# Effect of sample stacking on resolution, calibration graphs and pH in capillary zone electrophoresis

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## ABSTRACT

For the determination of components present in samples at very low concentrations, large injection volumes have to be applied in order to introduce a detectable amount of the analytes in capillary zone electrophoresis (CZE). To obtain a good resolution, the sample analytes have to be concentrated in narrow bands and therefore sample stacking is often applied. Sample stacking can lead to an increase in the electroosmotic flow and extra peak broadening during the analysis, through which the gain in resolution will be lost. Further, the presence of different electrolytes in the capillary can cause pH shifts. In this paper a model is given for the calculation of migration times of components applying sample stacking, and the effects of sample stacking in CZE on resolution, calibration graphs and pH are discussed

## INTRODUCTION

For the determination of components by capillary zone electrophoresis (CZE) [1,2], the components must be separated from their matrix and reliable linear calibration graphs with low detection limits must be obtained. For low detection limits, especially for components present in samples at low concentrations, large sample volumes have to be injected. In such a case, sample components must be concentrated, however, in order to obtain a high resolution and adequate detection. For this reason, sample stacking is often applied, *i.e.*, the sample is introduced at low ionic strength compared with that of the background electrolyte. Through the high local electric field strength, sample components migrate very quickly from the sampling zone, stack down in the background electrolyte owing to the much lower electric field strength and concentrate in very short sample zones. During the sample stacking procedure several interesting phenomena

occur. Huang and Ohms [3] discussed the effect of a non-uniform electrical field on the migration behaviour of different sample ions and the effect on sample injection in electrokinetic injection. Chien and Helmer [4] reported on electroosmotic properties and peak broadening in field-amplified capillary electrophoresis owing to a mismatch between the electroosmotic flow (EOF) in the sampling zone and background electrolyte in the capillary and concluded that the broadening mechanism will be a limiting factor for sample stacking in CZE. Burgi and Chien [5–7] found the preparation of a sample dissolved in a tenfold diluted background electrolyte to be an optimum condition for sample stacking and discussed field-amplified sample injection in CZE. No attention was paid to possible pH shifts due to the presence of different electrolytes in the capillary.

As a high electroosmotic flow leads to short analysis times, a low resolution can be the result and the linear character of the calibration graphs vanishes. In this paper the effect of sample stacking on resolution, calibration graphs and pH shifts with the injection of long sampling zones at low concentrations is considered.

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## EXPERIMENTAL

For all CZE experiments a P/ACE System 2000 HPCE instrument (Beckman, Palo Alto, CA, USA) was used. All experiments were carried out applying a Beckman eCAP capillary tubing (75 $\mu$m I.D.) with a total length of 46.7 cm and a distance between injection and detection of 40.0 cm. The wavelength of the UV detector was set at 214 nm. All experiments were carried out with application of a constant voltage of 10 kV and the operating temperature was 25°C. Sample introduction was performed by applying pressure injection where a 1-s pressure injection equals an injected amount of *ca.* 6 nl and an injected length of 0.136 cm. Data analysis was performed using the laboratory-written data analysis program CAESAR. These conditions were also used in all calculations.

## RESULTS AND DISCUSSION

### Effect of sample stacking on resolution

In sample stacking a long sampling zone at low ionic strength compared with that of the background electrolyte is introduced in a zone electrophoretic system, through which the local electric field strength will be very high compared with that of the background electrolyte. In Fig. 1a the capillary is filled to a length *l* with a sample dissolved in diluted background electrolyte S. The capillary length is $L_C$ and the length to the detector is $L_D$. If $E_S$, $\rho_S$, $m_{EOF,S}$, $E_B$, $\rho_B$ and $m_{EOF,B}$ are the electric field strengths (V m), the resistivities ($\Omega^{-1}$ m$^{-1}$) and mobilities of the EOF (m$^2$/V · s) of the diluted background electrolyte in the sampling zone S and of the background electrolyte B in the capillary, respectively, and if the capillary is filled to a fraction *x* ( = $l/L_C$) during the sampling procedure, for the overall velocity of the EOF, $v_{EOF,tot}$, can be derived [3,4,6]:

$$v_{EOF,tot} = x v_{EOF,S} + (1 - x) v_{EOF,B}$$
$$= x m_{EOF,S} E_S + (1 - x) m_{EOF,B} E_B \quad (1)$$

