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Stacking Ionizable Analytes in a Sample Matrix with High Salt by a Transient Moving Chemical Reaction Boundary Method in Capillary Zone Electrophoresis

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The paper presents a novel on-line transient moving chemical reaction boundary method (tMCRBM) for simply but efficiently stacking ionizable analytes in high-salt matrix in capillary zone electrophoresis (CZE). The powerful function and stability of the tMCRBM are elucidated with the ionizable test analytes of L-phenylalanine (Phe) and L-tryptophan (Trp) in the matrix with 85.6−165.6 mM sodium ion and further compared with the normal CZE of Phe and Trp samples dissolved in running buffer. The results verify that (1) the on-line tMCRBM mode can evidently increase separation efficiency, peak height, and resolution, (2) with the mode, the analytes in a 28-cm high-salt matrix plug can be stacked successfully and further separated well, (3) the values of relative standard deviation of peak height, peak area, and migrating time range from 3.9% to 6.1%; the results indicate the high stability of the technique of tMCRBM-CZE. The techniques implies obvious potential significance for those ionizable analytes, e.g., protein, peptide, and weak alkaline or acidic compound, in such matrixes as serum, urine, seawater, and wastewater, with high salt, which has a deleterious effect on isotachophoresis (ITP) and especially on electrostacking and field-amplified sample injection (FASI). The mechanism of stacking of zwitterionic analytes in a high-salt matrix by the tMCRBM relies on non-steady-state isoelectric focusing (IEF) but not on transient ITP, electrostacking, and FASI.

Many sample matrixes, such as serum, urine, seawater, wastewater, and eluting solution from liquid chromatography, contain a lot of salts such as sodium chloride in seawater and serum. Before electrophoresis, the high salt in the matrix should be removed because it may break down the stacking of analytes by transient isotachophoresis (ITP),[1−3] electrostacking,[4−7] and field-amplified sample injection (FASI)[8−12] in capillary zone electrophoresis (CZE) if no further pretreatments (e.g., sample extraction[13,14] or addition of acetonitrile to the sample matrix[15−21]) are used to avoid its harmful action in the matrix. The removal of salt from the matrix is worrisome and time-consuming. Thus, there will be significant interest if analytes in a high-salt matrix can be directly on-line stacked to achieve enhancement of sensitivity without any losses of the high separative efficiency and resolution of capillary electrophoresis (CE).

Numerous methods have been developed for stacking analytes in salt matrixes in CE. The first method is the "acetonitrile salt mixtures" mode invented by Shihabi et al.[15−21] The mechanism of sample stacking is presumably caused by a transient ITP or FASI.[16−21] Even here there is still salt in the sample matrix, but the harmful effect of the salt on ITP and FASI stacking can be counteracted by the addition of acetonitrile. Thus, it is no surprise that FASI can be performed in CE by adding 66% acetonitrile into

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the matrix with a high salt content. But the high salt may seriously break down the mechanism of normal FASI, because the low conductivity of the matrix is of key importance to a FASI run.[8−14] "Acetonitrile stacking" has been used for the enrichment of peptides,[16] insulin,[17] serum procainamide,[18,19] and weakly cationic compounds[20] in the presence of salt.

The second method of stacking analytes in a high-salt matrix is "pH-mediated sample concentration".[22−28] In this method, as described mainly by Lunte's group,[22−25] a sample is dissolved in a weakly acidic (or basic) buffer in the presence of salt; conversely, the running buffer is just the conjugate base (or acid) of the weakly acidic (or basic) buffer used in the sample. After a proper sample injection, there exists a moving reaction boundary between the weak acid (or base) in the matrix and the conjugate base (or acid) in the running buffer, when an electrical field is applied. The chemical reaction results in a progressive low-conductivity zone in the original matrix plug. The low-conductivity zone further leads to an action of pH-mediated reduced FASI stacking of the analytes in matrix.[23−27] The pH-mediated induced FASI has been used for the preconcentration of enantiomers of isoproterenol,[22] DNA sequencing,[23,26] anions in physiological samples,[25,27] and pharmaceutical cations.[28]

An analogous method, termed "velocity-difference induced focusing by dynamic pH junction", has recently been developed for the selective stacking of the analytes of interest in a high-salt matrix by Chen's group.[29−32] In this method, the pH values of sample and running buffers are normally both over (or less than) pH 7 simultaneously, but at the same time, the pH values are not equal to each other. In the dynamic pH junction system, the velocities of analytes of interest are greatly regulated by the pH change on the two sides of the pH junction and by the formation of a complex between the analytes and the background ions, such as borate ion, in running buffer, whereas the velocities of unwanted analytes in the matrix plug cannot be adjusted by the pH change and the absence of complex formation between the background ions and these analytes. Therefore, a selective focusing for the analyte of interest can be achieved in the dynamic pH junction system. A stacking of dynamic pH junction is not apparently affected by the presence of high salt, if the high salt does not influence the pH values of sample matrix and running buffers and the complex formation between the analytes of interest and background ions. By using the selective stacking method, Chen's group has successfully improved the sensitivity of CE for the analyses of epinephrine in dental anesthetic solutions,[29,30] catecholamines and acidic compounds,[31] and nucleotides.[32]

