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Electropherograms in capillary zone electrophoresis plotted as a function of the quantity of electric charge

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Abstract

We have plotted electropherograms in capillary zone electrophoresis (CZE) as a function of the quantity of electric charge (Q) in order to eliminate the dependency of the analyte peak areas, as well as that of the migration times, upon both the capillary temperature and the applied voltage. The procedure is based on an idea of a migration index (MI) and an adjusted migration index (AMI) which were originally proposed by Lee and Yeung. The value of Q is measured accurately and calculated easily because it is given by a product of the electrophoretic current and the migration times, where the index MI is derived by dividing the value of Q by the effective volume of the capillary. By calculating the CZE peak area from the newly plotted electropherogram, improvement in precision in quantitative analysis is expected. Concerning AMI, careful treatment is required in its application to analyte peaks whose migration time is close to that of the neutral marker. Experimental data and discussions concerning the migration indices are presented.

Keywords: Migration times; Electric charge; Reproducibility

1. Introduction

It is well known that capillary zone electrophoresis (CZE) is superior to other separation techniques in mass sensitivity, separation efficiency and speed of analysis [1,2]. In order to increase its popularity as a versatile analytical method, however, some problems to be overcome still remain, such as increase in the concentration sensitivity, improvement in the reproducibility of the injection volume and that of migration times, and improvement in precision in quantitative analysis [3].

Instability in migration times in CZE is mainly caused by the sensitive dependence of the electroosmotic flow (EOF) on temperature. The EOF is given by a function of the viscosity coefficient (η) of buffer solution, the zeta potential (ζ_c) between the buffer solution and the inner wall of the capillary, the electric field strength (E), and others. Among them, the temperature dependence of η is crucial. In order to improve the reproducibility in migration times, therefore, precise and accurate control of temperature throughout the CZE system is indispensable. In a real CZE instrument, however, maintaining temperature control along the whole capillary column including the sample vial and a part of the signal detection system is difficult, which means that the uniformity of temperature along the capillary column is not assured. Furthermore, temperature elevations by Joule heating due to electric current

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flowing through the buffer solution during a run are inevitable, causing local variations in temperature along the capillary column because of the smallness of the capillary volume and of the bending stress externally applied on the capillary. Such temperature variations disturb the thermal equilibrium in the capillary and then introduce unfavorable effects on the reproducibility of migration times and on precision of quantitative analysis. The value of ζ_c also depends on the capillary material and on the property of the buffer solution.

For the purpose of improving the reproducibility of migration times in CZE, Lee and Yeung [4] have introduced two kinds of novel indices instead of the conventional migration times (t_m): one is called the migration index (MI) and the other an adjusted migration index (AMI). By using the MI, the influence of the temperature dependence of η and those of the change of E, capillary length L, and inner diameter of the capillary can be eliminated. In addition, a mutual data comparison with and without gradient potentials can be achieved. Furthermore, by using the AMI, even the influence of ζ_c can be eliminated. By introducing the index AMI in CZE, they have demonstrated that mutual data transfer between different laboratories and/or different CZE instruments is facilitated.

We believe that the two indices proposed by Lee and Yeung are very important for the future of CZE as well as for the design of the CZE instrument. They, however, only calculated the values of MI and AMI for CZE peak positions. Precision in quantitative analysis or that in ordinate values in the electropherogram was the same as before. In order to improve the quantitative problem, we propose in the present paper to plot the whole CZE electropherogram as a function of MI and then calculate the peak areas. Here, the index MI can be calculated for any abscissa positions in the electropherogram as well as the peak positions. At the present instrumental stage, however, the problem concerning the injection volume is not negligible. As a primary study, therefore, we carried out experiments using the same CZE instrument equipped with the same capillary. In such a situation, the index MI could be replaced by the quantity of electric charge Q during the migration time. The value of Q is represented by a product of the effective volume of the capillary and the index MI. By using the quantity of electric charge, errors arising from the calculation of the volume of the capillary might be eliminated. We show that the dependency of the peak areas, as well as that of the migration times, on the temperature and the applied voltage is eliminated. Our other remarks concern the AMI. We have calculated the value of AMI for some analytes by introducing a neutral marker. However, the superiority of AMI to MI is not always recognized. In the present paper, we show experimental results and discuss the migration indices.

