Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2017, **9**, 6131

Separation and detection of free D- and L-amino acids in tea by off-line two-dimensional liquid chromatography†

Xiangyu Wang,‡[a] Huihui Wu,‡[b] Rongying Luo,[a] Donghai Xia,[a] Zhengjin Jiang*[a] and Hai Han*[a]

Received 26th June 2017
Accepted 2nd October 2017

DOI: 10.1039/c7ay01569k

rsc.li/methods

D-amino acids are currently paid attention to as new physiologically active substances. Foodstuffs and beverages containing D-amino acids are a matter of interest. Until now, the profiles of D-amino acids have not been reported in natural and fermented teas except theanine. In this study, an off-line 2D-HPLC method combining a Gemini C18 column and a CHIRALPAK® IC-3 column or a self-prepared poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary column was employed for the separation and detection of D-amino acids in tea samples with the co-existence of a large amount of L-amino acids. The free amino acid fractions in longjing, black, oolong, and pu-erh tea samples were separated and collected after pre-column derivatization using 9-fluorenylmethoxycarbonyl (FMOC) chloride in the reversed-phase mode, and then were concentrated and separated to D- and L-forms on a chiral column. Among them, the D-form of isoleucine (Ile) (1.0–1.6%), alanine (Ala) (0.1–0.8%), phenylalanine (Phe) (0.1–0.4%), valine (Val) (0.2–0.3%), serine (Ser) (0.1–0.2%), aspartic acid (Asp) (0.2%), and proline (Pro) (0.1%) were detected in longjing and oolong teas. Differences between natural tea and fermented tea in the profiles of D-amino acids as well as the total amino acids were also observed. The results provided useful information for the bio-function research of D-amino acids in tea.

Introduction

Tea, one of the most consumed beverages, is becoming increasingly popular in the world.[1] Depending on the manufacturing process attaining different levels of fermentation, tea is classified into green and white tea (unfermented), oolong tea (semi fermented), black tea (fully fermented) and pu-erh tea (post fermented).[2,3] That is how each tea with different fermentation treatments acquires its unique refreshing taste, attractive aroma, and potential health benefits.[4] These important physiological properties and potential health benefits were attributed to the presence of compounds such as amino acids, polyphenols, vitamins, carbohydrates, caffeine, and purine alkaloids.[4,5] Amino acids exist in large quantities in our foodstuffs, beverages and health products and play an important role in identification, classification, biological effects, quality control and optimizing process.[6–8] For instance, Alcazar *et al.* had classified five kinds of teas according to their free amino acids.[9] Moreover, a relationship between free amino acids and the fermentation level in pu-erh tea was studied by Zhu *et al.*.[10] It was found that the amino acid concentration increased after 7-day fermentation and then decreased gradually. The predominant amino acids (glutamine, arginine and theanine) in tea were also performed to select high-quality green tea leaf by Miyauchi *et al.*.[11]

D-Amino acids were thought to be harmful to human health, for example, the presence of D-amino acids in proteins led to a decrease in digestibility and the availability of the other amino acids.[12] However, their importance and physiological functions had been gradually realized. Kolodkin-Gal *et al.* found that D-amino acids could promote bacterial biofilm dispersion.[13] D-serine acts as a candidate molecule to improve the cerebral and cerebellar functions,[14] while D-aspartic acid plays an important role in hormonal synthesis and secretion.[15] It is worth mentioning that orally administered D-amino acids are distributed in the brain and peripheral tissues.[16] Therefore, the research and discovery of D-amino acid functional food products have been prevalent in recent years. Furthermore, D-amino acids were also widely used in other research fields besides their physiological function. For example, Tian *et al.* realized that milk origins could be differentiated based on the D-to L-amino acid ratio-based projection scores by principal component analysis.[17] Moreover, Bruckner *et al.* did a lot of research about D-amino acids in fruits, foods, trees and so on.[18,19] What's more,

[a]*Department of Pharmacy, Guangdong Province Key Laboratory of Pharmacodynamic Constituents of Traditional Chinese Medicine & New Drug Research, Jinan University, Guangzhou 510632, China. E-mail: thanhaik@jnu.edu.cn; jzjjackson@hotmail.com*

[b]*Anhui No. 2 Province People's Hospital, Hefei 230041, China*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ay01569k

‡ X. Y. Wang and H. H. Wu contributed equally to this work.

they realized that the fermentation degree would influence the percentage of D-amino acids.[20] On one hand, many D-amino acids were known as components of microbial peptide antibiotics. On the other hand, amino acids in food proteins under fermentation conditions changed their optical configuration from L to D through racemization or epimerization. In addition, there were lots of reports about D-amino acids in foodstuffs and beverages.[21–23] Most of the test samples were fermented products. However, few reports were available about the presence and changes of free D-amino acid profiles and their levels during fermentation of tea except D-theanine.[3] The main reason is that a large amount of L-amino acids will usually disturb the detection of their D-forms and it is difficult to achieve the chiral separation for many amino acids under the same chromatographic conditions.

