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Separation and on-line preconcentration by sweeping of charged analytes in electrokinetic chromatography with nonionic micelles

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Abstract

The separation and on-line preconcentration by sweeping of charged analytes in electrokinetic chromatography using a neutral pseudostationary phase is described. Under neutral or basic conditions, the electrophoretic migration of anionic analytes towards the anode is overcome by a high, cathode-directed electroosmotic flow; hence, they experience net migration towards the cathode, and the system is run at positive polarity mode. The separation and the retention factor, *k*, are dependent on both the analyte's electrophoretic mobility and its interaction with the pseudostationary phase. The versatility of the sweeping mechanism is then shown in this system. The charged analyte, prepared in a matrix free of the pseudostationary phase, penetrates the pseudostationary phase zone upon application of voltage. Analyte molecules are consequently accumulated and concentrated. As a demonstration, the separation and preconcentration of phenol derivatives using nonionic surfactants of the alkyl polyoxyethylene ether type (Brij 35 and Brij 58) yielded peak height enhancements up to 100-fold. The efficiency of sample stacking was also found to be improved with the use of a high viscosity background solution.

Keywords: Micellar electrokinetic chromatography; Sample stacking; Sweeping; Viscosity; Phenols; Chlorophenols; Alkylphenols

1. Introduction

Electrokinetic chromatography (EKC) is a mode of capillary electrophoresis (CE) that separates analytes based on partitioning to a pseudostationary phase [1,2]. EKC has been applied to solve many analytical separation problems because of the high efficiencies (i.e., >100 000 theoretical plates) attainable. Although initially conceived to separate neutral analytes [3], EKC has been extended to the separation of charged solutes that are difficult to separate by other CE modes [4–6]. The use of charged pseudostationary phases, like sodium dodecyl sulfate, is by far the most famous experimental form of EKC, however, the use of uncharged pseudostationary phases like nonionic surfactants have been proved to be useful as well for the separation of some interesting charged molecules (e.g., peptides and derivatized amino acids) [7–9]. Nonionic surfactants have the distinct advantage of not contributing appreciably to Joule heating, hence, they may be used at high concentrations [10].

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The analysis of low concentration analytes has always been a problem in CE due to the limited sample injection volume and the short pathlength for on-line UV–Vis detection. To address this in EKC, on-line preconcentration techniques like sample stacking and sweeping have been developed to improve detection sensitivity [11–17]. These techniques enable detection sensitivity improvement by permitting increase in the amount of sample loaded without compromising separation efficiency and resolution. This is achieved by narrowing the injected analyte zones. In sample stacking, the analyte zones are narrowed due to a change in effective electrophoretic velocity as the molecules incorporated in the pseudostationary phase pass the interface that separates regions of low and high electric fields. In sweeping, the analyte zones are narrowed due to a chromatographic or partitioning mechanism as the sample molecules experience the pseudostationary phase zone. The general condition for sweeping is that the sample be prepared in a matrix void of the pseudostationary phase used. Palmer et al. suggests a different mechanism under sweeping conditions in which the sample is prepared in a matrix with conductivity about two to three times greater than that of the micellar separation solution [18]. We believe, however, that a higher conductivity sample matrix does not significantly affect the focusing effect of sweeping [19]. This is corroborated by the recent work of Lin et al. in which it is shown that a sample matrix conductivity about two times greater than that of a cationic micellar separation solution is not necessary to provide significant preconcentration by sweeping [20]. Several tens to hundred-fold improvement in sensitivity have been reported with sample stacking, while several tens to several thousand-fold improvement in sensitivity has been obtained with sweeping. Moreover, a combination of sample stacking by electrokinetic injection in capillary zone electrophoresis (CZE) mode and sweeping in EKC mode afforded almost a million-fold increase in detection sensitivity of positively charged analytes [21]. More recently, the sweeping principle has been extended to CZE separations of neutral solutes involving complexation reaction of *cis*-diols with borate demonstrating the versatility and wide applicability of the sweeping technique [22].

In this report, we describe the separation and sweeping of charged analytes in EKC using neutral pseudostationary phases. Phenol derivatives are used as test samples and the nonionic micelles of Brij 35 and Brij 58 are used as neutral pseudostationary phases. The k equation for the current system is derived and the experimental k values are determined. Next, the sweeping equation is derived and experimentally evaluated. It is interesting to note that unlike previously reported sweeping mechanisms, where the pseudostationary phase or complexing agent penetrates the sample zone to effect preconcentration, here, the sample penetrates the pseudostationary phase zone where it is concentrated due to a partitioning mechanism. The effect of a high-viscosity background solution (BGS) on sample stacking is also briefly studied.

