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Comparison of linear and non-linear equations for univariate calibration

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Abstract

Univariate data accumulated for the purpose of calibration of chromatographic and spectroscopic methods often exhibit slight but definite curvature. In this paper the performance of a non-linear calibration equation with the capacity to account empirically for the curvature, $y = a + bx^m$, ($m \neq 1$) is compared with the commonly used linear equation, $y = a + bx$, as well as the quadratic equation, $y = a + bx + cx^2$. All equations were applied to high quality HPLC calibration data using unweighted least squares. Parameter estimates and their standard errors were calculated for each equation. Standard errors and 95% prediction intervals in analyte concentrations were estimated with the aid of the fitted equations and their respective covariance matrices. Results indicate that the non-linear and quadratic equations each provide a better fit than the linear equation to the data considered here, as judged by the Akaikes information criterion (AIC), the adjusted coefficient of multiple determination, the magnitude and scatter of residuals, standard errors in estimated analyte concentrations and lack of fit analysis of variance (ANOVA). While the difference between the equations $y = a + bx + cx^2$ and $y = a + bx^m$ as judged by the same criteria is more marginal, this work suggests that the non-linear calibration equation should be considered when a curve is required to be fitted to low noise calibration data which exhibit slight curvature.
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1. Introduction

Calibration is vital to the valid application of all chromatographic methods. The most usual method applied by chromatographers is the least squares method of linear regression. In many situations, the relationship between instrument response (y) and of analyte concentration (x) is close to linear [1], such that the relationship between x and y can adequately be described by the equation,

$$y = a + bx. \quad (1)$$

a and b are 'best estimates' of the true intercept and slope respectively of a straight line through the data.

Often, a and b are determined using unweighted least squares in which the sum of squares of residuals (SSR) is minimised [2]. In many circumstances in which a best straight line is required to be drawn through calibration data, the coefficient of determination, R^2, is in excess of 0.999, even when the data show signs of curvature [3]. Other equations may be fitted to the data, but it is important to be able to establish whether the fit is indeed better, as a reduction in SSR is to be expected if an equation to be fitted has more adjustable parameters than Eq. (1).

Many quantitative applications of analytical chemistry currently have very stringent requirements regarding the accuracy and precision of results. Reliable estimates of analyte concentrations and their uncertainties are needed. This is particularly true for applications that assay potentially toxic compounds or those coming under regulatory controls. Regulatory agencies such as the United States Food and Drug Administration (FDA) and the United States Environmental Protection Agency (EPA) require methods of chemical analysis to be fully validated, providing comprehensive method descriptions together with reliable uncertainty estimates. However, achieving good quality measurements is often difficult and this is demonstrated when inconsistent results are achieved by different laboratories [4]. Quality in analytical measurements can only be defined in terms of the relative performance of a method. The result of a measurement needs to be able to stand the test of comparison with the

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results of other measurements. This can only be achieved if the analytical result is independent of the method and the analyst [5]. A vital component of comparability is the ability to calibrate instrumentation using traceable reference materials. Much attention has been given to the production of primary reference materials to enable traceability. Yet the least squares technique for calibration of instruments to these reference materials to allow the prediction of sample concentration has previously been shown to have limitations [3]. Many analysts depend entirely on the use of a correlation coefficient with a value between 0.999 and 1.000 as an acceptability criterion when evaluating the least squares method. This is well known to be inadequate and many experts have expressed concern that publications are still accepted with this minimum statistic [6].

An attraction of fitting Eq. (1) to data is that much standard software is available to carry out this task. Nevertheless, there are situations in which close inspection reveals that slight, but definite, curvature exists in the calibration data suggesting that the fitting of Eq. (1) to the data is inappropriate. It is possible to reduce the concentration range in order to obtain an acceptable linear fit. However, in many situations it is not possible to anticipate analyte concentrations that may occur. In such situations there is merit in assuring that the calibration range is liberally chosen.

In this paper the fitting of Eq. (1) to 'real' calibration data is compared with that of a quadratic equation as well as a less commonly used non-linear equation. The data were drawn from HPLC studies on ibuprofen, genisten, pseudoephedrine, biochanin and sodium nitrate. In particular, a focus is brought to the issue of whether the quadratic and non-linear calibration equations offer a worthwhile improvement over the linear equation.

2. Theory

The instrument response is assumed affected by errors that are homoscedastic and normally distributed. The assumption is also made that the errors in analyte concentration used for calibration can be neglected. The approach adopted here can be extended to situations in which heteroscedasticity in data is prevalent.

2.1. Fit of linear equation to data

For values of analyte concentration, $x_1, x_2, x_i, \ldots, x_n$, the corresponding instrument response is given by, $y_1, y_2, y_i, \ldots, y_n$, where n is the number of data pairs. It is assumed that the relationship between x and y is given by Eq. (1). The a and b, respectively are established by minimising the sum of squares of residuals, SSR, given by,

$$\mathrm{SSR} = \sum (y_i - \hat{y}_i)^2 \tag{2}$$

where

$$\hat{y}_i = a + bx_i \tag{3}$$

In matrix form, a and b may be expressed as [7].

$$\begin{pmatrix} a \\ b \end{pmatrix} = \boldsymbol{p} = (\boldsymbol{X}^{\mathrm{T}}\boldsymbol{X})^{-1}\boldsymbol{X}^{\mathrm{T}}\boldsymbol{Y} \tag{4}$$

where

$$\boldsymbol{X} = \begin{pmatrix} 1 & x_1 \\ 1 & x_2 \\ 1 & x_3 \\ 1 & x_i \\ \vdots & \vdots \\ 1 & x_n \end{pmatrix}, \quad \boldsymbol{Y} = \begin{pmatrix} y_1 \\ y_2 \\ y_3 \\ y_i \\ \vdots \\ y_n \end{pmatrix} \quad \text{and } \boldsymbol{X}^{\mathrm{T}} \text{ is the transpose of } \boldsymbol{X}.$$

