SPECTROPHOTOMETRY

SPECTROPHOTOMETRY

- It is that technique that measures the amount of light absorbed or transmitted by a substance.
- It is an analytical technique that is applied to obtain valuable information, such as:
  - The identity of unknown compounds by their characteristic absorption spectra in the ultraviolet, visible or infrared regions (qualitative analysis) .
  - Determination of the unknown concentration of an analyte by measuring the light absorption at one or more wavelengths (quantitative analysis) .
- Enzyme catalyzed reactions can be followed by measuring the absorption of the substrate or product .

REGIONS OF THE ELECTROMAGNETIC SPECTRUM

Regions	X-Ray	Ultraviolet	Visible	Infrared	microwave
Wave length	0.1 -100 nm	100 - 400 nm	400 - 800 nm	800 nm - 100 µm	100 µm - 30 cm

The ultra violet and the visible regions are the ones that we usually use in the spectrophotometry.

1 nm = 10^{-9} m

1 µm = 10^{-6} m

λ is the symbol of wavelength.

THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER

1- Light source:

- It can be two kinds:
  - Tungsten lamp ; produces light at visible region.
  - Hydrogen lamp; produces light at ultraviolet region.

2- Collimator:

- It is a focusing device that transmits an intense straight beam of light.

3- Monochromator:

- It is a device that divides the light beam into it’s component wavelengths.

THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER

4- Selector:

- It selects the required wavelength.

5- Cuvette:

- It is a compartment in which the sample is placed.
- Three kinds:
  - Glass and plastic cuvettes; used in the visible region.
  - Quartz cuvettes; used in the ultraviolet region,

The glass cuvettes absorbs light in the ultraviolet region →Thus the amount of light measured by spectrophotometer will be the absorbance of sample + the glass cuvette.

THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER

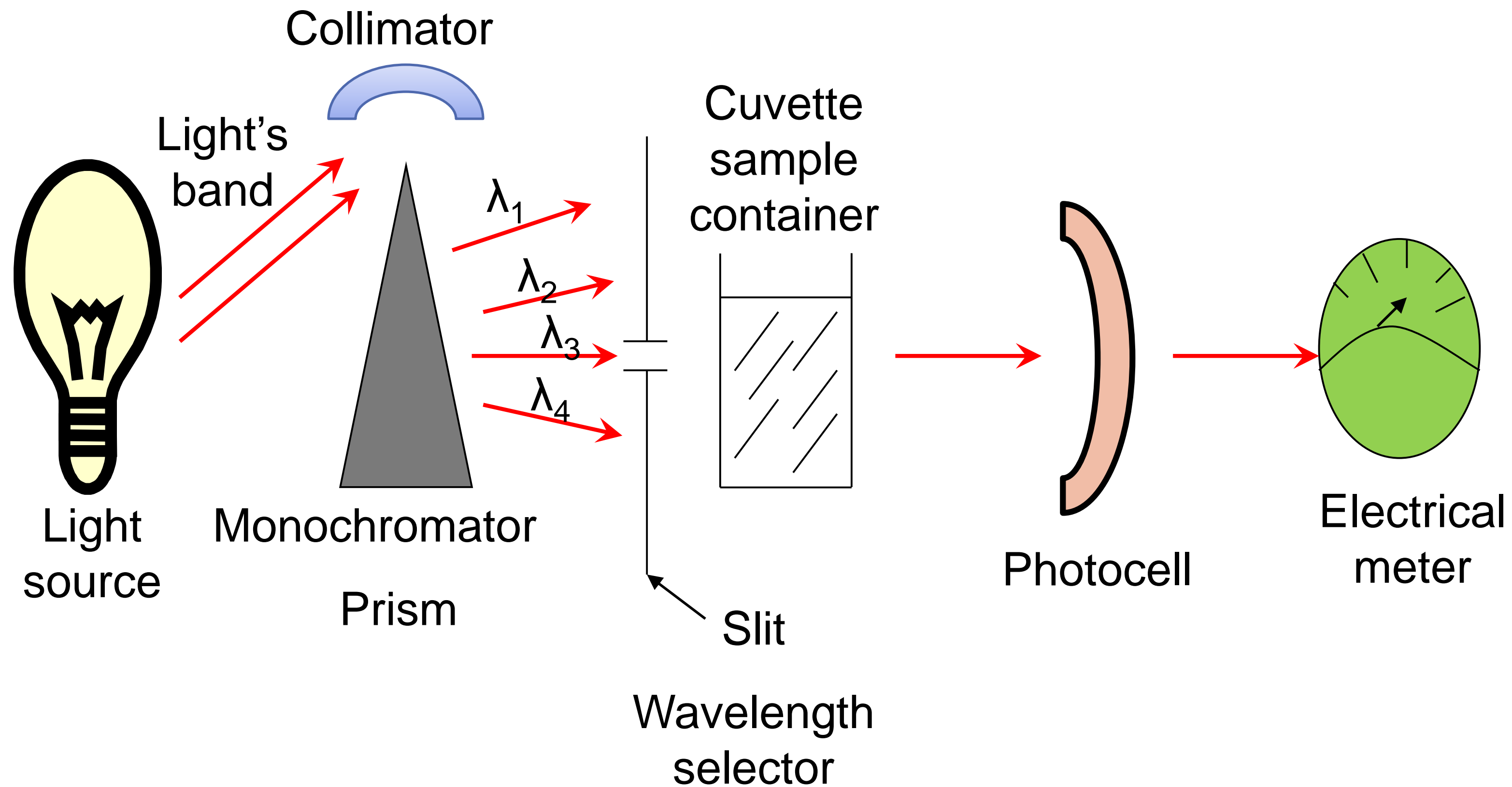
6- Photocell (photodetector):

