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An Updated Review of Fluorescence Spectroscopy and Fluorescence Microscopy

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ABSTRACT

A fluorescence spectroscopy is an accurate, sensitive reproducible method for the analysis of pharmaceutical dosage form. Fluorescence Spectroscopy is quick and sensitive tool for analyzing molecular environment. The fluorimetry technique is chosen for its exceptional sensitivity, high specificity, low cost. The field of florescence Microscopy is rapidly developing, and it increase image capabilities. Many new technologies and techniques have been developed during last decade that allowed for deeper, faster and high resolution. Fluorimetry is widely established approach that is employed in wide range of applications including Industrial, Medical diagnostics, DNA sequencing. It is excellent tool for both qualitative and quantitative investigations. Fluorescence spectroscopy is also used for Determination of ruthenium, Determination of glucose, Study of marine petroleum pollutants, Determination of zinc, Determination of cadmium, determination of aluminum in alloys, etc. Fluorescence Indicators like Eosin, Quinine sulphate are also useful for analysis. Because there is a choice of wavelengths not only for the radiation produced, but also for the light that stimulates it, the process is exceedingly sensitive and specific.

1. INTRODUCTION

Fluorometry is superior to spectrophotometry in terms of sensitivity and specificity. Fluorescence has a 10-1000 fold better sensitivity than absorbance studies.[1]

In fluorescence spectroscopy, fluorophores play a crucial function. Fluorophores are the parts of molecules that make them glow.[2]

The use of fluorescent compounds almost exclusively in the search for single-molecule detection is an indication of fluorometry's potential sensitivity. Furthermore, fluorometric analysis can incorporate multiple factors at once, such as excitation and emission wavelengths, lifespan, and polarization, making it a unique and versatile analytical instrument. Changes in the fluorescent compound's microenvironment have an impact on the above factors.[3]

Fluorescence spectroscopy takes advantage of the phenomenon of electron excitation upon collision with high energy particles such as photons and other excited electrons, as well as photon emission while lowering their energy to the ground state.

Fluorescence spectroscopy is a sensitive optical emission technique that uses a photon source to excite sample molecules. hose molecules that relax as a result of radiant emission can be identified later by measuring the intensity of that emission.

1.1.WHAT IS FLUORIMETRY

An analytical method for detecting and measuring fluorescence in compounds or targets such as cells, proteins, or nucleotides, or targets previously labeled with fluorescence agents.

1.2.WHAT ARE FLUOROPHORES

The molecules showing fluorescence activity are termed as fluorophores. A fluorophore is a molecule that will absorb energy of a specific wavelength and reemit energy at a different wavelength. Fluorophores are primarily molecules with aromatic rings, such as Tyrosine, Tryptophan, and Fluorescein. Fluorophores are used as a physical marker for structural studies of macromolecules such as proteins and nucleic acids in biophysical studies. Fluorophores can be extrinsic, such as radioactive probes and dyes, or intrinsic, such as specific amino acids in protein chains. Extrinsic fluorophores are more expensive and require foreign intervention, whereas intrinsic fluorophores do not.

1.3. PRINCIPLE [1,2]

The fluorescence refers to emission of electrons from the singlet ground state to the singlet excited state caused by the absorption of UV or visible radiation. The absorption of energy by incident light rays on molecules causes conformational changes, which leads to vibrational relaxation (the lowest vibrational level). If the aromatic molecule is rigid and cannot relax vibrationally to the ground state, it will reach the ground state through light emission. Fluorescence is caused by the emission of light. The electron systems of these compounds absorb the incident light first. The ground state (S0) of the system's electrons is excited to the excited energy level (S1). Furthermore, these electrons change vibrational levels in the excited state. The electrons move to the lowest vibrational energy level of the excited state by thermal energy expenditure. The state is not stable; it emits energy in the form of UV or visible radiation and returns to singlet ground state. Because these molecules can fluoresce, electrons can jump from the lowest energy level of the excited state to different vibrational energy levels of the ground state by emitting specific quantum of energy in the form of light. The vibrational level structures in the ground and excited electronic states are reflected in the fluorescence excitation and emission spectra, respectively.

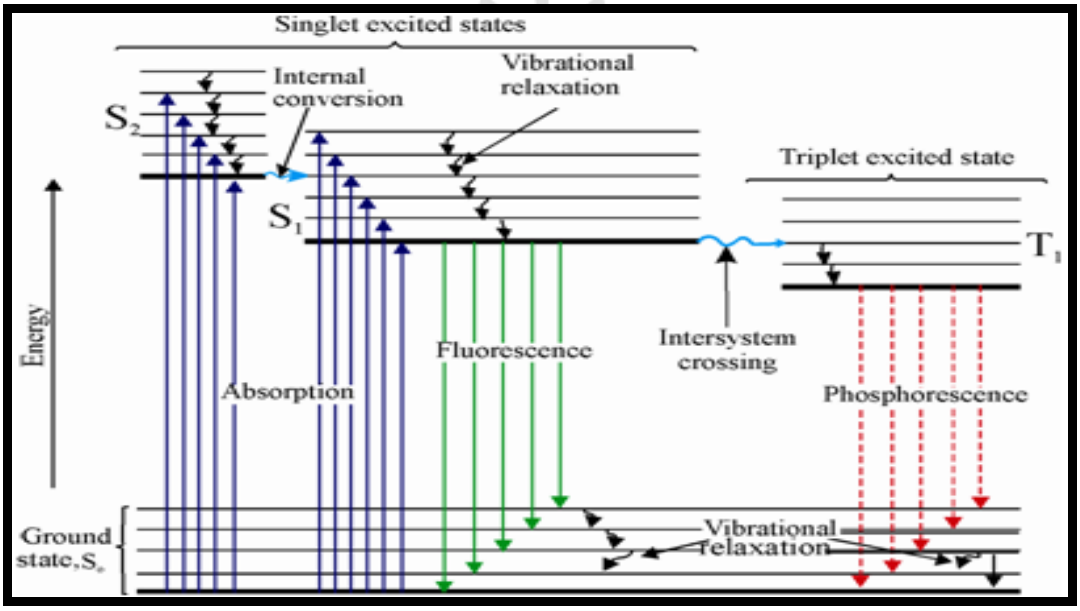

FIGURE 1 :- JANLONSKI DIAGRAM

Any fluorescent molecule has two characteristic spectra, the *excitation spectrum* and emission spectra. The excitation spectrum depicts the relative efficacy of various wavelengths of exciting light in causing fluorescence, whereas the emission spectrum depicts the relative

intensity of radiation released at different wavelengths. The wavelength of the exciting radiation has no effect on the shape of the emission spectrum.

