Linearity and the limitations of least squares calibration

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Abstract

The magnitude of errors that can arise in practice from the limitations of the least squares method of calibration is estimated. Data generated from $y = x^n$ ($0.7 \leq n \leq 1.3$ and $1 \leq x \leq 30$, or ≤ 60) was analysed by least squares regression. Each y-value was then presented to the linear model and an x-value predicted. The relative errors on small x-values reached 70% of the concentration value even when r^2 exceeded 0.999. Estimates of the error on each predicted x-value, determined from the standard errors of the slope and intercept failed to reveal large errors at small x-values. Reducing the range over which linear regression is performed improved the errors. Other data sets with a heteroscedastic error distribution show that linear regression by least squares can also lead to the rejection of methods that performed sufficiently well for their application. Heteroscedastic data may be treated by repeated measurements at the lower end of the range. Data from a validation of an HPLC method for isoflavones in legumes is used to show the errors in recovery when a check sample is presented to the instrument using a calibration which satisfies the linearity tests. It is recommended that both y- and relative x-residuals are inspected. It is proposed that over-reliance on linear calibration supported by r^2 may make a major contribution to large, hitherto unexplained, inter-laboratory errors.

Keywords: Least squares calibration; Linearity; Regression analysis; Chemometrics; Diadzen; Biochenin; Genistin

1. Introduction

The introduction of regulations to control the production of foods and pharmaceutical products and for environmental monitoring has led to the great interest in method validation and error estimation. In the USA and Europe, the number of regulations has increased considerably over the past 10 to 15 years. Regulatory agencies such as the United States Food and Drug Administration (USFDA) and the Environmental Protection Agency (EPA) require methods of chemical analysis to be fully validated, providing comprehensive method descriptions together with assured uncertainty estimates. With increasing globalization of trade and the formation of groups such as the European Union and the Asia Pacific

Economic Co-operation it has become increasingly important for national economies to trust in the quality of each other's analysis. The costs when this trust breaks down can be extensive. Recently USA regulatory agencies were critical of the analysis of beef for chemical residues in Australian abattoirs, and the subsequent bad publicity cost the country millions of dollars through lost trade. Because of this recognition of the critical role of analytical chemistry in everyday life, strict method validation protocols are no longer just the concern of the pharmaceutical analysts, but are becoming an integral part of all routine analytical chemistry.

Workshops have been established by international organisations to formulate international standards for quantifying uncertainty in analytical measurement [1] and there has been a fundamental change in the development of validation procedures employed by

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analysts. Despite these changes, a recent European study by De Bievre and coworkers has shown that there are still many fundamental problems in the application of analytical chemistry [2,3]. One hundred and sixty five laboratories, mostly throughout Europe, were requested to assay fourteen elements in synthetic (prepared by the US Geological Survey under the responsibility of the National Institute of Science and Technology) and natural water. The concentrations ranged from around 1 $\mu g/l$ for silver to 500 $\mu g/l$ for barium. The laboratories were asked to return the estimates of the concentrations and expanded uncertainty of each element, the method used and a statement as to whether the laboratory considered itself experienced in this analysis. The uncertainty of the estimates of concentration was expected to be about 4%. However, the results showed inaccuracies often above 20% and as high as 100% of the certified measured concentration. The estimated uncertainties given by the laboratories were considerably less than the observed errors, which suggests that the errors were due to bias rather than random uncertainty. Youden plots of the data provide evidence for both high random errors and considerable bias.

The De Bievre study mainly involved the application of atomic spectroscopy for trace analysis, however, the problems of inter-laboratory precision are well recognised for all analytical chemistry techniques, including HPLC. The USFDA has shown that within laboratory variation is often half the value of inter-laboratory variation [4], in other words inter-laboratory variations account for half of the total errors. Studies by one of the authors investigated methods of ruggedness testing in an attempt to improve inter-laboratory performance [5,6].

