**Introduction: Spectrophotometry and Fluorometry**

In this experiment you will use the SpectroVis Plus (a small computer controlled Spectrophotometer + Fluorometer) to:

- Measure the Transmission and Absorbance spectra of some common liquids from 380-950 nm.
- Measure the Fluorescence spectrum of Olive Oil for excitation at 405 and 500 nm.

In the process you will learn about:

- Beer’s law and verify its dependence on the number density of particles.
- Light scattering from small particles and molecules and how to detect it, as well as how to estimate the size of the scatterers.

**Absorbance, Transmittance and the Beer-Lambert Law**

**Transmittance**

The Transmittance is the Intensity of light going through a sample as a function of wavelength, so if 10% of the light going into a sample comes out the other side, then the transmission coefficient $T$ would be 0.1.

Mathematically the transmission coefficient $T$ is given by the ratio of the transmitted final intensity $I$ to the incident or initial intensity $I_0$ (often a percentage)

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

**Absorbance**

The Absorbance is basically the inverse of transmittance, whatever a material does not transmit, is absorbed.

The absorbance is written in terms of transmittance and Intensity by

$$A = \log_{10} \frac{1}{T} = \log_{10} \frac{I_0}{I}$$
Fluorescent molecules are compounds that absorb light of one wavelength, then re-emit light at a longer wavelength. Fluorescence is this process.

For example if we shine a violet (405nm) laser into a fluorescene mixture a bright green light will be transmitted. (Your instructor can demo this)

After an electron absorbs a high energy photon the system is excited electronically and vibrationally. The system relaxes vibrationally, and eventually fluoresces at a longer wavelength. In this lab you will observe the fluorescence of extra virgin Olive Oil and compare to its transmission and absorption spectrum.

**Sample Colors**

If we look at Absorbance over the visible spectrum we can determine what color an object will be, for example chlorophyll, a molecule found in plants, has the following absorption spectrum.
From this we see for chlorophyll that there is a dip in absorbance in 500-600nm range which means this range of light is what is being transmitted through the material to our eyes.

**Question 1:**

a.) What color should you expect chlorophyll a to be?
b.) What would be different about chlorophyll b?

**PART 1: Olive Oil Spectra**

In the first part of the experiment, you will measure the Transmission, Absorbance, and Fluorescence spectra using 405nm and 500nm excitation light of a sample of Extra Virgin Olive Oil and compare the various features of each type of spectrum.

Before you do anything you must calibrate the experiment and learn how to use it, jump to **Calibration and Equipment** and follow the steps.

**Procedure**

A.) Navigate to Experiment > Change Units > Transmittance and hit collect to take a transmittance spectrum

B.) Take a screenshot of the graph and put it in a word document to be printed. Be sure the entire graph is on the screen, you will likely have to change your y axis. Wait until the end to print this with the other 3

C.) Repeat step A and B for Absorbance and 405nm, 500nm fluorescence. Be sure to recollect each time, the graphs should all look very different, if ANY of them look the same call over your instructor.

**Analysis**

**Question 2.)**

Answer the following a, b for each graph:

a.) List any significant peaks or troughs shown.
b.) Discuss the meaning of these.
For the two fluorescence graphs also answer c.

c.) What wavelength is going into the substance, and what wavelength is being emitted?

Part II Beer-Lambert Law

For the Beer-Lambert Law we have the relation of the final intensity to the initial intensity is

\[ I = I_0 e^{-\alpha l} \]

where \( \alpha \) is the absorption coefficient (Specific to the material) and \( l \) is the path length in the absorbing/scattering medium.

The absorption coefficient can be written in terms of the number density \( n \) (number of particles/volume of material) and the absorption/scattering cross section \( \sigma \) which we’ll discuss in part 2. The absorption coefficient is

\[ \alpha = n\sigma \]

leaving the absorption as

\[ A = \log_{10} \frac{I_0}{I_0 e^{-\alpha l}} = n\sigma l(\log_{10} e) \]

\[ A = (0.4343 \sigma l)n \]

The importance of this last result is that it tells us that the absorbance is proportional to path length \( l \) and the number of particles per volume, \( n \). The standard cuvette length we will use is 1cm.

We can also use this in a linearization, substituting for \( n = \frac{N}{V} \) we have

\[ A = \frac{(0.4343 \sigma l)N}{V}, \]
\[
\frac{1}{A} = m \times V.
\]

So if we plot \(1/A\) against the volume of the dilution, which will be given to you, we should see a linear graph, with a slope equal to \(m = 1/0.4343\sigma lN\).

Procedure 2:
To test Beer’s law we will measure the absorbance of a solution of Tea and decrease the number density by diluting the solution by specific amounts. Hence for one specific wavelength through this material we should see a linear relationship between the number density and the measured absorbance.

