Optimization and Validation of the HPLC-Based Method for the Analysis of Gallic acid, Caffeine and 5 Catechins in Green Tea

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Abstract

A rapid and simple determination of gallic acid (G), caffeine (CF), (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and (+)-catechin (C) in green tea was developed and optimized. The optimized system consisted of a C18 reversed-phase column, an isocratic elution system (2.00 ml/min) of water:acetonitrile (87:13) containing 0.05% trifluoroacetic acid and diode array detector (210 nm). The tea catechins were identified by comparing absorption spectra and retention time with the reference standard under the identical conditions. The developed systems sufficiently separated G, EGC, C, EC, EGCG, CF and ECG within 7 min elution time at 30°C. The validation of this method showed that the detection limits of these compounds were 0.2 µg/ml with good linearity of up to 100 µg/ml. Assam green tea samples were analyzed using the developed systems and the levels of G, EGC, C, EC, EGCG, CF and ECG were reported.

Keywords: analysis, catechins, caffeine, gallic acid, HPLC

Introduction

Tea (*Camellia sinensis L.*), a widely consumed beverage in the world, comes in a variety of forms, scents and flavors. Green tea, oolong tea and black tea are made from the shoots of tea plants at different degrees of fermentation. Green tea, an unfermented tea, has been receiving much attention because it contains beneficial components, mainly polyphenols. Many biological functions of tea polyphenols have been discovered, including antioxidant activity (Martina, 2010; Kim et al., 2011), antimutagenic effects (Gupta et al., 2002; Wu et al., 2007), anti-obesity (Rains et al., 2011) and cardiovascular diseases prevention (Deka & Vita, 2011).

Tea polyphenols exist in the biologically active group of the tea components, especially certain catechins, which make up as much as 30% (w/w) of the dry mass of tea. The major green tea catechins are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC). The level of green tea catechins varies according to varieties, climates, cultivation process and manufacturing process (Chen et al., 2010; Jayasekera et al., 2011; Wei et al., 2011). Moreover, the level of catechins depends mainly on the methods of extraction and analysis (Labbé et al., 2006; Yang et al., 2007; Komes et al., 2010). Therefore, it is important to establish a simple and reliable analytical method for a routine determination of the levels of tea catechins. High performance liquid chromatography (HPLC) is normally used as the analytical technique to determine green tea catechins. The method has been optimized and validated as a simple and reliable analytical method (Khokhar et al., 1997; Bonoli et al., 2003; Wang et al., 2003). However, the time-consuming nature of the use of HPLC to determine the compounds in green tea remains a major problem for routine analysis. In this paper we have developed a simple, fast and reliable isocratic HPLC method for simultaneous separation, identification and quantification of gallic acid, caffeine, (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and (+)-catechin (C) in green tea.

Materials and Methods

Standards, chemicals and samples

Gallic acid (G), caffeine (CF), (-)-epigallocatechin (EGC), (+)-Catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Acetonitrile (ACN), trifluoroacetic acid (TFA) and methanol (MeOH) (HPLC-grade) were purchased from Fluka (Buchs, Switzerland). Green tea samples were purchased from the tea shops in Chiang Rai province.

Preparation of Standards

The individual standard solutions of G, EGC, C, EC, EGCG, CF and ECG were prepared by dissolving them in a small volume of methanol, to generate a stock solution with a concentration of 1000 μg/ml. The mixed standard stock solution was prepared by mixing an equal volume of each stock standard. Working standard solutions were prepared by the dilution of the mixed stock solution with water prior to HPLC analysis. Determination of dry matter content

A dry matter content of tea was determined using a hot-air oven as the international standard method (ISO 1573, 1981).

Sample extractions

The methods used for sample extraction were based on international standard (ISO 14502-1, 2005). A brief procedure is as follows. First, a tea sample was ground and accurately weighed (0.200 ±0.001 g) in a 10 ml extraction tube. A 5.0 ml of 70% (v/v) methanol was added and mixed on the vortex mixer. The extraction was performed in a water basin at 70°C for 10 min, after which the extracted content was centrifuged at 3,500 rpm (3,000 g) for 10 min. This was followed by a repeated extraction, combining the extract to make up the volume to 10 ml with 70% (v/v) methanol. The sample extract was filtered through a 0.45 μm PTFE filter prior to HPLC analysis. The sample was analyzed in duplicate.

HPLC analysis

HPLC analysis of standards and samples was conducted on Water 966 high performance liquid chromatography comprising a vacuum degasser, a quaternary pump, an auto-sampler, a thermostatted column compartment and a photo diode array detector. The column used was a Platinum EPS C18 reversed phase, 3 μm (53 mm x 7 mm) equipped with a guard column. The mobile phase eventually adopted for this study was water:acetonitrile (87:13) containing 0.05% (v/v) trifluoroacetic acid (TFA), with the flow rate of 2 ml/min. The absorption wavelength was selected at 210 nm. The column was operated at 30 °C. The sample injection volume was 20 μl. Peaks were identified by comparing their retention times and UV spectra in the range of 190-400 nm with standards, and by checking the purity of the peaks.

