CHAPTER 2: FLUORESCENCE SPECTROSCOPY

2.0 Introduction

Among instrumental techniques, fluorescence spectroscopy is recognized as one of the most sensitive. Therefore, fluorescence spectroscopy is a very important analytical technique in development of science especially in medicinal and biological study [23-25] since there are continually examining mechanisms and investigating the function of analytical reagents which are active in extremely low concentrations such as in tissues. [26-27]

2.1 Historical development

Phosphorescence history back to early 1500s, named after the Greek work for "light bearing". The term phosphor has been assigned to minerals that glow in the dark after exposure to light.

The term fluorescence was first discovered by Sir Gabriel Stokes, a physicist and professor of mathematics at Cambridge in the middle of the nineteenth century after the mineral *fluorspar* (Latin *fluo* = to flow + *spar* = a rock), which exhibit a blue-white fluorescence. First reported observation of fluorescence was made by a Spanish physician, Nicolas Monardes, in 1565 when he described peculiar blue color of an infusion of wood call *Lignum Nephriticum*. This wood was further investigated by other researchers but the phenomenon was not understood.

Stokes published a paper entitled 'On the refrangibility of light' [28] in 1852. He demonstrated that the phenomenon was an emission of light following absorption of light by moving a tube filled with a solution of quinine sulfate through the visible part of the spectrum and nothing happened. The solution remained transparent. But beyond the violet portion of the spectrum, i.e. in the non-visible zone corresponding to ultraviolet radiations, the solution glowed with a blue light. This experiment granted convincing evidence that

there was absorption of light followed by emission of light. Stokes stated that the emitted light is always of longer wavelength than the exciting light and this statement becomes later Stokes' law.

2.2 Theory of fluorescence

Light is a form of electromagnetic radiation (energy), the propagation of which is regarded as a wave phenomenon. It is characterized by a frequency (ν), and has a wavelength (λ) and, in vacuum, a constant velocity (c). These are related in the equation 1:

$$\upsilon = \frac{c}{\lambda} \qquad \textbf{(1)}$$

When light enters matter two things may happen to it:

1. It can pass through the matter with little absorption taking place. The material is then considered as being essentially transparent. In this case there is little loss of energy, but the velocity of the light is diminished to an appreciable extent. The refractive index of a substance is the ratio of the velocity of light in a vacuum to the velocity in that substance. In general, refractive index varies with wavelength so that light is separated into its component parts (spectrum) as it passes through a transparent prism.

2. The light may absorbed, either entirely or in part. In either case the absorption involves a transfer of energy to the medium. The absorption itself is highly specific phenomenon, and radiation of a particular energy can be absorbed only by characteristic molecular structures. Energy from light is absorbed in integral units, called quanta. The quanta-energy relationship can be expressed by the equation 2:

$$E = h\upsilon \quad \text{or} \quad E = \frac{hc}{\lambda} \qquad \textbf{(2)}$$

Where E is the energy, h is Planck's constant, 6.62 X 10^{-27}erg/seconds, c the velocity of light, ν the vibration frequency (second^{-1}), and λ the wavelength.

At room temperatures can be assumed that the molecule, when it absorbs the light, is in its lowest electronic level. The absorption of the quantum of light will then raise the energy by promoting the electron to a higher energy state (excited state). The simplified diagram below shows absorption by molecules to produce either the first, S1 or second S2, excited state as shown in Figure 2.1.

