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Proline-coated column for the capillary electrochromatographic separation of amino acids by in-column derivatization

With 3-trimethoxysilylpropyl chloride as the spacer, a proline-coated capillary column was prepared for the capillary electrochromatographic (CEC) separation of amino acids by in-column derivatization. Nine standard mixtures, including aspartic acid, glutamic acid, valine, phenylalanine, alanine, isoleucine, leucine, tyrosine, and tryptophan, were injected. *o*-Phthalaldehyde (OPA), OPA/2-mercaptoethanol (2-ME) and OPA/*N*-acetylcysteine (NAC) in borate buffer were tested as the derivatizing agent. Among them, OPA (50 mM) in borate buffer (pH 9.5, 50 mM) gave the best performance. The formation of isoindole could be detected by UV detection. The sandwich-type injection was carried out in hydrostatic mode (10 cm) with the program R(10 s)S(10 s) R(10 s)W(10 min) with R, S, and W being the reagent, sample, and waiting times. Mesityl oxide, benzyl alcohol, and acetone showed some interaction with the column. A current monitoring method was used instead of the determination of the electroosmotic flow (EOF). The direction of EOF was from anode to cathode even under acidic condition lower than the p*I* value (6.31) of the bonded group due to some unreacted silanol groups. Some parameters including pH, nature, and concentration of the mobile phase and the effect of organic modifier with regard to the CEC separation were investigated. With the proline-coated column (75 (50) cm $\times$ 75 μm ID) the best separation was performed in phosphate buffer (pH 4.00, 100 mM) with an applied voltage of -15 kV. The established method was also compared with those precolumn derivatized prior to the separation with proline-coated column as well as with in-capillary derivatization and separation with a bare fused-silica column.

Keywords: Amino acids / Capillary electrochromatography / In-column derivatization / Proline-coated column / Sandwich-type injection DOI 10.1002/elps.200406037

1 Introduction

Derivatization is a modification intended to give the analytes of interest more suitable characteristics. The most important ones are improvement of the detectability, sample preparation, separation, and stability. In recent years, capillary electrophoresis (CE) has gained popularity as a separation technique due to its great efficiency and the small sample volumes required. The procedures can be performed before, during, or after the electrophoretic separation [1, 2]. In 1992, Saito *et al.* [3] reported on an in-column derivatization HPLC method that involves simultaneous separation and derivatization on an octadecylsilica (ODS) column with a mobile phase of acetonitrile and borate buffer (pH 9.9) containing *o*-phthalaldehyde (OPA)/ *N*-acetylcysteine (NAC). Oguri *et al.* [4] used OPA and NAC for the determination of amino acids by an on-line mode in capillary derivatization HPCE method linked with fluorescence detection. The method was improved by including β-cyclodextrin and phosphate-borate buffer (pH 10) in the running buffer. Latorre *et al.* [5] have also reported that after in-capillary derivatization with 1,2-naphthoquinone-4-sulfonate, amino acids could be separated in a fused-silica capillary with a borate buffer using 2-propanol as organic modifier at an applied voltage of + 15 kV. Taga and co-workers [6] reported on the microreaction by various in-capillary derivatization methods. The proposed methods included at-inlet, throughout capillary [7], and zone-passing techniques [8]. They were evaluated using amino acids and OPA as a model system. The advantage of in-capillary derivatization is its use without changing the setup of the commercially available CE devices. The criteria for in-capillary derivatization

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Abbreviations: 2-ME, 2-mercaptoethanol; **NAC**, *N*-acetylcysteine; **OPA**, *o*-phthalaldehyde

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should be: kinetically fast, high reproducibility for the reaction, high molar absorptivity for the product, and complete resolution for the derivatized product and the reagent.

