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Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector

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Abstract

A simple and fast HPLC method using a photodiode array detector was developed for simultaneous determination of four major catechins, gallic acid and caffeine. After multiple extractions with aqueous methanol and acidic methanol solutions, tea extract was separated within 20 min using a methanol–acetate–water buffer gradient elution system on a C_{18} column. The sample extraction data demonstrated that the single extraction used in the previous studies with aqueous acetonitrile or methanol is not sufficient; the multiple extraction procedure is essential for the quantitative analysis of catechins, phenolic acids and caffeine in teas. Several green, Oolong, black and pu-erh teas were successfully analyzed by this method. The analytical results obtained indicated that green teas contain higher content of catechins [(−)-epigallocatechin gallate, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epicatechin] than both Oolong, pu-erh and black teas because fermentation process during the tea manufacturing reduced the levels of catechins significantly. The fermentation process also remarkably elevated the levels of gallic acid in full-fermented pu-erh and black teas. Another interesting finding is the low level of caffeine in Oolong teas, especially in Fujian Oolong tea.

Keywords: Green tea; Oolong tea; Pu-erh tea; Black tea; Catechins; Caffeine; Gallic acid; Flavonoids; Polyphenols; Gradient HPLC

1. Introduction

Tea (*Camellia sinensis*), originating in China, is the most widely consumed beverage in the world and has become an important agricultural product. Recent studies have shown that tea confers great beneficial effects to the health of consumers, including the effects of reduction of cholesterol, depression of hypertension, anti-oxidation, anti-microbial, protection against cardiovascular disease and cancer [1,2]. To understand the mechanisms involved in these beneficial effects, a great deal of scientific efforts has been contributed to isolate and identify the active components in various tea samples [3]. Polyphenols, especially catechins and phenolic acids, have been considered the main players in these beneficial

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effects on the human health [1–3]. The major tea catechins are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC). The main tea phenolic acid is gallic acids. Teas also contain certain amount of caffeine, a plant alkaloid occurring also in some other popular beverages such as coffee. Caffeine also has attracted much scientific and public attention during the past years due to its stimulatory effects. The health effects of caffeine have been discussed by Trevisanato and Kim [2].

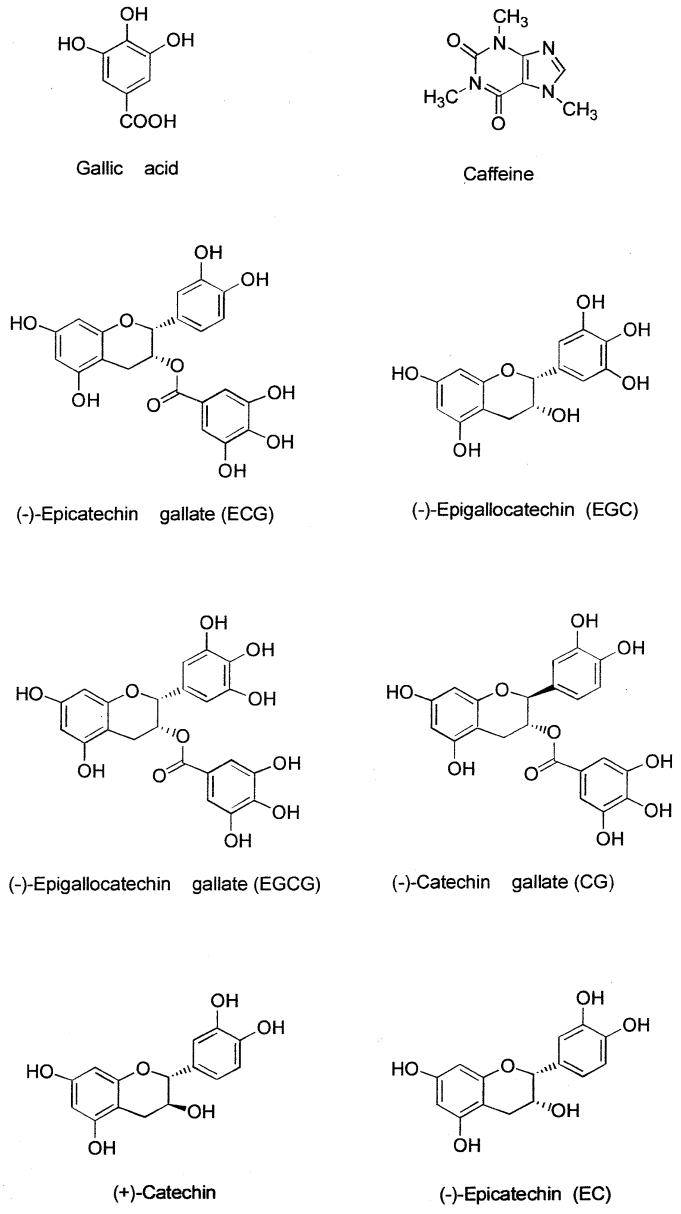

Fig. 1. Structures of catechins, gallic acid and caffeine.