Recently, a novel method has been advanced for stacking neutral (or charged) analytes in salt matrix in micellar electrokinetic chromatography (MEKC) by Palmer et al.[33−42] In the method, a moving interaction boundary exists between the analytes in the matrix and micellar molecules in the pseudostationary phase, as the moving boundary stacks the analytes together.[33,35] The method is so powerful that over 5000-fold concentration can be achieved in MEKC.[36] The method of stacking neutral analytes in a salt matrix is combined with the microchip electrophoresis.[37,38] The method has been further applied to the stacking of charged analytes in MEKC, such as deoxyguanosine,[39] phenol derivatives,[40] and seven plant hormones in tobacco flowers.[41] Moreover, the experimental conditions for stacking neutral analytes in MEKC are extended to the continuous conductivity conditions.[42]

ITP, the oldest electrophoretic technique,[43] has also been used to focus specific analytes of interest in the presence of high salt. The theoretical and experimental studies on transient ITP have been widely investigated for stacking analytes in a salt matrix during the past decade.[44−50] When specific analytes hold mobilities intermediate to those of leading and terminating ions, the stacking of analytes is induced. Generally, some background ions, used as stackers, are necessary for good stacking of analytes in a matrix.[46,48] Transient ITP is a well-rounded stacking technique in CZE as well as in slab or disk gel electrophoresis. For this reason, transient ITP possesses wide applications in numerous fields, particularly in biochemistry and biomedicine.[51−53]

Moving chemical reaction boundary (MCRB) is a new and useful boundary system of electrolytic solutions. The pioneer idea of MCRB, termed "precipitate reactive front", was evolved by Deman and Rigole,[54,55] and the valuable concept of "stationary neutralization reactive boundary" was advanced for electrically

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controlled electrofocusing in CE by Pospichal et al.[56] From the works above, the theory of MCRB has recently been developed by Cao et al.[57−60] The theory of MCRB for a strong electrolytic system has been proved by some experiments quantitatively.[61−66] Interestingly, the theory of MCRB become very ambiguous for this kind of boundary system created with weak (or strong) acid and strong (or weak) alkali, together with the presence of an excess of potassium chloride.[67,68] For example, if the boundary system is formed with acetic acid and sodium hydroxide in the presence of potassium chloride,[68] the original equation of MCRB can well predict the anodic movement of the boundary but cannot calculate the cathodic movement completely. The equations of MCRB should be individually corrected for the different experimental conditions.[67,68] In the experiments above, the moving chemical reaction boundary method (MCRBM) was developed for the investigation of the theory of MCRB.[61−68] The MCRBM has been used for dynamic investigation of classic isoelectric focusing (IEF),[63,69−71] contributed by Svensson[72,73] and by Vesterberg,[74] and the preparation of colloid in gel.[66]

In this paper, the method is modified as transient MCRBM, viz., tMCRBM, for the stacking of analytes in high-salt matrix in CZE. Thus, the purposes of the report are to describe the tMCRBM for simply but efficiently stacking ionizable analytes of Trp and Phe in high-salt matrix, to report the experimental results, and to discuss the mechanism of tMCRBM stacking qualitatively.

EXPERIMENTAL SECTION

Chemicals. Sodium hydroxide (Guaranteed Reagent grade, GR), 37% hydrochloric acid (GR), and sodium chloride (Analytical Reagent grade, AR) were purchased from the Shanghai Chemical Reagents Co. (Shanghai, China). Formic acid (AR) from the Shenyang Xinhua Chemical Reagents Factory (Shenyang, China). L-Tryptophan (Trp, Chrom pure) from the Lizhu Dongfeng Biotechnological Co. Ltd. (Shanghai, China). L-Phenylalanine (Phe, Chrom pure) from the Kangda Amino Acid Factory (Shanghai, China). The two amino acids hold a similar skeleton structure, their p*I*s are 5.48 (for Phe) and 5.89 (for Trp), and their molecular weights are 165.19 (for Phe) and 204.23 (for Trp). For those reasons, the two amino acids are difficult to separate.