The CE separation is mainly based on differences in solute size and charge at a given pH. Since the size and charge for most amino acids are similar, additional selectivity is frequently required to resolve complex mixtures. In our laboratory, a series of permanent wall-coated capillary columns have been prepared and employed as stationary phases of CEC [9–16]. Proline and hydroxyproline or their acylamide and *N*-alkyl derivatives have been used as BGE in the ligand-exchange mode of CE or MEKC for enantiomeric separation [17–21]. Up to now, to our knowledge, no wall-coated proline capillary column has been prepared as stationary phase of CEC, although copper complex with proline acylamide as chiral stationary phases, chemically modified on monolithic columns by CEC, has been reported by Chen *et al.* [21]. Proline could offer the hydrophobic force, hydrogen bonding, and steric effect for good separation of the analytes. Due to the lack of a primary amino group on the proline molecule, a competition reaction with the analytes toward the OPA reagent will not occur. Accordingly, proline was selected as the coated functional group of the capillary column. The prepared column was evaluated for its performance as the stationary phase for the CEC separation of amino acids after in-capillary derivatization with OPA by sandwich-type injection.

2 Materials and methods

2.1 Apparatus

All experiments were carried out in a laboratory-built unit. It consisted of a ±30 kV high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) and a UV-visible detector (Spectra System UV3000, Thermo Separation Products, CA, USA). Electrochromatograms were recorded and processed with a TSP ChromQuest (Thermo Separation Products, Fremont, CA, USA) and PC SISC-Lab data acquisition system (Scientific Information Service, Taiwan). Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) had 75μm ID and a total capillary length of 75 cm, with a distance of 50 cm between the injection end and the detection window.

2.2 Reagents and chemicals

Most chemicals were analytical reagent-grade from Merck (Darmstadt, Germany). Purified water (18 MΩ·cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions. Acetone, methanol, methyl sulfoxide, 2-mercaptoethanol (2-ME), NAC, phosphoric acid, 1-propanol, OPA, sodium phosphate monobasic, and toluene were from Acros (Geel, Belgium), benzyl alcohol, sodium phosphate dibasic, sodium phosphate tribasic from Merck, mesityl oxide from Ferak (Berlin, Germany), DL-amino acids from Sigma (St. Louis, MO, USA), ethanol and triethylamine (TEA) from Wako (Japan), and 3-trimethoxysilylpropyl chloride (TMS-Cl) was from TCI (Tokyo, Japan). Stock solutions of the amino acids (10 mM) were prepared in pure water and diluted appropriately prior to use. All solvents and solutions for CEC analysis were filtered through a 0.45 μm PTFE (Millipore) or cellulose acetate membrane (Adventec MFS, USA).

2.3 Functionalization of the capillary column

Fused-silica capillaries (75 μm ID) were first flushed with 0.2 M NaOH (30 min), then pure water (15 min), 0.1 M HCl (30 min), and pure water (15 min). Before silanization, the capillaries were rinsed with methanol (5 min) and then dried in a gas chromatography oven at 110°C for 1 h under a nitrogen flow of 2.0 kg·cm^{-2}. The capillaries were purged with nitrogen for 20 min, then dried at 110°C overnight. For functionalization of the capillary, the capillary column was first filled with 3-trimethoxysilylpropyl chloride (10% v/v) in toluene, then plugged with GC septa and reacted at 110°C for 12 h. The silylated capillary was rinsed with toluene to remove unreacted material. Then the solution of L-proline (48.5 mg) dissolved in a mix solvent containing methanol (400 μL) and triethylamine (20 μL) was used to fill the silylated capillary and reacted in the GC oven at 110°C for 12 h. The prepared column was further rinsed with acetone for 30 min and then purged with nitrogen.

2.4 At-inlet derivatization

The procedure was followed as described in [6]. A sample solution of selected amino acids and the reagent (OPA) solution were introduced successively to the proline-coated capillary column for various durations in the sandwich mode, and the resultant succession of plugs of the sample and reagent solution were allowed to stand for specified times.

2.5 Electrophoresis conditions

Before analysis, the proline-coated columns were preconditioned with the running buffer. They were rinsed with buffer between runs at 1 or 2 min intervals. The samples

CE and CEC

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were injected by siphoning at a height difference of 10 cm for 10 s. The samples were detected by UV light absorption measurement at 230 nm.

3 Results and discussion

3.1 Characterization of the prepared column

The procedures for the preparation of the permanent wall-coated proline in the fused-silica capillary are shown in Fig. 1, where 3-trimethoxysilylpropyl chloride was used for the activation of the inner wall of the fused-silica capillary and as spacer of the functional group. For characterization of the prepared column, both the direction and the magnitude of the EOF were measured. However, no peak could be detected within 120 min under the tested pH (3–5) regardless of whether mesityl oxide, benzyl alcohol, or acetone was used as neutral marker. This was the case whether the marker was injected from the anode or from the cathode. Some interaction of these compounds toward the coated column might be the reason. Hence, the current monitoring method developed by Huang *et al.* [22] was adopted for EOF measurement.

