SCANNING SPECTROPHOTOMETRY AND SPECTROPHOTOMETRIC DETERMINATION OF CONCENTRATION

Lab#1

BCH 333

Spectrophotometry :

- ***Spectrophotometry*** is the science of measuring the light-absorbing and light-transmitting characteristics of a substance.

- Molecules (or part of molecules) capable of absorbing light called *Chromophores.*

➔The basic principle is that molecules absorb and transmit light <u>of specific wavelengths</u> within the: ***ultraviolet*** (200 - 400 nm), ***visible*** (400 - 700 nm) and ***near-infrared*** (700 - 1000 nm) regions of the electromagnetic spectrum.

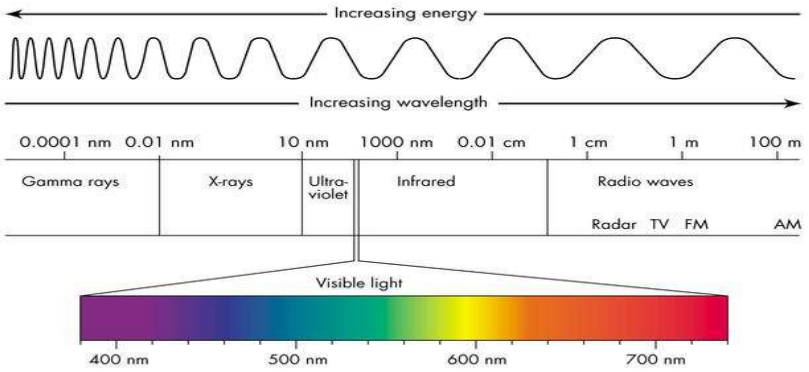


Figure 1. Electromagnetic spectrum.

Spectrophotometry cont':

- ***Spectrophotometer*** is an instrument used to measure the intensity of light that is transmitted or absorbed by a sample at a given wavelength.

- ***How the spectrophotometer works:***

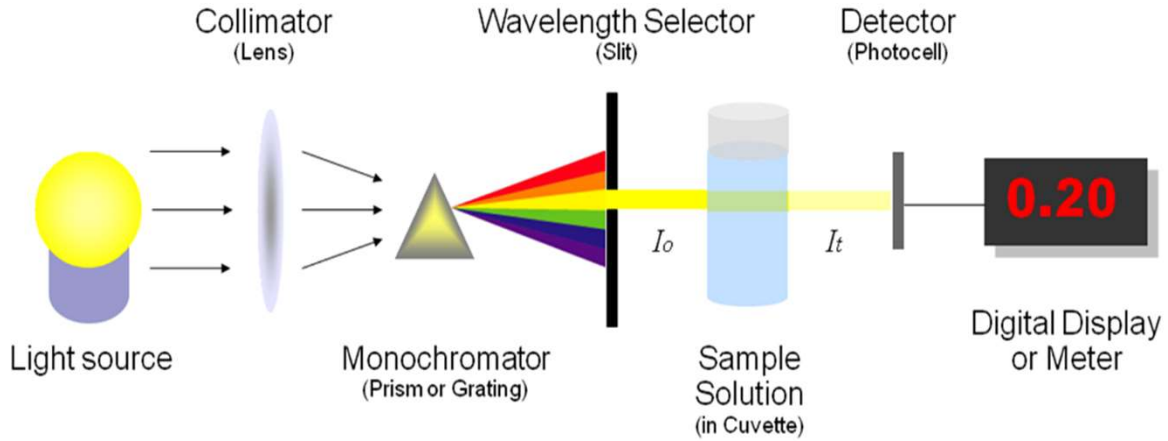


Figure 2. Basic structure of spectrophotometers.

- A nice video that show you how dose spectrophotometer work:
  http://www.youtube.com/watch?v=pxC6F7bK8CU

Spectrophotometry cont':

- The absorption phenomenon is described by *Beer-Lambert law* ***as:***

$$A_\lambda = \varepsilon.l.c$$

- A_λ= is the absorbance at specific wavelength, ε= extinction (absorption) coefficient , l= length of the light path through the solution, c= concentration of the absorbing substance.

