Chapter 26

Molecular Fluorescence and Phosphorescence Spectrometry

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Summary

General Uses

- Quantification of aromatic, or highly unsaturated, organic molecules present at trace concentrations, especially in biological and environmental samples
- Can be extended to a wide variety of organic and inorganic compounds via chemical labeling and derivatization procedures

Common Applications

- Determination of trace constituents in biological and environmental samples
- Detection in chromatography (especially high-performance liquid chromatography) and electrophoresis
- Immunoassay procedures for detection of specific constituents in biological systems
- Environmental remote sensing (hydrologic, aquatic, and atmospheric)

- In-situ analyses in biological systems (such as single cells) and cell sorting (flow cytometry)
- Studies of the molecular microenvironment of fluorescent molecules (fluorescent probe techniques)
- DNA sequencing
- Studies of ligand binding in biological systems
- Studies of macromolecular motions via polarized fluorescence measurement
- Fundamental studies of ultrafast chemical phenomena

Samples

State

Almost any solid, liquid, or gaseous sample can be analyzed, although solid samples may require a special sample compartment. Highly turbid liquid samples may cause difficulty.

Amount

Samples can be extremely small (for example, specialized techniques for examining nanoliter sample volumes have been developed).

Preparation

- It may be necessary to convert analyte to fluorescent derivative or tag analyte with a fluorescent compound.
- Use of high-purity solvents and clean glassware is essential.
- Complex mixtures may need extensive cleanup and separation before analysis.
- Turbid samples may require filtering or more extensive cleanup to minimize scatter background.
- Solvents that absorb strongly in the ultraviolet (UV) (such as toluene) must usually be avoided.

Analysis Time

Normally 1 to 10 minutes is needed to obtain a spectrum. Analysis time is determined primarily by time required for preliminary sample cleanup (which may be extensive and lengthy) rather than time required to obtain spectral data.

Limitations

General

- Analysis is limited to aromatic and highly unsaturated molecules unless derivatization or tagging procedure is used.
- Mixtures may need extensive cleanup before measurement.

- The possibility of quenching in mixtures means that care must be exercised in calibration (such as the use of standard additions).

Accuracy

Accuracy is highly dependent on the complexity of the sample and care must be used in calibration. Accuracies of 1% relative or better are possible if sufficient care is exercised.

Sensitivity and Detection Limits

Sensitivity is highly dependent on fluorescence quantum yield of analyte and the extent to which blank signals (such as impurity fluorescence and Rayleigh and Raman scatter) are minimized. For intensely fluorescent compounds (such as polycyclic aromatic hydrocarbons), detection limits of 10^{-11} to 10^{-12} *M* can readily be achieved using commercial instrumentation. Using specialized apparatus and great care, highly fluorescent nonphotosensitive analytes can be detected at or near single-molecule quantities.

Complementary or Related Techniques

- UV/visible (UV/Vis) absorption spectroscopy is much less sensitive but more nearly universal and often more accurate.
- Chemiluminescence is less widely applicable, but uses simpler apparatus than fluorescence, is more sensitive for some analytes, and often exhibits much lower blank because no light source is used.

Introduction

Photoluminescence is a type of optical spectroscopy in which a molecule is promoted to an electronically excited state by absorption of ultraviolet, visible, or near infrared radiation. The excited molecule then decays back to the ground state, or to a lower-lying excited electronic state, by emission of light. The emitted light is detected. Photoluminescence processes are subdivided into fluorescence and phosphorescence (1). For simplicity, we use the term *fluorescence* to mean both fluorescence and phosphorescence.

The key characteristic of fluorescence spectrometry is its high sensitivity. Fluorometry may achieve limits of detection several orders of magnitude lower than those of most other techniques. This is known as the fluorescence advantage. Limits of detection of 10^{-10} *M* or lower are possible for intensely fluorescent molecules; in favorable cases under stringently controlled conditions, the ultimate limit of detection (a single molecule) may be reached. Because of the low detection limits, fluorescence is widely used for quantification of trace constituents of biological and environmental samples. Fluorometry is also used as a detection method in separation techniques, especially liquid chromatography and electrophoresis. The use of fluorescent tags to detect nonfluorescent molecules is widespread and has numerous applications (such as DNA sequencing).

Because photons can travel through transparent media over large distances, fluorescence is applica-

ble to remote analyses. Atmospheric remote sensing is an example of this type of application. Remote sensing often requires the use of specialized apparatus (such as fiberoptics or laser sources).

The spectral range for most molecular fluorescence measurements is 200 to 1000 nm (10,000 to 50,000 cm^{-1}). Hence, optical materials used in UV/Vis absorption spectrometry are suitable for molecular fluorescence. Instrumentation for molecular fluorescence spectrometry is available from numerous manufacturers. Although commercial fluorescence spectrometers are useful in many situations, there are several important specialized applications (such as atmospheric remote sensing) for which truly suitable commercial instrumentation is not readily available.

Excitation of a molecule does not automatically produce fluorescence; many molecules exhibit very weak fluorescence. Most intensely fluorescent organic molecules contain large conjugated π-electron systems (1, 2). For example, most polycyclic aromatic hydrocarbons are intensely fluorescent. Very few saturated organic molecules, and relatively few inorganic molecules, exhibit intense fluorescence. To extend the applicability of fluorometry to the many compounds that do not exhibit intense native fluorescence, chemical reactions can be used to convert (derivatize) nonfluorescent molecules to fluorescent derivatives, or a nonfluorescent molecule may have chemically attached to it a fluorescent tag or label (3–6).

For a sample needing no preliminary cleanup, a fluorometric analysis can be carried out in 10 min or less. However, analyses of complex materials often require considerable preliminary cleanup. Fluorescence measurements may be carried out on liquid, gaseous, and solid samples. Solvents do not interfere unless they absorb at the wavelength used to excite the analyte or act to decrease the efficiency with which the excited analyte molecule fluoresces. However, fluorescent contaminants in solvents and laboratory glassware can be a major nuisance.

Fluorometry is more selective than UV/Vis absorption spectrometry for two reasons. First, many molecules absorb strongly in the UV or visible range but do not exhibit detectable fluorescence. Second, two wavelengths (excitation and emission) are available in fluorometry, but only one wavelength is available in absorptiometry. If two sample constituents with similar absorption spectra fluoresce at different wavelengths, they may be distinguished from one another by appropriate choice of emission wavelength. Similarly, two compounds that have similar fluorescence spectra but absorb strongly at different wavelengths may be distinguished by proper choice of excitation wavelength (selective excitation).

The selectivity of fluorometry is limited by the broad, featureless nature of the absorption and fluorescence spectra of most molecules. In UV/Vis absorption and fluorescence spectra, bandwidths of 25 nm or more are common, especially for polar or hydrogen-bonding molecules in polar solvents. The positions of spectral bands are not sensitive to the finer details of molecular structure, such as the presence of specific functional groups, and often cannot be predicted a priori with confidence. Hence, fluorometry is not generally useful for molecular identification.

The absorption and fluorescence spectra of a mixture of fluorescent compounds may be a jumble of overlapping bands. Such samples must be subjected to preliminary cleanup and separation (which may be quite time-consuming) or specialized sample-preparation and measurement techniques may be used; these may be instrumentally complex or time-consuming.

Because fluorescence measurements are rapid and use relatively inexpensive and rugged instrumentation, fluorescence can be used to screen samples, to generate preliminary data that allow an analyst to decide whether a sample requires detailed characterization, perhaps by a more expensive and complex technique such as gas chromatography/mass spectrometry (7). This is especially appropriate for environmental samples, which usually are very complex. Small, portable fluorometric instruments are suitable for performance of such screening operations in the field (8).

The fluorescence spectrum and intensity of a molecule often depend strongly on that molecule's environment. For example, changes in the "polarity" or hydrogen-bonding ability of a solvent may cause dramatic changes in the fluorescence behavior of a solute (6, 9). Thus, the fluorescence charac-

teristics of probe molecules may be used to make inferences about their immediate microenvironments. Fluorescent probe studies are a very important application of fluorometry, especially in biological and polymer science.

Molecular fluorescence spectrometry is usually blank-limited. Limits of detection are often governed not by one's ability to induce or detect the fluorescence of the analyte, but rather by the ability to distinguish the analyte fluorescence from Rayleigh and Raman scatter radiation generated by the sample, as well as from fluorescence of other sample constituents, fluorescent contaminants in the solvent, and fluorescent species adsorbed on the walls of the sample container. Whenever fluorescence spectrometry is used for trace analyses, scrupulous care must be devoted to maximizing the fluorescence signal produced by the analyte while minimizing blank fluorescence and scattering signals. Use of solvents (10) and sample containers that are as free as realistically possible of fluorescent contaminants is essential.

How It Works

Physical and Chemical Principles

The initial step in a fluorescence measurement is electronic excitation of an analyte molecule via absorption of a photon. Once formed, an excited molecule has available a variety of decay processes by which it can rid itself of the energy imparted to it by absorption. In addition to fluorescence (the desired decay route), there are nonradiative decay processes, leading to release of energy in the form of heat rather than light. Other sample constituents may interact with an excited analyte molecule in such a way as to prevent it from fluorescing; such processes are called quenching. Also, an electronically excited molecule may undergo chemical reaction (photodecomposition).

The fraction of electronically excited molecules that decay to the ground state by fluorescence is called the fluorescence quantum yield. The maximum possible value for the fluorescence quantum yield is 1.00. The number of molecules known to exhibit fluorescence quantum yields of unity can be counted on the fingers of one hand. Most intensely fluorescent molecules (that is, those with fluorescence efficiencies of 0.05 or greater) have extended π-electron systems (such as polycyclic aromatic hydrocarbons) (1, 2).

In fluorescence, the spin multiplicities of the ground and emissive excited states are the same. In most organic molecules, the ground state is a singlet state (all spins paired). Fluorescence occurs when a molecule has been promoted to an excited singlet state by absorption, and then decays back to the ground singlet state by emission. Fluorescence generally occurs only from the first excited singlet state (that is, the excited singlet state of lowest energy), irrespective of which excited singlet state was initially produced by absorption.

Phosphorescence is a light emission process in which the excited and ground states have different spin multiplicities. In an organic molecule whose ground state is a singlet, there are several energetically accessible triplet excited states (two unpaired spins). Following excitation into the manifold of singlet excited states by absorption, a molecule may undergo nonradiative decay (inter-system crossing) to the manifold of triplet states. The triplet state may emit a photon as the molecule decays back to the ground singlet state (phosphorescence).

Information Available from Fluorescence Measurements

Two basic types of spectra can be produced by a fluorescence spectrometer. In a fluorescence spectrum, or emission spectrum, the wavelength of the exciting radiation is held constant (at a wavelength at which the analyte absorbs) and the spectral distribution of the emitted radiation is measured. In an excitation spectrum, the fluorescence signal, at a fixed emission wavelength, is measured as the wavelength of the exciting radiation is varied. Because an analyte can fluoresce only after it has absorbed radiation, an excitation spectrum identifies the wavelengths of light that the analyte is able to absorb. Thus, subject to certain constraints, the excitation spectrum of a molecule should be the same as its UV/Vis absorption spectrum.

The excitation spectrum for a compound should not change as the emission wavelength is varied. Whenever the excitation spectrum varies with choice of emission wavelength, there is good reason to believe that two or more different substances are responsible for the observed fluorescence.

The maximum in the fluorescence spectrum of a compound occurs at longer wavelength than the maximum in the absorption spectrum. The wavelength difference between the absorption and fluorescence maxima is called the Stokes shift. Often, the Stokes shift is large (20 to 50 nm), especially for polar solutes in polar solvents. There is often some overlap, but not a great deal, between the absorption and fluorescence spectra of a compound. Both spectra may exhibit wavelength shifts whenever the solvent is changed; again, these effects are largest for polar solutes dissolved in polar, hydrogen-bonding solvents (6, 9).

