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Journal of Chromatography A, 700 (1995) 1-7

JOURNAL OF  
CHROMATOGRAPHY A

# Appropriate calibration functions for capillary electrophoresis I. Precision and sensitivity using peak areas and heights

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## Abstract

Calibration functions for CE can be calculated by using peak heights or areas. The relationship between peak heights and concentrations is non-linear, but can well be approximated by a parabolic function. The precision of peak heights is often better than of areas. However, the sensitivity of the calibration function decreases at higher concentrations. Thus calibration functions calculated by using areas lead to a better precision of the analytical result, which is estimated by the slope-normalized standard deviation.

## 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 the appropriate calibration functions with respect to precision, accuracy and data collection shall be considered by using data sets of preceding works [4,5].

## 2. Experimental

The CE experiments were performed with P/ACE 2050 and 2100 systems (Beckman, Palo Alto, CA, USA).

The capillaries were from fused silica, their standard measure was 30 cm length (inlet to detector)  $\times$  50  $\mu$ m I.D. Prior to their first use they were conditioned with 0.1 M NaOH for 30 min, heating to 50°C, and then equilibrated with running buffer for 40 min under the subsequent running conditions. Before each run the capillary

was rinsed with the running buffer for 1 min. The thermostat was set to 25°C.

If not stated otherwise, chemicals were of analytical-reagent grade, supplied by Merck (Darmstadt, Germany).

Pyridine-2,4-dicarboxylic acid [5]: the wavelength of detection was 254 nm. It was injected by pressure, 5 s with 0.5 p.s.i. (=34.5 hPa). The separation voltage was 25 kV (33  $\mu$ A, anode at the outlet buffer). Standard buffer citrate 60 mM, pH 3: 15 ml of a 60 mM (12.6084 g/l) solution of trisodium citrate (>99%; Riedel-de Haen) are filled up with a 60 mM solution (17.646 g/l) of citric acid to 100.0 ml.

N-Acetylcysteine [4]: the wavelength of detection was 214 nm. The samples were injected 4 s with 0.5 p.s.i. (=34.5 hPa). The separation potential was 20 kV. Borate buffer 90 mM, pH 8.55 with 5% polyethyleneglycol 20000 (PEG; Hoechst, Germany; pharmacopoeia quality): 329 mg boric acid, 351 mg sodium tetraborate and 5000 mg PEG are dissolved in and diluted to 100.0 ml with HPLC-grade water (Millipore, Eschborn, Germany). Sample pretreatment:

pharmaceutical formulations and standard substances were dissolved in degassed (ultrasonicated) HPLC-grade water. The solutions were immediately filled into sample vials and covered by a film of approximately 1 mm light mineral oil.

The program  $W_sA$ -statistics (see [6]) was partly used to evaluate the data sets shown in Figs. 1 and 2, and to calculate the data presented in Tables 1 and 2.

### 3. Results and discussion

The calibration data for N-acetylcysteine [4] and pyridine-2,4-dicarboxylic acid [5] are pre-

sented in Figs. 1 and 2, the corresponding electropherograms are shown in Figs. 3 and 4, respectively. In both cases the calibration function is a straight line, if corrected peak areas are used, but the function is curved when using peak heights. However, this curve can well be approximated by a parabola. This behavior of calibration functions is typical for CE.

When peak heights are used, the slope of the calibration function, that means its sensitivity, decreases at higher concentrations (Figs. 1 and 2, top). The standard deviation of analytical results [ $sdv(x)$ ] is dependent on the standard deviation of measurements [ $sdv(y)$ ], but also on the sensitivity of the calibration function (Fig. 5). This

