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Comparison of Some Extraction Methods for Isolation of Catechins and Caffeine from Turkish Green Tea

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Abstract: Effective extraction of anticancer and antioxidant principles from Turkish green tea were main purpose of this work. The pre-optimized experimental condition for liquid extraction was employed for comparative appraisal. Not only extraction methods also nature of the green tea samples (fresh, dried or frozen) and quantitative yields related to collection periods were investigated. After extraction of the green tea with various techniques the extract was partitioned with chloroform to remove caffeine, after that the extract was partitioned with ethyl acetate to obtain catechin mixture. Quantification of individual catechins was carried out by HPLC and analysis results proved that epigallocatechin gallate (EGCG) was main catechin specie present in all extracts. The results indicate that hot water extraction (at 80 ^{0}C) provides higher catechin yield when compared to other methods. The highest extract yields were obtained with dried leaves collected in second collection period. The crude catechin mixture contains high amount of EGCG and might be used as raw material for production of plant remedies at industrial scale.

Keywords: Turkish green tea; EGCG; extraction; HPLC; anticancer

1. Introduction

Tea (*Camellia sinensis*, *Theaceae*) is the second most consumed beverage in the world, next to the water, in addition to being the most widely consumed brew tea is the only food product known to contain substantial levels of the catechins (Bansal et al., 2012; Bronner and Beecher, 1998). There are five major types of tea in China based on the processing methods: white, green, oolong, black and Pu-erh (Wang et al., 2011). Green tea is produced when freshly reaped leaves of *Camellia sinensis* are subjected to decolorisation, and then they are pan-fried/steamed prior to rolling/shaping and drying (Wang et al., 2011). Recently the demand for green tea is attributed to sensory human health concerns and preference (Wang et al., 2011; Row and Jin, 2006). The main components present in green tea are polysaccharides, flavonoids, vitamins B,C,E, R-amino butyric acid, fluoride and caffeine (Row and Jin, 2006). Tea leaf contains 2-4% of caffeine which stimulates the cerebral cortex, and also causes irritation of gastrointestinal tract and sleeplessness for certain people (Dong et al., 2011). The four major catechin compounds of green tea are epigallocatechin (EGC), epicatechin (EC), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG), of which EGCG is the major

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constituent and the most abundant one, representing 50-80% of the total catechin content. Catechins contribute to the characteristic bitter an astringent taste (Yoshida et al., 1999).

Tea infusion not only gives specific taste and flavor, but also many essential dietary components for human health (El-Shahawi et al., 2012). Recently, catechin compounds of green tea have attracted significant attention, both in the scientific and in consumer communities for its multiple health benefits for a variety of disorders, ranging from cancer to weight loss and functionality such as anti-oxigenicity, antimutagenicity, and anti-carcinogenecity (Bansal et al., 2012; Yoshida et al., 1999).

Until today, published articles related to green tea were mainly concentrated on Chinese and Indian species. There are only a few published studies with Turkish green tea (Aksuner et al., 2012; Sarı and Velioğlu, 2011). Some of these articles mainly focused on antioxidant activity, total phenolic contents, vitamins, minerals, elemental contents, theaflavin, theanine constituents of different grades of black tea (Serpen et al., 2012). Turkey is the fifth tea producer in the world. Almost 1.5 million tone tea is produced in 2013 especially in north eastern part of Turkey. All produced material is mainly used for production of black tea which is traditional drink and only small part of the production is used for production of green tea. So there is a big demand for production of other chemicals from tea plant that is commercially valuable. Green tea extracts have been widely applied to different fields, particularly in food and beverage industries as additives. An inexpensive and easy method for the extraction and isolation of catechins and caffeine from Turkish green tea needs to be investigated.