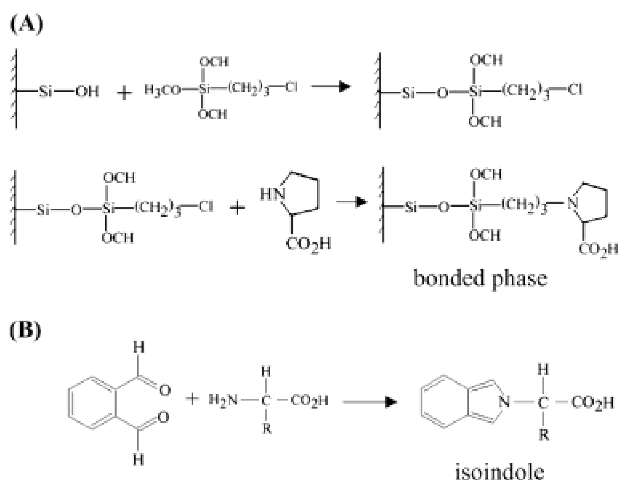

Figure 1. (A) Procedures for the covalent surface modification of the fused-silica capillary column. (B) Isoindole formation for the amino acids derivaized with OPA.

The experiment was carried out with the capillary column of 75 cm × 75 μm ID. With the polarity of the power supply being positive, 80 mM phosphate buffer was placed in the inlet end for replacing 100 mM phosphate buffer in the capillary tube. The inflection points for each curve and the EOFs at different pH values are summarized shown in Table 1. While with the polarity of the power supply being negative, a similar process was carried out. A different phenomenon was indicated where the current did not change throughout. The result illustrates that 80 mM buffer did not pass through the capillary tube. Based on the above experimental results, the direction of EOF was from anode to cathode.

Table 1. Current monitoring method for the determination of μ_{EOF}[a)]

pH	Current (μA)	Inflection point (min)	μ_{EOF}[d)] ($cm^2 \cdot V^{-1}s^{-1}$)
4.00	58[b)]–52[c)]	42.10	1.48×10^{-4}
4.50	50–40	35.04	1.78×10^{-4}
5.00	49–44	30.72	2.03×10^{-4}
6.00	58–48	26.88	2.33×10^{-4}
7.00	94–78	24.00	2.60×10^{-4}
8.00	144–122	23.00	2.71×10^{-4}

a) The experiment was carried out with a capillary column of 75 cm × 75 μm ID, with the polarity of the power supply being positive (+15 kV); 80 mM phosphate buffer was placed in the inlet end for replacing 100 mM phosphate buffer in the capillary tube.
b) Current at the starting point
c) Current at the plateau
d) $\mu_{EOF} = v_{EOF}/E = L_t^2/t_R \cdot V$ where v_{EOF} is the velocity of the EOF, E is the field strength, L_t is the column length, t_R is the retention time, and V is the applied voltage.

The p*I* value of proline is 6.30. Although the hydrogen of the imino group on the proline molecule has been substituted (Fig. 1A), the tertiary amino group still can be protonated. Therefore, the effective charge should be calculated from the protonated tertiary amino group and the carboxylic acid of the bonded phase. The p*I* value of the bonded phase is 6.31. It can be concluded that EOF reversal should be at a pH lower than its p*I* value. However, the current monitoring method indicated that direction of EOF is from anode to cathode. The contradictory results might be due to the presence of some unreacted silanol groups.

