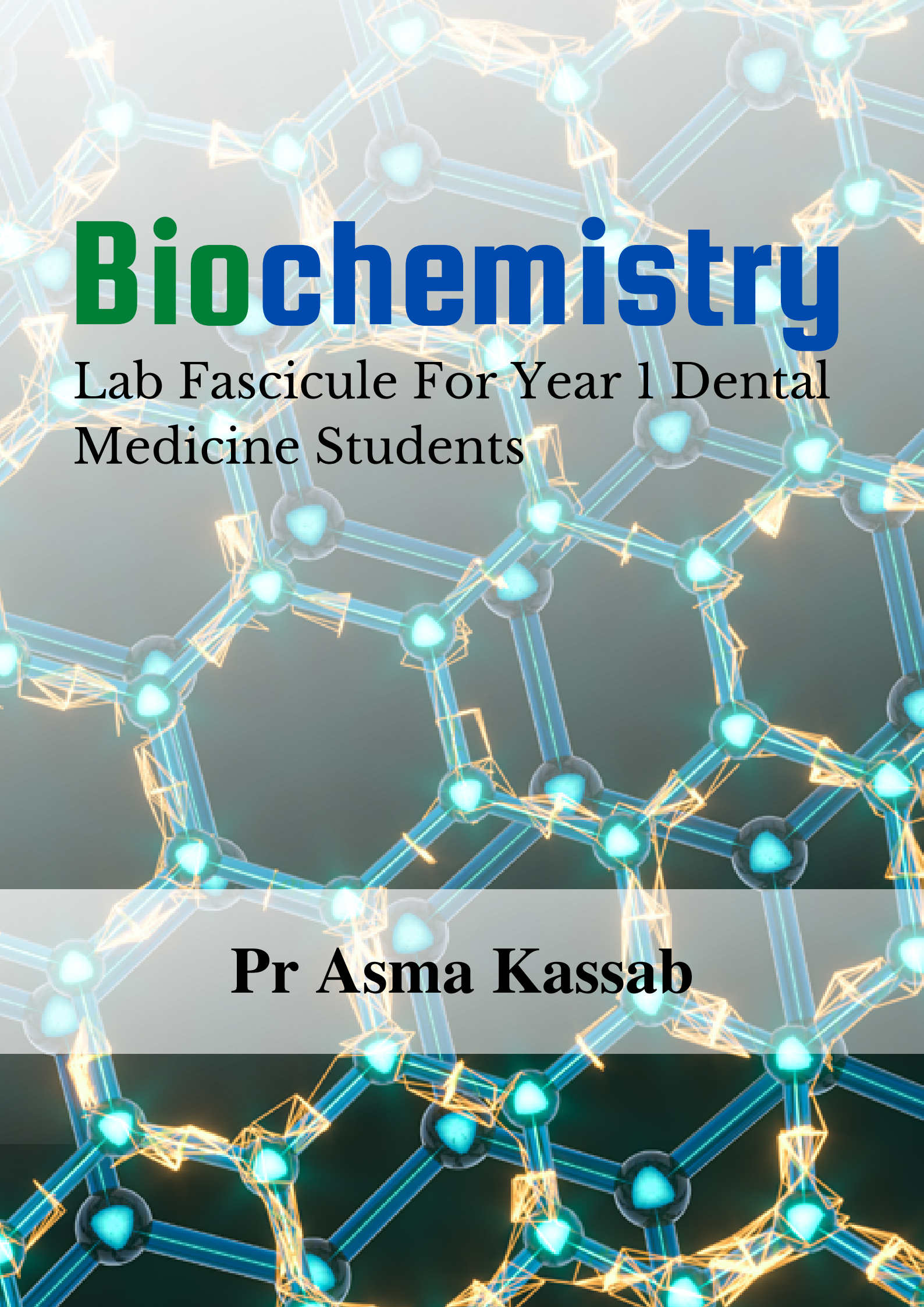


Biochemistry

Lab Fascicule For Year 1 Dental Medicine Students

Pr Asma Kassab

Biochemistry Lab Fascicule

EMPYREAL
PUBLISHING HOUSE

India | UAE | Nigeria | Uzbekistan | Montenegro

www.empyrealpublishinghouse.com

Biochemistry Lab Fascicule

By:

Pr Asma Kassab
Associate Professor

First Impression: November 2022

Biochemistry Lab Fascicule

ISBN : 978-93-93810-12-0

Rs. 650/- ($18)

Published by:
Empyreal Publishing House

PREFACE

The aim of the laboratory is to illustrate the theoretical concepts on an experimental level. These laboratories are oriented to familiarize you with a certain number of common techniques used daily in biochemistry research laboratories and allowing to separate a mixture, to prepare, to purify and eventually to identify substances or to verify some of their chemical or biological properties.

The laboratory should allow you to carry out in practice certain reactions that you have encountered in lecture or tutorials. This is why, in particular, in the reports we will try to link the theoretical and practical aspects of biochemistry as much as possible.

ACKNOWLEDGEMENT

The author acknowledge resources and support from the Dental Faculty, University of Monastir.

The author acknowledge Pr Jemni Ben Chibani for his critical review of the manuscript.

Pr Asma Kassab

Table of Contents

GENERAL RECOMMENDATIONS

1. Organization of the laboratory

Students will be divided into groups. Each group will do one session of 2 hours laboratory per week. The total duration of the biochemistry lab is 8 weeks.

In each group, the students work in pairs for the whole duration of the lab. The choice of the benches is final.

At the end of the 8 sessions, each student must take an exam including a practical test and a written test. Grades will be delivered for both parts.

2. Order and cleanliness

1. It is mandatory to wear a lab coat during the whole session.
2. It is essential to know the name of the used equipment.
3. It is essential to prepare the laboratory session: read carefully the text of the booklet corresponding to the manipulation prior to class.
4. Each student must clean his bench at the end of each session.

3. Precautions to take

1. Do not blow out the reagents by introducing dirty pipettes into the vials.
2. Recap the vials immediately after use.
3. Never pipette toxic or corrosive reagents (strong acids and bases and organic solvents) directly with the mouth. Use a pro-pipette for this purpose.
4. Set aside 2 minutes at the end of the session to tidy up and clean the bench.
5. Rinse pipettes with distilled water and place them at an angle on the edge of the tray.
6. Empty the burette and refill it with distilled water.
7. Wash the glassware with tap water (beakers, Erlenmeyer flasks, test tubes...) and rinse with distilled water.
8. Wipe the bench with a clean sponge.

9. Do not throw papers or reagents down the sinks.
10. No smoking in the lab.
11. Do not leave the room without permission.
12. In case of an accident, inform the teacher immediately.
13. Handling of toxic reagents must be done under the hood at the back of the room.

