Spectroscopy II - Transmission, Absorption, Fluorescence, Beer’s law and Scattering.

Spectrophotometry and Fluorometry

In this experiment you will use the Vernier SpectroVis Plus, a small, computer controlled Spectrophotometer + Fluorometer to measure the Transmission and Absorbance spectra of some common liquids from 380-950 nm. In addition, you will 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. You will also learn a little about light scattering and how to detect it by using the Fluorometer mode for non-fluorescent but scattering samples.
Vernier SpectroVis Plus Spectrophotometer + Fluorometer
(shown with sample cuvettes, not shown with USB cable)
How Does the SpectroVis Plus Work?

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.

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).
Specifications for the Spectro Vis Plus

Wavelength Range: 380 nm–950 nm

Support for fluorescence (two excitation sources centered at 405 nm and 500 nm)

Reported Wavelength Interval: 1 nm between reported values (collects 570 values)

Optical Resolution: ~ 2.5 nm

Dimensions: 15 cm x 9 cm x 4 cm

Light Sources: Incandescent white bulb, approximately 8000 hour lifetime, LED-based, approximately 100,000 hour lifetime

One-step calibration

No external power required
The transmission coefficient $T$ is given by the ratio of the transmitted intensity to the incident or initial intensity (often specified in percent as Transmittance) by

$$T = \frac{I}{I_0}$$  \hspace{1cm} (1.)

A schematic of the situation for spectrophotometry is

The absorbance is defined as

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right)$$  \hspace{1cm} (2.)
For the Beer Lambert law, the transmitted intensity $I$ is given in terms of the initial intensity $I_0$ by the relation

$$I = I_0 e^{-\alpha l}$$  \hspace{1cm} (3.)

where $\alpha$ is the absorption coefficient 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/volume) of particles and the absorption/scattering cross section $\sigma$ as $\alpha = n \sigma$, so that the absorbance can be written as

$$A = \log_{10}\left(\frac{1}{e^{-n \sigma l}}\right) = \log_{10}\left(e^{n \sigma l}\right) = n \sigma l \log_{10}(e)$$  \hspace{1cm} (4.)

or

$$A = 0.4343 n \sigma l$$  \hspace{1cm} (5.)

The importance of this last result is that it tells us that the absorbance is proportional to the path length $l$ and the number of particles per unit volume $n$. The standard cuvette length is $l = 1\text{ cm}$.

In the Beer’s law part of this experiment, we will decrease the number density by successive known dilutions of a solution of Green Tea, holding the number of molecules constant, and thus show that the absorbance is proportional to the number density, or equivalently, that the inverse absorbance, $1/A$, is proportional to the volume of the solution when the number of molecules is held constant.
The Beer-Lambert law helps to correlate the intensity of absorption of UV-visible radiation to the amount of substance present in a sample. The Beer-Lambert law has been widely used in many fields of pharmaceutical sciences, chemistry and quantification testing. It allows UV-visible spectroscopy to be useful as not just a qualitative but also a quantitative tool. In physics, it is often used to calculate the attenuation in optical fibers and materials, as well as the atmosphere.
The absorption peaks of chlorophyll \( a \) are at 665 nm and 465 nm. Chlorophyll \( a \) fluoresces at 673 nm (maximum) and 726 nm. The peak molar absorption coefficient of chlorophyll \( a \) exceeds 105 M\(^{-1}\) cm\(^{-1}\), which is among the highest for small-molecule organic compounds.
Fluorescent molecules are compounds that absorb light of one wavelength, then re-emit light at a longer wavelength. This emitted light can be quantified using fluorescence spectroscopy. Molecular and cellular biologists use fluorescent compounds to label proteins, gels, and even cellular organelles. In many ways, fluorescent compounds have revolutionized research in the life sciences. Fluorescence spectroscopy is also used in the characterization of laser materials.

Jablonski diagram. 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.

http://en.wikipedia.org/wiki/Fluorescence
Figure 3. (A) RA fluorescence spectra of the extra virgin olive oil O2 where $\lambda_{\text{exc}}$ is (a) 350 nm; (b) 320 nm; (c) 380 nm; (d) 410 nm; (e) 300 nm; (f) 280 nm; B) (a) 580 nm; (b) 550 nm; (c) 510 nm; (d) 620 nm; (e) 530 nm. The excitation and emission bandwidths are 1.5 and 3 nm, respectively.

