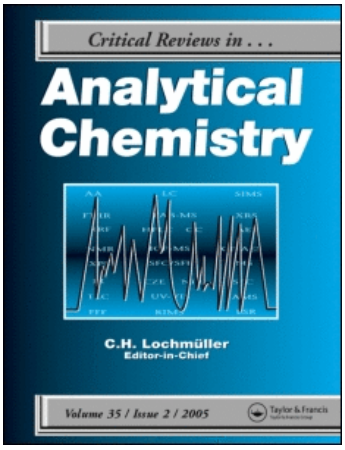

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FLUORESCENCE DERIVATIZATION

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I. INTRODUCTION

Luminescence derivatization procedures convert non- or weakly luminescent sample molecules to highly luminescent products. The usual motivation for forming luminescent derivatives is to increase the sensitivity with which samples can be detected. Analytical methods based on luminescence generally have detection limits from one to four orders of magnitude lower than corresponding methods based on absorption. Other advantages of luminescence methods relative to absorption include greater linear dynamic response ranges and greater selectivity, since both excitation and emission wavelengths can be chosen to maximize selectivity. The required instrumentation is relatively simple. More information on luminescence can be obtained from basic texts on this subject.[1-5] While luminescence includes fluorescence, phosphorescence, and chemiluminescence, most of the applications involve fluorescence. This review will be devoted exclusively to fluorescence. Applications of phosphorescence are limited, because phosphorescence is rarely observed from solution. Chemiluminescence is restricted in scope, because relatively few reactions give yield to efficient light production.

The specific requirements for a useful derivatization reaction depend on the intended application. Nevertheless, it is possible to generalize as to desirable properties for luminescence derivatization reactions. These include:

1. As with all types of derivatization, the reaction should be fast, quantitative, simple to perform, and specific for the compound of interest.
2. There must be some means of distinguishing fluorescence from the product of interest from fluorescence arising from other reactions involving the derivatizing reagents. In favorable cases, the excess reagent does not fluoresce itself or form fluorescent side products. In other cases the unwanted fluorescence may occur at sufficiently different wavelengths from that of the desired fluorescence so that they can be satisfactorily resolved. Finally, if reagents and sample have similar fluorescence, it should be possible to separate them by a simple procedure.
3. The derivative should have a high fluorescence efficiency for maximum sensitivity.
4. Excitation and emission wavelengths for the derivative should be greater than 330 nm. This permits the use of glass optics, which are less expensive and have less intrinsic fluorescence than quartz. If the fluorescent derivative is to be excited

by a mercury lamp, it is desirable that it absorb strongly at one of the mercury lines.

5. If derivatization is to be used for visualization, the derivative must fluoresce in the visible region of the spectra.
6. The derivative should be stable and not undergo rapid decomposition.

Fluorescence derivatization, strictly speaking, includes all procedures that involve reacting a compound of interest to yield a fluorescent product. In this review, we will be primarily interested in reactions that are generally useful for particular types of compounds. The review is not intended to be comprehensive. However, the most important and widely used reactions will be covered.

The rest of this review will itemize different types of applications of luminescence derivatization indicating the specific requirements for each kind of application. The analytical characteristics of fluorescence are briefly reviewed so that the reader may be aware both of the benefits and possible hazards of using fluorescent derivatives. A discussion of the relationship between molecular structure and fluorescence follows. Out of this discussion it will become clear that there are several distinct approaches to forming molecular structures with high fluorescence efficiencies. The remainder of the review will consider specific derivatization reactions organized according to the approach used to achieve fluorescence. It is hoped that this organization will make it clear why certain reactions are useful for fluorescence derivatization, as well as perhaps stimulating the development of new reactions for forming fluorescent derivatives.

II. TYPES OF APPLICATIONS

A. Direct Analysis

Direct analysis refers to those methods where the analyte is reacted to form a fluorophor with no separation, or only a single-stage separation required. Selectivity is very important, particularly if the analyte is in a complex matrix. Many direct methods involve simple operations such as oxidizing, reducing, or adding acid or base to induce analyte fluorescence. Most commonly, this type of method has been developed empirically. It is unique for the analyte. Many vitamin and drug assays fall into this category.

In this review we limit ourselves to reactions that are specific for some type of structure, either for individual functional groups (such as primary amines) or for two or more functional groups appropriately positioned relative to each other.

B. Thin Layer Chromatography (TLC) Detection

Fluorescence derivatization is frequently used to detect compounds separated by TLC. It is possible to perform the derivatization before the separation. In this case, the effect of derivatization on the chromatographic properties of the compounds to be separated must be considered. On the other hand, reagent luminescence is not a serious problem provided the chromatographic procedure successfully separates analyte luminescence from reagent luminescence. Alternatively, the derivatization can be performed after the separation by spraying the TLC plate with the fluorogenic reagents. In this case it is essential that the reagents do not luminesce. The more widely used fluorogenic spray reagents are summarized in Stahl's book on TLC.[6]

Often fluorescence derivatization is used only to render separated compounds visible to the eye. In this case derivatives emitting in the visible region are required, but reactions need not be quantitative. However, quantitative fluorometry, by measuring fluorescence intensity from the surface of the TLC plate, does require that reactions be quantitative or at least reproducible.

C. Liquid Chromatography (LC) Detection

Fluorescence detection in LC is a new and active research area. A number of fluorescence detectors have been reported in the literature[7-11] and several are available commercially. In general, fluorescence LC detection is one to three orders of magnitude more sensitive than detection based on UV-visible absorption. Fluorescence LC detectors are basically the same as conventional fluorescence instruments, except that they use a low-volume flow-through cell. The reason for the small volume is (of course) to minimize loss of chromatographic resolution within the detector. A typical volume would be 10 $\mu\ell$. This, of course, causes a reduction in sensitivity. Absolute detection limits are generally in the low picogram range for efficient fluorophors. Detectors using filters for wavelength resolution are available, as well as detectors using monochromators. The detectors with monochromators are more expensive, but offer greater flexibility in selecting excitation and emission wavelengths.

Derivatization can be performed before or after the LC separation. Precolumn derivatization is preferable provided the derivatives can still be satisfactorily separated. For example, catecholamines can be derivatized by reactions with dansyl chloride (l-dimethyl amino-naphthalene-5-sulfonyl chloride) and then separated with fluorescence detection. Post-column derivatization is also possible, although it generally involves some loss of chromatographic resolution. If the derivatization reaction is fast, the column effluent can be directly mixed with the necessary reagents and introduced into the detector. An example of such a system is the determination of amino acids using fluorescamine.[13] Alternatively, the effluent from the column can be segmented by air bubbles.[14] After segmenting, a variety of chemical operations can be performed without further loss of resolution. An example of such a system is the detection of various organic compounds by reacting them with Ce^{+4} to produce Ce^{+3}, which is fluorescent.[15]

D. Enzymatic Analysis

Enzymatic analysis refers both to methods for determining enzyme activities in the presence of excess substrate and the use of enzymes as highly selective reagents for substrates. Enzymes that can be made to catalyze the conversion of a nonluminescent substrate to a luminescent product or vice versa can be conveniently assayed by measuring the change in fluorescence. In this case, the derivatization reaction must be designed so that it can be catalyzed by the enzyme. Alternatively, fluorescent reactions can be coupled to enzymatic methods for substrates by reacting the product of the enzyme-catalyzed reaction to form a fluorescent derivative.

E. Fluorescence Polarization

Fluorescence polarization is a convenient means of estimating the size and overall dimensions of macromolecules labeled with a fluorophor. The macromolecule is excited with plane polarized radiation. Rotation of the macromolecule while the fluorophor is in an electronically excited state depolarizes the emission. The ratio of $I_{\parallel}$, the intensity polarized in the same plane as the excitation radiation, to $I_{\perp}$, the intensity polarized in the plane perpendicular to the excitation radiation can be related to the dimensions of the macromolecule. In this particular type of application, the derivatization reaction need not be complete or specific. What is important, is that it not affect the overall structure of the macromolecule. Also, the fluorescence lifetime of the fluorophor is an important parameter, since the longer the lifetime, the more time there is for depolarization.

F. Fluorescent Probes

Fluorescent probes are molecules used to study the binding sites of proteins. The

most popular choice as fluorescent probes are molecules whose luminescence behavior is a sensitive function of the molecular environment. For example, molecules whose fluorescence behavior varies significantly with solvent polarity are used to determine binding site "polarities." Molecules whose fluorescence behavior varies with molecular configuration can probe the geometry of binding sites. In general, the most important characteristic of a fluorescent probe is that its luminescence properties are able to provide some sort of information on binding sites.

G. Distance Measurements in Macromolecules

The phenomenon of "long-range energy transfer" is the basis for measuring the distance between two sites on a macromolecule. Energy transfer refers to a process by which an electronically excited donor molecule transfers its energy to an acceptor molecule. The condition for energy transfer is that the emission spectrum of the donor overlaps the absorption spectrum of the acceptor. The efficiency of energy transfer varies with $1/r^6$, where "r" is the distance between donor and acceptor.

To determine distances, one site is labeled with a donor while the other is labeled with acceptor. The donor is excited and the resulting emission is analyzed to determine the efficiency with which energy is transferred to acceptor. From this the distance between sites can be calculated.

For this application it is necessary to use a donor-acceptor pair with appropriate energy transfer characteristics.

H. Fluorescent Immunoassay

Immunoassay procedures involve competition between labeled antigen and unlabeled antigen for binding sites on an antibody. After equilibration, the bound and unbound fractions are separated and quantitated. One popular means of labeling the antigen is to bind a fluorophor to it. The completeness of the reaction is not critical since one normally runs standards along with the samples. What is important for this type of application, is that derivatization does not affect the antigen-antibody interaction that gives immunoassay methods their extreme specificity.

I. Fluorescence Microscopy

Fluorescence microscopy involves derivatizing or "staining" a component of a cell to render it fluorescent (and thus visible) when excited by UV light and viewed through a microscope. Fluorescent stains specific for certain types of cell components have been developed. However, a more generally useful method is to use an antibody to specifically react with the cell component of interest and to label the antibody with a fluorophor. As above, the primary requirement for the reaction used to derivatize the antibody is that it not affect antibody behavior.

III. ANALYTICAL CHARACTERISTICS OF FLUORESCENCE ANALYSIS

A. Fluorescence Intensity vs. Concentration

The relationship between fluorescence intensity and concentration has been considered in detail elsewhere.[16] When fluorescence is used for quantitative purposes, it is usual to work under conditions where the sample does not significantly absorb the excitation radiation, i.e., the intensity of the excitation radiation is equal throughout the sample. In this case, the equation relating intensity to concentration reduces to the following:

$$I_F = \phi_F K I_s \epsilon bc \qquad (1)$$

where I_F is the fluorescence intensity, ϕ_F is the fluorescence efficiency, ε is the molar absorptivity of the analyte at the excitation wavelength, b is the pathlength, c is analyte concentration, I_o is the intensity of the excitation source, and K is an instrumental constant.

If the excitation radiation is absorbed to a significant extent, then it is necessary to account for the fact that source intensity decreases with distance from the source in the sample. The relationship between concentration and fluorescence intensity ceases to be linear. At very high absorbances, there will be a point where fluorescence intensity either becomes independent of concentration or actually decreases with increasing concentration (depending on instrumental design). This effect is frequently referred to as the "inner filter effect". It is generally best handled by dilution. Because linear response is observed only at low analyte concentrations, fluorescence is most advantageous as a trace method. Concentrated samples can of course always be diluted; however, this often can lead to problems associated with working low concentrations, e.g., interferences from reagent contaminants and loss of sample due to adsorption on container walls. Thus, it is relatively unusual for fluorescence to be the method of choice when analyte concentration is high.

