Determination of free amino acids in tea by a novel method of reversed-phase high performance liquid chromatography applying 6-Aminoquinolyl-*N*-Hydroxysuccinimidyl carbamate reagent

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Abstract We describe a novel analytical method for quantification of free amino acids in tea using variable mobile phase pH, elution gradient and column temperature of reversed-phase high-performance liquid chromatography (RP-HPLC). The study of mobile phase pH 5.7 was chosen to simultaneous quantification of 19 free amino acids in tea, while it improved maximum resolution of glutamine, histidine and theanine. Elution gradient was adapted for enhancing the solution of free amino acids, mainly because of adjustment of mobile phase A and B. The column temperature of 40 °C was conducive to separate free amino acids in tea. The limit of detection (LOD) and limit of quantitation (LOQ) of this method were in the range of 0.097–0.228 nmol/mL and 0.323–0.761 nmol/mL, respectively. The relative standard deviation of intraday and interday ranged in 0.099–1.909% and 3.231–7.025%, respectively, indicating that the method was reproducible and precise, while recovery ranged between

81.06–112.78%, showing that the method had an acceptable accuracy. This method was applied for the quantification of free amino acids in six types of tea. Multivariate analysis identified serine, glutamine, theanine and leucine as the most influencing factor for classify among analyzed sample.

Keywords RP-HPLC · Tea · Free amino acids · Theanine · Glutamine

Introduction

Free amino acids are active compounds in tea which affect the quality of taste, aroma and colour of tea. Free amino acids such as theanine, glutamine and glutamic acid are responsible for a distinct umami taste of tea, which give tea a savory and brisk taste, and tryptophan and phenylalanine are contribute to the intensity of bitter and astringent taste of the tea, thus contents and quantity of free amino acids play a key role for determine the quality of tea (Mu et al. 2015; Liu et al. 2017; Lee et al. 2013). Moreover, theanine is a unique free amino acid in tea, and it has shown the protective effects on the prevention of neurodisorders (Khan and Mukhtar 2013). Histidine and leucine functions as antioxidant and branched-chain amino acid balance respectively (Wu 2009). Free amino acids are important for improve the quality of tea and health benefits. So it is interest in the development of a reliable and accurate method of free amino acids for assessing the composition and quality of six types of tea.

The determination using automated amino acid analyzer is a traditional method for free amino acids analysis. Free amino acids are separated by cation-exchange chromatography followed by post-column derivatization using *o*-

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phthalaldehyde (OPA) or ninhydrin (Bidleymeyer et al. 1984; Vasanits-Zsigrai et al. 2015). Both reagents enhanced the sensitivity of free amino acid analysis while the disadvantage of the method is post-column derivatization for long analysis times, low sensitivity and high instrument expenses (Masuda and Dohmae 2011; Azilawati et al. 2014). The classic analytical method for free amino acid was HPLC (high-performance liquid chromatography) system coupled to pre-column modification and reagents that generate fluorescent amino acid derivatives, including 9-fluorenyl-methyl chloroformate (FMOC-Cl), phenylisothiocyanate (PITC), 6-Aminoquinolyl-*N*-hydroxysuccinimidyl Carbamate (AQC) and *o*-phthalaldehyde (OPA) (Cohen and Michaud 1993; Hou et al. 2015; Báez et al. 2014; Chen et al. 2009). The OPA only reacts with primary amines and some fluorescent products such as glycine and lysine is unstable (Bidleymeyer et al. 1984; Hou et al. 2015). PITC reacts with primary and secondary amino acid, it also generates unstable derivatives, particularly glutamic acid and aspartic (Thippeswamy et al. 2006). The excess reagents must be removed prior to chromatographic analysis to avoid column contamination (Masuda and Dohmae 2011; Azilawati et al. 2014). AQC as an efficient amine derivatizing agent was introduced to detect free amino acid in the early 1990s (Cohen and Michaud 1993; Díaz et al. 1996). It reacts with primary and secondary amines which products are stable derivatives that are highly fluorescent, and AQC method avoids by-products removing steps (Cohen and Michaud 1993; Azilawati et al. 2016). So far, AQC method was chose to detect a lot of samples, such as beers, soils, grape juices, wines, fruit juices and vinegars (Kabelová et al. 2008; Hou et al. 2009; Hernández-Orte et al. 2003; Zeng et al. 2014; Callejón et al. 2008). In this study, Pre-column derivatization of free amino acids with AQC followed by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was used because it provides an efficient compositional analysis for free amino acids in tea.

Materials and methods

Chemicals and reagents

High-purity (> 98%) alanine, arginine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Theanine was supplied by Canspec China (Shanghai, China). A set of derivatizing reagent contains AccQ-Fluor reagent powder (6-Aminoquinolyl -*N*-hydroxysuccinimidyl Carbamate), AccQ-Fluor borate buffer and AccQ-Fluor

reagent diluents and AccQ-Tag Eluent A were purchased from the Waters Co. Ltd. (Milford, MA, USA). Acetonitrile and methanol of HPLC grade were procured from the Tedia Company (Fairfield, OH, USA). Sodium hydroxide and phosphoric acid of HPLC grade was obtained from the Weiqiboxing Company (Wuhan, China). Ultrapure water was obtained from a Milli-Q system of Millipore (Bedford, MA).