Figure 4. (a) Absorption spectrum of olive oil O2. (b) Absorption spectrum of a solution of maize oil in $n$-hexane (1:100) w/v. The spectrum intensity is multiplied by 100.
Space-filling model of the chlorophyll a molecule (from Wikipedia)

Structure of chlorophyll a
Fluorescence of Vegetable Oils: Olive Oils

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Fluorescence spectra of undiluted extra virgin olive oil obtained with the traditional setup (right-angle fluorescence) show considerable artifacts and deformations due to self-absorption phenomena, even when the spectra are corrected for inner filter effects. On the other side, front-face fluorescence spectra are much less affected by self-absorption. Front-face fluorescence of native olive oil reveals the presence of different fluorophores and can provide information about their amount. From the intense emission at ca. 315–330 nm, it is possible to detect fluorescent polyphenols and pheophytins and to evaluate their overall content. Low-intensity emission bands at 350–600 nm are correlated to vitamins and other important molecules. Among them, the fluorescence of the riboflavin fluorophore can be used to evaluate its concentration. The intense emission of chlorophyll derivatives, measured in the 640–800 nm spectral region, can provide information on their concentration.

KEYWORDS: Front-face fluorescence; olive oil; polyphenols; vitamins; pheophytins
Chlorophyll fluorescence

Chlorophyll fluorescence is light that has been re-emitted after being absorbed by chlorophyll molecules of plant leaves. By measuring the intensity and nature of this fluorescence, plant ecophysiology can be investigated.

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Assessing plant physiology with Chlorophyll fluorescence

Light energy that has been absorbed by a leaf will excite electrons in chlorophyll molecules. Energy in photosystem II can be converted to chemical energy to drive photosynthesis (photochemistry). If photochemistry is inefficient, excess energy can damage the leaf. Energy can be emitted (known as energy quenching) in the form of heat (called non-photochemical quenching) or emitted as chlorophyll fluorescence. These three processes are in competition, so fluorescence yield is high when less energy is emitted as heat or used in photochemistry.
Fluorescence spectroscopy

From Wikipedia, the free encyclopedia

**Fluorescence spectroscopy** aka fluorometry or spectrofluorometry, is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light; typically, but not necessarily, visible light. A complementary technique is absorption spectroscopy.

Devices that measure fluorescence are called fluorometers or fluorimeters.

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### Theory

**Main article: Fluorescence**

Molecules have various states referred to as energy levels. Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an excited electronic state of higher energy. Within each of these electronic states are various vibrational states.

In fluorescence spectroscopy, the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state. This process is often visualized with a Jablonski diagram.
Fluorescence

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation. It is a form of luminescence. In most cases, the emitted light has a longer wavelength, and therefore lower energy, than the absorbed radiation. However, when the absorbed electromagnetic radiation is intense, it is possible for one electron to absorb two photons; this two-photon absorption can lead to emission of radiation having a shorter wavelength than the absorbed radiation. The emitted radiation may also be of the same wavelength as the absorbed radiation, termed "resonance fluorescence".[1]

The most striking examples of fluorescence occur when the absorbed radiation is in the ultraviolet region of the spectrum, and thus invisible to the human eye, and the emitted light is in the visible region.

Fluorescence has many practical applications, including mineralogy, gemology, chemical sensors (fluorescence spectroscopy), fluorescent labelling, dyes, biological detectors, and, most commonly, fluorescent lamps.

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  2.4 Jablonski diagram
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What you are going to do

1.) 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. You should make some thoughtful comments and discussion on the differences between the graphs and what they mean. Read the referenced articles for additional information. What color is the Chlorophyll fluorescence? Note that shining a 405nm or 532nm laser on green plant leaves produces a bright chlorophyll fluorescence. Try this on spinach lettuce, regular lettuce, spinach juice, and some (living) green plant leaves.

To do this you just select from the Experiment menu > Change Units Spectrometer and choose the appropriate spectrum type, Transmission, Absorbance, Fluorescence 405nm or Fluorescence 500nm. Make sure you select calibration before taking any spectra. The software will prompt you to put in a blank cuvette.

Copy your screen graphs with appropriate ranges (make sure you vertically expand your vertical scales so that the features of the spectra are well displayed and take up most of the vertical scale) into a word document. Similarly, be sure you have wavelength plots over the entire 380-950nm range. If you want to examine a feature in more detail, you can expand the horizontal wavelength scale to be a smaller range around the feature you are interested in.
What you are going to do (more detailed procedure in following slides)

2.) In the second part of this experiment we will measure the absorbance spectrum of a solution of Green tea as a function of the volume of the solution, starting from a high concentration and adding known volumes of water to dilute it.