B. Sensitivity and Detection Limits

The sensitivity of fluorescence analysis depends on the factors appearing in Equation 2, ϕ_F, I_o, ε, b, and K. The dependence on fluorescence efficiency is self-evident. To increase sensitivity, most fluorescence instruments use mercury or xenon arc lamps, which are more intense than other common UV-visible sources. Laser sources have been used to further increase I_o, but they are not likely to achieve widespread use soon — mainly because of cost and limitations in wavelength selection and variation.[17] The pathlength b, is not normally used to increase sensitivity. One centimeter square cells are most common, although microcells are frequently used when the amount of sample is limited. The molar absorptivity is generally maximized by appropriate choice of excitation wavelength (although in specific situations a lower ε may be desirable to avoid interference, or so that the source radiation is not significantly absorbed by the sample). The instrumental factor, K, covers a variety of effects including the efficiency with which light is transmitted from the source to the sample, the efficiency with which fluorescence is transmitted from the sample to the detector, and the sensitivity of the detector to the fluorescence emission. Because of the instrumental factors affecting sensitivity, there is a considerable variation in sensitivity from instrument to instrument. Also, because source intensity and detector sensitivity are both wavelength dependent, the sensitivity of a given instrument varies with wavelength.

Detection limits depend on the variability and magnitude of the signal of interest relative to variability of the background signal. Quantitative treatment of detection limits for luminescence method has appeared elsewhere.[18] Sources of background variability include detector noise and variations in background emission. Background emission can arise from several sources including Raman scattered excitation radiation by the solvent, unwanted fluorescence from reagent contaminants, and stray light. Stray light background is much more likely to be a problem in samples containing large molecules such as proteins, because these molecules scatter light much more efficiently than do smaller molecules.

Detection limits vary from instrument to instrument although not to the same extent as sensitivity, since factors that enhance sensitivity also frequently enhance background. Most instrument specifications cite a detection limit for quinine sulfate that can be used as a basis for comparing instruments.

It is difficult to generalize detection limits for fluorescence. Many observed detection limits fall in the range of 10^{-7} to 10^{-9} *M*; however, quite frequently they go considerably

lower. Since fluorescence can be readily applied to small volumes, absolute detection limits, (i.e., in terms of amount rather than concentration) are also very low, frequently falling in the 0.01 to 1 pmol range. Response to concentration is generally linear up to the point where absorption of excitation radiation becomes significant.

The low detection limits account for the importance of fluorescence as an analytical method. Also important is the fact that the instrumentation required to achieve these detection limits is very modest in cost. Filter fluorometers are currently available for prices from $1000 to $2000. For only a few additional thousand dollars it is possible to purchase a low-resolution uncorrected spectrometer, which offers considerably greater flexibility in choosing analytical conditions. These low cost instruments achieve the same kind of detection limits as higher cost fluorescence instruments. (The additional cost is for greater resolution, recording capability, and automatic correction of spectra for the effect of source intensity and detector sensitivity variations with wavelength.)

C. Errors and Interferences

Quantitative fluorescence is subject to a variety of possible errors. The most important are itemized below:

Variations in source intensity — Many instruments compensate for source intensity variations by measuring the ratio of fluorescence intensity to source intensity, rather than directly measuring fluorescence.

Temperature variations — Increases in temperature typically cause the rate of internal conversion to increase, leading to a decrease in fluorescence efficiency that can amount to 1%/°C.

Photodecomposition — If the excited state responsible for emission undergoes a chemical reaction, the analyte will be gradually consumed. This normally manifests itself as a gradual decrease in fluorescence intensity with time. The rate of photodecomposition is proportional to source intensity, so it can be reduced by reducing source intensity — provided this does not lead to an intolerable loss of sensitivity.

Quenching — Quenching refers to processes by which other solutes interact with the first excited singlet state of an analyte and provide new paths by which the excited state may return to ground state radiationlessly. Quenching often involves collisional interaction between the quencher and analyte. This type of quenching normally is significant only at relatively high quencher concentrations, i.e., 10^{-2} to 10^{-3} *M*. Long range quenching can also occur. If the fluorescence emission spectrum of an excited molecule overlaps the absorption spectrum of an acceptor molecule, the excitation energy may be transferred to the acceptor over distances as great as 60 to 100 Å. This form of quenching can become significant at relatively low acceptor concentration. Long-range energy transfer has been used to measure distances between two binding sites on proteins if one site is bound to a donor while the other is bound to an acceptor (see Section II.G).

Contamination — Background fluorescence from reagent contaminants is a common problem in fluorescence analysis. Highest purity solvents and scrupulous cell cleaning techniques are essential.

Interferences from other fluorophors — Interferences can result if the sample contains molecules with absorption and fluorescence overlapping the absorption and fluorescence of the analyte. Sometimes sufficient selectivity can be obtained by choosing appropriate excitation and emission wavelengths. In other cases, a separation is required. Several techniques have been proposed to selectively measure one fluorescent component in the presence of other fluorophors. These include making measurements at more than one set of wavelengths and solving simultaneous equations, using differ-

ences in fluorescence lifetimes as a basis for distinguishing the analyte from interferences,[19] and using wavelength modulation.[20] The latter two techniques can be quite effective, but require specialized equipment. One can also (with the right equipment) obtain a complete set of emission and excitation spectra, which can then be analyzed to resolve various components.[21,22]

IV. RELATIONSHIP BETWEEN STRUCTURE AND FLUORESCENCE

The relationship between structure and fluorescence has been considered in detail by Becker[23] and Wehry.[24]

A. General Principles

Luminescence is light emission associated with a transition from an excited electronic energy level to a lower energy level. The processes involved in luminescence are illustrated in the energy-level diagram in Figure 1. Excited electronic energy levels are produced by absorption of radiation. Although absorption populates upper vibrational states and higher electronic levels, relaxation to the lowest vibrational level of the first excited singlet occurs extremely rapidly. Thus, with few exceptions, all absorbed photons ultimately lead to the first excited singlet. This state then undergoes one of three processes. Fluorescence is observed if the transition from the first excited singlet to the ground state is accompanied by the emission of a photon. The transition from the first singlet to the ground state may also occur radiationlessly. This process is known as internal conversion. The energy is released as heat. The third process that the first excited singlet can undergo is intersystem crossing to a triplet state. Although intersystem crossing is spin-forbidden, it still occurs at appreciable rates in many molecules. Once formed, the triplet can return to the ground state either radiationlessly or with the emission of a photon. Emission from a transition involving a change in spin is known as phosphorescence and is generally only observed in solid matrices where collisional interactions are minimized. In solution, radiationless deactivation of triplets occurs much more rapidly than luminescence.

The fluorescence efficiency, ϕ_F, is defined as the number of photons emitted as fluorescence, divided by the number of photons absorbed. The fluorescence efficiency reflects the rate of fluorescence relative to the rates of internal conversion and intersystem crossing:

$$\phi_F = \frac{k_F}{k_F + k_{IC} + k_{IX}} \qquad (2)$$

where k_F is the rate of fluorescence, K_{IC} is the rate of internal conversion, and k_{IX} is the rate of intersystem crossing. Changes in molecular structure affect fluorescence efficiency by affecting the rates of one or more of these three processes. To a first approximation, the rate of fluorescence is proportional to the molar absorptivity.

The rate of internal conversion reflects the degree to which a compound interacts with its medium. Factors such as molecular flexibility, increased solvation, and higher temperature tend to enhance the rate of internal conversion.

The rate of intersystem crossing reflects the probability of the transition from the lowest excited singlet to the triplet. Structural factors that reduce the forbiddenness of transitions involving a change in spin enhance the rate of intersystem crossing. These include the presence of paramagnetic species and the presence of high atomic number atoms — either incorporated into the luminescent structure or associated with the luminescence medium.

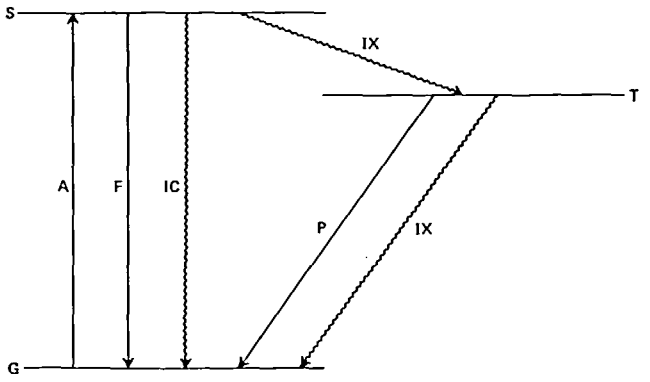


FIGURE 1. Simplified electronic energy diagram illustrating excited state processes. G = ground state, S = lowest excited singlet, T = lowest excited triplet, A = absorption, F = fluorescence, IC = internal conversion, P = phosphorescence, IX = intersystem crossing.

B. Structural Factors Affecting Luminescence

1. Extent of π-Electron System

Efficient fluorescence is only observed for molecules with extended π-electron systems, mainly aromatic compounds plus a few highly unsaturated aliphatic compounds. As the extent of the π-system increases, the fluorescence efficiency increases and the emission shifts to longer wavelengths. This is illustrated for linear aromatic hydrocarbons in Table 1. The data shown were taken at liquid nitrogen temperature in an organic glass rather than in solution; however, they serve to illustrate the effect. One practical consequence of this for fluorescence derivatization is that most important fluorescence derivatization reactions involve the formation of compounds with at least two fused aromatic ring systems. This generally leads to molecules for which excitation and emission can be accomplished at wavelengths longer than 330 nm, permitting the use of glass as an optical material, rather than quartz.

It should also be noted that for molecules like biphenyl, which have two aromatic systems that can rotate relative to each other, interaction between the two π-systems is greatest when the two π-systems are coplanar, and decreases as they become increasingly out of plane relative to each other. This type of molecule can be useful as a fluorescent probe of proteins, since luminescence properties are sensitive to small changes in molecular geometry.

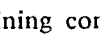
2. Nature of Lowest-Lying Transition

As previously noted, almost all absorbed photons lead to the first excited singlet. The nature of this transition is critical in determining the fluorescence behavior of molecules. For aromatic hydrocarbons, the lowest-lying transition always involves a transition from a π bonding orbital to a π* antibonding orbital. These transitions are characterized by high molar absorptivities and rapid rates of fluorescence. As a consequence, aromatic hydrocarbons are generally highly fluorescent (as illustrated by the data of Table 1).

If there is a heteroatom associated with the π-system, then one also has to consider the possibility of transitions involving the nonbonding electrons on the heteroatom, i.e., n-π* transitions. These transitions are characterized by relatively low molar absorptivities (typically 100 to 1000 *l*/mol/cm) and slow rates of fluorescence, because there is very little overlap between the n and π* orbitals. As a result, compounds with an n-π* lowest-lying singlet rarely fluoresce efficiently. This includes most azo com-

TABLE 1

Fluorescence Efficiencies of Linear Aromatic Hydrocarbons

Compound	Structure	ϕ_f	λ_{ex}(nm)	λ_{em}(nm)
Benzene		0.11	205	278
Naphthalene		0.29	286	321
Anthracene		0.46	365	400
Naphthacene		0.60	390	480

pounds and carbonyl-containing compounds and many nitrogen heterocycles with pyridine-type nitrogens. These compounds often undergo efficient intersystem crossing. In a solid phase many of them phosphoresce efficiently. In compounds containing pyrrole rings, however, (e.g., indole and carbazole) the orbital containing the nonbinding electrons is oriented perpendicular to the plane of the ring, enabling the nonbonding electrons to interact with the π-electrons of the carbon-carbon double bonds. As a result, transitions involving the nonbonding electrons in pyrrole rings behave like π-π* transitions and efficient fluorescence is frequently observed.