1.4. PURPOSE [4]

A fluorometer, sometimes known as a fluorimeter, is laboratory equipment used to measure the quantity of fluorescent chemicals in biological samples. Fluorescence spectroscopy can also be used to obtain information about the structure of macromolecules. Fluorometry is a high-sensitivity technique that allows measurements in a wide range of samples at trace levels. In addition, the approach has a high selectivity, allowing for interference-free analysis of complicated sample matrices.

2. INSTRUMENTATION[2,4,5]

A fluorometer or fluorimeter is a device used to measure fluorescence parameters such as intensity and wavelength of emission spectrum. These factors aid in the detection of individual molecules in a medium, as well as their quantity. Modern fluorometers are capable of detecting fluorescent molecule concentrations as low as 1 ppm.

There are two basic types of fluorometers, the filter fluorometer and the spectrofluorometer. The difference between them is the way they select the wavelengths of incident light. A filter fluorometer makes use of filters, whereas a spectrofluorometer makes use of grating monochromators. Filter fluorometers are less expensive to buy/build, but they are less sensitive and have lesser resolution than spectrofluorometers.

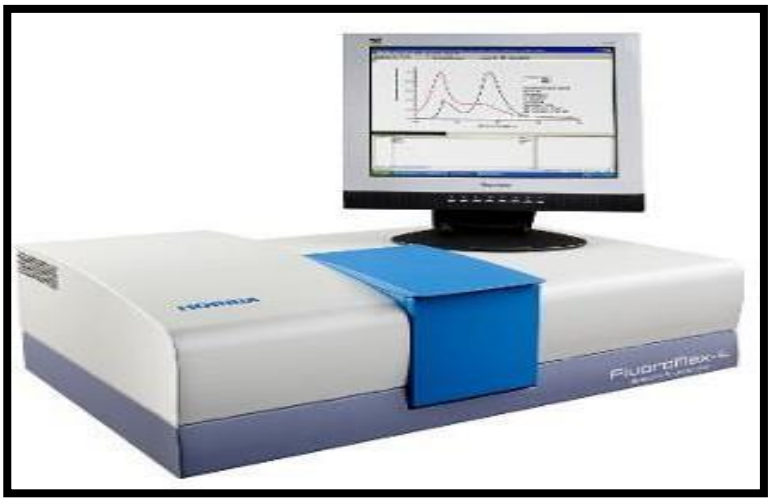

FIGURE 2 :- FLUORIMETER

The basic components of a filter fluorometer are similar to those of a photometer. It too makes use of filters to limit the wavelengths of the excitation and emission beams.

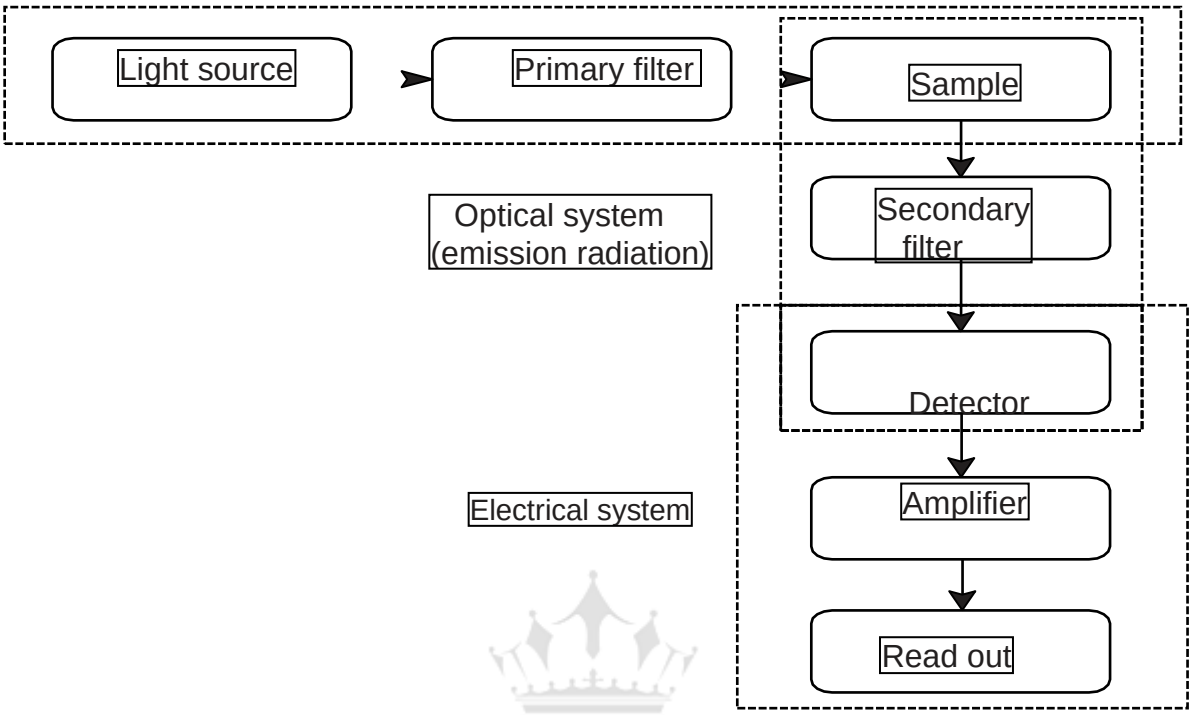

Fig 3 :- Schematic diagram of a typical fluorometer[5]

To limit the impact of variations and drift in source intensity and detector response, filter fluorometers typically employ a single beam arrangement with source intensity control.

Spectrofluorometer mainly consists of

A. Source of light

- Mercury vapor lamp
- Xenon arc lamp
- Tungsten film

B. Filters and monochromators

- Primary filters and secondary filters

- Excitation monochromators and Emission monochromators

C. Sample cells, Detectors

2.1 SOURCE OF LIGHT [4,5]

UV light is used to excite the molecules, so the excitation source is a high-pressure mercury discharge lamp /Tungsten lamp/ xenon arc lamp with a glass or fused silica envelope. The most relevant aspect of light sources for our present discussion is the useful wavelength range. In this regard, the xenon arc lamp is by far the most common light source in commercial instruments because it produces usable light ranging from ultraviolet to infrared. This range is more than adequate for most fluorescence studies on biological samples, because such studies are typically limited by the absorption characteristics of water at either end of this spectral range, as well as photodamage in the deep ultraviolet. An example of the light distribution from a xenon arc lamp, from 200 nm to 1100 nm, is shown in Figure 2. Xenon arc lamps provide significant illumination out to around 1300 nm. Obviously, the intensity of this light source is highly dependent on wavelength – a fact that has a significant impact on the excitation spectra.

