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Dual-Gradient Capillary Electrochromatography by Varying the Mobile Phase Composition and Applied Voltage

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Dual-gradient capillary electrochromatography (DG-CEC) was developed to provide superior performance with regard to the separation of ionized analytes; in this method, both the eluent composition and the applied voltage are varied during the separation procedure. As for the gradient in the eluent composition, a shift in the pH is employed to control not only the electrophoretic mobility, but also the retention factor of the analytes. The dual-gradient method was shown to be effective in increasing the resolution and reducing the chromatographic period of ionized analytes. Fourteen kinds of *o*-phthalaldehyde labeled amino acids were separated within 8 min using DG-CEC with multistage enlargement in the applied voltage. The separation efficiency increased particularly for highly retained amino acids in the dual-gradient, as compared to those in the ordinary single-gradient for the eluent.

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Introduction

Capillary electrochromatography (CEC) is a hybrid separation method that combines capillary liquid chromatography (CLC) and capillary electrophoresis (CE); the separations can be controlled both chromatographically and electrophoretically.[1-6] CEC can be categorized into two types: electroosmotic flow-driven CEC and a pressurized flow-driven CEC (pCEC). In the former CEC, the application of either a positive or negative voltage along a column can provide analyte migration toward the column outlet (detector) due to the electrophoretic or electroosmotic flow. In the case of pCEC, the analyte migration toward a detector is practically dominated by the pressurized flow. Thus, electrophoretic migration toward both the column inlet and the outlet (application of both positive and negative voltages) can be utilized to control the electrochromatographic separation. In our previous papers concerning pCEC, an enhancement of the separation performances induced by electrophoretic migration was demonstrated, *i.e.*, improvements in the separation, an alternation of the elution order, and a reduction of the separation period through a selective acceleration of analytes were achieved.[7,8]

Meanwhile, gradient elution is generally used in liquid chromatography (LC) to enhance separation. Various types of gradients have been developed in a mobile phase composition, such as the content of an organic modifier, the pH, the ionic strength, and their combination. Gradient elution in a mobile phase composition is also used in CEC and related techniques in order to control the electrochromatographic behavior of the analyte.[8-11]

As another type of gradient method for LC, a gradient in flow velocity was recently reported using a monolithic column, wherein the elution of highly retained analytes was gradually accelerated through an increase in the flow rate.[12,13] On the other hand, in the case of CEC, a gradient in the applied voltage was reported to control the electroosmotic flow velocity and the electrophoretic migration of an ionized analyte.[14,15] A gradient in the pressurized flow velocity identically varies the migration velocity of all the analytes in the mobile phase; however, the applied-voltage gradient can individually vary the electrophoretic velocity of each analyte, owing to the difference in their electrophoretic mobilities. Since the electrophoretic mobility of an analyte strongly depends on the property of the ambient fluid, the gradient in the eluent composition determines the electrophoretic migration behavior of the analyte. Thus, the electrophoretic velocity can be controlled by both the applied electric field and the eluent composition. In addition, the gradient in the eluent composition is quite effective in controlling the retention factor, as described above. Therefore, a suitable combination of gradients, both in the eluent composition and the applied voltage, should lead to high-performance separation.

In this study, we developed dual-gradient capillary electrochromatography (DG-CEC), wherein both the eluent composition and the applied voltage are varied during the separation procedure to achieve high-performance separation by controlling both the retention factor and the electrophoretic velocity. The performance of DG-CEC was demonstrated in terms of the improvement in the resolution and a reduction of the elution period through the separation of *o*-phthalaldehyde (OPA)-labeled amino acids.