Standard curves and limit of detection

The standard curves were made using Microsoft Excel 2007. Triplicate injections were made, and peak areas were plotted against the concentrations to generate the standard curves using linear regressions. The limit of detection (LOD) was determined as the ratio of signal to noise that was equal to (or higher than) three (S/N = 3). The limit of quantification (LOQ) was determined as the ratio of signal to noise that was equal to (or higher than) ten (S/N = 10).

Results and discussion

Development of analytical methods

Today's laboratories are under greater demand to analyze more samples in less time. To help meet this demand, the smaller particles packed in a HPLC column has been made to decrease analysis time and increase HPLC throughput. The use of the smaller particles offers two main improvements to the chromatographic separation-increased resolution and speed. In this study, rapid analysis was a main goal. Therefore, a column with a smaller particle size (3μm, 7 mm ID x 53 mm) was selected. We examined the optimal conditions for the analysis of the five catechins (EGC, C, EC, EGCG and ECG), gallic acid (G) and caffeine (CF) in green tea samples using a simple isocratic system for HPLC. In most studies, water/methanol/acid, water/acetonitrile/ethyl acetate and water/acetonitrile/acid methods have been used for catechin analysis (Yayabe et al., 1989; Goto et al., 1996; Wang et al., 2000; Nishitani & Sagesaka, 2004). In this study, three mobile phases widely used for tea analysis were tested. These are water/acetonitrile (87:13), water/acetonitrile/ethyl acetate (86:12:2) and water/methanol (87:13). It was found that the water/methanol (87:13) method provided poor resolution and low chromatographic efficiency (Figure 2). The use of water/acetonitrile (87:13) and water/acetonitrile/ethyl acetate (86:12:2) resulted in good separation. The optimal mobile phase is water/acetonitrile (87:13) due to the short running time consumed. Next, we studied the effect of acid on the chromatographic efficiency. It has been reported that the presence of acid is essential to complete separation (Dalluge et al., 1998). However, we found that 0.05% TFA did not benefit the separation of catechins but improved the peak sharpness (Figure 3). The elution order was G, EGC, C, EC, EGCG, CF and ECG, respectively.

Next, the compositions of the mobile phase, namely, water/acetonitrile containing 0.05% TFA, were varied (water:acetonitrile = 90:10, 87:13, 85:15 and 80:20). Figure 4 shows that the increase in acetonitrile resulted in short elution time. However, a peak overlap was found at 15% and 20% acetonitrile

(Figure 4 C and D). The use of 13% acetonitrile is possibly the optimal composition due to good resolution and short time. The flow rate of the mobile phase (water/acetonitrile (87:13) containing 0.05% TFA) was further optimized (Figure 5). We found the peak overlap at the flow rate of 2.5 ml/min (peaks of EGC/C and EC/EGCG). Good separation was achieved when the flow rates of 1.0, 1.5 and 2.0 ml/min were used. For good separation and rapid analysis, the flow rate of 2.0 ml/min was selected. At this flow rate, all components could be eluted within 7 min (Figure 5C), faster than the elution time previously reported by Horie & Kohata (2000), Wang et al. (2000), Zuo et al. (2002), Nishitani & Sagesaka (2004) and Sharma et al. (2005).

Column temperatures play an important role in the separation of compounds by HPLC. We subsequently investigated the effect of temperatures on chromatographic efficiency. It is clear, as Figure 6 shows, that the increase in temperatures (from 30 to 45 °C) resulted in shorter elution times; however, peak overlaps occurred (Figure 6 B, C and D). The optimal temperature was at 30 °C.

Wavelengths have been used for detection of catechins and caffeine in green tea, varying from 210 to 280 nm. (Goto et al., 1996; Dalluge et al., 1998; Wang et al., 2000; Horie & Kohata, 2000). Figure 7 shows the chromatograms of standard solution at 210, 254 and 280 nm. The absorbance of standards at 210 nm was obviously greater than those at 280 and 254 nm, respectively. Thus we selected 210 nm as the appropriate wavelength for detection of catechins, caffeine and garlic acid.

