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An HPLC Method for Detection of 17 Characteristic Components in Tea Extract

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Abstract: A HPLC method has been developed for the determination of tea for seven catechins ((+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-epicatechin gallate, (−)-gallocatechin and (−)-gallocatechin gallate), two flavonols (quercetin, kaempferol), seven organic acids (Gallic acid, ferulic acid, protocatechuic acid, p-hydroxybenzoic acid，p-coumaric acid, erucic acid， vanillic acid) and caffeine. Using HPLC with gradient elution and photodiode array detection, seventeen compounds were separated and detected by optimizing the column temperature, mobile phase ratio, and detection wavelength. The validity of this method was confirmed by the quantitative measurement of seventeen characteristic compounds. The correlation coefficient of all standards curves was 0.9967~1.0000 and the detection limits (s/N = 3) were 0.010~1.2 mg/L. The average recoveries of 17 characteristic components were between 81.50 and 124.80%. 59 green tea samples (origin: Hangzhou, China) were analyzed and 11 characteristic components were found in all samples and the content of different samples varied greatly. The catechins were the most abundant constituents in teas with up to 79.5~156.6 g/kg and the contents of (−)-epigallocatechin gallate were the highest. Quercetin and kaempferol were only found in some tea samples. Protocatechuic acid, p-hydroxybenzoic acid, ferulic acid, and erucic acid were not detected in the tea samples. This method is ideally suited for routine analysis for the determination of bioactive components in green tea with good repeatability and accuracy of results. Furthermore, this method can be applied to all kinds of tea and tea products and provided a detection method for establishing the HPLC fingerprint of a certain tea.

Keywords: HPLC, Tea, Catechins, Flavonols, Organic Acids, and Alkaloids Function

Introduction

Tea is rich in catechins, flavonols, organic acids, and alkaloids, which are important bioactive substances in tea. The content of catechins is the highest among them, which account for about 70% of the total tea polyphenols in tea (Zhao, 2002). Catechins contain 7 compounds namely (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-gallocatechins (GC) and (−)-Gallocatechin Gallate (GCG) (Sharangi, 2009). Catechins have antioxidant properties (Higdon and Frei, 2003; Kopjar *et al*., 2015), lowers serum uric acid (Zhu *et al*., 2017), maintain endothelial function and vascular homeostasis, and reduce the risk of atherogenesis and cardiovascular disease risk (Bruno *et al*., 2014), helps prevent neurodegeneration and delay brain function decline (Assuncao and Andrade, 2015), reduces liver steatosis and inflammation during nonalcoholic steatohepatitis (Li *et al*., 2016). Catechins can also influence sympathetic nervous system activity, increasing energy expenditure and promoting the oxidation of fat. The tea flavonols have antioxidant, anti-inflammatory, antiallergic, and anti-microbial effects （Sharangi, 2009). The organic acid in tea has many kinds and the content is about 3% of the total dry matter. It is one of the main components of aroma and taste, that takes part in the metabolism of the tea tree and usually be the intermediate product of sugar decomposition. Daily intake of beneficial organic acids can lessen the risk of cardiovascular diseases, improve cellular energy,

metabolism, digestion, and nutritive values and maintain a healthy gut (Gundogdu *et al*., 2014). Alkaloid is a natural product during tea tree metabolism. The main component of alkaloids is caffeine, it has a well-known diuretic effect (Barghouthy *et al*., 2020).

There are many varieties of tea and the price of tea varies greatly between different varieties and different producing areas. The quality attributes of teas are closely related to their inherent chemical constituents including polyphenols, pigments, organic acids, and alkaloids as well as volatile compounds (Nitin *et al*., 2006; Ouyang *et al*., 2020), in which catechins, caffeine, and flavonol glycosides are the source of astringency and bitterness in green tea infusions (Xu *et al*., 2018). The classification of tea varieties according to phenolic substances has provided excellent results (Chen *et al*., 2008; Gu *et al*., 2020). At present, the methods for detecting phenolic substances in tea mainly include High-Performance Liquid Chromatography (HPLC), Ultra-High-Performance Liquid Chromatography (UHPLC) (Jiang *et al*., 2015), High-Performance Capillary Electrophoresis (HPCE) (Bonoli *et al*., 2003) and high-performance liquid chromatography combined with Ultraviolet (UV), mass Spectrometric Detection (MS), Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (ESI-Q-TOF-MS/MS) (Li *et al*., 2017; Wu *et al*., 2021). But the MS equipment purchase cost and running cost are relatively high. High-performance liquid chromatographs with photodiode Array Detector (DAD) are widely used and the cost is low and suitable for promotion.

