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On-line concentration of neutral analytes for micellar electrokinetic chromatography
VI. Stacking using reverse migrating micelles and a water plug

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Abstract

Utility of a second enhanced field zone (water zone) is investigated for the on-line concentration of neutral analytes in micellar electrokinetic chromatography. Micellar solutions of sodium dodecyl sulfate prepared in acidic phosphate buffers are used as separation and sample solutions. Prior to long hydrodynamic injection of samples prepared in a low conductivity matrix, a long water plug is hydrodynamically injected to provide a second enhanced field zone. Practical and some fundamental considerations are presented. The technique is selective towards hydrophobic analytes. Notable detector response improvements (>100-fold) for several analytes are observed experimentally.

Keywords: Stacking; Neutral analytes

1. Introduction

Capillary electrophoresis (CE) has matured over the past few years into a powerful analytical tool used in diverse fields, and is considered orthogonal or complementary to high-performance liquid chromatography (HPLC). More popular modes are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis (CGE). Although separation efficiencies are far better with CE, concentration sensitivity is at least an order of magnitude poorer compared to HPLC. Vigorous attempts (i.e., on-line solid-phase extractors, membrane pre-concentrators [1], powerful detectors [2], sample stacking [3–8], etc.) have been done to solve the detection problem and thus further extend the use of CE for the analysis of samples in trace amounts.

Our research group is currently involved in the development of sample stacking techniques for neutral analytes in MEKC [3–7]. Sample stacking occurs at the boundary separating regions of low (high conductivity separation solution) and high (low conductivity sample solution or water zone) electric fields inside the capillary [8]. Samples are prepared in low conductivity matrices. Initially discovered for ionic analytes, sample stacking can be applied to nonionic analytes by proper manipulation of micelles and pertinent analytical parameters. Charged micelles will provide nonionic analytes effective mobilities necessary for both stacking and separation.

Reverse migration micellar electrokinetic chromatography (RM-MEKC) is characterised by micelles moving faster than the electroosmotic flow. Electroosmotic flow is either retarded by lowering the pH

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(below 5) [9] or by coating the capillary [10–12]. Sample stacking techniques in RM-MEKC mode using low pH phosphate buffers have been shown to be very good [5,7]. One mode is stacking with reverse migrating micelles (SRMM) [5], wherein samples prepared in water are injected using pressure into the capillary for long periods of time. To effect stacking and separation, voltages are simply applied at negative polarity after injection, with the separation solution in the anodic and cathodic vials. The other mode is field-enhanced sample injection with reverse migrating micelles (FESI-RMM) [7], wherein samples prepared in low conductivity micellar matrices are injected using voltage (negative polarity) into the capillary after injecting short plugs of water. The current during electrokinetic injection is monitored until 70–90% of the original current is reached. A vial containing the separation solution replaces the sample vial and voltages are again applied (negative polarity) to finish the analysis. Detector responses are increased up to more than 100-fold, plate numbers are still commendable, and optimisation schemes are simple.

In this article, another approach to concentrate neutral analytes inside the capillary in RM-MEKC is conveyed, stacking using reverse migrating micelles and a water plug (SRW). Several analytes (alkyl phenyl ketones, phenol derivatives, steroids and polycyclic aromatic hydrocarbons) are used to assess the new technique. Effects of the nature of the analyte, conductivity of the sample matrix, length of the sample solution, and length of the water plug are elucidated. A model to describe the technique and significant detector responses will be shown.

2. Theory

2.1. Stacking using reverse migrating micelles and a water plug (SRW) model

Fig. 1 depicts our model for SRW. The first step, after performing routine conditioning procedures, is to inject a long water plug into the capillary. The sample prepared in a low conductivity and low pH micellar matrix (S) is then injected into the capillary (Fig. 1A). Voltage is finally applied at negative polarity to effect stacking, removal of the sample matrix and water zone (Figs. 1B and C), and separation (Figs. 1C and D). The high conductivity and low pH micellar solutions (BGS) are found at both ends of the capillary during application of voltage.

To provide a zone inside the capillary with higher electric field compared to S, a water plug is injected prior to injection of S. Extra-band broadening effect due to the presence of micelles in S during stacking [5] can therefore be eliminated. Samples are prepared in low conductivity matrices such that most analytes in the S zone can reach the water zone faster. Also, more effective concentration can be gained since most analytes will reach the water zone with very small time differences. The low pH of S is adopted in order to reduce the surface charge of the capillary and thus to lower the bulk electroosmotic flow. Low bulk electroosmotic flow is preferred to prevent the loss of sample into the inlet vial.