4. Recommendations for writing your report

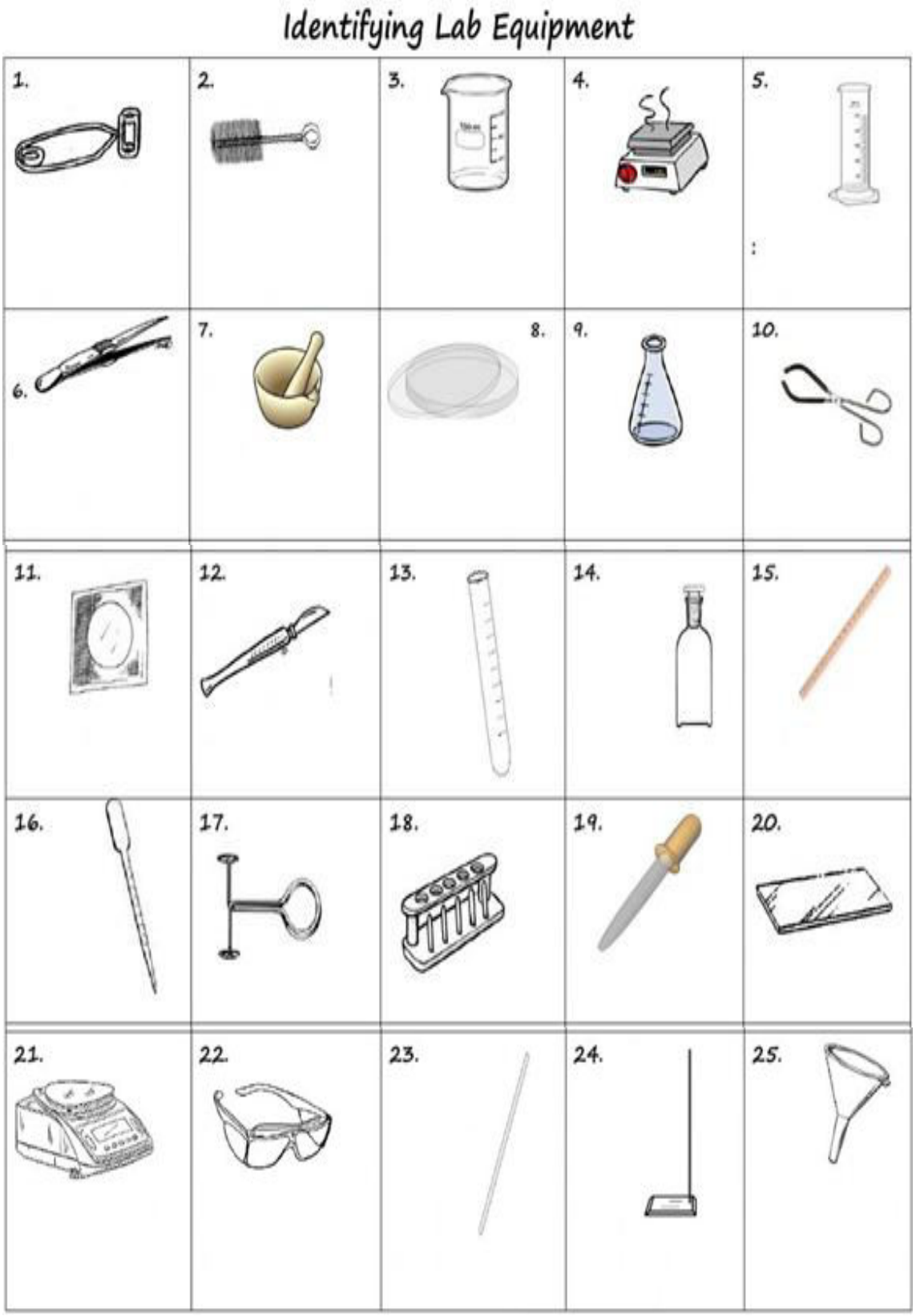
The report that you must give to the teacher at the end of each session will be evaluated and graded. It should include the full name, group number, date and title of the session at the top left of the first page.

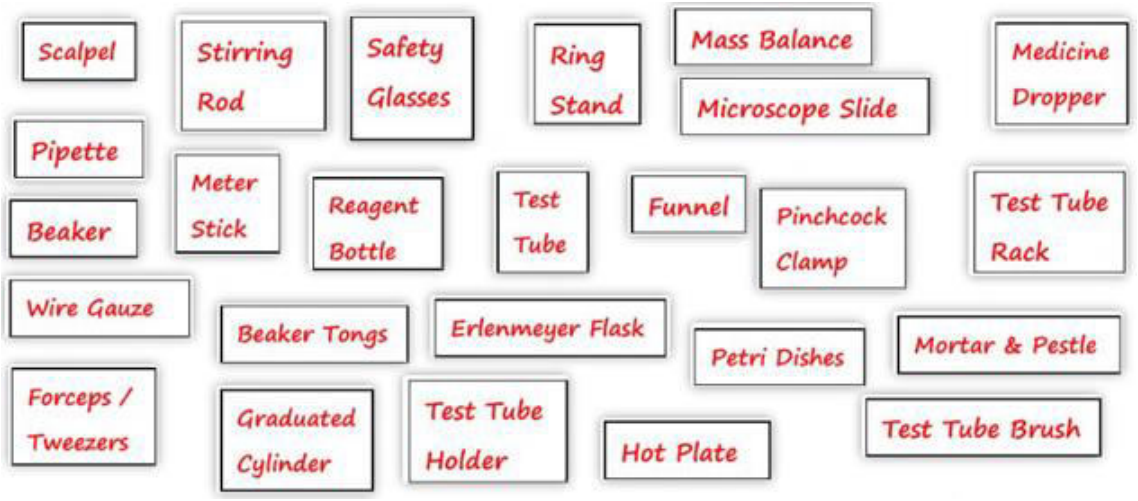
You must also take care in writing your report, the outline of which is predefined by the questions you will find at the end of each manipulation.

The evaluation of the report that is graded by a mark over /20 will be based on the following criteria:

1. Answers of the test-questions proposed at the beginning of the session, are marked with a grade over 5.
2. Answers of the manipulation questions should be given in the same proposed order.
3. Give precise, clear and concise answers.
4. The grade will take into account the care taken by the student in handling and tidying up the lab bench as well as in the presentation of the report.

Glassware and apparatus for students use during the labs




Scalpel
Stirring Rod
Safety Glasses
Ring Stand
Mass Balance
Microscope Slide
Medicine Dropper
Pipette
Meter Stick
Reagent Bottle
Test Tube
Funnel
Pinchcock Clamp
Test Tube Rack
Beaker
Wire Gauze
Beaker Tongs
Erlenmeyer Flask
Petri Dishes
Mortar & Pestle
Forceps / Tweezers
Graduated Cylinder
Test Tube Holder
Hot Plate
Test Tube Brush

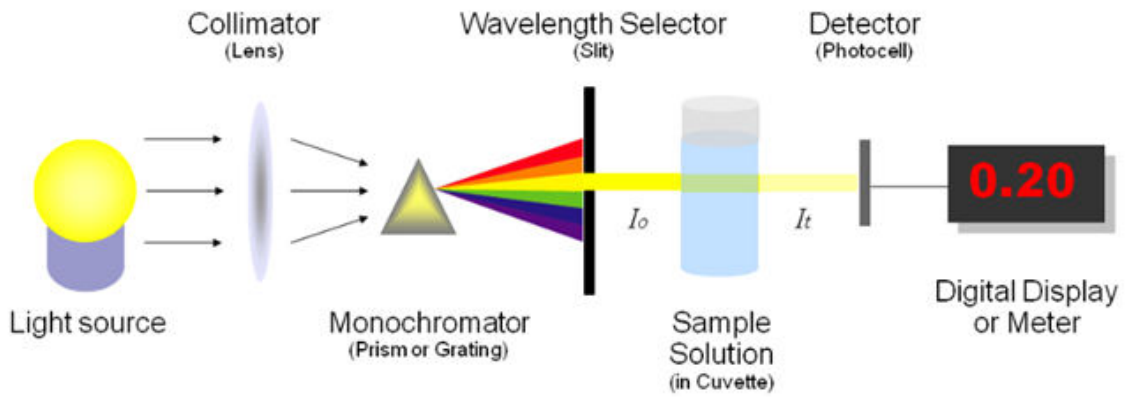
SESSION 1

Manipulation 1

Assessment of the concentration of a substance by spectrophotometric technique

1. Introduction

Spectrophotometric absorption technique is possible when a chemical reaction results in colored products dissolved in a non-absorbent solvent, by measuring the attenuation of the light after it has passed through this colored solution. This reduction in light intensity depends on the nature and quantity of the dissolved compound, the nature of the light and the thickness of the solution traversed.



