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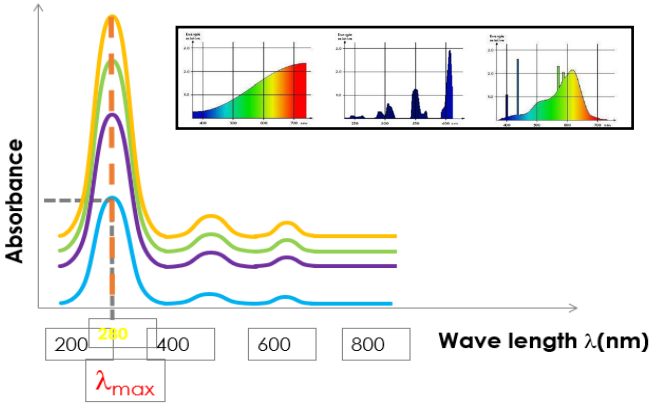
Biochemical Analysis Techniques I



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Summary

List of Figures

1. Solution and buffer

A solution (real solution) is a homogeneous combination of at least two components: a solute, which can be either a solid (salts, such as sodium chloride (NaCl), calcium chloride (CaCl2), liquid (alcohols, such as ethanol, ethanol...), gas (CO2, O2), or liquid and a solvent, which can be a liquid. The expansion, which takes place at the molecular or ionic level, produces homogenous products over the whole solution. The material that is present in the largest amount is the solvent (dispersion liquid for other chemical compounds). Any material added to the solvent and "dissolved" within is referred to as the solute.

Chemical solutions play a crucial role in biological analysis because they are used to prepare and manipulate biological samples for analysis. Here are some of the important uses of chemical solutions in biological analysis:

- Extraction of biomolecules: Chemical solutions such as buffers, acids, and bases are used to extract and isolate biomolecules such as DNA, RNA, and proteins from biological samples.
- Separation of biomolecules: Chemical solutions such as gels, chromatography columns, and electrophoresis buffers are used to separate and purify biomolecules based on their size, charge, or other physical properties.
- Detection of biomolecules: Chemical solutions such as reagents and stains are used to detect and quantify biomolecules in biological samples. For example, a colorimetric assay can be used to measure the concentration of a specific protein in a sample.
- Preservation of samples: Chemical solutions such as fixatives and preservatives are used to preserve biological samples for analysis. For example, formalin is used to fix tissues and prevent degradation of proteins and nucleic acids.

Overall, chemical solutions are essential tools in biological analysis because they enable researchers to prepare, manipulate, and analyse biological samples in a controlled manner.

- A true solution is a homogeneous mixture of at least two constituents: a solute and a solvent
  - A suspension is an heterogeneous liquid system = substance is not soluble in this liquid. Exp: sand +H2O
  - An emulsion is a mixture of two immiscible liquid substances such as water and oil

1.1. Solvent

A solvent is a substance that may combine with other chemicals to generate a homogenous solution. Thus, solvents can be used to extract (for the chemical, petrochemical, pharmaceutical, and food sectors), dissolve (for degreasing), suspend (for paintings), or change the physical characteristics of a substance. A solvent is a liquid that has the ability to dissolve and dilute other substances without chemically altering either them or the solvent itself. It is a liquid that enables the creation of a homogenous liquid phase after the addition of chemicals. Solvents that are organic molecules with carbon atoms are referred to as organic solvents. An organic solvent, according to Cohr, is a chemical substance or combination that is volatile,

liquid between 0°C and 200°C, and largely chemically inert. The most typical example of this is when you make tea in hot water (water is the most typical solvent), which is another way that solvents may be used to separate soluble chemicals from a mixture. One chemical species that makes up a liquid uniform phase solution is referred to as the "solvent" if it makes up a very significant majority of the solution—at least by a factor of 100. This is true of water in aqueous solutions, such as copper sulfate, where water serves as the solvent and the solutes, copper ions and sulfate, are the solutes.

1.1.1. Types of solvents

Organic solvents are classified into three families:

1.1.1.1. Hydrocarbon solvents

The category of organic solvents most frequently mentioned is that of hydrocarbons. The only two elements in the molecular structure of the solvents in this category are carbon and hydrogen. There are complicated petroleum mixes, aliphatic hydrocarbons, and aromatic hydrocarbons.

Aliphatic hydrocarbons (alkanes, alkene)

Saturated aliphatic hydrocarbons (alkanes), which only have single bonds, are distinguished from unsaturated hydrocarbons, which have at least one double bond. The formula for saturated compounds is C_nH2_{n+2}, although only molecules with five carbon atoms or more are liquid solvents at room temperature. Both branched (iso-pentane) and linear (nhexane) carbon chains are possible. Except for a few natural chemicals like terpenes, unsaturated molecules (alkenes) are less useful as solvents. Particularly in adhesives (such as hexane), aliphatic solvents are employed.

Aromatic hydrocarbons (benzene, toluene, xylene)

Aromatic hydrocarbons are naturally occurring compounds with a cyclic structure called an aromatic ring. These rings, in turn, are formed by bonded carbon atoms in symmetrical single- and double-bonded structures. The most common and well-known aromatic hydrocarbon is the six-carbon ring of benzene. Aromatic hydrocarbons exhibit unique chemical properties, including high stability and characteristic odors (smell). It is widely used in various industries such as petrochemicals, pharmaceuticals and plastics.

1.1.1.2. Oxygenated solvents

Oxygenated solvents, also known as oxygenated solvents, are a class of organic solvents that contain an oxygen atom in their molecular structure These solvents are derived from various oxygen-containing compounds such as alcohols, ketones, esters, ethers, and glycol ethers. The presence of oxygen in these compounds results in different chemical compositions compared to hydrocarbon-based compounds. Oxygenated dyes are commonly used in a wide variety of applications, including pigments and coatings, detergents, pharmaceuticals and specialty

chemicals Considered is valued for its cleaning properties, less toxicity than some other chemicals and relatively low environmental impact.

A) Alcohol

Alcoholic solvent refers to a solvent that is based on alcohol or contains alcohol as a major component. Alcohols are organic compounds that have a hydroxyl (-OH) functional group attached to their carbon atoms. Common examples of alcoholic chemicals are methanol (methyl alcohol), ethanol (ethyl alcohol), isopropanol (isopropyl alcohol), and butanol (butyl alcohol). Because of its ability to disperse a wide range of polar and non-polar materials, solvents are widely used in a variety of industries It is particularly effective for water-soluble materials, certain resins, greases and oils and polar compounds. Alcoholic solvents are used in pharmaceuticals, paints, coatings, personal care products, detergents, and many other industries. Furthermore, alcohol is relatively less toxic compared to some other chemicals, widely compatible with many substances, easily flammable but careful handling of alcohol is essential as it is flammable, and vapors can be harmful if inhaled in high concentrations, proper ventilation, safety It should also be followed with caution.

B) Glycols

Glycol solvent refers to a compound that has glycol as its main component. Glycols are organic compounds with two hydroxyl (-OH) functional groups attached to different carbon atoms. One of the most commonly used glycol solvents is ethylene glycol. Glycol solvents are valued for their ability to dissolve a wide range of substances, especially polar compounds. It is generally used where water mixing or low viscosity is desired. Glycol solvents are commonly used in industries such as paint coatings, printing inks, pigments, cleaners, antifreeze and formulas. Glycol solvents have many advantageous properties, such as low toxicity, high boiling point, ability to reduce the viscosity of water in solution and also exhibit good stability and compatibility with many materials. However, it is important to note that some glycol formulations can be harmful if ingested or in contact with the skin, so proper safety precautions should be followed when handling them.

C) Ketones

Ketone solvent refers to a solvent based on or containing ketones as a major component. Ketones are organic compounds, characterized by a carbonyl group (C=O) bound to two carbon atoms in a molecule. Common examples of ketone compounds are acetone (propanone), methyl-ethyl ketone (MEK), and cyclohexanone. Ketone solvents are widely used in a variety of industries and applications. They are valued for their ability to deposit a wide range of materials, including polar and non-polar materials. Ketone compounds are commonly used in paints and coatings,

adhesives, detergents, and as industrial chemical coatings. Due to their dissolving properties, they can also act as effective lubricants. Ketone solvents exhibit desirable properties such as low boiling points, good evaporation rate and low viscosity. They are usually sparkling water with little odour. However, it is important to handle ketone solvents with caution as they can be flammable and should be used in well-ventilated areas. Some ketone compounds, such as acetone, can also be absorbed by the skin and appropriate precautions may be needed to ensure safe use.

D) Esters

A chemical compound known as an ester solvent is composed of an acid with at least one hydroxyl group (-OH) substituted by a -O-alkyl (alkoxy) group. Strong hydrogen bonding and polarity are characteristics of ester solvents, which enable them to dissolve a variety of organic molecules, including living species or surroundings that may function as corrosion attack facilitators on metallic substrates. Ester solvents are also employed in situations when it is necessary to clean up petroleum spills from water bodies. They are an eco-friendly substitute for volatile, strictly regulated solvents for cleaning impurities from metallic items since they are non-toxic, non-volatile, and biodegradable.

E) Glycol ether

Glycol ether solvent is a type of organic solvent that contains both ether and alcohol groups in its structure. It is synthesized from glycols such as ethylene glycol or propylene glycol by reaction with alcohol. Glycol ether solvents are good solvents for many applications such as resins, oils, waxes, and dyes. They, too, have a lower boiling point and less toxicity. There are basically two types of glycol ether compounds: E-series, based on ethylene oxide, P-series, based on propylene oxide

1.1.1.3. Halogenated solvent

Halogenated compounds are organic chemical compounds that contain one or more halogen atoms such as chlorine, fluorine, bromine, or iodine. Halogenated solvents are used for a variety of purposes including dry cleaning, metal cleaning, degreasing, refrigeration and thermoplastic manufacturing. However, halogenated compounds can have adverse effects on human health and the environment. Some can be damaged by anger, depression, cancer, or heart disease. Some cause ozone depletion or pollute soil and water. Halogenated solvents should therefore be handled with care and properly disposed of. Chlorinated solvents are the most popular, followed by fluorinated ones. The majority of halogenated solvents come from aliphatic hydrocarbons. Apart from the smallest molecules which are gaseous (chloromethane, dichloromethane, chloroethane, chloroethylene, bromomethane), all commonly used halogenated

derivatives are colorless liquids. Chlorinated solvents are not or only slightly flammable, as are fluorinated derivatives. They all have higher boiling points and densities than the corresponding hydrocarbons. They are less volatile and insoluble in water but are excellent solvents for many synthetic polymers, mineral oils and greases. Halogenated solvents are widely used in vapor degreasing of metal surfaces (trichloroethylene). Other applications include paint stripping (dichloromethane) and dry cleaning (perchlorethylene).

1.1.1.4. Other classification

According to their molecular structure, solvents are also classified into:

A) Polar protic solvents (protogenic solvents):

having one or more hydrogen atoms capable of forming hydrogen bonds. For example, water, methanol, ethanol, etc.

B) Polar aprotic solvents:

having a non-zero dipole moment and devoid of hydrogen atoms capable of forming hydrogen bonds. For example, acetonitrile (CH3CN), dimethyl sulfoxide (DMSO, (CH3)2SO), tetrahydrofuran (THF, C4H8O), etc.

C) Non-polar aprotic solvents:

having a zero permanent dipole moment. For example, benzene, hydrocarbons: linear or branched alkanes, cyclic alkanes, alkenes, etc.

1.1.2. Physical and chemical attributes

Solvents are frequently clear liquids with a distinct smell. Water can dissolve some organic solvents. Others do not mix but instead create a distinct layer with a distinct border. Generally speaking, certain solvents have a low melting point, evaporate rapidly, and boil at low temperatures, while others do so more slowly and boil at high temperatures. Few organic solvents are denser than water, while the majority are less dense than water. The majority of solvents, with the exception of halogenated ones, are lighter than water.

Table 1. Common solvent used in laboratory

Solvent	Formula	MW	Boiling Point (°C)	melting point (°C)	density (g/mL)	Solubility[2]	Dielectric Constant[3]	flash point (°C)
acetic acid	$C_2H_4O_2$	60.052	118	16.6	1.0446	Miscible	6.20	39
acetone	C_3H_6O	58.079	56.05	-94.7	0.7845	Miscible	21.01	-20
acetonitrile	C_2H_3N	41.052	81.65	-43.8	0.7857	Miscible	36.64	6
benzene	C_6H_6	78.11	80.1	5.5	0.8765	0.18	2.28	-11
1-butanol	$C_4H_{10}O$	74.12	117.7	-88.6	0.8095	6.3	17.8	37
2-butanol	$C_4H_{10}O$	74.12	99.5	-88.5	0.8063	15	17.26	24
2-butanone	C_4H_8O	72.11	79.6	-86.6	0.7999	25.6	18.6	-9
t-butyl alcohol	$C_4H_{10}O$	74.12	82.4	25.7	0.7887	Miscible	12.5	11
carbon tetrachloride	CCl_4	153.82	76.8	-22.6	1.594	0.08	2.24	--
chlorobenzene	C_6H_5Cl	112.56	131.7	-45.3	1.1058	0.05	5.69	28
chloroform	$CHCl_3$	119.38	61.2	-63.4	1.4788	0.795	4.81	--
cyclohexane	C_6H_{12}	84.16	80.7	6.6	0.7739	0.0055	2.02	-20
1,2-dichloroethane	$C_2H_4Cl_2$	98.96	83.5	-35.7	1.245	0.861	10.42	13
diethylene glycol	$C_4H_{10}O_3$	106.12	246	-10	1.1197	10	31.8	124
diethyl ether	$C_4H_{10}O$	74.12	34.5	-116.2	0.713	7.5	4.267	-45
diglyme (diethylene glycol dimethyl ether)	$C_6H_{14}O_3$	134.17	162	-68	0.943	Miscible	7.23	67
1,2-dimethoxy-ethane (glyme, DME)	$C_4H_{10}O_2$	90.12	84.5	-69.2	0.8637	Miscible	7.3	-2
dimethyl-formamide (DMF)	C_3H_7NO	73.09	153	-60.48	0.9445	Miscible	38.25	58
dimethyl sulfoxide (DMSO)	C_2H_6OS	78.13	189	18.4	1.092	Miscible	47	95
1,4-dioxane	$C_4H_8O_2$	88.11	101.1	11.8	1.033	Miscible	2.21(25)	12
ethanol	C_2H_6O	46.07	78.5	-114.1	0.789	Miscible	24.6	13
ethyl acetate	$C_4H_8O_2$	88.11	77	-83.6	0.895	8.7	6(25)	-4
ethylene glycol	$C_2H_6O_2$	62.07	195	-13	1.115	Miscible	37.7	111
glycerin	$C_3H_8O_3$	92.09	290	17.8	1.261	Miscible	42.5	160
heptane	C_7H_{16}	100.20	98	-90.6	0.684	0.01	1.92	-4
hexamethylphosphoramide (HMPA)	$C_6H_{18}N_3OP$	179.20	232.5	7.2	1.03	Miscible	31.3	105
hexamethylphosphorous triamide (HMPT)	$C_6H_{18}N_3P$	163.20	150	-44	0.898	Miscible	??	26
hexane	C_6H_{14}	86.18	69	-95	0.659	0.0014	1.89	-22
methanol	CH_4O	32.04	64.6	-98	0.791	Miscible	32.6(25)	12
methyl *t*-butyl ether (MTBE)	$C_5H_{12}O$	88.15	55.2	-109	0.741	5.1	??	-28
methylene chloride	CH_2Cl_2	84.93	39.8	-96.7	1.326	1.32	9.08	--

N-methyl-2-pyrrolidinone (NMP)	CH_5H_9NO	99.13	202	-24	1.033	Miscible	32	91
nitromethane	CH_3NO_2	61.04	101.2	-29	1.382	9.50	35.9	35
pentane	C_5H_{12}	72.15	36.1	-129.7	0.626	0.04	1.84	-49
petroleum ether (ligroine)	--	--	30-60	-40	0.656	--	--	-30
1-propanol	C_3H_8O	60.10	97	-126	0.803	Miscible	20.1(25)	22
2-propanol	C_3H_8O	60.10	82.4	-88.5	0.785	Miscible	18.3(25)	12
pyridine	C_5H_5N	79.10	115.2	-41.6	0.982	Miscible	12.3(25)	17
tetrahydrofuran (THF)	C_4H_8O	72.106	65	-108.4	0.8833	soluble[2]	7.52	-14
toluene	C_7H_8	92.14	110.6	-93	0.867	0.05	2.38(25)	4
triethyl amine	$C_6H_{15}N$	101.19	88.9	-114.7	0.728	0.02	2.4	-11
water	H_2O	18.02	100.00	0.00	0.998	--	78.54	--
water, heavy	D_2O	20.03	101.3	4	1.107	Miscible	??	--
o-xylene	C_8H_{10}	106.17	144	-25.2	0.897	Insoluble	2.57	32
m-xylene	C_8H_{10}	106.17	139.1	-47.8	0.868	Insoluble	2.37	27
p-xylene	C_8H_{10}	106.17	138.4	13.3	0.861	Insoluble	2.27	27

1.2. Concept of concentration

When a substance (solute) is put into solution, the concentration of this substance can be expressed in various ways:

1.2.1. Concentration by weight per unit volume (weight)

It is defined in grams per litre of solution (g/l) exp. NaCl solution at 9g/l: 9g of NaCl was dissolved in water and then made up to the volume of 1 litre.

1.2.2. Percentage concentration (%)

It is defined as the number of grams of solute/100ml of solution. exp: 9% NaCl solution: 9 g of NaCl in 100ml of solution

1.2.3. Molar concentration (molarity)

Molar concentration is the most commonly used concentration scale. It translates the number of molecules grams or moles (n) in a volume V of 1 litre of solution: M=n/V.

1.3. Dilutions

1.3.1. Simple dilution

Diluting a solution means making it less concentrated (C2), so it involves pouring a certain quantity (an initial volume V1) of a concentrated solution (C1) and adding a certain quantity of solvent until the final volume is reached (V2). The equation which connects these terms is C1.V1=C2.V2 (Figure 1).

- C1: is the concentration of the concentrated solution (also called the stock solution, mother solution or initial solution).
- V1: is the volume taken from the concentrated solution.
- C2: is the concentration of the diluted solution (also called the daughter solution or final solution).
- V2: is the volume of the diluted solution.

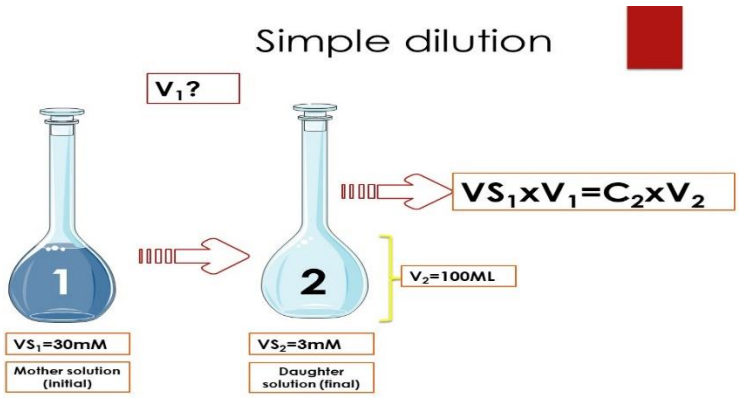


Figure. 1 : A simple dilution.

1.3.2. Successive dilution

To avoid preparing a stock solution containing only a few milligrams of solute per liter, or measuring tiny volumes of stock solution, we proceed by successive dilutions (Figure 2).

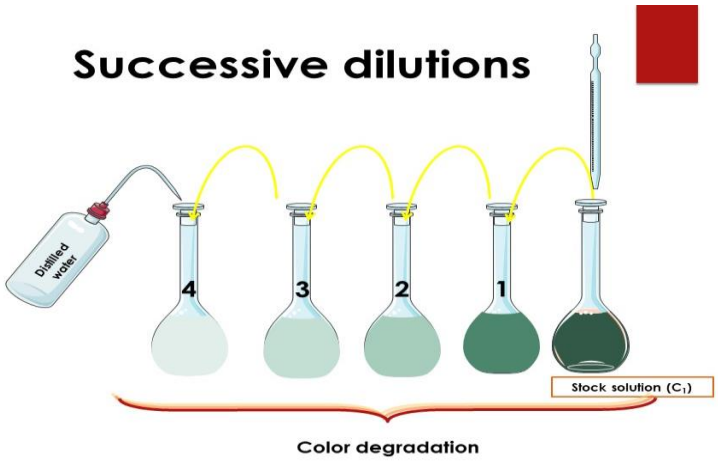


Figure. 2 : A successive dilutions.

1.3.3. The buffer solution

A buffer solution is a solution that resists change in pH upon the addition of a strong acid or strong base. This solution is prepared by two methods:

- Method 1: mixture of a weak acid and its conjugate base (ex: phosphate buffer)
- Method 2: mixture of a weak base and a little strong acid (eg: Tris-HCl buffer) or a weak acid and a little strong base.

2. Spectrometry

Electromagnetic radiation is in the form of an electromagnetic wave that propagates in a vacuum at the same speed as that of light. Only part of these waves is visible in the form of light. All of these waves, visible or not, form what we call the electromagnetic spectrum. The electromagnetic spectrum (Figure 3) extends from lower energy (or longer wavelength) radio frequencies to higher energy (or shorter wavelength) Gamma radiation. Electromagnetic radiation in the UV-VIS range is expressed by its wavelength λ, in nm. The UV range extends from 160 to 390 nm, the visible range from 390 to 770 nm (from blue to red) and the infrared (IR) range from 780 nm to 1mm.

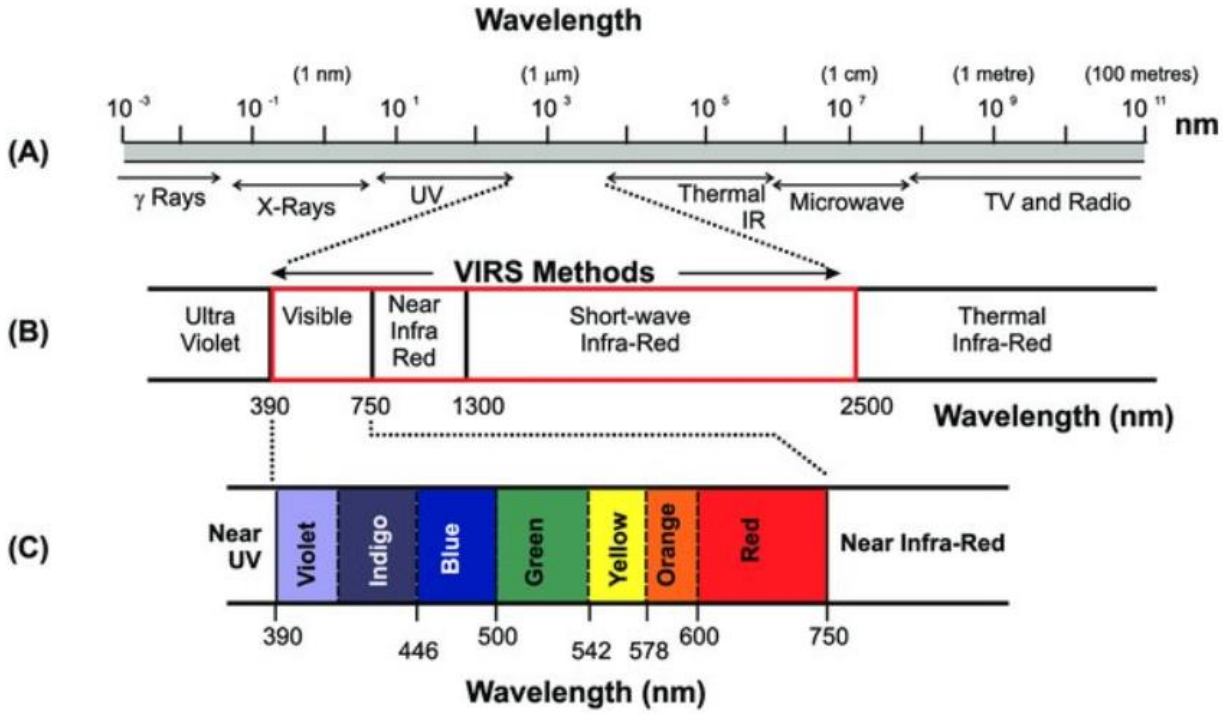


Figure. 3 : The electromagnetic spectrum.

Each of the particular areas of electromagnetic radiation corresponds to a type of spectroscopy which is based on a particular interaction of matter with this radiation. For the domain:

- From γ and RX, the radiation is extremely energetic and it will be able to affect the electrons of the atomic orbitals of the nucleus.
- UV and visible radiation is energetic and can affect electrons in peripheral atomic orbitals and/or molecular orbitals. These interactions are used in particular in atomic emission spectroscopy (SEA), atomic absorption spectroscopy (SAA) and molecular spectroscopy (UV-VIS).
- From IR, radiation is low energetic and can mainly only affect the vibration modes of molecules. These interactions are used in particular in IR spectroscopy and Raman spectroscopy.

- Radio, finally, the radiation is very low in energy and can only affect the rotation modes of molecules, modification of electronic spin states (electronic paramagnetic resonance, EPR) and modification of nuclear spin states (nuclear magnetic resonance, NMR).

2.1. Spectrum, spectroscopy and spectrometry

Spectroscopy is the study of the electromagnetic spectrum of a phenomenon, visually (hence the suffix -scopy). For some time now, the eye has been replaced by different types of photoelectric detectors, and it is then appropriate to speak of spectrometry (the suffix-metry indicating that we are carrying out a measurement and not a simple assessment of the phenomenon). It would be appropriate to replace the term "spectroscopy" with the more accurate term "spectrometry".

The spectrum is the distribution of energy, power, intensity, absorbance, transmission, etc. depending on wavelength or frequency. There are three types of spectra:

- Continuous spectra, for which there is a "signal" for each wavelength or frequency (figure 4).
- Discontinuous spectra, or line spectra, or even discrete spectra, which only have a signal for certain specific frequencies or wavelengths, characteristic of the irradiating or irradiated material (Figure 4b).
- The combined spectra which consist of a superposition of a continuous spectrum and a discrete spectrum (Figure 4c).

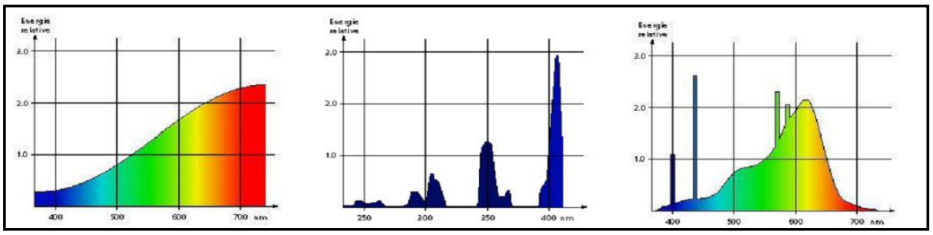


Figure. 4 : Different types of spectra. a: continuous spectrum, b: discontinuous spectrum, c: combined spectrum.

2.2. Absorption and emission

Energy exchanges between atoms and light are quantified: they take place in packets of energy called photons (Figure 5).

a. Absorption

An atom initially at rest at the fundamental energy level Ef can move to the higher excited energy level Ee, by absorbing energy $\Delta E = h.\nu = Ee-Ef$ (Planck's constant h = 6.63.10-34 Js, V is the frequency of the electromagnetic wave associated with the photon.

b. Spontaneous emission

Excited electronic states are not stable. More or less quickly, the atom falls back into the ground state by emitting a photon. The photon can be emitted in any direction. The energy hv of the emitted photon is equal to the energy difference Ee – Ef between the two atomic levels (e) and (f).

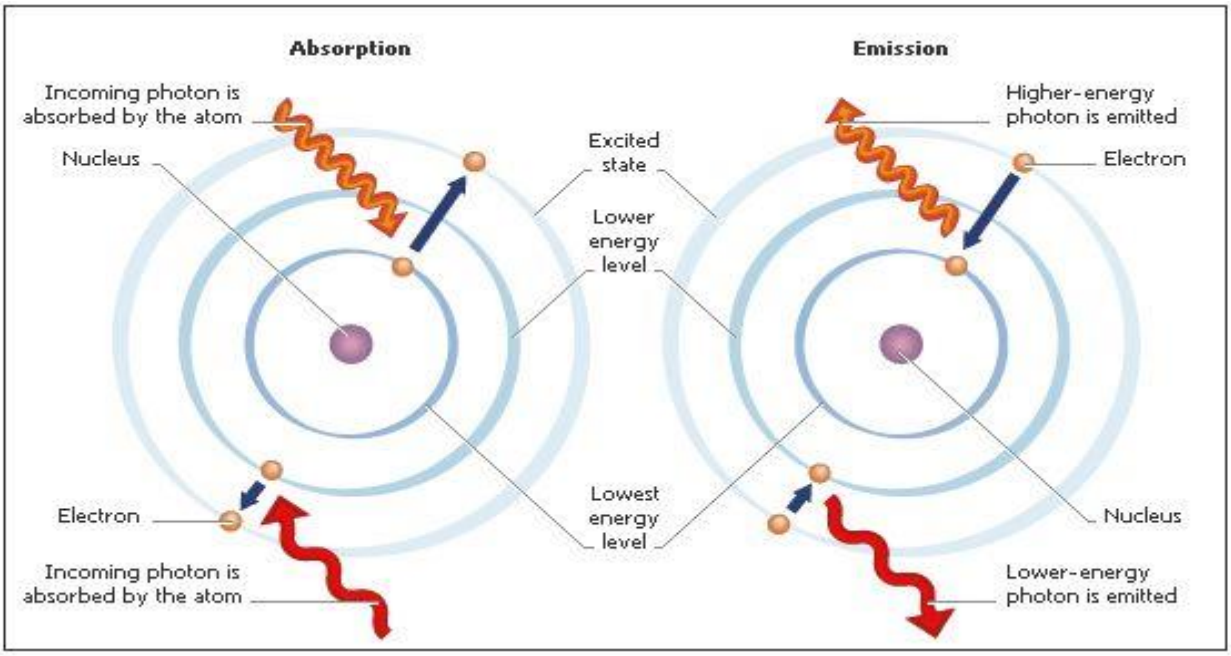


Figure. 5 : Absorption and emission.

2.3. Uv-VIs spectrometry

UV-VIS spectrophotometry relies on the interaction of electromagnetic radiation and matter. The absorbance of the compounds in the UV and visible is used in quantitative analysis by application of the Beer-Lambert law.

2.3.1. Principle

This type of spectrometry is mainly based on interactions between electromagnetic radiation and electrons in molecular orbitals. The absorption of radiation by molecules is due to the passage of an electron from one electronic level to another electronic level of higher energy.

If we place a solution containing a substance dissolved in a solvent, in a spectrophotometer. And we measure the absorbance at different wavelengths, we will obtain a graph of the peaks at defined wavelengths: this is the absorption spectrum. The absorbance maximum corresponds to λ max (Figure 6).

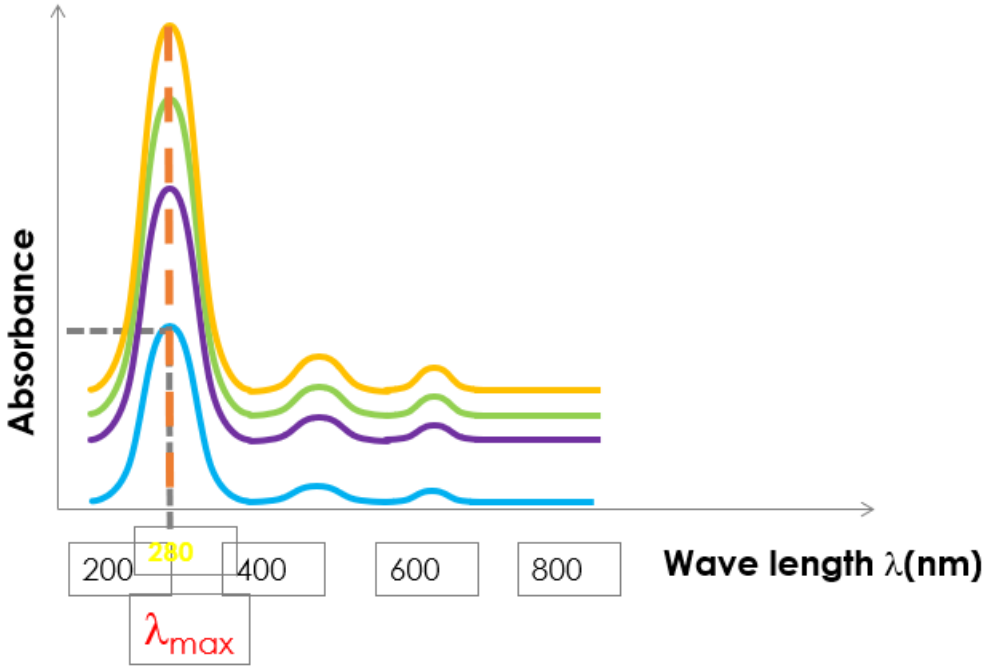


Figure. 6 : The absorption spectrum

The absorption of ultraviolet or visible radiation corresponds to an interaction of photons with electrons in the outer layers of atoms or molecules. The position of the absorption maximum corresponds to the wavelength of the radiation which causes the electronic transition.

2.3.2. The Beer-Lambert law

UV-VIS spectra give the transmittance or absorbance of the sample analyzed as a function of the wavelength of the radiation. The transmittance, denoted T, is given by:

$T = I/I_0$; 100

where I0 is the incident intensity and I, the transmitted intensity.

- If: I0= I, transparent medium
- If:I0> I, partially absorbent medium
- If: I= 0, opaque medium (total absorption)

The absorbance A is defined by: $A = -\log T$

This last quantity is very useful in quantitative analysis by application of the Beer-Lambert law which we will see later. The more absorbent a compound, the lower the transmittance and the higher the absorbance.

Consider a monochromatic light passing through an absorbing solution of concentration C contained in a tank of thickness l (Figure 7). Part of this radiation will be absorbed by the sample and part will be transmitted. Bouguer, Lambert and Beer studied the relationships that exist between I0 and I: the intensity of a monochromatic light passing through a medium where it is absorbed decreases exponentially: $I = I_0\, e^{-klC}$

Or' :

- l: the distance crossed by the light (thickness of the tank) (in cm)

- C: the concentration of absorbent species
- k: a characteristic constant of the sample.

This equation can be rewritten as log (I0/I) = klC/2.3 = ε l C. ε: is the molar extinction coefficient; it is a characteristic of the substance studied at a given wavelength. If C is the molarity, ε is in M-1.cm-1. We then obtain the relationship known as the Beer-Lambert law: $.A = -\log T = \varepsilon lc$

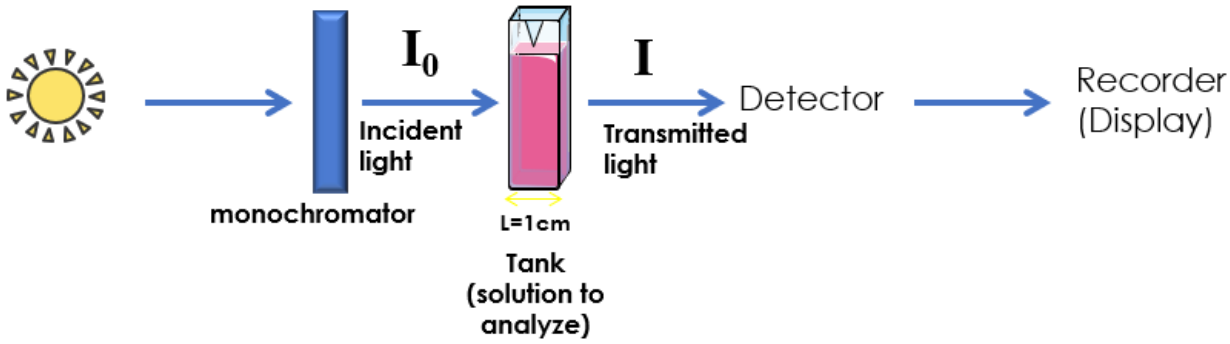


Figure. 7 : The absorbance of monochromatic light by a substance

The conditions of validity of the Beer-Lambert law:

- Monochromatism (a single wavelength λ max)
- Low concentrations (diluted)
- Stable temperature (depends on temperature)
- Ph stable
- Clarity of the middle

2.3.3. Origin of absorption

Radiation causes electronic transitions between different energy levels of molecules. Electronic transitions correspond to the passage of electrons from filled bonding and non-bonding molecular orbitals to unfilled anti-bonding molecular orbitals. The absorption wavelength depends on the nature of the orbitals involved. the electronic transitions generally observed in spectrophotometry: π π* and n π*. The following diagram illustrates this for orbitals of type σ, π and n (Figure 8):

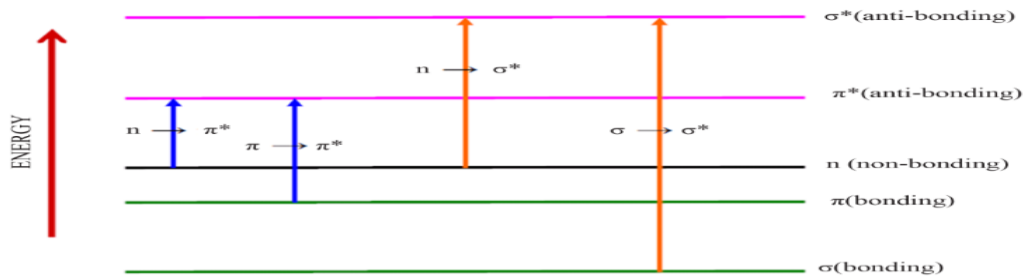


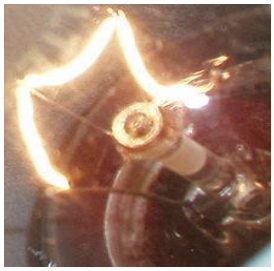
Figure. 8 : Diagram illustrates electromagnetic transitions.