- A plot of absorbance (A) against the wavelength (λ) is known as ***Absorption spectrum.***

Absorption spectrum:

- ***Definition:*** The curve that display the action or behaviour of absorption of molecule [chromophore] at different wavelengths (λ).

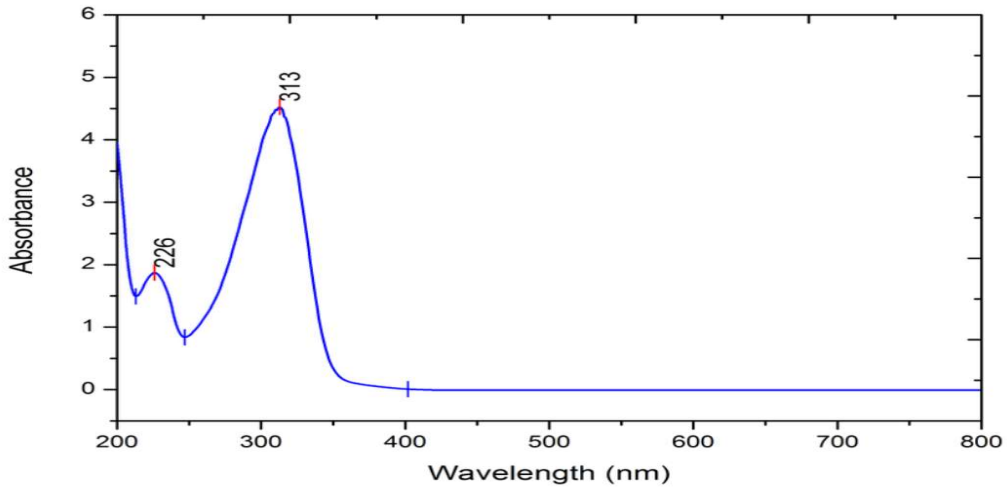


Figure 3. Absorption spectrum for a molecule.

- Every molecule has its own absorption spectrum, So it's considered as fingerprint for each molecule.

