PRINCIPLES AND APPLICATIONS OF SPECTROPHOTOMETERY

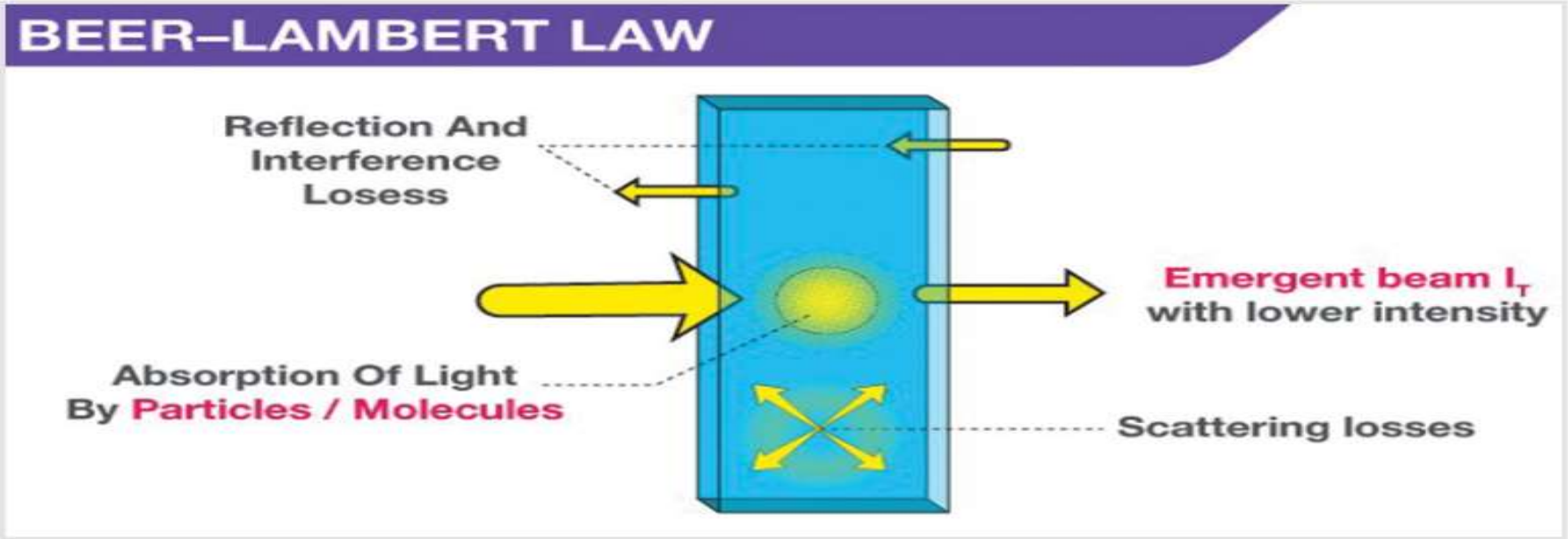
PRINCIPLES AND APPLICATIONS OF SpectrophotometerY

- Beer-lambert’s Law
- Absorption spectrum
- Spectrophotometer- Visible and UV spectrophotometer

- Introduction
- Principle
- Instrumentation
- Applications

What is the Beer-Lambert Law?

- The Beer-Lambert law, also known as Beer's law, the Lambert-Beer law, or the Beer-Lambert–Bouguer law relates the attenuation of light to the properties of the material through which the light is traveling.
- When a monochromatic light of initial intensity Io passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light I is less than Io.
- There is some loss of light intensity from scattering by particles in the solution and reflection at the interfaces, but mainly from absorption by the solution.
- The relationship between I and Io depends on the path length of the absorbing medium, l, and the concentration of the absorbing solution, c. These factors are related in the laws of Lambert and Beer.