The reports about chiral separation by two-dimensional liquid chromatography have been booming in the recent years.[24–26] There is no doubt that two-dimensional liquid chromatography is powerful in separation and analysis of complex biological samples. By a combination of different separation modes, this system maximizes the selectivity and peak capacity in the separation of complex samples.[27] In general, an on-line system offers the advantage of rapidity, good repeatability, high peak capacity and automation. However, it suffers from the limitation of analytical time in the second-dimension separation and the compatibility of the mobile phase.[28] Different from the on-line system, the off-line system still has the advantage of high peak capacity and strong separating force, but costs a large amount of labor and longer analytical time. In Gonzalez's studies, an off-line system was developed to separate polychlorinated biphenyl (PCB) chiral congeners.[29] The off-line approach also provides more flexibility for the selection of column dimensions and chromatographic conditions when we need to independently optimize the second-dimension analytical conditions for different fractions. What's more, the organic solvents in the first dimension were able to be evaporated, so that fractions could be concentrated or dissolved in a different solvent prior to reinjection.[30]

In this study, we described an off-line two-dimensional HPLC system and its application for the determination of the amino acid enantiomers in tea. Firstly, we extracted the amino acids from four different kinds of teas and separated them by HPLC using a Gemini C18 column (250 mm × 10 mm, I.D., 5 μm, Phenomenex) after the derivatization using FMOC-Cl in the first dimension. After enrichment and concentration, individual amino acids collected from oolong tea and longjing tea were run through a chiral column in the second dimension for the enantiomeric separation. Two chiral columns, *i.e.* a CHIRALPAK® IC-3 column (150 mm × 4.6 mm, 3 μm) and the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary column (180 mm × 100 μm I.D.),[31] were employed in order to further confirm the presence and percentage of several D-amino acids.

The objective of this work was to evaluate the free amino acids and investigate some of their enantiomers in tea by off-line two-dimensional chromatography. Furthermore, differences between natural tea and fermented tea in the amounts of D-amino acids as well as the total amino acids were investigated.

Experimental

Chemicals and reagents

2,2′-Azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl), methacrylate (γ-MAPS), ethylene dimethacrylate (EDMA), 2-hydroxyethyl methacrylate (HEMA), 11-dihydroquinidine (MQD), methanol (MeOH), quinidine, ammonium acetate, acetic acid, ethyl acetate, trifluoroacetic acid (TFA), boric acid, ammonium hydroxide, *n*-pentane, acetonitrile (ACN), acetic acid, tetrahydrofuran (THF), ammonium acetate and sodium acetate were all purchased from Aladdin Chemicals (Shanghai, China). Isopropanol and *n*-hexane were purchased from CNW (Shanghai, China). 9-Fluorenylmethoxycarbonyl (FMOC) chloride and all racemic amino acids (histidine (His), arginine (Arg), glutamine (Gln), asparagine (Asn), serine (Ser), methionine (Met), threonine (Thr), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), theanine (Thea), glycine (Gly), alanine (Ala), tyrosine (Tyr), proline (Pro), valine (Val), tryptophan (Trp), isoleucine (Ile), leucine (Leu), phenylalanine (Phe) and lysine (Lys)) and their single configurations were obtained from Energy Chemical (Shanghai, China). Distilled water was filtered through a 0.22 μm membrane before use. The fused-silica capillaries (375 μm O.D. × 100 μm I.D.) were supplied by Yongnian Ruifeng Chromatography Ltd. (Hebei, China).

Instruments

For all HPLC experiments, an UltiMate 3000 (Dionex, Germering, Germany) LC system was used and equipped with a ternary low-pressure-mixing gradient pump (LPG-3400SD), a membrane degasser unit (SRD-3600), CTC Combi Pal autosamplers and a six-port injection valve with a 90 μL sample loop, a temperature-controlled column oven with a flow manager (TCC-3000), and a variable wavelength detector (VWD-3100). All micro-HPLC experiments were carried out on a self-assembled nano-HPLC system which is composed of a Shimadzu SPD-15C UV detector (Kyoto, Japan) with a lab-made on-column detection cell, a four-port Valco injection valve with a 20 nL internal loop (Houston, USA) and a DiNa-S nano pump (Tokyo, Japan). Data acquisition and data handling were performed using a Unimicro TrisepTM Workstation 2003 (Shanghai, China) or ThermoFisher scientific Chromeleon 7.0. All chromatograms were converted to a text file and redrawn using Microcal Origin 8.5. A Jinghong DK-S22 water bath (Shanghai, China) was used for thermally initiated copolymerization. The pH values were monitored using a Sartorius PB-10 pH meter (Gottingen, Germany). The Greatwall R-1001VN rotary evaporator (Zhengzhou, China) was equipped with an EYELA A-1000S aspirator pump (Shanghai, China) and Yuhua CCA-20 refrigerated circulating bath (Gongyi, China) to maintain the temperature of the antifreeze.

Tea samples

Dry samples of pu-erh, keemun, oolong, longjing teas with sealed storage in the dark at 4 °C for less than a year (according to the production date) were used for the determination of amino acids. All leaf tea samples were purchased from a local tea market in Guangzhou.

Preparation of sample and standard solutions

To find out an optimum extraction method for tea samples, the efficiency of five different treatment methods was investigated.[32,33] Method 1 was achieved by adding 100 mL boiling water to 5 g oolong tea. The tea sample was allowed to brew for 1 h and then filtered through filter papers. 100 mL boiling water was added to the residue and it was brewed for 1 h again. All the filtrates were combined and cooled at room temperature. Then the solution pH was adjusted to 3.0 using hydrochloric acid and extracted using ethyl acetate; the aqueous fraction pH was adjusted to 9.0 using sodium bicarbonate. Finally, the sample extraction solution was dried using a rotary evaporator. In method 2, the extraction solution without adjusting pH was directly dried using a rotary evaporator and dissolved in methanol. In method 3, the filtrate was cooled at room temperature and extracted using ethyl acetate. In method 4, the extraction solution was dried without any processing after boiling. Method 5 was performed by adding 0.5 g magnesium oxide and 100 mL boiling water to 5 g oolong tea. The filtrate was cooled at room temperature and dried using a rotary evaporator. 15 mg of dried powder from the five methods were dissolved in 3 mL boric acid solution (0.2 M, pH 9.0) and then reacted with isometric FMOC reagent solution (20 mM) at room temperature.[34–36] Then, the solution was extracted using the same volume of *n*-pentane and the aqueous phase was used for HPLC analysis after filtering through a 0.22 μm nylon membrane filter.