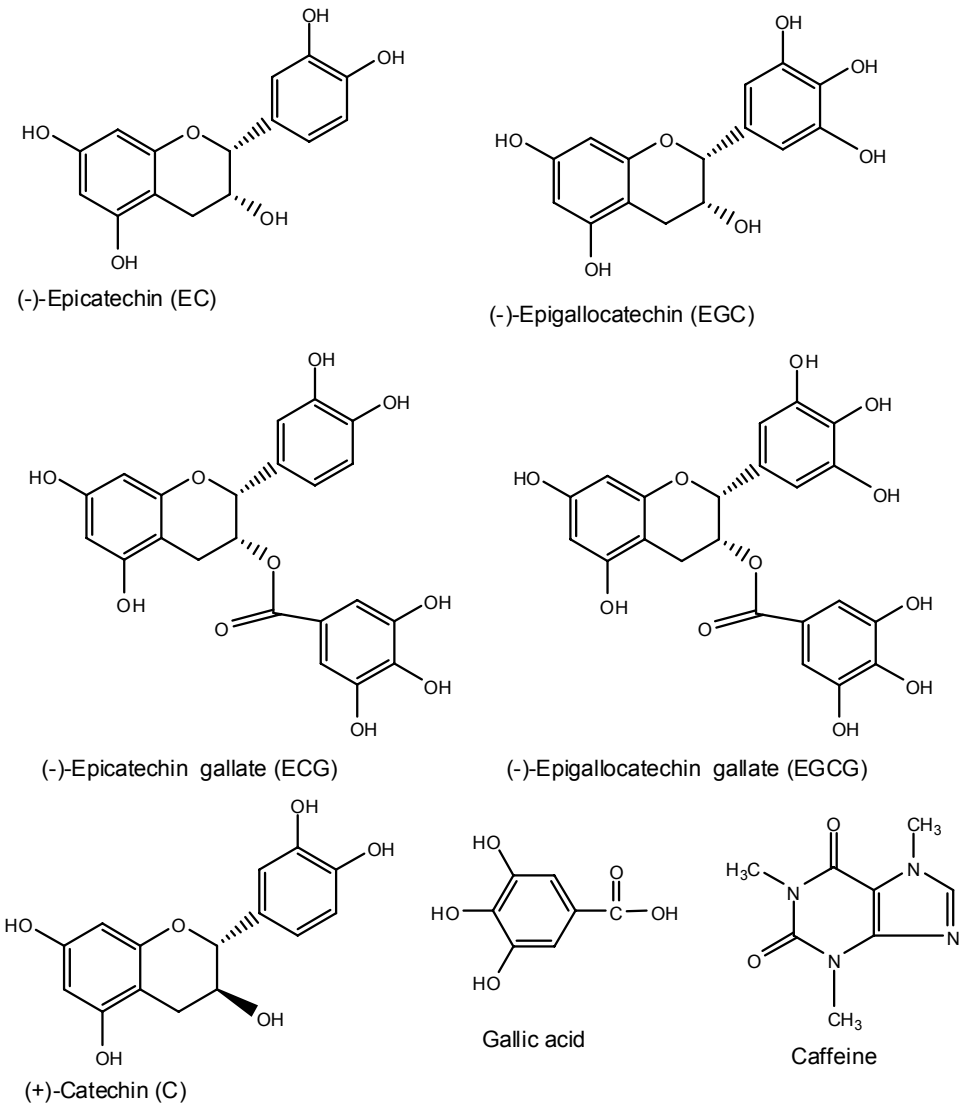

Figure 1 Structure of 5 catechins, gallic acid and caffeine.

