(2)

AD-A210 142

REPORT DOCUMENTATION PAGE

1a REPORT SECURITY CLASSIFICATION: Unclassified	1b RESTRICTIVE MARKINGS: None
SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release and sale. Distribution unlimited.
DECLASSIFICATION / DOWNGRADING SCHEDULE	
PERFORMING ORGANIZATION REPORT NUMBER(S): ONR Technical Report No. 15	5 MONITORING ORGANIZATION REPORT NUMBER(S)
NAME OF PERFORMING ORGANIZATION: University of Utah; 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION
ADDRESS (City, State, and ZIP Code): Department of Chemistry, Henry Eyring Building, Salt Lake City, UT 84112	7b ADDRESS (City, State, and ZIP Code)
NAME OF FUNDING / SPONSORING ORGANIZATION: Office of Naval Research; 8b OFFICE SYMBOL (If applicable)	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: N00014-89-J-1412
8c ADDRESS (City, State, and ZIP Code): Chemistry Program, Code 1113, 800 N. Quincy Street, Arlington, VA 22217	10 SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO; PROJECT NO; TASK NO; WORK UNIT ACCESSION NO

11 TITLE (Include Security Classification)
Quantitative Estimation of Component Amplitude in Multiexponential Data: Application to Time-Resolved Fluorescence Spectroscopy

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13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Technical	FROM 7/88 TO 7/89	July 1, 1989	31

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Regression methods for chemical kinetics, uncertainty in parameter estimates.

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Attached.

20 DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a NAME OF RESPONSIBLE INDIVIDUAL: Dr. Robert J. Nowak	22b TELEPHONE (Include Area Code): (202) 696-4410; 22c OFFICE SYMBOL

OFFICE OF NAVAL RESEARCH

Grant No: N00014-89-J-1412

R&T Code 413a005---03

Technical Report No. 15

Quantitative Estimation of Component Amplitude in Multiexponential Data: Application to Time-Resolved Fluorescence Spectroscopy

Prepared for publication in Analytical Chemistry

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July 1, 1989

QUANTITATIVE ESTIMATION OF COMPONENT AMPLITUDES IN MULTIEXPONENTIAL DATA: APPLICATION TO TIME-RESOLVED FLUORESCENCE SPECTROSCOPY

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ABSTRACT

Quantitative information of individual component contribution from multi-exponential data is obtained by a reiterative, linear least-squares algorithm. Uncertainty in the parameter estimates, arising from uncertainty in the data and overlap in the response, are predicted from first principles. The analysis method includes weighting to account for the Poisson error distribution arising from shot-noise limited signals, which increases the accuracy of the amplitude estimates. While the algorithm is applicable to a variety of kinetic methods, it is applied in the present work to the analysis of time-resolved fluorescence decay curves. A fluorescence decay curve, written as a row vector, $\underline{D}$, is decomposed into two factors: $\underline{A}$, a column vector containing the amplitude contribution of each component, and [C], a matrix which contains temporal behavioral of each component in its rows. The analysis uses linear least-squares to obtain estimates of $\underline{A}$, which increases the efficiency by reducing the number of parameters which are searched. The theory of error in linear least-squares allows the uncertainty of the component amplitudes to be determined from the [C] matrix, derived from best estimates of the temporal behavioral of the sample.

BRIEF

The individual component contributions to multiexponential data are estimated by a reiterative, linear least-squares algorithm, and the amplitude uncertainties are predicted from first principles.

Quantitative determination of individual component contributions in multicomponent spectroscopic data is a common and difficult analytical problem. For data which derive from first-order kinetic reactions, such as time-resolved fluorescence, metal ion complexation reactions, and the decay of radioisotopes, component contributions to the observed amplitude are particularly difficult to retrieve due to the similar temporal behavior of exponential decay curves. Computational approaches to reaction-rate methods of chemical analysis have been compared by Wentzell and Crouch (1) for reactions following first-order and pseudo first-order kinetics, and the computational difficulties commonly encountered were described. Mieling and Pardue (2) have developed a multiple-linear regression procedure to obtained the amplitudes of sample components simultaneously reacting at different rates. The method was similar to that examined by willis et al. (3) to follow complexation kinetics of alkaline earth complexes. The approach modelled the product concentration as the sum of the integrated first-order rate law for each component plus any other sources of product which are time-independent. The expression which is fit to the data is a sum of an offset and two or three exponentials of known decay constant, where the amplitudes are extracted by linear regression.

Time-resolved fluorescence spectroscopy, where the decay of intensity following pulsed excitation is generally obtained by time-correlated single photon counting techniques, produces data which also follow exponential relationships (4,5). Most data analysis schemes for time-resolved fluorescence (6-9) follow an approach similar to the method described above for chemical kinetics, except that the sensitivity of fluorescence lifetimes to the sample matrix does not generally allow the decay constants to be known in advance. As a result, fitting of the data requires a nonlinear least-squares method since

the observed intensity depends exponentially on the unknown decay constants; these nonlinear parameters appear in argument of the exponential time dependence.

In nonlinear least-squares, optimal values for each parameter are found by directly searching a parameter space while minimizing the squared deviations between theory and experimental results. A sequence of error-reducing steps is chosen where the best direction is reappraised after each step. A complication arises with this approach for fitting multicomponent systems, which results in large increases in computation time and uncertainty in evaluating parameters. Since each resolvable component in the fluorescence decay signal is described by an amplitude and a lifetime or decay constant, the number of parameters which must be optimized grows twice as fast as the number of components in the system. As a result, a three component systems requires that a six-dimensional parameter space be searched, which increases the complexity of the optimization problem and reduces the chances of converging on the solution.

In this work, we present a more efficient approach to the resolution of multiexponential data, where the decay constants are unknown. The method combines a linear regression step to obtain the amplitudes, similar to that described for multicomponent chemical kinetic data (2,3), within a nonlinear least-squares algorithm to determine the best fit decay constants. This approach was first used to resolve multicomponent fluorescence spectra from emission wavelength-decay time data matrices (10). We have modified the method to obtain single pre-exponential factors for each component and to account for the non-homogeneous variance of shot-noise limited data. Proper weighting of the observations is found to significantly influence the accuracy of the results. The theory of error in linear regression has allowed us to develop an

analytical expression which predicts quantitatively the precision of determining individual component contributions. Synthetic data, generated with multiexponential decay curves, and experimental fluorescence decay transients, obtained by time-correlated single photon counting, were used to evaluate the efficiency and accuracy of weighted regression algorithm and its predictions of the uncertainty for the component amplitudes.

