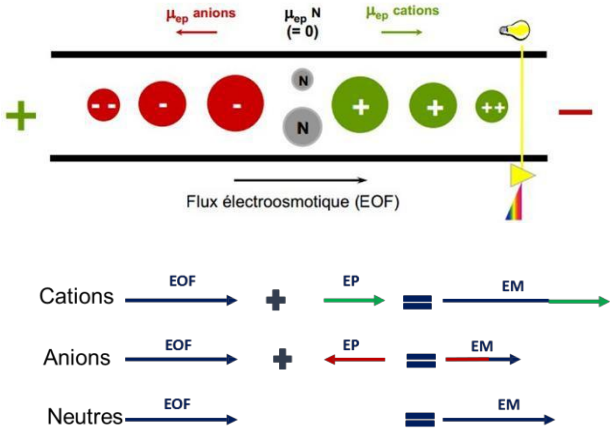


Figure 03: Representative diagram of the electrical double layer composed of the Stern layer, called the compact layer, and the Gouy-Chapman layer, called the diffuse layer. (Tanguy, 2009).P.31.

The migration speed of a molecule = electrophoretic velocity + electroendoosmotic velocity.

When it is a cation, the electrophoretic and electroosmotic mobilities have the same sign, resulting in a rapid migration of the cation toward the detector.

For an anion, the electrophoretic mobility is opposite in sign to that of the electroosmotic flow; thus, an anion will only migrate toward the cathode if its electrophoretic mobility is lower in absolute value than the electroosmotic mobility.



Neutral molecules all migrate at the speed of the electroosmotic flow and are not separated from each other.

III.3. Variants of Capillary Electrophoresis

III.3.1. Micellar Electrokinetic Chromatography (MEKC)

MEKC is a mode of separation in capillary electrophoresis where, in addition to the electrophoretic and electroosmotic migration phenomena, interactions occur between the analytes of interest and added compounds. The analytes are then separated based on their charge-to-size ratio and their types of interactions with the compounds added to the electrolyte, such as their hydrophobicity and/or chirality, for example.

As an example for the hydrophobicity factor, a surfactant (a chemical substance capable of reducing the surface tension between two substances) is added to the buffer at a concentration above its critical micellar concentration. This results in the formation of micelles, which have a hydrophobic core and a charged surface. In the case of an anionic surfactant such as sodium dodecyl sulfate, these micelles have their own electrophoretic mobility (μep) opposite in direction to the electroosmotic flow (EOF) and migrate toward the cathode more slowly than the EOF. Depending on their degree of hydrophobicity and thus their partition coefficient between the buffer and the micelles, the sample molecules are transported toward the cathode at varying speeds. The most hydrophilic molecules stay mainly in the buffer and migrate with the EOF, while the most hydrophobic molecules associate with the micelles and are retained longer in the capillary (Figure 04).

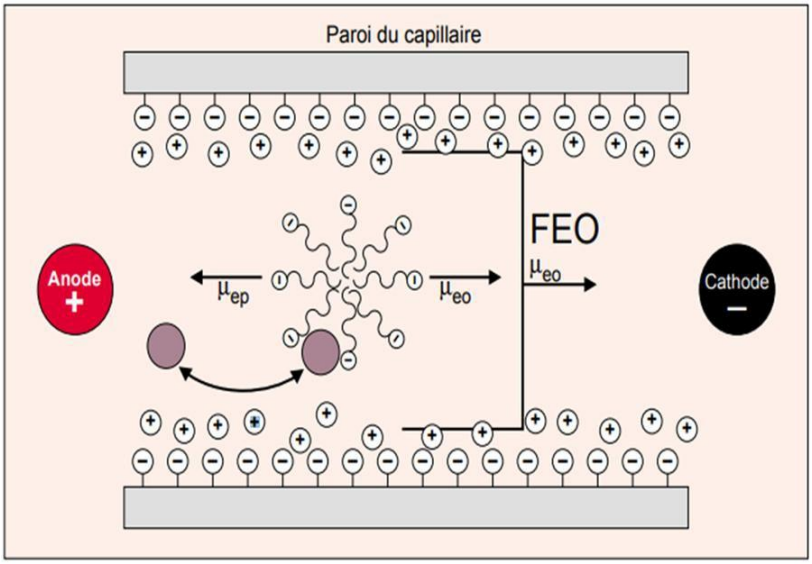


Figure 04: Principle of Separation of Molecules in Micellar Electrokinetic Chromatography (MEKC). Bistre circles: Molecules to be separated, interacting more or less with the micelle. Double arrows: Movement of molecules to be separated between the micelle and the solvent. (Feuilloley et al., 1999).

III.3.2. ary Gel Electrophoresis (CGE)

CGE allows the separation of molecules based on their size. It is widely used, particularly for the analysis of proteins and nucleic acids (Sanger sequencing) (Figure 05). This separation method is implemented using a capillary filled with a gel that acts as a molecular sieve.

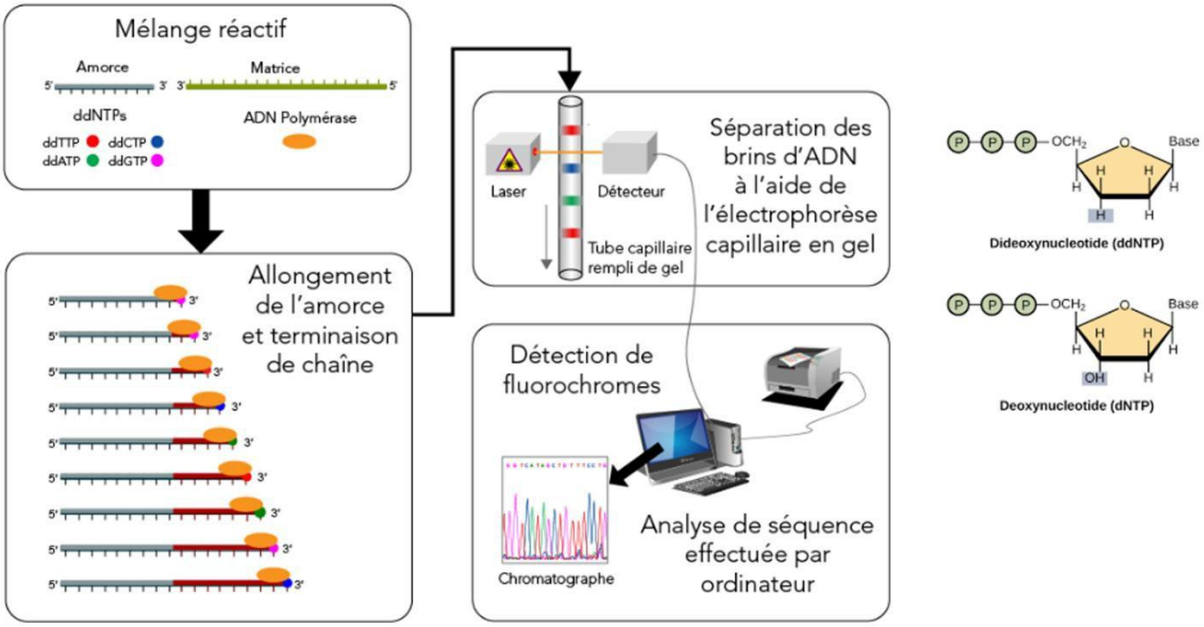


Figure 05: DNA Sequencing. Adapted from Estevezj (2012), Sanger-sequencing.svg, Wikimedia Commons (CC BY-SA 3.0).

The basic CZE principle can be modified to separate different types of analytes, other variants of CE are represented in Table 01.

III.4. Applications of Capillary Electrophoresis

CE is a versatile technique with applications across many fields:

- ✓ Biochemistry and Molecular Biology: Protein analysis (cIEF, CGE), DNA sequencing (CGE), study of protein-DNA interactions (ACE).
- ✓ Pharmaceutical Industry: Analysis of drug purity, chiral separations, determination of drug stability.
- ✓ Food and Beverage Industry: Analysis of vitamins, preservatives, organic acids, and contaminants.
- ✓ Environmental Analysis: Monitoring of pesticides, herbicides, and pollutants in water and soil.

- ✓ Clinical and Forensic Science: Analysis of body fluids for drugs of abuse, disease biomarkers, and DNA profiling.

Table 1: Common Capillary Electrophoresis Modes (Feuilloley et al., 1999).

Form	Abbreviation	Medium	Support	Application
1. Capillary Zone Electrophoresis	CZE	Aqueous buffer	None	Charged molecules
2. Micellar Electrokinetic Chromatography	MEKC	Aqueous buffer + surfactant	None	Hydrophobic molecules
3. Capillary Isoelectric Focusing	CIEF	Aqueous buffer + pH gradient	None	Charged molecules
4. Capillary Gel Electrophoresis	CGE	Aqueous buffer	Polyacrylamide gel	Charged molecules
5. Affinity Capillary Electrophoresis	ACE	Aqueous buffer + receptor or ligand	None	Binding studies, immunoassays
6. Capillary Electrochromatography	CEC	Aqueous buffer	Silica gel	Charged and hydrophobic molecules
7. Isotachophoresis	ITP	Buffers with variable electrophoretic mobility	None	Charged molecules (proteins)
8. Non-Aqueous Capillary Electrophoresis	NACE	Solvent + ions	None	Hydrophobic molecules
9. Single-Cell Capillary Electrophoresis	SC-CE	Cell survival medium (single living cell)	None	Hydrophilic metabolites

Lecture 02: Different types of electrophoresis (III)
Isoelectric Focusing- 2 D Electrophoresis
Membrane Transfer Techniques

I. Isoelectric Focusing (IEF)

Isoelectric focusing is an electrophoretic technique that separates proteins based on their isoelectric point (pI) , the pH at which a protein has no net charge (Righetti, 1983) (Figure 01).

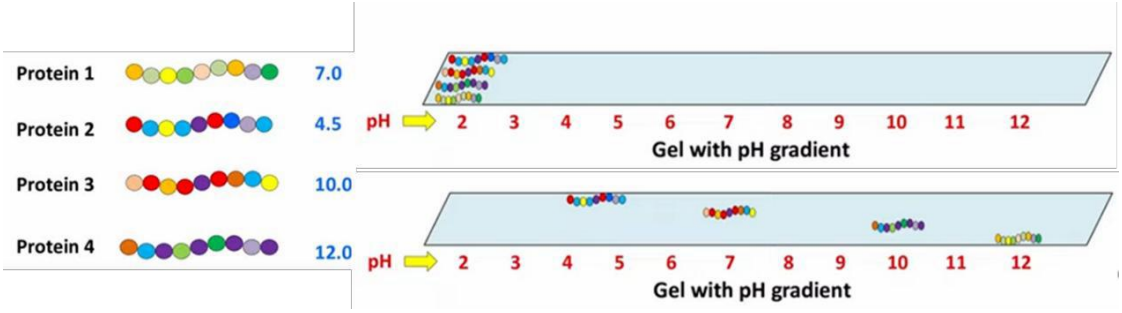


Figure 01: IEF principleAdapted from screenshots of Focalisation isoélectrique et électrophorèse sur gel 2D, by Quick Biochemistry Basics, (2022).

A stable pH gradient is established within a polyacrylamide gel (low %T to minimize sieving) using a mixture of low molecular weight amphoteric molecules called carrier ampholytes. When an electric field is applied, the ampholytes migrate to their respective pIs, creating a continuous pH gradient. A protein introduced into this system will migrate under the influence of the field until it reaches the point in the pH gradient where the pH equals its pI. At this point, its net charge becomes zero, and it ceases to migrate, becoming "focused" into a sharp band.

Modern IEF often uses immobilized pH gradient (IPG) strips, where the pH gradient is covalently incorporated into the polyacrylamide gel during polymerization. This provides greater stability and reproducibility than carrier ampholytes (Figure 02).

