INVESTIGATION OF THE EFFECTS OF GLUTEN-GREEN TEA PHENOLS INTERACTION ON IMMUNOGENIC GLUTEN PEPTIDES

GLUTEN-YEŞİL ÇAY FENOLLERİ ETKİLEŞİMİNİN İMMUNOJENİK GLUTEN PEPTİTLERİ ÜZERİNE ETKİLERİNİN İNCELENMESİ

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF GLUTEN-GREEN TEA PHENOLS INTERACTION ON IMMUNOGENIC GLUTEN PEPTIDES

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Celiac disease is an autoimmune disorder with the prevalence of 1% of the population and triggered by the consumption of gluten. Due to the high proline content of gluten, which makes it resistant to digestive enzymes, partial digestion results in the production of 33, 26, 19 amino acids-long gluten peptides. Subsequent to a series of reaction of gluten peptides, inflammation occurs through the epithelial cell wall. Therefore, gluten peptides produced as a result of partial digestion referred as 'immunogenic'.

Interaction between protein and polyphenols might take place through the covalent or non-covalent bonds. While the van der Waals interactions, hydrogen bonds, hydrophobic interactions are weaker than non-covalent interactions, covalent interaction takes place by electron pair sharing. Covalent interaction of protein with polyphenols occurs through the oxidation of polyphenols to electrophilic quinones and their subsequent binding to the protein via its amino, thiol side groups. Therefore, covalent interaction between protein

and phenol is stimulated under the alkaline condition which is suitable for the oxidation of polyphenols.

This study aimed understanding the effect of interaction of gluten with green tea extract (GTE) on immunogenic gluten peptides. For this purpose, gluten was treated for 2 and 3 hours with GTE under following conditions; 1% and 2% GTE concentration; pH 7 and pH 9; at 50 °C free to air exposure. Treatment of gluten with GTE was confirmed by monitoring the changes in total antioxidant capacity, free amino and thiol compounds, thermal stability. Gluten treated with GTE at pH 7 exhibited more radical scavenging activity than pH 9, indicating that the binding of GTE phenol to the gluten occurred through the thiol groups at pH 7, whereas through the amino side groups at pH 9.

Following the *in vitro* digestion of native gluten and gluten samples treated with GTE, bioaccessible fractions of modified gluten exhibited less antioxidant capacity than their initial content which might indicate the delivery of GTE phenolic compounds to the colon. Digestibility of gluten decreased with the treatment with 2% GTE at pH 9. Inhibition of gluten peptide release were provided by the treatment of gluten with GTE at pH 9, whereas treatment of gluten with GTE at pH 7 stimulate the release of immunogenic peptides. Considering the inhibition of 33-mer (57%), which is widely known immunogenic gluten peptide, the most effective gluten treatment parameters were found as 2% GTE concentration at pH 9 and 50 °C for 2 hours.

In this study, it has been also investigated how treatment of gluten with GTE affected the bread quality characteristics. For the preparation of breads, gluten treated under the most effective conditions with the lowest immunogenic peptide release was used. Texture profile analysis showed that the interaction of gluten with GTE decreased textural properties of bread. Therefore, the recipe was modified by using soy protein isolate and guar gum to improve the textural properties of bread prepared with modified gluten. The textural properties, porosity and browning indexes of bread prepared with gluten treated with GTE were improved becoming closer to the values of control bread, however, still was significantly different.

Keywords: Protein-phenol interaction, gluten, green tea extract, immunogenic gluten peptide, celiac disease, bread

ÖZET

GLUTEN-YEŞİL ÇAY FENOLLERİ ETKİLEŞİMİNİN İMMUNOJENİK GLUTEN PEPTİTLERİ ÜZERİNE ETKİLERİNİN İNCELENMESİ

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Çölyak hastalığı, popülasyonun %1'inde yaygın olarak görülen ve gluten tüketimi ile tetiklenen oto-immün bir hastalıktır. Gluten yüksek prolin içeriği nedeniyle sindirim enzimlerine dirençli hale gelmektedir. Bu nedenle glutenin kısmi sindirimi 33, 26, 19 amino asit büyüklüklerinde peptitlerin açığa çıkmasına neden olmaktadır. Gluten peptitlerinin bir dizi reaksiyonu, epitelyal hücre duvarında inflamasyon ile sonuçlanmaktadır. Bu nedenle, kısmi sindirim sonucu açığa çıkan gluten peptitleri, 'immünojenik' olarak kabul edilmektedir.

Protein ve polifenoller arasındaki etkileşim, kovalent veya kovalent olmayan bağlar yoluyla gerçekleşebilmektedir. Van der Waals etkileşimleri, hidrojen bağları, hidrofobik etkileşimler kovalent olmayan daha zayıf etkileşimler iken, kovalent etkileşimler elektron çifti paylaşımı ile gerçekleşmektedir. Proteinin polifenoller ile kovalent etkileşimi, polifenollerin elektrofilik kinonlara oksidasyonu ve ardından amino, tiyol yan grupları aracılığıyla proteine bağlanması yoluyla gerçekleşmektedir. Bu nedenle protein ve fenol

arasındaki kovalent etkileşim, polifenollerin oksidasyonu için uygun olan alkali koşullar altında arttırılmış olur.

Bu çalışmada glutenin yeşil çay ekstresi (YÇE) ile etkileşiminin immünojenik gluten peptitleri üzerindeki etkisinin anlaşılması amaçlanmıştır. Bu amaçla, gluten-YÇE etkileşimi 2 ve 3 saat boyunca %1 ve % 2 YÇE konsantrasyonu; pH 7 ve pH 9; 50ºC'de havaya maruz bırakılarak gerçekleştirilmiştir. Gluten-YÇE etkileşimi toplam antioksidan kapasite, serbest amino ve tiyol bileşikleri, termal stabilitedeki değişikliklerin izlenmesiyle doğrulanmıştır. pH 7'de YÇE ile muamele edilen gluten, pH 9'da etkileştirilene oranla daha fazla radikal süpürme aktivitesi sergilemiştir. Tiyol ve amino gruplarının pKa değerleri dikkate alındığında belirtilen pH'larda YÇE fenolünün glutene bağlanmasının pH 7'de tiyol grupları aracılığıyla gerçekleştiğini, pH 9'da ise amino yan gruplardan meydana geldiğini göstermiştir.

YÇE ile muamele edilen gluten örneklerinin ve doğal glutenin *in vitro* sindirimini takiben elde edilen biyo-yararlanılabilir fraksiyonların antioksidan kapasitesinin sindirim öncesi YÇE ile etkileştirilmiş glutenin antioksidan kapasitesine oranla daha düşük olduğu görülmüştür, bu da YÇE fenol bileşiklerinin sindirim sırasında erişilebilir hale gelmeden kolona ulaştığını göstermektedir. Ancak, pH 9'da % 2 YÇE ile yapılan etkileşim sonucunda gluten sindirilebilirliğinde azalma görülmüştür. Glutenin pH 7'de YÇE ile etkileşimi immünojenik peptitlerin salınımını teşvik ederken, pH 9'da YÇE ile etkileşim immunojenik gluten peptit salınımının inhibisyonunu sağlamıştır. Yaygın olarak bilinen immünojenik gluten peptidi olan 33-mer'in (% 57) inhibisyonu göz önüne alındığında, en etkili gluten-YÇE etkileşim parametreleri 2 saat boyunca pH 9 ve 50º C'de %2 YÇE konsantrasyonu olarak bulunmuştur.

Bu çalışmada ayrıca, gluten- YÇE ile etkileşiminin ekmek kalitesi özelliklerini nasıl etkilediği de araştırılmıştır. Ekmeklerin hazırlanmasında, en düşük immünojenik peptit salınımı için en etkili koşullar altında etkileştirilen gluten kullanılmıştır. Tekstür profili analizi, gluten-YÇE etkileşiminin ekmeğin tekstürel özelliklerini azalttığını göstermiştir. Bu nedenle modifiye gluten ile hazırlanan ekmeğin dokusal özelliklerini geliştirmek için formülasyon, soya proteini izolatı ve guar gam kullanılarak değiştirilmiştir. Bu sayede

YÇE ile muamele edilen gluten ile hazırlanan ekmeğin tekstürel özellikleri, porözite ve esmerleşme indeksleri kontrol ekmeğinin değerlerine yaklaştırılarak geliştirilmiştir.

Anahtar kelimeler: Protein-fenol etkileşimi, gluten, yeşil çay ekstraktı, immunojenik gluten peptitleri, çölyak hastalığı, ekmek.

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SYMBOLS AND ABBREVATIONS

Symbols

μ	Micro

Abbrevations

HMW-GS	High Molecular Weight- Glutenin Subunit
LMW-GS	Low Molecular Weight- Glutenin Subunit
HLA-DQ	Histocompatibility Leucocyte Antigens
MHC	Major Histocompatibility Complex
GS	Gluten Sensitivity
CD	Celiac Disease
IFN-γ	Interferon Gamma
TG	Transglutaminase
EGCG	Epigallo catechin gallate
EGC	Epigallo catechin
BLG	Beta Lactoglobulin
GTE	Green Tea Extract
PT	Persimmon Tannin
DPPH	2,2-diphenyl-1-picrylhydrazyl
TE	Trolox Equivalent
OPA	o-Phthalaldehyde
DTNB	5,5′-dithiobis-2-nitrobenzoic acid
DSC	Differential Scanning Calorimetry
SCFA	Short Chain Fatty Acid
Phe	Phenylalanine
Leu	Leucine
Ile	Isoleucine
Met	Methionine
Val	Valine
Trp	Tryptophan
Tyr	Tyrosine
Ala	Alanine
Ser	Serine
Gln	Glutamine
Asn	Asparagine
His	Histidine
Lys	Lysine
Arg	Arginine
Pro	Proline
DH	Degree of Hydrolysis
MR	Maillard Reaction
HILIC	Hydrophilic interaction chromatography
SSF	Simulated Salivary Fluid
SIF	Simulated Intestinal Fluid
SDF	Simulated Duodenal Fluid

TAC	Total Antioxidant Capacity

1. INTRODUCTION

Increasing numbers of patients with celiac disease and non-celiac gluten sensitivity have led to an increase in the consumption of gluten-free and low-gluten containing products recently. Due to the high proline content of gluten, which makes it resistant to digestive enzymes, partial digestion of gluten results in the production of immunogenic gluten peptides. Proline residues are prone to react with phenolic compounds in terms of protein-phenol interaction, on the other hand. Protein-phenol interactions might take place between proteins and phenolic compounds through covalent and non-covalent bonds. Under alkaline conditions, covalent interactions are stimulated through the addition of electrophilic quinones, which are produced during the oxidation of phenolic compounds, to the side chains of proteins such as amino and thiol compounds.

This study aims to stimulate the gluten-phenol interaction and investigate the possible effects on immunogenic gluten peptides. For this purpose, gluten was treated with green tea extract (GTE) under following conditions; 1% and 2% green tea extract concentration; pH 7 and pH 9; 2 hour and 3-hour treatment at 50^{o}C free to air exposure. Interaction between gluten and GTE was confirmed by monitoring the changes in the total antioxidant capacity, in the amount of amino and thiol groups, and in the thermal properties of the gluten. Following the *in vitro* digestion, digestive characteristics of native gluten and GTE-treated gluten samples were monitored through the changes in the antioxidant activity of bioaccessible fraction, degree of hydrolysis, total free amino acid content and gluten peptide content. Moreover, effect of interaction of gluten with GTE on bread characteristics such as texture profile, browning ratio and porosity were investigated.

2. GENERAL INFORMATION

2.1. Protein

Proteins are formed by the condensation reaction that takes place between the amino group of one amino acid and the carboxyl group of the other amino acid, resulting in peptides, oligopeptides, and polypeptides, respectively. The amino acid chain composed of 10 amino acids is called an "oligopeptide," >10 amino acids is called a "polypeptide," and >100 amino acids is called a "protein." The molecular masses of food proteins vary from 10 kDa (such as milk proteins) to 1 million kDa (such as wheat proteins). Polypeptides become unstable with the increase of the chain length as its free energy increases as well [1]. Polypeptide chains fold and create high order structures as a means of lowering this elevated free energy. These high order structures (secondary, tertiary, and quaternary) are often determined by the compositions of the amino acids in the primary structure, particularly by the amounts and distributions of hydrophilic (polar) and hydrophobic (nonpolar) amino acids. [1, 2]. For instance, two cysteine amino acids in a polypeptide chain can create a disulfide bridge and maintain protein structure. Hydrophobic amino acids also frequently interact with each other in aquatic environments [3]. Therefore, each protein has a distinctive, unique structure due to the content of the amino acids.

Since peptide bond is formed at C_α position of amino acid and bonds around C_α atom (C_α-C' and N- C_α), each peptide unit may rotate with different angles. N- C_α bond's rotation angle is called as phi (ϕ) and C_α-C' bond rotation angle is called as psi (ψ). Secondary structure is provided by the hydrogen bond between N-H (amid) group and C=O (carboxyl) group. In addition, in secondary structure, the rotation angles psi and phi are same between adjacent amino acid residues. α-helix and β-sheet are main motifs of secondary structures of proteins. α-helix structure is characterized by the formation of hydrogen bond between C=O residue and C-N residue of subsequent 4^{th} amino acid in the amino acid chain [3]. Proline does not have hydrogen, because its R-group is bonded

to N atom of amid group. Therefore, proline does not incline to hydrogen bond formation, provide steric hindrances to helices and so known as helices breaker [3]. Proline-rich proteins show random aperiodic structure [2]. In the β-sheet structure, hydrogen bonds are provided by arranging reciprocally the amino group of one peptide chain and the carboxy group of the other peptide chain [3].

Three dimensional tertiary structures of proteins are provided by hydrophobic, electrostatic, ionic, van der Waals interactions between different protein groups with secondary structure [1]. For example, when non-polar amino acid residues exposed to water, they avoid to interact with water and buried inside the molecule. This favors the hydrophobic and non-polar side chain interactions. In the case of interaction with water, the polar side chains are placed towards the surface of the molecule and participate in the hydrogen bond formation [2, 4].

When proteins contain subunits, various interactions and forces such as electrostatic, hydrophobic interactions, hydrogen bonding, and van der Waals forces encourage intermolecular and intramolecular folding. These interactions give proteins their quaternary structure [2]. Among other interactions, the disulfide bond is also effective in stabilizing the protein structure. A disulfide bond (S-S) is formed by the oxidation of free -SH groups (side chains of cysteine amino acids) in proteins by molecular oxygen. Disulfide bond might occur both in the same chain as intramolecular or between two chains as intermolecular [5].

2.1.1. Gluten

Gluten can be defined as the mass that remains as a result of removing starch and water-soluble components by washing the dough with water. Gluten consists of approximately 75% protein and 25% carbohydrates and lipids. Therefore, gluten is generally referred to as protein. Gluten proteins are unique in terms of glutamine, proline, hydrophobic amino acid contents, and properties provided by them. Glutamine accounts for 35% of gluten amino acid composition and 14% of gluten is proline. Due to the ring structure of the proline amino groups involved, proline cannot form an α-helix; therefore, proline act as

an α-helix breaker in gluten structure. In addition, hydrophobic amino acids (corresponding to 35% of gluten) increase the surface hydrophobicity of gluten and promote hydrophobic interactions. Due to the low charge density of gluten as a consequence of being poor in basic amino acids, the repulsion force within the protein is also low. This property encourages the interaction of proteins with each other. These aforementioned interactions between gluten proteins are effective in maintaining the stability of gluten structure, rheology and baking properties [6].

According to their solubility in alcohol-water solutions, gluten proteins are divided into two fractions; insoluble glutenin and soluble gliadin. The solubility of gluten proteins is associated with disulfide bonds. When disulfide bonds of insoluble glutenin fractions are reduced, produced subunits show solubility in aqueous alcohols [7]. Gluten proteins consist of 50-60% of gliadin, 40-50% of glutenin fractions [8].

When gluten hydrated and mechanical energy is supplied, gliadin and glutenin which present in flour form gluten polymer [9]. When hydrated, gliadin is extremely sticky and exhibit little or no resistance to extension and hence it is responsible for the dough's cohesiveness. On the other hand, glutenin has resistance to extension and gives dough elasticity [6]. Due to mechanical energy input during the mixing of the flour and water, the dough is subjected to uni- and biaxial deformations, and as a result, a continuous protein network is created via disulfide bond and thiol/disulfide interchange reactions. With the continuous energy input during kneading, protein interactions and covalent bond formation occur between them [9].

The balance between glutenin and gliadin provides unique viscoelastic property to dough and final products. Due to the metabolism of yeasts during fermentation, carbon dioxide is generated and the growth of air bubbles which are incorporated during mixing occurs. Kneading or punching during fermentation lead to release and distribution of these gas bubbles. Expanded and uniformly distributed gas bubbles determine the bread structure, its volume, porous structure, and texture [9]. Therefore, gluten network is crucial for retention of gas bubbles in the dough and the final characteristics of bread.

2.1.1.1. Glutenin

Glutenin consist of 20% HMW-GS (high molecular weight glutenin subunit) and %80 LMW-GS (low molecular weight glutenin subunit). HMW-GS's molecular weight is between 80-160kDa. HMW-GS consist of three structural domains; a non-repetitive N-terminal domain (A) which contain 80-100 residue (depends on being *x* or *y* type), a repetitive central domain (B) which contain 480-700 residues, a non-repetitive C-terminal domain contain 42 residues. They characterized by being rich in glutamine, proline, glycine, their total content is about 70% total amino acid residues. Therefore, it is considered that glutamine may stabilize the structure and interactions between subunits. LMW-GS have two domains; repetitive N-terminal domain and C- terminal domain. N-terminal domain of LMW-GS is rich in glutamine and proline, because contain repetitive units such as QQQPPFS.

2.1.1.2. Gliadin

Gliadins may be divided into α-, β-, γ-, ω- gliadins according to their mobility in electrophoresis, into ω5-, ω1,2-, α/β, γ-gliadins according to their amino acid compositions. While ω-gliadins' molecular weight range between 46-74kDa, other gliadins' molecular weight range between 30-45 kDa [8]. All gliadins have non-repetitive N and C terminal and repetitive central domain, but they differ in repetitive sequences in central domain. For instance, α/β- gliadins repeat dodecapeptides such as QPQPFPQQPYP for 5 times, γ-gliadins repeat heptapeptides such as QPQQPFP for 16 times [7, 8]. C-terminals of α/β- and γ-gliadins have 6 and 8 cysteines so that they have 3 and 4 intrachain disulphide bonds, respectively. ω-gliadins differ from other gliadins in terms of being rich in glutamine, proline, phenylalanine (because they consist of repetitive sequences such as PQQPFPQQ) but being poor in cysteine, therefore do not have disulphide bonds. At this point, the α-helix breaker property of proline amino acid affect secondary structures of gliadin proteins. Because N terminals of α/β- and γ-gliadins contain more proline and glutamine, they show β-reverse and β-turn structures like ω-gliadins. The rest C terminal domains of α/β- and γ-gliadins show α-helix and β-sheet structure [7].

