

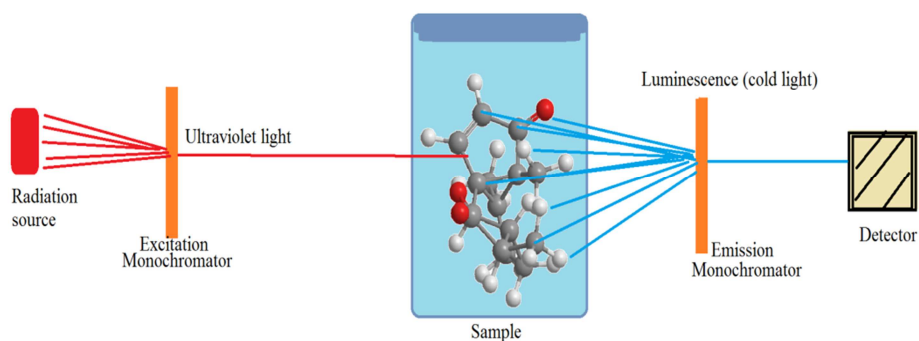


Program Name:	B. Pharm
Course Name:	Instrumental Methods of Analysis
Course Code:	BP 701 T
Semester:	VII th
Session:	2020-2021
Unit:	1 (Part-3)
Topic:	Fluorimetry
Sub-topic:	<ul style="list-style-type: none">• Introduction• Electronic states• Internal and external conversions• Factors affecting fluorescence• Quenching• Instrumentation• Applications

FLUORIMETRY

Introduction

It is an analytic method for detecting and measuring fluorescence 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. This process of emitting radiation with a longer wavelength than absorbed is known as luminescence (cold light).



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

Fluorescence

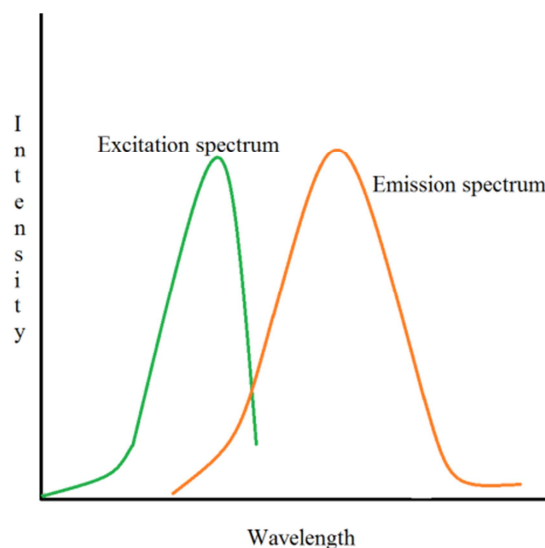
Fluorescence is a type of luminescence caused by photons exciting a molecule, raising it to an electronic excited state. It is an optical phenomenon in which the molecular absorption of energy in the form of photons triggers the emission of fluorescent photons with a longer wavelength.

Phosphorescence

Phosphorescence is a specific type of photoluminescence 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 fluorescence- Fluorochromes will only fluoresce 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

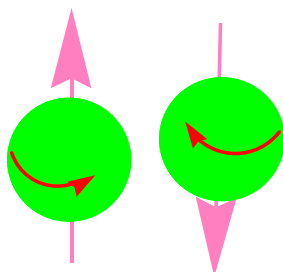


❖ ELECTRONIC STATES

Understanding the difference between fluorescence and phosphorescence requires the knowledge of electron spin and the differences between singlet and triplet states.

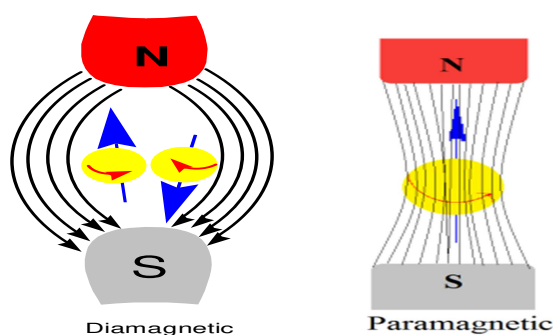
According to the Pauli Exclusion Principle, two electrons in an atom cannot have the same four quantum numbers {Principal (n), Azimuthal (ℓ), Magnetic (m_ℓ), and Spin quantum number (s)}.

