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Determination of underivatized amino acids to evaluate quality of beer by capillary electrophoresis with online sweeping technique

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*Tian Luo, Jing Ke, Yunfei Xie, Yuming Dong**

Institute of Pharmaceutical Analysis, School of Pharmacy, Lanzhou University, Lanzhou, Gansu Province 730000, PR China

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ABSTRACT

Capillary electrophoresis (CE) with ultraviolet detection was applied to determine underivatized amino acids in beer, based on the coordination interaction of copper ions and amino acids. An online sweeping technique was combined with CE to improve detection sensitivity. Using the United Nations Food Agriculture Organization/World Health Organization model of essential amino acid pattern and flavor of amino acids, the quality and taste in three kinds of beer were evaluated. It was found that Beer2 had higher quality than the other two kinds and the content of phenylalanine, proline, serine, and isoleucine was relatively large in all three kinds of beers with a great influence on beer flavor. Optimal conditions for separation were as follows: 50mM $CuSO_4$ at pH 4.40 as buffer; total length of fused silica capillary, 73 cm; effective length, 65 cm; separation voltage, 22.5 kV; and optimized sweeping condition, 70 seconds. In the appropriate range, linearity ($r^2 > 0.9989$), precision with a relative standard deviation $< 8.05\%$ ($n = 5$), limits of detection (0.13 −0.25 µg/mL), limit of quantification (0.43−0.83 µg/mL), and recovery (80.5−115.8%) were measured. This method was shown to be applicable to the separation of amino acids in beer and to perform quantitative analysis directly without derivatization for the first time.

1. Introduction

Amino acids, as the building blocks of proteins [1], are important life substances in nature, and they are essential nutrients in a series of samples, such as foods, beverages, plants, and pharmaceutical drugs. Therefore, they are often used to characterize foods [2] and beverages [3], as well as in quality control tests. Amino acids are also regarded as necessary ingredients in beer, because they play a key role in beer brewing [4], which can affect the taste and quality of beer [5]. There are many kinds of amino acids in beer, but their contents are low. Therefore, establishing a rapid and sensitive method for determination of amino acids in beer is helpful to control the quality and supervise the preparation process of beer.

At present, the analytical methods of determining amino acids in beer are gas chromatography (GC) [6], high-

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performance liquid chromatography (HPLC) [5,7–9], ^{1}HNMR spectroscopy [10], HPLC–mass spectrometry (HPLC–MS) [11–13], GC–MS [7], and capillary electrophoresis (CE) [4,14]. Among these methods, CE has advantages such as high efficiency, good resolution [15], and low consumption of samples and reagents [16], so it is widely used in the analysis of amino acids in beer. However, because most amino acids (except phenylalanine, tryptophan, and tyrosine) do not have strong chromospheres in their structures, they exhibit neither UV absorption nor fluorescence [17]. Consequently, amino acids are always determined by derivatization to increase detection sensitivity [18]. However, the derivative methods have some specific drawbacks, for instance, high requirements of reagents, complex operation process, and long derivatization time [14]. In addition to derivatization, the HPLC–MS and GC–MS methods can directly determine the amino acids in beer, but these techniques are more expensive than HPLC, GC, and CE, and not all laboratories are equipped with the related equipment to perform these analyses. Therefore, using a method of underivatization with commonly available technology is desirable.

Although the UV absorption of amino acids is very weak, they can form complexes $[Cu(AA)n]^{+2}$, where AA is amino acids, by coordination interaction with copper ions. This method was first proposed by Bazzanella and Bächmann [19] in 1998. The N and O atoms on the amino acid are used as coordination atoms [20]. The metal ions that have empty orbits and the ability to accept electrons act as center ions [21]. Under the effect of radiation, charges on the ligand orbitals transfer to copper tracks while producing an absorption band that has a strong molar absorptivity (ε) about 10^4. $[Cu(AA)n]^{+2}$ complexes have the strongest UV absorption under 254 nm [20,22], which greatly improves the sensitivity of determination of amino acids. Determination of amino acids according to the coordination interaction between amino acids and copper ions has been reported previously. Jiang et al [20] used this method to analyze amino acids in human saliva and green tea. In 2012, Xue and Hong-Mei [22] achieved effective separation of amino acids, also based on coordination interaction, in Radix Asparagi. In a similar way, Zhou and Shi [23] determined amino acids in plasma and nutritional supplements. However, there are no studies about using this method to analyze amino acids in beer. As mentioned earlier, beer is rich in amino acids and amino acids have a decisive effect on the fermentation of beer and on its taste and quality. In this study, we accurately and rapidly analyzed amino acids in beer according to the coordination interaction between amino acids and copper ions for the first time.