3.2 The choice of the derivatizing agent

In a preliminary experiment, several derivatization reagents, such as OPA (50 mM) in borate buffer (pH 9.5, 50 mM), OPA (50 mM)/2-ME (50 mM) in borate buffer (pH 9.5, 50 mM), and OPA (50 mM)/NAC (50 mM) in borate buffer (pH 9.5, 50 mM), were tested as reagent with sandwich-type injection at-inlet derivatization mode for the CEC separation. The results are shown in Fig. 2, where R, S, and W are the abbreviations for reagent, sample, and waiting time, respectively. The number in the parentheses indicate the duration for each process. Nine standard

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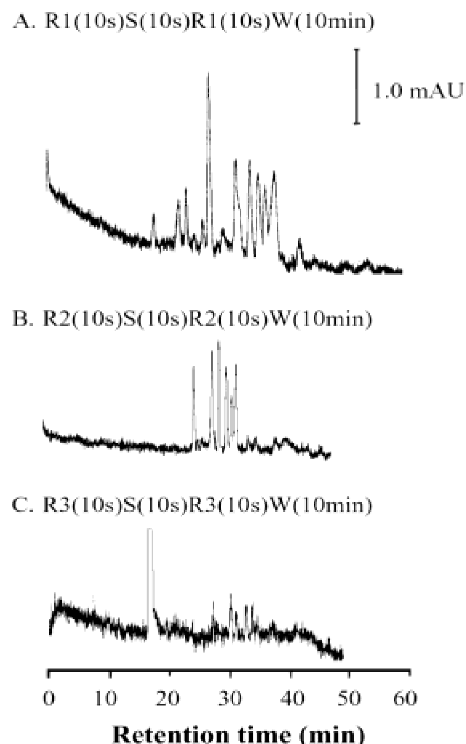

Figure 2. Electrochromatograms of amino acids derivatized with various reagents by the at-inlet injection technique using the sandwich program. Proline wall-coated column with 75 cm (50 cm) × 75 μm ID; sample concentration (mM): Ala, 1.61; Asp, 0.61; Glu, 0.48; Ile, 0.48; Leu, 0.52; Val, 0.50; Phe, 0.34; Trp, 0.45. Hydrostatic injection (10 cm) with R(10 s)S(10 s)R(10 s)W(10 min); mobile phase, phosphate buffer (pH 4.0, 100 mM); applied voltage, −15 kV; detection, UV 230 nm. (A) R_1 (50 mM OPA); (B) R_2 (50 mM OPA and 50 mM 2-ME); (C) R_3 (50 mM OPA and 50 mM NAC). All solutions in borate buffer (pH 9.5, 50 mM).

mixtures, including aspartic acid, glutamic acid, valine, phenylalanine, alanine, isoleucine, leucine, tyrosine, and tryptophan, were injected. OPA in borate buffer gave the best performance. A smaller peak intensity and lesser peak number were found for OPA and 2-ME. A greater signal-to-noise ratio and the poorest separation performance was found for OPA and NAC. For this reason, OPA in borate buffer (pH 9.5, 50 mM) was the first choice as derivatization reagent. The product, isoindole (Fig. 1B), could be analyzed with UV detection but not with fluorescence.

3.3 Effect of standing time

One key aspect of employing the derivatization reaction is allowing for an adequate period of time for the sample-reagent reaction to take place. Data illustrating the effect of reaction period are shown in Fig. 3. In all cases, the amount of reagent both before and after the sample plug was kept in large excess over that of the sample. At pH 4.0 the surface of the capillary column carries a negative charge (Table 1) and derivatized amino acids are mainly present as anionic species since the amino group is substituted by OPA and only the dissociation of the carboxyl group is involved (Fig. 1B). Thus, the sample was injected from the negative end. After the voltage is applied, the derivatives would migrate faster than the unreacted reagent. With a waiting time of 5 min for the derivatization, aspartic acid and glutamic acid were coeluted. Increasing the duration to 10 min gave complete resolution for the pair of aspartic acid and glutamic acid. Increased waiting time yielded a greater response of the derivatized product (Fig. 3), and also to longer retention times, especially for the late eluted analytes. Among the analytes, aspartic acid and glutamic acid both are polar with an extra carboxyl group. In other words, the greater effective charge and