THEORY

Modeling Fluorescence Decay Data. In absence of excited state-excited state interactions, the decay of fluorescence following pulsed excitation generally follows first-order kinetics where the response of an n-component sample is given by:

$$d_j = \sum_{i=1}^{n} (a_i/\tau) \exp(-j\Delta t/\tau_i) * I_j \qquad (1)$$

where the intensity, d_j, at time interval j is the sum of intensity contribution of the n fluorophores emitting in the sample. The parameters τ_i and a_i are the lifetime and integrated amplitude of the i^{th} component, respectively, and Δt is the time interval between data points. The asterisk, "*", represents the convolution integral (11), and indicates here that the exponential decay of fluorescence of each component is convoluted with the normalized instrument response function, I_j. Fluorescence decay data can be represented as a row vector, $\underline{D}$, of length t where t is the number of time intervals over which the fluorescence intensity is recorded. This data vector can be expressed conveniently as a product of a vector and a matrix containing the amplitude and time dependence of the components:

$$\underline{D} = \underline{A}\,[C] + \underline{R} \qquad (2)$$

where $\underline{A}$ is a row vector of length n containing the amplitudes or pre-exponential values of the individual fluorophores in the sample and [C] is an (n x t) matrix containing the time decay behavior of the excited state components in its rows. Since the observed data, $\underline{D}$, contains error associated with the observation, there is a difference between the observation and the theory, given by a vector of residuals, $\underline{R}$.

Combining Linear and Nonlinear Least-squares. An efficient method of combining linear least-squares in the analysis of time-resolved fluorescence decays has been described (10), based on assuming uniform variance in the residuals, $\underline{R}$. Given a time-resolved data vector $\underline{D}$, the method decomposes this matrix into it factors $\underline{A}$ and [C] which provide obtain quantitative and lifetime information respectively. In order to carry out this decomposition, a trial matrix $[\hat{C}]$ of normalized time decay curves is constructed row by row, by convolution of the exponential decay of the i^{th} component having an trial lifetime, $\hat{\tau}_i$, with the measured instrument response, I_j, which includes all contributions to temporal dispersion from the excitation pulse and detector response.

$$\hat{c}_{ij} = I_j * (1/\hat{\tau}_i) \quad \exp(j\Delta t/\tau_i) \tag{3}$$

This approach requires the assumption that the excited states of each component decay independently by first-order kinetics. This is an excellent approximation for dilute solutions and high repetition rate excitation, which result in infinitesimal concentrations of excited states.

The unweighted, linear least-squares solution for the vector of best component amplitude estimates $\hat{\underline{A}}$ is given by (12,13):

$$\hat{\underline{A}} = \underline{D}\,[\hat{C}]'([\hat{C}][\hat{C}]')^{-1} \tag{4}$$

where $[\hat{C}]'$ signifies the transpose of $[\hat{C}]$ and the superscript "-1" represents

the matrix inverse operation. A modeled data vector $\hat{\underline{D}}$ is calculated from the product of the amplitude estimates, $\hat{\underline{A}}$, and the trial matrix, $[\hat{C}]$.

$$\hat{\underline{D}} = \hat{\underline{A}}\,[\hat{C}] \tag{5}$$

Optimal parameter values are those which minimize the squared error or chi-square, χ^2, determined by sum of the squared difference between the current model and the observed data:

$$\chi^2 = (1/\sigma^2) \sum_{j=1}^{t} (d_{1j} - \hat{d}_{1j})^2 \;\geq\; (1/\sigma^2)\, \underline{R}\, \underline{R}' \tag{6}$$

χ^2 has a lower limit which depends on the residual error in the data, $\underline{R}$, which is assumed in this case to have uniform variance, σ^2.

Minimization of χ^2 requires an algorithm to search the n-dimensional parameter space of unknown lifetimes. The Nelder Mead SIMPLEX algorithm (14,15) relies on an iterative procedure which moves a simplex, a geometric figure having plane faces and n + 1 vertices in an n-dimensional space, where the vertices represent points at which χ^2 has been evaluated. The major advantage of the present algorithm, which adds a linear least-squares step of Equation 4 into a nonlinear least-squares search, is the reduction in the number of parameters which must be optimized by the nonlinear search procedure. This reduction represents a two-fold lowering in the dimensionality of the search when compared to the more common approach which uses nonlinear least-squares to search both lifetime and amplitude parameters (5).

Weighting Shot-Noise Limited Data. Application of the linear least-squares method of Equation 4 and minimization of the unweighted squared error, Equation 6, both require that the error in the data be described by a homogeneous error distribution of constant variance (12,13). In the case of shot-noise limited, fluorescence decay data, typical of time-correlated single

photon counting experiments (4), the dependence of the signal variance on its amplitude requires that the above solution be modified to account for differences in the expected magnitude of the residuals. Regression methods for single exponential decay data has been shown to be quite sensitive to the inhomogeneous variance of the observations (16), where the efficiency of extracting accurate parameter estimates is significantly degraded if weighting is neglected. The prior information concerning the expected variance of the individual observations, σ^2_j, required to properly weight the observations is readily available for fluorescence measurements made by single photon counting techniques (17), provided that the instrument introduces no excess or systematic errors. Under such conditions, the counting error is Poisson distributed, approaching a Gaussian distribution for a large number of counts (18). The variance, σ^2_j, of the observed data in the j^{th} time interval has an expected value equal to the mean number of counts for that interval (4). Although the mean number of counts is not available from single observation, if the number of counts is sufficiently large ($\geq$100), the variance may be estimated (with <20% error) by the observed number of counts in the time interval, $\sigma^2_j = d_j$. Therefore, the best estimate of the standard deviation of the noise in each channel is simply the square root of the counts in the respective channel. The correct description of the residual error vector, $\underline{R}$, in Equation 2 is a row of random numbers distributed about a zero mean, with varying standard deviation equal to the square root of the number of counts in corresponding element in the observed data vector, $\underline{D}$.

A convenient approach to the linear least-squares determination of the component amplitudes from such data is to multiply both sides of Equation 2 by a factor [W], such that the product of ($\underline{R}$ [W]) is a vector of uniform variance.

$$\underline{D}\,[W] = \underline{A}\,[C]\,[W] + \underline{R}\,[W] \tag{7}$$

A matrix, [W], which accomplishes this goal is a t-by-t diagonal matrix where the non-zero elements w_{jj} are:

$$w_{jj} = 1/\sigma_j = 1/(d_j)^{1/2} \tag{8}$$

The elements of the residuals vector, ($\underline{R}$ [W]), are drawn from a population having the same variance (equal to unity). The weighted squared error, or chi-square, also has a simple form, represented as:

$$\chi^2 = \sum_{j=1}^{t} (d_j - \hat{d}_j)^2/\sigma^2_j \;\geq\; \underline{R}\,[W]\,[W]'\,\underline{R}' \tag{9}$$

which is analogous to Equation 6.

The least-squares solution of Equation 7 for the vector of best estimated component amplitudes takes the same form as Equation 2:

$$\hat{\underline{A}} = \underline{D}\,[W]\,[W]'[\hat{C}]'([\hat{C}]\,[W]\,[W]'[\hat{C}]')^{-1} \tag{10}$$

Since the diagonal elements of the matrix ([W][W]') are equal to $1/\sigma^2_j = 1/d_j$, see Equation 8, then the product ($\underline{D}$ [W][W]') = $\underline{U}$, which is a unit row vector of length, t, where the elements are all equal to one. This substitution results in a simpler form for Equation 10:

$$\hat{\underline{A}} = \underline{U}\,[\hat{C}]'([\hat{C}]\,[W]\,[W]'[\hat{C}]')^{-1} \tag{11}$$

It is interesting to note that all of the information about the measured fluorescence intensity, used to estimate the component amplitudes in Equation 11, resides only in the product ([W][W]') within the inverse term.

