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Review

Electrokinetic chromatography

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Abstract

The important features of electrokinetic chromatography are critically reviewed. Special emphasis is given to systems using micelles as pseudostationary phase. Short and comprehensive overviews are given on the subjects of separation, comparison with capillary electrochromatography, on-line coupling with mass spectrometry, and developments that are expected in the future. A greater coverage on the subject of improvement of detection sensitivity, specifically by on-line concentration was also contributed.

Keywords: Electrokinetic chromatography; Reviews; Sample stacking; Pseudostationary phases; Detection, Electrophoresis

Contents

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1. Introduction

For more than a decade, scientists in different fields have been impressed at the ability of electrokinetic chromatography (EKC) — a mode of capillary electrophoresis (CE) — to solve many chemical analysis problems. Apparently, EKC has enjoyed an unprecedented growth in theory and applications since its appearance in 1984. Although it has matured as a quantitative separation technique and has been implemented in many laboratories, the potentials are still being realized. Striking aspects of EKC include ease of operation such that virtually any science graduate or student can perform an analysis and resolving power that is similar to capillary gas chromatography and better than high-performance liquid chromatography (HPLC).

EKC employs the experimental technique of capillary zone electrophoresis (CZE) or free solution capillary electrophoresis but utilizes the basic principles of chromatography for separation. The fundamental experimental set-up consists of a narrow bore capillary, a voltage delivery device equipped with proper paraphernalia, a detector equipped with data acquisition and manipulating gadgets, and separation solution reservoirs. State of the art instruments are now readily available from different vendors, which allow any imaginable experiment with great comfort. A narrow bore capillary permits applications of high voltages due to excellent dissipation of Joule heat and prevents zone broadening by lessening convective effects. In addition to the buffer used in CZE, a major component called pseudostationary phase is added. This then satisfies the definition of chromatography wherein two phases should exist between which the solute distributes itself. The electrokinetic phenomenon, including both electrophoresis and electroosmosis, is the means of transporting the pseudostationary phase and solutes inside the capillary [1].

The general procedure in performing EKC is as follows: (1) Conditioning the capillary with several capillary volumes of sodium hydroxide solution (0.1 to 1 *M*), followed by methanol, water, and finally the separation solution; (2) injection of the sample usually prepared in the separation solution; (3) application of a potential until peaks are detected; and (4) data acquisition and processing. Injections are done hydrodynamically (using pressure) or electrokinetically (using voltage). The capillary is usually replenished with a fresh separation solution to improve reproducibility. The separation solution is usually prepared by dilution of stock solutions of pseudostationary phases and buffers.

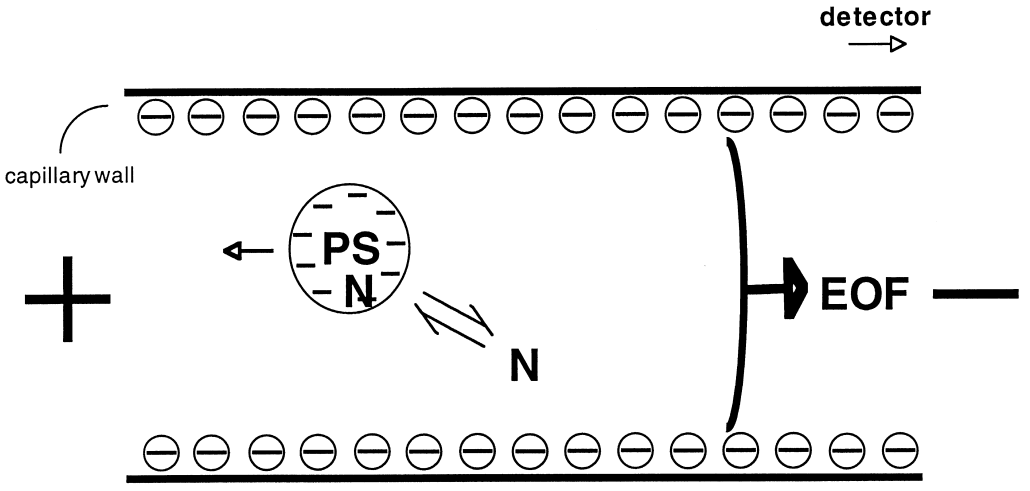

Fig. 1. The basic principle of electrokinetic chromatography. Components: anionic pseudostationary phase (PS), neutral analyte (N), electroosmotic flow (EOF); some explanations: the arrows near the PS and EOF show the magnitude of the electrophoretic velocities under an applied field, the EOF velocity is toward the cathode due to a negatively charged capillary wall, the PS velocity is toward the anode, the N partitions between the PS and the aqueous phase as depicted by the half arrows, and the migration velocity of N is toward the cathode due to a higher EOF velocity; separation of a mixture occurs due to differences in partitioning of analytes between the PS and surrounding liquid phase.

An elementary illustration on the principle of separation of EKC is given in Fig. 1. This figure considers a negatively charged pseudostationary phase (PS) contained in an aqueous buffer, a cathode-directed electroosmotic flow (EOF), and a neutral solute (N). The EOF velocity is greater than the PS velocity. Electrophoretic or electroosmotic velocities are products of the applied field (V/L = voltage applied/length of the capillary) and the electrophoretic or electroosmotic mobilities, respectively. The surface of the capillary carries a negative charge due to dissociation of silanol groups in a fused-silica capillary. The direction of electrophoretic velocities is dictated by the direction of the electric field, for example negatively charged molecules move toward the positive electrode. The direction of the electroosmotic velocity, for simplicity and as a rule of thumb, is directed toward the cathode or anode for negatively or positively charged capillary walls, respectively. Other scenarios will not be dealt at this moment.

The N partitions between the PS and the surrounding aqueous phase, and the degree of partitioning can be quantitated by the partition or distribution coefficient (K), given by Eq. (1):

$$K = \frac{c_{PS}}{c_S} \quad (1)$$

where c_{PS} and c_S are the molar concentration of a solute in the pseudostationary and surrounding phase, respectively. Separation of a mixture occurs due to differences in K values. In effect, N acquires an effective electrophoretic velocity that is slower than the electrophoretic velocity of the PS. The migration velocity of the PS or the solute, which is the summation of the (effective) electrophoretic and electroosmotic velocities, is then toward the cathode where the detector is closely situated.

2. Fundamentals

2.1. Modes of separation

2.1.1. Based on the nature of the pseudostationary phase

A variety of materials can be used as PS in EKC, for example, anionic, nonionic, zwitterionic, and cationic surfactants [2–10], microemulsions [11–13], macrocyclic and macromolecular phases [14,15], micelle polymers [16–20], polymer surfactants [21,22], vesicles [23], resorcarenes [24], dendrimers [25], and polymer ions [26,27]. Micellar electrokinetic chromatography (MEKC) is the technical term used when micelles or surfactants are used as PS. The name of the material added to the abbreviated term EKC, usually describes the other modes (for example, microemulsion EKC or MEEKC).

2.1.2. Based on the phase that will carry the solutes to the detector

Solutes can be brought to the detector either by the surrounding or pseudostationary phase, depending on the analytical conditions. The first and most common mode is normal migration EKC (NM-EKC), which is characterized by a faster moving EOF compared to the PS. The other is reversed migration EKC (RM-EKC), which is characterized by a faster moving PS compared to the EOF. In general, the solutes are brought to the detector by the faster moving phase. Intuitively, NM-EKC and RM-EKC are analogous to reversed-phase and normal-phase HPLC, respectively, in terms of migration order of polar and non-polar analytes [28]. Furthermore, a scientifically more appropriate nomenclature as suggested by Janini and co-workers are rheic and arheic EKC for NM-EKC and RM-EKC, respectively. Rheic and arheic EKC implies separations with and without EOF, respectively. However, RM-EKC can be performed with EOF and thus the above may not be suitable names for these techniques. It should be noted that all the analytes will not migrate in the same direction in RM-EKC, unless the EOF is completely zero. Weakly incorporated analyte by PS will migrate in the direction of EOF in RM-EKC.