2.2. Gluten Related Diseases

Wheat exposure (inhalation and/or digestion) is associated with many diseases such as IgE-mediated wheat allergy, gluten sensitivity and celiac disease. Unlike gluten sensitivity, wheat allergy and celiac disease are immune system-mediated diseases because T-cells play a role in inflammation occurrence. Wheat allergy is initiated by the recognition of wheat proteins by IgE antibodies and their crosslinking with IgE antibodies. These crosslinks of antibodies induce formation of mediators such as histamine, platelet activator factor, leukotrienes from mast cells and basophiles which cause allergenic inflammations [10-12]. Wheat can induce respiratory allergies (Baker's asthma) and food allergies [10-12]. Diagnostics of IgE-mediated wheat allergy are skin prick and wheat-specific IgE tests. According to epidemiologic studies, the prevalence of allergy to gluten or other wheat proteins varies between 0.2%-3.6% depending on country and age, with a higher prevalence in children [13]. Gluten sensitivity (GS) is generally defined as feeling distress after consumption of gluten containing foods. GS differs from both wheat allergy and celiac disease (CD) as it is not mediated by IgE antibodies and an immune response and being diagnosed by a negative wheat-specific IgE test and negative celiac disease serological test [10, 12].

2.2.1. Celiac Disease

Celiac disease is an autoimmune enteropathy which is triggered by ingestion of gluten in genetically susceptible individuals. CD has importance due to high consumption of gluten in many products, cause many clinical manifestations and have prevalence of 1% of general population. Classical clinical symptoms of CD include abdominal pain, bloating, vomiting, skin irritations. Additionally, CD is associated with many diseases such as malabsorption, malnutrition, osteoporosis, anemia due to destruction of intestinal mucosa, villi structure (villous atrophy) [14].

It is known that genetic factors have crucial role in CD, because HLA/DQ2 (Histocompatibility Leukocyte Antigens) gene are present in 95% of CD patients, and HLA/DQ8 gene are present in 5% of CD patients. Besides the genetic factors, environmental factors such as age of gluten introduction, breastfeeding, early infections, intestinal microbiota are also important to the onset of CD. Intestinal infections may result

in altered intestinal structure, increased intestinal permeability, and cytokine production, which all contribute to the pathogenesis of CD [15]. In addition, in viral infections, infection agents express proteins similar to protein structures produced by the host. These mechanism, called as molecular mimicry, lead to immune response and play role in CD pathogenesis [16]. Another environmental factor that affects CD is gut microbiota, which support the intestinal barrier and play role in tight junction formation by generating epithelial cells.

Normal protein digestion process in the gastrointestinal tract starts with the cleavage of proteins into smaller fragments by pepsin in stomach first and continues with the activity of pancreatic enzymes trypsin, chymotrypsin, carboxypeptidase in the small intestine. The trypsin cleaves the peptide bonds at the C-terminal of basic residues lysine and arginine; the chymotrypsin cleaves the peptide bonds from the aromatic amino acids like phenylalanine, tyrosine, tryptophan. These enzymes generate small peptides which contain 6-8 amino acids [17]. However, the high proline content and hydrophobic nature of gluten makes it more resistant to digestive enzymes. All these factors lead to production of peptides with different lengths during digestion. For instance, 18, 26, 33-long peptides are formed from gluten which are called as 18-mer, 26-mer, and 33-mer, respectively [17, 18]. These peptides are referred to 'immunogenic' because they contain certain sequences, which are called as epitopes that are recognized by HLA-DQ receptors [19]. Moreover, although proline cannot act as a hydrogen donor, multiple proline residues in epitopes provide selectivity filter for HLA-DQ receptor binding. In this way, gluten peptides bind to DQ molecules via hydrogen bonds [20]. It is known that there are 28 wheat epitopes and more than a hundred gluten peptides [19, 21]. The immunogenic characteristics of these gluten peptides vary according to the epitopes they contain and their numbers. In many studies, it has been stated that α-gliadin derived peptides cause more immune responses in celiac patients than other peptides. Although there are many immunogenic gliadin peptides, the α-gliadin derived 33-mer peptide stands out because it was the first identified and the most studied immunogenic peptide and it is immunodominant [21, 22]. This immunogenic 33-mer peptide present in the N terminal repetitive domain of α-gliadin and has molecular weight of 631.687g/mol, seven hydrogen bond donors, hydrogen bond acceptors [21]. In the amino acid sequence of the α-gliadin, 33mer, corresponds to a 57-89 amino acid residue range with sequence

LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF. This sequence contains six overlapping copies of three epitopes (PFPQPQLPY, PQPQLPYPQ and PYPQPQLPY) [23]. It is known that three epitopes (PFPQPQLPY, PQPQLPYPQ and PYPQPQLPY) of 33-mer peptide trigger T-cell proliferation and they are highly resistant to proteases, brush-border membrane enzymes and remain intact during digestion [24]. When gluten peptides reach intestinal epithelial barrier, they interact with zonulin, which regulates the permeability of tight junctions. Damage of permeability of tight junctions leads to transportation of gluten peptides into lamina propria [25]. Gluten derived peptides remain intact but inactive (non-immunogenic) until they reach lamina propria. When gluten derived peptides reached the lamina propria they become a good substrate for tissue transglutaminase (tTG) which catalyzes the deamination of glutamine amino acids because of being rich in glutamine. The TG makes the glutamine residues of gluten negatively charged by deamination. When the glutamine amino acids become negatively charged, have more affinity to HLA-DQ2/8, because HLA-DQ2/8 antigens favor binding polyproline II helical structure and negatively charged residues. The presentation of negatively charged gluten peptides to CD4+ T cells in the lamina propria by HLA molecules on antigen-presenting cells initiates the adaptive immune response which subsequent results in inflammation and intestinal mucosal damage (Figure 2.1) [25-27].

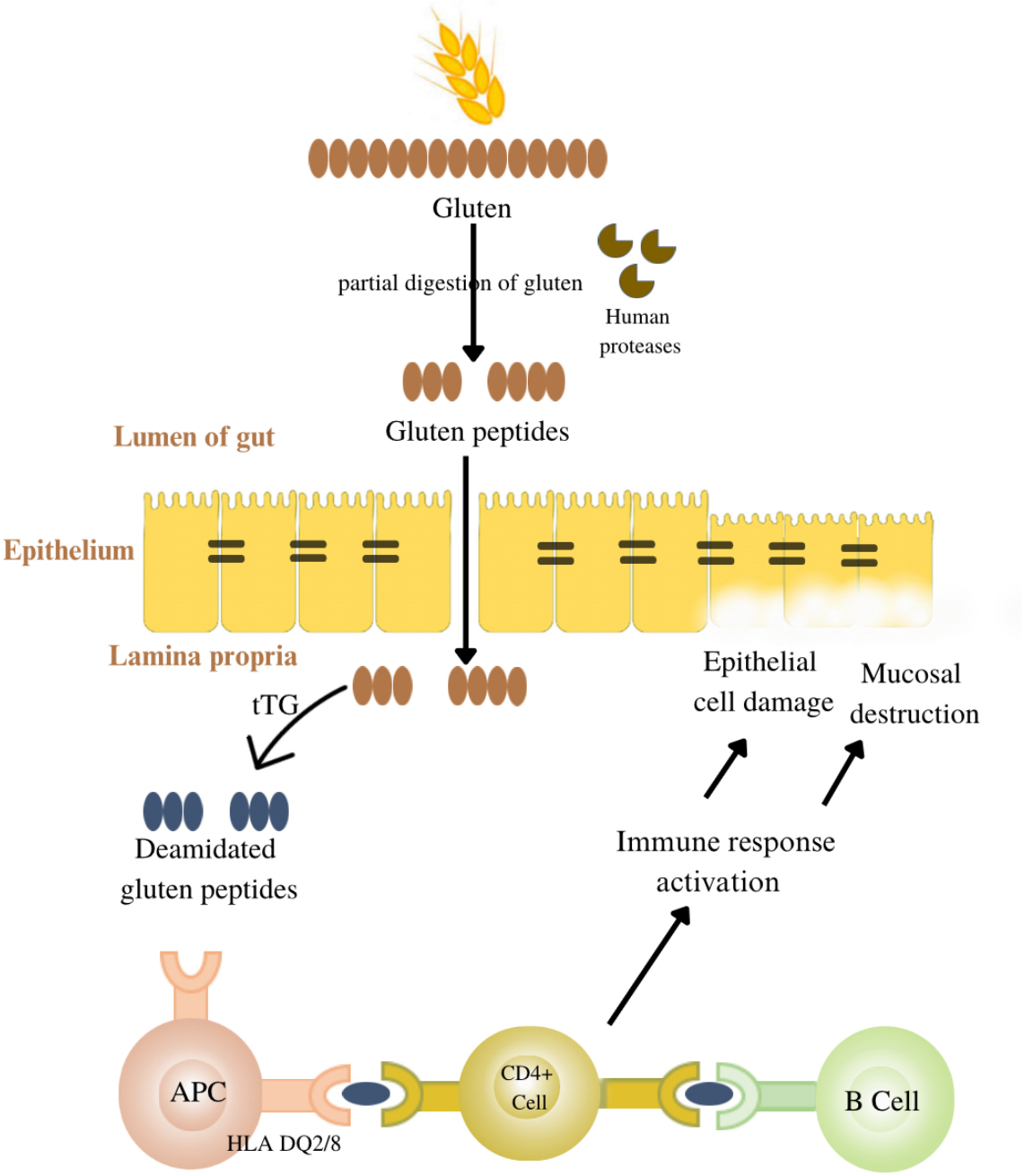


Figure 2. 1. Summary of celiac disease pathogenesis, adopted from [28, 29].

In digestive tract, enzymes cannot degrade gluten completely because of its high proline and glutamine content. For this reason, formulations or supplementations include microbial enzymes that cleaves the peptide bonds from proline and glutamine residues have been investigated in many works [30-32]. In addition, other microbial strategies aim supporting mucosal barrier, reducing immunogenic gluten peptide production by

digestion and thus reducing immune response. However, these microbial strategies are insufficient for the prevention of CD, and might be only complementary treatment [33].

Transglutaminase enzyme does not only catalyze the deamination of gluten derived peptide to glutamate such as in the case of CD pathogenesis, but also catalyzes crosslinking of glutamine and lysine residues. Therefore, using transglutaminase and mediating production of cross links is important to reduce gluten derived peptide levels and its immunogenic response. It has been reported that the TG–mediated transamidation with lysine or lysine methyl ester of gluten peptide or gliadin in alkaline conditions expressed less interferon, which is a cytokine that play role in inducing the adaptive an immune response, also, binding to DQ2 was reduced but not abolished [34]. On the other hand, Ogilvie et al. (2021), stated that the addition of TG to dough did not alter the formation of immunogenic gluten peptides [35]. Another strategy related to TG in respect to CD is its inhibition, which may prevent the deamination of gluten peptides. In a study, a chemical substance that inhibits TG is formulated as an oral capsule was administered for 6 weeks to patients with CD who consume 3 g of gluten daily. The treatment reduced the gluten-induced mucosal damage. However, when the dose of the substance was increased according to normal daily consumption of gluten, it showed side effects like headache, vomiting, and abdominal pain [36].

In literature, one of the common strategies to reduce immunogenic gluten peptides is sourdough fermentation. Sourdough fermentation includes lactic acid bacteria and various yeasts. Gluten hydrolysis in sourdough fermentation includes two pathways; first one is by the activation of endogenous flour enzymes due to acidification, second is by lactic acid bacteria proteases in starter culture [37]. Several works indicate that sourdough fermentation might be a strategy to eliminate/decrease gluten immunogenicity [37, 38]. However, complete degradation of gluten might be achieved by long fermentation process and combination of different starter cultures. Because, an individual microorganism is not capable of degrading all gluten peptides which contain resistant proline residues [38]. This was provided by another study of Ogilvie and Roberts (20021). They monitored the immunogenic gluten peptide content of both sourdough and conventional breads

commercially available in the market, and no difference was observed in these immunogenic gluten peptides content [37].

It is thought that processes which induce structural and conformational changes in gluten may alter its immunogenicity, antigenicity. Rahaman, Vasiljevic and Ramchandran (2016) investigated the effects of various pH (3 or 7), temperature (room temperature or 100 °C) and shear (0 or 1500 s−1) on digestibility and antigenicity of gluten and reported that temperature have remarkable effect on degree of hydrolysis of gluten, whereas pH and shear had no significant effect. Heat induced structural changes resulted in lower digestibility of gliadins and lower production of the potentially antigenic polypeptide fraction of gliadin [39]. Moreover, another process that results in heat-induced structural change might be microwave treatment. Lamacchia, Landriscina and Agnello (2016) expresssed that gliadins from microwave treated flour showed reduced antigenic capacity [40] whereas Mahroug et al. (2019) reported that microwave treatment of gluten resulted in increased amount of its potentially toxic epitopes released after digestion [41]. Because of these controversial results, microwave treatment cannot be an efficient solution for the patients suffer from CD. In conclusion, there is still a need for powerful strategies to eliminate immunogenicity of gluten.

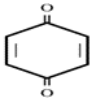
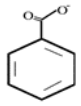
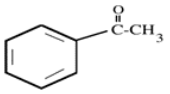
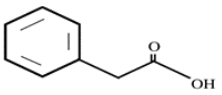
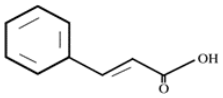
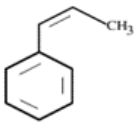
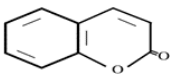
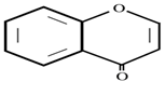
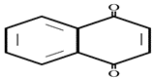
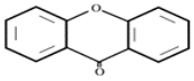
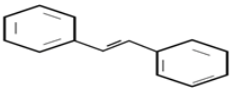
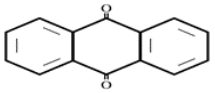
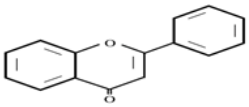
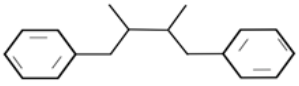
2.3. Phenolic Compounds

Phenolic compounds are secondary metabolites of plants. It is known that more than 10.000 phenolic molecules are present in nature and they are widely distributed in fruits, vegetables, legumes and beverages. Phenolic compound contents of fruits vary between 10-100 mg per 100 g of fruit, especially berries, lemon, kiwi rich in phenolic compounds. In addition, chocolate, coffee, green tea contain approximately of 350 mg, 190 mg, 80 mg phenolic compound per 100 g, respectively [42]. Phenolic compounds as secondary metabolites play a pivotal role in the defense mechanisms of plants under harsh conditions such as pathogen infections, high/low light exposure and temperature, and the presence of free radicals. In addition, phenolic compounds provide color and astringency and/or bitterness to fruits, vegetables, beverages etc. [43].

It is known that free radicals play a role in the formation of aging, cancer, diabetes, neurodegenerative and cardiovascular diseases in human. Phenolic compounds, on the other hand, are hydrogen donors thanks to the hydroxyl group in their structure, preventing the cycle of new free radical formation by quenching reactive oxygen species. Thus, they are associated with antioxidant, anti-cancer and anti-diabetes effects [44]. Phenolic compounds are characterized by one or more hydroxyl group attached to one or more aromatic rings. Phenolic compounds can be categorized according to their carbon skeleton which is shown in the Table 2.1 [45].

Tea has been one of the most consumed beverages in the world since ancient times and is produced from the plant *Camellia sinensis*. Tea beverages can be classified according to their production process; green (unfermented), white (lightly fermented), oolong (semi-fermented), or black (fermented). Green tea is produced by heating immediately after harvesting to inactivate polyphenol oxidase (PPO) enzyme, to prevent oxidation of tea phenolic compounds [46]. Therefore, green tea exhibit higher antioxidant activity compared to other teas [47]. Green tea phenolic compounds present up to 30% of the dry weight with especially flavanol, flavonol, phenolic acids [48, 49]. The major flavonoids of green tea are flavanols; epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin gallate. These flavonoid subclasses, flavanols, account for 70% of total phenolic content and provide 92% of antioxidant activity of green tea [49]. The presence of hydroxyl groups in positions 3' and 4' of the B ring, and free hydroxyl groups in rings A and C and a double bond between C2 and C3 carbons with a carbonyl group at C4 all contribute to flavonoids' strong antioxidant activity (Figure 2.2) [42]. When galloyl groups, free hydroxyl groups are considered, tea flavanols are especially stands out. Considering the wide consumption of green tea, its easy accessibility, its phenolic compound composition and their antioxidant activity, green tea is an important phenol and antioxidant source in daily life.

Table 2. 1. Classification of phenolic compounds [42].

Phenolic Compounds	Carbon Numbers	Basic Structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acid	C_6-C_1	
Acetophenones	C_6-C_2	
Phenyl acetic acid	C_6-C_2	
Hyrdoxycinamic acid	C_6-C_3	
Phenylpropene	C_6-C_3	
Coumarine, isocoumarine	C_6-C_3	
Chromone	C_6-C_3	
Naptoquinones	C_6-C_4	
Xanthone	C_6-C_1-C_6	
Stilbene	C_6-C_2-C_6	
Anthraquinone	C_6-C_1-C_6	
Flavonoid	C_6-C_3-C_6	
Lignan	$(C_6$-$C_3)_2$	

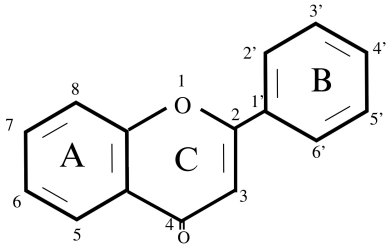


Figure 2. 2. Structure of flavonoid.