The composition of tea catechins, phenolic acids and caffeine in commercial teas varies with species, season, horticultural conditions and particularly with degree of fermentation during the manufacturing process [4,5]. It is, therefore, important to establish a simple and reliable analytical method for the simultaneous determination of the levels of these compounds in tea samples in developing high quality tea products. In most of previous studies, the tea catechins and caffeine have been analyzed separately by PC [4], TLC [6], GLC [7], HPLC [3,8–12] or spectrophotometric methods [13] with emphasis on individual catechins. But little research has been reported on the determination of free phenolic compounds, which are also important tea constituents with anti-oxidative properties [3]. Capillary electrophoretic techniques have been developed lately to separate catechins, phenolic and ascorbic acids [14–16]. Wang et al. [9] and Lin et al. [10] have used isocratic elution HPLC methods to separate catechins, and gallic acid in green tea infusions successfully. But the isocratic elution resulted in peak broadening and tailing of some less polar catechins. Recently, we have developed a successful gradient elution HPLC method for the quantitative separation and determination of phenolic acids in cranberry juices [17]. In this paper, we report a simple, rapid and precise gradient HPLC method with an economical mobile phase for the simultaneous separation, identification and quantitation of individual catechins, caffeine and phenolic acids in green, Oolong, black and pu-erh teas. Gallic acid was identified to be a main free phenolic acids; the four major catechins determined were EGCG, EGC, ECG, and EC as illustrated in Fig. 1.

2. Experimental

2.1. Chemicals

Gallic acid, 3,5-dihydroxybenzoic acid, 3,4,5-trimethoxycinnamic acid and myricetin were purchased from Acros Organics (Geel, Belglum, NJ). EC was obtained from ICN Biomedicals, INC. (Ohio). Caffeine and *p*-Anisic acid were pur-

chased from Eastman Organic Chemicals. EGC, EGCG, ECG, and (−)-catechin gallate (CG) were from Sigma Chemical Co. (St. Louis, MO). The methanol used for the mobile phase and the tea extraction was of HPLC grade reagent from Pharmco. Products INC. (Brookfield, CT). All solution preparations were made using distilled and then deionized water. All the other chemicals were of analytical reagent grade and were used without further purification.

2.2. Tea samples and preparation of tea extract

Eight commercial teas were purchased at a grocery store in Boston China Town. These are Meifoo green, Hangzhou Lung Ching, Fujian and Jiangxi Oolong, and Fujian black tea packaged in bags, loose leaves of Shanghai green, pu-erh and Jasmine tea produced in Fujian Province, China. All these tea samples were obtained in boxed can except bagged teas in paper box. Tea samples of 1.9–3.8 g was minced, ground and extracted three times with 20 ml 80% methanol for 3 h and then two times with 20 ml 80% methanol containing 0.15% HCl for 3 h. The extracts were combined and filtered through cotton to get rid of rough particles. The solution was further filtered through 0.45 μm of Nylon membrane filter. Fifty microliter of this extract was diluted to 1 ml with distilled–deionized water. A 10 μl of aliquot of this diluted solution was injected onto HPLC.

2.3. HPLC analysis

HPLC analysis was conducted on Beckman liquid chromatograph equipped with a Model 125 dual solvent pump, a 508 autosampler, a 168 photodiode array detector and Gold Nouveau Software. A C_{18} guard column and an Alltech adsorbosil C_{18} reversed-phase packing column (4.5 mm × 25 cm, 5 μm) were used for separation throughout this study. The PDA acquisition wavelength was set in the range of 200–400 nm, Analog output channel A at wavelength 280 nm and analog output channel B at 360 nm both with bandwidth 10 nm. A gradient elution was performed by varying the proportion of solvent A (water–acetic acid, 97:3 v/v) to solvent B (methanol), with a flow rate of 1 ml min^{-1}. The mobile phase composition started at 100% solvent A for 1 min, followed by a linear increase of solvent B to 63% in 27 min, and then bring mobile phase composition back to the initial conditions in 2 min for the next run. All the prepared solutions were filtered through 0.45 μm membranes (Fisher Scientific) and the mobile phase was degassed before injection onto HPLC.

3. Results and discussion

3.1. Sample extraction and effect of extraction solvent concentration on the HPLC separation

The critical step in the quantitation of catechins, phenolic acids and caffeine in teas is sample extraction. The extraction method must enable complete extraction of the compounds of interest and must avoid chemical modification. Three different extraction methods, (1) a normal tea brew with boiling water, (2) an acetonitrile or methanolic extract of ground tea and (3) the official German method for the determination of soluble solids (1 h boiling under reflux) [3,18,19], were used in the previous studies. The official German method led to decomposition of catechins while acetonitrile and methanolic extracts gave the highest yields. Thus, a single extraction with acetonitrile or methanol has been widely used in quantitative analysis of tea polyphenols [3,12,20]. However, our experimental results have shown that large amounts of polyphenols and caffeine still remained in the tea residues after a single methanolic extraction. In this study, a five times extraction, three times with 80% aqueous methanol and then two times with 80% aqueous methanol containing 0.15% HCl, was employed to extract catechins, phenolic acids and caffeine in variety of tea samples. The extraction results (Table 1) demonstrated that the second extracts generally contain more catechins and caffeine than the corresponding first extracts. Even the third and fourth extractions still contain significant amounts of catechins and caffeine. Thus, a multiple extraction procedure is essential for the quantitative analysis of catechins, phenolic acids and caffeine in teas.

