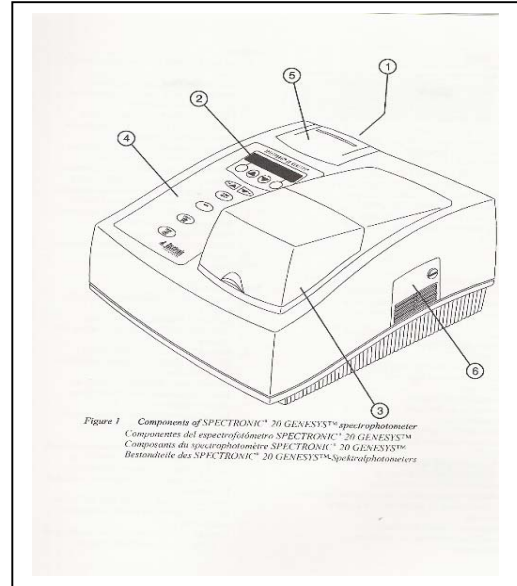


Assay using Spectrophotometry

Basics of Spectrophotometry

When visible light passes through a sample, some parts or **wavelengths** of the spectrum are absorbed and some are transmitted. **When a solution is colored, the color comes from the transmitted wavelength.** A solution appearing blue is actually transmitting blue, not absorbing it. Green plants actually absorb red light using it for photosynthesis. Orange soda looks orange because when it is hit by white light, the dye molecules in the soda absorb greenish-blue light and transmit the other wavelengths, which we see as orange (the complementary color.) **Each chemical has its own unique absorption spectrum,** or graph of absorbance against each wavelength in the spectrum.



A spectrophotometer is an instrument that can measure the amount of transmitted or absorbed light. It has a white light source that is beamed at a monochromator, which splits light into separate wavelengths. In turn the monochromator directs the specific wavelength towards the sample in a cuvette (sample container.) The sample can absorb or transmit the light directed at it. A light detector then measures the amount of light absorbed or transmitted. It provides a numerical reading as **Absorbance** or **Transmittance**. We can use this information to find the absorption spectrum of substances or to determine the quantity of a substance in solution.

Key to components

- | | |
|----------------------------|------------------------------|
| 1. On/off switch | 4. Keyboard |
| 2. LCD Display | 5. Optional Built in Printer |
| 3. Sample compartment door | 6. Lamp compartment door |

Basic Operation

Using the Spectronic® 20 genesys™ spectrophotometer you can perform absorbance and % transmittance measurements and determine concentrations using either a standard solution or a conversion factor. Regardless of the type of measurement you want to perform, you follow similar steps:

- Select the mode (A, %T, C)
- Set wavelength
- Measure the blank
- Enter the standard value or factor (concentration mode only)
- Measure the sample

Using spectrophotometry

In this exercise, you will make an **absorption spectrum** for bromophenol blue (a dye used in the laboratory for many purposes.) You will determine the **wavelength** where bromophenol blue absorbs the most light. This is called the A_{\max} . This will be used for detecting various **concentrations** of bromophenol blue.

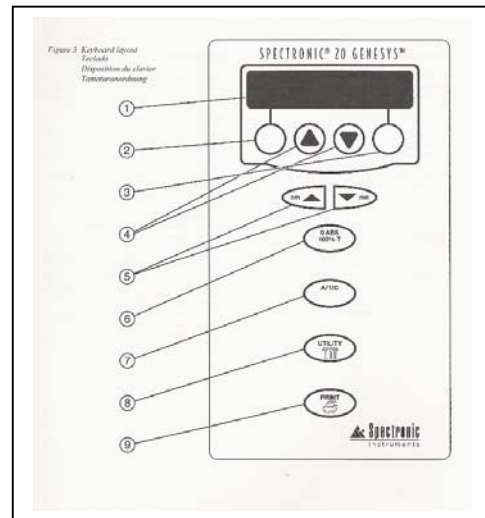
Determination of the A_{\max} of bromophenol blue

Materials

Beaker of distilled water
Bromophenol blue solution
Spectrophotometer
Cuvettes (or test tubes)
Micropipettor and tips

Method

Start by reading the SOP or Standard Operating Procedure for use of the Spec 20 Genesys. This spectrophotometer must warm up for thirty minutes before it is ready to be used.