The effective separation of underivatized amino acids was achieved by coordination interaction between amino acids and copper ions. However, the sensitivity of amino acids sample was reduced, as copper ions with ultraviolet absorption were added to the buffer, and the contents of amino acids in beer were low. Therefore, in this study, the sweeping technique was used to improve the detection sensitivity. Sweeping is an effective online preconcentration technique that utilizes the interaction of analytes with additives that include a pseudostationary phase in electrokinetic chromatography such as sodium dodecyl sulfate and a complex agent in capillary zone electrophoresis to concentrate the analytes into a narrow zone to enhance the detection sensitivity [15,24]. The role of copper ions is similar to additives. Under high voltage conditions, the effective mobilities of amino acids are slower than copper ions; copper ions are regarded as *besom* and concentrate amino acids into narrow bands within the capillary. Thus, the sensitivity of amino acids is greatly enhanced.

In this study, based on the coordination interaction between amino acids and copper ions, the sweeping technique was applied for direct separation of amino acids in beer for the first time. Using United Nations Food Agriculture Organization/World Health Organization's [25] model of essential amino acid pattern and flavor of amino acids, quality and taste in three kinds of beers were evaluated.

2.   Materials and methods

2.1.   Reagents and solutions

L-Lysine, L-histidine, L-aspartate, L-cysteine, L-tryptophan, L-serine, L-phenylalanine, L-glycine, L-valine, L-alanine, L-leucine, L-isoleucine, L-threonine, L-methionine, L-proline, and L-glutamic acid were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China; purity >99.5%). Copper sulfate and sodium acetate were purchased from Shuang Shuang Chemical Reagent Co., Ltd (Yantai, China). The purified water used to prepare various solutions was obtained from the GLP Laboratory of Lanzhou University (Lanzhou, China). Acetic acid (HPLC grade) was purchased from Shandong Yu Wang Pharmaceutical Co., Ltd. (Shandong, China). Three kinds of beers were purchased from a local supermarket (Lanzhou, China).

2.2.   Equipment

In this work, the CE was equipped with a K1060 system (KAIAO, Beijing, China) and a UV detector. A workstation with Easychrom-1000 software (Beijing, China) was used for data acquisition and evaluation. The uncoated fused silica capillaries (Hebei, China) were used to finish separation; the dimensions of the capillary were as follows: 50 μm i.d. × 375 μm o.d., 73 cm in total length, with length to the detector of 65 cm. The FE20 pH meter (Mettler Toledo Instrument Co., Ltd, Shanghai, China) was used to measure the pH of the buffer. Lac part analytical balance (Shanghai Ohaus Discovery professional analytical balance, Shanghai, China) was used for weighing the buffer solution.

2.3.   Solution and sample preparation

All 16 amino acids (0.00100 g) were dissolved in purified water to obtain a concentration of 1.00 mg/mL; stock solutions were stored at 4°C until use. The stock solutions were then diluted in different concentrations as needed prior to use. Stock buffer solutions of copper sulfate (100mM) were prepared with purified water daily. Beer samples were obtained from a local supermarket (Lan Zhou), degassed, and then subjected to direct sampling. All solutions and samples were filtered using 0.22-μm membranes prior to use.

2.4. Electrophoresis conditions

Before the experiment, the new capillary was rinsed in the following sequence: methanol for 3 minutes, water for 10 minutes, 1.0M HCl for 15 minutes, water for 3 minutes, 1.0M NaOH for 15 minutes, then water for 3 minutes, finally running buffer for 10 minutes. Between runs, the rinsed sequence includes water for 2 minutes, 1.0M NaOH for 3 minutes, water for 2 minutes, and running buffer for 2 minutes. At the end of experiment, the capillary was flushed with water for 10 minutes and air was injected into the capillary using an empty syringe.

3. Results and discussion

3.1. Optimization of copper(II) sulfate concentration

The concentration of copper ions has a significant influence on the separation of amino acids. The resolution of amino acids increased with the increasing of copper ions. This can be explained as follows: when the buffer concentration increased, strength of ions became stronger. At the same time, the interaction between the solute ions and capillary inner wall reduced, and so did the interaction between analytes, which greatly improved the separation of amino acids. However, if the concentration was too high, which lead to excessive ion intensity, a large Joule heat was produced. Therefore, as shown in Figure 1, 50mM was selected as the optimal concentration of copper(II) sulfate.

