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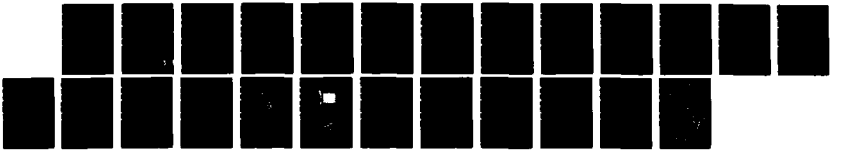

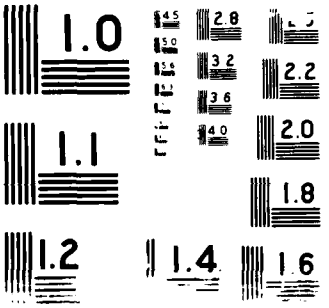
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The fluorescence spectra and intensity of anthracene in ethanol standards at concentrations ranging from 10^{-4} to 10^{-11} M are shown. The limit of detection measured is 10^{-12} M, or 227 parts-per-quadrillion. The linear dynamic range obtained with this instrument is 6 orders of magnitude with excellent linearity using 10 second signal integration time.

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Molecular Fluorescence Measurements With a Charge-Coupled Device Detector

by

P.M. Epperson, R.D. Jalkian, and M.B. Denton

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MOLECULAR FLUORESCENCE MEASUREMENTS WITH A CHARGE-COUPLED DEVICE DETECTOR

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BRIEF

The performance of a two-dimensional charge-coupled device detector based fluorescence spectrometer is demonstrated with anthracene as the fluorescence standard.

ABSTRACT

A novel fluorescence spectrometer is described using a two-dimensional charge-coupled device as the detector and a mercury pen lamp as the excitation source.

The fluorescence spectra and intensity of anthracene in ethanol standards at concentrations ranging from 10^{-4} to 10^{-11}M are shown. The limit of detection measured is 10^{-12}M, or 227 parts-per-quadrillion. The linear dynamic range obtained with this instrument is 6 orders of magnitude with excellent linearity using 10 second signal integration time.

There is a continuing demand for improved sensitivity and dynamic range of fluorescence spectrometers used for quantitative analysis of trace organic compounds. The sensitivity of a fluorescence instrument depends on the excitation source intensity and stability, the emission collection efficiency, the background fluorescence of the cuvettes and solvent blank, and the detector sensitivity. In the last decade, the majority of work aimed at increasing sensitivity has focussed on the development of more intense excitation sources. The most significant results were achieved by the use of laser excitation sources, often tunable dye lasers (1-3). However, the current cost and complexity of tunable dye laser systems make them impractical for use in most routine analytical instrumentation.

The development of sensitive detectors for fluorescence spectroscopy is an alternate method of increasing sensitivity that emphasizes the more efficient utilization of the available fluorescence emission from a sample. In principle, increasing the detector sensitivity is generally preferable to increasing source intensity, as the latter involves a larger perturbation to the sample with the possibility of problems due to photodecomposition and sample heating. Efforts at improving sensitivity by the use of new detectors has not been as successful as the work involving new excitation sources, due in part to the difficulty of improving upon the detector utilized for many years in conducting molecular fluorescence measurements - the photomultiplier tube. Multichannel array detectors such as vidicons and photodiode arrays offer a significant multichannel advantage over PMTs under certain conditions and are widely used for high light level absorbance spectroscopy (4-6). However, these detectors suffer from relatively high detector noise and dark current levels that limit their sensitivities when measuring low light levels.

This manuscript discusses the design and evaluation of a fluorescence spectrograph employing a charge-coupled device (CCD) detector for quantitative molecular fluorescence spectroscopy. The sensitivity and dynamic range of the instrument was evaluated by measuring solutions of anthracene in ethanol solvent at concentrations ranging between 10^{-4} to 10^{-12}M. Anthracene was chosen because it is commonly used to measure the sensitivity of fluorescence instruments allowing the results of this work to be compared against results from previous studies employing different detectors and excitation sources. Anthracene also has a distinctive fluorescence spectrum that aids in evaluating the spectral resolution of the instrument.

The emphasis of this study is on a practical method of improving the sensitivity of fluorescence measurements by making the best possible use of the fluorescence emission signal without resorting to the use of intense excitation sources. CCD detectors combine the multichannel advantages found in array detectors with the sensitivity normally associated with single-channel, photon-counting PMTs (7,8). Additionally, the CCD's ability to combine photogenerated charge packets from adjacent detector elements via a process known as "binning" allows the performance of the detector to be tailored to the needs of the spectroscopic measurement (7,9,10).

