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Stacking and separation of aspartic acid enantiomers under discontinuous system by capillary electrophoresis with light-emitting diode-induced fluorescence detection

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ABSTRACT

We describe the stacking and separation of D- and L-aspartic acid (Asp) by capillary electrophoresis (CE) with light-emitting diode-induced fluorescence detection (LEDIF). In the presence of cyanide, D- and L-Asp were derivatized with naphthalene-2,3-dicarboxaldehyde (NDA) to form fluorescent derivatives prior to CE-LEDIF. The separation of NDA-derivatized D- and L-Asp was accomplished using a discontinuous system – buffer vials contained a solution of 0.6% poly(ethylene oxide) (PEO), 150 mM sodium dodecyl sulfate (SDS), and 60 mM hydroxypropyl-β-cyclodextrin (Hp-β-CD), while a capillary was filled with a solution of 150 mM SDS and 60 mM Hp-β-CD. The role of PEO, Hp-β-CD, and SDS is to act as a concentrating media, as a chiral selector, and as a pseudostationary phase, respectively. This discontinuous system could be employed for the stacking of 600 nL of NDA-derivatized D- and L-Asp without the loss of chiral resolution. The stacking mechanism is mainly based on the difference in viscosity between sample zone and PEO as well as SDS sweeping. The limits of detection at signal-to-noise of 3 for D- and L-Asp were down to 2.4 and 2.5×10^{-10} M, respectively. Compared to normal sample injection volume (25 nL), this stacking approach provided a 100- and 110-fold improvement in the sensitivity of D- and L-Asp, respectively. This method was further applied for determining D- and L-Asp in cerebrospinal fluid, soymilk, and beer.

1. Introduction

Amino acids are essential compounds in a biological system because they are ubiquitous in cerebrospinal fluids, food products, and plant leaves [1–3]. Except for glycine, all amino acids exhibit optical activity. Although L-amino acids are mainly present in higher animals, some D-amino acids, such as D-serine and D-aspartic acid (D-Asp), are distributed in tissues and body fluids of vertebrates [4–6]. D-Asp is one of the most important D-amino acids, since the level of D-Asp in human body fluids are implicated in various diseases such as Alzheimer's disease, Parkinson's disease and renal disease [7–9]. Currently available methods for measuring D-Asp in biological tissues and fluids include an enzyme-linked immunosorbent assay [10], radionuclide labeling [11], gas chromatography [12,13], and high-performance liquid chromatography [14,15]. Since, the advantages of capillary electrophoresis (CE) include small injection volumes of sample, low consumption of solvents used, short analysis run time and high separation efficiency. Therefore it is a promising separation method for determining D-Asp [16,17]. When D- and L-Asp were derivatized with *o*-phthaldialdehyde (OPA) and N-acetyl-L-cysteine, the formed diastereomers could be easily separated by either capillary zone electrophoresis [18] or derivatized with (1/2)-1-(9-anthryl)-2-propyl chloroformate (APOC) by micellar electrokinetic chromatography (MEKC) [19]. The determination of D-Asp in the rat pineal gland was successfully achieved by adding 2,3,6-tri-*O*-methyl-β-cyclodextrin (chiral selector) to the background electrolyte (BGE) and using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD) as a derivatizing agent [20]. In contrast to OPA and NBD, the derivatization of amino acids with naphthalene-2,3-dicarboxaldehyde (NDA) offers several advantages, including rapid reaction kinetic, long excitation wavelength, high stability, and excellent sensitivity [21,22]. Due to these characteristics, Zhao et al. selected NDA to derivatize D- and L-Asp. The formed NDA derivatives were well separated by β-cyclodextrin-mediated MEKC [23]. Moreover, the combination of β-CD-mediated MEKC and NDA provided sufficient selectivity and sensitivity to monitor the D-Asp content in specific subregions of a single neuron [24]. Recently, the separation of 13 pairs of amino acid enantiomers, including

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D- and L-Asp, has been accomplished under low-pH conditions by adding a mixture of sulfated-β-CD and sodium dodecyl sulfate (SDS) to the BGE [25]. Moreover, sulfated-β-CD-mediated MEKC was further utilized for on-line sample stacking of D-serine, L-serine, D-glutamate, and L-glutamate. However, the application of this stacking technique to the biological samples was unsuccessful due to the high ionic strength of the sample. Besides, poor quantum yield of NDA derivatives was observed at low pH.To overcome these problems, the combination of poly(ethylene oxide)-based stacking and hydroxypropyl-β-CD-mediated MEKC was developed for on-line sample stacking and separation of 3 pairs of NDA-derivatized amino acid enantiomers in urine and plasma. During the separation, hydroxypropyl-β-CD (Hp-β-CD), SDS, and poly (ethylene oxide) (PEO) acted as a chiral selector, as a pseudostationary phase, and as a concentrated medium, respectively [26]. It should be noted that PEO-based stacking method has been demonstrated to be capable of concentrating a variety of analytes, such as DNA [27], RNA [28], proteins [29], and amino acids [30].

