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Appropriate calibration functions for capillary electrophoresis II. Heteroscedasticity and its consequences

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Abstract

If ordinary least squares regression methods are to be used, the standard deviation of the signal should not depend on the sample concentration, but this is not true in CE. Results indicate, that the signal standard deviation is approximately proportional to the sample concentration. Therefore weighted least squares regression must be used, if the standard deviation within the concentration range differs by more than the factor 50. It is advised to use this regression method down to the factor 5, where the difference to ordinary least squares calculations is still significant. This is demonstrated by comparing experimental and simulated data. These considerations are valid for other analytical techniques as well, if their characteristics of calibration and variance function are similar.

1. Introduction

The use of capillary electrophoresis (CE) as a quantitative analytical technique is becoming more and more important [1–3]. Thus the choice of appropriate calibration functions must be considered. The importance of the sensitivity and its meaning for the precision was discussed in Part I [4]. The consequences of heteroscedasticity (dependence of the signal standard deviation on the sample concentration) shall be discussed by using data sets of preceding works [4,5] and simulations.

2. Experimental

The CE experiments were described in Part I [4] and [5].

2.1. Simulations

Each simulated data set consists of six equidistant blocks at position x_i. The values of x_i are given in the tables. Eight y values are determined for each block, leading to 48 data pairs. In a first step the y values are calculated by Eq. 1:

$$y_i = \alpha_0 + \alpha_1 x_i \quad (1)$$

Straight lines with a slope of $\alpha_1 = 1$ and an intercept of $\alpha_0 = 0$ were used for simplicity. In the second step normal distributed noise was added. Usually the standard deviation of this noise was chosen as 10% of the signal (Eq. 2):

$$y_i = y_i + 0.1 \cdot y_i \varepsilon_i \quad (2)$$

Here ε_i a normal distributed random variable with a standard deviation of 1 [6]. Thus ε_i can be positive or negative.

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2.2. Weighted least squares algorithms

If the algorithm WLS/IV is used, the weighting factor w_i is defined as the inverse of the variance of y_i[var(y_i); Eq. 3]. In case of heteroscedasticity this variance depends on the position x_i of the data pair. Thus var(y_i) is defined as the variance of all y_i with identical x_i. Several data pairs with identical x_i must be available to estimate these weights [7–10].

$$w_i = \frac{1}{\mathrm{var}(y_i)} \tag{3}$$

The data of the weighted centroid ($\bar{x}_c$,$\bar{y}_c$) as well as of the slope a_1 are then obtained by the Eqs. 4–6:

$$\bar{x}_c = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i} \tag{4}$$

$$\bar{y}_c = \frac{\sum_{i=1}^{n} w_i y_i}{\sum_{i=1}^{n} w_i} \tag{5}$$

$$a_1 = \frac{\sum w_i \sum w_i x_i y_i - \sum w_i x_i \sum w_i y_i}{\sum w_i \sum w_i x_i^2 - \left(\sum w_i x_i\right)^2} \tag{6}$$

The intercept a_0 can be calculated by Eq. 7:

$$a_0 = \bar{y}_c - a_1 \bar{x}_c \tag{7}$$

The further developed algorithm GLS/VFE [7,8] is outlined in the following:

(1) Start with a preliminary estimation of the regression parameters by OLS regression (Eqs. 4–6, all $w_i = 1$). Set variance parameter $\Theta = 0$.

(2) Calculate sdv(y):

$$\mathrm{var}(y) = \frac{1}{n} \cdot \sum_{i=1}^{n} \frac{[y_i - \hat{y}(x_i)]^2}{\hat{y}(x_i)^{2\Theta}} \tag{8}$$

Here $\hat{y}(x)$ is the linear regression function, thus

$$\hat{y}(x_i) = \bar{y}_c + a_1(x_i - \bar{x}_c) \tag{9}$$

The standard deviation sdv(y) is simply the square root of var(y).

(3) The pseudo-maximum likelihood L is estimated using Eq. 10 [11]:

$$L = -n \log[\mathrm{sdv}(y)] - \sum_{i=1}^{n} \log[\hat{y}(x_i)^{\Theta}] \tag{10}$$

L is maximized by variation of Θ in the range between −0.3 and 1.5. The step width begins with 0.01, the interval where the highest L are found is further examined in steps of 0.001. Note that sdv(y) depends on Θ. For each calculation sdv(y) is again calculated using Eq. 8.