2.3.4. Materials

2.3.5. The light source:

the most used sources are:

- **The tungsten filament lamp**in a halogen atmosphere (iodine lamp) (Figure 9). It provides a continuous lamp spectrum usable between 350 and 700 nm (visible).
- **The arc lamp in a hydrogen or deuterium atmosphere** (Figure 9), called a hydrogen or deuterium lamp. It provides a continuous lamp spectrum usable between 200 and 360 nm. Thus many spectrophotometers combine deuterium lamp and iodine lamp to cover VIS and UV.
- **The arc lamp in a xenon atmosphere** (Figure 9). It provides a continuous lamp spectrum usable between 260 and 800 nm. It is a very popular lamp currently.Due to their broad spectrum (covering the UV and visible range) and their long lifespan.



Tungsten filament lamp in a halogen atmosphere.

Arc lamp in a deuterium a.

Arc lamp in xenon atmosphere.

Figure. 9 : Light sources.

2.3.6. Monochromators

The selection of the wavelength is carried out using the monochromator. It typically includes:

- An entry slot allowing access by a fine polychromatic beam.
- A dispersive network type system.
- An output slot, which, through precise motorization, will make it possible to select a precise wavelength (in fact a narrow bandwidth) dispersed and direct it towards the sample holder.

2.3.7. Sample holder system

Conventionally we use "cuvettes" with parallel optical faces, transparent and determining an optical path of 1 cm. The material of the tank containing the sample is fundamental:

- Single-use polystyrene tanks can only be used in visible areas (Figure 10).
- Certain plastics allow the creation of single-use tanks that can be used in the more or less distant UV.
- Glass tanks, to be cleaned after use, can only be used in visible areas and are generally abandoned in favor of single-use plastic tanks. But it should be noted that plastic is sometimes incompatible with certain solvents (Figure 10).
- Quartz cells (high cost) allow measurements in the UV up to 200 nm and also the visible (Figure 10, C).

Figure. 10 : Different tanks. a: polystyrene tank, b: glass tank, c: quartz tank.

2.3.8. Detectors and amplifiers

The basic principle of the detector is to transform the received optical signal into electric current or voltage. It is therefore a matter of counting the photons received per wavelength using the photoelectric effect.

However, as the output signal remains very weak, it requires amplification by a factor of at least 10 to be readable. Due to this limitation, we quickly moved on to an internal amplification system, these are photomultipliers.

2.3.9. Single-beam spectrophotometers

There are two possibilities depending on whether you work with a monochromatic beam or not (Figure 11):

- Inverted assembly: Light source, Sample, Polychromator, polychromatic detector.
- Direct assembly: Light source, Monochromator, Sample, detector.

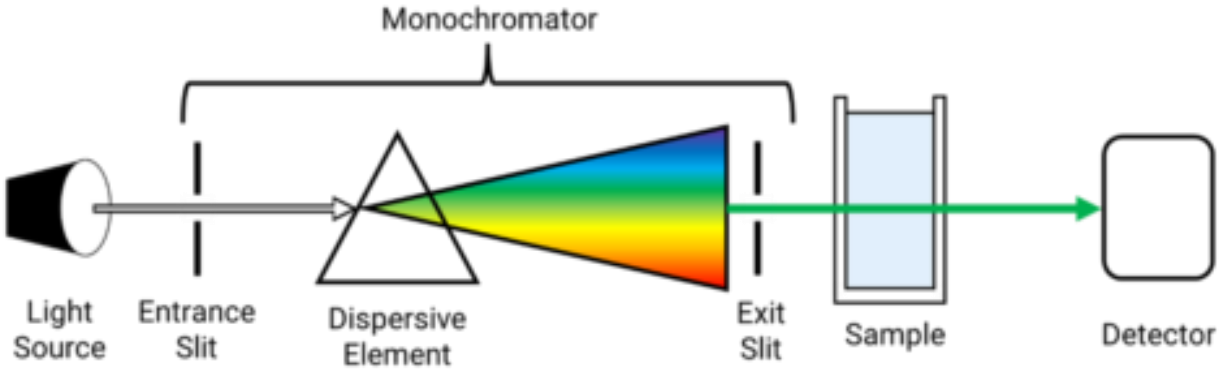


Figure. 11 : Single-beam spectrophotometer.

2.3.10. Double beam spectrophotometers

One beam passes through the sample compartment and the second the reference compartment (Figure 12). The subtraction of the blank is done automatically by data processing software.

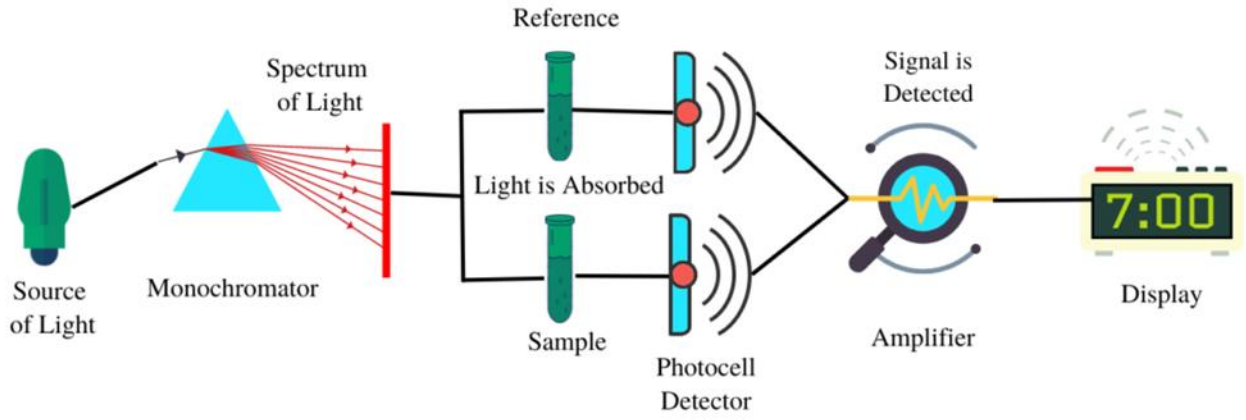


Figure. 12 : Double beam spectrophotometers.

2.3.11. Concept of white

When a chemical species is solubilized in a solvent and placed in a measuring cell, the absorption measured corresponds to the sum of three different absorptions:

- Absorption due to the cell which can be glass, quartz or polymer.
- Absorption due to the solvent.
- Absorption due to dissolved chemical species.

The first two absorptions are not due to the species analyzed. They must therefore be removed. To do this, we measure the absorbance of the cell with solvent and we subtract the absorbance thus obtained (the blank) from the absorbance measured with the species that we want to study. This is made possible by the additivity of the absorbance.

2.3.12. Applications of UV-VIS spectrometry

✓ **Quantitative analysis (determination of the concentration of an unknown substance):** UV-VIS spectrophotometry is used to measure the concentration of an unknown sample. To do this,

you must first choose your own absorption band. The example absorption spectrum of the sample may be available in the literature if it has already been studied. If not, double beam spectrophotometry must be performed to find out where its absorption band will lie. A suitable absorption band is selected. Two assay techniques are used in spectrophotometry:

- **the calibration range method**: The calibration range method consists of preparing solutions of known concentration and then the solution of unknown concentration. The absorbance measured for each of these solutions makes it possible to establish a graph where we draw A = f(C). All that remains is to place the absorbance of the unknown solution and by projection we obtain the unknown concentration (Figure 13).
- **The method of metered additions** consists of adding several times the same volume of a solution of known concentration to a given volume of unknown concentration. Graphical or mathematical work also based on the proportionality between A and C makes it possible to find the unknown concentration.

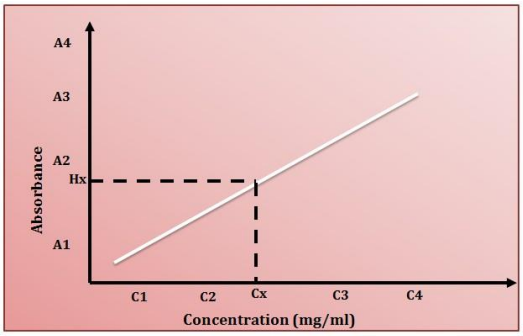


Figure. 13 : The calibration line.

- ✓ **Enzymatic assays**: the enzymatic activity can be easily and quickly calculated when the substrate or product absorbs light in the UV-Vis. In these cases, the rate of appearance or disappearance of a light-absorbing product or substrate can be measured using a spectrophotometer.
- ✓ **Turbidimetry:** any particles or even bacteria will make the solution cloudy. This is due to the Tyndall effect, which is caused by the scattering of light by particles. Turbidimetry is used in microbiology with the aim of carrying out a total bacterial count (both "dead" and living bacteria), by determining the biomass of a sample. The diffusion of light from the incident beam (of chosen wavelength) by bacteria (or other suspended bodies) in the medium is neglected. This is why we generally consider this measurement as a simple measurement of absorbance (although in reality it is a measurement of the degree of turbidity of the suspension). The measurement is therefore carried out with a “classic” spectrophotometer.

2.4. Atomic spectrometry

It is based on the property of atoms to emit or absorb electromagnetic radiation under certain physical conditions. It is one of the main techniques involving atomic spectroscopy in the UV-visible domain used in chemical analysis. It allows you to measure around sixty chemical elements (metals and non-metals). It is a very sensitive technique pg/L (ppm). Therefore, any atomic spectrochemical analysis requires evaporation and atomization of the sample. Two types of atomic spectroscopy are applied Atomic Absorption Spectrometry (AAS) and Atomic Emission Spectrometry (AES).

2.5. Atomic Emission Spectrometry (AES)

2.5.1. Principle

When a saline solution is heated in a flame at a constant flow rate, the water evaporates and the mineral salts remain in the state of atoms. The atoms are then brought to a high temperature and therefore capture part of the energy provided by the flame This puts them into an excited state. An excited atom necessarily returns to its basal state by releasing energy in the form of an emission of light. Each ion or atom is characterized by a specific wavelength. Exp: Na: yellow; Li: pink-red

$$\frac{Na^+}{Cl^-} \xrightarrow[\text{of solvent}]{\text{evaporation}} NaCl \xrightarrow{\text{atomization}} \frac{Na}{Cl} \xrightarrow[\text{of metal atoms}]{\text{excitation}} Na^* \xrightarrow[\text{ground state}]{\text{drop back to}} Na \xrightarrow[\text{emission}]{\text{light}}$$

Element	Emission Wavelength (nm)	Flame Colour
Sodium (Na)	589	Yellow
Potassium (K)	766	Violet
Barium (Ba)	554	Lime Green
Calcium (Ca)	622*	Orange
Lithium (Li)	670	Red

The intensity of the light is proportional to the concentration of the ion to be measured. Each element to be analyzed corresponds to a filter which will select the useful wavelength band It is then sufficient to establish a calibration curve: I=f(c). To determine the concentration of an unknown solution. For the simultaneous dosing of several elements, several filters and several detectors must be available at the same time.

2.5.2. Material

Flame photometer is composed of (Figure 14):

Nebulizer which allows you to create a “mist” with the solution to be analyzed

Burner: the gases used can be acetylene, butane or propane depending on the desired flame temperature

Filter or monochromator

Detector: photoelectric cell.

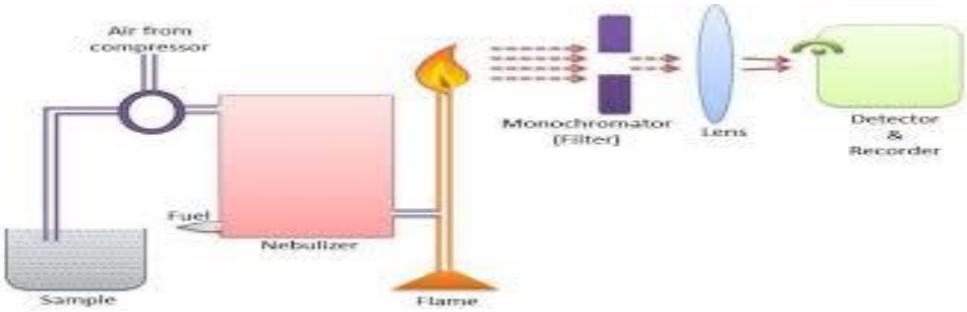


Figure. 14 : Atomic Emission Spectrometry.

2.5.3. Samples preparation

Samples must be in the form of an aqueous solution without debris or solid particles. The distilled water which is the solvent for our sample must be of very good quality in order to have precise results. Chemical species that can cause interference must be eliminated or present in the standard and the sample has the same concentration. Exp: elimination of calcium ions by precipitation with oxalic acid. Salts can be extracted from solid samples using distilled water or appropriate reagents. exp: Saturated CaSO4 for the extraction of sodium from the soil Using a grinder or agitator gives better results If the sample is of organic nature, the material organic must be eliminated by incineration (oven mitten). The oxides remaining below shape of ashes are solubilized next by strong acids.

2.5.4. Application

Dosages of elements alkaline or alkaline earth Na, K, Ca, Li, Fe, Ba... (which are in fact in ionized form Na^+, K^+, Ca^{2+}, Li^{2+}, Fr^{2+} with Ba^{2+}... in most samples:

- biological fluids such as blood plasma;
- pharmaceutical products;
- food products, drinks;
- industrial discharges, wastewater;
- mineral products used in metallurgy

2.5.5. Plasma torch spectrometry (Inductively coupled plasma atomic emission spectroscopy (ICP-AES)

Works on the same principle as flame photometry except that it uses a plasma torch (6000-10000 k) for atomization and excitation of the atoms in the sample. The ICP-AES method allows the detection and measurement of almost all chemical elements (except gases and certain non-metallic atoms C, N, F, O, H) (Figure 15)

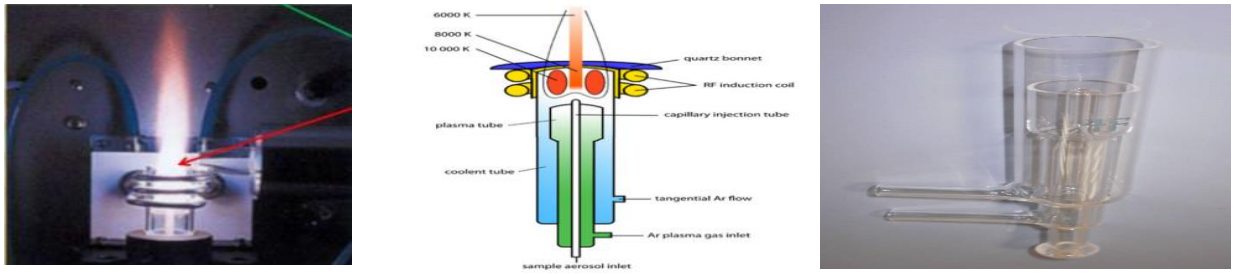


Figure. 15 : Plasma torch spectrometry.

2.6. Atomic absorption spectrometry (AAS)

It is a quantitative analysis method which allows the determination of the concentration of around sixty elements in solution (Ca, Fe, Mg, Li, Cu, Zn, Na, Pb, Hg, Cd, Sn, Bi, As, Cs, Ge, P, K, Rb, Se, Te, Th…….). This technique is very sensitive and allows trace detection (ppm) and based on the fact that an element in the atomic state is capable of absorbing radiation (defined lambda) that it itself is capable of emitting.

2.6.1. Principle

The solution to be analyzed is vaporized at constant flow in a flame and the elements are therefore in the state of atoms. the temperature of the flame (2000 – 3000°C) is lower than that of the atomic emission to avoid the excitation of the atoms. A light source provides light radiation (specific for the element to be analyzed) which passes through the cloud of atoms (figure 16). The quantity absorbed is proportional to the number of atoms present (concentration of the element) The concentration is determined using a previously established calibration curve. nb: Beer Lambert's law can be applied.

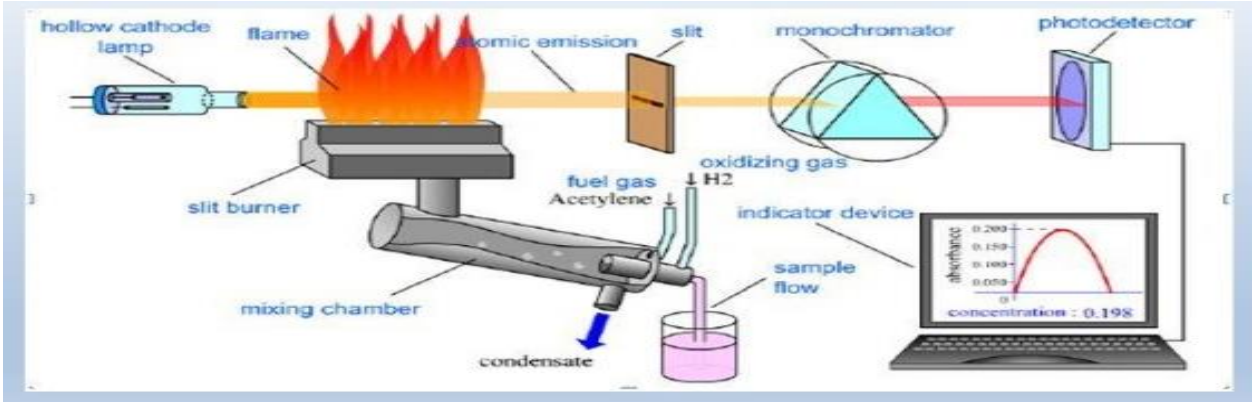


Figure. 16 : Atomic absorption Spectrometry

2.6.2. Material

2.6.3. light sources

a) hollow cathode lamp constituted: A cathode: cylinder hollowed out at one of its ends and made of the metal whose emission spectrum we want to produce. an anode; a sealed glass enclosure with

a window as transparent as possible at the wavelengths considered (quartz, borosilicate glass); this enclosure is filled with argon (Ar) or neon (Ne) (Figure 17)

Figure. 17 : hollow cathode lamp

When an electrical voltage is applied between the anode and the cathode (300 to 400 V), a few atoms of the filling neutral gas are ionized and accelerated towards the cathode. They thus tear off metallic atoms from the latter Shocks with Ar+ or Ne+ ions will then bring the metal atoms to an excited state (excitation phase) whose return to the ground state is accompanied by the emission of a characteristic light (emission phase).

b) Discharge lamp EDL (electrodeless discharge lamps). The lamp consists of a sealed quartz bulb in which there is a small quantity of element in the form of metal or salt. This bulb is placed inside a radio frequency coil. When current is applied to the coil, a radio frequency field is created. This vaporizes the atoms inside the bulb and brings them to an excited state. The emission of characteristic lines accompanies the return to the ground state (Figure 18).

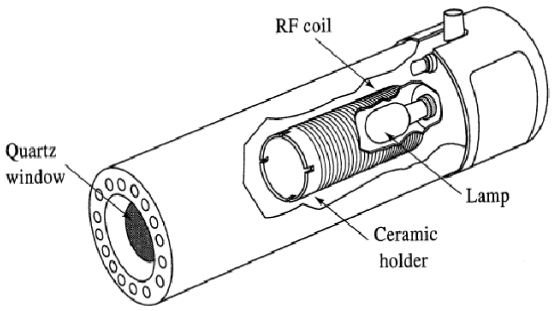


Figure 7. Schéma d'une lampe à décharge.

Figure. 18 : Discharge lamp.

2.7. Infra-red spectrometry (IR)

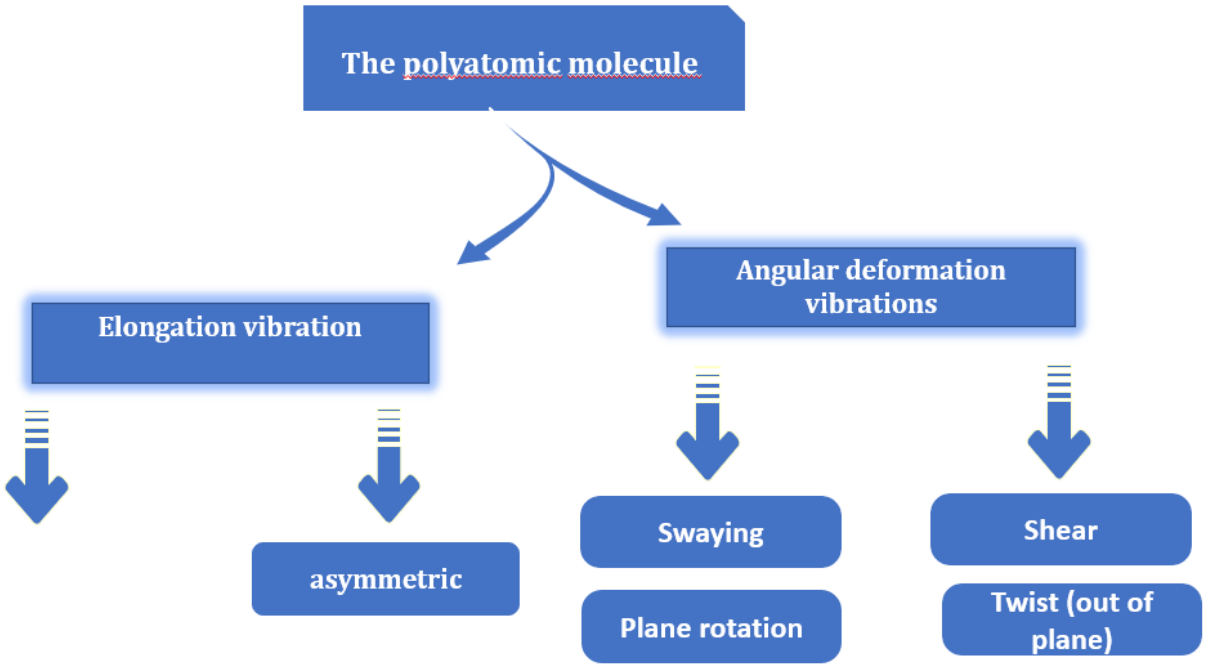
IR involves the examination of the interaction between infrared light and a molecule. The analysis can be conducted by three methods: absorption, emission, and reflection measurements. This approach is mostly employed in the fields of organic and inorganic chemistry. It is used by chemists to determine functional groups in molecules. Infrared spectroscopy quantifies the oscillations of atoms, enabling the identification of functional groups. In general, bonds that are

stronger and atoms that are lighter will exhibit a higher frequency of vibration in terms of stretching (measured in wavenumbers).

2.7.1. Vibration

IR affects the vibration modes of molecules. In the case of IR absorption spectroscopy, the radiation emitted by the source polychromatic is not enough to cause electronic transitions, but it does induce transitions between vibrational energy levels.

The motion of two particles in space can be separated into translational, vibrational, and rotational motions. Diatomic molecules (HH, H-Cl, C=O, etc.), only vibrate in one way, they move, as if they were attached by a spring, moving towards and away from each other.



2.7.1.1. Elongation vibration

Vibrations between two given atoms during which the interatomic distance varies along the axis of the bond. Vibrations between two given atoms during which the interatomic distance varies along the axis of the bond. In symmetrical vibration, the two atoms move toward and away from the central atom in concordance. In the asymmetric vibration, the two atoms move closer and further away from the central atom in discordance (Figure 19).

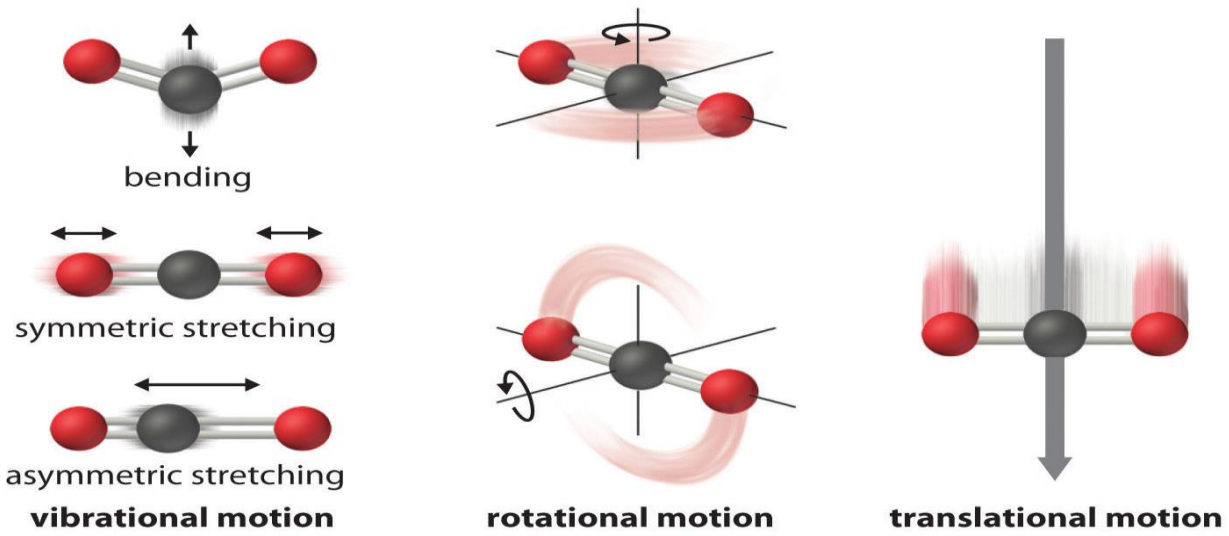


Figure. 19 : Different ways of vibration of molecule

2.7.1.2. Angular deformation vibration

Vibrations during which the angle formed by two adjacent bonds varies. During this movement, the interatomic distance remains constant. many types of movement can be observed: changing the bond angle (Shear), no changing in band angle (Swaying), simultaneous movement of the two atoms on each side of the plane (twist) or simultaneous movement of the two atoms out of the plane (Plane rotation).

2.7.2. IR equipment

There are two primary methods for obtaining IR spectra: scanning, which is the older technique, and Fourier transform (FT), often known as Fourier's Transform, which is the second method. An IR spectrometer consists of four basic components: an infrared radiation source, a radiation separation system or dispersive system (monochromator), a signal detector, and a recorder (Figure 20).

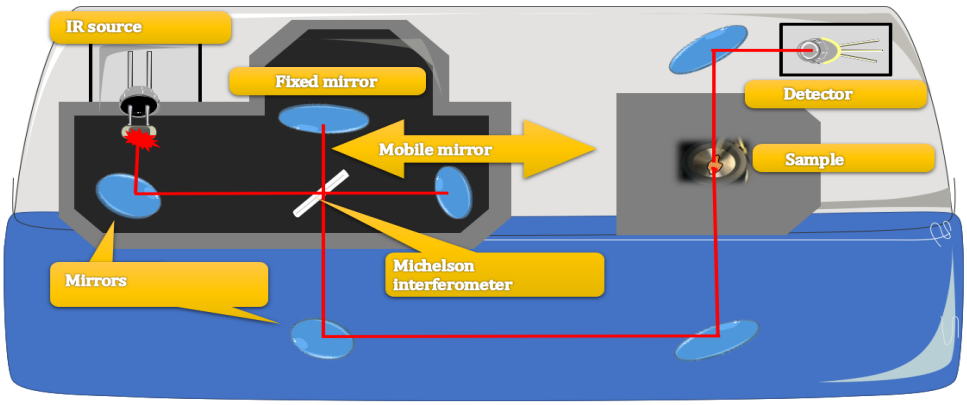


Figure. 20 : IR spectroscopy equipement.

The source: In most cases, we work in the mid-infrared region (4000 and 400 cm-1). We then use a Globar source based on silicon carbide.

The radiation separation system (monochromator): The sample is illuminated with polychromatic IR radiation. For scanning spectrometers, prisms or diffraction gratings are used as dispersive systems. For Fourier transform spectrometers, an interferometer (Michelson interferometer) is used.

The interferometer has three main components: a separator, a fixed mirror and a moving mirror.

The detector: The detection of the signal takes place by a component ensuring the conversion of the incident radiation into an electrical signal. The detector used is thermal type. It detects temperature variations and transforms them into intensity variations.

It is possible to produce IR spectra of solid, liquid or gaseous compounds. Depending on the physical state of the sample, the techniques differ. For the cells, you must choose a material that does not absorb IR: often NaCl or KBr. If the compound is liquid, a drop is placed between the windows of the cell to form a film of liquid. If it is solid, it can either be dissolved in a solvent or mixed with anhydrous KBr, the powder obtained then being subjected to strong pressure using a press to obtain a pellet. Finally, it can be placed in suspension in liquid paraffin.

2.7.3. Appearance of the IR spectrum

An infrared spectrum is often represented as transmittance (T%) versus wavenumber (cm-1). Each band is characterized by its value of $\tilde{\upsilon}$ ($\tilde{\upsilon}=1/\lambda$(cm-1)) at the absorption maximum.

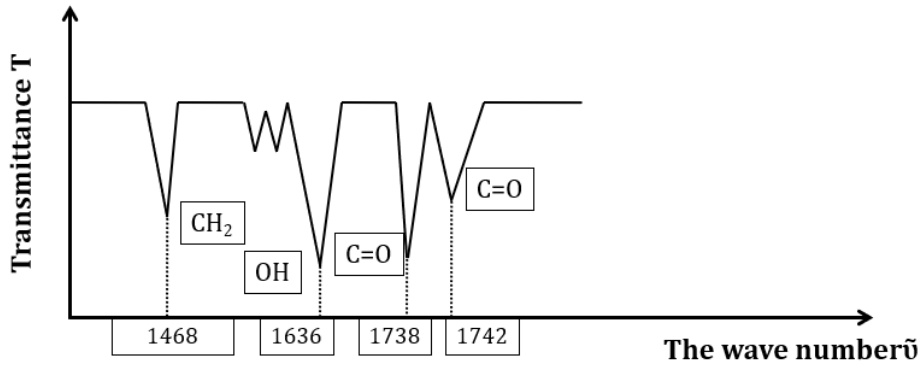
Despite the apparent complexity of IR spectra, due to the large number of absorption bands, there are absorptions at characteristic wavenumbers which make it possible to identify the different groups of a molecule (Figure 21, 22). Four main regions can be distinguished:

- 4000-2500 cm-1: X-H Elongations (O-H , N-H, C-H)

- 2500-1900-cm-1: Elongations of triple bonds C≡C and C≡N and cumulative double bonds X=Y=Z (allenes, isocyanates....)

- 1900-1500 cm-1: Elongations of double bonds (C=O, C=N, C =C, NO2)

- 1500-200 cm-1: Single bond lengths (C-N (NO2: strong at ≈ 1350 cm-1); C-O: strong between 1000 and 1300 cm-1...). This area, called the fingerprint region, is used to identify a compound with certainty and attest its purity.



Numberwave(cm^{-1})	Attribution	Molecule
1468	CH_2	Lipid
1636	OH	H_2O
1738	C=O	Phospholipid
1742	C=O	Triglyceride

Figure. 21 : IR spectrum 1

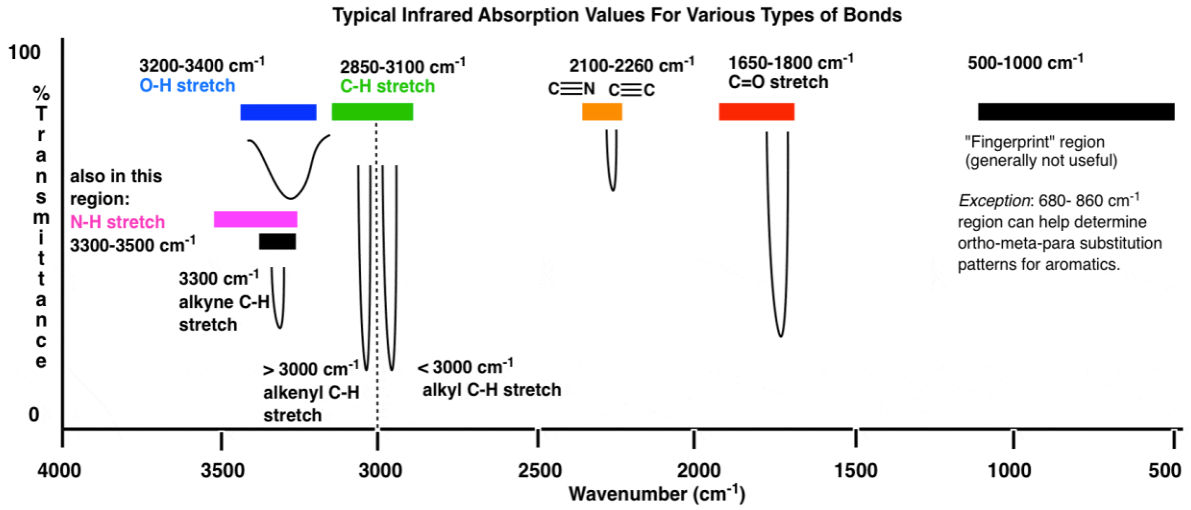


Figure. 22 : IR spectrum 2

2.7.4. IR analysis

When faced with a novel IR spectrum, optimise your time by posing two crucial inquiries:

Does a prominent, curved summit exist within the vicinity of 3400-3200 cm-1? Hydroxyl groups (OH) manifest at that location.

Does a prominent, intense peak exist in the spectral range of 1850-1630 cm-1? Carbonyl groups (C=O) appear in that location.

Initially, let us examine a few instances of hydroxyl group peaks within the range of 3400 cm-1 to 3200 cm-1, which Jon eloquently characterises as "tongues". All of the peaks below are

associated with alcohols. The presence of hydrogen bonding among hydroxyl groups causes changes in the strength of O-H bonds, leading to a spectrum of vibrational frequencies. The fluctuation leads to the formation of the wide peaks that are noticed (Figure 23).

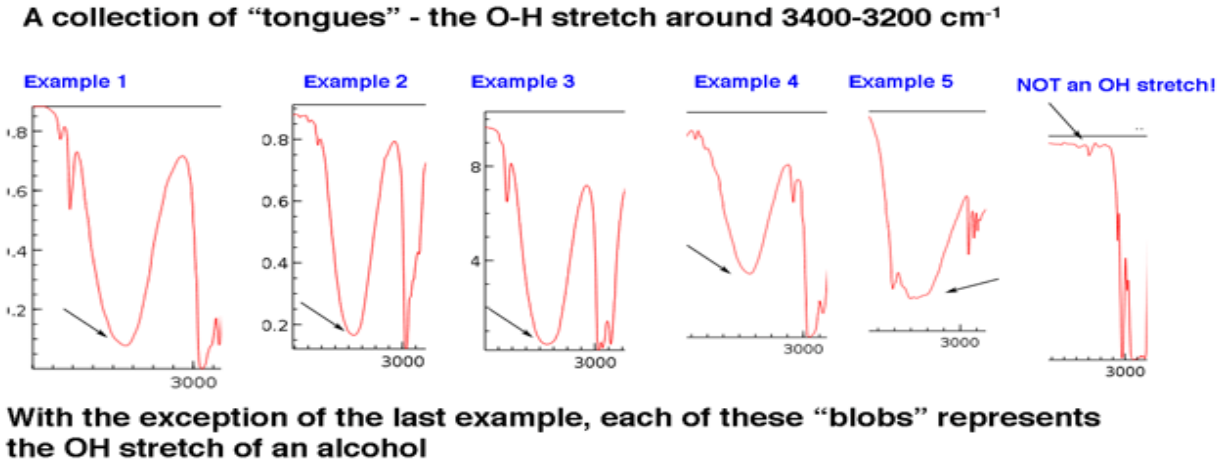


Figure. 23 : Collection of tongue O-H in IR spectrum

Typically, it is unnecessary to actively search for a hydroxyl group within the background noise.

While hydroxyl groups constitute the predominant form of broad peak in this region, N-H peaks can also appear in this area. They typically have a more distinct morphology and can manifest as either a single or double apex, contingent upon the quantity of N-H bonds present.

Now, let us examine a few instances of C=O peaks, namely in the spectral range of 1630-1800 cm-1. These peaks typically exhibit the highest intensity in the entire spectrum and have a very small width, resembling a sword (Figure 24).

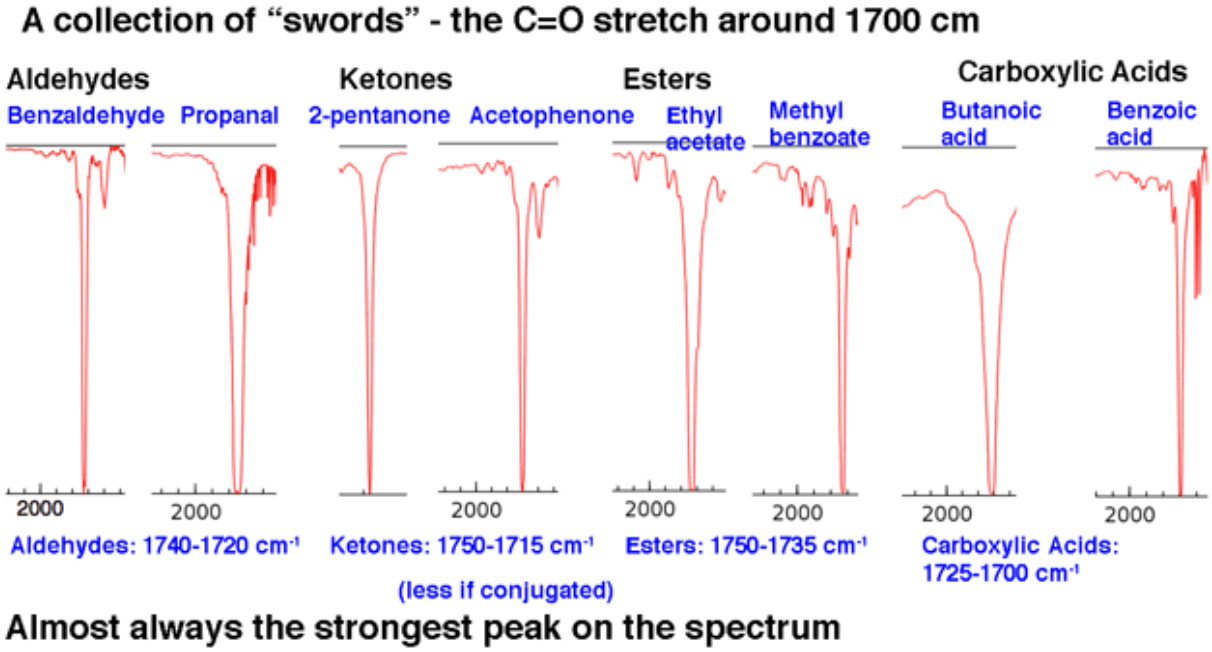


Figure. 24 : Collection of sword C=O bound in IR spectrum

The line at 3000 cm-1 serves as a convenient demarcation point between alkene C–H bonds (above 3000 cm-1) and alkane C–H bonds (below 3000 cm-1). This method can rapidly assist in identifying the presence of double bonds.

