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Continuous micropreparative trapping in carrier ampholyte-free isoelectric focusing

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Abstract

The micro-isolation of a minor ampholyte from a mixture of four ampholytes using carrier ampholyte-free isoelectric focusing (IEF) is described. The separation is achieved in two consecutive steps. In the first step, a concentrated mixed zone of minor ampholyte-containing major ampholytes is formed when the original mixture is loaded into the column by electromigration. During this loading, a flux of solvolytic ions from both ends of the column is set up in such a way that one end of the zone formed is immobilized in the column. It is achieved by selection of the pH range, which includes the p*I* value of the minor ampholyte but not the p*I* values of the major ampholytes. The minor ampholyte is captured in this zone completely, whereas the major ampholytes migrate through this zone to the outlet reservoir. After the zone has acquired a reasonable volume, the loading step is interrupted. In the second step, either the complete contents of the column are separated by conventional carrier ampholyte-free IEF in a pH range that includes the p*I* values of all the ampholytes, or using a smaller pH range the minor ampholyte can be separated and isolated completely. Zone formation was modelled by computer simulation for a two-ampholyte mixture and verified experimentally for a four-ampholyte mixture in an instrument for electrically controlled IEF by using coloured low-molecular-mass ampholytes.

1. Introduction

Modern capillary electrophoretic techniques, such as capillary zone electrophoresis (CZE), isotachophoresis (ITP) or capillary isoelectric focusing (CIEF), have outstanding separation abilities in terms of selectivity, resolution and speed of analysis [1]. The sophisticated detection techniques developed recently have decreased the detection limits to the level of single molecules [2].

Usually these techniques work in a one-dimensional mode only, which is frequently insufficient when complex biological mixtures are to be analysed. The presence of the large amounts of salts, the great complexity of biological samples and the high concentration ratio of the substances of interest adversely affect the analytical results. Therefore, the analysis of biological samples requires either an enhanced separation power of the method or sample pretreatment prior to analysis.

The use of two-dimensional analysis by coupling an electrophoretic technique with another separation technique, e.g., MS or HPLC [3,4], helps to resolve substances that cannot be resolved in one run. Surprisingly, the combination of two electrophoretic techniques can also sig-

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nificantly increase the resolution and this approach is frequently used. Combined ITP and CZE [5–8] is used mainly for the preseparation and preconcentration of samples. Moreover, the major bulk waste component can be easily eliminated from a CZE run using more sophisticated equipment, which results in simplification of the complex separated sample prior to proper analysis. The load used does not exceed the volume of the capillary [9], which does not need to be sufficient for the detection or preparation of minor components.

Such a simplification of the sample components with higher volumes of the load is used in non-capillary free-solution focusing techniques [10–12], where a range of the substances of close p*I* and/or pure substance themselves is the result of the separation run. These techniques are used for the preparation of purified samples. Bier et al. [10] used zwitterionic buffer mixtures in recycling IEF, where the sample was divided into three parts, anodic, cathodic and middle, where substances of interest were focused. Using buffers of higher concentration enhanced the solubility of proteins. Righetti et al. [11] introduced isoelectric membranes tailored for particular separations. The sample is introduced between two membranes, where substances with p*I* values different from the p*I* range of the membranes are flushed out by electromigration.

This paper describes another method of collecting an amphoteric compound A of interest from a sample containing higher concentrations of ampholytes B, C and D and KCl (electrolyte).

If a sample is placed in a load reservoir connected with a waste reservoir by a tube and electric current is passed through such a column, the minor ampholyte can be trapped in the tube as a stopped zone. Almost all major ampholytes leave the tube to the waste reservoir, passing through the created trapped zone of minor ampholyte.

In the trapped zone, under suitable conditions, the concentration of the originally minor ampholyte A is higher than those of the originally major ampholytes. In this way selective enrichment can be achieved.

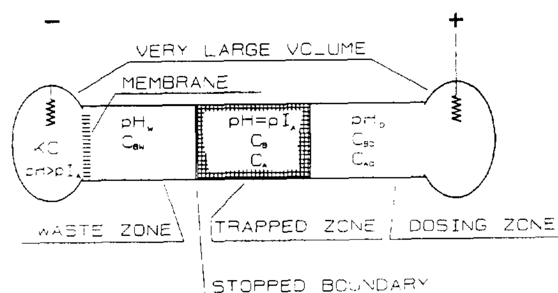

Fig. 1. Schematic diagram of the experimental arrangement. The loaded sample mixture is on the anodic and the waste zone on the cathodic side of the column.

2. Theory

A schematic diagram of the basic arrangement is shown in Fig. 1. The waste zone is at the cathodic side and the loading zone at the anodic side. If the waste zone has a pH value pH_W higher than the p*I* value of the minor ampholyte, pI_A, no minor ampholyte can enter the waste zone. On the opposite side, the slowly moving boundary between the trapped and loading zones must have a pH value from the side of the loading zone, pH_D, that is lower than pI_A.

