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1

Fluorescence spectroscopy and its applications: A Review

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Abstract

Fluorescence spectroscopy is a rapid, sensitive method for characterizing molecular environments and events samples. Fluorimetry is chosen for its extraordinary sensitivity, high specificity, simplicity and low cost as compared to other analytical techniques. It is widely accepted and powerful technique that is used for a variety of environmental, industrial, medical diagnostics, DNA sequencing, forensics, genetic analysis and biotechnology applications. It is a valuable analytical tool for both quantitative and qualitative analysis. This article presents a brief overview of the theory of fluorescence spectroscopy, together with examples of applications of this technique in organic and inorganic chemistry, medical diagnosis, medical science etc.

Keywords: Fluorimetry, DNA sequencing.

1. Introduction

Alzheimer's Fluorescence and phosphorescence are photon emission processes that occur during molecular relaxation from electronic excited states. These photonic processes involve transitions between electronic and vibrational states of polyatomic fluorescent molecules (fluorophores).

Fluorophores play the central role in fluorescence spectroscopy. Fluorophores are the components in molecules that cause them to fluorescence. Majorly fluorophores are the molecule which contains aromatic rings such as Tyrosine, Tryptophan, and Fluorescein etc.

Luminescence is emission of light by a substance not resulting from heat is thus a form of cold body radiation. It can be caused by chemical reactions, electrical energy, subatomic motions, or stress on a crystal. There are two pre-requisites for luminescence:

- The luminescent material must have a semiconductor structure with a nonzero band gap. [Metals do not provide luminescence if they have no band gap].
- The energy must be imparted to this material before luminescence can take place.

2. Types of luminescence

a) By Mechanism:
i) Fluorescence
ii) Phosphorescence

b) By Excitation Source:
i) Chemiluminescence
ii) Cathodoluminescence
iii) Electroluminescence
iv) Photoluminescence

Fluorescence spectroscopy is a sensitive optical emission technique in which sample molecules are excited with a photon source. Those molecules that relax by radiant emission can be subsequently detected by measuring the intensity of that emission. [1]

3. Principle of fluorescence spectroscopy [1,2]

Absorption of UV or visible radiation causes transition of electrons from singlet ground state to the singlet excited state. As this state is not stable, it emits energy in the form of UV or visible radiation and returns to singlet ground state. Fluorescence emission occurs as the fluorophore decay from the singlet electronic excited states to an allowable vibrational level in the electronic ground state.

The fluorescence excitation and emission spectra reflect the vibrational level structures in the ground and the excited electronic states respectively.
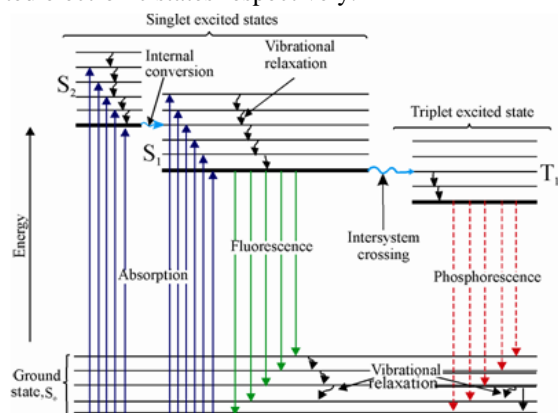

Figure 1: Jablonski diagram
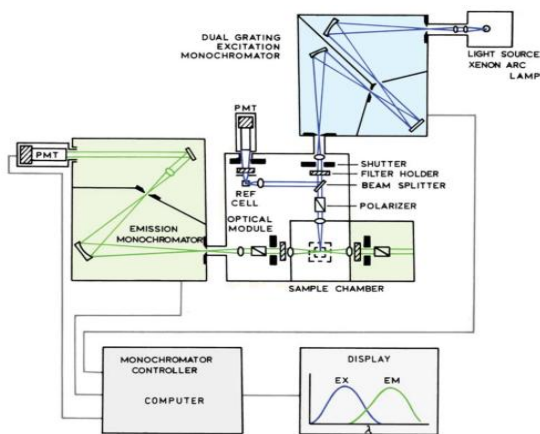
4. Instrumentation of spectrofluorometer