To attempt to explain the observed errors of the European study there are two approaches which can be taken. The first assumes that these are the actual error levels that can be achieved in practice, in which case the analytical community has greatly underestimated uncertainties. The consequences of unavoidable uncertainties in the region of ±20–100% would be catastrophic to the maintenance of quality produce and to the legal community attempting to convict on evidence produced by chemical analysis. The second approach starts from the premise that there are sources of bias that have not been discov-

ered, and therefore either corrected or used to contribute to the estimated uncertainty. The scale of the study and the consistent inaccuracies make it difficult to simply lay the blame on poor quality analytical chemistry. Whether or not poor quality analytical work was involved there was a clear need for better tools to monitor and estimate errors.

It is also noted that the "blind" analysis of samples with completely unknown concentrations posed considerably more problems than the analysis of samples (such as for a quality control method) where the analyst has a good expectation of the result. An unexpected concentration result is therefore taken as a symptom of a problem with instrumentation, which is then investigated. Many applications of analytical chemistry, such as environmental analysis and quality monitoring of unprocessed food, deal with samples over wide concentration ranges and thus do not have the same "early warning system" when an unusual result is obtained.

Early guidelines for method validation were laid down by Youden and Steiner [7], and the techniques set out in this manual are still used as the basis for most method validation [8–12]. There are many possible sources of inaccuracies in analytical chemistry including; the use of impure reference materials, poor recoveries and unsuitable data reduction techniques. A thorough method validation study should reveal errors due to many sources but the aim of the study reported here was to find errors which could go undetected by standard techniques for the treatment of data.

Most chromatographers wish their method to show uncertainties within certain acceptable percentage limits and HPLC methods are validated to quantify the uncertainties associated with the procedure. There are many excellent guides to method validation [8–15], all of which recommend a linearity study to assess the suitability of linear regression as the calibration technique. The linearity study stands alone in the myriad of validation tests carried out as the only one that is rarely examined for the magnitude of the errors it contributes to the overall uncertainty estimate. Many analysts depend entirely on the use of a r^2 (or r) value between 0.999 and 1.000 as an acceptability criterion. This is well known to be inadequate and many chemometrics experts have expressed concern that publications are

still accepted with this minimum data [15]. Very rarely is there an attempt to estimate the actual contribution of errors from the linear assumption to the overall error estimation. The unweighted least squares method assumes that the entire residual arises from random uncertainty in the ordinate, and that the data is homoscedastic, i.e., the magnitudes of the differences between calibration points and the fitted line are independent of concentration.

The work described in this paper was an attempt to quantify the magnitude of the errors due to these limitations of the least squares method. It is shown that very large percentage errors at the lower end of the concentration range can coexist with acceptable correlation r^2 and are grossly underestimated by confidence limits from an analysis of the errors in slope and intercept.

2. Theory

2.1. Linear regression

In linear least squares regression the line of best fit is chosen to minimise the sums of the squares of the residuals. The quality of the fit may be estimated by the square correlation coefficient [r^2; also (confusingly) named coefficient of the variation] which is the fraction of the variation in y that is explained by the linear model. The correlation coefficient (r) is also frequently quoted. However, a correlation coefficient does not give any indication of the errors associated with an individual measurement. The method of calculation of the confidence limits on an individual measurement is described as follows [16,17]. For a linear model from $\{x_i, y_i\}$ data pairs taken at n different levels of x:

$$y = a + bx \qquad (1)$$

where a is the intercept and b the slope, the following statistics are calculated

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

$$S_b = \frac{S_{y/x}}{\left\{ \sum_i (x_i - \bar{x})^2 \right\}^{1/2}} \qquad (3)$$

$$S_a = S_{y/x} \left\{ \frac{\sum_i x_i^2}{n \sum_i (x_i - \bar{x})^2} \right\}^{1/2} \qquad (4)$$

