Absorption spectra of plant pigments

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Objectives

By using UV-Visible absorption spectrophotometers you will measure absorption from pigments in solution (using Shimadzu 160A) and scattering free absorption spectra from leaves (using Hitachi). The aim of this exercise is to become familiar with Beers law and absorption spectrophotometry. It will be used for determination of some pigments in plants; chlorophylls and carotenoid. Furthermore, you will investigate photochemical reactions of chlorophylls in solution.

EXCERCISE

A. Determine the concentration of Chlorophyll a, Chlorophyll b and Carotenoids in a leaf extract using absorption spectrophotometry. Determine the rate of photobleaching of Chlorophyll a when the pigment extract is exposed to visible light irradiation.

B. Measure the absorption spectrum of an intact leaf (the same used for making the extract) using an absorption spectrophotometer equipped with an integrating sphere. Determine the rate of photobleaching of the pigments in the leaf subjected to similar irradiation as in the extract.

Procedure

Absorption of the extract

- Equipment and chemicals:
  - Pigment extract (chlorophyll (a+b) + ethanol
  - Solutions of Chlorophyll a and b
  - Ethanol
  - Shimadzu (UV-160A, an UV-Visible recording spectrophotometer)
  - Irradiator

Make a pigment extract of a leaf. This is done by taking pieces of a leaf into a glass flask containing ethanol. The pigments extracted from the leaf will contain chlorophylls and carotenoids
from the plant material. Transfer some of the solution, when it has obtained a green colour, to a
cuvette for measuring the absorption spectrum. Use Shimadzu for measuring the absorption
spectrum of the extract and premade solutions of Chlorophyll a and b in ethanol.
Find the concentration of chlorophyll a (Chl a) and Chl b in the extract. This is done by importing
the spectra in a worksheet (EXCEL or ORIGIN) and plotting of the measured absorption spectra.
In the worksheet, subtract a fraction of the pure Chla spectrum from that of the extract, so that you
get a straight line in the 665 nm region, where only Chla is absorbing. Do the similar operation on
the difference spectrum (containing only Chl b and Car) using the Chl b spectrum. Above 600 nm
only Chla b will contribute the absorption. Then you will get the carotenoid (Car) absorption
spectrum. You have decomposed the measured extract spectrum into a sum of three spectra,
\[ A_x = A_a + A_b + A_c \]

The absorption spectrum of the extract (A_x) is a sum of the contributions from Chla (A_a), Chl b
(A_b) and carotenoids (A_c). By reading out the absorbencies of the component spectra at the certain
wavelengths, you can find the concentrations. Then the extinction coefficients of the pigments are
needed;
- Chla: \( \varepsilon(656 \text{ nm}) = 0.81 \times 10^5 \text{ M}^{-1} \text{cm}^{-1} \), (at the peak absorption)
- Chlb: \( \varepsilon(648 \text{ nm}) = 0.46 \times 10^5 \text{ M}^{-1} \text{cm}^{-1} \). (at the peak absorption)
- Car: \( \varepsilon(450 \text{ nm}) = 1.2 \times 10^5 \text{ M}^{-1} \text{cm}^{-1} \). (at the peak absorption)

Irradiate the extract for 2, 4 and 6 and 8 minutes (white light) and measure the absorption spectrum
after each illumination. Plot the difference between the spectra before and after illumination.
The rate (R) of Chla photodecomposition is:
\[ R = \frac{\Delta c}{\Delta t} = \frac{\Delta A}{\Delta t} \cdot \frac{1}{\varepsilon \cdot l}, \]
where \( \Delta t \) is the time interval during which the change in absorbance (\( \Delta A \)) took place.
Discuss and explain your observations.

Absorption in scattering media

Equipment and chemicals:
- Intact plant leaves, used for making the extract
- Hitachi instrument, with integrating sphere

Measure the absorption spectrum of the other half of the leaf by measuring the reflection (R) and
its transmission (T) spectrum. Measure the absorption spectrum of the leaf after 8 minutes of the
same irradiation as given to the pigment extract. Compare and discuss the results.

