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Correlation between zone velocity and current in automated single capillary isotachophoresis–zone electrophoresis

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Abstract

In single capillary isotachophoresis–zone electrophoresis (ITP–CZE) the sample zone velocity is varying with its position in the capillary during the focusing step. When the voltage is kept constant, the current changes to the same extent. Correlation between the current and the sample zone velocity can therefore be used to calculate the velocity of the hydrodynamic flow that is needed to counterbalance the sample zone velocity. Measured data are in agreement with calculations implying that current monitoring can be used in an automated feedback system to regulate the hydrodynamic flow velocity during the focusing step. Conditions are described where automated anionic single capillary ITP–CZE can be performed without application of a hydrodynamic counterflow, extending its applicability to any commercially available CZE system.

Correlation between the ITP current and the sample zone position in the capillary was used to determine the moment for automatic switching from ITP to CZE. The reproducibility of the corresponding CZE migration times is investigated in addition to the effect of the remaining ITP terminator zone length on the CZE separation. A remaining terminator zone length of 10% of the total capillary length still resulted in an acceptable CZE performance.

1. Introduction

Capillary electrophoresis in combination with trace enrichment procedures has been proven to be a powerful analytical technique capable of highly efficient separations at low analyte concentrations [1,2]. Several methods of lowering determination limits in capillary zone electrophoresis (CZE) have been described [3]. Classical off-line sample pretreatment and preconcentration techniques, such as liquid–liquid extraction or solid-phase isolation, have been used in a number of bioassays. Although these procedures can be laborious and time consuming they offer flexibility [4]. On-line sample pretreatment offers the possibility of automation but has some restrictions with respect to the following step in the analytical method [5].

Electrophoretic analyte focusing procedures are a convenient way of lowering the determination limits that are typical for zone electrophoretic separations. Several modes have been described and successfully applied. A common feature of procedures such as stacking and sample self stacking or transient isotachophoretic (ITP) preconcentration, is that the analyte is concentrated at the boundary over which a

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difference in the electric field strength exists [3,6].

The combination of ITP with CZE has been successfully applied by several groups in a dual-capillary mode. In this mode the sample ions are transferred from the ITP system to the CZE capillary. In the single capillary mode as described by Reinhoud *et al.* [7,8] the process of ITP and CZE takes place in the same capillary, only the buffer vials are switched. Large sample volumes are injected, typically 10–90% of the total capillary volume. A hydrodynamic counterflow is used during the focusing step to keep the sample zones inside the capillary. The discontinuous ITP buffer is removed before the CZE step is started resulting in highly efficient separations. The method is automated, reproducible and takes place in a commercially available CZE apparatus without any modifications of the hardware. Determination limits are at least a factor hundred better than for conventional CZE. Similar results have been obtained in combination with electrospray mass spectrometry of β-agonists [9].

In this paper an equation is derived giving the correlation between the current and the sample zone velocity during the focusing step in single capillary ITP–CZE. With this linear relationship the pressure needed to counterbalance the sample zone velocity can be calculated. The position of the sample zone in the capillary is calculated from the current, compared with experimental data and applied in automated ITP–CZE procedures. The reproducibility is investigated for ITP–CZE using current monitoring for automated switching from the ITP to the CZE mode.

2. Theory

The coupling of ITP with CZE using a single capillary setup has been described for both anions and cations [7,8]. In both cases either the leading or terminating buffer can be used as background electrolyte for CZE, resulting in four modes of ITP–CZE. The focusing step is started after injection of a large sample zone in terminator buffer, typically 30–90% of the total capillary length. A counterflow is used to keep the sample zones in the capillary during the focusing process (Fig. 1). The front of the sample zone which forms a boundary with the leading buffer, is kept at a fixed position in the capillary by balancing the electrophoretic leading ion velocity with the hydrodynamic velocity. The rear boundary of the analyte zone is migrating in the direction of the front boundary resulting in a concentration of the analyte. After focusing the discontinuous buffer is removed isotachophoretically by either increasing or decreasing the hydrodynamic counterflow depending on the ITP–CZE mode that is used.

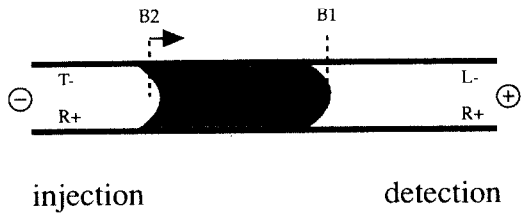

Fig. 1. Schematic representation of an anionic sample zone in the focusing step of ITP–CZE. The sample is solved in terminator buffer (T−, R+). The leading buffer (L−, R+) is also used as background electrolyte in the CZE step. The hydrodynamic flow velocity counterbalances the electro-osmotic and electrophoretic velocities of a sample zone at the front boundary (B1). The rear boundary (B2) migrates to B1 resulting in a concentration of the analyte.

2.1. Theoretical model

For the calculation of the zone velocity during the focusing step an isotachophoretic state is assumed. This assumption has been made to enable calculations of the local electric field strengths and the electrophoretic and electro-osmotic velocities. In the isotachophoretic state the velocities of all ions are the same with exception of the counter ion velocity [10]. For the analyte ions during the focusing step this assumption is not true, only when the focusing is completed they migrate with the same velocity as the leading and terminating ions. However, when low analyte concentrations in terminator buffer (*i.e.* $<0.1\%$ of the terminator buffer concentration) are considered the contribution to

the local electric field strength and conductivity in the terminator zone is negligible. Therefore, the velocity of the leading ions can be calculated without consideration of the migration of sample ions.

The terminator buffer is prepared at the isotachophoretic concentration. This concentration is given by:

$$\bar{c}_T = \frac{m_T}{m_L} \cdot \frac{m_L + m_R}{m_T + m_R} \cdot \bar{c}_L \quad (1)$$

where m_i is the absolute value of the ionic mobility of i. The subscripts L, T and R refer to the leading, terminating and counter ions, respectively. Because in buffer systems weak electrolytes are involved the analytical concentration of an analyte A is notated as $\bar{c}_A([HA] + [A^-])$ and the ionic concentration is notated as $c_A([A^-])$. Analogous, the mobility of the fully ionized analyte A is written as m_A and the effective mobility as $\bar{m}_A$. The relationship between the ionic and effective mobility is then given by $c_A m_A = \bar{c}_A \bar{m}_A$. An anionic system with univalent buffer ions will be considered, consisting of only one type of cations and anions (i.e. L^- and R^+, T^- and R^+). The leading buffer is also used as CZE background electrolyte.