Standard errors in a and b, written as σ_a and σ_b, respectively, may be found from the diagonal of the covariance matrix, $\boldsymbol{V}$, given by [8],

$$\boldsymbol{V} = \sigma^2(\boldsymbol{X}^{\mathrm{T}}\boldsymbol{X})^{-1} \tag{5}$$

where

$$\sigma^2 = \frac{\sum (y_i - \hat{y}_i)^2}{n-2} \tag{6}$$

Once fitting has been accomplished, an estimate of analyte concentration, $\hat{x}_0$, may be determined for a mean instrument response, $\bar{y}_0$, using:

$$\hat{x}_0 = \frac{\bar{y}_0 - a}{b} \tag{7}$$

It is assumed that errors in $\bar{y}_0$ are not correlated with errors in the parameter estimates. The variance in $\hat{x}_0$, written as $\sigma^2_{\hat{x}_0}$, is therefore given by [9]:

$$\sigma^2_{\hat{x}_0} = \left(\frac{\partial \hat{x}_0}{\partial \bar{y}_0}\sigma_{\bar{y}_0}\right)^2 + \boldsymbol{d}^{\mathrm{T}}_{\hat{x}_0}\boldsymbol{V}\boldsymbol{d}_{\hat{x}_0} \tag{8}$$

where

$$\boldsymbol{d}_{\hat{x}_0} = \begin{pmatrix} \dfrac{\partial \hat{x}_0}{\partial a} \\ \dfrac{\partial \hat{x}_0}{\partial b} \end{pmatrix} \tag{9}$$

Also,

$$\frac{\partial \hat{x}_0}{\partial \bar{y}_0} = \frac{1}{b}, \tag{10}$$

$$\frac{\partial \hat{x}_0}{\partial a} = -\frac{1}{b}, \tag{11}$$

$$\frac{\partial \hat{x}_0}{\partial b} = -\left(\frac{\bar{y}_0 - a}{b^2}\right) \tag{12}$$

2.2. Fit of quadratic calibration equation to data

In order to account for curvature in calibration data, it is possible to fit a quadratic equation to data. In this case the relationship between x and y is assumed to be:

$$y = a + bx + cx^2 \tag{13}$$

Eq. (13) can be fitted to data using linear least squares. The sum of squares of residuals SSR is given by Eq. (2), where

$$\hat{y}_i = a + bx_i + cx_i^2 \tag{14}$$

In matrix form, a, b and c may be expressed as,

$$\begin{pmatrix} a \\ b \\ c \end{pmatrix} = \boldsymbol{p} = (\boldsymbol{X}^{\mathrm{T}}\boldsymbol{X})^{-1}\boldsymbol{X}^{\mathrm{T}}\boldsymbol{X} \tag{15}$$

where

$$\boldsymbol{X} = \begin{pmatrix} 1 & x_1 & x_1^2 \\ 1 & x_2 & x_2^2 \\ 1 & x_3 & x_3^2 \\ 1 & x_i & x_i^2 \\ \vdots & \vdots & \vdots \\ 1 & x_n & x_n^2 \end{pmatrix}, \quad \boldsymbol{Y} = \begin{pmatrix} y_1 \\ y_2 \\ y_3 \\ y_i \\ \vdots \\ y_n \end{pmatrix}.$$

Standard errors in a, b and c, written as σ_a, σ_b and σ_c, respectively, may be found from the diagonal of the covariance matrix, $\boldsymbol{V}$, given by Eq. (5) where:

$$\sigma^2 = \frac{\sum \left(y_i - \hat{y}_i\right)^2}{n-3} \tag{16}$$

Once fitting has been accomplished, an estimate of analyte concentration, $\hat{x}_0$, may be determined for a mean instrument response, $\bar{y}_0$, using:

$$\hat{x}_0 = \frac{-b + \sqrt{b^2 - 4c(a - \bar{y}_0)}}{2c} \tag{17}$$

The variance in $\hat{x}_0$, written as $\sigma^2_{\hat{x}_0}$, is given by Eq. (8), where:

$$\boldsymbol{d}_{\hat{x}_0} = \begin{pmatrix} \dfrac{\partial \hat{x}_0}{\partial a} \\ \dfrac{\partial \hat{x}_0}{\partial b} \\ \dfrac{\partial \hat{x}_0}{\partial c} \end{pmatrix} \tag{18}$$

Also,

$$\frac{\partial \hat{x}_0}{\partial \bar{y}_0} = \frac{1}{\sqrt{b^2 - 4c(a - \bar{y}_0)}}, \tag{19}$$

$$\frac{\partial \hat{x}_0}{\partial a} = \frac{-1}{\sqrt{b^2 - 4c(a - \bar{y}_0)}} \tag{20}$$

$$\frac{\partial \hat{x}_0}{\partial b} = \frac{-1 + \left(b/\sqrt{b^2 - 4c(a - \bar{y}_0)}\right)}{2c} \tag{21}$$

$$\frac{\partial \hat{x}_0}{\partial c} = \frac{-(-b + \sqrt{b^2 - 4c(a - \bar{y}_0)})}{2c^2} - \frac{(a - \bar{y}_0)}{c\sqrt{b^2 - 4c(a - \bar{y}_0)}} \tag{22}$$

2.3. Fit of a non-linear equation to data

The relationship between x and y is assumed to be of the form,

$$y = a + bx^m \tag{23}$$

As Eq. (23) is non-linear in the parameters to be estimated, a, b and m cannot be found by the method of linear least squares. Instead, non-linear least squares is used to minimise SSR given by Eq. (2) [10], where

$$\hat{y}_i = a + bx_i^m \tag{24}$$

In this situation, the covariance matrix, $\boldsymbol{V}$, is given by,

$$\boldsymbol{V} = \sigma^2(\boldsymbol{D}^{\mathrm{T}}\boldsymbol{D})^{-1} \tag{25}$$

where σ^2 is given by Eq. (16), and

$$\boldsymbol{D} = \begin{pmatrix} \dfrac{\partial y_1}{\partial a} & \dfrac{\partial y_1}{\partial b} & \dfrac{\partial y_1}{\partial m} \\ \dfrac{\partial y_2}{\partial a} & \dfrac{\partial y_2}{\partial b} & \dfrac{\partial y_2}{\partial m} \\ \dfrac{\partial y_i}{\partial a} & \dfrac{\partial y_i}{\partial b} & \dfrac{\partial y_i}{\partial m} \\ \dfrac{\partial y_n}{\partial a} & \dfrac{\partial y_n}{\partial b} & \dfrac{\partial y_n}{\partial m} \end{pmatrix} \tag{26}$$

The partial derivatives in Eq. (26) are evaluated on completion of fitting by non-linear least squares.

