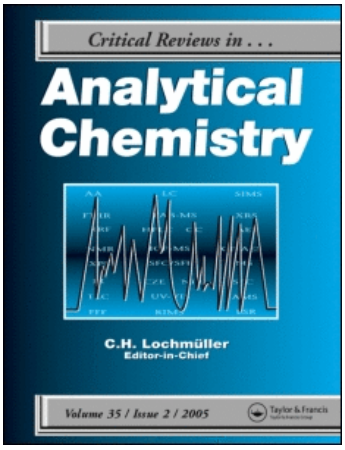

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Agustin G. Asuero[a]; Gustavo González[a]
[a] Department of Analytical Chemistry, Faculty of Pharmacy, The University of Seville, Seville, Spain

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Fitting Straight Lines with Replicated Observations by Linear Regression. III. Weighting Data

Agustin G. Asuero and Gustavo González
Department of Analytical Chemistry, Faculty of Pharmacy, The University of Seville, 41012-Seville, Spain

The purpose of this article is to stress the importance of weighting in fitting straight lines with replicated observations. Nevertheless, single response data are also taken into account. Although the concept of weighting is treated on chemometric texts, a detailed procedure is not given. For this reason the present review covers the information concerning this topic. Ignoring non-constant variance (heterocedasticity) often leads to improper estimation and inference in a statistical model which quantifies a given relationship. There are two main solutions to remedy this problem: transform the data or perform a weighted least-squares regression analysis. Weighting with replication in homocedastic and heterodedastic condition, including transformation depending weights, and normalization of the weights are considered. Weighting of observations, however, presents a more difficult problem that has commonly been recognized. The review covers briefly topics as random errors and noise, modelling the variance as a function of the independent variable and variation of precision with concentration. By transforming variable it is possible to introduce non-linear terms to the mathematical framework of linear regression, in order to improve fit as to satisfy the necessary assumptions such as homocedasticity. However, transformation data, the analysis of variance and summary data analysis will be the subject of a future report. A number of applications concerning the uses of weighting in analytical chemistry and weighted linear regression are given in tabular form. The analytical, pharmaceutical, biochemical and clinical literature has been thoroughly revised.

Keywords least squares method, replicated observations, weighting

INTRODUCTION

The least squares method is widely used to find or estimate the numerical values of the parameters to fit a function to a set of data (1–5) and to characterize the statistical properties of estimates (Table 1). In spite of this some difficulties and problems often arises to put the theory into practice, at least for a given application. Taylor and Schutsyer (9) quoted in 1986: "Although the theory concerning regression has since long been described, many errors can still be encountered when it is applied to solve problems in analytical chemistry."

The ordinary least squares which assumes the homocedastic condition (i.e., uniform variance) is widely used in physical and natural sciences. The least squares postulates have been examined in detail in part I of the series (3). The variance of the error term usually varies across observations, heterocedastic or non uniform variance condition occurs, and it may increase with the x value or in some more complex way (still assuming the independence of errors). Introductory material on weighted least squares can be found in several tutorials (10–15) and more specialized material on several reviews (16–21). The weighted straight line model with replication has been previously considered (4) in the part II of this series.

In many chemical, pharmaceutical and biological applications, such as assay development (15), and studies of enzyme kinetics and pharmacokinetics, regression models are utilized (7) to characterize a response variable Y and a predictor variable x; the constant variance assumption (homogeneity or homocedasticity) usually does not hold, which introduces some involvement as indicated above. The predictor may be (7) substrate concentration, mass, temperature, and the single responses y_{iv} may be peak area, velocity, radioactive count or some physical property.

A heterocedasticity refrain (22) states "It's a terrible thing that shouldn't be heteroscedastic. I fear it'll be the end of me." In fact, regression analysis is a tremendously powerful tool like electricity and just like electricity (23) if you aren't careful you can get badly burned! The appropriateness of the assumption of

Address correspondence to Agustin Asuero, Dept. of Analytical Chemistry, Faculty of Pharmacy, University of Seville, 41012-Seville, Spain. E-mail: asuero@us.es

TABLE 1
Examples on the application of regression analysis in chemical and pharmaceutical experimentation (6–8)*

Biochemical and chemical assay development
Pharmacological response over time
Stability prediction: concentration of drug versus time
Study of dose response relationship
Fitting linear portions of pharmacokinetics data
Best fit to linear physicochemical relationships
Calibration of analytical data
Plot of assay recovery versus known amount
Enzyme kinetics

*Examples in physical sciences indicate that the constant variance assumption may often be inappropriate.

equal variances errors can sometimes be checked after a model has been fitted by plotting (24–26) the residuals (or deviations from the fitted responses) versus the fitted values (Fig. 1), but it is much better (27) to have replications. Knowledge of the variance of experimental data is fundamental (28) to optimal design and proper analysis in many areas of investigation. With replications we can check the assumption before even fitting a model (29), and can in fact use the information obtained in choosing a form of weighting for weighted least squares (21). Alternatively we may use the replication averages and variances to determine a suitable variance-stabilizing transformation.

Curvilinear relationships between two variables frequently may be simplified by a transformation on either one or both of the variables. From an inference point of view, linear regression models are easy to implement in comparison with curvilinear or non-linear regression ones. The simplest model or the model with the minimum number of parameters that adequately fit the data in question is usually the best choice. This is well established as (27, 30) a form of Occam's razor: "Non sunt multiplicanda entia praeter necessitatem" (entities are not to be multiplied beyond that needed). Therefore, a straight line calibration curve should always be preferred (31, 32) over curvilinear or non-linear calibration models if equivalent results can be achieved.

If the form of a non-linear relationship between two variables is known, it is sometimes possible to make a transformation of one or both variables, such that the relationship between the transformed variables can be expressed as a straight line. We call such model linear transformable (32). Some authors use the term "intrinsically linear" for a special geometric property of non-linear models. Transformable linear models have some advantages in non-linear regression (NLR) analysis because it is easy to get starting values for some of the parameters.

There are basic reasons for transforming variables in regression. It affords possible remedies for either non-normality or heterogeneous variance, and may also simplify the relationship between the dependent and the independent variables. Nevertheless, if a non-linear model is meaningful and is readily interpreted, a transformation to linearize the model would not seem wise if it creates heterogeneous variance or nonnormality. However, transformation of data, the analysis of variance and summary data analysis will be the subject of a future report.

Non linear calibration curves sometimes curse in chemical analysis. In atomic absorption spectrophotometry (AAS), the initially linear relation according to Beer's law breaks down at higher absorbance owing to instrumental imperfections and the influence of atomic spectral line profiles (33). A commonly observed phenomenon in AAS is the ending of the calibration graph towards the concentration axis at elevated concentrations (34). It is generally known that electron capture detectors (EDCs), nitrogen-phosphorus and UV-photometric detectors,

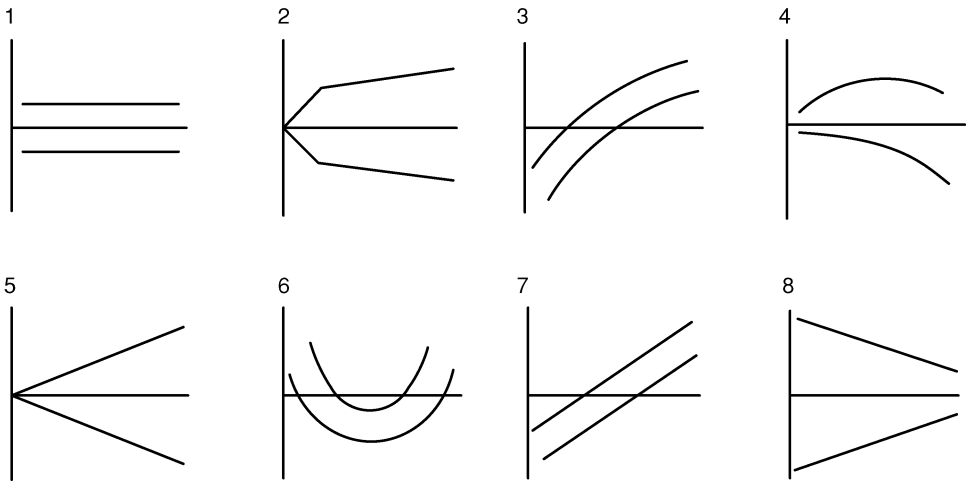

FIG. 1. Plot of typical residuals (26); difference between observed and nominal as % of nominal. 1- Variance uniform: weight 1; 2-Variation $\propto \sqrt{x}$: weight $1/\sqrt{x}$; 3-Additional linear term or quadratic function; 4-Non uniform variance wrong model; 5-Variation $\propto x$: weight $1/x^2$; 6-Variance uniform power curve; 7-Systematic analytical error; 8-variance decreases with x.

have a limited linear calibration range; in particular, curvilinear regression models usually lead to a better fit of the calibration data (35–37).

Tellinghuisen indicates (38) that, in most real problems, the response becomes non-linear when the range of the calibration data becomes sufficiently large. Sometimes, the standard response of the analyst to this situation is to curtail the range in order to employ linear method, being introduced clearly bias in the determination of this way as the selection of the "linear region" is arbitrary. In the case of some analytical quantification methods, quadratic curve fitting (31, 39–41) is more appropriate to the calibration data pairs than linear regression. In the field of LC-MS for instance, matrix-related non-linearity can be observed in several methods (41). Currently used operating conditions for analyzing volatile organic compounds (VOCs) by purge-and-trap gas chromatography/mass spectrometry (CG/MS) produced non-linear calibration curves with non-uniform variance (42). Nevertheless, a curved data set can be detector-related (e.g., GC-ECD or LC-ESD), but can also be an indication of problems with the assay such as adsorption, solubility or ion suppression (43).

Weighted least squares demands a large number of replicates than ordinary least squares; estimates about the minimum number of replicates by different authors range from 6 to 20 (3–4). This level of replicate is frequently difficult to obtain in practice (44, 45) because of different motives, as cost or availability of calibration, standard and reagents, or time demanded by previous operations or by, e.g., the chromatographic run.

Weighted least squares can be derived from maximum likelihood theory, provided that the measurement error variance is known and independent of the model parameters (46, 47) and the weights are calculated as the inverse of the measurement error variance. However, using measured values instead of predicted values to quantify the measurement error variance is approximately valid only when the noise in the data is (48) relatively low. This practice may thus introduce sampling variation in the resulting estimates, as weights can be seriously mis-specified.