NM-EKC or RM-EKC can be performed basically by the control of the EOF that drives the surrounding medium or buffer. For example using sodium dodecyl sulfate (SDS) as PS, the electrophoretic mobility of the micelle is greater than the EOF at pH below 5 [29]. RM-EKC can then be performed using buffers with pH lower than 5. A slower EOF occurs due to suppressed dissociation of silanol groups at the inner surface of the capillary that reduces the zeta potential. Highly acidic micelle polymers have shown similar characteristic [19]. A pH around 5

incurs almost similar SDS micelle electrophoretic and EOF mobilities resulting to impractical migration times and thus should be avoided. Similarly by coating the capillary with polyacrylamide or another suitable material, an almost zero EOF can be achieved and RM-EKC can be performed with any charged PS [30–32].

2.1.2.1. Neutral analytes

To visualize a separation in NM-EKC, consider a hypothetical mixture of four compounds, such as **W**, **X**, **Y**, and **Z**. **W**, **X**, and **Y** have no, weak, and moderate interaction with the PS, respectively, while **Z** is fully incorporated in the PS. The separation is depicted in Fig. 2A. The time at peak **W** corresponds to the migration time of the EOF (t_0), and **W** is usually termed as the marker of the EOF. The time at peak of **Z** corresponds to the migration time of the PS (t_{PS}), and **Z** is usually termed as the marker of the PS. The peak of **X** migrates faster than **Y** because it has more affinity toward the aqueous phase or has a lower K value. **X** and **Y** are usually the solutes being separated and their migration or retention time is given by t_r (Eq. (2)),

$$t_r = \frac{(1+k)}{1+(t_0/t_{PS})\,k} \cdot t_0 \qquad (2)$$

where k is the retention factor (number of molecules in the PS/number of molecules in the surrounding phase $= n_{PS}/n_S$). A retention time window between the marker of the EOF and PS is then evident, which means that a solute in NM-EKC is allowed to migrate only within this range.

Assuming a zero EOF, the separation in RM-MEKC of the hypothetical mixture above is depicted

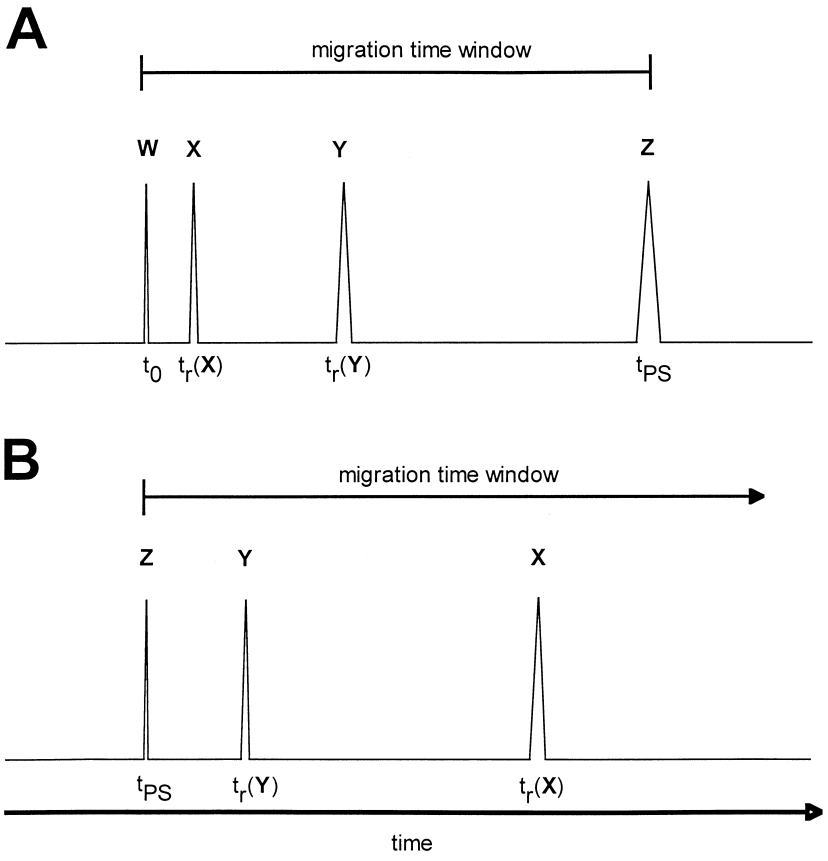

Fig. 2. Hypothetical separation of **W**, **X**, **Y**, and **Z** by NM-EKC (A) and RM-EKC (B). Explanations in Section 2.1.2.1.

in Fig. 2B, where the same symbols are applicable and the only difference is that the order of elution is reversed. Consequently, the marker of the EOF (**W**) will not be observed. Since t_0 is equal to infinity, Eq. (2) can then be rewritten as below.

$$t_r = \frac{k+1}{k} \cdot t_{PS} = \left(1 + \frac{1}{k}\right) \cdot t_{PS} \quad (3)$$

Under this condition, the PS corresponds to the mobile phase in conventional chromatography and the surrounding aqueous phase does the stationary phase. Also, under zero or highly suppressed EOF, a much wider retention time window (theoretically equal to infinity) compared to NM-EKC is provided (see Fig. 2). However, Eq. (2) holds true in the presence of EOF, even if the EOF velocity is lower than the electrophoretic velocity of the PS, and a proper usage of plus or minus signs is necessary to obtain the correct value.

A sample electropherogram showing a NM-MEKC (A) and RM-MEKC (B) analysis is depicted in Fig. 3 using some alkylphenyl ketones as test analytes. As discussed above, the migration time is reversed, that is hydrophobic analytes elute last in NM-MEKC while they elute first in RM-MEKC. Also, migration times were shortened in RM-MEKC without compromise to resolution, moreover, plate numbers obtained with RM-EKC for hydrophobic analytes are better than those obtained from NM-EKC [28]. The higher plate numbers could be explained in part by the faster migration time. It could be inferred here that NM-EKC and RM-EKC better analyze low to moderate and hydrophobic analytes, respectively.

2.1.2.2. Charged analytes

EKC was initially invented to extend the applicability of CE for neutral analytes, however, EKC also provides excellent separation of charged molecules that are sometimes impractical using CZE [33–35]. The separation behavior is dependent on micellar solubilization as well as electrophoretic migration. The same equations for neutral analytes can be used to describe migration of ionic analytes in EKC, except that the retention factor is calculated differently [34,35]. For example, the electrophoretic mobility of the charged analyte is considered and measured in a CZE system.

