CHAPTER 2: FLUORESCENCE SPECTROSCOPY

2.1 Introduction

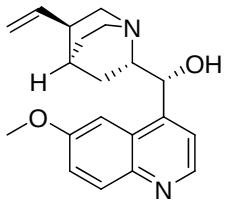
During the past 20 years there has been a remarkable growth in the use of fluorescence spectroscopy in the biological and chemical sciences and become among the most useful tools in experimental biology and chemistry. The measurements can provide information on a wide range of molecular processes, including the interactions of solvent molecules with fluorophores, conformational changes and binding interactions. This emphasis has changed and the used of fluorescence has been expanded.

Fluorescence is now used extensively in biotechnology, medical diagnostics, DNA sequencing and hybridisation[74], forensic and genetic analysis. The usefulness of fluorescence is being expanded by advances in technology for cellular and molecular imaging.[75,76] Fluorescence imaging can reveal the localisation and measurements of intracellular molecules and also single molecule detection.[77,78]

Fluorescence detection is highly sensitive, and there is no longer the need for the expense and difficulties of handling radioactive tracers for most biochemical measurements. No other instrumental methods available at comparable cost, can equal or surpass fluorimetry in analytical sensitivity. Concentrations of luminescing materials as low as 10^{-9} molar are routinely determined. This aspect is particularly desirable in the biomedical sciences where low concentrations of drugs, metabolites and toxins in blood serum and urine must routinely be monitored.

2.2 Historical development

The first observation of fluorescence from a quinine **(18)** solution in sunlight was reported by Sir John Frederick William Herschel in 1845.[79] It is evident from this early description that Sir Herschel recognised the presence of an unusual phenomenon that could not be explained by the scientific knowledge at that time.



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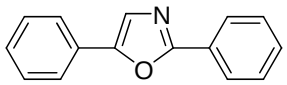
It is interesting to notice that the first known fluorophore, quinine **(18)**, was responsible for stimulating the development of the first spectrofluorometers that appeared in the 1950s. During World War II, the Department of War, USA was interested in monitoring antimalaria drugs, including quinine **(18)**. This early drug assay resulted in subsequent programmes at the National Institutes of Health, USA to develop the first practical spectrofluorometer.[80]

Instead of quinine, many other fluorophores are encountered in daily life. 2, 5-Diphenyloxazole **(19)** or PPO, one of the earliest fluorescent compounds known, was synthesised in 1896 by Emil Fischer[81], and today it is recognised as an efficient solute in scintillation solutions. Polynuclear aromatic hydrocarbons, such as anthracene **(20)** and perylene **(21)**, are also fluorescent and the emission from such species is used for environmental monitoring of oil pollution. An important feature of fluorescence spectrometer is its high sensitivity detection. The sensitivity of fluorescence was used in

1877 to demonstrate that the rivers Danube and Rhine were connected by underground streams.[82] This connection was demonstrated by placing fluorescein into the Danube which later its characteristic green fluorescence appeared in a small river that led to the Rhine. Fluorescein **(22)** was synthesised by Baeyer[83] in 1867.



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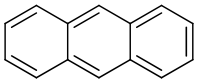

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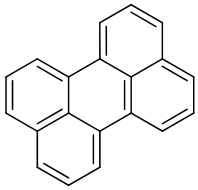

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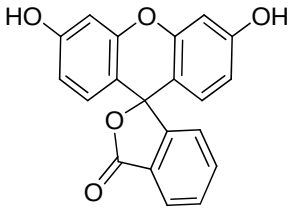

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The mechanism of the absorption-emission process was described by Sir George Stokes in 1852. He applied the scientific method to fluorescence and developed the 'Stokes Law of Fluorescence', which dictates that the wavelength of fluorescence emission must be greater than that of the exciting radiation. He was also responsible for naming fluorescence after the mineral fluorite (fluorspar), which exhibits a bluish-white fluorescence under ultraviolet irradiation. He published his historic paper on fluorescence of quinine **(18)**, chlorophyll and other plant materials in 1852.[84]

2.3 Theory of fluorescence

Fluorescence occurs in molecules as a result of a subsequent to a series of physical phenomena, normally beginning with the absorption of light. This phenomenon is derived from the electromagnetic nature of light, molecular electronic structure and the nature of the environment of the luminescent molecule. It is fairly obvious that an appreciation of this phenomenon is necessary for the understanding of the relationships between molecular structure and luminescence spectroscopy to chemical and biological problems.

