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# The Influence of Heat Treatment on Interaction of Green Tea Flavonoids with Milk Proteins

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**Abstract:** Several heat treatment norms are applied in the dairy industry during the manufacture of various products. The knowledge on the influence of temperature on the proteins-flavonoid binding is critical for optimization of process conditions. The aim of this study was to establish the effect of heat treatment on the interaction between milk proteins and flavonoids and also determine when to apply heat treatment. Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis revealed that free flavonoids decreased in the presence of milk proteins and a binding ratio (%) was calculated based on this decrease. Three different heat treatment norms were selected (80, 85 and 90 °C × 10 min). Analyses were carried out in both sodium caseinate and skimmed milk samples to observe the possible effect of milk serum proteins on the interaction. The binding ratio between flavonoids and proteins was found to be higher in the samples where green tea extract (GTE) was added before heat treatment than in the samples where GTE was added after heat treatment in both the skimmed milk and sodium caseinate systems. Results of protein surface hydrophobicity (PSH) index and protein partition also demonstrated that heat treatment must be applied after the addition of GTE to improve the amount of GTE binding to milk proteins.

**Key words:** Flavonoids, milk proteins, green tea, heat treatment, RP-HPLC.

## 1. Introduction

Tea (*Camellia sinensis*), is the second most consumed beverage in the world, well ahead of coffee, beer, wine and carbonated soft drinks [1]. The most common flavonoids in tea are the flavan-3-ols (flavanols or flavans), which are present in relatively large amounts in tea compared to their levels in other foods. The flavan-3-ol subclasses are ranked by degree of polymerization. Tea catechins are monomers and these form 20%-30% of the dry weight of green tea. The major catechins in fresh tea leaves and green tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC) [2]. The catechins represent nearly 80% of the total flavonoid content of green tea. With epidemiological and biological data supporting a potential protective role for tea and tea catechins, development of new products with tea as active ingredients has expanded [3].

Polyphenols have a significant affinity for proteins that lead to the formation of soluble complexes, which can grow in size and even form sediment. Many authors have developed models to explain protein-polyphenol complexes formation and precipitation [4-10]. Most of these models propose that protein-polyphenol complexes are formed by non-covalent interactions such as hydrogen bonds, hydrophobic interactions and van de Waals attractions between amino acids side chains and polyphenol aromatic rings. Sometimes these interactions could be complemented by hydrogen bonding, which could play an important role in reinforcing and stabilizing complexes. Additionally, each polyphenol is able to bind more than one protein, acting as a linker between two proteins [11].

Milk proteins have a common interest related to

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their cross-linking affinity and its effect on the texture as well as their nutritious properties. The reactions of milk proteins with some functional groups of other compounds in food have gained increasing attention in food research. The major milk proteins, caseins, have a micellar structure formed mainly via electrostatic interactions, and that is the main result of hydrophobic characters of casein fractions. The casein fractions are $\alpha_{s1}$-, $\alpha_{s2}$-, β- and κ-caseins, of which the molar ratio is about 4:1:4:1, respectively. Caseins are hydrophobic; they have a fairly high electrostatic charge, many prolines and a few cystine residues [12]. It is well-known that proline residues of proteins represent the primary sites for the binding of several polyphenolic compounds [13]. Caseins, having high proline content, show a tendency to react with other proteins and polyphenolic substance as well as other ligands [14]. The interaction between catechins and specific milk proteins has been studied including α-, β- and κ-casein and albumin. In general, these studies conclude that ionic, hydrophobic and hydrogen bonding forces are all important factors in catechin-protein interaction. Gallated catechins such as EGCG and ECG have previously been shown to have the strongest association with milk protein [3]. There are some studies on the formation of milk β-lactoglobulin-tea polyphenol complexes and the interaction of milk α- and β-caseins with tea polyphenols [15, 16]. EGCG binds to a wide variety of proteins, especially to nonglobular extended proteins, and particularly to proteins with a high content of proline. One such protein is β-casein, the second most abundant protein in milk [17]. The importance of the interaction between green tea catechins and proteins may have resulted from possible alterations of their bioavailability and functionality [18].

Green tea flavonoids are a major subject of studies related to the functional food area and milk is well accepted as a suitable vehicle in producing novel functional foods. Therefore research on the interaction of flavonoids with milk proteins comes to the forefront of food science. On the other hand, heat treatment is an essential step in the dairy industry for the production of various products. It is known that heat treatment can cause several structural changes in the protein molecules and these changes have been reported to affect the protein-flavonoid interactions [19-23]. The possible effect of whey proteins was also examined by using sodium caseinate and skimmed milk. Protein surface hydrophobicity (PSH) determined by using 1-anilinonaphthalene-8-sulfonic acid (ANS) as a fluorescence probe was another assay to observe changes in the hydrophobic character of milk proteins in the presence of green tea flavonoids.

The aim of this study was to contribute to the studies on the interaction of green tea flavonoids with milk proteins in terms of binding ratio by using reverse-phase high-performance liquid chromatography (RP-HPLC) and to determine the influence of heat treatment on the binding and the most efficient application stage of the heat treatment.