Formulae for calculating a_0 anda_1, and their standard errors by weighted linear regression with replication are given in Table 2. For additional details on this respect Part II of the series (4) should be consulted.

TABLE 2
Formulae for calculating statistics for weighted linear regression with replication data

Equation	Slope
$\hat{\bar{y}}_i = a_0 + a_1 x_i$	$a_1 = S_{XY}/S_{xx}$
Mean responses	Intercept
$\bar{y}_i = (\sum y_{i\upsilon})/n_i$	$a_0 = \bar{\bar{y}} - a_1\bar{x}$
Residual sum of squares	Weighted residuals
$SSE = \sum w_{\bar{y}_i}(\bar{y}_i - \hat{\bar{y}}_i)^2$	$w_{\bar{y}_i}^{1/2}(\bar{y}_i - \hat{\bar{y}}_i)$
Mean	Correlation coefficient
$\bar{x} = \sum w_{\bar{y}_i}x_i / \sum w_i$	$r = S_{XY}/\sqrt{S_{XX}S_{YY}}$
$\bar{\bar{y}} = \sum w_{\bar{y}_i}\bar{y}_i / \sum w_i$	Standard errors
Sum of squares about the mean	$s^2_{\bar{\bar{y}}/x} = \frac{SSE}{n-2} = \frac{S_{YY}-a_1^2 S_{XX}}{n-2}$
$S_{XX} = \sum w_{\bar{y}_i}(x_i - \bar{x})^2$	$s^2_{a_0} = s^2_{\bar{\bar{y}}/x}(\sum w_{\bar{y}_i}x_i^2)/$
$S_{YY} = \sum w_{\bar{y}_i}(y_i - \bar{\bar{y}})^2$	$(S_{XX}\sum w_{\bar{y}_i})$
$S_{XY} = \sum w_{\bar{y}_i}(x_i - \bar{x})(y_i - \bar{\bar{y}})$	$s^2_{a_1} = s^2_{\bar{\bar{y}}/x}/S_{XX}$
	$\mathrm{cov}(a_0, a_1) = -\bar{x}\ s^2_{\bar{\bar{y}}/x}/S_{XX}$

WEIGHTS

Rather little is known concerning about the distribution of error in experimental observations, being often depending on the size of measured variables. The variance of the so-called independent variable is not always small enough on this context to be neglected as happen in (3,4) the standard treatments. Nevertheless this later point will not be subject of consideration in that follows.

However, it is essential to use a regression analysis of the data in which appropriate weighting factors are incorporated (49–51). Weighting of observations presents a more difficult problem than has commonly been recognized (52). The idea behind the weighted least squares is to attach the most importance to the dates that are measured with the greater precision. The weighted least-squares procedure consists in (4) minimizing the weighted residuals (Table 2). The greater the departure from homocedasticity, the greater is the benefit to be expected from using a weighted least squares procedure (53).

In order to apply a weighted least-squares analysis one must assign weighting values w_i, to the various observations. However, w_i is a measure of the information present in the value y_i and is proportional (54) to the reciprocal of the variance of y_i. Thus, the results of a single assay without supplementary information scarcely contain enough information to adequately model the variance structure. Fortunately, the investigator frequently can choose the values of the independent variable at which measurements are to be made and usually can obtain replicates.

The treatment that follows is due to Deming (54). By definition

$$w_f = \frac{\sigma_0^2}{\sigma_f^2} \qquad [1]$$

σ_0^2 is simply a proportionality factor, i.e., the variance of a function of unit weight (55). Let f be $\bar{y}_i$ the mean of n_i observations $y_{i1}, y_{i2,}\ldots y_{in_i}$, which are random variates taken from a universe of standard deviationσ_0, and so each of unit weight. The variance of the mean $\bar{y}_i$ is given then by

$$\sigma^2_{\bar{y}_i} = \frac{\sigma_0^2}{n_i} \qquad [2]$$

and from Equation 1 we get

$$w_{\bar{y}_i} = \frac{\sigma_0^2}{\sigma^2_{\bar{y}_i}} = \frac{\sigma_0^2}{\frac{\sigma_0^2}{n_i}} = n_i \qquad [3]$$

Note that when single observations have unit weight, then the weights of the k means are given by $w_1 = n_1$, $w_2 = n_2, \ldots w_k = n_k$. So, we may in these cases consider the k samples means to be k observations of weights $n_1, n_2, \ldots n_k$.

As a matter of fact, weights are relative and not absolute, depending as they do on the arbitrary factor σ_0^2. If the n_i original variables were each of weight w_i instead of unity, the variance of single observations would be σ_0^2/w_i and the variance of $\bar{y}_i$ would be one nth as much. In this case

$$w_{\bar{y}_i} = \frac{\sigma_0^2}{\sigma_{\bar{y}_i}^2} = \frac{\sigma_0^2}{\frac{\sigma_0^2}{n_i w_i}} = n_i w_i \qquad [4]$$

or

$$\sigma_{\bar{y}_i}^2 = \frac{\sigma_0^2}{n_i} w_i \qquad [5]$$

being now $n_i w_i$ the weight. So as before, the weights of $\bar{y}_i$ is just n times the weight of a single observation.

Let $\bar{y}_i$ the mean of n_i observations from a population of standard deviation σ_i, the precision of a single observation varying now from one sample to another. Then, the variance of $\bar{y}_i$ will be σ_i^2/n_i. Note, that $\sigma_i (i = 1 \text{ to } k)$ need not all be equal. The weight of $\bar{y}_i$ is given now by

$$w_{\bar{y}_i} = \frac{\sigma_0^2}{\frac{\sigma_i^2}{n_i}} = n_i \frac{\sigma_0^2}{\sigma_i^2} \qquad [6]$$

Weighting factors must reflect both the number of replications n_i and also the variance at the locality of the point. The common practice in kinetic studies of replacing replicate measurements by their average causes a loss of information about the reliability of calculated rate constants as well as the agreement between experimental data and assumed reaction mechanism (56).

In most applications the true w_i values are not known. With sufficient replication at each concentration, at least ten replicates, an empirical estimation of w_i from the observed data can be obtained (3–4, 45). The biggest disadvantage of weighted least squares which many people are not aware of, is probably the fact that the theory behind this method is based on the assumption that the weights are known exactly (46, 47). This almost never the case in real applications, of course, so estimated weights must be used instead. Weighting factors as defined in Equation 6 should be modified for experimental purposes by taken the sample variances s^2 instead of the population σ^2 ones.

The effect of using estimated weights is difficult to assess, but experience indicates that small variations in the weights due to estimation do not often affect (57) a regression analysis or its interpretation. However, when the weights are estimated from small numbers of replicated observations, the results of an analysis can be very badly and unpredictably affected. This is especially likely to be the case when the weights for extreme values of the predictor or explanatory variable(s) are estimated using only a few observations. It is important to remain aware of this potential problem, and to use weighted least squares when the weights can be estimated precisely relative to one another.

A triplicate group of observations for example, may posses a small sample variance but a significant deviant mean. It is also possible for a group to posses a large sample variance but a mean value that is close to the correct one. In the first case, a weight estimated from the sample variance is too large, and in the second case is too small. In the limit, a group of observations may posses a zero variance, particularly if the values are rounded to a small number of significant figures, corresponding to an infinite weight. The fitted line is then constrained to pass through that group of observations, regardless of any contrary information contained in the rest of the data.

The general idea behind robust estimation is to reduce the weight given to outliers, so that they play a less significant role (58) in the determination of the parameters. Robustness can readily be incorporated into standard weighted least squares routines by a suitable choice of weights (59–63). If potential outliers are not investigated and dealt with appropriately, they will likely have a negative impact on the parameter estimation and other aspects of a weighted least squares analysis. If a weighted least squares regression actually increases the influence of an outlier, the results of the analysis may be even far inferior to an unweighted least squares analysis.

The weighting factors $w_{\bar{y}_i}$ are in correspondence with the data points $(\bar{y}_i, x_i)$. The more accurate a data point is known, the larger the value for the associated $w_{\bar{y}_i}$ should be. Therefore, the fitted curve should pass close to more accurately known points, and this is shown by the inclusion of the weighting factors in the model (4, 19, 21).

WEIGHTING SCHEMES

Although the general concept of weighting values is mentioned in several of the more complete texts on chemometrics (64–68), a detailed procedure is not given. For this reason, an overview on this topic is given below. It appears that the weighting procedure may have a large or a small effect in parameter estimation depending on the particular data set analysis. There is no doubt that in certain cases failure to weight the experimental points or incorrect calculation of the values of the weighting factors may lead to completely false constants. The solution to the problem depends on whether or not there are replicate observations at each sample point. With enzyme kinetic data, intuition may play a greater role (69).

The weighting factors can be given in a number of different ways (1, 18, 55, 70, 71), depending on the characteristics of the data set:

(a) Absolute Weights. In the absence of more complete information it is commonly assumed that equal weighting of all the points (x_i, y_i) is satisfactory, i.e., $w_i = 1$. These are absolute weights (71); that is, regression with constant standard deviation for the measured quantity (72). Note that this leads in replication cases to: $w_{\bar{y}_i} = n_i\, w_i = n_i$.

(b) Statistical Weights. The standard deviation might be properly described as being proportional to the signal or the concentration of analyte. However, it is not suitable to calculate the inverse of variance in laboratory routine, if we taking into account the fact that it requires several determinations for each calibration point and a fresh calibration line should be performed (44) each time the method is used. For these reasons other empirical weights based on x-variable (i.e., concentration) or y-variable (i.e. response) may provide a simple approximation of variance; i.e., weights such as $1/x^{0.5}$, $1/x$, $1/x^2$,$1/y^{0.5}1/y$, $1/y^2$ should be studied (73). These are called statistical weights (74). The best $w_i$will be that which gives rise to a narrow horizontal band of randomly distributed percent relative error around the concentration axis and present the least sum of the percent relative error across the whole concentration range (73).

The calculations are straightforward, but the equations are complex so the use of computer software is generally the best approach. In fact, some software packages for regression analysis allow one to enter an estimate of the functional dependence $\sigma_y = f\,[E\,(y)]$ and to carry out a suitable weighting with this function. The data system software for the instruments disposal at laboratory usually allow us to specify the curve fitting function to use (75); i.e., the usual integration programs for chromatography (76) contain possibility of weighting with,$1/x$,$1/x^2$ or $1/y$,$1/y^2$. If the first concentration is zero, then weighting by, $1/x$ or $1/x^2$, is impossible. Weighting using $y$seems more practical, because one assume that the standard deviation of y is more highly correlated to y than to x. However, because of the high correlation between y and x, there is only a slight difference between y and x weighting, especially in instances where there is no significant difference between the intercept and zero. The same is true for pharmacokinetics programs, which may include a weighting with complex models.

