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Approach to quasi stationary electrokinetic chromatography

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Abstract

A partial filling (PF) electrokinetic chromatography (EKC) system in combination with the application of weak counter pressures was built up by the combination of an UV-active polymeric dye, Poly R-478, used as additive in the separation buffer (SB) zone and an UV-permeable borate background buffer (BB). The electroosmotic flows of the buffers were equalized by matching their ionic strengths to achieve best efficiency. The influence of the pressure for the injection of the separation buffer and the effect of the counter pressure on the breakthrough of the separation buffer zone was investigated. The quasi stationary state of the separation buffer zone was evaluated by recording breakthrough curves. Based on these data the counter pressure was manipulated so that the separation buffer zone became quasi stationary and a large interference-free migration time window results. The system was optimized using a mixture of amino/nitroaromatics as test compounds.

Keywords: Micellar electrokinetic chromatography; Electrokinetic chromatography; Partial filling electrokinetic chromatography; Quasi stationary separation additive; Counter pressure; Polymeric dye

1. Introduction

During the last decade (micellar) electrokinetic chromatography [(M)EKC] became an interesting alternative and addition to high-performance liquid chromatography (HPLC) because of higher efficiency and the variability of separation additives in order to change selectivities. The high efficiencies of MEKC result from the flat electroosmotic flow profile and the small mass transfer contribution. Short diffusion distances are provided by the close vicinity of the micelles to the analytes. Therefore, the inclusion-exclusion kinetics is very fast. In contrast to HPLC, the selectivity of a EKC-system can be easily changed within a few minutes just by filling the capillary with a separation buffer (SB) containing a different separation additive. Because the needed amount of separation additive is small, the list of potential additives can also include rare and exotic additives offering completely different selectivities compared to typical alkyl chain surfactants. But even a small variation of the alkyl part of a surfactant can change the selectivity and may help resolve certain peak pairs remaining unresolved with the typical surfactant sodium dodecyl sulfate (SDS) [1,2].

But the method also includes some disadvantages. First, a lot of potential separation additives with interesting selectivities interfere with detection. Interferences can arise when using mass spectrometric (MS) or UV detection ([1] and literature cited therein). A lot of suitable separation additives, e.g. calixarenes, proteins and charge-transfer interacting additives, are UV-active. Because UV detection is the most common detection method in electrophoresis these additives are excluded when using common capillary electrophoresis (CE) equipment.

Secondly, separations in EKC suffer from a small migration time window not being sufficient for the separation of complex mixtures because the separation additive is not stationary but moves on its own. The migration time window is expressed by t_{add}/t_0, where t_{add} is the migration time of the micelle/separation additive and t_0 is the time of the electroosmotic flow. Peak capacity in EKC is limited because all components to be separated have to elute within the migration time window. Additionally, when close to t_{add}, the analytes

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undergo a relatively high band broadening and hence resolution gets worse.

But there are possibilities to overcome these problems. Our solution is the application of the partial filling (PF) technique with a parallel reduction of the electroosmotic flow. In PF-EKC, the capillary is first filled with an UV-permeable buffer solution (background buffer, BB), followed by the injection of the separation buffer (SB) containing the UV-active separation additive. Filling with the separation buffer has to be stopped before it reaches the detection window. In theory, the analytes are separated in the separation buffer zone, cross over in the background buffer zone, where they are detected without interferences.

But the partial filling technique is also involving some problems. Usually, the separation buffer zone gradually moves towards the detection window which reduces an interference-free detection. Therefore, the term "migration time window" from conventional (M)EKC, t_{add}/t_0, has to be modified to the term "interference-free migration time window", t_{SB}/t_0, meaning the window between the time of the EOF, t_0, and the breakthrough of the separation buffer zone, t_{SB}. The possibilities to enlarge the interference-free migration time window are the same as in conventional (M)EKC which are based on a reduction of the electroosmotic flow. The methods include the use of buffers with higher contents of organic modifiers [3,4], capillary surface deactivation ([5] and papers cited therein) and the application of moderate counter pressures at the cathodic side [1,6–11].