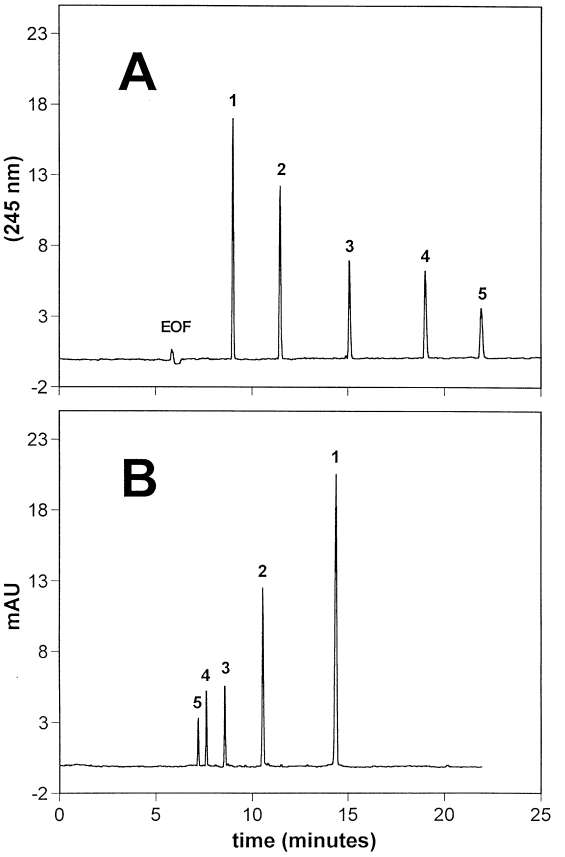

Fig. 3. Separation of five alkylphenyl ketones by NM-MEKC (A) and RM-MEKC (B). Conditions: separation solution, 25 m*M* SDS in 20 m*M* disodium hydrogenphosphate at pH 8.9 (A); 25 m*M* SDS in 20 m*M* phosphoric acid at pH 1.9 (B); sample solution, ~100 ppm alkyl phenyl ketones in methanol and the respective separation solution having a ratio of 1:5; injection, 50 mbar for 2 s; applied voltage, 20 kV (A), −20 kV (B); identity of peaks, acetophenone (1), propiophenone (2), butyrophenone (3), valerophenone (4), hexanophenone (5); capillary, 65 cm (effective length 56.5 cm) 50 μm I.D.×365 μm O.D.; capillary temperature, 20°C.

2.2. Resolution

The resolution (R_s) in NM-EKC is given by Eq. (4):

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k_2}{1 + k_2}\right) \cdot \left(1 - \frac{t_0}{t_{PS}}\right) \cdot \left(1 + \frac{t_0}{t_{PS}} k_1\right)^{-1} \quad (4)$$

where N is the theoretical plate number, α is the separation factor equal to k_2/k_1, and k_1 and k_2 are the retention factors of two closely eluting analytes 1 and 2, respectively. The resolution equation for RM-EKC when EOF is zero is then given by Eq. (5), which is similar to that in conventional or elution chromatography.

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha} \right) \cdot \left(\frac{k_2}{1 + k_2} \right) \quad (5)$$

Moreover, the last two terms in Eq. (4) show the effect of EOF on resolution, which was defined as the column availability (A_{col}) by Zhang et al. (Eq. (6)):

$$A_{col} = \left(1 - \frac{t_0}{t_{PS}} \right) \cdot \left(1 + \frac{t_0}{t_{PS}} \cdot k_1 \right)^{-1} = \frac{l'}{l} \quad (6)$$

where l' and l are the virtual and effective lengths of the capillary, respectively [36]. The value of A_{col} is equal to unity when the electroosmotic and electrophoretic velocities are balanced, just like in conventional chromatography. On the other hand, A_{col} is less than 1 ($l > l'$) when the electroosmotic velocity is greater than the electrophoretic velocity of the PS. Also, A_{col} is greater than 1 ($l' > l$) when the electroosmotic velocity is less than the electrophoretic velocity of the PS. In effect, the fraction of the capillary used for separation by an analyte that is related to l' is longer when EOF is zero or less than the electrophoretic velocity of the micelle. Along these lines, resolution in RM-EKC is therefore better than NM-EKC and resolution is even enhanced with the presence of a slow EOF.

2.3. Retention factor

The retention factor can be related to the K and phase ratio (volume of the pseudostationary phase/volume of the aqueous phase $= V_{PS}/V_{aq}$) using Eq. (7).

$$k = K \cdot \left(\frac{V_{PS}}{V_{aq}} \right) \quad (7)$$

In MEKC at low micellar concentrations, Eq. (7) can be rewritten as Eq. (8), where

$$k = K\nu(C_{PS} - \mathrm{CMC}) \quad (8)$$

ν is the partial specific volume of the PS and CMC is the critical micelle concentration. The linear relationship between k and C_{PS} observed experimentally could then be used to calculate K [2]. For monomolecular PS (e.g. micelle polymers), the plot of k and C_{PS} passes the origin because the CMC is equal to zero.

Using linear solvation energy relationships (LSERs), originated by Kamlet and Taft and coworkers to describe solvation effects on physicochemical processes, the retention factor in EKC (specifically MEKC) can be described in terms of several solute and PS descriptors. This can then be applied for the characterization of chemical selectivity of PS systems, which is perhaps the most robust method. Most LSER studies concluded that the ability of the micellar system to donate hydrogen bonds played the most critical role in determining selectivity while hydrophobicity that is related to solute size plays an important role in solute–micelle interaction in MEKC separations [37–40].

3. Electrokinetic chromatography versus capillary electrochromatography

Similar to EKC, capillary electrochromatography (CEC) combines the foremost features of HPLC and CE and has gathered enormous attention in the separation science community [41–50]. In CEC, a capillary is packed with a small-particle-size packing material (e.g. 3 μm), which is analogous to adding a PS to a buffer in EKC. From chromatographic theory, separation efficiency increases with the decrease in particle size, hence the trend toward developing separation technologies based on such particles can be brought to life in the CEC format. However, packing of capillaries in CEC requires skill from the analyst and could be troublesome and takes some time. Some problems are related to the preparation of frits at both ends of the packing that are used to prevent exiting from the capillary. The technical problems of frit preparation had been surmounted by the use of open tubular columns, monolithic packing technology, and sol–gel packing technology [45–49].

In general, EKC and CEC are competitive techniques. EKC and CZE or CEC and CZE are much more complementary analytical techniques. Although separation mechanisms for both EKC and CEC are unique and consequently different selectivities are observed, both techniques are primarily based on chromatographic principles. The wide variety of stationary phases used in HPLC, with well-characterized retention and selectivity mechanisms, are transferable to CEC. Mixed mode packing, use of two different stationary phases, has also been reported [50]. The wide variety of PSs amenable in EKC, as mentioned in a previous section, parallels this good trait of CEC. Logically, interactions between solute and various PSs are different, basically due to differing structures of PSs, thus providing unique selectivity. Other means of providing added selectivity in EKC include, using mixed PSs (usually of surfactants) [51–56], adjusting the capillary temperature [57,58], changing the buffer pH [33,59], and adding aqueous or PS phase modifiers (e.g. organic solvents and urea) [60–66]. Adding a shape selector to the mobile phase or using an enantioselective stationary phase performs enantiomeric separation in CEC [46,67,68]. In EKC, adding a shape selector to the separation solution or using an enantioselective PS can perform similar separations [69–71]. Shape selectors used in both CEC and EKC are usually charged or uncharged cyclodextrins or derivatives (singular or in mixtures).

Fig. 4 shows separations of 16 United States Environmental Protection Agency (EPA) priority polycyclic aromatic hydrocarbons (PAHs) with CEC using 1.5-μm ODS particles [43] (A) and MEKC using a polymerized surfactant [20] (B). Obviously, as seen in these figures, speed of analysis using CEC is better than MEKC. Efficiency of the two methods is comparable. In the CEC method, the nonporous nature of the particles used resulted in high flowrates responsible for the fast migration times. On one hand, according to Shamsi et al. [20], separation of these 16 EPA PAHs could be achieved within 15 min by MEKC, which is now comparable with the CEC system, if the percentage of acetonitrile is increased to 65% (v/v). On-line detection in the CEC system was done with UV-excited laser-induced fluorescence, due to the amount of sample that can be loaded to the system that necessitates high sensitivity detection. Furthermore, there is a loss of efficiency due to the frit and the change in mobile phase flow from the packed to the open fraction of the capillary where the detector window is situated. On-line detection in the packed fraction is not recommended due to disturbances caused by packing material. With the MEKC system, these problems are non-existent and a common UV detector can be used, although sensitivity of CEC and MEKC with UV detection is similar.

