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Simultaneous Determination of Seven Bioactive Components in Oolong Tea *Camellia sinensis*: Quality Control by Chemical Composition and HPLC Fingerprints

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ABSTRACT: A simple and reliable method of high-performance liquid chromatography (HPLC) was developed for the quality control of oolong tea (the dry leaves of *Camellia sinensis*): the quality control included the HPLC fingerprint and the quantitative determination of seven bioactive compounds chemicals, namely, (−)-gallocatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, caffeine, (−)-epicatechin, gallocatechin gallate, and (−)-epicatechin gallate. The developed analyses of the chemicals excelled in quantifying the chemicals in oolong tea. The chemical fingerprint of oolong tea was established using the raw materials of three main production sites in China, that is, Fujian (southern and northern parts), Taiwan, and Guangdong. The fingerprints from different cultivated sources were analyzed by hierarchical cluster analysis, similarity analysis, principal component analysis (PCA), analysis of variance (ANOVA), and discriminant analysis. The results indicated that the combination of chromatographic fingerprint and quantification analysEs could be used for the quality assessment of oolong tea and its derived products.

KEYWORDS: *oolong tea, HPLC-UV, fingerprint*

INTRODUCTION

Oolong tea is the partially fermented dried leaves of *Camellia sinensis* (L.) O. Ktze, and the main production sites are Guangdong province, Fujian province, and Taiwan province. This tea is popularly consumed in China, the United States, Japan, and elsewhere. Oolong tea has been demonstrated in possessing activities for antioxidant properties,[1] reduction of cholesterol,[2] depression of hypertension,[3] antimicrobial properties,[4] cardiovascular disease,[5] and cancer.[6]

Because of the great consumption of oolong tea worldwide, the quality control and safety of the tea, or its related products, have received a great deal of attention. The quality of tea is closely related to the composition of many of itschemical constituents, and indeed the levels of these chemicals greatly vary according to the environmental conditions of their production sites. Chemometrics coupled with the high-performance liquid chromatography (HPLC) fingerprint had become one of the most frequently applied approaches in assisting the recognition of the origin of production (area and manufacturer), which has been widely studied and applied to different phytomedicines in recent years.[7−9] The major chemical constituents of oolong tea are polyphenols, alkaloids, free amino acids, protein, and chlorophyll. Among these chemicals, polyphenols and alkaloids are well-studied bioactive components. The major phenolic compounds identified in oolong tea are (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin (EC), (−)-gallocatechin-3-gallate (GCG), and (−)-epicatechin gallate (ECG). In addition, the major alkaloid in oolong tea is caffeine.[10] Therefore, the identification and determination of polyphenols and/or alkaloids have been a focus for the chemical control of oolong tea.[11−14] Among different analytical methods, HPLC is the most powerful technology and shows good separations of the tea phenolic compounds.[15]

Here, we aimed to determine the parameters for quality control of oolong tea from three main productive areas in China. The chromatographic fingerprints of oolong tea from three sources were established and analyzed by different correlationship programs, including hierarchical cluster analysis (HCA), similarity analysis, and principal component analysis (PCA). In addition, a HPLC-UV method was developed for the simultaneous determination of the seven bioactive components in oolong tea. The combination of chromatographic fingerprint analysis and chemical determination of active ingradients offers a comprehensive strategy for the evaluation of oolong tea.

MATERIALS AND METHODS

Chemicals, Reagents, and Materials. Twenty-five samples of oolong tea were collected and are summarized in Table 1. The samples were authenticated by Ying Jia, Department of Pharmacognosy, Shenyang Pharmaceutical University, according to the morphological characteristics of oolong tea. Chemical standards were purchased from the Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and Mansitei Co. Ltd. (Chengdu, China). Methanol of HPLC grade was obtained from Yu-wang Chemical Factory (Shandong, China). Phosphoric acid of HPLC grade was obtained from Beijing Reagent Co. (Beijing, China). Distilled water, prepared from demineralized water, was used throughout the experiment.