The properties of the waste and loading zones determine the conditions for immobilization of the trapped zone. For the specific composition of the loaded sample mixture, there is only one proper composition of the waste zone.

To calculate the compositions of the waste and the trapped zones, a program developed and described earlier was used [13]. The program was upgraded for the calculation of two biprotic ampholytes in a loaded sample mixture containing also salts of strong and weak bases and acids as a background electrolyte. This program is based on: (1) equality of flows of the background electrolyte in all zones; (2) electroneutrality; (3) dependence of degree of dissociation of substances on pH; (4) constant omega function in all zones; (5) constant driving current in all zones; and (6) mass balance.

Let us first discuss the simplest part of the system, the neutralization boundary. It is created in the background electrolyte by partial replace-

ment (modification) of the cations and anions at the anodic and cathodic sides of the column with H^+ and OH^- ions, respectively. Flows of solvolytic ions H^+ and OH^- (J_H, J_{OH}) enter the neutralization boundary created in the background electrolyte from opposite sides. If these fluxes are equal, i.e.,

$$J_{H^+} = J_{OH^-} \quad (1)$$

the neutralization boundary stops and does not move. Then the sum of the flows of ions of the background electrolyte ($J_{B,H}$, $J_{B,OH}$) are also equal on both sides:

$$\sum J_{B,H^+} = \sum J_{B,OH} \quad (2)$$

Further, let us discuss the properties of the ampholyte zone. If the zone of the trapped ampholyte is created between two such modified zones which were stopped, it cannot move. The ampholyte is in its isoelectric state, and if the mobilities of its cationic and anionic forms are equal, it is also in the isoionic state. The amount of the dissociated fraction for the biprotic ampholyte is given by [14]

$$\alpha = \frac{2}{2 + 10^{pI - pK_1}} \quad (3)$$

where α is the degree of dissociation of both cationic and anionic forms of the ampholyte and pK_1 is the dissociation constant. Only the dissociated fraction can transfer electric charge (protons).

At the boundaries of the trapped zone with background electrolyte, a dynamic equilibrium exists, and the flows of the solvolytic ions are divided into two parts. The first part of the solvolytic flow neutralizes the oppositely charged ampholyte and solvolytic ions to the neutral form, creating water by neutralization. The second part of this flow enters the zone, ionizes the ampholyte and carries the charge to the other side of the zone, where the same effect with ions of opposite sign occurs. Assuming that the ampholyte is in the isoelectric state, only half of the solvolytic flow reaching the boundary enters the zone and is carried by the ampholyte to the opposite side of the zone.

In the trapped zone of the ampholyte, the flows of the solvolytic ions are also divided into two parts. They are partially carried by the ampholyte ($J_{A,A}$) and partially they continue as flows of free solvolytic ions ($J_{H,A}$, $J_{OH,A}$). The sum of these flows is equal to the flow of solvolytic ions entering from the background electrolyte, i.e.,

$$J_{H^+} = J_{OH^-} = J_{H,A} + J_{A,A} \quad (4)$$

Of course, the sum of flows ions of background electrolyte in the zone of ampholyte ($J_{B,A}$) must be equal to the flows of these ions in the modified background electrolyte ($J_{B,H}$; $J_{B,OH}$):

$$\sum J_{B,H^+} = \sum J_{B,OH^-} = \sum J_{B,A} \quad (5)$$

In other words, Eqs. 4 and 5 indicate that the amount of electricity carried by solvolytic and/or non-solvolytic ions is constant in each part of the whole system.

The flow of the ampholyte in the trapped zone ($J_{A,A}$) is given by

$$J_{A,A} = C_A U_A \alpha I / \kappa_A \quad (6)$$

where C_A is the analytical (total) concentration of the ampholyte is its zone, I is the current, U_A is the ionic mobility of the ionized form A and κ_A is the conductivity of the zone. Since in the isoelectric state the fraction of the ionized ampholyte α is constant (neglecting the influence of the ionic strength on the pK value), the flow of solvolytic ions from surroundings is balanced by setting its total concentration. Any change of the solvolytic flows from the surroundings results in a change in concentration of the ampholyte, i.e.,

$$\Delta J_H = J_{H,A} + J_{OH,A} + \Delta C_A U_A \alpha I / \kappa_A \quad (7)$$

From Eq. 7, it follows that the concentration of the ampholyte in the zone is proportional to the flow of the solvolytic ions and to its physicochemical properties, which also determine the amount of free solvolytic ions in the zone. The computer program is based on this idea.

Mobilities, pK values of all substances, con-

centration of the background electrolyte, concentrations of the ampholytes in the load and the chosen pH value of the waste zone are the input data for the program. From these data, the program calculates the compositions and the pH values of all zones that fulfil the conditions of the stopped boundary between the loading and trapped zones.

The procedure for computation is as follows. The compositions of the loading and waste zones are calculated first. The concentrations of both loaded ampholytes in the trapped zone are then obtained by iteration, until equal flows of background electrolyte in all zones are reached.

