amc technical brief

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Uncertainties in concentrations estimated from calibration experiments

Analytical chemists routinely use calibration data to evaluate the results of instrumental analysis. We subject a series of effectively matrix-matched standard materials of known concentrations to the same protocol as that used for test materials. We estimate the concentrations of analyte in the latter by interpolation, either graphically or by regression. But we can get important further information from the same data: the standard deviations and confidence limits of the estimated concentrations, and the conditions under which their uncertainties can be minimised. Often these uncertainties are disturbingly large. When the calibration graph is linear, straightforward equations are available to achieve these outcomes.

The line of regression of *y* on *x*

In most calibration experiments we make the assumption that the uncertainties in the concentrations (*x*) of the standards are negligible compared with those of the output signals (*y*) of the analytical instrument. The graph thus plotted is *the line of regression of y on x*, that is, the line $\hat{y} = a + bx$ which minimises, using the least squares criterion, the *y*-direction residuals in the calibration. From the slope and intercept of the graph, we use the instrument signal for an unknown test sample, y_0, to give the corresponding concentration, from $x_0 = (y_0 - a)/b$. It turns out that finding s_0 (the standard deviation of the unknown concentration x_0) is not a completely simple statistical problem because, in analytical work, we use the line of regression of *y* on *x* in an unusual way.

'Inverse regression'

We can illustrate this using a non-chemical example. If we determine the weights of a number of infants of known ages and plot them on a graph (for simplicity, we assume it to be linear) then, to use the line of regression of *y* on *x*, it will clearly be right to plot the weights as *y* and the ages as *x*. Different children of the same age do not all weigh the same, and there will be measurement errors too, whereas the infants' ages will be known exactly. The normal use of such a graph would be to estimate by interpolation the *average weight of a child of a given age*, i.e. we would find a *y*-value from an *x*-value. Such an estimate would naturally have an associated uncertainty, as the slope and intercept of the graph would be uncertain because of the scatter of the points. (The standard deviations of the slope and intercept are readily given by programs such as Excel®).

In analytical work, however, we use the same type of graph *to estimate x_0 from y_0*, the opposite process sometimes known as *inverse regression*. (This would be equivalent of estimating an infant's age from its weight.) So the estimate of s_0 is complicated by the fact that, in addition to the uncertainty in the position of the calibration line itself, the measured value of y_0 is also subject to uncertainty, so the value of s_0 must somehow reflect both these contributions (Figure 1).

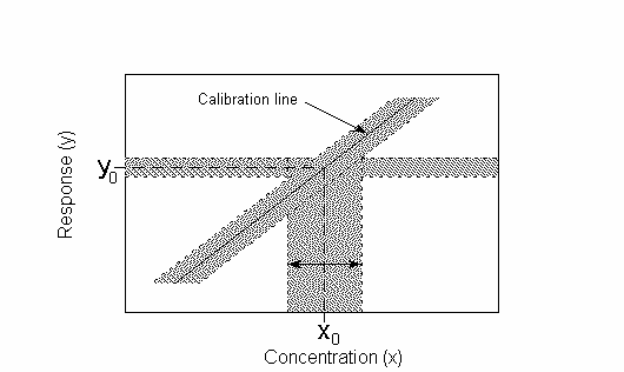

Figure 1. The confidence regions (shaded) in signal y_0 and the regression (calibration) line interact to give unexpectedly wide 'inverse confidence limits' (double arrow) for concentration x_0.

'Inverse confidence limits'

Because this interaction of error contributions is rather complex, we tend to use a simplified version of the necessary equations. If we use an *un-weighted* calibration approach (in which the *y*-direction random error is assumed to be the same for all *x* values, so that all the points on the graph have the same weight, or importance) then the equation for s_0 is:

$$s_0 = \frac{s_{y/x}}{b}\left\{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}\right\}^{\frac{1}{2}} \qquad (1)$$

In (1) the *n* calibrating points on the graph have means $\bar{x}$ and $\bar{y}$, the test material is measured *m* times giving a mean response value y_0, *b* is the slope of the graph, and $s_{y/x}$ is given by:

$$s_{y/x} = \left\{\frac{\sum_i (y_i - \hat{y}_i)^2}{(n-2)}\right\}^{\frac{1}{2}} \qquad (2)$$

where the $\hat{y}_i$ values are the *fitted y*-values, that is, the points on the calibration line at the standard values of x_i. Excel® calls $s_{y/x}$ the "standard error" of the line. Confidence limits for x_0 are obtained from $x_0 \pm ts_0$, the value *t*-value being taken at the required probability level and $(n - 2)$ degrees of freedom. These confidence limits have been called *inverse confidence limits*

(reflecting the use of inverse regression) or *fiducial limits* (Draper and Smith, 1998).