3.2. Optimization of the pH value of running buffer

In this study, acetic acid and sodium acetate were used to control the pH value of the buffer. pH can not only control the intensity of electroosmotic flow (EOF), but also has a great impact on the hydrolysis of Cu^{2+}. If the pH is too low, hydrolysis of copper ions can be restrained, as shown in Eq. (1).

$$Cu^{2+} + 2H_2O \leftrightharpoons Cu(OH)_2 + 2H^+ \tag{1}$$

However, the EOF will become smaller in too pH; even without EOF, a longer time is needed to finish the analysis. Thus, the pH of the buffer cannot be too low. By contrast, a higher pH (alkaline) is advantageous for the rapid analysis of the sample due to the larger EOF. However, chemical precipitation of copper sulfate will occur, eventually generating precipitates of copper hydroxide. In view of these factors, pH 4.40 was selected by the optimization of acidity. The results are shown in Figure 2.

3.3. Optimization of separation voltage

In CE, separation voltage is a very important parameter for EOF. A voltage too high or too low will not favor separation, as they will trigger the problem of Joule heat or reduction of separation efficiency, respectively. Therefore, we must strike a balance between the separation efficiency and Joule heat.

In this study, Ohm's law was used to select the appropriate voltage. According to the change of current, the
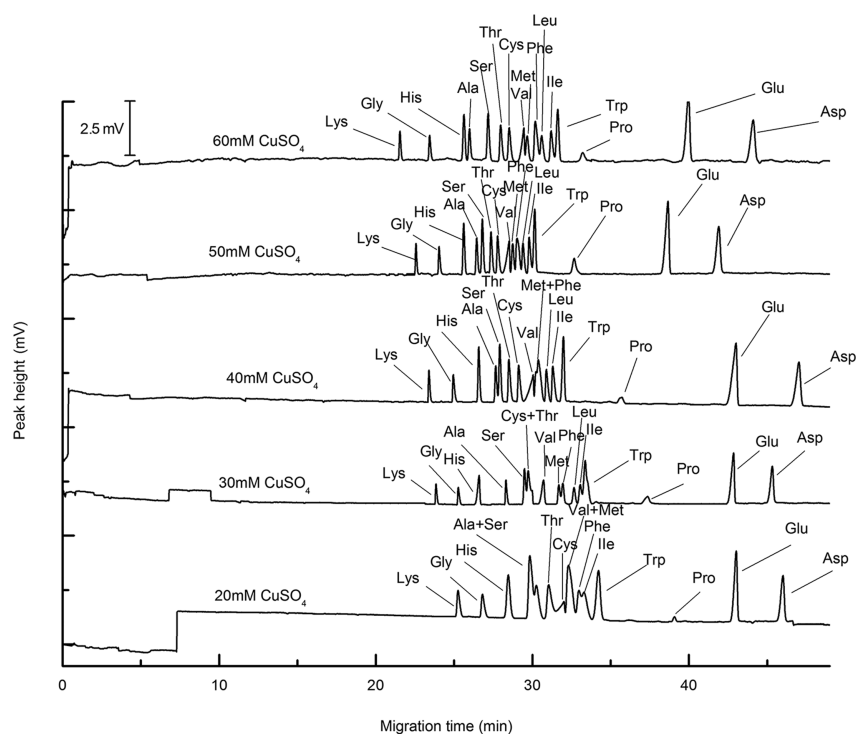
Figure 1 — Effects of copper(II) sulfate concentration on the separation. The running buffer contained 60 mmol/L, 50 mmol/L, 40 mmol/L, 30 mmol/L, and 20 mmol/mL CuSO₄ from top to bottom. The pH was 4.40 and separation voltage was 20 kV; other conditions were as described in the text. UV absorption at 254 nm.
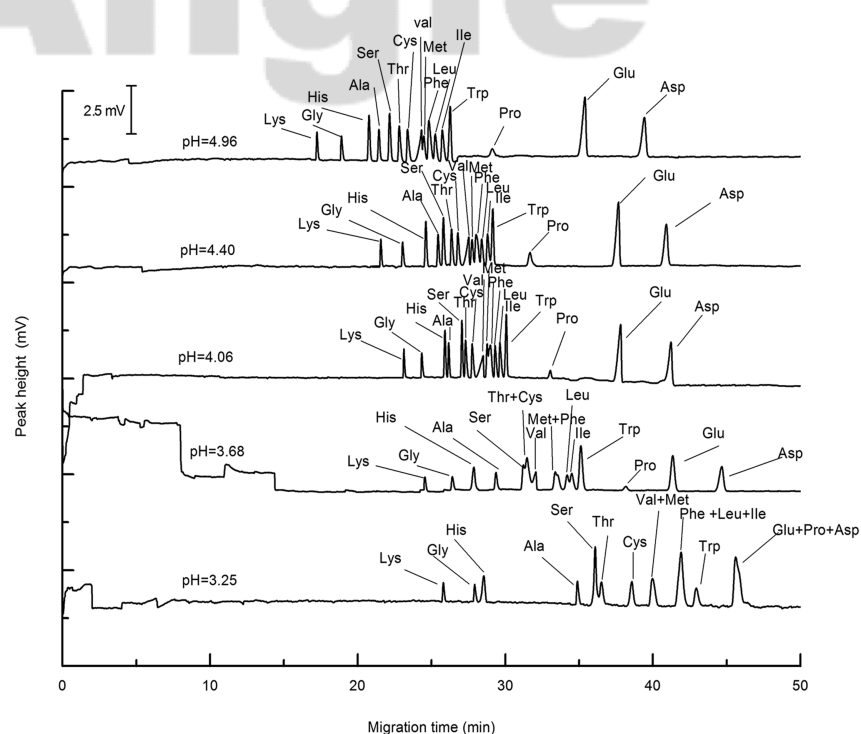
Figure 2 – Effect of pH on the separation. The running buffer contained 50 mmol/mL CuSO$_4$; from top to bottom, pH values were on the order of 4.96, 4.40, 4.06, 3.68, and 3.25. Separation voltage was 20 kV and other conditions were as described in the text. UV absorption at 254 nm.