The problem of a misleading regression coefficient arises from the fact that the large standard deviation of the points at the top of the curve dominates the calculations. To give the points at the lower end on the curve equal consideration, we must apply weighting (21, 64, 67). The most popular weighting schemes involve adjusting the data by a factor related to an inverse function of the concentration (Table 3).

Commonly, $1/x^0$(no weighting), $1/x^{0.5}$,$1/x$ and $1/x^2$ are applied. The choices to weight by $1/x$ or $1/x^2$, are useful when you want to weight the points at the left of the graph more than the points at the right (75, 109). Here, we are saying that the points with smaller values of y_i are known relatively accurately (x), and the points with larger values of y_i are less well known (110). Some authors (33, 111) have addressed the question of whether the inverse of the x_i^2, could not be a weighting factor with similar characteristics of weighting like the inverse of the variance.

Data transformation and weighting schemes are normally used to obtain the best-fit of standard curves in bioanalysis and the calibration model is usually selected during prevalidation (43). A comparison has been made between unweighted and weighted ($1/x$, $1/x^2$,$1/x^{0.5}$) regression models with or without an intercept in achieving the best-fit for the standard curve (112). In fact, bioanalysis should routinely test these models for their calibration curves as part of their assay validation not during prevalidation.

When the variance of residuals decrease with x, one can find the recommendation to consider the weight

$$w_i = \frac{1}{x_{\max} - x_i} \qquad [7]$$

where $x_{\max}$ is the largest value that x can possible assume (113).

In many cases, standard deviation rises approximately proportionally to the concentration, leading to a constant coefficient of variation (114). A method used for minimizing relative deviations rather than absolute deviations gives equal experimental weight to all measurements regardless of the range in which measurements are made. Thus, the deviation of experimental measurements ion the x-range no longer overshadow or swamp (115, 116) those in the low x-range. With this option, we minimize

$$Q = \sum w_i\,(y_i - \hat{y}_i)^2 = \sum \left(\frac{y_i - \hat{y}_i}{y_i}\right)^2 = \sum \left(1 - \left(\frac{a_0}{y_i} + a_1 \frac{x_i}{y_i}\right)\right)^2 \qquad [8]$$

Actually, in many typical applications, the experimental conditions are controlled so that the percentage error is constant. Nonconstant variance occurs when the variance of y_i depends on x_i; a peculiar case of heterocedasticity, important in analytical chemistry (3–4, 64, 67, 117), is that for many analytical methods relative standard deviations are reasonably constant over a considerable dynamic range.

Thus, weighted linear regression analysis can also be based on the square of the independent variable y_i^2. From a practical point of view, the two approaches $1/\sigma_i^2$ or $1/y_i^2$ yield the same result if the relative standard deviation is constant, as this results in σ_i being proportional to y_i Using the magnitude of the response rather than the variance does, however, void the generalized use of the weighted regression analysis, as this results in division by zero in calculation of the weights (118) in the case when $y_i = 0$.

Relative residuals give erroneous results in the case of experimental data with relatively large uncertainties and hence occasional large errors. In principle, the difficulty is present even when the errors are small. The situation can be corrected, for this case, by using for the relative residual (119) the absolute residual divided by the calculated value of the function, i.e.,

$$w_i = \frac{1}{y_i \hat{y}_i} \qquad [9]$$

"ever when another criterion is available, the inclusion of the factor $(y_i \hat{y}_i)^{-1}$ in the overall weighting factors may be beneficial" (119).

Weighting by $1/(y + \hat{y})^2$ tend towards weighting by $1/y^2$ as the variability of the data decreases, so that in the limit its

TABLE 3
Examples of some weighting schemes based on $1/y^n$ and $1/x^n$ ($n = 1,\ 2$)

Compound determined	Method	Calibration	Authors	Ref.
$1/y^2$ Weight				
Novel anticancer agent after liquid-liquid extraction with ethylacetate; pharmacokinetics profile	LC-MS/MS		Moreno-Farré et al., 2006	(77)
Aripiprazole and its main matabolite in human plasma	LC-MS/MS	0.1–100 ng/ml	Kubo et al., 2005	(78)
PCBs in fatty food samples	GC-ECD1		Loco et al., 2003	(35)
Triazine herbicides	TLC		Sarbu and Cobzac, 2000	(79)
Perifosine in human plasma with miltefosine as internal standard	HPLC/MS	4–2000 ng/ml	Knevel et al., 1999	(80)
Reproterol in human plasma utilizing a methylated structural analogue as internal standard	HPLC/MS	0.2–200 ng/ml	Knevel and Winkler, 1997	(81)
A collagenase inhibitor and its major metabolite from plasma and urine	HPLC-MS/MS	5–5000 ng/ml	Knebel et al., 1995	(82)
$1/y$ Weight				
Rofecoxib in human plasma	SPE with fluorescence detection	0.5–80 ng/ml	Matthews et al., 2002	(83)
Assay in human plasma and human urine of a compound which behaves as a specific inhibitor of the ezyme cyclooxygenase II	HPLC-postcolumn derivatization-fluorescent det.	5–500 ng/ml	Matthews et al., 2001	(84)
Indinavir (specific and potent HIV protease inhibitor): clinical monitoring in plasma of AIDS patients	SPE-reverse HPLC-UV detection	10–800 ng/ml	Poirier et al., 1999	(85)
Equilibrium constants and complex mobilities of porphyrins	Nonaqueous CE		Bowser et al., 1997	(86)
$1/x^2$ Weight				
Spironolactone (potassium-sparing diuretics) in paediatric plasma samples	SPE-HPLC	30–1000 ng/ml	Sandall et al., 2006	(87)
Cocaine and its metabolity in hair	GC-MS/MS	0.10–5 ng/ml 0.05–5 ng/ml	Cognard et al., 2005	(88)
Camptosar (anticancer agent) and its active metabolite in mouse plasma and tissues	LC-MS/MS	0.5–500 ng/ml	Bardin et al., 2005	(89)
Acrylamide in pig serum	LC-MS	10–5000 mg/ml	Feinberg and Laurentie, 2005	(90)
PNU-248686A matriz metalloproteinase inhibitor	LC-MS/MS	5.0–5000 ng/ml	Frigerio et al., 2003	(91)
Seven process related substances at ng ml-1 level in cetirizine tablets	LC-MS	2.5–250 ng/ml	Rudaz et al., 2003	(92)
Nemorubicin (broad spectrum antitumor activity) and its 13-OH metabolite in human plasma	LC-MS-MS	0.1–5 ng/ml	Fraier et al., 2002	(93)
Fosinoprilat (anti-hypertensive agent) in human serum	LC-MS	2.00 to 500 ng/ml	Jemal et al., 2000	(94)

(Continued on next page)

TABLE 3
Examples of some weighting schemes based on $1/y^n$ and $1/x^n$ ($n = 1,\ 2$) *(Continued)*

Compound determined	Method	Calibration	Authors	Ref.
Retigabine (novel class of potent anticonvulsant drug) and its acetyl metabolite	HPLC-MS	1–1000 ng/ml	Knebel et al., 2000	(95)
Triazine herbicides	TLC		Sarbu and Cobzac, 2000	(79)
Clopidogrel (platelet aggregation inhibitor) in human plasma	GC-MS	5–250 ng/ml; quadratic equation	Lagorce et al., 1998	(96)
Phenytoin, carbamazepine and 10,11-carbamazepine epoxide in human plasma	HPLC-UV detection	0.050–25 μg/ml	Batthi et al., 1998	(97)
Atovaquone (potential agent against malaria and toxoplasmosis) in plasma	Robotic based reverse phase HPLC	0.25–50 μg/ml	Studenberg et al., 1995	(98)
Non-linear behaviour of some chromatographic systems vs. weighted linear regression			Burrows and Watson, 1994	(37)
$1/x$ Weight				
Enkeohalins (neuropeptides) in cerebroesinal fluid	CE/MS	0.001–0.1 pmol/μl	Sinnaeve et al., 2005	(99)
Opiates morphine, codeine and their metabolites in hair	LC-ESI-MS/MS	25 to 4000 ng/ml	Murphy and Huestis, 2005	(100)
Synthetic opiate buprenorphine and its metabolites in human plasma	LC-ESI-MS/MS	0.6 to 50 ng/ml	Murphy and Huestis, 2005	(101)
Opiates, cocaine and metabolites in hair	LC-APCI-MS/MS	up to 5000 pg/mg	Scheidweiler and Huestis, 2004	(102)
Leucine-enkephalin(neuropeptide)	LC-MS	50 fmol/ml-10 pmol/ml	Sinnaeve and Boexlaer, 2004	(103)
Related substances at ng level in tablets containing Cetirizine	LC-ESI-MS	2.5–250 ng/ml	Rudax et al., 2003	(92)
Opiods, cocaine and metabolites in urine	LC/MS/MS	up to 10000 ng/ml	Dams et al., 2003	(104)
Rat plasma uridine (naturally occurring nucleoside and versatile therapeutic agent)	HPLC-ESI-MS	0.78 to 25 μM	Williams et al., 2003	(105)
Irinotecan (broad range antitumor activity) in beagle dog plasma	HPLC-fluorescent method	1.00–750 ng/ml	Guo et al., 2003	(106)
Sotalol in human plasma	LC	25–1000 ng/ml	Chiap et al., 2001	(107)
Captoril (antihypertensive) in plasma	HPLC	—	Wieling et al., 1996	(108)

use implies that the variance is proportional to the square of the observed variable, i.e., that the coefficient of variation is constant (120). This is a proposition put forward as a reasonable alternative to considered by many authors. Thus, there is an also reasonable ground for trying $1/(y + \hat{y})^2$ for any data without replicates for which weighting may be though to be necessary.