Various extraction procedures are available in the current literature. Vuong et al. (2011) published an important review related to extraction techniques of tea samples. All extraction techniques used for effective extraction of tea samples have been discussed in details. Apart from this important review other studies were published (Row and Jin, 2006; Liang et al., 2007; Bazinet and Labbe, 2007; Hu et al., 2009; Dong et al., 2011). In all these studies conventional liquid-liquid extraction (LLE) methods are used and optimized for an effective extraction of caffeine and catechins. For example, Row and Jin (2006) proposed a LLE method for Korean green tea samples. Green tea leaves were extracted in water at varying temperatures (50, 80, 100 ^{o}C) and extraction periods (4 hours, 40 minutes and 15 minutes). After filtration supernatant was extracted by chloroform (1:1 v/v) to separate caffeine then following this aqueous phase was re-extracted with ethyl acetate (1:1 v/v) to separate catechins. They report that the highest yield was obtained with extraction at 80 $^{\circ}$C for 40 minutes. Liang et al. (2007) report that hot water pre-treatment at 100 ^{o}C for 3 minutes provide better separation of caffeine and catechins. A two step water extraction (at 50 ^{o}C first then 80 ^{o}C) was another extraction procedure for effective separation of caffeine and catechins (Bazinet and Labbe, 2007). Additional to heat treatment at different temperatures some chemicals such as ethanol (Hu et al., 2009) or citric acid (Dong et al., 2011) had been used in extraction medium to enhance the extraction yields.

So, this study aims to determine of important chemical principles obtained from Turkish green tea. The goal of this research is to establish an efficient solvent extraction method for isolation of catechins and caffeine from green tea samples. All above optimized extraction methods will be employed for a comparative appraisal and this will be the first detailed study for Turkish green tea samples. Most of these studies report the quantitative data related to chromatographic analysis of tea infusion. But present study contains separation and quantification of catechines and caffeine after proper extraction from tea infusion. Not only extraction methods but also nature of the green tea samples (fresh, dried or frozen) and quantitative yields related to collection periods have been investigated. Quantification of individual catechins will be carried out by chromatographic techniques.

2. Material and Methods

Materials

Green tea samples. Green tea used in the experiments was supplied by Sürçay Co. Ltd. (Sürmene, Trabzon). In Turkey, tea is collected three times in a year. First collection is in May, second is in June, and the third is in July. Green tea samples used in our tests were collected from a tea garden at the same collection periods in 2012. Leaves on top branches of tea plants were collected, placed in a sealed plastic bag and immediately transferred to laboratory. Before extraction, an extensive literature search was carried out to find out the most effective and suitable extraction procedure. Three different sample preparation methods were designed. The first group green tea leaves were left drying at room temperature (defined as GT-D); second group was fresh green tea sample chopped in a blender just after collection (GT-F); the last group was freezed at -20 °C for a certain period then chopped as above (GT-FF).

Standards and chemicals. The standard chemicals of (−)epigallocatechin (EGC), (−)epicatechin (EC), (−)epigallocatechin gallate (EGCG), (−)epicatechin gallate (ECG) and caffeine were purchased from Sigma (ST Louis, MO, USA). N,N dimethylformamide, methanol, ethyl acetate and chloroform were analytical grade from Merck (Darmstadt, Germany).

HPLC analysis conditions. The instrument working conditions in this study were as follows: The composition of catechins in the extract was determined with a HPLC system (LC2010AHT; Shimadzu Corp., Kyoto, Japan) equipped with a Shim-pack VP-ODS C18 column (5 mm, 4.6 x 150 mm, 35 °C) at 278 nm. A gradient system was employed; solvents A (water) and B (N,N-dimethylformamide:methanol:acetic acid, 20:1:0.5, (v/v) were run in linear gradients with B increasing from 14% to 23% within 13 min, from 23% to 36% within next 12 min and maintained for 3 min, thereafter at a rate of 1.0 mL min^{-1}. Concentrations of catechins and caffeine were quantified by their peak areas against those of standards prepared from original compounds (Wang et al., 2011).

Extraction Methods.

Details of extraction methods of green tea samples are given below:

Citric acid water extraction (WE-C). 10 g of green tea was added into 300 mL water at 80 °C for 40 min. The extract was filtered through 110 mm filter paper. The supernatant was extracted with ethyl acetate three times using 1.5 L ethyl acetate in each extraction. Organic phases were re-extracted with 1.5 L aqueous citric acid solution three times to separate caffeine. Concentration of citric acid solution was 10 mg/L. The ethyl acetate phases containing caffeine were then dried and concentrated under reduced pressure (Dong et al., 2011) until dryness and weighted. Caffeine percentage was calculated from the formula given below.

% Caffeine (w/w)=(mass of caffeine extract/mass of tea sample) x 100

Aqueous extract was evaporated down to 300 mL then freez dried and the resulting solid was weighted. Extract percentage was calculated from the formula given below.