Experimental

Apparatus for dual-gradient CEC

Figure 1 shows a schematic diagram of DG-CEC; its arrangement is almost the same as that of the gradient elution pCEC shown in our previous report.[8] The apparatus is composed of two pumps (LC-10AD, Shimadzu; Kyoto, Japan),

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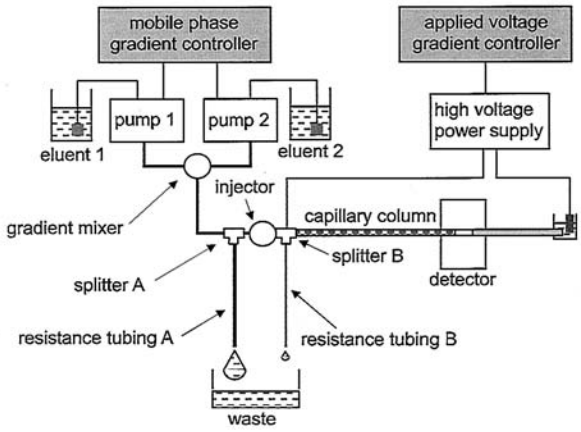

Fig. 1 Schematic diagram of dual-gradient capillary electrochromatography.

a pump controller for the mobile phase gradient (SCL-10A, Shimadzu), a mixer (volume, 0.1 mL; P/N228-35830-91, Shimadzu), two laboratory-made splitters, two resistance tubes (fused-silica capillary; i.d. 0.05 mm; length, 500 mm; supplied by GL Science, Tokyo, Japan), a UV detector (CE-1575, Jasco; Tokyo, Japan), an injector (Model 7520, Rheodyne; CA), a high-voltage power supply (HVA 4321, NF; Yokohama, Japan), and a laboratory-made capillary column (i.d. 0.15 mm) packed with ODS (dp., 5 μm; Develosil ODS-5, Nomura Chemical; Aichi, Japan). Splitters A and B are used to reduce the delay time for varying the eluent composition in the capillary column and for split injection, respectively. The high-voltage power supply was controlled by PC *via* a D/A converter (PCI-3176, Interface; Hiroshima, Japan), and the UV signal was recorded in PC using an A/D converter (K8DL-G16, Omron; Tokyo). High voltage was applied between the end of the column outlet and splitter B at the column inlet, which was grounded to ensure safety.

Measurement of electrophoretic mobility

The electrophoretic mobilities of anilines in a mixture of acetonitrile and 10 mM phosphate buffer were measured by capillary electrophoresis. A commercial CE system (CAPI-3100, Otsuka Electronics; Osaka, Japan) with an uncoated fused-silica capillary (i.d. 0.05 mm; effective length, 287 mm; total length, 406 mm) was used for an electrophoretic mobility measurement in order to exclude the chromatographic retention completely from the migration time. A high voltage (15 kV) was applied to generate the electrophoretic migration of anilines and an electroosmotic flow (EOF). The EOF velocity was estimated from the detection time of uracil (EOF marker).

Chemicals

Mixtures of uracil and anilines (aniline, *p*-ethylaniline, *p*-*n*-butylaniline, *p*-*n*-hexylaniline) were dissolved in a mixture of acetonitrile and distilled water (70/30) to prepare a sample solution. The aniline solutions (5 to 8 mM for each aniline) were used as a standard sample to study the performance of DG-CEC. Uracil was added as a marker for both t_0 and the neutral component.

Fourteen amino acids (Asp, Glu, Ser, His, Gly, Thr, Arg, Ala, Tyr, Val, Met, Trp, Leu, Lys) were labeled by OPA for UV detection at 280 nm by the following procedure.[16] A 1-mL volume of ethanol containing 54 mg of OPA was added to 10 mL of a 0.1 M borax aqueous solution. Before using the OPA solution, 40 μL of 2-mercaptoethanol was added to it; further, the OPA solution was mixed with an aqueous solution of amino acids (0.25 to 6.7 mM each) for the labeling reaction at room temperature. The unreacted OPA was removed from the solution by conducting solvent extraction with ethyl acetate thrice. The final water phase was used as the sample solution.

A mixture of acetonitrile and phosphate buffer was used as the eluent. All chemicals were purchased from Wako Pure Chemistry (Osaka, Japan).

