Ultraviolet-visible spectrophotometric analysis

Introduction:

- Ultraviolet (UV)–visible spectrophotometry is widely used in biochemistry, both for the determination of species and for studying biochemical processes.
- This technique enables the determination of micromolar concentrations of substances and has a broad scope of application in this field since most biochemical compounds absorb in the UV–visible region or can be converted into some absorbing derivative.
- On the other hand, this often results in interferences during the determination of the compound of interest, interferences that are minimized by using either derivative spectrophotometry, particularly in the presence of strong background absorption or, to a lesser extent, differential spectrophotometry.
- Spectrophotometric methods are usually less sensitive than fluorimetric ones, which accommodate concentrations of a few nanomoles per milliliter; however, their range of applications are much broader, including inorganic species, organic compounds, proteins, etc., since UV–visible absorption is more universal a property than fluorescence.
- Spectrophotometry is especially useful for monitoring enzymatic reactions, either to determine the reaction products directly or to measure the reaction rate.

What is UV-Vis Spectroscopy used for?

- Ultraviolet–visible (UV–visible) spectrophotometry is primarily a quantitative analytical technique concerned with the absorption of near-UV (180–390 nm) or visible (390–780 nm) radiation by chemical species in solution.
- It is routinely used in analytical chemistry for the quantitative determination of analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules
- UV–visible is used to :
  1. Identify unknown compounds,
  2. Provide information about the physical and electronic structures of organic and inorganic compounds.
  3. Determine the size and concentration .

How does a UV-Vis spectrophotometer work?

Whilst there are many variations on the UV-Vis spectrophotometer, to gain a better understanding of how an UV-Vis spectrophotometer works, let us consider the main components, depicted in Figure 1.

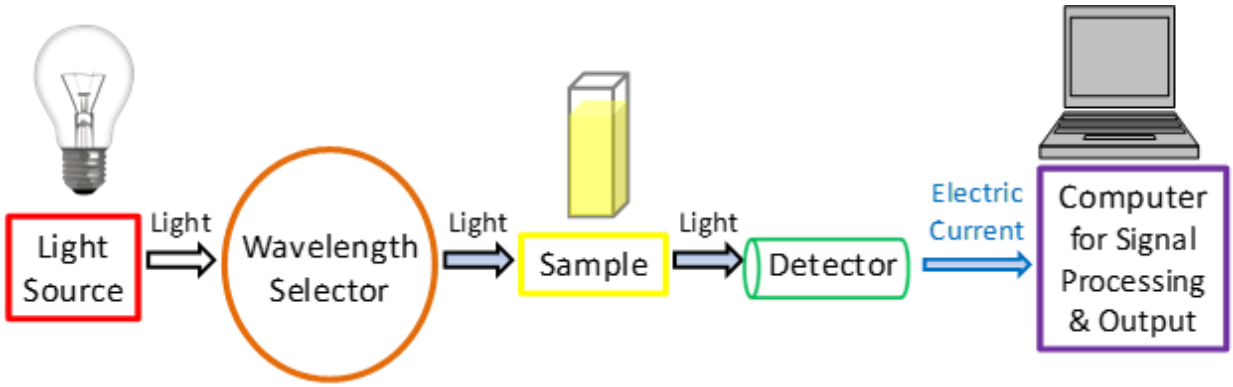

Figure 1: A simplified schematic of the main components in a UV-Vis spectrophotometer.

1- **Light source:** As a light-based technique, a steady source able to emit light across a wide range of wavelengths is essential. A single xenon lamp is commonly used as a high intensity light source for both UV and visible ranges. Xenon lamps are, however, associated with higher costs and are less stable in comparison to tungsten and halogen lamps.

2- **wavelength selection:** In the next step, certain wavelengths of light suited to the sample type and analyte for detection must be selected for sample examination from the broad wavelengths emitted by the light source. Available methods for this include:

Monochromators: A monochromator separates light into a narrow band of wavelengths.

Absorption filters - Absorption filters are commonly made of colored glass or plastic designed to absorb wavelengths of light.

Interference filters - Also called dichroic filters, these commonly used filters are made of many layers of dielectric material where interference occurs between the thin layers of materials.

Cutoff filters - Cutoff filters allow light either below (shortpass) or above (longpass) a certain wavelength to pass through. These are commonly implemented using interference filters.

Bandpass filters -Bandpass filters allow a range of wavelengths to pass through that can be implemented by combining shortpass and longpass filters together.

3- **Sample analysis:** Whichever wavelength selector is used in the spectrophotometer; the light then passes through a sample. For all analyses, measuring a reference sample, often referred to as the "blank sample", such as a cuvette filled with a similar solvent used to prepare the sample, is imperative. If an aqueous buffered solution containing the sample is used for measurements, then the aqueous buffered solution without the substance of interest is used as the reference.

4- **Detection:** After the light has passed through the sample, a detector is used to convert the light into a readable electronic signal. Generally, detectors are based on photoelectric coatings or semiconductors.

