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Improving separation efficiency of capillary zone electrophoresis of tryptophan and phenylalanine with the transient moving chemical reaction boundary method

Cheng-Xi Cao[a,b,*], You-Zhao He[b], Min Li[c], Yi-Tai Qian[b], Li Yang[b], Qi-Shu Qu[b], Shu-Lin Zhou[d], Wen-Kui Chen[d]

[a]*College of Life Science and Biotechnology, Shanghai Jiaotong University, 200030 Shanghai, China*
[b]*Department of Chemistry, University of Science Technology of China, 230026 Hefei, China*
[c]*Department of Chemsitry, Chuzhou Normal College, 239012 Chuzhou, China*
[d]*Institute of Allergy Reaction, Wannan Medical College, 241001 Wuhu, China*

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Abstract

A simple and convenient mode—moving chemical reaction boundary method–capillary zone electrophoresis (MCRBM–CZE)—was designed for the enhancement of separating efficiency of CZE. In this mode, the transient MCRBM is used for the on-line pre-treatment of sample. By analyses of tryptophan(Trp) and phenylalanine(Phe) as an example, the experiments by MCRBM–CZE were carried out and further compared with those by normal CZE without the transient MCRBM. The results reveal that by carefully selected appropriate electrolytes, a strong condensation effect can be achieved by using MCRBM–CZE; this effect can greatly improve the separation efficiency, resolution and peak height of Trp and Phe in CZE as compared with those of normal CZE of Trp and Phe. Even if the sample comprises high concentrations of salt, such as 80 m*M* NaCl(concentration of sodium ion up to 145.6 m*M*), the same condensation effect can also been observed; this implies obvious significance for biological samples like urine and serum. However, if the electrolytes was chosen inappropriately only a poor compression effect of sample was observed in the MCRBM–CZE runs.

Keywords: Moving chemical reaction boundary; Sample handling; Efficiency; Moving boundary system; Amino acids; Tryptophan; Phenylalanine

1. Introduction

Numerous methods have been used for the enhancement of separation efficiency and sample concentration in electrophoresis. In 1964, Ornstein [1] and Davis [2] introduced separately the mechanism of a discontinuous buffer system (DBS), viz., isotachophoresis (ITP), into polyacrylamide gel electrophoresis which was widely used in biochemistry and biomedicine later. In 1973, Jovin [3–5] advanced the theory of DBS for multi-zone electrophoresis from the theory of the moving boundary system (MBS)

*Corresponding author. College of Life Science and Biotechnology, Shanghai Jiaotong University, 1954# Huashan Road, 200030 Shanghai, China. Tel.: +86-021-5474-3341; fax: +86-021-6293-2772.

E-mail address: cxcao@mail1.sjtu.edu.cn (C.-X. Cao).

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developed during the 1920s–1950s as will be discussed below.

Owing to the great development of capillary electrophoresis (CE), the ITP mechanism of sample has been used for the enhancement of separation efficiency and pre-concentration in CE. In 1992, Schwer and Lottspeich [6] studied the improvement of separation efficiency with the DBS systemically. In their studies, the ITP mechanism was also used as shown in Fig. 1a–b of Ref. [6]. Whereas, Fig. 1c of Ref. [6] clearly exhibited a novel method of sample stacking—''sandwich mechanism'', because the sample was sandwiched between 0.1 *M* phosphoric acid and 0.1 *M* sodium hydroxide. Later, the ITP mechanism was further used for sample stacking in CE [7–10]. At the same time, the methods of electric stacking [11–13] and field amplification [14–17] were developed for the online pre-concentration of the sample in CE.