Figure 2: Schematic diagram of a spectrofluorometer

Spectroflourometer mainly consists of

A. Source of light
  ▪ Mercury vapour lamp
  ▪ Xenon arc lamp
  ▪ Tungsten film
B. Filters and monochromators
  ▪ Primary filters and secondary filters
  ▪ Excitation monochromators and Emission monochromators
C. Sample cells, Detectors[2-4]

5. Factors affecting fluorescence

5.1 Conjugation

Molecule must have unsaturation i.e. it must have π electrons so that UV/vis radiation can be absorbed. If there is no absorption of radiation, there will not be fluorescence.

5.2 Rigidity of structures

Rigid structures will produce more fluorescence, while flexible structure will produce less fluorescence.

5.3 Nature of substituent groups

Electron donating groups like amino, hydroxyl groups enhance fluorescence activity. Electron withdrawing groups like Nitro, carboxylic group reduce fluorescence. Groups like SO_3H or on NH_4^+ have no effect on fluorescence intensity.

5.4 Effect of temperature

Increase in temperature leads to increase in collisions of molecules and decrease in fluorescence intensity while decrease in temperature leads to decrease in collisions of molecules and increased fluorescence intensity.

5.6 Viscosity

Increase in viscosity leads to decreased collisions of molecules which will enhance fluorescence intensity while decrease in viscosity causes increased collisions of molecules which cause decreased fluorescence intensity.

5.7 Oxygen

Oxygen decreases the Fluorescence intensity in two ways: Oxidises fluorescence substances to non fluorescence substances. It quenches fluorescence because of paramagnetic properties.

5.8 Effect of pH

a. Aniline: Neutral or alkaline medium shows visible fluorescence while acidic conditions give fluorescence in UV region only.

b. Phenols : Acidic conditions do not give fluorescence while alkaline conditions gives good fluorescence.[2,4]

6. Effect of concentration on fluorescence intensity

Fluorescence intensity = Q x I_a

Where,

Q = Fluorescence efficiency

I_a = Intensity of absorbed light

Since emission is proportional to absorption, Ia has to be knownWhere,

I_o = Intensity of incident light and I_t = Intensity of incident light

It is known that,

$I_t = I_0 e^{-act}$ [from beer lamberts law]

$I_a = I_0 [1-e^{-act}]$

$I_a = I_0 \times act$

Fluorescence intensity = Q x I_a

$$F = QI_0 act$$

In this equation,

Q = Constant for a particular substance

I_a = Constant for an instrument

a = Molecular extinction coefficient

t = path length

c = concentration of substances

Fluorescence intensity is directly proportional to the substances. [2,4]

7. Advantages

- It's one of the newer methods and its potentialities are still largely unexplored.
- It also affects precision. Up to 1% can be achieved easily in Flourimetric.
- The method is very sensitive and also possesses specificity because there is a choice of wavelength not only for the radiation emitted, but also for the light which excites it.

8. Limitations

- Careful buffering is necessary as fluorescence intensity may be strongly dependent
- Ultraviolet light used for excitation may cause photochemical changes or destruction of the fluorescent molecule .
- The presence of dissolved oxygen may cause increased photochemical destruction.
- Traces of iodide and nitrogen oxides are efficient quenchers and therefore interfere.
- The method is not suited for determination of major constituents of a sample, because the accuracy is very less for large amounts.
- The extent of applicability of this technique is limited, because of the fact that all elements and compounds are unable to exhibit fluorescence.[13,15]

9. Precautions

- Fluorescence analysis is especially applicable to trace substances, care must be taken to eliminate contaminations of sample.
- 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 change between unknown and standard should be avoided.
- It is also not desirable to expose the solution to ultra violet radiation for longerperiods.[4]

10. Fluorescent Compounds

Table 1: Fluorescent compounds [4]

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
Chlor promazine	11	480	0.1
Cinchonidin	1	445	0.01
Cinchonine	1	420	0.01
Cyanocobalamine	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
Riboflavine	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