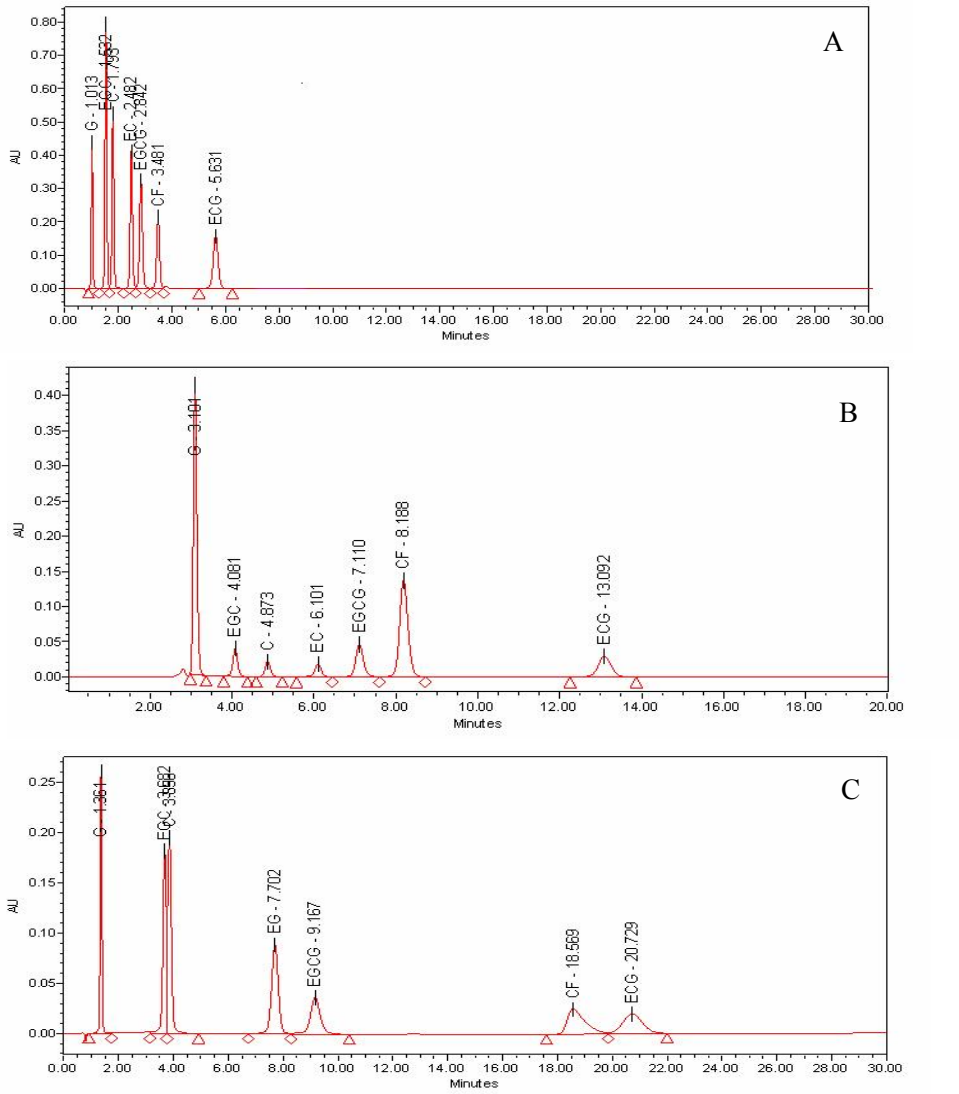

Figure 2 Effects of mobile phases on the separation of standard substance mixtures (100 µg/ml). The analysis was performed on Platinum EPS C18, 100 Å, 3 µm, 53 mm x 7mm, flow rate 2 ml/min, temperature 30°C, detection 254 nm, mobile phase (A) H_2O:ACN, 87:13 v/v, (B) H_2O:ACN:EtOAC, 86:12:2 v/v and (C) H_2O: MeOH, 87:13 v/v.

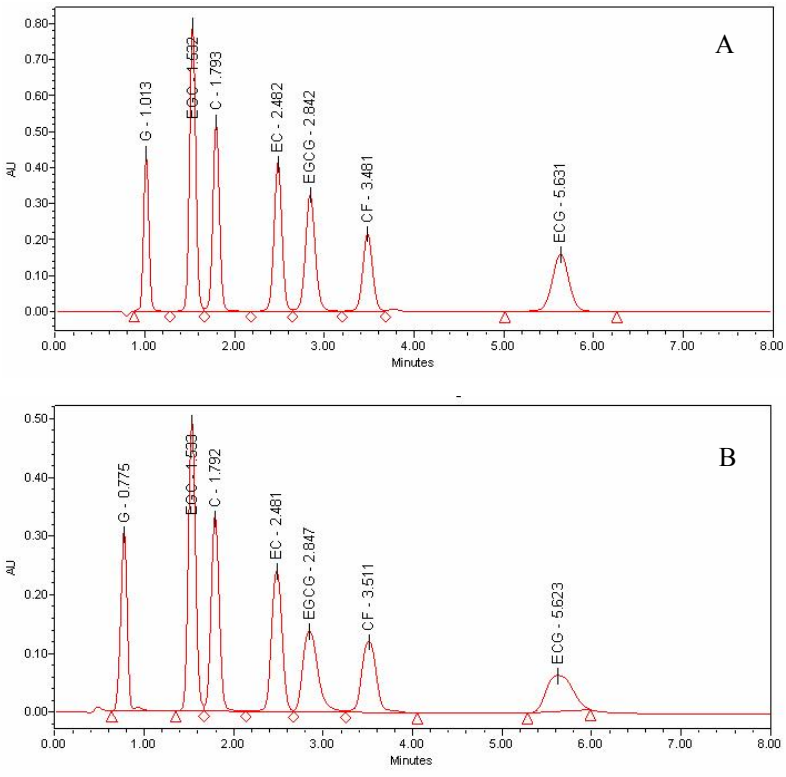

Figure 3 Effects of added acid on the separation of standard substance mixtures (100 µg /ml). The analysis was performed on Platinum EPS C18, 100 Å, 3 µm, 53 mm x 7 mm, flow rate 2 ml/min, temperature30°C, detection 210 nm, mobile phase H2O:ACN (87:13) in the presence (A) and absence (B) of trifluoroacetic acid.

