RAMA UNIVERSITY

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DEPARTMENT OF BIOTECHNOLOGY
FACULTY OF ENGINEERING & TECHNOLOGY

UV-Vis spectrophotometer

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UV-Vis spectrophotometer

Ultraviolet-visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. UV/Vis spectrophotometer is used in the quantitative determination of concentrations of the absorber in the solutions of transition metal ions and highly conjugated organic compounds

Basic Principle

The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length . Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorber in a solution. The absorbance changes with concentration. This can be taken from references (tables of molar extinction coefficients), or more accurately, determined from a calibration curve. The general Beer-lambert law is written as : **$A = \varepsilon cl$**

A= Absorbance

ε= molar absorption coefficient ($M^{-1}cm^{-1}$)

c = molar concentration (M)

l = optical path length (cm)

The Beer–Lambert law is valid for low concentrations only. Higher concentrations might lead to association of molecules and therefore cause deviations from the ideal behaviour.

•***What are transmittance and absorbance?***

•Consider monochromatic light transmitted through a solution; with an incident intensity of I_0 and a transmitted intensity of I

•The transmittance, T, of the solution is defined as the ratio of the transmitted intensity, I, over the incident intensity, I_0 and takes values between 0 and 1. However, it is more commonly expressed as a percentage transmittance

$$T = \frac{I}{I_0}$$

•The absorbance, A, of the solution is related to the transmittance and incident and transmitted intensities through the following relations:

$$A = \log_{10} \frac{I_0}{I}$$

$$A = -\log_{10} T$$

The absorbance has a logarithmic relationship to the transmittance; with an absorbance of 0 corresponding to a transmittance of 100% and an absorbance of 1 corresponding to 10% transmittance.

Reason for generation of absorption spectrum by UV-VIS

When UV-VIS region of electromagnetic region strikes molecules, it causes transition of molecular orbitals by interacting with Molecular (sub-) structures (called as chromophores). From the four possible transitions (n π*, π π*, n σ*, σ σ*), only two can be elicited with light from the UV/Vis spectrum for some biological molecules: n π* and π π*. The presence of several conjugated double bonds in organic molecules results in an extended π system of electrons which lowers the energy of the π* orbital through electron delocalisation. In many cases, such systems possess π π* transitions in the UV/Vis range of the electromagnetic spectrum. In proteins, there are three types of chromophores relevant for UV/Vis spectroscopy:

• peptide bonds (amide bond);

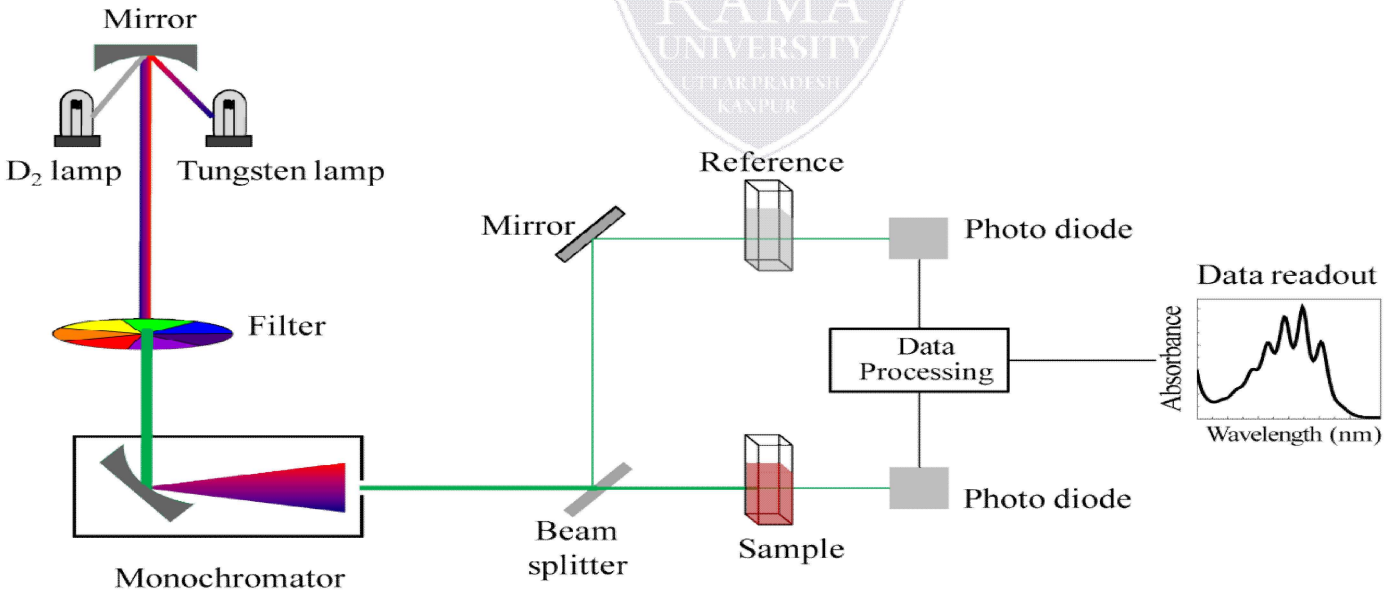
• certain amino acid side chains (mainly tryptophan and tyrosine); and