## 2. Materials and Methods

### *2.1 Preparation of Green Tea Extract (GTE) and Standards of Green Tea Flavonoids*

Decaffeinated GTEs were obtained from the GreenSelect ®, Indena (Italy). A GTE solution was prepared daily by dissolving GTEs in deionized water to concentrations of 0.1, 0.5 and 1.0 mg/mL.

Pure standards of green tea flavonoids [(+) catechin hydrate (#C1251), (-) epicatechin (#E4018), (-) epicatechin gallate (#E3893), (-) epigallocatechin (#E3768), (-) catechin gallate (#C0692), (-) epigallocatechin gallate (#4268), (-) gallocatechin gallate (#G6782)] were purchased from Sigma (Germany). All standards were dissolved in methanol and dilutions were performed with an acetic acid solution in deionized water (2%) to ensure the stability of green tea flavonoids [24].

*2.2 Preparation of Milk Protein Systems*

Sodium caseinate (Sigma, #C8654, Germany) solution was prepared by dissolving in deionized water to a concentration of 2.8%. Raw skimmed milk samples (S) were obtained from a local dairy (Atatürk Forest Farm Dairy Plant, Ankara, Turkey). GTE solution was added to sodium caseinate at given concentrations (0.1, 0.5 and 1.0 mg/mL) and added to raw and heat-treated skimmed milk samples at given concentration (1.0 mg/mL) (Table 1).

The total protein content of the samples was determined using the Bradford method [25].

*2.3 Determination of Green Tea Flavonoids by RP-HPLC*

Green tea flavonoids were determined using RP-HPLC [26]. The RP-HPLC analysis of green tea flavonoids was performed on an Agilent 1100 series HPLC system consisting of a quaternary pump (Agilent, G1311A), a manual injection block (Agilent, G1328B), a variable wavelength UV-detector (Agilent, G1314A), a column thermostat (Agilent, G1316A) and a degasser (Agilent, G1379A). The equipment was controlled using Agilent ChemStation software, which controlled the solvent gradient, data acquisition and data processing. The separation was performed with a silica-based C-18 RP-HPLC column (Thermo Hypersil ODS, 250 mm length × 4.6 mm i.d., particle size 5 μm and pore size 120°A).

Separations were performed using solvent A (2% of aqueous acetic acid in deionized water) and solvent B (acetonitrile) with a column temperature of 35 °C, a flow rate of 1.0 mL/min, and a detection wavelength of 280 nm. Sample solutions (20 μL) were injected onto the column and then subjected to a solvent gradient that started at 8% solvent B and then increased linearly to 30% solvent B over 25 min. The solvent composition was then returned to the initial conditions in 2.0 min.

Carrez clarification was carried out to determine the flavonoid content of samples including protein using RP-HPLC. Carrez I solution was prepared by dissolving 15 g of potassium hexacyanoferrate in 100 mL of water and Carrez II solution was prepared by dissolving 30 g of zinc sulfate in 100 mL of water. The acetic acid solution was added to the samples at a final concentration of 2%, and then aliquots of Carrez

**Table 1 Sample codes and preparation of the samples.**

| Sample codes | Preparation |
|---|---|
| F | 1 mg/mL green tea extract (GTE), non-heated |
| F80 °C | 1 mg/mL GTE, heat treated at 80 °C for 10 min |
| F85 °C | 1 mg/mL GTE, heat treated at 85 °C for 10 min |
| F90 °C | 1 mg/mL GTE, heat treated at 90 °C for 10 min |
| Cn + F | Sodium caseinate containing 1 mg/mL GTE, non-heated |
| (Cn + F)80 °C | Sodium caseinate containing 1 mg/mL GTE, heated at 80 °C for 10 min |
| (Cn + F)85 °C | Sodium caseinate containing 1 mg/mL GTE, heated at 85 °C for 10 min |
| (Cn + F)90 °C | Sodium caseinate containing 1 mg/mL GTE, heated at 90 °C for 10 min |
| Cn80 °C + F | Heat treated sodium caseinate at 80 °C × 10 min + GTE (final concentration is 1 mg/mL) |
| Cn85 °C + F | Heat treated sodium caseinate at 85 °C × 10 min + GTE (final concentration is 1 mg/mL) |
| Cn90 °C + F | Heat treated sodium caseinate at 90 °C × 10 min + GTE (final concentration is 1 mg/mL) |
| S + F | Skim milk containing 1 mg/mL GTE, non-heated |
| (S + F)80 °C | Skim milk containing 1 mg/mL GTE, heat treated at 80 °C for 10 min |
| (S + F)85 °C | Skim milk containing 1 mg/mL GTE, heat treated at 85 °C for 10 min |
| (S + F)90 °C | Skim milk containing 1 mg/mL GTE, heat treated at 80 °C for 10 min |
| S80 °C + F | Heat treated skim milk at 80 °C × 10 min + GTE (final concentration is 1 mg/mL) |
| S85 °C + F | Heat treated skim milk at 85 °C × 10 min + GTE (final concentration is 1 mg/mL) |
| S90 °C + F | Heat treated skim milk at 90 °C × 10 min + GTE (final concentration is 1 mg/mL) |

I (100 μL) and Carrez II (100 μL) were added to 1 mL samples and mixing using vortex. The precipitate was pelleted by centrifugation at 3,000× g and 20 °C for 15 min. The clear supernatant was filtered through an RC filter (0.45 μm, Sartorius, GmbH, Germany) before RP-HPLC injection.

