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TECHNICAL REPORT

Correction of Fluorescence Excitation Spectra.

Introduction

At the start of a recent research project in our group one of the objectives was the determination of the fluorescence excitation spectrum of C₇₀ over a broad wavelength range¹ to conclusively attribute the previously reported fluorescence spectra² to this chromophore. After overcoming trivial problems concerning the spectrometer configuration, solvent choice and concentration range the routinely "corrected" excitation spectra obtained on our SPEX Fluorolog 2 instrument still presented an interpretational problem. It appeared that visible excitation of C₇₀ produced fluorescence more efficiently than ultra violet excitation, as evidenced by differences in the peak intensity ratios in the absorption and fluorescence excitation spectra (at 340, 360, 380 and 470 nm). In fact wavelength dependent photophysical behaviour has been found for both C₆₀ and C₇₀ by other investigators^{3, 4, 5, 6} using different instrumentation and has been interpreted to indicate incomplete internal conversion from higher excited S_n states to the fluorescent S₁ state.

Upon further scrutiny however, we found that not a wavelength dependent photophysical behaviour of C₇₀, but incomplete correction for variation of the excitation photon flux with wavelength caused the apparent discrepancy between excitation and absorption spectra. (Recently it has been shown that C₆₀ also has a wavelength independent fluorescence quantum yield)^{7,8}. Thus, while "corrected" excitation spectra are on most commercial instruments now routinely obtained by ratioing the intensity of the emission signal to that of a quantum counter monitoring the excitation beam, additional correction is in general needed especially if the excitation spectrum has to be recorded over a broad wavelength range. This technical note gives a description of how these correction factors were obtained. Although manuals for spectrofluorimeters normally describe a procedure⁹ to obtain such correction factors, an exemplified description seemed appropriate here because of the rather exceptionally large excitation range involved and the consequently rather dramatic effects of the additional correction, the omission of which seems to be a recurring source

of poorly corrected excitation spectra inevitably leading to misinterpretation of the underlying mechanisms.

To clarify the problem a basic description of a steady state fluorimeter is useful. A typical lay-out⁹ of a modern spectrofluorimeter is given in fig. 1. In essence, the light from a lamp is dispersed by the excitation monochromator (M1) and directed to the sample (S) and partly to the reference quantum counter (which produces a signal R, proportional to the quantumflux impinging on it) by using a (quartz) beam splitter. The light directed to the sample is absorbed in part and emission is detected via M2 by a detector providing the emission signal F.

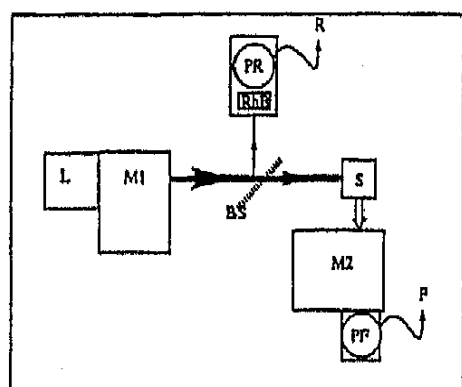


Fig. 1. Representation of a fluorimeter.
L : lamp; *M1*: excitation monochromator;
BS: beamsplitter; *RhB*: quantum-counter dye cell; *PR*: reference photomultiplier;
R: quantum-counter reference output;
S: sample; *M2*: emission monochromator;
PF: emission photomultiplier; *F*: sample emission signal.
 Note the difference in optical path from the lamp to quantumcounter *RhB* and to the sample (*S*).

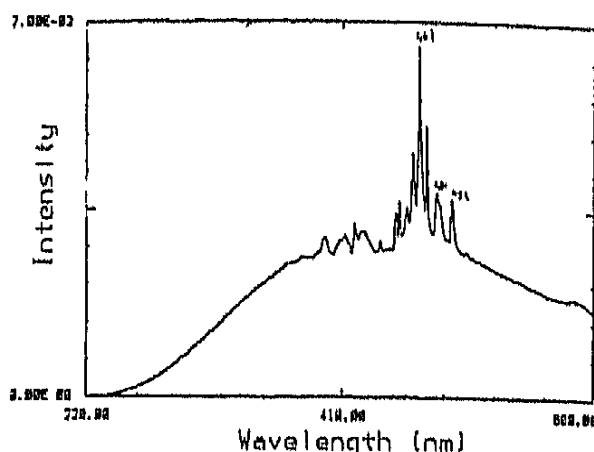


Fig. 2. The spectrum of a Xenon lamp, i.e. the signal *R* from the quantumcounter.