In many fluorescence spectrometers, one can simultaneously vary the wavelengths of the exciting and emitted radiation. Such measurements, commonly called synchronous scanning, are useful in the analysis of mixtures (11).

Fluorometry is a multidimensional technique (12). Several types of information (in addition to spectra and signal intensities) can be obtained. The fluorescence of a molecule may be partially or fully polarized. Measurements of fluorescence polarization can provide important information, particularly for macromolecules; the use of polarized fluorescence measurements is widespread in biological (13) and polymer (14) science.

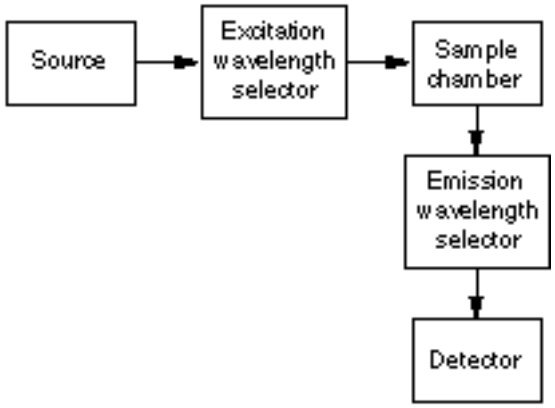
Also, the singlet excited states responsible for fluorescence of organic molecules have finite lifetimes, usually nanoseconds. (The triplet excited states responsible for phosphorescence of organic molecules have much longer lifetimes, often milliseconds or longer.) The fluorescence or phosphorescence rate of a molecule can be measured, and changes in fluorescence spectra as a function of time (time-resolved spectra) can be obtained. Measurements of time-resolved spectra or decay times can aid in analytical applications of fluorometry, and can also provide unique fundamental information in the study of very fast chemical and physical phenomena.

General-Purpose Instrumentation for Molecular Fluorescence Measurements

A block diagram of a fluorometer is shown in Fig. 26.1. In addition to the optical components shown, most fluorometers have dedicated computers, which control instrumental operating parameters (excitation and emission wavelengths, wavelength scan rates, monochromator slit widths, detector parameters) and the acquisition of spectral data, and also may be used for postprocessing of the data.

Sources

Figure 26.1 Block diagram of fluorescence spectrometer using conventional right-angle fluorescence collection.



The signal produced by an analyte is proportional to the number of excited analyte molecules formed per unit time. Thus, the source must produce high optical power (that is, a large number of photons per unit time). Because molecular absorption spectra usually are broad, a highly monochromatic source is not generally needed; an intense continuum source that emits throughout the UV, visible, and near infrared regions is adequate. The source used in most commercial fluorometers is the xenon arc lamp, the characteristics of which are described elsewhere (15).

For certain applications, it is preferable to use a laser excitation source (16). Few fluorescence spectrometers using laser sources are commercially available; most such instruments are intended for highly specific applications such as analyses of uranium in the nuclear industry (17). A Raman spectrometer (which uses a laser source) often can serve as an excellent, albeit expensive, instrument for laser-induced fluorescence spectrometry.

Wavelength Selectors

Portable, inexpensive fluorescence spectrometers use filters as wavelength selectors. Such instruments (filter fluorometers) are used when it is sufficient to measure fluorescence intensity at a single excitation and emission wavelength. These instruments are used in environmental field screening (8), hospital or clinical settings (18), and other applications in which low cost and small size are crucial. Moreover, filters can transmit a very large number of photons from source to sample and from sample to detector. Thus, filter instruments may be used in ultratrace analysis, wherein it is crucial to maximize the fluorescence signal that impinges on the detector, at the cost of decreased selectivity.

Most fluorometers in laboratory environments use grating monochromators as excitation and emission wavelength selectors. Usually, only moderate spectral resolution (1 to 2 nm) is needed. Parameters governing the choice of a monochromator for use in a fluorescence spectrometer are discussed in detail elsewhere (19).

Sample Illumination

The most common arrangement is the right-angle geometry in Fig. 26.1, wherein fluorescence is

viewed at a 90° angle relative to the direction of the exciting light beam. This geometry is suitable for weakly absorbing solution samples. For solutions that absorb strongly at the excitation wavelength, and for solids (or samples adsorbed on solid surfaces, such as thin-layer chromatography plates), a front-surface geometry often is preferable; here, fluorescence is viewed from the face of the sample on which the exciting radiation impinges.

For solution samples, rectangular 1-cm glass or fused silica cuvettes with four optical windows are usually used. For specialized applications, when very low limits of detection are required or it is necessary to illuminate a very small volume of solution, various flow (20, 21) or windowless (22) cells have been designed. Fiberoptics also are widely used in fluorometry.

Detectors

The fluorescence signal for an analyte present at low concentration is small; thus, a key requirement for a detector is its ability to detect weak optical signals. A photomultiplier tube (PMT) is used as the detector in most fluorescence spectrometers. PMTs used in fluorometry are chosen for low noise and high sensitivity, and are sometimes operated at subambient temperatures to improve their signal-to-noise ratios.

The main shortcoming of a PMT is that it is a single-channel detector. To obtain a spectrum, one must mechanically scan the appropriate monochromator across the wavelength range encompassed by the spectrum, which may be 50 nm or more. Thus, it is difficult to obtain spectra of transient species or analytes that remain in the observation region for a short time (such as eluents from chromatographic columns). It has long been recognized that a multichannel instrument using an array of detectors would be preferable for such applications because the entire spectrum could be viewed at once. UV/Vis absorption spectrometers with array detectors are commercially available and widely used.

Until recently, no electronic array detector has been competitive with a PMT in the detection of weak optical signals. That situation is changing as new classes of electronic array detectors are developed and improved. At present, the most promising electronic array detector for fluorometry is the charge-coupled device (CCD) (23). Fluorescence instruments using CCDs or other high-performance array detectors are not numerous, but will become more common in the future.

Corrected Spectra

Most fluorometers are single-beam instruments (see Fig. 26.1). Excitation and fluorescence spectra obtained using such an instrument are distorted, due to variation of source power or detector sensitivity with wavelength. Spectra of the same sample obtained using two different fluorometers may therefore be quite dissimilar (1); even changing the source or detector in a fluorometer may alter the apparent fluorescence or excitation spectrum of a compound. It is possible instrumentally to eliminate these artifacts, and several manufacturers offer instruments that can generate corrected spectra. Because most published fluorescence spectra are uncorrected, they cannot readily be reproduced by other investigators. Hence, there are few extensive and broadly useful data bases of fluorescence spectra.

That a fluorescence spectrometer is a single-beam instrument also means that fluctuations in the power output of the excitation source produce noise. This problem may be solved by splitting off a portion of the source output and viewing it with a second detector, and electronically ratioing the observed fluorescence signal to the output of the detector that is used to monitor the source power. High-performance commercial fluorometers have this capability.

More detailed discussions of instrumentation for measurement of fluorescence are available elsewhere (1, 6, 13, 24).

Specialized Types of Fluorescence Measurements

Several types of useful fluorescence measurements may not be possible using the simple fluorometer illustrated in Fig. 26.1. Some examples of more specialized fluorometric techniques are considered below.

Synchronous Fluorescence and Excitation–Emission Matrices

As noted above, it is possible to scan the excitation and emission monochromators simultaneously (synchronous fluorometry) (11). Often, synchronous fluorometry is carried out by scanning the excitation and emission monochromators at the same rate while keeping the wavelength difference (or offset) between them constant.

The main purpose of synchronous scanning is to generate spectra having decreased bandwidths. For example, Fig. 26.2(a) shows the excitation and fluorescence spectra of perylene, an aromatic hydrocarbon, in ethanol solution. The Stokes shift for perylene is relatively small (3 nm). If one carries out a synchronous scan with the wavelength offset between the excitation and emission monochromators held at 3 nm, one obtains the spectrum shown in Fig. 26.2(b) (25). The width of the synchronous spectrum is much smaller than that of either the excitation or fluorescence spectrum. When dealing with a mixture of fluorescent components, the beneficial effect of synchronous scanning is to greatly simplify the spectrum and decrease the extent of spectral overlaps (11, 25).

In some instances, it may be preferable to use a constant wavenumber (rather than wavelength) offset between the monochromators in synchronous fluorometry (26).

Because the synchronous spectrum for a compound depends on both the excitation and fluorescence spectra, it is strongly dependent on the wavelength (or wavenumber) offset. If one were to run many synchronous spectra for a particular compound, each at a different wavelength offset, one could acquire all information present in the absorption and fluorescence spectra of the compound. This information could then be set up in the form of a two-dimensional matrix called an excitation–emission matrix (27, 28). Acquiring an excitation–emission matrix by running many synchronous scans is slow; it can be generated much more rapidly by using an array-detector fluorometer designed expressly for this purpose (27).

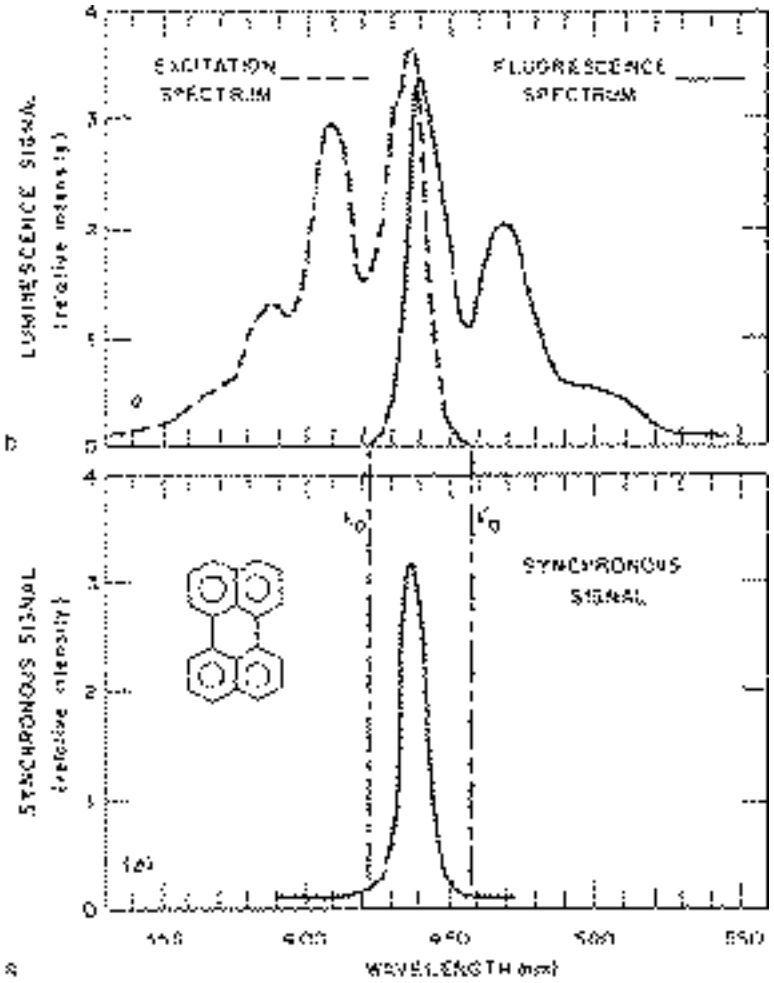
The data in an excitation–emission matrix are often visually presented in the form of a fluorescence contour plot, wherein points of equal detector signal are connected to produce what looks like a topographic map. Examples of such contour plots for several complex samples are shown in Fig. 26.3 (29). These plots can be quite useful for distinguishing complex samples from one another. Each one serves as a spectral fingerprint for a particular complex material (such as petroleum, coal-derived liquids, or biological fluids) (29, 30), although it does not directly identify the fluorescent constituents of the sample in any obvious way.

