Excitation Versus Emission Spectra as a Means to Examine Selective Fluorescence Quenching Agents

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To ascertain whether fluorescence quenching is best studied with the use of excitation or emission spectra, and to expand our existing PAH spectral data file, we have recorded excitation spectra of benzo[b]perylene, dibenzo[hi,wx]heptacene, tetrabenzo[de,hi,mn,qr]naphthacene, perylene, benzo[a]fluoranthene, benzo[def]indeno[1,2,3hi]chrysene, naphtho[2,1a]fluoranthene, naphtho[2,3b]fluoranthene, benzo[k]fluoranthene, 2-azapyrene, naphtho[8,1,2hij]pyreno[9,10,1def]phthalazine, indeno[1,-2,3ij]isoquinoline, benzo[lmn][3,8]phenanthroline, and 7-methyldibenzo[b,def]chrysene at various nitromethane concentrations. Results of these measurements verify our earlier observations concerning the nitromethane selective quenching rule and further illustrate the importance of considering inner-filtering artifacts in quenching studies.

Index Headings: **Fluorescence; Spectroscopic techniques.**

INTRODUCTION

Identification and quantification of unknown polycyclic aromatic hydrocarbon (PAH) mixtures require accurate fluorescence emission intensity measurements and availability of a large spectral data file for comparing the unknown's spectrum against PAH standards. Mixtures of environmental/industrial importance rarely contain a single component. The majority of mixtures commonly encountered contain several isomeric pairs or structurally similar PAHs, which emit in approximately the same spectral regions. Kalman filtering and Gaussian or other curve-fitting techniques,[1-6] alone or in combination with phase-resolved[7-9] or synchronous scanning[10-12] fluorescence spectroscopy, theoretically allow uncoupling of overlapped spectra. Such methods become less reliable, however, as the number of mixture components increases. High-performance liquid chromatographic (HPLC) separation prior to fluorimetric analysis affords a viable alternative, but again the method is extremely time consuming whenever large numbers of isomeric compounds are present. Blümer and Zander[13] suggested that nitromethane and/or nitrobenzene could be added to an aqueous/acetonitrile (20:80 percent by volume) binary mobile phase to selectively suppress fluorescence signals of alterant PAHs. Emission intensities of nonalternant PAHs would remain unchanged. Published studies[14-19] involving over 80 PAHs have identified dibenzo[hi,wx]fluoranthene as among the few exceptions to the so-called nitromethane selective quenching rule in the PAH6 benzenoid, fluorenoid, fluoranthenoid, and "methylene-bridged" cyclopenta-PAH subclasses. Nitromethane also quenches fluorescence emission of alkyl-substituted PAH6 benzenoids[20] and alternant unprotonated polycyclic aromatic nitrogen hetero-atoms[21,22] (PANHs); however, the number of PANH exceptions is considerable. Of the seventeen alternant monoaza- and diaza-PANH solutes studied, approximately one-third were listed as exceptions. Corrected emission intensities of the protonated PANHs remained essentially constant and were unaffected by nitromethane.

Utilization of selective quenching reagents can significantly simplify observed emission spectra. To prevent misidentification, experimentally determined spectra must be free of chemical and instrumental artifacts that might unexpectedly reduce emission intensities. Inner-filtering is a major problem associated with obtaining correct fluorescence data, which assumes that the sample is optically dilute (A cm$^{-1} \leq 0.01$) at all analytical wavelengths. Most commercial instruments employ right-angle fluorometry, which reduces stray radiation by placing the emission detector at 90° with respect to the incoming excitation beam (see Fig. 1). Only fluorescence emission originating from the center interrogation zone of the sample cell is actually collected. Attenuation of the excitation beam before it reaches the region viewed by the fluorescence detection optics (pre-filtering region) and goes through the interrogation volume element is denoted as primary inner-filtering. The correction factor, f_{prim}, for primary inner-filtering is given by the following expression:[23-25]

$$f_{prim} = \frac{F^{corr}}{F^{obs}} = \frac{2.303A(y - x)}{10^{-Ax} - 10^{-Ay}} \quad (1)$$