*2.4 Determination of Optimum Interaction Time between Green Tea Flavonoids and Milk Proteins*

Concentrations of 0.1, 0.5 and 1.0 mg/mL GTE were examined in sodium caseinate system to estimate optimum interaction time during 0, 1, 2, 3, 4, 5 and 10 h and to determine suitable concentration of GTE in order to obtain the maximum interaction level between green tea flavonoids and milk proteins.

Significant decreases in the peak areas of the green tea flavonoid in the presence of milk proteins indicated the formation of the interaction between green tea flavonoids and milk proteins. Percentage of binding (%) was calculated as follows:

$$\text{Binding (\%)} = [(1 - F_{(\text{GTE + protein})}/F_{\text{GTE}})] \times 100 \quad (1)$$

where $F_{(\text{GTE + protein})}$ is the concentration of flavonoids in the GTE and caseinate mixture (ppm), $F_{\text{GTE}}$ is the concentration of flavonoids in the GTE (ppm).

*2.5 Determination of the Effect of Heat Treatment on the Flavonoid-Protein Binding*

Heat treatment was applied for 10 min at 80, 85 and 90 °C to determine the effect of temperature on the binding between green tea flavonoids and milk proteins. GTE was added to the milk protein systems before and after heat treatment to determine the most suitable application method for binding. Given heat treatment conditions were also applied to GTE (as such, without protein) to observe possible changes in green tea flavonoids. GTE was added to raw milk and sodium caseinate that was adjusted to the same protein concentration. Green tea flavonoids were determined using RP-HPLC, PSH measurements and protein partition analyses were carried out in all samples.

*2.6 PSH Measurements*

The relative fluorescence intensity of the samples was measured using a Perkin Elmer Model LS50B spectrofluorimeter (UK) with a normal glass cell, at λex = 390 nm, λem = 480 nm. ANS (Merck Cat. No. 10762, Germany) was used as the fluorescent probe. Titration of the protein solutions with increasing concentration of ANS provides information on both the hydrophobic number and affinity of the binding sites. Before ANS titration, the fluorescence of the samples was measured as a blank. Milk samples or caseinate solutions do not show fluorescence or have the lowest level of fluorescence alone whereas "ANS-protein complex" has a remarkable fluorescence.

Kinetic data were obtained from ANS titration curves (not shown). All values were calculated as the average of four different kinetic approaches; Lineweaver-Burk, Hanes-Woolf, Eadie-Hofstee and Michaelis-Menten [27]. PSH is protein surface hydrophobicity index and it was calculated as:

$$\text{PSH} = F_{\max}/(K_d * P) \quad (2)$$

where $F_{\max}$ denotes the number of hydrophobic sites, $1/K_d$ gives the binding affinity of ANS to the protein and $P$ represents protein concentration (g/L).

*2.7 Protein Partition Analysis*

Protein partition assay was carried out according to Erdem (2000) [27]. A 9 mL sample containing milk protein was centrifuged at 10,000× g and 4 °C for 45 min. The precipitate was dispersed in 3 mL phosphate buffer (50 mM pH 6.8). The total protein content of the aliquots taken from the supernatant and dispersed precipitate was determined using the Bradford method [25]. The proportion of protein content of precipitate to supernatant was used as protein partition values.

*2.8 Statistical Evaluation*

Experimental data were analyzed by analysis of

variance and correlations between parameters. All the data in this study were processed by the SPSS® 22 Windows software (IBM, SPSS Inc., Chicago, IL, USA).

## 3. Results and Discussion

### *3.1 The Concentrations of Flavonoids of GTE*

The concentrations of green tea flavonoids [(+) catechin hydrate (C), (-) epicatechin (EC), (-) epicatechin gallate (ECG), (-) epigallocatechin (EGC), (-) catechin gallate (CG), (-) epigallocatechin gallate (EGCG), (-) gallocatechin gallate (GCG)] determined using the peak areas in the RP-HPLC chromatograms (Fig. 1a) are given in Table 2.

### *3.2 Flavonoid-Protein Binding in Sodium Caseinate System*

In order to obtain the interaction stoichiometry of green tea flavonoids with milk proteins, different concentrations of GTEs (0.1, 0.5 and 1.0 mg/mL) were

