

Appendix D: Absorbance Measurements with the Spectronic 20 Spectrophotometer

The concentration of colored solute in a solution is directly proportional to the intensity of its color, which in turn is proportional to the amount of absorbance of light at the wavelength that the color absorbs. The color, or absorbance, of a solution is also proportional to the path length that the light passes through. This is often expressed as the **Beer-Lambert Law, or Beer's Law**:

$$A = \epsilon C l$$

Where A is absorbance at a given wavelength of light,
 ϵ is the extinction coefficient (amount of color of the solute per mole)
C is the concentration of solute in the solution (doubling the concentration doubles the amount of light absorbed)
and l is the pathlength (if you double the length of the path of light, you double the opportunities for it to be absorbed)

There are generally two scales of measurement given on a spectrophotometer: one for absorbance and one for % transmittance. It is important to not confuse the two scales (the absorbance scale is the lower one if you are using an old analog Spec20 that has a dial indicator). If you are using a newer digital Spec20 model, there is no dual scale, but a digital display instead is toggled between absorbance and % transmittance by a switch on the top of the instrument.

Absorbance is essentially the opposite of transmittance: what light is not absorbed is transmitted. The percent transmittance is related to absorbance mathematically as:

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

Where A is absorbance at a given wavelength of light
% T is the percent transmittance, or $\frac{\text{light transmitted through a sample}}{\text{light transmitted through a blank}} \times 100$

The following table demonstrates how absorbance and % transmittance are related:

<u>Absorbance</u>	<u>% T</u>
∞ (infinity)	0
3	0.1
2	1.0
1	10.0
0	100

How a Spectronic 20 Works

You will be measuring the amount of light absorbed at a chosen wavelength. The Spectronic 20 is a typical absorption spectrophotometer, and it has the following basic parts:

- ◆ *A source of light.* For the Spectronic 20, this is a tungsten lamp. White light comes out of the bulb, and lenses focus it into a narrow, parallel beam.
- ◆ *A monochromator.* This is a device used to break up the light into several wavelengths and to block off all but the wavelength of interest. In your instrument, the light is broken up by a diffraction grating and sorted by an exit slit, which stops all but one wavelength band.
- ◆ *A cell holder to hold a cuvette or sample test tube.* In the Spectronic 20 there is an occluder to prevent light from passing through if there is no sample in the holder.
- ◆ *A photocell or other device used to “see” the light and to give a numerical value to its intensity.* The phototube will measure all the light remaining after the sample has done the absorbing.

How the Spectronic 20 Controls Do Their Job

The wavelength controller (on the top of the instrument) adjusts the position of the diffraction grating so that different colors of light are selected by the slit.

The zero % T control (on the left side of the front panel of the instrument) adjusts the electronics. With the sample compartment cover down and no sample in the cell holder, the zero control should be adjusted until the meter reads 0 for transmittance. This control adjusts for the so-called dark current, the current transmitted by the photocell in the dark.

The 100%T control (on the right side of the front panel of the instrument) adjusts the amount of light output while the blank cuvette is in the cell holder. A mechanical linkage is used to drive a wedge into the light beam for this adjustment. After inserting the blank cuvette into the cell holder, this knob should be adjusted until the transmittance is 100% (or the absorbance is zero). This allows you to correct for any stray absorbance resulting from the solvent alone.

In summary, there are three adjustments that must be made before the absorbance of a sample can be measured:

1. The wavelength must be selected by the knob on the top of the instrument.
2. The signal must be adjusted to 0% transmittance with no cuvette in the instrument by the knob to the left on the front of the instrument. (When the cuvette holder in the instrument is empty, this trippers a shutter to close inside.)
3. The signal must be adjusted to 100% transmittance (zero absorbance) with a “blank” cuvette containing only solvent by the knob to the right on the front of the instrument.

1. USE THESE INSTRUCTIONS FOR THE ANALOG Spec-20 ONLY!

- Notice that there are two measurement scales on the meter. The top (black) scale measures percent transmittance of light - the amount of light that passes through the sample. The lower (red) scale measures absorbance.
- Before you begin any measurements with the Spec-20, make a reference cuvette (sometimes called a control or blank). This cuvette often contains dH₂O, but may contain another liquid, such as a buffer or a dye used in all of the tested samples.
- Fingerprints and scratches on the cuvettes can render your measurements meaningless and ruin the cuvettes. Handle the cuvettes carefully. Before placing a cuvette into the sample compartment, grasp the cuvette at its top and wipe off with a Kimwipe. Never use test tube brushes or any scratchy or abrasive cleaner or scrub cloth on a cuvette. Rinse with dH₂O only!

