

LAB #5: Using a Spectrophotometer

Objectives:

- To learn the basics of spectrophotometry and colorimetry
- To learn how to use a spectrophotometer.
- To find an unknown concentration of protein using a standard curve.

Introduction:

Spectrophotometry

Many kinds of molecules interact with or absorb specific types of radiant energy in a predictable fashion. For example, when white light illuminates an object, the color that the eye perceives is determined by the absorption by the object of one or more of the colors from the source of the white light. The remaining wavelength(s) are reflected (or transmitted) as a specific color. Thus an object that appears red absorbs the blue or green colors of light (or both), but not the red.

The perception of color, as just described is qualitative. It indicates what is happening but says nothing about the extent to which the event is taking place. The eye is not a quantitative instrument. However, there are instruments, called spectrophotometers, that electronically quantify the amount and kinds of light that are absorbed by molecules in solution. In its simplest form, a spectrophotometer has a source of white light (for visible spectrophotometry—some use UV) that is focused on a prism or diffraction grating to separate the white light into its individual bands of radiant energy. Each wavelength (color) is then selectively focused through a narrow slit. The width of this slit is important to the precision of the measurement; the narrower the slit, the more closely absorption is related to a specific wavelength of light. Conversely, the broader the slit, the more light of different wavelengths passes through, which results in a reduction in the precision of the measurement. This monochromatic (single wavelength) beam of light, called the **incident beam** (I_0), then passes through the sample being measured. The sample, usually dissolved in a suitable solvent, is contained in an optically selected **cuvette**, which should be standardized to have a light path 1 cm across.

After passing through the sample, the selected wavelength of light (now referred to as the **transmitted beam**, I) strikes a photoelectric tube. If the substance in the cuvette has absorbed any of the incident light, the transmitted light will then be reduced in total energy content. If the substance in the sample container does not absorb any of the incident beam, the radiant energy of the transmitted beam will then be about the same amount as that of the incident beam. When the transmitted beam strikes the photoelectric tube, it generates an electric current proportional to the intensity of the light energy striking it. By connecting the photoelectric tube to a device that measures electric current (a galvanometer), a means of directly measuring the intensity of the transmitted beam is achieved. In the spectrophotometers that we will use (called the Spec-20), the galvanometer has two scales: one indicates the **% transmittance (%T)**, and the other, a logarithmic scale with unequal divisions graduated from 0.0 to 2.0, indicates the **absorbance (A)**.

Because most biological molecules are dissolved in a solvent before measurement, a source of error can be due to the possibility that the solvent itself absorbs light. To assure that the spectrophotometric measurement will reflect only the light absorption of the molecules being studied, a mechanism for “subtracting” the absorbance of the solvent is necessary. To achieve this, a “blank” (the solvent) is first inserted into the instrument, and the scale is set to read

100% transmittance (or 0.0 absorbance) for the solvent. The “sample,” containing the solute *plus* the solvent, is then inserted into the instrument. Any reading on the scale that is less than 100% T (or greater than 0.0 A) is considered to be *due to absorbance by the solute only*.

As mentioned earlier, spectrophotometers are not limited to detecting absorption of only visible light. Some also have a source of ultraviolet light (usually supplied by a hydrogen or mercury lamp), which has wavelengths that range from about 180 to 400 nm. Ultraviolet wavelengths ranging from 180 to 350 nm are particularly useful in studying such biological molecules as amino acids, proteins, and nucleic acids because each of these compounds have characteristic absorbances at different UV wavelengths. Other spectrophotometers use infrared radiation (from 780 to 25,000 nm) as well.

Units of Measurement

The following terminology is commonly used in spectrophotometry.

