The effect of pH of fluorescein solutions of the same concentration on the relative fluorescence intensity in a spectrometer

Introduction

During the initial research for my Physics Extended Essay, I came across the concept of fluorescence. I was intrigued by how a different wavelength of light is emitted when an incident light is shone on specific materials or compounds. Understanding that it was more closely related as an extension from the Electron Configuration unit in chemistry, I decided to investigate this effect in my IA. Upon researching the general methodology and the factors that influence fluorescence, I realized the extreme precision that this phenomenon requires, and decided to investigate how fluorescence intensity is affected by the pH level.

Research Question

What effect does pH 6, 7, 8, 9, 10 of fluorescein solutions at a concentration of 2.8 $\mu g/mL$ (ppm) at room temperature have on the relative fluorescence intensity measured using a spectrometer when the solution is excited at an excitation wavelength of 500nm?

Background Information

Fluorescence is a phenomenon that refers to the emissions of photons from a sample from the excited singlet state, following the absorption of photons. (ISS 2) When a fluorophore (a molecule that exhibits fluorescence properties) is excited to an excited state, it can exist in either the singlet or triplet state. The excited singlet state occurs when the spin of the excited electron is still paired with the spin of the ground state electron as illustrated in Figure 1. Since the excited electron is paired with the ground state electron, its return to the ground state is spin allowed. The excited electron can return to the ground state through the rapid emissions of photons. (Joseph 1-2) This process can be illustrated through a Jablonski diagram, which is an energy diagram that describes the entire process of fluorescence as shown in Figure 2.

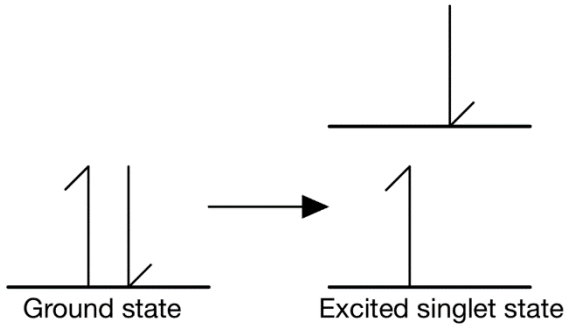

Figure 1. Hand drawn diagram showcasing the transition of the electrons from the ground state to an excited singlet state

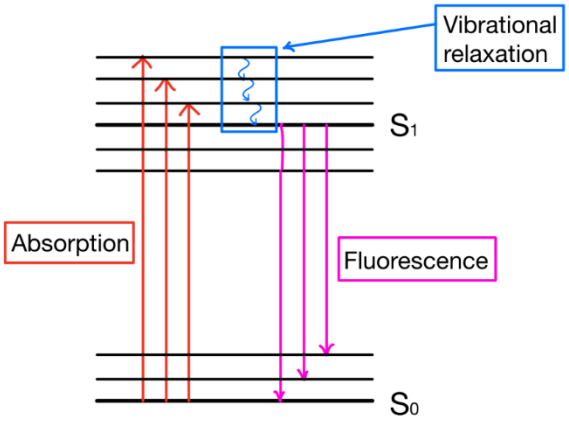

Figure 2. Jablonski Diagram of the process of fluorescence

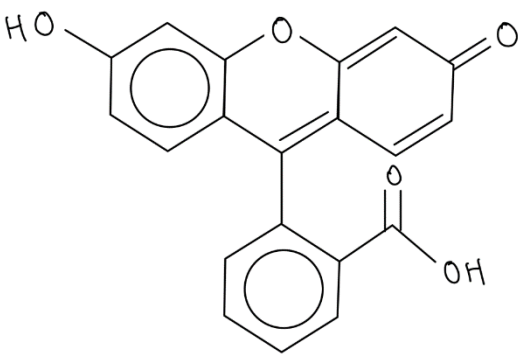

Figure 3. Molecular structure of fluorescein

Figure 2 consists of 2 distinct energy levels: the ground state (S_0) and the excited singlet state (S_1) and many different vibrational states (less bolded lines). When the fluorophore is excited by the absorption of photons, it transitions from S_0 to highly energetic and unstable vibrational states. The excess vibrational energy is quickly lost by a series of rapid vibrational relaxation, which is a nonradiative process, to the lower and more stable singlet state S_1. The electrons return to the ground state from its singlet excited state through the emission of photons, which when measured, produces a fluorescence spectrum.

