INCREASING THE TOTAL ANTIOXIDANT CAPACITY BOUND TO INSOLUBLE DIETARY FIBER

ÇÖZÜNMEZ BESİNSEL LİFE BAĞLI TOPLAM ANTİOKSİDAN KAPASİTENİN ARTTIRILMASI

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EZGİ DOĞAN

ABSTRACT

INCREASING THE TOTAL ANTIOXIDANT CAPACITY BOUND TO INSOLUBLE DIETARY FIBER

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Bound antioxidants in addition to dietary fibers have beneficial health effects. They reach the colon without being digested through the small intestine and contribute to the formation of reduced environment in the colon, while soluble antioxidants ingested with diet cause a rapid and short-term increase in plasma antioxidant capacity upon absorption in the small intestine. Therefore, increasing the antioxidant capacity bound to dietary fiber has a potential significance for human health.

The aim of this master thesis was to provide an in-depth investigation of the interaction between insoluble wheat bran and polyphenols and a demonstration for the digestion behaviors of antioxidant compounds bound to dietary fiber. Treatment with tannic acid, but not gallic acid, increased the bound antioxidant capacity of insoluble wheat bran depending on its aqueous concentration ($p < 0.05$). Among the beverages tested (white and red wines, black and green tea infusions), treatment with green tea infusion caused the highest increase in the

total antioxidant capacity. Temperature, time, air and pH were found to significantly affect the reaction between insoluble wheat bran and polyphenols. The bound antioxidant capacity of insoluble bran increased to above 100 mmol TE.kg^{-1} after treatment with green tea infusion at optimum conditions (50 °C, pH 9.0, no airflow). In this thesis, bound antioxidant capacity measurements were performed with QUENCHER procedure using ABTS$^{\bullet+}$ radical solution. Concentration of free amino groups available in wheat bran significantly decreased (62.6%) after the treatment. The results suggested that polyphenols are oxidized to quinones under alkaline conditions further bound to free amino groups available on the surface of wheat bran. The *in vitro* digestion results indicated that green tea treated wheat bran, which has more bound antioxidant compounds than insoluble wheat bran, shows 20 times more antioxidant activity through the colon than untreated insoluble wheat bran in its insoluble fraction.

Keywords: bound antioxidants, insoluble wheat bran, treatment with soluble phenolic compounds, QUENCHER, in vitro digestion

ÖZET

ÇÖZÜNMEZ BESİNSEL LİFE BAĞLI TOPLAM ANTİOKSİDAN KAPASİTENİN ARTTIRILMASI

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Son zamanlarda yapılan çalışmalar obezite, diyabet, kanser gibi bir çok hastalığın beslenme ile ilişkili olduğunu göstermiştir. Bu hastalıkların önlenmesi için otoriteler daha sağlıklı besinlerin tüketilmesi konusunda insanları teşvik etmektedir. Bu nedenle, klinik olarak etkileri kanıtlanmış besinsel lifler ve antioksidan bileşikler son zamanlarda dengeli ve sağlıklı beslenmede önemli bir yer tutmaktadır.

Antioksidanlar metabolik faaliyetler sonucunda insan vücudunda oluşan oksidatif strese karşı koruyucu etki gösterirler. Böylece oksidatif stresin sebep olduğu kanser, kalp damar hastalıkları, bağışıklık sistemi bozuklukları, yaşlanma gibi dejeneratif rahatsızlıkların önlenmesinde etkili olurlar. Ayrıca, antioksidanlar gıdalarda oksidasyonu geciktirerek veya engelleyerek oksidasyon sonucu meydana gelebilecek toksik maddelerin, kötü tat ve koku oluşumunun, temel besin madde kayıplarının önlenmesinde rol oynarlar.

Antioksidanlar gıdalarda serbest veya bağlı formlarda bulunabilirler. Yakın zamana kadar mevcut literatürde, meyve ve sebzeler içerdikleri serbest çözünür antioksidanlar sebebiyle önemli antioksidan kaynakları olarak görülürken , tahıllar düşük miktarda serbest çözünür antioksidan içerikleri sebebiyle antioksidan kaynağı olarak göz ardı edilmişlerdir. Ancak son zamanlarda, araştırmacılar tahılların içerdikleri antioksidan maddelerin büyük bir kısmının bağlı formda olduğunu ve bu bağlı antioksidanların toplam antioksidan kapasiteye büyük katkı sağlayacaklarını ifade etmişlerdir.

Antioksidanların yanında besinsel lifler de özellikle gastrointestinal sistemde önemli bir etkiye sahiptir. Besinsel lifler, ince bağırsak boyunca sindirime ve absorpsiyona direnç gösterirken, kalın bağırsakta kısmen veya tamamen fermente olurlar. Diğer bir deyişle, besinsel lifler sindirilmeden kolona kadar ulaşır ve burada kolon mikroflorası için fermente edilebilir substrat olarak işlev görürler.

Besinsel liflere bağlı antioksidanları içeren gıdalar ise hem antioksidan maddelerin hem besinsel liflerin etkilerini aynı anda gösterirler. Özellikle son yıllarda ‘besinsel lif-antioksidan madde kompleksi’ oldukça önem kazanmıştır. Tam tahıllar besinsel liflere bağlı antioksidan içerikleri ile bu tür gıdalara en iyi örnektir. Tam tahıllarda bağlı antioksidanlar çoğunlukla kepek fraksiyonunda bulunurlar.

Diyetle alınan çözünür antioksidan bileşikler ince bağırsakta hızla emilerek plazma antioksidan kapasitesini yükseltirler. Ancak çözünür antioksidan bileşiklerin bu etkileri birkaç saat gibi kısa bir süre içerisinde ortadan kaybolur. Oysa besinsel liflere bağlı durumdaki antioksidan bileşiklerin önemli bir bölümü ince bağırsaktan sindirilmeden geçerek kolona ulaşır ve burada indirgen ortam oluşumuna katkı yapar. Life bağlı antioksidanların bir kısmı kolondaki mikrobiyal flora vasıtasıyla serbest forma dönüştürülür ve vücut tarafından emilerek bazal plazma antioksidan

kapasitesinin yükselmesini sağlar. Dolayısıyla, besinsel liflere bağlı antioksidanlar çözünür antioksidanlara kıyasla vücutta çok daha uzun süre etki gösterirler. Bu nedenle diyetle alınan besinsel liflere bağlı antioksidanların miktarının arttırılması insan sağlığı açısından potansiyel bir öneme sahiptir.

Bu yüksek lisans tezinin amacı, çözünmez tahıl liflerinin toplam bağlı antioksidan kapasitesinin, çözünür antioksidanlarla modifiye edilerek yükseltilmesidir. Bu etkinin ortaya çıkması ve mekanizmasının aydınlatılması için çözünür antioksidan kaynağı olarak serbest fenolik bileşiklerce zengin yeşil çay, siyah çay infüzyonları ve kırmızı, beyaz şarap, besinsel lif kaynağı olarak ise çözünmez buğday, yulaf ve pirinç kepekleri kullanılmıştır. 25 °C de yarım saat boyunca gerçekleştirilen reaksiyon sonucunda buğday kepeğinin antioksidan kapasitesini en fazla arttıran çözünür antioksidan kaynağının yeşil çay infüzyonu (3g/100ml) olduğu görülmüştür. Serbest antioksidan bileşiklerin besinsel lif yüzeyine bağlanma reaksiyonu üzerine konsantrasyon, sıcaklık, süre, oksijen ve pH gibi faktörlerin etkileri ve en yüksek düzeyde bağlanmanın gerçekleştiği optimum reaksiyon koşulları (50 °C, pH 9.0, hava akışı yok, 1 saat süresince) belirlenmiştir. Optimum koşullarda yeşil çay ile farklı kepek türleri (yulaf, pirinç, buğday) arasında gerçekleştirilen reaksiyon sonucunda yulaf kepeği ve pirinç kepeğinin antioksidan kapasiteleri sırasıyla 7,65 ve 9,29 kat artarken, buğday kepeğinin antioksidan kapasitesi 17,34 kat artmıştır. Tez kapsamında, bağlı antioksidan kapasite, ekstraksiyona gerek kalmaksızın ölçüme olanak veren QUENCHER metodu yardımıyla belirlenmiştir. Yeşil çay fenoliklerinin buğday kepeği yüzeyine bağlanmış olduğu yapılan toplam fenolik madde ölçümü ile de kanıtlanmıştır. Buna göre, reaksiyon sonucunda buğday kepeğinin toplam bağlı fenolik madde miktarının 10,5 kat arttığı gözlenmiştir.

Reaksiyon mekanizmasının aydınlatılması amacıyla serbest amino grubu analizi yapılmış, yeşil çay ile muamele edilen buğday kepeğinin serbest amino grubu kontrol örneğine göre %62,6 azalmıştır. 3 saat boyunca gerçekleştirilen kinetik ölçüm sonuçlarına göre, çözünmez buğday kepeği ile çözünür fenolik bileşikler arasındaki reaksiyon 2 aşamada gerçekleşmektedir. İlk aşamada yeşil çay fenolikleri alkali koşullarda kinonlara dönüşür ve bu elektrofilik aktif formlar buğday kepeği yüzeyindeki serbest amino grupları ile etkileşir. İkinci aşamada yüzeyde fenoliklerin polimerleşmesi sonucunda bağlı antioksidan madde miktarı artar.