In principle the excitation intensity is proportional to the lamp spectrum multiplied by the transmission of *M1* and varies strongly as a function of wavelength (see Fig. 2). The most generally used quantum counter consists of a concentrated solution of Rhodamine B in 1,2-

propanediol (8 mg/ml), that over a wide range of wavelengths absorbs all light derived from the beam splitter, and converts it with a constant quantum yield to a fluorescence signal of which the spectral distribution is independent of the excitation wavelength and is detected by a reference photomultiplier or photodiode (output signal *R*). If carefully designed the signal *R* is thus proportional to the photon flux impinging on the quantum counter over the wavelength range in which the dye absorbs all light (~ 250-590nm for Rhodamine B). Thus in this range $R_{\lambda} \sim I_{\lambda}$ where I_{λ} stands for the photon intensity of the light impinging on the quantum counter and the subscript λ

refers to its wavelength.

At the other hand the main part of the excitation light is directed by the beam splitter to the sample solution, S, where it is converted into fluorescence detected as signal F (i.e. if M2 is set to a wavelength within the emission band of S and M1 is set to a wavelength within the absorption band). Neglecting reabsorption the fluorescence is proportional to the number of quanta absorbed and the quantum yield (Φ_f) i.e.:

$$F \sim I_\lambda (1 - 10^{-A_\lambda}) \Phi_f \quad (1)$$

In eqn (1) A_λ represents the absorbance of the sample.

If care is taken to work at sufficient dilution so that $A_\lambda \leq 0.1$ this can be simplified to:

$$F \sim I_\lambda A_\lambda \Phi_f \quad (1a)$$

Ideally the ratio F/R_λ then should be proportional to $A_\lambda \Phi_f$ and thus a plot of F/R_λ as a function of λ (the "corrected" excitation spectrum) should reproduce the absorption spectrum unless Φ_f is wavelength dependent. In practice however, an extra correction is needed on most instruments for the difference in optical path between the sample and the quantumcounter. A rather accurate and quite convenient measure for this effect can be obtained by measuring two identical concentrated solutions of Rhodamine B in 1,2-propanediol in quantumcounter and sample positions and record F/R_λ by scanning M1 with M2 set in the fluorescence band (~ 630 nm). In absence of an optical path difference F/R_λ should then be constant, in practice however it varies significantly with the excitation wavelength as demonstrated below.

Procedure for obtaining the additional correction factors

A SPEX Fluorolog 2 instrument in 211 configuration was used. A 10 ml solution of Rhodamine B in 1,2-propanediol (8 mg/ml) was divided over two quartz fluorescence cuvettes. These were placed in sample and quantum-counter position. The emission monochromator (M2) was set at 630 nm, a cut off filter (500 nm) was placed in the emission path to suppress the second order effect expected to arise upon 315 nm excitation and right angle observation was chosen for the sample position. Appropriate slits and photomultiplier voltages were chosen to optimize both R and F signals without overranging. The latter can be checked by putting the excitation monochromator (M1) at 470 nm (highest excitation intensity, see Fig. 2). On our instrument the following slits were used: 0.5

mm (4.5 nm), excitation path; 1.25 mm (4.5 nm), emission path. The F/R_λ values were then measured between 240 to 590 nm. This range is limited at short wavelengths because the lamp emission becomes too small, and at the high wavelength side because Rhodamine B does not absorb sufficiently beyond 590 nm. However, quantum counter dyes do exist that absorb at longer wavelengths (for instance HITC)¹⁰.

The resulting excitation "spectrum" should be of the form depicted in Fig. 3. Inverting the spectrum (R_λ/F) and normalising it by dividing it by the minimum value (in our case around 540 nm) results in the correction factors plotted in Fig. 4 and these are to be applied to a routinely corrected excitation spectrum (F/R) in order to obtain a "fully corrected" excitation spectrum (i.e. corrected for the optical path difference between sample and quantumcounter).

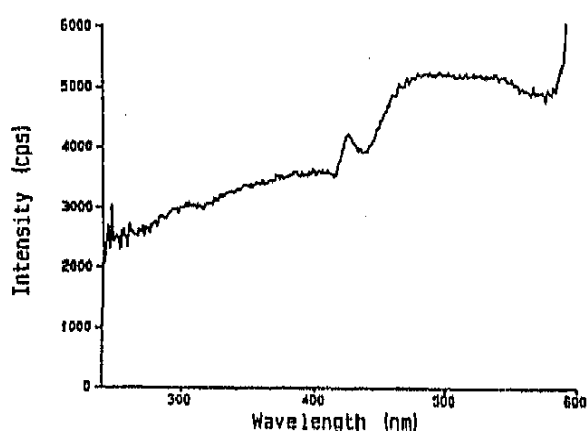


Fig. 3. Ratio of R and F signals obtained by placing two identical solutions of Rhodamine B in 1,2-propanediol at the quantum-counter and sample (right angle) positions

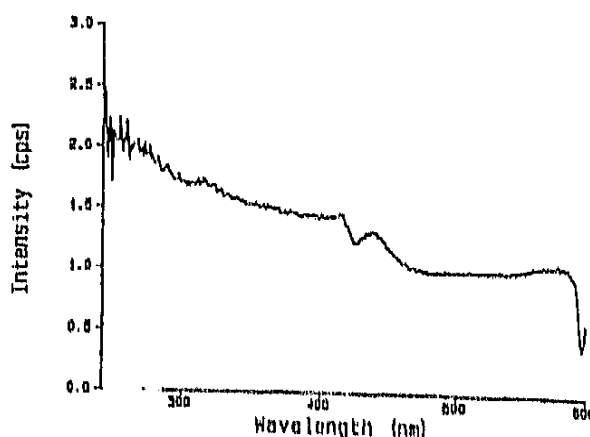


Fig. 4. Correction factors, obtained by inversion and normalisation of the "spectrum" in Fig. 3.