1.1. Retention of charged analytes in electrokinetic chromatography using a neutral pseudostationary phase

The retention factor, k, in EKC is the ratio of the number of moles of solute in the pseudostationary phase and in the aqueous phase [3]. The k is directly related to the affinity of the solute to the pseudostationary phase. For a charged analyte, a′, and using a neutral pseudostationary phase (NPS-EKC), k can be determined experimentally using Eq. (1), which is derived from the k equation for charged solutes in micellar EKC (MEKC) suggested by Terabe's [3,4] and by Khaledi's groups [5]:

$$k = \left| \frac{\mu_{ep}(a') - \mu(a')}{\mu(a')} \right| \quad (1)$$

where the electrophoretic mobility of the charged analyte, incorporated into the neutral pseudostationary phase, is assumed to be zero. The electrophoretic mobility of a′ in CZE and the effective electrophoretic mobility of a′ in EKC are $\mu_{ep}(a')$ and $\mu(a')$, respectively. The value of mobilities, $\mu(a')$ and $\mu_{ep}(a')$ can be determined experimentally using Eq. (2) [1]:

$$\mu = \left(\frac{1}{t_r} - \frac{1}{t_o} \right) \cdot \frac{Ll}{V} \quad (2)$$

The migration time of the analyte is represented by $t_{r,}$ while that of the unretained compound, as well as

that of the neutral pseudostationary phase, is given by t_o. The total length of the capillary is L while the length of the capillary from inlet end to detection window is l. The ratio between l and t_o defines the electroosmotic flow (EOF) and pseudostationary phase velocity.

1.2. Sweeping of charged analytes in NPS-EKC

The sweeping of a negatively charged solute, a′, in NPS-EKC is shown in Fig. 1. The injection of a long plug of sample (S, shaded area), prepared in a matrix with conductivity equal to that of the BGS but devoid of the pseudostationary phase, is depicted in Fig. 1A. A homogeneous electric field across the capillary is present since the conductivities of the liquids inside the capillary are equal. NPS-containing BGS is found at both ends of the capillary. Analyte molecules at the interfaces of S and BGS in the anode and cathode sides of the S zone are given by a'_a and a'_c, respectively. The first batch of NPS that will enter the capillary by virtue of EOF is given by ns_a. The NPS that is found near the S and BGS interface in the cathode end of the S zone is ns_c. The electrophoretic velocity of a′ and the EOF velocity are given by $v_{ep}(a')$ and v_{eof}, respectively. The electrokinetic velocities are assumed similar in both the S and BGS zones.

When voltage is applied, neutral pseudostationary phase will enter the capillary due to EOF. The reverse migrating a'_a molecules will penetrate the neutral pseudostationary phase zone (Fig. 1B), where they are subsequently accumulated (dark zone). The magnitude of analyte accumulation is primarily dependent on the affinity of the analyte to the neutral pseudostationary phase. Upon continued application of voltage, the last batch of analyte molecules, a'_c, will enter the neutral pseudostationary phase zone (Fig. 1C).

The final length of a′ zone after sweeping, l_{sweep} (Eq. (3)), may then be approximated by the difference in distances traveled by ns_a (d_{ns}, Eq. (4)) and a'_a ($d_{a'}$, Eq. (5)):

$$l_{sweep} = d_{ns} - d_{a'} \quad (3)$$

$$d_{ns} = v_{eof} t_{sweep} \quad (4)$$

$$d_{a'} = v_{a'}(\mathrm{EKC}) t_{sweep}; \; v_{a'}(\mathrm{EKC}) = v^{*}_{ep}(a') + v_{eof} \quad (5)$$

