

Lab 4 Determination of Quinine in Tonic Water by Fluorescence

Introduction to the Lab. In a previous lab, you determined the concentration of quinine in tonic water using UV-Visible absorbance spectroscopy. Benzoate, present as the sodium salt in tonic water, is used as a preservative. You will recall that benzoate absorbed strongly in the UV frequency range (see absorbance spectra in previous labs). Quinine, another component of tonic water, fluoresces strongly, allowing it to be detected at very low concentrations. Sensitivity is of course not a major limitation in this application because the concentration of quinine is not especially low in tonic water. Benzoate fluoresces similarly to toluene; the fluorescence spectrum of toluene is attached for comparison with quinine. By judicious selection of both the excitation λ_{ex} and emission λ_{em} wavelengths, you should be able to avoid any significant contribution from benzoate fluorescence when trying to excite and detect quinine in this experiment. This simplifies the experiment because now it is not necessary to independently determine the benzoate concentration. Fluorescence is, in general, a more sensitive analytical technique than absorbance, and you will also compare detection limits for the two techniques.

Background on Fluorescence. Ideally, in a fluorescence measurement the emission intensity F_i (labeled F_i in Fig. 4-1) is proportional to the incident power, P_0 , of the excitation source, as well as the molar absorptivity ϵ and the concentration c :

$$F = P_0 \Omega \epsilon c \quad 4-1$$

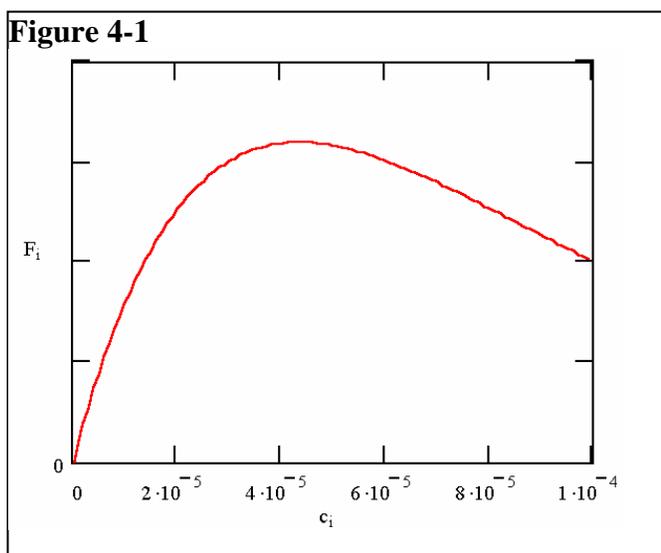
The parameter Ω represents a collection of terms, including the *quantum yield* and the collection and detection efficiencies.

When the sample is concentrated, as you know from *absorbance* spectroscopy in previous labs, then the power propagating through the sample decreases. The power propagating through the sample decreases exponentially with c and changes with F in accord with the Beer-Lambert Law (Beer's Law) as shown in Eq. 4-2.

$$F = P_0 10^{-\epsilon bc} \Omega \epsilon c \quad 4-2$$

In addition, the collection efficiency will also decrease due to the sample

reabsorbing its own fluorescence at λ_{em} (as opposed to absorbing the incident power at λ_{ex}), but we will neglect this effect here. A plot of Eq. 4-2 for simulated data is shown below, using $\epsilon = 10,000$. Notice that nonlinearity sets in at relatively low concentrations, and that F actually decreases for concentrations above $\sim 5 \times 10^{-5}$ M in this example. The upshot is that the calibration curve will become noticeably nonlinear when concentration becomes high. For example, when $c > 1/(10 \times \epsilon)$, then P is noticeably less than P_0 .



