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A STATISTICAL OVERVIEW ON UNIVARIATE CALIBRATION, INVERSE REGRESSION, AND DETECTION LIMITS: APPLICATION TO GAS CHROMATOGRAPHY/MASS SPECTROMETRY TECHNIQUE

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The paper summarizes critically the current approaches for the calculation of the limits of detection and quantification. In the context of the description of the method based on the calibration line, the arguments concerning the underlying experimental design, the choice of the appropriate model in the univariate regression, the effects of the dispersion characteristics of the data are deeply discussed. The effects of the scedasticity of the experimental data are taken into account in the obtainment of the calibration curve and in its utilization. To gain transparency, adaptability, and tutorial effectiveness the explicit formulas relevant to the use of straight line and quadratic models are reported. An application of the described procedures to GC-MS data is reported as an illustrative example.
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I. INTRODUCTION

Method validation, a major concern for analysts, requires that all the characteristics of an analytical method must be evaluated. Namely, specificity and/or selectivity, linear dynamic range, precision, accuracy, detection and quantification limits, recovery, proof of applicability have to be considered (Lindner & Wainer, 1996). In particular detection and quantification limits are two fundamental criteria of method validation but the existence of different approaches for their estimation can cause confusion and difficulty for effective comparisons. Table 1 lists for illustration the host of terms, symbols, and statistical items reported in the literature since the pioneering work of Kaiser (1966). To overcome the severe terminological and conceptual confusion surrounding these topics, the International Organization for Standardization (ISO) and the International Union of Pure and Applied Chemistry (IUPAC) developed revised documents bringing their nomenclature into essential agreement by 1995 (Currie, 1997).

Nevertheless two main points must still be remarked: in the first place the need of knowledge from the analyst of the basic statistical concepts underlying the different proposals for their proper use, and the convenience of the declaration of the approach chosen for the reliability of the data reported; second the quite cumbersome handling of the uncertainty of the results in the utilization of the calibration curve when the data do not fulfil the condition of uniform signal variance and the analytical technique gives a non-linear calibration curve.

The present work, therefore, in the spirit of a discussion- and application-oriented paper, aims to several purposes: (i) to recall the basic ideas underlying the most usual approaches in the definitions of the detection limits; (ii) to give rigorous guidelines to face the more cumbersome situations, that is, heteroscedasticity of the data and non-linearity in the calibration; (iii) to avoid any artlessness, like the use of calibration data too far away from the region of the blank value to calculate the detection limit (Mocak et al., 1997; Vial & Jardy, 1999) or the misunderstanding of the meaning of the residual standard deviation with the use of single data points or means of replicates; (iv) finally to remove the analyst from the blind use of black-box statistical packages allowing more transparency and unlimited applications.

Although the meaning of the detection limit is clear, in a qualitative sense, that is, it indicates the smallest concentration or amount of an analyte that can be reliably detected in a given sample by a chosen analytical procedure, two fundamental concepts must always be in mind: (i) one can operate in the signal domain, that of the instrumental responses, or in the analyte concentration/quantity domain with the passage from one domain to the other allowed by the calibration procedure; (ii) the detection of the analyte signal, that is, the statement of the presence of the real response of the analyte in a noisy background, is a problem at all different from the *a priori* forecast of unambiguously detecting the analyte signal when the analyte is effectively present in a sample at a defined concentration level.

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TABLE 1. Terms and symbols reported in the literature

Terms	Signal domain	Concentration domain	Reference
Limit of Detection	LOD	LOD	(IUPAC 1976)
	-	LOD	(ACS 1980)
	x_L	c_L	(Long et al. 1983)
Critical Level	L_C	x_C	(Oppenheimer et al. 1983)
	y_c	x_c	(Hubaux et al. 1970)
	Y_C	L_C	(Zorn et al. 1997)
Critical Value	L_C	x_C	(Currie 1995)
Threshold response value	y_p	x_1	(Clayton et al. 1987)
Method Detection Limit		MDL	(US Federal Register 1984)
Limit of guarantee for purity	x_G	c_G	(Kaiser 1966)
Limit of Identification	x_I	c_I	(Long et al. 1983)
Detection Level	L_D	x_D	(Oppenheimer et al. 1983)
Detection Limit	y_D	x_D	(Hubaux et al. 1970)
	Y_D	L_D	(Zorn et al. 1997)
Minimum Detectable Value	L_D	x_D	(Currie 1995)
Detection Limit with Assurance probability	-	x_d	(Clayton et al. 1987)
Limit of Quantification	-	LOQ	(ACS 1980)
	LOQ	-	(Long et al. 1983)
	Y_Q	L_Q	(Zorn et al. 1997)
Determination Limit	L_Q	x_Q	(Oppenheimer et al. 1983)
Minimum Quantifiable value	L_Q	x_Q	(Currie 1995)
Minimum Level	-	ML	(US EPA 1993)
Alternative Minimum Level	-	AML	(Zorn et al. 1997)

Non centrality parameter of the non central *t*-distribution	δ
False positive rate	α
False negative rate	β
Population standard deviation	σ
Estimated standard deviation	s
Residual standard deviation	$s_{y/x}$
Blank standard deviation estimated from noise magnitude	s_B
Confidence interval	
Regression band of a calibration line	
One-sided, and two-sided prediction and tolerance interval	

TABLE 2. Illustrative example: calibration data in terms of ratios of peak area of Chloromethane and of internal standard (Fluorobenzene) as a function of Chloromethane concentration

	Concentration Level (μg/L)								
No. replicates	0	0.03	0.1	0.2	0.4	0.8	1.6	3.2	4
1	0.009219	0.012867	0.024122	0.036817	0.051036	0.111975	0.174220	0.344967	0.355100
2	0.009101	0.012675	0.020211	0.038457	0.053503	0.084405	0.172282	0.297678	0.341706
3	0.006914	0.014311	0.020900	0.031085	0.064271	0.095427	0.168291	0.308669	0.365223
4	0.008310	0.012292	0.020327	0.036355	0.055831	0.118919	0.152625	0.277519	0.363193
5	0.007603	0.009007	0.023622	0.044505	0.071737	0.125506	0.229081	0.351525	0.417577
6	0.009011	0.011415	0.019576	0.037588	0.057600	0.089315	0.216992	0.302684	0.389765
7	0.006061	0.014701	0.026155	0.030706	0.075693	0.116848	0.186974	0.389644	0.411681
8	0.008032	0.013757	0.018471	0.034256	0.066599	0.138121	0.176933	0.323136	0.390485
9	0.005932	0.012900	0.030002	0.037076	0.059649	0.126417	0.242466	0.358242	0.465813
10	0.006034	0.012800	0.029385	0.042269	0.064498	0.105840	0.239470	0.366867	0.444202

The reported data for each concentration refer to ten replicates.

Stating analyte presence or absence and carefully estimating analyte concentration are primary goals which can be strictly linked to the calibration procedure. On this basis we first describe and thoroughly discuss the univariate calibration procedure, the inverse regression or discrimination, even if much material is available in standard tests, and then face the estimation of the detection and quantification limits.

In addition to the approach based on the dispersion characteristics of the calibration plot, also widely applied methods will be described and critically discussed pointing out the analogy of the concepts underlying the different approaches.

Finally, a practical application of the different procedures illustrated using GC-MS data is reported; any discussion about the qualitative identification-confirmatory step, necessary prerequisite for a meaningful quantification, will be omitted as outside the purpose of this paper.

II. CALIBRATION

A. Calibration Design

In establishing the univariate calibration function, defined as the functional relation between the expected instrumental responses and the analyte concentration/amount, the proper calibration design has to take into account whether the concentration of the calibration solutions is affected or not by significant errors. When the uncertainty in the concentration value x is negligible in respect to that of the instrumental response y, the usual assumptions in the regression analysis are valid and a very simple experimental design can be proposed. An uncontamined matrix aliquot is fortified at the jth standard concentration level x_j ($j = 1, 2, \ldots, k$) and m_j repeated measurements are made on the same solution to evaluate the instrumental uncertainty. Therefore in the calibration design the overall number of data points is given by $n = \sum_{j=1}^{k} m_j$.

If critical considerations of the characteristics of the apparatus and materials used in making up solutions indicate the presence of a non-negligible uncertainty in x and in the same time they allow to estimate the variance of x_j, $s_{x_j}^2$, a convenient approach is the maintainement of the previous calibration design adopting a weighted least-squares procedure with the weights containing the contributes of errors in x and y (Sharaf, Illman, & Kowalski, 1986). The estimates of the parameters of the model $y = \beta_0 + \beta_1 x + \varepsilon$ are then obtained minimizing, via a non-linear procedure, the sum

$$S = \sum_{j=1}^{k} \sum_{i=1}^{m_j} \frac{1}{s_j^2} (y_i - b_{0w} - b_{1w} x_j)^2 = \sum_{j=1}^{k} \sum_{i=1}^{m_j} \frac{(y_i - b_{0w} - b_{1w} x_j)^2}{s_{y_j}^2 + b_{1w}^2 s_{x_j}^2}$$

where s_j^2 is the overall variance of the responses at the level x_j, calculated for a straight line model via the error propagation as $s_j^2 = s_{y_j}^2 + b_{1w}^2 s_{x_j}^2$ ($s_{y_j}^2 =$ instrumental uncertainty; $s_{x_j}^2 =$ con-concentration uncertainty).

When $s_{x_j}^2$ is not calculable, the presence of significant errors in x_j can be tested by analysis of variance (Massart et al., 1988). Actually, analysis of variance of repeated measurements relative to different solutions of nominal equal concentration x_j evidentiates the effect of the "making up solutions" factor. If this factor is effective, again a weighted least-squares procedure must be employed with the weights given by $\frac{1}{Sj^2}$, where s_j^2 is comprehensive of the contributes of the instrumental variance $s_{y_j}^2$ and of the concentration variance $s_{x_j}^2$ (Mandel, 1967).

Concerning this experimental design it can be noted that if the different aliquots are samples of different matrices spiked at known analyte concentrations, the calibration design provides an overall calibration function applicable in the so-called "in-house validation" and in routine analysis when the uncertainty introduced by the different matrices is acceptable (Brueggemann, Morgenstern, & Wennrich, 2005).

Finally, when the error is concentrated in x instead of in y values, the conventional treatment can be applied simply using y as the independent variable.

When for insufficient instrumental or procedure reproducibility the variance s_j^2 results too large, the use of an internal standard is recommended. In the calibration plot the dependent variable is the ratio of the measured responses and the independent variable is the known molar concentration ratio.