Fig. 5 is another comparison between CEC (A) and EKC (B), in the separation of acidic, neutral, and basic compounds. These figures reveal large differences in selectivity between the two techniques and as pointed out by Lurie et al. [72], the use of these two techniques, would be very helpful for complex analysis like drug screening. The advantages of the CEC method over the present MEKC method are the infinite elution range and injection solvent compatibility with the separation system. Methanol is used as injection solvent in order to dissolve the more hydrophobic solutes, which resulted in peak splitting in the MEKC system. However, if a RM-MEKC method will be developed for these solutes, the advantages of CEC will be present in MEKC too. For example, the infinite elution range inherent in RM-MEKC and the increased solubility of the hydrophobic basic solutes (amphethamine, methamphetamine, and cocaine) by using low pH separation solutions — thus utilizing acid–base chemistry — as injection solvent. In our opinion, if one wishes to separate a mixture of low to moderately hydrophobic analytes, a NM-EKC method would be easier to develop than a CEC method. In the separation of hydrophobic analytes, a CEC method or a RM-EKC method may be chosen.

4. Improving detection

EKC, just like the other modes of CE, is restrained by low concentration sensitivity as a consequence of the short optical pathlength for on-capillary photometric detection and the small volume of sample solution that can be injected into the capillary. Thus, one of the challenging areas in EKC methods development is to increase detection sensitivity or

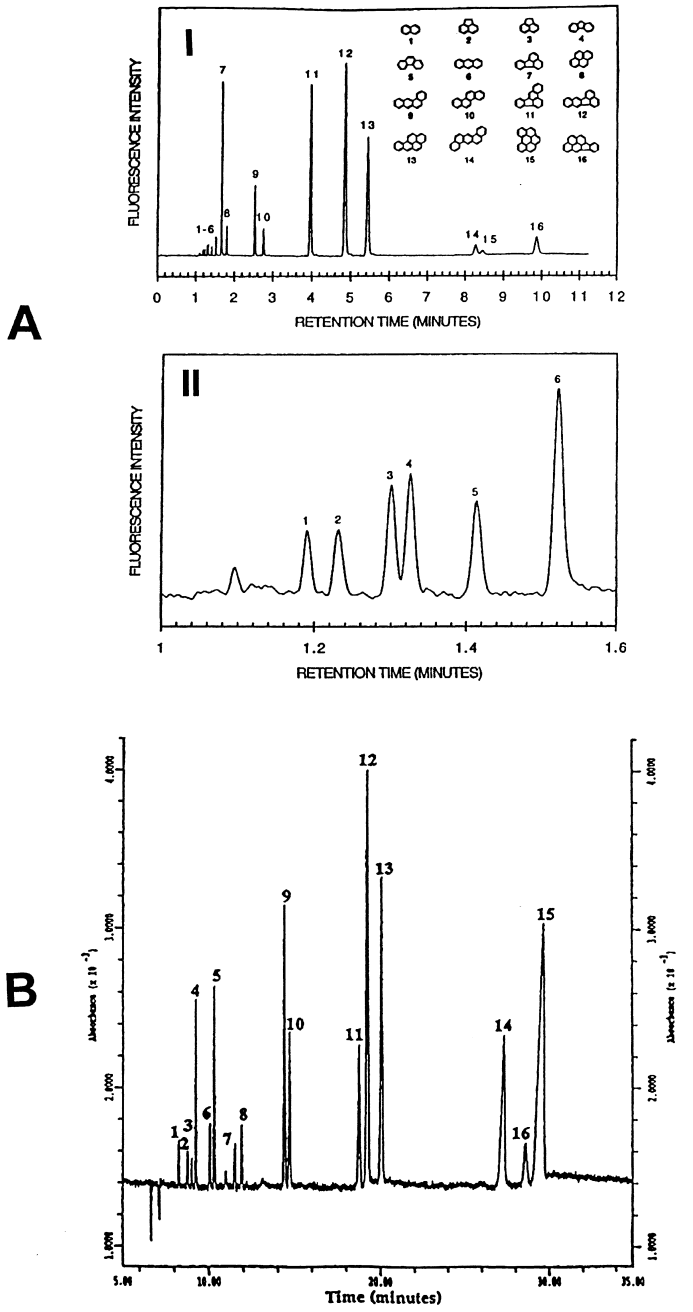

Fig. 4. Separation of 16 EPA PAHs by CEC (A) and MEKC (B). Conditions: (A) stationary phase, 1.5-μm nonporous ODS particles; column dimensions, 30 cm (packed length 20 cm)×100 μm I.D.; mobile phase, 65% acetonitrile in a 2 m*M* Tris solution; applied voltage, 29 kV; part II is an expanded version of part I showing the peaks between 1.0 to 1.6 min; (B) separation solution, 0.5% (w/v) poly(sodium undecylenic sulfate) in 12.5 m*M* each disodium hydrogenphosphate and sodium tetraborate (pH 9.2) with 40% acetonitrile (v/v); applied voltage, 30 kV; column dimensions, 47 cm (effective length 40 cm)×50 μm I.D.; identity of peaks, naphthalene (1), acenaphthylene (2), acenaphthene (3), fluorene (4), phenanthrene (5), anthracene (6), fluoranthene (7), pyrene (8), benz[*a*]anthracene (9), chrysene (10), benzo[*b*]fluoranthene (11), benzo[*k*]fluoranthene (12), benzo[*a*]pyrene (13), dibenz[*a*,*h*]anthracene (14), benzo[*ghi*]perylene (15), indeno[1,2,3-*cd*]pyrene (16). (Fig. 4A, reprinted with permission from Ref. [43], ©1998 American Chemical Society; Fig. 4B, reprinted with permission from Ref. [20], © 1998 American Chemical Society.)

