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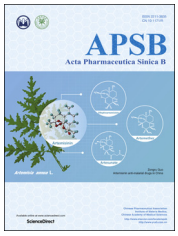

ORIGINAL ARTICLE

Investigation of free amino acid, total phenolics, antioxidant activity and purine alkaloids to assess the health properties of non-*Camellia* tea

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KEY WORDS

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Abstract To find novel functional beverages from folk teas, 33 species of frequently used non-*Camellia* tea (plants other than *Camellia*) were collected and compared with *Camellia* tea (green tea, pu-erh tea and black tea) for the first time. Data are reported here on the quantities of 20 free amino acids (FAAs) and three purine alkaloids (measured by UHPLC), total polyphenols (measured by Folin-Ciocalteu assay), and antioxidant activity (DPPH). The total amounts of FAAs in non-*Camellia* tea (0.62–18.99 mg/g) are generally less than that of *Camellia* tea (16.55–24.99 mg/g). However, for certain FAAs, the quantities were much higher in some non-*Camellia* teas, such as γ-aminobutyric acid in teas from *Ampelopsis grossedentata*, *Isodon serra* and *Hibiscus sabdariffa*. Interestingly, theanine was detected in tea from *Potentilla fruticosa* (1.16±0.81 mg/g). Furthermore, the content of polyphenols in teas from *A. grossedentata*, *Acer tataricum* subsp. *ginnala* are significantly higher than those from *Camellia* tea; teas from *I. serra*, *Pistacia chinensis* and *A. tataricum* subsp. *ginnala* have remarkable antioxidant activities similar to the activities from green tea (44.23 μg/mL). Purine alkaloids (caffeine, theobromine

Abbreviations: AABA, α-aminobutyric acid; AccQ, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; AMQ, 6-aminoquinoline; AQC, 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; EA, essential amino acid; EDTA, ethylene diamine tetraacetic acid; FAAs, free amino acids; F-C, Folin-Ciocalteu; GABA, γ-aminobutyric acid; GAE, gallic acid equivalents; HCA, hierarchical cluster analysis; HEA, half-essential amino acid; NEA, non-essential amino acid; PCA, principal component analysis; RSD, relative standard deviation; Thea, theanine; UHPLC, ultra-high performance liquid chromatography

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and theophylline) were not detected in non-*Camellia* teas. The investigation suggest some non-*Camellia* teas may be great functional natural products with potential for prevention of chronic diseases and aging, by providing with abundant polyphenols, antioxidants and specific FAAs.

1. Introduction

Our expected lifespan continues increasing, but many of us will lead a higher percentage of our lives in poor health conditions due to aging and increasing threats by many chronic diseases such as hypertension, hyperlipidemia, diabetes, chronic inflammation, and other stressors. As healthcare costs increase, preventive approaches (*e.g.*, supplemental diets, functional foods or drinks) are gaining in popularity. These alternatives represent inexpensive and readily applicable approaches to reduce the incidence of chronic diseases. Such preventive health products have gained a great deal of attention from both the scientific community and the general public. Additionally, exploration of the natural and sustainable resources for healthcare and supplementary nutrition has become crucial for the future of developing countries and for those with large populations such as China.

It is well-known that green teas prepared from leaves of *Camellia* plants have many important physiological properties and health benefits. Drinking green teas may reduce the risk of many diseases, such as cancers and cardiovascular diseases, as teas have a variety of biological activities including anti-tumor, anti-oxidation, and anti-obesity[1–3]. Previous studies have demonstrated that amino acids, polyphenols and purine alkaloids are important nutritional and active components in green teas. Examples include the nutritional roles for essential amino acids and the pharmacological effects of theanine and γ-aminobutyric acid (GABA). Theanine is a major amino acid uniquely found in green tea which can decrease norepinephrine and serotonin levels in the brain, lower blood pressure and produce neuroprotective and cognitive-enhancing actions[4,5]. GABA is an important inhibitory neurotransmitter in the mammalian central nervous system and is known to exhibit antihypertensive effects[6]. Teas rich in GABA can decrease blood pressure in rats[7]. Amino acids also participate in the biosynthesis of polyphenols and alkaloids[8,9]. The antioxidant and free radical-scavenging abilities of polyphenols in green tea may play an important role in the prevention of cardiovascular disease, chronic gastritis and some cancers[10]. However, purine alkaloids (such as caffeine, theobromine and theophylline), as additional key components of tea and coffee, may have negative impacts on our wellness when the intake is high[11].

A variety of folk teas originated from plants other than *Camellia* have been used by various indigenous and minority groups in China for centuries. Their potential nutritional and medical value in preventing chronic diseases has not been widely recognized or compared with common green teas until recent years. Folk teas are also a part of the traditional Chinese tea culture and are widely consumed as beverages and medicines in folk for disease prevention and treatment[12,13]. Recently, our team performed a systematic collection and some chemical and pharmacological investigations of these traditional teas and named them as non-*Camellia* teas (or in Chinese: Bie-yang-cha)[14–16]. Our previous studies demonstrated that many non-*Camellia* teas contain abundant polyphenols, show striking antioxidant effects *in vitro*, and have many other health benefits[17–20].

Due to the biological significance of the amino acids, polyphenols and alkaloids contained in green tea, for the first time we systematically investigated these important types of constituents and the antioxidant activity of extracts of plants for non-*Camellia* teas, in order to provide information on the health properties and promote the development of non-*Camellia* teas as functional beverages. In this study, 33 non-*Camellia* tea samples were collected based on these criteria: those teas (i) are not originated from *Camellia* plants; (ii) are popularly used for at least three hundred years in some minorities' areas or indigenous groups in China; and (iii) are presently consumed.

Several well-established biochemical methods were used in our study. The ultra-high performance liquid chromatography (UHPLC) method was used to determine the amino acid content. It is a rapid, modern, and effective method which has been used to detect amino acids in cheese and green tea[21,22]. Precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AccQ) is a widely used method for the derivatization of amino acids[21,23,24], but the use of UHPLC with precolumn derivatization with AccQ to detect 20 amino acids has not been previously reported. UHPLC was also applied to determine the purine alkaloid content. Lastly, the phenolic content and antioxidant activity of these tea products were determined using the Folin-Ciocalteu (F-C) assay and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method, respectively. The F-C assay and DPPH methods provide convenient, rapid and simple estimation of the total phenols content and antioxidant activity, respectively.

In the present paper, the content of the 20 free amino acids (FAAs), polyphenols, three purine alkaloids (caffeine, theobromine and theophylline), as well as the antioxidant activity, were investigated in 33 non-*Camellia* teas. The 20 investigated FAAs were: 9 essential amino acids (EA), threonine (Thr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), tryptophan (Trp) and histidine (His); 6 conditionally essential amino acids (HEA): arginine (Arg), cysteine (Cys), glycine (Gly), glutamine (Glu), proline (Pro) and tyrosine (Tyr); 3 dispensable or non-essential amino acids (NEA): alanine (Ala), aspartic acid (Asp), serine (Ser), and 2 activated amino acids (GABA and Thea).

2. Materials and methods

2.1. Collection of tea samples

The 33 non-*Camellia* teas (119 accessions) were collected in China from 2008 through 2013, the 9 *Camellia* teas (3 green teas, 3 pu-erh teas and 3 black teas, respectively) were purchased from

Table 1 Information on the tea samples.