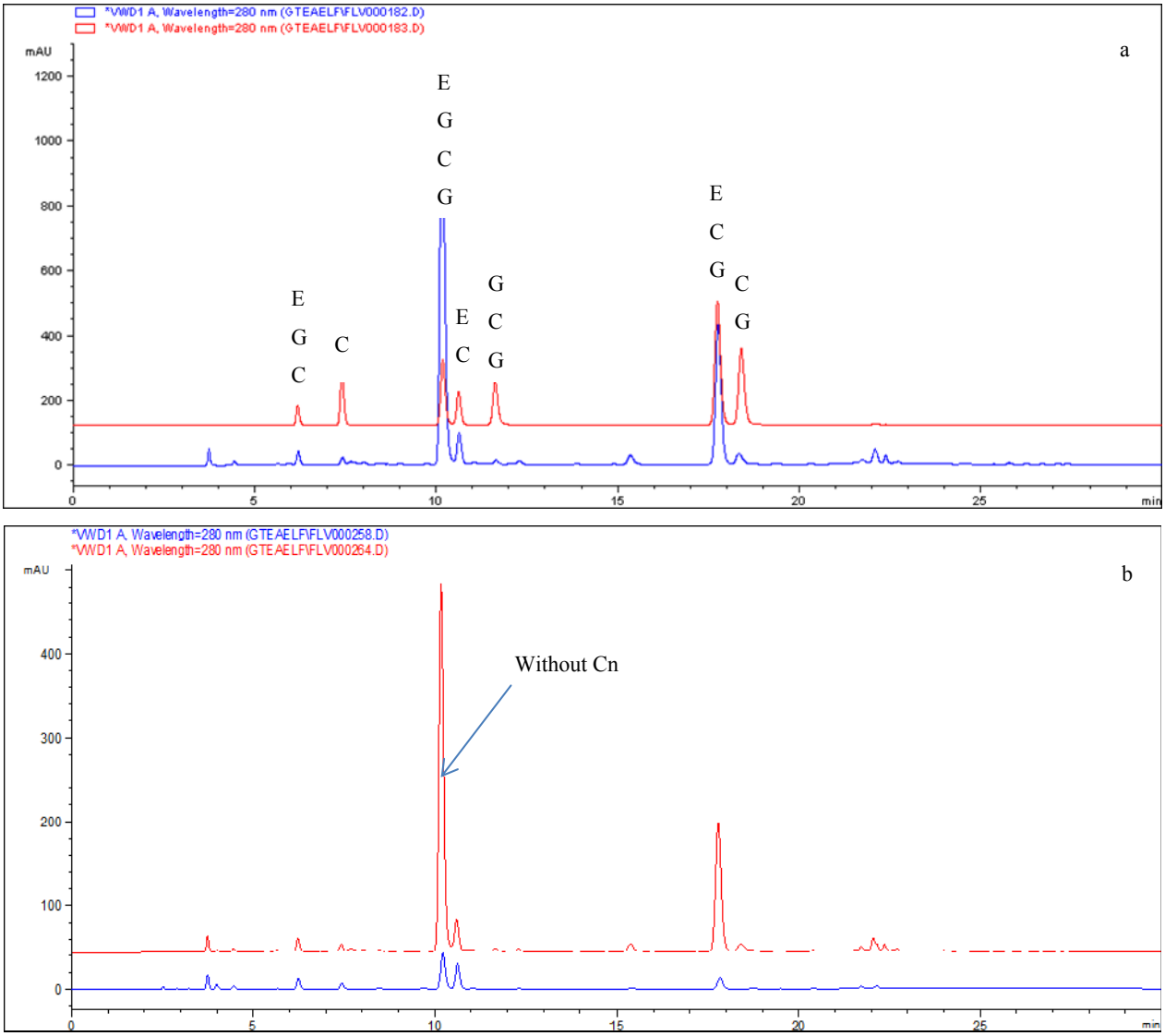


**Fig. 1 Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatograms of green tea flavonoids standards (red) and green tea extract (GTE) (blue) (a) and GTE with and without sodium caseinate (Cn) (b).**

Concentrations of EGC: (-) epigallocatechin, C: (+) catechin hydrate, EGCG: (-) epigallocatechin gallate, EC: (-) epicatechin, GCG: (-) gallocatechin gallate, ECG: (-) epicatechin gallate, CG: (-) catechin gallate are 100, 100, 50, 100, 50, 100 and 50 ppm, respectively, in the mixture of green tea flavonoids standards.

**Table 2 Flavanoid contents of decaffeinated GTE* (1.0 mg/mL).**

| Flavanoid | C | EC | ECG | EGC | CG | EGCG | GCG | Total flavanoid |
|---|---|---|---|---|---|---|---|---|
| Concentration (ppm) | 16.9 ± 0.2 | 89.5 ± 0.5 | 74.4 ± 0.3 | 57.8 ± 0.3 | 11.2 ± 0.2 | 189.7 ± 0.6 | 4.8 ± 0.2 | 444.4 |
| F (%) | 3.8 ± 0.1 | 20.1 ± 0.3 | 16.8 ± 0.3 | 13 ± 0.2 | 2.5 ± 0.2 | 42.7 ± 0.1 | 1.1 ± 0.1 | 100 |

EGC: (-) epigallocatechin, C: (+) catechin hydrate, EGCG: (-) epigallocatechin gallate, EC: (-) epicatechin, GCG: (-) gallocatechin gallate, ECG: (-) epicatechin gallate, CG: (-) catechin gallate.