Typically, y direction errors are greater at very low values and very high values of sample concentration than at optimal (for the

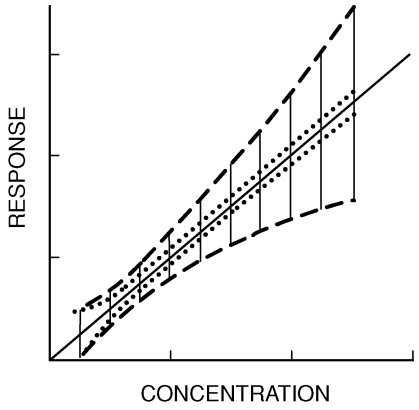

FIG. 2. Hypothetical concentration versus response relationship for typical serum analysis HPLC method (121). Solid line is mean response value. Dashed line is standard deviation of response. Dotted line is relative standard deviation of response.

method) sample concentration (121) values (Figure 2). In some cases, e.g., hormone assays, the analytical standard deviation becomes constant in the low range, resulting in an increased coefficient of variation (122, 123) in this area (Figure 3).

Experimental data is not always subject to a constant coefficient of variation, however. The error structure in real data usually lies somewhere on a continuous between a constant absolute error (homocedastic) at one extreme, and a constant coefficient of variation at the other. Between these two there is an error for which the standard deviation is proportional to the square root of the expected value (124–126). It has been observed from long experience that the measurement error of an analytical method, for example atomic absorption spectroscopy responds to the model later alluded. Over a range of concentrations near zero, the measurement error is seen to be constant. Over ranges of higher concentration, the measurement error is observed to be proportional to the concentration of analyte (127).

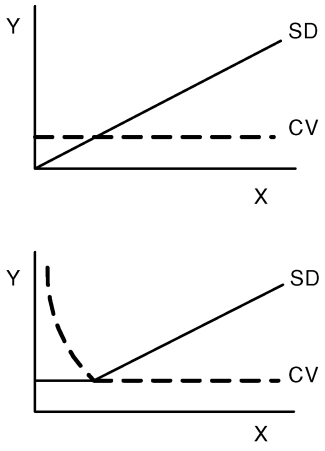

FIG. 3. Examples of relations between analyte concentrations (X), analytical standard deviation (SD), and coefficient of variation (CV).

So, the error structure was more complex than the classical assumptions of constant absolute or constant relative error. One can take the variance σ_i^2 of y_i, measured in a consistent way, either as a constant (i.e., simple errors)

$$\sigma_i^2 = \sigma_0^2 \qquad [10]$$

or as proportional to y_i^2 (i.e. relative errors)

$$\sigma_i^2 = \sigma_2^2 y_i^2 \qquad [11]$$

or as some combination of the two (128–130) resulting from the presence of additive simple and relative component (complex errors)

$$\sigma_i^2 = \sigma_0^2 + \sigma_2^2 y_i^2 \qquad [12]$$

It may be noted that many of the error models used can be regarded as simple approximations of the model described by Equation 12. For example, at low y_i values, Equation 12 degenerates to 10, and at high to 11. This later corresponds to constant relative error, one of the typical assumptions about experimental error. Statistical weights calculated in the case of ICP-MS (taking into account the various noise sources), for example, follows (131–134) a complex error model.

An account on the ways in which the variances change as the analyte concentration increases will given in a next section.

(c) Instrumental Weights (18). The primal conception of a weight is that of a repeated observation (54). A weighting scheme often used is appropriate where the uncertainties in the y_i values can be characterized by real standard deviations. In fact, one approach to data acquisition (135, 136) is to collect a relatively small number of data and to make replicates so that the standard deviation can be calculated and then used to assign individual weights to these data points. In these cases, the weights can normally be considered inversely related to the variance of the points. These are called instrumental weights.

In such a scheme, no assumptions about the precision of the various data points are necessary; such precision is often found to be nonuniform in a given data set, i.e., heterocedastic. This procedure, however, increases the cost of analysis and will be worthwhile only if additional data quality is required (44). At least 10 replicate measurements should be made at each x-value and used to calculate standard deviations. It is the task of the analyst to decide whether he must improve his method by using more sophisticated procedures. In the absence of a sufficient number of replicates, a functional relationship between variance and the independent variable can be assumed (19). In fact, regular cases of nonhomogeneity are characterized by a functional relationship between variance and the expected value of the responses (137).

(d) Transformation-Dependent Weights (18). Unequal weights may be introduced without being realized. A rather distinct approach and an also very often-practiced alternative when standard deviations are not available is to take a single

set of closely spaced data, in which insufficient information is available to justify the assignment of separate weights to the individual data points (135). However, it is sometimes possible to carry out a transformation of either one or both variables, in those cases in which the form of the non-linear relationship between the two variables is known, in such a way that the transformed variables are subject to a straight line relationship. These non-linear relationships are said to be intrinsically linear (32, 138).

The transformed data will not necessarily satisfy certain assumptions which are theoretically necessary to apply the regression analysis. It is important to note that all such transformations may affect the relative magnitudes of the errors at different points on the plot. Thus a non-linear plot with approximately equal errors at all values of x (homocedastic data) may be transformed into a linear plot with heterocedastic errors. In general, when experimental data z_i (dependent variable whose values are measured) are converted into transformed data y_i (dependent variable resulting from the linearization) for subsequent use in a least-squares analysis, one should introduce (18, 135,136) a weighting factor $w_i(\sigma_0^2 = 1)$ given by

$$w_i = \left(\frac{1}{(\partial y/\partial z)}\right)^2 \qquad [13]$$

These are called transformation-dependent weights. Weighted least squares are not always problem-free. Weighting factors given by Equation 13 are often even powers of the untransformed signal, such as y^2 or y^4, which are always positive even though the corresponding y-values may have zero average (2). As a consequence, random noise in regions where the signal is small may contribute significantly to the sum of squares, and may then distort the analysis.

(e) Mixed Instrumental Transformation-Dependent Weights. Use of the transformed weights with the transformed function and points will give unintended results (139). In order to maintain the proper relationship between the weights and the points being fit, we must also transform the weights. The random error propagation law (140–142), when applied to a function $y = f(z)$, gives (being $\sigma_0^2 = 1$)

$$\sigma_y^2 = \sigma_z^2 \left(\frac{\partial y}{\partial z}\right)^2 \qquad [14]$$

If the weights are transformed using the general error propagation law, we get

$$w_y = w_z \left(\frac{\partial y}{\partial z}\right)^{-2} = \frac{1}{\sigma_z^2 \left(\frac{\partial y}{\partial z}\right)^2} \qquad [15]$$

In other words, transformation-dependent weighting should be used in addition to any weighting based on the measurement of the standard deviation σ_z^2 of the individual data points. Nevertheless, in those cases in which the individual standard deviations remain unknown, global weighting scheme (d) is the best choice. Some typical examples of kind of weights are shown in Table 4. Some examples of application of weighting in analytical chemistry are compiled in Table 5.

Calculation of the weighting factors of the individual experimental points in accordance with the theory of mathematical statistics was first proposed and employed in complex chemistry by Hugus (180), due the difficulties associated with obtaining replicate measurements in complex processes.

WEIGHT NORMALIZATION

The numerator of the expression for w_i in Equation 1 is not strictly necessary, it could be replaced by any constant other than zero without altering the final fitted parameters, but a numerator of the type shown is a convenient normalization factor that ensures that the calculated weights do not differs (128) enormously from unity. The weights calculated as indicated in Equations 4 and 6 may be normalized (181,182). This can be accomplished by transforming each "old" weight $w_{\bar{y}_i}$ into a new weight $w^*_{\bar{y}_i}$

$$w^*_{\bar{y}_i} = \sigma_0^2 w_{\bar{y}_i} \qquad [16]$$

The choice of σ_0^2 is arbitrary, because σ_0 is a constant and it cancels from the normal equations (the weights are purely relative)

TABLE 4
Some kinds of weights*

Kind	Weight	Authors	Ref.
Absolute weights	1	Jurs, 1986	(139)
Statistical weights	$\frac{1}{y_i}$	Johnson, 1980	(74)
		Jurs, 1986	(139)
Assumption of constant percentage error	$\frac{1}{y_i^2}$	Anderson and Snow, 1967	(116)
		Smith and Mathews, 1967	(117)
Instrumental weights	$\frac{1}{s_i^2}$	Jurs, 1986	(139)
Transformation-dependent weights	$\frac{1}{(\frac{\partial y}{\partial z})^2}$	de Levie, 1986	(135)
		Meites, 1979	(1)
Mixed instrumental transformation depending weights	$\frac{1}{s_z(\frac{\partial y}{\partial z})^2}$	de Levie, 1986	(135)
		Meites, 1979	(1)

*s_i^2 is the estimate of σ_i^2.

TABLE 5
Some examples of weighting in analytical chemistry

Comments	Authors	Ref.
Weights in continuous variation data	Sayago and Asuero, 2006	(143)
Weights in mole ratio data	Boccio et al., 2006	(144)
Straight line weighted model with replication	Sayago and Asuero, 2004	(4)
Algorithm that calculates the sensitivity to the systematic error with application to weighting of experimental data	Baeza-Baeza and Ramis-Ramos, 2004	(145)
Weights in evaluation of dissociation constants from titration data	Meloun and Pluharova, 2000	(146)
Sensitivity weights in least squares fitting of linearized equations	Baeza-Baeza and Ramis-Ramos, 1995	(147)
Equilibrium constants from potentiometric data	Potwin, 1994	(148)
Pointwise resolution significance versus resolutive weights	Baeza-Baeza et al., 1992	(149)
Optimization of weighting in regression method by means of algorithms which evaluate useful information	Baeza-Baeza et al., 1990	(150)
Implicit and explicit methods of weighting titration data	May and Murray, 1988	(151)
Comparison of computer programs which differs in the statistical and weighting scheme	Casassas et al., 1986	(152)
Properly weighting in potentiometric acid-base titration	Kateman et al., 1983	(153)
Weighting and uncertainty in potentiometric titrations	Smit et al., 1983	(154)
A general weighting scheme is proposed for use in least-squares fit of data from acid-base titration	Avdeef et al., 1983	(155)
Weighting in potentiometry taking into account volume, pH and time	Wozniak and Nowogrocki	(156)
Selection of the error variable and need for weighting in the optimization of potentiometric titration data	Still, 1980	(157)
Weighting of the experimental point in least-squares procedures (part of a review)	Gaizer, 1979	(158)
Multiparametric refinement program MUCOMP for metal complex equilibria	Wozniak and Nowogrocki, 1978	(159)
Multiparametric refinement program MUPROT for the evaluation of acidity constants from titration data	Wozniak and Nowogrocki, 1978	(160)
Weighting taking into account the statistical uncertainties in both the pH and volume data	Schwartz and Gelb, 1978	(161)
Data in buffer region receive much greater weight than data near equivalent points	Avdeef and Bucher, 1978	(162)
Weighting taking into account both the pH and volume data	Avdeef et al., 1978	(163)
Weights function both of the variance of the individual observables and of the values of the parameters	Christian et al., 1974	(164)
Attention is given to appropriate weighting	Christian et al., 1974	(165)
Weighting factors in least squares	Sands, 1974	(166)
Formation constants from potentiometric data	Sabatini and Vacca, 1972	(167)
Attention is given to the introduction of weighting factors in the calculation of stability constants (review)	Rossotti, 1971	(168)
Weighting in connection with data transformation	Jurs, 1970	(139)
Weighting in potentiometric evaluation of equilibrium constants	Varga, 1969	(169)
Statistical analysis of kinetic data	Cleland, 1967	(170)
Weighting in polarography	Momoki et al., 1967	(171)
Weighting procedure applied to the Schwarzenbach method	Data and Grybowski, 1966	(172)
Stability constants of two-step overlapping equilibria from potentiometric data	Lansbury et al., 1965	(173)
Stability constants of acetylacetone complexes of V(II)	Schaefer, 1965	(174)
Provision for weighting experimental observations	Irving and Stacey, 1961	(175)
Weighted least squares to obtain equilibrium constants	Andereg, 1961	(176)
Effect of weighting procedures on equilibrium constants	Rydberg, 1960	(177)
Effect of weighting procedures on equilibrium constants	Rydberg and Sullivan, 1959	(178)
Weighting in the calculation of complexity constants	Sullivan et al., 1959	(179)