2. Theory

2.1. Migration indices

The concept of migration indices introduced by Lee and Yeung is outlined below briefly because details are described elsewhere [4]. In CZE, the net migration velocity (v_m) of an analyte is given by an arithmetic addition of the EOF velocity (v_{eo}) and the electrophoretic velocity (v_a):

$$v_m = v_{eo} + v_a = \frac{\varepsilon E}{\eta}\left[\zeta_c + \frac{2}{3}\zeta_a f(\kappa a)\right] \quad (1)$$

where ε is the dielectric constant of the medium, $E(=V/L)$ the local electric field strength, V the applied voltage, L the total length of capillary, ζ_c the ζ potential of the inner wall of the capillary, ζ_a that of the analyte, η the viscosity coefficient, κ the reciprocal of the analyte double layer thickness, a the radius of the analyte, and $f(\kappa a)$ is a function depending upon the shape and κa of the analyte in the buffer. It follows that the analyte migration time (t_m) is

$$t_m = \frac{L_{eff}}{v_m} = \frac{\eta L_{eff}}{\varepsilon E\left[\zeta_c + \frac{2}{3}\zeta_a f(\kappa a)\right]} \quad (2)$$

where L_{eff} is the effective length of capillary. As shown in Eq. (2), t_m is dependent upon E, L_{eff}, ζ_c and $\zeta_a f(\kappa a)$. Among those, η has the largest temperature dependence.

Now, the following relation holds between E and i [5]:

$$E = \frac{i}{k} \quad (3)$$

where i and k denote current density (in ampere cm^{-2}) and specific conductance (Ω^{-1} cm^{-1}), respectively. Substituting Eq. (3) into Eq. (2),

$$v_m = \frac{dx}{dt} = \frac{L_{eff}}{t_m} = \frac{\varepsilon i}{k\eta}\left[\zeta_c + \frac{2}{3}\zeta_a f(\kappa a)\right] \quad (4)$$

where x denotes the axial distance of the capillary beginning at the injection end and t the time. In Eq. (4), the product of k and η, called the Walden product, remains constant for small variations in temperature [6,7]. When we accept the Walden rule and define a migration index, MI, as the following equation, we can eliminate the temperature dependence of η:

$$MI \equiv \int_0^{t_m} \frac{i}{L_{eff}} dt = \frac{Q_m}{Vol} \quad (5)$$

$$= \frac{k\eta}{\varepsilon\left[\zeta_c + \frac{2}{3}\zeta_a f(\kappa a)\right]} \quad (6)$$

From Eq. (6), we can understand that MI is still dependent upon ζ_c and $\zeta_a f(\kappa a)$. Also, from Eq. (5), we can understand that the index MI is obtained by dividing Q_m by Vol($Q_m = MI \times Vol$), provided that the length, the inner diameter of the capillary, and the buffer conditions are fixed; where Q_m is the value of Q required during the migration time t_m and Vol (cm^3) is the effective volume of the capillary. The effective volume Vol is given by a product of L_{eff} and the value of the area of the cross section of the capillary column. When the value of Vol is kept constant, that of Q_m can be used as the same way as the index MI. In such a situation, we represent Q_m as MIC (migration index with the quantity of electric charge). A relation between MI and MIC is, therefore, given by MIC = MI × Vol. The index MIC can be easily calculated by monitoring the electric current though the capillary with a constant time interval (for instance, 1 s) and then integrating it. In the present study, we plotted the whole CZE electropherogram as a function of Q as mentioned before.

