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FLOURIMETRY – REVIEW ARTICLE

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Abstract:
Flourimetry is a measurement of fluorescence intensity at a particular wavelength involving the phenomena of emission of radiation when there is transition from singlet excited state to singlet ground state. The wavelength of absorbed radiation is called as excitation wavelength and that of emitted radiation is called as emission wavelength. Phosphorescence is a specific type of photo luminescence related to fluorescence. In fluorescence, the molecules of the substance from the ground state excite to singlet state and return back to the ground state. In phosphorescence molecules of the substance in triplet excited state return to ground state. The deuterium lamp was used as a source of light, primary and secondary filter are built in it, quartzs,cuvettes was used for handling the sample come photo multiplier tube was employed as the detector LED (Fluorimeter model LF-2) (QUANTALASE India). The percentage fluorescence intensity was determined. The results were analysed for precision, accuracy, specificity, robustness and ANOVA test was performed. It gave accurate results in microgram concentration in solution.

Keywords: *Flourescence, phosphorescence, deuterium lamp, photomultiplier tube, ANOVA,*

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INTRODUCTION:

It is an analytic method for detecting and measuring flourescence in compounds that uses ultraviolet light stimulating the compounds, causing them to emit visible light. The energy/light emitted by the substance has a longer wavelength than absorbed energy. This process of emitting radiation with a longer wavelength than absorbed energy is known as luminescence (cold light)

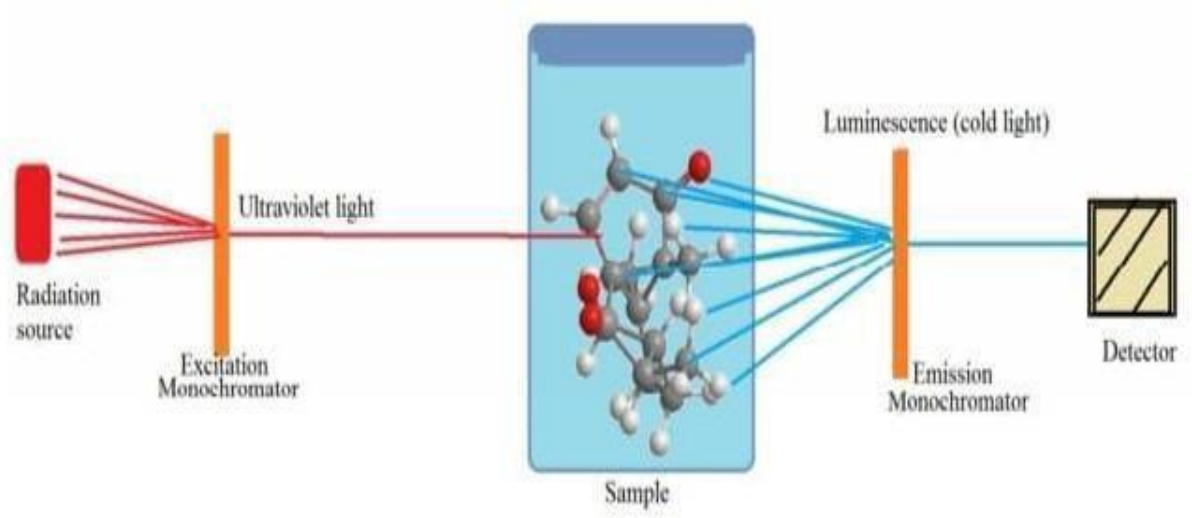


Figure No: 1 UV-Visible spectroscopy

Light consists of electromagnetic radiation of different wavelengths. Therefore, when the elements or their compounds are heated either on a flame or by an electric arc they emit energy in the form of light. Analysis of this light, with the help of a spectroscope gives us a discontinuous spectrum. A spectroscope or a spectrometer is an instrument which is used for separating the components of light, which have different wavelengths. The spectrum appears in a series of lines called the line spectrum. This line spectrum is called an atomic spectrum when it originates from an atom in elemental form. Each element has a different atomic spectrum. The production of line spectra by the atoms of an element indicate that an atom can radiate only a certain amount of energy. This leads to the conclusion that bound electrons cannot have just any amount of energy but only a certain amount of energy.

Emission spectroscopy is a spectroscopic technique which examines the wavelengths of photons emitted by atoms or molecules during their transition from an excited state to lower energy state.

THE MECHANISM OF PHOSPHORESCENCE:

As phosphorescing molecules can luminesce for a much longer time than fluorochromes, there must be a difference in the way they store the excitation energy. The basis for this discrepancy is found in the two forms of excitation levels, the singlet excited state and the triplet excited state, which are based on different spin alignments.

FLOURIMETRY:

It is measurement of fluorescence intensity at a particular Wavelength with the help of a filter Fluorimetry or a spectro fluorimeter

FLOURESCENCE:

Flourescence is a phenomenon of emission of radiation When the molecules are exited by radiation at certain Wavelength.

