THE FLUOROMETRIC ANALYSIS OF ASPIRIN

Introduction

Aspirin is acetylsalicylic acid (ASA), which hydolyzes to salicylic acid (SA) as follows

\[
\begin{align*}
\text{acetylsalicylic acid (ASA)} & \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{salicylic acid (ASA)} \\
\end{align*}
\]

Both of these compounds are fluorescent. Aspirin tablets will contain a small amount of hydrolyzed product. The purpose of this experiment is to determine the average concentration of SA in a bottle of aspirin tablets using molecular fluorescence. In order to verify the accuracy of your analysis, you will also perform a “blind test” to determine the concentration of SA in a standard solution.

Background

Origin of Molecular Fluorescence

When a molecule absorbs a photon in the ultraviolet or visible region of the electromagnetic spectrum, the energy of the molecule increases in two ways:

- the electronic energy of the molecule increases as a valence electron makes a transition from a lower-energy molecular orbital (usually the HOMO, the highest occupied molecular orbital) to a higher-energy molecular orbital (such as the LUMO, the lowest unoccupied molecular orbital).

- the vibrational energy of the molecule may also increase. After exciting the valence electron into the higher energy orbital, there may be enough energy “left over” to cause the molecule to vibrate more rapidly.

The next figure shows some possible transitions between energy levels for a molecule in whose ground state is a “singlet” electronic state, where every electron is paired.
You might wonder, “what eventually happens to the energy that was absorbed by the molecule from the photon?” The molecule does not remain in an excited state forever; eventually, the absorbed energy will be lost in some form. How is it lost? There are two common ways:

• if the molecule is in solution, some of the excess energy will be lost to the surroundings (usually the solvent molecules) in the form of heat.

• some of the excess energy may be lost as light: the molecule will spontaneously emit a second photon as it returns to the ground state. This light emission is called molecular luminescence; since the luminescence is caused by an initial absorption of light, in this case it can be referred to as photoluminescence.

The following figure shows some in a little detail some of the possible mechanisms of excess energy loss after a photon is absorbed.
Absorption of the incident photon results in excitation into an upper excited vibrational level of $S_1$ (arrow a) or $S_2$ (b). A portion of this energy is quickly lost (c) to the surrounding molecules, generally as thermal energy, leaving the molecule in the ground vibrational level of $S_1$, the lowest-energy excited singlet state. At this point, there are three things that can happen:

1. The remaining energy can also be lost to the surrounding molecules as thermal energy; we say that the molecule has undergone a *radiationless decay* from the excited state to the ground state. The dashed arrows in the figure represent radiationless loss of energy.

2. The molecule may spontaneously emit a photon as the valence electron collapses into its ground-state molecular orbital (the $S_1 \rightarrow S_0$ transition). This is a type of photoluminescence called *fluorescence*. The fluorescence transition may terminate in an excited vibrational level of the ground electronic state; a number of fluorescence transitions are possible.

3. The excited valence electron may spontaneously reverse its spin (undergo a “spin-flip”); this process is called *intersystem crossing*, and is represented by the arrow labeled ‘ISC’ in the figure. After the spin-flip, the molecule will quickly decay to the lowest vibrational level of $T_1$, the excited *triplet* state. From here, it is possible that a photon will spontaneously be emitted as the molecule relaxes to a vibrational level in its ground electronic state; this is a type of photoluminescence called *phosphorescence*.

Molecular fluorescence and phosphorescence are both examples of photoluminescence. By far the more common mechanism of relaxation, particularly in solution, is fluorescence. Phosphorescence is more typically observed in solids, and even these must usually be cooled to, say, liquid nitrogen temperature.

The following figure shows the sequence of events that typically occurs when a molecule fluoresces.
Figure 3. Typical sequence of events for molecular fluorescence. See text for explanation.

The following describes the steps numbered sequentially in figure 3.