Fiberoptic Sensors

An optical fiber is a light pipe that may be used to transmit light beams over long distances. A fiber may be used to transmit exciting light to a fluorescent analyte or transmit the emitted fluorescence to a detector. Thus, fiberoptics may be used to deal with extremely small objects (such as electrophoresis capillaries (31)), inaccessible samples (such as groundwater (32)), radioactive or otherwise hazardous materials (33), or materials that may be difficult to sample, store, and transmit to a laboratory (such as sea water (34)).

Numerous types of fiberoptic sensors based on fluorescence have been designed (35–37). These devices are sometimes called optrodes because they often are used for the same general purposes as ion-selective electrodes. It may be necessary to use in such a device a reagent that can incorporate chemical

Figure 26.2 Comparison of conventional excitation and Fluorescence spectra. (a) Perylene in ethanolic solution; (b) synchronous fluorescence spectrum using a 3-nm offset between excitation and emission monochromators. *(Reprinted with permission from T. Vo-Dinh et al., "Synchronous Spectroscopy for Analysis of Polynuclear Aromatic Compounds,"* Environmental Science & Technology, *12, pp. 1297–1302. Copyright 1978 American Chemical Society.)*



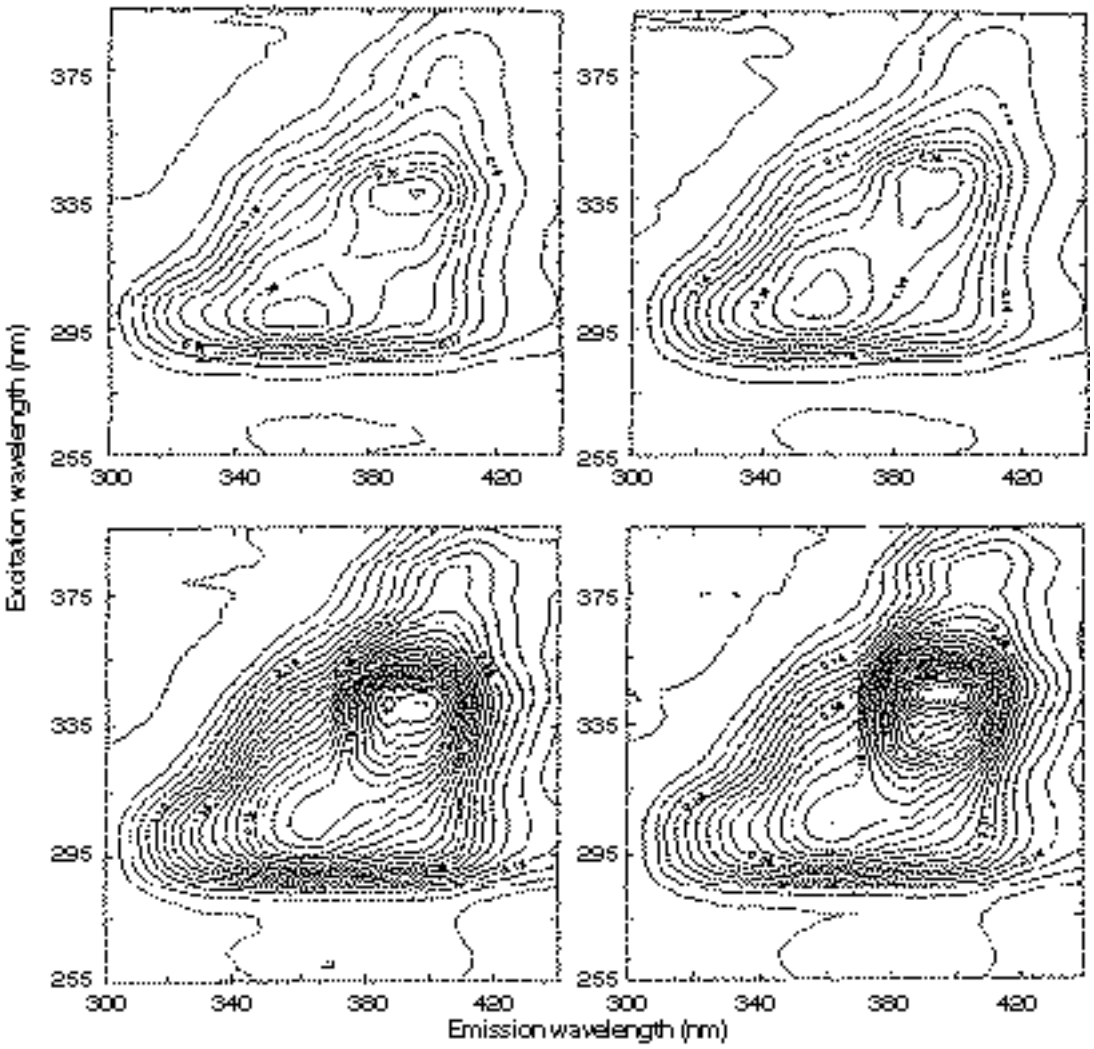
selectivity into the fluorometric analysis; because of their high selectivities, enzymes and antibodies may be used for this purpose (38).

Difficulties can be experienced in the use of fiberoptic sensors. For example, when a reagent is incorporated into a sensor, the sensing tip may not be as long-lived as one would wish, the reagent may not be sufficiently selective for the intended analytical use, and the chemical reactions may be slower than desirable.

Polarized Fluorescence Measurements

When a molecule is excited by polarized light, its fluorescence may be partially or fully polarized (13). A fluorometer can be used to measure fluorescence polarization by placing polarizing prisms or sheets in the excitation and emission beams. High-quality instrumentation for polarized fluorescence measure-

Figure 26.3 Fluorescence contour maps for four coal-derived liquids in ethanolic solution. The vertical axis is excitation wavelength; the horizontal axis is emission wavelength. *(Reprinted with permission from P. M. R. Hertz and L. B. McGown, "Organized Media for Fluorescence Analysis of Complex Samples: Comparison of Bile Salt and Conventional Detergent Micelles in Coal Liquids,"* Analytical Chemistry, *64, pp. 2920–2928. Copyright 1992 American Chemical Society.)*



ments is commercially available. Fluorescence polarization measurements are widely used for studying rotational motions of electronically excited molecules and to detect the binding of relatively small molecules to macromolecules. Polarized fluorescence measurements are often used in fluoroimmunoassay procedures that are widely used in the life sciences (39).

Laser-Induced Fluorescence

For measurements that require very high excitation source power, monochromatic exciting radiation, the ability to illuminate a very small sample volume, excitation with short light pulses, or propagation of the exciting light over large distances, it may be necessary to use a laser source (16, 40). For solution

samples, laser-induced fluorescence (LIF) tends to exhibit somewhat better limits of detection than lamp-excited fluorescence (41). However, the detection-limit advantage of LIF tends to be much less dramatic than one might expect (42) because of the blank-limited nature of fluorometry.

Because the wavelength of the exciting light must correspond to an absorption band of the analyte, fixed-wavelength lasers are not generally suitable for fluorescence spectrometry. A laser source for fluorometry should exhibit wavelength tunability over a fairly wide range in the UV or visible ranges. Thus, many applications of LIF use a dye laser (43) as the excitation source.

Most dye lasers are expensive (the pump source, usually another laser, may cost $20,000 to $65,000) and require some expertise to use effectively. The pump laser may consume a great deal of electrical power. Eventually, dye lasers may largely be replaced in fluorometry by compact solid-state lasers with low electricity requirements. Small solid-state lasers have been used in fluorometric analyses of compounds that absorb in the visible or near infrared (44). However, these devices cannot presently produce tunable UV (250 to 380 nm) output—needed for many applications of fluorometry—at high enough power to exploit the fluorescence advantage.

Laser sources (usually argon ion lasers) are used in fluorescence flow cytometers, which are commercial instruments used to count and sort biological cells and other particles (12, 45).

Fluorescence Decay Times and Time-Resolved Spectra

A fluorescence decay time is a measurement, at fixed wavelength, of fluorescence signal as a function of time. A time-resolved fluorescence spectrum is a spectrum measured within a narrow time window during the decay of the fluorescence of interest.

Two different approaches are used in such measurements. In pulse fluorometry, an excitation source is used that produces light pulses whose durations are short in comparison with the excited-state lifetime of the fluorescent molecule. The decay of the fluorescence is then measured directly, using a fast detector (1, 40). Because fluorescence decay times are often 1 ns or less, short pulses are needed. Lasers that can produce ultrashort pulses (10^{-9} to 10^{-12} sec or less) are commercially available, but tend to be expensive and touchy to operate. Also, fast detection systems are needed, and considerable care is needed in the choice of cables, connectors, and other ancillary components (46, 47).

An alternative technique, phase-modulation fluorometry (often called frequency-domain fluorometry) uses a source (lamp or laser) that is amplitude-modulated at one or more frequencies. Measurement of the phase or demodulation of the fluorescence signal can be used to generate fluorescence decay times and time-resolved fluorescence spectra (1, 48). Such instruments are generally simpler than those used for pulse fluorometry, although high modulation frequencies (1 GHz or greater) are needed to measure very fast fluorescence decays. Commercial instrumentation is available for these types of measurements.

Time-resolved measurements are instrumentally sophisticated, but they can improve both the sensitivity and selectivity of fluorometry. Measurements of fluorescence decay times or time- resolved spectra are used for several purposes, such as distinguishing sample constituents whose fluorescence spectra overlap one another (40, 49, 50) and distinguishing the fluorescence of an analyte from background scattering or luminescence of other sample constituents (44, 51); this approach to background suppression is widely used in fluorescence immunoassays (52, 53).

Low-Temperature Fluorometry

The absorption and fluorescence spectra of a molecule may undergo dramatic narrowing if the molecule is inserted in a solid matrix at low temperature (77 °K or lower) (16, 54) or expanded, in the gas phase, to form a supersonic free jet (55). These techniques exhibit much greater selectivity than conventional fluorescence measurements in liquid solution. Often, use of a laser source is required to exploit fully

the opportunities for increased selectivity offered by the low-temperature techniques. Use of the methods also entails an investment in cryogenic apparatus (for the solid-state techniques) or vacuum hardware (supersonic jet spectroscopy), and the time required to obtain a spectrum can be substantial. However, these methods can effect major savings in sample pretreatment before measurement of fluorescence.

What It Does

Analytical Information: The Fluorescence Advantage

The main analytical application of molecular fluorescence spectrometry is detection and quantitation of species present at concentrations so low that most other techniques are not useful. The origin of the fluorescence advantage in detection limit capabilities can be understood in the following way. Consider Beer's law, the fundamental relationship in quantitative absorption spectrometry:

$$A = \varepsilon bc = \log (P_0/P) = \log P_0 - \log P \tag{26.1}$$

where ε is the absorptivity of the analyte, b is the optical pathlength of the sample, c is the concentration of the analyte, P_0 is the excitation power (photons sec^{-1}) incident on the sample, and P is the power transmitted by the sample. P is the quantity that varies with c: As c decreases, P increases. When c is small, P is slightly smaller than P_0; thus, one must measure a small difference between two large numbers.