As in the above case, the standards solutions and reagent blank sample (without amino acids) were prepared by dissolving in boric acid solution, derivatization and filtration, respectively. Then all the solutions for 20 standards were equally mixed. The final concentration of the standard was 1.5 mM for all the amino acids.

Optimization of the off-line system with the 20 amino acid standards

On one hand, 20 amino acid standards were separated in the first dimension according to a previously reported method with proper modification.[37] To achieve the best separation of free amino acids, the influence of column length (Agela C18 (250 mm × 4.6 mm I.D., 5 μm) and Phenomenex C18 (150 mm × 4.6 mm I.D, 5 μm)) and mobile phase composition (TFA, inorganic salt and pH) were investigated. On the other hand, chiral separation of standard samples was also studied according to a previously reported method with proper modification.[38] Because different amino acids had different characteristics, chromatographic conditions were properly adjusted, especially for the mobile phase ratio.

HPLC system for the determination of free amino acids in tea samples

Free amino acids in tea were determined on a Venusil MP C18 analytical column (250 mm × 4.6 mm, I.D., 5 μm) (Agela, Tianjin, China) maintained at 40 °C. The mobile phase was composed of (A) ACN and (B) TFA in water (0.05%, v/v). The elution was performed using a linear gradient as shown in Table 1. The UV detection wavelength was 265 nm. The sample injection was 10 μL. The total run time was 60 min. A flow rate of 1 mL min^{-1} was used for all steps.

Table 1 The optimized gradient elution program for first dimension separation

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	32	68
16	43	57
26	43	57
36	52	48
45	52	48
55	100	0
56	32	68
60	32	68

Off-line two dimensional HPLC separation of amino acid enantiomers in tea samples

The first dimension (1-D) HPLC separation was performed on a Gemini C18 (250 mm × 10 mm, I.D., 5 μm, Phenomenex). The mobile phase was composed of (A) ACN and (B) TFA in water (0.05%, v/v) using an optimized gradient elution as shown in Table 1 and the flow rate was 2 mL min^{-1} and the mobile phase was filtered through a 0.22 μm nylon membrane filter and degassed prior to use. The column temperature was maintained at 40 °C. The UV detection wavelength was 265 nm. The sample injection volume was 50 μL. From 12 to 56 min, 10 fractions were collected. The fractions were concentrated to dryness with a rotary evaporator, and all the fractions were re-dissolved in 40 μL isopropanol, individually.

The second dimension (2-D) HPLC separation was performed on a CHIRALPAK® IC-3 (150 mm × 4.6 mm, I.D., 3 μm) or the self-prepared poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary column (180 mm × 100 μm I.D.) at room temperature. For the former, the mobile phase was isopropanol/*n*-hexane (10/90, v/v) with 0.1% TFA with a flow rate of 0.8 mL min^{-1}. The

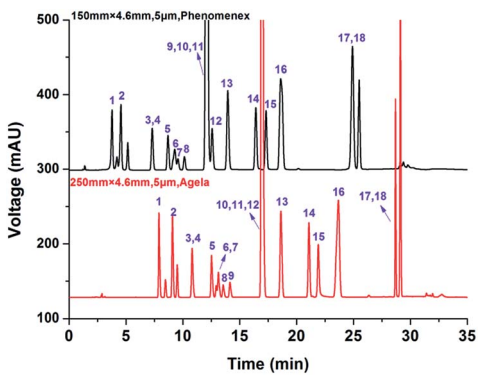

Fig. 1 Separation of standard FMOC-amino acids on two different columns. Experimental conditions: mobile phase A: 0.05% TFA, mobile phase B: ACN, gradient elution (see Table S1†); flow rate: 1 mL min^{-1}; column temperature: 40 °C; detection wavelength: 265 nm; injection volume: 10 μL. Red, Agela C18 (250 mm × 4.6 mm I.D., 5 μm); black, Phenomenex C18 (150 mm × 4.6 mm I.D, 5 μm). Samples: 1. histidine, 2. arginine, 3. asparagine, 4. glutamine, 5. serine, 6. aspartic acid, 7. glutamic acid, 8. threonine, 9. glycine, 10. theanine, 11. GABA, 12. alanine, 13. tyrosine, 14. proline, 15. valine, 16. tryptophan, 17. isoleucine, and 18. leucine.

UV detection wavelength was 254 nm. The sample injection volume was 10 μL. For the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary, the mobile phase was 0.1 M ammonium acetate/ACN (50/50, v/v) (apparent pH = 6.0) with a flow rate of 600 nL min^{-1}. The sample injection volume was 20 nL. The UV detection wavelength was 254 nm. Prior to the detection of L- and D-amino acids in tea samples, a reagent blank solution and amino acid standards were injected in order to test the interference of the off-line system and to investigate the chromatographic behaviour of amino acid enantiomers. The relative peak height percentage of D-amino acids (% D/(D + L)) in tea samples was calculated (eqn (1)).

$$\%\ \mathrm{D}/(\mathrm{D}+\mathrm{L}) = H_{\mathrm{D}}/(H_{\mathrm{D}} + H_{\mathrm{L}}) \quad (1)$$

H: peak height.