Besides those procedures of sample stacking mentioned above, some on-line stacking techniques, which are to some extent similar to the mechanism of the transient moving chemical reaction boundary method (MCRBM) as will be introduced below, have been developed for on-line pre-concentration of samples. In 1998, Xiong et al. [18] invented pH-mediated on-line sample stacking of DNA matrix, which was reduced by low conductivity due to gradual chemical reaction. At the same time, Shihabi and Friedberg [19] developed a similar technique for the insulin on-line stacking in capillary zone electrophoresis (CZE), even a sample matrix with a high concentration of salt. In 2000, Chen and co-workers [20,21] further investigated this kind of sample stacking by chemical reaction between the sample buffer and running buffer and used this method for the focusing of nucleotides and the selective stacking of catecholamine and weakly acidic compounds in CE. During 1998–2000, Quirino et al. [22–24] and Landers and co-workers [25,26] evolved the very powerful sweeping technique of analytes for stacking neutral analytes in micellar electrokinetic capillary chromatography (MEKC). In the sweeping procedure, a moving interaction boundary occurs between the neutral analytes and micelle molecules, the moving interaction boundary sweeps the analytes together. The sweeping method is so powerful that even over 5000-fold concentration can be achieved in MEKC [22] and those samples with high salt concentrations can be well concentrated [23–26].

MBS is an important boundary which had been widely investigated during the 1920s–1950s [27–34], and the corresponding experimental method—moving boundary method (MBM) [27,28,32]—had been used in displacement electrophoresis or ITP in the electrophoretic field [35]. If a MBS is formed with weak electrolytes, a moving reaction boundary may occur due to the (de-)protonation of weak electrolytes at the two sides of moving boundary. This kind of chemical reaction of (de-)protonation was recognized by Alberty in 1948 [31]. In the 1980s, Bocek and co-workers [36–39] greatly developed the theory of moving reaction boundary in a MBS formed with weak electrolytes. The moving reaction boundary in a MBS holds some common characteristics with the moving chemical reaction boundary (MCRB) [40–44], which need some further investigation. Off course, the moving reaction boundary, which occurs in a MBS formed with weak electrolytes, is different from MCRB. This has been shown by the comparisons between the equations for MBS and MCRB in Ref. [42].

In contrast to MBS, MCRB is a new boundary system [42]. The theory on MCRB had been developed by Cao and co-workers [40–43] from the pioneer idea of ''precipitate reactive front'' evolved by Deman and Rigole [44,45] and that of ''stationary neutralization reactive boundary advanced by Bocek and co-worker [46]. The theory of MCRB has been partially proved with some experiments performed by Deman and Rigole [44,45], by Pospichal et al. [46], and by Cao and co-workers [47–51]. Interestingly, if a MCRB is created with weak acid and alkali together with an excess of background electrolyte KCl, the boundary is very ambiguous [52,53]. Since under the excess existence of KCl as background electrolyte, the original MCRBE for a weak electrolyte system cannot predict the movement of a MNRB and hence should be individually corrected under different experimental conditions [52,53]. In those studies [47–53], the moving neutralization reaction boundary method (MNRBM) was developed for the MNRB research itself and also for CZE as shown in this paper.

In this paper, the MNRBM was combined with CZE to improve the separation of amino acids. As

will be shown, the separating resolution, peak height and theoretical plate number of CZE are intensely improved by the MCRBM. Here, we describe MCRBM–CZE and report the relative experimental results of amino acid analyses by this technique.

2. Experimental

The reagents used are sodium hydroxide, 37% hydrochloric acid, sodium chloride, 88.9% formic acid (HAc). The former two are guaranteed reagent (GR) grade and the latter two analytical-reagent grade. The former three were purchased from Shanghai Chemical Reagents (Shanghai, China), the latter one was from the Shenyang Xinhua Chemical Reagents Factory (Shenyang, China). L-Tryptophan (Trp) (from Lizhu Dongfeng Biotechnological Co., Shanghai, China) and L-phenylalanine (Phe) (from the Kangda Amino Acid Factory, Shanghai, China) are biochemical reagent, both of them are Chrom pure grade. The isoelectric points (p*I*) of Phe and Trp are respectively, 5.48 and 5.89—very near to each other, their molecular masses are 204.23 and 165.19, respectively, and they hold the same structure of benzene ring. Owing to those reasons, the two kinds of amino acids are difficult to be separated well especially at low pH values.

