Kinetic Fluorescence Determination of Vitamin B₁

Adapted from Nathan W. Bower. Journal of Chemical Education, 1982, Vol.59, No. 11, p. 975

**Background**

Luminescent substances are quantified readily by sensitive fluorescence and phosphorescence techniques. Kinetic fluorescent methods extend the sensitivity advantage of fluorescence analysis to non-fluorescing samples that can be converted chemically to fluorescent species. This experiment employs kinetic fluorescence to quantify a non-fluorescent species, namely thiamine (vitamin B₁) in solution. Fluorescence intensity is monitored as non-fluorescing thiamine is oxidized to fluorescent thiochrome. A quantitative relationship is established between the (initial) rates of increase of fluorescent intensity (i.e. initial rate of formation of thiochrome), which is directly related) to the thiamin concentration. The initial reaction rate is used to create the calibration curve.

Ryan and Ingle, who developed the fluorometric reaction-rate method for thiamine determination, found Hg$^{2+}$ to be an excellent oxidizing agent. The reaction is

\[ \text{thiamine} \rightarrow \text{thiochrome} \]

**Method**

**Wear protective gear; gloves and goggles.**

The following three solutions are prepared in advance.

Reagent grade mercuric chloride (10.068 g) is dissolved in conc. HCl, and then diluted to 100 ml to make a 500 ppm Hg$^{2+}$ solution.

The pH=12.2 buffer is prepared from reagent grade Na$_3$PO$_4$.12H$_2$O (1.92g) and Na$_2$HPO$_4$
(2.12g) in 100mL of water to make a 0.2M buffer. Sodium hydroxide or phosphoric acid is added to bring the pH to 12.2.

A standard thiamine stock solution (25 mg/L in water, pH=3-4) was prepared and stored in the dark.

Prepare five working standards with concentrations ranging from 0.25 ppm to 2.5 ppm using the stock solution in water (pH 3-4).

Pulverize a vitamin tablet in a mortar with a pestle and dilute to 1 L with acidified water. Make sure to filter any binder of the tablet if present. A further 1:10 dilution (or more) may be required to reduce the fluoresce signal and bring the signal into the calibration region and/or precipitation of the Hg\(^2+\) to an acceptable level.

**Calibrate the Spectrofluorometer (see below).**

**Kinetic run instrument setup**

Click [Experiment] menu button; select Kinetics

Set up the kinetic runs with excitation and emission wavelengths 365 nm and 444 nm respectively, and a 10nm spectral bandpass at for both slits; time increment = 5 s, total time = 300 s.

Perform kinetic runs for the standards and ‘unknown’. The stepwise procedure at this point is as follows. Usually the first run is for "practice". *Timing is important.*

i. Place 1.00mL of the standard (or unknown) to be analyzed is placed in the (fluorescence) sample cuvette.

ii. Add 1.00mL of the 500 ppm Hg\(^2+\) into the cuvette.

iii. Add 1.00mL of the 0.2 M, 12.2 pH buffer immediately.

iv. Cap the cuvette quickly and invert a few times to ensure adequate mixing and then place in the spectrofluorometer. Close the sample compartment. The reaction is then followed over time for 300 seconds, by way of the fluorescence intensity generated by the thiochrome produced.

v. Click [Run]. At the end of the run, the plot will appear on the origin window. Extract the slope of the intensity vs. time plot.

Repeat the five steps (above) for all the working standards.

Subject the ‘unknown’ prepared to the same procedure as above and determine the corresponding slope of the intensity vs. time plot.
Treatment of Results

(1) Generate a Table with thiamine concentration and slope (Reaction)

(2) With the data from (1) plot a calibration curve.

(3) Determine the mass of thiamine in the tablet.

Calibration of the Spectrofluorometer

For $\lambda_{ex} = 397$nm, water generates a Raman emission spectrum with a $\lambda_{em} = 467$nm. This water-Raman line is used for the spectrfluorometer calibration. Introduce the cuvette containing pure water, close the sample compartment.

Click [Experiment] menu button

Spectra>Experiment Type>Excitation>Next
Spectral acquisition window opens.

Click (Detectors) – verify reference detector R is active.

Click (Monos) – view monochromator settings
Set Ex. Mono; Start=200 nm, End=600, Inc=1nm, Slit=1nm
Set Em. Mono; 360nm, Slit=1nm

Click [Run] – excitation spectrum generated and eventually added to the origin window. Expand the data window, top bar right side; Click [Zoom] – enlarge the peak region
Click [Cursor] – select peak, note the current cursor position ($X=467$);
if $X \neq 467$, Click [Previous Experiment] button
Click [RTC] – in the Excitation Section enter current X value, [Enter]
Click (Monos) – in the Excitation Tab – Click [Calibration Excitation]
enter 467

Click [OK] > [Cancel] – Excitation Monochromator calibrated.

[Run] > [Zoom]; check peak value. :)

Click [Experiment] menu button
Spectra>Experiment Type>Emission>Next
Spectral acquisition window opens.
Click (Monos) – view monochromator settings
    Set Ex. Mono; 350nm, Slit=5nm
    Set Em. Mono; Start=365 nm, End=450, Inc=1nm, Slit=5nm
Click (Detectors) – verify S channel detector is active

Click [Run] – emission spectrum generated and eventually added to the origin window.
    Expand the data window
    Click [Zoom] – enlarge the peak region
    Click [Cursor] – select peak, note current cursor position (X=397); [OK]
        if X ≠ 397, Click [Previous Experiment] button
    Click (Detectors) – verify S channel detector is active
    Click [RTC] – in the Emission Section enter current X value, [Enter]
    Click (Monos) – in the Emission Tab – Click [Calibration Excitation]
        enter 397

Click [OK] > [Cancel] – Emission Monochromator calibrated.

[Run] > [Zoom]; check peak value :)