Sample preparation

Six types of tea were collected from local tea market. Green tea including Guapian (GT1), Maofeng (GT2) and Xihulongjing (GT3). Black tea including Qihong (BT1), Jingyangqing (BT2) and Tanyanggongfu (BT3). White tea including Baihaoyinzhen (WT1), Baimudan (WT2) and Shoumei (WT3). Oolong tea including Tieguanyin (OT1), Minnansezhong (OT2) and Wuyiyancha (OT3). Yellow tea including Laoganhong (YT1), Huangyatea (YT2) and Junshanyinzhen (YT3). Dark tea including Yiyangfuzhuan (DT1) and Qingzhuan (DT2). Samples were crushed as three repeats. Each sample was crushed into tea powder and kept in a sealed pack at -20°C .

Tea infusions were prepared. Free amino acids were extracted from 1.5 g of tea powder by in 250 mL boiling ultrapure water for 10 min refer to the common method (Horanni and Engelhardt 2013; Chen et al. 2009). The supernatant was collected after cooling to room temperature. Two mL of the extract was filtered through a $0.45\text{ }\mu\text{m}$ cellulose acetate membrane prior to derivatization. Three replicates of each sample were processed in parallel on the same day. Sample derivatization was performed following the protocol. Ten microliters of the sample was selected to derivatization. Seventy microliters of AccQ-Fluor borate buffer was added to sample followed by $20\text{ }\mu\text{L}$ of AccQ-Fluor reagent. The derivatized compound must be well homogenized and heated at 55°C for 10 min prior to chromatographic analysis.

Standard sample preparation

Aqueous 2.5 mM stock solutions of glutamine and theanine were prepared in ultrapure water. Then, $100\text{ }\mu\text{L}$ of the stock solution and $100\text{ }\mu\text{L}$ 2.5 mM Sigma standard mix (containing 17 amino acids) were mixed with $800\text{ }\mu\text{L}$ ultrapure water to a final concentration of 0.25 mM per amino acid.

RP-HPLC analysis

The RP-HPLC was performed using Waters Alliance System equipped with Waters 2475 Multi- λ Fluorescence Detector at 254 nm excitation and 395 nm emissions. Free

amino acid separation was achieved on AccQ-Tag C18 amino acid analysis column (3.9 mm × 150 mm i.d., 4 µm). A gradient mixture of 1% AccQ-Tag eluent A in ultrapure water (A), acetonitrile (B) and ultrapure water (C) was used as the mobile phase at a flow rate of 1.0 mL/min (Supplementary Table 1).

Method validation

Calibration curves for different concentrations, ranging from 5 to 200 nmol/mL of individual free amino acid were determined, with each concentration measured in triplicate. The linearity was evaluated by the calibration curves for each standard and least-squares regression lines relating the absorbance peak area at 254 nm. The detection limit (LOD) and quantitation limit (LOQ) were calculated based on the signal to noise ratio of 3 and 10 at 254 nm, respectively. Free amino acids standard mixture solution with 0.5, 1 or 1.5 multiple free amino acids content in dry tea powder was spiked into tea sample to evaluate the recovery of each free amino acid. The mean values of three replicates were reported together with the relative standard deviations (RSDs). Intraday variation of the analytical response was assessed on aliquots of each infusion six times on the same day. Inter-day variation of the analytical response was evaluated on freshly thawed aliquots of each infusion in triplicate on 3 consecutive days.

Statistical and multivariate analysis

Free amino acids were measured in triplicate and results were made clear with the mean SD of three values. Statistical analysis was performed using Student's *t* test with $P < 0.05$ indicating a significant difference between data sets. Cluster analysis (CA) obtained after calculations using the software SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Moreover, data of free amino acids in six types of tea was subjected to multivariate analysis with SIMCA-P 13 software (Umetrics AB, Umea, Sweden). Principal component analysis (PCA) and score plot were used to visualise the relationships among samples in the model plane. Then the corresponding loading plot was run for identifying variables important for the class separation.

Results and discussion

Optimization of RP-HPLC

Free amino acids in tea are important for quantitation of tea. Our initial goal was to develop and validate free amino acids method using AQC derivatization from quantitation of free amino acids in six types of tea. The method of AQC

for precolumn derivatization of free amino acids was introduced by Cohen and Michaud (1993) that could analyze 17 free amino acids using ternary eluent system (Cohen and Michaud 1993). We used this method to analyze free amino acids in six types of tea but we could not detect other free amino acids especially glutamine and theanine. It reduced the accuracy of free amino acids analysis. RP-HPLC method with AQC was developed in order to analyze free amino acid in beers, soil, grape juices, wines, fruit juices and vinegars (Hernández-Orte et al. 2003; Callejón et al. 2008; Zeng et al. 2014; Hou et al. 2009; Kabelová et al. 2008). The development and validation of RP-HPLC method with AQC derivatization was necessary for precise measurement of free amino acids in tea. Although a quaternary gradient system attempt to analysis free amino acids in tea, we have not obtained effective separation of free amino acids. Subsequently, ternary eluent system was introduced to determined free amino acids in tea. Meanwhile, triethylamine was used as a counter ion for improving the hydrophobicity and retention of the free amino acids. The mobile phase gradient, organic solvent concentration, pH of eluent A and column temperature were also optimized to obtain an effective separation of free amino acids.