As reported [9,10], in partial filling EKC the buffer solutions and buffer zone lengths have to be chosen carefully. In cases the electroosmotic flows of background buffer and separation buffer are different, the flat electroosmotic flow profile will be parabolically distorted by an intersegmental pressure arising at the boundary between BB and SB. In those cases efficiency is dramatically reduced compared to the case when the electroosmotic flows of the buffer zones are equal. Efficiency is maximum in the case of equalized electroosmotic flows of both zones [9] even when a weak counter pressure is applied.

2. Enlargement of the migration time window in EKC to infinity

Partial filling electrokinetic chromatography with the aid of weak counter pressures is an ideal combination to avoid interference with detection of UV-active or MS impairing separation additives and to achieve a reasonable migration time window. Most promising would be the case where the border between SB and BB becomes stationary and hence t_{add}/t_0 becomes infinite. This situation will result when the effective velocity of the separation additive, $u_{eff,add}$, becomes zero.

Because $u_{eff,add}$ results from a superposition of the electrophoretic movement of the (negatively charged) separation additive towards the anode, $u_{ep,add}$, and the velocity of the electroosmotic flow, u_{eo}, towards the cathode

$$u_{eff,add} = u_{eo} + u_{ep,add} \quad (1)$$

it results for $u_{eff,add} = 0$

$$u_{eo} = -u_{ep,add} \quad (2)$$

In other words, one has to manipulate the electroosmotic flow such that the values of u_{eo} and $u_{ep,add}$ are equal. Then, t_{add} becomes infinite, simplifying the equation for the retention factor $\tilde{k}$ according to Terabe et al. [12]

$$\tilde{k} = \frac{t_R - t_0}{t_0(1 - (t_R/t_{add}))} \quad (3)$$

to the well known equation for k in conventional chromatography

$$t_{add} \to \infty \Rightarrow \tilde{k} = \frac{t_R - t_0}{t_0} \equiv k \quad (4)$$

Under these conditions the partial filling system would be equivalent to a real chromatographic system with a real stationary phase. In the following, this will be called quasi stationary PF-EKC.

We describe in this paper the necessities for such a quasi stationary partial filling system using the UV-active polymeric dye Poly R-478 (Fig. 1) [8] as separation additive. The dye is a typical representative for separation additives which interfere with UV detection. In a previous paper it was shown that Poly R-478 offers interesting alternative selectivities compared to SDS [8].

In the present work Poly R-478 was used as an example to show exemplarily the optimization strategy for a quasi stationary PF system on the basis of optimized buffer concentrations [9] and the manipulation of the electroosmotic flow by counter pressure [6–11]. The general proceeding was demonstrated with a test mixture of amino/nitroaromatics.

The consequence of the filling velocity of the separation buffer for the system performance is another aspect which is

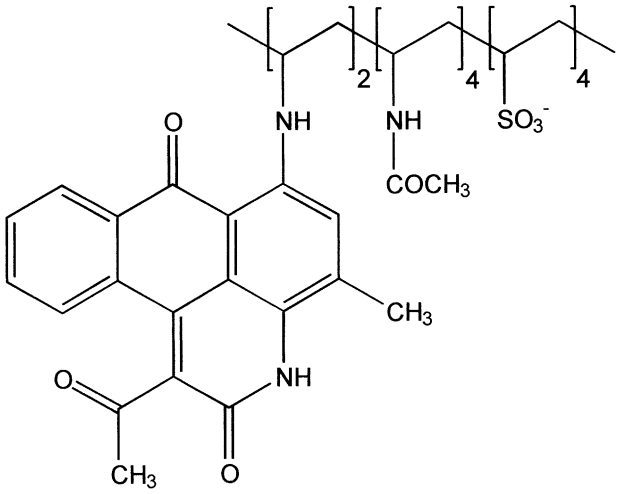

Fig. 1. Structure of the polymeric dye Poly R-478.

almost unnoticed and not yet investigated in PF-EKC. The filling velocity is controlled by the injection pressure. Dependent on the injection pressure a more or less pronounced parabolic distortion results at the border between the background and the separation buffer zone. Hence, studies on the influence of the injection pressure on the breakthrough behaviour of the zone and on the efficiency were included in this work.