To determine the absorbance of several samples of the same wavelength:

1. Turn on the Spec-20 using the power switch, set the desired wavelength and allow to it warm up at least 15 minutes.
2. With the sample compartment closed and empty, use the power switch/zero control knob to adjust the transmittance to zero (left knob- zero to left of meter)
3. Place the blank into the sample compartment with the vertical line on the cuvette aligned with the mark on the front edge of the sample compartment, and close the lid.
4. Using the transmittance/absorbance control knob, set the meter to zero absorbance (right knob - zero to right of meter).
5. Remove the blank from the sample compartment.
6. Place a cuvette containing your sample into the sample compartment, close the lid, and read the absorbance from the meter. Remove your sample from the Spec-20.
7. Repeat step 6 for your remaining samples. Re-zero the Spec-20 every 15-20 minutes (or if you notice that the meter no longer reads zero transmittance when the sample compartment is empty with the lid closed).

To determine the absorbance of one sample at several wavelengths:

1. To make the first reading, follow steps 1 through 6 above..
2. For the next reading, change the wavelength to the next one needed. Re-zero the Spec-20 following steps 2 through 5 above.
3. Place your sample back into the sample compartment and read the absorbance. Repeat until all readings are taken at all of the specified wavelengths.

To determine the absorbance of several samples at several wavelengths:

1. Zero the Spec-20 at your first wavelength and measure all of the samples at that wavelength.
2. Change the wavelength setting, re-zero, and make the next set of measurements.
3. Repeat until all samples have been measured at all wavelengths.

II. USE THESE INSTRUCTIONS FOR THE DIGITAL Spec-20 ONLY!

- Familiarize yourself with all parts of the Spec-20D before you begin.
- Notice the button labeled "Mode". You will zero the Spec-20D using the "Transmittance" mode. When you take an absorbance reading of a sample, you will switch to "Absorbance" mode using this button.
- Before you begin any measurements with the Spec-20, make a reference cuvette (sometimes called a control or blank). This cuvette often contains dH₂O, but may contain another liquid, such as a buffer or a dye used in all of the tested samples.
- Fingerprints and scratches on the cuvettes can render your measurements meaningless and ruin the cuvettes. Handle the cuvettes carefully. Before placing a cuvette into the sample compartment, grasp the cuvette at its top and wipe off with a Kimwipe. Never use test tube brushes or any scratchy or abrasive cleaner or scrub cloth on a cuvette. Rinse with dH₂O only!

To determine the absorbance of several samples of the same wavelength:

1. Turn on the Spec-20D using the power switch, set the desired wavelength and allow to warm up at least 15 minutes.
2. With the sample compartment closed and empty, use the power switch/zero control knob to adjust the transmittance to zero.
3. Place the blank into the sample compartment with the vertical line on the cuvette aligned with the mark on the front edge of the sample compartment, and close the lid.
4. Using the transmittance/absorbance control knob set the meter to 100% transmittance.
5. Remove the blank from the sample compartment. Change to "Absorbance" using the "mode" button.
6. Place a cuvette containing your sample into the sample compartment, close the lid, and read the absorbance from the meter. Remove your sample from the Spec-20D.

7. Repeat step 6 for your remaining samples. Re-zero the Spec-20 every 15-20 minutes (or if you notice that the meter no longer reads zero transmittance when the sample compartment is empty with the lid closed).

To determine the absorbance of one sample at several wavelengths:

1. To make the first reading, follow steps 1 through 6 above.
2. For the next reading, change the wavelength to the next one needed. Re-zero the Spec-20D following steps 2 through 5 above.
3. Change the mode to "Absorbance", place your sample back into the sample compartment and read the absorbance. Repeat until readings are taken at all of the specified wavelengths.

To determine the absorbance of several samples at several wavelengths:

1. Zero the Spec-20 at your first wavelength and measure all of the samples at that wavelength.
2. Change the wavelength setting, re-zero, and make the next set of measurements.
3. Repeat until all samples have been measured at all wavelengths.