Optimization of mobile phase pH and gradient

Separation of the major free amino acids depended on the mobile phase pH and gradient which affected the retention of certain free amino acid. The system of RP-HPLC including mobile phase A, B and C. Mobile phase A, AccQ-Tag eluent A adjusted to pH with sodium hydroxide or phosphoric acid. Mobile phase B and mobile phase C was cetonitrile and ultrapure water, respectively. The pH of the mobile phase A was crucial for influencing the separation of free amino acids due to the difference of iso-electric point in free amino acids. It can be seen in Fig. 1 that variations of pH from 5.0 to 5.8 caused fundamental modifications in the separation of free amino acids. Finally, an optimum pH of 5.70 was identified. A higher pH value seemed to be appropriate for the separation of serine, glutamine, glycine and citrulline (Sharma et al. 2014). As pH of mobile phase A was 5.00, the elution time of glycine was retained longer and interfered in the determination of glutamine and histidine (Fig. 1a). So this lower pH was not exact for the separation of free amino acids. The peak of glycine was separated off first while the peak pair of glutamine and histidine was overlapped when the pH of mobile phase A was increased 5.3 (Fig. 1b), 5.6 (Fig. 1c), 5.75 (Fig. 1e), and 5.8 (Fig. 1f). If the pH of mobile phase A was adjusted to 5.7, the peaks of Gln and His centered at 21.5 min and 22.5 min, respectively and glycine, glutamine and histidine were separated completely (Fig. 1d).

Fig. 1 Chromatograms for free amino acids standards with different pH of mobile phase. The mobile phase was used at pH = 5.00 (a), 5.30 (b), 5.60 (c), 5.70 (d), 5.75 (e) and 5.80 (f). Elution order as follows: 1. Asp, 2. Ser, 3. Glu, 4. Gly, 5. Gln, 6. His, 7. Arg, 8. Thr, 9.

Ala, 10. Pro, 11. Thea, 12. Tyr, 13. Cys, 14. Val, 15. Met, 16. Ile, 17. Lys, 18. Leu, and 19. Phe. * represents AMQ, ∇ represents NH₃, and ◇ represents the solvent peak

The results demonstrated that the optimum pH was 5.70 to separate 19 free amino acids, especially glutamine and histidine since Gln is a polar amino acid (pI 5.65) and His is an alkaline amino acid (pI 7.59). The ionization, charge,

hydrophobicity and retention behavior of AQC-amino acids were highly related to the mobile phase pH (Cohen and Michaud 1993; Hong 1994). Glutamine and histidine produced negative ions and positive ions, respectively,

when the mobile phase pH was 5.7 that resulted in the changes of hydrophobicity and retention time. The separation of glutamine and histidine was significant when the pH of the mobile phase was increased from 5.0 to 5.7. Songmei has reported that 17 amino acid AQC-derivatives was separated in soil through the adjustment of mobile phase pH (Hou et al. 2009). Satisfactory separation of amino acids in wine was achieved with the increase of mobile phase pH (Hernández-Orte et al. 2003). The mobile phase pH is a critical factor for separation of free amino acid.

More precise gradients and organic solvent concentrations were adjusted to improve separation efficiency of free amino acids. The optimized mobile phase gradient and retention times can be seen in supplementary Table 1. The separation of 19 free amino acids was achieved through the refined analysis of retention times, peak patterns and online fluorescent spectra (Fig. 1d).

Optimization of column temperature

The column temperature was one of critical influences on the separation of free amino acids (Liu et al. 1998). Co-eluting Lys-Leu was not separated at the common temperature of 37 °C (Supplementary Fig. 1b). The retention time of Lys-Leu did not vary significantly at different temperatures but the resolution of Lys-Leu was better at 40 °C, therefore 40 °C was selected as the working temperature. Subsequently, the satisfactory separation of the 19 free amino acids including glutamine, histidine, lysine and leucine was obtained at a column temperature of 40 °C (Supplementary Fig. 1a). Similarly, a high column temperature of 40 °C was obtained to determination amino acids in fruit juice (Zeng et al. 2014). Lower column temperature was suitable for four mobile phases with lower pH in the study of grape juices and wines (Hernández-Orte et al. 2003). Therefore, the effective separation of free amino acids requires trading off factors such as mobile phase pH, gradient and column temperature. In summary, satisfactory identification and quantitation of 19 free amino acids including glutamine and histidine were obtained with the optimized mobile phase gradient, pH 5.7 of eluent A and column temperature of 40 °C in this research.