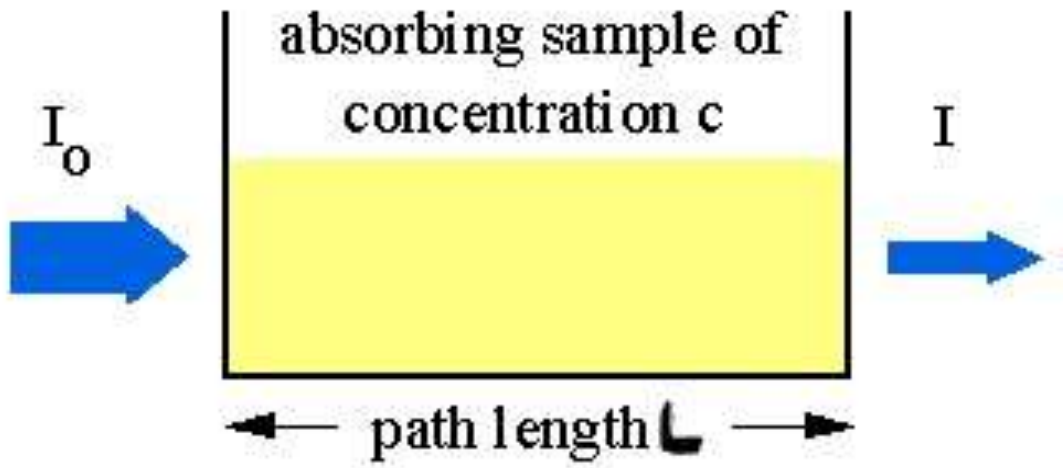


- The Beer-Lambert law is a linquisimal relation between the absorbance, the concentration of the solution, its molar absorption coefficient, and the optical coefficient in a solution.

  $A = \varepsilon * L * c$ or $A = \varepsilon * b * c$

  Where,
- A is the amount of light absorbed for a particular wavelength by the sample
- ε is the molar extinction coefficient
- L / b is the distance covered by the light through the solution
- c is the concentration of the absorbing species
- The absorption coefficient of molar is a property that depends on the sample that measures how powerful an absorber the sample is when it comes to the wavelength at which it is. The concentration is one mole (M) in the solution of the material dissolving in the solution. it is measured by the length of the cuvette that is used to measure absorbance, which typically is 1 centimeter.

- Absorption of light by a sample;



Instrumentation of Beer-Lambert Law

- The majority of measurements in experimental research are done using the term transmittance (T) which is described as:

$$T = I / Io \quad \text{or} \quad = (I_t / Io)$$

Io = Intensity of initial light

I_t = Intensity of transmitted light

- in which I represents the intensity of light that it reaches after passing through the sample, and Io is the light intensity. The relationship between T and A is:

$$A = -\log T = -\log (I / Io) \quad \text{or} \quad -\log (I_t / Io)$$

$$A = \log(I_o/I) = \log(1/T) = \varepsilon cL$$

Where

[L] = 1 cm,

[c] = 1 mol /dm^3

[ε] = 1 dm^3 /mol/ cm

ε is the molar absorption coefficient (also molar extinction coefficient).

A is the absorbance of the sample, which is displayed on the spectrophotometer.

- The Beer-Lambert law declares the existence of a linear relation between the amount of solution and its absorbance that allows that the amount of substance to be determined by taking the measurement of its absorbance. To show this linear dependence, the absorbance of five different solution solutions for Rhodamine B in water were determined by using the DS5 Dual Beam Spectrophotometer (Figure a) and from the absorption spectra, an linear calibration curve for the absorption versus the concentration was drawn (Figure b). By using this calibration curve, to determine the amount of an unidentified Rhodamine B solution can be measured by measuring its absorbance which is the primary purpose in this law

