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Original Article

Simultaneous determination of amino acids in different teas using supercritical fluid chromatography coupled with single quadrupole mass spectrometry

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ABSTRACT

Tea is a widely consumed beverage and has many important physiological properties and potential health benefits. In this study, a novel method based on supercritical fluid chromatography coupled with mass spectrometry (SFC-MS) was developed to simultaneously determine 11 amino acids in different types of tea (green teas, Oolong tea, black tea and Pu-erh tea). The separation conditions for the analysis of the selected amino acids including the column type, temperature and backpressure as well as the type of additive, were carefully optimized. The best separation of the 11 amino acids was obtained by adding water (5%, v/v) and trifluoroacetic acid (0.4%, v/v) to the organic modifier (methanol). Finally, the developed SFC-MS method was fully validated and successfully applied to the determination of these amino acids in six different tea samples. Good linearity ($r \geq 0.993$), precision (RSDs $\leq 2.99\%$), accuracy (91.95%–107.09%) as well as good sample stability were observed. The limits of detection ranged from 1.42 to 14.69 ng/mL, while the limits of quantification were between 4.53 and 47.0 ng/mL. The results indicate that the contents of the 11 amino acids in the six different tea samples are greatly influenced by the degree of fermentation. The proposed SFC-MS method shows a great potential for further investigation of tea varieties.

1. Introduction

Tea (*Camellia sinensis* L.), containing many compounds beneficial for health such as polyphenols, amino acids, vitamins, carbohydrates, and alkaloids, has been used as popular beverage and natural medicine for over thousand years [1]. Generally, teas are commercially classified into several major categories according to their degree of fermentation: non-fermented (green tea), semi-fermented (Oolong tea), fully fermented (black tea) and post-fermented (Pu-erh tea) [2]. During the fermentation process, enzymatic oxidation could take place, leading to formation of some constituents, which are responsible for the taste and color of tea. Various studies have demonstrated that amino acids play an important role in the delicate taste and characteristic flavor of tea [3–5]. For instance, theanine has a major effect on the taste of green tea due to its high content (about 50% of the total amino acid content) [6]. In addition, amino acids are indispensable elements for human body and possess many physiological activities such as disease prevention, aid to relaxation, blood pressure lowing, anti-tumor activity enhancement, etc. [1]. Various methods have been developed to determine amino acids in teas, such as gas chromatography (GC) [7], liquid chromatography (LC) [8], capillary electrophoresis (CE) [9] and near infrared spectroscopy (NIR) [10].

Supercritical fluid chromatography (SFC) utilizes supercritical carbon dioxide (sCO_2) as a major mobile phase component, providing some interesting features such as fast analysis, low consumption of organic solvents and environmentally friendless [11]. It has been widely used for the analysis of a broad range of analytes such as lipids, flavonoids, phenolics, alkaloids, saponins,

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and carbohydrates [12]. SFC applications to amino acid analysis have also been reported, but these studies were mostly focused on chiral separations [13–16]. So far, only a few reports have been devoted to the achiral analysis of amino acids by SFC [17,18]. Several amino acids were investigated in recent studies regarding them as a representative set of metabolites, for instance, lysine [17], phenylalanine, threonine, tryptophan, methionine, and glutamic acid [18]. The results demonstrated the potential of SFC for the achiral analysis of amino acids. Because of the lack of a suitable chromophore in most amino acids, MS was often used for their detection instead of pre- or post-column derivatization which was indispensable for UV detection [6,17–20]. To the best of our knowledge, the application of SFC-MS to the analysis of amino acids in teas has not yet been reported. In this study, an SFC-MS method was developed for the analysis of 11 amino acids in teas with different degrees of fermentation. Various experimental parameters including the types of stationary phase and additive, pressure and temperature were optimized in order to obtain a satisfactory SFC separation. Moreover, the developed method was used for the quantification of 11 amino acids in green, Oolong, black and Pu-erh teas.