It should be stressed that the diffusion and depletion of the primary background electrolyte due to the presence of the neutralization reaction on the boundaries is neglected. This is acceptable at a small difference in pH between the waste zones and the loading zone. By comparison with simulation data obtained from the program by Thormann and co-workers [15,16], only a minor difference in the concentrations of ampholytes was found, not exceeding 5%. In that program the mentioned effects were not neglected.

Calculated data for ampholyte A ($pI_A = 7.4$, $pK_1 = 6.9$, $pK_2 = 7.9$; $\mu_1 = \mu_2 = 30 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}) and for ampholyte B ($pI_B = 7.9$, $pK_1 = 7.4$, $pK_2 = 8.4$; $\mu_1 = \mu_2 = 30 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}) are given in Table 1. In all calculations, 10 m*M* KCl was used as the primary electrolyte. The concentration of the loaded major ampholyte was always 10^{-3} *M* and the concentration of the minor ampholyte A varied from 10^{-5} to 10^{-3} *M*. The transfer number of the trapped ampholyte in the loaded mixture varied from $2.1 \cdot 10^{-4}$ to $2.43 \cdot 10^{-2}$ and represents the amount of trapped ampholyte per unit of electric charge passed through the column.

The value of C_A^* represents the concentration of the trapped ampholyte in the case when no major ampholyte is loaded. The most important fact is shown in the middle column. The widely varying ratio of the concentration of the minor to major ampholyte in the loading zone (from 1:1 to 1:100) results in a nearly constant concentration of minor ampholyte in the trapped zone. The ratio C_A/C_B here varies from 1:0.81 to 1:0.55. If the concentration of minor ampholyte in the loading mixture is decreased by two orders of magnitude, its concentration in the trapped zone decreases by about 9%. In this way, a mixture enriched in the minor ampholyte is obtained.

For verification of the accuracy of the calculated data, a computer simulation was performed. A simulated column was filled with a mixture of previously calculated composition (see Table 2). The initial conditions are that the continuously loading (source) zone is placed at the anodic side and the continuously depleting waste zone on the cathodic side. Development of the concentration, pH and conductivity profiles along the column with time is shown in Fig. 2. The concentrations in the simulated trapped zone ($c_A = 2.23$ m*M*, $c_B = 1.24$ m*M*) are in good agreement with the calculated values ($c_A = 2.45$ m*M*, $c_B = 1.37$ m*M*), and their ratios are 1.798 and 1.788, respectively.

Table 1
Calculated data for model mixture containing two ampholytes, A and B, in 10 m*M* KCl

Loaded mixture			Trapped zone (pH = pI_A)			Non-trapped zone		Transfer number, $10^3 T_A$
c_A (mmol)	c_B (mmol)	pH	c_A (mmol)	c_B (mmol)	c_A^* (mmol)	c_B (mmol)	pH	
0.01	1	3.81	2.45	1.37	4.012	4.0	7.74	0.21
0.1	1	3.83	2.48	1.38	4.087	4.0	7.74	2.11
0.5	1	4.02	2.26	1.53	4.043	3.9	7.72	11.2
1	1	4.38	2.09	1.71	4.088	4.0	7.70	24.3

Table 2
Compositions of electrolytes used for simulation

Parameter	Loading zone	Waste zone
pH	3.83	7.74
Ampholyte A	1 m*M*	0 m*M*
Ampholyte B	10 m*M*	40 m*M*
K^+	7.952 m*M*	4.539 m*M*
Cl^-	9.199 m*M*	5.107 m*M*

3. Experimental

3.1. Chemicals

Low-molecular-mass p*I* markers of p*I* 5.3, 6.2, 8.6 and 10.1 were obtained from Dr. K. Šlais (Institute of Analytical Chemistry, Brno, Czech Republic), their chemical compositions and properties have been published elsewhere [17]. They are readily soluble in water and are coloured.

The apparatus used for the focusing is a modification of the previously used laboratory-made four-pole column [13] and will be described more precisely in a subsequent paper. The apparatus has four electrode chambers connected with a separation channel and one pair of electrode chambers on each side of the separation capillary. In each pair of equal polarity, one chamber is filled with the primary (background) electrolyte (here 0.01 *M* KCl) and the other with a modification electrolyte or loaded mixture. A solution of a strong base serves as a cathodic modification electrolyte and the loaded mixture contains the ampholytes dissolved in primary electrolyte with a properly adjusted pH value.

The flows of ions from the electrode chambers pass through the washed membranes into the separation channel, which is a quartz capillary (120 mm × 0.35 mm I.D.). The capillary is efficiently cooled by a stream of air from a flat nozzle, mounted on the supporting rack.

