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Automated isotachophoretic analyte focusing for capillary zone electrophoresis in a single capillary using hydrodynamic back-pressure programming

N.J. Reinhoud, U.R. Tjaden* and J. van der Greef

Division of Analytical Chemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden (Netherlands)

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ABSTRACT

An automated isotachophoretic (ITP) analyte focusing procedure prior to capillary zone electrophoresis (CZE) is described. The ITP focusing step is carried out in the same capillary as the CZE. Hydrodynamic back-pressure programming resulted in a reduction in the effects of the electroosmotic flow-rate during the ITP step and allowed the removal of terminating buffer before the CZE run was started. The characteristics of this on-line focusing procedure were studied for several anionic test compounds.

INTRODUCTION

Capillary electrophoresis (CE) has become an important separation technique complementary to high-performance liquid chromatography (HPLC). Several reviews have described the role of capillary electrophoretic separation methods in analytical chemistry [1–3]. Improvements in detection techniques resulted in the detection of extremely small amounts of analyte. Nevertheless, owing to the small dimensions of the separation system and the small injection volumes, the corresponding concentration detection limits are relatively high.

Different on- and off-line methods to improve the concentration detection limits in CE have been described. Off-line sample pretreatment and preconcentration, such as concentrating liquid–liquid or solid-phase extraction, offer flexibility in the choice of buffers and the amount of sample to be pretreated. These procedures are laborious and time consuming. On-line sample pretreatment offers the possibility of automation but has some restrictions with respect to the following step in the analytical method. Examples of on-line sample pretreatment and/or preconcentration are the combination of LC and CE [4–6] and of isotachophoresis (ITP) and capillary zone electrophoresis (CZE) [7–10].

An elegant way of lowering the determination limits that is typical for zone electrophoretic separations is the application field-amplified on-capillary sample concentration or stacking. This can be done in several ways, by using low-conductivity sample buffers, by using sample self-stacking, by addition of an electrolyte to the sample, allowing a transient isotachophoretic preconcentration, or by combining some of these methods [11–16].

In this paper we describe a procedure for the automated coupling of ITP with CZE using a single open capillary in a commercially available electrophoresis system without modification of the system. By using hydrodynamic back-pressure programming during the focusing step the

* Corresponding author.

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terminating buffer could be removed before the CZE step, resulting in highly efficient separations.

EXPERIMENTAL

Untreated fused-silica (100 μm I.D.) (SGE, Ringwood, Victoria, Australia) and UV-transparent (75 μm I.D.) (Polymicro Technologies, Phoenix, AZ, USA) capillaries were used. A programmable injection system for CE (PRINCE; Lauerlabs, Emmen, Netherlands) equipped with a reversible polarity power supply and the possibility of pressurized and/or electrokinetic injection was used for the analyte-focusing process.

On-capillary laser-induced fluorescence (LIF) detection took place using a water-cooled argon ion laser (Model 2025-03; Spectra-Physics, Mountain View, CA, USA) lasing at multiple wavelengths of 351.1 and 363.8 nm at 20 mW for excitation. The excitation wavelengths were filtered through a 350-nm band pass filter (10-nm band width). Fluorescence light was transported to the photomultiplier tube (PMT) through a liquid light guide (1000 × 5.0 mm I.D.) (Model 77556; Oriel, Stratford, CT, USA) equipped with a plano-convex fused-silica lens ($D = 11$ mm, focal length = 19 mm) (Model 41210; Oriel) at each end. The fluorescence light was directed on to a 450-nm band pass interference filter (10-nm band width, Type 53830; Oriel) for *o*-phthaldialdehyde derivatives or on to a 525-nm interference filter (10-nm band width) for fluorescein and analogues. The PMT (Model 9635B; Thorn EMI, Ruislip, Middlesex, UK) was operated at 800 V (Model 244 power supply; Keithley Instruments, Cleveland, OH, USA).

The signal was amplified by a current amplifier (Model 427; Keithley Instruments) and registered on a chart recorder (Kipp and Zonen, Delft, Netherlands) or digitized using a laboratory-made 12-bit A/D converter operating at a frequency of 20 Hz. The A/D converter was connected to a computer (Mega ST4; Atari, Sunnyvale, CA, USA) controlling the sampling frequency of the converter and the data handling.