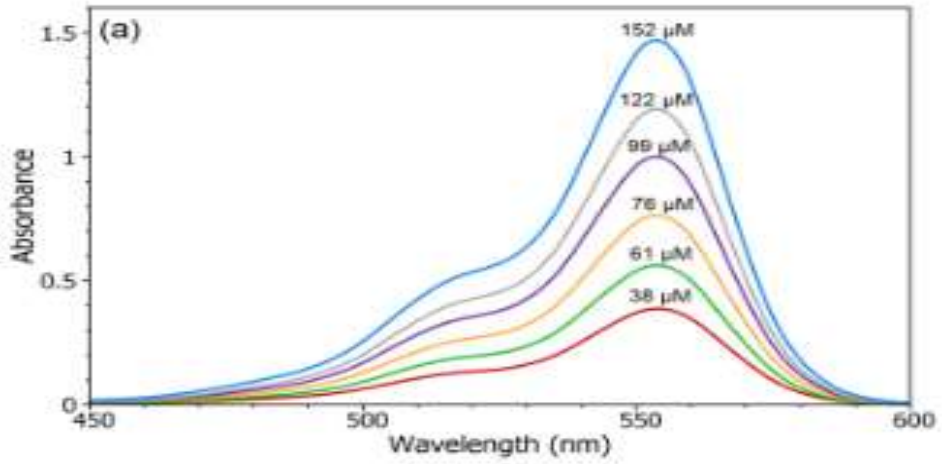


Figure: (a): Absorption spectra of Rhodamine B solutions with different concentrations in water measured using the DS5 Dual Beam Spectrophotometer. | Source: www.edinst.com

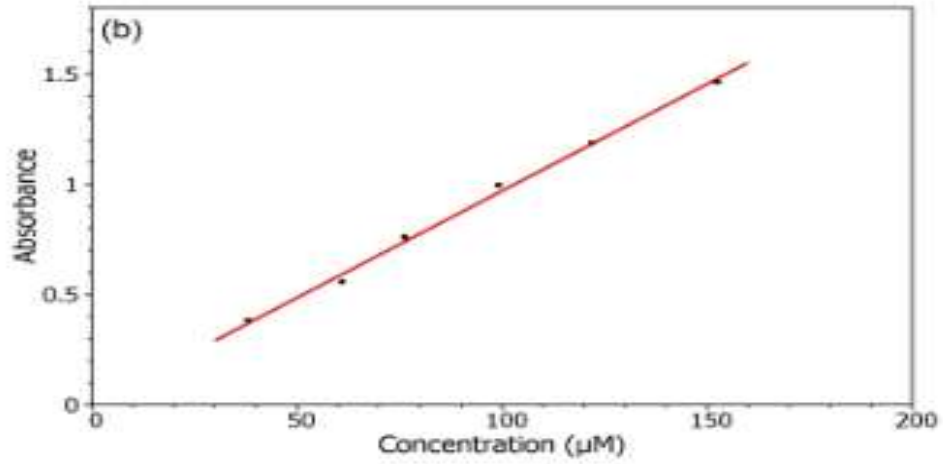


Figure: (b) Calibration curve of Rhodamine B in the water at measured at λmax. | Source: www.edinst.com

- Modern absorption instruments typically display the data in one of three ways: transmittance, %-transmittance or absorbance. A concentration that is not known for an analyte is determined by taking measurements of the quantity of light the sample absorbs, and then employing Beer's law. If the absorptivity coefficient of the sample is not known, the undetermined concentration can be determined by using the absorption curve against concentration calculated from standards.

Limitations of Beer-Lambert law

- The linearity of the Beer-Lambert law is limited by chemical and instrumental factors.
- Causes of non-linearity of the law occur in the following conditions:
- deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity
- scattering of light due to particulates in the sample
- fluoresecence or phosphorescence of the sample
- changes in refractive index at high analyte concentration
- shifts in chemical equilibria as a function of concentration
- non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band

Applications of Beer-Lambert Law

1. Application in Analytical chemistry

This study focuses on its separation and quantification and characterization of matter through the spectrophotometry. It does not require lengthy pre-processing of the sample to obtain the result. For example, the count of bilirubin in blood samples can be measured by using the spectrophotometer.

