**The Simultaneous Determination of Chloride and Bromide Ions**

**Using Fluorescence Quenching**

Need Help with the items in red especially the calculations and graphs. I only have to show an example under calculations. I am struggling with the class and really need some help --- thanks

I spoke to my professor and TA and they told me I had to use my numbers. So can you please finish helping me with the calcs and graphs? I would also like you to check my Stern Volmer graph. The things I need help with are in purple and highlighted.

I will still need to send to you for a final review. Thanks

**Objectives**:

1. To become familiar with basic fluorimetry concepts and instrumentation

2. To develop a spectrophotometric assay for determining the composition of a multi-component mixture.

3. To analyze the quenching of quinine by NaCl and KBr

4. To construct Stern-Volmer plots and determine quenching constants for chloride and bromide ions for quinine and acridine

**Text Reference**:

Skoog, Holler, and Nieman *Principles of Instrumental Analysis*, 5th edition, Saunders College Publishing, Fort Worth, TX **1992**, Ch 9.

Lakowicz, Joseph R., *Principles of Fluorescence Spectroscopy*, 3rd edition, Springer Publishing, New York, NY **2006.**

**Introduction**:

Fluorescence spectroscopy is an extremely versatile and sensitive experimental technique used in identification and quantification of many environmentally important compounds: polycyclic aromatic hydrocarbons, polycyclic aromatic nitrogen heterocycles, and polycyclic aromatic sulfur heterocycles. Through judicious selection of excitation and emission wavelengths, a single desired fluorophore can often be analyzed in complex unknown mixtures containing several absorbing and fluorescing species.

The determination of analyte concentrations based upon standard spectrofluorometric methods, assumes that the observed emission intensity, F, is directly proportional to the molar concentration of the analyte fluorophore.

 F = K’[fluor] (1)

where the proportionality constant, K’, depends upon the quantum efficiency of the fluorescent process, response of the photodetector at the emission wavelength, and solute molar extinction coefficient, which should remain constant during any given chemical analysis. Analyte concentrations are determined from a working-curve plot of the measured fluorescence intensity versus the known molar concentration of standard solutions.

While these experimental methods utilize fluorescence instrumentation, the data analysis is based on applications of the Beer-Lambert law. This type of analysis has been applied previously in CHEM 2211 in the evaluation of UV-Vis spectrophotometric data to determine cobalt and chromium concentrations in solution. The experiment presented here involves simple fluorescence-quenching measurements which the amount of chloride and bromide in an unknown mixture is determined simultaneously. The experimental method involves an unusual application of fluorescence: emission-quenching is monitored as the analytical procedure. The experiment provides a very convenient analytical method for determining chloride and bromide anion concentrations. There are very few standard analytical methods in the chemical literature for halide anions.

Quenching, a decrease in fluorescence intensity, of a fluorophore can be described by the Stern-Volmer equation:

$\frac{F\_{0}}{F}=K\_{SV}\left[Q\right]+1$ (2)

where F0 and F are the fluorescence intensity in the absence and presence of a quencher, KSV is the Stern-Volmer quenching constant, and [Q] is the concentration of the quencher. If the value of KSV is known, the concentration of the quencher can be determined when F0 and F are measured.

*Effect of Two Quenching Agents Upon Fluorescence Emission:*

Quenching agents decrease fluorescence emission through collisional deactivation involving the excited fluorophore molecule (dynamic quenching) or by formation of non-fluorescent quencher-fluorophore ground-state complexes (static quenching). Both processes give similar mathematical expressions; however, only the case of collisional deactivation by two quenching agents, Cl- and Br-, will be considered in this experiment.

*Dynamic Quenching:*

fluorophore**\*** + quencher 1 fluorophore + quencher 1

fluorophore**\*** + quencher 2 fluorophore + quencher 2

where $k\_{Q1}$and $k\_{Q2}$refer to the second-order rate constants for quenching and fluorophore**\*** is the excited-state fluorophore.