Experimental

The single beam instrument constructed for the molecular fluorescence measurements is mechanically simple and reliable. The design emphasizes the efficient use of all the spectral information available from the excitation of the analyte molecules in ethanol by simultaneously measuring the fluorescence emission as well as the elastically and Raman

scattered light from the solvent. The scattered light intensities are used to correct for source and detector drift between the sample and blank measurements.

Fluorescence spectrograph

A diagram of the fluorescence spectrograph built for these studies is shown in Figure 1. The excitation source is a mercury pen lamp (Ultra-violet Products, Inc). The 254 nm mercury line is selected by a 250 nm bandpass interference filter (Ealing Electro-optics, Inc.), that has a 12% transmittance at 254 nm. The filtered light passes into a 10 mm by 10 mm by 45 mm quartz cuvette placed approximately 10 mm from the bandpass filter. Fluorescence emission enters the spectrograph through the 250 μm by 5 mm tall slits of the 200 groove/mm, 200 mm focal length, f/3 concave holographic grating spectrograph (Model UFS-200, Instruments SA). The spectral coverage across the 320 columns of the CCD is approximately 240 nm. Two front surface mirrors are fixed to the cuvette holder on the sides opposite the excitation source and the entrance slit. The integration time is computer controlled in 100 ms intervals by an electro-mechanical shutter (#1 Synchro, Ilex Optical Co., Rochester, NY). Approximately 2 ml of solution are used in each cuvette; however, based on geometrical optics (11) the effective volume of sample from which fluorescence emission is collected is approximately 1 ml.

CCD detector system

The two-dimensional array detector employed in these studies is a RCA-SID501EX 512 by 320 element CCD with 30 x 30 μm square detector elements operated in a CH181 CCD detector system (Photometrics Ltd., Tucson, AZ).

This CCD is a backside illuminated device in which the protective glass substrate has been removed by the manufacturer to increase the quantum effiency of the detector in the 200 to 400 nm spectral region. The CCD is contained in a liquid nitrogen cooled Dewar and operated at 133 K to reduce the dark current. Photogenerated charge packets are digitized to 14 bits and the digitized images are stored and processed by a Motorola 68000 based computer (12). The CCD is operated either in a normal readout or a 200 fold parallel binned readout mode depending on the signal level. Parallel binning of charge involves the transfer and summation of photogenerated charge packets in the parallel shift direction along a column (7,10). The electro-optical properties of the RCA family of CCDs has been well characterized (11,13-17).

Reagents

Reagent grade anthracene (98% pure, Aldrich Chemical Co.) was used without further purification. 100% ethanol was redistilled once in an all glass distillation apparatus, with the first and last 100 ml of ethanol distillate was discarded. Solutions below 10^{-7}M were used within several hours of dilution from stock solutions. The solutions were not degassed Glassware was cleaned in 50% nitric acid followed by repeated rinsing with the redistilled ethanol.

Procedure

The fluorescence and scattered light is focused onto the CCD with the slit dimension perpendicular to the serial register as shown in Figure 2. The spectrum consists of the four anthracene fluorescence peaks between 370 and 460 nm, the Raman scattered peak from ethanol at

approximately 274 nm, and the elastically scattered peak at 254 nm. A spectrum from a 10^{-9}M solution is shown in Figure 3. For each exposure, the intensities from three subarrays of the CCD corresponding to the regions of the fluorescence emission, the elastically scattered light, and the Raman scattered light are recorded. The blank and sample intensities are measured 10 times at each concentration.

Photogenerated charge from each subarray is read using the normal or the binned readout modes depending on the intensity of the spectrum. Binning of charge 200 fold in the parallel direction for concentrations below 10^{-9}M is used to increase the S/N by reducing the total detector read noise contribution (7,10). Binning in the parallel direction serves to compress the intensity information in the slit dimension into a single 320 element row of the CCD. At high concentrations, the photogenerated charge is read in a normal readout mode to avoid oversaturating the charge holding capacity of the serial register. Figure 4 shows the spectra from 10^{-6} to 10^{-11}M solutions of anthracence. The spectra are normalized regarding intensity and offset for clarity. Each spectra is from a 10 second integration of the ethanol blank solution subtracted from a 10 second integration of the anthracene solution. A 17 point Savitsky-Golay smoothed fluorescence spectrum from a 100 second integration of a 10^{-11}M anthracene solution is shown in Figure 5 and illustrates the spectral quality obtainable at this trace concentration.

