A REVIEW ON SPECTROFLUORIMETRY

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ABSTRACT:

Fluorescence spectroscopy stands out as a swift and highly sensitive approach for assessing molecular surroundings and occurrences within samples. Its selection is predicated on its exceptional sensitivity, notable specificity, ease of use, and cost-effectiveness when juxtaposed with alternative analytical methods. Widely acknowledged and potent, this technique finds extensive employment across various domains such as environmental studies, industrial processes, medical diagnostics, DNA sequencing, forensics, genetic analysis, and biotechnology. Serving as a pivotal analytical instrument, it facilitates both quantitative and qualitative assessments. This exposition offers a concise insight into the principles underlying fluorescence spectroscopy, supplemented with illustrative instances of its utilization in fields ranging from organic and inorganic chemistry to medical diagnosis and scientific research.

Key words: fluorescence spectroscopy, DNA sequencing, forensics, sensitivity.

1. INTRODUCTION

Spectrofluorimetry is a widely used analytical method known for its sensitivity and accuracy through which we can detect / identify the compounds and also determine their concentration at ng or lower level.

When molecules absorbs electromagnetic radiation, the electrons gets excited from the ground state to excited state (unstable). As they return to ground state upon excitation, it emits a certain radiation called photoluminescence.

Photoluminescence is categorized into five types:

Based on mechanism:

- Fluorescence
- Phosphorescence

Based on type of excitation source:

- Chemiluminescence
- Cathadoluminescence
- Electroluminescence

1.1 Fluorescence

When a beam of light is passed through a substance, then it absorbs the photons and the electrons in it gets excited to the higher energy states from the ground state. As they tend to return to the ground state from the excited state, they lose some of the energy in the form of radiation of probably higher wavelength. This optical phenomenon where molecular absorption of energy in the form of photons stimulate the emission of fluorescent photons with a longer wavelength is called as fluorescence.

There is no change in the electron spin which results in short live electrons (-10^{-5} sec) in the excited state of fluorescence. Fluorescent material only emits the radiation when the radiation source is present. Not all the molecules which absorb UV or visible light are fluorescent and it is useful to quantify the extent to which a particular molecule fluoresces. This is done by means of quantum yield (ϕ_f) which is defined as the fraction of incident radiation which is re-emitted as fluorescence. [1]

$$\phi_f = \frac{no\ of\ photons\ emitted}{no\ of\ photons\ absorbed} = \frac{quantity\ of\ light\ emitted}{quantity\ of\ light\ absorbed}$$

1.2 Phosphorescence

It is a type of delayed fluorescence as it lasts for long time because of a change in electron spin, which results in longer life time of the excited state (seconds to minutes). It emits radiation even after the radiation source is removed. It is not as instantaneous as fluorescence because the re-emission is associated with forbidden energy states transition in quantum mechanisms.

Photoluminescence involves both photoexcitation and emission processes.

Photoexcitation

It may occur by absorption of one of the following forms of radiant energy:

- Sunlight
- Visible radiation
- UV rays
- X-rays

If the molecules absorb any of the above radiation, the electrons at the ground state gets excited. Absorption of a photon of suitable energy causes the molecule to get excited from the ground state to one of the excited states. This is called excitation or activation and is governed by FRANK-CONDON PRINCIPLE.

Emission process

After excitation electrons returns to ground state by a process called relaxation or deactivation and emits a certain radiation of probably higher wavelength. This deactivation process can be broadly categorized into two types:

1. Non-radiative deactivation
2. Radiative deactivation

In molecular species, energy transition may occur between different vibrational levels of particular excited state because the energy of the vibrational levels of excited state matches with energy of vibrational level of ground state. In this energy transition, some energy is lost as heat which is considered as a non-radiative transition. When an electron return from higher energy excited state to lower energy ground state, then the energy is lost/ emitted as radiation with higher wavelength than that of absorbed radiation. [2,3]

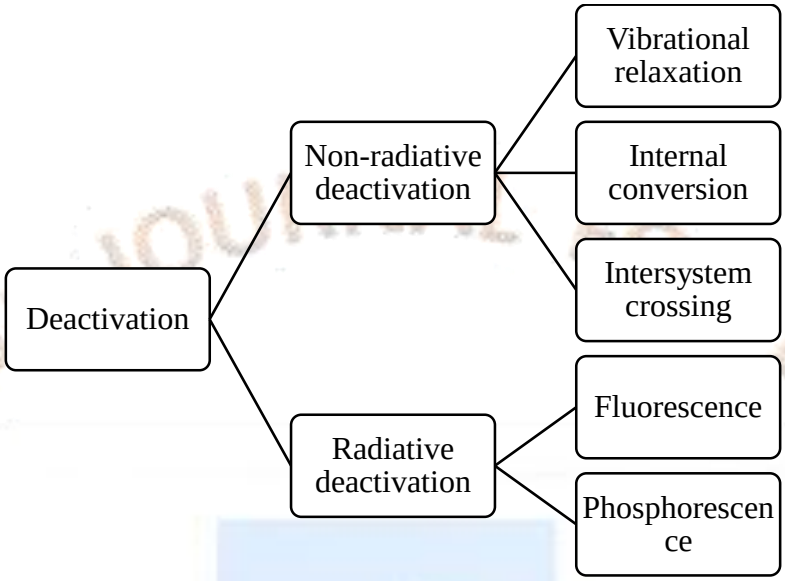


Fig.1: Types of deactivation or emission processes

Stokes (1862) observed that in fluorescence the light is absorbed at certain wavelength and is emitted at a greater wavelength and this is due to the fact that the electron after absorption of photon jumps to higher energy excited states and returns to ground state in step by step process by losing some energy. Since the relaxation takes place in steps by losing some of its energy, the wavelength of the emitted radiation must be of greater wavelength.

