VNIVERSITAT DE VALÈNCIA

# BIOCHEMISTRY AND MOLECULAR BIOLOGY
# ACADEMIC YEAR 2022- 2023

## Department of Biochemistry and Molecular Biology

## Faculty of Medicine and Dentistry


**TAUGHT BY:**

Eulalia Alonso Iglesias
Herminia González Navarro
Elena Ruiz García-Trevijano
Juan Saus Mas
Elena Ortiz Zapater
Mar Orzáez Calatayud
Gema Hurtado Genovés
Elena Jiménez Martí

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## SCHEDULE

| Week | Class | Classroom |
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| 19/09 - 23/09 | Seminar 1 | Seminar |
| 26/09 - 30/09 | Practical session 1 | Laboratory |
| 03/10 - 07/10 | Seminar 2 | Seminar |
| 17/10 - 21/10 | Seminar 3 | Seminar |
| 24/10 - 28/10 | Practical session 2 | Laboratory |
| 07/11 - 11/11 | Practical session 3 | Laboratory |
| 14/11 - 18/11 | Practical session 4 | Laboratory |
| 21/11 - 25/11 | Practical session 5 | Laboratory |
| 28/11 - 2/12 | Seminar 4 | Seminar |
| 12/12 - 16/12 | Practical session 6 | Laboratory |
| 19/12 - 23/12 | Seminar 5 | Seminar |

# 1

# 1. INTRODUCTION TO THE BIOCHEMISTRY LABORATORY

## I. SAFETY IN THE LABORATORY

Laboratories are, in general, hazardous workplaces and users should be aware of the potential risks they may face, how to prevent them and how to act in case of emergency.

### I.1. Types of hazards

Although the use of materials, chemicals, and instruments that pose a significant risk of contamination, poisoning or accident for students and lecturers is kept to a minimum in the Biochemistry practical sessions, students must be aware of the general hazards present in a laboratory of this type. **The best way of ensuring safety in the laboratory is through knowledge of the materials being used, and being attentive and careful and responsible**.

**a) Biohazards:**

Human clinical materials are biohazards, as they may carry viruses or bacteria, or even tumour cell lines of human origin, which can enter and develop in the human organism. The risk is accentuated by pipetting with the mouth, the presence of wounds on the skin or mucous membranes, and contamination of the hands.

The risk these materials pose can be minimized by using automatic pipettes, a mask, and gloves. Samples of human origin must be properly labelled and disposed of together with non-recyclable material that has been in contact with them in special containers.

**b) Chemical hazards:**

Reagents, such as solvents (ethanol, methanol, acetone, chloroform, etc.), reaction substrates, acids and alkalis, dyes (ninhydrin), and bleaches (acetic acid, etc.) are all chemical hazards. Some of these products may be toxic if

ingested or inhaled, and others are caustic. Most of the colouring products stain skin and clothing intensely.

The risks posed by these hazards can be minimized using gowns, pipette tips, automatic pipettes and, if necessary, safety goggles and gloves. If you are splashed with one of these chemicals, especially in the eyes, wash quickly and abundantly with water using any of the taps on the training tables. In case of ingestion, notify the lecturer in charge of the session, indicating the product ingested.

**c) Electrical hazards:**

Devices that generate intense electric fields for their operation (e.g. power supplies for electrophoresis) pose electrical hazards, as can the accidental contact of any electrical device, even those of moderate voltage, with water. The risk can be minimised by ensuring that plugs, sockets, electrodes, accumulators, capacitors and, in general, any electrical or electronic apparatus in the laboratory cannot be accidentally manipulated. It is essential to avoid handling liquids in the vicinity of electrical instruments other than those specifically designed for this purpose (thermostatic baths, hotplates, etc.).

Immediately move away from the electrical equipment if there is a liquid spillage in its vicinity and immediately notify the lecturer. In the event of an electric shock, avoid touching the person suffering the shock and try to disconnect the equipment or the general power supply, but without exposing yourself to the same hazard. In any case, notify the lecturer immediately.

**d) Thermal hazards:**

Heat sources (thermostatic baths and hotplates) used to raise the temperature (sometimes to over 100°C) in the course of chemical reactions or in the preparation of materials (e.g. melting of agar) are significant hazards. Incidents can occur by inadvertent handling or contact with hot surfaces of equipment, by handling or contact with tubes and vessels that have been heated, or by the splashing of hot liquids.

The risk can be minimised by avoiding handling heated materials without first checking their temperature (the heat may have been switched off, but the materials may still be hot!) and by careful handling of pipes and vessels attached to heating systems: use tongs, cloths or, if necessary, protective gloves and take care to avoid dropping any vessels or spilling any liquid.

## I.2 Safety rules

Because of all the above hazards, it is necessary (and obligatory) to always

wear a lab coat in the laboratory. Safety goggles, gloves and a face mask may also be used when necessary.

Smoking, eating, or drinking in the laboratory is forbidden, to avoid the danger of poisoning or contagion by accidental contact with a toxic product or pathogenic agent.

Check the labelling of solutions and products before use. When using glass or plastic pipettes, always use a pipette and never pipette by mouth. Do not pipette directly from the distilled water bottle or from solutions shared with other groups: pipette from a quantity of solution previously transferred to a beaker. Care must be taken when handling hazardous substances: acids, alkalis, organic solvents, and toxic products. Before pouring a solution into the sink, the tap must be turned on and the water allowed to run afterwards.

The materials must be cleaned before and after use. Glassware should be handled with care to avoid breakages.

## II. BASIC EQUIPMENT IN THE LABORATORY

**TEST TUBES**: used for the measurement of volumes with relative accuracy. They can be made of glass or plastic, with volumes usually between 10 mL and 2 L (Figure 1).

**VOLUMETRIC FLASKS**: used to measure volumes with high accuracy, as they are calibrated. The volume is indicated by a graduation mark. They typically measure volumes from 5 mL to 1L (Figure 1).

**ERLENMEYER FLASKS**: used for the preparation of solutions to be shaken. They are not used for measuring volumes. They can be made of glass or plastic, with volumes typically between 25 mL and 2 L (Figure 1).

**KITASATO FLASKS**: also known as Büchner flasks, they are used to create a vacuum when connected to a suction system. They are used to collect liquid filtrates, to wash insoluble solids (with an intermediate filter) and to degas solutions (Figure 1).

**BEAKERS**: used for dissolving substances, as containers for liquids and never for measuring volumes. Glass and plastic beakers are available in volumes from 5 mL to 3 L (Figure 1).

**BOTTLES AND FLASKS**: they are used as containers for liquids. There are topaz-coloured ones, specially designed to protect their contents from light. They can be made of glass or plastic and their use depends on the physicochemical characteristics of the solution to be stored. The range of volumes is very wide (Fig 1).

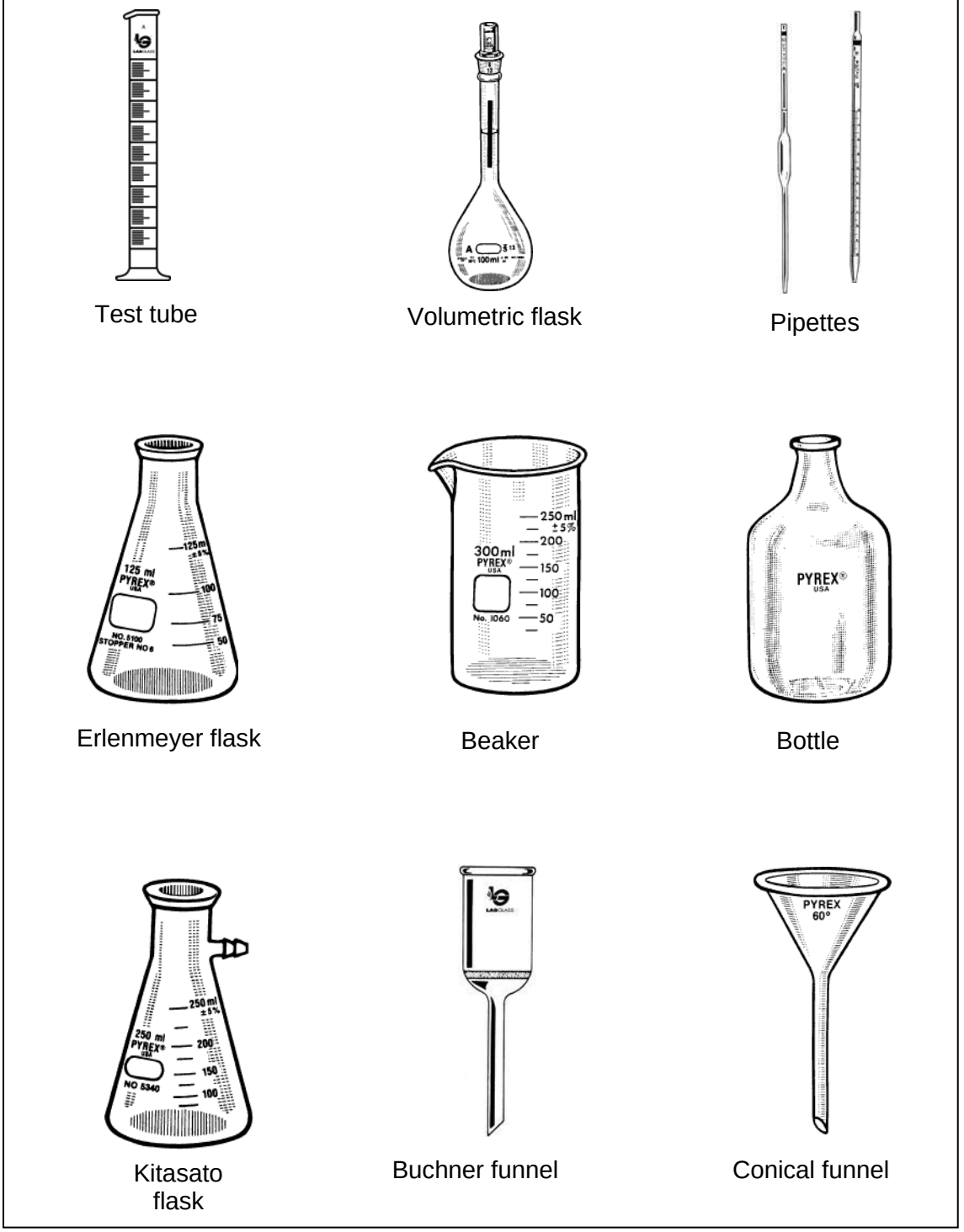



**Figure 1.** Laboratory equipment.

**WASH BOTTLES**: used for rinsing, adding water, washing, etc. They operate by manual pressure. They generally contain distilled water as this is the only water used in the biochemistry laboratory. For higher purity preparations, the water must be distilled several times or undergo other purification treatments.

**FUNNELS**: used to filter solutions, wash solutes, etc., at atmospheric pressure, through a paper or glass wool filter (Figure 1).

**TONGS**: used to insert and remove test tubes from high temperature baths.

**MANUAL PIPETTES**: are used to measure and transfer small volumes of solutions accurately. Their volume is precisely graduated, and they are available in sizes from 0.1 mL to 10 mL. They can be made of glass, which is reusable, or plastic, which is single use. The pipette can be filled by mouth aspiration (not recommended) or by a mechanical system (pipette). The latter should always be used, especially as the material to be aspirated may be toxic, pathogenic, or hazardous (Figure 1).

**PIPETTE**: a mechanical suction device attached to the top of the pipette to prevent accidental ingestion of toxic substances.

**AUTOMATIC PIPETTES**: these are high-precision mechanical devices that allow very small volumes of solutions to be measured and transferred with high accuracy. They are essential instruments in the biochemistry laboratory. Therefore, their handling and care are described in more detail in a special section (Figure 2).

**AUTOMATIC PIPETTE TIPS**: these are the disposable containers of the volume to be measured with these pipettes. They are available in different sizes and volumes, colour coded: white (0.1-10 µL), yellow (2-20 and 20-200 µL) and blue (200-1000 µL) (Figure 2).

**TEST TUBES**: the biochemical reactions studied in the laboratory are usually carried out using these containers and are made of glass or plastic depending on the substances involved. Their capacity is specified in terms of the internal diameter of the opening and the length of the tube (e.g. 12 x 75 mm).

**EPPENDORF TUBES**: these are small conical vials, fitted with lids, typically used for high-speed centrifugation of small volumes or for storing small quantities of solutions.

**RACKS**: These metal or plastic structures allow several test tubes to be arranged vertically so that they can be conveniently handled during the experiment.

**SPECTROPHOTOMETER CUVETTES**: these are small containers with two transparent sides placed opposite one another. Solutions are placed into these containers and then their light absorption or colour can be determined by means of a spectrophotometer. They are made of plastic, glass or quartz, depending on the wavelength to be measured. They can also be of various volumes, with the most common being 1 mL and 3 mL. Cuvettes should always be handled by holding the opaque sides.

**BUNSEN BURNER**: this is a heat source, burning centrally-piped gas, and it is used to heat the solutions. In general, glass tubes and containers should not be exposed directly to the flame, but heated using a liquid bath (water or oil) in a suitable container.

**THERMOSTATIC BATH**: a water vessel of relatively large volume (up to 50 L), whose temperature can be regulated and kept constant with great precision, thanks to a thermostat. It is used to carry out reactions that require strict temperature control.

**MAGNETS**: these are small magnetic rods, coated with Teflon, which are placed at the bottom of vessels in which a solute is dissolved. These rods rotate via the effect of a magnetic rotor underneath the vessel.

**MAGNETIC STIRRER**: instrument for stirring solutions. The stirrer has a powerful magnet that rotates and turns a small magnet inside a container.

**INCUBATION OVEN**: a closed cabinet with air temperature control for incubation.

**CENTRIFUGES**: Apparatus for settling suspended particles within a liquid by subjecting them to high centrifugal forces, achieved by the rapid rotation of the liquid in a container.

**PRECISION BALANCES**: These are used for the accurate weighing of reagents and substances.

## II.1. Handling and care of automated micropipettes

Automatic pipetting devices enable the repeated dispensing of equal volumes. Most of the automatic pipettes used in the biochemistry laboratory are of the manual type.

Automatic micropipettes operate by displacement of air by means of a plunger with a certain stroke length, which then draws a certain volume of liquid back to the point of origin by suction. The pipettes are adjustable, so that the measured volume can be controlled by precisely varying the length of the plunger stroke. Several types of adjustable pipettes are available, depending on the range of volume required: 0.5-10 µL, 2 to 20 µL, 10-100 µL, 20 to 200 µL and 200 to 1000 µL.

The required liquid volume is selected by means of a calibrated wheel. The solution is drawn into an interchangeable polypropylene tip which fits on the end of the pipette. Depending on their volume, the tips are white (0.1- 10 µL), yellow (2 to 200 µL) or blue (more than 200 µL). These tips can be ejected mechanically from the pipette after use without having to touch them with the fingers, thus avoiding contact with harmful substances.

Automatic pipettes are high-precision instruments, whose perfect functioning, accuracy and durability depend on their careful use, taking into account several factors:

- The volume to be taken should be carefully measured, avoiding reading errors on the graduated scale of each type of pipette.
- Place the plunger at the end of its stroke before inserting the pipette

tip into the solution to be taken, thus avoiding the formation of bubbles in the solution.