3. Effect of Metal Complexation

In metal-organic complexes, the nonbonding electrons of a heteroatom associate with the metal ion. This stabilizes the nonbonding electrons so it takes more energy to promote them to π* antibonding orbitals. As a result, metal complexation can cause the lowest excited singlet to change from n-π* to π-π* and cause a non- or weakly fluorescent ligand to become strongly fluorescent. This is useful both for metal analysis and for analysis of otherwise nonfluorescent organics.

There are other effects associated with metal complexation. If the metal is paramagnetic and/or has a high atomic number, this reduces the forbiddenness of transitions involving a change in spin, and thus causes an increase in the rate of intersystem crossing and a decrease in fluorescence (see below).

4. "Heavy Atom" Effect

The heavy atom effect refers to the observation that the presence of atoms with high atomic numbers causes a decrease in fluorescence and an increase in intersystem crossing. Table 2 shows data for the halogen-substituted naphthalenes illustrating this effect. The reason is that the heavy atom perturbs the electron spins — thus causing singlet states to have a certain amount of triplet character — thereby reducing the forbiddenness of intersystem crossing. The effect is also observed when the heavy atom is associated with the luminescence medium. For example, ethyl iodide has been used as a solvent to enhance intersystem crossing and, therefore, increase the sensitivity of phosphorescence analysis.[25]

5. Effect of Paramagnetic Molecules

The electrical fields associated with unpaired electrons also perturb electron spins and promote intersystem crossing. For example, metal complexes involving paramagnetic ions do not fluoresce. Oxygen quenching, which is frequently observed in nonpolar solvents, may reflect the fact that oxygen is paramagnetic.

6. Substituent Effects

Substituent effects are summarized in Table 3. The general rule is that electron-donating substituents such as NH_2, NHR, NR_2, OH, and OR enhance fluorescence efficiency and cause the fluorescence to shift to longer wavelength, while electron with-

TABLE 2

Internal Heavy Atom Effect Illustrated for 1-Substituted Naphthalenes (measurements in ethanol-ether at 77K)

Compound	ϕ_F	λ_F (nm)	ϕ_P	λ_P(nm)
Naphthalene	0.55	325	0.051	469.5
1-Fluoronaphthalene	0.84	316	0.056	473
1-Chloronaphthalene	0.058	319	0.30	483
1-Bromonaphthalene	0.0016	320	0.27	483
1-Iodonaphthalene	<0.0005	—	0.38	480

drawing substituents cause a reduction in fluorescence efficiency. Several common electron withdrawing groups such as

$$\text{COOH, COOR, } \overset{\overset{O}{\|}}{C}\text{–H, } \overset{\overset{O}{\|}}{C}\text{–R, and } -NO_2$$

introduce n-π^* transitions into the molecule. If the n-π^* transition is the lowest lying excited singlet, then there is a large decrease in fluorescence. The main effect observed with halogen substituents is the heavy atom effect. Other substituents (such as alkyl and sulfonate groups) have relatively little effect on fluorescence.

7. Complex Structures

Many of the fluorescent structures that arise through derivatization reactions involve more than one substituent and/or one or more heteroatoms. It is difficult to predict with any degree of certainty whether such structures will fluoresce efficiently. However, in general, increasing the size of the π-electron system and introducing electron-donating substituents can lower the energy associated with π-π^* transitions below that of n-π^* transitions, thus leading to efficient fluorescence from heteroatom-containing structures that might otherwise be expected to have low fluorescence efficiencies.

C. Approaches to Forming Fluorescent Derivatives

From the preceding discussion of the relationship between fluorescence and structure, it is possible to see several types of reactions that would involve reacting a nonfluorescent analyte to form a fluorescent product. These include:

1. Reactions that lead to the formation of an extended aromatic π-electron system
2. Reactions that change the nature of a substituent so as to render a nonfluorescent structure fluorescent
3. Complex formation reactions that tie up nonbonding electrons, thus rendering a nonfluorescent structure fluorescent.
4. Reactions that involve dissociating a structure from a heavy atom, thus rendering a nonfluorescent structure fluorescent

For all types of reactions, it is also possible to proceed in the opposite direction and measure a decrease in fluorescence; however, this is less common.

In addition to these possibilities, there is also the possibility of binding a fluorescent structure to another molecule through a side chain functional group where the reaction itself does not significantly influence the luminescence characteristics of the basic fluorescent structure. This, of course, requires a separation.

TABLE 3

Effect of Substituents on the Fluorescence of Aromatics

Substituent	Effect on frequency	Effect on intensity
Alkyl	None	Slight increase or decrease
OH, OCH_3, OC_2H_5	Decrease	Increase
O O O O ‖ ‖ ‖ ‖ CO–H, CH, CR, C–OR	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

D. Environmental Effects

The interaction between a molecule and its environment also has important effects on luminescence behavior. For molecules with either acidic or basic functional groups, pH is a critical variable. In general, the loss or gain of a proton will cause changes in both the efficiency and energy of emission. The situation is complicated by the fact that the acidity or basicity of the first excited singlet often differs from ground state acidity or basicity by as much as 6 or 7 pK units. For example, for 2-naphthol between pH 3 and 9, one observes a component of fluorescence characteristic of the 2-naphtholate ion, even though the ground state pKa is 9.5.[26] Since protonation reaction rates and rates of fluorescence are on an equivalent time scale, frequently one observes fluorescence from both protonated and unprotonated excited states. In practice, pH is a variable that must be properly adjusted prior to observing luminescence. In some cases, it is possible to exploit differences in luminescence vs. pH behavior to selectively analyze a mixture of two or more components with overlapping excitation and emission spectra.

Another important environmental consideration is solvent polarity. Increasing solvent polarity stabilizes the energy levels involved in fluorescence. For n-π^* transitions, the ground state is usually more polar than the excited state. Thus, the ground state is more effectively stabilized by polar solvents than the excited state — leading to an increase in the energy of n-π^* emissions and, therefore a shift in fluorescence to shorter wavelengths. For π-π^* transitions, the excited state is more polar and is more effectively stabilized by increased solvent polarity. As a result, the energies of π-π^* transitions decrease in polar solvents and fluorescence shifts to longer wavelengths. In some molecules the nature of the lowest lying singlet changes with solvent polarity, leading to a large change in fluorescence efficiency. Such compounds can serve as probes of the polarity of binding sites on proteins. This effect has also been used to detect polar compounds separated by TLC on cellulose.[27] The compounds were sprayed with flavones, and intense fluorescence was observed only in the vicinity of polar compounds.

The solvent can also interact with fluorophors to form excited state complexes or "exciplexes," which do not fluoresce. Choosing a solvent system to minimize exciplex formation can lead to an enhancement in fluorescence. This effect has been shown to increase the intensity for several important fluorescent derivatives.[28-30]

V. DERIVATIZATION REACTIONS

A. Other Sources of Information

The most complete and up-to-date information source for fluorescence derivatization reactions is the biannual reviews on *Luminescence in Analytical Chemistry*.[31] Passwater has abstracted much of the fluorescence literature.[32-34] Guilbault's book[2] includes an extremely large number of references covering all types of applications of fluorescence. Pesez and Bartos[35] provide detailed procedures for fluorescence derivatization of various organic functional groups. If this book is not available, much of the same material is covered in the authors' review article in *Talanta*.[36] Reactions of interest for medical or biological assays are covered in detail by Udenfriend.[37,38] The book by White and Argauer is particularly strong on fluorescence methods for inorganic ions.[5] It should be noted that all the above references are organized according to the nature of the compound being assayed. In this review, reactions are arranged according to the nature of derivatization reaction. Most reactions fit straightforwardly into a particular category; however, some reactions are not as readily classified, or can be fitted into more than one category.

B. Reactions Involving the Formation of Extended π-Systems

Table 4 lists 21 reactions used in forming fluorescent derivatives. The first 20 of these involve cyclization to form an aromatic ring leading to an extended π-system. In all cases except reactions 18 and 19, the reaction involves the extension of an already existing π-system. Reaction 18 differs from the other reactions in that the analyte, a tertiary amine, is not directly involved in the derivatization reaction. Instead it serves as a basic catalyst for condensation of malonic acid. In an acetylating medium, primary and secondary amines lose their ability to serve as basic catalysts. Thus this reaction is specific for tertiary amines, but it does not discriminate between different tertiary amines in any way. Reaction 19, the Hantzsch reaction, is also unique in that a ring system forms from small nonaromatic starting materials. Methods for amines, aldehydes, and β-diketones have all been developed based on this reaction. While these methods are not particularly sensitive as fluorescence methods go, they are quite selective.

The first eight reactions all involve the same principle; specifically an aromatic hydrocarbon with two adjacent substituents, both of which couple to the analyte, forming a new ring. In other reactions, 9 and 13 to 15, the analyte couples to a substituent and to an unsubstituted position on an aromatic ring. Electron-donating substituents can be used to make the aromatic ring more reactive and to the direct position of attack on the ring. Electron-donating substituents also help to enhance the fluorescence of the ultimate product. Reactions 11 and 12 involve the same principles, except that the substituted aromatic system is the analyte in these two reactions.

The catechol amines are an example of a class of compounds uniquely suited for fluorescence analysis, because they involve a long side chain on a substituted benzene which is readily cyclized to give a highly fluorescent indole derivative. These assays are widely used and there are many more references in addition to those cited in the table. Of the two variations of the methods cited for catechol amines, the trihydroxy indole method is more specific, while the ethylene diamine condensation method is more sensitive.

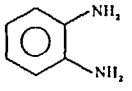
The steroids also represent a special case. These compounds have a fused aliphatic ring system with four rings. (In the estrogens, one ring is aromatic.) In strong sulfuric acid, condensation occurs to give polynuclear aromatics that are highly fluorescent.