2. Application In atmosphere

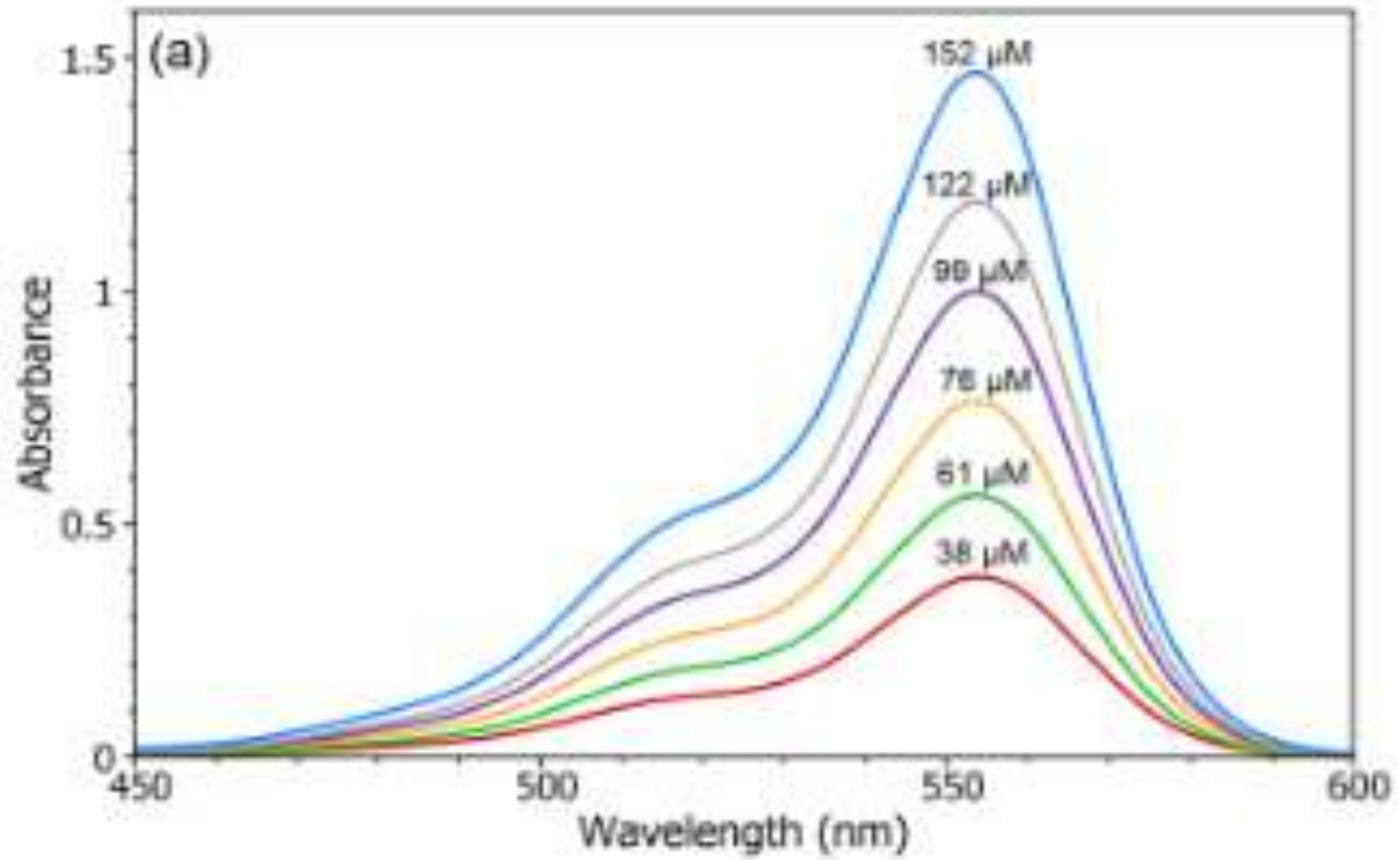
The solar or stellar radiation that occurs in the atmospheric layer can be described with this law.

ABSORPTION SPECTRUM

The absorption spectrum is the plot of absorbance versus the wavelength (or the frequency). The wavelength of maximal absorption λ_{max} is the wavelength for which a maximum is observed in the absorption spectrum and at which the sample possesses a corresponding molar extinction coefficient of

$$\varepsilon_{max} = A_{max} /(c \times L)$$

These wavelengths and molar extinction coefficients can allow identification of a given compound in a given solvent. However, such identification is rather limited and qualitative analysis is only possible for systems where appropriate features and parameters are known, such as identification of certain classes of compounds both in the pure state and in biological mixtures (e.g. protein-bound). Most commonly, this type of spectroscopy is used for quantification of biological samples either directly or via colorimetric assays. In many cases, proteins can be quantified directly using their intrinsic chromophores, tyrosine and tryptophan. Protein spectra are acquired by scanning from 500 to 210 nm. The characteristic features in a protein spectrum are a band at 278/280 nm and another at 190 nm. The region from 500 to 300 nm provides valuable information about the presence of any prosthetic groups or coenzymes.


