Three-Way Analysis of Fluorescence Spectra of Polycyclic Aromatic Hydrocarbons with Quenching by Nitromethane

Peter D. Wentzell,* Sreeja S. Nair, and Robert D. Guy

Trace Analysis Research Centre, Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J3

The application of trilinear decomposition (TLD) to the analysis of fluorescence excitation−emission matrices of mixtures of polycyclic aromatic hydrocarbons (PAHs) is described. The variables constituting the third-order tensor are excitation wavelength, emission wavelength, and concentration of a fluorescence quencher (nitromethane). The addition of a quencher to PAH mixtures selectively reduces the fluorescence intensity of mixture components according to the Stern−Volmer equation. TLD allows the three-way matrix to be decomposed to give unique solutions for the excitation spectrum, emission spectrum, and quenching profiles for each component. The availability of spectra and calculated Stern−Volmer constants can aid in the identification of unknown components. Preprocessing of the data to correct for Rayleigh/Raman scatter and primary absorption by the quencher is necessary. Both three-component (anthracene, pyrene, 1-methylpyrene) and four-component (fluoranthene, anthracene, pyrene, 2,3-benzofluorene) synthetic mixtures are successfully resolved by TLD using quencher concentrations up to 100 mM. Results are compared using both alternating least-squares and direct trilinear decomposition algorithms. The reproducibility of extracted Stern−Volmer constants is determined from replicate experiments. To illustrate the application of TLD to a real sample, a chromatographic cut from the analysis of a light gas oil sample was used. Analysis of the TLD extracted spectra and quenching constants suggests the presence of three classes of polycyclic aromatic hydrocarbons consistent with data from a second dimension of chromatography and mass spectrometry.

The utility of high-order tensors of data in the analysis of complex mixtures has been well established in the literature.[1−4] For second-order tensors (i.e., matrices of data), techniques for multivariate calibration, exploratory data analysis, and curve resolution are now routinely employed. In the case of curve resolution methods, the objective is to obtain the spectra and/or concentration profiles of individual components in a mixture. For second-order bilinear data, methods such as self-modeling curve resolution and iterative target-testing factor analysis[5] can be of great value in estimating these profiles. However, these methods usually present solutions that are not unique due to a rotational ambiguity in the spectral vectors, although this ambiguity can be restricted with the introduction of appropriate constraints.

The problem of ambiguity in curve resolution problems can be addressed by utilizing third-order tensors, or three-way data, which is a natural extension of the second-order matrix into a three-dimensional "cube" of data. Of particular utility to analytical chemists are third-order data sets which follow the trilinear or parallel factor analysis (PARAFAC) model.[6−9] In this model, the three-dimensional matrix is represented as the sum of a finite series of tensor products of three vectors. A major advantage of data following this model is that the decomposition is unique, permitting vectors associated with individual components of the mixture (spectra, concentration profiles, etc.) to be extracted directly. This permits identification and quantitation of species in mixtures where separation is difficult or time-consuming. Although the effectiveness of trilinear decomposition (TLD) has been demonstrated in the literature, applications have been somewhat sparse so far. One reason for this is the limited availability of data sets that follow a trilinear model. Although the widespread use of hyphenated techniques has improved the accessibility of such data, the extra dimension required can be a complicating factor. A second deterrent to the implementation of TLD has been the development of the algorithms themselves, but these have now matured to the point where their application is relatively routine.

Table 1 presents a sampling of TLD applications that have appeared in the literature, subdivided according to the type of measurement used for each order. The review is not intended to be comprehensive, but gives a cross section of the kinds of systems that have been examined. In most applications; the first order is the spectroscopic domain, with measurements being made as a function of wavelength. (Note that the term "first" is used merely for classification purposes and there is no mathematical significance to the sequence of measurement domains, even

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Table 1. Overview of Some Applications of Trilinear Decomposition

first order	second order	third order	refs
absorbance or fluorescence (wavelength)	chromatography (elution time)	sample composition (concentrations)	10−15
		kinetics (reaction time)	16
	titration (pH)	sample composition (concentrations)	17
	kinetics (reaction time)	sample composition (concentration)	18−19
fluorescence excitation (wavelength)	fluorescence emission (wavelength)	sample composition (concentration)	6, 20−23
		chromatography (elution time)	24
		titration (pH)	25
		polarization (angle)	26
		lifetime (frequency)	27
		quencher/modifier (concentrations)	7, 28−31

though there may be a natural physical sequence.) For the second order, applications can be broadly classified into those that use fluorescence excitation spectra paired with fluorescence emission spectra in the first order and those that use other techniques. In the latter category, chromatography and related techniques are natural choices for the second order. These methods lead to mixture component contributions that vary with elution time and generate a bilinear spectrochromatogram. Unfortunately, methods based on separation methods suffer from a number of drawbacks, not the least of which is a high sensitivity to retention time reproducibility (i.e., variations in time registration) when multiple samples are used as the third order. The pH of the system is another parameter that can be varied for the second order, but of course this requires components that are sensitive to acidity. Reaction kinetics have also been exploited in the second order by measuring the response as a function of reaction time, but this strategy suffers from the disadvantage that it only leads to a trilinear structure in certain cases.