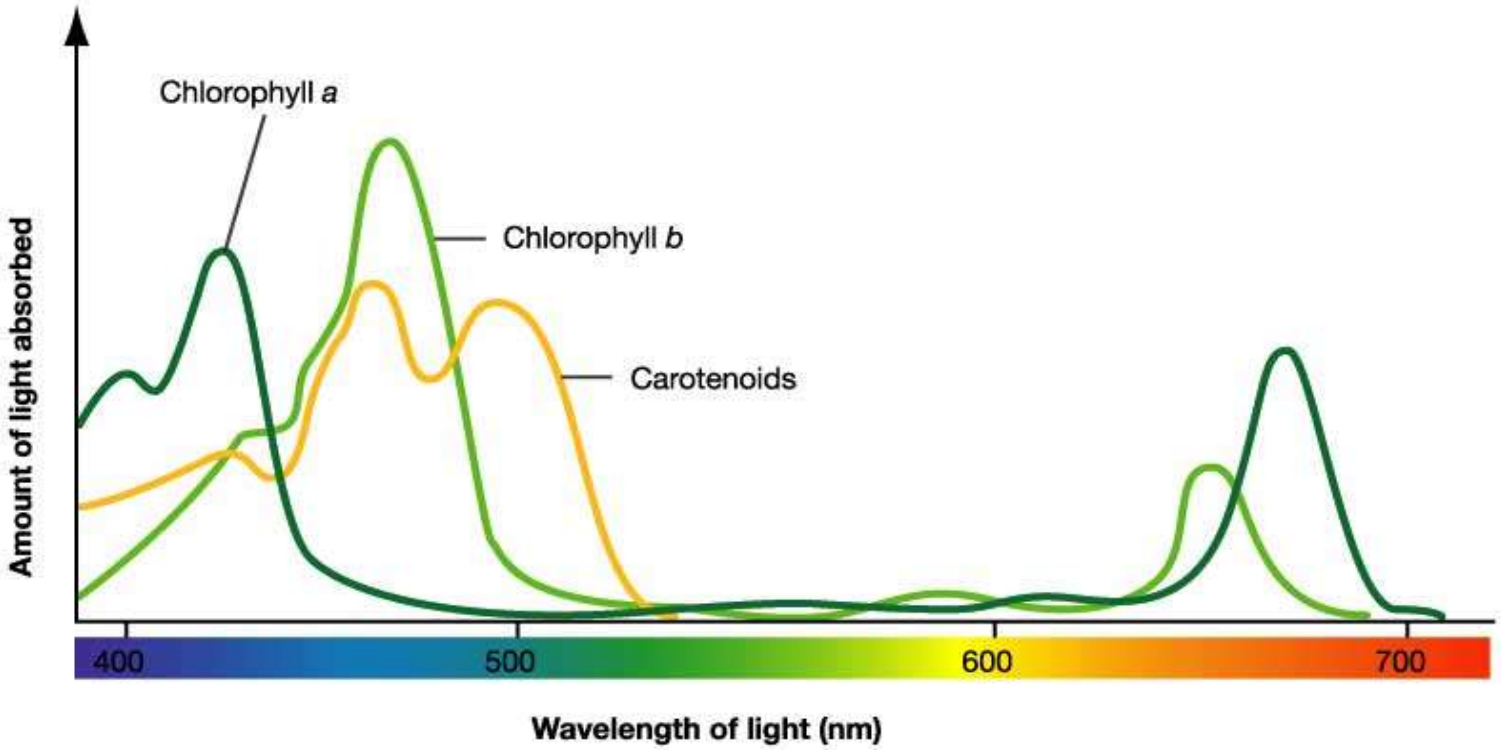
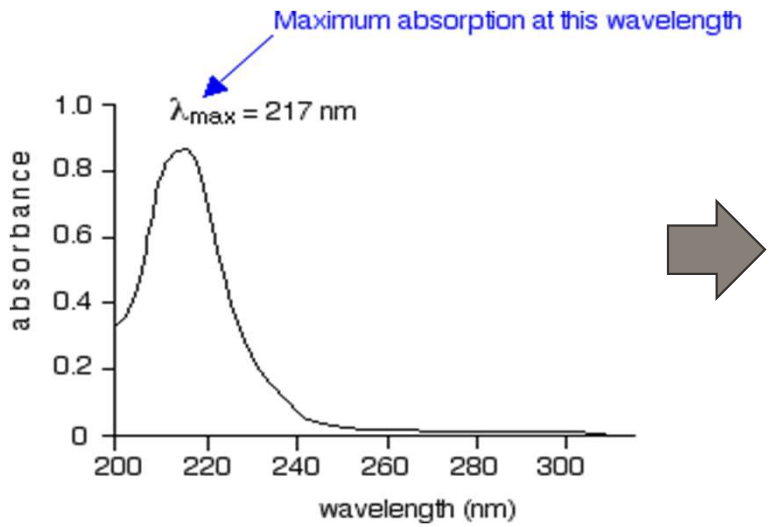


Figure 4. Absorption spectrum curve for different molecules.

λmax:

- ***Definition:*** it is the wavelength, at which the molecule has ***the maximum absorbance,*** and it can be determined from absorption spectrum curve.

[The wavelength of maximum absorption (λmax)]



You will see that absorption peaks at a value of 217 nm

Figure 5. Determination of λmax from the absorption spectrum.