This study expands upon our prior publication and show that the combination of Hp-β-CD-mediated MEKC and PEO-based stacking can be used to detect D- and L-Asp in biological and beverage samples. Several important electrophoretic parameters, such as SDS concentration, Hp-β-CD concentration, and injection volume, were evaluated in terms of separation resolution and stacking efficiency. The practicality of the proposed approach was validated by the determination of D- and L-Asp in cerebrospinal fluid (CSF), beer, and soymilk.

2. Experimental

2.1. Chemicals and preparation

DL-Asp, NaCN, $Na_2B_4O_7$, SDS, Hp-β-CD, PEO (M_w 8,000,000 g/mol), NaOH, methanol, and acetonitrile were obtained from Sigma–Aldrich (St. Louis, MO, USA). NDA was purchased from Tokyo Chemical Industry (Tokyo, Japan) and prepared in methanol. Tris was a product from J.T. Baker (Phillipsburg, NJ, USA). Boric acid was purchased from Riedel-deHaën (Buchs, Switzerland). A stock solution of 400 mM Tris-borate (TB) was prepared by dissolving 24.23 g of Tris in 500-mL aqueous solution that was adjusted with suitable amounts of boric acid to pH 9.0. Unless otherwise noted, the molarity of Tris represents that of TB buffer. PEO (0.1–1.0%, w/v) was gradually added to each of prepared 150 mM TB solutions at pH 9.0. During the addition of PEO, a magnetic stirring rod was used to produce a well-homogeneous suspension. After the addition was completed, the solutions were stirred for at least 12 h. Before CE separation, the solutions were degassed with a vacuum system in an ultrasonic tank for 30 min. All the prepared solutions were stored at 4 °C and used within a week. All other chemicals were of analytical grade.

2.2. CE-LEDIF

A CE with LED-induced fluorescence (CE-LEDIF) system (CE/LED-IF, Model: 2100) was purchased from Pebio Scientific Company (Taipei, Taiwan). A violet LED (InGaN; type, No M053UVC; Monarchal Electronics; price: <2.0 US$) was purchased on the Taipei electronic market; its luminous intensity is 300 mcd (operating current: 20 mA; viewing angle: $2\theta_{1/2} = 30^\circ$; peak emission wavelength: 410 nm; spectral half width: 15 nm). The fluorescence was collected through a 10× objective (numerical aperture: 0.25). One interference filter (488 nm) was used to block scattered light before the emitted light reached the photomultiplier tube (R928, Hamamatsu Photonics K. K., Shizuoka-Ken, Japan). The fluorescence signal was transferred directly through a 10-kO resistor to a 24-bit A/D interface at 10 Hz (Borwin, JMBS Developments, Le Fontanil, France) and stored in a personal computer. Before CE analysis, the capillaries (Polymicro Technologies, Phoenix, AZ, USA; 75 μm I.D. and 360 μm O.D.) were treated with 1 M NaOH solution overnight to obtain a high electroosmotic flow (EOF) [31]. The capillary was filled with a solution containing 150 mM TB, 70–165 mM SDS and/or 30–66 mM Hp-β-CD, while the sample was injected by raising the capillary inlet 20-cm height for a period of time up to 180 s. Subsequently, the ends of the capillary were immersed in the cathodic and anodic vials containing 150 mM TB, 70–165 mM SDS, 30–66 mM Hp-β-CD, and/or 0.6% (w/v) PEO solutions. By applying electric field, the PEO solution entered into the capillary from the buffer vial with the help of the EOF. The EOF was larger than the effective electrophoretic mobilities of analytes so that the detection window was located at 10 cm from the cathode end. After separation, PEO molecules adsorbed on the capillary wall were flushed out. The capillary was re-equilibrated with 1 M NaOH at 1 kV for 10 min for the next run. Chang's group has reported that the EOF and migration time of analyte is highly reproducible when the capillary have been treated with 0.5 M NaOH [27].

