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Ultraviolet detection of amino acids based on their on-column conjugation with cupric cation using a disposable electrophoresis microdevice

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ABSTRACT

A method for fast sensitive ultraviolet detection of amino acids was developed with a disposable electrophoresis microdevice. The microdevice was conveniently constructed by fixing a fused-silica capillary with a sampling fracture to a printed circuit board. During the separation process, the on-column conjugation of amino acids with cupric cation led to the ultraviolet absorption at 232 nm that could be directly used for fast analysis of amino acids. Using 20 mM boric acid (pH 5.3) containing 5 mM cupric cation and 0.015% Tween 20 as running buffer, this method could completely separate lysine, glutamine and serine at a sampling time of 2 s at +210 V and a separation voltage of +1800 V (240 V/cm). The theoretical plate numbers were from 140,000 to 205,000 plates/m. The linear ranges were from 10 to 500 μ M for lysine, 20–1000 μ M for glutamine and serine. The novel protocol had been successfully used to detect amino acids in beverage samples with recovery more than 85.0%, indicating its advantages and potential analytical application in different fields.

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1. Introduction

Microfluidic electrophoresis device (MED) is a powerful analytical tool due to its low consumption of reagents, short separation time, high separation efficiency, and low cost [1–3]. Many efforts have been made for the development of MED in different fields, including the design of cheap disposable device and the establishment of fast, sensitive and selective detection methods. Compared to those of glass or quartz MEDs, the utilization of polymer materials such as polydimethylsiloxane [4], polymethylmethacrylate (PMMA) [5], and polycarbonate [6] reduces the cost of MED preparation. However, these polymer materials are unfavorable to fabricating MED for ultraviolet (UV) detection. Our previous works used a fused-silica capillary as separation channel to develop a cheap hybrid quartz capillary/PMMA MED by using PMMA as the support model of quartz capillary and a printed circuit board (PCB) as the support substrate of PMMA and electrophoresis electrodes [7,8]. The hybrid MED utilized fracture sampling technique to obtain high separation efficiency and high detection sensitivity. This work designed a hybrid quartz/PCB MED (HQPM) by fixing directly a quartz capillary on a PCB for convenient construction and developed a novel method for fast UV detection of amino acids.

The analysis of amino acids has attracted considerable attention because of their important physical functions, broad distribution in nature and wide applications in human life. Due to the lack of UV absorption, fluorescent emission or electroactive group of these compounds, the detection of amino acids in their native forms is difficult [9]. Although derivatization methods of amino acids have been developed to improve the detection sensitivity of amino acids with fluorescence [10–12], or electrochemistry [13–15] in MED, 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 [16,17], have exhibited attractive advantages for direct determination of amino acids due to the high sensitivity and easy to miniaturization. However, the high voltage needed for the fast separation generally affects the amperometric detection. To avoid the derivatization of amino acids indirect UV [18] or fluorescent [19] detection methods have been developed. The sensitivity of these methods still needs to be further improved for their practical application. Several methods based on the conjugation of cupric cation with amino acids for their chemiluminescence [20] and UV detection [21–23] have been reported. By combining the newly designed HQPM this work proposed an on-column conjugation procedure of amino acids with cupric cation in separation buffer to produce UV absorption relative to the amount of amino acids. This method could achieve the baseline separation and UV detection of several amino acids within 3 min. This protocol could be successfully applied to detect amino acids in practical samples.

