CHM 317H1S
Winter 2021

Section C - Molecular Fluorescence

D: Fluorescence Spectroscopy

1. List of Experiments

1. Determination of Quinine in Tonic Water
2. Critical Micelle Concentration of Surfactants

2. Locker Inventory

Glassware for each experiment may be found in designated drawers in room LM6. These should include the following items; please report any missing or broken items to your lab TA or the lab coordinator.

✓	Quantity	Item (4 drawers)
	6	100 mL polyethylene beakers
	2	250 mL glass beaker
	4	50 & 100 mL glass beakers
	12 each	10 and 25 mL volumetric flasks with stoppers
	6	100 mL volumetric flasks with stoppers
	1	250 mL volumetric flask with stopper
	1	10 mL graduated cylinder
	2 each	1, 2, 5, and 10 mL transfer pipettes
	2 each	1, 5 and 10 mL Mohr (graduated) pipettes
	20	Pasteur pipettes with bulbs
	3	3-Way bulb pipette fillers
	6	100 mL polyethylene storage bottles
	2	Wash bottles
	1 pack	Paper towel
	1 box	KimWipes™

Extra items for D1:

Two each large & small spatulas, powder funnel, filter funnel, and glass stirring rod.

One 1.00 L volumetric flask with stopper

Aluminium foil (lab supplies)

3. Technique Overview

Students should already be familiar with the theory and practice of UV-visible absorption spectrophotometry. When a molecule undergoes electronic excitation through photon absorption, the resulting excited state can revert to the ground state via several mechanisms including photon emission by resonance, fluorescence, and/or phosphorescence. These are often described with reference to the electronic and vibrational energy levels involved by referring to a Jablonski diagram (analogous to the Grotrian diagram students should be familiar with from atomic spectroscopy.) The main distinctions between these mechanisms are the lifetime of the excited state and the energy (wavelength) of the emitted photon.

Fluorescence spectrophotometry makes use of a high intensity continuous light source and a monochromator to excite molecules in the sample with specific energy photons (the excitation wavelength, λ_{ex}). This light is modulated to provide alternate light and dark periods. The instrument is typically arranged so that emission from the sample is measured during the dark cycle along an optical path at right angles to the excitation. A second monochromator selects for a specific emission wavelength (λ_{em}) of light, which is measured using a sensitive detector such as a photomultiplier tube.

Fluorescence spectra can be recorded in a number of ways:

- **Excitation spectrum**: λ_{ex} is varied while λ_{em} is held fixed
- **Emission spectrum**: λ_{em} is varied while λ_{ex} is held fixed
- **Synchronous scanning**: λ_{ex} and λ_{em} are varied simultaneously with either a fixed or variable offset between them

Excitation spectra are analogous to absorption spectra (although there are some difference), while emission spectra often resemble a mirror image of the corresponding excitation spectrum.

Because fluorescence measurements involve the use of two distinct wavelengths rather than one, they are generally more selective than absorption measurements when multiple species are present. Because they involve the measurement of photons against a dark background, they are also more sensitive than absorption measurements on the same solution. Conversely, molecular emission is more susceptible to structural and environmental factors that influence both emission wavelengths and intensities; for this reason, fluorescence is widely used as a probe of environmental conditions.

4. Instrumentation

You will be using one of two Perkin-Elmer luminescence spectrophotometers located in LM6 that are capable of measuring fluorescence, phosphorescence, and chemi– or bioluminescence. These use a pulsed xenon flash lamp as the source, and separate Monk-Gillieson excitation and emission monochromators covering the wavelength range 200–800 nm with a wavelength accuracy of ±1.0 nm and reproducibility of ±0.5 nm. Both monochromators have slit widths that can be varied over the range 2.5–20 nm in 0.1 nm increments. All excitation spectra are corrected using an internal rhodamine cell. Instrument control and data acquisition is achieved using the FL WinLab software loaded on the adjacent computers. Sample cells are standard 10 mm pathlength fluorescence cells, having windows on all four sides. These are expensive: handle with care!

The following pages provide general information on using the luminescence spectrophotometers in the Analest laboratory. Please take time to read through these instructions carefully before coming to the laboratory.