Only two electrons can occupy each orbital where they must have opposite spin states. These opposite spin states are called spin pairing.



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.



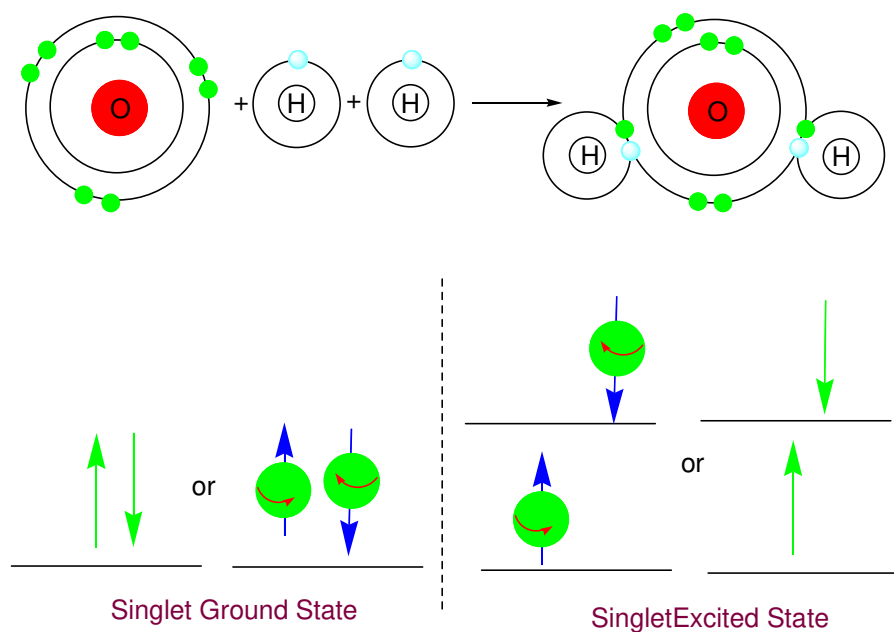
❖ Singlet state

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 ($n=0$),

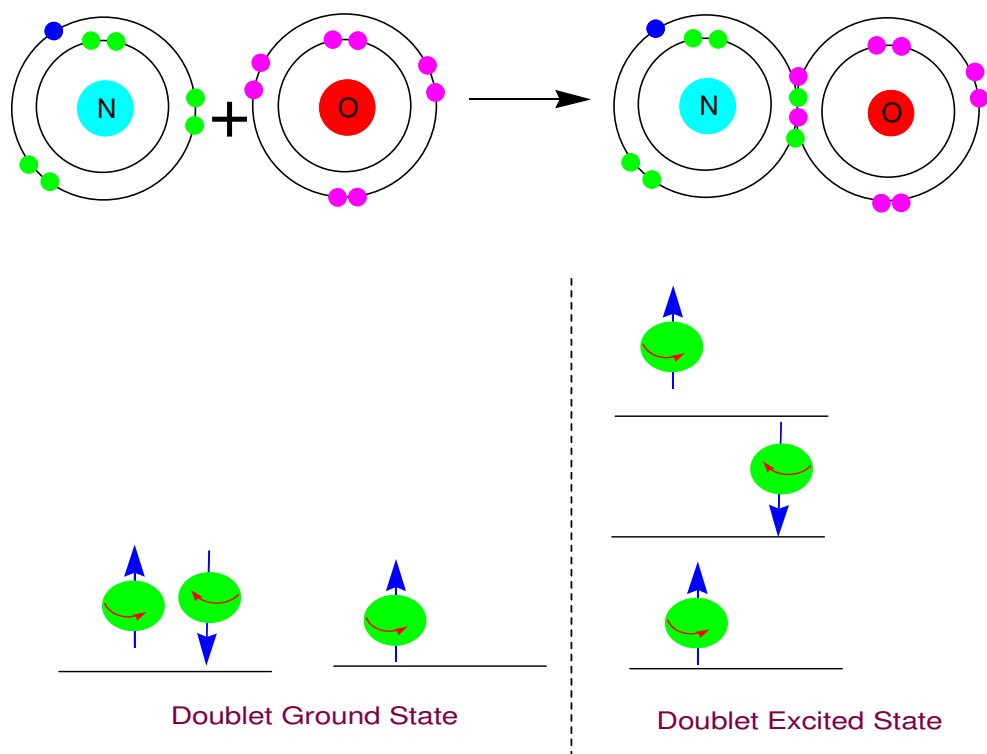
According to the formula: $n+1$,

$$0+1 = 1 \text{ spin state (singlet state)}$$



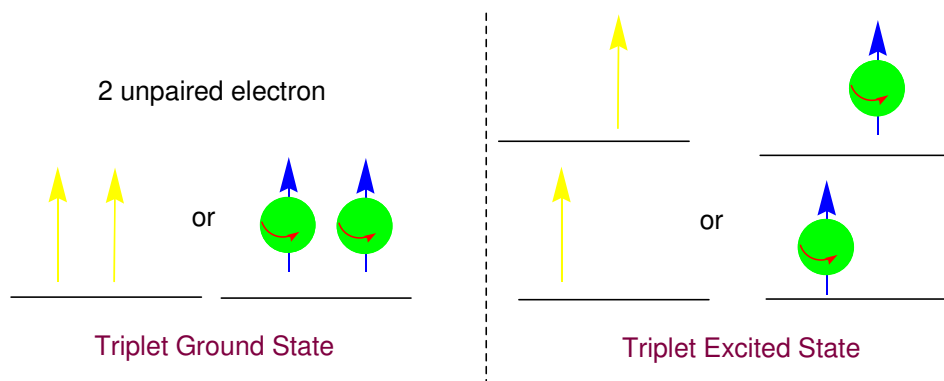
❖ Doublet state

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.



