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Micellar electrokinetic chromatography of aromatic anions and non-ionic aromatic compounds with stepwise changes of the concentration of cetyltrimethylammonium chloride

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ABSTRACT

Micellar electrokinetic chromatography in which the concentration of cetyltrimetylammmonium chloride (CTAC) was sequentially changed in the separation system was investigated using 10 aromatic anions and 11 non-ionic aromatic compounds as model analytes. All separations were performed in the absence of electroosmotic flow (EOF), and thus, analytes were detected in the order of their strength of interaction with micelles in the system. In isocratic elutions without EOF, the model analytes could be separated better with lower concentrations of CTAC but migration times of the analytes possessing relatively higher polarities increased markedly, and thus, long analysis times were required. Therefore, we attempted to increase the concentration of CTAC during a single measurement to reduce the analysis time without hindering the resultant separation of analytes obtained with lower concentrations. Briefly, the present surfactant stepwise elution can be performed by a sequential increase in CTAC concentrations of the running solution in the anodic reservoir from 30 to 50 mM for the anions and from 20 to 50 mM for the non-ionic compounds. Additionally, to perform expected gradient separations with good reproducibility, each running solution with a different CTAC concentration was treated with tetraethylammmonium chloride as an additive to adjust electric conductivities of each running solution to be equal. Under this condition, CTAC micelles of each zone of different CTAC concentrations would migrate with practically the same velocity. Consequently, by the present stepwise method, both the 10 anionic analytes and the 11 non-ionic analytes were well separated within reasonable periods which corresponded approximately to two-third and less than half of those by the isocratic elutions, respectively.

1. Introduction

In chromatography, several gradient methods in which retention factors (k) of each analyte are varied during a single measurement have been introduced to achieve better separations in shorter analysis times. Thermal gradient methods are employed in gas chromatography (GC) on a routine basis; in these methods, changes in the distribution coefficient (K) of solutes between mobile and stationary phases, and as a result, changes in k, occurred with the alteration of the surrounding temperature. Gradient elution methods of reversed phase liquid chromatography (RPLC), changing the composition of solvents in the mobile phases, are based on changes in K by fundamentally modifying polarities of mobile phases and are often employed to analyze samples comprising compounds having a wide range of hydrophobicities. In micellar electrokinetic chromatography (MEKC) that was devised by Terabe et al. [1] and is performed with capillary electrophoretic systems, some gradient methods, including those of electric field [2–4], temperature [4], and solvent composition [5–11], were reported in early studies. Solvent gradient methods in MEKC are based on mechanisms similar to those in RPLC. On the other hand, to our knowledge, there are no gradient MEKC methods in which concentrations and/or compositions of surfactants are changed during the single run, except those of our groups [12,13].

In electrokinetic chromatography (EKC), in contrast to liquid chromatography (LC), both distribution phases, the bulk phase and pseudo-stationary phase, can move freely in separation systems. In principle, it is possible to modify both features of the bulk phase (e.g. compositions of solvents and pH) and the pseudo-stationary phase (e.g. compositions of carriers and their concentrations) during a single run, and the latter modification looks especially interesting. In our previous work, benzoate anions were successfully separated using mixed micellar systems comprising "cationic"

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cetyltrimetylammmonium chloride (CTAC) and non-ionic surfactants with polyoxyethylene chains (NS-POEs), such as Tween 20 and Brij 35, by changing concentrations of NS-POEs in mixed systems during a single run in a manner such that, during the early period of the measurement, NS-POE-rich micelles were used to complete the separation, and then the concentration of NS-POEs decreased gradually to remarkably reduce the separation time [12]. However, changing of the NS-POEs' concentration in the mixed system had slight effects on the separation of non-ionic analytes, which are inherent targets of MEKC, because of the slight change in the affinity of these non-ionic analytes to the mixed micelles with an increase in the concentration of NS-POEs compared with that of the anionic benzoates [12]. Surfactant gradient methods changing the concentration of ionic surfactants in their pure systems promise to achieve better and faster separations of non-ionic compounds. Such changes in the concentration of micelles imply a direct change in the phase ratio of chromatography, leading to a change in the magnitude of k. In LC and GC, we are unaware of any gradient method in which phase ratios are changed to optimize separations and such gradient methods are possible in EKC only with these developments. In this report, we will demonstrate a gradient method of MEKC employing unmixed CTAC systems in which the surfactant concentration was changed. The adjustment of electric conductivities of each running solution by using an adequate electrolyte was required to realize stable and reproducible operations of the present method in which ionic CTAC zones of remarkably different concentrations were arranged in series in the capillary, a phenomenon that we discuss in detail in this report.