4.1 Starting the Instrument:

Turn on the instrument using the main switch on the left-hand side, at the bottom and towards the rear of the case. Log on to the computer using the appropriate username and password:

Username: chm317LS55 and password LS55 (right side of the bench)

Username: chm317fl6500 and password fl6500 (left side of the bench)

Once Windows has started, locate and double-click on the shortcut for the **FLWinLab** or **Fluorescence** software.

4.2 Configuring the Instrument via Software **FLWinLab**:

(a) ***Instrument Overview:*** Select **Setup** from the **Applications** menu. In the resulting **Status** window you will see icons representing the different components of the instrument. You can check and alter the settings for each part of the instrument by clicking on the appropriate icon. Check that the following parameters are set:

1. Source Mode: fluor ExCorr: on
2. Under **Ex. Mono:** (excitation monochromator)
   Excitation Filter: clear
3. Under **Em. Mono:** (emission monochromator)
   Emission Filter: clear

Minimise the **Status** window.

(b) ***Configuration:*** Select **Configuration…** from the **Utilities** menu; uncheck the "**Auto run methods**" check box, then click **OK**. This will prevent the software automatically starting to collect data whenever you select a method.

(c) ***Method:*** Load an appropriate method from the **Methods** window. You can use the drop-down menu at the bottom of this window to limit the display to the particular type of method needed, *e.g.* a scan or concentration method. Double-click on the desired method. A new window will open (either **Scan** or **Concentration**) containing several tabs and a set of icon-buttons in the window's toolbar.

➔ **Do not** close either the **Scan** or **Concentration** window until you have printed out your report, or you will lose all your data!

(d) Click on the **Setup parameters** tab in the resulting method window review the method parameters and set a default base **Destination filename** for your data.

WARNING: The software is old and extremely limited. Do **not** use more than 5 characters in your file name; do ***not*** use spaces, punctuation marks, or special characters; ***avoid*** ending it with a number. Failure to observe these limitations will result in you being unable to print or export your files!

Make sure that the **Auto increment filenames** box is checked; this will automatically append each successive data file with #01, #02, *etc.*

➔ Pay particular attention to the slit width settings. When obtaining spectra, you should use a narrower slit on the scanning side, and a larger slit on the fixed side. For example, to obtain an emission spectrum you might use an excitation slit width of 5 – 10 nm in order to increase the incident light intensity stimulating the emission, and narrower emission slits (e.g. 4 - 5 nm) in order to limit the effects of polychromatic radiation on the resulting spectrum. If either slit is too wide for the concentration of fluorophore being studied, the detector will saturate and you will not obtain accurate data at the peak wavelength(s). If either slit is too narrow when studying low concentrations of fluorophore, the signal will be noisy and weak.

(c) ***Sample Cell:*** Handle the fluorescence cell with care – these are expensive to replace! Hold the cell by grasping either opposite top corners, and support it underneath when walking between the instrument and the bench.

Use a wash bottle to rinse the cell with ultrapure water after every sample or standard; collect the washings in a large, marked beaker for eventual transfer to the appropriate waste container. Drain the cell by placing it upside down on a KimWipe™, making sure that it is safely placed well away from the edge of the bench!

Fill the cell using a clean Pasteur (dropping) pipette: rinse the cell rinse at least three times with small portions (~0.5 mL) of the solution first, collecting the washings in a waste beaker as before, then fill the cell from the bottom up *i.e.* place the tip of the pipette in the bottom of the cell and gently expel the solution from the pipette. You will need to fill the cell to between 2/3rds and 4/5ths of its volume. Carefully wipe off any liquid from the outside faces of the cell with a KimWipe™. If there are air bubbles in your sample, dislodge them by ***gently*** drawing and replacing solution with the Pasteur pipette.