The use of fluorescence excitation−emission matrices (EEMs) for the first two orders is advantageous because (1) the measurements can be made on a single instrument with consistent channel registration, (2) EEMs exhibit good sensitivity, selectivity, and bilinearity, and (3) a range of options is available for the third order. For other methods, the most common approach to introducing the third order is through variations in the sample composition. While this is effective, it requires a prior knowledge of mixture components in order to produce standard mixtures (unless one can ensure that the component concentrations vary independently by other means). Thus, the qualitative information provided by TLD is redundant. While sample composition has been used as the third order with fluorescence EEMs, other options, such as polarization and lifetime measurements, are also available. Under these circumstances, qualitative information (spectra and other properties) about individual components can be extracted from what is essentially an unknown mixture with virtually no prior information. One of the more interesting approaches is to add varying amounts of a component to the solution that differentially alters the fluorescence yield of the other mixture components.[7,28−31] The use of fluorescence quenchers is one way that this can be accomplished, since the fluorescent yield

of individual components will depend on their Stern−Volmer constants. This has been demonstrated in the literature, although for fairly specific systems with a limited number of components.[7]

The goal of this paper is to demonstrate the use of fluorescence quenching in conjunction with EEMs as a means to extract compositional information from mixtures of polycyclic aromatic hydrocarbons (PAHs). PAHs are a large and important class of compounds whose health effects have been extensively documented.[32] The characterization of PAHs in complex mixtures is a challenging analytical problem. In this work, we show how fluorescence measurements coupled with three-way analysis can be used to provide qualitative information about components in PAH mixtures. This study differs from earlier work based on fluorescence quenching in that it is more generally applicable to a wider range of compounds and is applied to more complex mixtures. Both synthetic and real mixtures are employed to examine the limitations of the technique.

THEORY

Trilinear Model. The theory and application of three-way methods have been treated in the literature, but a brief overview

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$$\underline{\mathbf{D}} = \mathbf{x}_1 \otimes \mathbf{y}_1 \otimes \mathbf{z}_1 + \mathbf{x}_2 \otimes \mathbf{y}_2 \otimes \mathbf{z}_2 + \ldots$$
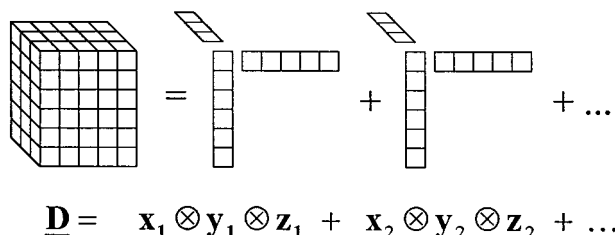
Figure 1. Trilinear decomposition.

will be presented here in the context of the present application. Although a variety of models exist for the decomposition of three-way arrays, the trilinear model is perhaps the most straightforward since it is analogous to the bilinear decomposition of matrices by principal components analysis (PCA). In this decomposition, the original data cube ($\underline{\mathbf{D}}$) is represented as the sum of tensor products of three vectors plus a residual error term ($\underline{\mathbf{E}}$):

$$\underline{\mathbf{D}} = \mathbf{x}_1 \otimes \mathbf{y}_1 \otimes \mathbf{z}_1 + \mathbf{x}_2 \otimes \mathbf{y}_2 \otimes \mathbf{z}_2 + \cdots + \underline{\mathbf{E}} \qquad (1)$$

In this representation, $\mathbf{x}$, $\mathbf{y}$, and $\mathbf{z}$ represent vectors of appropriate dimensions, as indicated in Figure 1, and $\otimes$ indicates the tensor product. Note that, throughout this paper, scalars are shown in italics, vectors are bold lowercase letters, two-way matrices are bold uppercase letters, and three-way arrays are underscored bold uppercase letters. Generally, in chemical applications, each group of vectors in eq 1 is associated with a particular observable component in the mixture and each vector represents a particular property, such as a spectrum, of that component. One objective of TLD is to extract the vectors to aid in the characterization of the mixture components. The principal advantage of trilinear over bilinear decomposition is that the former results in a unique set of vectors, while the vectors extracted by the latter have a rotational ambiguity. Both methods suffer from an ambiguity of scale such that the absolute magnitude of individual vectors is indeterminate without additional information, but this does not impede their application to quantitative measurements as long as an appropriate reference scale is chosen. In this paper, we are primarily interested in the qualitative interpretation of the vectors, and for that purpose the vectors are usually normalized to a convenient scale.

Algorithms for performing TLD are now well-established. Two approaches are commonly used: alternating least squares (ALS) and direct trilinear decomposition (DTLD). Although the objectives of both are the same and the results are usually comparable, ALS is based on a least-squares minimization while DTLD is formulated as an eigenvalue problem, leading to some differences in certain cases. Both methods have been used in this work, but the ALS algorithm was found to be somewhat more reliable.