Chemicals

Phosphoric acid, barium hydroxide, glutamic acid (Glu), aspartic acid (Asp) and bromophenol blue were obtained from Merck (Darmstadt, Germany). Sodium cacodylate and fluorescein (F) were purchased from Janssen Chimica (Beerse, Belgium). Hydroxypropylmethylcellulose (HPMC) was supplied by Sigma (St. Louis, MO, USA). The viscosity of a 2% aqueous HPMC solution is 4000 cP. Fluorescein isothiocyanate isomer I (FITC), *o*-phthaldialdehyde (OPA) and mercaptoethanol (ME) were obtained from Aldrich Chemie (Steinheim, Germany). In all experiments deionized water was used, obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

OPA derivatives of amino acids were prepared by adding 1 ml of amino acid dissolved in leading buffer to an OPA solution of 1 mg/ml in leading buffer containing 0.1% ME. Unless mentioned otherwise, the OPA derivatives were analysed after 5 min. Sample solutions of fluoresceins were prepared in leading buffer unless mentioned otherwise.

RESULTS AND DISCUSSION

Analyte focusing after hydrodynamic sample introduction

A discontinuous buffer system was used to permit ITP sample preconcentration (Fig. 1). The capillary and ITP anode vial (which is also the CZE cathode vial) are filled with leading buffer consisting of 10 mmol/l sodium phosphate (pH 9.4). To the leading buffer 0.05% (w/v) of HPMC was added. The HPMC only reduced the electroosmotic flow in the fused-silica capillary to such an extent that acceptable preconcentration runs could be made. The leading buffer was also used as the CZE buffer.

Step 1. Injections were made hydrodynamically at a pressure of 10–600 mbar. After injection, a terminating buffer vial containing 10 mmol/l sodium cacodylate buffer (pH 8.2) with 0.05% (w/v) HPMC was placed at the capillary inlet. For the analysis of OPA derivatives the pH was raised to 9.4 using $Ba(OH)_2$.

Step 2. The analyte focusing started by applying a voltage of −25 kV in conjunction with a

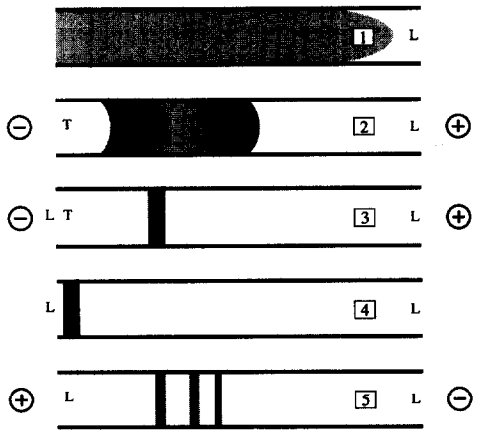

Fig. 1. Schematic representation of ITP–CZE in a single capillary. The leading buffer (L) is also used as electrophoresis buffer in the CZE step. The terminator buffer (T) is removed from the capillary in step 3. See text for further details.

hydrodynamic back-pressure of 40 mbar. This negative voltage resulted in an electroosmotic flow in the direction of the capillary inlet. To prevent the analyte from migrating out of the capillary a hydrodynamic back-pressure was applied. Because of the self-correcting properties of the ITP zones, the hydrodynamic back-pressure did not result in peak broadening. This is in contrast to field-amplified injection techniques where electroosmotic convections are a major source of peak broadening of large injection plugs [11].

Step 3. After 5–20 min of focusing, depending on the volume injected, the cacodylate buffer vial was replaced with the CZE buffer vial. A voltage of −20 kV was applied without a hydrodynamic back-pressure. As the capillary still contained cacodylate buffer, this step was carried out under real isotachophoretic conditions, although a vial containing phosphate buffer was placed at the terminator end. The sample zones were sandwiched between the anodic leading buffer and the terminating buffer plug, which is in the capillary. The electroosmotic flow-rate was higher than the mobility of the phosphate ions in the vial at the terminator end, thus preventing these ions from entering the capillary. Simultaneously the plug of terminating buffer was removed from the capillary. It is important for the next step that the CZE buffer vial is already in position.