An estimate of the analyte concentration, $\hat{x}_0$, may be established for a mean instrument response, $\bar{y}_0$, using,

$$\hat{x}_0 = \left(\frac{\bar{y}_0 - a}{b}\right)^{1/m} \tag{27}$$

The variance in $\hat{x}_0$, $\sigma^2_{\hat{x}_0}$, is given by Eq. (8), where

$$\boldsymbol{d}_{\hat{x}_0} = \begin{pmatrix} \dfrac{\partial \hat{x}_0}{\partial a} \\ \dfrac{\partial \hat{x}_0}{\partial b} \\ \dfrac{\partial \hat{x}_0}{\partial m} \end{pmatrix} \tag{28}$$

Partially differentiating Eq. (27) with respect to $\bar{y}_0$, a, b and m in turn gives,

$$\frac{\partial \hat{x}_0}{\partial \bar{y}_0} = \frac{1}{bm}\left(\frac{\bar{y}_0 - a}{b}\right)^{(1-m)/m} \tag{29}$$

$$\frac{\partial \hat{x}_0}{\partial a} = -\frac{1}{bm}\left(\frac{\bar{y}_0 - a}{b}\right)^{(1-m)/m} \tag{30}$$

$$\frac{\partial \hat{x}_0}{\partial b} = -\frac{1}{bm}\left(\frac{\bar{y}_0 - a}{b}\right)^{(1/m)} \tag{31}$$

$$\frac{\partial \hat{x}_0}{\partial m} = -\frac{1}{m^2}\left(\frac{\bar{y}_0 - a}{b}\right)^{(1/m)} \ln\left(\frac{\bar{y}_0 - a}{b}\right) \tag{32}$$

2.4. Goodness of fit statistics

As both Eqs. (13) and (23) contain three adjustable parameters and Eq. (1) only two, the SSR obtained when optimum values are found for *a*, *b* and *c* in Eq. (13) and for *a*, *b* and *m* in Eq. (23), are less than the SSR when Eq. (1) is fitted to the same data.

There are several statistical methods that can be used to compare the fit of Eqs. (1), (13) and (23) to data [11]. In this work we report statistics which are able to compensate for the number of adjustable parameters in an equation fitted to data.

2.4.1. Akaikes information criterion (AIC) and adjusted coefficient of multiple determination

An effective way of comparing two (or more) equations fitted to data where the equations have different numbers of parameters is to use the Akaikes information criterion [12]. This criterion takes into account the SSR, but also includes a term proportional to the number of parameters used. AIC may be written

$$\mathrm{AIC} = n \ln \mathrm{SSR} + 2M \tag{33}$$

where n is the number of data and M is the number of parameters in the equation.

If the addition of another parameter in an equation reduces SSR, then the first term on the right hand side of Eq. (33) is reduced. However, the second term on the right hand side of the equation increases by two for every additional parameter used. A modest decrease in SSR which occurs when an extra term is introduced into an equation may be more than offset by the increase in AIC by using another parameter. If two or more equations are fitted to data, then the equation producing the *smallest* value for AIC is preferred.

Another useful measure of goodness of fit when each equation has a different number of adjustable parameters is the adjusted coefficient of multiple determination, R^2_{ADJ}, given by [13],

$$R^2_{\mathrm{ADJ}} = \frac{(n-1)R^2 - (M-1)}{n-M} \tag{34}$$

where R^2 is the coefficient of multiple determination given by:

$$R^2 = 1 - \frac{\sum(y_i - \hat{y}_i)^2}{\sum(\bar{y} - \hat{y}_i)^2} \tag{35}$$

and

$$\bar{y} = \sum \frac{y_i}{n} \tag{36}$$

Once R^2_{ADJ} is calculated for each equation fitted to data, the equation is preferred that has the larger value of R^2_{ADJ}.

2.4.2. Comparing equations fitted to data using analysis of variance (ANOVA)

A routine statistical test which offers evidence regarding the quality of the fit of an equation to experimental data is a 'goodness of fit' ANOVA [14]. As an example, in the case of Eq. (1), the ANOVA is able to establish whether the slope, *b*, is important to the fit. This ANOVA may be adapted to allow for the comparison of two equations fitted to data, for example Eqs. (1) and (13). The mean values of SSR for each equation fitted to data are used as a basis for an *F*-test which indicates whether the equation with more parameters provides a significantly improved fit.

2.4.3. Lack of fit ANOVA

When repeat measurements are made at a particular value of *x*, scatter will be observed in the *y* values. The scatter over all the measured *x* values can be expressed as the sum of squares of the pure error (SSPE)[15]. An equivalent measure of the scatter based on an equation fitted to data is obtained from SSR, as given by Eq. (2). A lack of fit sum of squares (SSLOF) can be formed which is given by:

$$\mathrm{SSLOF} = \mathrm{SSR} - \mathrm{SSPE} \tag{37}$$

If an equation is a good fit to data, it is expected that SSLOF will be small. Whether SSR and SSPE differ significantly (suggesting an inappropriate equation has been fitted to data) is tested formally through the test statistic F_{LOF}, where:

$$F_{\mathrm{LOF}} = \frac{\mathrm{SSLOF}/\mathrm{d.f.}_{\mathrm{LOF}}}{\mathrm{SSPE}/\mathrm{d.f.}_{\mathrm{PE}}} \tag{38}$$

$\mathrm{d.f.}_{\mathrm{LOF}}$ and $\mathrm{d.f.}_{\mathrm{PE}}$ are the degrees of freedom associated with the SSLOF and SSPE, respectively.