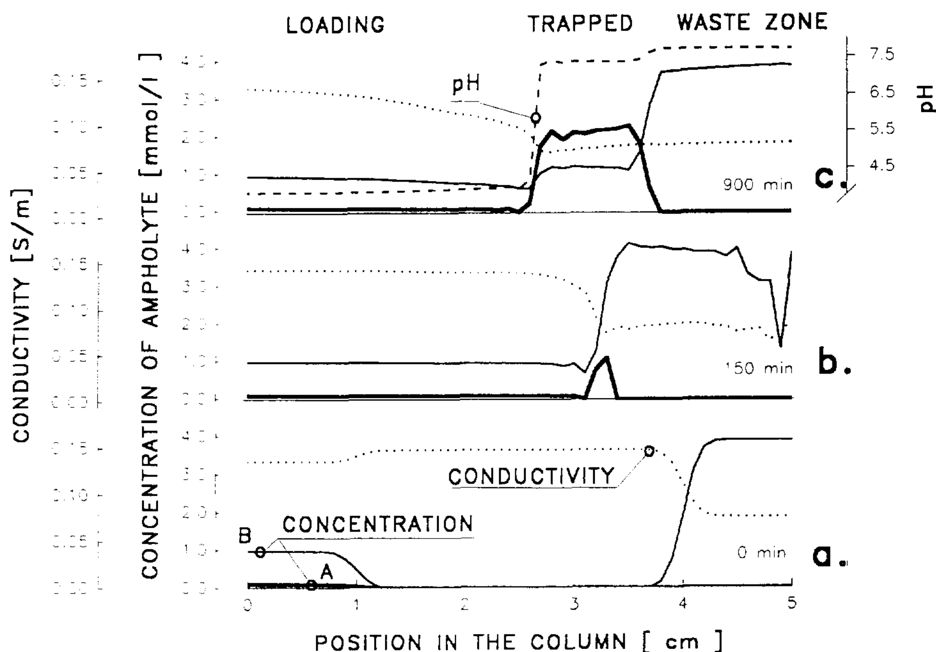

Fig. 2. Simulated time course of trapping. (a) Concentration profiles of the minor and major ampholytes (full thick and thin lines, respectively) and conductivity profile (dotted line) at the start of trapping; (b) developed profiles after 150 min; (c) profiles after 900 min; dashed line pH profile. Current density, 20 A m^{-2}.

3.2. Trapping procedure

After filling the appropriate electrode chambers with solutions of the loaded mixture and basic modification and neutral primary electrolytes, the membrane washing is started to keep the composition of the electrolytes constant during the run. The capillary is filled with the loaded mixture. The separation is started by setting the ratio of the driving currents on both regulators and switching on the main driving current. Solvolytic ions and the loaded mixture ions continuously penetrate the capillary, where the trapped ampholyte creates a zone. When the creation of the trapped zone starts, some fine readjustment of the driving current, depending on the position and velocity of the zone in the column, is obviously necessary. Non-trapped ampholytes migrate via the trapped zone to a waste zone and subsequently through a membrane of the primary electrolyte out of the column and into the electrode drain solution. After some time, the whole capillary is filled with the trapped zone, which contains substantially enriched trapped ampholyte. Subsequent interruption of the continuous loading and decreasing the pH range cause the major ampholytes to leave the trapped zone. In the capillary only the pure trapped ampholyte remains.

3.3. Micropreparation

The driving current is switched off and a thin needle (quartz capillary) is inserted into the column through the opened filling valve on one side of the column. The contents of the column between the membrane and the trapped zone are removed by suction and replaced with air. A small bubble of air on the other side of the trapped zone is inserted, to prevent remixing with other possible zones. After repositioning the needle to the centre of the zone, the zone is drawn into the needle using an attached microsyringe. The needle is removed from the column and the microprepared zone (ca. 5 μl) is transferred into a microvial, where it is stored at 0°C.

Analytical control was effected by capillary isoelectric focusing with electroosmotic displacement of zones (CIEF). CIEF was performed by using a laboratory-made capillary electrophoretic device. It features a 70 cm effective length, 90 cm total length × 75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), a CZE 1000R electric power supply (Spellmann, New York, USA) and a Spectra Focus fast-scanning UV–Vis spectrophotometric detector (Thermo Separation Products). Results were recorded in the wavelength range 370–600 nm and the spectra of the individual peaks were used for identification of the coloured ampholytes.

All experiments were performed with 10 mM phosphoric acid as anolyte and 20 mM sodium hydroxide containing 0.1% hydroxypropylmethylcellulose (Sigma, St. Louis, MO, USA) as catholyte at 20 kV constant voltage. The sample ampholytes were dissolved in a 2% solution of the carrier ampholyte Servalyt 3–10 (Serva, Heidelberg, Germany) and were injected gravitationally into the anodic end of capillary (raising it to 70 cm for 5 min). The experimental procedure has been described in detail elsewhere [18,19].

4. Results

To demonstrate the performance of the method for more complicated loaded mixtures, a model mixture of four ampholytes (low-molecular-mass pI markers) of pI 5.3, 6.2, 8.6 and 10.1 was chosen at concentrations of 10^{-3}, 10^{-4}, 10^{-3} and 10^{-3} M, respectively. The minor ampholyte of pI 6.4 is red and the others are yellow. This model mixture, dissolved in 0.01 M KCl (primary electrolyte) with pH adjusted to 5.5, served as the loading mixture in subsequent experiments.