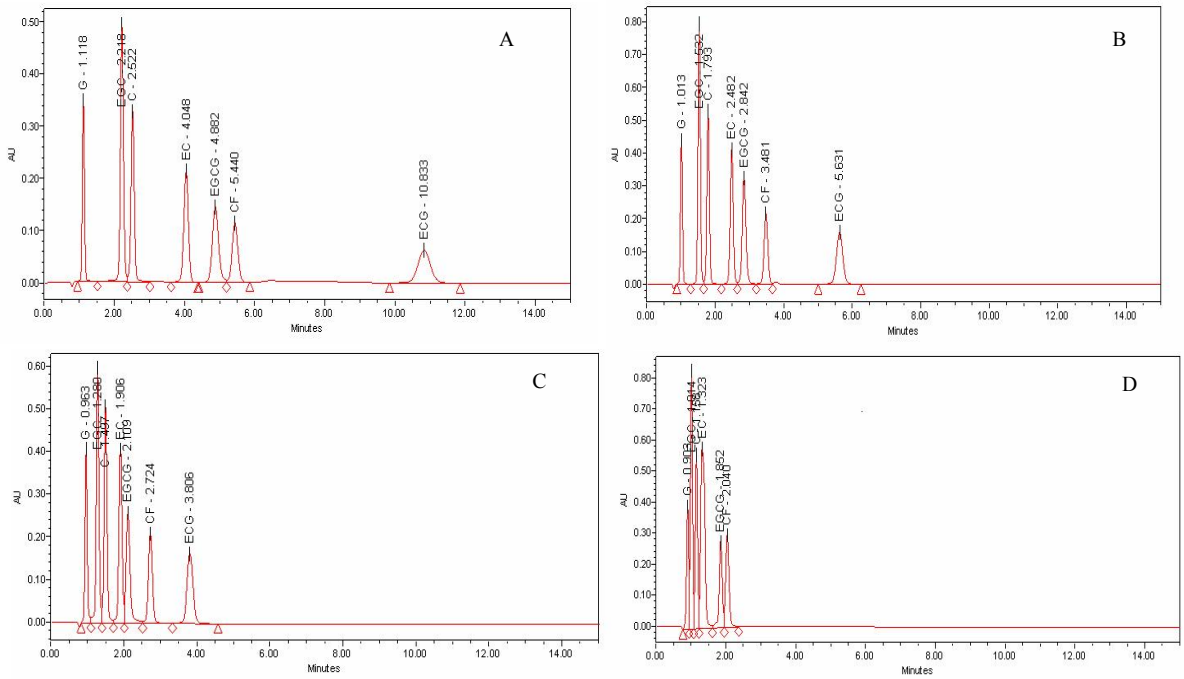

Figure 4 Effects of mobile phase compositions on the separation of standard substance mixtures. The analysis was performed on Platinum EPS C18, 100 Å, 3 µm, 53 mm x 7mm, flow rate 2 ml/min, temperature 30°C, detection 210 nm, mobile phase H2O:ACN (A) 90:10, (B) 87:13, (C) 85:15 and (D) 80:20.

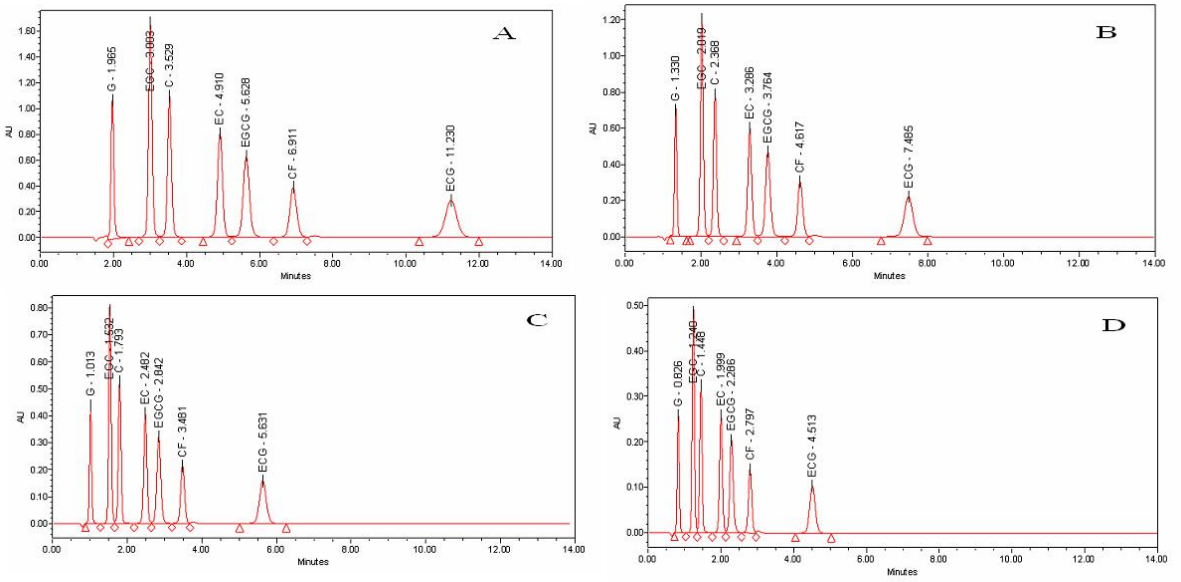

Figure 5 Effects of flow rate of mobile phase on the separation of standard substance mixtures. The analysis was performed on Platinum EPS C18, 100 Å, 3 µm, 53 mm x 7mm, mobile phase water:acetonitrile 87:13% containing 0.05% TFA, temperature 30°C, detection 210 nm, and flow rate of (A) 1.0, (B) 1.5, (C) 2.0 and (D) 2.5 ml/min.