2. Materials and methods

CTAC was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tetraethylammonium chloride (Et_4NCl) was purchased from Nakarai Tesque (Kyoto, Japan). N,N-dimetylacrylamide (a), 3-(trimethoxysilyl)propyl methacrylate (b), tetramethylethylenediamine (c), and ammonium peroxodisulfate (d) for the inner wall coating of capillaries were obtained from Aldrich Chemical (WI, USA, for a and b) and Nacalai Tesque (for c and d). As model analytes, benzoic acid, salicylic acid, 4-toluic acid, 4-dimethylaminobenzoic acid, 4-acetoxybenzoic acid, 2-phthalaldehydic acid, 4-phtalaldehydic acid, 4-hydroxybenzoic acid, 4-acetamidebenzoic acid, benzenesulfonic acid, 4-chloro-2-nitrophenol, 4-ethylphenol, 4-nitrophenol, 4-cresol, 4-toluidine, acetotoluidine, anisole, aniline, acetanilide, 1,4-hydroquinone, and 4-hydroacetanilide were purchased from Wako Chemical (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), or Tokyo Chemical Industry (Tokyo, Japan). Other chemicals were of analytical grade.

MEKC and capillary zone electrophoresis (CZE) were performed using a system designed in our laboratory; this system comprised a Matsusada HCZE-30PNO high-voltage supply (Siga, Japan) and a Jasco MD-2010 multi-wavelength detector (Tokyo, Japan) equipped with an EPSON Pro-720L personal computer (Nagano, Japan). Fused silica capillaries (inside diameter = 0.05 mm and outside diameter = 0.375 mm) were purchased from GL Science (Tokyo, Japan). The total column length was 600 mm of which the effective length was 300 mm. Inner walls of capillaries were coated with poly(N,N-dimethylacrylamide) (PDMA) according to the method by Wan et al. [14].

PDMA-coated capillaries were rinsed with distilled water for 10 min by using an MAS-1 aspirator, purchased from AS ONE (Osaka, Japan), before beginning daily experiments, and before each run, were further rinsed with water and running solutions to be used initially for 5 and 2 min, respectively. Running solutions were 10 mM phosphate buffer solutions (pH 7.2) and 50 mM acetate buffer solutions (pH 4.8) containing CTAC and Et_4NCl for aromatic anions and non-ionic aromatic compounds, respectively. The applied voltage was fixed at −12 kV or −10 kV for aromatic anions and non-ionic aromatic compounds, respectively. Electroosmotic flow (EOF) was almost completely suppressed by the aforementioned inner wall coating with PDMA. Each concentration of analytes in sample solutions was prepared to be approximately 0.1 mM. Sample solutions were injected on the anodic side by siphoning at a height of 10 cm for 30 s. Aliquots of the anthracene-saturated methanol solution were added to sample solutions to measure the migration velocities of CTAC micelles. To estimate electrophoretic mobilities of the four alkylammonium ions, we performed CZE measurements of the four cations (injected in their chloride or bromide solutions) and detected them at 195 nm.

The stepwise change in the concentration of CTAC was achieved by the immediate replacement of the anodic reservoir with another of a higher concentration of CTAC without switching off the HV supply. The electrode in the anodic reservoirs was grounded to facilitate the changing of reservoirs during the application of voltage. The alignment of fluid levels of both reservoirs of running solutions before each measurement was meticulously done to minimize the possibility of the flow caused by a gravitational pressure difference to achieve the reproducibility of an acceptable level.

To form a uniform electric field, electric conductivities of each running solution were adjusted as follows. Judging from operating currents, the 10 mM phosphate buffer solution (pH 7.2), containing 100 mM CTAC (Solution A), indicated almost the same electric conductivity as that containing 40 mM Et_4NCl (Solution B). Next, we combined Solution A and Solution B to prepare desired concentration of CTAC; for example, 50 mM CTAC (and 20 mM Et_4NCl) solution was made of Solution A and Solution B in a 1:1 ratio. Adjustment of conductivities of the acetic buffer solutions (pH 4.8) for separation of the 11 aromatic compounds was also done in the same way. This method enabled us to make solutions of any concentrations of CTAC, indicating the same electric conductivity. Conductivities of each running solution were confirmed with operating currents. When we observed almost a fixed current during a stepwise elution, a uniform electric field remained, and CTAC micelles migrated with the same velocity in all areas of the capillary. In fact, currents with the present adjustment method were stable in stepwise elutions.