In general, the reactions listed in Table 4 are performed in strong acid at elevated

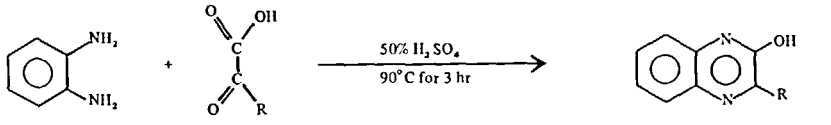
TABLE 4

Fluorescence Derivatization Reactions Based on Forming an Extended π-Electron System

1. o-Phenylene-diamine



Typical reaction:

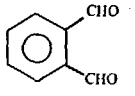


λ_{ex} = 360 nm, λ_{em} = 490 nm

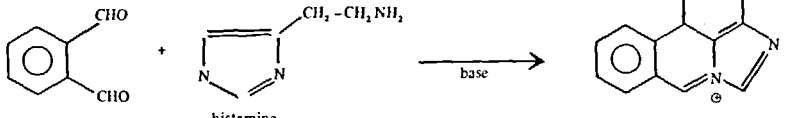
Compounds forming fluorescent derivatives:

α-Keto-acids[39]
Carbohydrates[40]
Imidazoles including histamine and histidine after treatment with *N*-bromo succinamide[41]
Alloxan[42]
Tocopherol[43,44]

2. *o*-Phthalaldehyde



Typical reaction:



histamine

λ_{ex} = 360 nm, λ_{em} = 490 nm

Compounds forming fluorescent derivatives:

Histamine[45,46]
Histidine[47]
Polyamines[48]
Most amino acids[49-51]
Indoles[52,53]
Peptides[54,55]
Proteins[56-58]

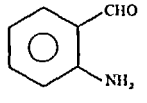
3. o-Aminothiophenol

Compounds forming fluorescent derivatives:

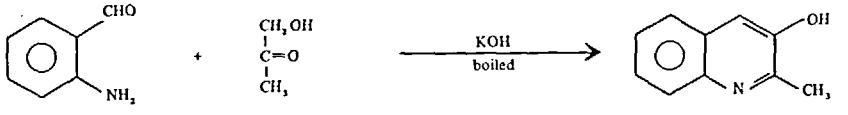
red-violet fluorescence

Furfural, pentoses, hexoses[59,60]
Aldehydes[61]

4. *o*-Aminobenzaldehyde



Typical reaction:



Compounds forming fluorescent derivatives:

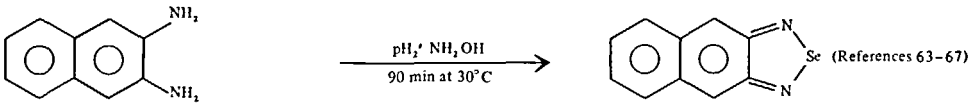
blue emission

Carbohydrates distilled to produce acetol[62]

TABLE 4 (cont.)

5. 2,3-Diaminonaphthalene (for Se)

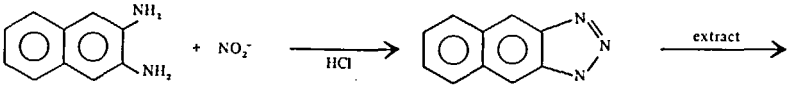
Typical reaction:



λ_{ex} = 366 nm, λ_{em} = 520 nm

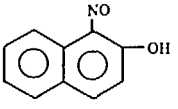
6. 2,3-Diaminonaphthalene (for NO_2^- and NO_3^-) (68, 69)

Typical reaction:

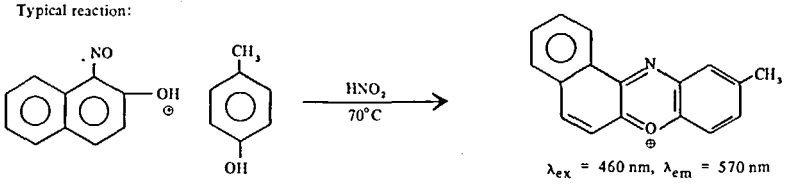


λ_{ex} = 355 nm, λ_{em} = 410 nm

7. 1-Nitroso-2-naphthol



Typical reaction:



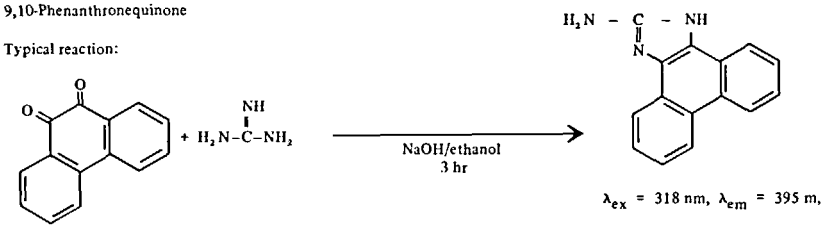
λ_{ex} = 460 nm, λ_{em} = 570 nm

Compounds forming fluorescent derivatives:

Para-substituted phenols[70]
Tyrosine[71–74]
Tyramine[74]
Tyrosine-containing peptides[75]

8. 9,10-Phenanthronequinone

Typical reaction:



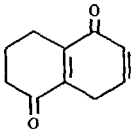
λ_{ex} = 318 nm, λ_{em} = 395 m,

Compounds forming fluorescent derivatives:

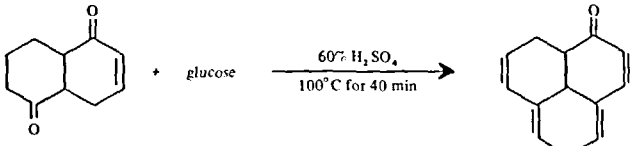
Guanidines[76,77]

TABLE 4 (cont.)

9. 5-Hydroxy-1-tetralone



Typical reaction:

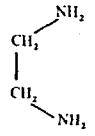


Compounds forming fluorescent derivatives:

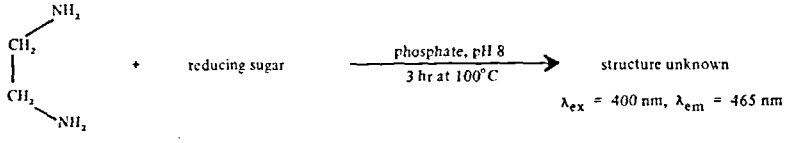
Hexoses,[78,79] particularly glucose[78,80]

λ_{ex} = 470 nm, λ_{em} = 550 nm

10. Ethylene diamine



Typical reaction:



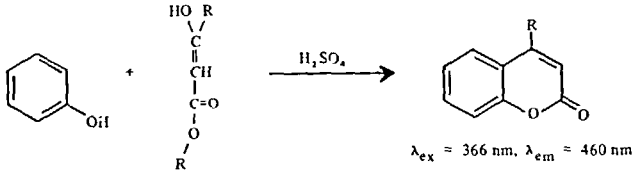
λ_{ex} = 400 nm, λ_{em} = 465 nm

Compounds forming fluorescent derivatives:

All reducing sugars–including aldoses, ketoses, deoxy sugars, amino sugars, and uronic acids[81,82]

11. Ethyl acetoacetate

Typical reaction:



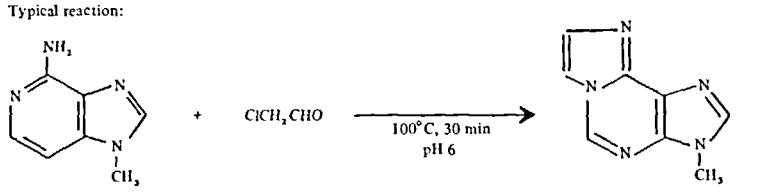
λ_{ex} = 366 nm, λ_{em} = 460 nm

Compounds forming fluorescent derivatives:

Phenols[83]

12. Chloroacetaldehyde

Typical reaction:



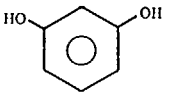
Compounds forming fluorescent derivatives:

λ_{ex} = 310 nm, λ_{em} = 410 nm

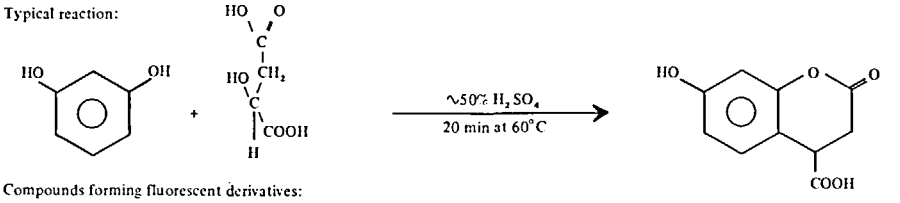
Adenine compounds[84-87]
Adenosine monophosphate[88]
Adenosine triphosphate[89]
Flavinadenine dinucleotide[90]
Adenosine and cytidine[91]

TABLE 4 (cont.)

13. Resorcinol



Typical reaction:

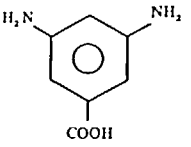


λ_{ex} = 430 nm, λ_{em} = 480 nm

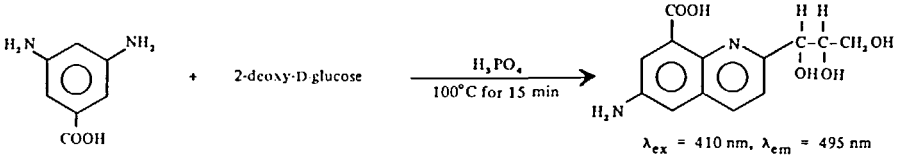
Compounds forming fluorescent derivatives:

Ketohexoses[92]
Carbohydrates[93]
β-Keto acids[94]
Polycarboxylic acids[95]
Dicarboxylic acids[96,97]
Spermine, spermidine, allylamine, acrolein (at pH 7)[98]

14. 3,5-Diaminobenzoic acid



Typical reaction:

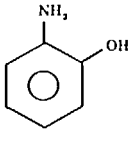


λ_{ex} = 410 nm, λ_{em} = 495 nm

Compounds forming fluorescent derivatives:

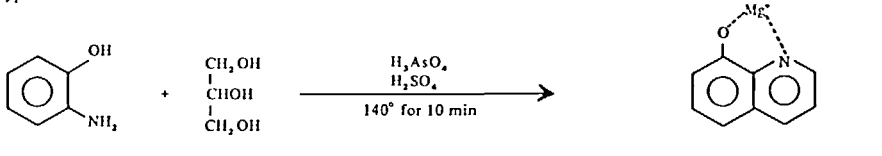
Aldehydes of type R–CH_2–CHO including

Acetaldehyde[99]
2-Deoxy glucose[100]
Succinic semialdehyde[101]
DNA[102-104]



15. *o*-Aminophenol

Typical reaction:



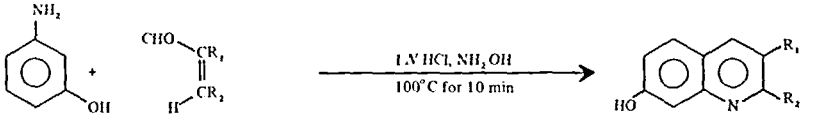
λ_{ex} = 390 nm, λ_{em} = 540 nm

Compounds forming fluorescent derivatives:

Glycerol[105]
Triglycerides after hydrolysis[105]

TABLE 4 (cont.)

16. *m*-Aminophenol

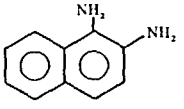


λ_{ex} = 350 nm, λ_{em} = 505 nm

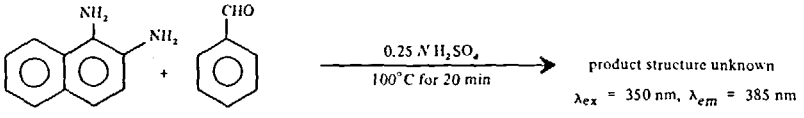
Compounds forming fluorescent derivatives:

α–β unsaturated carbonyl compounds[106]

17. 1,2-Diaminonaphthalene



Typical reaction:



Compounds forming fluorescent derivatives:

Aliphatic aldehydes[107]
Aromatic aldehydes including benzaldehyde, cinnamaldehyde, 4-dimethylamino benzaldehyde, and furfural[107]

Compounds not forming fluorescent derivatives:

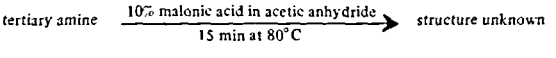
Aromatic aldehydes with hydroxyl, amino, or nitro substituents[107]

Application:

Monoamine oxidase activity[108]

18. Malonic acid/Acetic anhydride

Typical reaction:



base-catalyzed condensation

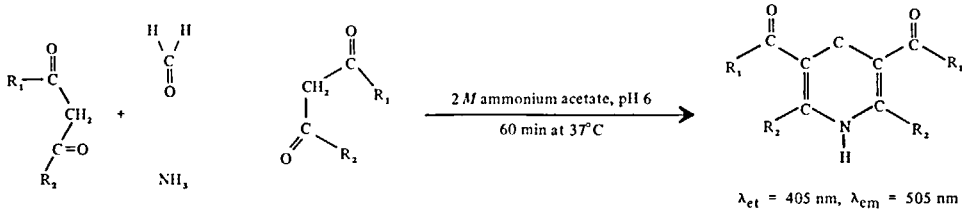
Compounds forming Fluorescent Derivatives:

Tertiary amines[109,110]

TABLE 4 (cont.)