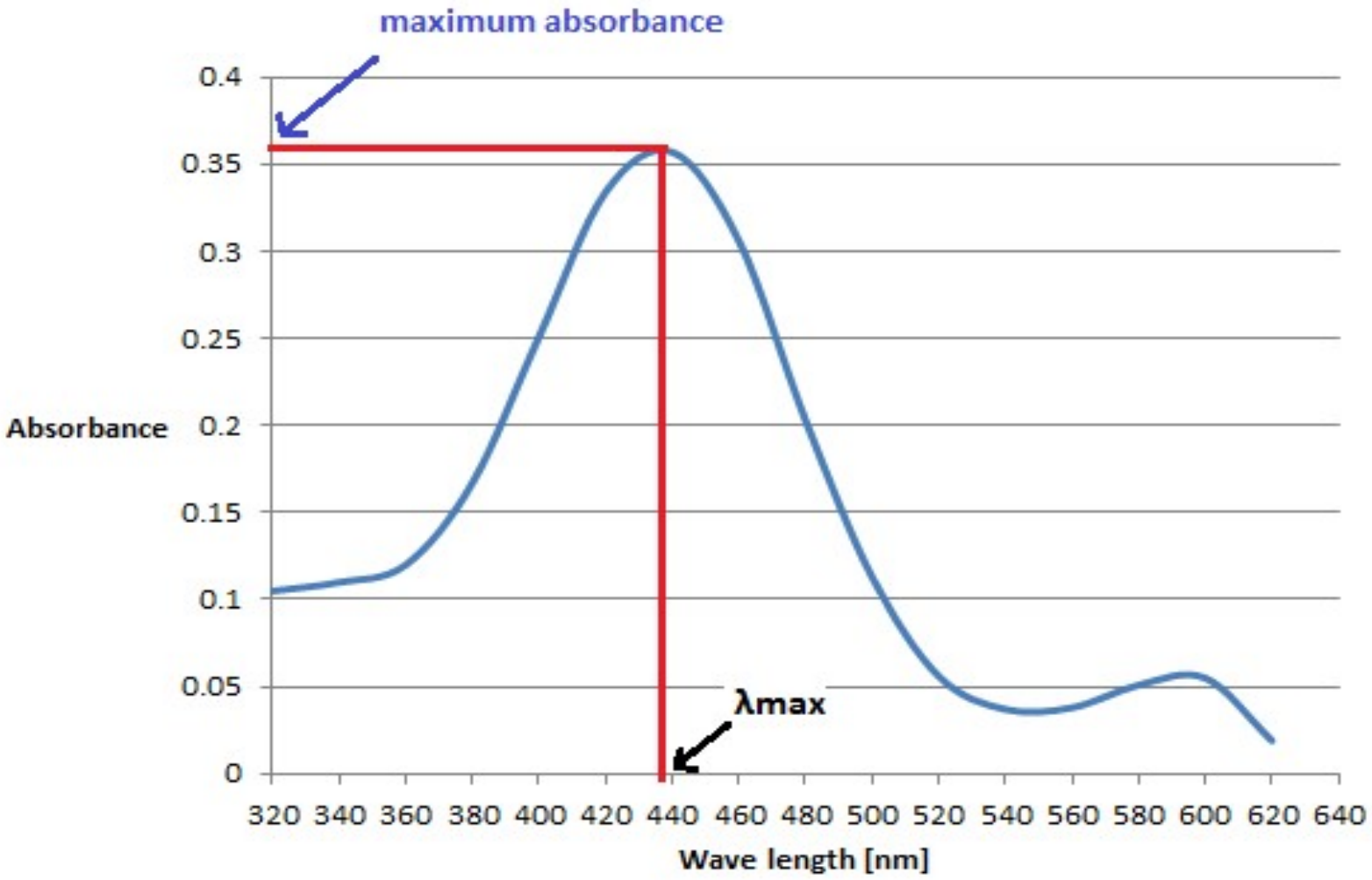


Figure 6. Absorption spectrum curve and determination of λmax.

Absorption spectrum cont':

- The absorption spectrum for a chromophore is affected by:
  1. Its structure.
  2. Environmental factors: pH, solvent polarity, orientation.

- These effects mean that it is essential to determine the absorption spectrum under defined conditions of pH and solvent compositions.

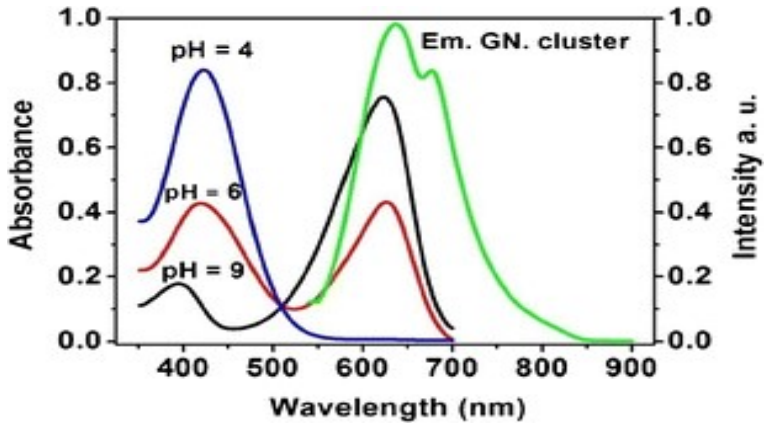


Figure 7. Absorption spectra of bromothymol blue at different pH.

