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On-line sample preconcentration by sweeping and poly(ethylene oxide)-mediated stacking for simultaneous analysis of nine pairs of amino acid enantiomers in capillary electrophoresis

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ABSTRACT

This study proposes a sensitive method for the simultaneous separation and concentration of 9 pairs of amino acid enantiomers by combining poly(ethylene oxide) (PEO)-based stacking, β-cyclodextrin (β-CD)-mediated micellar electrokinetic chromatography (MEKC), and 9-fluoroenylmethyl chloroformate (FMOC) derivatization. The 9 pairs of FMOC-derivatized amino acid enantiomers were baseline separated using a discontinuous system, and the buffer vials contained a solution of 150 mM Tris-borate (TB), 12.5% (v/v) isopropanol (IPA), 0.5% (w/v) PEO, 35 mM sodium taurodeoxycholate (STDC), and 35 mM β-CD, and the capillary was filled with a solution of 1.5 M TB, 12.5% (v/v) IPA, 35 mM STDC, and 35 mM β-CD. Based on the difference in viscosity between the sample zone and PEO solution and because of the STDC sweeping, the discontinuous system effectively stacked 670 nL of the 9 pairs of FMOC-derivatized amino acid enantiomers without losing chiral resolution. Consequently, the limits of detection for the 9 pairs of FMOC-derivatized amino acid enantiomers were reduced to 40–60 nM. This method was successfully used to determine D-Tryptophan (Trp), L-Trp, D-Phenylalanine (Phe), L-Phe, D-Glutamic acid (Glu), and L-Glu in various types of beers.

1. Introduction

Amino acids are one of the most important biological compounds because they are ubiquitous in biological fluids, food product, and plant. All amino acids are chiral molecules except for glycine. Recently, D-amino acids are found to play an important role in fermented foods [1–3], such as beer, wine, vinegar, cheese, yogurt, fermented milk and fish sauce. For example, in fermented foods, D-alanine (D-Ala), D-glutamic acid (Glu), D-phenylalanine (Phe), and D-aspartic acid (Asp) provide important information about adulteration and quality of food products [4]. Also, the level of D-Glu, D-serine (ser), and D-Asp in cerebrospinal fluid were highly implicated with mental diseases, such Alzheimer's disease and Parkinson's disease [5,6]. Due to their biological and clinical significance, a rapid, convenient, and sensitive method is required for routine analysis of D-amino acids in food and human body fluids.

Current methods for the determination of chiral amino acids in biological samples include gas chromatography [7,8], high performance liquid chromatography [9,10], and supercritical fluid chromatography [11,12]. However, the high cost chiral stationary phase is a prerequisite of these separation techniques. Capillary electrophoresis (CE) is an alternative method for determining amino acid enantiomers that has the advantages of small injection volumes of the sample, low solvent consumption, rapid analysis, and high separation efficiency [13]. Two major modes of CE, including capillary zone electrophoresis (CZE) [14,15] and micellar electrokinetic chromatography (MEKC) [16,17] are widely used for the separation of chiral amino acids. However, amino acids lack chromophores and fluorophores, except for 3 aromatic amino acids (tryptophan (Trp), tyrosine, and Phe). Various reagents, such as o-phthaldialdehyde (OPA) [18], naphthalene-2,3-dicarboxaldehyde (NDA), [19] fluorescein isothiocyanate (FITC) [20], dansyl chloride [21], and 9-fluoroenylmethyl chloroformate (FMOC) [22], have been synthesized for the derivatization of amino acids. Among these organic dyes, FMOC has distinct advantages, including low cost of chemicals, a short reaction time, high stable derivatives, and high yield derivatives [23]. Furthermore, Chen et al. achieved the simultaneous baseline separation of 9 pairs of FMOC-derivatized amino acid enantiomers using a mixture of β-cyclodextrin (β-CD) and sodium taurodeoxycholate (STDC) [24]. Han and Chen used β-CD-mediated MEKC to separate a mixture of 8 pairs of FMOC-derivatized amino acid enantiomers [22]. Although FMOC derivatization is beneficial to the chiral separation of amino acid enantiomers, the detection of FMOC-derivatized amino acids using CE with UV absorbance results in poor sensitivity.

Numerous concentration methods, including field-amplified sample stacking (FASS) [19,25], acetonitrile stacking [26], large volume sample stacking (LVSS) [27,28], single-drop microextraction [29], CD-

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sweeping [30] and sweeping [31], have been used to enhance the sensitivity of amino acid enantiomers in the CE methods. For example, 3 pairs of DL-amino acids were baseline separated by combining LVSS-CD-mediated MEKC with FITC derivatization [27]. Kirschner, et al. baseline separated four pairs of NDA-derivatized amino acid enantiomers using sulfated β-CD as the chiral selector with a low pH and reverse polarity [30]. Recently, the use of poly (ethylene oxide) (PEO) as an additive in CE is well-suited for the on-line stacking and separation of amino acids in [32–36]. This stacking mechanism is chiefly enabled by the viscosity difference between the PEO and sample solution, and because of the local electric field difference between the TB buffer and sample solution. This stacking method provides an approximately 100 to 1000-fold improvement in sensitivity. The combination of PEO-based stacking and hydroxypropyl-β-CD-mediated MEKC was developed for the on-line concentration and separation of 3 pairs of amino acid enantiomers in various biological fluids [19]. Although the mentioned methods have high sensitivity toward amino acid enantiomers, the simultaneous separation and stacking of numerous amino acid enantiomers is challenging.

