Republic of Iraq
Ministry of Higher Education & Scientific research
Al-Mustaqbal University
Science College
Biochemistry Department

Analytical Chemistry Instrumental Analysis

For

Second Year Student/course 1

Lecture 7

By

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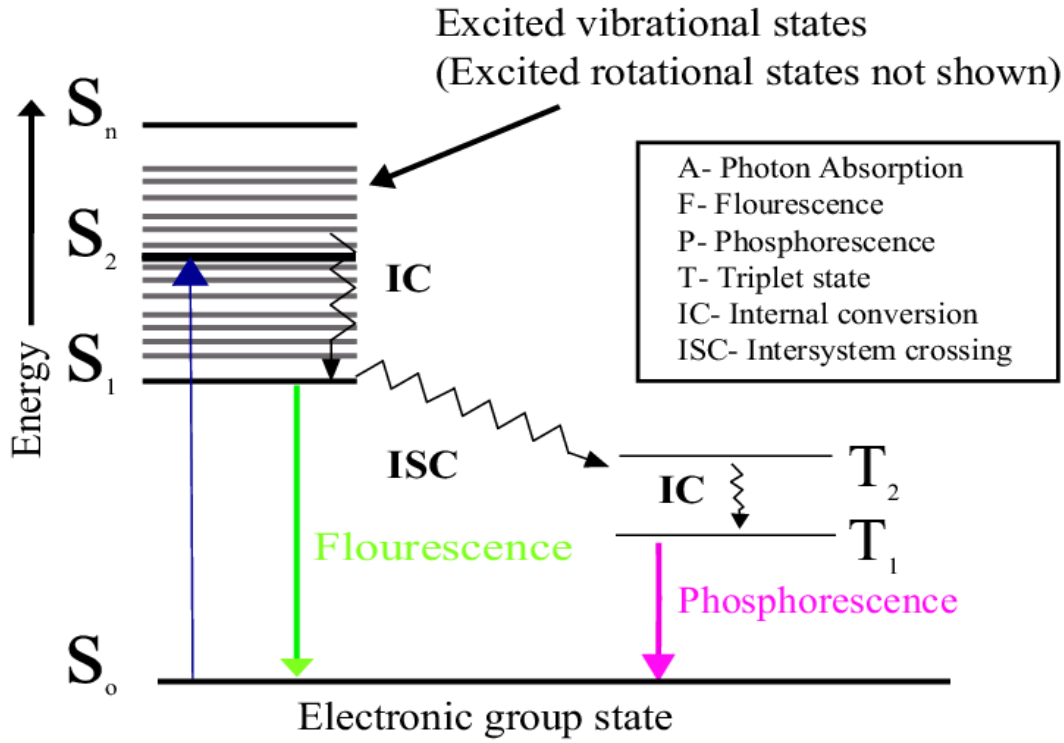
principles of Fluorescence

- ✓ Fluorescence is an emission phenomenon, the energy transition from a higher to a lower state within the molecule concerned being measured by the detection of this emitted radiation rather than the absorption.
- ✓ A molecule absorbs light at one wavelength and emits light at a longer wavelength.
- ✓ An atom or molecule that fluoresces is termed a fluorophore.
- ✓ Fluorometry is defined as the measurement of emitted fluorescent light.

Types of emission

- **Fluorescence** – return from excited singlet state to ground state; does not require a change in electron spin orientation (more common form of relaxation).
- **Phosphorescence** – return from a triplet excited state to a ground state; electron requires a change in its spin orientation.