2.4. Protein-Phenol Interactions

Through numerous chemical processes like oxidation, polymerization, and degradation, protein-phenol interaction can take place in food and plants. Also, it plays role in oral processing as providing astringent sensation. In the digestive tract, protein-phenol interactions also occur between digestive enzymes and phenolic compounds, which may inhibit the digestive enzymes' activity [50-52]. Interaction between phenolic compounds and proteins might be reversible (weak interaction) or irreversible (strong interactions). While covalent bonding is strong because it involves sharing electron pairs, it is irreversible; non-covalent interactions such as van der Waals interactions, hydrogen bonding, hydrophobic interactions, pi stacking are reversible [51]. Hydrogen bonds occur between a hydrogen atom attached to an electronegative atom and another electronegative atom [50-52]. Hydrogen bonds between peptides and phenolic compounds are formed thanks to hydroxyl groups in phenols and carbonyl group of peptides. While hydrophobic interactions might occur between two aromatic rings, thanks to charged groups, electrostatic interactions occur [51, 52]. There are three stages in the formation of these interactions. In the first stage, addition of polyphenol, several polyphenol molecules may initially bind with the proteins with different driving forces as mentioned previously. Later, higher concentration of polyphenol can cause the formation of polyphenol-coated dimers, which will result in precipitation. Finally, as the number of molecules increases, larger complexes may be formed [51]. The covalent interaction between proteins and phenolic compounds begins with the enzymatic or non-enzymatic (under alkaline conditions) oxidation of the phenols, resulting in the production of quinones. The oxidation reaction of phenolic compounds involves two steps: first, the hydroxylation of monophenols into o-diphenols, then the oxidation of o-diphenols into o-quinones under alkaline conditions and/or in the presence of oxidative enzymes, oxygen, and metals.

Quinones are reactive electrophilic intermediates that can attack nucleophilic side chains such as amino groups of lysine, indole groups of tryptophan, thiols, etc. In this way, nucleophilic addition occurs (Figure 2.3) [53–55]. Upon this reaction, cross-linked protein polymers may be formed by a further addition reaction [55].

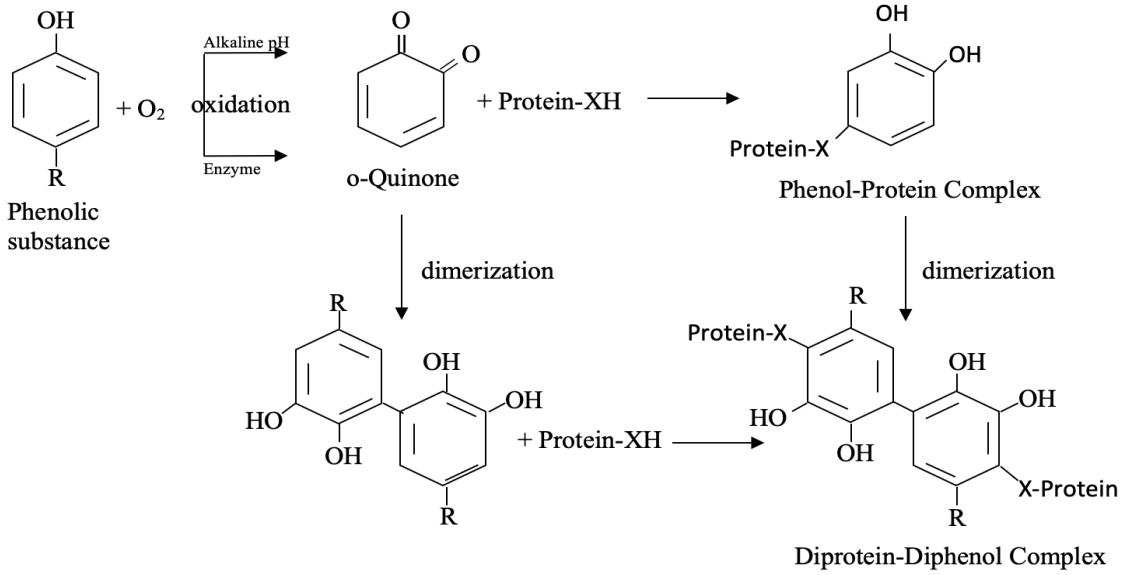


Figure 2. 3. Summary of mechanism of protein-phenol interaction via oxidation and nucleophilic addition [53].

The reaction between proteins and phenols is affected by factors such as types and structures of protein and phenols, pH, temperature, presence/absence of polyphenol oxidase, oxygen etc. The effect of pH on protein-phenol interaction is related to both the isoelectric points of proteins and the oxidation reactions of phenols. At isoelectric pH, proteins have more binding sites and protein-phenol interactions occur with maximum yield but non-covalently. At alkaline conditions, covalent protein-phenol interaction occurs via autooxidation of phenols and so quinone formation [52]. Therefore, pH has a high impact on the type of interaction, covalent or non-covalent [53]. In addition, pH also influences the electrophilic behavior of polyphenols. Awad et al. (2002), investigated the mechanism of the pH-dependent chemistry of glutathione adducts of flavonoid quinone/quinone methides and stated that pH influences electrophilic behavior of quercetin and the quinone adduct formation shifts from C ring at pH 3.5, to the A ring at 7.0, to the B ring at pH 9.5 [54].

Protein-phenol interactions are also affected by the protein structure in terms of its hydrophobic interactions, isoelectric point, and amino acid composition. Amino acid composition of proteins, especially proline residues and the number of its repeats, has been reported to play an important role as potential binding sites of proteins for phenolic compounds. For example, while gelatin has a higher affinity for flavonoids than casein [58], β-casein shows a higher affinity for phenolics because of its higher proline content [55]. Besides the proline residues, binding of polyphenols via amino acid residues such as histidine, arginine, phenylalanine, tyrosine, leucine has also been reported in the literature [53].

Protein-phenol interaction is also affected by the number of phenolic compounds, positions of its hydroxyl group, its molecular weight and flexibility. The more hydroxyl group phenol has, the more protein-phenol interaction occurs. Also, increase in hydroxyl group of phenolic compounds results in stronger binding. The effect of flexibility varies according to the phenol and its interaction with protein [52, 56].

2.4.1. Effects of Protein-Phenol Interaction on Protein

Protein-phenol interactions cause changes in the structure, antioxidant activity, digestibility, allergenicity, emulsifying, foaming, gelling and solubility properties of proteins [57-60]. Interaction of proteins with phenolic compounds via covalent or non-covalent bonds might result in the formation of a highly ordered or disordered structure. An increase in α-helix structure demonstrates that the formation of more ordered structure, while a decrease in α-helix referred to as the breaking of intermolecular S-S, disruption of hydrogen bonding, protein unfolding, and rearrangement of protein [61]. The primary mechanism sustaining the α-helix structure is hydrogen bonding. Therefore, the breakdown of hydrogen bonding or the binding of polyphenols through the hydrophobic pocket of proteins may cause conformational changes in the proteins' α-helices structure [62]. Zhou et al. (2020), investigated that both non-covalent and covalent interactions of epigallocatechin gallate and soy protein isolate under conditions pH 7 (non-covalent interaction) and pH 9 (covalent interaction) in their study. Non-covalent

interaction resulted in the increase in α-helix, β-turn and random coil, decrease in β-sheet; covalent interaction resulted in increase in α-helix and random coil, decrease in β-turn. These results were associated with the strength of the covalent bond and the enhanced protein denaturation with high phenolic compound concentrations [63]. On the other hand, *Cinnamomum camphora* seed proteins more ordered structure with the increase in β-sheet (from 19.81 to 21.39%) and a decrease in random coil (from 26.0 to 24.87%) as a result of its interaction with phenolic compounds at pH 9 [64]. In conclusion, types of proteins and phenolic compounds, conditions of interaction between them, driving force and strength of interaction effect the structure of protein distinctively.

Structural changes of protein as a result of its interaction with polyphenols lead to change in its thermal stability. According to previous reports, protein- phenolic compounds interaction can increase or decrease the thermal stability of proteins. Xu et al. (2019), reported that covalent conjugation of whey protein isolate with chlorogenic acid decreased its thermal stability from 89.5 to 86.2 °C which indicates decreased tertiary conformation stability of whey protein isolate after covalent interaction [65] . On the other hand, Yan et al. (2021) observed that covalent modification by phenolic extract increased the thermal stability of a protein isolate from *Cinnamomum camphora* seed kernel, which was associated with the incorporation of carboxylic and hydroxyl groups [66]. In a study comparing the covalent and non-covalent interactions of zein and polyphenols, it was stated that non-covalently interacted samples showed lower thermal stability than their correspond covalent due to the loss of the α-helix structure [71].

When phenolic compounds complexed with protein, they become main electron donator of protein which result in increased antioxidant capacity of proteins [67-69]. In a study, soy protein isolate was interacted with EGCG for 24 through both covalent (at pH 9) and non-covalent (at pH 7) interactions. These resulted in increase of total antioxidant capacity from 9.50 to 272.14 μg Trolox/g protein for covalent and 282.57 μg Trolox/g protein for non-covalent interaction [63]. Interaction of gelatin with tannic acid increased the radical scavenging activity of gelation from to 249.87 μmol TE/mg to 491.68 μmol TE/mg [70]. The increase in antioxidant activity in the protein-polyphenol complexes or conjugates also affected by the polyphenol concentration and pH of the interaction [62].

For example, rice protein exhibits stronger radical scavenging activity after the interaction with anthocyanin at pH 3. However, interaction at pH 7 and pH 9 provided less radical scavenging activity than pH 3 [71]. In addition, as a result of the interaction of whey protein isolate and casein with chlorogenic acid (20, 120 and 240 μmol/g protein), proteins exhibited synergistic radical scavenging capacity in dose-dependent manner [72].

The interaction of proteins with phenolic compounds via side groups of proteins such as thiol, amino, tryptophan etc. of protein results in blocking of these side chains and decreased in their amounts [73]. In the study of Rawel et al. (2022), phenolic compounds and related substances were interacted with soy glycinin at pH 9 for 24 hours. This led to reduction of free amino group of soy glycinin from 507.3 ± 5.4 nmol.mg^{-1}protein to 319.8 ± 0.8 nmol.mg^{-1}protein due to its interaction with caffeic acid. Moreover, interaction of soy glycinin with quercetin decreased its free -SH groups from 271.6 ± 25.5 nmol.mg^{-1} protein to 136.3 ± 24.5 nmol.mg^{-1} protein [74]. In another study in which the interaction of gluten with tannin were take place for 1 hour in distilled water, free thiol and amino content decreased while thiol content had a more pronounced decline [61]. Therefore, changes in amino and thiol groups of native proteins and phenol-protein complexes provide information about whether protein-phenol interaction takes place [61, 67, 73, 75].

The changes in structure and amino acid content of proteins and the formation of insoluble complexes as a consequence of protein-phenol interaction result in changes in the digestibility and bio-accessibility of proteins [52, 56, 76]. As it was mentioned before, trypsin favors cleaving basic amino acid residues such as lysine, arginine and chymotrypsin favors cleaving aromatic amino acid residues such as tryptophan, tyrosine, phenylalanine. Therefore, the changes in amino acid content and structure may induce structural hindrance of proteins to digestive enzymes. On the other hand, due to the unfolding of protein structure, proteins may become more sensitive to digestive enzymes. In a study in which interaction of soy glycinin with different phenolic acids and flavonoids was studied, it is observed that the tryptic, chymotryptic and pancreatic hydrolysis was stimulated (only flavone did not showed any effect), whereas the peptic digestion remained almost unaffected [75]. Addition of chlorogenic acid to casein and

whey protein isolate increase the protein digestibility due to protein unfolding and increased their susceptibility to digestive enzymes [72]. A study investigated the change in the digestibility of β-lactoglobulin as a result of its interaction with various phenolic extracts (green tea, black tea, coffee, cocoa) in the gastrointestinal tract (under different pH conditions). In this study, it is shown that gastric digestion decayed and the presence of cocoa and coffee phenolic extracts pancreatic digestion delayed in the presence of phenolic extracts [59]. The effect of phenol supplementation to bread on digestibility of gluten is also investigated. Onion skin supplemented bread had lower digestibility, as indigestible protein-phenol complexes were formed [77].

Similar to protein digestibility, protein-phenol interaction can also cause changes in protein allergenicity. Effect of protein-phenol interaction on allergenicity is based on several mechanisms; complete destruction of Ig-E binding epitopes by digestive enzymes because of unfolded protein structure, inability to recognition by allergen-specific-Ig-E, binding of phenols directly to allergenic IgE epitopes [78, 79]. In a study, flavonoids are mostly bound to β-sheet and β-turn (epitopes regions of β-lactoglobulin) structures and reduce allergenicity of β-lactoglobulin [80]. ELISA results show that Ig-E binding capacity of ovalbumin-quercetin conjugates was lower than native ovalbumin [81], while similar results were obtained with tannic acid-peanut proteins interaction [82].

In literature, there are several studies on interaction of phenolic compounds with gluten, gliadin, glutenin and peptides derived from them to investigate the effects of interaction on gluten' high order structure, digestibility, functionality etc. The interactions between the phenolic compounds and gluten, gliadin, glutenin occur with different driving forces for each, even if the interaction occurs with the same phenol source. For example, whereas the interaction of condensed tannin with gluten takes place via both hydrogen bonding and hydrophobic interaction, interaction with gliadin takes place via hydrogen bonding. Moreover, proanthocyanins crosslink with HMW-GS more than LMW-GS and with ω-gliadin than to α-/β-gliadin. This is due to the higher molecular size of HMW-GS and ω-gliadin and their glutamine-rich repetitive domains that promote hydrogen bond formation [83].

Interaction of gluten with phenolic compounds result in change in its textural, functional properties. Du et al. (2020) investigated the effects of tannin on gluten. For this, interaction between gluten and tannin took place for 1 hour within the tannin solution which was prepared with distilled water. It has been reported that high tannin concentration (8% tannin w/w to gluten) increased the hardness and viscoelasticity of gluten significantly and amplified the formation of compact structure of gluten via conformational changes. In addition, Pan et al. (2021) hypothesized that catechins would prevent the development of the gluten network structure according to the rheological features (decreased elastic modulus, increased viscous modulus). This result obtained from the increase in free -SH group as result of destruction of SS bonds due to interaction with catechins [84]. As opposed to this, Tian et al. (2021) observed that addition tea polyphenols, EGCG and ECG monomers to gluten increased both elastic modulus and viscous modulus which result from increased dough viscoelasticity [85].

2.4.2. Gluten-Phenol Interaction in Celiac Disease Perspective

There are several studies that investigate interaction of gluten, gliadin, gluten derived peptides with different phenolic sources to prevent/reduce immune responses caused by them in terms of celiac disease. It is thought that the gluten-phenol interaction may cause blocking of the celiac disease pathogenesis, in this way may prevent the celiac disease and/or reduce its responses. These prevention/reducing mechanisms may include, a) prevention of the interaction of gluten peptides with zonulin, prevention of loosening of intestinal tight junctions, and thus prevention of the passage of gluten peptides into the lamina propria due to structural modifications b) prevention of the transportation of gluten peptides through intestinal epithelial tight junctions due to the increase in molecular size of gluten peptides, c) prevention of the interaction of gluten peptides passed into the lamina propria with the tTG enzyme, d) reduction/inhibition of recognition of gluten peptides by HLA-DQ receptors [86]. In a work, to investigate the impact of green tea extract (GTE) on digestion of gliadin, GTE is added to digestion process of gliadin. GTE inhibits pepsin/trypsin-mediated digestion. Also, increase in GTE concentration gave rise to the levels of higher molecular weight gliadins. The physical interaction between GTE and gliadin is thought to bring reduced digestibility of gliadin. It is also possible that pepsin and trypsin are directly inhibited due to their interaction with GTE, which would result in the reduced gliadin digestion. Also, addition of GTE

prevented permeability of gliadin-derived peptides, decreased the production of inflammatory markers (IL-6 and IL-8) [87]. Dias et al. (2018, 2019), investigated the binding mechanisms of procyanidin B3, trimer C2 and EGCG to 32-mer (the first leucine residue of 33-mer peptide lack) peptide and their effect of transepithelial translocation. The binding of procyanidin B3 and trimer C2 through especially its leucine, tyrosine and phenylalanine residues take place via hydrogen bonding, and both hydrophobic and hydrophilic contacts. The interaction of EGCG with 32-mer peptide may take place via hydrophobic interactions between the aromatic and galloyl moieties of EGCG and the aromatic side chains of tyrosine and phenylalanine Also, interaction takes place through the hydrophobic sections of proline, leucine and glutamine residues in a non-selective way. Although their different binding affinities to gliadin, both procyanidins and EGCG were able to decrease the apical-to-basolateral translocation of 32-mer peptide in intestinal epithelial barrier [88, 89]. When procyanidin B3 was mixed with gliadin before digestion process, it was observed that the immunological properties of gliadin peptides decreased, although the interaction between them did not affect the digestive system enzymes [90]. Also, the interaction of procyanidins and gliadin derived peptides form stable complex which have potential to prevent the accessibility of gluten derived peptides, blocking their effects on the intestinal mucosa [91].

3. MATERIAL AND METHODS

3.1. Chemicals and Consumables

Ammonium bicarbonate, sodium bicarbonate, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2 carboxylic acid (Trolox) (97%), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (98%), cellulose, sodium tetraborate decahydrate, o-phthalaldehyde (OPA), ethyl alcohol (96%), sodium dodecyl sulfate (SDS) (98.5%), Tris(hydroxymethyl)aminomethane (TRIS) (99.9%), Urea (99.0-100.5%), 1,4-dithiothreitol (DTT) (97%), hydrochloric acid, methanol, acetonitrile, L-serine, L-cysteine, Amino acid standard mix solution (2.5 mM each), were purchased from Sigma- Aldrich Chemie (Steinheim, Germany). Formic acid (98%) was purchased from J. T. Baker (Deventer, The Netherlands). α-amylase (≥10 U/mg solid), from porcine pancreas, Pepsin (≥250 U/mg solid) from porcine gastric mucosa, pancreatin (4×USP) from porcine pancreas, lipase from porcine pancreas and bile extract were also purchased from Sigma Aldrich (Deisenhofer, Germany). Sodium hydroxide, calcium chloride were purchased from Merck (Darmstadt, Germany). ZIC-HILIC (150 x 4.6 mm, 3.5 μm) column were purchased from MerckSeQuant (Darmstadt, Germany). Nylon syringe filters (0.45 μm) were from IsoLab (İstanbul, Turkey). Sep-Pak Accell Plus QMA 1 cc Vac cartridge were from Waters (Milford, MA). Alumminum lid and pans were purchased from TA Instruments (New Castle, USA). Gluten peptides were purchased from Elabscience (Texas, USA). Deionized water (5.6 μS/m) was used throughout the analysis and sample preparation. Green tea extract (GTE), commercial gluten, starch, yeast, sugar, salt, shortening were purchased from local market.

3.2. Experimental Plan

Experimental plan is shown in Figure 3.1.

