

## LAB \_\_\_\_\_. THE SPECTROPHOTOMETER

## INTRODUCTION:

A spectrophotometer is a valuable instrument in biology. It allows us to measure and compare the concentration of materials in solution with relative ease. It is therefore important for you to understand how the spectrophotometer works so that you understand the data that you collect from it.

## WHAT DOES A SPECTROPHOTOMETER DO?

A spectrophotometer is an instrument that measures either the **transmittance** of light through a solution or the **absorbance** of light by a solution.



Light can either pass through an object or be stopped by an object which is in its path. We can easily see this if we try looking at a light through a piece of clear plastic — all of it is transmitted through and none of it is absorbed. Then try to see the same light through a piece of cardboard — all of it is absorbed by the cardboard and none of it is transmitted through.

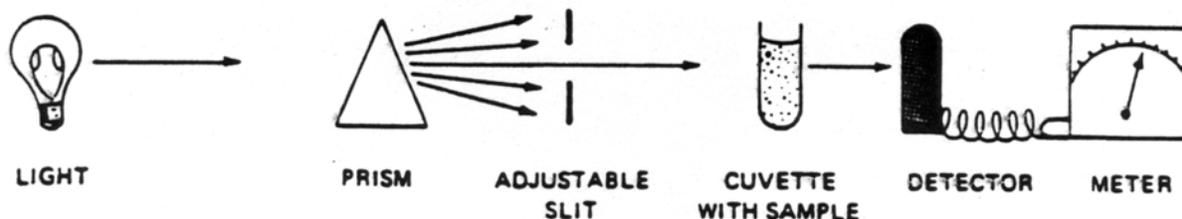
In this way, you can now see that **transmittance** refers to the light which passes through a solution. Whereas, **absorbance** refers to the light which cannot pass through a solution, but rather is absorbed by the solution.

For example, if we pass a beam of white light through a blue glass, not all the light passes through the glass to the other side — some of it is absorbed by the glass. The light which does pass through the glass is said to be transmitted and its measure is called transmittance. So, if your beam of white light is 40% absorbed by the glass, it can also be said that the light is 60% transmitted.

It is now clear (pardon the pun) that the darker or denser a substance is the more light it will absorb and the less light it will transmit.

## WHAT ARE THE PARTS OF A SPECTROPHOTOMETER?

Here is a diagram of the main parts of the instrument:



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### WHAT ARE THE SCALES ON A SPECTROPHOTOMETER?

#### Transmittance

Transmittance is an arithmetic scale that is divided into one hundred equal parts, so transmittance can be expressed as a percent. A value of 100% transmittance means that all the light has passed through the material in the cuvette and none was absorbed. A value of 0% transmittance means that no light passed through the sample in the cuvette.

#### Absorbance

Absorbance is a logarithmic scale with unequal divisions. A value of 0.0 absorbance means that no light has been absorbed by the material and all the light has been transmitted. A reading all the way at the other end of the scale means that all the light has been absorbed by the material.

### WHAT IS THE “BLANK” AND WHY DO WE HAVE TO USE IT?

All substances, clear or not, absorb *some* light. Therefore, if we wish to measure the absorbance (or the transmittance) of a material like protein in solution, we must not only consider the light absorbed by the protein, but also the light absorbed by the cuvette and the light absorbed by the solution in which the protein is dissolved.

In order to measure *only* the absorbance of the protein in the solution, we must “subtract” out the absorbance due to the cuvette and the solvent in which the protein is dissolved.

If our protein is dissolved in water, and we are trying to assess how much protein is in solution, we would ultimately want to measure how much light is absorbed by the protein alone and not the how much light is absorbed by either the water or the cuvette. In order to subtract out these factors, we prepare another cuvette with only water in it. This is called the “**blank**.” In general, the blank is a cuvette which contains everything that is in the sample (or experimental) cuvette, except the one material whose absorbance we are measuring.

To use the blank, you could measure your experimental cuvette, then measure your blank cuvette and find the difference between them. However the spectrophotometer has a built in calibration system that allows you to avoid this calculation and its much like zeroing an electronic scale. Follow this procedure:

1. Before you measure a solution in an experimental cuvette, first insert the blank cuvette into the machine. The spectrophotometer will register an absorbance value for the blank.
2. Now adjust the lower right hand dial to manually set the meter back to zero absorbance.
3. Take the blank out and now you are ready to read the absorbance of the material in solution without having your data affected by the absorbance of the cuvette or the solvent.
4. Also be aware that fingerprints on the cuvettes can affect your readings, so all cuvettes need to be wiped clean with a Kimwipe before inserting them into the spectrophotometer.