(4) Calculate new weights (Eq. 11); use Θ of maximized L:

$$w_i = \frac{1}{\hat{y}(x_i)^{2\Theta}} \tag{11}$$

Note that GLS/VFE can estimate weights without having several data pairs (x_i,y_i) with identical x_i.

(5) Estimate new regression parameters by Eqs. 4–6 using the new weights. Estimate variance of this iteration step k by Eq. 12; this time the loss in the degrees of freedom is considered:

$$\mathrm{var}(y)_k = \frac{1}{n-2} \cdot \sum_{i=1}^{n} \frac{[y_i - \hat{y}(x_i)]^2}{\hat{y}(x_i)^{2\Theta}} \tag{12}$$

EXIT IF condition 13 holds true:

$$\frac{\mathrm{var}(y)_{k-1}}{\mathrm{var}(y)_k} < 1.05 \tag{13}$$

ELSE continue with step 2.

3. Results and discussion

3.1. The limitations of OLS regression for heteroscedastic CE calibration data

Calibration lines are usually calculated by ordinary least squares (OLS) regressions. Actually the use of OLS calculations should be restricted to cases, where the signal standard deviation is independent of the signal itself (homoscedasticity). A systematic error occurs, if heteroscedastic calibration data are evaluated by using OLS regressions. This error can be avoided by using weighted least squares (WLS) regressions [7,8,10–12], compare Experimental).

It is well known that calibration data are heteroscedastic in CE. This does not cause problems, if the concentration range is small. In this case statistical tests will fail to prove heteroscedasticity, and the systematic error will remain insignificant. If the expected concentration is rather well known, the use of the external standard evaluation is an interesting alternative [13].

However, there are cases where one wants to calibrate over a concentration range of one order of magnitude or more, e.g. if drug concentrations in urine or other body fluids shall be investigated. Here it is important to consider the magnitude of the systematic error that will occur by ignoring heteroscedasticity and using OLS estimations. Therefore calibration functions were calculated by using OLS and WLS for the same set of data. The results in Fig. 1 demonstrate that there is a clear difference between these methods. It is also well known from statistical theory that WLS gives the better estimation. However, the difference between the calibration functions still does not reveal the magnitude of the systematic error that is made by OLS regression. The line estimated by WLS could be a very good approximation of the true relationship. In this case the whole difference observed would be caused by the systematic error using OLS calculations. However, both lines are influenced by random error. Thus both deviate from the true relationship. If they deviate to different sides, the difference between them could become very large, although their deviation from the true relationship would be similar.

The true relationship is never known when experimental data are considered. Thus simulated sets of data must be used to clarify the amount of error made by OLS estimations. Here the true relationship is known by definition. The simulated data sets must be designed with similar properties like real CE calibration data.

3.2. *Properties of CE calibration data*

The linear relationship between sample concentration and peak area is well known in CE. However, although the dependence of the signal standard deviation on the sample concentration was recognized, this dependence was never systematically investigated. Thus the degree of heteroscedasticity for typical CE calibration data was unknown. The signal standard deviation is approximately proportional to the signal itself for the data set shown in Fig. 1 (Fig. 2). A

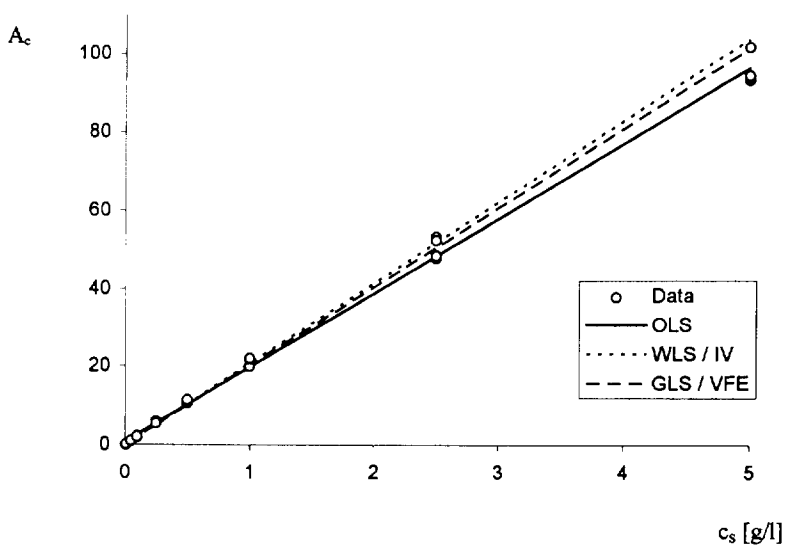

Fig. 1. Significant difference of regression lines, calculated using weighted least squares (GLS/VFE, WLS/IV) and ordinary least squares (OLS). Same data as in Fig. 2 of Part I [4] were used.