When light impinges upon matter, two things can happen. It can pass through the matter with no absorption taking place, or it can be absorbed either entirely or in part. Later, the energy is transferred to the molecule in the absorption process. The quanta-energy relationship can be expressed by the equation,

$$E = h\nu = \frac{hc}{\lambda}$$

where h, ν, c and λ are respectively, Planck's constant, the frequency of radiation, the velocity of light and the wavelength.

When light from external source hit the molecules and the energy absorbed is sufficient, the molecules may be excited by absorption of a photon to produce a transition from the ground state to an excited state. This process is known as excitation. Under normal conditions, the surplus energy of an excited molecule is invariably lost, with an ultimate return to the lowest vibrational level of the ground state. Several mechanisms may be involved in this process.

From this point, the molecule may return to the ground state by emission of photon (fluorescence), or by generation of heat (internal conversion), or may change to an excited triplet state (intersystem crossing) and then return to the ground state by emission of photon (phosphorescence), or may undergo chemical change. The processes that occur between the absorption and emission of light are usually illustrated by the Jablonski[85] diagram as shown in Figure 2.1.

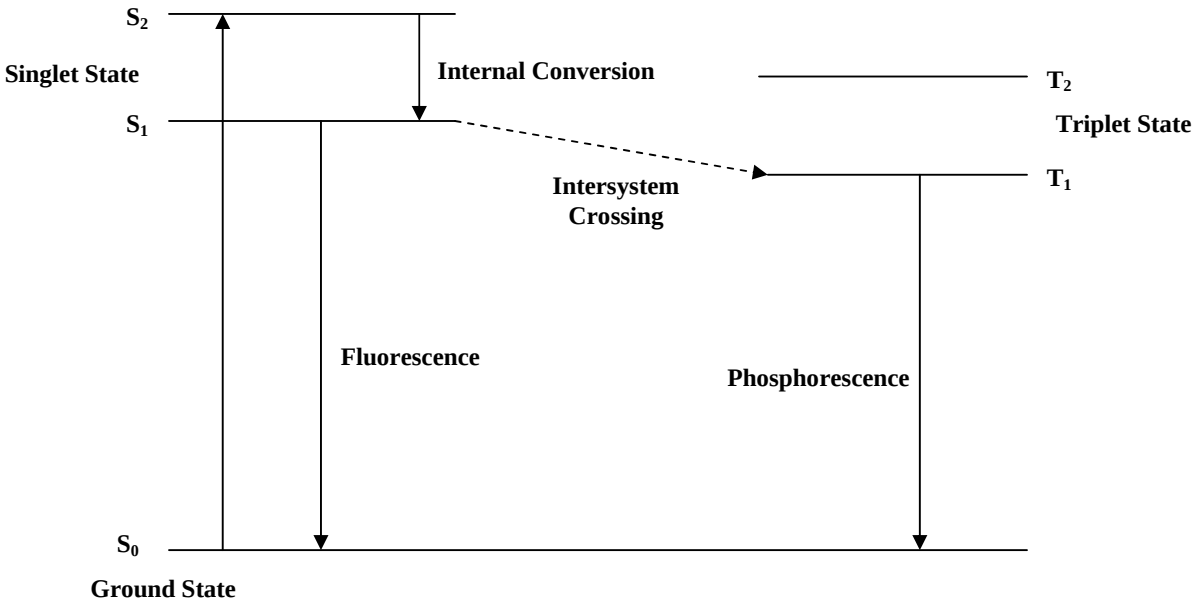


Figure 2.1: Jablonski diagram

Fluorescence is almost always the result of a transition between the lowest energy level of the first excited state (S_1) and some of the ground state (S_0). The part of the molecule responsible for the fluorescence is known as the fluorophore. The lifetime of an excited singlet state, and therefore the decay time of fluorescence is in the range 10^{-9} to 10^{-8} seconds.