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 state, the electron that is promoted has the same spin orientation (parallel) to the other unpaired electron.



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 ($s = -1/2$).

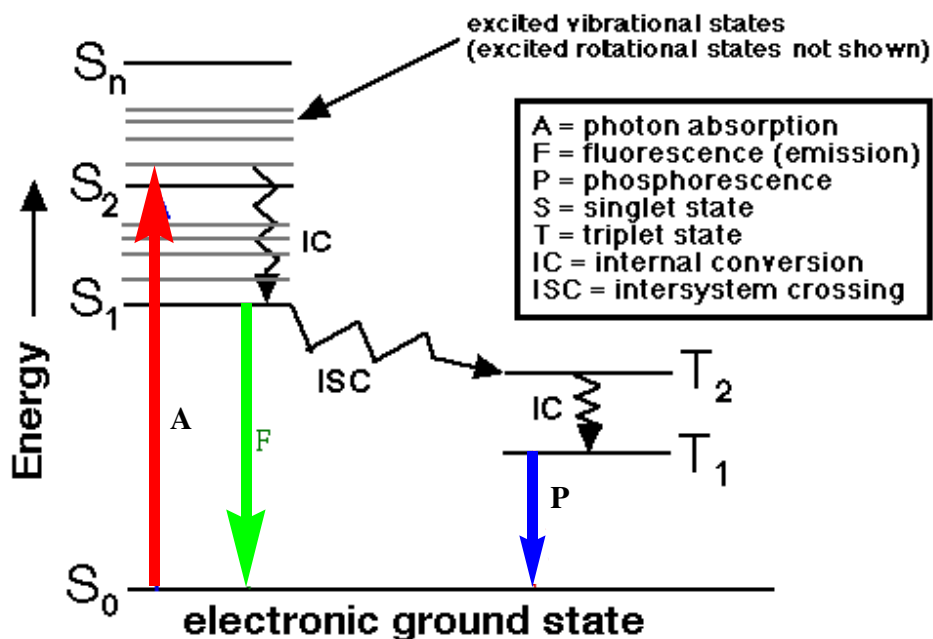
If we were to calculate the S for the excited singlet state, the equation would be $2(+1/2 + -1/2) + 1 = 2(0) + 1 = 1$, therefore making the center orbital in the figure a singlet state.

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.