2.3. Precolumn derivatization

According to the previous study, 50 μL of Asp enantiomers was mixed with a solution containing $Na_2B_4O_7$ (10 mM, 50 μL), NaCN (100 mM, 50 μL), and NDA (25 mM, 50 μL). After 20 min, NDA-derivatized Asp enantiomers (200 μL) were diluted to 500 μL with deionized water and ready to be analyzed by CE-LEDIF.

2.4. Analysis of DL-Asp in biological samples

CSF (lumbar) was kindly provided by Prof. Po-Ling Chang (Department of Chemistry, Tunghai University, Taiwan). Prior to analysis, a CSF sample was stored at −80 °C. Soymilk (Golden beans soymilk, Taiwan) and beer (Taiwan beer, Taiwan) were purchased from local market. These samples were diluted to 50-fold with deionized water. The diluted samples were spiked without and with standard D-Asp and L-Asp (1–100 nM). The resulting mixtures were derivatized with a solution consisting of $Na_2B_4O_7$ (10 mM, 50 μL), NaCN (100 mM, 50 μL), and NDA (25 mM, 50 μL). After gentle shaking, the solution reacted at room temperature for 30 min. The NDA-derivatized samples were ready to be analyzed by CE-LEDIF.

3. Results and discussion

3.1. Separation of D- and L-Asp by Hp-β-CD-mediated-MEKC in the presence of PEO

Prior to on-line sample stacking, we preliminarily investigated the role of the SDS micelle, Hp-β-CD, and PEO in the separation of D- and L-Asp. Compared to the analysis of D- and L-Asp without the use of additives (Fig. 1A), adding 150 mM SDS to the BGE caused an increase in migration time of D- and L-Asp (Fig. 1B). This result reveals that D- and L-Asp partitioned into negatively charged SDS micelles, thereby increasing their electrophoretic mobilities from −1.44 to -3.67×10^{-4} cm^2 V^{-1} s^{-1}. In contrast, adding 60 mM Hp-β-CD to the BGE resulted in decreased migration time of D- and L-Asp (Fig. 1C). This finding reflects that neutral Hp-β-CD can include NDA-derivatized D- and L-Asp, thereby lowering their electrophoretic mobilities (-1.23×10^{-4} cm^2 V^{-1} s^{-1}). Fig. 1D shows that a mixture of D- and L-Asp was partially resolved in the presence of 150 mM SDS and 60 mM Hp-β-CD. Under this separation condition, the electrophoretic mobilities of D- and L-Asp were −2.55 and -2.56×10^{-4} cm^2 V^{-1} s^{-1}, respectively. This