Tea No.	Sample No.	Origin	Family	Chinese name	Source provinces or regions	Collection time
1	BYC 1–4	*Engelhardtia roxburghiana* Lindl.	Juglandaceae	Luo-han-cha	Lingyun and Jinxiu, Guangxi	2011–2012
2	BYC 5–7	*Elsholtzia bodinieri* Vaniot	Lamiaceae	Feng-wei-cha	Yunnan	2009–2010
3	BYC 8–13	*Mallotus peltatus* (Geiseler) Müll.Arg.	Euphorbiaceae	Zhe-gu-cha	Hainan	2009–2012
4	BYC 14–16	*Chimonanthus nitens* Oliv.	Calycanthaceae	Xiang-feng-cha	Zhejiang	2011–2012
5	BYC 17–19	*Hibiscus sabdariffa* L.	Malvaceae	Mei-gui-qie-cha	Guangxi	2012–2013
6	BYC 20–22	*Rubus chingii* var. *suavissimus* (S.K.Lee) L.T.Lu	Rosaceae	Tian-cha	Medicinal plant garden and Jinxiu, Guangxi	2010–2013
7	BYC 23–25	*Fagopyrum tataricum* (L.) Gaertn.	Polygonaceae	Ku-qiao-cha	Shanxi	2011–2012
8	BYC 26–28	*Thamnolia vermicularis* (Sw.) Ach.[a]	Thamnoliaceae	Tai-bai-cha	Shaanxi	2008–2009
9	BYC 29–34	*Apocynum venetum* L.	Apocynaceae	Luo-bu-ma-cha	Cangzhou, Hebei; Fenglingdu and Pinglu, Shanxi; Liaoning; Alatai, Xinjiang	2009–2011
10	BYC 35–39	*Ampelopsis grossedentata* (Hand.-Mazz.) W.T.Wang	Vitaceae	Teng-cha	Hunan; Guizhou; Shaowu, Fujian	2010–2011
11	BYC 40–45	*Ligustrum robustum* (Roxb.) Blume	Oleaceae	Xiao-ye-ku-ding-cha	Yuqing, Junnian and Yuqing, Guizhou; Zhaotong, Yunnan; Hainan	2008–2009
12	BYC 46–48	*Adinandra nitida* Merr. ex H.L.Li	Pentaphylacaceae	Shi-ya-cha	Jinxiu and Shentangshan, Guangxi	2011–2012
13	BYC 49–52	*Ilex latifolia* Thunb.	Aquifoliaceae	Da-ye-ku-ding-cha	Emei, Sichuan; Zhejiang; Guangxi; Wuzhishan, Hainan	2008–2010
14	BYC 53–56	*Litsea coreana* var. *lanuginosa* (Migo) Yang & Huang[a]	Lauraceae	Lao-yin-cha	Sichuan; Guizhou; Hangzhou, Zhejiang	2008–2011
15	BYC 57–59	*Sarcandra glabra* (Thunb.) Nakai	Chloranthaceae	Jiu-jie-cha	Jiangxi	2011–2012
16	BYC 60–62	*Cassia obtusifolia* (L.) H.S.Irwin & Barneby	Leguminosae	Jue-ming-zi-cha	Linxia	2010
17	BYC 63–65	*Isodon serra* (Maxim.) Kudô	Lamiaceae	Xi-huang-cao-cha	Shaoguan, Guangdong	2011–2012
18	BYC 66–68	*Forsythia suspensa* (Thunb.) Vahl	Oleaceae	Lian-qiao-ye-cha	Shandong	2012
19	BYC 69–71	*Chrysanthemum indicum* L.	Compositae	Ye-ju-hua-cha	Zhejiang	2011–2012
20	BYC 72–74	*Dendranthema morifolium* (Ramat.) Tzvelev	Compositae	Ju-hua-cha	Huangshan, Anhui	2011–2012
21	BYC 75–80	*Potentilla fruticosa* L.[a]	Rosaceae	Yao-wang-cha	Shaanxi; Jilin	2008–2012
22	BYC 81–83	*Malus hupehensis* (Pamp.) Rehder	Rosaceae	Hua-hong-cha	Xiangyang, Hubei	2012–2013
23	BYC 84–86	*Gynostemma pentaphyllum* (Thunb.) Makino	Cucurbitaceae	Jiao-gu-lan-cha	Jinxiu, Guangnxi	2012–2013
24	BYC 87–89	*Cratoxylum cochinchinense* (Lour.) Blume	Hypericaceae	Huang-niu-cha	Xinyi, Guangdong	2012–2013
25	BYC 90–93	*Lycium barbarum* L.	Solanaceae	Gou-qi-ye-cha	Yinchuan, Linxia	2011–2013
26	BYC 94–96	*Scoparia dulcis* L.	Plantaginaceae	Si-shi-cha	Fujian	2012–2013
27	BYC 97–99	*Cyclocarya paliurus* (Batalin) Iljinsk.	Juglandaceae	Qin-qian-liu-cha	Suining, Hunan	2010–2011
28	BYC 100–102	*Acer tataricum* subsp. *ginnala* (Maxim.) Wesm.	Sapindaceae	Ku-jin-cha	Jilin; Liaoning	2011–2013

Table 1 (*continued*)

Tea No.	Sample No.	Origin	Family	Chinese name	Source provinces or regions	Collection time
29	BYC 103–105	*Pistacia chinensis* Bunge	Anacardiaceae	Huang-li-ya-cha	Loudi, Hunan	2013
30	BYC 106–108	*Coreopsis tinctoria* Nutt.	Compositae	Kun-lun-xue-ju-cha	Xinjiang	2012–2013
31	BYC 109–111	*Orthosiphon aristatus* (Blume) Miq.	Lamiaceae	Shen-cha	Yunnan	2011–2012
32	BYC 112–116	*Scutellaria baicalensis* Georgi	Lamiaceae	Huang-qin-cha	Hebei; Beijing; Chengde, Hebei; Zhuozi and Yakeshi, Neimenggu	2008–2012
33	BYC 117–119	*Lithocarpus litseifolius* (Hance) Chun	Fagaceae	Duo-sui-ke-cha	Mashan, Guangxi	2012
34	BYC 120–122	*Camellia sinensis* (L.) Kuntze	Theaceae	Green tea	Emei, Sichuan	2010–2013
35	BYC 123–125	*Camellia sinensis* (L.) Kuntze	Theaceae	Black tea	Fujian	2010–2013
36	BYC 126–128	*Camellia sinensis* (L.) Kuntze	Theaceae	Pu-erh tea	Yunnan	2010–2013

Most Latin names and families of original plants were identified in TPL (www.theplantlist.org), except the plants signed with '[a]' which were identified according to Flora of China (2000).

local retailor shops (Table 1). Except for the teas from *Hibiscus sabdariffa*, *Chrysanthemum indicum*, *Dendranthema morifolium* and *Coreopsis tinctoria* are derived from flowers, *Fagopyrum tataricum* and *Cassia obtusifolia* are from seeds, the plant part of all other teas are from leaves. All samples were kept sealed and stored in dry and cool places before testing.

The plant species of the tea samples were authenticated by Dr. Peigen Xiao and all species were validated taxonomically as described on the web site www.theplantlist.org. The voucher specimens were deposited in Xiao's laboratory at the Institute of Medicinal Plant Development of the Chinese Academy of Medical Sciences in Beijing, China.