Stokes shift

The difference between wavelength of absorption maxima (a) and the emission maxima (b). Stokes shift: b-a

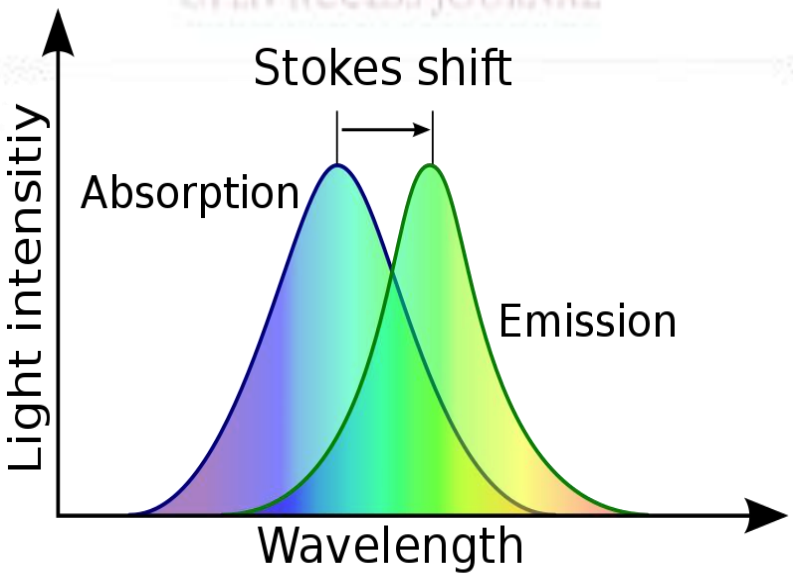


Fig.2: Fluorescence spectra showing stokes shift.

Stokes shift is indirectly proportional to overlapping of excitation and emission spectra i.e., greater the stokes shift, lesser will be the interference as the emission and excitation spectra do not overlap. Thus the good results of fluorescence are achieved with the compounds having the greater stokes shift.

1.3 Fluorophores

Presence of fluorophores is important for a substance to exhibit fluorescence. They are organic molecules of 20-100 Daltons. Fluorophores are categorized into two types:

Table 1: Types of fluorophores

Intrinsic fluorophores	Extrinsic fluorophores
Occurs naturally	Synthetically made
Originates from aromatic acids, neurotransmitters, porphyrins and green fluorescent proteins. e.g.: FMN, FAD, NAD cofactors.	Dyes and synthetic or modified biochemical which are added to specimen to produce fluorescence. e.g.: fluorescein (for protein) ethidium bromide and acridine orange (for DNA)

2. PRINCIPLE

When UV or visible light is absorbed by the molecule, it promotes an electron to excited state from ground state. Due to instability, the electrons returns to the ground state by emitting the radiation of higher wavelength as it loses some of its energy due to relaxation process. Fluorescence emission occurs as the fluorophore decay from the singlet electronic excited states to an allowable vibrational level in the electronic ground state. The fluorescence excitation and emission spectra reflect the vibrational level structures in the ground and the excited electronic states respectively.

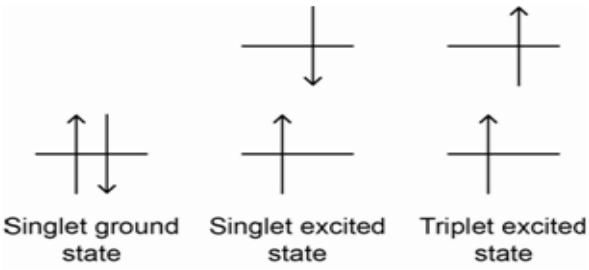
This can be explained better by Jablonski diagram, a graphical representation used in fluorescence spectroscopy used to illustrate electronic transitions that occur in the molecule after absorption of light. [4]

2.1 Jablonski diagram

Electronic states

- Ground state: It is a state of electron which has lowest energy and highly stable.
- Excited state: It is a state of electron which has higher energy and less stable.
- Singlet state: All the electron spins are paired in this molecular electronic state.
- Doublet state: An unpaired electron is present in this electronic state which gives two possible orientations.
- Triplet state: It is the electronic state which consists of unpaired electron with same spin.

- Singlet excited state: It is the excited state consisting of unpaired electron in orbital with opposite spin.
- Triplet excited state: It is the excited state which consists of unpaired electrons with same spin.



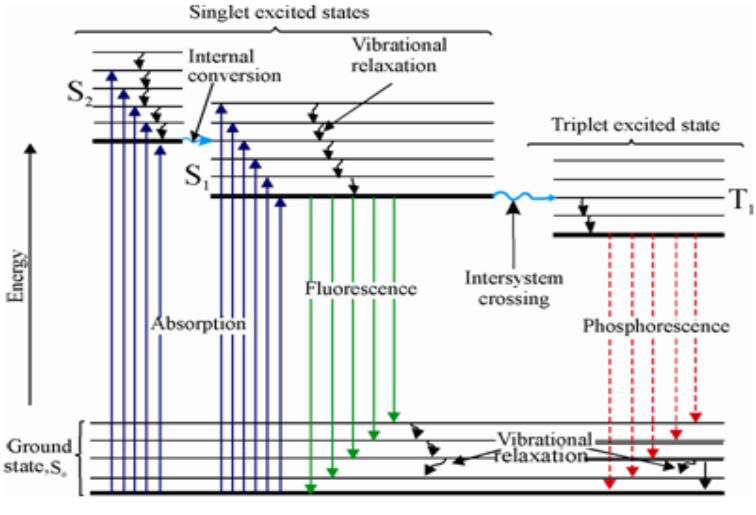


Fig.3: Jablonski diagram

2.2 Internal conversion

After excitation, an intermolecular process occurs by which excited electron returns to the lowest vibrational energy level of excited state without emitting any radiation. This process is called internal conversion.

