Determination of 18 Kinds of Amino Acids in Fresh Tea Leaves by HPLC Coupled with Pre-column Derivatization

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Abstract A rapid and accurate quantitative method of high performance liquid chromatography (HPLC) with fluorescence detector has been developed for the analysis of 18 kinds of amino acids in fresh tea leaves. The samples were minced and mixed, and extracted with ultra pure water at 90℃ for 20 min. The 6-aminoquinolyl N-hydroxy-succinimidyl carbamate (AQC) was used as pre-column derivatization reagent. Gradient HPLC separation was performed on a C_{18} column (Symmetry C_{18}, 3.9 mm × 15 cm, 4 μm). Good linearity between concentrations and peak areas was achieved in the concentration range of 5.0 - 250 μmol/L for 18 kinds of amino acids. The method was validated by the analysis of five replicates. The 18 kinds of amino acid standards were spiked in fresh tea leaf samples and the average recovery rate was 86.25% - 109.05% with relative standard deviations ($n = 5$) ranging from 6.03% to 10.56%. The limit of detection (LOD) for the analytes was 0.05 - 1.27 μmol/L. The method was successfully applied to the analysis of the 18 kinds of amino acids in fresh tea leaves from east Dongting and west Dongting mountains in Suzhou. The results indicate that the method is simple, rapid, precise and reliable.

Key words Fresh tea leaves, Free amino acids, Pre-column derivatization, High performance liquid chromatography (HPLC)

1 Introduction

As one of the world's three major natural drinks, tea has unique aroma, rich nutrition and health effects[1-2], and thus is favored by consumers. Tea is rich in amino acids, which are important components of tea and directly influence the quality of tea. Amino acids in tea are mainly present in two forms, one in free form and the other is bound form present in peptides and proteins[3]. Free amino acids, such as theanine, glutamic acid, arginine and serine, are important nitrogenous substances in tea. They are not only the basic units synthesizing tea leaf protein, but also substances synthesizing physiologically active substances related to metabolism. Their composition, content, and degradation products and conversion products during the processing and the changes in these components have a direct effect on the tea aroma and taste[4], and play an important role in the tea quality. Therefore, analyzing and studying the free amino acids in tea is of great significance to the development and utilization, process control, quality identification and nutritional value evaluation of tea.

At present, the studies on analytical methods of free amino acids in tea are concentrated on dry samples. For example, in the national standard GB/T8303-2013[5], tea samples need to be first ground and then put in the electric oven to heat and remove moisture to constant weight, so as to determine the content of amino acids. The dry samples are usually obtained through high temperature heat treating, drying, and crushing. However, in these processes, high temperature will change the nature of protein, and the biological activity of protease will lose. It will lead to degradation of protein and increase of free amino acids. Besides, heat treating and drying steps may lead to conversion of various biochemical components of tea[3]. As a result, the measured data will not reflect the actual level of amino acids in tea and accordingly generate the detection error.

There are many methods of determination of amino acids in tea, mainly including ninhydrin colorimetry[6-7], gas chromatography (GC) and mass spectrometry (MS)[8], capillary electrophoresis[9], and high performance liquid chromatography (HPLC)[10-11]. Classical amino acid analysis methods generally apply an amino acid analyzer, and use ninhydrin as a derivatization reagent for post-column derivatization detection. However, amino acid analyzers are expensive, they take a long time to analyze and the specificity is high, they can only be used for the analysis of amino acids, which limits their wide application. Compared with other methods, pre-column derivatization-high performance liquid chromatography (HPLC) does not require any special reaction device and has the advantages of high instrument popularization rate, rapid analysis, flexible and diverse characteristics, high sensitivity and easy for popularization, and thus has gradually become a routine method of amino acid detection. Chinese invention patent " application of reversed-phase high performance liquid chromatography in the detection of free amino acids in tea" (Patent No.: ZL201510109474.4)[12] took AQC as the pre-column derivatization reagent, and used the amino acid-specific analytical column to make gradient elution. Coupled with the reverse-phase high performance liquid chromatography, it realized the quantitative analysis of 19 kinds of amino acids such as glutamine in tea. However, the present invention uses chromatographic column special for amino acid separation, it needs complex gradient separation, the cost is high and the operation cycle is long (about one

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hour). In the process of large sample analysis, it takes a long time, not favorable for rapid analysis and popularization of amino acid. Besides, this invention did not make necessary methodological tests on the accuracy, sensitivity, and precision of the method, and the technical effects can not be effectively guaranteed.

