Chun Zhai
Wei Qiang
Jianping Lei
Huangxian Ju

Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing, P. R. China

Received July 21, 2008
Revised December 9, 2008
Accepted December 10, 2008

Research Article

Highly resolved separation and sensitive amperometric detection of amino acids with an assembled microfluidic device

An assembled microfluidic device (AMD) was constructed for highly resolved separation and sensitive amperometric detection of amino acids. In the AMD three polymer retainers were integrated with a quartz capillary and a copper microdisk electrode. A facile ultrasonic method was suggested for the preparation of sampling fracture on the capillary. The fracture led to separation efficiency up to 340 000 plates/m and improved greatly the sensitivity for amino acid detection in an 8 cm long quartz capillary. At a sampling voltage of 100 V for 2 s and a separation voltage of 1800 V (225 V/cm) within 300 s, the linear ranges of 12 amino acids determination at +0.8 V (*versus* Ag/AgCl) were from 12, 20, 17, 27, 31, 44, 25 and 45 to 750 μM for tryptophan, phenylalanine, methionine, valine, threonine, alanine, serine and glycin, and 4.6, 17, 16 and 49 μM to 1.0 mM for lysine, proline, leucine and tyrosine with the detection limit ($S/N = 3$) down to 1.4 μM for lysine. The designed AMD could be successfully applied to analyze amino acids in beverage samples with recovery from 97.8 to 105.2%, indicating its advantages and potential analytical application in different fields.

Keywords:
Amino acids / Amperometric detection / CE / Fracture sampling / Microfluidic device
DOI 10.1002/elps.200800581

1 Introduction

Amino acids as essential elements play key roles in life science, such as transfer nerve information, regulation metabolic activity, biosynthesis of protein and peptide [1]. Many efforts have been devoted to search cheap and fast analytical protocols for separation and determination of amino acids. Microfluidic electrophoresis device (MED) is one of the best candidates for the analysis of amino acids due to the small volume and fast separation [2]. However, compared with conventional CE [3, 4], the resolution of MEDs for simultaneous detection of amino acids is poor [5–20], which limits their practical application in real life samples. Therefore, many strategies have been involved to improve the separation efficiency of MEDs.

Culbertson *et al.* [21] extended the separation channel to 25 cm and used MEKC to separate nineteen tetramethylrhodamine-labeled amino acids with a separation field strength of 1170 V/cm and the minimum resolution of 1.2. The relatively complicated manufacture and regeneration process of the spiral-shaped channel and additional derivatization process limited its application. Jacobson *et al.* [22] and Wallenborg and Bailey [23] proposed a pinched injection method to improve the separation efficiency by forming a short sample plug. However, the injection time was comparable to or even longer than the separation time. Moreover, this method involved cumbersome voltage program for leakage control [24]. Sample stacking technique, which was used to compress sample zone [25], has been introduced to microchip CE for obtaining good resolution accompanied with sample concentration [26–28]. This technique needs long sampling time, complex chip structure and thus troublesome operation. Our previous work proposed a fracture sampling method to introduce a very narrow sample plug for separation and amperometric detection of eight carbohydrates [29]. The sampling fracture was prepared on the column of the fused-silica capillary. Different from the conventional methods, the sampling method introduced sample from the ultra-narrow fracture directly, thus allowing very narrow sample plug to be

Correspondence: Professor Huangxian Ju, Key Laboratory of Analytical Chemistry for Life Science (Ministry of Education of China), Department of Chemistry, Nanjing University, Nanjing 210093, P. R. China
E-mail: hxju@nju.edu.cn
Fax: +86-25-83593593