3. Experimental

3.1. Chemicals and reagents

All reagents used were of analytical grade; mobile phase solvents were of HPLC grade. All standard solutions were filtered through 0.45 μm Millex-HV filter discs (Millipore) prior to injection on the HPLC system.

3.1.1. Pseudoephedrine and ibuprofen

Pseudoephedrine and ibuprofen were obtained from Pfizers, Caringbah, Sydney, Australia. The reference standard solutions were prepared with an extraction solvent containing solvent A (see below)—acetonitrile (50:50) (Rhone Poulenc). Three combined reference standard solutions were accurately prepared to contain the following approximate concentrations: 0.036–0.044 mg/ml of ibuprofen, 0.054–0.066 mg/ml of pseudoephedrine· HCl.

3.1.2. Genisten and biochanin

Genisten and biochanin A, were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) and acetic acid were purchased from Rhone Poulenc. Millipore water was used for all mobile phases.

3.1.3. Sodium nitrate

Calibration solutions for sodium nitrate from Sigma were prepared from 1 to 25 mg/l using Milli-Q water (Millipore).

3.2. Instrumentation

3.2.1. Pseudoephedrine and ibuprofen

The aqueous mobile phase was 8.25 mM octanesulphonic acid, 0.1% glacial acetic acid at pH 3.70 (solvent A). The mobile phase was prepared by weighing 1.8 g of octane sulphonic acid (Biolab Scientific, Clayton, Australia), into a 1 l volumetric flask and dissolve in approximately 500 ml of Milli-Q water. 1 ml of glacial acetic acid was then added, and diluted to volume with Milli-Q water. The pH was adjusted to pH 3.70 ± 0.05 with 2 M sodium hydroxide solution (AnalaR, grade, BDH, Merck, Australia) and filtered through a 0.45 μm nylon membrane filter (47 mm, Activon).

Acetonitrile (Mallinckrodt, ChromAR HPLC grade) was used as the organic phase. The HPLC used was the Waters Alliance 2690 Separations Module with 996 photo diode array detection, controlled via Millenium 3.2 software (Waters Australia, Rydalmere, Australia). Twenty microlitre of sample and standard solutions were, respectively injected onto a C_8 bonded reversed-phase HPLC column (Waters Symmetry C_8, 5 μm, 150 mm × 3.9 mm with guard column). The column temperature was held at 40 ± 2 °C. The actives were separated using ion-pair chromatography and gradient elution of the aqueous-acetonitrile mobile phase. The gradient used was linear in several steps (see Table 1 for gradient mobile phase composition). The optimum flow rate was 1.0 ml/min.

3.2.2. Genisten and biochanin

The HPLC analysis was carried out on a Waters liquid chromatograph with an auto sampler model 717 plus and a model 600 controller pump connected to a photo diode array detector model 996 (Waters Australia). The column used was a Waters Nova-Pak C_{18} (150 mm × 3.9 mm i.d.; 4 μm) reversed-phase (Waters, Milford, MA, USA). The optimised mobile phase was 33% acetonitrile (A) and 67% water–acetic acid (99:1, v/v) (B) at a flow rate of 0.80 ml/min. The injection volume was 5 μl. The wavelength ranged from 200 to 400 nm throughout the chromatogram and each peak was plotted using the wavelength that provided a maximum response. The maximum absorption for genisten and biochanin A was 259.2 nm.

Table 1
The gradient conditions for HPLC assay of ibuprofen and pseudoephedrine

Time (min)	% Mobile phase A (aqueous)	% Mobile Phase B (organic)
0.00	75	25
7.00	30	70
10.00	30	70
12.00	90	10
15.00	90	10
17.00	75	25

3.2.3. Sodium nitrate

The HPLC analysis was carried out on a Waters liquid chromatograph with an auto sampler model 717 plus and a model 600 controller pump connected to a Waters 430 conductivity detector with thermostated five-electrode flow cell (Waters Australia). The column used was a 50 mm × 5.6 mm Waters Anion 10 μm diameter porous polymethylmethacrylate polymer with quaternary ammonium functional groups, exchange capacity 32 μeq./g (Waters). The column temperature was 40 °C, 20 μl of each solution was eluted at a flow rate of 0.8 ml/min using a mobile phase of 1.3 mM borate–1.3 mM gluconate aqueous buffer, 0.5% glycerin, 2% *n*-butanol and 12.5% acetonitrile.

4. Results and discussion

Ibuprofen, genisten, biochanin, pseudoephedrine and sodium nitrate calibration data shown in Table 2 were gathered in studies of linearity in HPLC. For illustrative purposes, the ibuprofen data are analysed in detail. Summary information concerning the other analytes is provided in Section 4.3.

Eqs. (1), (13) and (23) were fitted to data in Table 2 using the Excel spreadsheet package by Microsoft. Excel's Regression tool [16] was used to fit Eqs. (1) and (13) to the data in Table 2. This tool returns useful information including parameters estimates, their standard errors, SSR, adjusted coefficient of multiple determination and ANOVA. The determination of the covariance matrix, $\boldsymbol{V}$, given by Eq. (5) was realised using Excel's built in matrix functions. Fitting by non-linear least squares (which is an iterative process and cannot be done using the Regression tool) was accomplished with the assistance of the Solver utility in Excel [17]. Solver is an optimisation tool which may be applied to least squares problems [18]. An Excel worksheet may be constructed which calculates SSR as given by Eq. (2). Solver is able to iteratively alter values of the parameters appearing in the equation to be fitted to data until SSR is minimised [19]. The standard errors in parameter estimates may be obtained with the assistance of the matrix functions in Excel [20].

