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Study of 'Fingerprints' for Green Tea from Different Planting Areas in Eastern China

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ABSTRACT

Green tea is one of the main teas in China, which is unfermented and retains more natural substances of fresh tea leaves. This is the preliminary study of application of 'fingerprints' based on differences in component composition of green tea. Five green teas from different areas in eastern China are analyzed, which are processed by microwave-assisted solvent (ethanol) extraction method to obtain tea polyphenols, flavonoids, polysaccharides, pigments (thearubigins, theaflavins, theabrownins). The results show that the component composition of five green teas are varied from each other; based on these contents varieties, we have constructed a 'fingerprint' and applied linear discriminant analysis (LDA) and hierarchical cluster analysis (HCA) to assist in the identification of these five green teas. This method does not require large, expensive instruments (such as high performance liquid chromatograph, gas chromatograph, mass spectrometer, etc.), and is easy to use, which provides a new avenue for the identification of tea.

KEYWORDS

Green tea, contents varieties, identification, 'fingerprints'.

1. Introduction

Tea is currently the most popular and healthy non-alcoholic beverage in the world, its consumption is second only to potable water.[1] Tea contains a variety of natural active ingredients, such as polyphenols, polysaccharides, pigments, flavonoids, alkaloids, proteins, amino acids, etc.,[2] which might be the reason for its antioxidant activity,[3] antibacterial activity,[4] anti-obesity,[5] anti-cancer,[6] and other biological activities.[7] Green tea is an unfermented tea with the lowest degree of oxidation and is particularly effective in fighting cancer and cardiovascular diseases.[8]

Green teas from different regions are varied in climate, soil, altitude, harvesting season, etc., thus their contents of active ingredients will change dramatically, which will affect the quality of tea.[9] The analysis of green tea has recently garnered attention because of its importance in the pharmaceuticals, cosmetics, food additives and functional food.[10–11] Many methods are available for green tea detection, such as high performance liquid chromatography (HPLC),[12] gas chromatography (GC),[13] mass spectrometry (MS),[14] GC-MS,[15] gene mapping,[16] spectroscopic fingerprint,[17] and so on.[18–20] All these methods have high selectivity and great sensitivity for tea detection. However, these methods often require more complex sample pretreatment, environmentally unfriendly chemicals, expensive equipment, professional technicians, which limits their application in the simple and rapid analysis of green tea.

Here, we report a novel 'fingerprint' based on the variety of bioactive ingredients to discriminate green teas from different regions, which can provide different patterns to realize the selectivity. Green teas from five different regions are processed by microwave-assisted solvent extraction, and then the extracts are evaluated to obtain the contents of bioactive ingredients as polyphenols, flavonoids, pigments (thearubigins, theaflavins, theabrownins) and polysaccharides. In addition, linear discriminant analysis (LDA) and hierarchical cluster analysis (HCA) are applied for comprehensive consideration of interference from various factors to improve the applicability and specificity of the method. The method described herein has several advantages as follows: (1) it is simple and cost-effective, which requires no skilled operators and sophisticated instruments; (2) the determination can be achieved easily by extracting bioactive ingredients from different green teas, which are reusable to discover their nutritional values; (3) it is safe and green, no need of environmentally unfriendly chemicals. Based on these advantages, our method may provide a new way for the detection of biological samples.

2. Materials and Methods

2.1. Reagents and Instruments

All the reagents used were of analytical-reagent grade. Water was deionized and further purified with a thermo scientific water purification system (Lab Tower EDI 15, Sweden). Green teas of five different regions (high-grade green tea of Wuxi in Jiangsu province, Mingqian maojian of Mount Wuyi in Fujian province, Mingyuan spring buds of Jurong in Jiangsu province, Biluochun of Dongting Hill in Jiangsu province, Jinzhai Cuimei of Ta-pieh Mountains in Anhui province) were bought at a supermarket in Jurong, China. Absolute ethanol and sodium nitrite were obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Rutin hydrate, epigallocatechin gallate (EGCG), gallocatechin (EC), catechin (C), aluminum nitrate nonahydrate and gallic acid were from Macklin Biochemical Co., Ltd. (Shanghai, China). Sodium hydroxide, hydrogen peroxide 30 %, ethyl acetate, folinol reagent, sodium carbonate, sodium bicarbonate, oxalic acid, phenol, sulfuric acid were from

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XiLong Chemical Co., Ltd. (Shantou, China). The epigallocatechin (EGC) and epicatechin gallate (ECG) were from Aladdin Reagent Co., Ltd. (Shanghai, China). The methyl alcohol was from TEDIA company Inc. (Fairfield, USA). The acetonitrile was from Merck KGaA (Darmstadt, Germany)

The UV-vis absorption data were recorded using a UV-visible spectrophotometer (755B, Shanghai Precision Instrument Co., Ltd., China). The centrifugation was conducted by a high-speed centrifuge (TG 16-WS, Shanghai Lu Xiangyi Centrifuge Instrument Co., Ltd., China). The microwave process was completed with a multifunctional microwave chemical reactor (MWave-5000, Shanghai Sineo Microwave Chemistry Technology Co., Ltd., China). The identification and quantification of polyphenols was achieved by a high performance liquid chromatograph (ACQUITY Arc, Waters Technology Co., Ltd., USA). The evaporation and concentration were done by a rotary evaporator (RE-5203, Shanghai Yarong Biochemistry Instrument Factory, China).

2.2. Preparation of Tea Extractions

One gram of each green tea powder was processed with microwave-assisted ethanol solvent extraction, respectively. Under specific conditions (microwave chemical reactor power 400 W, liquid-solid ratio 35 mL g^{-1}, temperature 65°C and time 3 min), the bioactive ingredients were extracted; after the extraction process, the solutions were centrifuged at 4000 rpm for 10 min; the precipitates were processed for a second time under the same conditions; finally the two supernatants were collected in a reagent bottle, concentrated and vacuum-dried to obtain the extracts.