Table 1. Contents of Two Kinds of Basic Salt Matrixes of Trp and Phe

basic salt matrix (no.)	Trp (μg/mL)	Phe (μg/mL)	sodium formate (mM)	sodium chloride (mM)	sodium ion (mM)
1	15	40	65.6	20.0	185.6
2	15	40	65.6	40.0	105.6
3	15	40	65.6	60.0	125.6
4	15	40	65.6	80.0	145.6
5	15	40	65.6	100.0	165.6
6	0.15	0.60	82.0	80.0	162.0

Apparatus. A capillary electrophoretic apparatus (Bingda 1229, Beijing New Technology Institute, Beijing, China) was used. The apparatus was equipped with an electric power supply (up to constant voltage 30 kV), an UV detector set at 214 nm, and an automatic recorder. Two fused-silica capillaries with an i.d. of 75 μm (from the Factory of Yongnian Optical Fiber, Hebei, China) were used. The first was a capillary with total length of 60 cm and an effective length of 40 cm, the detector was placed at 20 cm from the cathodic end of the capillary, which was used for CZE with 20−60-s 13-mbar pressure injection (viz., small-volume sample plug). The second was a capillary with a total length of 75 cm and an effective length of 65 cm, the detector was set at 10 cm from the cathodic end, and this capillary was used for CZE with up to 660-s 13-mbar injections (viz., large-volume sample plug). The runs were carried out at 26 °C. Before each run, the new capillaries were conditioned by rinsing with 0.1 M HCl for 20 min, distilled water for 10 min, 0.1 M NaOH for 20 min, and running buffer for 30 min, in order.

Buffer and Sample Matrixes. The running buffer was pH 2.85 32.8 mM formic acid/sodium formate. Before use, the buffer was degassed and filtered with a 0.47-μm filter membrane. Three kinds of sample matrixes were prepared. The first was an acidic sample matrix used for a normal CZE; the sample matrix contained 15 μg/mL Trp and 40 μg/mL Phe in the running buffer. The second was an alkaline salt sample used for the runs of tMCRBM to improve the separation of CZE (see matrixes 1−5 in Table 1). The third was the diluted alkaline salt matrix 6 in Table 1 used for large-volume sample injection.

General Procedure of tMCRBM-CZE. First, the anode end of the capillary was, as shown in Figure 1a, inserted into an alkaline salt sample shown in Table 1. Then, the 13-mbar pressure was used for the injection (see Figure 1b). Next, the sample cell was displaced with the anodic vial holding the running buffer (see Figure 1c). After step c, the power supply was turned on, and a transient MCRB indicated by the "B" was created between the pH 2.85 acidic running buffer (holding H^+) in the anodic vial and the pH 8.34 alkaline salt sample (holding OH^-) in the capillary. After the reaction between the H^+ and OH^-, the original sodium formate in the matrix plug was gradually neutralized by the conjunct acid pH 2.85 formic buffer. The transient MCRB was designed to move toward the cathode, and its velocity was well controlled. So the boundary could effectively stack the ionizable analytes (Phe and Trp) moving toward the cathode all together as shown in Figure 1d. After the end of transient MCRB, the stacked zones of Trp and Phe electrically migrated as a manner

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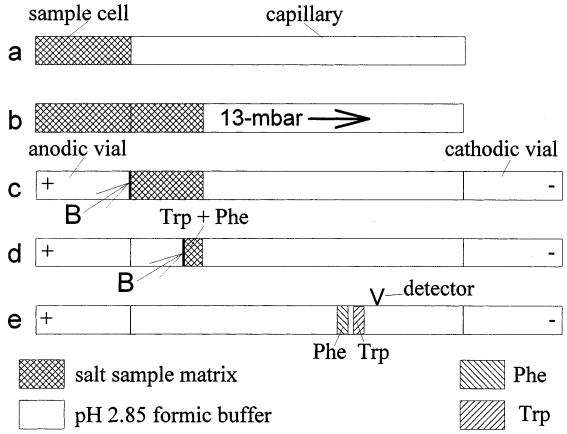

Figure 1. General procedure of tMCRBM for stacking ionizable analytes in sample matrix with high salt (at pH 2.85, electroosmostic flow (EOF) is very low, so the EOF is omitted): (a) Putting the anodic end of capillary into the salt sample cell; (b) applying 13 mbar for pressure injection; (c) displacing the sample with the anodic vial holding pH 2.85 32.8 mM formic buffer; (d) applying electric field for the tMCRBM stacking of analytes in the salt matrix plug; (e) CZE separation of Trp and Phe. The symbols + and − indicate the anode and cathode, respectively, the horizontal arrow implies the injection, the "B" marked by the arrows means the transient boundary, and "V" is the detector.

of CZE, further were separated in accordance with their mobilities, and passed through the detector (see Figure 1e). The mechanism of stacking analytes Trp and Phe by the transient MCRB will be discussed in detail as shown in Figure 7. During the experiments, the high-voltage power supply should be operated with extreme care to avoid electric shock. In addition, one ought to avoid the harmful action of sodium hydroxide and hydrochloric acid to one's eyes and skin.