3. Experimental

3.1. Buffer systems and chemicals

Buffer solutions were prepared from analytical grade chemicals (Merck, Darmstadt, Germany). Borate buffer was exclusively used. Two stock solutions were prepared having a concentration of 0.1 M with boric acid and adjusted to pH 9.4 and 10.7, respectively, with 1 M sodium hydroxide solution.

The separation buffer was prepared by diluting the borate buffer "pH 9.4" to a concentration of 20 mM with water–methanol (60:40, v/v), then Poly R-478 (Sigma, Steinheim, Germany) was added to give a concentration of 10 mg/mL. For the background buffers the borate buffer "pH 9.4" was diluted to concentrations of 10, 20, 30 and 40 mM with water and methanol 60:40 (v/v), respectively. For flushing purposes, buffers having the same concentrations were prepared from the stock buffer solution "pH 10.7".

For partial filling measurements with equalized electroosmotic flow velocities the separation buffer was used as above. The necessary background buffer concentration having the same electroosmotic flow as the separation buffer was estimated from the series of differently concentrated borate buffers prepared according to the procedure described in ref. [9]. As a result of this procedure the background buffer was prepared from the stock buffer solution "pH 9.4" to have a concentration of 23.0 mM borate in water/methanol 60:40 (v/v).

Thiourea (Merck, Darmstadt, Germany) was used as EOF marker for the background buffers. The electroosmotic flow velocity of the separation buffer was estimated with methanol as marker. 2,4-Diaminotoluene (2,4-DAT); 2,6-dinitrotoluene (2,6-DNT), 2,3-dinitrotoluene (2,3-DNT); 4-nitrotoluene (4-NT); 2,4,6-trinitrotoluene (2,4,6-TNT); 4-nitroaniline (4-NA) and 2,4-dinitrotoluene (2,4-DNT) were obtained from Promochem (Wesel, Germany) and injected at concentrations of about 200–600 μg/mL in buffer (20 mM borate, pH 9.4 in water/methanol 60:40 (v/v)).

All solutions were prepared using water purified by an Elgastat-UHQPS system (USF Elga, Ransbach-Baumbach, Germany), and filtered with 0.2 μm syringe filters (Schleicher & Schuell, Dassel, Germany).

3.2. CE system

All experiments were performed with a Prince system (injection device with high voltage power supply; Prince Technologies, Emmen, The Netherlands). Accurate counter pressures were achieved using an ER 3000 electropneumatic pressure controller (Dräger Tescom, Lübeck, Germany). It was controlled by the appropriate software on a PC.

For detection of the amino/nitroaromatics at 220 nm, a Jasco 875-CE UV detector (Jasco, Tokyo, Japan) and for data evaluation the PC-based integration software Gynkosoft Version 4.22 (Dionex Softron, Germering, Germany) were used.

Plain fused-silica capillaries, 63 cm × 50 μm i.d. × 365 μm o.d. (MicroQuartz, Munich, Germany) were applied. The effective capillary length was 41.5 cm.

3.3. Procedures

The determination of the necessary injection time of the separation buffer for different filling degrees and different injection pressures was carried out as follows: The capillary was first flushed with the background buffer "pH 10.7" for 3 min at 1000 mbar followed by rinsing with the background buffer "pH 9.4" for 3 min at 1000 mbar. Subsequently, the Poly R-478 separation buffer was injected by applying different injection pressures. At the same time the baseline was monitored until the breakthrough of the separation buffer at the detection window occurred. The breakthrough could be detected by an steeply increasing baseline. Breakthrough times were estimated to 1.92 min (450 mbar injection pressure), 4.64 min (200 mbar) and 18.11 min (50 mbar). Based on these breakthrough times the injection times necessary to provide different filling degrees were calculated.

