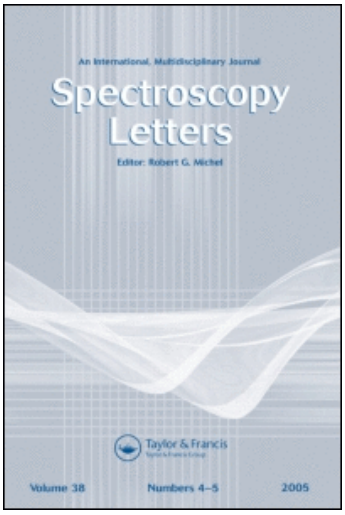

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

In Situ Measurement of Excitation Intensity for Fluorescence Excitation Spectroscopy

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To cite this Article Naqvi, K. Razi(1998) '*In Situ* Measurement of Excitation Intensity for Fluorescence Excitation Spectroscopy', Spectroscopy Letters, 31: 1, 147 — 155
To link to this Article: DOI: 10.1080/00387019808006768
URL: http://dx.doi.org/10.1080/00387019808006768

SPECTROSCOPY LETTERS, 31(1), 147-155 (1998)

IN SITU MEASUREMENT OF EXCITATION INTENSITY FOR FLUORESCENCE EXCITATION SPECTROSCOPY

Key words: Fluorescence spectroscopy; Fluorescence excitation spectroscopy, Spectral correction method, Photosynthetic specimens.

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Abstract. It is argued that the double-beam method for the automatic correction of fluorescence excitation spectra, which proved to be a great boon in the days of analog instrumentation, has become, after the advent of digital techniques for acquiring and manipulating spectral data, more of a hindrance than a help; the benefits of reverting to a single beam device and making *in situ* measurement of the excitation intensity are pointed out.

Introduction

The fluorescence excitation spectrum[1-3] of a sample must be corrected, if it is to be compared with the corresponding spectrum recorded on a different instrument, by dividing $F(\lambda)$, the output of the detector used for monitoring the fluorescence emitted by the sample within a given solid angle, by a reference signal whose amplitude is proportional to $E(\lambda)$ or $I_0(\lambda) \equiv E(\lambda)\lambda$, the energy or the internist (respectively) of the beam used for exciting the specimen; here λ denotes the wavelength of the exciting light. In most instruments $F(\lambda)$ and $E(\lambda)$ or $I_0(\lambda)$ are recorded in parallel by employing a dual-beam arrangement[1-5] which serves to share the excitation beam spatially or temporally between the sample and a reference detector, a device whose output is proportional to $E(\lambda)$ or $I_0(\lambda)$.

It is easy to see why dual beam arrangements for recording various spectra (absorption spectra, fluorescence excitation spectra, photoacoustic spectra[6]) were adopted in the era of analog instrumentation, when the calculations of ratios and logarithms were carried out with the help of operational amplifiers, and spectra were automatically plotted on a chart recorder or an *X-Y* recorder; a single-beam arrangement, entailing point-by-point division of two spectra (each of which was automatically plotted) and subsequent manual replotting of the ratio, would have amounted to throwing away the chief benefit of automatic recording. "The correction of the excitation spectrum can be tedious," Parker commented in 1961, "particularly if there are several maxima, and the value of the method in analytical chemistry would be greatly increased if the spectrofluorimeter could be made to record directly the true excitation spectrum". One can also see why an energy detector, such as a thermopile or a Golay detector, was considered, until the introduction of a compact function generator based on

a tapped helical potentiometer,[7] an impediment to the automatic recording of true excitation spectra. To cite Parker[1] once again: "To obtain the true excitation spectrum the intensity of the exciting light, I_0, must be determined as a function of frequency. This may be done by means of a sensitive thermopile ... or a fluorescence screen of constant quantum efficiency. The thermopile reading gives the relative intensities in energy units (E), and each reading must be divided by the frequency (or multiplied by the wavelength) to give relative intensities in terms of quanta. ... With the fluorescent screen the results obtained are proportional to quantum intensity and require no further correction". I will adhere to current usage and refer, in what follows, to a $X(\lambda)$-*versus*-λ plot as a corrected excitation spectrum (where $X(\lambda) \equiv NF(\lambda)/I_0(\lambda)$, and N is a multiplicative constant), and call a combination of a fluorescent screen and a photomultiplier, which are usually placed in tandem, a quantum counter (QC, for short).