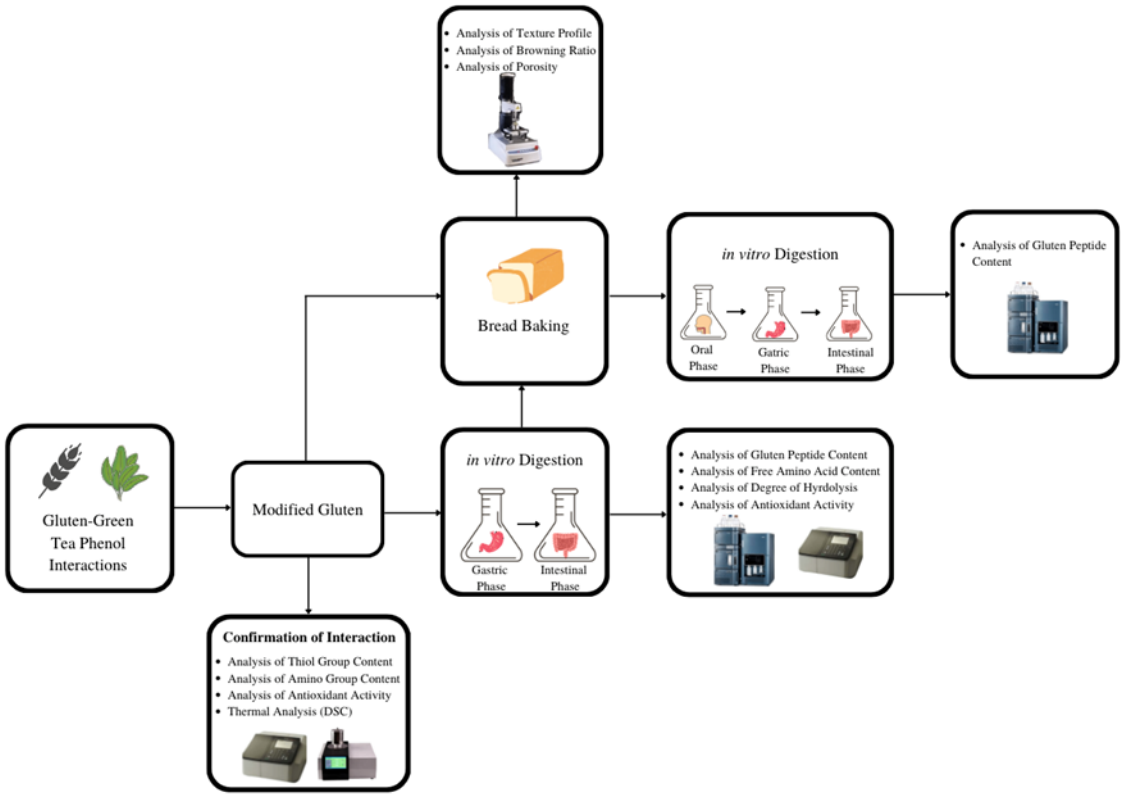


Figure 3. 1. Experimental plan of the study.

3.3. Confirmation of Gluten-GTE Phenol Interaction

3.3.1. Sample Preparation/ Gluten-Green Tea Interaction

Gluten was treated with GTE under following conditions; 1% and 2% GTE concentration; pH 7 and pH 9; 2 h and 3 h treatment at 50 ˚C. In this study interaction of gluten and GTE were carried out at 50 ˚C, according to a study which investigate the interaction of cereal bran with different beverages. In this study, cereal brans were treated with green tea infusion, and the results indicated that the temperature showed positive impact on antioxidant activities of cereal bran until it reaches 50 ˚C, but then it led to decrease in antioxidant activities of cereal brans [92]. The highest increase in antioxidant activity at 50˚ C indicated its more pronouncing effect. Sample preparation was summarized in

Figure 3.2. The amount of GTE which was used for the interactions with gluten was expressed as the percentage of the gluten weight. Also, the amounts of gluten, GTE which were used in gluten-GTE phenol interaction and conditions of interactions given in Table 3.2. Briefly, 15 g of gluten and 1% or 2% (w:w gluten, so 150 and 300 mg GTE, respectively) were added to 500 mL of ammonium bicarbonate and sodium carbonate-bicarbonate buffer solutions [93, 94]. These buffer solutions were preferred as they are food-grade. The amount of gluten (15 g) were chosen according to pre-trials for all interactions which took place under different conditions. Gluten-GTE interactions were carried out by mixing continuously for 2 or 3 hours by using a magnetic stirrer keeping the temperature at 50 °C, free exposure to air. After treatments were completed, gluten was sedimented and washed 10 times with 300 mL water in order to remove the excess GTE. Then, modified gluten samples were lyophilized and kept at 4 °C for further analysis. All the treatments of gluten with GTE took place under different conditions were replicated for two times.

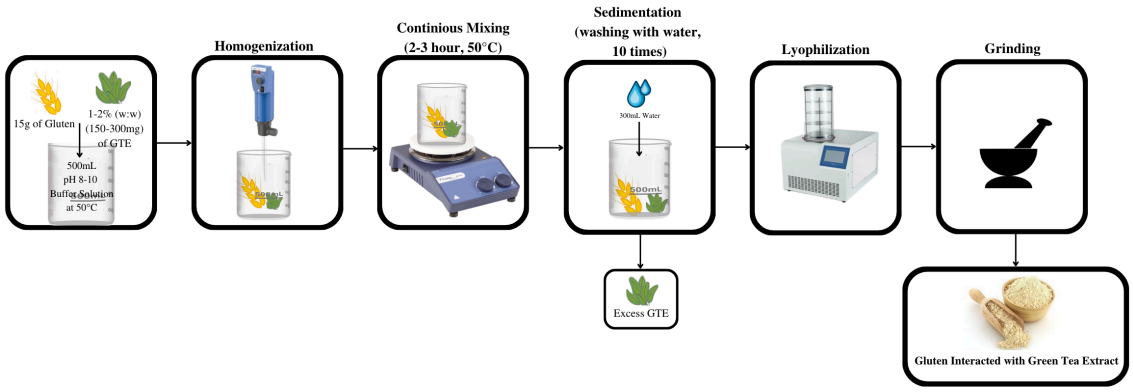


Figure 3. 2. Schematic presentation of the sample preparation.

Table 3. 1. Nomenclatures and interaction conditions of the samples.

	Interaction Parameters			
Sample	**The amount of gluten (g)**	**GTE Concentration (mg, %)**	**pH**	**Time (h)**
1% GTE x pH 7 x 2h	15	150, 1%	7	2
1% GTE x pH 7 x 3h	15	150, 1%	7	3
2% GTE x pH 7 x 2h	15	300, 2%	7	2
2% GTE x pH 7 x 3h	15	300, 2%	7	3
1% GTE x pH 9 x 2h	15	150, 1%	9	2
1% GTE x pH 9 x 3h	15	150, 1%	9	3
2% GTE x pH 9 x 2h	15	300, 2%	9	2
2% GTE x pH 9 x 3h	15	300, 2%	9	3

3.3.2. Analysis of Antioxidant Activity

Total antioxidant capacities of modified and native gluten samples were measured with using $DPPH^{\bullet+}$ radical solution by QUENCHER method reported by Serpen, Gökmen and Fogliano [95]. Briefly, 10 mg of native gluten and gluten treated with GTE were weighed into test tube and radical quenching reaction was started by adding 10 mL of $DPPH^{\bullet+}$. Reactions were carried out of for 27 min in dark with vigorous shake in an orbital shaker at 400 rpm. After 27 min, tubes are centrifuged 6080×g for 2 min, optically clear supernatants were transferred into cuvettes and absorbances were measured by UV-Visible spectrophotometer at 525 nm (Shimadzu Corp., Kyoto, Japan). All measurements applied were duplicated. Trolox was used to build the calibration curve and results were given in μg Trolox Equivalent (TE)/g dried weight.

3.3.3. Analysis of Amino Content

Before the analysis of amino and thiol content, native gluten and modified gluten samples were treated with 8 M urea for 2 hours at a concentration of 10 mg protein/ml 8M urea to make them solubilize. Amino content analysis were conducted according to the procedure reported by Nielsen, Petersen [96]. In this procedure, 3 mL OPA (o-phytaldialdehyde) reagent was added to 400 μL serine standard/blank/sample and mixed for 5 seconds. After mixture was stood for exact 2 minutes, absorbance value was read at 340 nm by using UV-Visible spectrophotometer (Shimadzu Corp., Kyoto, Japan). The results were calculated against a serine standard curve and given as μg Serine Equivalent/ g sample.

3.3.4. Analysis of Thiol Content

Thiol content of native gluten and modified gluten samples were determined by derivatization with DTNB (Elman's reagent, 5,5'dithiobis (2- nitrobenzoic acid) and following spectrophotometric quantification by comparison with a standard curve of L-cysteine. Cysteine standards were prepared from 5mM cysteine stock (in Tris Buffer) according to Table 3.2.

Table 3. 2. Preparation of cysteine standards.

STD Number		Standard Volume (μL)	SDS Buffer Volume (μL)	Cys (μM)	Cys in cuvette (μM)
1	Stock	50	950	250	41.7
2	Stock	20	980	100	16.7
3	Stock	10	990	50	8.33
4	STD3	5	995	25	4.17
5	STD3	100	900	10	1.7
6	STD3	25	975	2.5	0.4
Blank	-	-	1000	0	0

For this; firstly, samples were centrifuged 8000×g for 3 min, then 500 µL clear supernatant/cysteine standard/blank were transferred into tube, 2 mL SDS Buffer was added and the absorbance is measured at 412 nm. These absorbance values were referred as Abs_{before}. After that, 500 µL DTNB was added and samples were incubated for 30 min at room temperature and in the dark. After exact 30 min incubation, absorbances were measured at 412 nm referred as Abs_{after}. Thiol contents were calculated as follow;

$$Abs_{DTNB} \times Correction\ Abs = Abs_{STD} - Abs_{BLANK}$$

With this equation, Correction Abs is calculated for each standard and Correction Abs vs Thiol Content (R-SH, µM) graph was plotted.

$$[R-SH] = \frac{Abs_{After} - Abs_{Before} - Abs_{DTNB} - b}{a}$$

a is slope, b is intersection point of graph. The thiol concentrations were obtained by multiplying by [R-SH] with dilution factor. Results were given in µ mol/g sample [97, 98].

3.3.5. Thermal Analysis

The denaturation temperature of native gluten and modified gluten samples were assessed by using Differential Scanning Calorimetry (DSC) (TA Instruments, New Castle, USA). After 1-3 mg of samples are weighed to aluminum pan, aluminum lid and pan were hermetically sealed. Hermetically sealed empty aluminum lid and pan was also used as reference. Thermogram was recorded between 25-200 °C with 10 °C /min heating rate under dry nitrogen atmosphere with 30 mL/min flow rate.

3.4. Effects of Gluten-GTE Phenol Interaction on Digestive Properties

3.4.1. *In vitro* Peptic and Pancreatic Digestion of Native and Modified Gluten

250 mg of native gluten and modified gluten were weighted into tube and 5 mL of 10 mM HCl was added and these mixtures were incubated at 37 °C for 30 min to unfold protein structure. Following incubation, 125 µL pepsin 0.1mg/mL 10mM HCl was added and incubated at 37 °C for 2 hours for gastric phase. After pepsin treatment, 410 µL 1.43 M sodium bicarbonate was added to obtain pH 7.5 and 75 µL of 50 mg pancreatin/mL

pancreatin buffer was added and incubated at 37 °C for 2 hours for intestinal phase [99]. Digested samples were immediately cooled at ice bath and tubes were centrifuged 8000×g for 3 min, supernatants were transferred another tube and stored at -18°C for further analysis. *In vitro* digestions were carried out in two replicates.

3.4.2. Determination of Degree of Hydrolysis

Following the *in vitro* digestion of native gluten and gluten samples treated with GTE, and degree of hydrolysis (DH) were determined by the procedure reported by Nielsen, Petersen and Dambmann [96] as given in Section 3.3.3.

Calculation of %DH was made according to the equations given below;

$$Serine - NH_2 = \frac{ABS_{sample} - ABS_{blank}}{ABS_{std} - ABS_{blank}} \times \frac{0.9516meqv}{L} \times 0.1 \times \frac{100}{X \times P}$$

where serine-NH_2 = meqv serine NH_2/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in liter (L).

$$h = \frac{Serine - NH_2 - \beta}{\alpha}$$

where α is 1.00; β is 0.4 for gluten.

$$DH(\%) = \frac{h}{h_{total}} \times 100$$

Where h_{total} is 8.3 for gluten.

3.4.3. Analysis of Total Free Amino Acid

in vitro digested samples were centrifuged and filtered through 0.45 μm syringe filter into autosampler vial. Free amino acids were analyzed by Waters Acquity TQD LC/MS-MS (Waters, USA). Chromatographic separation of free amino acids was performed on a ZIC-HILIC column (150 × 4.6 mm i.d., 3.5 μm) by using a gradient elution of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 1 mL/min at 30 °C. The gradient elution starting with 20% A held for 4 min and then linearly increased to 80% in 3 min and held for 3 min. Then, it was decreased to the initial conditions (20% A) in 1 min and held for 4 min. Total chromatographic run time was 15 min. The injection

volume was 5 µL. The electrospray source had the following settings: capillary voltage of 5 kV; cone voltage of 20 V; extractor voltage of 3 V; source temperature of 120 °C; desolation temperature of 400 °C; desolvation gas (N_2) flow of 900 L/h and cone gas (N_2) flow of 50 L/h. Amino acids were identified by multiple reaction monitoring (MRM) using the parameters given in Salman, Yılmaz, Gökmen and Özdemir [100]. Concentrations of amino acids were calculated by means of external calibration curves built for individual amino acids in a range between 2 and 100 µM.

3.4.4. Immunogenic Peptide Analysis for Native Gluten and Gluten Treated with GTE

Before the quantification of gluten peptides, native gluten samples and gluten treated with GTE subjected to INFOGEST *in vitro* digestion procedure. After the intestinal phase, digests were centrifuged and clean-up procedure was applied by Solid Phase Extraction method using Sep-Pak Accell Plus QMA 1 cc Vac Cartridge. For this 250 µL gluten sample, 740 µL water with 0,1% formic acid and 10 µL internal standard were added into a tube and then centrifuged. The cartridge was preconditioned with 1 mL of methanol and then 1 mL of deionized water. 1 mL of supernatant was loaded onto preconditioned cartridge. Following the washing of cartridge with 1 mL of water, sample was eluted with 1 mL acetonitrile. Eluted sample was evaporated under the nitrogen until dryness and the residue was dissolved in 500 µL water in an autosampler vial. Immunogenic gluten peptides were analyzed by Waters Acquity TQD LC/MS-MS (Waters, USA). Chromatographic separation was performed on a ZIC-HILIC column (150 × 4.6 mm i.d., 3.5 µm) by using a gradient elution of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 1 mL/min at 30 °C. The gradient program for mobile phase B was: 0-4 min 80%, 4-8 min 80 to 40%, 8-12 min 40% to 20%, 12-14 min held at 20%, 14-16 min from 20% to 40%, 16-18 min from 40% to 80%, held for 4 min. Total chromatographic run time was 22 min. The injection volume was 10 µL. The electrospray source had the following settings: capillary voltage of 2,97 kV; cone voltage of 25 V; extractor voltage of 3 V; source temperature of 130 °C; desolation temperature of 350 °C; desolvation gas (N_2) flow of 550 L/h and cone gas (N_2) flow of 50 L/h. Amino acids were identified by multiple reaction monitoring (MRM) using the parameters given in Table 3.3.

Table 3.3. Immunogenic gluten peptides' amino acid sequences and MRM parameters [101].

Peptide Name	Amino acid Sequence	Molecular Weight (kDa)	Precursor Ion m/z (Charge State)	Fragmented Ions m/z	Retention time (min)
19-mer	LQLQPFPQPQLPYPQPQPF	2263	755.068 (+3)	488.251	8.34
26-mer	LQLQPFPQPQLPYPQPQLPYPQPQPF	3087	1029.543 (+3)	263.139	8.84
33-mer	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF	3912	979 (4+)	225.4	8.22

3.4.5. Antioxidant Activity Assay of Digested Samples

1 mL of DPPH solution was added to 200 μL digested sample and discoloration (radical quenching) reaction was carried out during centrifugation for 8000×g for 3 min. After centrifugation, optically clear supernatants are transferred into cuvettes and the readings were performed as mentioned already in the Section 3.3.2.

3.5. Effects of Gluten-GTE Phenol Interaction on Bread Properties

3.5.1. Bread Preparation

In this study, control bread was prepared using AACC (American Association of Cereal Chemists) Method 10-10.03 (1999) with some modifications in formulation. Bread preparation according to method based on 100 g of flour. In our study, flour was substituted with the 75 g of starch, 13 g of gluten and 12 g of water. However, 12 g of water is added during mixing process. Additionally, 28 mL of water (so the total amount of water 40 mL), 11 mL of sugar−salt solution containing 6 g of sugar and 1.5 g of salt, 20 mL of yeast suspension composed of 5.3 g of instant yeast were added. Mixing was carried out using KitchenAid Artisan Model 5KSM 150 mixer at the slowest rate. Dough was obtained by mixing of flour mix (75 g of starch and 13 g of native or modified gluten), yeast suspension and sucrose-salt solution for 15 sec and addition of 10 mL water in every 30 sec for 4 times and mixing was continued for 5 min. Dough was fermented 3 times for 52, 25, 13 min, respectively, in total 90 min at 30°C, 85% RH and punched between fermentation processes. After the last punch, dough was placed into commercial pans for 1-lb loaves and panned for 33 min. Therefore, the total fermentation process was completed at 123 min. Following panning, dough was baked at 220°C for 24 min in Memmert Oven (UNE 400, Germany). For the preparation of bread by using gluten

treated with GTE, gluten was treated with 2% of GTE at pH 9 for 2 h because the greatest decrease in immunogenic gluten peptides was obtained under these conditions.

To improve the characteristics of breads prepared with modified gluten, AACC formulation was modified to include soy protein isolate and guar gum. Bread formulation consisted of 13 g of gluten, 65 g of starch, 10 g of soy protein isolate, 5 g of guar gum, 11 mL of sugar−salt solution containing 6 g of sugar and 1.5 g of salt, 20 mL of yeast suspension composed of 5.3 g of instant yeast and 94 mL of water. Dough mixing was carried out using KitchenAid Artisan Model 5KSM 150 mixer at the slowest rate. Firstly, dry ingredients were mixed for 15 sec and then 20 mL of water was added in every 30 sec while continuously mixing until the total water content reach 94 mL. Dough was mixed for 5 minutes. Dough was fermented for 73 min and panned for 50 min at 30°C, 85% RH. Following panning, dough was baked at 220°C for 24 min in Memmert Oven (UNE 400, Germany). Gluten is substituted with gluten treated with 2% of GTE at pH 9 for 2 h for the preparation of modified bread samples.

3.5.2. Texture Profile Analysis

Mechanical characteristic of bread samples in a double compression cycle was recorded in texture analyzer (LLOYD Instruments, TA plus Ametek, UK**)** with a maximal load 1000 N, 40% penetration depth with 20 mm diameter probe on 25 mm-thick- sliced bread samples. Measurements were taken 1 h after baking. The hardness, firmness, springiness, cohesiveness parameters were measured twice for each sample.