The follow equations were respectively used for the computation of plate number[76] and resolution[77]

$$N = 2\pi(t_m h/A)^2 \quad (1)$$

$$R = 2(t_{m2} - t_{m1})/(w_2 + w_1) \quad (2)$$

where N is the plate number, A the peak area, t_m the migrating time, h the height of peak, and R the resolution of adjoining peaks, t_{m2} and t_{m1} are respectively the times of peaks 1 and 2 (in s) and w_1 and w_2 the baseline widths of peaks 1 and 2 (in s), respectively.

RESULTS AND DISCUSSION

To show the powerful function of tMCRBM, we first carried out the normal CZE of Trp and Phe. In the runs, the sample matrix was prepared in the running buffer, it contains 15 μg/mL Trp, 40 μg/mL Phe, and pH 2.85 32.8 mM formic buffer, and the results are given in Figure 2. From traces a−c in Figure 2, the 13-mbar injection times are 20, 40, and 60 s, respectively, corresponding to 0.80-, 1.60-, and 2.40-cm sample plugs. As shown in Figure 2, the separation efficiency and resolution become poorer and poorer

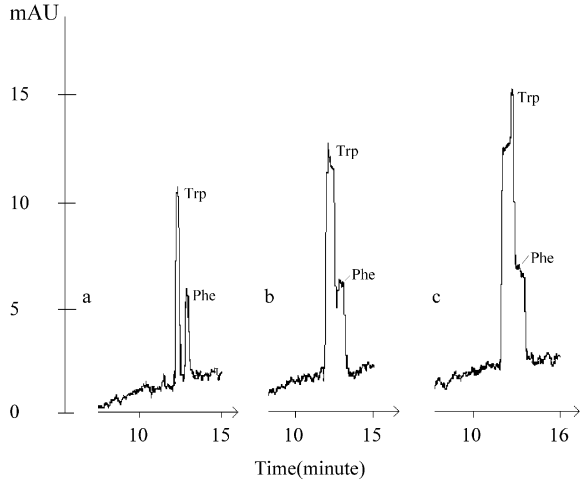

Figure 2. Electropherogram of normal CZE of Trp and Phe without the on-line tMCRBM stacking of the analytes for (a) 20-, (b) 40-, and (c) 60-s 13-mbar injection time. Conditions: running buffer pH 2.85 32.8 mM formic acid/sodium formate, normal sample matrix containing 15 μg/mL Trp + 40 μg/mL Phe in the pH 2.85 32.8 mM running buffer, total length 60 cm, effective length 40 cm, and i.d. 75 μm capillary, 20 kV, 16 μA, UV detector (at 214 nm), and 26 °C.

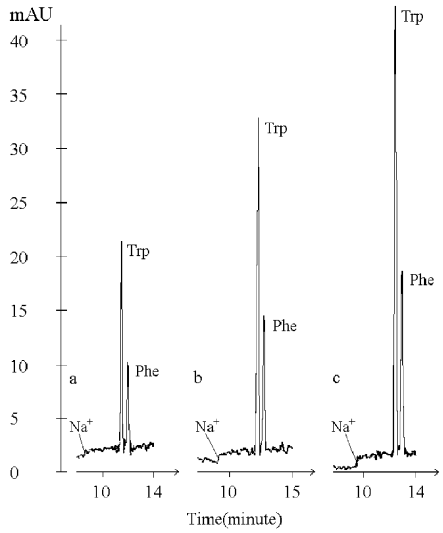

Figure 3. Electropherogram of CZE with the tMCRBM stacking of analytes in the alkaline salt matrix (no. 3 in Table 1) under (a) 20-, (b) 40-, and (c) 60-s 13-mbar injection time. The step marked by Na^+ is the sodium ion. Conditions: alkaline salt matrix containing 15 μg/mL Trp + 40 μg/mL Phe + 60 mM NaCl + 65.6 mM sodium formate. Other conditions are the same as those in Figure 2.

as the injection time increases from 20, to 40, and to 60 s. The quantitative analyses for Figure 2 show that the plate number (see eq 1) decreases from 1.57×10^4, to 3.49×10^3, and to 2.20×10^3, and the resolution (see eq 2) between Trp and Phe declines from 1.1, to 0.7, and to 0.3, accompanied with little enhancement of peak height, if injection time is elongated from 20, to 40, and to 60 s.