Error Estimation. An additional benefit of a linear least-squares determination of the component amplitudes is that errors associated with their estimation can be predicted from first principles. These uncertainties in the estimated component amplitudes are collected into a variance-covariance matrix,

[V], the diagonal elements of which are the variances associated with the amplitude estimates, and the off-diagonal elements are the covariances between parameter estimates. For a two component system the variance-covariance matrix is:

$$[V(a_i)] = \begin{vmatrix} \sigma^2_{a_1} & \sigma^2_{a_1 a_2} \\ \sigma^2_{a_1 a_2} & \sigma^2_{a_2} \end{vmatrix} \quad (12)$$

where $\sigma^2_{a_1}$ and $\sigma^2_{a_2}$ are the variance of the individual, estimated amplitudes of components 1 and 2, respectively, and $\sigma^2_{a_1 a_2}$ is the covariance between them. For data of uniform variance, where the least-squares amplitude estimates are given by Equation 5, the variance-covariance matrix is given by (12,13):

$$[V] = \sigma^2 ([C][C]')^{-1} \quad (13)$$

where σ^2 (a scalar) is the variance of the data, d_j, which for shot-noise limited signals is approximated by the average photon counts in each time channel averaged over the data vector, $\underline{D}$. The magnitude of the variance and covariance terms in [V], thus depends proportionally on the uncertainty in the data multiplied by the correlation in the rows of [C] as reflected in the inverse of [C][C]' (19). Lifetime values of individual components in a mixture, known prior to the analysis or obtained through a non-linear least-squares fit of the data, are required to construct [C] using Equation 1. Confidence bounds on the concentrations estimated using these lifetime values can then be determined using this expression.

When $\hat{\underline{A}}$ is obtained from a weighted linear least-squared method, Equation 11, the variance-covariance matrix is obtained by evaluating (13):

$$[V] = ([C]\ [W][W]'[C]')^{-1} \quad (14)$$

The estimated variance, $\sigma^2_{a_i}$, and covariance, $\sigma^2_{a_i a_j}$, of the ith and jth components depends not only upon the component lifetimes which are used to

construct [C], but also the particular data set which was observed, since the diagonal elements of [W][W]' are equal to $1/d_j$. Unlike linear least-squares for constant variance data (19), weighted least-squares requires that the error estimates be evaluated with each observed set of data, even when the component lifetimes are unchanged, since the relative intensity affects weight of an observation on the estimated results; see Equation 11.

EXPERIMENTAL SECTION

Modeling and data analyses on synthetic and experimental data were performed on a DEC 20/60 computer and a Compac 386 PC using FORTRAN. Subroutines from the IMSL and LINPACK libraries were called in the algorithms. Synthetic fluorescence decay curves were generated numerically by convoluting a Gaussian function of desired width with the decay law given in Equation 3. Random noise vectors, $\underline{R}$, were derived from a Gaussian distribution, the mean of which was zero and standard deviation of which proportional to the square root of the signal. The final data vector $\underline{D}$ was obtained according to Equation 2, by adding the random noise to the fluorescence decay law generated by Equation 1.

Time-resolved, fluorescence decay curves were experimentally obtained using a pulsed laser fluorometer shown in Figure 1. Excitation was accomplished by a mode-locked argon ion laser (Spectra Physics, Model 2000) synchronously pumping a rhodamine 6G dye laser (Spectra Physics, Model 375). The pulse rate of the dye laser output was maintained at 400 kHz by a cavity dumper (Spectra Physics, Model 454). The 604 nm emission from the dye laser was frequency doubled with a KDP crystal (Quantum Technology) and subsequently filtered with a Corning 7-54 UV-transmitting filter. The beam was then

directed onto a sample, and fluorescence was detected at a 90o geometry using a photomultiplier. Data were recorded using Phillips discriminators (Model 6915), LeCroy time-to-amplitude converter and multichannel analyzer (Models 4204 and 3588) controlled by a Leading Edge Model D personal computer via a GPIB interface.

The samples used were prepared by dissolving naphthalene (Aldrich gold labeled) in cyclohexane (Omnisolv). To avoid the uncertainty of preparing multicomponent samples, experimental data of multicomponent systems were generated by first recording transients of single component samples with differing fluorescence lifetimes, and then subsequently adding various transients together to obtain multicomponent fluorescence decay transients. Different fluorescence lifetimes for the naphthalene samples were obtained by varying the concentration of oxygen in the sample, which acts as a quencher. Oxygen was removed by freeze-pump-thawing samples of naphthalene in cyclohexane to a base pressure of <15 millitorr. Data sets with different amplitude parameters were acquired by varying the collection time for individual samples, thus varying the total number of counts in a given data set. The generation of multicomponent data sets by adding together transients of single component systems together not only avoided the uncertainty in sample preparation, but also guaranteed that the parameters describing the individual component behavior in those samples were accurately known.

The performance of each data analysis algorithm were compared by evaluating synthetically generated, fluorescence decay data. The number of iterations, required to reach a condition where chi-squared was changed by less than a designated value, was used as a criterion to judge the efficiency of each method, where an iteration is defined as a successful step in the SIMPLEX

algorithm (14). The effects of ignoring proper weighting factors were determined by comparison of the two linear least-squares algorithms. Equations 4, 11, 13 and 14 were evaluated by solving for $\hat{\underline{A}}$ and [V] using correct lifetime values, and by a simplex search for the lifetimes for 1, 2, and 3 component systems. The resulting estimates of component amplitudes and uncertainties obtained by unweighted and weighted least-squares were compared. Initial starting parameters were kept constant in comparing these methods. The predictions of amplitude variances given by Equations 13 and 14 were first evaluated using synthetic data. Comparison of the predicted component amplitude variance, $\sigma^2_{a_i}$, to the observed variance, $s^2_{a_i}$, obtained by fitting N different synthetic data sets tests the predicting capabilities of this expression. The observed amplitude variance, $s^2_{a_i}$, is determined from N least-squares estimates, $\hat{A}i$, by:

$$s^2_{a_i} = \sum_{i=1}^{N} (\hat{a}_i - \bar{a}_i)^2/N \qquad (17)$$

where a_i is the known or "true" value of the synthetic data set. Different fluorescence decay data were generated by varying both lifetime and amplitude parameters and the number of components in each sample. A total of ten different combinations of fluorescence decay parameters were used to generate 80 sets of data which were analyzed. The criterion used to determine whether the observed variance for synthetic data determined by Equation 17 could be statistically distinguished from the value predicted by Equations 13 or 14 is the F-test (18). The ratio of larger to smaller variance, F, was compared to the critical level for 95 and 99% confidence.