% Catechin (w/w)=(mass of catechin extract/mass of tea sample) x 100

Ethanol extraction (WE-E). 10 g of green tea sample was brewed in 75% ethanol at 30°C for 10 min in a thermostated water bath (tea:solvent ratio was 1:60, w/v). The infusion was filtered and caffeine was extracted by ethyl acetate. The filtrate was concentrated by a rotary evaporator under reduced pressure and finally dried by using vacuum concentration at 55 ± 2 °C (Hu et al., 2009) to obtain catechines. Caffeine and catechin percentages were calculated as above.

Two- step water extraction (WE-T). 10 g of green tea was initially brewed in 300 mL of distilled water. The first brewing step was carried out at 50 °C for 10 min in a thermostated water bath. Tea leaves were removed for a while and then re-soaked into a fresh portion of water for another 10 minutes at 80 °C and aqueous infusions were combined. The two-step extraction procedure was repeated three times. The filtered samples were initially partitioned with chloroform to separate caffeine. Aqueous phase was then extracted with ethyl acetate to obtain catechin mixture (Bazinet and Labbe, 2007). Caffeine and catechin percentages were calculated as above.

High temperature pretreatment water extraction (WE-HT). 10g of green tea leaves were soaked into 300 mL water at 100 ^{0}C for 3 minutes. The aim of this process was to remove the most of the caffeine from the leaves. Then same leaves were removed and were immediately soaked into a new 200 mL portion of water at 80^{0}C for another 40 minutes. The water phase was separated and extracted with chloroform. After separation of the phases, aqueous layer was re-extracted with ethyl acetate to obtain catechin mixture (Liang et al., 2007). Caffeine and catechin percentages were calculated as above.

Water extraction at 80 ^{o}C (WE). 10 g of ground leaves of green tea were extracted with 300 mL of pure water at 80 °C for 40 min. Leaves were removed by filtration using a filter paper (pore size: 5 µm). Aqueous tea infusion was initially partitioned with chloroform then second partition was carried out with ethyl acetate (Row and Jin, 2006). Caffeine and catechin percentages were calculated as above.

3. Results and Discussion

Catechin and caffeine yields. A previous report reveals that the content of caffeine in green tea is approximately 2.66%, and the total content of catechin compounds are 7.65%, while EGC, EC, EGCG and ECG were about 2.26%, 0.78%, 3.44%, and 1.01% respectively (Row and Jin, 2006).

In this study, we used 5 different extraction methods for isolation of catechin and caffeine for each collection period. The average yields of catechin and caffeine from first collection period in May are given in the Figure 1.

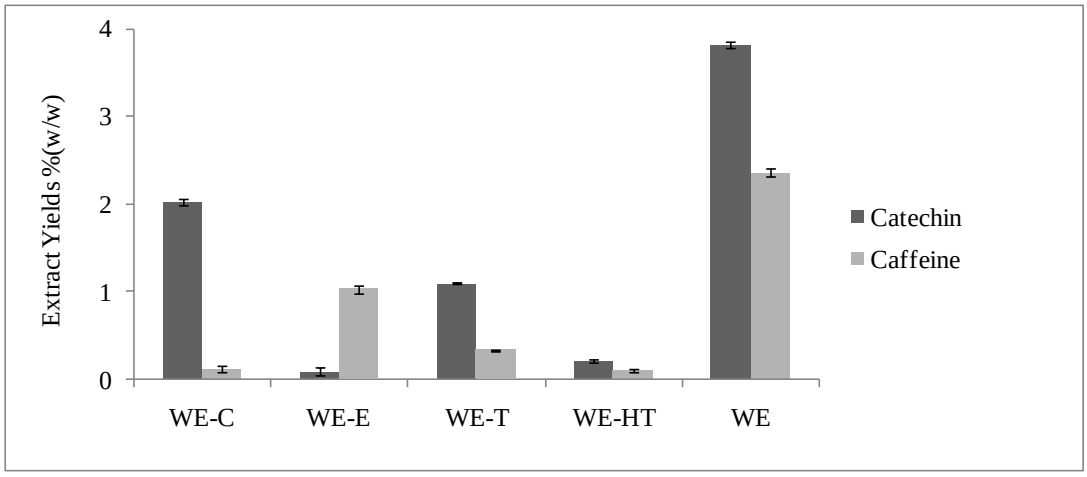

Fig. 1. Catechin and caffeine yields (%, w/w) of first collection period samples (May 2012).