Before each run in the partial filling mode, the capillary was rinsed with background buffer "pH 10.7" for 3 min and background buffer "pH 9.4" for 3 min at 1000 mbar followed by the injection of the separation buffer at different pressures and times. During the run, the inlet-vial contained the separation buffer and the outlet-vial contained the background buffer in order to avoid further zone boundaries. The buffer in the outlet-vial was replaced before each run. Injection of the sample was carried out hydrodynamically (10 mbar/0.1 min). The field strength was 476 V/cm throughout all measurements.

The effect of different injection pressures of the separation buffer was compared for 450, 200 and 50 mbar. For that purpose the amino/nitroaromatics were separated at filling degrees of 60 and 70% for each injection pressure (all these measurements were carried out without counter pressure). Estimation of the quasi stationary state was carried out with a filling degree of 80% (injection of the separation buffer at 200 mbar for 3.71 min) and different counter pressures.

4. Results and discussion

4.1. Effect of different injection pressures

The partial filling technique is inevitably connected with a boundary between the background buffer zone and the sep-

aration buffer zone which may give rise to additional band broadening. Even if the electroosmotic flows of the buffers are equalized so that no parabolic distortion by an intersegmental pressure occurs in the buffer zones, the boundary will have a slight parabolic shape due to the hydrodynamic injection of the separation buffer.

In open capillary tubing, pressure induces a laminar flow of a liquid, u_{hd}, showing a parabolic flow profile according to the equation of Hagen–Poiseuille [13]:

$$u_{hd} = \frac{\Delta p}{4\eta L}(R^2 - r^2) \quad (6)$$

where Δp is the pressure drop along the capillary, L is the total capillary length, R is the radius of the capillary and r is the radial position. The equation shows that at a given length and radius, the magnitude of the parabolic profile depends only on the pressure. Consequently, the parabolic profile at the zone boundary must decrease with decreasing injection pressure of the separation buffer. In practice, the parabolic deformation of the injection profile should be as small as possible because a steeper slope of the baseline increase at the breakthrough of the separation buffer zone and a lower broadening of the analyte bands due to zone crossing from SB to BB will result.

In order to scrutinize these considerations, the separation buffer was injected with pressures of 450 and 200 mbar to give filling degrees of 60 and 70%, respectively. Then the EKC separation of the amino/nitroaromatics mixture was performed without counter pressure to indicate the effect. The electropherograms are shown in Fig. 2. Using 200 mbar injection pressure, the increase of the baseline starts later compared to 450 mbar injection pressure and the slope is steeper. Both observations confirm the thesis that the parabolic deformation of the injection profile decreases with decreasing injection pressure. This is also illustrated by Fig. 3, where the separation of the amino/nitroaromatics at a filling degree of 80% and a counter pressure of 12 mbar is shown. At an injection pressure of 450 mbar, the deformation of the injection profile is as large that all components elute on the border zone between background buffer and separation buffer. At an injection pressure of 50 mbar, the same separation is almost not affected by an increasing baseline, because the parabolic distortion of the border is much smaller.

But with decreasing injection pressure the injection time increases inversely. Therefore, a compromise has to be made between injection pressure and injection time.

4.2. Estimation of the quasi stationary state

The influence of the counter pressure on the breakthrough of the separation buffer zone was investigated using a partial filling system with 80% filling degree. The separation buffer was injected with 200 mbar. Applying different counter pressures of 17–25 mbar the test mixture of amino/nitroaromatics was separated and the baseline was tracked for 60 min. The corresponding electropherograms are shown in Fig. 4. It can