Basic structure of spectrophotometers

(CC BY-4.0; Heesung Shim via LibreTexts)

Light absorption law: Beer-Lambert law

It is a law that links the intensity I of the beam, after its passage through the colored medium, to the intensity I_0, before this passage.

The decrease in the light intensity (absorption) after passing through the colored substance depends on two variables: the concentration C of the colored substance and the thickness l traversed by the beam. This absorption varies exponentially with each of these two factors. The expression will be:

$I = I0 \times 10^{-\square.l..C}$

Thus the Optic Density is DO = Log_I_0_ = ε.l.C

I

- The constant **ε**, called molar extinction coefficient is characteristic of the dissolved substance and the wavelength used.

It is expressed in liter×mol^{-1}×cm-1

- OD: absorbance of the solution without unit
- l : length of cell crossed by the light in cm
- C: molar concentration in mol×liter-1.

The use of the concept of optic density (OD) has two fundamental advantages:

- The OD is a linear function of concentration.
- The graphical representation of the OD as a function of concentration is a straight line whose slope expresses the molar extinction coefficient. Therefore, this slope is specific to the nature of the absorbent substance.

Notes:

- The measurement of an absorbance is given by a spectrophotometer, which measures the optical density. The more colored the solution is, the harder it is for light to pass through, so the more important absorbance should the solution have.
- Some colorless substances do not absorb light. To visualize one of their chemical or biological reactions, a specific "complexation" technique is often used. One of the products of this reaction can react with a colored reagent, the complexation being proportional to the amount of product of the reaction.

General conditions for carrying out colorimetric assays

To perform a colorimetric assay, certain conditions must be met:

- The reaction must give a color or opacity proportional to the concentration;
- The color or opacity must be stable while the measurements are being made;

- A calibration range shall be carried out under the same physico-chemical conditions as the tests;
- The wavelength of the spectrophotometer shall be that which allows the highest possible absorbance.

Conducting a calibration range

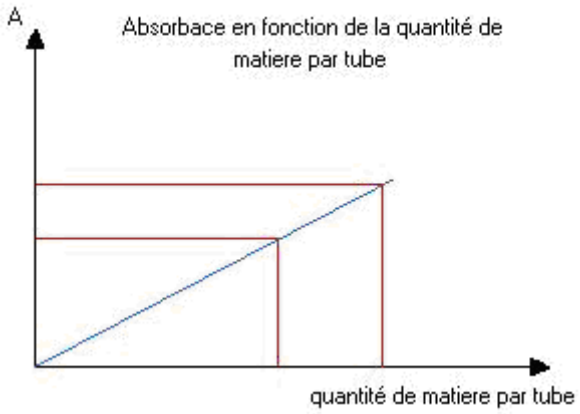
The calibration range is used to determine an absorbance at a given wavelength, for a spectrophotometer vessel of a given length wave and for a known concentration solution. It is thus necessary to prepare increasing concentrations of the element to be determined.

To perform the calibration range and the assay, the final volume of liquid in each tube must be **identical**. However, the volumes of reagents must remain constant to allow the reaction to take place, and the quantities (related to the concentrations) of compound to be determined must vary in each tube. In some assay methods, distilled water is added to adjust the same volume.

To adjust the spectrophotometer, **a 0 or blank tube** must be made to cancel the absorbance due to the reagents themselves. This tube contains all the reaction medium except the substance to be determined.

The realization of a calibration range requires a lot of precision and must be, preferably, carried out under the same conditions as the tests and by the same operator.

Translated with www.DeepL.com/Translator (free version)



After obtaining the absorbance values of the calibration range, the absorption curve can be drawn OD=f(Quantity). A straight line must pass through the landmark origin and be as close as possible to most calibration points. The absorbance value of the test is plotted on this line to determine the amount of compound present. In order to find the concentration of the test compound present in the initial medium, the volume of the test sample and any dilution shall be taken into account.

2. Principle

Maltose is treated with 3,5 dinitrosalysilic acid (DNS) resulting in its oxidation. During this reaction, a red coloration appears that can be followed by spectrophotometry, at a wavelength λ= 530 nm.

3. Reagents

- 3,5 dinitrosalysilic acid (DNS)
- Maltose etalon (1mg/ml)

4. Procedure

- Prepare the following test tubes:

Tubes	**0**	**1**	**2**	**3**	**4**	**5**	**6**
Standard maltose (ml)	0	0,3	0,5	0,8	1	-	-
Unknown solution (ml)	-	-	-	-	-	0,5	0,5
H_2O (ml)	1	0,7	0,5	0,2	0	0,5	0,5

- Add 1 ml of DNS to each tube, place in a boiling water bath for 5 min;
- Add 8 ml of distilled water to each tube;
- Shake by vortex and read the OD at 530 nm for each tube.

5. Report

a- Knowing that the concentration of standard maltose is 1 mg/ml, plot the optical density versus the amount of maltose (in mg).

b- What will be the concentration of maltose of the unknown solution in g/l?

c- What will be the concentration of maltose of the unknown solution in mole/l knowing that the MW of maltose = 360 g/mole.

d- Determine the molar extinction coefficient of maltose under these conditions.

SESSION 2

Manipulation 2

Preparation of aqueous solutions, titration,

Calculation of concentrations

I. Introduction

Most of biological reactions take place in aqueous solutions. To prepare a solution, we need either a product to weigh or a solution to dilute:

- Product to be weighed:

Use the molecular weight (MW) in g/mol indicated on the bottle.

In % Weight/Volume (W/V): for example a solution X at 5% (W/V) contains 5g of a product diluted in 100 ml of suitable solution (distilled water, acid, etc.)

- Solution to be diluted:

Know how to make dilutions to ½, 1/5th, 1/10th, etc.

On the bottle, use the data: % of product/liter of solution, the MW and the density (Kg/l).

Solutions are characterized by a concentration C defined as the quantity of a molecule per unit volume.

C = Quantity of a molecule/Unit of volume

The concentration can be expressed in unit of mass per unit of volume (g/L) therefore it is called mass concentration or in number of moles per unit of volume (mol/L) and so it is called molar concentration.

II. Molarity or molar concentration

M = Number of moles in the solution/Total volume of the solution

A molar solution of a product contains MW g/l of solution.

