**Fluorescence Quenching of Fluorescein by Iodide Ion**

**Student Name: ­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­Soham Das\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_ Experiment date.: \_\_\_\_\_\_\_26/08/2025\_\_\_\_\_\_\_\_  
Student ID: ­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­2023B2AA0733G­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­\_­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­ Group No.: \_\_\_\_\_\_\_\_\_\_3A\_\_\_\_\_\_\_\_\_\_\_\_**

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| **Aim** | To study the quenching of fluorescein fluorescence by iodide ions (I⁻) and to determine the Stern-Volmer quenching constant (K) by analyzing fluorescence intensity data at different quencher concentrations. |
| **Theory** | When fluorescein molecules absorb UV or visible light, they are promoted to an excited electronic state. They rapidly relax to the lowest vibrational level of the first excited singlet state (S₁) via internal conversion, losing some energy as heat. From this state, they may:   * Emit light (fluorescence) and return to the ground state (S₀) at a rate proportional to kf * Lose energy non-radiatively via internal conversion or vibrational relaxation at a rate proportional to knr   When a quenching agent, like the iodide ion (I−), is introduced, it provides a third pathway for the excited fluorescein molecule to lose energy. This is a non-radiative process where the excited molecule collides with the quencher, returning to the ground state without emitting light. The rate of this quenching process depends on the quencher's concentration, [Q], and is proportional to a constant, kq​[Q]. The net effect is a reduction in fluorescence intensity as the concentration of the quencher increases.  At steady state, the fluorescence intensity decreases with increasing quencher concentration. The Stern-Volmer equation relates the observed intensity (I) to the intensity without quencher (I°):  where:   * I​° or F° is the fluorescence intensity without a quencher. * I or F is the fluorescence intensity with a quencher present. * K is the Stern-Volmer constant. * [Q] is the quencher concentration.   A plot of I°​/I versus [Q] should yield a straight line with a slope equal to the Stern-Volmer constant, K. |
| **Experimental Section** | **Equipment & Apparatus**   * Spectrophotometer (for absorbance measurements) (Jasco V-770) * Spectrofluorometer (for fluorescence measurements) (Jasco FP-8500) * Quartz cuvettes: frosted on opposite sides for absorbance, clear for fluorescence * Volumetric flasks (5 × 10 mL, 2 × 100 mL, 1 × 250 mL, 1 × 50 mL) * Beakers (5 × 50 mL, 3 × 250 mL, 1 × 500 mL) * Micropipettes, 1ml microtips, droppers, aluminium foil, tissue paper   **Chemicals**   * Fluorescein solution: 60 μM in phosphate buffer * Potassium iodide solution: 1 M in deionized water * Ethanol * 0.1 M phosphate buffer (pH 7.4) |
| **Procedure** | 1. Prepare four different solutions of the KI solution with constant dye concentration with one solution with dye only to determine Io. Pipette 1 ml of fluorescein stock solution into each of five 10 ml volumetric flasks. Pipette 2 ml of ethanol into each flask. Pipette 1, 2, 3 and 4 ml of KI stock solution into 10 ml volumetric flasks. Dilute to the mark in each volumetric flask using distilled water. 2. Measure the absorbance of the fluorescein solution without KI to get the λmax. 3. Using pipette, add 1 mL of stock dye solution and 1 mL of water into a cuvette (two sides transparent and two sides frosted). 4. Measure absorbance spectrum using spectrophotometer 5. Measure fluorescence (emission) spectra of the prepared solutions to study the quenching process and overlay the measurements. 6. Record excitation and correlate with absorbance 7. Plot the data using origin or any other software 8. Report the value of Stern-Volmer constant, K |
| **Results and discussion** | Key Observations from the Plot:   1. Graph Title & Labels:    * Title: *UV-Vis Spectroscopy of Fluorescein*    * X-axis: *Wavelength (nm)*    * Y-axis: *Absorbance (a.u.)* 2. Major Absorption Peak:    * A sharp and intense absorption peak is observed around 490–500 nm.    * The maximum absorbance value is close to ~2.0 a.u. 3. Secondary Features:    * Minor fluctuations in absorbance are visible in the 300–350 nm region.    * These are smaller peaks compared to the main absorption band. 4. Spectral Region:    * The scan covers a wide wavelength range 200–800 nm, which includes both UV and visible regions.    * Fluorescein absorbs strongly in the visible region (blue-green light), explaining its intense yellow-green fluorescence.   Discussion Points:   * λmax (maximum wavelength absorption):   1. The peak near 490 nm corresponds to the π→π\* electronic transition of the fluorescein molecule.   