Abbreviations: **Ala,** alanine; **AMD,** assembled microfluidic device; **BR,** buffer reservoir; **DR,** detection reservoir; **E,** electric field strength; **EC,** electrochemical detection; **Gly,** glycin; **I_p,** peak currents; **Leu,** leucine; **Lys,** lysine; **MED,** microfluidic electrophoresis device; **Met,** methionine; **Phe,** phenylalanine; **Pro,** proline; **R_s,** resolution; **Ser,** serine; **SR,** sampling reservoir; **STB,** sodium tetraborate; **t_M,** migration times; **Thr,** threonine; **Trp,** tryptophan; **Tyr,** tyrosine; **Val,** valine; **WE,** working electrode

injected into the separation channel, which leads to high separation efficiency. Moreover, the ultra-narrow sampling channel could suppress sample leakage, thus avoiding additional equipment and programs usually applied in MCE for leakage control. However, the separation efficiency is insufficient for separation of amino acids due to the slow heat dissipation during the separation process. In order to use the fracture sampling technique in amino acid analysis, this work designed an assembled microfluidic device (AMD) by integrating three polymer retainers with a fused-silica (quartz) capillary and proposed a facile ultrasonic method instead of handwork for preparation of the sampling fracture on the capillary. The structure including three short retainers is favorable to dispersion of Joule heat. Thus, the newly designed AMD with a sampling fracture showed highly resolved separation for the detection of amino acids.

The detection of amino acids is also difficult in their native forms due to the lack of ultraviolet absorption, fluorescent emission or electroactive group of these compounds [2]. Although derivatization methods of amino acids have been developed to improve the detection sensitivity of amino acids with fluorescence [5–7], or electrochemistry [8–10] in microchip CE, the time-consuming derivatization process and by-products formed in the process limit the application of these methods. Electrochemical methods, especially amperometric detection based on the catalytic oxidation mechanism [30], have exhibited attractive advantages for direct determination of amino acids due to the high sensitivity, selectivity and easy to miniaturization. Based on the catalysis of copper electrode toward electro-oxidation of amino acids, this work integrated a copper microdisk electrode against the end of separation channel to develop a method for direct amperometric detection of amino acids. The newly designed AMD possessed reliable and convenient preparation, high separation efficiency and resolution, and low detection limit for fast simultaneous determination of 12 amino acids. This method could be successfully applied to detect various amino acids in practical samples.

2 Materials and methods

2.1 Reagents

The 99% purity of lysine (Lys), proline (Pro), tryptophan (Trp), leucine (Leu), phenylalanine (Phe), valine (Val), methionine (Met), threonine (Thr), alanine (Ala), serine (Ser), glycin (Gly), and tyrosine (Tyr) were purchased from Acros. Beverage for real sample detection was from Ichimore (Shanghai, China), which was a commercially artificial drink. Sylgard 184 silicone elastomer and curing agent were purchased from Dow Corning (Midland, MI). All aqueous solutions were prepared using $\geq 18\,M\Omega\,cm$ ultrapure water (Milli-Q, Millipore). The BGE containing sodium tetraborate (STB) and Tween 20 (polyoxyethylene (20) sorbitan monolaurate, $m = 20$) (Nanjing Chemicals, Nanjing, China) at different pHs adjusted by adding 1.0 M NaOH solution were passed through a membrane filter (0.2 μm pore size) prior to use. Stock solutions of amino acids were prepared daily. All solutions used for electrophoresis were dealt with ultrasonic for removing air bubble. Fused-silica capillaries (360 μm od, 25 μm id) were obtained from Yongnian Optical Fiber Factory (Hebei, China); 250-μm-diameter Cu wires were from Wuxi Cable Factory (Wuxi, China).

2.2 Equipment

Two laboratory-built high-voltage power supplies controlled automatically by a computer were used to supply separation voltage between 0 and +3000 V and sampling voltage between 0 and +1000 V, respectively. Electrochemical measurements were performed on a CHI 812 electrochemical station (CH Instruments, USA). The copper microdisk working electrode (WE) was prepared by inserting a 250-μm-diameter Cu wire into a glass capillary (400-μm od, 300-μm id), which was then sealed with 705 glue (Nanjing Chemicals). The electrode was successively polished with 2000-mesh and 4000-mesh sandpapers until a mirror-like appearance was observed, followed with washing and sonication in water bath. The WE was then oxidized in an air-oven at 100°C for more than 4 h and stored in a desiccator before use [29]. This procedure could ensure the reproducibility of the copper microelectrodes preparation. A 40-multiple light microscope (Nanjing Optics Instruments Factory, China) was employed to monitor the position of WE. Ultrasonic disintegrator with a 2 mm od probe from Ningbo Scientz Biotechnology (Ningbo, China) was used to prepare the sampling fracture.