Examples:

1 molar solution of HCl (1M) contains 1 mole/liter or 36.5g/l of HCl; MW of HCl=36.5g

1 molar solution of H_2SO_4 (1M) contains 1 mole/liter or 98g/l of H_2SO_4; MW of H_2SO_4=98g

1 molar solution of H_3PO_4(1M) contains 1 mole/liter or 98g/l of H_3PO_4; MW of H_3PO_4=98g

III. Normality or number of gram equivalents per liter

Normality is the third way to express concentration, but this time in number of active particles or number of gram equivalents per volume unit.

N = Number of gram equivalents of a substance/Unit of volume

Normality is related to the number of gram equivalents and the types of chemical reactions; such as :

- In acid-base reactions, the gram equivalent number is equal to the number of H+ proton or OH-ions exchanged.

- In redox reactions, the gram equivalent number is equal to the number of e- electron exchanged.

Examples:

1 normal solution of HCl contains MW/1 g/l of solution.

1 normal solution of H_2SO_4 contains MW/2 g/l of solution.

1 normal solution of H_3PO_4 contains MW/3 g/l of solution.

IV. Relationship between molarity and normality

Molarity and normality are related by the following relationship:

N= n x M

Such as:

- N: normality

- M: molarity

- n: number of gram equivalents

V. Acid-base equivalence:

Na x Va = Nb x Vb

na Ma x Va = nb Mb x Vb

Where:

- a: acid

- b: base

VI. Preparation of a dilute solution from a concentrated solution

This is a relationship mainly used when preparing dilute solutions from concentrated stock solutions:

Ci x Vi = Cf x Vf

Where:

- i: initial (concentrated solution)

- f : final (diluted solution)

Manipulation 3**:**

Determination of sulfuric acid H_2SO_4 **by sodium base NaOH**

1- Procedure

- Put 25 ml of H_2SO_4 in an Erlenmeyer flask;

- Add 2 drops of helianthine (colored indicator);

- Fill the burette with the 0.1M NaOH solution;

- Titrate the H_2SO_4 solution with NaOH;

- the end of the reaction is marked by the passage of the colored indicator from red to yellow. This is the acid-base equivalence point.

2- Report

a- What is the purpose of the experiment?

b- Determine the quantity of sodium in mg necessary to prepare a 0.5 liter solution of a 0.1 M solution (MW_{NaOH}=40g/mol).

c- Determine the volume of sodium required to neutralize the sulfuric acid.

d- Determine the molarity of the H_2SO_4 solution.

3- Applications

1- What volume must be taken from a solution A of HCl of concentration 0.3 M (mol/l) to have a solution B of HCl of concentration 0.12 M (mol/l)?

2- To have a 5% sodium solution, how much sodium must be weighed to have 200 ml of this solution?

3- Knowing that the molecular weight of NaCl is equal to 58 g/mol, how much NaCl must be weighed to have a solution of concentration 2.5 M (mol/l)?

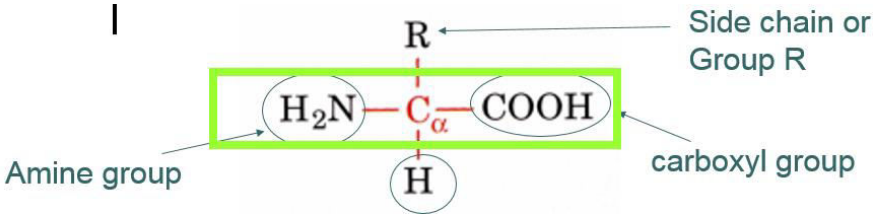
SESSION 3

Manipulation 3:

Characterization of amino acids by color reactions

I. Introduction

Amino acids are organic compounds with two functional groups: a carboxyl group -COOH and an amine group -NH2 are attached to the same carbon (carbon α). They have a general structure as follows:



The 20 amino acids correspond to this general structure except for proline (Pro) which constitutes a group apart; because its carboxyl and amine group are engaged in a ring.

Based on the composition of their side chain, we can classify the amino acids in different groups.

The identification and determination of amino acids contained in a protein (after hydrolysis) or in biological material can be done in different ways:

By fractionation methods:

- Electrophoresis, which mainly allows a separation into groups (neutral amino acids, acidic and basic amino acids),

- Chromatographic techniques of partition on column, or on paper, or on thin layer, or ion exchange chromatography with elution gradient.

By chemical and biological methods:

- Based in particular on colored reactions.

II. Manipulations

II.1 Reaction engaging amine and carboxylic groups: reaction with ninhydrin

a- Principle

This is a general reaction which requires the simultaneous presence of a carboxyl and an amine. The reaction is carried out in neutral medium: the ninhydrin gives, with the amino acids, purple compounds except for the proline, it gives a yellow compound.

b- Procedure

- In a test tube, put :

- 2ml of the amino acid solution (for example glycine)

- 3 drops of the 2% ninhydrin solution.

- Bring to boil for 1 min.

- Note the new coloration.

- Make in parallel and in the same way a test with H_2O, as a blank test, and a test with each of the unknown solutions.

II.2 Specific properties and reaction of the "R" radical of amino acids

II.2.1. Characterization of basic amino acids (Arg, His, Lys)

a- Principle

In the presence of phosphotungstic acid, the basic amino acids precipitate specifically.

b- Procedure

-In a test tube, put:

- 1 ml of a basic amino acid solution

- 4 ml of water

- 1 ml of a solution of phosphotungstic acid at 20%.

The immediate appearance of a white precipitate indicates the presence of a basic amino acid.

- Make in parallel and in the same way a test with H_2O, as a blank test, and a test with each of the unknown solutions.

II.2.2. Characterization of the sulphur radicals (Cysteine, Cystine, Methionine)

a- Principle

The sulfur amino acids in alkaline medium with lead salts give a black coloration due to the formation of lead sulfide.

b- Procedure

-In a test tube, put:

- 2 ml of an amino acid solution having a sulfur-containing radical

- 1 ml of the lead acetate solution

- Gradually add a few drops of the soda lye to the wall of the tube until the lead hydroxide precipitate is completely dissolved.

- Boil in a water bath for 10 min and observe the coloration.

- Make in parallel and in the same way a test with H_2O, as a blank test, and a test with each of the unknown solutions.

II.2.3. Characterization of the aromatic radicals (Try, Tyr, Phe)

a- Principle

The cyclic compounds, heated in the presence of nitric acid, give a yellow coloration (nitrated derivative) which turns to orange in alkaline medium.

b- Procedure

- In a test tube, put :

- 2 ml of a solution of aromatic amino acid.

- 0.5 ml of nitric acid

- Bring to boil in a water bath for 5 min and observe the coloration.

- Allow to cool then slowly add 1.5 ml of ammonia. Observe the new coloration

- Make in parallel and in the same way a test with H_2O, as a blank test, and a test with each of the unknown solutions.

III. Report

a- Summarize the 4 reactions in a table, precise for each one the aim, the result and the conclusion

b- Give the general characteristics of the tested amino acid.

Manipulation 4

Determination of the pHi of glycine

1. Introduction

Amino acids carry at least two ionizable groups: the -NH2 group and the -COOH group, ionizable to $-NH3^+$ and $-COO^-$. They therefore have an amphoteric character because they can be both electron donors and electron acceptors.

