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Investigations on factors that influence the moving neutralization reaction boundary method for capillary electrophoresis and isoelectric focusing

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Abstract

We investigated several factors, such as temperature, current intensity (i), time (t) and the product (mA min mm^{-2}, viz., C mm^{-2}) of i and t, etc., that obviously affect the moving neutralization reaction boundary method (MNRBM). The results manifest that the temperature and the product ti have a strong influence on the movement rate of the boundary. The data prove that about 0.6 C mm^{-2} (being equivalent to 10 mA min mm^{-2}) is a critical point. If the product ti is lower than the critical point, a good quantitative agreement exists between the observed and theoretical values, but if it is higher than the critical point, the agreements are poor. The optimized experimental conditions are: (1) 18–20 °C room temperature, (2) 0.6–0.8 mA mm^{-2}, (3) less than 10 mA min mm^{-2}, (4) 1% agarose gel, (5) daily prepared solution and gel containing NaOH. The optimized MNRBM is of benefit for the studies on MNRB itself, isoelectric focusing and capillary zone electrophoresis as will be partially shown in this paper.

Keywords: Moving chemical reaction boundary; Moving boundary system; Isoelectric focusing; Current intensity; Temperature effects

1. Introduction

In 1970, Deman and Rigole [1,2] advanced the important idea of the ''precipitate reaction front''—the prototype of the concept of the moving chemical reaction boundary (MCRB), and performed the experiment of ''precipitate reaction front'' created with cobaltic and hydroxyl ions. In 1993, Pospichal et al. [3] evolved the concept of stationary neutralization reaction boundary (SNRB) and used the concept for the study of isoelectric focusing (IEF) in capillary electrophoretic apparatus [4,5]. In 1997–1999, we [6–8] developed the concept of MCRB, formulated the MCRB equations, coupled with judgment expressions for IEF and MCRB.

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Moreover, in the series of papers entitled ''Moving Chemical Reaction Boundary and Isoelectric Focusing: . . . '', Cao [9–11] unveiled the relationships between the MCRB and IEF greatly contributed to by Svensson [12,13] and Vesterberg [14]. The studies directly demonstrate that Svensson's IEF relies on the concept of MCRB (exactly SNRB). Hence, the research on MCRB, especially on neutralization reaction boundary, possesses obvious significance to Svensson's IEF.

The theory of MCRB has been partially proved by some experiments. The experiments by Deman and Rigole [1,2] excellently proved the concept of MCRB. The experiments by Pospichal et al. [3] clearly demonstrated the idea of SNRB. And those by us [15,16] quantitatively manifested the validity of the MCRB theory for the MCRB formed with cobaltic and hydroxyl ions. Recent experiments by the authors showed both the validity of the MCRB theory for a moving neutralization reaction boundary (MNRB) created with the strong acid and alkali [17,18].

However, the new experimental results revealed the non-validity of the original MCRB equations for the MNRB created with weak acid and alkali [19] or with strong acid and weak alkali [20] with KCl in high concentration. The corrected MCRB equations were directly advanced for the given experimental conditions and were further proved by the experiments quantitatively [19,20].

In those studies [17–20], we observed numerous influence factors and evident physical chemical dependencies, e.g., boundary migration rate changes as the function of C mm^{-2} [current intensity (i)× time (t), see Figs. 2 and 3 and Table 1]. Those dependencies, as well as other impact factors, have not been reported yet in our previous studies, and optimized conditions have not been filtered out from various experiments. Thus, in this paper the authors investigated the numerous influent factors, especially displayed the dependencies of boundary migration rate on the product of *it*, viz., C mm^{-2}, that could not be well understood with the present theory, and gave the optimized experimental conditions.

The studies of this paper possess obvious significances for MNRB itself, capillary zone electrophoresis (CZE) and IEF, those will be further discussed in detail in the Discussion section.

Table 1
The relations among the i, time (t) and product (it), viz., C mm^{-2} [a]

	Current intensity, i (mA mm^{-2})					
	0.4	0.6	0.8	1.0	1.2	1.4
Time (min)	25	15	12.5	10	7.5	7.5
Critical product (min mA mm^{-2})	10	9	10	10	9	10.5

[a] The experimental conditions are given in Fig. 3.