11. Compounds readily converted to fluorescent derivatives

Table 2: Compounds readily converted to fluorescent derivatives. [4]

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
Primaryamines and secondary amines	Flourescamine	390	485

12. Fluorescent indicators

The intensity and colour of many fluorescent substances depends on pH. Some substances are so sensitive to pH that they can be used as pH indicators. These are termed as fluorescent or luminescent indicators. Those substances which fluorescence in ultra violet light and change in colour or have their fluorescence quenched with change in pH can be used as fluorescent indicators in acid base indicators .The merit of such indicators is that they can be employed in the titration of coloured solution in which the colour change of usual indicators would be masked.

**Table 3: Some important fluorescent indicators [2,7]**

Indicators	pH	Colour change
Eosin	3.0 -4.0	Colourless to green
Fluorescence	4.0 -6.0	Colourless to green
Quinine sulphate	3.0 -5.0	Blue to violet
Acridine	5.2 -6.6	Green to violet blue
2 naptha quinine	4.4 -8.3	Blue to colourless

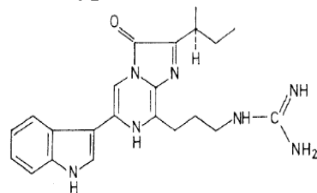## 13. Chemistry of bioluminescence

The luciferin—luciferase reaction is the emission of light as a result of the enzyme (a luciferase) -catalysed oxidation (generally with oxygen) of a substrate (a luciferin).

At present there are only three luciferins whose structures have been elucidated; namely.
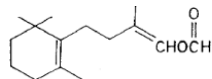
Firefly luciferin,
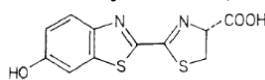
Cypridina luciferin

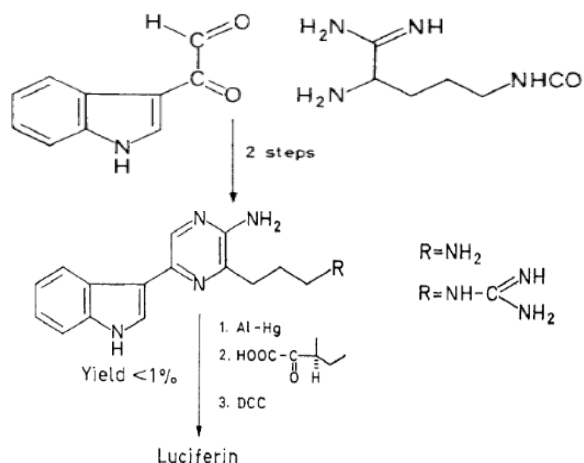**Latia luciferin**

13.1 Synthesis of luciferin

Condensation of 3-indolyiglyoxal with the a-amino-amidine gives the 2-aminopyrazine derivative.

14. Applications
14.1 Applications in inorganic chemistry
14.1.1. Determination of ruthenium

It is determined in the presence of platinum metal. Palladium forms a precipitate with the reagent which can be removed by centrifuging. Iron should be absent as it forms a complex that quenches the fluorescence. Any other element of the platinum group can be present to the extent of atleast $30m\mu.mL^{-1}$, without interfering the determination of ruthenium in the range of 0.3 -2.0$m\mu.mL^{-1.}$

14.1.2. Determination of boron in steel

It is determined by means of complex formed with benzoin. The boron present in the acid solution of the sample is first converted in to boric acid, which is then separated from the other constituents by co-distillation with methyl alcohol. The resulting distillate containing boric acid is neutralised by NaOH and methyl alcohol is evaporated off.

14.1.3. Determination of aluminium in alloys

The reagent used is dye pontachrome 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 aluminium in steel. The principle is the formation of complex of aluminium with azo dye 2,2-dihydroxy – 1,1 azo naphthalene – 4 sulphonic acid, sodium salt. After removal of aluminium and other interferences by mercury cathode electrolysis, the fluorescence of the complex is measured at 4.9 p H.