Bağlı antioksidan kapasitesi yükseltilen besinsel liflerin sahip olduğu fonksiyonel özellikleri sindirim sisteminde uzun süre sergilemesi vücudun antioksidan savunma mekanizması açısından önemlidir. Bu nedenle tez kapsamında besinsel lif yapısına bağlanan antioksidan bileşiklerin simüle edilmiş *in vitro* sindirim koşullarında sindirim davranışları incelenmiştir. Buna göre, buğday kepeğinin yapısında bulunan bağlı antioksidanların bir kısmı gastrik fazdan sonra çözünür hale geçerken, kalan kısmı duodenal ve kolon fazında değişime uğramadan antioksidan aktivite göstermeye devam etmiştir. Yeşil çay ile muamele edilen buğday kepeği antioksidan aktivitesinin yaklaşık % 17 sini çözünmez formda kolon sonuna kadar taşımıştır. Yeşil çay reaksiyonu sonucunda çözünmez buğday kepeğinin bağlı antioksidan kapasitesinin yükselmesine bağlı olarak, bu örneğin kolon sonunda gösterdiği antioksidan aktivite çözünmez buğday kepeğinin kolon sonundaki antioksidan aktivitesinin yaklaşık 20 katına eşit olduğu görülmüştür.

Anahtar Kelimeler: Bağlı antioksidanlar, çözünmez buğday kepeği, çözünür fenolik bileşenlerle muamele, QUENCHER, in vitro sindirim

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ABBREVIATIONS

ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
BTI	Black Tea Infusion
CUPRAC	Cupric Reducing Antioxidant Capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
ET	Electron Transfer
EGCG	Epigallocatechin gallate
FRAP	Ferric ion Reducing Antioxidant Power
GA	Gallic Acid
GTI	Green Tea Infusion
GTE	Green Tea Extract
HAT	Hydrogen Atom Transfer
HPLC	High Performance Liquid Chromatography
LE	Leucine Equivalent
ORAC	Oxygen Radical Absorbance Capacity
ROS	Reactive Oxygen Species
SPSS	Statistical Package for the Social Sciences
TE	Trolox Equivalent
TEAC	Trolox Equivalence Antioxidant Capacity
TNBSA	2,4,6-trinitrobenzene sulfonic acid
TRAP	Total Radical Trapping Antioxidant Parameter
TROLOX	6-hydroxy-2,5,7,8 tetramethylchroman-2 carboxylic acid

1. INTRODUCTION

Recently, researchers have emphasized that most of diseases, in particular obesity, diabetes, some cancers, are linked with human diet. Therefore, dietary guidelines promote to consume more healthful foods and beverages leading to achieve and maintain a healthy weight, promote health, and prevent diet related diseases like obesity, type 2 diabetes, certain cancers and cardiovascular diseases. That's why, people prefer consuming the functional foods that contain biologically active compounds providing a clinically proven and documented health benefits. Antioxidant compounds and dietary fibers have come into prominence due to their functional properties.

Antioxidant compounds have crucial role in human body by means of counteracting the oxidative stress which plays a major role in the development of chronic and degenerative ailments such as cancer, cardiovascular diseases, arthritis, aging, autoimmune disorders and neurodegenerative diseases [1-5]. In addition, antioxidant compounds delay or inhibit the oxidation of oxidizable substrates in foods and prevent formation of off-flavor and toxic compounds or destruction of essential nutrients by oxidative damage.

Antioxidants may generally be found in free and bound forms in food. According to the previous literature, free soluble antioxidant compounds in fruits and vegetables are outstanding sources of antioxidants, while cereals are underestimated by the reason of their low levels of antioxidant capacity. However, in recent years, researchers have revealed that it is compulsory to bear in mind the contribution of bound antioxidant compounds to total antioxidant capacity of cereals since cereal grains include most of antioxidant compounds in bound forms.

Besides antioxidants, dietary fibers have also potential importance in human body especially in gastrointestinal tract. Dietary fibers are defined as compounds resistant to digestion and absorption through small intestine with complete or partial fermentation in the large intestine [6, 7]. Therefore, they reach to the colon without being digested and provide fermentable substrates for colon microbiota.

Foods including bound antioxidants in addition to dietary fibers have beneficial health effects of both dietary fiber and antioxidant compounds and, therefore, 'dietary fiber-antioxidant compounds complex' has become important in recent years [8-11]. Whole grains are great example to that as includes the antioxidant compounds in addition to dietary fiber. In whole grains, bound antioxidant compounds are mostly located in the bran fraction. Among other cereals, wheat bran is the most preferred cereal bran in human diet. It has a significant role in human health because of the content of bound antioxidant compounds in addition to cell wall materials.

Soluble antioxidants that are ingested with diet cause a rapid increase in plasma antioxidant capacity upon absorption in the small intestine, then, this effect of soluble antioxidant compounds disappears within a few hours [12]. On the other hand, a significant part of the antioxidant compounds bound to dietary fiber reach the colon without being digested through the small intestine and contribute to the formation of reduced environment in the colon [13, 14]. Some part of the bound antioxidants is converted to free form by the colon microbiota, and is released slowly and continuously [15]. It provides an increase in the basal plasma antioxidant capacity via absorption through the system [16]. Thus, antioxidants bound to dietary fibers exert their effects much longer than soluble antioxidants in living organisms. The continuous presence of a relatively low concentration of

antioxidant compounds may have a higher potential benefit for body defense than the peak of plasma antioxidant concentration observed immediately after the ingestion of a free antioxidant compound-rich food [15]. From this point of view, cereal dietary fiber-phenolic compounds complex are particularly useful.

Main objective of this master thesis was to develop a new functional dietary fiber with high bound antioxidant capacity and to investigate in-depth the mechanism of interaction between the insoluble wheat bran fraction and antioxidant compounds. To achieve this, insoluble wheat bran reacted with polyphenols from different sources under different conditions to better understand the reaction mechanism leading to increased bound antioxidant capacity. In addition, *in vitro* digestion behavior of wheat bran with high bound antioxidant capacity was investigated within the scope of this thesis.

2. GENERAL INFORMATION

This part gives the fundamental literature on the topics covered in the thesis. As the main topic covered within thesis is antioxidant compounds bound to dietary fiber, general literature information about mainly antioxidants and dietary fibers, importance of antioxidant compounds in addition to dietary fibers, their digestion behavior and health benefits will be discussed throughout this part.

2.1. Antioxidants

Oxygen, an indispensable element for life, has deleterious effects on the human body under certain conditions [17, 18]. When human cells use oxygen to generate energy, free radicals are created as a consequence of metabolism. As a result of the cellular redox process and external factors, such as irradiation and xenobiotics, by-products that are generally reactive oxygen species (ROS) formed in body [19]. These highly reactive molecules react with other molecules due to their unpaired electrons and generate oxidative stress that cause damaging of all cell structures. Oxidative stress brings about the development of chronic and degenerative ailments such as cancer, cardiovascular diseases, arthritis, aging, autoimmune disorders and neurodegenerative diseases [1-5, 20]. Preventing and repairing damages caused by ROS, are essential to obtain and preserve a good health by enhancing the immune defense and lower the risk of cancer and degenerative diseases [21, 22]. Fortunately, the human body has several defense mechanisms including enzymatic, metal chelating and free radical scavenging activities to counteract oxidative stress. Superoxide dismutase, catalase, glutathione systems are examples for antioxidant enzymatic defense system in human body. Antioxidants naturally found or produced as well as externally

supplied through foods, beverages or supplements, have a vital role in human defense system.

Endogenous and exogenous antioxidants inhibit the oxidation by different mechanisms such as scavenging free radicals, chelating prooxidative metals, quenching singlet oxygen and photosensitizers, and inactivating lipoxygenase [23]. During radical scavenging, as a primary reaction mechanism of antioxidants, antioxidants react with free radical molecules and neutralize them by donating electron(s) while antioxidants themselves are converted to free radicals which are less active, less harmful and more stable forms. They are also neutralized by other antioxidants compounds and mechanisms [24].

2.1.1. Antioxidants in Foods

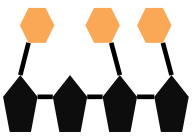
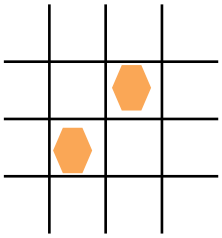
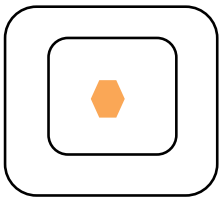
Food tissues are generally exposed to oxidative stress by the free radicals, reactive oxygen species, and prooxidants as they are living organism. Oxidative damage by free radicals also causes significant problems in foods either by producing off-flavor and toxic compounds or by destroying essential nutrients. For this reason, as a biological system, many of these tissues have developed antioxidant defense systems to control oxidation [25-28]. Antioxidants delay or inhibit the oxidation of oxidizable substrates in foods [29]. Antioxidant compounds in foods can be naturally present but also formed during processing. Most common dietary antioxidant compounds in foods are vitamins (C and E), carotenoids, chlorophylls and a wide variety of phytochemicals such as phenolic compounds, flavonoids, and complex polyphenols [30-32]. While green tea, fruits and vegetables are rich in flavonoids, cereals are rich in phenolic compounds especially phenolic acids. In coffee and bread crust, brown, nitrogenous

antioxidant macromolecules (melanoidins, melanoproteins) are occurred during thermal processing [33].