Table 1
Extraction efficiency of catechins, gallic acid and caffeine extracted from various teas with 80% aqueous methanol (1st–3rd extractions) and 80% methanol containing 0.15% HCl (4th and 5th extractions) at 20 °C, each extraction for 3 h

Tea (mg ml^{-1})	Extract	GA	EGC	EGCG	EC	ECG	CA	CG
Pu-erh	1st	0.085	0.063	0.032	0.032	0.022	0.36	–
	2nd	0.133	0.133	0.039	0.062	0.029	0.47	–
	3rd	0.076	0.089	0.035	0.041	0.025	0.28	–
	4th	0.062	0.095	0.040	0.042	0.025	0.29	–
	5th	0.025	0.051	0.034	0.017	0.016	0.16	–
Meifoo green tea	1st	0.028	1.17	1.75	0.39	0.76	0.99	–
	2nd	0.026	1.11	1.97	0.39	0.85	0.96	–
	3rd	0.016	0.47	0.96	0.170	0.39	0.49	–
	4th	0.0082	0.31	0.60	0.099	0.22	0.28	–
	5th	0.0028	0.147	0.31	0.047	0.104	0.119	–
Shanghai green tea	1st	0.0054	0.37	0.51	0.093	0.121	0.38	–
	2nd	0.0102	0.94	1.44	0.208	0.332	0.68	–
	3rd	0.0034	0.23	0.40	0.063	0.092	0.19	–
	4th	0.0028	0.22	0.37	0.049	0.079	0.144	–
	5th	0.0018	0.18	0.31	0.035	0.067	0.117	–
Hangzhou Lung Ching	1st	0.079	0.98	1.72	0.218	0.444	0.79	0.030
	2nd	0.057	1.16	1.95	0.244	0.523	0.87	0.023
	3rd	0.035	0.68	1.06	0.158	0.264	0.38	0.0096
	4th	0.015	0.58	0.71	0.063	0.162	0.27	0.0058
	5th	0.018	0.59	0.71	0.055	0.140	0.22	0.0044
Jasmine	1st	0.0088	0.20	0.28	0.041	0.087	0.31	–
	2nd	0.0282	0.76	1.31	0.168	0.41	0.74	–
	3rd	0.0108	0.29	0.52	0.067	0.149	0.29	–
	4th	0.0098	0.29	0.54	0.067	0.143	0.26	–
	5th	0.0036	0.154	0.30	0.034	0.079	0.128	–
Fujian Oolong	1st	0.034	0.151	0.23	0.041	0.068	0.044	0.0012
	2nd	0.44	1.72	1.24	0.31	0.252	1.21	0.0226
	3rd	0.038	0.191	0.40	0.049	0.114	0.30	0.0074
	4th	0.032	0.202	0.35	0.039	0.094	0.26	0.0066
	5th	0.021	0.138	0.24	0.029	0.053	0.16	0.0018
Jiangxi Oolong	Combined	0.033	0.32	0.56	0.059	0.129	0.37	–
Fujian black	Combined	0.041	0.114	0.076	0.027	0.089	0.43	–

It should be noted that strong solvents (80% aqueous methanol) have been used in the sample extraction. Severe peak splitting, tailing, and early elution can occur if these extracts are directly injected onto an HPLC system without further treatment because the sample solvent is of higher strength than the HPLC mobile phase. However, this does not present a problem once the extracts are diluted with distilled water as described in Section 2. The diluted tea extracts can be easily determined using HPLC–DAD due to its high sensitivity.

3.2. Chromatographic separation of standard and tea catechins, phenolic acids and caffeine

Both isocratic and gradient elution program with acetonitrile–acetate buffer or methanol–acetate buffer mobile phases were tested. Although isocratic elution could separate all catechins of

interest, the separation time was generally long (about 60 min) and several late-eluting components were broadened and tailed. A gradient elution program using methanol–acetic acid–water as solvent was finally chosen. Fig. 2 depicted a chromatogram of a standard mixture of 11 catechins, phenolic acids and caffeine. All these compounds were successfully separated within 25 min. Figs. 3–6 illustrated the representative separations of catechins, phenolic acids and caffeine in green, Oolong, pu-erh and black tea, respectively. The actual separation times for tea extracts are less than 20 min. It is not easy to separate (+)-catechin and ECG under the described conditions. However, the peak purity study has shown that signal ratios (relative absorbances at different wavelengths) were constant across the peak profile of ECG and other components, indicating that no significant amount of (+)-catechin occurs in all tea samples studied in agreement with previous studies [3,9,10]. EGCG, EGC, ECG, EC, caffeine, and gallic acid were identified as major components in all green, Oolong, pu-erh and black tea samples based on comparisons of chromatographic retention time and UV–Vis absorbance spectra of tea extracts with those of authentic standards. Trace amounts of CG were also observed in both Hangzhou Lung Ching and Fujian Oolong teas.