In our model the temperature in the leading and terminating zone is considered to be the same and constant. Most of the considered parameters are dependent on temperature. This means that the model is only applicable for systems where the Joule heat is efficiently dissipated. When the terminator zone is removed from the capillary, the local electrical field strength in the terminator zone increases considerably resulting in an increased heat development. Therefore, at a small terminating buffer zone length a deviation of the measured velocities from the calculated velocities may occur. However, in ITP–CZE usually large injection volumes are applied. After the focusing step, when the terminator buffer is removed, the accurate magnitude of the velocity is usually no longer relevant. In the Results and Discussion section the assumption of a constant axial temperature is verified.

2.2. The hydrodynamic counterflow

The counterflow to keep the boundary of the sample zone with the leading buffer zone on a fixed position in the capillary (Fig. 1) is given by:

$$v_L + v_{hd} = 0 \quad (2)$$

where v_L is the velocity of the leading ions and v_{hd} is the hydrodynamic flow velocity in the opposite direction. The velocity of the leading ions is given by the sum of the electrophoretic velocity ($v_{el,L}$) and the bulk electroosmotic velocity (v_{eof})

$$v_L = v_{el,L} + v_{eof} \quad (3)$$

The electrophoretic velocity of the leading ions is given by

$$v_{el,L} = \bar{m}_L E_L \quad (4)$$

where $\bar{m}_L$ is the effective electrophoretic mobility of the leading ions and E_L is the electric field strength in the leading buffer zone.

The electroosmotic velocity is weighted over the fraction of capillary filled with leading buffer (x) and with terminating buffer ($1 - x$) [6]

$$v_{eof} = x v_{eof,L} + (1 - x) v_{eof,T} \quad (5)$$

where v_{eof} is the bulk electroosmotic velocity, $v_{eof,L}$ is the electroosmotic velocity in the leading zone and $v_{eof,T}$ is the electroosmotic velocity in the terminating zone. Combination of Eqs. 5 and 3 gives for the velocity of the leading ions

$$v_L = x v_{eof,L} + (1 - x) v_{eof,T} + v_{el,L} \quad (6)$$

The hydrodynamic flow velocity caused by a pressure difference over a capillary is given by the Poiseuille equation:

$$v_{hd} = \frac{d^2}{32 \eta L_0} \cdot \Delta p \quad (7)$$

where L_0 is the total capillary length, η is the viscosity ($9.93 \cdot 10^{-4}$ kg m^{-1} s^{-1}), d the capillary diameter and Δp the pressure difference. Combination of Eqs. 2, 6 and 7 gives the pressure difference that results in a hydrodynamic velocity that counterbalances the velocity of the boundary between the leading and analyte zone

$$\Delta p = \frac{32\eta L_0}{d^2} \cdot [x v_{eof,L} + (1-x) v_{eof,T} + v_{el,L}] \quad (8)$$

where the electrophoretic velocity ($v_{el,L}$) has a negative value for anions. The fraction of the capillary that is filled with leading buffer (x) depends on the injected volume and changes when the focusing procedure proceeds. When the capillary is filled for 75% with sample in terminating buffer, the value of x is 0.25. This fraction will increase to 1.0 when the terminating buffer is removed from the capillary.

From Eq. 3 it can be seen that when the electroosmotic velocity is smaller than the velocity of the leading ions ($v_{el,L} + v_{eof} > 0$) the direction of the leading zone velocity reverses. Therefore, the counterflow velocity balancing the leading zone velocity (Eq. 1) is also reversed. This means that in stead of an increased pressure a reduced pressure is needed for complete removal of the anionic ITP buffer. The four ITP–CZE procedures as described previously [8] can all be performed in absence as well as in presence of electroosmotic flow using the appropriate pressure difference given by Eq. 8.

When the electrophoretic mobility is approximately the same as the electroosmotic mobility, there will be one value of x for which the leading zone velocity (v_L) is zero. Without applying a hydrodynamic flow, the boundary of the sample zone and leading zone will migrate in the electric field until this particular leading zone length is reached and the velocity is zero. When all parameters are kept constant, the position of this boundary will not change in time. When the position of this boundary is close to the capillary inlet, the ITP step can be stopped and the CZE step can be started without removing the remaining terminator zone. For untreated fused silica with an electroosmotic mobility of the buffer of *ca.* $60 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ this may be the case when chlorate ($m = 63 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$) or chloride ($m = 75 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$) is used as leading ion.

2.3. Determination of parameters

In the isotachophoretic steady state the electric field strength in the leading buffer zone (E_L) is by definition lower than in the terminating buffer zone (E_T). The local electric field strength depends on the total electric field applied over the capillary (E_0), the conductivities of the buffers and the length over which the capillary is filled with leading or terminating buffer. The total electric field strength is given by the running voltage (V_0) divided by the total capillary length (L_0). The electric field strength in the leading zone is given by [6]

$$E_L = \frac{\gamma E_0}{[\gamma x + (1-x)]} \quad (9)$$

and in the terminating zone

$$E_T = \frac{E_0}{[\gamma x + (1-x)]} \quad (10)$$

where γ is the conductivity ratio of the terminating and the leading zone (κ_T/κ_L) and x is the fraction of the capillary that is filled with leading buffer (L_L/L_0). The fraction that is filled with terminator buffer ($1-x$) is equal to L_T/L_0. When the sample is solved in terminator buffer at the isotachophoretic concentration (Eq. 1) the length of the injection zone is considered to be L_T.

The conductivity (κ_i in $S\ m^{-1}$) can be measured in a capillary filled with buffer i using Eq. 11:

$$\kappa_i = \frac{I}{\pi r^2 E} \quad (11)$$

where I is the electric current and r is the capillary radius. Alternatively, when the electrophoretic mobilities are known, the conductivity for weak univalent electrolytes can be calculated by [11]

$$\kappa_i = F \bar{c}_A (\bar{m}_A + \alpha_A m_R) \quad (12)$$

where F is the Faraday constant (96 485 $C\ mol^{-1}$) and α_A the mole fraction of A that is in the ionic form. In case of univalent buffer ions the conductivity ratio γ is given by

$$\frac{\kappa_T}{\kappa_L} = \frac{\bar{c}_T(\bar{m}_T + \alpha_T m_R)}{\bar{c}_L(\bar{m}_L + \alpha_L m_R)} \quad (13)$$

The concentration $\bar{c}_T$ is calculated via the ITP equation (Eq. 1). The ionic mobility m_R is

assumed to be the same in the leading and terminating zone. Although the ionic mobility depends on the ionic strength which differs in the leading and terminating zone, for the conductivity ratio this is negligible.