2.1.1.1. Location and Solubility of Antioxidant Compounds in Foods

Antioxidants may be generally found in two forms in foods as free (I) and bound forms (II, III, IV). More specifically, their location in foods may be divided into five groups: (I) free from chemical or physical interaction with other macromolecules; (II) chemically bound to other macromolecules that are insoluble antioxidant material (usually of high molecular weight); (III) ionically bound to food matrice; (IV) physically entrapped in food matrice and; (V) physically entrapped into different cellular structure (Table 2.1) [34].

Table 2.1. Location of antioxidant compounds in food structure

Free (I)	**Bound (II, III, IV, V)**			
	Chemically bound to macromolecules (II)	Ionic bound to food matrice (III)	Physically entrapped in food matrice (IV)	Physically entrapped within intact cell (V)
				

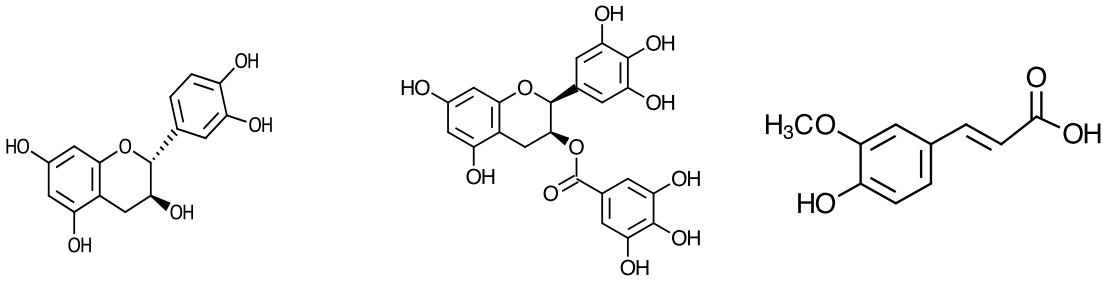
Antioxidants differ with respect to their chemical structures, solubility behaviors and locations in food structure. The solubility of the antioxidant compounds depend on the macromolecule that they are linked with and affect the compound's properties and activities. Interestingly, the same antioxidant compound might be present in both forms (free or bound forms) in different foods. For example, ferulic

acid is present in free form in many fruits while it is linked to cell wall polysaccharides through ester bonds in cereals [34]. Cereals represent the most typical examples of foods rich in the insoluble fractions of antioxidant compounds especially phenolic compounds [35], while most of fruits and vegetables represent the free soluble phenolic compounds rich foods. Because of the differences in structure, location and solubility of antioxidant compounds, the antioxidant activities, functional properties and health benefits of these beverages and cereals differ from each other. According to the previous literature, free soluble antioxidant compounds in fruits and vegetables are excellent sources of antioxidants, whereas grains are underestimated due to the relatively low levels of antioxidant activity. However, in recent years, researchers have revealed that cereals have significantly higher antioxidant capacity due to their insoluble bound phenolic compounds and these bound antioxidant compounds have potential role in human health [36, 37].

2.1.1.1.1.Soluble Antioxidants

Soluble antioxidant compounds in foods are divided into two groups as lipid-soluble and water-soluble. In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation [38]. Tocopherols and tocotrienols are example for lipid-soluble antioxidant compounds, whereas many water-soluble natural antioxidants, such as ascorbic acid, apple polyphenols, and tea polyphenols have been reported in the literature. Tea is one of the most consumed beverages throughout the world and it is rich in phenolic compounds [39]. While green tea is rich in flavan-3-ols, commonly named catechins (in particular epigallocatechin gallate (EGCG) (Figure 2.1)), black tea are rich in thearubigins,

which are the polymeric forms of flavan-3-ols. There is no doubt that soluble antioxidants are significant in preventing oxidative damage in foods or in human body; still the importance of bound insoluble antioxidant compounds will be emphasized throughout this thesis.



Catechin

Epigallogatechin gallate (EGCG)

Ferulic Acid

Figure 2.1. Structure of some phenolic compounds

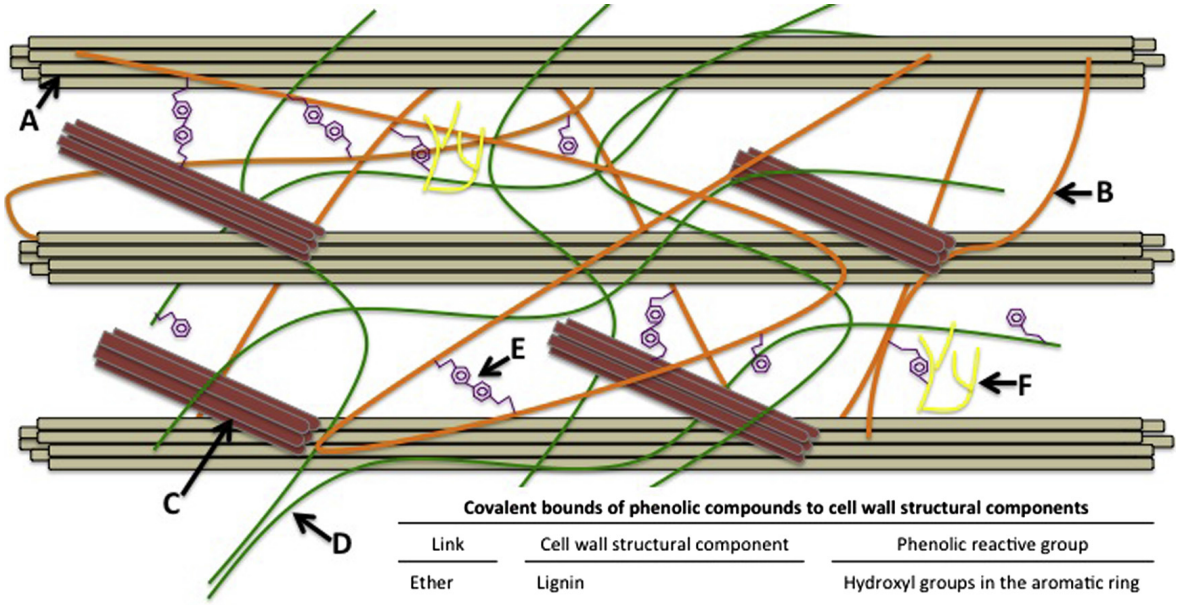
2.1.1.1.2.Bound Antioxidants

Fruits and vegetables contain mostly free and conjugated soluble phenolic compounds while approximately 24% of the total phenolics are in bound forms in these food matrices [40, 41]. Apple (*Malus domestica*), orange (*Citrus sinensis*) and onion (*Allium cepa*) contain 6.50%, 24.30% and 9.70% insoluble bound phenolics, respectively [41, 42]. Conversely, most of the phenolic compounds associated with whole cereal grains are in the insoluble bound forms [43]. About 85, 75, and 62% of the total phenolics are found in the insoluble bound forms in corn, wheat, and rice, respectively [40].

Phenolics in insoluble bound forms are covalently bound to cell wall materials such as cellulose, hemicellulose (arabinoxylan units) and proteins as shown in Figure 2.2. [43]. Phenolic acids, especially hydroxycinnamic acids (ferulic acid, sinapic

acid, cafeic acid etc.) form ether linkages with lignin through their hydroxyl groups and ester linkages with proteins and carbohydrates through their carboxyl groups [37, 44, 45]. These bound phenolic compounds have significant role as providing physical and chemical barriers, antioxidant activity, and antimicrobial functions [44, 46]. In addition, bound phenolic compounds have a vital position in human health owing to their releasing and absorption behavior in gastrointestinal system. However, these important properties will be discusses in-depth in section 2.3.2.

Because of the significant amount of antioxidant compounds are found in bound form in cereals, it is obligatory to consider the contribution to the total antioxidant capacity of cereals [47-52].



Covalent bounds of phenolic compounds to cell wall structural components

Link	Cell wall structural component	Phenolic reactive group
Ether	Lignin	Hydroxyl groups in the aromatic ring
Ester	Structural carbohydrates Proteins	Carboxylic group

Figure 2.2. Primary cell wall structure of plant material and cross-linking between structural components and phenolic compounds (A) Cellulose (B) Hemicellulose (C) Structural proteins (D) Pectin (E) Phenolic acids (F) Lignin [43]

2.1.2. Antioxidant Capacity Measurement

2.1.2.1. Classical Methods

There are several antioxidant capacity measurement methods that have similar principles [53-59]. In these methods, radicals are generated and the antioxidant capacity of the sample against radicals is measured [60]. The mechanism between radicals and antioxidant compounds are based on oxidation-reduction reactions. The scavenging or reducing activity of the antioxidant compounds is measured spectrophotometrically. Generally, antioxidant capacity measurements are divided into two main groups according to their transfer types as hydrogen atom (HAT) or electron transfers (ET) [61]. While oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP) assays are based on hydrogen atom transfer, Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) assays based on electron transfer. Most common antioxidant capacity measurement method for food samples is TEAC with ABTS or DPPH [62, 63]. This method principally measures the color changes as a result of the reduction of oxidant by antioxidant sample. All of these assays assume that all the antioxidant compounds present in foods are completely extracted by different extraction procedures [34]. However, complex structure of foods together with location and solubility of antioxidant compounds affect extraction efficiency. Most foods contain both lipophilic and hydrophilic antioxidant compounds that are soluble or insoluble forms [64, 65]. Therefore, there is no unique solvent or mixture to solve all of the antioxidant compounds in food structure and the resulting extracts are not representative of the exact antioxidant capacity. In summary, due to the diverse polarity of antioxidants, and most of them are covalently bound to insoluble food

matrice, measurement of the true ‘‘total antioxidant capacity’’ is still a challenge with classical methods [34].

