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Precise quantitative capillary electrophoresis

Methodological and instrumental aspects

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ABSTRACT

The separation efficiency of capillary electrophoresis (CE) is said to be unrivalled by liquid chromatography for a number of applications. However, the quantitative precision, which is essential for its use in routine analysis, has been commented upon more critically. The reproducibility of CE is dependent on a number of parameters. The relative standard deviation is predominantly dependent on the absolute sample concentration. Its optimization is very important, and the use of higher concentrations is found to be more favourable. The problem of the non-linear relationship between concentration and peak data, caused by column overload, is less critical. The buffer concentrations are adjusted to the optimum sample concentrations. Validated buffer recipes and rinsing steps will lead to more stable conditions, and the trends that occur can be diminished by the stepwise use of an external standard. Much of the precision is dependent on instrumental aspects. Seven set-ups from different manufacturers were tested. Thermostating proved to be the most important parameter in achieving a reproducible dosage. Ion mobility and buffer viscosity are controlled by the temperature. These parameters influence the amount of sample that is injected by electrokinetic or hydrodynamic injection. The recording of temperature, current, voltage and power is necessary for proper documentation. Short capillaries should be usable for achieving a short analysis time and thus a large number of repetitions per unit time, which is important for statistical certainty.

INTRODUCTION

Over 2 years ago, Steuer and Grant [1] published a very interesting paper that reviewed important facts about capillary electrophoresis (CE) and gave an overview of several CE instruments. Although this paper still contains much up-to-date information, the emergence of new trends in the field of CE necessitates an update of CE instrumentation. Many improvements have been made during the last 2 years, and the importance of precise quantitative results has been increasingly placed in the foreground. Whilst CE is used as a routine method, e.g., in the pharmaceutical industry [2], these quantitative aspects will be stressed in this paper. Fundamentals and developments of CE instrumentation [1,3–9] and a more detailed description of instrumentation [10] can be found elsewhere. The first part of this paper briefly outlines method development in CE with the aim of obtaining precise analytical data. The second part demonstrates a very precise and reliable method for testing CE instruments. The description of some important technical details will help one to make a selection quickly.

EXPERIMENTAL

Instrumentation

Instruments from seven companies were tested at the authors' laboratory. Subsequently an enquiry form was designed for completion by the companies involved. Reported data on reproducibility and detection limits were processed by the companies by a standard procedure (see below) on reference samples which were sent to them. The standard proce-

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dure was developed on a P/ACE 2100 instrument (Beckman) [10].

Test procedure

The following standard method was sent to the companies. It describes the preparation of the buffer and sample solutions and how the experiments should be carried out. The reproducibility data given in Table I were provided by this procedure.

Borate buffer, 100 mM (pH 8.5). Boric acid (6.183 g) is dissolved in 100 ml of HPLC-grade water (Millipore, Eschborn, Germany). The pH of this solution is adjusted to 8.50 by addition of 1 *M* sodium hydroxide. This solution is diluted to 1 1 with water in a volumetric flask.

Sample solution. Approximately (±2 mg) 90 mg of acetaminophen (Degussa, Homburg, Germany), 150 mg of acetylsalicylic acid (Bayer, Leverkusen, Germany), 60 mg of 3.5-dihydroxybenzoic acid (Serva, Heidelberg, Germany) and 120 mg of nicotinic acid (Ciba, Wehr, Germany) are weighed into a 100-ml volumetric flask and dissolved in and diluted to volume with the above borate buffer. All other chemicals were of analytical-reagent grade, supplied by Merck (Darmstadt, Germany).

Capillary electrophoresis. The experiment is performed with a 50-cm (capillary inlet to detection window) fused-silica capillary of 75 μm I.D. (Beckman, Munich, Germany). The capillary is conditioned by rinsing with 0.1 A4 sodium hydroxide for 30 min, then it is filled with the buffer and equilibrated for another 2 h. During this time a voltage of 25 kV is applied.