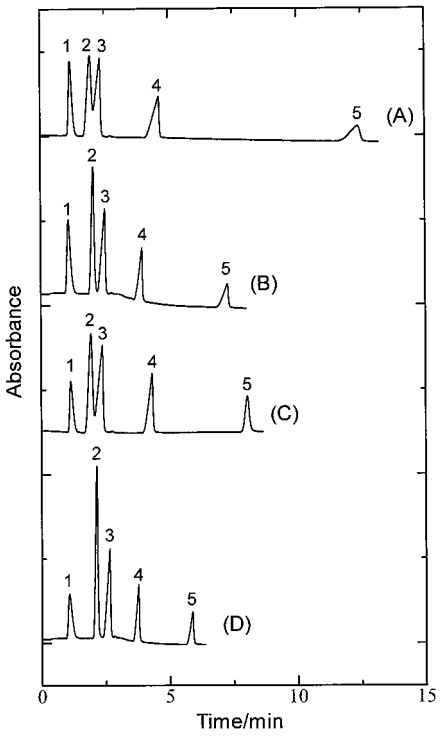

Fig. 2 Separation of anilines with various gradients. Column: i.d., 0.15 mm; packed length, 130 mm; total length, 210 mm. Eluent: (I) acetonitrile/10 mM phosphate buffer (pH 3.3) (45/55), (II) acetonitrile/10 mM phosphate buffer (pH 2.4) (45/55). Linear gradient for (C, D): 0 to 7 min, 0% (II) to 100% (II). Voltage gradient for (B, D): from +10 to –10 kV in 3 min then maintained constantly at –10 kV. Detection: UV 210 nm. Samples: (1) uracil, (2) aniline, (3) *p*-ethylaniline, (4) *p*-*n*-butylaniline, (5) *p*-*n*-hexylaniline. (A) Without gradient using eluent (I), (B) with voltage gradient using eluent (I), (C) with eluent composition gradient without applied voltage, (D) with dual gradient.

Results and Discussion

Dual-gradient separation of anilines

Figure 2 shows the separations of anilines (aniline (2), *p*-ethylaniline (3), *p*-*n*-butylaniline (4), and *p*-*n*-hexylaniline (5)) under various gradient conditions using the DG-CEC system (Fig. 1). Figure 2(A) shows the separation without the use of any gradient (separation in isocratic LC) as a reference. Figure 2(A) shows that complete baseline separation for aniline (peak 2) and *p*-ethylaniline (peak 3) was not achieved, and the elution of *p*-*n*-hexylaniline (peak 5) took 12.4 min. In this acidic eluent (a mixture of acetonitrile and 10 mM phosphate buffer of pH 3.3, 45/55), all anilines were partially protonated. Table 1 lists the electrophoretic mobilities of anilines under various buffer pHs. Since the electrophoretic mobility of *p*-ethylaniline was larger than that of aniline under acidic conditions, the

Table 1 Electrophoretic mobility (m^2/sV) of anilines in various buffer pHs

	pH 2.4	pH 2.8	pH 3.3
Aniline	2.45×10^{-8}	1.83×10^{-8}	1.10×10^{-8}
p-Ethylaniline	2.79×10^{-8}	2.25×10^{-8}	1.35×10^{-8}
p-n-Butylaniline	2.59×10^{-8}	2.20×10^{-8}	1.24×10^{-8}
p-n-Hexylaniline	2.40×10^{-8}	1.98×10^{-8}	1.02×10^{-8}

Medium, mixture of acetonitrile and 10 mM phosphate buffer (45/55); capillary i.d., 0.05 mm; total length, 406 mm; effective length, 287 mm; applied voltage, 15 kV; detection, UV 210 nm.

application of a positive voltage to the outlet end of the column would be effective for improving their insufficient separation. On the other hand, the application of a negative voltage would be suitable to reduce the elution time of *p-n*-hexylaniline (peak 5). In order to meet these conflicting requirements, a gradient in the applied voltage was employed. Figure 2(B) shows the separation of anilines by employing a gradient in the applied voltage, wherein the initial voltage, +10 kV, was decreased to –10 kV in 3 min and maintained constantly at –10 kV. In this case, the larger electrophoretic velocity of *p*-ethylaniline (peak 3) toward the column inlet at an early stage improved its separation from the aniline (peak 2). Their resolutions, R_{es}, shown in Figs. 2(A) and (B), were 1.1 and 1.9, respectively. Furthermore, the application of a negative voltage at a later stage reduced the elution time of *p-n*-hexylaniline (peak 5) to 7.3 min (59% of that obtained from Fig. 2(A)). This result demonstrates that the voltage gradient (which controls the electrophoretic velocity of analytes) effectively regulates the separation in CEC.

