EXPERIMENT 4
ABSORPTION SPECTROPHOTOMETRY: DIRECT-MEASUREMENT OPTION

This is a group experiment. People in each group will share one set of calibration data. Each person will process an individual unknown.

I. INTRODUCTION/PURPOSES
This experiment introduces the direct-measurement approach to absorption spectrophotometry. Principal purposes of the study are:

• to understand quantitative relationships between transmittance, absorbance and concentration,
• to use spectrophotometric data to quantify the ferrous iron concentration in an unknown sample and
• to understand relationships between measurement errors, sensitivity and concentration errors.

II. OVERVIEW
In absorption spectroscopy, one measures the intensity of radiant energy transmitted through a sample and relates the measured intensity to concentration or some other property of interest. Applications of absorption spectroscopy involve most of the regions of the electromagnetic spectrum ranging from high-energy processes such as the absorption of x-rays to low-energy processes such as the microwave region. Although this experiment will focus on absorption in the visible region, most of the principles you will discover are valid for the absorption of energy throughout the electromagnetic spectrum.

A. Example reaction
The reaction chosen for this study involves the complex ion formed between 1,10-orthophenanthroline (phen) and iron (II) (ferrous iron).

\[
\text{Fe(II)} + 3 \text{phen} \leftrightarrow \text{Fe(phen)}_3^{2+} \quad \text{(Red, } \lambda_{\text{max}} = 508 \text{ nm)} \quad \text{(1)}
\]

By using an excess of phen at an appropriate pH it is possible to force the reaction to the right converting virtually all of the Fe(II) to the complex ion. Because both reactants are colorless, the amount of light absorbed near 505 nm is related to the Fe(II) concentration.

B. Quantitative relationships
The quantity “measured” in this experiment is the percent transmittance, T(%). Percent transmittance is converted to transmittance, T, by dividing by 100.

\[
\text{T} = \frac{T(\%)}{100} \quad \text{(2)}
\]

Transmittance is related to a quantity called absorbance as follows.

\[
A = -\log T \quad \text{or} \quad T = 10^{-A} \quad \text{(3)}
\]
Under controlled conditions, the absorbance is expected to vary linearly with concentration $C$ as follows

$$A = \alpha + \beta C$$

(4)

The relationship between transmittance and concentration obtained by combining Equations 3 and 4 is

$$T = 10^{-A} = 10^{-(\alpha + \beta C)}$$

(5a)

Noting that $10 = e^{\ln(10)}$, Eq. 5a can be written as

$$T = e^{-\ln(10)\left(\alpha + \beta C\right)}$$

(5b)

where $\ln(10) = 2.3026$. This equation is used in the photometric error equations attached to this experiment. The photometric error equations will be used to analyze data obtained in this experiment.

C. Generalization

Although the Lambert/Beer/Bouguer law (Eq. 4 above) was described above in the context of absorption of visible light, it applies as well to the absorption of radiant energy throughout the electromagnetic spectrum ranging from very short wavelengths in the X-ray region to very long wavelengths in the microwave region. Accordingly, most of what you discover in this exercise is applicable to absorption methods in virtually all regions of the electromagnetic spectrum.

III. INSTRUMENTATION AND SOLUTIONS

A. Extra glassware

In addition to glassware in your equipment drawer, each group will need one 500-mL volumetric flask, ten 100-mL volumetric flasks and a box of twelve cuvets for the spectrophotometer. Each person in a group will need to use their own 100-mL volumetric flask.

B. Instrumentation

Each group will be assigned one spectrophotometer (Spectronic Genesys.). The spectrophotometer should be placed in the location where it will be used and turned on at least 20 minutes prior to use so that the circuitry and internal temperature will stabilize before taking measurements.

C. Solutions

All solutions are to be prepared in deionized water. Because you will be working with dilute solutions of iron in this experiment and because tap water contains significant concentrations of iron, you should be very careful to avoid contamination of your solutions with tap water. In other words, all glassware should be rinsed thoroughly with deionized water before use.