- **Transmittance (T)**: the ratio of the transmitted light (I) of the sample to the incident light (I_0) on the sample.

$$T = \frac{I}{I_0}$$

This value is multiplied by 100 to derive the % T. For example:

$$\% T = \frac{75}{100} = 0.75$$

- **Absorbance (A)**: logarithm to the base 10 of the reciprocal of the transmittance:

$$A = \log_{10} \frac{1}{T}$$

For example:

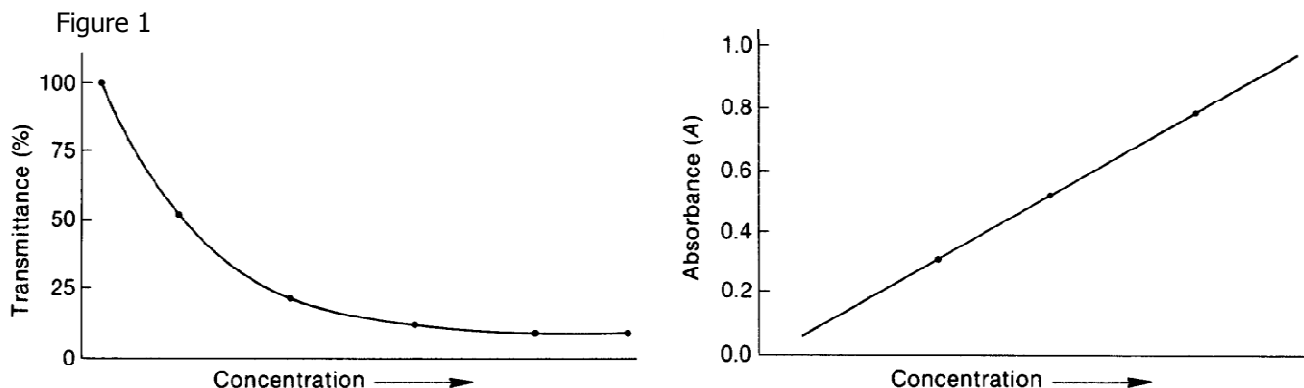
1. Suppose a % T of 50 was recorded (equivalent to $T = 0.50$).
2. Then $A = \log_{10} (1/0.50) = \log_{10} 2.0$.
3. Thus $\log_{10} 2.0 = 0.301$ (A equivalent to a % T of 50).

Similarly a % T of 25 = 0.602 A; a % T of 75 = 0.125 A; and so forth.

The absorbance scale is normally present along with the transmittance scale on spectrophotometers. The chief usefulness of absorbance lies in the fact that it is a logarithmic rather than arithmetic function, allowing the use of the Lambert-Beer law (Beer’s Law), which states that for a given concentration range the concentration of solute molecules is directly proportional to absorbance. This law can be expressed as

$$\log_{10} \frac{I_0}{I} = A$$

in which I_0 is the intensity of the incident light; I is the intensity of the transmitted light.



The usefulness of absorbance can be seen in the graphs shown in **Figure 1**, one graph showing the percent transmittance plotted against concentration and the other showing absorbance plotted against concentration. Using the Lambert-Beer relationship, it is necessary to plot only three or four points to obtain the straight-line relationship shown in the bottom graph of the two. However, certain conditions must prevail for the Lambert-Beer relationship to hold:

1. Monochromatic light is used.
2. A_{\max} is used (i.e., the wavelength maximally absorbed by the substance being analyzed).
3. The quantitative relationship between absorbance and concentration can be established.

The first condition can be met by using a prism or diffraction grating or other device that can disperse visible light into its spectra.

The second condition can be met by determining the absorption spectrum of the compound. This is done by plotting the absorbance of the substance at a number of different wavelengths. The wavelength at which absorbance is greatest is called the A_{\max} (or λ_{\max}) and is the most satisfactory wavelength to use because, on the slope, absorbance changes rapidly with slight wavelength deviations, whereas at the maximum absorbance, changes in wavelength alter absorbance less. **Figure 2** shows an absorption spectrum of a hypothetical substance having an A_{\max} of approximately 650 nm. Some compounds, however, can have several peaks both in the visible spectrum and in the ultraviolet range. An example of this is shown in **Figure 3** for riboflavin.