The electroosmotic flow velocities are calculated using

$$v_{eof,L} = m_{eof,L} E_L \quad (14)$$

and

$$v_{eof,T} = m_{eof,T} E_T \quad (15)$$

where m_{eof} is the electroosmotic mobility (in $m^2\ V^{-1}\ s^{-1}$). The bulk electroosmotic mobility is the same in both zones; however, local differences in the electroosmotic mobility in the leading and terminating zone exists. The electroosmotic mobility decreases as the ionic strength of the buffer increases. Because of a lower buffer concentration in the terminator zone, the electroosmotic mobility will be higher than in the leading buffer zone. The local difference in flow-rate with respect to the bulk flow-rate, is compensated for by a convective flow in the buffer zones. The electroosmotic velocity ($v_{eof,L}$) can be measured in the CZE mode, when the leading buffer is used as background electrolyte. The electroosmotic velocity of the terminator zone $v_{eof,T}$ can be measured in the capillary filled with terminating buffer, using the buffer concentration calculated using the ITP equation (Eq. 1).

From Eqs. 9 and 10 it follows that the electric field strength is considerably influenced by the length of the leading zone, which results in varying electrophoretic and electroosmotic velocities during the focusing procedure. Substituting Eqs. 9, 10, 14 and 15 in Eq. 8 gives the full impact of the fractional leading zone length (x) on the pressure difference.

$$\Delta p = \frac{32\eta L_0}{d^2} \cdot \frac{\gamma E_0}{[\gamma x + (1-x)]} \cdot \left[x m_{eof,L} + \frac{(1-x)}{\gamma} \cdot m_{eof,T} + \bar{m}_L \right] \quad (16)$$

where the effective mobility of the leading ions ($\bar{m}_L$) has a negative value for anions. Both Eqs. 16 and 8 can be used to calculate the pressure difference that results in a hydrodynamic flow that counterbalances the velocity of the boundary between the leading and analyte zone. Eq. 16 gives insight in all the parameters that are affecting the pressure difference.

When the conductivities of the leading and terminating buffer are measured or calculated (Eq. 12), the conductivity ratio γ (Eq. 13) can be calculated. Via experimentally determined electrophoretic and electroosmotic mobilities the corresponding velocities can be calculated using Eqs. 4, 14 and 15. The total velocity of the boundary of the leading buffer zone with the sample zone (v_L) is then calculated with Eq. 6. The pressure that results in a hydrodynamic flow velocity that counterbalances v_L is then given by Eq. 8.

When it is not possible to apply a pressure during the focusing procedure, a height difference can be used. The calculated pressure difference can be converted to a height difference using

$$\Delta h = \Delta p / \rho g \quad (17)$$

where Δp is the pressure difference (bar), ρ is the buffer density (1000 $kg\ m^{-3}$) and g is the gravitational force (9.8 $m\ s^{-2}$). A pressure difference of 20 mbar corresponds to a height difference of approximately 20 cm. This conversion is independent of the capillary diameter.

2.4. Current monitoring

When a constant voltage is applied during the focusing step, and the leading buffer is used as background electrolyte in the CZE step, the current will increase as more leading buffer enters the capillary. Just before a complete removal of the terminator buffer the ITP is stopped and the CZE is started. Monitoring of the current can be used to determine the moment for automated switching from ITP to CZE.

The total current in an ITP process is determined by Ohm's Law as the ratio of the applied voltage and the electrical resistance of the liquid in the capillary. The total resistance in the ITP capillary is given by the sum of the resistances of the leading, terminating and sample zones. For the ITP of low concentrations of analyte the contribution of the sample zones to

the total resistance of the capillary will be negligible so that

$$I = V_0/(R_L + R_T) \tag{18}$$

The electrical resistance of zone i (R_i) depends on the capillary radius (r), the length (L_i) and the conductivity (κ_i) of the zone.

$$R_i = L_i/(\pi r^2 \kappa_i) \tag{19}$$

Substituting Eq. 19 in Eq. 18 gives

$$I = \frac{V_0 \pi r^2}{\left(\frac{L_L}{\kappa_L} + \frac{L_T}{\kappa_T}\right)} \tag{20}$$

When the conductivities are known the variation of current during the focusing step can be calculated using Eq. 20 for a given leading and terminating zone length. The current after removal of the terminator zone in single capillary ITP–CZE is given by Eq. 20 for $L_T = 0$, *i.e.* when the capillary is filled with leading buffer only. In practice always a small zone of terminator will remain in the capillary when switching from ITP to CZE.

In ITP the composition of the leading zone also determines the composition of the terminating zone. Thus when the composition of the leading zone is known and the mobilities of the leading and terminating ions and of the counter ions are known, the change in current can be calculated. The conductivity in a zone is given by the sum of the product of concentration, mobility and charge of all ionic species. Combining Eqs. 12 and 20 for a system with univalent ions gives

$$I = \frac{V_0 \pi r^2 F}{\left[\frac{L_L}{\bar{c}_L(\bar{m}_L + \alpha_L m_R)} + \frac{L_T}{\bar{c}_T(\bar{m}_T + \alpha_T m_R)}\right]} \tag{21}$$

When in single capillary ITP–CZE a given remaining zone length of the terminator buffer is allowed, the corresponding ITP current is then calculated using Eq. 20. This is the threshold of the current that can be used to program the CZE apparatus to switch automatically from the ITP step to the CZE step. Although Eq. 21 gives a better insight in all parameters that are affecting the change in current, for precise determination of the current profile during the ITP step it is advisable to use Eq. 20 in conjunction with conductivity measurements of the leading and terminating buffers.

2.5. Counterflow and current

The hydrodynamic flow velocity needed to counterbalance the sample zone velocity depends on the position of the sample zones in the capillary (Eq. 16). This position can be calculated from the current (Eqs. 20 and 21). Combining these equations makes it possible to calculate the hydrodynamic flow velocity for a given current. The current is measured during the analysis and will be constant as long as the sample zones are not moving. Using Eq. 20, $L_L = xL_0$ and $L_T = (1-x)L_0$, the relative leading zone length x can be written as

$$x = \frac{\pi r^2 E_0 \kappa_T}{I(\gamma - 1)} - \frac{1}{(\gamma - 1)} \tag{22}$$

Substituting the expression for the current density $I/(\pi r^2) = E_L \kappa_L$ (Eq. 11) in Eq. 9 gives

$$\frac{\gamma E_0}{[\gamma x + (1-x)]} = \frac{I}{\pi r^2 \kappa_L} \tag{23}$$

Substituting Eqs. 22 and 23 in Eq. 16 results in the linear equation

$$\Delta p = a + bI \tag{24}$$

which gives the pressure difference for a given current. The slope b and intercept a are given by

$$a = \frac{32\eta V_0}{d^2} \cdot \left[\frac{\kappa_T m_{eof,L} - \kappa_L m_{eof,T}}{\kappa_T - \kappa_L}\right] \tag{25}$$

$$b = \frac{128\eta L_0}{d^4 \kappa_L \pi} \cdot \left[\frac{1}{\gamma} \cdot \left(1 + \frac{1}{\gamma - 1}\right) \cdot m_{eof,T} - \left(\frac{1}{\gamma - 1}\right) \cdot m_{eof,L} + \bar{m}_L\right] \tag{26}$$

where the effective mobility of the leading ions ($\bar{m}_L$) has a negative value for anions. Eq. 24 implies that with current monitoring not only the

moment to switch from ITP to CZE can be determined, but also the pressure needed in single capillary ITP–CZE.