The sample is injected under pressure or via vacuum. The product of pressure (or vacuum) and time should be about 17 250 Pa s (1000 Pa ≈ 0.145 psi). If the injection is hydrodynamic, the product of height difference and time should be about 172.5 cm

TABLE I

TECHNICAL DATA FOR CE INSTRUMENTS

For more details, see also refs. 1 and 10.

Characteristic		Favourable	Typical	Unfavourable
High voltage:				
Voltage range (kV)			0–30	
Capillary:				
Minimum length (cm)		17	30	40
Temperature control:				
Temperature range (°C)		15–60	20–50	None
Detection:				
Wavelength range (nm)		190–800	200-360	
Autosampler:				
Number of sample positions		80	40	None
Number of different buffers available[b]		80	4	1
Sample volume range (μl)		14500	10–1500	75–500
Reproducibility:'				
Repeatability (10 subsequent injections)	Height	0.5	0.7	1.0
	Area	0.7	0.9	1.2
Day-to-day precision (measurement during 2 days)	Height	0.8	1.5	2.4
	Area	1.6	2.0	2.4
Amount corresponding to the smallest detectable signal'		0.06	0.2	1.17
List price (DM)[d]		29 800	80 000	114600

[a] Inlet to detector cell.

[b] Sometimes only one at the capillary outlet.

[c] According to the standard test procedure described in the text, reproducibility in R.S.D., amount corresponding to the smallest detectable signal in mg l^{-1} 3,5-dihydroxybenzoic acid at pH 8.5.

[d] Winter 1992, without taxes and without computer ware.

s (1 cm ≈ 100 Pa). By this means the same amount will be injected with all instruments. The detection wavelength is set at 254 nm and the rise time at 1 s.

The length of time of the experiment is less than 10 min. The electropherogram is expected to show a pattern like that in Fig. 1. If not, the capillary should be treated again by rinsing with sodium hydroxide; possibly together with capillary heating, and equilibrated. Once the separation and the pattern in Fig. 1 have been established, the sample solution is injected several times until the migration times of five consecutive runs do not differ by more than 1%.

Repeatability. The sample solution is measured ten times. If an autosampler is used, it is crucial that the sample solution is injected from a different vial