The occurrence of a peak within the range of 2200 cm-1 to 2050 cm-1 is a modest indication that a triple bond [C≡N or C≡C] is present. No other objects or phenomena are seen in this area.

000-3000 cm^{-1}						
3700-3584	medium	sharp	O-H	stretching	alcohol	free
3550-3200	strong	broad	O-H	stretching	alcohol	intermolecular bonded
3500- 3400	medium	-	N-H	stretching	primary amine	-
3400-3300 3330-3250	medium	-	N-H	stretching	aliphatic primary amine	-
3350-3310	medium	-	N-H	stretching	secondary amine	-
3300-2500	strong	broad	O-H	stretching	carboxylic acid	usually centered on 3000 cm^{-1}
3200-2700	weak	broad	O-H	stretching	alcohol	intramolecular bonded
3000-2800	strong	broad	N-H	stretching	amine salt	-
3000-2500 cm^{-1}						
3333-3267	strong	sharp	C-H	stretching	alkyne	-
3100-3000	medium	-	C-H	stretching	alkene	-
3000-2840	medium	-	C-H	stretching	alkane	-
2830-2695	medium	-	C-H	stretching	aldehyde	doublet
2600-2550	weak	-	S-H	stretching	thiol	-
2400-2000 cm^{-1}						
2349	strong	-	O=C=O	stretching	carbon dioxide	-
2275-2250	strong	broad	N=C=O	stretching	isocyanate	-
2260-2222	weak	-	C≡N	stretching	nitrile	-
2260-2190	weak	-	C≡C	stretching	alkyne	disubstituted
2175-2140	strong	-	S-C≡N	stretching	thiocyanate	-
2160-2120	strong	-	N=N=N	stretching	azide	-
2150	-	-	C=C=O	stretching	ketene	-
2145-2120	strong	-	N=C=N	stretching	carbodiimide	-
2140-2100	weak	-	C≡C	stretching	alkyne	monosubstituted
2140-1990	strong	-	N=C=S	stretching	isothiocyanate	-
2000-1900	medium	-	C=C=C	stretching	allene	-
2000	-	-	C=C=N	stretching	ketenimine	-
2000-1650 cm^{-1}						

2000-1650	weak	-	C-H	bending	aromatic compound	overtone
1870-1540 cm^{-1}						
1818 1750	strong	-	C=O	stretching	anhydride	-
1815-1785	strong	-	C=O	stretching	acid halide	-
1800-1770	strong	-	C=O	stretching	conjugated acid halide	-
1775 1720	strong	-	C=O	stretching	conjugated anhydride	-
1770-1780	strong	-	C=O	stretching	vinyl / phenyl ester	-
1760	strong	-	C=O	stretching	carboxylic acid	monomer
1750-1735	strong	-	C=O	stretching	esters	6-membered lactone
1750-1735	strong	-	C=O	stretching	δ-lactone	γ: 1770
1745	strong	-	C=O	stretching	cyclopentanone	-
1740-1720	strong	-	C=O	stretching	aldehyde	-
1730-1715	strong	-	C=O	stretching	α,β-unsaturated ester	or formates
1725-1705	strong	-	C=O	stretching	aliphatic ketone	or cyclohexanone or cyclopentenone
1720-1706	strong	-	C=O	stretching	carboxylic acid	dimer
1710-1680	strong	-	C=O	stretching	conjugated acid	dimer
1710-1685	strong	-	C=O	stretching	conjugated aldehyde	-
1690	strong	-	C=O	stretching	primary amide	free (associated: 1650)
1690-1640	medium	-	C=N	stretching	imine / oxime	-
1685-1666	strong	-	C=O	stretching	conjugated ketone	-
1680	strong	-	C=O	stretching	secondary amide	free (associated: 1640)
1680	strong	-	C=O	stretching	tertiary amide	free (associated: 1630)
1650	strong	-	C=O	stretching	δ-lactam	γ: 1750-1700 β: 1760-1730

1670-1600 cm^{-1}						
1678-1668	weak	-	C=C	stretching	alkene	disubstituted (trans)
1675-1665	weak	-	C=C	stretching	alkene	trisubstituted
1675-1665	weak	-	C=C	stretching	alkene	tetrasubstituted
1662-1626	medium	-	C=C	stretching	alkene	disubstituted (cis)
1658-1648	medium	-	C=C	stretching	alkene	vinylidene
1650-1600	medium	-	C=C	stretching	conjugated alkene	-
1650-1580	medium	-	N-H	bending	amine	-
1650-1566	medium	-	C=C	stretching	cyclic alkene	-
1648-1638	strong	-	C=C	stretching	alkene	monosubstituted
1620-1610	strong	-	C=C	stretching	α,β-unsaturated ketone	-
1600-1300 cm^{-1}						
1550-1500	strong	-	N-O	stretching	nitro compound	-
1372-1290						
1465	medium	-	C-H	bending	alkane	methylene group
1450	medium	-	C-H	bending	alkane	methyl group
1375						
1390-1380	medium	-	C-H	bending	aldehyde	-
1385-1380	medium	-	C-H	bending	alkane	gem dimethyl
1370-1365						
1400-1000 cm^{-1}						
1440-1395	medium	-	O-H	bending	carboxylic acid	-
1420-1330	medium	-	O-H	bending	alcohol	-
1415-1380	strong	-	S=O	stretching	sulfate	-
1200-1185						
1410-1380	strong	-	S=O	stretching	sulfonyl chloride	-
1204-1177						
1400-1000	strong	-	C-F	stretching	fluoro compound	-
1390-1310	medium	-	O-H	bending	phenol	-
1372-1335	strong	-	S=O	stretching	sulfonate	-
1195-1168						

1370-1335 1170-1155	strong	-	S=O	stretching	sulfonamide	-
1350-1342 1165-1150	strong	-	S=O	stretching	sulfonic acid	anhydrous hydrate: 1230- 1120
1350-1300 1160-1120	strong	-	S=O	stretching	sulfone	-
1342-1266	strong	-	C-N	stretching	aromatic amine	-
1310-1250	strong	-	C-O	stretching	aromatic ester	-
1275-1200 1075-1020	strong	-	C-O	stretching	alkyl aryl ether	-
1250-1020	medium	-	C-N	stretching	amine	-
1225-1200 1075-1020	strong	-	C-O	stretching	vinyl ether	-
1210-1163	strong	-	C-O	stretching	ester	-
1205-1124	strong	-	C-O	stretching	tertiary alcohol	-
1150-1085	strong	-	C-O	stretching	aliphatic ether	-
1124-1087	strong	-	C-O	stretching	secondary alcohol	-
1085-1050	strong	-	C-O	stretching	primary alcohol	-
1070-1030	strong	-	S=O	stretching	sulfoxide	-
1050-1040	strong	broad	CO-O-CO	stretching	anhydride	-
1000-650 cm^{-1}						
995-985 915-905	strong	-	C=C	bending	alkene	monosubstituted
980-960	strong	-	C=C	bending	alkene	disubstituted (trans)
895-885	strong	-	C=C	bending	alkene	vinylidene
850-550	strong	-	C-Cl	stretching	halo compound	-
840-790	medium	-	C=C	bending	alkene	trisubstituted
730-665	strong	-	C=C	bending	alkene	disubstituted (cis)
690-515	strong	-	C-Br	stretching	halo compound	-
600-500	strong	-	C-I	stretching	halo compound	-
900-700 cm^{-1}						
880 ± 20 810 ± 20	strong	-	C-H	bending	1,2,4- trisubstituted	-

880 ± 20 780 ± 20 (700 ± 20)	strong	-	C-H	bending	1,3-disubstituted	-
810 ± 20	strong	-	C-H	bending	1,4-disubstituted or 1,2,3,4-tetrasubstituted	-
780 ± 20 (700 ± 20)	strong	-	C-H	bending	1,2,3-trisubstituted	-
755 ± 20	strong	-	C-H	bending	1,2-disubstituted	-
750 ± 20 700 ± 20	strong	-	C-H	bending	monosubstituted benzene derivative	-

2.8. Nuclear magnetic resonance (NMR)

NMR is a spectroscopic technique that relies on the magnetism of the nucleus. It is based on the measurement of the absorption of radiation in the radio frequency domain by an atomic nucleus in a strong magnetic field. It constitutes one of the most powerful methods for determining the structure of both organic and inorganic species. ^{1}H NMR spectroscopy makes it possible to identify H atoms of a molecules and provide information on their chemical environment. We obtain information on the carbon chain of the molecules. However, ^{13}C NMR spectroscopy makes it possible to identify H atoms of a molecules.

2.8.1. Principle

In the absence of an external magnetic field, the spin magnetic moments are randomly oriented. On the other hand, under the action of a static magnetic field H_0, these moments will align according to the direction of the imposed field. According to the laws of quantum mechanics, only certain discrete orientations of these vectors are allowed. In the case of the proton, two orientations are permitted: parallel and antiparallel (Figure 25).

The principle of proton NMR (1H NMR) consists of:

(1) use a magnetic field H_0 to orient the nuclear "spins" of the atoms,

(2) excite these spins by a radio wave at the resonance frequency, which makes switch some spins,

(3) after excitation, the spins return to their initial state (relaxation).

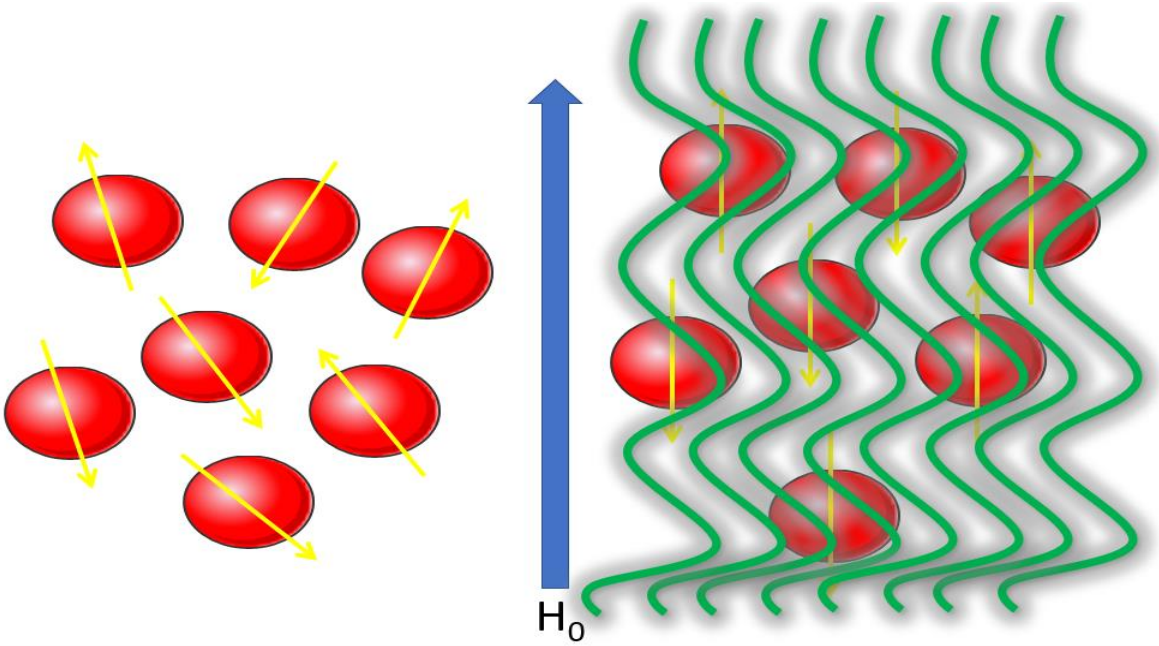


Figure. 25 : NMR principle

2.8.2. Material

There are 2 types of spectrometers, the scanning or continuous wave spectrometer (continuous-wave, cw), and the Fourier transform spectrometer (FT-NMR).

The following elements are essential to constitute a spectrometer:

- A magnet to produce the static field H_0.
- A source of electromagnetic radiation of appropriate frequency (generator).
- A frequency scanning unit throughout the absorption domain.
- A cell containing the sample.
- A detector (radio frequency receiver) which measures the quantity of radiation absorbed by the cell.
- A recorder that traces the energy absorbed as a function of frequency (Figure 26).

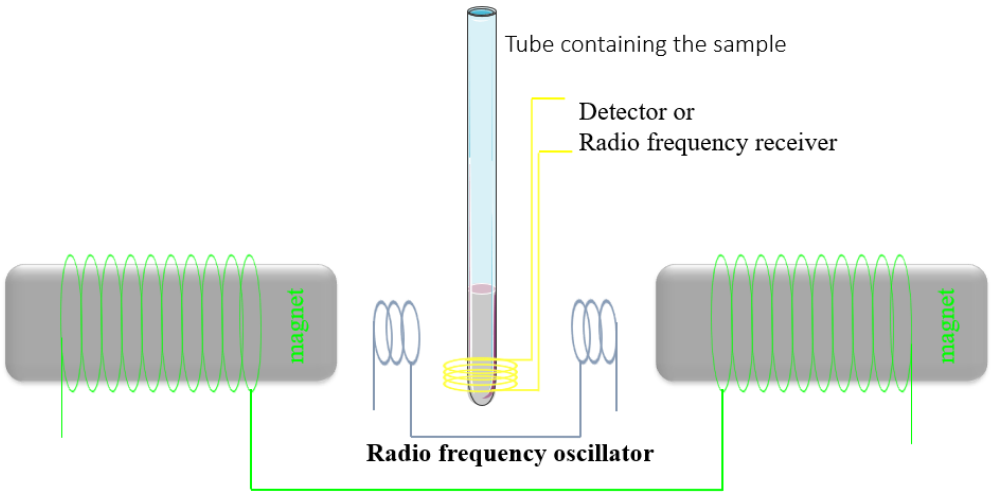


Figure. 26 : NMR spectroscopy

2.8.3. Samples

For the solution study, the sample is dissolved in a solvent. The quantity of product required for proton NMR is 10 to 50 mg. The sample is placed in a glass tube rotated in the center of a magnetic coil. The chosen solvent must be free of hydrogens. In fact, the protons of the solvent do not must not mask the protons of the sample examined. Solvents used: CCl4, CDCl3; CD3COCD3; CD3OD3; C5D5N; D2O; DMSO-d6

2.8.4. Molecules movement

The position of the different lines of the NMR spectrum is determined relative to a reference. In the case of the proton, the tetramethylsilane Si(CH3)4 (denoted TMs) is used. For convenience, we use a notation scale: the chemical shift noted δi, expressed in parts per million (ppm).

$$\delta_{ppm} = \frac{\nu_i - \nu_{ref}}{\nu_0}.10^6$$

υi: resonance frequency of nuclei i

υref: reference resonance frequency (TMS)

υ0: frequency of the static field H0

δ is characteristic of the proton environment. Protons likewise environment are said to be magnetically equivalent and have the same δ. The kernels having different environments are said to be magnetically different. If a signal comes out at a field close to that of the reference (TMS), we say that it comes out at strong field: it is shielded. Conversely, if a signal comes out at a chemical shift high, we say that it comes out at low field: the signal is deshielded (Figure 27).

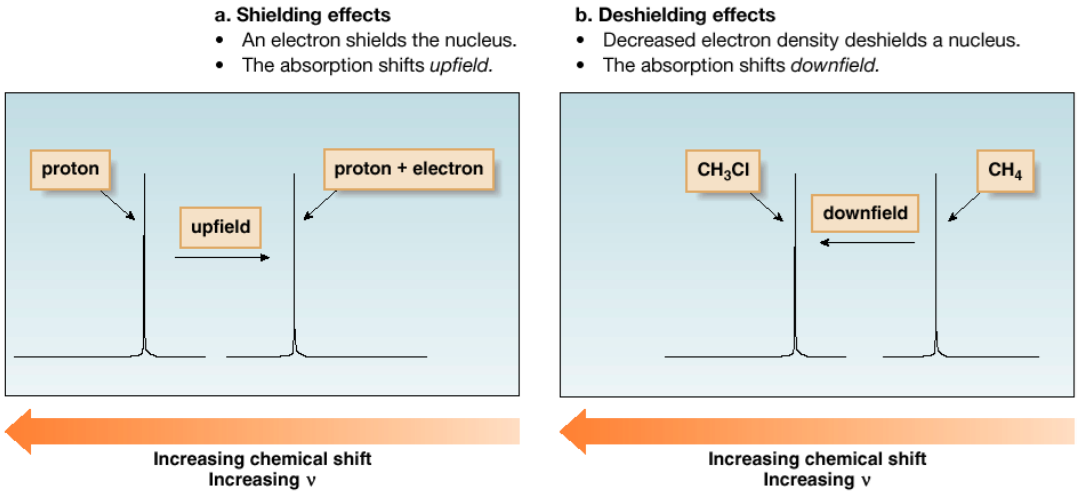


Figure. 27 : Shielding and deshielding effects

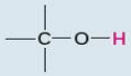
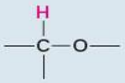
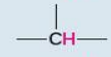
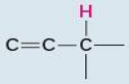
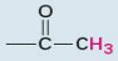
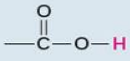
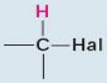
The chemical shift of a proton depends on:

- essentially the nature of the atom which carries it (carbon, nitrogen or oxygen most often)
- substituents carried by this atom
- the nature of adjacent atoms and the substituents carried by them

(OH, Cl, NO2…)

Attractor effects (inductive or mesomeric) acting on a carbon carrying H induce deshielding. A donor effect will, on the contrary, induce shielding. A second important effect is the presence of electrons π in the vicinity of the proton studied (aromatic ring or multiple bond). Chemical shifts therefore give information about the environment chemical of the group to which the proton in question belongs. Thus, will be able to identify groups of protons from the value of δ. Tables give the ranges of these movements depending on various environments.

Table 13.3 | Correlation of ^1H Chemical Shift with Environment

Type of hydrogen		Chemical shift (δ)	Type of hydrogen		Chemical shift (δ)
Reference	$Si(CH_3)_4$	0	Alcohol		2.5–5.0
Alkyl (primary)	$-CH_3$	0.7–1.3			
Alkyl (secondary)	$-CH_2-$	1.2–1.6	Alcohol, ether		3.3–4.5
Alkyl (tertiary)		1.4–1.8			
Allylic		1.6–2.2	Vinylic		4.5–6.5
			Aryl	Ar—H	
Methyl ketone		2.0–2.4	Aldehyde		9.7–10.0
Aromatic methyl	$Ar-CH_3$	2.4–2.7			
Alkynyl	$-C\equiv C-H$	2.5–3.0	Carboxylic acid		11.0–12.0
Alkyl halide		2.5–4.0			

2.8.5. Number of signals

The "chemical shift" of a proton NMR signal corresponds to the protection of a hydrogen nucleus by the electrons surrounding it. The two protons in acetylene possess similar electrical surroundings. It should be noted that acetylene can undergo a 180-degree flip or rotation along its axis, resulting in the full indistinguishability of its two protons. Consequently, the two protons will exhibit identical chemical shifts. [Please note that it will be integrated into two separate entities.]

This phenomenon is referred to as chemical shift equivalence. If two or more protons are chemically identical, they will provide a solitary signal at a precise chemical shift in a 1H NMR spectrum. In the case where three hydrogens on a molecule are chemically identical, rather of generating three distinct signals in the spectrum, only a single signal will be generated to represent all three.

The chemical shift equivalence arises from the interchangeability of hydrogen nuclei due to symmetry operations (such as planes of symmetry) or quick intramolecular processes (such as bond rotation or tautomerization). Hydrogens exhibiting this equivalence can be either homotopic (indistinguishable) or enantiotopic (equivalent in solvents that lack chirality). The number of peaks observed in an NMR spectrum is directly proportional to the amount of protons present in distinct chemical environments.

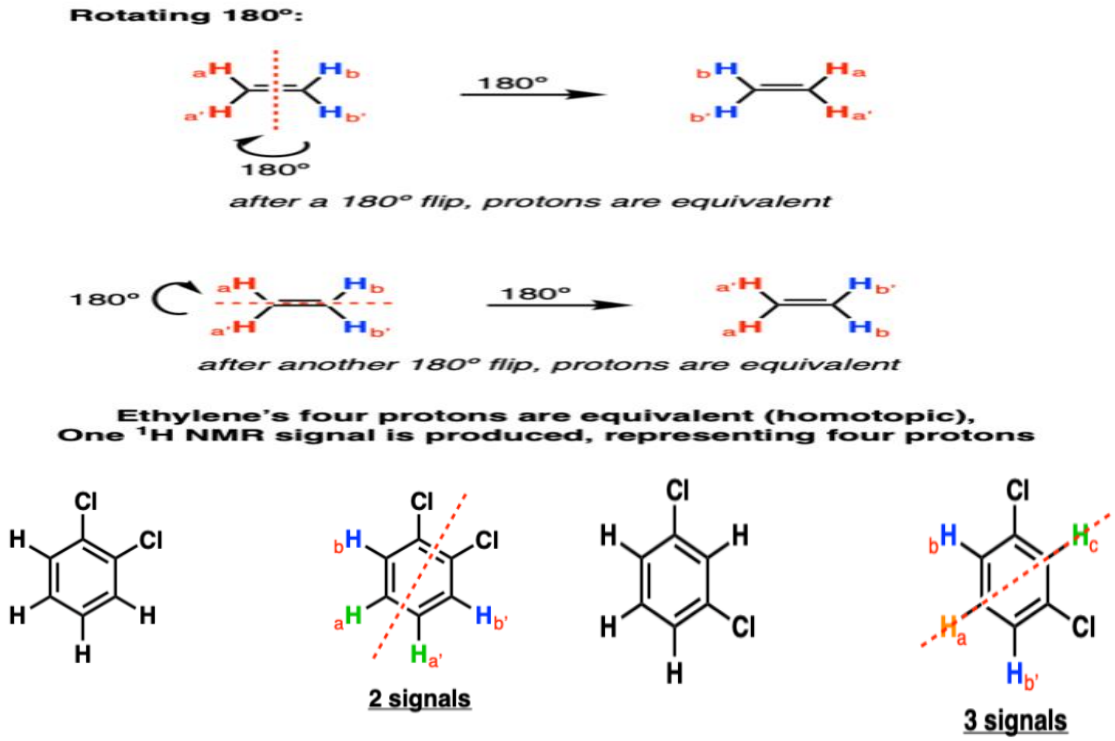


Figure. 28 : Signal number of molecules

2.8.6. Integration curve

In an NMR spectrum, the intensity of a signal is measured by its surface area. The integrations of signal surfaces are presented in the form of a series of levels. The height of each level is proportional to the number of corresponding H (Figure 29).

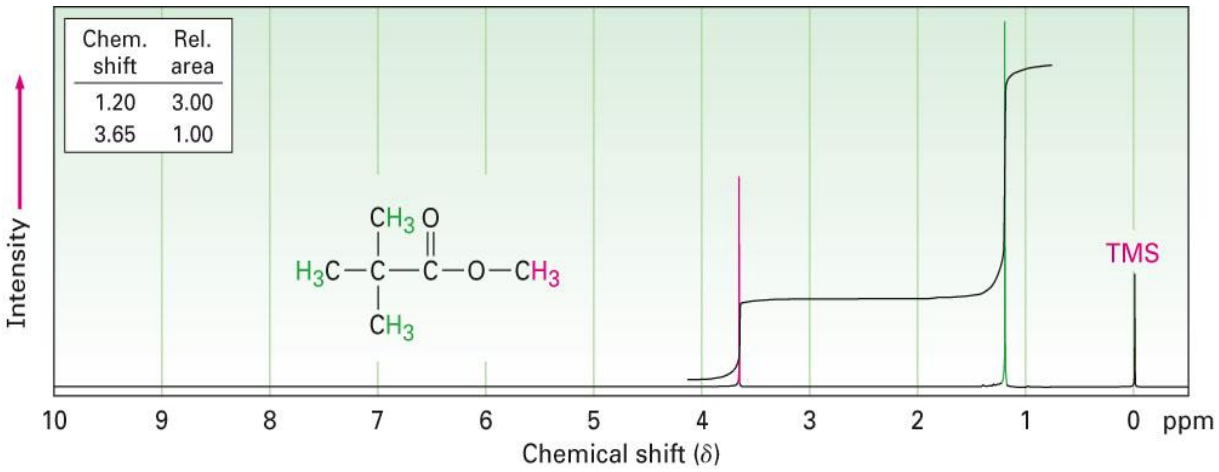


Figure. 29 : Integration curve (HNMR)

2.8.7. Fine structure - spin-spin coupling

The local field to which the proton is subjected depends on its environment electronic. This local field is also influenced by the presence and orientation of spins of other nuclei of the molecule when this spin is different from zero. Let Ha be the resonant proton and let Hb be a proton which disturbs the field magnetic necessary to obtain the Ha resonance. The local field at neighborhood

of Ha will be influenced by the two possible orientations of the spin of Hb. Ha will therefore undergo the effect of two magnetic fields. Its NMR signal will be in the form of a doublet whose peaks are of equal intensity, because the probability of the Hb proton having a spin +½ or -½ is approximately the same (Figure 30).

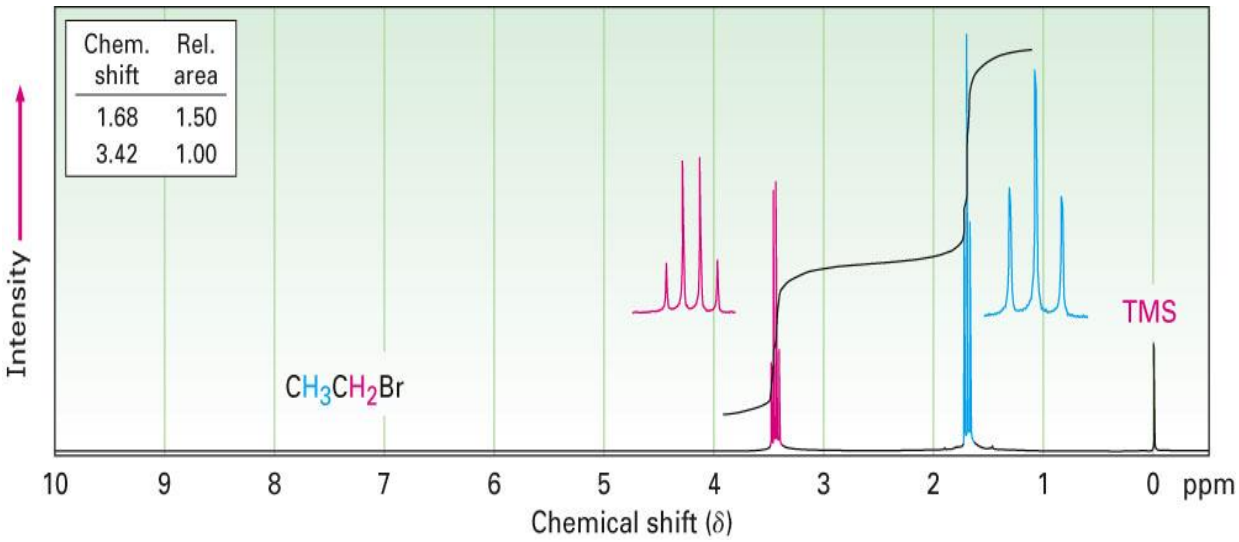


Figure. 30 : Spin-spin Splitting

Peaks are often split into multiple peaks due to magnetic interactions between non equivalent protons on adjacent carbons, The process is called spin-spin splitting. The splitting is into one more peak than the number of H's on the adjacent carbon(s), This is the "n+1 rule". The relative intensities are in proportion of a binomial distribution given by Pascal's Triangle. The set of peaks is a multiplet (2 = doublet, 3 = triplet, 4 = quartet, 5=pentet, 6=hextet, 7=heptet…..)

2.9. Fluorescence spectrometry

Fluorescence spectrometry is a highly effective analytical technique employed in many disciplines such as chemistry, biology, and environmental research. Fluorescence is the result of a substance absorbing light at a particular wavelength and subsequently emitting light at a longer wavelength. This approach yields essential insights into the molecular structure, content, and characteristics.

Fluorescence spectrometry provides numerous benefits compared to alternative analytical methods. First and foremost, it possesses a high level of sensitivity, enabling the identification of even minuscule quantities of a drug within a sample. Furthermore, this procedure is non-invasive, ensuring that the material remains undamaged during the examination process. This is especially crucial when examining fragile or costly specimens. In addition, fluorescence spectrometry exhibits a high level of selectivity due to the distinct luminous properties shown by different compounds. This enables researchers to discern and measure distinct components within an

intricate amalgamation. Fluorescence spectrometry is a very adaptable and potent instrument in scientific investigation and examination.

2.9.1. Principle

The primary tenet of fluorescence spectrometry is the assimilation of light. When a molecule absorbs light at a certain wavelength, it acquires energy and transitions to an excited state. The excited state is inherently unstable and rapidly transitions back to the ground state by the emission of light. Fluorescence occurs when the emitted light possesses a greater wavelength and lesser energy compared to the absorbed light. The Stokes shift governs this process, representing the disparity between the excitation and emission wavelengths.

The second concept pertains to the excitation and emission spectra. The excitation spectrum refers to the range of light wavelengths that a molecule can absorb, whereas the emission spectrum indicates the wavelengths of light that are emitted during fluorescence. Through the examination of these spectra, scientists are able to ascertain the precise chemical and quantify its content in a given sample. Moreover, the brightness of the produced light is directly related to the amount of the molecule present, enabling precise measurement and analysis.

The third principle pertains to the fluorescence lifespan. Every molecule possesses a distinctive fluorescence lifetime, which represents the average duration it spends in an excited state before returning to its original ground state. The duration of its existence can be affected by multiple factors, including the chemical surroundings and interactions with other molecules. Through the measurement of fluorescence lifetime, scientists can obtain valuable information on the molecular dynamics and interactions taking place within a given sample.

2.9.2. Types of fluoresence

An essential facet of fluorescence spectrometry pertains to the several possible types of fluorescence spectrometers. These instruments exhibit a range of differences in their design and functionality, enabling scientists to select the best appropriate one for their particular requirements. The predominant categories comprise of steady-state fluorescence spectrometers, time-resolved fluorescence spectrometers, and fluorescence microscopes.

Steady-state fluorescence spectrometers, which are widely employed in everyday analysis, represent the fundamental and simplest form of this technology. Their method involves quantifying the fluorescence intensity emitted by a sample in relation to the excitation wavelength. This enables scientists to ascertain the strength of fluorescence and the specific wavelength at which the sample produces the highest level of fluorescence. Operating these spectrometers is rather straightforward and they offer valuable insights about the existence and abundance of fluorescent chemicals in a given sample.

In contrast, time-resolved fluorescence spectrometers offer more comprehensive insights on the fluorescence lifespan of a given material. The term "lifetimes" refers to the duration required for a molecule to revert back to its lowest energy state following excitation. Scientists can gather insights about the molecular dynamics and interactions of the sample by quantifying the decrease in fluorescence intensity over a period of time. These spectrometers are highly valuable for analysing intricate biological systems and dynamic processes.

Fluorescence microscopes integrate the functionalities of fluorescence spectrometry and microscopy imaging. Scientists utilise these tools to visually observe fluorescent molecules within a sample and analyse their specific location and distribution within cells or tissues. This is accomplished by affixing fluorescent probes to certain molecules of interest, which thereafter exhibit fluorescence upon stimulation by the microscope's light source. Fluorescence microscopes are highly valuable instruments in the field of cell biology and biomedical research. They allow scientists to examine the intricate details and operations of biological systems at a microscopic scale.

Phosphorescence is a form of fluorescence. Phosphorescent substances emit light at a longer wavelength after absorbing light of a shorter wavelength. Phosphorescent materials differ from fluorescent materials in that they do not promptly release the absorbed radiation. Contrarily, a phosphorescent substance assimilates a portion of the radiation energy and then emits it for an extended duration after the radiation source has been eliminated. Phosphorescence can be generated through two distinct mechanisms: triplet phosphorescence (also known as phosphorescence) and persistent phosphorescence (or persistent luminescence). Triplet phosphorescence happens when an atom absorbs a photon with high energy, and this energy gets trapped in the spin multiplicity of the electrons. As a result, the atom transitions from a faster emitting "singlet state" to a slower emitting "triplet state". The longer durations of the reemission are linked to "prohibited" energy state transitions in quantum mechanics. Due to the gradual nature of these transitions in specific materials, absorbed radiation is subsequently released at a reduced intensity, with a time delay ranging from a few microseconds to up to one second after the excitation is terminated.

2.9.3. Material

The basic outline of a conventional fluorescence spectrophotometer is depicted in the Figure 31. The system comprises a light source, an excitation and emission monochromator (with grooves/mm), polarizers (prisms), a sample chamber, and a detector (such as a photomultiplier tube). Steady state measurements typically employ a 450W xenon arc lamp as the light source, whereas time-resolved measurements utilise a nanosecond flash lamp. Typically, most basic spectrometers share a similar structure, however additional detectors and/or light sources are

sometimes added, resulting in a T- or X-geometry. The light source emits photons across a wide range of energies, typically spanning from 200 to 900 nm. Photons collide with the excitation monochromator, which filters and allows just a specific band of light centred on the designated excitation wavelength to pass through. The transmitted light is directed through adjustable slits, which regulate both the intensity and precision by narrowing down the range of transmitted light. The attenuated light enters the sample cell, inducing fluorescence from the fluorophores present in the sample. The emitted light is directed into the emission monochromator, which is positioned perpendicular to the excitation light path in order to eliminate any background signal and reduce noise caused by stray light. Once again, the light that is produced is transmitted within a limited region, focused around the specific wavelength of emission. It then passes through slits that may be adjusted, before ultimately entering the photomultiplier tube (PMT). The signal undergoes amplification, resulting in the generation of a voltage that is directly proportional to the intensity of the emitted measurement. The PMT is the main source of noise in the counting process. Hence, the spectral resolution and signal-to-noise ratio are closely correlated with the chosen slit widths (Figure 31).

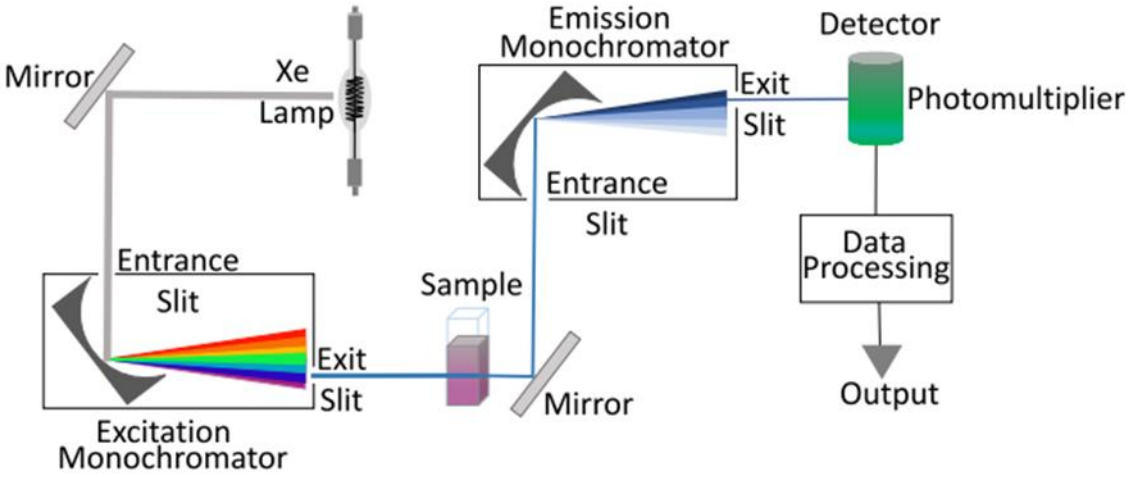


Figure. 31 : Fluorescence spectroscopy instrument

Multiple environmental conditions have an impact on the qualities of fluorescence. Fluorophores experience changes in intensity due to temperature, pH of the surrounding liquid, and the polarity of solvents. Typically, the fluorescence intensity falls as the temperature rises because there are more frequent molecule collisions at higher temperatures.

2.10. Mass spectroscopy

Mass spectrometry is an analytical method used for determining and identify compounds by measuring their mass. Furthermore, it enables the characterization of the chemical structure of molecules through fragmentation and subsequent quantitative analysis. thresholds as low as sub-nanogram (10-9 g) as well as picogram (10-12 g) levels are frequently achieved. Mass spectrometry is employed across a wide range of scientific disciplines: The subjects I am interested in are organic chemistry, dosages, astrophysics, biology, and medicine.

2.10.1. Electron impact ionisation principle

Within the spectrometer, a stream of highly charged electrons collides with the sample. Following the ionisation of the molecules, a radical cation known as a molecular ion is formed. It represents the molar mass of the substance. It undergoes fragmentation, resulting in the formation of cations with reduced masses, known as ion fragments, as well as neutral fragments such as radicals or molecules. Fragment ions are categorised based on their m/z ratio and quantified based on their relative abundance (Figure 33).

2.10.2. The Sample Inlet

The sample is held in a large reservoir and molecules are transported to the ionisation chamber at low pressure in a continuous flow through a small opening known as a "Molecular leak".

2.10.2.1. Ionisation

refers to the process of converting an atom or molecule into an ion by adding or removing one or more electrons. Atoms undergo ionisation when they are struck by a stream of electrons, causing one or more electrons to be dislodged and resulting in the formation of positive ions. The majority of the positive ions that are generated will possess a charge of +1. Ionisation can be accomplished by the following methods:

Electron Ionisation Mass Spectrometry (EI-MS)

Chemical Ionisation Mass Spectrometry (CI-MS)

Desorption Technique (FAB) is a method used to remove molecules off a surface by applying a high-energy beam of particles (Figure 32).