3.3. Quantitative analysis

Calibration curves were obtained at a detection wavelength of 280 nm for 5 catechins, gallic acid and caffeine using a series of standard solutions over the concentration range from 0.19 to 78.0 mg l^{-1}, as shown in Fig. 7. All calibration curves were linear over the concentration ranges tested with correlation coefficients ≥ 0.996. Although the sensitivity of the analysis could be enhanced up to three-time by using a shorter detection wavelength, the potential for interference by other components in complex tea extracts and mobile phase could also increase significantly. Considered the levels of catechins, gallic acid and caffeine in tea leaves, we have

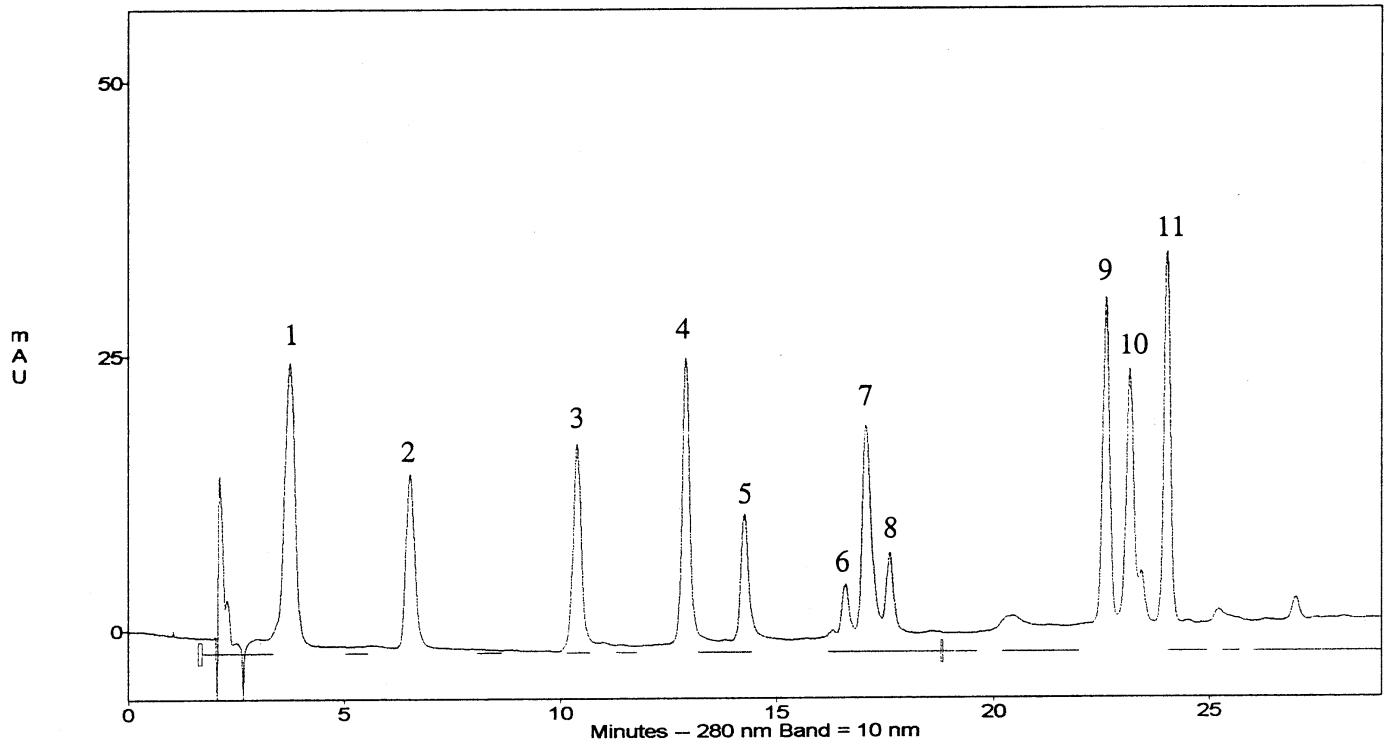

Fig. 2. HPLC chromatogram of catechin, phenolic acid and caffeine standards at 280 nm. Peaks: 1—gallic acid (GA); 2—3,5-dihydroxybenzoic acid; 3—(−)-epigallocatechin (EGC); 4—(−)-epigallocatechin gallate (EGCG); 5—(−)-epicatechin (EC); 6—(−)-epicatechin gallate (ECG); 7—caffeine (CA); 8—(−)-catechin gallate (CG); 9—*p*-anisic acid; 10—myricetin; 11—3,4,5-trimethoxycinnamic acid.