The stacking of tMCRBM mode was performed with alkaline salt matrix 3 in Table 1. In the runs, the concentrations of Trp and Phe in the salt matrix are equal to those in the salt-free matrix of Figure 2 and the injection times in Figure 3 are the same as those in Figure 2. The weakly alkali pH 8.34 65.6 mM sodium formate in the salt matrix, including the pH 2.85 running buffer in the anodic vial, is used to create a transient MCRB. As

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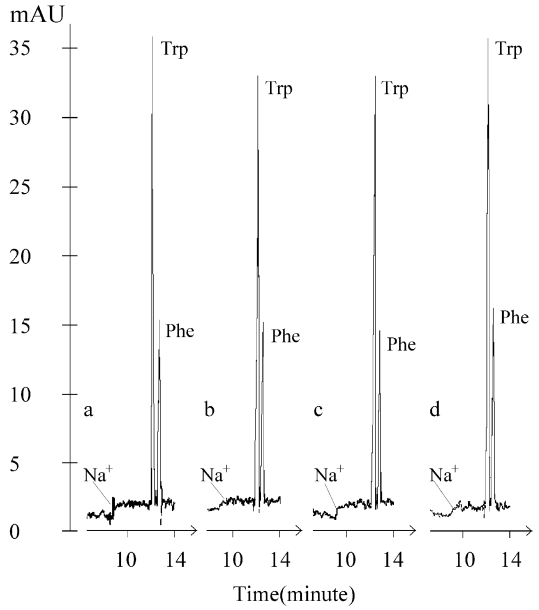

Figure 4. Electropherogram of CZE with the tMCRBM stacking of analytes in the alkaline salt matrixes with (a) 20, (b) 40, (c) 60, and (d) 80 mM sodium chloride (see nos. 1−4 in Table 1). The step indicated by Na^+ is the high concentration sodium ion. Conditions: 40-s 13-mbar injection. Other conditions are the same as those in Figure 2.

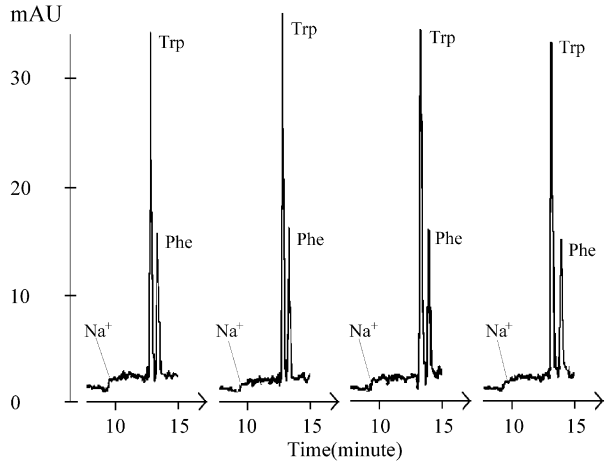

Figure 5. Repeatability test of tMCRBM stacking of analytes in the alkaline salt matrix 5 in Table 1. The step marked by Na^+ is sodium ion. Conditions: alkaline salt matrix holding 15 μg/mL Trp + 40 μg/mL Phe + 100 mM NaCl + 65.6 mM sodium formate, and 40-s 13-mbar injection. Other conditions are the same as those in Figure 2.

compared with the results in Figure 2, the peaks are more narrower, peak heights are obviously enhanced and accompanied by no loss of resolution between Trp and Phe in Figure 3.

The quantitative analyses for Figure 3 were calculated. For traces a−c in Figure 3, the plate numbers for Trp are 3.87×10^4, 3.07×10^4, and 2.95×10^4, respectively, the peak heights of Trp are respectively 18.5, 30.5, and 41 mAU, and the resolutions are 1.2, 1.1, and 1.1, respectively. Clearly, the resolutions are almost constant; the reason for this is that the tMCRBM can focus the plugs of analytes with different widths to a sharp zone under the given experimental conditions. The plate number, peak height, and resolution in Figure 3 were quantitatively compared with those in Figure 2. The results of the comparison are that (1) the plate number of Figure 3 is increased 2.5−13-fold as compared with that of the normal CZE mode of Figure 2, (2) peak height is raised 2.1−3.6-fold, and (3) resolution is improved to 1.1−3.3-fold. All of the data demonstrate that the tMCRBM mode can greatly enhance the separation efficiency, peak height, and resolution of CZE for the analytes in the high-salt matrix (up to 125.6 mM Na^+). The results given below will verify that significant improvements of stacking analytes can be realized whether the concentration of sodium chloride in the matrix is low (down to 20 mM) or high (up to 80 and 100 mM).

Figure 4 presents the results of tMCRBM mode for the stacking of analytes in the salt matrixes (nos. 1−4 in Table 1) containing 20, 40, 60, and 80 mM sodium chloride for traces a−d, respectively. The concentrations of analytes in the salt matrixes are also equal to those in the salt-free matrix of Figure 2; the injection times are all 40 s for the four runs in Figure 4. It is evident from Figure 4 that the separation efficiency, peak height, and resolution are almost constant, whether the sodium chloride in the matrixes is set at low (e.g., 20 and 40 mM), medium (e.g., 60 and 80 mM), or high concentration (e.g., 100 mM in Figure 5). All of the results demonstrate the absence of a salt effect on the tMCRBM mode for the stacking of analytes in salt matrixes under the given experimental conditions.