To assess the capabilities of Equations 13 and 14 to predict the uncertainty in amplitude estimates from laboratory data, fluorescence decays of naphthalene dissolved in cyclohexane were also evaluated using two-component

data sets. The fluorescence lifetime and amplitude were first determined for the single component vectors which make up these two-component systems using the weighted least-squares algorithm. Taking these values to represent the "true" or best estimate of the concentration and lifetime parameters (a_i, τ_i), we compared the component amplitudes estimated by linear least-squares analysis of the multicomponent data sets using the "true" lifetime values to build [C]. Results from 10 single component decay transients of naphthalene produced 9 reconstructed multicomponent data vectors which were analyzed, and the accuracy of the results were compared with the error predictions of Equation 14.

RESULTS AND DISCUSSION

Three methods of quantitative analysis of multicomponent fluorescence decay data are evaluated in this study, representing two different approaches to obtaining the fit to a multiexponential model. A direct search approach, in which both the lifetime and amplitude parameters are optimized using the nonlinear least-squares (5,18) to minimize the squared differences between the experimental data and the model, is compared to a second approach in which a linear least-squares step to determine the amplitudes is carried out within a nonlinear least-squares search for the optimum lifetimes. This second, linear least-squares approach is further expanded into unweighted and weighted least-squares methods, which are also compared.

A comparison of efficiency of the three data analysis methods is summarized in Table I, where the numbers of iterations required to converge on the optimal set of fitted parameters are compared. To illustrate the typical quality of fit, Figure 2 shows an example two-component synthetic data transient, along with the predicted fit and weighted residuals obtained by the

weighted linear least-squares algorithm. While all three methods perform adequately when the initial parameter estimates are close (or equal) to the true values, the direct search algorithm for amplitudes and lifetimes is much more sensitive to errors in the initial estimates. Prior information, therefore, is required for the direct search algorithm to successfully converge, particularly when the number of components in the sample is greater than two. For one- and two-component samples, substituting a linear least-squares determination of the amplitudes, which reduces the number of parameters to be search by the SIMPLEX algorithm by a factor two, increases the efficiency of convergence, on average, by about a factor two. From the results in Table I, weighting the linear least-squares fit for the Poisson-distributed error in the data does not appear to systematically affect the efficiency of convergence. Interestingly, the quality of fits as judged by the chi-square values (Equation 9) at the optimum as determined by unweighted and weighted linear least-squares searching show only slight differences. χ^2 was consistently smaller for the weighted least-squares fit by 1.0 to 2.0%.

While the rate of convergence and the apparent quality of fit do not depend strongly on weighting, the accuracy of the amplitudes which are returned by the analysis are sensitive to proper weighting of the observations. This behavior is summarized in Table II, where the accuracy of the component amplitudes which result from unweighted versus weighted least-squares analysis are compared for one-, two-, and three-component synthetic data sets. The relative error in the amplitude estimates is 1.9-times greater on average when $\underline{A}$ is obtained by unweighted least-squares, Equation 4, rather than by proper weighting of the photon shot-noise using Equation 11. The largest improvement provided by properly weighting the data is observed when the fluorescence decay

is dominated by amplitude from shorter-lived components. Over the fixed 500 ns observation time, short-lived components result in a greater dynamic range in the observed fluorescence intensity which creates a correspondingly greater range of measurement variance in the data. While a 1.9-fold improvement in amplitude precision provided by weighting the least-squares fit does not seem too significant, one would need to increase the signal counts by more than 3.5-times to achieve comparable improvements in precision.

An additional advantage of a linear least-squares determination of the component amplitudes is that the uncertainties of the parameters may be predicted from first principles, using Equations 13 and 14 presented above. The variance of the amplitudes determined by the diagonal terms of [V] from these equations were compared with the precision of fitting synthetic data sets, representing a wide range of concentration and decay time values. These comparisons are reported in Table III, along with the corresponding values of the F-statistic used to determine whether there is a systematic difference between the predictions and the observed uncertainties. Interestingly, both the unweighted and weighted least-squares methods of estimating the amplitude precision are equally valid in predicting their corresponding errors, according to these results. Both algorithms produce several F-values which are outside the expected bounds for 95% confidence (not unlikely for 16 results), while both sets of the results are within the 99% confidence bounds. We conclude, therefore, that while unweighted linear least-squares produced somewhat inferior results with respect to the amplitude accuracy compared to proper weighting of the shot noise (see above), amplitude reproducibility can be predicted reliably using this simpler algorithm.

The principle advantage of unweighted linear least-squares is that the

variance-covariance matrix depends only on the lifetimes of the components in [C], scaled by the average variance in the data vector given by the average number counts in each channel for shot-noise limited data; see Equation 13. As a result, this method requires that the [V] be evaluated only once for a given system of component lifetimes, being independent of the relative amplitudes of the components. By contrast, the weighted least-squares strategy uses the measured data vector to define the weighting factors in [W], which are included in the prediction of the amplitude errors in Equation 14. Thus, this equation must be fully reevaluated, and not just rescaled, for every new observation of D, even when the lifetimes of the components do not change.

Since the uncertainty in the component amplitudes determined by linear least-squares can be predicted accurately from first principles by evaluating [V], this theory can be a powerful tool for modeling errors in the analysis of multiexponential data. For example, one can predict a priori how the error in determining the component amplitudes is affected by similarity of lifetimes of the components. Such a prediction provided by Equation 14 is illustrated in Figure 3 for a two-component determination, where the relative standard deviations of the component amplitudes are plotted a function of the ratio of the decay times for two different component amplitudes. Note that the pre-exponential factor in Equation 1 is (a_i/τ_i), so that a_i is the total number of counts independent of the lifetime of emission. The results show the effect of the time-dependence and relative amplitude of the components on the error in determining the amplitudes. As the lifetimes approach the same value, the rows of [C] become identical, and the uncertainty in extracting the amplitudes from a decay transient increases without bound. As the lifetime ratio increases, the uncertainty of extracting each component decreases since the time behavior

of the components becomes more distinguishable.

When the amplitudes of the two components are equal, as shown by the dashed line of Figure 3, the errors in estimating the amplitudes are virtually identical. This is a somewhat surprising result, since one might anticipate that the longer-lived component could be determined with better precision since its intensity persists for a longer time when the first component has decayed away. The reason that the two components have equivalent amplitude error, however, is that the uncertainty of the amplitude determination is dominated by covariance. That is, the error one encounters in this analysis is related to partitioning the amplitude of the observed signal between the two components. Since the actual magnitude of the two amplitudes is the same in this case, the relative errors are equal. When the amplitude of one of the components is larger, as shown in the solid lines in Figure 3, the relative uncertainty of estimating this component decreases due to its proportionally smaller shot-noise, while the smaller-amplitude component exhibits larger uncertainty due to the added background noise from the larger component.