A capillary electrophoretic apparatus (Bingda-1229, Beijing New Technology Institute, Beijing, China) is used for the CZE of Trp and Phe. The apparatus is equipped with an electric power supply (up to constant voltage 30 kV), an ultraviolet-absorbance detector set at 214 nm and an automatic recorder. A quartz capillary (from the Factory of Rongnian Light-fiber, Hebei, China), is 60 cm (length to the detector 40 cm)×75 μm I.D., is used. Prior to the first run, the capillary is rinsed with 0.1 mol/l NaOH for 40 min, next with distilled water rinse for 10 min, followed by 0.1 mol/l HCl washing

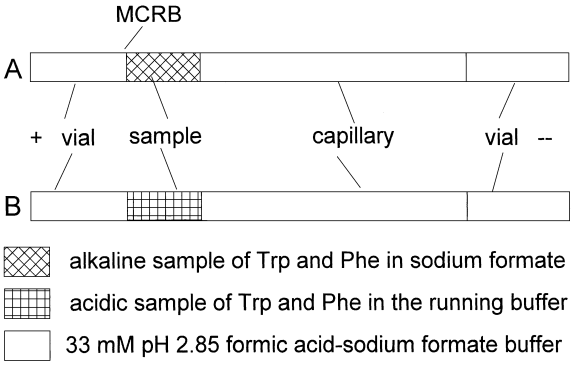

Fig. 1. The procedure diagrams of the Trp and Phe analyses of CZE. (**A**) the procedure of MCRBM–CZE of Trp and Phe; (**B**) normal CZE of Trp and Phe without the on-line pre-treatment of MCRBM. The symbols of + and − indicate the anode and cathode, respectively. For other symbols see the text.

for 40 min, and last by the 120 min equilibration with pH 2.85 32.8 m*M* formic acid buffer. Amongst different runs, the capillary is washed with the buffer for 2 min.

The running buffer is composed with 32.8 m*M* pH 2.85 formic acid-sodium formate. During a run, the anodic and cathodic cells and the capillary are filled with the running buffer.

Two kinds of samples are prepared. The first is an acidic sample used for normal CZE without the pre-treatment of MCRBM, the sample contains 15 μg/ml Trp and 40 μg/ml Phe in 32.8 m*M* pH 2.85 formic buffer. The second kind is an alkaline sample used for the experiments of MCRBM–CZE of Trp and Phe. As shown in Table 1, there are three alkaline samples, each containing 15 μg/ml Trp and 40 μg/ml Phe in 32.8/or 65.6 m*M* sodium formate; furthermore the former two contain no NaCl, but the latter contains NaCl in concentrations up to 80 m*M* (145.6 m*M* Na^+).

The MCRBM–CZE is performed as follows. Firstly, the anode end of the capillary is, as shown in Fig. 1A, inserted into one of the alkaline samples

Table 1
The contents of three alkaline samples used for the runs of MCRBM–CZE of Trp and Phe

Alkaline samples	Trp (μg/ml)	Phe (μg/ml)	Sodium formate (m*M*)	NaCl (m*M*)	Na^+ (m*M*)
1	15	40	32.8	0	32.8
2	15	40	65.6	0	65.6
3	15	40	65.6	80	145.6

(see Table 1). Then, the 13.5 cm gravity is used for the sample injection. After the injection, the run of MCRBM–CZE is performed as normal CZE as shown in Fig. 1B in which the same gravity injection is also used.