Steps 4 and 5. At the time that the sample zone was approaching the capillary inlet the voltage was reversed and the CZE run was started at a positive voltage of 30 kV. The correct timing of the voltage switching is important. If the ITP process is continued for too long, the sample zones will migrate out of the capillary into the inlet buffer vial. If the voltage is switched too early a plug of terminating buffer is still in the capillary and will disturb the homogeneity of the electric field necessary for CZE, resulting in peak broadening. Initially bromophenol blue was used as a visible marker. When reproducible ITP run times were measured, automated programming was applied and the marker could be omitted. When the use of a visible marker is inconvenient because of hardware incompatibility, timing can be done by monitoring the current. Applying a constant voltage, the current increases as long as the terminating ions leave the capillary.

In principle, all terminating ions and sample ions with mobilities below that of the analyte ion can be removed by the described procedure. This is an important difference from transient-like isotachophoretic preconcentration [12,15]. Another advantage of focusing under isotachophoretic conditions using a hydrodynamic back-pressure is that the time needed for focusing can in principle be as long as necessary for a given analysis without peak broadening or loss of analyte. An optimum can be found between the determination limit of an analyte for a given injection volume and the time needed for focusing.

The analyte focusing was studied for OPA derivatives of Glu and Asp derivatized before injection. The derivatives have an excitation maximum at 340 nm fitting with the 351.1/363.8 nm lasing wavelengths of the argon ion laser. The laser beam was filtered with a 350-nm band pass filter before focusing on to the capillary. This reduced considerably the background at 450 nm, the emission maximum of the OPA derivatives. Because OPA derivatives are not stable with time [17], some characteristics of the system were investigated with F and FITC. These com-

pounds have native fluorescence with an excitation maximum around 490 nm. Although this excitation maximum fits perfectly with the 488-nm lasing wavelength of the argon ion laser, a good signal was obtained using the 351.1/363.8 nm lasing wavelengths for excitation. For convenience the laser optics were not changed and all experiments were done using the same laser system, except for the emission wavelength, which was 525 nm for the fluoresceins. The effect of analyte focusing was compared with CZE runs without focusing.

Loadability and linearity for OPA derivatives

When analyte focusing was carried out for injection volumes similar to those in CZE runs, the improvement in detectability was limited (Fig. 2A and B). An explanation for this effect is that in CZE the analytes migrate through a background electrolyte whereas in ITP the analytes are sandwiched as distinct zones between leading and terminating electrolytes. In ITP–CZE, when the focusing procedure has been completed and the CZE is started, mixing of the analyte zones with the background electrolyte occurs. This results in some band broadening and dilution. In Fig. 2B the ITP preconcentration of analyte is levelled by this mixing process, resulting in comparable peak heights. Fig. 2C shows that increasing the injection volume by a factor of 5 results in an increase in the signal by a factor of 5.

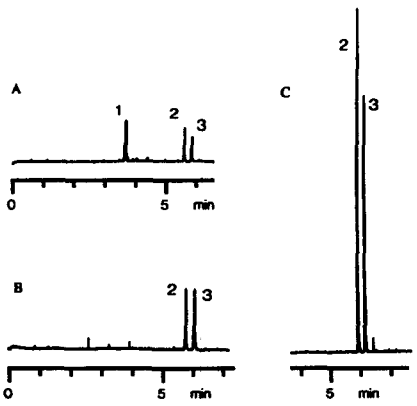

Fig. 2. Electropherograms of a mixture of 200 ng/ml OPA derivatives of (1) Phe, (2) Glu and (3) Asp. Injection volume of (A) 70 nl using CZE compared with (B) 70 nl and (C) 350 nl using ITP–CZE. A 700 × 0.1 mm I.D. fused-silica capillary was used. The derivative of Phe had a lower mobility as the terminator and was removed from the capillary in ITP–CZE.

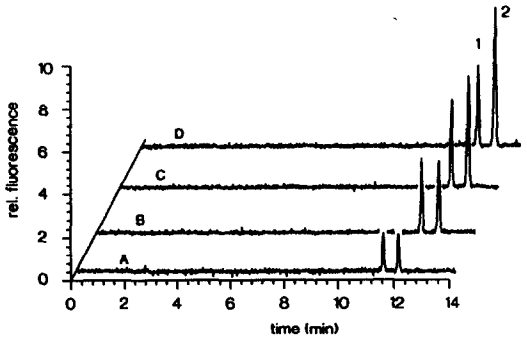

Fig. 3. Effect of injection volume on the analysis of a derivatization mixture of 1 μg/ml of (1) Glu and (2) Asp with OPA. Injection volume: (A) 0.2; (B) 0.7; (C) 1.4; (D) 2.4 μl. A 700 × 0.1 mm I.D. fused-silica capillary was used.

