

Lab Week 2 - Spectrophotometry

Purpose: Introduce students to the use of spectrophotometry for qualitative (what is it) and quantitative (how much is there of it) analysis of biological samples and molecules.

Key Concepts/Terms:

Absorbance (A)	Colorimetry	Transmittance (T)
Absorbance spectrum	Extinction coefficient (ϵ)	UV vs. visible radiation
Beer-Lambert Law	Lambda max (λ_{\max})	Wavelength (λ)
Blank vs. sample cuvette	Qualitative vs. quantitative analysis	
Calibration curve		

Laboratory Skills:

- (i) How to determine absorbance spectrum of unknown compounds (qualitative analysis).
- (ii) How to determine the λ_{\max} of a compound.
- (iii) How to use an absorbance spectrum to identify a pure compound.
- (iv) How to use the Beer-Lambert Law to determine the concentration of compounds in solution.
- (v) How to prepare and graph standard or calibration curves for quantitative analysis using colorimetry.

Background

Basic Laws of Light Absorption. For a uniform absorbing medium (solution: solvent and solute molecules that absorb light) the proportion of light radiation passing through it is called the transmittance, **T**, and the proportion of light absorbed by molecules in the medium is absorbance, **Abs**. Transmittance is defined as:

$$T = I/I_0 \quad \text{where: } I_0 = \text{intensity of the incident radiation entering the medium.}$$
$$I = \text{intensity of the transmitted radiation leaving the medium.}$$

T is usually expressed as percent transmittance, %T:

$$\%T = I/I_0 \times 100$$

The relationship between percent transmittance (%T) and absorbance (A) is given by the following equation:

$$A = 2 - \log (\%T)$$

On most spectrophotometers two scales are present, %T and Abs. Absorbance has no units (Why?) and varies from 0 to 2 (linear region for most substances is from 0.05 to 0.7). The **Beer-Lambert Law** states that **Abs** is proportional to the concentration (**c**) of the absorbing molecules, the length of light-path through the medium and the molar extinction coefficient:

$$A = \epsilon cl \quad \text{where: } \epsilon = \text{molar extinction coefficient for the absorbing material at}$$

wavelength in units of 1/(mol x cm)

$$c = \text{concentration of the absorbing solution (molar)}$$
$$l = \text{light path in the absorbing material (l=1 cm for our purposes)}$$

The Beer-Lambert Law may not be applicable to all solutions since solutions can ionize/polymerize at higher concentrations, or precipitate to give a turbid suspension that may increase or decrease the apparent absorbance. Further, the Beer-Lambert Law is most accurate between Abs of 0.05 to 0.70. Above 0.70, the measured Abs tends to underestimate the real Abs. Below 0.02 Abs many instruments are not accurate (remember that Abs is the log of a ratio). For example, a protein solution had an Abs of 1.42, but when it was diluted 1:4 it had an Abs of 0.45 meaning that the original, undiluted solution had a read Abs of $4 \times 0.45 = 1.80$ Abs.

UV/Visible Spectroscopy. The absorption spectrum (plural, spectra), or more correctly the absolute absorption spectrum, of a compound may be shown as a plot of the light absorbed by that compound against wavelength. Such a plot for a colored compound will have one or more absorption maxima (λ_{max} 's) in the visible region of the spectrum (400 to 700 nm).

Absorption spectra in the ultraviolet (200 to 400 nm) and visible regions are due to energy transitions of both bonding and nonbonding outer electrons of the molecule. Usually delocalized electrons are involved such as the π bonding electrons of $\text{C}=\text{C}$ and the lone pairs of nitrogen and oxygen. Since most of the electrons in a molecule are in the ground state at room temperature, spectra in this region give information about this state and the next higher one. As the wavelengths of light absorbed are determined by the actual transitions occurring, specific absorption peaks may be recorded and related to known molecular substructures. The term chromophore is given to that part of a molecule that gives rise independently to distinct parts of an absorption spectrum, for example the carbonyl group. Conjugated double bonds lowers the energy required for electronic transitions and results in an increase in the wavelength at which a chromophore absorbs. This is referred to as a bathochromic shift, whereas a decrease in conjugation, caused for example by protonating a ring nitrogen atom, causes a hypochromic shift which leads to a decrease in wavelength. Hyperchromic and hypochromic effects refer to an increase and a decrease in absorbance respectively.

Instrumentation. To obtain an absorption spectrum, the absorbance of a substance must be measured at a series of wavelengths. Absorption in the visible and ultraviolet regions can be measured by a UV/visible spectrophotometer.