2.6. The focusing step

An important aspect in the focusing step is the focusing time of the analytes. The time to focus the injection zone is given by the sample zone length divided by the total velocity of the slowest analyte ion in the ITP separation window, $t = L_{inj}/v_{A,f}$. The subscript f refers to focusing conditions. The total velocity of the analyte ions under focusing conditions is given by

$$v_{A,f} = xv_{eof,L} + (1-x)v_{eof,T} + v_{el,A} + v_{hd} \quad (27)$$

where $v_{el,A}$ is the electrophoretic velocity of the slowest analyte A. Because A is migrating in the terminator zone, the electrophoretic velocity of A can be written as $v_{el,A} = \bar{m}_A E_T$. The velocity of the terminating ions under focusing conditions can be written as

$$v_{T,f} = xv_{eof,L} + (1-x)v_{eof,T} + v_{el,T} + v_{hd} = 0 \quad (28)$$

Assuming ITP conditions the terminator ion velocity is counterbalanced by the hydrodynamic velocity, $v_L = v_T = -v_{hd}$. Combining this with Eqs. 27 and 28 gives for the analyte velocity under focusing conditions

$$v_{A,f} = v_{el,A} - v_{el,T} = (\bar{m}_A - \bar{m}_T)E_T \quad (29)$$

The focusing time is then given by

$$t = L_{inj}/[(\bar{m}_A - m_T)E_T] \quad (30)$$

The focusing time is thus independent from the electroosmotic and hydrodynamic velocity. When the effective electrophoretic mobilities of the analyte and the terminator ion are similar the focusing time will increase to infinity.

In practice the focusing step and the isotachophoretic removal of the terminator buffer can be combined thus reducing the analysis time. The focusing step is started at the appropriate pressure, given by Eq. 8 and after a few minutes the pressure is lowered. As a result the terminating zone length decreases slowly and the electrical field strength E_T increases. This can be repeated several times, thus increasing E_T and reducing the focusing time.

The described focusing procedure as illustrated in Fig. 1 offers several alternatives. In stead of counterbalancing the sample zone boundary with the leading buffer (the front boundary) the boundary of the slowest sample ion with the terminator zone (rear boundary) can be counterbalanced. Analogous to Eq. 8 the pressure needed to induce an appropriate counterflow is given by

$$\Delta p = \frac{32\eta L_0}{d^2} \cdot [xv_{eof,L} + (1-x)v_{eof,T} + v_{el,A}] \quad (31)$$

where $v_{el,A}$ is given by the effective electrophoretic mobility of the slowest analyte A and the electric field strength in the terminator zone (Eq. 4).

Solving the sample in leading buffer is another option. When the boundary of the terminator buffer with the sample zone in leading buffer (rear boundary) is counterbalanced (using Eq. 8), the focusing time is given analogous to Eq. 30 by

$$t = L_{inj}/[(\bar{m}_L - m_{\bar{A}})E_L] \quad (32)$$

In most cases it is unlikely that this will reduce the analysis time because the electrical field strength in the leading buffer is always lower than in the terminating buffer. However, when the effective electrophoretic mobility of the analyte and the terminator ion are similar the focusing time can be reduced by solving the sample in leading buffer.

Another possibility in reducing the focusing time is focusing under unsteady state conditions by solving the sample in a lower terminator concentration than given by Eq. 1 which results in an increased E_T. However, under unsteady state conditions care must be taken that no analyte is lost.

2.7. Composition of the sample

For the calculation of the velocities at the start of the focusing procedure, it is assumed that

low-concentrations analyte are solved in terminating buffer. When the terminating buffer is at the ITP concentration (Eq. 1) the sample zone length is considered to be L_T. The conductivity of the sample is considered to be the same as the terminating buffer. In that case all equations can be used immediately from the start of the focusing procedure. Another advantage of working under these well defined conditions is that the velocities of analyte ions are known and by using the appropriate counterflow no loss of analyte occurs.

When the sample is solved in a matrix other than the terminator buffer or when high concentrations of matrix constituents are present in the sample, the applicability of the derived equations is limited to the ITP steady state. Only the zone velocity at the start of the focusing step can be calculated with the sample zone conductivity. The focusing step then proceeds under unsteady state conditions. The sample zone velocity is not only changing with its position in the capillary but also because of local changes in electric field strength as a result of the migration of matrix ions. Under unsteady state conditions the possibility exists that analyte ions migrate out of the capillary. A full discussion on unsteady state migration is given by Foret *et al.* [12].

One way to overcome incompatibility of the sample matrix with the ITP conditions is a sample pretreatment where an excess of matrix components is removed and the analytes are transferred to a well defined matrix. In trace analysis (nanomolar sample concentration range and lower) of analytes in complex matrix it is unlikely that ITP–CZE or CE in general can be used without an additional pretreatment step. An additional sample pretreatment usually improves the performance of ITP–CZE with respect to reproducibility, selectivity and ITP focusing time [7,8].

3. Experimental

3.1. Chemicals

Acetic acid (HAc) was from Merck (Darmstadt, Germany). Fluorescein (F) and triethanolamine (TEtOHA) (97%) were purchased from Janssen Chimica (Beerse, Belgium). Fluoresceinisothiocyanate isomer I (FITC) was from Aldrich Chemie (Steinheim, Germany). The food colorant brilliant acid green (E142) was from Morton (Amersfoort, Netherlands). Hydroxypropylmethylcellulose (HPMC) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) came from Sigma (St. Louis, MO, USA). The viscosity of a 2% aqueous HPMC solution is 4000 cP. In all experiments deionised water was used (Milli-Q system; Millipore, Bedford, MA, USA).