Figs. 3 and 4 show that the capillary can be filled to 50% (2.4 μl) without band broadening. For the OPA derivative of Glu there is an optimum in the loadability with respect to peak height, whereas the peak height of the Asp derivative does not increase linearly with the injection volume. At larger injection volumes longer focusing times were necessary, resulting in degradation of the unstable derivatives. The focusing time increased from 4 min for a 0.2-μl to 20 min for a 2.4-μl injection volume. To eliminate these effects we studied the loadability, linearity and reproducibility with F and FITC.

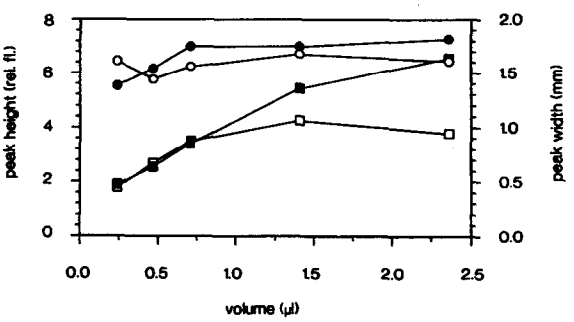

Fig. 4. The peak height of (■) Asp increases whereas the peak widths of (●) Asp and (○) Glu remain the same with increasing injection volume. The peak height of (□) Glu shows an optimum. The peak width is given as half of the peak width measured at 60% of the peak height. The corresponding electropherograms are shown in Fig. 3.

Loadability, linearity and reproducibility for fluoresceins

F and FITC are more stable than OPA derivatives under alkaline conditions. FITC is a well known fluorescent probe for the derivatization of amines. The reaction products are usually anionic because of the charges on FITC. It is therefore expected that the reaction products can be analysed in a similar way using ITP–CZE.

As can be seen from Fig. 5, the increase in detectability of F corresponds to the increase in injection volume. The total analysis time for the CZE run was 10 min, whereas the ITP–CZE run took 20–24 min. This included a flush of 1 min with 10 mmol/l KOH, 1 min with leading buffer and an ITP focusing step of 10 min. The flushing was necessary to obtain reproducible migration times.

Loading of the capillary with large sample plugs changes the condition of the capillary wall, especially when a dynamic coating of HPMC is used. For relatively clean samples and constant injection volumes, this resulted in a small shift in the migration time. However, when the injection volumes are increased the effects on the capillary wall are more severe. In Fig. 5 the migration times in the CZE step increased by *ca.* 65% for both analytes. In the study of fluoresceins a buffer of pH 8.1 was used, implying that at longer focusing times the pH in the capillary decreases. As a result, the electroosmotic flow in the CZE decreased, resulting in increased migration times. This shift did not occur with the OPA derivatives (Fig. 3). For the OPA derivatives a terminator buffer at the same pH as the leading buffer (pH 9.4) was used. The use of a buffering counter ion (*e.g.* $Tris^+$) is therefore advisable. However, for our test compounds the CZE performance was better with sodium phosphate buffer.

In Fig. 6 the effect of the focusing time on the performance in ITP–CZE is demonstrated. A focusing time of less than 5 min results in a decrease in signal because of incomplete focusing. However, increasing the focusing time from 10 to 34 min did not result in a change in resolution or efficiency. This is an important observation because it demonstrates that band broadening is independent of time in the focusing procedure, although a hydrodynamic back-pressure is used. Everaerts *et al.* [18] used a counterflow of electrolyte in ITP to increase the effective length of the separation system. They measured a considerable disturbance when a counterflow higher than 30% of the mobility of the sample zones was applied. They concluded that the optimum counterflow depended on, amongst other things, the capillary diameter, the viscosity and the temperature in the capillary. In our ITP–CZE system no peak broadening was seen at a back-pressure which is 100% of the analyte mobility. In principle this means that the effective length of our ITP system has been increased to infinity. However, the analysis time increases similarly. Therefore, an optimum has