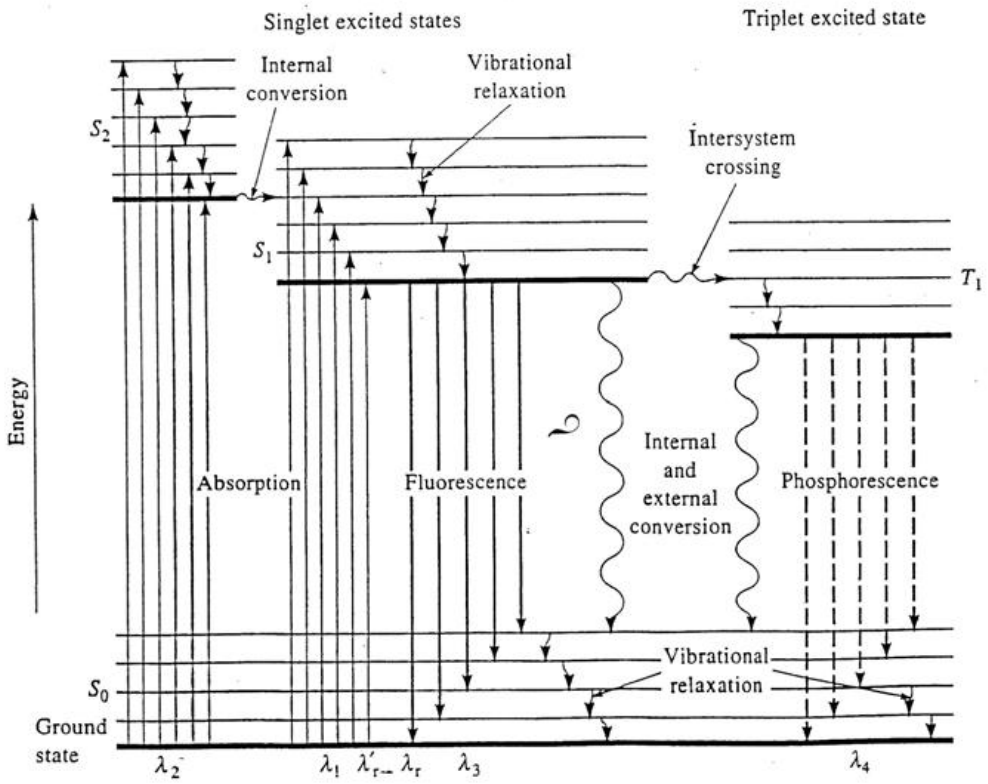


Figure 2.1: Transition giving rise to absorption and fluorescence emission spectra

Excitation can result in the molecule reaching any of the vibrational sub-levels associated with each electronic state. Since the energy is absorbed as discrete quanta, this should result in a series of distinct absorption bands. However, the simple diagram above neglects the rotational levels associated with each vibrational level and which normally increase the number of possible absorption bands to such an extent that it becomes impossible to resolve individual transitions.

Therefore, most compounds have broad absorption spectra except for those where rotational levels are restricted (for example, planar, aromatic compounds). Having absorbed energy and reached one of the higher vibrational levels of an excited state, the molecule rapidly loses its excess of vibrational energy by collision and falls to the lowest vibrational level of the excited state. In addition, almost all molecule occupying an electronic state higher than the second undergo internal conversion and pass from the lowest vibrational level of the upper state to higher vibrational level of a lower excited state which has the same energy. From there the molecules again lose energy until the lowest vibrational level of the first excited state reached.

From this level, the molecule can return to any of the vibrational levels of the ground state, emitting its energy in the form of fluorescence. If this process takes place for all the molecules that absorbed light, then the quantum efficiency of the solution will be at maximum.

In the production of excited states by promotion of an electron into a higher orbital, the direction of the spin of the electron is preserved. Since most molecules have an even of electrons and these are normally arranged in pairs of opposite spin, the promotion of an electron does not disturb this parity.

However, it is possible for the spin of the promoted electron to be reversed so that it is no longer paired and the molecule has two independent electrons of the same spin in different orbital. Quantum theory predicts that such a molecule can exist in three forms of very slightly differing, but normally indistinguishable, and the molecule is said to exist in a triplet state. The indirect process of conversion from the excited state produced by absorption of energy, the singlet state, to a triplet state, is known as intersystem crossing shown in Figure 2.1 and can occur in many substances when the lowest vibrational level of

the excited singlet state, S1, has the same energy level as an upper vibrational level of the triplet state.

2.3 Factors that effect the fluorescence characteristic

2.3.1 Environmental effects

The fluorescence of organic molecules is dependent upon environmental factor such as solvents, [29-30] pH [31-32] and molecular oxygen.[33] Molecular environment constitutes an important parameter which can be used by the analyst to increase the sensitivity and selectivity of fluorimetry. Some of the environmental factor will be discuss here.