$\hat{y}_i$ is the estimated value of y_i, and $\bar{x}$ the mean of the x data. S_a and S_b are known as the standard error on the intercept and slope, respectively. If m measurements of y are made with mean y_0 to yield an estimated $\hat{x}_0$, the standard error of $\hat{x}_0$ is

$$S_{\hat{x}_0} = \frac{S_{y/x}}{b} \left\{ \frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2} \right\} \qquad (5)$$

$\bar{y}$ is the mean of the y data. The confidence limits on $\hat{x}_0$ are

$$\hat{x}_0 = \frac{(y_0 - a)}{b} \pm t_{\alpha'',n-2} S_{x_0} \qquad (6)$$

where $t_{\alpha'',n-2}$ is the two-tailed Student-t value at the $100(1-\alpha)\%$ level for $n-2$ degrees of freedom.

2.2. Non-linear data

In this study the linear model was applied to data of the form $y = x^n$, where n was given values between 0.7 to 1.3. The model was then tested by calculating the x-residuals, i.e., the differences between the concentrations and the estimated concentrations, as a percentage of the concentration and by performing standard tests of fit. The function x^n coincides with x at $x = 1$ and progressively deviates thereafter. This models data that may arise in practice with increasing non-linearity as the ordinate increases. For example deviations from Beer's law in spectroscopy would follow this scheme, although the linearity would be over a range with a break at higher absorbencies.

3. Experimental

3.1. Software

Standard errors in the slope and intercept and the

r^2 were calculated using the linear regression data analysis tools of Microsoft Excel with Office 95. A spreadsheet was programmed to generate the confidence limits and the error in the concentration calculated using the linear fit.

3.2. Simulations

There were two stages to this work. The first studied the effects of deviations in the linearity in the relationship between concentration and the response.

Many chromatographic detectors exhibit a linear response over a limited concentration range, particularly spectroscopic methods of detection, and deviations from linearity can be expected for many applications. To study the ability of the correlation coefficient to reveal significant errors due to non-linearity a simulation study was carried out. Simulated abscissa ("concentration") ranges were used from $x = 1$ to $x = 30$, or to $x = 60$. The ordinate ("response"), y, was calculated, at 30 to 60 evenly spaced points over the concentration range, from the non linear relationship $y = x^n$ with $0.7 \leq n \leq 1.3$. It is to be noted that the simulation data was only numerical without units being specified, but we use the nomenclature "concentration" and "response" to make the parallels with typical analytical practice. The concentrations were then estimated ($\hat{x}_i$) using the line calculated by the least squares method at each y-value. The error in the estimation of the concentration (Δx_i) is therefore

$$\Delta x_i = \hat{x}_i - x_i = \frac{x_i^n - a}{b} - x_i \tag{7}$$

An error analysis was performed on the linear regression to calculate the 95% confidence limits of the estimates as discussed above.

The second part of this work sought to investigate the magnitude of errors when the data had an error distribution that was heteroscedastic, i.e., the errors are not of equal magnitude over the concentration range. The most common type of heteroscedasticity in practice occurs when the errors are of a constant relative size over the range. Data sets were created with 6 points at each level with a relative standard deviation of 1% and 4%. Between 1 and 6 of these points were randomly selected as the data set for the

linear regression for the experiments described in Section 4.1 Section 4.2 Section 4.3, and the errors were then treated as before.

3.3. HPLC study

3.3.1. Preparation of standards

Duplicate standard stock solutions containing 100 mg/50 ml of each of Diadzen, Biochenin and Genistin were each diluted 7 times to give a sample concentration range between 10 and 0.2 mg/50 ml. Each standard was injected three times.

3.3.2. HPLC conditions

20 µl of each of the standard solutions were injected onto a 10 cm×4.5 mm I.D. NovaPak C_{18} 5 µm column (Waters, Australia). A mobile phase of acetonitrile–water–acetic acid (60:40:1) was pumped at a flow-rate of 0.8 ml/min using a Waters Model 510 HPLC pump with a Waters intelligent sample processor (WISP) and a Waters Model 484 tunable absorbance detector. Integration was performed using the SMADCHROM integration program by Morgan Kennedy.