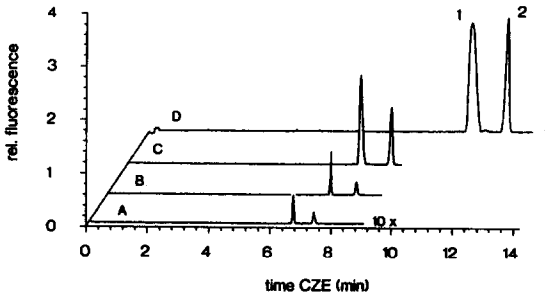

Fig. 5. Loadability in ITP–CZE of a mixture of (1) FITC and (2) F at a concentration of 500 ng/ml in leading buffer. Injection volume: (A) 14; (B) 140; (C) 750; (D) 1500 nl. The peak height of F and the peak area of F and FITC showed a linear increase with increasing injection volume. A 700 × 0.075 mm I.D. fused-silica capillary was used.

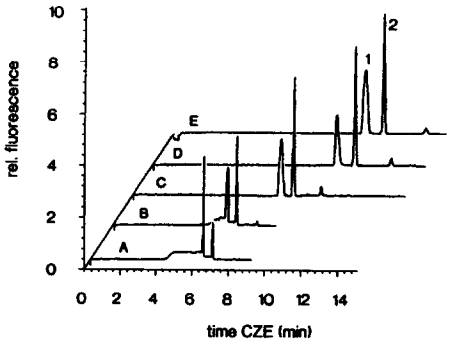

Fig. 6. Effect of focusing time on the performance in ITP–CZE of a mixture of 50 ng/ml of (1) FITC and (2) F. Injection volume, 1.5 μl. A 700 × 0.075 mm I.D. fused-silica capillary was used. Focusing time: (A) 1.8; (B) 4.5; (C) 10; (D) 18; (E) 34 min.

to be found between the injection volume, the complexity of the sample, the determination limit and the time needed for focusing of large injection plugs.

A study of the reproducibility in CZE and ITP–CZE of a mixture of FITC with F is shown in Fig. 7. Peak areas gave better results than peak heights. In ITP the zone length varies with the concentration. As a result, it is expected in ITP–CZE that the peak area will give a better correlation than peak height with analyte concentration. The relative standard deviations (R.S.D.s) of peak areas for CZE were 3.5% and 5.3% for F and FITC, respectively (Fig. 7A), and those of peak heights were 8.4% and 8.6%, respectively. For ITP–CZE R.S.D.s of peak areas of 6.5% and 5.3% were measured for F and FITC, respectively (Fig. 7B); the corresponding values were 9.8% and 18% for peak heights. When the ratio of peak areas was used R.S.D.s of 4.6% in CZE and 4.8% in ITP–CZE were found. The improvement in the R.S.D.s for peak-area ratios demonstrates the necessity to use an internal standard in quantitative analysis with ITP–CZE.

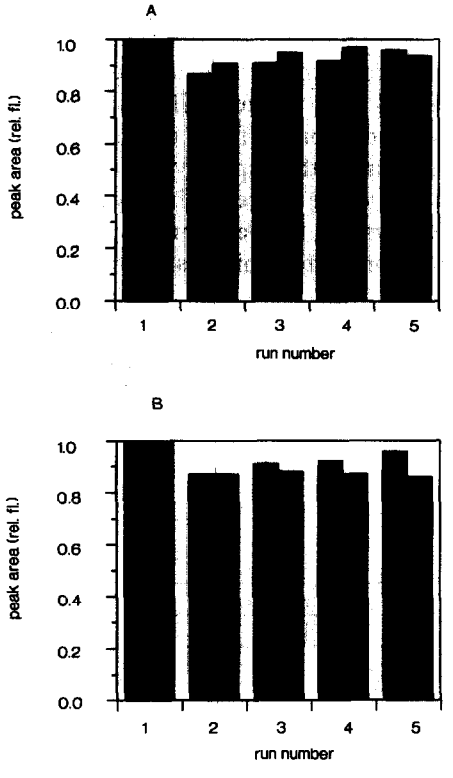

Fig. 7. (A) Reproducibility of peak areas of five CZE runs of a mixture of 1 μg/ml of F (grey bars) and 0.5 μg/ml of FITC (black bars) at an injection volume of 30 nl. (B) Reproducibility of peak areas of five ITP–CZE runs of a mixture of 10 ng/ml of F (grey bars) and 5 ng/ml of FITC (black bars) at an injection volume of 1.7 μl.