The quantum energy of the emitted photon is equal to the difference in energy between these two levels. It follows that the quantum energy and therefore wavelength of the emission are independent of the wavelength of the photon producing excitation. Examination of the Jablonski diagram reveals that the energy of the emission is typically less than that of absorption. Because of that, the wavelength of the emission is longer than that of excitation. This phenomenon was first observed by Sir G. G. Stokes in 1852 at the University of Cambridge.[84]

From the Jablonski diagram, although it is rare for molecules to enter an excited triplet state directly from the ground state, in many molecules there is an efficient process whereby an excited singlet state may be converted to an excited triplet state. This process is called intersystem crossing. It is immediately followed by vibrational relaxation whereby the molecule falls and return to the ground state from the triplet state by emission of a photon. This is called as phosphorescence. Phosphorescence decay is very much longer, typically milliseconds to seconds, therefore the wavelength of phosphorescence is generally longer than fluorescence.

Another process which has similar spectral but different temporal distribution[86] is called delayed fluorescence. The delay is due to a double intersystem crossing, from singlet to triplet and back to singlet. It is a form of luminescence which takes place over a time scale similar to the phosphorescence but otherwise has the nature of fluorescence.

2.4 Fluorescence properties of organic compounds

The fluorescence of a molecule is dependent upon the structure of the molecule and upon the environment in which it is situated. Many organic molecules are fluorescent. In relation to organic compounds, fluorescence is restricted to compounds possessing a fairly large conjugated system, in which electrons less strongly bound than σ electrons, can be promoted to π* antibonding orbitals by absorption of photon energy without extensive disruption of bonding as shown in Table 2.1.

Table 2.1: Transitions involved in the absorption process[101]

Transition	Molar Absorptivity	Comments
$n \longrightarrow \pi^*$	100	Observed in fluorometry
$\pi \longrightarrow \pi^*$	12000	Observed in fluorometry
$\sigma \longrightarrow \pi^*$	200	Not observed (high energy)

Furthermore, π to π* transitions in most aromatic hydrocarbons are strongly allowed. The combination of these two factors signifies that aromatic compounds possessing low lying (π, π*) singlet states usually fluoresce strongly. This means that the majority of usefully fluorescent organic compounds are aromatic or heterocyclic. Fluorescence commonly does not occur in compounds that have an n, π* singlet state as the lowest excited singlet state. The reason for this is that almost complete intersystem crossing takes place from the lowest n, π* singlet state to a triplet, from which phosphorescence occurs. There are several factors to be considered in the study of the fluorescence of organic compounds.

2.4.1 Environmental effects

A large number of environmental effects are of importance and only several effects such as nature of solvent, pH, oxygen and presence of other solutes will be discussed here. These multiple effects provide many opportunities to probe the local environment surrounding a fluorophore. However, it can be difficult to know which effect is dominant in a particular experimental system, and typically more than one effect will simultaneously affect the fluorophore.

2.4.1.1 Solvent effects

Although the fluorescence of gases[87,88] and solids[89] have been measured, the vast majority of fluorescence spectra have been measured in solution. Solvent effects are observed in fluorescence measurements which manifested by wavelength shifts or quenching of fluorescence. Fluorescence solvent effects are less well known, nevertheless, several groups of compounds are known in which change of solvent brings about remarkable fluorescence changes.

An important aspect in the examination of solvent effects in absorption and fluorescence spectra is the Franck-Condon principle.[90] In the original Franck-Condon principle, after the electronic transition, the molecules which end up in higher vibrational states immediately begin to relax to the lowest vibrational state. In the case of solvation, the solvent molecules will immediately try to rearrange themselves in order to minimize the interaction energy.

It follows from the Franck-Condon principle that there may be a difference in solvation energy of the ground and excited states and this will be reflected in changes in absorption and emission spectra. Figure 2.2 illustrates the Franck-Condon principle applied to solvation.

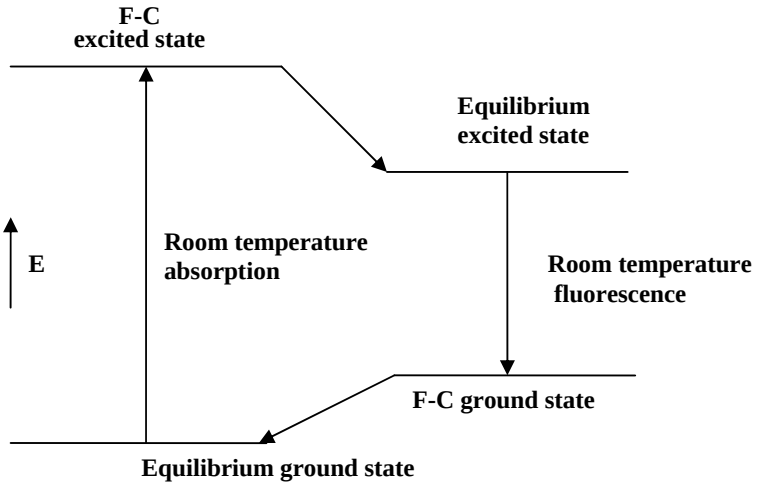


Figure 2.2: Schematic representation of equilibrium and Franck-Condon (F-C) electronic states