voltage–current profile was generated to determine the reasonable voltage. As shown in Figure 3, when the separation voltage was 25 kV, the relationship between the current and voltage deviated from Ohm's law, and the current increased greatly. This phenomenon indicated that high Joule heat has been produced, which would affect the separation of samples, under this voltage [26]. Through a series of experiments, the separation voltage of 22.5 kV was used.
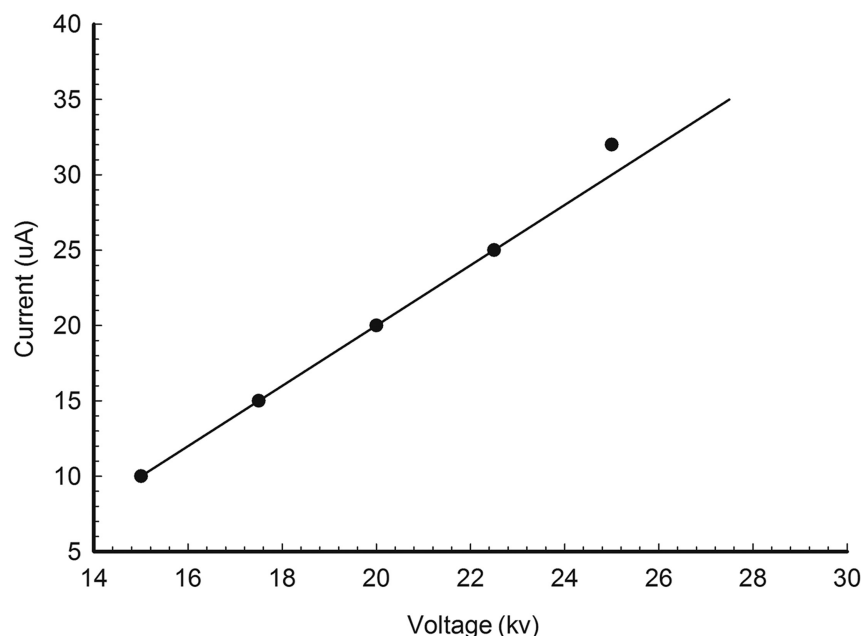
Figure 3 – Voltage–current profile. The running buffer contained 50 mmol/L CuSO$_4$. The pH was 4.40 and separation voltages were 15 kV, 17.5 kV, 20 kV, 22.5 kV, and 25 kV; other conditions were as described in the text. UV absorption at 254 nm.

3.4.   Effects of capillary diameter

Electrodispersion is the most pronounced band-broadening factor for relatively long migration time and low resolution, especially in large internal diameter capillaries. Using narrower capillaries, the passive influences of laminar flow can be reduced. However, while the best separation efficiency can be obtained on the narrower capillary, reduction in optical path length will lead to a significant loss in sensitivity. Considering these, we selected 50μm diameter and solved the problem of sensitivity by sweeping technology.