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Table 1. Different Cultivation Regions of Oolong Tea

sample	brand	production	similarity[a]
S1	Tieguanyin 1	Fujian (southern)	0.987
S2	Tieguanyin 2	Fujian (southern)	0.993
S3	Foshou	Fujian (southern)	0.986
S4	Maoxie	Fujian (southern)	0.987
S5	Meizhan	Fujian (southern)	0.989
S6	Qilan	Fujian (southern)	0.997
S7	Toutianxiang	Fujian (southern)	0.999
S8	Rougui	Fujian (northern)	0.998
S9	Dahongpao 1	Fujian (northern)	0.997
S10	Dahongpao 2	Fujian (northern)	0.991
S11	Shuixian	Fujian (northern)	0.997
S12	Bailv	Fujian (northern)	0.985
S13	Dongdingwulong	Taiwan	0.989
S14	Gaoshanwulong	Taiwan	0.996
S15	Renshenwulong	Taiwan	0.987
S16	Guihuawulong	Taiwan	0.992
S17	Alishanwulong	Taiwan	0.989
S18	Dongfangmeiren	Taiwan	0.997
S19	Naixiangwulong	Taiwan	0.998
S20	Fenghuangshuixian	Guangdong	0.982
S21	Huangzhixiang	Guangdong	0.975
S22	Songzhong	Guangdong	0.947
S23	Yulanxiang	Guangdong	0.979
S24	Zhilanxiang	Guangdong	0.981
S25	Milanxiang	Guangdong	0.948

[a]The similarity was calculated by similarity analysis, each fingerprint (from Figure 2) was compared the consensus chromatogram "R". All samples showed a similarity of at least >0.94.

Sample Preparation. The reference standards of the target compounds, that is, GC, EGC, EGCG, CAF, EC, GCG, and ECG, were accurately weighed and dissolved in methanol at 9.92, 14.68, 100.4, 59.64, 9.796, 5.988, and 20.04 μg/mL, respectively. The standards were then diluted to appropriate concentration ranges for the establishment of calibration curves. These stock solutions were stored at 4 °C. The oolong tea samples were powdered to a homogeneous size in a mill, and the accurately weighed powder (0.5 g) was brewed for 10 min with 100 mL of 100 °C water, and a final 0.05% phosphoric acid was added to the solution. This was followed by heating at 90 °C for 10 min in a water bath. The extracts was filtered through a 0.45 μm membrane prior to an injection into the HPLC system

Apparatus and Chromatographic Conditions. The assay was performed on a Hitachi (Japan) HPLC system equipped with an L-2400 UV detector and an L-2130 pump. The chromatographic separation was carried out on a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm; Zhonghuida Co. Ltd., China), maintained at 35 °C. The UV absorbance was monitored at 278 nm. The mobile phase was methanol (A) and 0.05% phosphoric acid aqueous solution (B) with a gradient program as follows: 0−18 min, linear gradient 5−16% A; 18−25 min, linear gradient 16−21% A; 25−40 min, linear gradient 21−23% A; 25−40 min, linear gradient 21−23% A; 40−65 min, linear gradient 23−38% A; 65−75 min, linear gradient 38−57% A; and 75−85 min, linear gradient 57−70% A at a flow rate of 1 mL/min. All injection volumes of samples and standard solutions were 20 μL.

Method Validation. Precision, repeatability, stability, and recovery were all carried out to validate the HPLC method, following an International Conference on Harmonization (ICH) guideline.[16] Standard stock solutions containing seven analytes were prepared and diluted with methanol to appropriate concentrations for plotting the calibration curves. Six concentrations of the seven analyte solutions were analyzed, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte.

Precision was determined by analyzing known concentrations of the seven analytes in six replicates during a single day. To confirm the repeatability, six different sample solutions prepared from the same sample were analyzed. The relative standard deviation (RSD) was taken as a measure of precision and repeatability. The stability of sample solutions was tested at roomtemperature. The sample solution was analyzed every 2 h within the 12 h period. Recovery tests were carried out to investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of the tea samples. The samples were then extracted and analyzed with the described method. The average percentage recoveries were evaluated by calculating the ratio of detected amount versus the added amount.