In most polar molecules, the excited state is more polar than the ground state. Hence an increase in the polarity of the solvent produces a greater stabilisation of the excited state than of the ground state. Consequently a shift in both absorbance and fluorescence spectra to lower energy or longer wavelength is usually observed as the dielectric constant of the solvent increases. The greater the polarity of the solvent, the lower will be the energy of the Franck-Condon excited state. This type of behaviour is characteristic of most π to π^* and intramolecular charge-transfer transitions.

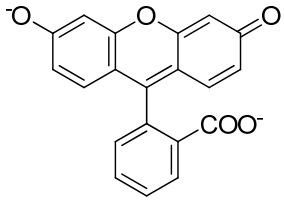
The effect of the dielectric constant of the solvent was used in explaining some fluorescence solvent effects.[91-94] Besides the dielectric constant, hydrogen bonding has been invoked in other instances.[95,96] For example, chlorophyll, in non polar solvents such as benzene shows very little fluorescence.[97] Addition of water, a polar solvent, causes a remarkable enhancement of fluorescence. This phenomenon is probably due to hydrogen bonding between the polar solvent.[98]

Hydrogen bonding and the dielectric constant of the solvent are frequently involved to explain the solvent-fluorescence relationship. However, these effects are highly complex because it may either reinforce or cancel one another and are largely unpredictable. Thus, more studies need to be done to understand this phenomenon.

2.4.1.2 Influence of pH

The basicity or acidity of a molecule is determined by its electronic structure and this may undergo detailed changes during excitation from the ground state by the absorption of light. A difference in the basicity or acidity of the ground and excited states will be reflected in differences between the absorption and fluorescence spectra with change in pH. The nature of the changes will depend on whether the basicity is increased or decreased during ionisation.

The effect of pH is particularly strong in some compounds, such as fluorescein, which can therefore be used as pH indicators because of a marked change from strong to weak fluorescence or a change in colour according to pH. The structure of the fluorescein molecule in alkaline or neutral environment **(23)** differs markedly from its structure in acid environment **(22).**[47]



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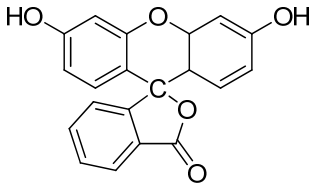

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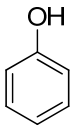
Most compounds of biological interest are ionisable and can therefore exist in aqueous solution in various stages of dissociation, depending upon the hydrogen ion concentration. Williams[99] has reported their observations on the relationships between molecular dissociation and fluorescence. The fluorescence characteristics of some monosubstituted benzene at different pH are shown in Table 2.2.

Table 2.2: Characteristics of some monosubstituted benzene at different pH

C_6H_5R	Excitation maxima (nm)	Fluorescence maxima (nm)	pH at which fluorescence is	
			Maximal	Zero[a]
OH	270	310	1	13
OCH_3	270	300	1-14	
NH_2	280	340	7-10	1
$NHCH_3$	290	360	7-10	1
NO_2, COOH, $N(CH_3)_3$+, $NHCOCH_3$	nonfluorescent at all pH values			

[a] In some instances fluorescence may be minimal rather than zero.
Data taken after Williams[99]

It has been reported that phenol **(24)** fluoresced maximally at pH 1 and the fluorescence diminished as the pH is raised and is essentially zero at pH 13.[99] This suggests that the fluorescent species is the unionized phenol molecule. The phenolate ion **(25)**, in aqueous solution, does not fluoresce when excited at any wavelength.[99]



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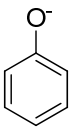

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2.4.1.3 Effect of oxygen

One of the infamous quenchers is molecular oxygen, which causes a reduction in fluorescence and completely abolishes phosphorescence. Quenching is the reduction in fluorescence by a competing deactivating process resulting from the presence of other molecules in the system. It has been reported that oxygen in solution at a concentration of 1 mM typically reduces the fluorescence intensity by about 20%.[100]