2.2. Chemicals and standards

AccQ · Tag™ Ultra UPLC™ amino acid analysis derivatization kits were purchased from Waters (Milford, MA, USA). The 20 FAAs, caffeine, theobromine, theophylline and gallic acid standards were purchased from Winherb Medical Technology Co., Ltd. (Shanghai, China). Mixed amino acid standard stock solutions of 2.5 mmol/L were prepared in 0.1 mol/L HCl and stored at 4 °C. Diluted solutions were freshly prepared weekly.

HPLC-grade acetonitrile was purchased from Honeywell Burdick & Jackson (Morristown, NJ, USA). HPLC-grade methanol was from Fisher Scientific (Atlanta, GA, USA) and hydrochloric acid, formic acid, and ortho-phosphoric acid were from CNW Technologies GmbH (Dusseldorf, Germany). Sodium acetate and triethylamine were purchased from Guangfu Technology Development Co. (Tianjin, China). Sodium azide, α-aminobutyric acid (AABA, internal standard), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and Folin-Ciocalteu phenol reagent were from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate was purchased from Beijing Chemical Works (Beijing, China). Trolox solution (10 mmol/L) was purchased from Beyotime Biotechnology (Haimen, Jiangsu, China). All reagents used were of analytical grade. All solutions were made with Milli-Q water (Millipore, Bedford, MA). Eluent A concentrate for gradient elution contained sodium acetate (1.4 mol/L), sodium azide (1.5 mmol/L), disodium EDTA (2.6 mmol/L) and tri-ethyl amine (170 mmol/L) in water, and was titrated to pH 4.95 with phosphoric acid.

2.3. Preparation of tea samples

Tea samples were prepared as follows: 250 mg (for amino acid and antioxidant analysis) and 150 mg (for total phenolics and purine alkaloids analysis) of teas were extracted with 10 mL of distilled water at 80 °C for 30 min respectively. After the tea water extract was cooled to room temperature, the volume was brought back to 10 mL with water. The sample solutions were filtered through a 0.22 μm nylon membranes purchased from Jinteng experiment equipment Co., Ltd. (Tianjin, China).

2.4. Analysis of total polyphenols

The total phenolic content of the tea samples was determined according to a procedure described by Ainsworth and Gillespie[25] with slight modifications. One hundred microliters of each filtered sample solution was mixed with 200 μL of 10% (*v/v*) F-C phenol reagent and 800 μL of 700 mmol/L sodium carbonate solution in a 2-mL microtube for 2 h before spectrometric analysis. Finally, 200 μL of the sample, standard or blank was transferred to clear 96-well microplates, and the absorbances read at 765 nm using the Quant MQX200 microplate reader from Biotek Instruments, Inc. (Winooski, VT, USA). Gallic acid was used as standards for quantification, and the results were expressed as percent gallic acid equivalents (GAE).

2.5. Antioxidant assay

The antioxidant activities of the tea samples were evaluated by DPPH method as previously reported, with some modifications[26–28]. Briefly, 7 μL of the diluted sample or Trolox

(six different concentrations) or ethanol was added to 193 μL of 0.2 mmol/L DPPH (freshly prepared prior to assay), then left at ambient temperature in the dark for 30 min. The absorbance was measured at 517 nm. Trolox and ethanol were used as the reference and control, respectively. The DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH Radical Scavenging Activity } (\%) = \left[(A_0 - A_1)/A_0\right] \times 100 \quad (1)$$

where A_1 is the absorbance of the test sample, and A_0 is the absorbance of the control. Results are expressed as EC_{50} and values are referred to the concentration of the tea infusions required for the 50% of the antioxidant activity (g/mL).

2.6. Derivatization reaction of free amino acids

Precolumn 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC) derivatization of FAAs was accomplished using a Waters AccQ · Tag™ reagent kit. Amino acid standards or tea samples (10 μL) were derivatized directly by mixing with 70 μL AccQ · Tag™ borate buffer. After adding 20 μL derivatizing reagent (10 mmol/L AccQ · Tag™ reagent), the mixtures were immediately vortexed, left to rest for 1 min at room temperature and finally heated for 10 min at 55 °C to complete the derivatization. Derivatized sample solutions were then subjected to chromatographic analysis. The concentration level of each analyte was approximately as follows: 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 200 and 400 pmol/μL.

2.7. UHPLC instrument and chromatographic conditions

The analysis of FAAs and alkaloids was carried out by using the DIONEX Ultimate 3000 UHPLC system equipped with a HPG3400 RS Pump, SRD-3400 degasser, WPS-3000T RS Auto-Sampler, TCC-3000RS Column Compartment, DAD-3000RS Diode Array Detector and Chromeleon chromatography software package (6.8 version).

UHPLC separation of AQC-derivatized amino acids was performed with a Waters Acquity UPLC™ column (BEH C18, 100 mm × 2.1 mm, 1.7 μm). The mobile phase A was Eluent A concentrate diluted 1:10 with ultrapure water while B was 60% (*v/v*) acetonitrile. The temperature of the column oven was set at 37 °C. The elution program is described in Table 2. After the program, the initial conditions were regenerated within 0.5 min and maintained for another 4.5 min, resulting in a net separation time of 11.5 min and a 16 min total cycle time. The injection volume was 2 μL, and UV detection of AQC amino acid derivatives was performed at 254 nm. Data were archived using Chromeleon chromatography software package. All of the operations and the data acquisition were controlled by a DIONEX ChemStation.

The caffeine, theobromine and theophylline content of the tea samples were determined according to our previously reported procedure[29]. Briefly, UHPLC was performed with DIONEX Acclaim PA2 column (150 mm × 2.1 mm, 2.2 μm). The mobile phase A was acetonitrile, while B was ultrapure water. The flow rate was set to 0.5 mL/min, and the temperature of column oven was set at 30 °C. Elution was accomplished with a linear gradient of phase A from 5% to 15% in 10 min. The injection volume was 10 μL, and UV detection of these alkaloids was performed at 272 nm.

2.8. UHPLC method validation

The precision, repeatability, stability, detection and quantification limits, linearity ranges and recovery of the proposed method were validated following the guideline of the International Conference on Harmonisation (ICH)[30]. To evaluate the precision of the method, a standard solution was injected six times successively. To get the repeatability of the method, six replicate analyses of a standard solution were performed to determine both the retention time and the peak area of the amino acid standards. The stability was assessed by injecting a standard solution six times within 0, 2, 4, 6, 8, 16, and 24 h, respectively. Detection and quantification limits (LOD and LOQ) were calculated at the respective signal-to-noise ratios of 3 and 10. Linear calibration curves were calculated over a concentration range of 0.001–1600 pmol per 2 μL injection volume. The recovery was assessed by the experiments in which the known amounts of AMQ (6-aminoquinoline)–amino acid standards (0.01, 0.50 and 1 mmol/L) were added to the tea samples. Each standard was analyzed in triplicate, and the peak area was plotted against the corresponding concentrations.

Table 2 Elution ternary gradient for UHPLC determination of amino acid concentrations.

Time (min)	Flow rate (mL/min)	Eluent A	Eluent B
0	0.40	88.0	12.0
1	0.20	96.0	4.0
2	0.20	93.0	7.0
3	0.15	90.0	10.0
3.5	0.30	83.0	17.0
4	0.30	82.0	18.0
4.5	0.50	78.0	22.0
6	0.20	76.0	24.0
7	0.20	76.0	24.0
8	0.50	65.0	35.0
10	0.50	65.0	35.0
10.1	0.50	0.0	100.0
11	0.50	0.0	100.0

Eluent A: contained sodium acetate (140 mmol/L), sodium azide (0.15 mmol/L), disodium EDTA (0.26 mmol/L) and tri-ethyl amine (17 mmol/L), in water, and was titrated to pH 4.95 with phosphoric acid.
Eluent B: 60% (*v/v*) acetonitrile.