(a)
152 µM
122 µM
99 µM
76 µM
61 µM
38 µM
Absorbance
1.5
1
0.5
0
450
500
550
600
Wavelength (nm)

Absorption spectroscopy

Absorption spectroscopy is a spectroscopic technique that measures the frequency of wavelength of absorbed light as a result of the interaction between the light and a sample.

Principle of Absorption spectroscopy

- Absorption spectroscopy is based on the principle that materials have an absorption spectrum which is a range of radiation absorbed by the material at different frequencies.
- The absorption spectrum of materials depends on the atomic and molecular composition of that material.
- The frequency of light radiation absorbed by a material is dependent on the energy difference between the two energy states of the molecules.
- The absorption results in the formation of an absorption line, which, together with other lines, form an absorption spectrum.
- Thus, when a photon with sufficient energy reaches an object, the energy is absorbed by the electrons causing them to bump into a higher energy state.
- The amount of photon (radiation) absorbed results in an absorption spectrum which can then be measured in terms of absorbance.
- The absorbance of a sample is dependent on the number of excited electrons which in turn is dependent on the concentration of molecules in the sample.

Steps of Absorption spectroscopy

- Solvent liquid and the sample solution are taken in two transport vessels, also termed as cuvettes.
- The vessel with solvent liquid is then placed in the spectrometer to determine the light loss due to scattering and absorbance by the solvent. Any absorbance observed in this process is to be subtracted from the absorbance of the sample.
- The cuvette with the sample solution is then placed in the spectrometer.
- The absorbance of the sample is noted in different frequencies which usually ranges from 200-800 nm.
- A similar spectrum is formed from a different concentration of the samples.
- A graph of the absorbance measured against the concentration of the sample is plotted, which can then be used for the determination of the unknown concentration of the sample.

Uses of Absorption spectroscopy

- Absorption spectroscopy is used to determine the presence of a particular substance in a sample and then to quantify the present substance.
- This technique has been applied in remote sensing, which allows the determination of the concentration of hazardous substances without the direct interaction of the instrument and the sample.
- Absorption spectroscopy is also used for the determination of the atomic and molecular structure of various substances.

What is a spectrophotometer?

•A spectrophotometer is an instrument that measures the amount of light absorbed by a sample.

•Spectrophotometer techniques are mostly used to measure the concentration of solutes in solution by measuring the amount of the light that is absorbed by the solution in a cuvette placed in the spectrophotometer.

•Scientist Arnold J. Beckman and his colleagues at the National Technologies Laboratory (NTL) invented the Beckman DU spectrophotometer in 1940.