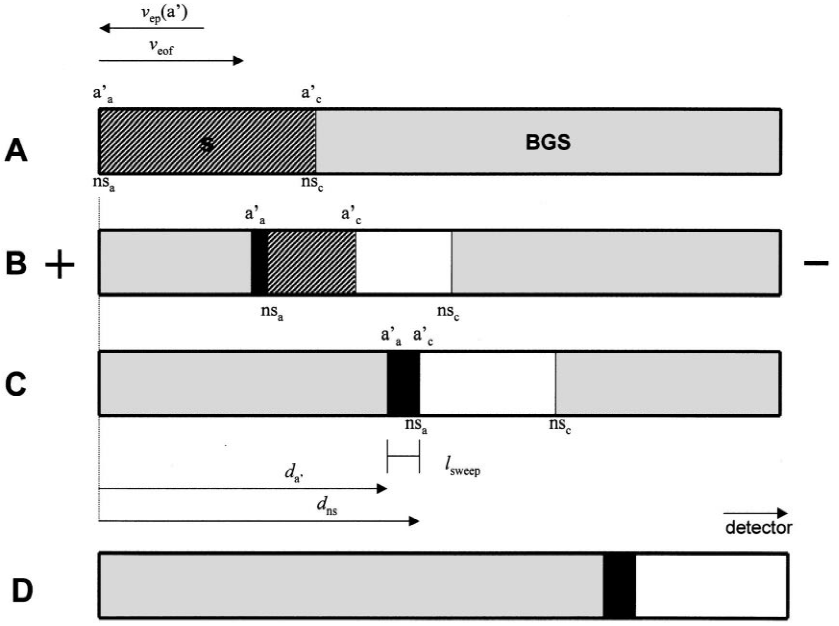

Fig. 1. Sweeping of a charged analyte in electrokinetic chromatography with a neutral pseudostationary phase.

The migration velocity of a′ in NPS-EKC is $v_{a'}$(EKC), in which v^*_{ep}(a′), the effective electrophoretic velocity of a′ (Eq. (6)), is derived from Ghowsi et al.'s [23] equation:

$$v^*_{ep}(a') = \frac{1}{1+k} v_{ep}(a') \quad (6)$$

The electrophoretic velocity of a′ in the sample zone and the time required for a'_c to reach ns_a are v_{ep}(a′) and t_{sweep}, respectively. These two terms are related by the injection plug length, l_{inj}, as shown in Eq. (7):

$$t_{sweep} = -\frac{l_{inj}}{v_{ep}(a')} \quad (7)$$

Substitution of Eqs. 4, 5, 6, and 7 to Eq. (3) yields the equation for l_{sweep}, defined in terms of injection plug length and retention factor (Eq. (8)):

$$l_{sweep} = l_{inj}\left(\frac{1}{1+k}\right) \quad (8)$$

This suggests that high k analytes will result in narrower and more concentrated zones than low k analytes after sweeping. Interestingly, the length of the resulting charged analyte zones after sweeping with a neutral pseudostationary phase is similar to those obtained for uncharged and charged analyte zones using a charged pseudostationary phase.

Finally, the swept a′ zone migrates to the detector due to EOF and its affinity to the neutral pseudostationary phase (Fig. 1D).

2. Experimental

All capillary electrophoresis experiments were carried out with a Beckman P/ACE 2000 System (Fullerton, CA, USA) equipped with 50 μm I.D.× 375 μm O.D. fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA). Capillary effective (inlet-to-detector) length was 50 cm while total length was 57 cm. Separation voltage was 20 kV at the inlet and capillary liquid coolant temperature was set at 25°C. Pressure injections were carried out at 3450 Pa (0.5 p.s.i.). Detection wavelength used was 214 nm. In order to approximate the length of the sample plug injection, the velocity of the liquid was determined as follows: a neutral marker was injected into a capillary filled with neutral pseudostationary phase-containing BGS and the plug was brought to the detector using pressure at 3450 Pa. The effective length (cm) was then divided by the migration time (min) of the marker to obtain the velocity of the liquid during injection. Indirect viscosity measurements were made in the same manner since it is known that viscosity varies inversely with velocity. The velocity of a sample matrix, which was desired to be adjusted in terms of viscosity, was determined, and polyethylene glycol 20 000 was gradually added until its velocity was approximately equal to that of the BGS of interest. Conductivities were measured with a Horiba ES-12 conductivity meter (Kyoto, Japan). The pH of solutions was measured and adjusted with the aid of a Beckman Φ 34 pH meter. Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA)

Reagents of the highest grade available were used. The phenol derivatives and dimethyl sulfoxide were from Nacalai Tesque (Kyoto, Japan); polyoxyethylene (23) lauryl ether (Brij 35) and polyethylene glycol 20 000 were from Wako (Osaka, Japan); disodium hydrogenphosphate was from Kanto (Tokyo, Japan); sodium tetraborate decahydrate was from Fluka (Switzerland); and polyoxyethylene 20 cetyl ether (Brij 58) was from Sigma (St. Louis, MO, USA). Stock solutions of the phenols were prepared with purified water. Appropriate amounts of the stock solutions were combined and diluted with water or neutral pseudostationary phase-free buffer to obtain the sample solutions. Background solutions were prepared every day to prevent reproducibility problems. All solutions were filtered through 0.45 μm filters from Toyo Roshi (Tokyo, Japan) prior to use.