14.1.4. Determination of chromium and manganese in steel

Steel is dissolved in acid and the solution is oxidised with persulphate.CrO_7^{2-} and MnO_4^{-} ions, which absorb in the violet and yellow green respectively. There is slight overlapping of absorption and, but if a portion is treated with $NaNO_2$ which reduces MnO_4^- but not, $Cr_2O_7^{2-}$, a difference measurement can be used to estimate Mn. A measurement at $\lambda = 4100$ A^O on the reduced portion will give chromium.

14.1.5. Determination of uranium salts

The sample is first boiled with nitric acid and then fused with sodium fluoride and uranium fluoride .Upon cooling, the melt solidifies in to glass which can be examined directly in a specially designed fluorometer. Palladium forms a precipitate with the reagent which can be removed by centrifuging .Iron should be absent because it forms a complex that quenches the fluorescence.

14.1.6. Estimation of rare earth terbium

Fluorescent complex formation with EDTA and sulpho salicylic acid.The excitation spectrum corresponds to the absorption spectrum of sulpho salicylic acid.The fluorescent spectrum shows peaks at 4850, 5450, 6300A^0 which corresponds to the ion Tb.

14.1.7. Estimation of bismuth

The solutions are evaporated in an argon hydrogen flame and then irradiated with iodine emission line at $\lambda = 2061.63$ A^0 which is very close to the bismuth line at $\lambda = 2061.70$ A^0 to absorb the radiation.

14.1.8. Determination of beryllium in silicates

The formation of fluorescent complex of beryllium with morin. The interferences such as iron and rare earths are removed by mercury cathode electrolysis; the fluorescence of the complex is complexing with triethanolamine and diethylene triamine penta acetate.

14.1.9. Estimation of 3,4 benzpyrene

This carcinogenic material is extracted by solution from tobacco or from the tobacco smoke deposits and separated out by the chromatography [Al_2O_3] and subsequent elution. The resulting fluorescent solution is then placed in a glass cell and irradiated with the mercury lamp and glass filter.[4]

14.1.10. Determination of zinc

The zinc complex of oxime fluoresces in ultra violet light and this forms the basis of the following method. By means of calibrated burette, 5.0, 10.0 ,15.0 ,20.0 ,25.0 ml of standard zinc solution in to separate 100ml volumetric flask .To each flask add 10 ml of ammonium acetate solution ,4ml of the gum Arabic solution, dilute to 45 ml with distilled water and mix by swirling. Now add exactly 0.40 ml of oxime solution and dilute to the mark with distilled water. Shake gently and transfer immediately to the cell of fluorimeter for measurement. Employ dichloro fluorescein solution as the standard. Commence measurements with most concentrated zinc solution. Plot instrument readings against zinc contents [mg/ml]

14.1.11. Determination of cadmium

Cadmium may be precipitated quantitatively in alkaline solution in the   presence of tartarate 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 fluorimetric determination of cadmium

Use an aqueous solution of the sample [25-50ml] containing from 0.1-2.0mg of cadmium and about 0.1 g of ammonium tartarate. Add an equal volume of 95% ethanol, warm to 60^0C, treat with excess of reagent solution. Adjust the pH to 9-11digest at 60^0C for 15 minutes, filter, wash with 20-25 ml of 95% ethanol containing a trace of ammonia and dry the precipitate at 13^0C for 30-35 minutes. Dissolve the precipitate in 50.0ml of glacial acetic acid, and measure the fluorescence of the solution. Evaluate the cadmium content. [7]

14.2 Application in organic chemistry

Assay of thiamine: Assayed by blue fluorescence of its oxidation product, thiochrome. The sample such as meat, cereals etc is first treated with acid and extract is treated with enzyme phosphatase. The latter causes hydrolysis of phosphate esters of thiamine which are present in the food materials. An oxidising agent such as $K_4Fe[CN]_6$ is added

to the first, while equal amount of NaOH and isobutyl alcohol are added to the both the aliquots. After shaking aqueous layer is removed and alcoholic layer is examined in a flurimeter.[4]

Estimation of quinine sulphate by fluorimetry

- *For the construction of calibration curve*: In to a series of 10 ml volumetric flasks transfer 1ml, 2ml, 3ml, 4ml and 5ml of standard solution of quinine sulphate [1µg/ml] and dilute to mark with 0.1N sulphuric acid. Select the proper excitation and emission filters [365nm and 459 nm]. Measure the fluorescence with a fluorometer.