In this study, in view of the low speed, low efficiency, high cost, and difficult popularization of existing analysis methods of amino acids in tea, we established a rapid, practical, high efficient, and accurate free amino acid separation and analysis method. Using fresh tea leaf samples to replace the dry tea samples, selecting ordinary Symmetry C_{18} column to replace the chromatographic column special for amino acid separation, adopting AQC as the pre-column derivatization reagent, and using high performance liquid chromatography-fluorescence detector for separation and analysis, we realized the simultaneous determination of 18 kinds of free amino acids in fresh tea leaves, to provide technical support for quality evaluation, grading, and fingerprint map construction.

2 Materials and methods

2.1 Materials and reagents

2.1.1 Instruments and equipment. In this experiment, we used 2695 high performance liquid chromatography equipped with 2475 fluorescence detector (American Waters Corporation), TG16-WS tabletop high speed centrifuge (Hunan Xiangyi Centrifuge Instrument Co., Ltd.), K600 crusher (German Braun), LE-3000 electroheating thermostatic water bath (Shanghai Yuejin Medical Instruments Factory), and Direct-Q 5 UV Ultra-pure water purification system (American Millipore Company).

2.1.2 Chemicals and reagents. Amino acid standards: aspartic acid, serine, glutamic acid, histidine, glycine, arginine, threonine, alanine, proline, theanine, cystine, tyrosine, valine, methionine, lysine, isoleucine, leucine, and phenylalanine (Sigma Company) and 17 kinds of amino acid mixed standards (the concentration of cystine was 1.25 mmol/L, and the others were 2.5 mmol/L); the derivatization reagent was AQC (American Waters Corporation); the acetonitrile was HPLC grade, and water was ultra pure water (resistivity: 18.4 MΩ at 25℃).

2.1.3 Samples. Fresh tea leaves were collected from Biluochun tea gardens of east Dongting and west Dongting mountains in Suzhou City.

2.2 Experimental methods

2.2.1 Preparation of solution. Amino acid standard solution: precisely weighed proper amount of amino acid standard, placed in 25 mL volumetric flask, added the ultrapure water, diluted and fixed the volume to the desired scale, obtained the single standard solution of amino acid, the concentration of theanine was 12.5 μmol/L and stored in a refrigerator at −20℃.

Took 40 μL of 17 amino acid mixed standard solution, added 40 μL of the theanine standard solution with concentration of 12.5 μmol/L, and diluted to 1 mL with ultrapure water to obtain the amino acid mixed standard solution containing 500 μmol/L theanine, 50 μmol/L cystine, and 100 μmol/L other amino acids.

Phosphate buffer: weighed 19.0 g sodium acetate trihydrate and 1.72 g triethylamine, dissolved in 1 000 mL water, adjusted the pH to 5.05 with phosphoric acid, added the appropriate amount of EDTA, and filtered with 0.45 μm filter membrane.

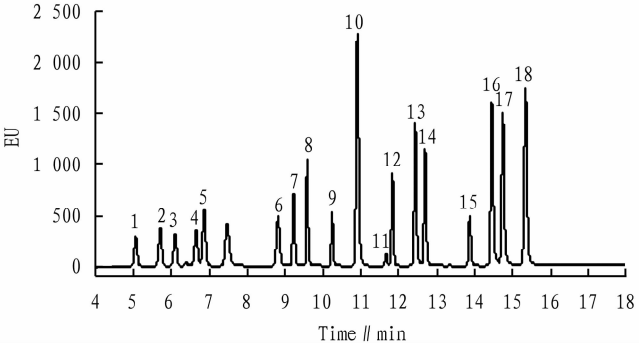
AQC derivatization reagent: added 1.25 mL of acetonitrile to AQC powder vial, mixed, and heated at 55℃ to dissolve.