In the absence of quenching agents, the change in the molar concentration of the excited fluorophore species with time can be approximated by:

$\frac{d[fluor^{\*}]}{dt}=k\_{abs}\left[fluor\right]- k\_{fluor}\left[fluor^{\*}\right]- k\_{int conv}[fluor^{\*}]$ (3)

In the presence of the two quenching agents, two additional terms are needed to describe the change in molar concentration of the fluorophore species:

$\frac{d[fluor^{\*}]}{dt}=k\_{abs}\left[fluor\right]- k\_{fluor}\left[fluor^{\*}\right]- k\_{int conv}\left[fluor^{\*}\right]- k\_{Q\_{1}}\left[quencher 1\right]\* [fluor^{\*}]- k\_{Q\_{2}}[quencher 2]\*[fluor^{\*}]$ (4)

 Under constant illumination (steady-state conditions), the fluorescence is constant:

$\frac{d[fluor^{\*}]}{dt}=0$ (5)

Since *d*[fluor\*]/d*t* = 0, Equation 4 can be solved in terms of the molar concentration of the excited fluorophore:

$\left[fluor^{\*}\right]=\frac{k\_{abs}\left[fluor\right]}{(k\_{fluor}+ k\_{int conv})\* \left\{1+\frac{k\_{Q1}}{(k\_{fluor}+ k\_{int conv})}\left[quencher 1\right]+ \frac{k\_{Q2}}{(k\_{fluor}+ k\_{int conv})}[quencher 2]\right\}}$ (6)

This must be directly proportional to the emission signal F because the fluorescence process begins with absorption of excitation radiation. Through suitable mathematical manipulation of Equation 1, a relatively simple expression is derived for relating the measured fluorescence emission intensity to both quencher concentrations:

 $\frac{F\_{0}}{F}-1 =\frac{k\_{Q1}}{(k\_{fluor}+ k\_{int conv})}\left[quencher 1\right]+ \frac{k\_{Q2}}{(k\_{fluor}+ k\_{int conv})}[quencher 2] $ (7)

where F0 and F are the fluorescence intensities in the absence and presence of a concentration of quencher, respectively.

Combining the rate constants gives:

$\frac{F\_{0}}{F}-1=K\_{Q1}\left[Q1\right]+K\_{Q2}\left[Q2\right]$ (8)

where $K\_{Q1}$is the Stern-Volmer quenching constant for quencher 1 and $K\_{Q2}$ is the Stern-Volmer quenching constant for quencher 2. Please note that $k\_{Q1}$ is not the same as $K\_{Q1}$ and$ k\_{Q2}$ is not the same as $K\_{Q2}$.

*Monitoring Fluorescence Emission*:

Numerical values for the two $K\_{Q1}$ and $K\_{Q2}$ coefficients are determined by preparing two sets of standard solutions having known quencher concentrations. This is similar to experimentally determining the molar absorptivity coefficient in the Beer-Lambert equation (*A=*ε*bC*), except that fluorescence emission is monitored instead of the absorbance of the solution.

Inherent in the above treatment is the assumption that the stoichiometric concentration of the fluorophore is constant for all of the solutions, and that the quenching process results from collisions between the excited fluorophore and quenching reagents.

**Procedure:**

1. **Before making any solutions, rinse all glassware (included pipettes and volumetric flasks) with 0.5 M H2SO4 to prevent contamination. Rinse glassware with 0.5 M H2SO4 again, if needed.**
2. You are provided with a solution of potassium bromide and sodium chloride of unknown concentration dissolved in 0.5 M H2SO4
3. To determine the $K\_{Q1}$and $K\_{Q2}$ coefficients, you are provided with the following:

a. 0.5 M H2SO4 (for dilutions)

 b. Approximately 1.8 x 10-2 M NaC1 dissolved in 0.5 M H2SO4

 c. Approximately 1.8 x 10-2 M KBr dissolved in 0.5 M H2SO4

d. A stock solution of containing approximately 4.0 ppm quinine dissolved in 0.5 M H2SO4