Fluorescence Quenching. For TLD to be successfully applied to a three-way array, the data set should follow a trilinear model. To demonstrate that this is the case for the measurement of fluorescence EEMs in the presence of quenching, consider a series of mixtures, each consisting of the same N fluorophores, but with different concentrations of a quencher, Q. For the fluorescence EEM of a given mixture j, such as that shown in Figure 2, it can be shown that fluorescence intensity at an excitation wavelength channel k and an emission wavelength
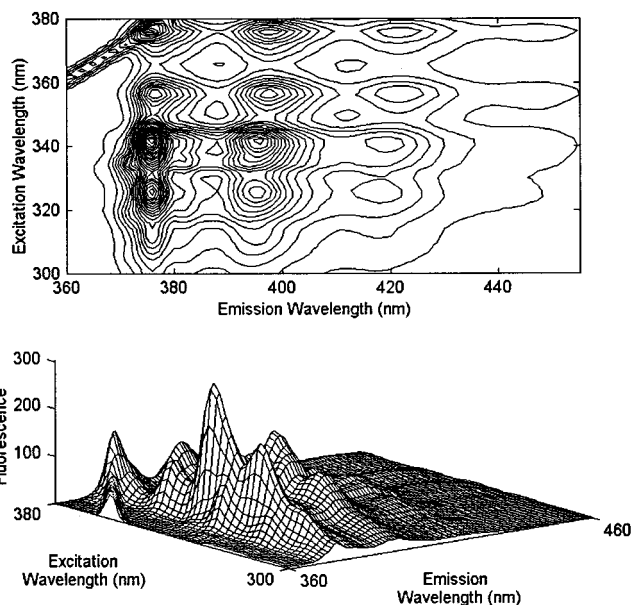

Figure 2. Typical fluorescence excitation−emission spectrum for synthetic four-component mixture.

channel l can be given as

$$F_{jkl} = b\, I_k R_l \sum_{i=1}^{N} c_{ij} \epsilon_{ik} \pi_{il} \phi_{ij} \qquad (2)$$

This equation assumes that we are in the linear region of fluorescence response (i.e., there are no primary or secondary absorption effects). The variable b is the cell path length, I_k is the excitation source intensity incident on the cell at channel k, R_l is a factor to account for detector sensitivity and collection efficiency at emission channel l, c_{ij} is the concentration of fluorophore i in mixture j, ϵ_{ik} is the molar absorptivity of fluorophore i at excitation channel k, π_{il} is the normalized fluorescence emission intensity of fluorophore i at emission channel l (normalized to unit absorbance, source intensity, and quantum yield), and ϕ_{ij} is the fluorescence quantum yield of fluorophore i in mixture j. Assuming that quenching characteristics follow the commonly observed Stern−Volmer relationship, the quantum yield for fluorophore i in solution j is given by

$$\phi_{ij} = \frac{\phi_i^{o}}{1 + k_i[Q]_j} \qquad (3)$$

where ϕ_i^{o} is the fluorescence quantum yield of fluorophore i in the absence of quencher, k_i is the Stern−Volmer constant for fluorophore i for quencher Q, and $[Q]_j$ is the concentration of the quencher in mixture j. In eq 2, we can make the following substitutions: (1) $c'_{ij} = c_{ij}\phi_{ij}$ is the effective concentration of fluorophore i in mixture j, (2) $\epsilon'_{ik} = bI_k\epsilon_{ik}$ is the corrected absorption cross section of fluorophore i at excitation wavelength channel k, and (3) $\pi'_{il} = R_l\pi_{il}$ is the corrected emission intensity of fluorophore i at emission wavelength channel l. This gives

$$F_{jkl} = \sum_{i=1}^{N} c'_{ij}\epsilon'_{ik}\pi'_{il} \qquad (4)$$

or, in tensor notation

$$\underline{\mathbf{F}} = \sum_{i=1}^{N} \mathbf{c}'_i \otimes \epsilon'_i \otimes \pi'_i \qquad (5)$$

where $\mathbf{c}'$, ϵ', and π' are vectors. It is clear that this is a trilinear model and that vectors corresponding to the effective concentration, excitation, and emission profiles of each component should be available by TLD. Note, however, that there is an ambiguity of scale and only relative magnitudes can be extracted.

Nonidealities and Data Preprocessing. Equation 5 gives the ideal trilinear model for fluorescence EEMs in the presence of quenching. Unfortunately, real measurements can give rise to nonideal behavior that can invalidate the trilinearity. In the present work, two of these are of particular importance: (1) Rayleigh and Raman scatter and (2) absorption by the quencher.

