Objective: The goal of this experiment is to determine an unknown concentration of ascorbic acid (AA) by monitoring the quenching of iron(III) hexacyanide by fluorescence spectroscopy. The analysis will require excitation of 365 nm and monitoring the emission at 418 nm (range of 400 to 440 nm). The analysis requires a calibration curve to determine the concentration of ascorbic acid in an unknown sample.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Chemicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorolog Spex-3</td>
<td>Ultra-high purity water</td>
</tr>
<tr>
<td>25-mL and 10mL Vol flask</td>
<td>0.01M HNO₃</td>
</tr>
<tr>
<td>Quartz Cells</td>
<td>2.0 mM K₃[Fe(CN)₆]₃</td>
</tr>
<tr>
<td>Various Vol pipette</td>
<td>2.0 mM NaNO₃</td>
</tr>
<tr>
<td></td>
<td>Ascorbic Acid</td>
</tr>
<tr>
<td></td>
<td>0.001% EDTA</td>
</tr>
</tbody>
</table>

Safety and Waste Disposal
Wear gloves when performing this experiment
Wash hands and expose skin after handling with iron(III) hexacyanide and all chemicals handled in this experiment.

Discussion: In this experiment, the amount of ascorbic acid in an unknown be measured by Fluorescence. Ascorbic acid (AA) or Vitamin-C does not fluoresce. Ascorbic acid can be oxidized by hexacyanoferrate(III) ions to dehydroascorbic acid. The overall stoichiometry has been found to be as follow:

\[
H_2\text{Asc} + 2[\text{FeCN}_6]^{3-} \rightarrow \text{Asc} + 2[\text{FeCN}_6]^{4-} + 2H^+
\]

The kinetics of this reaction involves four steps:

1. The first is the initial ionization of ascorbic acid:
   \[
   H_2\text{Asc} \rightarrow H\text{Asc}^- + H^+
   \]
2. The second is the first oxidation step (the slow step):
   \[
   H\text{Asc}^- + [\text{FeCN}_6]^{3-} \rightarrow H\text{Asc}^- + [\text{FeCN}_6]^{4-}
   \]
3. The third is the second ionization step:
   \[
   H\text{Asc}^- \rightarrow \text{Asc}^- + H^+
   \]
4. Finally, the fourth is the second oxidation step:
   \[
   \text{Asc}^- + [\text{FeCN}_6]^{3-} \rightarrow \text{Asc} + 2[\text{FeCN}_6]^{4-}
   \]

Again, the slow step is the first oxidation step of ascorbic acid (2). For this reason, the reaction needs to run its course before measurements are carried out.

Fluorescence is the subsequent emission of radiation after the absorption of radiant energy. Although all molecules absorb radiant energy, not all molecules fluoresce to get back to the ground electronic state. In the absorption process, the electron in the molecule gets excited from the ground electronic state to an excited electronic state. Although molecules absorb a range of wavelengths, in fluorescence spectroscopy, only one wavelength is chosen as the excitation wavelength. Therefore, electrons are excited to a specific higher energy level. Upon excitation, the molecule undergoes vibrational relaxation to the lowest vibrational level of the excited state. From here, the electron can emit radiation (fluoresce) to get back to the ground electronic state. Like the excited electronic state, the ground electronic state has a series of vibrational levels. Therefore, the molecule can fluoresce (give off light) to any of these vibrational levels of the ground electronic state. However, in fluorescence spectroscopy, the emission is monitored at only one specific wavelength known as the emission wavelength.

A schematic of this process as it occurs in the Fluorescence spectrometer is shown in the Jablonski Energy Diagram. Notice in the diagram that there are 6 emissions, but only the emission resulting in the return to the ground excited state is being monitored by the Fluorescence spectrometer. At low concentrations, the intensity of the fluorescence is proportional to concentration. At high concentrations, however, the compound will absorb some of the fluoresced light, resulting in a non-linear dependence of the fluorescence intensity on the concentration. Another reason not to use high concentrated sample is that the spectrometer has an upper limit on the signal it can handle. (A way to decrease the intensity without diluting the sample is to narrow the slit setting).

In this experiment, the fluorescence bands of potassium iron(III) hexacyanide can be monitored at 418 nm (scan between 400 - 440 nm) upon excitation at 365nm. The emission peak of iron(III) hexacyano complex at 418 nm decreases due to the reduction reaction of ascorbic acid.