**Step 1**: the molecule absorbs a photon, which transfers most of its energy to a valence electron, exciting it from the $S_0$ molecular orbital to a higher energy $S_1$ orbital. Some of the photon’s energy is also be transferred into vibrational energy, which causes the molecule to vibrate more rapidly. The wavelength of the incident photon must be such that the photon energy matches the energy difference between two vibronic energy levels of the molecule.

**Step 2**: the molecule’s excess vibrational energy is rapidly lost to the surroundings as heat.

**Step 3**: at this point, the molecule still has excess energy due to its excited electron. This electron spontaneously makes a transition back to the ground-state orbital, $S_0$. What happens to the energy? Some of the energy is lost through the emission of a photon (i.e., fluorescence), and the rest causes the molecule to vibrate more rapidly.

**Step 4**: At this point, the molecule is in its ground-state electronic configuration, but still has extra vibrational energy. As in step 2, this vibrational energy is rapidly lost to the surroundings as heat.

In summary, when a molecule is exposed to light, it may absorb photons if the photon energy matches the difference in energy between two vibronic states. After the molecule absorbs a photon, it may lose a portion of the energy it gains by emitting a fluorescence photon. The rest of the energy is lost to the surroundings (e.g., to solvent molecules) as heat.

**Types of Fluorescence Spectra**

Molecular fluorescence is a two-photon process: the molecule absorbs one photon of a certain energy, and then releases some of that energy by emitting a separate photon. Any instrument that is to control and measure this process must have a means of characterizing the wavelengths of both of these photons, as well as measuring the intensity of the emitted fluorescence. The following figure shows a schematic of a spectrofluorometer, which is used to collect fluorescence spectra.
Figure 4. Simple schematic of a spectrofluorometer. Light collected (by lens L1) from a light source passes through a monochromator, which isolates a single wavelength (actually, a narrow band of wavelengths) from the source. This light is focused (by lens L2) into the sample solution, which absorbs the light and emits fluorescence photons in all directions. Some of this fluorescence is collected (by L3) and passed into another monochromator, which isolates a single wavelength of the emitted light. A photomultiplier (PMT) detects the light that passes through the emission monochromator.

The spectrofluorometer in the figure consists of the following main components:

- a light source, which emits intense light in the ultraviolet and visible spectral regions;
- an excitation monochromator, which isolates one particular wavelength (the excitation wavelength) from the light source;
- a quartz cuvette to hold the sample solution;
- an emission monochromator, which isolates one particular wavelength (the emission wavelength) of the fluorescence emitted by the sample solution;
- a photomultiplier tube (PMT), a light detector.

In addition, various optical elements, such as lenses (L1-L3 in the figure) or mirrors, are used to direct the light in the spectrofluorometer.

Unlike any other analytical technique discussed in this course, fluorescence spectroscopy is a multichannel technique that contains two independently adjustable parameters, instead of just one. Either excitation or emission monochromator (or both!) may be set to any desired wavelength, or may be scanned to obtain fluorescence spectra.

There are three common types of fluorescence spectra:

1. Fluorescence excitation spectra, where the excitation monochromator is scanned while the emission monochromator is held at a constant wavelength;
2. Fluorescence emission spectra, where the emission monochromator is scanned while the excitation monochromator is held at a constant wavelength;
3. Fluorescence *synchronous spectra*, where both monochromators are scanned simultaneously with a constant wavelength difference. In this experiment we will obtain excitation and emission spectra; we will not discuss synchronous spectra further.

The following figure shows the excitation and emission fluorescence spectra of the analyte for this experiment, salicylic acid (SA).

![Fluorescence Spectra of Salicylic Acid](image)

**Figure 5.** Fluorescence excitation and emission spectra (solid lines) and the absorbance spectrum (dotted line) of salicylic acid. The emitted fluorescence light is at longer wavelengths than the excitation light absorbed by the molecule. The fluorescence excitation and absorbance spectra have similar shapes.