- $M + e^- \rightarrow M(*)^{+\bullet} + 2e^-$
- However, M^+ typically has extra energy and undergoes further decomposition/fragmentation: $M^{*+} \rightarrow X^+ + Y^\bullet$ (where X and Y are fragments) but often if $M^{*+} \rightarrow X^+ + Y^\bullet$, it also may form $X^\bullet + Y^+$

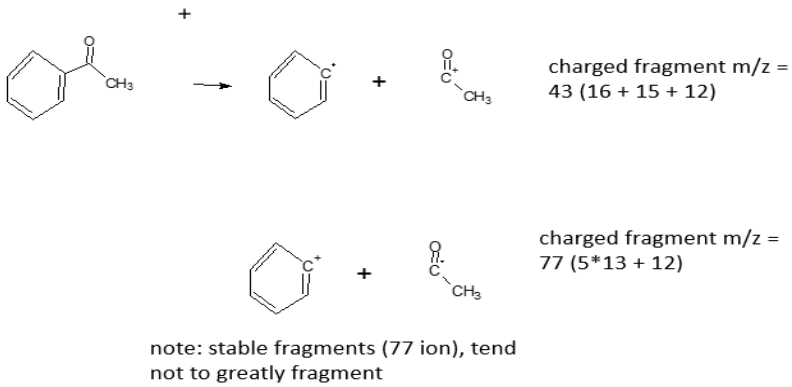


Figure. 32 : Ionisation in Mass spectroscopy

2.10.2.2. Acceleration

Ions are uniformly accelerated to ensure they possess same kinetic energy. Cations traverse three slits with a voltage gradient in a descending sequence. The middle slit conducts intermediate and final stages at zero volts.

2.10.2.3. Deflection

refers to the bending or deviation of an object from its original position or path. Ions experience deflection when exposed to a magnetic field as a result of disparities in their masses. As the mass decreases, the deflection increases. The deflection of an ion is also influenced by the magnitude of its positive charge; the greater the positive charge, the greater the deflection.

2.10.2.4. Detection

The detector identifies the beam of ions flowing through the mass analyzer based on the m/z ratio. Upon collision with the metal box, the ion's charge is nullified as an electron transfer from the metal to the ion.

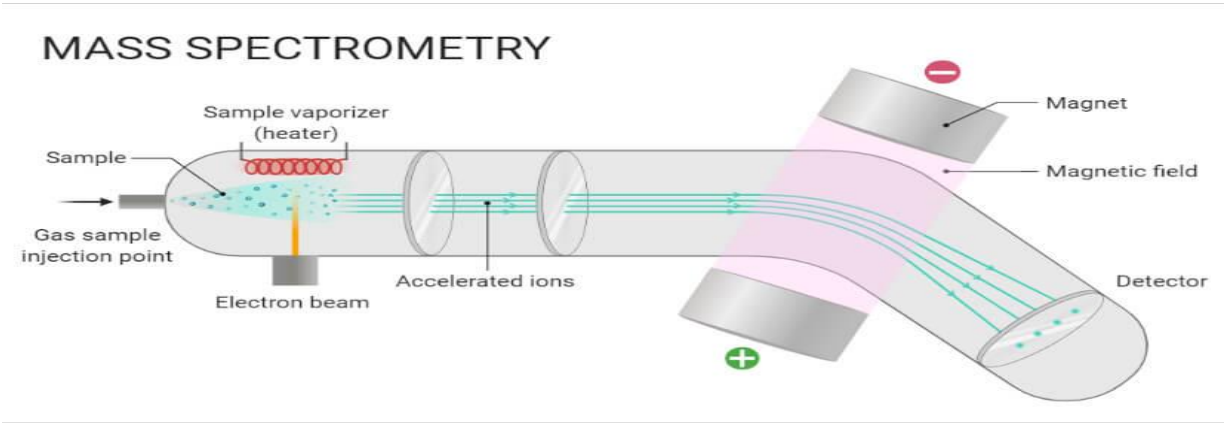


Figure. 33 : Mass spectroscopy instrument

2.10.3. Categories of analyzers

A mass analyzer is alternatively referred to as a mass filter. It is located at the core of every mass spectrometer. A mass analyzer is a device that separates molecular ions and their fragments in the gas phase based on their velocity, mass, and/or mass-to-charge (m/z) ratios. This separation is achieved by accelerating the ions and subjecting them to a combined electric and magnetic field. The isolated ions are then propelled towards the detector for identification and then transformed into a digital signal for analysis in a data system.

Magnetic sector mass analyzers are instruments used for analysing the mass of particles based on their magnetic properties. J.J. Thompson's initial mass spectrometer was constructed using a magnetic sector analyzer. Magnetic sector mass spectrometers, also known as spectrographs, are the earliest form of mass spectrometers that utilised photographic plates as ion detectors. Magnetic sector analyzers employ a magnetic field to induce circular motion in ion beams originating from the ion source. The ion beam traverses a magnetic field, experiencing a magnetic force that acts at a right angle to the direction of the ion's trajectory. As per the laws of physics, the perpendicular acceleration force induces the ion beam to move in a circular trajectory. The magnetic sector analyzers deflect the ion beam along a curved path. The radius (r) of this arc can be influenced by various factors, such as the ion's momentum, ion charge (C), and magnetic field intensity (B). A magnetic sector can exclusively separate ions based on their mass-to-charge ratio (m/z). However, in order to attain a greater level of detail and carry out tandem mass spectrometry, it is crucial to use an electric sector in conjunction with a magnetic sector. Mass spectrometers that have both magnetic and electrostatic sectors are commonly known as double-focusing magnetic sector MS. The inaugural double-focusing magnetic sector MS was unveiled in 1936 by A. Dempster, K. Bainbridge, and J. Mattauch. The utilisation of double-focusing magnetic sector MS was widespread during the 1950s and 1960s due to its capability to generate data with high resolution.

An electrostatic sector mass analyzer is a device used for analysing the mass of charged particles based on their electrostatic properties. The electrostatic sector mass analyzer comprises two curved plates that are arranged concentrically and subjected to equal and opposing voltages. As the ion beam passes through the analyzer, it undergoes deflection as a result of the potential difference across the plates. The ions undergo deflection in the presence of an electric field, where ions possessing same kinetic energy are concentrated and ions with varying kinetic energy are scattered. The radius (r) of the ion's flight orbit is dictated by the kinetic energy (V) of the ion and the voltage (E) applied across the plates. The electrostatic sector mass analyzer functions as a kinetic energy analyzer by separating ions according to their disparities in kinetic energy. Conversely, the magnetic sector segregates ions based on their momentum, thereby facilitating the separation of ions that have been accelerated to a consistent kinetic energy. Combining an

electrostatic sector with a magnetic sector in sequence can significantly enhance mass resolution. A mass spectrometer apparatus that combines an electrostatic sector and a magnetic sector is referred to as a **double-focusing mass spectrometer**.

Quadrupole mass analyzers: often known as the Quadrupole mass spectrometer (MS), was initially developed in 1953 by Wolfgang Paul and Helmut Steinwedel, who were scientists from West Germany. The instrument is considered the most basic form of mass spectrometer (MS). The term "quadrupole MS" is derived from its composition of four parallel rods with circular or hyperbolic cross-sections. The rods are aligned in parallel with the ion flight trajectories that are measured by a mass spectrometer. Every rod is linked to a radio frequency alternating current (RF) source and a stationary direct current source. The quadrupole mass analyzer selectively separates ions based on their mass-to-charge ratio (m/z) while they move along the axis of charged rods or poles. Quadrupole mass analyzers are commonly known as mass filters because they selectively separate ions based on their m/z value. Quadrupole mass analyzers can be found in different configurations, including single quadrupole MS (SQMS), triple quadrupole MS (TQMS) which consists of three quadrupole analyzers in a series, and quadrupole time-of-flight MS (QTOFMS) which is essentially a triple quadrupole MS but with the third quadrupole analyzer replaced by a time-of-flight (TOF) mass analyzer.

Time of Flight analyzers, often known as TOF analyzers: segregates ions based on the duration it takes for ions to traverse a predetermined length "flight tube" before reaching the detector. As per the principles of physics, ions with less mass will exhibit higher velocity and arrive at the detection before ions with more mass, which will move at a slower pace and reach the detector later. duration-of-flight mass spectrometers (TOF-MS) determine the mass-to-charge ratio (m/z) of ions by measuring the flight duration of the ions. The majority of TOFMS devices utilise multichannel plate detectors (MCP), which are extremely sensitive with reaction times below 1 nanosecond. Time-of-flight mass spectrometers (TOF-MS) can be built in different configurations, including single-stage TOFMS, multi-stage TOF/TOFMS, and hybrid quadrupole-TOFMS (QTOFMS). Both Time-of-Flight/Time-of-Flight Mass Spectrometry (TOF/TOF-MS) and Quadrupole Time-of-Flight Mass Spectrometry (QTOFMS) variations operate as tandem mass spectrometry (MS/MS) systems.

An ion trap mass analyzer comprises an ion trapping region enclosed by several electrodes. The combination of radiofrequency (RF) voltage and direct current (DC) voltages creates an electric field that effectively confines ions inside the designated trapping area. has the ability to control and store ions using several procedures, including separation and fragmentation. A trap may contain many outlets for the expulsion of ions. Upon leaving the trap, ions are propelled towards the detector based on their mass-to-charge ratio (m/z). Ion trap mass spectrometers has

the capability to execute multi-staged MS, which confers a significant advantage. Multi-stage mass spectrometry can yield useful insights into the ionic structure. Ion trap mass analyzers can be designed with several architectures, including linear ion traps (2-D traps), 3-D ion traps, electrostatic traps (Orbitraps), and magnetic field-based traps, also known as ion cyclotron resonance (ICR) traps. A notable characteristic of ion trap mass spectrometers is their seamless compatibility with other technologies, such as gas chromatography (GC) and liquid chromatography (LC).

An ion cyclotron analyzer is a device used to analyse ions based on their cyclotron motion.

The Quadrupole Ion Trap (QIT) is a type of mass analyzer. Wolfgang Paul, a renowned Nobel Prize laureate, pioneered the development of the fundamental principles of a quadrupole ion trap (QIT) mass analyzer during the early 1950s. He concurrently built the QIT mass analyzer with a quadrupole mass analyzer. The QIT mass analyzer functions as a three-dimensional ion storage device capable of effectively storing and holding charged particles or gaseous ions for extended periods. The device includes three hyperbolic electrodes: a circular electrode and two electrodes at the entrance and exit. By applying suitable voltages to the three electrodes, a trapping potential is created, resulting in the development of a cavity capable of capturing ions. The ring electrodes generate radiofrequency (RF) voltages, which create a three-dimensional quadrupole electric field in the trapping space. The trapping potential undergoes a switch at radio frequency (RF), which is why the trap is also known as a "radio frequency" trap. The quadrupolar field induces continuous oscillations in the confined ions. The movement of the oscillating ions is influenced by the voltages supplied and their corresponding mass-to-charge (m/z) ratios. Modifying ion-trapping voltages results in the selective ejection of ions in increasing order of their mass-to-charge ratio (m/z) from the trapping potential cavity through the endcap. Subsequently, the influx of ions is directed towards a detector that produces a mass spectrum. The utilisation of the trapping and ejection principle enables quadrupole ion trap (QIT) mass analyzers to effectively carry out multi-stage mass spectrometry (MSn). QIT mass spectrometers are advantageous instruments for various applications because to their selectivity, high sensitivity, MSn capabilities, and compact size.

2.10.4. Mass spectrometry spectrum

The sample introduction system is responsible for transporting the sample into the spectrometer. The ion source is the apparatus responsible for the ionisation of molecules. Multiple ionisation techniques exist. Electronic influence is the most prevalent. The analyzer employs a magnetic and/or electric field to segregate ions based on their (m/z) ratio. The detector captures the ions exiting the analyzer and quantifies them based on their respective abundance. A data processing

computer system that enables the conversion of information obtained by the mass spectrum detector.

The mass spectrum is a graphical representation that displays the m/z ratios of the measured ions. In electronic impact, where z = 1, the m/z values are given in Dalton units (Figure 34).

The ordinate axis represents the relative abundance of these ions. The highest peak is arbitrarily assigned a value of 100. The various categories of peaks detected in a mass spectrum include:

The base peak refers to the peak in the spectrum that has the highest intensity. It represents the ion that is the most prevalent and thus the most stable.

The molecular peak, also known as the parent peak, represents the ion that has a mass number equivalent to the molecular mass of the material.

- Fragment peaks represent distinct fragment ions.

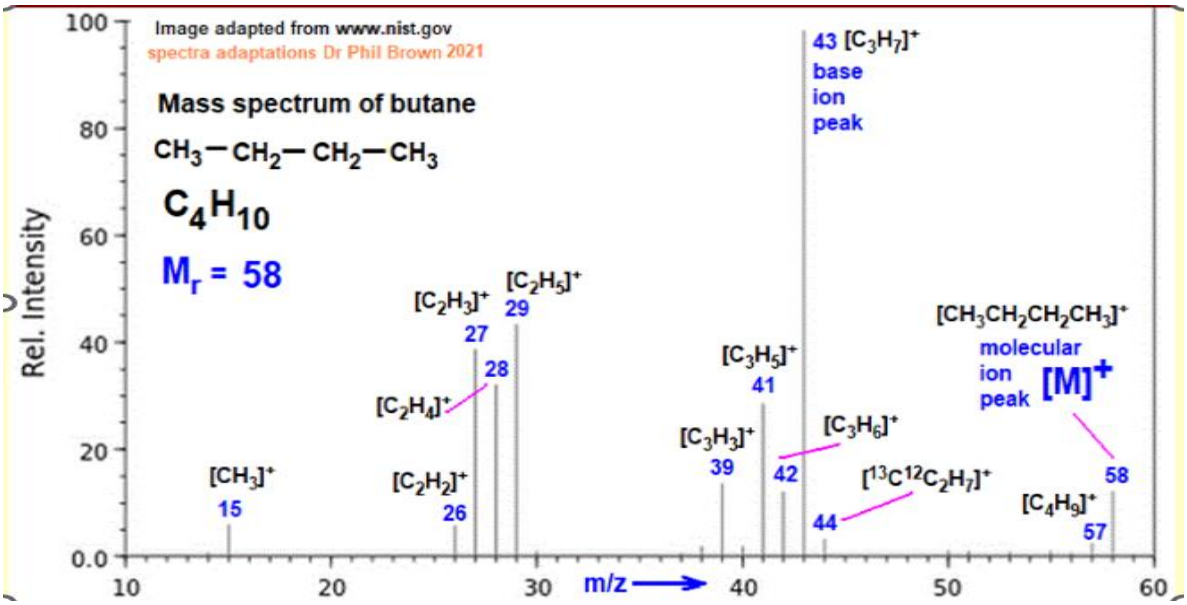


Figure. 34 : Mass spectrum analysis

To conduct an analysis of a mass spectrum, one can proceed by adhering to the following steps:

Search for the peak corresponding to the molecular ion. If this peak is present, it will be the highest peak in the spectrum, excluding the peaks caused by isotopes. The nominal molecular weight, which refers to a rounded-off value, will be an even number for compounds that consist solely of carbon (C), hydrogen (H), oxygen (O), sulphur (S), and silicon (Si).

Calculate the ratio of the mass to the charge (m/z) of the peak corresponding to the molecular ion. The value represents the ratio of the compound's molecular weight to the charge on the ion 1.

Determine the pattern of fragmentation in the mass spectrum. The fragmentation pattern arises when organic molecules are introduced into a mass spectrometer. Upon entering the ionisation chamber of a mass spectrometer, the vaporised organic sample is subjected to an electron beam bombardment. These electrons possess sufficient energy to dislodge an electron from an organic

molecule, resulting in the formation of a positively charged ion. The ion in question is referred to as the molecular ion, sometimes known as the parent ion, and is commonly denoted by the symbols M+ or M+. Molecular ions exhibit inherent energy instability, leading to their fragmentation into smaller constituents. In the most basic scenario, a molecule ion undergoes fragmentation, resulting in the formation of two distinct entities. One of these entities is a positively charged ion, while the other is an uncharged free radical. The uncharged free radical will not form a line on the mass spectrum.

Utilise the fragmentation pattern observed in a certain mass spectrum to aid in the identification of a relatively uncomplicated, unidentified chemical (such as an unknown alkane).

3. Extraction methods

Commonly, we want to separate organelles or molecules from tissues or cells, either to measure their concentration or study their characteristics or functioning. This step requires breaking the biological material to release the desired substances or structures. This is an initial step in many experimental procedures in biochemistry and is called homogenization. Several types of devices can be used to break cells. by physical (mechanical) or chemical or even enzymatic methods.

3.1. Mechanical techniques

3.1.1. Mechanical crusher

The mortar took the form of a test tube, more or less widened at the mouth, better suited to retaining liquids. The pestle, or "piston", consists of a rod with a bulge at its base. The latter has a diameter barely smaller than that of the interior of the mortar. By sliding the pestle to the bottom of the mortar, we force the material to pass through the very small space between the bulge of the pestle and the internal wall of the mortar, shearing and breaking it. After a few compression cycles, the material is homogenized. To obtain good homogenization, the distance between the surface of the piston and the internal wall of the mortar ("clearance") must be very small, less than a tenth of a millimeter (Figure 35).

There are several types of these devices (Figure 35):

- **a.** The most widespread is surely the "Potter-Eveljhem" device, it is a pestle made up of a steel rod and a Teflon bulge as well as a thick glass mortar.
- **b.** The other devices are generally entirely made of glass: "Dounce", etc. The piston bulge and mortar grinding area are often made of sintered glass. These models are relatively fragile, particularly the pestle rod.

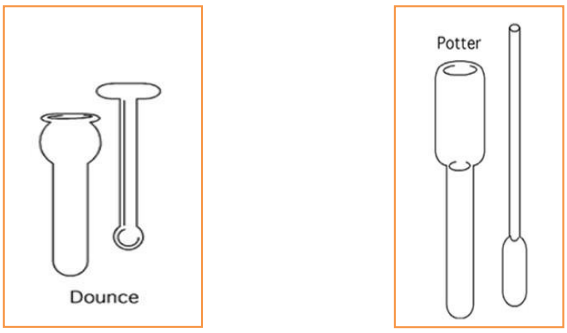


Figure. 35 : The different types of crusher.

3.1.2. Gas homogenizers

It consists of a metal chamber in which the cells are treated with nitrogen at high pressure. The pressure forces the nitrogen to dissolve in liquids. The pressure is then suddenly released, the nitrogen in solution returns to its gaseous state, forms bubbles inside the cells and the facts explode (Figure 36).

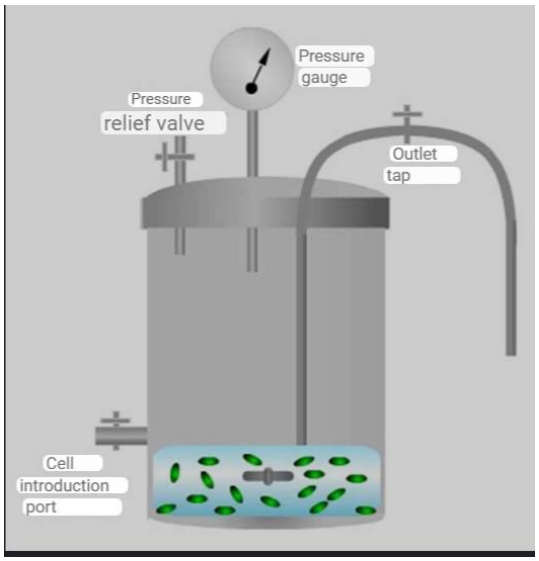


Figure. 36 : Gas homogenizers.

3.1.3. French's press

The cell suspension is poured into the cylinder. The piston is installed, a powerful one begins to press it into the cylinder.

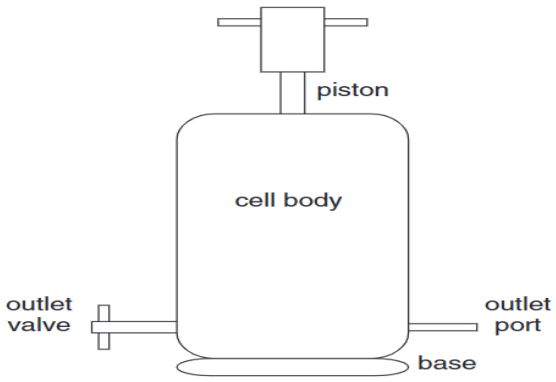


Figure. 37 : French's press

As there is only little air in the cylinder. The sinking of the piston very quickly increases the pressure on the cylinder walls. When the valve is slightly opened, the cells are cut. The higher the pressure in the cylinder, the more lysis is total (Figure 37).

3.1.4. Sonication

The cells are subjected to an ultrasound source. Ultrasound is generated by an “ultrasonic generator” which transforms electrical energy (50 to 60 Hz) into ultrasonic energy (20 kHz).

The movement of the ultrasound is transmitted to the liquid by a probe which causes alternating compressions and decompressions in the liquid. The formation of "cavitations", i.e. microscopic bubbles which cause the disintegration of the bacterial membrane (Figure 38).

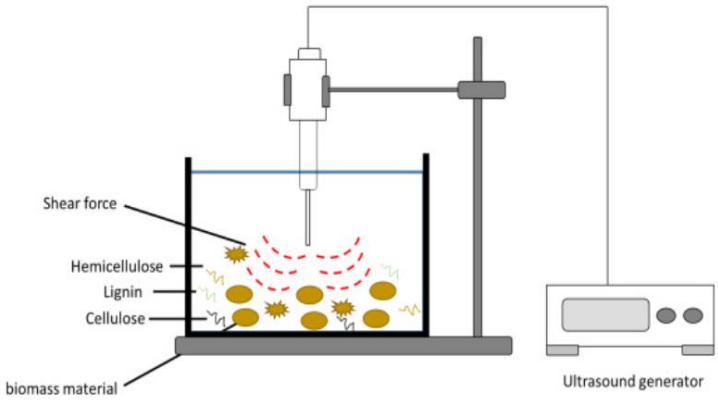


Figure. 38 : The mechanism of sonication.

3.1.5. Freezing-thawing

The bacterial suspension can be put through freezing (-20°C) and thawing (37°C) cycles which will break the membranes. During freezing ice crystals are formed which causes the disintegration of the cell membrane.

3.2. Chemical and enzymatic techniques

3.2.1. Lysis or osmotic shock

By creating a hypotonicity of the medium by dilution, the cells are caused to burst. But there is the risk, in this operation, of bursting the organelles. Water entering the cell causes it to swell until the lipid membranes rupture and allow their contents to pass into the medium.

3.2.2. Change in ionic force or pH

The ionic force of the medium is modified by adding ions or changing the pH. For certain types of cells, this treatment can make the membranes more permeable to the constituents of the medium. This will result in rupture of the plasma membrane.

3.2.3. Enzymatic lysis

With yeasts, plants, bacteria, we must take into account the cell wall which protects the plasma membrane. If we do not opt for a mechanical and violent method of extraction, we will first have to destroy this wall before attacking the rest of the cell.

3.2.4. Use of detergents

Detergents are capable of disrupting membrane lipids. They therefore weaken the interactions between the different constituents of the membrane. The result is membrane rupture. The action of detergents is difficult to control. The technique therefore requires precise focusing which can be tedious. A mild detergent (Triton, Tween, etc., sometimes deoxycholate) can be used to release compounds from within the compartments by dissolving the compartment membranes.

4. Splitting (separating) methods

4.1. Filtration

Filtration is a separation process that makes it possible to separate the constituents of a mixture which has a phase liquid and a phase solid through an environment porous. Filtration is an operation in which the solid particles remain on the filter and we recover a homogeneous mixture. The use of a filter allows heterogeneous mixture particles to be retained which are larger than the filter holes (porosity). The liquid that has undergone filtration is called filtrate, and what the filter retains is called residue.

4.1.1. Principle of filtration

Filtration is in principle sieving, i.e. separation according to the diameter of solid particles of different sizes. A pressure difference forces the liquid to pass through the filter while the solid particles are retained on the surface (Figure 39).

Furthermore, filtration is often accompanied by accessory phenomena, which are:

a. **Clogging** is caused by particles which are deposited on the surface of the filter but especially by their penetration into the interstices of the filtering material. This clogging completely modifies the porosity and slows down filtration.

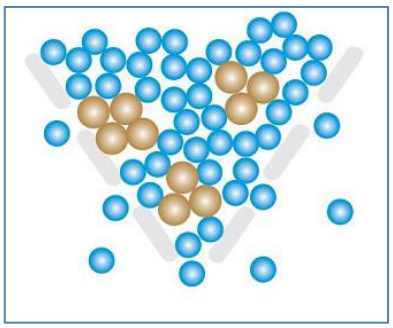


Figure. 39 : Principle of filtration.

b. Adsorption: the filter material has a certain electrical charge. Thus, certain products can be retained although their dimensions allow their passage through the pores of the filter.

4.1.2. Filtration equipment

1. The filters

There are two types of filter, depth filters (thick or thick) and surface filters (membrane).

✓ **Thickness filters**

Made up of fibrous substances (paper, asbestos, cellulose, cotton, fiberglass, etc.) or agglomerated substances (sintered glass, sand, coal, etc.). The effectiveness of a depth filter increases with its thickness but decreases when the pressure applied to the filter increases.

The materials used in depth filters are:

- Classic filter paper: there are several qualities of paper which differ in their shape (in rectangular, circular, pleated sheets, etc.), their texture (loose, fine), their porosity, their purity (raw, purified, ashless, etc.).
- Textile: gauze, cotton, wool, nylon, etc.
- Fibers: glass wool, asbestos.
- clays and porcelain.
- Sintered material: sintered glass obtained by compression at controlled temperature of glass microbeads with variable porosities and shapes.

✓ **Surface filters**

The most used are Millipores membranes. These are very thin sheets made of cellulose polymer with a very large number of rigorously calibrated pores. The dimensions of these pores vary depending on the filters from 0.025 µm to 8 µm.

2. Funnels

Funnels are conical shaped instruments intended to receive filtering material. We distinguish two types

✓ **Ordinary funnels**: glass, porcelain or polycarbonate (Figure 40).

✓ **Special funnels:** which can be made of glass or porcelain with a bottom plate pierced with holes, on which a round of filter paper is placed which covers this bottom exactly. In this category we distinguish between BUCHNER funnels, when fairly large quantities of solid are filtered, and HIRSCH funnels, for recovering small quantities of solid.

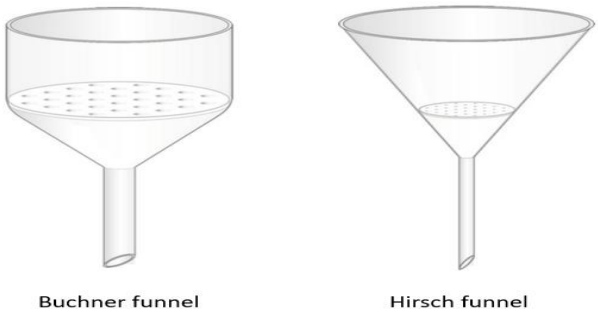


Figure. 40 : Funnels.

4.1.3. Laboratory filtration methods

4.1.3.1. Gravity filtration

In this method a filter and a container to receive the filter are used. The simplest device for this method is the simple funnel equipped with a paper filter placed on an Erlenmeyer flask to recover the filtrate (Figure 41. The pressure difference is created by the height of the liquid on the filter. For this method, two elements are necessary:

- The shape of the container should encourage the flow of liquid through the filter. It must be avoided that this creates an obstacle to the flow of the liquid. Which gives the shape of the funnel a certain importance.
- The opening angle of the funnel must be close to 60°.

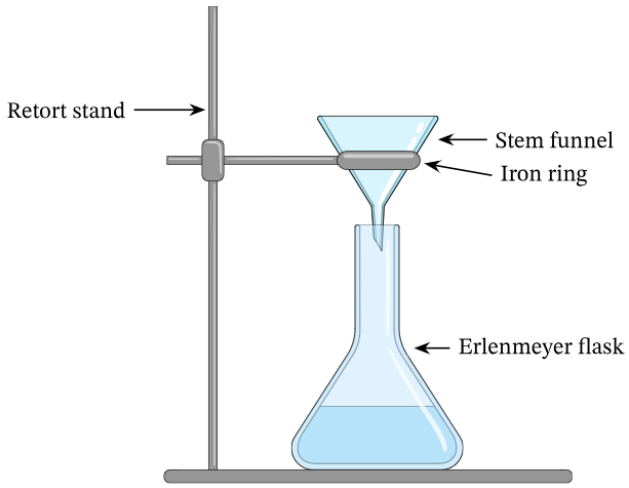


Figure. 41 : Gravity filtration.

This type of filtration has some disadvantages:

- Fairly slow filtration
- Difficulty in recovering the isolated solid phase, especially when it is not abundant (it is dispersed over a fairly large surface of filter paper, to which the grains stick).
- Incomplete separation: the solid retains a significant quantity of liquid.

4.1.3.2. Vacuum filtration

The filtration speed is increased by causing a depression downstream of the filter material. To carry out vacuum filtration, special funnels are used which are adapted to a "suction flask", in which a depression is created. The adaptation of the funnel to the flask is generally done via a rubber cone, which will "stick" to the flask and the funnel when the depression is established (Figure 42).

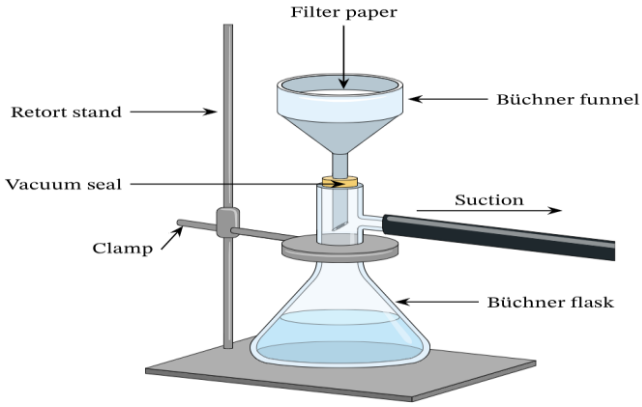


Figure. 42 : Vacuum filtration.

4.1.3.3. Pressure filtration

Pressure filtration prevents foaming and evaporation of the solvent. In the laboratory, sterilizing microfiltration using the Millipore device is pressure filtration. This device is made up of two plastic parts, which are screwed onto each other, enclosing a membrane filter (Figure 43).

Figure. 43 : A Millipore filter.

4.1.3.4. Ultrafiltration

Ultrafiltration covers a very wide area equivalent to a molecular mass range going from 500 to 1,000,000 Daltons. It is a membrane with such low porosity that it can retain proteins and nucleic acids. These filters can have pores as small as 25 nm which can retain most proteins. Ultrafiltration makes it possible to:

- Concentrate solutions of macromolecules.
- Eliminate most small molecular mass contaminants (salt, carbohydrates, etc.).

We use membranes with porosity allowing certain substances to pass through (salts, small molecules, small proteins). These membranes are most often sold as filtration units (Figure 44), which consist of a tube whose lower part is separated from the upper part by the membrane. The proteins are deposited in the upper

part of the tube, and rest on the membrane. The tube is then centrifuged to force the liquid and small molecules through it under the effect of centrifugal force.

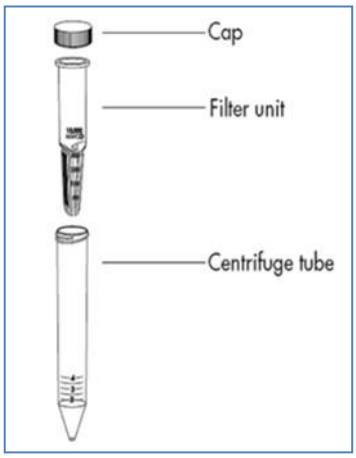


Figure. 44 : Ultrafiltration unit.

Several applications can be cited:

- **The clarification:** obtaining a liquid freed from solid particles.
- **Sterilization:** many biological molecules are sensitive to heat and cannot be sterilized in an autoclave. The solution to this problem is cold sterilization. The surface filters we use have pores of 0.45µm or 0.22µm in diameter. The bacteria are all retained by a 0.45µm filter. Those of 0.22µm stop many viruses and mycoplasts.
- **Desalination**: eliminate the salts used during a purification step for example.
- **Concentration**: concentrate a diluted sample.

4.2. Centrifugation

The word centrifugation is constructed from the verb "centrifuge" which comes from the Latin fugere which means "to flee" and "center", to which is added the suffix -action indicating an action.

Centrifugation is a separation process compounds depending on their difference in weight or density by subjecting them to a centrifugal force. The mixture to be separated can consist either of two liquid phases or of solid particles suspended in a fluid.

We can thus divide a preparation into a sediment (or "pellet"), made up of material more or less solidly packed in the bottom of the centrifuge tube, and into a supernatant which will be the residual liquid above the sediment (Figure 45).

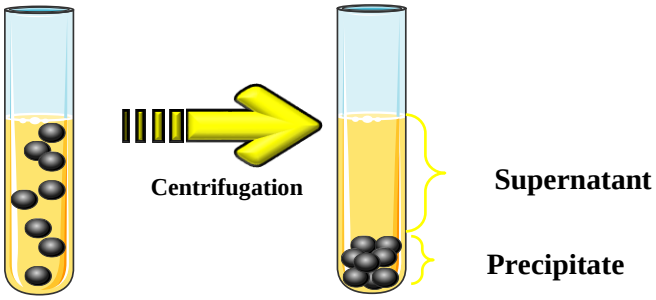


Figure. 45 : Centrifugation

4.2.1. Principle

All constituents contained in a solution are subject to:

- Gravity: force exerted from top to bottom
- Archimedes' thrust: force exerted from bottom to top

By rotating the sample, a new force appears, the centrifugal force, which is an acceleration exerted outwards, which causes it to settle or rise (Figure 46).

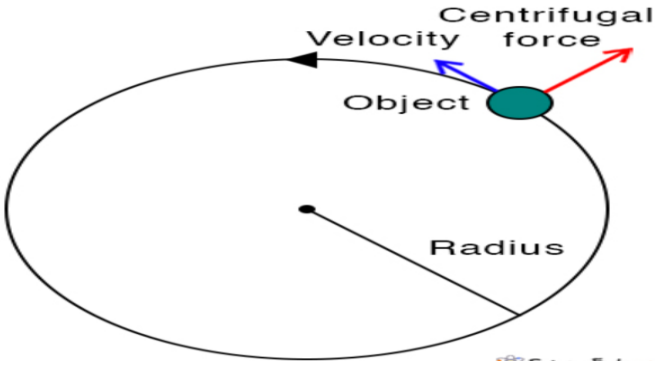


Figure. 46 : The centrifugal force

4.2.2. Calculation of gravitational force

In a centrifugation, you need to know the relative force of centrifugation (force of relative gravity, FGR) in "g" or the acceleration g. However, for a given rotation speed, each rotor has a different FGR since the radius of rotation is different. You must therefore be able to convert the rotation speed (RPM, rotations per minute) into FGR.

The mathematical formula for conversion (G and rpm): $g = 1.119 .10^{-5} \times rpm^2 \times r$

Where r is the rotor rotation radius (in cm) and rpm (rotation per minute) expresses the rotation speed.

Example : A rotor has a maximum rotation radius of 10 cm. How many revolutions will it take to obtain an acceleration of 100,000 g?

We have: g =100,000, r =10cm

We also have the equation: $g = 1.119 \times 10^{-5} \times rpm^2 \times r$.

$$rpm = \sqrt{\frac{g}{r \times 1.119 \times 10^{-5}}}$$

$$rpm = \sqrt{\frac{100\,000}{10 \times 1.119 \times 10^{-5}}}$$

rpm = 29894 ≈ 30000

4.2.3. Types of centrifugations

Differential centrifugation

In a first centrifugation at low acceleration, the most massive elements will sediment and form a pellet at the bottom of the tube. All the other elements will remain in the liquid fraction then called supernatant. The supernatant and the pellet are then recovered separately, which amounts to having separated the constituents which compose them. A second centrifugation cycle can be started again with the **previous supernatant**, but with **greater acceleration**. Gradually, we separate the different constituents, ending with the smallest elements and having the least difference in density with the solvent (Figure 47).

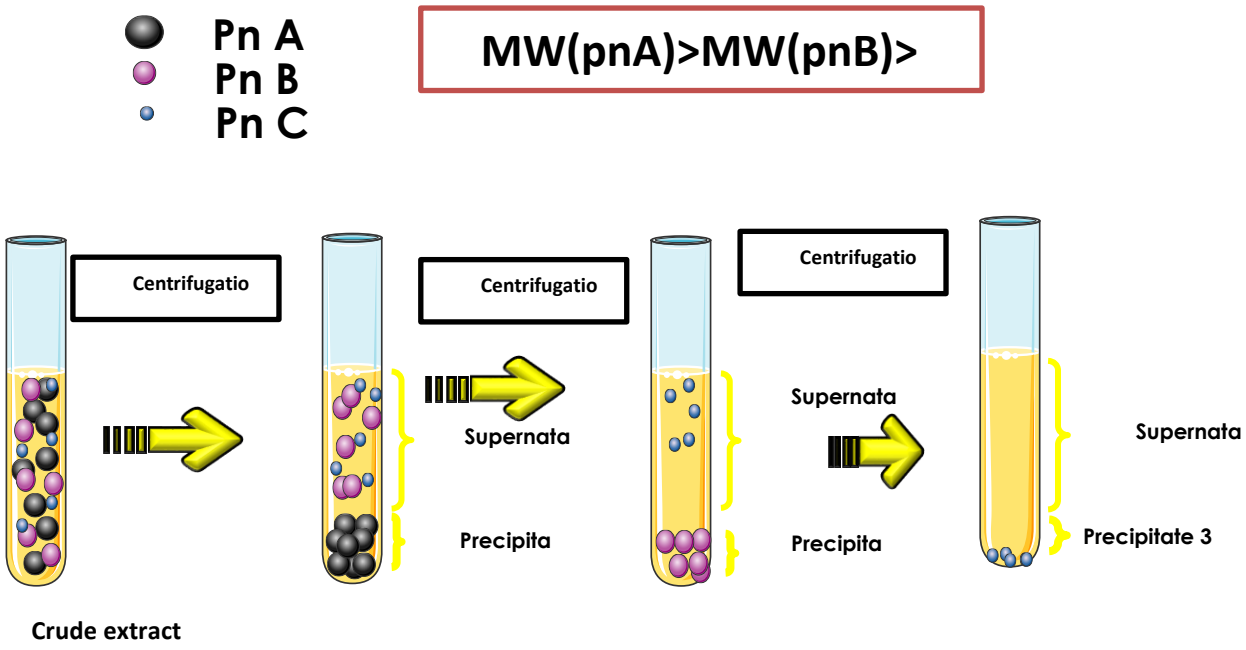


Figure. 47 : Steps of differential centrifugation.

