Experiment-1

Fundamentals of Spectrophotometer

Assay of tetracycline by calibration curve method

Outcomes: -

After completing this experiment, the student should be able to:

1. Prepare standard solutions of tetracycline.
2. Construct calibration curve based on Beer's Law.
3. Use Beer's Law to determine molar absorptive.
4. Explain the fundamental principal behind spectrophotometric analysis

Introduction: -

Most analytical methods require **calibration** {a process that relates the measured analytical signal to the concentration of **analyte** (the substance to be analyzed)}, the three most common analysis methods include the preparation and use of a **calibration curve**, the **standard addition method**, and the **internal standard method**. In this experiment we will use spectrophotometer to prepare a calibration curve for the quantitative analysis of tetracycline.

Spectrophotometer: -

It is a technique that uses the absorbance of light by an analyte at a certain wavelength to determine the analyte concentration, UV/VIS (ultra violet/visible) spectrophotometer uses light in UV and visible part of the electromagnetic spectrum, light of this wavelength is able to effect the excitation of electrons in the atomic or molecular ground state to higher energy levels, giving rise to an absorbance at wavelengths specific to each molecule, the complex formed between tetracycline and Fe^{3+} is intensely orange-brown colored, and therefore can be determined by spectrophotometer using the visible part of the electromagnetic spectrum.

The human eye is able to "see' light in the wavelength range 400-700 nm (nanometer or 10^{-9} m), to the human eye, wavelength appears as color, as shown in the following table: -

Wavelength, nm	Color	Complementary color
400-430	Violet	Yellow-green
430-480	Blue	Yellow
480-490	Green-blue	Orange
490-500	Blue-green	Red
500-550	Green	Purple
550-575	Yellow-green	Violet
575-590	Yellow	Blue
590-625	Orange	Green-blue
630-700	Red	Blue-green

Table: Correlation between wavelength, color, and complementary color

When we see an object as purple, in fact it absorbs light in the "green" region of the spectrum: only blue and red wavelengths reach the eye, which we experience as the purple color, the ASA-Fe^{3+} complex formed in the analysis of aspirin absorbs wavelengths in the blue, green and yellow regions of the spectrum, as shown in figure 1. Only violet and red light are "transmitted", as a result, the human eye sees the transmitted light as a mixture of violet and red, which we experience as a deep purple, a spectrum shows how much light is absorbed for a range of wavelengths, in UV/VIS spectrophotometer we plot the **Absorbance** (**A)** of a solution against the wavelength of the light reaching the solution (**λ**), this is called the **absorption spectrum**, the higher the analyte concentration, the more light at a certain wavelength will be absorbed, the relation between Absorbance **A** and analyte concentration **C** is given in equation (Beer's Law):- $\mathbf{A = \varepsilon\, l\, C}$

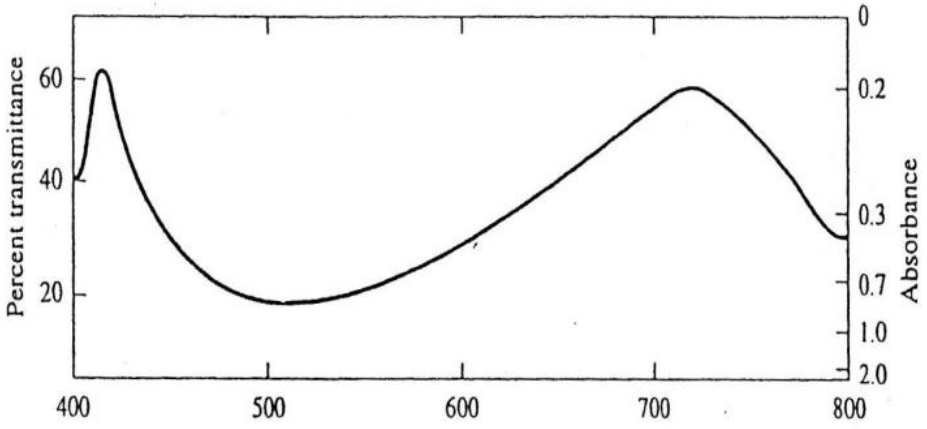


Figure 1: The absorption spectrum of the Fe^{3+}-salicylate complex.

