Chemistry 3211

Experiment #5

The Simultaneous Determination of Chloride and Bromide Ions

Using Fluorescence Quenching

18 June 2013

**ABSTRACT**

 The fluorescence quenching method was used to analyze an unknown sample using fluorescent indicators quinine and acrdine to determine the weight percentages of NaCl and KBr.

**INTRODUCTION**

 The composition of a multi-component mixture was determined by developing a spectrophotometric assay. Also, the quenching of quinine by sodium chloride (NaCl) and potassium bromide (KBr) was analyzed and Stern-Volmer plots were constructed for chloride and bromide ions for quinine and acridine. Basic fluorimetry concepts and instrumentation was demonstrated for familiarization.

 Fluorescence quenching has a wide application in biochemistry as a means to study proteins and membranes. It is used to identify the features of macromolecular assembly and associated structure changes. The basic principle is a high energy photon is absorbed and the molecule is raised to an excited state. The excess energy is released into the molecular environment as the excited molecule returns to the ground state. The fluorescence light is emitted as the same or lower frequency than the excited frequency. Collision quenching theorizes that fluorescent intensity is reduced by quenchers proportionally to the concentration of the quencher as summarized by the Stern-Volmer equation: $Fo/F = 1 +Ksv[Q] $ where Fo is the intensity in the absence of a quencher, F is the intensity in the presence of a quencher, K*sv* is the Stern-Volmer quenching constant and Q is the concentration. The major advantage to fluorescence quenching is it can separate compound two ways, excitation or emission spectra, as compared to a single spectra therefore it has a low signal to noise factor. Fluorscence quenching has a variety of applications. It has been determined that carbamazepine is useful in the treatment of epilepsy and bipolar depression. However when it is found in aquatic environments in high concentrations it can negatively impact aquatic animals such as the mussel’s immune system or the rainbow trout’s liver. To investigate the biogeochemical cycling fluorescence spectroscopy was employed due to its sensitivity and simplicity in use, fluorescence is used to provide information on humic substances (HS) and trace metals, compounds and pesticides. Specifically, carbamazepine and HS binding behavior was investigated and it was determined that carbamazepine and HS can increase its solubility in aquatic environments (1). Similarly, a growing environmental concern is the increasing number of reports of endocrine-disrupting compounds (EDCs) in water resulting from sewage treatment plants releasing estrogenic compounds into aquatic environments. It has been suggested that suspended solids as well as humic acid can be absorbed by EDCs. Further copper which is common in aquatic environments can interact with EDCs resulting in changes of aquatic toxicity. A study conducted by Yong and et.al. entitled “Effect of Copper Complexation on the Estrogenic Activities of the Endocrine Disrupting Compounds Using E-Screen Bioassay” employed fluorescence quenching methodology in their research. The research suggested that Cu reduces the combination effect of EDCs and should be taken into consideration when estrogenic activities are evaluated in aquatic environments (2). Fluorescence quenching has been used to identify denatured proteins’ residual structures. The residual structures are important since this is the location of the protein-folding initiation sites. Fluorescence quenching methodology proved effective in locating residual structures of tryptophan in denatured proteins which provide insight into transport of proteins across membranes (3).