Two aspects of this figure are noteworthy. First of all, the emission spectrum is at longer wavelengths than the excitation spectrum. This is due to the fact that the absorbed photon always possesses at least as much (and usually more) energy than the emitted photon, with the balance of the absorbed energy given off as heat. Secondly, the absorption spectrum and the fluorescence excitation spectrum occur at the same wavelengths and have very similar shapes. This is because the same phenomenon is responsible for both spectra: absorption of a photon from the ground state into a vibronic level associated with a higher-energy molecular orbital.

In this experiment, the sample solution will contain salicylic acid (the analyte) in the presence of a much higher concentration of acetylsalicylic acid. The fluorescence spectra of acetylsalicylic acid are depicted in the next figure.
Figure 6. Fluorescence excitation and emission spectra (solid lines) and the absorbance spectrum (dotted line) of acetylsalicylic acid. The absorbance and excitation spectra show the same two electronic bands (but with different relative intensities).

In analyzing the solution mixture by fluorescence spectroscopy, the excitation and emission wavelengths must be chosen so that the signal of the analyte (salicylic acid) is maximized relative to that of the interferent (acetylsalicylic acid). In addition, as we will see in the next section, we must be certain that the measurements are within the linear dynamic range of the analyte response.

**Quantitative Aspects of Fluorometry**

**Basic Relationship**

The following figure shows the situation for the fluorometric analysis of solutions.

Figure 7. Fluorescence occurs along the path by which the excitation light travels through the sample solution, resulting in a “ribbon” of fluorescence in the cuvette.
Fluorometric Analysis of Aspirin

Background

Only the portion of the analyte solution that is actually exposed to the incident light can absorb photons and subsequently emit fluorescence photons. Under most conditions, the intensity of fluorescence is linearly proportional to the rate at which photons are absorbed in the solution:

\[ P_f = \phi_f P_A \]

where \( P_A \) and \( P_f \) are the rates (in units of J/s or photons/s) of absorbed and fluorescent photons, and \( \phi_f \) is the fluorescence quantum efficiency. The quantum efficiency is an intrinsic property of the molecule undergoing fluorescence, as well as the wavelengths of the absorbed photons.

The rate of photon absorption in the solution is the difference in rates between photons entering, \( P_0 \), and leaving the solution, \( P \):

\[ P_A = P_0 - P \]

If we assume only a single solute – the analyte – is absorbing and emitting photons, then according to Beer’s Law,

\[ P = P_0 10^{-abC_A} \]

where \( a \) is the analyte absorptivity, \( b \) is the cuvette pathlength, and \( C_A \) is the analyte concentration in the solution. Substituting into the original expression and rearranging yields an expression for the rate of fluorescence emission from the solution:

\[ total\ analyte\ fluorescence\quad P_f = \phi_f P_0 (1 - 10^{-abC_A}) \quad [1] \]

This equation gives the rate of fluorescence emission by all the analyte molecules in the solution (those exposed to the incident light, at any rate).

Equation 1 predicts that the relationship between fluorescence intensity and analyte concentration is inherently nonlinear. As the analyte concentration increases, the antilog term \( 10^{-abC_A} \) grows smaller; thus, the fluorescence intensity eventually approaches a constant value of \( \phi_f P_0 \). The equation also predicts that the fluorescence intensity depends linearly on the intensity of the incident light – unlike the case for absorbance, which is independent of the intensity of the incident light.

If the analyte concentration is small enough that \( abC_A = A < 0.05 \), then it can be shown that the following expression gives a reasonable approximation for the fluorescence intensity

\[ P_f \approx 2.303\phi_f P_0 abC_A = K \cdot C_A \quad [2] \]

where \( K \) is a constant. Thus, the total fluorescence intensity is linearly proportional to analyte concentration so long as the concentration is small and \( K \) is constant. In practice, observed linear dynamic ranges in fluorometric analysis are usually 3-4 orders of magnitude – comparable to absorbance spectrometry – and even wider ranges of linear behavior are observed for strong fluorophores.