2.3.1.1 Solvent effects

The position and structure of the spectrum of many aromatic molecules is strongly depending on the solvent. Electronic transitions occur at rapid rates relative to the rates of inter-nuclear motion in molecules. Hence, during an electronic transition (absorption or emission), the nuclei remain essentially stationary (the Franck-Condon principle). Accordingly, when a molecule in its ground state absorbs a photon, it finds itself in a metastable excited state ("Franck-Condon" excited state), in which the molecular geometry and solvent configuration are those characteristic of the ground state. Solvent reorientation then occur approximately 10^{-11} to 10^{-12} sec after excitation, producing an "equilibrium" excited state, in which the solvent configuration is optimal for the geometry and electron distribution of the molecule. Emission from equilibrium excited state, forming a Franck-Condon ground state. Solvent relaxation then occurs, forming the equilibrium ground state. The energy relationship of the various equilibrium and Franck-Condon states are represented in Figure 2.2.

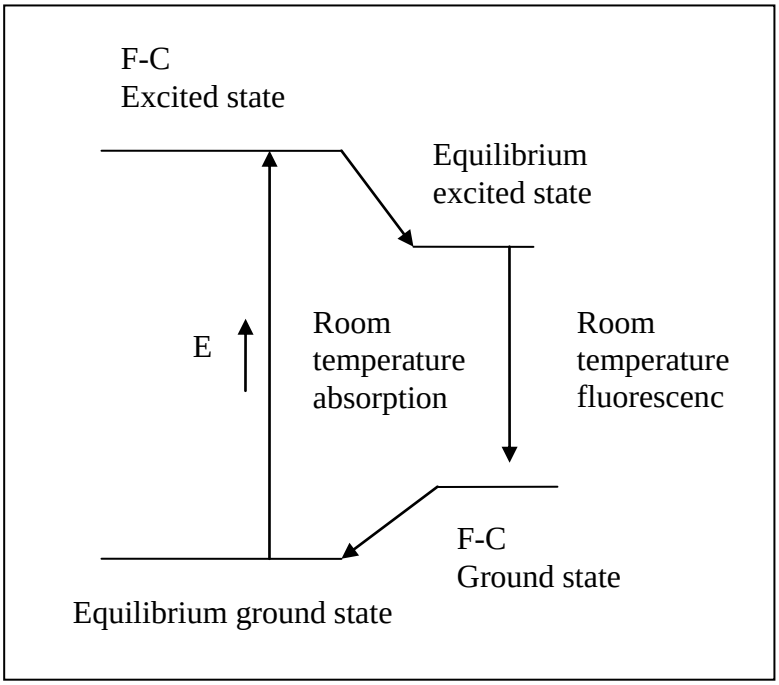


Figure 2.2: Schematic representation of equilibrium and Franck-Condon (F-C) electronic states

If the solute molecule becomes more polar in the excited state, this will be greater electronic stabilization of the excited state relative to the ground state by interaction with the polar solvent. The greater the polarity of the solvent, the lower will be the energy of the Franck-Condon excited state. This type of behavior is characteristic of the most $\pi \rightarrow \pi^*$ transition and is observed as shift to longer wavelength of the fluorescence and absorption band with increasing solvent polarity. For example indole shows a pronounced bathochromatic shift in fluorescence wavelength with increasing polarity of the solvents.

Absorption transitions of the $n \rightarrow \pi^*$ type are usually more affected by hydrogen bond donor properties of the solvent than by solvent polarity. If the nonbonding pair on a solute molecule is bond by hydrogen atom of the solvent, the hydrogen bonding interaction stabilizes the ground state molecule as well as the $n \rightarrow \pi^*$ excited state of the solute. However, because the ground state molecule has two electrons in the non bonding orbital

and the excited state has only one, the stabilization of the ground state is greatest. As a result, the energies of n→π* absorption increases with increasing solvent hydrogen bond donor capacity.

For example, acridine is virtually non-fluorescent in hexane but moderately so in water and moist ethanol. In hexane, the lack of fluorescence of acridine may be ascribed to the fact that the latter's longest absorption wavelength corresponds to n→π* transition, whereas in water the lone pair of electrons are hydrogen bonded, and the longest absorption wavelength is due to n→π* excitation, which favors fluorescence.