2.3. Content Determination of Active Ingredients

The content determination of polyphenols, total flavonoids, pigments, polysaccharides in extracts were carried out referring to Folin–Ciocalteu colorimetry,[21] aluminum nitrate complex spectrophotometry,[22] Roberts assay,[23] phenol-sulfuric acid assay.[24] All the raw data of standard curve for the quantification are shown in the Supplementary Material (Fig. S1, S2 and S3). Each experiment was conducted in five replicates.

2.4. Identification and Quantification of Polyphenols with High Performance Liquid Chromatography (HPLC)

Chromatographic conditions: The separation was achieved using a CORTECS-C18 column (2.7 μm, 100 × 3.0 mm) with a UV-visible light detector at wavelength of 280 nm. The mobile phase for gradient elution consisted of a acetonitrile-water system, wherein mobile phase A is ultrapure water, and mobile phase B is acetonitrile. The conditions of the gradient elution were 0–3.0 min, 9.0 % B; 3.0–6.0 min, 9.0–20 % B; 6.0–8.0 min, 20.0–30.0 % B; 8.0–9.0 min, 30.0–9.0 % B; 9.0–20.0 min, 9.0 % B. The volume of injection was 10 μL, the column temperature was 30 °C, and the system delivered a constant flow of 300 μL min^{-1}.

3. Results and Discussion

3.1. 'Fingerprints' of Five Green Teas from Different Regions

According to the method in Section 2.3, the bioactive ingredients' contents of five green teas (high-grade green tea, Mingqian maojian, Mingyuan spring buds, Biluochun, and Jinzhai Cuimei) were obtained. The raw data are shown in Table S1. In Fig. 1, the differently coloured squares represent different bioactive ingredients' contents (using mean values, n = 5), and a column of coloured squares constitute a 'fingerprint' map for each green tea, which is similar to the DNA fingerprints in molecular biology. It can also be seen clearly that the five green teas contain a lot of polyphenols; there are more theabrownins than polysaccharides only in Mingqian maojian; the content of theabrowins, pigments and thearubigins is almost equal in high-grade green tea, and Jinzhai Cuimei and Mingyuan spring buds have the similar contents of pigments and thearubigins. Above all, the 'fingerprints' of five green teas are different and could be used for the identification.

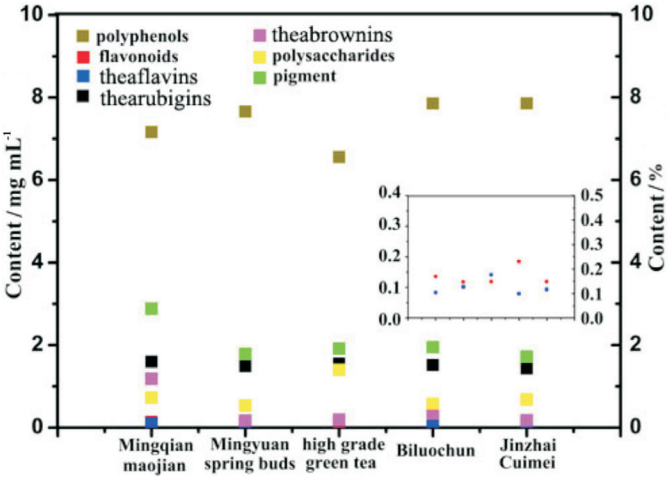

Figure 1 'Fingerprints' of five target green teas (using mean values, n = 5); the insert shows the partial enlargement.

3.2. Linear Discriminant Analysis (LDA)

In order to make the tea discrimination more simple, the initial data of the 'fingerprints' (Fig. 1) were processed by LDA, according to a multi-dimensional matrix (5 green teas × 7 ingredients × 5 replicates). As a classical linear discrimination method, LDA can transform sample data into a three-dimensional or two-dimensional version displayed as a scatter plot.[25] In the plot, data of the same specimen gather around a centre to form a cluster (Fig. 2); clusters of various specimens stay far from each other. Through this method, the separation of the specimens can be enhanced. From Fig. 2, it can be seen that each tea has its own spherical-cluster and distant from others (spheres represent sample data generated from 'fingerprints' of teas). The qualitative information on the teas can be achieved based on the position of the clusters presented in the plot. Five green teas are

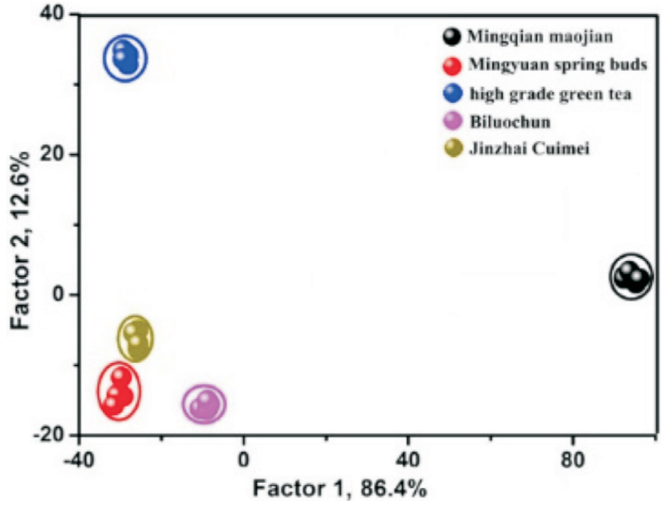

Figure 2 Canonical score plot for the five teas, which is provided by LDA according to a multi-dimensional matrix (5 green teas × 7 ingredients × 5 replicates).

successfully differentiated in the LDA, with a classification accuracy of 100 % according to a leave-one-out classification matrix (Table S2 and S3, Supplementary Material).