For the calculation of a reliable calibration model some recommendations are often reported in the literature: (i) the number of concentration levels must range between seven and ten (Garden, Mitchell, & Mills, 1980); (ii) the replicates must be at least eight to ten to verify the normality of the data by the Shapiro–Wilk test (Shapiro & Wilk, 1965), for instance, and to ascertain their scedasticity; (iii) the calibration design must tune the problem in hand: the estimation of detection limits requires calibration points near the hypothesized values of the limits, whereas for accurate quantitative analysis the standard solutions must bracket the unknown one; (iv) the calibration measurements are to be run in blocks, each block containing one replicate of each standard, randomly chosen to avoid the effect of any systematic error, and blanks to avoid carryover effects; (v) finally, the blank solution response must be inserted in the regression procedure when the detection limits are determined (Mocak et al., 1997; Vial & Jardy, 1999) to decrease the difference between the experimentally measured blank and the intercept of the regression line.

B. Homoscedastic and Heteroscedastic Data

Calibration data may be homoscedastic, that is, of uniform variance, or heteroscedastic, that is, of non-uniform variance. In the former case the correct calibration model is calculated by the ordinary least squares (OLS) regression, whereas in the latter one weighted least squares (WLS) regression is the appropriate choice. It is noticeable that the two procedures furnish quite similar parameter estimates but the choice becomes important in the calculation of the uncertainty of a concentration value obtained via inverse regression and on the detection limit evaluation (Schwartz, 1979; Vial & Jardy, 1999).

Standard procedures can be followed to test whether homoscedasticity or heteroscedasticity holds: (i) the plot of the residuals of the un-weighted least squares regression versus the predicted values: a funnel shape trend with the increasing responses indicates an increasing variability with the concentration (Fig. 1). More immediately the plot of the differences among the replicates and their mean at each concentration furnishes analogous results; (ii) the Bartlett's test, which compares the variances of the replicates at each concentrations or a F-test between the largest and the smallest variance of the replicates

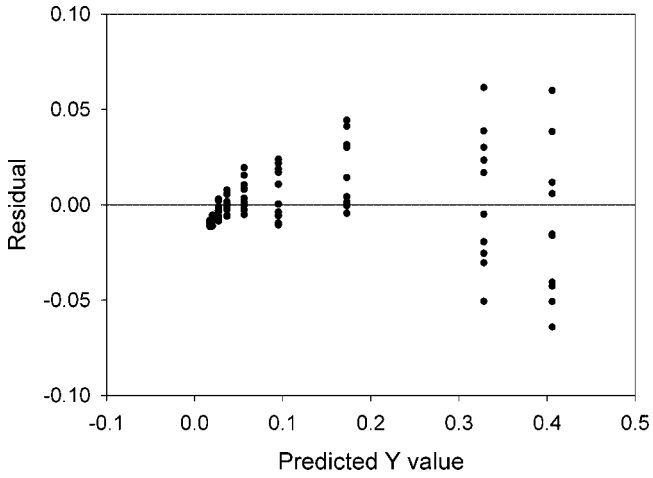

FIGURE 1. Plot of residual values for heteroscedastic data (Table 2) when ordinary least-squares regression is performed using a straight line model, $y = b_0 + b_1 x$.

(Massart et al., 1988). In this context the Hartley's F_{max}-test could be also mentioned (Hartley, 1950).

Under heteroscedastic conditions the plot of the experimental variances versus concentration and the calculation of the relevant model give some opportunities: the raw variance values coming from few repeated measurements are smoothed; the availability of the relationship between the variance values and the concentration level makes easier the calculation of the WLS regression and of its inverse as the weighting factors are the inverse of the variances at each concentration level; finally a comparison of the signal precision obtainable at differently defined detection limits is immediate (Vial & Jardy, 1999). It must be remarked that in this particular case the variance model must be drawn from experimental data belonging to the region of the detection limit including also the zero concentration (Wilson et al., 2004).

C. Calibration Models

Two models will be considered: the straight line, by far the most popular one,

$$y = \beta_0 + \beta_1 x + \varepsilon \tag{1}$$

and the non-linear, quadratic, calibration function, particularly useful, for example, when wide dynamic ranges and/or not isotopically pure internal standards are considered (Millard, 1978)

$$y = \beta_0 + \beta_1 x + \beta_2 x^2 + \varepsilon \tag{2}$$

The independent variable x is assumed unaffected by error; β_0, β_1, and β_2 are the parameters of the model and ε represents a normally distributed random error, with mean zero and constant variance σ^2 (homoscedastic condition), or non-constant variance (heteroscedastic condition): $\varepsilon \sim N(0, \sigma^2)$.

The signal y, therefore, is thought to be composed of a deterministic component predictable by the model and of a random component ε: $y \sim N(\beta_0 + \beta_1 x,\ \sigma^2)$ or $y \sim N(\beta_0 + \beta_1 x,$

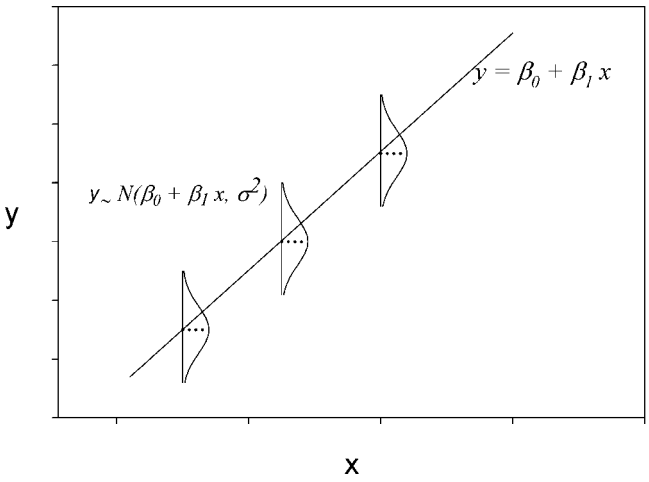

FIGURE 2. Illustration of the hypotheses for linear regression in the case of homoscedasticity.

$\beta_2 x^2, \sigma^2$). Figure 2 illustrates these assumptions in the case of a straight line for homoscedastic data.

The β parameters are unknown and the unweighted or weighted least-squares regression furnishes their estimates b by using a set of experimental data points (x_i, y_i). Thus one writes

$$\hat{y} = b_0 + b_1 x \tag{1'}$$

or

$$\hat{y} = b_0 + b_1 x + b_2 x^2 \tag{2'}$$

where $\hat{y}$ represents the predicted response of y for a given x.

Even if it is common practice to use software packages to calculate estimates of the parameters and of any other statistics of interest, here the explicit formulas for the two models considered are reported. This choice will allow to face any particular requirements like simultaneous prediction intervals and tolerance intervals, which usually are not provided by commercially available software packages.

III. CALIBRATION CURVE VIA UNWEIGHTED REGRESSION

A. Straight Line Calibration Curve

The least-squares estimates of parameters b_0 and b_1 in Equation (1′), of their variances $s^2_{b_0}$ and $s^2_{b_1}$ and of the variance of the y values $s^2_{y/x}$ are given by

$$b_0 = \bar{y} - b_1 \bar{x} \tag{3}$$

$$b_1 = \frac{\sum_{i=1}^{n} (x_i - \bar{x}) y_i}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \tag{4}$$

$$s^2_{y/x} = \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n-2} \tag{5}$$

$$s^2_{b_0} = s^2_{b/x} \left(\frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right) \tag{6}$$

$$s^2_{b_1} = \frac{s^2_{y/x}}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \tag{7}$$

where n is the overall data point number ($n = \sum_{j=1}^{k} m_j$, k = number of the concentration levels, m_j = number of replicates at the level j), $\bar{x} = \sum_{i=1}^{n} x_i / n$ and $\bar{y} = \sum_{i=1}^{n} y_i / n$. The statistic $s^2_{y/x}$ is called the residual variance of the regression and represents an estimate of the error variance σ^2 if the model is correct.

The adequacy of the model can be tested in several ways: (i) by the evaluation of the correlation coefficient $r = (n\Sigma xy - \Sigma x \Sigma y)/(n\Sigma x^2 - (\Sigma x)^2)^{1/2} (n\Sigma y^2 - (\Sigma y)^2)^{1/2}$; (ii) by the use of an analysis-of-variance technique which, in the absence of replicate data, implies an F-test on the regression significance (Mocak et al., 1997) while with replicate data is the so called *lack of fit* test (Analytical Method Committee, 1994); (iii) by inspection of the behavior of the residuals versus the predicted values. The first procedure is to be discouraged since a value of r close to unity not necessarily indicating a linear calibration function can lead to misinterpretation; the second one is effective when replicates at each concentration level are available; the third is graphical, easy to do and very revealing whether the assumptions on the errors ε and the model are correct.

The problem of confidence-banding the unknown true straight line with a fixed $(1-\alpha)$ probability is solved taking into account the joint uncertainties of b_0 and b_1. This leads to define a region in the plane $x - y$ bounded by the two functions

$$y^{\pm} = b_0 + b_1 x \pm (2F^{\alpha}_{2,n-2})^{1/2} s_{y/x} \left(\frac{1}{n} + \frac{(x - \bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} \tag{8a}$$

where F represents the chosen critical value of the Fisher statistic.

When the interest is in the calculation of a confidence interval on y at a particular point x instead of on the entire x value range, the critical constant $(2F^{\alpha}_{2,n-2})^{1/2}$ changes to the critical Student constant $t_{(1-\alpha/2,\, n-2)}$ (Wilson et al., 2004) to give

$$y^{\pm} = b_0 + b_1 x \pm t_{(1-\alpha/2,\, n-2)}\, s_{y/x} \left(\frac{1}{n} + \frac{(x - \bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} \tag{8b}$$

where $t_{(1-\alpha/2,\ n-2)}$ is $(1-\alpha/2)100\%$ point of Student's t-distribution on $n-2$ degrees of freedom.

The uncertainty of a future observation y predictable at a single x value has two contributes: the uncertainty of the estimates b_0 and b_1, which implies the non-uniqueness of the regression line, and the uncertainty of the single measurement or of the average of m ($m \geq 1$) replicates (Miller, 1993). This

uncertainty can be expressed by means of the $(1-\alpha)100\%$ two sided prediction intervals whose limits are

$$\bar{y}_m^{\pm} = b_0 + b_1 x \pm t_{(1-\alpha/2,\, n-2)}\; s_{y/x} \left(\frac{1}{m} + \frac{1}{n} + \frac{(x-\bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} \tag{9a}$$

If m is very large, $m \to \infty$, Equation (9a) collapses to

$$\bar{y}_{m\to\infty}^{\pm} = b_0 + b_1 x \pm t_{(1-\alpha/2,\, n-2)}\; s_{y/x} \left(\frac{1}{n} + \frac{(x-\bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} \tag{9b}$$

which coincides with Equation (8b).