In recent years, chromatographic fingerprint techniques have been widely applied in the quality control of food and drug. HPLC fingerprints can be used as an alternative method, which is easy to use and widely available（Peng *et al*., 2021). Therefore, for tea quality identification and product classification, it is particularly important to establish an HPLC method for the simultaneous detection of multiple characteristic components. At present, a lot of research focused on the methods for the simultaneous detection of more than 10 kinds of related substances in tea. The HPLC-DAD method established by Jiang *et al.* (2015) Was used for the determination of flavonol glycosides in tea for 77 min. developed an HPLC method with an amide-C_{16} column to determine 18 major active ingredients in black tea, including theanine, gallic acid, four purine alkaloids, eight catechins, and four theaflflavins, but it took 86 min. While the HPLC-DAD method established by Jiang *et al.* (2015) Was used for the determination of flavonol glycosides in tea for 77 min.

The HPLC-DAD methods by Samanidou *et al*. (2012) and can detect 15 and 20 characteristic components in tea respectively and the running time was less than 30 min. However, only two organic acids were detected by these two methods.

Developed a fast HPLC method with DAD and fluorescence detector for the analysis of 19 phenolic compounds, in which five organic acids were detected. This method requires two detectors. Therefore, HPLC methods for the simultaneous determination of various active ingredients in tea require further improvement.

The objective of this study was to establish an effective HPLC method for analyzing characteristic components in tea, which includes seven catechins (C, EC, EGC, EGCG, ECG, GC, GCG), two flavonols (quercetin, kaempferol), seven organic acids (Gallic acid, ferulic acid, protocatechuic acid, vanillic acid, p-coumaric acid, erucic acid，p-hydroxybenzoic acid) and an alkaloid named caffeine. The contents of 17 components in 59 tea samples (origin: Hangzhou, China) were determined， which provided a detection method for establishing the HPLC fingerprint of a certain tea and a reference for further establishing the method for evaluating the quality of tea from different producing areas in Hangzhou.

Materials and Methods

Chemicals and Reagents

Standards substance for 17 bioactive components: Gallic Acid (GA) (98.0%, purchased from Acros Organics Co., Belgium); caffeine(CA) (99.0%, purchased from Dr. Ehrenstorfer GmbH Co., Germany); Kaempferol (≥99%, purchased from Shanghai Nature Standard Biotechnology Co., Ltd.); Standards of Catechin (C) (≥98%), (−)-epigallocatechin gallate(EGCG)(≥95%), (−)-epicatechin (EC)(≥98%), (−)-gallocatechin (GC) (≥95%), (−)-epigallocatechin(EGC) (≥95%), (−)-epicatechin gallate(ECG) (≥98%), (−)-gallocatechin gallate(GCG) (≥98%) were purchased from Sigma(St. Louis, MO, USA); Standards of quercetin (≥98%), protocatechuic acid (≥98%), ferulic acid (≥98%), p-coumaric acid (≥98%), vanillic acid (98%), erucic acid (98%) and p-hydroxybenzoic acid (99%) were purchased from the National Research Center for Standard Materials(Beijing, China).

HPLC grade methanol, acetonitrile, formic acid, and ethanol were obtained from Merck (Darmstadt, Germany). Deionized water from the Milli-Q purification system (Millipore Co., LTD. American) was filtered through a 0.45 μM nylon membrane before use in HPLC analysis.

Tea Samples and Preparation of Tea Extract

59 samples of the green tea which Includes three types, were used in the experiment obtained from different manufacturers in Hangzhou and were collected in April. Among them, 54 tea samples were Jingshan tea (origin: Yuhang District, Hangzhou, China), 3 tea samples were steamed green tea (origin: Yuhang District, Hangzhou, China) and 2 tea samples were Qiantang Longjing (origin: Hangzhou, China). They were crushed and sieved through 60 mesh sieves respectively. Weigh 1.0000±0.0001g of

sample into 50 mL of 50% ethanol solution for ultrasonic extraction for 30 min the extraction solution is finally fixed to 100 mL in a volumetric flask and the clear supernatant is filtered by a 0.22 μM organic filter membrane and ready for HPLC detection.