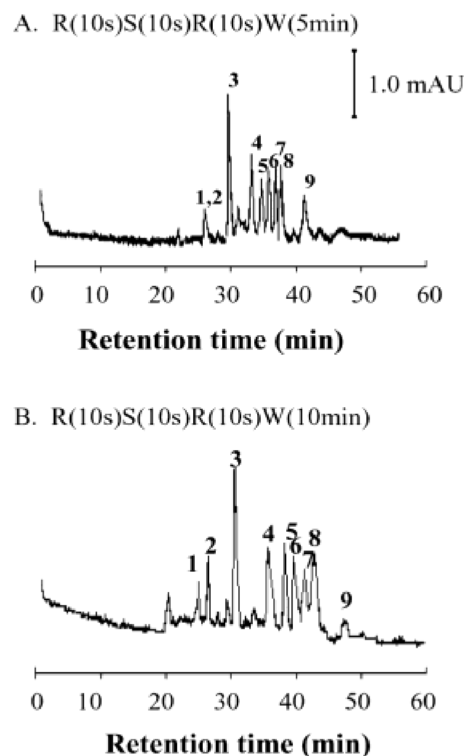

Figure 3. Electrochromatograms of amino acids derivatized with OPA by variation the waiting time of sandwich-type injection. Proline wall-coated column with 75 cm (50 cm) × 75 μm ID; sample concentration (mM): Ala, 1.61; Asp, 0.61; Glu, 0.48; Ile, 0.48; Leu, 0.52; Val, 0.50; Phe, 0.34; Trp, 0.45. Mobile phase, phosphate buffer (pH 4.0, 100 mM); applied voltage, −15 kV; detection, UV 230 nm; hydrostatic injection (10 cm) with waiting time: R(10 s)S(10 s)R(10 s)W(× min): (A) 5 min, (B) 10 min. Peak identification: 1, Asp; 2, Glu; 3, Val; 4, Phe; 5, Ala; 6, Ile; 7, Leu; 8, Tyr; 9, Trp.

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the similar structure led to early coelution. However, with increasing waiting time, more derivatized products would form and they are separated more easily than the parent compound due to more hydrophobic interaction with the bonded proline group. With a reaction time longer than 10 min, no significant different retention behavior for these analytes was seen. Thus, a waiting time of 10 min was chosen for further work.

3.4 Effect of the amount of the reagent

Initially the injection program was R(10 s)S(10 s)R(10 s) W(10 min). With more reagent after the sample plug: R(10 s)S(10 s)R(20 s)W(10 min), a greater response of the analyte was found, but coelution of leucine and tyrosine was found (Fig. 4). With injection of more reagent before sample introduction: R(20 s)S(10 s)R(10 s)W(10 min), a greater signal noise was found. Although better resolution for all the analytes resulted, longer analysis time was needed due to the more neutral reagent, blocking the migration of the derivatives. From these results it can be stated that the best separation for the mixture was obtained using the R(10 s)S(10 s)R(10 s)W(10 min) injection program.

3.5 pH of the mobile phase

The proline coating on the surface of the inner wall of the fused-silica capillary provides the fixed charge sites for generation of EOF under CEC conditions. Since analytes migrate in the counter direction of EOF, only a weak acidic condition was tested to see the retention behavior. At pH 3.50, all analytes could be resolved by injecting nine standard mixtures. Increasing the pH from 3.5 to 4.0 gave a wider range of the elution time (Fig. 5), probably due to a greater EOF and more fractions of the dissociated compound when the pH was increased. By further increasing the pH to 4.29, overlapping between glutamic acid and valine as well as leucine and tyrosine was found. Finally the pH was increased to 4.50 and 5.00, resulting in longer retention times. More peak overlappings were observed (Figs. 5D and E). It is well-known that the siloxane bond is stable over the pH range 4–7 for long-term use. Although a pH range of 3.50–4.00 could provide better performance of the CEC separation, a pH of 4.00 was chosen as the testing condition.

3.6 Concentration of the mobile phase

With regard to the effect of the mobile phase concentration on the retention behavior of the analyte, the concentration of mobile phase over the range from 50 to 200 mM was studied. In phosphate buffer (pH 4.0, 100 mM), complete separation of the nine amino acids was achieved (Fig. 5B). But on decreasing the phosphate buffer to 50 mM, slower migration of the analytes was indicated, especially for the early-eluted ions. Some overlapping for alanine and isoleucine was shown. Since the analytes migrate in the counter direction with the EOF, the above-mentioned phenomenon might be due to greater EOF velocity and