The real significance of Equation (9a) and (9b) is as follows: they give the limits of the intervals which contain the mean responses $\bar{y}_m$, or $\bar{y}_{m\to\infty}$ at a fixed x, with probability of $1-\alpha$ (see Fig. 3).

Actually one can be interested to define an interval bounding not a single measurement or a mean of m future measurements, but a proportion P of the entire population of y at a fixed x, with probability $1-\alpha$. This interval, called non-simultaneous tolerance interval, is given by

$$y^{\pm} = b_0 + b_1 x \pm s_{y/x} \left\{ t_{(1-\alpha/4,\, n-2)} \left(\frac{1}{n} + \frac{(x-\bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} + N(P) \left(\frac{n-2}{{}^{\alpha/2}\chi^2_{n-2}} \right)^{1/2} \right\}$$

where $N(P)$ is the two-sided P percentile point of the standardized normal distribution and ${}^{\alpha/2}\chi^2_{n-2}$ is the lower $\alpha/2$ percentile point of the χ^2-distribution with $n-2$ degrees of freedom (Miller, 1966; Zorn, Gibbons, & Sonzogni, 1997).

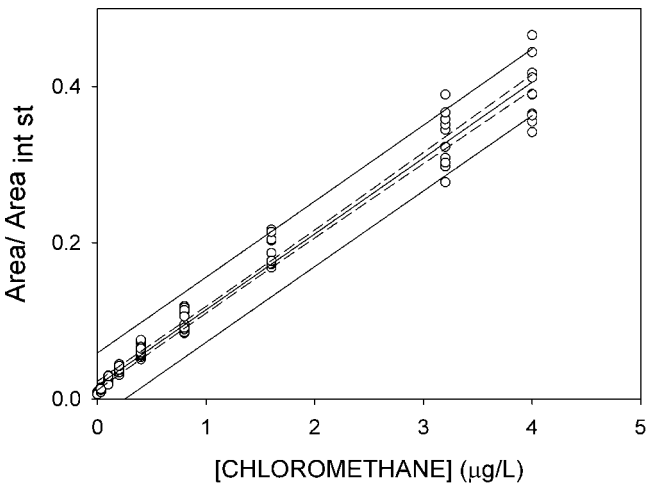

FIGURE 3. Illustrative example: (o) calibration data as summarized in Table 2; calibration line $y = b_0 + b_1 x$, regression bands (broken line), and prediction functions (continuous line) when a ordinary least-square regression is performed.

Prediction intervals or non-simultaneous tolerance intervals are the basis of a detection limit theory with the latter ones more appropriate with a large or unknown number of future detection decisions. More clearly, if a single sample is m times examined to detect the presence of the analyte, the use of the prediction interval at $x=0$ is the proper choice; alternatively, when an unknown number of samples have to be examined the use of the non-simultaneous tolerance interval at $x=0$ is appropriate.

When the number of prediction intervals to be handled simultaneously is large or unknown or when tolerance intervals are directly required, the use of simultaneous tolerance intervals is useful. In this case the limits bounding with probability at least $(1-\alpha)$ are given by

$$y^{\pm} = b_0 + b_1 x \pm s_{y/x} \left\{ (2F^{\alpha/2}_{2,n-2})^{1/2} \left(\frac{1}{n} + \frac{(x-\bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} + N(P) \left(\frac{n-2}{{}^{\alpha/2}\chi^2_{n-2}} \right)^{1/2} \right\}$$

This situation occurs when the inverse regression step is many times executed to quantify the content of the analyte in very numerous samples using always the same calibration line.

B. Outliers in Regression

In the context of OLS regression, several statistical approaches have been proposed to face the problem of outliers, that is, values which seem to be substantially different from the others. The identification of a data point as an outlier implies the decision of rejecting it before the calculation of the accepted regression line.

The question, particularly important when restrictions of time and/or standard material preclude further measurements, is cumbersome for two reasons: (1) the apparent presence can depend on the model chosen in the regression analysis; (2) even a single outlier can affect deeply the estimates of the regression coefficients calculated by the weak OLS method (Miller, 1993).

The residual diagnostic-based methods for identifying outliers can be of different complexity depending on whether the leverage value of the suspected point is considered.

A very simple test considers as outliers the points whose standardized residuals (mean zero and standard deviation unity) are larger than 2 or less than (-2) (Miller, 1993). Since this test suffers from the disadvantage that the residuals are not independent, its use deserves some caution particularly when the number of data points is small. Among the methods overcoming this drawback the so-called jackknife approach can be cited (Belsley, Kuh, & Welsch, 1980). It calculates at the ith suspected point the residual

$$r_{-i} = \frac{e_i}{s_i (1-h_i)^{1/2}},$$

where

$$s_i^2 = \frac{(n-2)s^2_{y/x} - \frac{e_i^2}{1-h_i}}{n-3}$$

and h_i, the leverage value of the i-th point, is defined by

$$h_i = \frac{1}{n} + \frac{(x_i - \bar{x})^2}{\sum (x_i - \bar{x})^2}$$

.

The property of the jackknife residuals of approximately following a t-distribution allows the easy identification of an outlier when $|r_{-i}| > t_{(1-\alpha/2,\, n-3)}$, at any chosen $1-\alpha$ value.

C. Inverse Regression

The analytical application of the calibration curve is the inverse regression, called also discrimination (Miller, 1966; Garden, Mitchell, & Mills, 1980), that is, the obtainment of x from an instrumental response y with the confidence interval for the true value of x (Brownlee, 1960). This interval depends on two factors: the uncertainty of b_0 and b_1 and the uncertainty of the experimental response reading, which can be a single or the mean of m replicate measurements. A common way to take into account these two sources of error is the application of the error propagation to the estimated concentration $\hat{x}_0 = (\bar{y}_{0m} - b_0)/b_1$ or better $\hat{x}_0 = \bar{x} + \frac{1}{b_1}(\bar{y}_{0m} - \bar{y})$ where $\bar{y}_{0m}$ is the mean of m measurements. The variance of $\hat{x}_0$ results to be

$$s^2_{\hat{x}_0} = \frac{s^2_{y/x}}{b_1^2}\left(\frac{1}{m} + \frac{1}{n} + \frac{(\hat{x}_0 - \bar{x})^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}\right) \tag{10}$$

Assuming $\hat{x}_0$ as approximately normal (Currie, 1997), the limits of the $(1-\alpha)100\%$ confidence interval for the true value of x_0 corresponding to the response average $\bar{y}_{0m}$ are (Massart et al., 1988)

$$\hat{x}_0^{\pm} = \hat{x}_0 \pm t_{(1-\alpha/2,\, n-2)} s_{\hat{x}_0} \tag{11}$$

Graphically this finding corresponds to select two limits $\hat{x}_0^-$ and $\hat{x}_0^+$, whose corresponding y values coincide with the limits of the prediction interval for y at the discriminated $\hat{x}_0$ value (Fig. 4a) (method I):

$$y^{\pm} = b_0 + b_1\hat{x}_0 \pm t_{(1-\alpha/2,\, n-2)}\; s_{y/x}\left(\frac{1}{m} + \frac{1}{n} + \frac{(\hat{x}_0 - \bar{x})^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}\right)^{1/2} \tag{12}$$

Consequently the limits $\hat{x}_0^-$ and $\hat{x}_0^+$ are given by the equation $\hat{x}_0^{\pm} = (y^{\pm} - b_0)/b_1$, which coincides with Equation (11) when $s^2_{\hat{x}_0}$ is given by Equation (10).

Equation 12 was also theoretically derived under the hypothesis that the function $g = t^2 s^2_{b_1}/b_1^2$ has a value less than 0.05 (Brownlee, 1960; Miller, 1991).

Another approach for the calculation of the limits $\hat{x}_0^-$ and $\hat{x}_0^+$ originates from the use of the $(1-\alpha)100\%$ two sided prediction band by intersecting it with a straight line $y = \bar{y}_{0m}$ (Fig. 4b)

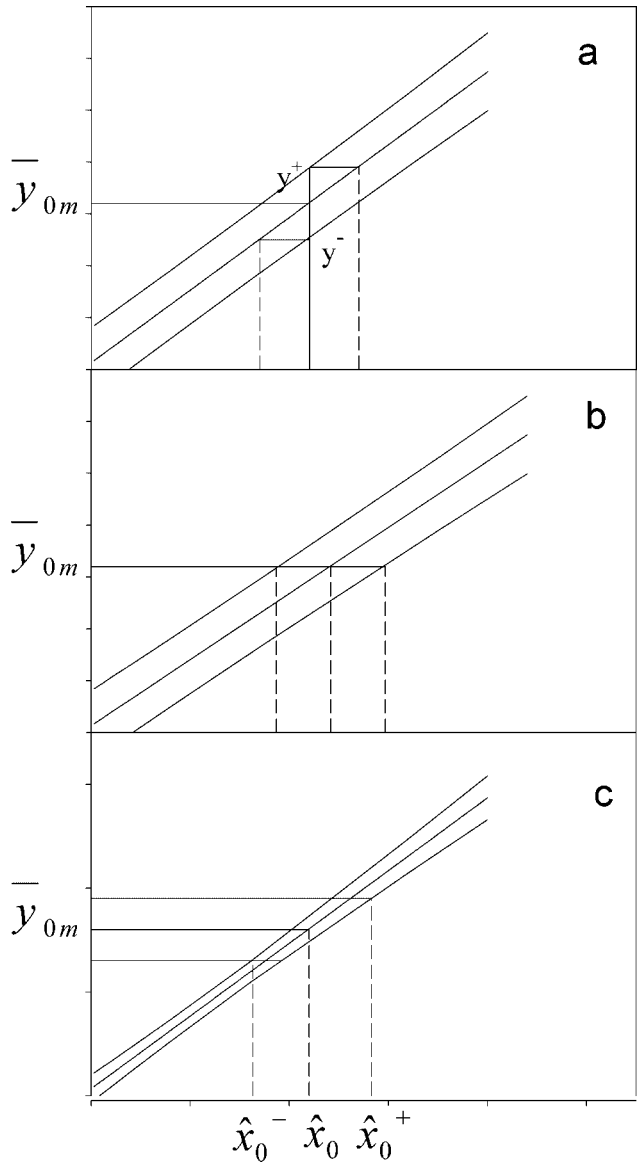

FIGURE 4. Graphical solution for the discriminated value $\hat{x}_0$ and its confidence limits $\hat{x}_0^-$ and $\hat{x}_0^+$, in correspondence to a mean response obtained from m repeated measurements, using the methods I (**a**), II (**b**), and III (**c**). The middle line in the a–c is the calibration line; the bounding lines are prediction functions in the a and b, and regression bands in the c. c: the regression bands, together with the confidence interval on $\bar{y}_{0m}$, individuate the confidence interval on x.

