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A High-Performance Liquid Chromatographic Method for Simultaneous Determination of 21 Free Amino Acids in Tea

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Abstract Free amino acids are closely related to the savory taste and beneficial effects of tea, and high-performance liquid chromatography (HPLC) is the most widespread analytical approach for simultaneous determination of free amino acids in tea. However, the reported HPLC methods have drawbacks such as long run times, low resolution, or poor efficiency. In this study, a special amino acid analysis column was used to separate and verify 21 amino acids including L-theanine, the predominant amino acid in tea. The mobile phases, including the sodium acetate and acetic acid concentration in buffer B, and the pH and concentration of sodium acetate in buffer A were optimized. The elution gradients were optimized using DryLab 2000 Plus software. In this way, an online o-phthaldialdehyde precolumn derivatization HPLC-fluorescence detection method was developed for simultaneous determination of 21 amino acids. Comparison to other HPLC methods for simultaneous determination of free amino acids in tea showed that our method is easy (automated derivatization), quick (30 min), inexpensive, and green (using a minimum of solution). It has good resolution (≥1.8) and high selectivity (interpark time≥0.5 min). Free amino acids in six tea samples were analyzed. This work provides an HPLC method to simultaneously measure 21 amino acids in tea and potential in other food products.

Keywords Free amino acids · Simultaneous determination · Tea

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Abbreviations

ACN	Acetonitrile
Ala	Alanine
Arg	Arginine
Asn	Asparagines
Asp	Aspartic acid
Cys	Cystine
DAD	Diode array detector
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
FLD	Fluorescence detection
GABA	γ-Amino butyric acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
HPLC	High-performance liquid chromatography
His	Histidine
Ile	Isoleucine
Leu	Leucine
LOD	Limit of detection
LOQ	Limit of quantification
Lys	Lysine
MeOH	Methanol
Met	Methionine
OPA	o-Phthaldialdehyde
PA	Peak area
Phe	Phenylalanine
Pro	Proline
RSD	Relative standard deviation
Rs	Resolution
RT	Retention time
Ser	Serine
NaAc	Sodium acetate
THF	Tetrahydrofuran
Thr	Threonine

TEA	Triethylamine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Introduction

Tea is a beverage made from the leaves and buds of *Camellia sinensis* (L.) O. Kuntze and has been safely consumed for thousands of years. It is currently the second most widely consumed beverage in the world after water and is considered to be refreshing, have an attractive aroma and taste, and have potential health benefits (Wan et al. 2009a). Tea contains many chemical components such as amino acids, polyphenols (catechins and flavonoids), polysaccharides, volatile oils, vitamins, minerals, and alkaloids (e.g., caffeine) (Loto 2011; Syu et al. 2008). The free amino acids are particularly interesting because they are not only responsible for the savory taste of tea infusions, but also have various beneficial effects. In tea plants, 26 varieties of amino acids have been identified (Wang 1984), accounting for about 1–4 % of the dry weight (Xu et al. 2011). Many of the free amino acids are involved in producing the distinctive aroma and taste of tea. For example, the brothy, sweet, umami taste of green tea is due to free amino acids, especially the predominant L-theanine (Thippeswamy et al. 2006). Theanine is a nonprotein amino acid that occurs naturally in the tea plant, accounting for 1–2 % of the dry weight of leaves and over 50 % of the total free amino acids (Wan et al. 2009b). Some of the free amino acids have physiological and pharmacological activities. For example, theanine is claimed to have effects such as enhancing relaxation and improving concentration and learning ability, preventing certain cancers and cardiovascular disease, promoting weight loss, and enhancing immune system performance (Vuong et al. 2011); another nonprotein amino acid, γ-aminobutyric acid (GABA), is a neurotransmitter, which induces hypertensive and has diuretic and tranquilizing effects (Zhao et al. 2011).