2.3.1.2 Influence of pH

The fluorescence properties of most aromatic compounds containing acidic or basic functional group are very sensitive to the pH change and the ability to form hydrogen bonding with the solvent. Proton-transfer reactions in polar solvents are very fast and acid-base reactions can occur during the life-time of an excited-singlet state of an aromatic molecule. For example, the acid-base properties of 2-napthol whereas this compound exhibits a single broad fluorescence peak in aqueous solution at 395 nm, while the 2-naptholate anion exhibits a fluorescence peak at 429 nm. This large energy separation of these fluorescence spectra makes it easy to determine the occurrence of acid-base reactions in the excited state. The pKa of the ground state of 2-napthol is about 9.5. When measuring of 2-napthol in a medium of pH < 9.5, the neutral molecular form predominates in the ground state. Observation of the fluorescence of the anionic form of 2-napthol at pH values much smaller than 9.5, indicating that the electronically excited 2-napthol molecule is a substantially stronger acid in its first excited singlet state than it is in the ground state. The observation of fluorescence from 2-naptholate in solutions of pH < 9.5 also indicates that

the excited-state proton-transfer reaction is rapid relative to the rate of decay of the 2-napthol singlet. [34]

In the pH range in which neutral 2-napthol is the predominant protolytic form in the ground state, may be represented the overall excitation-reaction sequence as shown in the following Figure 2.3:

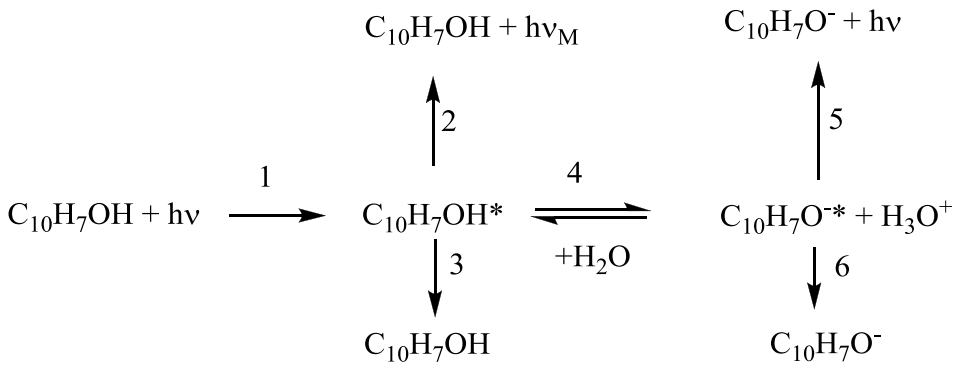


Figure 2.3: Overall excitation-reaction sequence of phenol

The steps in reaction sequence can be explained as follows:

1. Absorption of radiant energy to produce the excited molecule. The pH is less than 9.5, and so neutral 2-napthol predominates.

2. Deactivation of the excited 2-napthol molecule by molecular fluorescence (359 nm).

3. Radiationless deactivation of the excited neutral molecule.

4. Dissociation of the excited molecule, producing a proton and an excited anion.

5. Deactivation of the naptholate anion by fluorescence (429 nm).

6. Radiationless deactivation of the excited anion.

By measuring the relative fluorescence intensities for the neutral molecule and the anion as a function of pH, it can be determined that the pKa for 2-napthol is about 3.1; that is, the excited singlet exhibits an acid strength that is more than 10^6 times greater than that of the ground state. Techniques for measuring pKa values for electronically excited molecules have been described and compared by Vander Donckt [35] and by Schulman.[36] In the case of

2-napthol it is clear that the pH of the solution must be less than 3.5 before the molecular form predominates over the anionic form in the fluorescence spectrum. Since the fluorescence quantum efficiency of neutral 2-napthol is substantially larger than that of the naptholate anion, it is clear that, to obtain maximum sensitivity in fluorometric analyses of 2-napthol, the pH must be not less than 3.5. Knowledge of the equilibrium constants for excited-state protolysis, coupled with knowledge of the relative fluorescence efficiencies of the two protic forms, can thus be of great value in enhancing the sensitivity of fluorometric analyses of solutes containing dissociable functional groups.[36]

2.3.1.3 Effect of oxygen

Molecular oxygen is one of the quencher in fluorescence and phosphorescence. Fluorescence quenching by O_2 can be very serious problem in liquid solution because its will quench excited-singlet of organic molecules. This is because dissolved O_2 molecules have a very large diffusion coefficient, especially in polar solvents.