Dissolved oxygen molecules which have a large diffusion coefficient are very efficient at quenching the excited-singlet states of organic molecules, especially in polar solvents. While the ground state of oxygen is a triplet but it is no longer believed that "paramagnetic" effects play a generally important role in oxygen quenching. This has been confirmed by Laser-Flash Spectroscopy.[101]

Quenching by oxygen[102] is thought to occur by intersystem crossing. An encounter with a triplet oxygen molecule is thought to cause the excited singlet state to become an excited triplet. This does not mean that dissolved oxygen can enhance the sensitivity of phosphorometry, since oxygen is also a very effective triplet quencher.[103] Since the triplet state has a much longer life time than the excited singlet, it is much susceptible to collisional quenching processes involving impurities such as oxygen itself or impurities produced by photodecomposition of the solute.

2.4.2 Chemical structure

In order to effectively utilise fluorescence as an analytical tool, it is necessary that the researchers know the basic effects of structure on the emission process. Theoretical organic chemists are making more and more use of fluorescence in their attempts to elucidate molecular structure and mechanisms of molecular interaction.

As for analyst, a most practical reason for studying fluorescence-structure relationships is to enable the person to predict which molecules will fluoresce, under what conditions they will fluoresce and what their fluorescence characteristics will be.

2.4.2.1 Carbon skeleton

In liquid solution, most unsubstituted aromatic hydrocarbons exhibit quite strong fluorescence in the ultraviolet or visible region. As the degree of conjugation increases, the intensity of fluorescence often increases and shift to longer wavelengths (bathochromic shift) .Table 2.3 shows luminescence properties of selected condensed linear aromatics.

Table 2.3: Luminescence of Condensed Linear Aromatics in EPA[a] Glass at 77°K.[100] [a]A mixture of diethyl ether, isopentane and ethanol, 5:5:2 (v/v/v)

Compound	Excitation wavelength (nm)	Fluorescence wavelength (nm)
Benzene	205	278
Naphthalene	286	321
Anthracene	365	400
Tetracene	390	480
Pentacene	580	640

It can be seen from the table that the benzene (**26**) and naphthalene (**27**) fluoresce in the ultraviolet, tetracene (**28**) in the green and pentacene (**29**) in the red. It shows that increasing the size of conjugated system usually results in shifting of absorption as well as the fluorescence wavelength towards the red end of the spectrum.[100]

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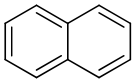

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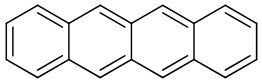

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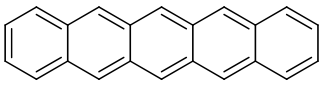

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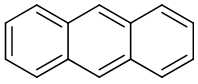
Generally, linear ring systems tend to fluoresce at longer wavelength than nonlinear systems. This can be seen in the case of anthracene (**30**), which has a fluorescence efficiency higher than 1,2-benzathracene (**31**).[104] This is probably because in the linear ring system there is a free flow of π electrons around the molecule, while in nonlinear systems the rings act as some sort of barrier or obstacle to the π electrons to move.



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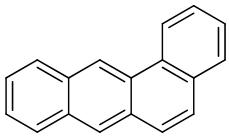

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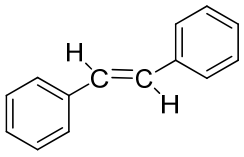
2.4.2.2 The geometrical arrangement of the molecules

Aromatic compounds consisting of two aryl groups separated an alkene chain, for example stilbene exhibit cis-trans isomerism. In such systems it is usually observed that the planar trans isomer **(32a)** is intensely fluorescent, whereas the cis isomer **(32b)** is non-fluorescent. It can be seen in **(32b)** that the cis isomer cannot have a planar configuration since molecular overcrowding of its ortho-hydrogen occurs.[105]



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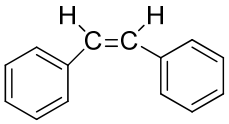
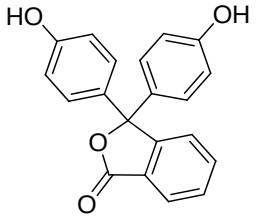


32b

The molecular rigidity also plays an important role. The most strongly fluorescent aromatic compounds are usually characterised by rigid, planar structures. For example, fluorescein **(22)** has intense green fluorescence (highly fluorescent), whereas the phenolphthalein **(33)** is non-fluorescent. The fluorescein **(22)** molecule has

a large, planar, conjugated region, whereas phenolphthalein has three smaller conjugated regions which are not coplanar.