PHOSPHORESCENCE:

Phosphorescence is a specific type of photo luminescence related to fluorescence. Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs. The slower time scales of the re-emission are associated with "forbidden" energy state transitions in quantum mechanics. As these transitions occur very slowly in certain materials, absorbed radiation may be re-emitted at a lower intensity for up to several hours after the original excitation.

THE MECHANISM OF FLOURESCENCE:

Fluorochromes will only flourescence if they are illuminated with light of the corresponding wavelength. The wavelength depends on the absorption spectrum of the fluorophore and it has to be

ensured that an appropriate quantity of energy is delivered to elevate the electrons to the excited state. After the electrons are excited they can dwell in this high energy state for a very short time only. When the electrons relax to their ground state or another state with a lower energy level, energy is released as a photon. As some of the energy is lost during this process, light with an increased wavelength and lower energy is emitted by the fluorochrome compared to the absorbed light.
F spectroscopy provides two types of spectrum.

(1) Excitation or absorption spectrum &

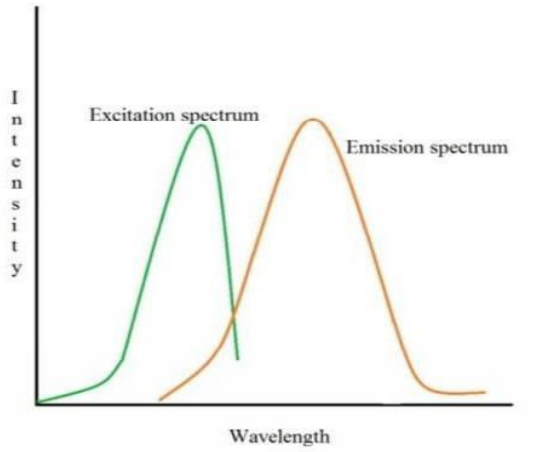
(2) Emission spectrum.

1. EXCITATION OR ABSORPTION SPECTRUM:

In an excitation spectrum, the emission monochromator is set to some Wavelength Where the sample is known to emit radiation and the excitation monochromator is scanned through the different Wavelengths.

2. EMISSION SPECTRUM:

The Emission Spectrum of a chemical element or chemical compound is the spectrum of frequencies of



electromagnetic radiation emitted due to electrons making a transition from high energy state to lower energy state.

Molecule contains σelectrons,∏ electrons and nonbonding (n) electron. The electrons may be present in bonding molecular orbital. It is called as highest occupied molecular orbital (HOMO). It has least energy and more stable. When the molecules absorb radiant energy from a light source, the bonding electrons may be promoted to anti bonding molecular orbital (LUMO). It has more energy and hence less stable.

HOMO Stands for "Highest occupied molecular orbital".

LUMO Stands for "Least unoccupied molecular orbital".

PRINCIPLE

Flourescence is the phenomena of emission of radiation when there is transition from ↑singlet excited state to singlet ground state. The wavelength of absorbed radiation is called as excitation wavelength and that of emitted radiation is called as emission wavelength. These two wavelengths are specific or characteristic for a given substance under ideal conditions.
Before understanding the principle, it is important to understand some electronic states.

1. SINGLET GROUND STATE:

A state in which all the electrons in a molecule are paired.

2. DOUBLET STATE:

A state in which an unpaired electron is present (eg) free radical .

3. TRIPLET STATE:

A state in which unpaired electrons of same spin present. (unpaired and same spin)

4. SINGLET EXCITED STATE:

A state in which electrons are unpaired but of opposite spin like (unpaired and opposite spin).
Absorption of uv/visible radiation causes transition from singlet ground state to singlet excited state. As this excited state is not stable, it emits the excess energy and returns back to ground state. To achieve this transition there are 3 possibilities**:**

1. COLLISIONAL DEACTIVATION:

It is a process which the entire energy is lost due to collisional deactivation and no radiation is emitted.

2. FLOURESCENCE:

A part of energy is lost due to vibrational transitions and the remaining energy is emitted as uv/ visible radiation of longer wavelength than the incident light. This is because the energy of emitted radiation is lesser than that of incident or absorbed radiation, because a part of energy is lost due to vibrational(collisional process) Hence the emitted radiation has longer wavelength than the absorbed radiation.
The different electronic transitions, energy level, states and time delay are mentioned in the following diagram.