Because of the contribution to the taste and healthful benefits of tea, free amino acid content correlates with tea quality. Therefore, simultaneous determination of levels of free amino acids in tea products is important. Several methods have been developed to determine the presence of various amino acids in teas using amino acid analyzers (Tsushida and Murai 1987; Ohtsuki et al. 1987; Wang et al. 2006; Wang et al. 2010a; Xu et al. 2011; Yuan et al. 2011), capillary electrophoresis (Kato et al. 2003; Hsieh and Chen 2007), ultra performance liquid chromatography (Kim et al. 2009), and high-performance liquid chromatography (HPLC) (Alcázar et al. 2007; Syu et al. 2008; Wang and Ruan 2009; Wang et al. 2010b; Tan et al. 2011; Yang et al. 2011). The combination of HPLC separation and prederivatization followed by fluorescence detection (FLD) or diode array detector (DAD) is the most convenient and widespread analytical approach. However, the six reported methods for simultaneous determination of free amino acids in tea by HPLC have drawbacks such as long run times, low resolution, or poor efficiency. For example, the method of Syu requires 115 min (Syu et al. 2008), and the method of Yang requires of 60 min (Yang et al. 2011) good separation between alanine (Ala) and theanine is not achieved in the method developed by Wang (Wang et al. 2010b), and good separations of critical pairs (Gly and Thr; Phe and Ile) are not achieved in Tan's method (Tan et al. 2011); only eight amino acids in Wang's method and 13 amino acids in Alcázar's method were determined, respectively, (Wang and Ruan 2009; Alcázar et al. 2007). Therefore, HPLC methods for simultaneous determination of free amino acids in tea require further improvements.

Hypersil AA-ODS column is designed for amino derivate analysis (Agilent Technologies 2005). In this work, this column was used for simultaneous determination of free amino acids in tea; however, high-resolution separation of several amino acids was not achieved using the manufacturer's instructions (Fig. 1a). Therefore, this method was improved. A good separation with high resolution and selectivity for 21 amino acids was achieved by optimizing the mobile phases and gradients.

Materials and Methods

Chemicals and Reagents

A standard solution of 16 amino acids, including aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), alanine (Ala), arginine (Arg), tyrosine (Tyr), cystine (Cys), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys), and three other amino acid standards, asparagine (Asn), glutamine (Gln), and tryptophan (Trp) were from Agilent (Agilent Technologies, Palo Alto, CA). L-Theanine and GABA were from Sigma-Aldrich (St. Louis, MO).

Acetonitrile (ACN) and methanol (MeOH) for the mobile phases were HPLC-grade reagents from Tedia Co., Inc., (Fairfield, OH). Sodium acetate (NaAc), triethylamine (TEA), tetrahydrofuran (THF), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and acetic acid used in the mobile phases were analytical reagent grade and were used without further purification. OPA regent (10 mg/mL) and borate buffer (0.4 M, pH 10.4) used for derivatization were from Agilent (Agilent Technologies, Palo Alto, CA). Deionized water was used in the EPED-T purification system (Yi Pu Yi Da Co., Nanjing, Jiangshu, China) and was degassed under vacuum and filtered through a 0.45 μm nylon membrane before use for HPLC analysis.

