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Reading:
1. Skoog, Holler and Crouch: Chapter 15, sections 15A-15C
2. Handout on Instrument DESCRIPTION
3. Handout on Instrument OPERATION

A.   INTRODUCTION

Fluorescence is a spectrochemical method of analysis where the molecules of the analyte are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis. As shown in Figure 1(reference 2, page 401), when light of an appropriate wavelength is absorbed by a molecule (i.e., excitation), the electronic state of the molecule changes from the ground state to one of many vibrational levels in one of the excited electronic states. The excited electronic state is usually the first excited singlet state, S1 (Figure 1). Once the molecule is in this excited state, relaxation can occur via several processes. Fluorescence is one of these processes and results in the emission of light (Refer to Figure 1 during the following discussion).
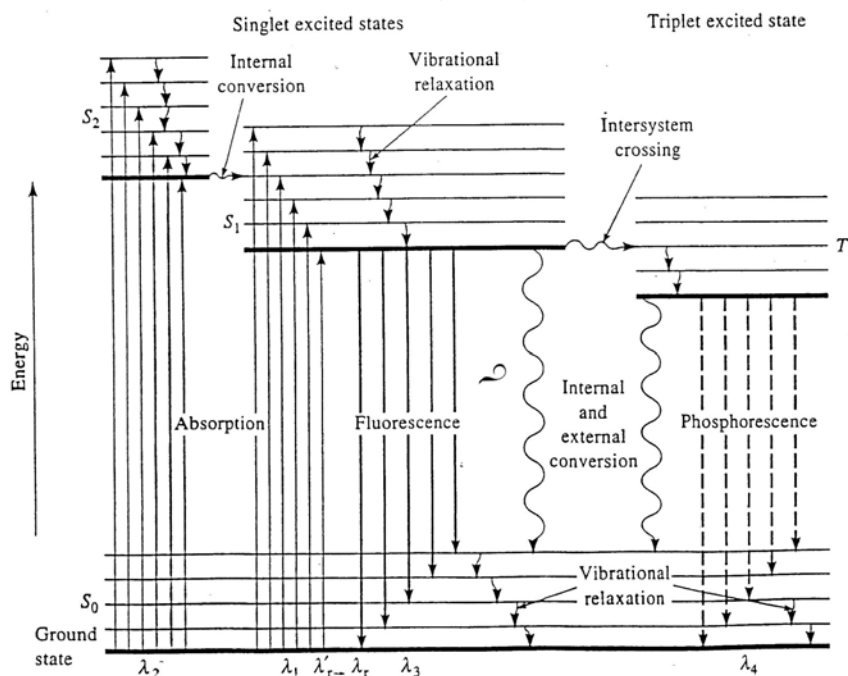

Figure 1: Electronic transition energy level diagram.

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Following absorption, a number of vibrational levels of the excited state are populated. Molecules in these higher vibrational levels then relax to the lowest vibrational level of the excited state (vibrational relaxation). From the lowest vibrational level, several processes can cause the molecule to relax to its ground state. The most important pathways are:

1. Collisional deactivation (external conversion) leading to nonradiative relaxation.
2. Intersystem Crossing (10^{-9}s): In this process, if the energy states of the singlet state overlaps those of the triplet state, as illustrated in Figure 1, vibrational coupling can occur between the two states. Molecules in the single excited state can cross over to the triplet excited state.
3. Phosphorescence: This is the relaxation of the molecule from the triplet excited state to the singlet ground state with emission of light. Because this is a classically forbidden transition, the triplet state has a long lifetime and the rate of phosphorescence is slow (10^{-2} to 100 sec).
4. Fluorescence: Corresponds to the relaxation of the molecule from the singlet excited state to the singlet ground state with emission of light. Fluorescence has short lifetime (~10^{-8} sec) so that in many molecules it can compete favorably with collisional deactivation, intersystem crossing and phosphorescence. The wavelength (and thus the energy) of the light emitted is dependent on the energy gap between the ground state and the singlet excited state. An overall energy balance for the fluorescence process could be written as:

$$E_{fluor} = E_{abs} - E_{vib} - E_{solv.relax.} \quad (1)$$

where E_{fluor} is the energy of the emitted light, E_{abs} is the energy of the light absorbed by the molecule during excitation, and E_{vib} is the energy lost by the molecule from vibrational relaxation. The $E_{solv.relax}$ term arises from the need for the solvent cage of the molecule to reorient itself in the excited state and then again when the molecule relaxes to the ground state. As can be seen from Equation (1), fluorescence energy is always less than the absorption energy for a given molecule. Thus the emitted light is observed at longer wavelengths than the excitation.