2. Experimental

2.1. Reagents

The reagents used here are HCl, NaOH, KCl and bromphenol blue, the former three are analytical-reagent grade purchased from Tianjin Chemical (Tianjin, China), Shanghai Chemical Reagents (Shanghai, China), and Nanjing Chemical Factory (Nanjing, China), respectively, and the latter is chemical-reagent grade purchased from Shanghai Chemical Reagents. The agarose is biochemical-reagent grade (Shanghai Huang-Hua Pharmaceutical Factory, Shanghai, China). The agarose gel is better than agar gel, since in agarose gel there is nearly complete absence of electroosmostic flow (EOF) [21].

2.2. Apparatus and procedure

The apparatus for the MNRB experiment was described in Refs. [15] and [16], and the MNRBM was given in Refs. [17] and [18].

In the experiments reported here, the anolyte holds 0.004–0.014 mol l^{-1} HCl and 0.1 mol l^{-1} KCl, the agarose gel in the tube comprises 0.004–0.014 mol l^{-1} NaOH, 0.1 mol l^{-1} KCl and 0.1% (w/v) bromphenol blue, and the catholyte holds 0.004–0.014 mol l^{-1} NaOH and 0.1 mol l^{-1} KCl. As has been shown in Refs. [17] and [18], the bromphenol in the gel (or tube) is used as the mark distinguishing the yellow acidic zone, the blue alkali zone and the boundary indicated by the arrows in Fig. 1. The

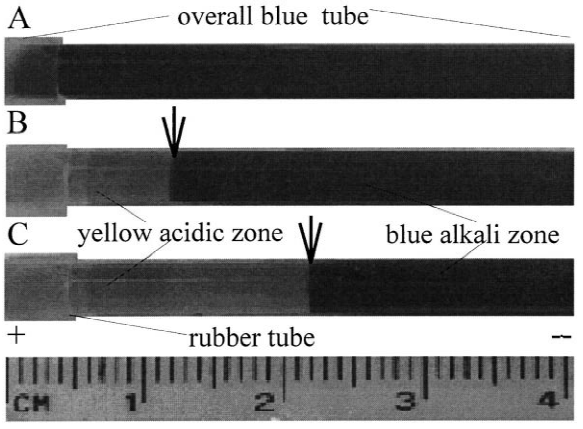

Fig. 1. The moving neutralization reaction boundary created with HCl and NaOH in 1% agarose gel. (A) Just the start of the run; (B) 5 min after the run; (C) 10 min after the run. The arrows indicate the boundary created with the acid and base; the symbols + and − mean the anode and cathode, respectively; the yellow acidic zone ranges from the symbol + to the arrow; the blue alkaline zone is from the arrow to the symbol −. Conditions: 0.01 mol l^{-1} HCl, 0.01 mol l^{-1} NaOH, 1% (w/v) agarose gel, 0.1 mol l^{-1} KCl, 0.1% (w/v) bromphenol blue, flow-rate 0.6 ml min^{-1}, current intensity 0.6 mA mm^{-2}, I.D. 4.5 mm and length of tube 90 mm, circumstance temperature 18–20 °C.

direction of boundary is designed to migrate electrically towards the cathode. Hence, before the run, the overall tube of Fig. 1A is blue. But, when the run begins, the color of the left side gel in the tube changes from its original blue to yellow (see Fig. 1B). The yellow zone becomes longer and otherwise the blue zone turns shorter when the run continues, as compared in Fig. 1B and C.

Those changes can be photographed at any time. After that, one can determine the length of displacement. And then one can calculate the observed velocity of MNRB with:

$$\mu_{obs}^{\alpha\beta} = l_{obs}/t \tag{1}$$

where l_{obs} and t are the displacement of boundary and run time, respectively. The theoretical velocity of boundary is computed with [6,7,15]:

$$\mu_{the}^{\alpha\beta} = \frac{m_{H+}^{\alpha} c_{H+}^{\alpha} E^{\alpha} - m_{OH-}^{\beta} c_{OH-}^{\beta} E^{\beta}}{c_{H+}^{\alpha} - c_{OH-}^{\beta}} \tag{2a}$$

$$E = i/\kappa \tag{2b}$$

coupled with the empirical equation of ionic mobility [22–26]:

$$m_{act} = m_0 \exp(-\eta\sqrt{zI})$$
$$(\eta = 0.77 \text{ if } z \geq 2; \eta = 0.50 \text{ if } z = 1) \tag{3}$$