Figure 2

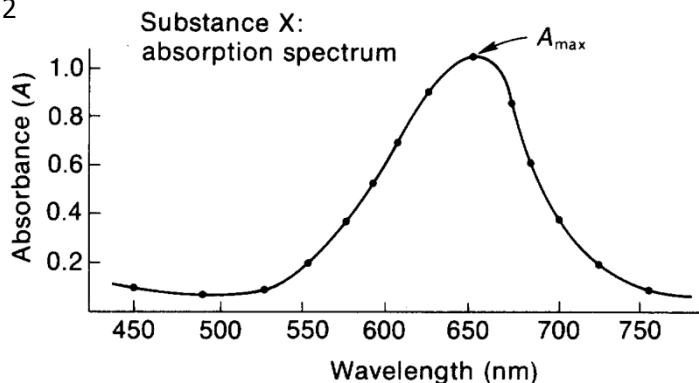
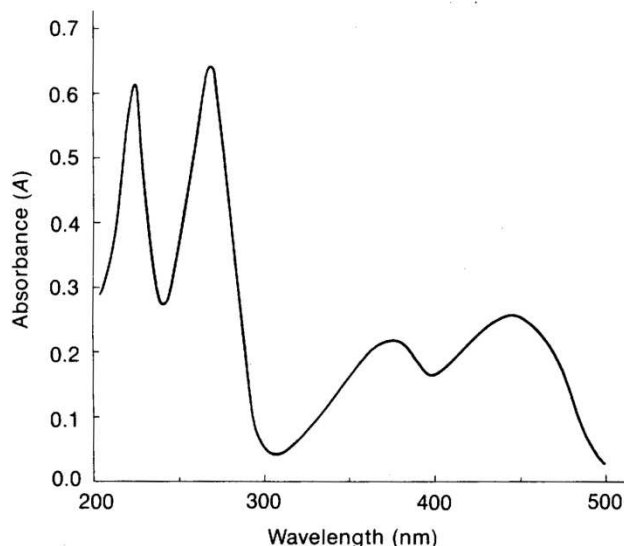
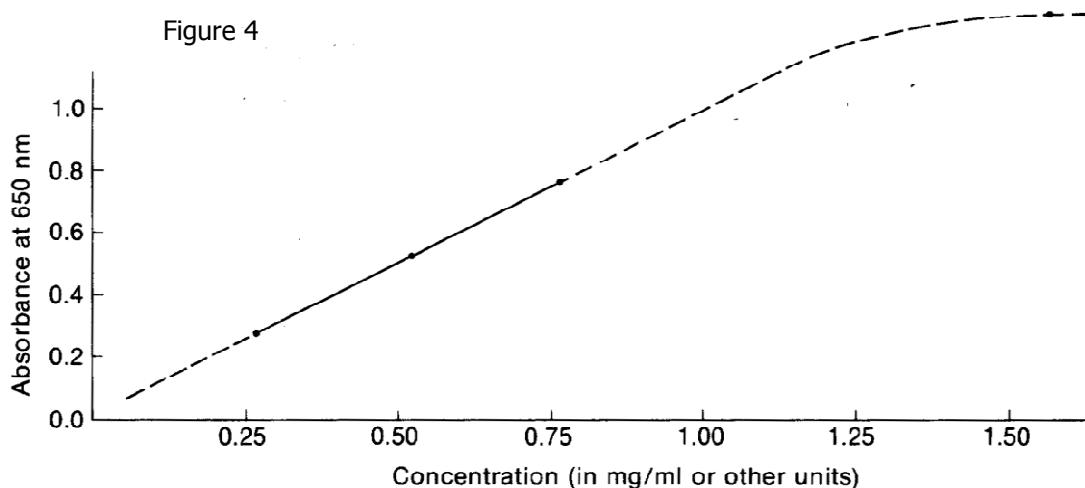