Inner Filter Effects

Equation 1 describes the rate of fluorescence emission of all analyte molecules exposed to the excitation light. Experimentally, however, it is sometimes only possible to observe a portion of the “ribbon” of fluorescence that is shown in figure 7. As shown in figure 8, typically only photons
originating from the middle portion of the fluorescent solution volume are actually collected into the emission monochromator. We shall call this volume the *detection region* of the sample solution.

![TOP VIEW](image)

**Figure 8.** Common optical geometry in fluorometry, as seen from the top view of the cuvette. Only the middle portion of the cuvette is imaged onto the entrance slit of the emission monochromator. Thus, only fluorescence light originating from the volume represented by the shaded area (the *detection region*) can actually give rise to measured fluorescence signal.

Referring to figure 8, there are two possible situations that result in a decrease in the measured fluorescence signal relative to the value predicted by eqns 1-2.

- incident light is absorbed before it reaches the detection region, so that $P_0^* < P_0$. Smaller incident light intensity means fewer absorbed photons within the detection region, and hence a smaller measured signal. This mechanism of signal reduction is called the **prefilter effect**.

- emitted fluorescence light is absorbed by the solution before it exits the cuvette. This reduction mechanism is the **postfilter effect**.

Together, these effects are referred to as the *inner filter* effect. In a solution that contains only the analyte, the postfilter effect is not usually unimportant, since there is usually little overlap between excitation and emission spectra. The following expression, which ignores the postfilter effect, gives the rate of fluorescence, $P_0^*$, from the detection region (i.e., the shaded region in fig 8) in a solution that contains the analyte as the only solute.

\[
P_0^* = \phi_F P_0 10^{-ab_1 C_A} (1 - 10^{-ab^* C_A})
\]\[3\]

You should compare this expression to that in eqn 1; also refer to figure 8 for the meaning of the pathlength terms $b_1$ and $b^*$.

This equation predicts that, as the analyte concentration increases, the measured fluorescence intensity will pass through a maximum and then decrease. At very high analyte concentrations, the measured fluorescence signal will be zero because essentially all of the incident light is absorbed before it reaches the detection region. The shape of the calibration curve predicted by eqn 3 is shown in fig 9.
Figure 9. Typical fluorometric calibration curve, including inner filter effects. The measured fluorescence signal is a nonlinear function of analyte concentration, although the relationship is approximately linear for small analyte concentrations (see inset). The calibration curve exhibits a maximum, beyond which an increase in analyte concentration actually results in decreasing fluorescence signals. This decrease generally occurs because of the prefilter effect, where the excitation light is greatly attenuated before it reaches the region that is “seen” by the emission monochromator.

Inner filter effects – both prefilter and postfilter – can also be caused by solutes other than the analyte, even if these solutes are not fluorescent themselves. One way in which inner filter effects can be detected is by their effects on the shapes of fluorescence excitation and emission spectra. Prefilter effects deform the excitation spectrum, while postfilter effects alter the shape of the emission spectrum. This phenomenon is depicted in figure 10, which shows the excitation spectra of anthracene solutions of varying concentration in the “rising” portion (where signal increases with concentration) and the “falling” (decreasing signal with increasing concentration) portion of the analyte calibration curve.
Excitation Scans during the "Falling" Phase

Figure 10. Prefilter effects revealed by excitation scans. Scans 1-6 are of increasingly greater analyte concentrations. The left three scans are taken in the “linear” region of the calibration curve, when the fluorescence signal increases linearly with analyte concentration. All three spectra have the same general shape. The right three scans are taken at higher analyte concentrations, where the fluorescence signal is decreasing with concentration. The prefilter effect results in peak inversion: the most strongly absorbed light (the peaks in the left three scans) give the smallest signals.

Inner filter effects may be corrected by diluting the sample solution and obtaining fluorescence measurements at properly selected wavelengths. If uncorrected, these effects will cause bias when the analyte concentration is estimated from the linear portion of the calibration curve. In order to detect inner filter effects – whether due to high concentrations of the analyte or due to the presence of other solutes – it is important to obtain both excitation and emission spectra of the sample solution.