II. Two-Dimensional Electrophoresis (2D-PAGE)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the highest-resolution technique for analyzing complex protein mixtures, such as whole-cell lysates (O'Farrell, 1975). It combines two orthogonal separation principles (Figure 03):

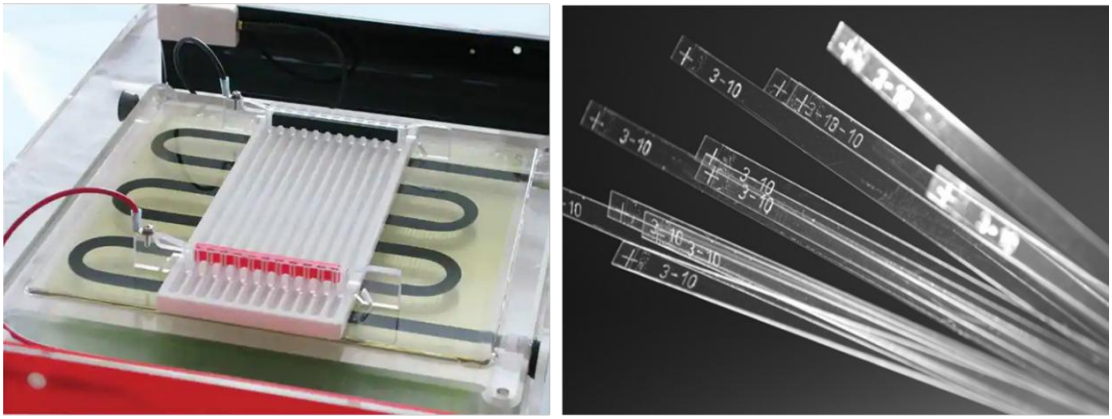


Figure 02: IPG strips (right) IEF System for IPG Strips and Gels (left). Adapted from Cleaver Scientific Système IEF by Fisher Scientific, (n.d).

First Dimension (IEF): Proteins are separated based on their isoelectric point (pI) using an IPG strip.

Second Dimension (SDS-PAGE): The IPG strip, now containing focused proteins, is equilibrated with SDS and placed on top of a standard SDS-polyacrylamide gel. Proteins are then separated perpendicularly based on their molecular weight.

The result is a 2D gel where proteins are displayed as individual spots, with thousands of different protein species potentially resolved. This technique is a cornerstone of proteomics for comparing protein expression patterns between different samples (e.g., healthy vs. diseased tissue).

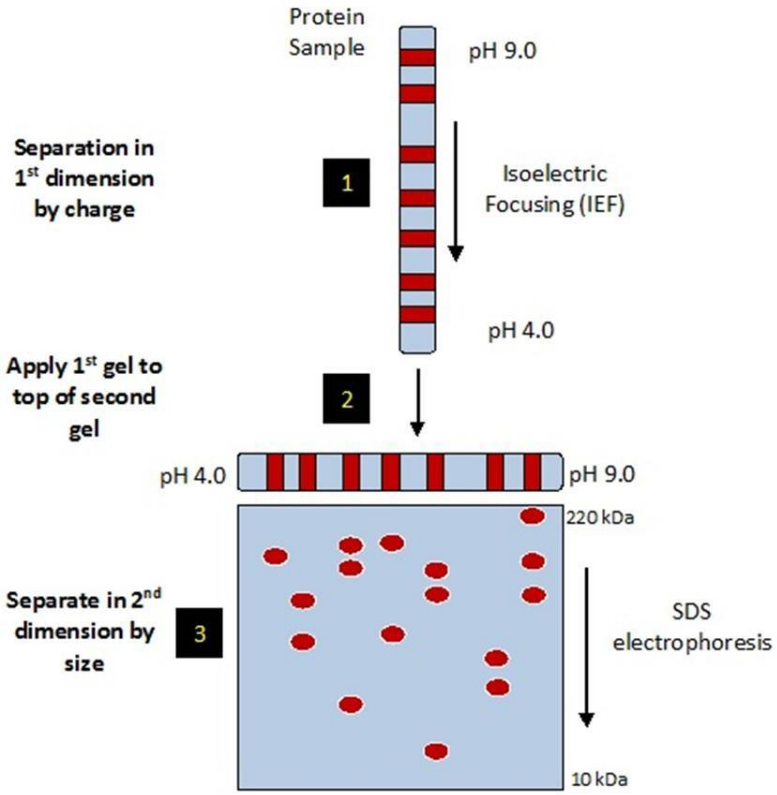


Figure 03: 2 D Electrophoresis principle. Adapted from 2D Overview, by Kendrick Labs, Inc., (2020).

III. Membrane Transfer Techniques (Blotting)

Blotting techniques are used to transfer DNA, RNA, or proteins from an electrophoretic gel to a solid membrane support for subsequent detection and analysis. This process immobilizes the molecules, making them accessible to probes or antibodies.

III.1. Molecular Hybridization: Southern and Northern Blot

In these two types of experiments, the aim is to locate a specific fragment either in the genome or in the mRNA of a cell, such as a particular gene. To do this, a labeled DNA probe is used, whose sequence is complementary to the target fragment. The "detection" is carried out by hybridization between the labeled probe and the corresponding DNA or mRNA fragment. The labeling of the probe with a radioisotope or a fluorochrome allows for visualization and quantification of the hybridization.

III.1.1. Southern Blot

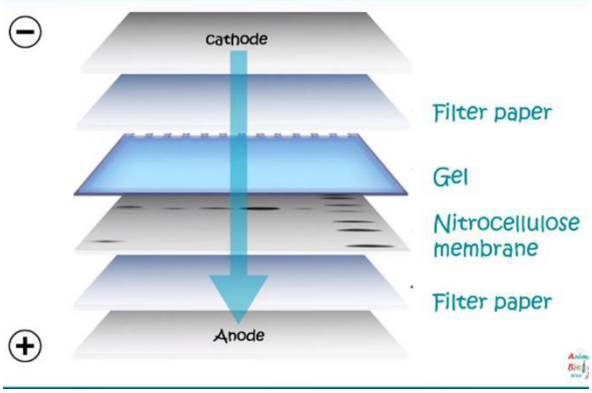
This is a molecular biology method used to analyze DNA. It was invented in 1975 by Edwin Southern to search for DNA fragments by hybridizing them with a complementary probe.

The name "Southern" has, by wordplay, inspired the naming of other techniques such as Northern Blot and Western Blot.

- **Procedure**

DNA Extraction

- Restriction of the DNA using restriction enzymes
- Separation of the restriction fragments by agarose gel electrophoresis
- Denaturation of the DNA (to obtain single-stranded DNA)
- Transfer and fixation of the DNA fragments onto a nitrocellulose membrane
- Hybridization of the membrane with a labeled probe
- Elimination of unpaired probes by washing
- The fragments hybridized with the probe appear on the autoradiograph.



III.1.2. Northern Blot

The Northern blot (a play on words from Southern blot) is analogous to the Southern blot, but it is used to detect specific RNA sequences among a mixture of RNAs (Alwine, Kemp, & Stark, 1977). RNA is separated by size on a denaturing agarose gel (to prevent secondary structure formation), transferred to a membrane, and detected using a labeled DNA or RNA probe.

Northern blotting is widely used to study gene expression levels by measuring specific mRNA transcripts. The workflow of Northen blot is represented in Figure 04.

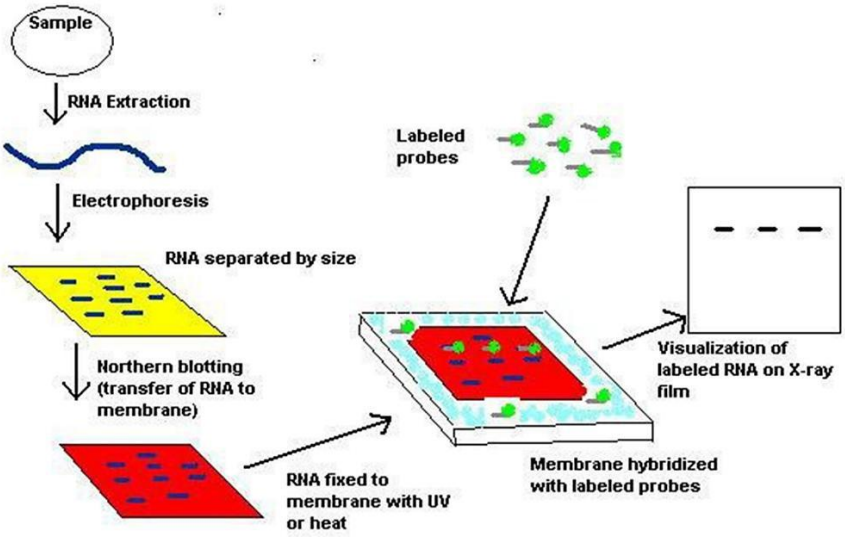


Figure 04: Northen blot workflow. Adapted from Ilewieszoośmiornicach (2017), Northern blot diagram

III.2. Western Blot (Immunoblotting)

Western blotting, also known as immunoblotting, is a powerful and widely used technique for detecting a specific protein within a complex protein mixture (e.g., a cell lysate) (Towbin, Staehelin, & Gordon, 1979; Burnette, 1981). It combines the resolving power of gel electrophoresis with the specificity of antibody-antigen interactions. This technique allows for the separation and identification of a specific protein of interest within a complex mixture of proteins, such as a cell lysate. With applications in diagnostics, biotechnology, molecular biology, proteomics, and more, the Western Blot technique is widely used to evaluate protein expression levels in cells, as well as variations in size and other properties.

III.2.1. Principle

In the context of Western Blot, the protein mixture is subjected to gel electrophoresis on a carrier matrix (e.g., SDS-PAGE, native PAGE, isoelectric focusing, etc.) in order to sort proteins based on size, charge, or any other differences within individual protein bands. The separated protein bands are then transferred to a carrier membrane (e.g., nitrocellulose, nylon).

This process is called transfer. The proteins adhere to the membrane in the same way they were separated due to charge interactions. These immunotransferred proteins can then be used to bind to antibodies for detection purposes.

3.2.2. Procedure

The Western blot workflow consists of several key steps (see figure 05):

Protein Separation: Proteins in the sample are denatured and separated by SDS-PAGE (or other electrophoretic methods like native PAGE or IEF).

Transfer (Blotting): The gel is placed in contact with a membrane, and an electric current is applied perpendicular to the gel surface. This causes the proteins to migrate out of the gel and onto the membrane, where they become immobilized through hydrophobic and electrostatic interactions. The membrane now contains a replica of the protein pattern from the gel.

Blocking: To prevent the antibodies from binding non-specifically to the membrane itself, the membrane is incubated in a blocking buffer containing a protein solution (e.g., non-fat dry milk, bovine serum albumin (BSA), or casein). This blocks all remaining binding sites on the membrane.

*Primary Antibody Incubation***:** The membrane is incubated with a solution containing a primary antibody specific to the target protein. The antibody binds only to its target antigen.

Washing: Unbound primary antibody is removed by washing the membrane with a buffer containing a mild detergent (e.g., Tween-20).

Secondary Antibody Incubation: The membrane is incubated with a secondary antibody that recognizes and binds to the primary antibody. Secondary antibodies are typically conjugated to a reporter molecule, such as an enzyme (e.g., horseradish peroxidase (HRP) or alkaline phosphatase (AP)) or a fluorophore.

Washing: Unbound secondary antibody is washed away.

Detection: The bound secondary antibody is visualized by a method appropriate for its conjugate.