Rayleigh and Raman scattering by solvent molecules leads to relatively strong emissions in the neighborhood of the excitation wavelength. In a fluorescence EEM, this leads to a diagonal band, such as that appearing in the upper left corner of the contour plot in Figure 2, and destroys the bilinear (and consequently the trilinear) structure of the data. Several solutions to this problem have been employed. One is to subtract a background EEM of the solvent from subsequent spectra. Unfortunately, the subtraction of two large values can leave a significant residual and this approach is not very reliable. A second approach is to restrict data collection (or data analysis) to regions where the emission and excitation ranges do not overlap. However, this leads to a loss of information that we have found is critical in multicomponent analysis. A third method is to weight the data points in such a way that the scattering band is inconsequential in the trilinear decomposition. JiJi and Booksh[33] have examined various weighting strategies used in conjunction with the ALS algorithm for TLD. The solution employed in this work is similar to the weighting strategy but eliminates the scattering problem before TLD is carried out. In the first step, the cube of data is "unfolded". This consists of placing the individual EEMs end to end (with either the excitation or emission channels shared) to form a large matrix. Weighted PCA is then used to decompose the resulting matrix, with weights adjusted to ignore the scattering regions. If the trilinear model (with the exception of the scatter) is valid, bilinear decomposition of the unfolded matrix by PCA will also be valid, since it is a less restricted model. The desired number of components can then be selected and the unfolded matrix can be reconstructed using the truncated PCA solution. Once this is done, the matrix can be refolded to give the third-order tensor, without scattering interference, ready for TLD. In this work, maximum likelihood PCA (MLPCA)[34−36] is used to carry out the

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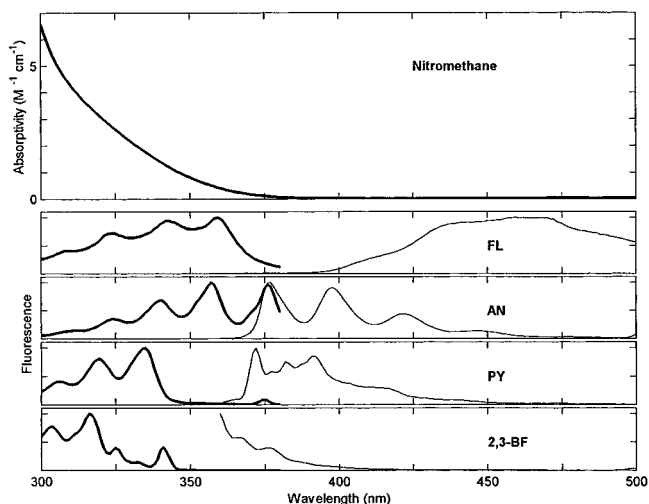(36) Andrews, D. T.; Wentzell, P. D. *Anal. Chim. Acta* **1997**, *350*, 341−352.

Figure 3. Comparison of absorption profile of nitromethane (top) with the fluorescence excitation (thick lines) and emission (thin lines) profiles of fluoranthene (FL), anthracene (AN), pyrene (PY), and 2,3-benzofluorene (2,3-BF).

weighted decomposition, but this is simply a generalized form of weighted PCA. There are several potential advantages to this strategy over the weighted ALS algorithms: (1) the weighting is applied only once, rather than at each iteration, (2) use of PCA as a preprocessing step helps to reduce the noise in the data and thus may enhance the application of TLD, and (3) since it is employed as a pretreatment, either ALS or DTLD can be used for the TLD. It is not expected, however, that the results of this approach will be substantially different from those of weighted ALS.

A second problem that arises in the analysis of experimental data is primary absorption effects due to high concentrations of the quencher. As the concentration of the quencher is increased, its absorption becomes more significant and it will reduce the amount of light reaching the fluorophore at particular wavelengths, selectively decreasing the emission intensity in a nonlinear fashion. Ideally, one would like to employ a quencher with an absorption spectrum that does not overlap with those of the fluorophores, but this cannot always be arranged. Figure 3 shows the absorption spectrum of nitromethane, the quencher used in this work, together with the fluorescence excitation and emission spectra of selected PAHs. Clearly, the absorption by nitromethane will contribute to destroying the trilinear structure of the data. To correct for this, the experimental data are adjusted to account for primary absorption effects using eq 6. In this equation, which

$$F_{\text{corr}} = \frac{2.303\epsilon_Q[Q]}{(1 - e^{-2.303\epsilon_Q[Q]b})}F_{\text{exp}} \qquad (6)$$

applies at a particular excitation wavelength, F_{corr} is the corrected fluorescence intensity, F_{exp} is the measured fluorescence intensity, ϵ_Q is the molar absorptivity of the quencher, $[Q]$ is the molar concentration of the quencher, and b is the path length (in cm). Of course, this correction requires that the concentration of the quencher and its spectrum be accurately known. Uncertainties can introduce errors when the quencher concentration becomes high. Nevertheless, it has been found to be effective in the present application.

Other sources of nonideality, such as secondary absorption effects and wavelength-dependent Stern−Volmer constants, may also arise in the analysis experimental data, but these have not been found to be significant problems for the system under study.

EXPERIMENTAL SECTION

Reagents and Samples. Anthracene (AN; 99%), fluoranthene (FL; 98%), and 2,3-benzofluorene (2,3-BF; 98%) were used as received from Aldrich. Pyrene (PY) was also purchased from Aldrich and recrystallized prior to use. 1-Methylpyrene (1-MPY) and 9-methylphenanthrene (9-MPA) were synthesized in-house and purified by recrystallization. Methanol (spectrophotometric grade, 99.9%) and nitromethane (spectrophotometric grade, 96%) were obtained from Aldrich.