3. Results and discussion

3.1. Isocratic CTAC-MEKC separations of aromatic anions and non-ionic aromatic compounds

First, isocratic MEKC separations of 10 aromatic anions (benzoate, salicylate, 4-toluate, 4-dimethylaminobenzoate, 4-acetoxybenzoate, 2-phthalaldehydate, 4-phtalaldehydate, 4-hydroxybenzoate, 4-acetamidebenzoate, and benzenesulfonate) and those of 11 aromatic compounds (4-chloro-2-nitrophenol, 4-ethylphenol, 4-nitrophenol, 4-cresol, 4-toluidine, acetotoluidine, anisole, aniline, acetanilide, 1,4-hydroquinone, and 4-hydroacetanilide) were performed with different concentrations of CTAC in the absence of EOF. Analytes were detected on the cathodic side of the capillary in the order of their partition coefficients (K) in the CTAC micellar system. Aromatic anions were, in general, expected to possess extremely large values of K because of both the hydrophobic interaction and the electrostatic interaction with CTAC occurring in a synergistic manner, as mentioned in our previous paper [12]. With 50 mM CTAC, as shown in Fig. 1a, most aromatic anions were predominantly distributed to CTAC micelles, and thus, eluted within 2 min with poor separation (peaks 2–8). On the other hand, a decreasing concentration of CTAC could reduce the distribution of analytes to moderate levels,

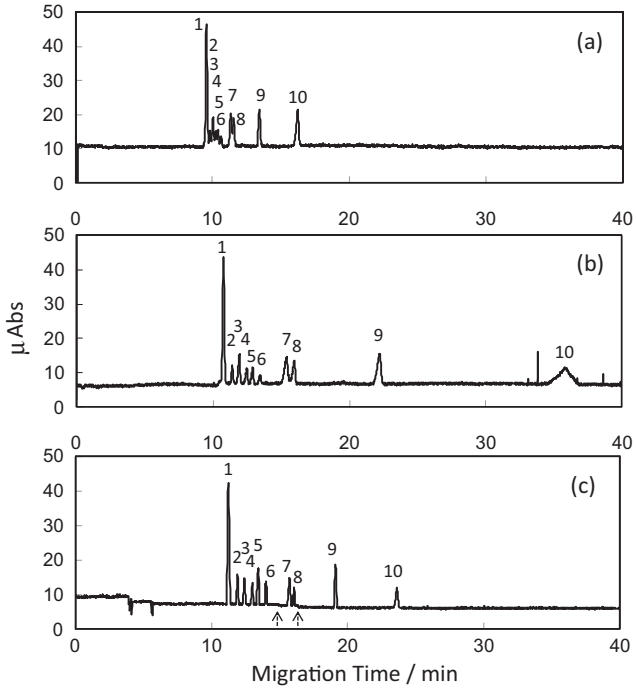

Fig. 1. MEKC separation of the 10 aromatic anions in the absence of EOF with isocratic methods (a and b) and with a stepwise gradient program of the concentration of CTAC in the inlet reservoir (c). *Conditions*: A poly(N,N-dimethylacrylamide)-coated capillary, 0.05 mm (inside diameter) × 600 mm (effective length = 300 mm); applied potential (current), −12 kV [17 μA]; running solution, 10 mM phosphate buffer (pH 7.2) containing (a) 50 mM CTAC and 20 mM Et_4NCl, (b) 30 mM CTAC and 28 mM Et_4NCl and (c) 30–50 mM CTAC and 20–28 mM Et_4NCl, respectively. The conductivity of each running solution was adjusted to be the same value by adding Et_4NCl; see text (Section 3.3) for details. In (c), the concentration of CTAC in the inlet reservoir was changed from 30 mM (initial) to 40 mM (at 4 min) and 50 mM (at 5.5 min) while those in the outlet reservoirs were fixed at 30 mM. The arrows in (c) indicate CTAC concentration gaps at the detection point; injection, from anodic side for 30 s at a height of 10 cm; detection, 205 nm. *Peak assignment*: 1, salicylate; 2, 4-toluate; 3, 4-dimethylaminobenzoate; 4, benzene sulfonate; 5, 4-acetoxybenzoate; 6, benzoate; 7, 4-phtalaldehydate; 8, 2-phthalaldehydate; 9, 4-hydroxybenzoate; and 10, 4-acetamidebenzoate.