2.1.2.2. QUENCHER Method

Above-mentioned classical methods are insufficient to measure the real antioxidant capacity of food. ‘QUENCHER’ method has been introduced to overcome the drawbacks of extraction methods. In this method, there is no need for extraction and hydrolysis processes. It is based on the direct measurement of solid samples by mixing them with the free radicals followed by a subsequent spectrometric measurement [34]. The principle of this method is the direct interaction between solid and radical solution such as $ABTS^{\bullet+}$ radical solution (Figure 2.3). In this assay, not only the soluble antioxidant compounds interact with radical solution according to the liquid-liquid type reaction but also insoluble antioxidant compounds come into contact with radical solution by means of the surface solid-liquid type reaction [34, 66]. In summary, QUENCHER method provides direct measurement of the antioxidant capacities of both insoluble and soluble compounds without an extraction procedure. This method is important especially for cereals, as their antioxidant activities are largely dependent on insoluble parts [67]. Chemical mechanism behind the classical methods can be adapted to direct QUENCHER procedure.

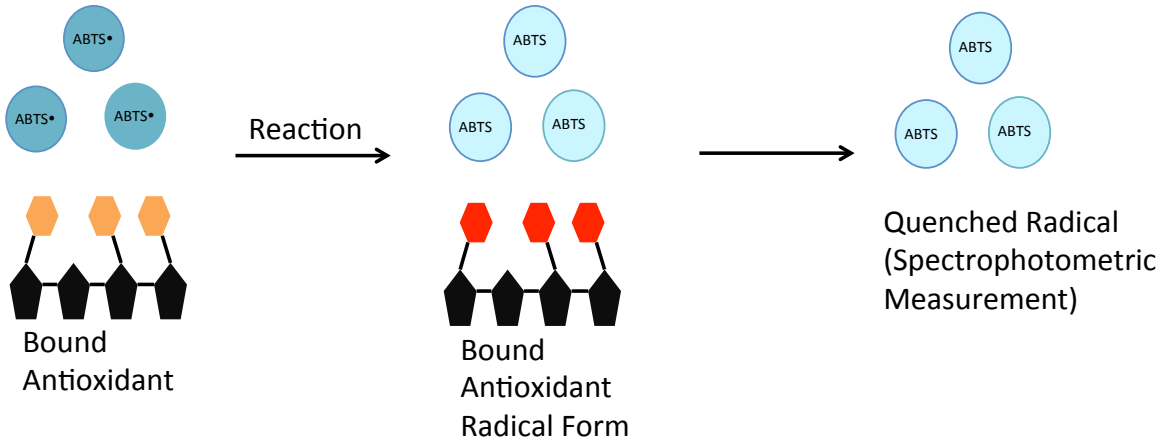


Figure 2.3. Mechanism of QUENCHER procedure

2.2. Dietary Fibers

Dietary fiber is generally defined as: ‘‘Dietary fiber consists of the remnants of edible plant cell, polysaccharides, lignin, and associated substances resistant to digestion and absorption through small intestine with complete or partial fermentation in the large intestine’’ [6, 7].

Cellulose, hemicellulose, lignins, pectins, and a variety of gums are typical components of dietary fiber. Raw and processed cereals, vegetables, legumes, and fruits are among the dietary fiber-rich foods in human diet. Because of their higher water content, fruits and vegetables provide less dietary fiber than the whole grains and cereal brans [68].

2.2.1. Soluble and Insoluble Dietary Fibers

Dietary fibers are divided into two groups based on their solubility in water: soluble dietary fiber and insoluble dietary fiber [69]. Soluble dietary fibers delay the gastric emptying by attracting with water and forming a gel, which slow down digestion [70]. Slowing the gastric emptying cause not only control the blood sugar level but also control the weight. In addition, they regulate the insulin sensivity, leading to control diabetes. Soluble fibers also reduce the LDL blood cholesterol level via

preventing the absorption of dietary cholesterol. Pectin and some types of hemicelluloses are main soluble dietary fibers present in foods, such as oatmeal, oat cereal, apples, oranges, pears, oat bran, strawberries, nuts, flaxseeds, beans, dried peas, blueberries, and carrots. Insoluble fibers pass through the gastrointestinal tract relatively intact, and speed up the passage of food and waste through the gut. In addition, they add bulk to faeces and prevent constipation. Insoluble fibers are mainly found in cereals and whole grains such as whole wheat, wheat bran, corn bran, seeds, nuts, barley, brown rice.

2.2.2. The Importance of Dietary Fibers in Human Health

Dietary fibers have a significant role in human health. Numerous studies indicate that dietary fiber consumption reduces the risk for diseases such as diabetes, coronary hearth disease, hypertension, obesity and especially gastrointestinal intestinal disorders [70-75]. Moreover, increasing the dietary fiber intake in human diet improves serum lipid concentration, lowers blood pressure, controls blood glucose level, improves weight lose and immune function [70, 76-80].

Dietary fibers have potential importance especially in gastrointestinal tract. Foods including high dietary fiber have lower energy density and it takes longer time to eat [70]. Soluble fibers disperse in water and can increase the volume and viscosity of intestinal contents [81-83]. Due to the non-digestible properties of dietary fibers, they increase the dry weight of intestinal contents and subsequently the amount of material passed into the large intestine [81]. In addition, dietary fibers can bind bile acids and impede micelle formation, thus increasing fecal excretion of bile acids and cholesterol [82, 84, 85].

The human intestinal tract is colonized by a complex microbiota, containing both potentially health-promoting and harmful microbes [86], and renders multiple

benefits such as immune regulation and degradation of indigestible food compounds [86, 87]. Dietary fibers also show prebiotic effects as “an indigestible food compounds that selectively stimulate the growth and activity of one or a limited number of microbial genus in the gut that confer health benefits to the host” [88]. Prebiotic effects in the gut can be evaluated based on the growth of bacteria that are generally regarded as beneficial such as lactobacilli and bifidobacteria. In addition to polysaccharides, dietary fibers can also be degraded by microbial action and subsequently some healthful products are produced such as short chain fatty acids (SCFA) [89]. SCFA including acetate, propionate, and butyrate are major anions in human colon and specific SCFA may reduce the risk of developing gastrointestinal disorders, cancer, and cardiovascular disease. While butyrate is the major energy source for colonocytes, propionate is largely taken up by the liver and acetate enters the peripheral circulation to be metabolized by peripheral tissues [90]. Ogue-Bon et al. showed that rice bran induced the bifidobacterium growth and SCFA production [91].

2.3. Antioxidants Bound to Dietary Fiber

Dietary fibers and antioxidant compounds have significant roles in human health. Foods including bound antioxidant in addition to dietary fiber show health effects of both dietary fiber and antioxidant compounds [92] and, therefore, ‘dietary fiber-antioxidant compounds complex’ has become important in recent years. Whole grains are great example to that as includes the antioxidant compounds in addition to dietary fiber [8-11]. In whole grains, bound antioxidant compounds are generally located in the bran fraction [13]. Liyana et al. showed that bound phenolics are approximately 40, 60, and 80% in flours, whole grains, and brans of wheat, respectively [37].

2.3.1. Wheat Bran Structure: As a Source of Antioxidant Bound to Dietary Fiber

Among other cereals, wheat bran is the most consumed cereal bran in human diet and has a significant role in human health because of the content of antioxidant compounds in addition to cell wall materials. The bran fractions consist of the pericarp, testa, and hyaline and aleurone layers. Nutritionally, bran fractions produced by milling are rich in fiber, minerals, vitamin B_6, thiamine, folate and vitamin E and some phytochemicals, in particular antioxidants such as phenolic compounds [93, 94]. Dietary fiber and phenolic compounds constitute 44.6% and 2.2% of the bioactive compounds in wheat bran [95]. The physiological effects of wheat bran can be split into nutritional effects (from the nutrients present), mechanical effects (mainly on the gastrointestinal tract, due to the fiber content) and antioxidant effects (root in the phytochemicals such as phenolic acid and alkylresorcinols) [94]. Studies have reported that non-endospermic tissues of wheat particularly the pericarp and testa contain high concentration of arabinoxylan units as an insoluble dietary fiber. Arabinoxylan units consist of α-(1,4)-linked D-xylopyranosyl residues with α-L-arabinofuranose side chains, potentially substituted with ferulic acid as an antioxidant compounds which accounts for up to 60% of wheat bran antioxidant capacity [96, 97]. Ferulic acids in addition to arabinoxylan unit are able to cross-link to other arabinoxylan units via diferulates [98, 99]. Dimerization of ferulates occurs by phytochemical coupling reactions or radical coupling reactions [99, 100] and allows the cell wall to build more stable structure [99]. Therefore, the solubility of arabinoxylan units decrease [15] and leading to be more resistant to digestive enzyme.

2.3.2. Digestion Behavior of Antioxidants Bound to Dietary Fiber

There are some doubts about in vivo antioxidant activity of antioxidant compounds bound to dietary fiber, due to the fact that dietary fibers may affect the release and absorption of some molecules, including phenolic compounds, and bioavailability of the antioxidants [101]. Therefore, it is necessary to understand the digestion behavior of antioxidant compounds associated with insoluble dietary fibers.