**Fluorometric Analysis of Aspirin: Procedure**

The purpose of this experiment is to assess the extent of aspirin hydrolysis by determining the concentration of hydrolysis product, salicylic acid (SA), in aspirin. A composite sample will be collected for this purpose. During the analysis, you will correct for incomplete extraction or loss of analyte during sample preparation. Finally, in order to assess the accuracy of the analytical measurement procedure, you will analyze a standardized “test” solution of the analyte.

**Materials**

**Chemicals**

- solvent: 1% acetic acid in chloroform.
- a stock solution (approximately 1000 µg/mL) of salicylic acid in solvent
- calibration standards of salicylic acid (see labels for concentrations)
- a “test sample” of salicylic acid to assess the accuracy/precision of the analysis
- aspirin in bottles

**Precautions**

Please observe the following general precautions during this experiment.

1. **Be careful with the cuvettes** – they are easy to break and they are expensive. To avoid dropping the cuvette during normal handling, carry the cuvette around in a small beaker that has been padded with a chemwipe. This “cuvette holder” also makes it easy to add a sample solution to the cuvette without tipping it. In this experiment, you will be using a microcuvette with a 2mm pathlength to reduce the prefilter effect (absorption of the excitation light). However, you should be aware that these microcuvettes are even more expensive and more difficult to rinse than normal cuvettes.

2. As much as possible, **protect your solutions from the room light**. Many fluorescent compounds are light-sensitive, so that the analyte concentration decreases upon standing in a lit room at room temperature.

3. **Do not leave solutions uncovered**. The solvent in this experiment (chloroform) is quite volatile. Evaporation will increase the analyte concentration.

4. Use organic solvents, such as acetone or methanol, for rinsing purposes; do not use water.

5. Accurate pipetting is more difficult with organic solvents than it is for aqueous solutions. Most organic solvents have a lower surface tension than water, and so tend to drip out of pipettes more readily.
Solution Preparation

The lamp needs about 15 min to stabilize completely: have the instructor turn on the instrument to allow it to warm up. Thoroughly clean the mortar and pestle with soap and sponge (do not use a wire brush.) and then rinse with acetone. Likewise, rinse all glassware with acetone and finally with your solvent (try not to use too much solvent for rinsing). You have been provided with calibration standards, but your “test” sample solution may not be in the concentration range of your calibration standards. In light of the typical behavior of fluorescence calibration curves (where the fluorescence intensity first increases then decreases with increasing concentration), it is advisable to make at least one dilution of the unknown.

Using the mortar and pestle, grind five aspirin tablets and mix them well. You should give some thought into how you choose your aspirin tablets – remember that you will want a representative sample of the “population” of aspirin in the bottle. When grinding, make the powder as fine as possible to facilitate rapid and efficient extraction.

From this composite sample, obtain a subsample weighing approximately 400 mg (use the analytical balance). In a 100 mL beaker, extract the subsample in about 20 mL of 1% acetic acid/chloroform. Stir the solution well for about three minutes; the aspirin will not all dissolve. Using an aspirator, quickly suction-filter the aspirin solution through a Whatman #1 filter. During the transfer, rinse the beaker and stirring rod with several small portions of the chloroform solvent; wash the undissolved sample several times with small amounts of solvent. Quantitatively transfer the filtered solution into a 50 mL volumetric flask and dilute to the mark.

Repeat this procedure with three more 400 mg subsamples, so that you will end up with four solutions of aspirin extracts.

In order to measure the analyte recovery, repeat the entire extraction procedure without the aspirin. Instead, spike the “blank” (i.e., 20 mL of extracting solvent) with 100 µL of the stock SA solution. Otherwise treat this solution exactly like a sample extracts: i.e., during subsequent filtration and transfer.