The difference between a molecule in the ground and the excited state is that the electrons are diamagnetic in the ground state and paramagnetic in the triplet state. This difference in the spin state makes the transition from singlet to a triplet (or triplet to singlet) more improbable than the singlet-to-singlet transitions. This singlet to triplet (or reverse) transition involves a change in the electronic state. Due to that, the lifetime of the triplet state is longer the singlet state by approximately 10 seconds fold difference.

The radiation that induced the transition from ground to excited triplet state has a low probability of occurring, thus their absorption bands are less intense than singlet-singlet state absorption. The excited triplet state can be populated from the excited singlet state of certain molecules which results in phosphorescence.

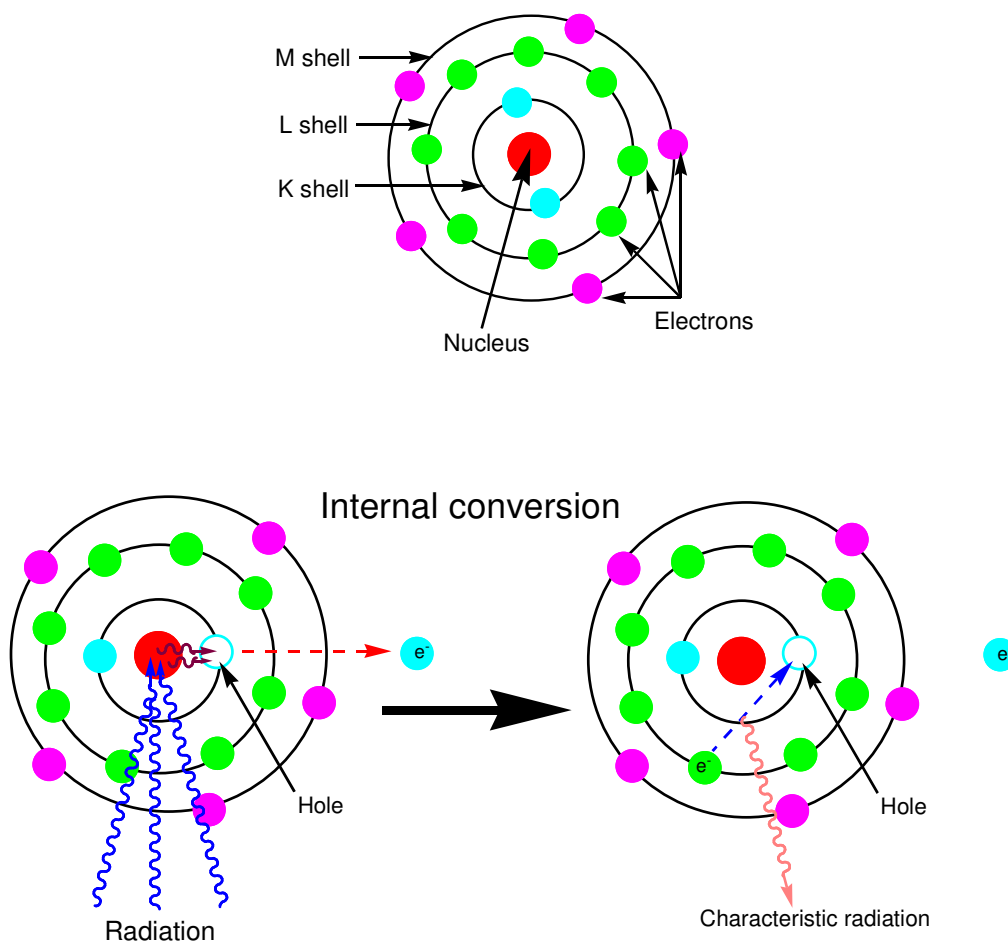
These spin multiplicities in the ground and excited states can be used to explain the transition in photoluminescence molecules by the Jablonski diagram.



