Spectrophotometry

Spectrophotometry

- Spectrophotometry is the measurement of light absorption or transmission .

It is an analytical technique that is applied to obtain valuable information , such as the identity of an unknown compound by their characteristic absorption spectra (qualitative analysis) , and determination of the unknown concentration of an analyte (quantitative analysis) .

Spectrophotometry is used for both and quantitative and qualitative analysis .

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

Regions of the electromagnetic spectrum ;

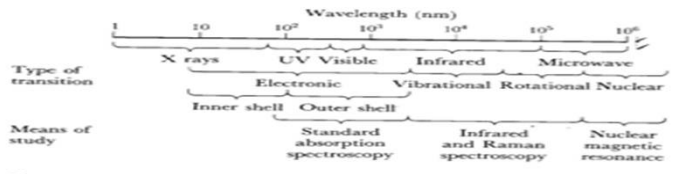
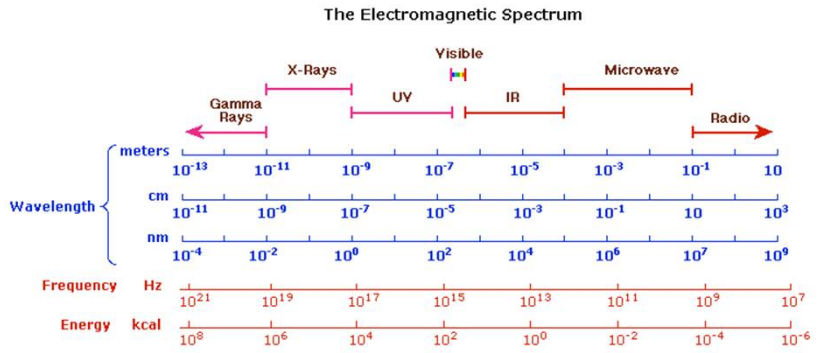


Figure 14-3
The part of the electromagnetic spectrum that is relevant to physical biochemistry.

Regions of the electromagnetic spectrum



Spectrophotometer

A Spectrophotometer is an instrument used to measure the amount of light transmitted or absorbed by a sample .

Components of the Spectrophotometer include :

1- a light source .

2- a collimator or focusing device , that transmits an intense beam of light .

3- a monochromator , that divides the light beam into its component wave-lengths .

4-A selector device for selecting the desired wavelength .

5- A compartment in which the sample is placed (cuvette).

6- A photodetector .

7-An electrical meter to record the output of the detector .

Spectrophotometer

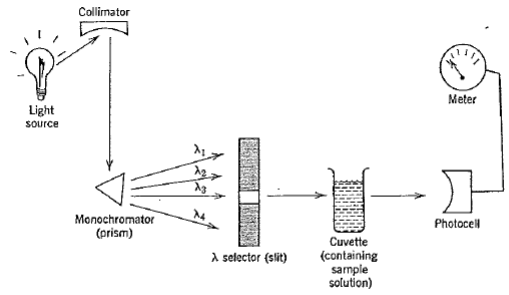


Figure 5-1 Essential components of a spectrophotometer.

Beer – Lambert Law

The fraction of the incident light that is absorbed by a solution depends on

- the thickness of the sample (path length l).
- The concentration of the absorbing compound (C) .
- The chemical nature of the absorbing compound .

The relationship between the concentration C , path length of light l , and the light absorbed by a substance is expressed mathematically in the Beer – Lambert law .

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

I = Transmitted light .

$I_\circ$ = Incident light .

A = Absorbance or optical density O.D.

a= Absorption coefficient or extinction coefficient for a particular absorbing compound .

Light absorption follows an exponential rather than a linear law .

If the concentration is expressed in Molarity " a " becomes the molar absorption coefficient a_m, or Molar extinction coefficient ε .

