Lectrue 7-8

SPECTROFLUOROMETRY

By

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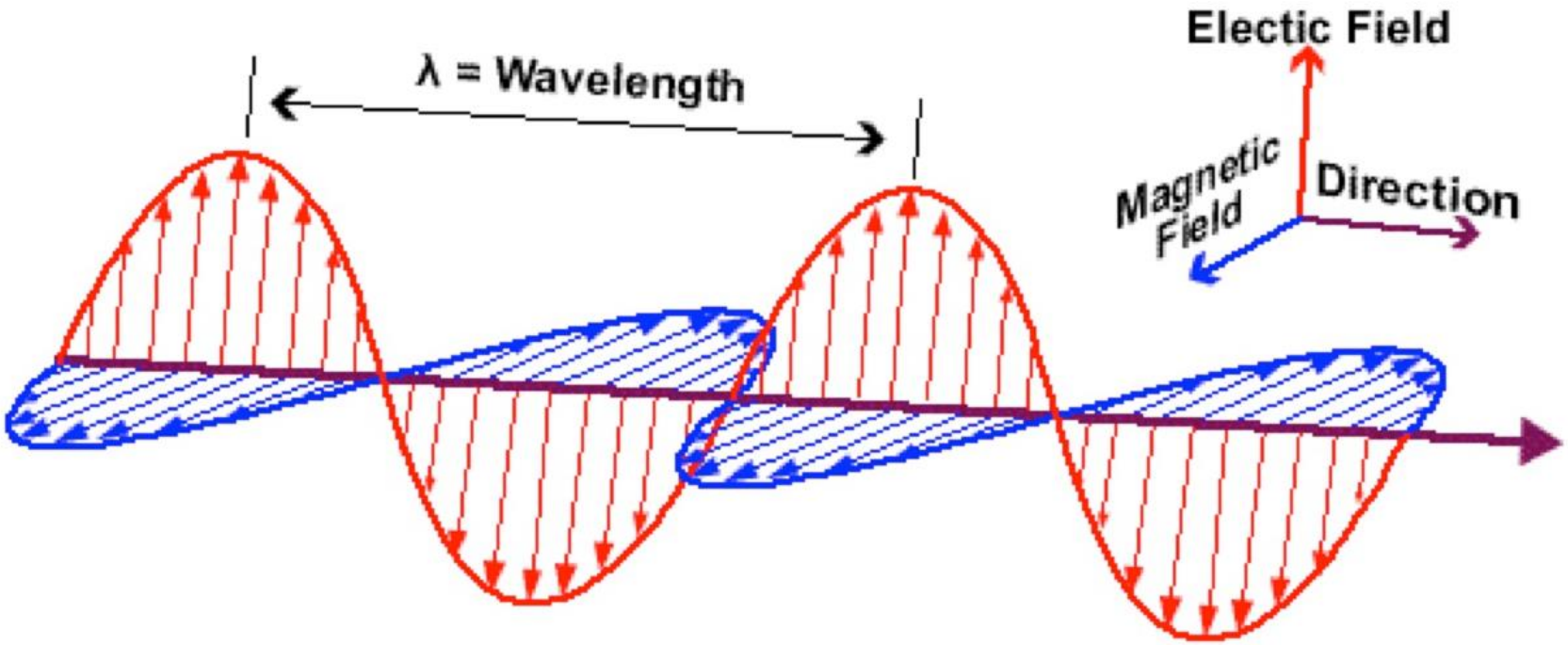
Electromagnetic Radiation

Properties :
as waves



a) wave properties

properties

wavelength ,

wave number ,

frequency ,

amplitude ,

intensity

b) particle

E = h v

E = hC/λ

E = hcʊ,

ABSORPTION OF RADIATION

EMR + matter → Absorption
Transmission
Emission
Reflection
Refraction
Scattering

M + hv → M* (excitation)

M* → M + heat (relaxation)
M + emission of radiation (fluorescence)

- **Absorbance** is the firststep in Fluorescence
- **FLUORESCENCE** is the light emitted by an atom or molecule after a finite duration subsequent to the absorption of electromagnetic energy.
- It is an electronic transition that promotes an electron from the ground state to an unoccupied orbital after absorption of a photon

Energy levels in molecules

$$E_{n,v,r} = E_n + E_v + E_r$$

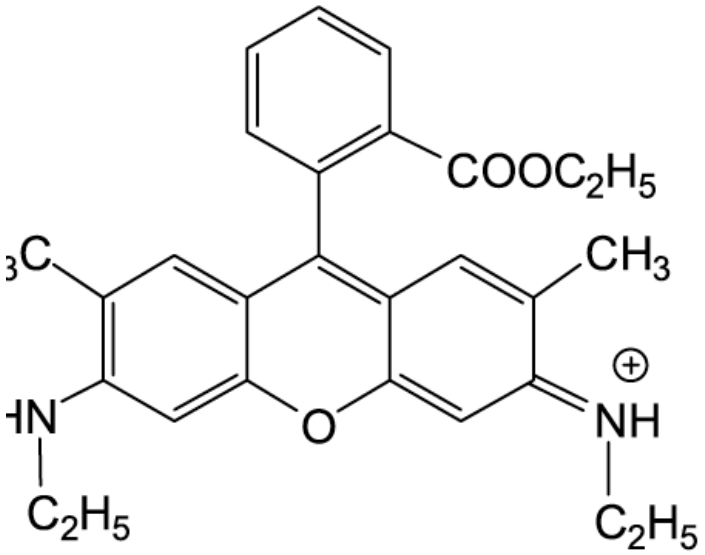
- where E_n *is the electronic energy,* E_v *the vibrational energy,* and E_r *the rotational energy*
- $E_n >> E_v >> E_r$
- E_n *often in the visible range,* E_v *in the IR range,* E_r *in the* microwave range.

