

Spectrophotometry

UV-Vis (ultraviolet-visible) Spectrophotometry is a technique by which the concentration of solutes can be determined based upon their ability to absorb light of a specific wavelength. The basic components of a UV-Vis spec are as follows:

Light Source

Monochromator (separates light into specific wavelengths)

Sample inside cuvette

Detector (phototube)

Read out (screen or printer)

As light passes through the sample, some of the photons may be absorbed by the component(s) of the sample. The detector indicates how much light has been transmitted through the sample and how much was absorbed. The amount of light absorbed is related to the concentration of the absorbing molecules. This relationship is described by the Beer-Lambert law described below.

Beer-Lambert Law $A = \epsilon l c$

A = absorbance

E = absorption coefficient of the material in the sample (ϵ is the molar absorption coefficient)

l = path length of light in sample (width of cuvette)

c = concentration of absorbing material in the sample

Using this relationship and a known absorption coefficient, the concentration of any substance in solution can be calculated from its absorbance.

What typically absorbs light? Functional groups that are cyclic/aromatic typically have the greatest absorption capabilities. Each group absorbs light of a specific wavelength. This can be used to monitor the concentrations of specific molecules in solution. Common biomolecules with absorption capabilities include: **proteins** (due to the amino acids tyrosine and tryptophan), **DNA and RNA** (due to purine and pyrimidine bases), **NAD, NADH, FMN, FMNH₂, and certain substrates and products of enzyme catalyzed reactions.**

Applications of UV-Vis spectrophotometry. It should be obvious that we can simply determine the concentration of biomolecules such as proteins and nucleic acids by reading their absorbencies. We can also monitor the progress of chemical reactions by monitoring the appearance of absorbing products or the disappearance of absorbing reactants. This is a common way of monitoring reaction kinetics (a process we will discuss and work with later in the course).

Exercise 1: Determining the concentration of proteins by $A_{280/260}$

The optimal absorbance by proteins occurs at a wavelength of 280 nm. Because other materials might be absorbing at that wavelength in a sample (such as nucleic acids) we adjust for that by making a measurement at 260 nm as well. The following equation was developed by Warburg and Christian in 1942 based on a spectrophotometric analysis of the protein enolase and yeast RNA.

$$\text{Protein concentration (mg/mL)} = 1.55A_{280} - 0.76A_{260}$$

You will be supplied with a sample containing a protein (albumin) in solution at an unknown concentration (tube labeled X). Use the spectrophotometer and the “Protein concentration” equation above to determine the concentration of albumin in the sample by taking measurements of the sample at **260 and 280 nm** wavelengths. Use 1 ml of sample for the measurements. Return unknown sample to original tube.

Exercise 2: Determining the concentration of a protein with a dye-binding assay.

A more sensitive method for determining protein concentration is to react the protein with a dye. The Bradford assay uses Coomassie blue binding to assess protein concentration. Coomassie blue changes its maximal absorption wavelength from 465 nm to 595 nm when bound to protein. So, measuring A_{595} in the presence of the dye is a way to monitor protein concentration with great sensitivity.

Establishing a standard curve: To effectively use a dye-binding assay, you must first generate a standard concentration curve with which to compare your unknown sample.

1. Set up 8 glass test tubes (7 for your standard curve and one for your unknown sample).
2. Use the 2.0 mg/mL standard albumin solution provided to you and deionized water to make up seven standard curve tubes with the following albumin concentrations: 0.0, 0.2, 0.6, 0.8, 1.2, 1.5, 2.0 mg/mL (50 μ L final volume).
3. Add 50 μ L of “unknown” from exercise 1 to your eighth tube.
4. Add 2.5 mL dye reagent to each tube and mix. Do this at the same time for the standard curve and unknown samples. Allow the samples to incubate for at least 10 minutes at room temperature (but no longer than 1 hour).
5. Measure the absorbance at **595 nm** of 1 mL of sample from each tube.
5. Plot absorbance at 595 (y or vertical axis) versus protein concentration (mg/mL) (x or horizontal axis) for the standards.
6. Determine the concentration of your unknown protein based on comparison to the standard curve.

Using the Spectrophotometer

Turn on power (let warm up for 5 minutes).

Press **“Mode”** to get to main menu (if not already there)

Choose “Photometric” from the menu by pressing the number **“1”** on the keypad.

To set a particular wavelength, press **“GOTO wl”**.

Type in desired wavelength on the keypad and press **“enter”**. (it takes a few seconds for the lamp to reset to the new wavelength).

To zero the spec, place blank sample in the cuvette holder nearest you (the other cuvette holder will also have a blank sample), close lid and press **“AUTO ZERO”**.

To measure an experimental sample:

Place experimental sample in cuvette holder nearest you and close the lid.

Read the absorbance (ABS) of your sample from the screen.

Opening and closing the lid starts a new reading.