In Fig.1note that Transmittance percentage (**T%**) is plotted on the left (linear scale), Absorbance (**A**) on the right (logarithmic scale), the absorbance maximum is at approximately 510 nm (λ_{max}), the spectrophotometer takes the place of the human eye by accurately measuring the intensity of the light transmitted at each wavelength, for spectrophotometer in the visible region the **light source** is a common tungsten light bulb emitting "white" light, the light is collimated and focused on an entrance slit (via a system of mirrors) the light falls on a **monochromator**, that separates the white light in its constituent wavelengths, the monochromator can be a **glass prism**, but in modern instruments it will be a **grating**, from the monochromator the light is sent through the sample holder a **cuvette** , and finally reaches a **photocell (detector)** that measures the intensity of the light at each specific wavelength, and recorded as absorbance **A** or the transmission **T** (or **%T**) vs (**λ**) or (**C**) by output (**readout)**.

Spectrophotometers have many different designs, in the simplest instrument (a single beam) for each wavelength **λ** set by the monochromator, the user first inserts

a **blank** solution in place of the sample a **cuvette**, the photocell then records the intensity at that wavelength, this intensity is called $\mathbf{I_o}$, next the blank solution is replaced by the sample solution, the photometer measures the new intensity called **I**, the transmittance (**T**) is then displayed on the screen or spectrophotometer output:

$$\mathbf{T = \frac{I}{I_o}} \quad \textbf{or} \quad \mathbf{T\% = \frac{I}{I_o} x100}$$

This procedure is then repeated for a number of wavelengths; more sophisticated instruments "scan" the spectrum over the required wavelength range automatically, and record the transmittance as a function of wavelength, in a **double beam** scanning spectrophotometer, part of the light is reflected to a separate blank cell (intensities "$\mathbf{I_o}$") and sample cell (intensities "**I**") are measured simultaneously at each wavelength, and automatically compared to yield a direct output of **T** vs **λ**, another modern form of the UV/VIS spectrophotometer is the "Diode Array" spectrophotometer, in this instrument the photocell, which measures light intensity at one wavelength at a time, is replaced by a CCD detector (**charge-coupled device**) similar to the detector in your digital camera, and the instrument can record the spectrum over the full wavelength range (typically 200−700 nm) within one second!

In each fractional layer of the sample the intensity of the incoming light will decrease proportionally to the concentration of the analyte, as a result, the Transmittance (**T)** decreases exponentially with increasing path-length **l**, this is expressed in the **Beer Lambert law**, which states**:**

$$\mathbf{-\log \frac{I}{I_o} = \varepsilon\, l\, C}$$

ε is called the "Molar Absorptivity" absorption factor of the compound, it is a function of wavelength specific for each molecule, with the path-length **l** normally given in cm, and **C** in Molarity units, mol/L **ε** has the units $L.mol^{-1}cm^{-1}$, if alternatively **C** is in mol/mL, then **ε** will have the units cm^2mol^{-1}. The Absorbance (**A)** is defined as:- $\mathbf{A = -\log \frac{I}{I_o}}$

Thus, the Beer-Lambert law, more commonly called **Beer's law**, can be written as**:** $\mathbf{A = \varepsilon\, l\, C}$ Finally, the relation between **A** and **T** are given by:

$$\mathbf{A = -\log T}$$

Note that **A** is a dimensionless quantity, because **A** is directly proportional to the analyte concentration, it is more often used than **T** or **T%,** most spectrometers can record either **A, T, or T%** for a given wavelength.

The absorption spectrum:-

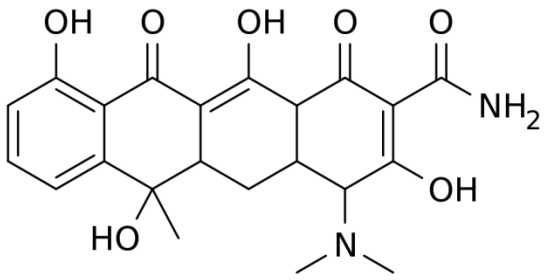
We place a cuvette filled with sample solution in the cell holder (blank solution than sample solution), the instrument will record light intensity and absorbance relative to the light intensity passes through blank solution alone (the Absorbance to zero), this must be repeated at different wavelengths to obtain the spectrum of the dissolved solute (**A** vs. **λ**) **absorption spectrum**, from **absorption spectrum** we find the wavelength with the highest absorbance, the wavelength of the absorption peak (λ_{max}), at this wavelength the spectrophotometric method is most sensitive for the analyte, next we determine the absorbance (**A**) at λ_{max} for a number of standard solutions of different concentration.