and for the electric field strengths in the zones S and B, respectively:

$$E_S = \frac{\rho_S V_{tot}}{[x \rho_S + (1 - x) \rho_B] L_C}$$

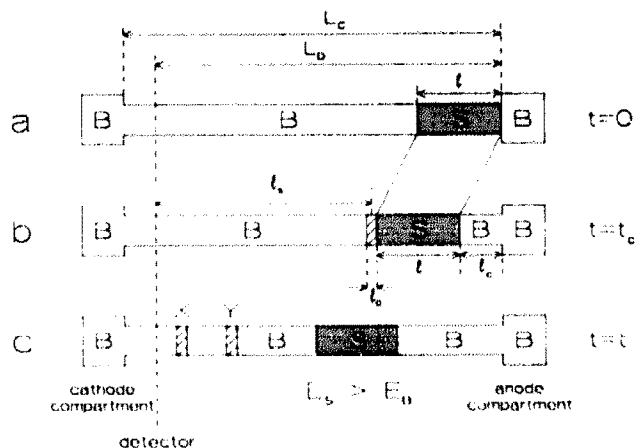$$E_B = \frac{\rho_B V_{tot}}{[x \rho_S + (1 - x) \rho_B] L_C} \quad (0 < x < 1) \quad (2)$$



Fig. 1. Effect of sample stacking. (a) Original situation with sampling zone S (sample components dissolved in dilute background electrolyte) behind background electrolyte B. (b) After a short concentration time $t_c$, the cationic sample components are concentrated in narrow zones with a length $l_p$ between background electrolyte B and the sampling zone S. The electric field strength $E_S$ is larger than $E_B$. The boundary between sampling zone S and background electrolyte B moves at the velocity of the EOF. (c) The separation of the components X and Y occurs in background B. $L_C$ = capillary length; $l_D$ = distance between injection and detection; $l$ = length of injected sampling zone S; $l_c$ = displacement of sampling zone during stacking procedure; $l_p$ = peak width of sample component after stacking procedure; $l_s$ = separation length after stacking procedure; B = background electrolyte; S = diluted background electrolyte in sampling zone.

whereby $V_{tot}$ is the total applied voltage (V) over the capillary with length $L_C$. For all calculations and experiments the composition of the background electrolyte B and the dilution factor K are given, where *K* is the ratio of the concentrations of the background electrolyte B and diluted background electrolyte S, which is approximately equal to the ratios $E_S/E_B$ and $\rho_S/\rho_B$.

From eqns. 1 and 2 it can be concluded that for increasing values of *x* and $\rho_S$, $v_{EOF,tot}$ will be determined to an increasing extent by the properties of the diluted background electrolyte in the sampling zone S. The migration times $t_1$ of the front and $t_2$ of the backside of the sampling zone S to reach the detector can be calculated according to

$$t_1 = \frac{L_D - l}{v_{EOF,tot}}; \quad t_2 = \frac{L_D}{v_{EOF,tot}} \quad (3)$$

Sample components (the equations given are valid for cationic species, where the EOF is in the direc-

tion of the cathode) will migrate quickly out of the sampling zone and stack down between sampling zone S and background electrolyte B (see Fig. 1b). After a short concentration time $t_{c,i}$ sample component $i$ will be concentrated to a very short zone, with length $l_{p,i}$ (see Fig. 1b) according to

$$t_{c,i} = \frac{l}{m_i E_S}; \; l_{p,i} = t_{c,i} m_i E_B = l \cdot \frac{E_B}{E_S} = l \cdot \frac{\rho_B}{\rho_S} \quad (4)$$

Eqn. 4 shows that the concentration factor $l/l_{p,i}$ in sample stacking is equal to the $K$ value.