An adjusted migration index (AMI) can be defined in the following manner [4]:

$$AMI \equiv \frac{MI_{eo} MI}{MI_{eo} - MI} \quad (7)$$

$$= \frac{3k\eta}{2\varepsilon\zeta_a f(\kappa a)} \quad (8)$$

where MI_{eo} is a value of MI for a neutral marker which is given by the following equation:

$$MI_{eo} = \frac{k\eta}{\varepsilon\zeta_c} \quad (9)$$

From Eq. (8), we can understand that AMI is dependent upon only $\zeta_a f(\kappa a)$ that is determined by the nature of the analyte alone. As the same manner, we can define AMIC (AMI with the quantity of electric charge) as follows:

$$AMIC \equiv \frac{MIC_{eo} MIC}{MIC_{eo} - MIC} \quad (10)$$

where MIC_{eo} is a value of MIC for the neutral marker, which is given by $MIC_{eo} = MI_{eo} \times Vol$. The relation between AMI and AMIC is given by AMIC = AMI × Vol.

3. Experimental

3.1. Chemicals

We used the following reagents: benzyl alcohol (BA), pyridoxal (PL) and adenosine-5′-monophosphate (AMP) (Wako, Osaka, Japan); pyridoxamine (PM) and adenosine 3′:5′-cyclic-monophosphate (c-AMP) (Sigma, St. Louis, MO, USA). Water was purified by deionization, followed by distillation. All other reagents were of analytical grade.

3.2. Apparatus

We used a fully automated CE-900 System (Jasco, Tokyo, Japan) with a 60 cm (effective length 45 cm) × 50 μm I.D. × 375 μm O.D. untreated fused-silica capillary column (GL Science, Tokyo, Japan). The detection of a CE peak was accomplished by using an on-column UV–Vis detector CE-970 (Jasco)

which monitored the instantaneous absorption intensity of the analyte at a wavelength of 265 nm. This is because compounds of the vitamin B_6 group (PM and PL) were expected to be unstable under the illumination of the shorter wavelength although the absorption maximum for each analyte is below 265 nm.

3.3. Data acquisition

An NB-MIO-16 A/D converter board (National Instruments, TX, USA) was configured in a two-channel data-collection mode with a Macintosh IIfx personal computer, which simultaneously acquire signals from the analog output of the UV detector and from the current monitor output of the CE-900 system. The data collection procedure was triggered by an injection signal from the system. The data acquisition interval was fixed at 0.1 s. After the completion of each run, the data were smoothed and downsampled to a data interval of 1.0 s. All the data processing program including the calculation of the indices MIC and AMIC were laboratory made on LabVIEW software (National Instruments).

3.4. Electrophoretic procedures

The separation buffer was 40 m*M* sodium phosphate, pH 8.0. The applied voltage for the capillary column was changed between 5 kV and 30 kV with a 5 kV step. A part of the capillary column, about 20 cm in the effective length of 45 cm, was maintained at a constant temperature between 35°C and 60°C. Prior to the first use, a new capillary was subjected to a standard wash cycle [(1) separation buffer for 1 min under 2000 mbar; (2) 0.1 *M* NaOH for 1 min under 2000 mbar; (3) separation buffer for 1 min under 2000 mbar; (4) run for 0.5 min under 5 kV], four times repeatedly. Before the first injection on the day and between injections the capillary was washed with the standard wash cycle once. During the run, the temperature of the sample vial set on a autosampler was kept at 10°C. An analyte was injected at the anode side by a dynamic compression injection method hydrodynamically (20 mbar, 0.1 min) [1]. The individual concentrations of analytes were 2.5 mg/ml (PM), 2% (v/v) (BA), 0.83 mg/ml (PL), 0.33 mg/ml (c-AMP) and 0.33 mg/ml (AMP), respectively. The mixture of the analytes were dissolved in separation buffer.

