Establishing a sensitive capillary electrophoresis-UV method for direct determination of amino acids to evaluate vinegar quality

Tian Luo\textsuperscript{1}, Yue Dong\textsuperscript{1}, Shujuan He\textsuperscript{1}, Jing Yang\textsuperscript{1}, Yuming Dong\textsuperscript{1,2*}

\textsuperscript{1}Institute of Pharmaceutical Analysis, School of Pharmacy, Lanzhou University, Lanzhou, Gansu Province 730000, P. R. China

\textsuperscript{2}Lanzhou Universty-Techcomp (China) Ltd Joint Laboratory of Pharmaceutical Analysis, Lanzhou, Gansu Province 730000, P. R. China

*Corresponding author:
Professor Yu-ming Dong, School of Pharmacy, Lanzhou University, Lanzhou 730000, P.R. China

Tell: 86-931-8915685
Fax: +86-931-8915686
E-mail address: dongym@lzu.edu.cn

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Abstract

In this study, the capillary electrophoresis method with ultraviolet detection was established to directly determine amino acids in vinegar, according to the coordination interaction between amino acids and copper ions. The online sweeping technique was combined to improve the detection sensitivity. The quality of vinegar was evaluated with amino acids as parameters by United Nations Food Agriculture Organization/World Health Organization amino acids model and principal component analysis. Optimum conditions were obtained under 50 mM CuSO₄ and adjusted pH 4.40 with 8mM acetate, 70s injection time, 22.5 kV separation voltage, 254nm detected wavelength. Method validation, indicating good linearity ($r^2>0.9989$), precision with a relative standard deviation less than 8.0% (n=5), limits of detection (0.13-0.25µg/mL), limit of quantification (0.43-0.83µg/mL) and recovery (80.5-112.6%). Under the optimal conditions, amino acids in vinegar can be directly separated which is propitious for the quality evaluation of vinegar.

Keywords

Amino acids; Capillary electrophoresis; Copper; Sweeping technique; Vinegar; Principal component analysis
1. Introduction

Amino acids (AAs), as the building blocks of life [1], exist in a variety of food [2]. They play an important role in the quality control of food. Therefore, the analysis of AAs in food is very important. Commonly used methods of AAs determination include high performance liquid chromatography (HPLC) [3-5], Capillary electrophoresis (CE) [6-10] and Amino Acid Analyzer [11, 12]. CE is widely used in the analysis of AAs in food [13] due to its low consumption of samples and reagents, high efficiency and good resolution [14, 15]. The most of AAs, except tryptophan, phenylalanine and tyrosine, lack a strong chromophore, such that they have neither ultraviolet (UV) absorption nor fluorescence [16, 17]. Thus AAs are always determined by derivatization to increase detection sensitivity, however, it also has some specific drawbacks with regard to complex derivatization procedure, expensive reagents, low stability and so on [18]. Although CE-electrochemical detection (CE-ECD) [19], CE-capacitively coupled contactless conductivity detection (CE-C4D) [20] and CE with indirect UV method can directly determine AAs [21], the electrode of electrochemical detector is sensitive to the change of temperature and flow rate, the selectivity of CE-C4D is poorer than that of CE-UV and the stability of method with indirect UV detection is poor. Therefore, using an underivative method with available technology is desirable.

Although most of AAs have no UV absorbance, coordination complexes of [Cu(AA)ₙ]²⁺ between AAs and Cu²⁺ can provide strong UV absorption [22]. When the lone pair electrons on
the nitrogen atom of amino group and oxygen atom of carboxyl group transfer to the empty orbital of Cu$^{2+}$ [23], the coordination complexes of [Cu(AA)$_n$]$^{2+}$ are formed. In this way, the AAs are directly detected by UV without derivatization. Compared with derivative method, this method not only saves time and reagents required for derivatization, but also greatly simplifies the tedious operation.

Effective coordination interaction between AAs and Cu$^{2+}$ can be achieved, but the Cu$^{2+}$ in the background electrolyte causes the decline in sensitivity because of UV absorption of Cu$^{2+}$. To improve the detection sensitivity, the sweeping technique was used in this study. Sweeping is one of effective online sample preconcentration techniques. It is the picking and accumulation of analytes by the analyte carriers (such as surfactants, microemulsions, polymers, dendrimers, complexing agent and transition metals) [24, 25]. In this study, Cu$^{2+}$ was also used as analyte carriers according to the interaction with AAs to achieve preconcentration of AAs. Thus, the sensitivity of AAs was greatly enhanced.