According to above results extraction at 80 ^{o}C for 40 minutes (WE) provided the highest extraction yield. So, this method was chosen to extract all different type sampling and collection periods. Extracted catechin percentage is lower than the catechin constituent determined by HPLC methods since liquid-liquid extraction limits the complete separation of

catechines. For industrial applications quantitative amount of separable catechin is more important than its actual amount in tea infusion. However, it should be noted that citric acid-water extraction seems to provide higher catechin fraction than caffeine. Thus, it might be used for selective extraction of catechins.

Second part of this study is to evaluate the impact of nature of the sample prior to extraction. Green tea leaves used as dry (GT-D), fresh frozen (GT-FF) or fresh (GT-F) for extraction, that collected at the same collection periods (called as first, second and third circulation) and extracted at 80 ^{o}C for 40 minutes (WE). These results are given in Figure 2 for catechin content and Figure 3 for caffeine content.

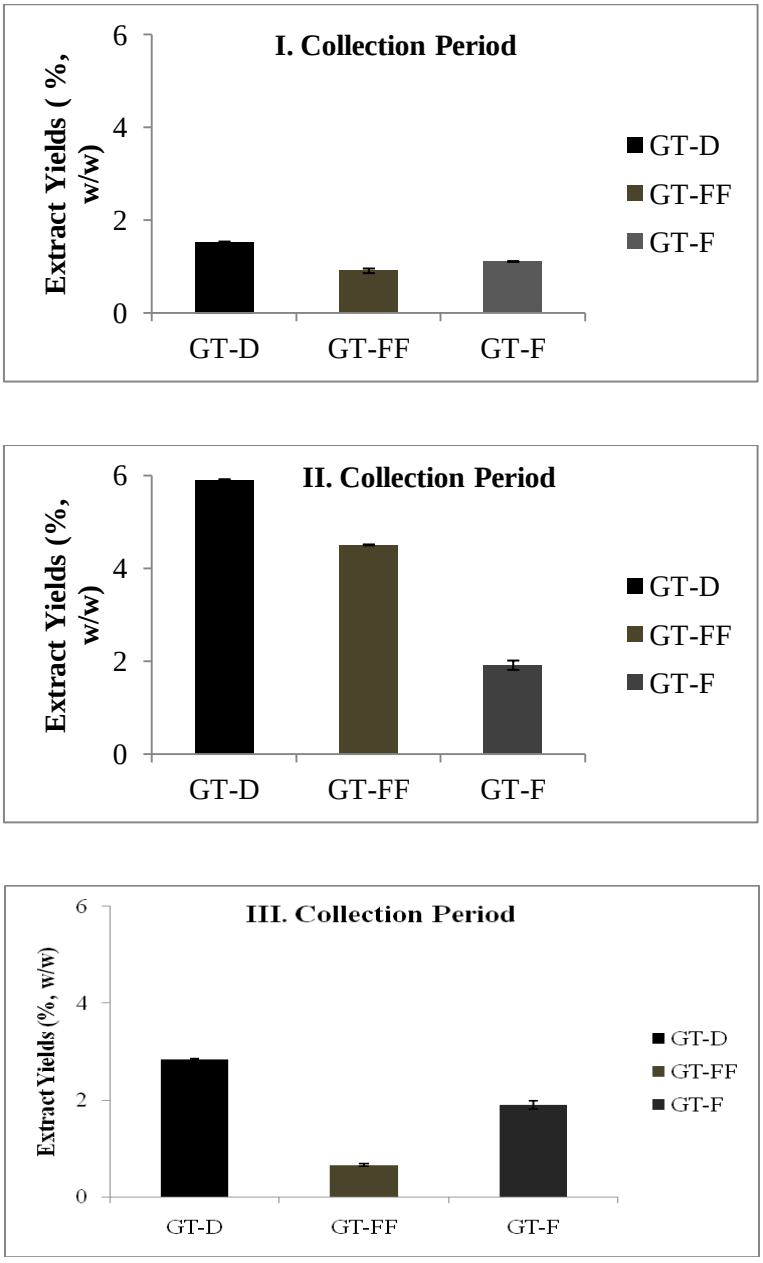

Fig. 2. Comparison of catechin extract percentage (%, w/w) of the dry, frozen, and fresh green tea leaf in three collection periods.