2. Experimental

2.1. Chemicals and materials

Food grade liquid carbon dioxide (99.5% purity) for SFC separations was supplied by Yinglai Gas Company (Guangzhou, China). Leucine (Leu), valine (Val), γ-aminobutyric acid (GABA), phenylalanine (Phe), glycine (Gly), L-theanine (The), threonine (Thr), serine (Ser), glutamine (Gln), asparagine (Asn), histidine (His) were all purchased from Aladdin Chemicals (Shanghai, China). Ammonium hydroxide, methanol (MeOH), formic acid, trifluoroacetic acid (TFA) and ammonium acetate, all of HPLC grade, were obtained from Merck (Shanghai, China). The distilled water was filtered using 0.22 μm membrane before use. All tea samples (Pu-erh tea, Oolong tea, black tea, green tea-Maojian, green tea-Biluochun, and green tea-Longjing) were purchased in a local supermarket (Guangzhou, China).

2.2. Instrumentation

All SFC-MS experiments were performed on a 1260 Infinity Hybrid SFC/UHPLC analytical system coupled with a 6130 single quadruple MS detector (Agilent Technologies, Santa Clara, CA, USA). The 1260 Infinity Hybrid SFC/UHPLC analytical system consisted of an Infinity SFC binary pump, an Aurora A5 Fusion Module, a degasser, an autosampler with 5 μL loop, a column oven, a make-up flow pump and a backpressure regulator. The operating software was Agilent OpenLab ChemStation Edition C.01.05. All chromatograms were converted into txt. files and then redrawn using Microcal Origin 8.5. Ultrasonication extraction was performed using a KQ-250B ultrasonic water bath (Kunshan Ultrasonic Instrument Co., Ltd, Jiangsu, China).

Ten different columns were tested during method development, namely, ZORBAX RX-SIL, ZORBAX C_{18} (Agilent Technologies, Santa Clara, USA), Unitary Diol, Unitary NH_2, Unitary XAmide (Acchrom Technologies, Beijing, China), Venusil NP, Venusil PFP, Venusil Imidazolyl, Venusil HILIC (Bonna-Agela, Tianjin, China) and Cosmosil 5X-SIL (Nacalai Tesque Inc, Kyoto, Japan). Except for ZORBAX RX-SIL, ZORBAX C_{18} and Unitary XAmide (150 mm × 4.6 mm, 5 μm), the dimensions of all columns were 250 mm × 4.6 mm with 5 μm particle size.

2.3. Sample preparation

Stock solutions of the 11 amino acids were prepared at 1.0 mg/mL concentration in MeOH/H_2O (7/3, v/v) and stored at 4 °C. All the tea leaves were cut into small pieces, and five grams of each tea sample were accurately weighted. After ultrasonic extraction with 50 mL water for 30 min, the samples were centrifuged (3500 rpm, 10 min). The supernatants were then transferred into a 50 mL volumetric flask, diluted with water to volume, and mixed thoroughly. All sample solutions were filtered through 0.22 μm membrane (Bonna-Agela Technologies, Tianjin, China) before use.

2.4. Chromatographic and MS conditions

The SFC separation of the 11 amino acids in teas was performed on Unitary XAmide column using gradient elution mode with sCO_2 and the organic modifier as mobile phase components A and B, respectively (0–15 min/17% B, 20–30 min/23% B, and 40 min/50% B). The mobile phase component B consisted of MeOH containing 5 mM ammonium acetate, 0.4% (v/v) TFA and 5% (v/v) water. The column temperature was 25 °C and the backpressure was 100 bar. The injected sample volume and flow rate were set at 5 μL and 3 mL/min, respectively. A make-up solvent made of MeOH containing 5 mM ammonium acetate was delivered at a flow rate of 0.3 mL/min. MS analysis was performed using electrospray ionization (ESI) in positive mode. The flow rate of nebulizer gas (N_2) was 11 L/min, the temperature 300 °C and the pressure 35 psi. Air was used as curtain gas. The capillary voltage was set at 4.0 kV and the scan range was 50–500 Da. The analyses were performed in selected ion monitoring mode using the precursor ions ($[M + H]^+$) (Table S1).