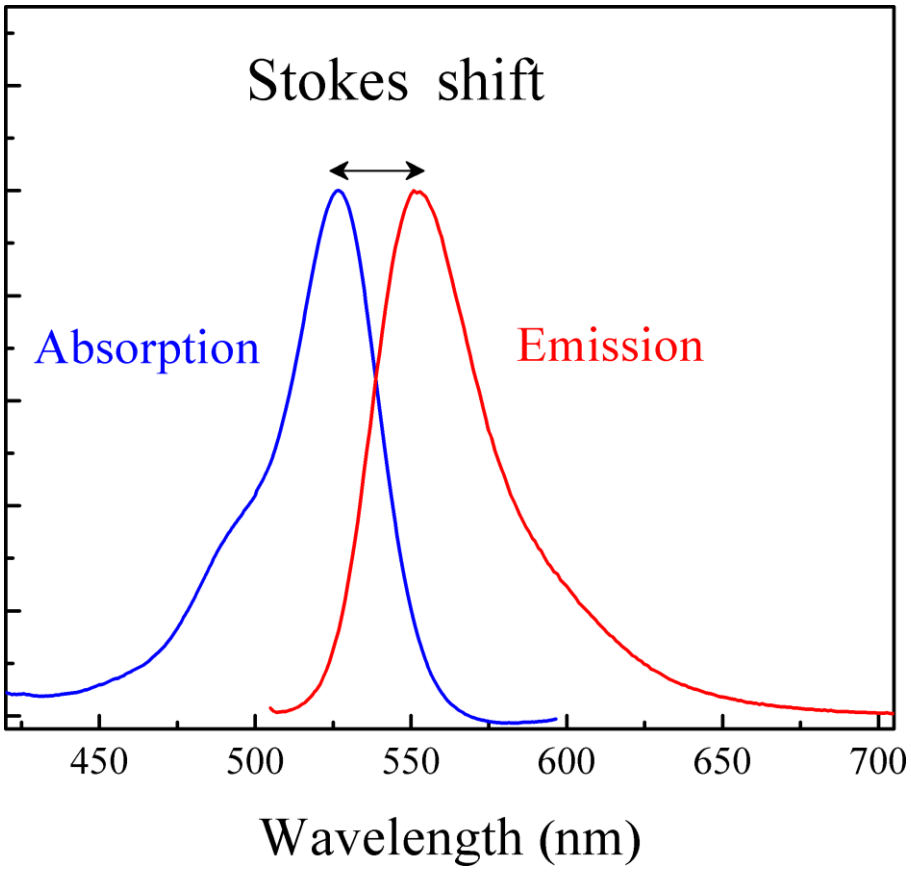


Internal conversion Vs Intersystem Crossing

Internal conversion is a transition occurring between states of the same multiplicity and it takes place at a time scale of 10^{-12} s (faster than that of fluorescence process).

Intersystem Crossing

- **Intersystem crossing** refers to a non-radiative transition between states of different multiplicity.
- It occurs via inversion of the spin of the excited electron, resulting in two unpaired electrons with the same spin orientation. This creates a state with Spin=1 and a multiplicity of 3 (triplet state).



- The difference between the maximum wavelength of the excitation light and the maximum wavelength of the emitted fluorescence light is a constant referred to as the **Stokes shift**.
- It is a measure of the energy lost during the lifetime of the excited state (through radiation-less vibrational deactivation) before returning to the ground singlet level (via fluorescence emission).
- The best results are obtained from compounds involving large shifts.

Theory

- Fluorescence spectroscopy is a **fast**, **simple**, **and inexpensive** method for determining the concentration of an analyte in a solution.
- It is used for the **quantitative analysis** of known compounds to find their concentration.
- Its main application is for measuring compounds **in solution**.

In fluorescence spectroscopy:

- A beam of light with a wavelength ranging between 180 and ~800 nm passes through a solution in a cuvette.
- The light emitted by the sample is then measured from an angle.

Measurements:

- **Excitation spectrum:** (the light absorbed by the sample).
- **And/or emission spectrum:** (the light emitted by the sample).