Density gradient centrifugation

In density gradient centrifugation, the different constituents reach a position from which they will no longer move, because they are in equilibrium. But equilibrium is reached when the density of a particle is equal to the density of the solvent, which means that the gravitational force is equal to the Archimedean thrust. We will therefore use a solvent whose density will vary depending on

the position in the tube allowing the different constituents to reach the zone of density equivalent to its own: we speak of a gradient.

Separation methods can be enhanced or refined by centrifugation in a concentration gradient. Indeed, one of the factors which influences the sedimentation speed is the difference between the density of the particle and that of the solvent. We can therefore modulate this speed by varying this density difference in a way by creating a density gradient.

- If the density of the particle is greater than that of the medium, it will sediment. The greater the difference in density, the faster the sedimentation.
- If there is no difference in density, there will be no sedimentation, regardless of acceleration.
- If the particle is less dense than the middle one, it will rise in the tube until it reaches a density level equal to its own or, if necessary, until it floats on the surface.

- **Gradient preparation**

Density variations are obtained by varying the concentration of a chemical in the solution. Various products can be used to make these gradients. They must be very soluble in aqueous solution, which makes it possible to obtain sufficient densities. We are also looking for products that are relatively inert, inexpensive, easy to handle, non-toxic, etc. Obviously, no product combines all these qualities and we must choose taking into account experimental constraints.

The most used gradients are:

- **Sucrose** ("sucrose") is very often used. It makes it possible to achieve fairly high densities, of the order of 1.3 g/ml with 2.5 M sucrose. This product has the advantage of being inexpensive, electrically neutral and rather inert for most cellular fractions. .

- **Cesium chloride** (CsCl): This is another commonly used product, particularly in the separation of nucleic acids.

This salt can reach a very high density, of the order of 1.9 g/mL to 7.5 mol/L. This ability to achieve such high densities in aqueous solution is its main advantage. Its cost and the fact that it is a salt, therefore charged molecules, reduce its scope of application. Other cesium salts can also be used such as CsSO4.

Other products can also be used to separate cells or organelles by density gradient. However, they are often restricted to particular applications. Their cost can also be prohibitive.

- **Metrizamide** is an iodobenzamido derivative of glucose. It is very soluble and uncharged. It makes it possible to achieve fairly high densities of around 1.46.

- **Ficoll Epichlorohydrin** is a polymer of glucose. Its main advantage is that it can produce high densities without generating high osmotic pressures. However, it is restricted to certain specific uses, especially the separation of entire cells, because of its cost.

4.2.4. Centrifuges

A centrifuge is a machine equipped with a rotation axis enclosed in an enclosure. Except for benchtop centrifuges whose rotation speed and typical operating time are relatively limited, it is necessary to prevent samples from heating. For this, the enclosure is refrigerated and often subjected to a high vacuum to avoid friction. A range of devices have been developed according to experimental needs, particularly in terms of the required accelerations, the volumes of material to be centrifuged, the working temperature, etc. :

- The tabletop centrifuges
- The Floor Centrifuges
- Ultracentrifuges
- Microcentrifuges

4.3. Dialysis

It often happens that a preparation of macromolecules contains different products that we want to get rid of. These products, salts, carbohydrates or other small molecules, were present in the initial preparation or were introduced during a purification step. (Exp. extraction or precipitation),A simple way to eliminate these small molecules is to dialysis.

4.3.1. Principle

Dialysis is based on the phenomenon of diffusion through a semi-permeable membrane. Two mechanisms enter into this process:

1. The diffusible molecules will cross the membrane according to the concentration gradient. There will therefore be a net movement of molecules from the more concentrated side to the less concentrated side.
2. At equilibrium, the concentrations of each diffusible species will be equal on both sides (Figure 48).

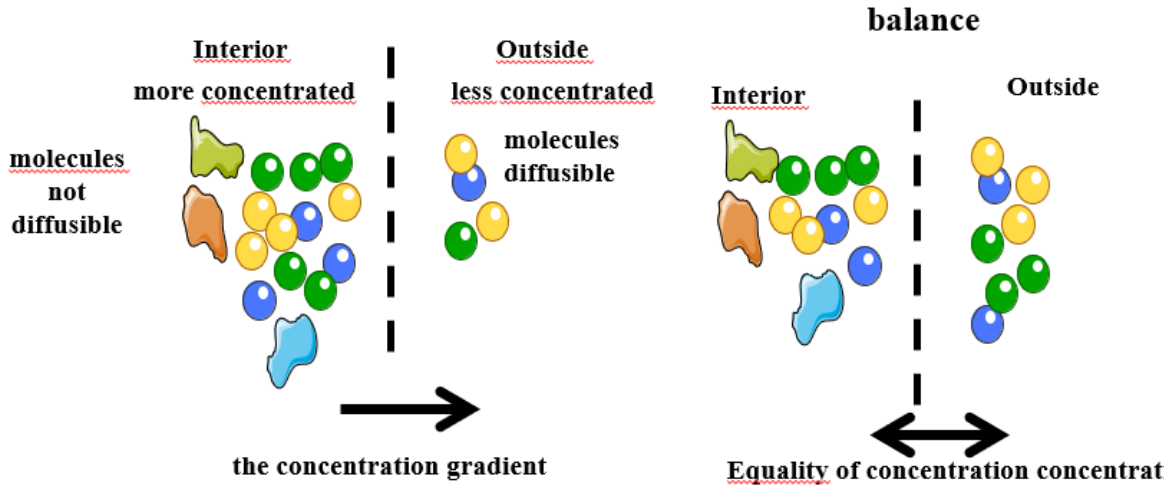


Figure. 48 : The two mechanisms that accompany the dialysis process.

4.3.2. Factors influencing dialysis

Several factors linked to the characteristics of the dialysis membrane, the counter-dialysis solution and the solution to be dialyzed can affect the smooth running of the dialysis:

- **The electrical charge of the dialysis membrane:** the membrane must not be electrically charged in order to minimize the phenomena of repulsion by identical charge or adsorption on the membrane of the product to be dialyzed.
- **Temperature:** the rate of diffusion of a substance across the membrane increases with increasing temperature which amplifies the agitation of the molecules and thus increases the probability for a given molecule to cross the membrane.
- **The membrane surface / volume ratio of the solution to be dialyzed:** for a given volume of solution to be dialyzed, the speed increases with the surface area of the membrane.
- **Presence of chemical substances modifying the dialysis speed by inhibiting it or increasing the speed:** The hormone adrenocorticotropin from the pituitary gland is dialyzed against distilled water. If the water is replaced by a 0.15M ammonium solution, dialysis is practically inhibited due to the appearance of non-dialyzable aggregates, while the addition of urea increases the dialysis rate.

4.3.3. Dialysis equipment

1. The membranes

There are several materials used to manufacture dialysis membranes, the most commonly used are cellulose esters or other cellulose derivatives (Figure 49). Membranes can be obtained with varying levels of porosity but easy to control during the manufacturing process, from 100 to one

million daltons (in terms of protein size). Some manufacturers have developed micro-dialysis systems to treat volumes as small as 250µl.

Figure. 49 : The dialysis membrane

2. Equipment

A dialyzer is designed using a large surface area and thin membrane; on the other hand, the concentration of the diffusing substance to be dialyzed in the counter-dialysis liquid is maintained at zero, either by using a large volume of this liquid, or by constantly renewing it. There are several types of dialyzers; The first dialyzer built was the GRAHAM dialyzer, followed by the MONOD dialyzer.

4.3.3.1. Methodology

1. The solution to be dialyzed is placed in a tube made of a semi-permeable membrane, often cellulose.
2. We close the two ends of this tube, forming a “sausage”.
3. We place this sausage in the solution against which we want to carry out the dialysis in the dialysis buffer

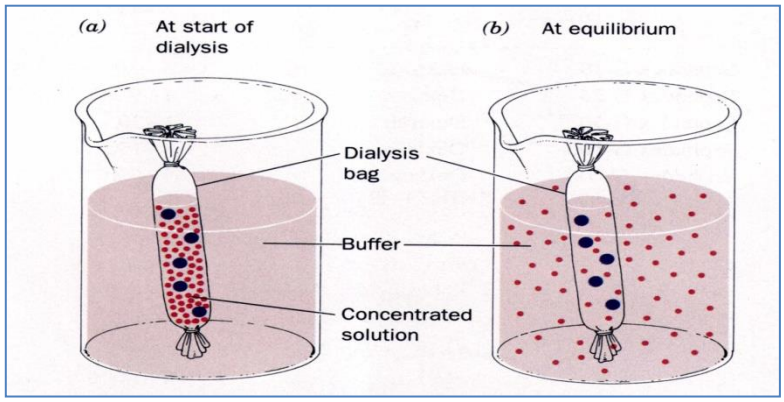


Figure. 50 : The dialysis process.

In order to speed up the dialysis process or avoid the tendency of salts to balance their concentration on either side of the membrane:

- The dialysis buffer should be changed often.
- A much larger volume of dialysis buffer than the protein solution should be used.
- The dialysis tube must be stirred.

4.3.4. Electrodialysis

The elimination of mineral salts thanks to a specific assembly, two peripheral compartments separated from the central compartment by special semi-permeable membranes. The establishment of a direct current of a few milliamperes allows the passage of anions towards the anode and cations towards the cathode. Mineral ions are thus eliminated without loss of organic products. The thermal rise produced by the current does not allow this use with thermolabile products.

4.3.5. Applications of dialysis

- ✓ Eliminate diffusible products contained in protein solutions.
- ✓ Concentrate a protein solution either by placing the dialysis rod containing this solution in a stream of air, or by placing the rod in powdered polyethylene glycol (PEG). The water leaves the tube to solubilize the PEG which is non-diffusible.
- ✓ Electrodialysis allows samples to be desalted before carrying out chromatography on paper.

4.3.6. Precipitation

The cell contains a very large quantity of proteins, among which the one that interests us is most likely a very small minority. It is possible to get rid of the majority of proteins that do not interest us. One of the quickest ways is to rush them.

4.3.6.1. The different types of precipitation

The total precipitation

As its name suggests, total protein precipitation methods aim to remove all proteins from a solution and irreversibly denature the proteins. Total precipitation is used when we want to separate proteins from other small contaminating molecules, amino acids, sugars, etc., or, sometimes, from other macromolecules. This approach is therefore incompatible with procedures where we want to recover an intact and functional protein. Several total precipitation methods are used:

- Precipitation with ethanol or acetone.
- Phenol precipitation.
- Trichloroacetic acid (TCA) precipitation.
- Perchloric acid precipitation (PCA).

Differential precipitation

Differential precipitation, or fractional precipitation, is much gentler than total precipitation. Differential precipitation does not denature proteins. This technique uses the differential solubility of proteins. As each protein is more or less soluble in solution depending on its composition, we can separate several according to their tendency to precipitate more or less quickly when we change the ionic force of the solution.

The electrolyte most used for differential precipitation is ammonium sulfate. This salt is very soluble in aqueous solution and allows very high ionic strengths to be achieved. It is very hydrophilic and competes effectively with proteins for water causing their dehydration. Sulfate and ammonium ions are relatively small and can easily approach charged residues in proteins to neutralize them. This salt also has the advantage of little denaturing of proteins and makes it possible to maximize the production of biologically active proteins.

4.4. Chromatographic methods

4.4.1. Vocabulary

- Chromatography = Techniques for separating the constituents of a homogeneous mixture which is based on a differential migration process, where the analytes are distributed into 2 phases, one mobile relative to the other (stationary phase and mobile phase).
- Chromatogram = signal recorded as a function of elution volume.
- Stationary phase = phase which remains in place in a column or on a plate.
- Mobile phase = eluent phase which moves on or through the stationary phase, it carries the constituents to be analyzed.
- Eluat = solution collected at the outlet of the column. Chromatography allows the identification and dosage of substances.
- Elution= process during which the phases are separated.

4.4.2. Definitions and classification

Chromatography is a physicochemical analysis method that separates the constituents of a mixture into several fractions. This separation is done by driving a mobile phase along a stationary phase. There is therefore a distribution or partition of components between these two phases. In any chromatographic separation, the sample is carried by a mobile phase, a gas, a liquid, or a supercritical fluid. The flow of this mobile phase is forced through an immiscible stationary phase immobilized in a column or on a solid surface. Molecules strongly retained by the stationary phase move much more slowly than the mobile phase while, in contrast, those that are weakly retained move more quickly, their speed, however, can never exceed that of the mobile phase. This

difference in mobility causes the constituents of the sample to separate into discrete bands or zones that can be analyzed qualitatively or quantitatively.

Chromatographic methods bring together very varied techniques which can be classified according to three different modalities:

- Classification according to the physical nature of the phases.
- Classification according to the phenomenon implemented.
- Classification according to the operating process.

We can define two main types of chromatographic techniques according to the nature of their mobile phase:

- Gas chromatography (GC) using a gas as the mobile phase
- Liquid chromatography (LC) where a liquid fulfills the role of mobile phase

Depending on the practical implementation of the method we will distinguish in the latter:

- Surface chromatography on paper or thin layer (TlC)
- Low pressure or high-pressure column chromatography also called High Performance Liquid Chromatography (HPLC)

Depending on the phenomena involved to achieve the separation, we will distinguish within the two techniques:

- Partition chromatography (GC and CL) when the separation is based on the differences in solubility of the molecules to be separated in the stationary liquid phase which permeates a solid support.
- Adsorption chromatography (GC and CL) when the stationary phase is an adsorbent solid, the separation being based on the differences in adsorption of the components of the mixture by the stationary phase.
- Ion exchange chromatography (CL) where the stationary phase is an ion exchanger, that is to say a solid containing ions and capable of exchanging them with those of the solution with which it is in contact
- Exclusion chromatography (CL) also known as gel permeation chromatography (or filtration). The fixed phase is a porous solid whose pore size is close to that of certain molecules in the mixture to be separated. The molecules of the mixture whose dimension is greater than that of the pores are excluded from the fixed phase and are first eluted, those

which can enter are carried away with a certain delay. This delay is all the greater as they easily penetrate into the pores.

4.4.3. Adsorption chromatography

This liquid-solid chromatography is based on the distribution of solutes between the fixed stationary phase (the adsorbent) and the mobile liquid phase (the eluent). Each of the solutes is subjected to a retention force (by adsorption) and a driving force by the mobile phase. The resulting equilibrium results in a differential migration of the solutes in the sample to be analyzed, which allows their separation.

1. Adsorption

It is the fixation of dissolved molecules by the stationary phase. This fixation is due to the establishment of secondary surface bonds between the adsorbent and the adsorbed molecule: dipole-ion bond, or dipole-dipole or Van der Waals bond.

2. Adsorbents

These are very divided solids (adsorption being a surface phenomenon, the adsorbent must have the largest possible useful surface area). It is characterized by the following criteria:

- Adsorption capacity: we distinguish between weak adsorbents (with low adsorption capacity), such as talc or sodium carbonate, and strong adsorbents (with high adsorption capacity) such as silica gel or alumina.
- Polarity: some adsorbents have a strong polarity, such as silica gel or alumina, while others, on the contrary, have a weak polarity such as activated carbon.
- Particle size: an adsorbent with a small particle size ensures better separation but, on the other hand, this occurs much more slowly.

Silica gel is the most widely used and cost-effective adsorbent to try first for separating neutral compounds containing one or two functional groups. On the other hand, to separate bases, it is preferable to use alumina that is more basic than silica. These two adsorbents are available commercially with the addition of a substance which allows the deposits to be visualized under UV light.

3. Solvents or eluents

For a given chromatographic system, the eluting power depends on the polarity of the solvent. The more polar an eluent is, the more easily it will entrain a polar substance. On the other hand, a non-polar solvent will have a poor eluting power with respect to polar substances but will easily carry away a non-polar constituent. Either a pure solvent or a mixture of several solvents is used in order to “adjust” its eluting power to the chromatographic system studied.

4. The interaction of substance-adsorbent and substance-eluent

Substance-adsorbent interactions essentially correspond to the establishment of dipole-dipole, dipole-ion or Van der Waals type bonds. In general, the more polar an adsorbent is, the more polar substances it binds.

Substance-eluent interactions are of two types:

- Dissolution of the substance by the eluent: substances tend to dissolve in the eluent and migrate with it. It is this phenomenon which is preponderant in the case of slightly polar solvents (hydrocarbons, ether, carbonyl compounds).
- Displacement of molecules adsorbed by the eluent: the eluent molecules seek the same adsorption sites as the molecules of the adsorbed substance and "displace" the latter. It is this phenomenon which is preponderant in the case of polar and protogenic solvents (alcohols, water, acids).

Adsorption chromatography is applied using different techniques:

- on a thin layer: the adsorbent gel (cellulose, silica) is poured onto a plate (glass, aluminum, plastic), mixed with a binder (plaster);
- on paper: in ascending or radial chromatography; in this technique the paper constitutes the fixed phase;
- on column: open at ambient pressure, in flash chromatography, at medium pressure, in HPLC.

4.4.4. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is mainly based on adsorption phenomena: the mobile phase is a solvent or a mixture of solvents, which progresses along a stationary phase fixed on a glass plate or on a semi-sheet. rigid plastic or aluminum material. After the sample has been deposited on the stationary phase, the substances migrate at a speed which depends on their nature and that of the solvent.

The main elements of a thin layer chromatographic separation are (Figure 51):

- The chromatographic tank: a container usually made of glass, of variable shape, closed by a waterproof lid;
- The stationary phase: a layer of approximately 0.25 mm of silica gel or another adsorbent is fixed on a glass plate using a binder such as hydrated calcium sulfate (plaster of paris) l starch or an organic polymer;
- The sample: approximately one microliter (µl) of diluted solution (2 - 5%) of the mixture to be analyzed, placed at a reference point located above the surface of the eluent;

- The eluent: a pure solvent or mixture that slowly migrates along the plate carrying the sample components

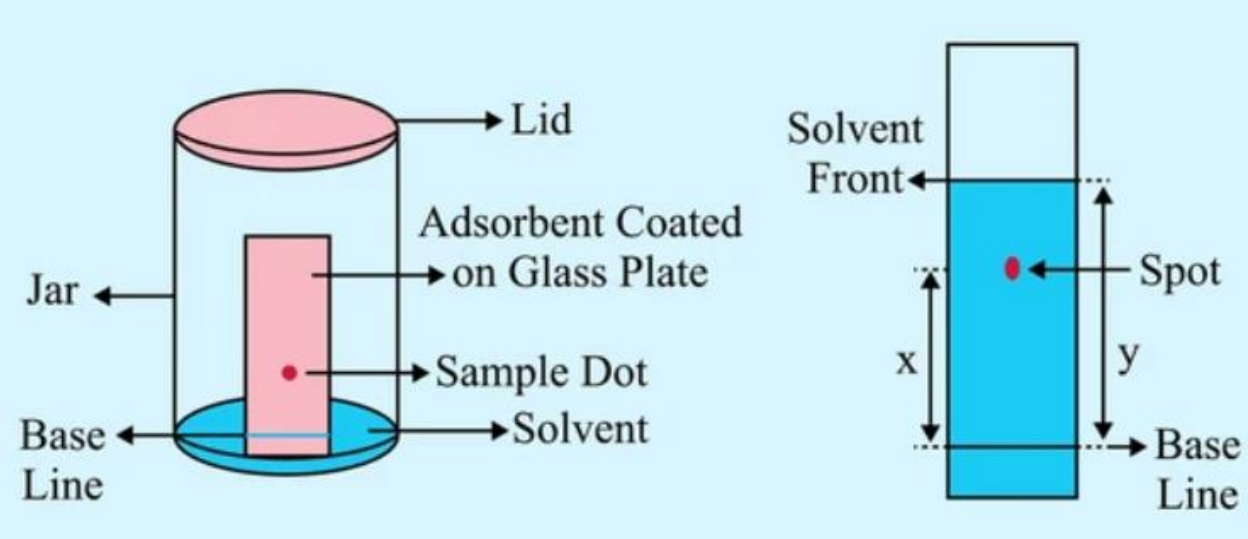


Figure. 51 : The main elements of a TLC separation.

3.7.5.1. Principle of the technique

When the plate on which the sample was placed is placed in the tank, the eluent rises through the stationary phase, essentially by capillarity. Additionally, each component of the sample moves at its own speed behind the solvent front. This speed depends on the one hand, on the electrostatic forces holding the component on the stationary plate and, on the other hand, on its solubility in the mobile phase. The compounds therefore move alternately from the stationary phase to the mobile phase, the retention action of the stationary phase being mainly controlled by adsorption phenomena. Generally, in thin layer chromatography, substances with low polarity migrate faster than polar components.

4.4.5. Column chromatography

While other chromatographic methods are usually employed for the analysis and separation of very small quantities of products, column chromatography can be a preparative method; it allows the separation of the constituents of a mixture and their isolation, from samples whose mass can reach several grams.

However, it has several disadvantages:

- Large amounts of solvent are required for elution;
- The duration of the elution is generally very long;
- Detection of compounds requires constant attention.

It is suitable for the purification of small quantities of product, when the operating conditions are perfect. However, the method being very empirical, its development often requires numerous tests.

It is a technique based on adsorption phenomena. The solid phase, most often alumina or silica, fills a column of variable length and section; the sample, in concentrated solution, is deposited at the top of the column and the separation of the components results from the continuous flow of an eluent, crossing the column by gravity or under the effect of low pressure. A single solvent can be used as eluent or the polarity of the eluent can be gradually increased so as to accelerate the movement of the compounds.

The molecules are carried downward at varying speeds depending on their affinity for the adsorbent and their solubility in the eluent. The chromatogram develops by forming a succession of cylindrical zones which separate by migrating downwards (Fig. 52).

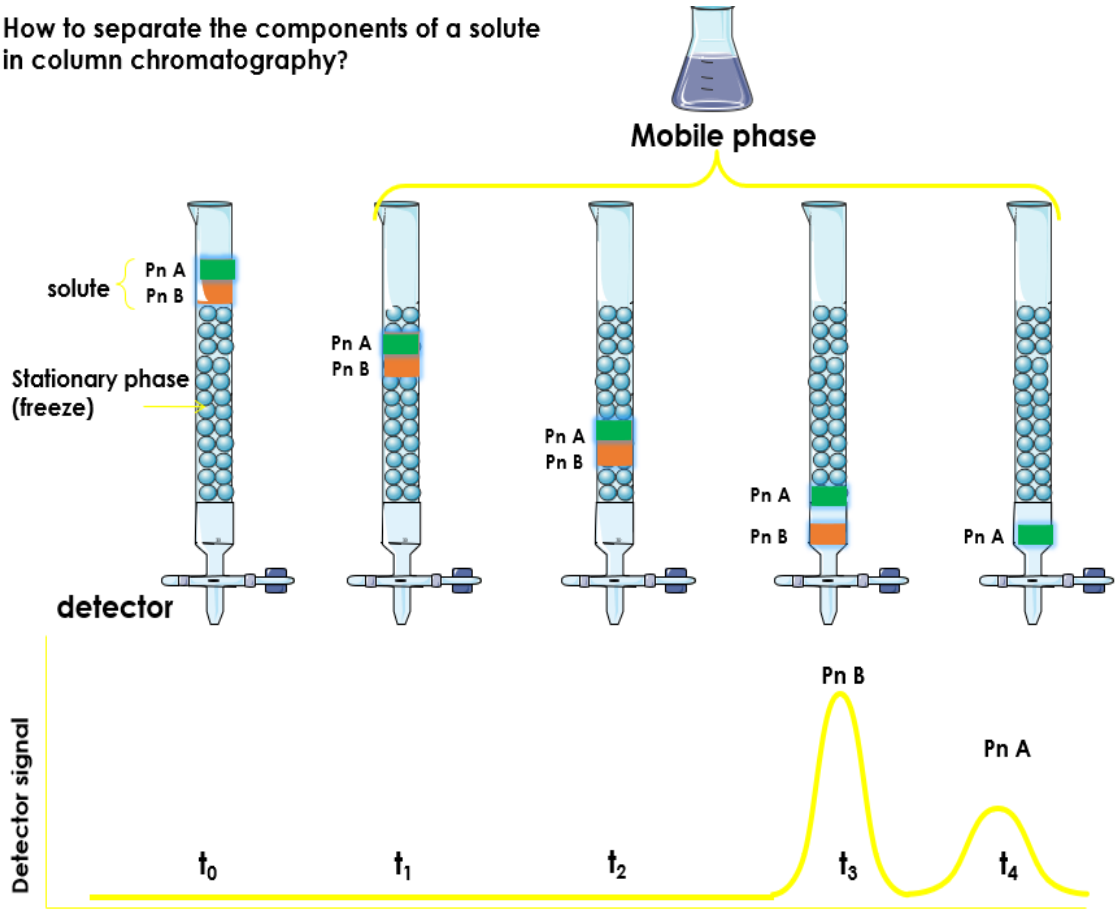


Figure. 52 : Separation of proteins in column chromatography.

4.4.6. Affinity chromatography

Affinity chromatography is a purification method based on reversible biospecific interactions between an effector fixed on a matrix and its affinity partner in solution. Very often, the attached molecule will be the substrate, the ligand, or the antibody. This will purify the enzyme, receptor or antigen, respectively.

In this type of chromatography, the stationary phase is a chemically inert macromolecular support onto which is grafted an effector which has a biological affinity for a compound of the sample to be analyzed. It must be hydrophilic, porous and provided with reactive functional groups

allowing the attachment of the effector or a spacer arm. The spacer arm is a carbon chain of 4 to 8 carbons whose role is to allow better interaction between the effector and its affinity partner. The spacer arm must have two functional groups (Figure 53).

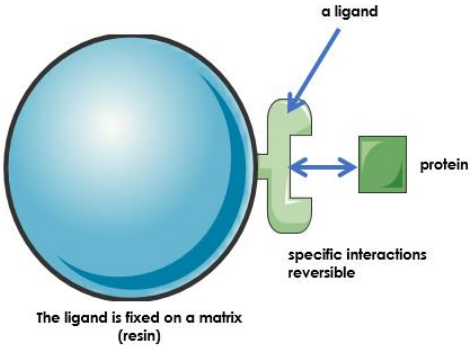


Figure. 53 : The elements that constitute affinity chromatography

The effector must be of high purity as it must contain a functional group. The ligand is attached to the matrix directly or indirectly using a coupling method. The latter must never denature the ligand (Figure 54).

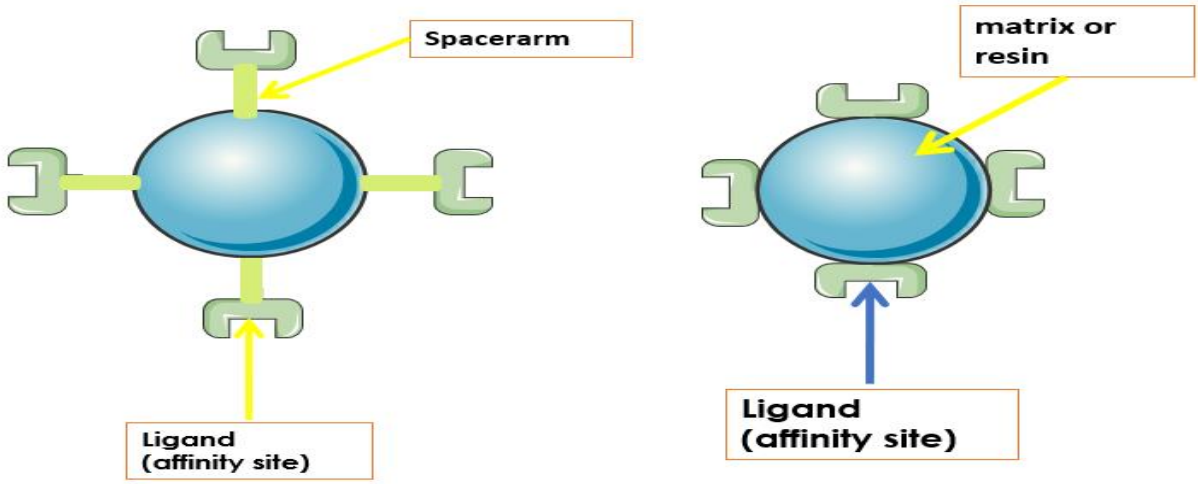


Figure. 54 : The effector can be attached directly (a) or indirectly (b).

1. The stationary phase:

It consists of an effector covalently attached to a support (carboxymethylcellulose, Sephadex, polyacrylamide gel) either directly or indirectly.

Examples derivatives of carboxymethylcellulose or CM-cellulose

- CM-aminohexyl: allows the fixation of an effector with a carboxyl function: -O-CH2-CO-NH-(CH2)6-NH2 (long spacer)
- CM-hydrazide: allows the fixation of an effector with a carboxyl function: -O-CH2-CO-NH-NH2 (short spacer)
- CM-aminohexyl (or aminododecyl) succinylated: allows the fixation of an effector with reactive -NH2 function. -O-CH2-CO-NH-(CH2)n-NH-CO - CH2-CH2-COOH (n = 6 or 12, long spacer).

2. Effectors

- **Enzyme-substrate affinity**: substrates, analogues, reversible inhibitors, allosteric effectors, coenzymes.
- **Ligand-receptor affinity**: hormones, peptides, peptide analogues
- **Antigen-antibody affinity**: haptens, antigens, antibodies.

3. Operating mode

1. Choice of mobile phase
2. Filling the column
3. Column balancing
4. Sample injection
5. Washing the column to remove unadsorbed substances
6. Elution of adsorbed or fixed substances:

- **Non-specific elution**: This is increasing the salt concentration (Fi), or changing the pH.
- **Specific elution**: The use of an elution buffer which contains an "unfixed ligand" competitor

7. Recovery of fractions
8. Chromatogram analysis
9. Gel regeneration

4.4.7. Size exclusion chromatography

Exclusion chromatography, also called gel permeation chromatography or gel filtration, applies well to high molecular mass species. The material used as a basis is a gel, composed of small, very regular and well-calibrated particles (microbeads, grains, balls or very porous gel beads). The structure of the gel results from the bonding of macromolecules assembled together so as to form a regularly cross-linked assembly.

We distinguish between highly crosslinked gels whose meshes are very narrow, moderately crosslinked gels whose meshes are medium and gels with a low degree of crosslinking whose meshes are open. Placed in the presence of the solvent, these grains swell by penetration of the mobile phase inside the grains.

1. Principle

The porosity of the gel allows the molecules to be separated according to their molecular weight. This involves separating proteins according to their size or shape using a molecular sieve. It is a chromatography whose column is filled with a porous gel, the diameter of the pores is close to the size of the molecules to be separated.

Large molecules whose diameter is greater than that of the pores are excluded and therefore eluted first. Small molecules and medium-sized molecules are eluted later, they penetrate the pores of the gel (are included in the gel), their migration is therefore delayed (Figure 55).

A given gel has a specific fractionation domain within which proteins will take a time proportional to their size (weight) to exit the gel. Within this margin, molecules can be separated from each other. Molecules larger than the upper fractionation limit (exclusion limit) will come out together. Whereas, the smallest molecules (the lower limit of the fractionation domain) will come out together much later. In these two cases it is obviously impossible to separate the molecules according to their mass. Molecules with a mass between these two limits can therefore be separated from each other (Figure 55).

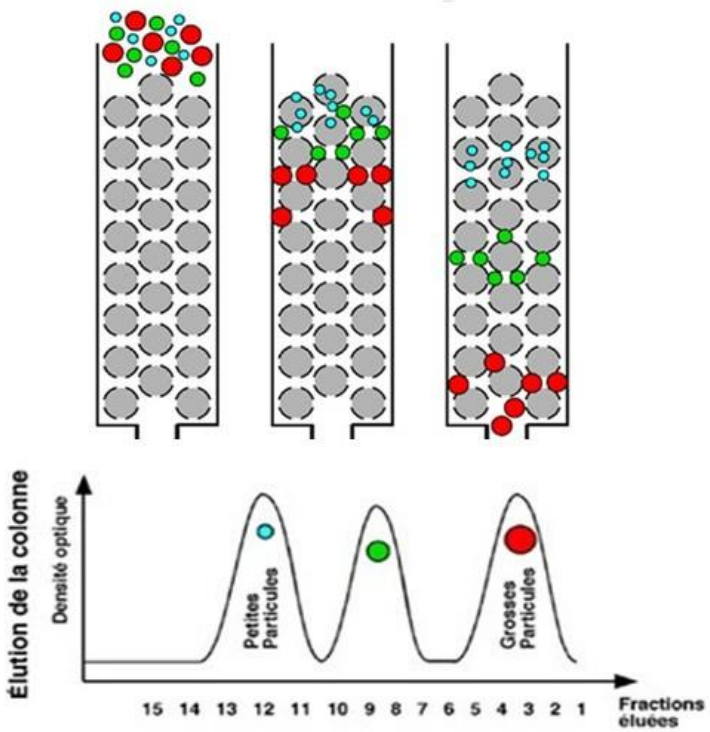


Figure. 55 : Chromatogram obtained by size exclusion chromatography.

2. Chromatographic equipment

It is composed of: a sample, the mobile phase, the column, the pump, the detector, the fraction collector and the recorder (Figure 56):

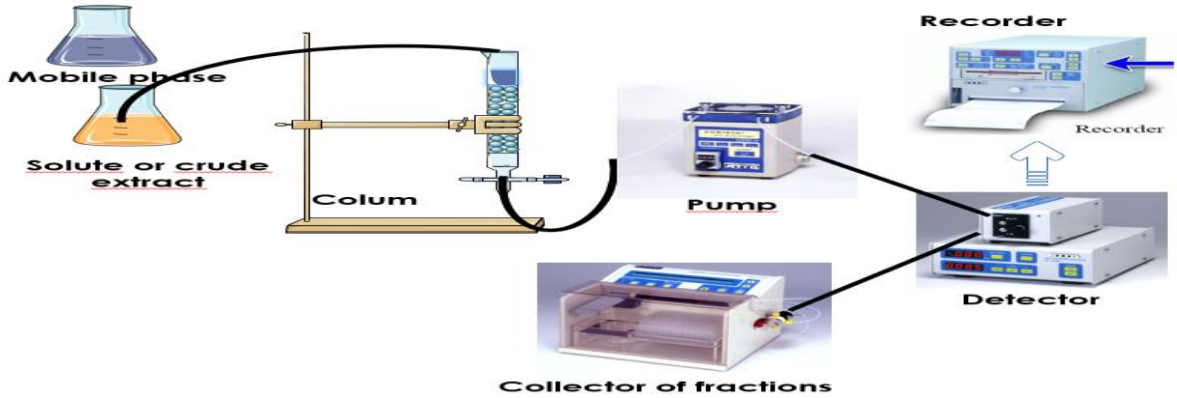


Figure. 56 : The constituents of chromatographic equipment.

a) **Sample:** solution which contains the different cellular constituents that we want to purify**.**

b) **The stationary phase**: fixed phase or gel (made up of microbeads or granules) is in a column,

c) **The mobile phase:** it is a liquid or buffer that passes and moves through the stationary phase, entraining the constituents of the solute.

d) **The column**: it can be made of glass, plastic or stainless steel (high pressure), the size is variable. It is the support which carries the stationary phase (gel or resin) through this gel, the mobile phase passes.

e) **The pump :** allows you to adjust the flow rate of the mobile phase through the column.

f) **The detector**: at the exit of the column, the compounds encounter an essential element which is called the detector. This element evaluates the quantity of each of the separated constituents. The detector sends an electronic signal to a recorder

g) **The recorder:** will draw the peaks according to their intensity, their elution volume or retention time. The set of peaks is called a chromatogram.

h) **The fraction collector:** it is a device which allows the different fractions of the solute to be collected (for example: 2ml/tube).

3. The stationary phase

The stationary phase is generally a porous polymer (dextran, polyacrylamide, agarose) whose pores have dimensions chosen in relation to the size of the species to be separated. We create a kind of sieve at the molecular scale, called selective permeation. CSE gels are characterized by their separation interval (separation limit), this interval is dictated by the porosity of the gel. These gels are characterized by:

- **Porosity**: determines the filtering power (there are microporous or macroporous gels).
- **Chemical inertia**: no adsorbent power with respect to solutes or interaction with the mobile phase.
- **Physicochemical stability**: resistance to T° and pressure (rigid gel: 5-20 atm, soft gel, pressure <1atm).
- **Granulometry**: the size of the gel granules (10-120µM: for more or less large particles, <40µM: fine particles).
- **The form**: the shape which ensures uniform flow of the mobile phase is the spherical shape).

4. The mobile phase

It is a solvent that induces swelling of the stationary phase and dissolves the sample. Water is used for hydrophilic gels and organic solvents if the gel is hydrophobic.

5. Operating mode

- Preparation of the gel (dry gel): dry gels must undergo swelling beforehand. This inflation can be carried out at 20°C (72h) or at 90°C (2h).
- Choice of column length: When the molecular distribution is very distinct, the column length should not exceed 30 cm. On the other hand, if the MM are very close, the length of the column must be 60 to 100 cm.
- Mobile phase degassing: Gel filtration resins are rather fragile, especially those with a high exclusion limit because they have high porosity. Flow rates and pressure must therefore be carefully controlled to prevent the gel beads from bursting.
- Introduction of the gel inside the column.
- Connect the pump: it drives the mobile phase with a flow rate allowing the homogenization of the gel.
- Equilibration of the gel by the mobile phase: the stationary phase is balanced by passing the mobile phase 2 to 3 times its volume.
- Injection of controls and sample.
- Collection of fractions.
- Regeneration of the column, or storage of the column at low temperature in the presence of an antibacterial agent.
- Use of chromatograms and biochemical analyzes of the collected fractions.

Gel filtrations can be carried out without problem at room temperature. However, they are often done at 4°C, in a cold room or a chromatography refrigerator, to avoid protein degradation. This is obviously the case during protein purification where protein stability is crucial.