The new capillary was flushed (at 138 kPa) with 1 *M* NaOH (30 min), followed by methanol (30 min), 0.1 *M* NaOH (30 min), purified water (30 min) and, finally, with the BGS (5 min). To ensure repeatability, the capillary was flushed between consecutive analyses with 0.1 *M* NaOH (1 min), methanol (1 min), purified water (2 min) and, finally, with the CZE buffer or BGS (5 min).

3. Results and discussion

3.1. Separation of phenol derivatives by CZE and NPS-EKC using a nonionic surfactant

Fig. 2A and 2B shows the separation of the three isomers of nitrophenol in CZE and NPS-EKC modes, respectively. In CZE, separation is brought about by the difference in the analytes' electrophoretic mobilities, and the order of elution is largely governed by their pK_a values. Under the conditions employed here, in which the analytes migrate electrophoretically against a high, cathode-directed EOF, the *m*-isomer, which has the highest pK_a, eluted first since it bears the least negative charge. The *o*- and *p*-isomers were not resolved, and they migrated as a single peak since their pK_a values are too close (Fig. 2A). In contrast, in NPS-EKC, all three isomers were fully resolved (Fig. 2B). Incorporation of a neutral pseudostationary phase, in this case, Brij 58, in the BGS provides an additional dimension for separation based on different affinities for the neutral pseudostationary phase. Nonionic micelles of the type used here possess a hydrophobic core and a hydrophilic

