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REFERENCE MATERIALS FOR FLUORESCENCE MEASUREMENT

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Reference materials for fluorescence measurement

INTRODUCTION

Measurement of the fluorescence properties of a molecule (fluorescence spectrum, excitation spectrum, quantum yield and fluorescence lifetime) is critical to the development of an understanding of the photophysics and photochemistry of the singlet excited state(s) of the species. Reliable and standard methodology is required if published data are to be of use to the photochemical community at large. The purpose of this document is to recommend standard practices and materials for these measurements and to discuss the avoidance of experimental inadequacies attendant on their application in practical use. This is not a comprehensive review of the many techniques advocated as solutions to each aspect of fluorescence measurement but rather it recommends appropriate methods which strike a balance between ease of application, generality of use, and reliability. For a review of general considerations see Demas and Crosby [1], Birks [2] and several general texts [3-6] as well as a thorough discussion of fluorescence standards prepared by the Ultraviolet Spectrometry Group [7]. For a description of terms used here, see the "Glossary of Terms in Photochemistry," submitted for provisional approval to the IUPAC [8]. Luminescence spectroscopic terms are discussed in a detailed IUPAC publication [9].

The following topics will be addressed:

I. General Considerations
II. Corrected Spectra
    A. Excitation Spectra
    B. Emission Spectra
III. Fluorescence Quantum Yields
IV. Fluorescence Lifetimes

I. GENERAL CONSIDERATIONS

General experimental methods should conform to the recommendations of Lamola and Wrighton [10]. Care should be taken to ensure the cleanliness of optical elements, to eliminate dust as much as possible from the environment, and to use clean solvents, glassware and other reagents. When handling optical cells, avoid fingerprints, which fluoresce. Use lint-free tissues only to wipe optical surfaces. Cell cleaning must be thorough, but cleaning materials must be shown not to introduce contamination or to interfere with the measurement. Filtration of solutions through micropore filters is recommended. Degassing procedures should be rigorous. Experience indicates five freeze-pump-thaw cycles in a vacuum system to ~10^{-2} Pa to be excellent. Aqueous solvents must be degassed without freezing because they expand. In these cases thorough argon purging is recommended using water-saturated argon.

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II. CORRECTED SPECTRA

A. Excitation corrections

Reliable use of an emission apparatus requires knowledge of the spectral photon irradiance (number of quanta/unit area/unit area/bandwidth unit) of the excitation system (exciting lamp plus monochromator) as a function of wavelength. Several techniques for excitation correction are in use:

- thermal detectors
- actinometers
- quantum counters

The recommended method, and the most common, is the use of quantum counters. For the wavelength region 250-600 nm, the recommended standard is rhodamine 101 [11]. This compound is suitable for both 90° and front surface emission observations. Rhodamine B has been recommended by Melhuish [12] (emission wavelength 610-620 nm), but it is not preferred because it is sensitive to temperature flucuations, and because it is unstable to absorbed light below 340 nm unless it is very pure. Quinine bisulfate is a good quantum counter below 340 nm [13]. A sodium salicylate screen or solution can be used from 250 nm into the vacuum ultraviolet.

Another method which will undoubtedly become more common is the use of continuous correction spectrophotometers in which the manufacturer provides the spectral correction. Such instruments are likely to require smaller excitation corrections but they should still be performed for the collection of primary data.

Reliable excitation spectra can only be obtained if the absorbance of the sample is small (< 0.05) over the wavelength region examined.

B. Emission corrections

Corrected emission spectra are required in order to determine true emission yields and bandshapes. Calibration of the emission system can be done by use of a calibrated light source or by reference to series of compounds of known emission. The latter method, when coupled with computer controlled normalization of an "experimental" curve by a stored, "corrected" curve, can provide accurate data in a reproducible manner. Many commercial systems include this feature.

a. Calibrated light sources

Standard lamps are available but many precautions are necessary to assure proper use of these standards in calibrations. If it is desired to use light source calibration we recommend the use of a calibrated excitation source (lamp plus monochromator) which has been calibrated by means of quantum counters. The calibrated source is then directed into the emission system (monochromator and photomultiplier) using a light scatterer or reflector whose reflectivity (or scattering function) is independent of wavelength. A $BaSO_4$ or MgO reflector is recommended.

b. Emission standards

For the range 400-600 nm a suitable standard is quinine bisulfate in acidic solution, excited in the range 260-360 nm. For excitation wavelengths beyond 360 nm the spectrum shifts to lower energies. The corrected spectrum is published [12, 13]. Melhuish [12] also presented a corrected spectrum for 2-aminopyridine in 1 **N** H_2SO_4 (excitation 280-330 nm, emission 340-450 nm). This standard is recommended in that wavelength region by Testa [14]. β-Carboline has also been proposed as a standard for the 450 nm range [15].

