

SPECTROPHOTOMETRY

SPECTROPHOTOMETRY

It is that technique that measures the amount of light absorbed or transmitted by a substance.

It is one of the most important technique in analytical biochemistry.

Unknown compounds can be identified by their characteristic absorption spectra in the ultraviolet, visible or infrared regions.

Concentrations of known compounds in solutions can be determined by measuring the light absorption at one or more wavelengths.

SPECTROPHOTOMETRY CONT'D

Spectrophotometry is used for both quantitative and qualitative analysis .

Enzyme catalyzed reactions can be followed by measuring the absorption of the substrate or product .

SPECTROPHOTOMETRY

CONT'D

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.

$$1 \text{ nm} = 10^{-9} \text{ m}$$

$$1 \text{ \AA} = 10^{-10} \text{ m}$$

$$1 \text{ } \mu\text{m} = 10^{-6} \text{ m}$$

λ is the symbol of wavelength.

SPECTROPHOTOMETRY CONT'D

Wave number is the reciprocal of the wavelength

$$\text{wave number} = \frac{1}{\text{wavelength}} \quad \text{cm}^{-1}$$

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 CONT'D

4- Selector:

- It selects the required wavelength.

5- Cuvette:

- It is a compartment in which the sample is placed.
- Two kinds:
 - Glass 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 CONT'D

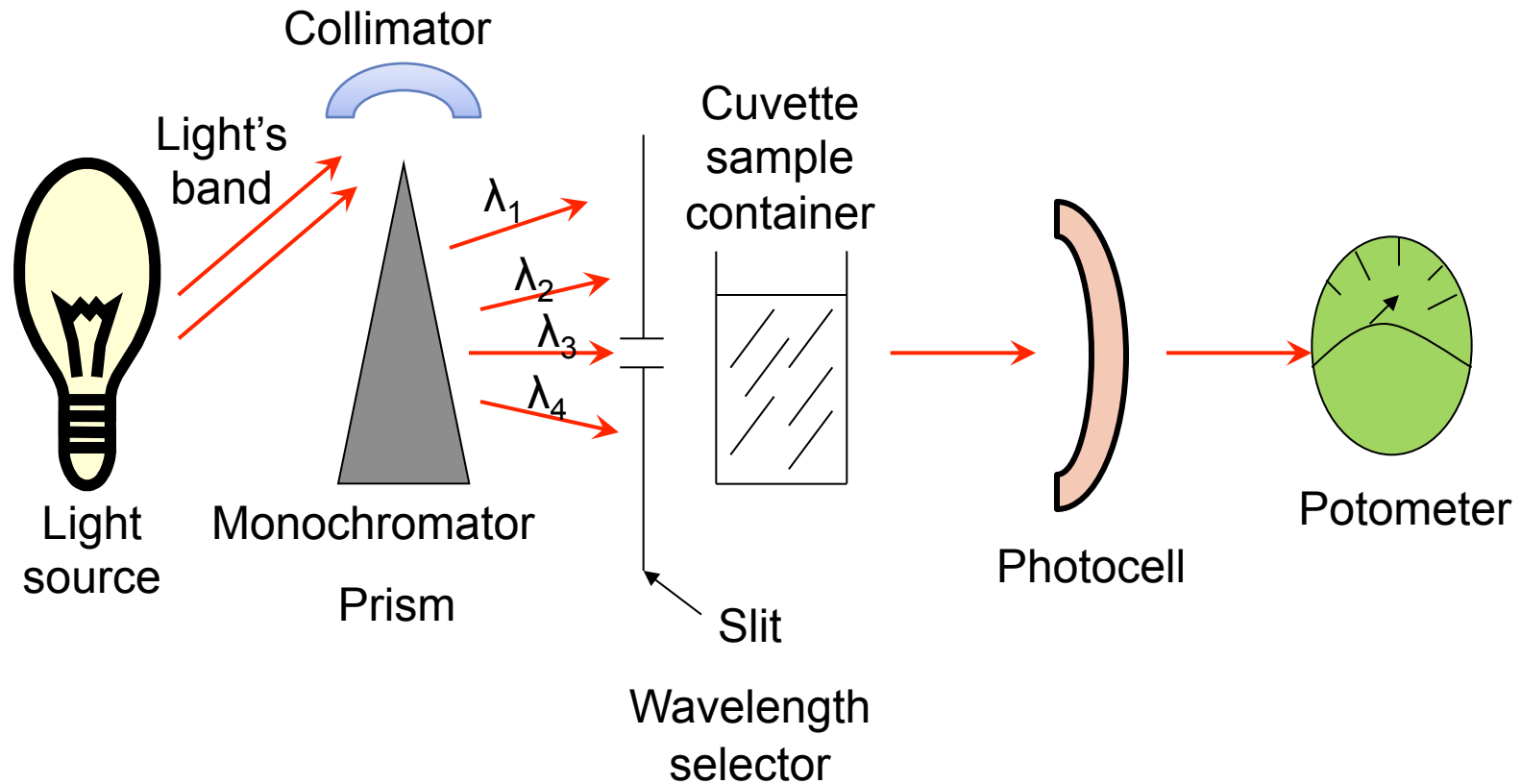
6- Photocell (photodetector):