and its choice does not affect the values of the estimates a_0 and a_1 (Table 2) of α_0 and α_1 (3–4) and is evidently the variance of a function of unit weight. The variance and covariance formula for the weighted linear least squares case show that σ_0 does not cancel, instead,

$$s^2_{\bar{y}/x} = \frac{\sum w^*_{\bar{y}_i} \varepsilon_i^2}{k-2} = \frac{\sigma_0^2 \sum w_{\bar{y}_i} \varepsilon_i^2}{k-2} \qquad [17]$$

hence its value is not arbitrary in estimating the variance ratios

If we make the reasonable requirement that the numerical estimates of the parameter ratios $\sigma^2_{a_0}/\sigma^2$ and $\sigma^2_{a_1}/\sigma^2$ for a weighted regression to be identical to those for unweighted regression when they are all equal, this means that

$$w_{\bar{y}_i} = n_i \qquad [18]$$

$$\sum w_{\bar{y}_i} = \sum n_i \qquad [19]$$

therefore with this choice

$$\sum w_{\bar{y}_i} = \sum n_i \frac{\sigma_0^2}{\sigma_i^2} = \sigma_0^2 \sum \frac{n_i}{\sigma_i^2} = \sum n_i \qquad [20]$$

$$\sigma_0^2 = \frac{\sum n_i}{\sum \frac{n_i}{\sigma_i^2}} \qquad [21]$$

Thus, from Equations 6 and 21 we have

$$w_{\bar{y}_i} = n_i \frac{\sum n_i}{\sigma_i^2 \sum \frac{n_i}{\sigma_i^2}} \qquad [22]$$

The convention has the feature that the weights in absence of replication add up to k, that is the weights have been scaled so that their sum is equal to the number of points on the graph

$$w_i = \frac{\frac{k}{\sigma_i^2}}{\sum \left(\frac{1}{\sigma_i^2}\right)} \qquad [23]$$

which they do also in unweighted regression for which they are all equal to unity. Other conventions are commonly used. For example, in the case (183) of no replicate observations:

(i) Let $\sigma_0^2 = 1$ (184, 185). Then $w_i^* = w_i$

$$w_i^* = \frac{1}{\sigma_i^2} \qquad [24]$$

(ii) Let $\sum w_i^* = 1$. Then $\sigma^2 = 1/\sum(1/\sigma^2)$ and

$$w_i = \frac{\frac{1}{\sigma_i^2}}{\sum (\frac{1}{\sigma_i^2})} \qquad [25]$$

Though no strictly necessary, as it could be replaced by any constant other than zero without altering the final parameters, it is advisable to normalize the weights (128, 181, 183, 186). This ensures as above indicated that the calculated weights do not differ enormously from unity, because in precise measurements w_i as well as $\sum w_i$ may take high values and generate round-off errors and spurious results on this way, when only limited number of significant figures are carried out on calculators or computers.

If the variances are known, the weighted least squares estimator with Equation 25 weights is the optimal one, and in case of normally distributed errors it is maximum likelihood and efficient (46, 47). The relative weights w_i may not be known exactly, so that an approximate analysis must be performed with estimated weights (17, 46, 47, 187). In some situations a more accurate analysis must be obtained by repeating the entire process with improved estimates of the weights supplied by the first analysis. More than a few repetitions of the process are seldom necessary, however, as a check.

ITERATIVELY WEIGHTS

An iterative procedure is necessary in those cases in which the weights applied to a linear model are themselves a function of the expected value (mean), and this is the case for the iteratively reweighted least squares. Let, $y_i = \alpha_0 + \alpha_1 x + \varepsilon_i$, where the ε_i are independently distributed as $N(0, \sigma_0^2 g(\theta_i))$ being g a positive function, and the weights

$$w_i = \frac{1}{g(\theta_i)} \qquad [26]$$

now unknown.

There are then two methods available for estimating a_0 and a_1: the maximum likehood method, and an iterative least-squares method. This later technique estimates the weights $w_i = 1/g(a_0 + a_1 x_i)$ from trial estimates of a_0 and a_1, say, the unweighted least-squares estimates firstly (which are unbiased), and then solving for new estimates of a_0 and a_1. These new values may be used for recalculating the w_i, and the process can be repeated. As a great accuracy is not necessary in the calculation of the weights to obtain accurate estimates of a_0 and a_1, only a few cycles of iterations (usually two or three) are required. The variance-covariance matrix of the least squares estimates is approximately the covariance matrix of weighted least squares estimators of α_0 and α_1, if we ignore the fact that the estimated w_i are strictly random variables (46). For this reason, and for computational simplicity, the iteratively least squares method is often preferred to the maximum likelihood approach (46, 47).

In a next section we will explain with more detail how the weights can be modelled as a function of the independent or dependent variable.

RANDOM ERRORS AND NOISE

Random errors and standard deviation play a most important role (188, 189) in analytical chemistry and the interpretation of experimental results. Random error is caused by noise and noise (190–196) sources may be a function of signal or concentration or other factors.

In spectrochemical analysis the precision of intensity measurements (197, 198) is frequently restricted by noise sources which may be classified into three groups: shot, flicker and detector noise. Shot noise is related to the rate of amount of ions

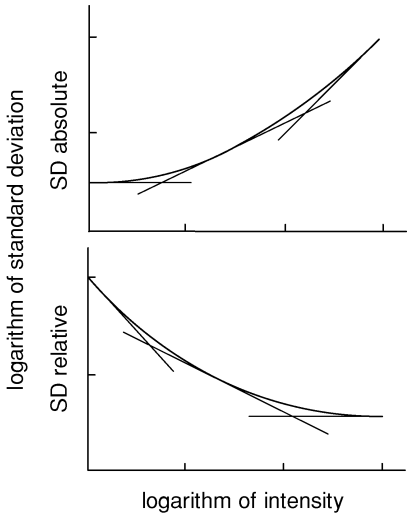

FIG. 4. The precision as depending on the intensity measurements, represented by the absolute (top) and the relative (bottom) standard deviation (SD). The three different components of the noise are characterized by the three straight lines.

at the detector and it is described by Poisson statistics. Flicker noise is related to the nebulization process and the fluctuations associated with the source, being proportional to the magnitude of the signal. Detector noise refers to the dark count noise arising from the detector and electronic involved. Accordingly, the variance includes three different components:

1. One component which is independent upon intensity and which, in general, is dominating at smaller intensity values originating from the measuring system (detector plus measuring electronics); i.e., constant terms, independent of all parameters that are typically varied in a given experiment (199).
2. Another component which is proportional to the intensity value and is often determined by the quantum noise. Terms those are Poisson in nature.
3. A third component which is proportional to the square of the intensity and is mainly caused by variations of the radiation source. This flicker noise is dominating at greater intensity values.

Total noise can be expressed (132, 200) as:

$$s_t = \sqrt{s_{shot}^2 + s_{flic}^2 + s_{\text{det}}^2} \qquad [27]$$

Typical graphs of precision as a function of intensity measurements are shown in Figure 4.

Many analytical methods have additional noise sources of their own. Noise introduced at the read-out stage, e.g., from the recorder dead band or the width of the tracing, is commonly small but need not be insignificant (190). If a recorder or other analog device is used, noise from this source is constant within each range, but between ranges it varies directly with the full-scale reading. If the readout is digital, the noise is half of the least-significant digit or bit; as with analog devices if is constant within ranges. When signals are converted to absorbances, the relationship between noise and concentration becomes even more complicated (190).

In spectrophotometric procedures, the analytical signal, y, may be an absorbance, a blanked absorbance or in kinetic methods (191), a change in absorbance. From theoretical grounds, the bias in the absorbance A may be expressed as

$$dA = UA + V + WA^2 \qquad [28]$$

where U,V, and $W$are the coefficients of proportional, constant and quadratic error (188). Though photometric errors in absorbance (191) have an exponential dependence on A, Equation 28 is a reasonable approximation when A is small. The three types of signal errors can often be interpreted in terms related to the analytical process as exemplified in Table 6. Sources of analytical signal fluctuations are given in Table 7.

The noise of chromatographic baselines has been investigated in connection to the detector, the nature and extent of filtering or smoothing, and the methodologies of qualitative and quantitative assessment, with regard to the detection limit (195).

MODELLING THE VARIANCES AS A FUNCTION OF THE DEPENDENT OR INDEPENDENT VARIABLE

Applying weighting factors implies knowledge about the variances of each data point. This in general is not cost effective as requires a large number of replicates (44). Alternatively, since variability usually changes smoothing with the response level,

TABLE 6
Likely analytical causes of the three types of signal errors (188, 33, 35–36, 41–42)

Constant error	Proportional error	Quadratic error
Sample turbidity	Volumetric error	Decay/dissociation of product
Reagent absorbance	Gravimetric error	Reagent depletion
Nonspecificity	Incomplete separation or	Instrumental non-linearity
Zero error/drift	derivation	• matrix-related non-linearity in CG-MS
Carryover	Matrix evaporation	• purge and trap GC-MS
Contamination	Matrix evaporation	• electron capture detector
	Error in timed processes	

TABLE 7
Sources of analytical signal fluctuation

Noise sources	Fluctuations	Variance
Photomultiplier detectors	Shot noise	$\propto \sqrt{y}$
Output of the light source	Fluctuations	$\propto y$
Instrument electronics	Nature of the circuit	Constant variance, or variance may change with signal in various ways
Non-linear noises of a quantum mechanical type	Fluctuations	$\infty y^{3/2}$
Non-linear noises of classical character		∞y^2
Sample cell turbulence		$\propto c$
Flame noise		$\propto c$

the variance can be modelled as a function of x (or a function of y) (7, 19). Thus, the variance models can be proposed to accommodate for scientist's insight into a wide range of physical processes resulting in improved inference and estimation (7, 201).