HPLC Analysis

The HPLC analyses were carried out on an Agilent 1260 system (Agilent Technologies, Inc., Santa Clara, CA, USA); consisting of a quad pump, diode array detector, and auto-sampler. Separation of compounds was carried out on the Hypersil GOLD PFP column (100 mm × 4.6 mm, 3 μm). The column temperature was maintained at 32℃ and the flow rate was 1 mL/min. The mobile phase was composed of ethanol (solvent A) and water (1% formic acid) (solvent B). Gradient profile: 0~5 min, 5% A; 5~15 min, 5~10% A; 15~25 min, 10%~15%A; 25~45 min, 15%~30%A; 45~70 min, 30%~65%A; 70~71 min, 65%~5%A; 71~72 min, 5% A。Injection volume was 10 μL. The absorbance detection was carried out using 280 nm. Peak identification was performed both by retention times and by spectral information provided by the DAD.

Reference standards for 17 bioactive components were mixed with methanol to obtain a standard solution with 2.00 g/L respectively. These solutions were configured to mix standard solutions of 0.01, 0.02, 0.03, 0.04 and 0.05 g/L. The mixed standard solutions with different concentrations would be filtered with a 0.22 μm organic filter membrane and prepared for analysis.

Standard Preparation and Method Evaluation

0.0020 g of each solid standard was accurately weighed and the volume was fixed to 10 mL with ethanol to prepare the standard stock solution with the concentration of each standard material of 2.000 mg/mL. The standard stock solutions of different volumes were accurately absorbed and placed in a 50 mL volumetric flask. Add water to dilute to the scale, shake well and prepare 0.01, 0.02, 0.03, 0.04, and 0.05 mg/mL mixed standard working solutions respectively. After being filtered by an organic filter membrane of 0.22 μm, the analysis test was carried out according to the chromatographic conditions.

The optimal determination conditions were studied from the detection wavelength, mobile phase, gradient elution program, and column temperature. The precision and recovery tests were carried out.

Precision test: Take the same sample and prepare the sample solution according to the "Tea samples and preparation of tea extract" method, inject 6 times continuously, 10 μL each time, and analyze the content of the components under the same chromatographic conditions and calculate the RSD value.

Stability test: Take the freshly prepared tea extract, inject 10 μL every 0 h, 2 h, 4 h, 8 h, 12, and 24 h, and calculate the RSD value according to the content of the detected components in the sample solution.

Recovery test: 0.5 mg and 1.0 mg of each of the 17 standard solutions were added to a 0.5 g sample of tea powder and prepared according to the "Tea samples and preparation of tea extract" method and analyzed according to the above HPLC detection methods. Parallel detection 3 times.

The LOD, the smallest amount of the compound that resulted in a detection S/N = 3, was determined using the standard solutions.

Data Analysis

Data Processing System (DPS) software v18.10 and SPSS 19.0 (Yeruva *et al*., 2021) were applied to analyze the data.

Results

Optimization of Wavelength

High-Performance Liquid Chromatography (HPLC) combined with a diode array detector can scan the separated components in the range of UV-Vis wavelength to obtain their absorption spectrum. In this experiment, 17 reference materials were scanned at 200-700 nm. It was found that catechins and caffeine have high sensitivity at 280 nm, the maximum absorption wavelength of organic acids is about 320 nm and the maximum absorption wavelength of flavonols is 360 nm. However, the UV absorption of organic acids and flavonols at 280 nm is relatively high and stable. Considering comprehensively, the detection wavelength of 280 nm can effectively guarantee the detection sensitivity of each component, which was consistent with Nishitani *et al.* (2004) research results.

Optimization of Mobile Phase

The chromatographic column is mainly composed of silica gel matrix and the low pH value can help inhibit the activity of silica hydroxyl, reduce tailing, and improve the peak shape and separation. In the analysis of organic acids, the chromatographic column is more stable and the peak has a better shape when the pH value is lower than 3. In this experiment, the effects of 0.1% formic acid (pH2.65) and 1% formic acid (pH2.10) as flow relative separation were compared. When 0.1% formic acid was used as the mobile phase, baseline separation of EGC and caffeine was not achieved and some peaks appeared fronting phenomenon; when 1% formic acid was used as the mobile phase, the separation effect is better, without the tailing phenomenon. When acetonitrile was used as mobile phase A, catechin and EGC could not be separated and the peak of organic acid was too early. When methanol was used as mobile phase A, 17 substances could be separated. Therefore, methanol and 1% formic acid were chosen as the

mobile phase.