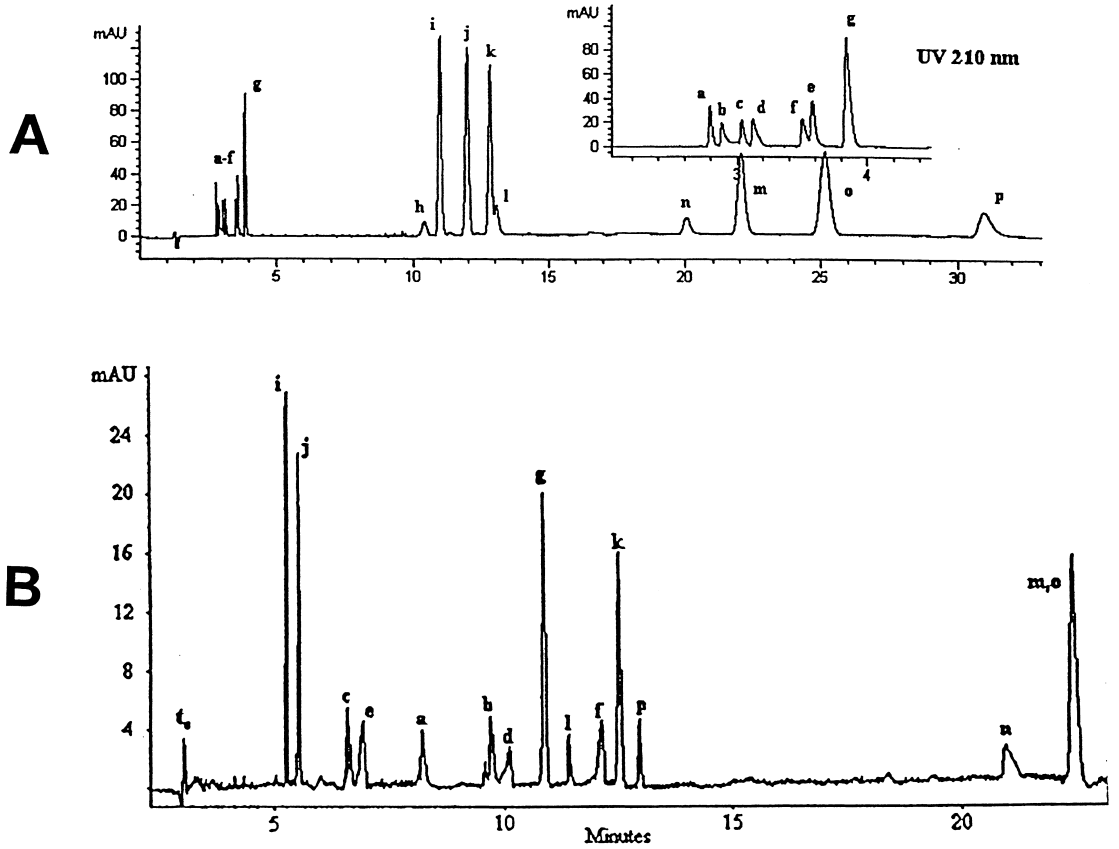

Fig. 5. Separation of basic, neutral, and acidic compounds by CEC (A) and MEKC (B). Conditions: (A) stationary phase, 3-μm Hypersil C_8 particles; column dimensions, 34 cm (25 cm packed length)×100 μm I.D.; mobile phase (step gradient), (for first minute) acetonitrile–25 m*M* phosphate buffer pH 2.5 (60:40) with 2 μl/ml hexylamine, (final condition) acetonitrile–25 m*M* phosphate buffer pH 2.5 (75:25) with 2 μl/ml hexylamine; applied voltage, 25 kV; (B) separation solution, acetonitrile–50 m*M* SDS–20 m*M* phosphate buffer pH 8.5 (15:85); applied voltage, 25 kV; column dimensions, 57 cm (effective length 50 cm)×50 μm I.D.; identity of peaks, amphetamine (a), methamphetamine (b), procaine (c), cocaine (d), heroin (e), quinine (f), noscapine (g), thiourea (h), phenobarbital (i), diazepam (j), testosterone (l), cannabinol (m), testosterone propionate (n), 9-tetrahydrocannabinol (o), 9-tetrahydrocannabinolic acid (p). (Reprinted with permission from Ref. [72], © 1998 American Chemical Society.)

lower limits of detection (LODs). With usual injection and UV detector, EKC LODs are at the mg/l levels or at least an order higher than HPLC. Solutions to this problem include the use of powerful detectors, use of extended pathlength cells, off-line preconcentration, and on-line concentration techniques.

4.1. Powerful detectors, detection cell manipulation, and off-line concentration

The most frequently used powerful detection scheme in EKC is laser-induced fluorescence [73,74]. Other modes include amperometric [75], electrochemical detection [76], and fiber optic sensor [77]. LODs for these detection modes are at the μg/l level or lower or at least an order of magnitude lower than UV detection. For solutes that contain no chromophore, indirect detection or derivatization prior to direct or indirect photometric or fluorescence detection have also been reported [78–82]. However, in an ordinary laboratory, these detectors may not be affordable and UV detection is frequently used.

Some investigations focused on increasing the pathlength for photometric detection by manipulating the detector cell configuration guided by Beer's law, wherein absorbance increases with the length through which light passes through a sample solu-

tion. Bubble cell, sleeve cell, and z-cell detector configurations have been introduced, which afforded from two-fold to more than ten-fold improvement in detector response [83–85].

Similar to HPLC, a successful EKC analysis would often rely on a dedicated sample preparation regimen (for example, liquid–liquid and/or solid–phase extraction) [86,87]. Sample preparation regimens usually increase the concentration of solutes before injection into the capillary, making usual photometric detectors practical for analysis. Furthermore, this is usually required in real sample analysis to prevent interference emanating from the matrix. However, EKC has the advantage of direct injection (with or without dilution) in cases where the solute concentration is sufficient for detection [88,89]. Column fouling occurs in HPLC and CEC if direct injection is performed, and a column guard may be helpful in HPLC. Furthermore, capillary columns used in EKC are much cheaper compared to columns used in HPLC.

4.2. On-line concentration

Of considerable interest to improve concentration sensitivity in EKC is the development of on-line concentration techniques, which are isotachophoresis, sample stacking, and sweeping that are performed individually or in combination. On-line techniques are easily transferable technologies because of their simplicity and economy. These techniques can be applied to charged and neutral analytes, except that isotachophoresis cannot be applied to neutral ones. Isotachophoretic preconcentration of charged solutes is performed by the proper choice of leading and terminating background electrolyte. This is usually followed by separation using CZE [90] but MEKC has also been applied in a non-ionic surfactant system [91].

4.2.1. Sample stacking

Sample stacking was initially discovered for charged analytes in CZE, which is basically performed by preparing the sample in a matrix having a resistance considerably higher or a conductivity considerably lower than that of the background solution [92–101]. Theoretically, the greater the difference in conductivity or resistivity, the greater the concentrating effect. Sample stacking is defined as the movement of ions across a boundary that separates regions of low and high electric fields. This boundary where focusing occurs is termed the stacking boundary (SB), which is pseudostationary and moves with the direction and magnitude of the EOF [102,103]. The sample and separation regions are the high- and low-electric-field regions, respectively. Sample ions have greater electrophoretic velocity in the sample region compared to the separation region, therefore, when the sample passes through, the SB will slow down and focus into thin zones. Hydrodynamic and electrokinetic injection schemes can be performed. From ten- to more than 1000-fold improvements in detector response have been documented.

In hydrodynamic injection, a dispersive effect brought by the mismatch of local EOF velocities limits the injection lengths [94,95,102]. To alleviate this and maximize the amount of sample solution that can be injected, a polarity-switching step was introduced to remove the sample matrix during sample stacking (large volume sample stacking, LVSS) [95]. The polarity-switching step, which is not accessible in every commercial instrument and can cause irreproducibility, can be surpassed by using an electroosmotic flow modifier or/and adjusting the pH for the analysis of anions [96,97] and cations [98] separately. In electrokinetic injection, a water plug is introduced before sample injection to cause an enhanced field at the injection tip that improved the focusing effect [99]. Furthermore, using a polarity-switching scheme, both positive and negative solutes can be injected and focused [100].

