Theory of spectrophotometry

Many compounds absorb light, but not all absorb the same amount, or the same colors (wavelength). Some absorb mainly in the visible spectrum (see below) while others absorb mainly in the ultraviolet or infrared range. Here we are mainly interested in measuring absorbance in the visible range.

\[
%T = 100 \times \left(\frac{I}{I_0}\right)
\]

where \( I \) is the light reaching the photocell and \( I_0 \) is the light striking the cuvette.

The exponential decrease of transmittance with concentration is awkward to work with: straight lines are more convenient than curved ones. Since transmittance decreases exponentially with concentration, \( \log 1/T \) increases linearly with increasing concentration. The second scale on the spectrophotometer reads absorbance (A), according to the equation \( A = \log 1/T \), with T expressed as a decimal fraction. This can also be expressed as:
A = - log T or A = 2 - log %T, with T expressed as a percentage.

The relationship between absorbance and concentration is known as the Beer-Lambert law: \( A = e l C \), where \( e \) is the extinction coefficient (a property of the light-absorbing substance), \( l \) is the light path in cm and \( C \) is the concentration of the light-absorbing substance. Since we will always use the same size cuvette then, for any given substance, the Absorbance will be proportional to the concentration.

There are three sources of absorbance, the container (usually a clear glass test-tube or cuvette), the solvent which is nearly always water, and the dissolved molecules. In most cases it is only the last that is of interest. To ensure that the container and solvent do not interfere with our readings we use a 'blank' which is a tube containing only solvent as a reference point. By defining the amount of light that passes through the blank as 100% transmission we can assume that any reduction in the sample tube is due to the dissolved molecules.

Each type of molecule absorbs each wavelength of light in a characteristic pattern. In general you can make a good guess about a solution's absorbance pattern by looking at the color of the solution: the color it appears is the color that is NOT absorbed. So a blue solution is absorbing all the colors except blue. As you will find out, this does not mean that all blues are identical, nor that the absorbance of other colors is necessarily complete.
How spectrophotometers work

The basic components are

A light source (in the Spec 20D it emits all colors of the visible spectrum). More complex machines may emit also UV light.

A method of selecting a specific wavelength (color). Most machines use a diffraction grating.

A sample chamber which will contain the solution to be measured

A photodetector that measures how much light passes through the sample

Before you can use the spectrophotometer, you need to set the limits it can recognize. First, with the sample chamber empty and closed. Under this condition no light passes through the chamber and you will define this as zero light (0% T)
Second, you need to let the machine see what happens to the chosen wavelength of light when just the cuvette and solvent are present. This should be the maximum amount of light that will pass through the chamber and will be defined as 100% T.

Finally, you insert your sample and measure how much of the same wavelength of light gets absorbed by the dissolved material.

As long as you do not change the wavelength, you can read other samples just by placing them in the chamber. However, if you change wavelength, then you must reset to 100% T with the blank, since it will absorb each wavelength differently.
Using the Spec 20D

1. Turn on (Power Switch) and allow to warm up 5-10 minutes

2. Set the wavelength to the required value. If above 600 nm, adjust the wavelength lever accordingly

3. Set to Transmission Mode using the Mode Selector button

4. With nothing in the sample compartment, use the Power/Zero Set knob to set transmission to zero

5. Place the Blank tube in the machine. Use the Transmittance/Absorbance Control to set Transmittance to 100%

6. Remove the Blank, change Mode to Absorbance

7. Insert the sample and read absorbance

8. Repeat for all samples to be read at this wavelength.

9. If you need to read at a different wavelength, go to step 2 and repeat
Using Micropipets

1) Choosing and setting the pipet (=pipette)
   • Choose a pipet with a maximal capacity closest to, but larger than, the volume you need to transfer. For example, if you wish to measure 60 µL, use a 10-100 µL or 40-200 µL capacity pipet.
   • Loosen the locking device (if present), rotate the adjustment knob to dial in the correct digital reading (NEVER force the knob beyond its limits, as this can permanently ruin the pipette); be sure to note the decimal point, if one is present on the digital display.
   • Choose the appropriate tip (sometimes color coded, but not always) and gently press it on to the end of the pipet (a slight twist helps). DO NOT FORCE IT ON.

2) Transferring liquid
   • Press down on the plunger until a slight hesitation is felt (= first “stop”)
   • Insert the end of the tip under the liquid
   • Slowly let up on the plunger (Don’t release plunger suddenly!).
   • Remove the pipette from the liquid and go to the receiving container.
   • Press down on the plunger to the second stop (Note: Pressing too hard may dislodge the tip); all liquid should be dispensed from the tip.
   • Pump up and down a few times, to be sure all material has been transferred
   • Press down on the plunger to the second stop and remove the pipette from the container before releasing the plunger.
   • Once you finish with that solution, discard the tip into a waste container by pressing on the release button.