The sampling zone S moves in time $t_{c,i}$ over a distance of

$$l_{c,i} = t_{c,i} v_{EOF,tot} = \frac{l}{m_i E_S} \cdot v_{EOF,tot} \quad (5)$$

After this stacking procedure the components will migrate and be separated according to the zone electrophoretic principle (see Fig. 1c). The separation process takes place for the largest part in the background electrolyte B, at an electric field strength of $E_B$, with an effective separation length $l_S$. The total migration time of a component $i$ will therefore be

$$t_{tot,i} = t_{c,i} + \frac{l_S}{v_i} = t_{c,i} + \frac{L_D - l - l_{c,i} - \frac{1}{2} \cdot l_{p,i}}{m_i E_B + v_{EOF,tot}} \quad (6)$$

To check the given model, for several dilutions of different background electrolytes the mobilities of the EOF were measured and with eqns. 1–3 the corresponding $t_1$ and $t_2$ values were calculated as a function of the injected length. The experimental values can easily be obtained from the dip in the UV signal in the electropherograms. As an example, in Fig. 2 the calculated relationships between migration times $t_1$ and $t_2$ and the injected length and measured values are given for background electrolytes of 0.02 $M$ tris(hydroxymethyl)aminomethane (Tris) adjusted to pH 4.5 by adding acetic acid with $K = 4$ (dashed lines) and 0.02 $M$ histidine–0.02 $M$ 2-(N-morpholino)ethanesulphonic acid (MES) with $K = 5$ (solid lines). The experimental values agree with calculated values although for small injected lengths the experimental values are higher than the calculated values, i.e., the effect of the low concentration in the sampling zone will be partially cancelled by diffusion.
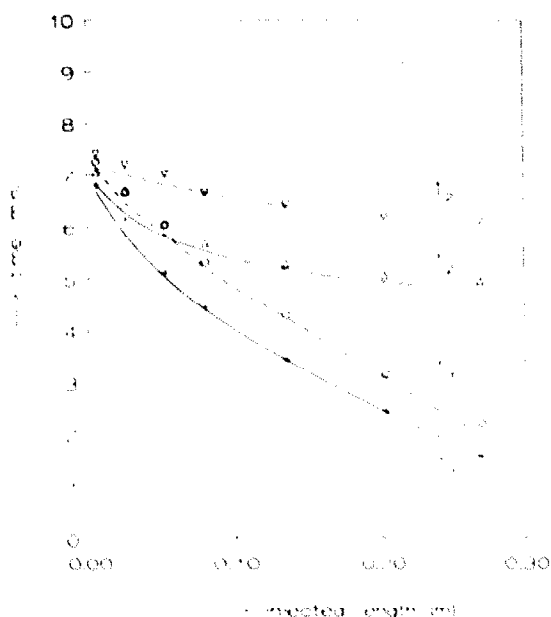


Fig. 2. Calculated relationships between migration times $t_1$ of the front and $t_2$ of the rear sides of the sampling zone and injected length for a background electrolyte of 0.02 $M$ Tris acetate at pH 4.5 with $K = 4$ (dashed lines) and 0.02 $M$ histidine–0.02 $M$ MES with $K = 5$ (solid lines) and measured ( ) $t_2$ and ( ) $t_1$ values in Tris acetate and ( ) $t_2$ and (+) $t_1$ values in 0.02 $M$ histidine–0.02 $M$ MES.

In Fig. 3 the calculated relationships between $t_1$ and $t_2$ values and the total migration times of histidine and imidazole (lines) and measured values are given, applying the background electrolyte 0.02 $M$



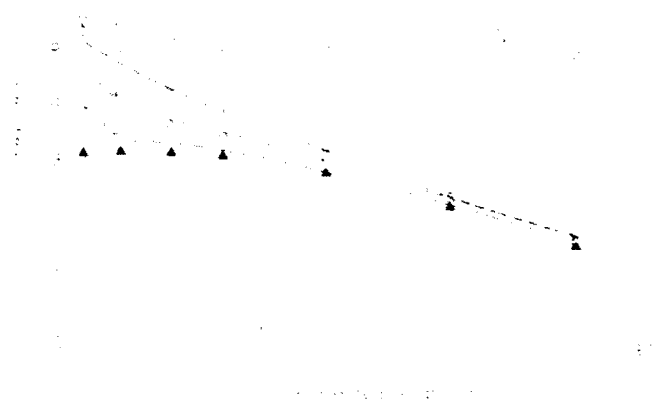Fig. 3. Relationships between calculated (lines) and measured values of ( ) $t_2$ and ( ) $t_1$ and migration times of ( ) histidine and (▲) imidazole versus injected length applying a background electrolyte of 0.02 $M$ β-alanine–0.02 $M$ MES ($K = 10$).