### WHAT WAVELENGTH OF LIGHT DO I USE?

Most of the material we see around due is colored due to light reflection.

Why do we see a blue sky? White light hits the layers of the atmosphere and all the colors of the spectrum are absorbed, except the color blue, which is reflected back to our eyes.

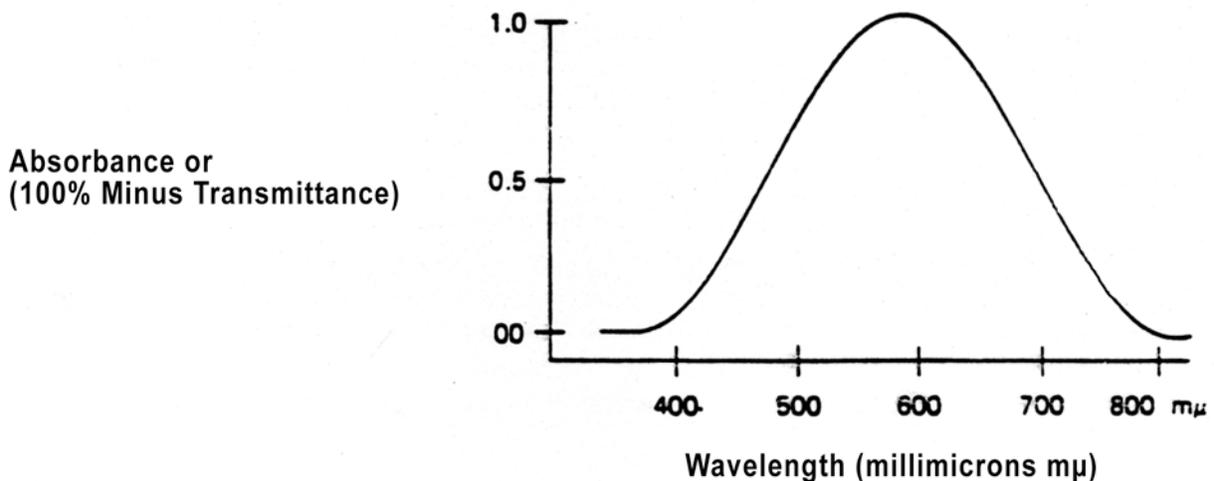
Why is your shirt red? Because white light hits the shirt fabric and all the colors of the spectrum are absorbed, except the color red, which is reflected back to our eyes.

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Everything around us absorbs certain colors (wavelengths of light) better than other colors. Since the spectrophotometer measures absorbance of light it would work the best in an experiment when we use the color light that is best absorbed by the material we are examining. In other words, the spectrophotometer shines specific wavelengths of light through the cuvette. We will get more accurate readings in our experiment, if we set the spectrophotometer to shine through the cuvette the wavelength of light that the material in the cuvette absorbs the most.

How do we know which color (wavelength) of light is absorbed best by a the material we are testing for in the cuvettes? To answer that question, we have to complete a test called an "Absorption Spectrum" on the material.

How do we run an "Absorption Spectrum"? Just measure the absorbance of the material over a range of wavelengths. Then plot a graph of absorbance vs. wavelength of light and we are likely to get a curve which looks like this:



What does this tell us?

The graph shows us that the material we are studying absorbs very little light between 400-500nm or between 700-800nm, so it would be difficult to get accurate measurements of how much material is in solution in our cuvette if the spectrophotometer was set in those wavelength ranges. But the absorbance of the material goes up very rapidly between 500-600nm and reaches a peak at about 600nm. Therefore, when we want to measure how much of this material we have in solution, it would be best if the spectrophotometer were set to this wavelength so it only shined light of wavelength 600nm through the cuvette.

Luckily, absorbance spectra have been done with most material we will test for in biology, so we don't have to develop one each time we use the spectrophotometer.