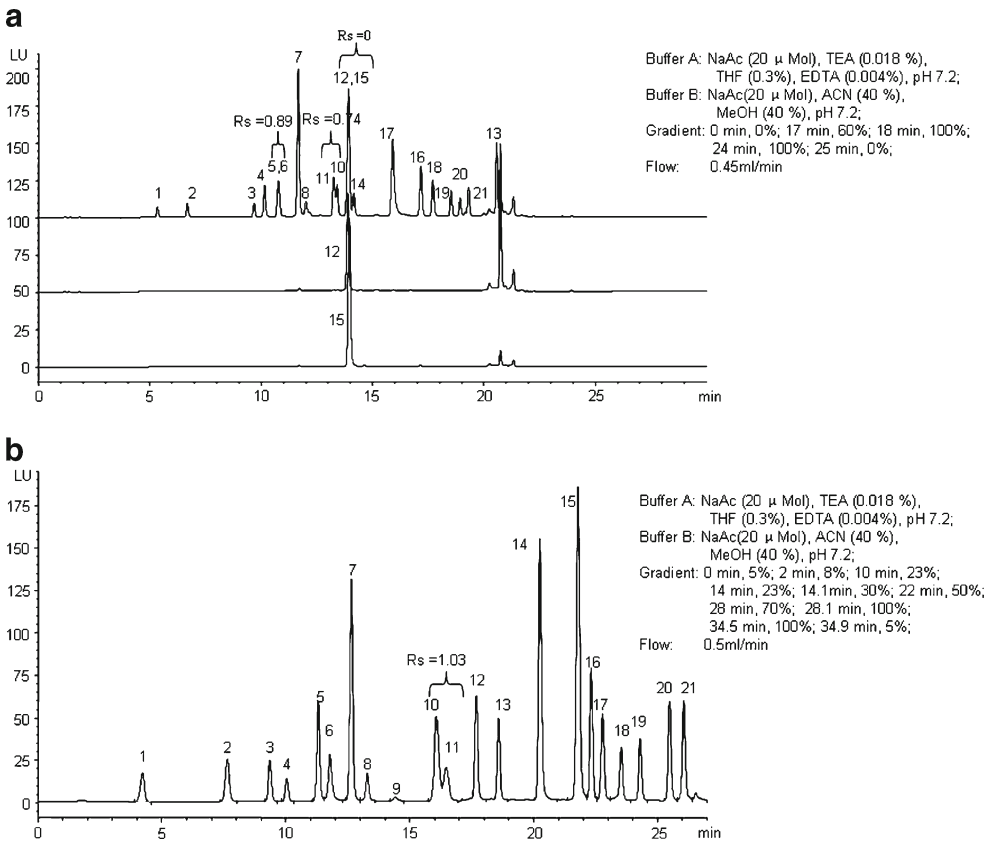


Fig. 1 Chromatograms of amino acid standard derivatives with OPA using Agilent amino acid method (**a**) and improved elution gradient method (**b**). *1* Asp, *2* Glu, *3* Asn, *4* Ser, *5* Gln, *6* His, *7* Gly, *8* Thr, *9* Cys, *10* Arg, *11* Ala, *12* Thean, *13* Lys, *14* Try, *15* GABA, *16* Met, *17* Val, *18* Trp, *19* Phe, *20* Ile, *21* Leu

Agilent Amino Acid HPLC Method

The Agilent amino acid HPLC method was developed according the Hypersil AA-ODS (2.1×200 mm, 5 μm, Agilent) instructions using an Agilent 1200 series HPLC system consisting of an LC-20AB solvent delivery unit, a SIL-20A autosampler, a CTO-20A column oven (40 °C), a G1321A FLD (Ex, 230 nm; Em, 450 nm), and an LC Ver1.23 workstation (Agilent Technologies). Separation was completed using the Hypersil AA-ODS column fitted with a guard column (Hypersil ODS, 20×2.1) (Agilent Technologies, Palo Alto, CA). The mobile phases were solvents A (20 μM NaAc, 0.018 % TEA, 0.3 % THF, 0.004 % EDTA, pH 7.2) and solvent B (20 μM NaAc, 40 % ACN, 40 % MeOH, pH 7.2). Elution conditions and flow rates were: 0–17 min, solvent A was reduced from 100 to 40 % and B from 0 to 60 % (linear gradient); 17–18 min, solvent B increased to 100 % (linear gradient); 18–24 min, solvent B was kept at 100 %; 24–25 min, solvent A increased from 0 to 100 % (linear gradient); 0–18 min, flow rate 0.45 mL/min; 18.1 min, flow rate increased to 0.8 mL/min; at 24 min, flow 0.45 mL/min (Gratzfeld-Huesgen 2002).