The nature of fluorescein, the compound being examined, is experimentally determined to have an excitation peak at approximately 500nm (AAT Bioquest). This excitation wavelength will follow the fluorescence process discussed above and emit photons with wavelength of approximately 520nm. This change in wavelength between its absorption and emission is known as the Stokes Shift. This is a phenomenon illustrated in Figure 2, as there is usually a difference in the energy absorbed and energy emitted due to vibrational relaxation. The nature of fluorescein exhibits small stokes shift, with the change in wavelength, $\Delta\lambda \leq 70nm$ (Gao 1).

Fluorescence spectrometry is a highly sensitive measurement process, where the molecular structure of the fluorophore or chemical environment can alter whether it will or will not fluoresce and its fluorescence intensity. The only factor that we are concerned about in this investigation is the pH effect. In most cases, aromatic compounds with acidic or basic ring substituents are very pH

dependent. The changes in the fluorescence properties arise from the different number of resonance structures associated with the acidic and basic forms (Douglas et al 368-369). The different resonance structures of the compound allow it to exist in a more stable form, or a more stable excited state, therefore giving it fluorescence properties. Fluorescein has a molecular formula of $C_{20}H_{12}O_5$, which can be drawn as shown in Figure 3. It has a hydroxyl and carboxyl group, which can act as a proton acceptor or donor. The protonated and deprotonated forms of the molecule at different pH levels will result in different fluorescence emissions. Protonated and deprotonated refers to a gaining and losing of an H+ ion respectively, and an example of the protonated and deprotonated forms of the fluorescein can be seen in Figure 4.

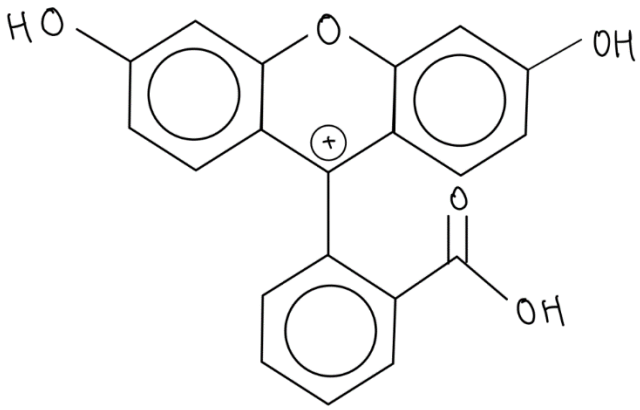

Figure 4 (a) Protonated form of the fluorescein compound

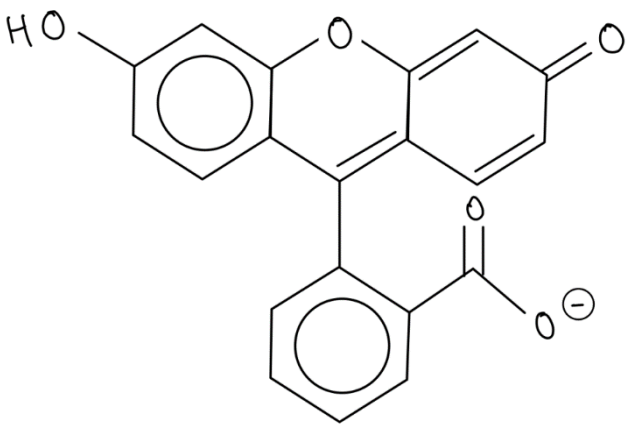

Figure 4 (b) Deprotonated form of the fluorescein compound

Figure 4(a) shows the protonated form of fluorescein as a cation, whereas Figure 4(b) shows the deprotonated form of fluorescein as an anion. These are only one of the many protonated or deprotonated forms of the fluorescein, and in these forms, the electronic transitions for the electrons are changed and thus have an influence on the fluorescence properties.

Preliminary Trials

Testing the ranges of pH levels

Initial research was conducted regarding the range of pH levels that give rise to fluorescence in the visible range. An experiment that yielded positive results had a pH range of 6.9 – 10.4 (Zhu. H et al 1), therefore it was decided to test the two extreme ends of the pH range – pH 6 and 10. The corresponding buffer solutions were made as well as a fluorescein solution of roughly 500ppm. Roughly 1mL of the fluorescein solution was pipetted into the buffer solutions and its fluorescence spectrum measured using the spectrometer. The results showed an increase in fluorescence relative intensity between the two pH levels; however, the initial sampling time produced a spectrum with its intensity way beyond 1, which required lowering the sampling time such that the peaks were visible.