3.3. Hierarchical Cluster Analysis (HCA)

In addition, we have applied HCA to further explore the intrinsic similarities between different green teas. HCA can deeply explore the intrinsic relationship between data, and gradually aggregate the samples according to the similarity of their qualities. The ones with the highest similarity are preferentially aggregated together, showing the inter-class spacing (Euclidean distance) smaller; the least similar is then aggregated together, showing a larger inter-class spacing.[26] As shown in Fig. 3, the samples are obviously separated into two groups: A and B; Jinzhai Cuimei and Biluochun have a minimum euclidean distance less than 5, followed by Mingyuan spring buds and high-grade green tea as the euclidean distance close to 10. The reason might be that Jinzhai Cuimei and Biluochun are both from mountainous areas and affected by a similar topography, temperature and humidity; high-grade green tea and Mingyuan spring buds are from Wuxi and Jurong, respectively, which are hilly terrain and affected by similar climatic conditions; with the influence of various comprehensive factors, such as climate, topography, soil conditions, etc., it eventually leads to other far-euclidean distances.

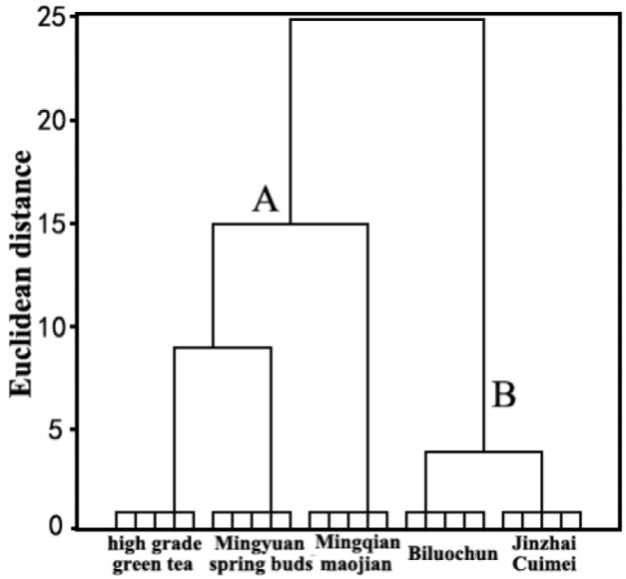

Figure 3 Hierarchical cluster analysis of five target green teas, according to a multi-dimensional matrix (5 green teas × 7 ingredients × 5 replicates).

3.4. HPLC Analysis

Recently, more and more studies show that green tea contains a variety of bioactive ingredients, including polyphenols, flavonoids, pigments, polysaccharides, etc.[2,27] Kodama's group have found that catechins are one of the major tea polyphenols, which account for 20–30 % of the dry weight, such as (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-gallocatechin (EC) and (+)-catechin (C).[27] Herein, HPLC analysis was carried out to determine the composition of polyphenols in green tea. The results are shown in Table 1, indicating that high-grade green tea contains a large amount of C, and a certain amount of ECG, EGC and EGCG; Mingyuan spring buds consisted of C, EGCG and ECG; there are four kinds of polyphenols in Biluochun and Mingqian maojian, such as C, EGCG, ECG and EGC; there are a lot of C/EGCG and a small amount of ECG in Jinzhai cuimei. These results are in consistent with former ones obtained by Folin–Ciocalteu colorimetry to a certain degree. Each experiment was conducted in triplicate. Certainly, there are some other polyphenols, which will be studied in our later research. All the raw data are shown in Fig. S4–9 in the Supplementary Material. The specific components and other characterizations of our findings need further research in the future. More studies on other samples will also be done in future.

4. Conclusion

In the present study, the discrimination of five green teas from different regions based on 'fingerprints' map was established based on the contents of active ingredients [polyphenols, flavonoids, polysaccharides, pigments (thearubigins, theaflavins, theabrownins)] in green teas, which is green, simple and cost-effective; in addition, linear discriminant analysis (LDA) and hierarchical cluster analysis (HCA) were applied to discrimi-

Table 1 Contents of polyphenols (mg mL^{-1}) in the green teas.

Samples	Component	Retention time	Content /mg mL^{-1}	Samples	Component	Retention time	Content /mg mL^{-1}
High grade green tea	EGC	3.85	0.26 ± 0.04	Mingqian maojian	EGC	3.80	0.29 ± 0.05
	C	4.78	3.80 ± 0.21		C	4.73	3.08 ± 0.26
	EGCG	9.38	1.29 ± 0.03		EGCG	9.30	1.47 ± 0.15
	ECG	11.51	0.44 ± 0.03		ECG	11.48	0.45 ± 0.06
	Total	–	5.79		Total	–	5.29
Jinzhai cuimei	C	4.76	3.68 ± 0.23	Mingyuan spring buds	C	4.69	4.58 ± 0.22
	EGCG	9.32	3.06 ± 0.25		EGCG	9.25	1.42 ± 0.14
	ECG	11.49	0.50 ± 0.02		ECG	11.46	0.55 ± 0.03
	Total	–	7.24		Total	–	6.55
Biluochun	EGC	3.76	0.31 ± 0.03				
	C	4.70	4.51 ± 0.28				
	ECG	11.47	0.61 ± 0.06				
	EGCG	9.25	2.07 ± 0.18				
	Total	–	7.50				

Note: the retention time of standards are as follows (EGC: 3.85 min, C: 4.70 min, EC: 8.79 min, EGCG: 9.30 min, ECG: 11.48 min); if the retention time of a component in the sample solution differs from that of a standard solution within ±0.1 min, the component can be identified as the standard.

nate green teas more intuitively, which laid a foundation for the later analysis of more 'fingerprints' mapping of tea in China.