19. Ammonia, formaldehyde, acetyl acetone (Hantzsch reaction)

Typical reaction:



λ_{ex} = 405 nm, λ_{em} = 505 nm

Compounds forming fluorescent derivatives:

a. β-Diketones-ethyl acetoacetate, 2,4-pentanedione, 1,3-cyclohexane dione

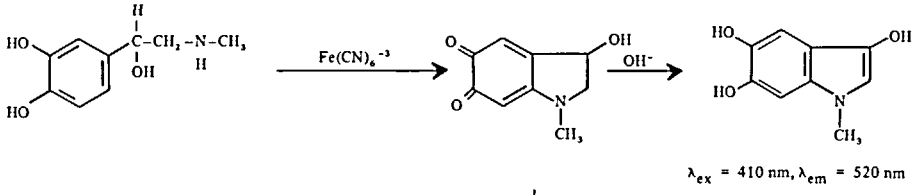
b. Formaldehyde[83,111,112]
Compounds oxidized to formaldehyde including 1,2-diols, hexitols, primary α-aminoalcohols, 17-ketosteroids, ethylenic compounds of type R–CH=CH_2[83,111,113]

c. Other aliphatic aldehydes[83,111,113]
Compounds oxidized to aldehydes – primary alcohols[110]
Ketoses[115,116]

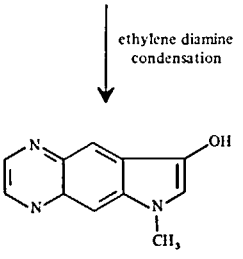
d. Primary amines, α-amino acids,[83,110,117] nitrites reduced to primary amines[83]

20. Catechol amines

Typical reaction:



λ_{ex} = 410 nm, λ_{em} = 520 nm

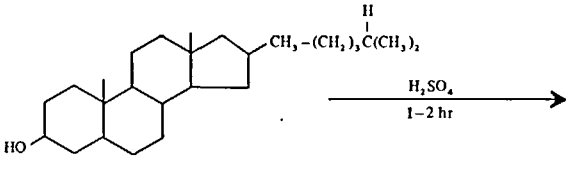


(References 118–125)

λ_{ex} = 420 nm, λ_{em} = 525 nm

21. Steroids

Typical reaction:



structure unknown
(References 126–131)

temperatures for extended intervals of time. They are suitable for derivatizing compounds separated by TLC, although they are not convenient for post-column derivatization in LC. Essentially, no work has been done to examine the effect of variations of reagent structure on the derivatization reactions. In several cases, the structure of the fluorescent product is not known at all, or is only hypothesized without definite proof.

Most of the reactions involve the formation of heterocyclic aromatics, creating the possibility of n-π^* transitions. (This is a lot easier than forming an aromatic hydrocarbon without any heteroatoms.) However, the π-system is sufficiently large and electron-donating substituents are present, causing the lowest excited-singlet to be π-π^*, thus leading to efficient fluorescence. In the case of *o*-aminophenol, the product of derivatization reaction is 8-hydroxyquinoline. The fluorescence is enhanced by adding Mg^{+2} to form a complex. (See Section V.D.2.)

The specificity of many of these reactions is quite good, since they require that the analyte have two adjacent functional groups that will couple to the reactions. Even though the reagents themselves are fluorescent in many cases, the extension of the π-system causes the fluorescence to shift to longer wavelengths. Thus there is no need to separate the product from the reactants to avoid reagent luminescence. There may, however, be other reasons for extracting the product.

C. Derivatization via Substituent Modification

1. Fluorescent Labels

Fluorescence derivatization reactions that involve only substituents can be subdivided into a number of categories. In one type of reaction, the substituent serves mainly as a means of coupling a molecule of interest to a fluorescent "label" under mild conditions. Important examples of this type of reaction are listed in Table 5. The two substituents that are most useful for this type of coupling are sulfonyl chloride and isothiocyanate, as illustrated in the table. The first two reagents in Table 5 are widely used to label proteins for fluorescence microscopy and fluorescence immunoassay. Excess reagent can be easily separated on the basis of size. Dansyl chloride and fluoroscein isothiocyanate are widely used as labels for fluorescence polarization measurements. The 9-isothiocyanatoacridine is considerably less important in biochemistry, but is included in the table because it has been evaluated for some analytical applications. The subject of fluorescent protein tracing is considered in detail by Nairn.[132] Reactions 1 through 4 and 9 couple to amine substituents in the compound to be labeled. The dansyl reaction can also be used to label phenols and thiols. Reaction 8 can be used for phenolic-OH. Reactions 5 and 6 are designed to selectively label sulfhydryl and carbonyl groups, respectively, with what is essentially a dansyl group.

These reactions can also be used to improve the detectability of compounds separated by liquid or thin-layer chromatography. Derivatization is generally performed prior to separation. The dansyl reaction is particularly widely used for this purpose. Many of these applications have been covered in an extensive review.[137] Because the dansyl group is smaller than the other "labels" in Table 5, it is less likely to interfere with the separability of the compounds to be determined. The dansyl derivatives also have the interesting property that fluorescence efficiency and emission wavelengths are highly solvent dependent. As one goes from nonpolar to polar solvents, the color of the fluorescence changes from blue to yellow and the efficiency decreases. As a result, dansyl derivatives have been widely used as fluorescent probes to study the polarity of protein binding sites. There has been some work with similar types of analytical applications using fluorescein isothiocyanate and 9-isothiocyanatoacridine.

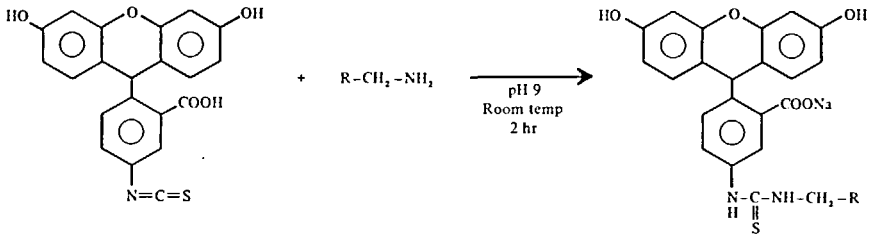
It should be noted that fluorescamine has also been used for the above types of

TABLE 5

Fluorescence Labeling Reactions

1. Fluorescein isothiocyanate

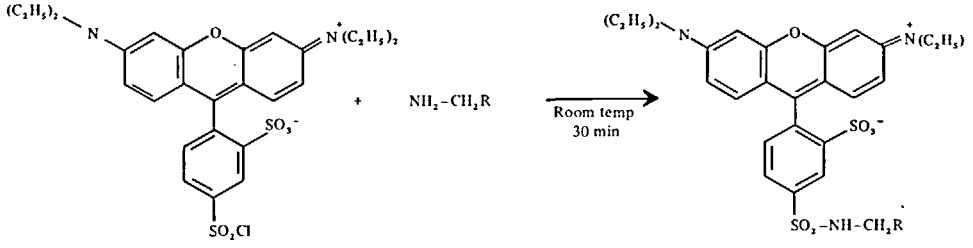
Typical reaction:



Applications:

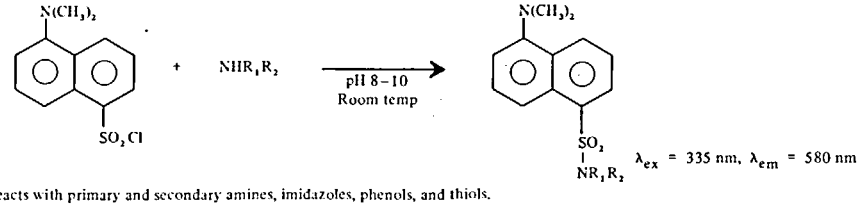
Identifying terminal amino acids[133]
Labeling amino acids prior to TLC separation[134]
Enzyme Labeling[135]
Stability[136]

2. Lissamine rhodamine B



3. Dansyl chloride (1-Dimethyl amino-naphthalene-5-sulfonyl chloride)

Typical reaction:



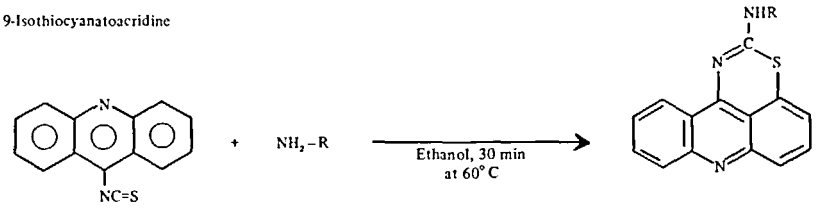
Reacts with primary and secondary amines, imidazoles, phenols, and thiols.

Analytical applications:

Amino acid analysis[137-139]
End group reagent for peptides and proteins[137]
Labeling reagent for fluorescence polarization[137]
Study active site of proteins[137]
Analysis of amines[137,140,141]
Analysis of phenols[137]
Analysis of catechols[137]
Analysis of amino sugars[137]
Hydroxybiphenyls[142]
Carbamate pesticides[143]
Protein[144,145]
Alkaloids[146]
Pesticides and herbicides[147,148]
Catecholamines[12,149,150]
Use in aprotic solvents[151]

TABLE 5 (cont.)

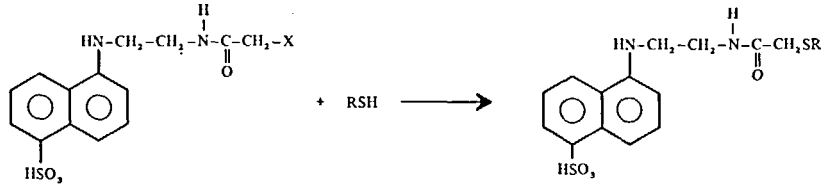
4. 9-Isothiocyanatoacridine



Analytical applications:

Analysis of primary and secondary amines[152-154]

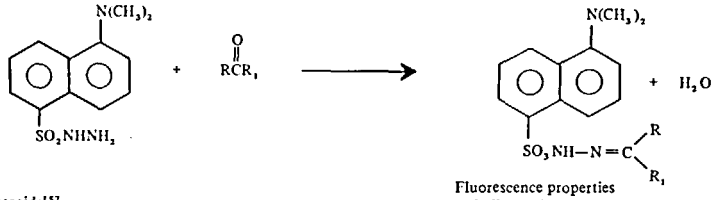
5. *N*-(Iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid



Fluorescence properties similar to dansyl derivatives

Specific reagent for thiols[155,156]

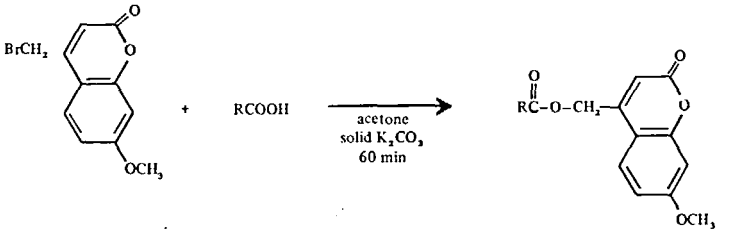
6. Dansyl hydrazine



Keto steroids[157]
Glycoproteins[158]

Fluorescence properties similar to dansyl derivatives

7. 4-Bromomethyl-7-methoxycoumarin



λ_{ex} = 328 nm, λ_{em} = 380 nm

Fatty acids[159]

TABLE 5 (cont.)