UV/Vis spectrometers consist of three basic components, (i) a light source and a mechanism to select a specific wavelength of light in the UV/visible region of the spectrum, (ii) a chamber where a cuvette containing a test solution can be introduced into the light path, and (iii) a photocell that can determine the amount of light absorbed by the sample (or the intensity of light transmitted through the sample).

The light source is usually a tungsten lamp for the visible region of the spectrum, and either a hydrogen or deuterium lamp for ultraviolet wavelengths.

Cuvettes are optically transparent cells that hold the material(s) under study and are used to introduce samples into the light path. A reference cuvette optically identical to, and containing the same solvent (and impurities) as the test cuvette is always required for setting the spectrophotometer to read zero absorbance at each wavelength used. For accurate work, the optical matching of the two cuvettes should always be checked. Glass and plastic absorb strongly below 310 nm and are not useful for measuring absorbance below that wavelength. Quartz or silica cells are used when measuring absorption of ultraviolet wavelengths by a solution since they are transparent to wavelengths greater than 180 nm.

When a cuvette is positioned in the light path it becomes an integral part of the instrument's optical system, so it should be treated with the same care given to other optical components. The use of scratched or contaminated cuvettes should be avoided since they reflect and/or absorb radiation that

will give you inaccurate measurements. Also, bubbles, turbidity, fingerprints, or condensation on, or inside, cuvettes should be avoided since they will diminish the accuracy of readings.

Note: The cuvettes commonly used for accurate work have an optical path length of 1 cm and require 2.5 to 3.0 ml of sample for all accurate reading. This applies to the Spec 20 tubes that you will be using in this laboratory.

Colorimetry. Many substances that do not absorb or have a very low absorbance in the visible region of the spectrum will react quantitatively with specific reagents to give a colored product that can be measured in the spectrophotometer. The absorbance (A) of such solutions can be plotted against the quantity or concentration (c) of the test substance producing the color. This graph is known as the calibration curve (or standard curve). Since it is often possible to produce comparatively high absorbances with relatively small amounts of material, colorimetry is widely used in biochemistry to assay a wide range of biologically important molecules.

Some important points to bear in mind when carrying out colorimetry are as follows:

- (i) unlike straightforward spectrophotometry, colorimetry is a destructive technique; *i.e.*, once reacted, the sample cannot be recovered;
- (ii) a chromophore reflects the complementary color(s) that it absorbs, *i.e.* a yellow compound appears yellow because it absorbs blue light and therefore it must be estimated in the blue region of the spectrum;
- (iii) colorimetric assays are usually most sensitive at the λ_{\max} of the chromophore produced. If this is not known, the spectrum needs to be determined before commencing the assay;
- (iv) reference cuvettes (*i.e.*, blanks) should contain everything except the substance being assayed – *i.e.*, all the reagents in the same concentrations as in the test cuvettes;
- (v) the reference cuvette and its contents require, if anything, more care because any error in these will be reflected in all of the values obtained;
- (vi) assays should normally be performed in duplicate or triplicate, and individual values, not means, should be plotted. This procedure allows the experimenter to justifiably omit erroneous values from the calibration curve;
- (vii) the “line of best fit” should be drawn through the data points, not necessarily the line that passes through the origin and the other points.
- (viii) calibration curves may vary as batches of reagents and standards vary. Therefore, new calibration curves should be prepared each time an assay is run;
- (ix) calibration curves should never be extrapolated beyond the highest absorbance value measured. It is always more accurate to repeat an assay at a concentration which falls within the most accurate region of the calibration curve (*i.e.*, you may need to dilute a solution to accomplish this). This is usually from 0.05 to 0.70.

Lab Exercises

Materials

Spectronic 20 spectrophotometer
Spec 20 cuvettes
Bradford's reagent – a protein-binding dye (Coomassie Brilliant Blue) reagent
Bovine Serum Albumin (BSA) standard 10 mg/ml (mw 66,000 g/mol or daltons)
DNP-glutamic acid - MW 313.2 g/mol (or daltons)
Cytochrome c - MW 12,327 g/mol
Blue dextran - MW 2,000,000 g/mol
Potassium acetate buffer (pH 6.0; 20 mM)
Commercial Beef, Chicken or Pork Bouillon, 1 cube/cup of distilled water or to package directions (for Bradford Assay).