Validation of the novel method by RP-HPLC

Linearity and range

The calibration curve was constructed within the linear concentration range of 5–200 nmol/mL which based on the regression equation ($y = ax + b$) and the least squares correlation coefficient (R), with x and y being the concentration in nmol/mL and peak area, respectively. Each

concentration was conducted in three independent replicates. The linear concentration ranges were sufficient to determine the free amino acids in tea. Linearity was validated using the correlation coefficient of the regression line that response to the concentration of free amino acid. The correlation coefficients were in the range of 0.9989–1.0000 that showed excellent linearity (Table 1).

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ in the method were determined based on the amount of free amino acids that gave a response three times and ten times the background noise of the chromatogram basal line, respectively. LODs were defined as the lowest concentration of free amino acids in sample which can be detected, but not quantified. LOQs were determined as the lowest concentration of free amino acids in sample which can be quantified with acceptable precision and accuracy under the normal operating conditions of the method. The LOD appears within a range of 0.097–0.228 nmol/mL. Ile was detected at the highest detection, while Tyr was detected at the lowest detection (Table 1). Quantitation limits of free amino acids were in the range of 0.323–0.761 nmol/mL (Table 1). The LOD and LOQ obtained in this work were consistent with the previous results on HPLC (Hernández-Orte et al. 2003; Hou et al. 2009).

Precision and accuracy

The precision of the method were calculated by examining the relative standard deviation for the intraday and interday. The intraday was defined as six consecutive repetition of standard mixture solution of 19 derivatized free amino acids in 1 day (intraday, $n = 6$). The relative standard deviation for the intraday analysis was ranged in 0.099–1.909% (Table 2). The intraday was defined as six times repetition of standard mixture solution of 19 derivatized free amino acids in three different days (interday, $n = 6$). The relative standard deviation for the interday analysis was ranged in 3.231–7.025% (Table 2). The results indicated that the precision was reasonable. Standard solutions of tea for examining the recovery were prepared for the accuracy study. The standard solutions were prepared in triplicate. The recoveries of free amino acids were within the range of 81.06–112.78% followed a $RSD < 7.85\%$. The recoveries were considered acceptable data to the method. Therefore, the novel RP-HPLC method possess the characteristic of effective repeatability, reproducibility, and recovery and supplied sufficient evidence for analysis of free amino acids in tea.

Table 1 Calibration linearity, precision, the detection limit and the quantitation limit of the novel method

Peak	Analyte	Regression equation	R ²	LOD (nmol/mL)	LOQ (nmol/mL)	Precision (RSD%, n = 6)	
						Intraday	Interday
1	D-Asp	Y = 864.7x – 1025.6	1.000	0.218	0.726	1.909	6.472
2	S-Ser	Y = 990.33x – 1273.1	0.9996	0.129	0.430	1.223	5.187
3	E-Glu	Y = 1567.7x – 1745.4	0.9995	0.196	0.653	0.887	4.581
4	G-Gly	Y = 2043.9x + 2738.5	0.9994	0.227	0.759	0.736	5.510
5	Q-Gln	Y = 1518.8x – 1691.2	0.9999	0.156	0.521	0.619	4.814
6	H-His	Y = 2975.4x + 1220.6	0.9997	0.164	0.548	0.600	4.237
7	R-Arg	Y = 2134.6x – 3372.8	0.9993	0.221	0.737	0.690	3.231
8	T-Thr	Y = 1766.1x + 1873.2	0.9990	0.117	0.389	0.720	4.443
9	A-Ala	Y = 1369.5x – 2310.7	0.9991	0.162	0.539	0.817	5.845
10	P-Pro	Y = 867.39x + 774.33	0.9995	0.181	0.603	0.290	4.740
11	Thea	Y = 3470.95x + 15433.09	0.9989	0.214	0.712	0.196	4.617
12	Y-Tyr	Y = 2580.3x + 6009.9	0.9995	0.097	0.323	0.147	4.753
13	C-Cys	Y = 323.3x + 1082.8	0.9991	0.212	0.707	0.099	5.721
14	V-Val	Y = 3184.9x + 6681.4	0.9994	0.216	0.719	0.153	4.680
15	M-Met	Y = 2882.1x + 5247.7	0.9991	0.202	0.674	0.149	5.211
16	I-Ile	Y = 3759.9x + 5119.2	0.9992	0.228	0.761	0.114	4.558
17	K-Lys	Y = 1607.7x + 1120.9	0.9990	0.108	0.362	0.082	7.025
18	L-Leu	Y = 3994x + 1134.9	0.9990	0.140	0.467	0.111	4.649
19	F-Phe	Y = 6751.6x + 4499.1	0.9996	0.113	0.375	0.099	4.282