8. 2-*p* Chlorosulfophenyl-3-phenyl indone

Amines[160]
Amino acids[161]
Amino sugars[162]
Tetrahydrocannabinol (thru phenolic OH)[163]

applications. However, fluorescamine offers an additional advantage — because the reagent itself does not fluoresce and is not easily hydrolyzed to fluorescent products. It is considered below.

2. Fluorogenic Substrates for Enzymes

If an enzyme can catalyze a reaction involving a change in either fluorescence wavelength or efficiency, the enzyme activity can be related to the change in fluorescence with time measured under controlled conditions. Fluorogenic substrates typically consist of a substituent that the enzyme likes to act upon, coupled to an aromatic system whose luminescence properties are significantly modified by the substituent. By cleaving the substituent, the enzyme causes a significant change in fluorescence properties. A fairly complete listing of fluorogenic enzyme substrates is presented in Table 6. Examination of this table shows that Pluorogenic substrates are generally applicable to various hydrolytic enzymes including esterases, phosphatases, and peptidases. From the point of view of fluorescence derivatization, it is necessary to find an aromatic system whose fluorescence properties are highly substituent dependent. Several such systems are listed in Table 6. It is also necessary that the aromatic system does not inhibit the enzyme. Otherwise the enzyme catalysis will not proceed and the approach will not work. In general, enzyme activity using a fluorogenic substrate differs from enzyme activity based on the natural substrate. This must be accounted for in determining activities.

The use of fluorogenic substrates has been particularly useful in situations where enzyme activities are low, since these methods are considerably more sensitive than other methods for measuring enzyme activities.

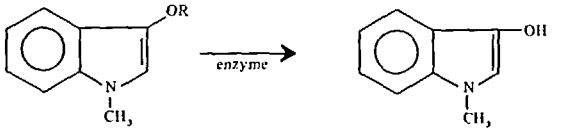
3. Other Reactions Based on Substituent Effects

Table 7 lists other reactions used for fluorescence derivatization that involve changing the nature of a substituent on an aromatic structure. Reaction 1, fluorescamine, is not readily classified since it also involves a change in the reagent ring system. This reaction is, however, as close to a perfect derivatization as can be found. It is fast under moderate conditions, and it is completely specific for primary amines. The reagent itself does not fluoresce and does not hydrolyze to fluorescent products. As a consequence, fluorescamine is well suited for all types of applications from protein labeling to chromatographic detection, with derivatization performed either before or after separation. This is evidenced by the list of applications in Table 7.

TABLE 6

Fluorogenic Enzyme Substrates

1. *N*-methyl indoxyl

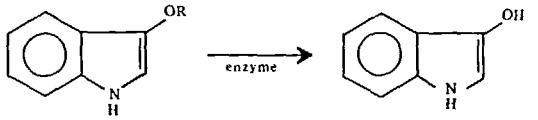


Nonfluorescent

$\lambda_{ex} \approx 430$ nm, $\lambda_{em} = 500$ nm

Enzymes determined:	R:
Cholinesterase[164,165]	Acetate (best), propionate, butyrate
Lipase[166]	Myristate (best), hexanoate, heptanoate, octanoate, nonoate

1a. Indoxyl

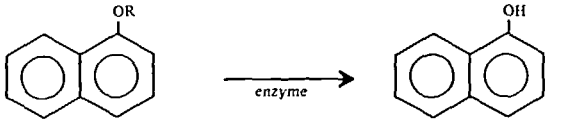


Nonfluorescent

$\lambda_{ex} = 395$, $\lambda_{em} = 470$

Enzymes determined:	R:
Cholinesterase[167]	Acetate
Sulfatase[166]	Sulfate
Hyaluronidase[168]	Acetate

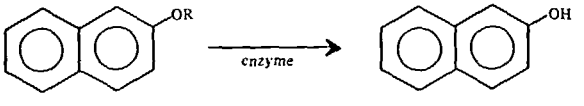
2. 1-Naphthol



$\lambda_{ex} = 345$ nm, $\lambda_{em} = 455$ nm

Enzymes determined:	R:
Cholinesterase[169]	Acetate, butyrate
Cellulase[170]	Acetate
N-acetyl-β-D-glucosaminidase [171]	2-Acetamido-2-deoxy-β-D-glucopyranoside
β-D-glucuronidase[172]	β-D-glucuronide
Acid and alkaline phosphatase[173-175]	Phosphate

3. 2-Naphthol

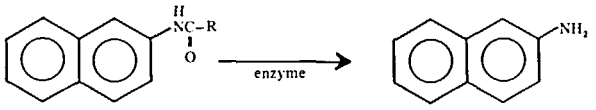


$\lambda_{ex} = 320$ nm, $\lambda_{em} = 410$

Enzymes determined:	R:
Cholinesterase[167]	Acetate, butyrate
Cellulase[170]	Acetate
β-D-glucuronidase[176]	β-D-glucuronide
Alkaline phosphatase acid phosphatase[175]	Phosphate
Plasmin, streptokinase[177]	α-*N*-methyl α-*N*-tosyl-L-lysine

TABLE 6 (cont.)

4. 2-Naphthylamine

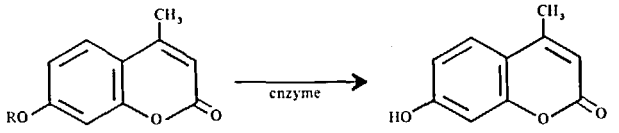


Fluoresces at 350 nm

λ_{ex} = 340 nm, λ_{em} = 400 nm

Enzymes determined:	R:
Leucine aminopeptidase[178-180]	L-lencyl
Trypsin, trypsiniline amidase[181,182]	α-Benzoyl-L-arginine
Peptidase[175,183]	Phenyl alanyl

5. 4-Methylumbelliferone

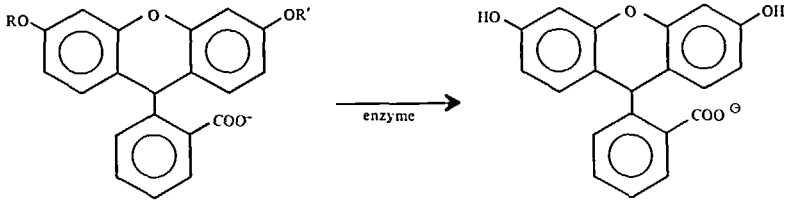


Nonfluorescent

λ_{ex} = 330 nm, λ_{em} = 450 nm

Enzymes determined:	R:
Lipase[164,165,184]	Heptanoate
Sulfatase[165]	Sulfate
Cholinesterase[164]	Acetate, butyrate
β-Glucuronidase[185]	Glucuronide
β-Glucosidase[186]	Glucoside
Hexosaminidase[187]	β-D-glucosaminide
α-Galactosidase[188]	α-Galactoside
α-L-Fucosidase[189,190]	α-L-fucoside
Acrosin[191]	p-Guanidinobenzoate
Chymotrypsin[192]	Glutaryl phenyl alanimide

6. Fluorescein

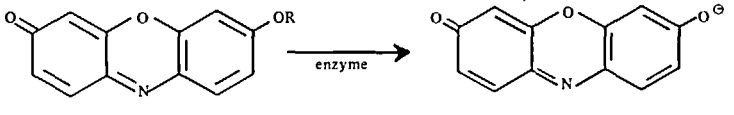


Nonfluorescent

λ_{ex} = 470 nm, λ_{em} = 510 nm

Enzymes determined:	RR':
Lipase[193,194]	Dibutyryl
Lipase, acylase α- and γ-Chymotrypsin[194]	Diacetyl, dibutyryl
Lipase[195,196]	Monodecanoate
Acid and alkaline phosphotase[197]	Methyl, phosphate
β-D-Galactosidase[198]	Di-β-D-galactophyranoside

7. Resorufin



Nonfluorescent

λ_{ex} = 540 nm, λ_{em} = 580 nm

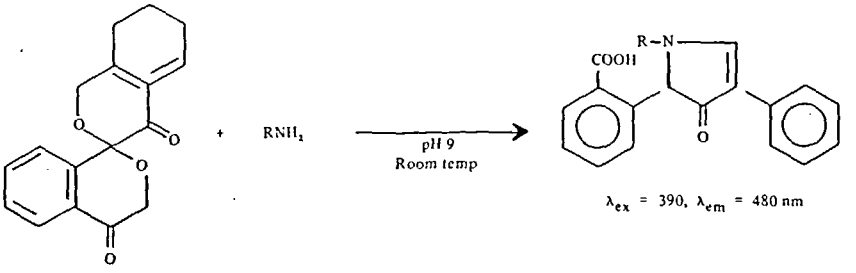
Enzymes determined:	R:
Cholinesterase[167]	Butyrate
Cellulase[170]	Acetate

TABLE 7

Fluorescence Derivatization Based on Substituent Modification

1. Fluorescamine (4-phenyl spiro [furan-2(3H),1'-phthalan]-3,3' dione)

Typical reaction:



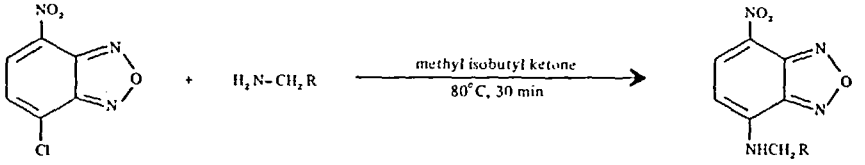
λ_{ex} = 390, λ_{em} = 480 nm

Compounds forming fluorescent derivatives:

Drugs[199,200]
Peptides[58] compared to *o*-phthalaldehyde[201–203]
Amines[204–208]
Antibody labeling[209–211]
Amino acids[13,212,213]
Protein assay[214]
Secondary amino acids[215]
Protein-labeling[209,216,217]
Amphetamine[218]
Live cells[219]

2. 7-Chloro-4-nitro-benzoxadiazole

Typical reaction:

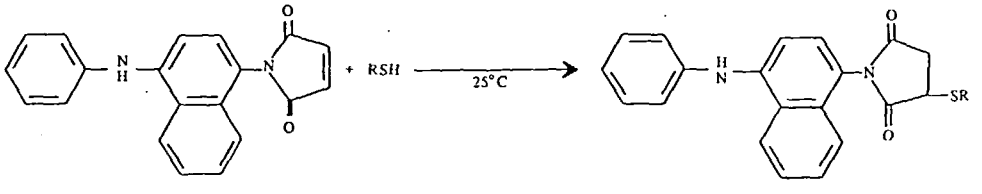


λ_{ex} = 465 nm, λ_{em} = 525 nm

Compounds forming fluorescent derivatives:

Amphetamines[220]
Amino acids[221]
Carbamate pesticides[222]
Amines[223,224]
Nitrosamines[225]
Gentamicin[226]

3. *N*-(1-anilino naphthy-4)maleimide



Nonfluorescent

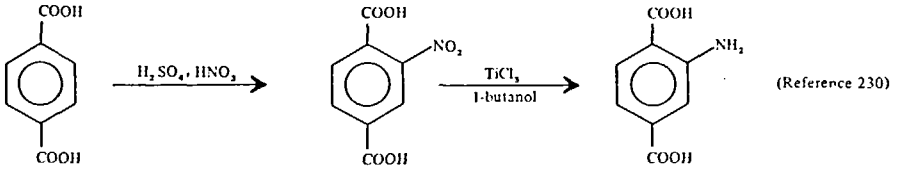
λ_{ex} = 355 nm, λ_{em} = 448 nm

Compounds forming fluorescent derivatives:

Thiols[227,228]
Analogous reaction with umbelliferone derivative in place of aminonaphthyl group[229]

TABLE 7 (cont.)