This study proposes a combined PEO-based stacking method, β-CD-mediated MEKC, and FMOC derivatization for the on-line concentration and separation of 9 pairs of amino acid enantiomers. The PEO, β-CD, STDC, and FMOC were the concentrating media, a chiral selector, a pseudostationary phase, and a resolution enhancer, respectively. We evaluated the effects of the derivatizing agent, β-CD, STDC, PEO, and injection volume on the separation and stacking of FMOC-derivatized amino acid enantiomers.

2. Experimental

2.1. Chemicals and preparation

The proteinogenic D-, L-Asparagine (Asn), D-, L- Histidine (His), D-, L-Valine (Val), D-, L-Leucine (Leu), D-, L-Trp, D-, L-Phe, D-, L- Glu, D-, L-Asp, D-, L-Lysine (Lys), isopropanol (IPA), 9-fluorenylmethylchloroformate (FMOC), sodium taurodeoxycholate (STDC), β-CD, poly(ethylene oxide) (PEO) (M_w 8,000,000 g/mol), NaOH, methanol, acetonitrile (ACN), O-phthalaldehyde (OPA) and FMOC were obtained from Sigma-Aldrich (St Louis, MO, USA). Naphthalene-2,3-dicarboxaldehyde (NDA) was purchased from Tokyo Chemical Industry (Tokyo, Japan) and prepared in ACN. Tris and isopropanol (IPA) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Boric acid was purchased from Riedel-deHaën (Buchs, Switzerland). A stock solution of 2000 or 400 mM Tris-borate (TB) buffer was prepared by dissolving 121.15 or 24.23 g of Tris in 500-mL aqueous solution that was adjusted with suitable amounts of boric acid range from pH 8.5 to 10.0. Unless otherwise noted, the molarity of Tris represents that of TB buffer. The PEO (0.1–1.0% w/v) was gradually added to each of prepared 150 mM TB solutions at pH 8.5. 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. Prior to use for CE separation, the solutions were degassed with a vacuum system in an ultrasonic tank for 10 min [37]. All the prepared solutions were stored at 4 °C and used within a week. All other chemicals were of analytical grade.

2.2. Instrument

A commercial UV absorbance detector (ECOM, Germany) was performed at 260 nm for analytes. Electrophoresis was driven by a high-voltage power supply (Bertan, Hicksville, NY, USA). The high-voltage end of the separation system was put in a laboratory-made plexiglass box for safety. Data acquisition (10 Hz) and control were performed using DataApex Software (DataApex, Prague, Czech Republic). The fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 50 cm length (10-cm to detector) with 75 μm I.D. and 365 μm O.D.

2.3. Precolumn derivatization

The derivatization of amino acid with FMOC, OPA, and NDA was performed according to the previous study [19,22,38]. Briefly, FMOC (300 μL, 10 mM) reacted with amino acid (300 μL, 0.4 −200 μM) in 70 mM borate buffer (pH 9.0) at room temperature for 2 min. For OPA derivatization, a solution (15 μL) containing OPA (4 mM) and 2-ME (4 mM) reacted with amino acid (10 μL, 1 mM) in 75 μL sodium tetraborate buffer (100 mM, pH 9.3) at room temperature for 2 min. For NDA derivatization, a solution (500 μL) containing 10 mM $Na_2B_4O_7$ (50 μL), 100 μM NaCN (50 μL), and 25 mM NDA was incubated with amino acid (5 μL, 10 mM) at room temperature for 30 min. The derivatized amino acid was detected by the proposed method.

2.4. On-line concentration and separation

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) [19]. The capillary was filled with a solution containing 150∼2000 mM TB, 10–50 mM STDC and/or 10–50 mM β-CD, while the sample was injected by raising the capillary inlet 20-cm height for a period of time up to 240 s. Subsequently, the ends of the capillary were immersed in the cathodic and anodic vials containing 150 mM TB, 10–50 mM STDC, 10–50 mM β-CD, and/or 0.5% w/v PEO solutions. 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 [35].

2.5. Analysis of DL-amino acids in biological samples

Three kinds of beer (Taiwan, Japan, Netherlands, USA) were purchased from local market. We spiked samples of beer with standard solution (4-12 μM) of DL-amino acids and derivatized the resulting solution with FMOC at room temperature for 2 min. Note that samples of beer were diluted to 10-fold through sample preparation procedure. The derivatized amino acid was detected by the proposed method.