Borate buffer: weighed 12.36 g of boric acid, added 400 mL of water, adjusted pH to 8.8 with 400 g/L sodium hydroxide solution and diluted to 500 mL with water to obtain 0.4 mol/L borate buffer solution.

2.2.2 Preparation of samples. Took fresh tea leaf samples, chopped, mixed well, weighed 0.25 g, added 10 mL of boiling water, soaked in a water bath at 90℃ for 20 min, cooled to room temperature, centrifuged at 3 000 r/min for 5 min, added water to supernatant to fix the volume to 10 mL, filtered with 0.45 μm microporous membrane for use.

2.2.3 Derivatization reaction. Precisely weighed 10 μL standard solution or fresh tea leaf sample solution, placed in automatic sample bottle, added 70 μL borate buffer, and mixed. Took 20 μL of AQC derivatization solution, added to the sample bottle in vortex state, mixed 10 − 20 s, placed 1 min, then heated at 55℃ for 10 min, took out and cooled to the room temperature for analysis.

2.2.4 Chromatographic conditions. Chromatographic column: Symmetry C_{18} column (3.9 mm × 15 cm, 4 μm). Column temperature: 37℃; flow rate: 2.0 mL/min; fluorescence detector: excitation wavelength: 250 nm, emission wavelength: 395 nm; injection volume: 10 μL. Mobile phase: A was phosphate buffer, diluted with ultrapure water at 1∶10; B was 100% acetonitrile; C was 100% ultrapure water. Gradient elution procedure: 0 min, 100% A; 0.5 min, 98% A + 2.0 B%; 0.5 − 9.0 min, 96.5% A + 3.5% B; 9.0 − 9.5 min, 95.0% A + 5.0% B; 9.5 − 11.5 min, 91.5% A + 8.5% B; 11.5 − 13.0 min, 83.0% A + 17.0% B (retention for 4 min); 17.0 min, 60.0% B + 40% C (retention for 2 min); 19 − 23 min, 100% A.



Note: 1. aspartic acid, 2. serine, 3. glutamic acid, 4. histidine, 5. glycine, 6. arginine, 7. threonine, 8. alanine, 9. proline, 10. theanine, 11. cystine, 12. tyrosine, 13. valine, 14. methionine, 15. lysine, 16. isoleucine, 17. leucine, and 18. phenylalanine

Fig. 1 HPLC chromatogram of 18 amino acid mixed standard solution

2.2.5 Quantitative determination. According to the retention time of amino acid standard in the sample, we made qualitative

determination of amino acids in the sample, and calculated the content of each amino acid using the external standard curve.

3 Methodology survey

3.1 Chromatographic separation of amino acid standard solution Fig. 1 is a HPLC chromatogram of 18 amino acid mixed standard solution. According to Fig. 1, the separation between the amino acids was good, the peak was compact and symmetrical, the analysis period was 23 min, and it guaranteed the separation effect and realized the purpose of rapid analysis.

The peak sequence of 18 amino acids was: aspartic acid, serine, glutamic acid, histidine, glycine, arginine, threonine, alanine, proline, theanine, cystine, tyrosine, valine, methionine, lysine, isoleucine, leucine, and phenylalanine.

3.2 Regression equation, correlation coefficient, and limit of detection We prepared the amino acid mixed standard solution at concentration of 5, 10, 50, 100, 200 and 250 μmol/L respectively. After derivatization, we measured the samples. Taking the amino acid solution concentration (X) as the abscissa and the corresponding peak area (Y) as the coordinate, we plotted the standard working curve and calculated the correlation coefficient. The results showed that in the range of 5 – 250 μmol/L, the linear relationship between amino acid concentration and its peak area was good, and the correlation coefficient was 0.997 8 – 0.999 9. At the 3 times the signal to noise ratio, we calculated the limit of detection of the method. The results are shown in Table 1.