2.5. Method validation

The developed SFC-MS method was validated according to ICH guidelines [21]. Linearity was determined using external standard calibration curves with triplicate injections of working solutions containing the 11 amino acids at seven different concentration levels, which were prepared by serial dilution in the ratio of 1:1 with MeOH/H_2O (7/3, v/v). The limits of detection (LODs) and quantitation (LOQs) were defined as the analyte concentrations that produced a signal-to-noise ratio of 3 and 10, respectively. The intra-day and inter-day precision values were evaluated and expressed as relative standard deviations (RSDs) by determining six sample solutions of green tea (Longjing) within the same day and three consecutive days. Accuracy was tested by spiking green tea (Longjing) with standard solutions of the 11 amino acids at known initial concentrations and by comparing the peak areas of spiked samples with those of unspiked tea and standard amino acid solutions treated in the same way (cf. section 2.3). Besides, the stability of the sample solutions (green tea-Longjing) treated as in Section 2.3 was investigated within 24 h at room temperature.

3. Results and discussion

3.1. Optimization of the SFC-MS method

The selection of an appropriate stationary phase is a critical step for developing a proper SFC method. The gradient elution programs listed in Table 1 were initially used for evaluating the separation performance for all ten stationary phases. The mixture of the 11 amino acid standards was used as test sample. C_{18} columns were often employed for the analysis of amino acids in reversed phase HPLC with satisfactory separation [4,6]. In this study, a ZORBAX C_{18} column was also tested, but as expected it did not show any

Table 1
Elution gradient selected for the different stationary phases.

Stationary phases	Nature of stationary phases	Elution gradient
Unitary XAmide	Amide-bonded silica	0–15 min/17% B, 20–30 min/23% B, 40 min/50% B.
Cosmosil 5X-SIL	Bare silica	0–15 min/12% B, 20 min/20% B, 30 min/40% B, 35–45 min/50% B.
ZORBAX RX-SIL	Bare silica	0 min/20% B, 20 min/40% B, 35–45 min/50% B.
Venusil HILIC	Amide-bonded silica	0 min/15% B, 35 min/30% B, 45 min/45% B.
Unitary Diol	Propanediol-bonded silica	0 min/15% B, 35 min/30% B, 45 min/45% B.
Venusil NP	Nitrobenzamide-bonded silica	0 min/15% B, 35 min/25% B, 45 min/45% B.
Venusil PFP	Pentafluorophenyl- bonded silica	0 min/5% B, 10 min/35% B, 30 min/45% B, 50 min/50% B.
ZORBAX C_{18}	Octadecylsiloxane-bonded silica	0 min/15% B, 15 min/30% B, 30–40 min/50% B.
Unitary NH_2	Amino-bonded silica	0 min/20% B, 40 min/30% B, 50 min/45% B.
Venusil Imidazolyl	Imidazole-bonded silica	0 min/5% B, 10 min/15% B, 20 min/25% B, 40 min/50% B.

Conditions: mobile phase: (A) sCO_2; (B) MeOH containing 5 mM ammonium acetate, 0.4% (v/v) TFA and 5% (v/v) water; temperature: 25 °C; backpressure: 140 bar; flow rate: 3 mL/min; make-up solvent: MeOH (containing 5 mM ammonium acetate); flow rate of make-up solvent: 0.3 mL/min; injection volume: 5 μL.

retention for these highly polar amino acids (Fig. S1). Further investigations were carried out on several polar stationary phases. Although the retention for all the analytes significantly increased on Venusil PFP, Venusil NP and Venusil Imidazolyl columns, serious peak tailing and co-elution of several peaks were observed. The other six hydrophilic stationary phases, including ZORBAX RX-SIL, Cosmosil 5X-SIL, Unitary Diol, Venusil HILIC, Unitary XAmide and Unitary NH_2, exhibited better separation performance for the selected amino acids in terms of retention, peak shape and resolution. Among them, almost all the 11 amino acids could be baseline separated on the Unitary XAmide column (corresponding chromatographic parameters for the 11 analytes are shown in Table S2), and therefore the latter was selected for further optimization.