The proportion of methanol in the mobile phase has a great influence on the retention time and component separation. When the concentration of methanol is 5%~10%, the chromatographic peaks of most organic acids can be separated; when the concentration of methanol is from 10 to 25%， it was suitable for the separation of catechins. For flavonoid components, the retention time of flavonoid aglycones was late. when the concentration of the methanol ratio was 30~65%, it can ensure the complete separation of flavonoid components as far as possible.

Optimization of Column Temperature

The effects of column temperature at 25, 30°C, and 35°C on the separation of components were studied. It was found that EGC and caffeine could not be separated when column temperature was 25°C; the peaks of EGC and caffeine still overlapped partially when column temperature was 30°C; EGC and caffeine were separated from baseline when column temperature was 35°C, but the peaks of EGCG and ferulic acid were overlapped; 17 components were separated from baseline at 32°C. Therefore, a column temperature of 32°C is better. As the column temperature increased, the retention time of biochemicals decreased. Similar observations were recorded by Sharma *et al.* (2005). However, the study only compared the five temperature ranges of 16, 30, 35, 40, and 45°C and finally obtained the analysis effect at 35°C. In this experiment, it was reduced by 1°C each time based on 35°C and finally obtained that the separation effect of 32°C was the best.

The Establishment of Standard Methods

The optimized operating conditions of HPLC have been Established: The absorbance detection was 280 nm; the mobile phase was composed of ethanol (solvent A) and water (1% formic acid) (solvent B). Gradient profile: 0~5 min, 5% A; 5~15 min, 5~10% A; 15~25 min, 10~15% A; 25~45 min, 15~30%A; 45~70 min, 30~65% A; 70~71 min, 65~5%A; 71~72 min, 5% A; the flow rate was 1 mL/min and the column temperature were 32℃. HPLC chromatograms of standards under optimized operating conditions were shown in Fig. 1A.

Under the optimized experimental conditions, the mass concentration of 17 characteristic components in the range of 10~50 mg/L has a good linear relationship with their peak area, the correlation coefficient is 0.9967~1.0000 and the detection limit (s/N = 3) is 0.010~1.2 mg/L. For the determination of the 20mg/L mixed standard sample, repeat the injection 6 times and the Relative Standard Deviation (RSD) is 0.096~2.64% (Table 1). The average recoveries of 17 characteristic components were between 81.50 and 124.80% (Table 1).

Analysis of Tea Products

59 green tea samples were analyzed using the Optimized HPLC method and the typical HPLC chromatograms of tea samples were shown in Fig. 1B.

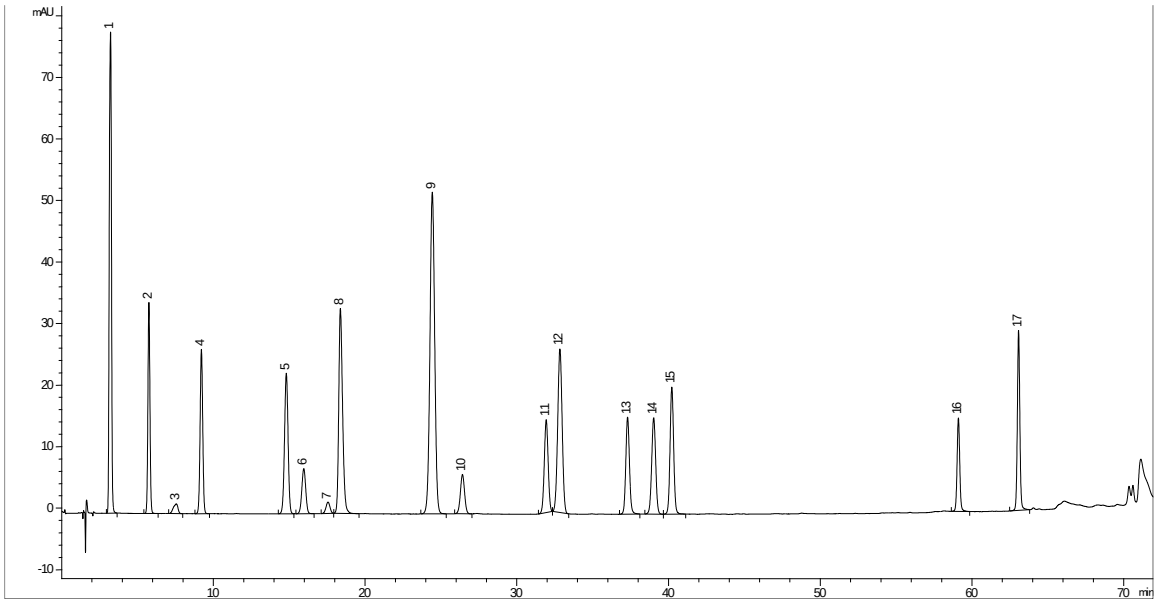
According to the retention time and UV-Vis absorption spectrum of 17 standards, the characteristic components in tea were analyzed qualitatively, which were shown in Table 2. It was found that there were 11 bioactive components in all collected tea samples, including gallic acid, GC, vanillic acid, C, EGC, caffeine, p-coumaric acid, EC, EGCG, GCG, and ECG. All tea samples contain large amounts of caffeine.