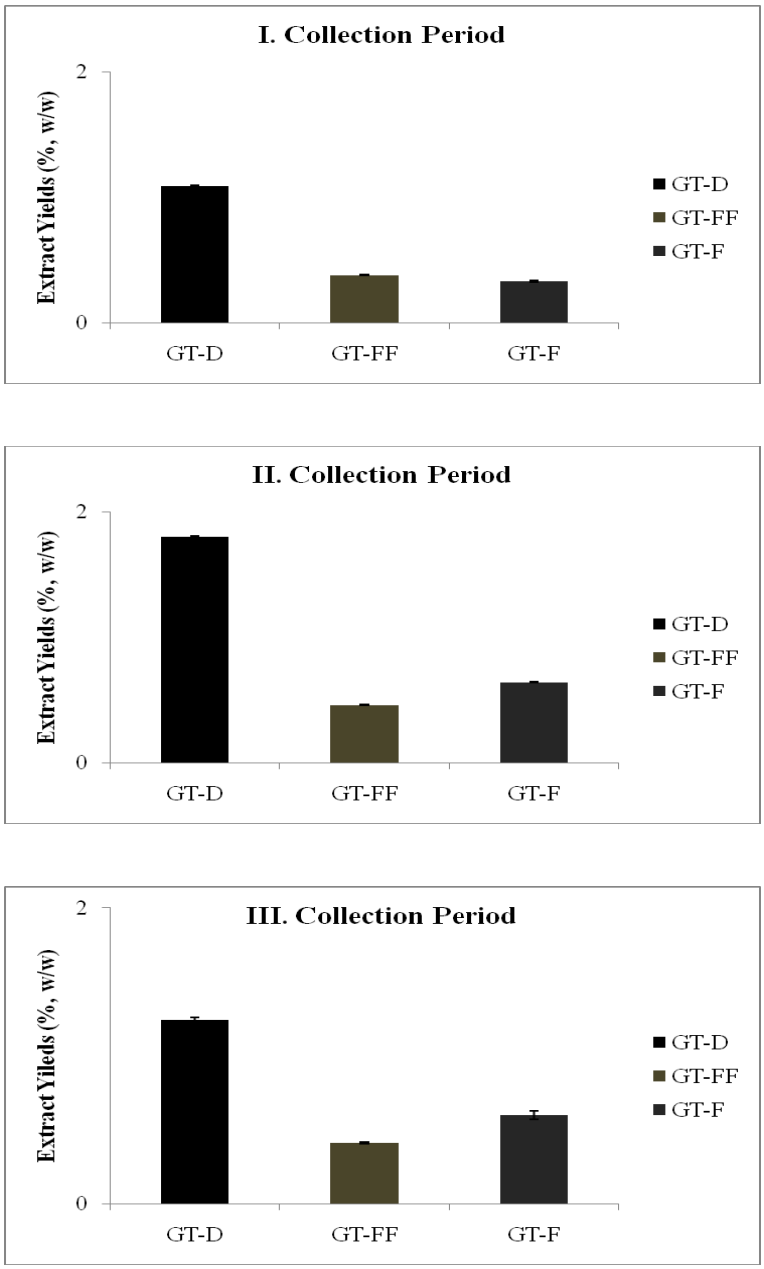

Fig. 3. Comparison of caffeine extract percentage (%, w/w) of the dry, frozen, and fresh green tea leaf in three collection periods.

As seen from the results given in figures when dried leaves used for the extraction the highest extract yields were obtained. This is expected, since drying decreases the water content and increases the catechin constituent. Drying naturally increases the amount of chemical present in leaves since 10 grams of dried leaves were equal to 30 grams of fresh leaves. However catechin or caffeine constituents of dried samples were not always three times higher than fresh or frozen samples. It is clear that fresh processing especially the chopping step is more effective to extract the active chemicals from destroyed cells. Frozen and fresh leaves gave closer yields to each other in terms of catechin and caffeine contents. Catechin constituent was found extremely low for the first collection period. It reached to 5.9% (±0.002) for the second collection period (July) and decreased to 2.9% (±0.002) following collection period (September). When collection periods are compared with second period (July), it was the most fruitful time, providing higher catechin and caffeine employing