Figure 5 displays the results of the tMCRBM mode for the stacking of the analytes in salt matrix 5 (Table 1). The results in Figure 5 show that even there is 165.6 mM sodium ion in the matrix, good separation efficiency and resolution are achieved by the stacking of tMCRBM. Further statistical analyses of Figure 5 data show that (1) the relative standard deviation (RSD) values for peak heights of Trp and Phe are 4.4% and 3.9%, respectively, for peak areas of Trp and Phe are 4.4% and 6.1%, respectively, and (3) for migrating times of Trp and Phe are 4.5% and 5.0%, respectively. The data quantitatively imply quite good repeatability achieved by the tMCRBM mode for the stacking of the ionizable analytes of Phe and Trp in the high-salt matrix.

The results above surely manifest that the stacking by tMCRBM mode can be used for the obvious enhancement of separation efficiency and resolution of ionizable analytes in a small matrix plug (less than 2.4 cm) with high salt in CZE, and good stability can be achieved by the stacking mode (see Figure 5). Furthermore, the results below substantiate that the analytes in a large-volume matrix plug (up to 28 cm) can be excellently stacked by using the tMCRBM without any loss of separation efficiency and resolution. In Figure 6, the injection time is not the 20−60 s used above, but 660 s, which corresponds to ∼28-cm matrix plug with 80 mM sodium chloride; this plug occupies ∼43% of the effective length of capillary. The matrix composition is given in no. 6 of Table 1. As compared with the salt matrixes (nos. 1−5 in Table 1) used above, the analytes of Trp and Phe in the salt matrix 6 in Table 1 are respectively diluted 100- and 67-fold. As shown in Figure 6, the analytes in the large-volume matrix plug are sharply condensed and the resolution of analytes is apparently better than those shown in Figures 3−5. It is calculated that ∼200-fold stacking of the analytes Trp and Phe in the matrix with 162 mM sodium ion can be achieved by using the tMCRBM mode.

However, the current of 84 μA is high, and the migrating time is doubled, as compared with that in Figures 2−5. The high

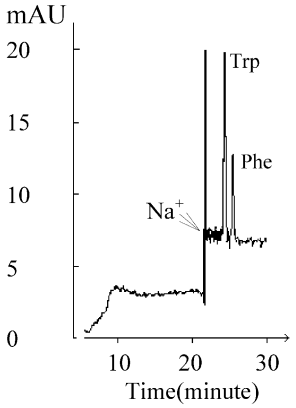

Figure 6. Electropherogram of CZE with the tMCRBM stacking of the diluted analytes in the alkaline salt matrix (no. 6 in Table 1). The step indicated by Na^+ is sodium ion. Conditions: 660-s 13-mbar injection, alkaline salt matrix holding 0.15 μg/mL Trp + 0.60 μg/mL Phe + 80 mM NaCl + pH 8.34 82 mM sodium formate; before sample injection, 103 mM sodium formate in the anodic vial is injected for 30 s by 13-mbar pressure; running buffer pH 2.85 32.8 mM formic buffer; total length 75 cm, effective length 65 cm, and i.d. 75 μm capillary, 24 kV, 84 μA; UV detector (214 nm); and 26 °C.

current of 84 μA is mainly caused by the large-volume matrix plug (~28 cm) with high salt (viz., high ionic strength and high conductivity) that greatly decreases the resistance of the capillary. The delay of Trp and Phe is principally because (1) the effective length of capillary is about doubled and (2) about half of the electrophoretic time is spent on the stacking of analytes in the 28-cm salt matrix plug, which needs to be progressively neutralized by the hydrogen ion in pH 2.85 32.8 mM formic buffer.

Evidently, the performance of the tMCRBM-CZE mode is analogous to a normal CZE. In addition, the sample matrix can be easily prepared as shown in Table 1. Thus, the technique of tMCRBM-CZE is very simple. Even so, the technique is of high efficiency for enhancing theoretical plate number and resolution of CZE, a powerful function for stacking ionizable analytes in large-volume high-salt matrix plug, and good stability for performing the tMCRBM stacking mode, whether the matrix holds low (20 mM NaCl in Figure 4a), medium (40−80 mM in Figures 3 and 4b−d), or high salt (100 mM in Figure 5).

What is the mechanism of the tMCRBM mode that results in the highly efficient stacking of ionizable analytes in the salt matrix? To elucidate the mechanism, it is necessary to review the principle of MCRB briefly. Figure 7a shows the foundation model of MCRB created by the left two phases, viz., phases α and β which correspond to the weak acid of the running buffer and the weak base of sodium formate, respectively (*Note: the right two phases, viz., phases β and γ, cannot create a MCRB due to their converse arrangement of the electrolytes in contrast to the arrangement of phases α and β.*). Thus, under the action of an electric field, the hydrogen ion in phase α and the hydroxyl ion in phase β migrate in opposite directions and react as water when they meet. This kind of electromigration chemical reaction has been proved by the numerous experiments[54−56,61−71] and the velocity of its boundary can be designed with the equations of MCRB,[57−60] including the corrected equations of MCRB for special cases.[67,68] Evidently, the necessary conditions to form a MCRB are (1) the presence of positive and negative reaction ions, e.g., the hydrogen and hydroxyl ions in Figure 7a, which exist in two phases and may react with each other if they meet; (2) the electric field that is used to force the motion of positive and negative reaction ions *in opposite direction* as shown in phases α and β of Figure 7a.