Discussion

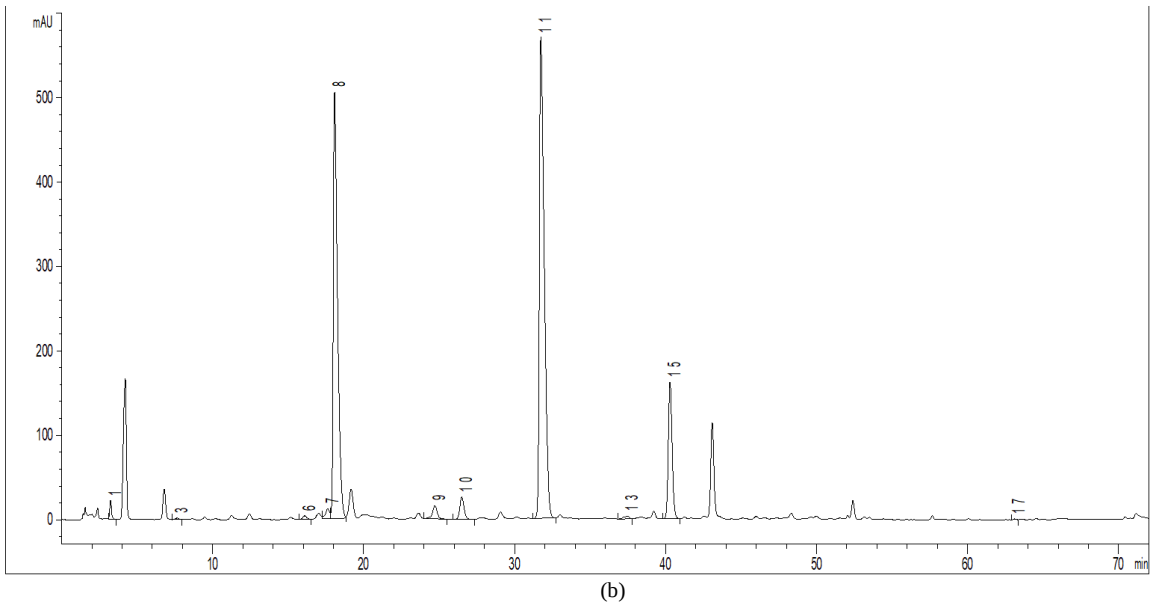
The retention time of some chromatographic peaks of the sample is close to that of p-hydroxybenzoic acid, ferulic acid, and erucic acid standards, but the UV-Vis absorption spectrum and the maximum absorption wavelength are inconsistent with the standards, so the possibility of its existence in the sample is excluded. Gallic acid, p-coumaric acid, and vanillic acid are the main organic acids observed in green teas that were produced in Hangzhou, China. While four organic acids, including protocatechuic acid, p-hydroxybenzoic acid, ferulic acid, and erucic acid， were not detected. A trace amount of erucic acid and a small amount of ferulic acid and protocatechuic acid were detected in 3 green tea soups by HPLC-MS/MS (Jeszka-Skowron *et al*., 2015). Unfortunately, this study did not indicate the variety of green teas. The inconsistent results may be due to the lower detection limit of HPLC-MS/MS and different varieties of green tea. The organic acids not detected in these green teas may be detected in other kinds of tea. For example, the 7 organic acids (gallic acid, ferulic acid, protocatechuic acid, vanillic acid, p-coumaric acid, erucic acid, p-hydroxybenzoic acid) were all detected in unfermented rooibos teas from South Africa (Krafczyk *et al*., 2008). Therefore, we guess the HPLC method can be also applied to the detection of bioactive substances from the teas of different tree species, such as rooibos teas.