The mobile phase composition in SFC-MS affects the peak shape, retention, selectivity and MS response [22]. The effect of several volatile additives (0.1% (v/v) formic acid, 0.1% (v/v) ammonium hydroxide and 5 mM ammonium acetate in mobile phase component B) on the separation and detection performance was studied and compared with that of pure MeOH (data are not shown). It was found that the addition of 5 mM ammonium acetate in MeOH could significantly enhance the ionization of the tested amino acids and hence the MS response. Therefore, ammonium acetate (5 mM in B) was chosen as the first additive.

TFA is a commonly used mobile phase additive for proteins or small peptide analysis [23]. In order to further improve the separation of the 11 amino acids, various amounts of TFA (0–0.5%, v/v) were also added into mobile phase component B. As shown in Fig. 1, by increasing the content of TFA in mobile phase B from 0 to 0.4% (v/v), both the resolution (*Rs*) values and peak shape were clearly improved. A further increase of the TFA content to 0.5% (v/v) did not lead to any significant improvement. Therefore, TFA (0.4%, v/v) was selected as a second additive in mobile phase B.

Water has been proved to be an interesting additive for the SFC analysis of polar analytes [24]. The benefit of the addition of water on the separation performance was also investigated by varying its proportion in mobile phase B from 0 to 10% (v/v). Fig. 2 shows that the addition of water not only shortened the retention of all analytes, but also significantly affected their *Rs* values. As the water content increased from 0 to 5% (v/v), the *Rs* values increased and a baseline separation of all the 11 amino acids was achieved. Surprisingly, a further increase of the water content to 10% (v/v) caused the distortion of peak shape and a decrease in resolution. For example, the *Rs* value of the pair of amino acids Gln and Asn (peaks 9 and 10) decreased from 1.90 to 1.70 when the water content increased from 5% to 7%. Therefore, a water content of 5% (v/v) in mobile phase component B was finally selected for further investigations.

Both the column temperature and the backpressure were also investigated in order to further improve the separation of the

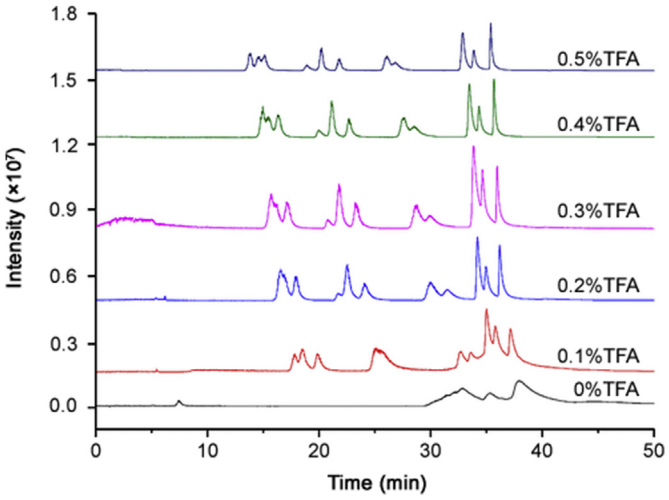

Fig. 1. Effect of concentration of TFA in the mobile phase on the separation of amino acids. Experimental conditions: column: Unitary XAmide column (250 mm × 4.6 mm, 5 μm); mobile phase: (A) sCO_2; (B) MeOH (containing 5 mM ammonium acetate and different percentage of the TFA): gradient: 0–15 min/17% B, 20–30 min/23% B, 40 min/50% B; temperature: 25 °C; backpressure: 140 bar; injection volume: 5 μL; flow rate: 3 mL/min; detection mode: SIM using the precursor ions ($[M+H]^+$) in ESI^+ mode; compounds: 11 amino acid standards (Leu; Val; GABA; Phe; Gly; The; Thr; Ser; Gln; Asn; His).