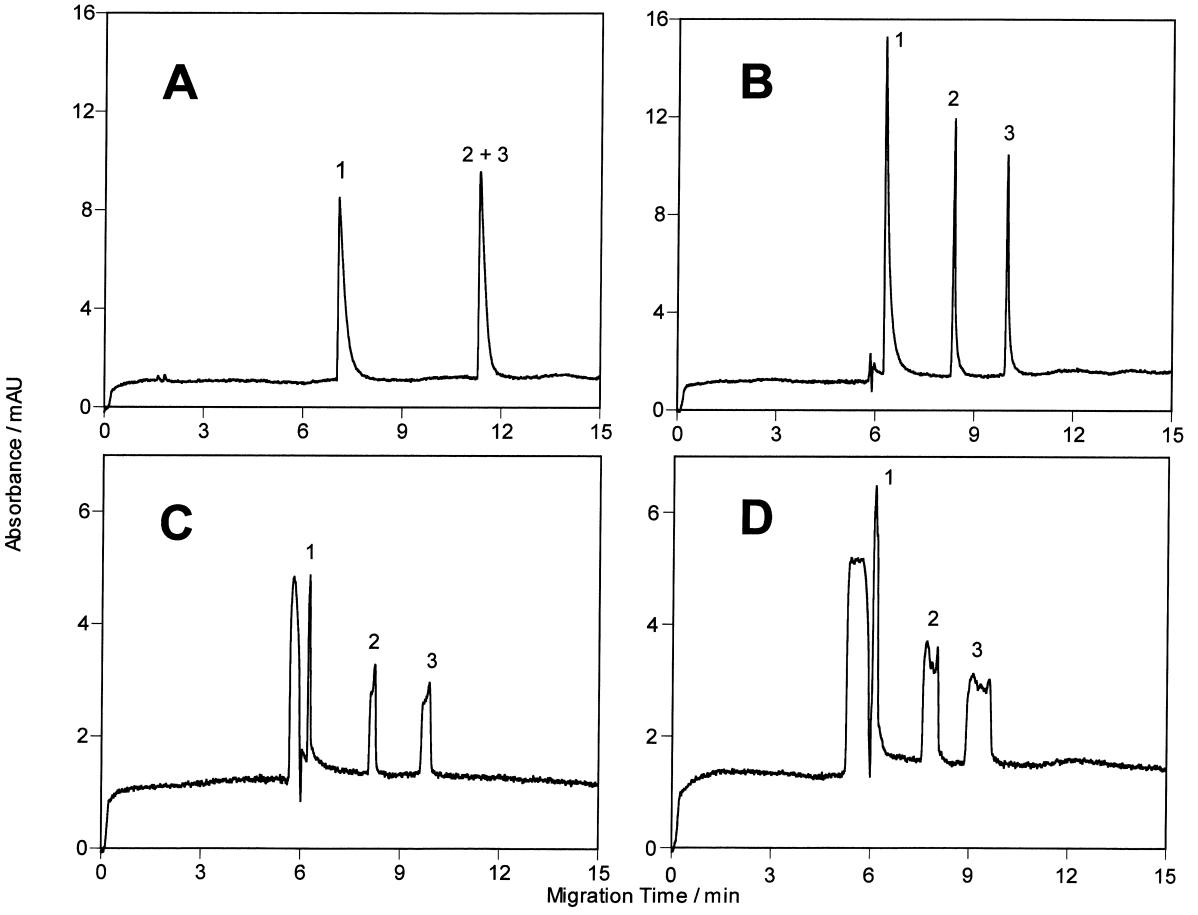

Fig. 2. Separation of nitrophenol isomers by CZE (A) and NPS-EKC (B, C and D). Conditions: BGS, 50 m*M* phosphate buffer, pH 8.50 (A) and 50 m*M* Brij 58 in 50 m*M* phosphate buffer, pH 8.50 (B, C and D); samples, 3-nitrophenol (peak 1, pK_a = 8.28), 4-nitrophenol (peak 2, pK_a = 7.15), and 2-nitrophenol (peak 3, pK_a = 7.17); concentration, approximately 300 ppm each (A and B) and approximately 6 ppm each (C and D); injection plug length, 0.7 mm (A), 0.4 mm (B), 1.9 cm (C), and 5.1 cm (D). pK_a values are taken from Ref. [27]. Other conditions are the same as given in the Experimental Section.

polyoxyethylene surface. Since the nitrophenols are charged and are reasonably hydrophobic, analyte–micelle interaction may be thought of as a combination of adsorption on the polar surface as well as inclusion in the hydrophobic core. For the separation of the *o*- and *p*-isomers, the difference in hydrophobicities may be much more important since they bear approximately equal charges. The values of the logarithm of the *n*-octanol–water partition coefficient, log $P_{o/w}$, of the *ortho* and *para* forms are 1.77 and 1.91 [25], respectively. The more hydrophobic *p*-isomer will interact more strongly with the nonionic micelle and migrate faster than the *o*-isomer. That these three positional isomers possess differing affinities to the micelle is emphasized even more with the results obtained from sweeping experiments (Fig. 2C and 2D). At an injection plug length of about 1.9 cm (Fig. 2C), the *o*-nitrophenol peak was already broad, the *p*-nitrophenol peak began to broaden, while the *m*-nitrophenol peak remained reasonably sharp. Focusing of meta form was good until an injection plug length of about 5.1 cm (Fig. 2D), giving rise to an enhancement factor, in terms of peak height, of approximately 15-fold.

In some instances, the presence of a neutral pseudostationary phase in the BGS does not only result in improved resolution but a change in elution order as well. Table 1 shows the migration times of three other phenol derivatives in CZE and NPS-EKC modes. In the CZE mode, 3-methylphenol eluted ahead of 4-chlorophenol, whereas the converse is true for the NPS-EKC mode. The *k* values for the three analytes, calculated using Eq. (1), are also shown. It is not surprising that 4-ethylphenol has the highest *k* value since it is the most hydrophobic of the three analytes, based on the values of log $P_{o/w}$.

3.2. On-line preconcentration using nonionic surfactant

Fig. 3A shows a typical 0.2 mm injection of a mixture of three phenols. To assess the efficiency of sample stacking as an on-line preconcentration technique, a thirty-fold dilution of the mixed samples in water was injected as a 5.5 cm plug (Fig. 3B). Sample stacking results from the movement of sample ions from a high electric field sample zone to a low electric field BGS zone. The change in electrophoretic velocity causes sample enrichment. Overly long injection plug lengths do not produce good results. This is because in sample stacking with hydrodynamic injection, the sample plug length is limited by the broadening effect caused by the mismatch of local EOF velocities [13,24]. The local EOF velocity of the low conductivity sample plug is greater than the local EOF velocity of the high conductivity BGS. The longer the sample-plug length, the greater the broadening effect caused by this mismatch of velocities. As may be inferred from the electropherograms, from around 35- to 45-fold improvement in detector response, in terms of peak

Table 1
Migration times of phenols in CZE and NPS-EKC with Brij 35, pK_a, log $P_{o/w,}$ and *k* for NPS-EKC

Analyte	Migration time[a]/min		pK_a [d]	Log $P_{o/w}$ [e]	*k* [f]
	CZE[b]	NPS-EKC[c]			
Dimethyl sulfoxide (EOF marker)	4.86 (0.18)	7.49 (1.53)			
4-Ethylphenol	8.57 (1.39)	8.03 (1.32)	10.00	2.58	8.82
3-Methylphenol	9.70 (1.56)	9.68 (1.86)	10.01	1.98	2.39
4-Chlorophenol	11.51 (0.88)	8.95 (1.35)	9.18	2.40	4.43

[a] The values specified for migration time are the means of five replicates and based on a 0.2 mm injection of approximately 300 ppm of each sample. The values in parentheses are the % RSDs.
[b] BGS, 20 m*M* borate, pH 11.25. Other conditions are the same as given in the Experimental Section.
[c] BGS, 100 m*M* Brij 35 in 20 m*M* borate, pH 11.25. Other conditions are the same as given in the Experimental Section.
[d] pK_a value of 4-ethylphenol is from Ref. [26]. Others from Ref. [27].
[e] Data from Ref. [28].
[f] Calculated using Eq. (1).