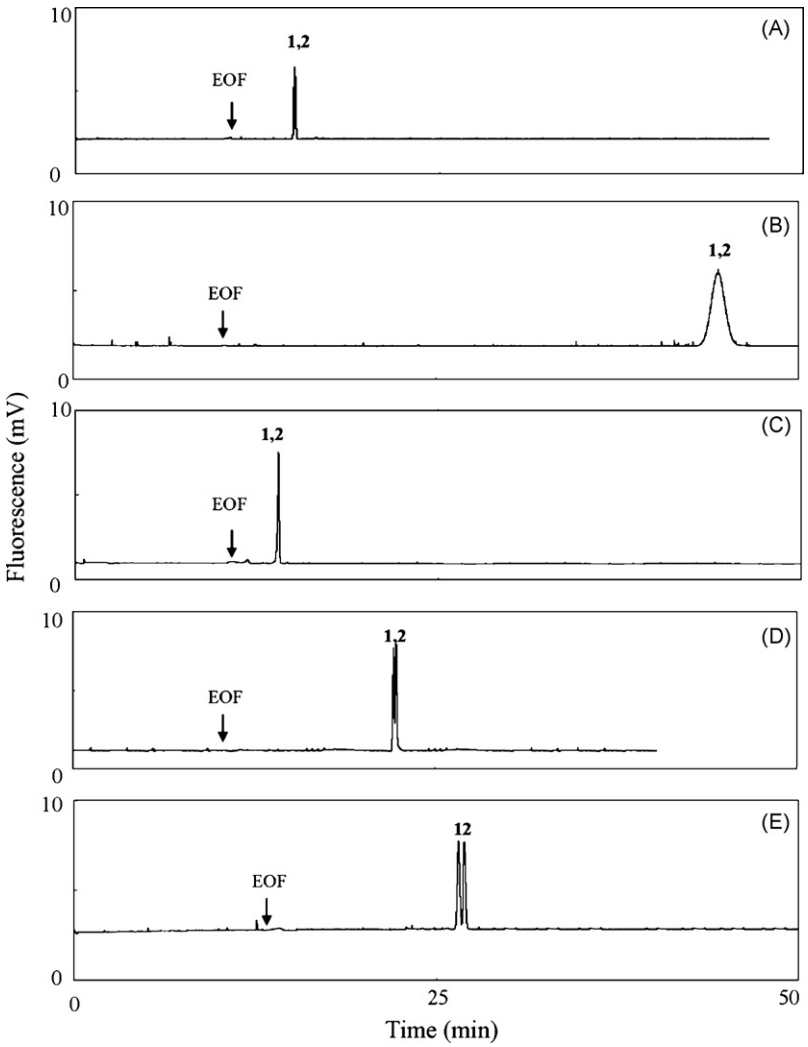

Fig. 1. Effect of additives on the separation of 1.0 μM Asp enantiomers. The capillary and buffer vial both contain (A) no additive, (B) 150 mM SDS, (C) 60 mM Hp-β-CD, and (D) 60 mM Hp-β-CD and 150 mM SDS. (E) Buffer vial contains 60 mM Hp-β-CD, 150 mM SDS, and 0.6% (w/v) PEO while the capillary was filled with 60 mM Hp-β-CD and 150 mM SDS. Electrophoresis conditions: 50-cm capillary (10 cm to detector); BGE, 150 mM TB at pH 9.0; applied voltage, 6.7 kV; hydrodynamic injection at 20-cm height for 10 s.

result implies that SDS and Hp-β-CD both interacted with D- and L-Asp. Importantly, Hp-β-CD-mediated MEKC provided better resolution than just using SDS micelles or Hp-β-CD in CE. Two separate peaks corresponding to D- and L-Asp were observed when the BGE contains 0.6% PEO, 150 mM SDS and 60 mM Hp-β-CD (Fig. 1E). Because of the adsorption of PEO on the capillary wall, bulk EOF mobility gradually decreased during separation [31]. A reduction in EOF may provide more opportunity for Hp-β-CD/SDS to include D- and L-Asp, resulting in the enhanced resolution [26]. Moreover, compared to Hp-β-CD-mediated MEKC, the electrophoretic mobilities of D-Asp (-1.8×10^{-4} cm^2 V^{-1} s^{-1}) and L-Asp (-1.83×10^{-4} cm^2 V^{-1} s^{-1}) were relatively small in the presence of three additives (PEO, SDS, and Hp-β-CD). This result indicates that highly viscous PEO molecules are capable of lowering the electrophoretic mobilities of D- and L-Asp.

3.2. Effect of the ratio of SDS to Hp-β-CD and their total concentration

Because SDS and Hp-β-CD both include hydrophobic molecules, the partition equilibrium of DL-Asp and the SDS micelle compete with that of DL-Asp and the Hp-β-CD cavity. Thus, we investigated the effect of the ratio of SDS to Hp-β-CD on the chiral resolution when the total concentration of Hp-β-CD and SDS was fixed at 210 mM. In the presence of 0.6% PEO, the resolution between D-Asp and L-Asp reached maximum at the molar ratio of Hp-β-CD to SDS of 2:5 (Fig. 2A). The previous study showed that three pairs of amino acid enantiomers, (valine, leucine, and isoleucine) were baseline separated at the molar ratio of Hp-β-CD to SDS of 1: 3 in the presence of 0.6% PEO [26]. We suggest that the separation of the enantiomers of other amino acids is probably achieved by varying the ratio of SDS to Hp-β-CD in the presence of PEO. Meanwhile, the total concentration of SDS and HP-β-CD was another important parameter for the separation of the enantiomers of Asp. The molar ratio of Hp-β-CD to SDS was fixed to 2:5 in the presence of 0.6% PEO. Fig. 2B reveals that the resolution between D- and L-Asp gradually increased with raising the total concentration of Hp-β-CD and SDS. Enhanced resolution is probably attributed to that a higher total concentration of Hp-β-CD and SDS provides more opportunity for D- and L-Asp to interact with Hp-β-CD and SDS micelles. The resolution between D- and L-Asp reached a plateau in the presence of 150 mM SDS, 60 mM Hp-β-CD and 0.6% PEO. This optimum separation condition was capable of separating 6 pairs of amino acid enantiomers, including DL-threonine, DL-histidine, DL-valine, DL-Asp, DL-isoleucine, and DL-leucine (Fig. 3).