Improvement of the HPLC Method

Four variables of the mobile phases, concentration of NaAc (0, 16, and 20 μM) and acetic acid (0–0.2 %) in buffer B and pH (6.9, 7.2, and 7.5) and concentration of NaAc (0, 15, 20, 25, and 30 μM) in buffer A, were optimized based on the resolution. The gradients were further optimized with DryLab 2000 Plus software (Molnar Institute) using the LC gradient (two runs) mode according to the DryLab manual. Two linear gradient runs of 5–100 % B in 20 and 60 min were performed. After data were imported, the linear gradient was optimized using "gradient editor" to provide the "highest" in silico resolution. The predicted linear gradient run was performed and improved manually to find acceptable resolutions (minimum Rs of ≥1.5) for each amino acid park.

Validation of HPLC-FLD Analysis

HPLC-FLD validation tests were carried out using tests of precision, selectivity, limit of detection (LOD), and limit of quantification (LOQ). Precision (reproducibility) of the method was determined by calculating the relative standard deviation (RSD) from repeated injections of the amino acid standard solution. Intraday precision was determined using five replicate injections, and interday precision was determined from five injections for 5 days, for both retention times (RT) and peak areas (PA). RSDs were calibrated using the formula RSD (%)=(SD/mean)×100. The selectivity criterion was that all amino acid peaks should have a chromatographic baseline with acceptable resolution (minimum

Rs of ≥1.5) and a minimum of interpark time greater than about 0.5 min. The LOD and LOQ were calculated at signal to noise ratios (S/N) of 3 and 10, respectively.

Determination of Amino Acids in Tea Samples

Six Chinese teas, including three green teas and three black teas, were purchased from local markets. Tea infusions were prepared to accord to our previous report (Zhao et al. 2011) as follow: tea samples of 5.0 g were ground into powder using a grinder (Joyoung Co., Jinan, Shandong, China); 2 g of the tea powder was extracted with 100 mL of distilled water for 2 h in a water bath (85 °C); the tea water extract was centrifuged at 12,000 rpm for 10 min at room temperature; 1,000 μL of the supernatant added with 200 μL of $CHCl_3$ was transferred to a 1.5 mL Eppendorf tube and then was centrifuged at 12,000 rpm for 10 min; and 800 μL of supernatants were filtered through a 0.2-μm PTFE filter for HPLC analysis using the optimized method.

Statistical Analysis

Results are expressed as means±SD. All data were analyzed using SPSS 11.0 software packages (SPSS Inc., Chicago, IL).

Results and Discussion

Determination of Amino Acids Using the Agilent Method

Standard solutions of 21 amino acids were separated on the amino acid analysis column according to the manufacturer's instructions (Fig. 1a). However, good separation between Gln and His (Rs=0.89), Ala and Arg (Rs=0.74), GABA and theanine (Rs=0), and theanine and Ala (Rs=0) was not achieved. Elution gradients were optimized with DryLab 2000 Plus software. A complex program with five gradients was developed, but good separation between Arg and Ala (Rs=1.03) was not achieved (Fig. 1b). This suggested that Agilent method needed a comprehensive improvement of elution buffers and gradient program.

Improvement of the HPLC Method

Good separation was not achieved after improvement of the elution program, so improvement of both elution buffers and the gradient program were performed. The concentration of NaAc (0, 16, and 20 μM) and acetic acid (0–0.2 %) in buffer B and the elution program were optimized. The optimized buffer B contained ACN (40 %), MeOH (40 %), and acetic acid (0.125 %). The optimized elution program and chromatogram of amino acid standard derivatives with OPA are in Fig. 2a.