Internal conversion through overlapping of the vibrational levels is more probable than the loss of energy by fluorescence from a higher excited state. When an electron moves from the higher electronic state to upper vibrational state of the lower electronic state, rupturing of bond takes place as the probability of the existence of the bonds with the strength less than that of the electronic excitation energy of the fluorophores is more. So predissociation also leads to internal conversion. [4, 5]

2.3 Intersystem crossing

A phenomenon where a non-radiative transition occurs between two isoenergetic vibration levels belonging to electronic states of different multiplicities. The difference between the spin states makes the transition from singlet to triplet or reverse (forbidden transitions) more improbable that singlet to singlet transitions.

This singlet to triplet or reverse transitions involves a change in the electronic state, due to which the lifetime of the triplet state is longer than singlet state by approximately 10 seconds fold difference. This event is relatively rare but ultimately results in phosphorescence or delayed fluorescence. [4]

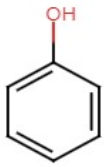
2.4 EXTERNAL CONVERSION

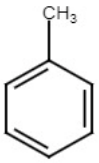
During vibrational relaxation, loss of energy occurs due to the collision with other molecules or by conversion of excess energy into heat which is absorbed by neighboring solvent molecules upon colliding with excited state fluorophores. External conversion is influenced by temperature, solvent viscosity and its composition. [4]

3. MOLECULAR STRUCTURE AND FLUORESCENCE [5]

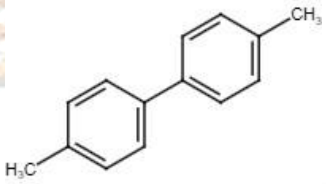
Molecular structure is a key parameter to determine the possibility and extent of fluorescence in a chemical species, but only few molecules shows fluorescence.

- Aromatic and heterocyclic compounds shows fluorescence due to the presence of certain substituted groups (electron donating groups) such as $–NH_2$, -OH, $-OCH_3$, on resonating nucleus.
  Electron withdrawing groups like –COOH, NO_2, -N=N, -Cl, -Br, -I diminishes fluorescence.
- The relative intensity of fluorescence increases with increase in the substitution of oxygenated species.
  Example: phenol, anisole

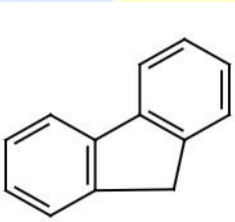




- Molecules with high rigidity shows high fluorescence than that of flexible molecules.
  e.g.; biphenyl shows low fluorescence than fluorene.

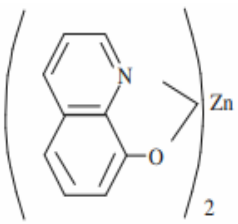


Biphenyl



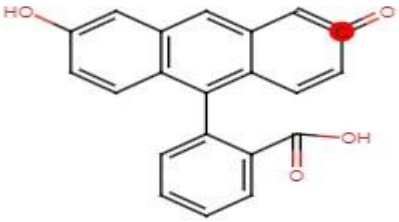
Fluorene

- Complex formation also leads to rigidity in molecules and shows higher fluorescence.
  e.g.; 8-Hydroxy quinolone complexed with zinc ions.

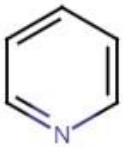


- Compounds with fused ring shows fluorescence. Extent of fluorescence is directly proportional to number of ring in the molecule.

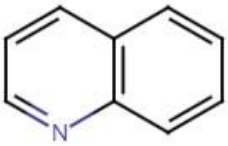
  e.g.; fluorescein



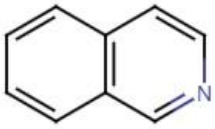
- Aliphatic and alicyclic carbonyl compounds or highly conjugated double bond structures also shows fluorescence.
- Simple heterocyclic compounds like pyridine, furan, thiophene and pyrrole does not show any fluorescence, but when fused with a benzene ring, they become highly fluorescent. E.g.; pyridine+ benzene ring derivatives



Pyridine



Quinolone



Isoquinoline

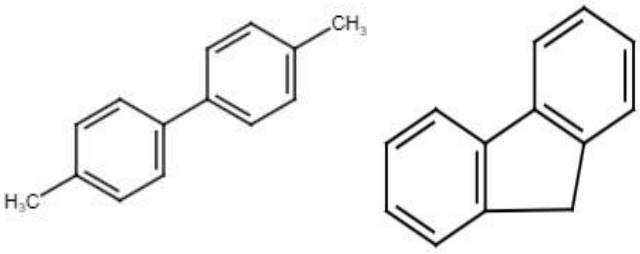
4. FACTORS AFFECTING FLUORESCENCE [6]

Fluorescence is a very sensitive phenomenon exhibited by a substance in a given solution. There are number of factors which directly and indirectly affect fluorescence and its intensity and sensitivity which is termed as "quenching".

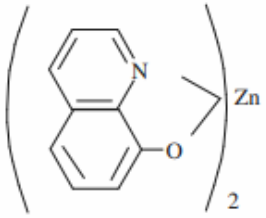
4.1 Effect of Structural rigidity

It is found that fluorescence is particularly favored in molecules with rigid structures.