Exercise 1) Checking accuracy and repeatability
   • Each member of the group should do this:
   • Add 3 ml of water to a test tube.
   • Add 50 µL of concentrated (in separate bottle) red dye to the tube and mix.
   • Determine the absorbance at 500 nm.
   • Write your results on the white board. You will get the entire class results next day.
   • Compare the class results: calculate the mean and standard deviation. This is a measure of the variation that comes from errors in handling. Keep this in mind when looking at results in later experiments.

Throughout the semester you will be held responsible for the equipment at your location, and will be penalized if it is left dirty, damaged or mislaid. Be sure to check everything at the beginning of each session and report any problems at that time, so you are not held responsible.
Using the Spectronic 20D spectrophotometer

Exercise 2: Colors and wavelengths
- Half fill a disposable glass test tube with tap water.
- Add a pinch of chalk dust and mix by shaking.
- Set the wavelength to 450 nm.
- Insert the test tube into the sample holder, but leave the cover open.
- Turn the right hand control knob clockwise as far as it will go. Turn it back when finished this exercise.
- Look straight down into the test tube: note the color. (Note: Dimming the room lights and shielding the opening to the sample holder will make it easier to see the colors.)
- Increase the wavelength by 50 nm and note the color; continue to 650 nm.
- At what wavelength(s) of visible light do you perceive the color Blue? Green? Yellow? Orange? Red?

Exercise 3. Absorption spectra
- Use one tube of red dye, one of blue dye, and one of water.
- Set the wavelength to 475 nm.
- Follow the Using the Spec 20D sheet to zero the instrument and measure the absorbance of the red and blue dyes at 475 nm.
- Increase the wavelength by 25 nm and repeat. Remember: every time you change wavelength you must rezero to 100%T.
- When you get to 600 nm, slide the filter lever over.
- Continue reading the absorbances up to 650 nm.
- For next week: Plot the absorption spectra (abs vs. wavelength) for both dyes. Look at the high and low areas. What do they tell you about light interacting with the dyes? How do you explain the red and blue colors of the dye?

Exercise 4. Transmittance vs Absorbance
- Remeasure the absorbance of the red dye at 500 nm, then switch to % Transmittance and record that value.
- Perform serial dilutions, as shown on the next page.
- For next week: Plot Dye Concentration (expressed as %) on the X axis and % Transmission and Absorbance values on the two Y axes. Draw smooth curves to connect the points for your two graphs.
- Which is closer to a straight line? Which is easier to work with, a curved line or a straight line relationship? From now on, when using a spectrophotometer you will always measure and report Absorbance values, not % Transmittance values - why?
Serial Dilution

Start with 2mls water/tube

Measure Abs and %T

Original dye

2mls

Measure Abs and %T

2mls

Measure Abs and %T

2mls

Measure Abs and %T

2mls

Measure Abs and %T
Answer these questions, and email the answers to me by 8:00 a.m. of the day of the lab

What is the reason for initially setting the empty machine’s transmission to zero? What is the reason for setting the transmission to 100% with the blank?

1) Chlorophyll is green mainly because it:
   a) comes from green plants
   b) absorbs all the green light
   c) absorbs red light
   d) absorbs blue light
   e) absorbs all the colors except green

2) You have a solution of dye that has an absorbance of 1.0 OD (10% transmittance) at 600 nm. If you diluted it to one quarter of its current concentration:
   a) it will have a transmittance of 2.5%
   b) it will have a transmittance of 40%
   c) it will have an absorbance of 0.25 OD
   d) it will have an absorbance of 4.0 OD
   e) it will absorb at 150 nm

3) You have just measured the absorbance of a solution at 450 nm, and found it to be 0.45 OD. You want to find its absorbance at 550 nm, so you:
   a) change the wavelength to 550 nm and read the absorbance
   b) change the wavelength to 550 nm and reset the transmittance to 100% with the blank
   c) set the machine to zero transmission with nothing in it
   d) multiply 0.45 by 550/450 to get the new absorbance
   e) take the antilog of 0.45 and multiply by 550/450
4) You have two samples of the same material, both dissolved in water. The first you know is at a concentration of 5 mg/ml, but the second was made by someone else, and you don't know the concentration. When both are read at the same wavelength, yours gives a reading of 0.7 OD (=20% transmittance) while the other reads 0.3 OD (=50% transmittance). You would suspect that the concentration in the second sample is:
   a) 0.3 mg/ml
   b) 2.1 mg/ml
   c) 5.0 mg/ml
   d) 11.7 mg/ml
   e) you just can't say

5) In spectrophotometry, nanometers are used as a measure of:
   a) Wavelength
   b) Concentration
   c) Absorbance
   d) Transmission
   e) Blanking