which differs slightly from the approximate form[26]

$$f_{prim} \approx 10^{0.5A} \quad (2)$$

where F^{corr} and F^{obs} refer to the corrected and observed fluorescence emission signal, respectively; A is the absorbance per centimeter of pathlength at the excitation wavelength; and x and y denote distances from the boundaries of the interrogation zone to the excitation plane, as shown in Fig. 1. Failure to correct the observed intensities may lead to erroneous conclusions regarding PAH identification (alternant vs. nonalternant), particularly if excitation wavelengths of 300 nm or less are employed. Many of the PAHs have excitation wavelengths in the 300–320 nm spectral region, and a few drops of nitromethane (or nitrobenzene) give solutions of appreciable absorbances. Selective quenching experiments are less prone to secondary inner-filtering artifacts. PAH emission bands often appear in the 370–550 nm spectral region where nitromethane is nearly "optically transparent." Readers are reminded that only a few

Received 8 May 1992.
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0003-7028/92/4609-1388$2.00/0
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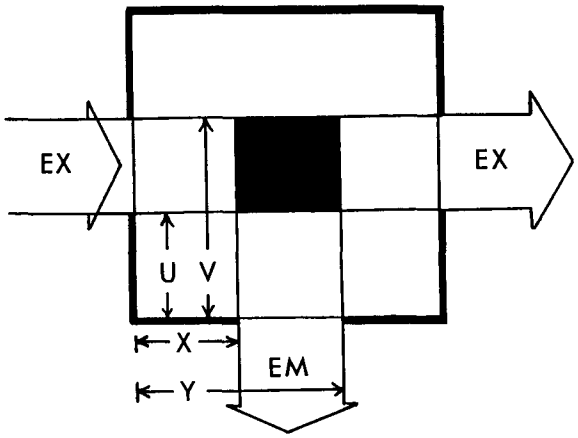

FIG. 1. Typical cell configuration for right-angle fluorometry. Window parameters (x,y) and (u,v) are determined by masking apertures or some other limiting aperture in emission and excitation beam, respectively.

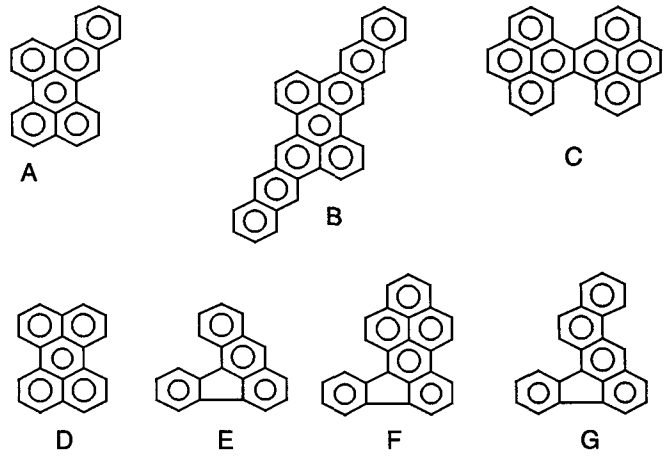

FIG. 2. Molecular structures of polycyclic aromatic compounds: (**A**) benzo[b]perylene; (**B**) dibenzo[hi,wx]heptacene; (**C**) tetrabenzo-[de,hi,mn,qr]naphthacene; (**D**) perylene; (**E**) benzo[a]fluoranthene; (**F**) benzo[def]indeno[1,2,3hi]chrysene; (**G**) naphtho[2,1a]fluoranthene.

drops of quenching reagent are used in this type of experiment.