In contrast, the relationship between a measured fluorescence signal (F, in photons sec^{-1}) to the analyte concentration is

$$F = k\varphi_F P_0(1 - 10^{-\varepsilon bc}) \tag{26.2}$$

where φ_F is the fluorescence quantum yield, k is the fraction of the photons emitted by excited analyte molecules that actually are detected (often 0.10 or less), and the other symbols have the same meanings as in Eq. (26.1). If the product $\varepsilon bc \leq 0.02$, as is often the case in analytical applications of fluorometry, Eq. (26.2) simplifies to

$$F = k\varphi_F P_0 \varepsilon bc \tag{26.3}$$

According to Eq. (26.3), if the analyte concentration is 0, the measured fluorescence signal is 0. If the analyte concentration is small, F is a small number. Hence, when the analyte concentration is low, the measurement situation in fluorometry—distinguishing between a small signal and zero signal—is more favorable than that encountered in absorption spectroscopy (measuring a small difference between two large numbers).

Eq. (26.3) predicts the fluorescence signal to be linear in the analyte concentration and the excitation power, P_0. The product $\varepsilon\varphi_F P_0$ determines the sensitivity of fluorometry for the analyte. In the most favorable case, the analyte absorbs strongly at the excitation wavelength, the source generates a large number of photons per unit time at that wavelength, and the excited analyte molecules so produced exhibit a high probability of decaying via fluorescence. If all three of these conditions are satisfied and the detector exhibits high sensitivity at the wavelength at which the analyte emits, then it is possible to achieve extremely low limits of detection for the analyte, much lower than can be achieved by absorption spectroscopy. This is the fluorescence advantage.

In real life, when the analyte concentration is zero, the observed signal is greater than zero, due to

background signals from fluorescence of other constituents of the sample or contaminants in the solvent or sample cell. Rayleigh or Raman scattering of source radiation onto the detector also generates background. Thus, fluorescence spectrometry is ordinarily blank-limited (42). Therefore, achieving low limits of detection in fluorometry is possible only if great care is taken to minimize the background arising from unwanted fluorescence or scattering, and to distinguish the analyte fluorescence from the background (10, 51, 56).

The sensitivity of fluorometry also depends on the efficiency with which light emitted by the sample is collected and caused to impinge on the detector. This, in part, determines the value of k in Eq. (26.2). The collection efficiency often is low (less than 10%). Sample compartments and cuvettes for commercial fluorometers can be modified to increase the fraction of emitted light that is actually collected (by placing mirrors behind the cell, for example). Because such alterations also increase the amount of scatter and spurious fluorescence that reach the detector, in practice they may be of little real benefit.

The low sensitivity implied by a small value of k can be improved by exploiting the fact that an analyte molecule may be excited more than once (thus, one may obtain many emitted photons from one analyte molecule (57); this is sometimes called the photon-burst effect). This opportunity works out strongly to the analyst's advantage only if the analyte exhibits a very low quantum yield for photodecomposition (56, 57); photosensitive molecules will be destroyed before they have the chance to emit many photons.

Interferences

It is useful to consider interferences in two classes: additive and multiplicative (58). In additive interference, background fluorescence is emitted by concomitants in the sample or contaminants in solvents or glassware, causing a finite blank. In multiplicative interference, the interferent does not itself fluoresce, but causes the fluorescence signal for the analyte to be smaller or (occasionally) larger than that observed in the absence of the interferent. One important phenomenon—quenching—can simultaneously cause additive and multiplicative interference. It is generally easier to correct for multiplicative interference (by standard additions (59), for example) than additive interference.

Additive Interference: Background Fluorescence and Scattering

Molecular absorption and fluorescence bands tend to be broad (25 nm or more) and featureless, especially in solution. In multicomponent samples, therefore, the absorption and fluorescence spectra of the various sample constituents may overlap. Background fluorescence (additive interference) from other sample constituents, or contaminants in the solvent or sample cell, can be a major problem, especially in biological and environmental samples. Several steps can be taken to deal with the problem:

- Using solvents and sample cells that are free of fluorescent contaminants is essential. Care must be exercised to avoid contamination of laboratory glassware by fluorescent substances (such as detergents used to clean glassware).
- Whenever possible, analyte fluorescence should be excited and measured at wavelengths at which other sample constituents do not absorb or fluoresce. Most organic molecules absorb and fluoresce in the UV or visible range (200 to 550 nm). Hence, fluorescence background often can be decreased by exciting and measuring analyte fluorescence at longer wavelengths in the near infrared (600 nm or more) (60, 61). Numerous fluorescent tags and derivatives have been developed that absorb and emit in the near infrared (60, 62).
- Synchronous scanning often is helpful in dealing with overlapping absorption and fluorescence

spectra of mixture constituents.

- The background fluorescence may have a different decay time from that of the analyte. Time resolution can be used to distinguish the analyte fluorescence from the background. This is most successful if the analyte fluorescence is longer-lived than the background. In such a situation, one simply waits until the interfering fluorescence and scattering have decayed away to negligible levels, and then measures the remaining analyte fluorescence (44, 49, 52). Fortunately, the background fluorescence observed in many samples is relatively short-lived (fluorescence from trace contaminants in many solvents has a decay time of 2 ns or less (10) and that in many biological samples has a decay time of 20 ns or less (44)). Moreover, Rayleigh and Raman scatter background is instantaneous.
- There may be an excitation wavelength at which only the analyte absorbs appreciably. If so, its fluorescence can be selectively excited. Use of low-temperature techniques greatly increases the possibility of achieving selective excitation of analyte fluorescence in complex mixtures (16, 54, 55).
- Fluorescent sample constituents may be eliminated by separating the analyte from the interferents before measurement of fluorescence. Accordingly, fluorometric analyses of complex samples often are preceded by, or coupled directly to, chromatographic or electrophoretic separations.
- Mathematical (chemometric) techniques may in some cases be able to decompose spectra that consist of overlapping bands, produced by several different compounds, into contributions from the individual sample constituents (63).

Combinations of the above techniques may be especially useful. For example, spectral overlaps may be dealt with more effectively by combined use of synchronous scanning and time resolution than by either method used individually (50).

Multiplicative Interference: Inner-Filter Effects and Quenching

The fluorescence signal generated by an analyte may be altered—perhaps even totally suppressed—by other sample constituents. One form of multiplicative interference is the inner-filter effect, in which an interferent that absorbs in the same wavelength range as the analyte decreases the radiant power available to excite the analyte. Another form of inner-filter effect occurs when an interferent absorbs at the wavelength at which the analyte fluoresces, thus causing the number of emitted photons that escape the sample and reach the detector to diminish. Instrumental correction for inner-filter effects is possible (64); however, such procedures may be difficult to implement in some commercial fluorometers.

Quenching is another type of multiplicative interference. Quenching is any process in which a sample constituent decreases the fluorescence quantum yield for the analyte. Among the most common fluorescence quenchers is O_2; removal of oxygen from a sample before fluorometric analysis (65) is often advisable.

One way that fluorescence quenching can occur is intermolecular electronic energy transfer:

$$M^* + Q \rightarrow M + Q^* \tag{26.4}$$

Here an excited analyte molecule (M^*) transfers excitation energy to a quencher molecule Q, causing de-excitation of M and forming an excited quencher molecule, Q^*. If Q^* is a fluorescent species, its fluorescence (called sensitized fluorescence) may then be observed. This phenomenon can allow one to observe fluorescence from a molecule (Q) that may be difficult to excite directly. More often, however, these processes are a nuisance. Not only do they cause a decrease in the fluorescence signal observed for a given concentration of analyte (M) in the sample, but they may produce unwanted background fluorescence signals; that is, Q may act both as a multiplicative and an additive interfer-

ence.

Quenching often follows the Stern–Volmer equation:

$$\frac{\varphi_F^0}{\varphi_F} = 1 + K_{SV}(Q) \tag{26.5}$$

where φ_F^0 and φ_F are the fluorescence quantum yields for the analyte in the absence of quencher and presence of quencher at concentration (Q), respectively, and K is the quenching constant (a measure of the efficiency with which Q quenches analyte fluorescence). Because the analyte fluorescence signal depends on the quencher concentration, one can determine Q, via its quenching action, in a sample that contains a fluorescent compound. Numerous procedures that use fluorescence quenching to determine species (most notably O_2) that are not themselves fluorescent but can act as efficient fluorescence quenchers have been devised (66, 67).

Equation (26.5) shows that the effect of a quencher decreases as the sample is diluted. Thus, quenching can be circumvented simply by diluting the sample, provided that the diluent is not itself a quencher and assuming that one does not thereby decrease the analyte concentration below its limit of detection.

Figure 26.4 is a dramatic example of the influence of inner-filter effects and intermolecular energy transfer on fluorescence spectra in mixtures (68). The sample, obtained in an industrial setting, is a complex mixture of polycyclic aromatic hydrocarbons. At the higher concentration in Fig. 26.4, the spectrum is badly perturbed by inner-filter effects and intermolecular energy transfer, causing it to be depleted of contributions from compounds that emit at high energy (short wavelength). The effect of energy transfer is to quench fluorescence from sample constituents that fluoresce at shorter wavelengths and sensitize fluorescence from the compounds that fluoresce at longer wavelengths. When the sample is diluted sufficiently, the various quenching phenomena cease to occur and the appearance of the spectrum changes dramatically. Checking for the occurrence of such phenomena by measuring fluorescence spectra of complex mixtures before and after dilution is a useful precaution.

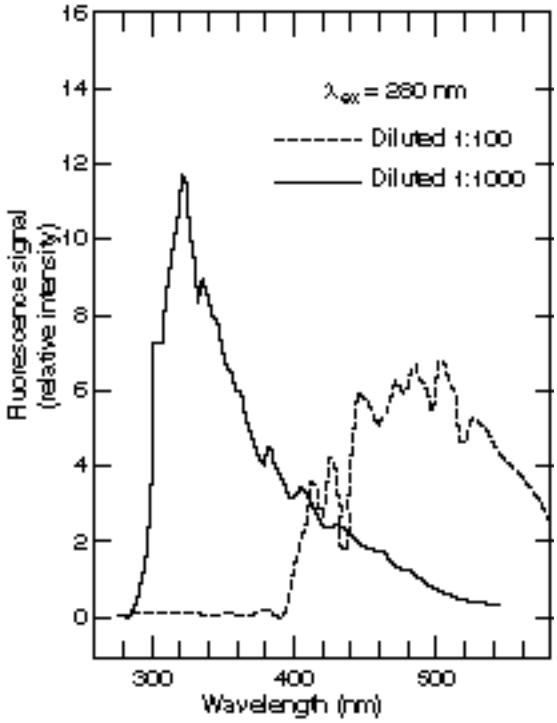
The fluorescence quantum yield for a given compound can vary dramatically from one sample to another, much more so than the molar absorptivity. Accordingly, the accuracy of fluorometric analysis is much more susceptible to errors caused by multiplicative interferences (and, thus, by improper or inadequate calibration) than is UV/Vis absorption spectrometry. Most complex samples contain one or more components that can quench the fluorescence of the analyte. Thus, it often is necessary to subject complex samples to be analyzed by fluorometry to extensive prior cleanup to remove potential quenchers.

Alternatively, one may try to provide a uniform microenvironment for the analyte (and thus a reproducible fluorescence yield) from sample to sample by any of several ploys, such as adding a micelle-forming surfactant to each sample (29). This is based on the fact that fluorescent molecules in solution may be partially or fully hidden from quenchers by incorporating them into organized media such as surfactant micelles (29) or cyclodextrin cavities (69).

Triplet states of organic molecules have much longer lifetimes than singlet excited states, and thus are more susceptible to quenching. Hence, phosphorescence is much more likely to be quenched than is fluorescence, so the experimental conditions needed to observe phosphorescence are more stringent than those required to detect fluorescence. Observation of phosphorescence of useful intensity from solutes in liquid-solution samples is rare.