Results and discussion

Analytical working curve

The sensitivity at low concentrations is determined by the ability of the instrument to accurately subtract the background signal of the blank from the background and fluorescence signal of the sample. Essentially the measurement involves the subtraction of a weak background signal from an even weaker analyte signal superimposed on the background. The accurate subtraction of the background requires correcting for any source or readout system drift between the measurement of the blank and the sample. This instrument utilizes the scattered light signal at 254 nm resulting from Rayleigh, Tyndall, and stray excitation light, and the signal at 274 nm resulting from Raman scattering to correct for instrumental drift.

The three intensities recorded for every image correspond to photogenerated charge from the portions of the CCD illuminated by: the fluorescence emission from the sample, I_{fs}; the elastically scattered excitation light, I_{es}; and the Raman scattered light, I_{rs}. The intensities of the same three regions of the CCD are recorded for a 10 second exposure of the blank to obtain the signals corresponding to: the blank fluorescence, I_{fb}; blank elastically scattered, I_{eb}; and the blank Raman scattered intensities, I_{rb}. The fluorescence due to anthracene is equal to the sample fluorescence minus the blank fluorescence corrected for any drift between the two measurements. The drift correction is accomplished by normalizing the fluorescence signal by either the elastically or Raman scattered signal according to:

$$I_{\text{elastically scattered corrected}} = I_{fs}/I_{es} \cdot I_{fb}/I_{eb} \qquad \text{Eq. 1}$$

$$I_{\text{Raman scattered corrected}} = I_{fs}/I_{rs} \cdot I_{fb}/I_{br} \qquad \text{Eq. 2}$$

Although either equation 1 or 2 can be used to correct for source or detector drift at low concentrations, the best results were obtained from using the elastically scattered peak. The intensity of the elastically scattered peak was approximately 10 times the Raman scattered peak. The correction is most significant at low concentrations when the background fluorescence is equal to or greater than the fluorescence emission due to the analyte molecules. The working curve covering the concentration range of 10^{-4} to 10^{-12}M is shown in Figure 6. At concentrations above 10^{-6} the curve is no longer linear due to reabsorption of the short wavelength fluorescence and poor collection of fluorescence emission from the wall of the cuvette near the source. The linear correlation coefficient of the log-log curve in the 10^{-7} to 10^{-12}M region is 0.99993 with a slope of 1.014. It is important to note that the CCD easily measured fluorescence intensities ranging over 6 orders of magnitude without changing the exposure time.

Limit of Detection

The concentration limit of detection (LOD) at a S/N of two for a 10 s integration determined using the background correction technique described by equation 1 is 1 x 10^{-12}M (227 parts-per-quadrillion, assuming the ethanol temperature to be 25°C, then the solvent density is 0.785 g/ml). Taking into account the volume of solution effectively sampled by the entrance slit and grating, the LOD corresponds to 1 femptomole, or

approximately $6x10^8$ molecules. This LOD is approximately 340 times lower than work done employing a 60 watt deuterium lamp and a PMT operated in the photon counting mode (18) and approximately 25 times lower than work done employing a tunable dye laser excitation source and a gated PMT (2) Without employing the correction technique for source and detector electronics drift described in equations 1 and 2, the LOD is 10^{-11}M

The 10-second integrated signal due to the fluorescence emission of 10^{-12}M anthracene is 2,800 photogenerated e- superimposed on the background intensity of 260,000 photogenerated e-. The dominant source of noise at the detection limit is the uncertainty in the background intensity. The standard deviation in the background intensity from 10 measurements with 10-second integrations of the blank using the correction method described by equation 1 is approximately 1,400 e-, or 0.5% of the total intensity. Thus, the minimum detectable signal is approximately 100 times less than the associated background. It is important to note that the instrument does not achieve its sensitivity at the expense of spectral resolution as illustrated by the quality of the spectrum in Figure 5.

Conclusions

The results of this work demonstrate the improvements in sensitivity possible through the use of a low noise, integrating multichannel array detector used in conjunction with a relatively weak and inexpensive excitation source. In addition to gain in sensitivity, the instrument also acquires the complete molecular spectrum thereby increasing the qualitative information obtained with every measurement. The ability to normalize the fluorescence signal to the scattered light signal results in a direct method of correcting for source and readout detector drift that is less prone to

errors than methods employing beam splitters and auxiliary detectors. The results of this work point to the use of the CCD fluorescence spectrograph as a practical detector for use in high performance liquid chromatography and flow-injection analysis. Although the sample volume was relatively large compared to the volume encountered in typical flow cells, with the addition of a source and collection lenses, the fluorescence spectrograph could be readily adapted for use in HPLC and FIA systems.