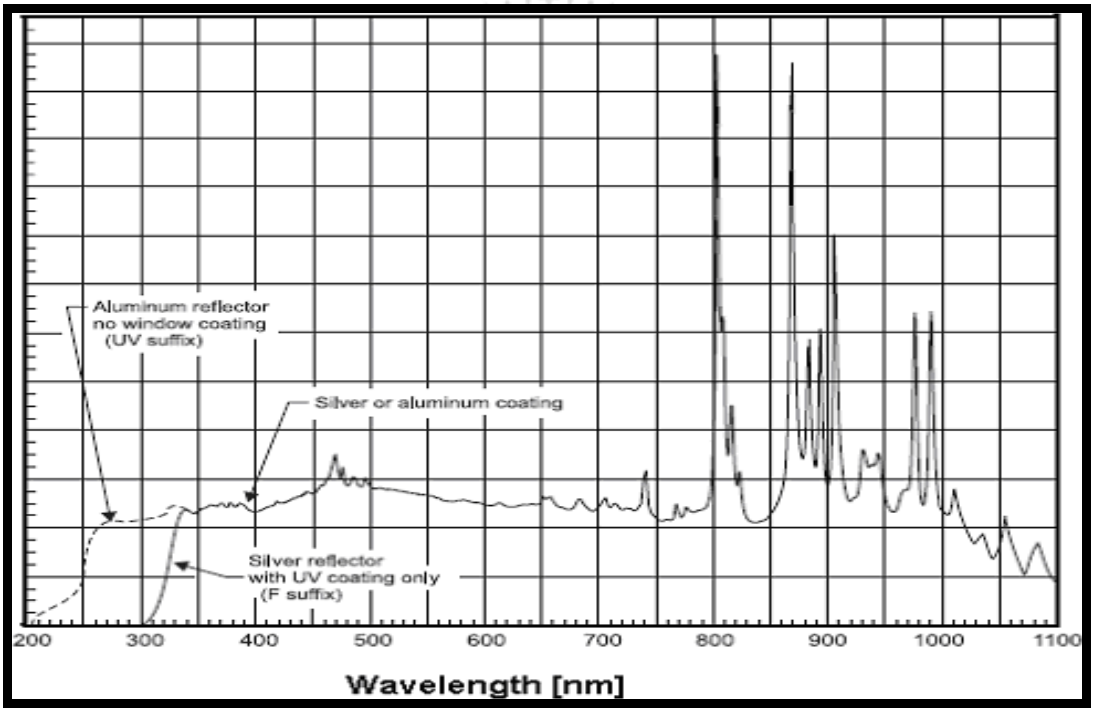

FIGURE 4:- SPECTRAL OUTPUT OF A XENON LAMP USED IN SPECTROFLUOROMETERS

These sources can produce intense radiation at 254 nm. The lasers or LEDs have also been used in some modern devices. Lamps emit a broad range of light, i.e., more wavelengths than those required to excite the compound, whereas LEDs and lasers emit more specific wavelengths. For example, blue LEDs emit radiation at 450–475 nm and are suitable forsome fluorescence studies.

The exciting wavelength is chosen by inserting a primary filter into the incident beam. This aids in the isolation of one of the main lines at 365, 405, 436, 546, or 579 nm, resulting in an intense line spectrum with high spectral purity. Interference and absorption type filters can be used in fluorometers for wavelength selection in both the excitation and fluorescence radiation path. Excitation filters that transmit a rather broad band of wavelengths are generally bandpass types.

2.2. FILTERS AND MONOCHROMATORS [5]

Monochromators are the most common and versatile devices for separating specific Monochromators work by dispersing incident light – most people are familiar with a prism's light-dispersing properties wavelengths of light from broad-band sources like xenon-arc lamps. The spectral region chosen by a monochromator is determined by the monochromator's design and, ultimately, by the physical size of the monochromator slits; the key consideration here is the monochromator's dispersion, which allows one to convert the physical width of a slit (e.g., in millimeters) to the FWHM of the spectral region passed.

Different monochromators of course have different dispersion factors but the common feature is that the smaller the slit, the higher will be the spectral resolution. This resolution comes at a cost, namely a drop in light intensity – a two-fold reduction in either slit width (entrance or exit) results in a light intensity reduction of approximately four-fold. Prism-based monochromators are not often employed in commercial spectrofluorometers, for a variety of reasons, one of which is that a linear scan of the prism assembly does not result in a linear dispersion of wavelengths.

The efficiency with which a monochromator transmits light will be wavelength and polarization dependent, regardless of the type of grating used.

2.3. SAMPLE CELLS[4]

Both cylindrical and rectangular cells constructed of glass or silica are employed in fluorescence measurements. Fluorometers typically employ 10 mm^2 cuvettes, 13 mm, or 25 mm test tubes. Because the size of the sample cell influences the measurement, the greater the cell's path length or diameter, the lower the concentration that can be read. Flow cells in the instrument sample chamber, through which the samples are pumped, are used for continuous online monitoring of samples for fluorescence studies. When sample volumes are limited, low volume microcells are used.

2.4.DETECTORS[5]

For detection and quantification of emitted light, most modern instruments employ photomultiplier tubes (PMTs). These devices are, of course, based on the photoelectric effect, which is the ejection of electrons from metallic surfaces as a result of incident light. The first phototubes used a basic arrangement to collect emitted photoelectrons and generate an electric current that could subsequently be measured.

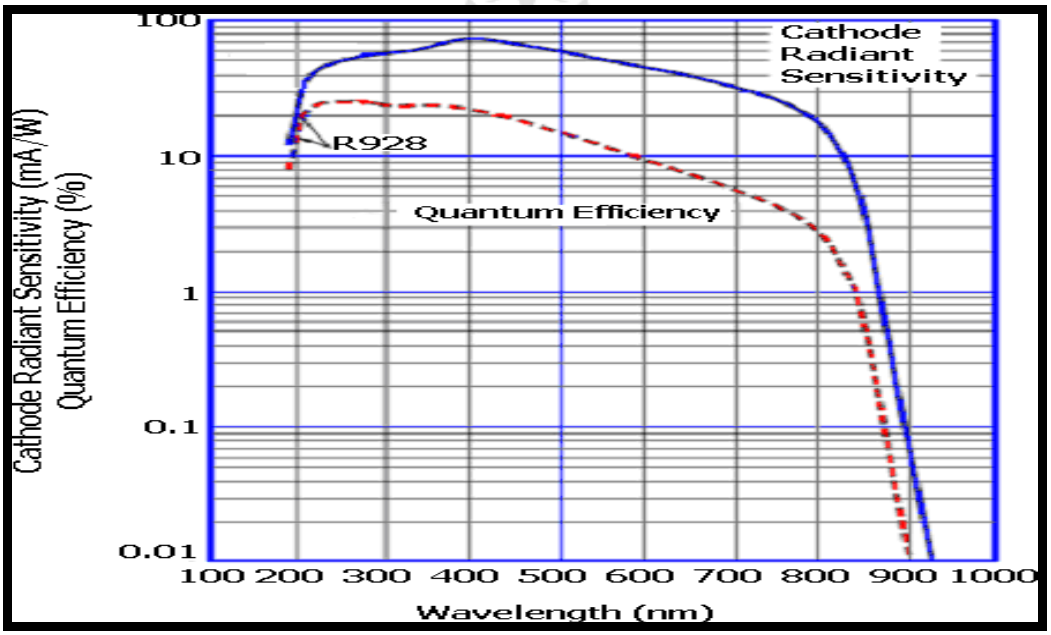

FIGURE 5:- SENSITIVITY AND QUANTUM EFFICIENCY FOR THE PMT MODE[5]

As a result, multiply the effect by a factor of ten (practical gains above 109 anode electrons per photoelectron can be achieved for short light pulses through continuous gains of around 107 are typical due to thermal loading in the final dynodes).