In the beginning, the experiment was carried out with a capillary diameter of 75 μm. However, using this capillary, we managed to separate only 11 amino acids; in other words, complete separation of 16 amino acids was not achieved. As shown in Figure S1 (see Supplementary Material online), in the 75μm capillary diameter, the peaks that overlapped together corresponded to alanine, serine, and threonine, cysteine, and valine; in addition, peaks of methionine, phenylalanine, isoleucine, and leucine were also seen. These peaks were not separated completely. Therefore, we selected 50 μm as the capillary diameter.

3.5.   Optimization of sample injection time

In this study, length of sample injection time can affect the separation and sensitivity of amino acids. The sensitivity was increased with the increasing of injection time, but when the sample injection time was more than 70 seconds, the sensitivity was no longer increased and sample peaks broadened; meanwhile, the resolution of analytes was affected. Therefore, we selected 70 seconds as the injection time. Before the preconcentration phase, proline was not detected, and detection sensitivity was improved by about 25–35-fold (Figure 4) after using the sweeping technique. Because the copper ions that exhibited UV absorption were added into the background electrolyte, detection limits were not very low. The problem of low sensitivity was properly resolved using this technique, and amino acids in beer samples that contain free amino acids with lower content were determined.
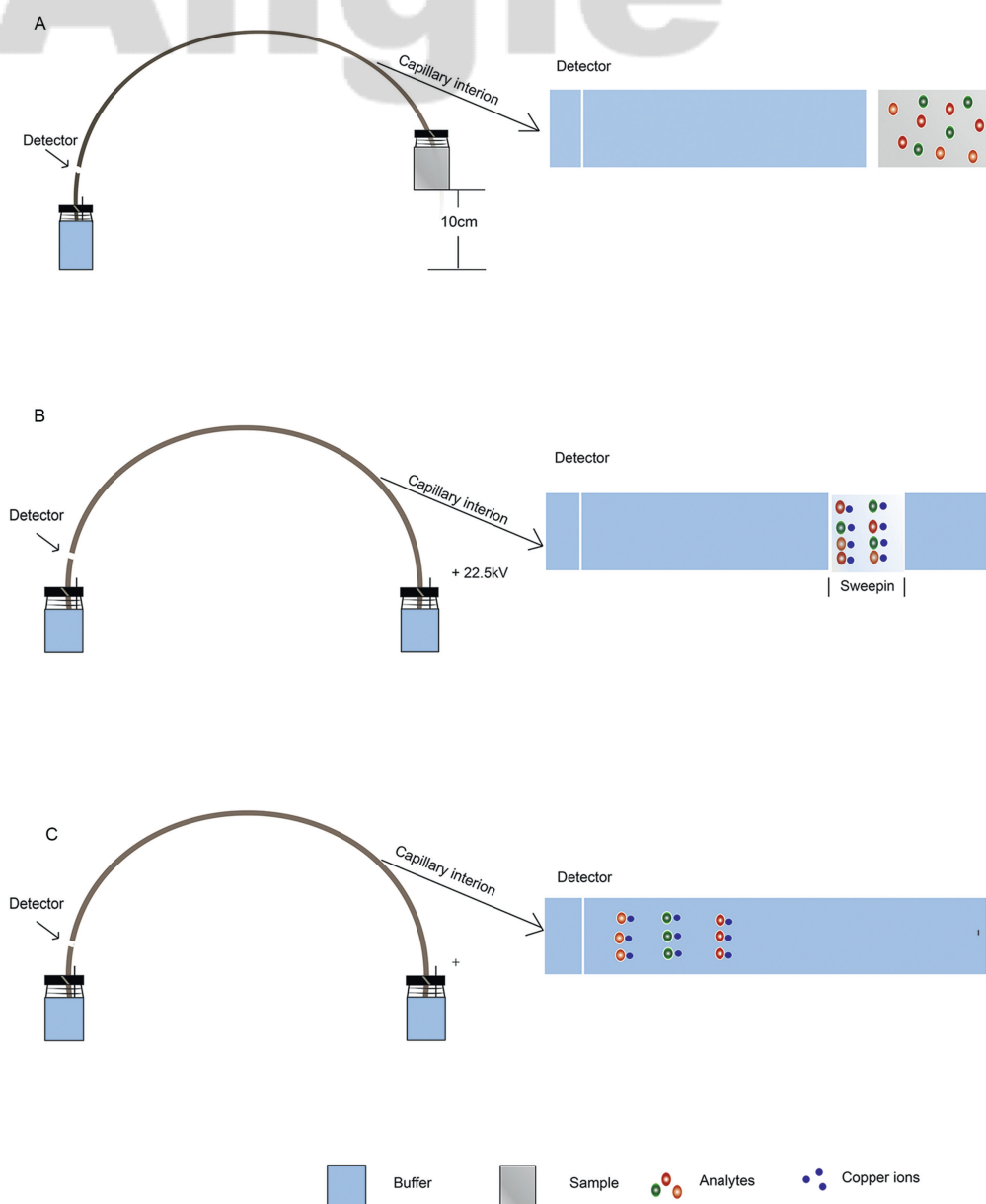
3.6.   Mechanism of online sample preconcentration sweeping technique and separation