For many physical properties, variance seems to be systematically related to the mean or other variables. The explicit description of this relationship by a function leads to the variance function model (Table 8). Different types of variance function and methods for estimation of them have been introduced (7, 202–205). In each case the estimated value of the weights w_i are completely determined by the values $\{\mu_i\}$ of the mean response at x_i, so the weights are often estimated by inserting an estimate for μ_i. Typical estimates for μ_i are the sample mean $\bar{y}_i$ based on the replicate responses at $x_i$or the predicted value $\hat{y}_i$ based on an initial fit to the data by least squares. For assay development, i.e., Model IV in Table 8, θ may be unknown. Since the weights depend on both μ and θ, the true value of θ must be estimated.

When the number of replicates is greater than one, a simple approach to estimation is to note that (206, 207)

$$\log[\mathrm{Var}(Y_{ij})]^{1/2} = \log \sigma_0 + \theta \log \mu_i \qquad [29]$$

Then the slope of least squares regression of $\log s_i$on $\log \bar{y}_i$ provides an estimate $\hat{\theta}$. The estimated weights would then be $\bar{y}_i^{-2\hat{\theta}}$. The power of the mean variance function is of the most

TABLE 8
Variante funtion estimation

	$Var(Y_{ij})$	w_i	Comments
I	$\sigma_0^2\,\mu_i^2$	μ_i^{-2}	Constant CV equal to σ; reasonable approach in e.g., HPLC, as long as the limits of assay sensitivity are not approached too closely
II	$\sigma_0^2\,\mu_i$	μ_i^{-1}	Quite useful from count data for which a Poisson assumption implies that $Var(Y_{ij}) = \mu_i$
III	$\sigma_0^2\,(\alpha + \mu_i)^2$	$(\alpha + \mu_i)^{-2}$	α known
IV	$\sigma_0^2\,\mu_i^{2\theta}$	$\mu_i^{-2\theta}$	General model to accommodate overdispersion; θ often falls in the range $0.6 \le \theta \le 0.9$. Poisson model if $\sigma_0 = 1$ and $\theta = 0.5$. Power of the mean variance function, which is likely to be of the most importance in chromatographic and capillary electrophoresis applications. Plot of $\log \lvert r_{ij} \rvert$ versus log of the predicted value gives a straight line
V	$\sigma_0^2\,(\theta_1 + \mu_i^{\theta_2})$	$(\theta_1 + \mu_i^{\theta_2})^{-1}$	θ_1 describes the imprecision of measurement that dominates at small response value and θ_2 the relationship between mean and variance that dominates at larger response values
VI	$\sigma_0^2 \exp(2\,\theta\,\mu_i)$		The variability increase very quickly with the mean Plot of $\log \lvert r_{ij} \rvert$ versus predicted value $\hat{\mu}_i$ show a linear relationship
VII	$\sigma_0^2\ (1 + \theta_1\,\mu_i + \theta_2\,\mu_i^2)^2$		The Standard deviation is thought to be a quadratic function of the dependent variable Plot of $\log \lvert r_{ij} \rvert$ versus y_i shows a quadratic relationship
VIII	$\sigma_0^2\ (1 + \theta_1\,\mu_i + \theta_2\,\mu_i^2)$		
IX	$\sigma_0^2\,g^2(\mu_i,\ z_i,\ \theta)$	$[g^2(\mu_i, z_i, \theta)]^{-1}$	g is the variance function general model

importance in chromatographic and capillary electrophoresis applications (19, 208).

Although we may have evidence and knowledge to support a model such as V of Table 8, he/she may not be sufficiently confident to choose values for θ_1 and θ_2 a priori. There is not restriction of the variance function approach. In some situations it is realistic to think about variability as a function of a predictor or some other variable not necessarily in the mean model. The most flexible approach to modelling variability should be the variance function to depend on the particular experimental situation. Thus, a general model for the response

$$Y_{ij} = a_0 + a_1 x_i + \sigma_0 g(\mu_i, z_i, \theta)\varepsilon_{ij} \quad i = 1, \ldots, k \quad j = 1, \ldots, n_i \qquad [30]$$

where g is a function which may depend on the mean response, on a vector z of known variables containing possible some or all the values in x and $q\theta$-parameters that may be known or unknown. The advantage of a general model IX (Table 8) is that it encourages analysts to think about the relationship between the variance and other factors for a given experimental situation (7) instead of relying on a few fixed relationships for describing variance with little basis beyond standard usage. The general model also allows additional flexibility because the values of the components of θcan be estimated from the data values (201) when necessary.

The terminology "generalised least squares" refers to use of weighted least squares (WLS) with estimated weights. When the variance function model holds and θ is known, then the weights $w_i = 1/g^2(\mu_i, z_i, \theta)$ are known except for the values of the μ_i, If the mean response model is well established, it makes sense to construct estimates of the weights using estimates $\hat{y}_i$. Details for the implementation of the iterative procedure may be consulted in the appropriate references (7, 201–205). However, for many problems, only two or three iterations are necessary for the algorithm to converge within a reasonable tolerance. Full iteration is a version of iteratively reviewed least squares.

The proper estimation of the variance function is still a challenge. Outliers can influence its estimation strongly (208). In general, models in which independent sources of experimental error add as variances rather than as contribution to σ, should be close to physical reality. However, many experimental techniques also have identifiable error sources that give variances proportional to signal (e.g., from counting detectors) (204).

Whenever heterocedasticity and non-linearity present concurrently, the use of generalized least squares regression with variance function estimation can lead to better estimates of model parameters and hence better estimation for concentration in test samples than the weighted least-squares method using the usual weight factors (205).

Generalized least squares perform better than weighted least squares and also extended least squares (209). With extended least squares (209–213), the variance of the error becomes a function of the expected (model-predicted) values at each time point, rather than a function of the corresponding data values. It has been pointed out that the $w_i = y_i^{-1}$ and $w_i = y_i^{-2}$ schemes are not robust and should be (213) avoided.

VARIATION OF PRECISION WITH CONCENTRATION

Several models for the standard deviation can be used (214–217); i.e., constant (the procedure reduces to regression with equal weights factors) or proportional to the concentration. A mixture of these two models to cover both ranges of concentration may also be used.

The precision of an analytical system can be expressed as a function of the concentration of an analyte (215, 218, 219). There are different mathematical models that have been proposed to estimate the change of analytical precision as a function of analyte concentration (Table 9), depending on the particular context. The most simple and effective model is the lineal one where the standard deviation of the analytical measurement, s, at concentration c, s_c, increases linearly with concentration (model II of Table 9), where $s_0$and k are the standard deviation at zero concentration and a proportionality constant, respectively. Model II of Table 9 has been used for routine quality control of within-laboratory geochemical data produced by atomic absorption spectrometry and spectrography (221–224). Because model II represents the sum of analytical errors of two independent terms, it should be theoretically more satisfactory to combine for this purpose the variances rather than the standard deviations. Thus, either the variance model V or VI (Table 9) may describe more correctly the variation of precision with the concentration. It is recognized that the standard deviation increases with concentration (but the coefficient of variation decreases). In fact, the widely applicable assumption of model II (244) (variability of the measurement error depends linearly on concentration) implies that

$$CV(u) = \theta + \frac{\sigma_0}{u} \qquad [31]$$

which is consistent with the evidence (245) gathered for different authors.

Note that the variance of the signal is constant, proportional to the signal or proportional to the square of the signal. Thus, the weights of the weighted linear regression method may be estimated with an iterative procedure as described in the ISO 11843-2 standard (235). This procedure assumes that the variance is linearly dependent on the concentration, and the weights being estimating according to

$$w_i = \frac{1}{(mx_i + q)^2} \qquad [32]$$

To give a realistic estimation for uncertainty of the analytical result, its precision must be known not only as a single value; it has to be estimated over the entire range of concentrations of interest. Especially, in the field of environmental research (238) it is very important to be able to predict the precision at any given concentration level to compare the analytical performance of the

TABLE 9
Typical examples of component error model

Model	Function	Authors	Ref.
		ISO 5725	(219)
I	$s = k\sqrt{c}$	Hughes and Hurley	(220)
II	$s_c = s_0 + k\ c$	Thompson and Howarth, 1973	(218)
	$\sigma_c = p\ c + q$	Thompson and Howarth, 1976	(221)
		Howarth and Thompson, 1976	(222)
		Thompson and Howarth, 1978	(223)
		Thompson, 1978	(224)
		Thompson, 1988	(215)
		Lee and Ramsey, 2001	(217)
III	$s_x = a_0 + a_1 x + a_2 x^2$	Oppenheimer et al., 1983	(225)
		Watters et al., 1987	(226)
		Zorn et al., 1996	(53)
IV	$s_c = A_0 + A_1 c + A_2 c^2 + A_3 c^3$	Modamio et al., 1996	(227–234)
V	$s_x = \sqrt{a_0 + a_1 x}$	ISO 11843-2, 2000	(235)
VI	$s_x = \sqrt{a_0 + a_1 x^2}$	Zitter and God, 1971	(236)
	$s_x = \sqrt{s_0^2 + k^2 c^2}$	Thompson, 1988	(215)
	$\sigma_x = \sqrt{p^2 c^2 + q^2}$	Rocke and Lorenzato, 1995	(237)
	$u_x = \sqrt{s_0^2 + (x s_i)^2}$	Lee and Ramsay, 2001	(217)
		Rocke et al., 2003	(238)
		Wilson et al., 2004	(128)
		EURACHEM/CITAT Guide, 2002	(239)
		Heydorn and Anglow, 2002	(240)
VII	$\sigma_x = \sqrt{c_0 + c_1 x + c_2 x^2}$	Watter et al., 1987	(226)
	$\sigma_y = \sqrt{c_0 + c_1 y + c_2 y^2}$	Schwartz, 1978	(241)
		Boumans et al., 1981	(242)
		Bubert and Klockenkämper, 1983	(197)
		Oppenheimer et al., 1983	(225)
VIII	$\sigma = bc^d$	ISO 5725	(219)
		Hughes and Hurtley, 1987	(220)
	$s_x = a_0 e^{a_1 x}$	Zorn et al., 1997	(53)
	$\sigma_c = pc^k + q$	Desimoni, 1999	(243)
	$\sigma_y^2 = Ay^b$	Prudnikov and Shapkings, 1984	(189)
	$\sigma_x^2 = A\,(x+1)^b$	Oppenheimer et al., 1983	(225)

method with the analytical requirements (246). The model VI incorporates both types of error that are observed in practice in a single model and provides an obvious advantage over existing models by describing the precision of measurements across the entire range.