The methodology of chromatographic fingerprinting was validated for its precision, repeatability, and stability. The precision was evaluated by the analysis of six injections of the same testing sample consecutively. The repeatability was examined by determination of six different samples prepared from the same botanical sample. The stability was examined by analysis of sample solution at different time points, that is, 0, 2, 4, 6, 8, and 12 h.

Data Analysis. Hierarchical cluster analysis is a multivariate analysis technique that is used to sort samples into groups.[17] Here, differnet samples of oolong tea were analyzed by using SPSS 16.0 software, and the results were subjected to HCA. Similarity tests were performed on the basis of the relative retention time and relative peak area (angle cosin method to calculate relative peak area), using the professional software named Similarity Evaluation System for Chromatographic Fingerprint (2009).[18,19]

PCA was used for separating interrelationships into statistically independent, basic components: this analysis was useful in regression analysis to mitigate the problem of multicollinearity and to explore the relationships among the independent variables, which allowed the identification of the primary predictors with minimal multicollinearity.[20−22] Here, the PCA was performed on the common chromatographic peaks in the HPLC fingerprints using software of SIMCA-P. Analysis of variance (ANOVA) was performed by SPSS 16.0 software. Discriminant analysis is a technique for classifying a set of observations into predefined classes, to determine the class of an observation based on a set of variables known as predictors or input variables. Discriminant analysis was performed by SPSS 16.0 software.

RESULTS AND DISCUSSION

Optimized Extraction Conditions. Various extraction methods (e.g reflux, sonication, boiling), solvents (e.g., water, methanol, ethanol), and number of times of extraction were evaluated to obtain the optimized extraction efficiency. A one-factor circulation method was used for optimizing conditions. In terms of the extractioin efficiency, there were no differences between brewing and other extraction methods, and thus brewing was used here. An aqueous solution of 0.05% phosphoric acid exhibited better extraction for all major constituents. Single extraction was sufficient.

Optimization of HPLC Conditions. The analytical conditions were optimized mainly on the basis of peak resolution, baseline, elution time, and the number of characteristic peaks in the chromatograms. The detection wavelength was set at 278 nm, at which all of the compounds had adequate absorption and no interference. The mobile phases (methanol−water and acetonitrile−water with different modifiers, including acetic acid, formic acid, and phosphoric acid) and column temperatures (30, 35, or 40 °C) were examined and compared. A representative HPLC profile for the seven active chemicals, GC, EGC, EGCG, CAF, EC, GCG, and ECG, is shown in Figure 1A.

Method Validation. *Calibration Curves.* The calculated results are given in Table 2. All of the analytes showed good linearity (r^2 > 0.999) in a wide range of concentrations.

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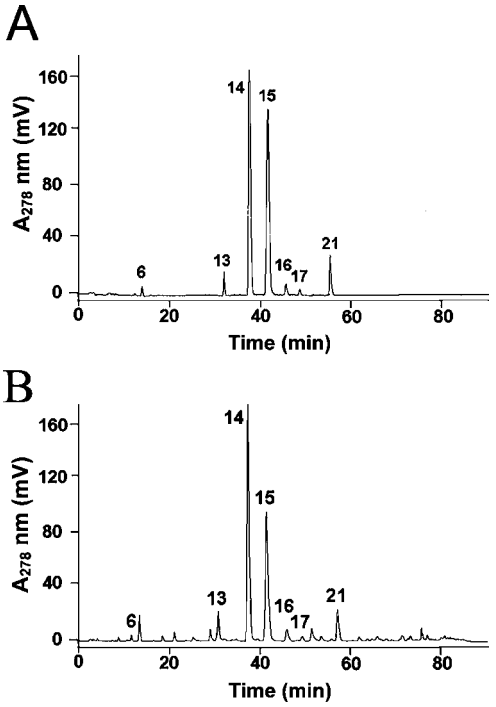

Figure 1.

Precision, Repeatability, and Stability. The results are shown in Table 2. The RSD values of the sven compounds were all <2.4%, which showed the system was excellent in the chemical analysis of oolong tea.

Recovery. The recovery of the method was in the range of 98.8−104.4%, with RSD < 3.8% as shown in Table 2.