III. FLUORESCENCE QUANTUM YIELDS

Determination of a fluorescence quantum yield requires the prior correction of the emission spectrum of the material or the use of a fluorescent standard whose emission spectral properties closely match those of the unknown. A number of experimental pitfalls exist which must be considered explicitly before a determined quantum yield value (ϕ) can be considered reliable. These include:

- Inner filter (self-absorption) effects
- Possible wavelength effects on ϕ
- Refractive index corrections
- Polarization effects
- Temperature effects
- Impurity effects
- Photochemical stability
- Raman scattering

Both primary and secondary methods are commonly used. Primary methods include the use of scattering surfaces or solutions to calibrate the detector/excitation system absolutely, the use of actinometers in place of, and surrounding the sample, and the use of integrating spheres or calorimetric techniques. Secondary methods are much preferred by most experimentalists. These involve the use of standard materials and rely on comparison of the integrated areas under the fluorescence spectra of the standard and the unknown under identical conditions of incident irradiance. It must be noted, however, that there are no suitable standards for spectral correction in the red region.

A. Primary methods

The use of scattering solutions, e.g. colloidal silica, or reflective surfaces such as $BaSO_4$ is recommended if this method is used.[4] Refractive index corrections and wavelength sensitivity factors must be considered when using this approach. The use of actinometric methods is not recommended for use with dilute solutions, and therefore is of little value.

B. Secondary methods

This is the method of choice for solution determinations. In the same apparatus, the quantum yield of an unknown is related to that of a standard by Eqn. 1,

$$\phi_U = [(A_S F_U n^2)/(A_U F_S n_o^2)] \phi_S \qquad (1)$$

where: the *u* subscript refers to the unknown and *s* to the standard and other symbols have the following meanings: ϕ is quantum yield, A is absorbance at the excitation wavelength, F the integrated emission area across the band and n's are respectively index of refraction of the solvent containing the unknown (n) and the standard (n_o) at the sodium D line and the temperature of the emission measurement. (The D line is used assuming dispersion among standard solvents to be small.) Absorbances of sample and standard must be similar and small (e.g. below 0.10). It is most desirable that both unkown and standard be in the same solvent. If this is not possible, then corrections for the difference in refractive indeces of the solvents must be made according to eq. 1 (or by other methods; see Miller [7]). Monochromatic excitation of both sample and standard must be used, and the excitation intensity impinging on both must be equal.

The need for refractive index corrections arises from two sources. As radiation passes from the solution into air (i.e., from high to low index region), its intensity changes because of the refraction. Second, internal reflection within a cell can occur. Errors may be severe if different solvents are used for sample and standard. For a discussion of the effects, see Miller [7].

Suitable standards for comparative determination of quantum yields of fluorescence in solution are given in Table I. All concentrations should be below 10^{-5}M in the specified solvent (absorbance < 0.1). All solutions should be degassed. Solvents should be of spectral grade and must be checked for spurious emission. Temperature should be kept constant during the measurement and should be reported. The values in Table I are for 20±1 °C unless indicated (e.g., tryptophan).

Far red and near infrared standards are not known. Two transition metal complexes can be used with caution. *Tris*-(4,4'-bipyridyl)ruthenium(II) dichloride emits near 620 with ϕ = 0.042± 0.002 in deoxygenated water, but the emission is temperature dependent [16]. *Tris*-(1,10-phenanthroline)osmium(II) di(hexafluorophosphate) emits with a maximum near 700 nm with low quantum yield (ϕ = 0.016±0.002) in degassed acetonitrile [17]. It is subject to self-absorption. The

TABLE I. Recommended Fluorescence Quantum Yield References In Various Emission Ranges.†

Region	Compound	Solvent	Φ_f	Ref.
270-300 nm	Benzene	Cyclohexane	0.05±0.02	a
300-380 nm	Tryptophan*	H_2O (pH 7.2)	0.14±0.02	b
300-400 nm	Naphthalene	Cyclohexane	0.23±0.02	c
315-480 nm	2-Aminopyridine	0.1 **N** H_2SO_4	0.60±0.05	d
360-480 nm	Anthracene	Ethanol	0.27±0.03	a,e
400-500 nm	9,10-Diphenyl-anthracene	Cyclohexane	0.90±0.02	f, g
400-600 nm	Quinine Bisulfate	1 **N** H_2SO_4	0.546	e, g
600-650 nm	Rhodamine 101	Ethanol	1.0±0.02	h
600-650 nm	Cresyl Violet	Methanol	0.54±0.03	i

† Errors quoted are those provided by the authors; see the individual references for their significance. Where no error is given, precision is implied by significant figures.
* At 25° C; this yield is very temperature dependent.