UV-Vis spectroscopy analysis, absorption spectrum and absorbance units

UV-Vis spectroscopy information may be presented as a graph of absorbance, optical density or transmittance as a function of wavelength. However, the information is more often presented as a graph of absorbance on the vertical y axis and wavelength on the horizontal x axis. This graph is typically referred to as an absorption spectrum; an example is shown in Figure2.

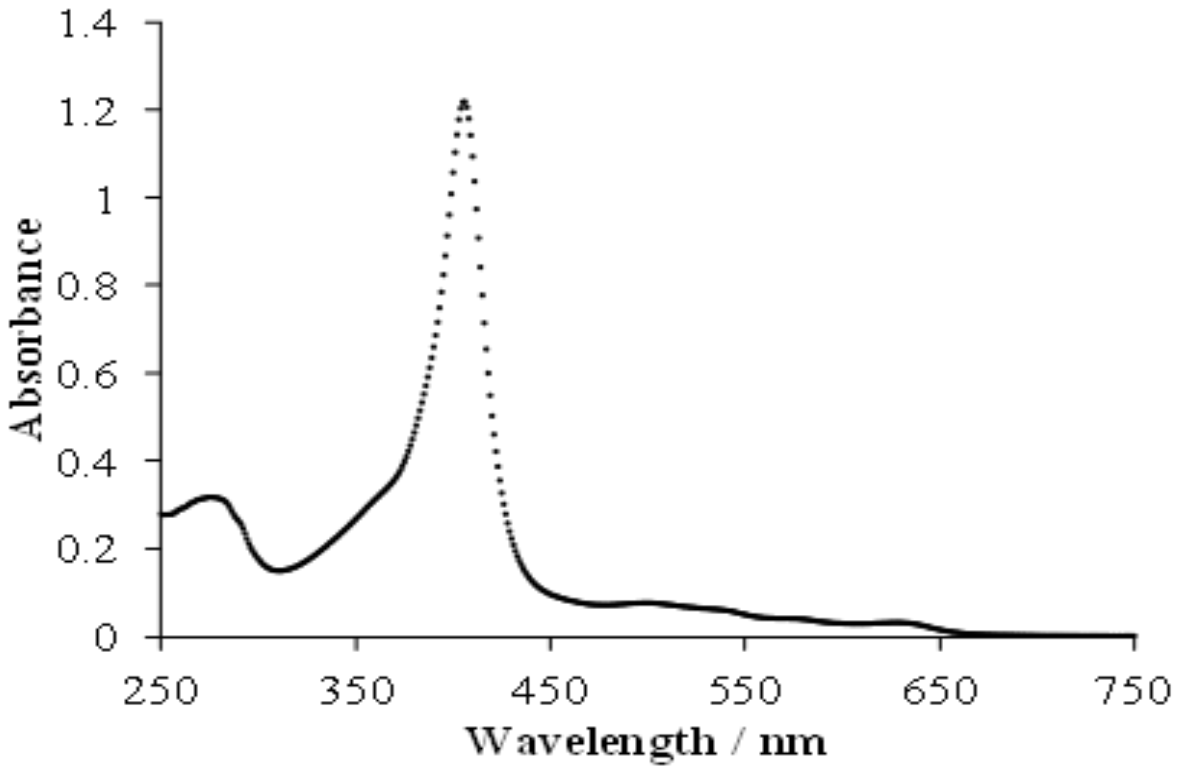

Figure2: An example absorption spectrum taken from a UV-Vis spectrophotometer.

Strengths and limitations of UV-Vis spectroscopy:

No single technique is perfect and UV-Vis spectroscopy is no exception. The technique does, however, have a few main strengths listed below that make it popular.

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

Although the strengths of this technique seem overwhelming, there are also certain weaknesses:

1. **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,1 possibly causing serious measurement errors.

2. **Light scattering** - Light scattering is often caused by suspended solids in liquid samples, which may cause serious measurement errors.

3. **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.

4. **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 of UV-Vis spectroscopy

UV-Vis has found itself applied to many uses and situations including but not limited to:

DNA and RNA analysis:

Quickly verifying the purity and concentration of RNA and DNA is one particularly widespread application. A summary of the wavelengths used in their analysis and what they indicate are given in Table 1.

Wavelength used in absorbance analysis in nanometers	What does UV absorbance at this wavelength indicate the presence of?	What causes UV absorbance at this wavelength?
230	Protein	Protein shape[10]
260	DNA and RNA	Adenine, guanine, cytosine, thymine, uracil
280	Protein	Mostly tryptophan and tyrosine

Table 1: Summary of useful UV absorbance when determining 260/280 and 260/230 absorbance ratios

When preparing DNA or RNA samples, for example for downstream applications such as sequencing, it is often important to verify that there is no contamination of one with the other, or with protein or chemicals carried over from the isolation process.

The 260 nm/280 nm absorbance (260/280) ratio is useful for revealing possible contamination in nucleic acid samples, summarized in Table 2.

Absorbance ratio	Typical values
260/280	1.8 absorbance ratio typical for pure DNA 2.0 absorbance ratio typical for pure RNA
260/230	Absorbance ratio varies; 2.15 to 2.50 typical for RNA and DNA[11]

Table 2: Summary of expected UV absorbance ratios for DNA and RNA analysis.

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.