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A NON-LINEAR LEAST-SQUARES APPROACH TO THE RESOLUTION OF HETEROGENEOUS FLUORESCENCE FROM MULTITRYPTOPHAN PROTEINS

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Protein heterogeneous fluorescence results from the different microenvironment of each emitting chromophore. The structural and dynamic information contained in this emission can be extracted to some extent by selective quenching experiments. In this work, graphical and numerical methods are described for the analysis of protein emission in terms of three separated contributions: a fluorescence fraction which is not accessible to the quencher and two additional fractions with different solvent exposure. 'Static quenching' deviations from Stern-Volmer behaviour are also discussed. The application of these methods is exemplified on simulated quenching experiments and real data on acrylamide quenching of lysozyme fluorescence.

1. Introduction

The emission properties of electronically excited states are very sensitive to their immediate [1] environment. In proteins, where a significant tryptophan ultraviolet fluorescence can often be detected, the presence of multiple chromophores leads to heterogeneity [2] in the spectral distribution and lifetime of their emission. Thus, if the individual contributions to the total emission could be resolved, it should be possible to obtain some information on the particular environment of each chromophore. Lifetime-resolved fluorescence spectroscopy is certainly a powerful tool for handling these questions [3], but unfortunately it is not without limitations. For example, in proteins with more than two chromophores the assignment is quite uncertain [4,5]. Even for single-tryptophan proteins [6], the finding of multi-exponential decay in the isolated tryptophan [7-9] has complicated the interpretation of lifetime analysis.

On the other hand, there is a semiquantitative approach to the problems of separating the different emissions of proteins, namely, the selective quenching of tryptophan fluorescence [10-12]. Several charged [13] and uncharged [14] quencher molecules have been proposed. This method, which is experimentally simple, can be employed to determine changes in the degree of exposure of emitting residues or to probe some details of the protein dynamics. Unfortunately, for proteins containing more than one tryptophan residue, the experimental data usually reflect averages, which hinder any structural interpretation.

In this work, we wish to demonstrate that selective quenching experiments have the capability of resolving the heterogeneous emission from a multichromophore protein. Graphical and numerical methods are proposed in which the emission is separated into three fractions, according to their quencher accessibilities. One of them, non-accessible fluorescence, is easily determined, with or without 'static quenching' mixing. Moreover, an

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attempt is made to identify the experimental parameters that should be optimized in designing studies of this kind.

2. Problem formulation

The quenching of a multifluorophore protein, assuming that the emitters are independent, is usually expressed by a modified Stern-Volumer equation:

$$\frac{F}{F_0}=\sum\frac{f_i}{1+K_iQ} \qquad (1)$$

where F_0 is the fluorescence intensity of the sample in the absence of quencher; F the residual fluorescence intensity at a quencher concentration Q; f_i the fractional contribution to the total emission of the fluorophore i and K_i its particular quenching constant.

A modified form of this equation was derived by Lehrer [11,15] in order to obtain the fractional accessible fluorescence and 'effective' quenching constants:

$$\frac{\Delta F}{F_0}=\sum\frac{f_iK_iQ}{1+K_iQ} \qquad (2)$$

where $\Delta F=F_0-F$.

Graphical methods to solve eq. 2 were described by Lehrer [11] himself. They are based on linearization procedures similar to those used in binding studies. The limitations of this approach were clearly exposed [16] by the same author. Thus, we describe here an alternative analytical way of solving eq. 1, now made possible by the increased computing facilities available to the biochemical laboratory.

The modified Stern-Volmer expression, eq. 1, is fitted to the experimental quenching data by an iterative non-linear least-squares method. In order to handle the lowest number of fitting parameters, the heterogeneous fluorescence is arbitrarily separated into a maximum of three fluorescence fractions. The first one, f_0, corresponds to the emission which is non-accessible to quencher molecules (i.e., $K_0=0$); f_1 and f_2 are assigned to fluorescence emissions with different quencher accessibilities.