We don’t really expect any contamination (assuming you’ve been using clean glassware!), so don’t bother to obtain a “reagent blank.”

At this point, you should have the following solutions: four extracts of aspirin samples; one spiked “blank” extract; four calibration standards; and a solvent blank. You will take these solutions and obtain your fluorescence measurements in a different laboratory (N312) – let your instructor know when you are ready to proceed to the next phase of the experiment.

Fluorescence Measurements

Choosing Excitation and Emission Wavelengths

Your instructor will explain how the fluorometer works, and how to collect fluorescence spectra. If you must wait for further instructions, take time to review the background material on molecular fluorescence.
After rinsing the microcuvette thoroughly, collect excitation and emission fluorescence spectra of your most concentrated calibration standard. All measurements should be corrected for variations in lamp intensity by using the S/R measurement option. Use 1 mm slits and scan at 120 nm/min. Display both spectra on the same screen and print it out. Determine the maximum excitation and emission wavelengths from your fluorescence spectra.

Now you will check the composition of your aspirin extracts by obtaining various excitation and emission spectra of two solutions (i) one of your undiluted extracts; and (ii) your diluted aspirin extract. You will need your instructor to assist you in collecting these spectra.

Based on the spectra you have collected, choose the excitation and emission wavelengths most appropriate for quantitative analysis of SA in your aspirin extracts. You will need to choose wavelengths that allow you to avoid interference and filter effects due to the presence of the high concentration of ASA in the sample solutions.

**Quantitative Measurements**

You will obtain steady-state fluorescence measurements at the chosen wavelength values for the following solutions: a solvent blank, the calibration standards (beginning with the most dilute), the “test” solution and its dilution(s), the four undiluted aspirin extracts, and your spiked “extract.” Be sure to rinse the microcuvette thoroughly between measurements. Your instructor will assist you on setting up the software for these measurements. Use the S/R measurement option to obtain “corrected” measurements. Obtain the average and standard deviation of thirty 1s measurements for each solution.
**Fluorometric Analysis of Aspirin: Data Sheet**

Name: ___________________________  Unknown #: ___________

Stock solution conc (µg/mL): ___________

Excitation wavelength (nm): ___________

Emission wavelength (nm): ___________

**Fluorescence Measurements**

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<th>signal avg, cps</th>
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**Results**

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FLUOROMETRIC ANALYSIS OF ASPIRIN: DATA TREATMENT

Two results are required: concentration of analyte in the “test” solution, and the concentration of analyte in the aspirin tablets. In calculating a confidence interval for the analyte in the aspirin tablets, you should consider the main source of the uncertainty in your point estimate. Here are some possibilities:

- measurement error, due to random error in the measurements of the extract solutions
- calibration error, due to random error in the measurements of the calibration standards
- sample preparation error, due to uncertainty in the extraction procedure, and associated lab operations that may change the analyte concentration
- sampling error, due to variability of actual analyte concentration in the aspirin sample that is extracted.

In many analytical procedures, the error due to the last two sources – sampling and sample preparation – dominates the overall error. In such cases, you can simplify the procedure for calculating the standard error of the point estimate of analyte concentration by ignoring the effects of measurement and calibration error. Consider this fact when you construct your confidence interval for the concentration of SA in the aspirin tablets.

In calculating the concentration of analyte in the aspirin, you may need to account for bias due to incomplete analyte recovery during sample preparation. If you are careful, then you should achieve approximately 100% analyte recovery. You can verify this assumption by using the fluorescence measurement of your spiked blank solution:

\[
\text{percent recovery} = \frac{\text{estimated conc of spike solution}}{\text{expected conc of spike solution}} \times 100\%
\]

Use propagation of error to calculate the standard error in this point estimate of analyte recovery; report your estimate as a 95% confidence interval on the DATA SHEET. A hypothesis test can be used to check if an initial assumption of 100% recovery is consistent with your results. If it is not, then apply the appropriate correction factor in calculating the concentration of analyte in the aspirin.