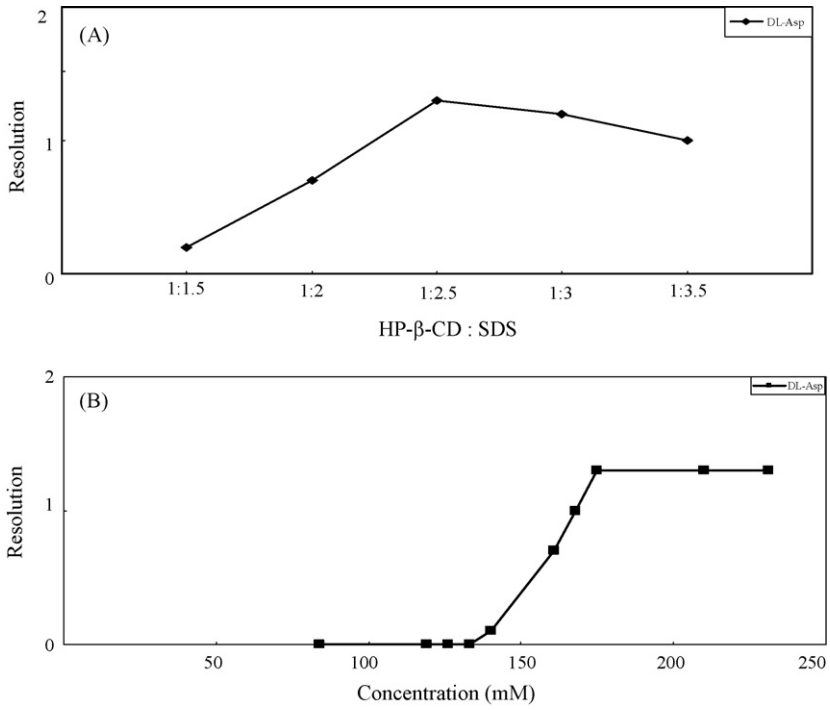

Fig. 2. Effects of (A) the molar ratio of Hp-β-CD to SDS and (B) total concentration of Hp-β-CD and SDS on the resolution between D-Asp and L-Asp. (A) The total concentration of Hp-β-CD and SDS was 210 mM. (B) The molar ratio of Hp-β-CD to SDS was 2:5. Buffer vial contains 70–165 mM SDS, 30–66 mM Hp-β-CD, and 0.6% PEO while the capillary was filled with 30–66 mM Hp-β-CD and 70–165 mM SDS. The other conditions are the same as Fig. 1.

3.3. Stacking, sensitivity, and reproducibility

The PEO and Hp-β-CD are both electrically neutral and thus have no electrophoretic mobility. In contrast, the electrophoretic migration of anionic SDS micelles is in the anode direction. In the initial stage, the capillary inside was filled with 150 mM TB (pH 9) containing 150 mM SDS and 60 mM Hp-β-CD (Fig. 4A). After a large-volume sample was hydrodynamically injected into the capillary at the anodic end, the negatively charged SDS micelles migrated into the sample zone and swept the slower moving analytes (Fig. 4B). The formed SDS–analyte complexes accumulated and migrated toward the anode. Meanwhile, a solution containing 0.6% PEO, 150 mM SDS and 60 mM Hp-β-CD entered into the capillary with the help of the EOF. The accumulated complexes slowed down when they entered into the PEO zone (Fig. 4C). As a result, they were stacked through the viscosity difference between PEO and the sample zone [32]. Finally, the separation of D- and L-Asp was conducted in the presence of 0.6% PEO, 150 mM SDS and 60 mM Hp-β-CD (Fig. 4D).