All samples were prepared in methanol. Two sets of synthetic samples were prepared. Set A consisted of fluoranthene, anthracene, pyrene, and 2,3-benzofluorene, while set B was prepared from anthracene, pyrene, and 1-methylpyrene. In all cases, the final concentration of each PAH in the measured solutions was $\sim 5 \times 10^{-7}$ M. The concentrations of the nitromethane in the quenching experiments were approximately 0, 1, 10, 20, 30, 40, 50, and 100 mM (precisely measured). (The purity of the nitromethane was assumed to be 100%. While this was not the case, the same bottle was used for all absorption and quenching measurements, so this would have no effect on the extracted spectra and only a minor effect on the absolute accuracy of the Stern−Volmer constants.)

To examine the application of the method to a practical problem, a fractionated light gas oil sample was employed. A 20-μL aliquot of the oil sample, obtained from Syncrude Canada Ltd., was diluted in hexane and separated by HPLC on a silica column. Elution was monitored by absorption at 336 nm and a fraction known to contain methylpyrene isomers, as well as other PAHs, was collected. This procedure was repeated several times to provide sufficient sample for further analysis. For the samples analyzed in fluorescence quenching experiments, the collected fraction was diluted by a factor of ~ 50.

Instrumentation. Fluorescence EEMs were obtained from samples in a 1-cm quartz cuvette on a Shimadzu RF-301PC spectrofluorometer with a xenon lamp excitation source. An excitation wavelength range of 300−380 nm in steps of 2 nm was employed, while the emission range was 360−500 nm in steps of 1 nm. A "medium" scan speed was used, and excitation and emission slits were both set at 3 nm. Automation of the combined excitation−emission scanning was facilitated through in-house software written in Microsoft Visual Basic. Preliminary experiments were conducted to confirm negligible photodegradation of the sample under the conditions used.

Further characterization of the light gas oil fraction was undertaken to confirm the results of the analysis by TLD. A second dimension of chromatography utilizing two 25-cm monomeric C18 reversed-phase columns (Supelco, RP18) coupled in series, was performed. The columns were maintained at 0 °C and the eluent was acetonitrile. In one experiment, detection was performed using a custom-built laser system consisting of a Kimmon IK series He−Cd continuous wave laser for excitation at 325 nm, a Princeton Instruments TE/CCD-1024-EM/1 CCD camera with a model ST130S controller, a SpectraPro-150 imaging dual-grating monochromator, and an 8-μL flow cell. This allowed continuous

collection of fluorescence emission spectra to aid in the identification of mixture components.

In a second experiment, mass spectrometry was used as the detection method in the second dimension of chromatography. A Micro Mass Fisons Model Quattro mass spectrometer equipped with a Quattro 2 source and an APCI probe was used for this purpose. Because sensitivity was low for this system, the fraction was initially characterized using a direct insertion probe. The instrument was then used in selected ion monitoring mode over several runs to provide more information on important ions.

Procedures. For each sample set, a series of solutions with different quencher concentrations was prepared. Fluorescence EEMs were collected for each of these and translated into a suitable format. Correction for primary absorption by the quencher was then carried out. Following this, the three-way data were unfolded to form a matrix, decomposed by MLPCA to remove Rayleigh and Raman scatter, reconstructed, and refolded. The rank used for MLPCA was set to the known number of solution components for the synthetic mixtures and to a value of 3 (suggested by analysis of the residuals) for the oil fraction. Regions to be eliminated (down-weighted) in this analysis were determined by setting a threshold slightly above the baseline in the EEM corresponding to a solvent blank. The reconstructed matrix was then analyzed by TLD.

Computational Aspects. Data analysis was carried out in MatLab v 5.2 (The Math Works Inc, Natick, MA) on a Sun Ultra 60 workstation with 2×300 MHz processors and 512 MB of RAM. The ALS algorithm for TLD was obtained from the PLS_Toolbox v 2.0 (Eigenvector Research, Inc., Manson, WA), while the algorithm for DTLD was written in-house.

RESULTS AND DISCUSSION

Four-Component Mixture. The components of the first synthetic mixture (set A) were selected to provide a range of quenching characteristics and spectral profiles. Stern−Volmer constants, as determined from separate experiments on pure component solutions, ranged from 0 to 174. Results of the TLD analysis, comparing normalized excitation and emission spectra extracted for the four components, are shown in Figure 4. Both ALS and DTLD algorithms were employed in this analysis and were found to produce comparable (often nearly identical) results. In general, visual comparisons of spectra suggested slightly better matches with ALS results, and DTLD occasionally generated spectra with negative excursions. Therefore, the ALS profiles are presented here and throughout the remainder of this paper. Figure 4 shows that there is very good agreement between the spectra extracted from the mixtures by TLD and those obtained from pure component solutions. The match of the emission spectra is perfect for anthracene and 2,3-benzofluorene and nearly perfect for the other two components. The agreement is not quite as good for the excitation spectra, but the largest discrepancies come at short wavelengths where the deficiencies of the correction for primary absorption are likely to be most apparent. The poorest matches are for pyrene and 2,3-benzofluorene, which have similar spectra and Stern−Volmer constants. Even in these cases, however, qualitative identification of the fluorophores is not difficult.