On the other hand, the usual data quality does not allow an accurate choice among the various possible combinations of f and K values, unless the initial values for the iterative procedure are quite close to the actual parameters. Moreover, model computations showed that the number of alternative combinations was drastically reduced if f_0 was prefixed and introduced as a constant. Therefore, a sequential path was designed: In the first place, the value of f_0 is independently determined by a graphical method; secondly, a simple set of linear equations is used for calculating a close estimation of f_1, K_1, f_2 and K_2; the values of these four parameters are finally refined by an iterative non-linear least-squares computation.

3. Determination of the non-accessible fraction

For high quencher concentrations, eq. 2 can be expressed by (see Appendix I):

$$\frac{F_0}{\Delta F}=\frac{1}{\sum f_i}+\frac{1}{Q}\,\frac{\sum(f_i/K_i)}{\left(\sum f_i\right)^2} \qquad (3)$$

On the other hand, it can be easily shown that:

$$\lim_{Q\to\infty}\frac{\Delta F}{F_0}=\sum_{i\neq 0} f_i=1-f_0 \qquad (4)$$

Hence, a plot of $F_0/\Delta F$ vs. $1/Q$ should extrapolate to an intercept $(1-f_0)^{-1}$.

Fig. 1 represents a model 'experiment' of tryptophan fluorescence quenching by acrylamide. Values chosen for K_1 and K_2 might represent [12] the quenching constants of two emissions; the first one would be partially shielded and the second completely exposed to the quencher solution. Fig. 1b is a representation of the last four points of fig. 1a in an $F_0/\Delta F$ vs. $1/Q$ plot; the intercept for $Q\to\infty$ (1.25) yields the expected value of f_0. Moreover, the acrylamide concentrations involved in that extrapolation (0.5–1 M) are not extremely high. To increase the accuracy of this graphical method in a real experiment, as many fluorescence readings as practical should be recorded in the high quencher concentration range.

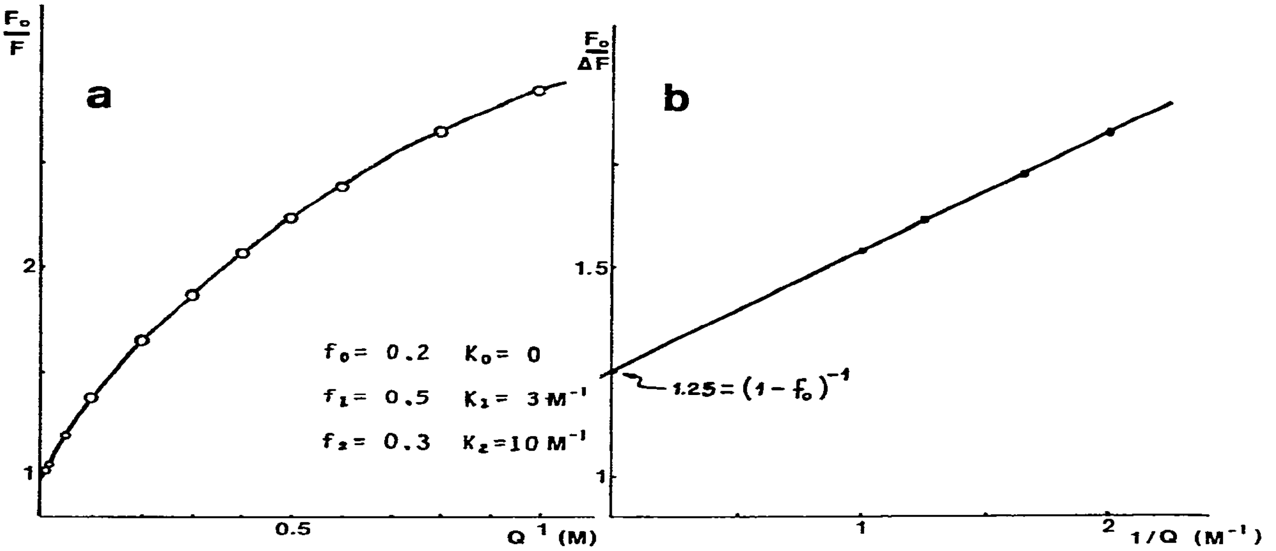

Fig. 1. (a) Stern-Volmer representation of a theoretical quenching experiment calculated with the parameters indicated therein. (b) Last four points of a in an inverse plot. From the intercept the f_0 value is evaluated.

