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Field-amplified sample stacking in micellar electrokinetic chromatography for on-column sample concentration of neutral molecules

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Abstract

On-column concentration of neutral molecules was achieved for the first time in micellar electrokinetic chromatography by means of field-amplified sample stacking. The stacking process was accomplished by dissolving the neutral analytes in a low-concentration micellar solution that was still above the critical micelle concentration. The lower total ionic strength in the sample buffer compared to the electrophoresis buffer allowed the negatively charged micelles to migrate rapidly into the boundary between the sample and the running buffer where they slow down. This field-amplified sample stacking was achieved by using normal or reversed electrode polarity and produced a 75–85-fold increase in sensitivity for 1,2,4,7- and 1,2,4,8-tetrachlorodibenzo-*p*-dioxins. The peak area counts obtained from the sample stacking process were proportional to the sample volume injected, and the stacking efficiency was dependent on the micellar concentration. The best stacking efficiency was obtained when the micelle concentration was slightly higher than the critical micelle concentration. When the injection volume was relatively small, the normal-polarity stacking procedure produced a higher stacking efficiency. However, when the injection volume was large, reversed polarity produced a higher stacking efficiency because the non-uniform distribution of the electrical field strength had been eliminated.

1. Introduction

In micellar electrokinetic capillary chromatography (MEKC), a micellar pseudo stationary phase, such as sodium dodecyl sulfate (SDS) and an aqueous buffer as the mobile phase are used to separate both charged and neutral molecules. An untreated fused-silica capillary surface is negatively charged, so that the bulk electroosmotic flow is toward the negative electrode, whereas the micelles, formed from an anionic surfactant, migrate in the opposite direction, toward the positive electrode. However, when standard MEKC conditions are used with anionic micelles, the dominant electroosmotic flow still drives the negatively charged micelles toward the negative end of the capillary.

Species having the same charge as that of the micelle do not interact with the micelle, while those having the opposite charge strongly interact with the micelle. The separation of charged

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species depends on the species' difference in electrophoretic mobility. On the other hand, the formation of micelles provides a unique chromatographic process for the separation of neutral molecules, whereby solute liquid–liquid differential partitioning between the micellar pseudo stationary phase and the electroosmotically pumped aqueous phase takes place [1]. Several published articles have described applications of MEKC for the separation of neutral molecules [2–4] and of charged species [5,6]. MEKC has become a popular microcolumn separation technique because of its high separation efficiency. Isotopically substituted compounds [7] and chiral molecules [8] have been separated by MEKC when organic modifiers were added to the mobile phase.

The high resolution of MEKC requires a small sample-injection volume. Small sample volumes, however, make the detection of low-concentration samples difficult. For example, in the case of UV-absorbance detection, typical concentration limits of detection (LODs) are of the order of 10^{-6} M [9], which is inadequate for the analysis of low-concentration constituents in biological samples, such as dioxins in human serum.

Sample stacking of charged species with discontinuous buffer systems has been used extensively in many areas of electrophoresis [9–11] to enhance the sensitivity of the measurements. When a sample is dissolved in a buffer with lower electrical conductivity than that of the electrophoresis running buffer, concentration or solute stacking occurs when the sample is injected electrokinetically. Because the electrical field strength in the sample medium is higher than that in the running buffer, the electrophoretic velocity is accelerated and the ions migrate rapidly to the boundary between the lower and higher conductivity zone. At this boundary the electrophoretic velocity of the ions is reduced, and the ions stack into a narrow zone.

Field-amplified sample injection is another way of achieving on-column concentration of charged species in capillary electrophoresis. In this procedure, a small plug of water is introduced at the inlet end of the capillary prior to electrokinetic sample injection. When a high voltage is applied across the capillary, a higher electric field strength is established across the water plug. Because the electroosmotic velocity of the bulk solution is slower than the electrophoretic velocity of the sample ions under the enhanced field strength, both positive and negative ions can be concentrated by switching the electrode polarity at the proper time [12]. Field-amplified sample injection can also be achieved by directly injecting low-conductivity sample buffers without using the water plug [13]. Researchers have shown that with this technique concentration-detection sensitivity can be increased several hundred fold [13].

In capillary electrophoresis, charged species have been shown to stack when extremely large injection volumes are used and when the sample buffer is pumped electroosmotically from the capillary while the stacking is in progress [14]. Although sample stacking can be effective for both positively and negatively charged species at high electroosmotic velocity or by polarity switching, neutral molecules are not affected by these techniques. In this report, we present a simple technique for on-column sample concentration of neutral molecules by using field-amplified sample stacking in MEKC media.