- Note the two stops on the piston stroke. The first stop (offering slight resistance to the finger) indicates the exact volume to be taken. The second stop (extra stroke) is only used to expel the entire liquid. If the pipette is filled up to the last stop, more volume is taken than has been measured, resulting in an error due to excess.
- Gently return the plunger to its original position when loading the solution. This prevents air bubbles from entering the pipette tip, which will cause a default error. In addition, and very importantly, the sudden return of the plunger to its original position can draw liquid into the pipette, which can damage the delicate mechanism.
- Do not invert or tilt the pipette excessively when it is filled with liquid.
- Do not knock or drop the pipette.
- Do not touch the pipette tip with your fingers when inserting the pipette, as this may contaminate the solution to be measured (many metabolites are dissolved in sweat).
- Dispose of used tips in the containers provided for this purpose on all tables.

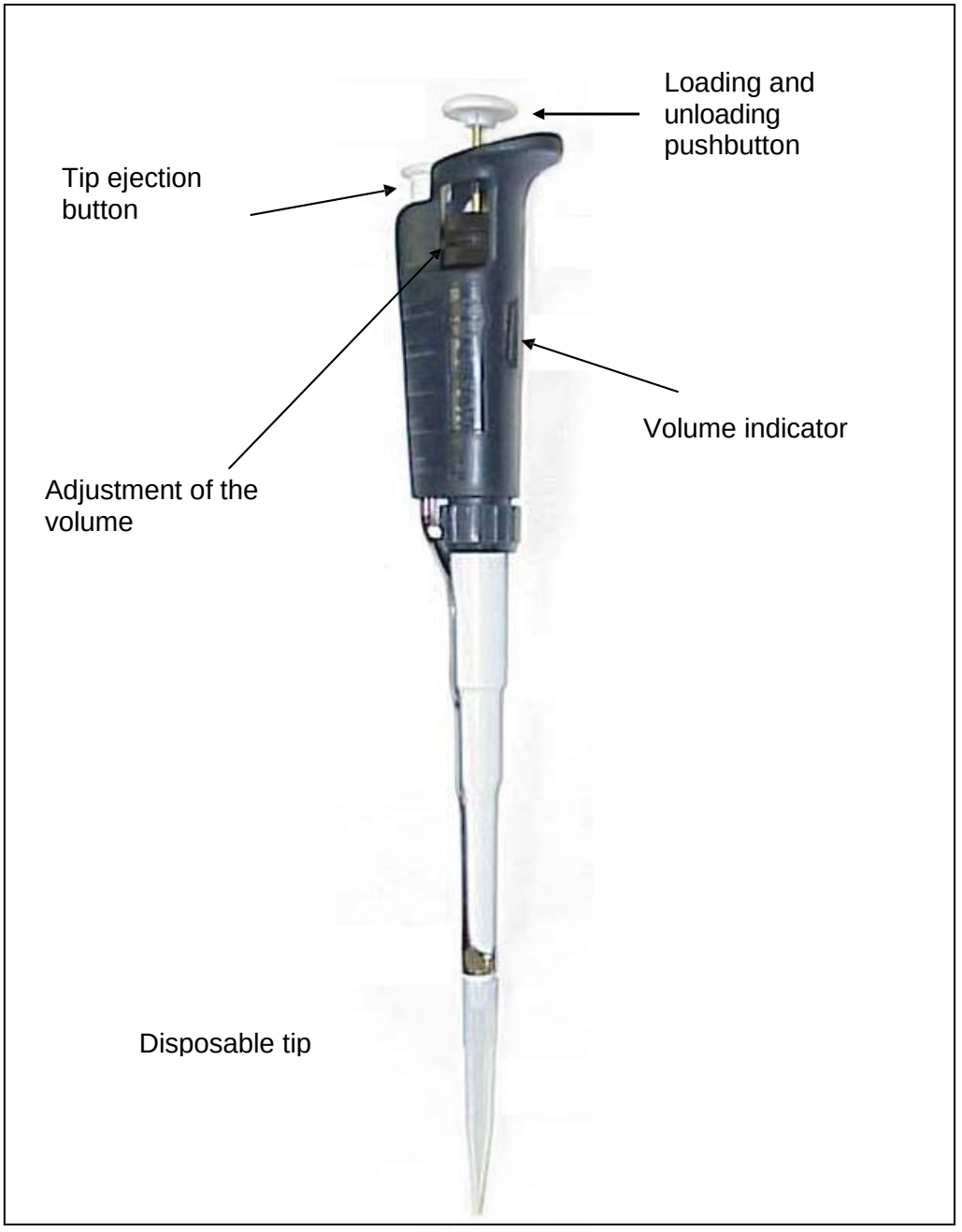



**Figure 2.** Automatic micropipette

# III. PREPARATION OF SOLUTIONS

***a)*** **For solids:**

– Calculate the required amount of solute, taking into account the characteristics of the initial product in terms of purity, hydration, etc.

– Weigh into a beaker using a spatula.

– Add a quantity of solvent (usually water) to the beaker that is less than the

volume of solution to be prepared.

- Dissolve the solute with the aid of a glass rod or with mechanical/magnetic stirring.
- Pour the solution into a volumetric flask, or into a measuring cylinder when high precision is not required. Add a little solvent to the beaker to collect the residue of the solution and pour it also into the volumetric flask or measuring cylinder.
- Add solvent until the bottom of the meniscus coincides with the final volume of the solution (up to the graduation mark).

***b)*** **For liquids:**

- Calculate the required quantity of liquid, taking into account the characteristics of the product (purity, density, etc.).
- Measure the volume using a pipette or measuring cylinder.
- Pour the liquid into a measuring cylinder or volumetric flask.
- Add solvent to make up the final volume of the solution.

The concentration of the solution is expressed in units as described in the following section.

## III.1. Most frequent units of concentration

A **solution** is the result of completely interposing the molecules of a solid in a liquid or of a liquid in another liquid. The most abundant component is called the solvent and the minority component is known as the solute.

All biochemical reactions take place in aqueous media, which is why the preparation and knowledge of the physicochemical properties of solutions in this medium are of great importance in the laboratory. Thus, biological fluids (blood, serum, plasma, urine, cerebrospinal fluid, etc.) are solutions of metabolites and mineral salts in water. In addition, most of the reagents used are also solutions.

The **concentration** of a solution is a measure of the amount of solute present in the solution. It can be expressed in various forms, the most common being Molarity, Normality, Weight/Volume and Volume/Volume.

**MOLARITY ($M$)**: equals the number of moles of solute per litre of final solution. A mole is the molecular weight ($\mathbf{MW}$) of the solute expressed in grams. Thus, a 1 M solution of sodium chloride (NaCl, MW=58.5 g) is one that contains 58.5 grams of this salt per litre of solution.

$$moles = \frac{masa\ (g)}{PM\ (\frac{g}{mol})}$$

$$M = \frac{moles}{Vol\ (L)}$$

When studying small concentrations, such as those of the metabolites in living organisms, submultiples of this unit are used (Table 1):

**TABLE 1.** Submultiples of concentration units.

| QUANTITY | CONCENTRATION | EQUIVALENCES |
|---|---|---|
| mmol (millimol) | mM | 0.001 M ($10^{-3}$ M) |
| mol (micromol) | μM | $10^{-6}$ M, $10^{-3}$ mM |
| nmol (nanomol) | nM | $10^{-9}$ M, $10^{-6}$ mM, $10^{-3}$ μM |
| pmol (picomol) | pM | $10^{-12}$ M, $10^{-9}$ mM, $10^{-6}$ μM, $10^{-3}$ nM |

**NORMALITY (N)**: equals the number of equivalents (***Eq***) of solute per litre of final solution. The number of equivalents of solute is equal to its mass in grams divided by the equivalent weight. In turn, the equivalent weight (***Eq***) of a substance is equal to its molecular weight divided by its valence. For example, for sulphuric acid ($H_2SO_4$ ; MW = 98 g/mol; its valence as an acid is 2 as it gives up two $H^+$ ) the Eq is 98/2 = 49 g. However, 1 Eq of hydrochloric acid (HCl, PM=36 g/mol, valence 1) is 36/1 = 36 g.

Submultiples of N are not commonly used, although the term mEq (= 0.001 Eq) is sometimes used.

$$PE = \frac{PM\ (g/mol)}{valencia}; \qquad Eq = \frac{masa\ (g)}{PE\ (g/mol)}; \qquad N = \frac{Eq}{Vol\ (L)}$$

**WEIGHT/VOLUME**: expresses the mass of the solute, in grams or submultiples thereof, per unit volume (litre or submultiples). This type of unit is widely used in clinical biochemistry. However, the current trend is to express analytical results in units of molarity. The most commonly used Weight/Volume units are g/L, mg/mL, $mg/cm^3$ , g/dL, % w/v (g/100 mL of solution), etc.

**VOLUME/VOLUME**: used when the solution is prepared by mixing two liquids. The most common units are mL/L and % v/v (mL/100mL of solution).

## III.2. Dilutions

To dilute a solution is to decrease its concentration. This can be done by simply increasing its volume, but it is usually done by removing a small volume

of the concentrated solution and then adding a certain amount of solvent to this quantity until the desired concentration is achieved. To determine the volume ***V1*** of concentrated solution, with a concentration ***C1***, which is needed to prepare a more dilute solution of concentration ***C2*** and volume ***V2***, the relationship is used:

$$V_1 \ x \ C_{1=} \ V_2 \ x \ C_2$$

# IV. PROCESSING OF DATA

## IV.1. Measurement error

**Precision and accuracy** are not synonymous concepts. A method is **precise** when repeating the measurement in independent tests and always gives similar results. When a method is precise, it is also said to be reproducible. However, a measurement is **accurate** when it is very close to the true value. Therefore, a method will be accurate when there is a high probability of obtaining measurements close to the theoretical value.

A device can give very precise but inaccurate measurements: for example, when the device is not properly calibrated.

No measurement made in the laboratory is exact. The **error** of a measurement is the difference between the measured value (***Xi***) and the exact or correct value, also called the theoretical value (***Xt***).

Errors arise from statistical variations and, although unavoidable, they can be minimised by working carefully. They originate either in the measuring instrument, either because of inaccuracy or calibration defects, or in the handling of the samples. In the latter case, we are talking about human error, which most frequently occurs when taking scale readings.

To guard against as far as possible the effect of error, the measurements are repeated a certain number of times and the **arithmetic mean** value ( $\bar{X}$ ) of the values obtained is used. The set of values is called the sample.

*Sample of n measurements:* $X_1, X_2, X_3, \ldots, X_n$

$$Error \ = X_i \ - \ X_t$$

$$\bar{X} = \frac{\sum_{Xi=1}^{Xi=n} X_i}{n}$$

The overall sample error is expressed by the **standard deviation**, ***SD***,

defined as:

$$DS = \sqrt{\frac{\sum(Xi - \bar{X})^2}{(n-1)}}$$

Also used is the **coefficient of variation**, ***Cv***, which represents the fraction of the standard deviation from the mean, usually represented as a percentage.

$$C_v(\%) = \frac{DS}{\bar{X}} \cdot 100$$

## IV.2. Calibration curves (standard curve)

In order to determine the concentration of a substance in a test solution, a **standard curve** must be constructed to show how a measurable physical property (e.g. colour) varies, either directly or after reaction with a reagent, as a function of the concentration of the substance. For this purpose, **standard solutions** of known concentrations are used, which must be treated identically to the solution to be titrated.

The value obtained in the quantification of the **test solution** must be within the limit values of the known solutions. In addition, a control, usually called a **blank**, must be carried out in which the test substance is replaced by distilled water (see Figure 3).

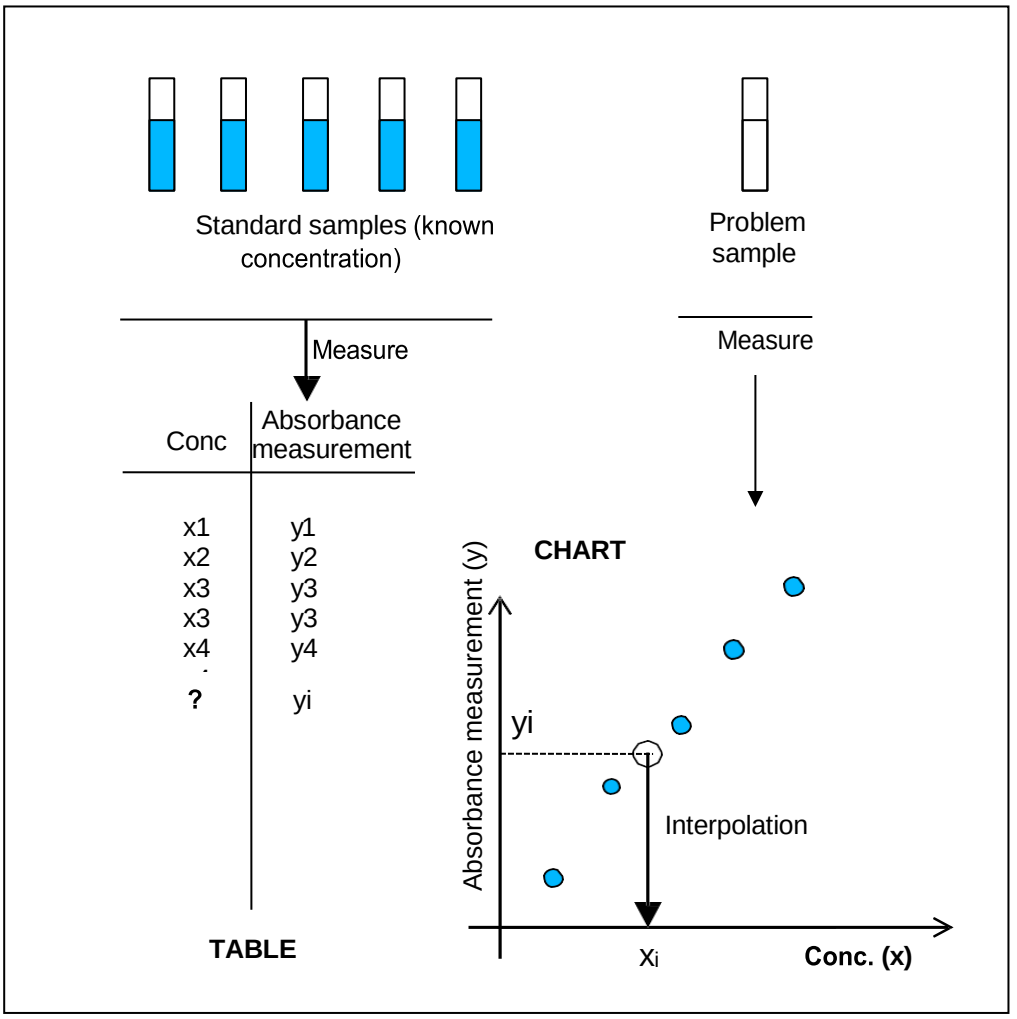



**Figure 3.** Calibration curve and problem interpolation

## V. CARRYING OUT LABORATORY WORK AND PRESENTATION OF RESULTS

The aim of laboratory work is the communication of ideas and results in a comprehensible way. The writing of a practical paper (objectives, method, results, discussion and conclusions) is a good exercise for the writing of a scientific publication.

You should have a laboratory notebook, which is rigid and lined. Loose-leaf (ring-bound) notebooks are practical, as they allow for the insertion of experimental results, but there is a risk of losing pages. Experiments should be written in sufficient detail to allow for experimental reproduction if necessary. The presence of diagrams of apparatus or purification processes, together with figures illustrating experimental results (e.g. electrophoresis) can give much more information than a long description in the text.

The experimental results should be summarised in the form of a table or graph.