- It detects the amount of light transmitted.

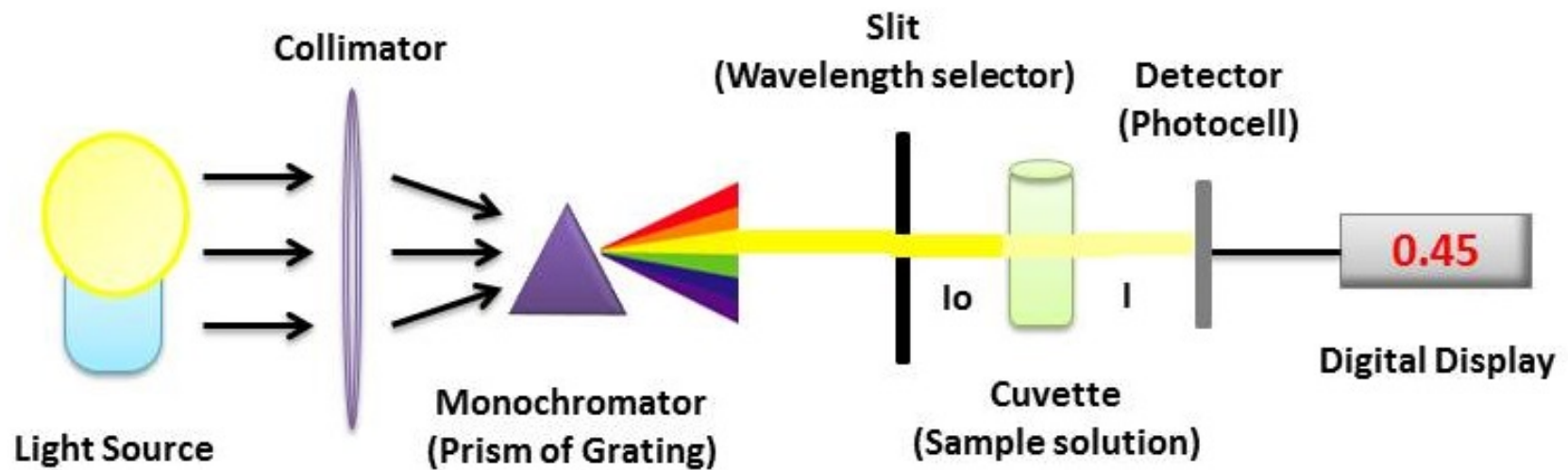
7- Electrical meter:

- It records the output of the detector.

THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER CONT'D



THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER CONT'D



Basic Instrumentation of a Spectrophotometer

THE ESSENTIAL COMPONENTS OF SPECTROPHOTOMETER CONT'D

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 LAW

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

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

I_0 = intensity of the **incident** light

I = intensity of the **transmitted** light

a = a **constant** for every compound at a specific wavelength

c = **concentration** of absorbing compound

BEER LAW CONT'D

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.

$$\text{Log } I_0 / I \propto a L$$

L = is the path **length**

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

LAMBERT-BEER LAW

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

a = is the **extinction coefficient** which is a constant for each substance but it differs at different wavelength and it is constant at a specific wavelength

- Molar absorption coefficient (a_m); in M
- Specific absorption coefficient (a_s); in g/l
- a_{m340} = is the molar absorption coefficient of a substance at a wavelength = 340 nm
- $a_m = a_s Mwt$

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 $M^{-1} \text{ cm}^{-1}$.