Sample Analysis. The developed assay method was subsequently applied to simultaneous determination of GC, EGC, EGCG, CAF, EC, GCG, and ECG in oolong tea, and the peaks corresponding to each chemcial were well-separated (Figure 1A). Different batches of oolong tea were collected from different cultivation regions. Twenty-five samples from three major regions of cultivation were collected here, including Fujian (southern and northern parts), Taiwan, and Guangdong of China (Table 1). Besides, the HPLC fingerprints of the 25 tea samples were also generated at the UV absoption of 278 nm: they were grouped as in Figure 2. The average chromatogram from the 25 batches was regarded as the consensus fingerprint of oolong tea. Peaks existing in all chromatograms of the samples were assigned as "common peaks". Thirty-six common characteristic peaks in the 25 chromatograms were selected. Peak 14 (RT = 44.3 min,

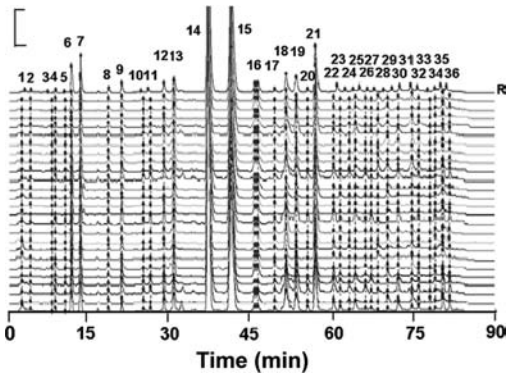

Figure 2.

EGCG) was chosen as the internal reference peak because it was a maximum peak present in the middle of the chromatogram. By comparison to the consensus fingerprint, all tea samples showed a similarity of at least >0.94 (Table 1); that is, these teas showed very similar chemical compositions.

The amounts of GC, EGC, EGCG, CAF, EC, GCG, and ECG were determined here for the 25 tea samples. The amount of EGCG was the highest, and GCG was the lowest, among the tested seven chemicals as described here. In general, the oolong teas from Guangdong contained the greatest abundance of the seven chemicals, in particular, the amounts of CAF and EGCG (Table 3). The pharmacological properties of oolong tea have been mainly attributed to all of these bioactive components. Thus, the current results could be taken into the consideration of the best quality of oolong tea.

Correlationship Analyses. Hierarchical cluster analysis was carried out following the basis of 36 peak values of the HPLC fingerprints. The result showed clearly that these samples were appropriately divided into three main clusters (Figure 3). The samples from Guangdong province (S20−S25) were grouped as one distinct cluster (group III). The samples from Fujian and Taiwan (S1−S19) shared a close similarity; however, a detailed analysis revealed that another two clusters were developed. The samples from Fujian (southern) from S1 to S7 could be grouped as one cluster (group I), and the samples from Fujian (northern) and Taiwan could be clustered (group II), that is, S8−S19. Small differences between Fujian (southern) samples and Taiwan samples were still revealed here.

PCA was employed here to analyze the relationship of the 25 oolong tea samples from different sources. The three-dimensional graphics of PCA scores (Figure 4A) showed that all of the samples could be divided into three parts, Fujian

Table 2. Calibration Curves, Precision, Repeatability, and Recoveries of Bioactive Compounds in Oolong Tea

analyte	regression eq	r^2	linear range (μg mL^{-1})	precision RSD[a] (%)	repeatability RSD (%)	stability RSD (%)	recovery[b] (%)
GC	$Y = 2.079 \times 10^4X - 2.066 \times 10^4$	0.9994	0.4960−9.920	1.0	2.2	1.5	98.0 ± 0.3
EGC	$Y = 7.679 \times 10^4X + 1.716 \times 10^4$	0.9995	0.7340−14.68	1.2	1.8	1.8	99.1 ± 1.1
EGCG	$Y = 5.208 \times 10^5X - 6.742 \times 10^4$	0.9997	5.020−100.4	0.7	2.0	1.2	98.3 ± 0.3
CAF	$Y = 1.470 \times 10^5X - 3.842 \times 10^4$	0.9993	2.982−59.64	0.9	1.2	2.0	98.9 ± 1.2
EC	$Y = 8.347 \times 10^4X + 9.938 \times 10^3$	0.9992	0.4898−9.796	0.8	1.8	2.1	97.6 ± 0.1
GCG	$Y = 1.382 \times 10^5X - 5.026 \times 10^4$	0.9995	0.2994−5.988	1.2	2.4	1.6	97.7 ± 0.6
ECG	$Y = 1.796 \times 10^5X - 9.739 \times 10^4$	0.9994	1.002−20.04	1.0	2.2	1.7	98.4 ± 0.2