4. Polynomial estimation of quenching parameters f_i and K_i

The method is based on a simple Gauss algorithm [17] which is described in detail in Appendix II. The application to the data set of the example of fig. 1 is presented here.

From eq. 2 the following expression can be obtained:

$$\frac{\Delta F}{F_0}=\frac{f_1K_1Q}{1+K_1Q}+\frac{f_2K_2Q}{1+K_2Q}$$

$$=\frac{(f_1K_1+f_2K_2)Q+(f_1+f_2)K_1K_2Q^2}{1+(K_1+K_2)Q+K_1K_2Q^2} \tag{5}$$

which is equivalent to

$$\frac{\Delta F}{F_0}=\frac{a_0+a_1Q+a_2Q^2}{b_0+b_1Q+b_2Q^2} \tag{6}$$

where $a_0=0$ and $b_0=1$.

From the raw experimental values of F and quencher concentrations, the coefficients a_i and b_i can be computed (see Appendix II) by solving the four-equations, four-unknowns system:

$$\begin{bmatrix} r_1 & r_2 & s_1 & s_2 \\ r_2 & r_3 & s_2 & s_3 \\ s_1 & s_2 & t_1 & t_2 \\ s_2 & s_3 & t_2 & t_3 \end{bmatrix} \cdot \begin{bmatrix} a_1 \\ a_2 \\ -b_1 \\ -b_2 \end{bmatrix} = \begin{bmatrix} s_0 \\ s_1 \\ t_0 \\ t_1 \end{bmatrix} \tag{7}$$

where

$$r_l=\sum_{j=1}^{N} Q_j^l,\quad s_l=\sum_{j=1}^{N}\left(\frac{\Delta F}{F_0}\right)_j Q_j^l \text{ and } t_l=\sum_{j=1}^{N}\left(\frac{\Delta F}{F_0}\right)_j^2 Q_j^l$$

The values of the quenching constants K_1 and K_2 are easily evaluated from the denominator Q roots in eq. 6, q_1 and q_2, as follows:

$$K_1=-q_1^{-1} \quad K_2=-q_2^{-1} \tag{8}$$

The same eq. 6 can now be used to compute f_1 and f_2. Finally, a value for f_0 can be obtained by subtraction, and compared with the value estimated in the previous section.

This particular method may be applied to other modified representations of the Stern-Volmer equation. On the other hand, different sets of equations such as eq. 7 can be used with any of the