Just after 20 kV voltage is turned on, a transient MCRB is created between the running buffer in the anodic cell and the alkaline sample in the capillary. Since the anodic cell is filled with acidic buffer while the sample zone holds the weak alkali of sodium formate (pH 8.34), under the action of electric field, an electromigration chemical reaction occurs between the hydrogen ion in the buffer of anodic cell and the hydroxyl ion in the alkaline sample. After the reaction between the hydrogen and hydroxyl ions, the alkali of sodium formate in the sample zone is neutralized by the acidic buffer. The velocity of the MCRB is carefully selected with the theory of MCRB [42,52]. The MCRB is designed to move towards the cathode. This kind of design leads to the condensation of the sample zone of Trp and Phe in the capillary during the electromigration chemical reaction between hydrogen and hydroxyl ions. After the completion of the chemical reaction, the condensed sample zone of Trp and Phe electrically migrates as the manner of CZE.

Because the zone of alkaline sample is thinned by the MCRBM, the theoretical plate number and resolution are improved (see Table 2 and Figs. 3 and 4). The computation of theoretical plate number is performed with the equation [54]:

$$N = 2\pi\left(\frac{t_m h}{A}\right)^2 \quad (1)$$

where N is the theoretical plate number, A the peak area, t_m the electromigration time and h the height of peak. And the resolution of adjoining peaks is calculated with the equation [55]:

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

where R_s is the resolution of adjoining peaks, viz., peaks 1 and 2, t_{m2} and t_{m1} are respectively the electromigration times of peaks 1 and 2 (in second), w_1 and w_2 are respectively the baseline widths of peaks 1 and 2 expressed as time (in s).

3. Results

Both normal CZE and MCRBM–CZE separations of Trp and Phe were carried out. The relative results are given in Figs. 2–5 and Table 2.

Fig. 2 shows the results of the normal CZE of Trp and Phe, the times of 13.5 cm gravity injection are 20, 40, 60 and 90 s for Fig. 2A, B, C and D, respectively. From the results in Fig. 2, it is obvious that peak 1(Trp) and peak 2(Phe) become wider and wider, and even emerge together as the injection time is prolonged to 60 and 90 s. The separation efficiency is extremely poor and resolution becomes very bad when 90 s injection time is used, as exhibited in Table 2

However, the wide sample zone can be successfully condensed by using the on-line transient MCRBM selected with the relative theory carefully. Fig. 3 displays the experimental results of MCRBM–CZE of Trp and Phe. The sample injection times from Fig. 3A–D are the same as those in Fig. 2. It is shown in Fig. 3 that peaks 1(Trp) and 2(Phe) are sharp, high and well separated; even with 90 s injection time (see Fig. 3D), peaks 1 and 2 are still separated clearly. The condensing effect is so strong that peak

Table 2
The comparisons of theoretical plate numbers (N), peak height (PH) and resolution (R) among normal CZE and MCRBM–CZE of Trp and Phe[a]

Fig. 2D			Fig. 3D		
N (peak 1)[b]	PH (peak 1)	R[c]	N (peak 1)[b]	PH (peak 1)	R_s[c]
$7.95\cdot10^2$	28	0.20	$2.65\cdot10^4$	115	0.98

[a] The comparisons was performed with experiments of 90 s injection of samples, viz., Figs. 2D and 3D.
[b] The theoretical plate number N is computed with Eq. (1) given by Terabe et al. [54].
[c] The resolution R_s is calculated according to Eq. (2) defined by Snyder and Kirkland [55].

