ROBUST ESTIMATION IN PULSE FLUOROMETRY

A Study of the Method of Moments and Least Squares

IRVIN ISENBERG
Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

ABSTRACT Most laboratories use least-squares iterative reconvolution (LSIR) as a routine method for estimating decay parameters in pulse fluorometric data. It is shown here, however, that LSIR is very sensitive to small amounts of error in the data whenever two decays become too close to one another, or whenever analyses of three decays are attempted. In such cases, inferior methods of estimating integrals, small zero point shifts, or small errors in the measured exciting light will result in failures of least squares, where the method of moments, with moment index displacement and λ invariance testing, will succeed. The method of moments is therefore robust with respect to such errors while least squares is not.

INTRODUCTION

In 1960, in a now-classic paper (1), Tukey showed that estimation of a location[1] by least squares was a very sensitive function of how the noise is distributed. While least squares is good if the noise exactly satisfies a Gaussian distribution, very small deviations from that distribution, so small as to almost always occur in practice, lead to the deterioration of least squares as a desirable estimator.

In 1972, Andrews et al. (2) published an extensive study of location estimators and concluded that, of 65 different estimators, least squares was generally the worst, except when the noise distribution was strictly Gaussian. On the other hand, a number of other estimators gave results that were orders of magnitude better than least squares under a variety of distributions, and were almost as good as least squares when the distribution was strictly Gaussian. Such estimators are said to be robust; more specifically they are robust with respect to the underlying distribution.

In general, a robust method of estimating parameters from a set of data is one that is insensitive to small deviations from the assumptions underlying the choice of the estimator (3). As one is never certain that all of the assumptions are satisfied exactly, robustness is a property that any good method should have.

Statisticians have devoted considerable attention to robustness in the presence of outliers, and robustness with respect to deviations in the underlying noise distribution, but, of course, one can consider robustness with respect to other perturbations. Thus, for example, robustness has been considered in signal processing by filters in which it is desirable to protect the output against uncertainties in the input signal (4).

In the present paper we consider robustness with respect to nonrandom errors in fluorescence time decay. We shall show that least squares iterative reconvolution (LSIR) becomes nonrobust with respect to such errors under conditions in which the method of moments maintains robustness, and that important circumstances exist where poor parameter estimation results from using LSIR, a nonrobust method, rather than from any inherent property of the raw data itself.

The study of robust methods has a long history (5), and in the last 25 years it has undergone an extensive development. Many types of estimation problems have now been studied, and there is a large, and growing, literature on the subject (see for example references 3, 7, and 8, and references therein). It is now firmly established that least-squares methods are generally not robust, and this realization has prompted workers to develop better methods for handling data for a variety of cases: linear regression, fitting to polynomials, and even general nonlinear problems (3–12).

In addition to not being robust, least-squares estimation has other serious faults: In general, it is not resistant (8), which means that for many problems the estimated parameters are often sensitive to small changes in the data set. As a result of its nonresistance and nonrobustness, residual plots will tend to look random even when the estimated parameters are poor (10, 12). Thus, in many cases where least-squares estimation is used, residual plots will not be trustworthy guides for judging the acceptability of estimated parameters.

Robust methods have become widely developed in engineering, but have as yet had only minimal impact in biophysics. Most biophysicists are unfamiliar with the

[1] A location estimator is one which estimates the value of a constant from a set of measurements. For location estimates the least-squares estimate is the same as the arithmetical average.

concept of robustness; most do not know that an extensive literature on robustness exists, and, in fact, most generally hold least-squares methods in high esteem. An anomalous situation therefore exists: Many scientists hold an opinion diametrically opposed to conclusions firmly established in the literature. Work on the estimation of fluorescence decay parameters has not escaped from this anomaly.

In 1974, Grinvald and Steinberg (13) published an LSIR procedure for analyzing fluorescence decay data. Somewhat later, two papers appeared that concluded that estimation by LSIR was generally the best method available (14, 15). Today, most laboratories use LSIR routinely. However, we will show here that there are important cases in which LSIR is not the method of choice. If used, it will artificially limit the capabilities of pulse fluorometry. When a better method is used, one can obtain results that cannot be obtained with least squares.