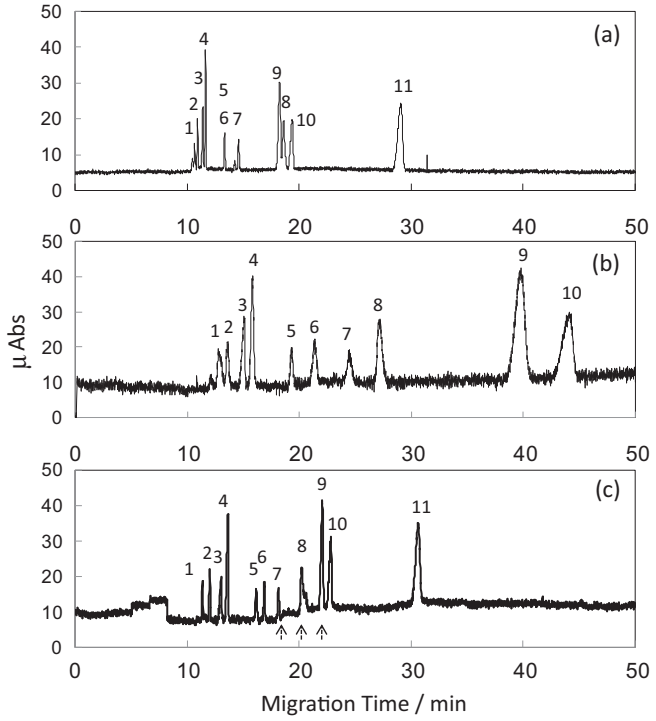

Fig. 2. MEKC separation of the 11 substituted benzenes in the absence of EOF with isocratic methods (a and b) and with a stepwise gradient program of the concentration of CTAC in the inlet reservoir (c) by using running solutions including (a) 50 mM CTAC and (b) 20 mM CTAC. *Conditions*: A poly(N,N-dimethylacrylamide)-coated capillary, 0.05 mm (inside diameter) × 600 mm (effective length = 300 mm); applied potential (current), −10 kV (14 μA); running solution, 50 mM acetate buffer (pH 4.8) containing (a) 50 mM CTAC and 20 mM Et_4NCl, (b) 20 mM CTAC and 32 mM Et_4NCl, and (c) 20–50 mM CTAC and 32–20 mM Et_4NCl. The conductivity of each running solution was adjusted to the same value as well as in Fig. 1. In (c), the concentration of CTAC in the inlet reservoir was changed from 20 mM (initial) to 30 mM (at 5 min), 40 mM (at 6.5 min), and 50 mM (at 8 min) while those in the outlet reservoirs were fixed at 20 mM. The arrows in (c) indicate CTAC concentration gaps at the detection point; injection, from anodic side for 30 s at a height of 10 cm; detection, 195 nm. *Peak assignment*: 1, 4-chloro-2-nitrophenol; 2, 4-ethylphenol; 3, 4-nitrophenol; 4, 4-cresol, 5, 4-toluidine; 6, acetotoluidine; 7, anisole; 8, aniline; 9, acetanilide; 10, 1,4-hydroquinone; and 11, 4-hydroacetanilide.

and a complete separation of aromatic anions was obtained with a running solution of 30 mM CTAC, as shown in Fig. 1b. However, this complete separation needed a considerably longer analysis time. To achieve a better separation of anions within a reasonable analysis time, an increase in the concentration of CTAC during the separation would be necessary. In the gradient method, relatively hydrophobic aromatic anions detected at an early period are sufficiently separated by CTAC micelles of lower concentrations, and relatively hydrophilic aromatic anions detected later are separated in less time by CTAC micelles of higher concentrations.

As reported in our previous paper [12], the addition of a non-ionic surfactant with polyoxyethylene chains (NS-POEs), Tween 20 or Brij 35, to CTAC systems could decrease the distribution to micelles of aromatic anions similarly. While this effect was useful to optimize the separation of anions, the addition of the NS-POEs could slightly change the distribution of "non-ionic" anisole because effects of the NS-POEs to decrease the transfer of anions are based entirely on the suppression of electrostatic interactions with CTAC [12]. Marked effects of the addition of the NS-POEs in improving the separation of the present 11 non-ionic aromatic compounds could not be observed as anticipated (data not shown). On the other hand, the change in the concentration of CTAC meant a change in the phase ratio of the chromatography and, in principle, was expected to drastically change retention factors of all solutes. Fig. 2a and b illustrates separations of the 11 non-ionic aromatic compounds by using 50 mM acetate buffer (pH 4.8) solutions containing CTAC of 50 mM and 20 mM, respectively. The separation using 50 mM CTAC in Fig. 2a was insufficient, and complete separation occurred with 20 mM CTAC, as shown in Fig. 2b. However, a considerably longer duration of time was required for the complete separation and the last peak, peak 11, did not appear within 1 h. The change in the concentration of CTAC during a single run is a possible way to achieve a complete separation within a reasonable analysis time for non-ionic aromatic compounds as well as for aromatic anions.