2.9. Statistical analysis

Mean values of each sample were obtained from at least three replications. The hierarchical cluster analysis was conducted by R 3.1.0 with package Pheatmap 0.7.7 (Free Software Foundation). The Python 2.7.8 software (Python Software Foundation) was used for principal component analysis (PCA). Pearson correlation was performed by SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Validation of FAA analysis method

The gradient elution program of the UHPLC method was optimized, and the final analytical method achieved a high resolution allowing the identification of target compounds in a short time (Fig. 1A). All analyzed AQC derivatives exhibited appropriate linearity ($R^2 \geq 0.9950$) within the applied calibration range (10–400 pmol per 2 μL injection volume) at 254 nm.

For most of the amino acids, the instrumentation precision of relative standard deviation (RSD) of amino acid standards varied $<3\%$, whereas the repeatability (RSD) was $<5\%$ for all analytes. The stability of amino acid standards (RSD) varied $<4\%$. Limits of detection and quantification (LOD and LOQ) were calculated at the respective signal-to-noise ratios of 3 and 10, with on-column amounts of AQC derivatives ranging between 0.05–0.10 pmol and 0.10–0.50 pmol (each per 2 μL injection volume), respectively. The recovery rate was calculated by comparing the obtained amounts with those added, and values ranged between 80% and 105%. Therefore, the overall UHPLC analytical procedure was fast, accurate and suitable for the quantitative analysis of a large number of samples.

3.2. Quantitative analysis of selected FAAs in various non-Camellia teas

The new analytical method was subsequently applied to simultaneously determine 20 FAAs levels of 119 samples from 33 non-*Camellia* teas representing a variety of types. Green tea (34), black tea (35) and pu-erh tea (36) from *Camellia* leaves were also analyzed at the same time for comparison. The individual contents of the 20 FAAs in these samples are listed in Table S1.

The results showed that most non-*Camellia* teas had relatively moderate levels of the 20 FAAs (Table S2). The total amino acid content in green tea (34) (24.99 mg/g) was the highest followed by pu-erh tea (36), black tea (35), teas from *Lycium barbarum* (25), *H. sabdariffa* (5), *Gynostemma pentaphyllum* (23) and *Scutellaria*

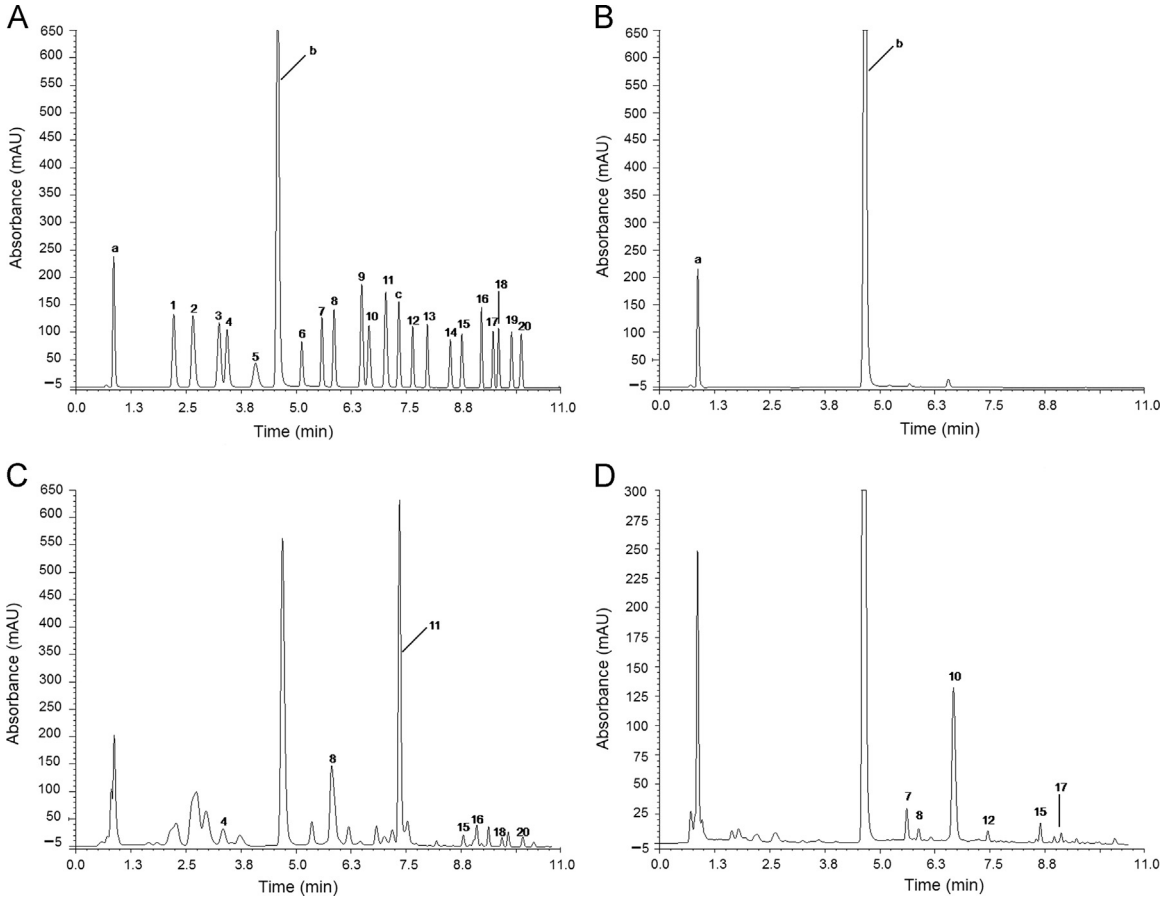

Figure 1 Representative UHPLC chromatograms of amino acids. (A) Standard solution (400 μmol/L): 1, aspartic acid (Asp); 2, glutamine (Glu); 3, serine (Ser); 4, histidine (His); 5, glycine (Gly); 6, arginine (Arg); 7, threonine (Thr); 8, alanine (Ala); 9, proline (Pro); 10, γ-aminobutyric acid (GABA); 11, theanine (Thea); 12, cysteine (Cys); 13, tyrosine (Tyr); 14, valine (Val); 15, methionine (Met); 16, lysine (Lys); 17, isoleucine (Ile); 18, leucine (Leu); 19, phenylalanine (Phe); 20, tryptophan (Trp). a, Solvent; b, 6-aminoquinoline (AMQ); c, α-aminobutyric acid (AABA). (B) Blank sample (Do not add tea sample, only add derivatization reagent); 2 tea samples: (C) green tea and (D) *Ampelopsis grossedentata*.

baicalensis (32), with contents ranging from 10.41 to 18.69 mg/g. Teas from *C. tinctoria* (30), *Pistacia chinensis* (29), *Forsythia suspensa* (18), *Litsea coreana* var. *lanuginose* (14), *Adinandra nitida* (12), *Acer tataricum* subsp. *ginnala* (28), *D. morifolium* (20), *Rubus chingii* var. *suavissimus* (6), *C. indicum* (19), and *Scoparia dulcis* (26) were at intermediate levels ranging from 5.24 to 9.70 mg/g. The other teas had lower concentrations ranging from 0.62 to 4.67 mg/g (Fig. 2A). Moreover, essential amino acid (EAAs) concentrations were high in black tea (35, 18.29 mg/g). Pu-erh tea (36), teas from *H. sabdariffa* (5), *L. barbarum* (25), *P. chinensis* (29), *A. nitida* (12) and green tea (34) with intermediate levels (4.62–8.17 mg/g). Other teas had lower levels (less than 4.50 mg/g) (Fig. 2B). HEAs are considered conditionally essential in the human diet, meaning their synthesis can be limited under special pathophysiological conditions. The content of HEAs was highest in tea from *L. barbarum* (25, 6.24 mg/g), and