[a]Relative standard deviation, where $n = 6$. [b]Recovery (%) = 100 × (amount found − original amount)/amount spiked. The data are presented as the mean ± SD, where $n = 6$.

Table 3. Chemical Composition of Oolong Tea from Different Cultivation Regions in China

sample[a]	GC[b]	EGC	EGCG	CAF	EC	GCG	ECG
S1	0.546	1.251	2.509	3.144	0.697	0.191	0.456
S2	0.594	1.601	2.510	3.520	0.753	0.125	0.476
S3	0.578	1.484	2.435	3.161	0.681	0.199	0.439
S4	0.740	1.893	2.643	3.715	1.247	0.234	1.447
S5	0.853	1.412	2.761	3.909	0.595	0.318	1.039
S6	0.508	1.440	2.868	3.452	0.541	0.339	1.235
S7	0.553	1.421	2.426	3.149	0.673	0.198	0.428
S8	0.204	1.123	2.940	7.279	0.730	0.296	0.899
S9	0.523	1.380	2.665	4.232	0.674	0.146	0.683
S10	0.501	1.321	2.704	4.456	0.734	0.241	0.719
S11	0.513	0.853	2.894	6.518	0.560	0.111	0.631
S12	0.517	0.916	2.939	6.724	0.607	0.189	0.730
S13	0.678	1.624	2.514	7.429	1.138	0.149	1.480
S14	0.490	0.989	2.555	6.427	0.745	0.092	1.364
S15	0.727	1.658	2.872	6.383	1.035	0.129	0.928
S16	0.628	1.704	2.836	5.236	0.971	0.123	0.893
S17	0.518	1.154	2.633	6.776	0.771	0.244	1.383
S18	0.711	1.904	2.546	7.689	1.069	0.220	1.492
S19	0.198	1.130	2.960	8.383	0.716	0.295	1.880
S20	1.601	0.999	4.832	9.117	0.228	0.373	1.462
S21	0.477	0.733	3.427	9.969	0.251	0.383	1.341
S22	0.437	0.710	3.219	8.853	0.503	0.144	1.252
S23	0.984	0.451	4.124	8.538	0.217	0.251	1.672
S24	0.664	1.046	4.885	8.774	0.317	0.441	1.619
S25	0.948	0.513	4.004	8.205	0.249	0.124	1.681

[a]Sample number as listed in Table 1 and Figure 2. [b]Values in mg/g of dry tea; and in mean ± SD, n = 3. The SD was <5% of the mean, which is not shown for clarity.

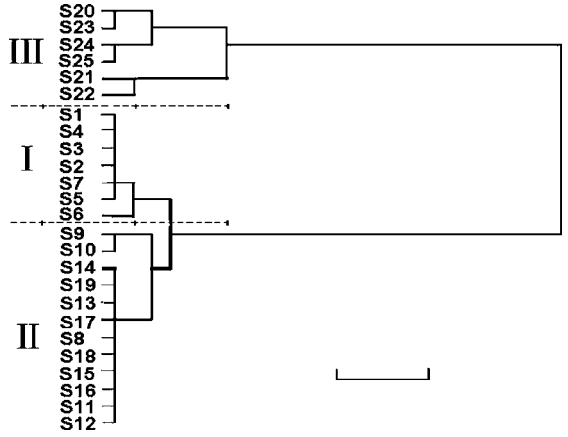

Figure 3.