The approximation inherent in equation (1) is valid if:

$$\frac{t^2 s_{y/x}^2}{b^2 \sum_i (x_i - \bar{x})^2} \leq 0.05 \qquad (3)$$

The *t*-statistic is used as above. In analytical calibrations, equation (3) is almost always valid: unless the data are very poor the function often has a value of <0.01.

Example
We can apply these equations to a simple and typical example of a good-quality calibration graph:

y	0.099	0.187	0.274	0.347	0.426	0.489
x	0	5	10	15	20	25

The data and regression line are shown in Figure 2. It is easy to show that in this case $b = 0.0157$, $s_{y/x} = 0.00894$, and $\sum (x_i - \bar{x})^2 = 437.5$. The 2-tailed *t*-value for $p = 0.05$ and 4 degrees of freedom is 2.78, so the function in equation (3) has the value 0.0057. Equation (1) can thus be applied with confidence. If we further assume that y_0 is measured once only with a value of 0.400, corresponding to an x_0 value of 18.6, we also find that $s_{y/x}/b = 0.569$, and that the three terms inside the brackets in (1) are respectively 1, 1/6, and 0.0855. The value of s_0 is thus $0.569 \times \sqrt{1.252} = 0.637$. The 95% confidence limits for x_0 are thus 18.6 ± 1.8 (Figure 2). This example is typical of good calibration graphs with modest values of *n*: in such cases the confidence interval is a good deal wider than we might naively expect, and if the value of x_0 is close to the limit of detection of the method the interval might conceivably include zero.

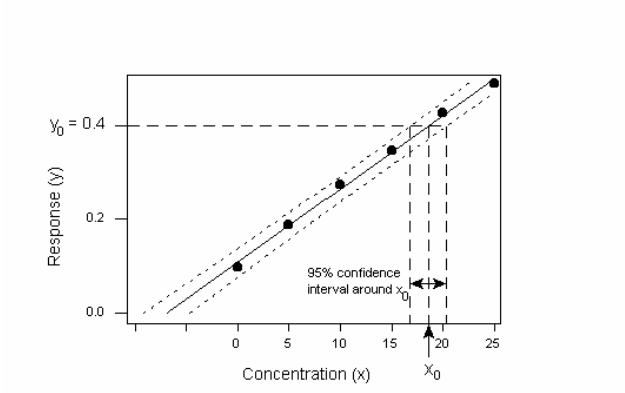

Figure 2 Confidence interval on a concentration estimated from a response of 0.4 from the example data.

Minimising s_0 and the confidence interval of x_0
By studying equation (1) we can deduce the conditions in which s_0 can be minimised, presumably a major aim of a calibration-based method. Unsurprisingly this standard deviation is small when *b* is substantial (that is, the line is "well determined") and when $s_{y/x}$ is small, which we see when the calibration points lie close to the regression line. In most cases if $m = 1$ this is the largest term of the three inside the brackets of equation (1), so a major reduction in s_0 is achieved simply by making multiple measurements of y_0. For example if $m = 4$ in our example, the value of s_0 falls from 0.637 to 0.403. If the number of measurements, $(m + n)$, is limited by considerations of time, costs, etc, then $(1/m + 1/n)$ is minimised by making $m = n$. However this may not produce the best (narrowest) confidence limits for x_0, as the value of *t* rises sharply as *n* falls. Thus it is often recommended that *n* is at least 6. Finally it is clear that the third term inside the brackets of (3) is minimised if $y_0 \approx \bar{y}$, that is, we obtain the smallest uncertainty near the centroid of the calibration graph. In such cases the third term may well be the smallest term in (1), but in our example, if y_0 is 0.1, then the third term becomes 0.3859, substantially higher than $1/n$. In principle it is feasible to reduce the third term further, maximising $\sum (x_i - \bar{x})^2$ by having two clusters of calibration points at high and low values of *x*. However this discards much information on the characteristics of the calibration, and is discouraged.

Standard additions
When a calibration experiment is carried out using the method of standard additions to minimise some matrix effects, the value of x_0 is effectively determined by extrapolating the line to the value $y_0 = 0$. Equation (1) must then be modified to give:

$$s_0 = \frac{s_{y/x}}{b} \left\{ \frac{1}{n} + \frac{\bar{y}^2}{b^2 \sum_i (x_i - \bar{x})^2} \right\}^{\frac{1}{2}} \qquad (4)$$

Available software
A Minitab® macro for calculating inverse regression confidence intervals is available through the Resources section of the program's Web site: www.Minitab.com. Since Excel® provides $s_{y/x}$ and *b* values automatically in its regression function, calculations of s_0 etc using this spreadsheet are also relatively simple.

Further reading

- N.R. Draper and H. Smith, *Applied Regression Analysis*, 3rd edn., Wiley-Interscience, New York, 1998.
- D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi and J. Smeyers-Verbeke, *Handbook of Chemometrics and Qualimetrics*, Part A, Elsevier, Amsterdam, 1997.

This Technical Brief was prepared for the Analytical Methods Committee by the Statistical Subcommittee and drafted by J N Miller.

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