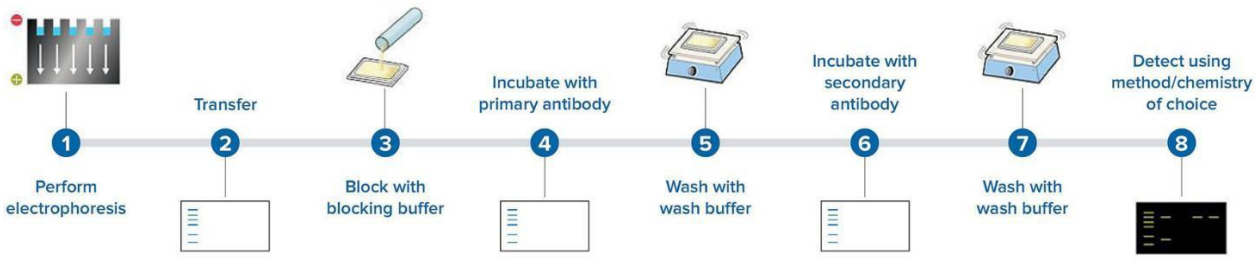


Figure 05: Summarized western blot workflow. Adapted from Western Blot, by Molecular Devices, (2019).

III.2.3. Detection Methods

The choice of detection depends on the label attached to the secondary antibody. Three types of detection can be used (Figure 06):

Colorimetric Detection: An enzyme-conjugated secondary antibody (e.g., HRP or AP) catalyzes a reaction with a soluble substrate, producing a colored precipitate that deposits on the membrane, forming visible bands.

Chemiluminescent Detection: An enzyme-conjugated secondary antibody (usually HRP) catalyzes the oxidation of a luminol-based substrate, emitting light. This light is captured on X-ray film or by a digital imaging system. This is the most sensitive and common detection method.

Fluorescent Detection: A fluorophore-conjugated secondary antibody (e.g., Alexa Fluor, Cy dyes) is used. The membrane is scanned with a fluorescence imager at specific excitation wavelengths. This allows for multiplexing (detecting multiple targets simultaneously) and direct quantification without a substrate reaction.

The final result is a membrane with visible bands corresponding to the target protein(s), allowing for the determination of protein presence, size, and relative abundance.

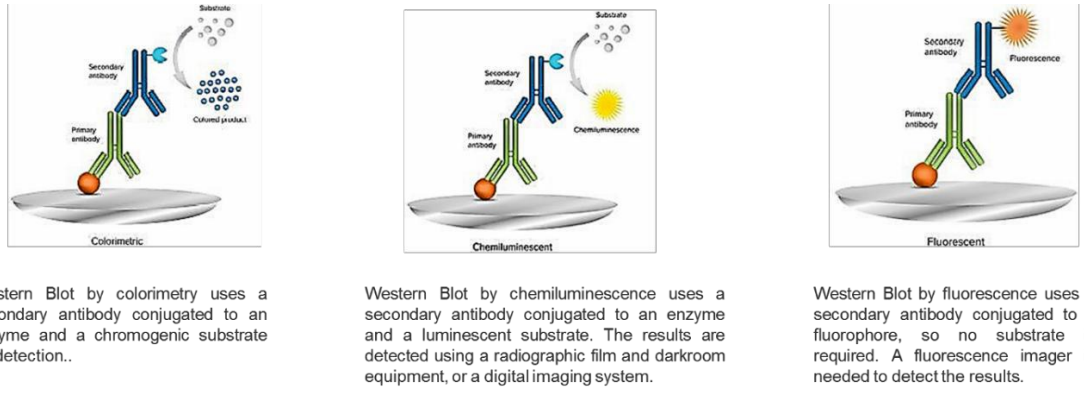


Figure06: Different types of detection used in Western blot. Adapted from Western Blot, by Molecular Devices, (2019).

Chapter Summary

Electrophoresis is a technique used to separate charged molecules (proteins, nucleic acids) based on their migration in an electric field.

Electrophoretic mobility (μ) of a molecule depends on its charge, size, shape, and the viscosity of the medium.

Zone electrophoresis involves applying the sample as a narrow zone on a support medium. **Gel electrophoresis** uses a gel matrix (polyacrylamide or agarose) as a molecular sieve, adding a size-based separation mechanism.

Polyacrylamide Gel: Gels are formed by polymerization of acrylamide and bisacrylamide, initiated by APS and TEMED. Pore size is controlled by adjusting %T (total monomer concentration) and %C (cross-linker concentration).

Buffer systems:

Continuous system: Same buffer in gel and electrodes; simpler but lower resolution.

Discontinuous system: Different buffers and a two-gel system (stacking gel and resolving gel); provides sharper bands and higher resolution.

Native PAGE separates proteins in their native conformation, preserving biological activity. However, migration depends on both charge and size, making molecular weight determination difficult.

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) is the most common method for protein analysis. SDS denatures proteins and gives them a uniform negative charge, allowing separation based solely on molecular weight. Reducing agents (e.g., DTT, β-mercaptoethanol) break disulfide bonds for complete denaturation.

Agarose gel electrophoresis is the standard method for separating nucleic acids (DNA, RNA). Agarose concentration determines pore size: lower concentrations for large fragments, higher concentrations for small fragments. Ethidium bromide (EtBr) or other fluorescent dyes are used for visualization under UV light.

Electrophoretic Mobility Shift Assay (EMSA) detects protein-nucleic acid interactions. A protein-bound DNA/RNA fragment migrates more slowly than the free fragment, producing a "shifted" band.

Capillary Zone Electrophoresis (CZE) performs separations in narrow capillaries, allowing high voltages and rapid, high-resolution analyses. Separation results from two phenomena:

Electrophoretic mobility: Movement of charged species in an electric field.

Electroosmotic flow (EOF): Bulk flow of buffer due to charged capillary walls, which transports all molecules (cations, anions, and neutrals) toward the detector.

Variants of CE include:

Micellar Electrokinetic Chromatography (MEKC): Uses surfactants to form micelles, allowing separation of neutral and hydrophobic molecules.

Capillary Gel Electrophoresis (CGE): Uses a gel-filled capillary for size-based separation (e.g., DNA sequencing).

Isoelectric Focusing (IEF) separates proteins based on their **isoelectric point (pI)** . Proteins migrate in a pH gradient until they reach the pH where their net charge is zero and stop focusing.

Two-Dimensional Electrophoresis (2D-PAGE) combines IEF (first dimension, separation by pI) and SDS-PAGE (second dimension, separation by MW). It is the highest-resolution technique for analyzing complex protein mixtures and is widely used in proteomics.

Blotting techniques transfer molecules from a gel to a membrane for detection:

Southern blot: Detects specific DNA sequences using a labeled DNA probe.

Northern blot: Detects specific RNA sequences using a labeled DNA or RNA probe.

Western blot (immunoblotting): Detects specific proteins using antibodies.

Review Questions

1. What are the key factors that influence the electrophoretic mobility of a molecule?
2. Describe the difference between a continuous buffer system and a discontinuous buffer system in PAGE. Why does the discontinuous system provide better resolution?
3. Define %T and %C in polyacrylamide gel chemistry. How does increasing %T affect the pore size of the gel and the migration of proteins?
4. After running an SDS-PAGE gel, describe how you would determine the molecular weight of an unknown protein using a molecular weight marker.
5. Explain why nucleic acids are suitable for separation by agarose gel electrophoresis without the need for a denaturing agent like SDS.
6. A student runs a DNA sample on a 1% agarose gel and observes that the bands are smeared and poorly resolved. What adjustments could be made to improve the separation?
7. In CZE, why do cations, anions, and neutral molecules all eventually pass through the detector when it is placed near the cathode?
8. What is the main difference between Micellar Electrokinetic Chromatography (MEKC) and conventional CZE? What type of molecules is MEKC particularly useful for separating?
9. A protein has an isoelectric point (pI) of 6.5. In an IEF gel with a pH gradient from 3 to 10, where will this protein focus? Explain why.
10. What is the purpose of the "blocking" step in a Western blot procedure? What would happen if this step were omitted?
11. You are investigating whether a specific transcription factor binds to a particular DNA sequence. Which two techniques covered in this chapter would be most appropriate to use? Briefly explain your choice for each.

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Chapter IV: Spectroscopic Techniques

Table of Contents

Learning Objectives

After reading this chapter, you should be able to understand the principles of interaction between electromagnetic radiation and matter. You will be able to distinguish between atomic spectroscopy, UV-Visible spectroscopy, infrared (IR) spectroscopy, mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. You will understand how each technique provides specific structural or quantitative information about biomolecules. Finally, you will be able to interpret basic spectra (IR, MS, and NMR) and select the appropriate spectroscopic method for identifying or quantifying a given compound.

Lecture 01: Atomic Spectroscopy-
UV-Visible Spectroscopy

I. Introduction to Spectroscopy and Spectrometry

Spectroscopic techniques are fundamental tools in the chemical and biological sciences, providing insights into the structure, composition, and dynamics of matter. These methods are based on the interaction between electromagnetic radiation and atoms or molecules.

It is important to distinguish between these two related terms spectroscopy and spectrometry.

Spectroscopy is the science that studies the interaction of different types of radiation with matter. It concerns the theory and the qualitative interpretation of these interactions.

Spectrometry refers to the measurement of the radiation's intensity and the quantitative determination of the relationship between the radiation and the properties of the matter. It encompasses the instrumentation and the methods used to obtain a spectrum.

I.1. The Electromagnetic Spectrum

Electromagnetic radiation spans a continuous range of wavelengths and frequencies, from high-energy gamma rays to low-energy radio waves. Different spectroscopic techniques utilize different regions of this spectrum to probe specific molecular or atomic properties (Figure 01):

- ✓ Gamma-ray spectroscopy: Studies nuclear transitions.
- ✓ X-ray spectroscopy: Probes core electronic transitions.
- ✓ UV-Visible spectroscopy: Investigates valence electronic transitions.
- ✓ Infrared (IR) spectroscopy: Examines molecular vibrations.
- ✓ Microwave spectroscopy: Studies molecular rotations.
- ✓ Radio wave spectroscopy (NMR): Probes nuclear spin transitions in a magnetic field.

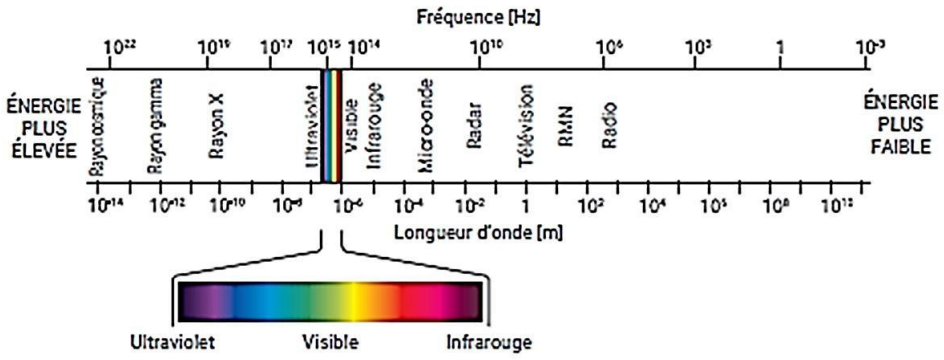

Figure 01: Electromagnetic spectrum. Adapted from Agilent Technologies (2021), The Basics of UV-Vis Spectrophotometry.

II. Atomic Spectroscopy

Atomic spectroscopic methods are used for the qualitative and quantitative determination of elemental composition in a sample. They focus on the behavior of free atoms in the gas phase, which have simple, well-defined energy levels (Bings, Bogaerts, & Broekaert, 2013).

II.1. Atom and Energy Transitions

An atom is composed of a nucleus of protons and neutrons, surrounded by a cloud of electrons. When energy is supplied, the electrons of an atom move from one energy level to a higher one (quantum jumps), causing the atom to transition from its ground state (stable) to an "excited" state (unstable). When the excitation ceases, the atom tends to return to the ground state by releasing the energy it had absorbed. This energy relaxation occurs in a very short time and is manifested by the emission of a light photon whose energy exactly corresponds to that of the quantum jump (Figure 02).