The dominant electroosmotic velocity of the buffer solution and the dragging force of the micelles in MEKC provide an excellent opportunity for stacking neutral molecules. To do so, we took a sample plug containing neutral molecules in a lower-conductivity micellar solution with the same surfactant as in the running buffer and introduced this plug hydrodynamically into the capillary filled with running buffer. Under high voltages, a higher electrical-field strength was established across the sample plug because of its higher resistivity. Stacking occurred when neutral molecules which were partitioned into the negatively charged micelles migrated rapidly into the boundary between the sample and the running buffer and slowed down. Using reversed electrode polarity during the sample stacking process, we were able to eliminate the non-uniform distribution of the electrical field by backing the sample buffer out of the capillary.

2. Experimental

An electrophoresis system similar to that described by Jorgenson and Lukacs [15] was constructed in our laboratory. The high-voltage power supply (0–60 kV) was from Glassman High Voltage (Whitehouse Station, NJ, USA). The high-voltage power supply can be configured to output either positive or negative voltages. Two power supplies configured with opposite polarity were used when reversed-electrode-polarity sample stacking was performed. Each end of the electrophoretic capillary was placed in a small glass reservoir containing the appropriate buffer and a platinum electrode connected to the power supply. The two reservoirs must be level for normal running conditions. A CV^4 UV detector from Isco (Lincoln, NB, USA) was operated at 230 nm, and the UV absorbance was recorded by a Shimadzu C-R3A integrator (Kyoto, Japan). The current running through the capillary was monitored on a chart recorder. An on-column optical detection cell was created by removing the polyimide coating from a short segment of the fused-silica capillary (10 cm from one end). The system, except for the detector and the integrator, was enclosed in a Plexiglass box with an interlock switch to protect analysts from high voltages. Timing and switching of the applied high voltage were accomplished by using a timer and a high-voltage relay, which was enclosed in a lead-impregnated plastic box to protect the operator.

Fused-silica capillary columns (50 μm I.D. × 360 μm O.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA) and used without surface modification. The total length of the capillary was 75 cm with an effective length of 65 cm. Ultrapure SDS was from Life Technologies (Gaithersburg, MD, USA). The 1,2,4,7- and 1,2,4,8-tetrachlorodibenzo-*p*-dioxins (TCDDs) used to characterize the stacking process were synthesized in our laboratory [16]. The polycyclic aromatic hydrocarbons (PAHs) were obtained from Aldrich (Milwaukee, WI, USA). All running and sample buffers were prepared with distilled water and filtered through a 0.45-μm disk filter (Alltech, Deerfield, IL, USA).

Sample injections were performed in one of two ways. When a very short injection band was desired (as in the case of a normal MEKC run without field-amplified sample stacking), the sample vial was placed 25 cm above the level of the buffer reservoir. The inlet end of the capillary was then inserted into the sample vial for a predetermined amount of time. An injection band length of approximately 0.2 cm was obtained with a 10-s injection. A 5-s injection resulted in an injection volume of approximately 2 nl. If a longer injection band was needed, a pressure of 10 p.s.i. (1 p.s.i. = 6894.76 Pa) was applied in the sample reservoir. With this method, we produced a rapid injection of various sample volumes onto the capillary by varying the injection time.

Sample stacking under reversed electroosmotic flow was monitored by measuring the electrical current through the capillary. Before the sample was introduced, the current through the capillary filled with the running buffer was measured. After the sample was introduced, the current decreased because of the lower electrical conductivity of the sample buffer. As the sample buffer was pumped out of the capillary by the reversed electroosmotic flow, the current increased gradually until it reached the initial measured value. At this point, the electrode polarity was set back to the normal configuration for the MEKC separation.