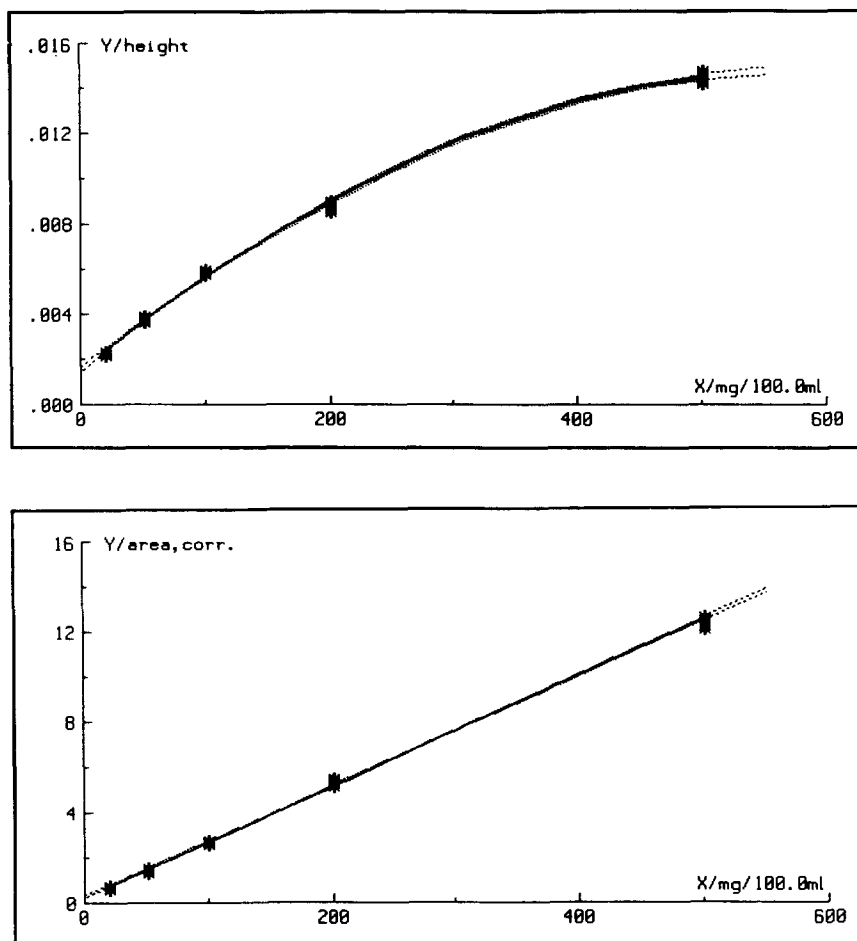


Fig. 1. Calibration functions of N-acetylcysteine ([4]; separation see Fig. 3). Peak heights (top) and corrected areas (bottom) were used. The data were taken from the same series, each concentration was measured eight times.

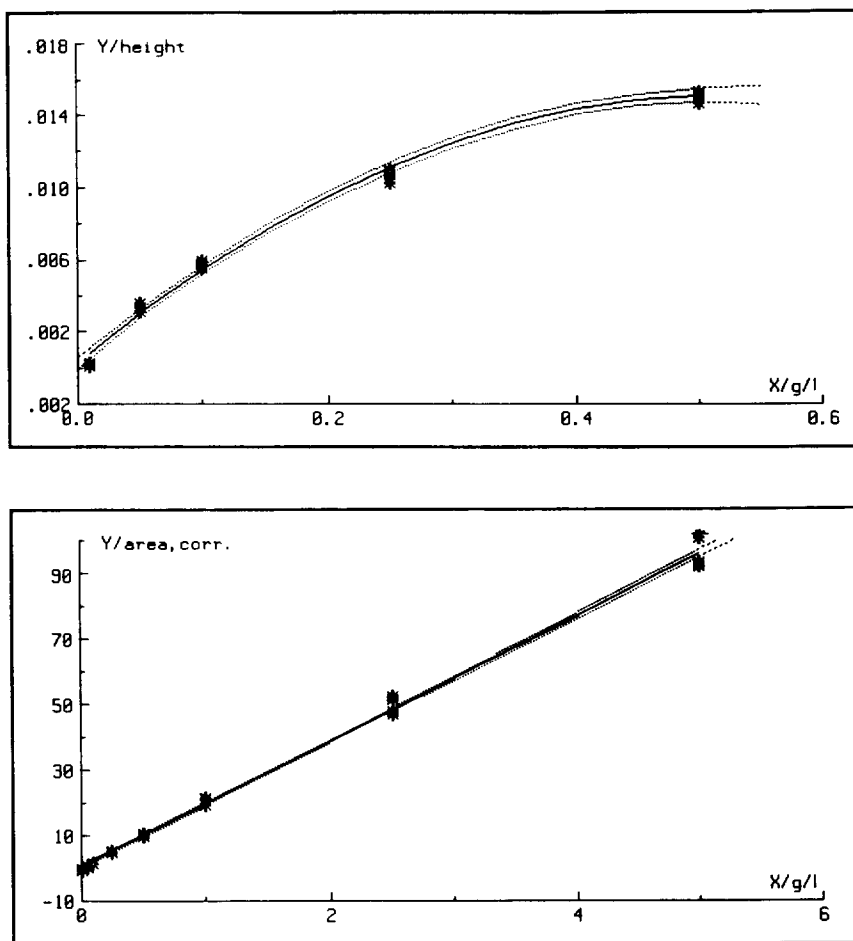


Fig. 2. Calibration functions of pyridine-2,4-dicarboxylic acid ([5]; separation see Fig. 4). Peak heights (top) and corrected areas (bottom) were used. The data was taken from the same series, each concentration was measured five times.

precision of the results, corresponding to  $sdv(x)$ , can be calculated by error propagation of the reciprocal of the calibration function (see Appendix).