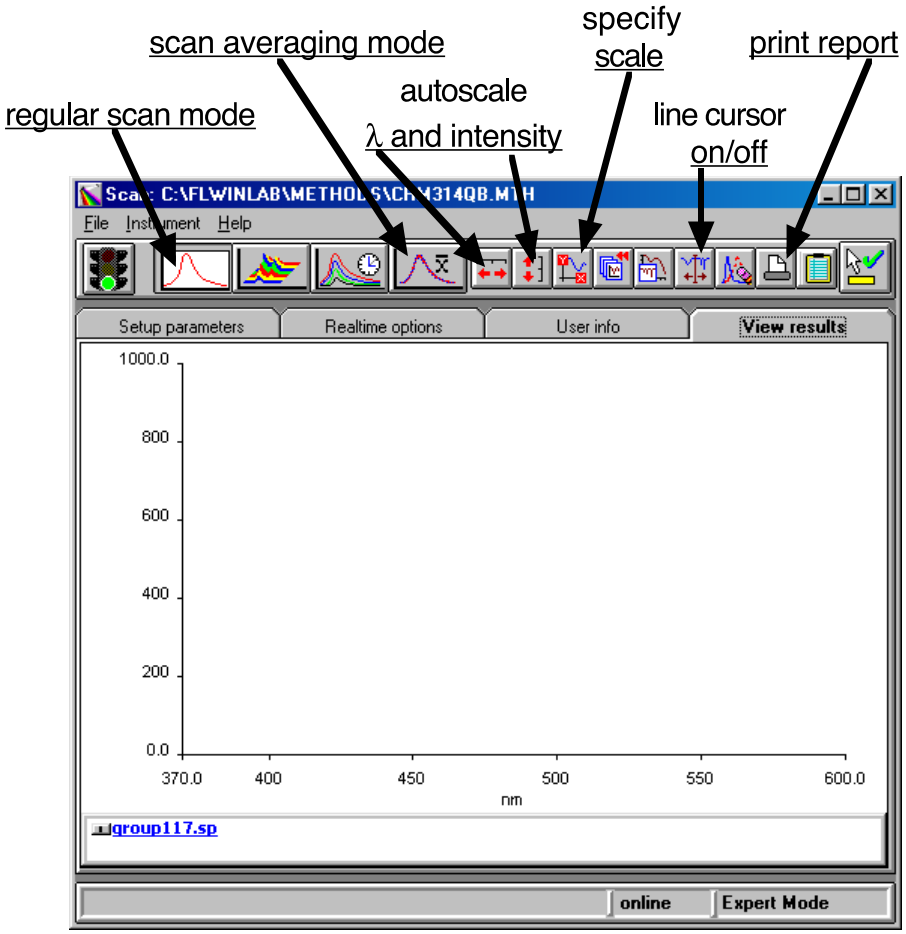
(d) ***Obtaining Spectra:*** Fill the fluorescence cell with your sample solution and carefully place it in the holder within the sample compartment, being sure to close the sample compartment lid.

➔ Since the cell may not be perfectly square, it is important to make sure you place it in the cell holder the same way round each time. Some cells will have a letter on the top corner indicating the cell material; use this as an orientation mark.

To protect the instrument, make sure that the sample compartment lid is closed whenever you are not inserting or removing the cell. Close the lid ***gently***; the catch is magnetic, so be careful not to bump it during measurements.

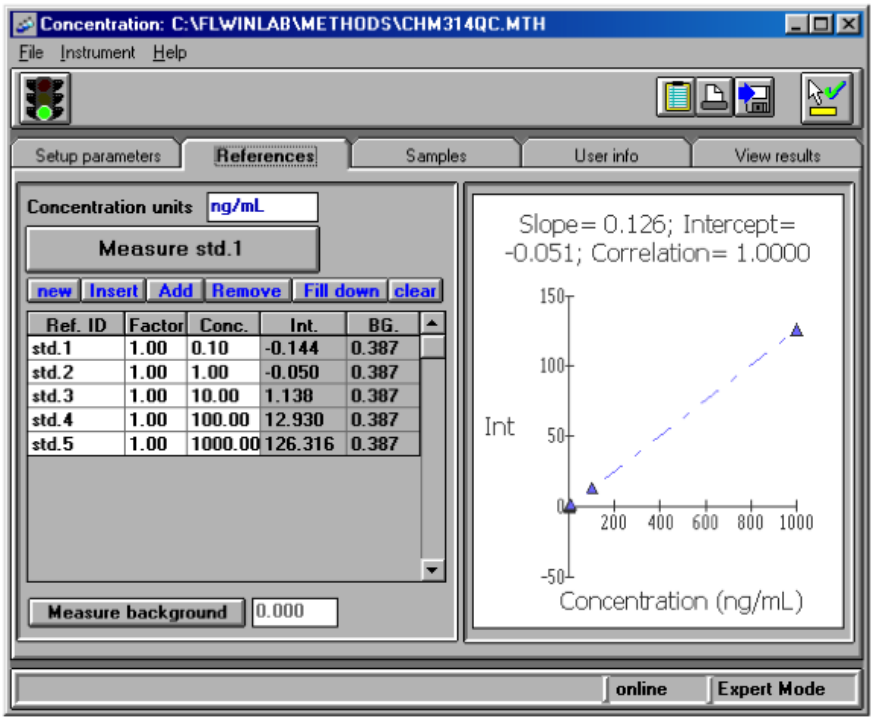
When ready, initiate the measurement by clicking on the traffic-light button in the toolbar. In the **Scan** window, click on the **View results** tab to see the fluorescence spectrum. When the scan is completed, the traffic-light button will revert to green. Once you have obtained all your scans, print out a report as outlined below.