UV-Visible spectrophotometer

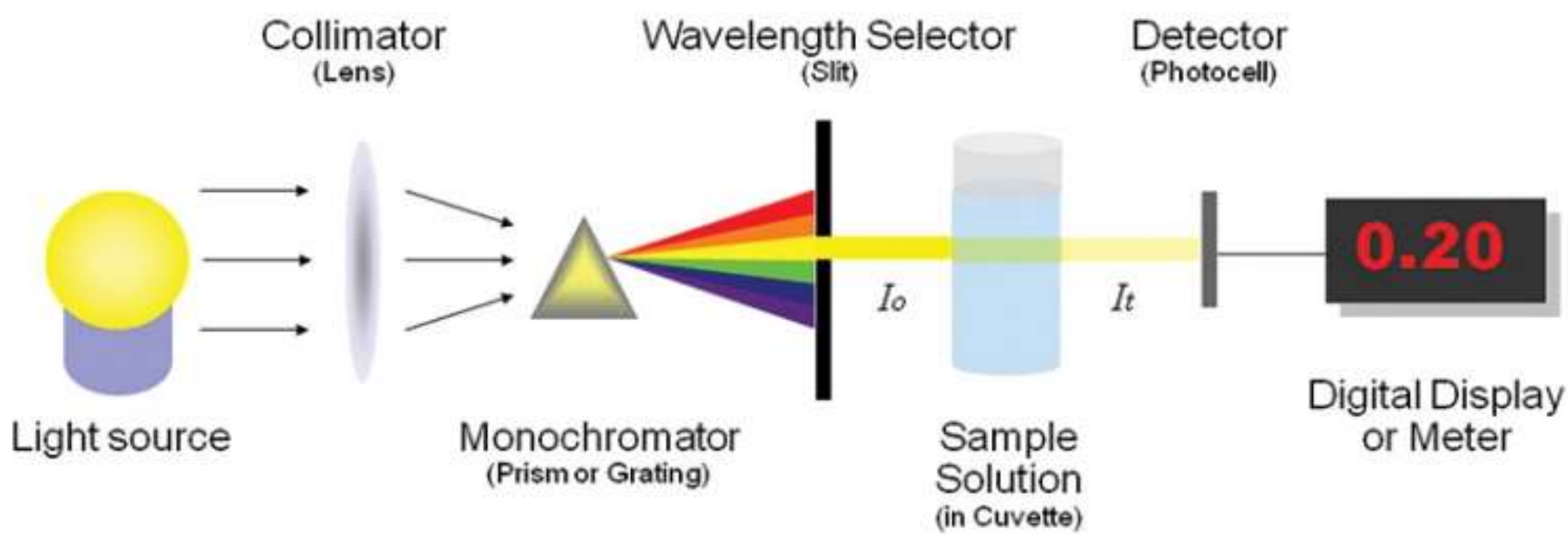
- UV- Visible spectrophotometer is equipment based on UV-Visible spectroscopic technique.
- UV-Vis spectroscopy is an analytical technique that measures the amount of discrete wavelengths of UV or visible light that are absorbed by or transmitted through a sample in comparison to a reference or blank sample. This property is influenced by the sample composition, potentially providing information on what is in the sample and at what concentration.

Properties of light

- Light has a certain amount of energy which is inversely proportional to its wavelength. Thus, shorter wavelengths of light carry more energy and longer wavelengths carry less energy.
- A specific amount of energy is needed to promote electrons in a substance to a higher energy state which we can detect as absorption.
- Electrons in different bonding environments in a substance require a different specific amount of energy to promote the electrons to a higher energy state. This is why the absorption of light occurs for different wavelengths in different substances.
- Humans are able to see a spectrum of visible light, from approximately 380 nm, which we see as violet, to 780 nm, which we see as red. UV light has wavelengths shorter than that of visible light to approximately 100 nm.
- Therefore, light can be described by its wavelength, which can be useful in UV-Vis spectroscopy to analyze or identify different substances by locating the specific wavelengths corresponding to maximum absorbance

Spectrophotometer

Principle, Instrumentation, Applications



Principle of Spectrophotometer

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device.

1.In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.

2.By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.

3.The spectrophotometer is useful for measuring the absorption spectrum of a

Instrumentation of Spectrophotometer

The essential components of spectrophotometer instrumentation include:

1.A table and cheap radiant energy source

•Materials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.

2.A monochromator, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.

•A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

Prisms:

•A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent

•Two types of Prisms are usually employed in commercial instruments. Namely, 600 cornu quartz prism and 300 Littrow Prism.

Grating:

3.Transport vessels (cuvettes), to hold the sample

•Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as “CUVETTES”.

•Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz.

4.A Photosensitive detector and an associated readout system

•Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it.

•Radiation detectors generate electronic signals which are proportional to the transmitter light.

•These signals need to be translated into a form that is easy to interpret.

•This is accomplished by using amplifiers, Ammeters, Potentiometers and

Advantages of UV-Visible Spectrophotometer

- The technique is **non-destructive**, allowing the sample to be reused or proceed to further processing or analyses.
- Measurements can be made **quickly**, allowing easy integration into experimental protocols.
- Instruments are **easy to use**, requiring little user training prior to use.
- Data analysis generally requires **minimal processing**, again meaning little user training is required.
- The instrument is generally **inexpensive** to acquire and operate, making it accessible for many laboratories.