In addition to fluorescence excitation and emission spectra, TLD also provides the effective concentration profiles (c' in eq 5) for each fluorophore. A plot of these values, normalized for
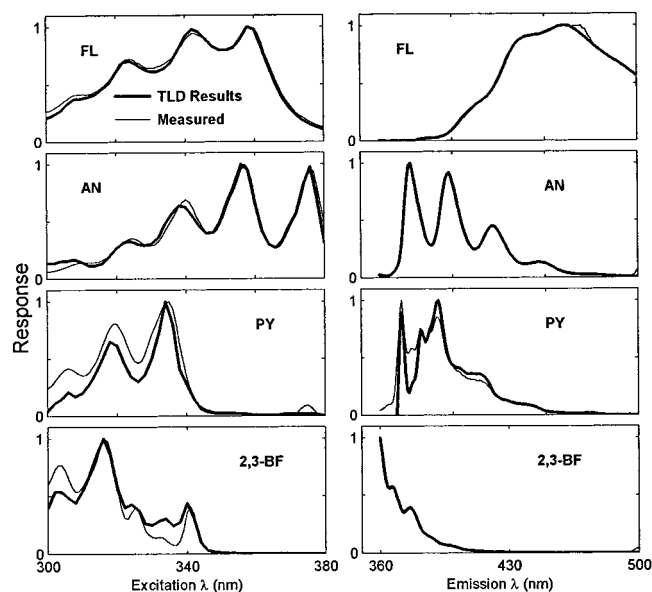
Figure 4. Comparison of TLD results for the synthetic four-component mixture with the excitation and emission spectra measured for the pure components (fluoranthene, anthracene, pyrene, 2,3-benzofluorene), presented in order of increasing Stern−Volmer constant (0, 44, 100, 174).
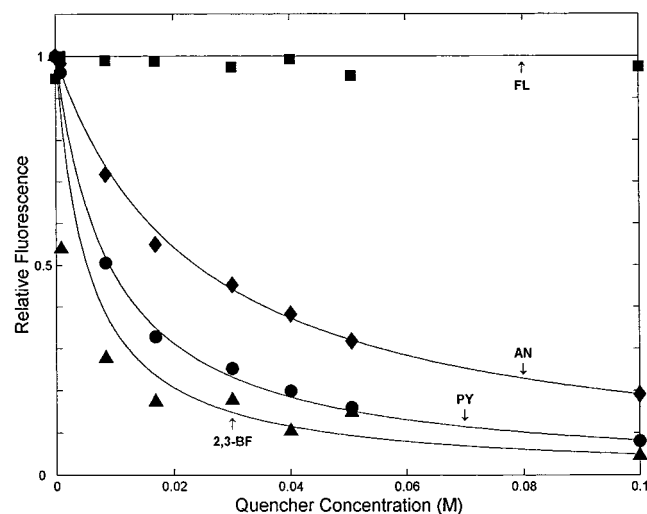

Figure 5. Normalized concentrations extracted by TLD for the synthetic four-component mixture, shown with the quenching curves based on Stern−Volmer constants estimated from the data (0, 42, 110, 191).

fluorophore concentration and initial concentration of unity, is shown in Figure 5. Because these values will be directly proportional to fluorescence quantum yield, they can be used to construct a Stern−Volmer plot (ϕ^{o}/ϕ vs [Q]) from which the Stern−Volmer constant can be obtained for each fluorophore to aid in its identification. Table 2 gives the constants obtained for the four-component mixture using both the ALS and DTLD algorithms, as well as the values obtained from a Stern−Volmer plot of pure component solutions. There is good general agreement in the values, suitable for qualitative confirmation of the identity of the fluorophores. The results from ALS tend to be better than DTLD in this case, and the largest errors are normally observed for the larger constants. This latter observation is not surprising, since the linearization of the Stern−Volmer equation results in larger
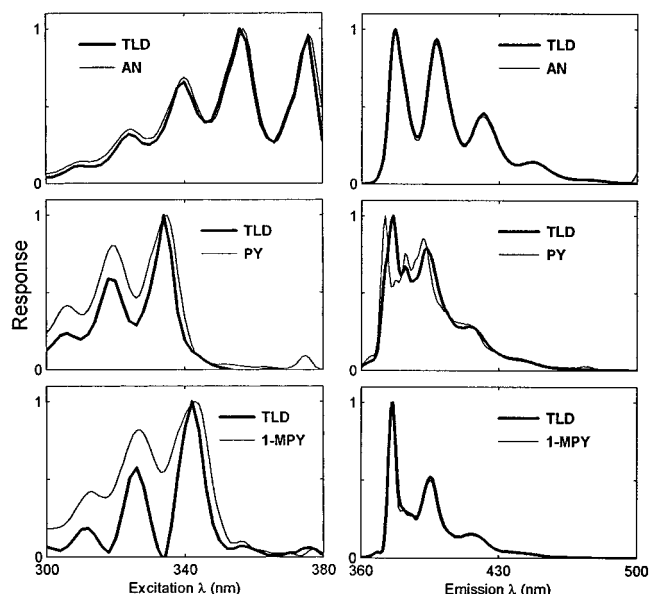

Figure 6. Comparison of TLD results for the synthetic three-component mixture with the excitation and emission spectra measured for the pure components (anthracene, pyrene, 1-methylpyrene), presented in order of increasing Stern−Volmer constants (44, 100, 120).