Fermented vinegar (FV) as a mellow condiment is greatly favored by people, but its cost is high caused by long production cycle. In order to get illegal benefit, unscrupulous merchant might use chemical vinegar (CV), which is generated by diluting acetic acid with water and adding dark reddish brown and other substances to water, to fake FV to reap fabulous profits [26]. CV has no nutritional value and is harmful to health [27]. As a dietary product, quality of vinegar is closely related to the life. Thus, identification of FV is particularly important.
China’s standards of vinegar, GB 18187—2000 “Fermented vinegar” and GB 2719—2003 “Hygienic standard for vinegar”, only used quality indexes to evaluate the quality of vinegar. However quality indexes cannot completely identify FV because CV can fake to FV by adding dark reddish brown, acetic acid and other substances to control the quality indexes and appearance in accord with FV. Although $^{14}$C decay method that was adopted in GB/T 22099—2008 “Identification method of fermented vinegar and synthetic vinegar” could distinguish FV and CV, equipment used in this method was expensive and operation was cumbersome [28]. In SB/T 10303—1999 “Mature vinegar quality standard” and SB/T 18623—2002 “Zhenjiang vinegar” standard, they indicate that the AAs were one of the important characteristics of FV [29], however, there was almost no AAs nitrogen in CV [30]. Hence FV can be identified by determination of AAs in FV. In addition to the identification of FV, AAs also have a very important relationship with the flavor of vinegar. In FV, the varieties of AAs are rich and they can produce different taste, such as sapor, sweet, bitter and aromatic [11], which make the vinegar has soft and mellow palate with layers of flavors.

So, establishing a rapid method for determining AAs in vinegar is helpful to identify FV and investigate taste of vinegar. Although the AAs in human saliva, plasma, green tea, beer, radix asparagi and nutritional supplements have been directly determined by CE-UV coupled with coordination interaction between AAs and Cu$^{2+}$ [31-34], there were no studies about using this method to analyze AAs in vinegar. In this study, based on the coordination interaction
between AAs and Cu\(^{2+}\), we achieved direct determination of AAs in vinegar by CE-UV for the first time.

Using AAs as analysis parameters, the quality of vinegar was evaluated according to principal component analysis (PCA) [35] and the recommendation values stipulated by United Nations Food Agriculture Organization/World Health Organization (FAO/WHO). The ratios of essential AAs (EAA) to total AAs (TAA) and EAA to non essential AAs (NEAA) were closer to 40% and 60%, respectively [36], the AAs will have the best nutritional value for human being in the stipulates of FAO/WHO. In addition, the contents of free flavor AAs were used to investigate the contribution for the vinegar taste.

2. Materials and methods

2.1 Chemicals and Reagents

Copper (II) sulfate, sodium acetate and lactic acid were analytical-grade and obtained from Shuangshuang Chemical Reagent Co., Ltd (Yantai, Shandong, China). Acetic acid (analytical-grade) was purchased from Shandong Yuwang Pharmaceutical Co., Ltd (Dezhou, Shandong, China). Ammonium sulfate was analytical-grade and purchased from the Tianjin k-stone Chemical Reagent Co., Ltd (Tianjin China). Glucose was obtained from Chemical Reagent Factory of Hunan Normal University (Changsha, Hunan, China). Distilled water was from the GLP lab of Lanzhou University (Lanzhou, Gansu, China) and was used to prepare all
solutions. Reference standards of AAs: L-cysteine (Cys), L-lysine (Lys), L-histidine (His),
L-glutamic acid (Glu), L-leucine (Leu), L-methionine (Met), L-aspartate (Asp), L-tryptophan
(Trp), L-proline (Pro), L-isoleucine (Ile), L-serine (Ser), L-phenylalanine (Phe), L-glycine
(Gly), L-valine (Val), L-alanine (Ala) and L-threonine (Thr) were purchased from Yuanye
Biological Technology Co., Ltd (Shanghai, China. High-purity≥99.5%). Different brands of
FV were purchased from a local supermarket (Lanzhou, Gansu, China).

2.2 Instrumentation

The experiment was run on K1060 CE (Kaiao, Beijing, China) instrumentation with a UV
detector and EasyChrom1000 workstation was used to acquire and analyze the experimental
data. The un-coated fused silica capillary (Yongnian, Hebei. China) (50 µm i.d. ×375 µm o.d.,
the total length was 73 cm and the effective length was 65 cm) was used in this study. FE20 pH
meter (Mettler Toledo Instrument Co., Ltd, Shanghai, China) was used to measure the pH
value of buffer solution.

