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Analytical aspects of carrier ampholyte-free isoelectric focusing☆

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Abstract

The applicability of carrier ampholyte-free isoelectric focusing (CAF-IEF) for analyses of ampholytes is demonstrated. The suggested method is based on the principle of both side regulated ionic matrix in CAF-IEF. A sharp step of pH is created in the column filled with a sample dissolved in a background electrolyte by influence of current and solvolytic fluxes. Here, ampholytes are focused upon. The magnitude of the step, its velocity and direction of its movement can be regulated electrically. In this manner, favorable separation properties of the system can be set up, even during the run. This brings several advantages over conventional methods. The principles of the separation can be easily changed, permitting selective pre-concentration (trapping) of minor components by processing large amounts of a sample to be preformed, effective isotachophoresis or IEF pre-separation and final electrophoretic analysis in one run. Advantages of these combinations are discussed together with the right choice of the working electrolyte. A 1000-fold increase in amount of substance in a column can be achieved for both isotachophoresis and capillary zone electrophoresis combined with CAF-IEF pre-concentration at reasonable working conditions. It enables a limit of detection at the nmol/l level with a concentration factor of about 10^7 to be reached. © 2001 Published by Elsevier Science B.V.

Keywords: Isoelectric focusing; Isotachophoresis–capillary zone electrophoresis; Sample preparation; Ampholytes

1. Introduction

Ampholytic substances like proteins, peptides, amino-acids, certain dyes and pesticides belong to a group of substances that play crucial roles in our lives. They compose a substantial part of biological materials and their metabolic pathways. They can be found as pollutants in the environment or as additives in food, etc. Ampholytes are nowadays intensively studied in different branches of science, including analytical chemistry. Analysis, separation, quantitation and identification can help us to understand them and reveal certain biological and environmental processes. These substances can be found in different and, often in very complex, environmental and biological matrices, such as water, soil, tissues and body fluids, which are hardly to analyze.

Common analysis of such a complex sample comprises mostly a sequence of individual steps (laboratory operational units), where the substance in question is isolated and pre-concentrated prior to its determination. Important analytical information can be lost during this procedure, owing to partial sample decomposition or adsorption of some compounds.

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This can be critical mainly for the determination of minor components.

An ideal method for the analysis of biological samples, having high variability of qualitative and quantitative compositions, is not available yet. However, a combination of suitable analytical methods can be the approximate. Analyses must be done in the presence of high concentrations of other compounds in most cases. Such analyses of complex mixtures requires the utilization of separation techniques in the first step since characterization of a new compound is not possible in a complex mixture. A separation method also has to produce a reasonable amount of the pure substance for further characterization. In the case of minor components it means that their concentration should be increased by several orders of magnitude. Moreover, the separation technique should not destroy the sample compounds.

All kinds of capillary electrophoretic methods, mainly capillary zone electrophoresis (CZE) [1], isotachophoresis (ITP) [2] and capillary isoelectric focusing (CIEF) [3], have been successfully adopted for the separation of ampholytes. Prospects for the use of electrophoretic methods in the comparison with chromatography are evident. These methods are fast, gentle and the adsorption of the sample is minimized.

ITP separates substances according to their effective mobilities. As a steady state method, it is perfect for pre-separation, pre-concentration and sample clean-up [4,5]. Usually it enables a relatively large amount of a sample to be injected. The typical volume is around 30 μl and in some special cases even 1000 μl [6]. The limit of detection (LOD) of ITP is in principle lower in comparison with the other techniques. When the steady state is reached, the substances migrate through the column in the form of consecutive adjacent rectangular zones which are not well resolved by the detector. To increase the LOD, spacers can be used, spatially separating the zones from each other. They transform the rectangular shape of the zones to a Gaussian form [7].

CZE in the comparison to ITP has higher resolution and better detection limits. As a stand-alone non-steady technique, it is not very convenient for the separation of analytes in more complicated samples with broader range of concentrations and for samples with low concentration of analytes. Typical amount of injected sample is low, in the range of hundreds of nanolitres, which puts a high demand on the detection.