- It detects the amount of light transmitted.

7- Electrical meter:

- It records the output of the detector.

THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER



BEER and LAMBERT LAWS

The fraction of the incident light I_0 that is absorbed by a solution depends on three factors:

1- The thickness of the sample or path length.

2- The concentration of the absorbing sample.

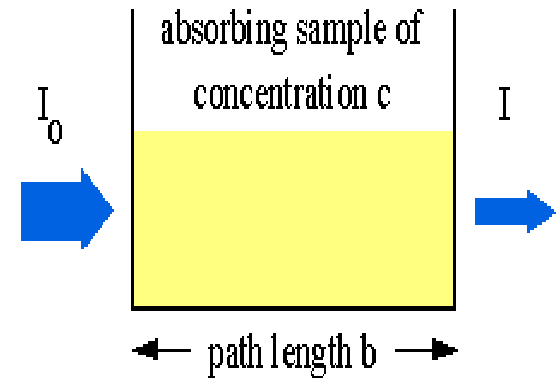
3- The chemical nature of the compound.

The relationship between the concentration, path length, and the amount of light absorbed or transmitted can be exposed mathematically in two laws: the Beer law and Lambert law.

BEER'S LAW

- **It states that the intensity of the light transmitted by an absorbing media decreases with increasing concentration of the absorbing compound.**

$\text{Log } I_0 / I \propto c$



I_0 = intensity of the incident light

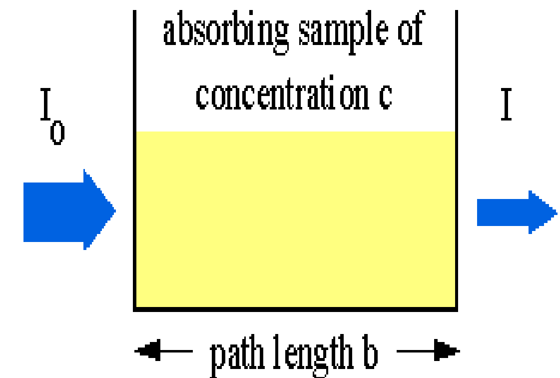
I = intensity of the transmitted light

c = concentration of absorbing compound

- **The amount of light absorbed = $I_0 - I$**
- **Log I_0 / I represents the fraction of light absorbed**

LAMBERT'S LAW

It states that the intensity of the light transmitted by an absorbing media decreases as the thickness or path length of the absorbing media increases.



$$\log I_0 / I \propto L$$

L = is the path length

BEER-LAMBERT LAW

The two law's can be combined in one law which is Beer-Lambert Law

$$A = \text{Log } I_0 / I = a\, c\, L$$

A = absorbance or optical density O.D.

a = is the extinction coefficient which is a constant for each substance but varies at different wavelength

If the concentration is expressed in :

- in Molaity →Molar absorption coefficient (a_m)
- in g/L → Specific absorption coefficient (a_s)
- a_{m340} = is the molar absorption coefficient of a substance at a wavelength = 340 nm

$$a_m = a_s \text{ Mwt}$$

a_m is most commonly used in biochemistry, and the path length *L* is almost always 1 cm, thus the units for a_m is M^{-1} cm^{-1}.