1. **Determine the absorption spectra of one of the standards distributed in lab** (*qualitative analysis*).
 - a. Warm-up Spec 20 – allow at least 20 min before taking readings.
 - b. Prepare a blank cuvette – place 3 ml of potassium acetate into a clean Spec20 tube. Use this cuvette to zero the instrument as described by your TA (directions are also available [here](#)).
 - c. Zero the Spec 20 – Set the wavelength dial on the Spec 20 to 350 nm. With the sample chamber empty, turn the left hand knob on the front of the spectrophotometer until the needle reads 0% Transmittance (upper scale). Next, place your reference blank into the sample compartment making sure to line up the marks on the cuvette and the sample compartment of the Spec 20. Adjust the right hand knob until the needle reads 100% Transmittance (this process cancels out background absorbance).
 - d. Measure the absorbance of your sample at 350 nm – Once the Spec 20 is zeroed, remove the blank cuvette and place your sample cuvette into the sample compartment. Read and record the absorbance value (A) on the **lower absorbance scale** (not the Transmittance or upper scale that you used to zero the instrument). If A is greater than 0.6, dilute your sample by 10 fold (*i.e.*, 1 volume of undiluted solution mixed with 9 volumes of diluent so that the final concentration is 1/10 of the original strength). Read and record A again. If the value is below 0.6, continue on to the next step. If not, you will need to do an additional dilution(s) before you can read and record the absorbance of the diluted sample. Keep a record of all dilutions as described below.
 - e. Determine the absorbance of the diluted sample at 50 nm intervals between 350-700 nm - this will give you a ballpark estimate of where the sample absorbs most (peaks) and least (valleys). If the absorbance of the sample is greater than 0.6 at any wavelength between 350-700 nm, dilute the solution until all of your absorbance readings fall below 0.6. If necessary, repeat all of your readings at the same dilution.
 - f. Establish the λ_{\max} of your sample - in regions where you are approaching absorption maxima (*i.e.*, absorbances increase to a peak), determine A at 10 nm rather than 50 nm intervals. The λ_{\max} of the sample is the λ that yields the highest A value. Keep in mind that the Spec20 has a bandwidth of about 10 nm, so there is no advantage in measuring the absorbance at shorter wavelength intervals (*e.g.*, 5 nm).
 - g. Plot your data on graph paper – plot absorbance (A) on the y-axis as a function of wavelength (λ) on the x-axis.

2. Calculate the extinction coefficient (ϵ) of the standards.

The extinction coefficient (ϵ) in the Beer-Lambert equation is a measure of how strongly a compound in solution absorbs light at a particular wavelength. In other words, at equal molar concentrations in solution, a compound that has an $\epsilon = 35,000$ at the λ_{\max} for that compound, absorbs light more strongly than another compound with $\epsilon = 5,000$ at its λ_{\max} . Extinction coefficients are useful numbers that allow researchers to calculate the concentration of a compound in solution (quantitative analysis). You will be using spectrophotometers to calculate the concentration of compounds in solution throughout the term, so the investment of time in this lab will save you time in the future.

The Beer-Lambert equation states that $A = \epsilon cl$ at the λ_{\max} of a compound. Therefore, if you know A at the λ_{\max} , c , and l (always equal to 1 cm for Spec 20's), you can calculate ϵ by rearranging the Beer-Lambert equation as follows:

$$\epsilon = A/cl$$

By the same reasoning, if you know A , ϵ and l , you will be able to calculate the molar concentration (c) of a compound in solution.

- a. Calculate the molar concentration (c) of your undiluted standard compound.
 - i) The concentration of each of the three standards is 10 mg/mL.
 - ii) The molecular weights of the three colored compounds are:
- b. Calculate the molar concentration of the diluted standard. Determine the molar concentration of standard that gave an $A \cong 0.6$ at the λ_{\max} . You will need to use the initial concentration of your standard (expressed in mol/L) and the dilution data from Part 1.
- c. Substitute your calculated values for A at λ_{\max} , c and l and solve for ϵ .

Note: Don't forget all the good stuff you learned about significant figures and error in previous labs. Report your estimate of ϵ with the number of significant figures you can justify.

Where would you expect the greatest amount of error to occur? During the dilution? During calculation of the molar concentration? During the absorbance measurement? Be sure to finish your calculations of the molar extinction coefficient BEFORE going on to the next part of the laboratory.

