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Research Article

Flavonoid contents and antioxidative effect of tea samples

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Abstract

A rapid GC method was developed for the separation and determination three flavonoids; desmodol (DES), triangularin (TRI) and 2′,4′-dihydroxychalcone (DIHY) in tea samples. The GC method was applied for the quantification of DES, TRI and DIHY in the tea samples collected from different local markets of Bangladesh. The results showed no significant variation in the amount of these markers in methanolic extracts of tea samples from different markets of Bangladesh. A variation in antioxidant activities, ranging from 62.82% to 89.04%, and variations in total phenolics, ranging from 7.14 to 9.71 mg caffeic acid/g dry weight of the methanol extracts, were observed. Anti-oxidative potency of the methanol extracts was comparable to that of pure quercetin and the synthetic antioxidant butylated hydroxylanisole (BHA).

Keywords: *Camellia sinensis;* Theaceae; tea samples; flavonoids; antioxidant activity; total phenol; GC-FID.

Introduction

Phenolics are a class of low molecular weight secondary plant metabolites found in most land plants. Phenolics (flavonoids) protect plants against ultraviolet radiation, pathogens and herbivores [1]. Although dietary intake varies considerably amongst geographic regions and cultures, the average daily consumption of flavonoids by humans is estimated to be 1 g [2]. Most of the protective effects of flavonoids in biological systems are ascribed to their antioxidant abilities, capacity to transfer electrons, free radicals and chelating abilities [3], to activate

antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases [4]. Recently, there has been considerable interest in finding naturally occurring antioxidants to replace synthetic antioxidants in food and medicine. Several studies have analyzed the antioxidant potential of a variety of herbs [5, 6] and amongst the different parts of plants studied, the leaves are reported to have highest antioxidant properties [7, 8], and the most active principle among the phytochemicals is the phenolic fraction [9, 10, 11]. The phenolics have *in vivo* antioxidant activities and have been used as natural antioxidants in food [12, 13].

The attractiveness of the tea samples to consumers is determined both by appearance and by internal attributes of firmness, taste and health benefits. Flavonoids, phenolic secondary plant metabolites, contribute to both fruit colour and human health. Flavonoids are widely believed to possess anti-oxidative, anti-microbial, anti-mutagenic and anti-carcinogenic properties [14, 15, 16, 17]. Epidemiological studies have shown an inverse relationship between the intake of fruit, vegetables and beverages rich in flavonoids and the incidence of coronary heart disease, however, the relationship with cancer is not clear [18]. Tea is one of the main sources for flavonoid intake in the European diet, after onion and apples [19].

Flavonoid compounds occur ubiquitously in food of plant origin and are highly diversified. They appear to have played a major role in the successful medical treatments of ancient times and their use has persevered up to now. In more recent time polyphenolic compounds have gained increasing interest because they exhibit beneficial health effects due to their potential antioxidant [20, 21, 22], anti-inflammatory [23] and cancer-preventive [24], activities. They are present in body cells and fluids as a result of ingestion of fruit, vegetables, and plant-derived food such as tea and chocolate [25].

Flavonoid compounds constitute a complex group of substances which are also found in medicinal plants such as *Orthosiphon stamineus* and in some citrus species [26, 27]. Because numerous studies have clearly demonstrated that flavonoids exhibit important quality properties, the quantification of flavonoid compounds is of particular interest. Many analytical procedures have been developed to study flavonoid compounds, which reflect their importance for undertaking analysis. The most successful approaches have been based on both chromatographic and spectrophotometric methods. Gas chromatography has been the method of choice because of its versatility, precision and relatively low cost.

The aim of this work is to compare the contents of bioactive DES, TRI and DIHY in tea samples collected from different local markets of Bangladesh and to evaluate their antioxidative properties.