3.2. Theoretical arrangement of the gradient method in concentrations of CTAC

First, a stepwise change in the CTAC concentration of the inlet running solution from 30 mM to 50 mM in a separation of the aromatic anions was tried without any additional treatments to the two running solutions except for CTAC concentrations; however, expected results were not observed. Insufficient separations were observed for peaks that emerged relatively faster, to make matters worse, with very poor reproducibility.

These results are attributed to the consequent difference in magnitudes of the electric field (E) in the zone of the 30 mM CTAC and in the zone of the 50 mM CTAC, and the situation precipitated a gradual breakdown of the zones in the capillary. This difference in E was caused by the difference in concentrations of CTAC as electrolytes.

The electric resistance of a zone of the capillary filled with a running solution (R) is expressed as follows [15]:

$$R = \frac{l}{\kappa A} \quad (1)$$

where l, A, and κ are the length of the zone of the capillary, its inner cross section, and the specific conductance of the solution, respectively. When the law of independent ionic migration is applicable in the present running solutions, κ is described as

$$\kappa = \sum \Lambda_i C_i$$

where Λ_i and C_i are the molar electric conductivity of an ionic species denoted by i and its concentration in the running solution, respectively. Here, we assume that values of Λ_i are constant under present conditions and that the κ of running solutions can be described as

$$\kappa = \Lambda_{\text{micelle}}(C - C_0) + \Lambda_{\text{free}}C_0 + \Lambda_{\text{Cl}}C + \kappa_{\text{buffer}}$$
$$= (\Lambda_{\text{micelle}} + \Lambda_{\text{Cl}})C + \text{const.}, \quad (C \geq C_0) \quad (2)$$

where C, C_0, Λ_{micelle}, Λ_{free}, Λ_{Cl}, and κ_{b} are the concentration of CTAC, the critical micellar concentration of CTAC, the electric conductivity of the cetyltrimethylammonium ion (CTA^+) forming the micelle, the electric conductivity of the free CTA^+, the electric conductivity of the chloride ion as the counter ion of CTA^+, and the specific conductance derived from buffer components in the solution, respectively.

With measurements under CTAC concentrations of 20, 30, 40, 50, and 60 mM, and the other conditions same as those of Fig. 1, operated currents (I [μA]) in isocratic MEKC in the absence of tetraethylammonium chloride had a linear relationship with the CTAC concentrations (C [mM]) expressed as $I = 1.6C + 3.2$ ($r^2 = 0.985$). The operated current, I, should be proportional to κ of the running solution, according to Eq. (1), and Ohm's Law, $V = IR$, where V is a potential applied to both sides of the zone. Thus, Eq. (2) consists with the experimental fact.

According to Eqs. (1) and (2), the magnitude of the electric field (E) in the zone filled with a running solution of a CTAC concentration (C) is expressed as

$$E = \frac{V}{l} = \frac{RI}{l} = \frac{I}{\{(\Lambda_{\text{micelle}} + \Lambda_{\text{Cl}})C + \text{const.}\}A}. \quad (3)$$

Thus, there should be a negative correlation between E and C.

A capillary in which several zones of different C exist in series will correspond to an electric circuit in which resistances of different strengths are connected in series. The current, I, flowing in each resistance is theoretically the same, and thus, different magnitudes of E exist simultaneously through the capillary according to Eq. (3). For example, if solutions of 30 mM and 50 mM CTAC are both inserted in series, E in the zone of the 30 mM CTAC should be greater than that of the 50 mM CTAC solution. Given such a situation, the migration of CTAC micelles in the lower concentration zone should be faster than that in the higher concentration zone, and thus, the boundary line between zones of different CTAC concentrations should break up. These phenomena should also lead to unexpected results and poor reproducibility, as aforementioned. For the realization of favorable elutions changing the concentration of CTAC, it is necessary to form stable zones of each CTAC concentration in the whole area of the capillary where CTAC micelles migrate with the same velocity during separation. Our strategy is the addition of another electrolyte to equalize E in all areas of the inner capillary.

