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van der Moolen, J.N.; Louwerse, D.J.; Poppe, H.; Smit, H.C.

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Correlation Capillary Zone Electrophoresis, a Novel Technique to Decrease Detection Limits

J. N. van der Moolen / D. J. Louwerse / H. Poppe / H. C. Smit*

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

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Summary
The application of multiple injections and correlation techniques in capillary zone electrophoresis, a method known for its high detection limits, is described. An experimental electrokinetic injection device, particularly suited for fast and reproducible multiple injections is used in combination with a conventional CZE system. The results show a considerable reduction of the detection limit, in agreement with the experience in the already fully developed correlation chromatographic methods, and in agreement with the theoretical predicted values.

Introduction
Capillary zone electrophoresis (CZE) allows high efficient separations of charged molecules in a short time. For this reason the popularity of this separation technique has increased drastically since the introduction around 1980 by Mikkers et al. [1] and Jorgenson and Lukacs [2].

In CZE, sample ions migrate as narrow zones through a background electrolyte (BGE) in a capillary under influence of an electric field. Sample ions with different charge and/or size migrate with different velocities. On-column UV-detection is most commonly used in CZE. One of the main restrictions is the high detection limit, in the 10^{-5}–10^{-6} mol/l range [3], as a result of the short available light path, the inner diameter of the capillary. Capillaries with diameters of 10–100 µm I.D. are used in order to make broadening of the sample zone caused by a temperature gradient across the capillary [2] negligible. This temperature gradient comes into being during the electrophoresis because heat is uniformly produced in the solution by the electrical current through the capillary, but can dissipate only through the capillary wall.

Besides the small diameter of the capillary, also the conductivity of the BGE has an upper limit because of this temperature effect. To prevent distortion of the electric field, the sample concentration has to be low compared to the BGE concentration: with increasing concentration, the sample causes significant changes in the local conductivity and pH, which lead to broadened, triangular shaped peaks [4]. This phenomenon, also known as concentration overloading, combined with the high detection limit results in a small linear range and constitutes a limit to the signal-to-noise (S/N) ratio.

Smit [5] established in 1970 the use of correlation techniques for the improvement of the S/N ratio in chromatographic trace analysis. This S/N ratio improvement is also known as the multiplex advantage. Recently it was demonstrated that the use of correlation techniques shows better results in comparison with pre-concentration for head-space GC [6]. This suggests the application of the correlation techniques in CZE, resulting in correlation capillary zone electrophoresis (CCZE). In this article a CCZE system will be introduced. It will be shown that the detection limit of CCZE can be improved considerably in comparison with conventional CZE.

Theory
Both the theory of CZE [1–4, 22, 28, 29] and the correlation technique have been extensively treated in literature. CCZE, however, is a new technique, so it appears to be useful to give a brief introduction to this chemometric technique for readers not familiar with it. The application of correlation techniques in chromatography for process control has been introduced in 1967 by Izawa et al. [7]. Instead of a single injection, multiple injections are applied semi-continuously according to a pseudo random binary sequence (PRBS).
The detector signal is a summation of all (noise-free) chromatograms plus (detector-) noise, so the signal increases while the noise level does not change. One of the possibilities to interpret the detector signal is to cross-correlate it with the input signal (the PRBS), what results in a so-called correlogram. A correlogram is comparable with the chromatogram but with an improved S/N ratio. Because in CCZE sample is injected while previous injected sample is eluting, this S/N enhancement is reached in less time than when ensemble averaging [8–13] of the electropherograms would have been used.

The main problem in correlation chromatography is the high demand on the injection system. Reproducible and fast injections over a long time are essential. Non-ideal injections lead to ghost peaks and in general to correlations noise [13, 14]. In CCZE, it is relatively easy to make changes to the injection system, because CZE is electrically rather than pressure driven. Moreover an electrical switch is faster than an air-actuated valve which is nowadays used in correlation HPLC [13].

Assuming linearity and stationarity, the digitized detector signal \( y(i) \) of \( g \) points can be described as the circular convolution of the discrete input signal \( x(i) \) and the discrete impulse response \( h(j) \) of the electrophoretic system, plus detector noise \( n(i) \):

\[
y(i) = \sum_{j=0}^{g-1} x(i-j) \cdot h(j) + n(i)
\]

which is abbreviated to \( y = x \ast h + n \).

The detector noise is assumed here as to be white noise with variance \( \sigma_n^2 \), and mean \( E[n(i)] = 0 \) (\( E \) is the expected value).