Better analytical results can be obtained by the combination of CZE with ITP, transient ITP or stacking. These combinations of the techniques are excellent for the purpose of pre-concentration, pre-separation and sample clean-up prior to CZE detection step. They are less convenient for analyses of ampholytes because bulk of ionic substances are also pre-concentrated and the sample must be cleaned up [8–10],

The use of (CIEF) — a quasi equilibrium technique — seems to be more satisfactory for the separation of ampholytes. A large volume of a sample can be introduced (typically the whole volume of the column) and the concentration of ampholytes is increased during analysis.

Isoelectric focusing has been accepted as a standard procedure to characterize and separate ampholytes from their mixtures [11]. Substances are separated according their isoelectric points (p*I*) in the pH gradient created by a mixture of synthetic carrier ampholytes (SCAMs) along the column. The position of a substance indicates its p*I*. The drawbacks of cIEF are the influence of inorganic salts present in the sample, the presence of SCAMs as UV absorbing background and the necessity to mobilize the sample prior to detection.

Recently, a series of papers has been published, where a carrier ampholyte free-isoelectric focusing (CAF-IEF) method was introduced, verified and used for pre-concentration, selective pre-separation and micro-preparation of proteins and ampholytes [12–15]. The method is based on the principle of both side electrically regulated ionic matrix in CAF-IEF. A sharp step of pH — neutralization reaction boundary (NRB) — is created in the column filled with sample dissolved in background electrolyte, e.g., 0.01 *M* KCl, due to the influence of current and solvolytic fluxes (H^+, OH^-). Here, ampholytes are focused upon. The magnitude of the pH step, its velocity and direction of movement can be regulated electrically. By this manner, favorable separation properties of the system can be set up. Properties of NRB were further studied. It was revealed that the

method is quasi steady state and the so-called Kohlraush omega function is not constant during the analysis [16–18].

CAF-IEF, like IEF, increases the concentrations of ampholytes, removes non-ampholytes, separates species according to their p*I*, uses the maximal volume of the sample (whole capillary) for separation and separated zones does not leave a separation space. On the other hand, it has some features of ITP. It creates adjacent sharp boundary zones of the constant composition that moves with the same velocity (zero). Every zone contains only simple ions of background electrolyte in addition to separated substance.

A continuous dosing procedure was developed for the increasing amount of analytes in ITP [2,19]. A sample was introduced by electromigration to the column during the analysis from the terminating electrolyte chamber. To obtain reasonable accumulation of analytes in the column, the velocity of the ITP boundary has to be suppressed by applying counter-flow of the leading electrolyte. The magnitude of the counter-flow should not exceed 30% of velocity of ITP boundary, i.e., the time of accumulation is limited. This is not necessary in the case of CAF-IEF, where the NRB does not move. This enables unlimited time of dosing in CAF-IEF from this point of view. Practically we are limited only by the time of stability of the NRB, which can be influenced by appropriate selection of pH range and concentration of the electrolyte.

Another advantage of CAF-IEF with continuous dosing is that NRB traps only ampholytes, while non-ampholytes pass through. This fact is very important from A practical point of view because no ionic impurities from the electrolyte and bulk component from the sample are accumulated. Accumulation of impurities is the greatest limiting factor of regular pre-concentrations methods.

As mentioned above, CAF-IEF is ideal for preseparation and pre-concentration of ampholytes. Detection of the non-moving zones is the principal problem that can be solved using a sliding detector [20] or better combining of CAF-IEF with another technique, e.g., ITP or CZE after mobilization of zones.

The aim of our paper is to introduce CAF-IEF as a powerful pre-concentration technique for the separation of ampholytes and to show the possibilities of combining it with the other electrophoretic techniques like ITP and CZE. The hyphenation serves here as a detection technique.

2. Theoretical

The main properties of the ampholyte zones are derived from the properties of the neutralization reaction boundary in the CAF-IEF. The boundary is created in the column filled with non-modified background electrolyte (BGE) in the position, where the opposite migrating fluxes of H^+ and OH^- ions from electrolyte chambers meet each other.

Only one NRB is created without the presence of sample ampholytes in the electrolyte, i.e., one part of the column is filled with acid modified background electrolyte while the second part is modified with base.