It has long been known (see, for example, references 16 and 17) that the estimation of decay parameters is an ill-conditioned problem. In any ill-conditioned estimation problem, the estimated parameters will be sensitive to errors, either random or nonrandom, and the effects of such errors will be severe if a nonrobust method of estimation is used. Furthermore, the effect of small errors will become more severe as the estimation problem becomes more difficult. Thus, analyses will become harder as decays get closer to one another, or as we treat problems with more parameters to find. Attempts to measure or compensate for errors are useful, of course, but such attempts cannot be a satisfactory substitute for the use of robust methods, since errors known to be present cannot be compensated for to an arbitrarily small extent, and there may be errors of unknown origin that cannot be measured at all.

In this paper we compare the method of moments with LSIR. Our primary aim is to investigate the influence of errors on the analyses, and we do so by computer simulation, because in such simulations the nature and magnitude of an error can be more carefully controlled than in real experiments. Two precautions must be taken, however. First, one must choose decays that represent cases that can be found in a real situation. To this end we have chosen as the main example decays of 8.8 and 12.8 ns, since one can prepare mixtures of dyes having these decays, collect data on such mixtures, and analyze them. Second, we have repeated the simulations a number of times, with different batches of random noise, to avoid the possibility of accidentally biased results on only one or two tries. We will show that least-squares estimation is very sensitive to errors and, if used, will set the bounds of what is feasible in pulse fluorometry. We will show that the method of moments permits a wider range of feasible experiments. With the method of moments, we will use moment index displacement (18–21), exponential depression (16), and lambda invariance tests (22, 23). The method as a whole is then insensitive to the existence of important nonrandom errors. It may be said to be robust with respect to such errors.

MATERIALS AND METHODS

2-aminonaphthalene-6-sulfonic acid and 5-dimethylaminonaphthalene-1-sulfonic acid were obtained from Molecular Probes. The purities were checked by thin layer chromatography. The solvent was Gold Shield absolute ethanol. Samples were made with absorbances at 300 nm of 0.018 for 2-aminonaphthalene-6-sulfonic acid and 0.063 for 5-dimethylaminonaphthalene-1-sulfonic acid. Fluorescence was excited at 300 nm and the emission observed through a Corning 0-52 cutoff filter (Corning Medical and Scientific, Corning Glass Works, Medfield, MA). Mixtures were made by taking equal volumes of the solutions. The exciting source was a Spectra-Physics Inc. (Mountain View, CA) picosecond laser assembly. A mode-locked 171-09 argon ion laser synchronously pumped a model 343-01 dye laser. A model 344S-01 cavity dumper dumped 600-nm pulses at a repetition rate of 400 kHz. The pulses had widths of 10 ps. The pulses were doubled in frequency using an Inrad angle phase-matched KDP crystal. Scatter was measured with a dilute solution of Ludox. Data were collected by monophoton fluorometry.

In analyses by LSIR we generally followed McKinnon et al. (14) except that, for reconvolution, some analyses were run with Simpson integration as well as the linear interpolation-integration scheme of McKinnon et al. Simpson integration is more precise than linear interpolation, but in some cases requires appreciably more computer time. Convergence was taken to be achieved when χ^2 changed by $<10^{-6}$ in two successive loops of fitting. Analyses were run on a PDP 11/34 computer (Digital Equipment Corp., Marlboro, MA).

In least-squares estimation, fitting was usually done over a range in which the fluorescence stayed above 30 counts, although, as discussed below, other ranges were sometimes used. A least-squares estimate was judged to be acceptable if it gave a χ^2 value below 2.00, and if both residual plots and autocorrelation functions of the residuals appeared random (24). An estimate by the method of moments was judged acceptable when it passed a lambda invariance test (22, 23). In the cases studied here, whenever an analysis passed a λ invariance test it also passed an MD incrementation test: Parameters estimated by $MD = 2$ agreed with those estimated by $MD = 1$. All analyses also passed a component incrementation test: When data known to have n decays were analyzed for $n + 1$ decays, the extra decay had a negligibly small amplitude. (In situations where the number of components is unknown, the component incrementation test determines that number within the resolution of the data [25]).