3.2. Conditions used in calculations

Zone velocity, current and counterflow (Figs. 2, 3 and 6) are calculated for an untreated fused-silica capillary of 500 mm × 100 μm I.D. An electroosmotic mobility of $60.0 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ is used for the leading buffer and $70.5 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ for the terminating buffer. The leading buffer consisted of 10 m*M* HAc set at pH 8.0 with TEtOHA and is also used as CZE background electrolyte. In the ITP step 7.25 m*M* HEPES at pH 8.0 is used as terminating buffer. The effective and ionic electrophoretic mobilities used in calculations are -42.0 and $-42.0 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ for acetate, -16.5 and $-22.0 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ for HEPES and 11.0 and $30.1 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ for TEtOHA. The ITP took place at a voltage of 10 kV. The calculated conductivities are 0.070 and 0.027 $S m^{-1}$ in respectively the leading and the terminating zone. The equations used for calculation of these parameters were given in the Theory section.

In most commercially available apparatus the pressure is applied at the capillary inlet. An increased pressure at the inlet is always considered a positive pressure and the resulting hydrodynamic flow has therefore a positive sign. A reduced pressure at the inlet results in a hydrodynamic flow in the opposite direction and has, according to our notation a negative sign.

During the ITP of anions the cathode is at the capillary inlet (Fig. 1), therefore the sign of the electrophoretic velocity of an anion A ($v_{el,A}$) in the direction of the anode is positive, analogous to the sign of a hydrodynamic flow. The sign of

the electroosmotic velocity (v_{eof}) in the direction of the cathode is negative for the same reason. The total velocity of the anion (v_A) in cases where the electroosmotic velocity predominates, is thus negative.

3.3. Experimental conditions

The conditions in Figs. 4 and 5 were similar to the conditions as given above with exception of the following. The capillary (520 mm × 0.100 mm I.D.; SGE, Ringwood, Australia) was pretreated by standing overnight with a solution of 0.05% HPMC and as a result the electrophoretic mobilities were 30 and $35 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ in the leading and the terminating zone, respectively. The effective mobility of acetate was $-39.7 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ which value was also used as the ionic mobility. A voltage of 15 kV was used and the currents were 13.1 and 6.0 μA for the leading and terminating buffer, respectively. Corresponding conductivities of 0.058 and 0.0265 S m^{-1} were used in calculations in Figs. 4 and 5.

For the counterflow and current monitoring experiments the food colorant brilliant acid green (E142) was used as visible test compound. For the reproducibility measurements and the variation of the terminator zone length (Fig. 7) fluoresceins were used with laser-induced fluorescence (LIF) detection. The LIF detection system has been described in detail elsewhere [7]. A programmable injection system for capillary electrophoresis (PRINCE; Lauerlabs, Emmen, Netherlands) equipped with a reversible polarity power supply and possibility for pressurized and electrokinetic injection was used for the automated ITP–CZE procedures.

3.4. Analyte focusing

The analyte focusing procedure consists of five steps [7,8]. In step 1 the injection takes place hydrodynamically at a pressure of 100 mbar. In the focusing step (step 2), the analyte focusing is started by applying a voltage in conjunction with a hydrodynamic pressure. The hydrodynamic pressure is used to prevent the sample zone from leaving the capillary. After 5–20 min of focusing, depending on the injected zone length, the focusing is completed. Step 3 is the ITP removal of the terminator buffer zone from the capillary. A voltage of 20 kV is applied without a hydrodynamic pressure. At the time that the sample zone is approaching the capillary inlet the terminator buffer vial is replaced for a vial containing the CZE background electrolyte (step 4), the voltage is reversed and the CZE run is started (step 5).

The current was monitored for precise timing of the moment to switch from the ITP to the CZE mode. When a constant voltage is applied the current increases as long as the terminating zone length decreases. The CZE equipment could be programmed so that at a defined threshold of the current the switching took place automatically. In principle, all terminating ions and sample ions with mobilities below that of the terminating ion, including the counter ions, are removed by the described procedure.

4. Results and discussion

Eq. 16 gives insight in all parameters that are affecting the zone velocity and the linear related counterbalancing pressure difference in the focusing and ITP step. Several of these parameters will now be discussed and compared with experimental data. Then the applicability of current monitoring for automated switching from ITP to CZE will be investigated in addition to the effect of the terminator zone length on the CZE performance.

4.1. Parameters affecting the sample zone velocity

In Fig. 2 the leading zone velocity is given for several electroosmotic flow-rates. As can be seen from Eq. 3 for a high electroosmotic flow-rate the zone velocity is always negative. However, when the electroosmotic mobility is similar or lower than the electrophoretic mobility the velocity v_L increases or becomes positive (increases in the opposite direction). As an example an injection zone length of 60% of the total capillary length will be considered. The leading zone velocity is counterbalanced so that the rear

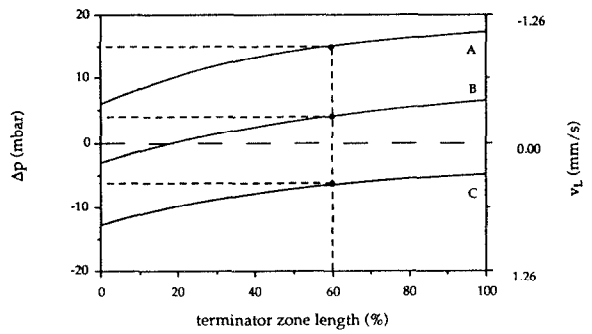

Fig. 2. The effect of the electroosmotic flow-rate on the leading zone velocity and the counterbalancing pressure in ITP–CZE. The lines are calculated values for an electroosmotic mobility in the leading zone of 60.0 (A), 30.0 (B) and 1.0 (C) $\cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$. All further parameters are given in the Experimental section.

boundary is moving to the fixed front boundary. As already mentioned in the Theory section several alternatives are possible.

Under conditions as illustrated in Fig. 2 line A, the focusing procedure is started at a pressure of 15 mbar (for conditions see the Experimental section). The front boundary of the sample zone is then focused at 60% terminator zone length. When the focusing step is completed the pressure is reduced to zero and the analyte zones migrate to the cathode. The analyte zones remain focused because of the ITP conditions and the terminator zone is removed from the capillary. The velocity of the sample zone as a function of the terminator zone length is described by line A. Just before complete removal of the terminator zone the voltage is reversed and the CZE is started.

For a similar injection under the conditions in Fig. 2 line B, the focusing procedure is started at a pressure of 4 mbar. After the focusing step the pressure is reduced to zero and the analyte zones will move to the point of 18% terminator zone length. At this point the electrophoretic mobility of the leading ions is counterbalanced by the electroosmotic mobility of the bulk. A reduced pressure (*i.e.* $\Delta p < -4$ mbar) is needed to remove the remaining terminator zone from the capillary.