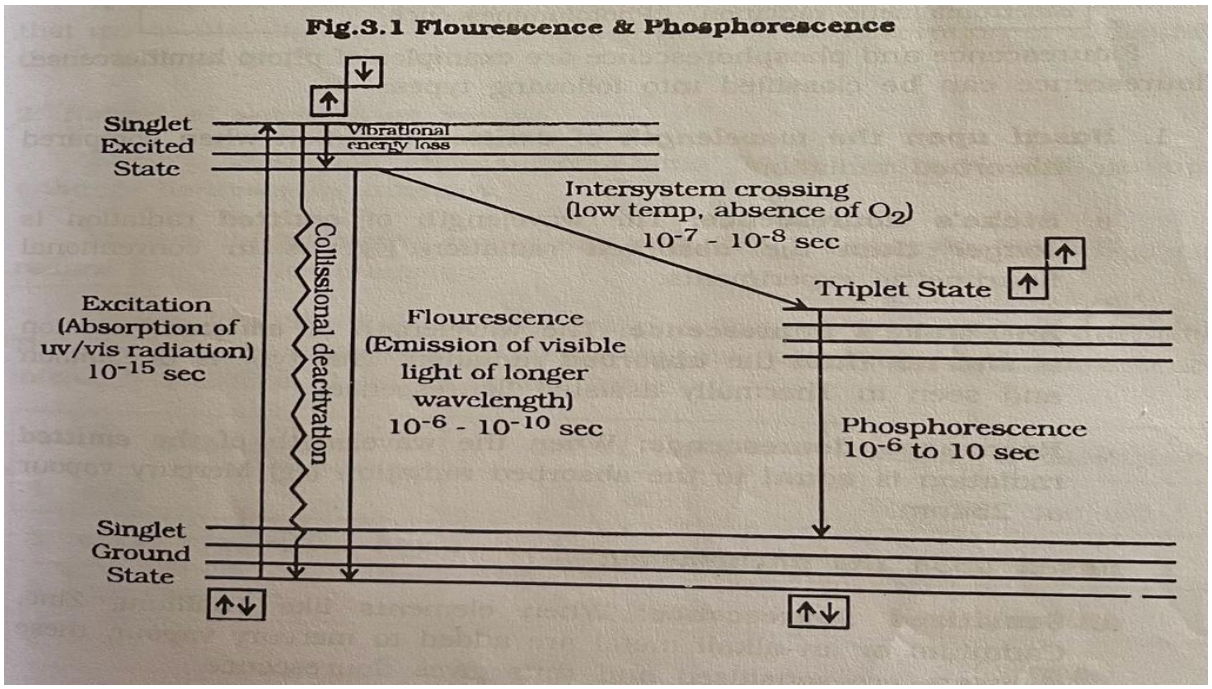


Figure No 2: Fluorescence and phosphorescence

TYPES OF FLOURESCENCE:

Luminescence is the phenomenon of emission of light radiation by substance, when excitation occurs in any form.

When excitation is by	**The phenomenon is called as**
Chemicals	Chemiluminescence
Electrochemical reactions	Electrochemiluminescence
Electromagnetic radiation	Photoluminescence

Flourescence and phosphorescence are examples of photoluminescence. flourescence can be classified into following types.

1. Based upon the wavelength of emitted radiation when compared to absorbed radiation.

a. STOKES FLOURESCENCE:
The wavelength of emitted radiation is longer than the absorbed radiation. [eg] as in conventional flourimetric experiments.

b. ANTI-STOKES FLOURESCENCE:
The wavelength of emitted radiation is shorter than the absorbed radiation . this type is uncommon and seen in thermally assisted flourescence.

c. RESONANCE FLOURESCENCE:
When the wavelength of the emitted radiation is equal to the absorbed radiation. [eg] mercury vapour at 254 nm

1. **UPON THE PHENOMENON**:

A. SENSITIZED FLOURESCENCE:
When elements like thallium , zinc , cadmium or an alkali metal are added to mercury vapour , these elements are sensitized and thus gives flourescence .

B. DIRECT LINE FLOURESCENCE :
Where , even after the emission of radiation , the molecules in metastable state and finally comes to ground state after loss of energy by vibration transition .

C. STEP WISE FLOURESCENCE:
These is nothing but the conventional type of flourescence , where a part of energy is lost by vibrational transition before the emission flourescent radiation.

D. THERMALLY ASSISTED FLOURESCENCE :

The excitation is partly by electromagnetic radiation and partly by thermal energy.

ELECTRONIC STATES:

Understanding the difference between flourescence and phosphorescence requires the knowledge of electron spin and the differences between singlet and triplet states. According to the Pauli Exclusion Principle, two electrons in an atom cannot have the same four quantum numbers {Principal (n), Azimuthal (ℓ), Magnetic (mℓ), and Spin quantum number (s)}. Only two electrons can occupy each orbital where they must have opposite spin states. These opposite spin states are called spin p airing.

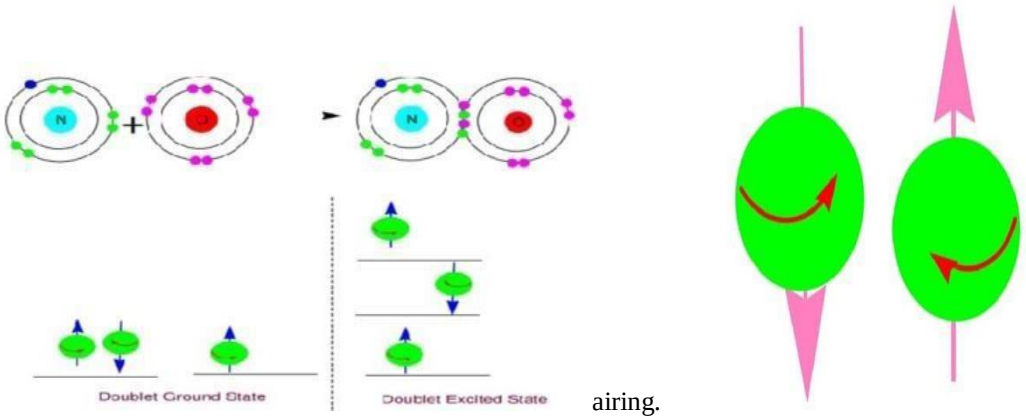


Figure No 3: Electronic States

Because of this spin pairing, most molecules do not exhibit a magnetic field and are diamagnetic.