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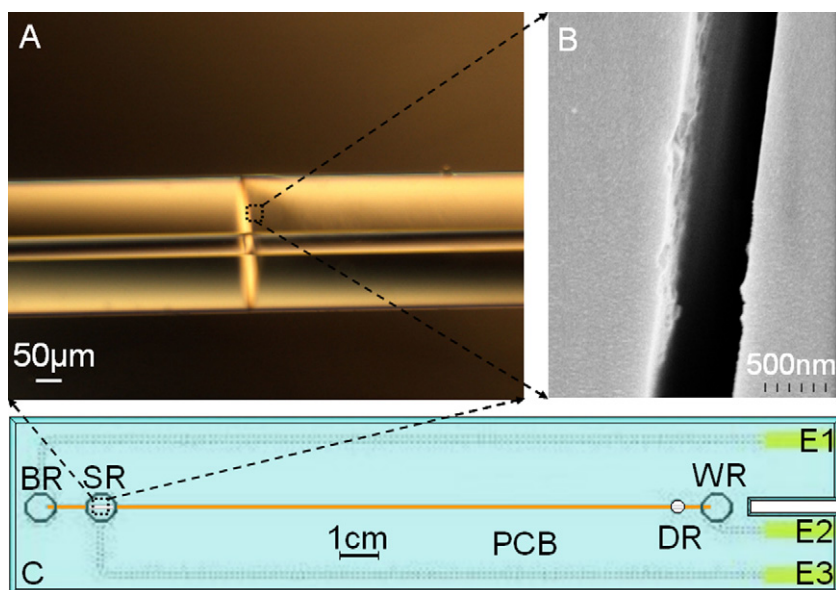


Fig. 1. Photo and SEM image of sampling fracture (A and B) on the HQPM (C). BR, buffer reservoir; SR, sample reservoir; DR, detection reservoir; WR, waste reservoir; E1, E2 and E3, electrodes for applying sampling and separation voltages.

2. Experimental

2.1. Reagents and materials

Glutamic acid (Glu), glutamine (Gln), leucine (Leu), lysine (Lys), proline (Pro) and serine (Ser) with the purity of 99% were purchased from Acros. Beverage for real sample detection was from Kirin (Shanghai, China), which was a commercially artificial amino supply drink. All solutions were prepared using $\geq 18 \text{ M}\Omega$ ultrapure water (Milli-Q, Millipore). Boric acid used for preparation of running buffer was passed through a membrane filter ($0.2 \mu\text{m}$ pore size) and dealt with ultrasonic for removing air bubbles prior to use. Fused-silica capillaries ($360 \mu\text{m}$ o.d., $50 \mu\text{m}$ i.d.) were obtained from Yongnian Optical Fiber Factory (Hebei, China). All other chemicals were of analytical grade.

2.2. Equipments

The UV microfluidic workstation employed in this work was home manufactured by cooperation with Beijing Cailu Scientific Instrument Limited Company. As described in our previous work [7], it was composed of a four-port high-voltage power supply, an UV detector and data processor. Ultrasonic disintegrator with a 2-mm o.d. probe from Ningbo Scientz Biotechnology Co., Ltd. (Ningbo, China) was used to prepare the sampling fracture. An inverted fluorescence microscope (Nikon Eclipse TE2000-U) was used to observe the fracture. Scanning electron microscopic (SEM) image of the sampling fracture were obtained on a Hitachi S-4800 scanning electron microscope (Japan).

2.3. Fabrication of HQPM

The HQPM was fabricated with a modified process reported previously [7]. Briefly, a PCB ($85 \text{ mm} \times 15 \text{ mm} \times 1 \text{ mm}$ for length \times width \times thickness) with a groove was firstly prepared with usual technique, and four holes were drilled on the PCB as buffer reservoir (BR, 3-mm i.d.), sample reservoir (SR, 3-mm i.d.), detection reservoir (DR, 1.5-mm i.d.) and waste reservoir (WR, 3-mm i.d.), respectively. A pretreated capillary was then fixed using 705 silicone glue in the groove on the PCB, where BR, SR and WR were respectively connected with E1, E2 and E3 for

applying separation and sampling voltages (Fig. 1). Before put into the groove, the fused-silica capillary (7.5-cm length) was cut with potsherd at 0.5 cm from one end, and the polymer coating at 0.6 cm from the other end was removed for UV detection. After the capillary was fixed on the groove, the cut at the position of sample reservoir was ultrasonated to form a perfect sampling fracture [17].

3. Results and discussion

3.1. Properties of HQPM

The ultrasonic method for formation of sampling fracture on a capillary excluded the subjective handling influence on the fracture quality [17]. Thus the preparation reproducibility of sampling fracture was good, which could be verified by the reproducible results for amino acids detection. The enlarged images of sampling fracture were shown as Fig. 1A and B, at which the width of the sampling fracture was measured to be about 800 nm, which was identical to the fracture width of quartz capillary/PMMA MED [8]. Sampling from the ultra narrow fracture could produce a very narrow sample plug, thus suppressing effectively sample leakage and zone broadening, improving greatly the separation efficiency, and reducing largely the sample consumption [24]. In addition, the fused-silica capillary could be easily peeled off from the HQPM. Thus the PCB could be conveniently recycled after use, which could reduce the detection cost. Compared with the PMMA MED, the preparation of HQPM avoided the use and assembly of PMMA to the PCB, thus was more simple and convenient.