The calibration curve method:-

To use the calibration curve technique, several standards containing exactly known concentrations of the analyte are introduced into instrument, and the instrumental response is recorded, ordinarily this response is corrected for instrument output obtained with a **blank**, ideally, the **blank** contains all of the components of the original sample except for the analyte, always starting with the lowest concentration, from these absorbance values, the resulting data are then plotted to give a graph of corrected instrument response versus analyte concentration, and this graph (**calibration curve**) in turn can be used to find the concentration of an unknown.

Materials and Equipment:-

UV/VIS spectrophotometer and polystyrene cuvettes or quartz cuvettes (Absorption cells), pipette, 10mL graduated cylinders, 250, 100, 50& 25mL volumetric flasks**,** beakers, Ferric chloride ($FeCl_3$), Hydrochloric acid (HCl) and tetracycline hydrochloride (TC).



Tetracycline (TC)

Procedure:-

Preparing the stock solution and standard solutions:-

1. Preparation of 250mL of (0.01N) HCl: Accurately transfer 25mL of (0.1N) HCl to volumetric flask (250mL) and fill to mark with D.W. How to prepare (0.1N) HCl from the concentrate solution of HCl?(H.W.)
2. Preparation of 50mL of (0.1%) $FeCl_3$: Accurately weigh 0.05g of solid $FeCl_3$, transfer quantitatively to a 50mL volumetric flask and fill to the mark with (0.01N) HCl, (**Fresh**).

3. Preparation of 100 mL of a stock standard solution of 0.25g/L (TC): Accurately weigh 25mg solid TC, Transfer quantitatively to a 100 mL volumetric flask and fill to the mark with (0.01N) HCl.
4. Prepare solution in 25mL volumetric flask, than fill to the mark with D.W., as following:-

Type of Soln.	Vol. of stock Soln. (mL)	Vol. of (0.1%) $FeCl_3$ Soln. (mL)	Vol. of (0.01N) HCl Soln. (mL)	Concentration (mg)	Absorbance (A)
Blank	**0**	**5**	**5**		
No.1	**1**	**5**	**4**		
No. 2	**2**	**5**	**3**		
No. 3	**3**	**5**	**2**		
No. 4	**4**	**5**	**1**		
Unknown 5mL	**0**	**5**	**0**		

Table (1) for calibration curve

Measuring the absorption spectrum and determining λ_{max}:-

This part of the experiment each pair of students should record all absorbance at each wavelength and draw the absorption spectrum.

1. Rinse one of the cuvettes with blank solution, put the cuvette in the sample compartment, this is the reference solution, set the wavelength to 400 nm, then set the Absorbance to zero.
2. Rinse a second cuvette standard solution No.4, place the cell in the sample compartment, measure the Absorbance at 350 nm and record in your notebook.

3. Repeat this procedure (steps 1 and 2 above) for the two cuvettes at wavelengths 360,370, 380, 390, 400, 420, --.600 nm, first setting A = 0 for the cuvette with blank, then measuring A for the cuvette with solution No.4, recording the absorbance at each wavelength, record in data table (at absorbance begin larger reduce wavelength intervals to 5nm).

Prepare a graph of absorbance (**A)** vs. wavelength (**λ)** and determine λ_{max} (maximum wavelength). Attach this graph to the lab report, (*Plotting* Use the program Excel to plot the absorption spectrum and determining λ_{max}).

The calibration curve:-

This part of the experiment must be done by each pair of students separately.

1. Set the wavelength at (λ_{max}), place the cuvette with blank in the cell compartment and again set the Absorbance to zero.
2. Measure and record the Absorbance of each of the four standard solutions & unknown, starting with the most dilute standard, after each measurement, rinse the cuvette with the next standard, not with blank!

Draw a plot having X-axis as concentration (mg/L) and Y-axis as Absorbance at λ_{max} (*Plotting* Use the program Excel to plot the calibration curve**).**

3. Use Beer's law to calculate **ε** for TC, given the cell width (path length l) to be 1 cm.
4. Use calibration curve to calculate concentration of unknown solution.
5. Find application for calibration curve equation, to calculate concentration of unknown solution?