*Values are means ± standard deviation; analyses were performed in triplicate.

added to sodium caseinate solution. RP-HPLC chromatograms of GTEs with and without sodium caseinate were given in Fig. 1b. Time-dependent changes of green tea flavonoids (1 mg/mL) at room temperature (21-24 °C) during 10 h are shown in Fig. 2a (concentrations of GTEs are not shown as the same trends were observed). Stability of green tea flavonoids at room temperature during 10 h was determined and this finding is compatible with the results of Chen *et al.* [28]. In brief, considering peak areas of the free flavonoids determined using RP-HPLC, it was envisaged that decreases in the peak areas of the flavonoids would result only from a flavonoid-protein interaction.

Significant decreases in the peak areas of the green tea flavonoids in the presence of sodium caseinate are shown in Fig. 1b ($p < 0.01$). Decreases in the peak areas of green tea flavonoids indicated the formation of links between GTE and milk proteins. Binding ratio values (%) of each green tea flavonoid are shown in Fig. 2b. Time-dependent changes of green tea flavonoids at room temperature are also shown in Fig. 2b.

The decrease in the content of green tea flavonoids, especially 90% reduction in EGCG and ECG, reveals the high-level interaction of green tea flavonoids and milk proteins. It was reported that the binding capacity of flavonoids increased with increasing polymerization degree [3, 15, 16]. Flavonoids containing gallate group have a higher binding affinity [5]. The interaction between green tea flavonoids and milk proteins was completed initially and no significant changes were observed during 10 h in the binding ratio values of all flavonoids especially for EGCG and ECG (Fig. 2b). These results have shown that there is no need for any time period in order for green tea flavonoids and milk proteins to interact and that also the binding is stable for 10 h.

Binding ratios of GTE flavonoids were evaluated for three different GTE concentrations at constant protein concentration (Fig. 3). No significant differences were observed in the binding ratio at the concentrations of 1.0 mg/mL and 0.5 mg/mL GTE ($p > 0.05$). The lowest binding ratio was obtained at the concentration of 0.1 mg/mL GTE especially for low polymerization degree of flavonoids. The binding ratio between GTE and milk proteins is approximately 10% in the sodium caseinate solution containing 0.1 mg/mL GTE. The binding ratio ranged between 40% and 90% in the presence of 1.0 mg/mL and 0.5 mg/mL GTE. The usual expectation was that the more the GTE concentration increased, the more the binding ratio would increase; however, it was exclusively seen in the results that the binding ratio for 0.5 mg/mL and 1.0 mg/mL GTE was almost the same. These unexpected results for the concentration of 0.1 mg/mL GTE may be explained by the solvation effect or increasing collision distance. It is thought that the interaction between flavonoids and proteins occurs adequately in the systems that GTE concentration is over a certain value and this interaction continues between proteins bonded with flavonoids as well. However, the interaction was at its lowest level in the system containing 0.1 mg/mL of GTE and resulted in the lowest binding ratio. The lowest binding ratio could possibly be explained by excess protein content and the higher distance between flavonoids and proteins. On the other hand, the same binding ratio values in

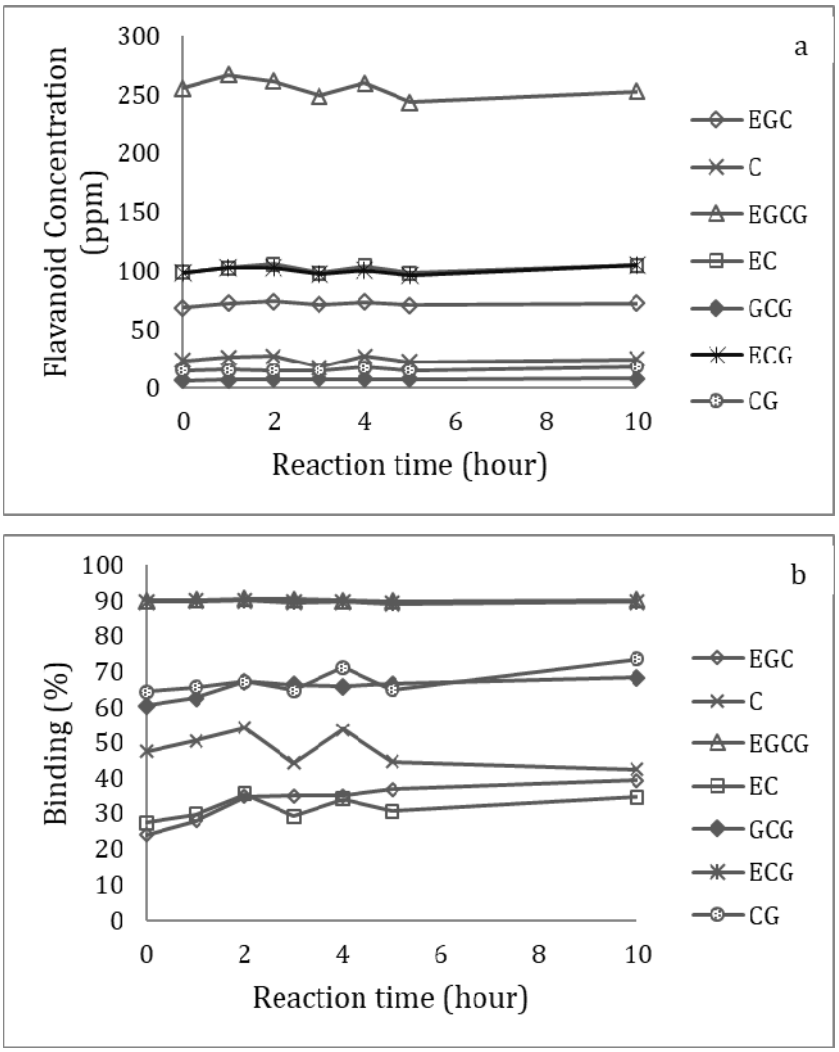


**Fig. 2 Variation of the concentration of free green tea flavonoids determined by RP-HPLC during the storage at room temperature (a) and the variation of binding ratios of each individual green tea flavonoids during the storage at room temperature (b).**