Fig. 4. Calculated relationships between $\Delta t_{tot,i}/t_{Av}$ and injected length for two components with mobilities of $50 \cdot 10^{-9}$ and $30 \cdot 10^{-9}$ m²/V · s in a background electrolyte of $0.02$ $M$ Tris–$0.02$ $M$ MES for $K = 1$–$50$.
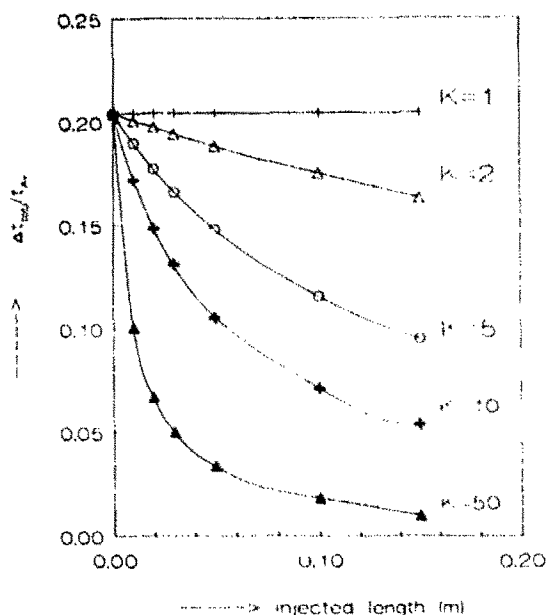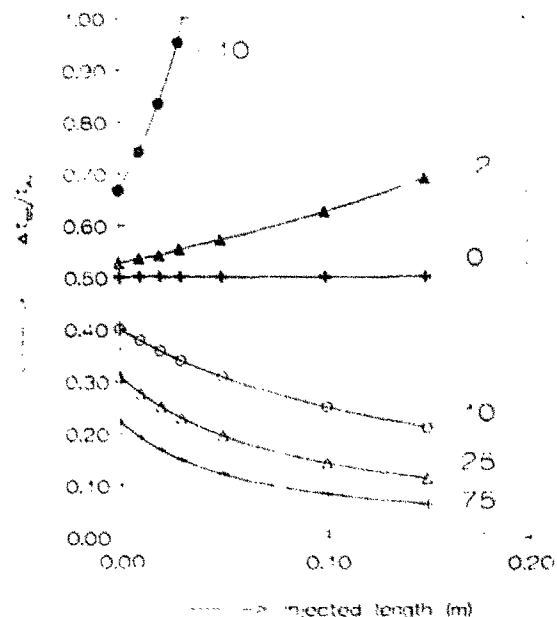


Fig. 5. Calculated relationships between $\Delta t_{tot,i}/t_{Av}$ and injected length for two components with mobilities of $50 \cdot 10^{-9}$ and $30 \cdot 10^{-9}$ m² V · s assuming several different mobilities of the EOF. For all calculations $K$ was 10 and $m_{EOF,S}$ was assumed to be $1.5 m_{EOF,B}$. The numbers on the right refer to $m_{EOF,B}$ ($10^{-9}$ m² V · s).

$\beta$-alanine–$0.01$ $M$ MES ($K = 10$). As can be seen, the calculated and measured values show good agreement. It is notable, however, that the migration times of imidazole and histidine, with a large difference in ionic mobility ($52.0 \cdot 10^{-9}$ and $29.6 \cdot 10^{-9}$ m²/V · s, respectively) hardly differ and they cannot be separated by injecting large sample volumes. They migrate just before the front of the sampling zone S ($t_1$).