Tables and graphs should be named in order of appearance in the text (Table 1, Table 2, etc.) and should have a title. In some cases, it may also be useful to give additional details in the form of a legend below the title. The units in which the results are expressed should appear at the top of each column, and never in each line of figures. These units should be chosen in such a way that they present a limited number of figures. For example, a concentration of 0.0072 mol/L is most easily written as 7.2 mmol/L or 72 x $10^{-4}$ mol/L.

In general, the values obtained are presented in the form of a graph rather than a table. To plot a graph, the values of the independent variable (the known parameter) are represented on the abscissa axis (x-axis) and those of the dependent variable (unknown parameter) on the ordinate axis (y-axis). To enable the correct identification of a graph, you should:

- Give it a simple and concise title.
- Clearly indicate quantities and units on the axes.
- Adjust the scales to obtain a slope of about 45°.
- Use simple numbers.
- Use clearly defined symbols.
- Join the points together with a continuous line or curve.

In your laboratory workbook, the units of magnitude and the standards recommended by the International System (SI) should be used. The symbols of the SI units must be written in lower case (except in the case of the liter, L). In the case of compound units which are formed by the multiplication of two or more units, they may be written without a full stop between them.

Similarly, if the relation between two or more units is one of division, the forms mol/L or mol·$L^{-1}$ are also correct. In addition, in order not to write very small numbers, the SI allows the use of submultiples of units, which is indicated by a prefix attached to the symbol of the unit they modify. The most used prefixes in biochemistry are milli- (m, $10^{-3}$), micro- (µ, $10^{-6}$) and nano- (n, $10^{-9}$).

# VI. CASE STUDY: DETERMINATION OF THE ERROR MADE WHEN MEASURING A VOLUME WITH A MICROPIPETTE.

Automatic micropipettes allow **accurate** measurement of small volumes. If they are also properly calibrated, we will measure volumes quite **accurately**. In this practical session, we will determine the error made when pipetting a 1000 mL volume of water with an automatic micropipette. To do this, we will assume that the mass measurement of each pipetted volume is not subject to error, since analytical balances are usually more accurate than micropipettes. We will also assume that the density of water, under laboratory conditions, is 1 g/cm$^3$.

## VI.1. Objectives

1) Familiarisation with the use of common biochemistry laboratory equipment.

2) Familiarisation with the handling of solutions and concentration units.

3) To understand the meaning of the experimental error, and to determine and analyse it.

## VI.2. Material required:

- Analytical balance.
- Flat-bottom vials.
- Micropipettes.
- Disposable tips.

## VI.3. Instructions for the exercise:

1) *Place an empty vial on the pan of the analytical balance.* ***Tare*** *the balance (it should read: 0.000 g).*
2) *Take a volume of 1800 mL of water with the appropriate micropipette and use the appropriate disposable tip (observe the range of volumes written on the top of the pipettes and note the colour coding of the tips).*
3) *Place this volume into the vial on the balance and record its mass value in Table 2.*
4) *Repeat steps 1-3 four more times.*
5) *Calculate the* ***errors*** *made in each measurement, the sample* ***mean****, the* ***standard deviation*** *and the* ***coefficient of variation****.*

**Table 2.** Values obtained from weighing**.**

| | MEASURED VOLUME | THEORETICAL VOLUME | ERROR |
|---|---|---|---|
| **1** | | | |
| **2** | | | |
| **3** | | | |
| **4** | | | |
| **5** | | | |

| MEAN | STANDARD DEVIATION | COEFF. VARIATION |
|---|---|---|
| | | |

# VII. QUESTIONS 1

NAME.....GRUPO.....

1) The concentration of a copper sulfate solution is 10 g/L. What is its molar concentration and its % w/v concentration? NOTE: The MW of $CuSO_4$ is 159.6 g/mol.

2) Calculate the necessary volume to prepare, from the solution of the previous question, dilute solutions of 1/2 v/v, 1/5 v/v, 1/10 v/v and 1/20 v/v, with a final volume of 5 mL each. Write down in Table 3 the volumes of concentrated solution and solvent required for each case.

**Table 3.** Volumes needed to prepare the dilute solutions

| | **DILUTION** | | | |
|---|---|---|---|---|
| **Volume (mL)** | 1/2 | 1/5 | 1/10 | 1/20 |
| **$CuSO_4$** | | | | |
| **H20** | | | | |
| **Total** | 5 | 5 | 5 | 5 |

3) What volume of 95% concentrated sulphuric acid (MW = 98.08 g/mol), with a density of 1.899 g/mL, should we take to prepare 100 mL of a 1 M solution of sulphuric acid?

4) The normal blood protein value is between 60-83 g/L. Plan a calibration curve (having as standard a BSA solution of 300 mg/mL) to be able to measure the protein value, both in normal samples and in pathological conditions of hyperproteinaemia or hypoproteinaemia. Plan the standard curve taking into account that we will have 200 μL of sample.The titration would be carried out in a final volume of 1 mL with the Bradford method by adding 0.2 mL of Bradford's reagent.

| Tube | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| | STANDARD CURVE | | | | | | | | | |
| BSA (mL) | | | | | | | | | -- | -- |
| [BSA] (μg/mL) | | | | | | | | | -- | -- |
| Sample (mL) | | | | | | | | | 0.2 | 0.2 |
| Water (mL) | | | | | | | | | 0.6 | 0.6 |
| Bradford (mL) | | | | | | | | | 0.2 | 0.2 |

# 2. Biochemical study of DNA (I): PURIFICATION, QUANTIFICATION AND AMPLIFICATION OF DNA.

## I. MOLECULAR BIOLOGY, GENETIC ENGINEERING AND RECOMBINANT DNA TECHNOLOGY.

The confluence of genetics and biochemistry was one of the factors that led to the birth of a new discipline: **molecular biology**. Three major events can be cited in the birth of this new discipline: the identification of deoxyribonucleic acid (DNA) as the universal genetic material of cells (Avery, McLeod and McCarthy, 1944); the determination of the physical structure of DNA (Watson and Crick, 1953) and, finally, the deciphering of the genetic code or information contained in the nucleotide sequences of DNA that is transcribed into messenger ribonucleic acid (RNA), which was made possible thanks to the research of the Spanish Nobel Prize winner Severo Ochoa.

The birth of molecular biology opened new expectations in the understanding and study of genetic diseases and cancer, at the same time as it provided many other disciplines with an excellent methodology that enabled great scientific advances. Today, Molecular Biology is understood as a multidisciplinary science that provides humanity with a range of concepts and methods that are very useful in many areas.

**Genetic Engineering** can be defined as a branch of Molecular Biology that concentrates on the study of DNA, via the *in vitro* manipulation of genes and the production of DNA molecules containing new genes or new combinations of genes. Ultimately, its aim is the genetic manipulation of organisms for a predetermined purpose.

Genetic engineering uses a range of techniques, including **recombinant DNA technology**. The term recombinant DNA refers to the creation of new combinations of DNA molecules that do not occur naturally together. Although natural recombination also produces recombinant DNA, this term is reserved for DNA molecules produced by the forced joining of fragments of diverse biological origin.

Recombinant DNA technology uses both methods derived from the biochemistry of nucleic acids and techniques that were originally developed for bacterial and viral research. This technology developed from the discovery of restriction enzymes and their action on nucleic acids. The principle of recombinant DNA technology is based on the isolation of a single DNA sequence

from a population of mixed sequences. Once this is achieved, we can insert a fragment of foreign DNA (e.g. a gene) into a cloning vector, such as a virus or a plasmid, and obtain a pure population of recombinant molecules (clones). The recombinant DNA obtained can be introduced into a host, resulting in the expression of the cloned gene in a different organism.

Genetic engineering has numerous applications in very diverse fields, ranging from medicine to industry. It is particularly useful in the study of genomes, in gene expression, in obtaining hormones (insulin, growth hormone), recombinant proteins (coagulation factors) and vaccines (hepatitis B), and in the creation of drugs or active peptides whose chemical synthesis is difficult. It is also used to obtain tissues or repair genetic abnormalities in humans (gene therapy). In agriculture and animal husbandry, it is also used to improve (non-traditional) plants and animals; and in the food industry to improve biotechnological processes for food production (bread, wine, beer, yoghurt, etc.).

# II. CASE STUDY: PURIFICATION AND QUANTIFICATION OF LIVER GENOMIC DNA

In order to carry out the studies described above, it is necessary to isolate and purify the DNA molecule so that it can be manipulated. To this end, first of all, a series of steps are carried out to obtain a cell extract or homogenate consisting of the cellular components in the presence of a solution. In eukaryotic cells, the DNA is located inside the nucleus, so for extraction, the cell membranes (cytoplasmic and nuclear membranes) must first be ruptured in the presence of components that preserve the integrity of the DNA required for the cell extract. The DNA obtained is then purified and concentrated, and finally, the degree of purity is quantified and determined.

The following steps will be carried out:

- To achieve cell **rupture** or **lysis** (and allow DNA to be released), the tissue is treated with an anionic detergent (SDS) capable of disrupting cell membranes, thereby releasing proteins and nucleic acids. DNA degradation by the action of DNases (which are activated in the presence of divalent cations) must be avoided during this process, and this is achieved by adding chelating agents (e.g. EDTA) to the medium.
- For DNA **isolation and purification** (removal of proteins and RNA from the medium), the extract is treated with proteolytic enzymes (e.g. proteinase K, which degrades most native proteins) resulting in small peptides that are also removed. The RNA molecules in the cell extract are degraded by the action of endogenous ribonucleases, which rapidly digest the RNA, converting it into ribonucleotides. The removal of the remaining proteins and peptides is carried out by a purification process using mini-columns. These columns have inside them a material that specifically binds with DNA by molecular interactions, but does not bind with contaminating proteins, peptides and ribonucleotides. The columns are washed several times to remove the proteins, peptides and ribonucleotides while the DNA remains bound. Once the contaminants have been removed, the DNA on the column is eluted with a buffer solution which is capable of breaking the interactions between the DNA and the column material. After elution, an isolated DNA preparation is obtained.
- To assess the **purity and quantify** the DNA, spectrophotometric assays are performed by assessing the absorbance of UV at 260 nm, which is directly proportional to the amount of DNA in the sample. An absorbance value of 1.0 at 260 nm corresponds to 50 g of double-stranded DNA. To control the purity of the DNA preparation, the ratio $_{A260}$ / $_{A280}$ is used. For a pure DNA sample, this ratio is between 1.7-1.9. Values lower than 1.7 indicate that the preparation is contaminated, either by proteins or by a component of the reagents used.

## II.1. Objectives

1) To become familiar with the terminology and concepts used in molecular biology.

2) To become aware of the importance of molecular biology techniques in medicine.

3) To learn to work with the volumes and quantities used in molecular biology techniques, as well as the basic rules to be observed.

## II.2. Material

- Automatic pipettes
- Autoclaved yellow and blue autoclaved tips
- Autoclaved Eppendorf tubes
- Falcon tubes
- Tube racks
- Gloves
- Spectrophotometer and quartz cuvettes
- Crushed ice
- Baths at 55ºC
- Thermoblock at 70ºC
- Pipettes
- Mini-columns
- 2 mL collection tubes
- Homogeniser
- Vortex mixer
- Centrifuge
- Balance

## II.3. Products and reagents

- Rat liver
- Extraction buffer
- Proteinase K (20 mg/mL).
- Binding buffer
- HBC buffer

- Wash buffer
- Elution buffer
- 100% ethanol

### II.4. Experimental procedure

The first part of this practical session involves the purification and quantification of DNA from eukaryotic tissue. In this practical session, we will use rat liver, but this procedure is standardised for the purification of genomic DNA from any tissue or cell preparation, following the same protocol.

**Stage 1: Tissue digestion and obtaining the homogenate**

*1. Homogenise the sample with the DNA extraction buffer (TL buffer). Place 30 mg of tissue in an Eppendorf tube with 200 µL of buffer.*

*2. Add 25 µL proteinase K and use the vortex mixer,*

*3. Incubate at 55°C. The average time is less than 3 hours, and can be done overnight. Vortex the sample every 20-30 minutes.*

*4. Centrifuge at maximum speed (≥10,000 x g) for 5 minutes.*

*5. Transfer the supernatant to a clean Eppendorf tube. Do not touch or disturb the sediment.*

***Note: Steps 1 to 3 above are carried out prior to the practical session by the technical staff of the department.***

**Stage 2: Extraction and purification of DNA**

*1) Take the supernatant obtained in the previous step and add 220 µL of binding buffer and vortex.*

*2) Incubate for 10 min at 70°C.*

*3) Add 220 µL of 100% ethanol and vortex.*

*4) Prepare the mini-column by placing it into the 2 mL collection tube.*

*5) Transfer ALL the sample from step 3), including any precipitates formed, to the mini-column and centrifuge at maximum speed for 1 minute.*

*6) Discard the collected liquid in the collection tube and place the column back into the collection tube.*

*7) Add 500 µL of HBC buffer to the column and centrifuge at maximum speed for 30 seconds.*

*8) Discard the collected liquid and collection tube and place the column into*

*a new 2 mL collection tube.*

*9) Add 700 µL of wash buffer.*

*10) Centrifuge at maximum speed for 30 seconds.*

*11) Discard the collected liquid in the collection tube and place the column back into the collection tube.*

*12) Repeat steps 10) and 11).*

*13) Place the empty mini-column back into the collection tube, and centrifuge at maximum speed this time for 2 min. Note: THIS IS AN IMPORTANT STEP TO ELIMINATE POSSIBLE ETHANOL INTERFERENCE.*

*14) Discard the collection tube and place the Mini column into a CLEAN Eppendorf tube.*

*15) Add 200 µL of elution buffer heated to 70°C.*

*16) Incubate at room temperature for 2 minutes.*

*17) Centrifuge at maximum speed for 1 minute.*

*18) Repeat steps 15), 16) and 17) without changing the tube column. The eluted liquid after the second centrifugation contains the DNA.*

**Stage 3: Quantification and measurement of purity**

*1) Make a 1:10 dilution of the DNA obtained and measure the absorbances at 260 and 280 nm in the spectrophotometer (1:10 dilution 100 µL DNA + 900 µL $H_2O$).*

*2) To calculate the amount of DNA obtained (in units of g DNA/mL), multiply the $A_{260}$ value by 50 and by the dilution factor (10).*

**DNA (g/mL) = $A_{260}$ x 50 x dilution**

# III. QUESTIONS 2

NAME.....GRUPO

1. Assess the amount of DNA obtained in the extraction.

2. Assess the purity of the extracted DNA.

3. Why is the DNA concentration calculated by measuring its absorbance at 260 nm and not at another wavelength? What is the numerical factor 50 which is used to convert absorbance into concentration units? Why is the purity of extracted DNA estimated using the A260/A280 ratio?

# 3. Biochemical study of DNA (II): DNA analysis by ELECTROPHORESIS.

## I. RESTRICTION ENZYMES

As mentioned above, DNA can be precisely manipulated, according to a premeditated design, using recombinant DNA technology. This requires enzymes that can cut, bind and replicate DNA and reverse transcribe RNA. Restriction enzymes are particularly useful as they allow very long DNA molecules to be cut into specific fragments that are easy to manipulate. Moreover, the available DNA ligases allow these DNA fragments to be used as modules that can be moved at will from one DNA molecule or vector to another. Recombinant DNA technology can therefore be said to be based on nucleic acid enzymology.