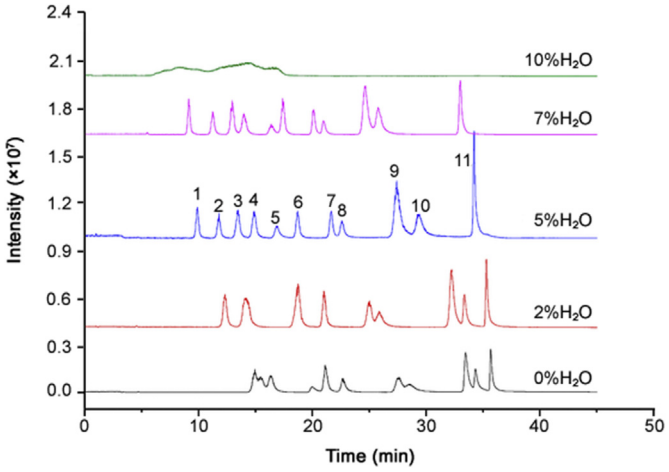

Fig. 2. Effect of concentration of water in the mobile phase on the separation of amino acids. Experimental conditions: mobile phase: (A) sCO_2; (B) MeOH (containing 5 mM ammonium acetate and 0.4% (v/v) TFA and different percentage of the water); amino acids: 1. Leu; 2. Val; 3. GABA; 4. Phe; 5. Gly; 6. The; 7. Thr; 8. Ser; 9. Gln; 10. Asn; 11. His; other conditions see Fig. 1.

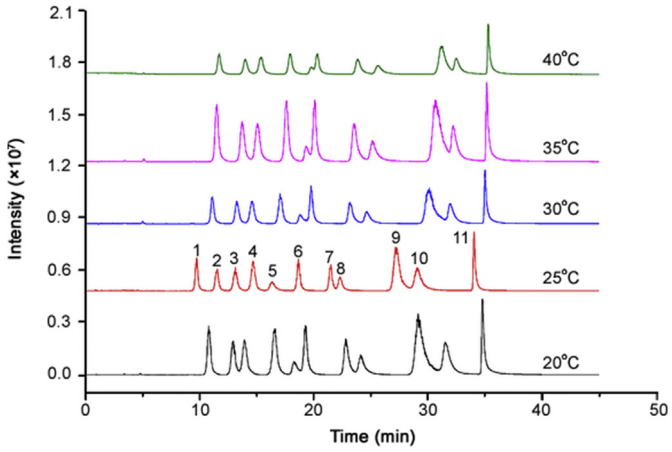

Fig. 3. Effect of temperature on the retention and resolution. Experimental Conditions: mobile phase: (A) sCO_2; (B) MeOH (containing 5 mM ammonium acetate, 0.4% (v/v) TFA and 5% (v/v) water); temperature: 20 °C, 25 °C, 30 °C, 35 °C and 40 °C; amino acids: 1. Leu; 2. Val; 3. GABA; 4. Phe; 5. Gly; 6. The; 7. Thr; 8. Ser; 9. Gln; 10. Asn; 11. His; other conditions see Fig. 1.