Testing the concentration of the fluorescein solution

The concentration of fluorescein sample will directly influence the fluorescence intensity as previously mentioned that at 500ppm the relative intensity goes way beyond the scale of 0-1. Adapting to the information in the research paper mentioned above, 0.003ppm of fluorescein solutions at pH 6 and 10 were made again. Upon testing the solutions in a spectrometer, the results yielded a near 0 intensity of the fluorescence emission peak. Despite increasing the concentration of the pH buffer solutions by adding more drops of fluorescein solutions (which can potentially alter the pH level) and increasing the sampling time, the intensity was still near 0. This gave a brief idea that the amount of fluorescein used must be increased significantly, in which later a rough 10 times the original amount of fluorescein was used.

Determining the Absorption Spectrum of the buffer solutions

The buffer solutions were made by dissolving the powder inside pre-made pH buffer capsules in 1000mL distilled water. However, concerning over the potential overlap of its absorption wavelength with that of the fluorescein solution, which could affect the fluorescence intensity. Therefore, an investigation was done. The capsules of pH 6 to 8 are made of varying ratios of sodium phosphate and potassium phosphate, while pH 9 and 10 are made of varying ratios of sodium bicarbonate and sodium carbonate. All these compounds are said to have absorption peaks in the UV range based on literature information, and upon testing in the spectrometer, produced near 0

peaks in the visible range. This confirmed that the absorption of the pH buffer compounds can be considered as negligible for the further investigations.

Investigating the potential time factor affecting fluorescein intensity

Whilst doing preliminary trials between days, the solutions have been kept under a box to prevent degradation of the fluorescein under ambient light. However, measurements from the spectrometer of the same fluorescein solutions have been increasing gradually, and the solutions are growing greener as time goes by. This strange phenomenon was investigated by creating 3 distinct fluorescein solutions of pH 10, where 3 timers were started as soon as the fluorescein droplets were dissolved in the pH buffers. The time between measurements were recorded, however between hours, the intensity did not change much. Thus, a hypothesis of that the fluorescein was not completely dissolved in the distilled water was proposed, as fluorescein is not very soluble in water. This also meant that the fluorescein slowly dissolved more in the buffer solutions over time, thus resulting in an increasing fluorescence intensity peak over time. This led to improvement in the method, where the fluorescein powder must be ensured to be fully dissolved.

Variables

Dependent Variable

The measurement that we are concerned with is the relative fluorescence intensity measured using a spectrometer (SpectroVis). We can ensure that the measurements are recorded properly by fixing the sampling time between trials to 30ms and ensure the sides of the cuvettes are cleaned properly.

Independent Variable

The variable that is changed is the pH levels of the fluorescein solutions. 5 different pH levels are used (pH 6, 7, 8, 9, 10), where their corresponding pH buffer solutions are made by dissolving the pre-made capsule powders in 100mL distilled water. Then 2mL of fluorescein solutions are added with a 1mL micropipette to the buffer solutions.

Controlled Variables

Variable controlled	Reasons for controlling the variable	Method of controlling the variable
Ambient temperature of the environment	The ambient temperature must be monitored to be at room temperature at roughly $20 - 23°C$. Fluorescence measurements are dependent on temperature due to the vibrations of the fluorophore molecules affecting the transitions between electronic states. It will also affect the pH level of the pH buffer solutions as they are temperature dependent. Therefore, temperature must be controlled to prevent it from affecting measurements.	Measuring the ambient temperature with a thermometer every 30 minutes of experimental work
Concentration of the fluorescein solutions	The concentration of the fluorescein solutions must be controlled as explored in the preliminary trials. Concentration has a direct effect on the fluorescence intensity and using a different concentration of fluorescein solutions will yield different fluorescence intensity measured.	The concentration of the fluorescein solutions can be controlled by ensuring that only 2mL of the fluorescein solutions are transferred to the pH buffer solution using a 1mL micropipette from the same stock fluorescein solution.
pH of the fluorescein solutions	Fluorescence intensity is also highly pH sensitive, where fluctuations in temperature or concentration of the solutions could easily change its pH level. Therefore, the pH of the fluorescein solutions must be controlled between trials to minimize random errors in the fluorescence intensity.	The pH of the fluorescein solutions can be controlled by measuring the ambient temperature and the pH of the solutions. Accurate pH measurements can be measured by actively stirring the solutions such that the solution kept inside the

		electrode of the calibrated pH probe is constantly in motion.
Type of Spectrometer used	Fluorescence intensity spectrum generated by the same spectrometer must be controlled. Different spectrometers have different sensitivity to the fluoresced light. Using a different spectrometer between trials could affect the measurements, leading to random errors.	The spectrometer can be controlled by using the Vernier SpectroVis spectrometer that mainly measures in the visible range.
Sampling time of the spectrometer	The sampling time of the spectrometer must be controlled as it directly influences the fluorescence intensity. Increasing the sampling time will expose the spectrometer's sensors to the fluoresced light for a longer period, potentially saturating the fluorescence emission peak. This applies the opposite way as well, which could affect the measured results between trials.	This can be controlled by setting the sampling time to 30ms, which was determined in the preliminary trials to be the optimal sampling time.