2.3 AA stock solution and sample preparation

AA stock solution preparation: each of 16 AAs reference standards was accurately
weighted and put it into volumetric flask, respectively, and they were then dissolved in water
and diluted with water about 1.0 mg/mL. They were stored until use under freeze at -20℃.
Subsequently the AAs stock solution was thawed at room temperature and diluted with water to different desired concentration, before the analysis.

Sample preparation: each of aliquot volume of different brands vinegar solution was put into volumetric flask, respectively, and diluted with water by 3-6 folds. Sample solution needed to be degassed and filtered with 0.22 µm membranes before use.

CV Samples were prepared as follow [21]: 0.6g of ammonium sulfate and 0.2g of glucose were accurately weighed and inserted into 20mL volumetric flask, and they were dissolved in water. Then 0.8ml of acetic acid and 0.3ml of lactic acid were added into this volumetric flask. After mixing, water was added to dilute above solution to graduated lines of volumetric flask. Before analysis, CV sample was degassed and filtered with 0.22 µm membranes.

2.4 CE conditions

Fifty mM copper (II) sulfate solution (adjusting pH value to 4.40 with 8mM acetate) was used as background electrolyte solution. Between runs the capillary was rinsed with background electrolyte solution. After every three runs, the background electrolyte solution in the vials was updated with new one and the capillary was rinsed in the following sequence: water for 2 min, 1.0 M NaOH for 3 min, water for 2 min and background electrolyte solution for 3 min. When the experiment was finished in every day, the capillary was flushed with water for 10 min, then the air was injected in capillary by empty syringe. The separation voltage was
22.5 kV and the detection wavelength was set at 254 nm. The injection was carried out by gravity at the injection end from a 12cm difference in heights for 70 s.

3. Results and discussion

3.1 Optimization of separation conditions

In this study, the solution of copper (II) sulfate that formed complexes of \([\text{Cu}(\text{AA})_n]^{2+}\) by coordination interaction with AAs was chosen as buffer solution, and acetic acid and sodium acetate were used to adjust pH. The resolution of AAs was improved along with the increase of the concentration of copper (II) sulfate solution. When the concentration of copper (II) sulfate solution was higher than 50 mM, Val, Met, Phe and Leu couldn’t be baseline separated (Fig S1(a)), and high concentration of copper sulfate solution might lead to excessive Joule heat. Therefore, 50 mM was selected as the optimal concentration of copper (II) sulfate solution.

The pH value of buffer solution is a key parameter in CE. It can control the intensity of electroosmotic flow (EOF) and has a great impact on \(\text{Cu}^{2+}\) hydrolysis. Low pH can restrain the \(\text{Cu}^{2+}\) hydrolysis, but lower pH is not conducive to separation because it can extend analysis time and cause other opposite effects. If the pH is higher in alkaline, the chemical precipitation reaction of copper (II) sulfate will occur. Taking these factors into account, 4.40 was selected as the optimized pH value (Fig S1 (b)).
After comprehensive consideration of separation efficiency and Joule heat, the 22.5 kV was used as separation voltage.

3.2 Effect of capillary inner diameter on the separation

Although large optical path length can be obtained on large internal diameter capillary, the radial dispersion and overlap of analytes in large internal diameter capillary will lead to low resolution. Under the optimized conditions, 16 AAs couldn’t be completely separated in the capillary of 75 µm inner diameter (Fig S2), some peaks were overlapped such as Ala, Ser and Thr; Cys and Val; Met and Phe; Ile and Leu. So, we used the capillary of 50 µm inner diameter to enhance resolution, and combined sweeping technology to solve the problem of low sensitivity that was caused by reduction in optical path length.

3.3 Optimization of sample injection time

In this study, Cu^{2+} serves an analyte carrier to form complexes of [Cu(AA)_n]^{2+} with AAs. Effective mobility of AAs in sample zone is very small due to they are neutral or partially charged, while the Cu^{2+} has strong effective mobility. Cu^{2+} can quickly migrate into the sample zone to form complexes of [Cu(AA)_n]^{2+} that have greater effective mobility than AAs. Therefore, AAs in sample can be focused into narrow bands within the capillary to achieve preconcentration. The absorbance of [Cu(AA)_n]^{2+} increased with the increase of injection time. The highest absorbance of [Cu(AA)_n]^{2+} was obtained when the injection time was 70s.
Detection sensitivity was improved about 11-20 fold by online sweeping. From Fig 1, we found that Pro was not detected and the peak areas of the rest of the AAs were very small before the preconcentration. After sweeping, Pro was easily detected and detection sensitivity was significantly improved.