If the magnitudes of fluxes of the H^+ and OH^- ions, J_H and J_{OH}, are equal:

$$J_H = J_{OH} = J_S \tag{1}$$

where the NRB has a fixed position in the column and does not move. In such a case a NRB becomes a source of ions for the BGE and its concentration falls down. The amount of depleted ions of BGE (ΔJ_C, ΔJ_A) is influenced by the magnitude of the solvolytic fluxes, J_S, and by the mobilities of all participating ions:

$$\Delta J_C = \Delta J_A = \frac{J_S u_A u_C (u_H + u_{OH})}{u_H u_{OH} (u_A + u_C)} \tag{2}$$

where u_A and u_C are mobilities of the anion and cation of the BGE and u_H and u_{OH} are the mobilities of the H^+ and OH^- ions.

The depletion of the electrolyte can be in the first approximation neglected for the calculation of the composition of the modified acidic and basic electrolyte that fulfills Eq. (1). The omega function is supposed to be constant in this case and it can be used for the calculation of the composition of uni-uni valent strong and weak electrolytes. For each pH of the acidic electrolyte at a given BGE, only one basic pH exists and can be easily calculated.

Unbalanced fluxes J_H and J_{OH} of the H^+ and OH^- ions cause the boundary starts to move. This phe-

nomena can be used for the mobilization of the stationary focused zones in the column, e.g., towards the detection site. The velocity of the movement can be calculated from the knowledge of composition of both acidic and basic electrolytes using a set of moving boundary equations for each ion presented in the system, including solvolytic ions. An equation for the ion c can be written as:

$$J_C^A - J_C^B = W(C_C^A - C_C^B) \quad (3)$$

where W is a volume velocity of the movement in the m^3 s per unit of electric charge and J_C^A, J_C^B, C_C^A, C_C^B are fluxes — total concentrations of ion c in the zone a and b, respectively. A third zone of the lower concentration originates behind the boundary during the movement of a NRB. Its composition depends on the concentration of the original electrolyte in the front of the NRB, pH values of both electrolytes and velocity of the boundary. For the calculation of the buffered electrolyte systems, a concentration of the associated solvolytic ions must be taken in the account as well.

A simple computer program in Power-Basic was written to evaluate the velocity of a boundary. All the mobilities, concentrations and dissociation constants of the basic and acid electrolyte are assigned at first. The magnitude of solvolytic fluxes is calculated. Composition, velocity and drop of omega function for the originating third zone is calculated based on its knowledge using parametrically chosen pH values. The computation stops, when the magnitude of the omega function obtained from the flux calculation and from the concentration of the third zones are equal.

For the calculation of the amount of the accumulated substance and estimation of the necessary dosing time the following equation can be used:

$$n = \frac{QT_A}{F} = \frac{ItC_A u_A}{\kappa_{DE} F} \quad (4)$$

where Q is the electric charge passed through the column, T_A is a transference number of the dosed substance a, F is Faraday constant, I is electric current, C_A is concentration of the dosed substance a, u_A is its effective mobility and κ_{DE} is the conductivity of the dosing electrolyte.

3. Experimental

3.1. Apparatus

A commercial isotachophoretic apparatus (CS Isotachophoretic Analyzer, Labeco, Slovak Republic) in the column-coupling configuration and with regular conductivity detection in the pre-separation and analytical column was used. The analytical column was moreover equipped with a fiber optic UV spectrophotometer (Knauer, Austria). UV detection was performed at 200 nm in the both, ITP and CZE, modes. The CSW collection software (Prague, Czech Republic) running on an IBM PC 486 personal computer was used for data acquisition.

3.2. Chemicals

Hydroxypropylmethylcellulose (HPMC) was obtained from the Serva and model synthetic low molecular mass p*I* marker PIM (p*I* 7.4) was obtained from Dr. K. Šlais, (Institute of Analytical Chemistry, Czech Academy of Sciences, Brno, Czech Republic) [21,22]. All other chemicals of analytical-grade were obtained from Lachema (Brno, Czech Republic).