BLANK SOLUTION

Is a solution that is necessary in all spectrophotometry studies.

It should contain all components of the assay or test solution *except the component who's absorbance is being measured*.

Purpose of the Blank:

The blank will cancel out the absorbance of the substances in the background so that the absorbance of the tests will be that of the compound under study only.

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

Example 1:

A solution containing 2 g/l of a light absorbing substance in a 1 cm cuvette transmits 75% of the incident light at 260 nm. Calculate the transmission of a solution containing

a) 4 g/l.

b) 6 g/l.

c) If the Mwt is 250, calculate a_m.

d) What type of cuvette should you use here? Why?

First, Calculate the A , and a_s

Note:

I_0 is always 100%→ as decimal (100/100= 1)

I = 75%→ as decimal (75/100= 0.75)

Absorbance (A) does not have a unit

$A = \text{Log } I_0 / I$

$A = \log 1.0 / 0.75 = 0.124$

$A = a_s \, c \, l$

$a_s = A / c \; l = 0.124 / 2 \times 1$

$a_s = 0.0625 \; (g/L)^{-1} \; cm^{-1}$

a) transmission of a solution containing 4 g/l.

$\log I_0 / I = a_s \, c \, l$

$\text{Log } 1.0 - \log I = 0.0625 \times 4 \times 1$

$0 - \log I = 0.25$

$-\log I = 0.25$ (divide by -1) → $\log I = -0.25$

$I = \text{antilog}(-0.25) = 0.562$ (multiply by 100) → 56.2%

b) $\text{Log } I_0 / I = a_s \, c \, l$

Log 1.0 - log I = 0.0625 x 6 x1.

- log I = 0.375

Log I = - 0.375

I = antilog - 0.375 = 0.422 → 42.2%

C) $a_m = a_s$ x Mwt = 0.0625 x 250 = 15.63 M^{-1} cm^{-1}

D) Quartz cuvettes should be used at the U.V range.

EXAMPLE 2

A solution containing 10^{-5} M ATP, has a transmission 0.702 (70.2%) at 260 nm in a 1 cm cuvette. Calculate:

a) The transmission of the solution in a 3 cm cuvette.

b) The absorbance of the solution in a 1 cm and 3 cm cuvette.

c) The absorbance if the concentration increased to 5x 10^{-5} M of ATP, in a 1 cm cuvette.

First, Calculate the A , and a_m

A = Log $I_\circ / I = a_m\, c\, l$

A = log 1.0 / 0.702 = 0.152

$0.152 = a_m \times 10^{-5} \times 1$

$a_m = 0.152 / 10^{-5} = 15200\ M^{-1}\ cm^{-1}$

a) transmission of the solution in a 3 cm cuvette.

$A = 15200 \times 10^{-5} \times 3 = 0.456$

A = Log $I_\circ / I$

0.456 = log 1.0 / I

0.456 = log1 – log I = 0 – log I = - log I

Thus I = antilog - 0.456 = 0.349 → 34.9%

b) A in a 1 cm cuvette.

$A = 15200 \times 10^{-5} \times 1 = 0.15$

A in a 3 cm cuvette.

$A = 15200 \times 10^{-5} \times 3 = 0.456$

c) A if the concentration increased to 5x 10^{-5} M of ATP, in a 1 cm cuvette.

$A = 15200 \times (5 \times 10^{-5}) \times 1 = 0.76$

PROTEIN DETERMINATIONS

Proteins in solutions can be determined spectrophotometrically by two methods:

a) Colorimetrical method: ***(e.g. Biuret method)***

it is based on the reaction of Cu^{2+} with peptides in an alkaline solution producing a purple complex that has an absorption maximum at 540 nm.

b) Direct spectrophotomety:

The absorbance at 280nm can be used to determine protein concentration in solutions.