Application of Absorption Spectrum:

- ***What is the benefit of studying the absorption spectrum :***

➔ Determine λ_{max}.

➔ Used to identify substances.

➔ It also can be used to know if there is any contamination with another molecule.

Example of how contamination affect the absorption spectrum of a chromophore

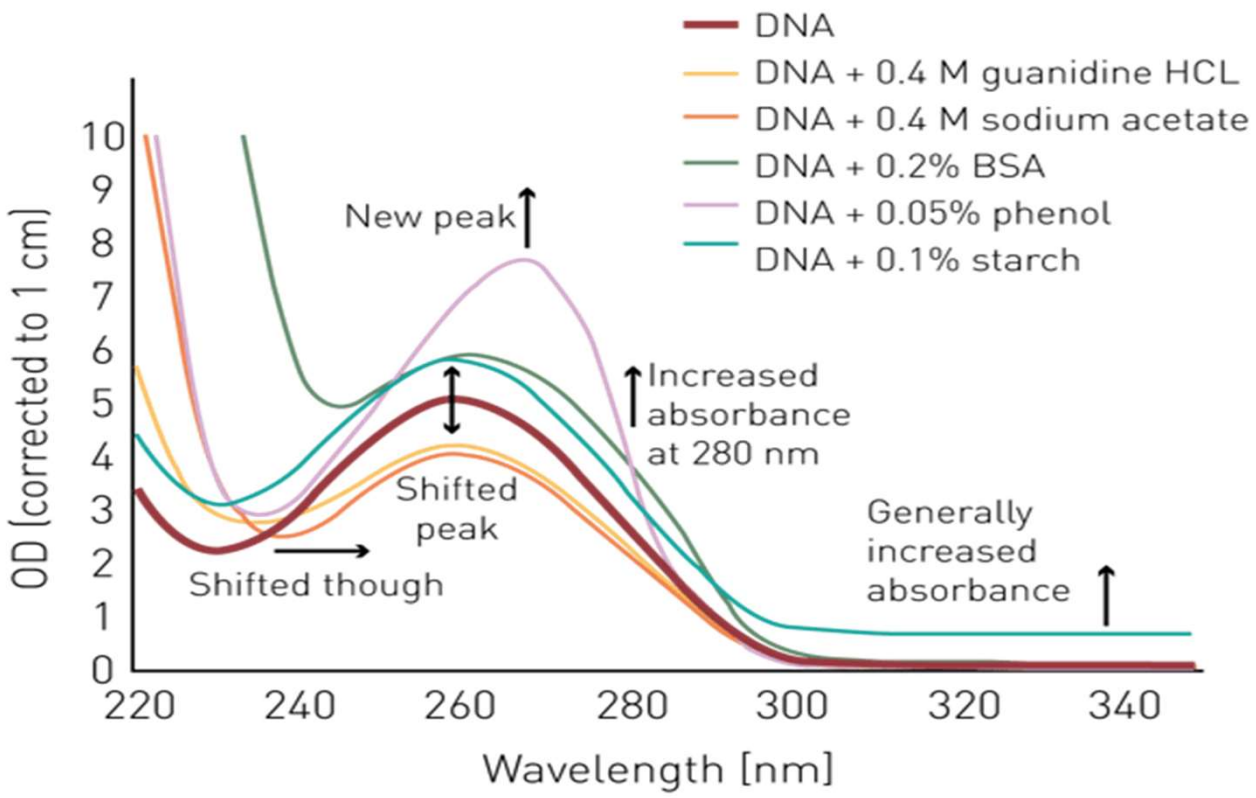


Figure 8. Absorbance spectra of DNA samples, pure or with potential contaminants.

Spectrophotometry for quantitative analysis:

- Spectrophotometry is widely used for quantitative analysis in various areas (e.g., chemistry, physics, biology, biochemistry, engineering, clinical applications, industrial applications, etc).

- By using the spectrophotometer, we can ***quantitatively measure absorbance***, and this information can be used to determine the concentration of the absorbing molecule [concentration of unknown sample].
- According to Beer-Lambert law:

More concentrated solution will ➔ ***absorb more*** light and ***transmits less***.