1. Solutions provided
   a. Diluted sulfuric acid, 0.7 M. This solution is prepared by adding 40 mL of concentrated sulfuric acid (BE CAREFUL) to approximately 1 L of deionized water in a beaker, mixing thoroughly and allowing to cool to room temperature. Obtain about 50 mL of this solution in a small beaker.
   b. Hydroxylamine hydrochloride, 10 g/100 mL. This solution is prepared by dissolving approximately 2.5 g of hydroxylamine hydrochloride ($\text{H}_2\text{NOH} \cdot \text{HCl}$) in approximately 25 mL of water in a small beaker. Obtain about 15 mL of this solution in a small beaker.
c. 1,10-phenanthroline, 0.1 g/100 mL (180 mL required). This solution is prepared by dissolving approximately 0.2 g of orthophenanthroline monohydrate in 200 mL of water. If necessary, the mixture is warmed gently and stirred to ensure complete dissolution. The solution stored in the dark until it is used. If the solution darkens at any step in the process, discard it and prepare another solution. Obtain about 200 mL of this solution in a small beaker.

d. Sodium acetate, 1.22 M. Obtain about 100 mL of this solution. This solution is prepared by dissolving approximately 10 g of sodium acetate in 100 mL of water in a small beaker. Obtain about 100 mL of this solution in a small beaker.

2. Solutions you must prepare
a. Standard ferrous ammonium sulfate solutions. Prepare two stock solutions of ferrous ammonium sulfate as described below. Each group should prepare standard solutions and each person should prepare her/his unknown sample.
   i. Stock 1. Weigh approximately 0.210 grams of reagent grade ferrous ammonium sulfate hexahydrate (FeSO₄(NH₄)₂SO₄·6H₂O, MM = 392.14) to the nearest tenth of a milligram, transfer the salt carefully to a small beaker and dissolve the salt in 12.5 mL (graduated cylinder) of the diluted sulfuric acid solution. When the salt is completely dissolved, transfer the solution quantitatively to a 500-mL volumetric flask using at least five rinses with small volumes of deionized water to ensure that all the solution is transferred to the volumetric flask. Dilute the solution in the volumetric flask to the calibrated mark and mix thoroughly.

   ii. Stock 2. Pipet 25.00 mL of the Stock 1 solution into a clean 250-mL volumetric flask, add 5 mL of the diluted sulfuric acid solution to the flask, dilute to volume and mix thoroughly.

b. Unknowns. Your unknown will be provided in a 250-mL volumetric flask. The unknown already contains sulfuric acid, dilute to volume with deionized water and mix thoroughly.

D. Reaction step (Color development)
Standard and unknown solutions prepared as described above will be used to develop the iron/phenanthroline complex ion in a blank, calibration standards and an aliquot of your unknown solution. Each group should prepare one blank and one set of standard solutions and each person should prepare her/his unknown sample.

1. Blank. To a clean but not necessarily dry 100-mL volumetric flask add 1 mL of the hydroxylamine hydrochloride solution, 10 mL of the sodium acetate solution and 20 mL of the orthophenanthroline solution, dilute the solution to volume and let stand at least 5 min before making measurements as described later.

   Note: For the standard solutions below, it isn’t necessary that you add the exact volumes of Stock 1 and Stock 2 solutions but it is important that you record the exact volumes added to the nearest 0.02 mL.

3
2. **Set 1 calibration standards.** Rinse and fill one clean 50-mL buret with *Stock 2* solution. Use this buret to add 4.00, 8.00, 16.0, 32.0 and 50.0 mL of the *Stock 2* solution to each of five clean but not necessarily dry 100-mL volumetric flasks. To each flask add 1 mL of the hydroxylamine hydrochloride solution, 10 mL of the sodium acetate solution and 20 mL of the orthophenanthroline solution, dilute the solutions to volume, mix well, and let stand at least 5 min before making measurements as described later. **Note and record the appearances of the different solutions.**

3. **Set 2 calibration standards.** Rinse and fill a second clean 50-mL buret with *Stock 1* solution. Following the same procedure as for *Group 1 calibration standards*, prepare a second set of calibration standards containing 5.00, 10.00, and 20.0 mL of *Stock 1* solution per 100 mL of diluted standard. **Note and record the appearances of these solutions.**

4. **Unknown sample.** Pipet a 25-mL aliquot of your diluted unknown solution into a clean 100-mL volumetric flask, add the color-forming reagents as described above for the standard solutions, dilute to volume and mix thoroughly.

**IV. MEASUREMENT STEP**

Two groups of data will be collected. The first group (Section A below) is used to prepare a calibration plot, to quantify the ferrous iron in your unknown samples and to evaluate the imprecision of replicate measurements. The second group (Section B below) is used to evaluate effects of stray light and wavelength error on results.

**A. Calibration/Reproducibility/Quantitation of unknowns**

This part of the experiment illustrates a calibration process and determination of unknown concentration. It is sufficient to obtain data for one calibration plot per group.

1. **Cuvets.** Although it would be best to use the same cuvet for all solutions, to save time, it is suggested that you use a separate cuvet for each solution (total of 10) prepared above.