Materials and Methods

Chemicals and reagents

Standard samples of DES, TRI and DIHY were purchased from Indofine Chemical Co. (Hillsborough, NJ USA). Solvents used for chromatography were methanol (GC grade) and water (GC grade), obtained from Merck (Darmstadt, Germany). Folin-Ciocalteu reagent, quercetin, butylated hydroxytoluene (BHT), Tween 20, β-carotene (95%) and linoleic acid (99%) were purchased from Sigma chemical Co. (St. Louis, MO). All other chemicals were of analytical grade or GC grade.

Sample collection

There are different varieties of tea samples available in Bangladeshi markets all the year round. Lipton tea is one of the most popular tea sample cultivars grown in the Indian region. For this experiment Lipton varieties were used. Lipton tea samples were collected from the local markets of different districts of Bangladesh. The tea samples were identified and voucher specimens were deposited with the Department of Botany, University of Dhaka, Bangladesh with number 039.

Flavonoids extraction

Tea samples (Lipton brand, 15 g) were collected from the local markets of different districts of Bangladesh. The samples were immersed in 30 ml of methanol and were kept for 24 h at room temperature. After vigorous shaking, the organic portion was filtered and the clear filtrate solution was evaporated until dry by using Kuderna-Danish evaporator. The crude dry product was dissolved in 2-3 ml of dichloromethane and evaporated until dry to remove the solvent and the residue left after evaporation. The extract was transferred to another tube before re-extraction of the tea samples with the same procedure as described above. The total extract (2 ml) from tea samples was filtered through a 0.45 µm Millex HA filter (Millipore, Molsheim, France) prior to GC analysis. The entire procedure was carried out at 4^{o}C and shaded from incident light.

Preparation of samples for GC analyses

The methanolic crude extract was diluted with 5 ml of dichloromethane and the samples were filtered through 0.45 µm membrane filters (Millipore, Molsheim, France) prior to GC analysis.

Identification and quantification of markers by GC

The GC analysis of the methanol crude extract of the tea samples was performed using a Varian gas chromatograph (model 3300, USA) equipped with flame ionization detector (FID). Signals were recorded on an integrator, Varian model 4290 (Varian, USA). Hydrogen generator (model 7526, Canberra Industries Inc., USA) was used for hydrogen production. A Megabore Column (USA), SE-54 phase, 30 m long, width 0.54 mm, i.d. 1.2 µ film thickness was used for the chromatographic separation. Operational conditions were column programmed temperature 90°C, detector temperature 300°C, injector temperature 250°C, carrier gas (nitrogen) with flow rate 18 ± 1 ml/min. Diluted samples (1/100, v/v, in dichloromethane) of 1 µl were manually injected into the GC column by using Hamilton micro syringe. Identification of compounds of the methanol crude extract was based on GC retention time on megabore column, matching with standards. The following reference compounds were used as markers: DES, TRI and DIHY. The markers were accurately weighed and dissolved in methanol to produce a series of concentrations. Standard calibration curves were established by plotting the areas of peaks against different concentrations of the reference compounds (varying from 0.5 to 10 µg on column for DES, TRI and DIHY). The external standard method was used for quantification of the markers in the tea sample extracts from different places.

The suitability of the method was evaluated by the intra- and inter-day precision and accuracy of replicates. The accuracy was evaluated through recovery studies by adding known amounts of the standard solution to the extract. Controls from all samples were prepared and analyzed. The recovery experiment was performed at three concentrations of the standards.

Determination of total phenolic contents of methanol extracts

The concentrations of total phenols in extracts were determined by using Folin-Ciocalteu reagent and external calibration with caffeic acid. Briefly, 0.2 ml of extract solution in a test tube and 0.2 ml of Folin-Ciocalteu reagent were added and the contents were mixed thoroughly. After 4 min, 1 ml of 15% Na_2CO_3 was added, and then the mixture was allowed to stand for 2 h at room

temperature. The absorbance was measured at 760 nm. The concentration of the total phenolics was determined as mg of caffeic acid equivalent by using an equation obtained from the caffeic acid calibration curve [28].