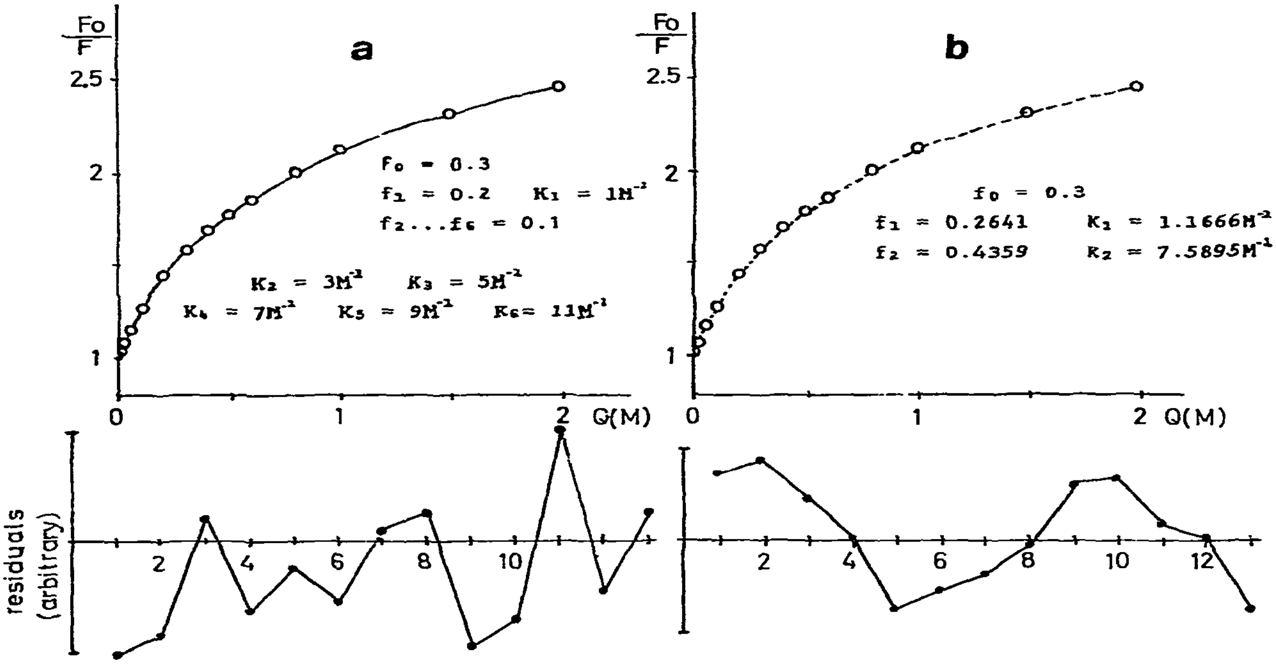

Fig. 2. (a) Stern-Volmer plot of a theoretical quenching experiment with seven different fluorescence fractions. (b) Circles: same as a. Dashed line: non-linear least-squares fit restricted to two accessible fractions. (a and b) below: Residual plots of the actual (a) and least-squares calculated (b) curves. See text for details.

representations. However, the method presented here produces better parameters in theoretical curves, so it is the one we routinely use.

Intermediate round-off errors may introduce severe distortions in the final results. Hence, it is convenient to keep as many significant digits as possible in the calculations. It may be necessary, in some instances, to 'normalize' the quencher concentrations, for the high powers of Q required may jeopardize the calculation.

5. Least-squares fit of the quenching data

In the two previous sections, the contribution of f_0, if any, and good estimates of f_1, K_1, f_2 and K_2 were obtained. In practice, this information may be enough to solve a great variety of structural and dynamic problems in biopolymer research. If more accurate values of f_i and K_i are needed, a standard non-linear least-squares fit [18,19] of the experimental data to any of the available quenching functions (F/F_0, F_0/F, $F_0/\Delta F$, etc.) is now straightforward. In practical terms, there is an advantage in using the Stern-Volmer expression (eq. 1), since convergence is achieved more rapidly. Moreover, this function is apparently free of local minima. The sample variance of the fit [18] is used as a convergence criterion within each fit (i.e., not for comparison between different fits):

$$s^2 = \frac{1}{N-n-1} \sum_{i=1}^{N} \left(y_i^{\text{obs}} - y_i^{\text{calc}}\right)^2 \qquad (9)$$

The method can be programmed * to be run in any desk-top computer.

The computed set of f and K values varies when

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* A non-linear least-squares program following the method of ref. 19 and written in BASIC is available on request.

varying the previously fixed f_0 value. That is, if this fraction of non-accessible fluorescence cannot be accurately defined, or the quality of the experimental data is very poor, different physical models may yield very similar s^2 values.

Inspection of the residual plot obtained after the fitting may be used to decide on the adequacy of the model. A random distribution of residuals is indicative [20] of a fit free of systematic errors. This technique, well known in the field of fluorescence lifetime deconvolution, requires at least 15–20 experimental points to give a significant display. In the example of fig. 2, a simulated experiment was constructed with the following parameters:

$f_0 = 0.3$

$f_1 = 0.2 \quad K_1 = 1\ \mathrm{M}^{-1}$

$f_2 \ldots f_6 = 0.1 \quad K_2 = 3\ \mathrm{M}^{-1} \quad K_3 = 5\ \mathrm{M}^{-1}$

$K_4 = 7\ \mathrm{M}^{-1} \quad K_5 = 9\ \mathrm{M}^{-1} \quad K_6 = 11\ \mathrm{M}^{-1}$