The importance of full excitation correction exemplified

The additional correction factors derived from Fig. 4 are minor as compared to the changes in excitation intensity with wavelength as derived from Fig. 2, thus indicating that the F/R_λ ratioing alone already provides a major correction. Nevertheless neglect of the additional correction factors may lead to severe problems especially if excitation scans are made over a broad spectral region¹¹.

This is exemplified by the fluorescence excitation spectra of C₇₀ with and without the application of the additional correction factors and comparison with the absorption spectrum as given in Fig. 5. As can be seen from Figs 4 and 5 the influence of the correction factors is most prominent on the ratios of peaks in the UV versus peaks in the VIS.

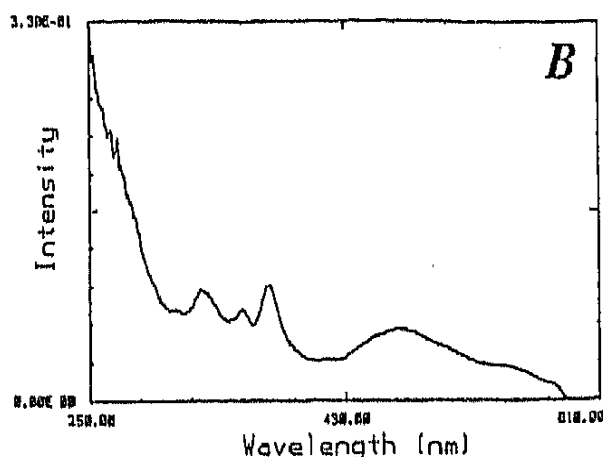
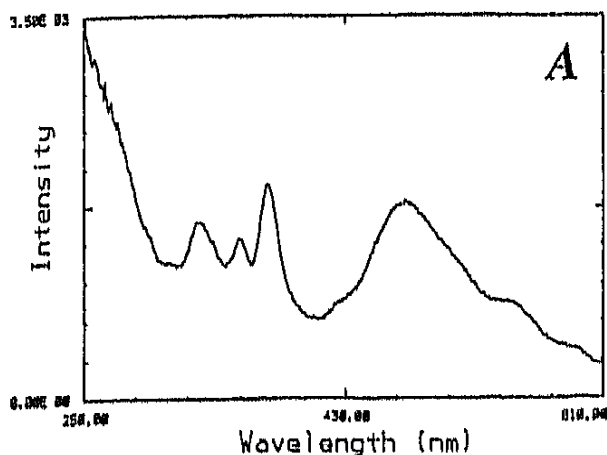
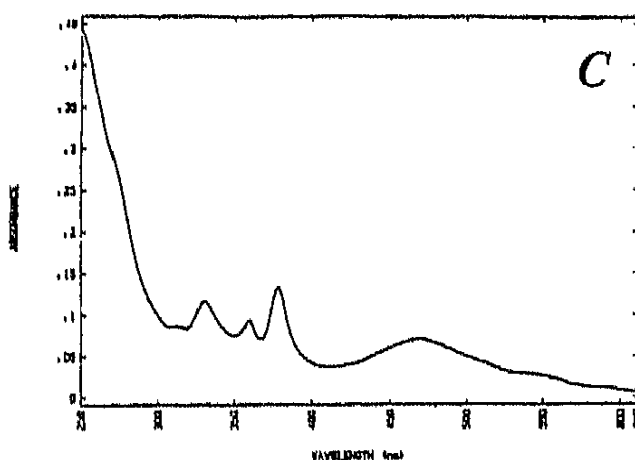


Fig. 5. Partly corrected (F/R_λ) (A) and fully corrected (B) fluorescence excitation spectra of C₇₀ together with an absorption spectrum (C) in n-hexane.



Conclusions

If a correct representation of fluorescence quantum efficiencies as a function of excitation wavelength is to be obtained, not only a correction for the excitation (lamp) intensity is needed but also a correction for the difference in optical path between sample and quantum counter.

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- 11 A rapid test for the reliability of the correction factors can be obtained by measuring the excitation spectrum of a solution of anthracene in cyclohexane (ca. $5 \cdot 10^{-7}$ M) and comparison of the results with the absorption spectrum. Here especially the ratio of the 252 and 375 nm peaks is indicative (ratio of ca. 25.88).

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