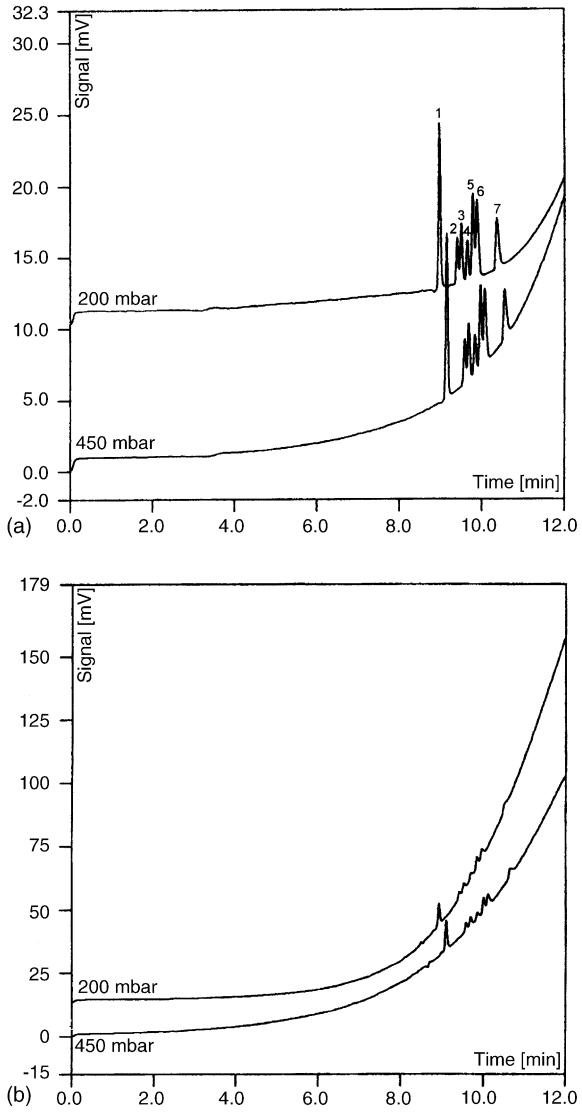

Fig. 2. Effects of different injection pressures for the separation buffer at filling degrees of (a) 60% and (b) 70%. Separation buffer: 10 mg/mL Poly R-478, 20 mM borate pH 9.4 in water/MeOH 60:40 (v/v). Background buffer: 23.0 mM borate pH 9.4 in water/MeOH 60:40 (v/v). Injection times at 450 mbar: 1.15/1.34 min (60/70% filling degree); injection times at 200 mbar: 2.78/3.25 min (60/70% filling degree). Peak identities: (1) 2,4-diaminotoluene; (2) 2,6-dinitrotoluene; (3) 2,3-dinitrotoluene; (4) 4-nitrotoluene; (5) 2,4,6-trinitrotoluene; (6) 4-nitroaniline and (7) 2,4-dinitrotoluene. Note the different scale of the *y*-axis.

be seen that an increase of the applied counter pressure enlarges the interference-free migration time window. At a counter pressure of 17 mbar the separation buffer zone breaks massively through within 60 min so that the baseline increase already impairs the electropherogram of the test mixture. With increasing counter pressures the increase of the baseline is more and more reduced to a very small elevation at 25 mbar. It might be possible that a certain amount of the separation additive always reaches the detection window along with the wall-near EOF because especially at higher counter pressures the difference between the EOF towards the detection window and the so-called central back flow [14,15]

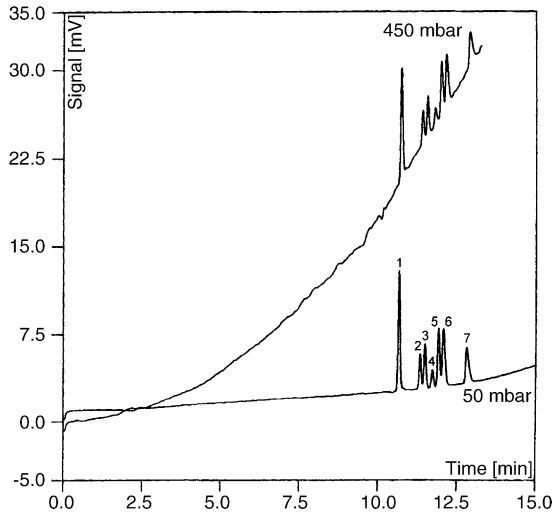

Fig. 3. Separation of some amino/nitroaromatics in a PF-EKC system with a filling degree of 80% and 12 mbar counter pressure using an injection pressure of 450 and 50 mbar. Separation and background buffer: as in Fig. 2. Injection of the separation buffer: 1.54 min/450 mbar and 14.49 min/50 mbar. Peak identities as in Fig. 2.

is intensified. This may explain the small elevation of the baseline.