Historically, phosphorescence measurements were made in low-temperature glasses formed by freezing liquid solutions. Phosphorescence received a major boost as an analytical technique when it was discovered that molecules in triplet states can be protected from quenching agents by adsorbing them on filter paper or other solid supports (70) or (as noted above for fluorescence) sequestering them in surfactant micelles or cyclodextrin cavities in liquid media. Room-temperature phosphorescence using these approaches has become a popular technique (70, 71), whereas the classic low-temperature pro-

Figure 26.4 Fluorescence spectrum of solvent extracts of a sample of particulate matter from an industrial environment. The samples are identical except that one was diluted 1:100 (dashed line) and the other 1:1000 (solid line) with ethanol. Extensive intermolecular energy transfer or inner-filter effects are occurring in the more concentrated sample.*(Reprinted with permission from T. Vo-Dinh, R. B. Gammage, and P. R. Martinez, "Analysis of a Workplace Air Particulate Sample by Synchronous Luminescence and Room-Temperature Phosphorescence,* Analytical Chemistry, *53, pp. 253–258. Copyright 1981 American Chemical Society.)*



cedure is now rarely used.

Accuracy and Precision

The accuracy and precision of analyses performed by molecular fluorescence can range from extremely high to abysmally poor, depending on the following factors:

- The care taken to prevent introduction of fluorescent contaminants into the sample (by use of high-quality solvents and clean glassware, for example).
- The complexity of the sample, number and types of interfering species present, and effectiveness of preliminary cleanup procedures. Loss of analyte, or contamination of the sample, during preliminary cleanup can cause large errors.

- The care exercised in calibration of the fluorescence measurement and use of appropriate mathematical techniques for evaluating the experimental data.
- The quality of the instrumentation used. For example, an instrument that monitors the power output of the source (and thus is able to correct measured fluorescence signals for fluctuations in P_0) will produce much more precise data than an instrument that does not do so.

Applications

General Considerations

Major classes of applications of fluorometry include the following:

- Detection and quantification of trace-level species, especially in biological-clinical (72–74) and environmental (75) samples.
- Detection in separation techniques, especially liquid (16, 76, 77) and thin-layer chromatography (77) and electrophoresis (16, 44, 78). Coupling of laser-induced fluorescence to electrophoresis for rapid base sequencing of DNA fragments (79, 80) may have considerable significance in biotechnology. Use of derivatization or labeling reactions to convert nonfluorescent compounds to fluorescent entities (3–6) may be necessary. Another way to deal with nonfluorescent species is indirect fluorescence, wherein a nonfluorescent analyte displaces a fluorescent molecule (which may be added to the mobile phase); the change in concentration (and hence in fluorescence intensity) of the fluorescent species is measured and related to the concentration of the nonfluorescent constituent (81).
- On-line analyses and remote sensing, using fiberoptic sensors or laser-induced fluorescence. Monographs (35) and review articles (36, 37, 82) on this subject are available.
- Detection and quantification in immunoassay. Fluoroimmunoassay procedures are discussed in detail elsewhere (39, 83, 84).
- Identifying, sorting, and counting particles (most notably biological cells) via fluorescence flow cytometry. Monographs (45) and review articles (16) on flow cytometry are available. Flow cytometers are also used to determine fluorescent analytes bound to particles or adsorbed on particulate surfaces (85).
- In-situ imaging, mapping, and quantification of species in biological systems, such as tissues and single cells. Detailed reviews of these types of applications are available (6, 86).
- Preliminary screening of complex mixtures (especially environmental samples), using the fluorometric data to decide whether it is appropriate to subject a sample to more detailed characterization (7, 8).
- Studies of the microenvironments of fluorescent probe molecules. Such properties of materials as viscosity, pH, “polarity,” and temperature may be inferred from measurements of fluorescence spectra, decay times, and polarization of suitable probe molecules. Fluorescent probes are widely used in biology and materials science to obtain information regarding the nature and accessibility of binding sites in biological macromolecules, dynamics of motions of polymer chains, homogeneities of polymer samples, the properties of micelles, the nature of domains on solid surfaces, the concentrations of specific ions in biological cells, and the spatial distribu-

tions of specific molecular species in biological membranes, to list a few of the possibilities. Reviews discussing the principles and practice of fluorescence probe studies, and precautions that must be exercised in the interpretation of these experiments, are available (6, 14, 87).

Selected Example: Determination of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), some of which can undergo metabolic conversion to carcinogens, are widely dispersed in the environment, often in small quantities. There are many different PAHs and PAH derivatives; many are intensely fluorescent. Thus, fluorometry is often used for the determination of PAHs and PAH derivatives in environmental and biological samples. Virtually every approach used in the fluorometric analysis of mixtures has been applied to PAHs. Hence, we can use the determination of PAHs to exemplify the strategies available for quantification of fluorescent analytes present in complex samples. Specific examples are considered below.

Fluorometry Preceded by Separation

An excellent example of this approach is described by May and Wise (88), who wished to determine PAHs sorbed on urban airborne particulate matter. The results were to serve as part of the data set to certify the sample as a standard reference material for 13 PAHs. Many other PAHs and PAH derivatives were present.

It was first necessary to remove the PAHs from the particulate samples via Soxhlet extraction. (Some sort of extraction is unavoidable; the extraction could be done with a supercritical fluid rather than a liquid solvent.) Then, each extract was concentrated and passed over a small silica gel column to remove the most polar compounds. Next, each extract was subjected to normal phase liquid chromatography (LC). This LC procedure separated an extract into several fractions, according to number of aromatic rings—three-ring, four-ring, and five-ring fractions were obtained—but isomeric PAHs were not separated from one another, nor were alkylated PAHs (such as the methylchrysenes) separated from their parents.

Each of the fractions obtained via the normal phase LC separation was then subjected to a second, reversed phase LC separation. Using this column, it was possible to separate isomeric PAHs from one another and alkylated PAHs from their parents. In the latter separation, a molecular fluorescence detector (using numerous excitation and emission wavelengths appropriate for the specific PAHs of interest) was used. The analyses were calibrated via internal standard techniques.

This procedure is typical in that interferences are minimized by carrying out a series of separations before the fluorescence measurement. The time needed to make the fluorescence measurement is trivial compared with that required for the various cleanup steps needed to get the sample to the point at which useful measurements can be made.

Synchronous Fluorometry

Synchronous scanning may decrease the extent of overlap in the spectra of mixtures of fluorescent compounds. This does not automatically eliminate the need for sample cleanup, but may enable the analyst to use fewer or less extensive cleanup steps than in conventional fluorometry.

A good example is described by Vo-Dinh and colleagues (68), who studied the PAH content of industrial airborne particulate matter. The sample was first subjected to solvent extraction to remove the PAHs from the particles, and the extract was then fractionated by LC. Seven LC fractions were produced (one fraction contained aliphatics, three contained primarily PAHs, and three contained primarily polar aromatics). One of the PAH fractions was examined by synchronous fluorometry, using a wave-

length offset of 3 nm; the conventional and synchronous fluorescence spectra of this fraction are compared in Fig. 26.5. Nine resolved features are observed in the synchronous spectrum; it is possible to identify and quantify (via standard additions) all nine PAHs responsible for these features.

In comparison with the separation approach of May and Wise, we note that use of synchronous fluorometry allowed Vo-Dinh and colleagues to omit one LC separation used by May and Wise; the LC step in question required at least 1 hr. Of course, several synchronous scans, at different wavelength offsets, may be needed to locate all sample constituents of interest. However, numerous synchronous scans can be run in the time needed for one LC separation in samples of this complexity. Thus, whenever one wishes to quantify a small number of fluorescent constituents of a complex sample, use of synchronous fluorometry may save time by decreasing the amount of sample cleanup needed to obtain useful fluorescence data.

Temporal Resolution

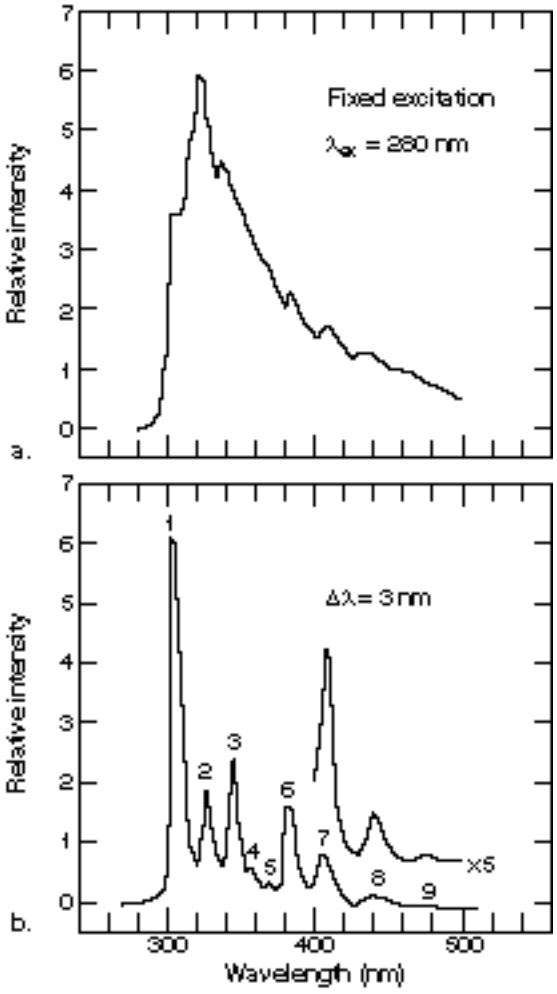
If two spectrally similar PAHs have different fluorescence decay times, they can be distinguished from one another via time resolution, provided that the decay time difference is sufficiently large. Structurally similar PAHs that are difficult to distinguish spectrally may also have similar fluorescence decay times. For example, the isomers benzo[a]pyrene and benzo[e]pyrene have fluorescence decay times in acetonitrile solution of 14.9 ns and 16.9 ns, respectively (89), making it quite difficult to distinguish between them solely on the basis of decay-time measurements. More encouraging examples can also be cited. For example, in acetonitrile solution the isomers dibenzo[a,h]pyrene, dibenzo[a,e]pyrene, and dibenzo[a,i]pyrene have decay times of 5.5, 18.5, and 26.5 ns, respectively (90) and the isomers benzo[k]fluoranthene and benzo[b]fluoranthene have decay times of 7.8 and 27.3 ns, respectively (89). Thus, even if time resolution by itself cannot realistically be expected to unravel a complex mixture of spectrally similar PAHs, it may add valuable selectivity to fluorometric procedures. For instance, decay-time data may be capable of distinguishing between PAHs that are not completely separated chromatographically, and may be used to ascertain if what appears to be a single peak in a chromatogram is actually due to two or more coeluting compounds (89, 91). Also, the fluorescence decay time may be used, in conjunction with chromatographic retention-time data, to identify fluorescent compounds as they elute; it may be easier experimentally to use decay times than emission wavelengths for this purpose (90). Of course, time resolution is also used in conjunction with fluorescence detection in liquid chromatography to reduce scatter or luminescence background interference.

Fiberoptic Sensors

Because PAHs are environmental contaminants, techniques have been developed for determining these compounds in natural waters, biological fluids, and industrial process streams and effluents. Fiberoptic sensors are a potentially attractive way of achieving rapid, sensitive determinations of PAHs in such samples. The obvious problem is how to achieve selectivity, given that most PAH-containing samples contain numerous fluorescent compounds.