In conventional CZE the input signal is a single injection, represented as a block \( b_w \) [15] of \( w \) points wide and value 1, which is completed with zero’s to \( g \) points (Figure 1a). The electropherogram is the result of the convolution of the block function with the impulse response:

\[
y = b_w \ast h + n
\]

The input function often used in correlation chromatography is a PRBS [5, 9, 11]. A PRBS resembles a type of binary white noise. The two levels of the PRBS are 1 (corresponding to a sample injection) and 0. It has a specific length of \( M = 2^m - 1 \) (\( m \) is an integer > 1) periods of width \( w \), controlled by a clock (Figure 1b). The clock period (CP) has to equal the width of the single injection to allow a good comparison between CZE and CCZE. The sequence length (\( w \cdot M \) points) has to be equal to or longer than the electropherogram length of a single injection between the less retained and the most retained component. For simplicity, it is assumed to have a length of \( g \) points, the length of the electropherogram (\( g = w \cdot M \) points). After the first sequence, the so-called pre-sequence, the noise-free detector signal becomes circular. This pre-sequence cannot be used for the correlation procedure, so at least two sequences are necessary.

Direct interpretation of the detector signal is not possible because it consists of a sum of shifted responses of the multiple injections. For interpretation a deconvolution procedure is required. Instead of a PRBS, a random binary sequence has been used as input signal [16, 17]. Deconvolution can be achieved by Fourier transformation. Due to the special properties of the PRBS, cross-correlation between the detector signal and the PRBS can be used instead of Fourier
Cross-correlation is preferred for simplicity [18]. The result is a so-called correlogram $h_{cc}$. Circular cross-correlation can be defined as

$$h_{cc}(k) = \sum_{i=0}^{g-1} p(k+i) \cdot y(i)$$

which is abbreviated to $h_{cc} = p \otimes y$.

For $w = 1$, the result of the cross-correlation is

$$h_{cc} = p \otimes y = p \otimes [p \ast h + n] = [p \otimes p] \ast h + p \otimes n = b_w \ast h + p \otimes n$$

It has been shown [19] that a Gaussian function can be described if the distance between two sample points is equal to (or smaller than) the standard deviation $\sigma$ of the peak. If the width of an injection is small compared to the standard deviation, a width of one point ($w = 1$) will be sufficient, because then the distance between two sample points is smaller than the standard deviation of the peak. However, in CZE the width of the (single) injection often is not small compared to the peak broadening in the capillary. The comparison between CCZE and CZE requires that the width of the CP equals the width of the single injection, so one point per CP is not enough. For more than one point per CP ($w > 1$) cross-correlation results in a correlogram with a broadened peak compared to a single injection. To get a correlogram which is comparable with the electropherogram ($b_w \ast h$), cross-correlation between the detector signal and a signal $p_k(i)$ is necessary [15]; the first point of every CP of $p_k(i)$ has a value of $\pm w \cdot M/I$, the other points are 0 (Figure 1c).

The number of injections $I$ is $(M+1)/2$. The result is

$$h_{cc} = p_k \otimes n = p_k \otimes [p \ast h + n] = [p_k \otimes p] \ast h + p_k \otimes n$$

An elaborated explanation of the procedures for the deconvolution of $y(i)$ can be found in literature [15, 20, 21].

The cross-correlation between $p_k(i)$ and $n(i)$ in Eq. (5) describes the noise contribution to the correlogram:

$$p_k \otimes n = \sum_{i=0}^{g-1} p_k(k+i) \cdot n(i)$$

Keeping in mind that the expected value operator $E$ is additive, and recalling that the mean of $n(i)$ is assumed to be zero, it can be derived easily that the mean of this noise contribution to the correlogram equals zero:

$$E \left[ \sum_{i=0}^{g-1} p_k(k+i) \cdot n(i) \right] = E \left[ \sum_{i=0}^{g-1} p_k(k+i) \cdot E[n(i)] \right] = 0$$

The variance of the noise in the correlogram ($\sigma^2_{n, cc}$) is reduced compared to the variance of the (white) noise in the electropherogram $\sigma_n$. In the next equation the variance is symbolized as var:

$$\text{var} \left[ \sum_{i=0}^{g-1} p_k(k+i) \cdot n(i) \right] = \sum_{i=0}^{g-1} \text{var}[p_k(k+i) \cdot n(i)]$$

$$= \sum_{i=0}^{g-1} \text{var}[p_k(k+i)] \cdot \text{var}[n(i)]$$

Only the first point of every CP of $p_k(i)$ has a value unequal to zero, i.e. $+g/I$ or $-g/I$. For both values the square is $(g/I)^2$, so Eq. (8) becomes:

$$\frac{1}{g^2} \sum_{i=0}^{g-1} p_k(k+i) \cdot \sigma_n^2 = \frac{1}{g^2} \sum_{i=0}^{M-1} \left( \frac{g}{M} \right)^2 \sigma_n^2$$

$$= \frac{1}{g^2} \left( \frac{M \cdot g^2}{l^2} \right) \sigma_n^2$$

Now the relation between the sigma of the noise in the correlogram ($\sigma_{n, cc}$) and the sigma of the noise in the electropherogram ($\sigma_n$) can be expressed as

$$\sigma_{n, cc} = \frac{\sqrt{M}}{l} \cdot \sigma_n = \frac{\sqrt{M}}{1/2 \cdot M} \cdot \sigma_n$$