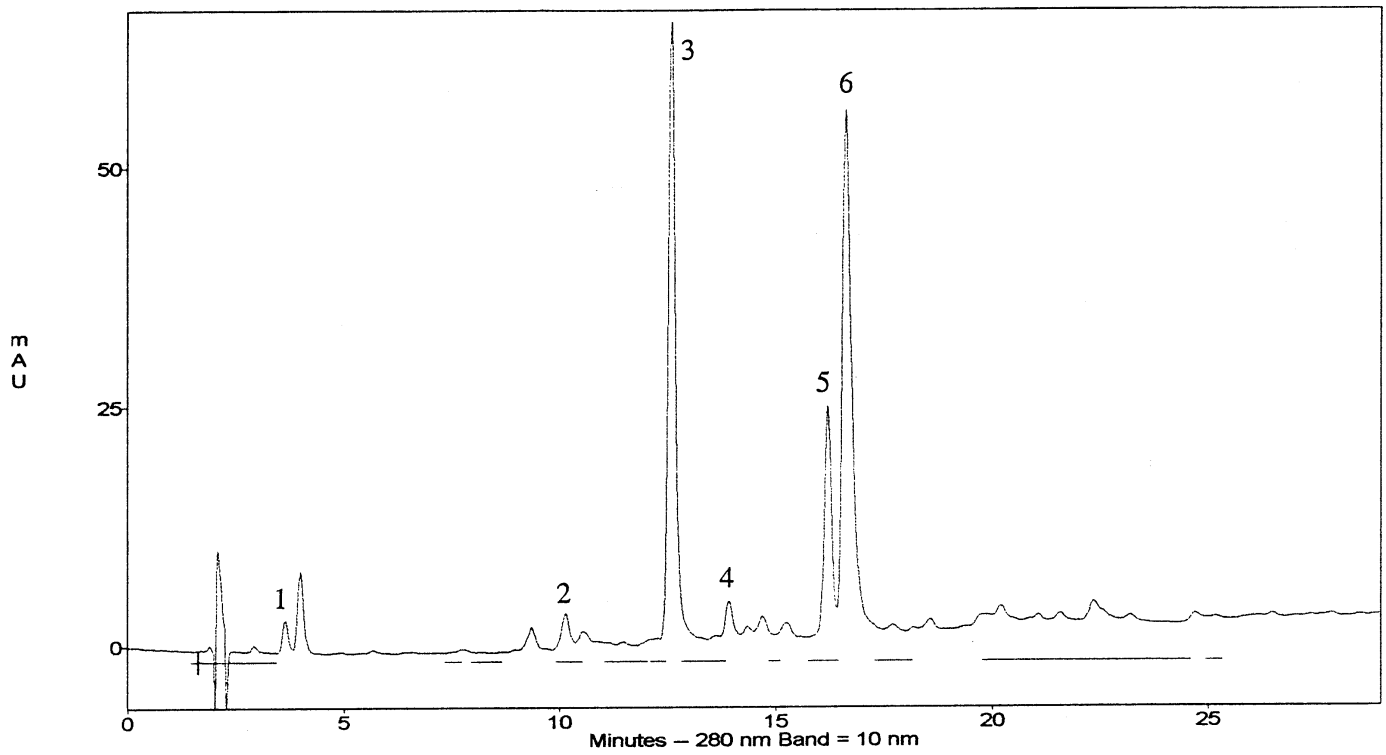

Fig. 3. HPLC chromatogram of jasmine (green) tea at 280 nm. Peaks: 1—gallic acid (GA); 2—(−)-epigallocatechin (EGC); 3—(−)-epigallocatechin gallate (EGCG); 4—(−)-epicatechin (EC); 5—(−)-epicatechin gallate (ECG); 6—caffeine (CA).