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Supplementary Material

Supplementary information is provided in the online supplement.

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Supplementary Material

For

Study on "Fingerprints" for Green Tea from Different Planting Areas in Eastern China

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Materials and methods

1. The standard curve for determination of polyphenols

The tea extracts' polyphenols were determined by using Folin-Ciocalteu colourimetry and using gallic acid as the standard. The gallic acid solution was diluted into a range of different concentrations, respectively mixed with some colour-forming reagents, and then the absorbance was measured at 765 nm. Three parallel tests were done at each concentration. The standard curve was prepared by plotting the concentration against the absorbance, shown in Figure S1.

The results showed that the standard curve equation is y=0.0165x+0.0102 (where x is the sample concentration, y is the mean absorbance of sample, r=0.9980).

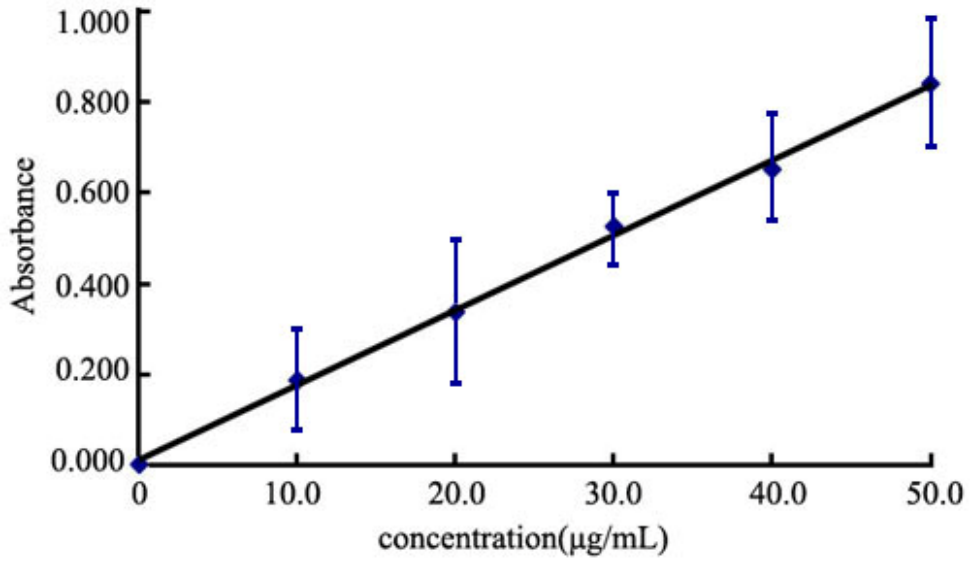

Figure S1. The standard curve of gallic acid, values are means ± RSD (%), n =3 tests at gallic acid concentrations of 0, 10.0, 20.0, 30.0, 40.0, and 50.0 μg/mL.

2. The standard curve for determination of total flavonoids

To quantify the flavonoids extracted from tea, we carried out the experiments to obtain the rutin standard curve. The rutin standard solution was diluted into a range of different concentrations, respectively mixed with some colour-forming reagents, and then measured the absorbance at 510 nm. By plotting concentration against absorbance, the standard curve was prepared, which is shown in Figure S2. It could be seen that the linear dynamic range of rutin was from 0 to 0.05 mg/mL, and the equation of the standard curve is y=9.07x-0.004 (where x is the sample concentration, y is the absorbance of sample, r=0.9987). All the data were taken in triplicate, the means and relative standard deviation (%) values were calculated.

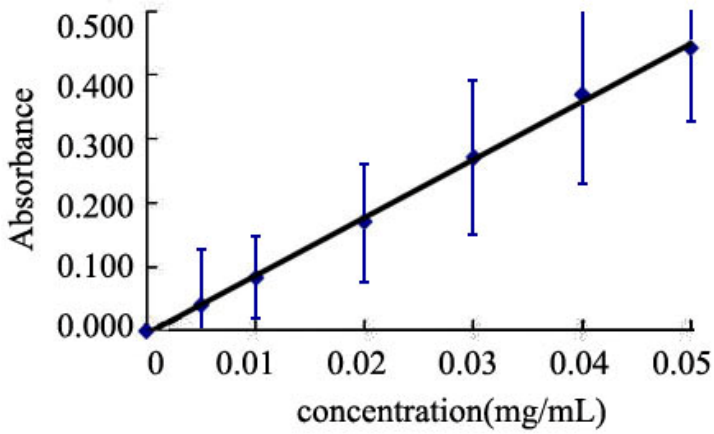

Figure S2. The standard curve of rutin, values are means ± RSD (%), n =3 tests at rutin.

concentrations of 0, 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 mg/mL.

3. The determination of pigments

The determination of pigments, such as total pigments, theaflavins (TFs), theabrownins (TBs), thearubigins (TRs), was referred to the Roberts assay with slight modification. 0.2 g of tea extract was diluted into 100 mL with water for preparing solutions A, B, C and D. Solution A: first pipette 25 ml of the former diluent, add 25 mL of ethyl acetate, then shake and stand for a few minutes to get 2 mL of ethyl acetate extract, lastly add 23mL of 95% ethanol to the ethyl acetate extract. Solution C: get the former ethyl acetate extract of 15mL, then add 15mL of 2.5% $NaHCO_3$ solution, then shake and stand for a few minutes to get 4 mL of ethyl acetate extract, at last add 21mL of 95% ethanol to the ethyl acetate extract. Solution B: take 2 mL of the former aqueous solution (from the process of making solution A), add 2 mL of the saturated oxalic acid solution and 6 mL of water, lastly add 15 mL of 95% ethanol to the solution. Solution D: pipette 25 mL of the former diluent, add 25 mL of n-butanol, then shake and let it stand for a few minutes to form a 2 mL water layer, add 2 mL of the saturated oxalic acid solution and 6 mL of water, lastly add 15 mL of 95% ethanol to the solution. Each solution's absorbance was measured at a wavelength of 380 nm by using 95% ethanol as a blank.