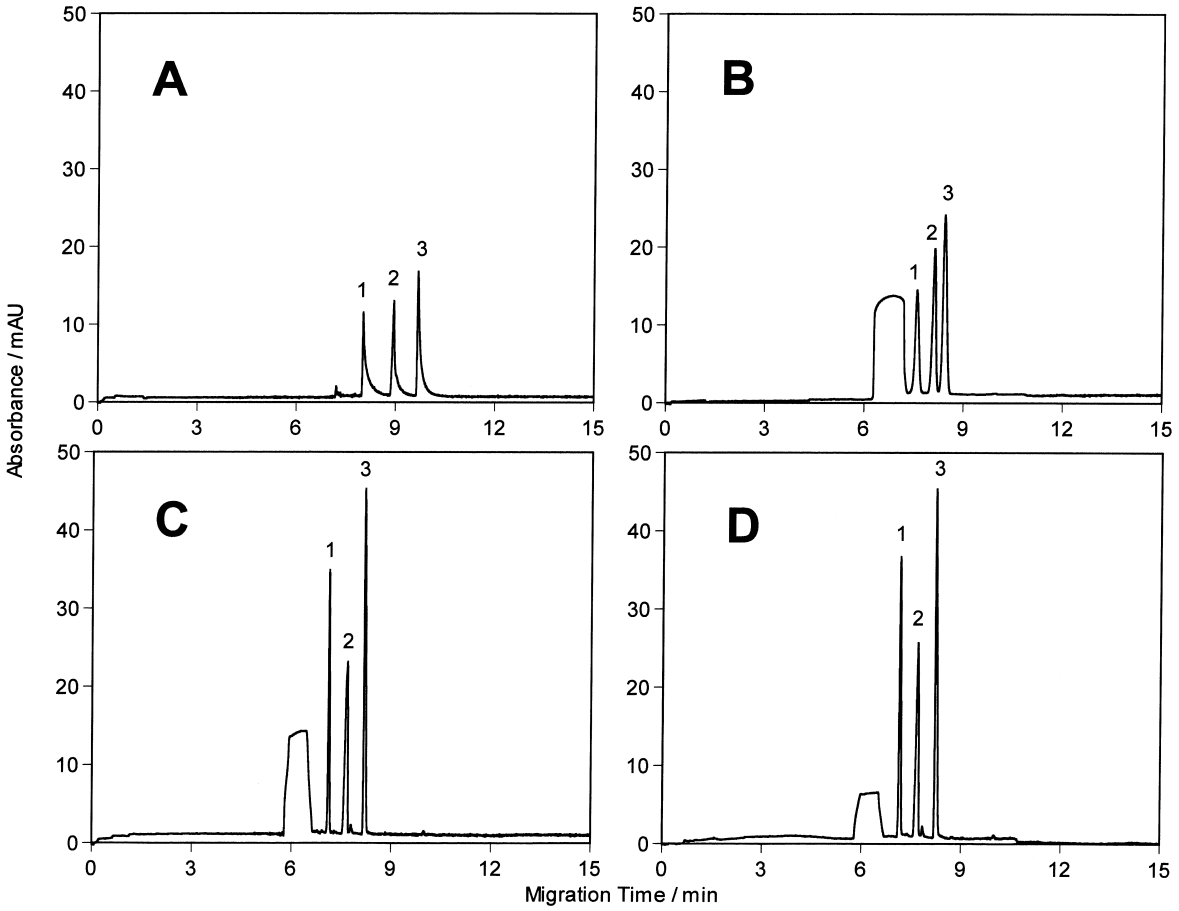

Fig. 3. NPS-EKC separation of three phenol derivatives. Conditions: BGS, 100 m*M* Brij 35 in 20 m*M* borate, pH 11.25; sample matrix, nonmicellar buffer adjusted to the conductivity of the BGS (A and C), water (B), and nonmicellar buffer adjusted to the conductivity and viscosity of the BGS (D); injection plug length, 0.2 mm (A) and 5.5 cm (B, C and D); sample concentration, approximately 300 ppm each (A) and approximately 10 ppm each (B, C and D); samples, 4-ethylphenol (peak 1), 4-chlorophenol (peak 2), and 3-methylphenol (peak 3). Other conditions are the same as given in the Experimental Section.

height, was attained compared with a typical injection.