Theoretically, it should be possible to calculate the necessary counter pressure for the quasi stationary state from a few breakthrough times at different counter pressures by a graph representing breakthrough time versus counter pressure. An interpolation of the breakthrough time to infinite should give the desired value. But to make this value reliable, measurements with relatively high breakthrough times have to be included. The procedure may last hours (compare Fig. 4). In addition, the calculation of the necessary counter pressure is difficult because the breakthrough curves are also influenced by the injection pressure of the separation buffer zone (compare Figs. 2 and 3) and by the ratio of u_{eo} and $u_{ep,add}$. Therefore, this method is not very suitable to determine the exact counter pressure for the quasi stationary state. It seems to be more practical to empirically find a compromise between an acceptable interference-free migration time window and a counter pressure as low as possible.

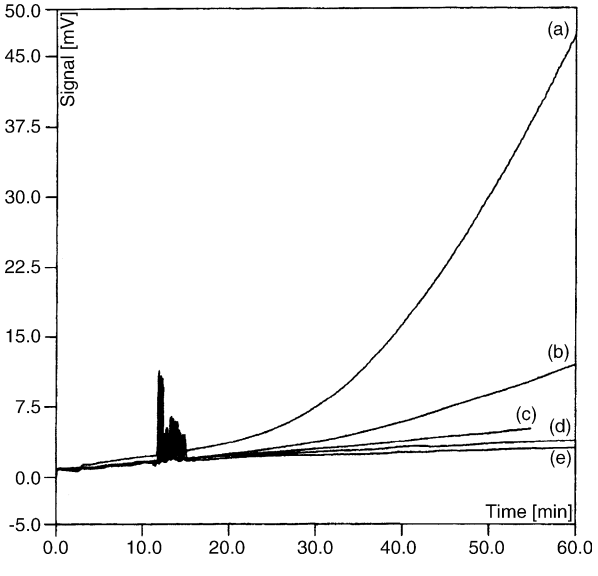

Fig. 4. Electropherograms showing the breakthrough of the separation buffer zone at different counter pressures at a filling degree of 80%. Counter pressure: (a) 17, (b) 19, (c) 21, (d) 23 and (e) 25 mbar as marked in the electropherograms. Separation and background buffer: as in Fig. 2. Injection of the separation buffer: 3.71 min/200 mbar.

4.3. *Efficiency and counter pressure in quasi stationary PF-EKC*

As discussed in the introduction and in references [9,11], increasing counter pressure decreases efficiency in PF-EKC but resolution follows a maximum curve because of an enlarged migration time window. To investigate these influences in quasi stationary PF-EKC the test mixture was separated applying a filling degree of the separation buffer of 80% and different counter pressures in the range given above (see Section 4.2). The separation buffer was injected with 50 mbar for 14.49 min in order to keep the deformation of the injection profile small.

Three exemplary electropherograms recorded at counter pressures of 18, 21 and 25 mbar are shown in Fig. 5. It can be seen that the separation quality of the test mixture is very similar in all three cases. At a counter pressure of 18 mbar, how-