Once a molecule has absorbed energy in the form of electromagnetic radiation (longer wavelength, that is upward-pointing, red arrow, $S_0 \rightarrow S_1, S_2, \dots, S_n$), there are a number of routes by which it can return to ground state. If the photon emission (short wavelength that is downward-pointing, green arrow) occurs between states of the same spin state ($S_1 \rightarrow S_0$) it is called fluorescence. If the spin state of the initial and final energy levels are different ($T_1 \rightarrow S_0$), the emission is called phosphorescence. In the diagram, this is depicted by a longer wavelength (lower energy) and therefore shorter length blue line. Since fluorescence is statistically much more likely than phosphorescence for most molecules, the lifetimes of fluorescent states are very short and phosphorescence somewhat longer. Three non-radiative deactivation processes are also significant here: internal conversion (IC), intersystem crossing (ISC), and vibrational relaxation.

❖ Internal conversion

It is an intermolecular process by which a molecule passes to a lower energy electronic state without emission of light. Overlap of vibrational energy levels in two electronic energy levels.



❖ External conversion

External conversion is a process in which excited molecules lose their energy due to collisions with other molecules or by transfer of their energy to solvent or other unexcited molecules. Therefore, the external conversion is influenced by temperature, solvent viscosity, as well as solvent composition.

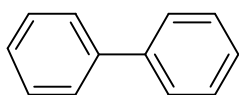
❖ Intersystem crossing

In this process spin of an excited electron is reversed and change in multiplicity results. Most common when vibrational manifold overlap exists and when the molecule has a heavy atom substituent (e.g. Br, I).

Factor affecting fluorescence

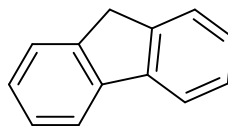
❖ Effect of Structural Nature

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 fluorescence quantum efficiency due to the flexible nature of the molecule while fluorine has high fluorescence quantum efficiency due to its rigidity.



1,1'-biphenyl

Flexible



9H-fluorene

Rigid

❖ Effect of Solvent Nature

Solvents affect the luminescent behavior 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 $P \rightarrow P^*$ 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, fluorescence quantum efficiency will decrease and phosphorescence will increase.

❖ Effect of Substitution

Substitution in the structure can also affect the fluorescence

Groups increase the fluorescence intensity	OH, OMe, OEt, CN, NHR, NH ₂ , NR ₂ , NO, NO ₂
Groups decrease fluorescence intensity	COOH, CHO, COR, COOR, SH, F, Cl, Br, I
Groups having no effect on fluorescence intensity	SO ₃ H, NH ₄ ⁺ , Alkyl grp

❖ Effect of Temperature

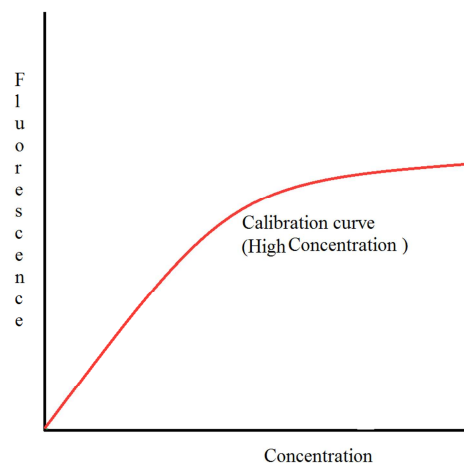
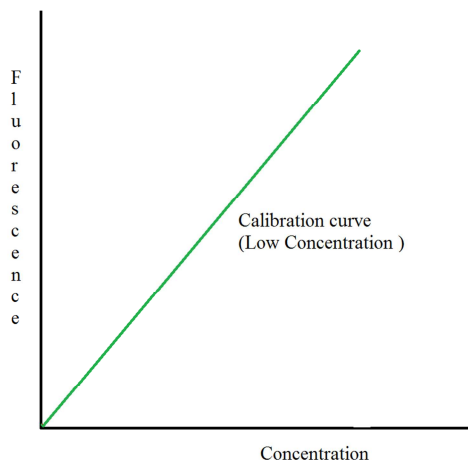
Molecule experiences larger collisional deactivation at high temperatures due to an increase in the movement and velocity of molecules. Therefore, lower temperatures are preferred for analysis.

❖ Effect of Dissolved Oxygen

Dissolved oxygen affects fluorescence at large scale. Molecules experience intersystem crossing due to its paramagnetic nature.