In diamagnetic molecules, electrons are not attracted or repelled by the static electric field. Free radicals are paramagnetic because they contain unpaired electrons that have magnetic moments that are attracted to the magnetic field.

1. SINGLET STATE:

When all the electron spins are paired in the molecular electronic state and the electronic energy levels do not split when the molecule is exposed to UV radiation. If there is n number of unpaired electrons, it means that (n+1) fold degeneracy (equal energy state) will be associated with the electron spin, regardless of the molecular orbital occupied. Thus if no unpaired electrons are present (n=0).

According to the formula: n+1, 0+1 = 1 spin state (singlet state)

2. DOUBLET STATE:

A doublet state occurs when there is an unpaired electron that gives two possible orientations when exposed to UV radiation and imparts different energy to the system.

TRIPLET STATE:

A singlet or a triplet can form when one electron is excited to a higher energy level. In an excited singlet state, the electron is promoted in the same spin orientation as it was in the ground state (paired).

In a triplet, excited stated, the electron that is promoted as the same spin orientation (parallel) to the other unpaired electron.

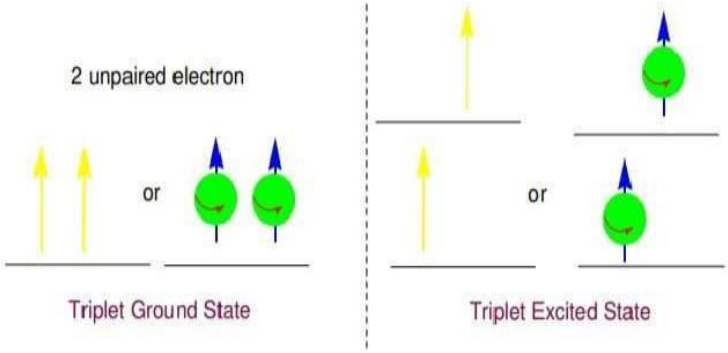


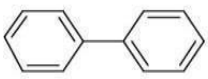
Figure No 4: Triplet State

Singlet, doublet, and triplet is derived using the equation for multiplicity, 2S+1, Where,
S is the total spin angular momentum (sum of all the electron spins). Individual spins are denoted as spin up (s = +1/2) or spin down (s = -1/2). If we were to calculate the S for the excited singlet state, the equation would be 2(+1/2 + -1/2) +1 = 2(0) +1 = 1, therefore making the center orbital in the figure a singlet state. If the spin multiplicity for the excited triplet state was calculated, we obtain 2(+1/2 + +1/2)+1 = 2(1)+1 =3, which gives a triplet state as expected.

- **FACTORS AFFECTING FLOURESCENCE**

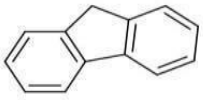
1. Effect of structural Nature
2. Effect of Solvent Nature
3. Effect of Substitution
4. Effect of Temperature
5. Effect of Dissolved Oxygen

1. Effect of Concentration EFFECT OF STRUCTURAL NATURE:
The nature of the chemical structure of a molecule in terms of flexibility and rigidity is of major influence on the fluorescence and phosphorescence signal. Molecules that have a high degree of flexibility will tend to decrease fluorescence due to higher collisional probability. However, more rigid structures have a lower probability of collisions and thus have more fluorescence potential.
For example, Biphenyl has very low flourescence quantum efficiency due to the flexible nature of the



1,1'-biphenyl

Flexible



9H-fluorene

Rigid

molecule while fluorine has high flourescence quantum efficiency due to its rigidity.

2. EFFECT OF SOLVENT NATURE:
Solvents affect the luminescent behaviour of molecules. There are three common effects can be recognized :

(1) **THE POLARITY OF SOLVENT** :
A polar solvent is preferred as the energy required for the P ---> P* is lowered.

(2) **THE VISCOSITY OF SOLVENT**:
Highly viscous solvent is preferred since collisional deactivation will be lowered at higher viscosities.

(3) HEAVY ATOMS IN SOLVENT :
If solvents contain heavy atoms, flourescence quantum efficiency will decrease and phosphorescence will increase.