Table 1 Linear equation, correlation coefficient and limit of detection of 18 kinds of amino acids

Amino acids	Linear equation	Correlation coefficient (r)	Limit of detection // μmol/L	Linear range // μmol/L
Aspartic acid	$Y=1.62\times10^8X+2.17\times10^4$	0.998 0	0.55	5 – 250
Serine	$Y=2.24\times10^8X+7.05\times10^3$	0.999 1	0.40	5 – 250
Glutamic acid	$Y=1.85\times10^8X+2.54\times10^4$	0.997 8	0.49	5 – 250
Histidine	$Y=3.31\times10^8X-1.17\times10^4$	0.999 8	0.30	5 – 250
Glycine	$Y=2.11\times10^8X-5.82\times10^4$	0.999 5	0.46	5 – 250
Arginine	$Y=3.15\times10^8X-1.37\times10^5$	0.999 6	0.35	5 – 250
Threonine	$Y=3.10\times10^8X+1.10\times10^5$	0.999 3	0.24	5 – 250
Alanine	$Y=3.50\times10^8X+5.48\times10^5$	0.997 9	0.14	5 – 250
Proline	$Y=1.82\times10^8X+1.51\times10^4$	0.999 0	0.30	5 – 250
Theanine	$Y=9.85\times10^7X+8.68\times10^5$	0.999 1	0.05	1 – 1 250
Cystine	$Y=9.61\times10^7X-2.80\times10^4$	0.999 9	1.27	2.5 – 125
Tyrosine	$Y=3.42\times10^8X+6.28\times10^4$	0.999 8	0.18	5 – 250
Valine	$Y=5.95\times10^8X+1.12\times10^5$	0.998 5	0.11	5 – 250
Methionine	$Y=4.95\times10^8X-1.28\times10^5$	0.999 4	0.14	5 – 250
Lysine	$Y=2.19\times10^8X+4.30\times10^5$	0.996 9	0.29	5 – 250
Isoleucine	$Y=7.71\times10^8X-1.57\times10^5$	0.998 6	0.10	5 – 250
Leucine	$Y=7.65\times10^8X-3.81\times10^5$	0.998 6	0.11	5 – 250
Phenylalanine	$Y=9.65\times10^8X-1.27\times10^5$	0.999 8	0.09	5 – 250

3.3 Recovery rate Precisely weighed 5 pieces of 0.25 g of fresh tea leaf samples with known amino acid content, added certain volume of amino acid mixed standard solution, made the measurement after derivatization reaction, and determined the content using the external standard method, calculated the recovery rate of amino acid and relative standard deviation (Table 2).

The recovery rate of 18 kinds of amino acids ranged from 86.25% to 109.05%, and the *RSD* was in the range of 6.03% – 10.56%, indicating that this method has high accuracy, good reproducibility and high reliability.

Table 2 Recovery rates and RSDs of amino acids from spiked fresh tea leaves

Name of amino acid	Added standard solution // μmol/L	Recovery rate // %	RSD // %	Name of amino acid	Added standard solution // μmol/L	Recovery rate // %	*RSD* // %
Aspartic acid	10	92.51	7.84	Theanine	50	87.91	9.02
Serine	10	89.21	6.03	Cystine	5	91.42	9.13
Glutamic acid	10	98.90	7.43	Tyrosine	10	109.05	8.04
Histidine	10	92.62	8.94	Valine	10	105.38	7.30
Glycine	10	88.02	6.97	Methionine	10	103.52	6.39
Arginine	10	86.25	10.56	Lysine	10	90.81	7.77
Threonine	10	93.54	9.57	Isoleucine	10	95.32	7.68
Alanine	10	95.81	8.33	Leucine	10	96.02	8.91
Proline	10	90.11	7.49	Phenylalanine	10	86.12	6.58

3.4 Precision Took the appropriate amount of 18 kinds of amino acid mixed standard solution, made analysis according to the above method, continuously injected 5 times, took the retention time and peak area of chromatographic peak as indicator to calculate the *RSD*, to examine the precision. The *RSD* of retention time of amino acid was 0.09% – 0.55%, the peak area *RSD* was 1.12% – 2.15%, indicating high precision of the method.