Significant progress has been made in the commercialization of PMTs with "extended red-response," which essentially means PMTs that can detect light at wavelengths above 800 nm.

3. FLUORESCENCE MICROSCOPY [2]

Fluorescence microscopy is a valuable tool for modern cell and molecular biologists, especially neurobiologists. It provides insight into the physiology of living cells at sub-cellular resolution. Fluorescence microscopy allows for the investigation of a wide range of processes, including protein localization and association, motility, and other phenomena such as ion transport and metabolism.

Modern fluorescence microscopy gives spatially accurate information on the location and density of fluorescent biomolecules on substrates and cells, but quantitative picture analysis is rarely done. The best use of fluorescence microscopy requires a basic understanding of the techniques' strengths and weaknesses, as well as an understanding of the fundamental trade-offs of the variables associated with fluorescent light collection.

In its most basic form, the ideal light microscopy experiment can be considered of as optimizing the competing properties of image resolution (in both the xy or lateral and z or axial dimensions), imaging speed (and/or acquisition time), and the amount of signal collected from the fluorescing sample. This is bounded by the limits imposed by photobleaching and/or phototoxicity. These factors are difficult to balance because of the limits, and they necessitate meticulous attention to detail and extensive empirical testing. Other secondary variables, such as the cost of necessary equipment and the difficulty of the procedure, can become important in addition to these basic variables.

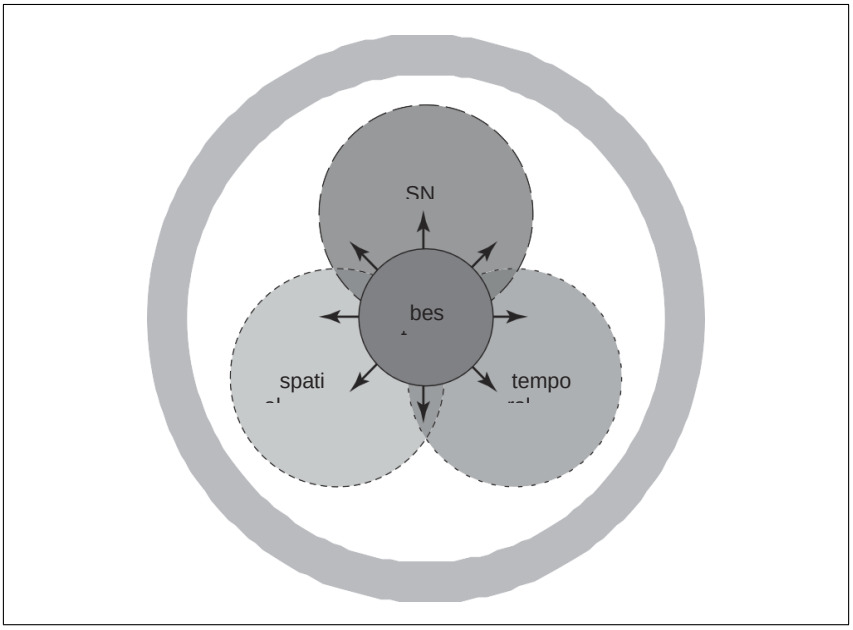

Fig 6 :- Diagram of some of the critical opposing factors in an imaging experiment [3]

3.1 Principle[6]

Fluorescence microscopy is a technique whereby fluorescent substances are examined in a microscope. It has a number of advantages over other forms of microscopy, offering high sensitivity and specificity.

In fluorescence microscopy, the specimen is illuminated (excited) with light of a relatively short wavelength, usually blue or ultraviolet (UV). A barrier filter is employed to analyses the specimen, which absorbs the short-wavelength light required for illumination while transmitting the fluorescence, which appears brilliant against a dark backdrop. Fluorescent constituents of the sample can be visible even in extremely small quantities since fluorescence is viewed as brightness on a dark background.

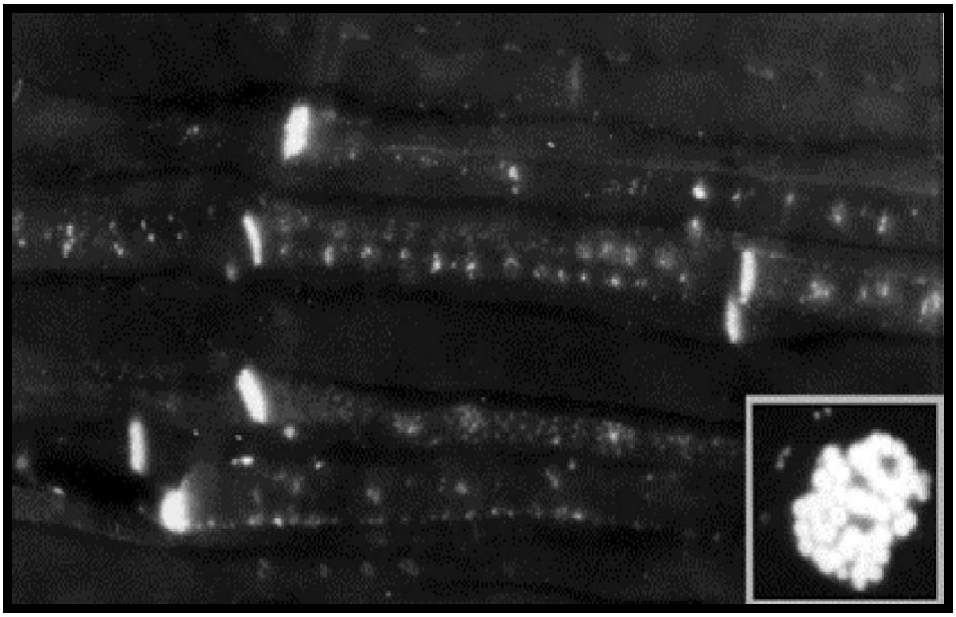

Fig 7 :- Fluorescence photomicrographs of a section of a plant stem[6]

In most modern fluorescence microscopes, epi-illumination is employed. This means that the light used for excitation is reflected onto the specimen through the objective, which acts as a condenser. Opaque or very thick objects can be examined using epi-illumination, even the skin of living people.