Antioxidant activity of extracts using β-carotene/linoleic acid system

The procedure for evaluating the antioxidant activity for the tea samples was modified from a method described by Taga, Miller and Pratt [29]. One ml of β-carotene (2 mg in 20 ml of chloroform) was added to a conical flask with 40 mg of linoleic acid and 400 mg of Tween-20. Chloroform was removed with a rotary evaporator at 40^{o}C and the mixture was dried with nitrogen. To the resulting residue, 100 ml of oxygenated distilled water was added and mixed and aliquots (3 ml) of the oxygenated β-carotene emulsion were placed in a tube containing 0.2 ml of the extracts (0.2 mg/ml) and the absorbance measured at 470 nm immediately, against a blank consisting of the emulsion without the β-carotene. The solution was incubated in a water bath at 50^{o}C to induce auto-oxidation. Taking absorbance at every 15 min intervals for 120 min, oxidation of the β-carotene emulsion was monitored. Absorbance of a control consisted of 0.2 ml of distilled water instead of the extract was also monitored. Quercetin (0.2 ml of 1.0 l M) and BHA (0.2 ml of 1.0 l M) were used as reference compounds [30]. The bleaching rate (R) of β-carotene was calculated using the equation:

$$R = \ln (a/b) \times 1/t, \quad (1)$$

Where ln=natural log, *a*=initial absorbance (470 nm), *b*= absorbance at 120 min interval and *t*=time (min). The antioxidant activity (AA) was determined as percent inhibition relative to control sample using the equation:

$$AA\ (\%) = [(R_{control} - R_{sample}) / R_{control}] \times 100: \quad (2)$$

Statistical analyses

Data are expressed as a means of triplicate measurements. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Means were compared by Tukey-HSD and LSD (least significant differences).

Results and Discussion

Collection of samples

Table 1 shows the locations in Bangladesh where the analysis samples were collected. The tea samples were collected in the late afternoon, when tea samples are less turgid and therefore less likely to be damaged and energy substrate levels are high to facilitate long storage life [31, 32].

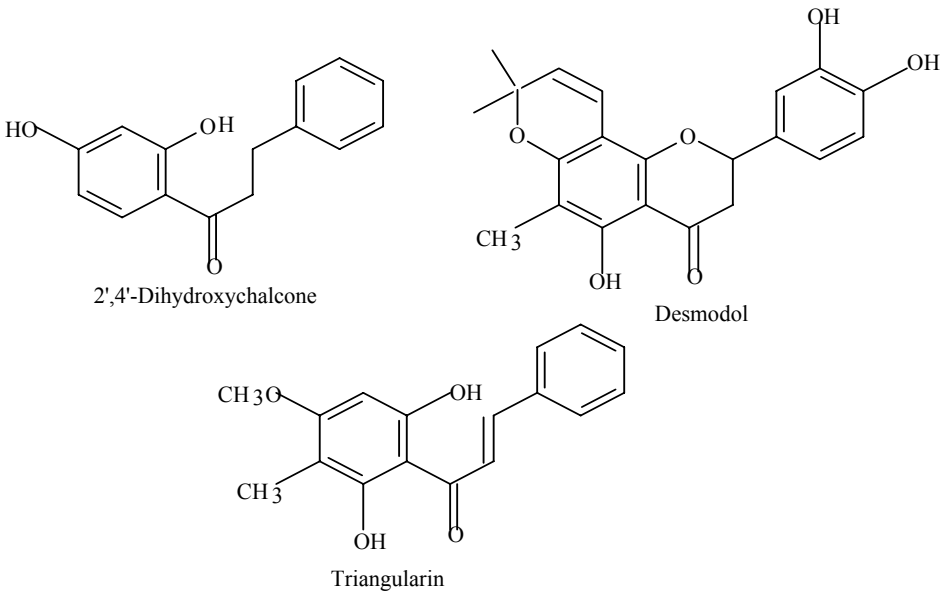
Concentrations of the markers in methanol extracts