Under the conditions in Fig. 2 line C, the focusing procedure is started at a pressure of −7 mbar to focus the analyte front boundary at 60% terminator zone length. After focusing the pressure is reduced (*i.e.* $\Delta p < -14$ mbar) to remove the remaining terminator zone from the capillary. All constants and variables used for Fig. 2 are given in the Experimental section.

Another parameter that is affecting the counterflow needed in the focusing procedure is the electric field strength. Increasing the electric field strength linearly increases all velocities (Eqs. 4, 9, 10, 14 and 15) and shortens the focusing time in ITP–CZE. As a result a linear increase of the counterflow velocity is needed. When the focusing voltage is doubled, a doubling of the pressure is needed to counterbalance the leading zone velocity. The time to complete the focusing will be reduced by a factor two (Eqs. 30 and 32).

High electric field strengths are not always favourable with respect to zone broadening. Increasing the electric field strength will increase the heat generation in the capillary, especially in the terminator zone where the electrical resistance is higher. When the terminator zone is almost removed from the capillary the electric field strength in the terminator zone increases to E_0/γ (Eq. 10, $x = 1$). At the same time, at a constant voltage the current through the capillary increases as more leading buffer is entering the capillary. The power in the terminator zone (W_T) increases with $W_T = V_T I$ resulting in a corresponding increase in heat development. Furthermore, the laminar flow profile that exists because of a mismatch of the electroosmotic velocities in the leading and the terminating zone [6] will be increased at higher electric field strengths. This is usually compensated for by the self-correcting properties of the ITP zones [7,8,10] but at high electric field strengths problems may arise. In order to avoid zone distortion or even disruption of the electrical current, it is might be necessary to reduce the voltage when the length of the terminating zone is getting smaller.

The ratio of mobility of the leading and terminator ions is important for the focusing time (Eqs. 30 and 32). When a zwitterionic buffer is used as terminator buffer a low conductivity can be obtained, resulting in a small value

of γ. The electroosmotic velocities in the leading and terminator zone change with the local electric field strengths E_T and E_L (Eqs. 9, 10, 14 and 15). A lower conductivity of the terminating zone results in an increase in E_T, especially at small terminator zone length. This results in a reduction of the focusing time. However, as mentioned, a high electrical field strength may lead to excessive heat development in the terminating zone.

The sample zone velocity is independent from the capillary diameter. However, the pressure (and height) difference is inversely dependent on the square of the capillary diameter. Capillaries with smaller inner diameters have better heat-dissipating properties and therefore higher electric field strengths are allowed. Because of a decreased loadability and detectability at small inner diameters an optimum can be found with respect to electric field strength, analysis time, capillary diameter and determination limits.

4.2. Single capillary ITP–CZE without a hydrodynamic counterflow

During the ITP step the electric field strength in the terminator (E_T) and leading buffer zone (E_L) and the corresponding zone velocities can be calculated. From Eqs. 9 and 10 it follows that when x (relative leading zone length) approaches unity, the terminating electric field strength increases to E_0/γ and the electric field strength in the leading zone increases to E_0. For the focusing step this means that during the removal of the terminating zone the electric field strength and the corresponding electrophoretic and electroosmotic velocities in the leading and the terminating zones increase. For anionic separations the electroosmotic and electrophoretic velocities increase in opposite directions. Under certain conditions the possibility exists that at a certain terminator length the electrophoretic velocity is counterbalanced by the electroosmotic velocity, without application of a pressure.

In Fig. 3A the calculated electrophoretic and electroosmotic velocities during the focusing procedure are shown for a given position in the capillary. Although the local electroosmotic velocities in the leading ($v_{eof,L}$) and terminating zone ($v_{eof,T}$) vary during the focusing procedure, the bulk electroosmotic flow velocity (v_{eof}, line A) is more or less constant. The electrophoretic velocity ($v_{el,L}$, line C) of the anionic leading ions increases in the opposite direction (gets more positive). This means that the total sample zone velocity (v_L, line B) reduces as the terminating buffer is removed from the capillary using a constant voltage.

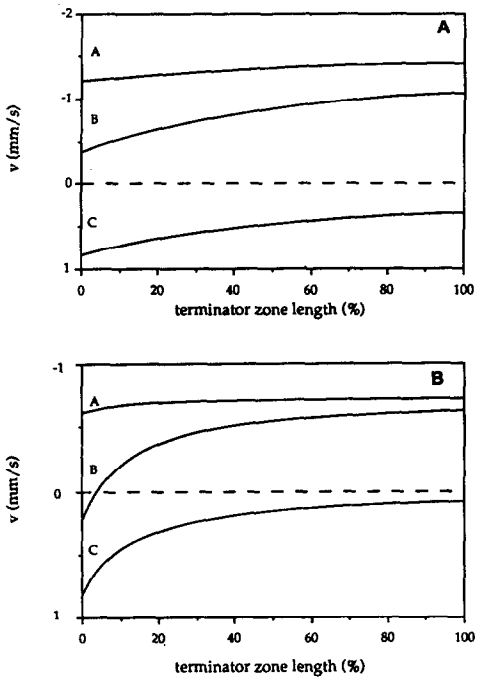

Fig. 3. (A) Variation in the bulk electroosmotic (line A, v_{eof}) and the electrophoretic (line C, $v_{el,L}$) flow velocity for a given position in the capillary. The total sample zone velocity (line B, v_L) decreases during the removal of the terminator zone. All further parameters are given in the Experimental section. (B) The same velocities as in (A) were calculated for an electroosmotic and electrophoretic mobility of respectively 30 and $-42 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$ and a γ of 0.1. At 4% terminator zone length the electrophoretic velocity is counterbalanced by the electroosmotic velocity. This implies that in principle anionic ITP–CZE can be carried out without a hydrodynamic counterflow.

When the electroosmotic mobility in the leading zone is approximately equal to the electrophoretic mobility of the leading ions, there is one value of x where the zone velocity is zero. A

reduced pressure is necessary to remove the remaining terminator buffer. The sample zone velocity will be reversed as the terminator zone length is getting smaller. Under these conditions (*i.e.* $-m_L \approx m_{eof,L}$) in principle the complete ITP–CZE procedure can be carried out without the application of a hydrodynamic counterflow.