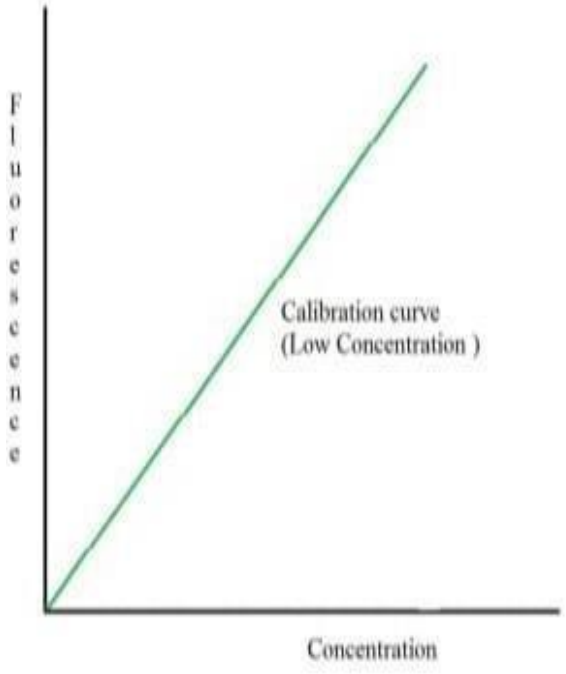
EFFECT OF SUBSTITUTION:
Substitution in the structure can also affect the fluorescence.

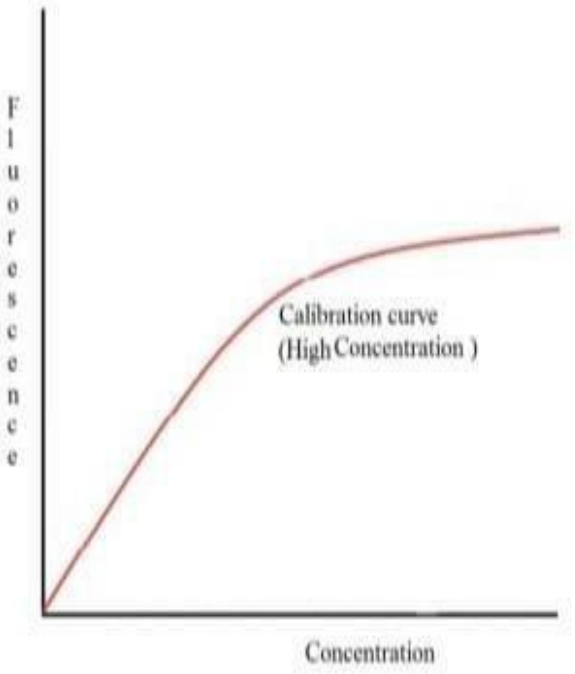
Groups increase the flourescence intensity	OH , Ome ,OEt , CN ,NH2 ,NR2 ,NO , NO2
Groups decrease flourescence intensity	COOH ,CHO ,COR ,COOR ,SH , F ,Cl ,Br , I
Groups having no effect on flourescence intensity	SO3 H ,NH4 + , Alkyl grp

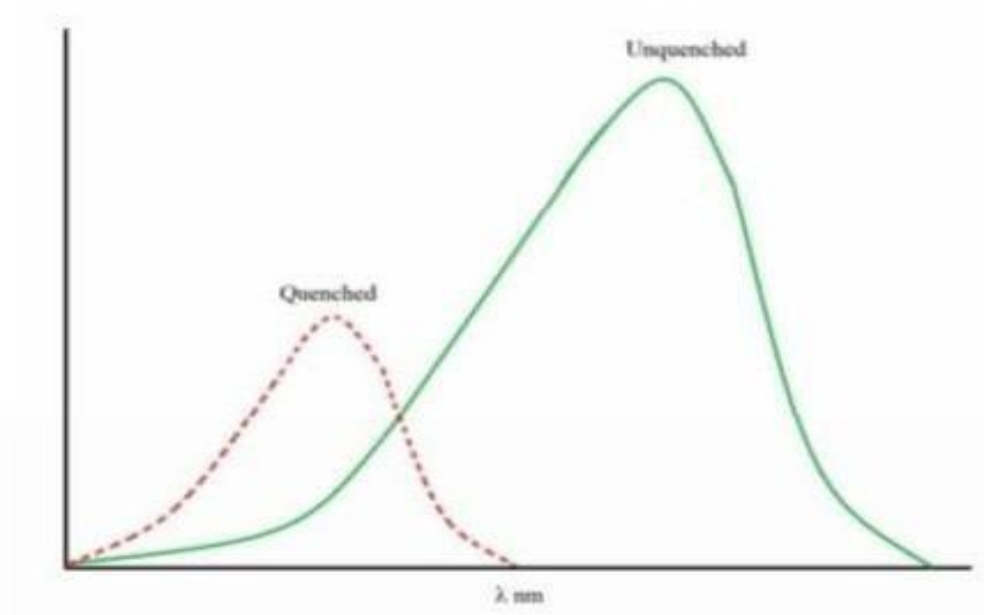
3. EFFECT OF TEMPERATURE:
Molecule experiences larger collisional deactivation at high temperatures due to an increase in the movement and velocity of molecules. Therefore, lower temperatures are preferred for analysis.