Since the electrophoretic velocity (v_{eph}) is represented by $v_{eph} = \mu_{eph}E$ (μ_{eph} and E are the electrophoretic mobility and electric potential gradient, respectively), a combination of appropriate controls in the applied voltage and the electrophoretic mobility would be effective for achieving a further enhancement in the separation efficiency. The acidic eluent will increase the electrophoretic mobility of anilines by enhancing the protonation of amino functional groups. In fact, the electrophoretic mobilities of anilines at pH 2.4 were approximately twice that of those at pH 3.3, as summarized in Table 1. The gradient in the eluent pH to an acidic condition will be effective for enhancing the difference between the electrophoretic mobility of aniline and *p*-ethylaniline during separation. Moreover, the increase in the electrophoretic mobility of *p-n*-hexylaniline will be suitable for efficiently reducing the elution time through electrophoretic migration. Meanwhile, the protonation of anilines also decreases the chromatographic retention to an OSD stationary phase. Therefore, both electrophoretic and chromatographic reductions of the elution time of *p-n*-hexylaniline were provided synergistically.

On the other hand, Fig. 2(C) shows the separation of anilines under an eluent composition gradient, in which the pH of the buffer in the eluent decreased from 3.3 to 2.4 in 7 min. The elution time of *p-n*-hexylaniline (peak 5) was shorter as compared to that of Fig. 2(A). However, the separation between aniline and *p*-ethylaniline was improved only slightly by the eluent gradient alone (the R_{es} increased to 1.4).

Figure 2(D) shows the separation of anilines under dual gradients of both the applied voltage and the eluent composition. In this case, the electrophoretic migration clearly improved the separation between aniline (peak 2) and *p*-ethylaniline (peak 3), and the resolution (2.5) was better than that for the voltage gradient alone, shown in Fig. 2(B). Furthermore, the peak width of aniline and *p*-ethylaniline decreased significantly, *i.e.*, the half widths of peaks 2 and 3 in Fig. 2(D) were 57 and 67% of those in Fig. 2(C), respectively. Similar reductions in the half width can also be observed in Fig. 2(B) (65 and 80% for peaks 2 and 3, respectively, of those in Fig. 2(A)). Although the mechanism for reducing the peak width is not sufficiently clear, the gradient in the applied voltage may have some important function. The electrophoretic velocity of a late-eluting analyte, such as *p-n*-hexylaniline (peak 5), toward the column inlet increased synergistically with increases in both the magnitude of the applied negative voltage and the electrophoretic mobility due to the eluent acidity. Thus, the elution time of *p-n*-hexylaniline (peak 5) under dual gradients was the shortest in Fig. 2 (47% of that in Fig. 2(A)). The relative standard deviations of the elution time of peaks 2 to 4 under dual gradients were 4.8, 8.6, 6.8, and 3.7% ($n = 3$), respectively. Although further study is requisite to improve the precision, the phenomena concerning the improvement in the resolution and the reduction of the analytical time were clearly achieved under the dual-gradient mode.