3. Apps

- Desalination
- Purification
- Virus elimination (sterilization)
- Determination ofMM(Appendix)

4.4.8. Ion exchange chromatography

Ion exchange chromatography (IEC) is a technique where the separation is a function of the electrical charge. The stationary phase is an ion exchanger consisting of a resin carrying negatively or positively ionized groups, exerting electrostatic (ionic) interactions with ionized proteins (Figure 57).

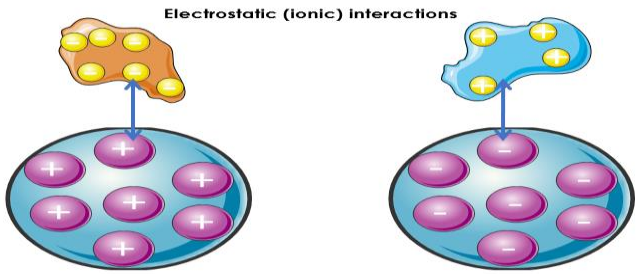


Figure. 57 : Electrostatic interactions exerted by proteins and resin.

4.4.8.1. Principle

In this chromatography, the column is filled with a stationary phase carrying negatively or positively ionized groups. It is a separation of proteins based on reversible ionic interactions between:

5. A stationary phase called ion exchangers
6. Exchangeable or mobile counter ions
7. A charged solute or protein

In an ion exchange column, proteins stick to charged groups on the resin through ionic interactions (Figure 58).

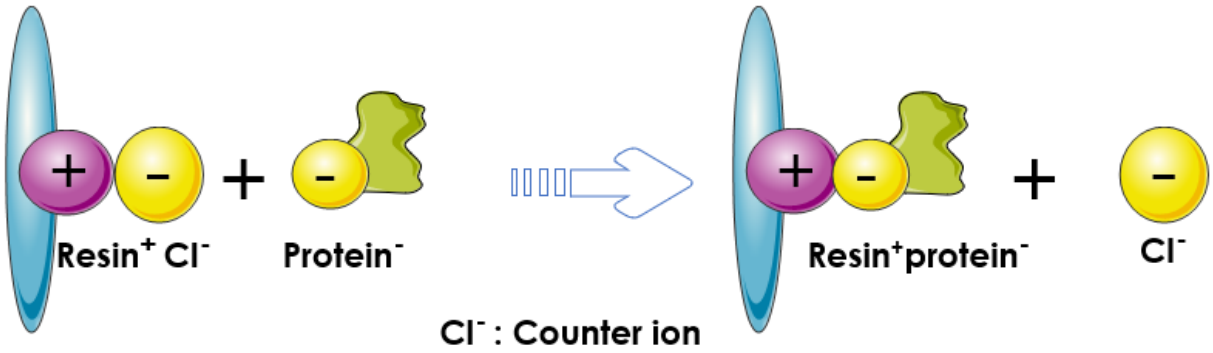


Figure. 58 : Principle of ion exchange chromatography.

4.4.8.2. Materials

a) The stationary phase

The stationary phase must be insoluble and chemically stable, the structure must be stable and its particles preferably spherical and uniform. It is a porous and insoluble solid support comprising ionized functional groups (positive or negative) allowing the retention of the species to be separated. Ion exchangers therefore include anions and cations. One of these ions is fixed on the support, while the other, called "counter-ion", is more or less labile and exchangeable depending on whether the exchanger is strong or weak (Figure 59).

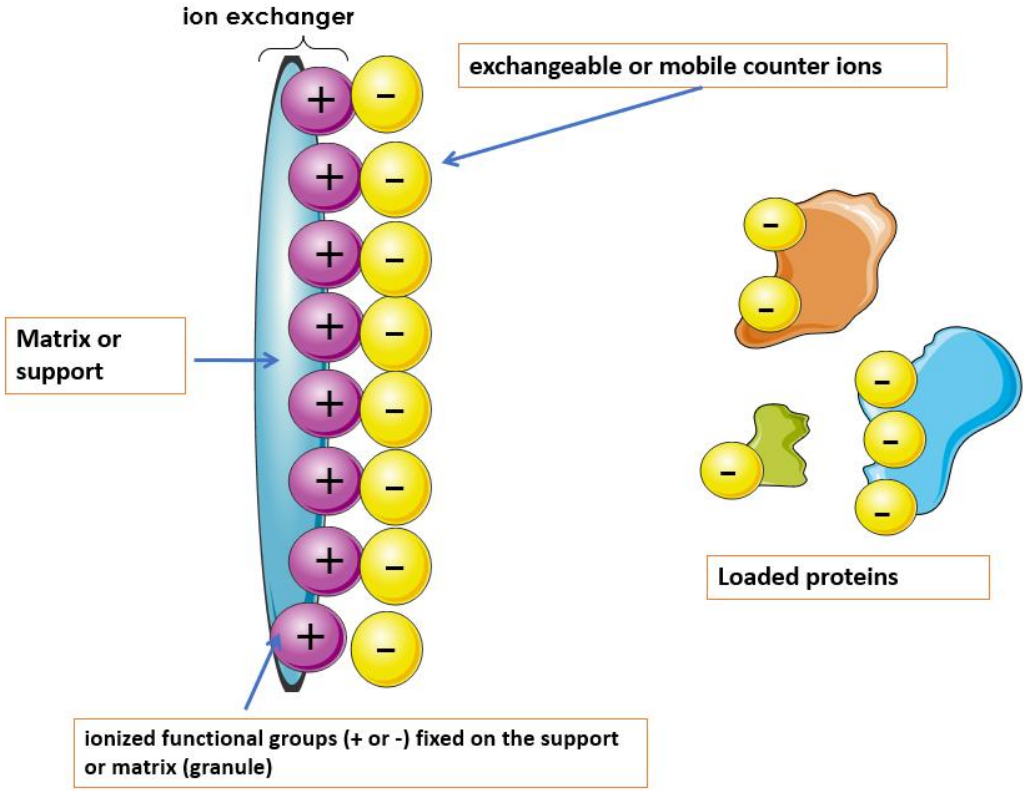


Figure. 59 : The main components of a CEI.

b) The different types of exchangers

- **Anion exchanger:** which reversibly exchanges anions. An anion exchanger is positively charged
- **Cation exchanger:** which reversibly exchanges cations. A cation exchanger is negatively charged

The most used groupings are:

- For cation exchangers (anionic resin): sulfonate (-SO3-), phosphonate (-PO32) and carboxylate (CO2-);
- For anion exchangers (cationic resin): quaternary (-NR3+), tertiary (-NR2+) or secondary (-NR+) ammonium.

Certain groups are called "strong" (sulfonate or quaternary ammonium) because their exchange capacity is constant and independent of pH. Other groups are called "weak" (the other groups) because at certain pHs, they are not ionized and therefore do not fulfill their role as exchangers.

c) The mobile phase and elution

Most separations using ion exchange resins are carried out with an aqueous solution due to the solubilization and ionization properties of water. However, it may be necessary to use mixtures of polar solvents (methanol-water) in order to optimize elution or facilitate the solubility of certain

solutes. Elution is ensured by shifting the balances**by changing the pH**or by increasing the ionic strength:

The pH influences the net charge of the protein (amphoteric character):

- **If pH>Pi: pn-:** To elute this protein, it is necessary to reduce the pH. The protein will be positively charged, it will detach from the resin.
- **If pH<Pi: pn+:** To elute this protein, it is necessary to increase the pH. The protein will be negatively charged, it will detach from the resin.

The ionic force exerts a competition effect (displacing the fixed "protein" ion by another ion which is highly charged and of higher concentration (e.g. Cl-, HO-, Na+, H+...).

The order of increasing effectiveness of counterions for cation exchange resins is as follows: monovalent cations: Li+ < H+ < Na+ < NH4+, divalent cations: Cd2+ < Mn2+ < Mg2+ < Zn2+ < Cu2+ < Ca2+ in addition, l The efficiency increases with the load: K+ < Ca2+ < Al3+.

4. Operating mode

1. Choice of gel
2. Choice of mobile phase
3. Adjustment of working conditions
4. Filling the column
5. Column equilibration (fixation of counter ions)
6. Sample injection (the protein fixation or adsorption step)
7. Elution (desorption step by Fi or pH)
8. Recovery of fractions
9. Chromatogram analysis
10. Gel regeneration

4. Applications of the IEC

- Water deionization (softeners)
- Separation
- Ion dosage.Gas chromatography

4.4.9. Gas Chromatography

Gas chromatography (GC) is a method for separating compounds that can be vaporized by heating (without decomposition). Separation is done in a column either by partitioning or by adsorption. She permits :

- Microanalysis (from µg to mg)
- Separation of complex mixtures
- Easy qualitative and quantitative analysis
- Analyzes in many application areas.

1. Principle

The separation principle is based on a difference in the distribution of the compounds in a mixture between two phases, a mobile phase (the carrier gas) and a stationary phase. The mixture to be analyzed is injected in the form of a fluid and is vaporized in the injector. The carrier gas carries it into the thermostatically controlled separation column. The compounds are distributed differently in the 2 phases, therefore move at different speeds then exit at different times. When they exit, they are detected and peaks appear on the recorder (Figure 60).

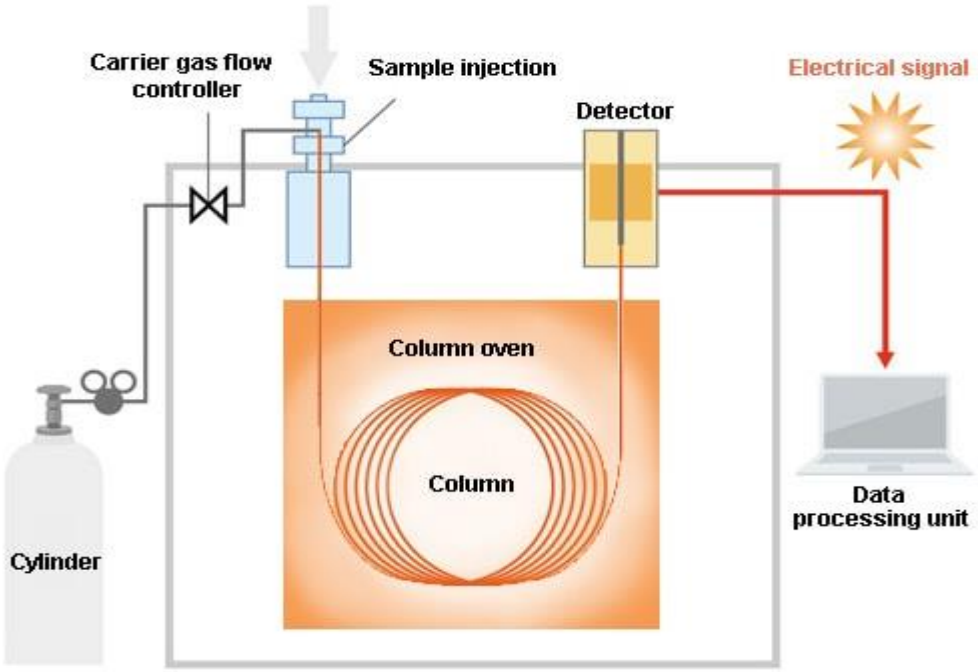


Figure. 60 : Principle of CPG.

2. Materials

A GC device schematically includes an injector, a column contained in a thermostatically controlled enclosure (oven) and a detector connected to an integrator or a computer on which the chromatogram appears (Figure 61).

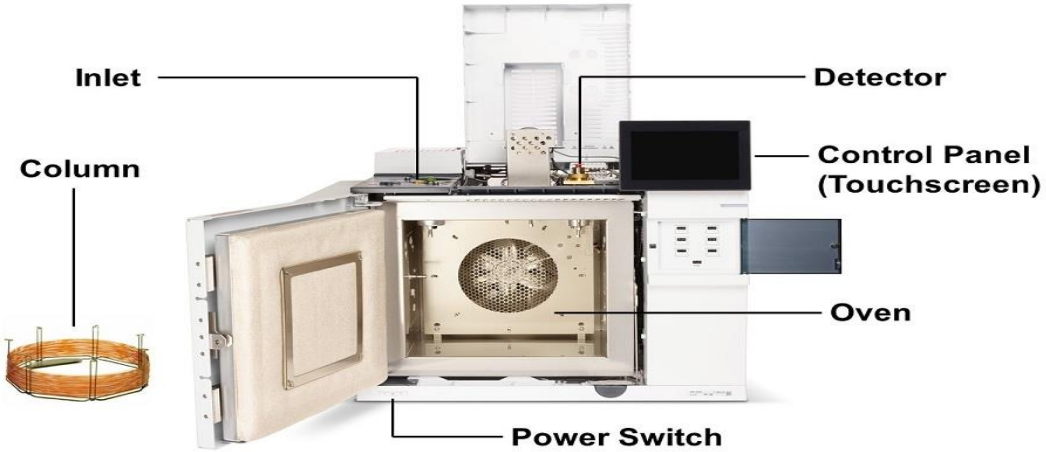


Figure. 61 : The CPG device.

a) Gas

Two types of gas are used in gas chromatography: carrier gas and combustion gas.

The carrier gas it is an inert and pure gas such as helium, dinitrogen or dihydrogen. The nature of the gas does not significantly modify the separation of the components due to the absence of interaction between the gas and the solutes, only the temperature factor is important. It is used to carry the solute from its injection until it leaves the column. The choice of carrier gas depends on the detector used. The flow rate of the carrier gas varies from:

30 to 40 ml/min: for classic columns.

0.2 to 2 ml/min: for capillary columns.

The most commonly used combustion gases are hydrogen and oxygen. These gases are used for the combustion of the organic matter leaving the column and detection

d) The oven

The oven is intended to receive the columns and bring them to the desired temperature (Figure 62). It is an air bath oven, equipped with heating elements and a ventilation and mixing system for temperature homogenization. The oven temperature may be:

- Stable and identical from start to finish of handling (= isothermal conditions)
- Programmed in successive stages (= in gradients)

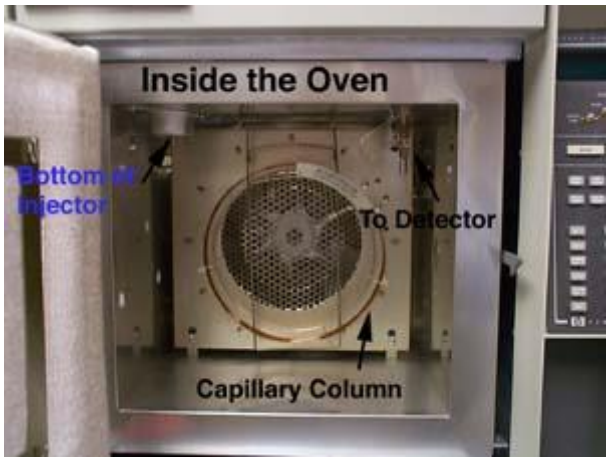


Figure. 62 : The oven.

e) The column

The stationary phase is in the form of thin coiled tubes. There are two types of columns:

- **Ordinary (filled) columns**: are filling columns, their diameter is 2 to 6 mm, their length is 1 to 5 m. They are made of steel or glass tubes. They are filled with a porous and inert support in the form of spherical grains (approximately 0.2 mm in diameter) on which the stationary phase is impregnated. Less resolving than capillary columns (Figure 63).
- **Capillary columns (open tube)**: are not filling columns, their diameter is 0.10 to 0.53 mm, their length is 10 to 100 m. They are made of stainless-steel tubes or fused silica; The

stationary phase is directly deposited on the internal wall of the column to a thickness of 0.05 to 5 µm (Figure 63).

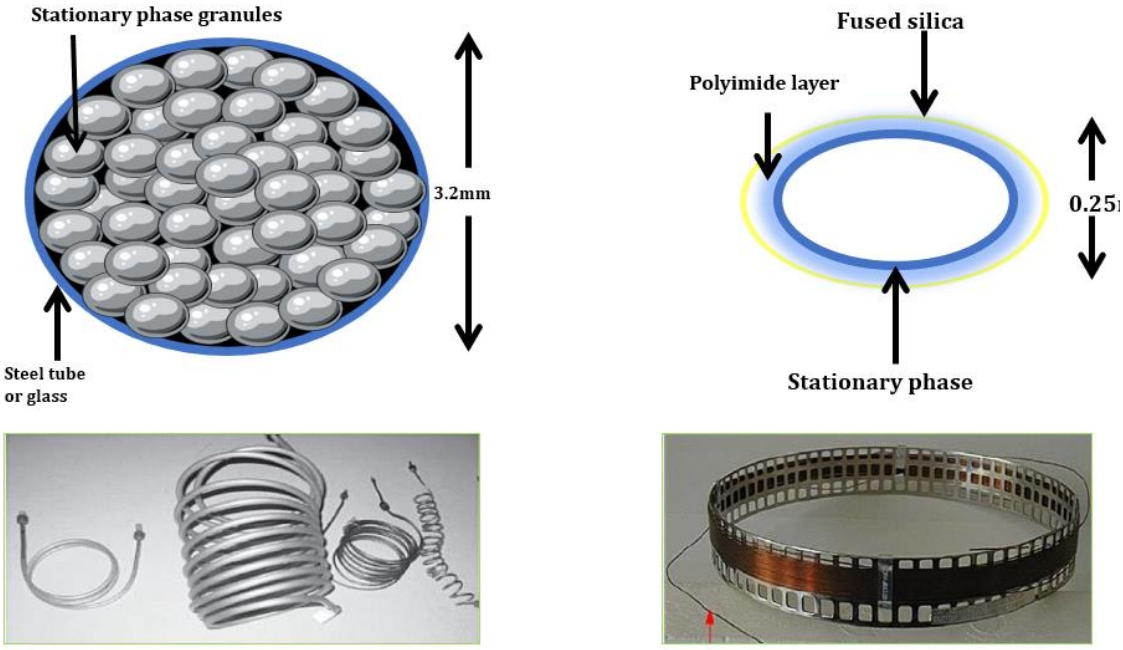


Figure. 63 : Filled column (a and b) and capillary column (b and c).

Several stationary phases are used in CPG:

- Solid stationary phases are made up of small, very homogeneous particles (alumino-silicate crystals).
- Liquid stationary phases are classified according to their polarity and the temperatures they are capable of withstanding: Polyether glycol (Carbowax) – polar phases based on polyethylene glycol – withstand up to 225°C. Squalanes – non-polar, saturated hydrocarbons – withstand up to 120°C.

f) The stationary phase

The choice of stationary phase is:

- An apolar phase will retain a compound the more it is apolar (and vice versa). Example: Squalane (apolar); Carbowax (fleece)
- A non-polar phase will retain the compounds in the order of their boiling temperature (therefore leaving the compounds in the order of their increasing boiling temperature)
- A phenyl phase will better retain an aromatic compound. Ex.: OV225 phases; DC550
- A fluorinated phase will retain ketones better. Ex.: phase QF1.

g) The injection system

Depending on the types of columns connected to the injectors, the characteristics of the injectors and their injection mode are different in order to optimize the quality of the separation:

- **Direct Spray Injector**

for packed columns and large diameter capillary columns. All the sample introduced by the syringe is entirely carried into the column

For low flow rate capillary columns, the amounts of compounds in the column must be very small to avoid saturation of the columns. The injected volumes must be very low (saturation even for v injected = 0.1µL). However, the injected volumes cannot be less than 0.1 µL and the use of very diluted solutions leads to disturbances due to excess solvent vapor. There are therefore different injectors to solve this problem.

- **Injector with leak system**

A large portion of the injected sample, vaporized and mixed with the carrier gas, is removed from the injector through a leak valve. Thus, a small fraction of the mixture enters the column. There are two modes depending on whether you are injecting and the leak valve is open (split mode) or valve closed for approximately 1 minute after injection (splitless mode) (Figure 64).

- **Programmable Temperature Injector (PTV)**

The mixture is introduced liquid into the cold injector then the injector is heated in split or splitless mode to vaporize the compounds

- **Cold injection into the column**

the mixture is injected directly cold and liquid into the column.

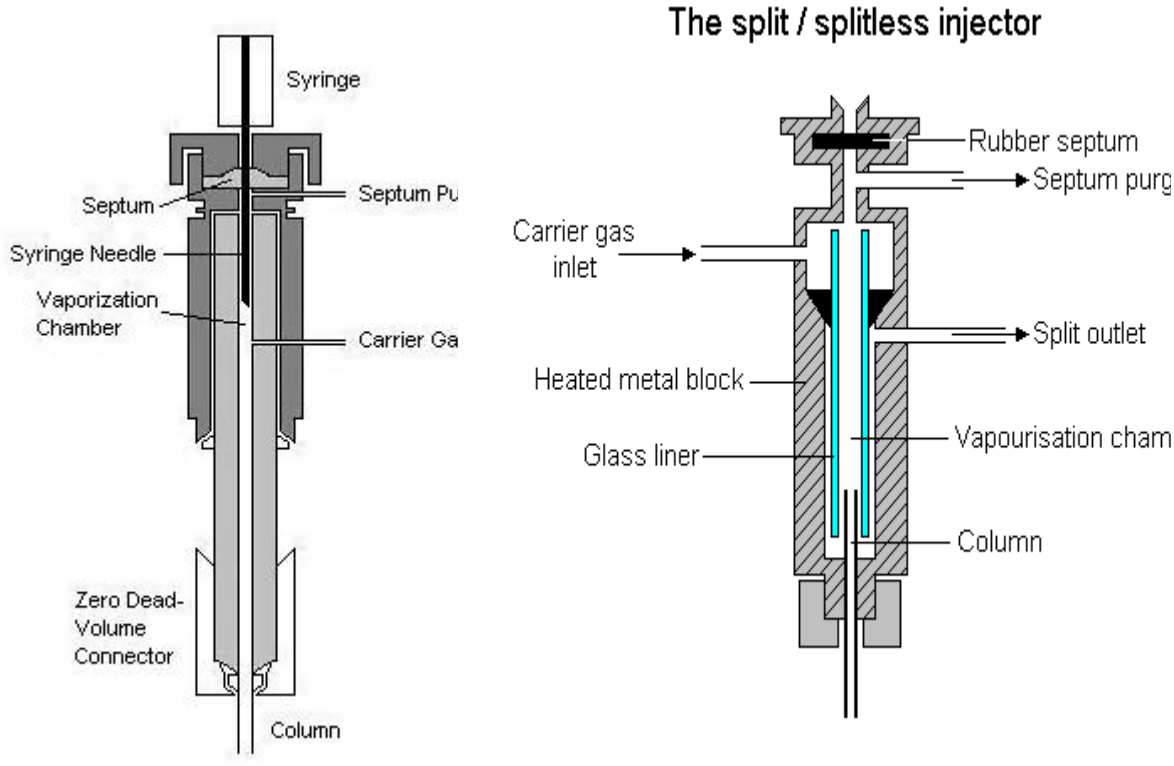


Figure. 64 : Direct spray injector (a) and Injector with leak system (b).

h) Detectors

After separation, the different constituents of the mixture are detected by detectors using various physical properties such as:

- Thermal conductivity (katharometer detector),
- The variation of the electrical properties of a flame (flame ionization detector) or
- The capture of electrons by the different separated molecular species (electron capture detector).

The table below shows the different types of detectors:

Tableau. 2 : The different types of detectors.

DETECTOR	Carrier gas	Sensitivity	Applications
Catharometer	H2/He	1 to 10 ng	All compounds
FID	He/N2	20 to 100 pg	Organic compounds
Email capture	N2	0.1 pg	Halogenated compounds
Thermionics	N2	P:1 pg / N:10 pg	Compounds with N or P
Flame photometry	N2/H2	P: 10 pg S: 1ng	Compounds with S or P

N: nitrogen compound, P: phosphorus compound.

4.4.10. High performance liquid chromatography

High-performance liquid chromatography or HPLC is liquid phase chromatography on a column, the performance of which, in terms of selectivity and resolution, is greatly improved. This chromatography uses a liquid mobile phase and a very finely divided stationary phase. To obtain a satisfactory flow rate, the eluent must be injected under pressures of several hundred bars.

4.4.10.1. Principle

HPLC uses a liquid mobile phase to separate the components of a mixture. These components are first dissolved in a solvent, then forced to flow through a chromatographic column under high pressure. Each solute is therefore subject to a retention force (exerted by the stationary phase) and a mobility force (due to the mobile phase).

4.4.10.2. Chromatographic equipment

The compounds to be separated (solutes) are dissolved in a solvent. This mixture is introduced into the liquid mobile phase (eluent). Depending on the nature of the molecules, they interact more or less with the stationary phase in a chromatographic column (Figure 65). The mobile phase pushed by a high pressure pump travels through the chromatographic system. At the column outlet, thanks

to an appropriate detector, the different solutes are characterized by peaks. The set of peaks recorded is called a chromatogram (Figure 66).

Figure. 65 : The HPLC device

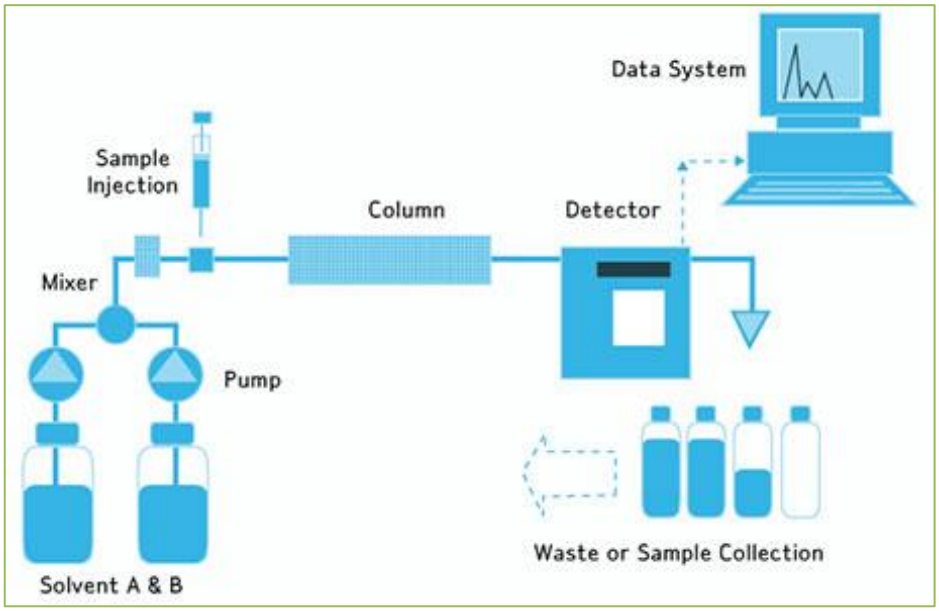


Figure. 66 : Chromatographic equipment.

- **Mobile phase tank**

It's a leak-proof bottle which contains the mobile phase (eluent) in sufficient quantity. Several bottles of eluent (solvents of different polarities) are available to be able to carry out elution gradients (Figure 67). Sometimes equipped with a degassing system (prevents air bubbles).

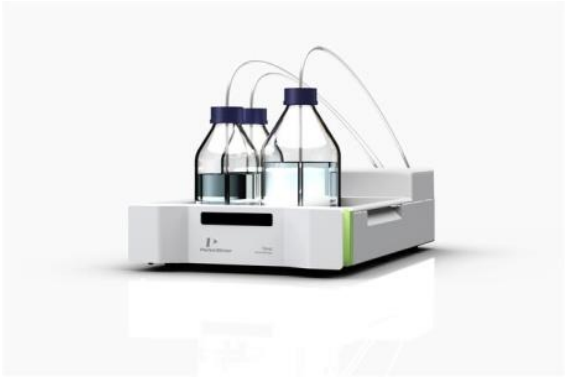


Figure. 67 : The mobile phase.

- **The pump**

The pump forces the mobile phase through the column. It is necessary to maintain an extremely high pressure (50 to 400 bars) upstream of the injector to overcome the pressure loss caused by the stationary phase and to allow the eluent to pass through the column.

It ensures a stable and adjustable flow rate. It must be resistant to corrosion regardless of the solvent used. The pumps are called binary, ternary or quaternary depending on the type of solvents it can mix.

There are two modes of operation (Figure 68):

- The isocratic mode for which the composition of the mobile phase is fixed.
- The solvent gradient mode for which the composition of the solvent being analyzed is varied in order to improve separations and especially to shorten analysis times.

Figure. 68 : The pump.

- **The injector**

The injection must be done very quickly so as not to disrupt the circulation of the solvent. The difficulty consists of introducing a volume of sample at the head of the column where the pressure reaches several tens of bars. For this, a multi-way high pressure valve (6 injection loop valve) is used (Figure 69). The operating principle of these valves is as follows: the sample is introduced with a syringe at atmospheric pressure into the loop (filling or load position (a)), then it is put into communication by rotation of the valve with the phase mobile and the column (inject position (b)) This system avoids sudden pressure variations in the device and is highly reproducible because there is little irregularity in the injections given that the quantity introduced is necessarily that of

the volume of the loop. The injected volumes are set by the capacity of the loop which has variable volumes ranging from microliter to millilitre.

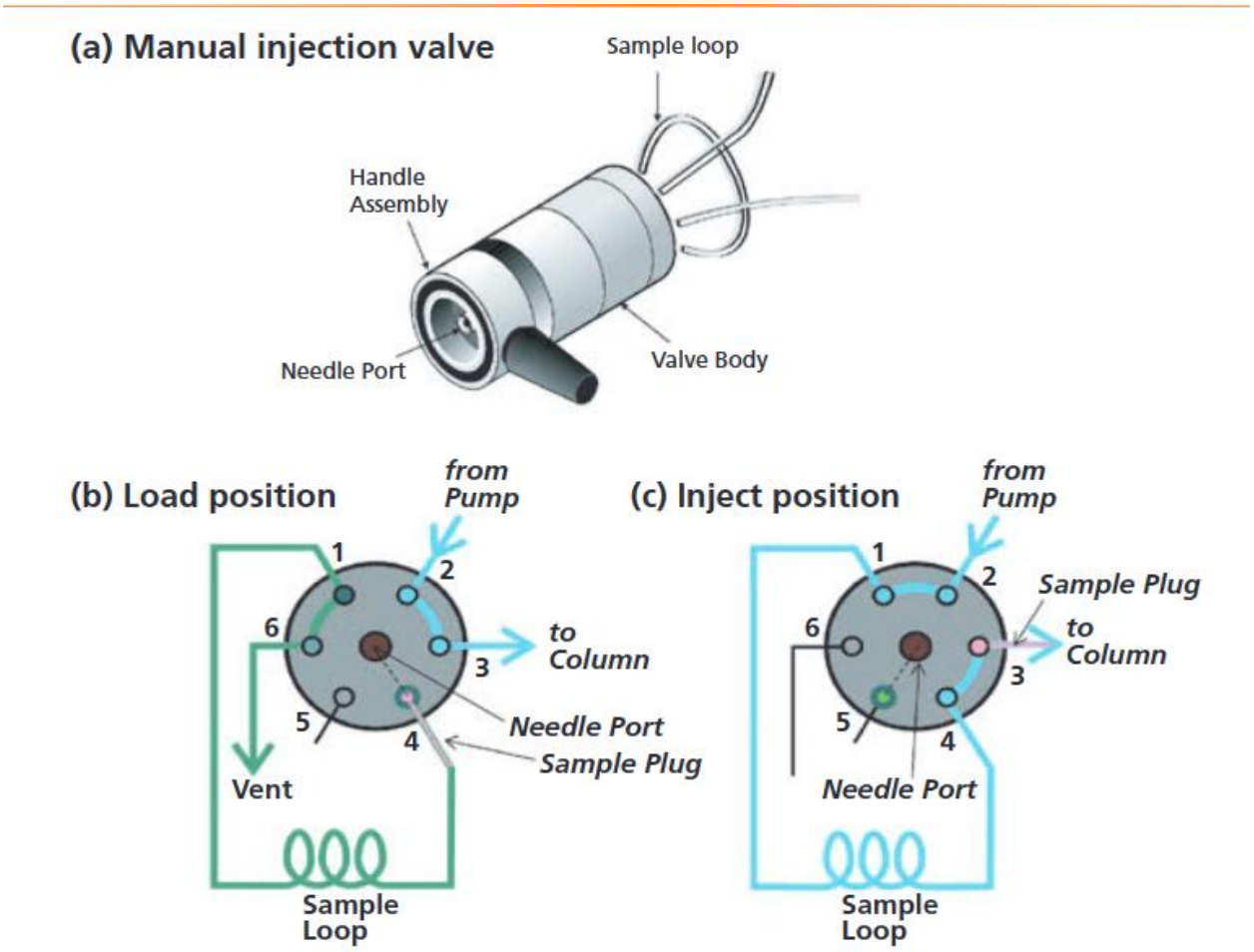


Figure. 69 : a) Injection with a loop. b) Filling the loop. In this step, the syringe is introduced to position 4; c) injection into the column

- **The column**

It is the active part of the system, it plays the preponderant role. The column is a calibrated cylinder generally made of stainless steel sometimes lined with an inert material (special glass or plastic). Standard columns have a length of between 10 and 30 cm with an internal diameter of 4 to 10 mm. They are filled with stationary phase with a particle size of 5 to 10 µm. There are also microcolumns with a length of 3 to 7 cm and an internal diameter of 1 to 5 mm, filled with stationary phase with a particle size of 3 to 5 µm. (Figure 70).

Generally, the column is preceded by a pre-column called a short guard column 0.4 to 1 cm filled with the same stationary phase to retain impurities and increase the life of the column.

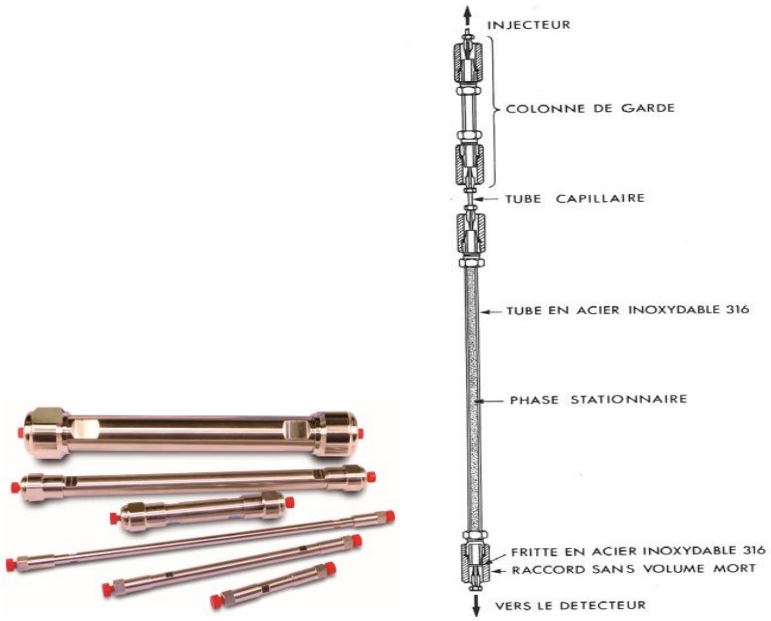


Figure. 70 : The columns.

- **The stationary phase**

Normal phase (normal HPLC) is made of silica gel, which is very polar. It is therefore necessary to use a non-polar eluent. Thus, when injecting a solution, the polar products are retained in the column, unlike the non-polar products which come out at the top.

The reverse phase (reverse phase HPLC) is mainly composed of silica grafted by linear chains of 8 or 18 carbon atoms (C8 and C18). This phase is nonpolar and therefore requires a polar eluent. In this case, the polar compounds will be eluted first.

- **The detector**

The purpose of the detector is to provide an electrical signal continuously reflecting variations in the composition of the eluent at the column outlet, which makes it possible to detect the passage of successive compounds.

- ✓ UV-Visible spectrophotometer.
- ✓ Fluorescence detector.
- ✓ Refractometric detector.

- **The recorder**

This is a computer which recovers all the data from the detectors, traces the chromatograms and integrates the surface of the peaks. It prints an analysis report giving the retention times and areas of each peak. It can be programmed so that it alone carries out the various calculations leading to the concentrations from standard chromatograms and the chromatograms of the mixtures analyzed.

When the peaks are poorly separated, we can use a Gaussian approximation to deduce the surface area of the individual peaks and therefore still evaluate the concentrations.A good separation will result in a distinct separation of the peaks corresponding to each of the products.

4.4.10.3. Application

- Qualitative analysis: the "retention time" (time after which a compound is eluted from the column and detected), qualitatively characterizes a substance.
- Quantitative analysis: the amplitude of these peaks, or the area limited by these peaks and the extension of the baseline makes it possible to measure the concentration of each substance in the injected mixture.

4.4.11. Partition Chromatography

This is liquid-liquid chromatography. It is based on the difference in solubility of the substances to be separated in two immiscible fluids. It is put into practice in paper chromatography. One of the fluids is a liquid retained on an inert support and constitutes the stationary phase. The other, moving liquid or gas, constitutes the mobile phase.

The main factor that intervenes is the partition coefficient between each phase. We can separate solutes whose partition coefficients between the two phases are different. Those that are more soluble in the mobile phase move more easily than those that are less so.

Another factor which intervenes is the polarity of the phase: we can use stationary phases which are slightly or non-polar, the mobile phase being polar (water or water - methanol mixture): we will then speak of partition chromatography with reversed phase polarity. Both in classic partition chromatography, we choose a polar stationary phase and a nonpolar mobile phase.

4.4.11.1. Materials

1. Support

All supports used in solid-liquid chromatography can be used provided they are made inert. We use either:

- Porous supports such as silica on which the liquid stationary phase is a multilayer linked to the support by hydrogen bonds or polar to silanol groups. There are film supports (porous on the surface).
- Non-polar porous supports such as silica in which the silanol groups have been transformed into non-polar trimethyl (or hexamethyl) groups by reaction with trimethylchlorosilane. This is reverse phase chromatography. The stationary phase will be linked to the support by hydrophobic interactions.

2. Stationary phase

It must line the walls of the pores of the support as uniformly as possible. This phase must be very poorly miscible with the mobile phase and its viscosity must be as low as possible so that mass transfer and diffusion phenomena are facilitated.