RESULTS

The decays of 2-aminonaphthalene-6-sulfonic acid and 5-dimethylaminonaphthalene-1-sulfonic acid in ethanol were found to be 8.8 and 12.8 ns, respectively. The decays from mixtures of the two fluorophores were also measured. These were analyzed by LSIR, using integration by linear interpolation (14), and by Simpson integration, and by the method of moments. Table I A shows the results for the method of moments. Fig. 1 shows the λ invariance plots for the method of moments analysis of sample 1. The curves for $MD = 1$ and $MD = 2$ show a region where the plots are essentially flat. $MD = 0$, which does not correct nonrandom errors, gives a sloping curve at all points. The λ invariance test (22, 23) states that one should choose the parameters where the plots are flat. The values of Table I *A* are those for $\lambda = 0.004$ and $MD = 1$. A component

TABLE IA
ANALYSES OF FLUORESCENCE OF MIXTURES

Sample	Counts in F	LSIR, Simpson α_1	τ_1	α_2	τ_2	χ^2	Method of Moments MD = 1 α_1	τ_1	α_2	τ_2
		10^{-3}	ns	10^{-3}	ns		10^{-3}	ns	10^{-3}	ns
1	8.8×10^6	7.97	11.79	4.94	11.79	603.5	4.09	8.54	3.71	12.78
2	8.9×10^6	7.71	11.80	4.77	11.80	1186	4.10	8.54	3.70	12.79
3	9.7×10^6	7.78	11.67	5.16	11.67	1068	4.05	8.59	3.50	12.83

Analyses of three sets of data on mixtures of 2-aminonapthalene-6-sulfonic acid and 5-dimethylnapthalene-1-sulfonic acid. Analysis was by LSIR using Simpson integration and by the method of moments. LSIR estimation was done by fitting over the entire range in which the fluorescence was 30 counts. Initial values were taken to be $\alpha_1 = 0.01$, $\tau_1 = 8.0$ ns, $\alpha_2 = 0.01$, $\tau_2 = 12.0$ ns, but the analyses were insensitive to the choice of initial values.

incrementation test is shown in Table I B. In each case a third component has negligibly small amplitude, thus verifying that the decay had two components.

LSIR analyses over the range in which the counts in the emission stayed above 30 all yielded χ^2 values that were much too high for any of the analyses to be acceptable (Table I A). It is clear that the data are distorted by an error of unknown origin that is corrected to a large extent by *MD*.

Estimations by LSIR were also made by using narrower fitting ranges. The results for sample 1 are shown in Table I *C*. Sample I had a fluorescence maximum at channel 58. The fluorescence stayed above 30 counts from channel 50 to channel 438, and in Table I C all ranges had an upper channel limit of 438.

The χ^2 values dropped below 2.00 only when the lower fitting limit was ≥ 58, the maximum of fluorescence. As the lower limit increased, the χ^2 value decreased. When it was in the vicinity of the maximum, the analyses were quite good. However, the residual and autocorrelation plots appear nonrandom over all ranges of fitting, including the range that gives a good analysis (Fig. 2), and unless one knew the answers beforehand, one could have no reason for preferring one over another.

To elucidate the problems involved in estimating the decay constants of a sample, when the actual decays are

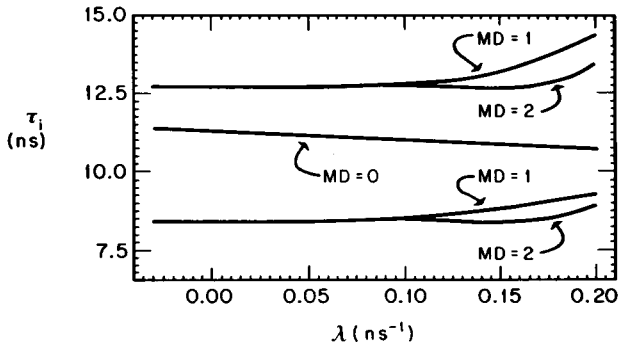

FIGURE 1 Lambda invariance test for the analysis of sample 1 of Table IA.

TABLE IB
METHOD OF MOMENTS—COMPONENT INCREMENTATION TEST

Sample	α_1	τ_1	α_2	τ_2	α_3	τ_3
	10^{-3}	ns	10^{-3}	ns	10^{-3}	ns
1	4.40	8.78	3.36	12.96	7.08×10^{-2}	2.65
2	3.98	8.54	3.59	12.78	-7.09×10^{-13}	−12.26
3	4.29	8.65	3.37	12.94	-3.5×10^{-6}	29.16

Component incrementation test on method of moments data of Table IA.