Table 2 Precision and accuracy of the novel method

Peak	Analyte	Green tea		Black tea		Yellow tea	
		Precision (RSD%)	Recovery (%)	Precision (RSD%)	Recovery (%)	Precision (RSD%)	Recovery (%)
1	D-Asp	2.94	98.87	3.02	96.86	0.79	103.72
2	S-Ser	3.81	90.19	2.04	87.93	0.92	102.27
3	E-Glu	2.79	109.74	2.05	97.67	1.85	103.40
4	G-Gly	1.87	105.58	0.24	109.33	1.35	91.12
5	Q-Gln	2.34	95.06	0.51	94.91	2.37	92.95
6	H-His	3.15	87.35	0.37	96.06	2.53	95.07
7	R-Arg	7.85	109.48	5.31	108.70	7.40	110.93
8	T-Thr	6.19	100.95	0.09	108.29	3.34	103.62
9	A-Ala	0.95	107.14	3.65	97.69	4.08	105.86
10	P-Pro	3.62	94.03	1.88	103.64	1.32	99.78
11	Thea	2.83	107.11	2.25	107.37	2.72	101.44
12	Y-Tyr	3.40	97.40	4.11	101.24	4.15	84.36
13	C-Cys	1.39	91.05	0.77	112.78	0.75	100.65
14	V-Val	2.68	108.48	3.87	109.30	3.67	109.28
15	M-Met	6.44	81.06	0.048	110.25	6.68	90.72
16	I-Ile	3.98	103.37	3.70	106.70	4.77	108.69
17	K-Lys	4.36	108.49	4.01	92.16	1.03	105.12
18	L-Leu	2.09	102.01	6.36	97.62	4.27	108.69
19	F-Phe	5.51	86.10	4.38	103.77	3.54	85.75

RSD suggested the relative standard deviations

Table 3 Free amino acids contents in six types of tea (nmol/mL)