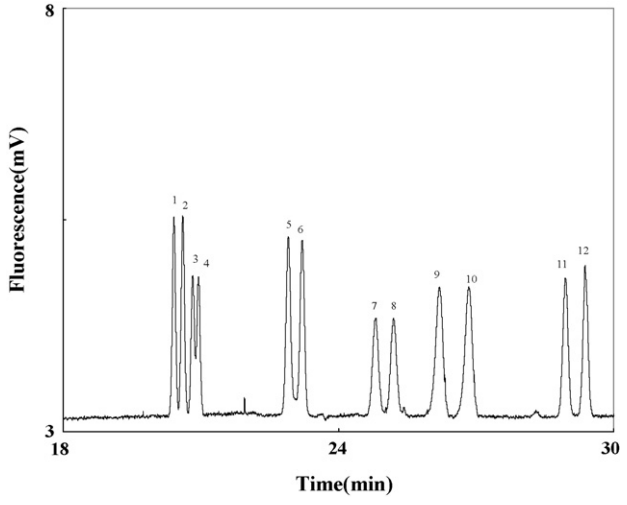

Fig. 3. Separation of 6 pairs of amino acid enantiomers by Hp-β-CD-mediated MEKC in the presence of 0.6% PEO. Buffer vial contains 60 mM Hp-β-CD, 150 mM SDS, and 0.6% (w/v) PEO while the capillary was filled with 60 mM Hp-β-CD and 150 mM SDS. Peak identifies: 1, D-Thr; 2, L-Thr; 3, D-His; 4, L-His; 5, D-Val; 6, L-Val; 7, D-Asp; 8, L-Asp 9, D-Ile 10, L-Ile 11, D-Leu 12, L-Leu. Electrophoresis conditions: 60-cm capillary (10 cm to detector); BGE, 150 mM TB at pH 9.0; applied voltage, 8.0 kV; hydrodynamic injection at 20-cm height for 10 s. The analyte concentrations are all 0.5 μM.

To test this proposed stacking mechanism, we investigated the effect of the sample volume (25–600 nL) on peak areas of D- and L-Asp under optimum separation conditions. The total capillary volume was calculated to be 2650 nL (60 cm length, 75-μm I.D.). Two tiny peaks corresponding to D- and L-Asp were observed by injecting 25 nL of 0.1 μM Asp enantiomers (Electropherogram a in Fig. 5A). The peak areas of D- and L-Asp gradually enhanced with increasing the sample volume (Electropherogram b–d in Fig. 5A). A plot between the peak area and the injection volume (25 – 600 nL) exhibited linearity ($R^2 > 0.99$), indicating that this method stacks Asp enantiomers well. Because the adsorption of PEO molecules on the capillary surface is relatively strong under low-ionic-strength conditions, an increased sample volume (prepared in 0.01 mM sodium tetraborate) facilitates the PEO adsorption process and thus resulted in a decreased EOF [26]. Thus, the migration times of D- and L-Asp took longer as more sample volume was injected into capillary. Additionally, when the injection volume exceeded 600 nL, the resolution between D-Asp and L-Asp decreased from 1.3 to 1.0. This reflects that an injection larger than 600 nL of sample volume (25% of capillary volume) could hamper the separation of Asp enantiomers. Under an injection of 600-nL sample volume, the quantification of D- and L-Asp was performed by Hp-β-CD-mediated MEKC in the presence of 0.6% PEO. When the concentration of D-Asp and L-Asp varied from 1 to 1000 nM, we found that the peak area linearly increased with increasing the concentration of D-Asp over the range of 1–100 nM ($R^2 = 0.9972$) and the concentration of L-Asp over the range of