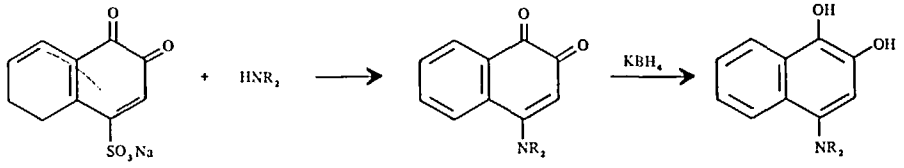
4. Terephthalic acid



(Reference 230)

λ_{ex} = 364 nm, λ_{em} = 438 nm

5. 1,2-Naphthoquinone-4-sulfonic acid

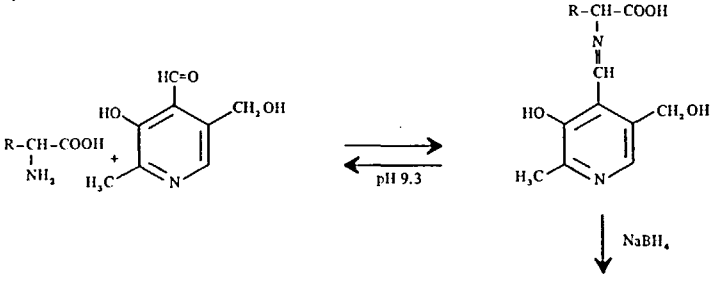


Extracted into CH_2Cl_2

Compounds forming fluorescent derivatives:

Primary and secondary alkyl amines[36]
Primary aryl amines[36]

6. Pyridoxal

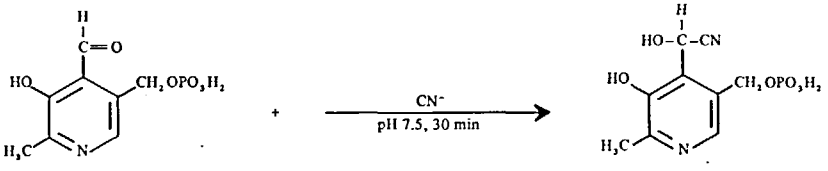


λ_{ex} = 332 nm, λ_{em} = 400 nm

Compounds forming fluorescent derivatives:

Amino acids[231]

7. Pyridoxal 5-phosphate



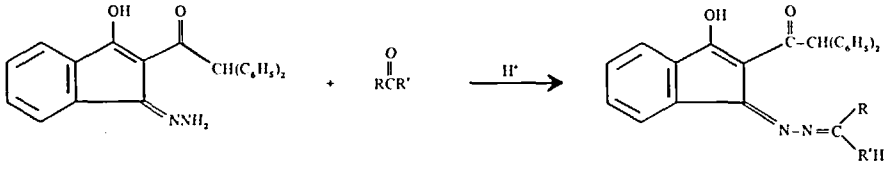
λ_{ex} = 315 nm, λ_{em} = 430 nm

For determining CN^- [232]

TABLE 7 (cont.)

8. 2-Diphenylacetyl-1,3-indanedione-1-hydrazine

Typical reaction:



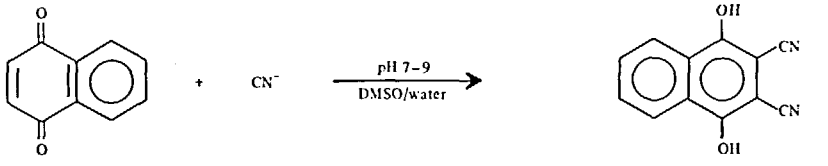
λ_{ex} = 300 nm, λ_{em} = 410 nm

Compounds forming fluorescent derivative:

Aldehydes, ketones[233-236]

Requires separating reagent from product.

9. Cyanide



λ_{ex} = 400 nm, λ_{em} = 470 nm

For quinones[237]

The second reaction in Table 7 involves converting a −Cl group to an electron-donating amine substituent, thus leading to efficient fluorescence. This reaction is somewhat surprising in that nitro compounds generally do not fluoresce.

The third reaction in the table is quite useful for thiols. However, it is not clear to the author why the product fluoresces efficiently while the reagent does not, since the derivatization reaction has the effect of removing the double bond from the π-system. Possibly removal of the double bond affects the molecule in some way, so that the lowest singlet does not involve the "n" electrons on the carbonyls.

Reactions 4 and 5 illustrate systems where addition of an electron-donating substituent causes a modification of fluorescence properties. Reactions 6 to 9 involve the modification of a substituent such that it can no longer introduce an n-π* transition into the basic aromatic structure.

D. Derivatization Based on Metal Complex Formation

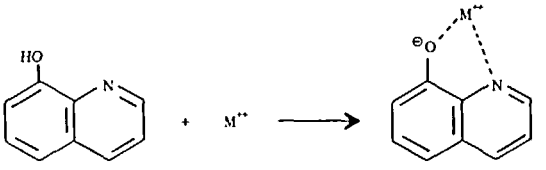
1. Methods for Metal Ion Determination

Complexation ties up nonbonding electrons causing them to be lower in energy. Thus, it is fairly common to have molecules that have n-π* lowest excited singlets when they are not complexed, and have π-π* lowest excited singlets when they complex with a metal ion. By changing the nature of the lowest excited singlet, complexation can cause a nonfluorescent structure to become fluorescent. This effect can be used for the analysis of either metal ion or organic substances, although considerably more work has been directed toward developing fluorescent reagents for metal determination. A listing of some of these reagents (along with metals forming fluorescent chelates) is presented in Table 8. For a more complete coverage of this area, the reader

TABLE 8

Reagents for Forming Fluorescent Metal Chelates

1. 8-Hydroxy quinoline (8-quinolinol, oxine)

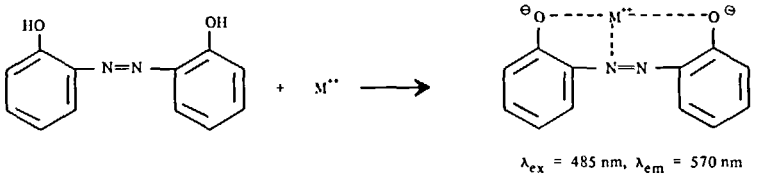


λ_{ex} = 380 nm, λ_{em} = 520 nm

Metals forming fluorescent chelates:

$Ag^{238,239}$
$Al^{239,240}$
Mg, $Ca^{239,241}$
Cd, Zn, Sn, Sr, Ba^{239}
La^{242}
Zn^{243}
Cd, Mg, Al, Ga, In, Tl, Pb, $Zn^{244,245}$
Li, Na, Pb, Cs, Mg, Ca, Sr, Ba, Sc, Y^{246}
Zn, Cd, Al, Ga, In, Ge, Sn^{246}
Mg, Ca, Sr^{247}
Be^{248}

2. 2,2′-dihydroxy azobenzene and derivatives



λ_{ex} = 485 nm, λ_{em} = 570 nm

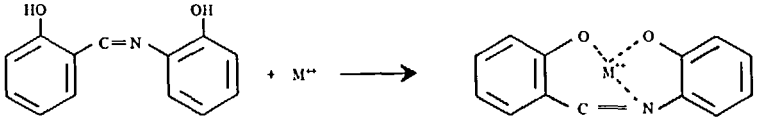
Including:

Acid Alizarin Garnet R (2,2′-dihydroxy azobenzene)
Mordant Blue 31 [2(2-hydroxy-5-sulfophenylazo)-1,8-dihydroxynaphthalene-3,6-) disulfonic acid]
Lumogallion [5-chloro-3-(2,4-dihydroxyphenylazo)-2-hydroxy benzene sulfonic acid]
Superchrome Garnet Y [3-(2,4-dihydroxyphenol azo)4-hydroxy benzene sulfonic acid]
Pontachrome Blue Black R (2,2′-dihydroxy-1,1′-azonaphthalene-4-sulfonic acid)

Metals forming fluorescent chelates:

Al, Ga, Se, Mg, In^{249}
Al, Ga, Sc, Y, La, Lu, Ln, Mg^{250}
Al, Ga, Sc, In^{251}
Al, Ga, Th, In, Mg, Zn, Be^{252}
Al, Ga, In, Be, Mg, Zn^{253}

3. Salicylidene-*o*-aminophenol



λ_{ex} = 405 nm, λ_{em} = 505 nm

and related compounds including:

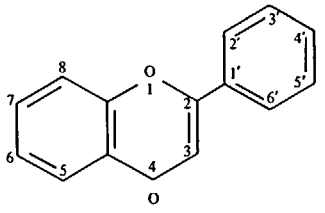
bis-salicylidene ethylene diamine
N-salicylidene-*o*-aminophenyl arsonic acid
N-salicylidene-4-aminobenzothiazole

Metals forming fluorescent complexes:

Be, Sc^{254}
Al, Ga^{255}
$Al^{256,257}$
Zn, Cd, Mg^{258}

TABLE 8 (cont.)

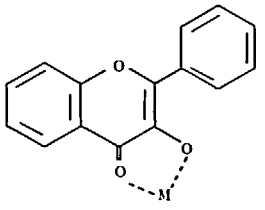
4. Polyhydroxy Flavones



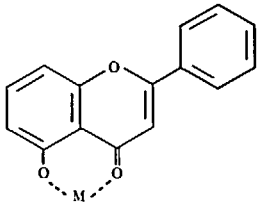
Including:

Morin (3,5,7,2'4' pentahydroxy flavone)
Quercetin (3,5,7,3'4' pentahydroxy flavone)
Flavonol (3-hydroxy-flavone)

Complexes formed:



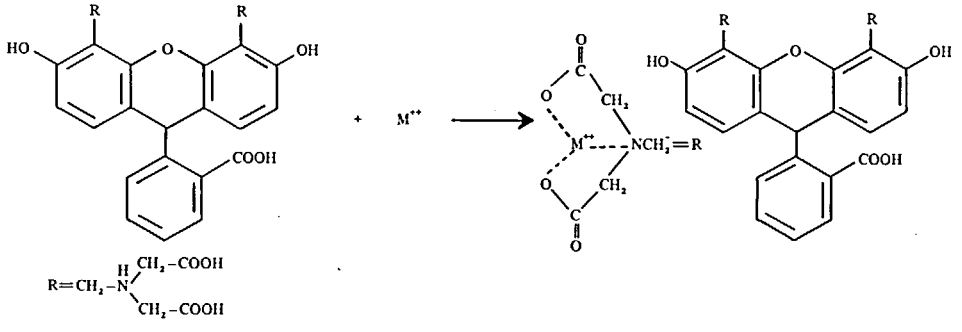
and



Metals forming fluorescent chelates:

Al, Be, B, G, Ge, Sc, Sn, Zn (References 259 and 260 and references therein)
Be[261]
Hf, W[262]
Sn[263]

5. Calcein (fluorescein iminodiacetic acid)

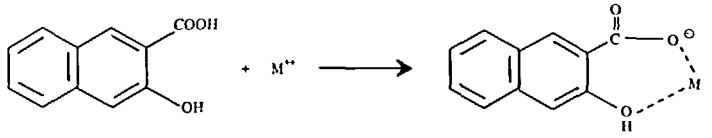


Metals forming fluorescent chelates:

Cd[264]
Ca[265-268]
Ca, Sr, Ba[269]

TABLE 8 (cont.)