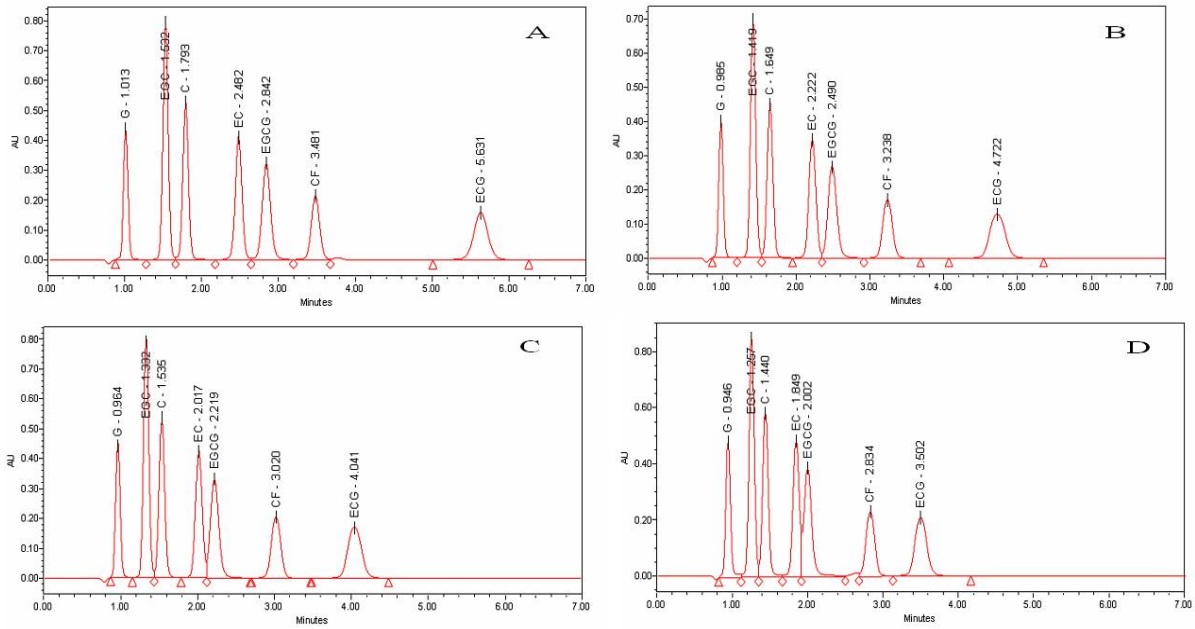

Figure 6 Effects of temperatures on the separation of standard substance mixtures. The analysis was performed on Platinum EPS C18, 100 Å, 3µm, 53 mm x 7mm, mobile phase water:acetonitrile 87:13% containing 0.05% TFA, flow rate 2.0 ml/min, detection 210 nm, at temperatures (A) 30, (B) 35, (C) 40 and (D) 45°C.

Validation of analytical methods

Calibration graphs for the catechins, caffeine and gallic acid were constructed using Microsoft Excel 2007 (Figure 8). All the analytes exhibited good linearity over the range tested, with a correlation coefficient (r) between 0.9958 and 0.9999 (Table 1). The linearity of all compounds was in the range of 1–100 µg/ml. The LOD and LOQ of all analytes were 0.2 and 1.0 µg/ml respectively. Green tea was extracted and analyzed for gallic acid, caffeine and 5 catechins using the developed method. A typical chromatogram of the green tea is shown in Figure 9. Precision and accuracy were evaluated using recovery tests (n = 7), adding known amounts of the standard solution (1, 20, 60 and 80 µg/ml) to a green tea extract. The recovery rates of all of the compounds were in the range of 84.7–103.7%. Relative standard deviations (%RSD) of all of the compounds were less than 10%. To check the specificity, we performed peak purity diode-array analysis and confirmed that each chromatographic peak

was attributable to a single component (data not shown). All these results suggested that the developed method was reliable.

Analysis of green tea samples

As the rapidly optimized method has been found applicable with acceptable accuracy and precision, this method was used for determining gallic acid, caffeine and 5 catechins in 6 Assam green tea samples produced from Camellia sinensis var. assamica. Table 2 shows that the average gallic acid was 1.23 %w/w dry basis. The caffeine content of six green tea samples ranged from 3.27% to 3.71% w/w, with a mean of 3.44%w/w. This was quite similar to the caffeine content in earlier reports, 2.94-3.62% (Nishitani & Sagesaka, 2004) and 2.29-3.82% (Wang et al., 2000). Table 2 indicates that all Assam green tea samples contain EGC, C, EC, EGCG and ECG. The major catechins were ECG, EGCG and EC, with a concentration of 2.94, 2.62 and 2.23%w/w, respectively. It is quite impractical to compare the individual catechins with other published works because many factors such as species, cultivating season, plucking standards or methods, horticultural conditions and processing can all influence the catechin contents (Chen et al., 2010; Jayasekera et al., 2011; Wei et al., 2011).