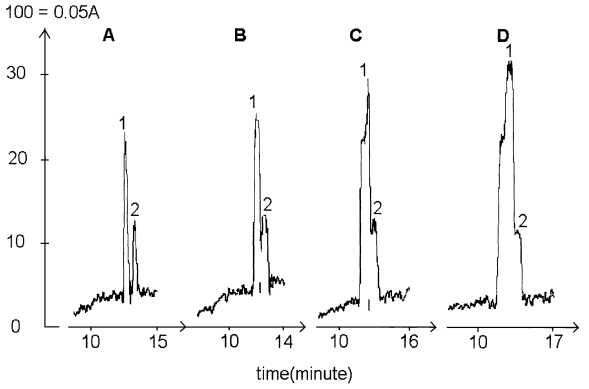

Fig. 2. Normal CZE electrophograms of Trp (peak 1) and Phe (peak 2) without the on-line pre-treatment of MCRBM. 13.5 cm gravity injection time: (**A**) 20 s, (**B**) 40 s, (**C**) 60 s, (**D**) 90 s. Conditions: running buffer = 32.8 m*M* pH 2.85 formic acid–sodium formate, sample = 15 μl/ml Trp + 40 μl/ml Phe in the running buffer, 60 cm (efficient length 40 cm) × 75 μm I.D. capillary, run voltage 20 kV, current 16 μA, UV detector (wavelength 214 nm), temperature 26 °C.

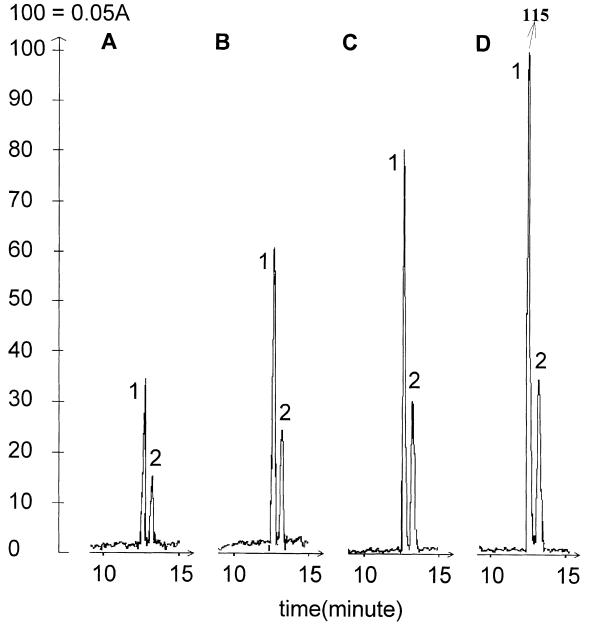

Fig. 3. MCRBM–CZE electrophograms of Trp (peak 1) and Phe (peak 2) alkaline sample in 65.6 m*M* sodium formate solution. 13.5 cm gravity injection time: (**A**) 20 s, (**B**) 40 s, (**C**) 60 s, (**D**) 90 s. Conditions: alkaline sample 2 (see Table 1), run voltage 20 kV, current 23–19 μA. Other conditions as in Fig. 2.

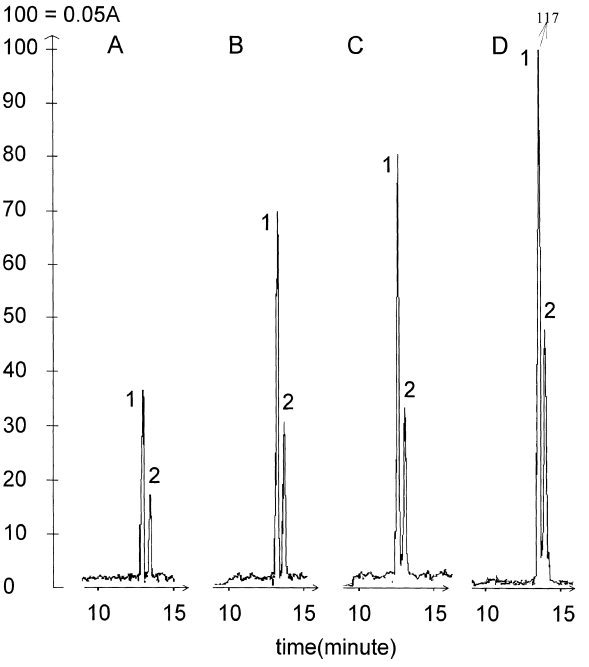

Fig. 4. MCRBM–CZE electrophograms of Trp (peak 1) and Phe (peak 2) alkaline sample with 80 m*M* NaCl and 65.6 m*M* sodium formate. 13.5 cm gravity injection time: (**A**) 20 s, (**B**) 40 s, (**C**) 60 s, (**D**) 90 s. Conditions: alkaline sample 3 (see Table 1), run voltage 20 kV, current 29–20 μA. Other conditions as in Fig. 2.