(method II) (Millard, 1978). The two limits $\hat{x}_0^-$ and $\hat{x}_0^+$ are defined by

$$\bar{y}_{0m} = b_0 + b_1\hat{x}_0^- + t_{(1-\alpha/2,\, n-2)}\; s_{y/x}\left(\frac{1}{m} + \frac{1}{n} + \frac{(\hat{x}_0^- - \bar{x})^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}\right)^{1/2} \tag{13a}$$

and

$$\bar{y}_{0m} = b_0 + b_1\hat{x}_0^+ - t_{(1-\alpha/2,\,n-2)}\; s_{y/x}\left(\frac{1}{m}+\frac{1}{n}+\frac{(\hat{x}_0^+-\bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2} \tag{13b}$$

These limits $\hat{x}_0^-$ and $\hat{x}_0^+$, whose $(1-\alpha)100\%$ prediction intervals for y yet have as upper and lower limits respectively the experimental response $\bar{y}_{0m}$, bracket the true value $x_0$with probability $1-\alpha$. It must be remarked that this confidence interval for x_0 can result asymmetric since normality is preserved in y-responses but no assumption is made on x.

In the case of unlimited applications of the calibration curve for discrimination, a suitable approach defines the $(1-\alpha)100\%$ confidence interval for x_0 by intersecting the $(1-\alpha/2)100\%$ confidence interval of $\bar{y}_{0m}$ with the two sided $(1-\alpha/2)100\%$ regression band of the straight line and projecting the intersections onto the x axis (Fig. 4c) (method III) (Garden, Mitchell, & Mills, 1980)

$$\bar{y}_{0m} - t_{(1-\alpha/4,\,m-1)}\, s_{\bar{y}_{0m}} = b_0 + b_1\hat{x}_0^- + \left(2F_{2,n-2}^{\alpha/2}\right)^{1/2} s_{y/x}\left(\frac{1}{n}+\frac{(\hat{x}_0^- - \bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2} \tag{14a}$$

and

$$\bar{y}_{0m} + t_{(1-\alpha/4,\,m-1)}\, s_{\bar{y}_{0m}} = b_0 + b_1\hat{x}_0^+ - \left(2F_{2,n-2}^{\alpha/2}\right)^{1/2} s_{y/x}\left(\frac{1}{n}+\frac{(\hat{x}_0^+ - \bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2} \tag{14b}$$

where $s_{\bar{y}_{0m}}$ is the standard deviation of the mean $\bar{y}_{0m}$ of m experimental responses. For few sample replicates m, the standard deviation of the mean $\bar{y}_{0m}$ can be substituted by $s_y/\sqrt{m}$.

The basic idea of this third approach is that all the points of the common area in Figure 4c have coordinates belonging jointly to the confidence intervals of $\bar{y}_{0m}$ and of the regression line.

Finally, the most conservative approach combines the regression band of the regression line and the tolerance interval on $\bar{y}_{0m}$ (method IV)

$$\bar{y}_{0m} - N(P)\left(\frac{n-2}{\alpha/2\chi^2_{n-2}}\right)^{1/2} s_{\bar{y}_{0m}} = b_0 + b_1\hat{x}_0^- + \left(2F_{2,n-2}^{\alpha/2}\right)^{1/2} s_{y/x}\left(\frac{1}{n}+\frac{(\hat{x}_0^- - \bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2} \tag{14c}$$

and

$$\bar{y}_{0m} + N(P)\left(\frac{n-2}{\alpha/2\chi^2_{n-2}}\right)^{1/2} s_{\bar{y}_{0m}} = b_0 + b_1\hat{x}_0^+ - \left(2F_{2,n-2}^{\alpha/2}\right)^{1/2} s_{y/x}\left(\frac{1}{n}+\frac{(\hat{x}_0^+ - \bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2} \tag{14d}$$

D. Detection and Quantification Limits

The availability of the calibration line and of its dispersion characteristics allows the calculation of the critical, detection and quantification limits. According to the literature (Hubaux & Vos, 1970; Long & Winefordner, 1983; Currie, 1995; Zorn, Gibbons, & Sonzogni, 1997), the critical level L_C is the assay signal above which a response is reliably attributed to the presence of analyte; the detection limit L_D is the signal corresponding to an analyte concentration level which may be *a priori* expected to be recognized; finally the quantification limit L_Q is a signal with a precision which satisfies an expected value.

The statistical definition of the critical level is based on the rejection of the null hypothesis, H_0: *concentration equal to zero*, at the significance level α, for example, $\alpha = 0.05$ (type I-error rate, false positive): $P(y > L_C|X=0) \le \alpha$. The numerical value of L_C can be easily calculated as the upper limit y^+ in Equation (9a) using $x=0$, $m=1$, and the $t_{(1-\alpha,\,n-2)}$ one-sided variate value

$$L_C = b_0 + t_{(1-\alpha,\,n-2)}\; s_{y/x}\left(1+\frac{1}{n}+\frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2}$$

The critical level in the concentration domain is $x_C = (L_C - b_0)/b_1$.

Figure 5 explains that the signal at $x=0$ has only 5% of proability of overcoming the critical level L_C. Consequently any response greater than L_C is to be attributed to the presence of analyte.

The detection limit L_D can be established invoking the type II-error, false negative error rate β. Actually, at the concentration x_C, L_C is the mean of the responses but a single response lies under L_C with probability β equal to 0.5. Therefore the detection limit L_D must be defined controlling the β-value which is usually set equal to the type I error rate α: $P(y < L_C|X=x_D) \le \beta$. Different values of α and β can be adopted when special circumstances, for example, a too high cost due to false negative error, suggest the choice.

The x_D value can be calculated as the abscissa of the intersection of the parallel line to the x axis passing through L_C, with the lower one-sided $(1-\beta)100\%$ prediction function

$$L_D = b_0 + b_1 x_D - t_{(1-\beta,\,n-2)}\; s_{y/x}\left(1+\frac{1}{n}+\frac{(x_D-\bar{x})^2}{\sum_{i=1}^{n}(x_i-\bar{x})^2}\right)^{1/2}$$

It is noticeable that in the equations giving L_C and L_D the terms inside the square root represent the two contributes of variance

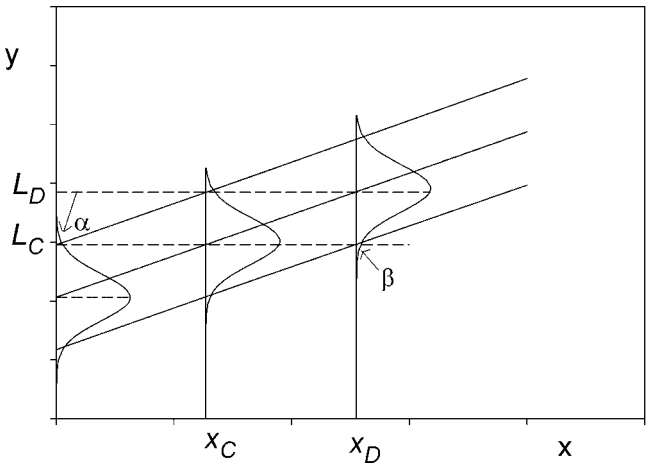

FIGURE 5. Graphical representation of the critical and detection limits in the signal and in the concentration domains with specified type I-error rate α and type II error rate β.

coming from the variability of the measurement and from the uncertainty of the calibration curve. The second contribute depends from the chosen experimental design so pointing out that the values of L_C and L_D depend on the experimental design adopted.

An alternative approach to calculate x_D is reported in the literature (Clayton, Hines, & Elkins, 1987; Currie, 1997; ISO, 1997). Remembering that the relations $P(y > L_C|X = 0) \leq \alpha$ and $P(y < L_C|X = x_D) \leq \beta$ are the theoretical basis of the underlying statistical tests, the critical level in the signal domain is calculated, as above, via a central *t*-distribution. The detection limit L_D is calculated by a non-central *t*-distribution taking the chosen protection against false negative error. The value of x_D in the concentration domain is immediately obtained by the calibration function as

$$x_D = \delta_{(\alpha,\beta,n-2)} \frac{s_{y/x}}{b_1} \left(1 + \frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2}$$

where $\delta_{\alpha,\beta,\ n-2}$ is the non-centrality parameter from the non-central *t*-distribution (Clayton, Hines, & Elkins, 1987).

Summarizing the former approach compares the background signal distribution with the analyte signal distribution at an unknown x_D using two central *t*-distributions; the latter, on the basis of the background signal distribution (central *t*-distribution) infers, via a non-central *t*-distribution, a signal L_D, and then x_D, which satisfies the type II error rate β.

Finally, the limits L_C and L_D can be calculated via the one-sided non-simultaneous (at a specified value of *x*) tolerance intervals:

$$L_C = b_0 + s_{y/x} \left\{ t_{(1-\alpha/2,\ n-2)} \left(\frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} + N(P) + \left(\frac{n-2}{\chi^2_{\alpha/2,\ n-2}} \right)^{1/2} \right\}$$

$$L_D = b_0 + b_1 x_D - s_{y/x} \left\{ t_{(1-\alpha/2,\ n-2)} \left(\frac{1}{n} + \frac{(x_D - \bar{x}^2)}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2} + N(P) + \left(\frac{n-2}{\chi^2_{\alpha/2,\ n-2}} \right)^{1/2} \right\}$$

The quantification limit L_Q is defined in different ways: (i) $(L_Q - b_0)/s_{L_C} = 10$: a net response equal to ten times the standard deviation at the lowest detectable signal L_C (Zorn, Gibbons, & Sonzogni, 1997); (ii)

$$\frac{L_Q}{s_{y/x} \left(1 + \frac{1}{n} + \frac{(x_Q - \bar{x})^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right)^{1/2}} = 10$$

a response equal to ten times the standard deviation of the prediction value at the concentration x_Q (Oppenheimer et al., 1983); (iii) $(L_Q - b_0)/s_{b_0} = 10$: a net response equal to ten times the standard deviation of the intercept (Miller & Miller, 1988; Vial & Jardy, 1999); (iv) finally, Eurachem (1993)) defines the quantification limit in the concentration domain as the analyte concentration x_Q for which the experimental relative standard deviation of the responses reaches a fixed level, for example, the level 0.1.

E. Quadratic Calibration Curve

When curvilinear calibration plots are obtained, the more usual model is

$$\hat{y} = b_0 + b_1 x + b_2 x^2$$

or equivalently

$$\hat{y} = \bar{y} + b_1 (x - \bar{x}) + b_2 (x^2 - \overline{x^2})$$

where $\bar{y} = \sum_{i=1}^{n} y_i/n$, $\bar{x} = \sum_{i=1}^{n} x_i/n$, and $\overline{x^2} = \sum_{i=1}^{n} x_i^2/n$.