(southern) (S1−S7), Fujian (northern)/ Taiwan (S8−S19), and Guangdong (S20−S25): this grouping was identical to that of the analysis by HCA. The PCA loading plot (Figure 4B) indicated that variable 14 (peak 14, EGCG) and variable 15 (peak 15, CAF) showed the greatest influence on the scores. Variable 21 (peak 21, ECG) and other variables (peaks 18, 19, 6, and 7) also affected the scores. Thus, the distinction of teas from different cultivation regions therefore could be revealed by the amounts of polyphenols and alkaloids.

On the basis of the results above, Fujian (southern), Fujian (northern)/Taiwan, and Guangdong were assigned as groups 1, 2, and 3, respectively. The data of determination were analyzed by ANOVA followed by an analysis of least significant

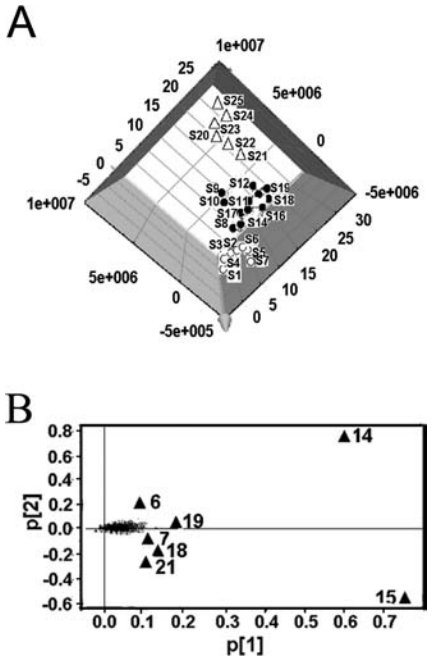

Figure 4.

difference (P < 0.05) among the means; the contents of GC (P < 0.05), EC (P < 0.05), EGCG (P < 0.05), CAF (P < 0.05), EGC (P < 0.05), and ECG (P < 0.05) in three groups of oolong tea were significantly different, except GCG (P > 0.05).

Further analysis was performed by discriminant analysis, and the results were similar; that is, 88.0% of the original grouped cases were correctly classified, and 83.0% of cross-validated grouped cases were correctly classified. By Fisher discriminant, the scatter plot was achieved (Figure 5): this result was

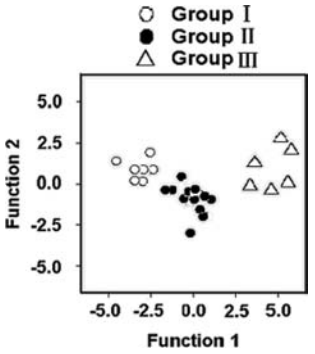

Figure 5.

identical to that of the PCA. Again, three groups of clusters were revealed, that is, group I (S1−S7), group II (S8−S19), and group III (S20−25). The following discriminant functions were obtained.

$$\text{function 1} = 2.352 \times \text{EGCG} + 0.985 \times \text{CAF} - 7.64 \times \text{GCG} - 11.534$$

$$\text{function 2} = 1.924 \times \text{EGCG} - 0.575 \times \text{CAF} + 3.653 \times \text{GCG} - 3.0$$

Using the aforementioned functions, an unknown oolong tea could be classified into one of those three groups (Fujian

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(southern), Fujian (northern)/Taiwan, and Guangdong). Increasing the database of different oolong teas could strengthen the correctness our developed cluster analysis.

The close relationship of Fujian and Taiwan teas could be accounted for by (i) the soil, climate, latitude, planting techniques, and production process being similar; and (ii) the seeds of oolong tea in Taiwan are known to come from Fujian, as recorded in the Qing Dynasty.[23] Guangdong teas have been planted at low latitude and had no modeling in production process, which resulted in less polyphenol loss.

In conclusion, the proposed HPLC fingerprint method combined with chemical quantitative analyses could provide an efficient and comprehensive tool for the quality evaluation of oolong tea in the consumer market. The chemometrics methods, including different cluster and statistical analyses, were proved to be satisfactory in the matching and discrimination of oolong tea from different regions of cultivation. Thus, this study provided an example for source identification and quality evaluation by using a combination of HPLC fingerprint and quantitative analyses.

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