The final goal of this study is to test the data analysis methodology on laboratory fluorescence decay data. To avoid the uncertainties in preparing multicomponent samples, experimental data for these systems were generated by recording transients of single-component naphthalene samples with differing fluorescence lifetimes as controlled by the partial pressure of oxygen. The single-component files were subsequently added together to obtain multi-component fluorescence decay transients having known individual component behavior. An example of such a transient, its fit to a biexponential model determined by the weighted least-squares algorithm, and the weighted residuals from the fit are shown in Figure 4. The relative errors in the component

amplitudes estimated by the weighted least-squares algorithm are compared to the relative standard deviation predicted by Equation 14 for 9 data sets, where the long-lived component amplitude was held constant and the short-lived component amplitude was varied. The results are plotted in Figure 5, showing the excellent agreement between the theoretical predictions and the observed errors. An F-test indicates that all of the observed results fall well within the 95% confidence limits.

In summary, we have developed an efficient method for resolving multiexponential data in the case where the decay constants are unknown. The method adds a linear least-squares step to the nonlinear least-squares search for the lifetime parameters. The linear least-squares algorithm was significantly more efficient in determining an optimal set of parameters compared to the more common, direct search procedure. In cases of three component data, the linear least-squares algorithm could tolerate over 75% error in the initial parameter estimates while the latter method would not converge on a solution unless the initial estimates were very near the true value. Correctly weighting of shot noise in fluorescence decay data was found to have a minimal impact on the rate of convergence but significantly improved the accuracy of the estimated component amplitudes. The linear least-squares formalism also allows one to predict quantitatively the magnitude of the errors to expect in the amplitude estimates. This capability can be powerful for modeling the accuracy of kinetic methods of analysis for multicomponent samples. While the data and error analysis methods were evaluated using time-resolved fluorescence decay data, the results are equally valid for optimizing the analysis and predicting amplitude errors for any method which produces a first-order kinetic response.

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CREDIT

This work was supported in part by the Office of Naval Research.

TABLE I. Number of Iterations Required to Satisfy Convergence Criteria [a]

Relative Error of Initial Parameter Estimate [b]	Direct Search Algorithm	Unweighted Linear Least Squares Algorithm	Weighted Linear Least Squares Algorithm
		one component system	
0%	108	82	98
50%	198	68	86
75%	188	64	62
		two component system	
0%	177	110	104
50%	<50% convergence [c]	111	122
75%	no convergence	144	120
		three component system	
0%	196	110	120
50%	no convergence	160	167

a Each result is an average of fitting 7 decay transients. Convergence was defined as an accepted SIMPLEX move which did not improve χ^2 by more than 1 part in 10^5.

b Relative error of the initial parameter estimate is the difference between the initial estimate and known value of fitting parameter divided by the known value.

c Convergence within 250 SIMPLEX moves occurred less than half the time.

Table II. Relative Error of Amplitude Estimates from Weighted and Unweighted Least Squares. [a]

Sample	τ_1	τ_2	τ_3	Avg. Amplitude Error (%), Weighted LS	Avg. Amplitude Error (%), Unweighted LS
One Component					
	100 ns			0.35 %	0.35 %
	150 ns			0.32 %	0.46 %
Two Component					
	100 ns	125 ns		3.4 %	2.1 %
	100 ns	150 ns		1.1 %	2.0 %
Three Component					
	75 ns	150 ns	250 ns	1.6 %	1.2 %
	50 ns	150 ns	250 ns	0.7 %	3.9 %

[a] Average of 4 trials, 500 point synthetic decay transients where Δt = 1.0 ns. Lifetimes used in the amplitude determination were the correct values. Component amplitude factors, a_i, ranged from 1.0×10^5 to 3.0×10^5 counts/ns.

Table III. Component Amplitude Variance Predicted (σ^2), Observed (s^2), and Compared by the F-Test. [a]

A(1)/A(2)	$\tau(1),\tau(2)$	$\sigma(1)$	$\sigma(2)$	$s^2(1)$	$s^2(2)$	F(1)	F(2)
			Unweighted Linear Least-Squares [b]				
1:1	150,200	0.9294	0.6865	0.6678	0.4917	1.39	1.40
1:1	90,150	0.3850	0.2182	0.7946	0.3466	2.06	1.59
1:1	210,280	0.9942	0.7517	1.3301	0.9679	1.34	1.29
2:3	100,200	0.3206	0.1087	0.2664	0.0857	1.20	1.27
3:7	100,200	0.2393	0.1129	0.1746	0.0625	1.37	1.81
3:7	200,400	0.2501	0.1325	0.0680	0.0413	3.68	3.21
3:7	300,600	0.3367	0.1993	0.1415	0.0548	2.38	3.64
3:7	150,300	0.2284	0.1140	0.2008	0.1190	1.14	1.04
			Weighted Linear Least-Squares [c]				
1:1	150,200	0.6160	0.3889	0.7188	0.5361	1.17	1.38
1:1	90,150	0.2539	0.1026	0.6282	0.2497	2.47	2.43
1:1	210,280	0.7601	0.5166	1.4292	1.0096	1.88	1.95
2:3	100,200	0.1828	0.0607	0.1695	0.0409	1.08	1.48
3:7	100,200	0.1945	0.0666	0.0964	0.0325	2.02	2.05
3:7	200,400	0.2245	0.1010	0.0686	0.0531	3.27	1.90
3:7	300,600	0.3156	0.1711	0.2586	0.1073	1.22	1.59
3:7	150,300	0.1967	0.0778	0.1882	0.1119	1.05	1.44

[a] A total of 10 decay profiles of 500 points were constructed synthetically for each combination of lifetime and amplitude.

[b] Component amplitudes found using Equation 4 and variances predicted by Equation 13.

[c] Component amplitudes found using Equation 11 and variances predicted by Equation 14.

FIGURE CAPTIONS

1. Block diagram of single photon counting fluorometer. PD - photodiode, PM - photomultiplier, S - sample.

2. Synthetically generated two-component fluorescence decay curve. Decays constructed from 500 points, where $a_1 = 1.0 \times 10^5$, $a_2 = 1.5 \times 10^5$ ns, $\tau_1 = 100$, $\tau_2 = 150$ ns, and $\Delta t = 1.0$. (a) fitted data, (b) weighted residuals, (c) histogram of weighted residuals and corresponding Gaussian distribution, having a zero mean, a unit standard deviation, and an area equal to the total number of counts.

3. Predicted variance for two-component fluorescence decay curve as a function of lifetime ratios. Synthetic data constructed from a 500 point decay, where $a_1 = 2.0 \times 10^5$, $a_2 = 1.0 \times 10^5$, and $\Delta t = 1.0$.

4. Experimental two-component fluorescence decay data fit to a biexponential model. Data derive from two samples of naphthalene, where $a_1 = 1.39 \times 10^4$, $a_2 = 2.08 \times 10^4$, $\tau_1 = 85.5$ ns, and $\tau_2 = 107.7$ ns. (a) fitted data, (b) weighted residuals, (c) histogram of weighted residuals and corresponding Gaussian distribution, having a zero mean, a unit standard deviation, and an area equal to the total number of counts.