Measurement of both solution absorbances and emission intensities is tedious if numerous solutes are to be examined. Excitation at longer wavelengths can minimize and perhaps even eliminate inner-filtering artifacts. Unfortunately, the minor excitation wavelengths were not obtained during the course of our earlier solvent polarity probe[27–34] and fluorescence quenching[14–16] investigations. To ascertain whether fluorescence quenching is best studied with the use of excitation or emission spectra, and to expand our existing PAH spectral data file, we have recorded excitation spectra of benzo[b]-perylene, dibenzo[hi,wx]heptacene, tetrabenzo[de,hi,-mn,qr]naphthacene, perylene, benzo[a]fluoranthene, benzo[def]indeno[1,2,3hi]chrysene, naphtho[2,1a]fluoranthene, naphtho[2,3b]fluoranthene, benzo[k]fluoranthene, 2-azapyrene, naphtho[8,1,2hij]pyreno-[9,10,1def]phthalazine, indeno[1,2,3ij]isoquinoline, benzo[lmn][3,8]phenanthroline, and 7-methyldibenzo-[b,def]chrysene at various nitromethane concentrations. Molecular structures of these solutes are depicted in Figs. 2 and 3. Results of these measurements verify our earlier observations concerning the nitromethane selective quenching rule and further illustrate the importance of considering inner-filtering artifacts in quenching studies.

MATERIALS AND METHODS

Sources and synthetic procedures of all compounds are identified in our earlier papers.[14–16,27–34] Stock solutions were prepared by dissolving the solutes in dichloromethane. Small aliquots of the stock solutions were transferred into test tubes, allowed to evaporate, and diluted with acetonitrile. Final solute concentrations were sufficiently diluted to minimize inner-filtering artifacts. Acetonitrile (HPLC grade), nitromethane, and 1,2,4-trimethoxybenzene were purchased from Aldrich Chemical Company and were used as received.

Absorption spectra were recorded on a Bausch and Lomb Spectronic 2000 in the usual manner with a 1-cm^2 quartz cuvette. The fluorescence spectra were run on a Shimadzu RF-5000U spectrofluorometer with the detector set at high sensitivity. Solute excitation spectra were examined as a function of nitromethane concentration at the emission wavelengths listed in Table I. All fluorescence data were accumulated in a quartz 1-cm^2 cuvette at 19°C, ambient room temperature, with excitation and emission slit width settings of 15 nm and 3 nm, respectively.

RESULTS AND DISCUSSION

Representative fluorescence excitation spectra of benzo[lmn][3,8]phenanthroline, benzo[a]fluoranthene, and naphtho[2,3b]fluoranthene dissolved in acetonitrile are shown in Figs. 4–6. Examination of the spectral data reveals that most polycyclic aromatic compounds (PACs) have more than one excitation band. For example, benzo[lmn][3,8]phenanthroline has a major excitation peak at 326 nm plus two minor excitation peaks at 360 and 378 nm. Normally one might choose the excitation band with the highest intensity, λ = 326 nm, to examine the emission spectra, but one must also take solvent effects

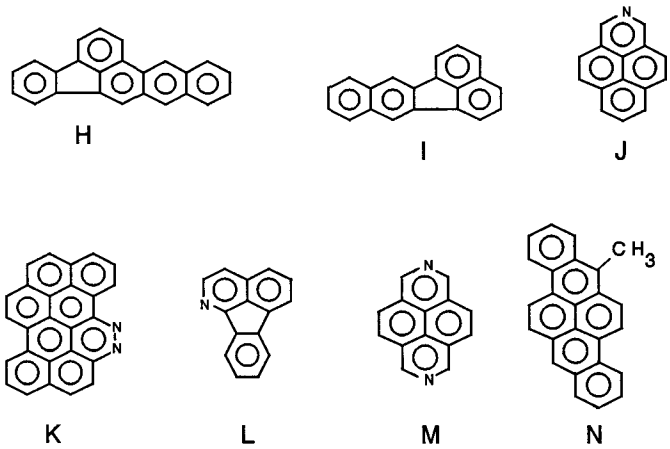

FIG. 3. Molecular structures of polycyclic aromatic compounds: (**H**) napahtho[2,3b]fluoranthene; (**I**) benzo[k]fluoranthene; (**J**) 2-azapyrene; (**K**) naphtho[8,1,2hij]pyreno[9,10,1def]phthalazine; (**L**) indeno[1,2,3ij]isoquinoline; (**M**) benzo[lmn][3,8]phenanthroline; (**N**) 7-methyldibenzo[b,def]chrysene.