Successful non-linear least squares fitting requires starting values be chosen for best estimates of parameters. Fitting equations to data by non-linear least squares can be problematical, as poor starting values or noisy data can cause the optimisation algorithm to become trapped in a local minimum in SSR. Parameter estimates obtained when a local minimum is located are not optimum and should be discarded. The identification of the global, rather than a local, minimum is facilitated by the use of good starting values for parameters estimates in Eq. (23). The similarity between Eqs. (1) and (23) is such that a and b as determined by linear least squares when Eq. (1) is fitted are conveniently adopted

Table 2
Calibration data for ibuprofen, genisten, biochanin, pseudoephedrine and sodium nitrate

Ibuprofen		Genisten		Biochanin		Pseudoephedrine		Sodium nitrate	
Concentration, (x) (mg per tablet)	Area, (y) (arbitrary units)	Concentration, (x) (mg/100 ml)	Area, (y) (arbitrary units)	Concentration, (x) (mg/100 ml)	Area, (y) (arbitrary units)	Concentration, (x) (mg per tablet)	Area, (y) (arbitrary units)	Concentration, (x) (mg/l)	Area, (y) (arbitrary units)
103.9	265 053	0.159	0.155598	0.158	0.121342	61.4	28 653	1.006	8 293
103.9	261 357	0.159	0.15508	0.158	0.121109	61.4	29 061	1.006	8 103
139.3	345 915	0.318	0.464125	0.315	0.40355	85.3	39 904	2.013	17 864
139.3	345 669	0.318	0.471655	0.315	0.415226	85.3	39 614	2.013	17 424
180.1	445 684	0.635	2.02122	0.631	1.839583	107.7	50 418	5.032	42 300
180.1	445 753	0.635	2.028043	0.631	1.835114	107.7	50 052	5.032	43 783
200.3	494 700	1.27	4.17856	1.261	3.840554	120.3	56 255	7.548	65 198
200.3	493 846	1.27	4.204061	1.261	3.846146	120.3	56 098	7.548	66 525
219.9	540 221	2.54	9.132732	2.522	8.523561	132.4	61 233	10.06	90 158
219.9	539 610	2.54	9.133685	2.522	8.539992	132.4	61 462	10.06	90 976
278.1	683 881	5.08	17.748132	5.045	16.80701	150.0	69 656	15.10	135 869
278.1	683 991	5.08	17.701031	5.045	16.6986	150.0	69 744	15.10	137 498
305.7	755 890	10.16	35.217533	10.09	34.06871	181.5	85 194	25.16	228 223
305.7	754 901	10.16	35.183699	10.09	33.91678	181.5	85 241	25.16	233 405

Table 3
Parameter estimates and statistics for ibuprofen data in Table 2

	Equation fitted to data		
	$y = a + bx$	$y = a + bx + cx^2$	$y = a + bx^m$
Parameter estimates	$a = 7186$, $b = 2437$	$a = 24477$, $b = 2250$, $c = 0.4505$	$a = 37523$, $b = 1546$, $m = 1.071$
Standard errors in estimates	$\sigma_a = 2090$, $\sigma_b = 9.744$	$\sigma_a = 3768$, $\sigma_b = 38.82$, $\sigma_c = 0.09278$	$\sigma_a = 6289$, $\sigma_b = 150.3$, $\sigma_m = 0.01540$
σ	2421	1427	1478
R^2	0.999808	0.999939	0.999934
R^2_{ADJ}	0.999792	0.999928	0.999923
SSR	7.034×10^7	2.238×10^7	2.402×10^7
AIC	257.0	242.9	243.9

as starting values for a and b when Eq. (23) is fitted to the same data. As the calibration data considered here has slight curvature, a starting value of $m = 1$ is adequate.

4.1. Goodness of fit statistics

Table 3 contains parameter estimates, standard errors and other statistics that were returned when Eqs. (1), (13) and (23) were fitted to the ibuprofen data in Table 2. Statistical t-tests carried out on the parameter estimates in Table 3 indicate that all estimates are significant at the $\alpha = 0.05$ level of significance.

Two measures of goodness of fit favoured in this paper, namely AIC and R^2_{ADJ} confirm that there is an improvement in the fit by using Eqs. (13) and (23) rather than Eq. (1). This finding is supported by other statistical measures of goodness of fit.

Table 4 shows the output of an ANOVA used to compare of the fit of Eqs. (13) and (23) to that provided by Eq. (1). As judged by these, both Eqs. (13) and (23) provided a significantly better fit ($P < 0.001$) to the ibuprofen data than Eq. (1).

Table 5 shows the lack of fit ANOVA for Eqs. (1), (13) and (23) fitted to the ibuprofen data in Table 2. The p value for the lack of fit ANOVA indicates that the SSR and SSPE differ significantly for Eq. (1) fitted to data ($P < 0.01$), suggesting that the fit is not good. By contrast, the F-test for both Eqs. (13) and (23) fitted to data indicate no strong statistical evidence for concluding that either fit is unsatisfactory ($P > 0.05$).

Table 4
Comparison of fitting equations to ibuprofen data in Table 2 using ANOVA

	d.f.	SS	MS	F	P value
Residual for Eq. (1)	12	70 363 425			
Gain from Eq. (13)	1	47 978 882	47 978 882	23.58	0.00051
Residual for Eq. (13)	11	22 384 542	2 034 958		
Gain from Eq. (23)	1	46 341 252	46 341 252	21.22	0.00076
Residual for Eq. (23)	11	24 022 172	2 183 834		

Table 5
Lack of fit (LOF) ANOVA for equations fitted to ibuprofen data in Table 2

	d.f.	SS	MS	F	P value
LOF for Eq. (1)	5	62 454 149	12 490 830	11.05	0.00322
LOF for Eq. (13)	4	14 475 267	3 618 817	3.203	0.08557
LOF for Eq. (23)	4	16 112 897	4 028 224	3.565	0.06856
Pure error	7	7 909 276	1 129 897		