PRE-LAB ASSIGNMENT

1. You have the fluorescence emission spectrum of quinine from your first two labs. For maximizing sensitivity and restricting the signal to just quinine, choose the excitation λ_{ex} and emission λ_{em} wavelengths you should use. Explain how you arrived at these values.
2. Using Excel or your favorite plotting software, plot Eq. 4-2 for quinine using the correct molar absorptivity and the same range of concentrations you used in Lab 1 for studying the limiting high value for the absorbance spectrometer. Use any arbitrary values of Ω and P_0 because these will not affect the shape of the curve. Plot Eq. 4-2 again after also including a range of concentrations that is ten-times lower. Based on these plots of Eq. 4-2, decide whether you should dilute your quinine sample to get it on the linear range of the calibration curve.
3. If you conclude that a diluted sample of tonic water must be used to achieve linearity, decide what dilution factor ought to be used. To support your idea, make a new plot for the new range of concentrations of standards that bracket that of the diluted tonic water sample to show that these are on a more linear scale.
4. Explain your procedure for how you will estimate the detection limit for the fluorescence determination of quinine. Recall the other measurements you have made in previous labs.

EXPERIMENTAL

1. Measure the excitation and emission spectra of quinine at the appropriate concentrations. Using your arguments in the Pre-Lab and an iterative process in the lab if necessary, choose the excitation λ_{ex} and emission λ_{em} wavelengths you should use. When collecting the spectra, be sure to set the non-scanned wavelength to an appropriate value.
2. Prepare and use your set of fluorescence calibration standards to determine the concentration of quinine in the supplied diluted tonic water “unknown”.
3. Make measurements for your determination of the fluorescence detection limit for quinine.

WRITTEN REPORT

1. Discuss whether the experimental excitation and emission wavelengths agreed reasonably well with your predictions in the Pre-Lab.
2. Plot the calibration curve for quinine. Does it behave as you had predicted?
3. Calculate the concentration of quinine in tonic water and its 95% confidence interval. Compare this to the result you obtained from absorbance spectrometry in the previous labs.
4. Calculate the detection limit (in molar concentration units) for the fluorescence determination of quinine in tonic water. Compare your fluorescence and absorbance detection limits in molar concentration units for quinine.

This laboratory was created by Professor Mary J. Wirth, September, 2002. It was revised by Professor Thomas P. Beebe, Jr., September 2003. The enrichment materials were prepared by 2003 first-year TA Erinc Sahin. We welcome your comments on how to improve the learning experience of this lab. The best time to communicate these comments is when you are working on the lab.

We are painfully aware that the fluorescence equipment (and other equipment used in this lab) is older than you are, unreliable, and unlike anything you are likely to encounter in the “real world.” Our faculty has been working to obtain funding to upgrade this experiment with modern analytical instrumentation. If you wish to become involved by expressing your support for this upgrade, please contact me (beebe@udel.edu). Your support of this effort, perhaps in the form of a short e-letter, would probably carry a great deal of weight with the administration.

CHEM 438 – Instrumental Methods Laboratory
Enrichment Materials for Fluorescence Spectroscopy Experiment
Written and provided by 2003 first-year graduate student Eric Sahin

Purposes of the Experiment:

- To teach the theory of fluorescence and quenching phenomena.
- To teach the working principles of fluorescence spectrometer and different applications and techniques of fluorescence spectroscopy in protein chemistry/structure/interaction studies (with the emphasis on Forster Resonance Energy Transfer (FRET)).
- To relate some fluorescent materials with the applications in their *in vivo* and *in vitro* real-life uses.

List of Resources:

Journal References:

- Chatellier, D. S.; White, H. B. “What color is egg white? A biochemical demonstration of the formation of a vitamin-protein complex using fluorescence quenching” *Journal of Chemical Education* Vol 65 (1988), pp 814
- Chirio-Lebrun; M. Prats, M. “Fluorescence Resonance Energy Transfer (FRET): theory and experiments” *Biochemical Education* 26 (1998), pp 320-323
- Croney, J. C. *et al.* “Fluorescence spectroscopy in biochemistry: teaching basic principles with visual demonstrations” *Biochemistry and Molecular Biology Education* 29 (2001), pp 60-65
- Pandey, S. *et al.* “Comparison of Analytical Methods: Direct Emission versus First-Derivative Fluorometric Methods for Quinine Determination in Tonic Waters” *Journal of Chemical Education* Vol 76 – Issue 1 (1999), pp 85-87
- Royer, C. A. “Approaches to Teaching Fluorescence Spectroscopy” *Biophysical Journal* Vol 68 (1995), pp 1191-1195
- Shane, E. C. *et al.* “Fluorescence Measurement of Pyrene Wall Adsorption and Pyrene Association with Humic Acids An Experiment for Physical Chemistry or Instrumental Methods” *Journal of Chemical Education* Vol 77 – Issue 12 (2000), pp 1617-1618
- Wingen, L. M. *et al.* “Chromatography, Absorption, and Fluorescence: A New Instrumental Analysis Experiment on the Measurement of Polycyclic Aromatic Hydrocarbons in Cigarette Smoke” *Journal of Chemical Education* Vol 75 – Issue 12 (1998), pp 1599-1603