TABLE IC
LSIR ANALYSES AT VARIOUS DATA RANGES

Lower channel limits	α_1	τ_1	α_2	τ_2	χ^2
	10^{-3}	ns	10^{-3}	ns	
50	8.0	11.79	4.9	11.79	603.5
56	8.2	8.92	7.1	12.71	7.82
57	10.5	9.27	4.9	13.55	2.38
58	9.0	8.82	6.5	13.00	1.65
60	8.6	8.69	7.0	12.90	1.64
100	7.5	8.53	7.9	12.60	1.44
120	7.2	7.52	9.2	12.38	1.27
150	6.9	7.69	9.2	12.37	1.30
200	22.0	4.87	11.0	12.10	1.21

LSIR analyses of data of sample 1 using various ranges of fitting. The fluorescence remained above 30 counts from channel 50 to channel 438. In all analyses the upper limit was channel 438.

8.8 and 12.8 ns, and to see the robustness with respect to nonrandom errors, successive sets of data were synthesized with these decays, and noise added with different batches of multinomial random deviates. For a least-squares estimation it is necessary, of course, to make an initial guess of the parameters. For the analyses for which the expected values were $\alpha_1 = 5.76 \times 10^{-3}$, $\tau_1 = 8.8$ ns, $\alpha_2 = 3.98 \times 10^{-3}$, $\tau = 12.8$ ns, initial values were chosen to be $\alpha_1 = 4 \times 10^{-3}$, $\tau_1 = 8.0$ ns, $\alpha_2 = 4 \times 10^{-3}$, $\lambda_2 = 12.0$ ns.

Thirteen data sets were analyzed both by LSIR with

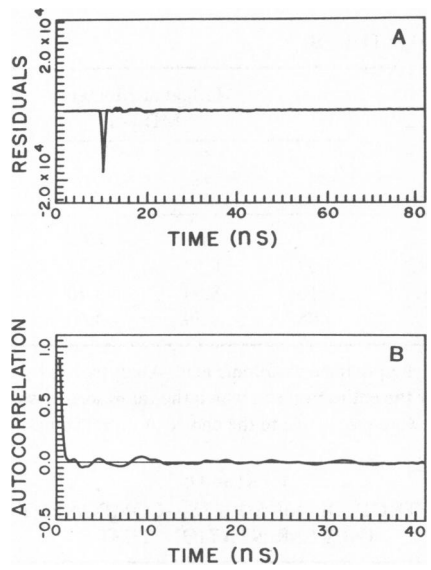

FIGURE 2 Residual plot (*A*) and autocorrelation plot (*B*) of analysis of range 58-438 of Table IC. Note the nonrandom nature of both plots.

Simpson integration, and by the method of moments. Three of the former were rejected because they did not converge; three analyses by the method of moments were rejected for not passing a λ invariance test. Seven were satisfactorily analyzed by both methods and the results are shown in Table II. Both methods give satisfactory parameters. However, LSIR does not yield satisfactory parameters when integration is by linear interpolation (14) (Table III). Integration by linear interpolation is satisfactory only if the decays are sufficiently separated from one another. Thus, for example, data synthesized with $\tau_1 = 5$ ns, $\tau_2 = 10$ ns, and equal amplitudes, analyzed well.

When small amounts of nonrandom error are present, analyses by LSIR become unsatisfactory even when Simpson integration is used. We introduced a 10 ps zero point shift into sets of synthesized data. Out of 18 sets of data, only 5 converged in 300 loops of iteration, and all 5 of these gave large values of χ^2. Table IV shows the results. Even though the decay times that were found would be satisfactory for many purposes, one would have no way of knowing this, since the χ^2 values are so high. One may contrast this situation with that of Table III where the χ^2 values are excellent even though the estimated parameters are poor.

It has been shown experimentally (19) that *MD* corrects analyses when the measured $E(t)$ differs from the true $E(t)$, provided of course that the differences are not too large. This effect had been predicted by a theoretical analysis (18) although we still do not have a complete theoretical understanding of this phenomenon.

To illustrate the use of the λ invariance test in this case, we mimicked the effect of having a slightly incorrect lamp profile by synthesizing data with an excitation

$$E(t) = Bt^{10}e^{-7.05t}$$

and analyzing it with

$$E(t) = Bt^{10}e^{-7.00t}.$$

The results are shown in Table V. Analyses by LSIR are poor; those by the method of moments are good.