Although with the foregoing model no resolution factors can be calculated, because peak broadening effects due to injection and the mismatch between the velocities of the EOF in sampling zone and background electrolyte are unknown, the ratio $\Delta t_{tot,i}/t_{Av}$ can be calculated and handled as parameter for the separation efficiency of two components with an average migration time $t_{Av}$. As an example, in Fig. 4 the calculated relationships, using the foregoing eqn. 6, between $\Delta t_{tot,i}/t_{Av}$ and injected length are given using measured values of the mobilities of the EOF for several dilutions of a background electrolyte of $0.02$ $M$ Tris–$0.02$ $M$ MES for two components with ionic mobilities of $50 \cdot 10^{-9}$ and $30 \cdot 10^{-9}$ m²/V · s, showing a dramatic decrease in separation efficiency on injecting large amounts of very dilute samples. The reason for this effect is easy to understand. The injection of a large sample volume reduces the effective separation length and the effective electric field strength over the background electrolyte B diminishes because the largest part of the applied voltage stands over the sampling zone, whereas the velocity of the EOF generally increases because the mobility of the EOF over the sampling zone is high. For $K = 1$ these effects seem to be negligible, but no concentration effect occurs. Note that in practice the resolution will be even more decreased than indicated in Fig. 4 owing to extra peak broadening effects. To show the effect of decreasing values of the mobility of the EOF, in Fig. 5 the calculated relationships between $\Delta t_{tot,i}/t_{Av}$ and injected length are given for two components with mobilities of $50 \cdot 10^{-9}$ and $30 \cdot 10^{-9}$ m²/V · s for several values of $m_{EOF}$. The numbers on the right in Fig. 5 are the $m_{EOF,B}$ values of the background electrolyte B, whereas the values of $m_{EOF,S}$ ($K = 10$) are always taken arbitrarily as $1.5 m_{EOF,B}$. As can be seen from Fig. 5, the decrease in separation power is smaller for lower $m_{EOF}$ values and the separation power even increases for reversed EOF, although in
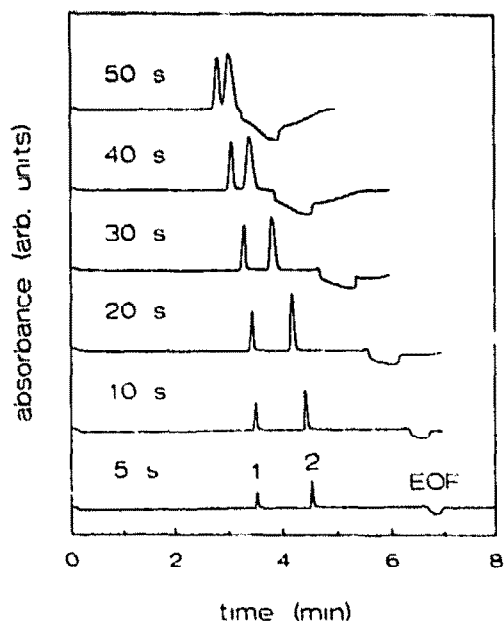
Fig. 6. Electropherograms for the separation of (1) imidazole and (2) histidine in water (0.0001 $M$) for different injection times applying as the background electrolyte 0.01 $M$ Tris acetate at pH 4.8. Decreasing resolution is obtained for longer injection times.

that event the migration times will be much higher. From Figs. 4 and 5 it can be concluded that injection of large sample volumes has a disastrous effect on the separation efficiency. This can be seen in Fig. 6, where the measured electropherograms are given for the separation of imidazole and histidine (both 0.0001 $M$ in water) applying a background electrolyte of 0.01 $M$ Tris adjusted to pH 4.8 by adding acetic acid using several different pressure injection times (a 10-s injection equals an injected length of 1.36 cm), showing the strong decrease in separation power.

*Effect of sample stacking on calibration graphs*

Strong variations in migration times due to variations in the velocity of the EOF can considerably affect the linear character of the calibration graphs of measured temporal peak area *versus* injected amount. To study the effect of introducing large sample volumes, experiments were performed with 0.01 $M$ Tris adjusted to pH 4.8 by adding acetic acid as background electrolyte and a sample consisting of imidazole and histidine both dissolved in background electrolyte and water (0.0001 $M$) for injec-