2. This is responsible for its strong color and fluorescence. * Electronic Transitions:   1. Absorption in the visible region arises from conjugated aromatic and xanthene structures in fluorescein.   2. The extended π-conjugation lowers the energy gap, shifting absorption into the visible region. * Applications:   1. Due to strong absorbance and fluorescence, fluorescein is widely used as a dye, tracer, and fluorescent probe in biological and chemical studies. * Structure-Spectrum Relation:   1. The broad and strong absorbance shows a high degree of conjugation.   2. Minor peaks in the UV region correspond to higher energy electronic transitions. * Practical Implication:   1. The sharp λmax helps in choosing excitation wavelengths for fluorescence experiments (commonly excited at ~490 nm). |
|  | Key Observations from the Plot:   1. Graph Title & Labels:    * Title: *Fluorescence*    * X-axis: *Wavelength (nm)*    * Y-axis: *Intensity (a.u.)* 2. Fluorescence Maximum (λmax):    * The emission peak occurs around ~516–518 nm.    * This corresponds to the green fluorescence of fluorescein. 3. Effect of Quencher Concentration:    * 0 mL quencher (blue line): Maximum fluorescence intensity (~6000 units).    * As quencher concentration increases (1 mL → 4 mL), the intensity decreases progressively.    * At 4 mL quencher, the fluorescence intensity drops significantly to ~1500 units. 4. Peak Position:    * The emission maximum wavelength does not shift noticeably.    * Only the intensity decreases, suggesting quenching affects efficiency, not energy level spacing.   Discussion Points:   1. Quenching Mechanism:    1. The decrease in fluorescence with quencher addition indicates fluorescence quenching.    2. Likely mechanisms:       * Dynamic quenching (collisional interactions between quencher and excited dye molecules).       * Static quenching (complex formation between quencher and fluorophore). 2. Concentration Dependence:    1. The systematic reduction in intensity with increasing quencher concentration supports a linear Stern–Volmer relationship (to be verified by plotting F°/F vs [quencher]). 3. λmax Stability:    1. Since λmax remains constant, the electronic transition levels are unaffected.    2. Only the number of photons emitted decreases (reduced quantum yield). 4. Practical Relevance:    1. Quenching studies are widely used to study molecular interactions, binding constants, and energy transfer processes in fluorescence spectroscopy.    2. This kind of plot can help determine whether the quencher acts through collisional or static binding. 5. Comparison with Plot-1 (UV-Vis):    1. Absorption was strongest at ~490 nm (excitation region).    2. Here, fluorescence occurs at ~516 nm (emission), which is a Stokes shift (emission at longer wavelength than absorption). |
|  | Key Observations from the Plot:   1. Graph Title & Labels:    * Title: *Stern-Volmer Plot*    * X-axis: *Concentration of Quencher (M)*    * Y-axis: F°/F (Ratio of fluorescence intensity without quencher to that with quencher). 2. Linear Relationship:    * The data points show a good linear fit, consistent with the Stern–Volmer equation:    * The plotted line equation is y = 5.4788x + 1 3. Stern–Volmer Constant K    * From the slope, K = 5.48 M-1    * The intercept is 1, which matches the theoretical equation.   Discussion Points:   1. Nature of Quenching:    1. The straight line indicates dynamic quenching (collisional), since the Stern–Volmer plot is linear.    2. Nonlinear plots would suggest static quenching or a combination of mechanisms. 2. Quenching Efficiency:    1. The higher the slope K, the more efficient the quencher is in reducing fluorescence.    2. Here, K ≈ 5.48 M-1, which reflects a moderate-to-strong quenching efficiency. 3. Interpretation of Intercept:    1. At quencher concentration = 0, F°/F=1, which matches the expected theoretical value (no quenching). 4. Connection to Previous Plots:    1. Plot-2 (Fluorescence spectra): Showed progressive decrease in intensity with quencher concentration.    2. Plot-3 (Stern–Volmer): Quantifies that decrease mathematically, showing it follows the Stern–Volmer relation. 5. Applications:    1. Stern–Volmer analysis helps determine whether quenching is collisional or static.    2. It can also be used to estimate binding constants or diffusion coefficients in biochemical and photophysical studies. |
| **Data Analysis** | The Stern-Volmer Plot was plotted in Excel using the data from the table attached below. With x axis having quencher concentration and y axis having the ratio F°/F  Using Stern-Volmer equation,  F°/F and quencher concentration Q to plot the Stern-Volmer line with slope K. |