The correction by *MD* is not due to the particular analytical form used for $E(t)$. For one thing, we know that *MD* corrects estimations due to real lamp errors (18, 19), where no analytical form for $E(t)$ is known, but where it is certainly not an exponential multiplied by a power of time. For another, we know from simulation studies that if the exponent used is 7.10 rather than 7.05, $MD = 1$ no longer corrects the estimation adequately, although $MD = 2$ does.

In Table VI, we show the results of three component analyses. Here LSIR takes a large amount of time. With Simpson integration and the conditions described in the Materials and Methods section, an analysis such as shown in Table VI does not converge in 2 h and the method is not

TABLE II
ANALYSES WITHOUT ADDED NONRANDOM ERROR

Sample	LSIR, Simpson				Method of moments *MD* = 1		
	τ_1	τ_2	α_2/α_1	χ^2	τ_1	τ_2	α_2/α_1
1	8.81	12.83	0.68	0.91	8.82	12.85	0.66
2	8.73	12.73	0.70	1.02	8.73	12.73	0.74
3	8.92	13.08	0.57	0.95	8.89	12.93	0.63
4	8.81	12.83	0.67	1.18	8.85	12.89	0.64
5	8.96	13.13	0.55	0.99	8.83	12.89	0.65
6	8.90	13.01	0.60	0.98	8.73	12.71	0.74
7	8.97	13.14	0.55	1.07	8.88	12.97	0.62
Mean	8.87	12.96	0.62		8.81	12.82	0.67
Standard deviation	±0.09	±0.16	±0.06		±0.06	±0.08	±0.05
Expected	8.80	12.80	0.69		8.80	12.80	0.69

Analyses of synthetic data by LSIR using Simpson integration, and by the methods of moments. 3×10^7 counts in excitation and emission.

TABLE III
ANALYSES WITHOUT ADDED NONRANDOM ERROR

Sample	LSIR, linear interpolation				Method of moments $MD = 1$		
	τ_1	τ_2	α_2/α_1	χ^2	τ_1	τ_2	α_2/α_1
1	8.06	12.00	1.49	1.12	8.54	12.55	0.86
2	8.31	12.21	1.18	1.22	8.68	12.62	0.80
3	8.27	12.15	1.24	1.21	8.81	12.81	0.68
4	8.08	12.00	1.47	1.14	8.54	12.43	0.94
5	8.24	12.15	1.25	1.37	8.83	12.84	0.67
6	8.05	11.97	1.52	1.15	8.98	13.08	0.63
7	8.00	11.95	1.56	1.04	8.77	12.72	0.73
8	8.18	12.11	1.28	1.18	8.97	13.17	0.52
9	8.10	12.06	1.41	1.12	8.73	12.71	0.74
10	8.17	12.10	1.33	1.17	8.76	12.74	0.72
Mean	8.15	12.07	1.37		8.76	12.77	0.73
Standard deviation	±0.10	0.09	±0.13		±0.15	±0.22	±0.12
Expected	8.80	12.80	0.69		8.80	12.80	0.69

Analyses of synthetic data using LSIR with integration by linear interpolation, and by the method of moments. 3×10^7 counts in excitation and emission.

practical. Using linear interpolation, the time is cut to ~7 min. However, as shown in Table VI, the results are very poor. On the other hand, the analyses obtained by the method of moments are excellent, and an analysis is rapid.

DISCUSSION

A number of examples have been given in which small amounts of nonrandom error destabilize an estimation by LSIR. Many more could be given if space permitted, but it is perhaps more fruitful to discuss general trends and their meaning.

We have shown that under certain conditions very small amounts of error destabilize estimation by LSIR. The more the ill conditioning, the easier it is for the estimation to be destabilized. Ill conditioning is increased when two decays are close to one another, or if one analyzes for three (or more) components. If the problem is not too ill conditioned, such as an analysis for two decays of 5 and 10 ns, then LSIR behaves well. However, when the decays are closer, extremely small amounts of error will cause LSIR to fail when the method of moments will not.

Of course, if one knew that a zero point shift error existed, and no other error did, then one could build a zero point shift variation into a least-squares calculation. However, one in general does not know that this is the case: one may have multiple errors or errors of unknown origin.

It is precisely the hallmark of a nonrobust estimator that it will work only under certain exact conditions. Thus, when one treats regression analyses, least-squares will be a good estimator when the noise is exactly Gaussian, something that almost never occurs.