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### PROCEDURE:

#### EXPERIMENT A: THE ABSORPTION SPECTRUM

1. Set the wavelength of the spectrophotometer to 400nm
2. Prepare a **blank** cuvette with water. The cuvette should always be  $\frac{3}{4}$  full. Zero the spectrophotometer.
3. Your teacher will assign **one** of the color dyes provided. You will use this **same color dye** throughout the rest of the lab.
4. Fill a **test** cuvette  $\frac{3}{4}$  full of your dye.
5. Insert the **test** cuvette (with the dye) in the spectrophotometer and record the absorbance at 400nm.
6. Take the **test** cuvette out. Reset the wavelength to 425nm
7. Using the **blank** cuvette zero the spectrophotometer again. Remember, every time we reset the wavelength, we must zero the spectrophotometer again.
8. Insert the **test** cuvette in the spectrophotometer and record the absorbance at 425nm.
9. Repeat the whole procedure again and take the absorbance at 450nm.
10. Continue to repeat the procedure for wavelengths at 25nm intervals through 625nm,
11. Record all your data in the data table provided. Record the color of your dye.
12. Graph the data on the semi-logarithmic paper: absorbance vs. wavelength. Draw a smooth curve connecting the points on the graph. Do **not** connect the points with straight lines. The Absorption Spectrum is a **smooth-curved lined!**
13. Determine the wavelength that produced the maximum absorption for your color dye.
14. Look at the data for other color dyes from other groups. Do other color dyes have peak absorbance at the same wavelength as your color dye?
15. Fill in the chart below

Dye color	Number of Peaks	Wavelength of maximum absorbance
Red		
Blue		
Green		

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### EXPERIMENT B: THE STANDARD CURVE

1. Although a spectrophotometer can only directly measure light absorbed or transmitted, it can indirectly measure quantity of a substance in solution. If we were given a test tube containing an unknown amount of a substance, we could use the spectrophotometer to determine how much of the material was in solution. But we could only do that if we first established the absorbance of different known concentrations of the substance and then construct a curve on graph paper — this is called a Standard Curve
2. To develop a standard curve of your dye, set up 5 cuvettes. Fill each cuvette with water to the same volume.
3. Using the same color as in Experiment A take an eye dropper and put the following number of drops of dye into the corresponding tube numbers.

Tube Number	Drops of Dye
1	3
2	6
3	9
4	12
5	15

4. Now you need to take each of these cuvettes with known amounts of dye and measure their absorbance on the spectrophotometer. Set the wavelength on the spectrophotometer to the wavelength which you found to be the maximum absorbance for the colored dye in Experiment A. (Remember that was the purpose of Experiment A — doing an absorbance spectrum — to determine at which wavelength of light the spectrophotometer will work the best with a particular substance.)
5. Remember at the start of this experiment to zero-in the spectrophotometer using your “blank” cuvette, You do not have to do this again between samples since you are not changing wavelength in this experiment.

	Drops of Dye	Absorbance
1	3	
2	6	
3	9	
4	12	
5	15	

6. Plot these data on standard graph paper with the relationship: Concentration of Dye vs. Absorbance. This is what is called a Standard Curve, because it is your standard to measure solutions of unknown concentration of your material. Connect the points with a straight line going through the origin (zero). **Do not just connect the points.** The plot should be a straight line on which all your points rest or closely approach.  
You now have a graph relating known concentrations with absorbance.

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7. Now you can use this Standard Curve and the spectrophotometer to determine unknown quantities. Have a group member “secretly” make up a cuvette with an amount of dye in it — from 1 to 15 drops.
8. Now, using the spectrophotometer determine the absorbance of the unknown cuvette.
9. Use the Standard Curve to determine the concentration of our unknown.