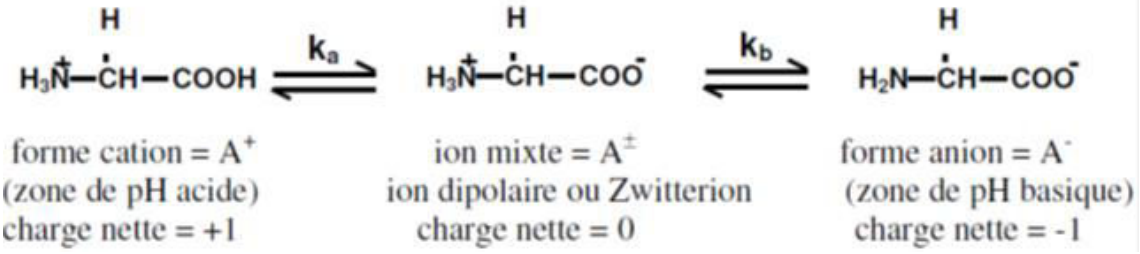
Amino acids in solution are always in charged forms. This charge depends on the pH of the meduim, the ionic strength and the dissociation constants of their functions.

- In acidic medium these amphoteric compounds gain protons. The function -COOH which has the strongest dissociation constant and therefore the lowest pK, will ionize first.

- The amino acid will carry both the positive and negative charge corresponding to the zwitterion form. At this moment when the + and - charges balance, the pH of the medium corresponds to the isoelectric point (pHi).

- In basic medium, the amino acid loses a proton and will be negatively charged.

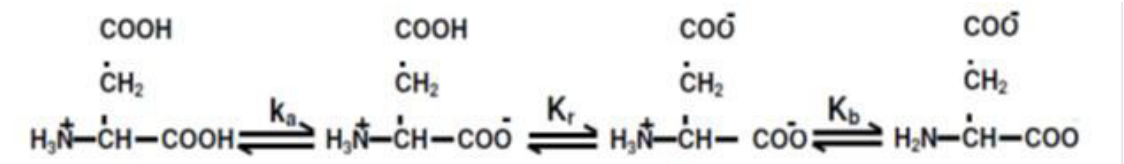
a- Case of a neutral amino acid: eg glycine (Gly)



The isoelectric pH, pH_i, is defined as the pH for which the net charge of the molecule is zero, i.e. the pH at which the dipolar ion or zwitterion A form will prevail. This pH is calculated from the dissociation constants K_a and K_b of the ionizable functions. Hence:

$$pHi = \frac{pk_a + pk_b}{2}$$

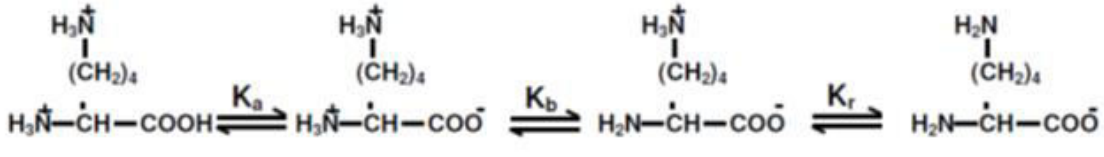
b- Case of an acidic amino acid: ex. aspartic acid (Asp)



Forme cation = A^+ Charge nette= +1 | ion mixte = $A^{\pm}$ charge nette= 0 | forme anion 1= A^- charge nette= -1 | forme anion 2= A^{--} charge nette= -2

$$pHi = \frac{pk_a + pk_r}{2}$$

c- Case of a basic amino acid : ex. Lysine



Forme cation 1 = A^{++} Charge nette= +2 | forme cation 2= A^+ charge nette= +1 | ion mixte= $A^{\pm}$ charge nette= 0 | forme anion = A^- charge nette= -1

$$pHi = \frac{pk_b + pk_r}{2}$$

2. Purpose:

Determination of the pk and pHi of the amino acid, glycine or glycocolle (Gly).

3. Principle:

The aim is to follow the evolution of the pH on a determined volume of an amino acid solution of pH equal to 2, according to the amount of sodium hydroxide NaOH added.

4. Materials and reagents:

- pH meter
- Beaker
- 20mM glycine solution (0.02M)
- Stirrer, magnetic bar
- 0.2N NaOH solution
- 2N HCl solution
- Burette

5. Procedure:

The Electrodes are very fragile and expensive, so please:

- Do not let the magnet bar hit them during agitation.

- Do not leave them dry.

- rinse them well before and after titration with distilled water.

- Measure 50 ml of the amino acid solution with a test tube.

- Pour the amino acid solution into a beaker.

- After calibrating the pH meter, rinse the pH meter electrode with distilled water and place it in the beaker containing the amino acid solution.

- Measure the starting pH of the solution and bring it to pH=2 with a few drops of 2N HCl.

- Place the 0.2 N NaOH solution in the burette.

- Titrate the amino acid solution by adding the sodium hydroxide NaOH solution by successive additions of 1 ml while stirring.

- Note the NaOH added volume and the corresponding pH value up to pH 10.5

6. Report

a) What is the isoelectric pH of the amino acid in the solution?

b) Draw the titration curve pH = f (V_{NaOH} in ml).

c) Deduce from this curve the different pK values and the pHi of this amino acid.

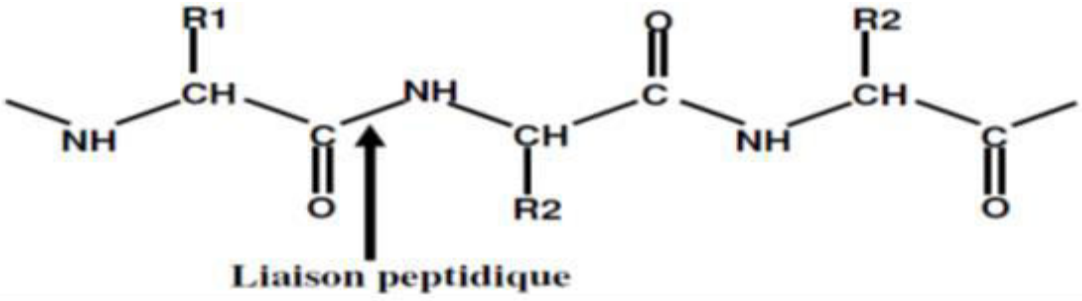
SESSION 4

Manipulation 5

Parameters influencing the structures of proteins

1. Introduction

Proteins are macromolecules made of amino acids linked to each other by amide bonds called peptide bonds.



Generally, a peptide sequence has two free ends NH2 and -COOH. By convention, this sequence is written with the NH2 end on the left and the -COOH end on the right.

Several factors can act on the structures of proteins yielding in their denaturation.

Denaturation is the destruction of quaternary, tertiary, secondary and even primary structures. This destruction can be definitive (irreversible), or not definitive, (reversible).The most important denaturant factors are heat and ionic strength.

Most proteins are less soluble at high salt concentrations. This effect is called salting out. The dependence of solubility on salt concentration differs from one protein to another. Thus, salting out can be used to fractionate proteins. It is also used to concentrate dilute protein solutions, including active fractions obtained in other purification steps. Dialysis can be used to remove salt.

2. Reagents:

Gornall's reagent containing : • $CuSO4, 5H_2O$: 1.5g • NaOH: 30g	- Na2SO4 27,2 % (27,2 g/100ml) - Na2SO4 19,9 % (19,9 g/100ml) - Na2SO4 15.75 % (15.75 g/100ml)

• KI: 1g • Double tartrate of Na and K: 6g • H_2O sufficient quantity for 1L	- BSA protein solution (3g/30ml) - 5 mg/mL BSA standard - Acetic acid diluted 1:10 - NaCl at 9 g/l

3. Influence of heat

a. Principle

Protein structures are stable at temperatures at or near physiological conditions.