3. Results and discussion

Fig. 1 is a schematic diagram showing the field-amplified sample-stacking process under normal and reversed electrode polarity. Fig. 1A shows the stacking process being carried out with the same electrode polarity as in the separation process. The injected sample plug contains neutral molecules and a lower concentration of SDS than the running-buffer SDS concentration. In the sample buffer, no electrolytes were added, so the electrical conductivity was much lower than that in the running buffer region. The neutral molecules incorporated into the negatively charged micelles experienced a much higher

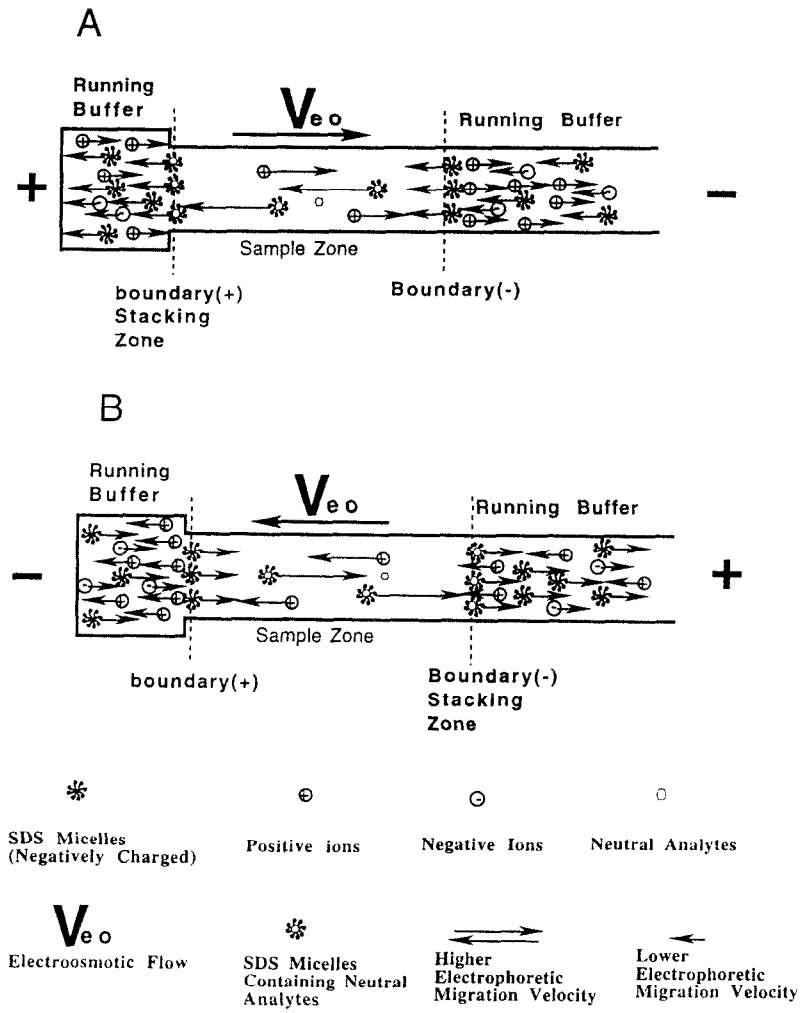

Fig. 1. Schematic diagrams showing the field-amplified sample-stacking process under normal (A) and reversed (B) electrode polarity.

electrical field strength and migrated rapidly toward the positive electrode. Their migration velocity was reduced when they encountered the boundary region where the electrical field strength was low. Therefore, the neutral molecules in the sample and running buffer boundary region were stacked toward the positive end of the electrical field across the sample zone. Since the bulk electroosmotic velocity (V_{eo}) was higher than the micelles electrophoretic migration velocity, the stacked sample zone migrated toward the negative electrode.

When a large volume of sample was introduced, the sample buffer had to be eliminated during stacking because a non-uniform distribution of the electrical field strength caused by the presence of the sample plug would deteriorate the chromatographic resolution. As depicted in Fig. 1B, neutral molecules in the sample can be stacked and buffer can be backed out of the capillary simultaneously by reversing the electrode polarity. This reversed polarity provides a possible procedure for introducing extremely large sample volumes into the capillary. In this

configuration, the neutral molecules incorporated within the negatively charged micelles are stacked toward the positive electrode while the direction of the bulk electroosmotic flow (V_{eo}) is reversed. The reversed electroosmotic flow causes the sample buffer to be pumped out the inlet end of the capillary while the stacking process is in progress. The stacking will be most efficient (higher number of theoretical plates) for neutral analytes with high partition coefficients into the micelles. On the other hand, neutral analytes with very low partition coefficients will be pumped out of the capillary under reversed electrode polarity.