Absorbance	Unknown Number of Drops of Dye

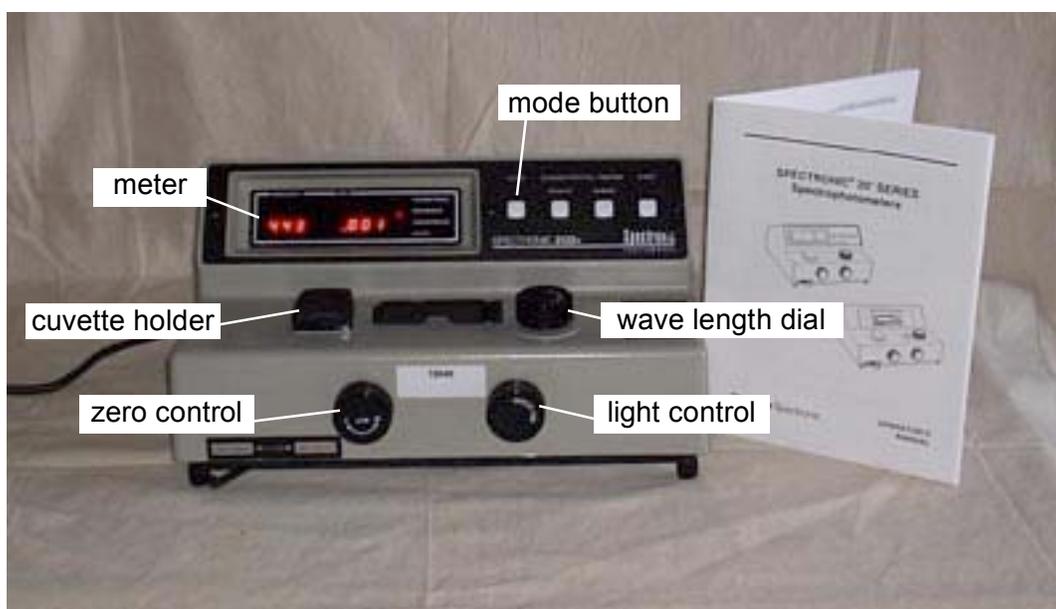
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### HOW TO OPERATE A SPECTRONIC 20D SPECTROPHOTOMETER

1. If the machine is not already warmed up, turn the machine on by rotating the **zero control knob** (left-hand knob) until the light goes on. Set your wavelength dial as instructed by your teacher. Always allow the machine to warm up for 15 minutes before using it for measurement.
2. After the machine is warmed up, use the **mode button** to set the mode to **transmittance** and turn the zero control knob (left-hand knob) until the digital meter reads zero (0% transmittance). Please make sure the cuvette holder cover is down tight and not letting any room light in.
3. Now clean your blank cuvette with soft tissue — like Kimwipes — to remove all fingerprints. Insert the blank into the cuvette holder. Make sure the mark on the cuvette is even with the mark on the front surface of the cuvette holder. Close the cuvette holder top.
4. With the blank cuvette in, rotate the **light control knob** (right-hand knob) until the meter scale reads 100% **transmittance**. Remove the blank, close the cuvette holder top. The meter should now read 0% transmittance.  
If the machine now reads 0% transmittance with the blank out and 100% transmittance with the blank in — the spectrophotometer has been “zeroed in.”

PLEASE REMEMBER THAT YOU MUST REPEAT THIS PROCEDURE AND ZERO IN THE MACHINE **EVERY** TIME YOU CHANGE THE WAVELENGTH.

5. Now insert your samples and read their absorbance or transmittance by selecting the correct mode with the mode button. From time to time it would be wise to recheck the instrument with the blank to make sure it is still zeroed in.