The bulk electroosmotic flow (towards the cathode) is the averaged electroosmotic flow of S, water zone and BGS. Equations describing the bulk electroosmotic flow of this three-zone system would consider the field strengths and surface charges of the capillary in each zone. We will not attempt, in this paper, to derive such complex equations. If the effective electrophoretic velocities of analytes in S zone and water zone are higher than the bulk electroosmotic flow, the migration of the analytes is toward the anode (Fig. 1B). The maximum fraction of the capillary filled with low conductivity zones that will allow migration of analytes to the anode is governed by the x^*_{max} [4]. Here, the value of x^*_{max} is the sum of the fractions of the sample solution zone and water zone inside the capillary. Basically, no sample is lost to the inlet vial, if the fraction of the capillary filled with low conductivity zones is lower than the x^*_{max}. Note also that the electrophoretic velocity is higher in the water zone because of a higher electric field.

It has been shown that the migration velocity of micelles in the BGS inside the capillary changes from the direction of the cathode to the anode [5]. Lowering of the bulk electroosmotic flow during sample matrix backout causes the change in the direction of migration velocity of the micelle. On top of that, change in the conductivity of the low conductivity zone(s) (water and S zone in this case) and increase in the field strength in the BGS zone contribute to this phenomenon. The migration ve-

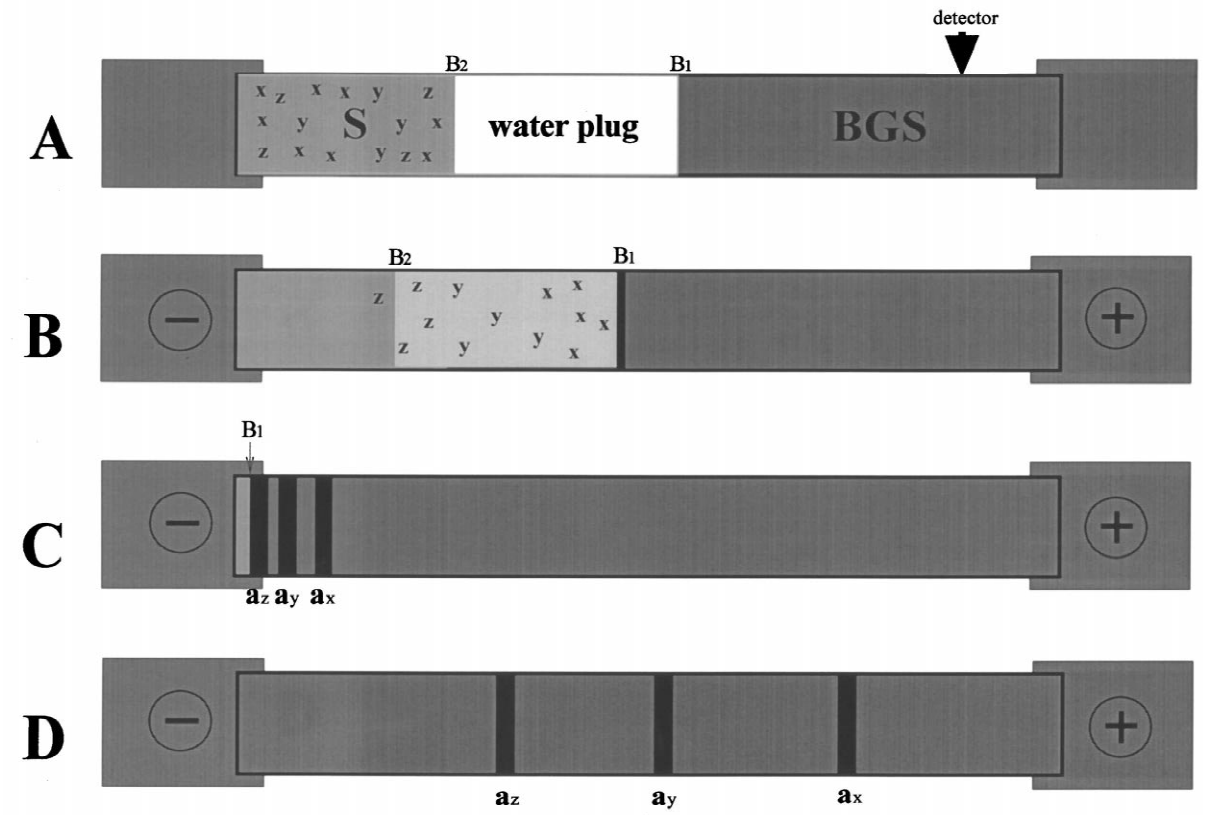

Fig. 1. Evolution of micelles and neutral analytes in the water zone, sample solution (S) and separation solution (BGS) zones during SRW MEKC analysis. (A) Hydrodynamic injection of water followed by S after conditioning the capillary with BGS (shaded parts indicate the presence of micelles); (B) application of voltage at negative polarity with the BGS in the inlet and outlet vials (neutral analytes solubilized in the micelles enter the water zone and stack in the concentration boundary in the order of decreasing retention factor $k(a_x)>k(a_y)>k(a_z)$); (C) separation of stacked zones prior to the total removal of the low conductivity zones; (D) focused bands separate by virtue of MEKC.