It is being concluded that the signal-to-noise ratio, as well as the detection limit, improves with a factor of $1/\sqrt{M}$.

For one point per CP, the same (theoretical) improvement of the signal-to-noise ratio has been deduced by Louwerse et al. [11].

**Experimental**

The system used for CCZE (Figure 2) is based on a home-made CZE system. The CZE system consists of a 75 $\mu$m I.D., 375 $\mu$m O.D. fused silica capillary, a high-voltage power supply, platinum electrodes in vials for sample and buffer (BGE) at both sides of the capillary, an UV-detector, and a recorder for current monitoring over a 10 kOhm resistance (Table I). The capillary, the electrodes and the vials, and the CZE-cell of the detector are placed in a safety box at a constant temperature (25–26 °C). Some modifications are necessary to be able to perform CCZE:
1) A high-voltage switch (Figure 3) is added. The switch includes a relay, a battery and electronics for controlling the relay, electronics for computer control, and a photodiode with a glass fibre to separate physically the high-voltage source from the computer electronics.

2) A microcomputer is used to control the injection procedure by means of the high-voltage switch, and to acquire the data obtained from the UV-detector. For this purpose also a PC-based system can be used as will be outlined in a future publication.

3) In order to make it possible to inject with short time-intervals, a connection between the capillary and both the sample and the buffer vial is made (Figure 2). The capillary is glued in a glass rod in which a hole of 30 μm was drilled perpendicular to the capillary. Two vials have been connected to this rod.

A 0.03 mol/l phosphate buffer (obtained from Merck) at pH 6.01 is used as BGE. Benzyl-trimethyl- ammonium-chloride (BTMA, obtained from Aldrich) and benzyl-tri-ethyl-ammonium-chloride (BTEA, obtained from Merck), dissolved in the BGE, are used as sample. The buffer as well as the sample solutions are filtered through a 0.45 μm filter. The capillary is rinsed every morning with sodium hydroxide.

With CCZE multiple injections are done, but the electric field has to be maintained during the injection procedure to prevent distortion of the separation of a previous injection. For that reason electro-kinetic injections are preferred, with the voltage of injection equal to the voltage of separation. The electrophoretic conditions are listed in Table II.

The data handling includes correction for linear drift of the detector signal, cross-correlation between the detector signal and the signal $P_k(i)$, and determination of the S/N ratio. To correct for the linear drift a manually determined straight line through the points has been subtracted from the detector signal. The cross-correlation procedure is done by a custom written program.

A common definition of the S/N ratio in separation methods is the ratio of the peak height and the standard deviation of the noise. Another possible definition is the ratio of the peak area and the standard deviation of the integrated noise. When using the peak area, it is possible to correct for the different velocities of different zones, by dividing the peak area by the migration time [22]. Nevertheless, the choice of the ratio of the peak height and the standard deviation of the noise will be adequate because of the aim of this article, the comparison between the two techniques, using experiments with only one component. Besides, the use of the ratio of the peak area and the standard deviation of the integrated noise is more complicated [23]. To determine the peak heights, a fitting procedure was applied to the total correlogram, respective electropherogram, using a non-linear regression software.
package with a second order base-line polynomial and a Fraser-Suzuki peak model [24]. Noise levels, expressed as standard deviations, were calculated from the entire baseline part of the correlogram c.q. the electropherogram.

Results and Discussion

The main goal of this work is to improve the S/N ratio. A correct comparison between CCZE and CZE is required. For simplification, experiments are done with only one component, because for this research the number of components is of less importance.

A typical detector signal corresponding to the second sequence of a PRBS injection of $10^{-5}$ mol/l BTMA is shown in Figure 4a. The (circular) PRBS sequence of 127 CP (Figure 4b) is recognizable, starting with the peak at approximately 610 s. The result of the cross-correlation (the correlogram) between this detector signal and the signal $p_k (i)$, is shown in Figure 5. With the same conditions an electropherogram of $10^{-5}$ mol/l BTMA has been obtained (Figure 6). As has been proved in the theory, a direct comparison between the correlogram and the electropherogram is allowed.