3.5.3. Image Analysis

Porosity index analysis and color measurements (L*, a*, b*) were performed with computer-based vision image technique reported by Mogol and Gökmen [102]. For color analysis whole bread, for porosity index 25 mm-thick-sliced bread samples were used. Digital images were taken at a distance of 25 cm from bread samples which placed on black background. The angle between the axis of the lens and the sources of illumination was adjusted to approximately 45°. Captured images were analyzed in MATLAB (The MathWorks, Inc., Natick, Massachusetts, United States).

3.5.4. *In Vitro* Digestion of Bread Samples

In vitro digestion was applied to bread samples according to the protocol reported by Minekus et al. (2014) [103] . For this; 400 μL Simulated Salivary Fluid (SSF), 50 μL salivary amylase (150 mg/ mL in water), 2.5 μL $CaCl_2$ and 480 μL water were added to 500 mg of bread and these mixtures incubated for 2 min at 37 °C for oral phase. After oral phase, 800 μL Simulated Gastric Fluid (SGF), 50 μL lipase (160mg/mL in water), 50 μL pepsin (160mg/mL in water) were added and pH was adjusted to approximately 3.0 using HCl. After HCl addition, water was added until total volume reaches 2 mL. This mixture was incubated 2 hours at 37°C for gastric phase. During gastric phase, pH readjusted to 3 in every 30 min. After gastric phase 850 μL Simulated Intestinal Fluid (SIF), 500 μL pancreatin (8 mg/mL in SIF), 250 μL bile (205.65 mg/mL SIF) and 4 μL $CaCl_2$ was added and pH was adjusted 7 with using NaOH. After NaOH addition, water was added until total volume reached 4 mL. During intestinal phase, pH readjusted to 7 in every 30 min. After digestion completed, digested samples were immediately cooled by placed in ice bath, centrifuged 8000×g for 3 min and frozen at -18°C.

3.5.5. Immunogenic Peptide Analysis for Bread

Immunogenic peptide quantification of bread samples subjected to *in vitro* digestion was done with the method reported in Section 3.4.5.

3.6. Statistical Analysis

All data were subjected to analysis of variance (one-way ANOVA) test in order to evaluate the statistically differences between mean values. Differences at $p < 0.05$ were considered significant by using Duncan's test. Also, in order to understand the effects of importance's of interaction parameters on binding of GTE to gluten analysis of covariance (ANCOVA) was applied. Principal Component Analysis (PCA) was performed to analyze possible relationships between samples. All statistical analysis conducted by using XLSTAT (Addinsoft, New York, USA).

4. RESULTS AND DISCUSSION

4.1. Confirmation of Gluten-Green Tea Phenol Interaction

4.1.1. Antioxidant Activity

When phenols are incorporated into proteins, their hydroxyl group connected with benzene ring remain to exert their antioxidant function which gives increase to the antioxidant activity of proteins [72]. Therefore, increase in antioxidant activity of proteins is one of the most important properties for the confirmation of protein-phenol interaction. Many studies revealed that protein-phenol interaction provided higher antioxidant activity to proteins [57, 70]. In the work of Aewsiri et al. (2010), gelatin, which is proline rich protein similar to gluten, was interacted with oxidized ferulic acid, caffeic acid and tannic acid at pH 9, at room temperature for 12h. With the incorporation of 5% oxidized caffeic acid, ferulic acid, and tannic acid, antioxidant capacities of treated gelatin increased to 420.69, 292.57, and 491.68 μmol TE/mg, respectively, whereas control gelatin was 249.87 μmol TE/mg [70]. In a study, interaction between pea protein isolate which is a plant-based protein like gluten, and catechin took place at pH 7 for 2 h at 25°C. DPPH radical scavenging rate (%) of pea protein isolate increased from 8.99% ± 0.24 to 70.42 % ± 0.51 at the 0.25% catechin concentration [57].

The total antioxidant capacity of native gluten, which is used as control in this study, and gluten samples modified with GTE under different conditions (%1 and %2 GTE concentration, pH 7 and pH 9, 2 h and 3 h) are given in the Figure 4.1. Antioxidant capacity of native gluten was found 11.27 ± 0.28 μg TE.g^{-1} and gluten samples which were interacted at different pH, time and GTE concentrations were found in the range between 55.83 ± 3.17 - 141.21 ± 7.01 μg TE.g^{-1}. It is obvious that gluten- GTE phenolic compounds interactions provided greater antioxidant capacity to native gluten (p<0.05). According to analysis of co-variance (ANCOVA), correlation coefficients of effects of GTE concentration, pH and interaction time (h) on total antioxidant capacity were found 0.691, 0.423 and 0.033, respectively (p<0.05). In this regard, the highest correlation coefficient corresponds to the most effective interaction parameter that affects total antioxidant capacity. Owing to the highest correlation coefficient (0.691), it can be seen

that GTE concentration was the most effective parameter in the interaction of gluten by GTE, in terms of total antioxidant capacity. Accordingly, the increase of GTE concentration led to more increase of total antioxidant capacity. Similar increase in total antioxidant capacity provided by a dose-dependent manner agrees with many of studies [72, 104]. In the study of de Morais et al. (2020), interaction of whey protein isolate and EGCG were carried out at pH 7 at 25ºC for 60 min with the different protein: phenol ratios from 1:1 to 1:0.1. The sample of whey protein isolate that interacted with EGCG at a 1:1 protein:phenol ratio showed approximately 75 μmol TE/g of sample, whereas the sample at a 1:0.1 protein:phenol ratio showed approximately 10 μmol TE/g of antioxidant capacity [68]. Therefore, in our study, the presence of a higher amount of GTE during modification of gluten, increases the phenolic groups incorporated to the protein structure and so total antioxidant capacity of gluten.

Another parameter that affects the protein-phenol interaction as well as total antioxidant capacity is pH of the medium. In this study, in order to understand the effect of pH on gluten-GTE interaction, One-Way ANOVA was applied to the samples. By doing so, the effects of GTE concentration and interaction time were omitted, where the pH of the gluten-GTE solution differed. The GTE-treated gluten samples with the same GTE concentration for the same interaction time at pH 9 showed lower antioxidant activity than the gluten samples treated at pH 7 (p<0.05). This outcome (higher antioxidant activity in the gluten samples treated at lower pH) is consistent with the results of Aewsiri et al. (2010) [70], Dai et. al (2022) [76] and Doğan Cömert and Gökmen [105]. When gelatin interacted with oxidized tannic acid at pH 9 or tannic acid at pH 7 and pH 9 antioxidant capacity of gelatin was increased from 10.52 μmol TE/g protein to 84, 94 and 88 μmol TE/g protein, respectively. This result indicated that the binding of unoxidized tannic acid provide higher antioxidant capacity to gelatin [70]. Therefore, in our study, stimulated oxidation of GTE phenolic compounds at pH 9 might result in lower increase in antioxidant activity of gluten than pH 7. Moreover, Rawel et al. (2005), observed higher binding affinity around the isoelectric point of bovine serum albumin pointing out the dominance of hydrophobic interactions [106]. Therefore, hydrophobic interactions of gluten amino acids, especially proline, with GTE phenolic compounds might be pronounced at pH 7 which might result in higher increase in antioxidant capacity of GTE-interacted gluten. In addition, Wang et al. (2020), in their study in which they investigate

the binding characteristics of gluten and quercetin, observed that once a hydroxyl group bonded to a specific site of the gluten, another hydroxyl group showed a steric hindrance reducing the affinity of protein binding site, increasing the binding distance. In addition to this, they reported that interaction distance of samples interacted at pH 9 were higher than pH 7 [107]. According to this, in our study, it can be expected that gluten interacted with GTE at pH 9 would have more binding distance which may result in lowered frequency of GTE phenolic compounds bonded to gluten. Moreover, it is well-known that the polymerization of quinones are stimulated under alkaline conditions. Therefore, in our study, under alkaline conditions, simultaneous formation and incorporation of polymerized GTE phenolic compounds during its interaction with gluten may lead to steric hindrance, preventing the binding of more phenolic moieties. Consequently, oxidation and polymerization of GTE phenolic compounds at higher pH may be the reason for the lower antioxidant activity of gluten ($p<0.05$).

According to analysis of co-variance, positive and moderate correlation between treatment time and the total antioxidant capacity of gluten was found with the correlation coefficient, 0.423. It was found that the total antioxidant capacities of treated gluten samples tend to decrease after 2 hours of treatment with GTE ($p>0.05$). It is thought that, due to the alkaline environment at pH 9, oxidation of GTE phenols occurred dominantly. Protein-phenol adducts might be formed by the nucleophilic addition of quinones, which are formed as a result of the oxidation of phenols. However, by prolonging the treatment time with GTE phenols, oxidized phenolic compounds might interact each other as well, leading to protein cross-linking, polymerization. In the study of Rohn, Rawel and Kroll (2004), interactions of bovine serum albumin and quercetin took place at pH 9 for 24 h with different bovine serum albumin:quercetin ratios from 20:1 to 2:1. With the increase in protein amount, the reactive quercetin sites were exposed to more and more crosslinking (polymerization) with the bovine serum albumin-quercetin complexes which resulted in lower antioxidant ability of complexes. Therefore, it has been reported that the crosslinking of protein-phenol interaction or polymerization is partly responsible from loss of antioxidant ability [69]. In our case, the treatment of gluten with GTE for 3 hours might possibly lead to more gluten-quinone-gluten cross-links which may result in lower free hydroxyl group of phenolic compounds of GTE. Therefore, loss of hydroxyl groups of phenolic compounds which provide radical scavenging activity might cause lower

antioxidant activity. Moreover, similar results were obtained in the work conducted by Doğan Cömert and Gökmen [105]. They stated that the increase in treatment time caused slight increase first, but then slight decrease in total antioxidant capacities of wheat bran, oat bran and rice bran treated with green tea infusion [105].

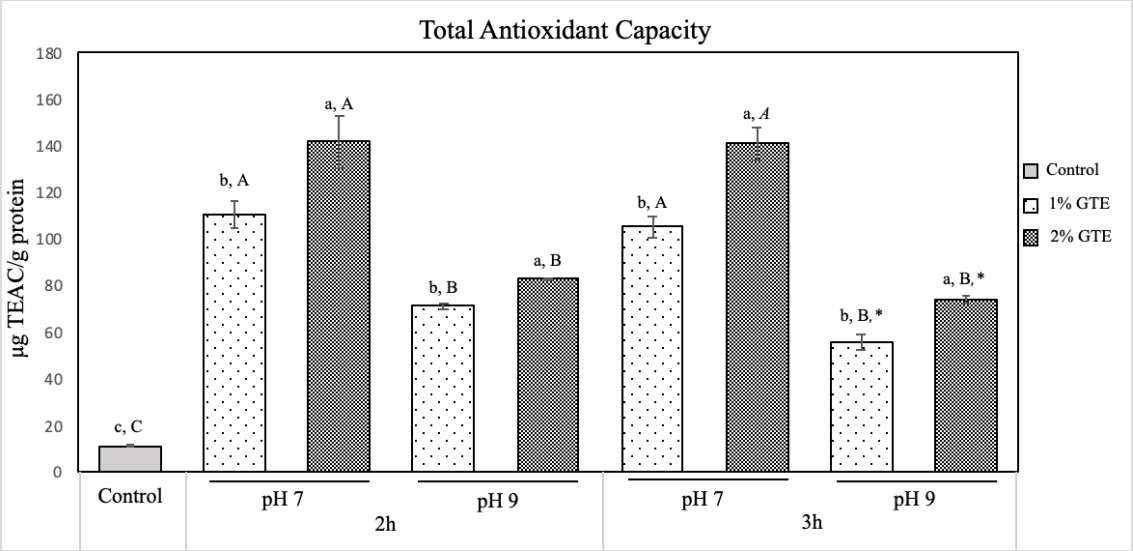


Figure 4. 1. The changes in total antioxidant capacity of gluten samples treated under different conditions.

*Lowercase letters refer to change in total antioxidant capacity according to GTE concentration of interaction, uppercase letters refer to change in antioxidant capacity according to interaction pH. Same letters indicate no statistical difference (p>0.05) and * indicate significant difference according to interaction time according to Duncan's test (p<0.05).

4.1.2. Amino Content

Reduction in the amount of free amino groups is one of the indicators to confirm the protein-phenol interaction. In the aforementioned study of Aewsiri et al. (2010), to obtain both covalent and non-covalent interactions, different interaction parameters such as oxidized or non-oxidized tannic acid and pH 7 or pH 9 were applied. To confirm the types of interactions whether they are covalent or non-covalent, the samples were treated with and without SDS first and then free amino content of samples were analyzed. Samples modified with tannic acid at pH 7 and pH 9 and treated with SDS showed less decrease in amino content when compared to those samples without SDS treatment. This differences after SDS treatment indicated that the interaction of gelatin with tannic acid

at pH 7 and pH 9 occur via both non-covalent and covalent interaction. Because SDS is a denaturing agent, treatment of samples with SDS lead to the destruction of non-covalent bonds, and therefore to the increase in amino content. On the other hand, sample modified with oxidized tannic acid at pH 9, which stimulates the covalent type interactions, did not show significant change in amino content after treated with SDS, as SDS cannot destroy the covalent interactions. These results showed that denaturing agents such as SDS or urea destroyed non-covalent protein interactions such as hydrogen bonds and some hydrophobic interactions [70]. In our study, samples were treated with 8M urea to solubilize the samples prior to free amino group analysis. Due to the destruction of non-covalent interactions by concentrated urea, it is assumed that most probably covalent interactions were measured. Therefore, the amino contents given in this study might represent the modification of amino residues as a result of covalent bonding.

Determination of the free amino group content is based on the reaction of OPA (o-phthaldial-dehyde) reagent with free amino groups of gluten resulting in color change [96]. The free amino group content of native gluten and gluten treated with GTE were given in Table 4.1. The amino contents of the samples were calculated in Serine Equivalent (SE). While the amino content of native gluten was found to be 178.91 ± 3.28 μg SE/g sample, the amino content decreased to 147.66 ± 0.47 μg SE/g in gluten treated with 2% GTE at pH 9 for 2 h (p<0.05). These conditions lead to the highest incorporation of GTE phenols via amino residues of gluten. According to co-variance analysis, correlation coefficients of effects of pH, time and GTE concentration parameters on free amino content of gluten samples treated with GTE were found -0.788, -0.455, -0.353. These results indicated that the relation between pH and amino contents of gluten samples were stronger than those of others. Also, all interaction parameters found to have negative correlation with free amino content which means all interaction parameters have supportive effect on binding of GTE phenolic compounds and so decrease in free amino content.

According to Table 4.1 all of the samples treated at pH 9 showed significant decrease in free amino content (p<0.05). This might indicate that the interaction between amino groups of gluten and GTE phenols were more pronounced at pH 9 which stimulates

covalent binding via nucleophilic addition reaction mostly. Even though higher antioxidant activities were obtained in gluten samples by treatment at pH 7, significant differences were not observed in free amino groups of some of the samples treated at pH 7, since non-covalent interactions such as hydrogen bonding, hydrophobic bonding and van der Waals forces between GTE phenolic compounds and gluten took place at pH 7. This might be related to the reactivity of amino acids depending on the pH of the solution. Because amino acids consist of both amino and carboxyl groups, and they can act as acid, base or zwitterion depending on pH of medium. This arise from ability of proton donation of carboxyl group and also ability of proton accepting of amino group. Proton donation (occur under alkaline condition) makes amino acids anion, whereas proton accepting (occur acidic condition) makes amino acids cation. Zwitterion form is the situation that carboxyl group of amino acid donated a proton, correspondingly amino group of amino acid accepted a proton. Therefore, in zwitterion form, amino acid is charged both negative and positive and have zero net charge. The pH that the amino acid become zwitterion is called as isoelectric point (pI). When the zwitterion is titrated with acid, COO^- group become protonated and turn into COOH. pK_{a1} is the value that COO^- and COOH concentrations are equal. When the zwitterion is titrated with base, NH_3^+ group become deprotonated and turn into NH_2. pK_{a2} is the value that NH_3^+ and NH_2 concentrations are equal. Asparagine, arginine and lysine are the amino acids that have ionizable amino side chain. pI values of these amino acids are 5.41, 10.76 and 9.74, respectively. pK_a value of ionizable side chain (pK_{a3}) of arginine is 12.48 whereas lysine 10.53. Amino acids loss H in their side groups at $pH>pK_a$, whereas retain at $pH<pK_a$ [5]; asparagine, arginine, lysine are still available toward the reactive quinones of GTE phenolic compounds at pH 9. Moreover, non-covalent protein-phenol interactions are more dominant at neutral pH [52].

Table 4. 1. The amino contents of control and gluten samples treated with GTE according to treatment conditions.

Interaction Parameters			
GTE Concentration	**pH**	**Time (h)**	**Amino Group Content (µg Serine Equivalent/ g sample)**
0	6.6	0	178.91 ± 3.28 b, *a*, A
1%	7	2	168.28 ± 5.16 ab, *ab*, A
1%	7	3	170.78 ± 2.97 b, *a*, A
2%	7	2	185.78 ± 1.09 a, *a*, A
2%	7	3	161.72 ± 5.16 b, *ab*, B
1%	9	2	159.18 ± 2.08 c, *b*, B
1%	9	3	154.84 ± 0.16 c, *b*, B
2%	9	2	147.66 ± 0.47 c, *b*, B
2%	9	3	148.44 ± 4.38 c, *b*, B

*The same lowercase letters following the mean values indicate no statistical difference ($p>0.05$) according to Duncan's test. The first line refers to control sample which is native gluten. Normal superscript letter refers to change in amino content according to GTE concentration, italic letters refers to change in amino content according to pH and uppercase letters refers to change according to time.

As given in the Table 4.1, in the gluten samples treated with 1% GTE at pH 7 for both 2 and 3 h there were no decrease in the amount of amino groups ($p>0.05$). The only significant decrease in the amount of amino groups of the samples could be observed in the gluten treated with 2% GTE for 3 h ($p<0.05$). This indicates that high GTE concentration and time are required for binding of GTE phenolic compounds to amino groups of gluten at pH 7. Moreover, although the treatment of gluten with GTE at pH 9 provided the decrease in free amino groups, the increase in GTE concentration did not affect those amino content. This might be explained by the mechanism proposed by Doğan Cömert and Gökmen [105]. Quinones which are oxidized forms of GTE phenolic compounds, might bind to the free amino groups of gluten, but they might be subsequently polymerized by binding on the top of another quinone on the surface. Therefore, their initial binding of phenolic compounds to the free amino groups of gluten may lead to decrease in amino content, and further bindings (polymerization) of quinones may lead to increase in antioxidant capacity without leading to decrease in free amino groups.