Fluorescence microscope is related to the specimen, to the microscope's optical system (particularly the filter combination) and to the observer's own optical and neurological characteristics.

The use of a narrow-band barrier filter, in particular, can be misleading because it makes everything appear the same color, whereas a wide-band or long (wavelength)-pass filter allows differentiation of different colors.

3.2 TYPES OF FLUORESCENCE MICROSCOPY

3.2.1. WIDE-FIELD FLUORESCENCE MICROSCOPY (WFFM) TECHNIQUE [7,8]

In its most basic form, wide-field fluorescence microscopy (WFFM) involves using a fluorescent light source, a microscope, excitation and emission filters, and an objective lens to excite the fluorophore(s) in the sample of interest. The resulting emitted light, which has a longer wavelength, is observed through the microscope eyepieces or by a camera, and is then computer digitized. The development of electron multiplied (EMCCD) and very low-noise, cooled CCD cameras have been one of the most significant achievements.

While maintaining high resolution, these cameras enable for fast detection of low-light fluorescence (EMCCD) or the steady accumulation of fluorescence signal to be integrated with little noise (cooled CCD). Both provide faster imaging and higher contrast at low signal levels, such as when the excitation light is intentionally reduced to avoid photobleaching or phototoxicity.

In addition to cameras, wide-field microscopy has also been improved by better light filters, mirrors, and objectives. Commercially available filters, have very high transmittance or reflection values enabled through new sputter-coating technologies.

Advantage: -

Wide-field microscopy has the advantage of constantly illuminating all portions of the picture, allowing for faster imaging. The rate of image acquisition, on the other hand, is normally dictated by the camera's or photomultiplier system's acquisition capability. A major advantage of the wide-field microscope is the low cost, simplicity, and flexibility of the system.

Disadvantage: -

Wide-field microscopy, on the other hand, has some drawbacks, such as low image resolution, the possibility of shading artifacts due to uneven illumination, and the need to align different cameras to ensure pixel registration when using multiple indicators.

3.2.2 MODERN CONFOCAL MICROSCOPY[7,8]

Many research efforts are seriously affected by the use of thin or sparse specimens, but when specimen thickness is added, increased out-of-focus light severely hinders imaging.

Fortunately, laser-scanning confocal microscopy can provide a solution (LSCM). The laser scanning confocal microscope (LSCM) remains a key piece of equipment in most imaging laboratories. Most modern LSCM systems offer a variety of advantages and are equipped with software to perform complex 3D ,4D or even 5D experiments.

These microscopes frequently include software to aid in the acquisition of data for complex methodologies such as spectral deconvolution, fluorescence recovery after photobleaching (FRAP), and fluorescence resonance energy transfer (FRET).

All of the major concepts of fluorescence excitation and emission also apply to LSCM. The major differences are the excitation source (a bright point source, i.e., laser), a sequential

scanning method of the point source of illumination and the detection of emitted light intensity with a photomultiplier tube (PMT).

Confocal microscopy's purpose is to eliminate out-of-focus light from the image. A pinhole aperture (Fig.) ensures that light reaching the detector only comes from the same (confocal) spot in the specimen where the excitation light was focused.

An image can be systematically built, pixel by pixel, by recording the fluorescence intensity at each spot by scanning the confocal excitation and detection point over the object.

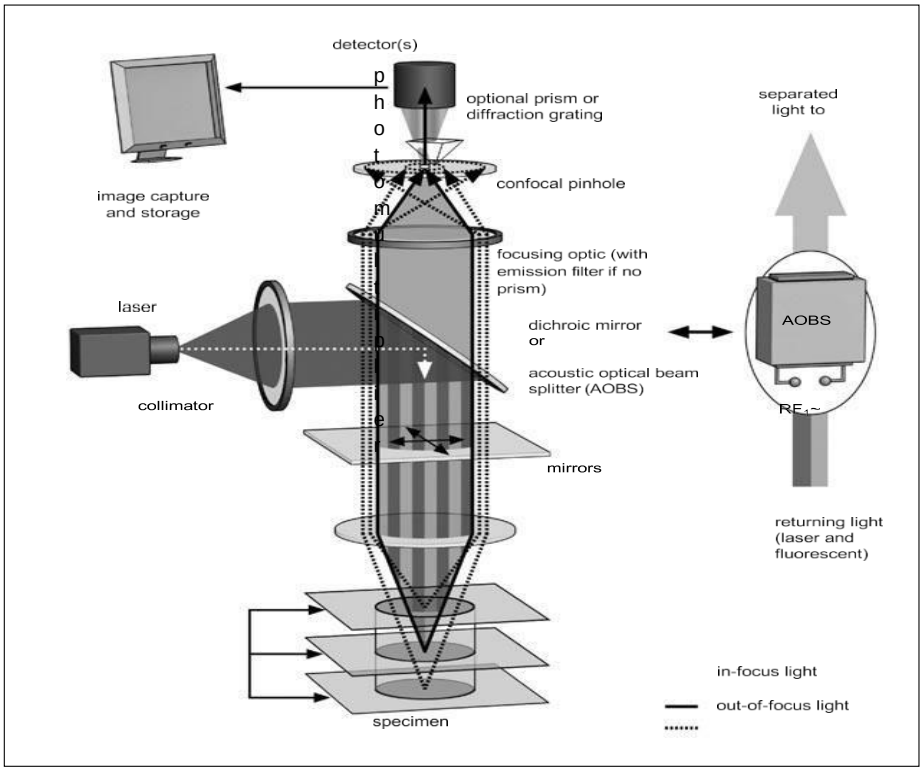

Fig 8:- BASIC ARCHITECTURE OF CONFOCAL MICROSCOPE [7]

This figure shows that the laser light is directed to the sample via collimating and beam-steering optics, scanning mirrors, and an objective that focuses the light to a diffraction-limited spot in the sample. Through a pinhole in the conjugate image plane to the point of focus in the sample, emission light from the sample is directed to light-sensing detectors. The light is perceived by the detectors after being spatially filtered by the pinhole, and a corresponding voltage is created and the sound was amplified and translated to digital levels and display and storage of images.

Advantage: -

The main advantage is that one may optically section while still doing complex experiments. Another advantage is the versatility of imaging capabilities and types of experiments they enable.

Disadvantage: -

The scan speed of a modern LSCM system is relatively slow, the price is pretty high, and the amount of light impinging on the sample is relatively large.