Chemical selectivity can be achieved by using, at the sensor tip, a reagent that selectively binds the analyte, thereby increasing its concentration (and decreasing the concentrations of interferents) at the probe tip. Immunochemical systems can provide exceptionally high selectivities. For example, Tromberg and colleagues (38) designed a fiberoptic sensor for a fluorescent carcinogenic metabolite of the PAH benzo[a]pyrene (BaP), in which the probe tip contains a monoclonal antibody for which the BaP metabolite is a hapten. The large equilibrium constant for formation of the antibody–hapten complex results in low limits of detection for the analyte, due to the preconcentration achieved at the fiber tip. Selectivity is enhanced by separating the reagent layer from the bulk sample by a membrane through which the analyte must diffuse; some potential interferents either may not penetrate the membrane or

Figure 26.5 Fluorescence spectra of extract of a particulate matter sample obtained in an industrial environment. (a) Conventional; (b) synchronous (wavelength offset: 3 nm). All peaks can be assigned to a single PAH constituent of the sample except peak 2, which is attributed to two PAHs. *(Reprinted with permission from T. Vo-Dinh, R. B. Gammage, and P. R. Martinez, "Analysis of a Workplace Air Particulate Sample by Synchronous Luminescence and Room-Temperature Phosphorescence,* Analytical Chemistry, *53, pp. 253–258. Copyright 1981 American Chemical Society.)*



may not be soluble in the solvent inside the reagent compartment.

A shortcoming of such sensors is the need to produce an appropriate antibody for each analyte of interest (not necessarily an easy or inexpensive task), and of course the method is inapplicable to any analyte for which no antibody exists. One must also worry about the stability of the antibody and the rates of formation of the antibody–antigen complex and diffusion of analyte through the membrane.

Temporal selectivity can be achieved in fiberoptic sensors for PAHs by using, as excitation source,

a pulsed laser and exploiting differences in fluorescence lifetimes for discriminating between PAHs, as in natural waters (92). A relatively inexpensive pulsed laser may be used, and no reagent layer is needed, simplifying the design and of the probe tip and eliminating the kinetic problems that may be encountered when designs including reagents at the fiber tip are used. This approach ultimately is limited by the fact, noted previously, that many PAHs have similar fluorescence decay times; it is most useful for PAHs (such as pyrene) with relatively long decay times.

Low-Temperature Fluorometry

In the best of all possible worlds, one could subject a complex sample to no (or minimal) cleanup and selectively excite fluorescence of the analyte (using an excitation wavelength at which only the analyte absorbs appreciably). In the absence of intermolecular energy transfer, one would obtain a fluorescence spectrum of the analyte that could be used directly for quantitative purposes.

Because of the broad, featureless absorption spectra of most molecules in liquid and gas phases, such a procedure is seldom possible for complex liquid or gaseous samples. However, the absorption and fluorescence spectra of sample constituents may undergo dramatic sharpening if the sample is incorporated into a low-temperature solid (16, 54) or expanded in the gas phase in a supersonic free jet (58). Such procedures have been applied extensively to the determination of PAHs in complex samples.

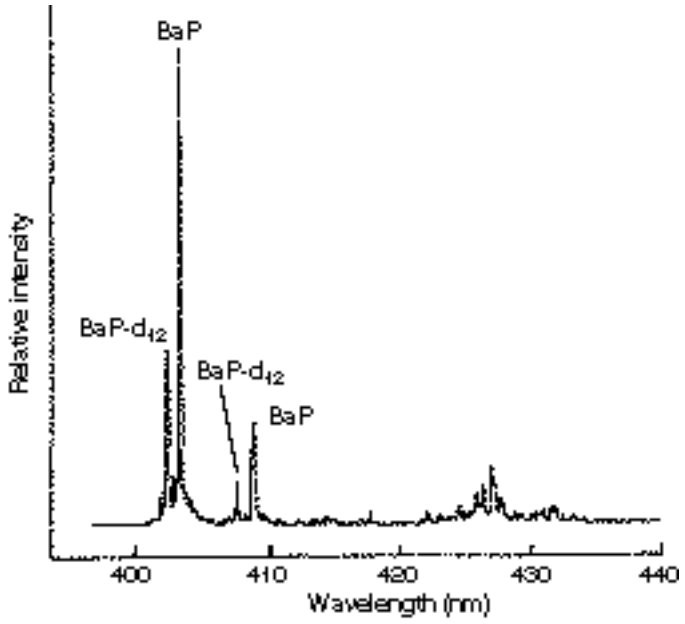
When PAHs are dissolved in n-alkane solvents that are then frozen at 77 °K or lower temperature, extraordinarily highly resolved absorption and fluorescence spectra may be obtained (the Shpol'skii effect (93)). The absorption spectra of sample constituents may be sharpened to the extent that an excitation wavelength can be found at which only the analyte absorbs appreciably. Also, in solid matrices, fluorescence quenching is less efficient than in solution. Thus, very high selectivity, freedom from additive and multiplicative interferences, and low limits of detection may be achieved with minimal sample cleanup.

For example, D'Silva, Fassel, and colleagues were able to identify and quantify individual PAHs in very complex materials, including crude petroleum and shale oils, without preliminary cleanup (93, 94). The samples were simply dissolved in a Shpol'skii solvent (such as n-octane), residual insoluble matter was filtered off, and the solutions were frozen rapidly to 77 °K. The spectral resolution is so high that a PAH often can be distinguished from its deuterated analog (see Fig. 26.6). Besides demonstrating the high selectivity of the technique, this fact shows that the deuterated analog can be used as an internal standard for quantitative purposes.

Even with this level of selectivity, preliminary sample cleanup may be needed in very demanding cases. For example, Garrigues and colleagues wished to determine each of the 12 possible isomeric methylbenzo[a]pyrenes in a coal tar extract (95). The effect of changing the position of a methyl group on the absorption and fluorescence spectra of these compounds is very small; thus, not only is very high spectral selectivity needed, but some degree of separation of the various isomers is required before measurement of fluorescence. Thus, the coal tar extract was subjected to a liquid chromatographic (LC) fractionation that produced several fractions, according to the number of aromatic rings. Then, the five-ring fraction was subjected to a second stage of LC, which separated the methylbenzo[a]pyrenes from the other five-ring PAHs and (to some extent) from each other. Chromatographic fractions were then diluted with a Shpol'skii solvent (n-octane) and cooled to 15 °K. As a result of this combination of chromatographic and spectroscopic selectivity, it was possible to quantify 8 of the 12 possible isomers (3 were not present in the sample, and 1 could not be distinguished from the parent PAH).

Many fluorescent molecules do not exhibit the Shpol'skii effect. For such compounds, chemical conversion to derivatives that exhibit highly resolved spectra in Shpol'skii matrices may be possible (96). Another approach using low-temperature solid matrices is fluorescence line narrowing (sometimes known as site selection), in which the choice of solvent is less critical than in Shpol'skii fluorometry. Very highly resolved fluorescence spectra may be obtained for molecules that do not exhibit the

Figure 26.6 Fluorescence spectra of benzo[a]pyrene (BaP), in a shale oil sample with 10 ppb perdeuterobenzo[a]pyrene (BaP-d_{12}) added as internal standard. The spectra were obtained in *n*-octane frozen solution (a Shpol'skii matrix) at 15 °K. *(Reprinted with permission from Y. Yang, A. P. D'Silva, and V. A. Fassel, "Deuterated Analogues as Internal Reference Compounds for the Direct Determination of Benzo[a]pyrene and Perylene in Liquid Fuels by Laser-Excited Shpol'skii Spectrometry,"* Analytical Chemistry, *53, pp. 2107–2109. Copyright 1983 American*



Shpol'skii effect. The principles of fluorescence line narrowing are described in detail elsewhere (16, 54, 97).

An impressive demonstration of the analytical capabilities of fluorescence line narrowing is a series of studies of adducts of carcinogenic PAH metabolites with DNA (97, 98). To detect, identify, and quantify these species in real samples requires extraordinary selectivity and sensitivity; numerous spectrally similar adducts of a particular metabolite may be present, the quantity of each adduct may be extremely small, and the scatter and fluorescence background interferences in biological materials are always a source of concern.

Shpol'skii fluorometry and fluorescence line narrowing use frozen liquid solution samples. An alternative approach is matrix isolation, wherein the sample is sublimed under vacuum and mixed with a gaseous diluent (the matrix). The resulting gas-phase mixture is deposited on a cold surface for spectroscopic examination as a solid (54, 99). The main advantage of matrix isolation is that analytes are dissolved in the solvent in the gas phase. Hence, solubility problems do not arise, and the solvent thus can be chosen for spectroscopic rather than chemical reasons. Shpol'skii matrices can be used in matrix isolation, and fluorescence line narrowing experiments also can be carried out.

Matrix isolation is difficult to apply to involatile analytes, but can be applied to extremely difficult samples. For example, Perry and colleagues detected and quantified individual PAHs in intractable solid solvent-refined coal samples by matrix-isolation Shpol'skii fluorometry without any preliminary sample cleanup (100). The extremely high selectivity that is possible is shown in Fig. 26.7, which compares the fluorescence spectrum of one sample constituent (7,10-dimethylbenzo[a]pyrene) with that of the pure compound. It is possible to combine low-temperature fluorometric measurements with time resolution

to achieve even higher selectivity in mixture analysis (49).

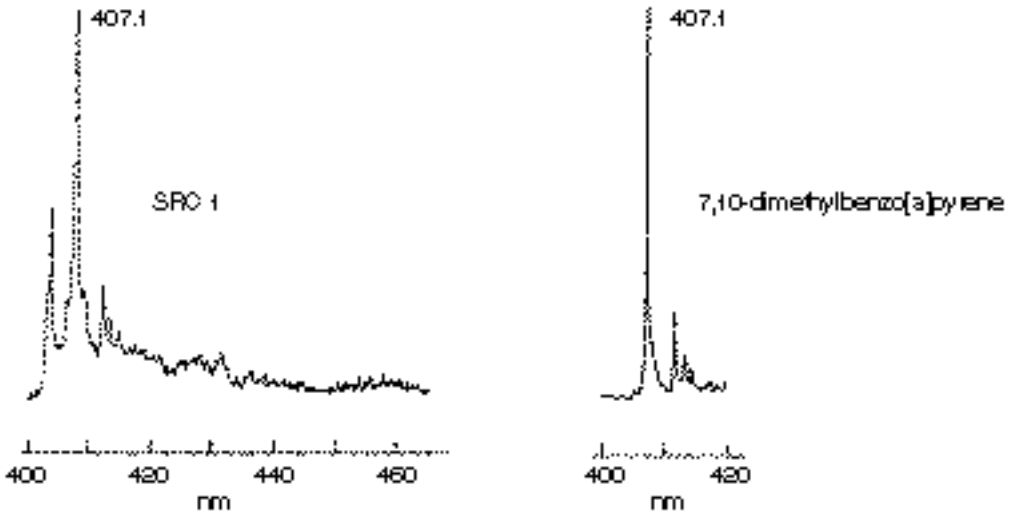
Selective and sensitive as these methods are, there are several caveats. Specialized, rather expensive apparatus may be needed to implement them properly. For example, it is difficult to exploit fully the selectivity of these techniques unless a laser is used as excitation source. The techniques may be time-consuming (although not necessarily more so than the separations often required to clean up a complex sample in conventional fluorometry) and require expertise and experience on the part of the analyst. Finally, authentic samples of the analytes should be available, so that the conditions for determining them (such as optimal excitation wavelengths) can be identified. Considerable trial and error may be required to identify the best conditions. Whether this process is more time-consuming than the separation steps that would be needed to eliminate interferences before carrying out a conventional fluorometric analysis varies from sample to sample.