locity of the concentration or stacking boundary (initially found between the water zone and the BGS in Fig. 1A, B_1), however, is equal to the bulk electroosmotic flow and towards the cathode [8]. More studies are underway to elucidate the concentration boundary in CE with sample stacking. B_2 is the concentration boundary between the water zone and S, which is also assumed to move with the direction and velocity of the bulk electroosmotic flow (Fig. 1B). B_2 is not important for stacking since analytes stack at B_1. Cations from the BGS on the anodic side will stack in B_2, which has no detrimental effect on the entire procedure.

During application of voltage, micelles from the S zone enter the water zone and carry with them neutral analytes. Micelles from the inlet vial enter the capillary, which is necessary for separation of stacked zones and stacking of molecules not done by micelles in the injected S zone. It is possible that when all micelles in the injected S zone reach B_1, some molecules of neutral analytes are still found in the low conductivity zone. Anionic components of the BGS enter as well. Once the analytes reach B_1, they concentrate into thin bands because of the difference in electric fields. The S and water zones are pumped out by the bulk electroosmotic flow toward the inlet vial and are replaced by the BGS coming from the outlet vial.

Effective electrophoretic velocities are dependent on the retention factor, thus high retention factor compounds reach and stack at B_1 before low retention factor compounds (Fig. 1B). Fig. 2 describes