The mechanical effects of mouth release phenolic compounds present in vacuole or weakly bound to cell wall. However, phenolic compounds strongly bound to cell wall materials are not affected by the acid conditions in gastric phase or small intestinal enzymes. Dietary fibers reduce the absorption of phenolic compounds as a primary effect in small intestine. There are two main reasons for that: (i) phenolic compounds may be entrapped with dietary fibers or (ii) phenolic compounds may not reach the intestinal wall as a consequence of increased viscosity in gastric phase by the insoluble dietary fibers [102]. Therefore, a significant part of the antioxidant compounds bound to dietary fiber reach the colon without being digested through the small intestine [13] and contribute to the formation of reduced environment in the colon [14]. Some part of the bound antioxidants is converted to free form by the colon microbiota, and is released slowly and continuously [15]. It provides an increase in the basal plasma antioxidant capacity via absorption through the system [16]. Thus, antioxidants bound to dietary fibers exert their effects much longer than soluble antioxidants in living organisms. Kroon et al. showed that over 95% of the total feruloyl groups take place in colonic fermentation, whereas only 2.6% of total feruloyl groups in the fiber are released after gastric and intestinal enzymatic treatment [15]. According to the study performed on rats, free ferulic acid ingested with diet cause rapid increase in plasma antioxidant activity but this effect fade away within 4 hours. However,

ferulic acids in addition to insoluble dietary fiber cause the plasma antioxidant capacity stable even after 24 hours [12].

To sum up, the continuous presence of a relatively low concentration of phenolic compounds may have a higher potential benefit for body defense than the peak of plasma antioxidant concentration observed immediately after the ingestion of a free phenolic compound-rich food [35]. From this point of view, cereal phenolic compounds bound to dietary fiber gain importance and are particularly useful for human health.

2.3.3. The Importance of Bound Antioxidant in Human Health

Epidemiological studies have shown that consumption of wheat bran reduce the risk of colorectal and gastric cancers [103]. The reason for this beneficial effect is most probably the content of insoluble dietary fiber and antioxidant compounds [104-106]. The mechanism under protection against carcinogenesis may be direct removal of the negative effects of this carcinogenesis such as binding carcinogens [107], absorbing water resulting in increased fecal bulk and shortened transit times [105, 108], affect indirectly by lowering colon pH via SCFA production and specific effect of butyrate[15, 109]. Therefore, it prevents carcinogenesis exposure of colon mucosa. In addition, ferulic acid bound to insoluble dietary fiber shows antioxidant effect by scavenging free radicals [110-112], inhibits carcinogenesis in animal models [113, 114], protects against the formation of nitroso compounds [115] and prevents DNA damaged in cultured cells [116]. Furthermore, ferulic acid units in addition to dietary fiber exert their antioxidant effects much longer than free ferulic acid by quenching the radicals continuously formed in intestinal tract and they maintain reducing environment in colon. In the colon, fermentable fibers induce health-promoting bacterial growth, such as lactobacilli and bifidobacteria, by acting

as prebiotics [117]. Fogliano et al. investigated the prebiotic activity of insoluble cocoa fiber. They showed that insoluble cocoa fraction cause increase in lactobacilli and bifidobacteria numbers, which is related not only to insoluble fiber content but also to the flavanol compounds [118].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Potassium peroxydisulfate, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8 tetramethylchroman-2 carboxylic acid (Trolox), 2,4,6-trinitrobenzene sulfonic acid (TNBSA), leucine, Folin–Ciocalteu reagent, acetonitrile, ferulic acid, tannic acid, gallic acid and sodium carbonate were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethyl alcohol, methanol, n-hexane, sodium hydroxide, hydrochloric acid and sodium bicarbonate, potassium chloride, sodium chloride, magnesium chloride hexahydrate, potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Bile extract, porcine and the enzymes: pepsin (≥250 U/mg solid) from porcine gastric mucosa, pancreatin (4 x USP) from porcine pancreas, protease from Streptomyces griseus (≥3.5 U/mg solid) and viscozyme L were purchased from Sigma–Aldrich (Deisenhofen, Germany).

Wheat bran, rice bran and oat bran, as a dietary fiber sources, were purchased from the local market. As soluble antioxidant sources, red wine, white wine, black tea and green tea were purchased from the local market. Powder of green tea extract was purchased from Danisco (Copenhagen, Denmark).

3.1.2. Preparation of Solutions for Antioxidant Capacity Measurement

$ABTS^{\bullet+}$ working solution, which was prepared from $ABTS^{\bullet+}$ stock solution, was used for antioxidant capacity measurements. ABTS solution was prepared by adding 5 mL of deionized water to 38.41 mg of ABTS. Potassium peroxydisulfate solution was prepared by mixing 5 mL of deionized water with 6.615 mg potassium peroxydisulfate. A total of 10 mL of stock solution of $ABTS^{\bullet+}$ was prepared by

reacting 5 mL of each solution described above which resulted in final concentrations of 7 mmol/L ABTS and 2.45 mmol/L potassium peroxydisulfate. The stock solution of $ABTS^{\bullet+}$ was allowed to stand in the dark at room temperature for 12–16 h before use [62]. The working solution of $ABTS^{\bullet+}$ having absorbance of 0.75–0.80 at 734 nm was prepared daily by diluting the 10 mL of stock $ABTS^{\bullet+}$ solution with approximately 800 mL of a water/ethanol (50:50, v/v) mixture.

3.1.3. Preparation of Insoluble Dietary Fiber Sources

In this study, insoluble fractions of wheat bran, oat bran and rice bran were used as dietary fiber sources. First of all, bran samples were ground using a grinder and washing cycles were performed to prepare the insoluble fractions of dietary fibers as described by Çelik et al. [119]. One gram of ground samples (wheat bran, oat bran, rice bran) was defatted two times by 5 mL of hexane to remove lipids and lipid-soluble substances from the samples. The mixture was homogenized for 3 min using an ultra turrax disperser (Heidolph Instruments GmbH, Schwabach, Germany). The mixture was further shaken at 350 rpm for 10 min using an orbital shaker (Edmund Bühler GmbH, Hechingen, Germany). The supernatant was removed after centrifugation at 6080g for 5 min. After that, the defatted samples were consecutively washed with water, ethyl alcohol, and water, respectively, to remove water and alcohol-soluble substances. The mixture was further shaken at 350 rpm for 10 min using an orbital shaker and the supernatant was removed after centrifugation at 6080g for 5 min. After three times washing cycles, the dietary fiber fractions, which are free of water and alcohol soluble substances as well as lipid phase and lipid soluble compounds, were lyophilized and passed through a sieve (Endecotts Test Sieve, London, U.K.) having 40 mesh size (425 μm) to obtain insoluble powder. The final insoluble powders were tested by measuring

antioxidant capacity of final water and found to be free of soluble antioxidant compounds. They were kept frozen (−18 °C) prior to analysis of AC.

3.1.4. Preparation of Beverages Rich in Antioxidants and Pure Solutions of Antioxidants

Red wine, white wine, black tea and green tea were selected as the beverages rich in soluble antioxidant compounds. Red wine and white wine were used in the treatments with insoluble wheat bran fraction as it is or after ten-fold dilution (v/v) with distilled water. Green tea and black tea were used after brewing. A total of 3 g tea was brewed in 100 ml of boiling water for 15 min. Tea infusions were filtered through a coarse filter paper and used in the treatments with insoluble wheat bran fraction as is or after ten-fold dilution (v/v) with distilled water.

Aqueous solutions of green tea extract were prepared at concentrations of 10%, 0.5% and 1.0% (w/v) by dissolving appropriate amounts in distilled water.

Aqueous solutions of tannic acid and gallic acid, representing polymeric and monomeric phenolic standard compounds, respectively, were prepared at concentrations of 10, 20, 50, 100 and 200 μmol L^{-1}.

Aqueous solution of ferulic acid was prepared at concentration of 1 mmol L^{-1} that was the equivalent concentration of phenolic compounds in reacted green tea extract.

3.2. Methods

3.2.1. Antioxidant Capacity Measurements

The total antioxidant capacity was measured using the direct QUENCHER procedure described elsewhere, and expressed as mmol Trolox equivalent kg^{-1} (mmol TE.kg^{-1}) [119, 120]. Ten milligrams of insoluble fraction was weighed into a test tube, and the reaction was initiated by adding 10 mL of $ABTS^{\bullet+}$ working solution. The tube was vigorously shaken in an orbital shaker (at 350 rpm for 27

min) in the dark and centrifuged at 6080g for 2 min. After 30 min reaction time, the optically clear supernatant (2 mL) was transferred into a cuvette, and measurement was performed at 734 nm (for ABTS assay) using a Shimadzu model 2100 variable wavelength UV−visible spectrophotometer (Shimadzu Corp., Kyoto, Japan). If the values of absorbance were below the linear response range of the radical discoloration/color formation due to the high antioxidant activity, preliminary dilution was necessary. Dilution was performed by mixing the ground samples with cellulose, an inert material against the radical solution, at different ratios (1:1, 1:10, 1:100 (w:w) etc.) in a tube depending on the total antioxidant capacity values. Trolox was used as a standard reference to convert the inhibition percentage of each sample to the Trolox equivalent antioxidant capacity (TEAC). The antioxidant capacity values were expressed as milimoles Trolox per kilogram insoluble dietary fiber. The standard solutions of Trolox were prepared in methanol at a concentration range between 0 and 600μg mL^{-1}. For each concentration, 0.1 mL standard solution was transferred into a test tube and mixed with 10 mL of $ABTS^{\bullet+}$ working solution. The mixtures were allowed to stand in the dark for 30 min, at room temperature. Subsequently, 2 mL of radical solution was transferred into a cuvette, and the absorbance was measured at 734 nm to build corresponding calibration curves.