(Because proteins have a distinct absorbance maximum at 280nm due to their aromatic amino acids).

EXAMPLE 3

A protein solution (0.3 ml) was diluted with 0.9 ml of water. To 0.5 ml of this diluted solution, 4.5 ml of biuret reagent was added and the color was allowed to develop.

The absorbance of the mixture at 540 nm was 0.18 in a 1 cm diameter cuvette. A standard solution (0.5 ml containing 4 mg of protein/ml) plus 4.5 ml of biuret reagent gave an absorbance of 0.12 in the same size cuvette.

a) **Calculate the protein concentration in the undiluted unknown solution.**

b) **What is the composition of the blank here ?**

A) Concentration of standard C_{st} = 4 mg/ml

Thus C_{st} = 4 g/L

$A_{standard} = a_s \times C \times l$

$0.12 = a_s \times 4 \times 1$

So $a_s = 0.12 / 4$

$= 0.03\ (g/L)^{-1}\ cm^{-1}$

First, find the value of a_s from the known values for standard solution
It is (a_s)→because conc. of sample is in **mg/ml**

-To convert from: **mg/ml → g/L** (divide by 1) since the ratio is the same (1mg = 0.001 g and 1mL = 0.001L)

Next, apply the value of a_s on beer-lambert law to find the concentration of test

$A_{test} = a_s \times C \times l$

↑Dilution factor

$0.18 = 0.03 \times C \times 1$

So $C_{test} = 0.18 / 0.03 = 6$ g/L = 6 mg/ml

The concentration of protein in the undiluted solution:

Df= total volume/ aliquot volume = (0.3+ 0.9)/ 0.3 = 4

$C_{undiluted}$ = 6 x 4= 24 mg/ml .

b) The blank should contain 4.5 ml of biuret and 0.5 ml of distilled water only.

EXAMPLE 4

Solutions Containing Two Absorbing Substance

A solution containing NAD^+ and NADH had an absorbance of 0.311 in a 1 cm cuvette at 340 nm, and 1.2 at 260 nm. Calculate the concentration of the oxidized and reduced forms of the coenzyme in the solution.

Both NAD^+ and NADH absorb at 260nm, but only NADH absorbs at 340nm.

a_m		
	260nm	**340nm**
NAD^+	18000	0.0
NADH	15000	6220

Note: Absorbance at 340nm represents the absorbance of NADH only since NAD^+ does not absorb at that wavelength → So the concentration of NADH can be obtained.

* At 340nm → $A_{NADH} = a_m \times C \times l$

$0.311 = 6220 \times C \times 1$

So $C_{NADH} = 0.311/6220 = 5 \times 10^{-5}$ M

*At 280nm → $A_{260nm} = A_{NADH} + A_{NAD+}$ (since both absorb at this wavelength)

$A_{NADH} = a_m \times C \times l = 15000 \times 5 \times 10^{-5} \times 1 = 0.75$

Thus $A_{NAD+} = A_{total} - A_{NADH} = 1.2 – 0.75 = 0.45$

Since $A_{NAD+} = a_m \times C_{NAD+} \times l$

$0.45 = 18000 \times C_{NAD+} \times 1$

$C_{NAD+} = 0.45 / 18000 = 2.5 \times 10^{-5}$ M

EXAMPLE 5

Solutions Containing Two Absorbing Substance

Ten grams of butter were saponified , the non-saponifiable fraction was extracted into 25ml of chloroform . The absorbance of the chloroform solution in a 1cm cuvette was 0.53 at 328nm and 0.48 at 458nm . Calculate the carotene and vitamin A content of the butter .

$a_{1\%}$		
	328nm	458nm
Carotene	340	2200
Vitamin A	1550	0.0

$a_{1\%}$ = absorption coefficient when concentration expressed in 1g/100ml .

Note: The absorbance at 458nm represents the absorbance of Carotene only , thus its concentration can be obtained .