Do not close the **Scan** window until you have printed out your report, or you may lose all your data (especially if you made a long file name such as that in the example below!)



(e) ***Measuring Concentrations:*** Fill the fluorescence cell with your blank solution and carefully place it in the holder within the sample compartment, being sure to close the sample compartment lid. In the **Concentration** window, click on the **Setup parameters** tab to check the instrument settings, then click on the **References** tab. A spreadsheet-style area and plot will appear: Add or remove entries to match the number of calibration samples you have, entering the appropriate concentration in ng/mL (ppb). List your calibration samples in order of increasing concentration.

Click on the **Measure background** button at the bottom of the window to record the background reading (mainly scatter from the solvent, cell, *etc*.) Remove the cell, rinse it, and then fill it with your first calibration solution. Click on **Measure std.1** to record the fluorescence intensity for the first (most dilute) standard.



The calibration window and tabs for determining concentration

Repeat this process as prompted by the software in order of increasing concentration until all your calibration solutions have been run. The software will construct the calibration curve in the area on the right-hand side of the window.

To determine the concentration of your unknowns, click on the **Samples** tab in the **Concentration** window and fill in the chart in the appropriate locations. Enter each unknown at least three times in order to obtain replicate measurements for each one; this provides greater accuracy and precision in the interpolated concentration value. Once you have measured all your calibration solutions and unknowns, print out a report as outlined below.

- **Do not** close the **Concentration** window until you have printed out your report, or you will lose all your data!

(f) **Printing a Report:** Click on the **View results** tab. You can rescale the plot, then either select **Print** from the **File** menu or click on the printer icon button in order to obtain a hard copy of your data.

4.3 Shutting Down the Instrument

Close all windows sequentially and exit the software. Log off from the computer, and turn off the optical bench. Make sure you remove, clean, and return the cell.

5. Experiment D1: Determination of Quinine in Tonic Water

This experiment is adapted from parts of expt. 10–1 from "Chemistry Experiments for Instrumental Methods" by Sawyer, Heineman, and Beebe. You will characterise the fluorescence spectrum of quinine, and perform a determination of quinine in a sample solution. Quinine is strongly fluorescent in dilute acidic solution, having one emission and two excitation peaks in its spectrum. As part of the characterisation, you will study the effect of pH on quinine fluorescence.

After performing this experiment, you should:

- Understand the connection between structure, pH, and fluorescence
- Understand the difference between excitation and emission spectra
- Be aware of the high concentration limits in fluorescence measurement
- Be aware of other optical phenomena that can be observed using fluorescence spectrophotometers

Chemicals:

- Quinine sulphate dihydrate
- 1 M sulphuric acid in deionised water
- 0.05 M sulphuric acid in deionised water
- Solution A (3.1 g boric acid + 2.65 g citric acid to 300 mL in pure water)
- Solution B (9.0 g disodium hydrogen phosphate-12-water + 1 g sodium hydroxide made to 300 mL in pure water)
- Chloride-free pH 7 buffer

Sample(s):

- A sample of tonic water will be provided

A. Instrument setup and solution preparation

1. ***Stock Solution:*** Make 1 L of a stock solution containing 100.0 mg/L of quinine: weigh out accurately (*i.e.* by difference on an analytical balance) approximately 100 mg of quinine (120 mg of quinine sulphate dihydrate) into a clean dry 250 mL beaker. As always, make sure you record the actual mass used in your lab notebook *when you do the actual weighing*!

   Fully dissolve the quinine in ~50 mL of 1 M sulphuric acid and ~100 mL of ultrapure water, then quantitatively transfer this solution to a 1.00 L volumetric flask and dilute to the mark with ultrapure water. Keep this solution protected from the light with aluminium foil.