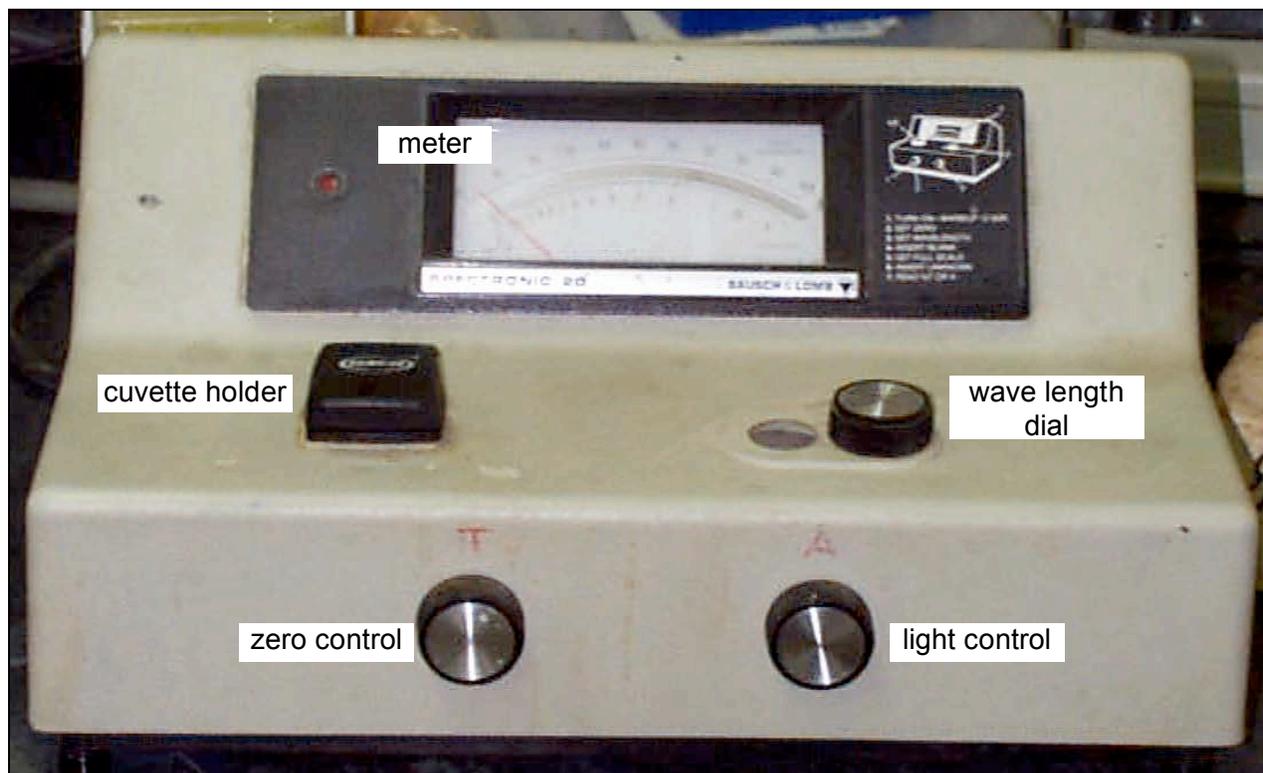
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### HOW TO OPERATE A SPECTRONIC 20 ANALOG SPECTROPHOTOMETER

6. If the machine is not already warmed up, turn the machine on by rotating the zero control knob (left-hand knob) until the light goes on. Set your wavelength dial as instructed by your teacher. Always allow the machine to warm up for 15 minutes before using it for measurement.
7. After the machine is warmed up, turn the zero control knob (left-hand knob) until the meter needle reads zero (0% transmittance). Please make sure the cuvette holder cover is down tight and not letting any room light in.
8. Clean your blank cuvette with soft tissue, like Kimwipes, to remove all fingerprints. Insert the blank into the cuvette holder. Make sure the mark on the cuvette top is even with the mark on the front surface of the cuvette holder. Close the cuvette holder top.
9. With the blank cuvette in, rotate the light control knob (right-hand knob) until the meter scale reads 100% transmittance. Remove the blank, close the cuvette holder top. The needle should now read 0% transmittance. If the machine reads 100% transmittance with the blank in and 0% transmittance with the blank out — the spectrophotometer has been “zeroed in.”

PLEASE REMEMBER THAT YOU MUST REPEAT THIS PROCEDURE AND ZERO IN THE MACHINE **EVERY** TIME YOU CHANGE THE WAVELENGTH.

10. You may now insert your samples and read their absorbance or transmittance. From time to time it would be wise to recheck the instrument with the blank to make sure it is still zeroed in.



SUMMARY QUESTIONS

1. Explain what an Absorption Spectrum is. Why did you perform that procedure first in this lab?

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2. Which pigment did you test? \_\_\_\_\_

3. Complete the chart by combining your data with your classmates' data:

Dye color	Number of Peaks	Wavelength of maximum absorbance
Red		
Blue		
Green		

4. Do the maximum wavelength of absorption data make sense for each pigment? Explain.

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5. Explain what a Standard Curve is. What was your goal in that procedure in this lab?