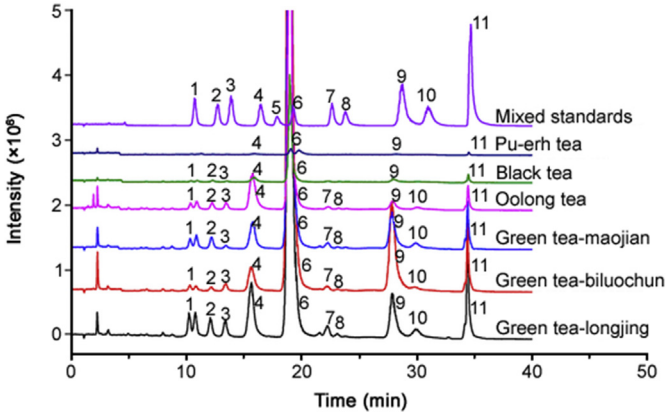

Fig. 4. SFC-MS chromatograms of different tea extracts and amino acid standards. Experimental conditions: column: Unitary XAmide; mobile phase: (A) sCO_2; (B) MeOH containing 5 mM ammonium acetate, 0.4% (v/v) TFA and 5% (v/v) water; temperature: 25 °C; backpressure: 100 bar; injection volume: 5 μL; flow rate: 3 mL/min; detection mode: SIM using the precursor ions ($[M+H]^+$) in ESI^+ mode; amino acids: 1. Leu; 2. Val; 3. GABA; 4. Phe; 5. Gly; 6. The; 7. Thr; 8. Ser; 9. Gln; 10. Asn; 11. His.

amino acids. As can be seen in Fig. 3, the *Rs* value between Gly and The (peaks 5 and 6) increased from 1.40 to 2.0 when the temperature increased from 20 °C to 25 °C, and then decreased progressively to 1.2 with a further increase of temperature up to 40 °C. The variation in backpressure from 220 to 100 bar only led to a slight decrease in the retention of these polar analytes (Fig. S2). This might be explained by the lower compressibility of mobile phase after adding a relatively high proportion of modifier to sCO_2 [24]. The highest *Rs* values with regard to all the amino acids were obtained at 25 °C and 100 bar.

3.2. Performance of the SFC method

The developed method was then validated in terms of linearity, LODs, LOQs, precision, accuracy and stability (Table 2). Good linearity ($r > 0.99$) was achieved for all amino acids. LODs ranged from 1.42 ng/mL (Leu) to 14.69 ng/mL (Gly), and LOQs were between 4.53 ng/mL (Leu) and 47.0 ng/mL (Gly), respectively. For the selected amino acids, RSDs for intra-day and inter-day reproducibility were lower than 3.0%, which indicates a good precision for the developed method. The recoveries of all the 11 amino acids were in the range of 91.95%–107.09%, indicating that the developed method was accurate. In addition, the results from Table 2 show that these 11 amino acids were stable within 24 h (RSDs not higher than 2.88%). The validation data demonstrated that the proposed method could be used for the simultaneous analysis of amino acids in real samples.

3.3. Real sample analysis

The developed SFC-MS method was subsequently applied to the determination of the 11 amino acids in different types of tea (Fig. 4). The corresponding contents of the amino acids in the six tea samples are listed in Table 3. Among the 11 amino acids, The was as expected the most abundant amino acid in all six tea samples. Leu, Val, GABA, Phe, The, Thr, Ser, Gln, Asn, and His could be effectively detected in both the green teas and Oolong tea, while only Leu, Val, GABA, Phe, The, Gln and His were found in the black tea. In Pu-erh tea, only four amino acids (Phe, The, Gln, His) could be identified. The highest total content of amino acids was found in the green teas, followed by Oolong tea, while a much lower content was present in the black and Pu-erh teas. A clear relationship between the amino acids content and the elaboration process of teas can be observed. This result was in good agreement with those reported by Alcazar et al. [25]. According to these reports, higher amounts of amino acids were present in unfermented teas (green) than those in fermented teas (Oolong, black and Pu-erh teas). These changes in amino acid concentrations probably affect the taste of the teas [25,26].

Table 2
Validation parameters of the developed SFC-MS method.