**EXPERIMENTAL SECTION**

**METHODS**

 The procedures established in the “The Simultaneous Determination of Chloride and Bromide Ions Using Fluorescence Quenching” protocol posted on T-square were used to conduct the experiment (4). To prevent contamination, all glassware was rinsed with 0.5 M of H2SO4. A solution of potassium bromide and sodium chloride of unknown concentration dissolved in 0.5 M H2SO4 was provided by the teaching assistant (TA). In addition, the TA provided the following solutions approximately 1.8 x 10-2 M NaC1 dissolved in 0.5 M H2SO4; approximately 1.8 x 10-2 M KBr dissolved in 0.5 M H2SO4; a stock solution of containing approximately 4.0 ppm quinine dissolved in 0.5 M H2SO4; and a stock solution of ~1.0 x 10-5 M acridine dissolved 0.5 M H2SO4 to determine the KQ1 and KQ2 coefficients. A series of eight quinine solutions were prepared by pipetting the appropriate quantities of stock solutions into 25 mL volumetric flasks and diluted to the mark with 0.5 M H2SO4. To prepare the eight quinine solutions we calculated how much quinine would be put into each volumetric flask which was determined to be 2ml of quinine for each flask. We also calculated how much NaCl and KBr was put into each for a,b,c,d it was .5, 1, 2, 3 (ml) of NaCl respectively and for e,f,g,h it was .5, 1, 2, 3 (ml) of KBr, respectively. These were the same numbers used for calculating the difference for 2ml of acridine in each.

Ex calculation: $\frac{\left(3.60\*10^{-4}\right)\*(x)}{\left[1.8\*10^{-2}\right)/25}$ =.5ml

A series of eight acridine solutions were prepared by pipetting the appropriate quantities of stock solutions into 25 mL volumetric flasks and diluted to the mark with 0.5 M H2SO4. The software for the RF-5301PC instrument was opened by double clicking the RF-5301PC icon. Upon initialization completion, the “configure” from the drop down menu was clicked and “parameters” was selected. The following parameters were set: spectrum type – emissions; EX wavelength – 350 nm; EM wavelength range – started at 400 and ended at 500; recording range – selected low at -50 and high 1000; scanned speed – fast; sampling interval: 1.0 nm; slit width – EX: 10 and EM: 3; sensitivity – high; and response time – auto, then “ok” was selected. The shutter was closed by pressing “shutter” button which caused a yellow circle to disappear. By pressing the “shutter” button again caused the shutter to open and the yellow circle shutter icon to reappear. Note, the shutter should be closed while opening the sample compartment. The quartz fluorescence cell was rinsed with a few aliquots of 0.5 M H2SO4, then the cuvette was filled with 0.5 M H2SO4. The shutter was checked to ensure it was closed then the cuvette was inserted into the fluorometer. The lide was closed and the shutter opened. The “auto zero” button was pressed and the shutter closed. Prior to each analysis the cuvette was rinsed with a few aliquots of the analyte solution. The concentration of the quencher was analyzed from lowest to highest and care was taken to ensure the shutter was closed while the sample chamber cover was open. The fluorescence intensity of the eight quinine solutions was measured at 450 nm with an excitation wavelength of 350 nm. The analysis of the samples was conducted by closing the shutter, inserting the cuvette into the sample holder, closing the lid, opening the lid and pressing “start”. When the dialog box appeared after the fluorescence spectrum was acquired the file was named and “save” was pressed. The maximum wavelength and intensity was determined by selecting the “manipulate” drop down menu and selecting “peak pick”. The dialog box was expanded and the λmax was displayed in nm and the intensity in arbitrary units and the values were recorded in the lab notebook. Next, the fluorescence intensity of the samples at the wavelength was determined by using the “point pick” feature located in the “manipulate” drop down menu. In the “point pick” dialogue box, the λmax was entered in box 1 and “OK” was pressed. The new dialog box was expanded and the wavelength and intensity was displayed. A message appeared “Warning! No More Channels Available!” after ten data points were collected which required the memory to be cleared. The memory was cleared by selecting “file” from the drop down menu and “channel” was selected and then “erase channel” was selected. The “all” was selected and “ok” was pressed. Upon seeing “Some Data Has NOT Been Saved To Disk! Do You Wish To Continue?”, the “yes” selected. After analyzing all the quinine samples the instrument parameters were reset for acridine analysis. The acridine parameters were set as follows: spectrum type – emission; EX wavelength – 360 nm; EM wavelength range – started at 400 and ended at 500; recording range – selected low at -50 and high 1000; scanned speed – fast; sampling interval: 1.0 nm; slit width – EX: 10 and EM: 3; sensitivity – high; and response time – auto. The above steps were repeated using acribine samples. The fluorescence intensity of the eight acridine solutions was measured at 472 nm with an excitation wavelength of 360 nm. Fluorescence intensity in the absence of quencher for all four fluorophore/quencher combinations was plotted from the measured intensities. Stern-Volmer plots were created for all four fluorphore/quencher combinations. An unknown solution was prepared by transferring 2 mL aliquots from the unknown sodium chloride/potassium bromide solution into two separate 25 mL volumetric flasks that contained 2mL quinine or 2 mL of the acridine stock solution, diluted to the mark with 0.5 M H2SO4. The fluorescence emission intensities were recorded for both quinine and acridine solutions using their respective spectrophotometer settings. The cuvette was rinsed with an aliquot of analyte solution prior to each analysis. The unknown solution was titled 6/18/2013.

