Bulgarian Chemical Communications, Volume 53, Issue 4 (pp. 405 - 411) 2021 *DOI: 10.34049/bcc.53.4.5341*

Comparison of antioxidant activity and determination of epigallocatechin gallate and trace elements of green tea samples manufactured and exported by Turkey

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Received: October 23, 2020; Revised: October 14, 2021

Green tea is one of the extensively used drinks in the world. Green tea extract contains several polyphenolic compounds and catechins. Epigallocatechin-3-gallate (EGCG) and epicatechin 3-gallate (ECG) are the most effective antioxidants. In this study, the relation between EGCG content and the antioxidant effect of samples purchased from four different manufacturers was investigated. The antioxidant effects of blended tea samples which contain additives (such as ginger, lemon) and pure green tea samples were compared *via* $DPHH^{\bullet}$ and $ABTS^{+\bullet}$ radical scavenging tests and *β*-carotene linoleic acid inhibition methods. The total ash content of green tea samples was determined according to the ISO 1575 test method. All samples were analyzed for trace elements by flame atomic absorption spectrometry (FAAS) after wet digestion. Some tea samples showed a higher (-)-EGCG content (e.g. 164.27 µg in 1 mL infusion for GT-A_7), nevertheless, all samples possessed good antioxidant activity. GT-A_6 showed the highest level of $DPPH^{\bullet}$ scavenging activity with 86.0 ± 0.1% inhibition. The TEAC value of GT-A_5 and GT-A_7 was determined to be 2.58 mmol/L/Trolox. The results of the total ash content (in the range of 5% to 7%) and of the trace elements contents suggest that all tea samples are of high quality.

Keywords: (-)-EGCG, green tea, trace element, antioxidant, LC-MS/MS.

INTRODUCTION

Tea is one of the most widely consumed beverages obtained from *Camellia sinensis* leaves and buds [1]. There are three types of tea like green tea, oolong tea and black tea depending on the level of oxidation. Green tea is an ancient beverage, which was consumed as a medicine and a healthful drink. This plant was used for headaches, body aches and pains, digestion, depression, detoxification in traditional Chinese medicine [2]. Numerous *in vitro* studies showed that fresh green tea leaves are very effective antioxidants. Subject to no fermentation, green tea leaves retain their green color and almost all of their original polyphenol content. Green tea phenolic compounds are mainly composed of catechins (epigallocatechin gallate ((-)-EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC), flavanols and phenolic acids [3]. Green tea also contains other compounds such as vitamins (B, C, E), volatile components and minerals but also some toxic metals such as cadmium (Cd) and lead (Pb) [4].

Green tea and black tea consumption has been increasing in Turkey in recent years, too. Turkey gives importance to tea agriculture and has an important place in the world tea export market. While the first three countries in tea exports are the Netherlands, Belgium and Germany, also Turkish tea is exported to many countries such as the USA, Georgia, Turkish Republic of Northern Cyprus, Singapore, England, Sweden and Mexico. Therefore, many different kinds of green tea assortments developed by tea companies are available in the Turkish market and are also exported abroad. It is stated that these teas sold in markets have high antioxidant activity and that the antioxidant activity of the tea samples is further enhanced by various additives like ginger, lemon, cinnamon, etc. But many factors can affect the quality of green tea during the manufacturing process (a certain grade of fermentation, heating, etc.). Also as a daily beverage, it is important to know the percentage of trace elements leaching into infusions. Therefore, in this study we aimed to: 1) determine the (-)-EGCG content because of its relationship to antioxidant activity; 2) investigate the antioxidant activities *via* radical scavenging (DPPH and ABTS) and *β*-carotene bleaching assays; 3) monitor the concentration of trace elements, particularly in view of allowable limits and total ash content. This is the first comparative study of antioxidant properties and trace element contents of green tea samples manufactured and exported by Turkey.

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MATERIAL AND METHODS

Plant material and reagents

In this study, 14 green tea bag samples: GT-A (from Firm 1), GT-A1(soft), GT-A2 (soft with jasmine), GT-A3 (bergamot), GT-A4 (jasmine), GT-A5 (ginger and lemon), GT-A6 (vitamins), GT-A7 (mint and lemon), GT-A8 (chai), GT-A9 (ginkgo), GT-B (from Firm 2), GT-C (from Firm 3), GT-D (from Firm 4), GT-D (from Firm 5), were purchased from Turkey markets. The tea bag samples were chosen from different firms; 10 samples were from one firm and all the others from different firms (Table 1). All chemicals were obtained from the Sigma Chemical Company (St. Louis, MO). Stock solutions (1000 mg/L) of the elements (Mn, Cu, Cr, Cd, Pb, Ni, Fe, Zn) were prepared by dissolving appropriate amounts of their nitrate salts in 1.0 % (v/v) HNO_3 and further diluted daily prior to use.