TABLE I. Summary of excitation wavelengths and percent reduction in the fluorescence intensity with successive drops of nitromethane for PACs.

Compound	λ_{em}	λ_{ex}	1 Drop[a] (% Redn)	2 Drops (% Redn)	3 Drops (% Redn)	5 Drops (% Redn)	10 Drops (% Redn)
A	466	314	54	88			
		406	38	65			
		432	38	65			
B	520	341	2		100		
		408	0		0		
		439	0		0		
C	439	320	72	93			
		387	61	83			
D	496	406	46				
		433	45				
E	518	s-298	45		78		100
		363	5		9		100
		406	1		1		3
		448	3		3		3
F	474	334				57	79
		409				2	2
G	491	298				88	98
		402				4	5
H	445	315	35		78		100
		358	6		21		40
		407	2		11		23
I	433	301	46		80		100
		378	4		9		30
		397	4		9		27
J	419	330	81				
		371	74				
K	470	314	33			100	
		394	2			6	
L	470	362	4			15	
M	426	326	45		80		
		360	33		62		
		378	33		60		
N	462	309	57		89		
		410	32		62		

[a] Solvent was acetonitrile in all quenching studies.

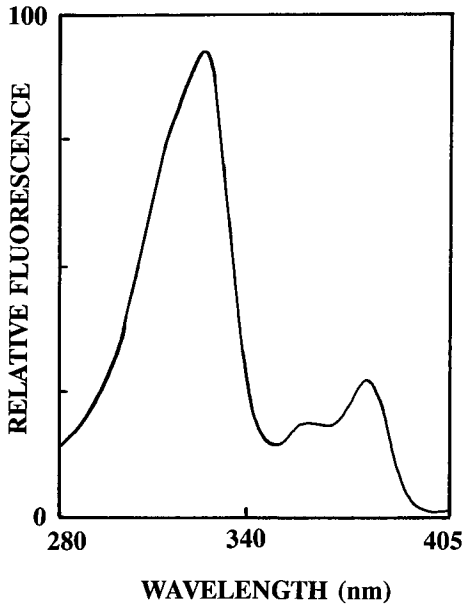

FIG. 4. Fluorescence excitation spectra of benzo[lmn][3,8]pehanthroline dissolved in acetonitrile. Major excitation bands occur at 326, 360, and 378 nm.

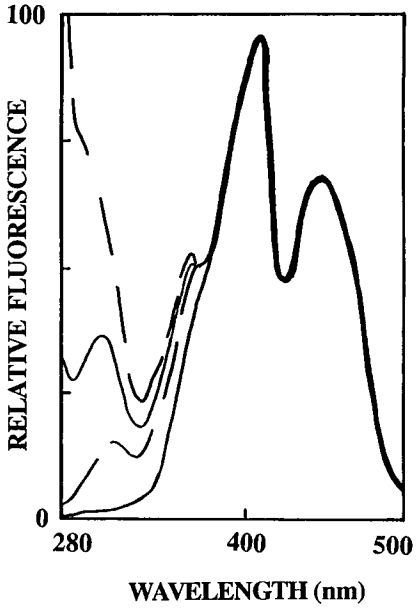

FIG. 5. Fluorescence excitation spectra of benzo[a]fluoranthene dissolved in acetonitrile at various nitromethane concentrations. From top to bottom, the curves correspond to 0, 1, 3, and 10 drops of nitromethane.

into account. Solvents like benzene give rise to an appreciable background when excited near 300 nm. A better choice for the benzo[lmn][3,8]phenanthroline might be the peak at 378 nm because it is a fairly intense excitation peak that does not excite most solvents.