5. Relative error in component amplitude from weighted least-squares fitting of experimental data. The amplitude of Component 1 was held constant, $a_1 = 4.28 \times 10^5$ counts, while the a_2 was varied as shown. The fluorescence lifetimes, τ_1 and τ_2, were 107.7 ns and 85.5 ns, respectively. Circles and rectangles are the observed relative errors for single determinations of a_1 and a_2, respectively, while the solid lines are the predicted relative standard deviations from the variance-covariance matrix, Equation 14.

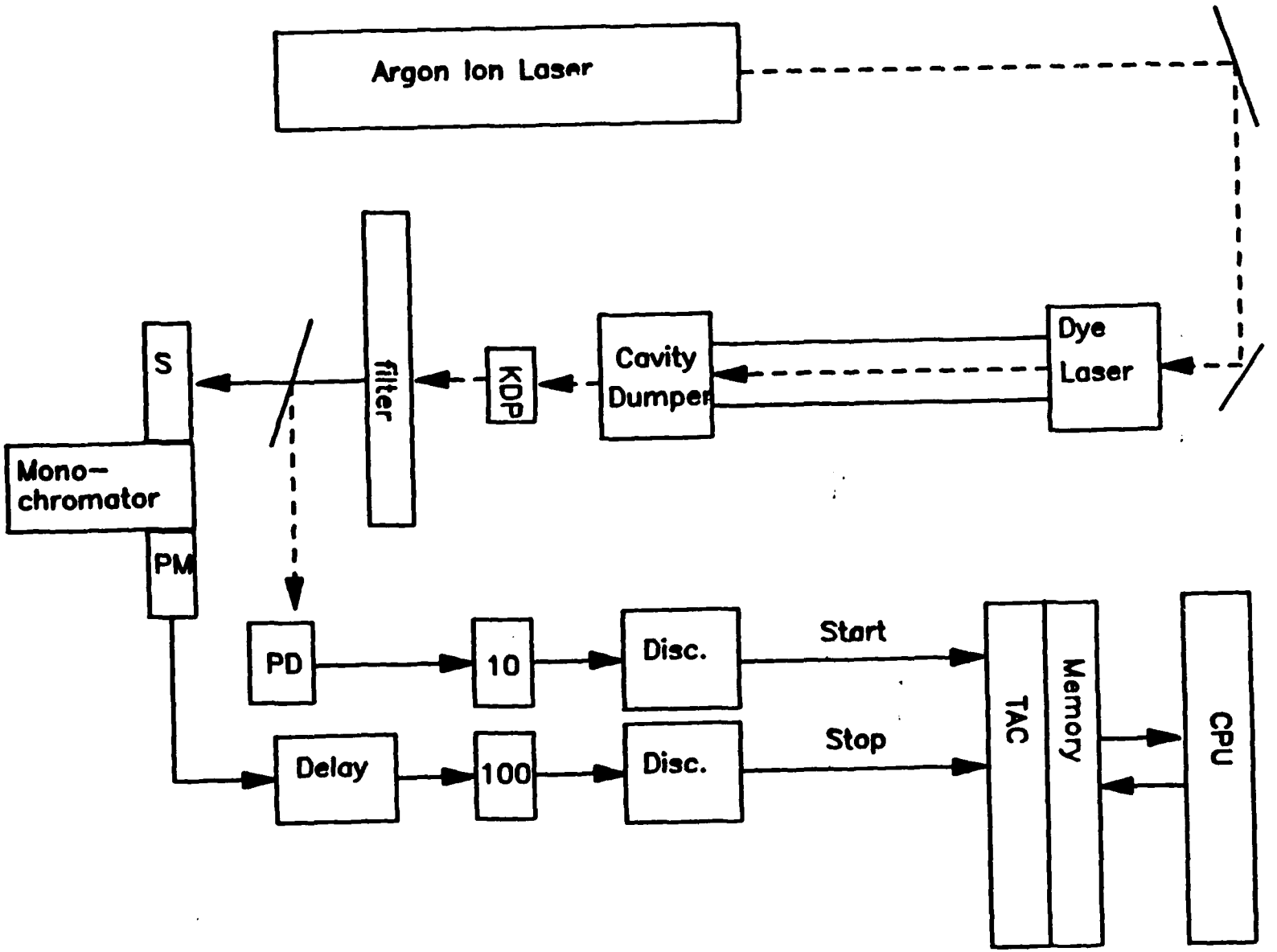
Argon Ion Laser
Dye
Laser
Cavity
Dumper
KDP
filter
S
Mono-
chromator
PM
PD
10
Disc.
Start
Delay
100
Disc.
Stop
TAC
Memory
CPU

FIGURE 1

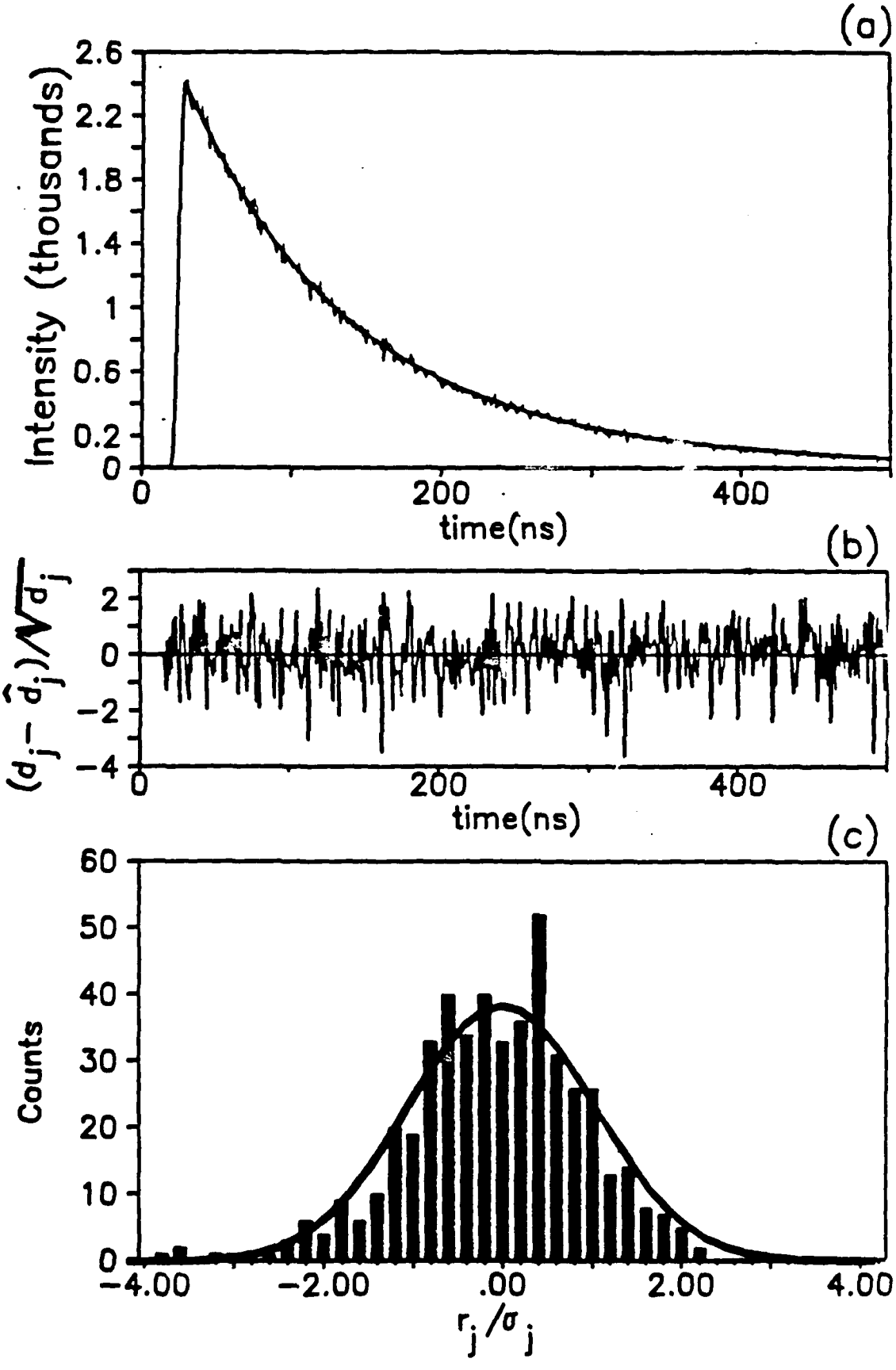
(a)
Intensity (thousands)
2.6
2.2
1.8
1.4
1
0.6
0.2
0
0
200
400
time(ns)
(b)
$(d_j - \hat{d}_j)/\sqrt{d_j}$
2
0
−2
−4
0
200
400
time(ns)
(c)
Counts
60
50
40
30
20
10
0
−4.00
−2.00
.00
2.00
4.00
r_j/σ_j