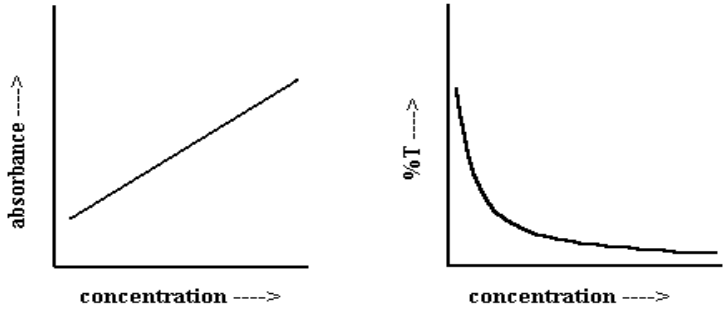


Figure 9. Graphs show the relationship between %T and concentration (right graph) and the relationship between absorbance and concentration (left graph).

Spectrophotometry for quantitative analysis cont' :

1. Beer-Lambert law:

➔ The Beer-Lambert law describes the relation of absorbance, path length and concentration of an absorbing substance:

$$A_\lambda = \varepsilon.l.c$$

- A_λ= is the absorbance at specific wavelength, ε= extinction (absorption) coefficient , l= length of the light path through the solution, c= concentration of the absorbing substance.

- There is a linear relationship between ***absorbance*** and ***concentration*** of an absorbing species.

➔ ***So, what does standard curve of concentrations mean?***

Spectrophotometry for quantitative analysis cont':

2. Standard curve for concentrations:

- ***Definition:*** It is a graph that shows the relationship between different known concentrations of a substance and the absorbance at a specific wavelength.

- Standard curves are commonly used to ***determine the concentration*** of a substance, using serial dilution of solutions of known concentrations called ***Standard Solutions.***

➔So, Standard Solution (reference) defined as a solution containing a precisely known concentration of an element or a substance.

- Based on the linear relationship between the ***concentration*** and the ***absorbance*** value at specific wavelength, the unknown concentration of a sample could be determined, by comparing the light absorbed by unknown with that of a standard solutions in the standard curve.

$$[X] \alpha A$$

➔Where ***[X] is the concentration of the unknown sample*** and ***A is absorbance***.

HOW?

- If the unknown concentration of a solution has absorbance value =0.45, the conc. From the curve will be …… ?