Figure 3

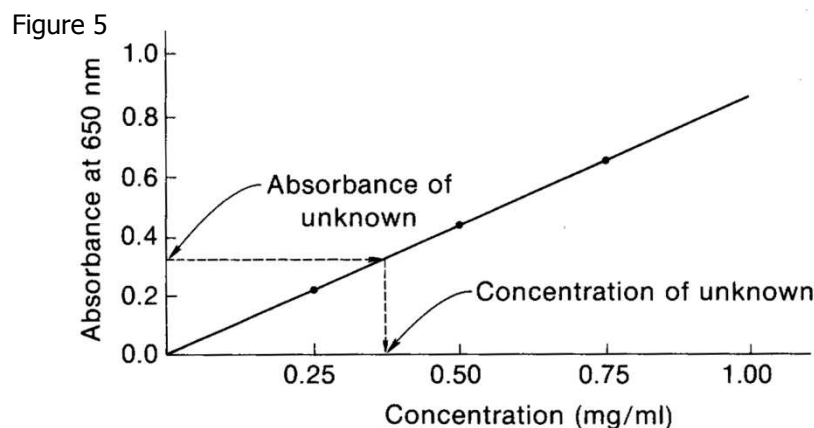


To establish the quantitative relationship between absorbance and concentration of the colored substance, it is necessary to prepare a series of standards of the substance analyzed in graded known concentrations (a.k.a. color standards). Because absorbance is *directly* proportional to concentration, a plot of absorbance versus concentration of the standard yields a straight line. Such a plot is called a **standard curve** or **calibration curve** as shown in **Figure 4**. After several points have been plotted, the intervening points can be extrapolated by connecting the known points with a straight line. It is not necessary to use dotted lines to indicate extrapolation on graphs; a dotted line is used in the illustration to indicate the parts of the line for which points were not determined but were presumed. When the Lambert-Beer law is followed, this is an acceptable and time-saving assumption; otherwise, points would be needed to be plotted throughout the entire line. In general, your graph should extend from a minimum of about 0.025 A to a maximum of about 1.0 A, or from 94% T to 10% T, this being the “readable” and reproducible values of absorbance. However, recall that the Lambert-Beer law operates at only certain concentrations. This is apparent in **Figure 4**, in which, at concentrations greater than 1.0 mg/mL, the curve slopes, indicating the loss of the concentration-absorbance relationship.

Figure 4



After a concentration curve for a given substance has been established, it is relatively easy to determine the quantity of that substance in a solution of unknown concentration by determining the absorbance of the unknown and locating it on the y -axis, or ordinate (**Figure 5**). A straight line is then drawn parallel to the x -axis, or abscissa, until it intersects with the experimental curve. A perpendicular is then dropped to the x -axis, the value at the point of intersection indicating the concentration of the unknown solution. In this example, the unknown absorbance is 0.32, which indicates a concentration of about 0.37 mg. Concentrations are commonly expressed either as micrograms per milliliter ($\mu\text{g/mL}$) or as milligrams per milliliter (mg/mL).



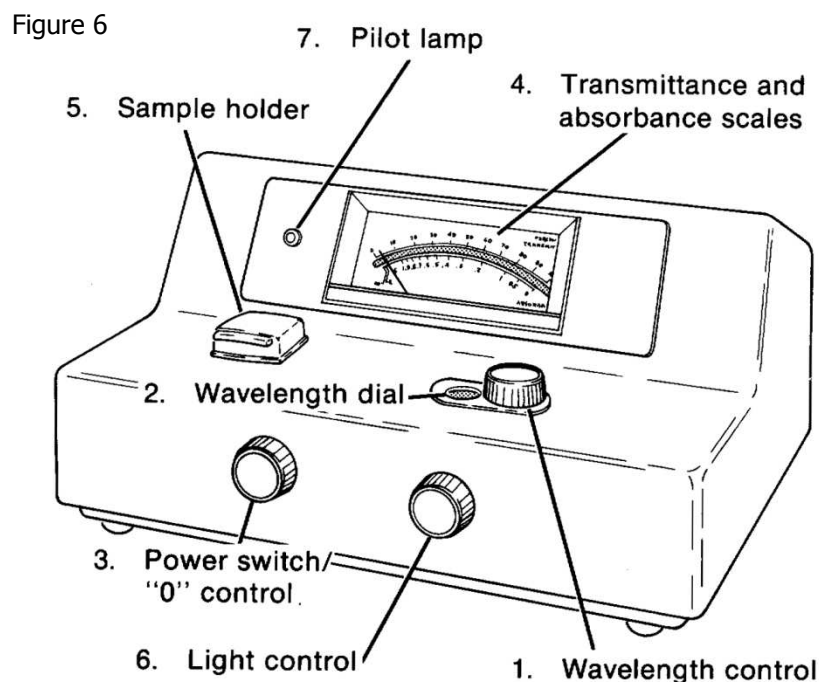
If the absorbance value of the unknown is such that the line drawn parallel to the x -axis intersects the experimental curve where it is curved (**Figure 4**), then you cannot accurately determine the concentration. In this event, dilute the unknown by some factor until the absorbance readings intersect with the straight-line part of the graph where concentration is proportional to absorbance. You can then determine the unknown concentration and multiply the value by the dilution factor.