tion times varying from 5 to 60 s, corresponding to sample lengths from 0.68 to about 8.13 cm. In these experiments the migration times of the zones for the
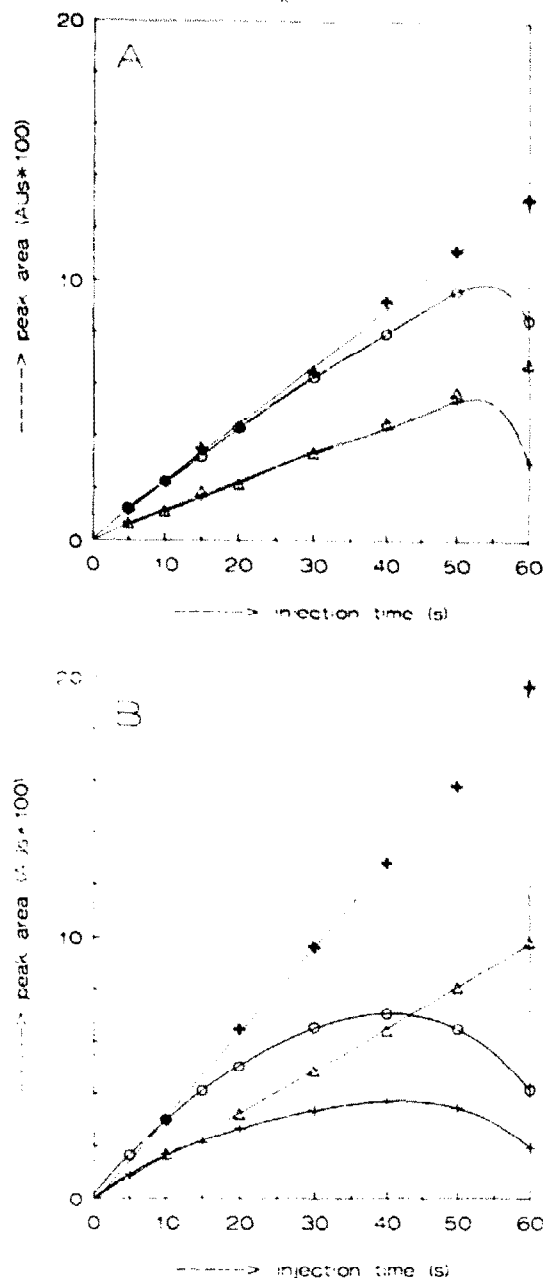


Fig. 7. Measured relationships between peak area and pressure injection time for ( + ) imidazole and ( ○ ) histidine dissolved in water (0.0001 $M$) and ( △ ) imidazole and ( + ) histidine dissolved in background electrolyte (0.0001 $M$) applying a background electrolyte of 0.01 $M$ Tris acetate at pH 4.8 and applying (A) a constant voltage of 10 kV and (B) a constant direct current of 5 μA.

sample dissolved in background electrolyte (measured from the rear side of the zones) were fairly constant, indicating constant migration velocities, through which temporal peak area can be handled in the calibration graphs. All peaks were rectangular owing to the absence of any concentrating effect and both temporal peak area and zone lengths show a linear relationship with injection times. In Fig. 7A the relationships between measured temporal peak area and injection time are given for histidine and imidazole dissolved in water and in background electrolyte and applying a constant voltage of 10 kV. For the samples dissolved in background electrolyte linear relationships are obtained, whereas the aqueous solutions deviate from linearity, especially for large injection times. To study the effect of working at a constant voltage compared with a constant direct current, we repeated the experiments in Fig. 7A but applying a constant current of 5 $\mu$A. The calibration graphs are shown in Fig. 7B. The non-linearity for the aqueous sample solutions was much stronger because at a constant current the electric field strengths increase, resulting in much higher velocities of the EOF.

### Effect of sample stacking on pH

If a capillary is partially filled with a buffer solution 2 with a different composition to that of the background electrolyte 1 (see Fig. 8), the mass balance of the hydrogen ions over solution 2 will be in imbalance, resulting in pH shifts. Roughly, four different cases can be distinguished, viz., the $E$ gradients in both electrolytes are equal but the pH in electrolyte 2 is (A) higher or (B) lower than those in electrolyte 1, and the cases where the pH values are equal but the $E$ gradient in electrolyte 2 is (C) higher or (D) lower than those of electrolyte 1.

The amount of hydrogen ions (across unit area per unit time) entering the electrolyte 2 (from the rear side) is $[H^+]_1 m_{11} E_1$ when the amount leaving this zone on the front is $[H^+]_2 m_{11} E_2$ (the subscripts 1 and 2 refer to the composition of the electrolytes 1 and 2, respectively). The extent of the flow of the hydrogen ions is indicated with an arrow in Fig. 8. If the entering and leaving amounts of hydrogen ions are not equal, pH shifts can be expected with time. If the entering flow of hydrogen ions is larger then the leaving flow, a pH decrease at the rear side and a higher pH in front of the concentration

boundaries between electrolytes 1 and 2 can be expected, as indicated in Fig. 8 by dotted lines. The size of the pH shift is difficult to define and depends on several factors, such as the length over which the capillary is filled with electrolyte 2, time of analysis, pH of the system and buffer capacity. At, e.g., a pH of 6 or higher the buffer capacity will be sufficient to compensate for the small differences in the entering and leaving flows of the hydrogen ions, but at low pH problems can be expected because the differences will be much larger.