The formulas for the determination of pigments concentrations were as follows:

TFs (%)=2.25×Ac×100%/m

TRs (%)=7.06×(2Aa+2Ad-Ac-2Ab)×100%/m

TBs (%)=2×Ad×7.06×100%/m

Here, m was the sample weight; Aa, Ab, Ac, Ad was the absorbance of solution A, B, C and D, respectively; 2.25 and 7.06 were the conversion factors under the same operating conditions.

4. The standard curve for determination of polysaccharides

The tea extracts' polysaccharides were determined by the phenol sulfuric acid assay method using glucose as the standard. The glucose solution was diluted into a range of different concentrations by mixing with some colour-forming reagents, and then the absorbance was measured at 490 nm. Three parallel tests were done at each concentration. By plotting the concentration against absorbance, the standard curve shown in Figure S3 was prepared.

The results showed that the standard curve equation is y=1.3618x-0.0197 (where x is the sample concentration, y is the mean absorbance of sample, r=0.9966).

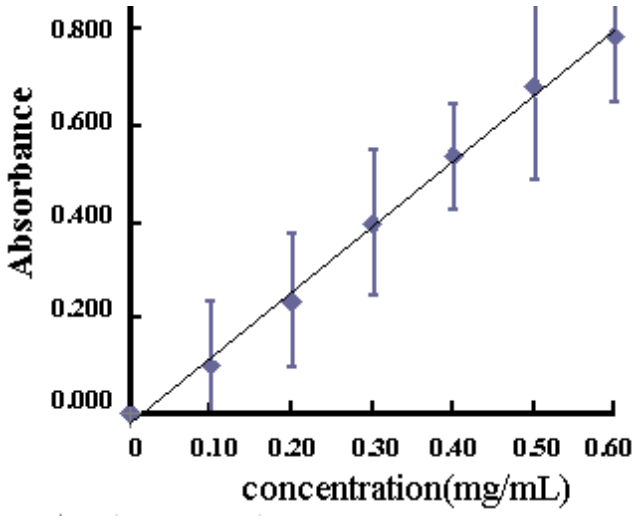

Figure S3. The standard curve of glucose, values are means ± RSD (%), n = 3 tests at gallic acid concentrations of 0, 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 mg/mL.

5. The raw data of the green tea active ingredients

Table S1. The contents of the green tea active ingredients.

	flavonoids	polyphenols	theaflavins	theabrownins	thearubigins	pigment	polysaccharides
	mg/mL	mg/mL	%	%	%	%	%
Mingqian maojian	0.1378	7.21	0.108	1.165	1.566	2.839	0.715
	0.1347	7.11	0.104	1.201	1.624	2.929	0.718
	0.1358	7.13	0.1	1.168	1.61	2.878	0.733
	0.1362	7.15	0.107	1.176	1.578	2.861	0.728
	0.135	7.19	0.102	1.198	1.597	2.897	0.723
Mingyuan spring buds	0.1072	7.58	0.118	0.154	1.49	1.742	0.519
	0.1162	7.62	0.123	0.172	1.48	1.775	0.515
	0.1264	7.63	0.134	0.174	1.491	1.799	0.528
	0.1198	7.77	0.129	0.168	1.501	1.798	0.523
	0.1203	7.68	0.132	0.176	1.483	1.791	0.578
high grade green tea	0.1285	6.6	0.177	0.194	1.539	1.91	1.382
	0.1187	6.54	0.169	0.184	1.542	1.895	1.405
	0.1092	6.54	0.176	0.186	1.536	1.898	1.392
	0.1199	6.43	0.179	0.189	1.562	1.93	1.412
	0.1204	6.67	0.182	0.193	1.553	1.928	1.409
Biluochun	0.1632	7.87	0.093	0.325	1.513	1.931	0.553
	0.2034	7.85	0.102	0.343	1.517	1.962	0.588
	0.1836	7.83	0.09	0.307	1.503	1.9	0.563
	0.179	7.92	0.099	0.336	1.52	1.955	0.572
	0.192	7.79	0.112	0.351	1.532	1.995	0.588
Jinzhai Cuimei	0.1354	7.83	0.117	0.169	1.404	1.69	0.643
	0.1043	7.89	0.114	0.171	1.423	1.708	0.643
	0.1038	7.83	0.111	0.167	1.436	1.714	0.703
	0.1225	7.79	0.121	0.175	1.454	1.75	0.711
	0.1305	7.93	0.119	0.182	1.442	1.743	0.693

6. The linear discriminant analysis (LDA) and cross-validation with leave-one-out classification matrix

Table S2. Training matrix obtained from the sensor array against 5 kinds of green tea samples. LDA was carried out and resulting in factors of the canonical scores and group generation. Cross-validation with leave-one-out classification showed the 100% correct classification