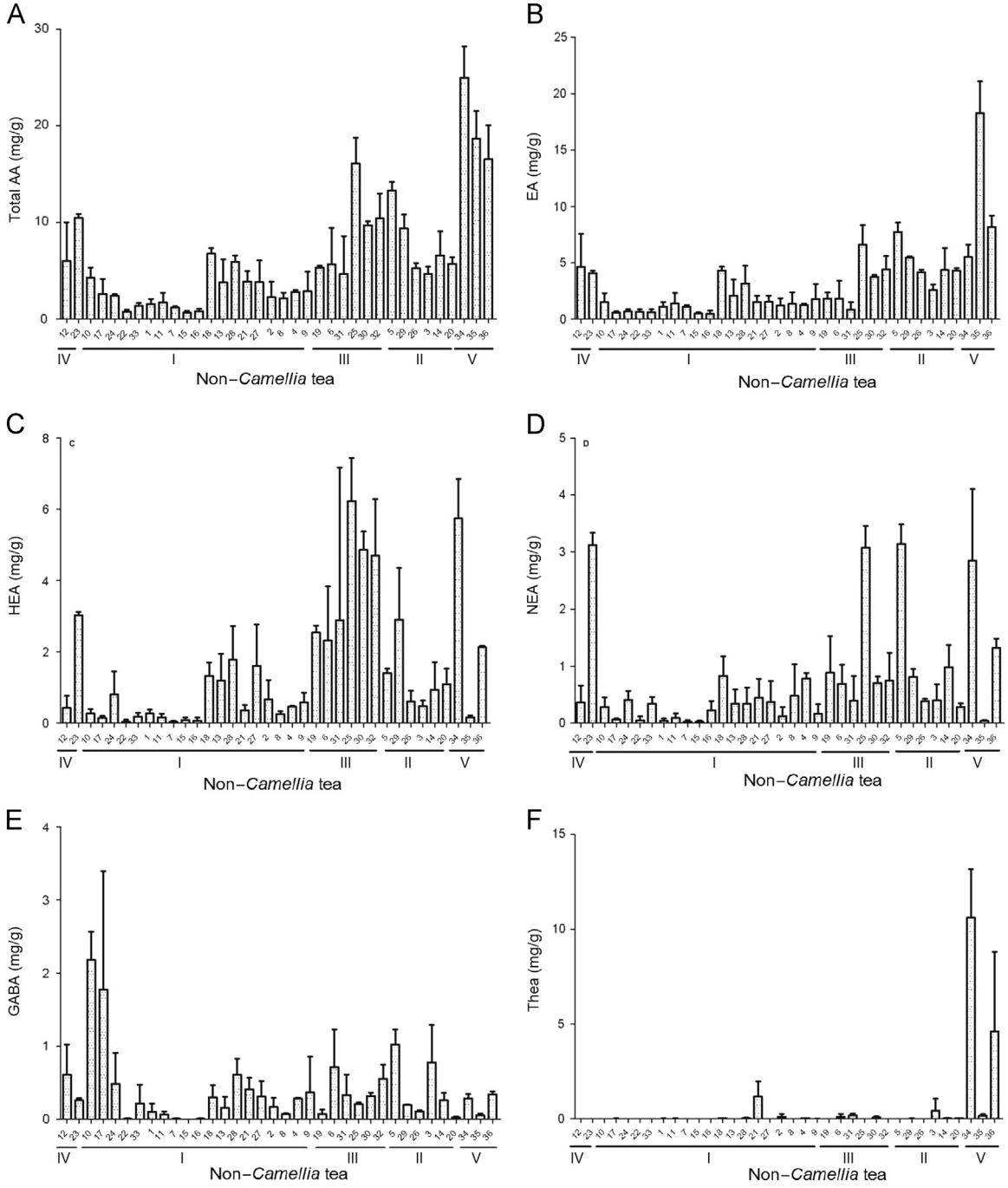

Figure 2 Content of amino acids in non-*Camellia* tea. (A) Total (total amino acid); (B) EA (essential amino acid, including His, Thr, Val, Met, Lys, Ile, Leu, Phe, Trp); (C) HEA (half-essential amino acid, including Glu, Gly, Arg, Pro, Cys, Tys); (D) NEA (non-essential amino acid including Asp, Ser, Ala); (E) GABA; (F) Thea. I, II, III, IV: corresponding to the 4 classes in Fig. 4, V: three *Camellia* tea (green tea, black tea and pu-erh tea).

followed by green tea (34, 5.74 mg/g), teas from *C. tinctoria* (30, 4.86 mg/g) and *S. baicalensis* (32, 4.70 mg/g). Teas from *G. pentaphyllum* (23, 3.03 mg/g), *P. chinensis* (29, 2.91 mg/g), *Orthosiphon aristatus* (31, 2.88 mg/g), *C. indicum* (19, 2.54 mg/g), *R. chingii* var. *suavissimus* (6, 2.31 mg/g). Pu-erh tea (36, 2.13 mg/g) contained relatively large amounts of HEA ranging from 2.13 to 3.03 mg/g, while other teas had less than 1.60 mg/g (Fig. 2C). The three NEAs, which are dispensable in humans and can be synthesized in the body, existed at relatively high amounts in teas from *L. barbarum* (25, 3.08 mg/g), *G. pentaphyllum* (23, 3.12 mg/g), *H. sabdariffa* (5, 3.14 mg/g) and green tea (34, 2.85 mg/g), and less than 1.5 mg/g in the other teas (Fig. 2D).

Although the total content of amino acids in each non-*Camellia* tea is lower than the content in the three common *Camellia* teas, the contents of some specific amino acids are significantly higher in non-*Camellia* teas than in green tea. In terms of the essential amino acids, the respective Thr contents of each of the non-*Camellia* were: *P. chinensis* (29, 3.85 mg/g), *L. barbarum* (25, 2.67 mg/g), *S. dulcis* (26, 3.66 mg/g), *S. baicalensis* (32, 2.68 mg/g), *L. coreana* var. *lanuginose* (14, 2.57 mg/g) and *D. morifolium* (20, 2.75 mg/g). The respective Val contents were *L. barbarum* (25, 0.60 mg/g), *S. baicalensis* (32, 0.76 mg/g), *R. chingii* var. *suavissimus* (6, 0.35 mg/g), and *A. nitida* (12, 0.52 mg/g). Met contents were *F. suspensa* (18, 2.09 mg/g). The Lys content in *F. suspensa* was (18, 0.51 mg/g). The Phe contents respectively were *L. barbarum* (25, 1.33 mg/g), *A. nitida* (12, 3.03 mg/g) and *G. pentaphyllum* (23, 1.65 mg/g), significantly higher than in green tea (34, 1.95 mg/g, 0.17 mg/g, 0.56 mg/g, 0.29 mg/g, 0.53 mg/g, respectively). In terms of NEA, the content of Asp in teas from *H. sabdariffa* (5, 2.61 mg/g) and *G. pentaphyllum* (23, 2.75 mg/g) were significantly higher than in green tea (34, 1.24 mg/g).