Now, with the explicit model of MCRB, we can discuss the movements of Trp and Phe in the transient MCRB system under the action of an electric field. Evidently, Parts a and b of Figure 7 can in detail represent Figure 1c and d, respectively. As has been discussed above, there is a transient MCRB between phases α and β, which moves toward the cathode as implied in Figure 7b. The pH values of phases α and β are 2.85 and 8.34, respectively, and the p*I*s of Trp and Phe are respectively 5.89 and 5.48. Thus, the partial analytes of Trp and Phe are negatively charged in phase β or positively charged in phase α (see Figure 7b). So if the pH 8.34 matrix plug in phase β is displaced by the pH 2.85 running buffer in phase α, the analytes of Trp and Phe in the original matrix plug fall into phase α, where the two analytes are positively charged immediately due to the low value of pH 2.85 and move toward the transient MCRB. It was selected that the velocities of positive-charge Trp and Phe in phase α are faster than the velocity of the transient MCRB moving toward the cathode. Thus, the positive-charge analytes can catch up with the motion of the transient MCRB and even migrate with the same velocity of the MCRB after their equilibrium of dissociation near the anodic side of the transient boundary. Clearly, as the transient MCRB is in progress, the boundary can stack the analytes in the original high-salt matrix all together and move coupled with the stacked positive charged analytes toward the cathode as shown in Figures 1d and 7b. On the another hand, the analytes in phase β all move toward the anode due to their partial negative charges. The effect can effectively suppress the diffusions of analytes into phase γ through the joining of phases β and γ and further enhance the stacking action to the analytes in phase β. Owing to the sharp MCRB as has been directly observed in the early experiments,[56,61−68] the stacked zones of analytes are very narrow as shown in Figures 3−6. As the concentrations of the stacked analytes increase, a progressive concentration gradation occurs between the stacked analytes and their circumstances. Thus, the stacked analytes may diffuse into phases α and β. If the stacked analytes diffuse into phase α completely, the analytes may carry more positive charges, which result in the acceleration of the analytes toward the cathode, and then the analytes in phase α catch up with the transient MCRB again. When the stacked analytes diffuse toward phase β, they may lose their positive charge or may be partially neutral moleculars or negative-charge ions. Clearly, the analytes appearing as neutral molecules or as negative-charge ions will be stacked again by the transient MCRB moving toward the cathode as shown in Figure 7b. After the end of transient MCRB, all of the analytes Trp and Phe are stacked together. Next, the stacked analytes electrically move as a manner of CZE further are separated in accordance with their mobilities (see Figure 1e).

From the discussions above, it is clear that the stacking of analytes by the tMCRBM mode is similar to the focusing of zwitterions in the classic IEF.[72−74] Of course, there is a difference between the staking of zwitterionic analytes and IEF of zwitterions, since, in the former case, the two analytes are focused as positive-charged ions and move toward the cathode with the transient boundary, while in the latter case, zwitterions are focused at their p*I*s, which indicate no motions.[72−74] Thus, the mechanism of