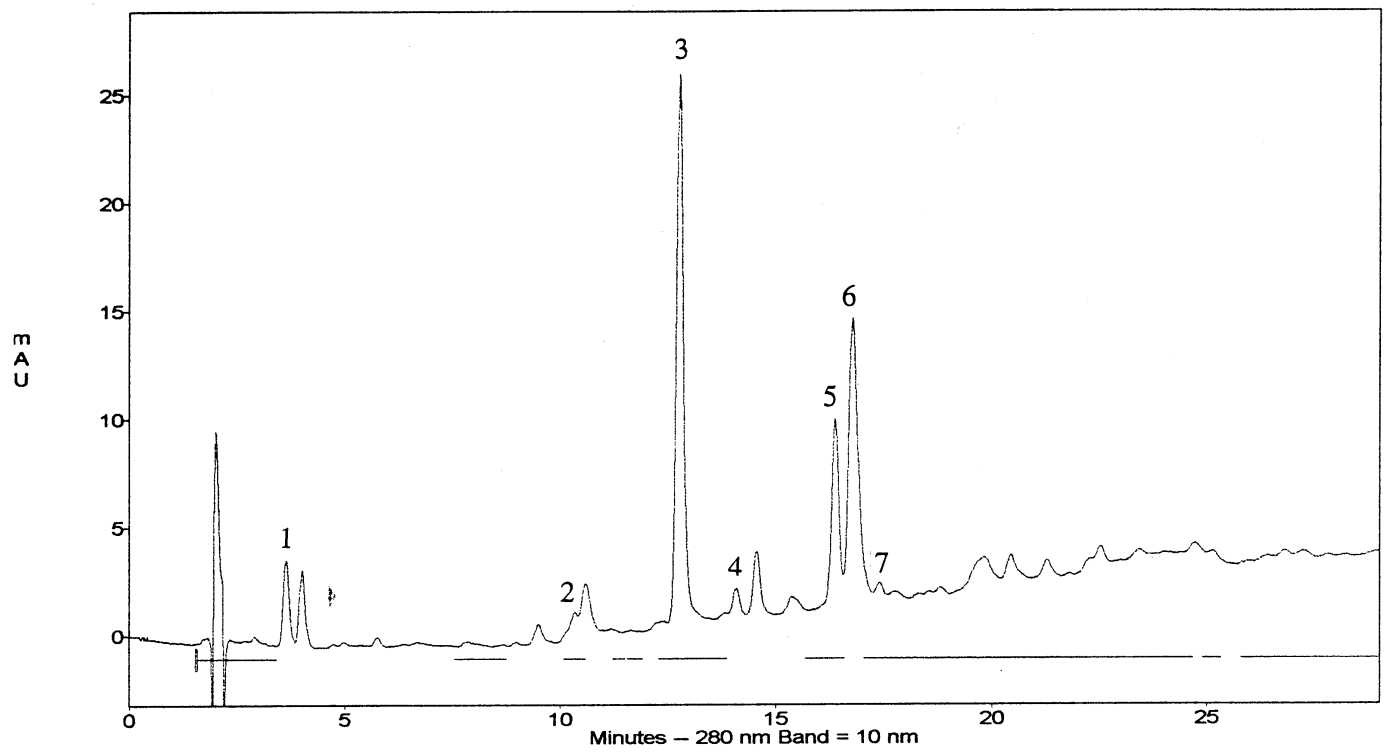

Fig. 4. HPLC chromatogram of Fujian Oolong tea at 280 nm. Peaks: 1—gallic acid (GA); 2—(−)-epigallocatechin (EGC); 3—(−)-epigallocatechin gallate (EGCG); 4—(−)-epicatechin (EC); 5—(−)-epicatechin gallate (ECG); 6—caffeine (CA); 7—(−)-catechin gallate (CG).

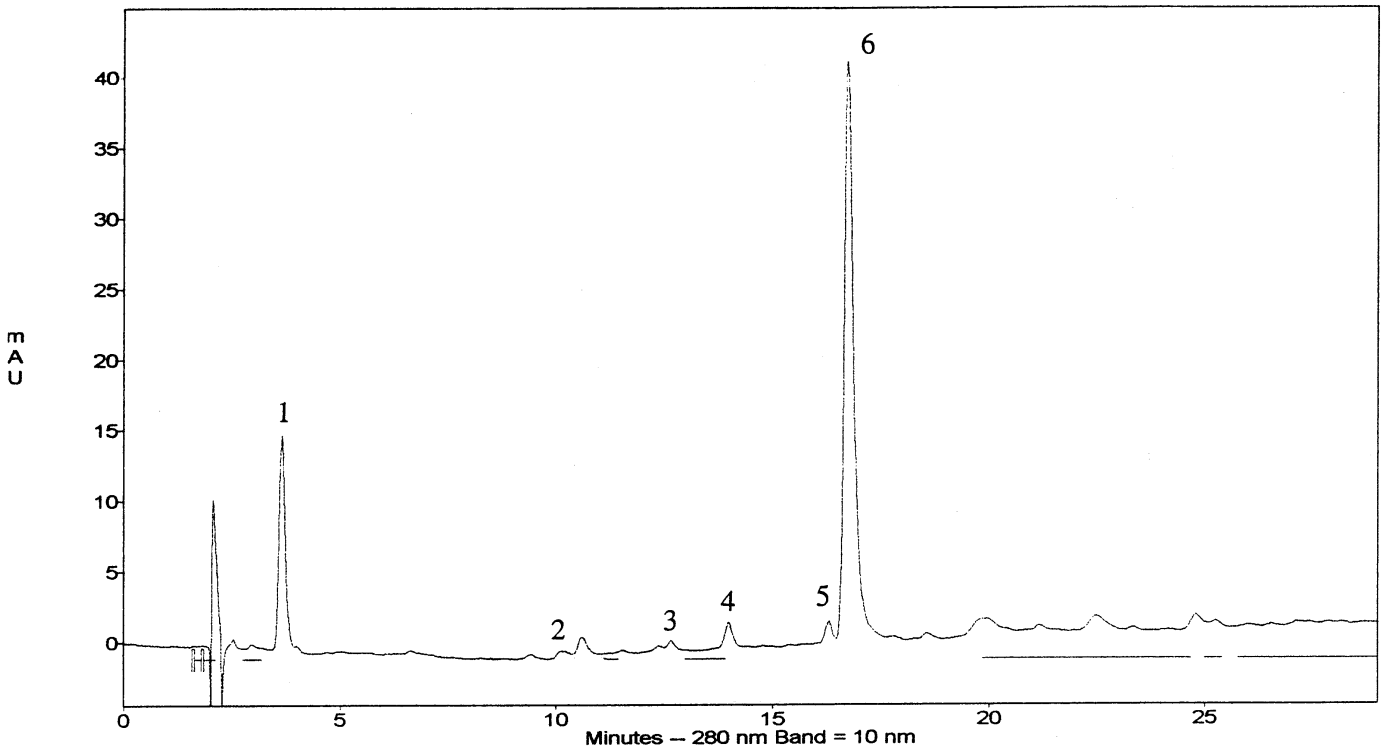

Fig. 5. HPLC chromatogram of pu-erh tea at 280 nm. Peaks: 1—gallic acid (GA); 2—(−)-epigallocatechin (EGC); 3—(−)-epigallocatechin gallate (EGCG); 4—(−)-epicatechin (EC); 5—(−)-epicatechin gallate (ECG); 6—caffeine (CA).