FIGURE 2

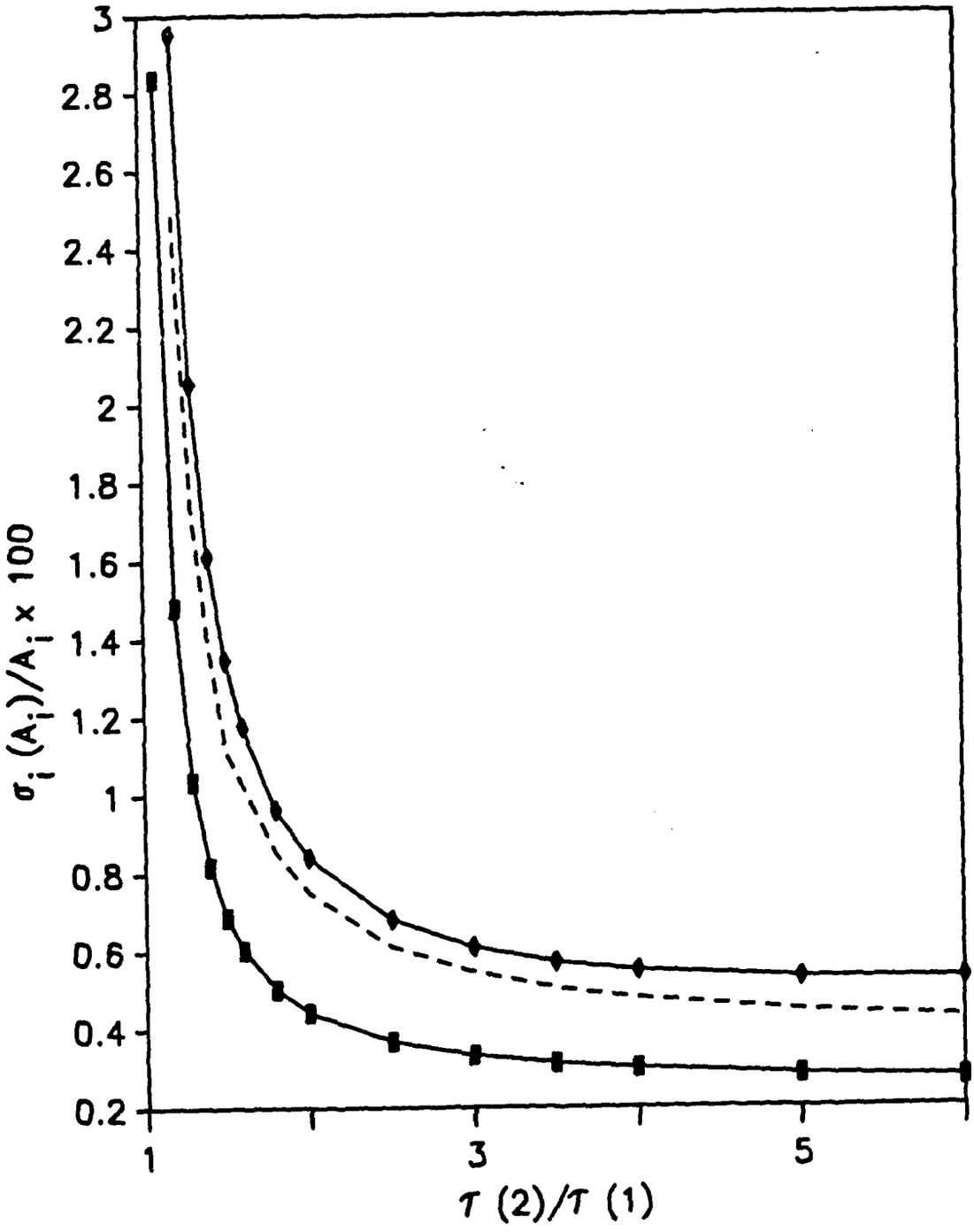
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
$\sigma_i (A_i)/A_i \times 100$
1
3
5
$T(2)/T(1)$