Beer – Lambert Law

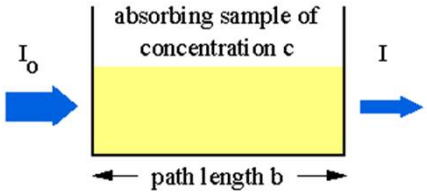
If the concentration is expressed in g/l " a" becomes the specific absorption coefficient

$a_m = a_s \times mw$.

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

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

a_{m340} refers to the molar absorption coefficient at 340nm .



Beer – Lambert Law

Blank solution:

Blank ; 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 .

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 .

Solutions Containing One Absorbing Substance

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

a) 4g/l ,

b) b) 6g/l .

c) If the mw is 250 calculate a_m , and calculate the absorbance in case a and b ,

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

Since A = Log I_o / I

A = log 1.0 /0.75 = 0.124

Since A = a_s c l , thus a_s = A /c l = 0.124/2 = 0.06 , so a_s = 0.06 .

a)Since log I_o / I = a_s c l

Log 1.0 – logI = 0.06 x c x l .

0 – log I = 0.06 x c x l .

- log I = 0.06 x 4 x 1 = - 0. 24

I = antilog - 0. 24 = 0.57 , 57%

b) log I_o / I = a_s c l

Log 1.0 – logI = 0.06 x 6 x1.

Solutions Containing One Absorbing Substance

-log I = 0.36

Log I = - 0.36

I = antilog - 0.36 = 0.436 .

C) a_m = a_s x mw = 0.06 x 250 = 15 .

d) quartz cuvettes should be used at the U.V range .

Absorbance in case b

A = 0.06 x 6 x 1 = 0.36 .

Example : A solution containing 10^{-5} M ATP , has a transmission 0.702 (70.2%)at 260 nm in a 1cm cuvette . Calculate a) the transmission of the solution in a 3cm cuvette .

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

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

a) A = Log I_o / I = a_m c l

A = log 1.0 / 0.702 = 0.152

0.152 = a_m x 10^{-5} x 1

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

Solutions Containing One Absorbing Substance

A = 15200 x 10^{-5} x 3 = 0.456

Since A = Log I_o / 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 x 10^{-5} x 1 = 0.15

c) A = 15200 x(5x 10^{-5}) x 1 = 0.76

Solutions Containing One Absorbing Substance

Protein determinations :

Proteins in solutions can be determined spectrophotometricaly by several methods for example :

a)Colorimetrical method such as :

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

Proteins + Biuret reagent ----- alkaline media ------> purple complex (max absorbance at 540nm) .

b) Direct spectrophotomety :

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

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

Solutions Containing One Absorbing Substance

Example : A protein solution (0.3ml) was diluted with 0.9ml of water . To 0.5ml of this diluted solution , 4.5ml of biuret reagent was added and the color was allowed to develop . The absorbance of the mixture at 540nm was 0.18 in a 1cm diameter tube . A standard solution (0.5ml containing 4mg 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 ?

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

Thus C_{st} = 4g/L .

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

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

So $a_s = 0.12 / 4 = 0.03$

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

$0.18 = 0.03 \times C \times 1$

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

The concentration of protein in the undiluted solution ,

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

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

Solutions Containing Two Absorbing Substance

Example : a solution containing NAD^+ and NADH had an absorbance of 0.311 in a 1cm cuvette at 340nm , and 1.2 at 260nm . 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 .

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

$0.311 = 6220 \times C \times 1$

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

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

Solutions Containing Two Absorbing Substance

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

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 : 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 .

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

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

Solutions Containing Two Absorbing Substance

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} --------> 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 = $0.052 / 10 = 5.2 \times 10^{-3}$ mg carotene / g of butter

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

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

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

Solutions Containing Two Absorbing Substance

$A_{\text{vitamin A}} = 0.53 - 0.0714 = 0.458$

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

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

$= 7.25 \times 10^{-5}$ g

= 0.073mg

The vitamin A content of the butter /g

= 0.073/10 = 0.0073mg / g of butter

= 7.3µg / g of butter .