If quantitative work is going to be done, then there must be some sort of relationship between Fluorescence intensity and concentration. The equation that describes this relationship is:

\[ P_F = \phi_F P_0 e_{bc} \]

In this equation, \( P_F \) is the radiant power of Fluorescence. Recall that power is in watts, which have units of J/sec. In this application, power is basically a measure of the number of photons being emitted from the sample. \( \phi_F \) is the quantum yield of fluorescence (photons emitted / photons absorbed), and \( P_0 \) is the radiant power directed at the sample. The product \( e_{bc} \) is the absorption of the Fluorescing species at the emission wavelength and have the usual definitions. This factor accounts for the molecules in the solution that absorb the Fluorescence before it can escape the solution. Because neighboring molecules in the sample can absorb the Fluorescence of a molecule, the linear relationship shown above does not hold at high concentrations. Specifically, the absorption (the \( e_{bc} \) factor) needs to be less than 0.05. To have such low absorptions, the solution needs to be very dilute.

Another factor in experimental design is that it is not possible to build a spherical detector to measure all of the emission from the sample. Instead, the emission is collected at a 90° angle to the excitation source. This angle minimizes interference from the excitation wavelength. Clearly, only part of the Fluorescence is actually measured.

Before working on the Fluorolog-3, go to the Miramar Instrument website and download the operation procedures. http://faculty.sdmiramar.edu/fgarces/LabMatters/Instrument.htm#FluoroLog

**Warning:** You will be issued matching sets of quartz cuvette. Do not damage these otherwise you will be billed $400.
Procedure

Please note that the quality of the result for this experiment depends on careful, precise preparation of each solution.

**Use deoxygenated Ultra-pure water in all the solution preps.**

1. **Ascorbic Acid (AA) Stock solution** - Prepare a fresh stock solution of AA (2.5×10⁻⁴M). It highly recommended that the solution be prepared on the same day that is to be used. This solution is prepared by dissolving the appropriate mass of AA in 250-mL with deoxygenated 0.010M HNO₃ and 0.001% disodium EDTA dihydrate solution. Measure the mass of AA to the precision of the Analytical balances and record in your lab notebook. Also calculate the concentration of this solution in ppm). Lower concentrations of AA is made by serial dilution with deoxygenated 0.010M HNO₃ and 0.001% disodium EDTA dihydrate solution. Store the solutions in amber-colored bottles in the dark.

2. **K₃[Fe(CN)₆] solution** - Stock solution of 2.0 mM K₃[Fe(CN)₆] solution in a 250-mL volumetric flask. This solution may be prepared for you by the lab technician.

3. **Preparation Sodium nitrate (NaNO₃) solution.** - Prepare 100mL of 2.0 mM solution of NaNO₃. This solution may be prepared for you by the lab technician.

4. **Unknown Ascorbic Acid** - Based on experiment 3 (iodometric titration of vitamin-C) determine an appropriate amount of vitamin-C unknown to prepare ~2.5×10⁻⁴M ascorbic acid solution. Follow the procedure in procedure step 1 to prepare this solution.

5. **HNO₃ + EDTA solvent** - Prepare 500-mL of 0.010M HNO₃ and 0.001% disodium EDTA dihydrate solution. This will be the solvent that will be use throughout this experiment.

Since ascorbic acid reacts slowly with dissolve oxygen, be sure the solution is deoxygenated with nitrogen gas. It is recommended that the solution is used the same day as the analysis. Solutions must be within ±1° of room temperature.

Calculate the ppm concentrations for K₃[Fe(CN)₆] and ascorbic acid solutions based on the five solutions below. Prepare five solutions based on the following solutions below.

<table>
<thead>
<tr>
<th>Solution</th>
<th>2.0 mM K₃[Fe(CN)₆] (mL)</th>
<th>2.0 mM NaNO₃ (mL)</th>
<th>Ascorbic acid (mL)</th>
<th>Total Vol (50mL) 0.010M HNO₃ + 0.001% EDTA (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.0</td>
<td>10.0</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>10.0</td>
<td>5.0</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>10.0</td>
<td>20.0</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>10.0</td>
<td>30.0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>10</td>
<td>10</td>
<td>Your discretion</td>
<td>Volume is 50-mL</td>
</tr>
</tbody>
</table>

Degas each solution for at least 5 min before measuring the emission but do so around 15 min after mixing the ascorbic acid into the bulk of the solution. Measure the emission again after one hour after mixing. Keep all solution away from oxygen.
Adjust the amount of unknown such that the emission is within the standards used.

Generate a table of AA concentration and Emission Intensities. Next overlay the graph and include it in your report. (See figure to right). Plot Ascorbic Acid concentration versus emission intensities. This is your calibration curve.