4. Results and discussion

We summarize in Table 1 (run Nos. 1–12) migration times measured and corresponding MICs calculated for individual separated peaks. The applied voltage was changed between 5 kV and 30 kV with a 5 kV step. The capillary column temperature was fixed at 35°C. Here, peak 1 is due to PM, peak 2 BA, peak 3 PL, peak 4 c-AMP and peak 5 AMP. Plotted in Fig. 1a are measured electropherograms as a function of the time. Fig. 1b shows fluctuations of electrophoretic currents as a function of the time during the corresponding runs, which were simultaneously monitored. As was expected, the migration time was shortened and the current value was increased with increase in the applied voltage. The separation condition at 30 kV must be fairly different from that at 5 kV because of the difference and the non-uniformity in the local Joule heating in the capillary column. This might explain the larger fluctuations in current at 30 kV than those at 5 kV. Using the two data shown in Fig. 1a and Fig. 1b, we reconstructed new electropherograms, abscissas of which are plotted by Q. This procedure was carried out by simply replacing the abscissa values of the times in Fig. 1a to the corresponding values of Q. Each value of Q is calculated from Fig. 1b by a numerical integration. The new reconstructed electropherograms are shown in Fig. 1c. We can find that six electropherograms almost coincide independent of the applied voltage.

Fig. 2 and Table 1 (run Nos. 13–15) show the same experimental results but the capillary column temperature was varied from 40°C to 60°C with a 10°C step for a fixed applied voltage of 15 kV. These results again show that the fluctuation in t_m is improved fairly well by using the index MIC. The relative standard deviation (R.S.D.) of the fluctuations of the migration times for individual peaks when introducing the index MIC for the runs 1–15 is 4.1% at most.

In order to eliminate the voltage and the temperature dependence of the individual peak areas, we calculated the peak areas in the two electropherog-

Table 1
Comparison of t_m and MIC obtained in various applied voltages and temperatures for individual peaks

Run No.	Applied voltage (kV)	Temperature (°C)	Peak 1[a]		Peak 2[b]		Peak 3[c]		Peak 4[d]		Peak 5[e]	
			t_m (s)	MIC ($\cdot 10^{-2}$ C)	t_m (s)	MIC ($\cdot 10^{-2}$ C)	t_m (s)	MIC ($\cdot 10^{-2}$ C)	t_m (s)	MIC ($\cdot 10^{-2}$ C)	t_m (s)	MIC ($\cdot 10^{-2}$ C)
1	30	35	86	0.994	92	1.068	115	1.351	136	1.612	181	2.169
2	30	35	86	0.996	92	1.069	114	1.341	136	1.616	180	2.166
3	25	35	117	0.947	131	1.064	153	1.247	195	1.602	260	2.150
4	25	35	117	0.950	131	1.068	153	1.252	195	1.604	260	2.150
5	20	35	164	0.925	190	1.073	214	1.211	284	1.615	380	2.167
6	20	35	164	0.925	188	1.070	213	1.214	282	1.614	378	2.171
7	15	35	250	0.940	289	1.088	324	1.221	435	1.643	587	2.222
8	15	35	252	0.945	291	1.092	327	1.228	438	1.649	592	2.232
9	10	35	386	0.924	454	1.088	503	1.206	683	1.641	918	2.210
10	10	35	393	0.923	463	1.088	512	1.204	699	1.648	940	2.221
11	5	35	775	0.905	923	1.073	1011	1.180	1388	1.618	1855	2.160
12	5	35	771	0.906	913	1.078	1003	1.179	1371	1.613	1827	2.192
13	15	60	210	0.916	243	1.077	275	1.203	365	1.628	486	2.142
14	15	50	221	0.907	259	1.065	288	1.186	389	1.609	519	2.154
15	15	40	234	0.909	277	1.061	305	1.187	417	1.604	558	2.184
R.S.D. (%)				3.0		0.9		4.1		1.0		1.3

L_{eff}=45 cm×50 μm I.D.; buffer: 40 m*M* sodium phosphate, pH 8.0.
[a] PM.
[b] BA.
[c] PL.
[d] c-AMP.
[e] AMP.