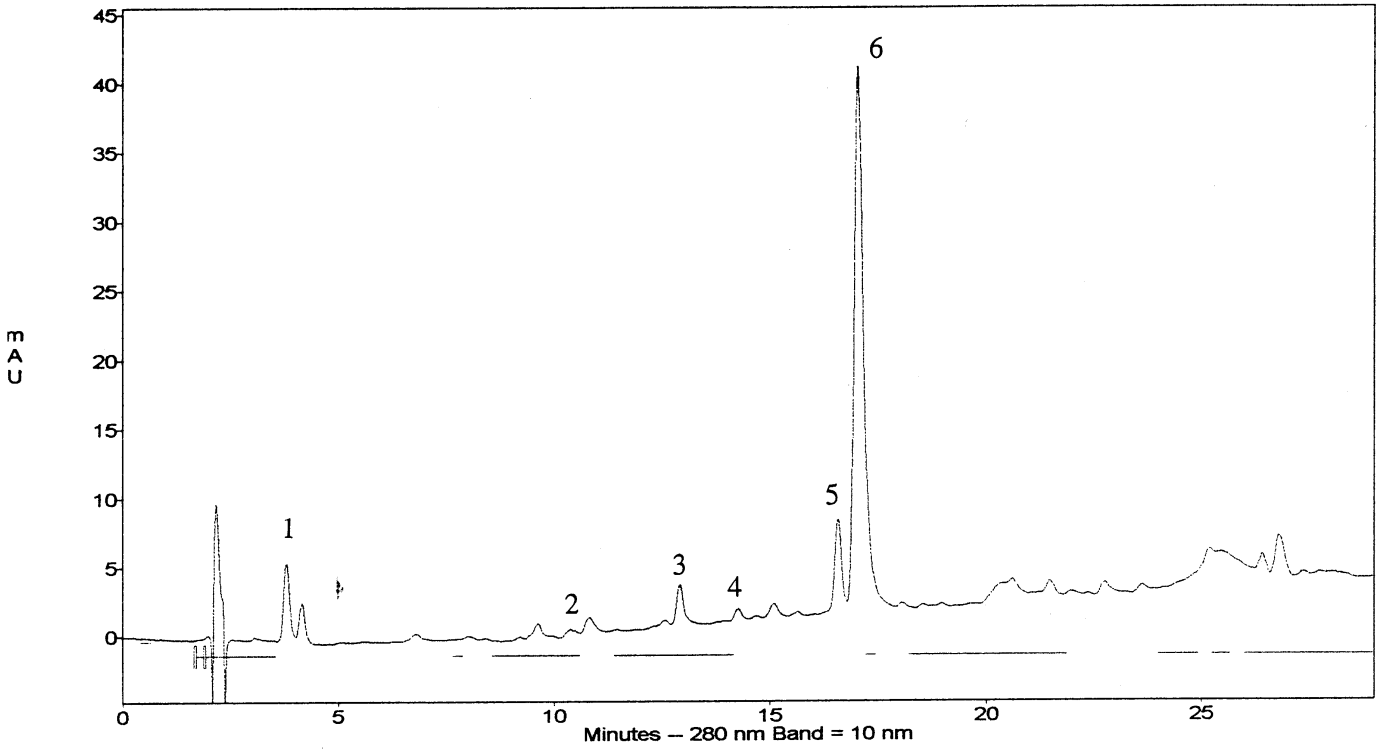

Fig. 6. HPLC chromatogram of black tea at 280 nm. Peaks: 1—gallic acid (GA); 2—(−)-epigallocatechin (EGC); 3—(−)-epigallocatechin gallate (EGCG); 4—(−)-epicatechin (EC); 5—(−)-epicatechin gallate (ECG); 6—caffeine (CA).