2.3 Fabrication of AMD

The mixture of silicone and curing agent with a quantity ratio of 10:1 was prepared, degassed and poured into a mold, in which a 360 μm od fused-silica capillary was placed as a template. After being cured at 70°C for 2 h and cooled at room temperature, the capillary was pulled out to obtain the PDMS matrix with an inner channel of 360 μm diameter, which was then divided into three segments as the retainers for the preparation of sampling reservoir (SR) (3 mm diameter), buffer reservoir (BR) (4 mm diameter) and detection reservoir (DR) (4 mm diameter), respectively (Fig. 1).

A fused-silica capillary (80 mm length) was used as the separation channel of the AMD without removing the cladding. Prior to assembly, one small scratch was made at the position of 8 mm from one end of the capillary. The

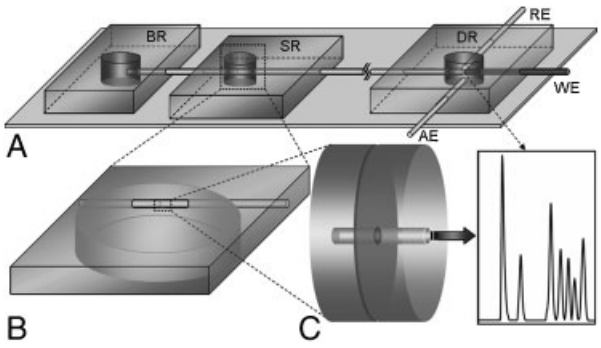

Figure 1. Scheme of an assembled microfluidic device with end-column amperometric detection (A), enlargement of SR with a fracture (B) and enlargement of the fracture for sampling (C). BR, buffer reservoir; SR, sample reservoir; DR, detection reservoir; WE, working electrode; RE, reference electrode; AE, auxiliary electrode.

capillary was then inserted into SR retainer until the scratch appeared in the area of SR, which was immersed in water and ultrasonicated with an ultrasonic probe at 150 W with an action frequency of 12 times/min and a distance of 2 mm for 1 min to obtain the sampling fracture (Fig. 1B). After the BR and DR retainers were assembled to the two sides of the SR retainer by the capillary, the WE was easily mounted in the PDMS guide channel of DR, exactly opposite to the end of the separation channel at an optimum distance of 15 ± 5 μm, at which the device showed excellent performance. Too small distance would cause large noise, while far distance produced band broadening. Then a Ag/AgCl reference electrode and a Pt wire as the auxiliary electrode were inserted to two sides of the DR due to the flexibility of PDMS matrix [29] to obtain an integrated three-electrode system for electrochemical detection (EC) of amino acids (Fig. 1A).

2.4 Analysis procedure

Aliquots containing 0.2 M NaOH, water and electrophoresis medium were individually added in BR to wash the separation channel at a voltage of 2000 V. Later, electrophoresis medium and sample solution were added in BR and SR, respectively. The fracture sampling was performed by applying a sampling voltage of 100 V between the SR and BR. To eliminate the electromagnetic interference to amperometric detection, the whole microdevice was placed in a Faraday cage. The separation voltage was then applied to the BR with the DR in grounded and the SR in floating by automatically switching the high-voltage contact, and the electropherogram was recorded on a CHI 812 using the ''amperometric i–t curve'' mode at +0.8 V (*versus* Ag/AgCl), at which the maximum ratio of signal to noise could be obtained. The electrodes used to apply the potentials were Pt wire, which were fixed in the reservoirs. All experiments were performed at room temperature.

3 Results and discussion

3.1 Properties of AMD and ultrasonic fabrication of sampling fracture

The AMD was composed of a flexible fused-silica capillary and three independent PDMS retainers (Fig. 1). From its fabrication process, other polymer materials could also be used to construct the retainers, and the separation channel could be integrated with an ultraviolet or fluorescence detection system for different analytes. More importantly, if necessary, the length and inner diameter of the separation channel could be conveniently regulated for obtaining the best separation performance. In this work an 8 cm capillary with an effective separation channel of 7.2 cm length was used for the separation of amino acids. Obviously, the preparation of AMD was easy and low cost, which provided a new path for fabrication of disposable and commercial MEDs.