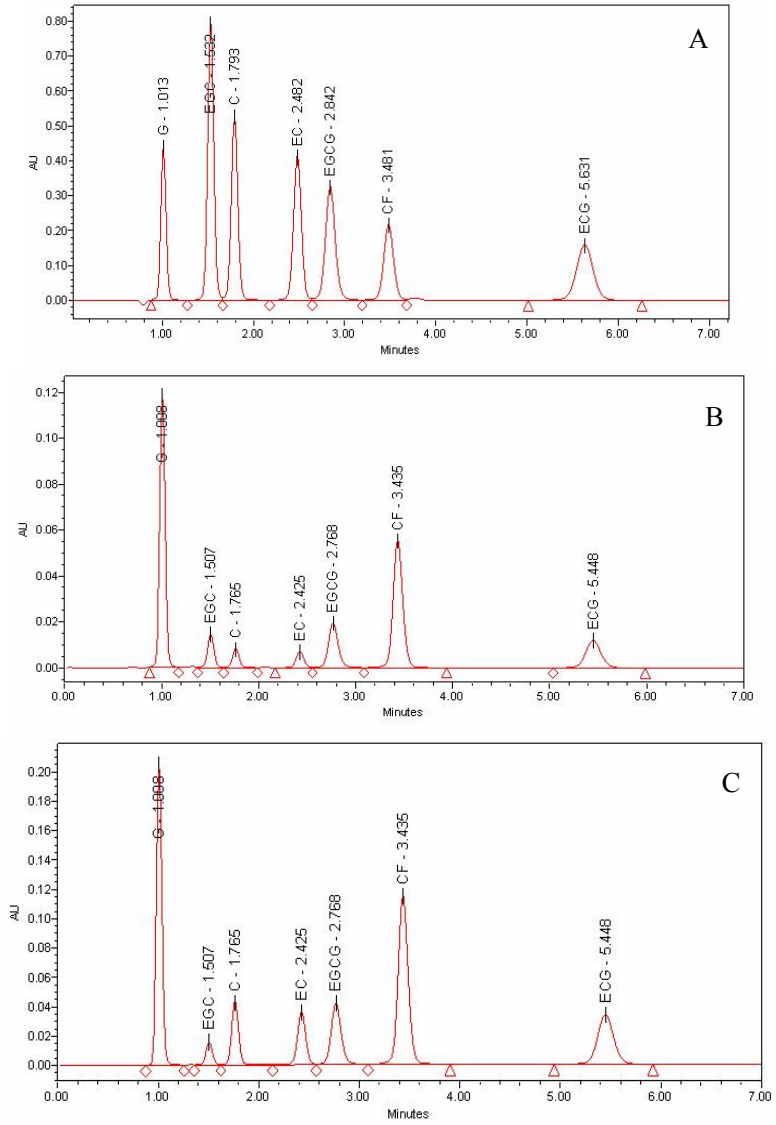

Figure 7 The chromatogram of standard substance mixtures. The analysis was performed on Platinum EPS C18, 100 Å, 3 µm, 53 mm x 7 mm, mobile phase water:acetonitrile 87:13% containing 0.05% TFA, flow rate 2.0 ml/min, temperature 30°C and detection wavelength at (A) 210, (B) 254, and (C) 280 nm.