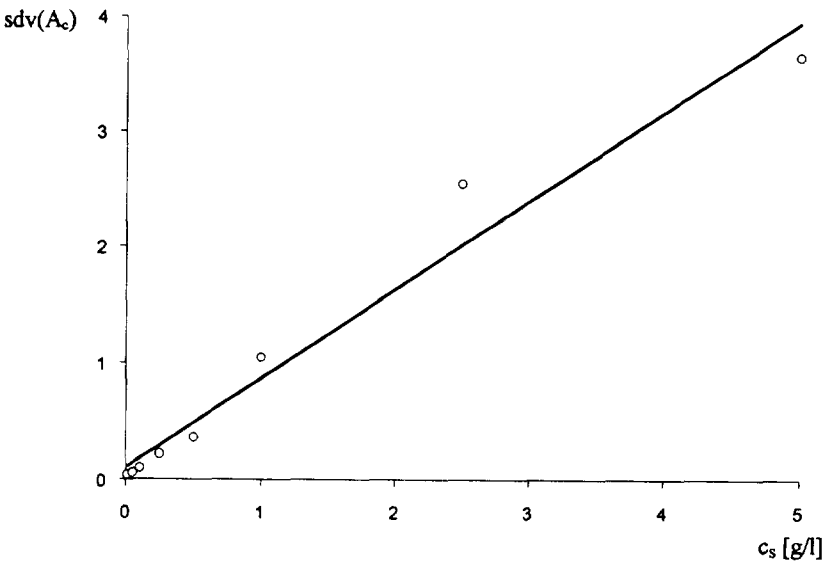

Fig. 2. Relationship between concentration and signal standard deviation for the data set presented in Fig. 1.

significant increase of the standard deviation with concentration was observed for other CE calibrations as well [5,14]. In these cases the standard deviation was a bit slower increasing than the signal, thus the relative standard deviation decreased with higher concentration.

The characteristics of the variance function are not only important for the following simulation experiments, but can also be used to estimate the contribution of different error sources to the total error in CE. Detection, integration, injection, dilution, weighting of substances and changing migration times from run to run are considered to be the main error sources in CE.

The error function for UV detection is well known [15–17]. After passing a minimum the detection error increases stronger than linear with the concentration (Eq. 14):

$$\text{relsdv}(A) = k \cdot \sqrt{1 + 10^{2A}} \qquad (14)$$

Here A is the absorbance. The constant factor k depends on the transmission of the reference solution and on its standard deviation.

The integration error of the considered data set can be neglected. The proper estimation of the baseline was visually controlled for all electropherograms. A few electropherograms at low concentrations had to be reintegrated.

The error is proportional to the sample concentration for injection, dilution and variation of migration times. For example, if a volume of 1 nl is injected with a standard deviation of 0.01 nl, the injected amount will be 1 ng and its standard deviation 10 pg, if a concentration of 1 g/l is injected, but only 10 pg with a standard deviation of 0.1 pg, if 10 mg/l are injected.

These errors, that are proportional to the concentration, seem to contribute the most to the total error. The detection error seems to have less influence.

There is another interesting consequence of this observed linearity of the variance function. The ratio of the standard deviations at the highest and the lowest concentration within the working range (R_{sdv}; Eq. 15) can be estimated from the ratio of the highest to the lowest concentration used for the calibration prior to the experiment. This is an important help to plan CE calibration experiments and evaluations.

$$R_{sdv} = \frac{sdv(y_{x=max})}{sdv(y_{x=min})} \quad (15)$$

3.3. Design of the simulation experiments

These simulations were designed to investigate the consequences of heteroscedasticity in general. Thus a linear functional relationship between x and y was simulated (Eq. 1). In CE x and y correspond to the sample concentration and the obtained signal, e.g. the peak area, respectively.

For simplicity the intercept α_0 was always chosen as 0, the slope α_1 as 1. Other parameters do not change the results. These are only influenced by the signal-to-noise ratio and the increase of the standard deviation with concentration.