3.3. Electrophoretic procedure

A continuous dosing of ampholytes was performed in the first step: an electrolyte system working in the CAF-IEF mode. The mixture of ampholytes dissolved in the dosing electrolyte was placed into a terminating chamber of a pre-separation column filled with the correspondingly modified primary electrolyte. After switching on the driving current, the ampholytes were dosed by electromigration into the separation capillary, where they were accumulated in the originating stationary neutralization boundary. A stationary non-moving boundary was attained by balancing fluxes of solvolytic ions from the dosing sample electrolyte and the modified primary electrolyte in the column. The zone of ampholytes grows during dosing in the column and when its volume reaches the detectable quantity, the dosing step could be interrupted.

Focused zones are mobilized in the second step. The dosing sample mixture in the terminating chamber is replaced with the electrolyte that can act

Table 1
Operational electrolytes

Parameter	Basic primary/LE	Acid primary/DE	Terminating-TE	BGE-CZE
Solvent	water	water	water	water
Cation	NH_4^+	NH_4^+	H^+	H^+
Concentration (*M*)	0.015	0.005	0.01	0.05
Anion	CH_3COO^-	CH_3COO^-	CH_3COO^-	CH_3COO^-
Concentration (*M*)	0.005	0.015	0.01	0.05
Additive	HMPC			HMPC
Concentration (%, w/w)	2			2
Sample		p*I* marker		

LE, leading electrolyte; DE, dosing electrolyte; TE, terminating electrolyte.

in the given arrangement as a terminating one. The zones of accumulated ampholytes in the column are mobilized and moved with constant speed in the ITP migration mode to the analytical column. The zones are transferred into an analytical column in the third step and they are detected in the ITP or CZE mode. The analytical column is filled with the leading and/or BGE electrolyte, respectively. The composition of the applied electrolytes is given in Table 1.

4. Results and discussion

4.1. Choice of the electrolyte system

The pH and, consequently, balance of the solvolytic fluxes in a non-buffered BGE is very sensitive to minor changes in the concentrations of ions, which can be caused, for example, by electrolysis in the electrolyte chambers. We focused our attention on the buffered electrolytes for this reason. Demands on the electrolytes applicable in the CAF-IEF with ITP and CZE detection are the following: anions and cations of the BGE should buffer in the acidic and alkaline pH region, respectively. The cation and/or the anion of BGE will serve as a leading ion for the anodic and/or cathodic mobilization, respectively. The mobility therefore should be higher than the mobility of the ampholytes.

The buffering counter-ion should guarantee low mobility of the solvolytic ion during the mobilization, H^+ in the case of the cathodic and OH^- in the case of anodic mobilization mode. Solvolytic ions can be advantageously used as terminators in such a case. This simplifies the electrolyte system used, provides the correct migration of the sample and prevents solvolytic ions penetrating from the electrolyte chambers to the separation column.

As mentioned above, the velocity of the movement of the NBR is an important characteristic of the electrolyte system in CAF-IEF. A theoretically calculated dependence of NRB velocity on the composition and pH of the acid and basic modified primary electrolyte is given in Fig. 1. Ammonium acetate prepared by mixing acetic acid with ammonium hydroxide to the desired pH serves as a primary electrolyte.

A three-dimensional plot where the composition of the acid and basic electrolyte are given on the *x* and *y* axes, respectively, and the NRB velocity is on the *z* axis is depicted in Fig. 1a. The velocities range from $+60–30\ m^3\ C^{-1}$ in the given electrolyte, i.e., the direction of migration changes from cathodic to anodic. The lines from point A to B and A to D mark the curves, where the electrolyte system works in the ITP mode. The ammonium or acetate ions serve as leading ions and the H^+ and OH^- ions serve as terminating ones in this case.

The domain of the electrolytes, which are characterized by the focusing properties, is localized in the area bounded by points A, B, C and D. The line connecting point A and C is the most important where the electrolytes' fulfilling condition of the equality of the fluxes lie, i.e., the velocity of the migration of the NRB is equal to zero. The area where the equal-velocity lines for the given electrolytes are extracted is better seen in Fig. 1b. These pairs of electrolytes can be used in the CAF-IEF.