Results and discussion

Optimization of chromatographic conditions

An optimization strategy for chromatographic conditions was performed initially with FMOC-derivatized amino acid standards to select the best separation conditions. Firstly, the two same packing columns with different lengths were compared under the same chromatographic conditions (Fig. 1). This indicated that the longer one had better resolution. According to related reports,[37,39,40] TFA was added to the aqueous mobile phase for chromatographic retention, resolution and peak shape. The mobile phase composition was also optimized by investigating the effects of TFA (Fig. 2A) and inorganic salt (Fig. 2B). From Fig. 2, it can be seen that amino acids had better separation when the concentration of TFA was increased from 0.02% to 0.05%, but there was no significant change when TFA was increased from 0.05% to 0.08%. The retention of most amino acids in only ammonium acetate solution was lower

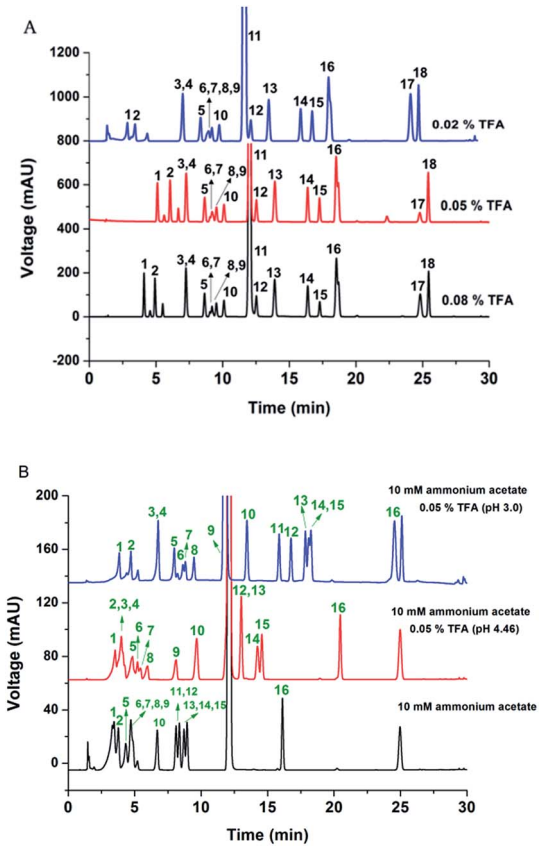

Fig. 2 The effects of TFA and inorganic salt in the mobile phase. Conditions: column: Phenomenex C18 (150 mm × 4.6 mm I.D., 5 μm, 100 Å); flow rate: 1 mL min^{-1}; column temperature: 40 °C; detection wavelength: 265 nm; injection volume: 10 μL. Gradient elution (see Table S1†). (A) The effects of TFA. black, 0.08%; red, 0.05%; blue, 0.02%. (B) The effects of inorganic salt. black, 10 mM ammonium acetate as mobile phase A; red, 10 mM ammonium acetate and 0.05% TFA (pH 4.46) as mobile phase A; blue, 10 mM ammonium acetate and 0.05% TFA (pH 3.0) as mobile phase A. Samples: 1. histidine, 2. arginine, 3. asparagine, 4. glutamine, 5. serine, 6. aspartic acid, 7. glutamic acid, 8. threonine, 9. glycine, 10. theanine, 11. GABA, 12. alanine, 13. tyrosine, 14. proline, 15. valine, 16. tryptophan, 17. isoleucine, and 18. leucine.

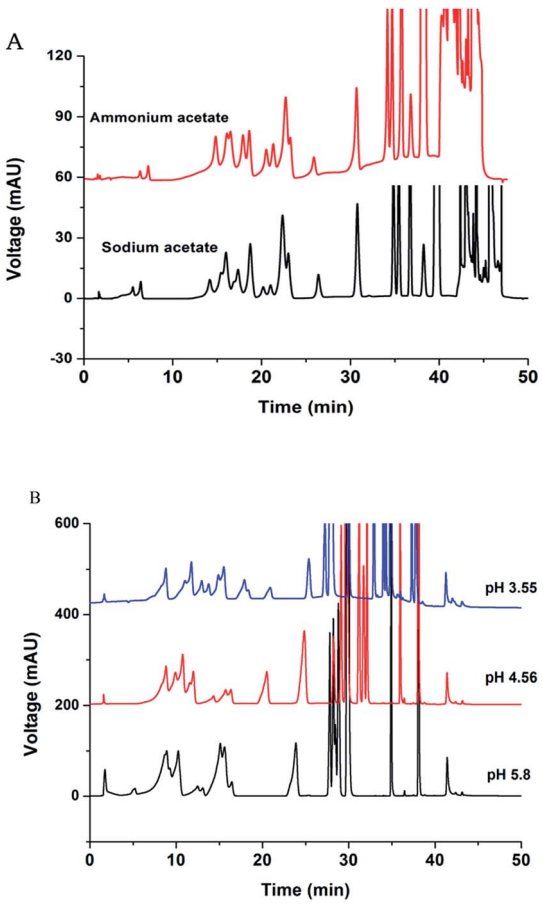

Fig. 3 The effects of different inorganic salts and pH values. Conditions: column: Phenomenex C18 (150 mm × 4.6 mm I.D., 5 μm, 100 Å); mobile phase A: ACN/H_2O (90/10, v/v); mobile phase B: inorganic salt/THF/ACN (86/4/10, v/v/v), gradient elution (see Table S2†); flow rate: 1 mL min^{-1}; column temperature: 40 °C; detection wavelength: 265 nm; injection volume: 10 μL. (A) The effects of two kinds of inorganic salts. (B) The effects of pH of sodium acetate. Mobile phase A: 0.05% TFA, 10 mM sodium acetate; mobile phase B: ACN/H_2O (80/20, v/v), gradient elution (see Table S3†).