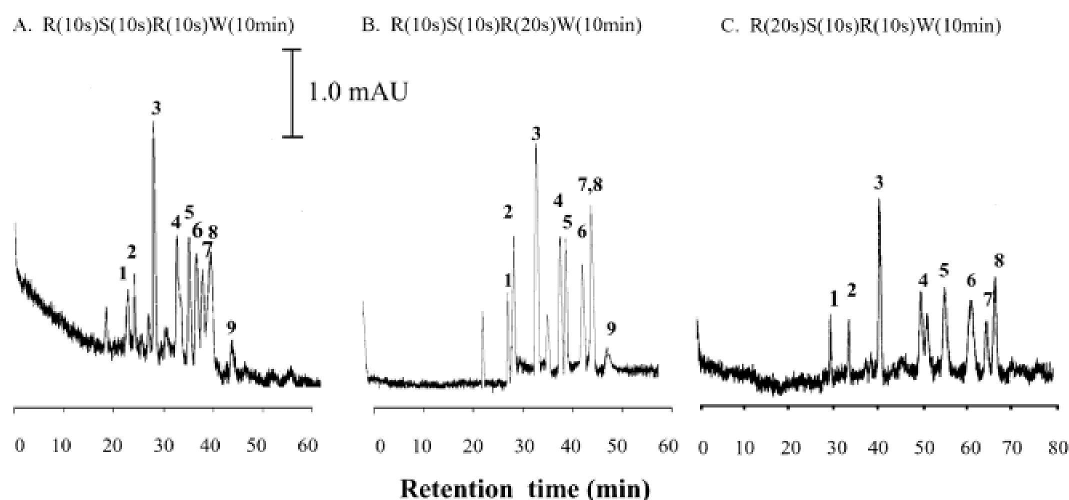

Figure 4. Electrochromatograms of amino acids derivatized with OPA by variation the reagent amount before and after the sample zone. Conditions as in Fig. 3, except (A) R(10 s)S(10 s) R(10 s)W(10 min); (B) R(10 s)S(10 s)R(20 s)W(10 min); (C) R(20 s)S(10 s)R(10 s)W(10 min). Peak identification as in Fig. 3.

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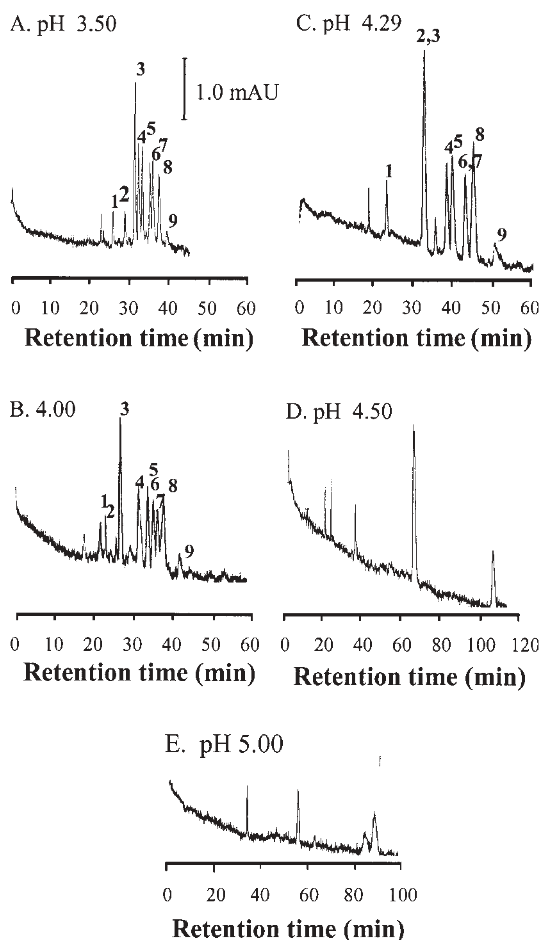

Figure 5. Electrochromatographic separation of amino acids derivatized with OPA at various pH values. Conditions as in Fig. 3, except injection as R(10 s)S(10 s)R(10 s) W(10 min). (A) pH 3.50; (B) pH 4.00; (C) pH 4.29; (D) pH 4.50; (E) pH 5.00. Peak identification as in Fig. 3.