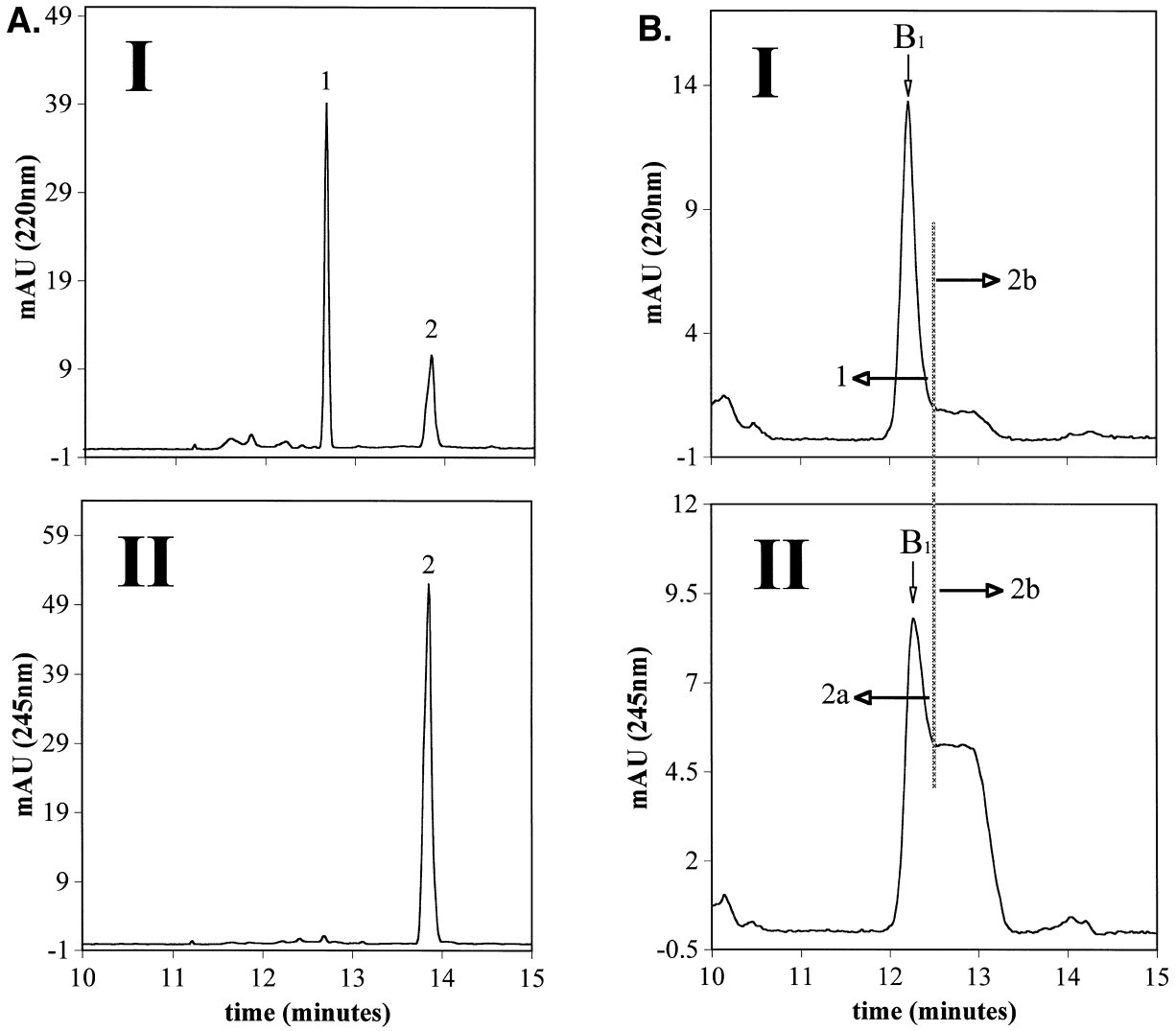

Fig. 2. Behaviour of neutral analytes during SRW. BGS, 50 m*M* SDS in 100 m*M* phosphate buffer (pH 2.0); sample matrix, 10 m*M* SDS in 4 m*M* phosphate buffer (pH 2.5); injection of S, 60 s; injection of water, 180 s; separation regimen, voltage (A), voltage for 2.5 min followed by pressure at 50 mbar (B) until all peaks are detected; applied voltage, −20 kV; peak identity, naphthalene (1), propionophenone (2), stacked propionophenone (2a), propionophenone in the low conductivity zone (2b); concentration of analytes, ~3–5 ppm.

this point. Fig. 2A shows the peaks of naphthalene and propionophenone obtained at two wavelengths (220 nm=naphthalene λ_{max} and 245 nm= propionophenone λ_{max}) after performing SRW. Retention factor of naphthalene is higher than that of propionophenone. To capture the analyte bands shown in Fig. 1B, a special experiment was performed. Voltage was applied for 2.5 min only and pressure (50 mbar) was used to drive the analyte bands to the detector (Fig. 2B). From Fig. 2, BI and an on-line recorded spectrum (not shown), 1 contains most of the naphthalene molecules that were considerably stacked. Although some molecules of propionophenone have stacked at B_1 (given by 2a in Fig. 2BII), most of them were contained in the low conductivity zone (given by 2b in Fig. 2BII). The characteristic spectrum of naphthalene was not found in the region around 2b.

Before the low conductivity zones are removed from the capillary, pre-mature separation of stacked

zones occur due to electrophoresis in the BGS [5]. Although this is not a very critical issue, it gives an idea that the field strengths in the zones are changing. Finally, separation of stacked zones continues with the application of voltage as depicted in Fig. 1D.

3. Experimental

3.1. Apparatus

All capillary electropherograms were obtained with a Hewlett-Packard 3D Capillary Electrophoresis System (Waldbronn, Germany). Electrophoresis and stacking were performed in fused-silica capillaries of 50 μm I.D. and 375 μm O.D. (64 cm, 56.5 cm to the detector) obtained from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were thermostated at 20°C. Wavelengths of detection for each analyte were selected using spectral absorbances curves recorded using a diode array detector. Conductivities were measured using a Horiba ES-12 conductivity meter (Kyoto, Japan).

3.2. Reagents and solutions

All reagents were purchased of the highest grade possible from Nacalai Tesque (Kyoto, Japan). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Stock solutions of 0.5 *M* sodium dodecyl sulfate (SDS) were prepared every week with purified water. Low pH micellar background solutions (BGS) were prepared by dilution of the SDS stock solution with appropriate phosphate buffers. Methanol (10–30%) was added to improve the separation of all test analytes. The BGSs were prepared every day to prevent migration time reproducibility problems, as pointed out in the previous paper [5].

Stock solutions of alkyl phenyl ketones (propionophenone, hexanophenone and valerophenone) were prepared in methanol. Stock solutions of polycyclic aromatic hydrocarbons (phenanthrene, fluorene and naphthalene) were prepared in acetonitrile. Concentrations of alkyl phenyl ketones and polycyclic aromatic hydrocarbons stock solutions ranged between 5200–6100 ppm. Particular care should be taken when handling polycyclic aromatic hydrocarbons as they are shown to be a health hazard. Stock solutions of phenols (amylphenol and butylphenol) were prepared with purified water. A few millilitres of ethanol (5–10%) were added to aid in the dissolution. Concentrations of phenol stock solutions were around 500 ppm. Stock solutions of steroids (testosterone, hydrocortisone and cortisone) were prepared in methanol at concentrations between 2100–2300 ppm.