3. Results and discussion

3.1. Role of derivatizing agent in β-CD-mediated MEKC

The resolution of D- and L-amino acids is highly dependent on the type of derivatizing agent [19,24,39]. We explored how 3 types of derivatizing agents, OPA, NDA, and FMOC, affected the resolution of D- and L-amino acids when β-CD-mediated MEKC was used for chiral separation in the presence of 0.5% w/v PEO. Fig. 1A shows that the chiral separation of a mixture of DL-Trp and DL-Phe was unsuccessful in the mode of the β-CD-mediated MEKC. A similar phenomenon was observed with the use of OPA for the derivatization of the same amino acids (Fig. 1B). The chiral separation of the DL-Trp and DL-Phe was accomplished when NDA and FMOC were used instead of OPA (Fig. 1C and D). Fig. 1 shows the following migration time trends: FMOC-derivatized amino acid > NDA-derivatized amino acid > OPA-derivatized amino acid > underivatized amino acid. This

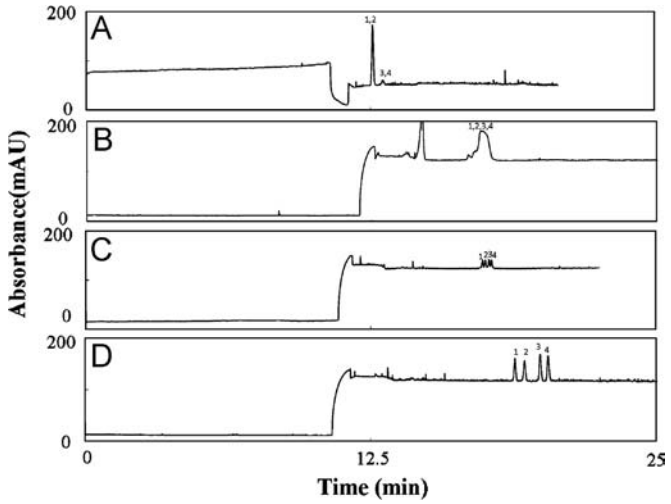


Fig. 1. Separation of 0.1 mM DL-Trp and DL-Phe derviatized with (A) no (B) OPA (C) NDA (D) FMOC by CE-UV. Before separation, the capillary was filled with a solution containing 1.5 M TB pH 10, 35 mM STDC, 35 mM β-CD and 12.5% (v/v) IPA, whereas buffer vials contain 150 mM TB pH 8.5, 35 mM STDC, 35 mM β-CD, 12.5% (v/v) IPA and 0.5% (w/v) PEO. Electrophoresis conditions: 55 cm capillary (10 cm to detector); applied voltage, 18 kv; hydrodynamic injection at 20 cm height for 10 s; the direct UV detection at (A) 220 (B) 355 (C) 420 nm (D) 260 nm. Peak identities: 1. D-Trp 2. L-Trp 3. D-Phe 4. L-Phe. All analyte concentrations are all 0.1 mM.

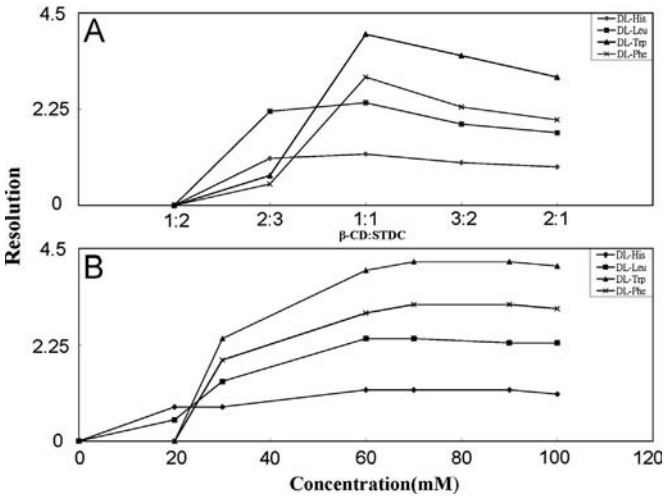


Fig. 2. Effects of (A) the molar ratio of β-CD to STDC and (B) total concentration of β-CD and STDC on the resolution between D-His, D-Leu, D-Trp, D-Phe and L-His, L-Leu, L-Trp, L-Phe. (A) The total concentration of β-CD and STDC was fixed at 70 mM. (B) The molar ratio of β-CD to STDC was fixed at 1:1. Buffer vial contains 0–50 mM STDC, 0–50 mM β-CD, 12.5% (v/v) IPA and 0.5% PEO while the capillary was filled with 0–50 mM β-CD, 0–50 mM STDC and 12.5% (v/v) IPA; applied voltage, 15.0 kV. The other conditions are the same as Fig. 1.

result indicates that the FMOC-derivatized amino acid interacted more strongly with the micellar phase and β-CD cavity than NDA-derivatized, OPA-derivatized, and underivatized amino acids. In chromatography, the resolution can be improved significantly by increasing the retention factor. Along a similar line, the FMOC-derivatized amino acid exhibited longer migration time, resulting in the enhancement of the resolution of DL-amino acid. In addition, FMOC can be directly used to derivatize DL-amino acid without using additional nucleophile, whereas the derivation of DL-amino acid with OPA and NDA requires toxic cyanide and 2-mercaptoethanol. The time for the derivatization of the DL-amino acid with FMOC was short (approximately 2 min) compared to the NDA [19] and OPA [39,40]. Consequently, FMOC was used in this study for the separation efficiency, derivatization procedure, and derivatization time.