The convenience of passing from a straight line to a quadratic model is indicated by proper tests. In addition to those cited previously, that is, plot of the residuals versus the predicted values, and the "*lack-of-fit*" test, the Mandel approach can be followed (Mandel, 1967): the residual variances obtained with the straight line model ($s^2_{y/x,I}$; I = first order) and with the quadratic model ($s^2_{y/x,II}$; II = second order) are used as mean squares M_I and M_{II}, respectively, to perform an ANOVA analysis. Table 3 summarizes the overall procedure. A value of the test-statistic $F = ((n-2)M_I - (n-3)M_{II})/M_{II}$ larger than the critical value with 1 and $n-3$ degrees of freedom, for the level of significance chosen, indicates the suitability of the quadratic model.

The parameters, estimated by least-squares procedure, are

$$b_1 = \frac{S_{ff} S_{xy} - S_{fx} S_{fy}}{\Delta}$$

$$b_2 = \frac{S_{xx} S_{fy} - S_{fx} S_{xy}}{\Delta}$$

TABLE 3. Analysis of variance to test the null-hypothesis H_0: $\beta_2 = 0$

Model	Number of parameters in model	Sum of squares of residuals	Degrees of freedom	Mean square
Straight line	2	SS_I	n-2	$M_I = \frac{SS_I}{n-2}$
Quadratic	3	SS_{II}	n-3	$M_{II} = \frac{SS_{II}}{n-3}$
Difference		$SS_I - SS_{II}$	1	$M_D = \frac{SS_I - SS_{II}}{1}$

where

$$\Delta = S_{xx}S_{ff} - S_{fx}^2$$

$$S_{xx} = \sum_{i=1}^{n} x_i^2 - n\bar{x}^2$$

$$S_{fx} = \sum_{i=1}^{n} x_i^3 - n\bar{x}\overline{x^2}$$

$$S_{ff} = \sum_{i=1}^{n} x_i^4 - n(\overline{x^2})^2$$

$$S_{xy} = \sum_{i=1}^{n} x_i\bar{y}_i - n\overline{xy}$$

$$S_{fy} = \sum_{i=1}^{n} x_i^2\bar{y}_i - n\overline{x^2}\bar{y}$$

n is the overall number of calibration points.

The parameter variances and covariances of interest are

$$s_{b0}^2 = \left(\frac{1}{n} + \frac{\bar{x}^2 S_{ff}}{\Delta} + \frac{\overline{x^2}^2 S_{xx}}{\Delta} - \frac{2\bar{x}\overline{x^2}S_{fx}}{\Delta}\right)s_{y/x}^2$$

$$s_{b_1}^2 = \frac{S_{ff}}{\Delta}S_{y/x}^2$$

$$s_{b_2}^2 = \frac{S_{xx}}{\Delta}S_{y/x}^2$$

$$s_{b_1,b_2}^2 = -\frac{S_{fx}}{\Delta}S_{y/x}^2$$

where $s_{y/x}^2 = \sum_{i=1}^{n}(y_i - \hat{y}_i)^2/n - 3$ estimates the measurement population variance σ^2.

Following the same arguments previously developed, the prediction and regression bands are

$$\bar{y}_m^{\pm} = \bar{y} + b_1(x - \bar{x}) + b_2(x^2 - \overline{x^2}) \pm t_{(1-\alpha/2,\,n-3)}s_{y/x}\left(\frac{1}{m} + U(x)\right)^{1/2} \quad (15)$$

$$\bar{y}_{m\to\infty}^{\pm} = \bar{y} + b_1(x - \bar{x}) + b_2(x^2 - \overline{x^2}) \pm t_{(1-\alpha/2,\,n-3)}s_{y/x}\left(\frac{1}{m} + U(x)\right)^{1/2} \quad (16)$$

where
$U(x) = \frac{1}{n} + (x - \bar{x})^2\frac{S_{ff}}{\Delta} + (x^2 - \overline{x^2})^2\frac{S_{xx}}{\Delta} - 2(x - \bar{x})(x^2 - \overline{x^2})\frac{S_{fx}}{\Delta}$.

Figure 6 shows a three-parameter parabolic calibration function together with the regression bands and two-sided prediction functions.

The calculation of an unknown x value, $\hat{x}_0$, with its confidence limits from an experimental $\bar{y}_{0m}$ response and of x_C, x_D, and x_Q is performed following the same guidelines used for the straight line model. In particular $L_C = (y^+)_{x=0}$ is given by

$$L_C = \bar{y} - b_1\bar{x} - b_2\overline{x^2} + t_{(1-\alpha,\,n-3)}s_{y/x}(1 + U(0))^{1/2}$$

and x_C is calculated from $L_C = \bar{y} + b_1(x_C - \bar{x}) + b_2(x_C^2 - \bar{x}^2)$. Again the analytical calculations can be replaced by a graphical procedure.

IV. CALIBRATION CURVE VIA WEIGHTED REGRESSION

Repeated measurements for each concentration level allow to test the scedasticity of the data. It is known (Schwartz, 1979; Currie, 1997) that the heteroscedasticity slightly modifies the estimates of the parameters but heavily affects confidence and detection limits. To account for the heteroscedasticity the weighted regression analysis is used suitably weighting the data to

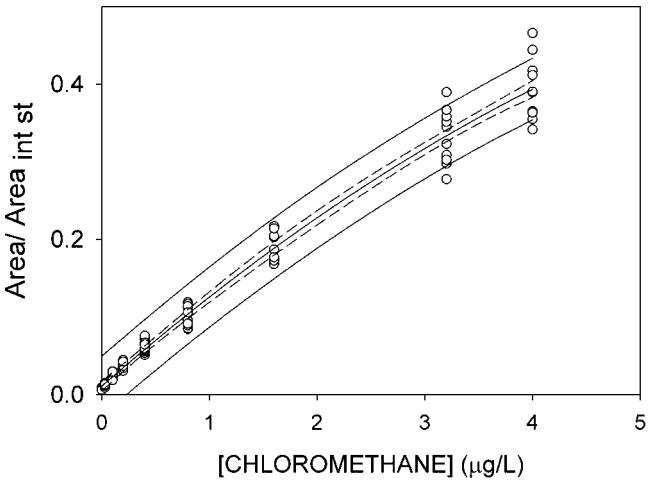

FIGURE 6. Illustrative example: (o) calibration data as summarized in Table 2; quadratic calibration function (middle line) with its regression bands (broken line) and prediction functions (continuous line) when OLS regression is performed.

obtain homoscedasticity. Each measurement relative to the concentration level x_j is multiplied by the factor

$$w_j^{1/2} = \left(\sigma^2/s_j^2\right)^{1/2},$$

where s_j^2 is the experimental variance of the replicate responses at x_j and σ^2 is the unknown common variance of all the weighted measurements (Garden, Mitchell, & Mills, 1980).

Some authors suggest the use of smoothed variance values obtained modeling the experimental variance values versus the concentration in place of the experimental ones (Schwartz, 1979; Analytical Method Committee, 1994; Zorn, Gibbons, & Sonzogni, 1997).

A. Weighted Straight Line Calibration Curve

Convenient weighted least-squares estimates of the intercept β_0 and of the slope β_1 and of their variances are given by the following formulas:

$$b_{0w} = \bar{y}_w - b_{1w}\bar{x}_w \tag{17}$$

$$b_{1w} = \frac{\sum_{i=1}^{n} w_i(x_i - \bar{x}_w)y_i}{\sum_{i=1}^{n} w_i(x_i - \bar{x}_w)^2} \tag{18}$$

$$s^2_{b_{0w}} = \left(\frac{1}{\sum_{i=1}^{n} w_i} + \frac{\bar{x}_w^2}{\sum_{i=1}^{n} w_i(x_i - \bar{x}_w)^2}\right)(s_{y/x})^2_w \tag{19}$$

$$s^2_{b_{1w}} = \frac{1}{\sum_{i=1}^{n} w_i(x_i - \bar{x}_w)^2}(s_{y/x})^2_w \tag{20}$$

$$(s_{y/x})^2_w = \frac{\sum_{i=1}^{n} w_i(y_i - \hat{y}_{iw})^2}{n-2} \tag{21}$$

where $\bar{x}_w = \sum w_i x_i / \sum w_i$, $\bar{y}_w = \sum w_i y_i / \sum w_i$, and $\hat{y}_{iw} = b_{0w} + b_{1w}x_i$.

The adoption of $1/s_i^2$ as the weighting factor w_i in the squared weighted residual $w_i(y_i - \hat{y}_{iw})^2$ gives a dimensionless residual standard deviation near unity, $(s_{y/x})_w \approx 1$ (Oppenheimer et al., 1983). Otherwise if a normalization factor is introduced in the weighting scheme,

$$w_i^* = n\frac{\frac{1}{s_i^2}}{\sum \frac{1}{s_i^2}},$$

which makes the sum of the normalized weights equal to the number n of observations, the weighted residual standard deviation $(s_{y/x})_w$ approximates the square root of the harmonic mean of the experimental variances of the data at any x_i.

The weighted prediction interval relevant to the mean $\bar{y}_{m_j}$ of m_j responses at concentration x_j is calculated as

$$(\bar{y}_{m_j})^{\pm}_{jw} = b_{0w} + b_{1w}x_j \pm t_{(1-\alpha/2,\, n-2)} (s_{y/x})_w \left(\frac{1}{m_j w_j} + \frac{1}{\sum_{i=1}^{n} w_i} + \frac{(x_j - \bar{x}_w)^2}{\sum_{i=1}^{n} w_i(x_i - \bar{x}_w)^2}\right)^{1/2} \tag{22}$$

The first term inside the parenthesis in Equation (22) is the variance of the mean of m_j responses at x_j. For $m_j \to \infty$ this term vanishes and Equation (22) then describes the regression band of a WLS straight line.

When the model of the variance is not available, an easy approach to obtain the prediction intervals is to calculate the limits $\bar{y}^{\pm}_{jw}$ at the calibration points inserting the experimentally available values $w_j = 1/s_j^2$ into Equation (22), and then interpolating between them.