Appropriate portions of the stock solutions were combined and diluted appropriately with methanol or separation solution to determine concentrations wherein analytes possess comparable peak heights. Portions of stock solutions were then combined and diluted to around 200 ppm (steroids and polycylic aromatic hydrocarbons) or 100 ppm (alkyl phenyl ketones and phenol derivatives) with methanol or water to yield the secondary stock solutions. Final dilutions (concentrations in the figures) were done with appropriate micellar phosphate buffer solutions for the optimisation processes. All solutions were filtered through 0.45 μm filters (Toyo Roshi, Japan) prior to capillary electrophoresis experiments.

3.3. SRW procedure

The capillary was rinsed prior to use with 1 *M* NaOH (10 min), followed by methanol (5 min), purified water (5 min), and finally with the BGS (5 min). A water plug was then introduced into the capillary at the cathodic end followed by injection of a sample solution using pressure (50 mbar). Voltage was applied at negative polarity with the BGS in the cathodic and anodic vials, until all peaks are detected. The capillary was flushed, between consecutive analyses to ensure repeatability, with 1 *M* NaOH (1 min), followed by methanol (1 min), 0.1 *M* NaOH (1 min), purified water (2 min), and finally with the BGS (2 min). Other conditions are specified in the text or figures.

4. Results and Discussions

4.1. Choosing the sample matrix

As pointed out in Section 2, low conductivity micellar solutions should be utilised as sample

matrix. Improvements of peak responses were lower when the BGS or high conductivity micellar matrices were used. Peak shapes were also distorted (similar to that observed with field enhanced sample injection with reverse migrating micelles [7]) for the less retained compounds. Longer water plugs significantly improved peak shapes, but improvements in peak responses are still not comparable to that obtained with low conductivity micellar matrices. The effect of using neutral and low conductivity micellar matrices was not investigated.

4.2. Effect of the length of the water zone and S

Keeping the ratio constant between the length of the water zone and S constant, the effect of increasing the length of the low conductivity zone (water zone+S zone) was first investigated. A ratio equal to one was chosen. Preliminary experiments have shown that at this ratio the peak shapes did not distort significantly with the increase in the lengths of the S and water zones. The sample steroids were used as test compounds. Fig. 3A and B show the effect of increasing the lengths of the low conductivity zones to stacking enhancement factors. Stacking enhancement factors [3] are calculated using the equations below, where the numerator is the peak height or corrected peak area obtained with stacking and the denominator is the peak height or corrected peak area obtained with the usual MEKC injection (2 s injection).

$$SE_{\text{height}} = \frac{H_{\text{stack}}}{H} \quad (1)$$

$$SE_{\text{area}} = \frac{A_{\text{corr,stack}}}{A_{\text{corr}}} \quad (2)$$

With the increase in the fraction of capillary filled with low conductivity zones, stacking enhancement factors increase only to a maximum (see Fig. 3). This is explained by the x^{*}_{max}, or the maximum fraction of the capillary filled with low conductivity zones, which will allow the migration of neutral analytes toward the stacking boundary. Longer injections of S and/or water are not useful in sample stacking for RM-MEKC (low pH), since the time to remove the sample matrix and/or water will take a long time, resulting in very long migration times. This is because of the protonation of silanol groups at low pH causing a low bulk electroosmotic flow.