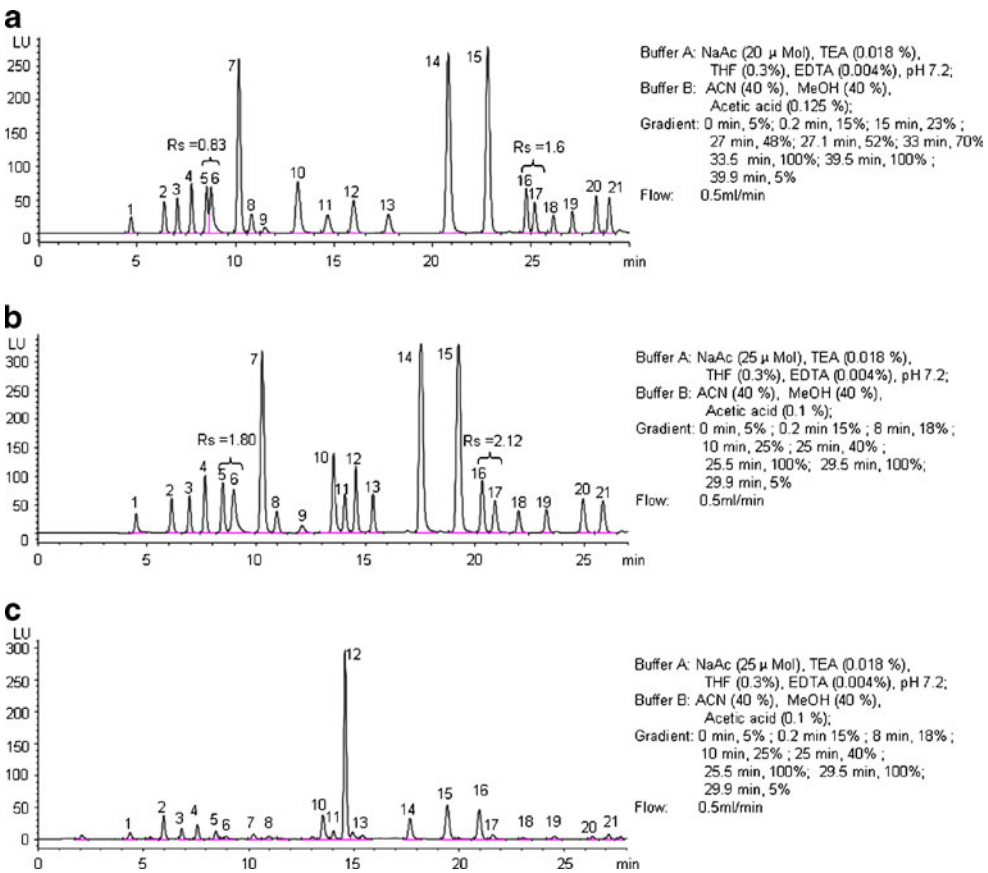


Fig. 2 Chromatograms of amino acid standards derivatives with OPA using improved buffer B and elution program (**a**) and final optimized approach (**b**), representative HPLC chromatograms of amino acid in tea (high-grade Yunnan black tea) (**c**). *1* Asp, *2* Glu, *3* Asn, *4* Ser, *5* Gln, *6* His, *7* Gly, *8* Thr, *9* Cys, *10* Arg, *11* Ala, *12* Thean, *13* Lys, *14* Try, *15* GABA, *16* Met, *17* Val, *18* Trp, *19* Phe, *20* Ile, *21* Leu