   Holding each cuvet near the top to avoid getting fingerprints in the area where the light beam will pass through it, rinse and fill the eleven cuvets to within about 1.5 cm of the top with the blank and calibration standards. Wipe the outside of each cuvet gently with a lint-free tissue to remove any fingerprints or solution that may be on the surface.

2. **Spectrophotometer settings (use the Spectronic Genesys spectrophotometer).** Some preliminary settings are needed before you begin making measurements with the spectrophotometer.

   a. **Mode and wavelength.** Set the mode control to percent transmittance by pressing the A/T/C button until 100%T shows in the display. Adjust the wavelength to 505 nm by pressing either the nm ▲ or nm ▼ buttons.

   b. **Percent transmittance (100 % T).** Place the cuvet containing the blank solution into the cuvet well with the vertical marking on the cuvet aligned with the reference point on the cuvet well, close the cover, and press the 0 ABS 100%T button which allows the instrument to read 100%T for the blank.
Observe the meter for a few minutes and record any variations in the 100 %T readout.

3. Transmittances of standards and unknowns. Measure and record the percent transmittance at 505 nm three times for each calibration standard. Repeat this process for each unknown using a separate cuvet for each. Each person should prepare a record of all readings for all standards and her/his unknown before leaving the laboratory. These data will be used to prepare calibration plots, to calculate the ferrous iron concentrations in your unknowns and to evaluate the imprecision of replicate measurements.

B. Effects of wavelength errors and stray light
The purpose of this section is to study effects of two variables, namely wavelength error and stray light, in absorption spectrophotometry.

1. Effects of wavelength error. Measure and record the percent transmittance at 505, 535 nm for all five Set 1 calibration standards. Then measure the transmittances at 508 and 538 nm for the solution containing 16.0 mL of Stock 2 solution. Don’t forget to use the blank to reset the 100 %T reading when you change from one wavelength to another.

2. Effects of stray light. To emulate the effect of the stray light we shall combine the results of the measurements at two different wavelengths. Set the wavelength to 580 nm, use the blank to reset the 100 %T reading, then measure the transmittance for each of the eight calibration samples.

C. Least significant digit (LSD)
The smallest difference in readings that can be resolved with a digital instrument is called the least significant digit (LSD). Results obtained with a digital instrument can be no better than ± one-half the least significant digit.
Note that the smallest change in percent transmittance that can be resolved on the digital readout is 0.1 (in units of %T). This means that the imprecision of transmittances read with this instrument can be no better than ± (1/2) (0.1 units of % T) = ± 0.05 units of % T. This is equivalent to ± 0.0005 in units of T.
V. DATA PROCESSING AND INTERPRETATION

You are encouraged to use a spreadsheet (e.g. Excel) to do calculations and Origin software to plot data and to fit models to your data. Worksheets should be arranged with the independent variable (Concentration) in Column A and values of dependent variables (e.g. transmittance and absorbance) in subsequent columns.

Before proceeding with other aspects of the data processing, convert all percent transmittances, T(%), to transmittances, T, by dividing by 100. This can be done either by moving the decimal place two places to the left as you enter your data in the spreadsheet or by dividing data by 100 in the spreadsheet.

A. Means and standard deviations
Calculate the mean and standard deviation of the transmittance for all standard solutions. Use the mean values of transmittance in all calculations/plots below and use the individual standard deviations to calculate the pooled standard deviation (s_{T,p}) of transmittance.

If the pooled standard deviation is greater than 0.0005 in units of T (one half the least significant digit) then use the pooled standard deviation as the standard deviation of transmittance (s_{T} = s_{T,p}) in calculations below. Conversely, if the pooled standard deviation is less than 0.0005 in units of T, then use 0.0005 as the standard deviation of transmittance in calculations below (s_{T} = (1/2) LSD = 0.0005).

B. Linear calibration plot
Use the mean values of the transmittances at each concentration to calculate absorbance at each concentration. Prepare a plot of absorbance vs. concentration for the five standard solutions in the Set 1 calibration standards. Do a linear least-squares fit of the data for absorbance vs. concentration and express the results as

\[ A = \alpha \pm s_\alpha + (\beta \pm s_\beta)C \] with \( s_r = ? \) and \( r = ? \).

in which \( \alpha \) and \( \beta \) are the intercept and slope, \( s_\alpha \) and \( s_\beta \) are the standard deviations of the intercept and slope, \( s_r \) is the standard error of the estimate, \( r \) is the correlation coefficient and \( C \) is concentration. Include the plot with the least-squares fit as Figure 1 in your report.