3.2.2. Treatment of Insoluble Dietary Fiber with Soluble Antioxidants

Insoluble wheat bran fractions were treated with beverages rich in soluble antioxidants (white wine, red wine, black tea and green tea) and aqueous solutions of pure antioxidants (tannic acid and gallic acid) prepared as explained above. A total of 50 mg insoluble wheat bran was weighed into a tube. The reaction was initiated by adding 5 ml of beverages or aqueous solutions of pure antioxidants

(treatment 1). The reaction tube was mixed using a magnetic stirrer at a speed of 350 rpm at 25 °C for 30 min. After reaction, the tube was centrifuged at 6080g for 2 min and the supernatant was removed. The remaining soluble antioxidant compounds that were not bound to insoluble wheat bran were removed by washing four times with ethanol and water, respectively. The antioxidant capacity of the water used in the last step was measured in order to be sure that final precipitate was free of soluble antioxidant compounds. Then, the final precipitate of insoluble wheat bran fraction was lyophilized prior to measurement of antioxidant capacity. According to results of antioxidant capacity measurements, the beverage which was the most efficient to increase antioxidant capacity of wheat bran was determined.

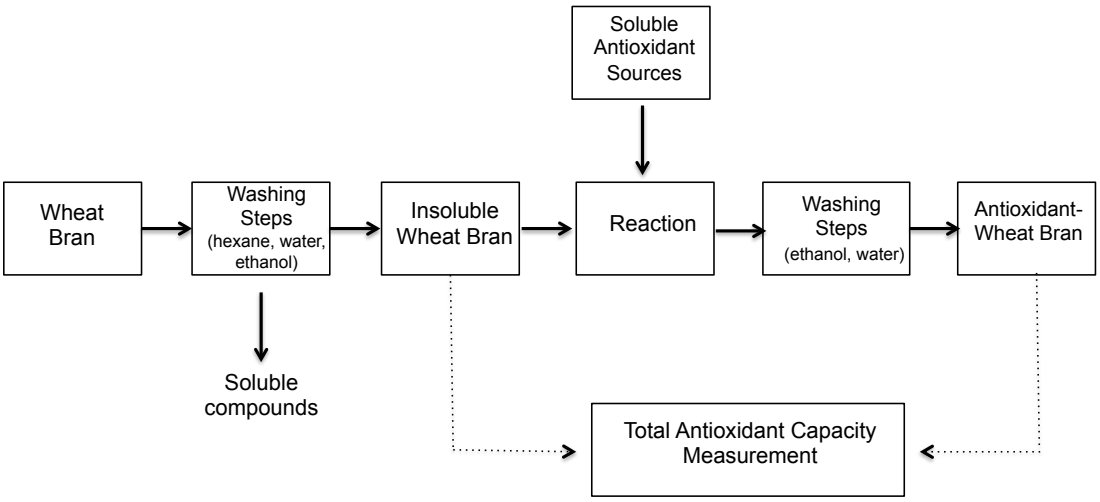


Figure 3.1. Scheme for treatment wheat bran with soluble antioxidant compounds (treatment 1)

3.2.2.1. Effect of Radical Formation on Wheat Bran Surface

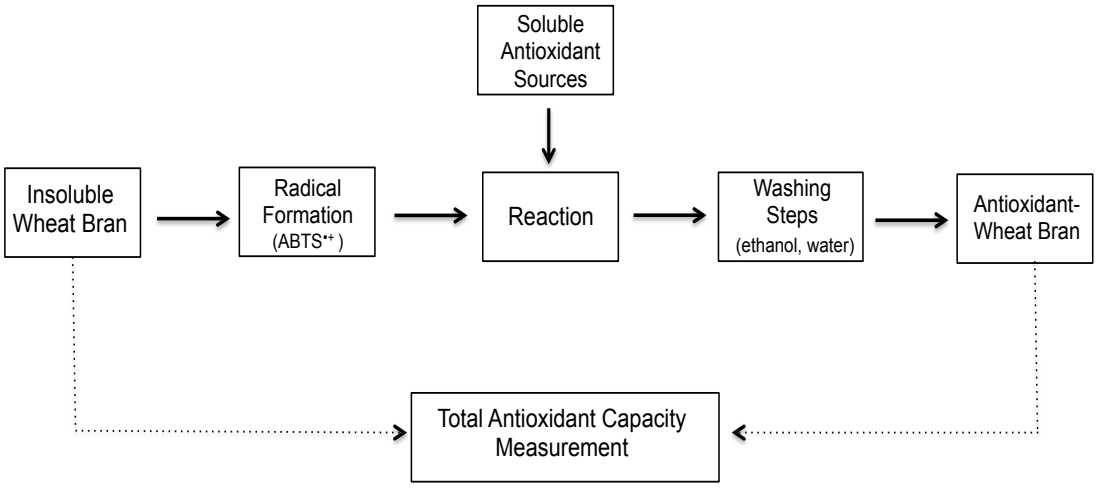
In order to understand the effect of phenoxy radical formation on the reaction between soluble phenolic compounds and insoluble dietary fiber, phenoxy radicals were formed by $ABTS^{\bullet+}$ radical solution before or during reaction. Firstly, to create phenoxy radicals on the surface of insoluble wheat bran (treatment 2) before its reaction with soluble antioxidants, 50 mg of insoluble wheat bran reacted with 5

mL of $ABTS^{\bullet+}$ radical solution at magnetic stirrer (at 350 rpm) for 30 min. After that, the supernatant was removed followed by centrifugation at 6080g for 2 min, and the residue was mixed with 5 mL of aqueous pure antioxidant solutions (gallic acid or tannic acid) at concentrations of 10, 20, 50, 100 and 200 μmol L^{-1}. The mixture was shaken for 30 min at room temperature using a magnetic stirrer at a speed of 350 rpm to interact the soluble antioxidants with pretreated insoluble wheat bran. Subsequently, the mixture was centrifuged at 6080g for 2 min, and the supernatant was removed. The residue was washed out four times with 10 mL of ethyl alcohol, and water to remove the remaining traces of the soluble antioxidant compounds. The last washing water was tested and found to be free of soluble antioxidant compounds. Then, the insoluble residue was lyophilized prior to antioxidant capacity measurement.

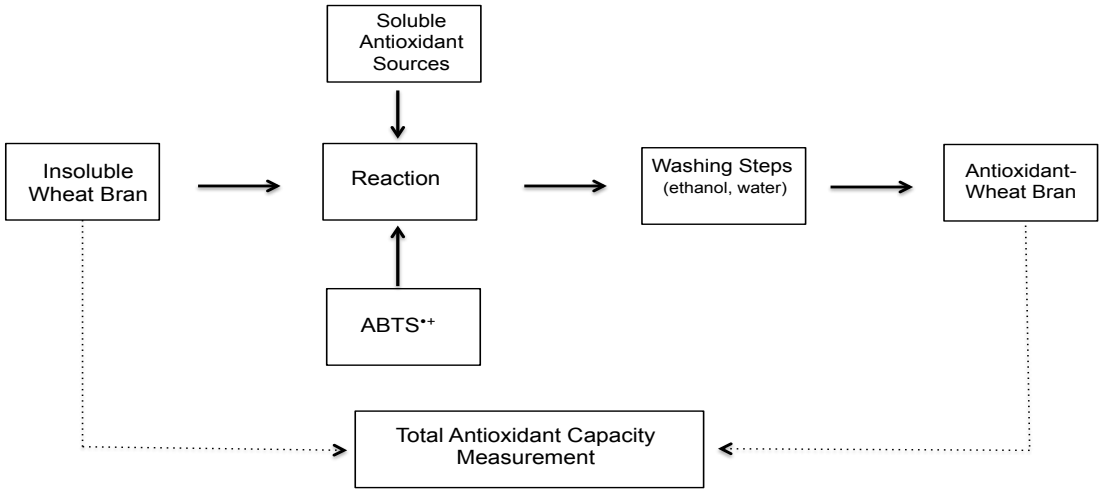
Secondly, 50 mg of insoluble wheat bran reacted with both 5 ml of $ABTS^{\bullet+}$ radical solution and 5 ml of aqueous solutions of gallic acid at the same time to create phenoxy radicals during reaction between insoluble wheat bran and soluble antioxidant compounds (treatment 3). The mixture of insoluble wheat bran, $ABTS^{\bullet+}$ radical solution and soluble antioxidant compounds (gallic or tannic acid) was shaken for 30 min at room temperature. After the same washing procedure was carried out as described above, antioxidant capacity of insoluble wheat bran was determined. As a result, antioxidant capacity values of insoluble wheat bran samples were compared with each other to determine if the radical formation had effect on the increase of antioxidant capacity of insoluble wheat bran.