Determination of total ash content

The total ash content of green tea samples was determined according to the ISO 1575:2015 test method. According to this method, the powdered green tea samples were weighed into a prepared dish and the test portion was heated at a temperature near 100°C until the moisture was expelled. The dish was transferred to a furnace and heated at 525±25 °C until the ash was visibly free from carbon particles. The cooled ash was moistened with distilled water, then dried on a steam bath and then on a hot-plate. The dish was returned to the furnace for 60 min, cooled in a desiccator and weighed. Then it was heated again in the furnace for 30 min, cooled and weighed. These operations were repeated until the difference between two successive weighings did not exceed 0.001 g. This test was done in triplicate and the mean ash content was determined.

Determination of trace elements in green tea samples

A Varian AA240 model atomic absorption spectrometer equipped with single element hollow cathode lamps and anair-acetylene burner was used for the determination of metals. The instrumental parameters were used according to the manufacturer's recommendations. The wet digestion method developed by Narin *et al.* (2004) was used for the sample preparation [5]. For the determination of trace elements, green tea samples (1.000 g) were leached with a mixture of concentrated nitric acid and hydrochloric acid (3:1 v/v). After digestion, samples were filtered and diluted to 10 mL with ultra-deionized water. Blank digestions were carried in the same way.

Quantitative determination of (-)-EGCG in green tea samples

LC-MS/MS experiments were carried out using a Shimadzu LC-MS/MS 8040 (Shimadzu, Japan). A pneumatically assisted electrospray ionization (ESI) ion source was used throughout the experiments. Multiple-reaction monitoring (MRM) conditions were established for standard. The following instrumental settings were used for MRM analysis: heat block temperature, 400 °C; DL temperature, 250 °C; nebulizing gas (N_2), 3 L/min; drying gas (N_2), 15 L/min; collision energy, 25.0, 12.0, 9; dwell time, 100 msec.

The mass spectrometric behavior of (-)-EGCG was studied using both positive-ion and negative-ion ESI. In this experiment, we observed that the negative-ion mode is more sensitive for the detection and analysis of (-)-EGCG.

A mixture of acetic acid-water (1:99, v/v) and acetic acid-methanol (1:99, v/v) was selected as the mobile phase. The mobile phase consisted of 50% acetic acid-water and 50% acetic acid-methanol solution at a flow rate of 0.4 mL/min, and 1 µL of the standard and samples was injected. 1000 µg/mL stock solution of (-)-EGCG was prepared by dissolving the reference standard in methanol and calibration standards (100, 50, 10, 5, 2.5 and 1 µg/mL) were prepared by dilution of the stock solution. For tea sample solutions, 100 µL of infusion was dissolved in 900 µL of ultra-deionized water.

Linearity of the methods was established by triplicate injections of each concentration of standard solutions. Response function of the standard calibration curve was $y = 50752x + 30595$. The correlation coefficient (r^2) of the calibration curve was 0.9997.

Evaluation of the antioxidant activity of the sample infusions

Each tea bag sample was infused with 250 mL of boiling water (the equivalent of one teacup) for 10 minutes and filtered. The antioxidant activity of tea infusions was evaluated using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+\bullet}$) radical scavenging and inhibition of *β*-carotene/linoleic acid co-oxidation methods.

$DPPH^{\bullet}$ radical scavenging method. $DPPH^{\bullet}$ scavenging abilities of the tea samples were determined using the method of Gyamfi *et al.* [6]. Catechin, epicatechin and epigallocatechin gallate

were used as positive standards. The % inhibition was calculated using equation (1):

$$\% \text{ inhibition} = (Abs_{control} - Abs_{sample}) / Abs_{control} \times 100 \quad (1)$$