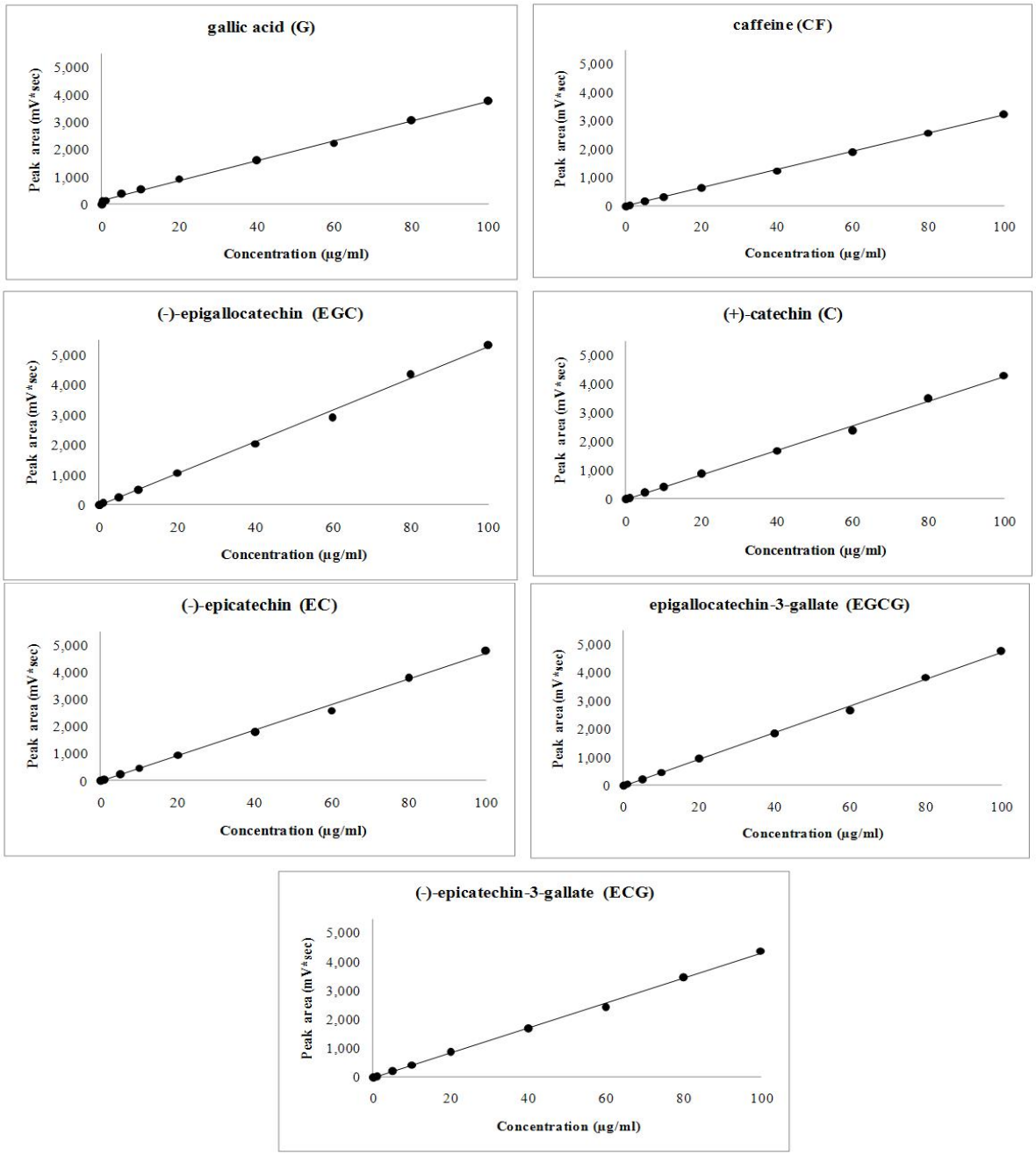

Figure 8 Calibration curves for gallic acid (G), caffeine (CF), (-)-epigallocatechin (EGC), (+)-Catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG).

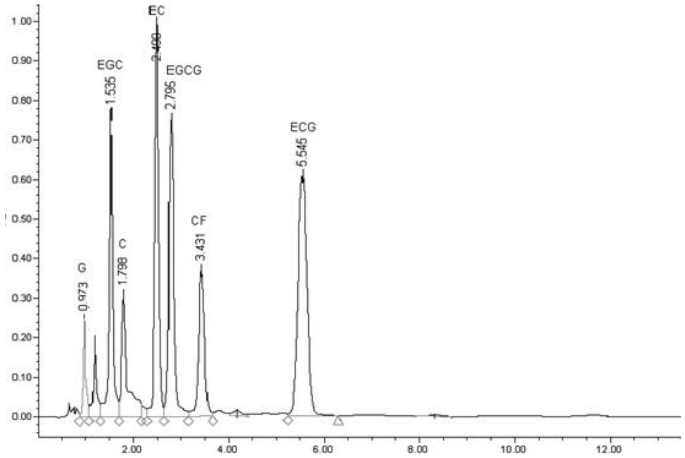

Figure 9 A typical HPLC chromatogram of Assam green tea. The analysis was performed on Platinum EPS C18, 100 Å, 3 µm, 53 mm x 7 mm, mobile phase water:acetonitrile 87:13% containing 0.05% TFA, flow rate 2.0 ml/min, temperature 30°C and detection wavelength at 210 nm.

Table 1 Results of validation of the analytical method (n = 7)

Parameters		Compounds						
		G	CF	EGC	C	EC	EGCG	ECG
Linearity	(µg/ml)	1-100	1-100	1-100	1-100	1-100	1-100	1-100
LOD	(µg/ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
LOQ	(µg/ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
r		0.9958	0.9999	0.9998	0.9998	0.9999	0.9994	9.9996
%Recovery	1.0 µg/ml	93.4±7.9	83.1±4.9	84.7±8.2	96.9±8.5	95.9±8.9	93.6±8.3	90.0±8.0
	20 µg/ml	98.4±8.0	95.4±4.4	93.9±8.6	96.1±6.9	89.8±8.1	103.8±3.8	94.1±4.3
	60 µg/ml	97.8±4.4	94.6±4.3	93.8±7.8	94.8±4.5	94.1±4.2	96.2±5.3	94.4±4.1
	80 µg/ml	98.6±4.3	99.9±1.1	94.7±4.3	94.9±4.2	93.9±3.9	95.4±3.2	97.7±1.2
%RSD	1.0 µg/ml	8.5	5.9	9.7	8.8	9.3	8.8	8.9
	20 µg/ml	8.1	4.6	9.2	7.2	8.9	3.7	4.5
	60 µg/ml	4.5	4.5	8.4	4.8	4.5	5.5	4.3
	80 µg/ml	4.4	1.1	4.6	4.4	4.1	4.3	1.2

Table 2 Contents of gallic acid, caffeine and 5 catechins in Assam green tea samples

Assam green tea	Content (%w/w dry basis)						
	G	CF	EGC	C	EC	EGCG	ECG
1	1.41	3.71	1.60	1.70	2.88	2.20	3.53
2	1.31	3.43	1.95	1.51	2.29	2.74	2.90
3	1.65	3.44	1.70	2.15	2.62	2.44	3.45
4	1.11	3.35	2.16	1.41	1.31	3.69	1.65
5	1.03	3.27	2.48	1.61	2.39	3.47	2.84
6	0.89	3.45	0.70	1.53	1.87	1.16	3.24
Min	0.89	3.27	0.70	1.41	1.31	1.16	1.65
Max	1.65	3.71	2.48	2.15	2.88	3.69	3.53
Mean	1.23	3.44	1.77	1.65	2.23	2.62	2.94

Conclusion

The developed isocratic HPLC method allows rapid and simultaneous analysis of gallic acid (G), caffeine (CF), (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) and (+)-catechin (C) in green tea within 7 min. This method is simple, rapid and can be used for fast routine analysis.

Acknowledgements

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