Photoluminescence

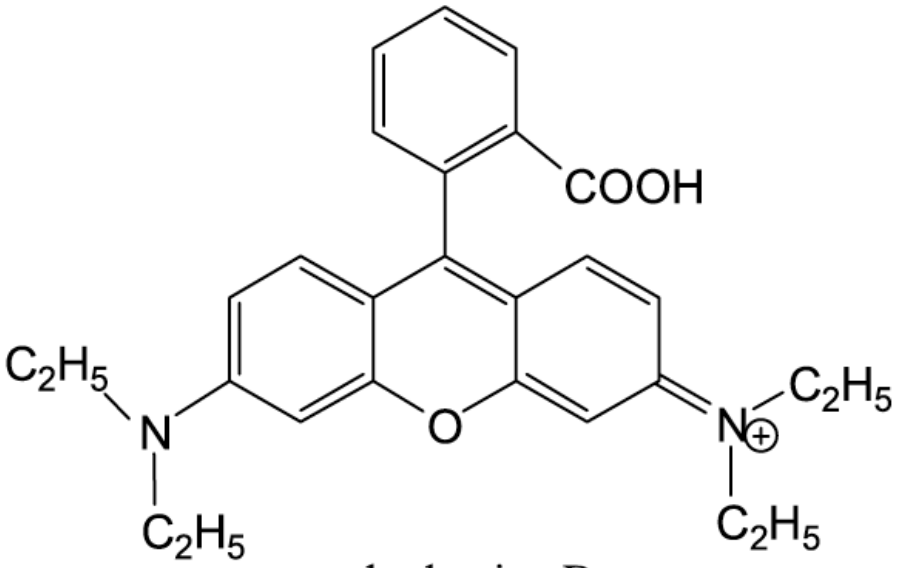
- Process that leads to excited molecules can be *physical (e.g. absorption of light)*, *mechanical (e.g. friction)*, or *chemical (e.g. reactions)*
- When excited molecular states decay back to the ground state, resulting in the emission of light, they are undergoing a *luminescence process*
- Generation of excited molecules by light absorption, that then decay emitting visible light, is *photoluminescence*
- Photoluminescence processes are divided into 2 classes:

  – Fluorescence and Phosphorescence

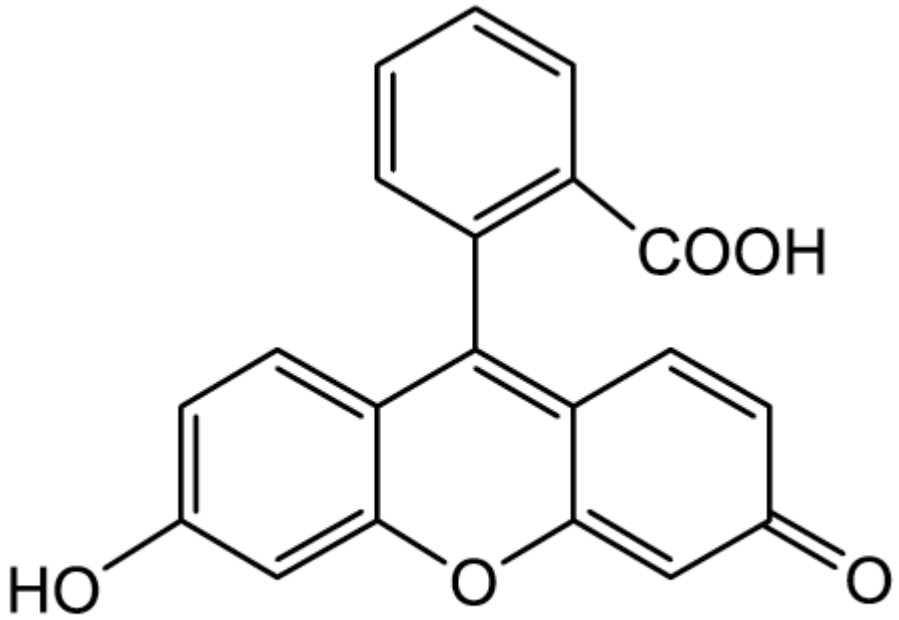
Common Fluorophores



rhodamine 6G



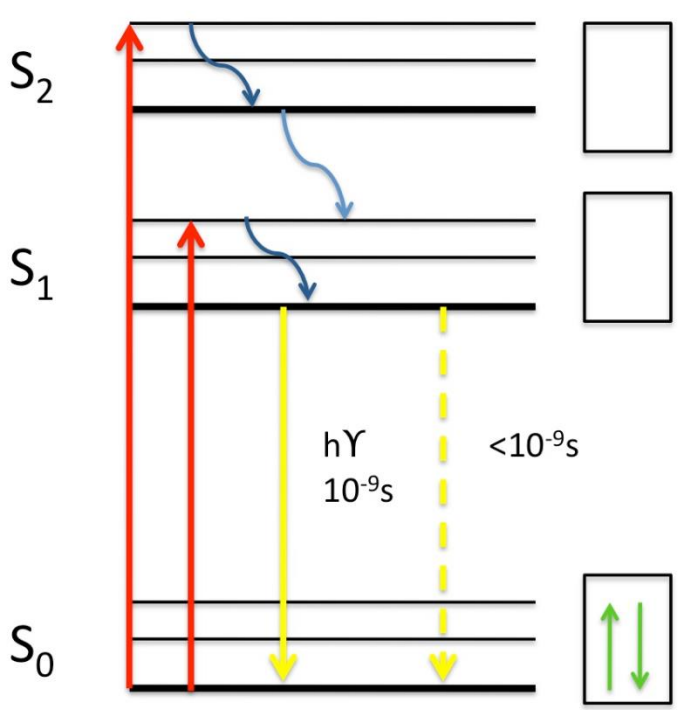
rhodamine B

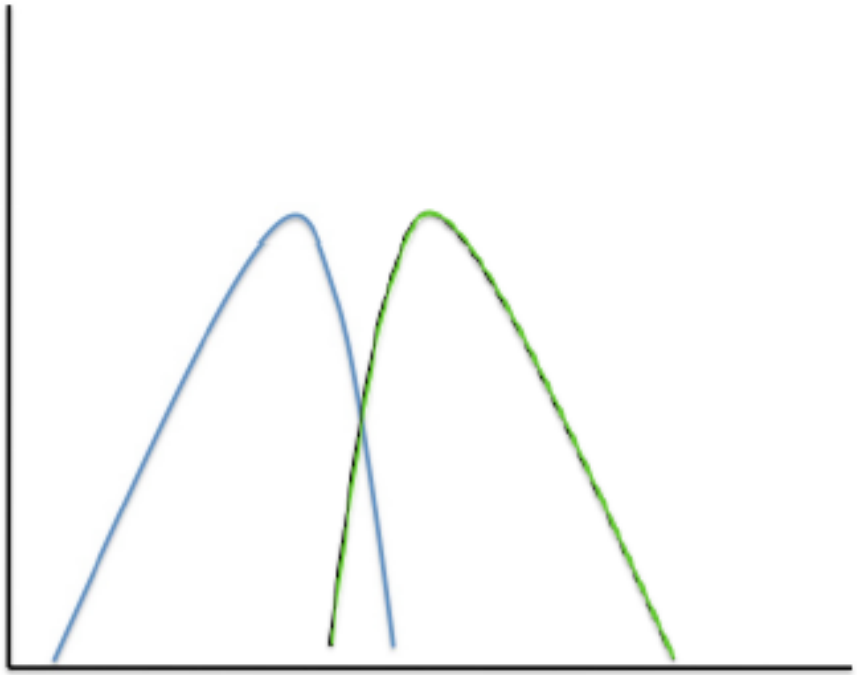


fluorescein

What is Fluorescence?

- FLUORESCENCE is the light emitted by an atom or molecule after a finite duration subsequent to the absorption of electromagnetic energy.





PRINCIPLES:

- When a molecule of substance absorbs EMR , the molecule is excited by absorption of photon (energy) . Part of this energy is usually lost as heat

  (deactivation of the molecule via collisions with certain molecules of solvent present) , then the excited electron drops back to the ground state by re-emitting a photon of lower energy (at longer wavelength) than was absorbed

The process:

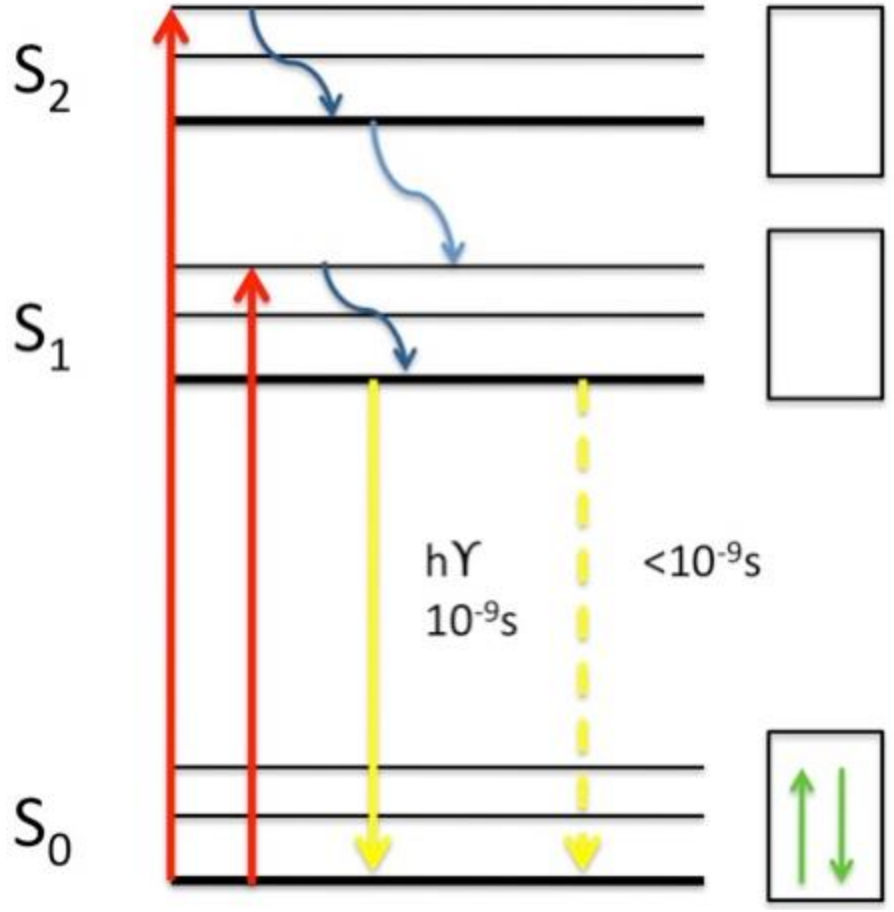
- **After absorption of a photon (a process takes 10^{-15} s) by the fluorophore , electronic transition to higher energy level (excited state) , (transition from the lowest vibrational level of the ground state to one of the vibrational levels of the electronic excited states (S1, S2) . Absorption spectrum of the molecule will observed .**
- **An internal conversion takes place when the excited molecule passes from the vibrational level of the higher excited electronic state to another higher vibrational level of S1 (iso energetic) with the excited state , this process is called vibrational relaxation**