It is important in this part of the experiment NOT TO SPILL OR LOSE ANY OF THE SOLUTION, OTHER THAN THE FIRST REDUCTION OF SOLUTION AFTER THE FIRST ABSORBANCE SPECTRUM TO A STANDARD VOLUME OF 2 ml. The experiment assumes that the number of molecules in the total volume of solution (that is cuvette plus graduated cylinder) remains constant while the total volume is increased.
Green Tea Absorbance vs. Wavelength

Initial 2 ml volume

Five fold dilution to 10 ml volume

Steve Mahrley
11/2/2012 data

Beer’s Law Experiment

Procedure:
Prepare a green tea solution of sufficient concentration to produce a peak absorbance of at least 1.5 around 397 nm. This should already be available in class. Verify your initial solution has sufficient absorbance.

Procedure... (from Steve Mahrley, 11/2/2012)

A cuvette holds approximately 3.6 mL.
- I initially ran Absorbance with full cuvette/concentration and saved the file as * 2 mL.
- I filled the (10 ml) Graduated Cylinder to 2 mL and dumped the rest of the cuvette contents down the drain (see note!). I added water to 4 mL, ran the program again and saved it as *.4 mL.
- I poured the entire cuvette into the GC, checked to make sure the volume was still 4 mL, then added water to 6mL.
- Ran the program, saved as 6 mL.
- Repeated this for 8mL and 10mL
- I did this for both green tea and Gatorade fruit punch

NOTE: After the first reduction of the initial cuvette volume to 2mL, DO NOT THROW AWAY OR LOSE ANY SOLUTION! The laboratory experiment assumes that the total number of molecules remains constant while the volume is increased.
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 (428.8 nm in the following example), and also for the peak wavelength of about 397 nm. (use your actual values)

<table>
<thead>
<tr>
<th>(off peak)</th>
<th>A</th>
<th>V(ml)</th>
<th>1/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>lambda(nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>428.8</td>
<td>0.996</td>
<td>2</td>
<td>1.004</td>
</tr>
<tr>
<td></td>
<td>0.526</td>
<td>4</td>
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<td></td>
<td>0.39</td>
<td>6</td>
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<td></td>
<td>0.292</td>
<td>8</td>
<td>3.425</td>
</tr>
<tr>
<td></td>
<td>0.218</td>
<td>10</td>
<td>4.587</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(peak)</th>
<th>A</th>
<th>V(ml)</th>
<th>1/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>lambda(nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>397</td>
<td>1.532</td>
<td>2</td>
<td>0.653</td>
</tr>
<tr>
<td></td>
<td>0.959</td>
<td>4</td>
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<tr>
<td></td>
<td>0.729</td>
<td>6</td>
<td>1.372</td>
</tr>
<tr>
<td></td>
<td>0.549</td>
<td>8</td>
<td>1.821</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td>10</td>
<td>2.381</td>
</tr>
</tbody>
</table>
To analyze the data on Absorbance as a function of dilution volume

Plot the $1/A$ vs. wavelength data from the tables for the off peak and peak wavelengths for each of the sample volumes, including the starting volume of 2ml (i.e. 2, 4, 6, 8, and 10 ml).

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

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, 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.
Inverse Absorbance vs. Dilution Volume
397 nm (on peak), Green Tea

\[ \frac{1}{A} = 0.2118V + 0.1834 \]
\[ R^2 = 0.9898 \]

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.
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.
There are at least six conditions that need to be fulfilled in order for Beer’s law to be valid. These are:

1.) The absorbers must act independently of each other;
2.) The absorbing medium must be homogeneous in the interaction volume
3.) The absorbing medium must not scatter the radiation – no turbidity
   (not true, can have single scattering, just not multiple scattering effects);
4.) The incident radiation must consist of parallel rays, each traversing the same length in the absorbing medium;
5.) The incident radiation should preferably be monochromatic, or have at least a width that is narrower than that of the absorbing transition; and
6.) The incident flux must not influence the atoms or molecules; it should only act as a non-invasive probe of the species under study. In particular, this implies that the light should not cause optical saturation or optical pumping, since such effects will deplete the lower level and possibly give rise to stimulated emission.

If any of these conditions are not fulfilled, there will be deviations from Beer’s law. Some of them are discussed in more detail here.