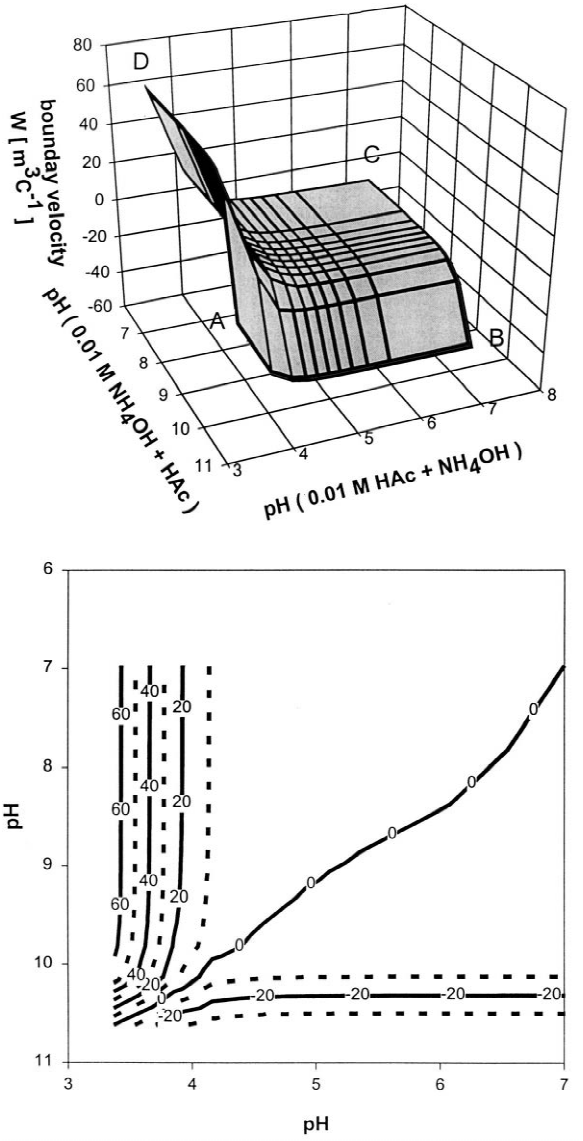

Fig. 1. Calculated dependence of neutralization reaction boundary velocity on the composition of adjacent electrolytes — their pH.

4.2. Estimation of the detection limits for classical ITP–ITP and ITP–CZE mode

Calibration curves of the low molecular p*I* marker of p*I* 7.4 were measured for the estimation of the LODs in the given electrolytes. The sample was injected via a 30-μl injection valve (ITP–ITP) or by micro-syringe (ITP–CZE). It was pre-separated in a pre-separation column working in the ITP mode and transferred into an analytical column. Ampholyte was detected in the ITP detection mode — using the same leading electrolyte as in the pre-separation column, or with the BGE in the CZE detection mode. In the ITP detection mode the zone length evaluated the zone property while the peak area was used in the CZE detection mode.

The calibration curve (slope = 197 058.7118, intercept = 1.3572, $r = 0.9998$) for the ITP–ITP combination is given in Fig. 2a and b. The zone length was plotted against the injected concentration in the range from 0.5 m*M* to 0.8 μ*M*. The amount of injected sample ranged from 15 nmol to 24 pmol. The estimated detection limit was 150 pmol or 5 μ*M* in the concentration terms for the 30-μl injection.