5. Internal Conversion: Direct vibrational coupling between the ground and excited electronic states (vibronic level overlap) and quantum mechanical tunneling (no direct vibronic overlap but small energy gap) are internal conversion processes. This is a rapid process (10^{-12} sec) relative to the average lifetime of the lowest excited singlet state (10^{-8} sec) and therefore competes effectively with fluorescence in most molecules.

Other processes, which may compete with fluorescence, are excited state isomerization, photoionization, photodissociation and acid-base equilibria. Fluorescence intensity may also be reduced or eliminated if the luminescing molecule forms ground or excited state complexes (quenching).

The quantum yield or quantum efficiency for fluorescence is therefore the ration of the number of molecules that luminesce to the total number of excited molecules. According to the previous discussion, the quantum yield (ϕ) for a compound is determined by the

$$\phi = \frac{k_f}{k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d} \quad (2)$$

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relative rate constants (k_x) for the processes which deactivate the lowest excited singlet states, namely, fluorescence (k_f), intersystem crossing (k_i), external conversion (k_{ec}),

internal conversion (k_{ic}), predissociation (k_{pd}), and dissociation (k_d).

B.   EXPERIMENT SUMMARY

In this experiment:

1. the excitation and emission spectra for the fluorescent dye fluorescein will be measured.
2. the effect of concentration and instrumental bandwidth on the fluorescent signal will be studied.
3. quinine in tonic water will be determined fluorimetrically using a calibration curve and standard addition.

C.   EQUIPMENT

The instrument used in this experiment is Perkin-Elmer MPF-2A Spectrofluorometer. Description of the instrument and operational procedures are outlined in the handouts: INSTRUMENT DESCRIPTION and OPERATION (in the drawer below the instrument).

Optical System

Figure 1-1 in the INSTRUMENT DESCRIPTION handout illustrates the optical system of the MPF-2A. A 150 W xenon lamp (1) is used as the light source. The bright spot of the xenon lamp is enlarged via the elliptical mirror (2), and after being collimated into a beam, is focused via a concave mirror (4) onto the excitation slit assembly (3) through the entrance slit (4). Part of the beam, which is then dispersed to a spectrum via the diffraction grating assembly (5), is directed out of the exit slit (6), passes through a collecting lens assembly (7), and impinges on the sample cell (8). For light source compensation, a portion of the excitation light is reflected by a beam splitter quartz plate (9) to a Teflon reflecting plate (10). The scattered light from Teflon plate is directed to a monitor photomultiplier (11). The emitted light from the cell is passed through a lens (12), and directed into the emission monochromator, consisting of the slit assembly (13) and a diffraction grating assembly (14). The spectral light is reflected from a convex mirror (15) and directed to the measurement photomultiplier (16).

Using the numbering system in the above text, label Figure 1-1 appropriately and submit the labeled Figure with your report.

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D.   EXPERIMENTAL

D.1. Start-Up

Use your handout on OPERATION (page 2-10) to start up the instrument.
Prepare solutions while the instrument is warming up.

D.2. Solutions

 i. Tonic water solutions

   1.Solution TW10:

   Dilute the tonic water by a factor of 10 in 0.1 M H_2SO_4 . Pipette 10.0 mL
   of tonic water into a clean 100 mL volumetric flask and fill to the mark
   with 0.1 M H_2SO_4.

   2.Solution TW 200:

   Pipette 5.0 mL TW10 into a clean 100 mL volumetric flask and filling to
   the mark with 0.1 M H_2SO_4 . Calculate how many times the original tonic
   water has been diluted.

 ii. Solutions for Standard Addition Method

   Pipette 5 mL of TW10 solution into each of five 100 mL volumetric
   Flasks. Dilute the first volumetric flask to volume with 0.1 M H_2SO_4 . Then
   pipette 1 mL of the stock 10 ppm quinine solution to the second
   volumetric flask and dilute with 0.1 M H_2SO_4. Repeat by pipetting 2, 3,
   and 5 mL to the third, fourth and fifth volumetric flask respectively and
   again dilute each to volume with 0.1 M H_2SO_4 . Calculate how many
   times the original tonic water has been diluted.
 iii. Fluorescein solution (1000 ppm in 95 % ethanol)

D.3. Excitation and Emission Spectra of Fluorescein

Fluorescence spectroscopy can yield low detection limits, high sensitivity and high
specificity. The high specificity is largely due to the fact that fluorophores exhibit
specific excitation (absorption) and emission (fluorescence) wavelengths. These
wavelengths can be determined via the collection of two spectra, an excitation spectrum
and an emission spectrum. Although the approximate excitation and emission
wavelengths for many molecules are known, these wavelengths should generally be
optimized for the specific conditions employed.