The time course of the experiment is shown in Figs. 3a, b and c, where the trapped zone (pI 6.2, the darker zone) was photographed after times of 10, 25 and 120 min, respectively. The zone grows, and by 120 min it is 20 mm long. The non-trapped ampholyte (pI 10.1 and 8.6,

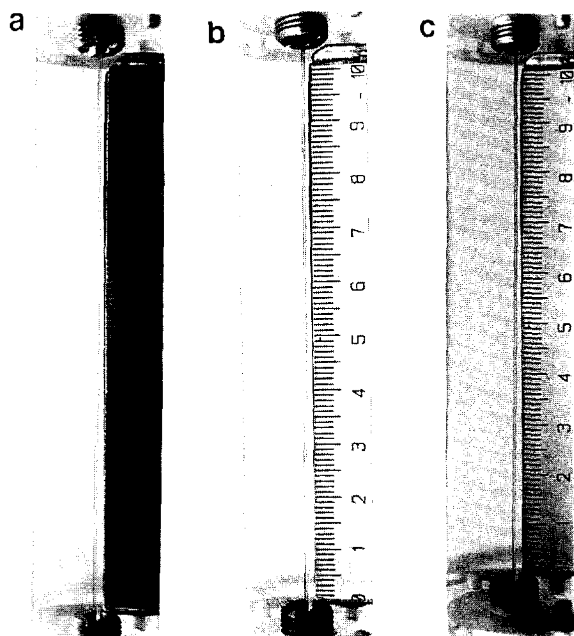

Fig. 3. Time course of trapping. Zones after (a) 10, (b) 25 and (c) 120 min. The zones of trapped ampholyte are in positions 39–40, 31–34, and 55–75, respectively. The driving current was 200 μA during the first 10 min, then decreased to 100 μA.

lighter zone) pass through the column to the waste electrode chamber and from it they are rinsed out by a continuous flow of fresh electrolyte. The equivalent length of the non-trapped zone without rinsing (under these conditions) should be ca. 400 mm.

The CIEF analysis of this mixture (wavelength 410 nm, maximum absorbance of major ampholytes) is shown in Fig. 4. The ampholytes migrate in order of their decreasing p*I* values, the first peak being ampholyte of p*I* 10.1 and the last peak ampholyte of p*I* 5.3. Fig. 5 shows the CIEF analysis of the trapped zone when continuous loading was not stopped. As can be seen, the ratio of the minor to major ampholyte concentration is dramatically changed. The minor ampholyte is more concentrated in the mixed trapped zone than in the original loading mixture, and originally major ampholytes are at lower concentration. Only a small amount of ampholyte of p*I* 5.3 is present in the trapped zone.

Fig. 6 shows the CIEF analysis of the isolated zone, refocused in the column, after the loading was stopped. It can be seen that the zone

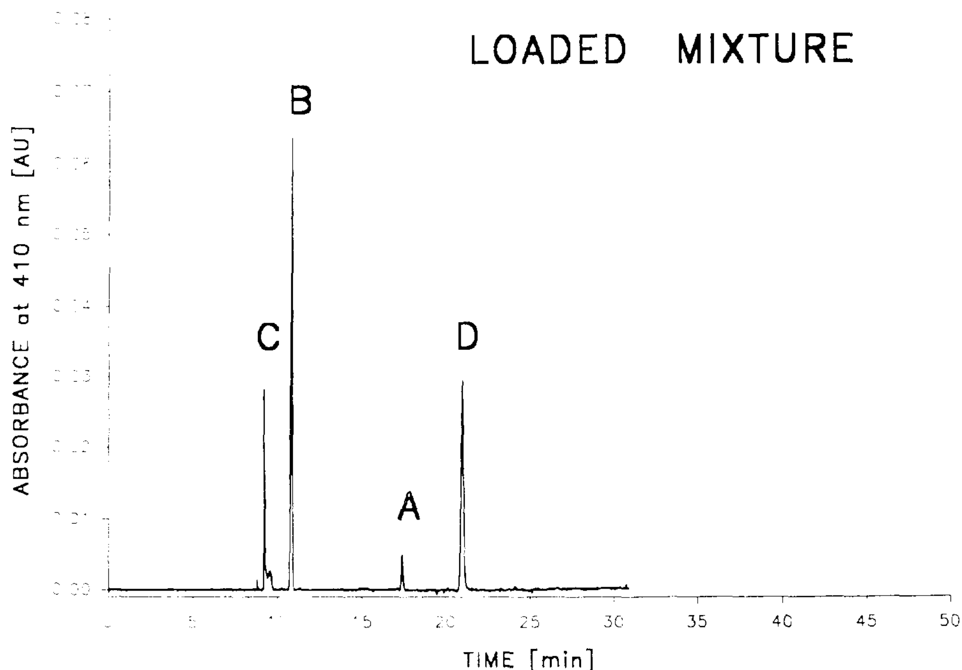

Fig. 4. CIEF analysis of the loaded mixture of four p*I* markers of p*I* (A) 6.2, (B) 8.6, (C) 10.1 and (D) 5.3.