1 runs out off the range of 0.05 A (= 100 relative absorbance in Figs. 2–4) and reaches up to 115 relative absorbance while 90 s sample injection time is used as shown in Fig. 3D.

The comparisons of separation efficiency and resolution are quantitatively performed between Fig. 2D and 3D. Table 2 collects the results of the comparisons, as well as the enhancement of peak height. As shown in Table 2, the theoretical plate number N is very poor ($=7.95\cdot10^2$, computed with peak 1 in Fig. 2D) in the experiment in accordance to normal CZE of Fig. 1B, the resolution R_s between peaks 1 and 2 is very bad ($\approx$0.20), and the peak height (PH) is low (= 28 relative absorbance, peak 1 in Fig. 2D). Moreover, in the experiments with MCRBM–CZE in Fig. 1A, N is up to $2.65\cdot10^4$ (computed with peak 1), R_s is 0.98, and PH equals 0.98. The ratio between the theoretical plate numbers of Fig. 3D and 2D is 33 and that between resolutions of Fig. 3D and 2D is about 5, and peak height is

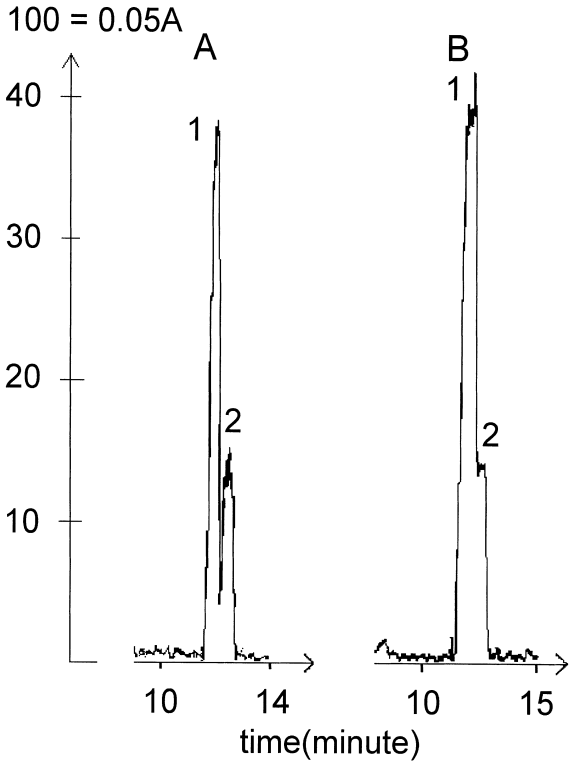

Fig. 5. MCRBM–CZE electrophograms of Trp (peak 1) and Phe (peak 2) alkaline sample in 32.8 m*M* sodium formate weak alkali. 13.5 cm gravity injection time: (**A**) 60 s, (**B**) 90 s. Conditions: alkaline sample 1 (see Table 1), 20 kV, current 18–16 μA. Other conditions as in Fig. 2.

increased four times. Those results manifest that a high condensation effect, excellent separation efficiency and good resolution can be achieved by using the method of MCRBM–CZE, even with long time of gravity sample injection.

More importantly, the alkaline sample containing NaCl in high concentrations up to 80 m*M* (see Table 1) can be well compressed as shown in Fig. 4. As compared with Fig. 3, the separation efficiency and peak height are further increased. Apparently, not only do the addition of NaCl un-interfere the analyses of Trp and Phe, but increases the separation efficiency and compression achieved by the transient MCRBM. Off course, the resolution is slightly lower as shown in Fig. 4D. The concentration of sodium ion in the sample is 145.6 m*M*, very near to that of 0.9% NaCl solution, or serum, or briny water. This gestates great significances. Many samples like biological ones containing high concentrations of salt, such as serum, urine and briny water, etc. can be directly analyzed without desalting by using the on-line pre-treatment of transient MCRBM.