2. ***Calibration Standards and Sample Solution:*** Prepare a set of calibration standards by serial dilution of your 100.0 mg/L (≡ 100.0 μg/mL ≡ 100 ppm) stock solution with 0.05 M sulphuric acid as follows: pipette 10.00 mL of your stock solution into a labelled 100.0 mL volumetric flask, and dilute to the mark with 0.05 M sulphuric acid. Now pipette 10.00 mL of the 10.00 mg/L solution into another 100.0 mL volumetric flask and dilute as before.

Repeat this process until you have five calibration solutions covering the range 1.000 mg/L to 0.100 μg/L (1 ppm – 0.1 ppb).

You will also need to dilute the tonic water sample by a factor of 1/1000 using the 0.05 M sulphuric acid solution before measuring it.

➔ The concentrations indicated here and throughout this experiment are the *nominal* concentrations based on an exact mass of 100 mg of quinine. Obviously, you should calculate your *actual* concentration, and use those values to construct your calibration curve and perform all calculations!

3. ***Excitation Spectrum of Quinine:*** In the **FLWinLab** software, load the method **CHM317QB**. Obtain excitation and emission spectra for the 0.05 M sulphuric acid and your 0.1 mg/L standard as follows: use an emission wavelength, λ_{em} = 450 nm with a 2.5 nm slit width, and an excitation range of 200 to 800 nm using a 2.5 nm slit width (see section 4.2(d)) and a scan speed of 250. Once you have the scan parameters configured in the software, obtain excitation spectra of both the acid and the quinine. Identify the peaks you see in the excitation spectrum.

➔ Always note the operating conditions and sample identify/concentration for each scan in your lab notebook together with the corresponding file name for the spectrum *as you obtain each spectrum*!

4. ***Emission Spectrum of Quinine:*** Now set the excitation wavelength, λ_{ex} to the wavelength of the excitation peak corresponding to the ground-to-first electronic excitation ($S_0 \rightarrow S_1$*) – **check** your choice with your demonstrator before continuing. Set the excitation slit width to 2.5 nm, and scan the emission spectra of the acid and quinine from 200 to 800 nm using a 2.5 nm emission slit width. Identify the peaks you see in the emission spectrum. Make sure you print out your spectra.

5. ***Concentration Calibration***: Click on the Quantitation icon on the right side of the window and select the **CHM317QC** method from the list. In the sample table, make sure you have enough rows for a blank, your standards, and three separate measurements of your same. Use the drop-down menu in the appropriate column to designate each row as either blank, standard, or sample, and enter the numeric values for your concentrations.

   Configure the instrument settings to use λ_{ex} = 350 nm, λ_{em} = 450 nm, and both slits set to 2.5 nm.

   Run a blank (0.05 M sulphuric acid), your calibration solutions, and you sample (three times) in sequence. You can check on the results in the relevant tab. Record all the fluorescence intensity values so that you can construct your own calibration curve and perform your own concentration calculations later.

6. ***Effect of pH*:** You will investigate the effect of pH on quinine fluorescence using both fluorescence and absorbance spectrophotometry. Quinine is dibasic, with the first base–conjugate acid equilibrium occurring over the pH range 2–7; you will therefore need to identify the **isosbestic wavelength** to use in subsequent fluorescence measurements.

   (a) UV-Visible spectra: First, prepare two solutions by pipetting 2.00 mL of your 100 mg/L quinine stock solution into separate 25.00 mL volumetric flasks. Make one to volume using the 0.05 M sulphuric acid and the other using halide-free pH 7 buffer; make sure you label the flasks. Follow the instructions provided by your TA to obtain the UV absorbance spectrum of your solutions over the range 260 to 500 nm, using deionised water as the

blank/reference. Use the line cursor to identify (a) **all** absorbance maxima and (b) **all** isosbestic wavelengths of the two forms; print copies of the resulting spectra for your reports. Compare the absorbance values at each λ_{max} with the corresponding peak intensities from the excitation spectra recorded in step 3. Which peak for the diprotonated (acidic solution) corresponds to the highest molar absorptivity? Which of the isosbestic wavelengths likewise corresponds to the highest molar absorptivity for the diprotonated and monoprotonated (neutral solution) forms?