Example, quantum efficiencies for fluorene and biphenyl are nearly 1.0 & 2.0, respectively, under similar conditions of measurement. The difference in behaviour is largely result of increased rigidity furnished by bridging methylene group in fluorene.



The influence of rigidity has also invoked to account for increase in fluorescence of certain organic chelating agents when they are complexed with a metal ion. For example, the fluorescence intensity of 8-hydroxyquinoline is much less than that of its zinc complex.



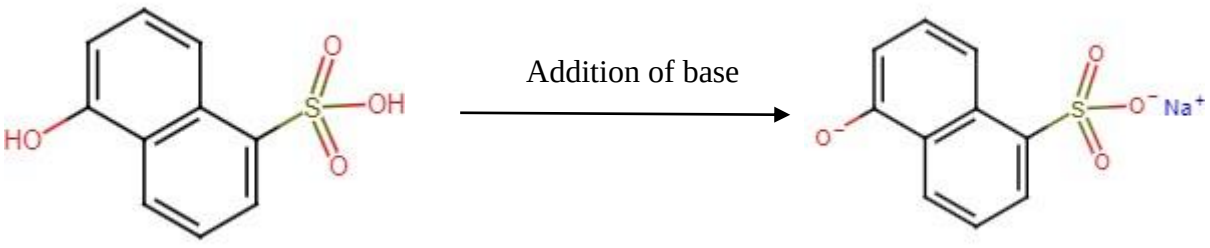
4.2 Temperature and viscosity

Increase in temperature and decrease in viscosity is likely to cause collision between the molecules and thus decrease in fluorescence by deactivation of excited molecules. Low temperature and appropriate dilute solutions are necessary for quantitative results. Similarly, many substances are not normally fluorescent at room temperature are capable of emitting light when excited at a low temperature or when in a viscous solvent or glassy matrix. The temperature coefficient of fluorescence are typically-1%°C increase in the temperature.

4.3 Effect of pH on Fluorescence

The fluorescence of certain compounds as a function of pH has been used for detection of end points in acid-base titrations.

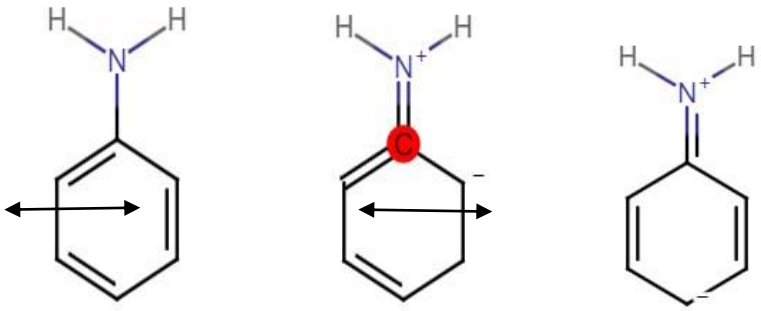
Example, Fluorescence of phenolic form of 1-napthol-4-sulfonic acid is not detectable by eye because it occurs in UV region when the compound is converted to phenolate ion by the addition of base, the emission band shifts to visible wavelength, where it can readily be seen.



1-napthol-4-sulfonic acid (Phenolic form) 1-napthol-4-sulfonic acid (Phenolate ion)

The fluorescence of aromatic compound with acidic or basic ring substituents is usually pH dependent both the wavelength and emission intensity are likely to be different for protonated and unprotonated forms of compound. The change in emission of compounds of this type arise from differing number of resonance species that are associated with acidic and basic forms of molecules.

Example, Aniline has several resonance forms, but anilinium has only one.



Resonance forms of aniline

4.4 Effect of Pol

The energy of n-π* transitions is often increased in polar solvents, while that of π-π* transitions is decreased in polar solvent. In some cases such shifts may be great enough to lower the energy of π-π* process below that of n-π* transitions and hence enhanced fluorescence results. Solvents containing heavy atoms/ other solutes with such atoms in their structure decrease fluorescence.

Example: carbon tetra chloride, ethyl iodide

The effect is similar to that observed when these species substituted into fluorescent compounds. The orbital interactions result in increase in rate of triplet formation and hence there is a decrease in fluorescence.

4.5 Presence of dissolved oxygen

This often reduces emission intensity of fluorescent solution probably due to photo chemically induced oxidation of fluorescent material. Quenching also occurs as a result of paramagnetic properties of molecular oxygen that can be expected to promote inter system crossing and conversion of excited molecules to triplet state.

4.6 Inter filter effect

There may be presence of non fluorogenic material in the sample which absorbs light and thus shows filter effect. The presence of such materials should be kept minimum and constant both in standard and test samples.

4.7 Photodecomposition

In absorption spectrophotometry, the intensity of the radiation passing through solution is weak by photochemical standards, although adequate for measurements; decomposition of the solute is therefore, not very likely. Spectrofluorimetry, on the other hand, requires high intensity illumination for irradiation, and the risk of photochemical change is thereby increased. An error up to 20% could quite easily arise. It may be

possible in unfavourable cases to select radiation of a wavelength which is not strongly absorbed so that the extent of photochemical change is reduced, at the same time adequate sensitivity is retained.

4.8 Effect of substituents

Substitutes have a significant impact on fluorescence. Fluorescence is frequently enhanced by a delocalizing electron substituent, such as the NH2, OH, F, OCH3, NHCH3, and N(CH3)2 groups, because these groups tend to boost the likelihood of a transition between the lowest excited singlet state and the ground state. Fluorescence is reduced or totally quenched by electron withdrawing groups comprising -Cl, Br, -1, NHCOCH3, NO2, or –COOH.