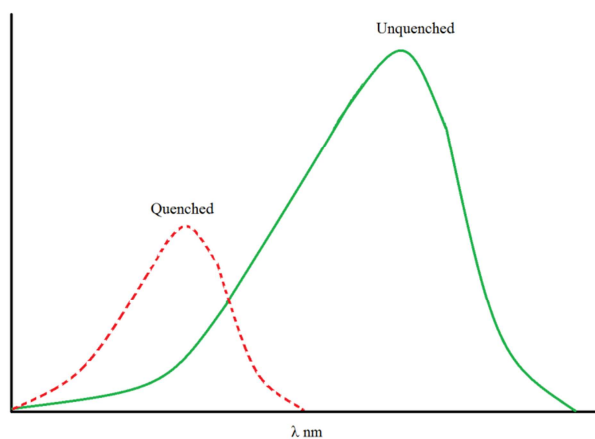
❖ Effect of Concentration

The fluorescence is directly proportional to the amount of absorbed radiation. When the concentration of the fluorescent molecules increases in a sample solution, the fluorescence intensity is reduced.



QUENCHING

It refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching. Like – molecular rearrangement, Static quenching, and collisional quenching, etc.

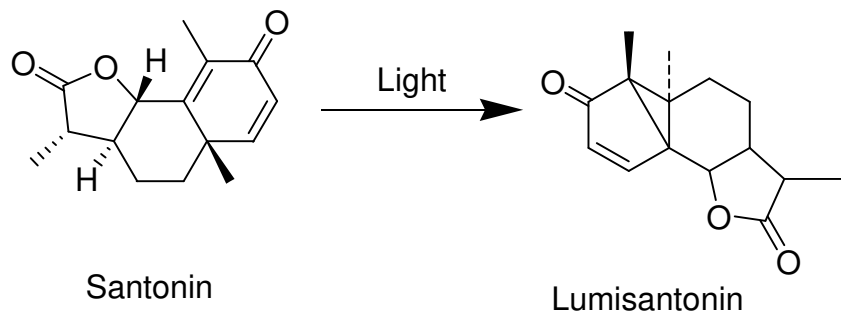


➤ Excited-State Reactions Quenching

Such reactions occur because light absorption frequently changes the electron distribution within a fluorophore, which in turn changes its chemical or physical properties. For example, a neutral solution of phenol can lose the phenolic proton in the excited state.

➤ 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. For example lumisantonin a photoproduct of santonin obtained via molecular rearrangement. The C-3 carbonyl group has moved to C-2, the C-4 methyl has moved to C-1, and the C-10 carbon has been inverted.

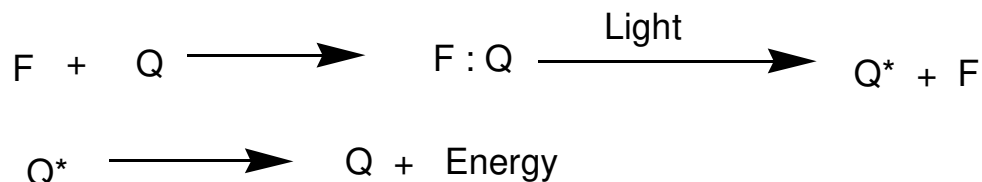


➤ Collisional Quenching

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.

➤ Static quenching

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



Here, a complex formation occurs between the fluorescing 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.

➤ Concentration

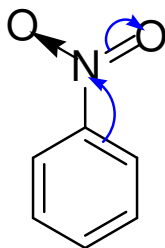
Concentration quenching is a kind of self-quenching. It occurs when the concentration of the fluorescing molecule increases in a sample solution. The fluorescence intensity is reduced in a highly concentrated solution (>50 µg/ml).

➤ 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 fluorescence when excited at 290 nm. But pH <5 or, pH >13 does not show any fluorescence.