The relationship between standard deviation $sdv(y)$ and x was also chosen linear for these simulations (Eq. 2). This was the strongest increase of the standard deviation so far observed in CE. The stronger this increase, the higher the systematic errors by using OLS. Thus these simulation demonstrate the systematic errors that must be expected in the worst case possible.

3.4. Performance of difference regression algorithms

The quality of a WLS regression depends on the knowledge about the variance function. The results are best, if the variances $var(y_i)$ are known. Thus the proper estimation of the variance function is very important. A number of different algorithms were designed for this purpose (reviewed in [7,8]). Mainly the two WLS algorithms WLS/IV and GLS/VFE were tested and compared to ordinary least squares (OLS) regression in this work.

Other algorithms, e.g. robust regression algorithms ([18,19]; cited in [20]), were considered in pilot studies. These algorithms also use weighting but to distinguish outliers from data belonging to the calibration set. They are very effective in doing this: their breakdown point is 50% outliers. These algorithms showed some slight advantages compared to OLS. However, they were by far inferior to the two mainly considered WLS algorithms. The calculation of robust regressions is very time-consuming. Thus it was decided not to include them in the main investigations.

The simple WLS/IV just uses inverse variances as weighting factors w_i (Eq. 3). The calculation of the regression parameters is analogous to OLS (Eqs. 4–6). However, the variance of a random sample is a disputable estimator for the variance of the whole set [11,12,21]. Even if the standard deviation is simulated as proportional to the signal, the standard deviation of a random sample corresponding to lower signals can be higher than at higher signals, caused by random errors. Thus the use of inverse variances as weighting factors can be misleading [8].

A further developed algorithm GLS/VFE (generalized least squares using variance function estimation) was recommended in preceding works [7,8]. The difficulty of estimating the variance is overcome by an iteration, where regression parameters and the variance function can be determined at the same time. The variance function is the dependence between signal y and the variance of the signal $var(y)$. This can often be expressed by Eq. 16.

$$var(y) = \sigma^2 y^{2\Theta} \quad (16)$$

Here σ^2 is the variance $var(y)$ at $y = 1$, and the variance parameter Θ represents the degree of heteroscedasticity. If Θ equals 0, then the variance is constant (homoscedasticity). If Θ equals 1, then the standard deviation of the signal is proportional to the signal itself. The algorithms WLS/IV and GLS/VFE were compared to WLS/SV (weighted least squares using simulated variances). Here the inverse of the known variances from the simulation procedure were used as weighting factors. The regression parameters were again calculated using Eqs. 4–6.

WLS/SV cannot be used in practice, because the true variance function is always unknown for experimental data. However, the error in es-

timating the simulated regression parameters is only caused by the random scatter of the simulation data, if this algorithm is used. The systematic error by erroneous estimation of the variances is avoided by using the true ones. Thus WLS/SV offers a possibility to recognize the amount of error which is caused by inprecise estimation of the variance function using other algorithms.

Some error measures were defined to quantify the systematic error that must be expected for the different algorithms in dependence on the ratio R_{sdv}. The mean squared error (MSE, Eq. 17) is a good measure for the average error that is caused by the estimation of the calibration function [7,11,12,22].

$$\mathrm{MSE} = \sum_{i=1}^{n} [(\alpha_0 + \alpha_1 x_i) - (a_0 + a_1 x_i)]^2 \qquad (17)$$

Here a_0 and a_1 are intercept and slope of the calibration function, α_0 and α_1 are the corresponding parameters of the simulated true function.

The true x values x_0 for a given y are known from the parameters of the simulated function (Eq. 18). The difference between the x value x_{estm} estimated using the calibration function (Eq. 19) and x_0 is a measure for the total error Err caused by the calibration function (Eq. 20, compare Fig. 3). This total error consists of the random error within the data pairs used for the calibration and the systematic error caused by incorrect weighting. Errors are considered at the lowest concentration (Err_{low}) and the highest concentration (Err_{hi}) used for the calibration.

$$x_0 = \frac{y - \alpha_0}{\alpha_1} \qquad (18)$$

$$x_{estm} = \frac{y - a_0}{a_1} \qquad (19)$$

$$\mathrm{Err} = \frac{|x_{estm} - x_0|}{x_0} \qquad (20)$$

The data in Table 1 show, that there is a big difference in performance between the different algorithms. The strongest distinction is observed for the systematic error at the lowest concentration, Err_{low}. This can be understood by looking at Fig. 4: if no weights are used, the data at high concentrations are given too much weight. Wrong estimations at high concentrations work like a lever and lead to a considerable systematic error at low concentrations.