Empirical models which have considered for radioligand assay (Models III and VIII) may be appropriate in other general situations; the weights must be estimated by modelling the standard deviation as a function of concentration. Model of standard deviation quadratic, exponential and a two-component model (237) have also been proposed.

Issues related to the choice of weighting methods are not entirely solved and there is no universal solution for all cases. Since there is normally no way of knowing in advance the data analysis for which weighing scheme to choose, selection is often subjective and somewhat arbitrary (227). Prediction of the analytical error function in the field of drug and pharmacokinetics analysis is not possible a priori, due to the error associated with the characteristics of the active principle, analytical method and technique used, and so this function must be determined. In order to establish the best function that would relates standard deviation (as dependent variable) and their concentration

values (as independent variables) the polynomial statistical Model IV has been assayed (227–234), from a validation procedure involving the use of calibration curves (six on the same days during five days). These analytical error functions are a useful alternative to the weighting methods used in parameter estimation of e.g., β-blockers (227). The selection was made by the stepwise forward procedure (65), which allows to discriminate the best fitting when the differences among selected statistical parameters (F value, standard error of estimate. . .) did not differ significantly.

NON-UNIFORM VARIANCE IN ANALYTICAL CHEMISTRY (WEIGHTED LINEAR REGRESSION APPLICATIONS)

Although calibration can be a complex procedure involving sophisticated statistical methods, most analytical work still relies on that workhorse of analysis, the straight line univariate classical calibration (38). Classical, i.e., non-weighted linear regression is by far the most widely used regression method (118). When measurements are obtained over a wide range of the x variable, however, the assumption of uniformity in the variance of y is not valid. Although the assumption of homocedasticity is valid for some analytical procedures, there are others for which it is not, including these methods based on (247) counting measurements either photon or radioactive and also spectrophotometric and chromatographic analysis (i.e., Table 3) under certain conditions. Little attention, in general, has been paid to this problem by either statisticians or analytical chemistry.

In analytical chemistry, the assumption of homocedasticity distribution will generally turn out to be justified within the calibration range (12). Peculiar cases of heterocedasticity occur when constant relative variance (counts: Poisson distribution) are involved (138, 206, 207, 225). Photometric absorbances obeying the Lambert-Beer law over a wide range may also tend to heterocedasticity. In spectrophotometric measurements the uncertainty in the absorbance depends strongly on the absorbance itself and other parameters of the experiment as well. In these cases, the introduction of weighting factors may be considered (194, 195, 199).

Determination of the concentration of analyte (drug, metabolite) in biological matrices (serum, plasma, urine) by high-performance liquid chromatography (HPLC) is used in clinical pharmacology for two applications (121): (i) therapeutic drug monitoring, and (ii) tracer studies (single dose and pulsed dose). Unlike the pharmaceutical analysis, the concentration range in the bioanalysis test samples (being influenced by many factors such as absorption, distribution, metabolism, excretion, etc.) is dynamic and broad, normally of the order of three or more (43, 205, 298).

Let us assume the deviations of a chromatographic process only come from the uncertainty of the injection procedure. As we have only volumetric deviations and sample preparation is normally a combination of volumetric steps, this is often the most variable part of the chromatographic method. When volumetric deviations dominate we may expect the same coefficient of variation at all concentrations; that is increasing absolute standard deviation with higher concentrations (75). Variability increases systematically with response level so that the usual assumption of constant variance across the response range is not satisfied.

Many calibration curves are straight lines at low concentrations and curve toward the concentration axis at high levels. Simple first- and high-order regression equations often (12, 64, 190) do not satisfactorily fit these types of data. The problem can be solved by limiting the dynamic range to straight-line concentrations, by using a multiple curve technique or by developing an appropriate mathematical model (249).

Although a first-order equation is appropriate for many analysis (e.g., absorption measurements at low absorbances), for many others the calibration graph is non-linear and usually convex (33–42, 250). Many chromatographic detector exhibit a linear response over a limited concentration range, particularly spectroscopic methods of detection and deviation from linearity can be expected (36, 37, 251) for many applications. Non-linearity of the analytical curve, i.e., deviation from Beer-Lambert law, is a well-known phenomenon in atomic absorption spectrometry as soon as absorbance values higher (252) than 0.5 to 1.0 are reached.

With respect to variance uniformity (homocedasticity) it has been shown that assumption of homocedasticity is only true (253) when the concentration values $c < 22\ LD$(*LD*: limit of detection), whereas the error varies with the concentration (heterocedasticity) if $c > 50\ LD$. For this case, in trace analysis, a linear dependence of the Model type II in Table 9 may be observed. Due to the small value of k (usually between 0.01 and 0.001 for best instrumental methods), when experimental values do not differ greatly in magnitude, for example a concentration range of ten to twenty-fold, it can be considered that the standard deviation does no differ significantly and homocedasticity is assumed (253).

In analytical chemistry least squares regression is usually applied without defining weights (254–256) to the calibration samples although the variances of the responses are known to be concentration dependent. This approach has an important drawback for lower concentrations because the estimated values for "unknown" samples have deviations from the real values which are unnecessarily high. Consequently also the estimated uncertainties for low concentrations are large and the mathematically related limit of detection (12, 21, 53, 64).

Methods with an extended range capability should be checked for homocedasticity (constancy of precision) over the actual range applied to a given dosage form (260–263). With calibration over moderate-to-wide-calibration ranges the assumption of constant variance (250, 264) is always false. For example, many atomic and molecular absorption procedures have constant relative standard deviation (RSDs) over absorbance ranges of about 0.1–0.7, and if the relative variance is constant, the absolute variance must increase with concentration. A common result of incorrectly assuming constant variance is gross error at low

TABLE 10
Examples of non-uniform variance (weighted least squares) in analytical chemistry

Method	Comments	Authors	Ref.
Counting measurements Radioligand assay (i.e., radioimmunoassay (RIA) and immunoradiometric assay (IRMA))	Nuclear analytical measurements (Poisson counting statistics)	Currie, 1968	(273)
	Numerous elements in moon rocks from the lunar analysis program of the U.S. National Aeronautics and Space Administration	Morrison, 1971	(274)
	Practical method for evaluating the magnitude of the random errors in radioimmunoassay dose response variables and the relationship between this error and position on the dose-response curve	Rodbarg, 1976	(207)
	Types of response curves usually used in quantitative estimates from radioimmunoassay	Finney, 1976	(275)
	Methods for calculating statistical uncertainties in the form of confidence limits for analyses determined by calibration of counting experiments for which the calibration curve is linear	Schwartz, 1978	(241)
	Program for the analysis of immunoassays or bioassays that have a logistic dose-response relationship	Das and Tydeman, 1980	(276)
	Data reduction	Cernosek, 1980	(277)
	Data reduction	Fischer, 1983	(278)
	Calibration curves (i.e., logistic given in relation to log dose), are fitted by WLS to observed counts directly using empirical weights proportional to the reciprocal of estimated counting variance	Gettys, Burrows, and Hennicks, 1986	(279)
X-Ray fluorescence analysis (XRF)	Zn in brass	Bubert and Klockenkamper, 1983	(197)
	Fe, Pb, Cu trace analysis of glass	Bubert et al., 1984	(280)
Activation analysis	Program applied to data processing	Taczanow, 1973	(281)
UV-visible Spectrophotometry	Copper chloride solutions	Schwartz, 1979	(247)
	Hypothetical data	Garden et al., 1980	(190)
	Confidence band statistics in WLS procedures	Mitchell, 1985	(264)
	Detection of systematic errors in Platinum determination	Doerffel and Hebisch, 1988	(282)
	Role of statistical weighting in the least squares analysis	Tellinghuisen, 2000	(283)
	NO2-N-standardlösungen	U. Hillebrand, 2000	(284)
Gas Chromatography (GC)	Calibration curves constructed by measuring the peak heights corresponding to injected molar charges of benzene	Bocek and Novak, 1970	(285)
	Calibration curves with non uniform variance	Schwartz, 1979	(247)
	Determination of estriol in urine from women in pregnancy	Maurin and Scholler, 1981	(286)
	Calibration graphs and logarithmic transformation data	Kurtz, 1983	(287)

(Continued on next page)

TABLE 10
Examples of non-uniform variance (weighted least squares) in analytical chemistry *(Continued)*

Method	Comments	Authors	Ref.
	Analyzing pesticides by gas chromatography	Kurtz, 1985	(288)
	Gas chromatography/mass spectrometry toluene (from 4.6 picograms to 15 nanograms)	Rocke and Lorenzato, 1995	(237)
	Analyzing several pesticides by GC: 16 polychlorinated biphenylds (PCBs)	Zorn, Gibbsons, and Zonzogui, 1997	(53)
	Weighted vs. unweighted least squares in the calculation of the limit of detection and quantitation	Zorn et al., 1999	(289)
	Proprionitrile by GC/MS	Wilson et al., 2004	(127)
Differential pulse anodic stripping voltammetry	Tl by standard addition	Frankle et al., 1978	(290)
Atomic absorption spectrometry	Zn by standard addition	Frankle et al., 1978	(290)
	Typical sample calibration data	Tyson, 1988	(291)
	Cd; graphite furnace 0–43 ppb	Rocke and Lorenzato, 1995	(237)
	Validation of the calibration procedure	Penninckx et al., 1996	(263)
	Indium in 0.24 M HNO_3	Desimoni, 1999	(243)
Adsortive cathodic stripping voltammetry	Cr(VI) in water and in-house reference soil	Desimoni, 1999	(243)
Inductive coupled plasma (ICP)	Determination of La in aqueous solution by ICP-OES	Bubert and Klockenkamper, 1983	(197)
	ICP-EAS calibration data: Be, 5 to 5000 μg/l	Taylor and Schutyser, 1986	(9)
	Replication calibration data for Ni.	Waters, Carroll, and Spiegelman, 1987	(226)
	ETV-ICP-MS calibration data for ^{239}Pu	AMC, 1994	(292)
	Mo, Cr, Co, Pb and Ni in sub-surface and drinking water by ICP-AES	Sarbu, 1995	(111)
	Non-constant variance in calibration ICP-MS	Ketkar and Bzik, 2000	(293)
	Zn in deionised water extract	Brüggemann and Wennich, 2002	(50)
Kinetic data	Estimation of Km and V in enzyme kinetics	Wilkinson, 1961	(187)
	Chymotripsin-catalysed hydrolysis of methyl hippurate	Elmore et al., 1963	(294)
	Study of linear transformations derived from Michaelis-Menten	Dow and Riggs, 1965	(295)
	Presentation of kinetic results and their uncertainties due to random and systematic errors	Cvetanovic, Singleton and Paraskevopoulos, 1979	(296)
	Pocket calculator program for computing the first order constant	Bossaert et al., 1985	(297)
	Kinetic data with butyrylcholinesterase	Mannernik et al., 1986	(28)
	Determination of Km and Vmax	Price and Dodds, 1989	(298)
	Exponential decay with random errors added to the points	Logan, 1995	(299)