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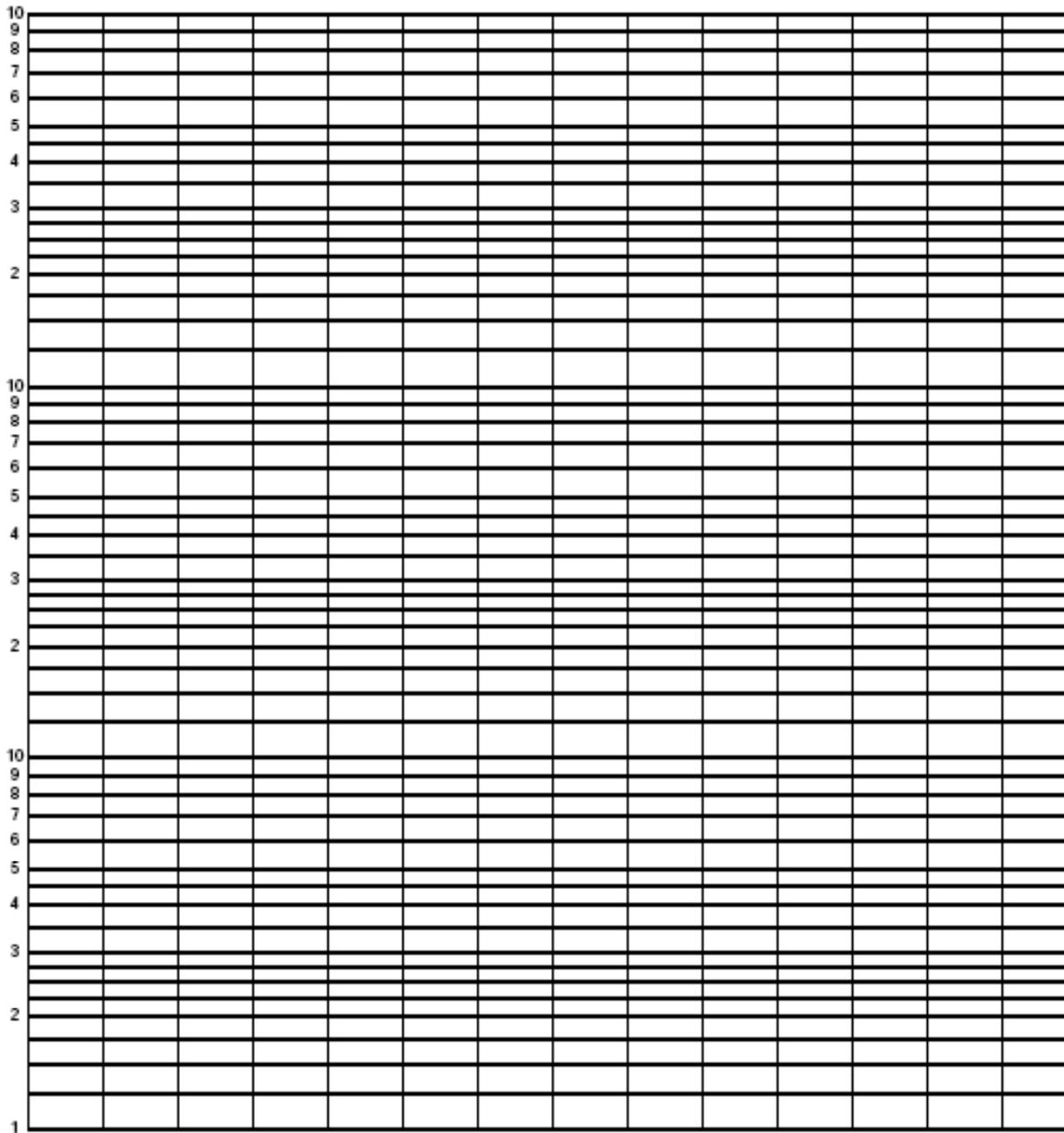
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6. Were you able to determine the concentration of your unknown cuvette? \_\_\_\_\_

Suggest possible sources of error. \_\_\_\_\_

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**Color of Dye** \_\_\_\_\_ **Maximum Absorbance** \_\_\_\_\_

<b>Wavelength</b>	<b>400</b>	<b>425</b>	<b>450</b>	<b>475</b>	<b>500</b>	<b>525</b>	<b>550</b>	<b>575</b>	<b>600</b>	<b>626</b>	<b>650</b>	<b>675</b>	<b>700</b>	<b>725</b>
<b>Absorbance</b>														