Analytes	Linear regression data			LOD (ng/mL)	LOQ (ng/mL)	Precision (%RSD, n=6)		Stability (%RSD, n=6)	Recovery (%, n=6) (%RSD)
	Regression curve	Range (ng/mL)	r			Intra-day	Inter-day		
Leu	$Y=1\times10^{10}X+380949$	4.5-910	0.997	1.42	4.53	2.39	2.92	2.41	105.62 (1.09)
Val	$Y=9\times10^{9}X+1\times10^{6}$	28.5-5710	0.997	8.92	28.55	1.52	1.81	2.63	100.85 (1.08)
GABA	$Y=1\times10^{10}X+826154$	9.1-1830	0.996	2.85	9.11	2.56	2.94	2.12	92.20 (0.82)
Phe	$Y=2\times10^{10}X+1\times10^{6}$	9.6-1930	0.997	3.02	9.67	0.65	2.65	1.01	95.95 (0.32)
Gly	$Y=1\times10^{9}X+35394$	47.0-9400	0.999	14.69	47.0	2.08	2.10	2.20	96.95 (0.28)
The	$Y=7\times10^{10}X+3\times10^{7}$	46.0-9250	0.993	1.44	4.62	1.25	2.99	2.52	107.09 (1.20)
Thr	$Y=1\times10^{9}X+157293$	9.1-1820	0.996	2.84	9.10	2.09	1.82	2.57	97.74 (0.56)
Ser	$Y=1\times10^{9}X+146449$	26.0-5290	0.998	8.28	26.50	0.59	2.45	2.58	100.87 (0.25)
Glu	$Y=2\times10^{10}X+2\times10^{6}$	22.5-4500	0.995	7.03	22.50	2.57	2.29	2.78	92.97 (0.26)
Asp	$Y=2\times10^{9}X+347536$	23.6-4727	0.996	7.38	23.60	2.52	1.91	2.88	97.84 (1.23)
His	$Y=1\times10^{10}X+7\times10^{6}$	46.0-9290	0.995	13.59	46.50	2.97	2.31	1.86	91.95 (0.26)

Table 3
The content of amino acids in different types of tea.

Samples	The content of the amino acids (μg/g±SD) (n=3)											
	Leu	Val	GABA	Phe	Gly	The	Thr	Ser	Glu	Asp	His	Total
Pu-erh tea	–	–	–	0.28±0.03	–	5.9±0.02	–	–	0.06±0.007	–	0.23±0.005	6.47
Black tea	0.11±0.01	0.35±0.07	0.10±0.01	0.54±0.07	–	14.0±0.01	–	–	0.42±0.03	–	0.36±0.03	15.88
Oolong tea	4.05±0.15	6.11±0.18	5.67±0.13	20.87±0.33	–	48.84±0.26	12.85±1.15	0.71±0.087	5.65±0.07	4.50±0.39	2.26±0.24	111.51
Green tea												
Maojian	8.15±0.05	10.82±0.22	2.37±0.03	20.5±0.32	–	97.95±0.49	12.69±0.32	1.09±0.71	17.33±0.17	30.05±0.95	12.42±0.37	213.39
Biluochun	2.46±0.13	1.82±0.10	2.32±0.08	18.8±0.16	–	176.92±1.08	14.92±0.38	4.98±0.22	41.47±0.63	10.31±0.19	12.76±0.05	286.81
Longjing	12.23±0.36	16.23±0.37	14.33±0.67	21.2±0.23	–	136.65±1.15	17.07±1.92	7.39±0.61	20.23±0.77	30.44±0.56	52.81±0.19	328.65

4. Conclusions

In this study, a green and efficient SFC-MS method was developed for the separation of 11 amino acids. After systematic optimization of the method, the 11 amino acids could be baseline separated on a Unitary XAmide column using gradient elution with MeOH containing ammonium acetate (5 mM), TFA (0.4%, v/v) and water (5%, v/v) as organic modifier. Moreover, the developed method was fully validated for the quantitative determination of 11 amino acids in green teas, Oolong, black and Pu-erh teas. The obtained results indicate that SFC-MS is an attractive method for the determination of amino acids in teas.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2019.05.001.

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