Since the output of modern instruments is stored digitally, and mathematical operations involving large data sets no longer daunt the experimenter, the time has come to reassess the pros and cons associated with a QC and with parallel recording of $F(\lambda)$ and the reference signal. Other considerations (such as the spectral range over which the correction is to be applied, convenience, cost, etc.), which have so far been considered subordinate to automatic ratioing, should now dictate the choice between a QC and an energy detector. The relative merits of parallel or serial recording have already been weighed in closely related contexts,[8,9] and all in all the serial option has been adjudged to be the better; the same reasoning is applicable to a fluorescence excitation spectrometer.

If the excitation beam is directed to the reference detector by means of a beam-splitter (a quartz plate), the dependence of the reflectivity of the

plate on the wavelength and the state of polarization of the excitation beam constitutes a large source of error.[5] Though it is possible to compensate for the selective reflectivity of the beamsplitter by placing a photodiode at the sample position and at the position of the reference detector and thereby correcting the output of the reference detector,[5] or to eliminate it altogether by using an instrument in which the excitation beam is time-shared between the sample and the reference detector through the use of a rotating chopper mirror,[4] one would be well advised, if one is constructing a new instrument, to abandon the dual-beam operation. If a split-beam spectrofluorimeter is already available, one should consider reverting to single beam operation by discarding the beamsplitter, measuring $F(\lambda)$, then replacing the cuvette holder with the reference detector, and measuring *in situ* $I_0(\lambda)$ or $E(\lambda)$; further data handling can then be consigned to a computer.

Materials and Methods

The measurements reported here were done by using a commercial spectrofluorimeter (Spex Fluorolog 2), which uses double monochromators both for isolating the excitation bandpass and for analyzing the fluorescence emitted by the sample; since this instrument has been described in considerable detail in a recent article[5] dealing with the correction of fluorescence spectra, it will be sufficient to mention here that it uses a beamsplitter and a quantum counter, and the software supplied with the instrument provides the two signals, $F(\lambda)$ and $I_0(\lambda)$, as well as their ratio $X(\lambda)$. Since the instrument is used almost exclusively for investigating photosynthetic pigment, a 250 W quartz-halogen lamp was substituted for the light source (a 450 W Xe arc) originally supplied with instrument. The fluorescence signal was sampled every 0.5 nm, and the excitation bandpass was 1.8 nm. Absorption spectra were acquired by means of a commercial

instrument (Shimadzu Model UV-160A), using a spectral bandpass of 2 nm and a sampling interval of 0.5 nm.

The energy detector used in this work is a commercial unit (Oriel Corporation, Model 7500 Golay Detector); though marketed as a detector of infrared radiation, we have found that it response maintains nonselective down to 430 nm. The chopper mirror supplied with the instrument was replaced by a slotted disc chopper (EG&G Model 9479), which was used to modulate the exciting light at 10 Hz with a 50% duty cycle; the output of the detector was fed to a lock-in amplifier (EG&G Ortholoc-SC 9505), whose output, to be identified with $E(\lambda)$, was digitized and stored in a personal computer. Subsequent data handling was done through a commercial spreadsheet program.