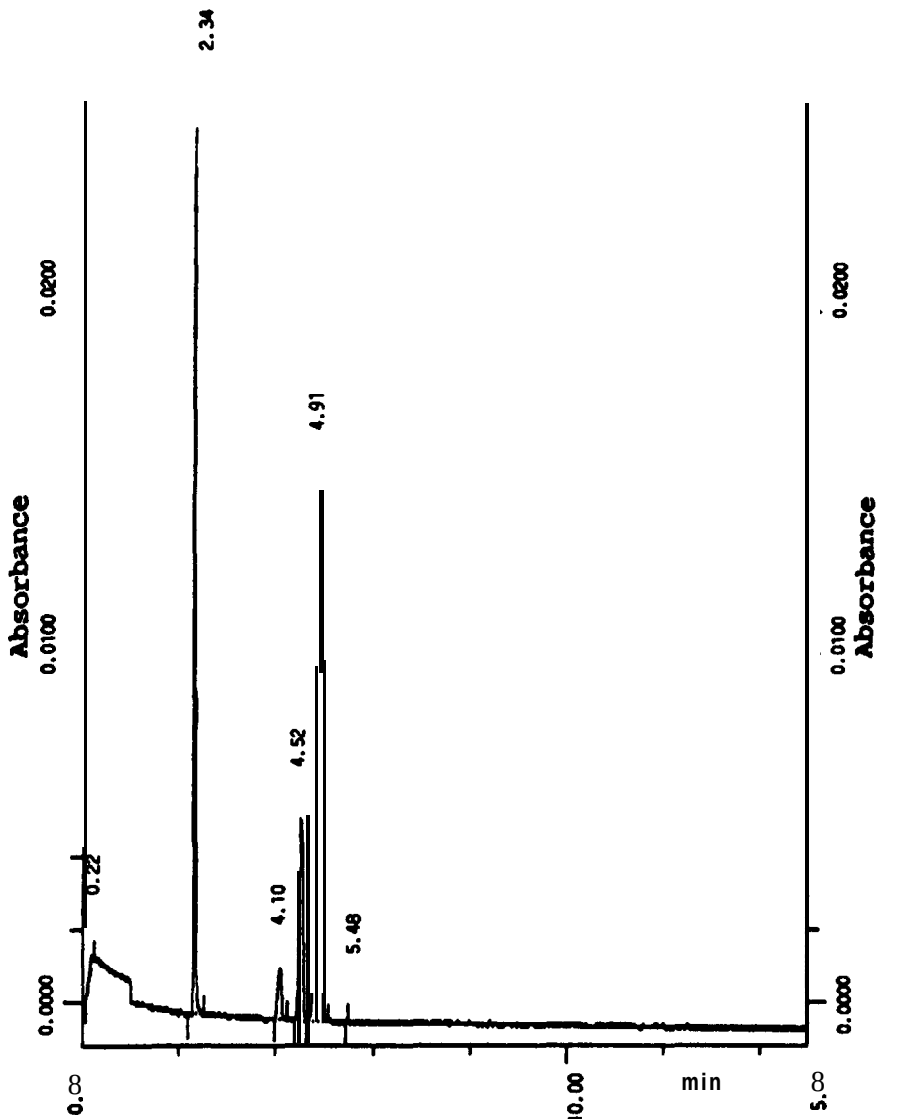

Fig. 1. Standard electropherogram. Elution order: acetaminophen (t_M = 2.34 min), nicotinic acid (4.10 min), acetylsalicylic acid (4.52 min), 3,5-dihydroxybenzoic acid (4.91 min). Wavelength, 214 nm; for other experimental conditions, see text.