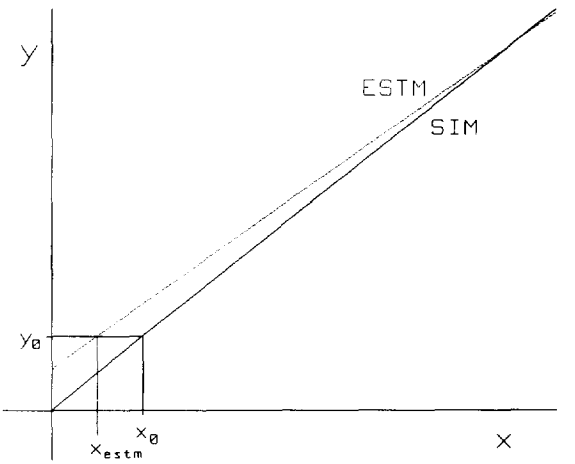

Fig. 3. Determination of the parameters x_0 (Eq. 18) and x_{estm} (Eq. 19), which are needed to estimate the error Err (Eq. 20) caused by the estimated calibration function ESTM at different x. The plain line represents the simulated function SIM. Especially at low x values the Err_{low} is strongly influenced by the error of the calibration function.

The error at low concentrations Err_{low} in dependence on R_{sdv} is the most important aspect of these investigations. The choice of the position of the x_i other than equidistant or of Θ other than 1 has only very minor effects [compare differences between sections (a) in Tables 1 and 2 as well as between sections (b) in Tables 1 and 2]. The results are independent of the signal-to-noise ratio [sdv(y)/y] used in the simulations.

This relationship between Err_{low} and R_{sdv} is presented in Table 3. If R_{sdv} equals 50, the error is increased by more than 10 fold, if OLS regression is used. Here the error caused by the wrong estimation of the calibration function is more than 3-fold compared to the random error at low concentrations [section (e) in Table 1, 0.33 compared to 0.1]. The error Err_{low} of OLS regressions is still more than 1.5-fold higher than of GLS/VFE, if R_{sdv} equals 5. The average error caused by the OLS estimation of the calibration

Table 1
Differences in errors for various R_{sdv}, if different regression algorithms are used

	Err_{low}	Err_{hi}	MSE
(a) $R_{sdv} = 2$, $x_i = 15, 18, 21, 24, 27, 30$			
WLS/SV	0.02321	0.01822	0.2067
GLS/VFE	0.02337	0.01840	0.2010
WLS/IV	0.02475	0.02001	0.2514
OLS	0.02456	0.01912	0.2235
Normalized to GLS/VFE			
WLS/SV	0.9929	0.9905	0.9865
GLS/VFE	1	1	1
WLS/IV	1.0587	1.0879	1.1997
OLS	1.0507	1.0395	1.0664
(b) $R_{sdv} = 3.16$, $x_i = 15.0, 21.5, 28.0, 34.4, 40.1, 47.4$			
WLS/SV	0.02487	0.01678	0.4071
GLS/VFE	0.02494	0.01690	0.4089
WLS/IV	0.02626	0.01844	0.4993
OLS	0.03071	0.01848	0.4852
Normalized to GLS/VFE			
WLS/SV	0.99972	0.9932	0.9957
GLS/VFE	1	1	1
WLS/IV	1.0533	1.0919	1.2210
OLS	1.2316	1.0934	1.1866
(c) $R_{sdv} = 5$, $x_i = 15, 27, 39, 51, 63, 75$			
WLS/SV	0.02621	0.01512	0.7823
GLS/VFE	0.02656	0.01533	0.7968
WLS/IV	0.02772	0.01693	0.9747
OLS	0.04187	0.01786	1.0560
Normalized to GLS/VFE			
WLS/SV	0.9870	0.9866	0.9818
GLS/VFE	1	1	1
WLS/IV	1.0435	1.1045	1.2232
OLS	1.5763	1.1652	1.3253
(d) $R_{sdv} = 10$, $x_i = 15, 42, 69, 96, 123, 150$			
WLS/SV	0.02774	0.01457	2.723
GLS/VFE	0.02780	0.01474	2.798
WLS/IV	0.02825	0.01625	3.426
OLS	0.07322	0.01787	4.081
Normalized to GLS/VFE			
WLS/SV	0.9980	0.9884	0.9733
GLS/VFE	1	1	1
WLS/IV	1.0163	1.1028	1.2245
OLS	2.6343	1.2123	1.4586
(e) $R_{sdv} = 50$, $x_i = 15, 162, 309, 459, 603, 750$			
WLS/SV	0.02841	0.01294	54.00
GLS/VFE	0.02841	0.01297	54.37
WLS/IV	0.02848	0.01447	68.47
OLS	0.33258	0.01740	95.10