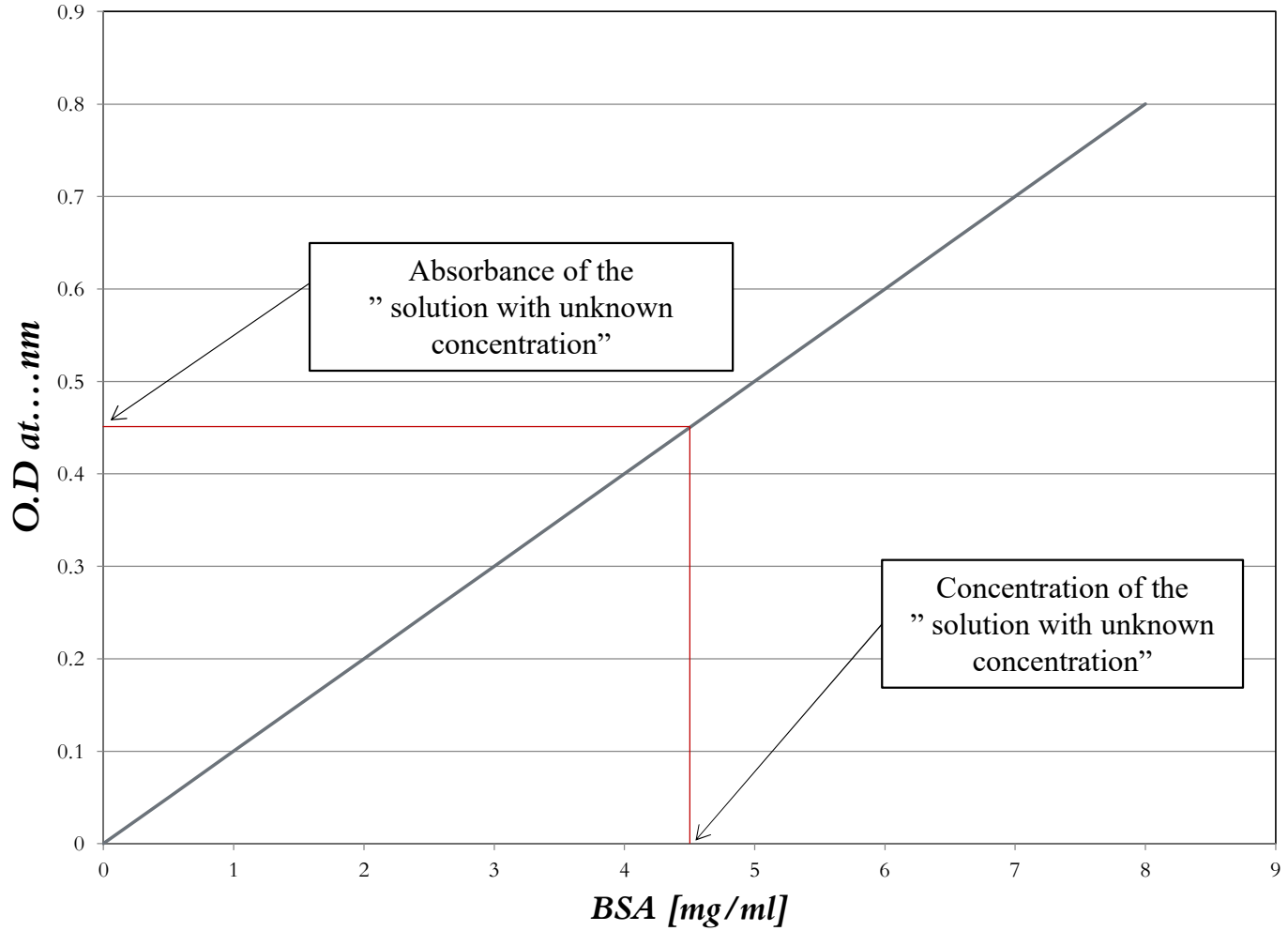


Figure 10. BSA standard curve of concentration

So, from the curve the concentration of the unknown is about 4.5 mg/ml.

Summery

To determine the concentration of a solution with "an unknown concentration":

From standard curve of concentration:

➔Measure the absorbance of the "solution with unknown concentration" in order to determine the concentration, from the curve.

Using Beer-Lambert law:

➔Using available information of any standard solution to determine the "ε", then using these information to get the unknown concentration. By: $A_\lambda = \varepsilon lc$

*Note: "ε" will change when the wavelength changed.

Calculating the unknown concentration by Beer-Lambert Law:

Example:

Guanosine has a maximum absorbance of 275 nm. $\varepsilon_{275}=8400\ M^{-1}cm^{-1}$ *and the path length is 1 cm. Using a spectrophotometer, you find that* $A_{275}=0.70$*. What is the concentration of guanosine?*

To solve this problem, you must use Beer's Law:

$$A_\lambda = \varepsilon.l.c$$

$$0.70 = 8400\ M^{-1}\ cm^{-1}.1\ cm.\ c$$

➔ $c= 8.33 \times 10^{-5}$ mol/L

Practical part

OBJECTIVES:

- Drawing the ***absorption spectrum*** of bromophenol blue at pH= 2.4 and determination of ***λmax.***
- Determination the concentration for unknown concentration of bromophenol blue using a ***standard curve.***

1
Absorption spectrum

METHOD:

1. Take a test tube and add the following reagents:

Reagent	*Volume (ml)*
0.1 M citrate buffer, pH 2.4	9.0
7.5 x 10^{-5} M bromophenol blue	0.2
95% ethanol	0.8

2. Mix and measure the absorbance of the solution from 320 to 620 nm at 20 nm intervals, using a scanning spectrophotometer, against a water blank.

*Note: Use suitable cuvettes at sets of wavelengths.

:RESULTS

Wavelength (nm)	*Absorbance*
320	
340	
360	
380	
400	
420	
440	
460	
480	
500	
520	
540	
560	
580	
600	
620	

- Plot a graph of absorbance against wavelength (absorption spectrum curve).
- From the graph (absorption spectrum curve), determine the ***λmax*** for bromophenol blue at pH 2.4.