4.1.3. Thiol Content

As the thiol groups are one of the target residues for the interaction of phenolic compounds with proteins, free thiol contents of the samples were analyzed. The thiol group amounts of native gluten and gluten samples interacted with GTE under different conditions given in the Table 4.2. In literature, thiol content of gluten reported to be between 0.16 to 12.37 μmol/g [39, 61, 108]. In our study, thiol content of native gluten was found to be 36.351 ± 2.56 μmol/g sample and it was reduced to 28. 29 ± 0.64 μmol/g sample after treating with 2% GTE at pH 7 for 3 h. The difference between thiol results and the literature might be because of different analysis methods applied. According to co-variance analysis, correlation coefficients of effects of pH, time and GTE concentration parameters on thiol content of modified gluten samples were found as 0.435, -0.161, and -0.184, respectively. These results showed that the thiol contents were mostly affected by pH in the GTE treated gluten samples. The positive and moderate correlation between pH and thiol group amounts indicated that GTE phenols could not be incorporated into gluten through thiol groups as pH increases. Secondly, negative and low correlations were found between the thiol group content and both GTE concentration and time. This outcome can indicate that the GTE concentration and interaction time have almost no effect on binding of GTE phenolic compounds to thiol group of gluten.

The decrease in thiol group content in gluten samples treated with GTE at pH 7 ($p<0.05$) points out that the incorporation of GTE phenolic compounds might occur via thiol side group at pH 7. Such significant decrease could not be observed in thiol groups of gluten treated at pH 9 in this study, indicating that GTE phenolic compounds could not bind to gluten through thiol groups ($p>0.05$). However, in the literature, the reduction in thiol groups with the interaction of protein and phenolic compounds under alkaline conditions has been reported [109, 110]. Interaction of soy protein isolate with anthocyanin at pH 8 for 2 h resulted in reduction of thiol group from 160 μmol/ g protein to 10 μmol/ g protein [109]. The covalent modification of flax seed protein by hydroxytyrosol (at pH 9 for 2 h) led to decrease in thiol group from 40.39 ± 1.30 to 2.02 ± 0.37 nmol/mg protein [110]. This might be due to the dominant preference of amino groups at pH 9. As discussed in Section 4.1.2, GTE phenolic compounds preferred to bind to gluten through amino groups at pH 9, whereas preferred through thiol groups at pH 7. This most probably arises from the negative net charges of amino groups of gluten at pH 9. In addition to this, Rahaman

et al. (2016), revealed that the exposure of thiol groups in gluten was highly affected by pH and the exposure of thiol group of gluten was maximum at pH close to isoelectric pH [39]. The pK_a value of thiol group is 8.5. Therefore, at pH 7 which is below pK_a value, thiol groups might remain protonated form to interact with GTE phenolic compounds which might result in decrease in thiol content. On the other hand, thiol groups (R-SH) might be deprotonated (loss of H^+) and form negatively charged thiolates ($R\text{-}S^-$) at pH 9, above pH 7, might prevent the binding of thiol groups with GTE phenolic compounds. Besides the interaction pH, as being the other interaction parameters, prolonging time and increasing GTE concentration at pH 7 did not exert any more reduction in thiol content of gluten. Moreover, correlation coefficient between antioxidant capacities and thiol contents of samples was found -0.791 which means that incorporation of GTE phenolic compounds led to reduction in thiol content of gluten and provide antioxidant activity.

Table 4. 2. The thiol contents of control and gluten samples treated with GTE according to treatment conditions.

Interaction Parameters			
GTE Concentration	**pH**	**Time (h)**	**Thiol Group Content (µmol / g sample)**
0	6.6	0	36.35 ± 2.56 [a]
1%	7	2	28.10 ± 0.64 [b]
1%	7	3	30.21 ± 0.83 [b]
2%	7	2	29.53 ± 0.18 [b]
2%	7	3	28.29 ± 0.64 [b]
1%	9	2	35.90 ± 0.55 [a]
1%	9	3	36.29 ± 0.09 [a]
2%	9	2	36.48 ± 1.56 [a]
2%	9	3	37.26 ± 2.48 [a]

*The same lowercase letters following the mean values indicate no statistical difference ($p>0.05$) according to Duncan's test. The sample in the first line refers to control sample which is native gluten.

4.1.4. Thermal Analysis

The interaction of proteins with phenolic compounds usually induces changes in structure, denaturation temperature, enthalpy of unfolding and heat capacity. These modifications result from the binding process and subsequent protein unfolding [111-113]. Therefore, monitoring the thermal stability of proteins is a method to understand whether they have interacted with phenolic compounds or not. Thermal analysis is performed by measuring the melting point of samples using Differential Scanning Calorimetry (DSC). In DSC analysis, maintaining the sample and reference pan at same temperature leads to differences in the input energy between reference and sample due to heat absorbed or released by the sample. Therefore, the principle of DSC is based on measuring the thermal power which is required to maintain reference and sample at the same temperature as a function of temperature or time. The midpoint of transition or melting temperature T_m is defined as the state of native protein and its denatured conformations are in equilibrium. Therefore, molecules/samples which have higher T_m value referred as more stable molecules [114]. Change in T_m value due to protein-phenol interactions indicates binding/incorporation of phenolic substances to protein structure [75, 115, 116]. In a study, thermal denaturation temperature increased from 93 °C to 99 °C as a result of interaction of soy protein with phenolic acids at pH 9 for 24 hours [74]. T_m values of native gluten and gluten treated with GTE were obtained from DSC thermogram and given in Table 4.7. While T_m value of native gluten was found as 82.7 °C which is consistent with previous reports [117, 118], T_m values of gluten treated with GTE varied between 82.81- 93.45 °C. This change in T_m values was an indication of the interactions between gluten and GTE phenols took place under different pH, time and GTE concentration conditions.

Table 4. 3. T_m (ºC) values of gluten samples interacted with GTE under different conditions.

Interaction Parameters			
GTE Concentration	**pH**	**Time (h)**	**T_m (ºC)**
0	6.6	0	82.70
1%	7	2	91.25
1%	7	3	89.82
2%	7	2	92.26
2%	7	3	85.70
1%	9	2	82.81
1%	9	3	84.68
2%	9	2	88.00
2%	9	3	93.45

*The first line refers to control sample which is native gluten.

4.2. Effects of Gluten-GTE Phenol Interaction on Digestive Properties

4.2.1. Degree of Hydrolysis for Protein Digestibility

Determination of the degree of hydrolysis is based on the measurement of the absorbance value of the colored compound formed as a result of the reaction between the amino groups of proteins and OPA reagent [96]. In this study, in order to have an information about the digestibility, this analysis was done in bioaccessible fractions of digested samples. As it is well-known, the digestive enzyme trypsin cleaves the peptide bonds of C-terminal of basic residues such as lysine, arginine and chymotrypsin cleave the aromatic residues like phenylalanine, tyrosine, tryptophan [17]. Therefore if these action sites are blocked by binding or incorporation of phenolic compounds into the protein, digestive enzymes might not reach to proteins to cleave and digest proteins [119]. In addition to this, incorporation of phenolic compounds to protein structure might cause steric or chemical hindrance for the protease enzyme activity. This might result in decreased degree of hydrolysis. On the other hand, incorporation of phenolic compounds may cause protein structural and conformational change, unfolding of proteins [68, 120-122]. This led to exposure of target enzyme action sites, eventually increased digestibility [72, 121, 123].

Degree of hydrolysis of native gluten and gluten treated with GTE is given in Table 4.4. Compared to gluten, the degree of hydrolysis of the gluten treated with 1% GTE at pH 9 increased and the digestibility decreased when the GTE concentration was increased to 2% (p<0.05). This result indicated that the gluten structure is actually unfolded at pH 9, however the concentration of GTE is not sufficient for incorporation of quinones into the gluten structure when it is 1%. With the unfolding of the high-order structure at pH 9, gluten become more susceptible to digestive enzymes because of the absence of phenolic compounds bound to these regions. However, these more accessible sites also enable efficient incorporation of phenolic compounds to gluten when the phenol concentration is increased to 2% (w:w). This might result in blockage of active sites for digestive enzymes in gluten and thus decrease of the digestibility. Similar to our study, soy protein isolate was interacted with EGCG at pH 9 for covalent interactions and it has been reported that these covalent interactions caused lower digestibility of soy protein isolate. On the other hand, soy protein isolate was also interacted with EGCG at pH 7 for non-covalent interactions, and the digestibility was improved. In addition to this, the EGCG concentration affected soy protein digestibility significantly, while protein digestibility decreased from 76.17 ± 1.56% to 27.87 ± 2.67% with the highest EGCG concentration [63]. In the study of Morais et al. (2020) interaction of whey protein with EGCG and caffeic acid at pH 3.5 and pH 7 was investigated. Caffeic acid showed relatively strong binding affinity to whey protein at pH 7, resulting in decreased digestibility with the percentage of 13% [68]. Considering the stronger modification of gluten at pH 9 through covalent interactions, decrease in degree of hydrolysis is inevitable. On the other hand, modification of gluten at pH 7 did not lead to decrease in degree of hydrolysis, most probably to weaker non-covalent interactions.

Table 4. 4. Degree of hydrolysis (%) of gluten samples interacted with GTE under different conditions.

Interaction Parameters			
GTE Concentration	**pH**	**Time (h)**	**Degree of Hydrolysis (%)**
0	6.6	0	30.51 ± 2.52 [b]
1%	7	2	29.20 ± 1.16 [b]
1%	7	3	38.08 ± 2.16 [a]
2%	7	2	41.52 ± 1.69 [a]
2%	7	3	30.51 ±1.94 [b]
1%	9	2	41.57 ± 1.76 [a]
1%	9	3	40.81 ± 2.63 [a]
2%	9	2	17.29 ± 0.42 [c]
2%	9	3	16.42 ± 7.20 [c]

*The same lowercase letters following the mean values indicate no statistical difference (p>0.05) according to Duncan's test.

4.2.2. Total Free Amino Acid Content

Free amino acid contents of native gluten as well as gluten treated with GTE were measured after *in vitro* digestion process. Therefore, changes in the amounts of free amino acids which were released from the food matrix with the action of the digestive enzymes were compared to see the bioaccessibility of amino acids. Total free amino acid, essential amino acid, total reactive amino acid contents of native gluten and the gluten treated with GTE were given in the Table 4.5. The total free amino acid content was found to be 219.24 ± 105.25 µg/L in the native gluten. Interestingly, the only decrease in total free amino content has been observed in the gluten treated with 2% GTE at pH 9 for 2h (p<0.05), while there is no significant decrease in the amount of free amino acid content in the rest of the samples (p>0.05). This indicated that gluten-GTE interaction did not have a negative effect on the bioaccessibility of amino acids. On the other hand, despite there was a significant reduction in the total free amino acid content of the gluten treated with 2% of GTE at pH 9 for 2h, (p<0.05), total essential amino acid content remained same (p>0.05). Essential amino acids take part in the synthesis of peptides and non-peptide substances, regulation of gene expression, cell signaling pathways, energy and nutrient metabolism, immune function in human body [124]. Therefore, considering these functions of essential amino acids, it is thought that it is important that the gluten-GTE interaction did not lead to decrease in the amount of essential amino acids.

Table 4. 5. Free and total amino acid concentrations (µg/L) of gluten samples interacted with GTE under different conditions.

Sample	PHE	LEU	ILE	MET	VAL	TRP	TYR	THR	ALA	SER	GLN	ASN	HİS	LYS	ARG	PRO	Total Free Amino Acid Content	Total Essential Amino Acid Conten	Total Reactive Amino Acid Content	Total Amino Acid that digestive enzymes act on Content
Control	9.30 ± 0.80 ^{c}	31.52 ± 6.01 ^{c,d}	14.88 ± 1.24 ^{b,c,d}	1.65 ± 0.19 ^{b}	6.24 ± 0.76 ^{a,b,c}	5.37 ± 0.25 ^{b,c}	6.48 ± 0.06 ^{c}	3.05 ± 0.33 ^{b}	4.34 ± 0.59 ^{a,b,c}	3.04 ± 0.13 ^{b}	21.46 ± 1.56 ^{e,f}	1.47 ± 0.06 ^{a,b,c,e}	2.02 ± 0.06 ^{c,d}	21.60 ± 1.14 ^{e,f}	7.24 ± 0.46 ^{c}	1.41 ± 0.1	219.24 ± 105.25 ^{c,d,e}	133.70 ± 49.62 ^{c,d}	45.84 ± 0.06 ^{c,d}	49.99 ± 0.43 ^{c,d}
%1 GTE x pH7 x2h	7.98 ± 0.44 ^{c}	36.37 ± 0.61 ^{b,c,d}	14.39 ± 3.45 ^{b,c,d}	1.32 ± 0.19 ^{b}	6.02 ± 1.37 ^{a,b,c}	4.09 ± 0.24 ^{c,d}	6.23 ± 036 ^{c}	2.29 ± 0.58 ^{b,c}	3.70 ± 1.10 ^{a,b,c}	2.62 ± 0.90 ^{b,c}	23.69 ± 1.42 ^{d,e}	1.07 ± 0.16 ^{c,d,e}	2.07 ± 0.50 ^{c,d}	24.34 ± 1.90 ^{d,e}	9.08 ± 0.61 ^{b,c}	n.d.	226.39 ± 124.60 ^{c,d}	136. 98 ± 59.92 ^{c,d}	48.22 ± 3.49 ^{c}	51.72 ± 2.19 ^{c}
%1 GTE x pH7 x 3h	12.48 ± 0.25 ^{b}	52.70 ± 1.49 ^{a}	19.30 ± 2.20 ^{a,b,c}	1.81 ± 0.52 ^{b}	8.68 ± 1.61 ^{a,b}	4.40 ± 0.36 ^{c}	8.86 ± 0.03 ^{b}	4.40 ± 1.01 ^{a}	6.02 ± 1.37 ^{a}	4.46 ± 0.85 ^{a}	40.14 ± 1.25 ^{a}	1.59 ± 0.14 ^{a,b}	3.08 ± 0.48 ^{a,b,c}	39.90 ± 1.16 ^{a}	10.61 ± 0.46 ^{b}	n.d.	336.53 ± 173.88 ^{a,b}	200.04 ± 79.29 ^{b}	70.26 ± 2.44 ^{b}	76.25 ± 1.05 ^{b}
%2 GTE x pH7 x 2h	7.61 ± 0.30 ^{c}	29.65 ± 3.71 ^{d}	9.24 ± 1.00 ^{d}	0.83 ± 0.10 ^{b}	4.54 ± 0.37 ^{c}	3.38 ± 0.66 ^{c,d}	4.20 ± 0.40 ^{d}	2.20 ± 0.01 ^{b,c}	2.99 ± 0.05 ^{b,c}	2.26 ± 0.13 ^{b,c}	25.53 ± 1.89 ^{d}	0.88 ± 0.05 ^{d,e}	1.44 ± 0.10 ^{d}	25.49 ± 1.89 ^{d}	4.96 ± 0.26 ^{d}	n.d.	194.63 ± 95.84 ^{d,e}	114.42 ± 39.45 ^{d}	41.20 ± 1.63 ^{d,e}	45.65 ± 2.20 ^{d,e}
%2 GTE x pH7 x 3h	8.00 ± 0.26 ^{c}	37.27 ± 3.61 ^{b,c,d}	15.02 ± 1.60 ^{b,c,d}	1.09 ± 0.20 ^{b}	6.59 ± 0.30 ^{a,b,c}	4.40 ± 0.36 ^{c}	6.30 ± 0.17 ^{c}	3.26 ± 0.14 ^{a,b}	4.47 ± 0.13 ^{a,b,c}	3.29 ± 0.17 ^{a,b}	26.58 ± 1.20 ^{d}	1.16 ± 0.16 ^{b,c,d,e}	2.30 ± 0.20 ^{b,c,d}	26.84 ± 0.69 ^{d}	7.97 ± 1.66 ^{c}	n.d.	238.91 ± 128.41 ^{c}	143.74 ± 61.55 ^{c}	50.07 ± 3.44 ^{c}	53.53 ± 3.14 ^{c}
%1 GTE x pH9 x 2h	18.43 ± 0.51 ^{a}	58.40 ± 2.32 ^{a}	24.65 ± 7.12 ^{a}	3.25 ± 1.10 ^{a}	8.32 ± 2.35 ^{a,b}	7.99 ± 2.08 ^{a}	10.57 ± 1.10 ^{a}	3.22 ± 0.86 ^{a,b}	5.52 ± 2.39 ^{a,b}	3.38 ± 1.09 ^{a,b}	34.83 ± 1.85 ^{b}	1.66 ± 0.50 ^{d,e}	4.00 ± 1.28 ^{a}	35.49 ± 1.84 ^{b}	17.90 ± 1.58 ^{a}	n.d.	372.88 ± 204.95 ^{a}	230.76 ± 104.80 ^{a}	80.85 ± 5.79 ^{a}	90.37 ± 3.42 ^{a}
%1 GTE x pH9 x 3h	13.57 ± 0.72 ^{b}	57.92 ± 0.81 ^{a}	22.31 ± 4.39 ^{a,b}	3.58 ± 0.84 ^{a}	9.40 ± 2.14 ^{a}	6.78 ± 0.60 ^{a,b}	8.01 ± 0.01 ^{b}	2.80 ± 0.51 ^{b,c}	4.25 ± 0.76 ^{a,b,c}	2.62 ± 0.39 ^{b,c}	30.68 ± 0.15 ^{c}	1.28 ± 0.01 ^{a}	3.37 ± 0.41 ^{a,b}	30.77 ± 0.79 ^{c}	16.38 ± 0.64 ^{a}	n.d.	329.39 ± 171.65 ^{b}	211.34 ± 93.18 ^{a,b}	70.18 ± 1.72 ^{b}	75.52 ± 0.27 ^{b}
%2 GTE x pH9 x 2h	7.88 ± 1.38 ^{c}	39.02 ± 0.32 ^{c}	11.60 ± 2.12 ^{c,d}	1.58 ± 0.29 ^{b}	3.87 ± 0.28 ^{c}	2.28 ± 0.39 ^{d}	4.91 ± 0.70 ^{d}	1.56 ± 0.14 ^{c}	2.32 ± 0.28 ^{c}	1.57 ± 0.22 ^{c}	16.58 ± 1.77 ^{g}	0.71 ± 0.1 ^{a,b,c,d}	1.70 ± 0.20 ^{d}	16.90 ± 1.82 ^{g}	8.24 ± 0.95 ^{c}	n.d.	187.82 ± 94.89 ^{f}	122.20 ± 50.18 ^{d}	36.34 ± 1.43 ^{e}	40.22 ± 3.32 ^{e}
%2 GTE x pH9 x 3h	7.83 ± 1.31 ^{c}	40.79 ± 5.14 ^{b}	13.55 ± 0.54 ^{c,d}	1.64 ± 0.33 ^{b}	4.47 ± 0.01 ^{c}	2.34 ± 0.52 ^{d}	4.27 ± 0.53 ^{d}	1.71 ± 0.03 ^{c}	2.96 ± 0.02 ^{b,c}	1.99 ± 0.29 ^{b,c}	18.32 ± 1.10 ^{f,g}	0.84 ± 0.04 ^{e}	1.93 ± 0.24 ^{c,d}	18.70 ± 1.12 ^{f,g}	8.40 ± 0.19 ^{c}	n.d.	201.69 ± 108.13 ^{d,e}	132.58 ± 61.78 ^{c,d}	38.12 ± 0.90 ^{e}	41.54 ± 2.27 ^{e}

*The values followed by the same lowercase letters are not statistically different within a column for each gluten sample interacted GTE under different conditions (p>0.05).

n.d. means not detected.