3.2.3 TWO-PHOTON MICROSCOPY[9,10]

Two-photon excitation microscopy is an alternative to confocal microscopy that provides advantages for three-dimensional and deep tissue imaging. TPFM is a type of laser scanning microscopy that is particularly useful for imaging thick samples both in vitro and in vivo. TPE is a nonlinear process that involves the absorption of two photons with enough combined energy to cause a chemical transition to an excited electronic state. The concept of two-photon excitation was first proposed theoretically by Maria Gopper Mayer in her doctoral dissertation. The units of two-photon absorption cross-section (GM) are named in her honor (1GM=10−50cm4sec-photon−1). The simultaneous absorption of two photons in a single event causes two-photon excitation. The energy of each photon is half that of a single-photon absorption event. Because the energy of a photon is inversely proportional to its wavelength, photons used in two-photon excitation should have a wavelength (2p) that is probably twice that of the photons used in one-photon excitation to accomplish an equivalent transition.

For example, if a fluorophore efficiently absorbs light at 400 nm under conventional excitation, it could be excited by two simultaneous photons at approximately 800 nm.

Thus, rather than fluorescence being excited by UV-visible illumination, it can be excited by infrared illumination. The resulting excited state from which emission occurs is the same singlet state as that excited during con ventional one-photon absorption. Thus, the fluorescence emission after two-photon excitation is exactly the same as that generated in normal one-photon excitation.

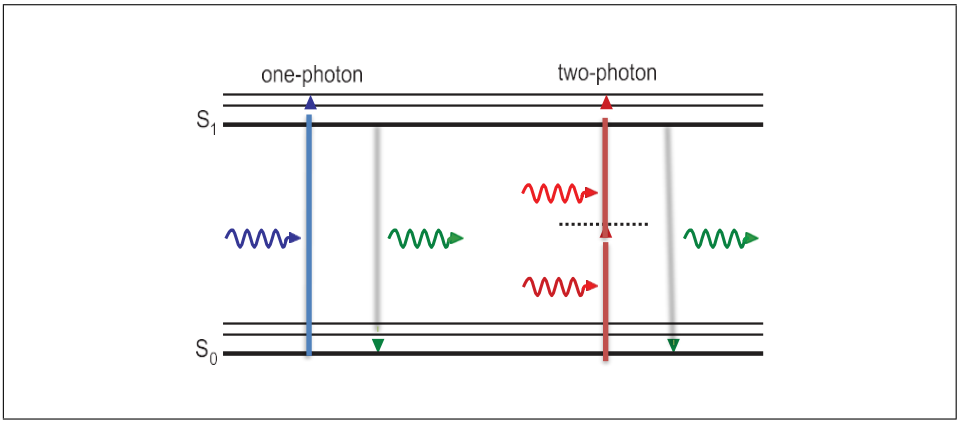

Fig 9 :- Jablonski (energy-level) diagram of conventional one-photon excitation (left) and nonlinear two-photon excitation (right) of fluorescence. In each case, the absorption of photon(s) generates an excited state from which the molecule can relax by emitting a fluorescent photon. Thus, the path to the excited state follows a different path under either one- or two-photon ab- sorption, leading to different absorption spectra. However, fluorescence is emitted from the same excited state producing identical emission spectra.[9]

Two-photon excitation arises from the simultaneous absorption of two photons in a single event. Each photon has half the energy as in the corresponding single-photon absorption event. The energy of a photon is inversely proportional to its wavelength; therefore, in two photon excitations, the photons should have a wavelength (λ2p) of approximately twice that of the photons required to achieve an equivalent transition under one-photon excitation.

Implications for Two-Photon Microscope Design

Scanning mirrors and PMTs work in the same way for image creation and detection. Because fluorescence only occurs in the focal plane, all photons recorded may be assigned to a single emission point source, independent of scatter. As a result, the photon-capturing PMTs don't need to be imaged and can be relocated closer to the object, enhancing the sensitivity of the instrument as more photons can be collected.

An aspect known as group velocity dispersion (GVD) is one element of two-photon excitation that can impact image intensity. GVD is caused by the inclusion of several optical elements in the excitation pathway, and it expands and reduces the peak power of the laser pulses. Fortunately, additional optical elements can be used to compensate for this effect. The

one- photon confocal microscope has the same controls and optical concerns as the two-photon confocal microscope.

Advantage: -

Using two-photon excitation is that many indicators have a broad excitation spectrum, allowing many indicators to be used at the same time without switching laser wavelengths or filters.

Disadvantage:-

Because a point-scanning microscope and a tunable pulsed Tisiphone laser are required, the cost is high. When a precompensation unit is included to correct dispersion in excitation pulse lengths, the cost increases slightly.

3.2.4 STIMULATED EMISSION DEPLETION (STED) FLUORESCENCE MICROSCOPY [7,11,12]

Stefan Hell and colleagues developed STED microscopy a new super resolution technique that has been proven to improve fluorescence microscopy resolution by an order of magnitude over classic diffraction limited techniques like LSCM. STED microscopy (stimulated emission depletion microscopy) is a type of far-field optical method that has been shown to achieve sub-diffraction resolution.

STED can achieve optical resolutions previously thought to be only possible with electron microscopy, and it has been used to investigate essential biological processes that no other technology could. STED imaging can be used in three separate fields: neurology, plasma membrane biophysics, and subcellular clinical diagnostics, all of which have a lot of potential for STED imaging research.

STED microscopy uses a reduction in the size of the excitation point to segregate indicators, resulting in individual indications being triggered individually. In STED microscopy, an excitation laser beam and a depletion laser beam are usually required. In the donut profile, the depletion laser beam is formed. The depletion laser beam is shaped to have a donut profile in the focal plane and overlapped with the excitation laser beam so that the STED process turns off the fluorophores at the outer rim of the excitation spot. This depletion reduces the size of the focal plane fluorescence spot, improving the lateral resolution significantly.

Disadvantage: -

The cost of the apparatus and the quantity of electricity that impinges on the sample are two factors in the STED technique. The system is relatively expensive because two pulsed lasers are required in addition to the already expensive laser scanning microscope system and extremely sensitive emission detectors. The amount of power used in a STED system is high.