4.2. Estimated analyte concentrations

The purpose of a calibration equation is to estimate analyte concentration, given instrument response. Table 6 shows the estimated concentrations, $\hat{x}_0$, of ibuprofen for the measured responses, $\bar{y}_0$, determined using Eqs. (7), (17) and (27). As the analyte concentrations, x_0, used in the calibration are assumed to have negligible error, the absolute error, $(x_0 - \hat{x}_0)$ and percentage error, given by $((x_0 - \hat{x}_0)/x_0)100\%$, may be determined and are included in Table 6. The standard errors in the estimates of the concentrations, $\sigma_{\hat{x}_0}$, were calculated using Eq. (8) for each equation fitted to data. Inspection of Table 6 indicates that the percentage errors are quite small for Eq. (1) fitted to data, with only one value exceeding 1%. On average, the percentage errors in estimates of analyte concentration are smaller for Eqs. (13) and (23) compared to Eq. (1). Specifically, when Eqs. (13) and (23) are fitted to the data in Table 2, the fitting yields absolute percentage errors which are less on average by 27 and 26%, respectively than the absolute percentage errors obtained upon fitting Eq. (1) to the same data.

4.2.1. 95% prediction intervals

For fitting using Eq. (1) to be abandoned in favour of Eqs. (13) or (23) there needs to be a reduction in the prediction interval that is of practical significance, and not just statistical significance.

Fig. 1 shows the 95% prediction intervals for Eqs. (1) and (23) fitted to the ibuprofen data. These are calculated using the standard errors in Table 6 and the critical values of the t-distribution at the 95% level of confidence. The prediction interval for Eq. (13) has been omitted from Fig. 1 due the fact that it is visually indistinguishable from that obtained when Eq. (23) is fitted to the data. To emphasise the difference between the prediction intervals when Eqs. (1) and (23) are fitted to data, a narrow range of $x = 190$–208 mg per tablet is shown in Fig. 1. Fig. 1 indicates that fitting Eq. (23) to

Table 6
Estimated concentrations, errors and standard errors for ibuprofen for calibration equations, $y = a + bx$, $y = a + bx + cx^2$ and $y = a + bx^m$ fitted to ibuprofen data in Table 2

x_0	Equation fitted to data											
	$y = a + bx$				$y = a + bx + cx^2$				$y = a + bx^m$			
	$\hat{x}_0$	$(x_0 - \hat{x}_0)$	% error	$\sigma_{\hat{x}_0}$	$\hat{x}_0$	$(x_0 - \hat{x}_0)$	% error	$\sigma_{\hat{x}_0}$	$\hat{x}_0$	$(x_0 - \hat{x}_0)$	% error	$\sigma_{\hat{x}_0}$
103.9	105.83	−1.93	−1.85	1.10	104.71	−0.81	−0.78	0.72	104.68	−0.78	−0.75	0.75
103.9	104.31	−0.41	−0.39	1.10	103.14	0.76	0.74	0.72	103.09	0.81	0.78	0.76
139.3	139.01	0.29	0.21	1.06	138.97	0.33	0.23	0.64	139.06	0.24	0.17	0.66
139.3	138.91	0.39	0.28	1.06	138.87	0.43	0.31	0.64	138.96	0.34	0.25	0.66
180.1	179.95	0.15	0.08	1.03	180.64	−0.54	−0.30	0.63	180.67	−0.57	−0.32	0.65
180.1	179.98	0.12	0.07	1.03	180.67	−0.57	−0.32	0.63	180.70	−0.60	−0.33	0.65
200.3	200.07	0.23	0.12	1.03	200.88	−0.58	−0.29	0.63	200.86	−0.56	−0.28	0.65
200.3	199.72	0.58	0.29	1.03	200.53	−0.23	−0.11	0.63	200.51	−0.21	−0.10	0.65
219.9	218.75	1.15	0.52	1.03	219.54	0.36	0.16	0.63	219.47	0.43	0.19	0.64
219.9	218.50	1.40	0.64	1.03	219.29	0.61	0.28	0.63	219.22	0.68	0.31	0.64
278.1	277.71	0.39	0.14	1.07	277.60	0.50	0.18	0.61	277.55	0.55	0.20	0.64
278.1	277.75	0.35	0.13	1.07	277.64	0.46	0.16	0.61	277.59	0.51	0.18	0.64
305.7	307.26	−1.56	−0.51	1.11	306.25	−0.55	−0.18	0.66	306.32	−0.62	−0.20	0.68
305.7	306.85	−1.15	−0.38	1.11	305.86	−0.16	−0.05	0.66	305.93	−0.23	−0.07	0.68

the ibuprofen data in Table 2 affords a reduction in the 95% prediction interval of approximately 35% compared to the interval determined when Eq. (1) is fitted to the same data.

4.2.2. Plot of residuals

Fig. 2 shows a plot of the difference between the estimated analyte concentration and the true analyte concentration, expressed as a percentage where estimates were determined following the fitting Eqs. (1), (13) and (23) fitted to data in Table 2.

Examination of Fig. 2 indicates that the percentage errors in the estimate of the analyte concentration obtained after fitting Eq. (1) show a trend of positive values to negative values back to positive values for ibuprofen concentrations between 103.9 and 305.7 mg per tablet. Such a characteristic trend is indicative of a model violation [20]. That is, the equation fitted is inadequate to properly describe the relationship between the estimated concentration of ibuprofen and the true concentration. A trend in the percentage residuals in Fig. 2 obtained after fitting Eqs. (13) and (23) to data is less obvious than when Eq. (1) is fitted to the same data, though there are too few calibration data to be able to draw the conclusion that there is no model violation.

4.2.3. Predicted concentrations using replicate measurements

As a means of assessing the predictive capabilities Eqs. (1), (13) and (23), six replicate measurements of response were made at a known concentration of ibuprofen of 200.3 mg per tablet. The replicate values were not used in the least squares fitting of Eqs. (1), (13) and (23) to data. Table 7 shows the predicted values based on each equation fitted to the data.