The improvements obtained here are higher than expected. At the concentration level Brij 35 is employed in for these experiments, the resulting solution is viscous, and it was thought that this high viscosity of the BGS may have been responsible for the improved results. In sample stacking, efficiency may be partly degraded because of the mixing of zones, which arises as a result of the disturbance generated by the mismatch of EOF velocities in the sample and BGS zones [13]. With a viscous BGS, however, mixing of zones appears to be less severe, as experimental results show (Fig. 4, overlaid traces shown on expanded time scale). A 15 s plug of BGS or water was injected before (Figs. 4B and 4D) or after (Figs. 4A and 4C) a 1 s injection of a marker. It is assumed here that peak broadening is a result of zone mixing. Evidently, peaks broadened more considerably with injection of a plug of water prior to or after the marker when the BGS contained 50 m*M* borate only as compared to that in which viscosity was enhanced by the addition of polyethylene glycol 20 000 into the 50 m*M* borate buffer. In addition, current was observed to be more stable with the latter compared to the former (overlaid current traces

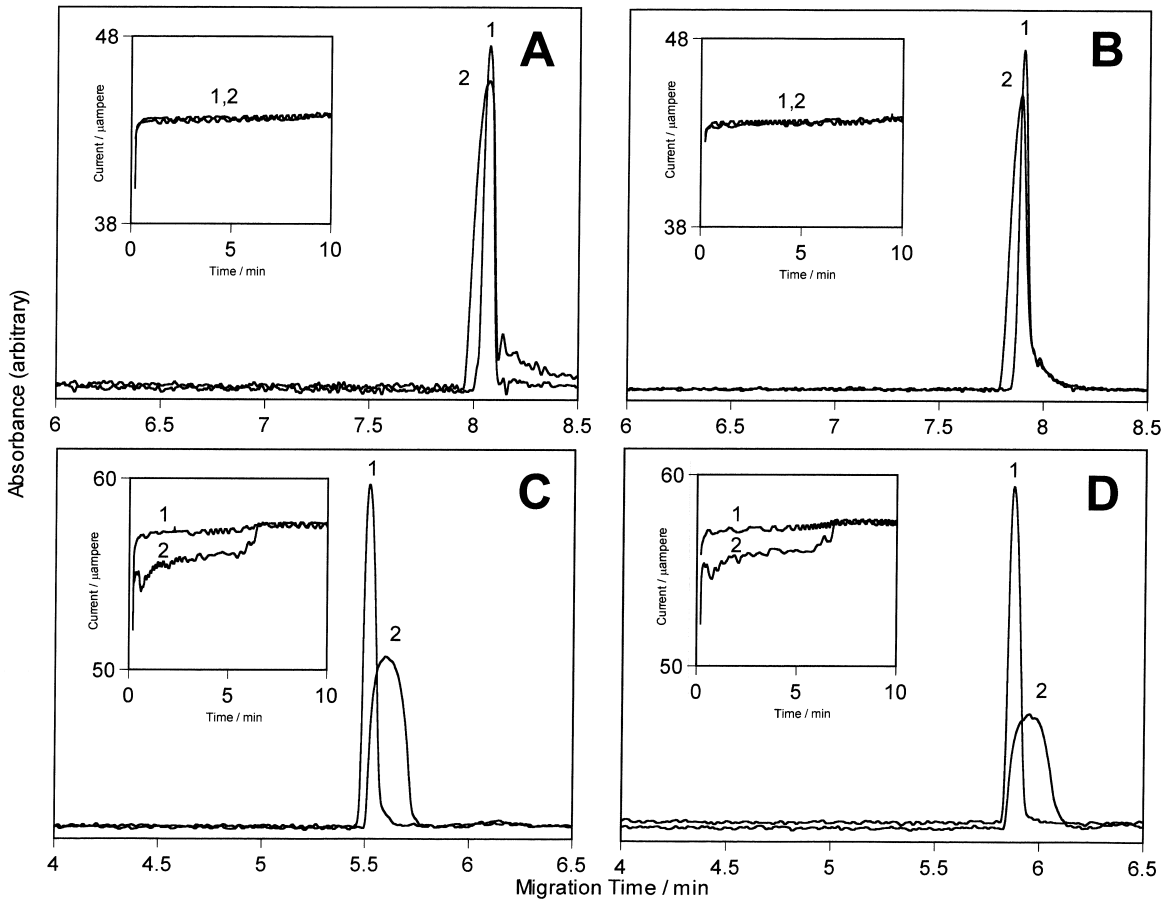

Fig. 4. Experimental verification of reduced zone mixing with a high viscosity BGS. Conditions: BGS, 7.5% polyethylene glycol 20 000 in 50 m*M* borate, pH 9.00 (A and B) and 50 m*M* borate, pH 9.00 (C and D). A 1 s injection of a marker, dimethyl sulfoxide was preceded (B and D) or followed (A and C) by a 15 s injection of BGS (peak 1) or water (peak 2). Shown inset are the current traces over 10 min runs. Other conditions are given in the Experimental Section.

shown inset). Consequently, the sharp difference in EOF velocities between the sample and BGS zones, on which sample stacking is predicated, remains pronounced.