FIGURE 3

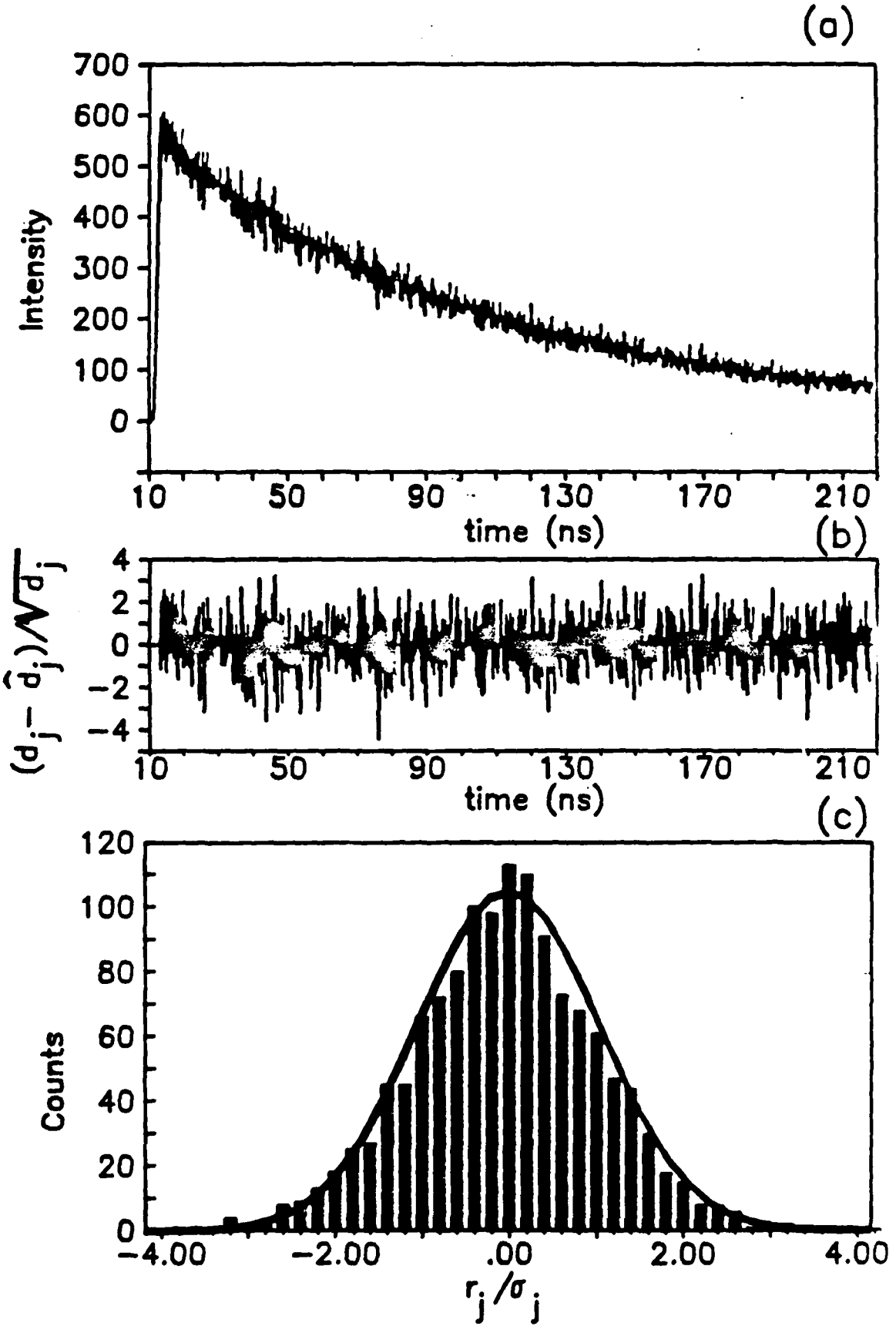
(a)
700
600
500
400
300
200
100
0
Intensity
10
50
90
130
170
210
time (ns)
(b)
4
2
0
−2
−4
$(d_j - \hat{d}_j)/\sqrt{d_j}$
10
50
90
130
170
210
time (ns)
(c)
120
100
80
60
40
20
0
Counts
−4.00
−2.00
.00
2.00
4.00
r_j/σ_j

FIGURE 4

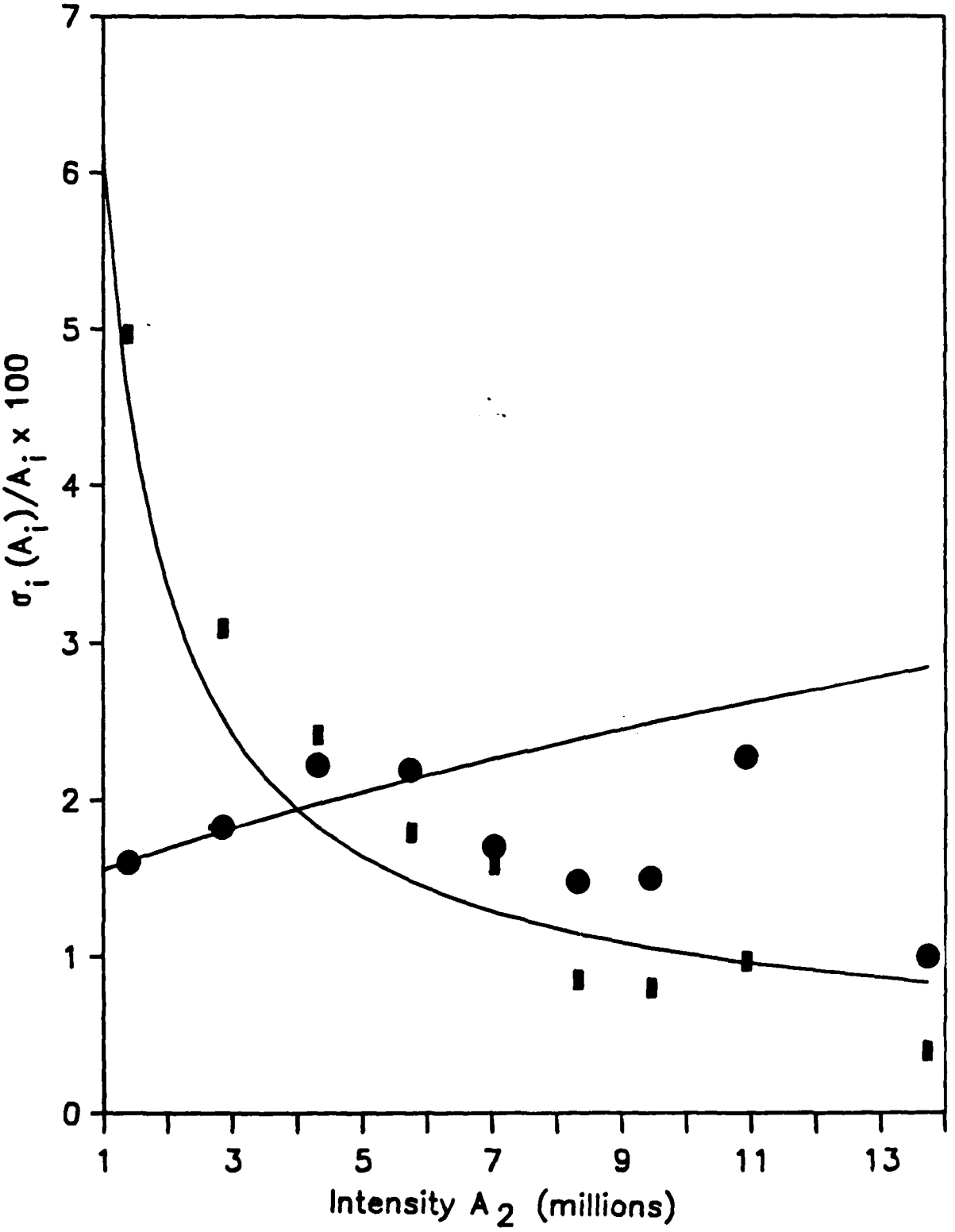
7
6
5
4
3
2
1
0
$\sigma_i(A_i)/A_i \times 100$
1
3
5
7
9
11
13
Intensity A_2 (millions)

FIGURE 5