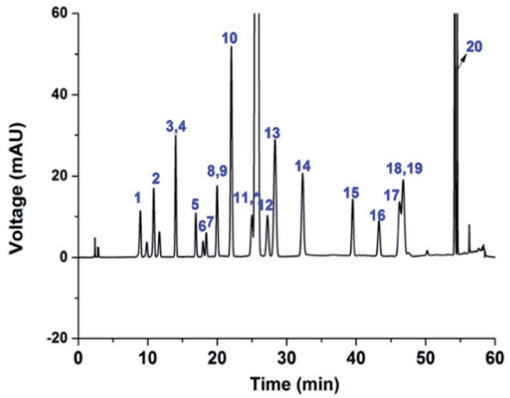

Fig. 4 Chromatogram of the FMOC-derivatives of standard amino acids. Conditions: column: Agela C18 (250 mm × 4.6 mm I.D., 5 μm, 100 Å); mobile phase A: 0.05% TFA, mobile phase B: ACN, gradient elution (see Table 1); flow rate: 1 mL min^{-1}; column temperature: 40 °C; detection wavelength: 265 nm; injection volume: 10 μL; samples: 1. histidine, 2. arginine, 3. asparagine, 4. glutamine, 5. serine, 6. aspartic acid, 7. glutamic acid, 8. threonine, 9. glycine, 10. theanine, 11. GABA, 12. alanine, 13. tyrosine, 14. proline, 15. valine, 16. tryptophan, 17. isoleucine, 18. leucine, 19. phenylalanine, and 20. lysine.

compared to that of the mobile phase with ammonium acetate and 0.05% TFA. Besides, lower pH could improve the separation. Furthermore, we exchanged the ammonium acetate to sodium acetate (Fig. 3A). The result suggested that sodium acetate was more suitable for separating FMOC-amino acids. On this basis, we adjusted the pH again; however, all the earlier eluted peaks had fronting peak shapes in different pH (Fig. 3B). Compared to Fig. 2 and 3, it was revealed that the better retention, selection and peak shape could not be obtained by adding inorganic salt. Finally, the Agela C18 column (250 mm × 4.6 mm I.D., 5 μm) was selected and the best first dimensional chromatogram was obtained (Fig. 4) under the optimized mobile phase containing ACN as phase A and 0.05% TFA as phase B by gradient elution (Table 1). This gave lower column pressure (93 bar), better peak shape and resolution, and yielded the most of the amino acids that could be resolved and detected in the extracts.

Due to amino acids having different chromatographic characteristics, it was difficult to chirally separate them under the same chromatographic conditions in the 2-D separation. Therefore, appropriate optimization was necessary including the mobile phase composition and flow rate for different amino acids. For Ala, Ser, Val, Asp, Phe, Ile, Pro, Thr and Trp, a better enantioseparation could be obtained when the composition of *n*-hexane/isopropanol (90/10, v/v) was used. But this was not suitable for all the amino acids, Ser, Asp and Pro needed a lower ratio of *n*-hexane of 80% to improve the enantioseparation. The resolution values of Ile, Ala, Phe, Val, Ser, Asp, and Pro enantiomers were between 2.0 and 6.8, which makes it possible to separate the low content D-amino acids from their L-forms after FMOC-derivatization (Table 2).

Optimization of extraction procedures

Five different sample preparation methods for the oolong tea sample were adopted and hot water was selected as the extraction solvent (Table 3). Then the five samples were analyzed by HPLC. It could be observed from Fig. 5 that the impurity peaks were reduced and the baseline was smoother in method 3 compared to that of method 4. After pH adjustment, the peaks of amino acids nearly disappeared in method 1. It was estimated that the original structures of amino acids were destroyed by the alkali or acid. Due to some amino acids having poor solubilities in methanol, the peak areas in method 2 were smaller. For method 5, the peak areas are also not large enough, even if having a smooth baseline. Therefore, method 3 was selected as the best extraction procedure for larger peak area and better resolution.

Table 2 Mobile phase conditions, retention times and resolution of different standard amino acid enantiomers on a CHIRALPAK® IC-3 column

Amino acids	Mobile phase (n-hexane : isopropanol)	t_R of L-form (min)	t_R of D-form (min)	R_s of L-form (min)
Ala	9 : 1	17.5	22.9	3.8
Ser	8 : 2	12.7	22.8	6.8
Val	9 : 1	13.1	16.8	2.0
Asp	8 : 2	10.6	15.0	5.0
Phe	9 : 1	20.7	25.7	3.2
Ile	9 : 1	13.5	19.0	3.0
Pro	8 : 2	16.0	22.5	3.4
Thr	9 : 1	25.2	40.4	1.9
Trp	9 : 1	32.8	37.9	1.4

Table 3 Five different sample treatment methods of oolong tea. (✓, processing; ✗, not processing)

	Method 1	Method 2	Method 3	Method 4	Method 5
Extraction (ethyl acetate)[a]	✗	✗	✓	✗	✗
Magnesium oxide[b]	✗	✗	✗	✗	✓
pH adjustment[c]	✓	✗	✗	✗	✗
Redissolved with methanol[d]	✗	✓	✗	✗	✗

[a] The extraction solution after boiling was extracted using ethyl acetate. [b] Magnesium oxide was added in the boiling process. [c] The extraction solution pH was adjusted to 3.0 using hydrochloric acid and then it extracted using ethyl acetate; the aqueous fraction pH was adjusted to 9.0 using sodium bicarbonate. [d] The extraction solution was dried using a rotary evaporator and redissolution in methanol.

Separation of free amino acids in four kinds of teas

Amino acid peaks in four kinds of tea samples (Fig. 6) were identified by comparing with retention times of the FMOC-amino acid standards. Comparing the contents and compositions of free amino acids in the four kinds of teas, the pu-erh was lowest and longjing was highest in types and content of free amino acids with keemun and oolong following behind. It was noteworthy that there was a relationship between the degree of fermentation and free amino acid contents. As a kind of natural

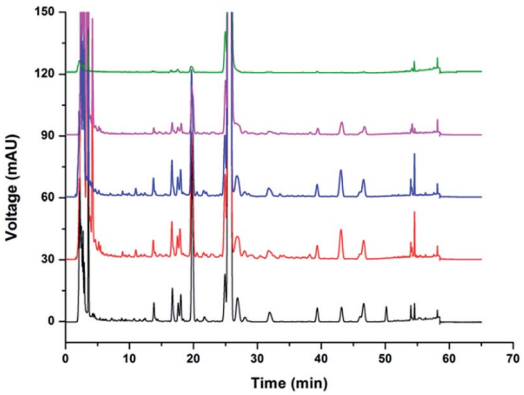

Fig. 5 The chromatograms of different extraction methods in the first dimension. Conditions: column: Agela C18 (250 mm × 4.6 mm I.D., 5 μm); mobile phase A: ACN, mobile phase B: 0.05% TFA, gradient elution (see Table 1); flow rate: 1 mL min^{-1}; detection wavelength: 265 nm; column temperature: 40 °C; injection volume: 10 μL. From top to bottom are the chromatograms from methods 1–5.