B. Inverse Regression

The arguments developed in the context of the Section "III. C. Inverse Regression" hold again. To discriminate an unknown value $\hat{x}_0$ and to determine the confidence limits $\hat{x}_0^-$ and $\hat{x}_0^+$ from the average value of m responses $\bar{y}_{0_m}$, the procedures previously described can be followed. Being $\hat{x}_0 = (\bar{y}_{0_m} - b_{0_w})/b_{1_w}$, the first approach gives $\hat{x}_0^{\pm} = \left(\bar{y}_w^{\pm} - b_{0_w}\right)/b_{1_w}$, where

$$\bar{y}_w^{\pm} = b_{0_w} + b_{1_w}\hat{x}_0 \pm t_{(1-\alpha/2,n-2)}(s_{y/x})_w \left(\frac{1}{m \times w_{\hat{x}_0}} + \frac{1}{\sum w_i} + \frac{(\hat{x}_0 - \bar{x}_w)^2}{\sum w_i(x_i - \bar{x}_w)^2}\right)^{1/2}.$$

The second procedure gives $\hat{x}_0^-$ and $\hat{x}_0^+$ as the solutions of the following equations:

$$\bar{y}_{0m} = b_{0w} + b_{1w}\hat{x}_0^- + t_{(1-\alpha/2,n-2)}(s_{y/x})_w \left(\frac{1}{m \times w_{x_0^-}} + \frac{1}{\sum w_i} + \frac{(\hat{x}_0^- - \bar{x}_w)^2}{\sum w_i(x_i - \bar{x}_w)^2}\right)^{1/2} \tag{23}$$

and

$$\bar{y}_{0m} = b_{0w} + b_{1w}\hat{x}_0^+ - t(1-\alpha/2, n-2)(s_{y/x})_w \left(\frac{1}{m \times w_{x_0^+}} + \frac{1}{\sum w_i} + \frac{(\hat{x}_0^+ - \bar{x}_w)^2}{\sum w_i (x_i - \bar{x}_w)^2}\right)^{1/2} \quad (24)$$

The equations

$$\bar{y}_{0m} + t_{(1-\alpha/4, m-1)} s_{\bar{y}_{0m}} = b_{0w} + b_{1w}\hat{x}_0 - (2F_{2,n-2}^{\alpha/2})^{1/2} (s_{y/x})_w \left(\frac{1}{\sum w_i} + \frac{(\hat{x}_0^+ - \bar{x}_w)^2}{\sum w_i (x_i - \bar{x}_w)^2}\right)^{1/2} \quad (25)$$

and

$$\bar{y}_{0m} - t_{(1-\alpha/4, m-1)} s_{\bar{y}_{0m}} = b_{0w} + b_{1w}\hat{x}_0^- + (2F_{2,n-2}^{\alpha/2})^{1/2} (s_{y/x})_w \left(\frac{1}{\sum w_i} + \frac{(\hat{x}_0^- - \bar{x}_w)^2}{\sum w_i (x_i - \bar{x}_w)^2}\right)^{1/2} \quad (26)$$

furnish the limits $\hat{x}_0^-$ and $\hat{x}_0^+$ according to the third procedure. In the left-hand side of Equations (25) and (26) the term $s_{\bar{y}_{0m}}$ is the experimental standard deviation of the mean $\bar{y}_{0m}$ of m measurements.

Finally, with the weighted tolerance interval approach (fourth procedure) the limits $\hat{x}_0^-$ and $\hat{x}_0^+$ are given by the following equations:

$$\bar{y}_{0m} - (s_{y/x})_w \left(\frac{1}{m \times w_{x_0^-}}\right)^{1/2} N(P) \left(\frac{n-2}{\alpha/2\chi^2_{n-2}}\right)^{\alpha/2} = b_{0w} + b_{1w}\hat{x}_0^- + (2F_{2,n-2}^{\alpha/2})^{1/2} (s_{y/x})_w \left(\frac{1}{\sum w_i} + \frac{(\hat{x}_0^- - \bar{x}_w)^2}{\sum w_i (x_i - \bar{x}_w)^2}\right)^{1/2}$$

$$\bar{y}_{0m} + (s_{y/x})_w \left(\frac{1}{m \times w_{x_0^-}}\right)^{1/2} N(P) \left(\frac{n-2}{\alpha/2\chi^2_{n-2}}\right)^{\alpha/2} = b_{0w} + b_{1w}\hat{x}_0^+ - (2F_{2,n-2}^{\alpha/2})^{1/2} (s_{y/x})_w \left(\frac{1}{\sum w_i} + \frac{(\hat{x}_0^+ - \bar{x}_w)^2}{\sum w_i (x_i - \bar{x}_w)^2}\right)^{1/2}$$

When the weights $w_{\hat{x}_0^-}$ and $w_{\hat{x}_0^+}$ are calculated from the model of the variance, the limits $\hat{x}_0^-$ and $\hat{x}_0^+$ can be obtained via an iterative procedure. More easily, the graphical procedure furnishes the limits $\hat{x}_0^-$ and $\hat{x}_0^+$.

C. Detection and Quantification Limits

Equation (22), with $x_j = 0$ and the $(1-\alpha)100\%$ point of Student's t distribution (one-sided interval), determines L_{Cw} and then

$$x_{Cw} = \frac{L_{Cw} - b_{0w}}{b_{1w}} \quad (27)$$

The obtainment of L_{Cw} requires the insertion of the weight w_0 at zero concentration by using either the variance calculated with the model of the variance (Zorn, Gibbons, & Sonzogni, 1997) or the experimental variance of the blank.

Intersection of the parallel line to the abscissa axis at the level L_{Cw} with the lower $(1-\beta)100\%$ one-sided prediction function gives the detection limit x_{Dw}. The graphical solution is immediate; otherwise a more cumbersome iterative approach requiring the correct value for w_{x_D} can be used. Further, the procedure based on the non-central t-distribution can be adopted (ISO, 1997).

About the calculation of x_{Qw} the same definitions reported in Section "III. D. Detection and Quantification Limits" hold. The starting equations are

$$\frac{L_{Qw} - b_{0w}}{s_{L_{cw}}} = 10 \quad (28)$$

$$\frac{L_{Qw}}{(s_{y/x})_w \left(\frac{1}{w_{x_{Qw}}} + \frac{1}{\Sigma w_i} + \frac{(x_{Qw} - \bar{x}_w)^2}{\Sigma w_i (x_i - \bar{x}_w)^2}\right)^{1/2}} = 10 \quad (29)$$

$$\frac{L_{Qw} - b_{0w}}{s_{b_{0w}}} = 10 \quad (30)$$

D. Weighted Quadratic Calibration Curve

With non-uniform variance data the estimates of the parameters b_{1w} and b_{2w} of a quadratic calibration curve

$$\hat{y}_w = \bar{y}_w + b_{1w}(x - \bar{x}_w) + b_{2w}(x^2 - \overline{x^2}_w) \quad (31)$$

are

$$b_{1w} = \frac{s_{ff} s_{xy} - s_{fx} s_{fy}}{\Delta}$$

and

$$b_{2w} = \frac{s_{xx} s_{fy} - s_{fx} s_{xy}}{\Delta}$$

where

$$\Delta = s_{xx} s_{ff} - s_{fx}^2$$

$$s_{xx} = \sum_{i=1}^{n} w_i x_i^2 - \left(\sum_{i=1}^{n} w_i\right) \bar{x}_w^2$$

$$s_{fx} = \sum_{i=1}^{n} w_i x_i^3 - \left(\sum_{i=1}^{n} w_i\right) \bar{x}_w \overline{x^2}_w$$

$$s_{ff} = \sum_{i=1}^{n} w_i x_i^4 - \left(\sum_{i=1}^{n} w_i\right) (\overline{x^2}_w)^2$$

$$s_{xy} = \sum_{i=1}^{n} w_i x_i y_i - \left(\sum_{i=1}^{n} w_i\right) \bar{x}_w \bar{y}_w$$

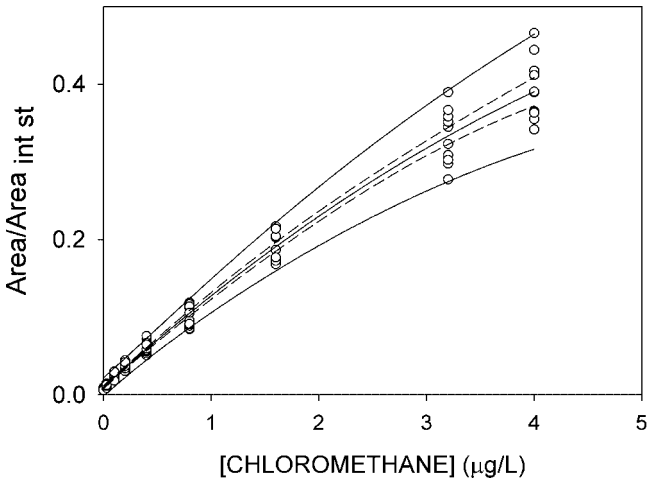

FIGURE 7. Illustrative example: (o) calibration data as summarized in Table 2; quadratic calibration function (middle line) with its regression bands (broken line) and prediction functions (continuous line) obtained with a WLS regression.

$$s_{fy} = \sum_{i=1}^{n} w_i x_i^2 y_i - \left(\sum_{i=1}^{n} w_i\right) \overline{x^2}_w \bar{y}_w$$

$$\bar{x}_w = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i},\ \overline{x^2}_w = \frac{\sum_{i=1}^{n} w_i x_i^2}{\sum_{i=1}^{n} w_i},\ \bar{y}_w = \frac{\sum_{i=1}^{n} w_i y_i}{\sum_{i=1}^{n} w_i}$$

and n is the overall number of calibration points.

The variances and covariances of interest are

$$s^2_{b_{1w}} = \frac{s_{ff}}{\Delta}(s^2_{y/x})_w$$

$$s^2_{b2w} = \frac{s_{xx}}{\Delta}(s^2_{y/x})_w$$

$$s^2_{b_1,b_2w} = -\frac{s_{fx}}{\Delta}(s^2_{y/x})_w$$

where

$$(s^2_{y/x})_w = \frac{\sum_{i=1}^{n} w_i (y_i - \hat{y}_{iw})^2}{n-3} \qquad (32)$$

The weighted prediction interval at x_j is given by:

$$(\bar{y}_{m_j})^{\pm}_{jw} = \bar{y}_w + b_{1w}(x_j - \bar{x}_w) + b_{2w}(x_j^2 - \overline{x^2}_w) \pm t_{(1-\alpha/2,n-3)}(s_{y/x})_w \left(\frac{1}{m_j w_j} + U_w(x_j)\right)^{1/2} \qquad (33)$$

$$(\bar{y}_{m_j\to\infty})^{\pm}_{jw} = \bar{y}_w + b_{1w}(x_j - \bar{x}_w) + b_{2w}(x_j^2 - \overline{x^2}_w) \pm t_{(1-\alpha/2,n-3)}(s_{y/x})_w (U_w(x_j))^{1/2} \qquad (34)$$

where

$$U_w(x_j) = \frac{1}{\sum_{i=1}^{n} w_i} + (x_j - \bar{x}_w)^2 \frac{s_{ff}}{\Delta} + (x_j^2 - \overline{x^2}_w)^2 \frac{s_{xx}}{\Delta} - 2(x_j - \bar{x}_w)(x_j^2 - \overline{x^2}_w)\frac{s_{fx}}{\Delta}$$

Figure 7 shows a quadratic calibration curve together with the regression band and the two-sided prediction curves calculated with $m_j = 1$.