• certain prosthetic groups and coenzymes (e.g. porphyrine groups such as in haem)

Problem to solve

1. Cytosine has a molar extinction coefficient of $6*10^3$ 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.
2. A protein with extinction coefficient $E^{1\%} = 16$ yields an absorbance of 0.73 when measured in a 0.5-cm cell.

Working and lens arrangement



•UV/Vis spectrophotometers are usually dual-beam spectrometers where the first channel contains the sample and the second channel holds the control (buffer) for correction. The light source is a tungsten filament bulb for the visible part of the spectrum, and a deuterium bulb for the UV region. Since the emitted light consists of many different wavelengths, a monochromator, consisting of either a prism or a rotating metal grid of high precision called grating, is placed between the light source and the sample. If wavelengths are selected by prisms or gratings, the technique is called spectrophotometry.

•A prism splits the incoming light into its components by refraction. Refraction occurs because radiation of different wavelengths travels along different paths in medium of higher density. Diffraction is a reflection phenomenon that occurs at a grid surface, in this case a series of engraved fine lines. The distance between the lines has to be of the same order of magnitude as the wavelength of the diffracted radiation. By varying the distance between the lines, different wavelengths are selected. This is achieved by rotating the grating perpendicular to the optical axis.

•The resolution achieved by gratings is much higher than the one available by prisms. Nowadays instruments almost exclusively contain gratings as monochromators as they can be reproducibly made in high quality by photoreproduction. In a dual-beam instrument, the incoming light beam is split into two parts by a half mirror. One beam passes through the sample, the other through a control (blank, reference). At about 350 nm most instruments require a change of the light source from visible to UV light. This is achieved by mechanically moving mirrors that direct the appropriate beam along the optical axis and divert the other.

•Since borosilicate glass and normal plastics absorb UV light, such cuvettes can only be used for applications in the visible range of the spectrum (up to 350 nm). For UV measurements, quartz cuvettes need to be used.

Applications

Qualitative and quantitative analysis

Qualitative analysis may be performed in the UV/Vis regions to identify 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.

Difference spectra

The main advantage of difference spectroscopy is its capacity to detect small absorbance changes in systems with high background absorbance. A difference spectrum is obtained by subtracting one absorption spectrum from another. Common applications for difference UV spectroscopy include the determination of the number of aromatic amino acids exposed to solvent, detection of conformational changes occurring in proteins, detection of aromatic amino acids in active sites of enzymes, and monitoring of reactions involving ‘catalytic’ chromophores (prosthetic groups, coenzymes).

Spectrophotometric and colorimetric assays

Testing for time or concentration dependent response either by measuring the product of primary reaction or by secondary reactions. Determination of protein concentration by Lowry or Bradford assays, where a secondary reaction is used to colour the protein. The more intense the colour, the more protein is present. These assays are called colorimetric assays.

Test your understanding

UV-Visible spectra can be used for

a. Determination of presence or absence of biological entity in sample

b. Determination of concentration of biological entity in sample

c. Both (a) and (b) are correct

d. None of the above

UV-Visible spectra can be used for measuring Cis-Trans Isomerism

a. FALSE

b. TRUE

UV- Visible rays arises in organic molecules due to

a. Triple bond in organic molecules

b. conjugated double bonds in organic molecules

c. Single bond in organic molecules

d. Diene structure in organic molecules

Chromophores relevant for UV/Vis spectroscopy is / are

a. peptide bonds (amide bond)

b. certain amino acid side chains (mainly tryptophan and tyrosine)

c. certain prosthetic groups and coenzymes

d. All the above