Proteins heated in a slightly acidic environment will be coagulated. This denaturation corresponds to the destruction of quaternary, tertiary and secondary structures. It is an irreversible denaturation.

b. Procedure

In a test tube, put:

- 1 ml of a protein solution of BSA (5mg/ml)

- Add 2 to 3 drops of acetic acid diluted to 1/10

- Add 5 ml of NaCl at 9g/l

- Heat the upper part of the tube. Compare with the unheated part

- Conclude

4. Influence of ionic strength: salt release

a. Principle:

Salts intervene according to their concentration and the charge of their ions on the solubility of proteins in solution. The ionic strength is defined as:

→

$Fi = 1/2 \times C \times Z^2$

When Fi increases, the solubility of the proteins decreases, the proteins precipitate. Each protein precipitates at a given Fi, thus at a given concentration of a salt species.

b. Purpose:

It is to fractionate and measure the concentration of β and δ globulins by salt release or precipitation: serum contains albumin.

b. Procedure:

i) Calibration

Tubes	**1**	**2**	**3**	**4**	**5**
NaCl (ml)	1	0,9	0,75	0,5	0,25
BSA standard (ml)	0	0,1	0,25	0,5	0,75
Gornall's reagent (ml)	2	2	2	2	2

- Mix the tubes by vortex.

- Let the tubes stand 15 min in the dark, then read the OD at 530 nm against tube 1.

- Draw the calibration curve Do= f (number of mg of protein).

- Keep tube 1.

ii) Saline loading

With a solution of:

- 15.75% Na_2SO_4 precipitates the δ globulins.

- Na_2SO_4 at 19.9% precipitates β and δ globulins.

- Na_2SO_4 at 27.2% precipitates all globulins.

Prepare 4 tubes:

Tubes	**FA**	**FB**	**FC**	**FD**
Serum (ml)	0,5	0,5	0,5	0,5
NaCl 9g/l (ml)	5	0	0	0
Na_2SO_4 at 27,2 % (ml)	0	5	0	0
Na_2SO_4 at 19,9 % (ml)	0	0	5	0
Na_2SO_4 at 15,75 % (ml)	0	0	0	5

- Mix, incubate at 37°C for 15 min.

- Filter the FB, FC and FD solutions, the filtrate must be clear.

iii) Assay

Tubes	**FA**	**FB**	**FC**	**FD**
Filtrate A (ml)	1	-	-	-
Filtrate B (ml)	-	1	-	-
Filtrate C (ml)	-	-	1	-
Filtrate D (ml)	-	-	-	1
Gornall reagent (ml)	3	3	3	3

- Leave the tubes for 15 min in the dark, then read the OD at 530 nm against tube 1 of the calibration.

5. Report

- Using the calibration curve, determine the amount in mg of protein in each of FA, FB, FC and FD tubes.

- Report this amount to 1 ml of serum.

- What types of proteins (albumin, □, β or δ globulins) are contained in each filtrate.

- Determine the amount of total albumin, □, β and δ globulin proteins per ml of serum.

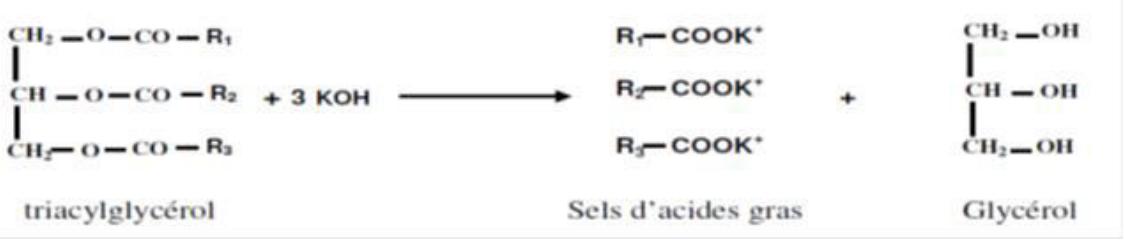
SESSION 5

Manipulation 6:

Determination of the saponification number of an oil

1. Introduction

Lipids are generally esters of alcohol and fatty acids. The nature of the alcohol is variable and the fatty acids are saturated or unsaturated, sometimes substituted by phosphoric acid and a nitrogenous base (phospholipids). Fats are generally rich in saturated fatty acids and oils in unsaturated fatty acids. The long paraffinic chains of the fatty acids make lipids insoluble in water and soluble in organic solvents.



The most abundant neutral lipids are the acylglycerols (formerly called glycerides), which are reserve substances consisting mainly of triacylglycerols.

Triacylglycerols result from the esterification of a trihydric alcohol, glycerol by 3 fatty acids, which are different in most cases (heterogeneous triacylglycerols).

There are exceptions of homogeneous triacylglycerols such as triolein (esters of glycerol and oleic acid) composing olive oil.

2. Principle

In hot and strongly basic triacylglycerols release glycerol and alkaline salts of fatty acids (stearic, oleic, palmitic acids) called soaps according to the reaction:

The saponification reaction takes place in the presence of a known volume and in excess of alcoholic KOH. The excess KOH is measured by a solution of sulfuric acid of known title. The saponification number is expressed as the amount of KOH (in mg) required to saponify one gram of oil.

3. Reagents

- Flask Erlenmeyer for saponification

- Ascending refrigerant

- Olive oil

- Potassium hydroxide (KOH) of unknown normality

- Sulfuric acid (H_2SO_4) at 0.25N

- Phenol phthalein

1. Procedure

ii) Oil test

In a Florence flask for saponification, introduce:

- 15 drops of olive oil (weight = 0.37 g).
- Measure 10 ml of alcoholic KOH using graduated cylinder, add them and then shake.
- Connect the Florence flask to the refrigerator (this allows to condense the vapors emitted during the heating of the solution).
- Connect the cold water circulation
- Heat moderately to bring the alcoholic solution to a gentle boil, continue saponification for 20 minutes.
- Remove the flask and cool it under a stream of tap water.
- Add 3 drops of phenol and measure the excess of NaOH by H2SO4 (V1).

ii) Blank test

While waiting for the end of the saponification, in another Erlenmeyer, prepare a blank test (without oil).

- Pour the same quantity of alcoholic KOH.

- Add 3 drops of phenol phthalein.

- Dose the potash with H_2SO_4(V2).

5. Report

1- Give the volumes of H_2SO_4 used in the blank test as well as in the one with oil.

2- Calculate the saponification number of the oil knowing that the molar weight of KOH = 56 g/mol.

SESSION 6

Manipulation 7

Carbohydrates

Identification and characterization by color reactions

I. Introduction:

Sugars, also called carbohydrates, of general formula $C_n(H_2O)_n$, are compounds with many hydroxyl groups (-OH) responsible for their very hydrophilic character. The presence of carbonyl (-C=O), aldehyde or ketone groups gives them a reducing character. They can be divided into two groups:

1. **Oses or monosaccharides:** The simplest sugars. Depending on the carbon atom number, we find: trioses, tetroses, pentoses, hexoses. All natural oses are of the D series (dextrorotatory).
2. **Osides or holosides:** the linking of several oses forms the osides. According to the number of oses they can be divided into :

a- Oligoholosides: are oligomers formed by a number n of bones linked together by glycosidic bond alpha or beta. By convention the number n varies from 2 to 10. The most important are disaccharides example: maltose, sucrose, lactose. The raffinose is a trisaccharide.

b- Polyholosides (sometimes called glycans, polyosides, polyholosides or complex carbohydrates or polysaccharides): are polymers consisting of a large number of bones that can reach hundreds linked together by osidic bonds. Example: Glycogen, starch, cellulose.