- *For the analysis of tablets:* weigh 20 tablets and reduce to a fine powder. Weigh accurately a quantity of the tablet powder equivalent to 100mg of quinine sulphate and mix with 75ml of 0.1 N sulphuric acid in a 100 ml volumetric flask. Mix the contents thoroughly to dissolve the drug and adjust the volume to 100 ml with 0.1N sulphuric acid and filter the contents.[6]

14.3 Special fluorimetric applications

14.3.1 Investigation of chemical structures and processes

Fluoremetric methods have successfully been applied in the investigation of hydrogen bonding, cis trans isomerism polymerisation, tautomerism and rates of reactions etc.

- The free radicles can best be detected with a spectrograph so that the whole spectrum of a short lived component may be photographed at the same time.

- Steric hindrance in diphenyl can be studied by constraining the two phenyl group to move out of a single plane ,by substituting CH_3 groups adjacent to the centre bond [ortho substitution].The molecule behaves like a two benzene-insulated chromophores.

14.3.2 Chemical analysis

This type analysis may be quantitative as well as qualitative

- *Detection of impurity* – solvents used for spectrofluorometry must be free of absorbing impurities. Thus , cyclohexane must be purified until it shows no trace of benzene bands at 2600 A^0.

- If absorbing impurities are present in the vitamin, they can be removed either by chemical method or utilizing the change of shape of absorbing band in the presence of an impurity.

- *Estimation of fluorescent intensity* – intensity of pure fluorescing component in sufficiently low concentration is proportional to concentration, but this condition is not always easy to get. It is therefore desirable to measure the intensity from a specimen.

 This method however, suffers some difficulties. They are;

- If some of the exciting radation is absorbed by the impurities, the amount of radation left   to actually

irradiate the specimen is reduced by unknown amount. This is called quenching.

- Impurities present may also deactivate the excited molecules by collision before they have time to radiate the fluorescence. This is called quenching.
- If a major part of the exciting radiation is absorbed by a specimen, further increase in concentration will cause very little increase in fluorescent intensity.[4]

14.3.3 Laser induced fluorescence spectroscopy of human tissues for cancer diagnosis

Cancer is one of the most dreaded disease. Early tumours often arise from tissue which have a rapid turnover of cells and are active in repair like transformed mucosa on the surface of hollow organs (oral cavity, gastrointestinal tract, female reproductive organs etc.). Laser spectroscopic techniques have the potential for in-situ, near real time diagnosis and the use of non-ionizing radiation ensures that the diagnosis can be made repeatedly without any adverse side effects. Laser Induced Fluorescence (LIF) has been used for diagnosing cancer in two ways.

One approach involves Systemic administration of a drug like hematoporphyrin derivative which is selectively retained by the tumour. When photo excited with light of appropriate wavelength the drug localized in the tumour fluoresces. This fluorescence is used for detection and imaging of the tumour.

Photo excitation also leads to populating the triplet state via intersystem crossing. The molecule in excited triplet state can directly react with bio-molecules or lead to generation of singlet oxygen which is toxic to the host tissue. The resulting destruction of the host tissue is exploited for photodynamic therapy of tumour.

14.3.4 Study of marine petroleum pollutants

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. Main components of any oil are hydrocarbons. The other components are primarily derivatives of hydrocarbons containing single atoms of sulfur, oxygen or nitrogen. Only few of hydrocarbons fluoresce, while the major of them show no ability to luminescence. The content of compounds able to fluorescence1 rarely exceeds 10% of the oil mass. At the same time the petroleum strongly absorbs radiation, especially the ultraviolet and blue light.

In spite of this petroleum is a luminescent medium and fluorescence is a phenomenon which allows testing oils. Fluorescence of oils has wavelength over then 260 nm and covers a spectral area of ultraviolet and visible light.