e. A stock solution of ~1.0 x 10-5 M acridine dissolved 0.5 M H2SO4

1. Prepare a series eight quinine solutions by pipetting appropriate quantities of stock solutions into 25 mL volumetric flasks and diluting to the mark with 0.5 M H2SO4.
	1. 3.60 x 10-4 M NaCl and 0.32 ppm quinine
	2. 7.20 x l0-4 M NaCl and 0.32 ppm quinine
	3. 1.44 x l0-3 M NaCl and 0.32 ppm quinine
	4. 2.16 x l0-3 M NaCl and 0.32 ppm quinine
	5. 3.60 x 10-4 M KBr and 0.32 ppm quinine
	6. 7.20 x l0-4 M KBr and 0.32 ppm quinine
	7. 1.44 x l0-3 M KBr and 0.32 ppm quinine
	8. 2.16 x l0-3 M KBr and 0.32 ppm quinine
2. Prepare a series eight acridine solutions by pipetting appropriate quantities of stock solutions into 25 mL volumetric flasks and diluting to the mark with 0.5 M H2SO4.
	1. 3.60 x 10-4 M NaCl and 8.0 x 10-7 M acridine
	2. 7.20 x l0-4 M NaCl and 8.0 x 10-7 M acridine
	3. 1.44 x l0-3 M NaCl and 8.0 x 10-7 M acridine
	4. 2.16 x l0-3 M NaCl and 8.0 x 10-7 M acridine
	5. 3.60 x 10-4 M KBr and 8.0 x 10-7 M acridine
	6. 7.20 x l0-4 M KBr and 8.0 x 10-7 M acridine
	7. 1.44 x l0-3 M KBr and 8.0 x 10-7 M acridine
	8. 2.16 x l0-3 M KBr and 8.0 x 10-7 M acridine
3. Open the software for the RF-5301PC instrument by double clicking on the RF-5301PC icon. The instrument may undergo an initialization routine that takes about one minute.
4. Once the initialization routine is complete, click on the **Configure** drop down menu, then select **Parameters**.
5. Set the parameters to the following then select **OK**.

 Spectrum Type: Emission

 EX Wavelength: 350 nm

 EM Wavelength Range: Start: 400 End: 500

 Recording Range: Low: -50 High: 1000

 Scanning Speed: Fast

 Sampling Interval (nm): 1.0

 Slit Width: EX: 10 EM: 3

 Sensitivity: High

 Response Time: Auto

1. Close the shutter. Do this by pressing the **Shutter** button in the lower left hand of the screen. When the shutter is closed, you will see the yellow circle disappear. Pressing the **Shutter** button again will open the shutter and expose the yellow circle in the shutter icon. The shutter should always be closed when opening the sample compartment. The type of lamp used for most fluorescence measurements emits very intense UV radiation. Closing the shutter protects you from this stray radiation.
2. Rinse the quartz fluorescence cell with a few aliquots of 0.5 M H2SO4, then fill the cuvette with the 0.5 M H2SO4.
3. Check to ensure the shutter is closed, then insert the cuvette into the fluorometer. Note the orientation of the markings on the cuvette. For the most reproducible results, these markings should always be in the same orientation throughout the experiment. Close the lid to the sample compartment, then open the shutter.
4. Auto zero your instrument by pressing the **Auto Zero** button in the lower left hand side of the screen. **This should be the only time you use Auto Zero.** One you have auto zeroed the instrument, close the shutter.
5. Stop and seriously consider how you will analyze your samples to maximize your accuracy, minimize cross contamination and reduce the effects of residual solution in the cuvette. Before each analysis, be certain to rinse the cuvette with a few aliquots of the analyte solution. Always analyze from lowest to highest concentration of quencher. Always be mindful that the shutter must be closed when the sample chamber cover is open.
6. Measure the fluorescence intensity of the eight quinine solutions at 450 nm, with excitation wavelength of 350 nm. To analyze your samples you need to close the shutter, insert the cuvette into the sample holder, close the lid, open the shutter and press **Start**. The **Start** button can be found in the lower left hand corner.
7. Once your fluorescence spectrum has been acquired, a dialogue box will appear. Name your file and press **Save**.
8. Determine the maximum wavelength and intensity by selecting the **Manipulate** drop down menu and selecting **Peak Pick**. **NOTE**: you will have a different λmax for your acridine and quinine solutions.
9. A dialogue box should appear. Expand this box FULLY. You will see your λmax displayed in nm and the intensity displayed in arbitrary units. Record these values in your notebook. You do not need to export the file. You do not need to include any spectra in your lab report.
	1. Once you have determined your λmax for your fluorophore you can determine the fluorescence intensity of your other samples at that wavelength by using the **Point Pick** feature, which is also found under the **Manipulate** drop down menu.
	2. In Box 1 of the the **Point Pick** dialogue box, enter the λmax determined in step 16. Press **OK**.
	3. A new dialogue box should open. Expand this box fully. Your wavelength and intensity will be displayed at the bottom of the box.
10. After you have collected 10 data points you will receive the following message:

 *Warning! No More Channels Available!*

You will need to clear some data from the memory.