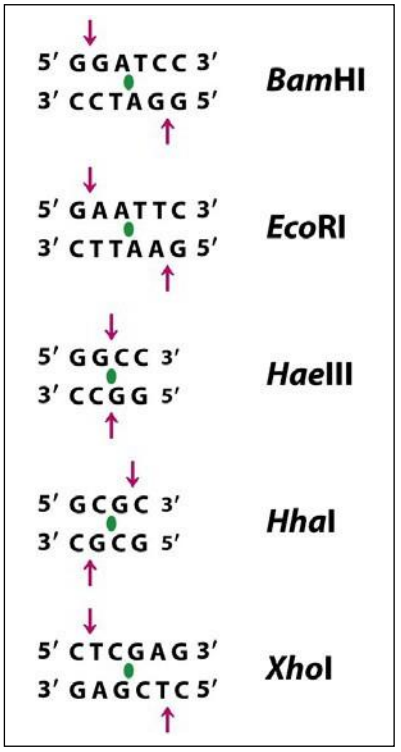



**Figure 4**. Examples of restriction enzyme recognition sequences.

**Restriction enzymes** are very precise molecular scalpels that allow the researcher to manipulate segments of DNA. These enzymes, also called restriction endonucleases, recognise a specific base sequence in double-helix DNA and cut it at specific points (Fig. 4).

Restriction enzymes have been found in a wide variety of prokaryotes. Their biological function is to destroy foreign DNA molecules. The cell's own DNA is not degraded because the sites recognised by its own restriction enzymes are methylated.

Many of the restriction enzymes recognize specific sequences between four and eight base pairs long and hydrolyze a phosphodiester bond on each DNA strand in this region. A notable feature of most of these cut sites is that they almost always possess binary rotational symmetry (they are palindromic sequences or inverted repeats). On the other hand, restriction enzymes, after cutting the DNA, can cause cohesive ends or blunt ends. Sticky ends are generated when the enzyme cuts the two strands asymmetrically, leaving the ends of each strand single-stranded and complementary to each other. On the other hand, the blunt ends are generated when the enzyme cuts the two strands

at the same place, generating two double-stranded ends (Fig. 5).

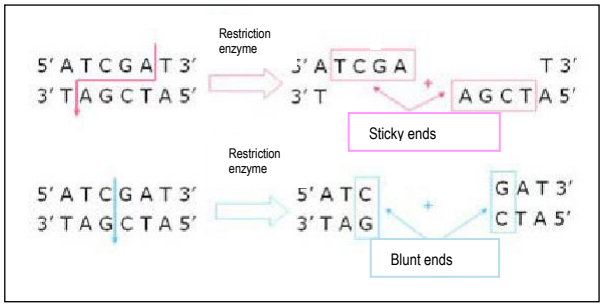



**Figure 5**. Example cuts with sticky or blunt ends.

The sequences recognised by most restriction enzymes contain a binary symmetry axis. The two strands of the region recognised by the restriction enzyme are related by a 180° rotation around the axis marked by the central point. The cut sites are indicated by arrows. To the right of each sequence, the abbreviated name of the enzyme that recognises it is given (Fig. 6).

Three/four-letter abbreviations refer to the host organism (e.g. Eco for *Escherichia coli*, Hind for *Haemophilus influenzae*, Hae for *Haemophilus aegyptius*). These abbreviations are followed by a Roman numeral specifying one of several restriction enzymes of the same strain.

Restriction enzymes are used to hydrolyse DNA molecules to obtain fragments of a specific size that can be easily analysed and manipulated to provide information about the molecule.

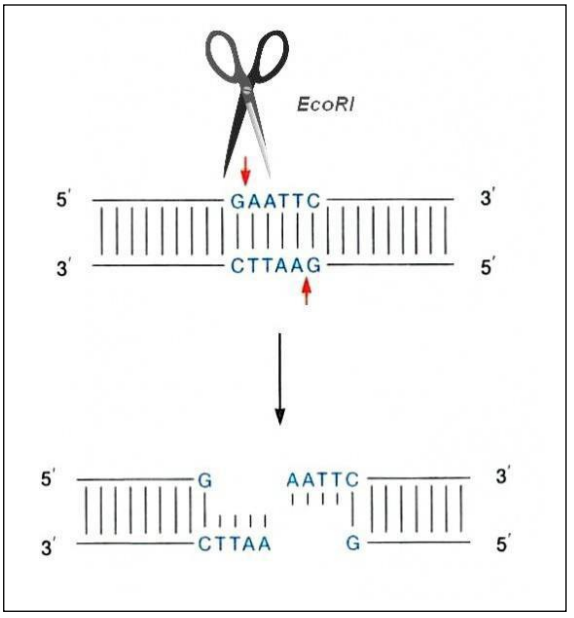



**Figure 6**. Enzymatic double-strand DNA breakage by *EcoRI*.

Fragments resulting from restriction analysis can be separated and visualised by **agarose gel electrophoresis**. The gels are stained with ethidium bromide, which, when bound to double-stranded DNA, confers an intense orange fluorescence. A band containing as little as 50 ng of DNA can be easily seen with this technique.

In these gels **the electrophoretic mobility of a fragment is inversely proportional to the logarithm of the number of base pairs**.

## II. RESTRICTION MAP

A DNA molecule cut by a given restriction enzyme will give rise to a series of fragments whose size depends on the number and location of recognition sites that exist for that enzyme in that DNA molecule. The combination of restriction enzyme digestion and gel electrophoresis is called **restriction analysis**. The

representation of a DNA molecule, either circular or linear, showing the restriction sites it possesses, is called a **restriction map**. In a restriction mapping experiment, the DNA is cut with various restriction enzymes, alone and in combination, to determine the number of cut sites and their relative positions on the DNA (Fig. 7).

The cut (or digested) DNA is separated by electrophoresis using an agarose gel and the sizes of the fragments are determined by comparison with the sizes of known fragments (the 'standards or markers') located in another ‘run’ of gel.

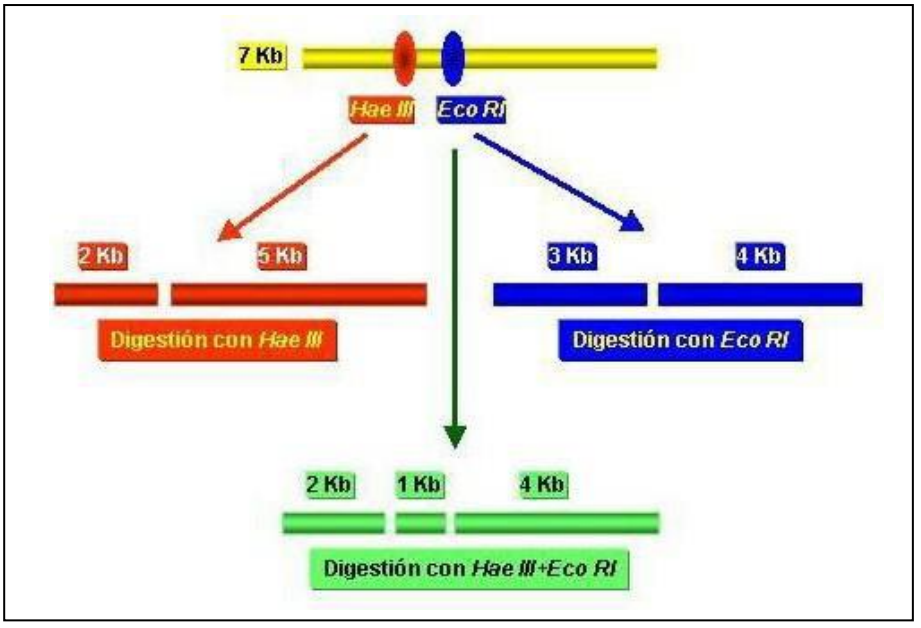



**Figure 7**. Example of restriction mapping of a DNA fragment.

The fragments are organised as a map by comparing the fragment sizes in runs where a single enzyme was cut (single digests) with those in runs where two enzymes were cut (double digests).

# III. CASE STUDY: RESTRICTION ANALYSIS USING ELECTROPHORESIS

## III.1. Objectives

1) To understand and perform restriction analysis of lambda phage DNA. Phage DNA fragments, after digestion with different restriction enzymes (individually and in combination), will be separated and analysed by agarose gel electrophoresis. The student will observe the banding pattern originating from each digestion and interpret the results.

## III.2. Procedure for the practical session

The enzymes used in the restriction analysis of lambda DNA are: XbaI, BamHI, EcoRI and HindIII. A double digestion with XbaI and HindIII and a reaction without enzymes will also be prepared to analyse intact lambda phage DNA.

**At each table:**

2) *Identify the Eppendorf tubes containing the lambda phage DNA digests. These reactions have been performed prior to the practical session, as indicated in the attached table:*

| V(microlitres) | 1. Control-Ø Non-Enzyme | 2. XhoI 1-Enzyme | 3. BamHI 1-Enzyme |
|---|---|---|---|
| **$H_2$ 0** | 24 | 20 | 20 |
| **Buffer (5X)** | 8 | 8 | 8 |
| **DNA λ (0.1 g/L)** | 8 | 8 | 8 |
| **Enzyme 1.5 U/L** | 0 | 4 | 4 |
| **$V_{TOTAL}$** | 40 | 40 | 40 |

| V(microlitres) | 4. EcoRI 1Enzyme | 5. HindIII 1-Enzyme | 6.XhoI-HindIII 2-Enzymes | |
|---|---|---|---|---|
| **$H_2$ 0** | 14 | 14 | 10 | |
| **Buffer (5X)** | 8 | 8 | 8 | |
| **DNA λ (0.1 g/L)** | 14 | 14 | 14 | |
| **Enzyme 1.5 U/L** | 8 | 8 | 4 | XhoI |
| | | | 4 | HindIII |
| **$V_{TOTAL}$** | 40 | 40 | 40 | |

For optimal enzyme activity, a medium with optimal conditions of ionic

strength, pH, cofactors, etc. must be prepared. This medium is different for each enzyme and therefore has a specific digestion buffer (5x means that the concentration of the buffer is 5 times higher than required, so the volume to be taken will be 1/5 of the total).

Tube 1, to which no enzyme is added, will serve as a control of intact, undigested lambda DNA.

These tubes with the reaction mixtures will have been incubated for 2 hours at 37°C.

3) *Add 8 µL of electrophoresis buffer to each tube and mix well by pipetting.*

This "electrophoresis buffer" contains a dense component, glycerol, to allow samples to settle to the bottom of the wells, and low molecular weight dyes, such as bromophenol blue, to observe the migration front during electrophoresis.

4) *Load 30 µL of each digestion mixture into the wells of the 0.75% agarose gel.*

The agarose gel contains ethidium bromide in order to "stain" and visualise the DNA. However, ethidium bromide is a mutagenic agent, so the agarose gel should not be handled without gloves.

The agarose gel must be correctly positioned in the electrophoresis cuvette as it should not be moved after loading the samples.

Each table loads its 6 samples in this order:

1-No-enzyme, **2-XhoI**, **3-BamHI**, **4-EcoRI**, **5-HindIII**, **6-XhoI-HindIII**.

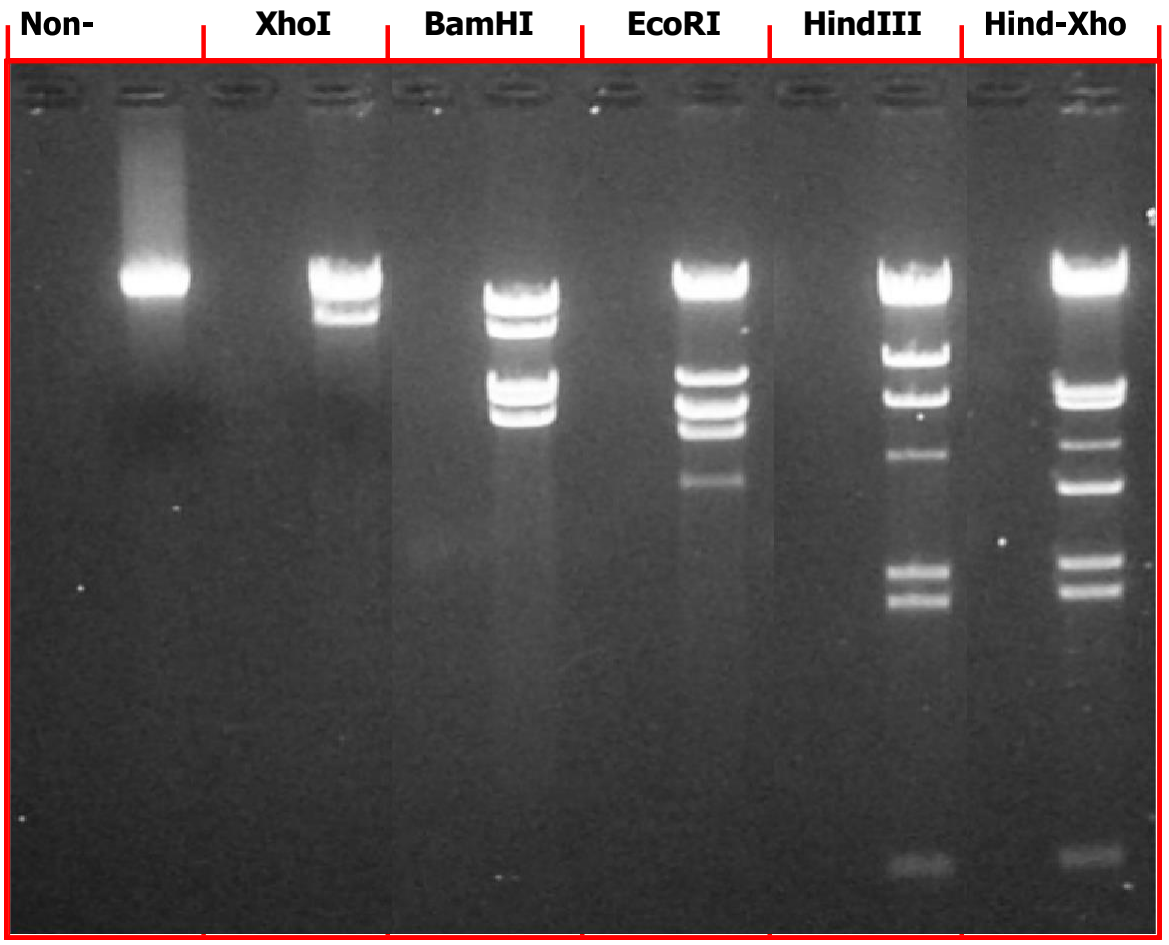
5) *Connect the electrophoresis cuvette to the power supply and switch on the equipment.*

6) *Apply a potential difference of 150 volts and let the samples migrate for 45-60 min.*

7) *Place the gel on a transilluminator emitting 300 nm wavelength ultraviolet radiation and observe the DNA banding pattern derived from the different restriction enzyme digestions.*

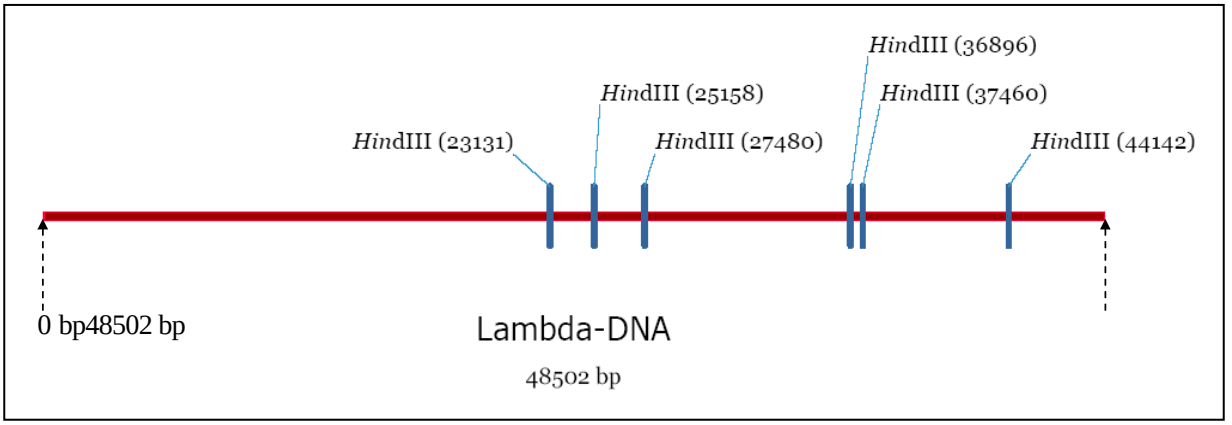
## IV. QUESTIONS 3

1) Bacteriophages (also called phages) are viruses that exclusively infect bacteria. They consist of a protein coat or capsid containing their genetic material which, in the case of the lambda phage, is a 48.5 kb linear DNA molecule. Digestion of lambda DNA with restriction enzymes and analysis by agarose gel electrophoresis gave the following result:




How many cutting sites does the lambda phage DNA have for each restriction enzyme?