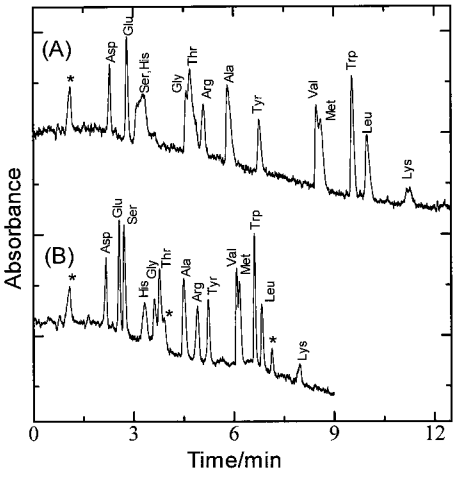

Fig. 3 Separation of fourteen OPA-labeled amino acids by (A) single gradient CEC in eluent composition alone and (B) DG-CEC in both the applied voltage and the eluent composition. Peaks marked with an asterisk (∗) are unknowns. Eluent: (I) acetonitrile/10 mM phosphate buffer (pH 4.8) (10/90), (II) acetonitrile/10 mM phosphate buffer (pH 7.8) (30/70). Linear gradient for (A, B): 0% (II) to 100% (II) in 8 min. Voltage gradient for (B): from +2 to +4 kV in the initial 3.5 min, to 6 kV from 3.5 to 5.0 min, to +10 kV from 5.0 to 6.7 min, and then maintained at +10 kV. Detection: UV 280 nm. The other conditions are the same as those in Fig. 2.

Separation of OPA-labeled amino acids by DG-CEC

The performance of DG-CEC by varying the applied voltage and the eluent pH is demonstrated in Fig. 3 for the separation of OPA-labeled amino acids, which were negatively charged through dissociation of the carboxyl group. Therefore, with regard to the pH level, the gradient in the pH of the mobile phase was varied from acidic to basic in order to increase the negative charge of the analytes. Moreover, the simultaneous gradient of the acetonitrile content of the aqueous eluent was used for the gradient to provide a more satisfactory decrease in the chromatographic retention. In this case, a multistage enlargement in the positive voltage was employed as the gradient in the applied voltage, which resulted in a further improvement of separation, as compared to the voltage gradient with polarity inversion.

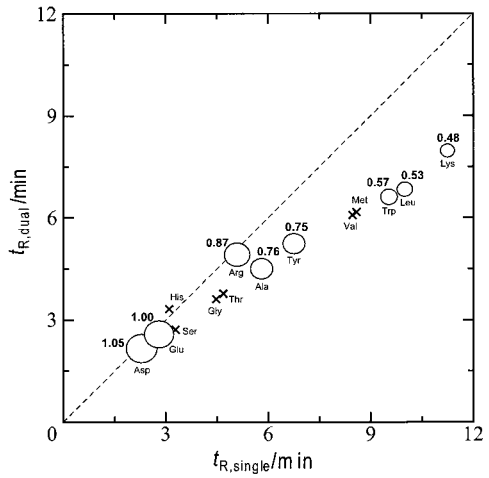

Fig. 4 Relationship between the elution time of amino acids with single and dual gradient. The size of the circle represents the relative half width ($w_{1/2,dual}/w_{1/2,single}$), the values of which are given alongside the circle. The cross mark was used when either one or both half widths were not measured. The chromatographic conditions are the same as those in Fig. 3.

Figure 3 shows the separations of OPA-labeled amino acids with (A) a single gradient in the mobile phase composition alone and (B) dual gradients in both the applied voltage and the eluent composition. The details of the gradients of both the eluent composition and the applied voltage are described in the figure captions. Figure 3(A) shows that the fourteen amino acids were almost separated by the eluent gradient LC, whereas the separations of Ser/His, Gly/Thr, and Val/Met were not achieved. In the dual-gradient mode (Fig. 3(B)), however, the peaks of Ser/His were separated at the baseline and the separations of Gly/Thr and Val/Met were also improved. Furthermore, the entire separation period was reduced.

The elution times of amino acid for single and dual-gradient modes are plotted in Fig. 4. When the half widths of an analyte can be measured under both single and dual gradients, the relative half width ($w_{1/2,dual}/w_{1/2,single}$) is represented by the size of the circle (the values of which are given alongside the circle); otherwise, cross marks are used. As shown in Fig. 4, the distance (shift) from the diagonal line ($y = x$) was enhanced by an increase in the elution time. In the early stages of the eluent gradient (acidic condition), the amino acids developed a smaller negative charge due to a partial dissociation of the carboxyl group, and a positive voltage applied initially to the outlet end of the capillary column was low. Therefore, a lesser electrophoretic migration produced a smaller difference between $t_{R,dual}$ and $t_{R,single}$, *i.e.*, plots with earlier elution lie around the diagonal line. With the progress of the gradient in the mobile phase, a higher pH in the eluent increased the electrophoretic mobility of the analytes through dissociation of the carboxyl group. Since the applied voltage also increased during the gradient procedure, the electrophoretic velocity of the analytes in the mobile phase was synergistically increased. Thus, the contribution of the electrophoretic migration became larger with the progress of the gradients, and the shift from the diagonal line increased.