Substitution in benzene ring cause shifts in wavelength of absorption maxima and cause changes in fluorescent peaks. Fluorescence is inhibited by the substitution of – COOH or > C= O on an aromatic ring. In these compounds the energy of the n, π* systems is less than in the π, π* systems.

4.9 Effect of concentration

For the quantitative studies there should be a definite relationship (linear) between the concentration of species and the fluorescent intensity which is emitted. Such a relationship exists for very dilute solutions and fluorescent intensity. The Beer's law can be applied and thus.

Where,

$F = K (Io – I_t)$

Io = Intensity of incident radiation

I_t = Intensitv of transmitted radiation

F = intensity of fluorescent radiation

K = proportionality constant

Now, according to Beer-Lambert's law

$$I_t = I_o .10^{-abc}$$

$$(Io – I_t) = I_o (1 - 10^{-abc})$$

$$\text{Log } F_o/ F_o – F = abc$$

If abc is small > 0.01 then F = K'c. Thus, for low concentrations fluorescence intensity is directly proportional to the concentration and it is also proportional to the intensity of the incident radiation. This equation holds for concentrations of the order of a few parts per million or less, depending on the substance. At higher concentrations, the fluorescent intensity generally decreases. According to equation it is assumed that the fluorescence is measured in the same path as the incident radiation. In practice, however, the fluorescence is measured at right angles to the incident light. The equation still holds true when b is replaced by b', the depth of cell in the direction of the detector.

5. QUENCHING [7]

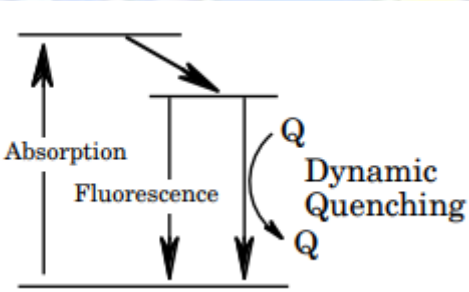
Decrease of fluorescence intensity by the interaction of the excited state of the fluorophores with its surroundings is called quenching. A variety of processes can result in quenching, such as excited state reactions, energy transfer, complex-formation and collisions. As a consequence, quenching is often heavily dependent on pressure and temperature. Molecular oxygen, iodine ions and acrylamide are common chemical quenchers. The chloride ion is a well-known quencher for quinine fluorescence. Quenching poses a problem for non- instant spectroscopic methods, such as laser induced fluorescence.

Table 2: Examples of quenching agents

Quenching agents	Typical fluorophores
1. Thiocyanate	Anthracene
2. Chloride	Quinine
3. Iodide	Tryptophan
4. Disulphide	Tyrosine
5. Nitric oxide	Naphthalene

5.1 Types of quenching

One experimentally useful type of quenching is due to collisions between quenching agents and fluorophores, and is called collisional or dynamic quenching. The dynamic quenching agent provides a non-radiative route for loss of the excited state energy.



A second type of quenching, sometimes confused with dynamic quenching, is static quenching, in which the quenching agent forms a non-fluorescent complex with the quenching agent.

$$\text{Fluorophore} + Q \rightleftharpoons \text{Fluor.Q (Complex)}$$

5.1.1 Static quenching

Static quenching (or contact quenching) of fluorescence is, when a Fluorophore **(F)** and a Quencher **(Q)** are creating a non-fluorescent complex **(FQ)** before excitation of F. The chemical equation therefore is:

$$F + Q \rightleftharpoons FQ$$

The chemical equilibrium (K_s) between the Fluorophore, the Quencher and the complex FQ is formed by the law of mass action and equal to the **Stern-Volmer constant (K_{sv})**. Therefore [FQ] stands for the concentration of the complex FQ, [F] for the concentration of the loose fluorophore and [Q] for the loose Quencher.

$$K_{SV} = K_s = \frac{[FQ]}{[F][Q]}$$

The **Stern-Volmer-equation** describes the dependence of the fluorescent intensity of a fluorescent dye on the concentration of a quenching material (Quencher). It was created by the two physical chemists Otto Stern and Max Volmer in 1919. There F_0 is the fluorescent intensity of the fluorescent dye without the quencher, F is the fluorescent intensity of fluorescent dye with the quencher. K_s stands for the Stern-Volmer-constant and [Q] for the concentration of the quencher.

$$\frac{F_0}{F} = 1 + K_{SV} \cdot [Q]$$

Example: Caffeine and related xanthine derivatives reduce the intensity of fluoroscence of riboflavin by complex formation.

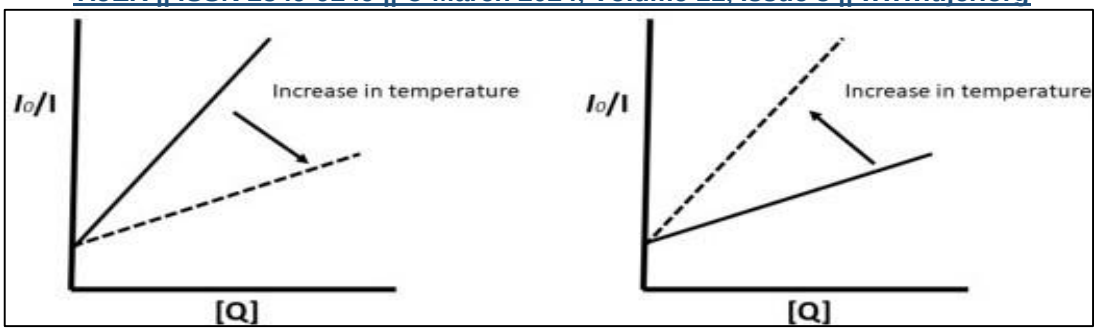
5.1.2 Dynamic quenching

Also called as collisional quenching which occurs between two light-sensitive FLUOROPHORES (called donor and acceptor fluorophores). Exchange of energy between donor and acceptor fluorophore happens after excitation – acceptor may either quench the energy or release a lower energy photon.