2) From the restriction map of the lambda phage DNA for the HindIII enzyme (see figure below), determine the size of the fragments (from the largest to the smallest, in number of base pairs) and identify the corresponding DNA bands in the agarose gel.




Band: 1 > 2 > 3 > 4 > 4 > 5 > 6 > 7

Size in bp:
(from highest to lowest)

3) For the HindIII run, measure the electrophoretic mobility of each band in mm. This distance is measured from the well of the gel to the DNA band. Complete the data in the table below:

| | Band size (bp) | Log size (bp) | Migration (mm) | 1/ Migration (mm) |
|---|---|---|---|---|
| 1 | | | | |
| 2 | | | | |
| 3 | | | | |
| 4 | | | | |
| 5 | | | | |
| 6 | | | | |
| 7 | | | | |

Given that the distance migrated is inversely proportional to the logarithm of

the DNA size, determine the standard line representing the logarithm of the size (log size bp - Y) versus the inverse of the migration distance (1/migration (mm) - X). (See APPENDIX 2 - page 66 - for the representation and calculation of the line in EXCEL).

| **RESULT STANDARD LINE** | | |
|---|---|---|
| x | **y** | **r** |
| | | |

Representation:

4) From the comparative analysis of the HindIII and HindIII-XhoI digests, estimate which of the HindIII fragments (1, 2, 3, 4, 5, 6 or 7) is cut by the XhoI enzyme. Roughly indicate the XhoI cleavage site on the DNA restriction map.

5) Apolipoprotein B is a high molecular weight protein found in chylomicrons and in VLDL and LDL lipoproteins. Certain ApoB variants (e.g. the E allele) are

associated with lower serum cholesterol levels, but also with an increased risk of cholelithiasis (gallstones).

From the sequence presented below, locate the altered nucleotide in the E allele of Apolipoprotein B and suggest an assay to identify it in the population using a restriction enzyme (see Table 1).

**WT allele of Apolipoprotein B**: Part of Exon 29 amplified by PCR:

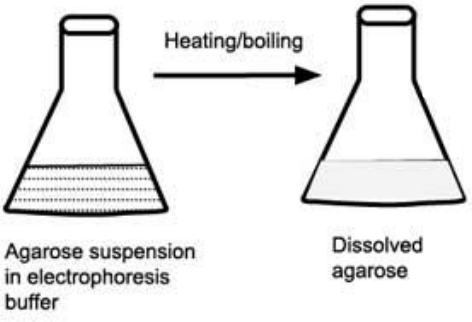
```
Phe  Gln Gly  Leu  Lys  Asp  Asn  Val  Phe  Asp  Gly  Leu  Val
TTC  CAG GGA  CTC  AAG  GAT  AAC  GTG  TTT  GAT  GGC  TTG  GTA

Arg  Val  Thr  Gln  Glu  Phe  His  Met  Lys  Val  Lys  His  Leu
CGA  GTT  ACT  CAA  GAA  TTC  CAT  ATG  AAA  GTC  AAG  CAT  CTG

Ile  Asp  To   Leu  Ile  Asp
          be
ATT  GAC  TCA  CTC  ATT  GAT
```

**Apolipoprotein B allele E**: Part of Exon 29 amplified by PCR:

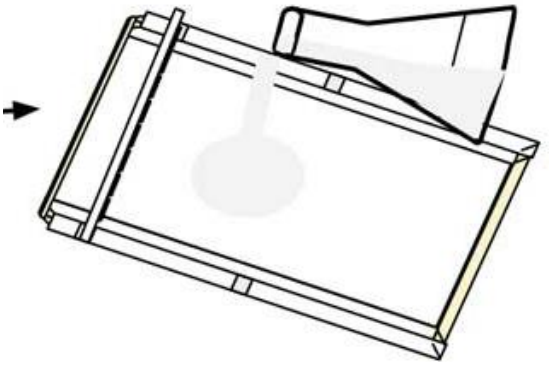
```
Phe  Gln Gly  Leu  Lys  Asp  Asn  Val  Phe  Asp  Gly  Leu Val
TTC  CAG GGA  CTC  AAG  GAT  AAC  GTG  TTT  GAT  GGC  TTG GTA

Arg  Val  Thr  Gln  Lys  Phe  His  Met  Lys  Val  Lys  His Leu
CGA  GTT  ACT  CAA  AAA  TTC  CAT  ATG  AAA  GTC  AAG  CAT CTG

Ile  Asp  To   Leu  Ile  Asp
          be
ATT  GAC  TCA  CTC  ATT  GAT
```

## ANNEX 1: Steps for DNA analysis using agarose gel electrophoresis:

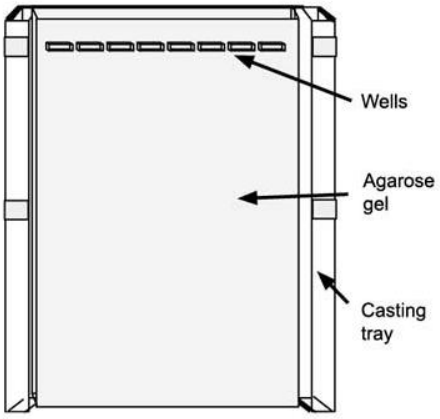
1. Heat the agarose to dissolve it:



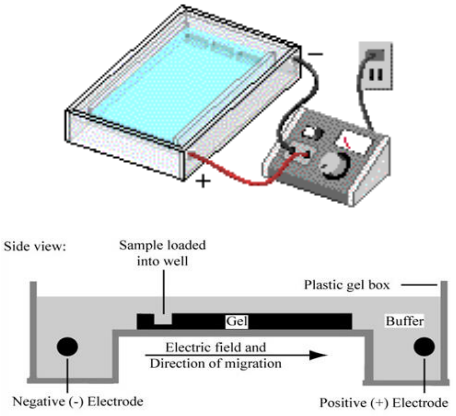

2) Fill the mould with the melted agarose



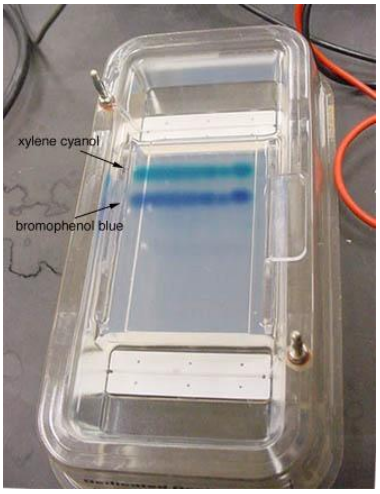
3) Pipette the samples into the wells:



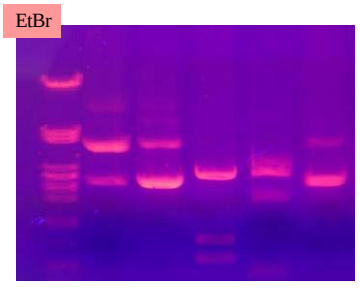

4) Start the electrophoresis:

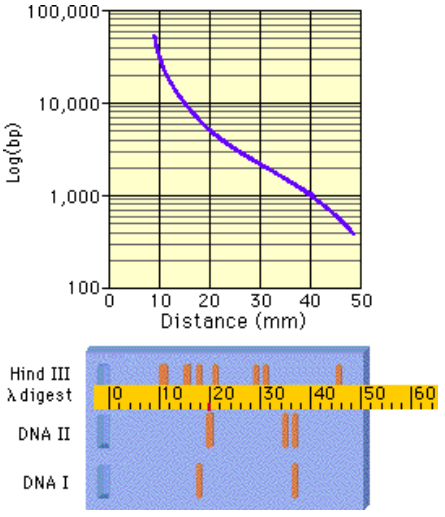



5) Observe the gel after migration:




6) View in the transilluminator and analyse the results:



# 4. SPECTROPHOTOMETRY

## I. INTRODUCTION

### I.1. Theoretical basis

Spectrophotometry is an analytical technique based on the interaction between matter and electromagnetic radiation. This interaction consists of the exchange of energy.

Both matter and electromagnetic radiation can be described by their energetic properties. Thus, the molecules of a sample can be found in different energy states. The lowest energy state is called the *ground state*.

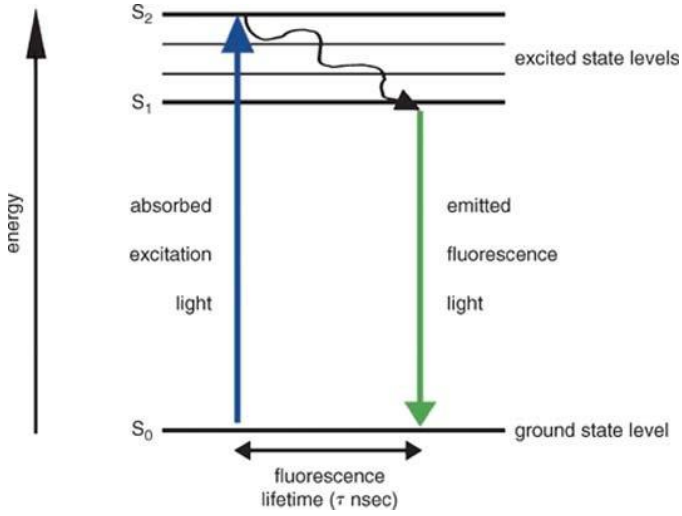



**Figure 8:** fluorescence fundamentals

Any state of higher energy than the fundamental state is called an *excited state*. The energy states of matter are represented in simple diagrams such as the one in Figure 8.

Electromagnetic radiation, whether conceived of in terms of its properties as a wave or as a particle, is characterised by its energy content. This energy is expressed by its wavelength (λ), or its frequency ($\nu$), or its wavenumber ($\bar{\nu}$). In any case, these three parameters are related to each other in a straightforward way:

$$E = h \cdot \nu$$

$$E = h \cdot \frac{c}{\lambda}$$

$$E = h \cdot c \cdot \nu$$

The intensity (***I***) of the radiation depends on its number of photons, each of which will have an associated energy equal to the energy of the radiation.

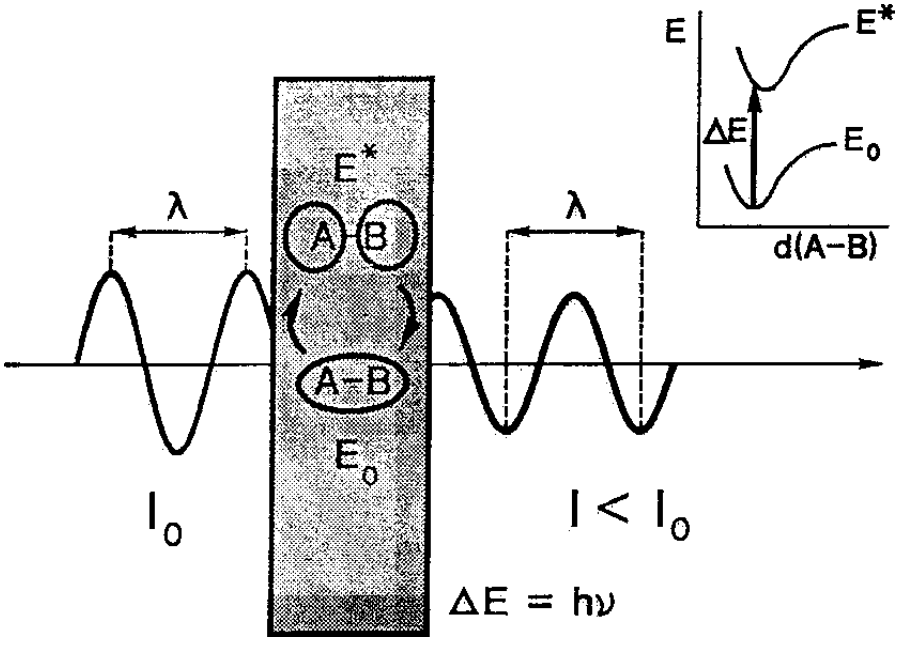



**Figure 9.** Absorption of radiation by a sample

When radiation of energy ***E*** is incident on a sample, some of its photons can be absorbed, provided that the energy difference between the ground state of the sample molecules and one of the excited states is equal to the energy of the radiation. The radiation passing out of the sample will have less intensity (fewer photons), but will be characterised by the same energy value as the incident wave (Figure 9).

## I.2. The spectrum of electromagnetic radiation

The most commonly used radiation in spectrophotometry is that of between 170 and 1000 nm in wavelength. This radiation is called Ultraviolet-Visible (UV-Vis), and includes the range of light visible to the human eye (350 - 1000 nm). UV-Vis radiation constitutes only a small part of the electromagnetic spectrum (Figure 10). Radiation of higher or lower energy than this is used in other types of spectroscopy.

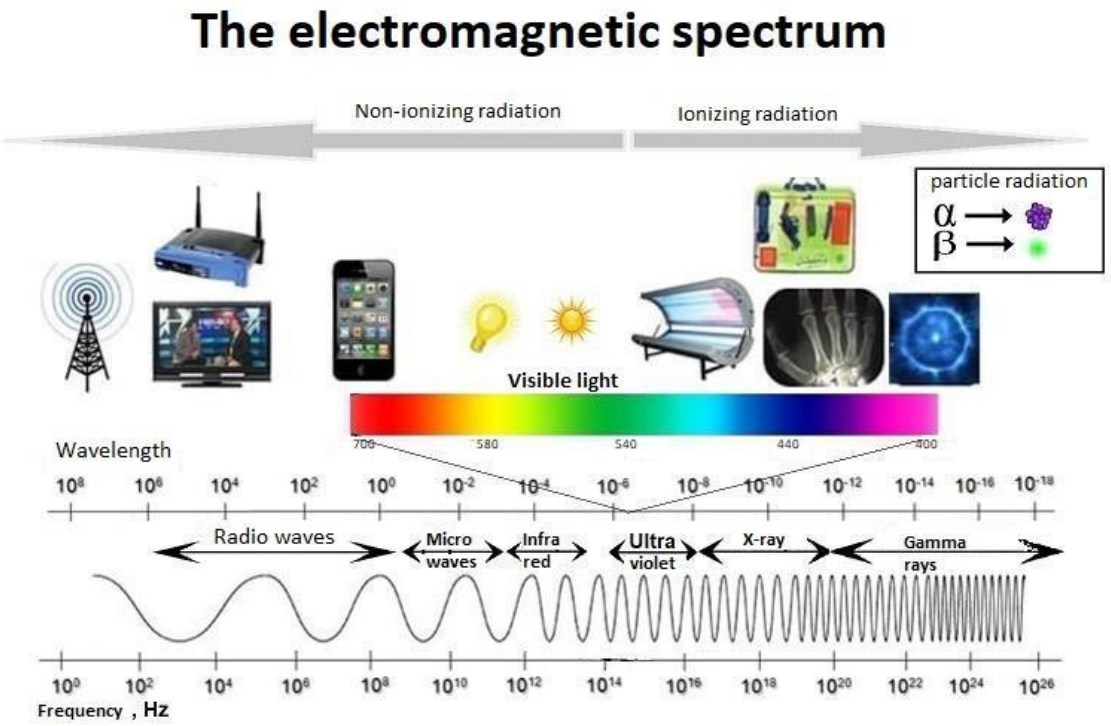



**Figure 10.** Spectrum of electromagnetic radiation. Wavelength (λ) in m.

Substances that absorb visible light generally have a visible colour, which is why their analysis is also called colorimetry. Similarly, molecules that absorb UV-Vis light are called chromophores.