* 1. To clear data from the memory, select the **File** drop down menu and then select **Channel**. From the **Channel** tab select **Erase Channel.**
	2. A dialogue box will appear. Select **All**, then press **OK**.
	3. Another dialogue box will open that states:

*Some Data Has NOT Been Saved To Disk! Do You Wish To Continue?*

* 1. If you are certain you have recorded all of the necessary data, select **Yes**.
1. When you have analyzed all of your quinine samples, you will need to modify the instrument parameters for analyzing acridine. The acridine parameters should be:

 Spectrum Type: Emission

 EX Wavelength: 360 nm

 EM Wavelength Range: Start: 400 End: 500

 Recording Range: Low: -50 High: 1000

 Scanning Speed: Fast

 Sampling Interval (nm): 1.0

 Slit Width: EX: 10 EM: 3

 Sensitivity: High

 Response Time: Auto

1. Repeat steps 13 through 18 for the acridine samples. Measure the fluorescence intensity of the eight acridine solutions at 472 nm, with an excitation wavelength of 360 nm.
2. From the measured intensities create plots to determine fluorescence intensity in the absence of quencher for all four fluorophore/quencher combinations.
3. **Next create Stern-Volmer plots for all four fluorophore/quencher combinations.**
4. Prepare an unknown solution by transferring 2 mL aliquots from the unknown sodium chloride/potassium bromide solution into two separate 25 mL volumetric flasks that contain either 0.32 ppm quinine or 8.0 x 10-7 M of the acridine stock solution, diluting to the mark with 0.5 M H2SO4. Record the fluorescence emission intensities in both quinine and acridine solutions, using their respective spectrophotometer settings above. Before each analysis, be certain to rinse the cuvette with an aliquot of the analyte solution.

**Calculations:**

Plot the fluorescence intensity for each fluorophore as a function of the concentration of each quencher.

I have plotted the concentration of quencher vs the fluorescence F as per equation 1 to determine the Fo value from the intercept. As per the graph, the intercept Fo = 299.91. This makes sense as the y-intercept value will be the intensity when the concentration of quencher = 0.

 Use Equation 2 to determine $K\_{Cl^{-}}$ and $K\_{Br^{-}}$ for both quinine and acridine. The value of F0 can be obtained by determining the intercept of the best-fit line through the raw data using Equation 1 (FIRST PAGE OF PROTOCOL)

Next, using the value of Fo determined from the 1st graph, we can calculate Fo/F in order to plot the 2nd graph. Check the 2nd plot. The slope of each graph will be the K values. Hence for Q solutions of NaCl, the KCl- = 88.98. Similarly after plotting the other graphs, the values obtained are the following.

Q solutions: KCl- = 88.98, KBr- = 107.51

A solutions: KCl- = does not look correct , KBr- = 309.86

It looks like your data for NaCl in Acridine is not correct. It seems quite erroneous compared to the other values.

The molar concentrations of bromide and chloride ions in the unknown mixture are computed by solving the following two fluorescence-quenching equations simultaneously.

For quinine:

$\frac{F\_{0}}{F\_{unk}}-1=K\_{Cl^{-}\_{quinine}}\left[Cl^{-}\right]+K\_{Br^{-}\_{quinine}}\left[Br^{-}\right]$(9)

$$\frac{288.244}{249.860}-1=88.98\left[Cl^{-}\right]+107.51\left[Br^{-}\right]$$

For acridine:

 $\frac{F\_{0}}{F\_{unk}}-1=K\_{Cl^{-}\_{acridine}}\left[Cl^{-}\right]+K\_{Br^{-}\_{acridine}}\left[Br^{-}\right]$ (10)

$$\frac{268.613}{206.788}-1=?\left[Cl^{-}\right]+309.86\left[Br^{-}\right]$$

**Report:**

Tabulate and report your raw data. The report should include plots of fluorescence intensity as a function of the concentration of each quencher and fluorophore concentration. (The plots are already as shown in excel) You should also include Stern-Volmer plots for each quencher and fluorophore combination. Tabulate the quenching constants and report the amount of each quencher in the unknown sample. In the discussion, be sure to identify any sources of error in the determination of the quenching constants.

**Questions:**

1. In a typical fluorometer configuration, the detector is oriented at a 90° angle relative to the excitation source. Why? How is this orientation different from a typical configuration in a single-beam UV/Vis spectrometer?
2. In this experiment, are the photons emitted by the fluorophore more or less energetic than the excitation photons. Why?
3. How does a fluorescence cuvette differ from one used in UV/Vis spectroscopy?
4. What generally has more energy: The incident photon absorbed by a fluorophore or the photon emitted by the fluorophore as fluorescence? Why? Use a diagram to justify your answer.