To find a discriminated $\hat{x}_0$ value together with its confidence limits and to calculate the critical value L_{Cw}, the detection L_{Dw} and the quantification L_{Qw} limits, the arguments and the approaches above described hold again.

V. OTHER APPROACHES IN THE SIGNAL DETECTION AND IN THE ESTIMATION OF THE DETECTION LIMIT

Other procedures to evaluate the detection limit are reported in the literature. We here mention three of them for their continuous and wide application.

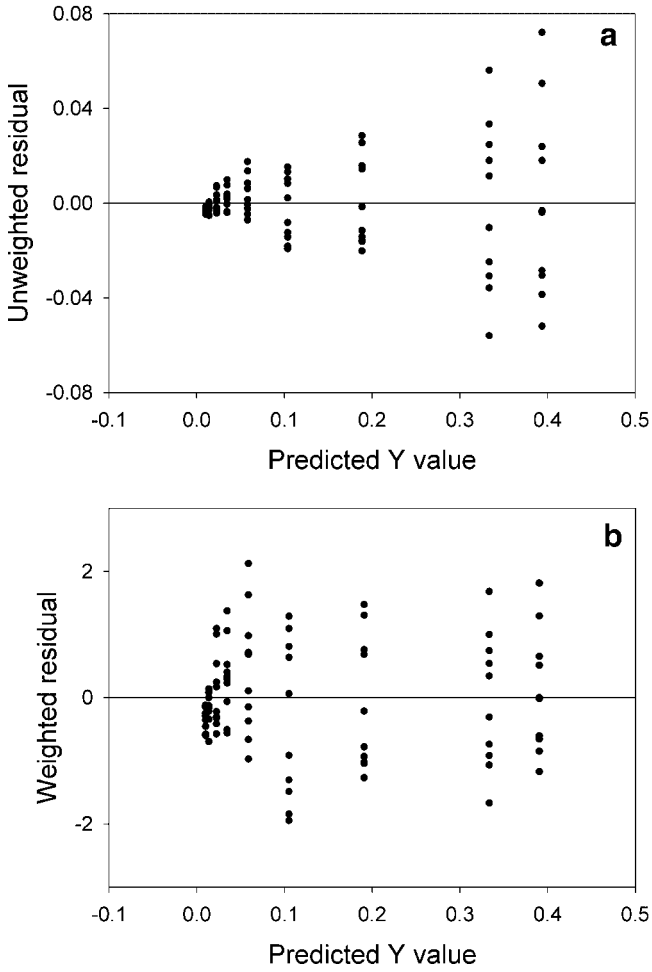

FIGURE 8. Plot of the residuals for the data of Table 2 fitted with the correct quadratic calibration model: (**a**) OLS, (**b**) WLS regression.

TABLE 4. Critical L_C and detection L_D limits in the signal domain from the signal-to-noise ratio approach

Parameters μ_B, σ_B^2 and σ_A^2 known and single analyte-concentration design ($n_A = 1$)

Homoscedastic data ($\sigma_A^2 = \sigma_B^2 = \sigma^2$)	Heteroscedastic data
$L_C = \mu_B + z_{1-\alpha}\sigma$	$L_C = \mu_B + z_{1-\alpha}\sigma_B$
$L_D = L_C + z_{1-\beta}\sigma$	$L_D = L_C + z_{1-\beta}\sigma_A^{(D)}$ (*)
$L_D = \mu_B + 2z_{1-\alpha}\sigma$ (if $\alpha = \beta$)	$L_D = \mu_B + z_{1-\alpha}(\sigma_B + \sigma_A^{(D)})$ (if $\alpha = \beta$)

Parameters μ_B, σ_B^2 and σ_A^2 unknown and multiple analyte-concentration design ($n_A > 1$)

Homoscedastic data ($\sigma^2 \cong s_p^2 = \dfrac{(n_B-1)s_B^2 + (n_A-1)s_A^2}{n_B + n_A - 2}$)

$$L_C = \bar{y}_B + t_{(1-\alpha,\upsilon)}\, s_p \left(\frac{1}{n_A} + \frac{1}{n_B}\right)^{\frac{1}{2}} \qquad (\upsilon = n_B + n_A - 2)$$

$$L_D = \bar{y}_B + t_{(1-\alpha,\upsilon)}\, s_p \left(\frac{1}{n_A} + \frac{1}{n_B}\right)^{\frac{1}{2}} + t_{(1-\beta,\upsilon)}\, s_p \left(\frac{1}{n_A} + \frac{1}{n_B}\right)^{\frac{1}{2}}$$

$$L_D = \bar{y}_B + 2\, t_{(1-\alpha,\upsilon)}\, s_p \left(\frac{1}{n_A} + \frac{1}{n_B}\right)^{\frac{1}{2}} \qquad (\text{if } \alpha = \beta)$$

$$L_D \cong \bar{y}_B + \delta_{\alpha,\beta,\upsilon}\, s_p \left(\frac{1}{n_A} + \frac{1}{n_B}\right)^{\frac{1}{2}} \text{ (**)}$$

Heteroscedastic data

$$L_C = \bar{y}_B + t_{(1-\alpha,\upsilon)} \left(\frac{s_A^{(C)2}}{n_A} + \frac{s_B^2}{n_B}\right)^{\frac{1}{2}} \text{ (***)} \qquad \left(\upsilon = \frac{(s_A^{(C)2}/n_A + s_B^2/n_B)^2}{(s_A^{(C)2}/n_A)^2/(n_A-1) + (s_B^2/n_B)^2/(n_B-1)} - 2\right) \text{ (****)}$$

$$L_D = \bar{y}_B + t_{(1-\alpha,\upsilon)} \left(\frac{s_A^{(C)2}}{n_A} + \frac{s_B^2}{n_B}\right)^{\frac{1}{2}} + t_{(1-\beta,\upsilon)} \left(\frac{s_A^{(D)2}}{n_A} + \frac{s_B^2}{n_B}\right)^{\frac{1}{2}} \text{ (*****)} \qquad (\text{if } s_A^{(C)} \cong s_A^{(D)})$$

$$L_D = \bar{y}_B + 2\, t_{(1-\alpha,\upsilon)} \left(\frac{s_A^{(C)2}}{n_A} + \frac{s_B^2}{n_B}\right)^{\frac{1}{2}} \qquad (\text{if } s_A^{(C)} \cong s_A^{(D)} \text{ and } \alpha = \beta)$$

(*) $\sigma_A^{(D)}$, standard deviation of the analyte response L_D.

(**) values of $\delta_{\alpha,\beta,\upsilon}$ for specified α, β, and υ are given by Clayton, Hines, & Elkins (1987).

(***) $s_A^{(C)}$, sample standard deviation of the analyte response L_C.

(****) from Sharaf, Illman, & Kowalski (1986).

(*****) $s_A^{(D)}$, sample standard deviation of the analyte response L_D.

In the first approach, OLS regression furnishes the calibration straight line $y = b_0 + b_1 x$ with the associated regression residual standard deviation $s_{y/x}$ and the standard deviation of the intercept s_{b0}. The limit of detection in the signal domain L_D is defined by the net response equal either to three times $s_{y/x}$ (Miller & Miller, 1988) or to three times s_{b0} (Vial & Jardy, 1999) and consequently by $3s_{y/x}/b_1$ or by $3s_{b0}/b_1$ in the concentration domain. The net response is obtained as difference of the gross signal and the blank signal estimated by the intercept of the regression straight line.

It is noticeable that the traditional value 3 is simply the rounding off of 3.29, that is, two times 1.645, which gives α and β rates of false positive and false negative errors both equal to 5%. For a Gaussian distribution the critical value of the one-tailed standardized variable z for $\alpha = 0.05$ is indeed 1.645. It can be observed that, even if not explicitly mentioned, a critical level L_C

TABLE 5. Regression parameters for the straight line and the quadratic model

	Ordinary least-squares		Weighted least-squares	
parameter	notation	estimate	notation	estimate
		Straight line		
slope	b_1	0.0971	b_{1w}	0.1045
intercept	b_0	0.0173	b_{0w}	0.0131
residual standard deviation	$s_{y/x}$	0.0208	$(s_{y/x})_w$	1.0259
			$(s_{y/x})_w'$	0.0092 *
		Quadratic model		
coefficient	b_2	-0.0064	b_{2w}	-0.0074
coefficient	b_1	0.1214	b_{1w}	0.1249
intercept	b_0	0.0106	b_{0w}	0.0100
residual standard deviation	$s_{y/x}$	0.0192	$(s_{y/x})_w$	0.8670
			$(s_{y/x})_w'$	0.0078 *

The dimensionless datum $(s_{y/x})_w$ is not comparable with $s_{y/x}$ because the weights chosen are not normalized so that their sum is not equal to the number n of observations (Vial & Jardy, 1999). Dividing the weighted residual standard deviation $(s_{y/x})_w$ by $(\sum w_i/n)^{1/2}$, one obtains a value $(s_{y/x})'_w$ which can be compared with $s_{y/x}$.

is introduced at the net signal level 1.645 times the chosen standard deviation. In the case of heteroscedastic data the procedure to calculate L_D is the same; however the use of the weighted residual standard deviation $(s_{y/x})_w$ requires that weighting factors normalized to their sum are employed in the weighted regression to save the dimensional significance of $(s_{y/x})_w$.

The second approach considers the signal-to-noise ratio in the signal domain. This procedure does aim to establish the presence of the analyte rather than to foresee whether a defined analyte concentration is detectable. In this context two operative ways are proposed on the basis of the independence (*Case A*) or dependence (*Case B*) of the measurements of the blank and of the sample (Sharaf, Illman, & Kowalski, 1986).

Case A. Be $\bar{y}_B$ the mean of n_B replicate measurements of the blank, $\bar{y}_A$ the mean of n_A replicate measurements of the analyte signal and $D = \bar{y}_A - \bar{y}_B$. Under the assumption of equal variances of the sample and blank measurements, ${\sigma_A}^2 = {\sigma_B}^2 = \sigma^2$, the variance of the difference D is $\sigma_D^2 = \sigma^2(\frac{1}{n_A} + \frac{1}{n_B})$. If only the estimates ${s_A}^2$ and ${s_B}^2$ are available and the F test proves their statistical equality (Massart et al., 1988), the estimate of σ^2 is the pooled value $s_p^2 = (n_A - 1)s_A^2 + (n_B - 1)s_B^2/(n_A + n_B - 2)$, and consequently $s_D^2 = s_p^2(\frac{1}{n_A} + \frac{1}{n_B})$.