3.2. Detection wavelength

No UV absorption of Lys in the wavelength range from 210 to 300 nm could be observed (Fig. 2A, curve 1). Although cupric cation showed obvious absorption in the wavelength range of 210–240 nm (Fig. 2A, curve 2), it was much lower than that of the mixture of Lys and cupric cation at the same concentrations (Fig. 2A, curve 3). The mixture showed a greatly enhanced absorption peak at 230 nm, indicating some degree of conjugation of amino acids with cupric cation. After subtracting the absorption of cupric cation, this peak shifted to 232 nm (Fig. 2A, curve 4), which could be used

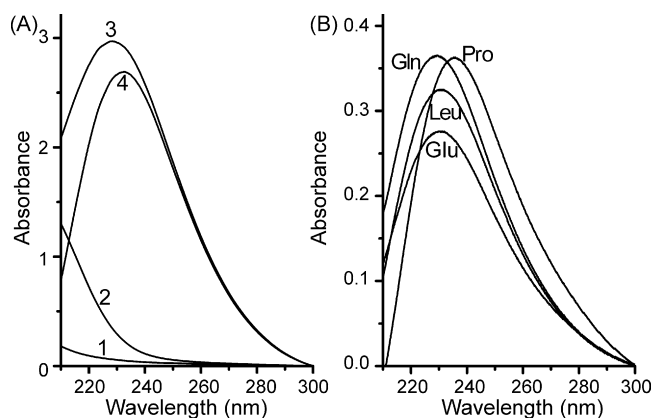


Fig. 2. UV absorption of (A) 1 mM Lys (1), 1 mM cupric cation (2), the mixture of 1 mM Lys and 1 mM cupric cation (3), and difference between 3 and 2 (4), and (B) differences of 0.5 mM Glu, Leu, Gln and Pro to 1 mM cupric cation.

as the detection wavelength of the complex of cupric-Lys in presence of cupric background in the running buffer. Besides Lys, several typical amino acids, Glu, Pro, Gln and Leu as the representatives of acidic, imine, amine, amide and neutral amino acids, also showed the UV absorption in the presence of cupric cation (Fig. 2B). Their maximum absorption peaks occurred in the range of 230–234 nm. Thus, 232 nm was chosen as the detection wavelength of amino acids, at which all amino acids showed the UV absorption more than 98.7% of maximum absorbance.

3.3. Optimization of running buffer

When solution pH is higher than 6, cupric cation can form deposition. Thus acetic acid or boric acid could be used as the candidate of running buffer. Compared with acetic acid, boric acid showed much weaker UV absorption around 232 nm, which was favorable to the UV detection of analytes in the running buffer. Thus, boric acid was selected as the running buffer.

High buffer concentration could suppress the electroosmotic flow (EOF) and thus resulted in long t_M [21]. Moreover, high concentration of running buffer would produce more Joule heat in the separation channel to worsen the separation and decrease the reproducibility of the analysis. However, when the concentration of running buffer was less than 20 mM, the buffer capacity was not enough for keeping a stable baseline. Considering the analytical time, stability of separation and buffer capacity, 20 mM of boric acid (pH 5.3) was used as the optimal running buffer, in which the copper complexes could be formed [25].

In the optimal running buffer Lys, Gln and Ser did not show the absorption signal. Upon addition of cupric cation into the buffer, their UV spectra showed obvious absorption. All the absorbance at 232 nm increased with the increasing concentration of cupric cation and trended the saturated values at the cupric concentrations of 4 mM for Lys and 1 mM for Gln and Ser (Fig. 3). When the concentration of cupric cation was higher than 5 mM, the baseline noise increased sharply. Thus, for obtaining a quantitative result, 5 mM of cupric cation was used as the additive to perform the chelation with amino acids, whose concentrations could be variable in samples.