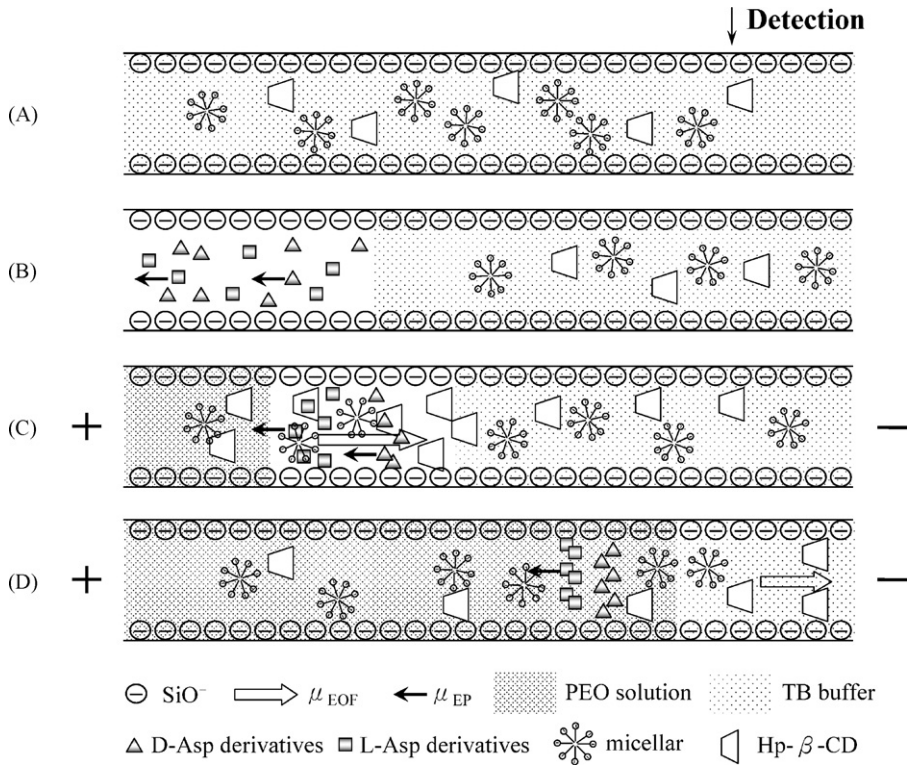

Fig. 4. Evolution of analyte zone in the separation and stacking of Asp enantiomers. (A) Filling of capillary with 60 mM Hp-β-CD, 150 mM SDS; (B) injection of a large volume of Asp enantiomers; (C) stacking of Asp enantiomers by SDS micelle and PEO; (D) separation of the stacking Asp enantiomers by Hp-β-CD-mediated MEKC. The μ_{EOF} and μ_{EP} represent the EOF mobility and the electrophoretic mobilities of Asp enantiomers, respectively.

1–100 nM (R^2 = 0.9981). The intra-day migration times of D- and L-Asp were 2.5% and 2.5% (n = 6), respectively, while the inter-day migration time of D- and L-Asp were 4.4% and 4.5% (n = 15), respectively. Estimated from the separation of 600 nL of 1 nM Asp enantiomers (Fig. 5B), the limits of detection at a signal-to-noise of 3 for D- and L-Asp were 2.51 and 2.53×10^{-10} M, respectively. Compared to the analysis of D-Asp by β-CD-mediated MEKC with LIF detection [24], the combination of Hp-β-CD-mediated MEKC and PEO with LED-induced fluorescence detection under unstacking condition with similar sensitivity, but by stacking can provide better sensitivity.

3.4. Analysis of D- and L-Asp in CSF, soymilk and beer

Fig. 6A shows the analysis of injected into 25 nL of 50-fold diluted CSF by Hp-β-CD-mediated MEKC in the presence of 0.6% PEO. There was no peak corresponding to D- and L-Asp. In comparison, two peaks corresponding to D- and L-Asp were observed in

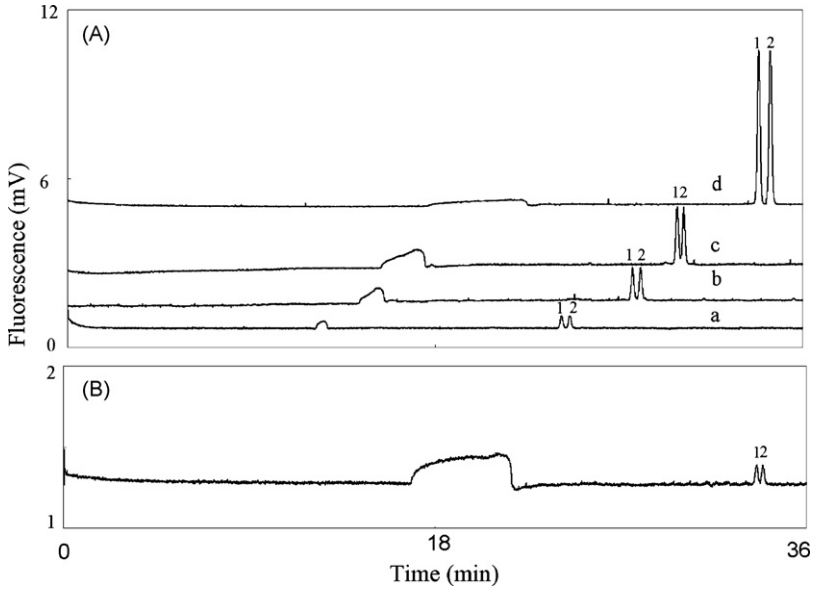

Fig. 5. (A) Stacking and separation of (a) 25, (b) 150, (c) 300, and (d) 600 nL Asp enantiomers (0.1 μM) by Hp-β-CD-mediated MEKC in the presence of 0.6% PEO. (B) Stacking and separation of 600 nL Asp enantiomers (1 nM) by Hp-β-CD-mediated MEKC in the presence of 0.6% PEO. The other conditions are the same as Fig. 3.