Sampling 13 points and adding 0.1% of random noise, the fit of fig. 2b was obtained, by following the methods described above constrained to three fluorescence fractions:

$f_0 = 0.3$

$f_1 = 0.2641 \quad K_1 = 1.1666\ \mathrm{M}^{-1}$

$f_2 = 0.4359 \quad K_2 = 7.5895\ \mathrm{M}^{-1}$

of which the s^2 is as low as 7.4×10^{-7}. However, the residuals of this fit (fig. 2b) are far from random, indicating the inadequacy of the model. Therefore, a low s^2 does not guarantee the validity of a model by itself; thus, it may be necessary to complement it with other criteria like the residual plot.

6. Static quenching and non-accessible fluorescence

To account for the static quenching term by numerical analysis, an exponential expression has been proposed [21,22] to be included in the Stern-Volmer equation. Eqs. 1 and 2 become:

$$\frac{F}{F_0} = \sum \frac{f_i}{\exp(V_i Q)(1 + K_i Q)} \qquad (10)$$

$$\frac{\Delta F}{F_0} = \sum \frac{f_i + f_i K_i Q - f_i \exp(-V_i Q)}{1 + K_i Q} \qquad (11)$$

where V is a static quenching constant.

So, two different quenching mechanisms have to be taken into account in these cases. On the other hand, two different types of non-accessible fractions can be distinguished, for there may be fractions non-accessible to static and collisional quenching, respectively. None of these fractions can be detected with the graphical method described above, since in all cases

$$\lim_{Q \to \infty} \frac{\Delta F}{F_0} = 1 \qquad (12)$$

If, however, there is a fraction which is non-accessible to both kinds of quenching,

$$\lim_{Q \to \infty} \frac{\Delta F}{F_0} = 1 - f_{00} \qquad (13)$$

where f_{00} is this non-accessible fraction.

Therefore, a plot of $F_0/\Delta F$ vs. $1/Q$, the intercept of which is not unity, indicates the presence of a fluorescence fraction unaffected by static and collisional quenchings.

Moreover, there is the possibility of estimating the dynamic quenching constants, K_i, regardless of the presence or absence of static quenching, from the polynomial method described above, by using the plot region of low Q concentrations, where the linear approximation to the exponential term holds:

$$\exp(-VQ) \simeq 1 - VQ \quad \text{for} \quad VQ < 0.1 \qquad (14)$$

In this case, eq. 11 becomes

$$\frac{\Delta F}{F_0} \simeq \sum \frac{f_i (K_i + V_i) Q}{1 + K_i Q} \qquad (15)$$

Using this expression, the polynomial calculation can be applied to evaluate K_i from the denominator coefficients.

7. An example with real data

The acrylamide quenching of indole derivatives occurs predominantly by a collisional process [22]. It has been used [14] to quench the tryptophan fluorescence of lysozyme, a protein containing six tryptophan residues [23]. What follows is an application of the methods presented here to compute