- *$ABTS^{+\bullet}$ radical scavenging method.* 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonate) radical ($ABTS^{+\bullet}$) test was used as an alternative for radical scavenging activity determination. Catechin, epicatechin and epigallocatechin gallate were used as positive standards at a concentration of 100 µg/mL. Absorbance was measured on a UV spectrophotometer at 734 nm [7].
- *Inhibition of β-carotene/linoleic acid co-oxidation method.* The *β*-carotene bleaching method [8] was performed to determine the antioxidant activity of tea samples. Absorbance was measured using a spectrophotometer at 470 nm. The antioxidant activity was calculated according to equation (2):

$$AAC = [(Abs^{120}_{sample} - Abs^{120}_{control}) / (Abs^{0}_{control} - Abs^{120}_{control})] \times 100 \quad (2)$$

RESULTS AND DISCUSSION

Determination of total ash content of samples

Ash content is an important quality control parameter of tea, a measure of inorganic inclusions and is linked to the mineral content that indicates the quality of the material. In this study, the total ash content was determined by the ISO 1575 test method. According to Turkish Food Codex requirements, the total ash content must be between 4% and 8% [9]. When the total ash content results of the test samples were taken into consideration, it was determined that all samples agree with the Turkish Food Codex in the range of 5% to 7%. These values indicate that no contamination occurred during processing and purport high quality and purity of the tea samples. The results of the tea samples are given in Table 1.

Table 1. Sample codes, total ash content, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging results and amount of (-)-EGCG in samples.

Samples	Additives	Total ash content	$DPPH^{\bullet}$	$ABTS^{\bullet+}$	(-)-EGCG content
		(%)	%Inhibition	TEAC (mmol/L/Trolox)	(µg/mL infusion)
GT-A (Firm 1)	-	7.41 ± 1.23	79.7 ± 3.9	2.54 ± 0.07	86.88 ± 0.59
GT-B (Firm 2)	-	5.52 ± 0.11	82.2 ± 1.7	2.57 ± 0.03	54.18 ± 0.59
GT-C (Firm 3)	-	5.49 ± 1.48	82.7 ± 1.8	2.47 ± 0.00	104.45 ± 1.97
GT-D (Firm 4)	-	6.04 ± 0.72	81.2 ± 1.0	2.58 ± 0.00	104.10 ± 1.45
GT-E (Firm 5)	-	5.38 ± 0.12	79.4 ± 0.8	2.34 ± 0.20	16.98 ± 0.33
$GT\text{-}A_1$	Soft	5.38 ± 0.09	81.2 ± 2.7	2.56 ± 0.04	84.90 ± 0.77
$GT\text{-}A_2$	Soft with jasmine	6.96 ± 0.63	78.5 ± 3.7	2.57 ± 0.00	85.57 ± 0.69
$GT\text{-}A_3$	Bergamot	5.47 ± 1.21	81.3 ± 2.7	2.55 ± 0.04	52.77 ± 5.18
$GT\text{-}A_4$	Jasmine	5.12 ± 0.02	81.4 ± 0.3	2.58 ± 0.00	85.90 ± 0.26
$GT\text{-}A_5$	Ginger and lemon	6.01 ± 0.02	82.5 ± 1.5	2.58 ± 0.00	79.06 ± 0.64
$GT\text{-}A_6$	Vitamins	7.10 ± 0.08	86.0 ± 0.1	2.57 ± 0.00	122.67 ± 1.38
$GT\text{-}A_7$	Mint and lemon	5.95 ± 0.17	84.4 ± 0.9	2.58 ± 0.01	164.27 ± 2.28
$GT\text{-}A_8$	Chai	6.88 ± 0.84	82.7 ± 2.8	2.55 ± 0.00	133.40 ± 2.40
$GT\text{-}A_9$	Ginkgo	6.19 ± 1.05	84.2 ± 0.4	2.51 ± 0.01	106.96 ± 0.18

Table 2. Levels of the investigated ions in the green tea samples.