- **At this state the excess energy of the excited molecule is removed by collision with solvent molecule (rapidly within 10^{-12} s) .**
- **Once the molecule reaches the first excited singlet and internal conversion occur (within 10^{-8}s) the electrons return to the ground state by emission of radiation (photons) at longer wavelength (less energy) , however the emitted radiation wavelengths is independent of the wavelength of excitation , but the intensity of the emitted radiation will be proportional to the intensity of incident radiation (i.e. number of photon absorbed). This emission process is known as "FLUORESCENCE " .**

- **Sometimes certain molecules exhibit intersystem crossing when the molecule in its excited state transfers to lower energy triplet state (one electron reverse its spin) , the electron can return to the ground state by emission of a photon , this process is referred to as "PHOSPHORESCENCE ". Phosphorescence is much longer – lived than fluorescence (› 10^{-4}s) .**
- **One can observe phosphorescent substance even when the excitation source is removed (it is not possible for the eye to observe fluorescence emission after removal of the excitation source (usually UV radiation).**

- **Fluorescence and phosphorescence are a like in that excitation is brought about by absorption of photons , but phosphorescence is much longer lived than fluorescence .**
- **The two phenomena are referred to by the term " PHOTOLUMINESCENCE ".**
- **"CHEMILUMINESCENCE" is based upon that the excited species is formed as a product of a chemical reaction .**
- **Measurement of the intensity of photoluminescent or chemiluminescent radiation permits quantitative determination of a varity of organic and inorganic species .**
- **Typically the emission spectrum is the same, regardless of the excitation wavelength.**
- **Emission spectrum is a mirror image of the absorption spectrum.**

Stokes' Shift



Photoluminescence

Photoluminescence processes are divided into 2 classes:

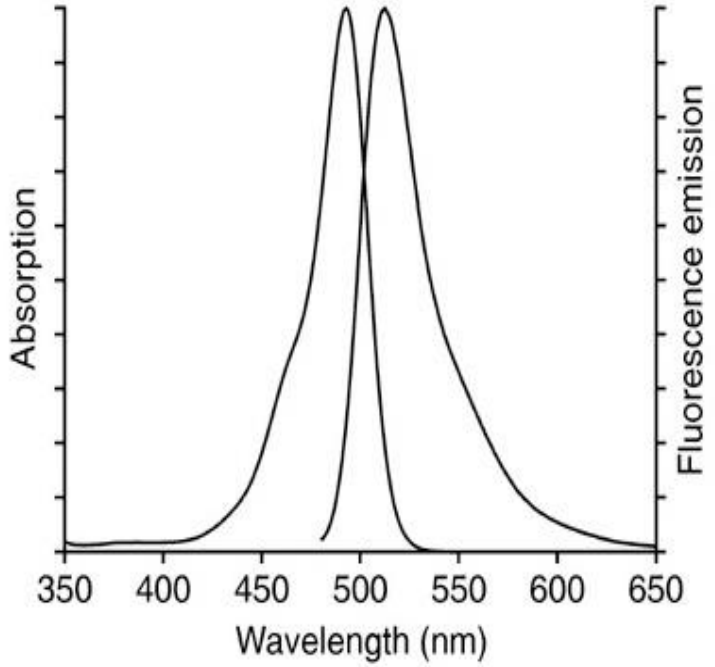
a- Absorption ***(*** **10^{-15} s)**

a– *Fluorescence (* **10^{-8} s)**

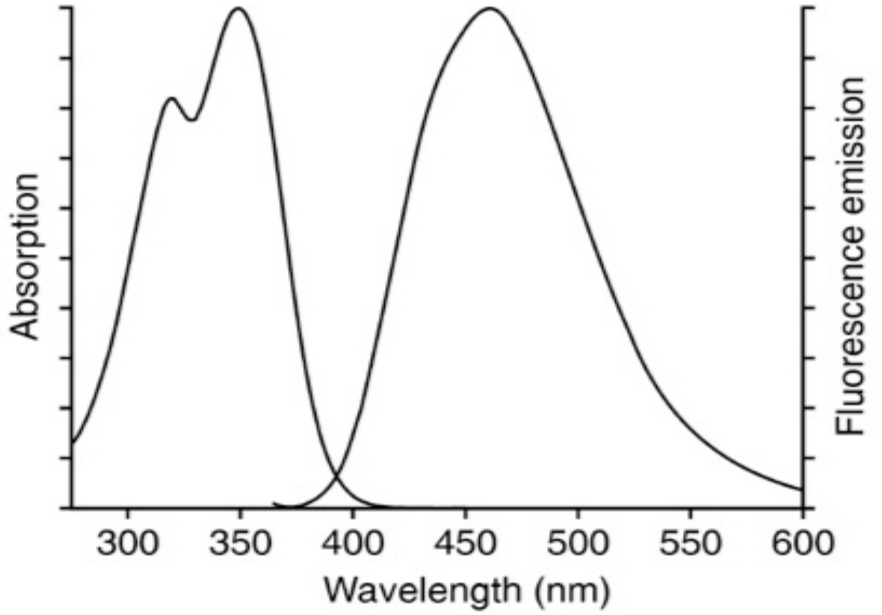
b- Phosphorescence (**(› 10^{-4} s)**

Stokes' shift

Fluorescein



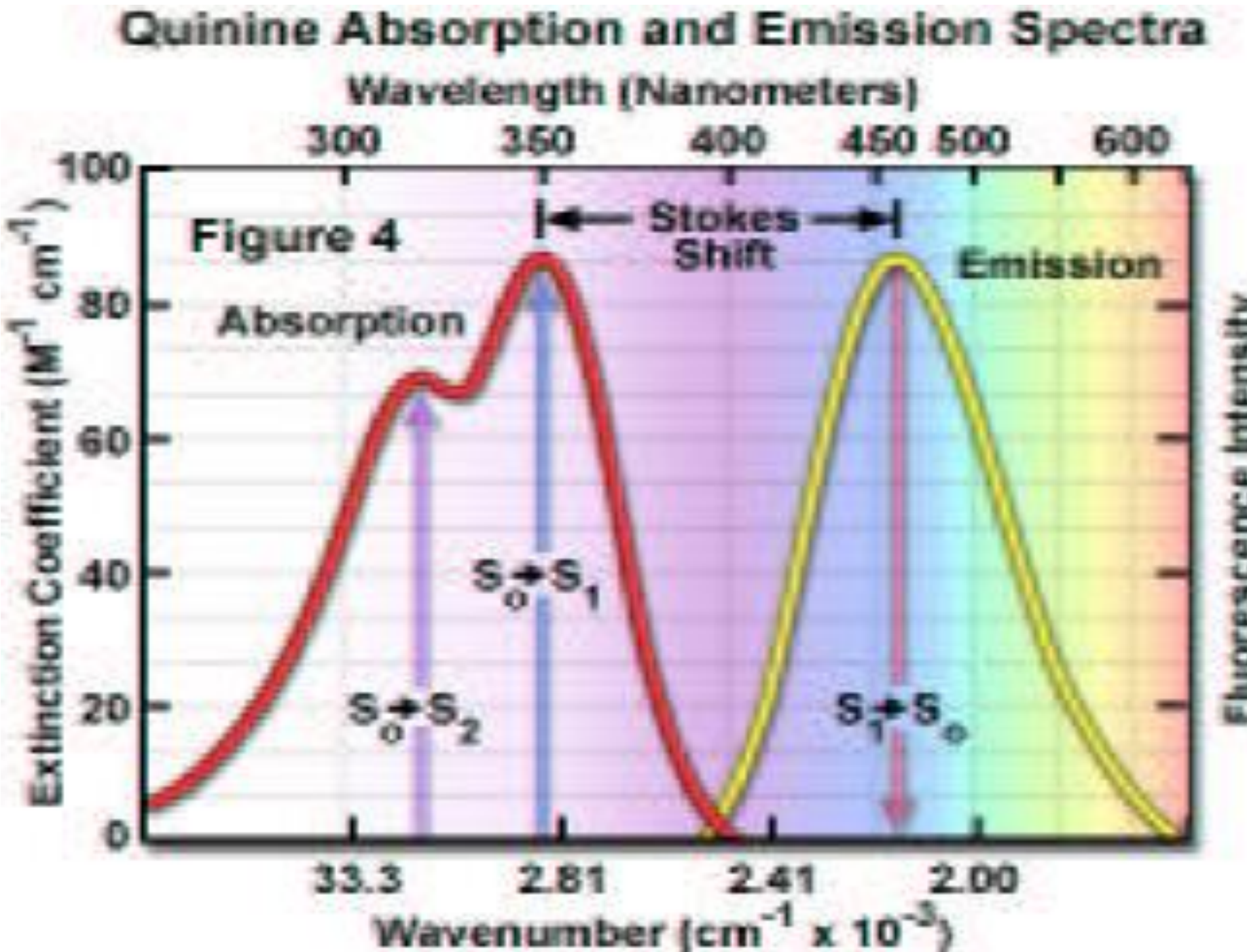
Quinine Sulfate



Stokes shift can be calculated from λmax of Abs and Fluorescence spectrum

Convert Abs λmax and Fluo. λmax to wavenumbers to get Δv

Fluorescein Δv = 1444 cm Quinine sulfate = 6100 cm


Quinine Absorption and Emission Spectra
Wavelength (Nanometers)
300
350
400
450
500
600
Figure 4
Stokes Shift
Absorption
Emission
$S_0 \rightarrow S_1$
$S_0 \rightarrow S_2$
$S_1 \rightarrow S_0$
Extinction Coefficient (M^{-1} cm^{-1})
Fluorescence Intensity
100
80
60
40
20
0
33.3
2.81
2.41
2.00
Wavenumber (cm^{-1} x 10^{-3})

Advantage of photoluminescence :

- 1 – It has higher sensitivity .
- 2 – It has high selectivity .
- 3 – It has large linear concentration range .

However the luminescent methods are much less widely applicable than absorption methods because of the relatively limited number of chemical systems that can be made to produce luminescent radiation.

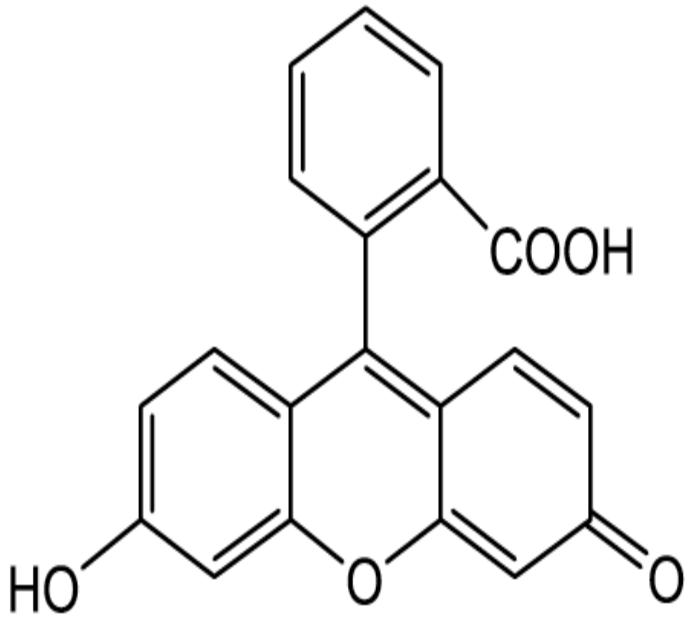
Variables that affect fluorescence and phosphorescence :