First, when sampling for 70 seconds at the capillary inlet that was 10 cm higher than outlet end, a sample zone was formed, as shown in Figure 5A. The capillary was then inserted into the buffer and 22.5kV voltage was applied to finish the separation. Because the mobilities of copper ions were greater than amino acids, under the high voltage condition, it was easier for the copper ions to catch up with amino acids and concentrate them into narrow bands; meanwhile, $[Cu(AA)n]^{+2}$ complexes were formed by the coordination interaction between copper ions and amino acids, which resulted in enhanced sensitivity (Figure 5B). During the enrichment and separation process, we found that there was a small increase in the current after applying voltage. The main reason for this was that the conductivity of amino acid was different from that of the buffer solution. At the beginning of the separation, the capillary inlet was filled with analytes; however, as the
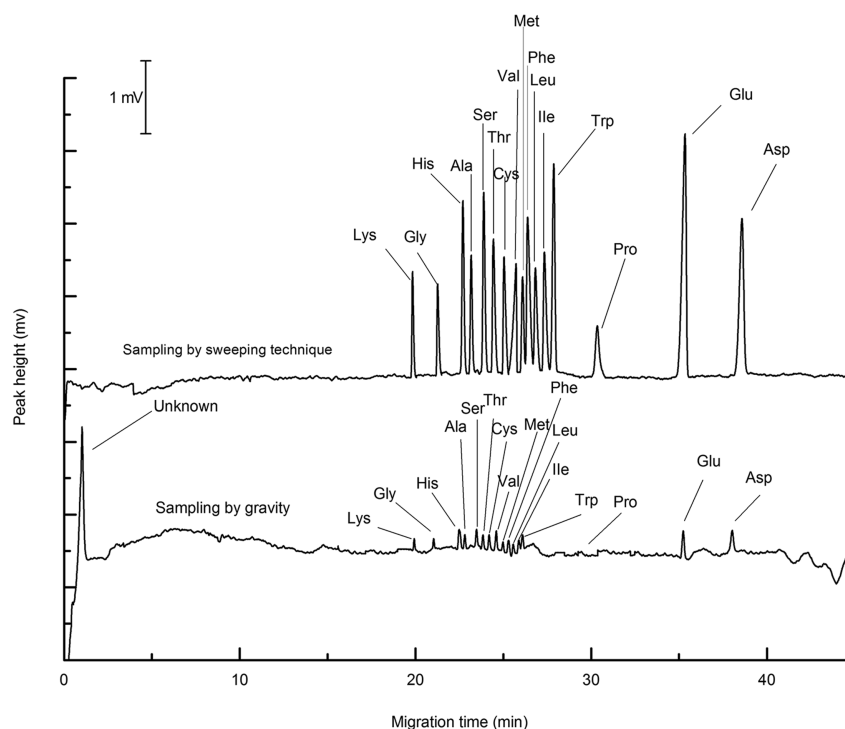
Figure 4 — Electrophoretograms of amino acids by sampling based on gravity and sampling based on the sweeping technique, respectively. (A) Sampling for 70 seconds; (B) sampling for 5 seconds. The running buffer contained 50 mmol/L CuSO₄, the pH was 4.40, and separation voltage was 22.5 kV; other conditions were as described in the text. UV absorption at 254 nm.