It is the mark of a robust estimator that the estimation is stable, or relatively stable, in the presence of errors that are not known precisely, or perhaps not known at all. Attempts to measure and compensate for errors are, of course, useful, but they are no substitute for the use of a robust

TABLE IV
ANALYSES WITH 10-ps ZERO POINT SHIFT

Sample	LSIR, Simpson				Method of moments $MD = 1$		
	τ_1	τ_2	α_2/α_1	χ^2	τ_1	τ_2	α_2/α_1
1	9.11	13.19	0.50	1.98	Rejected by λ test		
2	9.01	13.20	0.50	2.37	8.63	12.59	0.84
3	8.96	12.89	0.62	2.26	8.75	12.70	0.74
4	8.95	12.89	0.62	1.98	8.96	13.11	0.55
5	8.97	12.90	0.61	2.12	8.95	13.07	0.57
Mean	9.00	13.01	0.57		8.82	12.86	0.68
Standard Deviation	±0.06	±0.16	±0.06		±0.16	±0.26	±0.14
Expected	8.80	12.80	0.69		8.80	12.80	0.69

Analyses of synthetic data with a 10-ps zero point shift. 3×10^7 counts in excitation and emission.

TABLE V
ANALYSES WITH INCORRECT EXCITATION

Sample	LSIR, Simpson				Method of moments $MD = 1$		
	τ_1	τ_2	α_2/α_1	χ^2	τ_1	τ_2	α_2/α_1
1	7.82	11.95	1.62	1.91	8.86	12.94	0.63
2	7.83	11.95	1.62	1.80	8.63	12.57	0.84
3	7.79	11.91	1.68	2.09		Rejected by λ test	
4	7.85	11.96	1.59	1.67	8.77	12.73	0.72
5	7.70	11.86	1.80	1.79	8.41	12.33	1.05
6	7.65	11.85	1.84	1.73	8.37	12.28	1.10
7	7.75	11.90	1.71	1.83	8.52	12.42	0.95
8	7.63	11.82	1.88	1.81	8.48	12.41	0.97
9	7.79	11.92	1.67	1.80	8.72	12.67	0.76
10	7.95	12.05	1.46	1.89	8.91	12.99	0.60
Mean	7.78	11.92	1.69		8.63	12.59	0.85
Standard deviation	±0.10	±0.07	±0.13		±0.20	±0.26	±0.18
Expected	8.80	12.80	0.69		8.80	12.80	0.69

Analyses of synthetic data with a slightly incorrect excitation profile. See text for details. 3×10^7 counts in excitation and emission.

estimator, since all compensatory measurements themselves have limits.

We have shown that LSIR is not robust, and the method of moments is robust, with respect to important errors in pulse fluorometric measurements. By this, we do not mean to say that the method of moments is the "best" method for analyzing fluorescence decay data; other methods may, and probably will, arise that will outperform the method of moments. Any such method will have to be robust, but there is no reason to believe that other robust estimators cannot be designed.

The fact that LSIR is not robust means that its use in a routine manner will artifically and unnecessarily limit the use of pulse fluorometry. Many analyses can, of course, still be performed by LSIR, but only when the problem is not too ill conditioned. It is difficult to see, however, why one should use LSIR.

In addition to the deficiencies discussed, LSIR also suffers from the need for large amounts of central processing units (CPU) time. The method of moments needs only a negligible amount of CPU time; the real time for an analysis is determined entirely by the time needed to produce a λ invariance plot, and even this time is typically one or more orders of magnitude below the CPU time needed by LSIR. Of course, this latter time depends critically on the details of a LSIR calculation. It is a sensitive function of the initial choice of parameters and of the number and actual values of the parameters, and it depends critically on the criteria one chooses for saying that an analysis has converged. However, Eisenfeld et al. (27) found the CPU time for LSIR to be 1 to 2 orders of magnitude larger than that for the method of moments, even though the initial choice of parameters was excellent and the actual decay times were well separated (5, 15, and 30 ns).