The phenomenon is most significant in the 270–400 nm range.

14.3.5 Accurate determination of glucose

Glucose is considered as a major component of animal and plant carbohydrates in biological systems. Furthermore, blood glucose levels are also an indicator of human health conditions. The abnormal amount of glucose provides significant information of many diseases such as diabetes or hypoglycemia. Fluorophotometry was used widely owing to its operational simplicity and high sensitivity.

Recently bio-molecule-stabilized Au nanoclusters were demonstrated as a novel fluorescence probe for sensitive and selective detection of glucose.

Fluorescence spectroscopy is a rapid, sensitive method for characterizing molecular environments and events. Fluorescence output is linear to sample concentration over a very broad range.[1]

14.3.6 A highly sensitive fluorescent immunoassay based on avidin labeled nanocrystals

Nanocrystals of the fluorogenic precursor fluorescein diacetate (FDA) were applied as labels to enhance assay sensitivity. Each FDA nanocrystal can be converted into $\sim 2.6 \times 10^6$ fluorescein molecules, which is useful for improving sensitivity and limits of detection of immunoassays. There are four binding sites in each avidin molecule that can bind non-co-operatively up to four molecules of biotin with a very high affinity .The four binding sites together with the high affinity of the avidin-biotin interaction serve as an aid in amplifying the sensitivity of immunoassays .

The avidin-biotin complex is almost inseparable and even stable under strong chemically denaturant conditions over a wide pH range. The avidin-biotin technique is widely used to localize antigens in cells and tissues and to detect biomolecules in immunoassays and DNA hybridization techniques.

- In the immunoassay using the labeled avidin-biotin technique, biotinylated antibody and Neutr Avidin-labeled FDA were used.
- The biotinylated antibody was first incubated with the antigen.
- After washing, NeutrAvidin-labeled FDA was added.
- After further incubation and washing steps, the FDA associated with the antigen was hydrolyzed and dissolved. The fluorescence intensity was finally measured.
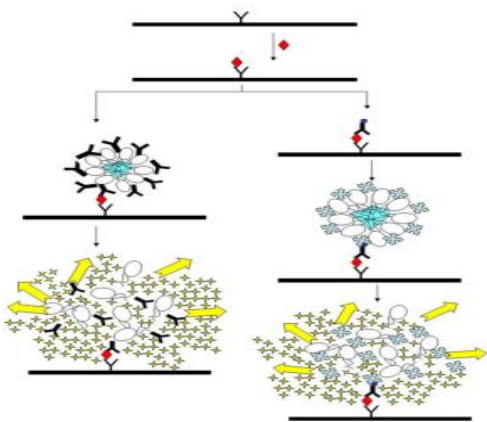
Figure no 4: Principle of a labelled avidin – biotin fluorescent immunoassay

The analyte was first incubated with the capture antibody preadsorbed on the microtiter plate and then exposed to the biotinylated-antibody followed by FDA-avidin conjugates.

High signal amplification was achieved after solubilization, release, and conversion of precursor FDA in to fluorescein molecules by the addition of DMSO and NaOH.

14.4 Flourescence polarisation immunoassay of mycotoxins

Immunoassays are routinely used in the screening of commodities and foods for fungal toxins (mycotoxins) constructed in competitive heterogeneous formats. Immunoassays have been realized in the development of major groups of mycotoxins, including aflatoxins, group B trichothecenes (primarily deoxynivalenol), ochratoxin A, and zearalenone. In typical competitive enzyme-linked immunosorbent assay (ELISA) formats the signal developed depends upon the presence of an enzymatic tracer.

Generally the tracer is either the toxin that has been labeled with an enzyme (often used in cases where antibody is immobilized) or antibody labeled with an enzyme (in cases where a toxin-protein conjugate is immobilized). The same two configurations have been used in many immunoassays and biosensors. Non-enzymatic labels such as fluorescence, radioisotopes, colloidal gold, etc. have also been used to facilitate detection of the competitive event. Assays of this nature, which require separation of the 'free' and 'bound' tracer are termed heterogeneous and encompass the vast majority of mycotoxin immunoassays.