4. Results and discussion

4.1. Regression of non-linear simulated data

A typical linear fit for data ($1 \leq x \leq 30$) generated from $y = x^{1.1}$ is shown in Fig. 1. The r^2 for the fit
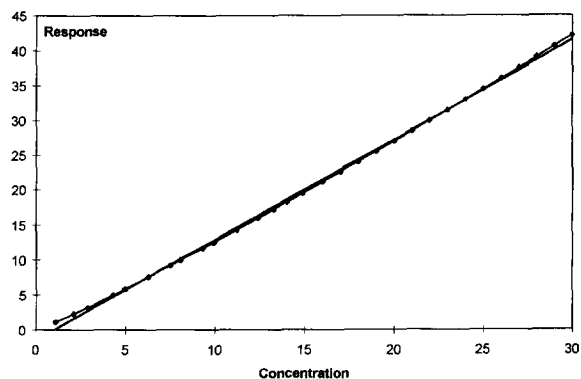

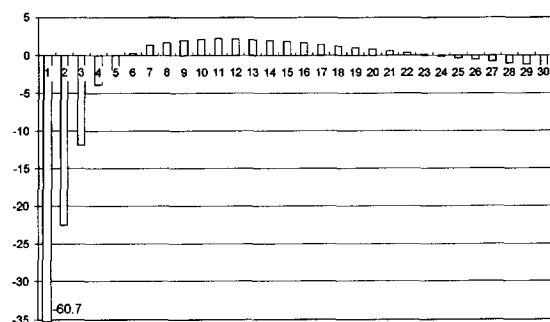Fig. 1. Simulated data, $y = x^{1.1}$ for $1 \leq x \leq 30$ at one unit intervals fitted to $y = 1.4318x - 1.4218$; $r^2 = 0.999$.

Fig. 2. Relative errors in estimated concentrations for the data of Fig. 1. Relative errors are given as 100 $\Delta x_i / x_i$ where Δx_i is calculated from Eq. (7).

was 0.999. However when each y-value was presented as a response to the linear equation and a concentration estimated, the errors found (Eq. (7)) were considerable when determined as a percentage of the concentration value (Fig. 2).

The procedure was repeated for data generated from $y = x^n$ where $0.7 \leq n \leq 1.3$. Table 1 contains information of the linear regression on the lines taken over the range. It is seen that acceptable, for validation purposes, fits are achieved for $0.9 \leq n \leq 1.1$ when $r^2 = 0.999$ or better.

However calculation of errors that are generated on estimated concentrations can be considerable. The distribution of the % errors calculated from Eq. (7) were analysed for the minimum and maximum

values, the mean and the median. The results are shown in Table 2. The range of the observed errors was much larger than should be expected for such a well established validation tool. This was particularly true for those simulations which gave a correlation coefficient greater than 0.999 and would often be accepted to show a very small contribution to the overall error estimate for the method.

The very large errors (10–80%) occur at the lowest part (1–15%) of the concentration range. This can be explained by the minimisation of residuals at the top of the range, which are of greater absolute magnitude, at the expense of those at the bottom of the range.

Further experiments in which the number of points over the concentration range was decreased showed similar errors in the calculated concentration. Below six points the errors increased.

This initial work demonstrated that the reliance on the correlation coefficient alone can lead to serious errors being overlooked. The next stage of this work was to investigate the utility of estimation of errors including those for the slope, intercept and individual concentration results. Table 2 shows the standard errors on the slope and the intercept for each data set. Apart from observing the magnitude of the intercept compared to the lowest data point ($x = 1$, $y = 1$), there is not much to be gained from these in terms of direct error estimation. However, the standard errors on slope and intercept are used to