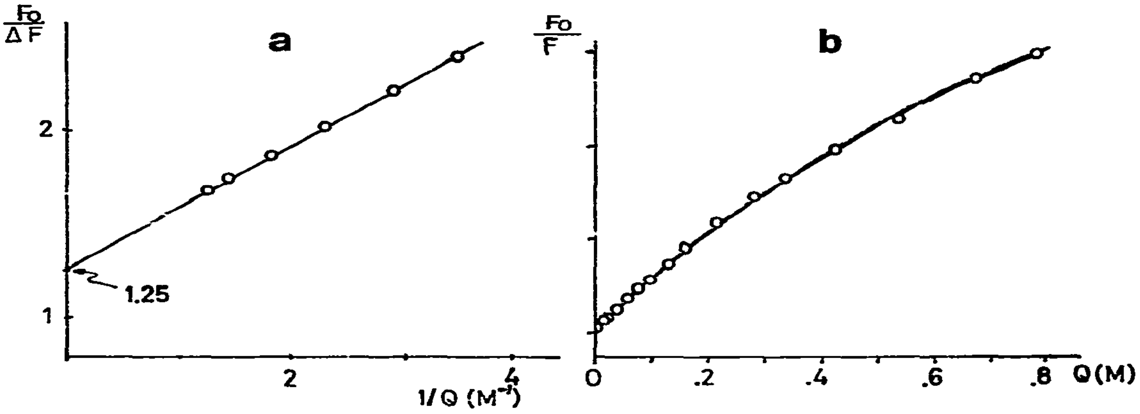

Fig. 3. (a) Inverse plot of lysozyme quenching by acrylamide in the high acrylamide concentration range. (b) Circles: experimental points of lysozyme quenching; these points have been obtained by digitizing fig. 4 of ref. 14. Solid line: least-squares fit of these points constructed with the following parameters: $f_0 = 0.2$, $f_1 = 0.8$ and $K_1 = 3.83$ M^{-1}.

the fluorescence fraction of lysozyme non-accessible to an aqueous solution of acrylamide. The experimental data were directly taken from fig. 4 of ref. 14. The graphical plot of eq. 3, for high quencher concentrations (Q between 0.3 and 0.8 M) (fig. 3a), is a good straight line with an intercept giving $f_0 = 0.20$. The polynomial estimation of the Stern-Volmer parameters yields:

$f_0 = 0.1998$
$f_1 = 0.8005 \quad K_1 = 3.006$ M^{-1}
$f_2 = -0.004 \quad K_2 = -7.552$ M^{-1}

The root q_2 of the denominator, corresponding to the negative K_2, cancels out with the numerator root. Consequently, f_2 is zero. If, in spite of this result, a non-linear least-squares fit to three fractions is attempted, using the above values as starting parameters for the iterations, the following set is obtained:

$f_0 = 0.200$
$f_1 = 0.8003 \quad K_1 = 3.830$ M^{-1}
$f_2 = -0.0003 \quad K_2 = -5.945$ M^{-1}
$s^2 = 1.1 \times 10^{-4}$

These parameters were used to produce the curve of fig. 3b. It is interesting to note that a fluorescence study of lysozyme [24] shows that Trp-62 and Trp-108 react with aqueous solutions of oxidants to give non-fluorescent derivatives. The remaining fluorescence of the modified lysozyme is just the 20% of its initial value. This assignment, however, is not definitive [25], and it is complicated by the possible electronic energy transfer between tryptophan residue. We think that the methods described here, although semiquantitative, have a potential utility for solving these and similar problems in protein and macromolecular research. For example, they can be extended to triplet quenching without major changes.

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Appendix I

From eq. 2 of the text

$$\frac{F_0}{\Delta F} = \frac{1}{\sum_i \frac{f_i K_i Q}{1 + k_i Q}} = \frac{1}{\sum_i \frac{f_i}{1 + \frac{1}{K_i Q}}}$$

where the sum includes all the fractions but f_0, the term of which is zero. Each term can be expressed by:

$$\frac{f_i}{1+\frac{1}{K_iQ}}=\frac{f_i\left(1-\frac{1}{K_iQ}\right)}{1-\left(\frac{1}{K_iQ}\right)^2}\simeq f_i\left(1-\frac{1}{K_iQ}\right)$$

if

$$\left(\frac{1}{K_iQ}\right)^2\ll 1.$$

Then

$$\frac{F_0}{\Delta F}\simeq\frac{1}{\sum_i f_i\left(1-\frac{1}{K_iQ}\right)}=\frac{1}{\sum_i f_i-\sum_i f_i\frac{1}{k_iQ}}$$

$$=\frac{1/\sum_i f_i}{1-\frac{1}{Q}\frac{\sum_i f_i/K_i}{\sum_i f_i}}$$

Multiplying numerator and denominator by

$$1+\frac{1}{Q}\frac{\sum_i (f_i/K_i)}{\sum_i f_i}$$

and neglecting the squared term:

$$\frac{F_0}{\Delta F}\simeq\frac{1}{\sum_i f_i}+\frac{1}{Q}\frac{\sum_i (f_i/K_i)}{\left(\sum_i f_i\right)^2}$$