Ionic analytes separated in a MEKC system were concentrated by sample stacking [104–106]. At first, sample stacking of neutral analytes was thought to be not amenable for EKC since such analytes are unaffected by an enhanced field [104,107]. However, with the proper manipulation of the PS that will provide effective electrophoretic mobilities to neutral solutes, several sample stacking techniques and applications were developed for EKC, using micelles [108–116], microemulsions, or charged cyclodextrin [117]. From around ten- to more than a 100-fold improvement in detector response has been reported. Combination of sample stacking and a z-cell afforded around 1000-fold improvement in detector

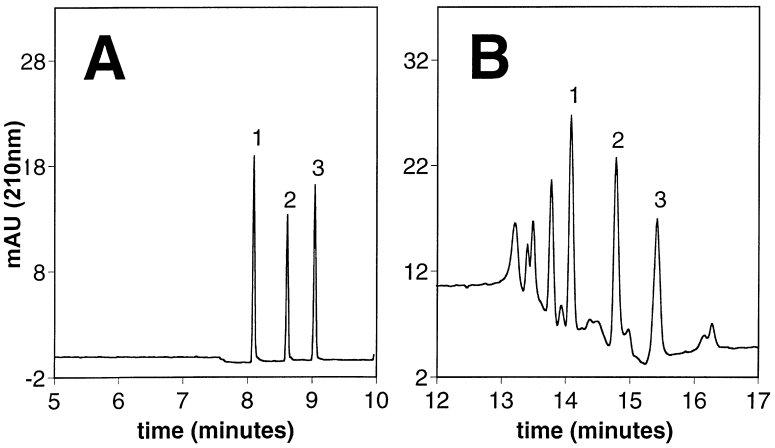

Fig. 6. Enhancing sensitivity 1000-fold using stacking (SRMM) and a z-cell. BGS, 100 m*M* SDS in 40 m*M* phosphate buffer (pH 2.5); injection, 1 s (A), 200 s (B); concentration of samples, A, 2,3,5-trimethylphenol (peak 1, 153 ppm), 4-ethylphenol (peak 2, 457 ppm), 3-chlorophenol (peak 3, 416 ppm), B, 1/1000 dilution of samples in A with water; capillary, 65 cm (effective length 56.5 cm) (A, normal cell, 50 μm I.D.; B, z-cell (3 mm), 70 μm I.D.), voltage, −22 kV (A), −16 kV (B). Reprinted with permission from Ref. [118], © 1997 International Scientific Communications.

response and LODs in the n*M* level (Fig. 6) [118]. Like in CZE, hydrodynamic [108–112] and electrokinetic [113–115] injection schemes are available.

Table 1 lists the different sample stacking techniques, their abbreviations, and maximum stacking enhancement factors recorded that were developed in our laboratory. Similar problems in CZE sample stacking were encountered in EKC sample stacking. For example with hydrodynamic injection in NM-EKC (normal stacking mode) [108,109], the length of sample that can be injected is limited, thus a polarity switching step was also evaluated in order to introduce more sample and increase detection sensitivity (reversed electrode polarity stacking mode, REPSM) [108,110]. However, in RM-EKC using micelles of SDS in a low pH buffer, narrowing of analyte bands and separation of focused analyte bands was achieved without polarity switching inherent in LVSS (stacking with reverse migrating micelles, SRMM) [111]. This is because only a nega-

Table 1
Maximum stacking enhancement factors achieved for each stacking technique in MEKC[a]

		SE_{height} (Maximum)[b]	SE_{area} (Maximum)[c]
A. Hydrodynamic injection			
(1)	Normal stacking mode (NSM)	14	37
(2)	Reversed electrode polarity stacking mode (REPSM)	45	~100
(3)	Stacking with reverse migrating micelles (SRMM)	>100	>100
(4)	Stacking using reverse migrating micelles and a water plug (SRW)	>100	~200
B. Electrokinetic injection			
(1)	Field enhanced sample injection (FESI)	23	~100
(2)	Field enhanced sample injection with reverse migrating micelles (FESI–RMM)	~100	~100

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[b] $SE_{height} = \frac{\text{peak height obtained with stacking}}{\text{peak height obtained with usual injection}}$.

[c] $SE_{area} = \frac{\text{corrected peak area (with migration time) obtained with stacking}}{\text{corrected peak area (with migration time) with usual injection}}$.

tive polarity is needed to focus, separate, and bring the solutes to the detector. In REPSM, a negative polarity is needed to focus the analyte bands and a positive polarity is needed to separate and detect the resulted focused bands. More than a 100-fold increase in detector response in terms of peak height was achieved for SRMM and only around 40 and ten for REPSM and NSM respectively.

Unlike charged solutes, neutral solutes have much lower solubility in aqueous solution, which is a problem source in sample stacking in EKC. Samples in EKC sample stacking with hydrodynamic injection (NSM, REPSM, and SRMM) should be prepared in a low conductivity non-micellar solution. Liu et al. [108] prescribed a low conductivity and micellar solution (just above the CMC) as sample matrix that improves the solubility of analytes. However, we found out that presence of micelles lessens the concentrating effect of sample stacking by causing a broadening effect [111]. Use of small amounts of organic solvents into the sample matrix is a possible solution to the solubility problem. Therefore, water soluble solutes are better concentrated using SRMM while those with limited solubility in water are better concentrated using other sample stacking techniques that utilize micellar solutions as sample matrix. These techniques will be introduced below. In SRMM, broadening with micelles in the matrix was explained by the time difference in which molecules found nearest to and farthest from the SB reach the SB.

Using a micellar solution to solve the solubility problem, hydrodynamic and electrokinetic injection sample stacking schemes were developed. In hydrodynamic injection RM-EKC, a water plug is injected prior to injection of the sample (stacking using reverse-migrating micelles and a water plug, SRW) that rendered sensitivity enhancements comparable to SRMM [112]. In electrokinetic injection NM-EKC, a water plug is injected prior to injection of sample at negative polarity followed by separation using positive polarity (field enhanced sample injection, FESI) [114]. By using a mixed micelle containing a cationic surfactant, electrokinetic injection can be performed at positive polarity [113]. In electrokinetic injection RM-EKC, no polarity switching is necessary and only the sample vial is replaced by a separation solution vial after the predetermined optimum injection time was reached (field enhanced sample injection with reverse migrating micelles, FESI-RMM) [115]. The water plug provides a high electric field zone for effective stacking. Increase in detector response (peak height) was around 20 for FESI and around 100 for FESI-RMM. Furthermore, the micellar solutions used in the aforementioned techniques should have a lower conductivity compared to the separation solution. For example, micellar concentration is only slightly above the CMC and buffer component concentration is much lower compared to the separation solution. This provides an enhanced field in the sample zone, which rendered greater enhancements in detector response.

4.2.2. Sweeping

Sweeping, a new concept in EKC, is defined as the picking and accumulating of analyte molecules by the PS that enters and fills the sample zone upon application of voltage [119]. This phenomenon, initially observed by Gilges [120] or other workers who have not documented it, however, was not well studied until this time. It occurs whenever the sample matrix is void of a charged carrier phase and it does not matter whether the sample matrix has a higher, similar, or lower conductivity compared to the background solution. As an on-line concentration technique, it has provided more than 5000-fold increases in detection sensitivity, which is greater than any other reported technique [119]. Moreover, it is independent from the EOF and works for charged or uncharged solutes making it an almighty technique. On top of that, additives like organic solvents may be added to the sample matrix to increase solubility or pH of sample and separation buffer can be adjusted to promote solubility and enhance focusing [121]. Figs. 7 and 8 show the on-line concentration using sweeping and separation of neutral and basic solutes in MEKC, respectively.