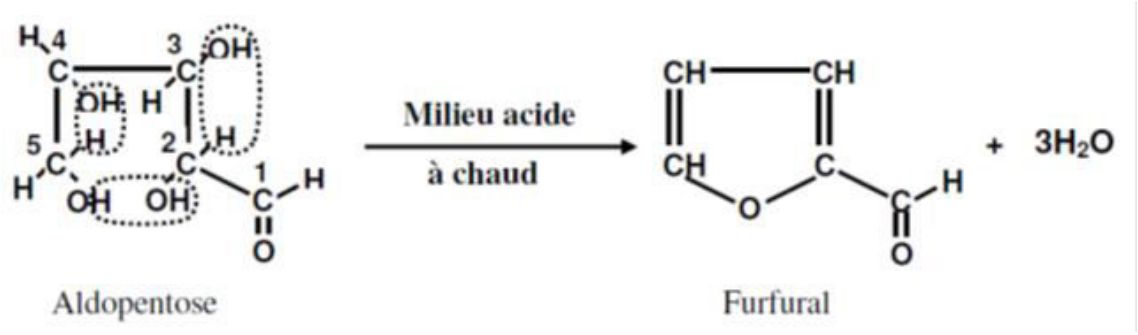
3. heterosides: consisting of one or more molecules of oses with non-carbohydrate molecules.

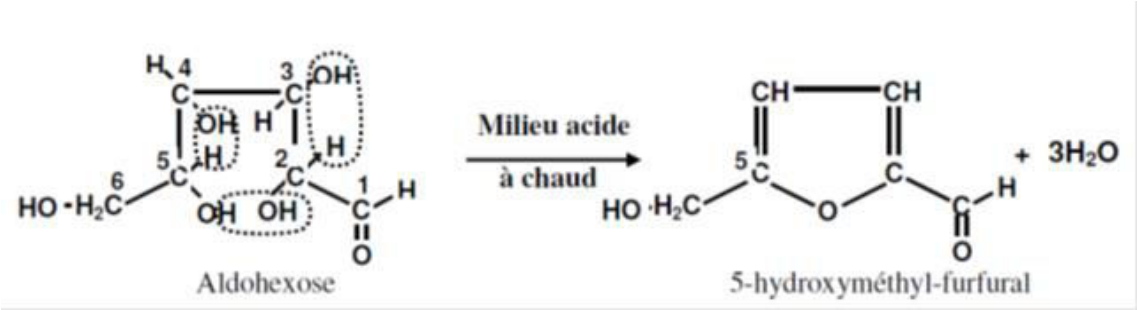
II. Furfuralic reactions

II.1 Principle

In a strongly acidic medium and at high temperature, the oses with at least 5 carbon atoms undergo dehydration and are transformed into furfural (if the ose is a pentose) or into a furfural derivative (if the ose is a hexose).

Furfural and its derivatives can condense with substances such as phenols, aromatic amines to form characteristic colored products.





II.2 Materials

- Test tubes

- Boiling water bath

- Pipettes

- Propipettes or suction bulbs

II.3 Reagents

- solutions of glucose, fructose, mannose, ribose, sucrose and lactose at 1% in distilled water.

- Concentrated HCl

- 1N HCl

- Concentrated H_2SO_4

- Lugol (mix 1g of iodine with 2g of potassium iodide in 100 ml of distilled water)

- Thymol reagent (2 g α-naphthol + 100 ml Ethanol)

- Sellivanoff's reagent (2 g resorcinol + 0.5 ml concentrated H_2SO_4 + 100 ml H2O)

- Bial's reagent (0.2 g orcinol + 100 ml concentrated HCl + 5 drops of 10% $FeCl_3$)

II.4 Procedure

II.4.1. Thymol reaction

This is a general reaction to all sugars (oses or osides). In the presence of sulfuric acid, sugars form a furfural derivative that condenses with alpha -naphthol to give a product colored pink-red ring.

Procedure (handle in a fume hood).

1. Prepare 3 test tubes: put in the first tube 1 ml of a glucose solution, in the second tube 1 ml of the unknown solution and in the third tube 1 ml of distilled water.

2. Add to each tube 2 to 3 drops of Thymol reagent

3. Shake the tubes to mix the contents

4. Gently pour 1 ml of concentrated H2SO4 down the wall of each tube

5. Observe and note the colorations obtained

II.4.2. Selivanoff reaction:

This reaction is characteristic of ketoses which, in the presence of concentrated hydrochloric acid and at high temperature, condense with resorcinol to form a red compound.

Procedure (Handle under the hood).

1. Prepare 3 test tubes: put in the first tube 1 ml of a fructose solution, in the second tube 1 ml of the unknown solution and in the third tube 1 ml of distilled water.

2. Add to each tube 1 ml of Selivanoff's reagent

3. Add to each tube 0.5 ml of concentrated HCl

4. Heat the tubes for 5 min in a boiling water bath and allow to cool

5. Observe and note the colorations obtained.

II.4.3. Bial reaction

This reaction is specific to pentoses. In concentrated HCl medium and in the presence of Fe+3 ions, the pentoses condense with orcinol to form a green colored compound.

Procedure

1. Prepare 3 test tubes: put in the first tube 1 ml of the ribose solution, in the second tube 1 ml of the unknown solution and in the third tube 1 ml of distilled water.

2. Add 1.5 ml of Bial's reagent

3. Bring the tubes to a boil for 3 minutes

4. Observe and record the colorations obtained

III. Report

1- Prepare a table in which you put the aim, the results and the conclusion for each reaction:

2- Coloration obtained in the Thymol reaction

Coloration obtained in the Selivanoff reaction.

Coloration observed in the Bial reaction

3- Specify for each test if it is a pentose or hexose, a ketose or aldose.

4- Give the general conclusion for the carbohydrate used solution.

SESSION 7

Manipulation 8

Determination of hemoglobin concentration

1. Introduction

Hemoglobin is a heteroprotein composed of a protein copula, globin, and a prosthetic (non-protein) group, heme, containing an iron atom.

Hemoglobin has the following characteristics:

- It is an elective transporter of oxygen from the lungs to the tissues and of carbon dioxide from the tissues to the buffers.

- It actively participates in the buffering capacity of blood.

- It has a peroxidase activity which is detected by measuring it in the blood or in the urine.

There is a large variety of hemoglobin depending on :

- the species, the tissues (examples: muscle hemoglobin or myoglobin)

- the age of the animal (embryonic, fetal and adult hemoglobin)

- and finally, the condition of the animal (normal hemoglobin or pathological hemoglobin).

2. Principle

The determination of hemoglobin is based on the absorption at 540 nm of one of its stable derivatives: cyanmethemoglobin. The latter is obtained following the transformation of hemoglobin under the action of potassium ferricyanide and potassium cyanide.