Impregnation can be carried out in several ways:

- By evaporation of the solvent: the stationary phase is dissolved in an appropriate solvent then mixed with the support. The solvent is then removed by evaporation.
- by filtration of the solvent: in this case the solvent of the stationary phase is eliminated by filtration.
- by percolation: the stationary phase is "dissolved" in a volatile solvent, then it is percolated through the column; the interstitial liquid is removed and the solvent is then evaporated by a gas stream. By varying the concentration of the stationary phase solutions, it is possible to vary the thickness of the film on the support.

3. The mobile phase

The choice of the mobile phase is empirical; we let ourselves be guided by the notion of polarity.

- If the polar support and the mobile phase less polar than the stationary phase: normal phase partition chromatography. The more polar a compound is, the more it will be retained, the later it will emerge.
- If the nonpolar support and the mobile phase more polar than the stationary phase: reversed phase partition chromatography. The more apolar a compound is, the more it will be retained, the later it will emerge.

4.4.12. Paper chromatography

4.4.12.1. Principle

The technique resembles that of TLC but the principle is based on sharing phenomena. The mobile phase is most often an organic solvent and water; the stationary phase consists of the water itself adsorbed on the cellulose of the paper or chemically linked to it. As in thin layer chromatography, the sample, put in solution, is deposited at a reference point on the paper and the solvent, which moves by capillary action, causes the components of the sample to migrate at variable speeds depending on their solubility. Generally, compounds that are more soluble in water or those that easily form hydrogen bond associations are strongly retained by the stationary phase and therefore migrate slowly.

When water is one of the solvents of the mobile phase, the organic solvent(s) must be fairly soluble in it. Products such as ethanoic acid, propanol, phenol or pyridine are the most frequently used solvents mixed with water to develop a chromatogram. Paper chromatography is used

primarily for the analysis of very polar compounds, such as amino acids, sugars and polyfunctional compounds. Its biggest disadvantages compared to TLC are:

- a much longer development time;
- generally less good separation.

2. Paper

Ordinary filter paper can be used, but it is preferable to obtain paper designed for this use, with a low level of impurities and with uniform characteristics. The main brands are Whatman, Schleicher and Schüll, Durieux, Arches. There are eight categories of Whatman paper, classified according to their thickness, the texture of their surface and the speed with which water diffuses through them. For example, Whatman No. 1 paper is the most used, but if you want a high flow speed, you will use No. 4; No. 20 paper is very slow, but it allows better separation, giving very dense and uniform spots. The description of the analysis by chromatography on paper is identical to that on thin layer.

4.4.13. Chromatogram analysis

4.4.13.1. Vocabulary

* Baseline - the part of the chromatogram where only the mobile phase is coming out of the column

* Peak maximum - the highest point of the peak

* Injection point - the time when the sample is placed on the column

* Dead point - the position of the peak maximum for a solute that does not get retained

* Dead time (to) - the time between the injection point and the dead point

* Dead volume (Vo) - the volume of mobile phase that passes through the column between the injection point and the dead point; Vo = d x to, where d is the flow rate in ml/min (Figure 71)

* Retention time (tr) - time elapsed between the injection point and the peak maximum of solute

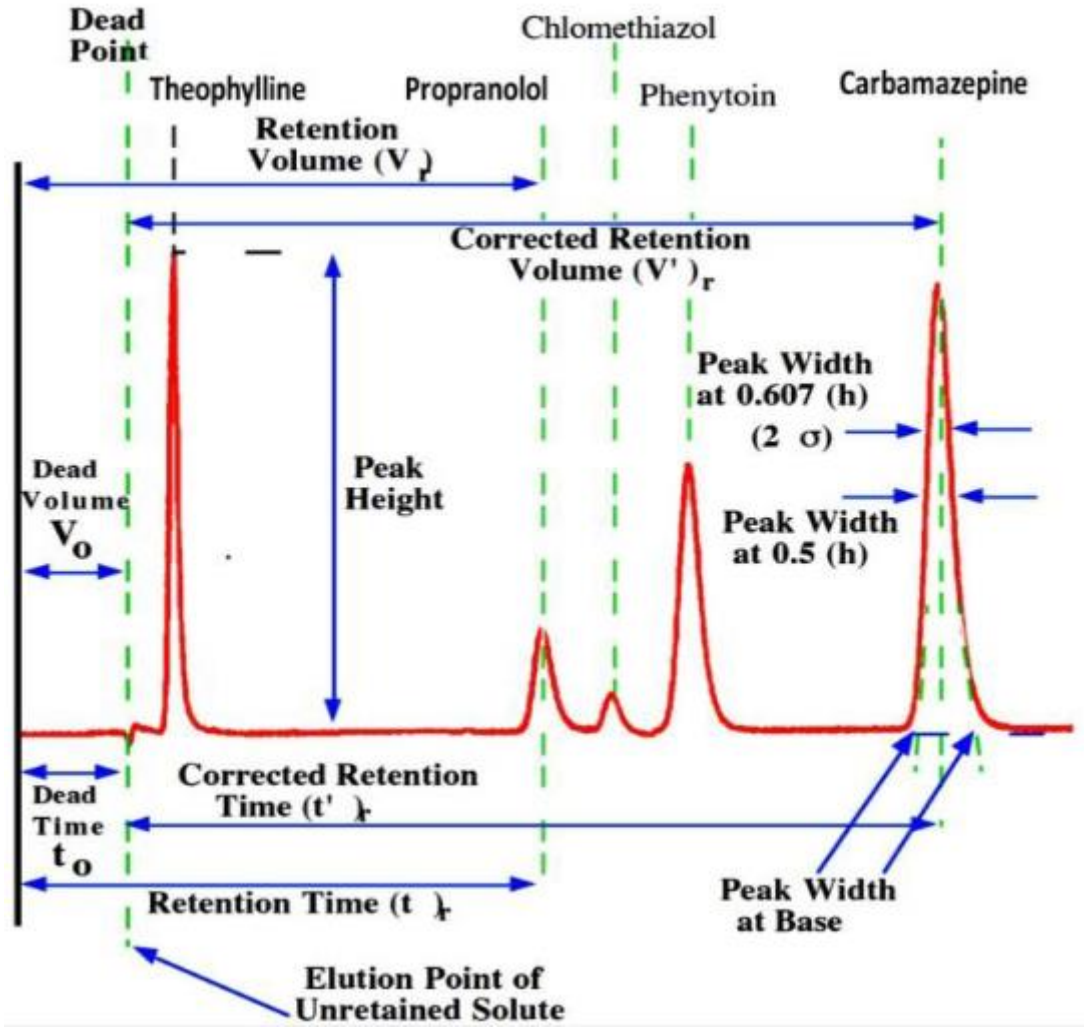


Figure. 71 : Chromatogram analysis vocabular

* Retention volume (Vr) - volume of mobile phase passed through the column between the injection point and the peak maximum of solute; Vr = Qtr

* Corrected/adjusted retention time (t'r) - time elapsed between the dead point and the peak maximum [t'r = tr - to]

* Corrected retention volume (V'r) - volume of mobile phase passed through the column between the dead point and the peak maximum. It will also be the retention volume minus the dead volume; [V'r = Vr - Vo = Q(tr - to) = Q t'r]

* Peak height (h) - distance between the peak maximum and the base line geometrically produced beneath the peak

* Peak width (w) - distance between each side of a peak measure at 0.6065 of the peak height. The peak width measured at this height is equivalent to two standard deviations (2s) of the Gaussian curve and thus has significance when dealing with chromatography theory

* Peak width at half height (w0.5) - distance between each side of a peak measured at half the peak height. The peak width measured at half height has no significance with respect to chromatography theory

* Peak width at the base (wB) - distance between the intersections of the tangents drawn to the sides of the peak and the peak base geometrically produced. The peak width at the base is equivalent to four standard deviations (4s) of the Gaussian curve and thus also has significance when dealing with chromatography theory

* Relative retention time - retention time for the analyte divided by that for the standard (Figure 72)

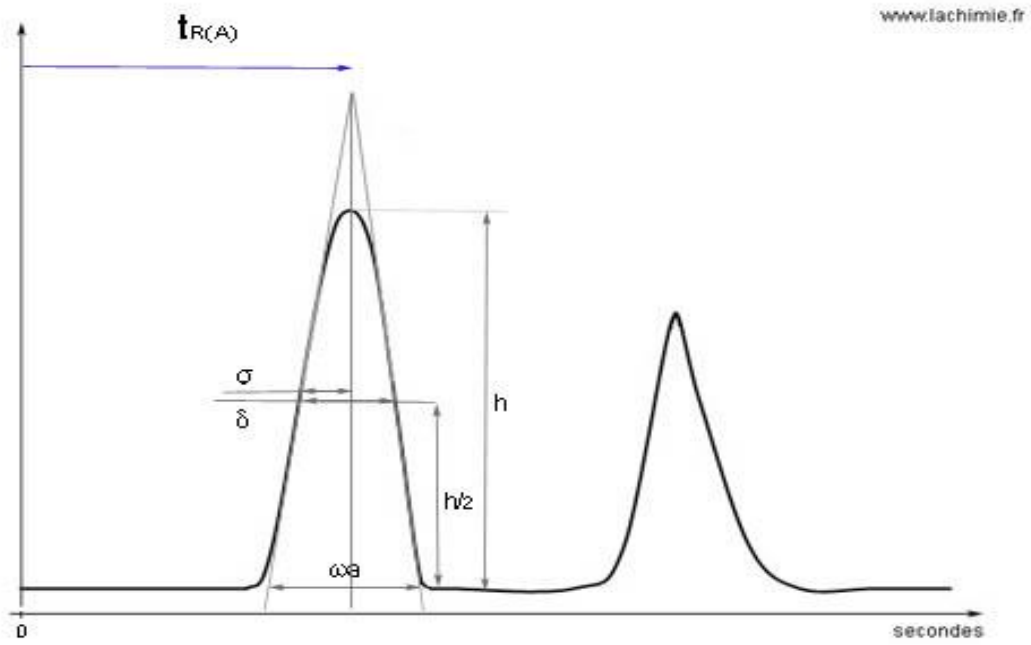


Figure. 72 : The electromagnetic spectrum.

*** Retention factor (capacity factor) (k)**

- additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase; **[k = (tr – t0)/ t0 = t'r/ t0]**. This tells that analyte in the stationary phase is k times the mobile phase at any point in the column at any time. It is evident, therefore, that k is related to the distribution coefficient of the analyte, which was defined as the relative concentrations of the analyte between the two phases. Since amount and concentration are related to volume, this can be written as. **k= t'r/ t0 = Cs/Cm = Kd x Vs/Vm MS** and Mm are the masses of analyte in the stationary and mobile phase, respectively VS and Vm are the volumes of analyte in the stationary and mobile phase, respectively VS/Vm - volumetric phase ratio (β) k = Kd β

*** Distribution or partition coefficient (Kd)**

- basis of all forms of chromatography, which explains how a compound distributes between two immiscible phases. Kd = Concentration in phase A/ Concentration in phase B Retention factor (k) changes with the change in both the distribution coefficient between the two phases and the volume of the stationary phase and it range from 1 to 10. Retention factors are important as they

are independent of the physical dimensions of the column and the flow rate of mobile phase through it. They are also a reflection of the selectivity of the system that in turn is a measure of its inherent ability to discriminate between two analytes.

*** Selectivity or Separation factor (α)**

– relative retention ratio for the two analytes

α = kA/kB = KdA/KdB = t'rA/ t'rB

*** Theoretical plate**

An imaginary functional unit of the column where equilibrium has been established between stationary and mobile phases. The length of column containing one theoretical plate makes up the plate height (H). Efficiency of a column is expressed by the number of theoretical plates in the column or HETP (Height Equivalent to a Theoretical Plate). N‟ is no. of plates per column. If HETP is less, greater the value N, the column is ↑ efficient, the narrower is the analyte peak. If HETP is more, lesser the value N, the column is ↓ efficient. The measure of the column efficiency is denoted by the plate number „N‟ given by L divided by the plate height.

H =L/N (N the average of N1, N2…….). Several relationships in the case of symmetrical (Gaussian) peaks make it possible to calculate the number of theoretical plateaus according to the retention quantities used: **$N= (tR/\sigma)^2 = 16(tR/\omega)^2 = 5.54 (tR/\delta)^2$**

*** Resolution**

is the measure of true separation of two consecutive peaks on a chromatogram. It is the measure of both column and solvent efficiencies. Resolution (RS) is defined as the ratio of the difference in retention time (ΔtR) between the two peaks (tRA and tRB) to the mean (wav) of their base widths (wA and wB) **R= 2 (Tr2 - Tr1)/ [(ωb1 +ωb2)]**

4.5. Electrophoretic methods (Analysis method

The term "electrophoresis" describes the migration of charged particles under the influence of an electric field. The prefix "electro" refers to electricity from the Greek èlektron which means yellow amber and the root "phoresis" comes from the Greek phoresis which means "to carry from one side to the other.

4.5.1. Principle

Electrophoresis is a qualitative and quantitative analysis method based on the differential migration of electrically charged particles under the influence of an electric field.

4.5.2. Parameter and condition of realization

Several factors can influence the electrophoresis of biological molecules:

1. Nature of molecules

The molecules to be separated play a very important role in their separation by electrophoresis due to three properties:

- **Load:** the greater the load, the greater the speed of movement.
- **The size:** the migration speed is inversely proportional to the molecular weight of the molecules to be separated. Molecules with a low MW migrate more quickly.
- **The form :** molecules have various shapes depending on their structures, linear, two or three-dimensional. On a support, molecules with the same charges and the same MM but with different shapes migrate with different speeds.

2. Electric field

Electrical mobility is the speed of movement of particles in the suspension, and it depends on the charge and geometry of the particle.

The dispersed particles have an external electric charge, on which an external electric field exerts an electrostatic force. For this reason, a particle of electric charge Q, placed in an electric field E, is subjected to a force F which drives it towards the electrode of opposite sign:

$$\mathbf{F = Q.E}$$

Friction forces f, due to the viscosity of the medium η (the viscosity coefficient depends on the temperature) oppose the migration of the particle, all the more so as the particle is large (r = radius) and that the migration speed (v) is high (Figure 73):

$$\mathbf{f = 6\pi.\eta.r.v}$$

There comes a point when these two forces balance, and the particle then moves at a constant speed; we can then write:

$$\mathbf{Q.E = 6\pi.\eta.r.v} \quad \textbf{soit} \quad \mathbf{v = Q.E/6\pi.\eta.r}$$

We define for each particle its mobility μ, independently of the electric field, by the relation:

$$\boldsymbol{\mu} = \frac{\mathbf{v}}{\mathbf{E}} \qquad \textbf{soit} \quad \boldsymbol{\mu} = \mathbf{Q/6\pi.\eta.r}$$

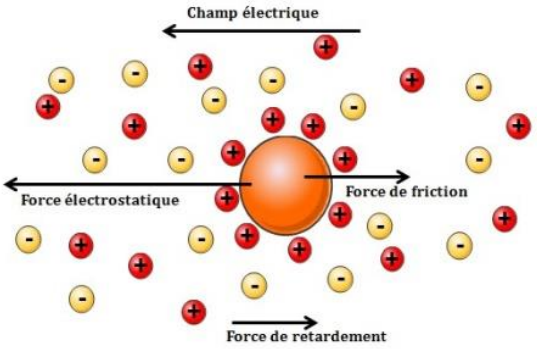


Figure. 73 : Illustration of electrophoresis.

- **Voltage:** the migration speed is proportional to the voltage according to the relationship: F=VQ/d where V= voltage, F= force exerted by the electric field, Q= charge of the molecule and d= distance between the two poles.
- **Amperage:** the electric current is carried by the ions of the buffer and the molecules to be separated. Consequently, the speed of migration and the distance traveled during a given time are proportional to the intensity of the current.
- **Resistance:** under an electric field the migration speed of the molecules is inversely proportional to the resistance which is, in turn, dependent on the length of the support and the concentration of the buffer ions.

3. Stamp: the buffer determines the ionic force and pH of the medium.

- **Buffer composition:** the buffer must be, by its composition, inert with respect to the sample and the support. The most commonly used buffers are veronal (barbital), phosphate, tris, pyridine, borate, acetate, etc.
- **Ionic fORCE:** the IF is very important because the electric current is carried by the ions of the buffer and the sample:
- **pH:** pH is very important since it determines the degree of ionization of the molecules contained in the sample. At acidic pH the molecules are positively charged and vice versa at basic pH.
  - If pH > pHi: net negative charge (anion), migration towards the anode.
  - If pH < pHi: net positive charge (cation), migration towards the cathode.
  - If pH = pHi: zero net charge, no migration.

4. Support

Although the support must be completely inert, we find, in practice, three phenomena in the different supports used.

- **Adsorption:** certain supports can adsorb to the compounds in the sample in a non-specific manner but different from one molecule to another. This adsorption will slow down these molecules and prevent their migration (Figure 74). Paper is the support which presents the most adsorption for the majority of biological molecules, which has pushed researchers to abandon it completely.

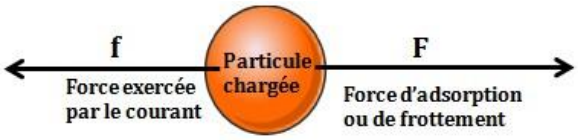


Figure. 74 : The adsorption phenomenon.

- **Electroendosmosis (Electro-osmosis):** under the experimental conditions, the support becomes negatively charged; a mobile layer of positive charges is formed in the solvent, in contact with the support and generally drives the liquid phase (essentially H3O+) towards the cathode. This current accelerates or slows down the migration of the molecules, depending on whether they migrate towards the cathode or towards the anode. (Figure 75).

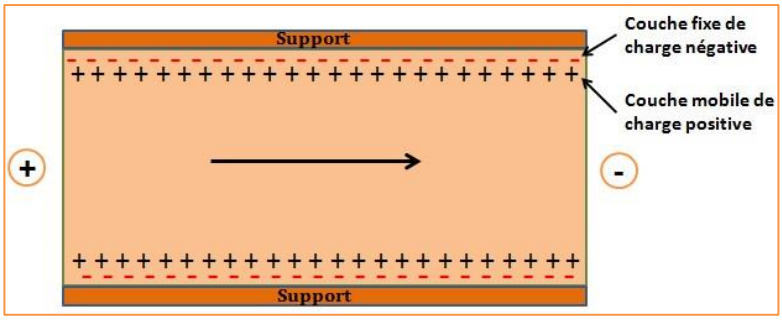


Figure. 75 : The phenomenon of Electroendosmosis.

- **Sieving:** the acrylamide (and starch) gel looks like a sieve. At a given percentage of the gel certain molecules are retained, others pass with difficulty and others easily. If the concentration increases, the sieving phenomenon also increases proportionally.

4.5.3. Materials

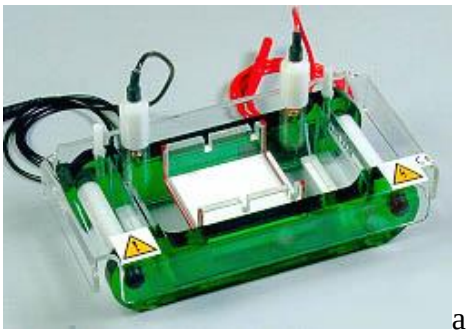
4.5.3.1. Horizontal mounting

This type of assembly (Figure 76) is mainly used for matrices such as cellulose acetate or paper where the samples move on its surface. The two ends of the matrix immerse in a solution of electrolytes, the ions move on the surface, creating the current which will drive the various molecules of the sample which will migrate according to their charge. Horizontal mounts are also used for agarose matrices during nucleic acid electrophoresis or immunoelectrophoresis.

4.5.3.2. Vertical mounting

This type of assembly (Figure 76) is mainly used for matrices such as polyacrylamide gels. Samples generally move within the matrix.

The matrix is in the form of gelled material, between two glass plates. It is often prepared shortly before use by forming a glass plate/gel/glass plate. During gelling, wells are made where samples will be deposited. Each end of the gel will be brought into contact with a buffer containing electrolytes which, when subjected to an electric potential, will allow the propagation of a current in the gel. This current will carry the molecules constituting the sample. This migration will allow the separation of the various molecular species which will migrate at different speeds.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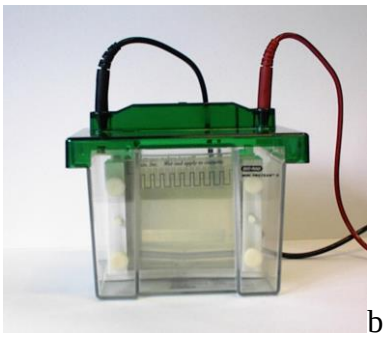
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Figure. 76 : Horizontal mounting and Vertical mounting.

4.5.3.3. Main electrophoresis matrices

There are several types of matrices which differ in their constitution (material with which they are made), the type of assembly in which they are used and their physicochemical characteristics:

1. Paper: usually used in a horizontal assembly, more rarely vertical, it was mainly used to separate amino acids or other small charged molecules (Figure 77). The deposition and migration of samples takes place on the surface. Among the disadvantages we can cite:

- Adsorption phenomena.
- Electro-osmosis.
- the large quantity of the sample.

Figure. 77 : A paper backing.

2. Cellulose acetate: These are very porous and fragile thin sheets. Deposition and migration take place on the surface on a horizontal assembly. The low resolution only allows the separation of large groups of proteins. Its low cost, speed and ease of use make it useful for the separation of serum proteins, particularly in clinical biochemistry for the diagnosis of diseases.

3. Silica or cellulose: silica (powder) or cellulose (also powder) is spread on a horizontal glass plate then moistened with a suitable buffer. After depositing the samples using syringes or fine-tipped pipettes, the two ends of the plate are connected to the electrophoresis buffer by filter paper soaked in the same buffer.

4. Starch

Rarely used today, this matrix forms a porous gel. Starch gels were used to separate proteins intended for enzymatic staining.

5. Agarose

Agarose does not polymerize by creating covalent bonds, it gels at low temperature (<38°C). This gelation is due to the formation of a multitude of hydrogen bonds between the long linear dextran molecules that make up the agarose. Agarose gels have large "pores" and are used primarily to separate large molecules with a molecular mass greater than 200 kDa. Its poor adhesion to glass generally requires this gel to be spread on a horizontal glass plate. This is the matrix often used for nucleic acid electrophoresis. Immunoelectrophoreses are also carried out on agarose gel (facilitates the diffusion of antibodies). Increasingly, this matrix is used for the separation of serum proteins.

6. Polyacrylamide

Polyacrylamide gel is polymerized between two glass plates or inside cylindrical glass tubes, where it will form a porous gel. The sample is placed on the top of this vertical gel. The molecules will then be able to migrate inside this gel. Polyacrylamide gels are commonly used for protein separation. They are also often used for small nucleic acids, particularly in sequencing.

4.5.3.4. Stamps

Efficient separation by agarose or polyacrylamide gel electrophoresis depends on effective maintenance of pH in the matrix.Therefore, buffers are an integral part of any electrophoresis technique.The different categories of buffer systems available for electrophoresis are:

- Denaturants and non-denaturants,
- Continuous and discontinuous.

The denaturing and non-denaturing system

When we maintain the integrity of the molecule and its net charge, the medium is said to be natural or non-dissociating. If we denature the molecules by dissociating them into unit chains or if we change their net charges the medium is called denaturing or dissociating. Among the dissociating and/or denaturing agents we find:

- **Sodium dodecyl sulfate (SDS):**is an anionic detergent that denatures secondary and tertiary structures (but not disulfide bonds), and applies a negative charge to each protein in proportion to its mass. As a result, proteins separate by mass only (Figure 78).

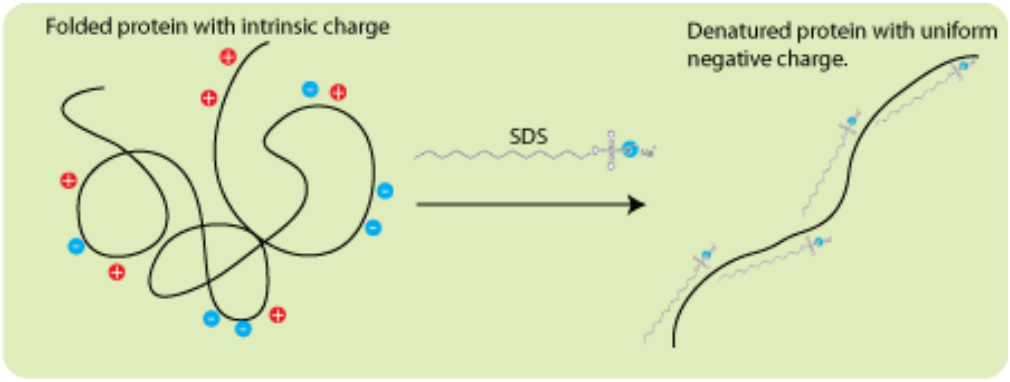

Figure. 78 : Mechanism of action of SDS.

- **Mercaptoethanol and dithiothreitol (DTT):** both of these agents are used to reduce inter-chain disulfide (SS) bridges to sulfhydryl (-SH) groups (Figure 79). This will allow the study of molecules composed of several chains. Used with SDS we can know the MM of the different chains of molecules
- **Urea:** the main property of urea is to break hydrogen bonds but keeps the net charges as they are. In general, concentrations of 2.5 to 6.25 M urea are used.Separation on the basis of molecular weight requires the inclusion of denaturing agents, which unwind the nucleic acid strands and eliminate the influence of shape on their mobility.

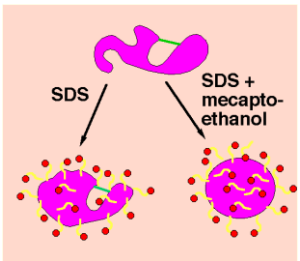

Figure. 79 : Mechanism of action of mercaptoethanol.

Continuous and discontinuous systems

In continuous buffer systems, the identity and concentration of the buffer components are the same in the gel and the vessel. The bands tend to be wider and consequently poorer resolution. These systems are used for most forms of DNA agarose gel electrophoresis,

Batch systems often use two different buffers in the gel. The gel is split into concentration gel (pH 6.8) at the top with low percentage of acrylamide and separation gel (pH 8.8) at the bottom with higher percentage of acrylamide (Figure 80).

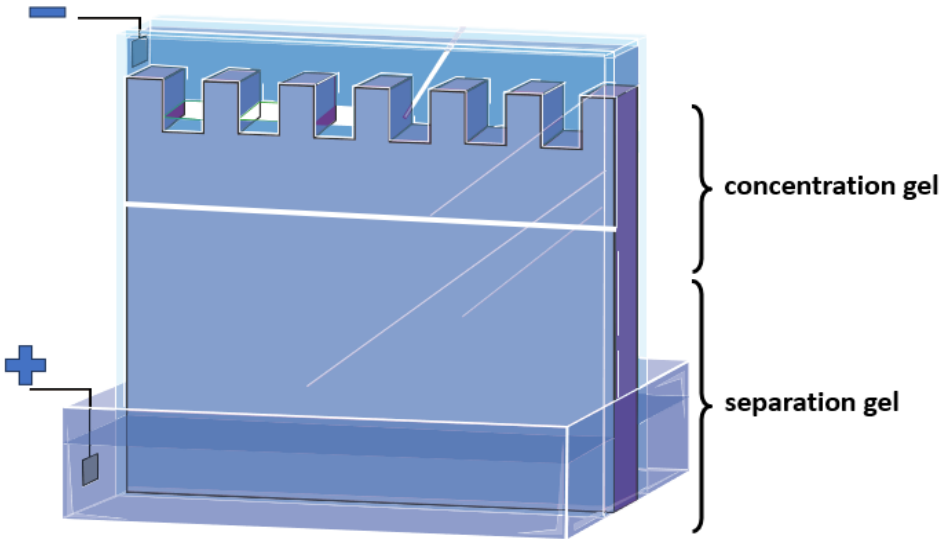

Figure. 80 : Concentration and separation gel

4.5.3.5. The sample solution

this solutioncontains glycerol or sucrose which makes the sample denser and therefore allows it to remain at the bottom of the loading well. it also contains dyes which make it possible to follow the migration of samples through the gel such as bromophenol blue, or cyanol xylene. Since these molecules are small, they migrate quickly through the gel during electrophoresis, thereby indicating the progress of the electrophoresis. we also find denaturing agents such as SDS, DTT, Urea, etc.

4.5.3.6. The applied current

a direct current generator connected to the electrodes of the tank (electrical cords: one black, one red) (Figure 81). This current creates an electric field which will allow the molecules to migrate. The current voltage is a function of the length of the fragments and the concentration of the gel. The higher it is, the faster the samples migrate. However, the high voltage causes a huge increase in buffer temperature and current in a very short time.Therefore, it is recommended not

to exceed 5-8 V/cm and 75 mA for standard size gels or 100 mA for mini gels. The current voltage and depending on the length of the fragments and the concentration of the gel.

Figure. 81 : The current generator

4.5.3.7. Visualization or coloring

The easiest way to visualize proteins is to color them. Before proceeding with this staining, the proteins must be fixed, by putting the gel in a diluted acid such as acetic acid or trichloroacetic acid 1 to 10%. The most commonly used dye is Coomassie blue (Figure 82). There is also Ponceau red, Amido-schwartz (black) and lissamine green. Decolorization is done by transferring the gel to an acidic methanolic solution. A transparent gel is obtained with the colored protein bands.

The most commonly used intercalating agent to make DNA bands visible under ultraviolet light is ethidium bromide. The dye can be included in both the buffer and the gel, the gel alone, or the gels can be stained after DNA separation. Silver staining is a highly sensitive method for visualizing nucleic acid and protein bands.

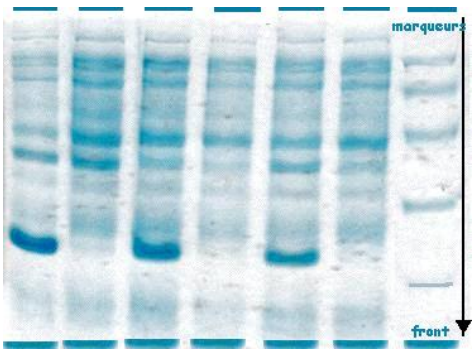

Figure. 82 : An acrylamide gel colored by coomassie blue

4.5.4. Different types of electrophoresis

4.5.4.1. Thin layer electrophoresis

The equipment used is very simple; consisting of a tank having two buffer compartments, a direct current generator and the glass or hard plastic support on which the matrix is placed (Figure 83).

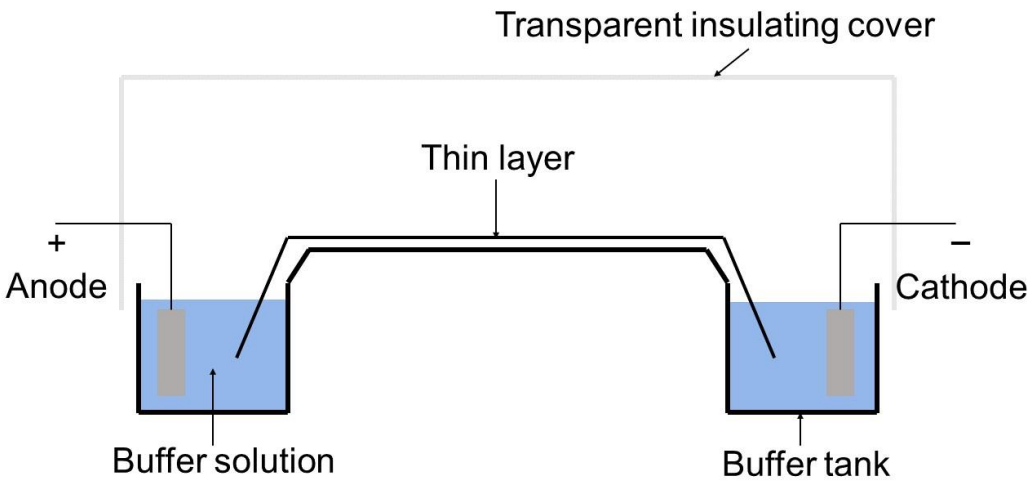

Figure. 83 : Thin layer electrophoresis

Operating mode (Figure 84)

- The support strip is impregnated with buffer then placed in the electrophoresis tank filled with buffer.
- The samples (only a few microliters) are then deposited in the center of the strip.
- An electric current is then applied to both sides of the tank (low intensity current must be used).
- Under the effect of the applied electric field, the constituents of the sample will migrate towards the anode or towards the cathode.
- The direction of migration and the speed (or distance) of migration of each constituent are determined by the charge of this constituent.
- This charge can vary depending on the pH, which is set by the experimenter through the use of a buffer solution.
- This therefore allows differential migration of the constituents of a sample depending on the charge.
- After a migration time the band is removed, the proteins are then fixed by drying, heating or by a strong acid to prevent any further diffusion.

Figure. 84 : The equipment for thin layer electrophoresis

4.5.4.2. Polyacrylamide gel electrophoresis (SDS-PAGE)

It is a technique for separating and analyzing charged particles by differential migration under the action of an electric field. In this type of electrophoresis, the migration speed is a function of size (the effect of charge is canceled). The gel is poured between glass plates fixed on a support. A comb is embedded between these plates. After polymerization of the gel, the comb is removed, thus forming wells. The size and number of teeth of the combs are variable, which makes it possible to deposit volumes ranging from 20 µL to 200 µL of protein sample to be separated.

Two gels can be prepared: a separation gel (tight pores) and a concentration gel (loose pores). Depending on the level of acrylamide and bis-acrylamide (Figure 64), TEMED and ammonium persulfate, the porosity of the gel varies. The polyacrylamide gel is a crosslinked gel obtained by polymerization of acrylamide which forms chains and bis-acrylamide which bridges the acrylamide chains, persulfate and TEMED catalyzes the polymerization reaction (Figure 85).

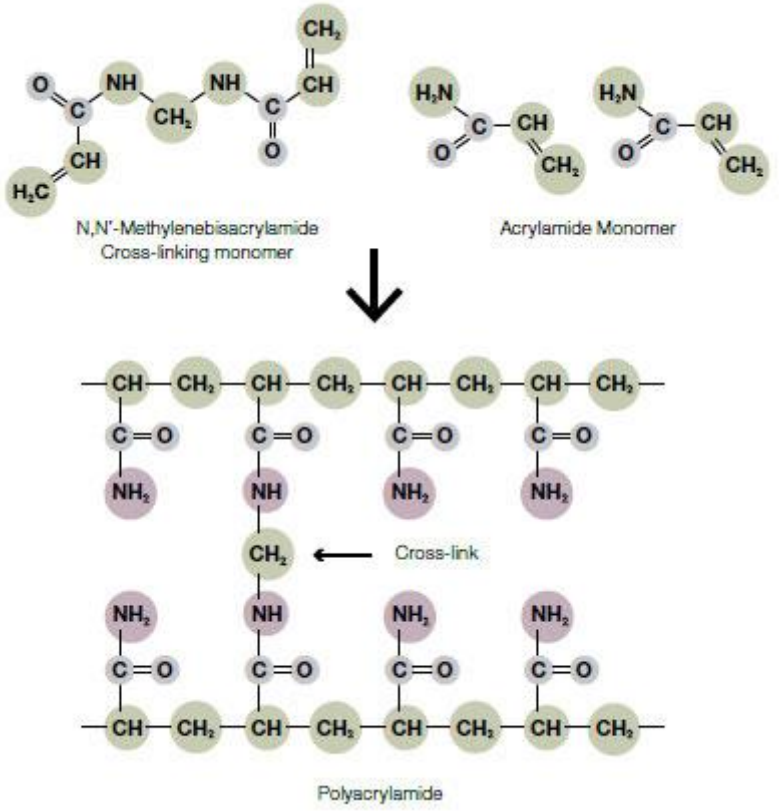

Figure. 85 : Acrylamide and bis-acrylamide.

The diameter of the pores (mesh) is inversely proportional to the concentration of the gel (weight of acrylamide + bisacrylamide in 100 ml of the gel). The choice of pore diameter depends on the MM of the molecules to be separated (Table 3).

Table 3. The diameter of the pores depends on the MW of the molecules.

Gel concentration (%)	MP (kDa)
2.5-5	300-1000
5-10	100-300
10-15	40-100
15-20	10-40
20-25	2-10

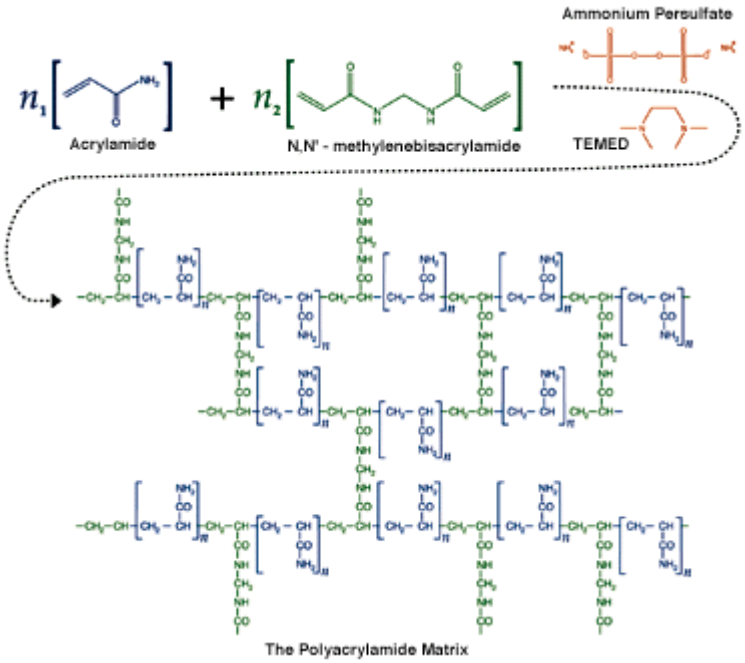

Figure. 86 : The polymerization reaction in the presence of TEMED and ammonium persulfate.

To designate the concentration of the gel we generally use the term T% and to express the bisa/acryl ratio we use the term C%.

T%= weight of acryl + weight of bisa in g per 100 ml of gel

C%= weight of bisa/acryl x 100

In practice, the bisa/acryl ratio being constant (between 2-3%), we talk about the percentage of the gel (or T%) by saying for example: electrophoresis on a 15% polyacrylamide gel or at T%=15%.