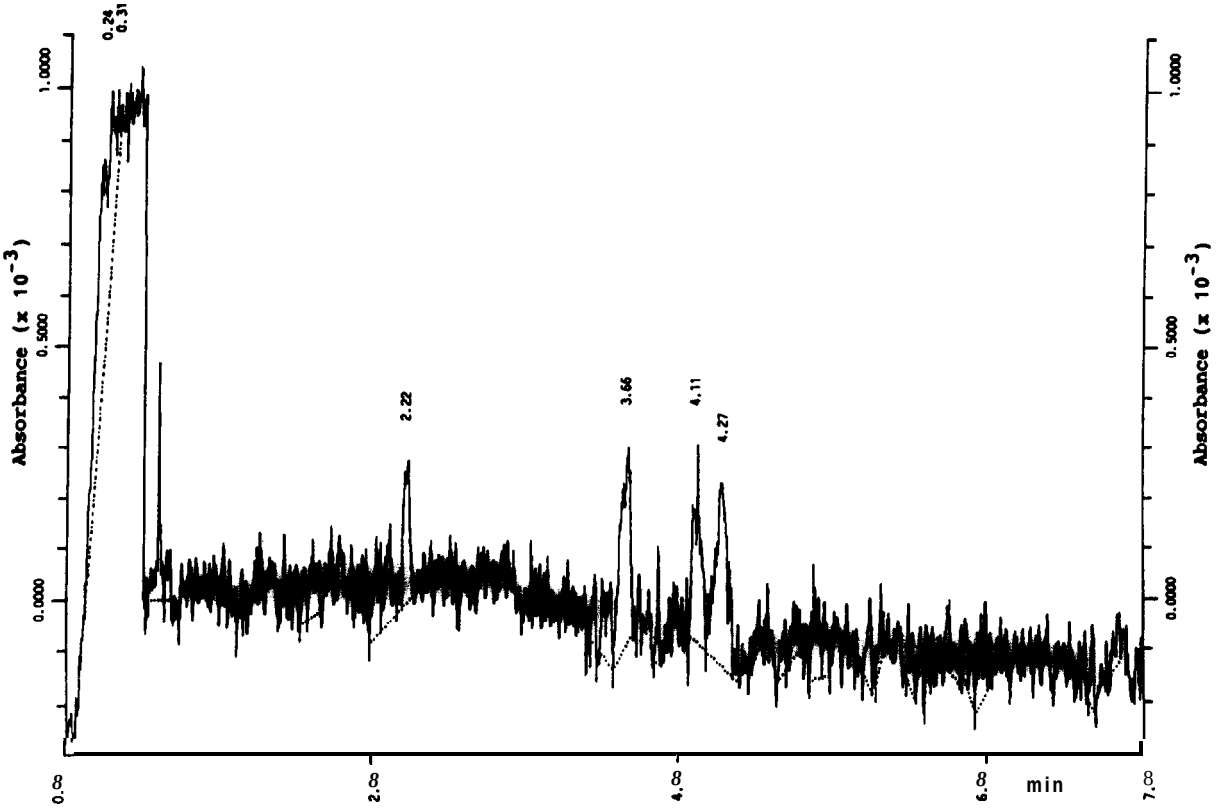

Fig. 2. Standard electropherogram for the calculation of the limit of detection. Elution order, acetaminophen (t_M = 2.22 min), nicotinic acid (3.66 min), acetylsalicylic acid (4.11 min), 3,5-dihydroxybenzoic acid (4.27 min). The lowest concentrated dilution should show four distinguishable peaks from the baseline, as given here. Wavelength, 200 nm.

each time. Before each run, the capillary is rinsed with running buffer until the capillary contents have been exchanged twice. The repeatability is reported as relative standard deviation of peak height and area. Instead of the area, the corrected area (area/migration time) may be used. This makes hardly any difference, because the variation in migration time is very small.

Day-to-day precision. During 48 h at least 60 samples are injected from a minimum of ten different sample vials. The relative standard deviations obtained correspond to the day-to-day precision.

Amount corresponding to the smallest detectable signal. Changing the conditions given above, the wavelength is 200 nm in the following experiment. The sample solution described above is diluted several-fold and the dilutions are injected. The amount corresponding to the smallest detectable signal is calculated from the lowest concentrated dilution. It is important that all four main peaks are distinguishable from the baseline noise (as in Fig. 2). The limit of detection is given as the concentration of 3,5-dihydroxybenzoic acid (α = 30.100 1 mol^{-1} cm^{-1} at 200 nm; this was determined in the CE buffer of pH 8.5) in mg l^{-1} and is calculated from the dilution steps.

RESULTS AND DISCUSSION

Quantitative CE: methodological aspects

Sample concentration is one of the most important parameters that can influence the reproducibility of CE experiments. It was found that the relative standard deviation of peak data depends on the