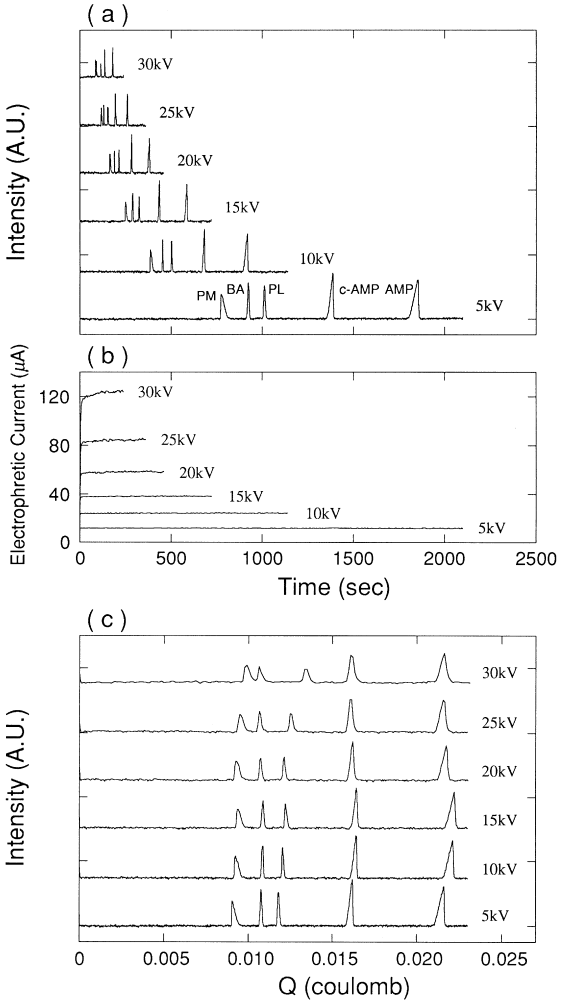

Fig. 1. (a) Electropherograms plotted as a function of the time for various applied voltages, run numbers of which correspond to Nos. 1, 3, 5, 7, 9 and 11 in Table 1, respectively. The first peak is due to PM, the second BA, the third PL, the fourth c-AMP, and the fifth AMP, respectively. The capillary column temperature was kept at 35°C. (b) Electrophoretic currents plotted as a function of the time which were simultaneously monitored. (c) Electropherograms plotted as a function of the quantity of electric charge (Q).