Risk Assessment

Fluorescein

Fluorescein is considered hazardous and can cause serious eye damage and eye irritation when comes to contact with it (fishersci). Proper lab goggles must be used to prevent fluorescein solutions or fluorescein powder from contacting the eyes. Since it is hazardous, fluorescein must be disposed into a waste beaker, where it could be brought to proper waste treatment.

pH Buffer solutions

The buffer solutions of pH 6, 7, 8, 9, 10 are classified to be nonhazardous (fishersci). Any skin contact with these buffer solutions only requires immediate rinsing with running water. Although specific disposal procedures were not mentioned, the safest method to prevent damage of the sink is to collect all the pH buffer solutions in a waste beaker and neutralize it to a roughly pH 7 before disposing in the sink with running water. Goggles and aprons must be used to prevent any contact of these chemicals with skin or clothing.

Apparatus and Methodology

Making buffer solutions

5 100cm^3 volumetric flask and their stoppers

5 labels

1 500cm^3 distilled water

1 pH 6, 7, 8, 9, 10 buffer capsules

The 5 pH buffer capsules (pH 6, 7, 8, 9, 10) were opened and poured into the respective labelled 100cm^3 volumetric flask before pouring 100cm^3 of distilled water to dissolve it. The remaining powder that was stuck to the walls of the flask was ensured to be washed off with distilled water and that the powder was fully dissolved by covering the flask with its stopper and inverting the flask back and forth until no residuals were seen floating in the solution.

Making fluorescein solution of different pH levels

1 1000cm^3 volumetric flask and stopper

1 spatula

1 0.035g of fluorescein powder

1 magnetic stir bar

1 250cm^3 beaker

1 stir station

1 weigh boat

1 1000cm^3 of distilled water

1 electronic balance

21 labels

1 50cm^3 graduated cylinder

20 50mL beakers

21 parafilm wraps

1 box

0.035g of fluorescein powder was measured on a weight boat on an electronic balance and was transferred to a 1000cm^3 volumetric flask, where any residual mass on the weight boat would be washed off with distilled water and poured back into the flask. 1000cm^3 volumetric flask was filled with distilled water and its stopper put on to

invert the flask back and forth in order to dissolve the powder. As explored in the preliminary trials, fluorescein powder is not very water soluble, therefore a magnetic stir bar was added to the flask and put on a stir station. The station was set to full power until no residuals were seen floating in the solution. Roughly 150cm^3 of the fluorescein solution was transferred to a 250cm^3 beaker as a stock solution for easier transfer with the micropipette. The previously made pH buffer solutions were divided into four 25cm^3 solutions in 50cm^3 labelled beakers. Using the 1cm^3 micropipette, 2cm^3 of fluorescein solution was transferred into three of the beakers, leaving one excess pH buffer solution for calibration. A parafilm was added to all the beakers and the ones with fluorescein were placed under a box to prevent degradation.

Measuring the actual pH levels of the buffers

1 pH probe	1 pH 10 solution	1 magnetic stir rod
1 pH 6 solution	1 stir station	

The excess 25mL of each buffer solutions were used to test the pH with. The pH probe was calibrated with pH 6 and 10 solutions. The probe was placed inside the pH 6 buffer with a magnetic stir rod in it and on a stir station. The station was turned on to a medium level and the probe ensured to be inside the solution of constant motion to provide accurate measurements. This was because the solution must be kept inside the electrode of the pH probe when in motion. The pH of the solutions were entered when the voltage across the electrode of the pH probe stopped or slowed down in fluctuations. This process was repeated for the pH 10 solution as well.

Measuring the fluorescence and absorbance spectrum

1 Vernier SpectroVis spectrometer	1 cuvette wipe	1 distilled water
3 cuvettes	1 micropipette	1 waste beaker

Roughly 2cm^3 of pH 6 solution was transferred to a blank cuvette and placed in the spectrometer for calibration of the absorbance spectrum. The pH 6 fluorescein solution was transferred to another blank cuvette where its fluorescence and absorbance spectrum were recorded. It was ensured that the excitation wavelength was set to 500nm, and the sampling time set to 30ms.