Table 1 Validation of the method

Amino acid	Intraday RSDs		Interday RSDs		Resolution	Interpark time (min)	LOD (μM)	LOQ (μM)
	Retention time (%)	Peak area (%)	Retention time (%)	Peak area (%)				
Asp	0.1	1.8	1.4	3.8	–	–	2.2	7.2
Glu	0.1	1.9	1.0	3.0	7.39	1.62	1.2	4.0
Asn	0.1	2.2	0.7	3.3	3.89	0.82	0.3	1.0
Ser	0.1	1.9	0.6	2.9	3.42	0.71	0.5	1.5
Gln	0.1	3.6	0.7	5.4	3.58	0.81	0.5	1.5
His	0.1	1.9	0.7	3.1	1.80	0.50	1.5	4.9
Gly	0.1	1.8	0.8	3.0	4.30	1.31	0.4	1.2
Thr	0.1	2.0	0.9	3.1	2.33	0.66	0.8	2.5
Cys	0.1	1.4	0.8	3.8	3.61	1.16	6.8	22.7
Arg	0.1	2.2	0.6	3.1	4.61	1.43	19.2	63.9
Ala	0.1	2.1	0.6	3.5	2.13	0.52	5.8	19.4
Theanine	0.1	2.2	0.6	3.3	2.27	0.50	0.8	2.7
Lys	0.1	2.4	0.6	3.4	3.69	0.79	2.2	7.4
Tyr	0.1	1.4	1.3	2.9	7.80	2.20	0.2	0.7
GABA	0.1	1.7	2.9	3.5	4.88	1.72	0.2	0.7
Met	0.1	2.0	1.8	3.3	3.48	1.09	1.4	4.7
Val	0.1	2.2	1.2	4.0	2.12	0.59	0.9	3.1
Trp	0.1	2.6	1.8	3.8	3.90	1.10	0.6	2.1
Phe	0.1	1.9	1.9	3.3	4.63	1.26	0.2	0.8
Ile	0.1	1.8	2.3	3.3	5.81	1.67	0.3	1.0
Leu	0.1	1.9	1.4	4.7	2.99	0.91	2.6	8.5

Good separation between Gln and His (Rs=0.83) was not achieved.

The pH (6.9, 7.2, and 7.5) and concentration of NaAc (0, 15, 20, 25, and 30 μM) in buffer A, the percent of acetic acid (0–0.125 %) in buffer B, and the elution programs were optimized. The final optimized buffer A (pH 7.2) contained NaAc (25.0 μM), TEA (0.018 %), THF (0.3 %), and EDTA (0.004 %). The final optimized buffer B contained ACN (40 %), MeOH (40 %), and acetic acid (0.1 %). The optimized elution program and amino acid standards chromatogram are in Fig. 2b. High resolution (Rs≥1.8) for each amino acid park was achieved (Fig. 2b).

Method Validation

The validation tests of precision and selectivity are in Table 1. The precision tests demonstrated that this method had a good reproducibility, with intraday RSDs of 0.1 and <3.0 % (excepting Gln) for retention time and <3.0 and <5.0 % (excepting Gln) for peak areas. Gln was not reproducible because it was degraded during analysis. Therefore, the standard solution containing Gln needed be made fresh every day. Validation tests showed that the method had a high selectivity with an interpark time greater than 0.5 min and good resolution (Rs≥1.8).

Analysis of Free Amino Acids in Tea Products

Free amino acids in six teas were detected using the improved HPLC method. The contents of 21 amino acids are in Table 2, and a representative HPLC chromatogram is in Fig. 2c. The chromatogram showed about 25 identified peaks, revealing other amino acids in tea. This is consistent with a previous report about 26 varieties of amino acids identified in tea plants (Wang 1984).

Comparison of HPLC Methods for Simultaneous Determination of Free Amino Acids in Tea

An HPLC approach for simultaneous determination of free amino acids in tea was developed in this work. Six previously reported HPLC methods simultaneously determine free amino acids in tea. A comparison of these HPLC methods with our method is in Table 3. This comparison showed that the automated online precolumn derivatization used by Tan (Tan et al. 2011), Yang (Yang et al. 2011), and us does not need manual manipulation for derivatization.