Figure 5 − Mechanism of online sample preconcentration sweeping technique and separation. **(A)** During sampling, a sample zone was formed in the capillary interior. **(B)** Copper ions and amino acids formed complexes and concentrated the sample of amino acids into narrow bands. **(C)** Complexes were separated.

conductivity of amino acids was lower than the buffer solution, at this moment, a low current was detected. With the separation of analytes, the current increased rapidly and then remained stable. This phenomenon helps to observe enrichment. During electrophoresis, we are able to observe whether the enrichment is carried out by the change of current. Finally, $[Cu(AA)n]^{+2}$ complexes were separated effectively according to the difference in mobilities, as shown in Figure 5C.

3.7.   Method validation

To evaluate the method completely, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy were validated.

3.7.1.   Linearity, LOD, and LOQ

Under the optimal CE conditions, the linearity was studied using diluted standard mixtures of amino acids at five different concentrations. The corresponding peak areas were linearly related to the concentration of amino acids. The LOD and LOQ values were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively. Under the optimal condition, each amino acid was analyzed in parallel five times on a single day for intraday precision and on 5 different days for interday precision. The precision of analysis was evaluated using relative standard deviation. The liner regression equation, correlation of determination, precision, LOD, and LOQ are shown in Table S1 (see Supplementary Material online).

3.7.2. Recovery

Recovery was evaluated by adding 80%, 100%, and 120% standard amino acid into the beer samples and calculated according to the following equation:

$$R(\%) = \frac{C_s - C_u}{C_a} \qquad (2)$$

where C_s is the concentration of spiked samples; C_u is the concentration of unspiked samples; and C_a is the concentration that was spiked. The recoveries of spiked samples are shown in Table S2 (see Supplementary Material online) and indicate a good recovery of this method.

3.8. Application to beer samples

Electrophorograms of the samples are shown in Figure 6. The amino acids were identified by the standard addition method. As can be seen, using this method, we have achieved the direct determination of underivatized amino acids in beer. Based on the essential amino acids pattern (EAA pattern) and proportion of essential amino acids in the total amino acids, we evaluated the quality of three kinds of beers. The EAA pattern is obtained by calculating the ratio of other essential amino acids to tryptophan whose content in protein is assumed to be 1; the closer the ratio of the essential amino acids to tryptophan in the sample, the better the quality of the sample. The sample whose proportion of essential amino acids in total amino acids is closer to 40% has good quality [27].

According to the EAA pattern and proportion of essential amino acids in the total amino acids, the three kinds of beers were compared. It can be seen from Table 1 that 13 kinds of amino acids were detected in Beer2, including seven kinds of essential amino acids (phenylalanine, leucine, isoleucine, lysine, methionine, tryptophan, and valine). There were 11

Table 1 – Amino acids determined in three beer samples under optimal conditions.			
Analytes	Beer1 (µg/mL)	Beer2 (µg/mL)	Beer3 (µg/mL)
Lys	10.98	15.56	18.71
Gly	22.95	28.29	21.83
His	14.71	17.88	11.57
Ala	36.90	14.10	15.18
Ser	49.20	38.27	40.48
Val	19.97	20.07	22.05
Met	N[a]	26.21	N[a]
Phe	109.03	54.89	39.44
Leu	N[a]	38.54	N[a]
IIe	54.06	40.37	52.48
Try	9.96	4.98	10.42
Pro	47.91	66.38	35.70
Glu	9.46	4.70	7.86
TAA	370.42	371.24	260.54
EAA	204	201.14	143.1
EAA/TAA (%)	55.07	53.93	54.92

EAA = essential amino acids; TAA = total amino acids.
[a] N indicates undetectable.