It is no longer believed that "paramagnetic" effects play a generally important role in O_2 quenching. The proposed mechanisms to account for quenching of excited-singlet states by O_2 are as following [35]:

1. Chemical oxidation of excited singlet: $^1A^* + O_2 \rightarrow A^+ + O_2^-$
2. Energy transfer from $^1A^*$ to O_2: $^1A^* + {}^3O_2 \rightarrow {}^3A^* + {}^1O^*_2$,

   $^1A^* + {}^3O_2 \rightarrow {}^1A + {}^1O^*_2$
3. Enhanced intersystem crossing in $^1A^*$: $^1A^* + {}^3O_2 \rightarrow {}^3A^* + {}^3O_2$
4. Enhanced internal conversion in $^1A^*$: $^1A^* + {}^3O_2 \rightarrow {}^1A + {}^3O_2$
5. Formation of a complex between O_2 and the ground state ^1A.

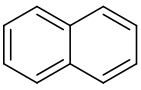
The effect of oxygen on fluorescence varies from compound to compound. Many substituted aromatic and some heterocyclic are almost insensitive to oxygen but on the

other hand the fluorescence from unsubstituted aromatic and aliphatic aldehydes and ketones is very sensitive to oxygen. In most cases, only 2% decrease in intensity is observed, so it is not usually worth the trouble to eliminate oxygen from the solution in practical fluorescence measurements.

2.3.2 Chemical structure

2.3.2.1 Carbon skeleton

Generally, an increase in the extent of the π-electron system (i.e. degree of conjugation) leads to a shift of the absorption and fluorescence spectra to longer wavelengths and to an increase in the fluorescence quantum yield. This simple rule is illustrated by the series of the linear aromatic hydrocarbons which are naphthalene **(13)**, anthracene **(14)**, naphthacene **(15)** and pentacene **(16)** emit fluorescence in ultraviolet, blue, green and red, respectively.



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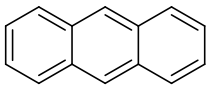

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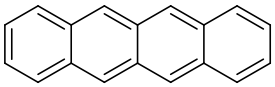

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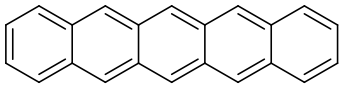

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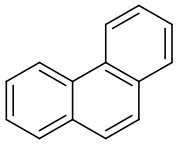
It is nearly always observed that linear ring system fluoresce at longer wavelength than nonlinear systems. Thus λ_{em} is at 400 nm for anthracene **(14)** and at 350 nm for phenanthrene **(17)**; similarly, λ_{em} is at 480 nm for napthacene **(15)** and at 380 nm for benz[a]anthracene **(18)**.



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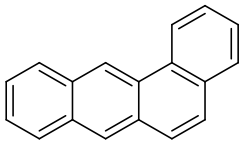

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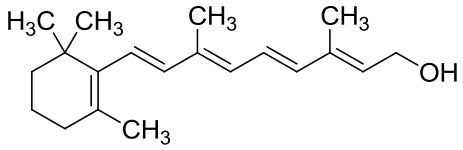
Molecules which contain conjugated double bonds in a cyclic structure are likely to fluoresce more than those containing a chain arrangement. This can be seen with naphthalene **(13)** and vitamin A **(19)** which posses a system of five conjugated double bond but the fluorescence efficiency of naphthalene is at least five time more than of vitamin A.



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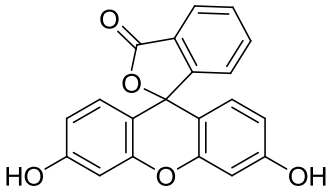
2.3.2.2 The geometrical arrangement of the molecules

Geometrical arrangement also seems to be important. The most strongly fluorescent aromatic compounds are usually characterized by rigid, planar structures. In a non-rigid molecule the absorbed energy can be readily dissipated as heat. The need for molecular rigidity explains the much greater fluorescence of cyclic compounds as compared with chain system. This can be illustrated by comparing the efficiencies of fluorescein **(20)** has intense green fluorescence, whereas the some what similar phenolphthalein **(21)** has only weak fluorescence. The fluorescein molecule has a large, planar, conjugated region, whereas phenolphthalein has three smaller conjugated regions which are not coplanar.