(Continued on next page)

TABLE 10
Examples of non uniform variance (weighted least squares) in analytical chemistry *(Continued)*

Method	Comments	Authors	Ref.
High Performance Liquid Chromatography (HPLC)	Spiked tissue samples (ivermectin: a broad spectrum antiparasitic agent) at various concentrations; 9.7 to 100 ppb	Oppenheimer et al., 1983	(225)
	Liquid solid isolation of drugs from complex matrices	Johnson et al., 1988	(300)
	Calibration curve: HPLC assay of a drug in blood	Davidian and Haalang, 1990	(7)
	Calibration and validation of linearity in biopharmaceutical analysis	Karnes and March, 1991	(301)
	WLS, alternatives in Clinical Pharmacology	Szabo et al., 1994	(121)
	Normal-phase HPTLC method for fumonisin in rise	Dawlatana et al., 1995	(302)
	Guiding principles for the evaluation of a method's overall performance	Bressolle	(303)
	Total captopril in plasma	Wieling et al., 1996	(108)
	CDRI compound 81/70 (anthelmintic) in cow milk validation	Nagaraja et al., 1999	(112)
	Biogenic amines by means of pre-column derivatization with dansyl chloride and separation of derivatives by RPLC	Castillo and Castells, 2001	(45)
	Determination of lamotrigine in biological fluids	Almeida and Castell-Bruno, 2002	(73)
	Selection of the weighting exponent	Kuss, 2003	(75)
	Determination of methotrexate in human plasma	Sadray et al., 2003	(205)
	Determination of lanthanides in synthetic standards	Santoyo et al., 2003	(304)
	Method validation of paclitaxel in pig serum	Kiser and Dolan, 2004	(76)
	Lantanides in international geochemical reference samples	Santoyo et al., 2006	(305)
Gravimetry	Sulphate determination, water analysis	Ferrus and Torrades, 1985	(306)
	Barium sulphate gravimetric	Torrades and Raurich, 1993	(307)
Electrophoresis	Regression models fitted by weighted least squares in the separation of peroxidase isoenzymes from two flaxgenotypes	Fieldes and Tyson, 1981	(308)
Capillary electrophoresis (CE)	Calculation of pKa values	Gluck and Cleveland	(309)
	Peak heights or areas in CE	Wátzig, 1995	(310)
	Heterocedastics and its consequences	Baumann and Wätzig, 1995	(208)
Mass spectrometry (MS)	Reduction of isotope dilution data by weighted linear regression	Schoeller, 1976	(16)
	LC/MS/MS of new substances and drug candidates over a concentration range of 1–1000 ng/l	Olah et al., 1997	(109)

concentrations (265). The least-squares procedure minimizes these errors, often rotating the line so that it does not pass through the origin, thus causing large relative errors at low concentrations (19, 21, 244). All analytical chemists should know that if a response curve has an intercept significantly different from zero, proper provision in the calculation (266) must be made.

Many applications in biology and medicine require the use of weighted least squares (267). Programs in Microsoft QuickBasic, BASIC and PASCAL have been devised for the estimation of parameter in a linear model by weighted least squares techniques using matrix procedures (268–270). A program has been described to establish calibration diagrams by weighted, linear, least squares regression of unbalanced response arrays (243). This program, prepared as a template in MATHCAD 7.02, avoids the use of sophisticated or robust techniques, and is validated by noise data (271) as done firstly with ordinary least squares of replicated data with unbalanced data set. A macro for weighted least squares use in an Excel spreadsheet (272) has also been devised.

A survey of the analytical applications of the use of weighted linear regression is given in Table 10.

RESIDUALS

A detailed examination of this topic is out the scope of this paper. Nevertheless some minor comments are described in the following. An interesting way for testing homocedasticity is to plot the residuals calculated from the straight line obtained by using the conventional least-squares method against x_i, or versus the fitted response values, because the residuals and the $y's$ are usually correlated (65). A horizontal band of residuals indicates constant variance and unweighted least squares regression is appropriate, that is, the residuals should be of believable size and more or less randomly distributed. A funnel shape opening toward larger values signifies increasing variability with concentration, resulting (53) in incorrect estimates of the intercept, slope and residual standard deviation, using unweighted least squares regression. An advantage of using standardized residuals is that (58) the vertical scale is in units of the assumed standard deviation of the data. Although least squares residual plots can be used for the identification of outliers, studentized residuals are more suitable for this purpose (24, 58).

When data are fitted to the wrong equation, the shape of the residual plot contain (24,25, 65) valuable information that can be utilized to determine the way in which the equation should be modified to achieve a better description of the data. If the residuals tend to follow a curve (Figure 1), the use of a curved calibration graph rather than a linear one is desirable. In the later case the signs (+ or −) of the residuals, which should be in random order if an appropriate statistical model has been used, will tend to occur in sequence ("runs"), being the number of runs significantly less than if the signs of the residuals has been + and − in random order.

The Wald–Wolfowitz method tests for the significance of the number of runs in a set of data by comparing the observed number of runs with tabulated data (311), but it cannot be used if these are fewer than nine points in the calibration graph. When the number of observations (signs) is greater than 19, the critical number can be approximately calculated from a formula (17). However, the number of accessible experimental points is frequently less, being these tests of restricted value. In practice, the use of y residual plots is recommended as they can provide a distinctive visualisation of both non-linearity and alert the analyst to potential problems. Outlier tests such as Cook's algorithm can also be applied to detect and remove value with large errors (40, 312–314).

In fits of models to experimental data it is usually assumed that the residuals have approximately a normal or Gaussian distribution. This is probably an acceptable general purpose assumption, except that these tend to be outliers due to accidents, such as misassignments, local perturbation, misidentified reference lines, transcription errors, and so on (59).

CONCLUDING REMARKS

In the annals of curve fitting, fitting a straight line has held a prominent place (315). The linear regression problem is certainly one of the most important data analysis, if not the most important (316). However, often problems arise when people unfamiliar with mathematical statistics attempt to put this theory into practice for a certain application. This is due to the difficulties encountered in interpreting the given formulae and due to the lack of insight into their statistical origin. Weights are often neglected in regression methods (199, 283). The analytical, pharmaceutical, biochemical and clinical literature has been thoroughly revised in searching for analytical applications which have been shown in tabular form.

The need for weighting depends on the nature of the uncertainties involved and results directly from the least-squares criterion, which is derived from the likelihood function (17, 32, 46, 47, 65), provided that the measurement error variance is known and independent of the model parameters and the weight are calculated as the inverse of the measurement error variance. The decision on weighted or unweighted least squares can be realized on the basis of a statistical test (4, 263) or on the basis of a theoretical model (317).

Random errors and standard deviations play a most important role in analytical chemistry and the interpretation of experimental results (189, 318). The variance of the signal is constant, proportional to the signal or proportional to the square of the signal (319, 320). Heterogeneous variances imply that some observations contain more information than others. Rational use of the data would require that more weight be given to those contain the most information. An experiment which includes replications allows further tests to be made on the appropriateness of assumptions (27, 117, 192).

Given that homocedasticity is often an unrealistic assumption or clearly violated based on the data available, the researcher should be sensitive to if and how the results obtained may be affected by heterocedasticity. The nature of the data in

bioanalytical calibration curve is such that it is very unlikely that the data are homocedastic (43, 112, 248). Since there is no objective criterion governing the choice of weigthing factors (227), in the absence of external information the choice must be an aesthetic one, namely a factor that appears to the experimenter to give the best fit of calculated values to experimental observations over the whole range, or a factor whose logic is pleasing (120). Apparent heterocedasticity may be caused by model mis specification. The main advantage that weighted least squares enjoy over other methods is the ability to handle regression in which the data points are of varying quality.

However, using measured values in lieu of predicted values to quantify the measurement error variance is approximately valid only when the noise in the data is relatively low. This practice may introduce sampling variation in the resulting estimates, as weights can be seriously affected, and also large estimated uncertainties for low concentrations and for the mathematically related limit of detection (225, 321,322). Caution must be exercised in assigning weights based on the variance from replicate measurements unless the number of measurements at each point is large. This procedure, however, increases the cost of the analysis and will be worthwhile only if additional data analysis is required (44).

An appealing alternative to replication is to assume that heterocedasticity is present due to some underlying, smooth variance function (7, 201, 204). The biggest disadvantage of weighted least squares is probably the fact that the theory behind this method is based on the assumption that the weights are known exactly (46,47, 57). This is almost never the case in real applications, of course, so estimated weights must be used instead. In cases in that homocedasticiy is not given, the estimated standard deviation s_y is frequently a function of the measured quantity $s_y = f(y)$, strictly speaking, $\sigma_y = f[E(y)]$. In this case the calibration system is heterocedastic and weighted least squares fitting is to be applied (317). Graphical displays can be constructed to investigate variance function models.

Although linear regression is, by definition, a process of linear modelling, it is possible to introduce non-linear terms to the linear mathematical framework by transforming variables. A basic rule of science says that (27, 30, 32), all other things being equal, the simplest model that describes the observed behaviour of the system should be adopted. Simple relationships are more easily understood and communicated to others. According to the FDA guidelines (43, 51) for bioanalytical method validation, "the selection of weighting and use of a complex regression equation should be justified."

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