In linear regression, methods robust with respect to the

TABLE VI
ANALYSES OF THREE-COMPONENT EMISSION

Sample	LSIR, Linear Interpolation						Method of Moments				
	τ_1	τ_2	τ_3	α_2/α_1	α_3/α_1	χ^2	τ_1	τ_2	τ_3	α_2/α_1	α_3/α_1
1	2.63	5.55	10.39	1.01	0.65	3.66	2.95	6.92	10.99	0.69	0.34
2	2.29	4.60	10.05	1.91	1.21	4.54	3.02	7.01	10.98	0.66	0.33
3	2.20	4.58	10.05	2.05	1.27	4.90	3.02	7.00	10.98	0.66	0.33
4	2.32	4.94	10.21	1.56	0.95	4.56	3.01	6.98	10.99	0.66	0.34
5	2.38	5.13	10.30	1.39	0.84	4.14	3.02	7.15	11.10	0.67	0.31
Mean	2.36	4.96	10.20	1.58	0.98		3.00	7.01	11.01	0.67	0.33
Standard deviation	±0.16	±0.40	±0.15	±0.42	±0.26		±0.03	±0.08	±0.05	±0.01	±0.01
Expected	3.00	7.00	11.00	0.67	0.34		3.00	7.00	11.00	0.67	0.34

Analyses of synthetic three components data. The initial values chosen were τ_i = 1.00, 5.00, 12.00; α_i = 0.02, 0.01, 0.01. Theoretical values were τ_i = 3.00, 7.00, 11.00; α_i = 0.0176, 0.0118, 0.0059. 3×10^7 counts in excitation and emission.

underlying distribution take much more computing time than least squares. Nevertheless, the added time, while a disadvantage, is generally considered to be a small price to pay for robustness (10, 11). In analyzing fluorescence decay data, the method of moments takes much less time than least-squares. Thus, there appears to be no advantage to using least-squares.

Our conclusions are contrary to those of two papers in the literature (14, 15). However, these papers appeared before the λ invariance test was presented. In some cases, the examples cited in the papers used niether *MD* nor exponential depression. In addition, they did not investigate the effect of small nonrandom errors on estimation of closely spaced decay constants.

One suspects that the widespread use of LSIR is due, at least in part, to a feeling among chemists and biophysicists that least-squares methods are generally the epitome of good estimation procedures, and to a lack of knowledge of the literature on robust estimation. In fact, Tukey has stated (10) that chemists and physicists are routinely poorly educated about least squares. Misconceptions abound. In the area of pulse fluorometry, one of the standard misconceptions has been stated by Grinvald 27. "If the proper statistical weights[2] for the data are known, the least-squares estimate for the decay parameters has the highest probability of representing the true values of the parameters. " No reference is given for this theorem, and as a general theorem it is false since the literature on robust statistics abounds with counter examples. There are indeed mathematical theorems that do relate to least squares, although they do not apply to nonlinear estimation. The Gauss-Markov theorem (6, 10) states that, if we restrict our estimators to be linear functions of the data (a very severe restriction), and if the components of noise have variances in a known ratio, then least squares with weights inversely proportional to the variances, will yield estimates with minimal variances. In addition, if the errors follow an exactly Gaussian distribution, then the estimate with the smallest variance will be a linear one. However, the deviations from a Gaussian that are needed for the second theorem not to be applicable are so small that in practice they will almost always occur (1). In any case, the situation is far worse when one uses nonlinear least squares: One does not even have these theorems to serve as a basis for the estimation.

MD introduces robustness with respect to certain nonrandom errors commonly found in real data. The λ invariance test provides a way of judging if an analysis is satisfactory. Before the advent of the λ invariance test it was necessary to have some a priori knowledge of the type of error that was present, but that appears to be no longer necessary. It should be added, however, that, while it may easily be shown that λ invariance is necessary for the estimated parameters to be acceptable, only a partial theoretical foundation for the sufficiency of the test is currently available (22, 23), although no counter example is as yet known. To be safe, therefore, one should not rely only on the λ invariance test, at least until we have a fuller theoretical understanding. To be acceptable, parameters should also satisfy a component incrementation test, as well as be invariant to an increment in *MD* (19, 20, 25). It is interesting, incidentally, that all of these are invariance tests of one form or another. All of the method of moments analyses in this paper satisfied all of these tests.

It should be emphasized that the λ invariance test is not restricted to the method of moments (22). It is a property of the convolution and may be used with any method that is statistically resistant and that takes only a small amount of computing time (22). Therefore, if other robust estimators are designed, λ invariance testing or another type of parameter testing may be used with them, and these may turn out to be superior to the method of moments. Meanwhile, however, it will not be necesary to restrict the application of pulse fluorometry by using LSIR.

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[2]By proper statistical weight Grinvald means a weight equal to the inverse of the number of counts in a channel. This is the weight customarily used in LSIR estimation.

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