On the other hand, the sampling fracture was prepared by ultrasonic instead of handwork under selected conditions, which could exclude subjective handling influence during the preparation of the fracture. The conditions, including the ultrasonic power, time, action frequency and the distance between the probe and capillary, could be experientially selected by examining the separation results. The optimal power, time, action frequency and distance were 150 W, 1 min, 12 times/min and 2 mm, respectively.

3.2 Sampling conditions

The designed AMD allowed injecting various volumes of sample by changing the sampling time or voltage. Trp was used as a model analyte for analyzing the effect of sampling time on the detection signal and separation efficiency. At a sampling voltage of 100 V, with the increasing sampling time the signal increased and trended to a constant peak current after 2 s, at which the peak became wider. Compared with the sampling time of 1 s, the separation efficiency decreased by 5 and 15% in theoretical plates at 2 and 3 s, respectively. Thus, the sampling time of 2 s at 100 V was used as the optimal sampling condition.

During the sampling process through the ultranarrow fracture a self-stacking phenomenon of analytes could be deduced due to the much less width of the fracture channel compared with separation channel (Fig. 2). The much less width led to much higher electric field strength (E) or EOF in the fracture channel than the separation channel [31]. Thus there existed a boundary of electric field strength between the fracture channel and the separation channel. Thus a stacking process occurred at the boundary, at which the migration rate of the sample suddenly slowed during the sampling. The stacking process resulted in a narrow sample plug, thus increasing the separation efficiency and the detection sensitivity.

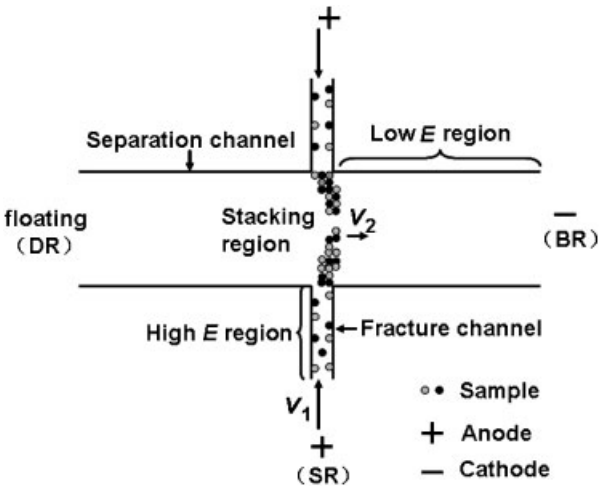

Figure 2. Cross section scheme of stacking process during the fracture sampling. v_1 and v_2 ($v_1 >> v_2$) are the migration rates of the sample in fracture and separation channels, respectively; *E*, electric field strength. Others are the same as in Fig. 1.

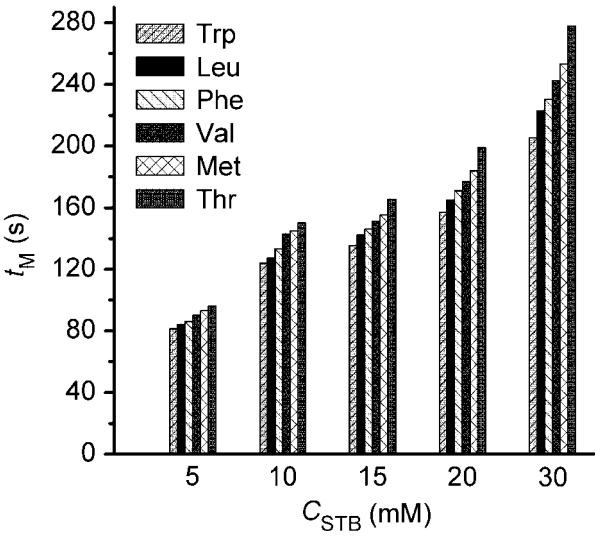

Figure 3. Effects of STB concentration on migration times of Trp, Leu, Phe, Met, Val and Thr at a separation voltage of 1800 V with a sampling voltage of 100 V for 2 s.