Fig. 3C shows the result of injecting a 5.5 cm plug of a thirty-fold dilution of mixed samples prepared in nonmicellar BGS. The peaks are sharper and higher than those obtained from sample stacking (Fig. 3B). The mechanism of preconcentration is sweeping as described earlier. As much as 100-fold improvement in detector response, by way of sensitivity enhancement factor in terms of peak height, SEF_{height}, was obtained. Linearity of peak height response as well as reproducibility of other peak descriptors were found to be acceptable (Table 2).

To determine the effect of viscosity on the efficiency of sweeping, the viscosity, as well as the conductivity of the sample matrix, was adjusted to approximate that of the BGS (Fig. 3D). Polyethylene glycol 20 000 was used as the viscosity-enhancing agent. Results show marginal improvements over those obtained from typical sweeping experiments. This suggests that while a high viscosity medium may result in much improved enhancement factors when there is a marked difference in the conductivities of the sample and the BGS, its effect is not as

Table 2
Calibration line, limit of detection (LOD), % relative standard deviations (%RSDs), and sensitivity enhancement factor in terms of peak height (SEF_{height})

	Analyte		
	4-Chlorophenol	4-Ethylphenol	3-Methyphenol
Calibration line			
Equation of the line[a]	$y = 1.1124x + 0.4389$	$y = 1.5338x + 0.3783$	$y = 1.6403x + 0.8575$
Correlation coefficient, R^2	0.9999	0.9998	0.9990
LOD ($S/N=3$)			
ppb	28	20	19
$\times 10^{-8}$ M	21.8	16.4	17.6
RSD (%, $n=5$)			
Migration time	1.0	0.9	0.8
Corrected peak area[b]	3.8	5.6	3.4
Peak height	2.6	4.2	4.4
SEF_{height}[c]	54	100	87

[a] Peak height (mAU) = slope × concentration (ppm) + y − intercept. Determined over a 100-fold concentration range (0.3 ppm–30 ppm).
[b] Corrected peak area = peak area/migration time.
[c] $SEF_{height} = \frac{\text{peak height with sweeping injection (5.5 cm)}}{\text{peak height with typical injection (0.2 mm)}} \cdot \text{dilution ratio}$.

pronounced when the conductivities of these two zones are already approximately equal.

3.3. Validation of l_{sweep} equation

The calculated k values were used to determine the predicted length of zones after sweeping. These were then compared with experimentally obtained results to evaluate the validity of the l_{sweep} equation, as shown in Table 3. Actual results for 4-ethylphenol and 4-chlorophenol are in good agreement with the predicted values. However, for reasons yet unknown, the actual length of swept zone for 3-methylphenol is considerably shorter than expected. This aberration is mirrored in the SEF_{height} obtained. Although the calculated k value for 4-chlorophenol is higher than that obtained for 3-methylphenol, the SEF_{height} for the latter is higher. It should be noted, however, that the difference in k values between the two analytes is not that high. k, therefore, may still be deemed to qualitatively predict the extent of preconcentration.

Table 3
Predicted and experimentally determined l_{sweep} values

Analyte	l_{sweep}/cm[a]	
	Predicted[b]	Experimental[c]
4-Ethylphenol	0.56	0.61
4-Chlorophenol	1.01	1.07
3-Methylphenol	1.61	0.99

[a] Based on a plug length of 5.5 cm.
[b] Calculated using Eq. (8).
[c] Conditions: BGS, 100 mM Brij 35 in 20 mM borate, pH 11.25; sample concentration, approximately 7.5 ppm each. Other conditions are the same as given in the Experimental Section.

4. Conclusion

The principle of sweeping has been extended to EKC separation involving neutral pseudostationary phases. The technique is limited to charged species that migrate electrophoretically against the EOF. Separation is dependent on both electrophoretic and chromatographic effects. Up to 100-fold improvement in detection sensitivity is achieved, which translates to two orders of magnitude increase in concentration sensitivity. Applicability of the sweeping technique in NPS-EKC to cations and other samples will be studied. Different additives that act

as a neutral pseudostationary phase in EKC are being investigated as well.

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