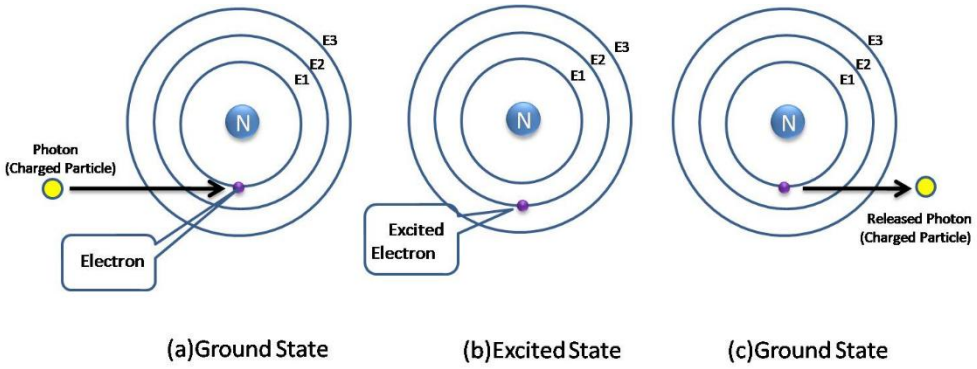

Figure 02: Energy transition in an atom
(https://upload.wikimedia.org/wikipedia/commons/2/2c/Atom_excitation.jpg)

II.2. Atomic Absorption Spectroscopy (AAS)

Atomic Absorption Spectroscopy is a quantitative technique used to measure the concentration of specific elements in a sample. A sample is atomized, typically by introducing it into a flame or a graphite furnace. A beam of light from a lamp that emits the characteristic spectrum of the target element (e.g., a hollow cathode lamp) is passed through the cloud of atoms. Ground-state atoms of that specific element will absorb light at their characteristic wavelengths, transitioning to an excited state. After passing through a monochromator, the light is dispersed, and all wavelengths appear except the one absorbed by the atom, resulting in the presence of dark lines (Figure 03) The amount of light absorbed is proportional to the number of atoms in the light path.

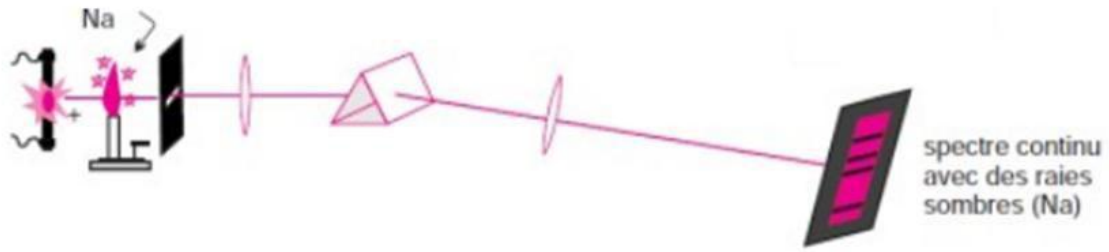

Figure 03: Schematic representation of atomic absorption spectroscopy principle. Adapted from screenshot of Spectroscopie d'absorption et d'émission atomique, by Dr Gouasmi, (2020)

The relationship between absorbance and concentration is described by the Beer-Lambert Law:

$$A = K.\ C$$

A: The absorbance.

C: The concentration of the element in the sample.

k: Coefficient specific to each element for the chosen wavelength.

NB. Linearity is only verified for low concentrations.

II.3. Atomic Emission Spectroscopy (AES)

Atomic Emission Spectroscopy measures the light emitted by atoms when they return to the ground state after being excited. A sample is subjected to a high-energy source, such as a flame (Hence the name Flame Emission Spectrometry: FES), an inductively coupled plasma (ICP), or an electric arc. This energy excites the atoms, which then emit light at characteristic wavelengths as they relax. The light is dispersed by a monochromator resulting in a spectrum of bright lines on a dark background (Emission Spectrum) (Figure 04), the intensity of the emitted light at specific wavelengths is measured by a detector.

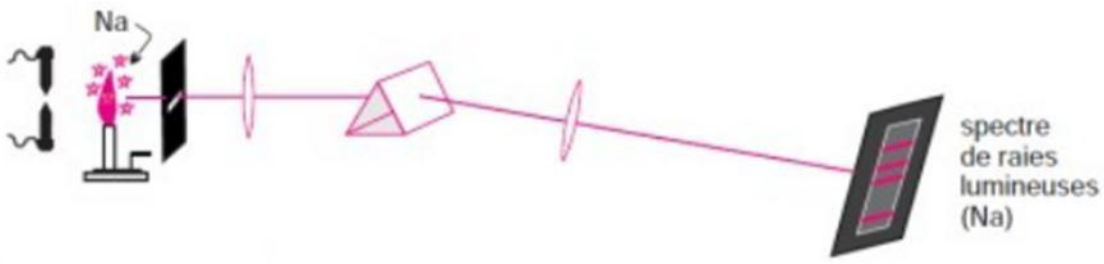

Figure 04: Schematic representation of atomic emission spectroscopy principle. Adapted from screenshot of Spectroscopie d'absorption et d'émission atomique, by Dr Gouasmi, (2020)

The intensity of the emitted light is directly proportional to the concentration of the element:

$$I_e = K.C$$

I_e: The intensity of the emitted radiation.

C: Concentration of the element

K: Coefficient specific to each element for the chosen wavelength.

AES is particularly useful for easily excitable elements like sodium, potassium, and calcium.

III. UV-Visible Spectroscopy

III.1. Principle

UV-Visible (UV-Vis) spectroscopy is one of the most widely used analytical techniques in biochemistry and molecular biology. It measures the absorption of light in the ultraviolet (200–400 nm) and visible (400–800 nm) regions of the electromagnetic spectrum (Perkampus, 1992). When UV or visible light passes through a molecule, photons with energy matching the energy gap between electronic energy levels can be absorbed. This energy promotes an electron from a lower-energy molecular orbital to a higher-energy orbital. These are called electronic transitions.

The types of bonds and functional groups that absorb UV-Vis light are often called *chromophores*. Common chromophores in biomolecules include:

- ✓ Carbonyl groups (C=O)
- ✓ Aromatic rings (e.g., in phenylalanine, tyrosine, tryptophan)
- ✓ Conjugated double-bond systems
- ✓ Peptide bonds (absorb in the far UV, around 190–220 nm)

III.2. Absorbance and Transmittance (Beer-Lambert Law)

When light passes through a sample or is reflected by it, the amount of light absorbed equals

the difference between the incident energy (I_0) and the transmitted energy (I). It is quantified by *absorbance* (A) or *transmittance* (T).

Transmittance is the fraction of incident light that passes through the sample:

$$\mathbf{T = I / Io \text{ or } \% T = I/Io \times 100}$$

Absorbance expresses the amount of light absorbed is the negative logarithm of transmittance

$$\mathbf{A = – logT = Log\ Io/I}$$

The fundamental law governing absorbance measurements is the Beer-Lambert Law:

$$\mathbf{A=\varepsilon \cdot l \cdot C}$$

where:

A: The absorbance (unitless),

ε (epsilon): The molar absorptivity or extinction coefficient ($M^{-1}cm^{-1}$), a constant for a given molecule at a specific wavelength,

l: The path length of the light through the sample (cm),

C: The concentration of the absorbing species (M).

This linear relationship between absorbance and concentration is the basis for quantitative analysis by UV-Vis spectroscopy.

III.3. Instrumentation

A UV-Vis spectrophotometer consists of several key components (Figure 05).

- Light Sources

- Deuterium Arc Lamp: Provides a stable, continuous spectrum in the UV region (185–400 nm).

-Tungsten Halogen Lamp: Provides a stable, continuous spectrum in the visible and near-infrared (NIR) regions (350–3000 nm).

- Xenon Flash Lamp: Emits intense, short flashes of light covering both UV and visible ranges (185–2500 nm). It requires no warm-up time and has a long lifespan.

Many instruments use both a deuterium and a tungsten lamp to cover the full UV-Vis range, switching between them automatically.

- Monochromators

A monochromator is a device that selects a specific wavelength or narrow band of wavelengths from a broad-spectrum light source. It typically consists of an entrance slit, a dispersive element (a prism or a diffraction grating), and an exit slit. By rotating the grating, different wavelengths are directed through the exit slit and onto the sample.

- Sample Holders (Cuvettes)

Liquid samples are typically placed in a cuvette, a small rectangular container with a precise optical path length (usually 1 cm). The material of the cuvette must be transparent to the wavelengths being measured (Table 01).

Table 01: Wavelength according to the cuvette material. Adapted from Agilent Technologies (2021), The Basics of UV-Vis Spectrophotometry.

Matériau	Longueur d'onde acceptable (nm)
Quartz	170-2700
Quartz Infrasil (NIR)	220-3800
Verre optique	334-2500
Polystyrène (jetable)	340-800

- Detectors

A detector converts the light signal into an electrical signal that can be processed and recorded. Common detectors include photomultiplier tubes (PMTs), photodiodes, and charge-coupled devices (CCDs).

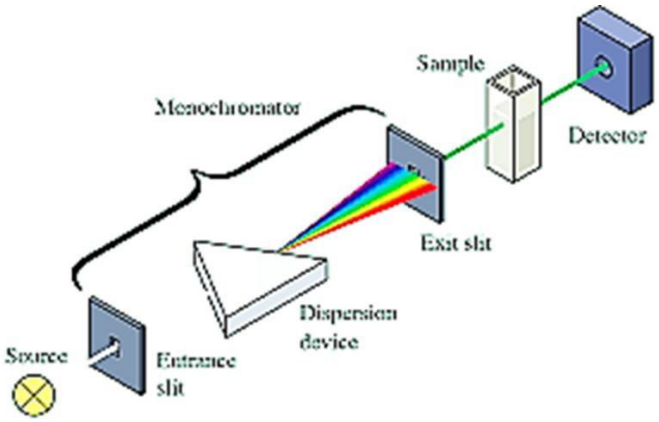

Figure 05: Schematic representation of the main components of UV-Visible spectrometer. Adapted from How does a spectrophotometer function?, by Shimadzu Corporation, (n.d).

III.4. Applications of UV-Vis Spectroscopy

UV-Vis spectroscopy has a vast range of applications in biological sciences:

- ✓ Quantification of biomolecules: Determining the concentration of nucleic acids (DNA, RNA) at 260 nm and proteins at 280 nm.
- ✓ Enzyme kinetics: Monitoring the appearance or disappearance of a chromophoric substrate or product over time (e.g., NADH oxidation at 340 nm).
- ✓ Determination of binding constants: Studying ligand-protein or protein-protein interactions.
- ✓ Purity assessment: The A260/A280 ratio is a classic indicator of nucleic acid purity (a ratio of ~1.8 for pure DNA, ~2.0 for pure RNA).
- ✓ Monitoring chemical reactions: Following the progress of a reaction that involves a change in absorbance.

Lecture 02: Fluorescence spectroscopy- Infrared (IR) Spectroscopy- Nuclear Magnetic Resonance (NMR) Spectroscopy

I. Fluorescence spectroscopy

I.1. Principle

Fluorescence spectroscopy is an optical spectroscopic method based on the emission of photons from a substance following excitation by light absorption. When a molecule in its ground state absorbs a photon of sufficient energy, an electron is promoted to a higher energy singlet level. Due to vibrational energy losses in non-radiative decay, the emitted light is of lower energy (longer wavelength) than the absorbed light, a phenomenon known as Stokes' shift. This process is illustrated by the Jablonski diagram, which shows the possible radiative transitions (absorption, fluorescence, phosphorescence) (Figure 01) and non-radiative transitions (vibrational relaxation, internal conversion, intersystem crossing). Fluorescence occurs when an electron from a singlet excited level decays radiatively to the ground singlet state within a characteristic decay time of 10^{-10} to 10^{-7} seconds. The emission spectrum and absorption spectrum of a compound are usually almost mirror images, attributed to the same vibrational level structures involved in each process (Zacharioudaki et al., 2022). The fluorescence intensity is proportional to the molecule's concentration in diluted solutions, as described by the equation:

$$I_F = k \cdot I_0 \cdot \Phi \cdot (\epsilon \cdot b \cdot C)$$

where

k: An instrument constant

I_0: The incident light intensity

Φ: The quantum yield

ϵ: The molar absorptivity,

b: The path length, and

C: The concentration.