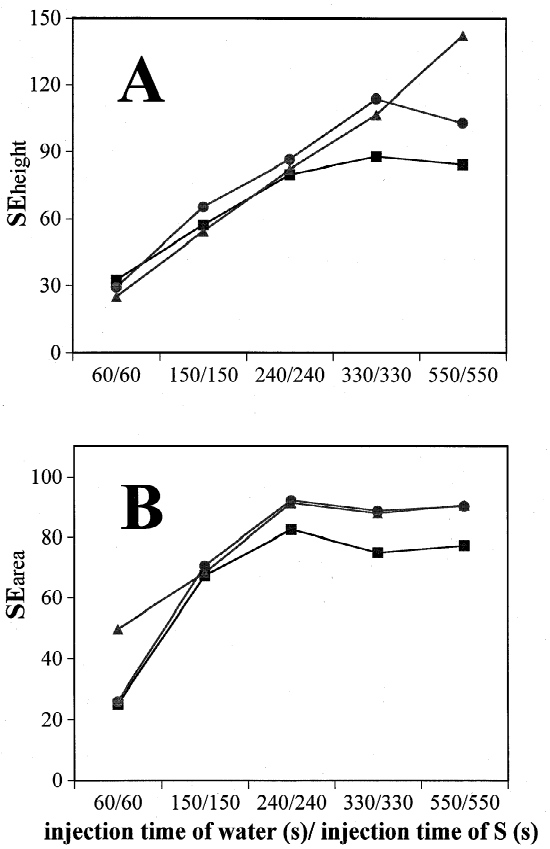

Fig. 3. Effect of the lengths of the low conductivity zones (water and S at a ratio equal to 1) on stacking enhancement factors (height, A and area, B). BGS, 50 m*M* SDS in 20% methanol/100 m*M* phosphate buffer (pH 2.0); sample matrix, 10 m*M* SDS in 4 m*M* phosphate buffer (pH 2.5); applied voltage, −25 kV; concentration of analytes, ~3 ppm; detection 247 nm.

A 240 s injection of S was then chosen. This injection time for S was regarded as the best value, related to the maximum amount of sample that can be injected and stacked into the capillary. The injection time of water was gradually lowered until a modest migration time was obtained without compromise to peak shapes. The optimised electropherogram of the test steroids is shown in Fig. 4 (notice the sharp peaks), wherein a 120 s injection of water was utilized. Commendable stacking enhancement factor values are obtained (Table 1A).

With the length of the S zone kept constant, the effect of the ratio between the length of the water and S zones (length of water zone/length of S

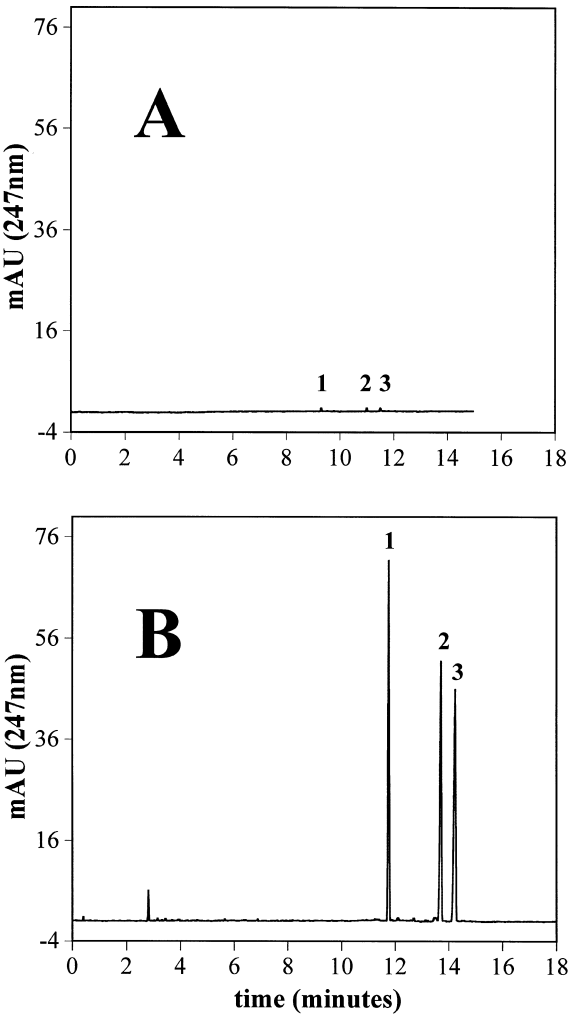

Fig. 4. Optimized SRW MEKC electropherogram of the test steroids. (A) 2 s injection of S; (B) 120 s injection of water and 240 s injection of S; for identification of peaks, see Table 1, part (A). Other conditions are the same as in Fig. 3.

zone = injection time of water / injection time of S) to peak shapes was studied. The sample polycyclic aromatic hydrocarbons were chosen as test analytes. It was found that when the ratio is low, peak shape of the lower retention factor analyte (naphthalene) becomes broad (Fig. 5B). If the ratio is high, however, peak shape of the higher retention factor analyte (phenanthrene) becomes broad (Fig. 5D). The exact mechanisms are unclear at this point. Probably with low ratios, the high electric field zone is short, giving low retention factor analytes less time to reach B_1. With high ratios, pre-mature separation of injected molecules found near B_1 could have caused the broad peaks.

Table 1
Stacking enhancement values obtained with SRW

	SE_{height}	SE_{area}
(A) Steroids		
(1) Testosterone	102	105
(2) Hydrocortisone	81	102
(3) Cortisone	72	106
(B) Polycyclic aromatic hydrocarbons		
(1) Phenanthrene	43	33
(2) Fluorene	41	49
(3) Naphthalene	28	32

Note: Conditions are the same as in Figs. 4 and 5 (parts A and C). The numbers above correspond to the peak numbers in the figures.