In dynamic quenching, the contact between the fluorophore and the quencher results in the loss of fluorescence pathway for return to ground state and it can happens by different mechanisms such as inducing intersystem crossing (oxygen and iodide are thought to quench by this method). Others, such as aromatic amines, appear to donate electrons to the excited state. Dynamic quenching exhibits a concentration dependence and is explained by Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q]$$

Example: Quenching of quinine drug by chloride ion and tryptophan by iodide ion.



(A) (B)

Static quenching Dynamic quenching

Fig.4: Types of quenching

5.1.3 Resonance energy transfer

Also called as fluorescence resonance energy transfer in which the energy from an excited state is transferred to an acceptor molecule. This transfer occurs without photon emission, but the process is related to absorbance in some respects. The rate of transfer depends on:

- The spectral overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor;
- The quantum yield of the donor;
- The relative orientation of the transition dipoles of the donor and acceptor;
- The distance between the donor and acceptor.

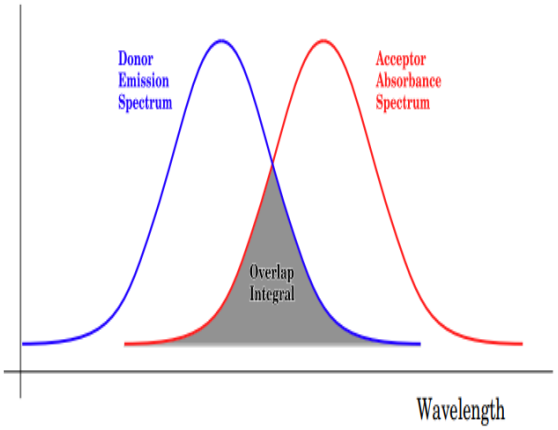


Fig.5: Fluorescence resonance energy transfer.

5.1.4 Chemical quenching

Chemical quenching is due to various factors like pH, presence of oxygen, halides and electron withdrawing groups, heavy metals.

- Change in pH: Aniline at pH (5-13) gives fluorescence when excited at 290nm. But pH < 5 or pH >13 it does not show fluorescence.
- Oxygen: oxygen lead to the oxidation of fluorescent substance to non-fluorescent substance and thus causes quenching.

- Halides and electron withdrawing groups: halides like chloride ions, iodide ions, electron withdrawing groups like - NO_2, - COOH, - CHO group lead to quenching.
- Heavy metals: Presence of heavy metals also lead to quenching because of collision and complex formation. [2]

5.1.5 Concentration quenching

Concentration quenching is a kind of self-quenching it occurs when the concentration of fluorescent molecule increases in a sample solution (>50 µg/ml) which results in curve deviation.

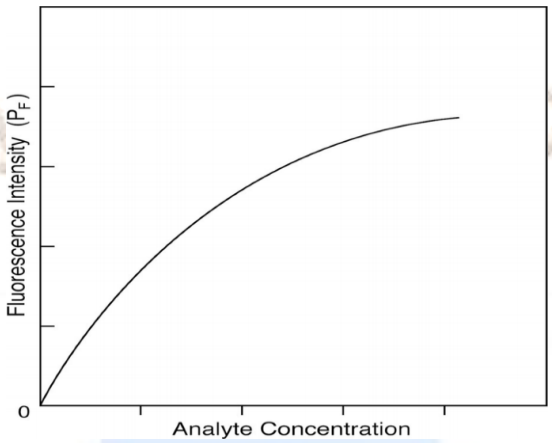


Fig.6: Self quenching

5.1.6 Excited state reactions quenching

It occurs because light absorption frequently changes the electron distribution within a fluorophore, which in turn changes its chemical or physical properties.

Example: A neutral solution of phenol can lose the phenolic proton in excited state.

5.1.7 Molecular rearrangement quenching

It involves the migration of a group or an atom from one center (migration origin) to another (migration terminus) due to light and heat within the same molecule.

Example: Lumisatonin, a photoproduct of satonin obtained via molecular rearrangement.

6. INSTRUMENTATION [7,8]

Spectrofluorimeter consists of mainly five components:

- Light source
- Filters and monochromators
- Sample cells
- Detectors
- Readout devices