Analytes	Results LDA (The first two factors)		Group
Green tea	Factor 1	Factor 2	
Mingqian maojian	92.47	2.68	1
Mingqian maojian	95.28	1.71	1
Mingqian maojian	92.32	2.36	1
Mingqian maojian	93.66	3.38	1
Mingqian maojian	96.11	2.32	1
Mingyuan spring buds	-30.34	-14.08	2
Mingyuan spring buds	-29.25	-14.42	2
Mingyuan spring buds	-30.80	-14.28	2
Mingyuan spring buds	-31.54	-15.72	2
Mingyuan spring buds	-29.70	-11.73	2
high grade green tea	-28.12	32.86	3
high grade green tea	-28.15	34.26	3
high grade green tea	-28.47	34.38	3
high grade green tea	-29.29	34.85	3
high grade green tea	-29.25	33.63	3
Biluochun	-9.03	-16.33	4
Biluochun	-8.25	-15.61	4
Biluochun	-10.58	-16.18	4
Biluochun	-8.82	-16.25	4
Biluochun	-8.81	-15.04	4
Jinzhai Cuimei	-25.69	-7.69	5
Jinzhai Cuimei	-25.59	-7.76	5
Jinzhai Cuimei	-25.75	-5.06	5
Jinzhai Cuimei	-26.76	-5.35	5
Jinzhai Cuimei	-25.65	-6.93	5

Table S3. The results of leave-one-out classification matrix [a,c]

			Predicted group membership					Total
		Group	1	2	3	4	5	
Original	Number	1	5	0	0	0	0	5
		2	0	5	0	0	0	5
		3	0	0	5	0	0	5
		4	0	0	0	5	0	5
		5	0	0	0	0	5	5
	%	1	100.0	0	0	0	0	100.0
		2	0	100.0	0	0	0	100.0
		3	0	0	100.0	0	0	100.0
		4	0	0	0	100.0	0	100.0
		5	0	0	0	0	100.0	100.0
Cross Validation[b]	Number	1	5	0	0	0	0	5
		2	0	5	0	0	0	5
		3	0	0	5	0	0	5
		4	0	0	0	5	0	5
		5	0	0	0	0	5	5
	%	1	100.0	0	0	0	0	100.0
		2	0	100.0	0	0	0	100.0
		3	0	0	100.0	0	0	100.0
		4	0	0	0	100.0	0	100.0
		5	0	0	0	0	100.0	100.0

a: 100% of the original grouping observations have been correctly classified;

b: Only those observations in the analysis are cross-validated. In Cross-validation, each observation is classified according to a function derived from all observations other than that observation;

c: 100% cross-validation of grouped observations classified correctly.

7. HPLC

7.1 The standard curve for determination of different polyphenols

The determination of different polyphenols in tea extracts was carried out by HPLC using an external standard method, taken EGC, C, EC, EGCG and ECG as examples. The standard solutions were diluted into a range of different concentrations (40-100 μg/mL), respectively. Three parallel tests were done at each concentration. The standard curves were prepared by plotting concentration against peak area, which are shown in Figure S4. The results show that the equation of EGC, C, EC, EGCG and ECG is y=-73760.97+6611.08x (r=0.99783), y=368298.98+11183.11x (r=0.99253), y=52217.22+24658.56x (r=0.99311), y=-312168.93+26823.54x (r=0.99168), y=-1.14728E6+71669.01x (r=0.99573) (where x is the sample concentration, y is the mean peak area of sample), respectively.