(*Continued on p. 16*)

Table 1 (*continued*)

	Err_{low}	Err_{hi}	MSE
Normalized to GLS/VFE			
WLS/SV	1.0000	0.9976	0.9932
GLS/VFE	1	1	1
WLS/IV	1.0012	1.1150	1.2594
OLS	11.7056	1.3409	1.7492

Number of data sets simulated for each condition: 2000. Each set consists of six equidistant blocks x_i of y-values ($n = 48$ data pairs). First all y get the value of their x_i (corresponds to a straight line with $\alpha_0 = 0$ and $\alpha_1 = 1$), then normal distributed noise is added ([6]; see Experimental). The standard deviation sdv of the noise is 0.1 (10%) of the corresponding signal y. The noise is simulated proportional to signal, that means $\Theta = 1$.

function equals 0.0419 [section (c) in Table 1]. However, this error will be the double in a number of cases. Thus this error cannot be neglected compared to 0.1 random noise.

These investigations demonstrate the high performance of the algorithm GLS/VFE. The systematic errors caused by this algorithm are negligible, as can be seen from comparison to WLS/SV.

Table 4 offers a surprise in the first place. All regression parameters are the same, if only two concentrations are used for the calibration (2-point calibration). The systematic error by over-estimation of high concentrations using OLS cannot take place. Thus Err_{low} remains small, even if OLS is used. However, the use of a 2-point calibration for a concentration range of one order of magnitude or more causes problems, too. It must be validated, whether the method is really linear over some orders of magnitude, and the used statistical tests must be applicable in case of heteroscedasticity.

4. Conclusions

Weighted least squares regression (WLS) methods are superior to ordinary least squares (OLS) methods. If the ratio of standard deviations within the concentration range R_{sdv} exceeds 5, the additional error by using OLS cannot be neglected; if R_{sdv} exceeds 50, the use of WLS is mandatory. However, it is advisable to use WLS for smaller R_{sdv} as well. The difference between OLS and WLS is less, but was found significant down to an R_{sdv} of 3, which is consistent to previous results [7]. Moreover results can never get worse using WLS.

In CE the error is approximately linear to the concentration. Thus the expected R_{sdv} can be estimated from the ratio of the highest and the lowest concentration. Therefore it is possible to decide if WLS will be necessary before the calibration is started.

WLS is best if the weights are known. In this work two methods to estimate weighting factors were investigated. The further developed algorithm GLS/VFE [7,8] is beneficial compared to WLS/IV for a number of reasons. It is slightly better in estimating the true regression coefficients, and it can be applied to all possible sets of data, whilst WLS/IV needs several values y_i with identical x_i to calculate the weights. Moreover, it is not possible to estimate the variance function properly, if the simpler WLS/IV is used. Thus confidence intervals cannot be calculated correctly, and the uncertainty of the calibration function can only be roughly estimated. Therefore the error of the analytical result is less precisely obtained. GLS/VFE can be easily implemented and shall be used in all cases, if heteroscedasticity cannot be refused. The use of WLS/IV is still much better than using OLS regression in these cases.

The proper estimation of the variance function is still a challenge. Outliers can influence its estimation strongly. Other methods to estimate different models of variance functions, especially weighted robust methods, shall be the issue of future work.