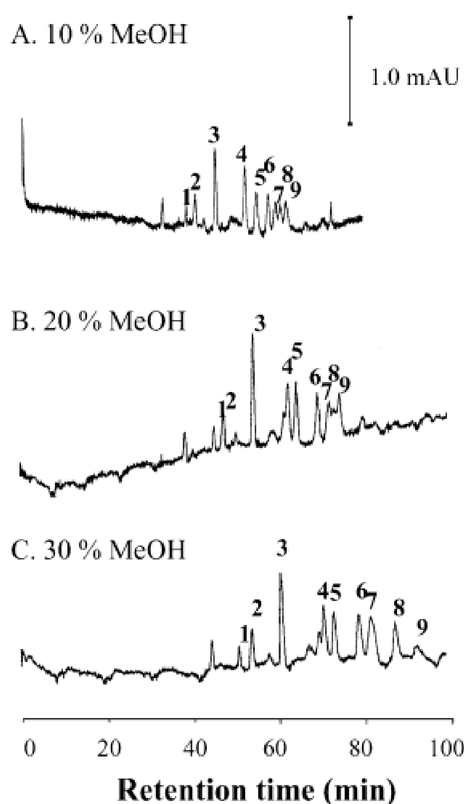

Figure 6. Electrochromatographic separation of amino acids in phosphate buffer with the addition of methanol. Conditions as in Fig. 3, except phosphate buffer (pH 4.00, 100 mM) with the addition of methanol. Peak identification as in Fig. 3.

less dissociation of the analyte as buffer concentration decrease. On increasing the phosphate buffer to 150 mM and further to 200 mM, as expected, faster migration and more peak overlapping were observed.

3.7 Organic modifier for the mobile phase

Besides the stationary phase, an important factor in any chromatographic separation is the mobile phase composition. We next investigated the effect of the addition of organic modifier on the CEC separation. The retention of the analytes increases with an increase in methanol concentration from 10% to 30% (Fig. 6). In addition, an improvement for the resolution of leucine and tyrosine was also indicated. When ethanol was used instead, complete separation was demonstrated but the retention time was much longer than that in methanol at the corresponding percentage. Even in the presence of 5% ethanol, the separation time was around 80 min. The viscosity of ethanol is greater than that of methanol but the polarity is similar. This would lead to smaller EOF and faster separation. The unexpected behavior might be due to more complicated hydrophobic interaction among the derivative analytes, mobile phase, and stationary phase.

3.8 Retention mechanism

Derivatization at the capillary inlet in HPCE has been investigated by Taga and Honda [6]. They reported that the separations of leucine and isoleucine, valine and threonine, and alanine and serine were difficult in an alkaline medium (pH 10.0) but lowering the pH to 6.8 and the addition of CHAPS, a cationic surfactant, to a concentration of 3.2 mM resulted in rather good separations. However, even under these conditions, the separation of leucine and isoleucine was not complete. The sandwich injection program they used was R(3 s)S(1 s)R(3 s) W(20 min). They claimed that the separation mechan-

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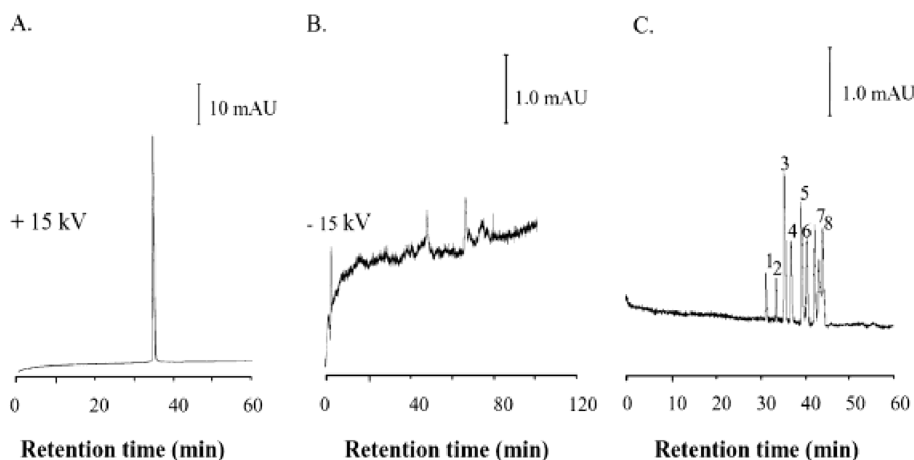

Figure 7. Separation of amino acids with other modes for the comparison with the method established in Fig. 5B. (A) All conditions as in Fig. 5B but with sandwich-type injection from the anodic end, and separated by the bare fused-silica column. (B) Condition was as in (A) but sample injection was from the cathodic end. (C) Conditions as in Fig. 5B except the sample was prepared by the precolumn derivatization method and then separated by proline wall-coated column. Peaks: 1, Asp; 2, Glu; 3, Val; 4, Phe; 5, Ala; 6, Ile; 7, Leu; 8, Tyr.

ism was mainly electrophoretic, whereas hydrophobic interaction and ion pair formation should also be considered.