## I.3. Measurement of absorption spectra

### *a) Lambert-Beer Law*

When there is absorption of UV-Vis radiation, the intensity of incident radiation is related to the intensity of emergent radiation in accordance with the Lambert-Beer law:

$$I = I_0 \cdot e^{-2{,}303 \cdot \varepsilon \cdot C \cdot l}$$

Where $\mathcal{E}$ is the extinction coefficient, $C$ is the concentration of the sample and $l$ is the distance through which the radiation passes (the thickness of the sample), which is usually 1 cm. If the concentration is molar and $l$ is measured in cm, it has units of $M^{-1}$ - $cm^{-1}$ .

Normally, it is not intensities that are measured directly, but a related parameter called *absorbance* (***A***), whose units are arbitrary (AU). Absorbance is defined as:

$$A = \log \frac{I}{I_0}$$

and substituting in the above expression we obtain:

$$A = \varepsilon \cdot C \cdot l$$

This expression is known as the Lambert-Beer equation. This simple relationship tells us that the absorbance of a sample depends linearly on the concentration of the chromophores and is directly proportional to the extinction coefficient. This coefficient is characteristic of each chromophore for a given wavelength.

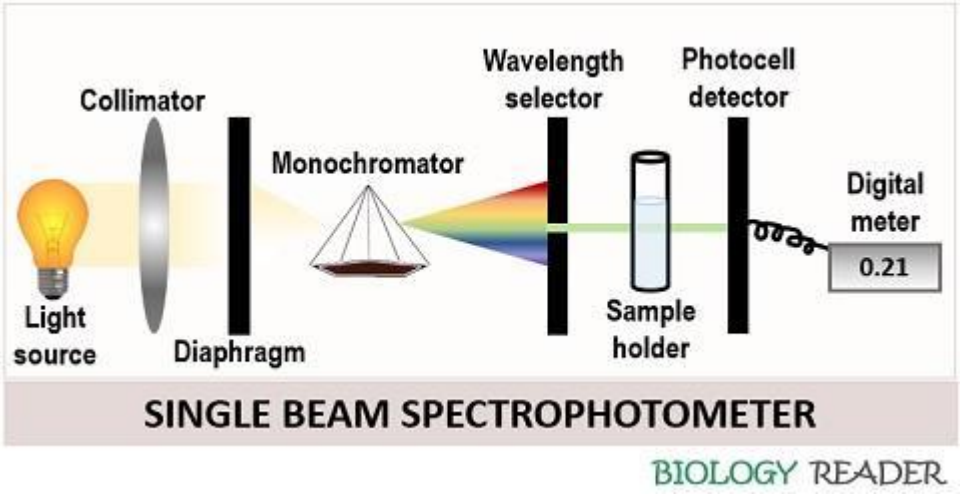
### *b) Absorption spectra*

An absorption or absorbance spectrum is a graphical record of the absorbance (***A***) of a sample at varying wavelengths (λ). It is sometimes also represented as ε versus λ. Absorption spectra consist of one or more bands (peaks), which are usually broad. In addition, each chromophore has a characteristic spectrum.

### *c) Diagram of a spectrophotometer*

The instrument for detecting the interaction between electromagnetic radiation and matter is called a spectrophotometer. The spectrophotometer consists of a light source (a bulb) that produces white light. The first slit selects a beam containing all the emitted frequencies. This beam passes through a glass prism which breaks down the white light into its different frequencies (from red to violet). A second slit selects one of the frequencies (monochromatic light) that will strike the cuvette containing the sample. The combination of the prism and the second slit is called a monochromator.

The monochromatic beam passing through the sample strikes the detector, which transfers the data to a computer system where the spectrum is generated. If the frequency selected on the second slit is not absorbed by the sample, a baseline point of the spectrum is produced. When the frequency of the radiation is such that it effects a transition (vibrational, electronic, etc.) an absorption peak is observed in the spectrum. Figure 11 shows a basic diagram of a single beam spectrophotometer.



## I.4. Applications in biological samples

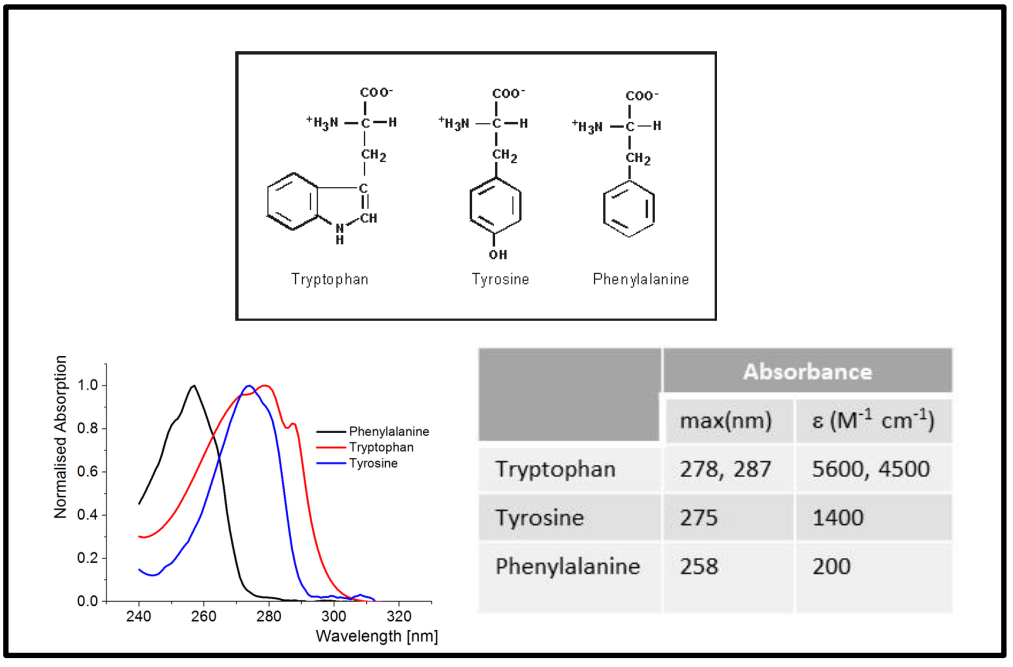
Since each chromophore has a characteristic spectrum, absorbance spectra are used as recognition standards for the molecules present in the sample. In addition, in some cases they are used to study different properties of the molecules such as structural variations, protonation states of some of their groups, interactions with other molecules, etc.

On the other hand, since absorbance depends on chromophore concentration, UV-Vis spectrophotometry is used to determine concentrations in solution.

### *a) Proteins as chromophores*

Several chromophore groups can exist in a protein, each with its own spectrophotometric characteristics. The main ones are:

- Peptide groups ($\lambda_{max}$ ~ 200 nm).
- Aromatic amino acids ($\lambda_{max}$ ~ 250-300 nm), mainly tryptophan which absorbs at ~280 nm. The latter absorption is responsible for the near-UV spectrum of most proteins (Figure 12).
- Some non-peptide prosthetic groups, such as the haeme group of haemoglobin.



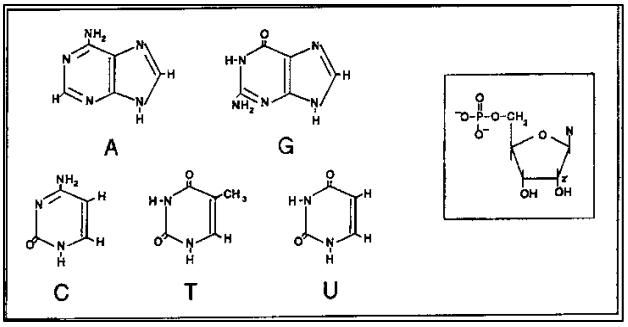

| | Absorbance | |
|---|---|---|
| | max(nm) | ε ($M^{-1}$ $cm^{-1}$) |
| Tryptophan | 278, 287 | 5600, 4500 |
| Tyrosine | 275 | 1400 |
| Phenylalanine | 258 | 200 |

**Figure 12.** Chromophores in proteins

### *b) Nucleic acids as chromophores*

In nucleic acids the chromophore groups are the nitrogenous bases, with an absorption maximum near 260 nm (Figure 13).

A)




B)

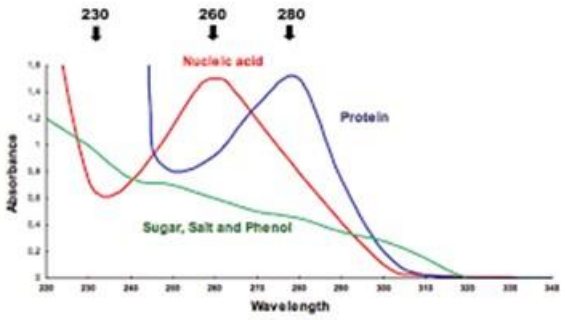



**Figure 13.** A) Chromophores in nucleic acids. B) Absorption maximum of nucleic acids ($\lambda$=260 nm) and proteins ($\lambda$= 280 nm).

# II. CASE STUDY: DETERMINATION OF TOTAL PROTEIN CONCENTRATION

The determination of the protein concentration in a sample can be carried out by following a modification of the **Lowry method or Lowry protein assay**. The technique is based on the formation of a coloured compound, created by reacting Folin-Ciocalteau's reagent with the phenolic (tyrosine) and indole (tryptophan) groups present in proteins.

As the absorbance varies linearly with protein concentration for a concentration range between 10 and 100 µg/mL, a standard line can be constructed with bovine serum albumin (BSA) concentrations of 0-100 µg/mL. The determination of the concentration of the test samples is obtained by interpolation on the standard line, once the absorbance at 660 nm is known.

## II.1. Objectives

*1)* Familiarisation with the use of a colorimeter and the application of Lambert-Beer's law.

2) To become familiar with the derivation and use of standard curves.

3) To use spectrophotometric measurements to determine protein concentrations.

## II.2. Reagents

***a) Prepared in advance of the day of the assay***

**REAGENT A**: $Na_2CO3$ 2%, NaOH 0.4%, Sodium tartrate 0.16%, Sodium dodecyl sulphate 1 %.

Dissolve all products except sodium dodecyl sulphate (SDS) in a volume of less than 1 litre; then add the SDS and place in a continuous shaking tray. When the SDS has completely dissolved (after about 2-3 hours), make up to 1 litre in a volumetric flask.

**REAGENT B**: $CuSO_4$-$5H_2O$ at 4%.

Reagents A and B are stable indefinitely at room temperature. Do not store reagent A cold because it will precipitate SDS.

**BSA SOLUTION:** BOVINE SEROALBUMIN 250 µg / mL.

Store in the refrigerator to avoid contamination.

***b) Prepare for the day of the assay:***

**REAGENT C:** *100* parts A + *1* part B, i.e. 10 mL A + 0.1 mL B (10 mL for each group).

**REAGENT D:** Folin-Ciocalteau's reagent in distilled water to a 1:1 ratio; i.e. 0.6 mL reagent + 0.6 mL water (1.2 mL/group in Eppendorf tubes).

**BSA SOLUTIONS FOR THE STANDARD CURVE:** 0 to 250 µg/mL in distilled water in a final volume of 0.3 mL (see summary on the next page).

## II.3. Procedure for the practical session

*1) Prepare the tubes with the standard solutions.*

Arrange the 6 tubes for the standard curve: each tube contains a volume of **0.3 mL** at the indicated BSA concentration (**0, 25, 50, 100, 175** or **250 µg/mL).**

*2) Prepare the problem solution.*

Pipette **0.3 mL of** the test serum into a separate tube.

*3) Add **0.9 mL of REAGENT C** to each tube (standards and test).*

*4) Shake and leave at room temperature **>** 10 minutes.*

*5) Add **90 µL of REAGENT D** to each tube.*

*6) Shake and leave at room temperature **>** 20 minutes.*

*7) Measure the absorbance of all tubes at **660 nm**. Record all absorbance data together with the corresponding concentrations in a table.*

*8) Construct the **BSA standard line** from the absorbance and concentration data of the standard samples.*

*9) Determine the protein concentration in the test solutions by interpolation on the standard line.*

## Preparation of BSA standard curve + Problem Sample (P)


| Volume (L) | | | | | | | (x 2) |
|---|---|---|---|---|---|---|---|
| BSA standard solution 250 g/mL | 0 | $V_2$ | 60 | $V_4$ | $V_5$ | 300 | -- |
| $H_2O$ | | $V_{H_2O}$ | 240 | $V_{H_2O}$ | $V_{H_2O}$ | 0 | -- |
| [BSA] final (g/mL) | 0 | 25 | 50 | 100 | 175 | | ¿? |
| **Problem sample** | -- | -- | -- | -- | -- | -- | |

$$Vi = \frac{C2 \cdot V2}{Ci}$$

Vi = ? L→ Ci = 250 g/mL

V2 = 300 mL→ C2 = 25 - 175 g/mL

## Reaction preparation

| Volume (L) | | | | | | | (x 2) |
|---|---|---|---|---|---|---|---|
| Final target/problem volume | 300 | 300 | 300 | 300 | 300 | 300 | 300 |
| Reagent C | 900 | 900 | 900 | 900 | 900 | 900 | 900 |
| Shake and wait 10 minutes | | | | | | | |
| Reagent D | 90 | 90 | 90 | 90 | 90 | 90 | 90 |
| Shake and wait 20 minutes | | | | | | | |

- Transfer the solution to a microcuvette.
- Read the absorbance at 660 nm in the spectrophotometer.
- Blank ONLY with the 1st standard solution (0 g/mL BSA).

Plot the graph, interpolate the problem value and calculate its concentration (see page 16).

# III. QUESTIONS 4

1) Standard curve. Calculate the parameters of the standard curve by means of a regression line (see ANNEX 2).

| **RESULT STANDARD SAMPLES** | | | |
|---|---|---|---|
| Tube | BSA CONCENTRATION (µg/mL) | AMOUNT OF PROTEIN (MG) | ABS |
| 1 | 0 | | |
| 2 | 25 | | |
| 3 | 50 | | |
| 4 | 100 | | |
| 5 | 175 | | |
| 6 | 250 | | |

| **RESULT STANDARD STRAIGHT LINE** | | |
|---|---|---|
| x | **y** | **r** |
| | | |

2) Numerical result

| **RESULT PROBLEM SAMPLES** | |
|---|---|
| Tubes | PROTEIN CONCENTRATION |
| 1 | |
| 2 | |

# 5

# 5. ELECTROPHORETIC ANALYSIS

## I. ELECTROPHORESIS

### I.1. Definition

Most biological molecules are electrically charged and are therefore able to migrate under the influence of an electric field. The movement of these molecules through a solvent under the action of an electric field is called electrophoresis.