(It should be noted that Eq. (3) is valid under the conditions: 25 °C temperature and less than 0.1 mol/l ionic strength). In Eq. (2), the superscripts α and β indicate phase α and β, respectively, the subscripts H+ and OH−, mean hydrogen and hydroxyl ions, respectively, m the mobility, c the equivalent concentration, E the electric strength, i the current intensity and κ the specific conductance of solutions. Note that m and c are positive if the ion carries net positive charge(s) or negative if net negative charge(s), as have been defined [6–9]. In Eq. (3), m_{act} and m_0 are the actual and absolute mobilities, respectively, the ionic strength (I) is given as:

$$I = 0.5\sum c_i z_i^2 \tag{4}$$

Multiplying the two sides of Eq. (2) with the time t, one gets the displacement expression:

$$l_{the} = \frac{m_{H+}^{\alpha} c_{H+}^{\alpha} E^{\alpha} - m_{OH-}^{\beta} c_{OH-}^{\beta} E^{\beta}}{c_{H+}^{\alpha} - c_{OH-}^{\beta}} \cdot t \tag{5}$$

The expression shows the theoretical displacement l_{the} is proportional to the run time t.

The relative difference (RD) between theoretical and observed displacement is calculated with:

$$RD = 2(l_{the} - l_{obs})/(l_{the} + l_{obs}) \tag{6}$$

3. Results

3.1. Influence of current intensity (i)

Fig. 2 proves that if 0.6 mA mm^{-2} current intensity is used, the linear relation between displacement and time *at high slope* holds for constant 15 min, even the electrolyte systems for different runs are different from each other. Similar results can be observed in other runs under different currents.

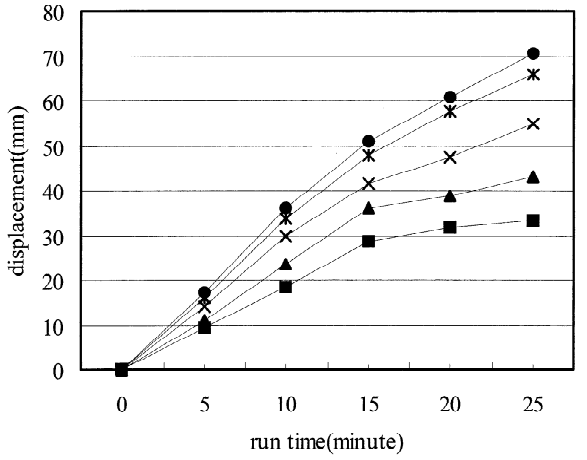

Fig. 2. The boundary displacements at constant current intensity of 0.6 mA mm^{-2}. Conditions: 0.006 mol/l NaOH; ♦–♦: 0.012, *–*: 0.01, ×–×: 0.008, ▲–▲: 0.006, and ■–■: 0.004 mol l^{-1} HCl. The other conditions are 0.1 mol l^{-1} KCl, 1% agarose gel, 0.1% bromphenol blue, I.D. 4.2–4.6 mm and length 90 mm, flow-rate 0.6 ml min^{-1}, and temperature 7–9 °C.