The optimised electropherogram with the optimum ratio is given in Fig. 5C. A 2 s injection is also included for comparison (Fig. 5A). Although polycyclic aromatic hydrocarbons are very hydrophobic, detector response improvements are not so great as expected (Table 1B) probably because of adsorption into the capillary walls. Improving the stacking of polycyclic aromatic hydrocarbons (environmentally important compounds) is currently being conducted in our laboratory.

4.3. Detector response improvements obtainable with SRW

Fig. 6 depicts the electropherograms obtained from a 2 s injection of sample solution (A), 2 s injection of sample solution diluted 100 times with sample matrix (B), and SRW of sample solution diluted 100 times with sample matrix (C). The sample phenols and alkyl phenyl ketones were used as test analytes. The peaks obtained in Fig. 6B are difficult to integrate since the peaks are as high as the background noise. The ratios of the responses obtained from SRW and A were then calculated to show how much stacking occurred (Eqs. (3) and (4)).

$$R_{\text{height}} = \frac{H_{\text{stack}} \text{ from S diluted 100 times with sample matrix}}{H \text{ from S}} \quad (3)$$

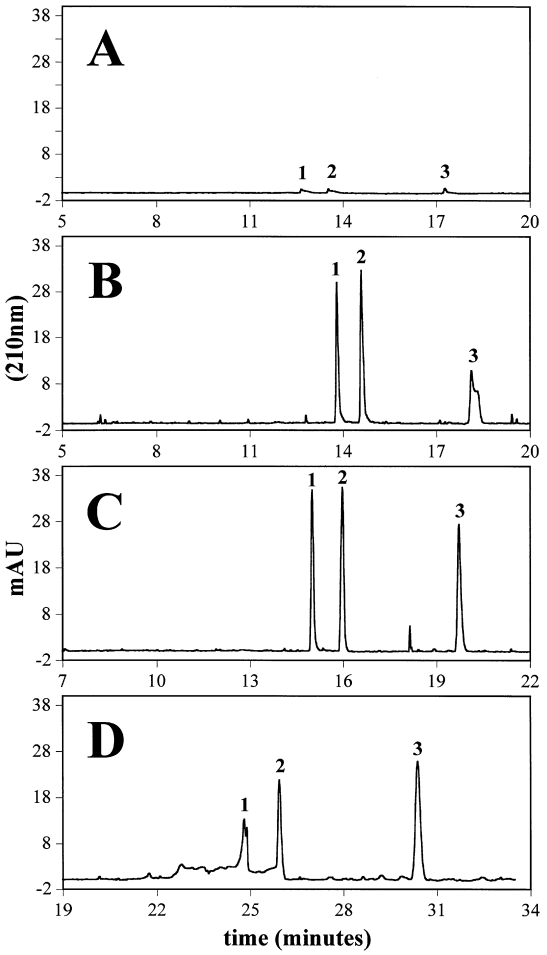

Fig. 5. Effect of the ratio of the length of the S and water zones on peak shapes. BGS, 50 m*M* SDS in 30% methanol/200 m*M* phosphate buffer (pH 2.0); sample matrix, 10 m*M* SDS in 10% methanol/4 m*M* phosphate buffer (pH 2.5); 2 s injection (A); ratios in terms of injection time (water/S), 30 s/120 s (B), 90 s/120 s (C), 360 s/120 s (D); applied voltage, −30 kV; concentration of analytes, ~1 ppm; detection, 210 nm; identification of peaks, see Table 1, part (B).