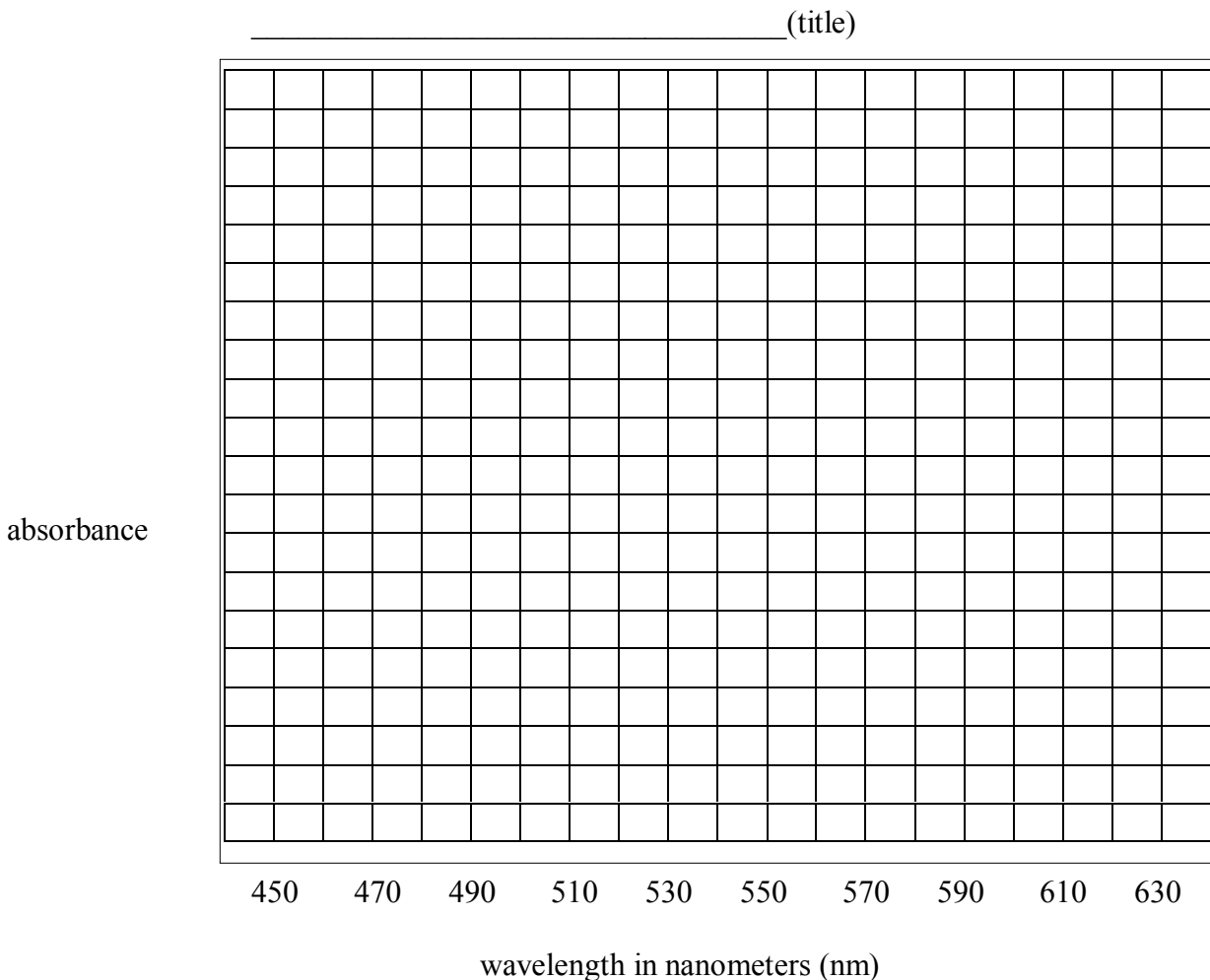


- Step 1. Fill a cuvette up to the white line with distilled water for the “blank” and another with the Bromophenol blue solution. (If you are using a test tube with no lines, use about 2.5 ml of fluid.)
- Step 2. Open the sample compartment door; insert the “blank,” press the A/T/C soft key until an A (for Absorption) reads on the LCD screen. Next, using the up or down key, set the wavelength for 480 nanometers (nm). Finally, press the 0 ABS key to set the blank.
- Step 3. Remove the blank and insert the cuvette containing the Bromophenol blue solution. A reading will appear on the LCD screen. Record that reading next to the wavelength of 480 nm in the chart below.
- Step 4. To determine the A_{\max} or the best wavelength for the Bromophenol blue solution continue this process using the range of wavelength in nanometers from 480nm to 620nm. Read the absorption of the compound every 10 nm. Record each reading in the chart. **Remember to zero the blank at each new wavelength between each reading.**
- Step 5. Rinse out cuvettes with distilled water when complete and allow to dry.