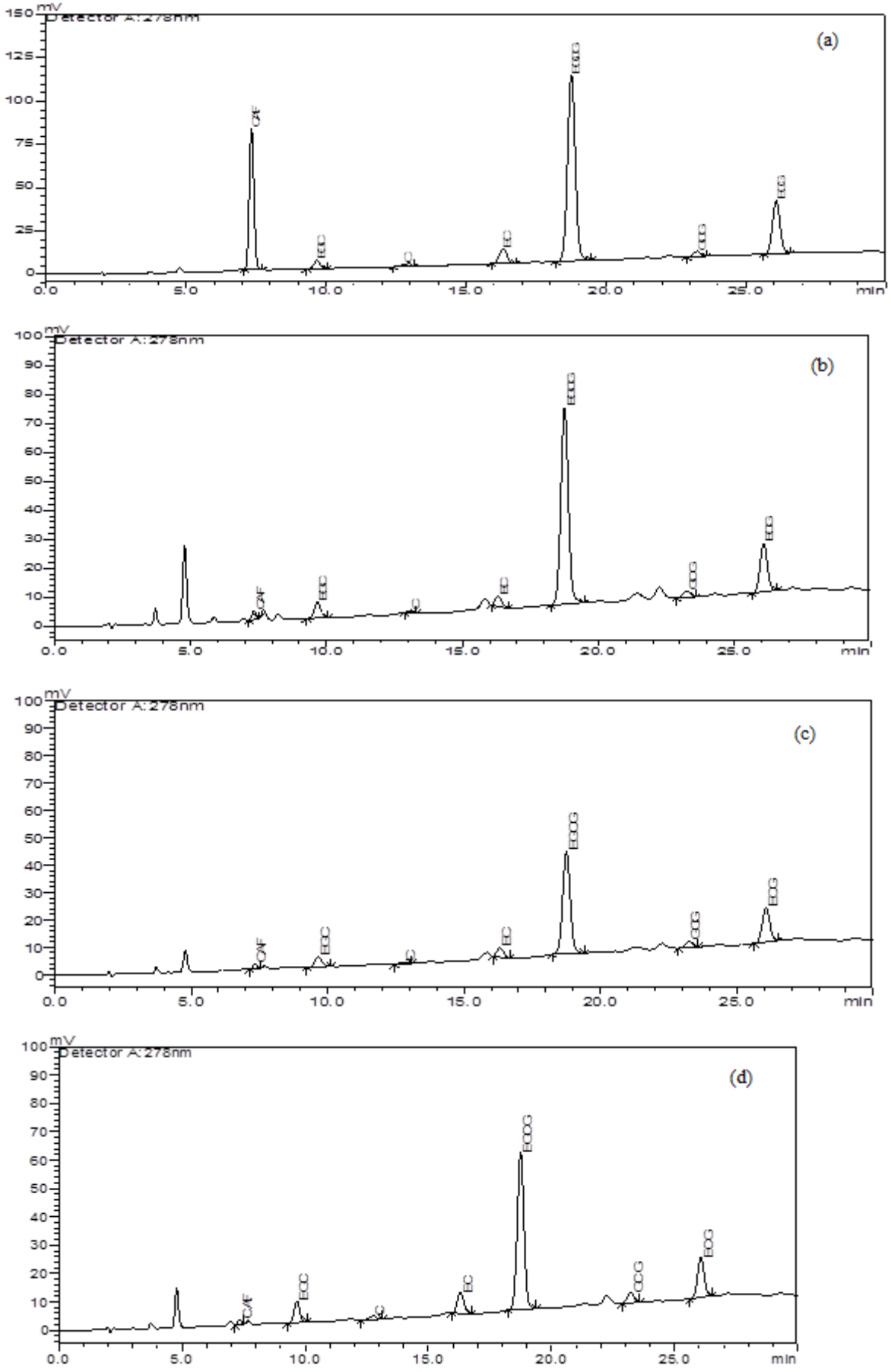

Fig. 4. HPLC chromatograms of standart mixture (**a**) and the extracts obtained from first collection period (**b-c-d**).

water extraction at 80°C at 40 minutes. It should be noted that catechin amount of Turkish green tea is quite low except for second collection period. This can be related to environmental conditions such as geographic structure, plant variety, growing conditions and climate.