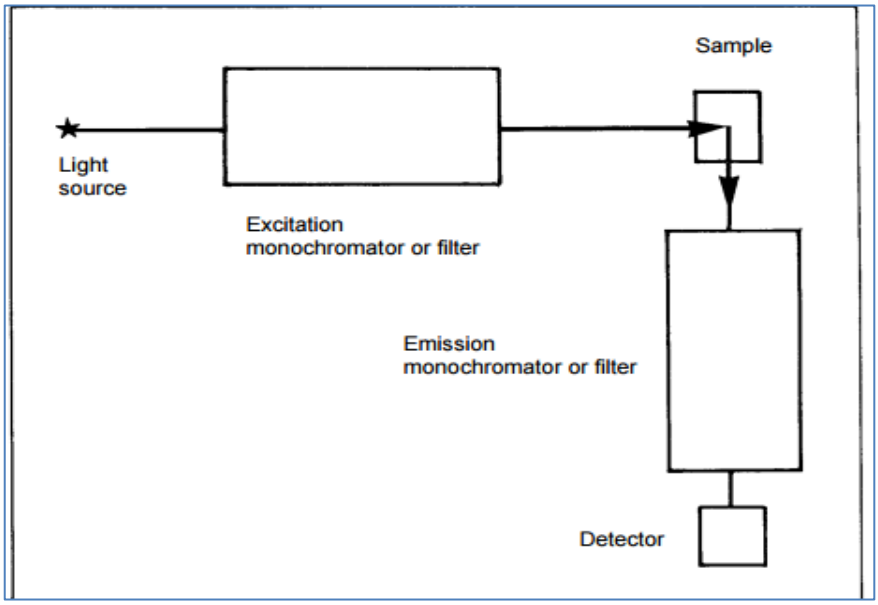
Key Principle:

- The concentration of the analyte is **directly proportional** to the intensity of the emission.

Factors influencing the emission spectrum:

The intensity and shape of the emission spectrum depend on:

- The excitation wavelength.
- The concentration of the analyte and the solvent.
- The path length of the cuvette.
- The self-absorption of the sample.

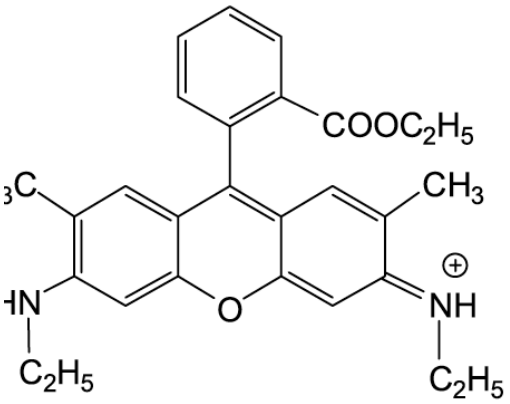


Mechanism of a Spectrofluorometer

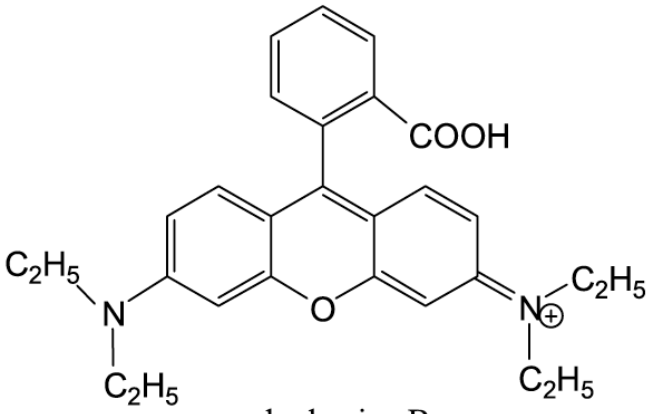
✓ The light from an excitation source passes through a filter or monochromator (1), and passes through the sample. Here some of it is absorbed, making some of the molecules in the sample fluoresce.

- ✓ A part of the fluorescent light is then focused on a filter or monochromator (2), which often is placed at a 90° angle to the excitation light.
- ✓ The light is then detected by a detection device.
- ✓ Various light sources may be used as excitation sources, such as: lasers, photodiodes, xenon arcs, and mercury-vapour lamps.