The difference in migration velocity of micelles within the sample and the running buffer is the key to achieving the stacking effect in MEKC. As shown in Fig. 2, no stacking occurs when the sample buffer and the running buffer are the same. Chromatograms A and B in Fig. 2

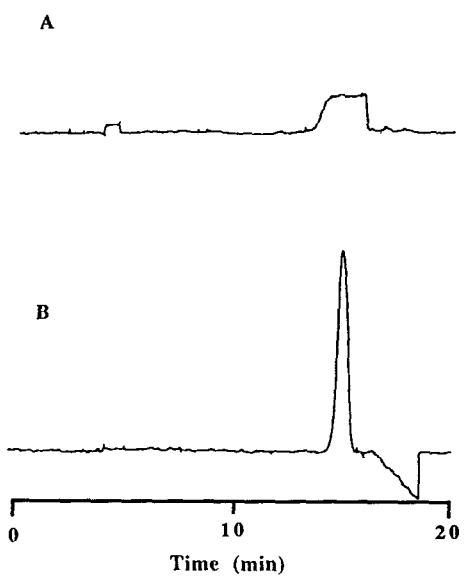

Fig. 2. Chromatograms obtained by using different sample buffers. (A) The running buffer consisted of 20 m*M* sodium phosphate, 40 m*M* SDS, and had a pH of 8.5; the sample buffer was the same as the running buffer. (B) Reversed-electrode-polarity sample stacking was performed using the running buffer shown in (A); the sample buffer was 9 m*M* SDS. The injection volume was 108 nl, applied voltage was 25 kV for both (A) and (B). Solutes were 1,2,4,7- and 1,2,4,8-TCDDs in both cases.

were obtained by injecting 108 nl of the sample into a 65 cm × 50 μm I.D. capillary. The sample shown in Fig. 2A was prepared by using the running buffer as the sample solvent. Because the electrical conductivity of the sample buffer was identical to that of the running buffer, there was no field enhancement, and sample stacking did not occur. The injected sample plug was not compressed, and it eluted as a square-shaped peak because of the extended length of the injection plug (5.5 cm). Under these conditions, reversed electrode polarity backed out the sample plug without stacking of the analytes. In contrast, when the sample was prepared in a lower-concentration SDS solution, the initial sample band was compressed by both normal- and reversed-polarity sample-stacking processes. The injection volume shown in Fig. 2B is the same as that in Fig. 2A. In Fig. 2B, however, the sample was prepared in a 9 m*M* SDS solution, and the resulting sample zone was compressed by a factor of approximately 4.

The three chromatograms in Fig. 3 show MEKC results following a normal injection and following field-amplified sample stacking under normal and reversed electrode polarity. Sample stacking allows a sample of relatively large volume to be injected without a significant effect on the chromatographic resolution. The UV response of the analyte was increased with either normal (Fig. 3B) or reversed (Fig. 3C) electrode polarity field-amplified stacking than with the non-stacking signal in Fig. 3A. As shown by the peak widths in Fig. 3B and C, at an injection volume of 32 nl, field-amplified sample stacking with the normal electrode polarity produced a higher stacking efficiency than reversed electrode polarity. This increased efficiency is due to the insignificant disturbance in the distribution of the electrical field strength across the capillary caused by the presence of a short sample plug (1.6 cm). The lower stacking efficiency for the reversed electrode polarity procedure (Fig. 3C) is not due to the sample buffer backing out from the injection end of the capillary but rather to the reversal of the electroosmotic flow during the stacking and the separation processes. The current laboratory-built system requires more than 1

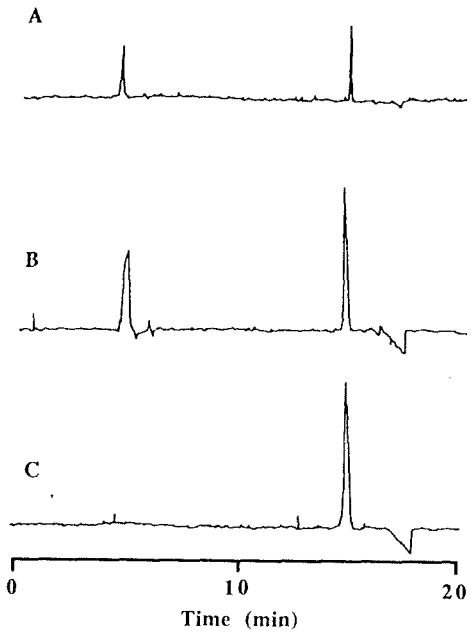

Fig. 3. Comparison chromatograms obtained with and without field-amplified sample stacking. Running buffer as in Fig. 2. The sample buffer was 9 m*M* SDS in all cases. Separation was carried out at 25 kV. (A) Gravity injection of 2 nl. (B) Pressure injection of 32 nl with normal-electrode-polarity sample stacking. (C) Pressure injection of 32 nl with reversed electrode polarity during sample stacking. Solutes were 1,2,4,7- and 1,2,4,8-TCDDs in all cases.

min to reverse the electrode polarity which allows additional time for peak diffusion and mixing effects to occur.