Operating mode of an SDS-PAGE

- Preparation of the concentration gel and the separation gel
- The separation gel is poured between glass plates fixed on a casting support followed by the concentration gel, a comb is placed between these plates.

- After polymerization of the gel, the comb is removed forming wells.
- place the sample and MM markers in the wells
- The glass plates containing the polymerized gel are placed in an electrophoresis tank.
- Fill the tank with the migration buffer
- Switch on the current generator
- At the end of migration, remove the gel from the plates, stain then bleach
- Gel analysis

Application

- **Check the purity of chromatography fractions**
- **Determining the MW of a protein**: The MW of proteins is determined using markers that are known MW standard proteins. It is necessary to determine: Frontal ratio (Rf) = migration distance of a compound/migration front (fm) then draw the line: log (MW) = f (Frontal ratio) which makes it possible to determine the MM of an unknown protein (Figure 87).

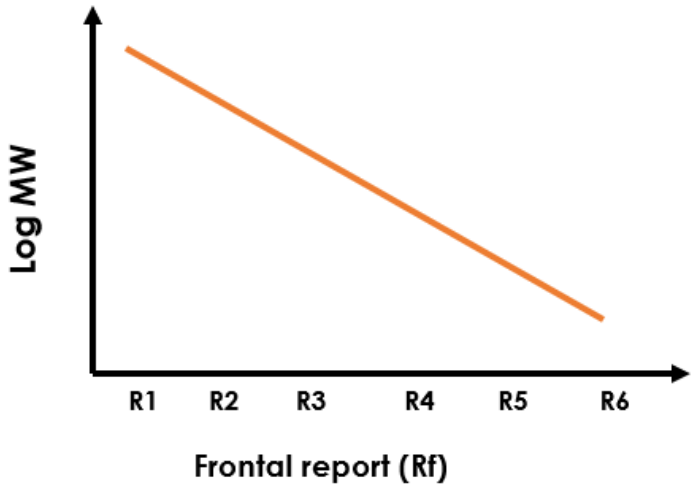

Figure. 87 : A calibration curve log (MW) = f (Frontal Ratio).

4.5.4.3. Electrical isofocusing (IEF)

We know that proteins have a charge that allows them to migrate in an electric field. The basic principle of isoelectric focusing (IEF) is to create a pH gradient in which proteins subjected to an electric field can move. The proteins will migrate in this electric field. Arriving at the pH corresponding to their pI, they will immobilize since their net charge will be zero. In this way, it is possible to separate proteins according to their pI (Figure 88).

The method consists of placing the two terminals of the gel or support in a solution of a strong acid at the anode and in a solution of a strong base at the cathode and adding to the

support ampholytes carrying a certain number of groups positively or negatively ionizable (generally polyamines, polycarboxyls or sulfates) and we connect the electric current the pH gradient is maintained by the fact that each ampholyte immobilizes at its pI. This is guaranteed by the fact that these ampholytes will distribute themselves in order of pI and their buffering capacity will help to maintain around them a small zone of pH equal to their pI. A series of ampholytes each having a pI covering a certain pH range will therefore create a continuous pH gradient. If we make a small quantity of proteins migrate into this system, after or during its formation,

These ampholytes can sweep a pH range:

- **Wide range** (Exp. pI between 3-10): allowing analysis of a large number of proteins.
- **Narrow range** (Exp. pI between 4-5 or 5-6.5): allow very fine separation for precise measurement. Using a range of molecules with known pIs we can draw a calibration line connecting the pIs to the distances traveled, we can, by projection, find the pIs of the proteins in the sample.

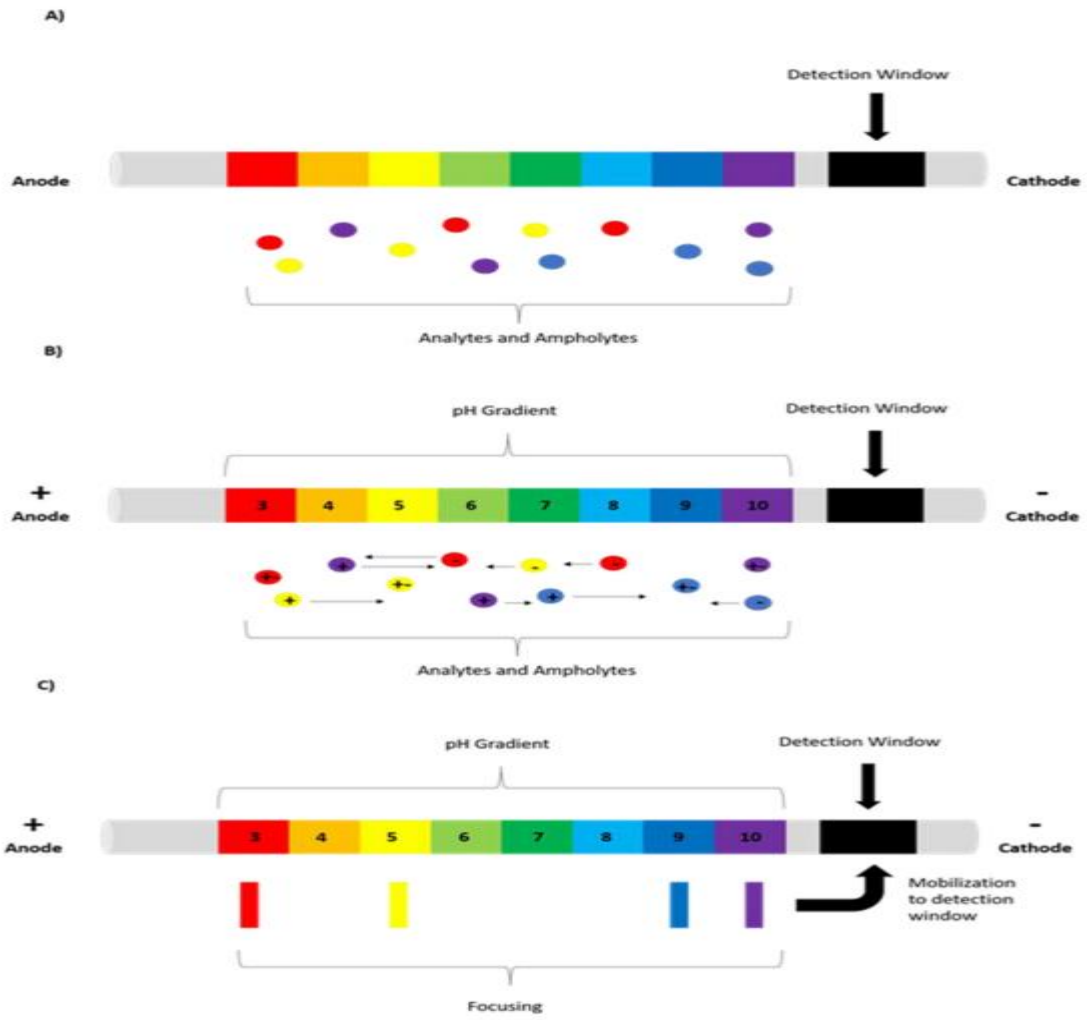

Figure. 88 : An IEF gel.

Operating mode of an IEF

1. Preparation of 6% polyacrylamide gel.
2. The separation gel is poured between glass plates fixed on a support
3. deposit the sample and pI markers
4. the polymerized gel is placed in a separation tank
5. Turn on the current generator (proteins are larger than ampholytes, they will migrate much more slowly)
6. At the end of migration, remove the gel from the plates,
7. Before coloring, the ampholytes must be removed because they can be colored by putting the gel in a washing solution.
8. coloring then bleaching
9. Gel analysis

4.5.4.4. Two-dimensional electrophoresis

The best technique for analyzing a complex mixture of proteins is two-dimensional electrophoresis. This is the combination of electrophoresis which separates according to pI (isoelectric focusing, followed by denaturing SDS electrophoresis, separating according to molecular weight.

Firstly, the mixture is separated according to the pI in a cylindrical or flat gel (sheet). The vertical strip of the flat gel is then cut out or the cylinder of gel is recovered. This band is then deposited horizontally on a concentration gel and electrophoresed, allowing separation according to molecular weight (Figure 89).

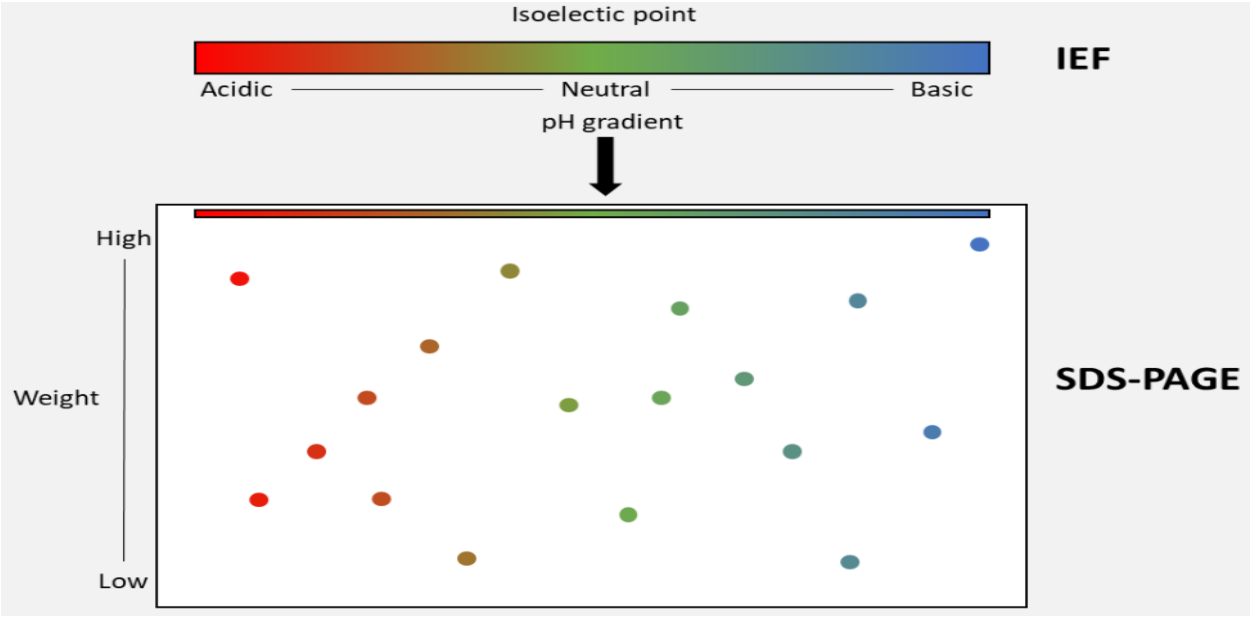

Figure. 89 : Two-dimensional electrophoresis

4.5.4.5. Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate nucleic acids or proteins based on their molecular weight. Agarose gels have large "pores" and are used primarily to separate large molecules with a molecular mass greater than 200 kD. It is used at concentrations of 0.5% to 2%.

Agarose gels are made by suspending dehydrated agarose powder in an aqueous buffer, then boiling the mixture and then pouring it into a mold. The gel is cooled to room temperature until a rigid gel is formed. There are a very varied number of stamps. The most often used are Tris/Acetate/EDTA (TAE).

4.5.4.6. Pulsed field electrophoresis

To separate DNA fragments larger than 20 kb. The principle of this electrophoresis consists of changing the orientation and/or polarity of the electric field alternately over time. Each time the field changes, the DNA molecule must reorient itself parallel to the new field. When the field is restored to its initial direction, the molecule must once again reorient itself (Figure 90). These reorientation times cause a delay in net migration that is proportional to the size of the molecule. The migration support is a 1% agarose gel and the size of the separated fragments is on the order of 50 kb to a few megabases.

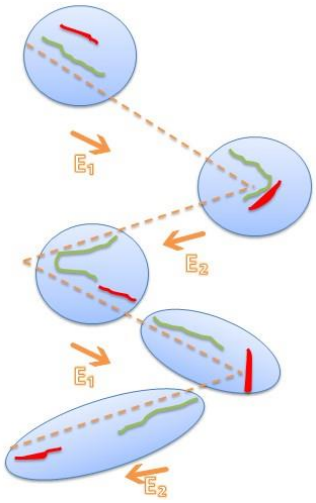

Figure. 90 : Principle of pulsed field electrophoresis

4.5.4.7. Capillary electrophoresis

It is a recent technique which is beginning to develop and which essentially offers the advantages of speed, very high resolution and, therefore, very high detection sensitivity. Electrophoresis uses a silica capillary with a diameter of approximately 50 µm and a length of 1 m (filled with buffer or gel), and high voltages (15 -30 kV). This results in very rapid migration

rates of the compounds in the capillaries and these are detected by UV absorption, fluorimetry or conductimetry directly on the capillary (Figure 91).

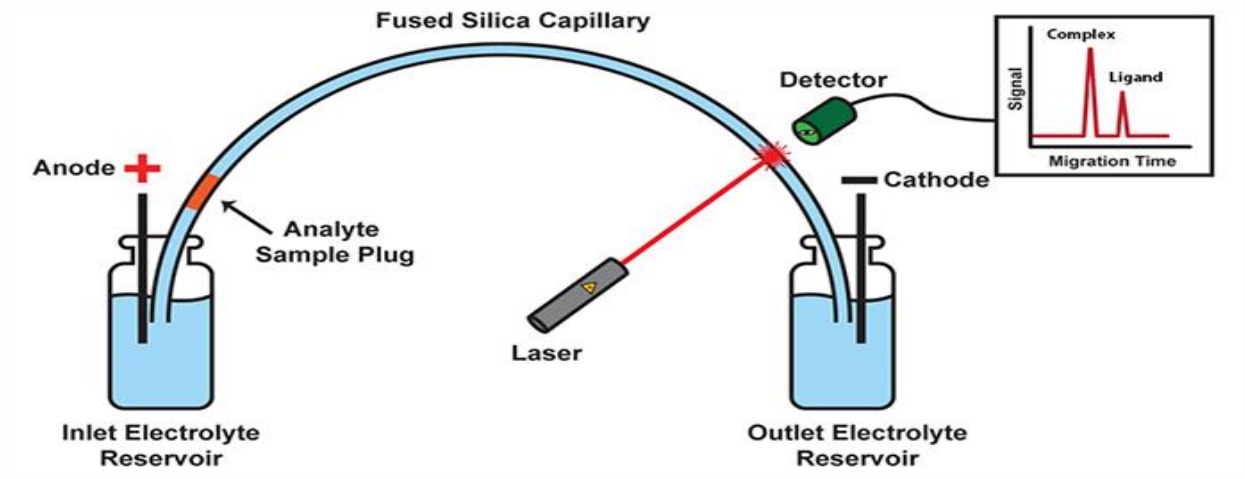

Figure. 91 : Principle of capillary electrophoresis.

The areas of application are a priori numerous: analysis of peptides, amino acids, oligonucleotides, etc. the number of plates is of the order of 500,000 per meter, which provides remarkable resolution The technique can also be used. apply to non-ionized molecules in the presence of appropriate detergent micelles.

4.5.4.8. Immunoelectrophoresis

Immunoelectrophoresisis a general name for a number of biochemical methods of separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require antibodies reacting with the proteins to be separated or characterized.

1% agarose gel buffered at high pH (approximately 8.6) is traditionally preferred for electrophoresis, as well as for reaction with antibodies. Agarose was chosen as a matrix because it has large pores allowing free passage and separation of proteins, but also provides an anchor for immunoprecipitated proteins and specific antibodies. The high pH was chosen because the antibodies are virtually immobile at high pH. Electrophoresis equipment with a horizontal plate is normally recommended for electrophoresis. Immunoprecipitates or Ac-Ag Complexes can be seen in the gel but are stained with protein dyes such as Coomassie blue.

A) Grabar and Williams immunoelectrophoresis

This technique is used for the separation and identification of a mixture of antigen (Ag). The support used is the agarose gel which has two types of wells: a point well for the deposition of the Ag (serum to be analyzed) and a channel-shaped well for the deposition of the antibody (Ac).

To begin with,a mixture of Ag is subjected to an electric field along which the constituents are distributed according to their electrophoretic mobility.

Secondly, the Ac is deposited in the slot. The latter diffuse in a direction perpendicular to that of the electrophoretic track whose fractions (separated Ag) diffuse in the opposite direction. When the Ag encounters its specific Ac, the Ac-Ag complex precipitates giving precipitation arcs. the plaque is subsequently washed with a saline solution. Revelation is done using the appropriate dye (Coomassie blue). Identification is carried out by comparison with a normal control serum treated under the same conditions (Figure 92-93).

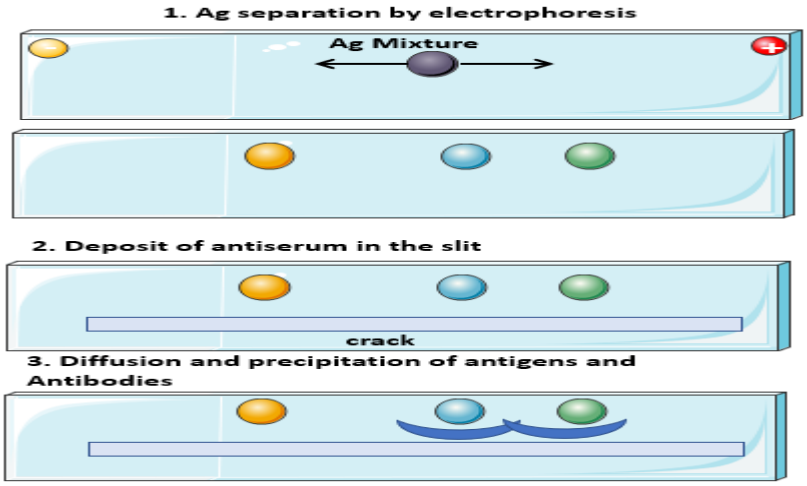

Figure. 92 : Grabar and Williams immunoelectrophoresis 1.

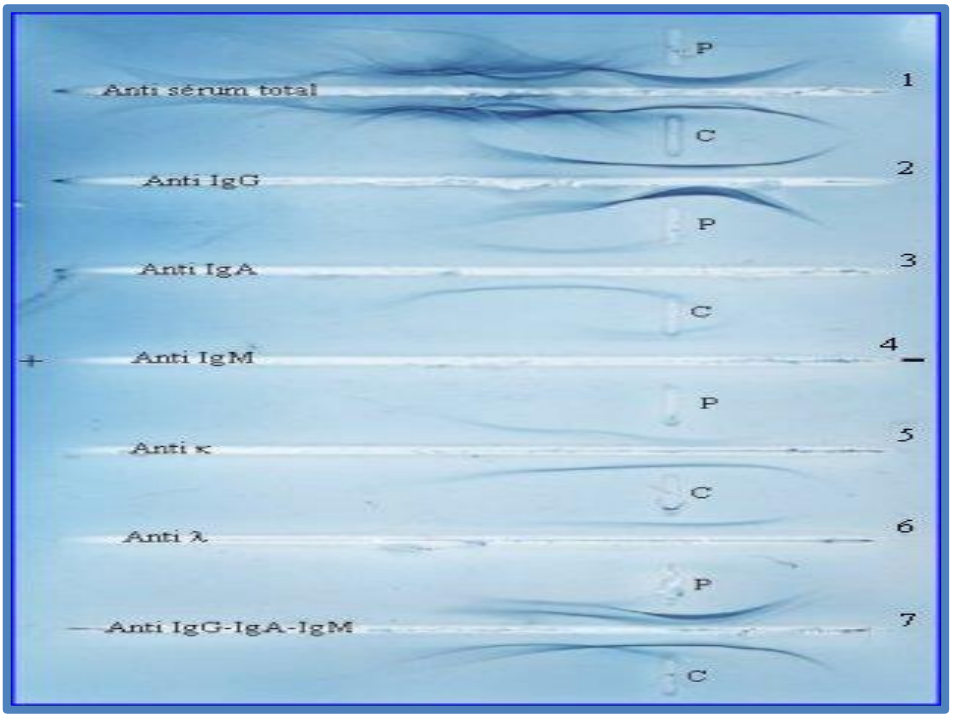

Figure. 93 : Grabar and Williams immunoelectrophoresis 2.

B) One-dimensional electro-immunodiffusion (Laurell)

This technique makes it possible to accelerate the diffusion time by forcing the Ag to migrate under the effect of an electric field, in a gel containing the Ac. The Ags move and encounter the antibodies which then form rocket-shaped precipitates called “rockets” (Figure 94). whose height is proportional to the protein concentration. We use part of the wells to make a calibration line.

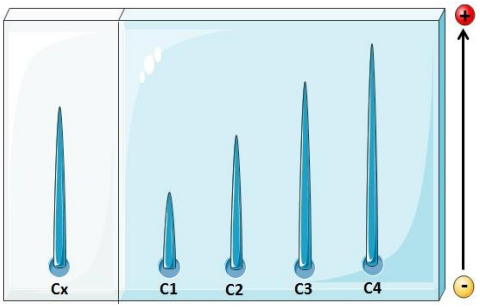

Figure. 94 : Grabar and Williams immunoelectrophoresis.

C) Two-dimensional electro-immunodiffusion

The proteins are separated in a first dimension in an agarose gel. A gel containing the antiserum is then poured, then the second dimension is produced.

D) Immunoblotting

Immunoblotting uses polyacrylamide gel electrophoresis to separate proteins. These proteins are then transferred from the gel to a nitrocellulose membrane (single diffusion), where they are exposed to an antibody specific for the protein of interest. The proteins are transferred from an acrylamide gel (the pores are too small) so that the antibodies diffuse easily to the surface of the nitrocellulose membrane and can easily reach the proteins.

The different stages of the transfer:

- Gel electrophoresis
- Transfer of proteins to nitrocellulose membrane (sandwich) (Figure 95)
- Incubation of a specific primary antibody solution
- Incubate another solution of labeled antibody (staining, fluorescence, radiography or chemiluminescence) (Figure 96).

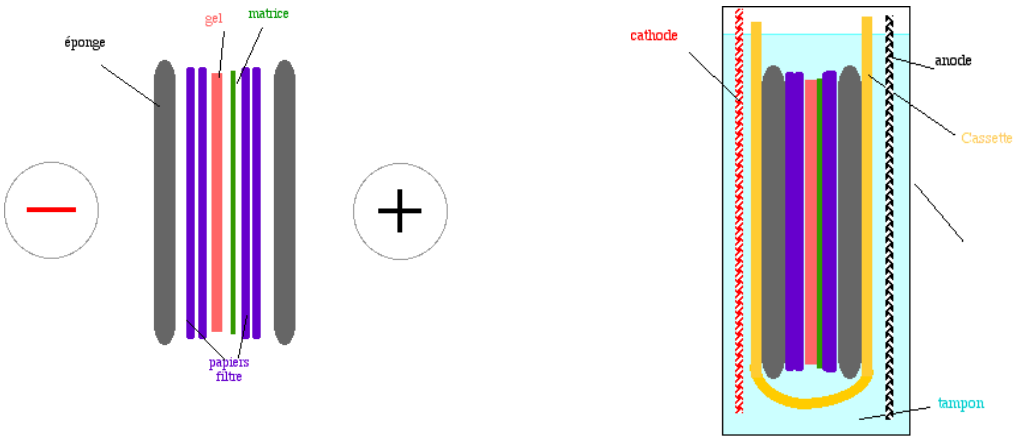

Figure. 95 : Transfer of proteins to nitrocellulose membrane (sandwich).

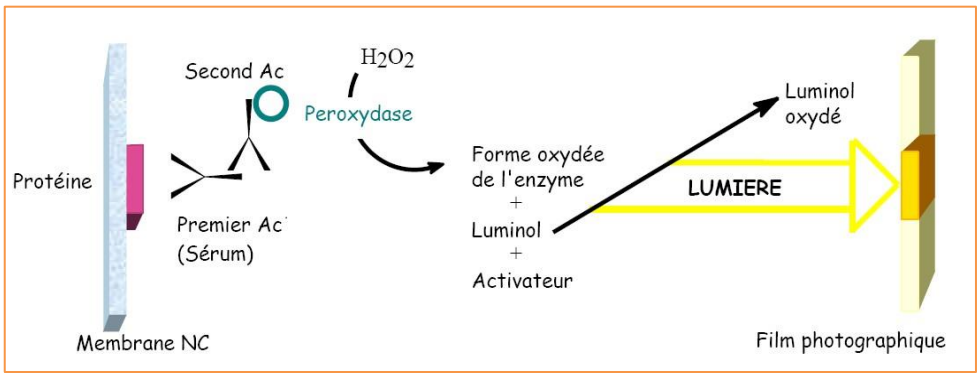

Figure. 96 : The incubation step with labeled primary and secondary antibodies.

4.5.4.7. Sequencing a protein

The steps involved in sequencing a protein are:

- Determination of the number of polypeptide chains (subunits) in the protein
- Cutting of disulfide bonds (inter- or intramolecular)
- Determination of the amino acid composition of each polypeptide chain
- Polypeptic chains are often too long to be directly sequenced; they must first be cut into smaller peptide fragments by specific reactions.
- Determination of the sequence of each of the peptide fragments by Edman degradation
- Elucidation of the sequence of each of the polypeptide chains by covering the sequences of the different fragments
- Determination of the structure of the entire protein, including disulfide bonds between subunits.

A) Breakdown of disulfide bridges

The breakdown of the disulphide bonds between the cysteines is above all a chemical denaturation, which is also essential for being able to separate the different groups of amino acids. For this purpose, reducing agents carrying thiol (–SH) functions are used, such as 2-mercaptoethanol or dithiothreitol (Cleland's reagent). There are also non-thiolated reducers such as TCEP (Tris (2-carboxyethyl) phosphine). You should also know that two cysteines are joined by their thiol group (–SH), and then form a molecule called cystine. Cysteine is an amino acid, so it can be denatured. The disulfide bridge is responsible for the tertiary structure of proteins, providing great stability to the latter.

B) Hydrolysis of peptide bonds

The hydrolysis of peptide bonds makes it possible to separate the different amino acids, because they are responsible for the primary structure, acting between the ketone group of an amino acid (C=O) and the amine group (NH2) of an AA (neighboring amino acid.

- **Total alkaline hydrolysis:**this is the use of sodium hydroxide (NaOH) at 4 mol/L hot (110°C) for approximately 24 hours. Total alkaline hydrolysis destroys serine, threonine, cysteine.
- **Total acid hydrolysis:**it is the use of hydrochloric acid (HCl) at 6 mol/L hot (110°C) and for approximately 24 hours. Total acid hydrolysis destroys tryptophan and transforms the amide functions of glutamine and asparagine into acid to give glutamate and aspartate. Total acid hydrolysis destroys fewer amino acids and is therefore the most widely used hydrolysis method.

C) Hydrolyzate analysis

After breaking the disulfide bonds, the hydrolyzate is deposited on an anion or cation exchange resin. Once the fractions have been recovered, they are stained with ninhydrin and the absorbance is measured at 570nm. We finally obtain an estimate of the percentage of each amino acid contained in the chain (% of alanine residues, etc.). The determination of this composition is not necessary to determine the sequence and is in fact less and less practiced.

D) Determining the sequence

E) Chemical marking of the N-terminus (NH2)

The first method uses the Sanger reaction (Figure 97). The 2-amine functions of amino acids and peptides react with 2,4-dinitrofluorobenzene to form yellow derivatives, 2,4-dinitrophenyl-peptides. When these compounds are subjected to acid hydrolysis, all peptide bonds are hydrolyzed, but the bond between the 2,4-dinitrophenyl function and the amine function of the N-

terminal amino acid remains stable. We can subsequently identify this amino acid, by chromatography for example.

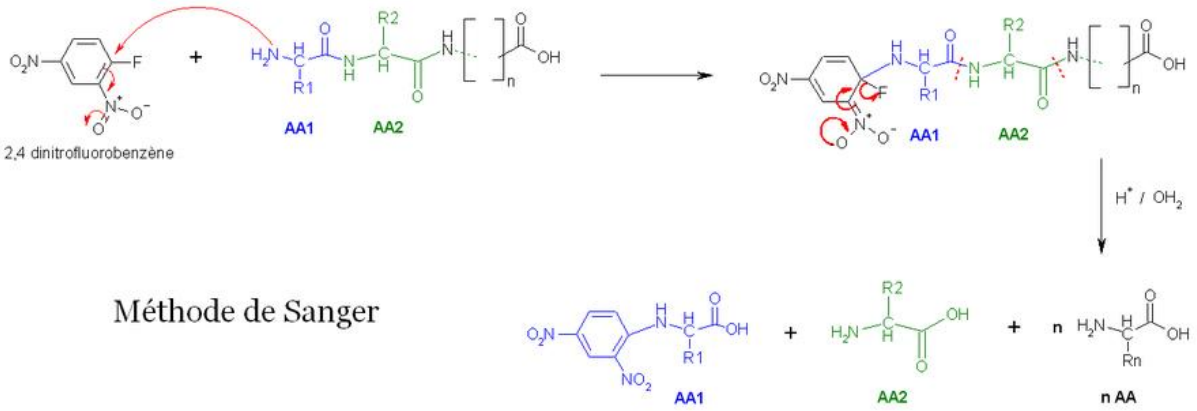

Figure. 97 : Sanger method

The second method uses dansyl chloride, which combined with total acid hydrolysis forms a Dansyl-Amino Acid1 (DNS-aa) which is fluorescent and a hydrolyzate (Figure 98).

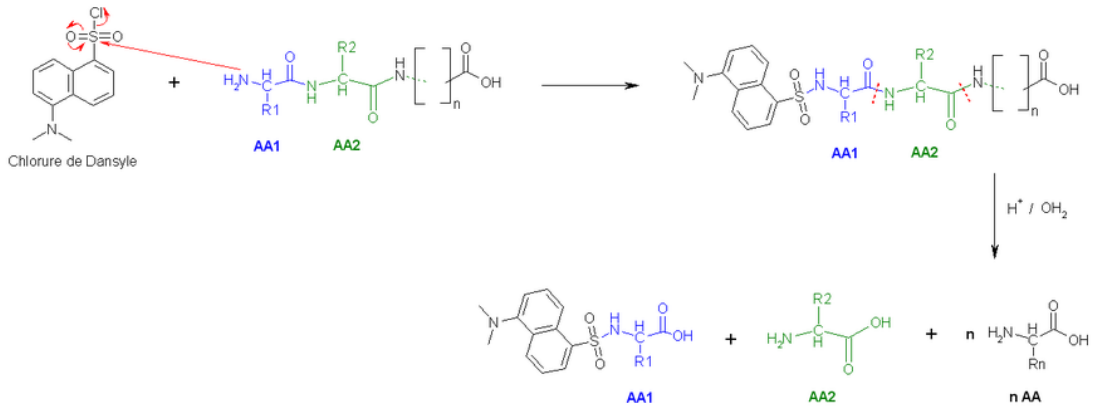

Figure. 98 : Dansyl chloride method

A third method is that of Edman, in which phenylisothiocyanate (PITC) reacts with the N-terminal amine function to give a complex which, after the action of an acid under mild conditions, releases a phenylthiohydantoin and the peptide remaining intact (Figure 99). The first two methods use total acid hydrolysis, the rest of the peptide chain sees its amino acids cleaved. The great advantage of the Edman method is that the peptide chain remains intact and can be recovered and resubmitted to treatment with PITC.

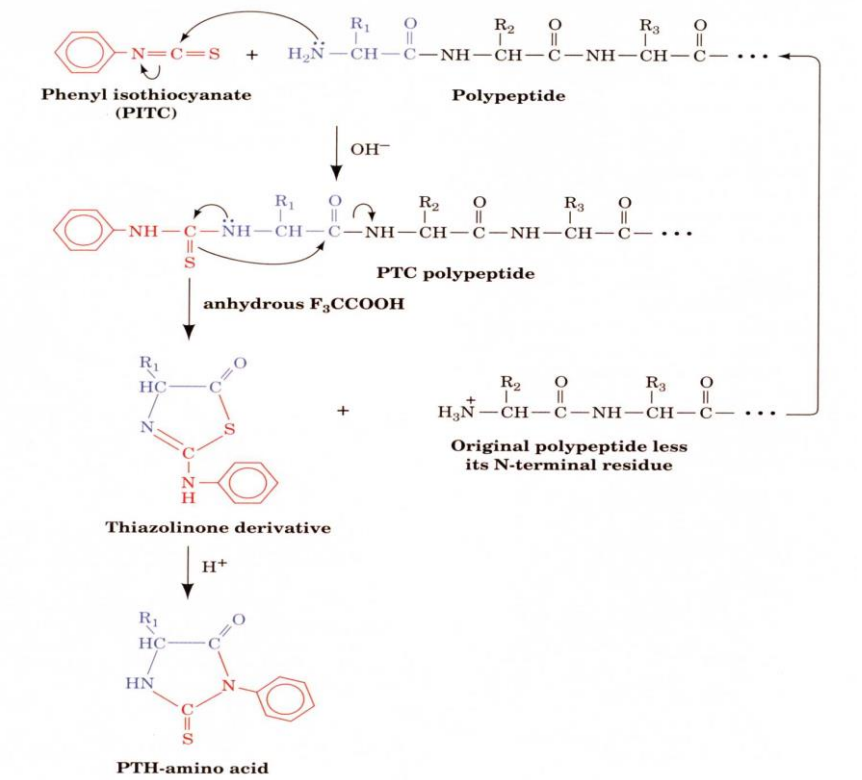

Figure. 99 : Edman's method

F) Identification of C-terminal amino acids

Hydrazinolysis is used, which cuts the bonds at 100°C and produces a free amino acid. We can also use carboxypeptidases which cut the C-terminal end, there are several types:

- Carboxypeptidase A, which cuts all amino acids at the C-terminal except lysine, arginine and proline. It is also blocked when the proline precedes the C-terminal amino acid.
- Carboxypeptidase B, which is specific for basic amino acids (Lys, Arg). It is blocked when the next amino acid is proline.

G) Enzymatic analysis of N- and C-termini

There are also biochemical methods using enzymes capable of cutting the first or last amino acid of the polypeptide chain, these are called exopeptidases. Aminopeptidases cleave the peptide bond located just after the first amino acid and release it. Symmetrically, carboxypeptidases cleave the peptide bond located just before the last amino acid.

There are several of these enzymes of varying specificities, for example among the carboxypeptidases, we find:

- Carboxypeptidase A cleaves the C-terminal amino acid when it is aromatic or aliphatic.
- Carboxypeptidase B cleaves the C-terminal amino acid when it is basic (Arginine or Lysine).
- Broad-spectrum carboxypeptidase Y.

By analyzing the amino acids released by these enzymes, it is possible to analyze the N or C-terminal sequence of the protein.

H) Fragmentation of the polypeptide chain

The polypeptide chain is cut into a series of small peptides by enzymatic or chemical hydrolysis.

Chemical method: The most commonly used chemical hydrolysis involves a reaction with cyanogen bromide, which cleaves peptide bonds in which the carboxyl function is provided by methionine residues (Figure 100).

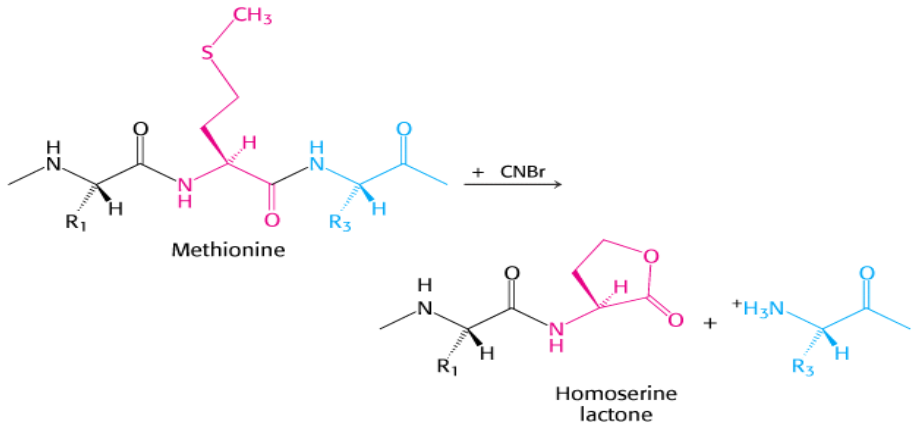

Figure. 100 : Chemical hydrolysis by cyanogen bromide

Enzymatic method (use of endopeptidases): Enzymatic hydrolysis uses proteases, enzymes that hydrolyze peptide bonds. Trypsin catalyzes the hydrolysis of peptide bonds in which the carboxyl function is provided by lysine or arginine, chymotrypsin allows the cleavage of the C-terminal side of aromatic amino acids (Phe, Trp and Tyr) (Table 4).

Table 4. Enzyme cutting sites

Enzyme	Amino acid	Cutoff site
Trypsin	Arg/Lily	C-ter
Chymotrypsin	Phe/Trp/Tyr	C-ter
Protease V8	Asp/Glu	C-ter
Pepsin	Phe/Trp/Tyr	N-ter
Thermolysin	Leu/Island/Trp/Tyr/Val/Ala/Phe	N-ter
Carboxypeptidase A	All aa in C-ter., except Pro, Arg/Lys	Releases the amino acid C-ter
Carboxypeptidase B	Only Arg/Lys quant C-ter.	Does not cut if Pro is the penultimate amino acid.

I) Sequence identification

The identification of the sequence is done logically based on the results obtained by using the different methods described previously.

G) Mass spectrometry sequencing

Mass spectrometry (MS) has become an important technique for characterizing and sequencing proteins. This technique makes it possible to precisely measure the mass/charge ratio (m/z) of ions in the gas phase:

Fragmentation by MS/MS mass spectrometry makes it possible to sequence short amino acid sequences (10 to 20). Coupled with the protein digestion techniques previously described, it is thus possible to know the sequence of a protein. However, this approach requires using different digestion protocols to hope to obtain the entire protein sequence.

For an organism whose genome is entirely known, a single type of digestion will be used (for example a trypsin digestion followed by an HPLC analysis coupled to an ESI-MS/MS type mass spectrometer), which allows to obtain up to 30% of the protein sequence (which is sufficient to characterize the protein).

For an organism whose genome is not entirely known, automated sequencing is first used (Edman sequencing for example).

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