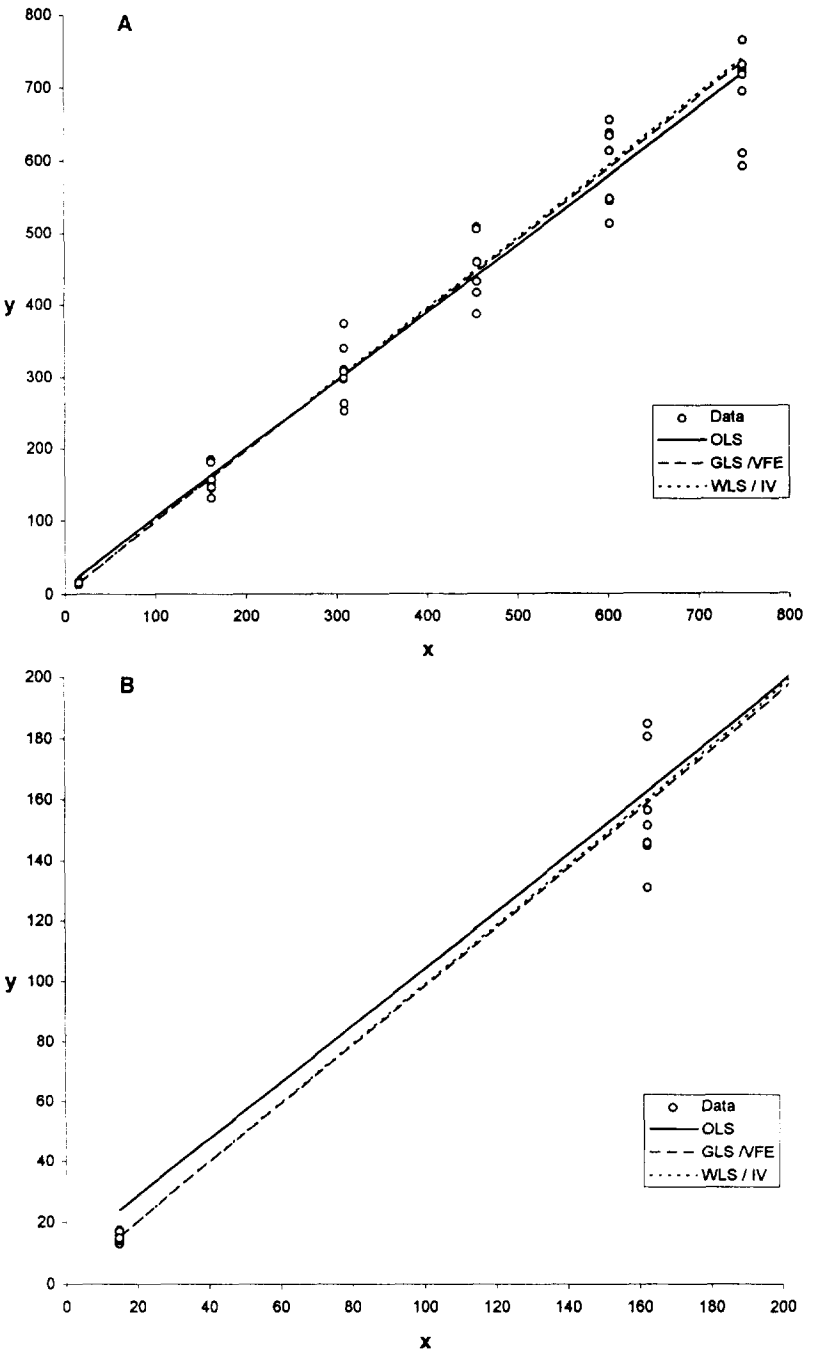

Fig. 4. Known linear functions were obtained by simulation. The signal standard deviation was chosen proportional to the signal [compare Experimental, same simulation parameters as for section (e) in Table 1]. In many simulations the data at high x values are strongly influenced by the random error. If no weighting factors are used, actually all data are equally weighted with the factor 1 (Eqs. 4–6). Thus the uncertain data at high concentration are given too much weight, if OLS is used (A). This leads to large deviations between OLS-estimated and simulated, thus true, functions at low x values. This is shown in (B), which is a magnification of (A). WLS algorithms provided a much better approximation. Over the whole range the lines estimated by WLS/IV, GLS/VFE and the simulated line ($\alpha_0 = 0$, α_1) can hardly be distinguished in this figure.

Table 2
Differences in errors for different regression algorithms for various R_{sdv}, if $\Theta \neq 1$; other conditions as in Table 1