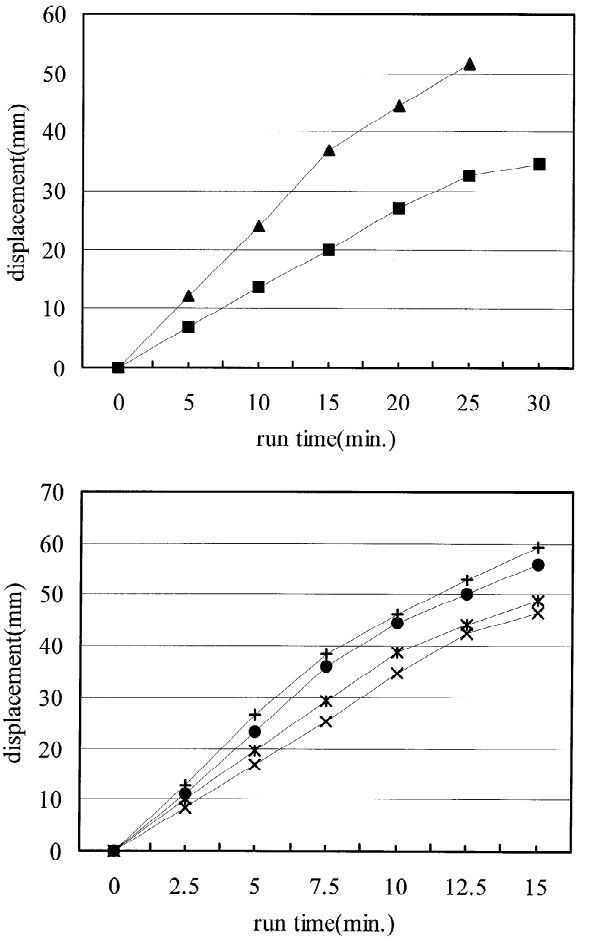

Fig. 3. The boundary displacements under different constant current intensity (*i*). (Top) Intervals of 5 min. ▲–▲: 0.6 mA mm^{-2}; ■–■: 0.4 mA mm^{-2}. (Bottom) intervals of 2.5 min +–+: 1.4 mA mm^{-2}; ♦–♦: 1.2 mA mm^{-2}; *–*: 1.0 mA mm^{-2}; ×–×: 0.8 mA mm^{-2}. Conditions: ■–■ in (Top) are 0.008 mol l^{-1} HCl and 0.01 mol l^{-1} NaOH; the remainder in (Top) and (Bottom) are 0.01 mol l^{-1} HCl and 0.014 mol l^{-1} NaOH. Other conditions as Fig. 2.

According to Eq. (2), the displacement of boundary is proportional to *i*, viz., the strength of electric field. As shown in Fig. 3A, the linear relation between the displacement and time *at high slope* exists for 25 min if 0.4 mA mm^{-2} current intensity is turned on, but for 15 min if 0.6 mA mm^{-2} is on. Fig. 3B shows the relation holds for 12.5 min if 0.8 mA mm^{-2} is turned on, and for 10 min if the 1.0 mA mm^{-2} is on, and for 7.5 min if the 1.2 and 1.4 mA mm^{-2} is on. All those show that the current possesses obvious influence on the movement rate of MNRB created with HCl and NaOH.

Fig. 3 further indicates that the higher *i* is the longer the displacement becomes within a given time. If the low *i* 0.4 mA mm^{-2} is used, the displacement of MNRB is very short. This can, we found, lead to obvious relative error of detection.

Thus, under most of our runs, *i* is set at 0.6 mA mm^{-2}. Under such an *i*, the displacement of boundary is longer, good linear relation is present between the time and displacement, and further good agreement exists between the theoretical and experimental results.

3.2. Influence of Coulomb (it)

More careful scrutiny shows that the coulomb per square millimeter (viz., the product *it*) is a critical influent factor on the movement of MNRB in Figs. 2 and 3. If *i* is set at 0.6 mA mm^{-2} (see Fig. 2 and ▲–▲ in Fig. 3A), 15 min linear relation exists *at*

Table 2
The comparisons between the theoretic and observed rates before/after the critical point of product in Table 1[a]

	Current intensity, i (mA mm^{-2})				
	0.6	0.8	1.0	1.2	1.4
Before the critical product					
Theoretical velocity (10^{-5} m s^{s-1})	5.51	7.34	9.18	11.01	12.85
Observed velocity (10^{-5} m s^{-1})	4.83	5.83	6.83	8.00	9.17
Relative difference (RD)	0.13	0.23	0.29	0.32	0.33
After the critical product					
Theoretical velocity (10^{-5} m s^{-1})	5.51	7.34	9.18	11.01	12.85
Observed velocity (10^{-5} m s^{-1})	2.42	2.6	3.33	4.75	4.83
Relative difference (RD)	0.78	0.95	0.93	0.79	0.91

[a] The RD is computed with Eq. (6), the experimental conditions are the same as those in Fig. 3.

high slope (with 15 min, the good agreements, it was found, exist between the experimental and theoretical results, see Table 2), after 15 min the linear relation holds at low slope (the bad agreements are present between the observed and theoretical, see Table 2). The same results are also observed in Fig. 2. The product of *it*, viz., C mm^{-2}, is 9 min mA mm^{-2}, as shown in Table 1.