3.2. Role of STDC, β-CD, and PEO.

Because negatively charged STDC and neutral β-CD both interact with hydrophobic molecules, the partition equilibriums of the DL-amino acid and the β-CD cavity competed with those of the DL-amino acid and STDC micelles. Previous studies have demonstrated that the successful separation of amino acid enantiomers relies on the molar ratio of the CD analog to anionic micelle in the mode of CD-mediated MEKC [19,41]. Therefore, this study tested the effects of the molar ratio of β-CD to STDC on the chiral separation of four amino acid enantiomers in the presence of 0.5% (w/v) PEO. When the total concentration of β-CD and STDC was fixed at 70 mM, the resolution of the four amino acid enantiomers achieved its maximum at the molar ratio of 1:1 (Fig. 2A). In addition, this study explored how the total concentration of β-CD to STDC influenced the chiral separation of the mentioned amino acids at a fixed molar ratio of β-CD to STDC of 1:1. Fig. 2B shows that the resolution of the FMOC-derivatized DL-amino acids gradually increased with the increasing total concentration of β-CD and STDC at the concentration range of 0–100 mM and plateaued in the presence of 35 mM STDC, 35 mM β-CD, and 0.5% (w/v) PEO. The improved resolution was caused by the higher total concentration of β-CD and STDC, which had a greater opportunity to interact with the FMOC-derivatized DL-amino acids.

Thereafter, the role of PEO in the separation of the FMOC-derivatized DL-amino acids was explored. Without PEO, the chiral

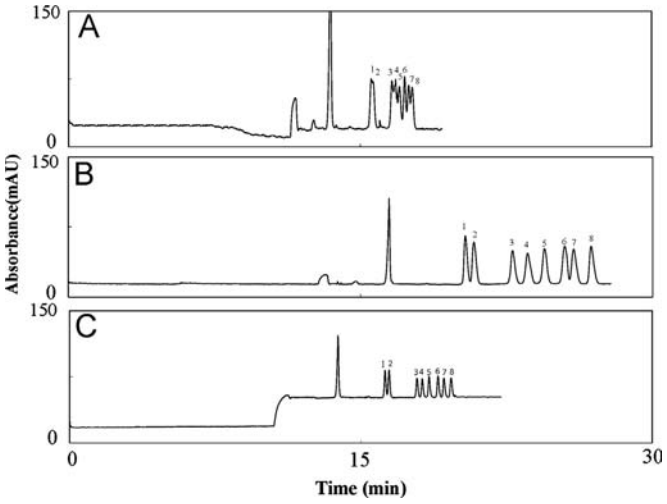
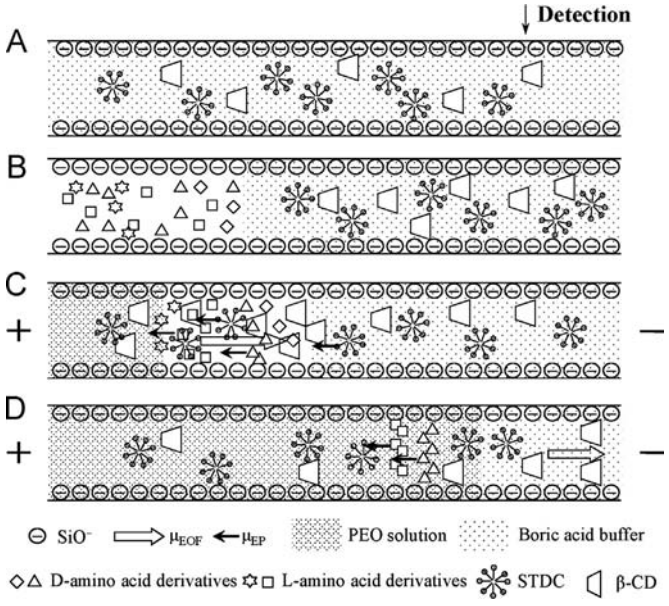


Fig. 3. Separation of 4 pairs of amino acid **enantiomers** by β-CD-mediated MEKC buffer vial in the absence of presence of PEO. Buffer vial contains 150 mM TB (pH 8.5), 35 mM β-CD, 35 mM STDC, and 12.5% (v/v) IPA in the (A) absence and (B and C) presence of 0.5% (w/v) PEO. The capillary was filled with a solution of (A, B) 150 mM TB (pH 8.5) and (C) 1.5 M TB (pH 10) containing 35 mM β-CD, 35 mM STDC and 12.5% IPA. Peak identifies: 1, D-His; 2, L-His; 3, D-Leu; 4, L-Leu; 5, D-Trp; 6, L-Trp; 7, D-Phe; 8, L-Phe Electrophoresis conditions: 45-cm capillary (10-cm to detector); applied voltage, 15.0 kV; hydrodynamic injection at 20-cm height for 10 s. The analyte concentrations are all 0.1 mM.