Quantitative Chemical Determination of Protein

In protein molecules, the successive amino acid molecules are bonded together between the carboxyl and amino groups of adjacent amino acids to form long, unbranched molecules. Such bonds are commonly called **peptide bonds**. The structures resulting from the formation of peptide bonds are called dipeptides, tripeptides, or polypeptides, depending on the number of amino acids involved. The individual amino acids are called **residues**.

Biuret, a simple molecule prepared from urea, contains what may be regarded as two peptide bonds and thus is structurally similar to simple peptides. This molecule when treated with copper sulfate in alkaline solution (the biuret reaction) gives an intense purple color. The reaction is based on the formation of a purple-colored complex between copper ions and two or more peptide bonds. Proteins give a particularly strong biuret reaction because they contain a large number of peptide bonds. The biuret reaction may be used to quantitate the concentration of proteins because peptide bonds occur with approximately the same frequency per gram of material for most proteins.

In this experiment, you will determine the concentration of unknown protein solutions by measuring colorimetrically the intensity of their color production in the biuret reaction as compared with the color produced by a known concentration of the protein albumin.

Procedure:**Calibration of the Spectrophotometer**

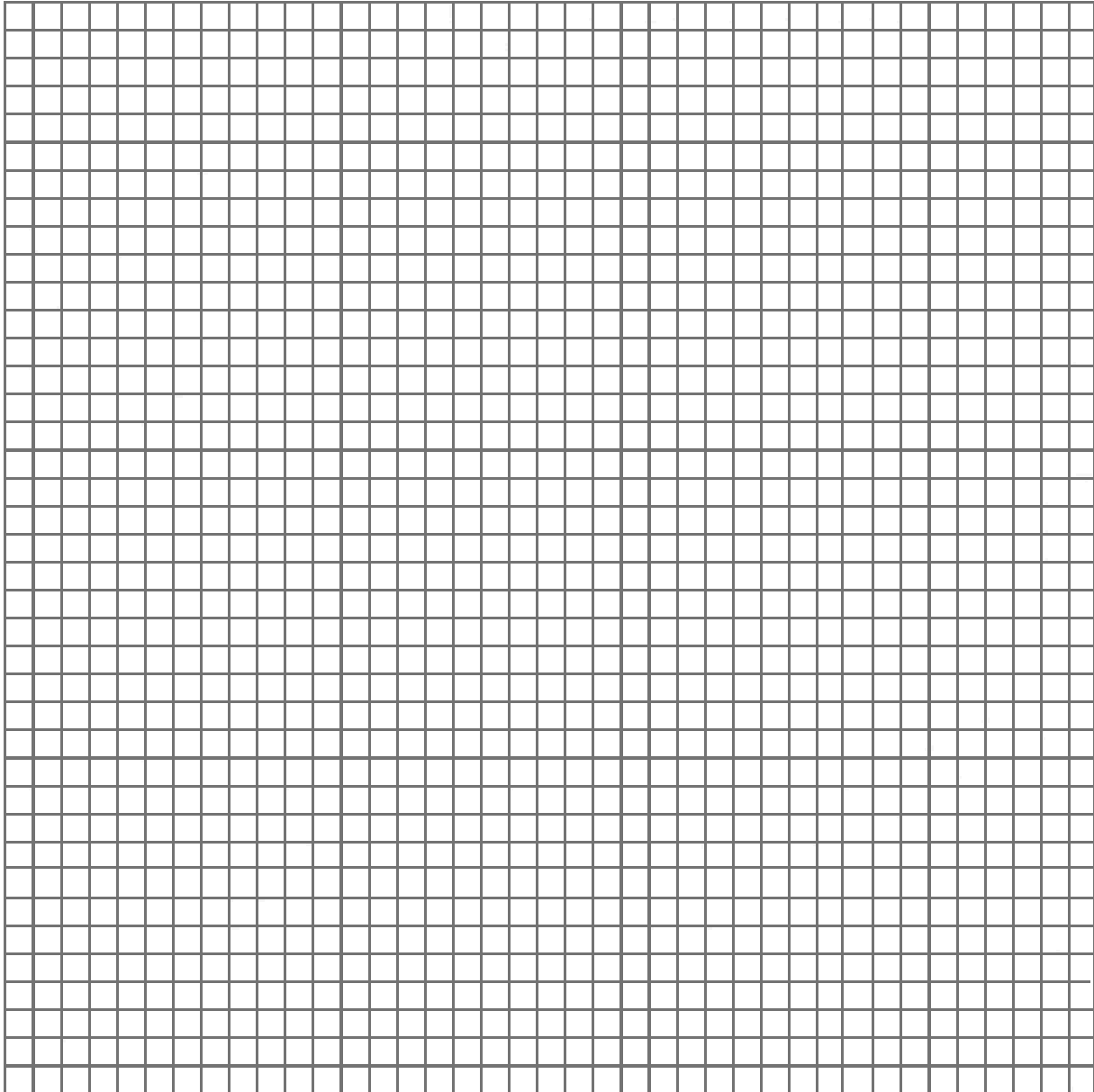
1. Rotate the wavelength control (1) shown in **Figure 6** until the desired wavelength is shown on the wavelength dial (2). The wavelength for a given substance can be found by referring to the literature or by determining it experimentally.
2. Turn the instrument on by rotating the "0" control (3) in a clockwise position. Allow at least 5 minutes for the instrument to warm up.
3. Adjust the "0" control with the cover of the sample holder closed (5) until the needle is at 0 on the transmittance scale (4).
4. Place a cuvette containing water or another solvent in the sample holder, and close the cover.
5. Rotate the light control (6) so that the needle is at 100 on the transmittance scale (0.0 absorbance). This control regulates the amount of light passing through the second slit through the phototube.
6. The unknown samples may then be placed in the tube holder, and the percentage of transmittance or absorbance can be read. The needle should always return to zero when the tube is removed. Check the 0% and 100% transmittance occasionally with the solvent tube in the sample holder to make certain the unit is calibrated. *Note:* Always check the wavelength scale to be certain that the desired wavelength is being used.