	Concentration (μg/g)[a]							
Sample	Mn	Cu	Cr	Cd	Pb	Ni	Fe	Zn
GT-A1	1288.6±23.7	11.4±0.5	< 2.0	1.0±0.1	6.2±0.2	9.7±0.3	333.7±39.3	24.6±1.0
GT-A2	1035.2±48.8	9.0±0.3	< 2.0	1.1±0.1	< 4.0	9.6±1.0	319.7±6.7	19.5±1.0
GT-A3	1158.5±62.5	11.0±0.2	< 2.0	< 0.1	< 4.0	10.3±0.3	352.1±19.9	25.6±03
GT-A4	1195.2±52.8	11.4±0.2	< 2.0	1.3±0.1	4.0	9.7±0.5	320.7±0.8	23.8±1.5
GT-A5	894.1±32.7	8.4±0.5	< 2.0	1.0±0.1	5.9±0.6	5.8±0.2	348.0±2.8	19.9±1,4
GT-A6	868.8±82.3	9.5±0.3	4.4± 0.3	< 0.1	< 4.0	9.4±1.0	286.4±10.9	22.8±0.0
GT-A7	1083.9±61.9	9.9±0.2	< 2.0	1.0±0.1	< 4.0	7.4±0.1	369.4±0.9	19.2±0.7
GT-A8	841.1±17.6	8.2±0.7	< 2.0	1.1±0.1	< 4.0	5.4±0.6	250.0±16.2	20.8±0.6
GT-A9	1305.7±48.4	12.6±1.0	< 2.0	1.1±0.0	< 4.0	8.6±0.4	355.9±27.7	22.3±1.0
GT-A	1083.4±20.6	9.0±0.5	< 2.0	1.1±0.1	< 4.0	5.2±0.6	253.4±2.3	18.7±1.2
GT-B	1083.1±4.2	7.6±0.3	< 2.0	1.2±0.1	< 4.0	4.0±0.3	179.0±16.6	15.1±1.4
GT-C	841.2±6.2	5.2±0.4	< 2.0	1.1±0.1	< 4.0	<3.0	240.1±3.4	13.4±0.9
GT-D	991.2±16.3	8.8±0.7	< 2.0	1.4±0.1	< 4.0	5.0±0.5	275.7±25.1	18.4±2.0
GT-E	933.4±4.5	7.8±0.4	8.1 ± 1.0	1.4±0.1	< 4.0	8.1±0.8	360.4±22.9	17.2±1.7

[a]Data are expressed as mean ± standard deviation (n=3).

Trace elements in green tea samples

The bio elements and toxic metal concentrations of tea samples are given in Table 2. Cadmium, which has a toxic effect on organs and systems such as kidneys, skeletal system and respiratory system, is classified as a human carcinogen. The Joint Expert Committee on Food Additives (JECFA) has determined the weekly tolerable cadmium level (PTWI) as 7 μg/kg body weight [10]. The levels of cadmium were in the range of 1.0-1.4 μg/g in our analyzed samples. The highest and lowest levels of cadmium were found in the GT-D and GT-A_1 samples, respectively. These results suggest that the green tea consumption does not exceed the PTWI recommendation for this metal. The levels of cadmium in the tea samples were under the detection limits of the method applied, hereby, it was concluded that green tea consumption is not dangerous for human health.

Lead toxicity is a particularly insidious hazard with the potential of causing irreversible health effects. It interferes with several body functions primarily affecting the central nervous, hematopoietic, hepatic and renal systems by producing serious disorders. The highest concentration of Pb was found in the GT-A_1 and GT-A_5 samples (6.2 ± 0.2; 5.9 ± 0.6 μg/g, respectively). Pb concentration in the other samples was lower than 4.0 μg/g. The temporary PTWI doses of Pb from all sources tolerated by a healthy human are established as 0.025 mg/kg body weight [11]. Considering the average concentration of Pb in the respective tea samples, it was determined that they did not threaten health.

The daily requirement for manganese has been established as 6–11 mg/day for a person aged 9-70 years [12]. The lowest manganese level was found in GT-A_8 as 841.1 μg/g while the highest was 1288.6 μg/g in GT-A_1. According to our results, a cup of tea provides a dietary intake of manganese in the range of 13.33 - 21.33%.

Copper is a trace element that can be found in almost every cell of a human organism. According to our analysis, the maximum concentration of Cu is in GT-A (12.6 μg/g) and the minimum concentration of Cu is in GT-C (5.2 μg/g). The daily requirement for copper has been established as 5-10 mg/day for a healthy person aged 9-70 years [12]. According to the results of our samples, one cup of tea per day provides approximately0.1-0.25%. These results suggest that green tea samples are not a rich source of Cu in the daily diet.

Chromium is an essential nutrient for the body (blood, urine, and body tissues) and is normally present in food. The FDA has determined that the chromium concentration in bottled drinking water should not exceed 0.1 mg/L. The highest concentration of chromium was found at 8.1 μg/g in GT-E and 4.4 μg/g in GT-A_6. Cr^{3+} concentration in the other samples was lower than 2.0 μg/g [12, 13]. When the results were examined, even a cup of tea with the highest amount of chromium (GT-E) consumed per day is within the limits determined by the FDA.