Data Analysis

Fluorescence and absorbance peak values were extracted from the recorded spectrum, allowing for further treatment and comparison between the experimental and literature results. Figure 5 shows the absorbance and fluorescence spectrum on the LoggerPro interface.

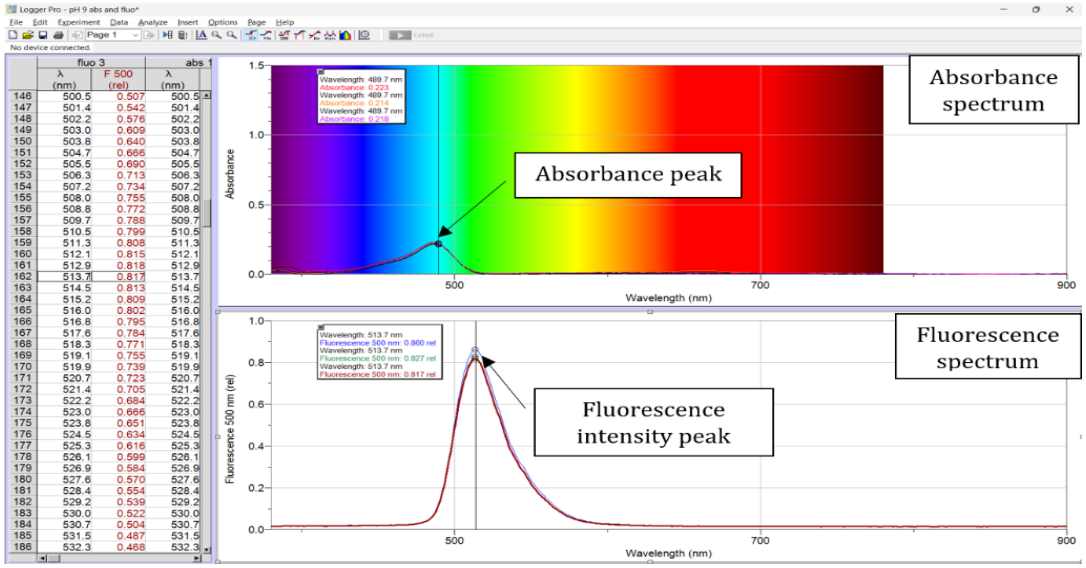

Figure 5. LoggerPro interface for pH 9, where the absorbance (top) and fluorescence (bottom) spectra are shown.

The absorbance peak is found by hovering the cursor over the regions of "absorbance peak", where the maximum value from the table was recorded on a table. This process was repeated for the fluorescence intensity peak, where all the recorded values from the trials are shown in Table 1.

Table 1. Recorded fluorescence and absorbance peaks and their corresponding wavelengths and the pH values

		Trial 1				Trial 2				Trial 3			
pH	actual pH ±0.2	$\lambda_{abs} \pm 4nm$	Abs_{max}	$\lambda_{fluo} \pm 4nm$	Flu_{max}	$\lambda_{abs} \pm 4nm$	Abs_{max}	$\lambda_{fluo} \pm 4nm$	Flu_{max}	$\lambda_{abs} \pm 4nm$	Abs_{max}	$\lambda_{fluo} \pm 4nm$	Flu_{max}
6	6.1	478	0.0975	512	0.2609	478	0.0993	513	0.2540	478	0.0958	513	0.2595
7	7.1	486	0.1980	513	0.7034	486	0.1935	513	0.7277	486	0.1890	514	0.6860
8	8.1	487	0.1428	514	0.6049	487	0.1380	513	0.5914	486	0.1920	513	0.7007
9	9.1	486	0.2317	513	0.8597	486	0.2226	514	0.8271	486	0.2264	513	0.8180
10	10.0	486	0.1749	513	0.6556	486	0.2319	513	0.8365	487	0.1659	514	0.8602

The table above used variables to represent the classes of numbers, where λ_{abs} refers to the wavelength of the maximum absorbance peak, and correspondingly λ_{fluo} represents the wavelength of the maximum relative intensity peak. Abs_{max} refers to the absorbance peak and Flu_{max} represents the maximum relative fluorescence intensity. These values obtained from these trials were averaged out to produce the following in Table 2.