Moreover, to investigate in-depth the effects of radical formation on increase of antioxidant capacity of wheat bran after treatment with two different beverages (green tea or black tea), radicals were formed with $ABTS^{\bullet+}$ radical solution in two different ways: 1) on the surface of insoluble wheat bran before treatment with

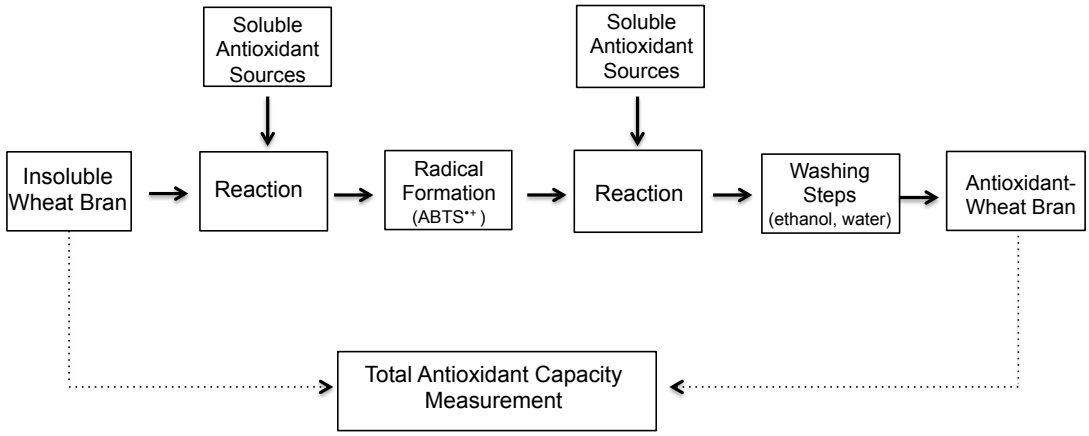
green tea or black tea infusion (treatment 2); 2) on the surface of insoluble wheat bran after treatment with green tea or black tea infusion (treatment 4). In the first way, phenoxy radicals were formed on the surface as mentioned in treatment 2. After removing of residual ABTS solution, black tea or green tea was added to the tube and reacted with pretreated wheat bran at magnetic stirrer (at 350 rpm) for 30 min. After supernatant was removed, the washing cycles were performed to remove residual soluble phenolic compounds. The antioxidant capacity of insoluble wheat bran was measured followed by the lyophilization of the residue. In the second way (treatment 4), 5 ml of green tea or black tea infusion reacted with 50 mg of insoluble wheat bran at room temperature for 30 min prior to radical formation. After removing of residual soluble phenolic compounds, radicals were formed as stated before. Following the removing of residual ABTS solution, 5 ml of green tea or black tea infusion was added to the tube once again; and after 30 min, the same procedure was performed for antioxidant capacity measurement.



(a)



(b)



(c)

Figure 3.2. Scheme for radical formation procedures (a) treatment 2; (b) treatment 3; (c) treatment 4

3.2.2.2. Effects of Different Parameters on the Reaction Between Insoluble Wheat Bran and Soluble Phenolic Compounds

Time, temperature, pH of reaction medium and oxygen flow in reaction tube can affect the interaction between insoluble bran and soluble antioxidant compounds. After determination of beverage that was the most efficient to increase the antioxidant capacity of wheat bran, the reaction conditions (time, temperature, pH, O_2 flow) were changed. In order to investigate the effects of different parameters, the reaction between soluble antioxidant compounds in beverage (green tea) and

insoluble wheat bran fraction was carried out at different temperatures (25°C or 50°C), times (0.5 or 1 h) and pH values (2, 4, 6, 7, 8, 9, 10,12) as well as with or without O_2 flow. In this manner, the optimum conditions for increasing the antioxidant capacity of insoluble wheat bran were determined. In addition, the reaction kinetics was investigated by treating insoluble wheat bran with two concentrations of green tea extract (0.5% and 1.0%) at optimum conditions. The reaction was carried out up to 3 h and the antioxidant capacity of wheat bran was measured after 0.5, 1, 2 and 3 h of treatment. After determination of optimum conditions to increase the antioxidant capacity of wheat bran, the reaction was performed at these conditions using oat bran and rice bran instead of wheat bran as an insoluble dietary fiber source.

3.2.3. Investigation of Mechanism

To investigate the mechanism for binding of phenolic compounds on to the surface of dietary fiber, different analyses were performed like ferulic acid analysis, free amino group analysis, total phenolic compounds analysis.

3.2.3.1. Ferulic Acid Analysis

In a previous study carried out by our group, it was reported that soluble antioxidant compounds might react with bound ferulic acid, which is consist of ninety percentages of phenolic compounds in the insoluble wheat bran [121]. To investigate if the binding of soluble phenolic compounds on to the surface of bran fraction was due to the interaction between soluble phenolic compounds and bound phenolic compounds (especially ferulic acid) in wheat bran, the treatment was performed between free ferulic acid (instead of wheat bran) and green tea extract at optimum conditions. For this reason, 4 mL of ferulic acid standard solution (1mmol L^{-1}) reacted with 4 mL of green tea extract (10%) at optimum

conditions, which were determined before. Ferulic acid standard solution reacted with water at the same conditions as a control. Before and after reaction (60 min), the mixture of ferulic acid and green tea extract samples were prepared for chromatographic analysis that was performed on an Agilent 1200 HPLC system consisting of photodiode array detector, quaternary pump, autosampler and column oven. Phenolic acids were separated on a Waters Atlantis C18 column (250 mm x 4.6 mm, 5 mm) using a isocratic mobile phase containing 80% of solvent A (formic acid/H_2O, 1:99, v/v) and 20% of solvent B (formic acid/ acetonitrile, 1:99, v/v) at a flow rate of 1 ml/min. Differences between the peak of controls and samples were compared with each other to determine if the green tea phenolic compounds were bound to ferulic acid. The quantitation was based on calibration curves built for each compound identified in the samples. The concentrations were expressed as mg per g of dry matter.

3.2.3.2. Free Amino Group Analysis

It has been previously reported that phenolic quinones, being reactive electrophilic intermediates, could readily attacked by nucleophiles such as lysine, methionine, cysteine and tryptophan moieties in a protein chain reversibly or irreversibly [122, 123], and the reason of binding might be the free amino groups in wheat bran. For this reason, insoluble wheat bran fractions, untreated and treated with green tea infusion at optimum conditions, were analyzed for the determination of free amino groups. Analyses were performed using TNBS assay as reported in Hermanson (1996) [124]. 10 mg of samples and 1 ml of 0.1 M sodium bicarbonate buffer solution (pH 8.5) were transferred to a test tube and the reaction was initiated by adding 0.5 ml of 0.01% (w/v) TNBSA. Following incubation at 37 °C for 2 h, the mixture was centrifuged at 6080g for 2 min. After that, optically clear supernatant and 0.25 ml of the 1 N HCl was transferred into a cuvette and the absorbance was

measured at 335 nm by using a Shimadzu model 2100 variable-wavelength UV-visible spectrophotometer (Shimadzu Corp., Kyoto, Japan). The free amino content of treated or untreated wheat bran was determined by means of a calibration curve prepared with leucine, and expressed as mmol leucine equivalent (LE) per kg on a dry basis.

3.2.3.3. Bound Phenolic Compounds Analysis

Insoluble wheat bran fractions, untreated and treated with green tea infusions at optimum conditions, were analyzed for bound phenolic compounds. Analyses were performed using the QUENCHER method with Folin–Ciocalteu reagent. Samples at 10 mg were transferred to a test tube and 0.8 ml of 0.2 M Folin–Ciocalteu reagent was added for oxidation. After 5 min, the mixture was neutralized with 1.25 ml of 20% aqueous Na_2CO_3 solution and kept in the dark for 1 h. After the mixture was centrifuged at 6080g for 2 min, the optically clear supernatant was transferred into a cuvette and the absorbance was measured at 765 nm against the solvent blank solvent using a Shimadzu model 2100 variable-wavelength UV-visible spectrophotometer (Shimadzu Corp., Kyoto, Japan). The bound phenolic content was determined by means of a calibration curve prepared with gallic acid, and expressed as mg of gallic acid equivalent per kg on a dry basis.

3.2.4. *In vitro* Digestion

To investigate the digestion behavior of bound antioxidant compounds, insoluble wheat bran and its green tea infusion treated form were digested in simulated *in vitro* digestion system. Digestion fluids mimicking the saliva, gastric and duodenal phases were used to simulate the conditions of gastrointestinal tract. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated duodenal fluid

(SDF) were prepared according to the procedure described by Minekus et al. (Table 3.1) [125]. *In vitro* digestion procedure was adapted from procedure reported by Papillo et al. [126]. A total of one gram dry ground samples was weighted into a glass flask followed by adding of 5 mL of SSF and the flask was shaken for 2 min to simulate the oral passage. After that, 5 mL of pepsin solution (12.5 mg/ml in 0.1 M HCl) and 10 mL of SGF were added and the mixture was adjusted to pH 2.0 to simulate the gastric phase. Then, the acidified mixture was incubated at 37^{o}C by shaking for 2 h at an agitation speed of 60 strokes per min. After bile salts were dissolved in the SDF solution to a concentration of 10 mg/mL, 20 mL of the mixture of SDF with bile salts and 5 mL of pancreatin solution (10 mg/ml in water) were added to the flask. Following the pH adjustment to 7.5, the mixture was incubated at 37^{o}C by shaking for 2 h at an agitation speed of 60 strokes per min to simulate the duodenal phase. The colon phase, which is separated two parts as ascending and descending colon, was simulated by a consecutive hydrolysis of proteins and polysaccharides in the sample. For ascending colon, 5 mL of protease solution (1mg/ml, pH 8.0) was added, and the mixture was incubated at 37^{o}C by shaking for 1 h. After 150 µL of Viscozyme L was added, the pH was adjusted to 4.0 and the mixture was incubated at 37^{o}C by shaking for 16 h at an agitation speed of 30 strokes per min to simulate the descending colon. All samples simulated gastric, duodenal and colon phases were lyophilized for the total antioxidant capacity measurement. Furthermore, to measure the antioxidant capacity of insoluble fractions, washing steps (with ethanol and water) were performed to remove the soluble substances from the lyophilized samples. Therefore, the antioxidant capacities of insoluble and soluble phases were determined.