Lysine, asparagine, tyrosine, methionine, histidine, tryptophan, arginine amino acids (Figure 4.2) have possible side groups which might interact with GTE phenolic compounds. Therefore, these amino acids considered as reactive in this study. pKa values of amino, phenolic, imidazole and guanidyl groups are 10.2, 9.6, 7.0, 13.8, respectively [5]. Considering the pKa values of the side chains of amino acids, interaction between amino acid side chains and electrophilic quinones might be more favorable at pH 9. Whereas the total free reactive amino acid content of native gluten was found to be 45.84 ± 0.06 µg/L, it has been decreased significantly (p<0.05) with the treatment of gluten with 2% GTE for 2 and 3 h. The decrease in total reactive amino acid content in gluten samples treated with 2% of GTE at pH 9 (p<0.05) indicate that the interaction between amino acid side chains and quinones occurred favorably under these conditions. On the other hand, total reactive amino acid content of gluten samples treated with 1% of GTE at pH 9 were increased (p<0.05). This result showed that GTE concentration was also important for binding, besides pH value.

Besides these amino acids, in literature, it has been also reported that the proline residues, proline repeats are one of the determinants of protein-phenol interactions. Moreover, in many studies, interaction between derived immunogenic peptides, proline rich proteins such as salivary protein, casein and several phenolic compounds have been shown [125-127]. Proline content of native gluten was found as 1.41 ± 0.1 µg/L, whereas proline contents of gluten samples treated with GTE were not detected. Therefore, reduction in the proline content of gluten as a result of the its treatment with GTE under all interaction conditions indicate that the proline residues also preferential sites for binding.

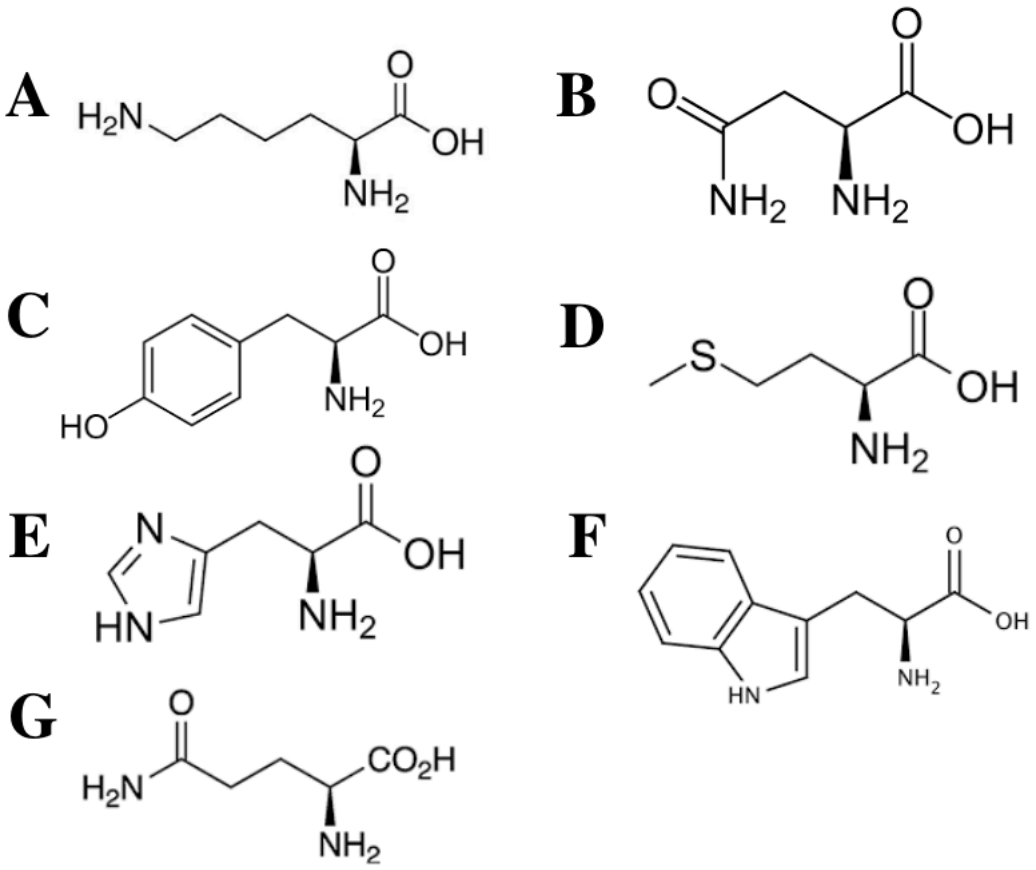


Figure 4. 2. Structures of amino acids.

*a) lysine, b) asparagine, c) tyrosine, d) methionine, e) histidine, f) tryptophan, g) arginine.

In the digestive process, trypsin cleaves the lysine and arginine residues, while chymotrypsin cleaves the phenylalanine, tyrosine and tryptophan residues [17]. Considering the total amounts of these amino acids, it can be seen that treatment of gluten with 2% GTE at pH 9 lead to significant decrease (p<0.05), compatible with the DH% results.

4.2.3. Immunogenic Gluten Peptides

Celiac disease is triggered by the formation of immunogenic peptides as a result of partial digestion of gluten. For this reason, immunogenic peptides were analyzed to understand the effect of GTE- gluten interactions on the formation of immunogenic peptides. In literature, there are lots of different long immunogenic gluten peptides quantified [21, 101]. In this study, the presence of some of these peptides, 33-mer, 26-mer and 19-mer, were confirmed. The concentration of 33-mer peptide after *in vitro* digestion of gluten

was found as 216.25 ± 11.39 µg/mL. As given in Table 4.6, there was an increase in the amount of gluten peptides in the samples treated with GTE phenolic compounds at pH 7 (p<0.05). On the other hand, treatment of gluten with GTE phenolic compounds at pH 9 resulted in less formation of gluten peptides. The highest decrease (57%) in the formation of gluten peptides (especially the most immunogenic one, 33-mer) was observed in the samples treated with %2 GTE at pH 9. These results suggested that the modifications of gluten at pH 9, which are most probably the covalent modifications, provide less immunogenic peptide formation. However, modifications of gluten at neutral pH, which are most probably non-covalent interactions dominantly, were not efficient in inhibition of immunogenic peptide formation. As it was mentioned before, immunogenic gluten peptides are rich in glutamine whereas does not contain cysteine residues. At pH 9, binding of GTE phenolic compounds could proceed through these amino residues, and therefore may lead to decrease in the release of gluten peptides. On the other hand, as it has been mentioned before, at pH 7, binding of GTE phenolic compounds to gluten occurred efficiently via thiol groups, without providing the elimination of immunogenic peptide release.

In literature there are a few studies investigating the interaction of gluten peptides in molecular level with EGCG which is one of the main phenolic compounds of GTE. The sequences of the gluten peptides represent potential binding sites for the phenolic compounds, such as aromatic side chains of tyrosine and phenylalanine and hydrophobic sections of proline, leucine, and glutamine. Van Buiten et al. (2019), reported that interaction of EGCG with 33-mer peptide were multi-phase reaction driven by non-specific binding. The first phase was endothermic reaction correspond to hydrophobic interactions, second phase was weak exothermic which suggests the formation of hydrogen bonds and crosslinking of protein-phenol complexes, third and fourth phases were endothermic reactions driven by polar interactions and hydrogen bonding until reaching a saturation point [128]. Similarly, Dias et al. (2018), revealed that the binding of EGCG to 32-mer peptide (the first leucine residue of 33-mer peptide lack) occurs spontaneously but with two types of binding events: the first with 2–3 and the second with approximately 5 EGCG binding sites. More importantly, they reported that the interaction of EGCG with 32-mer occurred through the different parts of the peptide, particularly in the regions have more leucine, proline and glutamine residues. Therefore,

those residues become more important for the interaction between gluten peptides and phenolic compounds. On the other hand, in our study, it has been revealed that pH-dependent selectivity of phenolic compounds for the binding sites took place during interaction of gluten with GTE phenolic compounds.

Celiac disease is initiated by translocation of gluten peptides which is generated by the partial digestion of gluten, to the lamina propria, subsequent a series of reactions which results in inflammation in epithelial wall. In this study, it has been revealed that the interaction gluten with GTE phenolic compounds at pH 9 could be considered as an efficient strategy to mitigate the release of immunogenic gluten peptides and reduce its immunogenicity in the perspective of celiac disease.

Table 4. 6. Changes in gluten peptides as a consequence of interaction of gluten with GTE phenols under different conditions.

Interaction Parameters			Change %		
GTE Concentration	**pH**	**Time (h)**	**33-mer**	**19-mer**	**26-mer**
1%	7	2	+56	+114	12
1%	7	3	+44	+69	+76
2%	7	2	+47	+213	+103
2%	7	3	+27	+241	+151
1%	9	2	-40	-69	-49.5
1%	9	3	-37	-30	-25.5
2%	9	2	-57	-32.5	-73
2%	9	3	-33	-3	-73

* Positive sign refers to increase in gluten peptides, whereas negative sign refers to decrease.

4.2.4. Bioaccessibility of Phenolic Compounds

Bioactivity has been defined as the ability of a compound that exhibit biological effect. To be defined as bioactive, the compounds must be absorbed through the intestinal epithelial layer/enterocytes and transported to target organ or tissues where they exert biological effect, health benefit. Bioaccessibility accounts to the release of compounds from ingested food, whereas bioavailability corresponds to absorption of these

compounds released from food matrix through intestinal epithelial layer, and their metabolism [129].

During digestion, compounds are released from food matrix by chewing, grinding, by the action of gastric acids, gastric and intestinal enzymes that hydrolyze lipids, proteins, carbohydrate. The fraction which is released from the food matrix during digestion in the gastrointestinal lumen, soluble in bulk aqueous phase, available for absorption by enterocytes and pass into blood stream called as bioaccessible. The undigested fraction that is not released from the food matrix, so could not become bioaccessible, reaches the colon to be further metabolized by colon microbiota [130].

in vitro digestion model consists of 3 main stages of gastric, intestinal and colon phase. The digest is centrifuged at the end of intestinal phase to separate the soluble (supernatant) and the insoluble (pellet) parts. The supernatant obtained by centrifugation corresponds to the bioaccessible fraction, whereas the remaining part corresponds to the fraction reaches colon [129]. In this work, native gluten and gluten treated with GTE were subjected to *in vitro* digestion, and the total antioxidant activity of bioaccessible fractions were measured to see the phenol bioaccessibility. During digestion, amino acids and peptides which exert antioxidant activity will be released as a result of protein hydrolysis. Despite this, it was found that bioaccessible fractions of samples exerted less antioxidant activity than their initial content (Table 4.7), indicating that the phenolic compounds could not be released from their complexes with gluten. This can be due to the strong binding of GTE phenolic compounds to gluten structure which provide increased stability of phenolic compounds during digestion and prevents the release of these phenolic compounds. In the study of Qie et al. (2022), even though the interaction of skimmed bovine milk-coffee interaction at 25 °C reduced the total phenolic content, skimmed bovine milk-coffee system had more total phenolic content during digestion compared with the milk-free coffee system. Therefore, interaction of coffee phenolic compounds with the skimmed milk protein showed protective effect on the phenolic compounds of coffee by reducing their loss during digestion [131].

Table 4. 7. Total antioxidant capacities of soluble fraction of digests.

Interaction Parameters			
GTE Concentration	**pH**	**Time (h)**	**Total Antioxidant Capacity (µg TEAC/g)**
0	6.6	0	12.05 ± 0.30 [e]
1%	7	2	23.36 ± 3.14 [d]
1%	7	3	40.06 ± 1.05 [b]
2%	7	2	42.62 ± 0.87 [b]
2%	7	3	48.55 ± 3.30 [a]
1%	9	2	27.78 ± 0.21 [c]
1%	9	3	27.95 ± 0.60 [c]
2%	9	2	27.91 ± 1.45 [c]
2%	9	3	26.24 ± 0.52 [c,d]

*The same lowercase letters following the mean values indicate no statistical difference (p>0.05) according to Duncan's test.

Less antioxidant activity found during *in vitro* digestion of treated gluten with GTE might indicate the incorporation of phenolic compounds into gluten provides the phenolic compounds to be retained in the non-bioaccessible fraction. Through further oxidation and polymerization, phenolic compounds might become larger and relatively insoluble, leading them to retain in the insoluble part of digests rather than soluble (bioaccessible) fraction [132]. Similar to protein-phenol interactions, interaction of phenolic compounds with other constituents of foods such as dietary fibers, pectin etc. might provide delivery of phenolic compounds to colon [133, 134]. Better stability of phenolic compounds because of protein-phenol interactions might provide their stable delivery to colon. Considering beneficial health effects of phenolic compounds on gut microbiota, this might be an important outcome of protein-phenol interactions. When polyphenols reached the colon, two-way interaction might take place; modulation of gut microbiota composition by polyphenols and catabolism of polyphenols by microbiota [135]. The composition of gut microbiome generally consists of phyla of Firmicutes, Bacteroidetes followed by Actinobacteria, Proteobacteria and Verrucomicrobia. The changes in gut microbiome through the increase in Firmicutes/Bacteroides ratio are accepted to be associated with metabolic disorders, obesity, diabetes, inflammatory bowel syndrome, irritable bowel syndrome, cardiovascular diseases etc. Studies demonstrate that the polyphenols which reach colon decrease this ratio and provide health benefits to host by prebiotic-like activity. Moreover, the undigested fraction is further undergone chemical

transformations by gut microbiome to generate metabolites which are bioavailable and able to modulate host metabolism [135, 136]. In the gut lumen, phenolic compounds are broken down by colon microbiota to generate absorbable bioactive metabolites especially called as short chain fatty acids (SCFA), such as acetate, propionate, butyrate. It has been shown that these components have influence on IL-10 production in B-cells, increase the number of protective IgA-secreting cells which means they can promote the tolerance of food antigens and modulate their response [138, 139]. Tian et al. (2021), showed that microbiota derived-memory related metabolites and neurotransmitters were significantly up-regulated by the administration of anchovy protein hydrolysate-catechin conjugates to mice [140].

As mentioned before, in celiac disease pathogenesis, interaction of tight junctions of intestinal epithelial with gluten peptide result in increased intestinal permeability. It has been reported that SCFAs can modulate tight junctions and improve their barrier function [139, 141]. Considering these information, gluten-phenol interaction might be a strategy to provide the phenols reach the colon, metabolized by colon microbiota and so benefit from gut-derived metabolites in the management of celiac disease, gut-related inflammatory diseases, food allergies, even though this is not the main purpose of this study.

4.3. Principal Component Analysis

Principal Component Analysis (PCA) was run to determine the differences between samples which were prepared by the treatment of gluten with GTE phenolic compounds under different conditions. PCA plot was given in the Figure 4.3. F1, explaining 59.10 % of the data variability, shows that the gluten samples treated at pH 9 form a distinct cluster to the right side, while the gluten samples treated at pH 7 are placed to the left. It has clearly seen that gluten treated with GTE phenolic compounds at pH 7 and pH 9 are differentiate each other with the percentages of 59.10%. As being closer to TAC and amino content vectors indicate that the gluten samples treated with GTE at pH 7 showed more antioxidant activity and had higher free amino content. On the other hand, as being closer to free thiol content vector means that the gluten samples had more thiol groups after treatment with GTE at pH 9. These highlighted once again that the interaction

between quinones and thiol groups of gluten were favored at pH 7, as well as the interaction between quinones and amino groups were favored at pH 9. Moreover, PCA showed that pH conditions of the treatment had an impact on DH%, total free amino acid (TFAA) as well as inhibition percentages of immunogenic gluten peptides. Considering the inhibition of immunogenic gluten peptides, gluten samples treated at pH 9 clustered closer to the vectors of gluten peptides inhibition, as opposite to the samples treated at pH 7. In addition to this, F2, explaining 22.40 % of the data variability, shows that at pH 9 the gluten samples treated with 2% GTE clustered below, whereas gluten samples treated with 1% GTE clustered above. This correlated well with DH% results, corresponding to the lower DH% in gluten samples treated with 2% GTE and higher DH% in gluten samples treated with 1% GTE.

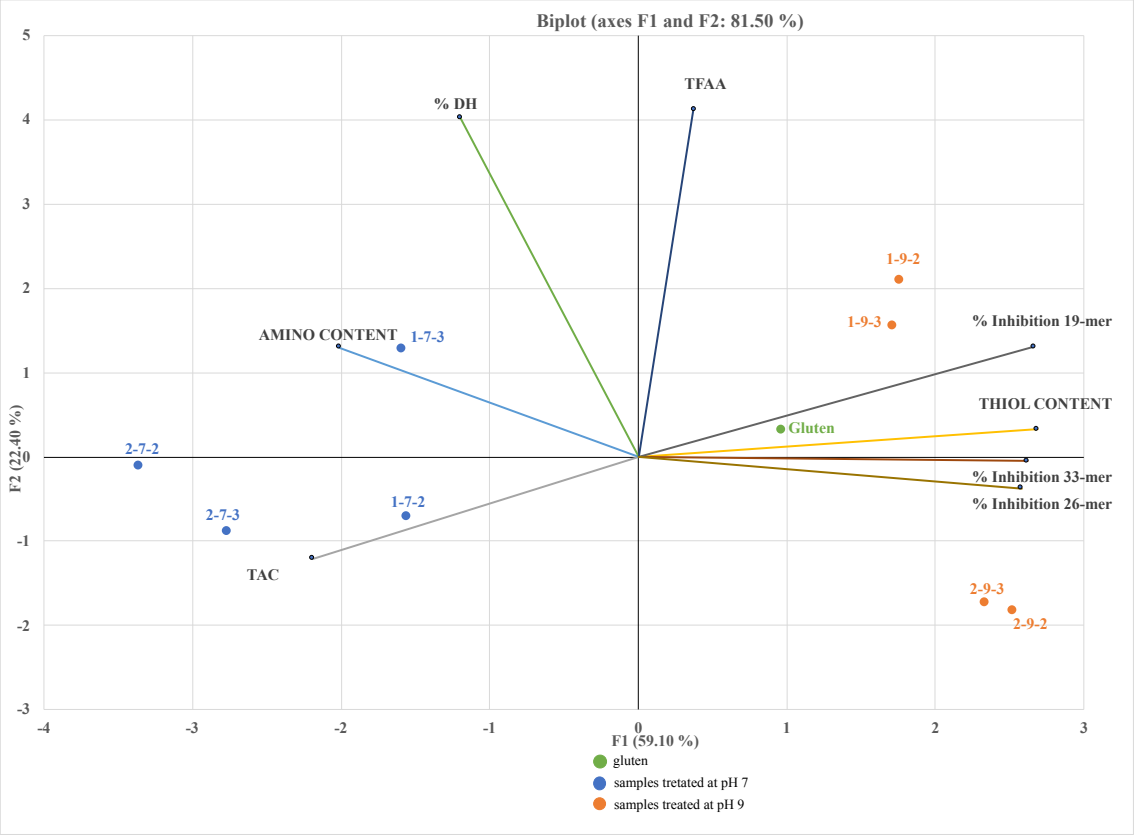


Figure 4. 3. Principal Component Analysis plot for the native gluten and gluten samples treated with GTE under different conditions.