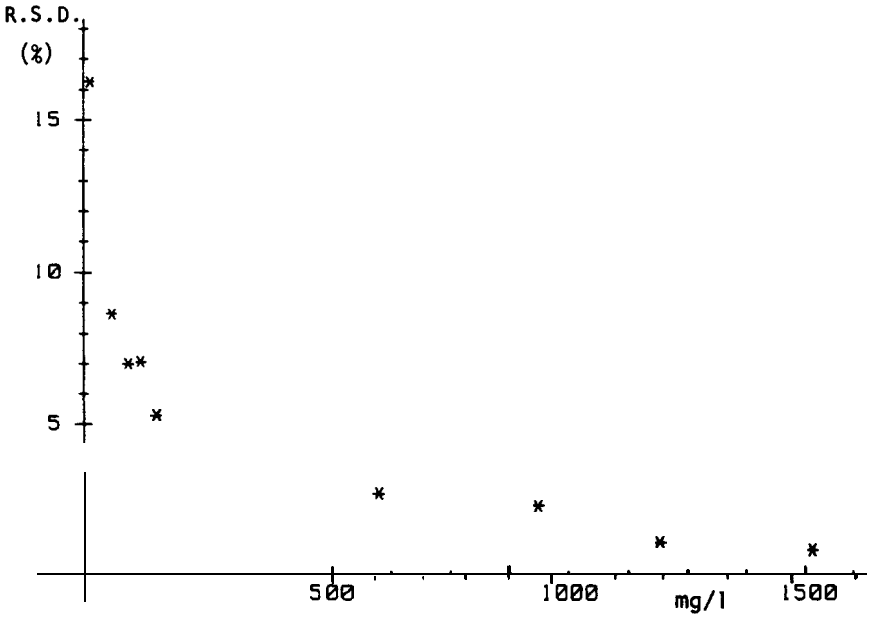

Fig. 3. Dependence of the relative standard deviation of peak height on sample concentration. Borate buffer (pH 8.5), 100 m*M*; benzoic acid sample was dissolved in this running buffer. Injection, 17 250 Pa s; separation in a 67 cm (effective length) × 75 μm I.D. fused-silica capillary; voltage, 25 kV; wavelength, 200 nm.

sample concentration to a large extent: the higher the concentration, the lower is the relative standard deviation (Fig. 3). In the meantime this rule has been confirmed for a number of different substances by CE. However, there are limitations to the use of high sample concentrations. Very high sample concentrations lead to column overload, and hence to a decrease in separation efficiency. This may cause overlapping of peaks and lead to incorrect results.

A more concentrated running buffer will cause a later occurring column overload, and thereby a higher sample concentration is possible. On the other hand, too high a buffer concentration may lead to an excess of thermal heating and thus thermal diffusion. Other methods of improving the reproducibility of quantitatively relevant peak data are to use buffer additives, which provide a better peak shape, e.g., by preventing capillary wall absorption, or optimization of the stacking conditions, if a high sample concentration cannot be used.

A large number of experiments are necessary to optimize these parameters. Twenty measurements for each experimental set-up are proposed for calculating the relative standard deviation. This number is needed in order to recognize trends in the data, and will lead to statistically relevant results. Although twenty measurements per series are used, relative standard deviations differing by less than the factor of 1.35 cannot be statistically distinguished. This uncertainty becomes more severe when smaller numbers of experiments are performed.

Note: if every experimental set-up needs twenty measurements, many of conditions are tested so that the optimum conditions can be found. Then a short duration of the analysis is required. This must be considered during the first steps of method development, and separation should be achieved within a short time.Therefore, the possibility of using short capillaries is a very helpful instrumental detail (see below).

Quantitative CE: instrumental aspects

Temperature control. The control of the capillary temperature is very important. First, the resistance of the buffer within the capillary leads to so-called Joule heating. This must be removed, otherwise overheating of the buffer inside the capillary could

lead to evaporation and thus to the breaking of the electrical circuit.

More important is the reproducibility of the dosage. If the sample is introduced into the capillary by pressure or vacuum, the injected volume V and hence the amount of sample can be described by the Hagen–Poiseuille rule [7]. The volumes injected do not depend only on the pressure difference Δp and the injection time t_i, but also on the column length L, the inner diameter d and the viscosity η of the liquid:

$$v = \frac{\Delta p \ \pi d^4 t_i}{128 \eta L} \quad (1)$$

These three parameters, however, are all dependent on temperature. Whereas widening of the capillary is less important because of the very small coefficient of expansion of silica [11], the changes in viscosity are enormous [12,13]. The viscosity of water is about 10% lower at 25°C than at 20°C. This can explain in part the bad long-term reproducibilities obtained with CE, whereas good values are reported in other instances (e.g., [13–18]).