Table 2 Contents of free amino acids in tea

Amino acid	High-grade Yunnan black tea (Dian hong)	Yunzhang Feng black tea	Long-run Gongfu black tea	Dalishu green tea	Dadugang green tea	Laomudeng green tea
	Contents (nmol/g)					
Asp	10.67±2.53	8.22±0.19	8.04±0.13	10.13±0.86	2.11±0.52	10.84±0.35
Glu	14.07±1.83	7.25±0.41	8.00±0.21	10.60±1.17	6.94±0.14	10.10±0.44
Asn	6.88±1.08	3.64±0.19	16.58±0.34	1.25±0.13	1.35±0.17	1.31±0.04
Ser	7.67±2.44	3.81±0.34	6.19±0.16	3.44±0.30	2.56±0.11	2.62±0.06
Gln	6.75±1.30	2.21±0.12	2.10±0.17	4.08±0.54	2.23±0.08	1.61±0.78
His	0.38±0.66	0.61±0.05	1.27±0.13	0.59±0.10	0.65±0.05	0
Gly	0.86±0.19	0.68±0.11	0.69±0.04	0.60±0.02	0.34±0.04	0.50±0.01
Thr	3.73±1.13	2.57±0.06	3.60±0.09	2.96±0.18	2.09±0.12	2.69±0.01
Cys	0	0	0	0	0	0
Arg	2.85±3.33	0.99±0.10	3.50±0.19	4.23±0.20	7.77±0.29	2.32±0.04
Ala	6.16±1.73	2.67±0.16	3.82±0.16	2.47±0.31	1.97±0.04	2.46±0.03
Thean	54.01±5.72	31.19±0.77	39.76±1.36	63.60±3.77	65.97±1.38	49.42±1.31
Lys	6.83±3.58	5.36±0.50	3.19±0.76	3.28±0.35	1.53±0.51	2.53±0.00
Tyr	1.90±0.14	1.81±0.09	2.85±0.18	0.98±0.03	1.05±0.05	0.98±0.03
GABA	2.42±0.81	1.18±0.02	1.69±0.12	1.51±0.21	0.75±0.03	0.49±0.01
Met	10.14±7.47	8.38±0.09	13.16±0.39	3.80±0.17	2.02±0.07	2.46±0.02
Val	4.34±1.90	3.86±0.12	4.22±0.19	0.96±0.09	1.64±0.07	1.11±0.04
Trp	1.42±0.38	1.42±0.17	2.16±0.12	0	0	0
Phe	3.16±0.24	4.48±0.11	5.23±0.13	1.34±0.07	1.41±0.06	2.44±0.01
Ile	3.07±0.97	2.85±0.10	4.70±0.95	1.46±0.12	1.01±0.03	0.87±0.04
Leu	3.63±0.88	3.86±0.16	4.23±0.18	3.35±0.66	1.08±0.01	1.62±0.03

The method was easy and had high reproducibility and high throughput. This method and the method of Syu (Syu et al. 2008) were effective for the measurement of 21 amino acids simultaneously in a single separation. Our approach had good resolution (Rs≥1.8) and high selectivity (interpark time of ≥0.5 min). The elution time of Alcázar et al. (2007) and our method were both 30 min, which is faster than other studies except for Tan (15 min) (Tan et al. 2011). Finally, our method used minimal amounts of solvent, so it is less expensive and less wasteful. Our method is quick and easy, inexpensive and green, and with good resolution and selectivity.

Table 3 Comparisons of HPLC methods for simultaneous determination of free amino acids in tea

Reference	Method	Number of amino acids	Selectivity		Elution time (min)	Solvent (mL)
			MinRs	MinIpt (min)		
Alcázar et al. 2007	Off-line OPA P-D HPLC-FLD	13	1.50	0.41	30	60
Syu et al. 2008	Off-line dabsylation P-D HPLC	21	–	–	115	–
Wang and Ruan 2009	HPLC-FLD	8	–	–	38	38
Wang et al. 2010b	Off-line OPA P-D HPLC-DAD	17	–[a]	–	35	35
Tan et al. 2011	Online OPA P-D HPLC-FLD	20	–[b]	0.08	15	30
Yang et al. 2011	Online OPA P-D HPLC-FLD	17	–	–	60	60
This work	Online OPA P-D HPLC-FLD	21	1.80	0.50	30	15

(–) unknown

MinRs minimum resolution, *MinIpt* minimum interpark, *P-D* precolumn derivatization

[a] Baseline separation between Ala and theanine was not achieved

[b] Baseline separation between Gly and Thr; Phe and Ile was not achieved

In conclusion, we report an easy, quick, inexpensive, and green HPLC method with good resolution and high selectivity for simultaneous determination of free amino acids in tea that has the potential to analyze the free amino acids in other food products.

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