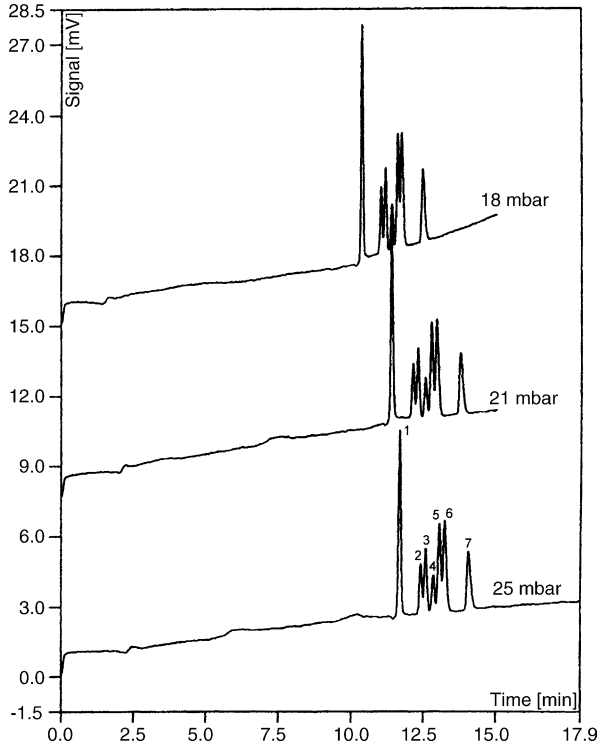

Fig. 5. Separation of some amino/nitroaromatics in a PF-EKC system at 80% filling degree. Counter pressure: 25, 21 and 18 mbar. Separation and background buffer: as in Fig. 2. Injection of the separation buffer: 14.49 min/50 mbar. Peak identities as in Fig. 2.

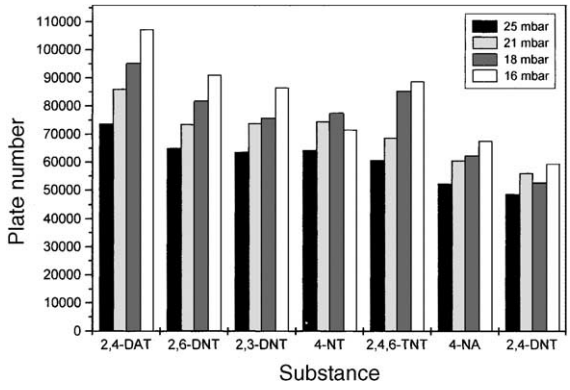

Fig. 6. Plate numbers vs. counter pressure for pressures of 25–16 mbar. Separation and background buffer: as in Fig. 2. Filling degree 80%. Injection of the separation buffer: 14.49 min/50 mbar.

ever the quasi stationary state of the separation buffer zone is not yet reached (increase of the baseline due to breakthrough of the separation additive Poly R-478). A counter pressure of 21 mbar seems to be ideal in order to approach the quasi stationary state and to keep the loss of efficiency as small as possible (see Fig. 6).

The resulting plate numbers for each component and counter pressures of 25, 21, 18, and 16 mbar are plotted as columns in Fig. 6. As expected, plate numbers are increasing with decreasing counter pressure. On the other hand resolution of the mixture is not significantly reduced (see Fig. 5) when increasing the counter pressure from 18 to 25 mbar because the increased migration time window compensates for the loss of efficiency [11]. This means that one is relatively free to adjust the counter pressure so that the interference-free migration time window is sufficiently large with respect to the given separation problem and the filling degree of the separation buffer.

5. Conclusion

The feasibility of a quasi stationary PF-EKC system with the application of weak counter pressures was investigated using an amino/nitroaromatic mixture as an example. The movement of the separation buffer zone was controlled by applying appropriate counter pressures. A quasi stationary state of the separation buffer zone could be realized by a fine tuning of the counter pressure. In order to realize a high efficiency in an electroosmotic flow equalized partial filling system the injection pressure and the counter pressure have to be kept as low as possible. However, a low counter pressure is less important because the loss of resolution is compensated by the larger migration time window at higher pressures. This means that the counter pressure can be freely adjusted so that the interference-free migration time window is sufficiently large for the given separation problem at the chosen filling degree of the separation buffer.

As an outlook we will try to detect the breakthrough of the separation buffer zone separately (at a wavelength different from the absorbing bands of the analytes) by a second detector before the analysing detector. This way the counter pressure can be automatically controlled to maintain the quasi stationary state of the separation buffer zone.

The described technique may be applied to electrokinetic chromatography of complex mixtures and to micropreparative separations in order to avoid a contamination of the analyte fractions by the separation buffer additive.

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