Using a Standard Curve to Determine an Unknown

7. Prepare a set of six test tubes, containing increasing amounts of a standard solution of albumin and 0.5 M KCl, as shown in **Table 1**.

Tube Number	Albumin 2.5 mg/mL (mL)	Total protein content (mg)	0.5 M KCl (mL)	Biuret reagent (mL)	%T	A ₅₄₀
1	0.0	0.0	5.0	0.5	100	0.0
2	1.0	2.5	4.0	0.5		
3	2.0	5.0	3.0	0.5		
4	3.0	7.5	2.0	0.5		
5	4.0	10.0	1.0	0.5		
6	5.0	12.5	0.0	0.5		
()			---	0.5		
()			---	0.5		

8. Add 0.5 mL of biuret reagent to each of the tubes, and mix thoroughly by rotating the tubes between your palms. The color is fully developed in 20 minutes and is stable for at least an hour. While waiting for the color to develop, calibrate your instrument at 540 nm using Tube 1—which is a blank containing all reagents except protein.
9. Read Tubes 2 through 6 against it. Record your readings in **Table 1**. Convert the % T to absorbance (A) for Tubes 1 through 6 using **Table 2** and then plot your data to obtain a standard curve for albumin. Do this on the graph paper provided, and also use Excel to do the same (for Excel, do a regression analysis to check the reliability of your standard curve).
10. Using the standard curve, determine the concentrations of your unknown protein solutions using both your handwritten graph and the Excel spreadsheet/graph.