The GC method applied is a modification of that reported by Stuart *et al*. [33] for the analysis of flavones present in tea samples. In the present study, programmed method was used for the simultaneous assay of the authentic markers, whose chemical structures are shown in Fig. 1. All the standards were determined in a single run of GC-FID. The standards were resolved and eluted at 3.07, 3.74 and 4.98 min, with respect to DES, TRI and DIHY (Fig. 2). The markers (0.5, 1, 2.5, 5 and 10 µg on column for DES, TRI and DIHY showed good linearity in the range from 0.5 to 10 µg in the calibration curves that were obtained by GC analysis. All the reference markers were present in the chromatographic profiles of the samples from various locations when the sample solution was analyzed by GC-FID (Fig. 3). The peaks of DES, TRI and DIHY were confirmed by comparison of the retention times with the reference standards.

Table 1. Percentage concentrations of marker flavonoids in the tea samples collected from markets of different districts of Bangladesh.

Location	Marker concentration (% of total dry weight)		
	Desmodol (DES)	Triangularin (TRI)	2′,4′-dihydroxychalcone (DIHY)
Kushtia	0.011	0.19	0.087
Pabna	0.014	0.21	0.071
Dhaka	0.013	0.21	0.098
Sylhet	0.110	0.31	0.106
Comilla	0.021	0.21	0.092
Khulna	0.017	0.17	0.046
Rajshahi	0.0188	0.19	0.083
Chittagong	0.0133	0.16	0.079

The experiments were performed in September 2004.



Scheme I

Figure 1. Chemical structure of markers.

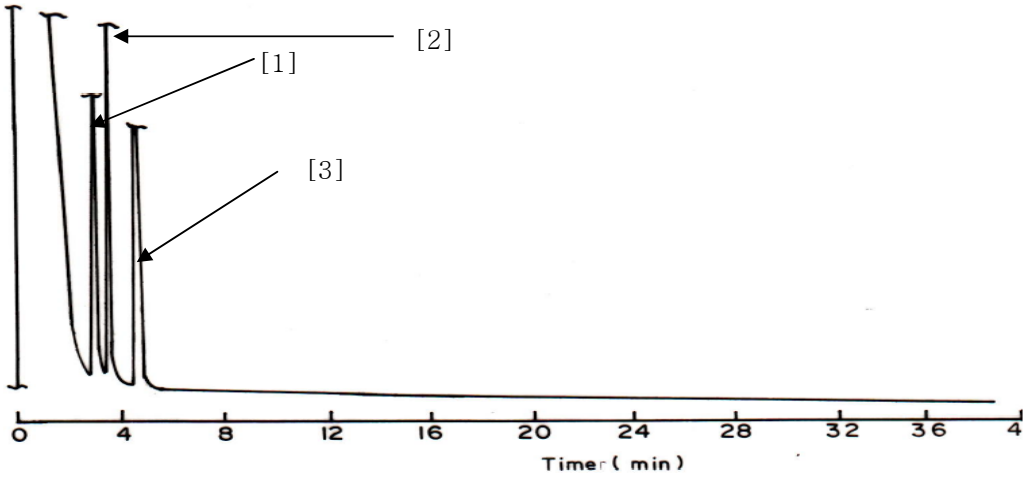


Figure 2. Chromatogram of the mixture of reference compounds, DES [1]; TRI [2] and DIHY [3].