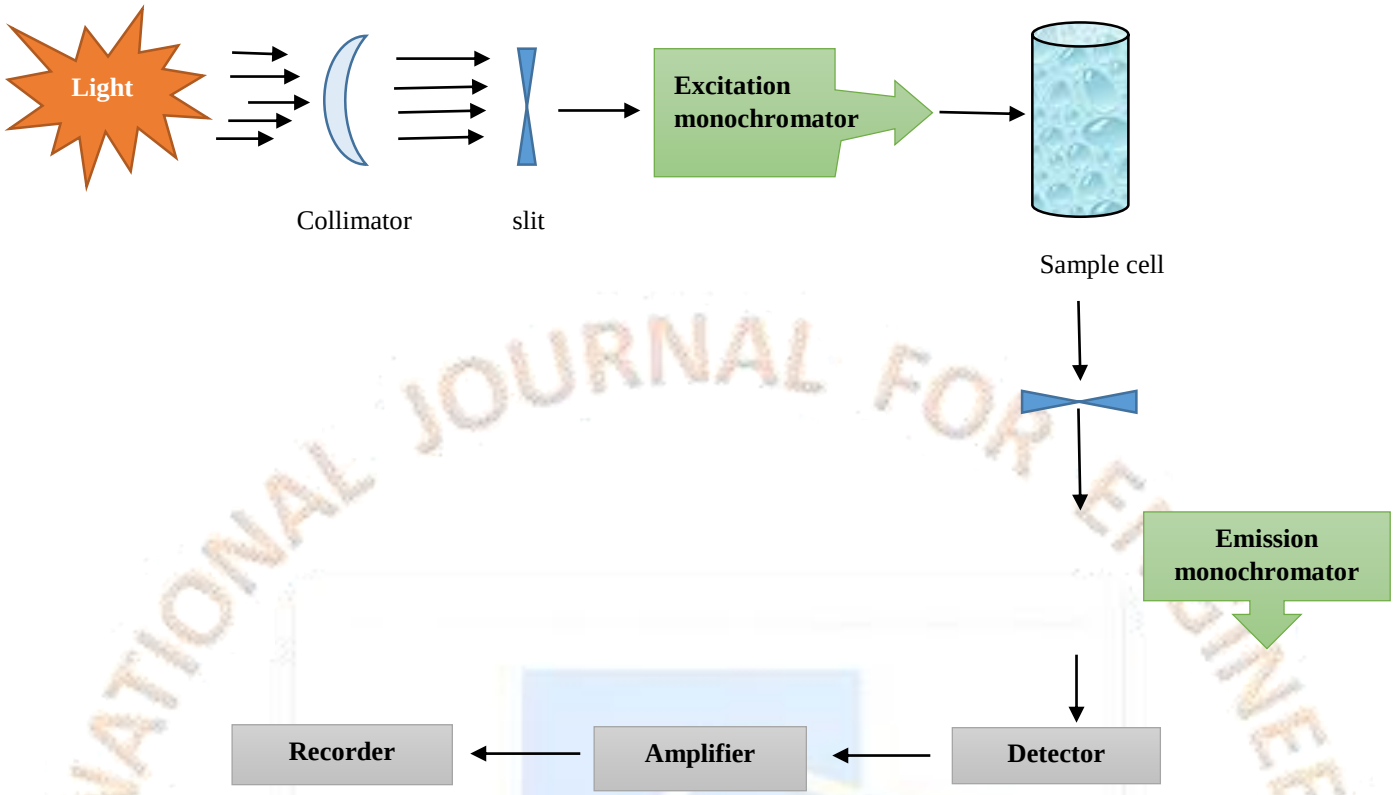


Fig.7: Instrumentation

6.1 Light source

Various light sources used in fluorimeter are:

- Mercury vapour lamp
- Xenon arc lamp
- Tungsten lamp
- Tunable dye lasers

Mercury vapour lamp

Filter fluorimeters often employ low pressure mercury arc lamps. This source produces intense lines at certain wavelengths. One of these lines will usually be suitable for excitation of a fluorescent sample. Mercury lamp emits light near peak wavelengths. Mercury lamps have the property that their spectral output depends upon the pressure of the filler gas. The output from a low-pressure mercury lamp is concentrated in the UV range, whereas the most commonly employed lamps, of medium and high pressure, have an output covering the whole UV-visible spectrum.

Xenon arc lamp

Spectrofluorometers, on the other hand, need a continuous radiation source, are often equipped with a 75-450 W high-pressure xenon arc lamp. These produce an intense continuum between about 250 and 600 nm. As the xenon arc lamp produces lot of heat, the lamp assembly needs to be cooled therefore, these instruments cannot be used for routine work. Xenon arc sources can be operated either on a continuous DC basis or stroboscopically; the latter method offers advantages in the size and cost of lamps. The output is essentially a continuum on which are superimposed a number of sharp lines, allowing any wavelength throughout the UV-visible region of the spectrum to be selected.

Tunable dye lasers

For certain applications, it is preferable to use a laser excitation source. A tuneable dye laser, using a pulsed nitrogen laser as the primary source can produce monochromatic radiation between 360 and 650 nm. Since the radiation produced is monochromatic, there is no need for an excitation monochromators. A few fluorescence spectrometers using laser sources are commercially available; most such instruments are intended for highly specific applications such as analysis of uranium in the nuclear industry.

6.2 Filters and Monochromators

Monochromators

Most common type of monochromators utilizes a diffraction grating. Two monochromators are employed in spectrofluorometers:

- Excitation monochromator: used for the selection of the excitation wavelength from incident beam and the excitation wavelengths which are frequently being selected are in the ultraviolet region
- Emission monochromator: used for determination of fluorescence spectrum and the emission wavelength is selected in the visible region.

The excitation monochromator selectively delivers a narrow band of wavelengths of excitation light that strikes the sample. A portion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The emitted light enters the emission monochromator, which is positioned at 90° angle with respect to excitation light path in order to minimize the risk of transmitted or reflected incident light reaching the detector.

Filters

Two types of filters are used:

- Primary filters: absorbs visible light and transmits UV light.
- Secondary filters: absorbs UV light and transmits visible light.

The simplest filter fluorimeters use fixed filters to isolate both the excited and emitted wavelengths. To isolate one particular wavelength from a source emitting a line spectrum, a pair of cut-off filters are required. These may be either glass filters or solutions in cuvettes. Recently, interference filters having high transmission (≈

40%) of a narrow range (10 – 15 nm) of wavelengths have become available and it is possible to purchase filters with a maximum transmission at any desired wavelength.

6.3 Sample cells

Cuvettes may be circular, square or rectangular (the latter being uncommon), and must be constructed of a material that will transmit both the incident and emitted light. Square cuvettes, or cells will be found to be most precise since the parameters of path length and parallelism are easier to maintain during manufacture. However, round cuvettes are suitable for many more routine applications and have the advantage of being less expensive.