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Credits

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Figure Captions

Figure 1. Spectrograph constructed for the molecular fluorescence measurements employing a 512 by 320 element CCD detector.

Figure 2. Orientation of anthracene spectrum of CCD. Wavelength is dispersed in the serial charge transfer direction and the slit is focused along the parallel transfer direction.

Figure 3. Spectrum from 10^{-9}M anthracene in ethanol for a 10 s integration. The anthracene fluorescence spectrum is centered about 400 nm. The elastically scattered light peak is at 254 nm and the Raman scattered light peak is at 274 nm.

Figure 4. Spectra of 10^{-6} to 10^{-11}M anthracene for a 10 s integrations. Spectra are normalized to constant intensity and offset for clarity.

Figure 5. Spectrum of 10^{-11}M (2.3 parts-per-trillion) anthracene in ethanol from a 100 s exposure. Spectrum has been smoothed by a 17 point Savitsky-Golay smoothing routine.

Figure 6. Analytical working curve of fluorescence emission intensity versus anthracene concentration from 10^{-4} to 10^{-12}M for 10 s integration.

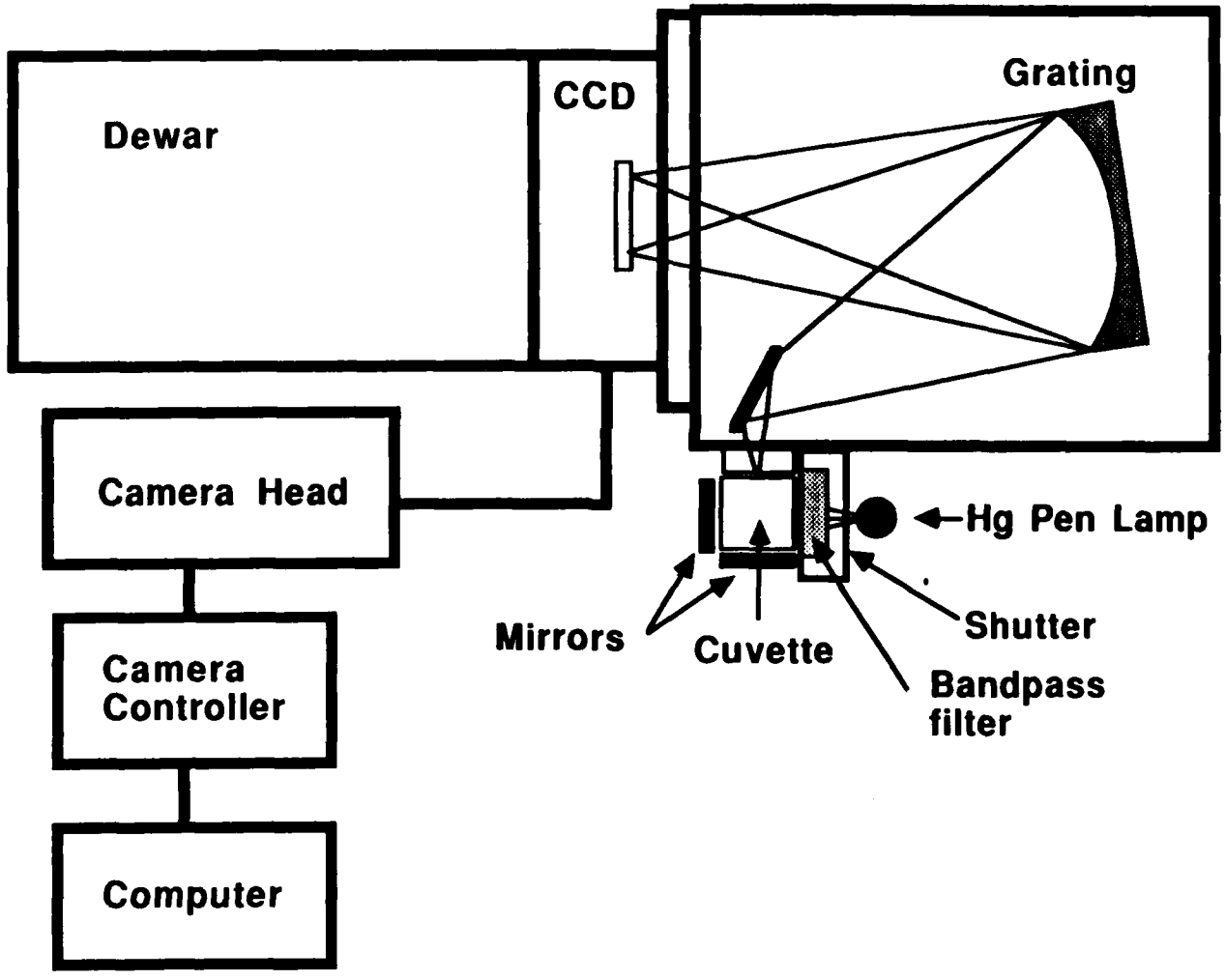
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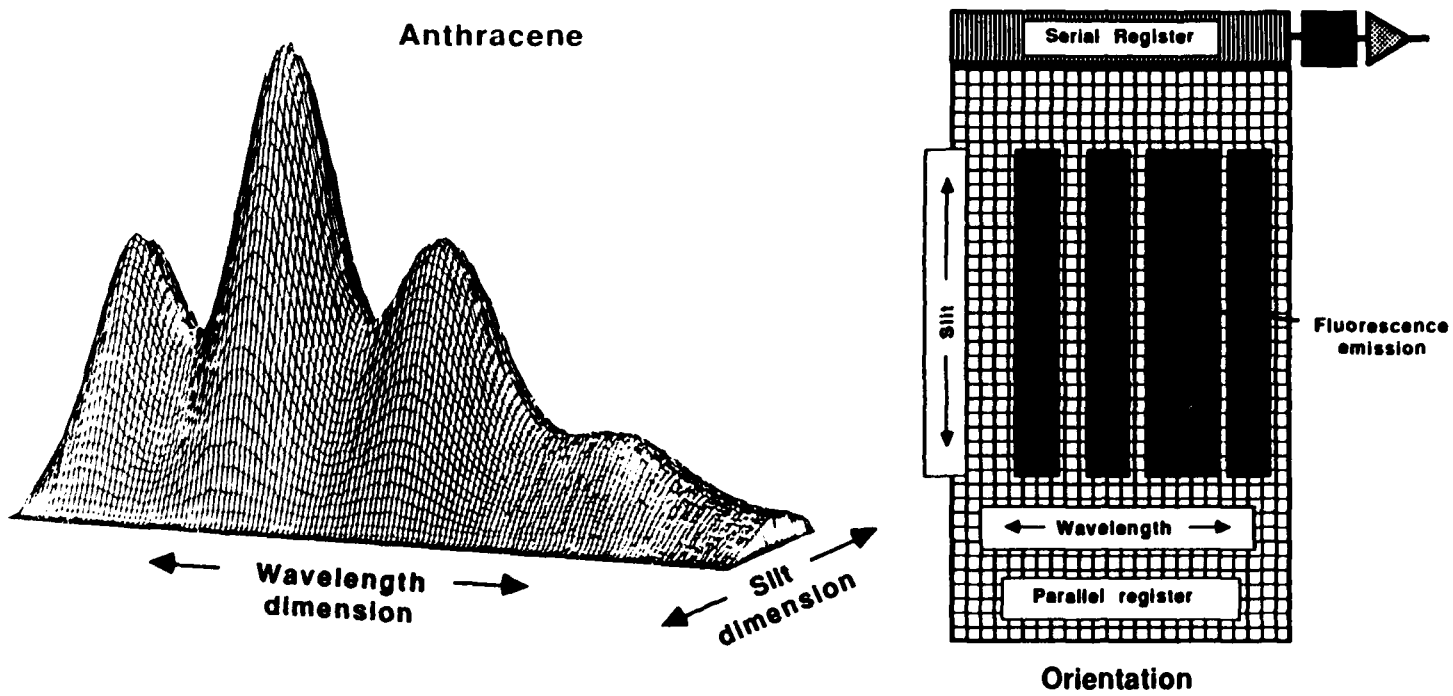
FIGURE 1