Selection of an appropriate emission peak must also take solvent and quencher spectral characteristics into account. Since this study examined the quenching of the excitation spectra by nitromethane and 1,2,4-trimethoxybenzene, one must look at where these molecules absorb radiation in order to minimize primary and/or secondary inner-filtering artifacts. Figure 7 indicates that

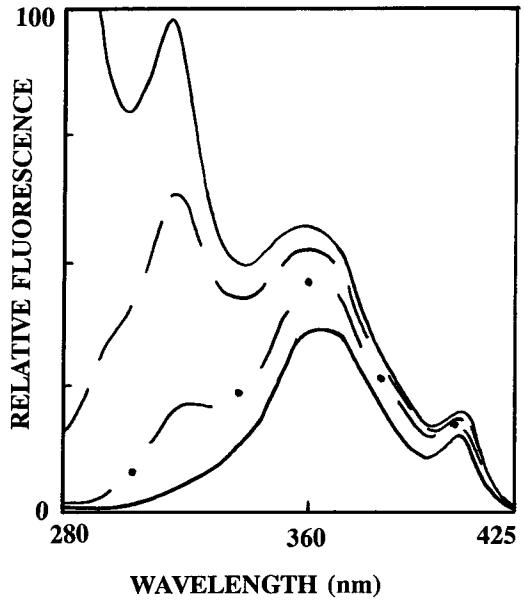

FIG. 6. Fluorescence excitation spectra of naphtho[2,3b]fluoranthene dissolved in acetonitrile at various nitromethane concentrations. From top to bottom, the curves correspond to 0, 1, 3, and 10 drops of nitromethane.

nitromethane and 1,2,4-trimethoxybenzene both have significant absorbance from 300 nm to approximately 365 nm. Any excitation or emission peaks in this region will demonstrate inner-filtering artifacts. For example, the first peak in the benzo[k]fluoranthene excitation spectra (Compound I in Fig. 7) is heavily inner-filtered by both selective quenching agents. The second and third excitation peaks at 378 and 397 nm should show minimal (if any) inner-filtering, and any decrease in observed fluorescence intensity must be the result of quenching. Examination of Table I shows that this is indeed the case. When one drop of nitromethane is added to a solution of benzo[k]fluoranthene dissolved in acetonitrile, the emission intensity corresponding to the 301-nm excitation wavelength is reduced by 40%, while intensities of the 378-nm and 397-nm peaks are only reduced by 4% each. After ten drops of nitromethane, the first peak is completely removed from the excitation spectra, and the second and third peaks are reduced by approximately 30%. Clearly, nitromethane does marginally quench benzo[k]fluoranthene's fluorescence emission, making this PAH solute an exception to the nitromethane selective quenching rule. Quenching is much more pronounced in the more polar aqueous/acetonitrile solvent mixture (20:80 by volume), which is the recommended solvent for the alterant vs. nonalternant PAH selective quenching studies. We have been substituting acetonitrile for the mixed solvent in order to solubilize the larger polycyclic aromatic compounds. With very few exceptions, we have obtained identical nitromethane quenching results irrespective of whether acetontrile or an aqueous/acetonitrile mixture was used as the solvent. For comparison purposes, Fig. 5 depicts the effect of nitromethane on the excitation peaks of an unquenched nonalternant polycyclic aromatic hydrocarbon. The first peak at 298 nm in the benzo[a]fluoranthene excitation spectra is so heavily inner-filtered that it ceases to exist after ten drops of nitromethane have been added. Peak intensities for excitation at 363, 406, and 446 nm, where nitromethane does not absorb, remain constant.

The "excitation method" for examining quenching phenomena requires that emission peaks must be greater than approximately 400 nm so that at least one (preferably two) of the excitation peaks in the 280–380 nm excitation range is not heavily inner-filtered. Indeno[1,2,3ij]isoquinoline (Compound L in Fig. 7) does have a useable emission peak above 400 nm; however, it would not be a good candidate for this method since its single excitation peak is at 362 nm. It is questionable whether inner-filtering or quenching artifacts predominate at this particular wavelength. Of the 14 polycyclic aromatic compounds listed in Table I, benzo[b]perylene, dibenzo[hi,wx]heptacene, perylene, benzo[a]fluoranthene, benzo[def]indeno[1,2,3hi]chrysene, naphtho[2,1a]fluoranthene, naphtho[2,3b]fluoranthene, benzo[k]fluoranthene, naphtho[8,1,2hij]pyreno[9,-10,1def]phthalazine, and 7-methyldibenzo[b,def]chrysene are probably the better suited fluorophores for this type of study as the emission peaks and at least one excitation peak are well out of the inner-filtering region. A decreased fluorescence signal at the higher excitation wavelength will clearly demonstrate only quenching effects.