6.4 Detectors

Mostly photomultiplier tubes are used in fluorimeters. They have high sensitivity and less noise. . The material from which the photocathode is made determines the spectral range of the photomultiplier and generally two tubes are required to cover the complete UV-visible range.

The spectral response of all photomultipliers varies with wavelength, but it is sometimes necessary to determine the actual quantum intensity of the incident radiation and a detector insensitive to changes in wavelength is required. A suitable quantum counter can be made from a concentrated solution of Rhodamine 101 in ethylene glycol which has the property of emitting the same number of quanta of light as it absorbs, but over a very wide wavelength range. Thus, by measuring the output of the quantum counter at one wavelength, the number of incident quanta over a wide wavelength range can be measured.

6.5 Readout devices

The output from the detector is suitably amplified and displayed on a read out device like a meter or digital display. The sensitivity of the amplifier can be changed so as to be able to analyse samples of varying concentrations.

Digital displays are most legible and free from misinterpretation. Improvement in precision is obtained by the use of integration techniques where the average value over a period of a few seconds is displayed as an unchanging signal. Microprocessor electronics provide outputs directly compatible with printer systems and computers, eliminating any possibility of operator error in transferring data.

7. APPLICATIONS [9,10]

Fluorescence spectroscopy has a wide range of applications in various fields due to its sensitivity and accuracy. It is widely used in the qualitative and quantitative analysis of inorganic compounds and has become extensively used spectroscopic technique in the fields of Biochemistry and Molecular Biophysics also. Despite the fact that the fluorescence measurements do not provide detailed structural information, the technique has become quite popular in these areas. This is due to its sensitivity to changes in the structural and dynamic properties of biomolecules and bio molecular complexes. The increased interest in fluorescence appears to be due to advances in time resolution, methods of data analysis, and improved instrumentation.

7.1 In inorganic chemistry

- Determination of ruthenium
- Determination of boron in steel
- Determination of aluminium in alloys
- Determination of chromium and manganese in steel
- Determination of uranium salts
- Estimation of rare earth terbium
- Estimation of bismuth
- Determination of beryllium in silicates
- Estimation of 3,4-benzopyrene
- Determination of zinc and cadmium
- Determination of selenium

7.2 In organic chemistry

- Assay of thiamine
- Estimation of quinine sulphate

7.3 In investigation of chemical structures and reactions

- Hydrogen bonding
- Cis and trans isomerism
- Polymerization
- Tautomerism
- Rate of reaction
- Free radicals
- Steric hindrance

7.4 In chemical analysis

- Detection of impurities
- Estimation of fluorescent intensity

7.5 Special applications

- Laser induced fluorescence spectroscopy of human tissue for cancer diagnosis
- Study of marine petroleum pollutants
- Accurate determination of glucose
- A highly sensitive fluorescent immunoassay based on Avidin labelled nanocrystals
- Fluorescence polarization immunoassay of mycotoxins.[11]

Quantitative and qualitative analysis by Spectrofluorimetry:

Quantitative analysis using spectrofluorimetry involves measuring the fluorescence emitted by a sample to determine the concentration of a specific analyte and Qualitative analysis involves identifying the presence or absence of specific compounds or analytes based on their characteristic fluorescence properties. Fluorescence spectroscopic measurements can be carried out from simple steady-state emission intensity to quite sophisticated time-resolved measurements. Although fluorescence measurements do not provide detailed structural information, fluorescence spectroscopy is gaining interest in many areas of science for quantitative analysis of complex mixtures with the help of advanced multivariate statistical tools.

Steps involved in quantitative analysis using spectrofluorimetry:

- Selection of suitable solvent for analysis of sample for fluorescence
- Preparation of standard solutions
- Measuring the fluorescence intensities of solutions
- Construction of calibration curve
- Validation

Steps involved in qualitative analysis using spectrofluorimetry:

- Selection of appropriate solvent for analysis
- Preparation of sample solutions
- Measurement of fluorescent spectra
- Comparison of obtained spectra with reference spectra
- Identification of unknown compound
- Interpretation of results
- Validation

Validation using Spectrofluorimetry [12]

Validation is done to know whether to confirm the method used is more reliable, accurate and precise. Validation is done according **ICH Q_2 R_1** guidelines. Typical validation characteristics which should be considered are listed below:

- Accuracy
- Precision
- Specificity
- Detection limit
- Quantitation limit
- Linearity
- Range
- Robustness
- System suitability

Fluorescence spectrometry, because of its rapid analysis, relatively inexpensive in addition to its ease of use, sensitivity, and selectivity, instrumental versatility, speed of analysis, and its non-destructive character, is a powerful analytical tool in various fields. Comparing with the other spectrometric analytical techniques [e.g., IR or ultraviolet (UV) molecular adsorption] fluorescence spectrometry has the higher sensitivity and can be used to analyze materials including endogenous fluorescent compounds even at deficient concentration levels.

8. CONCLUSION

Fluorescence spectroscopy is highly sensitive and selective analytical technique known for its ability to analyse chemical compounds to biological products. It is less tedious and less cumbersome when compared to other methods. The compounds can be analysed upto the levels of nanograms. It is widely used analytical tool for both quantitative and qualitative analysis. Its advancements such as multimodal imaging, high throughput screening, use of fluorescent probes and sensors, time resolved fluorimetry have made fluorimetry an indispensable tool in scientific research, medical diagnostics, environmental monitoring and industrial quality control. The reasons for choosing fluorimetry as a method of validation are its high sensitivity, specificity, wide applicability, speed, automation, and commercial availability.

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