Under 0.4 mA mm^{-2} (■–■ in Fig. 3A), 25 min linear relation is present between the boundary displacement and time; and the product between the current and time is 10 min mA mm^{-2} as given in Table 1. Fig. 3B shows if 0.8 mA mm^{-2} (×–×), the relation presents for 12.5 min, and the product is also 10 min mA mm^{-2} as given in Table 1. And if 1.0, 1.2 and 1.4 mA mm^{-2}, the products are equal to 10, 9 and 10.5 min mA mm^{-2}, respectively (see Table 1).

All of the data in Table 1 and Figs. 2 and 3 prove that the product $it \approx 10$ min mA mm^{-2} is a critical point. If the product is lower than critical point, good quantitative agreement exists between the observed and theoretical values as manifested by Table 2. But if it is higher than the critical point, as shown in Table 2, the agreements between the theoretical and observed are poor.

The reason why the C mm^{-2} value has a critical influence on the movement of MNRB is still completely unknown to our knowledge. The reason is possibly the Joule heat generated by the electric field. The Joule heat raises the temperature of the gel in the tube. It was found the tube was hot (about 30–35 °C) when a high i (1.4 mA mm^{-2}) was turned on, but cold (about 10–15 °C) if 0.6 mA mm^{-2} was used. As shown in Table 3, the temperature shows a great influence on the conductance and ionic mobility as will be discussed in the following section. The alteration of ionic mobility and conductance may further lead to the change of boundary velocity.

3.3. Influence of agarose gel

The agarose gel used here is of little of influence on the motion of MNRB. As shown in Fig. 4, if 0.5–1% agarose gel was used the relative difference (RD) between the theoretical and observed values is very low (near to 1%), but if 1.5–2% gel is used, the value of RD is high (near 10%).

But the gel is necessary under the given experimental conditions. As shown in Fig. 1, the

Table 3
The conductance of standard solutions of KCl at different temperatures[a]

KCl (w/w concentration)[b]	κ ($\cdot 10^{-3}$ S^{-1} m^{-1})		
	0° C	18° C	25° C
71.1352	0.65144	0.97790	1.11287
7.41913	0.071344	0.111612	0.128496
0.745263	0.0077326	0.0121992	0.0140807

[a] The data are cited from Ref. [27].
[b] Grams of KCl per 1 kg of solution in vacuum.

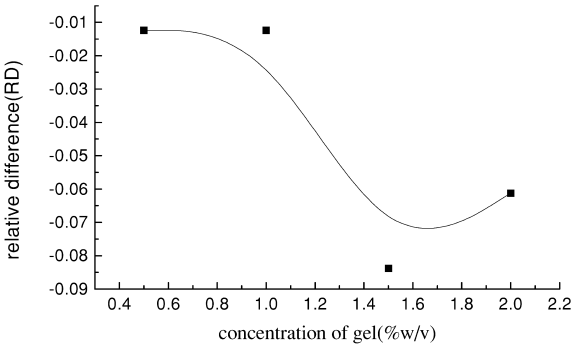

Fig. 4. The influence of agarose gel on the movement of MNRB. The value of RD is computed with Eq. (4). Conditions: 0.01 mol l^{-1} HCl and NaOH. Other conditions as in Fig. 2.