HPLC analysis of the extracts. Catechins are a group of chemicals and present in plants as epi structures mainly gallate forms. Therefore chemical constituents of the catechin extracts should be evaluated for better understanding of these classes of compounds. Each extract was analyzed by HPLC and chromatograms were used for identification of individual catechin and their quantitative amounts were determined. HPLC chromatograms of the extracts from first collection period are given in Figure 4a-d.

Table 1. Quantitative analysis of catechins

	I. Collection period					
	GT-D			**GT-FF**		**GT-F**
Catechin	**%***	**%****	**%***	**%****	**%***	**%****
EGC	8.304	0.126	7.351	0.067	13.116	0.146
C	0.399	0.006	0.480	0.004	1.204	0.013
EC	1.725	0.026	2.035	0.018	4.717	0.052
EGCG	20.552	0.312	13.582	0.123	19.201	0.214
GCG	0.820	0.012	1.016	0.009	1.664	0.018
ECG	3.826	0.058	3.519	0.032	3.739	0.041
	II. Collection period					
	GT-D			**GT-FF**		**GT-F**
Catechin	**%**	**%**	**%**	**%**	**%**	**%**
EGC	1.146	0.067	4.459	0.201	11.158	0.213
C	6.027	0.355	0.772	0.034	0.947	0.018
EC	0.422	0.024	1.983	0.089	2.782	0.053
EGCG	2.240	0.1322	13.646	0.617	30.148	0.577
GCG	0.652	0.038	2.636	0.119	4.796	0.091
ECG	3.063	0.180	6.407	0.289	6.359	0.121
	III. Collection period					
	GT-D			**GT-FF**		**GT-F**
Catechin	**%**	**%**	**%**	**%**	**%**	**%**
EGC	2.428	0.069	0.760	0.005	10.545	0.200
C	0.097	0.003	0.154	0.001	1.098	0.020
EC	0.814	0.023	0.592	0.003	4.272	0.081
EGCG	15.807	0.450	5.117	0.034	29.986	0.571
GCG	2.604	0.074	0.153	0.001	5.975	0.113
ECG	5.377	0.153	2.166	0.014	6.093	0.116

*Percentage of each catechin in the catechin extract.
**Percentage of each catechin in tea sample.

As seen from HPLC chromatograms trace amount caffeine are present in catechin extract. It was quantified and given in Table 2. Standart concentrations are; 8.537 mg EGC, 1.151 mg C, 6.234 mg EC, 41.289 mg EGCG, 1.4 mg GCG, 9.104 mg ECG, 10.197 mg caffeine in 100 mg mixture. Retention times: caffeine 7.33; EGC 9.66; C 12.79; EC 16.32; EGCG 18.76; GCG 23.24; ECG 26.07 min. Injection volume is 10 µL. The identification of catechins was carried out by comparison of their retention times to those of standards and amounts were calculated. The quantitative analysis results of catechins are given in Table 1. Chloroform extraction to separate caffeine from catechin seems to be quite successful. But chloroform is a toxic solvent and more appropriate extraction solvents should be considered for this process

Table 2. Quantitative analysis of caffeine present in catechin extracts.

	I.	***Collection period***
GT-D	**GT-FF**	**GT-F**
%*	**%***	**%***
0.253	0.178	0.183
	II.	***Collection period***
0.615	0.465	0.660
	III.	***Collection period***
1.101	1.177	0.520

*Percentage of caffeine in the catechine extract.

4. Conclusions

Researches usually use boiling water or organic solvents such as acetonitrile, methanol, ethyl acetate, and ethanol to extract catechins. Although water extraction is favorable for its cost and safety, the yield is lower compared with organic solvent extraction. These solvents are harmful to human, and the products could not be used in medicine, cosmetics and food industry. Water which is harmless, cheap and available in large quantity is more favorable solvent to extract catechins.

In this research the most prosperous extraction procedures to obtain catechin and caffeine compounds from green tea leaves were established. Amount of extracted catechin was highest when dried leaves extracted with water for 40 minutes at 80 ^{o}C in the second collection period (June). Catechin amount reaches to 0.590±0.002 g when 10 grams of dry green tea is extracted. The extract was partitioned between water-chloroform, to remove caffeine impurities. Finally, the resulting extract was partitioned with water-ethyl acetate to purify the catechin compounds of EGC, EC, EGCG, EGC, GCG, C. The overall sequence of the catechins in green tea followed the order of EGCG>EGC>ECG>EC>GCG>C in the water extracts.

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