In terms of HEA, Gly showed low contents in all the samples but tea from *H. sabdariffa* showed the highest content (0.21 mg/g). The content of Arg in tea from *P. chinensis* (29, 1.93 mg/g) was relatively higher than that in three *Camellia* teas. The content of Pro in teas from *R. chingii* var. *suavissimus* (6, 2.03 mg/g), *C. indicum* (19, 1.40 mg/g), *L. barbarum* (25, 3.91 mg/g), *C. tinctoria* (30, 4.41 mg/g), *Orthosiphon aristatus* (31, 2.48 mg/g) and *S. baicalensis* (32, 3.17 mg/g), were significantly higher than in three *Camellia* teas which ranged from 0.05 to 0.31 mg/g. Except for nine teas (including 4–8, 15, 16, 22, 33), Cys content in most non-*Camellia* teas also showed higher concentration than in the three *Camellia* teas.

Of note, GABA was detected in all teas except tea from *Sarcandra glabra* (15, Fig. 2E). Thea was detected in approximately half of the teas (Fig. 2F). The GABA content in teas from *Ampelopsis grossedentata* (10), *Isodon serra* (17) and *H. sabdariffa* (5) was the highest (all above 1.02 mg/g), much higher than that in green tea (34, 0.28 mg/g). For Thea, green tea (34) was found to have significantly higher content (10.61 mg/g) than the others. Pu-erh tea (36) was the second highest at approximately 4.59 mg/g. The only other tea with a Thea content above 1.0 mg/g was tea from *Potentilla fruticosa* (21, 1.16 mg/g). All others had less than 0.5 mg/g. We are the first group reporting these results, and further studies on more tea samples remain to be performed.

3.3. Principal component analysis (PCA) of teas

The potential utility of employing PCA using a combination of the key parameters and the contents of the 20 FAAs as a means of classifying the teas was explored. The PCA provided three eigenvalues, which were analyzed to obtain three factors (Z1–Z3, Table S3). The proportion of each eigenvalue was computed, and the cumulative proportion of the three factors was found to total 90.61%. The three factors were retained for further analyses because their cumulative proportions were higher than 90%, which is considered adequate for the estimation of FAAs patterns. The eigenvectors of the patterns of the 20 FAAs used to obtain the three factors are summarized in Table S4. The plot of the PCA scores, as shown in Fig. 3A, was readily divided into two relative clusters, which indicates that the content and distribution of the 20 FAAs are highly varied in the three *Camellia* teas and 33 non-*Camellia* teas. The plots of the PCA loadings were utilized to identify the differential FAAs for the discrimination of groups. Gly and Arg were largely influenced by the first factor (PC1), whereas His was dominant by the second factor (PC2). Try, Cys and Arg were mainly influenced by the third factor (PC3, Fig. 3B).

A similar PCA was performed to analyze 33 non-*Camellia* teas based on content of the 20 FAAs. The cumulative proportion of the three factors was found to total 82.51% (Table S5), and the eigenvectors of the patterns of the 20 FAAs used to obtain the three factors are summarized in Table S6. The plot of the PCA scores, as shown in Fig. 3C, was readily divided into four relative clusters, indicating that the content and distribution of the 20 FAAs is highly varied in the different non-*Camellia* teas. The plots of the PCA loadings were utilized to identify the differential FAAs for the discrimination of groups. Gly, Arg and Try are largely influenced by the first factor (PC1), whereas His and Thr are dominant by the second factor (PC2). Try and Arg are mainly influenced by the third factor (PC3, Fig. 3D).

3.4. Cluster analysis of non-Camellia tea based on FAA level

Hierarchical cluster analysis (HCA) was used to confirm the results of the PCA analysis. The content of the 20 FAAs obtained for the 33 non-*Camellia* teas was standardized into the Euclid equation to obtain the Euclidean distances between the samples. The cluster analysis provided a dendrogram, and Ward's minimum-variance cluster analysis (WMVCA) produced two large sub-clusters, which were denoted A and B (Fig. 4).

Cluster B was further divided into two sub-clusters, which were denoted II and III. Sub-cluster II, which is comprised of 3, 5, 14, 20, 26 and 29, was defined by relatively high contents of Gly and Thr. Sub-cluster III also consisted of six non-*Camellia* teas, including 6, 19, 25, 30, 31 and 32; this sub-clusters contained a high amount of Pro, Thr and Glu. Cluster I included 19 non-*Camellia* teas have relatively low content of the total amino acid (<4.0 mg/g) except 10, 18, and 28 (4.26, 6.77 and 5.91 mg/g, respectively). Among these teas, 10 and 17 with high content of GABA could be considered together for a separate class. Cluster IV included two non-*Camellia* teas (12 and 23) which exhibited relatively high contents of Phe, Asp and Glu.

HCA was also conducted to accurately describe the content characters among the 20 FAAs (Fig. 4). Thr was the FAA with the highest average content of (1.42 mg/g), followed by Pro (0.69 mg/g). GABA, Phe, Glu and Asp were at intermediate levels in all 20 FAAs ranging from 0.27 to 0.40 mg/g, while the other FAAs had a lower content ranging from 0.09 to 0.25 mg/g.