kinds of amino acids in both Beer1 and Beer3, which contained five kinds of essential amino acids (phenylalanine, isoleucine, lysine, tryptophan, and valine). In terms of the total contents of amino acids: Beer2 > Beer1 > Beer3. Concerning the total contents of essential amino acids, Beer1 was greater than Beer2, and Beer3 was the lowest. Comparing the proportion of essential amino acids in the total amino acids, we found that their proportions in Beer2, Beer3, and Beer1 were 53.93%, 54.92%, and 55.07%, respectively, and that they were greater than 40%. These results suggested that the quality of amino acids in the three kinds of beers was relatively low. When comparing
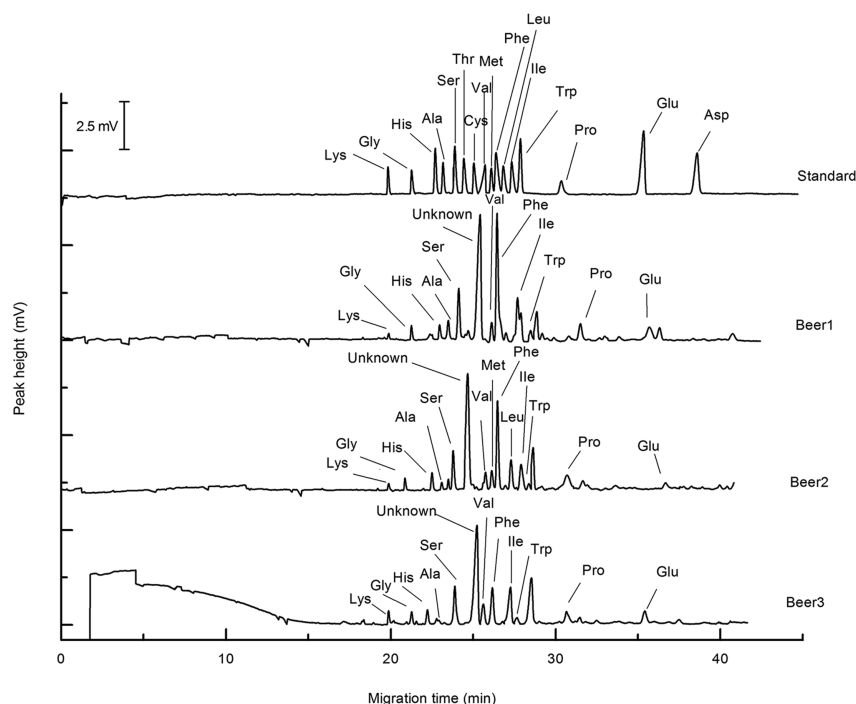
Figure 6 – Electrophorograms of the three beer samples under optimized conditions. The conditions were as in Figure 4.

Table 2 – Ratios of essential amino acids to tryptophan in three kinds of beers and values in the EAA pattern.

Analytes	Beer1	Beer2	Beer3	Values in WHO/FAO
Lys	1.10	3.12	1.20	5.5
Val	2.01	4.03	2.12	5
Met + Cys	N[a]	5.29	N[a]	3.5
Phe + Tyr	10.94	11.02	3.78	6
Leu	N[a]	7.73	N[a]	4
IIe	5.43	8.11	5.04	7

FAO = Food and Agricultural Organization; WHO = World Health Organization.
[a] N indicates undetectable.

between the three kinds of beers, the proportion of amino acid in Beer2 was relatively closer to 40%, followed by Beer3, finally Beer1. When comparing between the composition of essential amino acids in three beers with EAA pattern (Table 2), we found that in the ratio of Lys, Ala, and IIe, Beer2 sample was the most close to the EAA pattern; the other two kinds of beers did not show much difference; regarding the ratio of Met + Cys and Leu, Met + Cys in Beer2 was very close to the EAA pattern but Leu was about two times as much as the EAA pattern, and there were no Met + Cys and Leu in Beer3 and Beer1; in the ratio of Phe + Tyr, Beer3 was very much close to the EAA pattern, whereas the other two kinds of beers were far greater than the EAA pattern. Through a series of comparison, Beer2 was identified as the one most close to the EAA pattern, followed by Beer3 and Beer1, respectively. Therefore, among these three beers, the quality of amino acids in Beer2 was better than that in Beer3 and Beer1, with Beer1 having the lowest.

Beer is enjoyed by an increasing number of people worldwide, with many having a great relationship with its unique mellow flavor. In the three beer samples analyzed, the composition of phenylalanine, proline, serine, and isoleucine was relatively high. In general, amino acids influence the taste and flavor of beer; for example, phenylalanine and serine impart sweetness; proline imparts sweetness and the slightly bitter taste; and isoleucine imparts bitterness [28,29]. It is these amino acids that impart different flavors to make the beverage form a unique mellow taste.

In summary, for evaluating the quality and flavor of beer, determination of free amino acids is vital. In this study, based on the coordination interaction between amino acids and copper ions to achieve direct determination of underivatized amino acid in beer, and according to the EAA pattern and flavor of amino acids, we have found that the ratio of essential amino acids in Beer2 was very close to the EAA pattern; moreover, the contents of phenylalanine, proline, serine, and isoleucine were relatively high in all three kinds of beers, which had a great influence on beer flavor. Compared with other methods used to determine amino acids in beer, the proposed method can determine amino acids in beer directly and accurately; additionally, it is simpler, quick, convenient, much cheaper, and easier to operate.

Conflicts of interest

All authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2017.03.003.

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