3. Determine the concentration of proteins in solution using a colorimetry.

The Bradford assay can be used to detect and quantify the level of proteins in solution. Cytochrome *c* is a red colored protein that you analysed earlier by determining its spectrum in the visible range. It is red because it binds a porphyrin group similar to the heme in hemoglobin that makes your blood red. The protein portion of cytochrome *c* is colorless because it does not absorb in the visible range. Proteins containing tryptophan, phenylalanine and tyrosine absorb at about 280 nm due to the π electrons characteristic of the conjugated double bonds in their R groups. One way to estimate the concentration of proteins in solution is to measure absorbance at 280 nm. Unfortunately, this technique is extremely inaccurate for proteins because of the varying levels of tryptophan, phenylalanine and tyrosine in many proteins. Because of such inaccuracies, a variety of assays have

been developed to measure protein levels that do not depend on the presence of particular amino acids. The Bradford protein-dye binding assay is based on the color change of a dye when it goes from a polar to a nonpolar environment. Thus, since most proteins having tertiary/quaternary structure have nonpolar regions, they will bind the Bradford dye. Binding results in a color shift from a greenish brown to blue. Although the Bradford assay is the main technique used to measure the level of proteins in solution, you should keep in mind that it also has drawbacks. Amphipathic compounds such as fatty acids or detergents interfere with the Bradford assay and can cause a color change that is not related to protein concentration (i.e., a potential source of error).

You will use the Bradford assay to make a calibration curve that relates protein amount with color development (colorimetry) in a solution. You will use known concentrations of protein to make a standard curve that will be used in next weeks chromatography lab and several other labs during the semester – so learn this technique well. You will also determine the protein content of commercial beef, chicken or pork bouillon using this assay.

a. Quantitative analysis.

- i. Construct a standard curve. The Bradford assay can be used to determine how much protein is present in an unknown solution by comparing the absorbance of an unknown (following reaction with the Bradford reagent) with the absorbance of solutions containing known concentrations of protein. A standard curve relates the concentration of a protein in solution with the absorbance of that protein following reaction with the Bradford reagent.
 - ii. The Bradford assay is not linear over a wide range of protein concentrations. There are two forms of the assay used, one for low amounts (1- 25 μg) known as the microassay and one for higher amounts (20-150 μg) known as the standard assay. The description below is of the standard assay. You will be using the standard assay in this laboratory.
- b. The range of BSA amounts for the standard curve are
 - c. Dilute BSA standard 1:1 with buffer. This solution will serve as your stock solution (5 mg/ml = 5 $\mu\text{g}/\mu\text{l}$).
 - d. Prepare BSA samples from the stock solution - make duplicates test tubes containing the following protein concentrations 10, 20, 30, 40, 50, 60, 70, 100, and 140 μg protein in a total of 0.1 ml of buffer.
 - e. Prepare 4 1:10 dilutions of commercial bouillon using the acetate buffer. Add 100 μl of each dilution into each of 4 tubes.
 - f. Add 5 ml of 1:4 diluted Bradford reagent to each test tube.
 - g. Mix thoroughly - place Parafilm over the end of each test tube and agitate (avoid contaminating your solutions with proteins from your fingers).
 - h. Wait at least 5 min before reading the absorbance of the tubes in the Spec 20 at 595 nm.
 - i. Make sure that your A values fall between 0.02 and 0.7 absorbance units. If higher, you must dilute your sample until the reading is in the desired range.
 - j. Plot your standard curve – A (y-axis) vs. protein amount (x-axis). Then use the standard curve to determine the protein content of bouillon. Is this a good protein source?

Note: The Bradford Assay measures total protein in the 5 ml of each sample. Therefore, you are measuring the total amount of protein in the 5 ml sample – this is not a concentration!

APPENDIX

Spectra and their Biochemical Usefulness. Molecules interact differentially with radiation of different wavelengths (λ 's) – i.e., they absorb more strongly at specific λ 's and less at others. An absorbance spectrum may be represented as a graph of the amount of energy absorbed by a system plotted against λ . Different types of instruments are required for the study of different regions of the spectrum. Some of these regions yield information of great use to the biochemist and are used routinely, whereas other regions requiring more sophisticated instrumentation and expertise are used only in detailed research of biological macromolecules.

Visible and UV spectra arise due to the outer electrons of atoms changing between major electronic energy levels. When molecules absorb visible and ultraviolet light they change their rotational and vibrational energy levels (electrons are raised to unoccupied higher orbitals). These spectra are used routinely in biochemistry. Fluorescence spectra may also arise from when electrons that went to higher orbitals return to their ground state (lower) orbital, they release some of the energy as light. The rest is released as vibrational energy (heat).

Vibration rotation spectra are caused by changes in the vibration energy levels. They occur in the near infrared region and may be accompanied by changes in the rotational energy levels. Such spectra are sometimes used in studies of the detailed structure of biological macromolecules in non-aqueous environments.

Electron spin resonance spectra and nuclear magnetic resonance spectra arise due to changes in the direction of the spins of electrons and nuclei respectively in an intense magnetic field. These two types of spectra are valuable for studying the structure of biological molecules.

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