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In this section, the excitation and emission wavelengths for fluorescein will be determined by collecting excitation and emission spectra.

 Fluorescein (CAS No.: 2321-07-5, MW: 332.31)
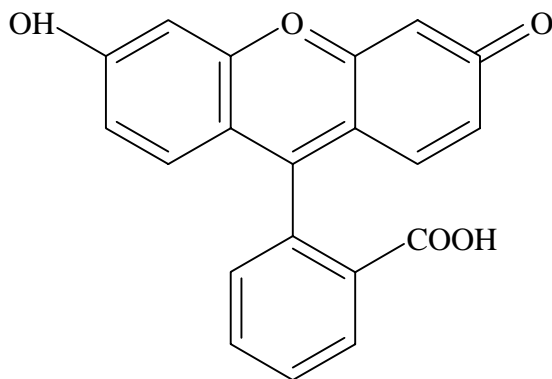
Synonym:
        Fluorescein 548;
        Fluorescein 27

Absorption max: 498 nm

Fluorescence max: 518 nm

D.3.1 Cell Handling

Absorption cells (cuvettes) should receive the same care given a lens or other optical component. The optical surfaces of cells that are placed in the light beam must be absolutely clean, or serious errors in spectrophotometric measurements will result.

In the handling of cells, the following well-known rules should be followed without exception.

1. Never touch the optical surfaces of the cell. Contact with the skin will invariably leave a film that, though invisible to the eye, will change the light transmission and reflection characteristics of the cell windows, especially in the ultraviolet region.
2. Handle cells only at the top portions of the side plates that do not face the optical axis.
3. When filling cells with sample solutions, a dropper, or preferably a pipette, should be used rather than direct pouring from a beaker or test tube.
4. Rinse the cell with several portions of the solution before filling. Avoid overfilling the cell.

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5. Do not spill liquid on the outside of a cell. Before inserting a cell into the holder, carefully wipe the cell windows with a clean lens tissue or suitable absorbent lint-free disposable wiper.
6. Always orient cells in the same direction in the cell holder. When using a matched pair of cells, always use the same cell for the reference.
7. For the disposable plastic cells, solvents like methanol and ethanol can be contained for a maximum time of 5 min. Never use the plastic cells for toluene.

D.3.2 Emission Spectrum

Note: For the fluorescein analysis, the plastic disposable cuvettes are used.

Find fluorescein's approximate absorption maximum (498 nm).

With the excitation wavelength fixed at 498 nm, obtain the emission spectrum between 350 and 670 nm.

Scanning Speed: medium

Slit Width:
    Ex = 3.5
    Em = 3.5
    You may need to adjust these.

Fill a plastic cuvette with the solution of 10-ppm fluorescein in 95% ethanol. Fill the cuvette with a disposable pipette. Place the cuvette into the sample compartment, and close the cover of the spectrofluorometer.

Follow instructions in the handout on operation to obtain the emission spectrum in direct mode (page 2-11).
If the spectrum is off-scale, discard the data and adjust the maximum of the sensitivity of the recorder. Run the spectrum under the new parameters. If the spectrum is still off-scale, consult your instructor.
Locate the exact wavelength of the maximum.

Emission maximum: _____

D.3.3 Excitation Spectrum

Repeat the same procedure as for the emission spectrum, ensuring that the following parameters have been adjusted.

Em Wavelength: the value you have just obtained.
Ex Wavelength: 350 - 670 nm
Once the parameters are correctly set, run the spectrum. Locate the excitation maximum.

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Excitation maximum: _____

Ensure that you have recorded both the excitation and emission maxima.

D.4. Inner Filter Effect

In this experiment, you will monitor the fluorescence intensity of Fluorescein as a function of concentration. Prepare the following solutions: 0.1, 1.0, 10 and 100 from the stock 1000 ppm Fluorescein in 95% ethanol.   You will measure the relative fluorescence intensity over a set period of time.