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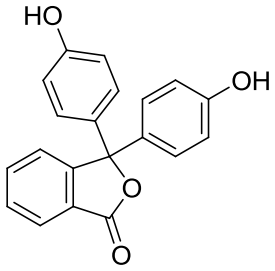

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2.3.2.3 The type and positions of substituents

A simple generalization is that ortho-para-directing substituents often enhance fluorescence, whereas meta-directing groups repress it. In general, electron-donating substituents such as –OH, -OR, $-NH_2$, -NHR, $-NR_2$ induces an increase in the molar absorption coefficient and a shift in both absorption and fluorescence spectra. Moreover, these spectrums are broad and often structureless compared to the parent aromatic hydrocarbons. The presence of lone pairs on the oxygen and nitrogen atoms does not changed the $\pi \rightarrow \pi^*$ nature of the transitions of the parent molecule. These lone pairs are indeed involved directly in π bonding with the aromatic system, in contrast to the lone pair of electrons of carbonyl substituents or heterocyclic nitrogen.

The nature of substituents plays an important role in the nature and extent of a molecule's fluorescence. Fluorescence yield (in this case intensity) of aromatic and heterocyclic hydrocarbons are usually altered by ring substitution.

Substituents which enhance π-electrons mobility will increase fluorescence, while those which decrease it will reduce fluorescence. Thus, in general, electron donating substituents tend to enhance fluorescence while electron withdrawing substituents to diminish or abolish it.

Stevenson [37] has studied the effects on the absorption spectrum of benzene by substituents. Alkyl substituents on aromatic ring generally produce a bathochromic

displacement in both the absorption and fluorescence spectrum. Thus the spectra of toluene are found to be displaced towards the red when compared with those of benzene.

Monosubstituted compounds containing OH, OCH_3, NH_2, $N(CH_3)_2F$ and CN are fluorescent, while those containing $NHCOCH_3$, Cl, Br, I, CO, NO_2, SO_3H and COOH are likely to be weakly or non fluorescent. The presence of bromine, iodine or heavy metal substituent in either mono or poly-substituted aromatic compounds invariably leads to a reduction in fluorescence by encouraging intersystem crossing from the excited state to the triplet state. The presence of NO_2 also reduces fluorescence, whether the molecule is simple or complex.

2.4 Instrumentation of fluorescence spectroscopy

Figure 2.4 below shows a schematic diagram of a typical fluorescence spectrometer that contains a light source, excitation monochromator, sample cell, emission monochromator, detector and recorder.

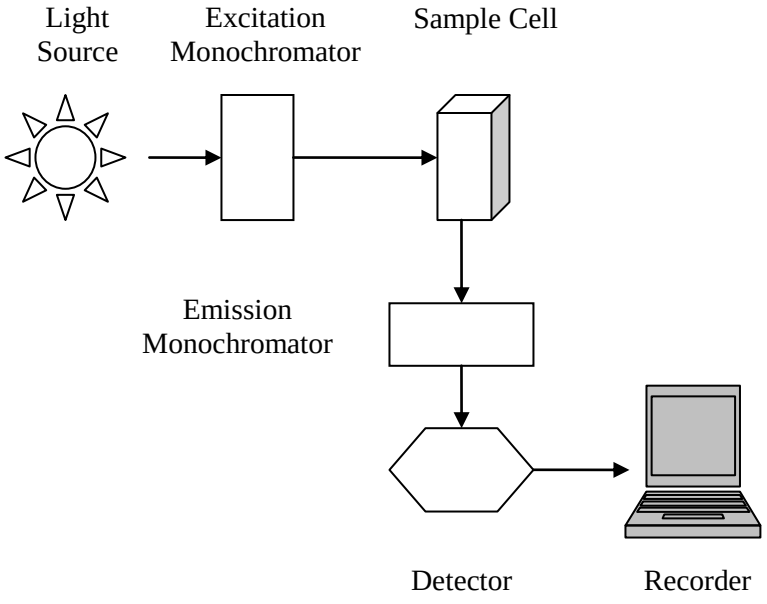

Figure 2.4: Schematic diagram of a typical fluorescence measuring devices

2.4.1 Light sources

There are a lot of type of excitation source can be used for spectroscopic studies including tungsten incandescent, mercury, xenon, hydrogen, and deuterium lamps. The type of light source chosen will depend primarily on whether a fluorescence excitation spectrum or emission is to be measured.[38]Most spectofluorometer make use of mercury-arc discharge lamps because of their high light intensity. Hydrogen discharge lamps also can be used. More recently, the xenon arc has been introduced.

2.4.2 Monochromators

Monochromator is used to isolate a narrow band of electromagnetic radiation from the source. It is a wavelength selector. There are two types of monochromator which are filters and grating.