Orientation of anthracene spectrum



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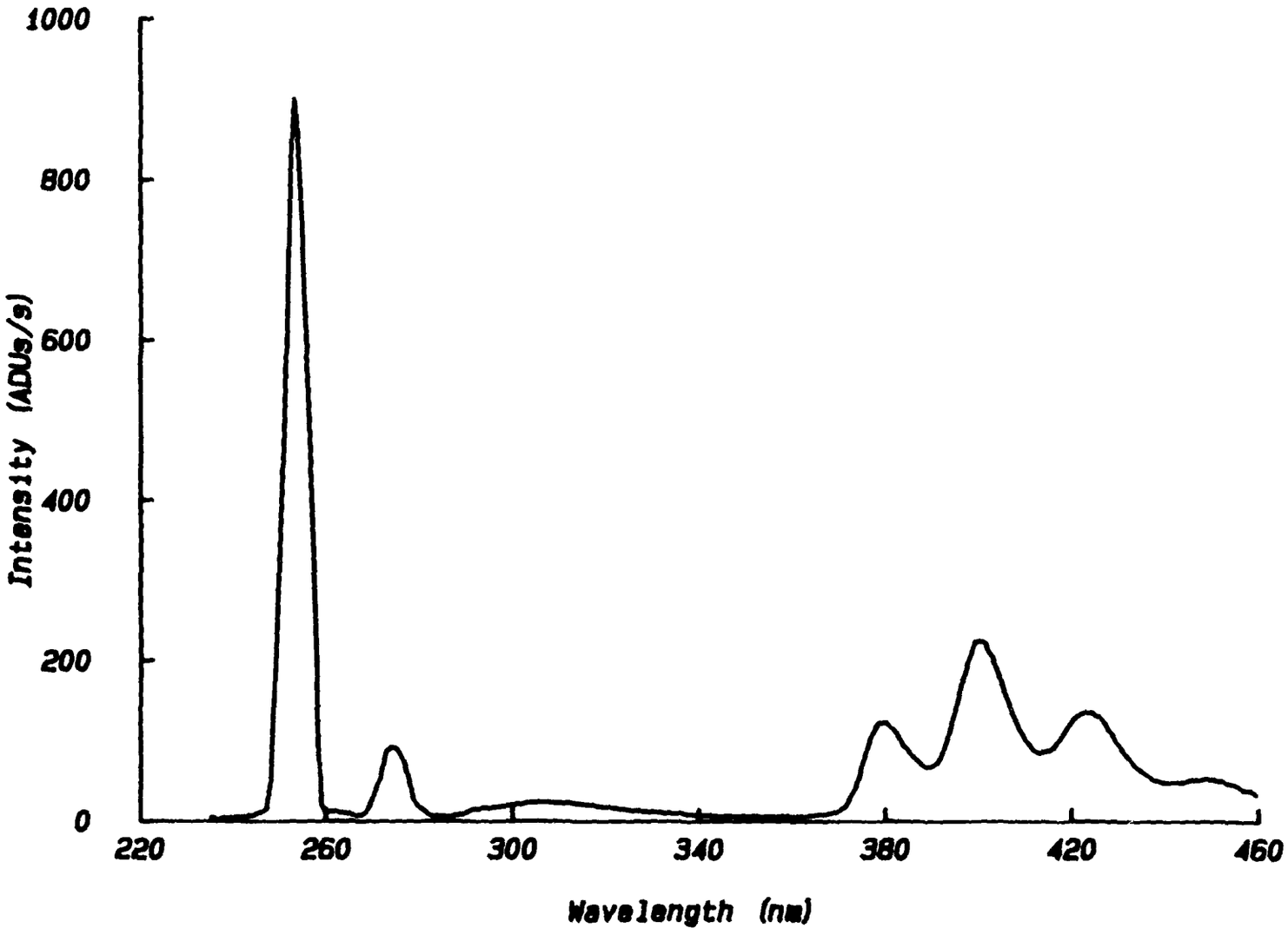
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