(b) Emission spectra: Take five 25.00 mL volumetric flasks and pipette 1.00 mL of your 10 mg/L quinine solution into each. Use graduated pipettes to dispense different volume ratios of buffer solutions A and B so that the total volume of both added to each flask is 5 mL; dilute the contents of each flask to the mark with ultrapure deionised water, and mix thoroughly by repeated inversion. The following table is a suggestion.

Flask:	1	2	3	4	5
Volume of 10 mg/L quinine standard (mL)	1.000	1.000	1.000	1.000	1.000
Vol. solution A (mL)	4.00	3.00	2.75	2.50	2.25
Vol. solution B (mL)	1.00	2.00	2.25	2.50	2.75

Reselect the wavelength scan icon and make sure you are still using the **CHM317QB** method. Record the emission spectrum of each solution (λ_{em} = 370 – 600 nm) using the isosbestic wavelength for λ_{ex}; use widths of 2.5 nm for excitation and 4.0 for emission monochromators. Also measure the actual pH of each solution using the pH meter provided LM6. You should note both the wavelengths and intensities of the fluorescence emission peaks as a function of pH. Make sure you print out your spectra.

➔ In fluorescence spectrophotometry, it is common practice to use a larger slit width on the emission rather than excitation monochromator, as this better optimises the signal-to-noise ratio in the measured fluorescence intensity.

7. **Clean-up:** Remove the fluorescence cell from the instrument, clean it, and return it to your TA. Shutdown the instrument following the procedure outlined in section 4.3. Dispose of all your solutions in the proper waste container. Used Pasteur pipettes should be rinsed thoroughly with distilled water before being disposed in the teal-coloured decontaminated glass bin. Clean all your glassware, using some alcohol on a KimWipe™ to remove any labels, and return it to the correct bench drawers.

➔ Please keep all the transfer and graduated pipettes in a different drawer to the rest of the glassware, in order to minimize accidental breakage

Check all areas where you have been working – balance, bench, and instrument – to make sure that they are clean and tidy, and that all chemicals have been returned to the correct shelves. When done, have your TA validate your lab notebooks before leaving.

8. **Data analysis:**

a. Construct the calibration curve from step 4 for yourself using Excel in order to determine the linear range and limits of detection and quantitation for the instrument and methodology used in this experiment. Comment on the LOD and LOQ, as well as the linear range in your discussion. Do you think they are reasonable, and what do they suggest to you about fluorescence as a quantitative technique?

b. Use this information from the calibration curve to determine the concentration of quinine in your tonic water sample.

c. From step 6, determine the effect of pH on quinine fluorescence. Why do the observed effects occur, and what is the optimum pH for quinine fluorescence measurements? Do you think using a different isosbestic wavelength would have made a difference? Why/why not?

d. Use the data you have collected to estimate the pK_a or pK_b for the equilibrium observed. Hint: what is a common equation that relates pH to pKa?

Experiment D2: Critical Micelle Concentration of Surfactants

In this experiment you will estimate the critical micelle concentration of sodium dodecyl sulphate (SDS), a common surfactant, using acridine orange (Sigma-Aldrich, catalogue number **318337)** as a fluorescent probe. Acridine orange is an example of a tricyclic basic dye (similar to methylene blue). As such, it exists in acidic or neutral solution as the protonated form (AOH^+); it exists as the neutral form (AO) in highly basic solution (pH > 12), and has a pK_a in the range 9.5–10. This experiment explores the effect of environment on fluorescence intensity, which can either increase or decrease as a consequence.

After performing this experiment, you should:

- Be aware of the importance of sample environment on fluorescence
- Understand the relationship between absorption and excitation spectra
- Understand the Stern-Volmer relationship in fluorescence quenching
- Be aware of the ways fluorescence can be used as a probe of different systems

Chemicals:

- 0.020 M sodium dodecyl sulphate in deionised water
- 3.0 - 4.0 × 10^{-5} M acridine orange in deionised water (note actual concentration)
- 1.00 M sodium bromide in deionised water

Preparation:

1. Look up the structures and MSDS information for the chemicals you will be using. List any specific safety concerns and handling instructions.
2. Research the terms surfactant, micelle, and critical micelle concentration. What are the stages in micelle formation?