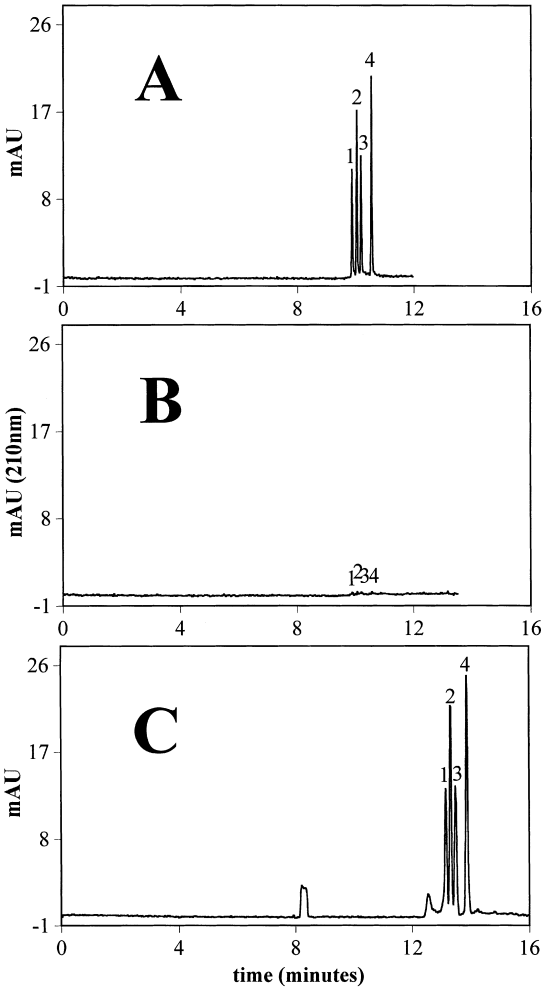

Fig. 6. Optimized SRW MEKC electropherogram of the test alkylphenyl ketones and phenols. BGS, 50 m*M* SDS in 10% methanol/200 m*M* phosphate buffer (pH 2.0); sample matrix, 10 m*M* SDS in 4 m*M* phosphate buffer (pH 2.5); injection, 2 s (A, B), SRW with 150 s injection of water and 240 s injection of S; concentration of analytes, ~100 ppm each (A), 1/100 dilution of the sample in A (B, C); applied voltage, −22 kV; detection, 210 nm; identification of peaks, see Table 2.

$$R_{\text{area}} = \frac{H_{\text{corr,stack}} \text{ from S diluted 100 times with sample matrix}}{A_{\text{corr}} \text{ from S}} \quad (4)$$

Values obtained are all more than one for all test analytes indicating that detector responses are improved more than 100-fold (Table 2). Resolution was a bit poorer with the SRW electropherogram compared to the 2 s injection electropherogram, due to overloading.

4.4. Effect of retention factors

Attempts were made to concentrate by SRW low retention factor phenols (e.g. phenol, methyphenols,

Table 2
Ratios obtained from SRW with a 100-fold dilution of sample solution and 2 s injection of sample solution

	R_{height}	R_{area}
(1) Amylphenol	1.18	2.92
(2) Hexanophenone	1.27	1.74
(3) Butylphenol	1.07	1.60
(4) Valerophenone	1.18	1.82

Note: Conditions are the same as in Fig. 6. The numbers above correspond to the peak numbers in Fig. 6.

acetophenone, etc.) but the results were not satisfactory. Injections of a micellar zone (low conductivity micellar sample matrix or BGS) after injection of the sample solution, slightly improved the stacking of low retention factor compounds. Stacking of low retention factor compounds is under investigation. Nevertheless, high retention factor analytes can be stacked effectively and with ease as shown above. The present technique is therefore selective for hydrophobic analytes, which is an advantage when dealing with real samples containing large amounts of neutral and polar substances, wherein the target analyte is a hydrophobic one. Compounds with moderate retention factors can also be analyzed using the present technique and the expected stacking enhancement factors are intermediate between those of low and high retention factor compounds. The extent of stacking in this middle group will also vary depending on their exact retention factors.

4.5. Application to quantitative analysis

Calibration curves within a concentration range of 1×10^{-5} *M* and 1×10^{-7} *M* (around 3.5 to 0.05 ppm) are constructed for the SRW MEKC analysis of steroids in Fig. 4B. Linear regression analysis equations [where y is the peak height (mAU), x is the concentration in ppm multiplied by 10, and *CL* is the concentration detection limit ($S/N=3$)] are as follows: $y=1.93x+3.61$, $r=0.995$ and $CL=15.5$ ppb (testosterone); $y=1.60x+1.97$, $r=0.998$ and $CL=18.7$ ppb (hydrocortisone); $y=1.39x+2.38$, $r=0.996$ and $CL=21.6$ ppb (cortisone). Note that responses are linear within two orders of concentration magnitude, which is not found in other stacking techniques reported by us [3–7]. Reproducibility of peak heights (%R.S.D., $n=5$) are 4.57% (testosterone), 3.00% (hydrocortisone) and 1.19% (cortisone). Reproducibility of migration time (%R.S.D., $n=5$) are 0.34% (testosterone), 0.27% (hydrocortisone) and 0.29% (cortisone). Reproducibility values were computed from five consecutive experiments. In summary, these statistical values are very favourable.

5. Conclusion

We have described how to use a second enhanced field zone to perform on-line concentration of neutral analytes in MEKC. Critical issues are the lengths of the sample solution and water zone, and the conductivity of the sample solution. An added feature of the technique is selectivity towards hydrophobic compounds, which could be both an advantage and disadvantage. More than 100-fold gain in detection sensitivity was demonstrated. Detailed mechanisms of the technique will be the subject of future studies.

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