3.3 Optimization of electrophoresis conditions

The effect of STB concentration on migration times (t_M) of Trp, Leu, Phe, Met, Val and Thr is shown in Fig. 3. With the increasing STB concentration (ionic strength), the electrical double-layer became thinner, leading to larger migration times of these amino acids. Furthermore, high concentration of STB resulted in large current in the separation channel, which increased the Joule heat and made the baseline worse. Considering the need of time for completely separating those amino acids, 20 mM of STB was used as the optimal concentration.

The dependence of migration time and resolution (R_s) between two adjacent amino acids on the pH of the separation buffer showed that 12 amino acids could be completely separated within 300 s at pH 11.5. Although a strong alkaline condition should be used for the catalytic oxidation of amino acids on copper electrode [30], high pH increased remarkably the migration time of amino acids. Moreover, when the pH was greater than 12, carbohydrates, the most frequent interferences in amino acid analysis, would interfere with the detection of amino acids due to the dissociation of hydroxyl group in carbohydrates to become negatively charged species [16]. Thus the buffer with pH 11.5 was used as the separation buffer.

Amino acids showed some degree of adsorption on the wall of the quartz separation channel due to the interaction of amino acids with the wall [32]. The adsorption could not be avoided at highly basic condition, but could be effectively depressed by adding nonionic surfactant (Tween 20) in the buffer [17]. Thus, 0.015% Tween 20 was used as an additive to suppress the adsorption of amino acids.

To evaluate the effect of separation voltage, the migration times of Trp, Leu, Phe, Met, Val and Thr and their resolutions were measured as a function of the separation

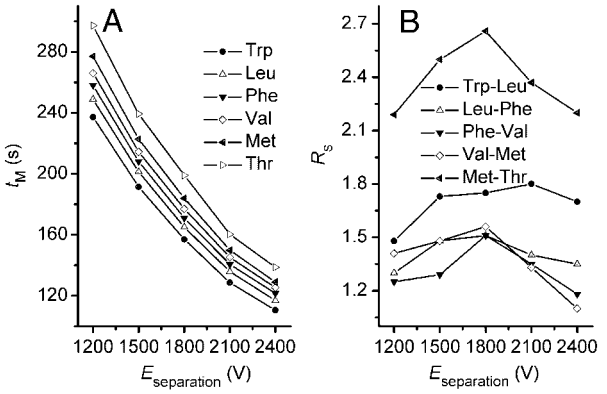

Figure 4. Effects of separation voltage on (A) migration times and (B) resolutions for Trp, Leu, Phe, Met, Val and Thr at 125 μM in 20 mM STB with a sampling voltage of 100 V for 2 s.

voltage between 1200 and 2400 V (Fig. 4). Here the resolution is defined as $2((t_M)_B-(t_M)_A)/(W_B+W_A)$, where *W* is the full peak width [33]. With the increasing separation voltage, the migration time decreased due to the increasing EOF. At the separation voltage of 1800 V, six analytes could be completely separated with R_s values more than 1.5, and the running time was less than 210 s.

3.4 Separation of amino acids

Figure 5 shows the electropherogram of 12 amino acids with the AMD under optimum conditions: sampling at 100 V for 2 s and separation at 1800 V with 20 mM STB solution (pH 11.5) containing 0.015% Tween 20. The baseline separation of these amino acids could be obtained within 300 s. All R_s values were greater than 1.5. The satisfactory separation greatly enhanced

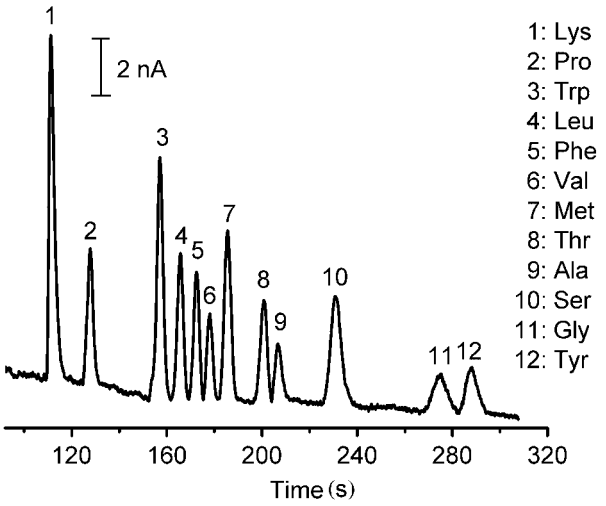

Figure 5. Electropherograms for 250 μM of 12 amino acids under optimal conditions.

from end-column detection. If an in-column detection mode were used, higher separation efficiency would be obtained, because end-column detection means loss of separation efficiency compared with in-column detection [9].