In this work, a column coated with proline was used for the CEC separation of the amino acids. The elution order was Asp (pK_a 2.09) > Glu (2.19) > Val (2.32) > Phe (1.83) > Ala (2.34) > Ile (2.36) > Leu (2.36) > Tyr (2.20) > Trp (2.38). In Taga's work, the elution order was His > Lys > Tyr > Leu > Ile > Val > Thr > Ser > Gly > $(Cys)_2$ > Glu > Asp. Aspartic acid was eluted last with a retention time of more than 160 min. In our case, aspartic acid was eluted first. The isomers, leucine and isoleucine, were well resolved. A different separation mechanism is evident. In order to further elucidate the retention mechanism, under the optimum conditions of our work, both bare fused-silica column with sandwich-type injection and precolumn methods were also compared (Fig. 7). Only one peak was observed for the nine compounds injected with the separation voltage of +15 kV. When the polarity of the separation voltage was changed into −15 kV, three small peaks were found. The phenomena indicated that electrophoresis could not be acting as the unique mechanism for the separation. Therefore, it can be concluded that the proline functional group coated on the surface of the fused-silica capillary played the main role in the separation mechanism. In other words, besides electrophoresis, hydrophobic and hydrogen bonding interaction between the derivatives and the proline coating might be the predominant force for the separation. By further considering the elution order, it mostly followed the order of the dissociation constant, except for phenylalanine and tyrosine. A greater hydrophobic force toward the proline coating for these amino acids with the aromatic ring was obvious. As compared with the precolumn derivatization method, the elution order was similar to the in-capillary derivatization method. The selectivity might mainly stem from the proline-coated column.

4 Concluding remarks

In this work, a proline-coated column was prepared for the CEC separation of an amino acid mixture by in-capillary derivatization with OPA (50 mM) in borate buffer (pH 9.5, 50 mM). In phosphate buffer (pH 4.00, 100 mM), with sandwich-type injection of R(10 s)S(10 s)R(10 s) W(10 min), applied voltage of −15 kV, mixture of nine amino acids including Asp, Glu, Val, Phe, Ala, the isomers Ile and Leu, Tyr, and Trp could be well separated. The reproducibility of the retention time (*n* = 4) expressed as the average of RSD was around 2.5% (Table 2). The column could be used continuously for longer than two months and no significant EOF change was shown.

Besides the electrophoretic mobility, hydrophobic interaction and hydrogen bonding played an important role in CEC separation. Although the resolution of the precolumn derivatization method is better than that of the in-capillary derivatization method, dramatically less reagent is required for the latter (OPA: 20 nL; sample: 10 nL) than the former (at least μL level of OPA and sample mixture for the batch preparation of the derivative) was needed. In other words, with almost no waste solution, short

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Table 2. Reproducibility for the retention behavior of amino acids derivatized in capillary with the sandwich-type injection[a)]

Analyte	t_R (min)[b)]	RSD (%)	t_R (min)[c)]	RSD (%)
Asp	25.90	0.77	24.90	1.92
Glu	25.90	0.77	26.40	2.84
Val	29.79	2.40	30.24	2.36
Phe	32.40	3.87	35.52	2.98
Ala	34.08	4.19	37.92	2.29
Ile	35.52	3.80	39.36	1.63
Leu	36.48	4.09	40.24	1.09
Tyr	37.44	3.65	42.24	2.43
Trp	40.08	2.23	47.04	3.66

a) Conditions as in Fig. 3; the data was obtained from the average of four measurements.
b) R(10 s)S(10 s)R(10 s)W(5 min)
c) R(10 s)S(10 s)R(10 s)W(10 min)

analysis time, and requirement for only small volumes of analytes, our method is especially advantageous for biological assays.

The authors thank the National Science Council of Taiwan for financial support.

Received March 15, 2004

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