I.2. Instrumentation

A fluorescence spectroscopy system typically consists of a light source, a sample compartment, monochromators or filters, and a detector (Figure 02).

Light sources include conventional lamps such as xenon (Xe) and mercury (Hg) arc lamps, which provide broadband UV-visible emissions, and deuterium lamps for UV range. Light-emitting diodes (LEDs) have also emerged as inexpensive, bright illumination sources.

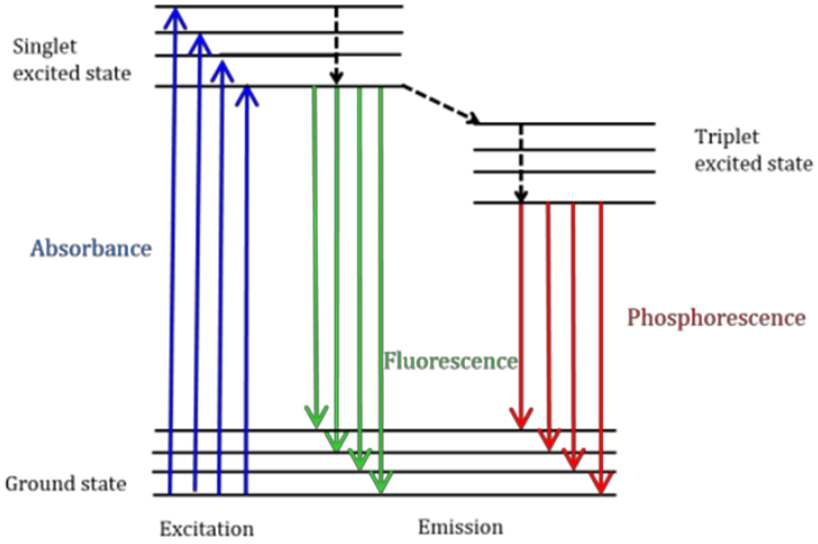

Figure 01: Jablonski diagram illustrating different transitions between a molecule's energy states (Listiaji, 2021).

Laser sources, used in laser-induced fluorescence (LIF), include solid-state lasers (e.g., Nd:YAG with harmonics at 532, 355, 266 nm), gas lasers (argon ion, HeNe, nitrogen, excimers), liquid dye lasers (tunable over a wide range), and diode lasers (compact, from visible to IR). The choice of laser depends on the required wavelength range and properties such as monochromaticity and directionality.

The sample compartment typically uses fused quartz cuvettes for liquid samples, with various accessories for temperature control, stirring, and automation. Fluorescence is usually detected at a right angle to the excitation beam to minimize scattered light.

Detectors include photomultiplier tubes (PMTs), which are highly sensitive and commonly used for low light intensity detection; avalanche photodiodes (APDs), which generate measurable signals even from few photons; and charge-coupled devices (CCDs), which allow the whole fluorescence spectrum to be analyzed at once without wavelength scanning (Zacharioudaki et al., 2022).

I.3. Applications

- **Environmental Monitoring**
  - ✓ Characterization of Dissolved Organic Matter (DOM): Identification of humic-like, fulvic-like, and protein-like components in natural waters using EEM-PARAFAC.

- ✓ Pollutant Detection: Monitoring of polycyclic aromatic hydrocarbons (PAHs), oils, pesticides, and heavy metals in water, soil, and sediments.
- ✓ Wastewater Treatment Monitoring: Tracking organic matter removal and detecting fecal contamination via tryptophan-like fluorescence.

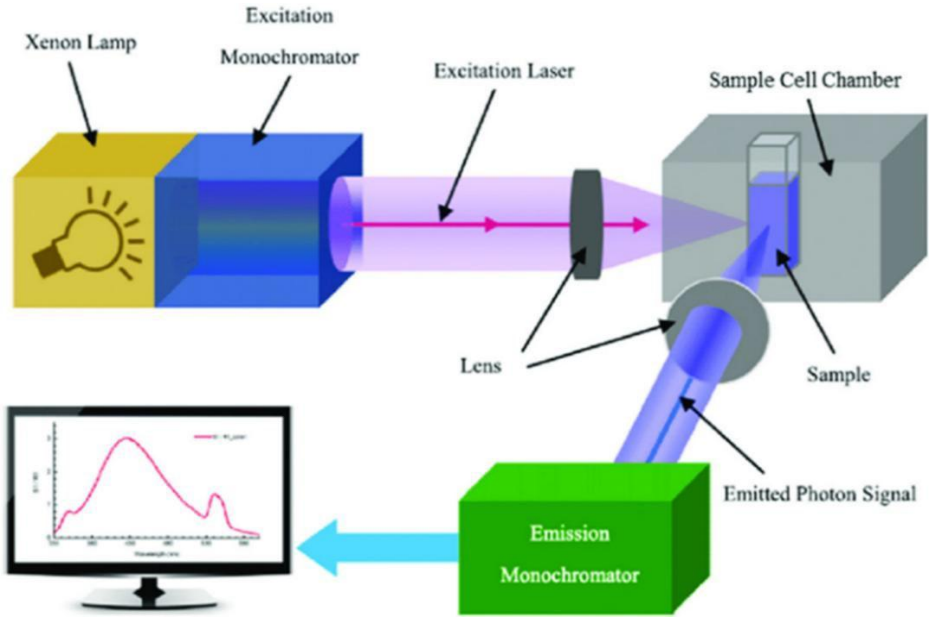

Figure 02 : Fluorometer diagram (Li & Yue, 2014).

- ➢ **Biochemical and Biomedical Applications**
  - ✓ Protein Analysis: Detection and quantification of aromatic amino acids (tryptophan, tyrosine), protein conformation studies, and protein-ligand interactions.
  - ✓ Enzyme Assays: Monitoring enzyme kinetics using fluorogenic substrates.
  - ✓ Medical Diagnostics: Detection of disease biomarkers, cancer imaging, and drug delivery monitoring.
- ➢ **Pharmaceutical and Food Industry**
  - ✓ Drug Discovery: High-throughput screening of drug candidates and studying drug-receptor binding.
  - ✓ Food Quality Control: Detection of contaminants (aflatoxins, pesticides), analysis of vitamins, and monitoring food freshness.
  - ✓ Beverage Analysis: Characterization of wines, beers, and juices based on fluorescent compounds.
- ➢ **Industrial and Material Science**
  - ✓ Polymer Characterization: Studying polymer structure, degradation, and additives.
  - ✓ Nanotechnology: Characterization of quantum dots and fluorescent nanoparticles.

II. Infrared (IR) Spectroscopy

Infrared (IR) spectroscopy is a powerful technique for identifying functional groups and determining the molecular structure of organic and inorganic compounds. It is based on the absorption of infrared radiation, which causes molecules to undergo vibrational transitions (Stuart, 2004).

II.1. Principle: Molecular Vibrations

Covalent bonds in molecules are not rigid; they constantly stretch, bend, and twist. When IR radiation of a specific frequency matches the natural vibrational frequency of a bond, the molecule absorbs that energy, and the amplitude of the vibration increases. For a vibration to be IR-active, it must involve a change in the dipole moment of the molecule.

There are two main types of molecular vibrations (Figure 03):

- **Stretching Vibrations**

A rhythmic movement along the bond axis, causing the interatomic distance to increase and decrease. Stretching vibrations can be *symmetric* (both bonds lengthen and shorten simultaneously) or *asymmetric* (one bond lengthens while the other shortens).

- **Bending Vibrations**

A change in the bond angle between two bonds, or the movement of a group of atoms relative to the rest of the molecule. Types of bending vibrations include *scissoring*, *rocking*, *wagging*, and *twisting*.

Stretching vibrations typically occur at higher frequencies (higher energy) than bending vibrations.

II.2. Factors Influencing Vibrational Frequencies

The exact frequency of a bond vibration is influenced by its chemical environment.

- ✓ Electron-withdrawing groups can alter the bond strength.
- ✓ Conjugation with a double bond or an aromatic ring can lower the frequency of a carbonyl (C=O) stretch.
- ✓ Hydrogen bonding (e.g., in O-H groups) broadens and lowers the frequency of the stretching vibration.
- ✓ Hybridization affects bond strength: a C-H bond on a sp-hybridized carbon (≡C-H) vibrates at a higher frequency than one on a sp^2-hybridized carbon (=C-H), which is higher than one on a sp^3-hybridized carbon (-C-H).

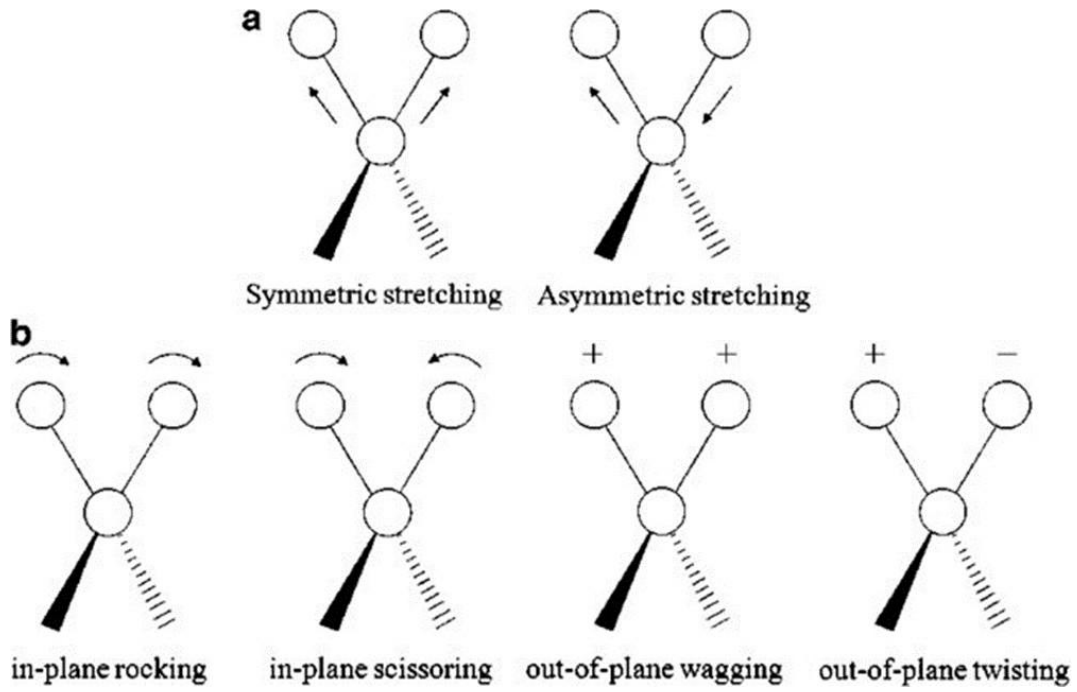

Figure 03: Types of molecular vibrations: (a) Stretching vibrations and (b) bending vibrations. "+" and "-" symbols indicate motion towards the paper and away from the paper, respectively (Ojeda & Dittrich, 2012).

II.3. Instrumentation

Two main types of IR spectrometers are used:

- **Dispersive IR Spectrometers**

These use a monochromator (prism or grating) to scan through the IR frequencies one by one. They are slower and have lower sensitivity.

- **Fourier Transform Infrared (FTIR) Spectrometers**

These are the modern standard. An FTIR spectrometer uses an interferometer to measure all IR frequencies simultaneously. The resulting signal, an interferogram, is then converted into a spectrum using a mathematical operation called a Fourier transform. FTIR offers significant advantages: higher speed, better signal-to-noise ratio, and higher resolution.

II.4. Interpretation of IR Spectra

An IR spectrum is a plot of transmittance (%) or absorbance (y-axis) versus wavenumber (cm^{-1}) (x-axis). Absorption bands (peaks pointing down in transmittance mode) correspond to specific molecular vibrations. The spectrum is divided into two main regions for interpretation (Example given in appendix 01).