6. 3-Hydroxy-2-naphthoic acid

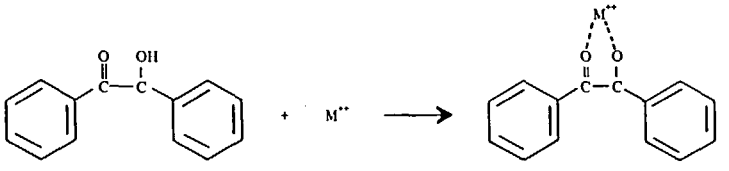


λ_{ex} = 370 nm, λ_{em} = 460 nm

Metals forming florescent chelates:

Al, Be[270]
Al, Be, Sc, Ga, Zn[271]

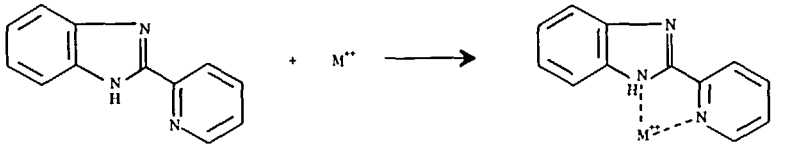
7. Benzoin (α-Hydroxy-α-phenyl-acetophenone)



Metals forming fluorescent chelates:

B[272,273]
Zn[274]

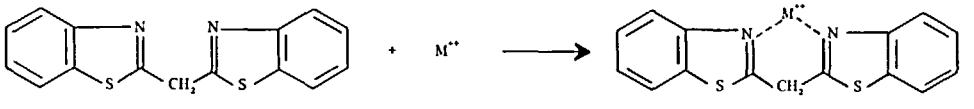
8. 2-(2[1]-Pyridyl)benzimidazole



Metals forming fluorescent chelates:

Ga, In, Zn[275]

9. 2,2'-Methylene dibenzothiazole



Metals forming fluorescent chelates:

Zn[276]

should consult the references cited in section V.A, or another recent reference.[277]

The first four reagents listed in Table 8 are the most widely studied. The others are included to provide some idea as to the many possibilities for designing fluorescent chelates. This has been an active research area and the list in Table 8 is only a small fraction of the total number of fluorescent chelates that have been evaluated for analytical purposes. Also, a considerable amount of work has been done to evaluate structural changes on the analytical properties of various chelates. For example, several derivatives of 2,2'-dihydroxyazo benzene (reaction 2) and polyhydroxy flavones (reaction 4) have been used as fluorescent metal reagents. The primary motivation for the extensive work on various complex structures has been to improve the selectivity for particular metals, and also to modify the extractability into organic solvents, rather than to modify fluorescence behavior.

Careful inspection of the table shows that most ligands form fluorescent chelates with the same set of metals. The metal ions that are best determined by fluorescence have low atomic numbers and are not transition elements. The other elements do not tend to form strongly fluorescent complexes, because the heavy atom effect and the presence of paramagnetic species both promote intersystem crossing and ultimately radiationless deactivation of the first excited singlet. This problem can be attacked by precipitating a sample into a solid matrix, in which case luminescence analysis for heavy metals is possible.[278]

It should be noted that calcein differs from other reagents. In the case of calcein, the metal ion does not tie up nonbonding electrons associated with the aromatic π-system; it merely associates with an iminodiacetate group attached to fluorescein; however, this complexation is sufficient to change the fluorescence behavior so that analysis for calcium is possible.

From several points of view, metal complexation reactions are very well suited for fluorescence derivatization. They are fast and can be driven to completion by adding an excess of reagent. Both pH control and solvent extraction can be used to vary selectivity; however, sufficient selectivity can be a problem. While fluorescence methods for metals are highly sensitive and have wide linear dynamic ranges, they often do not offer significant advantages over competing techniques, such as flame methods and X-ray fluorescence. It is paradoxical that the greatest research effort has been expended on the class of derivatization reactions that offers the fewest advantages over competitive methods.

In addition to direct metal analysis, indirect analysis for other ligands is possible if the ligand of interest pulls metal ions away from a fluorescent metal chelate causing a decrease in fluorescence. For example, phosphate causes the fluorescence of the aluminum-morin complex to decrease.

2. Methods for Organics Based on Complexation

It is possible to analyze for nonfluorescent organic ligands by adding metal ions that form fluorescent complexes. Clearly the reagent structures listed in Table 8 would be amenable to this type of analysis simply by adding an excess of metal ion. Four examples of analytical methods for organics that utilize metal complexation are listed in Table 9. Relatively little attention has been given to this type of derivatization, even though it would seem to offer considerable potential for the analysis of otherwise nonfluorescent aromatic compounds.

E. Methods Based on Heavy Atom Effect

The heavy atom effect has been used in a few fluorescence derivatization procedures. Table 10 cites three examples. The first reaction involves determining a heavy atom directly by incorporating it into a fluorescent molecule and measuring the resulting decrease in intensity. The other two reactions both involve indirect methods where the analyte takes a heavy metal ion out of a complex and the resulting ligand becomes fluorescent.

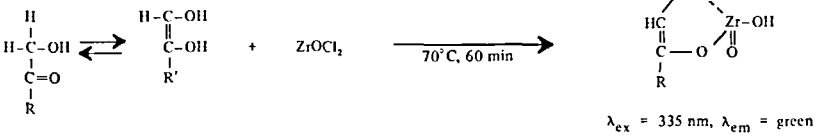
Because the heavy atom effect reduces fluorescence and enhances phosphorescence, it is more useful for phosphorescence derivatization than for fluorescence.

VI. SOME CONCLUDING REMARKS

Except in the area of complexing agents that form fluorescent metal chelates, very little systematic work has been done on fluorescence derivatization reactions. For some reactions, the structure of the fluorescent product is unknown. Other reactions have

TABLE 9

1. $ZrOCl_2$ for ketoses

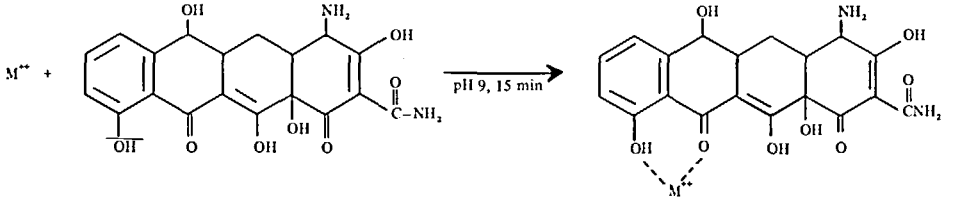


λ_{ex} = 335 nm, λ_{em} = green

Compounds forming fluorescent derivatives:

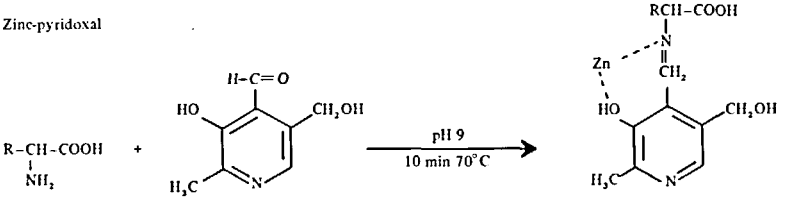
Ketoses[279,280]

2. Metal ions for tetracycline[281-284]



λ_{ex} = 400 nm, λ_{em} = 520 nm

3. Zinc-pyridoxal

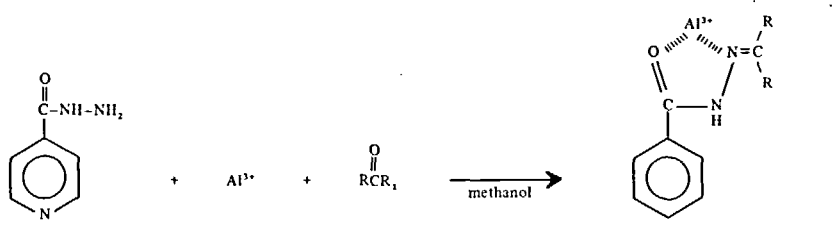


λ_{ex} = 365 nm, λ_{em} = 485 nm

Compounds forming fluorescent derivatives:

Amino acids, amino sugars[285-288]

4. Isonicotinic hydrazide with Al^{3+}



λ_{ex} = 370 nm, λ_{em} = 490 nm

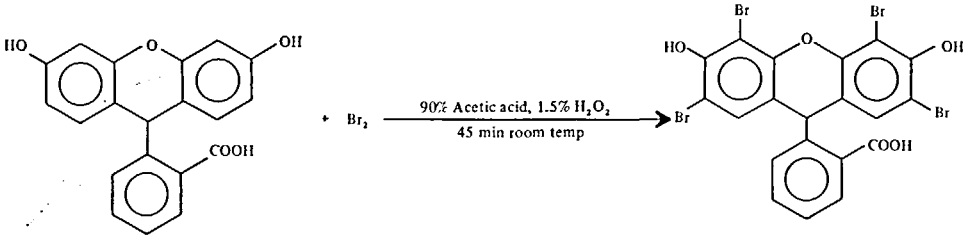
Ketosteroid analysis[289]

been discovered by accident. There are virtually no studies looking at the effect of structure variations on the characteristics of derivatization reactions. One reason for this is the fact that it is very difficult to accurately predict the fluorescence efficiencies of complex structures. Another reason is that most methods have been developed in response to a particular problem by scientists who were not interested in detailed eval-

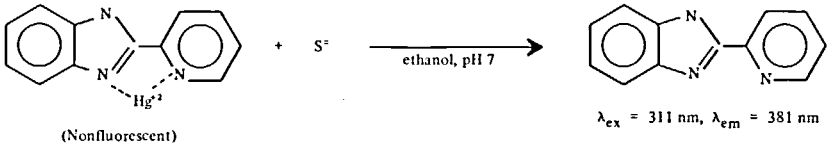
TABLE 10

Methods Based on Heavy Atom Effect

1. Determination of bromine



2. Determination of sulfide[291]



(Nonfluorescent)

3. Determination of organosulfur compounds[292-294]

Pd^{++} — Calcein + HSR ⟶ Pd^{++} — HRS + Calcein

(Nonfluorescent) (Fluorescent)

uation of the analytical possibilities of a reaction. At any rate, there is a lot of work to be done, both in understanding and optimizing present reactions, and in developing new reactions. The recent development of fluorescence detectors for liquid chromatography may help to stimulate interest in this area.

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