The separation can be achieved in various ways, from chromatographically (as in lateral flow test strips), washing (as in ELISAs), or reagent flow over a surface (as in certain biosensors).

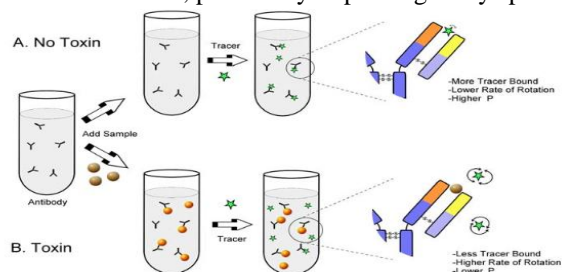Fluorescence polarization immunoassay (FPIA) differs from ELISA in that it is a homogeneous assay

conducted in solution phase. Unlike heterogeneous immunoassays, homogeneous assays do not require the separation of the free and bound tracer.

When a fluorophore in solution is exposed to plane-polarized light at its excitation wavelength the resulting emission is depolarized. The depolarization results from the motion of the fluorophore during the processes of excitation and emission. Because of this, the more rapid the motion of the fluorophore the more the emission is depolarized. The fluorescence emission can be segregated, using polarizers, into horizontal and vertical components.
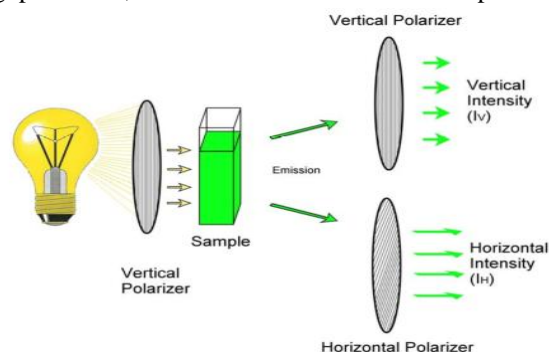
Figure 5: Measurement of fluorescent polarisation

In order to make the assay specific for a toxin, the toxin can be covalently linked to the fluorophore to make a fluorescent tracer .In this case the tracer competes with toxin (from the sample) for a limited amount of toxin-specific antibody. In the absence of toxin the antibody binds the tracer, restricting its motion and causing a high polarization. In the presence of toxin less of the tracer is bound to the antibody and a greater fraction exists unbound in solution, where it has a lower polarization. The polarization is inversely related to the toxin concentration. The advantage of this format, relative to a competitive ELISA format, is the lack of a need to separate the free from the bound tracer, potentially improving assay speed.

Figure 6: Fluorescent polarisation immunoassay

A cuvette is filled with dilute antibody solution, a portion of sample extract is added and the fluorescence intensities of the blank are obtained. The tracer is then added, mixed, and held for a period before re-introducing the cuvette into the instrument to obtain the fluorescence polarization measurement. The holding period, generally ranging from several seconds to several minutes can be an important factor in the assay.

Example

      Aflatoxins, because of their potency as carcinogens are of relevance to human and animal health at lower concentrations than many of the other mycotoxins, and the regulatory levels for these toxins in foods and feeds are also lower (ppb level). For this reason assays for these toxins likewise need to detect lower level.[8]

15. Conclusion

      Fluorescence spectroscopy is a sensitive optical emission technique in which sample molecules are excited with a photon source. Those molecules that relax by radiant emission can be subsequently detected by measuring the intensity of that emission. Fluorimetry is generally used if there is no colourimetric method sufficiently sensitive or selective for the substance to be determined. The important applications for determination of organic and inorganic compounds including immunoassays and chemistry of bioluminescence are reviewed.

      Special fluorimetric applications are also included in this study. At first glance it seems easy to perform fluorescence experiments. However, there are numerous factors that can compromise the data and invalidate the results. One needs to be constantly aware of the possibility of sample contamination, or contamination of the signal from scattered or stray light. Collection of emission spectra, and examination of blank samples, is essential for all experiments.

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