- **The Functional Group Region (4000–1500 cm^{-1})**

This region contains characteristic absorption bands for specific functional groups. It is the primary region for identifying what functional groups are present in a molecule (See table below).

Table 01: Functional groups and their characteristic bands

Functional Group / Bond	Characteristic Band (cm^{-1})	Appearance / Intensity
O–H (Alcohols, Carboxylic acids)	3200 – 3600	Broad, Strong
N–H (Amines, Amides)	3300 – 3500	One or two sharp band(s)
C–H (Alkanes)	2850 – 3000	Band(s)
C–H (Alkenes, Aromatics)	Just above 3000	Band(s)
C–H (Terminal Alkynes)	~ 3300	Band
C≡N (Nitriles)	2200 – 2260	Sharp, Strong
C≡C (Alkynes)	2100 – 2260	Sharp
C=O (Carbonyls: ketones, aldehydes, carboxylic acids, esters, amides)	1650 – 1850	Very strong, Sharp

- **The Fingerprint Region (1500–400 cm^{-1})**

This region is complex, containing many overlapping bands from bending and other skeletal vibrations. While difficult to assign individual bands, the overall pattern is highly unique for every molecule, like a human fingerprint. Comparing the fingerprint region of an unknown compound to that of a known standard provides definitive identification.

III. Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is the most powerful technique for determining the detailed structure of organic molecules, including complex biomolecules like proteins and nucleic acids. It exploits the magnetic properties of certain atomic nuclei (Keeler, 2010).

III.1. Principle of NMR

- **Nuclear Spin and Magnetic Moments**

Certain atomic nuclei, such as ^{1}H (proton) and ^{13}C, possess a property called spin, which gives them a nuclear magnetic moment. In the absence of an external magnetic field, these spins are randomly oriented. When placed in a strong, static external magnetic field (B_0), the nuclear

spins align either parallel (lower energy state) or anti-parallel (higher energy state) to the field.

- **Resonance Condition**

If the sample is now irradiated with a pulse of radiofrequency (RF) energy, nuclei in the lower energy state can absorb this energy and "flip" to the higher energy state. This absorption occurs only when the frequency of the RF radiation exactly matches the energy difference between the two spin states. This condition is called resonance. When the RF pulse ceases, the excited nuclei relax back to the lower energy state, emitting an RF signal that is detected and processed to produce an NMR spectrum.

III.2. Chemical Shift (δ)

The resonance frequency of a nucleus is not simply a constant; it is exquisitely sensitive to its electronic environment. The electrons surrounding a nucleus create tiny local magnetic fields that either shield or deshield the nucleus from the applied external field B_0.

- ✓ Shielding: A high electron density around a nucleus opposes B_0, meaning the nucleus experiences a slightly weaker effective field. It will therefore resonate at a slightly lower frequency.

- ✓ Deshielding: A low electron density means the nucleus experiences a slightly stronger effective field and resonates at a slightly higher frequency.

This variation in resonance frequency is called the chemical shift (δ) . It is measured in parts per million (ppm) relative to a reference compound (usually tetramethylsilane, TMS, for ^{1}H and ^{13}C NMR). A typical ^{1}H NMR spectrum spans from 0 to 12 ppm. Deshielded protons appear at higher ppm values (downfield), while shielded protons appear at lower ppm values (upfield).

III.3. Signal Multiplicity: Spin-Spin Coupling

A signal in a high-resolution ^{1}H NMR spectrum is often split into multiple peaks. This phenomenon, called spin-spin coupling (or J-coupling), arises from the interaction of the magnetic moments of nearby non-equivalent protons through the intervening bonding electrons.

- **The n+1 Rule**

The multiplicity of a signal from a proton (or a set of equivalent protons) is determined by the number of equivalent protons on the immediately adjacent carbon atom(s). If a proton has n equivalent neighboring protons, its signal will be split into n+1 peaks (Figure 04).

- ✓ 0 neighbors → singlet (s)
- ✓ 1 neighbor → doublet (d)
- ✓ 2 neighbors → triplet (t)

- ✓ 3 neighbors → quartet (q)
- ✓ 4 neighbors → quintet (quin)
- ✓ 5 neighbors → sextet (sxt)
- ✓ 6 neighbors → septet (spt)

The relative intensities of the peaks in a multiplet follow Pascal's triangle (e.g., triplet 1:2:1, quartet 1:3:3:1). Protons on heteroatoms like O or N (e.g., -OH, -NH) often do not couple due to rapid exchange and appear as broad singlets.

- ➢ **Coupling Constants (J)**

The distance between the peaks in a multiplet is constant and is called the coupling constant (J) , measured in Hertz (Hz). J is independent of the external magnetic field strength and is a characteristic of the interaction between the coupled nuclei.

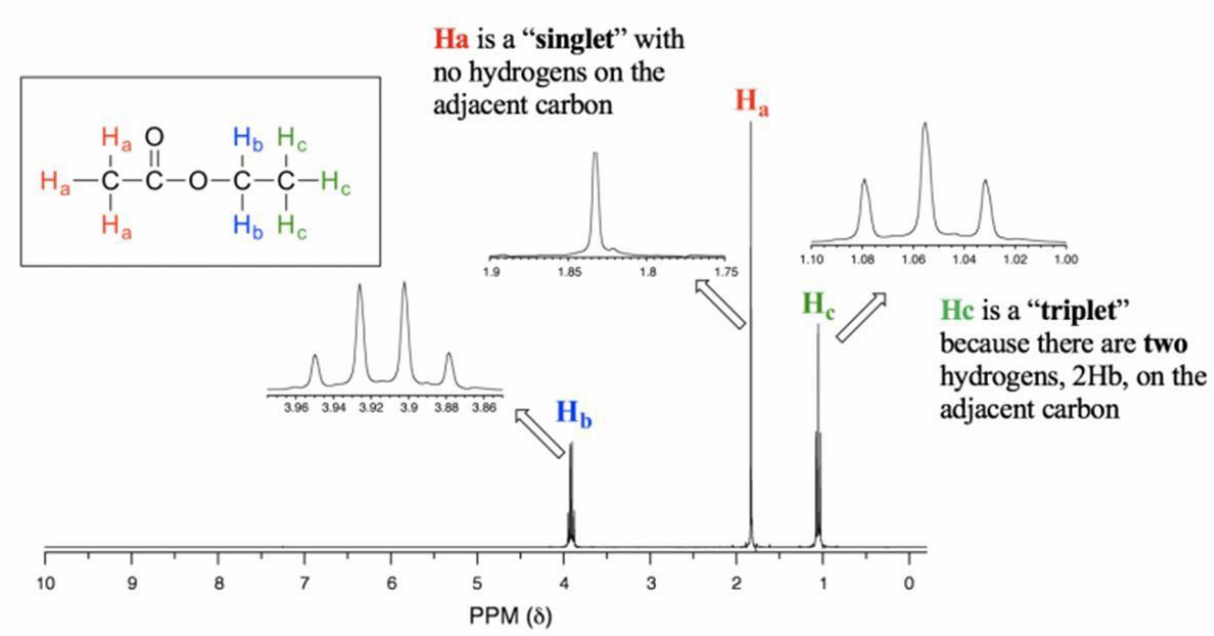

Figure 04: Example of The ^{1}H NMR spectrum of ethyl acetate with signals splitting. Adapted from ¹H NMR Spectra and Interpretation (Part II), by KPU, (2021).

The area under each peak or multiplet in a ¹H NMR spectrum is directly proportional to the number of protons giving rise to that signal. The spectrometer's software integrates these areas, producing a step curve (integral) or numerical values that indicate the relative number of protons for each signal.

III.4. Applications of NMR Spectroscopy

- ✓ Structural elucidation of organic and inorganic compounds.
- ✓ Conformational analysis and study of molecular dynamics.
- ✓ Study of biomolecular structure and interactions (protein NMR, DNA/RNA).
- ✓ Metabolomics and metabolic profiling of biofluids.
- ✓ Pharmaceutical analysis: Drug discovery, quality control, characterization of impurities.
- ✓ Materials science (polymers, zeolites).
- ✓ Medical imaging (MRI) – a macroscopic application of NMR.

Lecture 03: Mass spectroscopy

I. Introduction

Mass spectrometry (MS) is a sensitive, quantitative, and analytical technique used in environmental, pharmaceutical, medical, forensic, food, and other sciences.

This technique involves the separation of gaseous ions from the liquid or solid-state of the samples. After conversion into a gaseous state, these are separated based on their mobility in an electric and magnetic field. The detected ions or separated ions are analyzed in a mass spectrum.

When this technique is interfaced with high-performance liquid chromatography (HPLC) or gas-liquid chromatography (GLC), mass spectrometer functions as a powerful detector, which helps in detecting even trace amounts of a particular substance in a sample.

II. A simplified Principle of Working of Mass Spectrometers

- ✓ Sample (solid,liquid,gas) is ionized
- ✓ Sample's molecules might break into charged fragments during ionization
- ✓ Ions are separated according to their mass-to-charge ratio (m/z)
- ✓ Ions are detected by a mechanism capable of detecting charged particles (Figure 01)

Results are displayed as spectra of the relative abundance as a function of m/z ratio

Identification is done by correlating known masses to the identified masses or through a characteristic fragmentation pattern

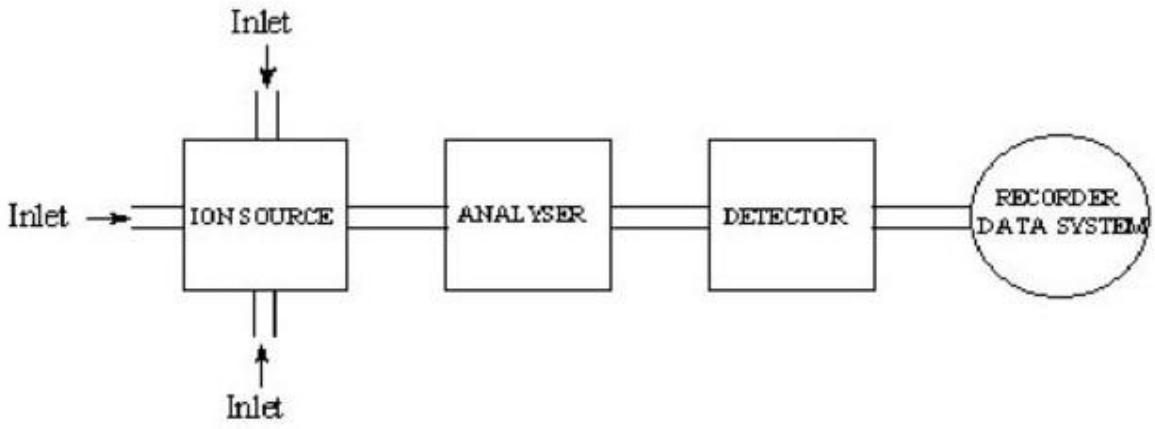

Figure 01: A simplified principle of working of mass spectrometers (Vairamani, 2003).

III. Ionization Techniques

III.1. Electron Ionization (EI)

EI was one of the very first mass spectrometry techniques developed. It is a popular hard ionization method in which high-energy electrons produce ions after interacting with atoms or molecules in a solid or gas phase.

Since it uses high energy to generate ions, it creates a lot of ion fragmentation which can be helpful for determining the structure of unknown compounds.

When EI is combined with other separation techniques, it can be used to detect other thermally stable and volatile compounds in solid, liquid, and gas states. EI has high ionization efficiency and sensitivity, and it can provide a lot of structural information.

- Analysis: Useful for organic compounds with molecular weights below 600 Da
- Detection: EI can only detect positive ions
- Applications: EI has many different applications including, but not limited to, environmental analysis, archaeological analysis, forensic analysis, and pharmaceutical analysis.