Under the conditions in Fig. 3B the leading zone velocity in is zero at 4% terminator zone length. This means that when ITP–CZE is carried out without application of a counterflow the sample zones will be focused at 4% terminator zone length. Under these conditions the velocity of the front boundary of the sample zone is not counterbalanced by a hydrodynamic flow. As a result the terminator zone length containing the analyte ions is reduced to 4% already during the focusing step. As mentioned in the Theory section, this means that a loss of analyte may occur. Therefore the sample should be solved in leading buffer so that the rear boundary of the sample zone is the boundary between leading and terminating buffer. During focusing this boundary moves to the 4% position and remains there. The front boundary will migrate to the rear boundary without loss of analyte. The boundary velocity and the focusing time are then given by Eqs. 6 and 32, respectively. After the focusing step the CZE can be started without the additional step of the removal of the terminator buffer, providing that the 4% zone does not disturb the CZE separation. Fine tuning of the leading ion velocity with respect to the electroosmotic flow velocity is one way of optimizing such an ITP–CZE procedure. This broadens the applicability of automated anionic single capillary ITP–CZE to all commercially available systems that are not capable of fine adjustment of a pressure.

4.3. Verification of sample zone velocity

To verify the derived Eq. 16 a counterflow experiment was carried out using height differences for focusing a zone of coloured dye on a fixed position in the capillary. The height difference was measured at two points, where the sample zone was slowly moving forward and where the sample zone was slowly moving backward. The mean value was considered the height difference where the sample zone velocity was zero. At height differences close to zero, where the direction of the sample zone velocity reversed, it was not possible to obtain reliable results. A hysteresis in the zone velocity was observed when counterbalancing it with a hydrodynamic velocity. Different results were obtained depending from which direction the height difference was changed. At height differences of 5 cm or more the data became reproducible. In Fig. 4A the calculated line and measured data points are shown. The deviation of the measured data points from the calculated line may be due to temperature effects which

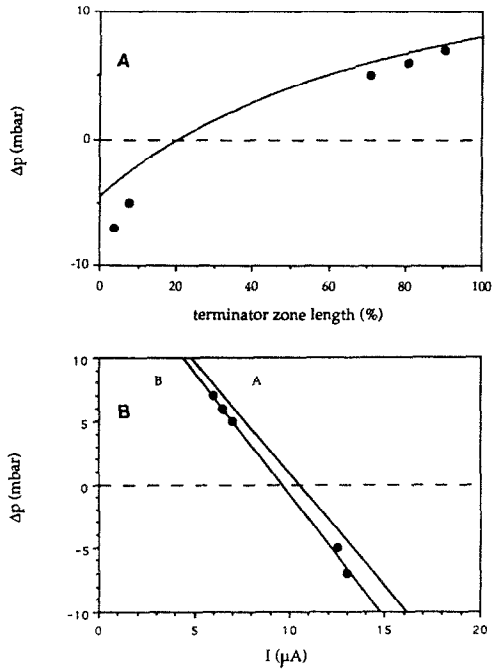

Fig. 4. (A) Calculated counterbalancing pressure difference in ITP–CZE (line) and the measured data points at several positions in the capillary. A negative pressure difference was needed for complete removal of the terminator buffer from the capillary. All parameters are given in the Experimental section. (B) For several positions of a sample zone in the capillary the running current was plotted against the counterbalancing pressure. Calculated (line A) and measured (line B) data result in straight lines as predicted by Eq. 24. The same data points as in (A) are used.

become more pronounced at small terminator zone lengths.

In Fig. 4B the calculated current is plotted against the pressure difference needed for a proper counterflow which is a straight line (Eq. 24). The same data points as in Fig. 4A are used in Fig. 4B. The regression line of the calculated data was $p = 18.4 - 1.76I$, the line of the measured data was $p = 18.5 - 1.92I$. The measured intercept is approximately similar to the calculated intercept. The calculated slope however is 9% below the value of the measured slope. Using the regression line for $p = 0$ the current at zero sample zone velocity (*i.e.* $-v_{el,L} = v_{eof}$) is calculated. For the calculated line this was at 10.5 μA, for the measured data this was 9.63 μA.

Fig. 4B demonstrates that relatively small differences in the used parameters may result in considerable differences in the calculated and the actually needed pressure, especially near the point of reversal of the pressure. However, it should be kept in mind that current monitoring will be a convenient tool in compensating for these differences. Only when the appropriate pressure is applied under ITP conditions the current will be constant. In the Experimental section all parameters and those used for calculations are given.

4.4. Temperature effects

One of the assumptions in the described model is that the temperature in the leading and terminating zone is the same and constant. For ITP this is by definition not true because the electrical field strength in the leading buffer is lower than in the terminating zone. However, in systems with efficient dissipation of the Joule heat the effects of axial temperature differences will be negligible. The temperature inside the capillary can be calculated using [13]

$$T = T_a + \frac{E^2 \kappa d_1^2}{8} \cdot \left[\frac{1}{k_1} \ln\left(\frac{d_2}{d_1}\right) + \frac{1}{k_2} \ln\left(\frac{d_3}{d_2}\right) + \frac{2}{h d_3} \right] \quad (33)$$

where d_1, d_2 and d_3 are the inside capillary diameter, the outside fused-silica diameter and the outside polyimide diameter, respectively. T_a is the working temperature and h is the heat-transfer coefficient to the surroundings. The thermal conductivities k_1 and k_2 are respectively 1.5 W m^{-1} K^{-1} for silica and 0.16 W m^{-1} K^{-1} for polyimide. For example, the temperature in the leading and terminating zone is calculated for a terminating zone length of 60% and 10% of the total capillary length under conditions as described for Fig. 2.

When the capillary is filled for 60% with sample in terminating buffer, the temperatures inside the leading and terminating zones are 293.3 and 293.8 K, respectively ($T_a = 293$, $E_L = 10.4$ kV m^{-1}, $E_T = 26.4$ kV m^{-1}, $\kappa_L = 0.070$ S m^{-1}, $\kappa_T = 0.027$ S m^{-1}, $d_1 = 100$ μm, $d_2 = 340$ μm, $d_3 = 355$ μm, $h = 180$ W m^{-2} K^{-1}, for other conditions see the Experimental section). The viscosity and electrophoretic mobility decrease 2.6% per degree K, which means a difference of 1.3% in viscosity and mobility is expected under these conditions. The effects on the calculated counterbalancing pressure will be negligible.

After the focusing step the terminator buffer is removed from the capillary. When the length of the terminating zone is 10% of the total capillary length the temperatures inside the leading and terminating zones are 293.8 and 295.1 K, respectively ($E_L = 17.3$ kV m^{-1}, $E_T = 44.1$ kV m^{-1}). When the running voltage is reduced to 6 kV ($E_0 = 12$ kV m^{-1}) the temperatures in the leading and terminating zones become 293.3 and 293.8 K, respectively ($T_a = 293$, $E_L = 10.4$ kV m^{-1}, $E_T = 26.5$ kV m^{-1}). As the terminating zone gets smaller the temperature will increase. In our experience, the corresponding decrease in viscosity does not interfere with automated ITP–CZE procedures.