Disadvantages of UV-Visible Spectrophotometer

- **Stray light -** In a real instrument, wavelength selectors are not perfect and a small amount of light from a wide wavelength range may still be transmitted from the light source, possibly causing serious measurement errors.Stray light may also come from the environment or a loosely fitted compartment in the instrument.
- **Light scattering -** Light scattering is often caused by suspended solids in liquid samples, which may cause serious measurement errors. The presence of bubbles in the cuvette or sample will scatter light, resulting in irreproducible results.
- **Interference from multiple absorbing species -** A sample may, for example, have multiple types of the green pigment chlorophyll. The different chlorophylls will have overlapping spectra when examined together in the same sample. For a proper quantitative analysis, each chemical species should be separated from the sample and examined individually.
- **Geometrical considerations -** Misaligned positioning of any one of the instrument's components, especially the cuvette holding the sample, may yield irreproducible and inaccurate results. Therefore, it is important that every component in the instrument is aligned in the same orientation and is placed in the same position for every measurement. Some basic user training is therefore generally recommended to avoid misuse.

Applications

Some of the major applications of spectrophotometers include the following:

- Detection of concentration of substances
- Detection of impurities
- Monitoring dissolved oxygen content in freshwater and marine ecosystems
- Characterization of proteins
- Respiratory gas analysis in hospitals
- Molecular weight determination of compounds
- The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

DNA and RNA analysis

- The 260 nm/280 nm absorbance (260/280) ratio is useful for revealing possible contamination in nucleic acid samples.
- Pure DNA typically has a 260/280 ratio of 1.8, while the ratio for pure RNA is usually 2.0.
- Pure DNA has a lower 260/280 ratio than RNA because thymine, which is replaced by uracil in RNA, has a lower 260/280 ratio than uracil.
- Samples contaminated with proteins will lower the 260/280 ratio due to higher absorbance at 280 nm.
- The 260 nm/230 nm absorbance (260/230) ratio is also useful for checking the purity of DNA and RNA samples and may reveal protein or chemical contamination. Proteins can absorb light at 230 nm, thus lowering the 260/230 ratio and indicating protein contamination in DNA and RNA samples.

Bacterial culture

- UV-Vis spectroscopy is often used in bacterial culturing. OD measurements are routinely and quickly taken using a wavelength of 600 nm to estimate the cell concentration and to track growth.
- 600 nm is commonly used and preferred due to the optical properties of bacterial culture media in which they are grown and to avoid damaging the cells in cases where they are required for continued experimentation.

Beverage analysis

- The identification of particular compounds in drinks is another common application of UV-Vis spectroscopy. Caffeine content must be within certain legal limits, for which UV light can facilitate quantification.
- Certain classes of colored substances, such as anthocyanin found in blueberries, raspberries, blackberries, and cherries, are easily identified by matching their known peak absorbance wavelengths in wine for quality control using UV-Vis absorbance.

Q1. Determine the relative amount of light that is absorbed by the sample if the absorbance of the sample is 1 at a specific wavelength.

According to the definition of absorbance;

$A = \log_{10} (I_0/I)$

The equation is rearranged to determine the relative loss of intensity

$10A = I_0/I$

$10\text{-}A = I/I_0$

Substituting the value of $A = 1$

$1\text{-}I/I_0 = 1 - 10\text{-}1 = 1 - 1/10 = 0.9$

Therefore, we can say that 90% of the light is absorbed and 10% of light is transmitted.

Q2. Cytosine has a molar extinction coefficient of 6*103 at 270 nm at pH 7. Calculate the absorbance and percent transmission of 1*10-4 and 1*10-3 M cytosine solution in a 1-mm cell.

Solution

For $1*10^{-4}M$, the absorbance **A** is given as,

$A = \log(I_0/I) = e * b * c = 6*10^3 * 0.1 * 1*10^{-4} = 0.06$

in percent transmission, $I/I_0 * 100$,

$A_{\%} = 10^{-0.06} * 100 = 10^{1.94}\ (\%) = 87.10\ \%$

Similarly, for $1*10^{-3}M$, the absorbance **A** is given as,

$A = \log(I_0/I) = e * b * c = 6*10^3 * 0.1 * 1*10^{-3} = 0.6$

in percent transmission, $I/I_0 * 100$,

$A_{\%} = 10^{-0.6} * 100 = 10^{1.4}\ (\%) = 25.12\ \%$

THANK YOU