Nuts and Bolts

Relative Costs

The simplest instruments for molecular fluorescence spectrometry (filter fluorometers or low- resolution scanning monochromator systems with no bells and whistles) cost less than $20,000. Instruments with reasonably high spectral resolution capable of synchronous scanning, generating corrected spectra, and computer postprocessing of spectra are usually in the $20,000–$30,000 price range. More sophisticated instruments offering more advanced components or capabilities (such as electronic array detector for rapid acquisition of spectra, high spectral resolution, accurate fluorescence polarization

Figure 26.7 Fluorescence spectra of pure 7,10-dimethylbenzo[a]pyrene (right) and the same PAH in a solid coal-derived material (left). Both spectra were obtained by matrix isolation in *n*-octane (a Shpol'skii matrix) at 15 °K. *(Reprinted with permission from M. B. Perry, E. L. Wehry, and G. Mamantov, "Determination of Polycyclic Aromatic Hydrocarbons in Unfractionated Solid Solvent-Refined Coal by Matrix Isolation Fluorescence Spectrometry,"* Analytical Chemistry, *55, pp. 1893–1896. Copyright 1983 American Chemical Society.)*



measurements, measurement of phosphorescence decay times, and spectral subtraction for some routine types of samples) can range from $30,000 to $75,000 or more. Instrumentation for fluorescence decay time and time-resolved fluorescence measurements (both pulsed and phase-modulation methods) is available; such instruments generally cost at least $50,000 and perhaps much more, depending on their capabilities. For certain specialized applications (such as laser-induced remote sensing or low-temperature fluorometry), assembly of an instrument from components (such as a laser, monochromator, and detector) may be necessary; in such cases it is easy to spend $100,000 or more.

A Raman spectrometer can double as a high-resolution, low stray-light fluorescence spectrometer; however, few (if any) laboratories would purchase a Raman spectrometer solely for fluorescence measurements!

Vendors for Instrumentation and Accessories

Many of these companies manufacture special-purpose fluorescence instrumentation, such as that used in polarized fluorescence measurements or determination of fluorescence lifetimes or time-resolved spectra.

Spectrometers

Buck Scientific Inc.
58 Fort Point St.
East Norwalk, CT 06855
phone: 800-562-5566
fax: 203-853-0569
email: 102456.1243@compuserve.com
Internet: http://ourworld.compuserve.com/homepages/Buck_Scientific

Hamamatsu Corp.
360 Foothill Rd.
Bridgewater, NJ 08807
phone: 800-524-0504
fax: 908-231-1218
email: hamacorp@interramp.com

Hitachi Instruments Inc.
3100 N. 1st St.
San Jose, CA 95134
phone: 800-455-4440
fax: 408-432-0704
email: info@hii.hitachi.com
Internet: http://www.hii.hitachi.com

Instruments S.A., Inc.
3880 Park Ave.
Edison, NJ 08820
phone: 800-438-7739
fax: 908-549-5125
email: john@isainc.com

ISS Inc.
2604 N. Mattis Ave.

Champaign, IL 61821
phone: 217-359-8681
fax: 217-359-7879

McPherson Inc.
530 Main St.
Acton, MA 01720
phone: 800-255-1055
fax: 508-263-1458
email: 72234.2257@compuserve.com

On-Line Instrument Systems Inc.
130 Conway Dr.
Bogart, GA 30622
phone: 800-852-3504
fax: 706-353-1972
email: olis@mindspring.com
Internet: http://www.olisweb.com

Perkin-Elmer Corporation
761 Main Ave.
Norwalk, CT 06859
phone: 800-762-4000
fax: 203-762-4228
email: info@perkin-elmer.com
Internet: http://www.perkin-elmer.com

Photon Technology International
1 Deerpark Dr.
South Brunswick, NJ 08852
phone: 908-329-0910
fax: 908-329-9069

Shimadzu
7102 Riverwood Dr.
Columbia, MD 21046
phone: 800-477-1227
fax: 410-381-1222
Internet: http://www.shimadzu.com

Spectronic Instruments
820 Linden Ave.
Rochester, NY 14625
phone: 800-654-9955
fax: 716-248-4014
email: info@spectronic.com

Turner Designs Inc.
845 W. Maude Ave.
Sunnyvale, CA 94086
phone: 408-749-0994
fax: 408-749-0998

Varian Instruments
P.O. Box 9000
San Fernando, CA 91340
phone: 800-926-3000

Cells

Buck Scientific (see listing above)

Hellma Cells Inc.
P.O. Box 544
Borough Hall Sta.
Jamaica, NY 11424
phone: 718-544-9534
fax: 718-263-6910

Wilmad Glass
Route 40 and Oak Rd.
Buena, NJ 08310
phone: 609-697-3000
fax: 609-697-0536
email: cs@wilmad.com
Internet: www.wilmad.com

Optical Parts

Esco Products Inc.
171 Oak Ridge Rd.
Oak Ridge, NJ 07438
phone: 201-697-3700
fax: 201-697-3011

Melles Griot
1770 Kettering St.
Irvine, CA 92714
phone: 800-835-2626
fax: 714-261-7589

Oriel Corp.
250 Long Beach Blvd.
Stratford, CT 06497
phone: 203-377-8262
fax: 203-378-2457
email: 73163.1321@compuserve.com

Detectors

Burle Industries Inc.
1000 New Holland Ave.
Lancaster, PA 17601
phone: 800-326-3270
fax: 717-295-6097

EG&G Reticon

345 Potrero Ave.
Sunnyvale, CA 94086
phone: 408-738-4266
fax: 408-738-6979

Hamamatsu Corp. (see listing above)

Princeton Instruments Inc.
3660 Quakerbridge Rd.
Trenton, NJ 08619
phone: 609-587-9797
fax: 609-587-1970
email: postmaster@prinst.com

For vendors of fluorescence detection systems for liquid chromatography and electrophoresis, see the chapters on those topics.

Consumables

Users of fluorometers often need to purchase sample cells and various optical parts (such as lamps, mirrors, gratings, detectors, polarizers, and fiberoptics). Although these can often be purchased from the manufacturer of the fluorometer, it may be advantageous (and cheaper) to procure these items from specialty vendors.

Solvents and chemicals may be purchased from any of the major chemical manufacturers and supply houses. As noted earlier, solvent purity is a key issue. Do not scrimp here. At the very least, spectrophotometric-grade solvents should be used; in some instances, HPLC-grade or equivalent solvents may be needed. The latter are expensive but their use may save much time and money in the long run.

Required Level of Training

The steepness of the learning curve for operating a fluorescence spectrometer is strongly dependent on the complexity of the instrument. Virtually all manufacturers will install the instrument at your site, check to ensure that the instrument meets specifications, and provide a rudimentary overview of instrument operation to prospective operators. Often, this is all that is needed; most instruments use menu-driven computer software, so that any operator familiar with the terminology of fluorometry can readily access the instrument's capabilities. Anyone who can operate a UV/Vis absorption spectrometer should be able to learn to operate a basic type of fluorescence spectrometer without significant difficulty. More complex instruments (especially those using laser sources) are correspondingly more difficult to operate.

Operation of basic instrumentation for routine, well-established fluorometric methods can be performed by a person with an associate degree. Adaptation of existing procedures for non-standard samples, or use of more complex instrumentation, requires a bachelor's degree background in analytical chemistry (and perhaps biochemistry or organic chemistry). Development of new instruments or new types of applications, or operation of highly sophisticated instruments (as in laser-excited fluorescence for atmospheric remote sensing) usually requires some graduate-level education.

Service and Maintenance

The key to long life and trouble-free operation of a fluorescence spectrometer is the same as that for any optical spectrometric instrument: House it in a climate-controlled environment free from dust, chemical fumes, and excessive heat, humidity, and vibration. If this is done, the only maintenance that should normally be required is periodic replacement of the source. Lamps (even those obtained from the same manufacturer) tend to be highly variable; some last for years, but others last only several months. Lamps age and their power output tends to decrease slowly with time. Periodic checks of instrumental sensitivity using samples (such as quinine or anthracene solutions) recommended in the manufacturer's instruction manual should be carried out; when the sensitivity has degraded to an unacceptably low level, the lamp probably must be replaced. High-pressure xenon lamps must be handled carefully to avoid breakage and possible injury. The manufacturer's instruction manual should explicitly describe the precautions to be taken in changing the lamp. Follow those precautions rigorously.

Although a manufacturer may offer a yearly service contract, these are probably unnecessary unless the instrument is to be used under very harsh conditions or the user's installation has virtually no capabilities for even minor instrument repairs.

Most manufacturers maintain service departments that can provide assistance over the telephone (instruction manuals tend to be lamentably inadequate in the area of troubleshooting) or, in case of severe difficulty, will dispatch a service engineer for on-site repairs. For a properly housed and properly used fluorometer, such service visits should be infrequent.

Suggested Readings

Books

GUILBAULT, G. G., *Practical Fluorescence,* 2nd ed. New York: Marcel Dekker, 1990. Multiauthor treatise accurately described by its title. The best available compendium of information on analytical applications of fluorometry.

PARKER, C. A., *Photoluminescence of Solutions.* Amsterdam: Elsevier, 1968. Despite its age, this is still an excellent introduction to the principles and practice of fluorometry. The discussion of applications, though useful, is very dated.

RENDELL, D., *Fluorescence and Phosphorescence Spectroscopy.* New York: Wiley, 1987. Open-learning introductory text; very readable and contains many useful examples and helpful hints.

SCHULMAN, S. G., *Molecular Luminescence Spectroscopy: Methods and Applications.* New York: Wiley, 1985, 1988, 1993. Three-volume multiauthor compendium of recent advances; many excellent chapters on specific topics.

SLAVIK, J., *Fluorescent Probes in Cellular and Molecular Biology.* Boca Raton, FL: CRC Press, 1994. Much less limited in scope than the title implies; contains much useful introductory material, especially on instrumentation.

Book Chapters and Review Articles

Analytical Chemistry, in even-numbered years, publishes a Fundamental Review Article on Molecular Fluorescence, Phosphorescence, and Chemiluminescence; the most recent such article is SOPER, S. A., L. B. MCGOWN, AND I. M. WARNER, *Analytical Chemistry,* 68 (1996), 73R. Recent developments in instrumentation and techniques are surveyed authoritatively in these articles. In odd-numbered years, some reviews survey applications of fluorescence; for example, see DIAMANDIS, E. P., *Analytical Chemistry,* 65 (1993), 454R.

HARRIS, T. D., AND F. E. LYTLE, "Analytical Applications of Laser Absorption and Emission Spectroscopy," in D. S. Kliger, ed., *Ultrasensitive Laser Spectroscopy.* New York: Academic Press, 1983. Authoritative (albeit somewhat dated) and readable survey of the virtues and shortcomings of laser-induced fluorescence tech-

niques.

HURTUBISE, R. J., "Trace Analysis by Luminescence Spectroscopy," in G. D. Christian and J. B. Callis, eds., *Trace Analysis: Spectroscopic Methods for Molecules.* New York: Wiley, 1986. Particularly strong on instrumentation and applications.

WEHRY, E. L., "Molecular Fluorescence and Phosphorescence Spectroscopy," in B. W. Rossiter and R. C. Baetzold, eds., *Determination of Electronic and Optical Properties.* 2nd ed., vol. 8. Physical Methods of Chemistry. New York: Wiley, 1993. Detailed coverage of instrumentation and techniques.