4.5. Current monitoring

The calculated change in current (Eq. 20) is in good agreement with the measured data (Fig. 5). The change in current at constant voltage during the focusing procedure is calculated for several other conductivities of the terminator buffer

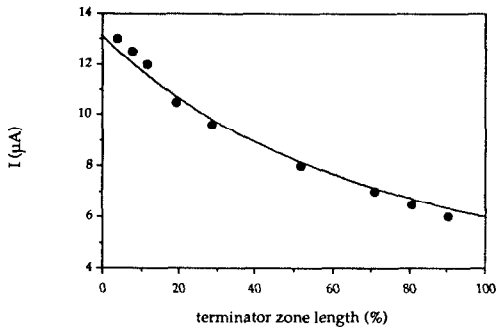

Fig. 5. Calculated change in current at a constant voltage during the focusing step in ITP–CZE (line) and the measured data points at several positions in the capillary. All parameters are given in the Experimental section.

(Fig. 6). The larger the difference in conductivity, the larger the change in current. In practice large differences in conductivity will make automated switching from ITP to CZE using current monitoring easier.

When the zone length of terminator buffer is 5% of the total capillary length, the current is at 95% of I_0 for a conductivity ratio of 0.48 (line A), where I_0 is the maximum current when the capillary is completely filled with leading buffer. For the same terminator zone length the current is at 50% of I_0 for a conductivity ratio of 0.048 (line C). In practice this means that at large differences in conductivities smaller terminator zone length will remain after automated switching from ITP to CZE. However, in case of similar conductivities of leading and terminator buffer, somewhat larger remaining terminator zone lengths are allowed. The effect on zone broadening in the CZE step caused by conductivity differences will consequently be less.

In Fig. 7 the effect of the length of the remaining terminator zone on the CZE separation is shown. When the current at the moment of switching is lower than 92% of I_0 zone broadening occurs. The corresponding remaining terminating zone length is 10% of the total

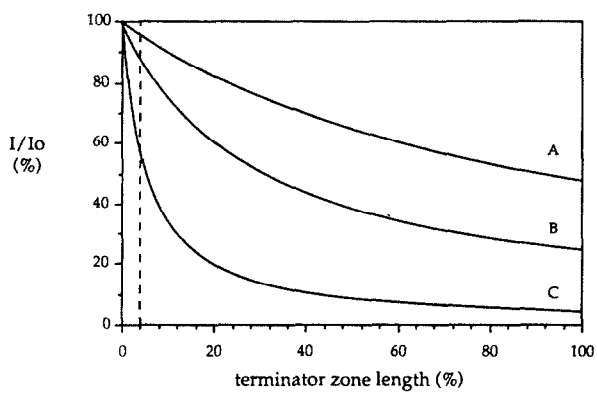

Fig. 6. The calculated change in current at a constant voltage during the focusing step in ITP–CZE. A conductivity ratio of terminating and leading buffer (γ) of 0.48 (A), 0.24 (B) and 0.048 (C) is used for calculation. The conductivity of the leading buffer (κ_L) was kept constant. The ionic mobility of the terminator ions was $-22.0 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$. All other parameters are given in the Experimental section.

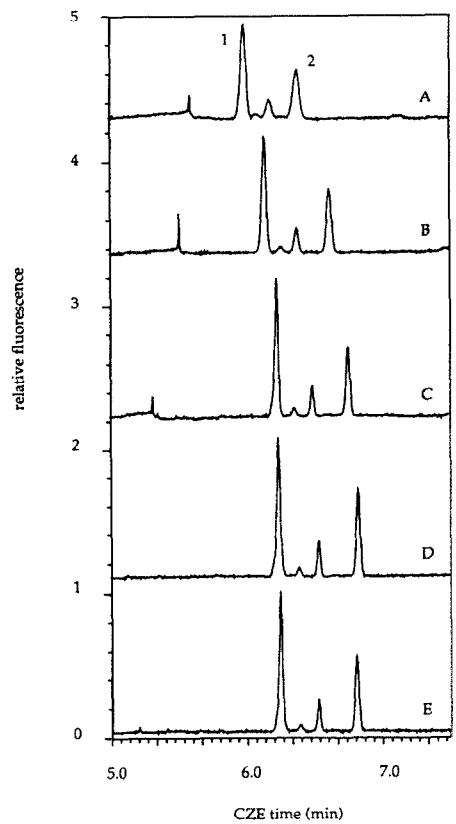

Fig. 7. The effect of the remaining terminator buffer zone length on the CZE separation of FITC (1) and F (2) in ITP–CZE. Other peaks are unknown degradation products of FITC. Automated switching using current monitoring was used at 88.2% (A), 90.9% (B), 93.6% (C), 96.3% (D) and 99.0% (E) of the maximum current I_0. Because of a decrease in the migration length in CZE a decreased migration time is notable as the remaining terminating zone gets larger.

capillary length. Although the 10% seems like an overload of the CZE, the effect on the peak shape is relatively small. Because of the conductivity ratio of 0.48 the disturbance of the homogeneity of the electric field by the remaining terminator zone is limited. A larger difference in conductivity would result in more peak broadening, but the consequently smaller conductivity ratio would make precise switching with small remaining terminator zones easier (Fig. 6, line C: $\gamma = 0.048$).

The reproducibility of CZE migration times in ITP–CZE with automated switching using current monitoring was investigated for six ITP–CZE runs. The current was programmed at 99% of I_0 at the switching time. The R.S.D. in migration times was 1.4%. This is approximately three times higher in comparison with CZE in a bioassay of anthracyclines where a R.S.D. of the migration times of 0.5% has been reported by our group [4].

5. Conclusions

Equations have been derived giving the zone velocity and the current for a given terminator zone length in single capillary ITP–CZE. Monitoring the current offers the possibility to calculate the hydrodynamic flow velocity that is needed to counterbalance the leading zone velocity. It is therefore expected that current monitoring can be used in an automated feedback mechanism to control the applied pressure. Correlation between current and the position of the sample zones in the capillary can be used for automated switching from ITP to CZE. This results in reproducible CZE migration times and will make the implementation of automated focusing procedures in bioassays easier.

Under certain conditions ITP–CZE separations can be carried out even without the application of a hydrodynamic counterflow. This extends the applicability of this procedure to equipment that is not capable of applying a hydrodynamic pressure during the focusing step.

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