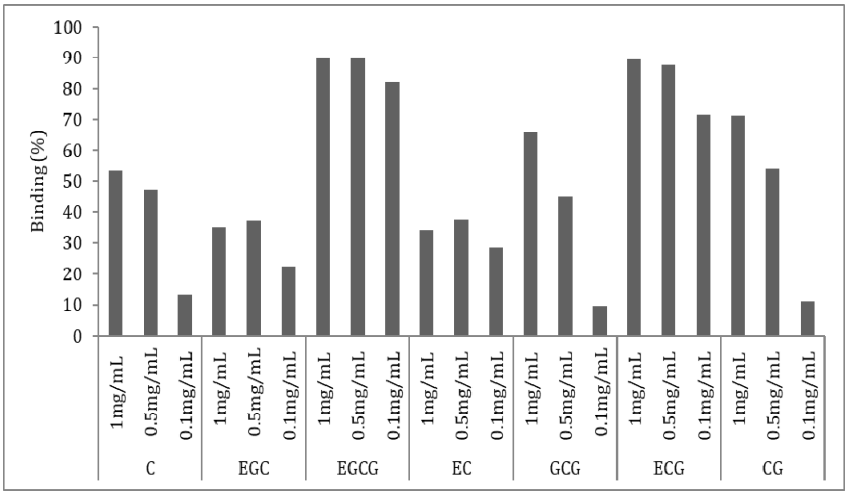


**Fig. 3 The effect of GTE concentration in the milk protein system on flavonoid-protein binding ratio.**

the systems containing 1.0 mg/mL and 0.5 mg/mL of GTE cannot be explained by these data. Further research needs to be carried out to discuss different binding models in this subject.

### *3.3 Effect of Heat Treatment on the Flavonoid-Protein Binding*

The addition stage of GTE to the milk protein systems was studied at three different heat treatment norms (80, 85 and 90 °C × 10 min). At first, GTE without protein was examined at these norms to investigate the effect of heat treatment on green tea flavonoids. It was found that heat treatment had no significant effect on flavonoids ($p > 0.05$) (not shown).

GTE was added to the milk protein systems before and after heat treatment. Same experimental conditions were applied in both the sodium caseinate-GTE and skimmed milk-GTE systems to also observe the possible effects of milk serum proteins. It was observed that flavonoid-protein interaction increased significantly when GTE was added before heat treatment ($p < 0.01$) (Figs. 4a and 4b). Especially for EGC and EC, the interaction increased with increasing temperature. As explained above, flavonoids containing gallate have a higher binding affinity. It was found that dependence of temperature is lower in the flavonoids containing gallate than in the flavonoids without gallate.

It was determined that the addition of GTE to the milk protein system before and after heat treatment has a significant effect on binding between green tea flavonoids and milk proteins. On the other hand, the addition of GTE to the heat-treated sodium caseinate and skimmed milk systems resulted in lower binding rates (Figs. 4c and 4d; total flavonoid content estimated using RP-HPLC). These lower binding rates were more significant for flavonoids without gallate although the same trend was observed for almost all flavonoids. However, flavonoid-protein interaction increased in all flavonoids especially in flavonoids without gallate when GTE was added before heat treatment. These results were possibly explained with decreasing of hydrophobic sites on the casein micelles caused by heat treatment [29]. In other words, casein micelles were transformed into a compact structure, and hydrophobic binding sites were masked.

It was thought that the presence of serum proteins would be a hint to understand the heat treatment effect. Contrary to expectations, serum proteins have no effect like masking the hydrophobic sites on casein micelles. It is thought that in an independent way from denatured serum proteins, decreasing of binding ratio due to heat treatment possibly results from more compact casein micelle structure resulting in masking of hydrophobic sites.

### *3.4 PSH Index and Protein Partition Results of GTE-Milk Protein Systems*

The characterization of binding interactions between green tea flavonoids and milk proteins was examined with the PSH measurement approach in the previous study [30]. In this study, the effects of heat treatments and the addition stage of GTE were also examined using PSH and protein partition assays to put forward the alterations of the hydrophobic character of casein micelles.

PSH values are given for sodium caseinate and skimmed milk in Figs. 5a and 5b, respectively. PSH index decreased in both the sodium caseinate and skimmed milk samples via interaction between the flavonoids and hydrophobic sites on the protein surface [30]. It was observed that the relationship between total binding and PSH was remarkable. PSH index decreased (Fig. 5a) with increasing binding ratio (Fig. 4c) especially in the sodium caseinate system. The protein system turned into a more compact system and the sites that ANS could bind blocked by flavonoid binding.