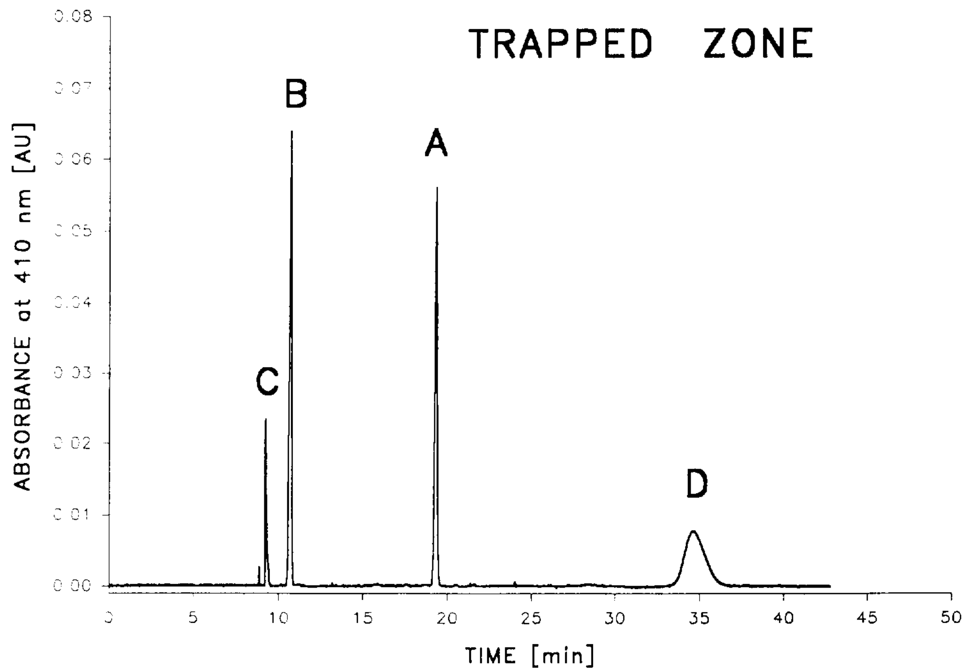

Fig. 5. CIEF analysis of the trapped zone. Details as in Fig. 4.

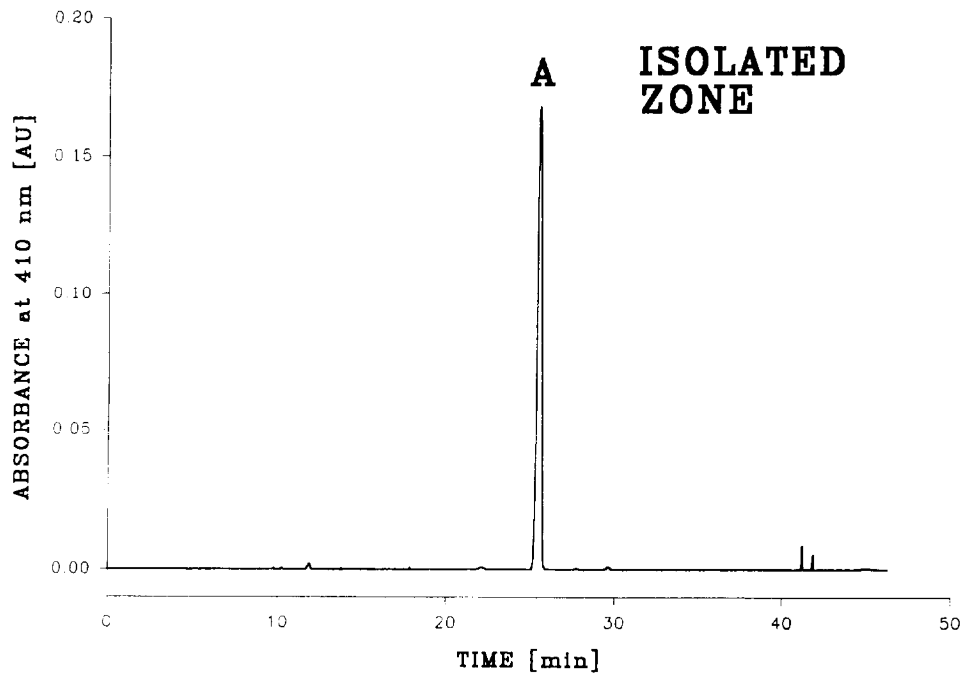

Fig. 6. CIEF analysis of the isolated zone. Details as in Fig. 4.

contains mainly minor ampholyte, the major ampholytes having left the zone. Ampholytes leaving the isolated zone to the cathodic side are shown in Fig. 7. Here, small amounts of ampholytes B and C were detected together with some impurities X of original product of ampholyte A. These impurities were also found in the original product of ampholyte A (Fig. 8). On the anodic side, no impurities were detected after refocusing of the isolated zone. CIEF analysis of the cathodic drain electrolyte shows only the presence of ampholytes B and C (Fig. 9), which confirms the theoretical considerations.