THEORY

Absorption spectroscopy and Beers law:

Light is absorbed by molecules. The energies of bound electrons in molecules are quantized, so
that only discrete electron energies are allowed. When the energy of an incoming photon, which is:

\[ E_{\text{phot}} = h \nu = h c / \lambda, \quad (\nu \text{ is the frequency- and } \lambda \text{ is the wavelength of the photon}) \]
corresponds to an energy difference in the molecule;

$$E_{foi} = E_i - E_0$$

(Ei an excited- and E0 is the ground state)

absorption of the quantum can take place (see Figure below). Since the energy levels are characteristic of a molecule, the absorption spectrum is typical of a molecule too. Absorption spectroscopy is hence a useful tool for studying and identifying molecules. The absorption event is visualized in the figure below.

Absorption of a photon by a molecule

By measuring the absorption spectrum of a sample, the concentration of an absorbing substance can be determined too. Then use is made of the so called Beers law, which is derived in the following way:

On differential form, Beers law states that inside a slab of the sample (between x and x+\Delta x in a cuvette of length l), containing absorbing molecules in concentration c, the reduction in light intensity, -\Delta I, due to absorption is:

$$- \Delta I = \epsilon' \cdot c \cdot I \cdot \Delta x$$

where I is the intensity at position x inside the cuvette. The law is saying that the number of quanta absorbed in a certain region (\Delta x) is in proportion to the number of molecules (c) and the light intensity I. \epsilon' is the proportionality constant. When this equation is rearranged and integrated from 0 to l, Beers law on the integral form is obtained:

$$\ln \left( \frac{I_0}{I_t} \right) = \epsilon' \cdot c \cdot l \text{, or: } \log \left( \frac{I_0}{I_t} \right) = \epsilon \cdot c \cdot l \text{, where: } \epsilon = \log \epsilon'$$

\epsilon is called the molar extinction coefficient. So, by recording the absorbance A, defined as the logarithm of the ratio between the incident (I0) and transmitted light intensity (It), one gets:
The absorbance is in proportion to the concentration of the absorbing molecules and the path length in the absorbing medium. The extinction coefficient is specific for the absorbing molecule, and indicates the energy differences and the transition probabilities between the various energy levels.

Energy levels and quantum transitions
The energy levels of a molecule is visualized in Figure 1. The manifold of energy levels are subdivided in *singlet*, in which the total electron spin is zero, and *triplet* levels, in which the overall electron spin is one. In addition to the electronic energies, shown as fat levels, there are vibrational levels too, due to the motion of the nuclei. As a first approximation, the nuclei are considered to be in a harmonic potential, so the energy levels due to nuclear motion are equally spaced.

The quantum transitions in the molecule due to absorption of a light quantum is visualized as vertical arrows. In the left part of figure 1 the corresponding absorption spectrum is visualized.

**Figure 1**: Typical energy diagram showing the ground state (S₀) and the first excited state (S₁) for a molecule. The vibrational levels are the thin lines. Absorption is illustrated in the figure by the solid, straight arrows. After excitation, there is loss of vibrational energy (dotted arrows) to the first excited state. From this S₁ state the molecule can return to the ground state by emitting a quantum of light (fluorescence) or by stepwise transition to the ground state using the ladder of vibrational levels (non-radiative transition). There may also be a transition from the S₁ state into the triplet manifold.

A plot of absorption versus wavelength is called an absorption spectrum.
**Instrumentation for measuring absorption**

Absorbance measurements are made by a spectrophotometer. The spectrophotometers vary in design, but they all have the same basic principles shown in figure 2:

![Figure 2: A spectrophotometer measuring absorbance.](image)

In a typical operation at a single wavelength, a measurement is made of the light transmitted by the solvent alone ($I_0$), followed by a measurement of that transmitted by the sample when dissolved in the same solvent ($I_t$). Then the ratio between the measured intensities is calculated, which in practice is done by the instrument. To obtain an absorption spectrum, this operation is repeated at many wavelengths, and in practice this is done by scanning the monochromator over the selected wavelength range.