Results of Absorbance Spectrum

Wavelength	Absorbance
480 nm	
490 nm	
500 nm	
510 nm	
520 nm	
530 nm	
540 nm	
550 nm	
560 nm	
570 nm	
580 nm	
590 nm	
600 nm	
610 nm	
620 nm	

Step 7. Graph the data on the attached sheet. The *independent variable is the thing you change* (wavelength). The independent variable is always plotted on the X-axis (horizontal). The *dependent variable is the thing you measure* (absorbance) and should be plotted on the Y-axis (vertical). Use the whole page of graph paper to make your graph easy to read and most accurate. Wavelength (nm) on X-axis, Absorbance readings on the Y-axis. A graph is incomplete without a title, be sure to add one.



Effect of Concentration on Absorbance

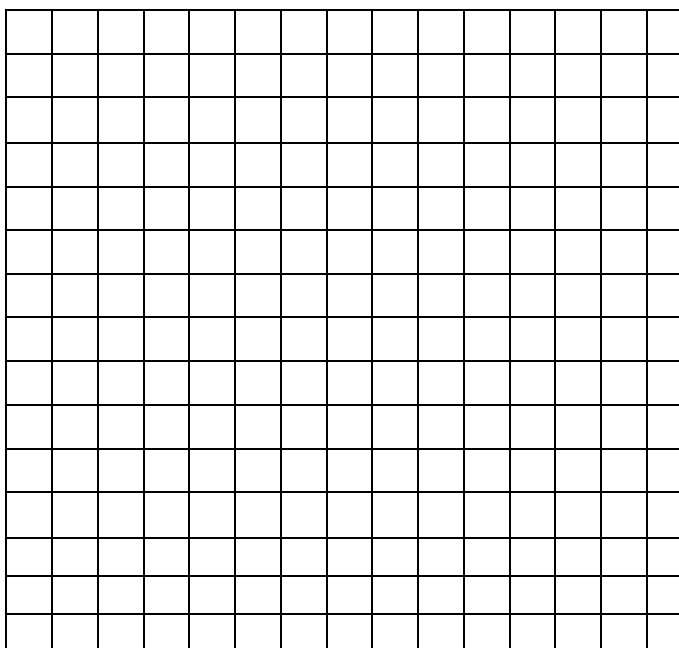
In this exercise, you will make what is known as *serial dilutions*, a very important lab technique. You will measure absorbance at A_{\max} (the wavelength you determined in part A, the peak on your graph.) Graph the data you collect from the dilutions. Use the spectrophotometer to determine the absorbance of an unknown of Bromophenol Blue, using the graph to determine its concentration.

Method

1. Obtain 4 test tubes and label them 1-4.
2. Use a P1000 micropipettor to transfer 3000 μ L of distilled water into each tube.
3. Add 3000 μ L of 18.6 μ M bromophenol blue to tube 1. This tube represents a $\frac{1}{2}$ dilution. Use a vortex mixer to mix the contents of the tube or mix thoroughly by sealing tube with parafilm and inverting several times to mix.
4. Pipette 3000 μ L from tube 1 into tube 2. This is a $\frac{1}{4}$ dilution ($\frac{1}{2}$ of $\frac{1}{2} = \frac{1}{4}$). Mix.
5. Pipette 3000 μ L from tube 2 into tube 3. Mix.
6. Pipette 3000 μ L from tube 3 into tube 4. Mix.
7. Remove 3000 μ L from tube 4 and discard down the sink.
8. Set the Spec 20 to the A_{\max} wavelength that you determined in the previous lab.
Remember to zero the spectrophotometer using your blank of distilled water.
9. Read the absorbance of tube 4 and record it in the chart below. Then read tube 3, 2 and 1, recording each (and blanking in between each!)

Tube number	Dilution	Concentration of BPB	Absorbance (nm)
1	$\frac{1}{2}$	9.3 μ M	
2	$\frac{1}{4}$		
3			
4			

10. Graph your results below. Plot the concentration on the X-axis, the absorbance on the Y-axis. Title your graph.



Identification of an unknown

Obtain a tube of Bromophenol Blue at an unknown concentration, read it on the spectrophotometer (remember to **zero by using the blank**) and use your graph (from 10 above) to determine the concentration of Bromophenol Blue in the unknown.

	Absorbance at A_{\max}	Concentration
Unknown		

Challenge Questions:

1. Suppose the reading of BPB was 1.6 which is outside the range of the graph. So you dilute the unknown in the following way: you take one mL of unknown and add it to 4 ml of distilled water, and then take a reading and get the result above. What would be the concentration of your original unknown? _____
2. How would you describe the relationship between absorbance and concentration?

3. In your graph from part B the effect of concentration on absorbance, which is:
The independent variable: _____
The dependent variable: _____
4. How do you recognize the independent variable? _____

5. List ways spectrophotometry might be useful in identifying chemicals.



Assay using Spectrophotometry

Blue is the color...

This exercise is a rewrite of one prepared by Professor John Urbance at Michigan State University posted with his permission.