The experimental conditions must be carefully chosen. It was predicted with the theory of MCRB that the condensing effect is badly decreased if the alkaline sample containing 32.8 m*M* sodium formate (see Table 1) is used to form a MCRB. The comparative investigations prove this conclusion. As compared with Fig. 2C and D, the separation efficiency and peak height in Fig. 5A and B, coupled with resolution, are slightly improved. However, the separation and resolution are extraordinary poor and the peak heights are very low, in contrast to those in Fig. 3C–D and Fig. 4C–D.

4. Discussions and conclusions

From the above results, it is evident that the mechanism of MCRB can greatly enhance the separation efficiency, peak height and resolution of Trp and Phe CZE, by using appropriate electrolytic arrangement. Even for the sample containing a high concentration of salt, 80 m*M* NaCl (Na^+ up to 145.6 m*M*, see Table 1), good resolution, much high-peaks and excellent theoretical plate number can easily achieved with the mode of MCRBM–CZE of amino acids.

The mode of MCRBM–CZE described is clearly different from the mechanism of ITP [6–10], electric stacking [11–13] and field-amplification injection [14–17]. ITP is based on the mechanism of MBS [27–35] and electric stacking [11–13], including field-amplification injection [14–17], relies on that of concentration boundary [35,56].

The mode reported here is also to some extent similar to, but different from the ''sandwich mechanism'' developed by Schwer and Lottspeich [6]. In Schwer and Lottspeich's procedure, the electrolytes are, from the anode through the capillary to the cathode, 20 m*M* pH 2.5 sodium phosphate buffer, 0.1 *M* phosphoric acid (650 nl), sample (500 nl), 0.1 *M* NaOH (300 nl) and 20 m*M* pH 2.5 sodium phosphate buffer. The sample is sandwiched between 0.1 *M* phosphoric acid and 0.1 *M* sodium hydroxide, a neutralization reaction boundary is, *indirectly,*

created within the sample between the acid of 0.1 *M* phosphoric acid and alkali of 0.1 *M* NaOH under the action of electric field. The sample AB is condensed from the two sides of the acid and alkali. But in this letter, a transient MCRB is directly created between 32.8 m*M* pH 2.85 formic acid–sodium formate buffer and sodium formate in the sample. The sample is mainly compressed by the side of MCRB between the running buffer and sample as indicated in Fig. 1A.

The mode is also analogous to, but different from the pH-mediated on-line sample stacking of analytes by Xiong et al. [18], by Shihabi and Friedberg [19] and by Chen and co-workers [20,21], which was reduced by low conductivity due to gradual chemical reaction, and to the very powerful sweeping technique of analytes for stacking neutral analytes in MEKC by Quirino et al. [22–24] and Landers and co-workers [25,26].

The mode of MCRBM–CZE is simple and convenient. As shown in Fig. 1A and B, the operation of MCRBM–CZE is the same as that of normal CZE. Only two solutions, viz., the running buffer and sample, are used, this is the same as that of normal CZE as implied in Fig. 1B.

The theory of MCRB, together with its MCRBM, is very useful. The theory of MCRB has been used for the studies on the mechanism of isoelectric focusing (IEF) [57,58]. It was revealed by Pospichal and co-workers [46,59] and Cao [60–62] that IEF is replied on the mechanism of neutralization reaction boundary. Recently, MCRBM has been used for the preparation of colloid in gel by Cao et al. [63]. In this letter, the transient MCRBM is successfully used for the on-line pre-treatment of sample of animo acids, and further for enhancement of separation efficiency and resolution of CZE of Trp and Phe. More importantly, the transient MCRB reported here can condense the sample containing sodium ion in high concentrations (up to 145.6 m*M*). This has great significance for many samples containing salts like serum, urine and briny water, etc.

Acknowledgements

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