separation of the 4 pairs of FMOC-derivatized amino acid enantiomers (DL-His, DL-Leu, DL-Trp, and DL-Phe) was ineffective using the β-CD-mediated MEKC (Fig. 3A). In contrast, the baseline separation of the 4 pairs of FMOC-derivatized amino acid enantiomers was achieved by combining the PEO and β-CD-mediated MEKC (Fig. 3B). The migration time of the FMOC-derivatized amino acid enantiomers that were obtained from the combination of the PEO and β-CD-mediated MEKC was slower than that of those obtained from the β-CD-mediated MEKC, indicating that the adsorption of PEO on the capillary surface occurred and EOF is reduced. The improvement in separation efficiency was probably caused by the hydrophobic interaction between the carboxylic group of the FMOC-derivatized amino acid and β-CD/STDC micelles, rather than the electroosmotic

flow (EOF) decay. To confirm this hypothesis, the capillary was filled with 1.5 M TB rather than with 150 mM TB. Previous studies have shown that filling the capillary with 1.5 M TB can reduce PEO adsorption and generate a high and reproducible EOF [42]. Compared to filling the capillary with 150 mM TB (Fig. 3B), the time for the separation of the 4 pairs of the FMOC-derivatized amino acid enantiomers was reduced from 27.5 to 20.4 min by filling the capillary with 1.5 M TB (Fig. 3C). The EOF mobility varied from 1.49 to 1.89×10^{-4} cm^2 V^{-1} s^{-1} when the concentration of TB was increased from 0.15 to 1.5 M.



Scheme 1. Evolution of analyte zone in the separation and stacking of neurochemicals. (A) Filling of capillary with 1.5 M TB (pH 10) containing 12.5% (v/v) IPA; (B) injection of a large volume of anlaytes solution; (C) stacking of sixteen analytes by PEO solution (150 mM TB pH 8.5, **3**5 mM STDC, 35 mM β-CD and 12.5% (v/v) IPA); (D) separation of the stacking CBI-DL-amino acids PEO. The μ_{EOF} and μ_{EP} represent the EOF mobility and the electrophoretic mobilities of cationic and anionic neurochemicals, respectively. The detection wavelength was set at 260 nm.

3.3. Stacking, sensitivity, and reproducibility

PEO and β-CD are both electrically neutral and, consequently, do not have electrophoretic mobility, whereas the electrophoretic migration of the anionic STDC micelles was in the direction of the anode. Scheme 1 shows that the mechanism for the analysis of the DL-amino acids was divided into four steps. (1) sample injection: the capillary was filled with 1.5 M of TB containing 35 mM β-CD and 35 mM STDC (Scheme 1A), and then a large-volume sample was hydrodynamically injected into the capillary filled with the STDC micelle and β-CD (Scheme 1B). Meanwhile, the buffer vial contained PEO, STDC micelle, and β-CD. (2) STDC-mediated sweeping: After voltage was applied to the capillary, the negatively charged STDC micelles migrated to the sample zone and swept the slower moving FMOC-derivatized DL-amino acids [19]. (3) PEO-mediated stacking: the formed complexes of the STDC micelles and FMOC-derivatized DL-amino acids migrated toward the anode until they encountered the PEO zone (i.e., they entered the capillary by EOF), where the complexes were stacked again by the viscosity difference between the PEO and sample zone (Scheme 1C) [41]. (4) β-CD-mediated chiral separation: the chiral separation of the FMOC-derivatized DL-amino acids was conducted through equilibrium distribution among the FMOC-derivatized DL-amino acids in the aqueous phase, the STDC micelle, and the β-CD cavity. Consequently, the complexes between the FMOC-DL-amino acids and STDC micelles/β-CD were detected at the cathode with the help of the EOF (Scheme 1D). Supplementary Fig. S1 reveals that on-line sample preconcentration and separation of D- and L-Trp was successful by combining PEO, β-CD, and STDC. Supplementary Fig. S2 shows that an increase in the concentration of sample buffer did not hamper the use of the proposed method for stacking D- and L-Trp. Thus, we excluded the contribution of FASS in the proposed method.

After the injection of 8 nL of sample, tiny peaks corresponding to 9 pairs of the FMOC-derivatized DL-amino acids were observed. The limits of detection (LODs) at *S*/*N* of 3 for the FMOC-derivatized DL-amino acids ranged from 4.4 to 8.9 μM, respectively (Table 1). Under optimal separation conditions (35 mM β-CD, 35 mM STDC, 0.5% (w/v) PEO, and filling the capillary with 1.5 M TB), the electropherograms (a–d) shown in Fig. 4A show that the peak intensities of the 18 peaks significantly increased with the increase in sample volume from 40 to 670 nL. There was good linearity

Table 1
On-line concentration and separation of 670 nL FMOC-DL-amino acids using 0.5% PEO buffer solutions.