Nickel is a nutritionally essential trace element but excessive Ni intake is harmful to humans [14]. The concentration of nickel was found lower than 3.0 µg/g in GT- and 10.3 µg/g in GT-A_3. The daily requirement for nickel has been established as 100-600 µg/day for an adult person [12]. When the analysis results were examined, it was clear that green tea samples are not a rich source of nickel.

Iron is a very important element for humans and animals. The daily requirement for iron is 40-45 mg/day. In the green tea samples, GT-B was found to contain the lowest concentration for iron of 179.0 µg/g and GT-A_7 was found to contain the highest concentration of 369.4 µg/g [12]. The results of the iron analysis in the tea samples are consistent with the literature and support the view that tea infusions are not an important source of Fe [15].

An adult person needs to intake 23-40 mg/day of zinc for daily dietary requirements. The lowest zinc concentration was found to be 13.4 µg/g in GT-C and the highest concentration was found to be 25.7 µg/g in GT-A_3 [16]. Zinc content in tea infusions was found to be low, and therefore cannot be regarded as a major dietary source.

Quantitative determination of (-)-EGCG in green tea samples

Fragmentation of $[M-H]^-$ ion (m/z 457) of (-)-EGCG resulted in three major ions at m/z 125, 169, and 305. The m/z 125 ion represents an unmodified A-ring, the m/z 169 ion is due to the loss of an intact gallic acid anion, while the ion at m/z 305 results from the neutral loss of gallic acid [17] (Fig.1). In our study, three of the most abundant product ions observed were m/z 125 and m/z 169 and 305. The LC-MS/MS spectrum of (-)-EGCG is presented in Fig. 1.

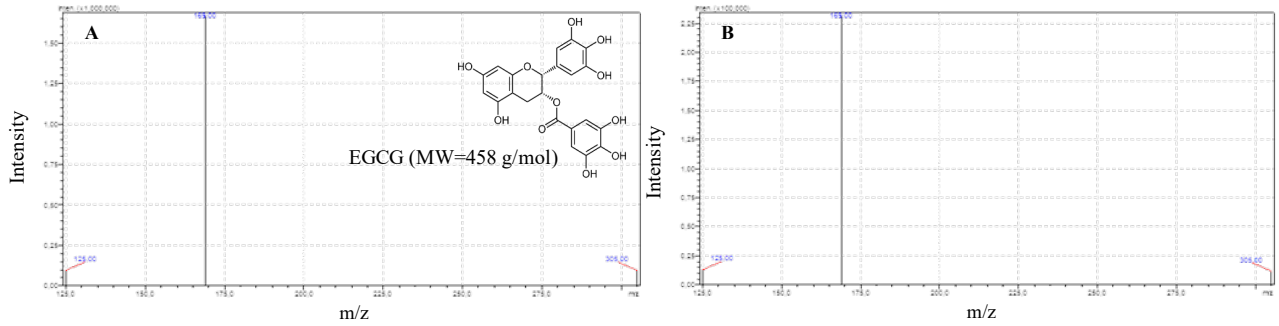

Figure 1. LC-MS/MS mass spectra of (-)-EGCG (A) and GT-A_7 (B)