$A_{458nm} = A_{carotene}$

$A_{458nm} = a_{1\%} \times C_{carotene} \times 1 = 2200 \times C_{carotene} \times 1$

$C_{carotene} = 0.48/2200 = 2.1 \times 10^{-4}$ g/100ml

Thus the carotene content in the 25ml of chloroform extract is

2.1×10^{-4} g --------> 100ml

? --------> 25ml

the carotene content in the 25ml of chloroform extract = $(25 \times 2.1 \times 10^{-4}) / 100$

$= 5.2 \times 10^{-5}$ g

$= 5.2 \times 10^{-2}$ mg .

The carotene content per gram of butter = 0.052 / 10 = 5.2×10^{-3} mg carotene / g of butter

Absorbance at 328nm is the absorbance of $A_{carotene} + A_{vitamin\ A}$

$A_{carotene} = a_{1\%} \times C \times l = 340 \times 2.1 \times 10^{-4} \times 1 = 0.0714$.

$A_{vitamin\ A} = A_{Total} - A_{carotene}$

$A_{vitamin\ A}$ = 0.53 – 0.0714 = 0.458

$C_{vitamin\ A}$ = $A / a_{1\%}$ x 1 = 0.458/1550 = 2.9×10^{-4} g/100ml

Thus vitamin A content in the 25ml of chloroform extract is

2.9×10^{-4} g --------> 100ml

? --------> 25ml

vitamin A content in the 25ml of chloroform extract = $(25 \times 2.9 \times 10^{-4}) / 100$

= 7.25×10^{-5} g

= 0.073mg

The vitamin A content per gram of butter = 0.073/10 = 0.0073mg / g of butter

COUPLED ASSAY

- **Many compounds of biological importance do not have a distinct absorption maximum λ_{max} .**
- **Their concentration can be determined if they can be linked to or coupled with a reaction that fulfills the following condition ..**

If they promote the formation of another compound that has a characteristic absorption peak.

- **In coupled assay reactions the product of the first reaction is the substrate of the following reaction ;**

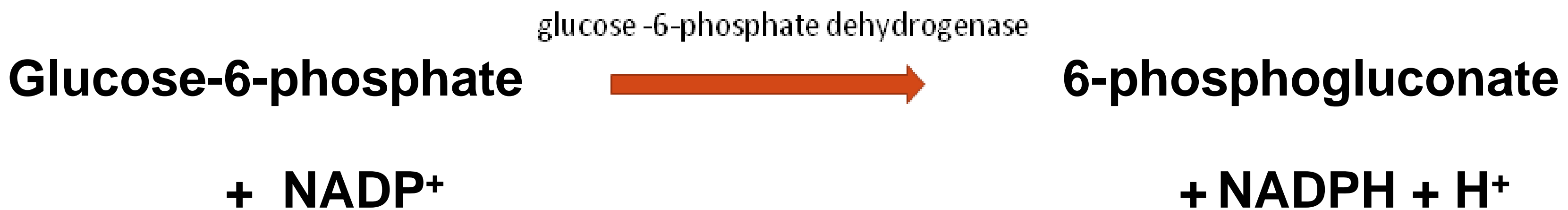
A = substance under study that does not have a distinct λ_{max}

F = has a distinct λ_{max}

→ *Thus A can be estimated by measuring the absorbance of F*

EXAMPLE

To 2.0 ml of a glucose solution; 1.0 ml of a solution containing excess ATP, $NADP^+$, $MgCl_2$, hexokinase and Glucose -6-phosphate dehydrogenase was added. Calculate the concentration of glucose in the original solution. Absorbance of final solution at 340 nm increased to 0.91, a_m= 6220, L = 1 cm.



Notes:

*Glucose has no absorption at 340 nm, but NADPH does!

*From the reaction: 1 mole of glucose produces 1 mole of NADPH, thus each number of NADPH moles produced originates from every mole of glucose in the original solution.

Absorbance at 340 nm is the absorbance of NADPH = 0.91

$A = a_m \times C \times L \rightarrow 0.91 = 6220 \times C \times 1$

$C_{NADPH} = 0.91 / 6220 = 1.46 \times 10^{-4}$ M

Thus there is 1.46×10^{-4} M of glucose present in the *test* solution

The glucose concentration in the *original* solution = $1.46 \times 10^{-4} \times (3/2)$ = 2.2×10^{-4} M

Dilution factor