4. EFFECT OF DISSOLVED OXYGEN:
Dissolved oxygen affects flourescence at large scale. Molecules experience intersystem crossing due to it is paramagnetic nature.

- **EFFECT OF CONCENTRATION:** The flourescence is directly proportional to the amount of absorbed radiation. When the concentration of the flourescent molecules increases in a sample solution, the flourescence intensity is reduced.
- **QUENCHING:** It refers to any process that decreases the flourescence intensity of a sample. A variety of molecular interactions can result in quenching. Like – molecular rearrangement, Static quenching, and collisional quenching, etc.


Fluorescence
Calibration curve
(Low Concentration)
Concentration


Fluorescence
Calibration curve
(High Concentration)
Concentration


Unquenched
Quenched
λ nm

TYPES OF QUENCHING:

1. Collisional Quenching
2. Static Quenching
3. Concentration Quenching
4. Chemical Quenching

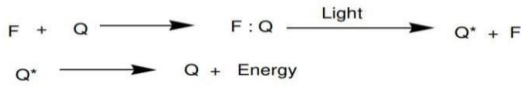
1. COLLISIONAL QUENCHING:

Collisional quenching occurs when the excited fluorophore experiences contact with an atom or molecule that can facilitate non-radiative transitions to the ground state. Common quenchers include O2, I-, Cs+, and acrylamide.

For example, quenching of quinine drug by chloride ion and quenching of tryptophan by iodide ion.

2. STATIC QUENCHING :

Static quenching occurs at the ground state of the flourescent molecule. It can be simplified by the following mechanism.



Here, a complex formation occurs between the flourescing molecule at the ground state (F) and the quencher molecule (Q) through a strong coupling. Such complex may not undergo excitation or, may be excited to a little extent reducing the fluorescence intensity of the molecule.

For example, Caffeine and related xanthines and purines reduce the intensity of riboflavin by the static mechanism.

3. CONCENTRATION QUENCHING:

Concentration quenching is a kind of self-quenching. It occurs when the concentration of the flourescing molecule increases in a sample solution. The flourescence intensity is reduced in a highly concentrated solution (>50 µg/ml).

4. CHEMICAL QUENCHING:

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

- **CHANGE IN PH :**

Aniline at pH (5-13) gives flourescence when excited at 290 nm. But pH 13 does not show any flourescence.

- **OXYGEN MOLECULES:**

Oxygen leads to the oxidation of flourescent substance to non-flourescent substance and thus, causes quenching.

- **HALIDES AND ELECTRON-WITHDRAWING GROUPS:**

Halides like chloride ions, iodide ions, and electron-withdrawing groups like -NO, -COOH, - CHO groups lead to quenching.

- **HEAVY METALS:**

The presence of heavy metals also lead to quenching because of collision and complex formation.

INSTRUMENTATION

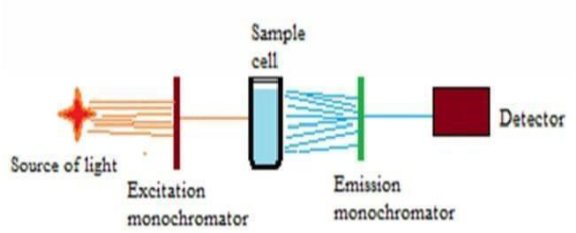
Figure No 5: Instrumentation

1. Source of light
2. Filter and Monochromators
3. Sample Hander or Cells or Cuvettes
4. Detectors

1. SOURCE OF LIGHT :

- Deuterium and Hydrogen lamp

- Xenon arc Lamp
- Tungsten Halogen Lamp&
- Mercury Vapor Lamp



Schematic Diagram of Single-Beam Fluorimeter

1. DEUTERIUM AND HYDROGEN LAMP:

A pair of electrodes is enclosed in a glass tube containing hydrogen or deuterium gas. When current is passed in electrodes electron discharge is occurring which exited the gas molecule which results in the emission of radiation (UV & Visible). Wavelength: 160-800 nmQuartz window must be employed

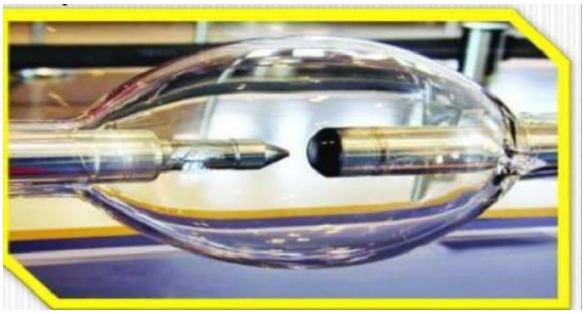


Figure No 6: Source of Light

2. XENON ARC LAMP :

It consists of two tungsten electrodes form an arc at a specific distance and xenon gas is stored (under pressure) in quartz or fused silica tube. It emits radiation with a higher intensity (500 nm) than a hydrogen discharge lamp.