Quercetin and kaempferol were found in some tea samples, the detection rates were 3.70 and 7.41% (Table 2). Jiang *et al.*（2015）detected quercetin, kaempferol and myricetin in 3 green teas and kaempferol had the highest content. However, this experiment detected 59 green tea samples and found that only a few green tea samples detected kaempferol and quercetin and the content was very low. This may be related to tea variety, origin, and

tea bud picking time. In this study, the contents of total catechin and caffeine in 59 green tea samples were 79.5~156.6 and 23.5~37.4 mg/g respectively. Through the detection of 116 green tea samples, Ning *et al*. (2016) concluded that the total catechins content was 10.14~15.39% and the caffeine content was 2.56-3.96 in green tea. It is similar to the results of this study. All seven catechins were detected and the contents of EGCG and ECG were the highest. A study found that C, EC, and EGC were bitter with a sweet aftertaste while ECG and EGCG were bitter and astringent (Senanayake, 2013). The average content of EGCG in 59 green tea samples is 80 mg/g, which is much higher than other characteristic components in green tea, indicating that EGCG may contribute more to the bitterness and astringency of green tea.



(a)



(b)

Fig.1: HPLC chromatograms of standards and tea samples. (A): HPLC chromatograms of standards. (B): HPLC chromatograms of tea samples. Note: (1) Gallic Acid (GA), (2) protocatechuic acid, (3) (−)-Gallocatechin (GC), (4) p-hydroxybenzoic acid, (5) vanillic acid, (6) (−)-catechin (C), (7) (-)-epigallocatechin (EGC), (8) caffeine, (9) p-coumaric acid, (10) (−)-epicatechin (EC), (11) (−)- Epigallocatechin Gallate (EGCG), (12) ferulic acid, (13) (−)-Gallocatechin Gallate (GCG), (14) erucic acid, (15) (−)-Epicatechin Gallate (ECG), (16) quercetin, (17) kaempferol

Table 1: Method validation and standard recovery

Compound	Linearity	Linear equation[a]	R^2	LOD mg/L	RSD n = 6	Adding content	Recovery R/%	Average Recovery rate
Gallic Acid (GA)	10-50	Y=39.47469X-1.86888	0.9999	0.010	0.099	0.50	98.40	99.20
						1.00	100.00	
Protocatechuic acid	10-50	Y=15.86840X-0.188013	1.0000	0.033	0.099	0.50	103.00	104.00
						1.00	105.00	
(−)-Gallocatechin (GC)	10-50	Y=1.4355X+1.73517	0.9979	0.940	0.460	0.50	109.20	99.00
						1.00	88.80	
p-hydroxybenzoic acid	10-50	Y=15.89457X-0.291553	0.9999	0.082	0.170	0.50	124.60	124.80
						1.00	125.00	
Vanillic acid	10-50	Y=18.62611X-0.112988	1.0000	0.097	0.110	0.50	83.20	81.50
						1.00	79.80	
(−)-Catechin (C)	10-50	Y=6.44652X+1.19194	0.9999	0.280	0.370	0.50	101.00	91.40
						1.00	81.90	
(-)-Epigallocatechin (EGC)	10-50	Y=1.59064X-0.774798	0.9967	1.200	2.640	0.50	100.40	101.20
						1.00	102.00	
Caffeine	10-50	Y=29.51172X-4.34513	0.9999	0.066	0.097	0.50	75.20	96.10
						1.00	117.00	
p-coumaric acid	10-50	Y=56.42393X-2.96858	0.9999	0.043	0.096	0.50	96.40	97.60
						1.00	98.90	
(−)-Epicatechin (EC)	10-50	Y=13.8925X-0.111222	1.0000	0.280	0.480	0.50	91.20	96.60
						1.00	102.00	
(−)- Epigallocatechin Gallate (EGCG)	10-50	Y=13.8925X-3.12894	0.9998	0.120	0.360	0.50	108.60	120.80
						1.00	133.00	
Ferulic acid	10-50	Y=27.85783X-5.93283	0.9999	0.260	0.150	0.50	92.20	98.60
						1.00	105.00	
(−)-Gallocatechin Gallate (GCG)	10-50	Y=13.19198X-0.30458	0.9999	0.450	0.450	0.50	97.80	97.00
						1.00	96.10	
erucic acid	10-50	Y=13.58444X+1.10103	0.9999	0.370	0.300	0.50	122.60	119.80
						1.00	117.00	
(−)-Epicatechin Gallate (ECG)	10-50	Y=17.16386X-2.02072	0.9999	0.280	0.350	0.50	121.60	120.30
						1.00	119.00	
Quercetin	10-50	Y=9.23867X-0.840224	0.9997	0.270	0.910	0.50	107.60	105.30
						1.00	103.00	
Kaempferol	10-50	Y=16.83619X-0.924957	0.9999	0.140	0.250	0.50	101.60	100.80
						1.00	100.00	