References:

1. M. Rujimethabhas and P. Wilairat, *J. Chem. Ed.*, **1978**, *55*, 342.
2. P. Montes-Navajas, A. Corma, and H. Garcia, *Chem. Phys. Chem.*, **2008**, *9*, 713-720.
3. M. Shaikh, J. Mohanty, P. K. Singh, W. M. Nau, and H. Pal, *Photochem. Photobio;. Sci.*, **2008**, *7*, 408-414.

A. Instrument setup and solution preparation

1. Make sure you record the *actual* concentrations, to the full number of significant figures provided, of acridine orange and sodium dodecyl sulphate (SDS) shown on the bottles in your lab notebook *at the start* of this experiment.

B. Fluorescence and Absorption Spectra of Acridine Orange

2. Take a clean, dry, 25.00 mL volumetric flask and accurately pipette 1.00 mL of the acridine orange stock solution into it. Dilute the contents to the mark with ultrapure deionised water, and label it clearly. **Use this diluted solution for all subsequent experiments.**

   One of you should use this solution to determine the fluorescence excitation and emission wavelengths of acridine orange while the other prepares the next set of solutions (from step 7 onwards).

3. Use the spectrofluorometer on the right for this experiment. Start by rinsing and then filling the fluorescence cell with your first acridine orange solution; note the actual concentration of this diluted solution in your lab notebook. Load the method **CHM317D2**, and click on the **Setup parameters** tab. See section 4.2 for details on handling the cell, configuring the software, and obtaining the spectra.

4. ***Excitation Spectrum of Acridine Orange*:** In the tab, click on the **Excitation** button. Set the emission wavelength (λ_{em}) to 530 nm with a slit width of 5 nm and the excitation wavelength (λ_{ex}) range as 250–510 nm with a slit width of 2.5 nm; record the excitation spectrum of your solution. Use the vertical cursor in the results window to identify the wavelengths of any peaks you see in the excitation spectrum, and record these in your lab notebook.

➔ Always note the operating conditions and sample identify/concentration for each scan in your lab notebook together with the corresponding file name for the spectrum *as you obtain each spectrum*!

5. ***Emission Spectrum of Acridine Orange:*** Now return to the **Setup parameters** tab and click on the **Emission** button. Keep the same slit widths as before, but set the excitation wavelength λ_{ex} to that for the most intense near-UV region peak in your excitation spectrum from step 4; set the emission wavelength (λ_{em}) range as 500–700 nm, and record the emission spectrum. Reset λ_{ex} to the most intense visible region peak in the excitation spectrum, and record this emission spectrum also. Note the emission wavelength(s) in your lab notebook, and print out the combined excitation and emission spectra.

6. ***Absorbance Spectrum of Acridine Orange*:** Your TA will have setup one of the UV/Visible spectrophotometers for you. Half-fill a UV cell with the stock (~30-40 μM) acridine orange solution, then fill it up with distilled or deionised water. Record the UV/Visible spectrum over the range 250–600 nm using distilled or deionised water as a blank. Determine the wavelengths of all key features in the absorbance spectrum, recording them in your lab notebook, and print a copy of the spectrum.

C. Effect of Surfactant and Determination of its CMC

7. To each of four of the flasks containing 1.00 mL of the **diluted** acridine orange solution, add 1.00, 5.00, 10.00 and 15.00 mL of the SDS solution, respectively. Make each one to volume using ultrapure water, being careful to label each flask as you make the solution.

8. Now set up the software to obtain emission spectra using λ_{ex} = 480 nm and λ_{em} = 500–650 nm using an excitation slit width of 2.5 nm and an emission slit width of 5 nm. Clear all existing spectra from the results window (note that this will *not* delete the actual files), and record the emission spectra for each of the five solutions prepared (with 0, 1, 5, 10, and 15

mL of SDS added). Use Excel to plot a graph of the maximum emission intensity *versus* the concentration of SDS.

➔ Start with the **highest concentration** of SDS first so that you can check that the fluorescence intensity will not be off-scale. If the peak intensity is ≥ 1000 units, you will need to adjust the excitation and emission slit widths; please consult your demonstrator for appropriate values, then collect a fresh emission spectrum using the new settings. Make sure you rinse the cell ***thoroughly*** with distilled water before running the lowest concentration sample next.

