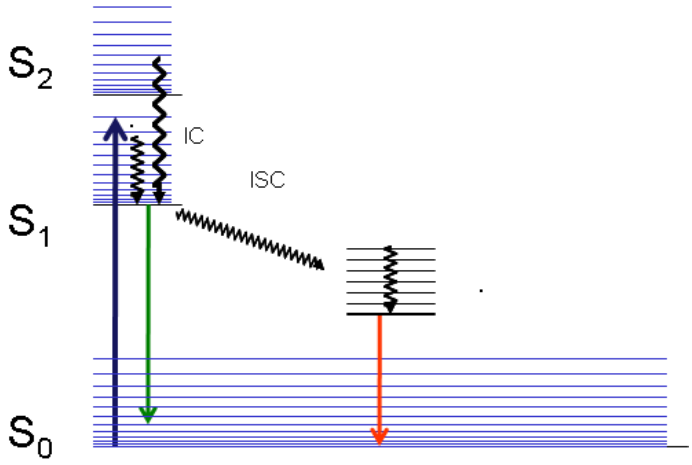
Quantification of Riboflavin (Vitamin B_2) by Fluorimetry

Objective

The purpose of this lab is to quantify of a fluorophore in solution. Riboflavin is a highly fluorescent molecule, and it will be quantified using fluorescence spectroscopy.

Background

Once a molecule is electronically excited it could relax via photon emission. If the transition to low energy states occurs between states of the same spin state (e.g. $S_1 \rightarrow S_0$) this is termed fluorescence If the spin state of the initial and final energy levels are different (e.g. $T_1 \rightarrow S_0$), the emission is called phosphorescence. Fluorescence is statistically much more likely than phosphorescence, the lifetimes of fluorescent states are very short (1×10^{-5} to 1×10^{-8} seconds) and phosphorescence lifetimes are longer (1×10^{-4} seconds to minutes or even hours).



The non-radiative relaxation processes are also of significance, they are;

a. vibrational relaxation
b. intersystem crossing (ISC)
c. vibrational relaxation..

Examples of the first two can be seen in the diagram. Internal conversion is the **radiation-less** transition between energy states of the same spin state. Intersystem crossing is a radiation less transition between different electronic spin states. Vibrational relaxation, the most common of the three-for most molecules, occurs very fast and is enhanced by collisions of the excited molecule with molecules wherein energy, in the form of vibrations and rotations, can be transferred. Thus, most excited state molecules never emit any energy because of these collisional deactivation processes.

Luminescence (Fluorescence and Phosphorescence) is the emission of light, radiative decay by a compound after it is excited by absorbing a high energy photon (low wavelength of light). The emission of radiation will occur at a longer wavelength than the light used to excite it.

To quantify the analyte in question at **low** concentrations, using either phosphorescence or fluorescence the following equation

$$P_f = 2.303\ k'\varepsilon bC\ P_{in}$$

is appropriate, where P_f is the fluorescent or the phosphorescent signal, k' the product of a geometry factor and the quantum efficiency of the fluorescent process, ε is the molar absorptivity of the molecule at the excitation wavelength, b the path length, C the concentration and P_{in} the radiant power of the excitation radiation.

Thus, provided low enough concentrations are measured, a plot of P_f versus [analyte] should be linear. The geometry factor and radiant power can be combined as a measured instrumental constant.

$$P_f = 2.303\ k'\varepsilon bC\ P_{in} = k\ C$$

$$\mathbf{P_f = k\ C}$$

Where $k = 2.303\ k'\varepsilon bP_{in}$. In this exercise a *synchronous* experiment is performed because such a mode of the experiment yields the lowest overlapping of the output signals. The functional form of the relationship between the signal and the concentration of analyte remains linear.

Procedure

A stock solution (~5 uM, note the actual concentration) of Vitamin B_2 in 1% acetic acid is provided (note the actual concentration). Make a series of standards, by mixing 1.00, 2.00, 3.00, 4.00, 5.00, 10.00, 15.00, 20.00, and 25.00 mL of stock solution provided, in 50-mL volumetric flasks with the solvent provided. (Make sure to use 1% acetic acid for diluting the stock solutions, molar mass of Vitamin B2 =376.3639 g/mol)

Weigh the Vitamin tablet. Prepare the unknown sample by dissolving 0.04-0.05g of the tablet (weighed accurately) in 10mL of ethanol and 50mL of 1% acetic acid in a 150mL beaker. Sonication facilitates the dissolution process. Filter into a ***clean*** filter flask; wash down all soluble B_2 with small aliquots of solvent. Transfer the filtrate ***quantitatively*** into 250mL volumetric flak. Dilute to the mark with 1% acetic acid. This is a laboratory/test sample (laboratory sample #1).

(If a cloudy suspension results, centrifuge about 10mL of the cloudy solution for about 5 min. Use the centrifuged solution for the next step, or filter 10 mL of the cloudy solution thro' a 45μ syringe filter, laboratory sample #1.)

Pipette 5.00mL of this solution (laboratory sample #1) into a 250-mL volumetric flask and dilute with the solvent. This is a laboratory/test sample (laboratory sample #2). You may have to further dilute this solution if the signal exceeds the linear range maximum (2×10^6 cps). Note all dilutions. Use a *four window cuvette* for this experiment.

Collecting spectra: Obtain the UV spectrum a standard solution from 200-700nm to determine the λ_{max} ($\cong\lambda_{ex}$) values (optional). With a reasonable wavelength for excitation (i.e. λ_{ex}) proceed to

determine a suitable λ_{em} for the experiment** using the fluorimeter, this is an iterative process. ***(See operating instructions of the instrument).***

The instrument automatically initializes the instrument components at the start of collecting spectra using the software package. If it does not initialize automatically, a manual initialization must be performed (from the Collect menu, please see the operating instructions).

Set up the parameters to collect the synchronous spectra with a suitable offset based on λ_{ex} and λ_{em} wavelengths. Keep other parameters as default values. Check the Detector; S, R and S/R must be active.

At this point, collect the *synchronous spectrum* of the most concentrated standard by placing the cuvette containing the standard in the sample holder, record the peak maximum and save the resulting spectra (real-time data is shown on the screen). It is recommended that you start with your most concentrated standard so as to have an idea of what the intensity of the spectrum looks like. ***Note: the highest intensity in counts per second (cps) must be less than two million to be in the linear range for the detector of this instrument.***

Use the rest of the standards to obtain the synchronous spectra, needed to generate a calibration plot. Record the peak values of the outputs (S/R) in your note book.

Obtain the synchronous spectrum and the signal height (S/R output) of the prepared laboratory samples #1 and #2 (which are the 'unknowns', in triplicate) made from the tablet using the same experimental parameters as those for the standards.

If necessary (i.e. the raw signal S (cps) > 2 million), make an appropriate dilution of the 'unknown' (and record the dilution factor) so that the signal falls within the linear portion of the calibration plot.

Using the calibration plot and any dilution factors used in the two laboratory samples #1 and #2, estimate the mass Vitamin B_2 in the tablet (mg/tablet).