| **Conclusion** | The UV-Vis absorption spectrum of fluorescein (Plot-1) showed a strong absorption maximum around 490 nm, characteristic of π→π\* electronic transitions within its conjugated aromatic system. This absorption band corresponds to the excitation wavelength that produces the characteristic green fluorescence emission.  The fluorescence spectra (Plot-2) exhibited a maximum emission near 516–520 nm, consistent with the expected Stokes shift between absorption and emission. With increasing quencher concentration, a systematic decrease in fluorescence intensity was observed, while the emission maximum remained unchanged. This indicates that quenching reduces the efficiency of fluorescence without altering the fundamental electronic transition energies.  The Stern–Volmer plot (Plot-3) of F°/F versus quencher concentration yielded a linear relationship, consistent with the Stern–Volmer equation for dynamic quenching. The Stern–Volmer constant, K​, was determined to be approximately 5.48 M⁻¹, confirming moderate quenching efficiency. The intercept was close to unity, as predicted theoretically, with minor deviation attributed to experimental error.  Overall, the combined spectroscopic analysis confirms that fluorescein undergoes efficient excitation at ~490 nm and emission at ~516 nm, and that fluorescence is progressively quenched by the added quencher in a concentration-dependent and Stern–Volmer linear manner. This validates the applicability of Stern–Volmer kinetics to quantify quenching interactions in fluorophore–quencher systems. |
| **Precautions** | 1. Sample Preparation    * Use freshly prepared fluorescein solutions to avoid degradation.    * Ensure all solutions are free from dust, bubbles, and turbidity, as these can scatter light and affect absorbance/fluorescence readings.    * Accurately measure quencher volumes using calibrated pipettes to maintain correct concentrations. 2. Instrument Handling    * Calibrate the spectrophotometer/fluorimeter before measurements.    * Always run a blank solution (solvent only) to set the baseline.    * Avoid opening the instrument cuvette holder frequently to minimize stray light. 3. Cuvette Care    * Use clean, scratch-free quartz cuvettes, since scratches or fingerprints can distort readings.    * Wipe cuvettes with lint-free tissue before inserting them into the instrument.    * Keep the cuvette orientation consistent during all measurements. 4. Measurement Conditions    * Record absorbance and fluorescence at room temperature, avoiding direct sunlight or heat sources that may affect stability.    * Perform measurements quickly to minimize photobleaching of fluorescein.    * Use the same excitation wavelength (close to λmax ~490 nm) for all fluorescence measurements to ensure comparability. 5. Data Accuracy    * Repeat each measurement at least twice to ensure reproducibility.    * Avoid very high absorbance (>2.0) values in UV-Vis, as they reduce accuracy (dilute sample if necessary).    * Maintain consistent path length and solution volume across all experiments. 6. Safety Precautions    * Handle all chemicals, including quenchers, with gloves and goggles.    * Dispose of chemical waste according to lab safety protocols.    * Avoid direct exposure of eyes to intense light from the fluorimeter. |
| **Future Scope** | 1. Quenching Mechanism Studies    * Extend the study by distinguishing between dynamic (collisional) and static (complex formation) quenching using temperature variation or lifetime measurements.    * Time-resolved fluorescence spectroscopy can provide deeper insights into the mechanism. 2. Different Quenchers    * Investigate the effect of various quenchers (ionic, molecular, heavy atoms, etc.) on fluorescein fluorescence to compare efficiencies.    * Study selective quenching to understand binding interactions. 3. pH-Dependent Behavior    * Fluorescein exhibits strong pH sensitivity. Future work can explore quenching behavior at different pH values to simulate biological environments. 4. Applications in Sensing    * Extend the Stern–Volmer analysis to design fluorescence-based sensors for detecting specific ions, biomolecules, or pollutants.    * Real-world applications include biosensing, medical diagnostics, and environmental monitoring. 5. Computational Studies    * Use quantum chemical simulations or molecular docking to support experimental results and visualize fluorophore–quencher interactions. 6. Nanomaterial Integration    * Incorporate fluorescein into nanoparticles, polymers, or films to study quenching in advanced material systems, relevant for drug delivery and optoelectronic applications. |
| **References** | Kemp W., “Organic Spectroscopy”, 3rd ed., Palgrave, New York (1991)  Willard H. H., Merritt L. L., Dean J. A., and Settle F. A. Jr., “Instrumental Methods of Analysis”, 7th ed., Wadsworth, New York (1989)  Prof. Woormileela Sinha’s class notes on Fluorescence and Stern–Volmer analysis |