III.2. Chemical Ionization (CI)

CI is a soft ionization technique that uses a reagent gas to ionize sample molecules through ion-molecule reactions in the gas phase. Samples to be analyzed must be in vapor form or vaporized before being introduced to the ion source.

The first step is for the reagent gas to undergo electron ionization, generating a molecular ion. This molecular ion will then fragment as it reacts with other reagent gas molecules and ions, creating analyte ions. It is important to select the most appropriate reagent ion as it increases the selectivity and response time of CI.

Due to having little excess energy from the ions, there is little fragmentation that occurs, and resultantly, little structural information is obtained. Because there are few fragments formed, the products are usually ions of the analyte, which makes it possible to get the exact molecular weight of the analyte.

- Analysis: Both small and large molecules of various polarities in more complex biological samples
- Detection: Molecules that typically fragment a lot under EI conditions
- Applications: Identification, structure elucidation, and quantification of organic compounds, and some use in biochemical analysis

III.3. Electrospray Ionization (ESI)

ESI has become the most popular ionization technique, It is a soft ionization method using electrospray to apply a high voltage to a liquid to produce an aerosol, producing multiply charged ions.

The electrospray is created by putting a high voltage on a flow of liquid at atmospheric pressure, sometimes this is assisted by a concurrent flow of gas.

The created spray is directed to an opening in the ESI spray vacuum system of the mass spectrometer, where the droplets are de-solvated by a combination of heat, vacuum and acceleration into gas by voltages.

Eventually the ions are ejected from the droplets and accelerated into the mass analyzer by voltages.

- Applications: Analysis of peptides, proteins, and nucleotides.

III.4. Matrix-Assisted Laser Desorption/Ionization (MALDI)

MALDI is a soft ionization, pulsed technique. The analyte is co-crystallized with an excess of a small organic molecule called the "matrix." A pulsed laser (usually UV) is fired at the sample. The matrix absorbs the laser energy, causing rapid heating and desorption of the matrix and analyte into the gas phase. The analyte is ionized by proton transfer in the hot plume. It produces mostly singly charged ions ($[M+H]^+$). It is most used for the analysis of large biomolecules (proteins, peptides, oligonucleotides), polymers, and for imaging mass spectrometry.

A Common combination: MALDI-TOF (MALDI coupled with a Time-of-Flight mass analyzer).

IV. Mass Analyzer

IV.1. Magnetic Sector Mass Analyzer

The sample for analysis in a mass spectrometer is subjected to high vapor pressure which causes the ions to fragment and ionize.

The ionized ions in the sample are accelerated by applying voltage so that they move according to their mass in a mass analyzer.

These ions are then made to pass through a magnetic force in the detector, which causes the relative ions with the same velocity to move in a perpendicular path or take a circular path. The charged ions when exposed to a magnetic field take a perpendicular path since the magnetic force provides a centripetal field (field towards the center).

Then the ions with the same magnetic and electric charges are made to pass through undeflected, reaching the data system for their recording. The mass spectrum obtained in this process helps in further analysis of substances in the sample (Figure 02).

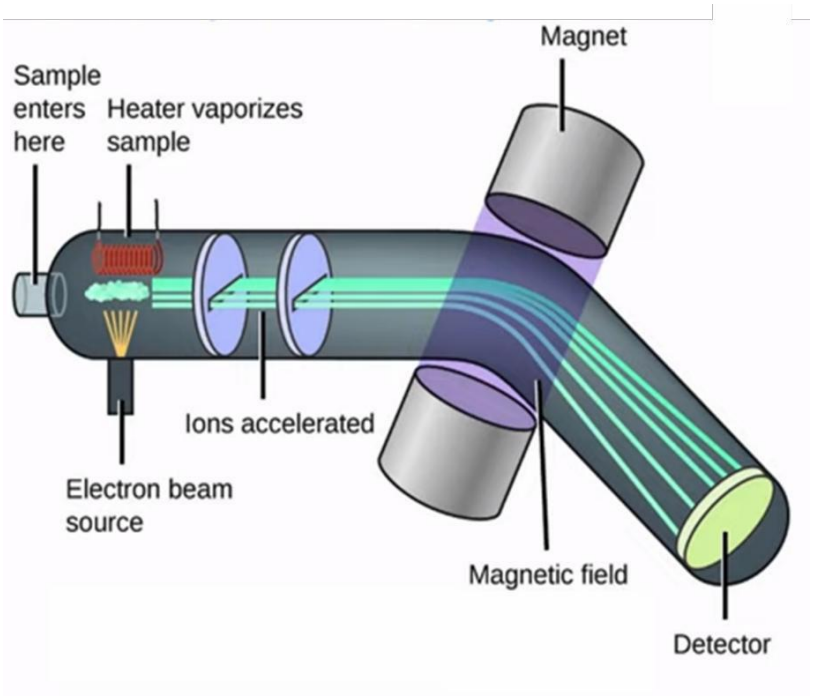

Figure 02: Schematic represenation of MS with Magnetic Sector Mass Analyzers Adapted from screenshot of Mass spectrometry | Atomic structure and properties | AP Chemistry, by Khan Academy, (2019)

IV.2. Time-of-Flight (TOF) Mass Analyzer

Ions are accelerated by a high voltage pulse into a field-free flight tube. Their velocity depends on their m/z (lighter ions travel faster). The time it takes for an ion to reach the detector at the end of the tube is measured. m/z is then calculated from this flight time:

$$m = A \cdot t^2$$

where A is a constant related to the acceleration energy and the flight distance. TOF analyzers have a high mass range and are very fast, making them ideal for MALDI.

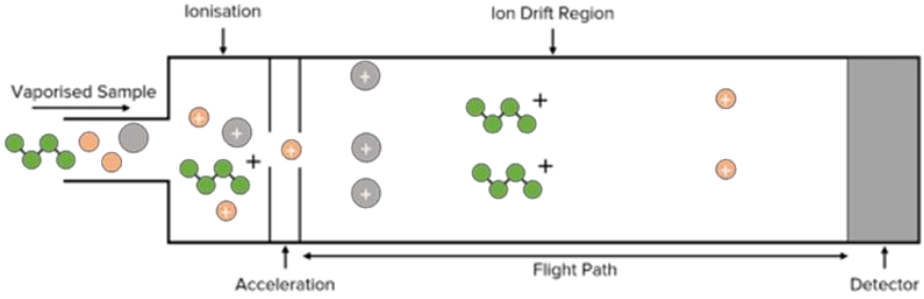

Figure 03: Schematic representation of TOF analyzer. Adapted from Time of Flight Mass Spectrometry, by MME Revise, (2024).

IV.3. uadrupole Mass Analyzer

Consists of four parallel rods. By applying a combination of RF and DC voltages, only ions of a specific m/z have a stable trajectory and pass through the rods to the detector. All other ions are ejected. Quadrupoles are robust, fast, and relatively inexpensive, making them workhorses for GC-MS and LC-MS (Figure 04).

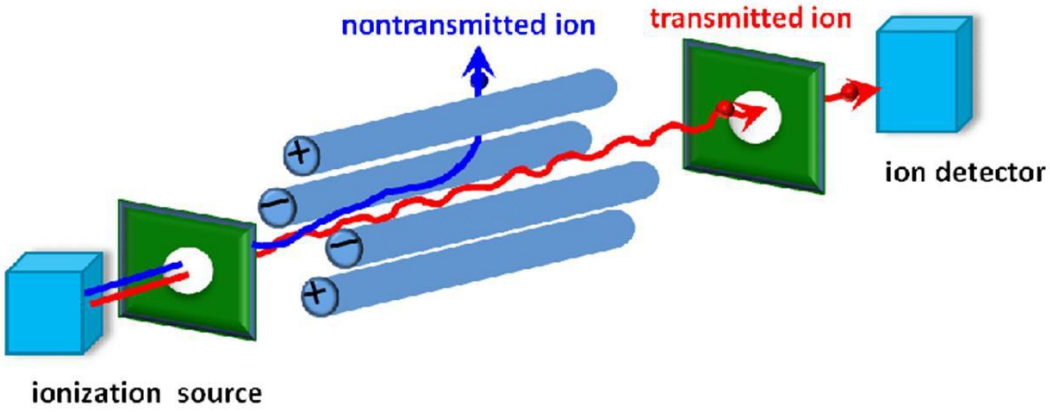

Figure 04: Schematic representation of quadrupole mass analyzer. From Domingues et al. (n.d)

V. Mass Spectrum

A mass spectrum is a two-dimensional representation of the ions detected.

V.1. The Molecular Ion (M^{+}•) and Radical Cations

A molecular ion is a charged species formed when a neutral molecule gains or loses one or more electrons, maintaining the original atom connectivity of the parent molecule (for at least a fleeting moment), but often with weakened bonds that lead to fragmentation. A radical cation is a positively charged ion (cation) that also contains an unpaired electron (radical).In mass spectrometry, it is often formed when a molecule loses a single electron during ionization, resulting in both a positive charge and an unpaired electron.

Example: When a molecule (M) loses one electron, it forms a radical cation: $M \rightarrow M^{\cdot +} + e^-$

It is usually in the hard type of ionization where ions are completely fragmented.

V.2. Fragmentation Patterns

The fragmentation of a molecular ion is often predictable and characteristic of certain functional groups or structural features (Figure 05). For example, alkanes often show clusters of peaks separated by 14 mass units (CH_2), corresponding to loss of alkyl fragments.

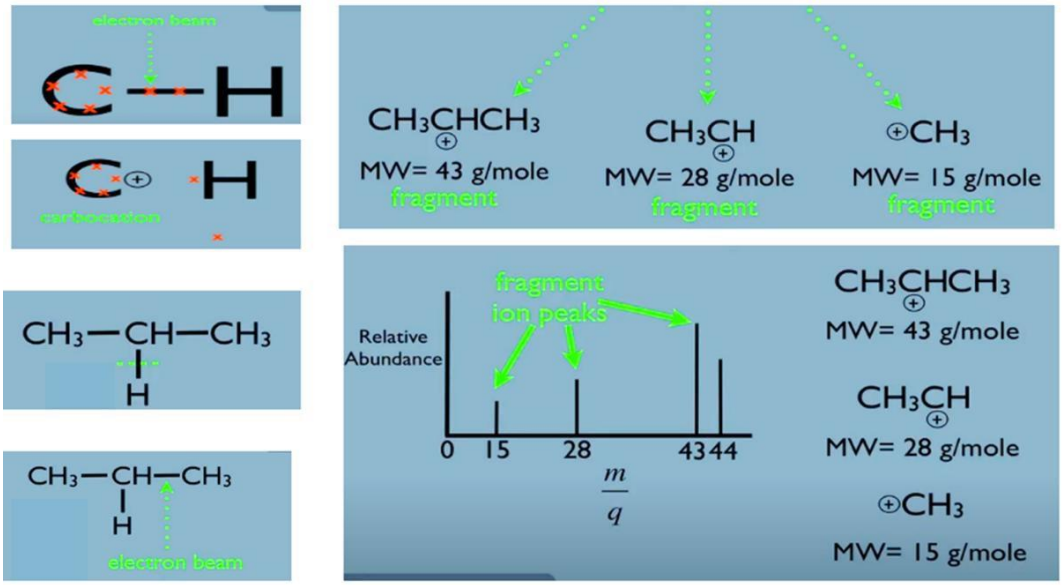

Figure 05: Example of propane fragmentation. Adapted from screenshot of Introduction to Mass Spectrometry, by Knowbee, (2015).

V.3. Isotopic Abundance: M, M+1, and M+2 Peaks

Elements exist as a mixture of isotopes. A mass spectrum will show peaks not only for the most abundant isotopic composition (the M peak) but also for ions containing heavier isotopes (e.g., M+1 for one ^{13}C, M+2 for one ^{34}S or one ^{37}Cl). The relative heights of these isotopic peaks are highly characteristic. For example, the presence of a chlorine atom (^{35}Cl:^{37}Cl ratio of ~3:1) will show a distinct M and M+2 peak in a 3:1 ratio. Bromine (^{79}Br:^{81}Br ratio of ~1:1) shows M and M+2 peaks of nearly equal height (Figure 06). Table 01 shows the relative abundance of some elements.