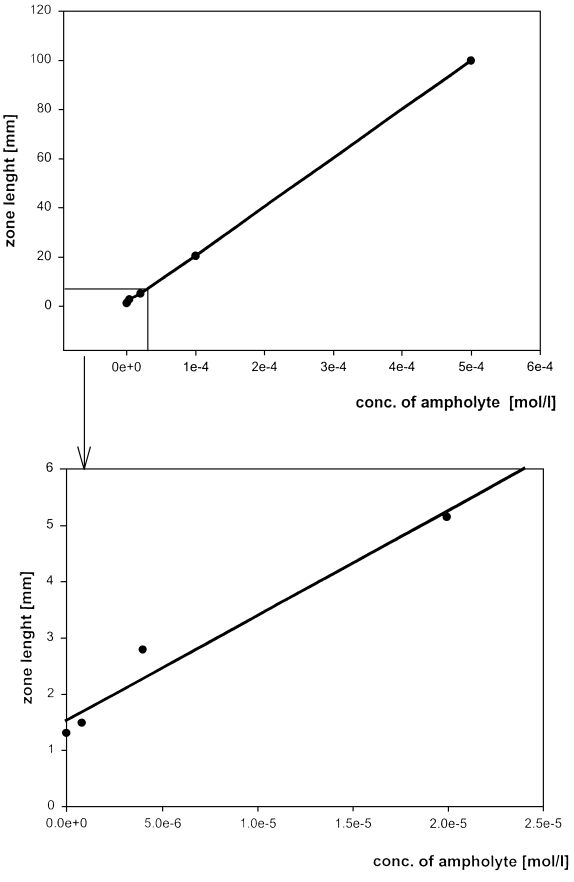

Fig. 2. (a,b) Dependence of the zone length of the ampholyte on the concentration for ITP–ITP combination. LE = 0.005 *M* NH_4Ac + 0.01 *M* NH_4OH, TE = 0.01 *M* HAc.

The calibration curve for the ITP–CZE is depicted in Fig. 3a and b. The zone area was plotted against the amount of injected sample. The calibration curve is a linear combination (slope = $7.0526 \cdot 10^{12}$, intercept = 78.0121, $r = 0.9997$) for the amount of injected sample, which ranged from 10 nmol to 10 pmol. The estimated LOD for this arrangement is about 30 pmol. The concentration LOD is about 1 μmol/l for the 30-μl injection.

4.3. Continuous dosing using combination CAF-IEF–ITP–ITP

A time dependence of zone length on the dosing time was measured for the experimental verification of the use of CAF-IEF for continuous dosing. The constant concentration of a sample ampholyte (6 μmol/l, i.e., slightly above concentration LOD) was used in the dosing electrolyte. The accrue of the zone of the ampholyte dosed from the dosing electrolyte mentioned in Table 1 and employing the dosing current of 400 μA, is given in Fig. 4. The dependence is fairly linear (slope = $5.5484 \cdot 10^{-3}$, intercept = 1.5050, $r = 0.9844$), achieved dosing speed of 2.11 nmol A^{-1} s^{-1}.

As mentioned in Section 2, the dosing speed can be increased by an increase of the transference number of the ampholyte, e.g., by lowering the conductivity of the DE. Dependence of zone length on the dosing time at ten times lower concentration of the DE and at the same concentration of the sample ampholyte (6 μmol/l) is given in Fig. 5. The dosing current was established at 200 μA owing the lower conductivity of the electrolyte. The dependence is fairly linear (slope = 0.0261, intercept = −0.8108, $r = 0.9829$). The achieved dosing speed is 19.88 nmol A^{-1} s^{-1} with the given equipment. It is possible to increase the amount of a dosed ampholyte 100 times in 3830 s.

4.4. Continuous dosing using a combination of CAF-IEF–ITP–CZE

The time dependence of the peak area on the

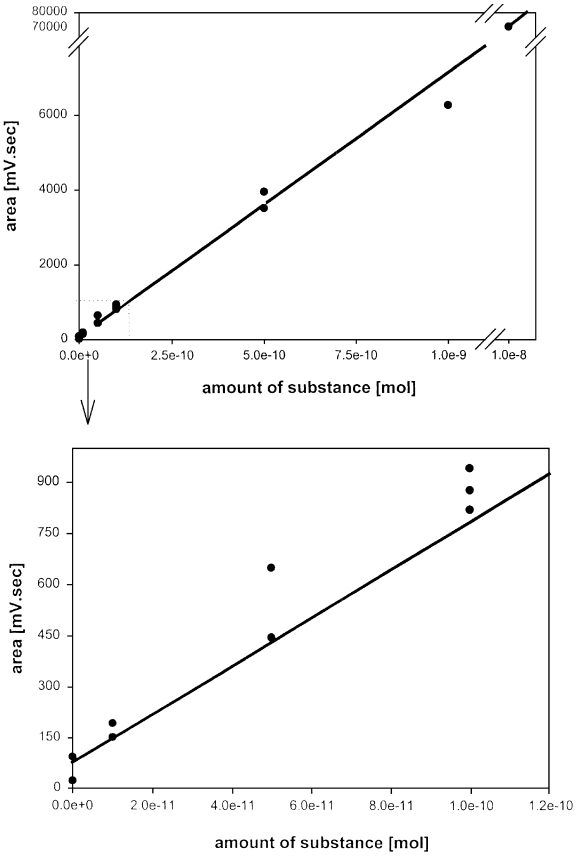

Fig. 3. Dependence of peak area on the amount of injected ampholyte for the ITP–CZE combination. LE = 0.005 *M* NH_4Ac + 0.01 *M* NH_4OH, TE = 0.01 *M* HAc, BGE = 0.05 *M* HAc.