Table 2. Averaged values of the absorbance and fluorescence peaks and the wavelengths across the three trials

pH	Measured pH ±0.2	$\lambda_{abs} \pm 4nm$	Abs_{max}	$\lambda_{fluo} \pm 4nm$	Flu_{max}
6	6.1	478	0.098±0.002	513	0.258±0.003
7	7.1	486	0.194±0.005	513	0.71±0.02
8	8.1	487	0.16±0.03	513	0.63±0.05
9	9.1	486	0.227±0.005	513	0.83±0.02
10	10.0	487	0.19±0.03	513	0.8±0.1

There were a few qualitative observations made during the experiment that could be stated. The fluorescein stock solutions glowed a bright green color under ambient light, however although it was left in the stir station to be dissolved for a long time, there were still residuals of the fluorescein powder remaining. When adding the fluorescein solutions into the pH buffer solutions, a distinct difference in pH 6 and 10 were seen as pH 10 immediately turned green after adding the droplets, while pH 6 barely had any change in color. The intermediate pH levels could not be distinguished very well, however the pH 6 and 10 had a huge difference when put into the spectrometer, where the excitation of blue light resulted in a strong green fluorescence color. The pH 10 was clearly much brighter in the green color than the pH 6, which would deem our initial literature information to be valid. Table 2 shows that the average fluorescence wavelength between the trials is consistently at 513nm, which is very close to the literature value of 512nm, providing a certain validity to our fluorescence spectrum. This can be seen in Figure 6, where all the averaged fluorescence intensity peaks are aligned.

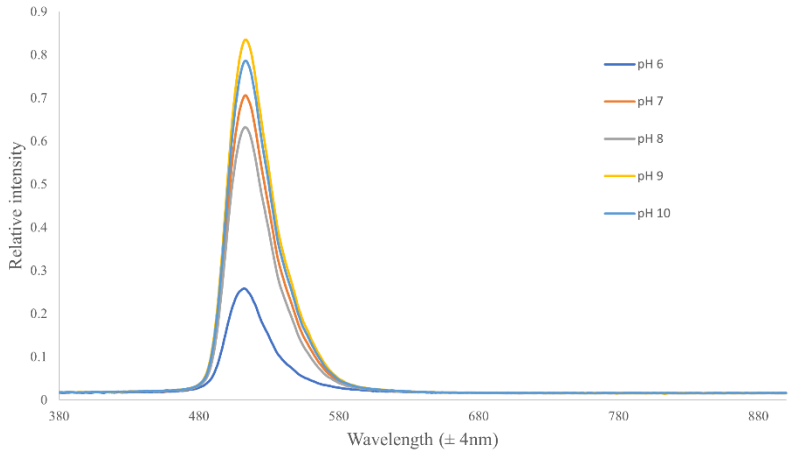

Figure 6. Averaged fluorescence intensity spectrum against wavelengths

By inspection, this graph does not correspond to the theory that the fluorescence intensity increases as pH increases. It can be noticed that pH 7 has a greater fluorescence intensity than pH 8, and pH 9 has a greater fluorescence intensity than pH 10. This can be investigated by looking at the range uncertainty between these trials for the potential random uncertainties that arise. This is shown in Figure 7, where the averaged fluorescence intensity is plotted against pH levels with their respective range uncertainties.

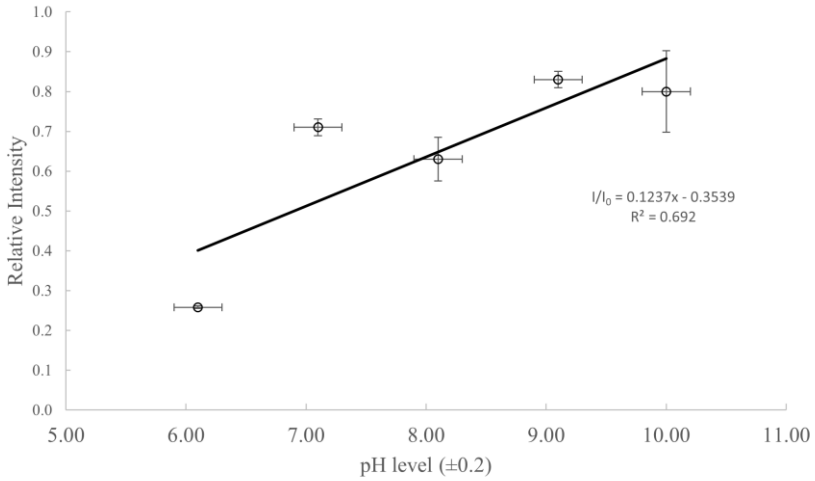

Figure 7. Averaged relative intensity against pH levels with their range uncertainties.