Wavelength: 750-1000 nm.

3.TUNGSTEN HALOGEN LAMP :

It is also known as a halogen lamp. It is an incandescent light source. It is consists of a filament made up of tungsten enclosed in a quartz vessel containing an inert gas and a small quantity of Iodine or bromine (Halogen).

Its 85% emitted light lies in IR and near IR region, 15 % in the visible region, and less than 1% in the UV region.

FILTERS AND MONOCHROMATORS:

- ❖ **FILTERS**
- Primary filters
- Secondary filters
- ❖ **MONOCHROMATORS**
- Excitation monochromators

2.FILTERS:

Filter is a device used to get selected wavelength. It allows the light pass through it but absorbed the light of different wavelength may partially and fully. A specific filter is used to obtain the desired wavelength for special analysis like Primary filter and Secondary filter.

1. PRIMARY FILTERS:

A Primary Filter absorbs visible radiation and transmit UV radiation.

2. SECONDARY FILTERS:

A Secondary Filter absorbs UV radiation and transmit visible radiation

Figure No 7: Filters

3. MONOCHROMATORS:

They convert polychromatic light into monochromatic light. They can isolate a specific range of wavelength or a particular wavelength of radiation from the source.

EXCITATION MONOCHROMATOR

- Excitation monochromators provides suitable radiation for excitation of molecules.

- **EMISSION MONOCHROMATORS:**
- Emission monochromators isolate only the radiation emitted by the fluorescent molecules.

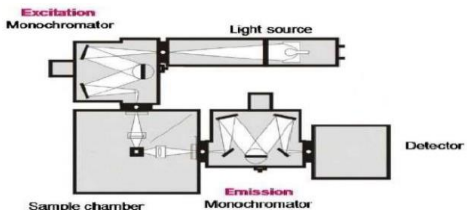


Figure No 8: Monochromator

Figure No 9: Cuvettes

1. SAMPLE HANDER OR CELLS OR CUVETTES:

Cuvettes are used for the handling of samples. These are made up of quartz and can have various shapes. Ex: Cylindrical or Rectangular etc.

Cuvettes are fabricated of silica or glass. They are meant for holding the diluted samples. Path length is about 10mm or 1 cm . Cells and other glassware used for fluorimetric analysis should be carefully cleaned, preferably by boiling in 50% nitric acid followed by thorough rinsing in Dectectors:

Detector is a device which transforms light energy into electrical signals that are observed on recorder. The characteristics of ideal detector is give quantitative response, high sensitivity, low noise, short response time, and response quantitative to wide spectrum of radiation received.

Some commonly used detectors are as follows :

- ❖ Barrier layer cell/Photovoltaic cells
- ❖ Phototubes/ Photo emissive tubes
- ❖ Photomultiplier tubes

1. BARRIER LAYER CELL/PHOTOVOLTAIC CELLS :

It is consist of a coated silver or gold thin layer of metallic film which acts as an electrode and another metal plate acts as another electrode. Both of the layers are separated by selenium layer that act as a semiconductor. When UV radiation falls on selenium layer, an electron become mobile and is taken up by transparent metal layer that results a potential difference between the electrodes & causes the flow of current. When it is connected to galvanometer, a flow of current observed which is proportional to the intensity and wavelength of light falling on it.

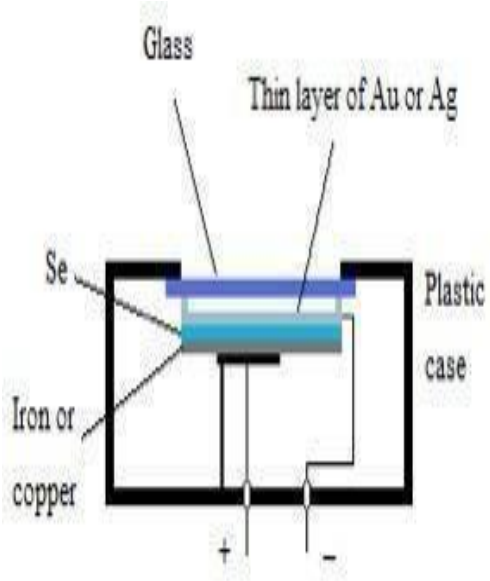


Figure No 10: Barrier Layer Cell

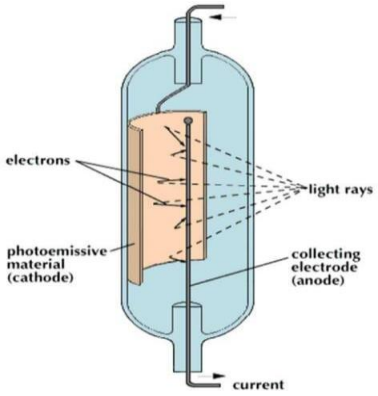


Figure No 11: Photo Multiplier Tube

The most common types are:

1. **SINGLE BEAM (FILTER) FLUORIMETERS**
2. **DOUBLE BEAM (FILTER) FLUORIMETERS**
3. **SPECTROFLUORIMETER (DOUBLE BEAM)**

1. SINGLE BEAM (FILTER) FLUORIMETERS :

It contains tungsten lamp as a source of light and has an optical system consists of primary filter.