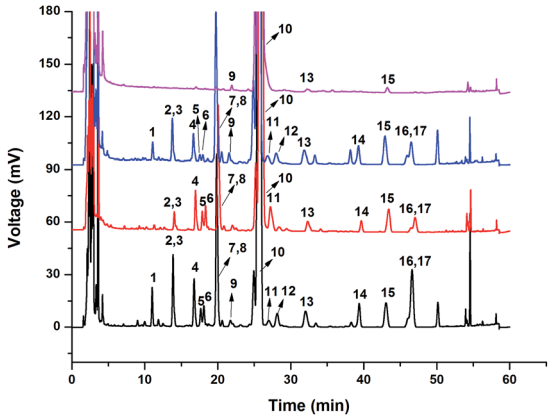

Fig. 6 Chromatograms of amino acids in different tea samples. Conditions: column: Agela C18 (250 mm × 4.6 mm I.D., 5 μm); mobile phase A: ACN, mobile phase B: 0.05% TFA, gradient elution (see Table 1); flow rate: 1 mL min^{-1}; detection wavelength: 265 nm; column temperature: 40 °C; injection volume: 10 μL. black, longjing; red, oolong; Blue, keemun; pink, pu-erh. Samples: 1. arginine, 2. asparagine, 3. glutamine, 4. serine, 5. aspartic acid, 6. glutamic acid, 7. threonine, 8. theanine, 9. glycine, 10. FMOC-OH, 11. alanine, 12. tyrosine, 13. proline, 14. valine, 15. tryptophan, 16. isoleucine, and 17. phenylalanine.

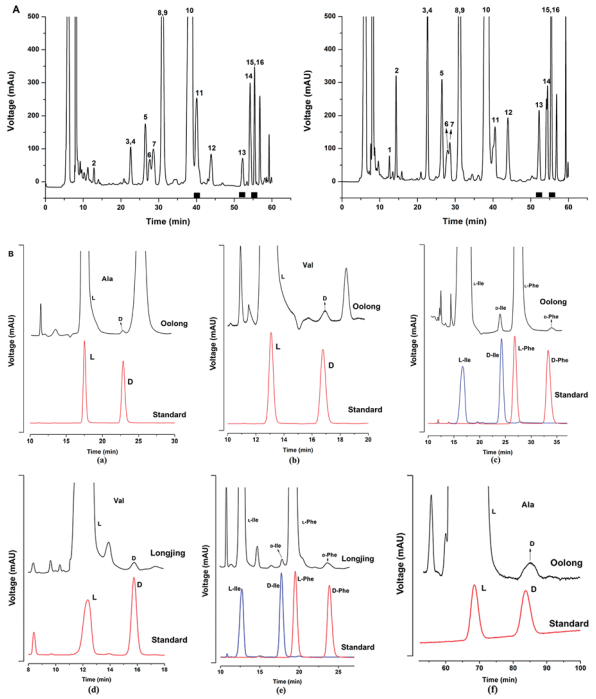

Fig. 7 Offline 2D-HPLC separation of various amino acid enantiomers in the oolong and longjing. 1-D conditions: column: Gemini C18 (250 mm × 10 mm I.D., 5 μm, Phenomenex); mobile phase A: ACN, mobile phase B: 0.05% TFA, gradient elution (see Table 1); flow rate: 2 mL min^{-1}; detection wavelength: 265 nm; column temperature: 40 °C; injection volume: 50 μL. 2-D conditions: column: CHIRALPAK® IC-3 (150 mm × 4.6 mm I.D., 3 μm); mobile phase: isopropanol/*n*-hexane (90/10, v/v) with 0.1% TFA; flow rate: 0.8 mL min^{-1}; detection wavelength: 254 nm; column temperature: room temperature; injection volume: 10 μL. Capillary chromatographic conditions: column dimension: 180 mm × 100 μm I.D.; mobile phase: 0.1 M ammonium acetate/ACN (50/50, v/v); flow rate: 600 nL min^{-1}; UV detection wavelength: 254 nm; injection volume: 20 nL. Red or blue, amino acid standards; black, amino acids in tea. (A); 1-D, separation of amino acids in oolong (left) and longjing (right) using a semi-preparative HPLC column. (B); 2-D, chiral separation using CHIRALPAK® IC-3 (a–e) and poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary columns (f). oolong, (a–c) and (f); longjing, (d and e).

tea, the amino acids in longjing had not been decomposed or transformed in the production process and were higher than others. However, the oolong, keemun and pu-erh had experienced the fermentation process in different degrees. A further fermentation process would result in the reduction of the total amount of amino acids.