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2.4.2.3 The type and positions of substituents

The nature of substituent groups especially chromophoric ones plays an important role in the nature and extent of a molecule's fluorescence. Substitutions may alter the fluorescence of a parent molecule by their effect on the location, quantum yield and lifetime of fluorescence. Substituent effects on the chemical and physical properties of organic molecules in their ground electronic state constitute a lively area of investigation at present. Furthermore only little is known about the influence of substituents on the behaviour of excited states.

One of the best studied examples to determine the effects of substitution on spectra is that of benzene. Alkyl substitution on both benzene and naphthalene causes small red shifts of the absorption bands and so correspondingly for the fluorescence. The quantum efficiency of toluene and the xylenes is higher than benzene. Multiple alkyl substitution causes differences in fluorescence quantum yields that again depend on the relative positions of substitutions.[106]

The monofluoro derivatives of aromatic hydrocarbons in general have their emissions near the same wavelength and their intensities are approximately the same as the parent hydrocarbon. For benzene, the fluorescence is shifted to higher wavelength and the intensity progressively decreases when going to chlorine and bromine as substituents. The iodobenzene compound is not fluorescent.[107] This is due to the probability of radiationless transitions to the lowest triplet state (intersystem crossing) increases with the atomic number of the halogen. A simple generalisation is that ortho-para directing substituents often enhance fluorescence, whereas meta directing group reduce it.

The effects of substituents on the fluorescence of aromatics are summarised in Table 2.4. It can be seen that substituents which enhance π electron mobility will increases fluorescence intensity, whereas those decreases it, reduces fluorescence. Therefore, in general, electron donating substituents tend to enhance fluorescence while electron withdrawing substituents tend to reduce it.

Table 2.4: Effects of Substituents on the Fluorescence of Aromatics[100]

Substituent	Effect on frequency of emission	Effect on intensity
Alkyl	None	Very slight increase or decrease
OH, OCH_3, OC_2H_5	Decrease	Increase
COOH	Decrease	Large decrease
NH_2, NHR, NR_2	Decrease	Increase
NO_2, NO	Large decrease	Large decrease
CN	None	Increase
SH	Decrease	Decrease
F ↓ Cl ↓ Br ↓ I	Decrease	Decrease
SO_3H	None	None

2.5 Instrumentation of fluorescence spectroscopy

Fluorescence measurements fall into two categories, the measurement of the emission spectra, called fluorimetry, and measurement of the time dependence of the emission, called fluorometry.[108]

All fluorescence instruments consist of light source, wavelength selectors, sample compartment and detector system. A schematic representation of essential components of fluorescence spectrometer is shown in Figure 2.3.