Use Excel to generate a calibration curve and then determine the concentration of ascorbic in your unknown solution. (Chapter 4 Harris). Following the example in chapter 4.7 - 4.9, p 83-89, use LINEST in your least square analysis to find $S_y$, $S_x$, $S_m$ and $S_b$. Determine the error (standard deviation and coefficient of variation) in your determination of the ascorbic acid concentration. See equation 4-27 and the example in chapter 5.3 (page 106, Harris 8th Ed)

Compare the results for your unknown with the results you determined in experiment 3 and experiment 4. Carry out a "Comparing Replicate Measurement" analysis. Compare experiment 6 with 3 and experiment 6 with 4. Also restate your results in the comparison of experiment 5 with 3. Are the set of results lie within experimental error? See p. 76 in your text (Harris, 8th Edition)

Calculate the percent concentration m:m of ascorbic of your unknown and determine the standard deviation, RSD and 95% CL for your results.

Print a hard copy of spread sheet that is properly formatted to fit a single page with proper labels and turn it in with your write-up.

Discussion (Talking points)

Discuss the "Comparing Replicate Measurement" analysis of ascorbic acid in this experiment with your other method of ascorbic acid analysis, experiment 3 (iodometric titration) and experiment 4 (cyclic voltammetry of ascorbic acid) Do the results significantly different or do the results lie within experimental error? Write an appropriate discussion on the technique of fluorescence spectroscopy for quantitative analysis. What is the function of $K_3[Fe(CN)_6]$ and EDTA in this experiment?

Postlab Question

1. Two fluorescent compounds A and B are present in a mixture. Using a spectrofluorimeter similar to the one used in this experiment, how would you determine the quantity of A in the mixture if-

   A absorbs radiation in a region where B does not.

   Both A and B absorb in the same region but A emits fluorescence at different wavelengths than B.

2. List at least two reasons fluorescence spectroscopy is far more sensitive than UV-Vis spectroscopy?
### Quantification of Ascorbic Acid by Fluorescence Spectroscopy

#### Analytical Chemistry 251

<table>
<thead>
<tr>
<th>#</th>
<th>CRITERIA (Tentative point distribution - may change depending on experiment)</th>
<th>pts %</th>
</tr>
</thead>
</table>
| 1 | **Introduction and Procedures**  
A. Introduction  
- Objective of Expt.  
- Background information on Fluorescence spectroscopy  
- Math relationship used in terms of fluorescence intensity and concentration and calibration curve setup  
B. Procedures  
- Outline of procedures in Expt.  
- Flow chart pictorial of procedures. Solution preparation and dilution procedure to be carried out.  
- Procedural changes.  
- Information (data) to be recorded during expt. (to be presented in Table form.)  
- Safety and disposal information.  
  
This portion of the report should be turned in before the start of lab class (prelab discussion). | 5 |
| 2 | **Data, Observe., Results and Calc.**  
C. Data and Observation  
- Write out how solutions are prepared and how the dilution procedure was carried out.  
- How is the instrument set up. What instrument settings were used. Write a detailed description on the important steps in using the instrument.  
- Data in **table form** & detail observation written in the table. Indicate the instrument settings as well.  
  
This portion of the report should be turned in before you leave the laboratory. | 5 |
| 3 | **Calculations & Results**  
D. Calculations  
- Data to be used in the in the calibration curve.  
- LINEST result and calculation of unknown concentration.  
- Statistical analysis of data and the standard deviation of the results  
E. Results  
- Final Result(s) in **table form**.  
  
In this section accuracy of results is very important as well as detailed calculation showing how the result was obtained. "Unknown" will also be included in this section. | 9 |
| 4 | **Discussion / Conclusions and Post-Lab Questions**  
F. Discussion  
- Discuss the percent error in your calculation. Write an appropriate discussion on the technique of fluorescence spectroscopy for quantitative analysis. What is the role of o-phenylenediamine in this experiment? Can this technique be applied to any chemical that do not fluoresce? How would this be altered if instead of using a calibration curve, standard addition was used in the analysis.  
G. Conclusion  
- Summary of the goal of the experiment, how does the result compared to the literature value and how that goal was achieved in the experiment.  
H. Post-lab questions from manual or class assignment  
- Complete well thought-out answers.  
  
This portion (Calculation and Discussion) is turned in at the beginning of class of the due-date. | 6 |
| 5 | **Overall Presentation (of lab notebook)**  
- Lab technique during experiment; example are, class preparation, safety glasses precautions and leaving the laboratory clean.  
- Report presentation: examples are the headings of each report that includes name, title, lab partner, date and section #.  
- Legibility of report. Is the report easy to read or is important information jotted down by small print in the corners of the lab report? The overall impression is important.  
Lab Technique  
- Safety: wear goggles, handle chemicals with caution, proper handling of lab equipment  
- Leave lab clean and tidy | 10 |
| **Total (This total may be adjusted depending on lab technique and student conduct in the experiment)** | 100% |

Unknown ______ 30pts