**INSTRUMENTATION**

 The RF-5301PC Spectrofluorophotometer manufactured by Shimadza of Kyoto, Japan was used in the experiment. Also, the Xenon short arc lamp made by Ushio, Inc. of Tokyo, Japan was used to conduct the experiment.

**RESULTS**

**RAW DATA**

Table 1. Quinine Solutions measured with no, NaCl and KBr concentrations with max wavelength of 450 nm with resulting intensities in a.u.

|  |  |  |  |
| --- | --- | --- | --- |
| Solution | Concentration (mL) | Max Wavelength (nm) | Intensity (a.u.) |
| None | 0 | 450 | 288.244 |
| NaCl | 0.00036 | 450 | 290.24 |
| 0.00072 | 450 | 286.53 |
| 0.00144 | 450 | 268.375 |
| 0.00216 | 450 | 252.398 |
| KBr | 0.00036 | 450 | 291.061 |
| 0.00072 | 450 | 279.342 |
| 0.00144 | 450 | 271.428 |
| 0.00216 | 450 | 242.47 |

Table 2. Acridine Solutions measured with no, NaCl and KBr concentrations with max wavelength of 472 nm with resulting intensities in a.u.

|  |  |  |  |
| --- | --- | --- | --- |
| Solution | Concentration (mL) | Max Wavelength (nm) | Intensity (a.u.) |
| None | 0 | 472 | 268.613 |
| NaCl | 0.00036 | 472 | 271.23 |
| 0.00072 | 472 | 275.235 |
| 0.00144 | 472 | 276.027 |
| 0.00216 | 472 | 272.125 |
| KBr | 0.00036 | 472 | 261.889 |
| 0.00072 | 472 | 236.971 |
| 0.00144 | 472 | 199.668 |
| 0.00216 | 472 | 171.076 |

Table 3. Unknowns with Quinine and Acridine with max wavelength (nm) and intensities (a.u.).

|  |  |  |
| --- | --- | --- |
| Solution | Max Wavelength (nm) | Intensity (a.u.) |
| Unknown with Quinine | 450 | 249.860 |
| Unknown with Acridine | 472 | 206.788 |

**GRAPHS**

Graph 1. Concentration versus Fluorescence Intensity for Quinine solution NaCl.

Graph 2. Fluorescence in absence of Quencher divided by Fluorescence with Quenchers Fluorescence Intensity versus Concentration for Quinine solution NaCl.

Graph 3. Concentration versus Fluorescence Intensity for Quinine solution KBr.

Graph 4. Fluorescence in absence of Quencher divided by Fluorescence with Quenchers Fluorescence Intensity versus Concentration for Quinine solution KBr.

Graph 5. Concentration versus Fluorescence Intensity for Acridine solution KBr

Graph 6. Fluorescence in absence of Quencher divided by Fluorescence with Quenchers Fluorescence Intensity versus Concentration for Acridine solution KBr.