Table 1
Electrophoretic mobilities of the four tetraalkylammonium ions and cetyltrimetylammonium chloride (CTAC) micelle.

Cations	Electrophoretic mobility ($cm^2\ min^{-1}\ V^{-1}$)
Tetramethylammonium	0.0225
Tetraethylammonium	0.0180
Tetrapropylammonium	0.0171
Tetrabutylammonium	0.0138
CTAC micelle	0.0175

Conditions: Same as those in Fig. 1, except for the running solution. A solution of 10 mM phosphate buffer (pH 7.2) without CTAC and tetraetylammonium chloride was used here.

3.3. Suitable electrolytes to adjust electrical conductivity of each zone in the capillary

In a preliminary examination, conductivities of each zone of a different CTAC concentration were adjusted to be almost the same by the addition of an adequate concentration of NaCl as an adjustor but the initial uniform electric field in the entire capillary gradually broke down. Reproducible stepwise separations could not be achieved with NaCl perhaps because Na^+ has a markedly larger electrophoretic mobility compared with that of a CTAC micelle.

To form a uniform electric field in a sustainable manner during a separation, adequate adjusting salts were needed, such as those comprising cations possessing the same electrophoretic mobility as that of a CTAC micelle and chloride ions, common counter anions of the cation and CTA^+. Given the sizes and the charges, the electrophoretic mobility of a CTAC micelle is lesser than those of most metal cations, and we focused on tetraalkylammmonium ions because they are generally larger than general metal cations and have a range of sizes available, depending on sizes of their alkyl groups. Tetraalkylammmonium ions having methyl, ethyl, propyl, and butyl groups as alkyl groups were examined, and the tetraethylammmonium ion (Et_4N^+) and the tetrapropylammonium (Pr_4N^+) ion showed electrophoretic mobilities similar to that of a CTAC micelle in a 10 mM phosphate buffer solution (pH 7.2), as indicated in Table 1, and became candidates. There was little attractive electrostatic interaction between tetraalkylammmonium ions and the CTAC micelle in the running solution because both of them have positive charges. However, hydrophobic interactions increased with an increase in sizes of alkyl groups of tetraalkylammmonium ions. Indeed, tetrabutylammonium ions (Bu_4N^+) added to the running solution provided apparent effects on the separation and peak shapes of solutes (data not shown), strongly suggesting that CTAC and Bu_4N^+ had an attractive interaction to form some type of a mixed micelle. In this work, we required additive ions to only adjust electric fields, and thus, from the two candidates, we chose Et_4N^+ because of its hydrophobicity was lower than that of Pr_4N^+.

3.4. Stepwise-elution MEKC of aromatic anions and the non-ionic aromatic compounds employing CTAC systems

Figs. 1c and 2c show MEKC separations of the 10 aromatic anions and the 11 non-ionic aromatic compounds, respectively, with optimized stepwise programs of changes in CTAC concentrations in running solutions of the anodic reservoir (see the captions of Figs. 1c and 2c for stepwise programs). In both electropherograms, complete separations and shortened analysis times were accomplished. The reproducibility of analysis for these gradient separations with optimized programs ($n = 5$) were studied briefly and the values of relative standard deviation (RSD) were obtained in the range of 3.04–3.57% (migration time) and

5.49–9.65% (peak area) for the 10 aromatic anions and, 1.83–3.03% (migration time) and 1.01–7.70% (peak area) for the 11 neutral aromatic compounds under the same conditions with those in Figs. 1c and 2c.

A similar effective separation was achieved for aromatic anions with the previous micellar composition gradient method employing the mixed systems of CTAC and NS-POEs [12]. However, the previous gradient method did not provide us with remarkable benefits in the separation of non-ionic aromatic compounds because of slight effects on the distribution of neutral analytes by a change in the concentrations of NS-POEs in the mixed systems, as aforementioned. The present method by using a change in the concentration of ionic surfactants enables us to perform a gradient separation of non-ionic compounds as regular analytes for MEKC.

4. Conclusions

The stepwise method in MEKC of changing concentrations of an ionic surfactant will be useful in separations of both organic ions and non-ionic organic compounds to achieve good separations within acceptable analysis times, which corresponded to approximately to two-third and less than half of those by isocratic method, respectively, in this work. In the method, adjusting the conductivity of each zone containing the ionic surfactant of a different concentration is necessary for creating a stable and uniform electric field over the capillary during single separations to realize expected and reproducible gradient elutions.

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