5. Conclusion

The developed continuous trapping method showed good results in simulations and experiments. The method allows trapping of a minor ampholyte component from a large-volume load. The concentration of the trapped substance is favourably high. The relative enrichment of the minor ampholyte in the trapped zone is sufficiently high.

The volume of the load from which the trapped ampholyte originates is more than one order of magnitude higher than the volume of the separation capillary. The method is based on carrier ampholyte-free IEF and the microprepared substance contains only ions of the background electrolyte.

Acknowledgement

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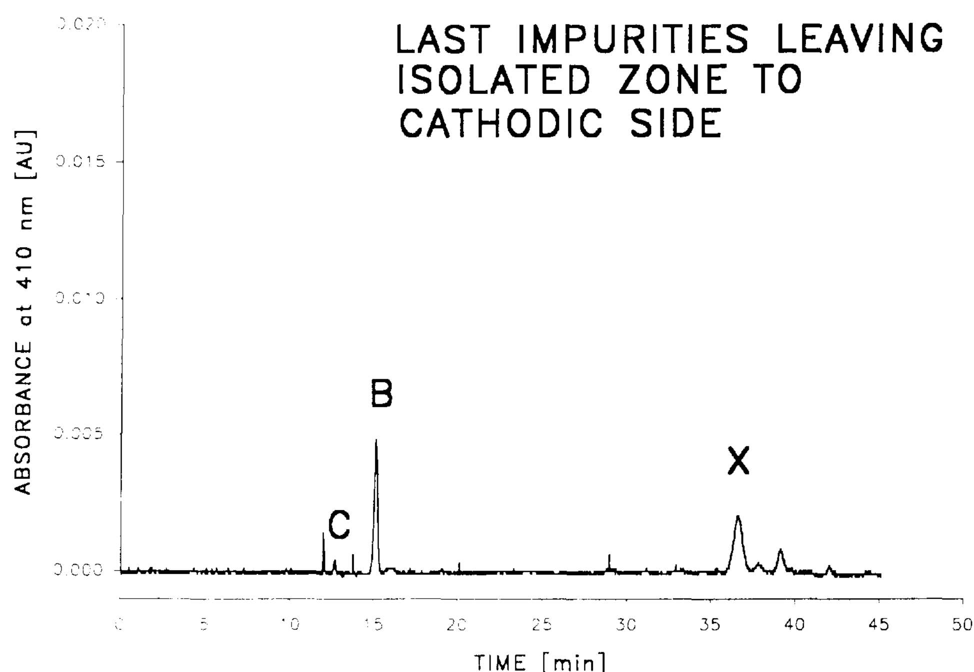

Fig. 7. CIEF analysis of the impurities leaving the isolated zone to the cathodic side. X denotes impurities of the original product A. Details as in Fig. 4.

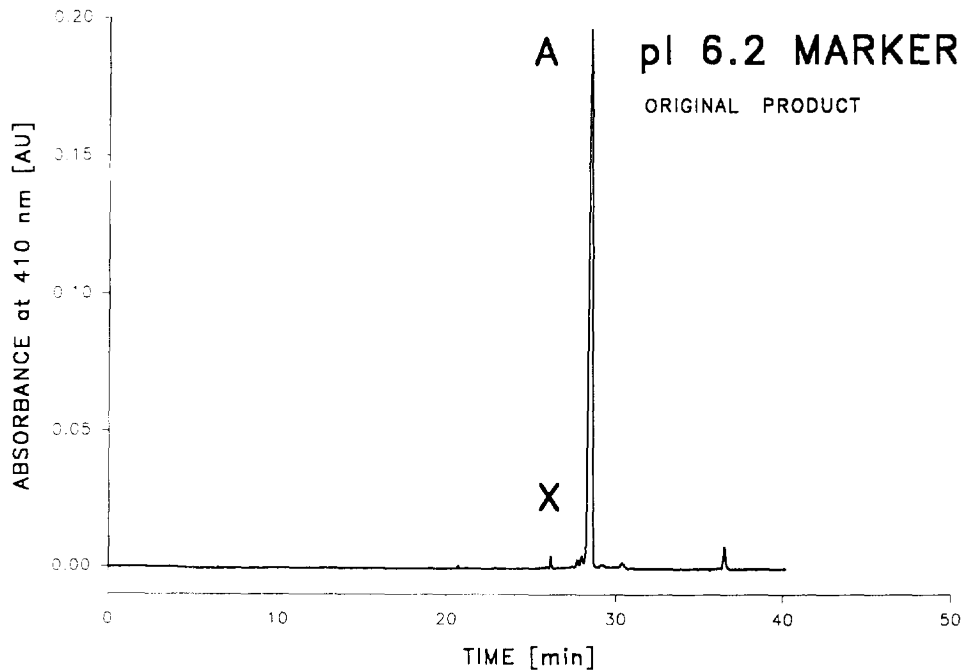

Fig. 8. CIEF analysis of the original product A. Details as in Fig. 4.