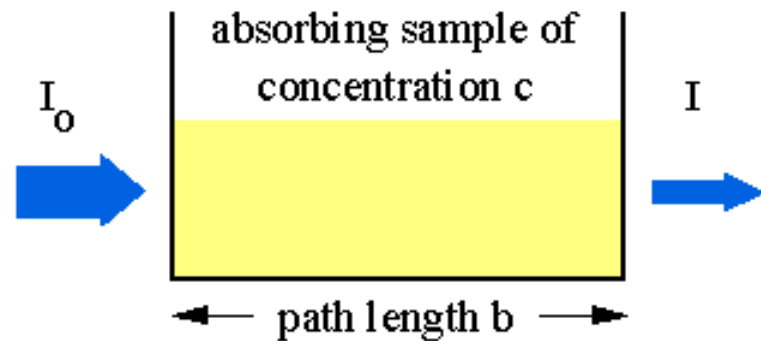
The absorption coefficient varies in different substances, it also varies with varying wave-lengths also.

LAMBERT-BEER LAW

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

- A = absorbance or optical density O.D.

➤ $A = a c l$



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 whose 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.

- **Note:** Glass cuvettes are not to be used in the U.V region, since the glass itself will absorb light thus leading to a false high result.
- In the U.V region Quartz cuvettes are to be used!

PROTEIN DETERMINATIONS

Proteins in solutions can be determined spectrophotometrically by two methods:

a) Colorimetric method:

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.

Proteins + Biuret reagent $\xrightarrow{\text{alkaline media}}$ purple complex (max absorbance at 540nm)

b) Direct spectrophotometry:

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).

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

Example:

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?**

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

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

$$A = \log 1.0 / 0.75 = 0.124$$

$$A = a_s c l$$

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

$$a_s = 0.0625$$

$$a) \log I_0 / I = a_s c l$$

$$\text{Log } 1.0 - \log I = 0.06 \times c \times l$$

$$0 - \log I = 0.0625 \times c \times l$$

$$- \log I = 0.0625 \times 4 \times 1 = - 0.25$$

$$I = \text{antilog } - 0.25 = 0.562 \rightarrow 56.2\%$$

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

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

$\text{Log } 1.0 - \log I = 0.0625 \times 6 \times 1.$

$-\log I = 0.375$

$\text{Log } I = -0.375$

$I = \text{antilog } -0.375 = 0.422 \rightarrow 42.2\%$

C) $a_m = a_s \times \text{Mwt} = 0.0625 \times 250 = 15.63$

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

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

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 5×10^{-5} M of ATP, in a 1 cm cuvette.**

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

a) $A = \text{Log } I_0 / I = a_m c l$

$$A = \log 1.0 / 0.702 = 0.152$$

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

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

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

➤ $A = \text{Log } I_0 / I$

➤ $0.456 = \log 1.0 / I$

$$0.456 = \log 1 - \log I = 0 - \log I = -\log I$$

$$\text{Thus } I = \text{antilog } -0.456 = 0.349 \rightarrow 34.9\%$$

b) A in a 1 cm cuvette.

$$A = 15200 \times 10^{-5} \times 1 = 0.152$$

A in a 3 cm cuvette.

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

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

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

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 tube. 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 test tube.

- a) Calculate the protein concentration in the undiluted unknown solution.
- b) What is the composition of the blank here ?

SOLUTIONS CONTAINING ONE ABSORBING SUBSTANCE

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

Thus $C_{st} = 4 \text{ g/L}$

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

$$0.12 = a_s \times 4 \times 1$$

$$\text{So } a_s = 0.12 / 4 = 0.03$$

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

$$0.18 = 0.03 \times C \times 1$$

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

The concentration of protein in the undiluted solution:

$$C_{\text{undiluted}} = 6 \times (1.2/0.3) = 24 \text{ mg/ml} .$$

↑ Dilution factor

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

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	
Compound	260nm	340nm
NAD^+	18000	0.0
NADH	15000	6220

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.

$$A_{340\text{nm}} = A_{\text{NADH}} = a_m \times C \times l$$

$$0.311 = 6220 \times C \times 1$$

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

$$A_{260\text{nm}} = A_{\text{NADH}} + A_{\text{NAD}^+} \text{ (since both absorb at this wavelength)}$$

SOLUTIONS CONTAINING TWO ABSORBING SUBSTANCE

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

$$\text{Thus } A_{\text{NAD}^+} = A_{\text{total}} - A_{\text{NADH}} = 1.2 - 0.75 = 0.45$$

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

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

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