3.4 Method validation

To validate the proposed method of CE-UV, the linearity between the concentration of the AAs and the corresponding peak areas, limit of detection (LOD), limit of quantification (LOQ), precision and recovery were investigated at the optimum conditions.

3.4.1 Linearity, precision, LOD and LOQ

The linearity was performed with the diluted standard mixtures of AAs at five different concentrations under the optimized conditions. As shown in table S1, the results indicated good linearity for each analyte. LOQ and LOD were estimated as the concentrations at signal-to-noise ratios of 10 and 3, respectively. Precision was evaluated by the relative standard deviation (RSD). The each AA was analyzed with parallel 5 times in a single day for intra-day precision and different five days for inter-day precision. The relative standard deviation of the peak area and migration time of each AA in intra-day was less than 5.0 % and less than 4.3%, respectively, in inter-day less than 7.1% and less than 8.0%, respectively. The temperature of capillary has a great effect on the precision of CE, and the K1060 CE instrumentation used in
this study did not have a temperature control device, which resulted in increased RSD values. In addition, manual injection was used in this study; there was no automatic sampling device. Accidental errors in manual operation also made the RSD values larger. Thus, RSDs of precision was high in this work.

3.4.2 Recovery

Using the standard addition method, three different concentration standard AAs were added into the vinegar samples to evaluate recovery. The results indicated a good recovery of this method (table S2).

3.5 Application in vinegar samples

The CE-UV method that achieved determination of AAs by coordination interaction between AAs and Cu$^{2+}$, provided a sensitive and direct method for the determination of AAs in vinegar. Thus, the proposed CE-UV method was propitious to assess the quality of vinegar.

3.5.1 Identification of CV and FV

FV is rich in many types of AAs, but CV nearly does not contain AAs, which can be used to distinguish FV and CV. Fig.2a was the electrophorogram of FV (Peaks were identified by
adding standard amino acids), Fig. 2b was the electrophorogram of CV and Fig. 2c was the electrophorogram of acetic acid solution. We found that FV contained rich AAs, but there was only the peak of acetic acid and no AAs in the CV and acetic acid solution. Thus, CV was distinguished from FV.

3.5.2 The total amount and composition of AAs

As shown in Table S2, the 14 samples were rich in AAs, and the contents of AAs were difference in different brands vinegar. There were 15 AAs in S11, in which included 7 essential AAs (Lys, Thr, Val, Ile, Leu, Phe and Trp). S1 and S2 contained 12 AAs, 6 of which were essential AAs (Lys, Thr, Val, Ile, Leu, and Phe). The 13 AAs were determined in S3, S5, S6, S12 and S13, including 6 essential AAs except S13 that included 7 essential AAs. The remaining samples had 14 kinds of AAs, which contained 7 essential AAs. In these samples, the total AAs content in S11 was the highest, followed by S12, S3 was the lowest. About the total content of EAAs, S11 was also the highest, S3 was the lowest.

From a point of view of food nutrition, nutritional value of AAs is largely related with the proportion of essential AAs. The values of EAA/TAA and EAA/NEAA in vinegar were closer to the 40% and 60% (which were stipulated by FAO/WHO), respectively, the more nutritional value of vinegar for human being. The values of EAA/TAA in these vinegars were close to
40%. About the EAA/NEAA value, the others were relatively close to 60% except S4 (80.9%). It showed that these vinegar samples (except sample S4) had good nutritional value.

As first essential AA in human body, Lys plays an important role in the metabolism of the human body. Contents of Lys in S1, S2, S4, S5, S9, S10, S11, and S12 were higher than others. Combining the results obtained by analysis of the EAA/TAA and EAA/NEAA, contents of Lys and amount of AAs in these vinegars, we found that the quality of S1, S2, S5, S9, S10, S11 and S12 were relativity high.

3.5.3 Flavor AAs

AAs also have a great relationship with the taste of vinegar. From the Fig 3, we found that the ratio of sweet AAs in total AAs was the largest, followed by bitter AAs and sapor AAs, finally to aromatic AAs. The results indicated that sweet AAs had greatest contribution to the taste of vinegar, and aromatic AAs were lowest. Despite contents were low, unique flavor of aromatic AAs gave special taste on vinegar. In terms of single AA, Ala, Leu, Glu and Phe had largest contribution in the sweet AAs, bitter AAs, palatable AAs and aromatic AAs, respectively. It was this different taste of AAs that gave pleasant aroma and mellow flavor for vinegar on the basis of acid.