Temperature changes between day and night will have a large influence. The (short-term) repeatability depends less on temperature and thus it is more acceptable. It is extremely important to have a constant temperature for all injections of a series of samples and standards. This can be obtained by using temperature-constant rooms or thermostated capillaries inside the instrument. During the injection there is no additional Joule heating, so the heat transport capacity of the system is of minor importance. However, the injection is the most important point for reproducibility. Therefore, it is a good idea to wait for a certain inner capillary temperature before starting the next injection.

It must be mentioned that a constant temperature is important for reproducible electrokinetic injections. The mobilities of the sample substances are temperature dependent. An average dependence of 2% per °C has been given [17,19]. This also affects the reproducibility of the peak areas A.

In contrast to chromatography, different samples do not pass the detector cell with the same velocity. Thus slower compounds will remain longer in the detection cell and give a longer response, resulting in larger peak areas. However, this effect can be compensated for by the use of corrected peak areas, cA [4], which again are proportional to the sample concentration [20,21]:

$$cA = A/t_M \quad (2)$$

A record of the temperature course would be an important feature to control and validate the experimental conditions. At least the temperature at the point of injection should be reported.

There is a benefit when heating the capillary to temperatures considerably above ambient. If the separations are irreproducible, the capillary can easily be re-equilibrated by rinsing with sodium hydroxide followed by rinsing with the CE buffer solution. The removal of troublesome substances with sodium hydroxide can be accelerated by heating the capillary. An increase in temperature can also be achieved by the application of a voltage to the sodium hydroxide-filled capillary.

Capillary. In CE it is advantageous to use capillaries with a short length L. These will give a very high field strength E (eqn. 3) and thus short migration times t_M (eqns. 4 and 5) [22] and a high separation efficiency, whilst the resolution remains almost the same [23]:

$$E = U/L \quad (3)$$

$$t_M = L/\mu \ E \quad (4)$$

$$t_M = L^2/\mu \ U \quad (5)$$

where μ includes both electroosmotic and electrophoretic mobility.

Some instruments are offered with capillary cartridges. If several capillaries are used at the same time, these become easier to handle. A cartridge can be changed more quickly than the whole capillary. However, the change of a capillary within the cartridge is typically more time consuming than installing the capillary in an instrument. Hence if one capillary is used after another it will be easier to work without cartridges.

Autosampler. Statistically reliable quantitative analysis needs many measured data and thus an autosampler. Of course, a large number of positions in the sampler tray is desirable, so that the analysis times in CE can be extremely short (typically less than 5 min). In some instruments operation occurs with only one buffer at the outlet end of the capillary. This is not troublesome in our experience. Un-

der extreme conditions the outlet buffer could be transformed by electrolysis. A resultant shift in pH would lead to altered separation conditions in this event.

Apart from this, there are two important features of autosamplers. First, it is important to cover the vials to prevent evaporation, otherwise the concentrations within the vials will increase during a longer sample time and give lower accuracy. An evaporation rate of 0.05 nl s^{-1} was given for sample volumes about 5 μl [17], which corresponds to more than 4 μl per day. However, evaporation can be slowed by keeping the micro-vials in a humid atmosphere. The relatively lower but absolutely higher evaporation from larger sample volumes makes it necessary to prevent it as much as possible. Second, it is advantageous if the sampler tray can be cooled and thermostated. Various samples in CE applications, such as proteins and peptides, are unstable. Thermostating of the sampler tray to higher temperatures than ambient may be sensible to observe reactions, e.g., to determine the decay of unstable substances or the kinetics of enzymatic reactions.

Although only fairly small amounts can be obtained with fraction collectors, they are often sufficient for carrying out another analytical technique, e.g., off-line mass spectrometry or DNA sequencing (e.g., [24]), electron microscopic analysis of protein conformations [25] and a blotting technique followed by an immunological reaction [26]. The combination with a polymerase chain reaction also seems useful.