It can be observed from Figure 7 that the fluorescence intensities at the pH levels that were supposedly theorized to be higher have greater range uncertainties. Those that have lower range uncertainties (pH 6, 7, 9) provide a good indication of where the true intensity would lie, as well as a good estimation of the trends between fluorescence intensity and pH levels. Although the experimental results do not provide a clear representation of the theory suggested due to the high uncertainties at pH 8 and 10, the general trend tends towards an increase in relative intensity against an increase in pH levels. The significant difference in intensity between pH 6 and 7 could be an extension to the literature findings that the fluorescence intensity increased only within the range of 6.9 – 8.4 (Zhu. H et al 6), while tending to become nearly constant beyond 8.4. This suggestion could not be verified with our experimental results, however due to the highly uncertain pH 8 and 10, this could be a possible theory to the trends. The literature does not include pH 6 in their experiment, however, with the drastic difference in relative intensity between pH 6 and 7, as well as this similar observation from the preliminary trials, suggest that the relationship between relative intensity and pH levels could be nonlinear. Furthermore, as shown in Table 2, the absorbance wavelength for the pH 6 samples is averaged out to be 478nm, which deviates from the rest of the absorbance wavelengths (486nm-487nm), providing an alternative explanation for the drastically lower fluorescence spectrum due to the greater difference from fluorescein's excitation wavelength, which is around 500nm. In addition to the weak correlation, the weak R^2 value of 0.692 also indicates that the relationship is not linear. However, since it was suggested that the trend tends to constant beyond pH 8.4, this could mean that it has a linear relationship at pH levels greater than 8.4, while any pH levels below that is a nonlinear relationship. The reasons behind this hypothesis could be beyond the current level of study but could be a great extension in the future.

Discussion and Evaluation

To provide an answer for the research question, the general trend does agree with the theory that fluorescence intensity increases with pH levels, however due to the highly uncertain pH 8 and 10, the trend could not be clearly observed. In addition, it was theorized that the relationship between fluorescence intensity and pH levels could be a linear relationship for pH levels beyond 8.4, while any pH levels below that could be a nonlinear relationship. This was evident through the great distinction between the pH 6 and 7 fluorescence intensities, as well as through the suggestion of literatures that yielded a similar theory and results. Therefore, with these proposed theories it is not exactly clear what relationship or what function of best fit lines are suitable for these two proposed regions (below pH 8.4 and above pH 8.4) unless further investigations are conducted.

Strengths

1) Calibration of pH probe
   The pH probe used to measure the pH levels of the fluorescein solutions was cleaned with distilled water thoroughly between each trial. The same pH probe was used to measure each solution's pH level and that the probe was calibrated using an accurately made pH 6 and 10 solutions. This careful process ensured that the fluorescein solutions were close to the desired pH levels, and as shown in Table 2, the pH levels were relatively close to the desired pH level.
2) Choice of pH buffer compounds
   The pH buffer compounds used were tested, through a spectrometer, and its absorbance spectrum evaluated. The underlying concern was that the pH buffer compounds would absorb at a similar wavelength to fluorescein, and that the varying ratios between the compounds would affect the fluorescence intensity between different pH levels. However, upon testing and confirmation with literature, all the pH buffer compounds are concluded to be absorbing in the ultra violet region, which will have a minimal effect when the fluorescein solution is excited at 500nm. Despite this, the pH buffer solution was still used as the calibration solution for the spectrometer before each trial to minimize the errors.
3) Storing the fluorescein solutions
   The fluorescein solutions were stored under a carboard box to prevent degradation under ambient light, especially the UV light from the sun. Exposure to UV sunlight leading to degradation of fluorescein will result in a loss in its fluorescence properties through the rapid decrease in absorption ability (Smith 3). The prevention of degradation by UV sunlight was taken by ensuring that the solutions were placed back under the box as soon as possible after uses, limiting their exposure to sunlight.

Weaknesses

There are many errors attributing to the deviation of the fluorescence intensity from the desired trends – an increase in pH levels lead to an increase in fluorescence intensity. Although it is not clear how significant these errors are to the investigation, they must be evaluated and an improvement must be provided to minimize their effects to improve the investigations in the future.