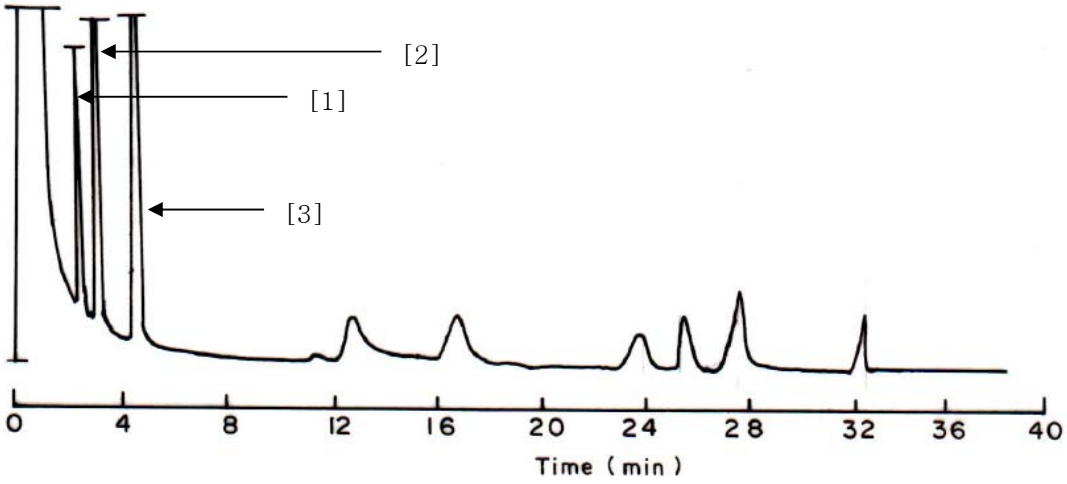


Figure 3. Typical chromatogram of the tea sample (Lipton brand, DES [1]; TRI [2] and DIHY [3]).

To assess the precision of these methods, standard solutions of DES, TRI and DIHY were determined six times on the same day and over a six-day period. The results showed very good precision, ranging from 1 to 100 µg/ml (Table 2). The accuracy of the method was evaluated through recovery studies. The recovery experiment was performed at three concentrations (1, 5 and 7.5 µg) of the standard added to sample solutions, in which the marker content had been determined, using the sample from Dhaka. The results for the recoveries of DES, TRI and DIH Y were in the range 62.82% to 89.04%. The limit of detection (LOD) of the GC-MS method, established at signals three times that of the noise for DES, TRI and DIHY were 4.0, 3.3 and 4.5 ng, respectively.

Table 2. Analytical characteristics of calibration curves for the marker flavonoids.

Constituent	Ret. Time (min)	Range (µg/ml)	Regression Equation	Detection Limit (ng)	R^2	Precision (%) (SD[a]) (n=5, 10ng/µl)	
						Inter-day	Intra-day
Desmodol (DES)	3.07	0.5 -10	0.1988x-0.3110	4.0	0.9999	0.74	0.32
Triangularin (TRI)	3.74	0.5 -10	0.0698x-0.0244	3.3	0.9987	0.79	0.48
2′,4′-dihydroxy chalcone (DIHY)	4.98	0.5 – 10	0.1220x+0.3653	4.5	0.9977	1.15	1.01

[a] SD = standard deviation.

The GC-FID procedure was applied to the determination of the markers in the tea samples from different regions. As shown in Table 1, all the analyzed samples showed no significant range in the concentrations of the markers, in samples from the same region and from different regions. The variation may be ascribed to environmental conditions and variation in sample sourcing. The tea samples selected for this experiment were of similar size, fresh weight and dry weight; however, the chemical composition of the tea samples could be affected by the soil fertility levels and age [34]. The values obtained for the markers appear to fall within the ranges previously reported for tea samples [35, 36, 37, 38]. However, the overall levels of the marker concentration were markedly higher in samples from Sylhet. TRI was comparatively the main component found in tea samples at concentrations ranging from 0.16 to 0.31% of total dry skin weight. Concentrations of DES and DUHY ranged from 0.011 to 0.110%, and 0.046 to 0.106%, respectively (Table 1). A literature search, to identify the unknown peak at 25.9 min in the GC finger-print, showed that it might be tetramethylscutellarein [39]. The GC results showed that the relative concentrations of the markers varied considerably. Based on this observation, the antioxidant activities of the extracts were evaluated to assess the effect of the variation of the phenolic content on the anti-oxidative potential.