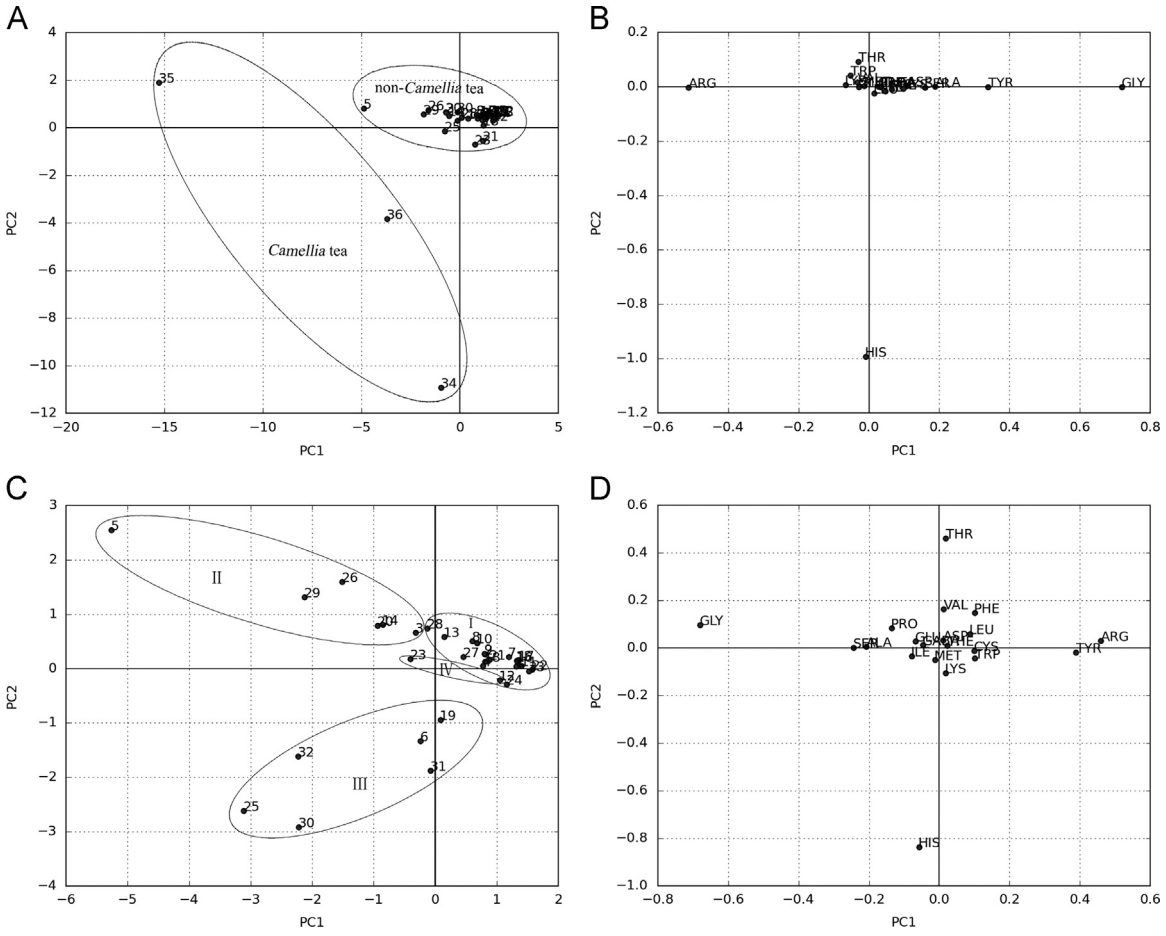

Figure 3 Principal component analysis of amino acids in teas. (A) Scores plot of 36 teas; (B) loading plot of 36 teas; (C) scores plot of 33 non-*Camellia* teas; (D) loading plot of 33 non-*Camellia* teas. I, II, III, IV: corresponding to the 4 classes in Fig. 4.

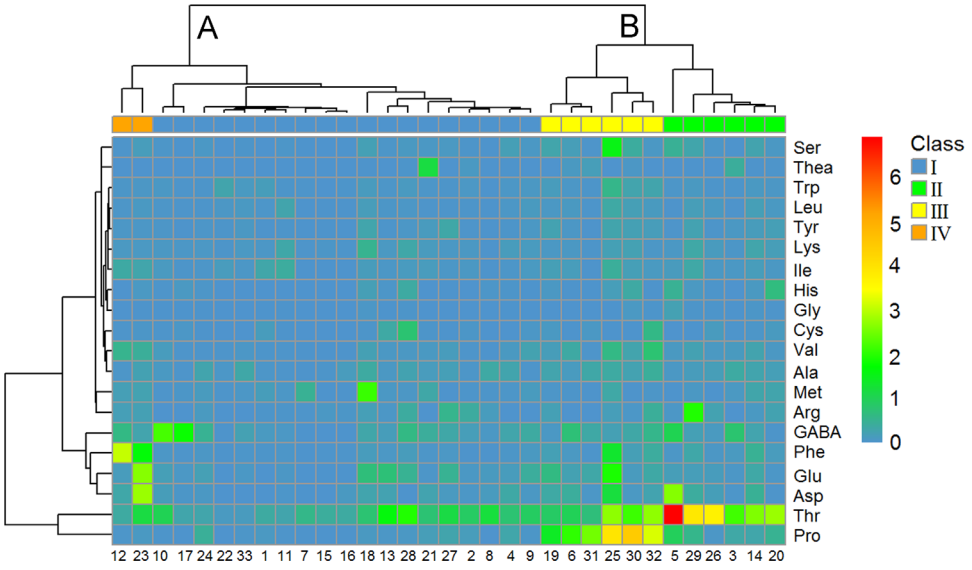

Figure 4 Hierarchical cluster dendritic diagram of 33 non-*Camellia* teas. Cluster (A) is divided into I and IV, cluster (B) is divided into II and III, respectively.

3.5. *Total phenolic content of non-Camellia tea*

Fig. 5A shows the total phenolic content of the non-*Camellia* tea samples (data presented in Table S7). Most of the samples had high polyphenol content. Tea from *A. grossedentata* (10) had the highest total phenolic content (177.25 mg GAE/g) among all of the non-*Camellia* teas. It had over 2 times higher phenolic content than green tea (34, 80.07 mg GAE/g), black tea (35, 39.77 mg GAE/g) and pu-erh tea (36, 67.82 mg GAE/g). Teas from *A. tataricum* subsp. *ginnala* (28), *Mallotus oblongifolius* (3) and *P. fruticosa* (21) all had a high phenolic content over 100.00 mg GAE/g. The total phenolic content of teas from *I. serra* (17, 97.84 mg GAE/g) and *P. chinensis* (29, 96.86 mg GAE/g) was relatively lower but still higher than that of green tea. Furthermore, teas from *C. tinctoria* (30, 81.92 mg GAE/g) and *Engelhardtia roxburghiana* (1, 82.62 mg GAE/g) had a similar level of polyphenolics as green tea. The polyphenol content of some samples of teas from *Ligustrum robustum* (11), *A. nitida* (12),

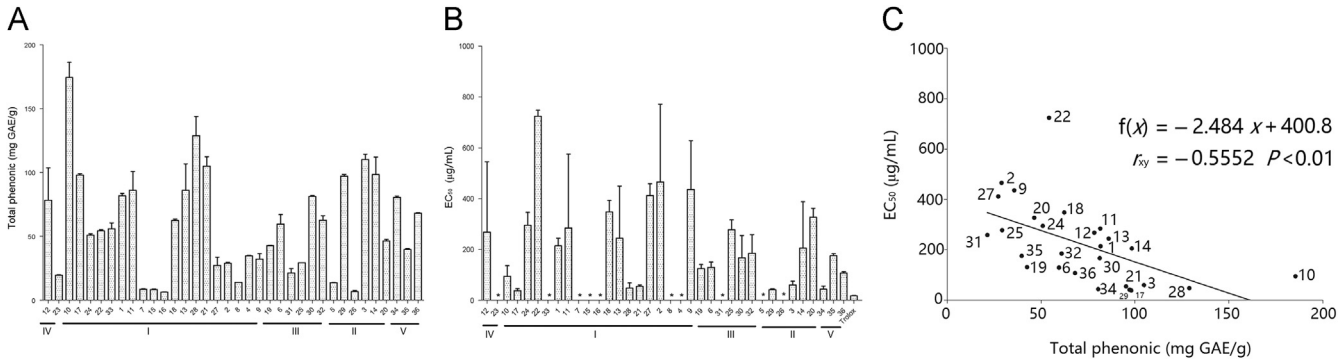

Figure 5 Total phenolic content, DPPH EC_{50} of non-*Camellia* tea and their relationship. (A) Total phenolic content of non-*Camellia* tea; (B) antioxidative activity of non-*Camellia* tea (mean ± SD); (C) Pearson correlation between total phenolic content and antioxidative activity of non-*Camellia* tea. I, II, III, IV: corresponding to the 4 classes in Fig. 4, V: 3 *Camellia* teas (green tea, black tea and pu-erh tea). *EC_{50} was too high to be detected in this study conditions.