Ex Wavelength: maximum measured above

 Em Wavelength: maximum measured above

Slit Width:
       Ex = 3.0
       Em = 3.0

Fill a disposable cuvette with the blank solution (i.e., EtOH) and insert it into the holder. Run the blank, which will be used to correct the remaining data for background radiation.

Follow instructions on page 2-16 of the Operation handout to record a fluorescence trace for each sample, for 1 minute, using the excitation and emission wavelengths that you have entered.

List the average fluorescence intensity for each concentration below. (Remember to record the sensitivity setting used for each solution)

Concentrationn (ppm)	Average Fluorescence Intensity
0.1	
1.0	
10	
100	
1000	

D.5. Bandwidth Effect on the Quality of the Spectrum

Note: For the bandwidth analysis, the quartz cuvette is used.

The bandwidth of a monochromator is defined as the span of monochromator settings (in units of wavelength) needed to move the image of the entrance slit across the exit slit [4]. The bandwidth of the spectrofluorometer can be changed by adjusting the width of the excitation and emission slits. For this study, the fluorescence intensity of 5000 ppm

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anthracene in toluene is measured at different slit widths to observe this effect. Fill the quartz cuvette with 5000 ppm anthracene in toluene, and insert it into the cell holder.

Spectrum Type: Emission

      Ex Wavelength: 380 nm
      Em Wavelength: 390 - 600 nm
      Scanning Speed: high
Obtain the emission spectrum using the following sets of slit widths.
Slit Width:
      Ex = 3
      Em = 3
Slit Width:
      Ex = 10
      Em = 3
Slit Width:
      Ex = 10
      Em = 10

D.6 Analysis of Quinine in Tonic Water- Calibration Curve Method

Note: For the quinine analysis, the plastic disposable cuvettes are used.

In this section, a calibration curve of quinine will be constructed using a series of standard solutions you will prepare.

The concentrations of these solutions are 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ppm.

The excitation and emission maximums for quinine are 350 and 445 nm respectively. The exact maximums may differ from those given above. You may want to determine what they are exactly under our experimental conditions.

Change to the following settings:

      Ex Wavelength: 350
      Em Wavelength: 445
      Slit Width:
            Ex = 3
            Em = 3

Run a blank solution sample before beginning with the standards.

Place the first standard in the cell holder and obtain an intensity measurement. Repeat with the remaining standards to complete the calibration curve. Then, insert the TW200

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example you prepared into the cell holder and obtain an intensity measurement. List the intensity values in a table.

D.7. Analysis of Quinine in Tonic Water – Standard Addition Method

Another method of quantification, standard addition, involves adding varying quantities of a standard to a constant concentration of unknown. These are the solutions that you prepared at the beginning of the lab. Adjust the parameters as follows:

     Ex Wavelength: 350
     Em Wavelength: 445
     Slit Width:
          Ex 3
          Em 3

Insert a blank solution in the sample holder and run the blank to correct for the background.
Measure the intensities of all five solutions that you have made using the same procedure described in section *D.4*.
List the intensity values in a table.

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E.   DATA PROCESSING AND QUESTIONS

1. Considering its molecular structure, why would you expect fluorescein to be highly fluorescent? (Reference 2, page 405).
2. What causes the inner filter effect for fluorescein? How can errors due to this effect be reduced or eliminated? (References 2 and 3).
3. What effects on the spectrum do the excitation and emission bandwidth have? Why?
4. From the values obtained in the calibration curve analysis, determine the concentration (in ppm) of quinine in the tonic water.
5. Starting with equation 15-7 in Skoog, Holler and Crouch, derive an expression relating fluorescence intensity to concentrations and volumes in the standard addition method.
6. Using the expression you derived, plot a linear curve with your standard addition data. Using the slope and intercept of the plot (and any other known values that you might need) calculate the concentration of quinine in the tonic water. Do not determine the concentration graphically!
7. Do the results from the calibration curve agree with those by standard addition? If so what does this prove? If not, what does this prove, and which do you think is correct?
8. When would standard addition be more suitable than a calibration curve for quantitative analysis?
9. In this experiment, you optimized literature excitation and emission wavelength maxima prior to actual analysis. How would you go about determining and optimizing excitation and emission wavelength maxima for an analyte without any literature data?

F.   REFERENCES

1. J. E. O'Reilly, J. Chem. Ed. 1975, 52, 610.
2. Skoog, Holler and Crouch. Chapter 15.
3. R. D. Baun, Introduction to Instrumental Analysis, McGraw-Hill, NY, 1987, Chapter 11.
4. Skoog, Holler and Crouch. Chapter 7.