To compare the results of CCZE and conventional CZE, the S/N ratio of the correlogram as well as the electropherogram of $10^{-5}$ mol/l BTMA have been determined. The results are summarized in Table III. As was shown in the theory section, cross-correlation between the detector signal and the signal $p_k (i)$ should result in a peak identical to the peak of a single injection. However, the peak in the correlogram (M.I.) is broadened compared with the peak in the electropherogram (S.I.). There is no explanation for this difference yet, but a study is in progress.

To show the possibilities of CCZE, the peak height ($H$) in the correlogram (M.I.) has been corrected ($H'$) for the difference in standard deviation ($\sigma$). Assuming a Gaussian peak, the area $A$ is a constant:

$$ A = \sqrt{2 \cdot \pi} \cdot \sigma_{\text{M.I.}} \cdot H = \sqrt{2 \cdot \pi} \cdot \sigma_{\text{S.I.}} \cdot H' \quad (11) $$

$$ H' = H \cdot \frac{\sigma_{\text{M.I.}}}{\sigma_{\text{S.I.}}} \quad (12) $$
The improvement of the S/N ratio by using the correlation technique is approximately 5.3 after the correction. This improvement has been achieved in two sequences, twice the time of a conventional electropherogram. It is being reminded that the presequence cannot be used for the correlation procedure. Signal averaging would require at least 28 experiments with conventional CZE to achieve the same improvement. When the length of the electropherogram is longer than the length between the less retained and the most retained component, the improvement by using the correlation technique can be reached in less than twice the time of a conventional electropherogram.

According to the theory it should be possible with a PRBS of 127 CP and 64 injections to obtain an improvement of the signal-to-noise ratio of:

\[ \frac{I}{\sqrt{M}} = \frac{64}{\sqrt{127}} = 5.7 \]

This theoretical improvement has been deduced assuming linearity, stationarity and the presence of white noise. It is not yet clear how this improvement is affected by the presence of non-white noise (this is more or less correlated noise, e.g. low frequency noise [25]) on the detector signal, but this is going to be studied in more detail. Linearity and stationarity of the system are very important in CCZE. The fact that CZE is linear in only a small concentration range and an influence between successive injections can cause non-linearities in CCZE. Variations in current or temperature can cause non-stationarities. Both non-linearities and non-stationarities result in extra baseline noise in the correlogram. This noise is generally called correlation noise and has been extensively discussed in literature [8, 14, 20, 26]. A special kind of correlation noise are ghost peaks. Ghost peaks are images of the real peak and are the result of an non-ideal injection pattern, while the deconvolution procedure is based on an ideal pattern [13, 15, 27]. Their position is characteristic for their origin and can be predicted theoretically [14]. As seen in Figure 5, no ghost peaks can be distinguished. This indicates that the injection system used does not cause large injection errors.

To study the potential possibilities of the CCZE system for separations of components, some experiments have been done with a separation of BTMA and BTEA. A resulting correlogram is shown in Figure 7, the corresponding electropherogram in Figure 8. Although the total number of points in the figures differ, the improvement in the S/N ratio is clearly visible. If the noise in Figure 7 is compared to the noise in Figure 5, it might be noticed that there is a large amount of correlation noise in Figure 5. The reason for this difference in noise will be studied.

The quality of the injection system can be determined by measuring the reproducibility of the injections. In general, the reproducibility of electro-kinetic injections is worse than that of hydro-dynamic injections [28].
With conventional CZE, injections are done by replacing the buffer vial by the sample vial and putting the high-voltage on the desired value. The major difficulty is to reproduce the duration of the injection [29]. With the injection system described in this publication, the main part of the separation capillary remains at the same voltage during the injection procedure, because it is not allowed to influence the separation of previous injections. Due to this, the reproducibility of the electro-kinetic injections is probably better than with the conventional injection procedure. Several experiments showed a peak area reproducibility better than 2% relative standard deviation, but some more experiments have to be done. A study of the possibility of a hydrodynamic injection device for CCZE is in progress.

Conclusion
Correlation techniques are useful in CZE. In contrast to the application of correlation techniques in chromatography, injection according to a PRBS in CCZE is simple in principle: it can be accomplished by an electrical switch. In chromatography it presents some experimental problems to switch a gas or liquid flow. The injection device described in this publication showed a good peak area reproducibility with electro-kinetic injections, and no ghost peaks can be distinguished.

As has been shown, it is possible to improve the S/N ratio, and as a result, to obtain a lower detection limit. An improvement of approximately 5.3 has been established by experiments with one component. The improvement is at the expense of time, but signal averaging would require much more experiments with conventional CZE to achieve the same enhancement.

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