Appendix II

Given a set of points (x_j, y_j), $j=1, 2, \ldots, N$, to minimize the sum of squared errors at each point, a rational function can be defined:

$$y_j=\frac{\sum_{i=0}^{m} a_i x_j^i}{\sum_{i=0}^{m} b_i x_j^i}$$

The 'error' of the j-th point can be defined:

$$\varepsilon_j=y_j\sum_{i=0}^{m} b_i x_j^i-\sum_{i=0}^{m} a_i x_j^i$$

and the sum of squared errors:

$$S=\sum_{j=1}^{N}\varepsilon_j^2=\sum_{j=1}^{N}\left(y_j\sum_{i=0}^{m} b_i x_j^i-\sum_{i=0}^{m} a_i x_j^i\right)^2$$

In order to minimize S with respect to the coefficients a_i and b_i, the following system is solved:

$$\frac{\partial S}{\partial a_p}=-2\sum_{j=1}^{N}\left[\left(y_j\sum_{i=0}^{m} b_i X_j^i-\sum_{i=0}^{m} a_i x_j^i\right)x_j^p\right]=0$$

$$\frac{\partial S}{\partial b_p}=2\sum_{j=1}^{N}\left[\left(y_j\sum_{i=0}^{m} b_i x_j^i-\sum_{i=0}^{m} a_i x_j^i\right)y_j x_j^p\right]=0$$

Permuting the i and j sums and defining the coefficients:

$$r_l=\sum_{j=1}^{N} x_j^l \quad S_l=\sum_{j=1}^{N} y_j x_j^l \quad t_l=\sum_{j=1}^{N} y_j^2 x_j^l$$

the expressions yield the following $2m$-order linear set of equations:

$$\sum_{i=0}^{m} a_i r_{i-p}-\sum_{i=0}^{m} b_i S_{i-p}=0$$

$$\sum_{i=0}^{m} a_i s_{i-p}-\sum_{i=0}^{m} b_i t_{i+p}=0$$

The homogeneity of this set of equations could be avoided if some of the parameters (a or b values) are known a priori. In order to keep round-off errors to a minimum, it is convenient to select the equations with lower powers of p.

For example, the expression

$$\frac{\Delta F}{F_0}=\sum_{i=0}^{m}\frac{f_iK_iQ}{1+K_iQ}$$

is equivalent to

$$\frac{\Delta F}{F_0}=\frac{a_0+a_1Q+Q_2Q^2+\ldots+a_mQ^m}{b_0+b_1Q+b_2Q^2+\ldots+b_mQ^m}$$

where homogeneity is excluded by substituting $b_0=1$. Considering also that $a_0=0$, the following equation set results:

$$\begin{bmatrix} r_1 & r_2 & \cdots & r_m & s_1 & \cdots & s_m \\ r_2 & r_3 & \cdots & r_{m+1} & s_2 & \cdots & s_{m+1} \\ \vdots & \vdots & \vdots & \vdots & \vdots & & \vdots \\ r_m & r_{m+1} & \cdots & r_{2m-2} & s_m & \cdots & s_{2m-1} \\ s_1 & s_2 & \cdots & s_m & t_1 & \cdots & t_m \\ \vdots & \vdots & \vdots & \vdots & \vdots & & \vdots \\ s_m & s_{m+1} & \cdots & s_{2m-1} & t_m & \cdots & t_{2m-1} \end{bmatrix}$$

$$\cdot \begin{bmatrix} a_1 \\ a_2 \\ \vdots \\ a_m \\ -b_1 \\ \vdots \\ -b_m \end{bmatrix} = \begin{bmatrix} s_0 \\ s_1 \\ \vdots \\ s_{m-1} \\ t_0 \\ \vdots \\ t_{m-1} \end{bmatrix}$$

where

$$r_l = \sum_{j=1}^{N} Q_j^l,\ S_l = \sum_{j=1}^{N} \left(\frac{\Delta F}{F_0}\right)_j Q_j^l \text{ and } t_l = \sum_{j=1}^{N} \left(\frac{\Delta F}{F_0}\right)_j^2 Q_j^l$$

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