Table 1
Slopes and intercepts and their standard errors of linear regressions on $y = x^n$ ($1 \leq x \leq 30$)

n	0.7	0.75	0.8	0.85	0.9	0.95	1.05	1.1	1.15	1.2	1.25	1.3
r^2	0.990	0.993	0.996	0.998	0.999	1.000	1.000	0.999	0.998	0.997	0.995	0.993
Slope	0.32	0.39	0.48	0.58	0.69	0.83	1.20	1.43	1.71	2.04	2.43	2.89
S_a	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.01	0.02	0.60	0.05
Intercept	1.53	1.44	1.31	1.12	0.85	0.83	−0.62	−1.43	−2.43	−3.71	−5.29	−7.26
S_b	0.11	0.11	0.10	0.09	0.07	0.00	0.06	0.01	0.01	0.40	0.03	0.05

Table 2
Relative (%) errors in concentrations arising from the use of linear regression on $y = x^n$ ($1 \leq x \leq 30$). Relative errors are given as 100 $\Delta x_i / x_i$ where Δx_i is calculated from Eq. (7)

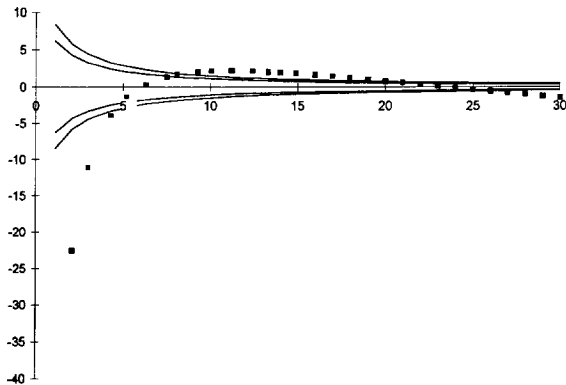| n	0.7	0.75	0.8	0.85	0.90	0.95	1.05	1.1	1.15	1.2	1.25	1.3
Mean % error	9.70	15.6	6.19	4.54	2.96	1.45	−1.39	−2.72	−4.00	−5.22	−6.40	−7.5
Median % error	−1.87	15.5	−1.27	−0.92	−0.57	−0.26	0.23	0.45	0.70	0.95	1.20	1.47
Minimum % error	−7.67	1.10	−4.89	−3.60	−2.36	−1.16	−31.2	−60.7	−88.7	−115	−140	−164
Maximum % error	229	30.0	144	105	68	33.0	1.12	2.20	3.24	4.25	5.22	6.15

Fig. 3. 95% and 99% confidence limits of the estimates of the x-values (lines) and errors (points) from linear regression on data generated from $y=x^{1.1}$. Data is plotted as % relative values.

calculate the confidence limits for the individual estimated x-values. Fig. 3 shows the calculated (Eqs. (5,6)) confidence limits (lines) plotted with the percentage errors (points) for estimates of concentration from the linear regression of data generated from $y=x^{1.1}$. Although the general trend of the percentage errors, increasing rapidly as the magnitude of x decreases, is revealed by the calculated confidence limits, it is clear that the confidence limit calculation was underestimating the true errors.

The magnitude of the errors and their significance are reduced as the concentration range taken for the regression analysis is made smaller. The percentage errors and correlation coefficients obtained for various ranges from estimates of concentrations from linear regressions on $y=x^{1.05}$ and $y=x^{0.7}$ are shown in Tables 3 and 4, respectively. It is clear that it is the absolute size of the range which influences the errors. For data generated with small deviations from linearity ($n=0.9-1.1$) a range from one to five times

Table 4
Relative (%) errors in concentrations arising from the use of linear regression on $y=x^{0.70}$ ($1 \leq x \leq 30$) as a function of the range of x values taken for the regression

Data Range	1–20	1–5	10–30	20–30
r^2	0.994	0.996	0.998	1.000
Maximum % error	−18.9	−5.65	−3.54	−0.310
Minimum % error	4.05	2.63	1.03	0.169
Median % error	0.605	−0.350	0.181	0.027
Mean % error	−1.03	0.270	−0.056	−0.001

the smallest concentration seems to be the largest that produces tolerable errors over the entire range of data. A range from one to two times the smallest concentration will tolerate larger deviations from linearity.