Amino acids	Linear regression[a]	Linear regression coefficient (R^2)	.LOD (μM) ($S/N=3$)[b]	LOD (nM) ($S/N=3$)[c]	Sensitivity enhancement[d]	Migration time (min; %RSD)
D-Asn	$y=0.81x+2.44$	0.9980	8.3	62.6	133	33.23 (1.3)
L-Asn	$y=0.73x+4.39$	0.9926	8.9	61.1	145	33.52 (1.3)
D-His	$y=0.68x+4.51$	0.9901	7.3	57.8	127	33.71 (1.3)
L-His	$y=0.66x+3.40$	0.9900	7.4	52.6	141	33.91 (1.3)
D-Val	$y=0.98x+1.60$	0.9995	4.4	41.6	106	34.24 (1.3)
L-Val	$y=0.98x+0.10$	0.9992	4.5	40.3	112	34.84 (1.5)
D-Leu	$y=0.73x+0.76$	0.9996	5.6	42.1	132	35.08 (1.4)
L-Leu	$y=0.79x+0.11$	0.9999	5.6	41.9	134	35.48 (1.5)
D-Trp	$y=0.97x+0.01$	0.9987	5.5	40.7	135	35.97 (1.4)
L-Trp	$y=0.94x+0.01$	0.9988	5.6	40.1	136	36.68 (1.5)
D-Phe	$y=0.93x+0.39$	0.9976	6.6	41.7	158	37.18 (1.4)
L-Phe	$y=0.85x+3.54$	0.9928	6.8	40.6	168	37.78 (1.5)
D-Glu	$y=0.68x+6.03$	0.9902	4.7	43.1	108	43.20 (2.1)
L-Glu	$y=0.75x+6.58$	0.9902	4.8	44.4	109	44.16 (2.6)
D-Asp	$y=0.82x+2.06$	0.9904	8.5	41.6	201	44.90 (2.6)
L-Asp	$y=0.82x+3.43$	0.9953	8.5	41.1	208	45.40 (2.5)
D-Lys	$y=0.89x+3.55$	0.9930	7.8	37.8	206	47.10 (2.0)
L-Lys	$y=1.05x+0.35$	0.9903	7.9	36.2	219	47.67 (2.1)

[a] y is peak height (mV); x is injection time (15 s∼240 s).
[b] Estimated form figure of the injection of 8 nL of sample.
[c] Estimated from Fig. 4B.
[d] Sensitivity enhancement=($LOD_{normal\ injection}/LOD_{stacking}$).

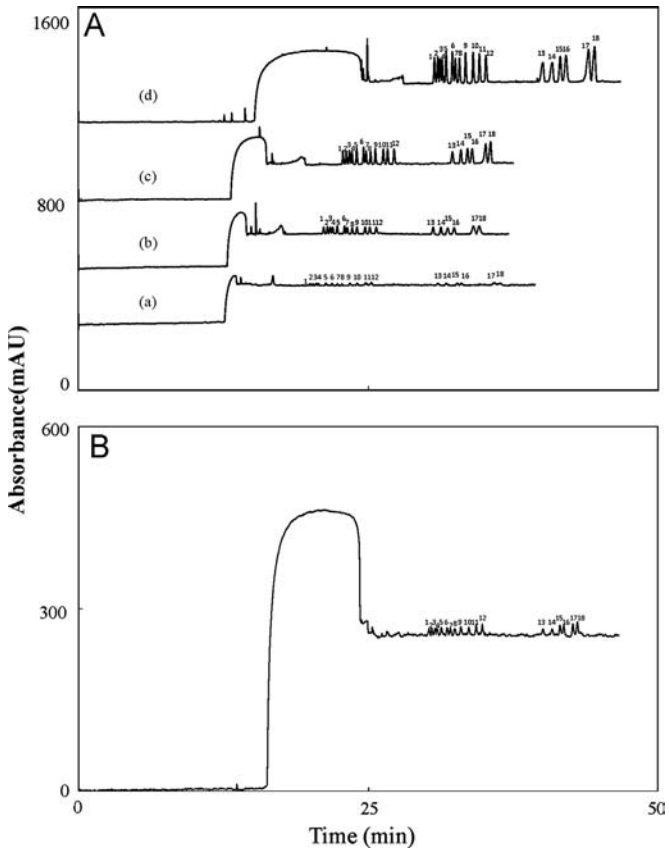


Fig. 4. (A) Stacking and separation of (a) 40, (b) 170, (c) 340, and (d) 670 nL FMOC-DL-amino acids **enantiomers (5 μM)** by β-CD-mediated MEKC in the presence of 0.5% (w/v) PEO. **(B)** Stacking and separation of 670 nL amino acid **enantiomers (0.2 μM)** by β-CD-mediated MEKC in the presence of 0.5% PEO. **Peak identifies**: 1. D-Asn, 2. L-Asn, 3. D-His, 4. L-His, 5. D-Val, 6. L-Val, 7. D-Leu, 8. L-Leu, 9. D-Trp, 10. L-Trp, 11. D-Phe, 12. L-Phe, 13. D-Glu, 14. L-Glu, 15. D-Asp, 16. L-Asp, 17. D-Lys and 18. L-Lys. The other conditions are the same as Fig. 1.