- **Both molecular structure and chemical environments are determine wither a substance will or will not fluoresce , also the intensity of emission .**

Quantum efficiency :

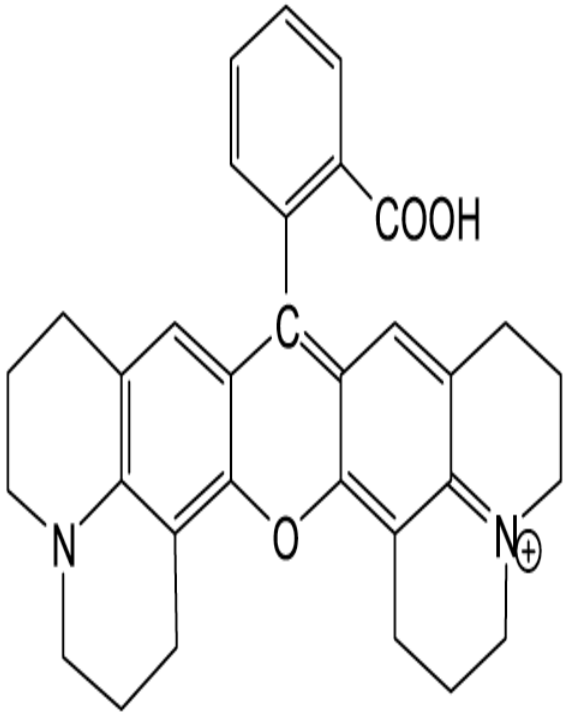
- **Quantum efficiency is the ratio of the number of molecules that fluoresce to the total number of excited molecules .**
- **The quantum efficiency may approach unity . Chemical species that do not fluoresce have quantum efficiency equal zero .**

Quantum Yield (Φ)



fluorescein

Φ = 0.79
At pH 12



rhodamine 101

Φ = ~1.00
EtOH + HCl

- **Transition types in fluorescence :**
- **Fluorescence seldom results from absorption of UV radiation of wavelengths lower than 250 nm because such radiation is highly energetic to cause deactivation of the excited state causing dissociation or rupture of bonds (σ – σ*) . Fluorescence arises most commonly from π – π* or n – π* transition , however fluorescence is more commonly associated with π – π* than with n – π* transition , because π – π* possess shorter lifetime .**

Chemical Structure and fluorescence :

1 – The most intense fluorescence is found in aromatic compounds (with low energy π – π* transition) .

2 - Aliphatic and alicyclic highly conjugated double bond structures may also exhibit fluorescence (less frequently than aromatic) .

3 – Unsubstituted aromatic hydrocarbons :

The quantum efficiency increase with increasing number of rings .The quantum efficiency increase with increasing their degree of condensation

4 – Simple heterocyclic (pyridine , furane , thiophene , pyrole) do not exhibit fluorescence (n – π* transition) .

5 – Heterocyclic compounds fused with benzene ring , do fluoresce (quinoline , indole) .

6 – Subsitution on benzene ring causes shifts in absorption wavelength and corresponding changes in fluorescence peaks , in addition to fluorescence efficiency .

7 – Halogen substitution decrease fluorescence by increase atomic number (I › Br › Cl) ; increase intersystem crossing to triplet state) .

8 – Carboxylic acid or carbonyl group on aromatic ring inhibits fluorescence .

9 –Fluorescence is particularly favored in molecules that possess rigid structure .

Temperature and solvent :

1 – Quantum efficiency decreases with increasing temperature (temperature improves the probability for deactivation by external conversion) .

2 – Decrease in solvent viscosity will decrease quantum efficiency .

3 - Fluorescence of molecules decrease by solvents containing heavy atoms (CBr4 , EtI)

4 – Polar solvents inhance fluorescence .

Effect of pH :

Aromatic compounds with acidic or basic substituents are usually pH dependent , bath wavelength and emission intensity are different for ionized and non ionized forms (fluorescent behavior as a function of pH has been used as acid – base indicator) .

Dissolved Oxygen :

Dissolved Oxygen reduces the emission intensity of fluorescent solution (quenching effect) .

Fluorescence Quenching :

- Quenching of fluorescent by substance that compete for the electronic excitation energy and decrease the quantum efficiency e.g. I^- and Br^- substituents . Such substances may be determined indirectly by measuring the extend of fluorescence quenching .
- A colored species in solution with fluorescent species may interfere by absorbing the fluorescent radiation . This phenomenon is called "Inner Filter Effect" e.g. $Cr_2O_7^{2-}$ solution has absorption peaks at 254 and 348 nm , these peaks overlap with excitation (275 nm) and emission (350 nm) peaks for tryptophane .
- The inner filter effect can also arise from too high concentration of the fluorophore itself (some of the analyte molecules will absorb the emitted radiation of others).

Practical considerations :

1 – The fluorometric analysis is extremely sensitive (up to ppb) .

2 – Dilute solution is less stable

3 – Adsorption onto the surface of the container (add polar solvent)

4 – Oxidation of trace substance may occur

5 – Photodecomposition is more likely to occur at low concentration . The measurements should be therefore made rapidly.

Emission and Excitation Spectra :

- **Excitation spectrum measure the luminescence intensity at fixed emission wavelength while the excitation wavelength is varied .**
- **Fluorescence and phosphorescence spectra**

These spectra measure the emission intensity at fixed excitation wavelength – as a function of wavelength .