3.5 Reproducibility Took 5 pieces of the same fresh tea leaf samples, extracted, derivatized, and measured using the above method. Taking the retention time and peak area of amino acids with the chromatographic peak area larger than 1% as the indicators, we calculated the *RSD*, and examined the reproducibility. The *RSD* of retention time of amino acids in fresh tea leaves was in the range of 0.55% – 1.98%, the peak area *RSD* was in the range of 2.08% – 3.71%, showing high reproducibility of the method.

3.6 Stability Took 5 pieces of the same fresh tea leaf samples, derivatized in accordance with the above method, and injected the samples at 0, 4, 8, 12, and 24 h. Taking the retention time and peak area of amino acids with the chromatographic peak area larger than 1% as the indicators, we calculated the *RSD*, and examined the stability of derivatized solution of amino acids in fresh tea leaves. The *RSD* of each amino acid derivatized product was in the range of 0.23% – 0.01% ($n=5$) and the peak area *RSD* was in the range of 1.18% – 3.91% ($n=5$), indicating that the derivatized solution of amino acid is stable for 24 h at room temperature.

4 Application of the method

Using the established method, we measured the free amino acids in fresh leaf samples of Biluochun tea collected from east and west Dongting mountains in Suzhou City, as presented in Table 3.

Table 3 Measurement results of free amino acids in fresh tea leaf samples

Amino acids	Content of amino acid//mg/g	
	East Dongting mountain	West Dongting mountain
Aspartic acid	4.56	2.92
Serine	3.77	1.29
Glutamic acid	3.50	2.60
Histidine	1.77	1.32
Glycine	0.23	0.05
Arginine	2.22	0.89
Threonine	0.95	0.73
Alanine	1.08	0.68
Proline	0.81	1.22
Theanine	35.12	27.88
Cystine	0.12	ND
Tyrosine	0.52	0.22
Valine	2.32	1.40
Methionine	0.31	0.22
Lysine	0.68	0.32
Isoleucine	0.53	0.28
Leucine	0.84	0.43
Phenylalanine	0.81	0.35

Note: ND denotes that the amino acid is lower than the limit of detection.

5 Conclusions

We established a rapid and accurate quantitative method of high performance liquid chromatography (HPLC) with fluorescence detector for the analysis of 18 amino acids in fresh tea leaves. The samples were minced and mixed, and extracted with ultra pure water at 90℃ for 20 min. The AQC was used as pre-column derivatization reagent. Gradient HPLC separation was performed on a C_{18} column (Symmetry C_{18}, 3.9 mm × 15 cm, 4 μm). Good linearity between concentrations and peak areas was achieved in the concentration range of 5.0 – 250 μmol/L for 18 amino acids. The method was validated by the analysis of five replicates. The 18 amino acid standards were spiked in fresh tea leaf samples and the average recoveries were 86.25% – 109.05% with relative standard deviations ($n = 5$) ranging from 6.03% to 10.56%. The limit of detection (LOD) for the analysis was 0.05 – 1.27 μmol/L. This proposed method has the advantages of rapid and simple sample preparation, low cost, high precision, accuracy and stability. The analysis process is rapid, sensitive and reproducible. It was shown to be a suitable method for determination of free amino acids in fresh tea leaves.

In this method, we used fresh tea leaves as samples to replace the dry samples in traditional methods. This can avoid the possible conversion of various biochemical components of tea resulted from high temperature and drying, and the detection error due to changes in the free amino content. Thus, the measured data are more accurate and reliable, and can truly reflect the content of amino acids in tea. Besides, we used the common C_{18} column (C_{18}) Symmetry column to take the place of the special column for amino acid to separate and analyze the amino acids. Through the technical optimization, we successfully separated the amino acid components and achieved the quantitative determination of 18 kinds of free amino acids such as theanine in tea. The entire process of method is only 23 min, it greatly increases the efficiency of the amino acid analysis, thus it can meet the requirements of high-volume sample analysis. In addition, the symmetry C_{18} column is cheap, not highly special, and can be used for testing of other substances, so this method greatly reduces the analysis cost, and it is especially suitable for popularization and application in grass-roots testing agencies and units.

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