1) Fluorescein solutions
   The fluorescein powder was dissolved in distilled water, however as seen in Figure 3, there are very little locations for hydrogen bonding. This leads to the property of fluorescein being only very slightly soluble in water. This was a problem in the experiment as despite having the fluorescein solution in a stir station for a long period of time, the fluorescein powder did not dissolve fully and that there are still residuals floating. This gave rise to the initial strange phenomenon of a potential time factor, where the fluorescein fluoresces more intensely overtime. This was already resolved because of solubility of fluorescein in water, and the improved method developed in the preliminary trials did not resolve the problem fully. The dissolving of the leftover fluorescein residuals in the solution over time is attributed to the random errors that arise in this investigation. Subtle changes in the concentration as more fluorescein residuals dissolve will affect the fluorescence intensity recorded by the spectrometer. Although it is not evident to conclude from the data how much this effect has affected the results, concentration is certainly a crucial aspect to the fluorescence intensity of fluorescein. One way to resolve this is to either use ethanol as the solvent or turn the fluorescein compound into an ionic form. Although ethanol is a polar solvent, it has locations of nonpolar regions (ethyl group) that allows for nonpolar organic molecules to interact through van der Waals forces, thus, fluorescein will have a higher solubility in it than distilled water. In addition, turning the fluorescein powder into an ionic salt (see Figure 4) will increase the solubility of it in water significantly. The partially positive hydrogen atoms in the water molecule can interact with the negatively charged regions of fluorescein easily, therefore providing a higher solubility in water.
2) pH difference
   The pH buffer solutions are said to have an uncertainty of ±0.02 when it is at 25 °C. Since temperature was considered as one of the controlled variables, being the means to ensure that the pH levels of the fluorescein solutions are as close to the desired levels as possible and also to prevent fluctuations in pH

levels between trials. Upon measuring the ambient temperature, the temperature varied between 21°C to 24°C, potentially leading to a greater uncertainty when the temperature starts to deviate from the mentioned one. The unpredictable fluctuations in room temperature could give rise to random uncertainties in the investigation as different temperatures at different times could change the pH levels between trials. Although its impact could not be accurately measured due to the sensitivity and difficulty of the nature of this investigation, this factor could be minimized in the future. The fluorescein solutions could be kept in a water bath that stays at a constant 25°C. This would allow the solutions to be at the desired temperature for a minimal level of uncertainty when measuring in the spectrometer.

3) Excitation wavelength
   The spectrometer is described to have a ±4nm uncertainty for wavelengths, meaning that although the 500nm fluorescence excitation light was said to be shone upon the fluorescein sample, it is not certain that it is the same for each trial. A difference in the excitation wavelength will affect the fluorescence intensity, and although a 4nm uncertainty is not likely to induce a drastic difference in the results, there remains a possibility due to the highly sensitive nature of fluorescence spectroscopy. Since the transition between the ground state and its first excited singlet state of the fluorophore relates to its fluorescence intensity, a deviation from the excitation wavelength would mean that less or more molecules are able to be excited and emit light of the fluorescence wavelength, thus, affecting the fluorescence intensity. This instrumental limitation would contribute as a systematic error as the excitation wavelength is said to have an uncertainty of 4nm. One method of reducing this error is to conduct the experiment on a more precise spectrometer; one that preferably has an uncertainty less than 1 to minimize the potential deviation in the excitation wavelength that would change the results of the fluorescence intensities.

Further Investigation

Fluorescein

Due to the instrumental limitation of the spectrometer, which only allows relative intensity to be measured, it prevented the exploration of pH levels that are below pH 6. This was due to the common grounds found for the specific concentration that prevents the relative fluorescence intensity peak to saturate beyond 1. This, therefore led to the unclear and limited idea of what the relationship between pH levels and fluorescence intensity is as a whole. Although it was theorized that for fluorescein, the pH levels beyond 8.4 is tending near constant, and that the pH levels lower than that has a nonlinear relationship, it was never confirmed based on this investigation and literatures. Therefore, an extension could look into using a spectrometer that measures the fluorescence intensity (not the relative intensity that compares the measured intensity to the incident intensity, which would saturate for more intense lights) for pH levels lower than 6. Although this investigation could encounter other problems, which include quenching (the process of absorbance of its own emitted light, potentially affecting measurements), a common ground must be found through trial and error for the concentration, such that it prevents quenching and have a relatively intense fluorescence for lower pH levels.

Another alternative is to conduct investigations for the mediate pH levels between 6 and 10 (could extend to pH 12). By conducting more trials of smaller step sizes, it would provide a clearer image of what exactly the function or relationship between pH levels and fluorescence intensity is. This could be an investigation to provide an answer to the theorized nonlinear and near constant regions mentioned above.

Other compounds

In addition to investigating the fluorescence nature of fluorescein, there are other compounds readily available to be investigated. For instance, the widely known quinine and pyranine. Both are compounds with fluorescence property, and one could investigate whether its fluorescence nature is similar or different from that of fluorescein, or whether their relationships between pH levels and fluorescence intensity resemble that of fluorescein. This could provide insights into how this exact relationship connects with the property of the compounds.

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