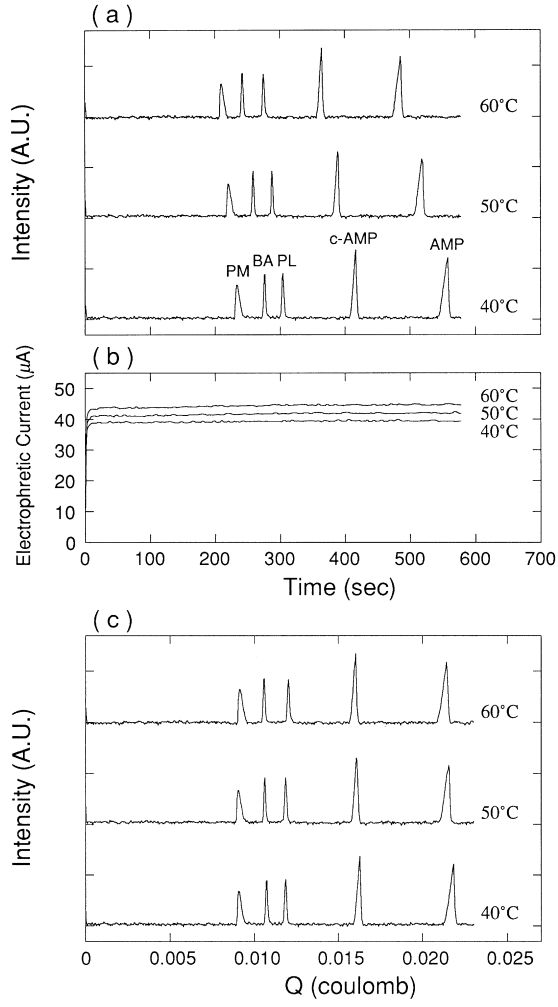

Fig. 2. (a) Electropherograms plotted as a function of the time for various capillary column temperatures, run numbers of which correspond to Nos. 13, 14 and 15 in Table 1, respectively. (b) Electrophoretic currents plotted as a function of the time which were simultaneously monitored. (c) Electropherograms plotted as a function of the quantity of electric charge (Q).

rams, abscissas of which are the time and Q, respectively. Table 2 shows individual results. Here, Area-t_m represents a peak area derived from the ''time-electropherogram'' and Area-MIC represents that derived from the ''Q-electropherogram''. The R.S.D. of the fluctuations of the peak areas for individual peaks when using the ''Q-electropherogram'' for runs 1–15 is less than 9.4%.

From above results, we find that the voltage and the temperature dependence of the peak areas, as well as that of the migration times, is eliminated by using the Q-electropherogram. It also indicates the improvement of precision in quantitative analysis, as well as that in qualitative analysis. The degree of improvement of precision in quantitative analysis is a bit worse than that in qualitative analysis. This might be due to errors in the injection volume.

Table 2
Comparison of peak areas obtained from the ''*t*-electropherogram'' and the ''*Q*-electropherogram''

Run No.	Applied voltage (kV)	Temperature (°C)	Peak 1		Peak 2		Peak 3		Peak 4		Peak 5	
			Area-t_m (s)	Area-MIC ($\cdot 10^{-4}$ C)	Area-t_m (s)	Area-MIC ($\cdot 10^{-4}$ C)	Area-t_m (s)	Area MIC ($\cdot 10^{-4}$ C)	Area-t_m (s)	Area-MIC ($\cdot 10^{-4}$ C)	Area-t_m (s)	Area-MIC ($\cdot 10^{-4}$ C)
1	30	35	1.52	1.862	0.86	1.054	1.24	1.534	2.55	3.130	3.05	3.764
2	30	35	1.53	1.873	0.96	1.178	1.27	1.569	2.11	2.629	2.71	3.398
3	25	35	2.24	1.866	1.55	1.288	1.70	1.425	3.59	3.016	4.50	3.824
4	25	35	2.22	1.849	1.30	1.106	1.71	1.417	3.28	2.757	4.43	3.716
5	20	35	3.37	1.923	2.18	1.257	2.12	1.215	4.99	2.871	6.33	3.650
6	20	35	3.31	1.908	2.31	1.332	2.05	1.193	4.92	2.867	6.32	3.680
7	15	35	4.49	1.697	3.09	1.174	3.52	1.333	7.77	2.966	9.53	3.645
8	15	35	4.98	1.877	3.03	1.144	3.62	1.360	7.30	2.761	9.64	3.646
9	10	35	7.27	1.743	5.13	1.231	5.13	1.241	11.95	2.883	15.56	3.753
10	10	35	7.59	1.793	5.37	1.270	5.49	1.293	12.47	2.967	16.33	3.917
11	5	35	15.67	1.819	11.35	1.322	11.54	1.336	26.96	3.112	34.04	3.945
12	5	35	16.15	1.902	10.69	1.218	11.55	1.350	26.52	3.175	34.85	4.111
13	15	60	4.76	2.108	2.93	1.300	3.18	1.408	7.44	3.308	9.14	4.081
14	15	50	4.49	1.876	2.96	1.233	2.84	1.186	7.51	3.143	9.12	3.835
15	15	40	4.76	1.863	3.17	1.246	2.95	1.164	7.78	3.064	10.04	3.946
R.S.D. (%)				5.5		6.7		9.4		6.0		4.7

Conditions as in Table 1.