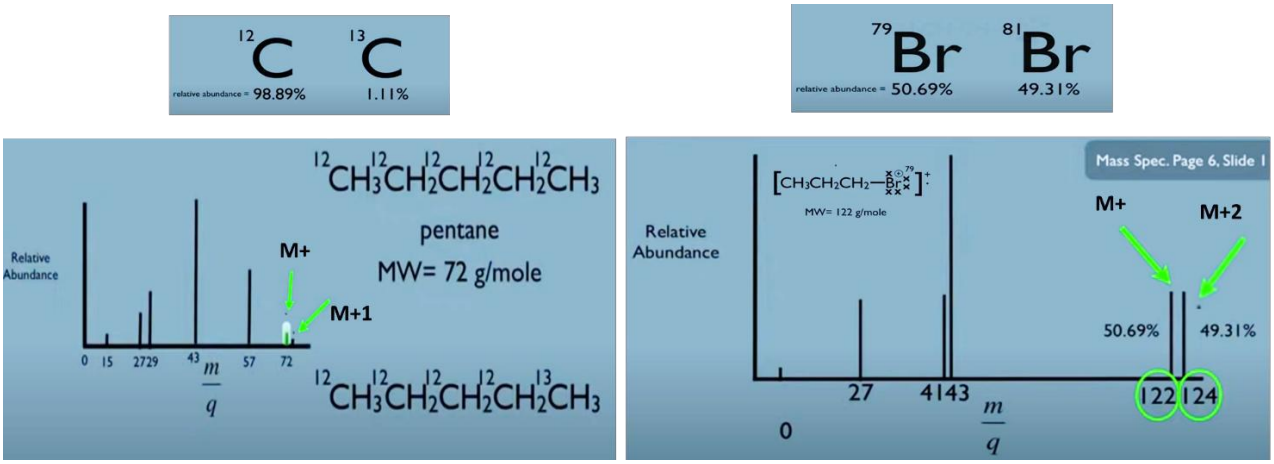

Figure 06: Isotopes peaks. Adapted from screenshot of Interpreting M+ Peaks in Mass Spectrometry, by Knowbee, (2015).

Table 01: Relative abundance of some elements. Adapted from screenshot of Interpreting M+ Peaks in Mass Spectrometry, by Knowbee, (2015).

element	M	M+1	M+2	M+4
C	^{12}C 98.89%	^{13}C 1.11%		
N	^{14}N 99.64%	^{15}N 0.01%		
O	^{16}O 99.76%	^{17}O 0.04%	^{18}O 0.20%	
S	^{32}S 95.0%	^{33}S 0.76%	^{34}S 4.22%	^{36}S 0.02%
F	^{19}F 100.0%			
Cl	^{35}Cl 75.77%		^{37}Cl 24.23%	
Br	^{79}Br 50.69%		^{81}Br 49.31%	

VI. Applications of Mass Spectrometry

- ✓ Molecular weight determination.
- ✓ Structural elucidation of organic and biomolecules.
- ✓ Proteomics: Protein identification, characterization of post-translational modifications (PTMs), and protein quantification.
- ✓ Metabolomics: Profiling small-molecule metabolites.
- ✓ Pharmaceutical analysis: Drug discovery, pharmacokinetics, impurity profiling.
- ✓ Clinical diagnostics: Newborn screening, therapeutic drug monitoring.
- ✓ Environmental analysis: Detection of pollutants and pesticides.
- ✓ Forensic science: Drug testing, explosives analysis.

Chapter Summary

Spectroscopy is the study of the interaction between electromagnetic radiation and matter.

Atomic Spectroscopy focuses on free atoms in the gas phase and includes:

Atomic Absorption Spectroscopy (AAS): Measures light absorbed by ground-state atoms at characteristic wavelengths. The absorbance is proportional to concentration.

Atomic Emission Spectroscopy (AES): Measures light emitted by excited atoms as they return to the ground state. The emission intensity is proportional to concentration.

UV-Visible Spectroscopy investigates electronic transitions in molecules. **Chromophores** (e.g., carbonyl groups, aromatic rings) absorb UV-Vis light.The **Beer-Lambert Law** ($A=\varepsilon \cdot l \cdot C$) relates absorbance to concentration.

Fluorescence Spectroscopy is based on the emission of light by a molecule after excitation Fluorescence intensity is proportional to concentration in dilute solutions.

The Stokes' shift: Emitted light is of longer wavelength than absorbed light.

The Jablonski diagram illustrates radiative (fluorescence, phosphorescence) and non-radiative transitions.

Infrared (IR) Spectroscopy probes molecular vibrations. **Stretching** (symmetric and asymmetric) and **bending** (scissoring, rocking, wagging, twisting) vibrations.

A vibration is IR-active if it involves a change in dipole moment. Vibrational frequencies are influenced by factors such as electron-withdrawing groups, conjugation, hydrogen bonding, and hybridization. IR spectra are divided into two regions:

Functional group region (4000–1500 cm^{-1}): For identifying functional groups.

Fingerprint region (1500–400 cm^{-1}): Unique pattern for each molecule, used for definitive identification.

Nuclear Magnetic Resonance (NMR) Spectroscopy exploits the magnetic properties of certain nuclei (e.g., ^{1}H, ^{13}C). In a strong magnetic field, nuclei align with or against the field. Radiofrequency pulses cause **resonance** (spin flip).

Chemical shift (δ) reflects the electronic environment of a nucleus. Shielded nuclei appear upfield (lower ppm), deshielded nuclei downfield (higher ppm).

Spin-spin coupling (J-coupling) causes signal splitting into multiple peaks. The **n+1 rule** predicts multiplicity based on the number of equivalent neighboring protons.

The area under a signal (**integration**) is proportional to the number of protons giving rise to that signal.

Mass Spectrometry (MS) measures the mass-to-charge ratio (m/z) of ions. Samples are ionized then pass through a mass analyzer to detector.

Ionization techniques

Electron Ionization (EI): Hard ionization, extensive fragmentation, useful for structural determination.

Chemical Ionization (CI): Soft ionization, little fragmentation, provides molecular weight information.

Electrospray Ionization (ESI): Soft ionization, produces multiply charged ions, ideal for large biomolecules.

Matrix-Assisted Laser Desorption/Ionization (MALDI): Soft ionization, produces mostly singly charged ions, used for large biomolecules and polymers.

Mass analyzers:

Magnetic sector: Ions are deflected by a magnetic field; separation based on m/z.

Time-of-Flight (TOF): Ions are accelerated and their flight time to the detector is measured; lighter ions arrive faster.

Quadrupole: Uses oscillating electric fields to filter ions based on m/z; robust and fast.

Mass spectrum interpretation:

Molecular ion (M^{+}•) gives the molecular weight.
Fragmentation patterns provide structural information.

Isotopic peaks (M+1, M+2) are characteristic of elements like C, Cl, Br, and S.

Review Questions

1. Explain the difference between atomic absorption spectroscopy (AAS) and atomic emission spectroscopy (AES). What type of information does each technique provide?
2. You need to measure the absorbance of a protein sample at 280 nm. What type of cuvette material would you choose? Justify your answer.
3. What is the Stokes' shift in fluorescence spectroscopy? Why does it occur?
4. Sketch a simple Jablonski diagram and use it to explain the difference between fluorescence and phosphorescence.
5. What type of molecular vibration is IR spectroscopy used to study? Differentiate between stretching and bending vibrations.
6. What are the two main regions of an IR spectrum? What kind of information is obtained from each region?
7. Why is an IR spectrum sometimes referred to as a molecular "fingerprint"?
8. A proton in an organic molecule has three equivalent neighboring protons. Into how many peaks will its NMR signal be split? What is this pattern called?
9. What information does the integration of an NMR signal provide?
10. Describe the basic principle of mass spectrometry. What are the three main components of a mass spectrometer?
11. Explain what is meant by the molecular ion ($M^{+}\bullet$) in a mass spectrum. What information can be obtained from it?
12. Why does a compound containing a bromine atom show two peaks of nearly equal intensity in its mass spectrum?
13. You are analyzing a protein sample. Which ionization technique and mass analyzer would be most suitable? Justify your choice.

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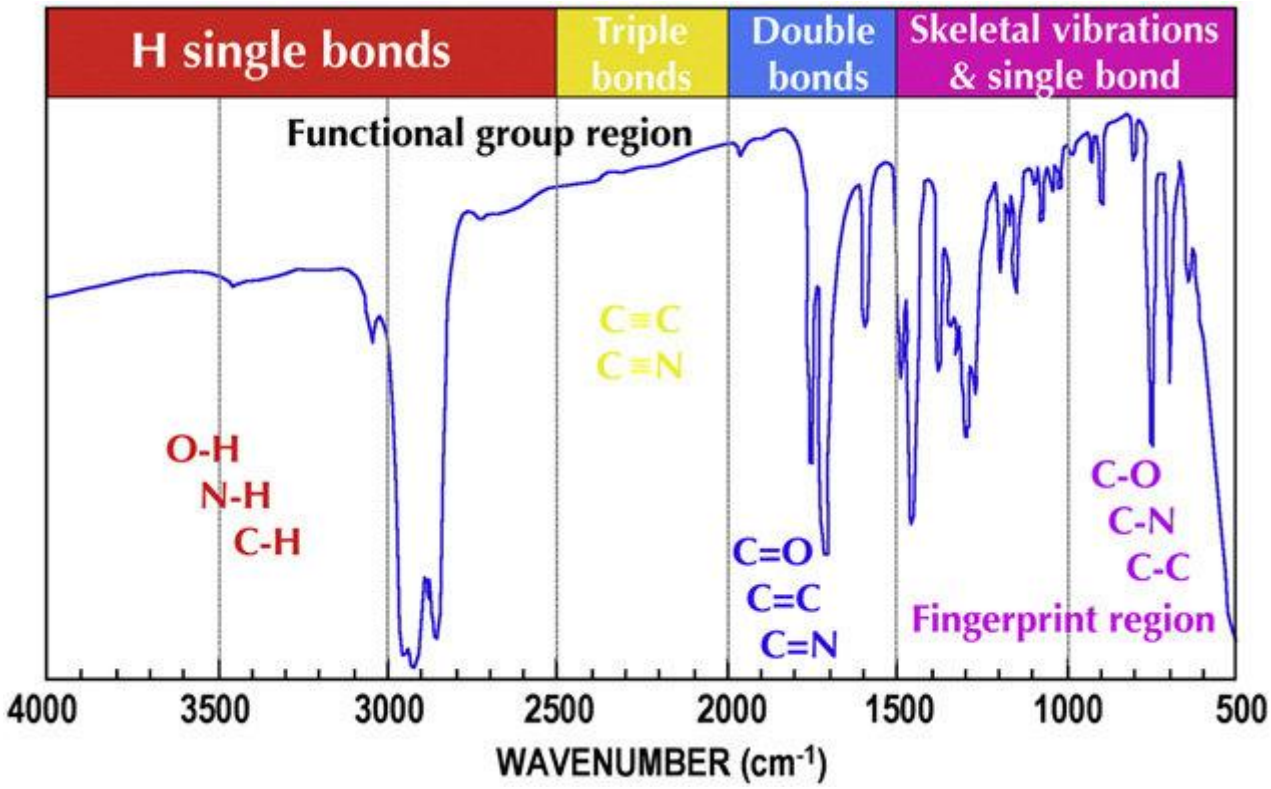
Appendix 01: Functional group and finger print regions in IR spectrum (Marcelli et al., 2012)



A typical mid-IR transmission spectrum showing in a schematic way typical absorptions lines associated to vibrational modes of molecules, organic components and others contributions of biological and non-biological nature.

Reference:

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