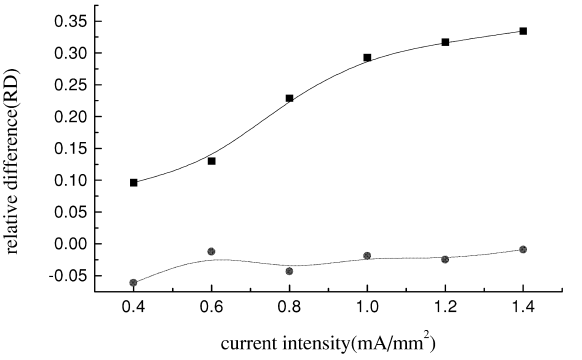

Fig. 5. The influence of circumstance temperature on the movement of MNRB under different current intensity (*i*). ●–●: the values of RD at 18–20 °C temperature and *i*. Conditions: 0.01 mol l^{-1} HCl and NaOH and the other conditions are as in Fig. 1. ■–■: the values of RD at 7–9 °C temperature and *i*. Conditions: 0.014 mol l^{-1} HCl, 0.01 mol l^{-1} NaOH, and the other conditions are as in Fig. 2.

boundary within the gel is straight, sharp and clear; but if the same boundary moves into gel-free solution, the boundary is unclear, confused and skewed severally (see Fig. 3A and B in Ref. [15]). This directly proves the good anti-convection of the gel.

We also found, if 0.5% (w/v) gel was used as anti-convection medium, the mechanical property of the gel is poor and unstable, as result the gel in tube is easily removable. Thus, in our runs, 1% (w/v) agarose gel was used for most of the runs.

3.4. Influence of temperature

The temperature, which directly decides the solution conductance as given in Table 3, has a strong influence on the movement of MNRB created with the strong acid and alkali.

We performed two groups of experiments under the temperatures of 18–20 °C and 7–9 °C. The values of relative difference (RD, see Eq. (6)) between the theoretical and observed values for the two groups are displayed in Figs. 5 and 6.

Fig. 5 shows the RD values at 18–20 °C (●–●) and 7–9 °C (■–■). It is clear in Fig. 5 that all of the RD values are slightly less than zero if the experiment was carried out at 18–20 °C (●–●); this implies a good agreement between the theoretical and observed values. However, if the runs were carried out at 7–9 °C (■–■), all of the values of RD are greatly over zero (up to 35%) as manifested in Fig. 5; this means the poor agreements between the theoretical and observed values.

The comparisons in Fig. 6 further demonstrate the obvious influence of temperature on the movement of MNRB created with strong acid and alkali. Fig. 6 show the values of RD are generally close to zero if the runs are performed at temperature 18–20 °C, this is similar to those in Fig. 5. But the values in Fig. 6 are over zero greatly if at 7–9 °C, this is similar to those in Fig. 5.

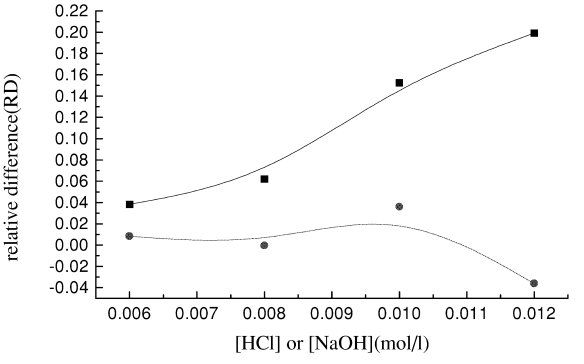

Fig. 6. The influence of circumstance temperature on the movement of MNRB created with different concentration of reactive electrolytes. ●–●: the values of RD at 18–20 °C and under different [NaOH]. Conditions: 0.01 mol l^{-1} HCl, 0.6 mA mm^{-2}, the other conditions are as in Fig. 1. ■–■: the values of RD at 7–9 °C and under different [HCl]. Conditions: 0.006 mol l^{-1} NaOH, 0.6 mA mm^{-2}, and the other conditions are as in Fig. 2.

Clearly the comparisons in Figs. 5 and 6 show the temperature can have an extremely strong influence on the motion of the boundary. Thus, it should be noted that one should choose a temperature of about 20 °C, which is less than, but close to 25 °C [only at which one can get the ionic actual mobility with the empirical equation (see Eq. (3))] for the calculation of the theoretical values of boundary movement, because ionic actual mobility at different temperature has not been obtained yet.

Two reasons may be responsible for the great influence of temperature on the movement of MNRB. One is temperature can strong modify the conductance of the electrolyte solution. Table 3 verifies that the conductance of KCl increases over 30% and even up to 40% if the temperature of KCl solution increases from 0 to 18 °C. Similar results can also be observed in other solutions like HCl [27].

