1.9 Fluorescence spectrophotometry

Fluorescence spectrophotometry is the measurement of the fluorescence, i.e. photoluminescence, emitted by a substance while it is being exposed to ultraviolet, visible, or other electromagnetic radiation. In general, the light emitted by fluorescent solutions is of maximum intensity at a wavelength longer than that of the absorption band causing excitation, usually by some 20 or 30 nm.

The intensity of the light emitted by a fluorescent solution is, in certain circumstances, a simple function of the concentration of the solute and can, therefore, be used for analysis. It is difficult, however, to measure absolute fluorescence intensity, and measurements are usually made by reference to dilutions of a properly selected reference substance. The general scheme in fluorescence spectroscopy is, therefore, to excite with radiation at the wavelength of maximum absorption, and to measure or compare the intensity of the fluorescent light with that of a reference solution. The fluorescent light should be carefully freed from scattered incident light.

Terms

Fluorescence intensity is an empirical expression of fluorescence activity, commonly given in terms of arbitrary units proportional to detector response.

The *fluorescence emission spectrum* is the relationship between the intensity of the emitted radiation and the wavelength and is frequently represented in a graphic form.

The *fluorescence excitation spectrum* is the relationship between the maximum intensity of radiation emitted by an activated substance and the wavelength of the incident radiation and is frequently represented in a graphic form.

Apparatus

Measurement of fluorescence intensity can be made with a simple filter fluorimeter (sometimes the term fluorometer is used). Such an instrument consists of a radiation source, a primary filter, a sample chamber, a secondary filter, and a fluorescence detection system. In most such fluorimeters, the detector is placed on an axis at 90° from that of the incident beam. This right-angle geometry permits the incident radiation to pass through the test solution without contaminating the output signal received by the fluorescence detector. However, the detector unavoidably receives some of the incident radiation as a result of the inherent scattering properties of the solutions themselves, or if dust or other solids are present. Filters are used to eliminate this residual scatter. The primary filter selects short wavelength radiation capable of causing excitation of the test substance, while the secondary filter is normally a sharp cut-off filter that allows the longer wavelength fluorescence to be transmitted but blocks the scattered excitated radiation.

Most fluorimeters use photomultiplier tubes as detectors; many types are available, each having special characteristics with respect to spectral region of maximum sensitivity, gain, and electrical noise. After amplification of the photocurrent its value is read visually on a measuring device or recorded.

A fluorescence spectrophotometer differs from a filter fluorimeter in that filters are replaced by monochromators, of either the prism or the grating type. For analytical purposes, the fluorescence spectrophotometer is superior to the filter fluorimeter in wavelength selectivity, flexibility, and convenience.

Many radiation sources are used in fluorimeters and fluorescence spectrophotometers. Mercury lamps are relatively stable and emit energy mainly at discrete wavelengths. Tungsten lamps provide an energy continuum in the visible region. The high pressure xenon arc lamp is often used in fluorescence spectrophotometers because it has an energy continuum extending from the ultraviolet into the infrared.

In fluorescence spectrophotometers the monochromators are equipped with slits. A narrow slit provides high resolution and spectral purity, while a large slit sacrifices these for high intensity. Choice of slit-width is determined by the wavelength separation between incident and emitted radiation, as well as by the degree of sensitivity needed.

The cells used in fluorescence measurements may be rectangular cells similar to those used in absorption spectrophotometers, except that they are polished on all 4 vertical sides and on the bottom, or cells in the shape of round tubes with flat polished bottoms may be used. A convenient size is 2-3 mL, but some instruments can be fitted with small cells holding 0.1-0.3 mL, or with a capillary holder requiring even less solution.

Standardization

Fluorimeters and fluorescence spectrophotometers should be standardized daily with a stable fluorophore to assure proper reproducibility of response. The changes are due to instrumental variables such as differences in lamp intensity and photomultiplier sensitivity. The fluorophore may be a pure specimen of the fluorescent substance under test or another readily purified fluorescent substance with absorption and fluorescence bands similar to those of the test substance. Quinine in dilute sulfuric acid is often a convenient fluorophore for blue fluorescence, sodium fluorescein for green fluorescence, and rhodamine for red fluorescence.

Calibration of the wavelength scale

The wavelength scale of the fluorescence spectrophotometer should be periodically calibrated.

Preparation of solution

The solvent used for the measurement should be properly selected. The solvent itself, its purity and its pH may markedly affect the intensity and spectral distribution of fluorescence.

Test solutions prepared for fluorescence spectrophotometry are usually 10 times to 100 times less concentrated than those used in absorption spectrophotometry. In analytical applications, it is necessary that the fluorescence intensity be linearly related to the concentration in the range used for measurements; but if a solution is too concentrated, a significant part of the incident light is absorbed by the substance near the cell surface, thus resulting in a reduction of the light reaching the centre. That is, the substance itself acts as an "inner filter". Fortunately, fluorescence spectrophotometry is inherently a very sensitive technique, and concentrations of 10^{-5}-10^{-7} mol/l are frequently used.

Owing to the usually very narrow range within which the fluorescence is proportional to the concentration of the fluorescent substance, the ratio $(c\text{-}d)/(a\text{-}b)$, where a is the fluorescence intensity read for the reference substance, b the reading for the corresponding blank, c the fluorescence intensity read for the test substance, and d the reading for the corresponding blank, should be not less than 0.40 and not more than 2.50. It is then necessary to make a working curve of fluorescence intensity corrected for a solvent blank versus concentration.

Measurement technique

Fluorescence measurements are sensitive to the presence of solid particles in the test solution. Such impurities may reduce the intensity of the exciting beam or give misleadingly high readings because of multiple reflections in the cell. It is, therefore, wise to eliminate solid particles by centrifugation; filtration may also be used, but some filter-papers may contain fluorescent impurities.

Oxygen dissolved in the solvent has a strong quenching effect. The intensity of fluorescence is, therefore, increased by use of a degassing procedure, such as bubbling nitrogen or another inert gas through the test solution.

Temperature regulation is often important in fluorescence spectrophotometry. For some substances, fluorescence efficiency may be reduced by as much as 1-2% per degree of temperature rise. In such cases, if maximum precision is desired, temperature-controlled sample cells are essential. For routine analysis, it may be sufficient to make measurements rapidly enough so that the test solution does not heat up appreciably from exposure to the intense light source.

Many fluorescent compounds are light-sensitive. Exposed in a fluorescence spectrophotometer, they may be photodegraded into more or less fluorescent products. Such effects may be detected by observing the detector response in relation to time, and may be reduced by attenuating the light source with filters or screens.