4.2. Heteroscedastic data

Linear regression by the least squares method assumes that each data point in the range has a constant absolute variation (i.e., is homoscedastic). However, many analytical methods produce data which are heteroscedastic in that the errors are a constant relative value (e.g., a 1% variation at each concentration). The use of least squares for this type of data is not commonly believed to lead to erroneous results but it is known to overestimate errors. This is demonstrated by using a data set that has heteroscedastic errors of 1% across the concentration range, generated as described earlier. The plot of y-residuals from the linear regression is shown in Fig. 4. Residual plots reveal heteroscedasticity by their gradual increase in absolute magnitude.

The simulated data was fitted with a r^2 of 0.999. Table 5 shows the errors in the estimation of the concentration together with the confidence limits

Table 3
Relative (%) errors in concentrations arising from the use of linear regression on $y=x^{1.05}$ ($1 \leq x \leq 60$) as a function of the range of x values taken for the regression

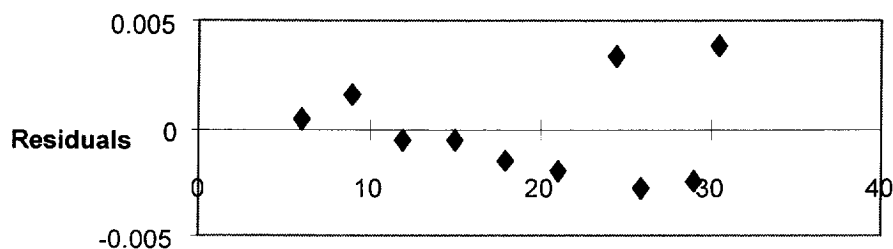| Data Range	1–60	1–40	1–20	1–10	1–5	20–30
r^2	0.999	0.999	0.999	0.999	0.999	0.999
Maximum % error	83.9	53.9	21.1	7.14	1.86	−0.042
Minimum % error	−1.36	−1.23	−1.13	−0.980	−0.640	0.080
Median % error	−0.240	−0.644	−0.230	−0.151	−0.072	0.001
Mean % error	3.47	2.61	1.16	0.522	0.169	−0.006

Fig. 4. Plot of y-residuals for simulated data ($y = 0.01x$) with a heteroscedastic errors of 1% across the data range.

determined from Eq. (6). The calculated confidence limits are much larger than those seen in practice. These could lead an analyst to reject a perfectly acceptable error contribution from the linearity assumption.

The use of weighted regression is usually recommended for heteroscedastic data. To apply weighted regression the analyst must establish the standard deviation of the response at each point across the concentration range. However, this study showed that this is not necessary as long as the concentration range is chosen with regard to the expected percentage errors. If the analyst expects errors of 4% the use of normal least squares will not contribute more than 4% over a range from one to 30 times the minimum concentration. However, for less than a 1% contribution from the regression the concentration range must be kept less than one to six times. This study did not consider errors less than 1% or more than 4%. These recommendations are based on the measurement of duplicates at each

concentration level. Larger concentration ranges are possible for 6 or 7 measurements at each level. Repeated measurements reduced the chance of extreme values at either end of the concentration distorting the calibration curve.