This reaction takes place in two steps:

- oxidation of hemoglobin and its derivatives into methemoglobin ;

- transformation into cyanmethemoglobin in the presence of potassium cyanide.

The molar extinction coefficient of this derivative is equal to 11 000L/moles/cm.

3. Sample

- Whole blood collected on EDTA.

4. Reagents

Drabkin's reagent (☠ toxic).

5. Procedure

- In a test tube, put:

- 5 ml of Drabkin's Reagent;

- 0.02 ml of whole blood (taken with the micropipette);

- Mix and leave for a few minutes at room temperature.

- Read the optical density at 540 nm against a white tube (Concentrated Drabkin Reagent).

6. Report

1- Calculate the concentration of hemoglobin in mole/l.

2- Knowing that the molar weight of hemoglobin is 16114 g/mol, deduce its concentration in g/l.

3- Conclude.

Usual values

Newborns: 195 ± 50 g/l (12 mmol/l)

1-year-old children: 112 g/l (6.95 mmol/l)

10-year-old children: 129 g/l (8 mmol/l)

Men: 160 ± 20 g/l (9.9 mmol/l)

Women: 140 ± 20 g/l (8.7 mmol/l)

Manipulation 9

Determination of total salivary proteins

by the BIURET method

1. Introduction

Human saliva is the product of gland secretion. This saliva is a colorless, transparent, slightly viscous liquid that foams when shaken.

Saliva is an aqueous solution containing organic substances and ions in the form of salts. The most important element is water (99.5%). Proteins and low molecular weight organic substances represent less than 0.5%.

Saliva is a buffered medium that ensures a constant pH: several buffer systems are present, such as the carbonic acid-bicarbonate system, the monopotassium-dipotassium phosphate system, and proteins and amphoteric amino acids.

The main salivary proteins are:

- Salivary amylase, which hydrolyzes cooked starch and slowly hydrolyzes raw starch.

- Maltase hydrolyzes maltose into glucose.

- Lysozymes, mycolytic and bacteriolytic enzymes that prevent the actions of fungi, bacteria and viruses respectively.

2. Principle

Proteins will be quantified by a colorimetric assay. The staining reagent used is the Gornall reagent, composed of:

- Copper sulfate, which gives the blue color of the reagent due to copper ions ;

- Sodium hydroxide solution (NaOH), which makes the medium basic;

- double tartrate of sodium and potassium, which "chelates" (traps) the Cu2+ ions and avoids their precipitation in basic medium in the form of insoluble copper hydroxide Cu(OH)2 ;

- Potassium iodide, to avoid the reduction of cupric ions before the determination.

Thus, in alkaline medium, serum proteins that have at least 2 peptide bonds form a blue-violet complex with copper II ions (Cu2+), the intensity of the color of which is proportional to the protein concentration. This coloration also varies according to the nature of the proteins to be determined, the alkalinity of the medium, the concentration

of copper sulfate and the temperature. A colorimetric determination is therefore possible at 540 nm.

3. Equipment

- Spectrophotometer at 540 nm

- 15 ml test tubes

- Pipettes (1ml, 2ml, 5ml)

- Racks for 10 test tubes

4. Reagents

- Physiological water: solution containing 9g of NaCl in 1L of distilled water

- Gornall's reagent containing:

- $CuSO_4$, $5H_2O$: 1.5g

- NaOH: 30g

- KI: 1g

- Double tartrate of Na and K (salt of seignette): 6g

- H_2O quantity sufficient for 1L

- Standard Bovine Serum Albumin at 5 mg/ml.

5. Procedure

- Prepare the following tubes:

Tubes	**White**	**Standard**	**Assay**
NaCl 9g/l (ml)	1	-	-
Pure saliva (ml)	-	-	1
BSA standard (ml)	-	1	-
Gornall's reagent (ml)	3	3	3

- Shake and let stand 30 min in the dark

- Read the OD at 540 nm against the white tube.

6. Report:

Determine the salivary protein concentration in mg/ml.

SESSION 8

Manipulation 10

Study of the amylase activity of saliva

(Determination of Vm and Km)

1. Introduction

Salivary proteins have an essentially enzymatic role. They play an important biological role in digestive processes.

Examples: amylase allows the transformation of starch into maltose and dextrins and maltase transforms maltose into glucose.

Amylase is the most quantitatively important enzyme in saliva. It comes mainly from the parotid glands. Amylase is a water-soluble protein, its molecular weight is 50,000 and it has a calcium in its active site, essential for its activity.

2. Principle

Filtered and suitably diluted saliva acts on a cooked starch, at pH 6.9 and at room temperature for variable periods. Amylase activity is monitored by determining the amount of reducing sugars that appear as a function of time:

- In alkaline medium, reducing sugars are oxidized by 3,5-dinitrosalicylic acid (DNS). During this reaction, a red coloration appears which follows the Beer-Lambert law.

- The amylase activity will be expressed as the amount of product formed (micromoles of maltose formed) per unit of enzyme (mg of salivary protein) and per unit of time (minute).

3. Reagents

- 1% starch starch in 0.02 M phosphate buffer, pH 6.9 and containing 6.7 mM NaCl.

- 3,5-Dinitrosalicylic acid (DNS)

4. Procedure

- Collect saliva on crushed gauze, placed on a funnel attached to a test tube

- Dilute the filtered saliva (0.1 ml is diluted in 50 ml of distilled water).

a- Calibration

You already have a calibration curve DO=f (number of micromoles of maltose)

b- Enzymatic kinetics

- At each step, shake the tubes gently

- DNS stops the enzymatic reaction; it must be added after each incubation time

For the time t=0, the DNS is added before the salivary enzyme.

- Each pair will take a volume x that can be: 0,5 or 1,5 or 2 ml.

Tubes	**0 min**	**1 min**	**2 min**	**4 min**	**6 min**	**10 min**	**15 min**
Starch (ml)	X	X	X	X	X	X	X
Diluted saliva (ml)	-	1	1	1	1	1	1
DNS (ml) after each time	2	2	2	2	2	2	2
Diluted saliva (ml)	1	-	-	-	-	-	-

- Mix and place all tubes in a boiling water bath for 5 min, then cool under running water.

H2O (ml)	17-x	17-x	17-x	17-x	17-x	17-x	17-x

- Read the OD at 530 nm against the 0 min tube.

5. Report

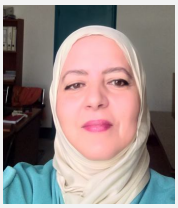
1- Using the calibration curve, determine the number of micromoles of reducing sugars for each incubation time.

2- Plot the number of micromoles of reducing sugars that appear as a function of time. Deduce the initial rate (Vi) of the reaction

3- Draw the curve Vi as a function of the amount of substrate (starch). Deduce the maximum speed (Vmax) and the Michaelis constant (Km).

4- Calculate the specific amylase activity, assuming that the concentration of salivary amylase is equal to the concentration of salivary proteins (determined in manipulation 10).

ABOUT THE AUTHOR



Pr Asma Kassab

Dr. Asma Kassab, PhD is a Biochemistry Associate Professor at the Dental Medicine, University of Monastir. She also serves as a peer reviewer in higher education at Arab universities. As a researcher, she writes articles published in well-recognized journals.

EMPYREAL PUBLISHING HOUSE

ISBN 978-93-93810-12-0

www.empyrealpublishinghouse.com