If possible, high sample concentrations should be used in CE to achieve optimal reproducibility [1,3,7]. However, at high sample concentrations the sensitivity of peak height calibrations is very low. Thus the relative standard deviation of the analytical result is much better, if peak area calibrations are used, although the standard deviation of peak heights may be lower (Tables 1 and 2). Therefore calibration functions using areas shall be preferred in most cases. There is only one exception: sometimes the determina-

tion of rather low sample concentrations cannot be avoided. In these cases the calibration curve of peak height may be sensitive enough, and the determination of peak heights may be more reproducible, because it is less influenced by integration errors than the determination of peak areas.

#### 4. Conclusions

Peak areas shall be preferred to peak heights, when calibration functions are calculated in CE. These considerations are valid for other ana-

Table 1  
Repeatability for the determination of N-acetylcysteine by CE.

	Concentration (mg/l)				
	205	513	1004	2010	5012
<b>Peak height</b>					
sdv(y) ( $\times 10^{-5}$ )	4.8	8.6	7.7	14.5	20.3
relsdv(y) (%)	2.1	2.2	1.3	1.6	1.4
dy/dx ( $10^{-6}$ l/mg)	4.3	4.1	3.7	3.0	0.7
sdv(x) (mg/l)	11.1	20.9	20.7	49.1	296.8
relsdv(x) (%)	5.40	4.08	2.06	2.44	5.92
<b>Corrected peak area</b>					
sdv(y)	0.05	0.055	0.036	0.104	0.168
relsdv(y) (%)	7.2	3.8	1.3	1.9	1.3
dy/dx = $a_1$ ( $10^{-3}$ l/mg)	2.4	2.4	2.4	2.4	2.4
sdv(x) (mg/l)	20.9	23.1	14.9	43.4	69.9
relsdv(x) (%)	10.2	4.5	1.5	2.2	1.4

Each concentration was measured eight times. The corresponding calibration functions are shown in Fig. 1, for calculations compare Appendix. rel = Relative.

Table 2  
Repeatability for the determination of pyridine-2,4-dicarboxylic acid by CE.

	Concentration (mg/l)				
	10	50	100	250	500
<b>Peak height</b>					
sdv(y) ( $\times 10^{-4}$ )	0.5	1.7	1.5	2.9	2.1
relsdv(y) (%)	20.0	4.8	2.6	2.7	1.4
dy/dx ( $10^{-5}$ l/mg)	5.6	5.2	4.6	3.0	0.2
sdv(x) (mg/l)	0.9	3.3	3.3	9.7	105.0
relsdv(x) (%)	9.0	6.6	3.3	3.8	21.0
<b>Corrected peak area</b>					
sdv(y)	0.037	0.054	0.096	0.219	0.359
relsdv(y) (%)	48.9	5.9	4.8	3.9	3.4 <sup>a</sup>
dy/dx = $a_1$ ( $10^{-3}$ l/mg)	21.6	21.6	21.6	21.6	21.6
sdv(x) (mg/l)	1.7	2.5	4.4	10.1	16.6
relsdv(x) (%)	17.0	5.0	4.4	4.0	3.3

Each concentration was measured five times. The corresponding calibration functions are shown in Fig. 2, for calculations compare Appendix and Table 1.

<sup>a</sup> Rather high sdv(y) compared to other measurement series.