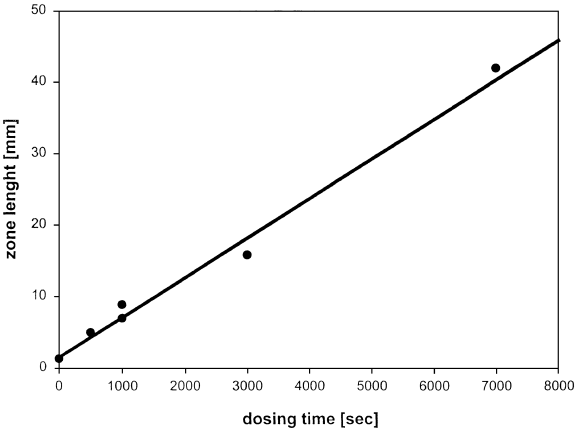

Fig. 4. Dependence of the zone length of the ampholyte on the dosing time for the CAF-IEF–ITP–ITP combination. LE = 0.005 *M* NH_4Ac + 0.01 *M* NH_4OH, DE = 0.005 *M* NH_4Ac + 0.01 *M* HAc, TE = 0.01 *M* HAc.

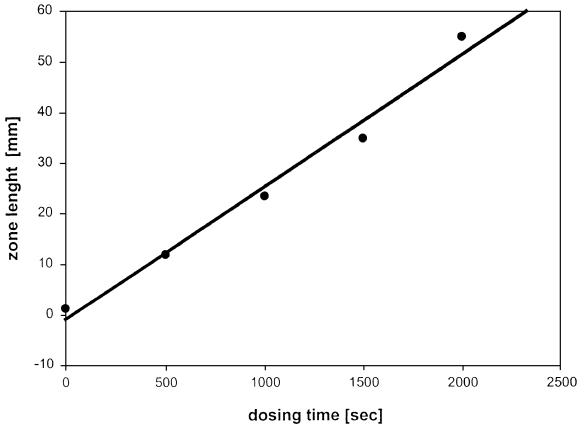

Fig. 5. Dependence of the zone length of the ampholyte on the dosing time for the CAF-IEF–ITP–ITP combination. LE = 0.005 *M* NH_4Ac + 0.001 *M* NH_4OH, DE = 0.0005 *M* NH_4Ac + 0.001 *M* HAc,TE = 0.01 *M* HAc.

dosing time was measured to verify the experimentally applicability of CAF-IEF with CZE for continuous dosing. A constant concentration of the sample ampholyte (1 μmol/l) in the dosing electrolyte was used. The accrue of the ampholyte zone dosed from the dosing electrolyte mentioned in Table 1 at a dosing current of 400 μA is given in Fig. 6. The dependence is fairly linear (slope = 1.3857, intercept = 46.8793, $r = 0.9989$). The achieved dosing speed is 0.491 n*M* A^{-1} s^{-1}. This agrees well with the

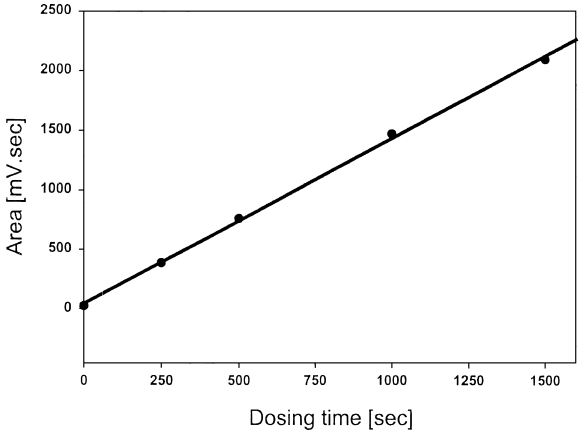

Fig. 6. Dependence of the peak area of the ampholyte on the dosing time for the CAF-IEF–ITP–CZE combination. LE = 0.005 *M* NH_4Ac + 0.01 *M* NH_4OH, TE = 0.01 *M* HAc, DE = 0.005 *M* NH_4Ac + 0.01 *M* HAc, BGE = 0.05 *M* HAc.