The linearity of the method was studied at a concentration level just above the detection limit. Calibration graphs were constructed for FITC between 0.2 and 18 ng/ml. F was used as internal standard at a concentration of 25 ng/ml. The peak-area ratios were used for linear regression. Table I gives the regression data calculated for linear regression as described by Miller [19].

Practical considerations

Several practical aspects are of importance for the applicability of the method to analyses in complex matrices such as urine or plasma samples.

First, a separation window exists in the ITP step within which the analyte of interest is focused. This separation window is an important tool in sample clean-up and can be tuned by the choice of the leading and terminating buffer. In the literature numerous ITP systems have been described [18].

The anions that are faster than the leading buffer ions migrate into the separation capillary already during the ITP step. Ions with lower mobilities than the terminator ions, including neutral species and cations, are removed from

TABLE I

REGRESSION DATA FOR ITP–CZE OF FITC USING F AS AN INTERNAL STANDARD

A 700 × 0.075 mm I.D. fused-silica capillary was used with an injection volume of 1.7 μl. Weighting factors were not necessary because calibration was carried out with five different concentrations with constant concentration intervals of 0.4 ng/ml for the low concentration range and 4.0 ng/ml for the higher concentration range.

Range (ng/ml)	Slope	S.D.	Intercept	S.D.	Correlation coefficient
0.2–1.8	0.137	0.010	−0.012	0.01	0.985
2.0–18.0	0.142	0.015	0.02	0.15	0.965

the capillary in the ITP step, resulting in a considerable sample clean-up. It is therefore important to choose a favourable set of buffers, *i.e.*, a leading buffer with a high mobility to reduce the number of matrix anions with a higher mobility. The electrophoretic mobility of the terminator buffer should be as close to that of the analyte as possible. The pH of the buffers is often an important parameter for fine tuning the mobilities of the analytes and the buffer ions.

Second, the separation order of anions in ITP is reversed with respect to CZE. Anions with a high electrophoretic mobility migrate, after focusing and reversal of the voltage, through the analyte zones with a lower electrophoretic mobility. In the analysis of, *e.g.*, urine samples, the possibility exists that a zone of matrix ions migrates as a spacer between two analyte zones. Depending on the zone length of the matrix ions, the migration times in CZE may change. With clean samples as described above, no difference in migration times in ITP–CZE with respect to CZE has been observed. Because a low concentration of analyte (*i.e.*, nanomolar range) is concentrated in the ITP step to approximately that of the leading buffer ions (*i.e.* millimolar range), the starting zone length will be reduced from decimetres to micrometres. A mixing of zones occurs as soon as the ITP has ended and the CZE has started, which is caused by diffusion, convection and dilution with background electrolyte. As a result, although strongly concentrated, the analyte ions start in the CZE process more or less as one mixed zone. In biological samples, however, a number of ions are present at a much higher concentration. Depending on their mobilities and of the separation window, these matrix ions may act as spacers for analyte ions. After switching from the ITP to the CZE mode, the analytes start the CZE run at different positions in the capillary, resulting in different migration lengths. In these cases a sample pretreatment might be necessary to obtain reproducible results. On the other hand, an excess of spacer ions can be added deliberately to the sample to improve the resolution in ITP–CZE separations.

Finally, in the ITP step a hydrodynamic backpressure is levelling the electroosmotic flow-rate. It is obvious that fluctuations in either of them would result in decreased reproducibility. It is therefore of crucial importance that the pressure can be monitored and controlled accurately during the process, which is the case with the described system. Further, the electroosmotic flow should not change from run to run. A constant pH is important with respect to the electroosmotic flow-rate and it is therefore advisable to use a buffering counter ion. Injection of large sample plugs of a different pH may cause local changes in the electroosmotic flow. In these cases the samples should be pH adjusted or pretreated. The same applies to samples with large differences in salt concentration. As a consequence, the method will be suitable especially for the analysis of compounds in a constant matrix with respect to pH and salt concentration, such as plasma.

CONCLUSIONS

An automated procedure has been developed for the isotachophoretic preconcentration of low concentrations of analyte using ITP–CZE in a single capillary in combination with hydrodynamic back-pressure programming. The described method is reproducible and linear at low concentrations. An improvement in the determination limit of more than 100 with respect to CZE has been demonstrated. The increase in loadability is only limited by the capillary volume. An optimum has to be found between the injection volume, the determination limit and the time needed for focusing of large injection plugs.

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