Table 3
AMIC values ($\cdot 10^{-2}$ C) for individual peaks obtained in various applied voltages and temperatures

Run No.	Applied voltage (kV)	Temperature (°C)	Peak 1	Peak 2	Peak 3	Peak 4
1	30	35	1.443	−0.510	−0.317	−0.210
2	30	35	1.446	−0.528	−0.316	−0.211
3	25	35	0.863	−0.723	−0.317	−0.211
4	25	35	0.865	−0.724	−0.319	−0.212
5	20	35	0.668	−0.944	−0.320	−0.213
6	20	35	0.716	−0.901	−0.317	−0.211
7	15	35	0.693	−1.002	−0.322	−0.213
8	15	35	0.701	−0.985	−0.323	−0.214
9	10	35	0.616	−1.111	−0.323	−0.214
10	10	35	0.608	−1.130	−0.320	−0.213
11	5	35	0.557	−1.240	−0.323	−0.215
12	5	35	0.581	−1.188	−0.320	−0.214
13	15	60	0.667	−0.901	−0.314	−0.210
14	15	50	0.612	−1.044	−0.315	−0.211
15	15	40	0.581	−1.165	−0.318	−0.213
R.S.D. (%)			36.2	23.4	0.9	0.7

Conditions as in Table 1.

Table 3 shows individual AMIC values calculated from Eq. (10) for individual peak positions, where peak 2 is used as a neutral marker. We can recognize the superiority of the index AMIC over MIC for peaks 3 and 4. For peaks 1 and 2, however, AMIC gives us worse results than MIC, especially for a high applied voltage. This was not indicated in the previous report [4]. Two reasons might be possible: (1) when the migration velocity of an analyte is close to that of a neutral marker, the measurement error is emphasized. This is because the denominator in Eq. (10) approaches to zero: $MIC_{eo} \approx MIC$. (2) Depending upon pH values of the buffer and analyte solutions, the degree of dissociation in the analyte fluctuates because of the local temperature variation along the capillary column. As mentioned before, the temperature variations become large for the higher applied voltage. In such a case, variations of ζ_a result in the ineffective use of MIC and AMIC.

From our measurements, we found that AMIC worked well for analytes whose migration time are very different from that of the neutral marker. For analytes whose migration time are close to that of the neutral marker, however, attention should be paid in using the index AMIC. It also should be noted that there might be a room for a discussion whether the fundamental assumption used in the derivation of MIC and AMIC, the Walden rule, is applicable.

To plot the electropherogram as a function of Q is essentially the same as to use a power supply with a constant-current mode [8]. The concept of the indices MIC and AMIC is, therefore, equivalent to use the constant-current power supply on the assumption that the Walden rule is hold on CZE. The constant current mode of operation, however, has some problems in a practical use: (1) it is difficult to construct a high-precision version and is troublesome to deal with because of the high impedance of the buffer solution in the capillary column. (2) It is difficult to derive the electrophoretic mobility of analyte. (3) Also, it is difficult to use a gradient potential in order to speed up the separation.

5. Conclusions

In the present paper, we reported the usefulness of electropherogram plotted as a function of the quantity of electric charge. By calculating the analyte peak areas from the reconstructed electropherogram, the temperature and the applied voltage dependence of

the peak areas are almost eliminated. It means improvement in precision in quantitative analysis. As was pointed out by Lee and Yeung [4], improvement in precision in qualitative analysis is also achieved. From a practical point of view, the procedure is quite useful. This is because deterioration in the day-to-day and/or successive reproducibility in both qualitative and quantitative analysis is mainly introduced by the fluctuations of the capillary temperature and the applied voltage.

We have also calculated the index AMIC. AMIC works well for an analyte whose migration time is far from that of a neutral marker. We found, however, that it have a problem for an analyte whose migration time is close to that of the neutral marker. In order to use AMIC effectively in a practical situation, we have to study it more systematically including the applicability of the Walden rule in CZE.

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