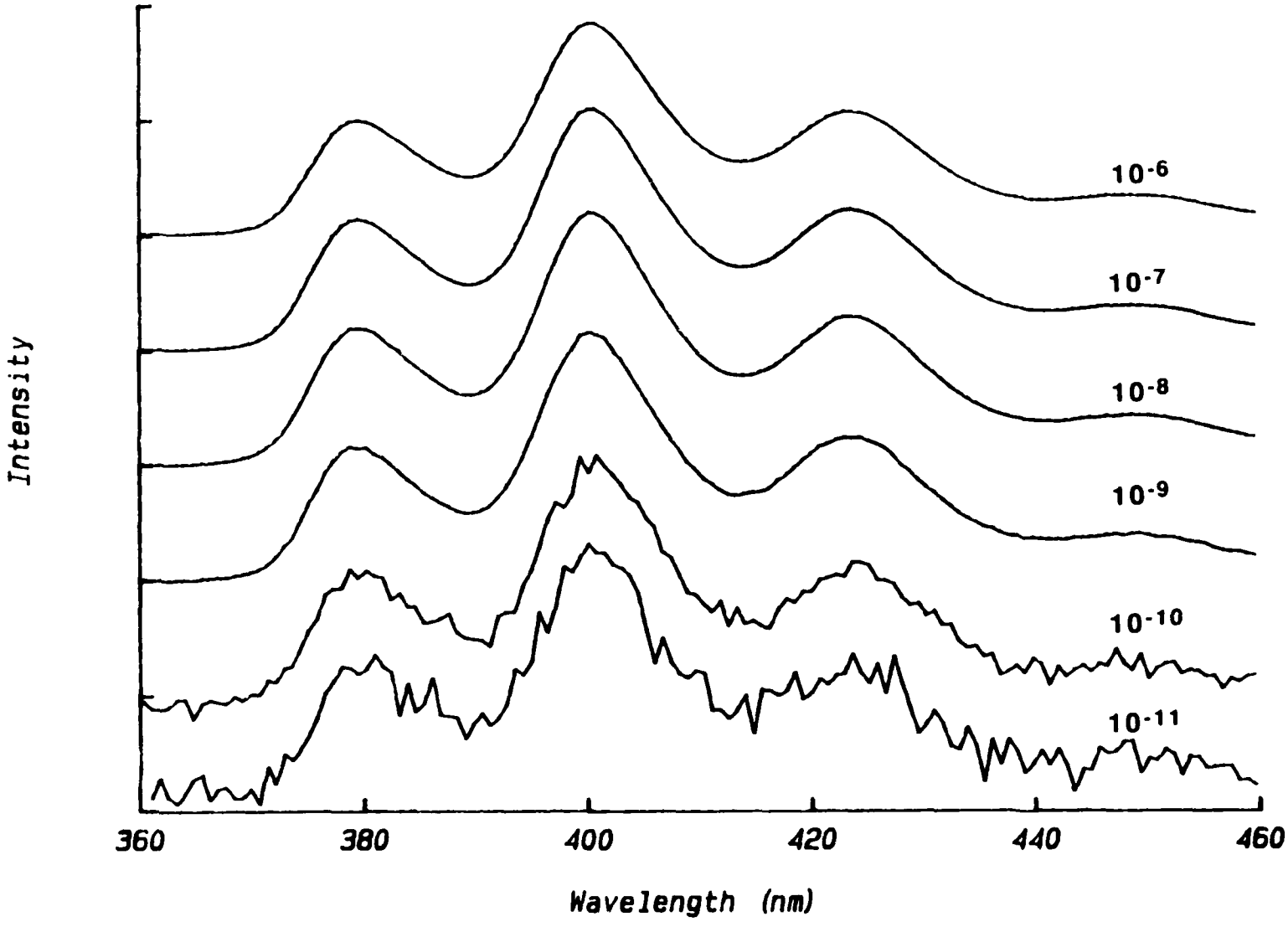
Wavelength (nm)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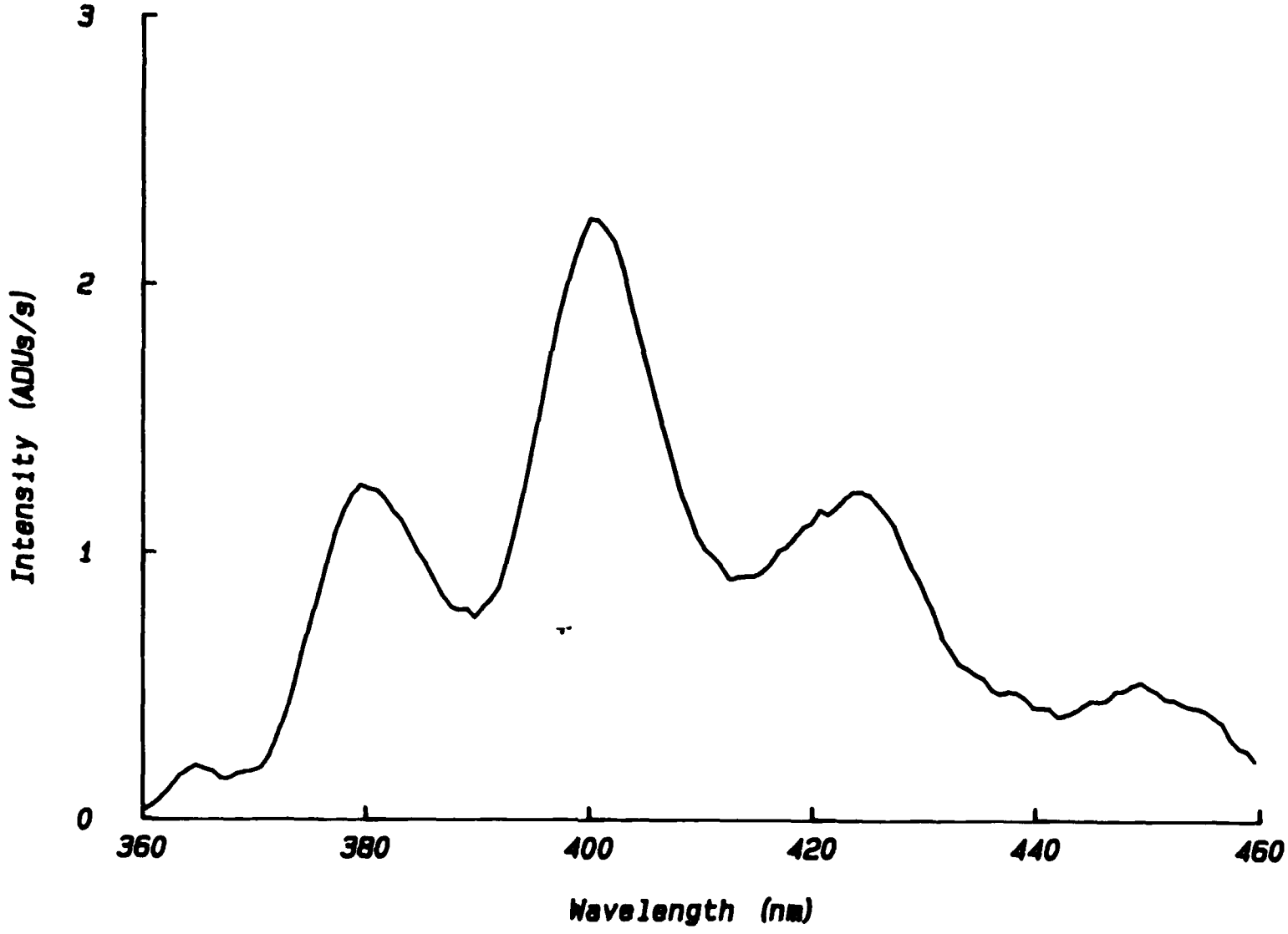
460
Wavelength (nm)