4. FLUORESCENT CONPUNDS [2]

TABLE 1:- FLUORESCENT COMPOUNDS [3]

Compound	pH	Wavelength[nm] Fluorescence	Minimum concentration
Adrenaline	1	335	0.1
Allyl morphine	1	355	0.1
Amylobarbitone	14	410	0.1
Chloroquine	11	400	0.05
Chlorpromazine	11	480	0.1
Cinchonidin	1	445	0.01
Cinchonine	1	420	0.01
Cyanocobalamin	7	305	0.003
Ergometrine	1	465	0.01
Folic acid	7	450	0.01
Noradrenaline	1	320	0.006
Oxytetracycline	11	520	0.05
Pamaquine	11	530	0.06
Procaine	11	345	0.01
Procainamide	11	385	0.01
Proflavine	1	510	0.01
Physostigmine	1	360	0.04
Quinine	1	450	0.002
Reserpine	1	375	0.008
Riboflavin	6	520	0.01
Salicylic acid	11	435	0.01
Thiopentone	13	530	0.1
Thymol	7	300	0.1
Vitamin A		470	0.01

5. COMPOUNDS CONVERTED TO FLUORESCENT DERIVATIVES [2]

TABLE 2 :- FLUORESCENT DERIVATIVES [3]

Compound	Reagent	Excitation Wavelength [nm]	Emission Wavelength [nm]
Adrenaline	I_2or NaOH	420	530
Chlordiazepoxide	Photo oxidation	380	480
Hydrocortisone	70% H_2SO_4	470	520
5Hydroxytryptamine	Phthalaldehyde	365	495
Zinc	Rhodamine B	366	580

6. FLUORESCENT INDICATORS [2,13]

Many fluorescent substances' intensity and color are affected by ph. Some substances are pH sensitive enough to be used as pH indicators. These are termed fluorescent or luminescent indicators. A fluorescent indicator interacts with its environment and goes through changes that affect its fluorescent properties. Greater sensitivity can be achieved by using a fluorescent compound because the detection limits for fluorescence are lower, by a factor of 102 –104, than those for colorimetric light absorption.

Fluorescent indicators in acid base indicators are substances that fluoresce in ultra violet light and change color or have their fluorescence quenched when the pH changes. The merit of such indicators is that they can be employed in the titration of colored solution in which the color change of usual indicators would be masked.

Organic molecules that fluoresce, such as polycyclic aromatics, tend to be planar, rigid and unsaturated, and often conjugated. When such molecules have adjacent groups capable of coordinating to metal centers, they will form complexes.

TABLE 3 :- SOME IMPORTANT FLUORESCENT INDICATORS [3]

Indicators	pH	Color change
Eosin	3.0 -4.0	Colorless to green
Fluorescence	4.0 -6.0	Colorless to green
Quinine sulphate	3.0 -5.0	Blue to violet
Acridine	5.2 -6.6	Green to violet blue
2 naphtha quinine	4.4 -8.3	Blue to colorless

ADVANTAGES AND LIMITATIONS

Advantages[2]

- It's one of the more recent approaches, and its potential is still completely unexplored.
- It also has an impact on precision. In Fluorometric, you may easily obtain up to 1% accuracy.
- The method is extremely sensitive and particular, as there is a choice of wavelengths not only for the radiation released, but also for the light that excites it.

Limitations [2]

- Buffering is essential because fluorescence intensity can be highly dependent.
- Excitation with ultraviolet light may result in photochemical changes or the destruction of the fluorescent molecule.
- Increased photochemical degradation may be caused by the presence of dissolved oxygen.
- Iodide traces and nitrogen oxides are effective quenchers and so interfere.
- Because the method's accuracy is low for big volumes, it's not suitable for determining a sample's key constituents.
- Due to the fact that not all elements and compounds can fluoresce, the technique's usefulness is limited.

7. PRECAUTIONS[2]

- Fluorescence analysis is especially applicable to trace substances, care must be taken to eliminate contaminations of samples.
- Rubber and cork stoppers contain fluorescent materials and these are extracted if the solvent touches them.
- Filter paper also contains fluorescent material which is extracted by solvents.
- Grease from stop cocks and other sources is a fluorescent contaminant.
- All glasses contain Al, Ca and SiO_2 which may be extracted.
- The most important consideration is the concentration of the reagent. Concentration must be expressed in micro molecules so that the ratio of the reagent to metal may be estimated easily.
- Large temperature changes between unknown and standard should be avoided.
- It is also not desirable to expose the solution to ultra violet radiation for longer periods.

8. APPLICATION

Determination Glucose by Fluorescence Spectroscopy [2,14–16]

Fluorescence is a promising alternative technology to electrochemistry and spectroscopy for accurate glucose analysis in diabetic patients. The determination of b-D-glucose is important in industrial quality control and processing applications, as well as in clinical diabetes diagnosis and treatment. In biological systems, glucose is a major component of animal and plant carbohydrates. Furthermore, blood glucose levels are a predictor of human health. The abnormal amount of glucose reveals important information about many diseases, including diabetes and hypoglycemia. Fluorophotometer was widely used due to its ease of use and high sensitivity. Among the various methods employed to this aim, those utilizing the enzyme glucose oxidase (GOD) are the most widespread. GOD is an oxidoreductase, which catalyses' the oxidation of glucose to gluconic acid.

Determination of zinc [2]

The zinc complex of oxime fluoresces in ultraviolet light, which serves as the foundation for the following method. Using a calibrated burette, pour 5.0, 10.0, 15.0, 20.0, and 25.0 ml of

standard zinc solution into a separate 100ml volumetric flask. Add 10 mL ammonium acetate solution, 4 mL gum Arabic solution to each flask, dilute to 45 mL with distilled water, and swirl to combine.

Now, add exactly 0.40 mL of oxime solution and dilute with distilled water to the mark. Shake gently and immediately transfer to the fluorimeter cell for measurement, as the standard, use dichlorofluorescein solution. Start with the most concentrated zinc solution and move up. Plot instrument measurements versus zinc concentrations in milligrams per milliliter (mg/ml).

Determination of uranium salts [2,17]

The effectiveness of the Hydrogeochemical and Stream Sediment Reconnaissance (HSSR) for the National Uranium Resources Evaluation depends on the reliable identification of uranium at concentrations in the low parts-per-billion (ppb) range in natural surface and ground waters. The sample is first boiled with nitric acid and then fused with sodium fluoride and uranium fluoride. The melt cools to the point where it hardens into glass, which may be studied directly in a fluorometer. With the reagent, palladium produces a precipitate that may be removed by centrifugation. Because iron generates a compound that quenches fluorescence, it should be avoided.

Determination of ascorbic acid [18,19]

Ascorbic acid (AA), generally known as Vitamin C, is a water-soluble vitamin that is essential for tissue growth and repair throughout the body. It investigated how ascorbic acid (AA) and 2-cyanoacetamide react fluorescently. The findings of the experiments revealed that around pH 12.9-13.3, AA can react with 2-cyanoacetamide to generate a luminous product that emits bright fluorescence. The fluorescence intensity was measured at excitation and emission wavelengths of 329 and 380nm, respectively. Another procedure is based on the reaction between AA and Methylene Blue (MB). The fluorescence intensity of MB was measured at excitation and emission of 664 and 682 nm, respectively.