9. Make additional solutions using 1.000 mL of acridine orange and different volumes of SDS in order to obtain a sigmoidal (*s*-shaped) curve with enough data points to be able to determine accurately the SDS concentration at the inflexion point of this graph – this is the critical micelle concentration (CMC). Make sure you record *all* concentrations and intensities in your lab notebook *as you acquire them*, and that you print a copy of both your spectra and your graph.

D. Effect of Heavy Atoms on Fluorescence Intensity

10. When you are satisfied that you have sufficient data from part C to determine the critical micelle concentration of SDS, empty your flasks into the appropriate waste container, rinse them thoroughly with distilled then ultrapure water, and proceed to make a set of solutions containing 1.00 mL of acridine orange stock solution with different volumes of 1.00 M sodium bromide solution (0, 5.00, 10.00, 15.00, and 20.00 mL), each made up to a final volume of 25.00 mL.
11. Record the emission spectra of these solutions in the same way as you did for the acridine orange–SDS solutions in part C, and plot a graph of emission intensity *versus* concentration of sodium bromide. Make sure you record *all* concentrations and intensities in your lab notebook *as you acquire them*, and that you print a copy of both your spectra and your graph.

E. Finishing Up

12. Remove the fluorescence cell from the instrument, clean it, and return it to your TA. Shutdown the instrument following the procedure outlined in section 4.3. Dispose of all your solutions in the proper waste container. Used Pasteur pipettes should be rinsed thoroughly with distilled water before being disposed in the teal-coloured decontaminated glass bin. Clean all your glassware, using some alcohol on a KimWipe™ to remove any labels, and return it to the correct bench drawers.

➔ Please keep all the transfer and graduated pipettes in a different drawer to the rest of the glassware, in order to minimize accidental breakage

Check all areas where you have been working – balance, bench, and instrument – to make sure that they are clean and tidy, and that all chemicals have been returned to the correct shelves. When done, have your TA validate your lab notebooks before leaving.

F. Data Analysis

13. **CMC:** Determine the mid-point of the sigmoidal curve, and report this as the critical micelle concentration (CMC). The literature value, as determined by a conductometric method, is 8.1 mM at 25°C. Sigmoidal curves, such as those observed here or during potentiometric titrations, are notoriously difficult to fit in Excel™ since the latter does not include an appropriate function. There are several approaches you can adopt:
    - Use other software to fit a sigmoidal function to the data, neglecting the initial points
    - Use a 1st or 2nd-derivative plot to find the inflexion point, just as you would for a pH titration.
    - Exclude the initial and final portions of the data, and fit a cubic (3rd-order polynomial) through the central portion of the data around the inflexion point; use this to solve for the inflexion point.

14. **Quenching:** Use a Stern-Volmer plot to determine the quenching constant K_q for acridine orange in the presence of sodium bromide. The Stern-Volmer equation may be written as $F_0/F = 1 + K_q[Q]$ where F_0 is the fluorescence intensity without quenching agent, F is the intensity with quenching, and $[Q]$ is the molar concentration of the quenching agent. Determine if there is any evidence of a limit to the linear range of this plot.

7. Elements for Report Discussion:

Your formal report for this technique should describe the experiments performed and present the results obtained, commenting on any special features observed in the spectra obtained. From these results, you should go on to discuss the factors that affect molecular fluorescence, and how such factors can be exploited. You should address the following questions in addition to any specific questions or findings from each experiment:

1. How are absorbance and fluorescence spectra related? What other optical phenomena can be observed in fluorescence emission and excitation spectra (particularly in relation to the first parts of experiment D1)? Why might these other optical phenomena pose a challenge in collecting quality emission and excitation spectra?
2. What are the important structural features of a molecule or coordination complex that determine whether or not it can undergo absorption and fluorescence? What other factors (instrumental or otherwise) can affect the intensity and wavelength of fluorescence emission?
3. Why is the curve recorded in experiment D2 sigmoidal (*s*-shaped)? What accounts for the dramatic difference in fluorescence intensity between the low and high SDS concentration portions of the graph?
4. DNA hybridization can be detected using fluorescent indicators known as *intercalating dyes*. How do such indicators function, and how does this relate to the effects observed in experiment D2?
5. Fluorescence is widely used in biological and clinical chemistry, either by exploiting the native fluorescence of proteins and other biological molecules, or by attaching fluorescent molecules to antibodies, enzymes, and short sections of DNA or RNA. What features of fluorescence account for its widespread use in this way?