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 [26]. The adsorption could be effectively depressed by adding nonionic surfactant (Tween 20) in the buffer [27]. Thus, 0.015% Tween 20 was used as additive to suppress the adsorption of amino acids.

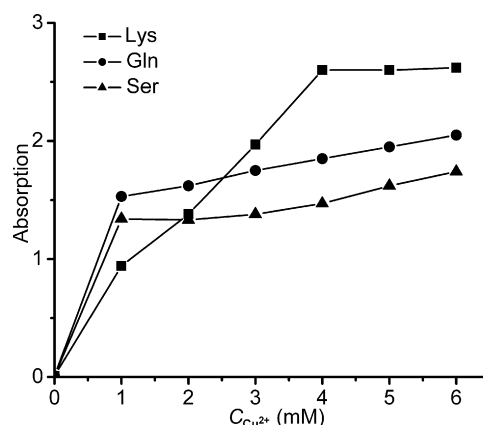


Fig. 3. Effect of cupric cation concentration on UV signals of Lys, Gln and Ser at 232 nm.

In summary, 20 mM boric acid (pH 5.3) containing 5 mM cupric cation and 0.015% Tween 20 were used as the optimal conditions of the running buffer.

3.4. Effects of separation voltage and sampling conditions

The separation voltage affected the migration time (t_M) and separation efficiency by alerting the EOF. Low separation voltage resulted in long t_M , while high separation voltage led to high Joule heat in separation channel. At the separation voltage of +1800 V (240 V/cm), a stable baseline could be obtained and the t_M of Lys was about 100 s (Fig. 4), thus, it was selected as the separation voltage to obtain fast separation.

The sampling voltage and time were related to the detection sensitivity and separation efficiency. Low sampling voltage and short sampling time were allowed in the fracture sampling technique [7,8,17,28], which indicated the utilization of low-cost power and the limitation for the sample zone diffusion. When the sampling voltage was higher than +210 V, the baseline became unstable. However at the sampling voltages less than +210 V, long sampling time was needed for sensitive detection, which was disadvantageous with the separation due to the serious broadening of sample zone. At the sampling voltage of +210 V the sampling time of 2 s was suitable for avoiding the broadening and obtaining high separation efficiency of amino acids.

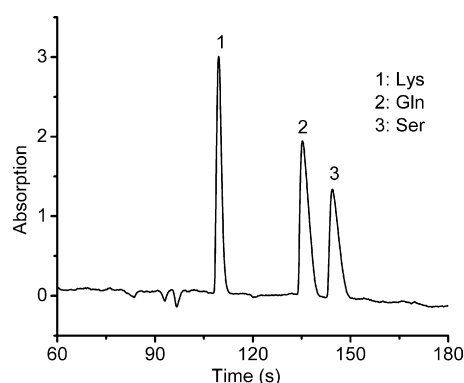


Fig. 4. Electropherogram for 50 μ M Lys, 100 μ M Gln and Ser under optimal conditions: separation voltage, +1800 V; sampling voltage, +210 V for 2 s; running buffer, 20 mM boric acid (pH 5.3) containing 5 mM cupric cation and 0.015% Tween 20; detection wavelength, 232 nm.

Table 1
Separation efficiency and reproducibility of HQPM-UV for amino acids ($n=6$).

Analytes	N (plates/m)	RSD (%) of t_M			RSD (%) of UV detection		
		Run-to-run	Day-to-day	Device-to-device	Run-to-run	Day-to-day	Device-to-device
Lys	205,000	1.2	1.5	1.6	4.2	5.6	6.2
Gln	158,000	1.3	1.4	1.9	4.9	6.7	8.9
Ser	140,000	1.5	1.7	2.0	5.8	6.9	8.3

3.5. Separation and detection of amino acids

Under optimum conditions the electropherogram of 50 μM Lys, 100 μM Gln and Ser showed baseline separation (Fig. 4) within 3 min. The theoretical plate numbers (N) and separation reproducibility of these amino acids were illustrated in Table 1. Here, N is defined as $16(t_M/W)^2$, and W is the full peak width [29]. The N values ranging from 140,000 to 205,000 plates/m were about one order magnitude higher than those of 12,000 plates/m [15], 14,870 and 29,730 plates/m [27], 12,000 and 8200 plates/m [30], and 12,577 and 17,354 plates/m [31] for amino acid separation on MEDs, showing much better separation efficiency of the proposed HQPM.