Start by measuring the absorbance spectrum for each dilution of tea, to do so do the following.

A.) Insert the Cuvette labeled “1” and click “run”. Allow the program to run long enough to get a stable graph and completed data table (about a minute.) Click “Stop” and carefully remove the cuvette.

B.) Repeat for cuvettes 2-5

Hint: When you click on “Run” again, you can choose to “store latest run” and it will conveniently put all the data on one graph.

Analysis

Table
First make data tables of Absorbance A vs. Volume V and plot \(1/A\) vs. the volume for a wavelength which starts out with an absorbance of about 1), and also for a wavelength around the peaks in your graph.

To do this Export your Logger Pro file to Excel (*.csv) by selecting file->export->csv and save the file. To get the data you need just pick a wavelength as said above and find the associated row for that wavelength, then take all 5 Absorbance values across the row.

Note that the order that logger pro will output is 5 1 2 3 4, your instructor can explain.

Here’s an example.

<table>
<thead>
<tr>
<th>Label #</th>
<th>V(mL)</th>
<th>A</th>
<th>(1/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>offpeak</td>
<td>lambda(nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>0.996</td>
<td>1.004</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.526</td>
<td>1.901</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>0.292</td>
<td>3.425</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>0.218</td>
<td>4.587</td>
</tr>
<tr>
<td>onpeak</td>
<td>lambda(nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.532</td>
<td>0.652742</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.599</td>
<td>1.042753</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.729</td>
<td>1.371742</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0.549</td>
<td>1.821494</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>0.42</td>
<td>2.380952</td>
</tr>
</tbody>
</table>

The examples used 428.8nm offpeak, and 397nm for on peak, but use whatever works best for your data.

Graphs
Next to analyze the data on Absorbance as a function of dilution volume Plot the 1/A vs. the diluted volume (V(mL)) for the specific wavelengths you’ve chosen. Use the table above to relate the labels on your cuvettes to the dilution volumes V(mL).

A.) For the off-peak wavelength, fit a linear trend line with the option “set intercept = 0” ENABLED. (this is a trendline option which forces the intercept to be zero)

B.) For the on-peak wavelength, fit a linear trend line with the option “set intercept = 0” DISABLED. (this is a trendline option, default does not force the intercept to zero).

Make sure your graphs are properly formatted and titled and the data vertical range adjusted so as to make the data spread out over most of the range of the axis, as in the following examples
Off peak wavelength obeys Beer’s law over this range of concentration. Note the forced fit with intercept of zero fits the data nicely.

On peak wavelength deviates noticeably from Beer’s law over this range of concentration. Note the non-zero intercept required to fit the data. Fit with zero intercept gives lower R^2 value with poor fit.

Comments

So the off peak wavelength produces a good fit to Beer’s law, while the peak wavelength requires a non-zero intercept, although the concentration dependence was still linear.

There are several factors which can produce deviations from Beer’s law. Under certain conditions Beer-Lambert law fails to maintain a linear relationship between absorbance and concentration of analyte. These deviations are classified into three categories:

- **Real Deviations** - These are fundamental deviations due to the limitations of the law itself.
- **Chemical Deviations** - These are deviations observed due to specific chemical species of the sample which is being analyzed.
- **Instrument Deviations** - These are deviations which occur due to how the absorbance measurements are made.

**Question 3**: Remember from the intro that Beer’s law can be rewritten as

\[
\frac{1}{A} = m \cdot V.
\]

a.) Did your graphs follow beer’s law well, explain why or why not using your R^2 coefficient.
b.) What deviations from above may have affected the quality of your graphs fit?
Calibration and Equipment

Equipment Info and Use

The Equipment you will use is the Vernier SpectroVis Plus Spectrophotometer + Fluorometer shown below

The figure on the right shows what’s going on inside the spectrophotometer. To work for absorption light from the LED and tungsten bulb light source passes through a solution. Emerging light goes through a high-quality diffraction grating, then the diffracted light is collected and sorted by the CCD array detector.

To measure Fluorescence, fluorescent light is scattered at right angles (RA fluorescence) to the excitation light sources

(LED’s) to minimize light detected at the excitation wavelength(s). Basically we try to make sure all the light being measured is coming from the molecule, not the source.

*Note that for this to work you must insert the flat face of the cuvette toward the absorption light and source, the grated side should face the two fluorescent lights top and bottom.

Calibration

To calibrate the device insert a blank cuvette into the SpectroVis as directed above. Then do the following:

1) Open Logger pro, if the device is properly connected you should see a graph overlayed with the visible spectrum of light.
2) In the top menu go to Experiment > Calibrate > Spectrometer 1
3) Run the calibration, the first time you must wait the 90s for the warm up, then hit finish calibration, after the first time you can skip this warmup. The device is ready.