The fluorescent sample whose excitation spectrum is reported here was kindly supplied by C. Büchel, who has described details of the isolation procedure and the pigment composition elsewhere.[10] Since the absorption spectrum of a suspension of thylakoids, obtained by placing the sample cell in the cuvette holder of a conventional spectrophotometer and scanning the instrument, suffers from distortions caused by scattering losses,[11] a strategy devised by Latimer and Eubanks[12] (which does not require the use of an integrating sphere, the traditional device for recording the absorption spectra of turbid samples) was employed; an appraisal of their approach and other methods for finding the true absorption spectrum of a scattering-and-absorbing specimen has been presented in two recent articles.[13,14]

Results and Discussion

The quality of the correction for the spectral variation of the intensity of the exciting light is usually judged by comparing $A(\lambda)$, the absorption spectrum of the sample, with its corrected fluorescence excitation spectrum

$X(\lambda)$, normalized so as to bring the two spectra into coincidence at an arbitrarily chosen wavelength.[1-3] For such a comparison to be meaningful, one must ensure that the emission quantum yield, ϕ, as well as the emission anisotropy, r, is independent of λ, and that the excitation spectrum is recorded by using an optically thin sample. The restriction about the wavelength-independence of r can be easily overcome by measuring a signal, S, which is proportional to the total fluorescence emitted by the sample in all directions. If the sample is isotropic (in the absence of illumination), S is measured most easily by inserting an appropriately oriented polarizer in the path of the emitted beam;[15] if, on the other hand, the unirradiated sample happens to be anisotropic, it becomes necessary to measure polarized fluorescence in three mutually perpendicular directions, or to surround the sample by an integrating sphere.[16] When a comparison between $A(\lambda)$ and $X(\lambda)$ was carried out, using dilute isotropic solutions (in 95% ethanol) of chlorophyll *a* and chlorophyll *b*, excellent agreement was observed between the absorption spectrum of the solute and its corrected excitation spectrum in the region where the response of the detector is flat ($\lambda \geq 430$ nm).

The main results of this investigation are summarized in Fig. 1, which displays the following plots: (*i*) $E(\lambda)$, the energy distribution of the exciting light, (*ii*) $F(\lambda)$, the uncorrected fluorescence excitation spectrum, (*iii*) $A(\lambda)$, the scattering-compensated absorption spectrum of the sample, and (*iv*) $X(\lambda)$. The close fit between $A(\lambda)$ and $X(\lambda)$ is sufficient to vouch for the reliability of the correction factor found by using the Golay cell.

Concluding Remarks

The adoption of a single-beam spectroscopic system in preference to a double-beam version might at first sight seem to be a regrade suggestion.

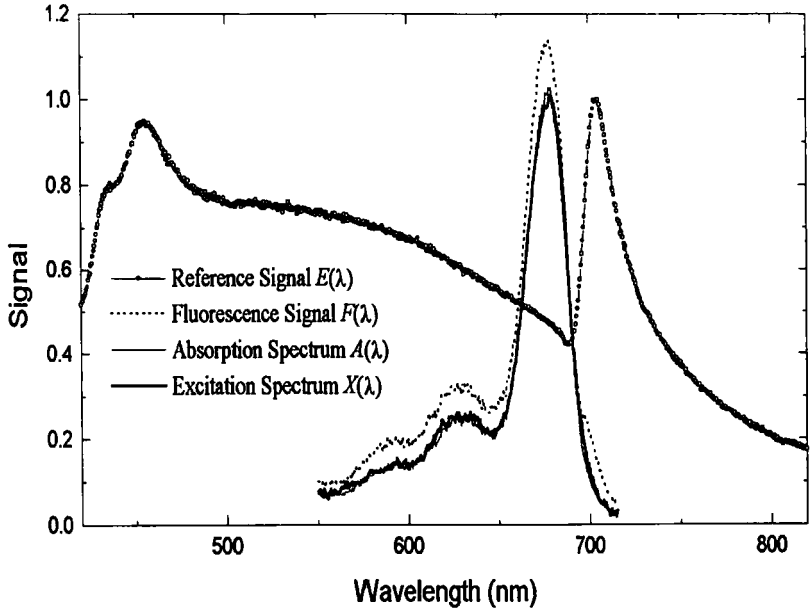

FIG. 1. Comparison of the scattering-compensated absorption spectrum, A(λ), and the corrected excitation spectrum, $X(\lambda)$; $F(\lambda)$ is the apparent excitation spectrum (the intensity of fluorescence emitted at 720 nm plotted as a function of λ), and $E(\lambda)$ is the energy distribution of the exciting light measured with a Golay cell.