The RSDs of migration times ($n = 3$) were up to 1.2% for run-to-run, 2.1% for day-to-day and 3.0% for chip-to-chip. Thus, both the AMD and the proposed method, including sampling and separation processes, had good reproducibility and stability.

3.5 Amperometric detection

The amperometric detection of these amino acids with a copper microdisk electrode at +0.8 V (*versus* Ag/AgCl) showed the linear ranges from 12, 20, 17, 27, 31, 44, 25 and 45 to 750 μM for Trp, Phe, Met, Val, Thr, Ala, Ser and Gly, and 4.6, 17, 16 and 49 μM to 1.0 mM for Lys, Pro, Leu and Tyr with the relative coefficients from 0.994 to 0.999.

Table 1. Separation efficiency and reproducibility of AMD-EC for amino acids at 250 μM ($n = 3$) in optimal conditions[a)]

Analytes	*N* (plates/m)	RSD (%) of t_M			RSD (%) of I_P		
		Run-to-run	Day-to-day	Chip-to-chip	Run-to-run	Day-to-day	Chip-to-chip
Lys	136 000	0.2	0.3	1.2	3.0	4.6	5.2
Pro	139 500	0.3	0.6	1.2	5.8	6.3	7.3
Trp	168 800	0.4	0.6	1.3	4.0	5.8	4.9
Leu	265 000	0.4	0.8	1.9	5.8	7.2	7.9
Phe	299 700	0.6	0.9	2.1	6.1	7.3	8.2
Val	333 100	0.6	0.9	2.6	4.5	5.2	4.7
Met	252 800	0.7	1.0	2.4	4.1	5.6	7.5
Thr	332 000	0.9	1.3	2.5	4.9	6.4	7.0
Ala	303 000	0.9	1.5	2.7	7.0	8.2	7.2
Ser	172 300	1.0	1.7	3.0	5.1	6.4	8.1
Gly	158 700	1.1	1.8	2.9	4.9	5.9	7.0
Tyr	227 900	1.2	2.1	3.0	5.4	7.0	8.7

a) Optimal conditions: sampling at 100 V for 2 s and separation at 1800 V with 20 mM STB solution (pH 11.5) containing 0.015% Tween 20.

the ability of the AMD to analyze complex amino acids samples, which was a typical obstacle for microdevices or chips in practical application. Moreover, the separation results of 12 amino acids were even better than the CE separation of 11 amino acids within 36 min with an 80 cm capillary [3] and 12 amino acids within 48 min with a 120 cm capillary [4].

The theoretical plate numbers (N) and separation reproducibility of these amino acids are summarized in Table 1. Here, N is defined as $16(t_M/W)^2$, where W is the full peak width [33]. The N values ranging from 136 000 to 333 100 plates/m were much greater than those of 12 000 plates/m for Phe and Gly [10], 14870 and 29730 plates/m for Pro and Thr, respectively [17], 12000 and 8200 plates/m for Trp and Gly, respectively [18], and 12577 and 17354 plates/m for Tyr and Trp, respectively [19], showing much better separation efficiency of the proposed AMD. It should be pointed out that the N values were obtained Compared with those obtained from conventional MEDs [8, 12, 15, 17] the results obtained from the AMD showed wider detection ranges by one order magnitude. The detection limits at the signals to the maximum baseline noise ratio of 3 were 1.4, 5.2, 3.8, 4.7, 6.2, 8.1, 5.1, 9.3, 13.2, 7.7, 13.6 and 14.8 μM for Lys, Pro, Trp, Leu, Phe, Val, Met, Thr, Ala, Ser, Gly and Tyr, respectively, which were lower than those of 15.3 μM for Pro and 12.8 μM for Thr with a copper electrode on PDMS microchip electrophoresis [17]. The detection sensitivity of these amino acids was in the range from 14.5 to 173 nA/μM/cm^2, which is much higher than those of 0.047–45.6 nA/μM/cm^2 by CE at a copper disk electrode [3]. The high sensitivity should be due to the self-stacking effect.