In a considerably homogenous electric field system, the width of the resulting zone after sweeping (l_{sweep}) is given by Eq. (9):

$$l_{sweep} = l_{inj} \cdot \frac{1}{1+k} \tag{9}$$

where l_{inj} is the length of the injected zone. The k

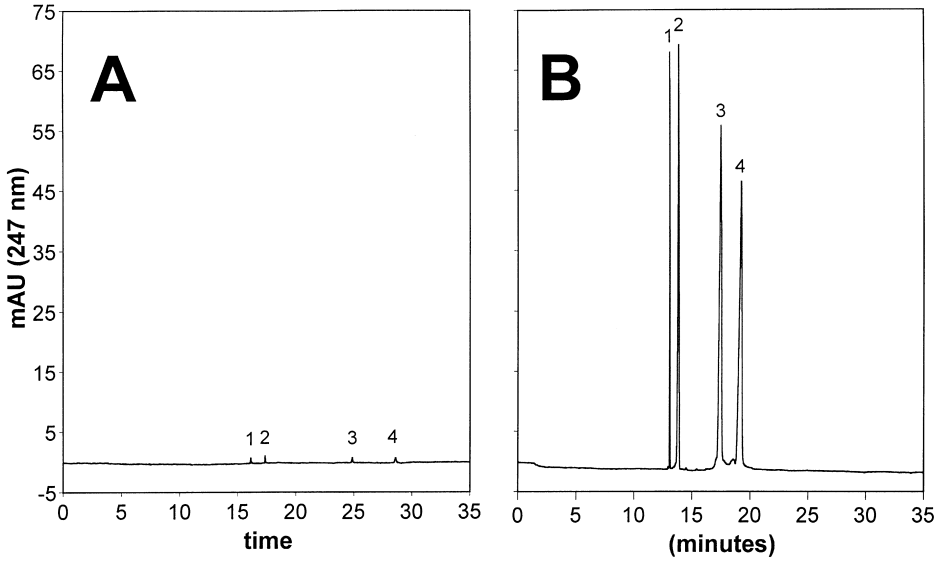

Fig. 7. Sweeping MEKC analysis of neutral solutes using a neutral capillary and a basic buffer. BGS, 50 m*M* SDS and 20% methanol in 20 m*M* borate buffer (pH 9.3); S, steroids in borate buffer adjusted to the conductivity of the BGS; injected length, 0.64 mm (A), 42 cm (B); applied voltage, −30 kV; concentration of analytes, ~20 ppm (A), ~2 ppm (B); identification of peaks, progesterone (1), testosterone (2), hydrocortisone (3), cortisone (4); capillary, Celect-N, 65.5 cm (effective length 57 cm). Reprinted with permission from Ref. [121], © 1999 American Chemical Society.

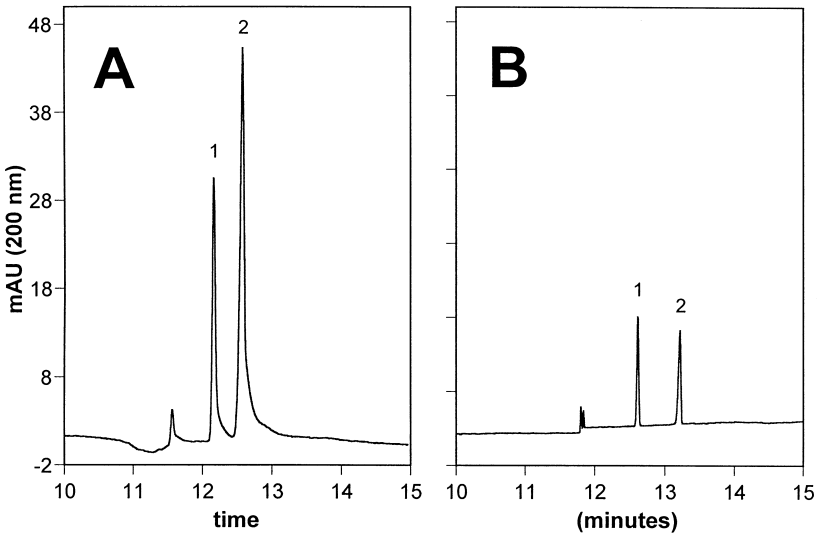

Fig. 8. Sweeping MEKC analysis of two positively chargeable compounds. BGS, 75 m*M* SDS and 10 m*M* triethanolamine in 20% methanol and 30 m*M* phosphoric acid (pH 1.9); S, amines in phosphoric acid adjusted to the conductivity of the BGS; injected length, 0.64 mm (A), 32 cm (B); applied voltage, −25 kV; concentration of analytes, ~300 ppm (A), 0.3 ppm (B); identification of peaks, 1-naphthylamine (1), 1-phenylethylamine (2); capillary, 65 cm (effective length 56.5 cm). Reprinted with permission from Ref. [121], © 1999 American Chemical Society.

values in the sample zone when filled with micelle are assumed equal to the k values in the separation zone. Sweeping is then basically dependent on the retention factor and the length of the initial zone. Therefore, this equation predicts an almost unlimited increase in detection sensitivity for analytes having k approaching infinity, or analytes having great affinities to the PS. In the absence of EOF (RM-EKC), l_{inj} can be almost the distance from the injection end to the detector window if separation of focused zones is possible. The inability of present instruments to apply more than 30 kV of voltage limits the use of longer capillaries and thus limits the capacity of sweeping to concentrate more dilute samples. In the presence of EOF (NM-EKC), l_{inj} is more limited if one considers the virtual length of the capillary for separation.

In a heterogenous electric field system, where the field in the sample region is lower than the separation region — done by preparing the sample in a high salt-containing matrix — l_{sweep} can be described by Eq. (9) too. In this case, the values of k are theoretically higher than those in a homogeneous system due to stacking of the micelle at the SB causing the higher concentration of micelle entering the sample region (consult Eq. (8)), and higher concentration factors should be expected [122,123]. However, studies from the authors revealed that preparing the sample in higher conductivity matrix does not have a profound beneficial effect on sweeping as an on-line concentration technique [123].

Furthermore, if the electric field in the sample region is higher than the separation region — done by preparing the sample in a low conductivity matrix — l_{sweep} can still be described by Eq. (9). In this case, the values of k are lower than in a homogeneous system due to the low concentration micelle, which enters the sample zone. This condition is similar to NSM and SRMM. Therefore, with the concept of sweeping, the concentrating mechanism in MEKC when the sample matrix is a high resistivity non-micellar aqueous solution is understood more clearly. It was found that the total focusing effect is brought by the cumulative effect of sweeping and sample stacking. The analyte zones were concentrated first by sweeping and once these swept zones reach the interface between the low- and high-electric-field regions they were then concentrated finally by sample stacking. It is amended that sample stacking and sweeping are two different phenomena for sample focusing or concentration, since many workers usually use stacking to describe an on-line sample concentration process [124].

4.2.3. Cation-selective electrokinetic injection-sweeping

Up to this time, with the advent of on-line concentration techniques specifically sweeping described above, the lowest LOD with a UV detector reached was in the ppb level or three orders of magnitude lower than usual injection. Recently in the authors' laboratory, a novel method that combines sample stacking in CZE with electrokinetic injection [field enhanced sample injection in CZE, FESI(CZE)] and sweeping, afforded the detection of positively chargeable and hydrophobic analytes at ppt levels (LOD as low as 4.1 ppt) with outstanding plate numbers. This translates to improvements in peak heights compared with usual injection approaching a million-fold [125]. The main idea is to selectively introduce by FESI(CZE) as many molecules of cationic analytes as possible from a very dilute sample solution and focus the resulting zone by sweeping. Other possibilities are still currently being explored, for example the use of cationic surfactants.