Online Resources:

Contains useful information and shockwave interactive animations about the physics of fluorescence:
<http://phys.educ.ksu.edu/vqm/html/fluorescence.html>

“Handbook of Fluorescent Probes and Research Products” together with technical information:

<http://www.probes.com/handbook/>

Laboratory for Fluorescence Dynamics: University of Illinois at Urbana-Champaign, Department of Physics:

<http://lfd.uiuc.edu/>

Valuable information about the theory of fluorescence by Photon Technology International. The site also contains applications and techniques using fluorescence as a tool:

<http://www.pti-nj.com/information.html>

Very useful table of chromophores, their excitation and emission wavelengths, molecular weights, quantum yields and miscellaneous properties:

<http://pingu.salk.edu/flow/fluo.html>

History of synthesis and use of fluorescent probes:

http://laxmi.nuc.ucla.edu:8248/M248_99/synprob/part1/history.html

Journal of Fluorescence:

<http://cfs.umbi.umd.edu/jf/>

University of Maryland at Baltimore School of Medicine, Center for Fluorescence Spectroscopy:

<http://cfs.umbi.umd.edu/cfs/>

Very interesting and informative site about light emitting (bioluminescent) organisms and including the chemistry, physiology of the phenomenon, myths and facts section and photographs: Bioluminescence Web Page:

<http://www.lifesci.ucsb.edu/~biolum/>

Interesting Questions and Subjects to increase laboratory interest:

“Real Life” uses of fluorescence which can be used in laboratory and/or lecture discussions:

- Bioluminescence: Why do organisms take the risk of being seen by their predators? Is shining an evolutionary wise selection?
- What are the differences between fluorescence, luminescence and phosphorescence (if any)?
- Scorpion hunt at night: use a “black light” (UV source) to see unusually “bright green” scorpions.
http://www.agpix.com/view_caption.php?image_id=18832&photog=1
Case study: Scorpions are not fluorescent under UV light right after changing their outermost layer as a part of their usual growth. It takes a few hours for them to shine again. Propose a mechanism for this time dependent gain of fluorescence using the fact that fluorescence is the result of fluorescence-active chemical centers called “fluorophore”.
- Use of fluorescent probes in crime scene investigation: molecules that specifically target blood proteins or DNA.

Fluorescence as a tool for biological science: Shiny cells and proteins:

- Fluorescent protein family as a useful tool in molecular biology and microscopy: Locating proteins *in vivo* by tagging them with a “light source”! Talking about an experiment design (making the students propose with instructor’s guidance) to determine “cell fate” in *Xenopus* embryos (injection of GFP into individual cells with microinjection during 2, 4, 8 cell stages of development).
- Importance of Trp residues in proteins in terms of fluorescence. Tracing conformational changes by fluorescence spectroscopy.
- How can we investigate protein-ligand interactions using fluorescence?
- Uses of FRET.
- Use of GFP (or other fluorescent proteins such as yellow FP, red FP, blue FP) containing specialized protein construct (by constructing the plasmid coding for it) as a generalized protein immobilization kit

(enzyme / signal peptide / antibiotic ...) with the advantage of visualization and quantification on functionalized polymeric surfaces. (CHEM438 TA Erinc Sahin's master thesis)

<http://people.sabanciuniv.edu/~taralp/presentations/ACS2003%20NewOrleansPoster%20Erinc.pdf>

Figure 4-2. Emission spectrum of toluene. This is provided as an example of a compound with an emission spectrum that should be similar to the emission spectrum of benzoic acid/benzoate. Notice that the upper horizontal axis is in wavelength units of Angstroms, labeled as "A", but more correctly should be "Å", where 10 Å equals 1 nm. Notice also that the top axis is increasing nonlinearly in a different direction than the output you will generate in the lab.