High voltage. The possibility of working with constant power or constant current instead of constant voltage is an interesting feature. High buffer concentrations and thus high electrical power should be used. In this instance the Joule heating causes a significant decrease in the buffer resistance. If the voltage is kept constant, the current will increase, and also the power, leading to additional heating. If the current is kept constant, the voltage will decrease owing to the loss of buffer resistance and a negative feed-back will result, which is easier to handle. All instruments can be used in reverse voltage, *i.e.*, with the anode at the buffer outlet. The only difference is the ease of switching the pole. The recording of power, current and voltage is also an important feature in controlling the stability of the experimental conditions. This is necessary for proper validation and in the case of failed experiments.

Other important aspects. All pressure or vacuum injection systems manage to produce reproducible pressure differences and thus precise quantitative results. This can be seen from the good repeatabilities obtained with all instruments (see Table I).

Multi-wavelength detection has the same benefits in CE as in LC: sample identification and validation of peak purity by spectral information (*e.g.*, refs. 27-3 1 and references cited therein). The use of laser fluorescence detectors, now commercially available, promises the advantages of better selectivity and much lower limits of detection for CE [32–36].

CONCLUSIONS

Comparison of quantitative results

The repeatability indicates the deviations of the injected amount due to pressure changes in the injection system or different injection vials. This is more realistic than repeated injection from only one vial. Pressure differences may be due to differences in placement of the vial caps or filling levels. The day-to-day precision describes long-term changes. For example, these changes are caused by evaporation of the sample solution or temperature changes. Neither measurements consider variations due to dilution, weighing or other influences. To summarize, it may be stated that the precision is very acceptable with most instruments and much better than reported elsewhere. This is because a high sample concentration was used.

The standard deviation for a random sample can differ considerably from the value for the entirety, as can be seen from Table I. There is a rather large scatter in this data. The F-test for the comparison of variances and standard deviations cannot distinguish between differences less than the factor 1.2 ($F_{60,60;0.1} = 1.4$ [37]; $\sqrt{1.4} \approx 1.2$). This must be considered when comparing the precision data achieved with different instruments.

According to these comments, it is obvious that there is hardly any difference, if any, in the repeatability data. The precision of the injection itself is very reliable for all instruments. However, there are differences in the day-to-day precision to some extent. Possibly these are caused by the different abilities of the instruments to keep the capillary temperature constant during the moment of injection (see

above). The amount corresponding to the smallest detectable signal depends strongly on the experimental conditions, e.g., the detection wavelength. Hence, because there were typically only minor differences between several instruments, it seems sensible to test this parameter under experimental conditions of particular interest.

Final remarks

All the CE instruments tested showed a very high technical standard. They are distinguisable from each other by various features that are important for individual purposes. The precision of the quantitative results is very acceptable. Hence this method can replace LC in a number of application fields, especially for peptides, proteins, DNA, amines and a number of other polar substances. New developments, e.g., MS interfaces, laser fluorescence detection and thermostated autosampler trays, will lead to the wider application of this efficient method.