The relative standard deviations (RSD) of migration times ($n=6$) were less than 1.5% for run-to-run, 2.5% for day-to-day, and 3.1% for device-to-device, indicating acceptable reproducibility of the separation and the fabrication of HQPM.

The RSD ($n=6$) of peak areas measured at the concentration of 50 μM of Lys, 100 μM of Gln and Ser were from 4.2% to 5.8% for run-to-run, 5.6–6.9% for day-to-day and 6.2–8.9% for device-to-device. These results indicated both the designed HQPM and the proposed method including the sampling fracture and UV microfluidic workstation had acceptable reproducibility and stability.

The calibration curves, plots of peak area vs. amino acid concentration, for UV detection of amino acids showed the linear ranges from 10 to 500 μM for Lys, 20–1000 μM for Gln and Ser with relative coefficients from 0.996 to 0.999. Compared with those obtained from conventional MEDs [13,27,32,33] the results obtained from the HQPM showed wider detection ranges by one order magnitude. The detection limits at the S/N ratio of 3 were 2.6, 4.0 and 4.8 μM for Lys, Gln and Ser, respectively, which were close to the results obtained by amperometric detection in our previous work [17], lower than those of 32.9 μM for indirect fluorescence detection [19], 7.1 μM for contact conductivity detection [33], 32–50 μM [34] and 5–10 μM for contactless conductivity detection [35]. The high sensitivity should be due to the self-stacking effect of the fracture sampling [17] and the strong enhancement of UV absorbance due to the chelating of amino acids with cupric cation.

3.6. Analysis of amino acids in beverage sample

The designed method was tested by analyzing amino acids in commercial available beverage samples. The sample solution was firstly diluted at 1:20 (v/v) with running buffer. The resulting electropherogram was illustrated in Fig. 5. In the presence of cupric cation one obvious peak occurred at the t_M value as the same as that of Lys. Furthermore, sugars which were abundant in the beverage samples (6.9 g/100 ml) did not show any interference with the UV detection of amino acids under the optimal conditions (Fig. 5, curve 2). In order to identify this peak, a standard solution of Lys was spiked into the sample, which led to an enhanced signal at the same t_M (Fig. 5, curve 3). The concentration of Lys in the sample was measured with the calibration curve to be 76.9 μM . Considering the dilution of the sample, the Lys content was calculated to be 28.1 mg/100 ml, which was similar to the given 30 mg/100 ml for Lys in the beverage. The recovery for the spiked Lys was calculated to be 85.6%, indicating satisfactory accuracy.

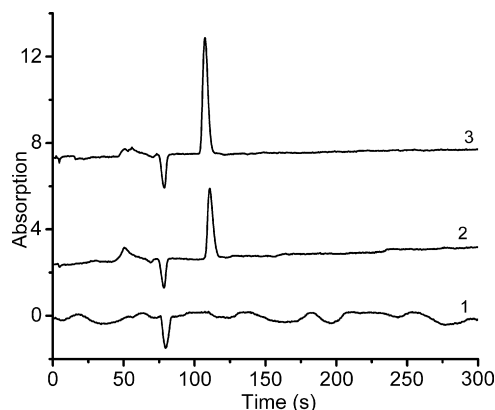


Fig. 5. Electropherograms for beverage sample in absence (1) and presence (2) of cupric cation and the sample spiked with 50 μM Lys (3). Other conditions are the same as Fig. 4.

4. Conclusions

A fast, sensitive, selective and low-cost approach was developed for the UV detection of amino acids using a disposable electrophoresis microdevice. The proposed strategy could directly detect amino acids and exclude the interference of sugars in the samples, thus avoided the trouble derivatization and pretreatment of samples. The ultrasonic-formed sampling fracture produced a very narrow sample plug to achieve highly efficient separation and good sampling and separation reproducibility. The low cost, convenient fabrication, wide linear ranges, low detection limits, good fabrication reproducibility and detection precision, and satisfactory accuracy showed that the HQPM and the proposed methodology have excellent analytical application in different fields.

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