An increased number of measurements at the low end of the concentration range can offset both the effects of non-linearity and heteroscedasticity as they will weight the least squares function in favour of reducing the residuals at this extreme of the calibration curve. A limited study carried out on the combined effect of heteroscedasticity and non linearity ($n = 1.1$) showed that doubling the number of determinations at the lowest two concentrations over a one to 30 times the minimum concentration range reduced the errors significantly from over 60% to less than 40% at the lowest concentration and from almost 10% down to 4% at the second lowest value. The errors increased slightly at the top of the concentration range but still remained less than 1% in relative terms. Fig. 5 shows the distinctive y-

Table 5
Linear regression on heteroscedastic data generated with a constant 1% relative standard deviation

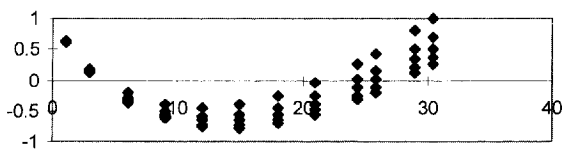| Concentration (x)	Response (y)	Relative (%) error in the estimated x	95% confidence limits on estimated x expressed as a % of x
6.022	0.0596	−0.83	16.34
9.038	0.0910	−1.81	10.89
11.979	0.1185	0.38	8.22
15.011	0.1489	0.33	6.56
17.991	0.1779	0.79	5.47
21.068	0.2084	0.91	4.67
24.352	0.2467	−1.38	4.04
25.844	0.2556	1.04	3.81
28.994	0.2876	0.80	3.39
30.412	0.3081	−1.26	3.24

Fig. 5. Plot of y-residuals from a linear regression on data generated from $y = x^{1.1}$ with 1% heteroscedastistic error distribution.

residuals plot for the combination of non-linearity and heteroscedasticity. The points and best fit line is shown in Fig. 6 to demonstrate just how difficult it can be to detect problems by the visual examination of the plot.

Errors introduced when applying the least squares method for heteroscedastic data were not significant if the concentration range was chosen with regard to the acceptable error requirement for the application.

4.3. HPLC study

Table 6 shows the complete data set obtained for a linearity study of the HPLC assay of Diadzen. Table 7 shows the regression statistics, r^2 was 0.999. The results were examined more closely by calculating the percentage difference between the weight added and the weight calculated using the linear fit, shown in column 3 in Table 6.

The 99% confidence limits were also calculated, shown in column 4 of Table 6. These results demonstrate errors of up to 30% on the calculation of
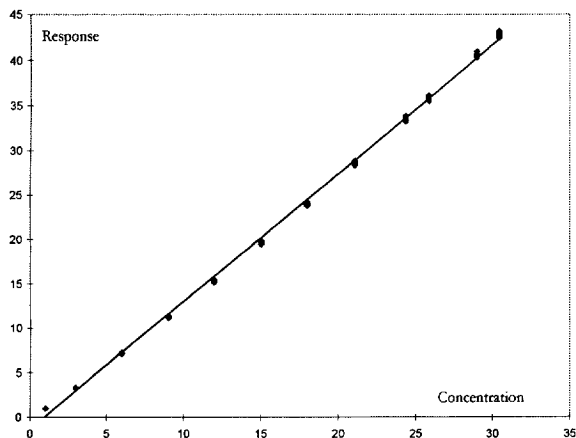

Fig. 6. Data generated from $y = x^{1.1}$ with 1% heteroscedastistic error distribution (points) fitted to $y = 1.4326x - 1.2982$.

weights despite an apparently acceptable r^2 value. It is also clear that the calculation of a confidence interval does not always reflect the real errors associated with the linear assumption. The plot of y-residuals for this data is shown in Fig. 7. This plot shows slight evidence of curvature but little evidence of heteroscedasticity. Similar results were obtained for Biochenin and Genisten assayed by this method.

4.4. Strategies for avoiding excessive errors

In practice, the use of y-residual plots are recommended as they can provide a distinctive visualisation of both non-linearity and heteroscedasticity and alert the analyst to potential problems. Outlier tests such as Cook's algorithm [18] can also be applied to detect and remove values with large errors, however they are limited in the same way as the least squares method by depending on absolute rather than relative errors.

The use of the regression line to estimate the concentration at each y value and thus determine the relative error (i.e., calculate "x-residuals") is a simple way of being aware of the error contribution from the linear regression. This calculation is already applied in analysis by inductively coupled plasma atomic emission spectroscopy where very great linear ranges are routinely used.