Fig. 3B and C show one advantage of using reversed electrode polarity during sample stacking to back out the sample buffer. The positive response at 4 min in Fig. 3B (which was due to the presence of the sample buffer) was not seen in Fig. 3C. The negative response in the chromatograms (Figs. 2B and 3B and C) after the analyte peak was caused by the excessive accumulation of SDS, which in turn was due to charge balance requirements.

We evaluated the stacking efficiency by measuring the theoretical plates at various injection volumes under both normal and reversed electrode polarity (Fig. 4). The highest stacking efficiency was obtained for a 2-nl injection volume. The stacking efficiency decreases with

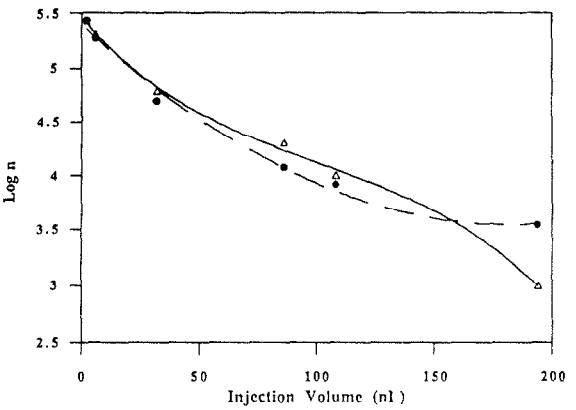

Fig. 4. Relationship between stacking efficiency and sample injection volume at different electrode polarities. Conditions as in Fig. 3B and C. The solid line is for normal electrode polarity, and the dashed line is for reversed electrode polarity. n = Number of theoretical plates.

increasing injection volumes (Fig. 4). At injection volumes smaller than 160 nl (8.2 cm injection band length) but greater than approximately 80 nl, normal electrode polarity gave a higher stacking efficiency even though the presence of the sample buffer, which is approximately 11 to 5.5% of the total length of the capillary, caused a non-uniform distribution of the electrical field. However, the extra peak-broadening effect caused by the reversal of the electrode polarity during the reversed electroosmotic flow had a greater negative effect on the resolution for injection volumes greater than 80 nl but less than 160 nl. When the injection volume was less than about 80 nl, the observed difference in stacking efficiency was marginal. However, when the injection volume was greater than 160 nl, the non-uniform electrical field distribution due to the longer injected band length caused the reversed-electrode-polarity method to give a higher stacking efficiency (Fig. 4). The actual value of the injection volume at which these two effects are equal needs to be experimentally determined for a particular chromatographic system. Depending on the injection volume, one can select whichever of the two modes for sample stacking that provides higher stacking efficiency.

We studied the effect of the micelles con-

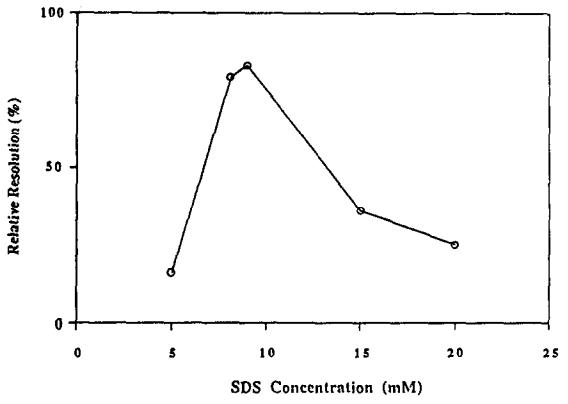

Fig. 5. Effect of SDS concentration on stacking efficiency. Conditions as in Fig. 3C. Injection volume was 32 nl. Resolution was normalized to the resolution obtained at 2 nl injection without stacking.

centration on the stacking efficiency over a range of SDS concentrations, and the results are summarized in Fig. 5. The first data point at a SDS concentration of 5 m*M* is below the critical micelle concentration; field-amplified sample stacking does not occur at this level. As expected, the best sample-stacking efficiency was obtained when the SDS concentration was at, or slightly higher than, its critical micelle concentration (8.1 m*M*). The electrical conductivity of the sample buffer increased with the increase of the SDS concentration. The stacking efficiency was proportional to the field-enhancement factor, which has been defined as the ratio of the electrical field strength of the running buffer to that of the sample buffer in capillary electrophoresis [17]. As shown in Fig. 5, this proportionality seemed true for MEKC measurements when field-amplified sample stacking was used, since the electrophoretic velocity of the micelles was proportional to the electrical field strength, which is in turn proportional to the stacking efficiency.