3.5.4 Principal component analysis
PCA can not only grasp main contradiction, but also simplifies work. Thus, in this study, PCA was used to evaluate the quality of the 14 samples.

In the PCA, If the great majority of the absolute values of the correlation coefficients between analysis indexes were greater than 0.3, these indexes were suitable for the PCA [37]. Before the performing, the correlation coefficient matrixes between 15 variables (the contents of 15 AAs) were calculated. As shown in Table S3, 84.5% absolute values of the correlation coefficients were greater than 0.3, which indicated these variables were suitable for the PCA.

3.5.4.1 Extraction and analysis of principal component

Appropriate principal components were selected according to the characteristic value and contribution rate of principal component [38]. The characteristic values of first three principal components were greater than 1 and the cumulative percentage reached 89% by the principal component analysis for 14 vinegars (Table 1), which showed that three principal components contained almost total information of all the parameters. Thus three principal components were selected in PCA.

The loads of principal component were offered to show the correlation coefficient between each variable and principal component (Table S4). The results showed that Thr, Val, Ile, Leu, Phe, Lys, Glu, Gly, Ser, Ala and Pro had larger loads on the first principal component, these indicated they had highly positive correlation with the first principal component. Because 68%
of the total contribution rates came from the first principal component, these AAs were considered as characteristic AAs of vinegar. They included abundant flavor AAs and seven kinds of human essential AAs (Thr, Val, Ile, Leu, Phe, Lys and Ser), therefore, characteristic AAs could be used to evaluate quality of vinegar.

3.5.4.4 Comprehensive evaluation

By the principal component analysis, the three principal components (F1, F2 and F3) were extracted, Thr, Val, Ile, Leu, Phe, Lys, Glu, Gly, Ser, Ala and Pro were regarded as characteristic AAs in vinegar. These characteristic AAs offered important reference for the quality evaluation of vinegar. In this study, comprehensive factor scores were got by linearity weighting method that took contribution rates of each principal component as weight. Comprehensive factor scores was used to evaluate quality of vinegar, according to the equation

\[ F = 0.6863F1 + 0.1216F2 + 0.0870F3 \ldots (1) \]

As shown in Table 2, S11 had the highest comprehensive scores, followed by S12, the S3 was the lowest. The results were consistent with the previous results of AAs analysis.

4. Conclusion

In this work, free AAs in vinegar were directly and sensitively determined by CE-UV through coordination interaction between AAs and Cu\(^{2+}\) coupled with sweeping technique to
identify FV for the first time. According to EAA/TAA and EAA/NEAA values and the the results of PCA, the quality of vinegar was evaluated. By comparison, ratios of AAs in sample S11 were the closest to stipulates and comprehensive scores were the highest. The flavor of free AAs was used to investigate the contribution for the vinegar taste. As a result, sweet AAs had greatest contribution to the taste of vinegar.

Acknowledgements

The authors have declared no conflict of interest.

5. References


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**Figure Captions**

**Figure 1.** (a) Electropherograms of amino acids by sampling based on gravity and sampling on the basis of sweeping technique. (b) histograms of amino acids by sampling based on gravity and sampling on the basis of sweeping technique. The background electrolyte contained 50 mM CuSO₄, the pH was 4.40, and separation voltage was 22.5 kV, other conditions were described in the text. UV absorption at 254 nm.
Figure 2. Electropherograms of fermented vinegar, chemical vinegar and acetic acid solution under optimized conditions. The conditions were as in Fig1.
**Figure 3.** (a) Contribution rate of individual amino acids. (b) Rates of sweet, bitter, aromatic, savor amino acids to total amino acids, respectively.
Table 1. Eigenvalue and contribution rate of principal factors

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<th>Number of principal factors</th>
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<th>Cumulative Contribution rate(%)</th>
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<td>68.625</td>
<td>68.243</td>
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<tr>
<td>2</td>
<td>1.824</td>
<td>12.158</td>
<td>80.783</td>
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<tr>
<td>3</td>
<td>1.304</td>
<td>8.694</td>
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Table 2. Component score and Comprehensive score of the tested sample

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<tr>
<th>Sample</th>
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<th>F2</th>
<th>F3</th>
<th>F</th>
<th>Ranking</th>
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<td>S12</td>
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