However, the results presented above demonstrate the convenience of the single-beam approach to the correction of fluorescence excitation spectra; clearly, analogous techniques, such as photoacoustic spectroscopy,[6] can also benefit from this approach. Though the particular detector used in this study cannot be used for wavelengths shorter than 430 nm, the use of an appropriate QC[5] or a pyroelectric detector[4,6] would extend the short-wavelength limit down to 200 nm. If the excitation source happens to be unstable, and one does not want to sacrifice the stability provided by a

ratioing system,[3] one can use a quartz plate to split a small fraction of the *undispersed* beam and use it for compensating for fluctuations in the intensity of the lamp.[9]

REFERENCES

1. Parker, C. A., Rees, W. T. Correction of Fluorescence Spectra and Measurement of Fluorescence Quantum Efficiency. Analyst 1960; 85: 587.

2. Parker, C. A. Fluorescence Excitation Spectra and Fluorescence Sensitivity. Photoelectric Spectrometry Group Bulletin 1961; No. 13: 334.

3. Parker, C. A. *Photoluminescence of Solutions*. Amsterdam, Elsevier, 1968: 249-251.

4. Landa, I., Kremen, J. C., Corrected and Automated Spectrophotofluorimeter Employing a Pyroelectric Detector for Correction. Anal. Chem. 1974; 46: 1694.

5. Hofstraat, J. W., Latuhihin, M. J. Correction of Fluorescence Spectra. Appl. Spectrosc. 1994; 48: 436.

6. Murphy, J. C., and L. C. Aamodt, L. C. The Photothermophone, a Device for Absolute Calibration of Photoacoustic Spectrometers, Appl. Phys. Lett. 1977; 31: 728.

7. Hamilton, T. D. S. Absolute Recording Spectrofluorimeter. J. Sci. Instrum. 1966; 43: 49.

8. Kollias, N., Baqer, A., Razi Naqvi, K. Fiber Optic Spectrophotometer for Noninvasive Transmission and Diffuse Reflection Studies, Spectrosc. Lett.1986; 19: 149.

9. Bishop, J. An Innovative UV-VIS Spectrophotometer Design, International Laboratory 1989; 19(4): 58 .

10. Büchel, C., Wilhelm, C. Isolations and Characterization of a Photosystem I-associated antenna (LHC I) and a photosystem I-core Complex from the Chlorophyll c-Containing Alga *Pleurochloris meiringensis*, J. Photochem. Photobiol. 1993; 20: 87.

11. Shreve, A. P., Trautman, J. K., Owens, T. G., Albrecht, A. C. A Femtosecond Study of Electronic State Dynamics of Fucoxanthin and Implications for Photosynthetic Carotenoid-to-Chlorophyll Energy Transfer Mechanisms. Chem. Phys. Lett. 1991; 154: 171.

12. Latimer, P., Eubanks, C. A. H. Absorption Spectrophotometry of Turbid Suspensions: A Method of Correcting for Large Systematic Distortions. Arch. Biochem. Biophys. 1962; 98: 274.

13.Razi Naqvi, K., Melø, T. B., Bangr Raju, B., Javorfi, T., Garab, G. Comparison of the Absorption Spectra of Trimers and Aggregates of Chlorophyll *a/b* Light-Harvesting Complex LHC II. Spectrochim. Acta A (in press).

14.Razi Naqvi, K. Measuring (without an Integrating Sphere) the Absorption Spectrum and the Selective Scattering Spectrum of a Turbid Suspension. (submitted for publication).

15.Poole, J. A., Findeisen, A. Polarization Effects in the Measurement of Luminescence Yields. J. Chem. Phys. 1977; 67: 5338.

16.Johansson, L. B.-Å., Lindblom, G., Razi Naqvi, K. Fluorescence Detected Linear Dichroism: A New Method for Studies of Molecular Orientation in Uniaxial Systems. J. Chem. Phys. 1981; 74: 3774.

Date Received: June 12, 1997
Date Accepted: August 1,1997