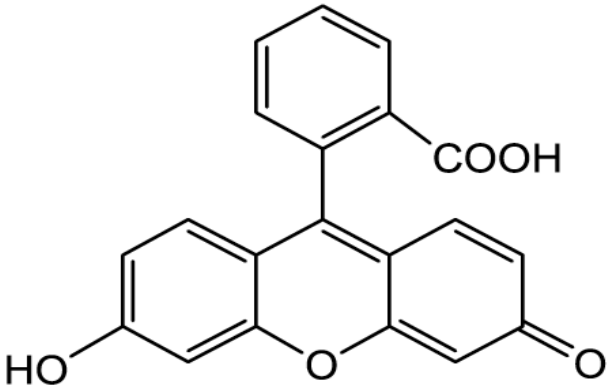
Common Fluorophores



rhodamine 6G



rhodamine B



fluorescein

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**

$$A = \log Po/P = abc$$

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

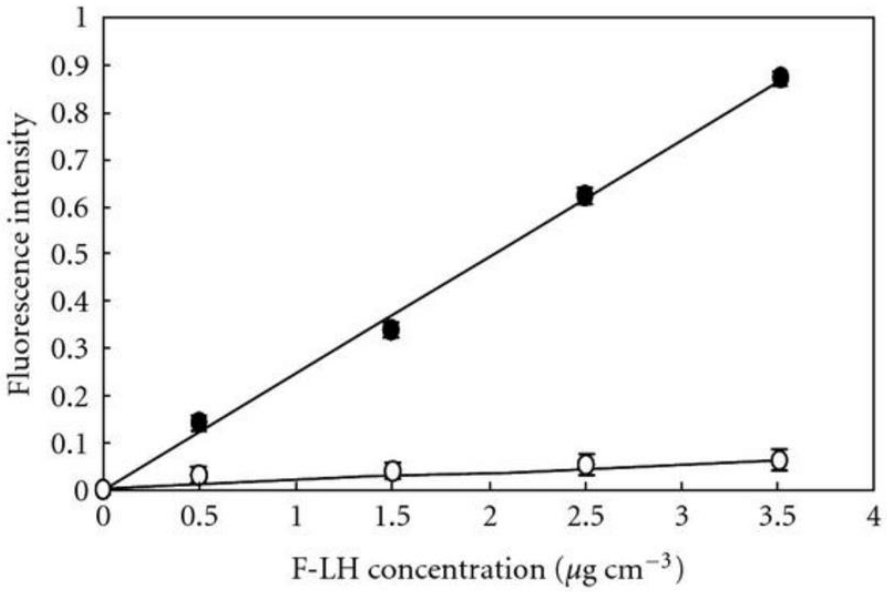
$$F = k\,Po\,(1 - 10^{-abc})$$

$$F = 2.3\,K\,abc\,Po \quad (\text{at dilute solution , } A < 0.05)$$

- **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)**



Factors that influence fluorescence measurements include:

- **Concentration effects**
  - Inner filter effects, concentration quenching
- **Background effects**
  - due to Rayleigh and Raman scattering
- **Solvent effects**
  - Interfering nonspecific fluorescence, quenching from the solvent
- **Sample effects**
  - Light scattering, interfering fluorescence, sample adsorption
- **Temperature effects**
- **Photodecomposition (bleaching) of the sample.**

Advantage of fluorescence spectroscopy

Sensitivity:

- It is more sensitive when the concentration is low, such as µg/ml or ng/ml.

Precision:

- Accuracy of up to 1% can be achieved.

Specificity:

- More specific than the absorption method, where two compounds may have the same absorption maxima.

Range Of Application:

  - Even non-fluorescent compounds can be converted into fluorescent compounds using chemical agents.

Disadvantages:

1. Not useful for identification.
2. Not all compounds fluoresce.
3. Contamination can quench the fluorescence and hence give false or no results.

Applications

Analytical Chemistry:

- To detect compounds from HPLC flow.
- TLC plates can be visualized if the compounds or a coloring reagent is fluorescent.
- Plant pigments, steroids, proteins, naphthols, etc., can be determined at low concentrations.

Biochemistry:

- Generally used as a non-destructive way of tracking or analyzing biological molecules (e.g., proteins).
- Enables direct or indirect analysis of aromatic amino acids (phenylalanine, tyrosine, tryptophan).

- Fingerprints can be visualized with fluorescent compounds such as ninhydrin.

Medicine

- Blood and other substances are sometimes detected using fluorescent reagents, particularly when their location is not previously known.
- There has also been a report of its use in differentiating malignant skin tumors from benign ones.

Pharmacy

Possible direct or indirect analysis of drugs such as:

- **Vitamins:** Vitamin A, Vitamin B2, Vitamin B6, Vitamin B12, Vitamin E, Folic Acid.
- **Catecholamines:** Dopamine, Norepinephrine.
- **Other Drugs:** Quinine, Salicylic Acid, Morphine, Barbiturates, Lysergic Acid Diethylamide (LSD).
- To measure the amount of impurities present in a sample.