AA	Green tea			Black tea			White tea		
	GT1	GT2	GT3	BT1	BT2	BT3	WT1	WT2	WT3
D-Asp	140.5 ± 11.9 ^c	144.4 ± 6.6 ^{bc}	159.9 ± 12.2 ^{ab}	44.0 ± 3.8 ^g	62.7 ± 0.9 ^{ef}	68.5 ± 4.8 ^e	108.9 ± 7.9 ^d	64.4 ± 2.9 ^{ef}	19.6 ± 0.6 ^{ij}
S-Ser	149.4 ± 13.7 ^c	132.5 ± 5.5 ^c	212.5 ± 22.5 ^a	61.8 ± 5.6 ^e	26.4 ± 1.6 ^f	61.0 ± 3.2 ^e	59.9 ± 5.7 ^e	62.7 ± 3.6 ^e	19.7 ± 1.4 ^{fg}
E-Glu	61.7 ± 5.2 ^{ef}	89.9 ± 4.1 ^d	123.5 ± 10.4 ^c	137.2 ± 12.1 ^c	137.1 ± 9.3 ^c	184.2 ± 17.1 ^b	260.2 ± 13.8 ^a	282.8 ± 15.6 ^a	42.6 ± 2.1 ^{fg}
G-Gly	3.4 ± 0.3 ^{ef}	6.3 ± 0.5 ^{cd}	6.4 ± 0.2 ^{cd}	6.1 ± 0.3 ^{cd}	6.9 ± 0.5 ^c	7.8 ± 0.7 ^c	23.6 ± 1.9 ^a	14.4 ± 1.3 ^b	2.8 ± 0.1 ^{fg}
Q-Gln	24.0 ± 0.2 ^f	167.6 ± 5.8 ^a	147.1 ± 12.6 ^b	98.5 ± 5.1 ^d	11.8 ± 1.1 ^{fgh}	44.2 ± 1.3 ^e	42.7 ± 1.3 ^e	92.5 ± 7.4 ^d	3.8 ± 0.1 ^h
H-His	2.1 ± 0.2 ^{gh}	7.3 ± 0.1 ^e	12.0 ± 0.6 ^c	3.4 ± 0.1 ^g	3.0 ± 0.1 ^{gh}	5.0 ± 0.3 ^f	20.8 ± 0.3 ^a	11.9 ± 0.7 ^c	Trace
R-Arg	72.5 ± 5.5 ^c	85.5 ± 1.2 ^b	104.1 ± 4.2 ^a	58.1 ± 2.9 ^d	35.4 ± 2.3 ^e	72.4 ± 4.4 ^c	113.5 ± 8 ^a	90.5 ± 7.2 ^b	11.1 ± .3 ^f
T-Thr	17.8 ± 1.9 ^f	29.2 ± 1.0 ^{bc}	29.4 ± 1.6 ^{bc}	24.2 ± 2.6 ^d	19.0 ± 0.4 ^{ef}	25.9 ± 1.8 ^{cd}	38.9 ± 1.4 ^a	29.0 ± 1.0 ^{bc}	Trace
A-Ala	22.9 ± 2.5 ^{ef}	22.8 ± 0.8 ^{ef}	31.8 ± 0.7 ^d	48.4 ± 2.9 ^{bc}	29.6 ± 2.0 ^{de}	47.5 ± 3.4 ^c	117.5 ± 1.6 ^a	116.2 ± 4.3 ^a	19.6 ± 1.5 ^{fg}
P-Pro	8.1 ± 0.2 ^f	17.8 ± 1.2 ^e	18.3 ± 0.9 ^e	29.7 ± 2.5 ^d	40.0 ± 3.9 ^c	34.6 ± 3.0 ^{cd}	75.2 ± 2.7 ^a	56.0 ± 5.0 ^b	8.4 ± 0.3 ^f
Thea	414.5 ± 27.7 ^e	673.9 ± 35.5 ^{ab}	685.0 ± 69.3 ^{ab}	459.1 ± 42.9 ^{de}	119.4 ± 12.3 ^{fg}	366.2 ± 25.0 ^e	620.8 ± 24.8 ^{bc}	717.8 ± 68.6 ^a	98.0 ± 3.7 ^{fgh}
Y-Tyr	7.4 ± 0.8 ^{fg}	14.1 ± 0.4 ^d	10.2 ± 0.9 ^e	22.2 ± 1.7 ^c	22.7 ± 1.4 ^c	20.5 ± 1.2 ^c	25.9 ± 0.1 ^b	36.1 ± 1.6 ^a	5.3 ± 0.5 ^{gh}
C-Cys	–	–	–	–	–	–	–	–	–
V-Val	9.0 ± 0.5 ^{ghi}	20.9 ± 0.9 ^e	17.2 ± 1.2 ^{ef}	33.4 ± 2.4 ^{cd}	34.1 ± 4.6 ^{cd}	37.2 ± 2.1 ^c	79.4 ± 5.4 ^a	54.7 ± 3.6 ^b	10.6 ± 0.4 ^{gh}
M-Met	–	–	–	–	–	–	–	–	–
I-Ile	5.7 ± 0.4 ^{ghi}	13.9 ± 0.6 ^c	10.6 ± 0.3 ^{ef}	23.1 ± 2.4 ^{cd}	21.5 ± 0.8 ^{cd}	24.7 ± 1.6 ^c	60.0 ± 3.8 ^a	39.0 ± 3.8 ^b	9.1 ± 0.3 ^{fg}
K-Lys	Trace	Trace	Trace	16.7 ± 1.1 ^{de}	20.5 ± 1.4 ^{cd}	21.8 ± 1.7 ^{cd}	70.6 ± 6.3 ^a	40.9 ± 2.4 ^b	5.4 ± 0.1 ^f
L-Leu	14.5 ± 1.3 ^e	31.0 ± 1.1 ^{cd}	28.5 ± 3.5 ^{cd}	28.0 ± 2.5 ^d	25.9 ± 1.5 ^d	33.9 ± 1.2 ^c	63.3 ± 4.3 ^a	43.2 ± 4.1 ^b	6.3 ± 0.2 ^f
F-Phe	10.4 ± 0.6 ^g	23.6 ± 0.3 ^{de}	20.3 ± 1.1 ^e	29.6 ± 1.9 ^c	26.9 ± 1.0 ^{cd}	24.0 ± 2.1 ^d	44.3 ± 1.5 ^b	63.2 ± 2.7 ^a	6.7 ± 0.2 ^h
AA	Oolong tea			Yellow tea			Dark tea		
	OT1	OT2	OT3	YT1	YT2	YT3	DT1	DT2	DT3
D-Asp	36.1 ± 2.0 ^{ghi}	37.7 ± 2.8 ^{gh}	21.4 ± 1.0 ^{hij}	48.6 ± 3.5 ^{fg}	163.1 ± 4.8 ^a	172.6 ± 2.0 ^a	23.1 ± 1.8 ^{hij}	9.6 ± 1.4 ^{jk}	–
S-Ser	87.0 ± 0.9 ^d	53.9 ± 2.1 ^e	6.7 ± 0.3 ^{fg}	12.7 ± 1.3 ^{fg}	182.3 ± 11.1 ^b	216.8 ± 1.6 ^a	21.3 ± 2.5 ^{fg}	7.4 ± 1.1 ^{fg}	–
E-Glu	39.3 ± 1.5 ^{fgh}	34.0 ± 0.7 ^{ghi}	14.6 ± 1.1 ^{hij}	20.3 ± 1.5 ^{ghij}	141.6 ± 14.4 ^c	72.2 ± 1.2 ^{de}	12.0 ± 1.6 ^{ij}	2.9 ± 0.1 ^j	–
G-Gly	1.6 ± 0.1 ^{fg}	Trace	1.9 ± 0.1 ^{fgh}	2.5 ± 0.1 ^{fgh}	7.3 ± 0.3 ^c	5.0 ± 0.3 ^{de}	Trace	Trace	–
Q-Gln	18.5 ± 0.9 ^{fg}	5.8 ± 0.2 ^{gh}	3.4 ± 0.2 ^h	6.6 ± 0.3 ^{gh}	98.9 ± 3.8 ^{cd}	112.9 ± 9.1 ^c	2.0 ± 0.1 ^h	–	–
H-His	2.6 ± 0.1 ^{gh}	1.9 ± 0.1 ^{hi}	Trace	Trace	13.6 ± 1.3 ^b	10.4 ± 0.7 ^d	Trace	–	–
R-Arg	5.4 ± 0.1 ^f	2.2 ± 0.1 ^f	4.4 ± 0.2 ^f	11.8 ± 0.6 ^f	107.9 ± 10.1 ^a	59.0 ± 2.9 ^d	6.1 ± 0.3 ^f	–	–
T-Thr	13.5 ± 0.6 ^g	7.7 ± 0.2 ^h	Trace	1.6 ± 0.1 ⁱ	22.6 ± 0.5 ^{de}	30.9 ± 2.3 ^b	Trace	–	–
A-Ala	55.5 ± 1.9 ^b	22.0 ± 1.9 ^{ef}	9.9 ± 0.6 ^h	12.7 ± 0.2 ^{gh}	29.3 ± 0.7 ^{de}	36.1 ± 1.2 ^d	10.4 ± 0.7 ^h	Trace	–
P-Pro	5.8 ± 0.5 ^{fg}	5.0 ± 0.4 ^{fg}	5.2 ± 0.3 ^{fg}	3.9 ± 0.2 ^{fg}	19.4 ± 1.4 ^e	9.5 ± 0.8 ^f	3.6 ± 0.1 ^g	Trace	–
Thea	176.8 ± 4.4 ^f	178.9 ± 13.2 ^f	15.5 ± 1.4 ^{hi}	49.4 ± 3.3 ^{ghi}	548.5 ± 37.4 ^{cd}	624.6 ± 27.6 ^{abc}	37.2 ± 2.6 ^{ghi}	14.4 ± 0.3 ^{hi}	–
Y-Tyr	5.2 ± 0.4 ^{gh}	2.8 ± 0.1 ^{hi}	4.3 ± 0.3 ^{hi}	3.1 ± 0.2 ^{hi}	7.6 ± 0.5 ^{fg}	9.2 ± 0.2 ^{ef}	1.8 ± 0.2 ⁱ	2.2 ± 0.3 ⁱ	–