Determination of D-amino acids in teas

To detect the low content of D-amino acids, the fractions after 1-D separation were concentrated to dryness with a rotary evaporator to improve the concentration of D-amino acids. In order to investigate the differences of D-amino acids between natural tea and fermented tea, the amino acids in longjing and oolong were collected respectively and successfully enantioseparated under different chromatographic conditions on a CHIRALPAK® IC-3 column (Fig. 7). From the chromatograms, owing to the high enantioselectivity of the chiral stationary phase, the retention times of D-amino acids were easily assigned by comparison with standards and the relative peak height percentage of D-amino acids (% D/(D + L)) is shown in Table 4. Until now, the existence of D-amino acids in teas has never been reported except D-theanine;[3] therefore, to confirm the D-amino acid percentage, sufficient enantiomeric separations of the same FMOC-amino acids in tea samples were obtained under two or three different mobile phase conditions (Fig. 8). The % D/(D + L) values of Val, Ser, Ala, Ile, Phe, Asp and Pro determined using different mobile phases were almost the same. This indicated the reliability of the present method and that some D-amino acids really exist in teas, such as D-Val, D-Ser, D-Ala, D-Ile,

Table 4 Amino acids and their % D(D + L) values in longjing and oolong

Amino acids	% D/(D + L) (oolong)	% D/(D + L) (longjing)
Ser	0.2	0.2
Ala	0.1	0.8
Val	0.2	0.3
Ile	1.6	1.0
Phe	0.1	0.4
Asp	0.2	—
Pro	0.1	—
Trp	—	—
Thr	—	—

and D-Phe in both kinds of teas and D-Asp and D-Pro in oolong. It was of interest to note that the D-amino acids in oolong were more diverse than those of longjing which had a higher total amino acid content. The results suggested that the configuration of some L-amino acids transforms into the D-form in the tea fermentation process. Meanwhile, the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary column was also proposed for the confirmation of D-amino acids in teas. The enantioseparation conditions, such as the organic modifier content, the buffer concentration, the flow rate and the apparent pH of the mobile phase, were also optimized (data not shown). Because the L-amino acids were much higher than their D-forms in tea samples and FMOC- L-amino acids eluted earlier on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary column, better enantiomeric resolution was emphasized. Therefore, Ala and Ser were selected for confirmation due to the high enantiomeric resolution value of their FMOC-derivatives. The % D/(D + L) values of Ala and Ser determined on the poly(MQD-*co*-HEMA-*co*-EDMA) monolith were also consistent with those obtained on the CHIRALPAK® IC-3 column. Fig. 8f shows that the D-Ala in the oolong tea sample was successfully separated and detected on the poly(MQD-*co*-HEMA-*co*-EDMA) monolithic capillary column.

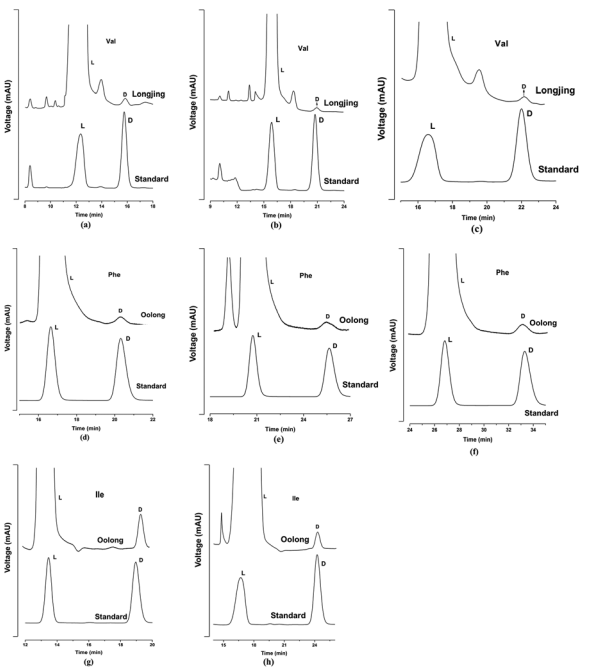

Fig. 8 The same FMOC-amino acids enantioseparated under two or three mobile phase conditions. Experimental conditions: column: CHIRALPAK® IC-3 (150 mm × 4.6 mm I.D., 3 μm); mobile phase A: *n*-hexane, mobile phase B: isopropanol; detection wavelength: 254 nm; flow rate: 0.8 mL min^{-1}; column temperature: room temperature; injection volume: 10 μL. (a) Val in longjing at *n*-hexane/isopropanol (88/12, v/v); (b) Val in longjing at *n*-hexane/isopropanol (90/10, v/v); (c) Val in longjing at *n*-hexane/isopropanol (92/8, v/v); (d) Phe in oolong at *n*-hexane/isopropanol (88/12, v/v); (e) Phe in oolong at *n*-hexane/isopropanol (90/10, v/v); (f) Phe in oolong at *n*-hexane/isopropanol (92/8, v/v); (g) Ile in oolong at *n*-hexane/isopropanol (90/10, v/v); (h) Ile in oolong at *n*-hexane/isopropanol (92/8, v/v).

Conclusions

In this study, by using the offline two-dimensional HPLC system combining reversed phase and enantioselective columns, the chiral amino acid analysis in longjing, black, oolong, and puerh teas was performed and D-Ile, D-Ala, D-Phe, D-Val, D-Ser were observed in the longjing tea sample (% D/(D + L) value ranged from 0.2–1.0) and D-Ile, D-Val, D-Ser, D-Asp, D-Phe, D-Ala and D-Pro (% D/(D + L) value ranged from 0.1–1.6) were observed in the oolong tea sample. These values were further confirmed under different column or mobile phase conditions. There are various D-amino acids which have not been reported to exist in natural and fermented teas except theanine. Meanwhile, we also found there was a difference between natural tea and fermented tea in the amounts of D-amino acids as well as the total amino acids. The results could provide useful information for the manipulation of fermentation according to the changes of D-amino acids in different teas and the research of bio-functional ingredients in foods and beverages.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We gratefully appreciate the financial support from the Science and Technology Planning Project of Guangdong province (2016A040403055).

Notes and references

1 Y. Clement, *Prev. Med.*, 2009, **49**, 83–87.
2 Z. Liu, Z. Chen, H. Guo, D. He, H. Zhao, Z. Wang, W. Zhang, L. Liao, C. Zhang and L. Ni, *Food Funct.*, 2016, **7**, 4869–4879.
3 R. Horanni and U. H. Engelhardt, *Eur. Food Res. Technol.*, 2015, **240**, 61–70.
4 Y. Kim, K. L. Goonder, J. D. Park, J. Choi and S. T. Talcott, *Food Chem.*, 2011, **129**, 1331–1342.
5 Z. Wu, J. Teng, L. Huang, N. Xia and B. Wei, *RSC Adv.*, 2015, **5**, 92089–92095.
6 S. A. Majidano, M. Y. Khuhawar, R. A. Zounr, A. H. Channar, T. M. Jahangir and M. Mughal, *Anal. Methods*, 2015, **7**, 3148–3156.