C. Unknown concentration
Use the slope and intercept of data from the fit in Figure 1 with the value of absorbance for your unknown to calculate the ferrous iron concentration (mol/L) in your unknown after dilution to 250 mL.

Use the standard error of the estimate and an appropriate equation from the least-squares discussion (Eq 8-18 in the text) to calculate the standard deviation of the unknown concentration. Calculate and report the **mass (grams) of iron in the 250 mL flask from which your unknown was taken.** (Don't forget to take account of the second dilution of your unknown.)
D. Transmittance vs. concentration
Prepare a scatter plot of mean transmittance vs. concentration including data for all calibration standards on one graph. Use an appropriate equation from the section on quantitative relationships to rationalize the shape of the plot.

Use a suitable program (e.g. Origin) to fit Eq. 5a to the plot of transmittance vs. concentration. The Origin syntax for this equation is

$$10^{-(P1 + P2 \times X)}$$

(7b)
The fitting parameters, P1 and P2, correspond to $\alpha$ and $\beta$ in Eq. 6. It will be helpful to use the values of $\alpha$ and $\beta$ from the fit of absorbance vs. concentration as initial estimates of these fitting parameters. Include the plot of scatter points and fitted curve in your report as Figure 2. Comment on the goodness of the fit of Eq. 5a to the data.

Note: You may find it helpful to review Part VI of the Origin tutorial at this point.

E. Standard deviation of absorbance ($s_A$)
Because of the nonlinear relationship between absorbance and transmittance (Eq. 3), the relationship between the imprecision of absorbance and transmittance is also nonlinear (See Eq. PE-8 below). Use the pooled standard deviation of transmittance selected above (Sec. V-A) and mean transmittances of the standards to calculate standard deviations of absorbance, $s_A$, for the eight calibration standards. Prepare a plot of absorbance error, $s_A$, vs. concentration and include this plot in your report as Figure 3. Pay careful attention to how absorbance error changes with concentration and discuss this in your report. Use Eq. PE-9 to rationalize the shape of this plot.

F. Concentration error vs. transmittance
This section emphasizes dependencies of absolute and relative concentration errors on concentration.

1. Absolute concentration error. Use mean values of transmittance, the standard deviation of transmittance, $s_T$, the values of $\alpha$ and $\beta$ obtained from a fit of absorbance vs. concentration (Fig. 2) with Eq. PE. 4, to calculate the concentration error, $s_C$, for each of the standard concentrations. Prepare a plot of concentration error vs. concentration and include this plot in your report as Figure 4. Comment on the shape of the plot and its significance in terms of minimizing absolute concentration errors in absorption spectroscopy.

2. Relative concentration error. Use Equation PE-5 with your experimental data to calculate the percentage concentration error for each standard concentration. Prepare a plot of percentage concentration error vs. concentration and include this plot in your report as Figure 5. Comment on the shape of the plot and its significance in terms of minimizing relative concentration errors in absorption spectroscopy.

3. Tabular summary. Include a table with columns corresponding to concentration, transmittance, absorbance, transmittance error, absorbance error, absolute concentration error and relative concentration error (%).
G. Effects of variables

1. Wavelength error. Data collected in Part IV-B-1 are to be used here. Calculate and plot absorbance vs. concentration for data collected at 505 and 535 nm on a single plot and do a least-squares fit of the data. Include the graph as Figure 6 and the least-squares calibration equations in your report. Comment on the effects of wavelength on the calibration sensitivity.

Calculate absorbances at 508 and 538 nm using transmittances measured at these wavelengths. Then use the absorbance at 508 nm with the calibration equation at 505 nm to calculate the iron concentration. Repeat the process using the absorbance at 538 nm with the calibration equation at 535 nm to calculate iron concentration.

Calculate and report the percentage difference between values calculated at each wavelength and the standard concentration used to obtain the data. Comment on the effect of a 3-nm error in wavelength on results determined at 535 vs 505 nm. Use the spectra attached at the end of this experiment to explain effects of a fixed wavelength error on the concentration error at these wavelengths.