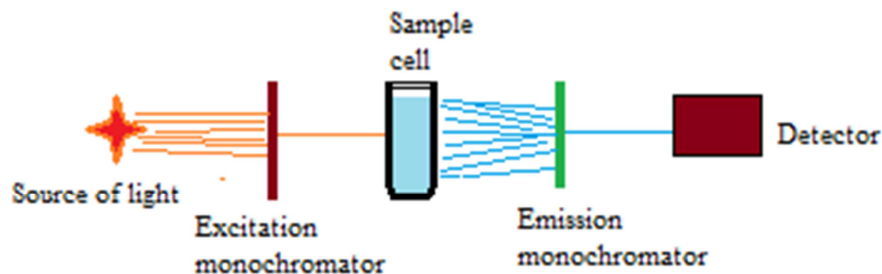
- **Oxygen molecules**- Oxygen leads to the oxidation of fluorescent substance to non-fluorescent substance and thus, causes quenching.
- **Halides and electron-withdrawing groups** - Halides like chloride ions, iodide ions, and electron-withdrawing groups like -NO, -COOH, -CHO groups lead to quenching.



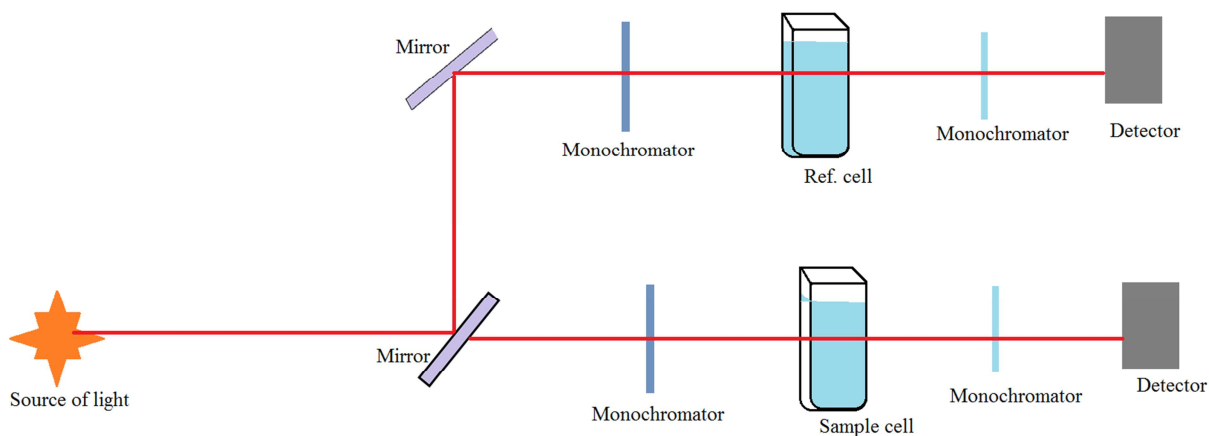
Electron withdrawing process
by *nitro* groups

- **Heavy metals**- the presence of heavy metals also lead to quenching because of collision and complex formation.

Instrumentation



Schematic Diagram of Single-Beam Fluorimeter



Schematic Diagram of Double-Beam Fluorimeter

SOURCE OF LIGHT

Deuterium and Hydrogen lamps

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 excited the gas molecule which results in the emission of radiation (UV & Visible).

Wavelength: 160-800 nm

Quartz window must be employed



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.



Xenon arc Lamp

Tungsten Halogen Lamp

It is also known as a halogen lamp. It is an incandescent light source. It 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.



Tungsten Halogen Lamps

Mercury Vapor Lamp

These lamps are ideal light sources that provide high-intensity light in the deep UV to visible regions. It consists of 2 alloys (tungsten) electrodes which are placed together in a medium containing mercury vapor and 25-50 torr of pure argon gas. These electrodes are enclosed in an elliptically shaped in a silica glass tube. It provides clear white light, high intensity with 24000 Hrs. of life.



Mercury Vapor Lamp

FILTER AND MONOCHROMATOR

Filter

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.

Primary filter:-absorbs visible radiation and transmit UV radiation.

Secondary filter:-absorbs UV radiation and transmit visible radiation.

Monochromator

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 monochromators: Provides suitable radiation for excitation of molecules.

Emission monochromators: Isolate only the radiation emitted by the fluorescent molecules.

SAMPLE HANDER OR CELLS OR CUVETTES

Cuvettes are used for the handling of samples. These are rectangular or cylindrical in shape with two rough and two smooth sides, and made up of glass, quartz or fused silica.