7 Q. F. Zhu, N. Zhang and M. J. Gong, *Anal. Methods*, 2017, **9**, 4520–4526.
8 M. Krstic, M. Stojadinovic, D. Stanic-Vucinic and T. Cirkovic Velickovic, *RSC Adv.*, 2015, **5**, 3260–3268.
9 A. Alcázar, O. Ballesteros, J. M. Jurado, F. Pablos, M. J. Martín, J. L. Vilches and A. Navalón, *J. Agric. Food Chem.*, 2007, **55**, 5960–5965.
10 Y. Zhu, Y. Luo, P. Wang, M. Zhao, L. Liu, X. Hu and F. Chen, *Food Chem.*, 2016, **194**, 643–649.
11 S. Miyauchi, T. Yuki, H. Fuji, K. Kojima, T. Yonetani, A. Tomio, T. Bamba and E. Fukusaki, *J. Biosci. Bioeng.*, 2014, **118**, 710–715.
12 J. Csapó, C. Albert and Z. Csapó-Kiss, *Acta Univ. Sapientiae, Aliment.*, 2009, **2**, 5–30.
13 I. Kolodkin-Gal, D. Romero, S. Cao, J. Clardy, R. Kolter and R. Losick, *Science*, 2010, **328**, 627–629.
14 S. H. Snyder and P. M. Kim, *Neurochem. Res.*, 2000, **25**, 553–560.
15 A. D'Aniello, *Brain Res. Rev.*, 2007, **53**, 215–234.
16 A. Morikawa, K. Hamase, T. Inoue, R. Konno and K. Zaitsu, *Amino Acids*, 2007, **32**, 13–20.
17 H. Tian, N. Zheng, S. L. Li, Y. D. Zhang, S. G. Zhao, F. Wen and J. Q. Wang, *Sci. Rep.*, 2017, **7**, 1–9.
18 H. Brückner and T. Westhauser, *Amino Acids*, 2003, **24**, 43–55.
19 R. Pätzold and H. Brückner, *Eur. Food Res. Technol.*, 2006, **223**, 347–354.
20 H. Brückner and M. Hausch, *J. High Resolut. Chromatogr.*, 1989, **12**, 680–684.
21 Y. Gogami, K. Okada and T. Oikawa, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 3259–3267.
22 S. Eto, M. Yamaguchi, M. Bounoshita, T. Mizukoshi and H. Miyano, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 3317–3325.
23 H. Brückner, M. Langer, M. Lüpke, T. Westhauser and H. Godel, *J. Chromatogr. A*, 1995, **697**, 229–245.
24 R. Koga, Y. Miyoshi, Y. Sato, M. Mita, R. Konno, W. Lindner and K. Hamase, *J. Chromatogr. A*, 2016, **1467**, 312–317.
25 L. M. Polo-Díez, M. J. Santos-Delgado, Y. Valencia-Cabrerizo and Y. León-Barrios, *Talanta*, 2015, **144**, 375–381.
26 H. Han, Y. Miyoshi, K. Ueno, C. Okamura, Y. Tojo, M. Mita, W. Lindner, K. Zaitsu and K. Hamase, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 3196–3202.
27 Z. Liang, K. Li, X. Wang, Y. Ke, Y. Jin and X. Liang, *J. Chromatogr. A*, 2012, **1224**, 61–69.
28 P. Donato, F. Rigano, F. Cacciola, M. Schure, S. Farnetti, M. Russo, P. Dugo and L. Mondello, *J. Chromatogr. A*, 2016, **1458**, 54–62.
29 L. Ramos, L. M. Hernandez and M. J. Gonzalez, *Anal. Chem.*, 1999, **71**, 70–77.
30 S. Eeltink, S. Dolman, G. Vivo-Truyols, P. Schoenmakers, R. Swart, M. Ursem and G. Desmet, *Anal. Chem.*, 2010, **82**, 7015–7020.
31 Q. Wang, P. Zhu, M. Ruan, H. Wu, K. Peng, H. Han, G. W. Somsen, J. Crommen and Z. Jiang, *J. Chromatogr. A*, 2016, **1444**, 64–73.
32 K. Y. Syu, C. L. Lin, H. C. Huang and J. K. Lin, *J. Agric. Food Chem.*, 2008, **56**, 7637–7643.
33 F. Sari and S. Y. Velioglu, *J. Food Compos. Anal.*, 2011, **24**, 1130–1135.
34 A. Jámbor and I. Molnár-Perl, *J. Chromatogr. A*, 2009, **1216**, 3064–3077.
35 Q. Wang, N. Cheng, W. Yi, S. Peng and X. Zou, *Bioorg. Med. Chem.*, 2014, **22**, 1515–1521.
36 Q. Wang, N. Cheng and X. Zheng, *Bioorg. Med. Chem.*, 2013, **21**, 4301.
37 S. Boonchiangma, P. Ratchakrut, S. Chanthai and S. Srijaranai, *Chromatographia*, 2015, **78**, 923–927.
38 J. Y. Jin and W. Lee, *Bull. Korean Chem. Soc.*, 2008, **29**, 491–493.
39 R. Horanni and H. U. Engelhardt, *J. Food Compos. Anal.*, 2013, **31**, 94–100.
40 J. L. Bernal, M. J. Nozal, L. Toribio, J. C. Diego and A. Ruiz, *J. Sep. Sci.*, 2005, **28**, 1039–1047.