We also conducted a calibration experiment to determine the peak area counts over a range of injection volumes from 2 to 194 nl (Table 1). The peak area was proportional to the sample volume injected for both polarity configurations in the stacking process. We obtained regression coefficients of 0.9897 for normal electrode polarity and 0.9961 for reversed electrode polarity. The sensitivity for 1,2,4,7- and 1,2,4,8-TCDDs

Table 1
Calibration of peak area counts against injection volume for 1,2,4,7- and 1,2,4,8-TCDDs

Injection volume (nl)	Band length (cm)	Peak area (counts)	
		Normal	Reversed
2	0.10	3 266	3 266
6	0.31	8 063	8 250
32	1.63	36 550	46 298
86	4.38	82 125	126 690
108	5.50	140 740	152 310
194	10.0	271 220	243 490
r		0.9897	0.9961
S_a		9771	5564
S_b		99.6	56.7
Maximum sensitivity enhancement factor		75	85

Conditions: the running buffer consisted of 20 m*M* sodium phosphate and 40 m*M* SDS at pH 8.5; sample buffer consisted of 9.0 m*M* SDS; capillary was 65 cm × 50 μm I.D. untreated fused silica; 25 kV was used for both stacking and separation processes. S_a and S_b are the standard error of the regression on the intercept and the slope, respectively.

under the conditions outlined in Table 1 was increased by a factor of 75 to 85 for the two stacking processes.

Fig. 6 shows the separation of a mixture of 16 PAHs using cyclodextrin-modified MEKC. A normal 2-nl injection of the low-concentration PAH standard solution shown in Fig. 6A produced very small peaks for only three of the PAHs. A 54-nl injection of the same low-concentration standard using normal-electrode-polarity sample stacking conditions produced a significant sensitivity enhancement while maintaining high chromatographic resolution (Fig. 6B).

In conclusion, during MEKC neutral molecules can be effectively concentrated on-column by field-amplified sample stacking. The stacking process can be performed in either normal or reversed electrode polarities depending on the injection volume. For a small injection volume (smaller than 160 nl) with an SDS concentration of 9 m*M* in the sample buffer, the use of normal electrode polarity during the sample-stacking process gives better stacking efficiency. For volumes of greater than 160 nl at the same SDS concentration, however, the use of reversed electrode polarity during the sample-stacking process provides a higher stacking efficiency. Because reversed polarity allows large sample volumes to be injected, the concentration detection limits for neutral molecules can be significantly improved.

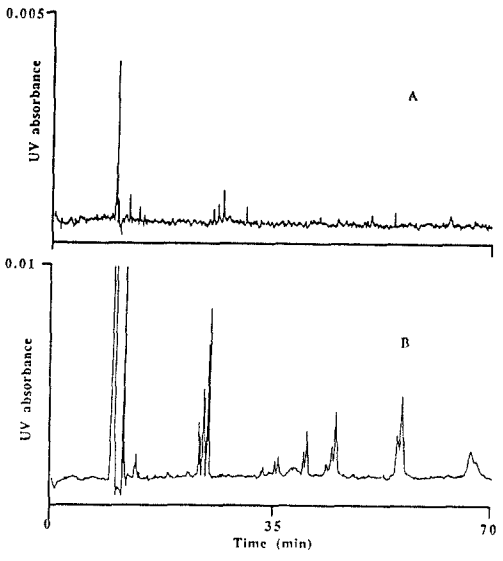

Fig. 6. MEKC chromatograms of a PAH mixture with and without field-amplified sample stacking: (A) 2-nl injection (0.005 absorbance range setting); (B) 54-nl injection (0.01 absorbance range setting) with normal electrode-polarity sample stacking. Conditions: The running buffer consisted of 100 m*M* sodium borate, 100 m*M* SDS, 5 *M* urea and 10 m*M* γ-cyclodextrin, and had a pH of 9.0. The sample buffer consisted of 9 m*M* SDS. The applied voltage was 27 kV.

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