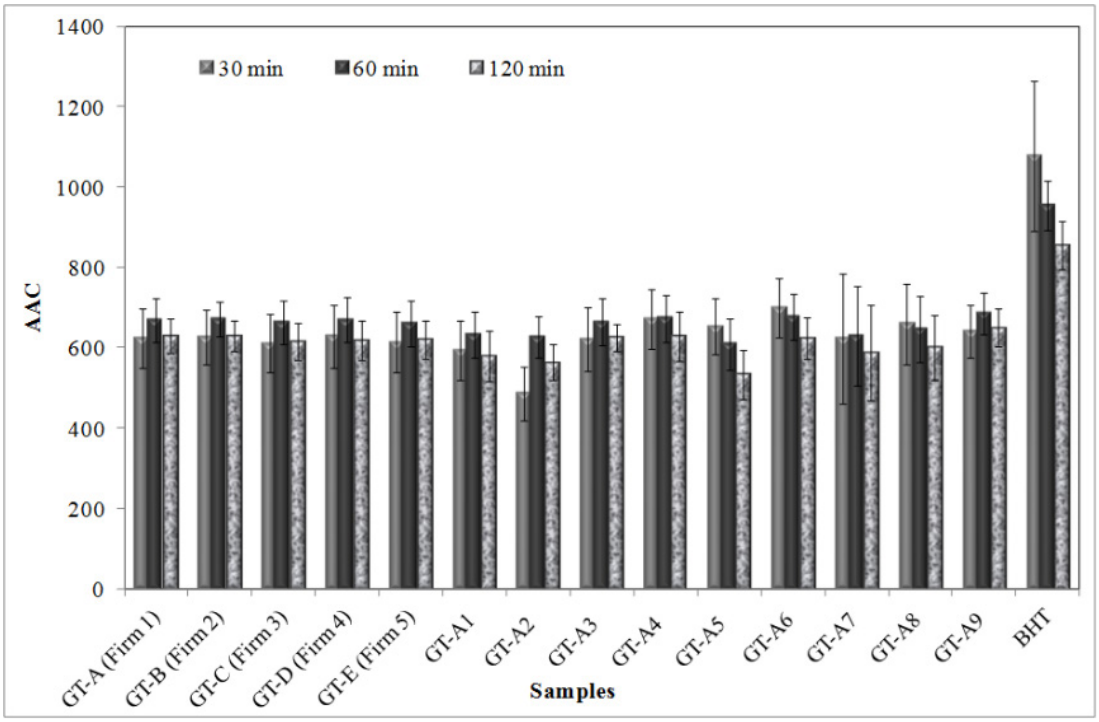

Figure 2. Effect of the samples and positive control (BHT) on *β*-carotene/linoleic acid co-oxidation. Data are expressed as mean ± standard deviation (n=3).

All samples were analyzed in triplicate. The results are shown in Table 1. It is observed that "blended" tea samples have a lower (-)-EGCG content than "pure" green tea samples. The tea sample named GT-A_7 presented the highest amount of (-)-EGCG (164.27 µg in 1 mL infusion). The LC-MS/MS spectrum of GT-A_7 is shown in Fig.2.GT-A_7 sample was found more active than catechin and epicatechin as positive controls in the ABTS test. This tea sample also showed the highest level of DPPH$^{\bullet}$ scavenging activity with 86.4 ± 0.9 % inhibition. So, it can be concluded that mint and lemon additives do not affect the antioxidant activity but the latter is generally affected by the amount of EGCG.

Evaluation of the antioxidant activity of the infusions

DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical scavenging inhibitions of samples are given in Table 1. Although there is no significant difference among tea samples, GT-A_6 showed the highest level of DPPH$^{\bullet}$ scavenging activity with 86.0 ± 0.1% inhibition. In this experiment, the addition of vitamins or other plants like jasmine, chai, ginkgo, etc. into the tea bags did not increase the antioxidant activity. The lowest activity was observed for the GT-A_2 sample with 78.5 ± 3.7% inhibition. Since the jasmine additive in the GT- A_2 sample will cause a decrease in the amount of green tea, this may be reflected in the results of scavenging activity.

In the ABTS$^{\bullet+}$ radical scavenging method, green tea samples without additives were generally found more active than the other samples. The TEAC value of GT-A_4, GT-A_5 and GT-A_7 was determined to be 2.58 mmol/L/Trolox and was found to be higher than those of catechin and epicatechin which were used as positive controls.

The oxidation-inhibiting effect of the samples was determined in a time-dependent manner, and time-dependent alteration was observed. The inhibition percentages of all extracts are given in Fig. 2. All samples managed to inhibit β-carotene bleaching but were not found as active as the synthetic antioxidant butylated hydroxytoluene (BHT). Dissolving tea samples in water may cause a decrease in the amount of nonpolar antioxidant compounds. In this method, nonpolar antioxidants were concentrated at the lipid-air interface and provided high protection in emulsions against polar antioxidants that exist in the aqueous phase [18,19].

CONCLUSION

In the present study, we aimed to compare the relation between EGCG content and antioxidant activity of pure and blended tea samples and also to determine the percentage of trace elements leached into infusions. The highest EGCG content was found in pure tea samples and tea blends. As a result of this study, antioxidant activities of the blended samples were found higher than those of pure samples. These results indicated that the antioxidant activity is not only affected by the amount of EGCG but also by different additives. The results of the total ash and trace elements contents suggest that all tea samples which are manufactured and exported by Turkey have high quality and purity. This study supports that consumption of green tea per day has a significant beneficial effect on health.

Acknowledgements: *We are grateful to the Doğadan Food Products Industrial and Marketing Incorporated for their support and Erciyes University Ziya Eren Drug Application and Research Center (ERFARMA) for providing LC/MS/MS facility.*

Conflict of interest: *The authors declare that they have no conflict of interest.*

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