Results of protein partition are given in Fig. 5c. Protein content in the precipitate was increased with increasing flavonoid-protein interaction. The amount of precipitable protein in skimmed milk was lower than that in the milk samples containing GTE. This

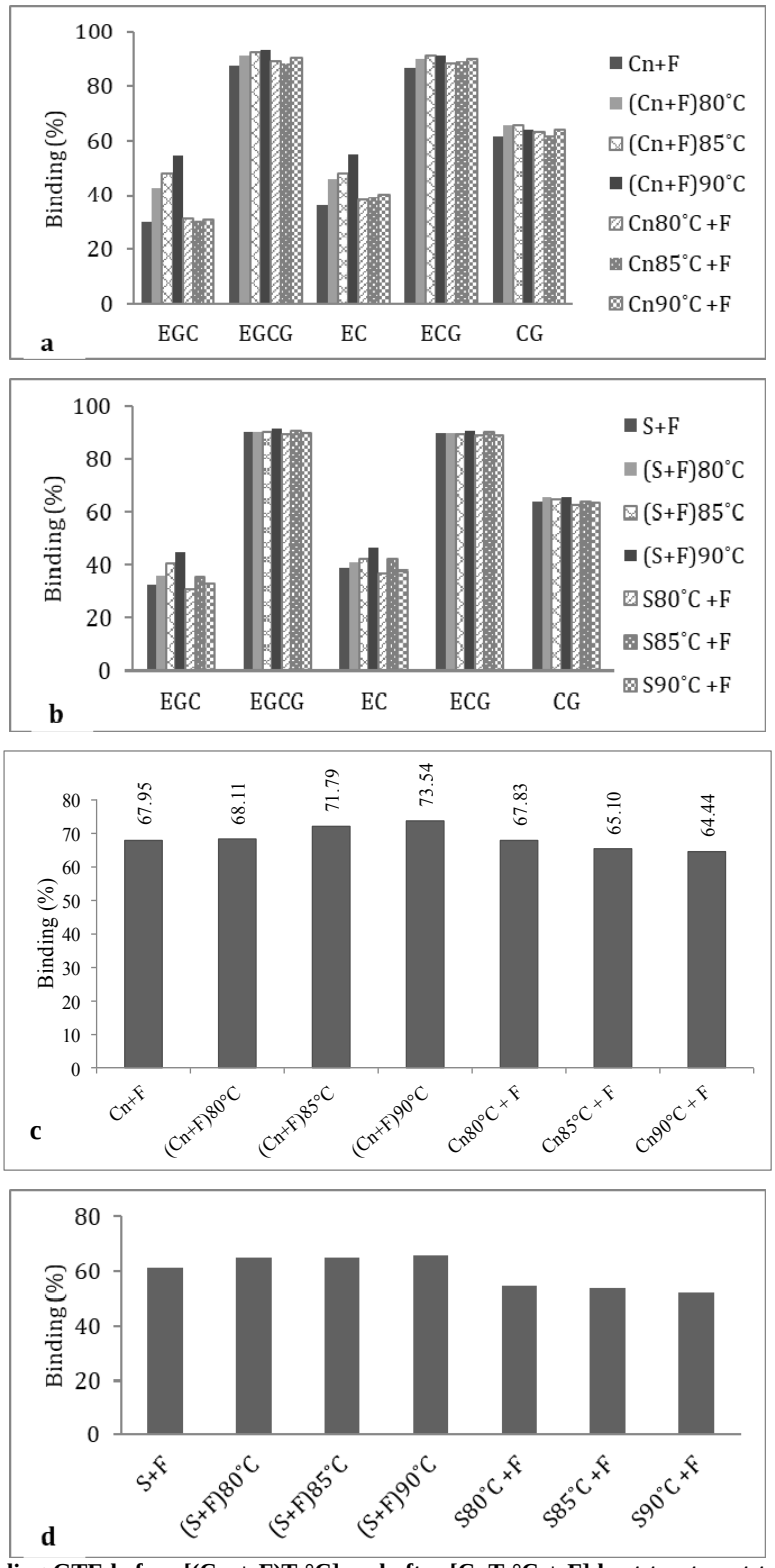


**Fig. 4 The effects of adding GTE before [(Cn + F)T °C] and after [CnT °C + F] heat treatment to the caseinate system (a) and adding of GTE before [(S + F)T °C] and after [ST °C + F] heat treatment to the milk system (b) at different heat treatment temperatures on the binding ratio. The effects of adding GTE before [(Cn + F)T °C] and after [CnT °C + F] heat treatment to the caseinate system (c) and adding of GTE before [(S + F)T °C] and after [ST °C + F] heat treatment to the milk system (d) at different heat treatment temperatures on the total flavonoid binding ratios of caseinate system.**