*Samples nomenclature according to interaction conditions of gluten with GTE. e.g., 1-7-2- refers to gluten treated with 1% GTE at pH 7 for 2 hours.

4.4. Effects of Gluten-GTE Phenol Interaction on Bread Properties

Gluten modified with GTE resulting in less formation of immunogenic peptides was used for breadmaking to compare the effect of modification on technological properties of gluten. For this purpose, texture profile analysis, color analysis and porosity and loaf volume analysis were performed in bread samples.

4.4.1. Texture Profile Analysis

When wheat flour is mixed with water and kneaded, several physical and chemical changes occur. During mixing of flour with water, water binds to hydrophilic and charged groups of gluten. Gliadins and glutenins interact with each other by formation of covalent and non-covalent bonds. During kneading process glutenin polypeptides undergo sulfhydryl-disulfide interchange reactions which result in threadlike polymer formation. These linear polymers tend to interact with each other via hydrogen bonding, hydrophobic associations, and disulfide cross-linking to form a network-like film (known as gluten network). Moreover, non-covalent interactions such as hydrogen bonds, ionic bonds, hydrophobic bonds and Van der Waals forces are effective in gluten network formation [5, 142]. Gluten network have capability of entrapping gas which is produced during fermentation. Therefore, it plays crucial role in breads' softness, air cell size and distribution, volume etc.

In this study, breads were prepared from both native gluten and the gluten treated with 2% GTE at pH 9 for 2 h, which provided the highest decrease in immunogenic peptides. Important properties for breads such as hardness, chewiness, cohesiveness were monitored by texture profile analysis and results were given in Table 4.8. In texture profile analysis, hardness corresponds to the force applied in the first compression and it is related to bite force [143]. Hardness of control bread which was prepared with native gluten was found to be 1.11 ± 0.16 N, and it increased to 4.94 ± 0.10 N in the bread sample prepared with gluten treated with GTE (p<0.05). In a study, addition of catechin mixture to the bread led to increase in hardness of bread from 1.22 ± 0.06 N to 1.73 ± 0.07 N, because they might impede the formation of the gluten network [144]. Therefore, change in textural properties of gluten is inevitable. Similarly, Du et al. (2020), observed that hardness of gluten increased from 117.12 ± 7.00 N to 173.59 ± 14.89 N as result of

interaction of gluten with 8% tannin (w:w of gluten) for 1 h in the distilled water [61]. As it is well-known, gluten is responsible for the softness in bread making process. Its interactions with GTE phenolic compounds might cause to reduction in gluten network in dough formation process, thus, weaker gas holding capacity and reduced softness. According to the study conducted by Pan et al. (2021), 1% catechin addition to gluten caused reduction in gluten macro polymer content, α-helices, induction in β-sheets, β-turns and antiparallel β-sheets. In this study, the change in the proportion of α-helices structure explained with protein unfolding [84]. Therefore, reduction in α-helices of gluten as a result of its interaction with GTE can explain the reduced softness of breads.

Table 4. 8. Textural properties of bread samples.

Sample	Hardness	Cohesiveness	Springiness Index	Chewiness
		Traditional recipe		
Control	1.11 ± 0.16 ^{c}	0.57 ± 0.02 ^{a}	0.88 ± 0.003 ^{a}	4.44 ± 0.53 ^{c}
Modified gluten	4.94 ± 0.10 ^{a}	0.37 ± 0.02 ^{b}	0.81 ± 0.013 ^{c}	14.81 ± 1.42 ^{a}
		Modified recipe		
Control	0.98 ± 0.07 ^{c}	0.55 ± 0.04 ^{a}	0.85 ± 0.06 ^{b}	4.65 ± 0.33 ^{c}
Modified gluten	2.87 ± 0.28 ^{b}	0.40 ± 0.02 ^{b}	0.81 ± 0.007 ^{c}	9.64 ± 1.08 ^{b}

*The same lowercase letters following the mean values indicate no statistical difference (p>0.05) according to Duncan's test.

Especially the hardness and other textural parameters of bread sample containing modified gluten were found significantly different than control (p<0.05). Therefore, modification of the recipe has been done to improve the textural properties. Gluten-free bread formulations basically based on naturally gluten-free flours, hydrocolloids which is used to improve water binding capacity, mimic viscoelastic and cohesive properties of gluten, give stability to product and protein which is used to improve non-gluten network/dough structure, gas holding capacity, bread volume with the induction of protein-protein interactions. In the literature, it has been reported that carboxy methyl cellulose, guar gum, sodium alginate, xanthan gum as hydrocolloids and egg proteins, milk proteins, soy proteins as alternative protein sources are frequently used to improve these properties in gluten-free bread formulations [145-147]. Bian et al. (2023), reported that 8% soy protein isolate substitution improved the specific volume, hardness and springiness of quinoa bread from 1.67 ± 0.009 to 2.29 ± 0.05 mL g−1, from 2671.45 ±

62.57 to 1496.47 ± 85.21 g and from 0.58 ± 0.02 to 0.71 ± 0.03%, respectively [148]. Also, Mohammadi et al. (2015), reported that the addition of guar gum ($30gkg^{-1}$) significantly decreased crumb hardness from 72.47 ± 2.42 to 26.54 ± 2.37 and increased specific volume from 1.76 ± 0.03 to 1.93 ± 0.06 of the gluten-free bread [149]. In our study, guar gum and soy protein isolate were used to improve textural properties of bread which prepared with gluten treated with GTE. Moreover, it is known that more water is usually added to gluten-free breads to observe similar consistency of dough to batter [146]. Considering this information, bread formulation was modified. The recipe modification by using these additives and change in water content did not cause significant differences between the breads prepared with two different recipes in terms of their hardness, cohesiveness and chewiness ($p>0.05$). Accordingly, similar hardness and the other textural parameters were provided by the modification in the recipe. By the addition of guar gum and soy protein isolate, hardness of the bread prepared by using gluten treated with GTE was reduced from 4.94 ± 0.10 N to 2.87 ± 0.28 N ($p<0.05$).

Cohesiveness refers to the tendency of molecules to remain together, strength of the internal bonds, internal resistance of food structure. It is calculated by the ratio of second compression (second chew) to first compression (first chew) during texture profile analysis. Breads with higher cohesiveness forms bolus instead of disintegration during mastication [93, 150]. Cohesiveness of the bread prepared by using gluten treated with GTE significantly decreased compared to control ($p<0.05$). Therefore, modified bread will disintegrate rather than forming bolus. Du et al. (2020), in their work, explained the altered texture of gluten, the decrease in cohesiveness, by the interaction between tannin with gliadin and glutenin [61]. Therefore, in our study, both covalent and non-covalent interactions between gluten and GTE phenols might cause weakening of internal bonds of gluten and decreased the tendency of molecules to remain together, namely cohesiveness. On the other hand, chewiness refers to the amount of energy needed for swallowing and it is associated with poor bread quality. According to the cohesiveness results, breads prepared with modified gluten have higher chewiness because they tend to disintegrate rather than forming bolus.

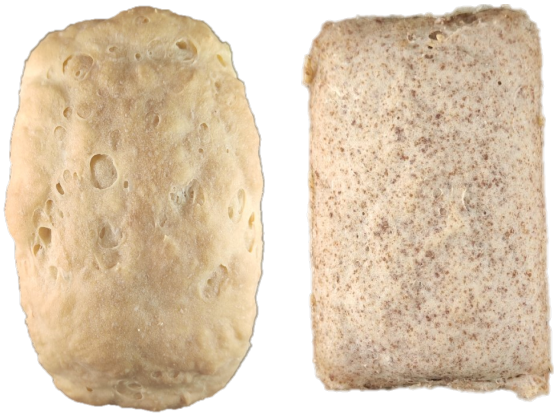
Springiness is defined as the rate of return to the state before deformation after removing the deforming force on the food. In the texture profile analysis, it corresponds to the time interval between the end of the first compression and then the beginning of the second compression. Springiness index is the ratio of the products recovery to its original height. These terms are related to the elasticity. It is known that glutenin macropolymer plays important role in elasticity of gluten [84]. Addition of polyphenols to gluten cause decrease in elastic solid content [94], depolymerization of gluten macropolymer [84]. In our study, similar result was obtained with the reduction in springiness of modified bread which result from gluten-GTE phenols interaction.

4.4.2. Color Analysis and Browning Index of Bread

Bread baking is a process which provide dough unique sensorial features. In general, the color and aspect of food product is the first quality parameter which is evaluated by consumer. Browning of baked products is provided by Maillard Reaction (MR), which results in the formation of brown colored pigments. Therefore, MR is one of the main reactions occurring in baked products and has an important role on the general consumer's acceptability. In this study, to monitor the changes in surface color, color parameters (L*, a*, b*) were measured by image processing. In the color measurements, L* refers to the lightness with values from 0 (black) to 100 (white), a* refers to red-green component of a color (+a and -a indicate red and green values, respectively) and b* refers to yellow-blue component of a color (+b and -b indicate yellow and blue values, respectively). Also, 100-L* values represents the browning index (BI) of bread [151]. In this study, color measurements were done for surface color of bread samples prepared according to modified recipe and their color parameters is given in the Table 4.9.

Bread containing GTE-treated gluten showed the greater L* values which means darker color formation. Therefore, browning index of this bread was less than the browning index of the control. Jannson et al. (2017), investigated the effect of GTE on MR in lactose-hydrolyzed ultrahigh temperature processed milk. They expressed that the binding of GTE polyphenols to amino acids and proteins might inhibit Strecker aldehyde formation [152]. Moreover, it is known that modification of amino groups of proteins by transglutaminase treatment, acetylation and succinylation inhibit MR and brown color

formation [153]. Therefore, the reduction in free amino groups of gluten due to its interaction with GTE phenolic compounds might mitigate MR and so lower browning ratio for the breads prepared with GTE-treated gluten. Moreover, bread crust sample containing GTE-treated gluten had greater a* value which indicates red color. On the other hand, control bread crust showed more yellowish color as indicated with the higher b* value. This can be explained by the formation of brown pigments as a result of oxidation of GTE phenolic compounds at pH 9 during its interaction with gluten [132]. Moreover, our results were consistent with the results of Pan et al. (2022) [144]



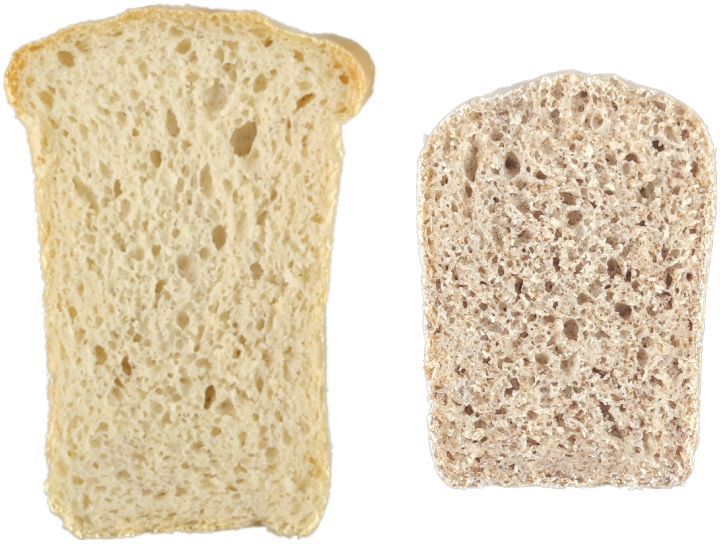
(a) (b)

Figure 4. 4. The photographs of bread samples prepared by using a) native gluten, b) gluten treated with GTE at pH 9 for 2 h with modified recipe.

Table 4. 9. Color parameters and browning indexes of breads prepared with modified recipe.

Sample	**L***	**a***	**b***	**BI**
Control	75.71	4.25	22.16	24.29
Modified gluten	77.53	4.35	15.32	22.47

4.4.3. Porosity and Brea Loaf Height



(a) (b)

Figure 4. 5. The photographs of crumbs of bread samples with modified recipe prepared by using a) native gluten, b) gluten treated with GTE at pH 9 for 2 h.

In bread making process, during mixing, air bubbles incorporate into dough. Carbon dioxide is released as a result of yeast metabolism during fermentation and air bubbles which previously incorporated grow and lead to expansion of the dough during proofing. Also, in the early stage of baking, yeasts show their greatest activity until the dough temperature reaches yeast inactivation temperature which is around 45°C, and so produce large amount of carbon dioxide gases. During baking, physical, chemical and structural changes take place as a result of simultaneous heat and mass transfer. The rising temperature also lead to thermal expansion of the vapor. Rising the temperature to 60°C of dough, its porous structure is set, transformed into crumb with the denaturation of proteins and gelatinization of starch [146, 154, 155]. Gelatinization of starch increases the dough viscosity and give resistance of dough to extension. In a study which starch: protein blends have different ratios were used as model flours for bread making process, bread prepared with the 15% of gluten concentration showed 24 ± 8 % porosity [156].

Treatment of gluten with GTE resulted in less porosity in bread, porosity ratios of breads prepared with modified recipe were found as 23.64 ± 6.88 % and 10.77 ± 1.41 % for the

control and GTE-treated gluten, respectively. This result was consistent with the results of Fu et al. (2018) [93] , and Wang et al. (2007) [157], which reports that the addition of GTE to the bread decreases its pore size and porosity. On the other hand, breads prepared with GTE treated gluten showed smaller pore sizes and denser crumb but also showed a more homogeneous pore distribution. Bread loaf height was measured as 101 mm for the control bread, whereas was 81 mm for the bread prepared by using GTE-treated gluten. Consequently, decrease in both porosity and loaf volume as a result of treatment of gluten with GTE were most probably due to the possible changes in high-order structure of gluten, as mentioned previously, which result in reduced capability of network forming and gas holding.

4.4.4. Gluten Peptide Analysis of Breads

As the main purpose of this study, it is also important to monitor the amount of immunogenic gluten peptides in the bread samples. The analysis of immunogenic gluten peptides, 33-mer, 26-mer and 19-mer, were done in the bread samples after *in vitro* digestion procedure was applied. Concentrations of the immunogenic gluten peptides in both control bread and bread prepared by using GTE treated gluten were found <LOD, which is 2 µg/mL. This result is compatible with the previous reports. Ogilvie et al. (2020) reported that the concentrations of 33-mer, 26-mer and 19-mer peptides after intestinal digestion of wheat bread were around 5 µg/mL, 2 µg/mL and 6 µg/mL, respectively [19]. In the study of Schalk et al. (2017), in which the immunogenic gluten peptide concentrations in flours obtained from different varieties of wheat has been investigated, it has been reported that the 33-mer peptide concentration ranges between 91–603 µg/g flour [23]. Considering the amount of gluten used for the bread making in this study, the amount of 33-mer is consistent with the results of this study, as well.

5. CONCLUSION

In this study, interaction of gluten and GTE were investigated in terms of celiac disease for the first time. The changes in the total antioxidant capacity, the amount of amino and thiol groups, and the thermal properties of the gluten confirmed that the interaction of gluten with GTE was successful under those conditions. Binding of GTE phenolic compounds to gluten at pH 7 took place through the thiol groups of gluten and provided more antioxidant activity than at pH 9, whereas binding of GTE phenolic compounds to gluten was favored via the amino groups of gluten at pH 9. Following the *in vitro* digestion, digestive characteristics of native gluten and GTE-treated gluten samples were monitored. The changes in the immunogenic gluten peptide concentrations indicated that the inhibition of immunogenic peptide formation can be achieved by the treatment of gluten with GTE at pH 9. The highest inhibition (57%) of 33-mer peptide, which is known as the most immunogenic gluten peptide, has been observed in the gluten treated with 2% GTE at pH 9 for 2 hours. These results revealed that the protein-phenol interaction can be a strategy to eliminate the immunogenic gluten peptide release. Moreover, soluble fractions of samples exerted less antioxidant activity than their initial content indicating that GTE phenolic compounds could not be released from its complexes with gluten during digestion and so reached the colon. Thanks to their complexation with gluten, delivery of GTE phenolic compounds to colon where SCFAs are generated by gut microbiota might be important for the prevention or reduction of gut-related diseases, even though this is not the main purpose of this study. On the other hand, protein-phenol interaction should be further investigated with the different phenolic compounds sources and different interaction parameters to achieve complete elimination of immunogenic gluten peptides.

Although there is a wide variety of gluten-free sources to use to produce gluten-free products, it comes with a high cost. Provided by the results of this study, GTE, an easily accessible ingredient, can be used for the interaction of wheat gluten, whose extraction is much easier and cheaper than other gluten-free ingredients. Gluten-free bread is still a challenge for celiac-disease patients, as it does not meet consumer expectations. In this study, the interaction of gluten with GTE under those conditions reduced the textural properties and browning indexes of bread. However, with the addition of guar gum and

soy protein isolate, textural properties and color measures of bread prepared with GTE-treated gluten were improved. As a result, in this study, it has been shown that gluten-GTE interaction is feasible to produce breads with reduced immunogenic peptide, acceptable textural properties as well as increased antioxidant activity for the celiac disease and/or non-celiac gluten sensitivity patients. Gluten treated with phenolic compounds can be used for the development of different gluten-free/ low-gluten products. Besides all of these, further in vivo studies are needed to reveal whether there are possible side effects and confirm the feasibility of gluten-phenol interaction in healthy food design.

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