5. Electrokinetic chromatography hyphenation with mass spectrometry

Very complex systems like biological samples contain vast amounts of information that should be measured individually with great accuracy, sensitivity, selectivity, and precision. One of the best answers to this problem is a dedicated separation technique like EKC coupled on-line with a detector that provides data not only concerning concentration but also the identity or structure of the analyte detected. At present, EKC-hyphenated techniques, like EKC–mass spectrometry (EKC–MS or EKC–MS–MS), is still at a young stage compared to other CE-hyphenated techniques like CZE–MS. CE coupling to mass spectrometry is one of the fast-growing areas of research, basically because of the great power of the

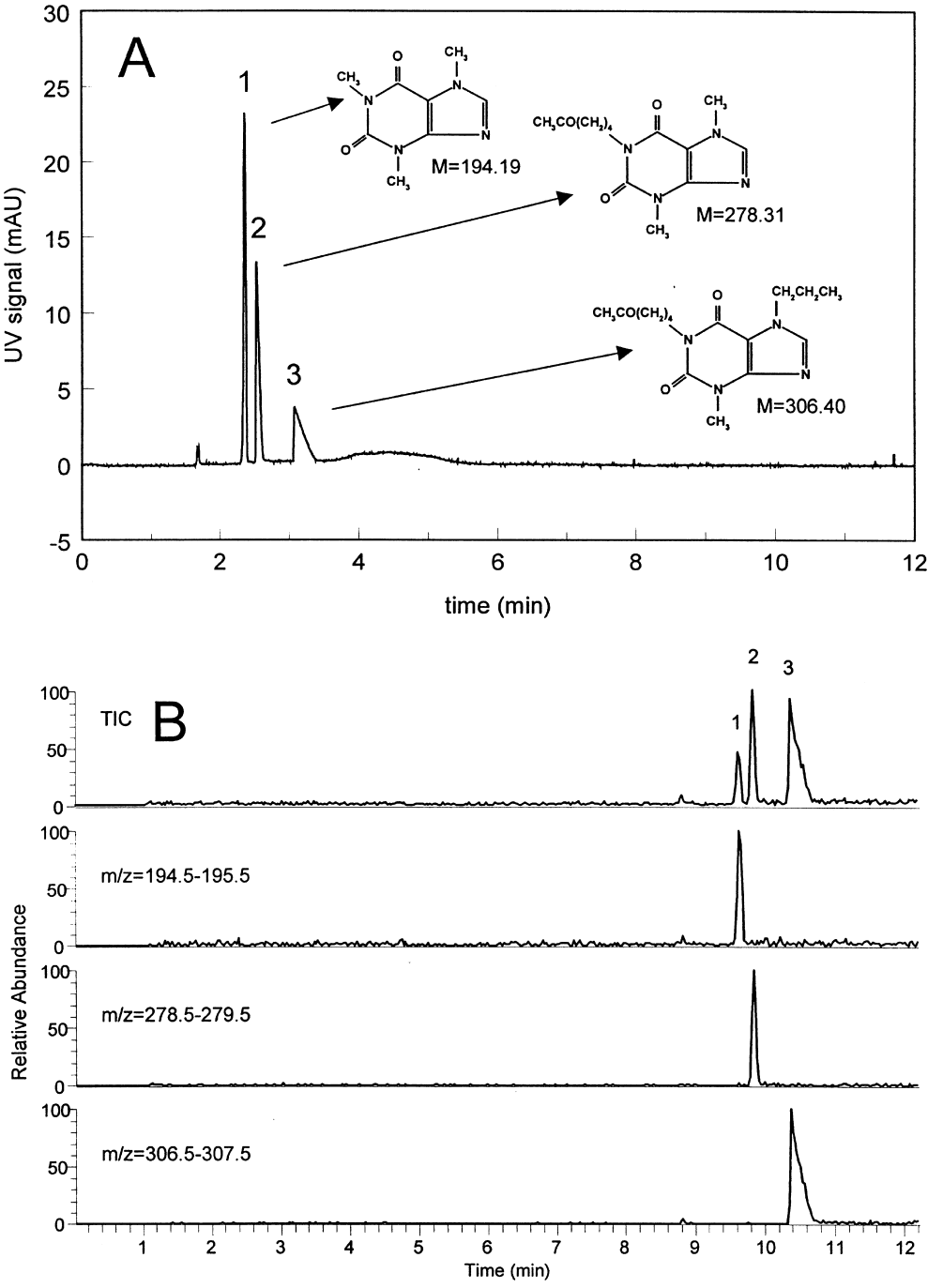

Fig. 9. Partial-filling MEKC–ESI–MS. (A) UV electropherogram and (B) total ion current (TIC) and selected ion electropherograms. Buffer, 10 m*M* ammonium acetate (pH 9.5); PS, 0.3% (w/v) poly(sodium undecylenic sulfate), injection 10 kV for 60 s; samples, (1) caffeine, (2) pentoxifylline, (3) propentofylline, each at 0.1 mg/ml, injection 50 mbar for 5 s; capillary, 50 μm I.D., 21.5 cm to UV detector, 88.0 cm to MS detector; CE voltage, 20 kV; ESI voltage, 3.5 kV; sheath liquid, methanol–water–acetic acid (50:50:1, v/v/v); equipment, HP^{3D}CE and Finnigan LCQ MS with ESI interface. (Performed by Dr. P.G. Muijselaar.)

technique especially useful in the biomedical fields (e.g. analyzing DNA adducts, peptide, and proteome research).

The major problem in EKC–MS with an electrospray ionization (ESI) interface is the incompatibility or introduction of a nonvolatile PS into the interface, which masks the ionization process necessary for detection and contaminates the ion source. From the chemical point of view, employing the partial-filling technique [7,126,127], anodically migrating PS technique [128], or using polymer surfactant [129] can solve this. In the partial filling technique, only a part of the capillary is filled with an electrolyte solution containing the PS, thus allowing separation without allowing the PS to enter the mass spectrometer. A partial filling MEKC–ESI–MS analysis is shown in Fig. 9. In the anodically migrating PS technique, by adjusting the pH of the buffer, the overall velocity of the PS (for example, SDS micelle) is toward the anode and thus will not enter the MS. Finally, use of a polymer surfactant at a very low concentration, which is sufficient for separation due to the zero CMC, will not deteriorate ionization efficiency significantly.

On the other hand, an atmospheric pressure chemical ionization interface can be used as substitute for an ESI interface, the former being less affected by high salt concentrations that enter the interface and thus more applicable for direct coupling to the mass spectrometer [130]. Furthermore, a coupled capillary set-up with voltage switching and buffer renewal system that allows heartcutting of sample zones with the subsequent transfer via a second capillary to the MS is another alternative [131].

6. Future directions

It would be terrible to list all the excellent applications of EKC and thus that will not be done here. With this in mind, EKC application has penetrated most areas of scientific research and will continue to expand. Now, we would like to focus on some of the exciting aspects of EKC in the future. Although much work had been done to elucidate the fundamental mechanisms on EKC, including selectivity and band broadening, a realm of exploration is still existent and is worth looking at. Precise real-time monitoring of the dynamic changes occurring inside the capillary is one of them, which could lead to more intimate understanding [132]. With the help of organic chemists, tailor-made surfactants with superb selectivity will be used as possible PSs. Molecular imprinting techniques would be an area of interest. For example, receptors could be mimicked with the possible application in chiral drug-receptor binding and pharmacokinetic studies. Also, cationic polymer micelles with an expected unique effect on capillary wall chemistry and EOF will be developed. Moreover, surfactants that interfere less with MS interfaces and detection will be useful.

MS detection using an ESI interface is applicable only to charged analytes. Application of on-line formation of charged coordination compounds from neutrals in EKC is an interesting field of study. Bayer et al. have reported the MS detection of terpenes, carotenoids, and vitamins A and D as silver complexes and sugars and saccharides as boron complexes [133]. More robust instrumental designs or innovations for MS interfaces should be expected too. Coupling to other detection schemes like NMR is also worth mentioning. Individual chemical and spatial structural properties of organic solutes can then be obtained. Furthermore, it will be preferred over HPLC–NMR since less samples and low solvent volumes are required.

Basic studies on the transfer of EKC from capillaries to microchip devices [134], especially utilizing the developed on-line concentration techniques described previously with special emphasis on sweeping, is very apparent. This will lead to some real-world applications with hand-carried instruments for on the spot analysis.

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