Another reason is that temperature adjusts ionic mobility intensely. Because of the almost constant ionic transference number (T) in electrolyte solution (see Fig. 17 in Ref. [28]), so ionic mobility, which is computed from equivalent conductance with the equation $m = T\lambda/F$ (m = ionic actual mobility, T = transference number, λ = equivalent of solution and F = Faraday's constant), is intensely affected by the temperature, due to the obvious dependence of conductance on temperature (see Table 3).

Therefore, the temperature greatly influences the movement of MNRB by its influences on the conductance of solution and ionic mobility of solution, which are important parameters in the calculations of a MNRB with Eq. (2). However, up to now one can only calculate ionic actual mobility at 25 °C (see Eq. (3)) at different ionic strength [22–26], but cannot obtain ionic actual mobility at different temperature.

3.5. Influence of carbon dioxide in the air

The carbon dioxide in the air also acts upon the movement of MNRB. We find that if the solution and agarose gel containing the alkali of NaOH are exposed to the air for a long time (such as a weak), the obvious difference between the theoretical and observed values can be observed.

And even in the day-to-day test, the slight difference is observed. As shown in Table 4, in the group of within-day experiments, the average displacements of MNRB are generally short in contrast to those of the day-to-day runs. The within-day relative standard deviation (RSD) is low when compared with that of the day-to-day RSD; this indicates the within-day repeatability is better than the day-to-day one.

This influence is obviously caused by the carbon dioxide in the air. Thus, it is recommended that new

Table 4
The comparisons between boundary displacements of within-day and day-to-day groups[a]

	Time, t (min)				
	5	10	15	20	25
Within-day group ($n=5$)					
$X \pm$SD (mm)[b]	9.7±0.42	20.0±0.71	30.4±0.79	37.1±1.67	40.0±1.98
RSD (%)[b]	4.4	3.5	2.6	4.5	4.9
Day-to-day group ($n=5$)					
$X \pm$SD (mm)[b]	10.1±0.55	19.8±0.98	31.6±1.82	38.8±1.36	43.5±2.55
RSD (%)[b]	5.4	4.9	5.7	3.5	5.9
P (t-test)[c]	0.146	0.818	0.260	0.138	0.055
	($P \gg 0.05$)	($P \gg 0.05$)	($P \gg 0.05$)	($P \gg 0.05$)	($P \approx 0.05$)

[a] 1000 ml catholyte and 1% agarose gel in 12 run tubes containing 0.01 mol/l NaOH, 0.1 mol/l KCl and 0.1% bromphenol blue are prepared. In the within-day group, five of the 12 tubes were used to run immediately, and in the day-to-day group, five of the remaining seven tubes were used to run under the same conditions next day. Conditions: 0.01 mol/l HCl and NaOH. Other conditions as in Fig. 2.

[b] $X \pm$SD = average ± standard deviation, RSD = relative standard deviation.

[c] P (t-test): the value of probability, viz., P, of t-test is performed under such parameters: double tails, paired t-test, $n=5$ for each group and $f = n_1 + n_2 - 2 = 8$ ($n_1 = n_2 = 5$).

solution and gel containing the alkali of NaOH should be prepared daily. Otherwise the solution and gel can absorb enough carbon dioxide from the air due to their exposure to the air for a long time Consequently, the actual concentrations of NaOH in the old solution and gel are lower than those just prepared. As result, the influence can be observed.

3.6. Good repeatability

Table 4 also shows good repeatability of the method. In the within-day group ($n=5$), the boundary displacements within different time are determined from their photographs. As shown in Table 4, the RSD values of displacement for the within-day group are between 2.6 and 4.9%.

In the day-to-day group, Table 4 manifests the RSD values of displacement are 3.5–5.9%, almost equal to those for the within-day group. Generally, the average displacements of the within-day group are very near to those of the day-to-day group. Except for the test of 25 min in column 6 that shows apparent difference between the within-day and day-to-day groups ($P=0.055$, very near to 0.05), the other comparisons from columns 2 to 5 verifies all of P values are greatly over 0.05.