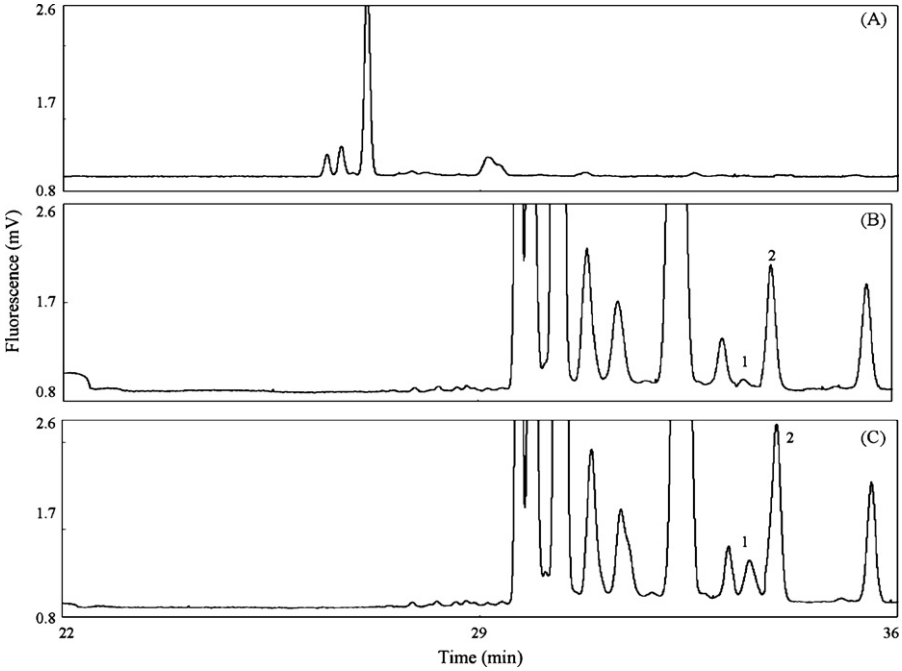

Fig. 6. Stacking and separation of a 50-fold diluted CSF samples by Hp-β-CD-mediated MEKC in the presence of 0.6% PEO. A 50-fold diluted CSF sample was spiked (A and B) without and (C) with 75 nM D-Asp and L-Asp. The sample was injected at 20-cm height for (A) 10 s and (B and C) 240 s. The other conditions are the same as Fig. 3.

Table 1
The quantifications of DL-CBI-Asp in CSF, soymilk, beer and tea samples by CE-LEDIF when the sample volume was injected up to 600 nL.

Sample	D-Asp, M (RSD%, $n=5$)	L-Asp, M (RSD%, $n=5$)	DL-Asp, M[b]	%D-Asp[a]
CSF	2.43×10^{-8} (5.87)	1.41×10^{-6} (7.00)	1.43×10^{-6}	1.69[33]
Beer	16.75×10^{-6} (2.76)	1.06×10^{-4} (4.38)	1.22×10^{-4}	13.6[34]
Soymilk	2.84×10^{-6} (5.24)	45.14×10^{-6} (4.63)	5.94×10^{-6}	4.63[35]

[a] $[D/(D+L)]\times100\%$.
[b] Refs. [33–35].

the analysis of injected into 600 nL of 50-fold diluted CSF (Fig. 6B). Two peaks were further confirmed by comparing the peak areas with (Fig. 6C) and without spiked standard D- and L-Asp (Fig. 6B). The mean recoveries for D- and L-Asp at three spiked levels (10, 50, 100 nM) ranged from 93% to 101%. By applying a standard addition method, the concentrations of D-Asp and L-Asp from a single CSF sample repeated measures five times were estimated to be 24.3 ± 1.4 nM and 1.4 ± 0.1 μM. This result is consistent with the reported values [33] (Table 1). Encouraged by this result, this method was also exploited for detecting D-Asp and L-Asp in soymilk and beer. This work used a standard addition method to determine the concentrations of D- and L-Asp in two beverage samples, listed in Table 1. The values of D- and L-Asp in soymilk and beer are in good agreement with the reported values [34,35].

4. Conclusion

We have demonstrated that PEO plays important roles in controlling EOF and stacking Asp enantiomers when Hp-β-CD-mediated MEKC is utilized for the separation of Asp enantiomers. The resolution between D-Asp and L-Asp is highly dependent of the molar ratio of SDS to Hp-β-CD as well as the total concentration of SDS and Hp-β-CD. Under an injection of 600 nL sample volume, the combination of PEO-base stacking and Hp-β-CD-mediated MEKC allowed detection of Asp enantiomers at the nM level. The successful example of the determination of Asp enantiomers in CSF samples shows great potential of this method for diagnosis of Alzheimer's and Parkinson's disease. This method could be also applied for detecting Asp enantiomers in soymilk and beer.

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