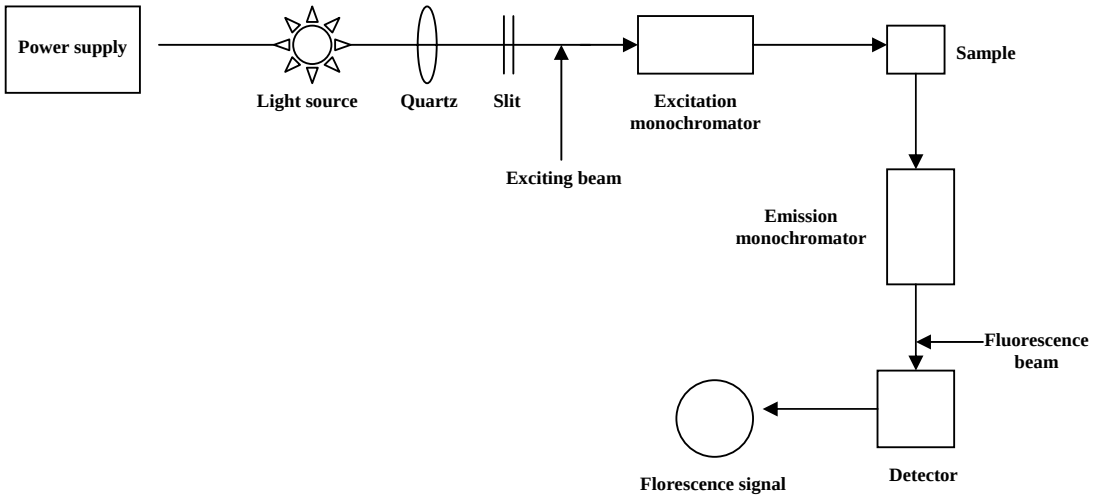

Figure 2.3: Essential components of fluorescence spectrometer

The light source produces light photons over a broad energy spectrum, typically ranging from 200 to 900 nm. Photons impinge on the excitation monochromator, which selectively transmits light about the specified excitation wavelength. The transmitted light passes through adjustable slits, then into the sample cell causing fluorescent emission by fluorophors within the sample. Emitted light enters the emission monochromator, which is positioned at a 90° angle from the excitation light path to

eliminate background signal and minimise noise due to stray light. Finally, the emitted light entering the photomultiplier tube which the signal is amplified and creates a voltage that is proportional to the measured emitted intensity.

In principle, the greatest sensitivity can be achieved by the use of filter or monochromator, together with the highest intensity source possible. In practice, to realise the full potential of the technique, only a small band of emitted wavelengths is examined and the incident light intensity is not made excessive, this is due to minimise the possible photodecomposition of the sample.

2.5.1 Light sources

Several types of light source are available for spectroscopic studies such as tungsten incandescent, mercury, xenon, hydrogen and deuterium lamps. Since the total fluorescence observed is proportional to the intensity of the source excitation, it is necessary to have a significant amount of energy available in the absorption region of the sample to be determined.

The type of light source chosen depends primarily on whether a fluorescence excitation spectrum or fluorescence emission is to be measured.[109] The most common incandescent lamp used in spectroscopic studies is the tungsten lamp. The disadvantages of incandescent lamps are their limited useful output which is covering only the visible range. Tungsten lamps therefore are useful for excitation in the visible range. However, since many compounds are excited by ultraviolet and short wavelength visible radiation, the tungsten lamp has found very little use in fluorescence instrumentation.

Xenon arc lamp on the other hand, employ a source whose output is a continuum on which are superimposed a number of sharp lines, allowing any wavelength throughout the ultraviolet and visible region of the spectrum to be selected. It is also the brightest and most powerful sources of ultra violet radiation.[100] In this particular work, a pulsed xenon was used because it produced a high output using a low voltage, resulting in longer lamp life with minimal ozone and heat production. Moreover, the pulsed source reduces potential photobleaching of the sample during analysis by several orders of magnitude over continuous sources.

2.5.2 Monochromators

The purpose of the monochromator is to disperse polychromatic or white light into the various colours or wavelengths. It is a wavelength selector. The performance specifications of a monochromator include dispersion, efficiency and stray light levels. Dispersion is usually given in nm/mm and this can be accomplished using prisms or diffraction gratings.

The monochromators in most spectrofluorometers use diffraction gratings rather than prisms. Prisms are not used in spectrofluorometers instruments because they give their greatest dispersion in ultraviolet and not the visible where most measurements are made. In addition, a larger prism would be needed to obtain adequate sensitivity and this would be very expensive.

Two types of monochromator are used in luminescence equipment, filters and gratings. Filters allow a relatively wide range of wavelengths to excite the sample and to pass through to the photodetector. Meanwhile, monochromators which are usually diffraction gratings with slit arrangements inherently allow the passage of a smaller range of wavelengths which is ultimately determined by the optical characteristics of the diffraction grating. However, it is possible to allow a wider range of wavelengths to pass a monochromator by manipulation of the slits.

2.5.3 Slits

The slit can best be described as volume controls for the fluorescence intensity. For optimal instrumental performance, the excitation slit width automatically controls the sample photomultiplier tube voltage. This control provides an optimum signal-to-noise ratio as function of sample intensity.

The slit width is the most important factor in determining the resolution of the instrument. The distribution of energy as a function of wavelength for the light passing through the exit slit of a monochromator can be represented as an isoceles triangle, if the entrance and exit slits are of equal width as shown in Figure 2.4. The middle wavelength (peak transmittance) is called the nominal wavelength and is the value read on the dial of the instrument.