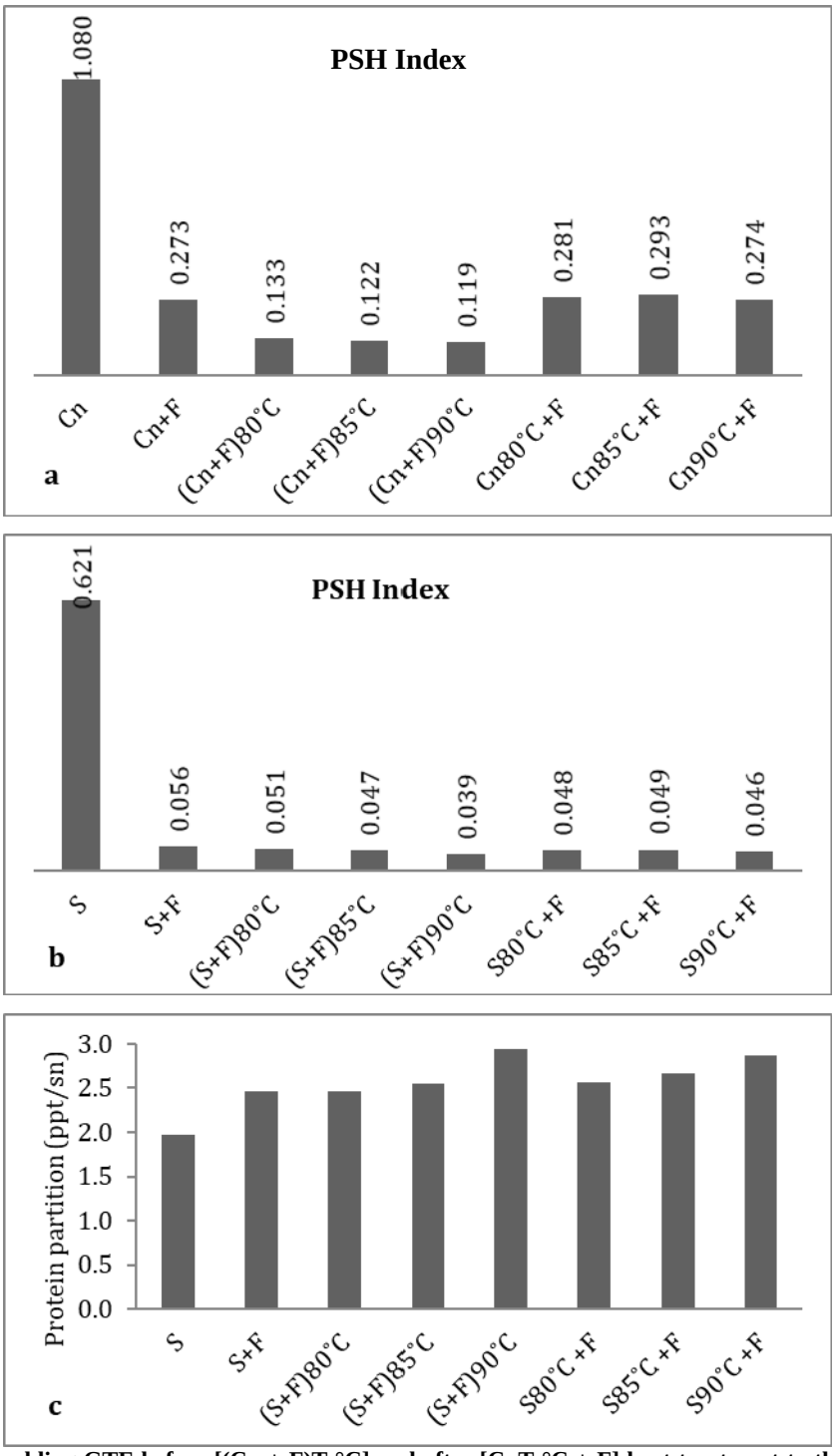


**Fig. 5 The effects of adding GTE before [(Cn + F)T °C] and after [CnT °C + F] heat treatment to the caseinate system (a) and adding of GTE before [(S + F)T °C] and after [ST °C + F] heat treatment to the milk system (b) at different heat treatment temperatures on protein surface hydrophobicity (PSH) index in the caseinate system, and changes of protein partition in skim milk system (c ).**

result showed that the formation of precipitable protein increased when binding between proteins and flavonoids formed. The addition of GTE to the protein system before heat treatment resulted in more protein precipitation, which in turn resulted from more flavonoid-protein binding. It was also found that the

amount of precipitable protein increased with increasing heat treatment norms.

## 4. Conclusions

Results of PSH index, protein partition and binding rates, which were calculated by using RP-HPLC, were found to be compatible with each other. These results demonstrate that heat treatment must be applied after the addition of GTE to improve the GTE amount binding to milk proteins. From food science and technology points of view, flavonoids are important compounds that influence both the quality and stability of food products. This study presents a holistic approach to examine the usage of functional compounds such as green tea flavonoids in dairy products. It is suggested that the binding of milk proteins by green tea flavonoids, or catechins, can be used for manufacturing of novel milk products. Therefore, interactions between green tea flavonoids and milk proteins and the effect of heat treatment on the interaction must be considered in this regard. Furthermore, the addition of green tea flavonoids to milk may affect the functionality of milk proteins. In further studies, these effects will be investigated in dairy products such as yoghurt and cheese.

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