FIGURE 4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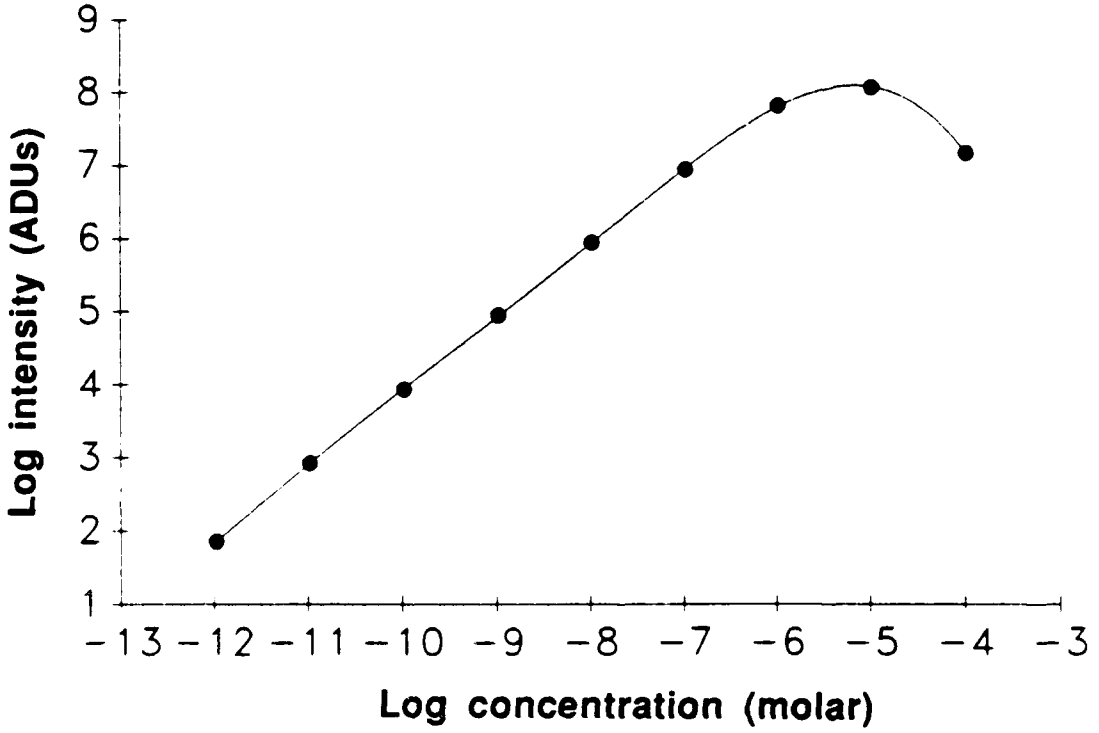
460
Wavelength (nm)


Log intensity (ADUs)
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-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3
Log concentration (molar)

FIGURE 6

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