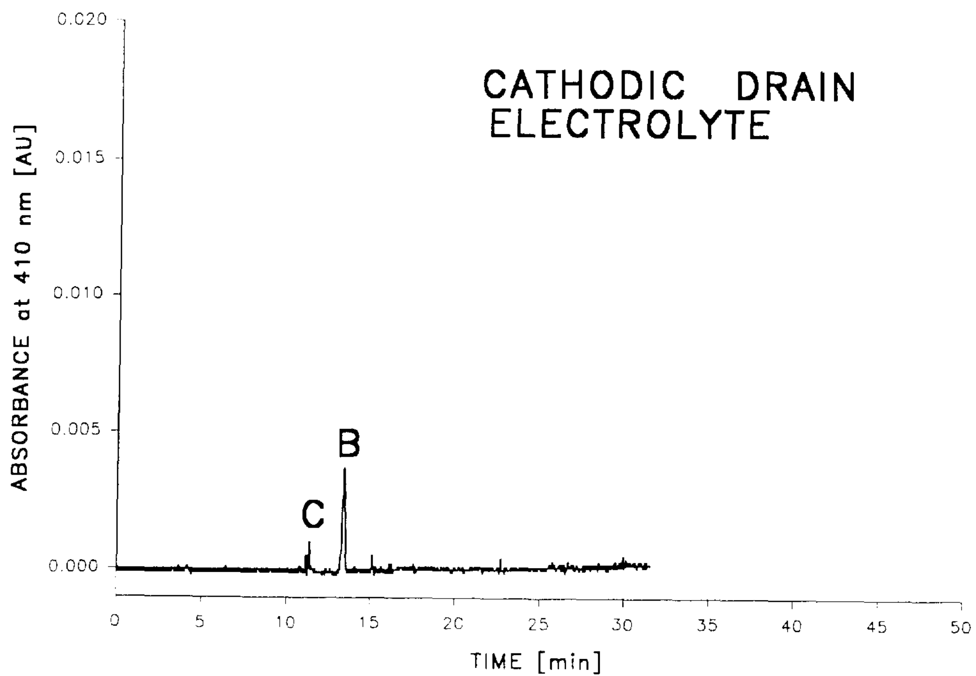

Fig. 9. CIEF analysis of cathodic drain electrolyte. Details as in Fig. 4.

References

[1] C.A. Monning and R.T. Kennedy, Anal. Chem., 66 (1994) R280.
[2] D.Y. Chen, K. Adelhem, X.L. Cheng and N.J. Dovichi, Analyst, 119 (1994) 349.
[3] M.A. Moseley, L.J. Deterding, K.B. Tomer and J.W. Jorgenson, J. Chromatogr., 480 (1989) 1989.
[4] M.M. Bushey and J.W. Jorgenson, Anal. Chem., 62 (1990) 978.
[5] M. Deml, F. Foret and P. Boćek, J. Chromatogr., 320 (1985) 159.
[6] V. Dolník, K.A. Cobb and M. Novotny, J. Microcol. Sep., 2 (1990) 127.
[7] D. Kaniansky and J. Marák, J. Chromatogr., 498 (1990) 191.
[8] F. Foret, V. Šustáček and P. Boćek, J. Microcol. Sep., 2 (1990) 229.
[9] S. Hjertén, J. Liao and R. Zhang, J. Chromatogr. A, 676 (1994) 409.
[10] M. Bier, J. Ostrem and R.B. Marguez, Electrophoresis, 14 (1993) 1011.
[11] P.G. Righetti, E. Wenisch, A. Jungbauer, H. Katinger and M. Faupel, J. Chromatogr., 500 (1990) 681.
[12] E. Wenish, K. Vorauer, A. Jungbauer, H. Katinger and P.G. Righetti, Electrophoresis, 15 (1994) 647.
[13] J. Pospíchal, M. Deml and P. Boček, J. Chromatogr., 638 (1993) 179.
[14] H. Svensson, Acta Chem. Scand., 15 (1961) 325.
[15] R.A. Mosher, D.A. Saville and W. Thormann, The Dynamics of Electrophoresis, VCH, Weinheim, 1992.
[16] R.A. Mosher, P. Gebauer, J. Caslavska and W. Thormann, Anal. Chem., 64 (1991) 2991.
[17] K. Šlais and Z. Friedl, J. Chromatogr. A, 661 (1994) 249.
[18] J. Chmelík and W. Thormann, J. Chromatogr., 632 (1993) 229.
[19] J. Caslavska, S. Molteni, K. Šlais, F. Matulík and W. Thormann, J. Chromatogr. A, 680 (1994) 549.