2. Stray light. To emulate the effect of the stray light, combine the data taken at the wavelength 505 nm with those taken at 580 nm, $T_{\text{stray}} = (T_{505\,\text{nm}} + T_{580\,\text{nm}})/2$. Note that this approach is only justified if the intensity of the source light is the same at 505 and 580 nm (a reasonable approximation for Genesys spectrophotometer). Plot the results for absorbance $A_{\text{stray}}$ vs. concentration, including the data from all eight calibration samples. In the same graph reproduce the plot $A_{505\,\text{nm}}$ vs. concentration. You should have one figure with two plots on it. Include this graph in your report as Figure 7 and comment on the effect of stray light on absorbance sensitivity, $\Phi_{A,C}$ as concentration increases.

| Note: To rationalize the result, assume that transmittance at the principal absorption wavelength changes with concentration as $T_{505\,\text{nm}} = 10^{-(\alpha + \beta |C|)}$, whereas the transmittance at the other wavelength does not change with concentration at all, $T_{580\,\text{nm}} = \text{const.}$ Under these assumptions, substitute $T_{\text{stray}}$ into Eq. (3) and consider what happens when $T_{505\,\text{nm}} \to 0$ at high analyte concentration while $T_s$ remains constant. |
PHOTOMETRIC ERROR EQUATIONS

This summary of photometric error equations describes how transmittance and absorbance errors are propagated to concentration errors.

Table PE-1. Summary of symbols used to describe photometric error equations.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>$s_T$</td>
<td>Transmittance error</td>
</tr>
<tr>
<td>$s_A$</td>
<td>Absorbance error</td>
</tr>
<tr>
<td>$s_C$</td>
<td>Concentration error</td>
</tr>
<tr>
<td>$\Phi_{T,C}$</td>
<td>Sensitivity of transmittance to changes in concentration, $dT/dC$</td>
</tr>
<tr>
<td>$\Phi_{T,A}$</td>
<td>Sensitivity of transmittance to changes in absorbance, $dT/dA$</td>
</tr>
<tr>
<td>$\Phi_{A,C}$</td>
<td>Sensitivity of absorbance to changes in concentration, $dA/dC$</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Intercept of a plot of absorbance vs. concentration</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Slope of a plot of absorbance vs. concentration</td>
</tr>
</tbody>
</table>

Useful derivative

$$\frac{d(e^u)}{dx} = e^u \frac{du}{dx} \quad (PE-1)$$

Absorbance/transmittance/concentration relationships

$$A = -\log T = \alpha + \beta C \quad (PE-2a)$$

Transmittance/concentration relationship

$$T = 10^{-A} = 10^{-(\alpha + \beta C)} = e^{-\ln(10)(\alpha + \beta C)} \quad (PE-2b)$$

Transmittance/concentration sensitivity

$$\Phi_{T,C} = \frac{dT}{dC} = -\ln(10)\beta e^{-\ln(10)(\alpha + \beta C)} = -\ln(10)\beta T \quad (PE-3)$$

Concentration error

$$s_C = \frac{s_T}{|\Phi_{T,C}|} = \frac{s_T}{\ln(10)\beta T} = \frac{10^{(\alpha + \beta C)}}{\ln(10)\beta} s_T \quad (PE-4)$$

Relative concentration error (multiply by 100 for percentage error)

$$\frac{s_C}{C} = \frac{s_T}{\ln(10)\beta CT} = \frac{10^{(\alpha + \beta C)}}{\ln(10)\beta C} s_T \quad (PE-5)$$

Transmittance/absorbance relationship

$$T = 10^{-A} = e^{-\ln(10)A} \quad (PE-6)$$

Transmittance/absorbance sensitivity

$$\Phi_{T,A} = \frac{dT}{dA} = -\ln(10) e^{-\ln(10)A} = -\ln(10)T \quad (PE-7)$$
Absorbance error vs. $s_T$ and $A$

$$s_A = \frac{s_T}{|\Phi_{T,A}|} = \frac{s_T}{\ln(10)T} = \frac{10^A}{\ln(10)s_T} \quad (PE-8)$$

Absorbance error vs. $s_T$ and $C$

$$s_A = \frac{10^{(\alpha+\beta C)}}{\ln(10)} s_T \quad (PE-9)$$

Absorbance/concentration sensitivity

$$\Phi_{A,C} = \frac{dA}{dC} = \beta \quad (PE-10)$$

Concentration error

$$s_C = \frac{s_A}{|\Phi_{A,C}|} = \frac{s_A}{\beta} = \frac{10^{(\alpha+\beta C)}}{\ln(10)\beta} s_T \quad (PE-11)$$

Notice that the concentration error in Eq. PE-11 derived using absorbance sensitivity is the same as that in Eq. PE-4 derived using transmittance sensitivity. The numeric value of $\ln(10)$ is 2.3026.

Figure 1. Absorption spectrum for iron(II)/o-phenanthroline complex.
Fe(II) concentration ($10^{-5}$ M): a) 1.25, b) 2.50, c) 5.0, d) 7.5, e) 10, f) 12.5