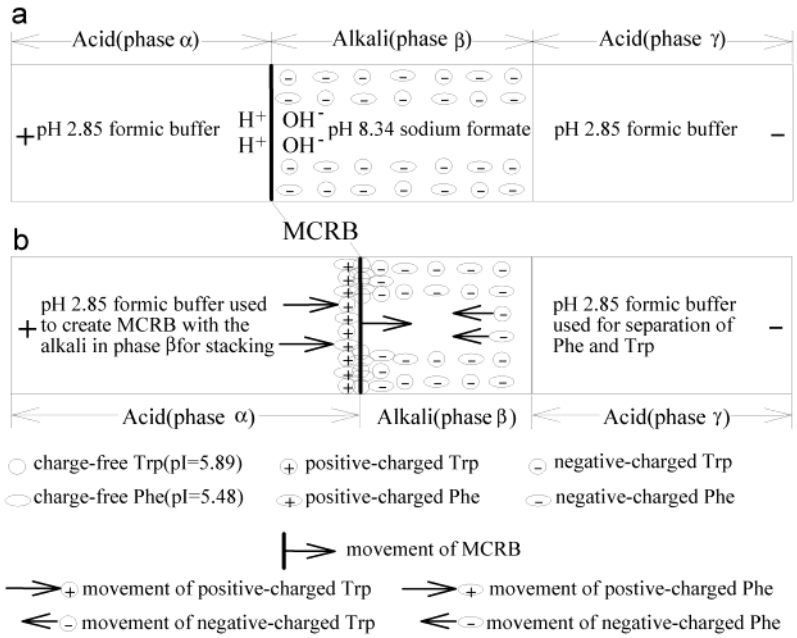

Figure 7. Mechanism of stacking zwitterionic analytes by tMCRBM (at pH 2.85, the EOF is very low, thus is omitted): (a) the initial arrangement of electrolytes and sample matrix; (b) the motions of the analytes and transient MCRB which stacks the analytes under electric field. The symbols + and − indicate the anode and cathode, respectively.

stacking the analytes Trp and Phe by tMCRBM is a non-steady-state IEF, as distinguished from the classic steady-state IEF.[72−74] This conclusion does not imply a conflict between the classic IEF and MCRB, since the theoretical and experimental results have undoubtedly demonstrated that the classic IEF[72−74] relies upon the stationary neutralization reaction boundary—the specific case of MCRB.[63,79,70]

The existence of salt mainly affects the conductivity of phase β but not the dissociation of two analytes Trp and Phe and the pH value of buffer in phase β. So the presence of salt does not affect the stacking of analytes by the tMCRBM obviously; this has been proved by the results in Figures 3−6.

The technique of tMCRBM for the stacking of ionizable analytes is clearly different from transient ITP,[1−3,43−49] electrostacking,[4−7] FASI,[8−12] and the "acetonitrile−salt" method.[7−21] ITP is in nature the same as the moving boundary method (MBM) originally used to determined ionic transference number,[43,51,78−81] and electrostacking and FASI can be considered to be caused by the concentration boundary that results in the enhancement of electric field in the phase with low or zero concentration electrolyte (water used in FASI can be roughly regarded as a zero concentration electrolyte),[4−12,52,82] and the "acetonitrile−salt" stacking is also an indirect ITP or FASI mechanism as pointed out by Shihabi et al.[17−21]

The technique is also dissimilar to the dynamic pH junction method developed by Chen's group[29−32] and the "sweeping procedure for neutral or charged analytes in MEKC".[33−42] In the dynamic pH junction method, the pH values of two phases on the two sides of the junction are both over or less than pH 7 simultanously, so there are no necessary conditions for the formation of a MCRB given in Figure 7a. Additionally, the stacking is replied on the formation of complex between the analytes and background ion and on the velocity changes of analytes through the pH junction. The stacking of neutral (or charged) analytes in MEKC[33−42] is directly caused by the moving interaction reaction between the neutral (or charged) analytes and the charged (or neutral) micellar molecules in the pseudostationary phase.

Of course, there is indeed a transient MCRB in the pH-mediated stacking by Lunte's group,[22−28] because all of the necessary conditions (see Figure 7a) for creating a transient MCRB are present in the pH-mediated system. Whereas, the transient MCRB is not directly used to stack analytes in the matrix but is used to cause a neutralization reaction between the weak acid (or base) and its conjugate base (or acid) and further to creat a low-conductivity zone in the original matrix, which leads to a FASI stacking of analytes.

CONCLUSIONS

From the results and discussion above, it is clear that the tMCRBM is a powerful tool for stacking of ionizable analytes in salt matrixes. First, the mode can be significantly used to improve the separation efficiency and resolution of CZE for the ionizable analytes in a high-salt matrix (see Figures 2−5). Second, the analytes in the large-volume sample matrixes with high salt can be sharply stacked and well separated by the method as shown in Figure 6. Third, the stability of the method is high as shown in Figure 5.

The tMCRBM is a novel method used for stacking ionizable analytes in a salt matrix; its mechanism relies upon non-steady-state IEF. The mode is evidently different from ITP, electrostacking, and FASI and also dissimilar to the dynamic pH junction[29−32] and sweeping neutral (or charged) analytes in MEKC.[33−42] There

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is a transient MCRB in the pH mediated-stacking,[22−28] but the transient MCRB is used to form a low-conductivity zone for a FASI stacking of analytes in the original matrix, but not directly used to stack analytes in matrix.

On-line tMCRBM possesses obvious potential significance for stacking ionizable analytes in a salt matrix, since many samples, such as serum, seawater, and urine as well as elution from chromatography, contain high salt. For example, there exists up to 146 mM sodium ion in seawater and serum. This paper shows that a matrix with 165.6 mM sodium ion can be directly stacked to obtain good separation efficiency and to notably enhance detection sensitivity in CE. Of course, it is necessary to do some further studies on the theory and procedure of tMCRBM stacking for such analytes as peptide, protein, and weakly basic or acidic drugs in salt matrixes, as well as its factual applications to some salt samples such as drugs in serum or urine and pollutants in seawater.

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