The dual-gradient also led to a reduction of the peak width. The size of the circle plotted in Fig. 4 became reduced with an increase in the elution time. Since the $w_{1/2,dual}/w_{1/2,single}$ values of Asp and Glu were over 1.00, the peak width reduction effect was not observed for the analytes eluted earlier. Meanwhile, it is interesting to note that the $w_{1/2,dual}/w_{1/2,single}$ values of amino acids eluted later (Trp, Leu, Lys; 0.57 to 0.48) were significantly smaller than their relative elution time ($t_{R,dual}/t_{R,single}$) of approximately 0.7. That is, the analytes eluted later were more sensitive to the peak width reduction as compared to those eluted earlier; *i.e.*, the separation efficiency increased for the analytes eluted later. The retention factor of the analyte around the column outlet end was higher than that around the column inlet end due to gradient elution. Therefore, the migration velocity of the analyte at the frontal zone was lower than that at the rearward zone, and this difference in the velocity would lead to a decrease in the peak width. Since the effect of electrophoretic migration was relatively larger for the analytes eluted later, a significant decrease in the peak width was observed with Trp, Leu, and Lys.

Conclusions

Dual-gradient capillary electrochromatography, in which both the eluent composition and the applied voltage are varied during the separation procedure, was developed in this study. The dual-gradient method was more effective in increasing the resolution and reducing the separation time of the ionized analyte than the single-gradient methods. Although a mechanism for increasing the separation efficiency has not been clarified sufficiently, the heterogeneous separation field, both in time and space, may have an important role. Further study of this field is currently in progress.

References

1. T. Tsuda (ed.), "*Electric Field Applications in Chromatography, Industrial and Chemical Processes*", **1995**, VCH, Weinheim.
2. F. Steiner and B. Scherer, *J. Chromatogr., A*, **2000**, *887*, 55.
3. I. S. Krull, A. Sebag, and R. Stevenson, *J. Chromatogr., A*, **2000**, *887*, 137.
4. S. Eeltink, G. P. Rozing, and W. T. Kok, *Electrophoresis*, **2003**, *24*, 3935.
5. F. Svec (ed.), *J. Chromatogr., A (Special issue for electrochromatography)*, **2004**, 1044.
6. J. M. Cintron and L. A. Colon, *J. Chromatogr., A*, **2006**, *1106*, 256.
7. S. Kitagawa, A. Tsuji, H. Watanabe, M. Nakashima, and T. Tsuda, *J. Microcol. Sep.*, **1997**, *9*, 347.
8. R. Nakashima, S. Kitagawa, T. Yoshida, and T. Tsuda, *J. Chromatogr., A*, **2004**, *1044*, 305.
9. C. A. Rimmer, S. M. Piraino, and J. G. Dorsey, *J. Chromatogr., A*, **2000**, *887*, 115.
10. J. P. Quirino, M. T. Dulay, and R. N. Zare, *Anal. Chem.*, **2001**, *73*, 5557.
11. K. Ohyama, M. Wada, N. Kishikawa, Y. Ohba, K. Nakashima, and N. Kuroda, *J. Chromatogr., A*, **2005**, *1064*, 255.
12. Y. Ueki, T. Umemura, J. Li, T. Odake, and K. Tsunoda, *Anal. Chem.*, **2004**, *76*, 7007.
13. P. N. Nesterenko and M. A. Rybalko, *J. Anal. Chem.*, **2005**, *60*, 349.
14. B. Xin and M. L. Lee, *J. Microcol. Sep.*, **1999**, *11*, 271.
15. E. F. Hilder, C. W. Klampfl, and P. R. Haddad, *J. Chromatogr., A*, **2000**, *890*, 337.
16. P. Lindroth and K. Mopper, *Anal. Chem.*, **1979**, *51*, 1667.