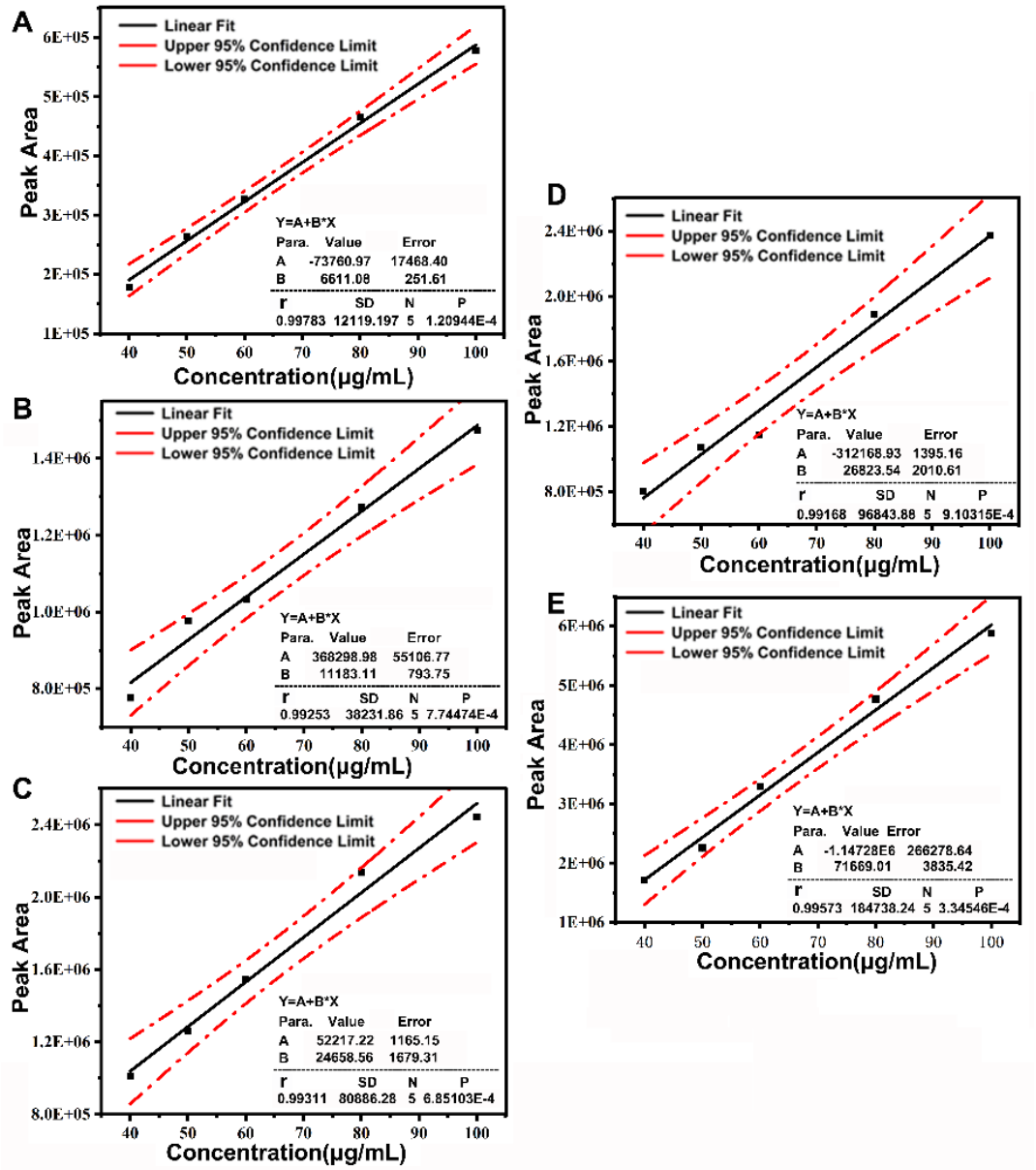

Figure S4. The standard curve of different polyphenols, values are means ± RSD (%), n =3 tests at concentrations of 40, 50, 60, 80 and 100 μg/mL. A: EGC; B: C; C: EC; D: EGCG; E: ECG.

7.2 The HPLC spectra of different teas

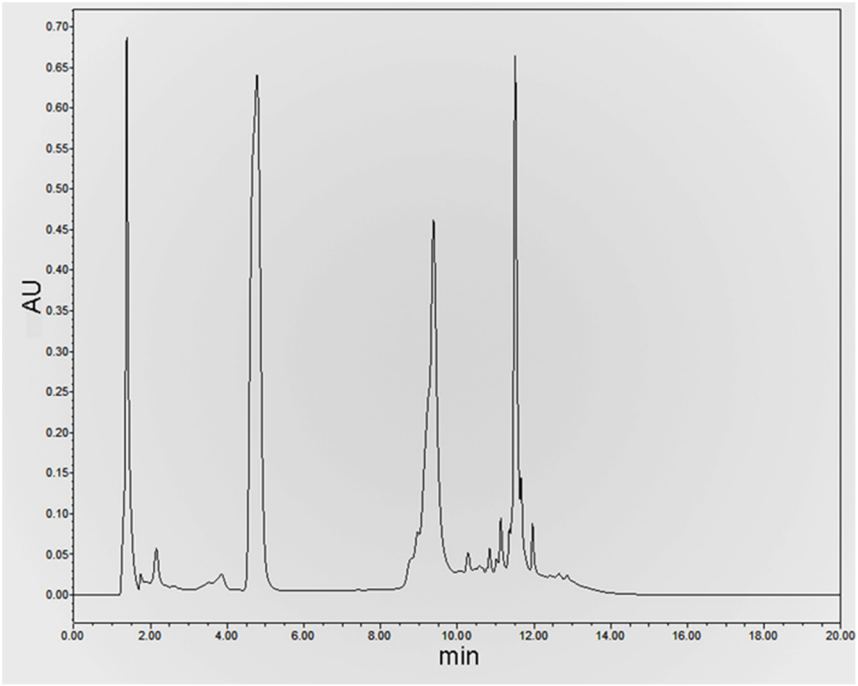

Figure S5. The HPLC spectrum of high-grade green tea.

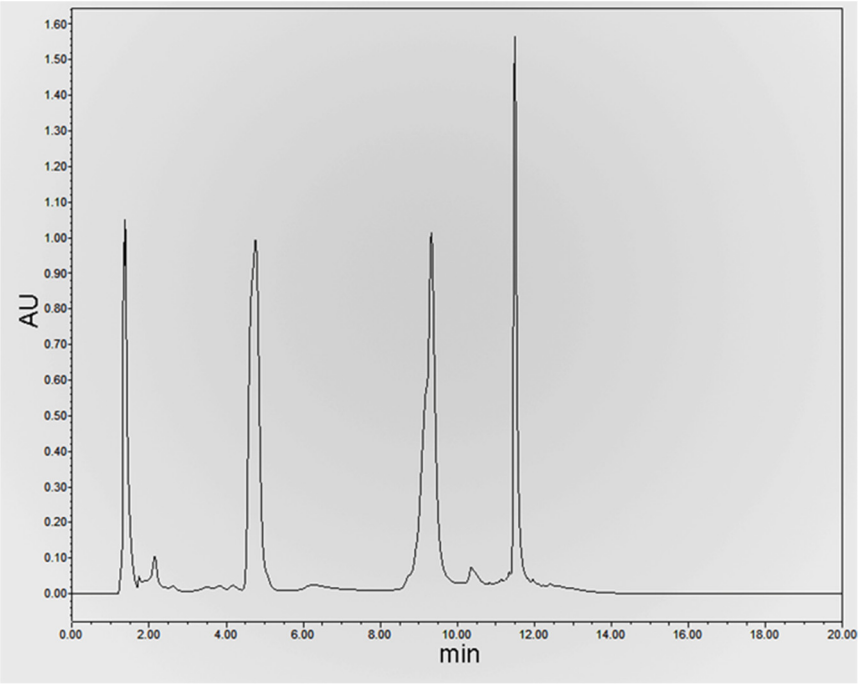

Figure S6. The HPLC spectrum of Jinzhai cuimei.