In order to test formally whether the mean value obtained for $\hat{x}_0$ from the replicate measurements is significantly different from the known value of 200.3 mg per tablet, the *t*-test statistic is calculated using:

$$t = \left(\frac{\bar{\hat{x}}_0 - x_0}{\sigma_{\bar{\hat{x}}_0}} \right) \tag{39}$$

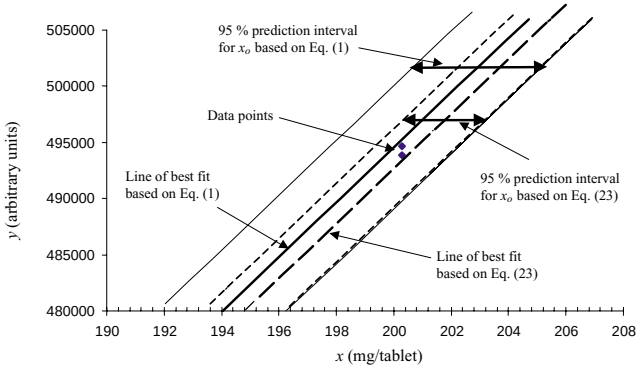

Fig. 1. Ninety-five percent prediction intervals for Eqs. (1) and (23) fitted to ibuprofen data in Table 2.

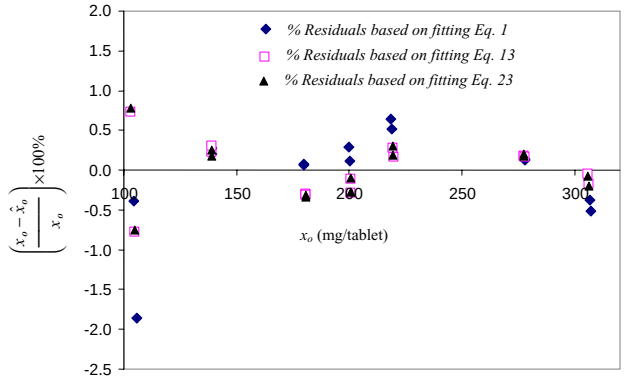

Fig. 2. Plot of residuals in the concentration values for the assay of ibuprofen.

Table 7
Estimated concentrations from six replicate measurements based on Eqs. (1), (13) and (23) fitted to ibuprofen data in Table 2

	Equation fitted to data			Area, y
	$y = a + bx$, $\hat{x}_0$	$y = a + bx + cx^2$, $\hat{x}_0$	$y = a + bx^m$, $\hat{x}_0$	
	198.926	199.734	199.712	491 914
	198.469	199.276	199.255	490 800
	199.495	200.304	200.281	493 299
	199.797	200.607	200.583	494 036
	198.699	199.506	199.485	491 360
	199.048	199.856	199.834	492 210
Mean, $\bar{\hat{x}}_0$	199.072	199.880	199.858	
$\sigma_{\bar{\hat{x}}_0}$	0.20240	0.20292	0.20242	
t	−6.066	−2.068	−2.182	
P	0.0018	0.0935	0.0811	

The known ibuprofen concentration is 200.3 mg per tablet.

where $\bar{\hat{x}}_0$ is the mean of estimated concentrations, $\sigma_{\bar{\hat{x}}_0}$ is the standard error in the mean and $x_0 = 200.3$ mg per tablet. Table 7 shows the P value for each equation fitted to the ibuprofen data. Examination of Table 7 indicates that the mean of estimated concentrations based on Eq. (1) fitted to data is statistically different from the nominal concentration of 200.3 mg per tablet ($P < 0.01$) indicating a bias in predicted concentrations. By contrast, the means of the estimated concentrations based on fitting Eqs. (13) and (23) to the same data reveals no significant difference between the mean of the estimated concentration and the nominal concentration ($P > 0.05$).

4.3. Fitting equations to other calibration data

In order to indicate which of the equations considered here provided the best fit to data as judged by the goodness of fit criteria adopted in this work, the analysis was extended to HPLC calibration data gathered from pharmaceutical, health and environmental research. More specifically, the analytes considered were genisten, pseudoephedrine, biochanin and sodium nitrate. A visual inspection of residuals indicated no strong evidence of heteroscedascity in the data, therefore Eqs. (1), (13) and (23) were fitted the data in Table 2 using unweighted least squares.

Table 8 shows the R^2, R^2_{ADJ} and AIC for the calibration data. As with the ibuprofen data in Table 2, calibration data for all the analytes shown in Table 8 consisted of two repeat measures of response at each of seven different concentrations. No effort was made to select calibration concentrations that would minimise the standard errors in the fitted parameters, though such choices are important in some circumstances [22]. Table 8 indicates that Eqs. (13) and (23) provide a better fit to data than Eq. (1) as measured by R_{ADJ} and AIC for all calibration data considered in this study. For three out of the five calibration data sets considered (i.e. for genisten, biochanin and sodium nitrate) the R_{ADJ} and AIC indicate that Eq. (23) is a superior fit to Eq. (13). The final