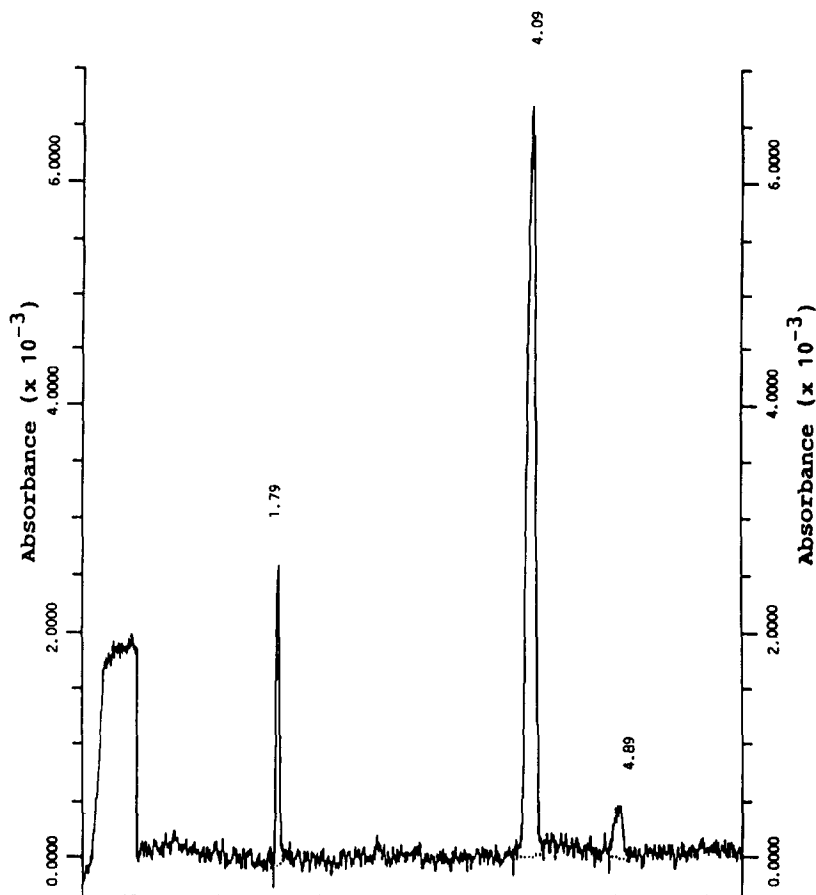


Fig. 3. Electropherogram of an aqueous solution of 1 g/l N-acetylcysteine. Migration order: acetanilide as marker of the endosmotic flow: 1.79 min, N-acetylcysteine: 4.09 min, N,N-diacetylcysteine: 4.89 min [4].

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

## Appendix

### Calculation of $sdv(x)$

The standard deviation of the analytical result is calculated by Eq. 1 [6,8,9]:

$$sdv(x) = \frac{sdv(y)}{\frac{\partial f}{\partial x}} \sqrt{\frac{1}{n_a} + \frac{1}{n_c} + \frac{(x_a - \bar{x}_c)^2}{S_{xx}}} \quad (1)$$

where

$$S_{xx} = \sum_{i=1}^{n_c} (x_i - \bar{x}_c)^2 \quad (2)$$

and

$$x_a = \frac{y_a - \bar{y}_c}{a_1} + \bar{x}_c \quad (3)$$

Thus  $x_a$  is the analytical result, estimated by the signal  $y_a$ . The value  $sdv(y)$  represents the standard deviation of the signal.  $\partial f/\partial x$  is the first derivative, that means the slope, of the calibration function  $f$ . The number  $n_a$  of measurements is used to obtain the mean  $y_a$  ( $n_a$  may