There are three types of filter:

1. Neutral tint. These give a nearly constant transmission over a wide range. They are designed to decrease the intensity of the fluorescence signal uniformly and are used with strongly fluorescing compounds.
2. Cut off filters. This type of filter is used to cut off stray or unwanted radiation since it produces a sharp cutoff with little or no transmission on the other.
3. Bandpass filters. These are composite filters constructed from sets of cutoff filters. One part consists of a long-wavelength sharp-cutoff filter (blue and green series), and other is a short-wavelength cutoff filter (red and yellow series).

A grating consists of a large number of parallel lines or grooves ruled at extremely close intervals (e.g, 30,000 lines per inch) on a highly polished surface, such as aluminum.

A master grating is used as a mold in the production of replica gratings. A film of parting compound is applied to the master, the film is aluminized, the crevices are filled with epoxy resin, and an optical flat is bonded by epoxy to the aluminum replica of the master-grating pattern. When the epoxy hardens, the replica grating, completely anodized, is separated from the master.

When a beam of monochromatic light is focused on a transmission grating, each line acts like a source of this radiation. At certain points on the opposite side of the grating the wavelengths reinforce other. At other points there is destructive interference and darkness. The result is a series of bright lines with dark region between them. Since each wavelength has its own diffraction angle through grating, the polychromatic radiation passing through is separated into a spectrum.

2.4.3 Slits

The slits in a fluorescence instrument are used to determine the bandpass and have a reflective effect on the amount of light that is passed by monochromator to the next component in the optical system. The slit width determines the resolution of a system.

Generally, there are three types of slits commonly used in fluorescence instruments: fixed slits, continuously variable unilateral slits, and continuously variable bilateral slits.

Fixed slits: a piece of metal is placed with a slit etched into it in the light path. The slit width is fixed by the manufacturer to provide a bandpass of such as 5 nm, 10 nm and etc. This arrangement is convenient and reasonably inexpensive, but reduces the flexibility available to the user.

Continuously variable unilateral slits: the slit can be selected in order to balances the sensitivity needs with the resolution requirement. In other words, if a given analysis requires a 9-nm bandpass and fixed-slit system is used, the analyst might have to use 5-nm

slits. Fortunately, in this system, the analyst could open the slits to 9 nm, thus improving the sensitivity. Continuously variable slits are more expensive and normally found in research grade units.

Continuously variable bilateral slits: in this system, both jaws will move as the analyst changes the slit width, while in unilateral system only one jaw moves. The bilateral system in better, as it will ensure that the image is focused directly on the center of the sample (for excitation monochromator) or the central point of the detector.

2.4.4 Sample cells

Pyrex cells are useful for measurements above 320 nm (which compose 95% of all common analyses). Only below 320 nm are quartz or fused-silica cells required. Hence for all practical purposes the large additional cost of quartz or fused silica is not justified.

Below 320 nm, where quartz is necessary, the researcher should consider the properties of the different kinds of quartz. Corning quartz possesses a lower native fluorescence than do other varieties. However, even this quartz possesses sufficient fluorescence to be detected on the spectrofluorometer at high sensitivity (λ_{ex} = 265 and 330 nm; λ_{em} = 500nm). Fused silica is preferred over fused quartz. Care should be given to the selection of sample cuvettes, especially with respect to scratches and surface flaws.

2.4.5 Detectors

Fluorescence spectrometer use photomultilier (PMT) as the detector. These devices include a photoactive material which creates a current. The photomultiplier has a dynode chain to amplify the signal; typically 6-12 dynodes are present. The potential across a photomultiplier may range up to 1000 V. The user should ensure that the voltage across the PMT is at least enough to maintain the PMT in the linear range. This value is dependent on the PMT but is usually 400-500 V. As the high voltage on the PMT increased, the signal

increases as does the noise (the increase is not linear). At high voltages the noise from a PMT can become very significant. It is important to ensure that the PMT does not saturate (i.e., is blasted by a very strong light source), especially when the high voltage is on, because it will exhibit a very noisy response for many hours. In many systems, the electronics automatically turn off the high voltage if the current rises above a preset level.

2.5 Objectives of the project

The aim of this project is to synthesis derivatives of quinoline starting with halogenated quinoline as the starting material react with various amines to form *N*-alkylamino and *N*-arylamino derivatives. The second part of the project is to investigate the fluorescence characteristics of all the compounds prepared with respect to:

i. solvents

ii. pHs

iii. concentrations

iv. time