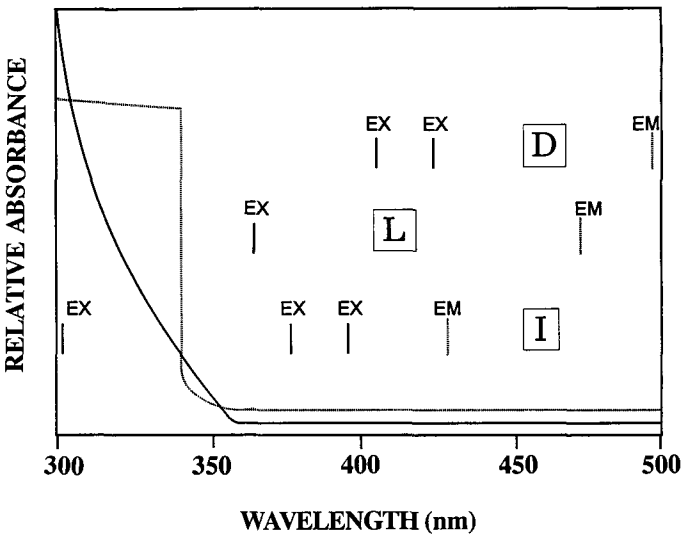

FIG. 7. Absorption spectra of nitromethane (solid line) and 1,2,4-trimethoxybenzene (broken line). Superimposed on the absorption spectra are the excitation (EX) and emission (EM) wavelengths of benzo[k]fluoranthene (Compound I), indeno[1,2,3ij]isoquinoline (Compound L) and perylene (Compound D). To minimize primary and secondary inner-filtering artifacts, the fluorophore's excitation and emission wavelengths must occur in a spectral region in which the quenching agent is "optically transparent."

Utilization of the excitation spectra for the examination of selective quenching by nitromethane gives results consistent with past studies[14–16] on the emission spectra of polycyclic aromatic compounds, but it is not without limitations. Compounds that were quenched by nitromethane in previous studies are still quenched by nitromethane and vice versa. Examination of the excitation spectra is less tedious and time-consuming than the emission spectra examination; however, several polycyclic aromatic compounds cannot be examined this way because the emission peaks are not far enough removed from the inner-filtering region. For example, pyrene, with emission peaks at 371, 382, and 391 nm, has its optimum excitation wavelength(s) in the 280–370 nm spectral region. Strong quencher absorbance would result in appreciable inner-filtering artifacts at all excitation wavelengths considered. For compounds having suitable emission peaks, the "excitation method" offers a very convenient, fast, and reliable method to study fluorescence quenching phenomena. The technique does require a prior knowledge of both the excitation and emission spectra.

It should be noted that the "excitation method" can lead to an erroneous conclusion regarding the ability of 1,2,4-trimethoxybenzene to selectively quench the fluorescence emission of nonalternant polycyclic aromatic hydrocarbons if one is not careful. Trimethoxybenzene has a fluorescence excitation and emission spectra at several wavelengths employed in Table I. When one or more Pasteur pipet drops of 1,2,4-trimethoxybenzene are added to the dissolved PAC solution, new peaks may appear in the observed fluorescence spectra. Making exact solutions of 1,2,4-trimethoxybenzene for the blank and solute + blank for back subtraction is tiresome and more time-consuming than gathering absorbance data and making inner-filtering corrections on the emission

spectra. Nitromethane did not pose this problem, as it does not fluoresce at the excitation and emission wavelengths utilized. We recommend using the "excitation method" to examine selective quenching when applicable, as it is easier and less time-consuming.

ACKNOWLEDGMENTS

This work is supported in part by grants from the National Science Foundation (Grant No. CTS-8922485), by the University of North Texas Research Council, and by a National Science Foundation Doctoral Research Fellowship awarded to S. A. Tucker.

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