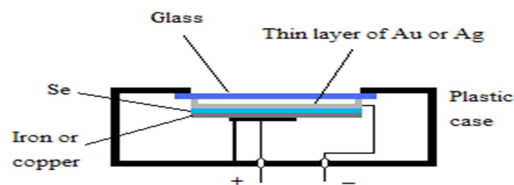
DETECTOR

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

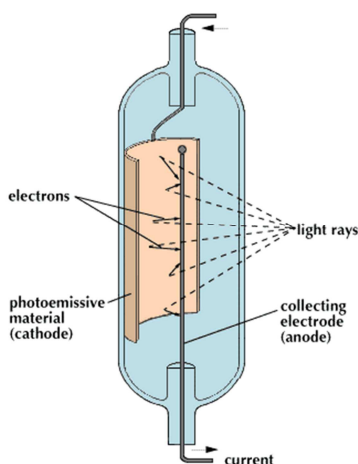
(1) Barrier layer cell/Photovoltaic cell

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.



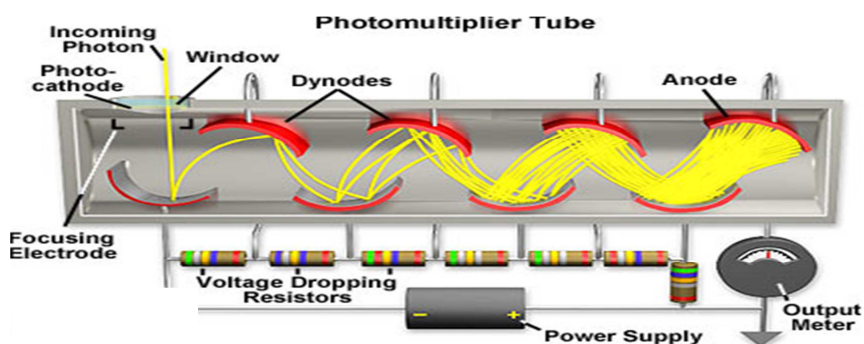
(2) Phototubes/ Photo emissive tube

It consists of an evacuated glass tube with a photocathode and a collector anode. The surface of the photocathode is coated with a layer of elements like cesium, silver oxide and its mixtures. When radiant energy falls on the photosensitive cathode, electrons are emitted which are attracted to the anode, causing a flow of current. It is more sensitive than a barrier layer cell.



(3) Photomultiplier tube

A photomultiplier tube multiplies photoelectrons through the secondary emission of electrons. A primary photo-cathode is fixed in a vacuum tube which receives radiation from the sample. Some 8 to 10 dynodes are fixed, each with an increasing potential of 75-100V higher than the preceding one. Near the last dynode, an electron collector electrode is fixed. It is extremely sensitive to light and can detect weaker or low radiation.



Applications

➤ **Applications in inorganic/ organic chemistry**

- ❖ Determination of ruthenium
- ❖ Determination of aluminum 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 benzpyrene

➤ **Investigation of chemical structures and reactions**

Applied in the investigation of-

- ❖ Hydrogen Bonding
- ❖ Cis and Trans isomerism
- ❖ Polymerization
- ❖ Tautomerism
- ❖ Rates of reactions etc.
- ❖ Free radicles: The free radicles can best be detected with a spectrograph so that the whole spectrum of a short lived component may be photographed at the same time.

➤ **Other Applications**

- ❖ Quantitative as well as qualitative analysis
- ❖ Human cancer diagnosis (Laser induced fluorescence spectroscopy)
- ❖ Study of marine petroleum pollutants
- ❖ Accurate determination of glucose
- ❖ Fluorescence polarization immunoassay of mycotoxins

- ❖ Determination of fluorescent drugs in low-dose formulations in the presence of non-fluorescent 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.

Reference:

- Skoog D.A., A Textbook of Principles of Instrumental Analysis, 6th edition.
- Beckett A.H. & Stenlake J.B., A Textbook of Practical Pharmaceutical Chemistry, volume 2
- Chatwal G. R., A Textbook of Instrumental Methods of Chemical Analysis