Graph 9. Stern-Volmer Plots for Concentration Quencher (mL) vs. Fluorescence Intensity (a.u.) for Fluorophores, Q-NaCl, Q-KBr, A-NaC and A-KBr.

**CALCULATIONS**

Intensity In the Presence of a Quencher and Intensity in the Absence of a Quencher

For Q solutions in the Presence of a Quencher example: Fo = Absence of a Quencher and

F = Quencher

Factors for None Factors for NaCl Concentration of 0.0036

Wavelength: 450 nm Wavelength: 450 nm

Intensity: 288.244 Intensity: 290.24

$\frac{Fo}{F}$ = $\frac{288.244}{290.24}$ = 0.993

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 is correct 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.

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

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:

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

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

For acridine:

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

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

**DISCUSSION**

*Statistical Analysis*:

*Analysis of Sources of Error*:

 Fluorescence quenching is a very sensitive technique and is highly susceptible to interference by contamination of any trace level of an organic chemical. To eliminate this error, rinsing the cuvette with an aliquot of analyte solution prior to each analysis is essential. An error can be attributed to pH and temperature. Errors associated with pH effects can be reduced or eliminated by using nonaqueous solvents. Though room temperature does not significantly affect fluorescence intensities, increased molecular collisions occur more frequently at higher temperatures which decrease fluorescence intensity. Maintaining a constant temperature will reduce this error. An error can result from selecting dyes that are similar in color causing the peaks to overlap. Potential interactions should be considered when choosing color combination. Nonfluorescent dyes can interfere with emitted light from the fluorescent dyes causing a decrease in the fluorescence signal and giving an inaccurate reading. To prevent this error, ensure fluorescent dyes are selected for the experiment.

*Significance*:

*Conclusion*:

**G. Points for Discussion**

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? The detector is oriented at 90o to eliminate background signal and minimize noise from stray light. The cuvette has a 1 cm path length. If the cuvette is not inserted at a 90o angle like in single-beam UV/Vis then the path length is shortened. Since absorbance is dependent on path length then the absorbance would be smaller than expected for a given concentration sample.
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? Fluorescence cuvettes must be clear on all four sides so when it is placed at a 90o angle the beam contributions are limited when the measurement is taken reducing signal and noise. The cuvette for a UV/Vis spectroscopy is clear on two sides and cloudy on the other two.
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. The photon absorbed by a fluorophore has more energy than the photon emitted by the fluorophore as fluorescence due to the energy dissipation during the excited state creating longer wavelengths.

<http://www.jacksonimmuno.com/technical/f-cy3-5.asp> (has a table verifying this statement)

<http://www.dkfz.de/Macromol/teaching/files/fcs_practical.pdf> (page 7 picture section 1.3.2)

**References**

1. Bai, Y, Fu, P, Li, W, Xing, B, Wu, F, Liu, C, & Guo, J 2008, 'Interaction between carbamazepine and humic substances: a fluorescence spectroscopy study', *Environmental Toxicology And Chemistry*, 27, 1, pp. 95-102, Agricola, EBSCO*host*, viewed 16 June 2013.

2. Jong Yol, P, Byoung Cheun, L, Jin Sung, R, Jiho, L, & Sang Don, K 2008, 'EFFECT OF COPPER COMPLEXATION ON THE ESTROGENIC ACTIVITIES OF ENDOCRINE-DISRUPTING COMPOUNDS USING E-SCREEN BIOASSAY', *Environmental Toxicology & Chemistry*, 27, 3, pp. 535-541, Environment Complete, EBSCO*host*, viewed 16 June 2013.

3. Kumar, S, & Swaminathan, R 2007, 'Employing the fluorescence anisotropy and quenching kinetics of tryptophan to hunt for residual structures in denatured proteins', *Journal Of Chemical Sciences*, 119, 2, pp. 141-145, Academic Search Complete, EBSCO*host*, viewed 18 June 2013