Table 3 continued

AA	Oolong tea			Yellow tea			Dark tea	
	OT1	OT2	OT3	YT1	YT2	YT3	DT1	DT2
C-Cys	–	–	–	–	–	–	–	–
V-Val	9.1 ± 0.4 ^{ghi}	7.2 ± 0.2 ^{bij}	3.9 ± 0.3 ^{ijk}	3.3 ± 0.1 ^{jk}	31.2 ± 0.8 ^d	13.9 ± 0.7 ^{fg}	1.1 ± 0.1 ^k	–
M-Met	–	–	–	–	–	–	–	–
I-Ile	3.8 ± 0.3 ^{ij}	4.2 ± 0.1 ^{bij}	2.0 ± 0.1 ^{ij}	1.6 ± 0.1 ^{ij}	19.1 ± 0.4 ^d	8.5 ± 0.5 ^{gh}	0.8 ± 0.3 ^j	Trace
K-Lys	7.0 ± 0.1 ^f	5.4 ± 0.1 ^f	2.2 ± 0.1 ^f	1.3 ± 0.1 ^f	23.2 ± 0.5 ^c	11.9 ± 0.8 ^e	–	Trace
L-Leu	4.8 ± 0.4 ^{fg}	4.3 ± 0.3 ^{fg}	2.6 ± 0.2 ^{fg}	2.9 ± 0.1 ^{fg}	30.1 ± 1.7 ^{cd}	18.9 ± 0.7 ^e	1.4 ± 0.3 ^{fg}	Trace
F-Phe	11.2 ± 0.8 ^g	5.2 ± 0.1 ^{hi}	3.6 ± 0.2 ^{hij}	2.8 ± 0.2 ^{ijk}	14.8 ± 0.7 ^f	10.2 ± 0.5 ^g	Trace	Trace

Data are average ± SD (n = 3) on a dry weight basis

Values in the same row with differing superscripts are significantly different ($p < 0.05$)*Analysis of free amino acids in six types of tea*