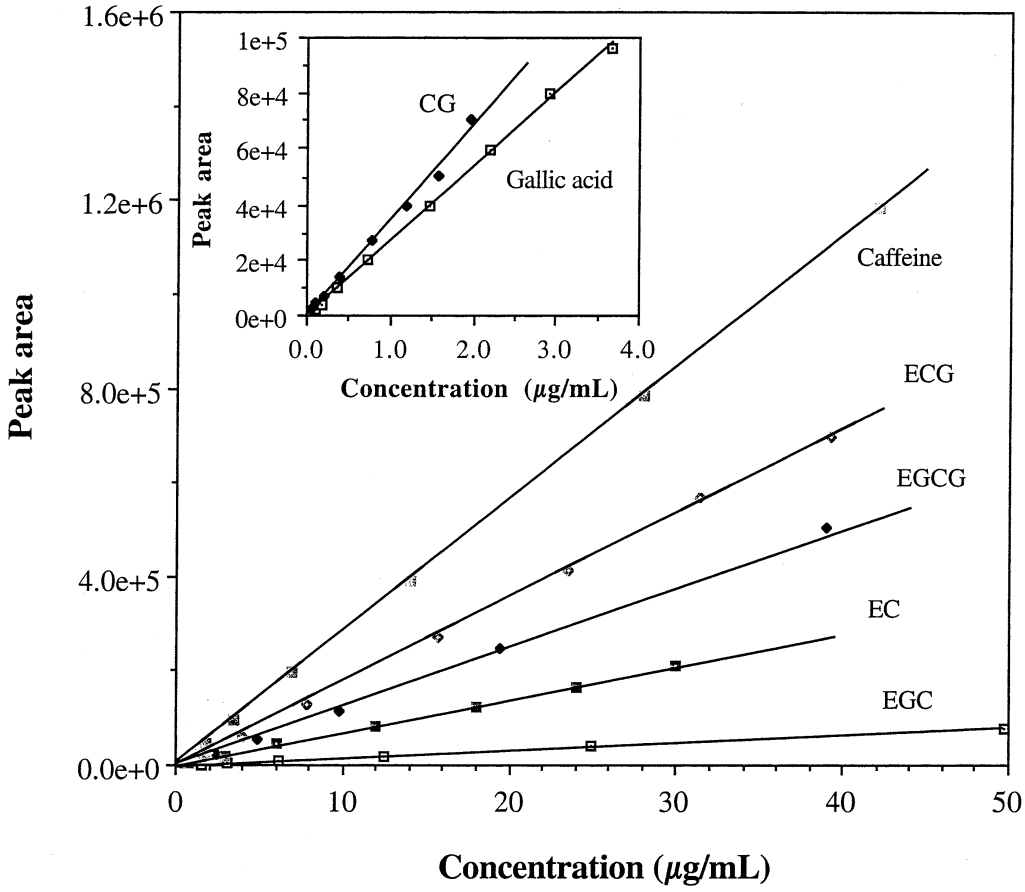

Fig. 7. Calibration curves for: gallic acid (GA); (−)-epigallocatechin (EGC); (−)-epigallocatechin gallate (EGCG); (−)-epicatechin (EC); (−)-epicatechin gallate (ECG); caffeine (CA); (−)-catechin gallate (CG).

Table 2
The contents of individual catechins, gallic acid and caffeine in teas

Content (mg g^{-1} tea)	GA	EGC	EGCG	EC	ECG	CA	CG
Pu-erh	5.53	6.23	1.99	3.24	1.32	22.4	–
Meifoo green tea	0.74	27.7	52.7	10.3	21.8	26.8	–
Shanghai green tea	0.37	30.8	51.1	7.25	11.3	23.0	–
Hangzhou Lung Ching	1.84	37.6	62.4	6.60	16.3	28.5	0.81
Jasmine	1.13	27.6	54.2	6.90	15.8	29.6	–
Fujian Oolong	1.42	10.0	22.2	2.63	6.06	7.44	0.27
Jiangxi Oolong	1.67	15.9	28.2	2.96	6.45	18.7	–
Fujian black	2.06	5.71	3.79	1.36	4.45	21.6	–

selected 280 nm as a good detection wavelength for these chemicals.

A total eight types of tea samples, four green teas (Meifoo green, Shanghai green, Hangzhou Lung Ching and Jasmine tea), two Oolong teas (Fujian and Jiangxi), one pu-erh and one black tea were analyzed for the content of individual catechins, gallic acid and caffeine by using the proposed method. The results obtained are presented in Table 2. Slightly lower concentrations in Table 2 than in Table 1 for some tea components could be due to the unstability of these chemicals even though acidic methanol solution had been used as extraction solvent, which increased the

stability of catechins and other polyphenolic compounds [3,20,21]. In general, four green teas contain higher levels of catechins than Oolong teas; the catechin contents of pu-erh and black teas are very low, which is in agreement with the degree of fermentation during manufacturing process [22]. Green tea is derived directly from drying and steaming the fresh tea leaves and, thus, no fermentation (i.e. oxidation) occurs. Oolong tea is derived when the fresh leaves are subjected to a partial fermentation stage before drying. Both black and pu-erh teas undergo a full fermentation stage before drying and steaming although the fermentation of black tea is oxidation and that of pu-erh tea is fermented using microorganisms. During the fermentation, tea catechins are oxidized or condensed to other large polyphenolic molecules such as theaflavins and thearubigins [22]. The health effect of these oxidized products is not well understood yet. The fermentation process also increases the liberation of gallic acids from CGs as indicated by the remarkably high levels of this acid in both pu-erh and black teas (Table 2). In contrast to previous reports, we have observed much reduced caffeine level in Oolong teas, especially, Fujian Oolong tea. The causes of this reduction by biochemical mechanism or other factors are interesting and further studies on this topic are warranted.

4. Conclusion

The developed gradient HPLC method allows rapid and simultaneous determinations of individual catechins, gallic acid and caffeine in green, Oolong, pu-erh and black teas with an economical mobile phase. The major catechins have been found to be EGCG, EGC, ECG, EC. Gallic acid is the main phenolic acid observed in tea extracts. All tea samples contain large amounts of caffeine. The contents of tea catechins, gallic acid and caffeine are related to quality of tea leaves and degree of fermentation during tea manufacturing. The described technique can, thus, also be used as an ideal analytical method in the quality control process during tea manufacturing.

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