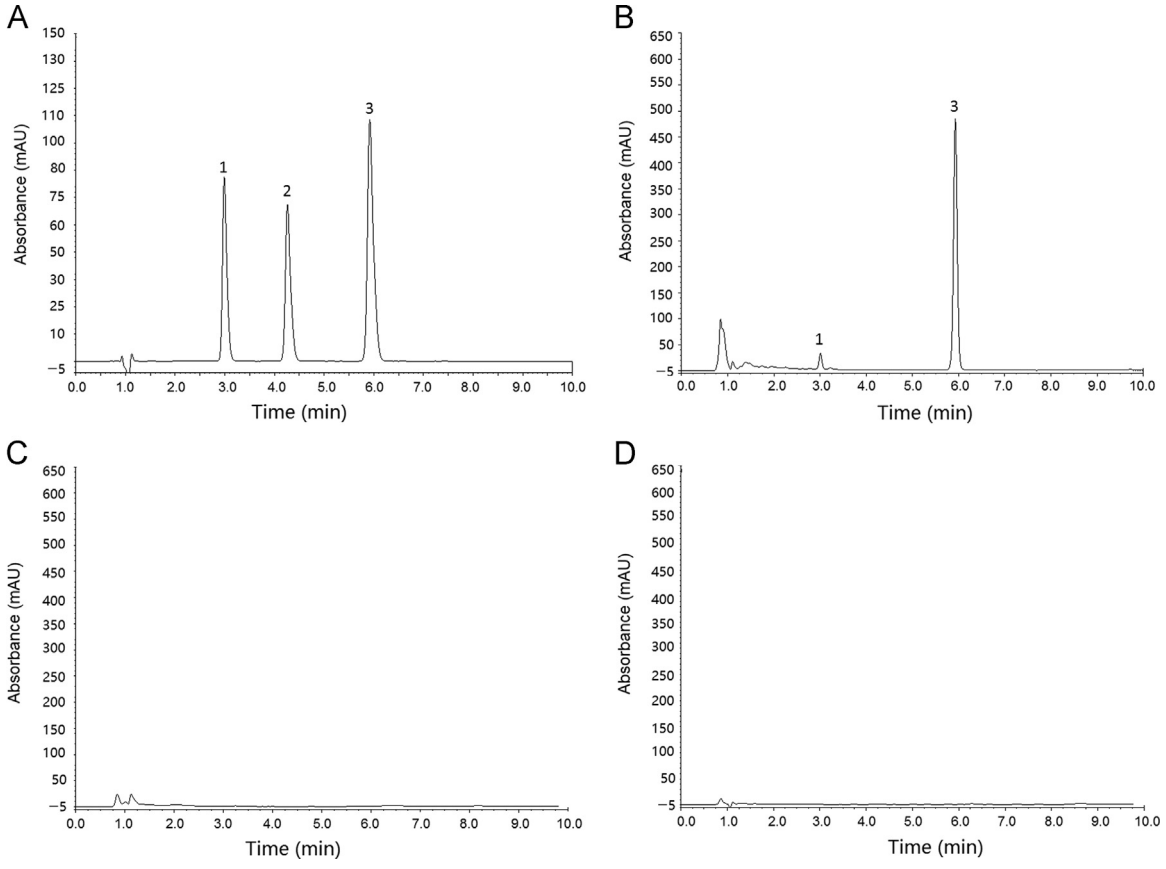

Figure 6 Chromatograms of three purine alkaloids in standard solution and different tea samples. (A) Three purine alkaloid standard solution: 1, theobromine (0.76 μmol/L); 2, theophylline (0.78 μmol/L); 3, caffeine (1.6 μmol/L); (B) green tea; (C) *Ampelopsis grossedentata*; (D) *Mallotus oblongifolius*.

Ilex latifolia (13) and *L. coreana* var. *lanuginose* (14) were much higher than comparable values from green tea, potentially due to different collection times or/and areas. The polyphenol content of teas from *R. chingii* var. *suavissimus* (6), *F. suspensa* (18), *Malus hupehensis* (22), *Cratoxylum cochinchinense* (24), *S. baicalensis* (32) and *Lithocarpus litseifolius* (33) was slightly lower than that of green tea. The other teas had much lower polyphenol content than found in green tea. Tea from *C. obtusifolia* (16) had the lowest polyphenol content (6.27 mg GAE/g).

3.6. Antioxidant potential of non-Camellia tea

As shown in Fig. 5B (and Table S7), there were remarkable differences in the DPPH-scavenging capacity among non-*Camellia* teas. The EC_{50} values varied from 38.41 μg/mL to 724.13 μg/mL. According to their antioxidant power, 33 non-*Camellia* teas can be divided in three groups: (a) strong ($EC_{50} < 100$ μg/mL); (b) intermediate (EC_{50}, 100–500 μg/mL); (c) weak ($EC_{50} > 500$ μg/mL). The EC_{50} values of teas from *I. serra* (17, 38.41 μg/mL), *P. chinensis* (29, 41.83 μg/mL), and *A. tataricum* subsp. *ginnala* (28, 48.27 μg/mL) were significantly lower than those of the remaining non-*Camellia* teas and two *Camellia* teas, black tea (35, 176.23 μg/mL), pu-erh tea (36, 108.10 μg/mL), and had no significant difference with the green tea (34, 44.23 μg/mL) and the Trolox (17.67 μg/mL). In addition, correlation analysis showed that the DPPH-scavenging capacity of the non-*Camellia* teas was significantly correlated with their phenolic content ($r = -0.5552$; $P < 0.01$) (Fig. 5C).

3.7. Caffeine, theobromine, and theophylline in non-Camellia tea

The presence of caffeine, theobromine, and theophylline in the non-*Camellia* tea samples was determined by comparison to alkaloid standard chromatograms. The results show that these alkaloids were not present in any of the non-*Camellia* tea samples. In contrast, in the three common *Camellia* teas, caffeine and theobromine were detected, but theophylline was not detected (Fig. 6). Therefore, non-*Camellia* tea may be a more suitable beverage in some cases than *Camellia* tea due to the very low amounts of addictive and potentially harmful purine alkaloids.

4. Conclusions

A variety of teas from non-*Camellia* plant are popularly consumed in many regions of China for centuries. In this study, we report for the first time, that some non-*Camellia* teas contain higher amount of some specific amino acids (such as Arg, Pro, Cys, Thea and GABA), polyphenols (particularly, teas from *A. grossedentata*, *A. tataricum* subsp. *ginnala*, *M. oblongifolius* and *P. fruticosa*, *I. serra* and *P. chinensis*) than found in *Camellia* tea, Some non-*Camellia* teas also have remarkable antioxidant activities (particularly, teas from *I. serra*, *P. chinensis*, *A. tataricum* subsp. *ginnala*, and *M. oblongifolius*). They do not contain addictive substances such as caffeine, theobromine and theophylline. Furthermore, a UHPLC method with precolumn derivatization with AccQ to detect 20 amino acids in teas has been established. This method compares favorably against the HPLC or UHPLC methods in which less than 20 amino acids are quantified.

In a summary, the discovery that some non-*Camellia* teas contain abundant amino acids and polyphenols, which have significant antioxidant activities, but lack caffeine, demonstrating that some of these teas have potential to prevent many chronic diseases. Additionally, non-*Camellia* teas have been frequently used for centuries throughout many regions of China, indicating that they are quite safe to use. Furthermore, since the non-*Camellia* teas are derived from various families of plants, the unique types of polyphenols and corresponding bioactivities are highly diverse and can be their characteristic features different from common *Camellia* tea. Some bioactivity profiles of several non-*Camellia* teas have been recently characterized by several preliminary chemical and pharmacological studies[31,32]. Therefore, our study suggests that non-*Camellia* teas might be more valuable as a functional beverage than *Camellia* tea, and could be a beneficial supplement or alternative beverage to *Camellia* tea. Exploration of the non-*Camellia* teas should provide a variety of beverage choices for health benefits.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.apsb.2015.11.003.

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