All the results mentioned above show good repeatability of the method.

3.7. Optimized experimental conditions

The above results show that numerous factors have obvious influence on the movement of MNRB. The most strong factors are temperature and the product of *it*, viz., C mm^{-2}. The former mechanism is clear as discussed in Section 3.4. But the physical–chemical mechanism of the latter is still unclear, it cannot be well understood, whereas the latter hold critical influence on the movement of the MNRB as shown in Tables 1 and 2 and Figs. 2 and 3. Hence it is necessary for us to pay much attention on the investigation of physical chemical relation of product of the current intensity and time.

Nevertheless the existences of much unclear physical chemical relations, the investigations in above section still give the following optimized experimental conditions: (1) 0.6–0.8 mA mm^2 *i*; (2) less than about 10 min mA mm^{-2} product between the time and *i*; (3) about 18–20 °C circumstance temperature which is slightly less than the standard temperature 25 °C; (4) 1% agarose gel; and (5) daily prepared solution and gel containing the alkali of NaOH.

4. Discussion

Evidently, the purpose in this report is to investigate the influent factors on the moving rate of MNRB which holds many applications in electrophoresis as discussed below, but not directly on electrophoresis. Even such factors like current, gel concentration and temperature, etc., have been observed since the beginning of electrophoresis, whereas those influent factors on MNRB rate have not been explored yet. More importantly, the product of *it*, viz., C mm^{-2}, which has critical effect on MNRB as shown in Tables 1 and 2, has never been described in both MCRB and electrophoresis, to the authors' knowledge.

The studies have an obvious significance for electrophoresis. At first, the optimized conditions can be directly used for the further experimental investigations of the MNRB. Secondly, MNRBM can be directly used for the on-line condensation of sample and enhancement of separation efficiency in CZE. As shown in the accompanying paper [29], the theory of MCRB is used for the semi-quantitative selection of experiment conditions, and the MCRBM performed in agarose gel in large electrophoretic tube [16–20] can be directly used to improve the theoretical plate number, peak height and resolution of animo acids in CZE. The design of experiment of MCRBM–CZE [29] is the same as that of MCRBM in large tubes [16–20], the difference is that the former is a transient MCRB with an animo acid sample in its system but the latter is a continuous MCRB without any sample in its system.

And in fact, many online sample stacking techniques being similar to the transient MCRBM have been developed and used for CE, including CZE and micellar electrokinetic capillary chromatography

(MEKC). In 1992, Schwer and Lottspeich [30] invented the ''sandwich method''-acid (set near to the anode)-sample-base (set near to the cathode)-for enhancement of separation efficiency in CE (see Fig. 1c in Ref. [30]). In 1998, Xiong et al. [31] designed pH-mediated sample concentration, which was reduced by low conductivity due to gradual chemical reaction, for the analyses of DNA sequencing. Separately, Shihabi and Friedberg [32] developed a similar technique for the insulin on-line stacking in CZE, even if the sample contains high concentrations of salt. In 2000, this kind of sample stacking by chemical reaction between the sample buffer and running buffer was further investigated by Britz-McKibbin and co-workers [33,34] and used for the focusing of nucleotides and the selective stacking of catecholamine and weakly acidic compounds in CE. In order to stacking neutral analytes, powerful sweeping technique of analytes has been advanced for MEKC by Quirino et al. [35–37] and Landers and co-workers [38,39]. In the sweeping procedure, a moving chemical reaction occurs between the neutral analytes and micellar moleculars. The sweeping method is so powerful that even over 5000-fold concentration can be achieved in MEKE as clearly demonstrated by Quirino and Terabe [35].

And at last, MNRBM can be used for the studies on IEF. The procedure reported here is developed from the electrically controlled electrofocusing in a capillary apparatus by Pospichal et al. [3]. Pospichal et al. [3] have used the procedure for the investigation on mechanism of IEF [3] and for the carrier ampholyte-free IEF [5]. It has been unveiled that IEF relies on the MNRB as shown in Refs. [3–5] and [9–11]. Clearly this study, coupled with others [16–20], is of benefit for further experimental investigations on the dynamic mechanism of IEF which is still completely unclear [9,40–42].

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