- The emitted radiations is measured at 900 by using a secondary filter and detector. Primary filter absorbs visible radiation and transmit uv radiation which excites the molecule present in sample cell.
- In stead of 90 if we use 180 geometry as in colorimetry secondary filter has to be highly efficient otherwise both the unabsorbed uv radiation and fluorescent radiation will produce detector response and give false result.
- Single beam instruments are simple in construction cheaper and easy to operate.

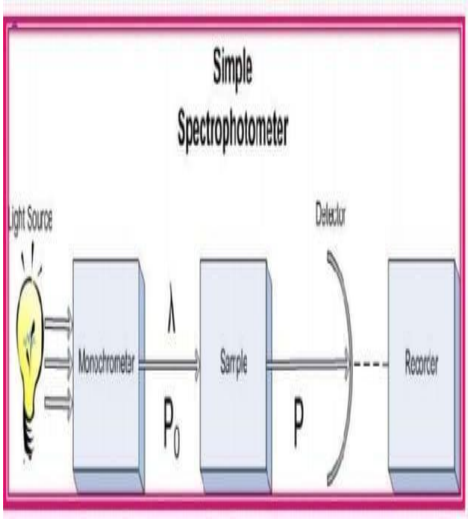


Figure No 12: Single Beam Fluorimeter

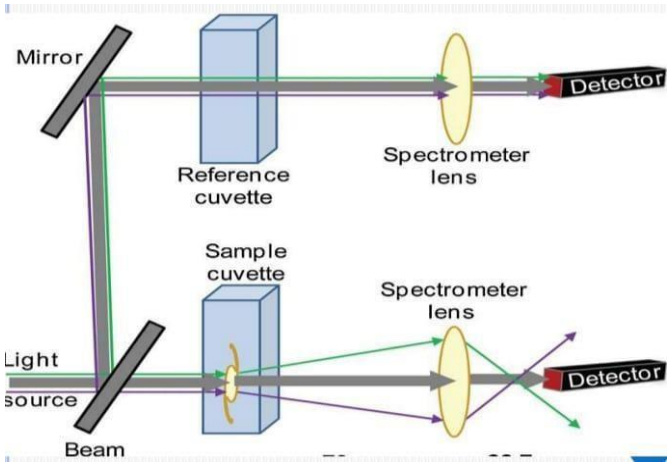


Figure No 13: Double Beam Fluorimeter

2. DOUBLE BEAM (FILTER) FLUORIMETERS:

It is similar to single beam except that the two incident beams from a single light source pass through primary filters separately and fall on the another reference solution. Then the emitted radiations from the sample or reference sample pass separately through secondary filter and produce response combily on a detector.

3. SPECTROFLUORIMETER (DOUBLE BEAM) :

- In this primary filter in double beam fluorimeter is replaced by excitation monochromator and the secondary filter is replaced by emission monochromator.
- Incident beam is split into sample and reference beam by using beam splitter.

APPLICATIONS

✓ **APPLICATIONS IN INORGANIC/ ORGANIC CHEMISTRY:**

- ❖ Determination of ruthenium.
- ❖ 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 .
- ❖ Determination of cadmium.
- ❖ Assay of thiamine .
- ❖ Estimation of quinine sulphate .
- ❖ Estimation of 3,4 benzopyrene.

✓ **OTHER APPLICATIONS:**

- ❖ Quantitative as well as qualitative analysis .
- ❖ Human cancer diagnosis (Laser induced fluorescence spectroscopy) .
- ❖ Study of marine petroleum pollutants .
- ❖ Accurate determination of glucose
- ❖ Flourescence polarization immunoassay of mycotoxins
- ❖ Determination of flourescent drugs in low-dose formulations in the presence of non-flourescent excipients.
- ❖ Determination of impurities where the impurity is fluorescent.
- ❖ Study of the drugs complex formulations.
- ❖ Widely used in bio-analysis for measuring small amounts of drug and for studying drug-protein binding.
- ❖ Estimation of traces of boron in steel by means of the complex formed with benzene.
- ❖ Estimation of calcium by fluorimetry with a calcium solution.

CONCLUSION:

Fluorimetry is a sensitive technique in which trial molecules are excited with a photon source that resulting emission of cold light. The molecule being tested can be affected by concentration, binding, solvent, pH value, structure type, and quenching effect. The chief applications of this technique are determination and study of organic and inorganic

compounds, immunoassays, cancer cell diagnosis, Study of pollutants and drugs complex formulations etc. However, there are many factors that can compromise your data and invalidate your results. We should always be aware of possible sample contamination and signal contamination by stray or scattered light. Emission spectrum collection and blank inspection are essential for all experiments.

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