($R^2 > 0.9900$) between the peak height and injection time (15–240 s), indicating that analytes stacked well (Table 1). Because the adsorption of the PEO on the capillary surface was strong under the low-ionic strength conditions, the increased sample volume facilitated the PEO adsorption process and caused an increase in the migration time of the 18 peaks [19]. When the sample volume exceeded 670 nL, the peak efficiency of the FMOC-DL-amino acids was substantially reduced. The sample volume was optimized as 670 nL for this study. Table 2 shows that the calibration curves that were obtained by plotting the peak areas of the FMOC-derivatized DL-amino acids against their concentrations were linear ($R^2 > 0.9900$). The relative standard deviations (RSD) of the migration times and peak areas for the FMOC-derivatized DL-amino acids were less than 2.9% and 3.3%, respectively. The LODs for the 9 pairs of the FMOC-derivatized DL-amino acids ranged from 36.2–62.6 nM (Fig. 4B). A comparison of the LODs that were obtained from normal injection (8 nL) and that were obtained from stacking condition (Fig. 4B) resulted in a 110 −220-fold improvement in sensitivity. Compared to the previous studies (Supplementary Table S1), this is the first example for simultaneous stacking and separation of nine pairs of amino acid enantiomers by the use of CE-based system [19,20,22,29,43].

3.4. Analysis of the DL-amino acids in various types of beer

To demonstrate the practicality of the proposed method, we determined the DL-amino acids in various types of beers, beer samples was performed by combining the PEO-based stacking method, β-CD-mediated MEKC, and FMOC derivatization. Fig. 5A shows that 3 small peaks that corresponded to L-Trp, L-Phe, and L-Glu were observed in the analysis of a 10-fold dilution of Taiwan beer without PEO-based stacking. When the sample volume was increased to 500 nL, six peaks corresponding to DL-Trp, DL-Phe, and DL-Glu were detected using the proposed method (Fig. 5B). To confirm these results, this study identified them by comparing the peak areas with (Fig. 5C) and without (Fig. 5B) spiked-standard DL-Trp, DL-Phe, and DL-Glu. Using standard addition methods, the concentrations of the D-Trp, L-Trp, D-Phe, L-Phe, D-Glu, and L-Glu in Taiwan, American and Netherland beer samples were estimated to be shown in Table 3. These results are consistent with previously reported values [44,45]. Table 3 shows that the proposed method had good linearity for plotting the peak

Table 2
The quantification of DL-Asn, DL-His, DL-Val, DL-Leu, DL-Trp, DL-Phe, DL-Glu, DL-Asp and DL- Lys when the sample volume was injected up to 670 nL.

Amino acids	Linear regression	Spiked concentration (μM)	R^2	Mean recovery (%)[a]	Variation (%)	
					Intraday[b]	Interday[c]
D-Asn	$y=3.14x+0.04$	0.2–20	0.9972	102	1.7	5.9
L-Asn	$y=2.95x+0.03$	0.2–20	0.9900	100	1.7	5.8
D-His	$y=1.70x+0.05$	0.2–20	0.9922	99	1.8	5.7
L-His	$y=2.36x+0.05$	0.2–20	0.9907	101	2.0	5.7
D-Val	$y=4.11x+0.22$	0.2–20	0.9902	100	2.2	5.7
L-Val	$y=4.10x+0.09$	0.2–20	0.9929	101	2.0	5.6
D-Leu	$y=3.18x+0.08$	0.2–20	0.9954	100	2.2	5.6
L-Leu	$y=3.31x+0.57$	0.2–20	0.9928	103	2.1	5.6
D-Trp	$y=4.19x+0.24$	0.2–20	0.9955	101	2.4	5.5
L-Trp	$y=3.93x+0.18$	0.2–20	0.9929	102	2.3	5.5
D-Phe	$y=3.86x+0.83$	0.2–20	0.9972	101	2.6	6.1
L-Phe	$y=3.41x+3.75$	0.2–20	0.9991	102	2.6	6.1
D-Glu	$y=2.53x+0.26$	0.2–20	0.9984	103	2.7	5.5
L-Glu	$y=2.52x+0.65$	0.2–20	0.9994	101	2.8	5.6
D-Asp	$y=3.77x+0.03$	0.2–20	0.9997	101	2.9	5.6
L-Asp	$y=3.75x+0.22$	0.2–20	0.9997	101	3.0	5.6
D-Lys	$y=4.08x+0.05$	0.2–20	0.9925	102	3.1	5.2
L-Lys	$y=4.73x+0.18$	0.2–20	0.9986	101	3.3	5.2

[a] The recoveries were determined in triplicate at the spiked concentrations of 0.2, 10.0, 20.0 μM.
[b] The samples were analyzed eighteen consecutive times in 1 day (intraday, $n=5$).
[c] The samples were analyzed five consecutive times of five different days (interday, $n=25$).