References

1. E. L. Wehry, in B. W. Rossiter and R. C. Baetzold, eds., *Physical Methods of Chemistry,* vol. 8 (New York: Wiley, 1993), p. 109.
2. E. L. Wehry, in G. G. Guilbault, ed., *Practical Fluorescence,* 2nd ed. (New York: Marcel Dekker, 1990), p. 75.
3. W. R. Seitz, *Crit. Rev. Anal. Chem.,* 8 (1980), 367.
4. D. W. Fink, *Trends Anal. Chem.,* 1 (1982), 254.
5. H. Lingemann and others, *J. Liq. Chromatogr.,* 8 (1985), 789.
6. J. Slavik, *Fluorescent Proves in Cellular and Molecular Biology* (Boca Raton, FL: CRC Press, 1994).
7. M. M. Krahn and others, *Environ. Sci. Technol.,* 27 (1993), 699.
8. J. P. Alarie and others, *Rev. Sci. Instrum.,* 64 (1993), 2541.
9. E. L. Wehry, in G. G. Guilbault, ed., *Practical Fluorescence,* 2nd ed. (New York: Marcel Dekker, 1990), p. 127.
10. T. G. Matthews and F. E. Lytle, *Analytical Chemistry,* 51 (1979), 583.
11. T. Vo-Dinh, in E. L. Wehry, ed., *Modern Fluorescence Spectroscopy,* vol. 4 (New York: Plenum, 1981), p. 167.
12. T. Ndou and I. M. Warner, *Chemical Reviews,* 91 (1991), 493.
13. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* (New York: Plenum, 1983).
14. M. A. Winnik, *Photophysical and Photochemical Tools in Polymer Science* (Dordrecht, The Netherlands: Riedel, 1986).
15. R. Phillips, *Sources and Applications of Ultraviolet Radiation* (New York: Academic Press, 1983).
16. J. W. Hofstraat, C. Gooijer, and N. H. Velthorst, in S. G. Schulman, ed., *Molecular Luminescence Spectroscopy: Methods and Applications,* vol. 3 (New York: Wiley, 1993), p. 323.
17. R. Brina and A. G. Miller, *Analytical Chemistry,* 64 (1992), 1413.
18. J. F. Brennan and others, *Appl. Spectrosc.,* 47 (1993), 2081.
19. C. A. Parker, *Photoluminescence of Solutions* (Amsterdam: Elsevier, 1968), p. 131.
20. J. M. Harris, *Analytical Chemistry,* 54 (1982), 2337.
21. L. W. Hershberger, J. B. Callis, and G. D. Christian, *Analytical Chemistry,* 51 (1979), 1444.
22. G. J. Diebold and R. N. Zare, *Science,* 196 (1977), 1439.
23. P. M. Epperson, R. D. Jalkian, and M. B. Denton, *Analytical Chemistry,* 61 (1989), 282.
24. C. A. Parker, *Photoluminescence of Solutions* (Amsterdam: Elsevier, 1968), p. 128.
25. T. Vo-Dinh and others, *Environ. Sci. Technol.,* 12 (1978), 1297.

26. E. L. Inman, Jr., and J. D. Winefordner, *Analytical Chemistry,* 54 (1982), 2018.
27. G. D. Christian, J. B. Callis, and E. R. Davidson, in E. L. Wehry, ed., *Modern Fluorescence Spectroscopy,* vol. 4 (New York: Plenum, 1981), p. 111.
28. T. Vo-Dinh, *Appl. Spectrosc.,* 36 (1982), 576.
29. P. M. R. Hertz and L. B. McGown, *Analytical Chemistry,* 64 (1992), 2920.
30. D. Eastwood, in E. L. Wehry, ed., *Modern Fluorescence Spectroscopy,* vol. 4 (New York: Plenum, 1981), 251.
31. J. A. Taylor and E. S. Yeung, *Analytical Chemistry,* 64 (1992), 1741.
32. S. Barnard and D. R. Walt, *Environ. Sci. Technol.,* 25 (1991), 1301.
33. C. Moulin and others, *Appl. Spectrosc.,* 47 (1993), 2007.
34. R. B. Thompson and E. R. Jones, *Analytical Chemistry,* 65 (1993), 730.
35. O. S. Wolfbeis, *Fiber Optic Chemical Sensors and Biosensors* (Boca Raton, FL: CRC Press, 1991).
36. W. R. Seitz, *Crit. Rev. Anal. Chem.,* 19 (1988), 135.
37. M. J. Sepaniak, B. J. Tromberg, and T. Vo-Dinh, *Progr. Anal. Spectrosc.,* 11 (1988), 481.
38. B. J. Tromberg and others, *Analytical Chemistry,* 60 (1988), 1901.
39. M. C. Gutierrez, A. Gomez-Hens, and D. Perez-Bendito, *Talanta,* 36 (1989), 1187.
40. T. D. Harris and F. E. Lytle, in D. S. Kliger, ed., *Ultrasensitive Laser Spectroscopy* (New York: Academic Press, 1983), p. 369.
41. R. J. van de Nesse and others, *Anal. Chim. Acta,* 281 (1993), 373.
42. F. E. Lytle, *J. Chem. Educ.,* 59 (1982), 915.
43. J. Hecht, *The Laser Guidebook* (New York: McGraw-Hill, 1992), p. 263.
44. K. J. Miller and F. E. Lytle, *J. Chromatogr.,* 648 (1993), p. 245.
45. H. M. Shapiro, *Practical Flow Cytometry* (New York: Liss, 1988).
46. J. N. Demas, *Excited State Lifetime Measurements* (New York: Academic Press, 1983).
47. F. E. Lytle, *Analytical Chemistry,* 46 (1974), 545A, 817A.
48. L. B. McGown and K. Nithipatikom, in T. Vo-Dinh, ed., *Chemical Analysis of Polycyclic Aromatic Compounds* (New York: Wiley, 1989), p. 201.
49. R. B. Dickinson, Jr., and E. L. Wehry, *Analytical Chemistry,* 51 (1979), 778.
50. K. Nithipatikom and L. B. McGown, *Appl. Spectrosc.,* 41 (1987), 395.
51. J. N. Demas and R. A. Keller, *Analytical Chemistry,* 57 (1985), 538.
52. E. P. Diamandis and T. K. Christopoulos, *Analytical Chemistry,* 62 (1990), 1149A.
53. N. T. Azumi and others, *Appl. Spectrosc.,* 46 (1992), 994.
54. E. L. Wehry, in E. H. Piepmeier, ed., *Analytical Applications of Lasers* (New York: Wiley, 1986), p. 211.
55. J. M. Hayes and G. J. Small, *Analytical Chemistry,* 55 (1983), 565A.
56. S. A. Soper and others, *Analytical Chemistry,* 63 (1991), 432.
57. M. D. Barnes and others, *Analytical Chemistry,* 65 (1993), 2360.
58. T. C. O'Haver, in J. D. Winefordner, ed., *Trace Analysis: Spectroscopic Methods for Elements* (New York: Wiley, 1976), p. 15.

59. G. L. Campi and J. D. Ingle, Jr., *Anal. Chim. Acta,* 224 (1989), 225.
60. G. Patonay and M. D. Antoine, *Analytical Chemistry,* 63 (1991), 321A.
61. S. A. Soper, Q. L. Mattingly, and P. Vegunta, *Analytical Chemistry,* 65 (1993), 740.
62. A. J. G. Mank and others, *Analytical Chemistry,* 65 (1993), 2197.
63. J. Zhang and others, *Anal. Chim. Acta,* 279 (1993), 281.
64. D. R. Christman, S. R. Crouch, and A. Timnick, *Analytical Chemistry,* 53 (1981), 2040.
65. M. E. Rollie, G. Patonay, and I. M. Warner, *Analytical Chemistry,* 59 (1987), 180.
66. W. Xu and others, *Analytical Chemistry,* 66 (1994), 4133.
67. R. L. Plant and D. H. Burns, *Appl. Spectrosc.,* 47 (1993), 1594.
68. T. Vo-Dinh, R. B. Gammage, and P. R. Martinez, *Analytical Chemistry,* 53 (1981), 253.
69. R. P. Frankewich, K. N. Thimmiah, and W. L. Hinze, *Analytical Chemistry,* 63 (1991), 2924.
70. R. J. Hurtubise, in G. G. Guilbault, ed., *Practical Fluorescence,* 2nd ed. (New York: Marcel Dekker, 1990), p. 431.
71. T. Vo-Dinh, *Room-Temperature Phosphorimetry for Chemical Analysis* (New York: Wiley, 1984).
72. W. R. G. Baeyens, in S. G. Schulman, ed., *Molecular Luminescence Spectroscopy: Methods and Applications* (New York: Wiley, 1985), p. 29.
73. G. G. Guilbault, *Practical Fluorescence,* 2nd ed. (New York: Marcel Dekker, 1990).
74. E. P. Diamandis, *Analytical Chemistry,* 65 (1993), 454R.
75. E. L. Wehry, in G. G. Guilbault, ed., *Practical Fluorescence,* 2nd ed. (New York: Marcel Dekker, 1990), p. 367.
76. P. Froehlich and E. L. Wehry, in E. L. Wehry, ed., *Modern Fluorescence Spectroscopy,* vol. 3 (New York: Plenum, 1981), p. 35.
77. A. Hulshoff and H. Lingeman, in S. G. Schulman, ed., *Molecular Luminescence Spectroscopy: Methods and Applications,* vol. 1 (New York: Wiley, 1985), p. 621.
78. E. Arriaga and others, *J. Chromatogr.,* 652 (1993), 347.
79. X. C. Huang, M. A. Quesada, and R. A. Mathies, *Analytical Chemistry,* 64 (1992), 2149.
80. K. Ueno and E. S. Yeung, *Analytical Chemistry,* 66 (1994), 1424.
81. S. I. Mho and E. S. Yeung, *Analytical Chemistry,* 57 (1985), 2253.
82. D. R. Walt, *Proc. IEEE,* 80 (1992), 903.
83. I. A. Hemmila, *Applications of Fluorescence in Immunoassay* (New York: Wiley, 1991).
84. H. T. Karnes, J. S. O'Neal, and S. G. Schulman, in S. G. Schulman, ed., *Molecular Luminescence Spectroscopy: Methods and Applications,* vol. 1 (New York: Wiley, 1985), p. 717.
85. J. Frengen and others, *Clin. Chem.,* 39 (1993), 2174.
86. R. Y. Tsien, *ACS Symp. Ser.,* 538 (1993), 130.
87. B. Valeur, in S. G. Schulman, ed., *Molecular Luminescence Spectroscopy: Methods and Applications,* vol. 3 (New York: Wiley, 1993), p. 25.
88. W. E. May and S. A. Wise, *Analytical Chemistry,* 56 (1984), 225.
89. W. T. Cobb and L. B. McGown, *Analytical Chemistry,* 62 (1990), 186.
90. D. J. Desilets, P. T. Kissinger, and F. E. Lytle, *Analytical Chemistry,* 59 (1987), 1830.

91. L. B. McGown, *Analytical Chemistry*, 61 (1989), 839A.
92. R. Niessner, U. Panne, and H. Schroder, *Anal. Chem. Acta.*, 255 (1991), 231.
93. A. P. D'Silva and V. A. Fassel, *Analytical Chemistry*, 56 (1984), 985A.
94. Y. Yang, A. P. D'Silva, and V. A. Fassel, *Analytical Chemistry*, 53 (1981), 2107.
95. P. Garrigues, J. Bellocq, and S. A. Wise, *Fresenius' J. Anal. Chem.*, 336 (1990), 106.
96. S. Weeks and others, *Analytical Chemistry*, 62 (1990), 1472.
97. R. Jankowiak and G. J. Small, *Analytical Chemistry*, 61 (1989), 1023A.
98. P. D. Devanesan and others, *Chem. Res. Toxicol.*, 5 (1992), 302.
99. E. L. Wehry and G. Mamantov, *Analytical Chemistry*, 51 (1979), 643A.
100. M. B. Perry, E. L. Wehry, and G. Mamantov, *Analytical Chemistry*, 55 (1983), 1893.