Antioxidant activity

The total phenols analysis of tea samples from different locations are given in Table 3. The results showed that the total phenolic content of the methanol extracts varied from 7.14 mg (Khulna) to 9.71 mg caffeic acid/g dry weight (Sylhet). There was no significant difference between the means of the methanolic extracts from different locations with respect to total phenolic contents, as the values are almost identical for a number of cases. The test used to evaluate the potency of the extracts as antioxidants is a well-established model system; based on β-carotene coupled with auto-oxidized linoleic acid, there was a gradual decrease at A470 with β-carotene bleaching. The decrease in absorbance of β-carotene in the presence of methanolic extracts of the tea samples from eight different localities, with the oxidation of β-carotene and linoleic acid, is also shown in Table 3. A variation in antioxidant activities ranging from 62.82% (Chittagong) to 89.04% (Sylhet) was observed. The antioxidant activity, calculated from data of all the extracts tested in relation to one another can be seen in Table 3. Antioxidant activities of the tea samples extracts tested using the β-carotene bleaching method decreased in the order Sylhet>Pabna>Khulna>Dhaka>Comilla> Kushtia>Rajshahi>Chittagong.

Table 3. Radical scavenging activity and total phenolic contents of methanolic extracts of the tea samples.

Location	Total phenolics (mg g^{-1}dry wt)	Antioxidant activity (%)
Kushtia	9.32	76.98
Pabna	7.44	88.68
Dhaka	8.88	87.38
Comilla	9.11	83.75
Khulna	7.14	88.49
Rajshahi	9.22	76.88
Chittagong	9.36	62.82
Sylhet	9.71	89.04
BHA[a]	-	58.4
Quercetin[a]	-	72.3
Control		0.00

Data are expressed as means of triplicate measurements.
The experiments were performed in September 2004.
[a]Reference.

Flavonoid acids have been implicated as natural antioxidants in plants, fruit and vegetables. Lipophilic flavone derivatives were identified in the tea samples and quantified by GC-FID. Lipophilic flavones were the predominant phenolics in the methanol extract (Table 1) and their antioxidant properties have been well documented [40, 41]. However, there was no relationship found between the antioxidant activity and the total phenolic content (r_{xy} = 0:048), on the individual phenolics DES (r_{xy}= 0:003), TRI (r_{xy} = 0:044) and DIHY (r_{xy} = 0:052). The almost similar results on relationship between antioxidant activity of plant extracts and phenolic content have been previously reported [42, 43]. Antioxidant activity of plant extracts is not limited to phenolics; the presence of different antioxidant components in the extracts such as sugars and other compounds that function as hydrogen donors, may erroneously contribute to the concentration of the total phenols determined with Folin–Ciocalteau reagent [44]. Therefore, there is no simple relationship between the concentration of total phenol and the antioxidant activity when comparing plant extracts.

Conclusions

The GC-FID chromatographic profiles of the tea samples collected from different locations were qualitatively similar but the results showed variations in the concentration of the markers, DES, TRI and DIHY. All the methanolic extracts of samples from different locations showed considerable variation in antioxidant activities, which could be ascribed to soil fertility levels, age of the plants and variation in sample sourcing. Though the antioxidant pattern is generally complex, thus making assessment of a plant extract based on compositional data difficult, the concentration of individual antioxidants in tea samples extracts determined by GC-FID is the preferred way to provide standardized information. The GC-FID finger-printing could be used in authentication of tea samples and formulations. Radical scavenging activity and in vivo antioxidant studies of the tea samples are being investigated in the laboratory of the Department of Pharmacy, Dhaka University, Bangladesh.

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