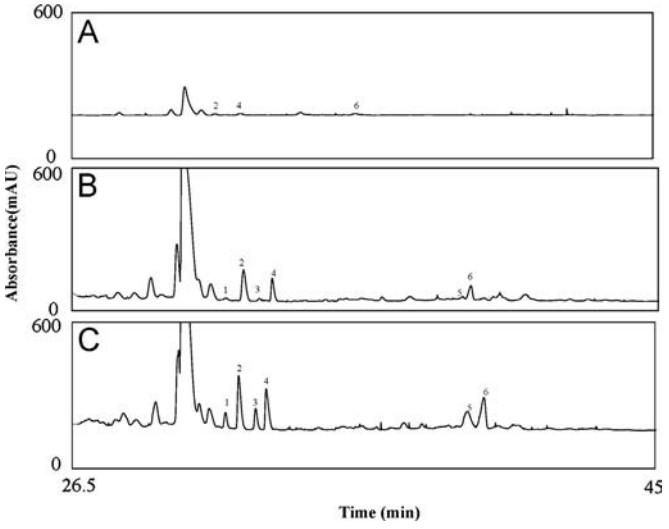


Fig. 5. On-line concentration and chiral separation of a 10-fold diluted beer sample by CE-UV. A beer sample was spiked (A and B) without and (c) with 7 μM FMOC-DL-amino acids. The sample was injected volume for (A) 20 nL and (B, C) 500 nL. Peak identities: 1, D-Trp.; 2, L-Trp.; 3. D-Phe.; 4. L-Phe.; 5. D-Glu 6. L-Glu. Other conditions are as the same as in Fig. 1.

Table 3
Comparison of the concentration of amino acids in three different beer beverages.

Beer beverage	Amino acids	Regression[a]	Spiked concentration (μM)	Correlation coefficient (R^2)	Concentration (μM) (RSD%) ($n=5$)[b]
Taiwan beer	D-Trp	$y=14.25x+16.54$	4.0–12.0	0.9965	11.61 ± 0.24 (2.00)
	L-Trp	$y=4.41x+160.69$	4.0–12.0	0.9910	364.38 ± 8.06 (2.16)
	D-Phe	$y=20.77x+13.06$	4.0–12.0	0.9956	6.29 ± 0.15 (2.34)
	L-Phe	$y=8.62x+121.03$	4.0–12.0	0.9904	140.41 ± 3.65 (2.52)
	D-Glu	$y=21.00x+19.82$	4.0–12.0	0.9941	9.44 ± 0.24 (2.66)
	L-Glu	$y=9.12x+73.69$	4.0–12.0	0.9967	80.80 ± 2.39 (2.99)
American beer	D-Trp	$y=19.28x+4.54$	4.0–12.0	0.9991	2.35 ± 0.08 (3.39)
	L-Trp	$y=15.20x+38.58$	4.0–12.0	0.9992	25.38 ± 0.83 (3.17)
	D-Phe	$y=25.44x+3.10$	4.0–12.0	0.9999	1.22 ± 0.05 (4.41)
	L-Phe	$y=15.10x+33.27$	4.0–12.0	0.9947	22.03 ± 0.72 (3.13)
	D-Glu	$y=14.01x+35.28$	4.0–12.0	0.9998	25.18 ± 1.08 (4.15)
	L-Glu	$y=11.98x+100.42$	4.0–12.0	0.9916	83.82 ± 3.51 (4.11)
Netherland beer	D-Trp	$y=14.67x+3.39$	4.0–12.0	0.9912	2.31 ± 0.07 (3.17)
	L-Trp	$y=4.33x+72.71$	4.0–12.0	0.9974	167.92 ± 5.75 (3.39)
	D-Phe	$y=17.83x+14.11$	4.0–12.0	0.9995	7.91 ± 0.30 (3.70)
	L-Phe	$y=4.54x+96.43$	4.0–12.0	0.9948	212.40 ± 7.97 (3.70)
	D-Glu	$y=14.32x+23.07$	4.0–12.0	0.9932	16.11 ± 0.69 (4.19)
	L-Glu	$y=3.86x+35.45$	4.0–12.0	0.9928	91.83 ± 4.11 (4.40)

[a] y represents peak height (v) and x represents the concentrations of spiked analytes.
[b] Refs. [44,45].

areas of the FMOC-derivatized DL-amino acids against the spike concentrations, had good recovery for the FMOC-derivatized DL-amino acids at five spiked levels, and had excellent reproducibility for migration time and peak area.

4. Conclusion

In this study, on-line concentration and chiral separation of 9 pairs of FMOC-derivatized DL-amino acids have been accomplished by discontinuous conditions: (a) the capillary was filled with a solution of 1.5 M TB (pH 8.5), 12.5% (v/v) IPA, 35 mM β-CD, and 35 mM STDC; (b) the buffer vial contained 150 mM TB (pH 8.5), 35 mM β-CD, 35 mM STDC, 12.5% (v/v) IPA and 0.5% w/v PEO. The use of FMOC for the derivatization of amino acid enantiomers enhanced the resolution of the FMOC-derivatized DL-amino acids. The baseline separation of the FMOC-derivatized DL-amino acids was involved in the interplay between β-CD and STDC. The stacking of the FMOC-derivatized amino acid enantiomers was performed using the differences in viscosity among the sample zone, PEO solution, and STDC sweeping. Under optimal separation and stacking conditions, the proposed method enabled detection at the 36 nM level. Determining 3 pairs of FMOC-derivatized DL-amino acids in 3 types of beer demonstrated the potential of this method for food analysis.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.039.

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