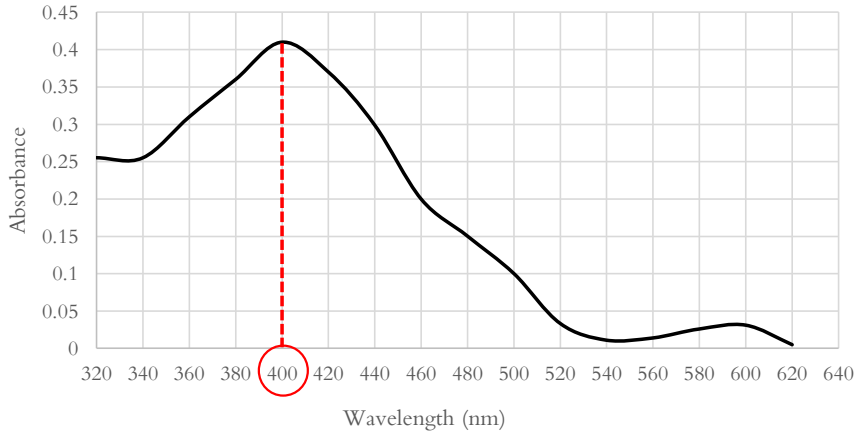


Figure 11. Absorption spectrum curve of substance X and determination of λmax.

2
Standard curve and determination of unknown concentration

METHOD:

1. Set up 7 test tubes as following:

Tube No.	*0.1 M citrate buffer [pH 2.4] (ml)*	*7.5x10 $^{-4}$M bromophenol blue (ml)*	*95% ethanol (ml)*	*Unknown sample (ml)*	*Molar concentration of bromophenol blue x 10 $^{-5}$*
1	9 . 0	0.1	0 . 9	-	0.75
2	9 . 0	0.2	0.8	-	1.5
3	9 . 0	0.4	0.6	-	3
4	9 . 0	0.6	0.4	-	4.5
5	9 . 0	0.8	0.2	-	6
6	9 . 0	1.0	-	-	7.5
Unknown	9 . 0	-	-	1.0	*?*

Standard Solutions
C1 x V1 = C2 x V2

Unknown sample From the standard curve

2. Mix and measure the absorbance of all the tubes at 430 nm against a water blank.

RESULTS:

Tube No.	*Molar concentration of bromophenol blue x 10 - 5*	*Absorbance at 430 nm*
1	0.75	
2	1.5	
3	3	
4	4.5	
5	6	
6	7.5	
Unknown	?	

- Plot a standard curve of absorbance against molar concentration of bromophenol blue.
- From the curve determine the molar concentration of the unknown (using TREND formula).

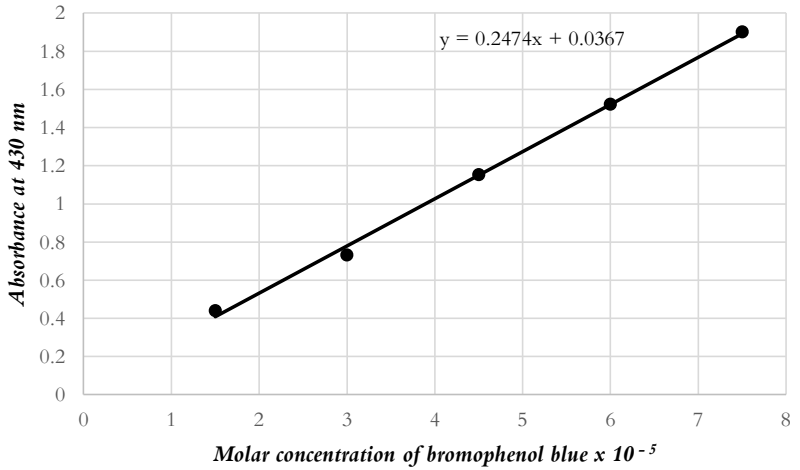


Figure 12. Bromophenol blue standard curve of concentration

Spectrophotometry Introduction:

http://www.youtube.com/watch?v=qbCZbP6_j48

Spectrophotometry for quantitative analysis :

http://www.youtube.com/watch?v=VqAa_cmZ7OY&feature=relmfu