A common way of characterising biological macromolecules is by measuring their mobility when subjected to an electric field. This property can be used to determine the molecular weight of a protein, to distinguish molecules according to their net charge, their shape, or their charge/mass ratio, to detect changes in their amino acid sequence, and to separate different molecular species.

### I.2. Electrophoresis theory

If a particle with charge $q$ suspended in an insulating medium is in an electric field $E$, the particle will move at a constant velocity $v$, determined by the balance between the electric force and that resulting from friction with the medium. Under these conditions, electrophoretic mobility is defined as the velocity of the charged particle per unit of electric field:

$$\mu = v / E$$

The direction of the moving particle in the electric field depends on the sign of its charge, so that:

– Molecules with a net **negative** charge (**ANIONS**) are directed towards the positive pole, the **ANODE**.

– The molecules with a net **positive** charge (**CATIONS**) are directed towards the negative pole, the **CATHODE**.

The degree of mobility depends on several factors, including:

***a)*** **Electric field factors:**

– The electrical potential, ***V***, in volts (the higher the ***V,*** the greater the μ).

– The current intensity ***I***, in amperes, which is related to the resistance, ***R***, in accordance with Ohm's law.

$$V = I \cdot R$$

– The temperature, which will increase as ***R*** increases, due to the *Joule effect*.

***b)*** **Factors deriving from the charged molecule**:

The most important are the value of its charge, its size and its molecular shape. The charge will be conferred by the ionised groups of the molecule. In the case of proteins, these groups are the side chains of some amino acids (Glu, Asp, Arg, Lys, Cys, His, Tyr) and the N- and C-terminal groups.

***c)*** **Factors deriving from the buffer solution used:**

The buffer acts as a dispersing phase for the particles and as a conductor for the electric current. Its properties depend on:

– the pH

– ionic strength

– the nature of the ions in the solution

The pH of the buffer solution is very important in the case of protein electrophoresis, since the ionic state of the amino acids depends on it, which in turn is responsible for the charge of the molecule.

When the **pH is equal to the isoelectric point of a protein**, the number of its positive charges is equal to the number of its negative charges, so the protein will not exhibit electrophoretic mobility:

$(^{+}H3N)_n - R - (COO^{-})_m$ n = m protein with no net charge

At **pH values of the solution which are lower (more acidic) than the isoelectric point of the** protein, the positively charged groups will predominate, so the protein will migrate towards the cathode.

$(^{+}H3N)_n - R - (COO^{-})_m$ n > m protein with net positive charge

**At pH values which are more basic than the isoelectric point**, negatively charged groups will predominate, so the protein will migrate towards the anode:

$(^{+}H3N)_n - R - (COO^{-})_m$ n < m negatively charged protein

***d)*** **Particle-solvent interaction factors:**

When the solvent contains ions in significant concentration, solvation of the solute molecules can occur and they become surrounded by these ions or by water molecules, thus changing the migration properties of the particle.

## I.3. Types of electrophoresis

There are several types of electrophoresis, which can be classified according to different parameters:

**MOVING-FRONT ELECTROPHORESIS**: The macromolecules are present throughout the solution and their position is determined by a complex optical system (Schlieren optics). This method is currently rarely used, as it has many disadvantages, such as the large amount of sample required, the optical equipment needed for mobility detection, etc.

**ZONE ELECTROPHORESIS**: In this type of electrophoresis, the buffer solution is stabilised on a support that is as inert as possible, so that the components are perfectly separated into delimited zones. It is the most widely used as it has great advantages in terms of technical simplicity and low sample requirements.

There are several variants of this technique:

- **USING PAPER**: this is no longer used, as it presents certain problems, such as the presence of microchannels, in which electrophoretic mobility is distorted (chromatographic effect), the presence of adsorption phenomena and the high temperature produced by the passage of the electric current.
- **USING CELLULOSE ACETATE**: this is one of the most widely used techniques due to its advantages of simplicity and analytical quality. Adsorption problems are avoided due to the presence of cellulose esters, which do not interact with organic macromolecules. This is the technique we will use in the practical session.
- **GELS**: Polyacrylamide gels are currently used more frequently. In the presence of the detergent sodium dodecyl sulphate, the target proteins lose their three-dimensional structure and greater sensitivity is obtained for the determination of their molecular weight.

Finally, there are a number of techniques derived from electrophoresis that introduce certain technical aspects that increase its sensitivity and resolving power.

**IMMUNOELECTROPHORESIS**: This combines the principles of zone electrophoresis with those of immunodiffusion. As a result, you can observe a precipitate at the point where the antigen (usually the mobile phase) reacts with the specific antibody.

**ISOELECTRIC FOCUSING**: This is performed on a pH gradient, so that proteins are separated according to their isoelectric point. It is a high-resolution technique for separating proteins.

## I.4. Instrumentation

***a)*** **Power supply**:

A current rectifier system, capable of producing the required range of electrical voltages. They can be of constant voltage or constant current.

In conventional electrophoresis, the necessary potential gradient is 5-10 V/cm, so the equipment must be equipped with a cooling and safety system.

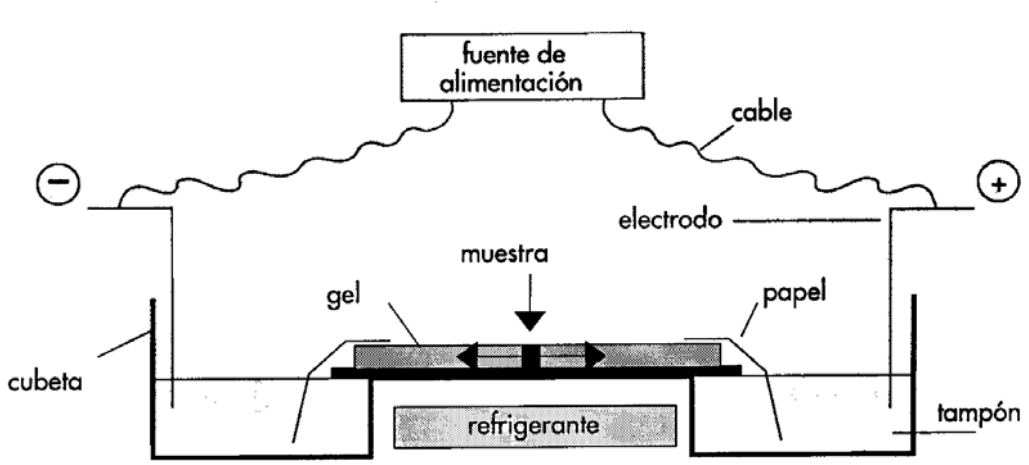



**Figure 14.** Assembly of an electrophoresis system

***b)*** **Electrophoretic chamber or cuvette**:

This is the container in which the electrophoresis will be carried out (Fig. 14). It consists of two compartments for the electrodes and a central compartment, where the support on which the electrophoresis is to be performed is placed. They are made of non-conductive material.

***c)*** **Electrodes:**

They are responsible for transmitting the current to the electrophoresis solution. They can be made of various materials: graphite, stainless steel or platinum (Fig. 14).

# II. SERUM PROTEIN ELECTROPHORESIS

## II.1. Proteins and Enzymes in the serum

Although all body fluids contain proteins, it is the proteins found in the blood serum that are the ones that are most frequently used to establish clinical diagnoses, as the concentration in the blood of many proteins changes characteristically in certain pathophysiological conditions.

There are more than 100 proteins with a physiological function in serum (see Table 4 for a summary of some of them). The most abundant serum protein is albumin, and most of the rest are collectively known as globulins. Some serum proteins are enzymes, e.g. coagulation factors.

**Table 4.** Selected serum proteins and their functions

| **Function** | **Protein** |
|---|---|
| Transport | Apolipoproteins, Albumin, Transferrin |
| Immunity | Immunoglobulins |
| Osmotic pressure maintenance | All, particularly Albumin |
| Enzymes | Renin, Coagulation factors |
| Protease inhibitors | Antitrypsin 1 |
| pH regulation (buffer) | All |

Knowledge of serum proteins has been achieved largely through the application of electrophoretic techniques. Cellulose acetate electrophoresis of normal serum reveals a pattern of five distinct bands, which can be quantified by densitometry (Figure 15). They are the immunoglobulins. In order of proximity to the anode, i.e. from fastest to slowest migration speed, we can distinguish the following main bands: Albumin, Globulin $\alpha$1, Globulin $\alpha$2, Globulins $\beta$ and Globulins $\gamma$.

The results of electrophoresis (see Figure 15 below) enable the characteristic patterns of some pathologies to be relatively quickly and easily observed, by identifying variation in the normal distribution of band intensities. The abundance of each of the fractions can be determined by densitometry on an automated reader system, which will produce a characteristic pattern of distribution profiles. For example, the abnormally low presence of albumin (*hypoalbuminaemia*) or $\gamma$-globulins (*immunosuppression* or *hypogammaglobulinemia*) can be easily detected, serving as the basis for the diagnosis of certain diseases.

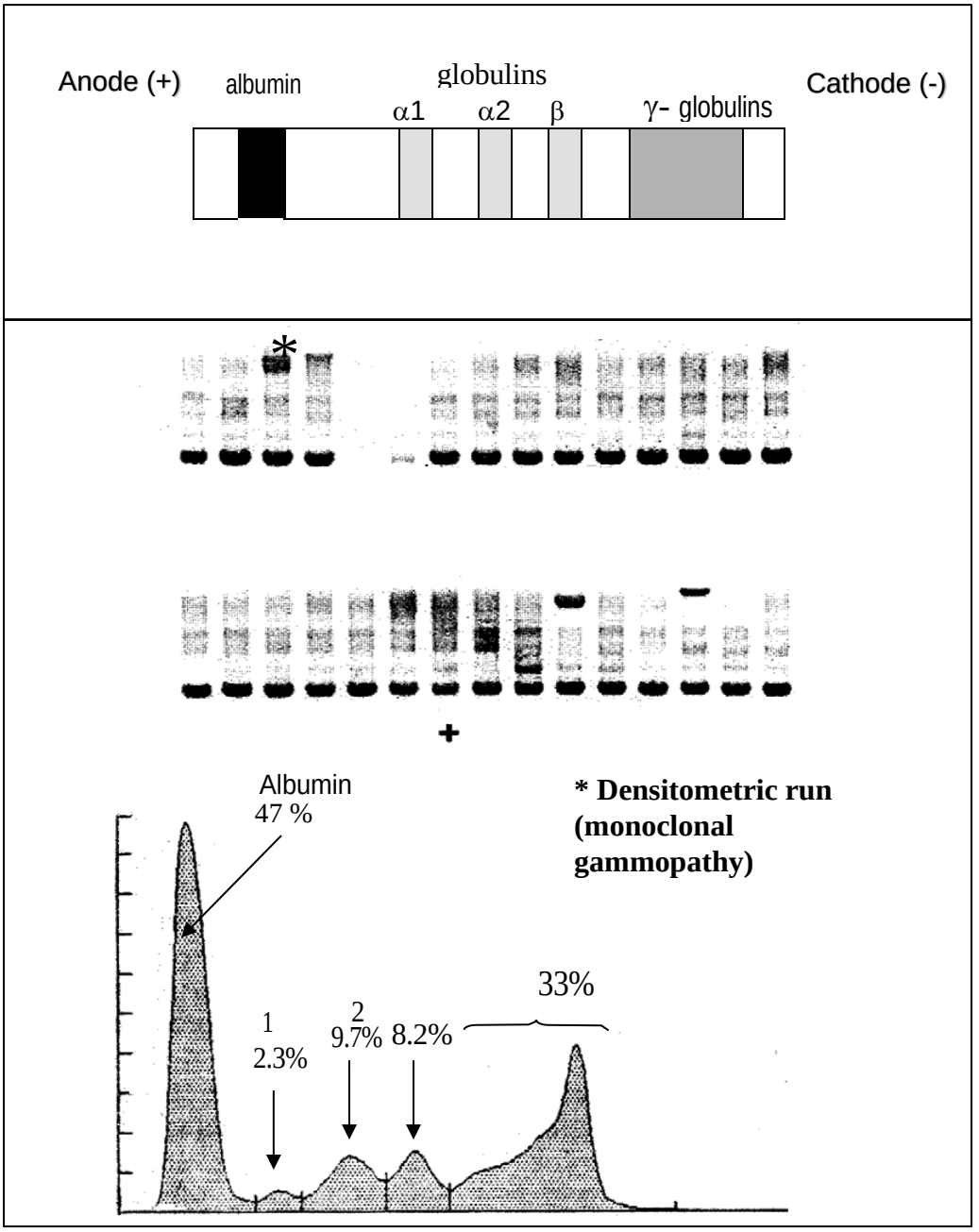



**Figure 15.** Elec-trophoresis of serum in c-ellulose acetate.

# III. CASE STUDY: ELECTROPHORESIS OF SERUM PROTEINS USING CELLULOSE ACETATE

## III.1. Objectives

1) To carry out serum protein analysis by electrophoresis.

2) To understand its usefulness and limitations.

3) To provide a clinical interpretation of the results.

## III.2. Material required:

- Cellulose acetate strips
- Veronal Buffer pH 8.6
- 2-10 L applicator or pipette
- Power supply and electrophoresis cuvette
- Protein staining solution: Ponceau Red S in 5% TCA
- Support bleaching solution
- Transparency solution
- Densitometer

## III.3. Procedure for the practical session

1) *Preparation of acetate strips:*

Prepare the alkaline buffer (pH 8.6), so that the anionic form of the proteins predominates. Place the strips on the holders of the cuvette so that their ends are immersed in buffer.

2) *Sample application:*

With a micropipette, deposit a small volume (2 L) of serum near the cathode (negative pole), as the pH of the buffer will make the proteins migrate towards the anode.

3) *Migration:*

Connect the power supply, following the instructions of the lecturer leading the session. Remember that a ***high voltage*** will be produced, so ***care*** must be taken with the cuvettes. The container must also be covered to prevent evaporation of the buffer. When instructed, disconnect the power supply and carefully remove the strip, taking care not to wrinkle or break it.

4) *Colouring:*

Immerse the strip (5-10 minutes) in staining reagent; this is a protein-specific dye, in a strongly acidic medium, which fixes and colours the samples.

5) *Discolouration:*

Immerse the stained strip (5-10 minutes) in the decolourisation solution in

order to remove the dye bound to the strip and to differentiate the banding from the separated proteins attached to it.

6) *Transparency:*

Place the discoloured strip (5-10 minutes) in the transparency reagent to until it is completely transparent, allowing visualisation or analysis with quantitative optical systems.

7) *Drying:*

Place the transparent strip (5-10 minutes) in the oven at 60ºC to dry it and make it easier to handle.

8) *Quantification:*

Following the teacher's instructions, place the strip into the densitometer, which measures the intensity of the colouring, proportional to the protein content of each band.

# IV. QUESTIONS 5

1) What enables the different molecules to be separated by electrophoresis?

2) Describes the normal pattern of separation of serum proteins by cellulose acetate electrophoresis.

3) What type of proteins differed from the normal pattern in the problem serum assigned to your group? Why?