**Absorption in scattering media**

When light is falling on a surface, some of the light is reflected, some is absorbed by the molecules in the material and some is transmitted through the material. The principle for measuring the light transmitted by a scattering medium is shown in figure 4. The transmitted light enters an integrating sphere with almost perfectly reflecting walls, so that every ray will eventually reach the light detector connected to the sphere. In the case of scattering media, in order to get the incident light, the reflected light has to be subtracted. The light reflected by the surface can be measured by the same integrating sphere by mounting the surface to the rear side of the integrating sphere. Then the absorbance of the scattering material can be obtained as:

$$A = \lg \left( \frac{(I_0 - I_R)}{I_T} \right) = \lg \left( \frac{1 - R}{T} \right),$$

where $T = I_T/I_0$ and $R = I_R/I_0$, where $T$ and $R$ is called the transmission- and reflection coefficients of the material, respectively.

![Figure 4: Principle for detecting all light transmitted in a scattering sample](image)
The expression for absorption is similar to a non scattering sample introduced earlier, however, the incident light is considered to be the difference between the actual- and the light reflected by the leaf.

**Photochemical reactions**

The triplet level is often the starting point for photochemical reactions. When light is absorbed by molecules, the triplet state will become populated in some of the molecules, due to intersystem crossing. The ground state of oxygen molecules, which are one of the major constituents of our atmosphere, is a triplet. A molecule (called sensitizer) excited to the triplet state has the same spin as a ground state oxygen molecule, and hence the triplet energy of the sensitizer can be transferred to molecular oxygen, which becomes excited to a singlet state (singlet oxygen). Singlet oxygen is very reactive and may oxidize neighbouring molecules. This is what happens when the chlorophyll extract is subjected to prolonged illumination. A fraction of the chlorophyll molecules enters the triplet state, and their energy can be transferred to dissolved oxygen. The singlet oxygen molecules which are formed diffuses and reacts with chlorophyll molecules in the solution. Photooxidized chlorophylls are formed which result in a loss of Chla absorption and the appearance of new absorption bands due to the photoproducts. The new absorption spectrum will be a mixture of the absorption spectrum of the orginal- and the photobleached pigments. Photobleaching of the chlorophylls in the leaf is prevented by the presence of carotenoids. These carotenoids are acceptors of triplet energy from chlorophylls and hence they prevent the formation of singlet oxygen. These reactions are written in the following way:

\[
\begin{align*}
M + h\nu & \xrightarrow{\text{abs}} M^* \xrightarrow{\text{isc}} M^* \quad (\text{absorption and intersystem crossing in molecule } M) \\
3M^* + 3O_2 & \xrightarrow{\text{coll}} M + 3O_2^* \quad (\text{collision and energy transfer, spin change in } O_2) \\
T + 3O_2^* & \xrightarrow{\text{oxidation}} TO_2 \quad (\text{oxidation of target molecule, which may lethal}) \\
3M^* + Car & \xrightarrow{\text{coll and ET}} M + 3Car \quad (\text{collision and energy transfer to a carotenoid})
\end{align*}
\]

When a carotenoid is introduced into the system, there is compeating energy transfer from the M molecule in the triplet state to oxygen and the carotenoid. In this way, less harmful singlet oxygen is produced.

**How to use Origin**

Double click (dc) on the Origin 5.0 icon.
Use File/Import/Ascii on the Menu line.
Use Column/add new columns from the menu line (add for instance 5).
Tag the Column (put the cursor on top of the column), and move it to last (use Column/Move to last).
Label the column, by dc on the the column top (for.inst. abs of extract)
For making an aritmetic operation, tag the column, and use column, set column values.
For making a plot, tag the column, and use plot/line.