a. W. R. Dawson and M. W. Windsor, *J. Phys. Chem.*, **72**, 3251 (1968).
b. E. P. Kirby and R. F. Steiner, *Ibid.*, **74**, 4480 (1970).
c. I. B. Berlman, "Handbook of Fluorescence Spectra of Aromatic Molecules", Academic Press: London, 1965.
d. R. Rusakowicz and A. C. Testa, *J. Phys. Chem.*, **72**, 2680 (1968).
e. W. H. Melhuish, *Ibid.*, **65**, 229 (1961).
f. S. Hamai and F. Hirayama, *Ibid.*, **87**, 83 (1983).
g. S. R. Meech and D. Phillips, *J. Photochem.*, **23**, 193 (1983).
h. T. Karstens and K. Kobs, *J. Phys. Chem.*, **84**, 1871 (1980).
i. D. Magde, J. H. Brannon, T. L. Cramers and J. Olmsted, III, *Ibid.*, **83**, 696 (1979).

organic dye 1,3-*bis*-(*p*-dimethylaminophenyl)squaraine emits with high yield (ϕ = 0.65 in CH_2Cl_2) in the range 650-700 nm, but the bandshape varies with temperature and is sensitive to the presence of complexing impurities, *e.g.* alcohols [18].

C. Standards in matrices or low temperature glasses

Very few standards are available for use at low temperature. Great care must be taken to avoid scattering losses and other optical inhomogeneities caused by cracking of the glass. Polarization effects may also be severe; see Miller [7]. Dilute solutions ($<10^{-5}$M) of 9,10-diphenylanthracene in degassed methylcyclohexane glass at 77 K have fluorescence yield of 0.90-1.0. Accuracy greater than ±15% cannot be expected. An alternative method which may have wide applicability, is to use optically dense (>2), thin (~3 mm) samples and front surface detection. 9,10-Diphenylanthracene and 2,5-diphenyl-1,3,4-oxadiazole were observed to have equal emission yields by this method and a value of ϕ = 1 was assumed for these compounds. They were then employed in this way to determine the fluorescence yield of naphthalene in a nitrogen matrix (0.14) at 10 K [19].

D. Standards for low intensity emitters

No good standards exist. The recommended method is to choose a known standard in the wavelength region of interest (see Section II. B) and prepare a series of solutions containing an effective fluorescence quencher (e.g. dibromomethane). Using fluorescence lifetimes techniques, measure the lifetime diminution and calculate an operational emission quantum yield. Quenching ratios between the intensity of emission without and with quencher (I_0/I) or between the lifetime of emitting species without and with quencher (τ_0/τ) should not exceed 10.

IV. FLUORESCENCE LIFETIMES

Fluorescence lifetimes can be determined using phase shift techniques or by direct observation of emission intensity decay following pulse excitation. Each method has merit. Discussion of each technique can be found in Lakowicz [3], and O'Connor and Phillips [6], respectively.

Intensity decay profiles are observed following excitation by sources such as nitrogen or hydrogen (or deuterium) flashlamps, and pulsed laser sources. Close attention must be paid to instrumental response limits if direct decay profiles are used to derive lifetimes. The use of single-photon counting electronics and mathematical deconvolution represent the best method for accurate work.

With phase fluorimetric techniques, the presence of multicomponent decay can be revealed, even when observing at only one modulation frequency, by comparing the measured lifetime from the phase shift to that obtained from the modulation ratio. Agreement of the two values is evidence for a single lifetime.

Solution standards must be stipulated according to concentration, purity, solvent used and the presence (and concentration) of any known quenchers (e.g. oxygen). Suitable standards recommended to check an apparatus for use in various ranges of fluorescence decay time and emission wavelength are given in Table II.

TABLE II. Recommended Fluorescence Lifetime References†

Wavelength (nm) exc.	em.	Compound	Lifetime	Solvent	Ref.
295	580	Erythrosin	66 ps± 8	Water	a
295	580	Rose bengal	800 ps ± 30 550 ps ± 20	Ethanol MeOH	a a
310	370	2,5-Diphenyl-oxazole	1.27 ns	Cyclohexane	b
580	600	Rhodamine B	2.85 ns	Ethanol	b
295	460	Coumarin 450	4.3 ns ±0.2	Ethanol	a
365	>400	Anthracene	5.24 ns 5.1 ns	Cyclohexane Ethanol	b b
365	>400	9,10-Diphenyl-anthracene	7.7 ns	Cyclohexane	b
310	370	N-Methylcarbazole	18.3 ns 16.0 ns	Cyclohexane Ethanol	c c
280	325	1-Cyanonaphthalene	18.2 ns	Hexane	b
280	320	2-Methylnaphthalene	59 ns	Cyclohexane	c
280	320	Naphthalene	100 ns	Cyclohexane	c, e
310	390	Pyrene ($<10^{-5}$M)	410 ns	Ethanol	d, e

† Errors quoted are those provided by the authors; see the individual references for their significance. Where no error is given, precision is implied by significant figures.

a. H. E. Zimmerman, J. H. Penn and C. W. Carpenter, *Proc. Nat. Acad. Sci. USA*, **79**, 2128 (1982).
b. R. A. Lampert, L. A. Chewter, D. Phillips, D. V. O'Connor, A. J. Roberts and S. R. Meech, *Anal. Chem.*, **55**, 68 (1983).
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e. Long lifetimes are extremely sensitive to the environment; extreme caution should be used to determine such lifetimes.

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