Table 2. Comparison of Extracted and Measured Stern−Volmer Constants for Four-Component Mixture

	Stern−Volmer constant (M^{-1})		
compound	measured	extracted (ALS)	extracted (DTLD)
fluoranthene	0	0	−1
anthracene	44	42	43
pyrene	100	110	114
2,3-benzofluorene	174	191	221

errors for the points with greater quenching, and it is these points which have the greatest leverage in the fit. Weighted regression or nonlinear fitting might improve the estimates.

Three-Component Mixture. Normally, the natural progression might be to start with a three-component mixture and progress to four components, but in this work, the three-component mixture (data set B) was regarded as the more difficult problem because it contained pyrene and 1-methylpyrene, which had similar spectra ($R_{ex} = 0.79$, $R_{em} = 0.83$) and quenching constants ($k_{SV} = 100$ and 120, respectively). Because it was anticipated that TLD might have difficulty resolving this pair, the mixture was limited to only three components. In hindsight, the performance was good enough that additional mixture components might have been included.

Figure 6 shows the spectra extracted by TLD for this mixture, along with the measured pure-component spectra as before. Once again, the agreement is very good and the largest deviations occur at short wavelengths in the excitation spectra. Table 3 gives the extracted Stern−Volmer constants (using the ALS algorithm) for the mixture components. In this case, the constants are averages obtained from four replicate experiments carried out over several days. The uncertainties represent the 95% confidence interval around the mean and indicate good repeatability. The agreement between the extracted values and those measured for the pure

Table 3. Comparison of Extracted and Measured Stern−Volmer Constants for Three-Component Mixture

	Stern−Volmer constant (M^{-1})	
compound	measured	extracted (ALS)
anthracene	44	41 ±2
pyrene	100	103 ±5
1-methylpyrene	120	126 ±2

component solutions is also very good. Although the reference values are not always encompassed by the confidence intervals, it should be noted that the reference values were obtained from a single experiment in each case and so are not shown with confidence intervals, which would likely overlap those of the extracted values. In addition, systematic sources of differences, such as those that might arise from the absorption correction, have not been included. In view of the similarities among spectra and quenching constants, the qualitative information provided was better than anticipated.

Light Gas Oil Fraction. The analysis of the oil fraction in this work is intended to illustrate a practical application of the TLD fluorescence quenching method. Such petroleum-derived samples are very complex and the large number of PAHs they contain prohibits a complete analysis in a single stage or even multiple stages. Any method that can increase the amount of information provided while reducing the number and/or complexity of the analytical steps represents an improvement. This example provides a comparison of the qualitative information extracted by the TLD analysis to that obtained by more traditional analytical methods which require an additional separation step.

The particular fraction chosen for analysis here was selected because it had been employed in an earlier (unrelated) study and was therefore well-characterized. In the previous study,[37] Lohnes et al. concluded that this fraction contained the three isomers of methylpyrene. Although other PAHs were also known to be present in the fraction, they were not detected in that study because of the fluorescence excitation wavelength used.

In the present work, rank analysis of the fluorescence EEMs for this fraction suggested a pseudorank of 3. Although it is known that there are more than three fluorescent constituents in the fraction, this result was not unexpected since compounds that are structurally similar (and hence have similar spectra and quenching characteristics) may be mathematically indistinguishable. In some respects, this is a useful feature of the TLD method, since it provides information based on compound class rather than individual constituents and is therefore applicable to mixtures whose true complexity might otherwise make the mathematical decomposition unfeasible. The same arguments could be made for soft ionization methods in mass spectrometry which cannot distinguish structural isomers. An important distinction is that the fluorescence quenching may distinguish among structural isomers and, conversely, may group together nonisomers with minor structural differences (e.g., in the length of an alkyl substituent).

TLD of the fluorescence EEMs of the oil fraction, assuming a rank of 3, produced the spectra shown in Figure 7. Extracted
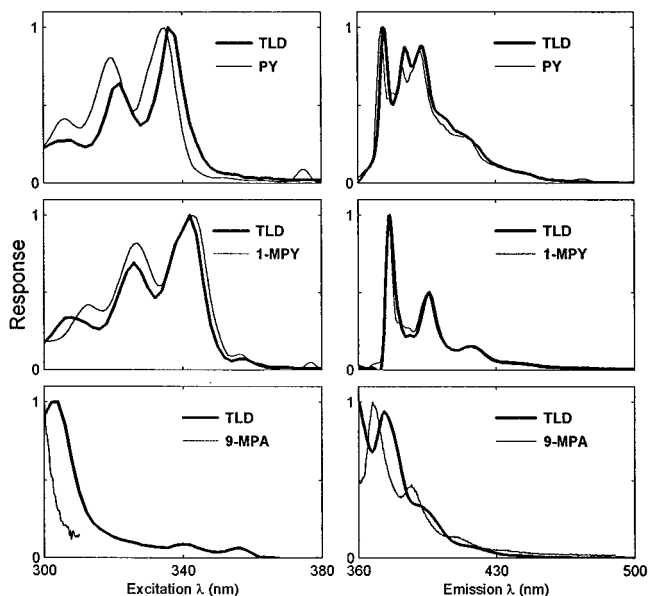
(37) Lohnes, M. T.; Guy, R. D.; Wentzell, P. D. *Anal. Chim. Acta* **1999**, *389*, 95−113.