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REFERENCES

1 W. Steuer and I. Grant, *Nachr. Chem. Tech. Lab., 38 (1990)* M1.
2 D. M. Goodall, S. J. Williams and D. K. Lloyd, ***Trends Anal. Chem.,*** 10 (1991) 272.
3 J. Snopek, I. Jelinek and E. Smolková-Keulemansová, *J. Chromatogr., 452 (1988) 571.*
4 A. G. Ewing, R. A. Wallingford and T. M. Olefirowicz, ***Anal.*** Chem., 61 (1989) 292A.
5 R. A. Wallingford and A. G. Ewing, ***Adv. Chromatogr., 29 (1989)*** 1.
6 W. G. Kuhr, ***Anal. Chem., 62*** (1990) 403R.
7 D. M. Goodall, D. K. Lloyd and S. J. Williams, *LC* GC, 3 (1990) 28.
8 D. Perrett, G. Ross and D. M. Goodall, ***Capillary Electrophoresis: a Bibliography,*** Chromatographic Society, Nottingham, 1991.
9 W. G. Kuhr and C. A. Monnig, ***Anal.*** Chem., 64 (1992) 389R.
10 H. Wätzig and C. Dette, ***Fresenius' Z. Anal. Chem.,*** in press.
11 H. Remy, ***Lehrbuch der Anorgamschen Chemie,*** Vol. I, Akademische Verlagsgesellschaft, Leipzig, 13th ed., 1970, p. 602.
12 H. Remy, ***Lehrbuch der Anorganischen Chemie,*** Vol. I, Akademische Verlagsgesellschaft, Leipzig, 13th ed., 1970, p. 62.
13 D. S. Burgi, K. Salomon and R.-L. Chien, ***J. Liq. Chromatogr., 14 (1991) 847.***
14 K. Otsuka, S. Terabe and T. Ando, ***J. Chromatogr., 396 (1987) 350.***
15 ***S.*** Fujiwara and S. Honda, ***Anal.*** Chem., 59 (1987) 2773.
16 R. G. Nielsen and E. C. Rickard, ***Analytical Biotechnology: Capillary Electrophoresis and Chromatography (ACS Symp. Ser., No. 434),*** American Chemical Society, Washington, DC, 1990, p. 36.
17 S. E. Moring, J.C. Colburn, P. D. Grossman and H. H. Lauer, *LC* CC, 8 (1990) 34.
18 D. J. Rose, Jr. and J. W. Jorgenson, ***Anal.*** Chem., 60 (1988) 642.
19 S. Hjerten, ***Chromatogr. Rev., 9 (1967) 122.***
20 ***X.*** Huang, W. J. Coleman and R. N. Zare, ***J. Chromatogr., 480 (1989) 95.***
21 H. Wätzig and C. Dette, ***Pharmazie,*** in press.
22 J. W. Jorgenson and K. D. Lukacs, ***J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 230.***
23 J. W. Jorgenson and K. D. Lukacs, ***Anal.*** Chem., 53 (1981) 1298.
24 G. M. Harthaway, ***Biosyst. Rep., 17 (1992) 3.***
25 A. B. Hodgson, A. W. Robards and D. M. Goodall, ***Electron Microsc., 3 (1992) 69.***
26 K.-O. Eriksson, A. Palm and S. Hjertén, ***Anal. Biochem., 201 (1992) 211.***
27 S. Ebel, W. Miick and A. Werner-Busse, ***Fresenius' Z. Anal.*** Chem., 327 (1987) 794.
28 S. Ebel and W. Mück, *Fresenius'* ***Z. Anal.*** Chem., 331 (1988) 351.
29 S. Ebel and W. Mück, ***Fresenius' Z. Anal.*** Chem., 331 (1988) 359.
30 S. Ebel and W. Miick, ***Chromatographia, 25 (1988) 1039.***
31 ***S.*** Ebel and W. Mück, ***Chromatographia, 25 (1988) 1075.***
32 A. A. A. M. van de Goor, B. J. Wanders and W. J. Henzel, ***Technical Information Bulletin DS-824***, Beckman Instruments, Palo Alto, CA, 1992.
33 B. J. Wanders and A. A. A. M. van de Goor, ***Technical Information Bulletin DS-826,*** Beckman Instruments, Palo Alto, CA, 1992.
34 S. Wu and N. J. Dovichi, ***J. Chromatogr., 480 (1989) 141.***
35 ***C.*** Toulas and L. Hernandez, *LC* GC, 5 (1992) 27.
36 L. Hernandez, J. Escalona, N. Joshi and N. Guzman, ***J. Chromatogr., 559 (1991) 183.***
37 L. Sachs, ***Angewandte Statistik,*** Springer, Berlin, Heidelberg, 6th ed., 1984.