Determination of boron in steel [2]

A compound generated with benzoin is used to determine it. The boron in the sample's acid solution is first transformed into boric acid, which is then co-distilled with methyl alcohol to separate it from the other ingredients. The boric acid-containing distillate is neutralized with NaOH, and the methyl alcohol is evaporated.

Study of marine petroleum pollutants [2,20]

Fluorescence spectroscopy is one of the good techniques to detection of oil slicks on the water surface, determination of petroleum contaminants in seawater and determination of particular petroleum derivative compounds as well as identification of pollution sources. This approach involves obtaining a hexane extract of a water sample and measuring the intensity of light emitted by the extract. The intensity of luminescence of a pattern – solution of an artificially aged crude Sea oil is then compared to the outcome of this measurement. Hydrocarbons are the primary constituents of any oil. The remaining components are mostly hydrocarbon derivatives with single Sulphur, oxygen, or nitrogen atoms. Only a small percentage of hydrocarbons glow, while the vast majority do not. Fluorescence of oils has wavelength over than 260 nm and covers a spectral area of ultraviolet and visible light.

Determination of aluminum in alloys[2]

The reagent used is dye Ponta chrome blue black F, which is used at p H of 4.8 in buffered solution. It is suitable for the range of 0.01 – 1.00 % of acid-soluble aluminum in steel. The principle is the formation of complex of aluminum with azo dye 2,2-dihydroxy – 1,1 azo naphthalene – 4 sulphonic acid, sodium salt. After removal of aluminum and other interferences by mercury cathode electrolysis, the fluorescence of the complex is measured at 4.9 p H.

Determination of ruthenium[2]

In the presence of platinum metal, it is determined. With the reagent, palladium produces a precipitate that may be removed by centrifugation. Because iron generates a compound that quenches fluorescence, it should be avoided. Any other element of the platinum group can be present to the extent of at least 30mμ.mL-1, without interfering the determination of ruthenium in the range of 0.3 -2.0 mμ. mL^{-1}.

Determination of cadmium[2]

Cadmium may be precipitated quantitatively in an alkaline solution in the presence of tartrate by 2- [0-hydroxy phenyl]-benzoxazole. The complex dissolves readily in glacial acetic acid, giving a solution with an orange tint and a bright blue fluorescence in UV light. The acetic acid solution is used as a basis for fluorometric determination of cadmium.

Use an aqueous solution of the sample [25-50ml] containing 0.1-2.0mg of cadmium and about 0.1 g of ammonium tartrate. Add an equal volume of 95% ethanol, warm to 6℃, treat

with excess of reagent solution. Adjust the pH to 9-11digest at for 15 minutes, filter, wash with 20-25 ml of 95% ethanol containing a trace of ammonia and dry the precipitate for 6℃, 30-35 minutes. Dissolve the precipitate in 50.0ml of glacial acetic acid, and measure the fluorescence of the solution. Evaluate the cadmium content.

9. DRUGS MEASURED BY FLUORESCENCE SPECTROSCOPY

SR. NO.	DRUG NAME AND COMBINATION	METHOD	DESCRIPTION	REFRENCE NO.
1	Lisinopril	Fluorescence spectroscopy	Complex between Lisinopril and Fluorescein, Excitation wavelength:- 366 nm Emission wavelength:- 475 nm	21
2	Avanafil	Fluorescence spectroscopy	Avanafil in methanol Excitation wavelength:- 314 nm Emission wavelength:- 367nm	22
3	Paracetamol	Fluorescence spectroscopy	Paracetamol in potassium chlorate Excitation wavelength:- 360nm Emission wavelength:- 446nm	23
4	Montelukast	Fluorescence spectroscopy	Montelukast in methanol Excitation wavelength:- 340 nm Emission wavelength:- 390nm	24
5	Telmisartan	Fluorescence spectroscopy	Telmisartan in methanol and alkali solutions like Sodium hydroxide Excitation wavelength:- 366nm Emission wavelength:- 475nm	25
6	Diclofenac	Fluorescence spectroscopy	diclofenac in cerium (IV) Excitation wavelength:- 250nm Emission wavelength:- 356nm	26
7	Metformin	Fluorescence spectroscopy	Metformin in ninhydrin Excitation wavelength:- 250nm Emission wavelength:- 356nm	27
8	Dapagliflozin	Fluorescence spectroscopy	Dapagliflozin in methanol Excitation wavelength:- 278nm Emission wavelength:- 303nm	28

9	Citalopram	Fluorescence spectroscopy	Citalopram in methanol and sulphuric acid Excitation wavelength:- 239nm Emission wavelength:- 300nm	29
10	Nifedipine	Fluorescence spectroscopy	Nifedipine in cerium (IV) Excitation wavelength:- 255nm Emission wavelength:- 354nm	30
11	Naproxen	Fluorescence spectroscopy	Naproxen in NaOH Excitation wavelength:- 271nm Emission wavelength:- 353nm	31
12	Coumarin	Fluorescence spectroscopy	Coumarin in acetonitrile Excitation wavelength:- 310nm Emission wavelength:- 390nm	32
13	Gemifloxacin mesylate and linezolid	Fluorescence spectroscopy	Gemifloxacin in acetonitrile Excitation wavelength:- 260nm Emission wavelength:- 441nm Linezolid in methanol Excitation wavelength:- 260nm Emission wavelength:- 380nm	33
14	Thiabendazole	Fluorescence spectroscopy	Thiabendazole in ethanol Excitation wavelength:- 370nm Emission wavelength:- 428.8nm	34
15	Famotidine fluconazole ketoconazole	Fluorescence spectroscopy	Famotidine in methanol Excitation wavelength:- 230-360 nm Emission wavelength: - 330 - 460 nm fluconazole in methanol Excitation wavelength: - 220 - 320 nm Emission wavelength: - 250 - 350 nm ketoconazole in water Excitation wavelength: - 210 - 310 nm Emission wavelength: - 335 - 435 nm	35

10. CONCLUSION

Fluorescence spectroscopy is a spectroscopy method that determines the concentration of an analyte in a sample by analyzing its fluorescence properties. This technique is widely used for measuring compounds in a solution, and it is a simple method to use. Fluorescence spectroscopy is a quick and sensitive way of characterizing molecular environments and event samples. When compared to other analytical techniques, fluorimetry is chosen for its high sensitivity, high specificity, simplicity, and low cost. It is a useful analytical tool for both quantitative and qualitative research. It is a widely used and effective technique for a wide range of environmental, industrial, medical diagnostics, DNA sequencing, forensics, genetic analysis, and biotechnology applications. The field of fluorescence microscopy is rapidly expanding, providing cell and neurobiologists with ever-increasing imaging capabilities.

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