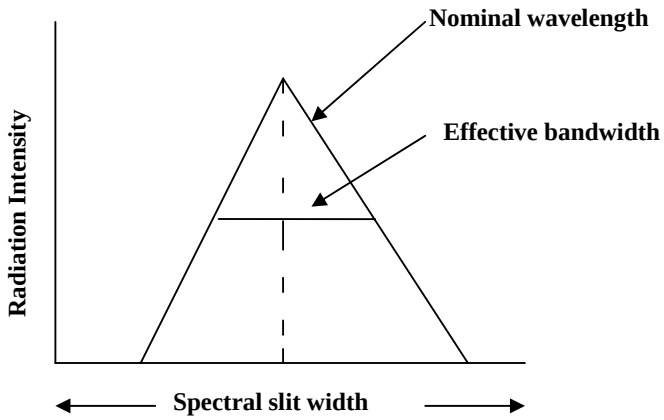

Figure 2.4: Distribution of radiant energy emerging from a slit as a function of wavelength[101]

The smaller the slit widths employed, the narrower is the range of spectral bandpass and the greater is the analytical selectivity. However, a smaller slit width results in a decrease in the intensity of the transmitted light and therefore results in a decrease in the analytical sensitivity. For weakly fluorescing samples it is advantageous to increase the bandpass and collect more light. For highly fluorescent samples the narrow bandpass is recommended to avoid exposing the detector to too high signal levels.

Generally, a wider slit setting causes higher fluorescence signal measurements. However, because of the fluorescence ratioing system used in the fluorescence machine, widening the excitation slit width will not increase the reported fluorescent signal ratio, but does increase sample fluorescence signal, resulting in an improved signal-to-noise ratio.

2.5.4 Sample cells

The majority of fluorescence assays are carried out in solution, the final measurement being made upon the sample contained in a cuvette or in a flowcell. The material of the sample cell must be fluorescence free. Cuvettes may be circular, square or rectangular, and must be constructed of a material that will transmit both the incident and emitted light. Square cuvettes, or cells were found to be most precise since the parameters of pathlength and parallelism are easier to maintain during manufacture. Figure 2.5 shows the method of measuring fluorescence. The cuvette reader excites the sample over the entire path length and reads the emitted light at right angles.

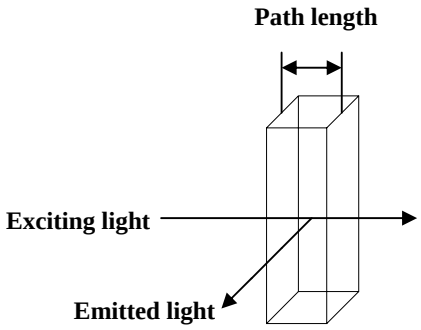

Figure 2.5: Cuvette Reader

Pyrex cells are useful for measurements above 320 nm which compose 95% of all common analyses. The pyrex cells are inexpensive compared to quartz or silica cells. For measurements involving ultraviolet radiation, quartz or silica cells are necessary. Parker[110] has shown that various types of quartz cuvettes emit an appreciable amount of fluorescence. In this work, quartz cells were used instead of silica and glass cuvettes. This is because those materials have some fluorescence which may contribute appreciably to routine measurements.

2.5.5 Detectors

All commercial fluorescence instruments use photomultiplier tubes as detectors because of their extreme sensitivity and fast response. A photomultiplier is a phototube capable of effecting photocathode emission with multiple cascade stages of electron amplification to achieve a large and linear amplification of primary photocurrent within the tube itself. Upon exposure to light, the electric current produced by a photomultiplier is then amplified by an amplifier to a measurable level.

The photomultiplier surface contains a thin layer of one or more elements possessing a low ionization potential. As a result, valence electrons are easily released when struck by photons. The alkali metals are commonly used and are plated on an Ag / Ag_2O cathode. Photomultiplier tubes can be made to respond to different wavelengths by varying the elements of the photosensitive surface.

The limit of sensitivity of a photomultiplier is normally governed by the level of dark current which is the signal derived from the tube with no light falling on it. A photomultiplier dark current is acquired prior to the onset lamp pulse and is subtracted from that pulse for correction of phototube dark current. The instrument measures and corrects every flash of the lamp to improve sensitivity at low levels of fluorescence, making it possible to measure samples in room light, thus freeing the user from working through septa in light-tight compartments.