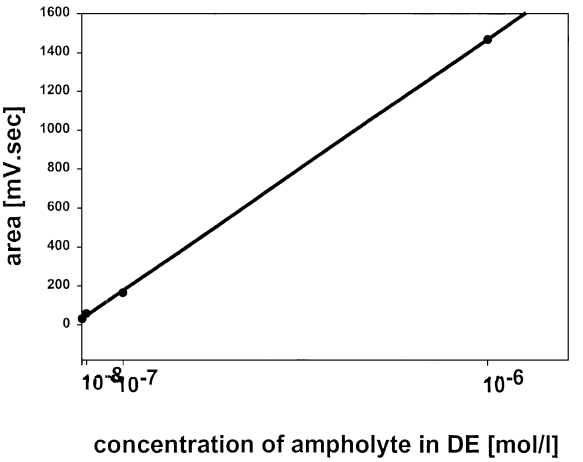

Fig. 7. Dependence of the peak area of the ampholyte on the concentration for the CAF-IEF–ITP–CZE combination. LE = 0.005 *M* NH_4Ac + 0.01 *M* NH_4OH, DE = 0.005 *M* NH_4Ac + 0.01 *M* HAc, TE = 0.01 *M* HAc, BGE = 0.05 *M* HAc.

estimated dosing speed of 0.5 n*M* A^{-1} s^{-1}, which was calculated from the conductivity of the DE.

A dependence of the peak area on the concentration of the ampholyte in DE was measured in the range from μmol/l to nmol/l at a constant dosing time of 1000 s to estimate the concentration limit of the method. As can be seen from the Fig. 7, it is still possible to detect ampholyte at concentrations 10^{-8} mol/l, two orders of magnitude below the LOD common for the ITP–CZE combination. The corresponding electrophoregrams are shown in Fig. 8.

Next is the possibility of how to increase the accumulation of the zone (during the given time 1000 s) and, thus, the concentration LOD is to dose from both the acid and basic electrolyte and to fill the whole column with sample dissolved in working electrolyte. The resulting electrophoregram is shown in Fig. 8. At this condition ampholyte at the nmol/l concentration level can be still detected.

5. Conclusion

The suggested combination of CAF-IEF with ITP or CZE was shown to be successful for the preconcentration of ampholytes from diluted samples. Up to 1000-fold increases in the amount of a substance in a column can be achieved for both ITP

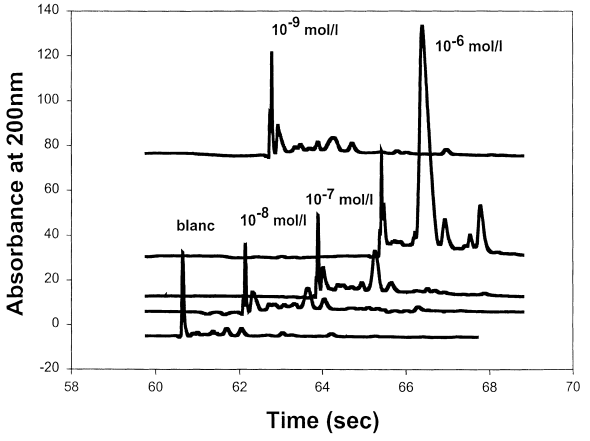

Fig. 8. Electrophoregrams of the accumulated ampholyte using different the concentrations in DE. Dosing time = 1000 s. CAF-IEF–ITP–CZE combination.

and CZE detection modes at reasonable working conditions. Concentrations on the nmol/l level can be detected at concentration factors of about 10^7. A buffered electrolyte enables commercially available equipment to be used.

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