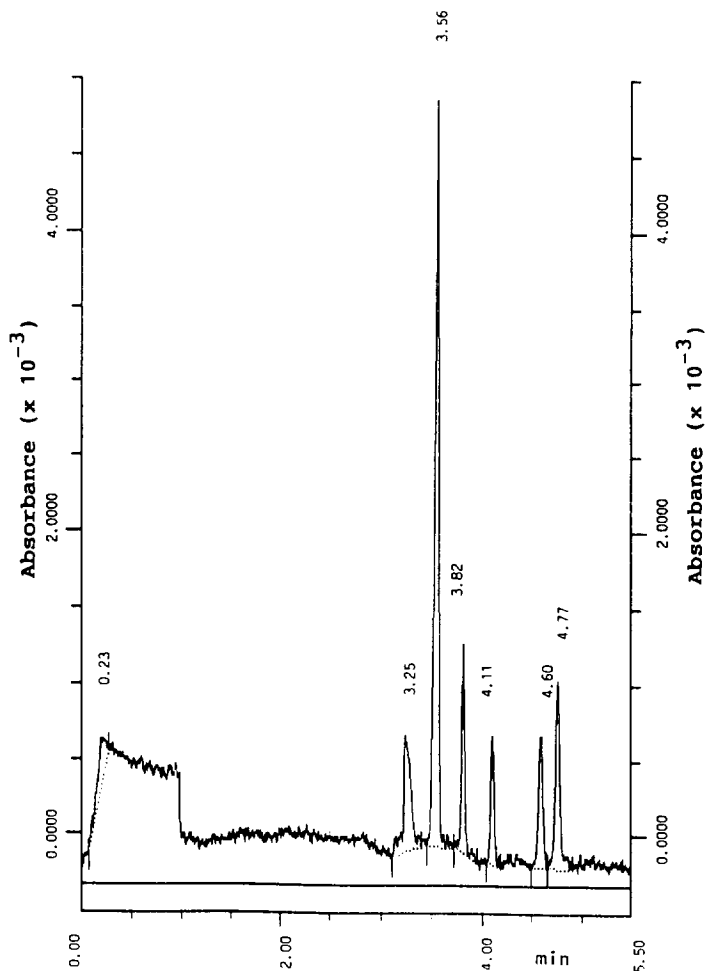


Fig. 4. Optimal separation of pyridine-dicarboxylic acids: citrate buffer pH 3.0, 60 mM. Sample concentration: 100 mg/l pyridine-2,4-dicarboxylic acid ( $t_M = 3.56$  min), 20 mg/l for the added isomers. Elution order: pyridine-2,6; -2,4; -2,5; -2,3; -3,4 and -3,5-carboxylic acids [5].

equal 1), the number of measurements used to estimate the calibration function is called  $n_c$ .

A simplified formula (Eq. 4) may be used, because the value of the square root is close to unity and the same for calibrations using peak heights or areas. The relative standard deviation  $\text{relsdv}(x)$  is easily obtained from  $\text{sdv}(x)$  (Eq. 5):

$$\text{sdv}(x) = \frac{\text{sdv}(y)}{\frac{\partial f}{\partial x}} \quad (4)$$

$$\text{relsdv}(x) = \frac{\text{sdv}(x)}{x_a} \quad (5)$$

The calculations for Table 1 are demonstrated in the following:

(1) Peak heights: the calibration function (Fig. 1) is:

$$f(x) = a_0 + a_1x + a_2x^2$$

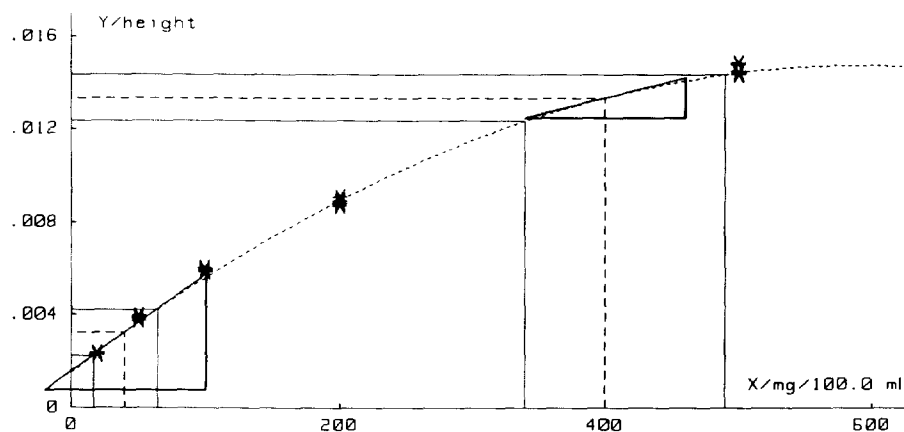


Fig. 5. The standard deviation of the analytical result [sdv(x)] is dependent on the standard deviation of the measurement [sdv(y)], but also on the sensitivity, that means the slope, of the calibration function. Same data as in Fig. 1 (top).

with  $a_0 = 1.5 \cdot 10^{-3}$ ,  $a_1 = 4.47 \cdot 10^{-6}$  l/mg and  $a_2 = -3.78 \cdot 10^{-10}$  l<sup>2</sup>/mg<sup>2</sup>. Hence:  $\partial f / \partial x = 4.47 \cdot 10^{-6}$  l/mg  $- 7.56 \cdot 10^{-10}$  l<sup>2</sup>/mg<sup>2</sup>  $\cdot x$  for  $x = 1004$  mg/l:  $\partial f / \partial x = 3.714 \cdot 10^{-6}$  l/mg, thus:

$$\text{sdv}(x) = \frac{\text{sdv}(y)}{\frac{\partial f}{\partial x}} = \frac{7.67 \cdot 10^{-5}}{3.714 \cdot 10^{-6} \text{ l/mg}} = 20.65 \text{ mg/l}$$

(2) Corrected peak areas: the calibration function is:

$$f(x) = a_0 + a_1 x$$

with  $a_0 = 0.2686$  and  $a_1 = 2.44 \cdot 10^{-3}$  l/mg. The first derivative is the slope of the calibration line, thus for  $x = 1004$  mg/l:

$$\text{sdv}(x) = \frac{\text{sdv}(y)}{\frac{\partial f}{\partial x}} = \frac{3.63 \cdot 10^{-2}}{2.44 \cdot 10^{-3} \text{ l/mg}} = 14.87 \text{ mg/l}$$

## Acknowledgements

The author wishes to thank Dr. C. Dette for his support.

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