Figure 7. Comparison of TLD results for the light gas oil fraction with the excitation and emission spectra of nearest available standards (pyrene, 1-methylpyrene, 9-methylphenanthrene). See Table 4 for possible assignments.

Table 4. Extracted Stern−Volmer Constants for Light Gas Oil Fraction and Probable Identity of Components

	Stern−Volmer constant (M^{-1})		
compo-nent	extracted (ALS)	extracted (DTLD)	possible assignment
1	112	106	2- and 4-methylpyrene (k_{SV} (pyrene) = 100)
2	132	123	1-methylpyrene (k_{SV} (1-methylpyrene) = 120)
3	144	146	substituted phenanthrenes (k_{SV} (9-methylphenanthrene) $\approx$ 146)

Stern−Volmer constants are given in Table 4, along with the tentative identification of the associated components. This identification is based on the spectral and quenching characteristics extracted by TLD and confirmation by a second dimension of chromatography with fluorescence and mass spectrometry detection. The second dimension of chromatography indicated that the fraction contained the three methylpyrene isomers as well as an assortment of trimethylphenathrenes and ring-substituted phenanthrenes. (Although other alkyl substitutions isomeric with the trimethylphenanthrenes are technically possible, extensive chromatographic characterization of this fraction has indicated that these do not appear in this fraction.) With regard to the TLD results, component 2 has been identified with a high degree of certainty to be 1-methylpyrene onthe basis of the close agreement of the spectra and Stern−Volmer constants, measured to be 120 using a pure component solution. Component 1 gives a pyrene-like spectrum and Stern−Volmer constant but is anticipated to be a composite of the other two methylpyrene isomers based on the fact that pyrene appears in a different fraction and the spectral characteristics of these two isomers are known from earlier work to differ somewhat from 1-methylpyrene. Unfortunately, standards for 2- and 4-methylpyrene were not available for more complete confirmation, so the spectra and the quenching constant for pyrene

are presented for comparison. Component 3 gives spectra and quenching constants consistent with substituted phenathrenes. Again, the trimethylphenathrene standards were not available, but for comparison, the spectra of 9-methylphenanthrene (the most similar available standard) are shown. The range of excitation wavelengths used in this experiment was just at the edge of the phenanthrene absorption and in the region of high quencher absorption, so this may account for some of the differences in the excitation spectra. The agreement in the emission spectra is especially good in view of the fact that the extracted spectrum is likely a composite of the substituted phenanthrenes. The quenching constant of 9-methylphenanthrene was determined in a separate experiment to be ~146, but this value is likely to have a substantial uncertainty because of the strong overlap of the excitation spectrum with the absorption profile of the quencher.

TLD was also carried out with the assumption of a pseudorank of 4. The fourth component extracted in this case had featureless spectra and a small quenching constant, indicating it was likely only compensating for residual variance, which was not of significant consequence.

These results demonstrate that the TLD fluorescence quenching method can provide qualitative information about the composition of real mixtures without the need for further physical separation. The example also shows that, in certain cases, the TLD will distinguish some isomers from others (i.e., the methylpyrenes) while, in other cases, isomers and structurally similar nonisomers may be grouped together (i.e., the substituted phenanthrenes). Since the goal in the analysis of complex mixtures is often characterization by broad classes rather than an individual breakdown of constituents, this may prove to be a useful feature of this technique.

CONCLUSIONS

This work has demonstrated the utility of TLD for the analysis of fluorescence quenching data from mixtures of fluorophores. Qualitative information is obtained in the form of excitation and emission spectra of mixture components, as well as their Stern−Volmer constants. Features of fluorescence quenching as the third-order measurement for TLD are that no prior knowledge of mixture composition is required and measurements can be made in the steady state. Although fluorescence quenching has been used in conjunction with TLD in the past, the present study differs from earlier work in its more general applicability to a broader class of compounds. In relatively complex mixtures, it was found that components tend to be grouped into classes that have similar spectral and quenching characteristics.

Synthetic mixtures of up to four components were examined in this study, and components as similar as pyrene and 1-methylpyrene were readily resolved. More work needs to be carried out to systematically examine the limitations of the method. The effects of experimental factors such as the number of components present, spectral similarity, relative concentrations, quenching constants, and measurement noise need to be investigated in greater detail. The results presented here suggest that a current limitation is the need to correct for the primary absorbance of the quencher, and better methods may be available for this. The use of different quenchers might not only reduce this problem but should also expand the versatility of the method by providing greater analytical selectivity. Although such aspects remain to be studied, this work has clearly demonstrated the potential benefits of third-order methods in the analysis of complex mixtures.