In sample stacking, we will have generally case C, whereby electrolyte 1 is a background electrolyte at high concentration and the sample is introduced in a large zone at low concentration. If a long sampling zone is introduced, the concentration time of the components will be long and if during that time the pH at the rear side of the sampling zone increases, through which weak cationic species ac-
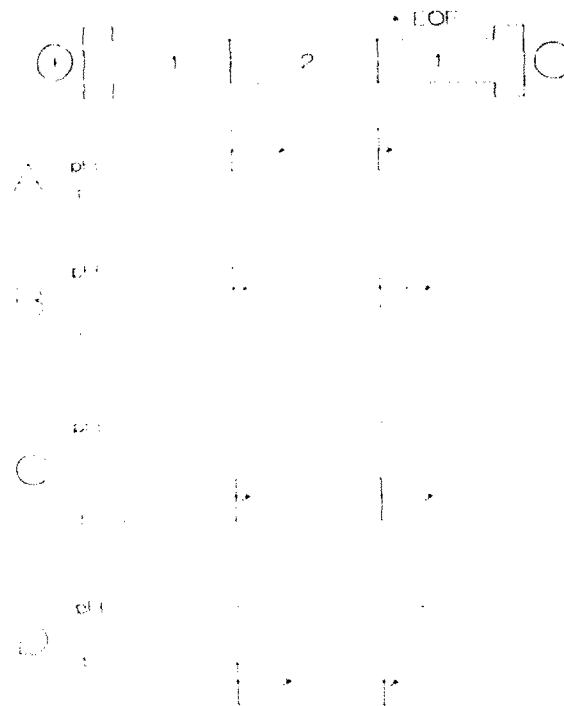


Fig. 8. Introducing two different electrolytes 1 and 2 into a capillary can result in pH shifts. Four different cases can be distinguished, viz., the $E$ gradients in both electrolytes are equal but the pH in electrolyte 2 is (A) higher or (B) lower than those in electrolyte 1 and the pH values are equal but the $E$ gradient in electrolyte 2 is (C) higher or (D) lower than those of electrolyte 1. Expected pH shifts are indicated by dotted lines. The arrows represent the entering and leaving flows of the hydrogen ions in zone 2.

Fig. 9. Electropherograms for the separation of (1) creatinine and (2) clenbuterol (0.0005 M in water) with different electro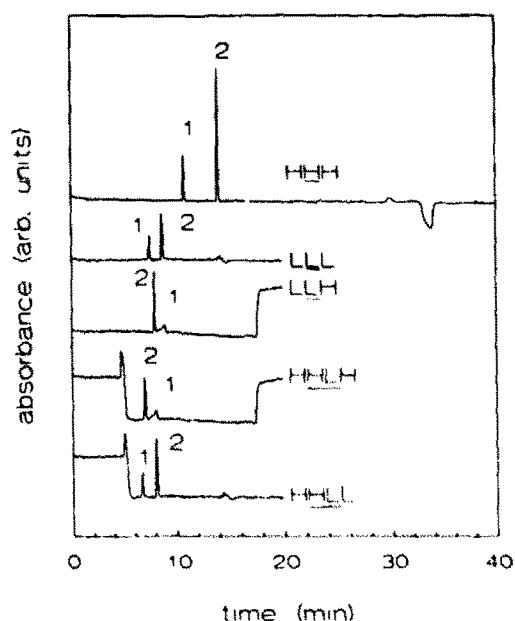lytes in the cathode and anode compartments and separation capillary. The electrolytes applied are 0.02 M Tris (electrolyte H) and 0.002 M Tris (electrolyte L) adjusted to pH 4.0 by adding formic acid. The electropherograms are labelled with letters, where the first and last letters indicate the applied electrolyte in the cathode and anode compartments respectively, and the underlined letters indicate the capillary content. Hence, the label HHLH means that the cathode compartment is filled with electrolyte H, the capillary is partially filled with electrolyte H on the cathode side and with electrolyte L on the anode side and the anode compartment is filled with electrolyte H. Applying a normal CZE system (HHH and LLL), creatinine migrates in front of clenbuterol. Application of solution H at the rearside of an L zone (LLH and HHLH) results in reversed migration. The concentration boundaries between H and L electrolytes are indicated by a strong UV shift, owing to differences in UV absorbances of the electrolytes.