%T	Absorbance (A)				%T	Absorbance (A)			
	.25	.50	.75			.25	.50	.75	
1	2.000	1.903	1.824	1.757	51	.2924	.2903	.2882	.2861
2	1.699	1.648	1.602	1.561	52	.2840	.2819	.2798	.2777
3	1.532	1.488	1.456	1.426	53	.2756	.2736	.2716	.2696
4	1.398	1.372	1.347	1.323	54	.2676	.2656	.2636	.2616
5	1.301	1.280	1.260	1.240	55	.2596	.2577	.2557	.2537
6	1.222	1.204	1.187	1.171	56	.2518	.2499	.2480	.2460
7	1.155	1.140	1.126	1.112	57	.2441	.2422	.2403	.2384
8	1.097	1.083	1.071	1.059	58	.2366	.2347	.2328	.2319
9	1.046	1.034	1.022	1.011	59	.2291	.2273	.2255	.2236
10	1.000	.989	.979	.969	60	.2218	.2200	.2182	.2164
11	.959	.949	.939	.930	61	.2147	.2129	.2111	.2093
12	.921	.912	.903	.894	62	.2076	.2059	.2041	.2024
13	.886	.878	.870	.862	63	.2007	.1990	.1973	.1956
14	.854	.846	.838	.831	64	.1939	.1922	.1905	.1888
15	.824	.817	.810	.803	65	.1871	.1855	.1838	.1821
16	.796	.789	.782	.776	66	.1805	.1788	.1772	.1756
17	.770	.763	.757	.751	67	.1739	.1723	.1707	.1691
18	.745	.739	.733	.727	68	.1675	.1659	.1643	.1627
19	.721	.716	.710	.704	69	.1612	.1596	.1580	.1565
20	.699	.694	.688	.683	70	.1549	.1534	.1517	.1503
21	.678	.673	.668	.663	71	.1487	.1472	.1457	.1442
22	.658	.653	.648	.643	72	.1427	.1412	.1397	.1382
23	.638	.634	.629	.624	73	.1367	.1652	.1337	.1322
24	.620	.615	.611	.606	74	.1308	.1293	.1278	.1264
25	.602	.598	.594	.589	75	.1249	.1235	.1221	.1206
26	.585	.581	.577	.573	76	.1192	.1177	.1163	.1149
27	.569	.565	.561	.557	77	.1135	.1121	.1107	.1093
28	.553	.549	.545	.542	78	.1079	.1065	.1051	.1037
29	.538	.534	.530	.527	79	.1024	.1010	.0996	.0982
30	.532	.520	.516	.512	80	.0969	.0955	.0942	.0928
31	.509	.505	.502	.496	81	.0975	.0901	.0888	.0875
32	.495	.491	.488	.485	82	.0862	.0848	.0835	.0822
33	.482	.478	.475	.472	83	.0809	.0796	.0783	.0770
34	.469	.465	.462	.459	84	.0757	.0744	.0731	.0718
35	.456	.453	.450	.447	85	.0706	.0693	.0680	.0667
36	.444	.441	.438	.435	86	.0655	.0672	.0630	.0617
37	.432	.429	.426	.423	87	.0605	.0593	.0580	.0568
38	.420	.417	.414	.412	88	.0555	.0543	.0531	.0518
39	.409	.406	.403	.401	89	.0505	.0494	.0482	.0470
40	.398	.395	.392	.390	90	.0458	.0446	.0434	.0422
41	.387	.385	.385	.380	91	.0410	.0396	.0386	.0374
42	.377	.374	.372	.369	92	.0362	.0351	.0339	.0327
43	.367	.364	.362	.359	93	.0315	.0304	.0292	.0281
44	.357	.354	.352	.349	94	.0269	.0257	.0246	.0235
45	.347	.344	.342	.340	95	.0223	.0212	.0200	.0188
46	.337	.335	.332	.330	96	.0177	.0166	.0155	.0144
47	.328	.325	.323	.321	97	.0132	.0121	.0110	.0099
48	.319	.317	.314	.312	98	.0088	.0077	.0066	.0055
49	.310	.308	.305	.303	99	.0044	.0033	.0022	.0011
50	.301	.299	.297	.295	100	.0000	.0000	.0000	.0000

Note: Intermediate values may be arrived at by using the .25, .50, and .75 columns. For example, if %T equals 85, the absorbance equals .0706. If %T equals 85.75 the absorbance equals .0067