Table 8
Comparison of fit of calibration equations for several analytes

Analyte	Equation	c	σ_c	$\lvert t_c \rvert$	p_c	m	σ_m	$\lvert t_m \rvert$	p_m	R^2	R^2_{ADJ}	AIC	ΔPI (%)
Ibuprofen	$a + bx$	–	–	–	–	–	–	–	–	0.999808	0.999797	257	–
	$a + bx + cx^2$	0.4505	0.0928	4.86	0.0005	–	–	–	–	0.999939	0.999928	243	−39
	$a + bx^m$	–	–	–	–	1.0708	0.0154	4.60	0.0008	0.999934	0.999923	244	−37
Pseudoephedrine	$a + bx$	–	–	–	–	–	–	–	–	0.999663	0.999635	202	–
	$a + bx + cx^2$	0.1532	0.0461	3.33	0.0067	–	–	–	–	0.999832	0.999802	195	−24
	$a + bx^m$	–	–	–	–	1.0693	0.0240	2.89	0.0147	0.999817	0.999784	196	−20
Genisten	$a + bx$	–	–	–	–	–	–	–	–	0.999523	0.999483	3.05	–
	$a + bx + cx^2$	−0.02195	0.00553	3.97	0.0022	–	–	–	–	0.999804	0.999768	−7.38	−32
	$a + bx^m$	–	–	–	–	0.9525	0.0096	4.95	0.0004	0.999848	0.999821	−11.0	−42
Biochanin	$a + bx$	–	–	–	–	–	–	–	–	0.999780	0.999761	−8.73	–
	$a + bx + cx^2$	−0.007074	0.00533	1.32	0.2136	–	–	–	–	0.999810	0.999776	−8.80	−0.8
	$a + bx^m$	–	–	–	–	0.9818	0.0105	1.73	0.1116	0.999826	0.999795	−10.1	−6.2
Sodium nitrate	$a + bx$	–	–	–	–	–	–	–	–	0.999554	0.999517	246	–
	$a + bx + cx^2$	13.42	6.79	1.98	0.0732	–	–	–	–	0.999671	0.999611	244	−6.7
	$a + bx^m$	–	–	–	–	1.032	0.0142	2.25	0.0459	0.999694	0.999638	243	−9.2

column in Table 8 shows the mean percentage change in the prediction interval, ΔPI, when Eqs. (13) and (23) are fitted to data, compared to fitting Eq. (1) to the same data.

The c parameter estimate in Eq. (13) may not be significant for data exhibiting slight curvature. The significance of c can be tested formally by calculating the magnitude of t-test statistic, t_c, given by,

$$|t_c| = \frac{c}{\sigma_c} \tag{40}$$

In order to establish whether the best estimate for the m parameter in Eq. (23) is significantly different from unity (unity corresponds to the linear case given by Eq. (1)), the magnitude of the t-test statistic, $|t_m|$ was determined for each data set where:

$$|t_m| = \left|\frac{m-1}{\sigma_m}\right| \tag{41}$$

Examination of Table 8 reveals that the P value for the t-test statistic, t_m, for the m parameter estimate for biochanin is greater than 0.05. Similarly, the P value for the test statistic, t_c, for the c parameter estimate for biochanin and sodium nitrate is greater than 0.05. In these instances the t test statistic offers no strong evidence for favouring Eqs. (13) and (23) over Eq. (1).

5. Conclusion

The extent to which slight curvature in calibration data should be accounted for depends upon the analyst's requirements and desired constraints over such quantities as the prediction interval for estimated analyte concentrations. These needs are context specific and are not the province of statistical analysis. Nevertheless, the prevalence of slight curvature in calibration data means that there is merit in establishing whether fitting a calibration equation other than the customary linear equation does indeed provide improved statistics.

The equations considered in this study that are able to account for curvature in data (Eqs. (13) and (23)) provided a better fit to data sourced from a diverse range of HPLC calibrations than the conventional linear equation. This conclusion is based on consideration of several statistics including lack of fit ANOVA and the standard errors in the estimated analyte concentrations. Application of the AIC and R^2_{ADJ} permitted a fair comparison of the goodness of fit for all equations. Owing to the ease with which AIC may be calculated and interpreted, it is advised that it be adopted as a statistic to assist in deciding whether Eqs. (13) or (23) (or any other equation, such as $y = bx$ or $y = a+bx+cx^2+dx^3$) offers a worthwhile improvement over Eq. (1).

The marginal nature of the difference between Eqs. (13) and (23) when fitted to data reported here, indicates that it is not possible to anticipate which equation should be favoured. It is recommended that each equation be trialed when an calibration equation is to be fitted to data exhibiting slight curvature.

The expense of moving from calibration using a linear equation to one employing a non-linear equation such as Eq. (23) includes the extra complexity that is inherent in non-linear fitting. In particular, in order to establish standard errors in estimates of analyte concentration, any computer based package must allow access to the covariance matrix as given by Eqs. (5) and (25). Many packages offering fitting by linear and non-linear least squares do not provide this access. The inbuilt facilities of Excel allow for the convenient fitting of equations and the determination of standards errors in estimates of analyte concentrations.

Fitting by non-linear least squares can be challenging, especially when data are noisy or when it is difficult to establish good starting values for parameters. However, the similarity between Eqs. (1) and (23), allied to the inherent low noise of much chromatographic calibration data, reduces the probability of the fitting routine becoming trapped in a local minimum. Nevertheless, once any fitting by least squares has been completed, it is prudent to view response residuals (e.g. $(y_i - \hat{y}_i)$ versus x), as well as the relative size of the standard errors in parameters estimates, as these give valuable clues as to the success, or otherwise, of the fitting procedure [21].

Fitting can be extended to data which exhibit heteroscedasticity through the introduction of a weight matrix, $\boldsymbol{W}$, so that account can be taken of the variance of response at each analyte concentration [23]. Fitting an equation with several adjustable parameters, such as Eqs. (13) and (23), becomes less defensible as the number of calibration data decreases.

6. Nomenclature

a, b, c, m	parameter estimates
AIC	akaikes information criterion
d.f.	degrees of freedom
M	number of parameters in fitted equation
MS	mean of sum of squares
R^2	coefficient of multiple determination
R^2_{ADJ}	adjusted coefficient of multiple determination
SSLOF	lack of fit sum of squares
SSPE	sum of squares pure error
SSR	sum of squares of residuals
$\boldsymbol{V}$	covariance matrix
$\boldsymbol{W}$	weight matrix
$\hat{x}_0$	estimated analyte concentration
$\bar{y}_0$	mean instrument response,
ΔPI	mean percentage change in prediction interval
σ	standard deviation of residuals of the response variable about the fitted line
σ_a, σ_b, σ_c, σ_m	standard errors in parameter estimates
$\sigma_{\hat{x}_o}$	standard error in estimate of analyte concentration

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