The threshold value or the critical level L_C, defined as the minimum signal-to-noise ratio detectable, comes from $D = t_{(1-\alpha,v)}S_D$ and results to be

$$L_C = \bar{y}_B + t_{(1-\alpha,\upsilon)}\, s_p \left(\frac{1}{n_A} + \frac{1}{n_B}\right)^{1/2}$$

where $t_{(1-\alpha,\upsilon)}$ is the $(1-\alpha)100\%$ point of the Student's t-test distribution on $v = n_A + n_B - 2$ degrees of freedom.

Some observations can be advanced: (i) the critical level L_C can be lowered making more and more measurements, that is, suitably changing the experimental design; (ii) if n_A is equal to unity, the variability of the signal is drawn from n_B measurements of the blank and the limit L_C becomes

$$L_C = \bar{y}_B + t_{(1-\alpha,n_B-1)}\, s_B \left(1 + \frac{1}{n_B}\right)^{1/2}$$

(iii) if $n_B \to \infty$, ${s_B}^2$ and $\bar{y}_B$ approximate the parameters ${\sigma_B}^2$ and μ_B, respectively, and L_C becomes

$$L_C = \mu_B + z_{1-\alpha}\sigma_B$$

the early definition of the limit of detection; (iv) a deep analogy exists between the relationship

$$L_C = \bar{y}_B + t_{(1-\alpha,n_B-1)}\, s_B \left(1 + \frac{1}{n_B}\right)^{1/2}$$

and

$$L_C = b_0 + t_{(1-\alpha,n-2)}s_{y/x}\left(1 + \frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}\right)^{1/2}$$

drawn from the straight line calibration approach. The correspondence between the different estimates is as follows: the mean $\bar{y}_B$ with the intercept b_0, the variance s_B^2/n_B of the mean of the

TABLE 6. Illustrative example: GC-MS measurements of Chloromethane in water

	x_0	$(\hat{x}_0^-, \hat{x}_0^+)^a$	$(\hat{x}_0^-, \hat{x}_0^+)^b$	$(\hat{x}_0^-, \hat{x}_0^+)^c$	$(\hat{x}_0^-, \hat{x}_0^+)^d$
			Ordinary least-squares		
Straight line	1.86	(1.72, 2.01)	(1.72, 2.01)	(1.74, 2.00)	(1.65, 2.10)
Quadratic model	1.70	(1.55, 1.85)	(1.57, 1.86)	(1.51, 1.94)	(1.42, 2.03)
			Weighted least-squares		
Straight line	1.77	(1.64, 1.91)	(1.65, 1.91)	(1.65, 1.92)	(1.55, 2.01)
Quadratic model	1.67	(1.56, 1.79)	(1.57, 1.82)	(1.54, 1.87)	(1.47, 1.95)

Discriminated $\hat{x}_0$ value, with 95% confidence limits $\hat{x}_0^-$ and $\hat{x}_0^+$, obtained with ordinary and weighted least-squares approaches using a straight line and a quadratic calibration model. The $\hat{x}_0$ value comes from the response $\bar{y}_{0m} = 0.1983$, average value of $m = 10$ measurements taken on ten replicate samples of nominal Chloromethane concentration equal to 1.60 μg/L. All x values are expressed as μg/L. For simplicity, the notation of the limits is the same in the ordinary and weighted least-squares method.

[a]method I.
[b]method II.
[c]method III.
[d]method IV.

blank measurements with the variance of the intercept

$$s_{y/x}^2 \left(\frac{1}{n} + \frac{\bar{x}^2}{\sum_{i=1}^{n} (x_i - \bar{x})^2} \right),$$

the variance of a single response s_B^2 with $s_{y/x}^2$. In both cases the statistic $t = (u - \bar{u})/[\mathrm{Var}(u - \bar{u})]^{1/2}$, where $\bar{u}$ and $\mathrm{Var}(u - \bar{u})$ are the mean value of u, and the variance of $u - \bar{u}$, is the underlying concept for the calculation of the prediction value u.

Up to now only a type I error has been taken into account. To infer the detection limit L_D, protection against the type II error, false negative, must be considered resorting to the normal distribution when variances of the blank and of the sample signal are known and to central or non-central t-distributions when only estimated variances are available. Table 4 summarizes the different situations occurring.

Case B. When the measurements of the blank and of the sample are not independent owing to measurement procedure required by matrix effect, the so called paired-data procedure must be considered: each measurement of the blank signal $y_{B,i}$ is followed by a measurement of the sample signal $y_{A,i}$. The net signal is therefore $d_i = y_{A,i} - y_{B,i}$ with variance $s_d^2 = \sum (d_i - \bar{d})^2/(n-1)$, where $\bar{d}$ is the mean value of the differences and n is the number of the pair of signals. The mean value $\bar{d}$ of n net signals is considered significantly different from zero if $(\bar{d} - 0)/(s_d/\sqrt{n}) > t_{(1-\alpha,n-1)}$. The critical level in terms of net signal is $\bar{d}_C = t_{(1-\alpha,n-1)}(s_d/\sqrt{n})$ and therefore $L_C = \bar{y}_B + t_{(1-\alpha,n-1)}(s_d/\sqrt{n})$. The detection limit is given by $L_D = L_C + t_{(1-\beta,n-1)}(s_d/\sqrt{n})$.

In *Case A* and *Case B* the discussion has been developed in the signal domain. The passage to the concentration-quantity domain requires once again the use of the calibration curve so introducing new sources of uncertainty.

Finally, in the third procedure, adopted by United States Environmental Protection Agency (US EPA, 1993), a limit, called method detection limit (MDL), is defined as

$$\mathrm{MDL} = t_{(a=0.01,n-1=6)}\, s = 3.14\, s$$

where s is the standard deviation of a sample of $n = 7$ replicates in which the analyte is spiked at a concentration of two to five times the suspected MDL, and t is the single-sided 99%, six degrees of freedom variate of Student's distribution. The mean response value at the spiked concentration permits the immediate calculation of x_{MDL} admitting null response at null concentration level.

VI. ILLUSTRATIVE EXAMPLE

In this section we present, as a comprehensive example of the theory reported above, an application relative to GC-MS measurements of Chloromethane in water. The data reported in Table 2 and shown in Figures 3, 6, and 7 are treated to draw the most suitable calibration model from 0 to 4 μg/L, to calculate calibration based critical, detection and quantification limits and, finally, to determine an unknown concentration with its confidence limits.

The experimental data were collected following the recommendations of the EPA method (Munch, 1995; Lavagnini, Favaro, & Magno, 2004). The calibration design procedure implied the preparation of ten replicate samples at each of the nine concentration levels chosen and the recording of a single GC-MS peak in total ion current acquisition mode. The replicate

TABLE 7. Illustrative example: GC-MS measurements of Chloromethane in water

	Straight line				Quadratic model			
Ordinary least-squares								
x_C	0.36 [a]	0.55 [b]			0.27 [a]	0.42 [b]		
x_D	0.72 [a]	1.10 [b]	0.72 [c]	0.64 [d]	0.55 [a]	0.90 [b]	0.55 [c]	0.49 [d]
x_Q	2.15 [e]			2.15 [f]	1.74 [e]			1.74 [f]
Weighted least-squares								
x_C	0.11 [a]	0.18 [b]			0.08 [a]	0.13 [b]		
x_D	0.23 [a]	0.38 [b]	0.23 [c]	0.26 [g]	0.16 [a]	0.28 [b]	0.16 [c]	0.19 [g]
x_Q	0.66 [e]	0.68 [e]		0.88 [h]	0.57 [e]	0.58 [e]		0.65 [h]

Critical limit x_C, detection limit x_D and quantification limit x_Q, obtained with ordinary and weighted least-squares approaches using a straight line and a quadratic calibration model. Detection limits obtained with other approaches are also reported. All x values are expressed as μg/L.

[a]Values obtained by using the method based on the one-sided prediction interval ($\alpha = \beta = 0.05$).

[b]Values obtained by using the method based on the one-sided 95% non-simultaneous tolerance interval.

[c]Values obtained by using the method based on the non-central t-distribution ($\alpha = \beta = 0.05$).

[d]x value computed from the net response equal to 3 $s_{y/x}$.

[e]x_Q is the concentration level whose response L_Q is defined by the relationship $(L_Q - b_0)/s_{L_C} = 10$, where L_C is the critical response (Zorn, Gibbons, & Sonzogni, 1997).

[f]x value computed from the net response equal to 10 $s_{y/x}$.

[g]x value computed from the net response equal to 3 $(s_{y/x})'_w$, where $(s_{y/x})'_w = (s_{y/x})_w/(\sum w_i/n))^{1/2}$.

[h]x value computed for the net response equal to 10 $(s_{y/x})'_w$.

solutions were randomly analyzed at each of the nine concentrations to encompass instrumental and dilution variability. Moreover, carryover effects were annihilated inserting blanks in the sequence. The employed hyphenated instrument was made up of the following modules: (i) AquaTek 70 Liquid vial autosampler (Tekmar, Mason, OH); (ii) Tekmar HP76795 purge-and-trap with cryomodule; (iii) HP6890 gas chromatograph equipped with a J&W DB 624 capillary column (60 m × 0.32 mm, 1.40 μm film thickness; Agilent, Palo Alto, CA); and (iv) Agilent HP5973 quadrupole mass spectrometer. The instrument worked under TekLink 3100 and HP Enhanced Chemstation Control software.

To illustrate the effects of the use of incorrect and correct calibration models for the data reported in Table 2, we used the unweighted/weighted straight line and quadratic approaches. Table 5 shows the parameters of the four calibration models. Table 6 shows the discriminated values for the same instrumental response for each calibration model and Table 7 shows the critical, the detection, and the quantification limits. It appears that the heteroscedasticity slightly affects the estimates of the parameters for a given model (see Table 5) and slightly modifies the discriminated value $\hat{x}_0$ for a given response y_0 (see Table 6). On the contrary its effect is heavy on the values of the detection limits (see Table 7). The confidence limits for $\hat{x}_0$ are found symmetric with the methods I and II, and asymmetric with the approaches III and IV. Moreover the amplitudes of the confidence intervals calculated with the I and II methods are very similar and narrower than those obtained by the III and IV procedures.

The trend of the residuals in Figure 1 proves that the straight line model is incorrect and the increasing variances with the predicted y values indicate the absence of homoscedasticity. The use of the quadratic model gives a more symmetric distribution of the residuals around the zero values (Fig. 8a) and finally the WLS quadratic regression also accounts for the heteroscedasticity (Fig. 8b). A further confirmation of the suitability of the quadratic weighted calibration curve instead of the weighted straight line can be obtained by the Mandel test (Mandel, 1967). The resulting F-value, equal to 36.2, is found to be significant at the 5% level ($F_{1,87} = 3.99$).

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