The novel method was applied to quantify the free amino acid in green, black, white, oolong, yellow and dark teas. Quantification of free amino acids was prepared in triplicate and performed based on the mean value of the peak area. As shown in Table 3, significant differences ($P < 0.05$) existed among six types of tea. Green and white teas contained the highest contents of amino acids (on average 1444.7 and 1343.7 nmol/mL, respectively), followed by black and yellow teas (on average 1139.4 and 1000.9 nmol/mL, respectively). Oolong and dark teas, with contents of 361.3 and 65.2 nmol/mL, were the lowest (Table 3). The content of free amino acids in green, white, black and yellow tea samples much higher compared to the content in oolong and dark tea sample may be on account of the natural variation of tea cultivation and manufacturing, especially the fermentation degree in processing has a distinct influence on the amounts of free amino acids (Alasalvar et al. 2012; Kocadağlı et al. 2013; Horanni and Engelhardt 2013; Gokmen et al. 2012). The most abundant free amino acids was theanine in green tea, black tea, white tea, oolong tea, yellow tea and dark tea, especially maofeng, xihulongjing, qihong, tanyanggongfu, baihaoyinzhen, baimudan, huangyatea and junshanyinzhen, which had a concentration over 300 nmol/mL (Table 3). Approximately same content of theanine in green and black tea was assessed as in previous investigations, while it was higher than reported by Kocadağlı in 2013 and Wang in 2010 (Horanni and Engelhardt 2013; Jin Yan et al. 2014; Kocadağlı et al. 2013; Wang et al. 2010). The varied content of theanine in different type tea samples was due to the grade of fresh leaves, plucking time of fresh leaves, growing environment of tea plant, processing technique, and tea grade. The content of theanine in white tea and green tea was higher than other four types of tea (Table 3). Similarly, umami taste of white tea and green tea was associated with abundant of theanine (Mu et al. 2015). The glutamine that was separated from histidine was also detected in six types of tea, and the amounts ranged from 2.0 to 167.6 nmol/mL, which were higher than the contents of histidine ranged from 1.9 to 20.8 nmol/mL. Glutamic acid and alanine were detected in most teas at concentrations in the ranges of 2.9–282.8 nmol/mL and 9.85–117.5 nmol/mL, respectively (Table 3), which were consisted with the content in green, black, white, oolong tea that reported by Rouba and his co-workers in 2013. In the theanine biosynthetic pathway, glutamine is the precursor of theanine, and glutamic acid. Due to the absence of a valid method for free amino acids especially glutamine analysis, the research on theanine biosynthetic pathway in tea has been limited. Fortunately, with the novel RP-HPLC method, the accurate quantitation of glutamine, theanine,

Fig. 2 Cluster analysis using the Wards method and Euclidean distance for studied samples of six types of tea

alanine and glutamic acid in six types of tea provided adequate technology support and theoretical foundation in studying theanine biosynthesis (Liu et al. 2017; Deng et al. 2012). The concentrations of aspartic acid, serine and arginine were abundant in most samples, having ranges of 9.6–172.6 nmol/mL, 7.4–216.8 nmol/mL and 2.2–113.5 nmol/mL, respectively (Table 3). Moreover, the contents of glycine, threonine, proline, tyrosine, valine, isoleucine, leucine and phenylalanine in green, black, white, yellow and oolong teas were within the ranges of 1.6–23.6 nmol/mL, 1.6–38.9 nmol/mL, 3.6–75.2 nmol/mL, 1.8–36.1 nmol/mL, 1.1–79.4 nmol/mL, 0.8–60.0 nmol/mL, 1.4–63.3 nmol/mL, 2.8–63.2 nmol/mL, respectively (Table 3). The significant variation of content of free amino acids was found in six types of tea for different tea material and manufacture. Phenylalanine is associated with the intensity of bitter and astringent taste of the tea (Scharbert and Hofmann 2005; Lee et al. 2013). The content of phenylalanine in green tea, black tea and white tea was higher than yellow tea and dark tea. It showed that quantity of phenylalanine could lay a foundation to assess bitter and astringent taste of the tea. Lysine was found in oolong, black, white and yellow teas within the range of

1.3–70.6 nmol/mL, but its concentration was too low to be quantified in green tea (Table 3). Meanwhile, cysteine and methionine were not detected for the low content in six types of tea, which agreed with the report that there was not cysteine and methionine in green tea and seven grades of black tea (Gokmen et al. 2012; Jin Yan et al. 2014). In addition, dark tea contained fewer free amino acids, which might be attributable to the coarse leaves and stems, the microbial fermentation processing technique and the storage time.

Cluster analysis was performed on free amino acids obtained from investigated samples to understand the subtle changes in the occurrence and abundance of specific compounds. Therefore, the dendrogram obtained after calculations using the Wards method and Euclidean distance is shown in Fig. 2. There are five clear clusters groups including dark tea (I), oolong tea (II), white tea (III), black tea (IV) and green tea (V), respectively. However, OT3 and YT1 were classified as I cluster, and WT3 was divided into II cluster (Fig. 2). Principal component analysis showed that black tea, green tea, oolong tea and dark tea fell within the lower right quadrant plot, upper of the plot, the lower left quadrant plot, respectively, whereas,

Fig. 3 PCA score plot and loading plot of free amino acids in study samples of six types of tea

WT3 and YT1 remaining dispersal (Fig. 3a). As shown in Fig. 3b, a broad range of metabolites was established, suggesting that Gln, Thea, Ser and Leu were the most important contributors to influence class separation. So the novel RP-HPLC method not only beneficial for the analysis of free amino acid in six types of tea and the hygienical function of tea, but also apply theoretical basis for cluster of tea.

Conclusion

A novel liquid chromatographic method was established for quantification of 19 free amino acids in tea, also offering the possibility of simultaneous quantification with glutamine and histidine. The method demonstrated good precision, reproducibility and repeatability based on the optimization of mobile phase gradient, pH and column temperature and assessment of linearity, limit of detection, limit of quantitation, replication and recovery for all free amino acids. In addition, the method permitted that glutamine, theanine, serine, and leucine were key determinants for free amino acids of six types of tea. We provide a potential valuable tool to analyze free amino acids of tea.

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