The RSDs of peak currents (I_p) measured at the concentration of 250 μM analytes ($n = 3$) were from 3.0 to

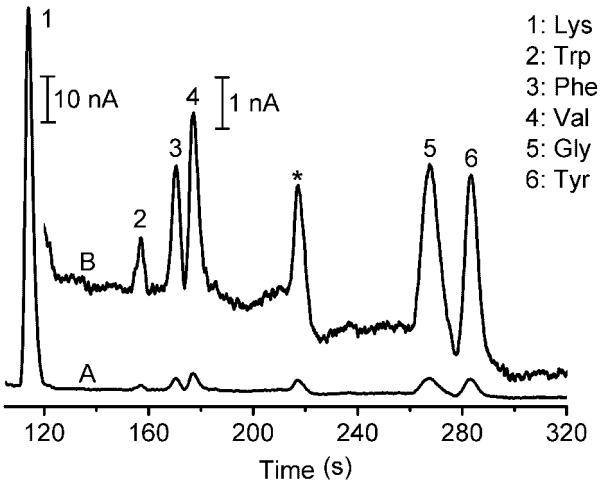

Figure 6. Electropherograms for a diluted beverage sample. Curve B is the enlargement of curve A between 120 and 320 s. The peak labeled with asterisk means an unknown component.

7.0% for run-to-run, from 4.6 to 7.3% for day-to-day and from 4.7 to 8.7% for chip-to-chip, indicating that the AMD including the used copper microdisk electrode and the fracture sampling had good fabrication reproducibility.

3.6 Analysis of amino acids in beverage

The AMD with amperometric detector was used for the analysis of amino acids in beverage. The beverage sample was firstly diluted at 1:20 v/v with electrophoresis buffer. The resulting electropherogram is illustrated in Fig. 6. Six components observed from the electropherogram corresponded to Lys, Trp, Phe, Val, Gly and Tyr, which were confirmed by adding standard solutions of amino acids to the sample. The fifth peak occurred in the electropherogram was an unknown component in the sample, which showed much different migration time from amino acids. The other amino acids were not seen in the sample. The concentrations of Lys, Trp, Phe, Val, Gly and Tyr in the sample were measured with the calibration curves to be 959.8, 24.0, 136.2, 250.1, 395.6 and 493.9 μM with the RSD ($n = 3$) of 3.3, 3.6, 4.5, 6.4, 6.2 and 7.5%, respectively. The total concentration of detectable amino acids in the beverage was calculated to be 702.4 mg/100 mL. In comparison with the given 743 mg/100 mL for total amino acids in the beverage, the relative deviation was 5.5%. The recoveries of amino acids added to the beverage sample were from 97.8 to 105.2%.

4 Concluding remarks

An assembled and disposable microfluidic device with a sampling fracture has been developed by integrating three PDMS retainers with a fused-silica (quartz) capillary for baseline separation and sensitive amperometric detection of 12 amino acids. An ultrasonic method is proposed to form an ultranarrow fracture on the capillary. The fracture sampling with a low sampling voltage produces an on-column sample self-stacking effect to narrow the sample plug, thus leading to high separation efficiency and detection sensitivity. The low cost, convenient fabrication, high integration, wide linear ranges, low detection limits, good fabrication reproducibility and detection precision show that the AMD and the proposed methodology have excellent analytical application in different fields.

We gratefully acknowledge the financial support of the National S&T Pillar Program (2007BAK26B06, 2006BAK09B05) from the Ministry of S&T, and the National Science Fund for Creative Research Groups (20521503), Key Program (20535010), Major Research Plan (90713015) and General Program (20745003, 20705012) from NSFC.

The authors have declared no conflict of interest.

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