quire a lower mobility, the concentration effect will be lost. In such instances bad peak shapes can be the result. Even double peaks were obtained on the electropherograms.

Applying field-amplified CZE, the sample components are introduced behind an electrolyte at low concentration (electrolyte 2). To indicate that pH shifts can occur and can lead to a different migration behaviour and bad peak shapes, several electropherograms are given for the separation of a solution of 0.0005 M in water of (1) creatinine and (2) clenbuterol applying different electrolyte systems.

At pH 4 clenbuterol can be considered to be completely protonated whereas creatinine is partially protonated and small pH changes will change its effective mobility considerably (its p$K$ value is 4.83). The electrolytes applied are always solutions of 0.02 and 0.002 M Tris, both adjusted to a pH of 4.0 by adding formic acid. The electrolyte at a concentration 0.02 M Tris formate will be indicated by H and that a concentration of 0.002 M Tris formate by L. The electropherograms in Fig. 9 are labelled with a letter system, in which the first and last letters indicate the applied electrolyte in the cathode and anode compartments, respectively, and the underlined letters indicate the capillary content. Hence, the label HHLH means that the cathode compartment is filled with electrolyte H, the capillary is partially filled with electrolyte H on the cathode side and with electrolyte L on the anode side and the anode compartment is filled with electrolyte H.

In Fig. 9, the electropherograms HHH and LLL are given for application of normal CZE systems with the electrolyte H and L, respectively, and in both electrolytes creatinine shows a higher effective mobility than clenbuterol. If the cathode compartment and separation capillary are filled with electrolyte L and the anode compartment with electrolyte H (electropherogram LLH), the $E$ gradient at the rear side is much lower, through which the hydrogen flow entering at the rear side is too small to maintain a pH of 4 and the pH in the separation capillary will increase. In the electropherogram the migration order of clenbuterol and creatinine is reversed, confirming the pH shift. In the electropherogram HHLH, field-amplified CZE was applied. The separation capillary was partially filled with electrolyte L and the sample was introduced between electrolyte L and H (anode compartment). In zone L a pH increase can be expected, through which clenbuterol and creatinine migrate in the reverse order again. If, however, the anode compartment is also filled with electrolyte L (electropherogram HHLL), the pH of electrolyte L will be constant and clenbuterol and creatinine again migrate in the normal order. From Fig. 9 it can be concluded that the presence of different electrolytes in the capillary and electrode compartments, e.g., in sample stacking or field-amplified CZE, can lead to pH shifts resulting in a different migration order, bad peak shapes and loss of separation power.

## CONCLUSIONS

Applying sample stacking, migration times of sample components can be calculated with the given model. Although the given model is fairly simple, e.g., diffusion effects are neglected, the calculated and measured values agree fairly well. Calculations with this model indicate a dramatic decrease in separation power owing to an increasing contribution of the electroosmotic flow to the net migration of sample components, whereas the electric field strength in the separation compartment strongly decreases, in contrast with electrokinetic injection [6]. Also, calibration graphs can be far from linear, by introducing large volumes of very dilute samples and undesirable pH shifts can cause a different migration behaviour if the capillary is filled with different electrolytes.

Although by the application of sample stacking and field-amplified CZE the separation efficiency can be increased owing to the concentration effect, it may only be applicable for the injection of small sampling zones. Another approach for handling ex-

tremely large injection volumes in CZE was given by Chien and Burgi [8], whereby the whole separation capillary is filled with sample. By applying a high voltage with reversed polarity, the sample buffer is removed, whereafter the ZE separation can be carried out. A better resolution can also be obtained by suppressing the EOF or applying closed systems. Although the separation length will be decreased on injecting a long sampling plug, this length is then fully used for the separation of the analytes, as the system does not migrate through the capillary owing to the EOF.

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