Fluorescence usually occurs at wavelengths that are longer than the excitation wavelength .

- **Phosphorescence bands are generally found at higher wavelengths than fluorescence bands , because the excited triplet state is lower in energy than the corresponding singlet state .**
- **The excitation spectrum usually corresponds closely in shape to the absorption spectrum of the molecule**
- **There is frequently (but not necessarily) a close relationship between the structure of the excitation spectrum and the structure of the emission spectrum (mirror image) .**
- **Both the quantum efficiency and the shape of the emission spectrum are independent of the wavelength of the exciting light.**
- **Only those molecules that will absorb radiation , usually in UV- radiation (› 250 nm) , can fluoresce .**
- **The emission radiation is usually in the visible region .**

Fluorescence – Concentration relationship :

- The fluorescence intensity "F" is proportional to the radiant power of the excitation beam that absorbed by the fluorescent species :

  $F = K (Po – P)$

  where K is constant dependant on quantum efficiency
- From Beer;s law

  $A = \log Po/P = abc$

  $P/Po = 10^{-abc}$

  $F = k Po (1 - 10^{-abc})$

  $F = 2.3 K abc Po$ (at dilute solution , A ‹ 0.05)
- At constant Po

  $$F = KC$$
- Plot of fluorescence intensity F of a solution versus concentration of the emitted species , C , should be linear (at low concentration , where A ‹ 0.05)

Deviation :

1 – At higher concentration

2 – Self quenching (collesion between excited molecules)

3 – Self absorption (when wavelength of emission and absorption are overlapped) .

Instrument : Spectrofluorometer

1 – UV – source: mercury vapor lamp : giving line source (365 nm , 520 nm) High pressure xenon lamp: used for scanning spectrum

2 – Filter1 : used to filter the wavelengths close to wavelengths of the emission (scatter radiation) , excitation filter

3 – Cuvets : it is better to use quartz cells as the glass cells will

pass appreciable amount of the excitation radiation (365 nm) , some instruments use glass cells + filter

4 – Filter 2 : emission filter to give the monochromatic radiation that detected by the detector

5 – Detector

6 – Read-out Device (Recorder)

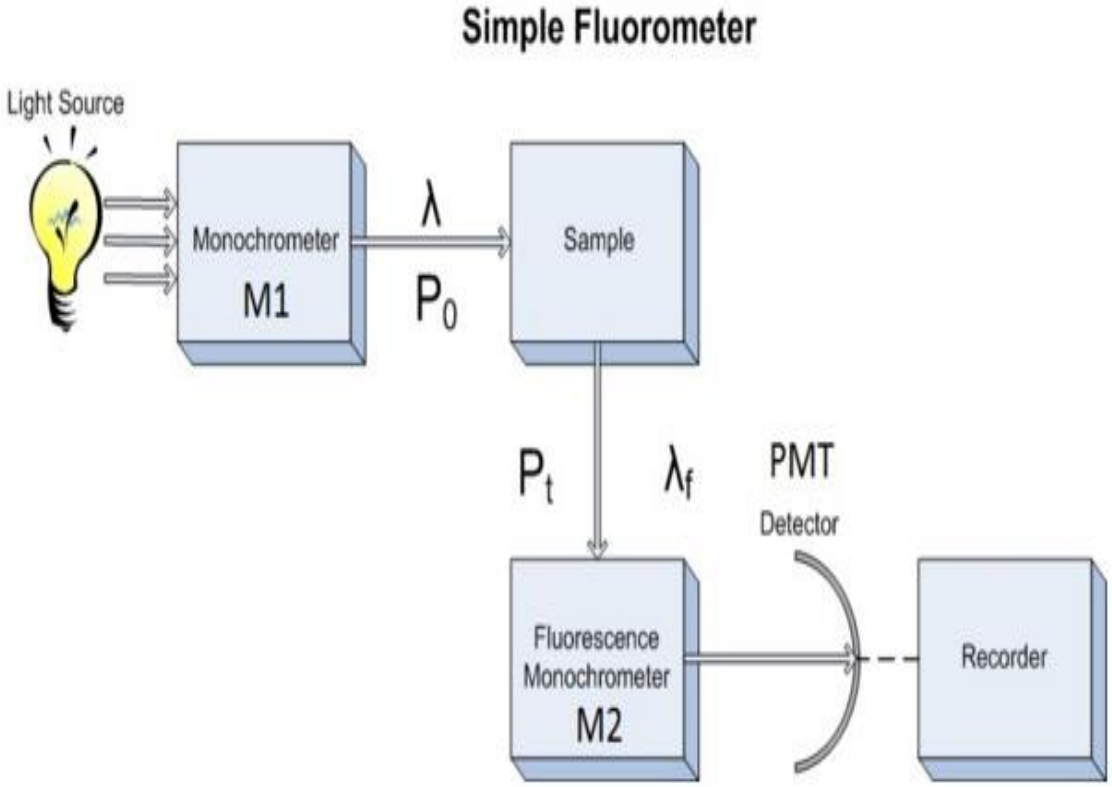


***Figure 4.2**: Schematic Diagram of Fluorescence Spectrometer. M1 = excitation monochrometer, M2 emission monochrometer, L light source. s = sample cell, PMT photo multiplier tube detector.*