	Err_{low}	Err_{hi}	MSE
(a) $R_{sdv} = 1.78$, $x_i = 15, 42, 69, 96, 123, 150$, $\Theta = 0.25$, that means the standard deviation of the noise at block x_i can be calculated as $sdv(x_i) = 0.1 \cdot x_i^{0.25}$			
WLS/SV	0.003005	0.000446	0.003355
GLS/VFE	0.003040	0.000447	0.003393
WLS/IV	0.003255	0.000490	0.004064
OLS	0.003182	0.000451	0.003515
Normalized to GLS/VFE			
WLS/SV	0.9884	0.9966	0.9886
GLS/VFE	1	1	1
WLS/IV	1.0707	1.0950	1.1976
OLS	1.0467	1.0087	1.0358
(b) $R_{sdv} = 3.16$, $x_i = 15, 42, 69, 96, 123, 150$. $\Theta = 0.5$, that means $sdv(x_i) = 0.1 \cdot x_i^{0.5}$			
WLS/SV	0.006536	0.001400	0.02893
GLS/VFE	0.006586	0.001410	0.02918
WLS/IV	0.006955	0.001553	0.03551
OLS	0.008366	0.001518	0.03378
Normalized to GLS/VFE			
WLS/SV	0.9924	0.9932	0.9915
GLS/VFE	1	1	1
WLS/IV	1.0560	1.1021	1.2171
OLS	1.2703	1.0778	1.1579

Possibly the use of 2-point calibrations is an interesting alternative to WLS regression. A systematic error is also avoided in our simulations. However, the use of 2-point calibrations over more than one order of magnitude sounds rather exotic for the practitioner. In the simulations considered the true function was strictly linear. If 2-point calibrations should be used, this must be true for the analytical method as well. Moreover, linearity has to be validated, which seems to be not trivial, especially in the heteroscedastic case. This another topic for further investigations.

Those considerations are valid for other ana-

Table 3
The difference in performance between the algorithms OLS and GLS/VFE is most pronounced for the total error caused by the calibration function at low x, Err_{low} (Eq. 20, Fig. 3); this error is considered as a function of the degree of heteroscedasticity, which is expressed as R_{sdv} (Eq. 15)

R_{sdv}	Normalized Err_{low} of OLS	Compare table
2	1.05	1a
3.16	1.23	1b
5	1.57	1c
10	2.63	1d
50	11.71	1e

The conditions of the simulations are described in Table 1.

Table 4
Two-point calibrations

	Err_{low}	Err_{hi}	MSE
WLS/SV	0.01626	0.01669	4.943
GLS/VFE	0.01626	0.01669	4.943
WLS/IV	0.01626	0.01669	4.943
OLS	0.01626	0.01669	4.943
Normalized to GLS/VFE			
WLS/SV	1.0000	1.0000	1.0000
GLS/VFE	1	1	1
WLS/IV	1.0000	1.0000	1.0000
OLS	1.0000	1.0000	1.0000

If 2-point calibrations are used, there is no additional error caused by heteroscedasticity. This is not surprising: a line is defined by two points, thus there is only one possibility. Systematic errors can only appear, if there are at least three x values: now one value can be given too much weight. Possibly the use of 2-point calibrations is an interesting alternative to WLS regression. However, linearity has to be validated, which seems to be not trivial, especially in the heteroscedastic case. Simulation conditions like in Table 1, but only two equidistant blocks x_i of 24 y values were simulated, $R_{sdv} = 10$, $x_i = 15, 150$.

lytical techniques as well, if their characteristics of calibration and variance function are similar.

Symbols and abbreviations

a_0	intercept of estimated regression line
a_1	slope of estimated regression line
α_0	intercept of simulated line
α_1	slope of simulated line
A	absorbance
A_c	corrected peak area
CE	capillary electrophoresis
c_s	sample concentration
ε_i	normal distributed random variable
Err_{hi}	total error caused by the calibration function at high x values
Err_{low}	total error caused by the calibration function at low x values
GLS/VFE	algorithm: generalized least squares using variance function estimations
k	iteration number
L	likelihood
MSE	mean squared error
n	number of data pairs per set
OLS	algorithm: ordinary least squares
relsdv	relative standard deviation
R_{sdv}	ratio of the standard deviations at the highest and the lowest concentration within the working range (Eq. 15)
σ^2	variance at y = 1
sdv	standard deviation
Θ	variance parameter
var	variance
w_i	weighting factor
WLS/IV	algorithm: weighted least squares using inverse variances
WLS/SV	algorithm: weighted least squares using simulated variances
x_0	true x at given y
$\bar{x}_c$	coordinate of the centroid
x_{estm}	x estimated by regression parameters a_0 and a_1 at given y
x_i	coordinate of simulated data pair; corresponds to sample concentration in CE
$\bar{y}_c$	coordinate of the centroid
y_i	coordinate of simulated data pair; corresponds to signal in CE
$\hat{y}(x)$	estimated regression function

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