4) How can the presence of a specific protein be detected and quantified by electrophoresis?

5) Indicate two types of alterations that can be diagnosed by means of the serum protein electrophoresis. Give reasons for your answer.

# 6. ENZYMOLOGY

## I. INTRODUCTION

In 1860, Berthelot isolated the enzyme saccharase from baker's yeast. This enzyme catalyses the hydrolysis of the glycosidic bond between carbon 1 of glucose and carbon 2 of fructose causing the reversal of the rotational power of the sucrose solution, which is why this enzyme is also known as invertase (Fig. 16). The scientific name of the enzyme is β-D-fructofuranosidase or β-D-fructofuranoside-fructohydrolase (E.C. 3.2.1.26). In the lab, we will use a commercial enzyme.

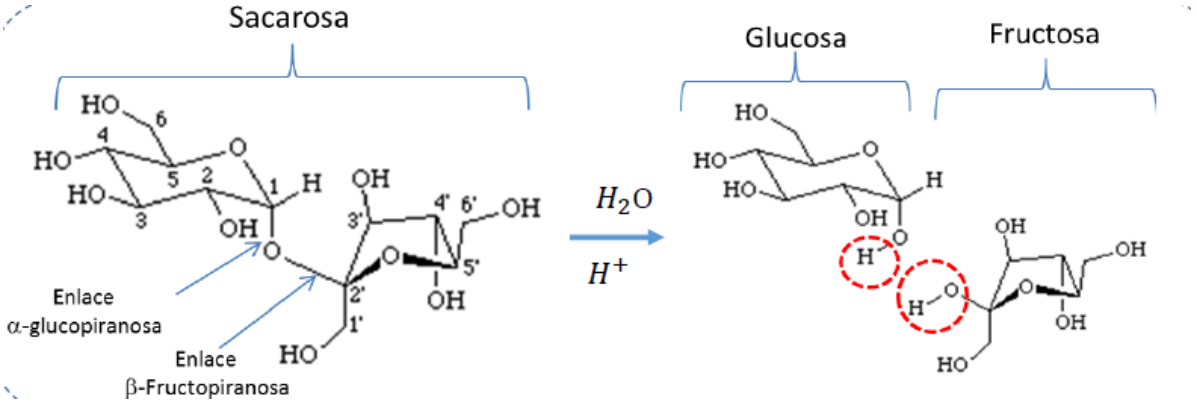



**Figure 16.** The sucrose hydrolysis reaction catalysed by invertase.

# II. CASE STUDY: ASSESSMENT OF ENZYMATIC ACTIVITY

The chosen method is based on the measurement of the amount of reducing sugars (glucose + fructose) released during sucrose hydrolysis catalysed by the enzyme invertase. For this purpose, the reagent 3,5-dinitrosalicylate (DNS; this reagent was introduced by Sumner in 1921 for the determination of reducing substances in blood and urine) will be used. In the presence of reducing sugars, 3,5-dinitrosalicylate is reduced to 3,5-diaminosalicylate. This compound has an absorption at a maximum of 530 nm and therefore the absorbance value at this wavelength will be proportional to the concentration of reducing sugars (glucose + fructose) present in the medium.

## II.1. Objectives

1) To gain an introduction to enzymes as elements for analysis in the biochemistry laboratory.

2) To know the basic fundamentals of the measurement of enzyme activity (reaction step and development step).

3) To apply theoretical knowledge of enzymology in a practical study of the factors affecting enzyme activity.

## II.2. Reagents

- 0.3 M sucrose used as enzyme substrate.
- 0.05 M sodium acetate buffer of pHs 3.5, 5, 6.5 and 8.5 (pH 5 is normally used as this is the optimum) to keep the pH of the reaction medium under control.
- Aqueous solution of glucose + fructose (0.005 M each) to be used as a control for reducing sugars for the construction of the standard curve.
- 3,5-Dinitrosalicylate, the colour reagent. The DNS solution is prepared as follows: 2.5 g of DNS is dissolved in 50 mL of 2 M NaOH at 80°C, and 75 g of potassium sodium tartrate is dissolved in 125 mL of distilled water and the two solutions are mixed while hot, with distilled water being added to create a 250 mL solution.

## II.3. Standard curve

The standard curve will make it possible to relate the absorbance at 530 nm of

the tubes to the quantity of moles of glucose and fructose present in the solution and, therefore, by making the corresponding calculation, it will be possible to estimate the speed at which the enzyme has functioned (moles of sucrose hydrolysed by the enzyme per unit of time), i.e. its activity.

**PATTERN CURVE**

| Tube no. | B | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| Glucose + Fructose (0.005M) (mL) | - | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| Distilled water (mL) | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | - |
| 0.3 M sucrose (mL) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Buffer pH 5.0 (mL) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| 3,5-DNS (mL) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 100ºC, 5 min | | | | | | |
| Distilled water (mL) | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Vortex the tubes | | | | | | |
| Absorbance at 530 nm | | | | | | |

## II.4. Effect of temperature

All enzymes show a maximum activity at a characteristic temperature known as the optimum temperature. The optimum temperature of invertase can be determined by measuring its activity at different temperatures.

**EFFECT OF TEMPERATURE**

| Tube no. | B | 1 | | | | 5 |
|---|---|---|---|---|---|---|
| Distilled water (mL) | 0.5 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| 0.3 M sucrose (mL) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Buffer pH 5.0 (mL) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Enzyme (mL) | - | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Incubate for 5 min at the indicated temperature. | | | | | | |
| Incubation temperature | RT | RT | 37ºC | 50ºC | 65ºC | 80ºC |
| 3,5-DNS (mL) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 100ºC, 5 min | | | | | | |
| Distilled water (mL) | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Vortex the tubes | | | | | | |
| Absorbance at 530 nm | | | | | | |

## II.5. Effect of pH

Enzymes generally have a pH optimum at which their activity is maximal. The pH optimum of invertase can be determined by measuring its activity at different pHs.

**EFFECT OF pH**

| **Tube no.** | **B** | **1** | | | |
|---|---|---|---|---|---|
| **Distilled water (mL)** | 0.5 | 0.4 | 0.4 | 0.4 | 0.4 |
| **0.3 M sucrose (mL)** | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| **Buffer (mL)** | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| **pH of added buffer** | **(3.5)** | **(3.5)** | **(5.0)** | **(6.5)** | **(8.5)** |
| **Enzyme (mL)** | - | 0.1 | 0.1 | 0.1 | 0.1 |
| **Incubate 5 min at room temperature** | | | | | |
| **3,5-DNS (mL)** | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| **100ºC 5 min** | | | | | |
| **Distilled water (mL)** | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| **Vortex the tubes** | | | | | |
| **Absorbance at 530 nm** | | | | | |

## II.6. Effect of substrate concentration

The rate of an enzymatic reaction increases with increasing substrate concentration until a point is reached where the substrate concentration is high enough to saturate the enzyme. At this concentration, the maximum rate of the reaction is reached, and the rate becomes independent of the substrate concentration.

The relationship between the rate of an enzymatic reaction (*V0*) and the substrate concentration is expressed by the Michaelis-Menten equation:

$$V_0 = \frac{V_{máx} \cdot [S]}{K_M + [S]}$$

where Vmax is the maximum velocity, $_{Km}$ is the Michaelis-Menten constant and Σ represents the substrate concentration. The Vmax and $_{Km}$ of invertase will be calculated for the substrate sucrose. For this purpose, the invertase activity at different sucrose concentrations will be assessed.

## EFFECT OF SUBSTRATE CONCENTRATION

| Tube no. | B | 1 | | | | 5 |
|---|---|---|---|---|---|---|
| Distilled water (mL) | 0.5 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Sucrose (mL) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Molarity of added sucrose | 0.3 | 0.3 | 0.15 | 0.075 | 0.038 | 0.019 |
| Buffer pH 5.0 (mL) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Enzyme (mL) | - | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Incubate 5 min at room temperature | | | | | | |
| 3,5-DNS (mL) | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| 100ºC 5 min | | | | | | |
| Distilled water (mL) | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Vortex the tubes | | | | | | |
| Absorbance at 530 nm | | | | | | |

# III. QUESTIONS 6

1. Indicate by means of a diagram the rationale for the method used to measure invertase activity.

2. Why is it necessary to process a "B" tube parallel to the experimental tubes in each of the experiments you have carried out in this practical session?

3. STANDARD CURVE: create a graph to represent the **absorbance** of each of the tubes as a function of the **quantity of product** put in each of them. Why is it necessary to make a standard curve?

| **Tube** | **1** | | | | **5** |
|---|---|---|---|---|---|
| **Absorbance** | | | | | |
| **Product (moles)** | | | | | |

4. EFFECT OF TEMPERATURE: plot the **reaction rate** of each tube as a function of **incubation temperature**. What is the optimum temperature for the enzyme?

| **Tube** | **1** | | | | **5** |
|---|---|---|---|---|---|
| **Absorbance** | | | | | |
| **Speed (moles/minute)** | | | | | |
| **Temperature** | **T.A. (25ºC)** | **37ºC** | **50ºC** | **65ºC** | **80ºC** |

5. EFFECT OF pH: plot the **reaction rate** of each tube as a function of the **pH of the incubation medium**. What is the optimum pH of the enzyme?

| **Tube** | **1** | | | |
|---|---|---|---|---|
| **Absorbance** | | | | |
| **Speed (moles/minute)** | | | | |
| **pH** | **3.5** | **5.0** | **6.5** | **8.5** |

6. EFFECT OF SUBSTRATE CONCENTRATION:

A. Plot the **reaction rate** of each tube as a function of the **sucrose concentration** in the incubation medium.

B. Plot the **inverse of the reaction rate** of each tube as a function of the **inverse of the sucrose concentration** present in the incubation medium.

| **Tube** | **1** | | | | **5** |
|---|---|---|---|---|---|
| **Absorbance** | | | | | |
| **Speed (moles/minute)** | | | | | |
| **1/v (minute/mol)** | | | | | |
| **Sucrose (mM)** | | | | | |
| **1/ Sucrose ($mM^{-1}$)** | | | | | |

Using the graphs you have created:

1. Calculate Km and Vmax.

2. What is the name given to the double-reciprocal plot? Why is this representation used and not the direct representation for the calculation of Km and Vmax?

7. The activity of an enzyme plus an amino acid decarboxylase can be assayed manometrically by monitoring the release of $CO_2$. In an experiment with this enzyme in the presence or absence of a hydroxy acid (HA) (0.05 M), the following results were obtained:

| **[aa] (mM) (-$10^{-3}$ )** | | 12.5 | 16.67 | 25 | 50 | 100 |
|---|---|---|---|---|---|---|
| **V (mol/min-mL)** | **- HA** | 13.3 | 17.2 | 22.2 | 33.3 | 40 |
| | **+ HA** | -- | 4.6 | 6.4 | 11.8 | 20 |

a) indicate the type of inhibition exerted by the hydroxy acid on the enzymatic reaction, and b) calculate the values of Km and Vmax for each of the cases.

## ANNEX 2. CALCULATION OF A REGRESSION LINE IN EXCEL

1. Enter the data in the spreadsheet. First column the X values and in the next column the Y values.

2. Select data

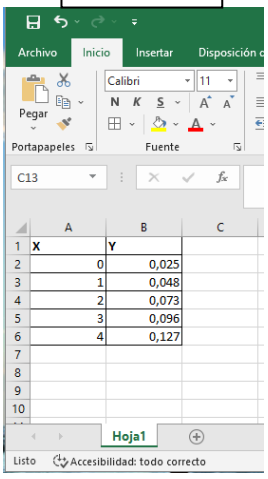
3. Go to insert and choose Charts → Insert Scatter Chart. The graph is then created. You can add the titles of the axes, the units, the title of the graph, change the scale, etc.

4. Select the points on the line and click with the right mouse button. A menu will appear, select: Add trendline

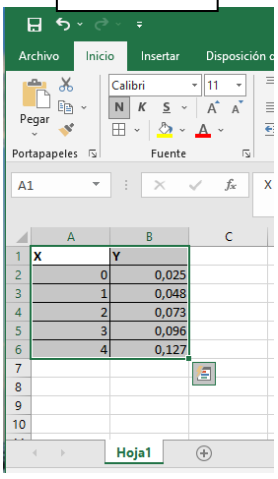
5. From the options it gives, select: Linear trend and select the boxes for: Display equation on graph and Display R-squared value on chart.

6. The corresponding regression line and the values of the equation of the line and R-squared will appear inside the graph.

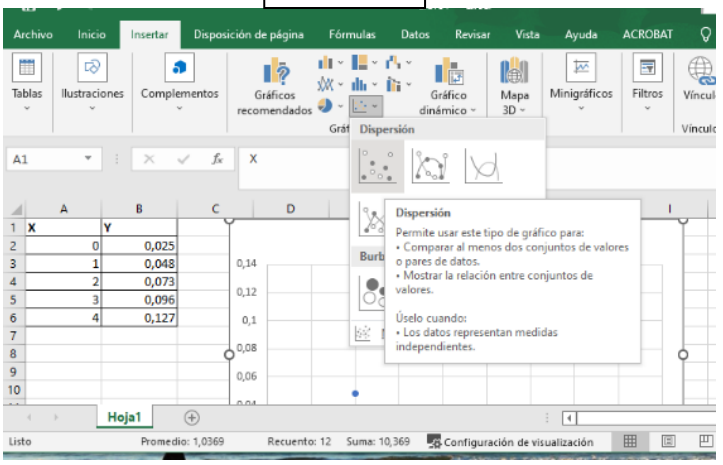
STEP 1



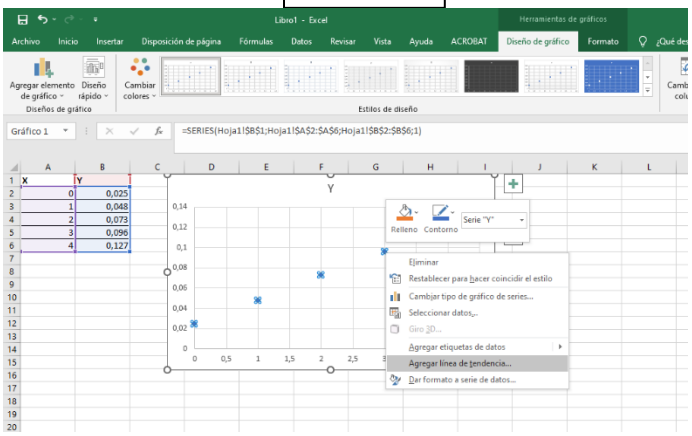

STEP 2



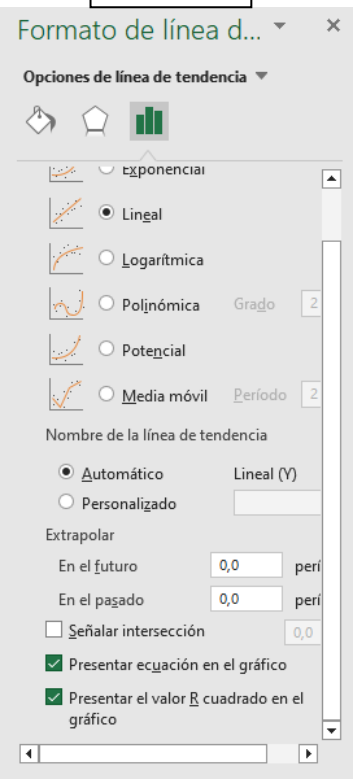

STEP 3



STEP 4




STEP 5




STEP 6