Coupling the above techniques with a relationship between fluorescence intensity and concentration would be exceptionally useful. Such a relationship can be derived from Beer's Law, which states that the fraction of light intensity transmitted by a sample is

$$\frac{I}{I_o} = 10^{-\varepsilon bc} \qquad (4.2.1)$$

where

Instrumentation

Introduction to Fluorescence Spectroscopy

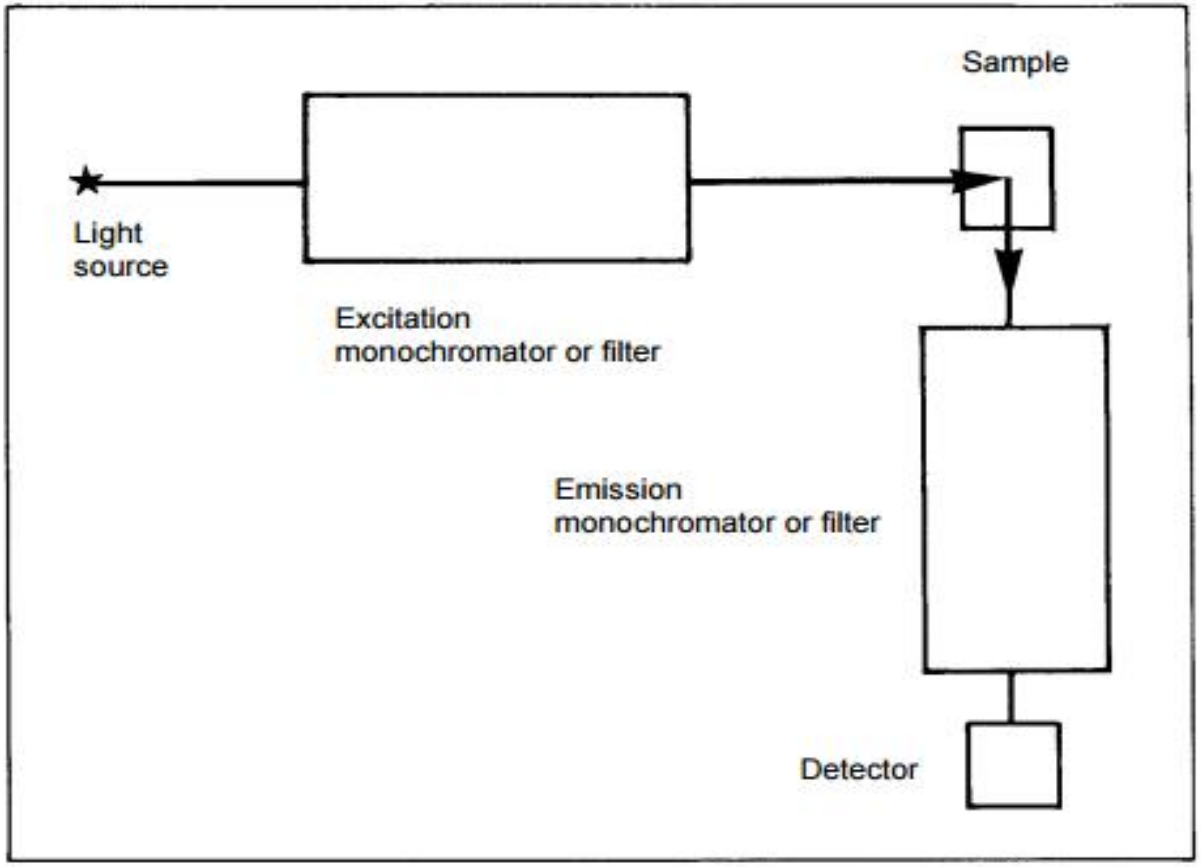


Figure 5 Essential components of a fluorescence spectrometer

- **The measurement is made at a right angel to the direction of the incident radiation , to separate them from the emission radiation . The fluorescence radiation emitted in all directions but the incident radiation passes through the solution straight.**
- **Therefore filter 2 is used to separate the emission radiation from the excitation radiation (to isolate the fluorescent emission spectrum) .**

Fluorescence Spectra

- To an analyst, the outstanding feature of luminescence methods is their sensitivity. With appropriate compounds picogram concentrations can be detected using fluorimetric and phosphorimetric methods, using chemiluminescence .
- In the analysis of a very dilute solution, absorptiometry involves detecting a small change in a large light intensity, a very difficult thing to do; in contrast, using the right-angled optics familiar in fluorimetry , the small fluorescence signal is detected against a background which is (ideally) zero.
- In chemiluminescence spectrometers, which fundamentally consist of a cuvette holder and an adjacent photomultiplier, the light gathering is very efficient and the sensitivity correspondingly greater.

- **procedures**
- The availability of two monochromators in most fluorescence spectrometers permits the collection of two simple types of spectra. Scanning the excitation monochromator at a constant emission wavelength yields the excitation spectrum; scanning the emission monochromator at a fixed excitation wavelength provides the fluorescence or emission spectrum, which occurs at longer wavelengths than the excitation spectrum

Applications

- Fluorometry is generally used if there is no colorimetric method sufficiently sensitive or selective for the substance to be determined.

1 – analysis of metals: eg.Al^{3+} forms fluorescent complex with eriochrome blue black, 8-hydroxy quinoln.

2 - analysis of nonmetals: eg.condensation reaction between boric acid and benzoin forms fluorescent complex.

3 – organic applications: determination of quinine , riboflavin , thiamine, amino acids .

Applications of fluorometric methods to organic compounds

Diverse compounds as adenine, anthranilic acid, aromatic polycyclic hydrocarbons, cysteine, guanine, isoniazid, naphthols, salicylic acid, skatole, trypotophan, uric acid, and warfarin.

Many medicinal agents that can be determined fluorometrically are listed, including adrenaline, morphine, penicillin, phenobarbital, procaine reserpine, and lysergic acid diethylamide (LSD).

The most important applications of fluorometry include the analysis of food products, pharmaceuticals, clinical samples, and natural products.