Table 2: Contents of the 17 compounds in 59 tea samples

Compound	Range of content (mg/g)	Average content (mg/g)	Detection rate %
Gallic Acid (GA)	0.296~1.060	0.567	100
protocatechuic acid	nd	nd	nd
(−)-Gallocatechin (GC)	0.476~2.090	1.340	100
p-hydroxybenzoic acid	nd	nd	nd
Vanillic acid	0.0812~0.266	0.164	100
(−)-Catechin (C)	0.603~1.970	1.210	100
(-)-Epigallocatechin (EGC)	2.260~24.200	11.000	100
Caffeine	23.500~37.400	30.100	100
p-coumaric acid	0.218~1.460	0.718	100
(−)-Epicatechin (EC)	5.050~12.200	7.540	100
(−)- Epigallocatechin Gallate (EGCG)	52.300~113.000	80.000	100
ferulic acid	nd	nd	nd
(−)-Gallocatechin Gallate (GCG)	0.121~1.430	0.594	100
erucic acid	nd	nd	nd
(−)-Epicatechin Gallate (ECG)	13.000~49.100	21.300	100
quercetin	0.0852~0.101	0.0931	3.70
Kaempferol	0.0702~0.110	0.0915	7.41
Total catechins	79.5~156.6	121.9	100

Conclusion

The HPLC method with 17 characteristic components in tea has been established, including gallic acid, protocatechuic acid, ferulic acid, vanillic acid, p-coumaric acid, erucic acid, p-hydroxybenzoic acid, C, EC, EGC, EGCG, ECG, GC, GCG, quercetin, kaempferol, and caffeine.

The correlation coefficient of all standards curves for 17 components was 0.9967~1.0000 and the detection limits (s/N = 3) were 0.010~1.2 mg/L. The average recoveries of 17 characteristic components were between 81.50 and 124.80%. 59 green tea samples were analyzed by the established HPLC method. It shows that this method can well separate and detect organic acids,

catechins, alkaloids, and flavonols. Therefore, this simple detection method for similar bioactive components widely distributed in plants, foods, and beverages has wide application potential.

Through the analysis of green tea samples, 11 characteristic components were found in all samples and the content of different samples varied greatly. The contents of total catechins in 59 green tea samples were 79.5~156.6 mg/g and the contents of EGCG were the highest at 52.3~113.8 mg/g. Although the samples are all green tea produced in Hangzhou, the detection results of characteristic components of tea samples vary greatly due to different tea varieties, production technology, planting area, and picking time. The content of chemical components can be related to the quality of tea, to provide technical support for the identification of famous and high-quality native teas. The method provided a detection method for establishing the HPLC fingerprint of a certain tea and a reference for further establishing the method for evaluating the quality of tea from different producing areas in Hangzhou.

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Author's Contributions

Ying Liu and Dan Wu: Participated in all experiments and wrote the manuscript.

Ping Tang: Data-analysis

Xingqian Ye: Grammar checking and modification.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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