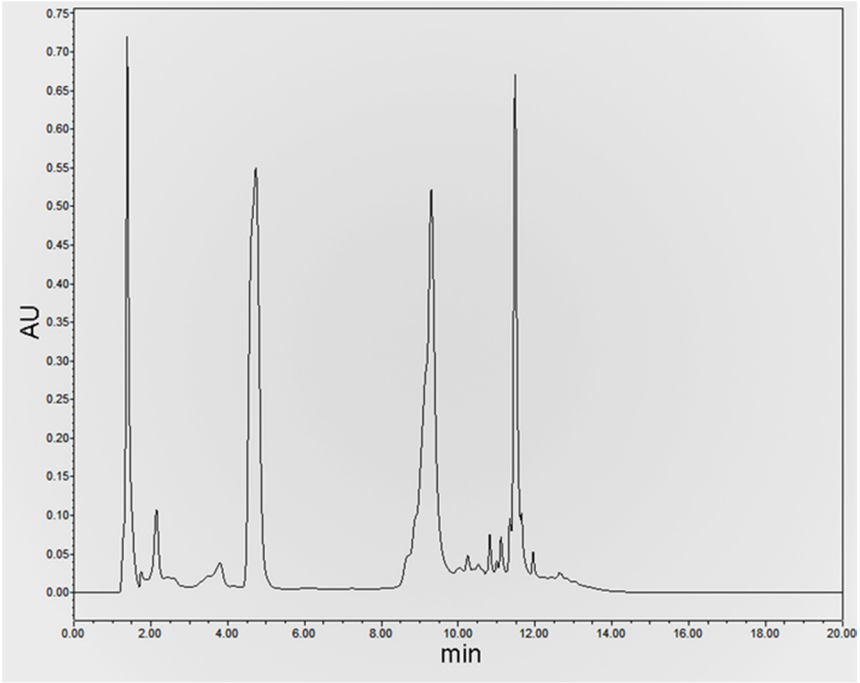

Figure S7. The HPLC spectrum of Mingqian maojian.

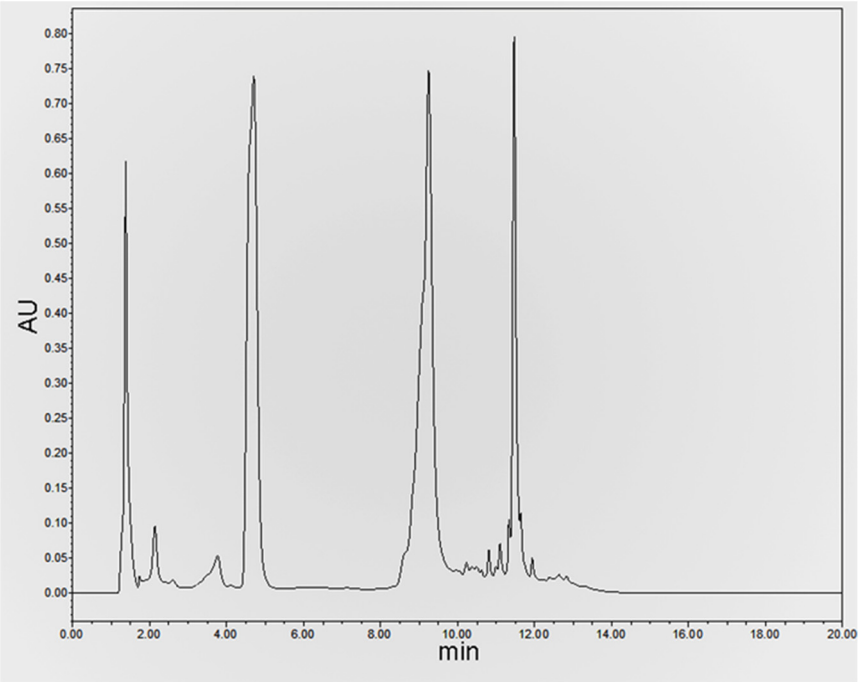

Figure S8. The HPLC spectrum of Biluochun.

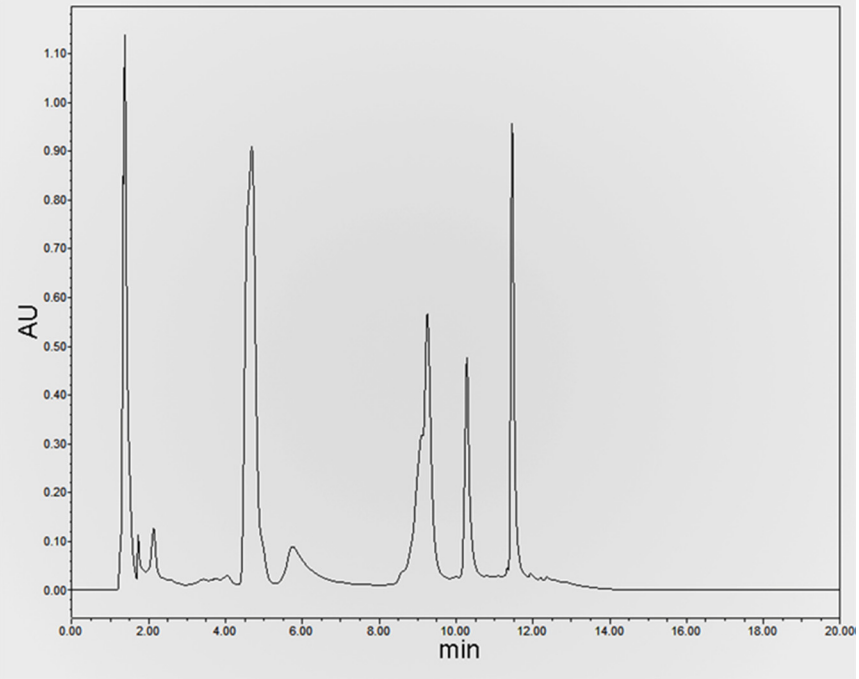

Figure S9. The HPLC spectrum of Mingyuan spring buds.