We note, too, the long standing, but essentially unexplained, observation of the "horn" curve in which the r^2 value increases according to an inverse power law of the concentration [19]. Without knowledge of the calibration ranges taken in the many studies published over the years it is not possible to assess the rapid increase in r^2 at low concentrations in terms of the theory presented here, but the possibility that a significant part of the error may arise from inappropriate use of linear regression in calibration is one that should be investigated further.

5. Conclusions

This study investigated error estimation routines for linear regression by the least squares method. It has shown that a correlation coefficient of 0.999 or greater can hide exceptionally large relative errors when data generated from a function that becomes

Table 6
Peak areas and calculated errors together with confidence intervals expressed as a percentage of the value from a linearity study of the HPLC analysis of Diadzen

Concentration mg/50 ml	Peak area	% Error	99% confidence interval (±%)
0.162	0.243	9.06	35.14
0.162	0.236	10.52	35.71
0.162	0.239	9.91	35.47
0.171	0.204	21.80	38.71
0.171	0.210	20.73	38.19
0.324	0.596	15.82	18.97
0.324	0.606	14.72	18.72
0.324	0.615	13.77	18.52
0.343	0.515	28.92	21.22
0.343	0.519	28.50	21.10
0.343	0.515	28.88	21.21
0.647	1.153	27.33	10.99
0.647	1.143	27.86	11.07
0.647	1.155	27.19	10.97
0.685	1.974	−11.20	6.78
0.685	1.965	−10.74	6.80
0.685	1.973	−11.15	6.78
1.295	3.425	1.43	4.04
1.295	3.457	0.55	4.00
1.295	3.396	2.20	4.07
1.37	3.939	−6.51	3.53
1.37	3.942	−6.57	3.53
1.37	3.914	−5.85	3.55
2.59	7.251	−1.71	1.95
2.59	7.238	−1.54	1.95
2.59	7.204	−1.06	1.96
2.74	7.966	−5.40	1.78
2.74	7.879	−4.28	1.80
2.74	7.921	−4.83	1.79
5.48	15.798	−3.42	0.91
5.48	15.767	−3.22	0.91
5.48	15.772	−3.25	0.91
10.36	29.209	−0.64	0.52
10.36	29.281	−0.89	0.52
10.36	28.611	1.41	0.53
10.96	30.152	1.82	0.51
10.96	30.750	−0.12	0.50
10.96	30.105	1.97	0.51

Table 7
Statistics of the linear regression of the HPLC analysis of Diadzen

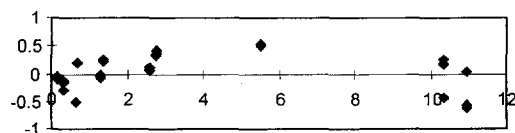|	
r^2	0.999
Intercept	−0.172
Standard error of intercept	0.066
Slope	2.818
Standard error of slope	0.014

Residuals

Fig. 7. Plot of y-residuals on linear regression on data obtained from a linearity study on the HPLC assay of Diadzen.

increasingly non-linear as the concentration increases, is presented to least squares linear regression. Many analytical methods, especially spectroscopic methods, exhibit a linear response over a limited concentration range and deviations from linearity at higher concentrations can be expected for many applications. The study also showed that calculating confidence limits on the estimated x-values from standard errors of the slope and intercept failed to predict the magnitudes of the errors. Studies on the effect of the concentration range on the errors arising from non-linearity showed that the smaller the range the more rugged the method was to non-linearity. This is an obvious conclusion which is clear from the assumptions employed by the least squares method.

Inspection of y- and x-residuals is recommended to alert the analyst to problems of high relative error at small concentration values being masked by seemingly good regression statistics.

The results of this study demonstrate the need for the continued efforts of chemometricians to develop and evaluate alternative data reduction methods for use in analytical chemistry. There is a clear need for further research into tools for method validation and routine error monitoring.

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