Table 3.1. Preparation of stock solutions of simulated digestion fluids (each volume values are in total 500 mL of simulated fluids) [125]

	SSF (pH 3.0)		SGF (pH 7.5)		SDF (pH 8.0)	
Chemicals	Concentration ($g\ L^{-1}$)	Volume (mL)	Concentration ($g\ L^{-1}$)	Volume (mL)	Concentration ($g\ L^{-1}$)	Volume (mL)
KCl	46.7	10	46.7	28	46.7	5.4
KH_2PO_4	68	20	68	0.9	68	0.8
$NaHCO_3$	84	4	168	6.5	42.5	42.5
NaCl	120	1	120	10	120	8
$MgCl_2.(H_2O)_6$	30	1	30	2	30	1.1
pH adjustment	Concentration ($mole\ L^{-1}$)	Volume (mL)	Concentration ($mole\ L^{-1}$)	Volume (mL)	Concentration ($mole\ L^{-1}$)	Volume (mL)
NaOH	1	4	12	3	1	0.5
HCl	1	1	-	-	1	0.3

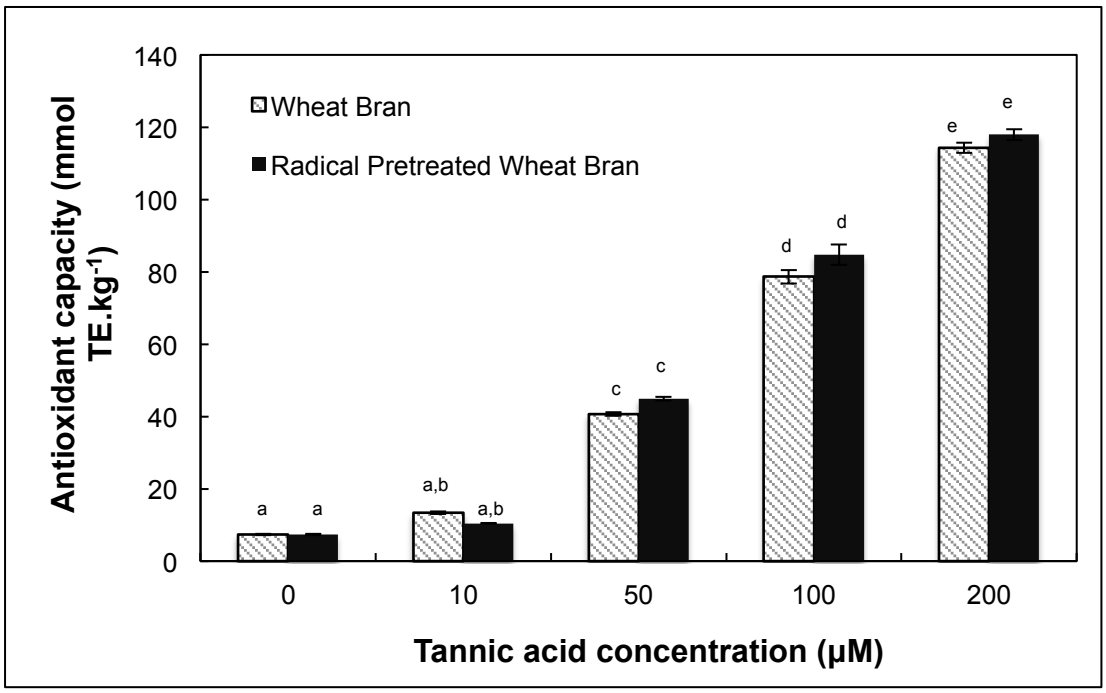
3.2.5. Statistical Analysis

The analytical data were reported as the mean ± standard deviation of duplicate independent measurements and were subjected to one-way ANOVA. The significance of mean differences was determined by Tukey HSDa post hoc test using SPSS version 17.0. p values of <0.05 were considered statistically significant.

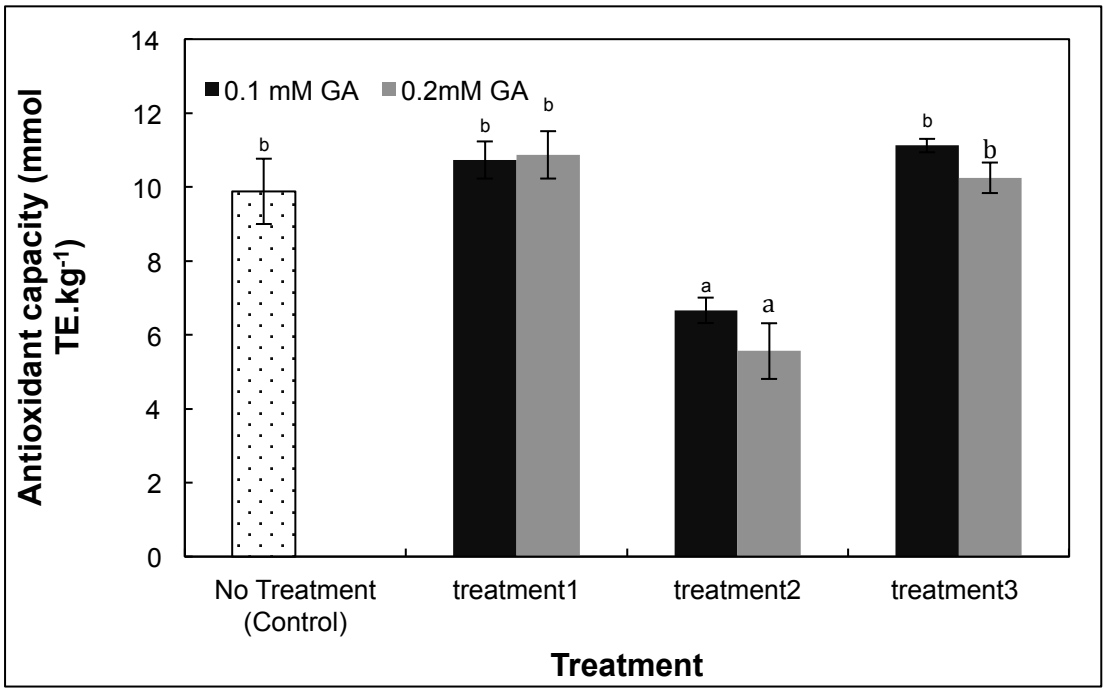
4. RESULTS AND DISCUSSION

4.1. Interaction of Insoluble Wheat Bran with Standard Solutions

The insoluble wheat bran used in this study was found to have an initial total antioxidant capacity of 6.8 ± 0.2 mmol TE.kg^{-1}. This insoluble fraction of wheat bran was treated with phenolic compounds in order to understand the mechanisms of reaction between insoluble wheat bran and soluble antioxidant compounds. As shown in Figure 4.1.a, increasing the concentration of tannic acid during the treatment (30 min at room temperature) led to a significant increase ($p < 0.05$) in the antioxidant capacity of insoluble wheat bran. However, there were no statistically significant differences ($p > 0.05$) in increasing antioxidant capacity when wheat bran itself or its radical pretreated form was used as the insoluble material. This indicated that the reason for the increased antioxidant capacity was not the radicals that formed on the surface of insoluble wheat bran. Under similar conditions, treatment with gallic acid, a monomeric form of tannic acid, did not cause any significant increase in the antioxidant capacity. This was probably due to the fact that tannic acid, as a polymeric compound, produced more quinones that enhanced its binding ability to bran matrix but gallic acid could not. Moreover, the radical formation during reaction between gallic acid and insoluble wheat bran does not affect the antioxidant capacity of insoluble wheat bran. However, radical formation prior to reaction with gallic acid caused to decrease in antioxidant capacity of insoluble wheat bran due to the loss of activity of its functional groups via the radical scavenging of phenolic compounds in wheat bran (Figure 4.1.b).



(a)



(b)

Figure 4.1. Increase of the antioxidant capacity of insoluble wheat bran during treatment with (a) different concentrations of tannic acid, and (b) gallic acid (GA) at room temperature for 30 min (treatment 1: with GA; treatment 2: with ABTS•+ radical solution prior to GA treatment; treatment 3: with GA and ABTS•+ radical solution at the same time.

4.2. Interaction of Insoluble Wheat Bran with Beverages

After standard solutions treatment, insoluble wheat bran was treated with different antioxidant-rich beverages to understand the interaction of soluble antioxidant and insoluble fiber. As shown in Figure 4.2, treatment with green tea infusion (3 g/100 ml) significantly increased ($p < 0.05$) the antioxidant capacity of insoluble wheat bran from 6.8 ± 0.2 mmol TE.kg^{-1} (control) to 18.8 ± 0.4 mmol TE.kg^{-1}. It is known that green tea infusions are rich in flavan-3-ols (epicatechin, epigallocatechin and epigallocatechin gallate) that can be oxidized to quinones and polymerize easily during treatment [127]. However, treatment with white wine had no statistically significant effect ($p > 0.05$) on the antioxidant capacity of insoluble wheat bran. Compared to controls, treatment with black tea infusion and red wine also caused statistically significant increases ($p < 0.05$) in the antioxidant capacity of insoluble wheat bran, most probably due to the presence of polymeric phenolic compounds in high quantities. The effects of red wine, black tea and green tea infusions were found to be dependent on concentration. In fact, diluted forms of these beverages were significantly less effective ($p < 0.05$) than their undiluted forms (Figure 4.2).

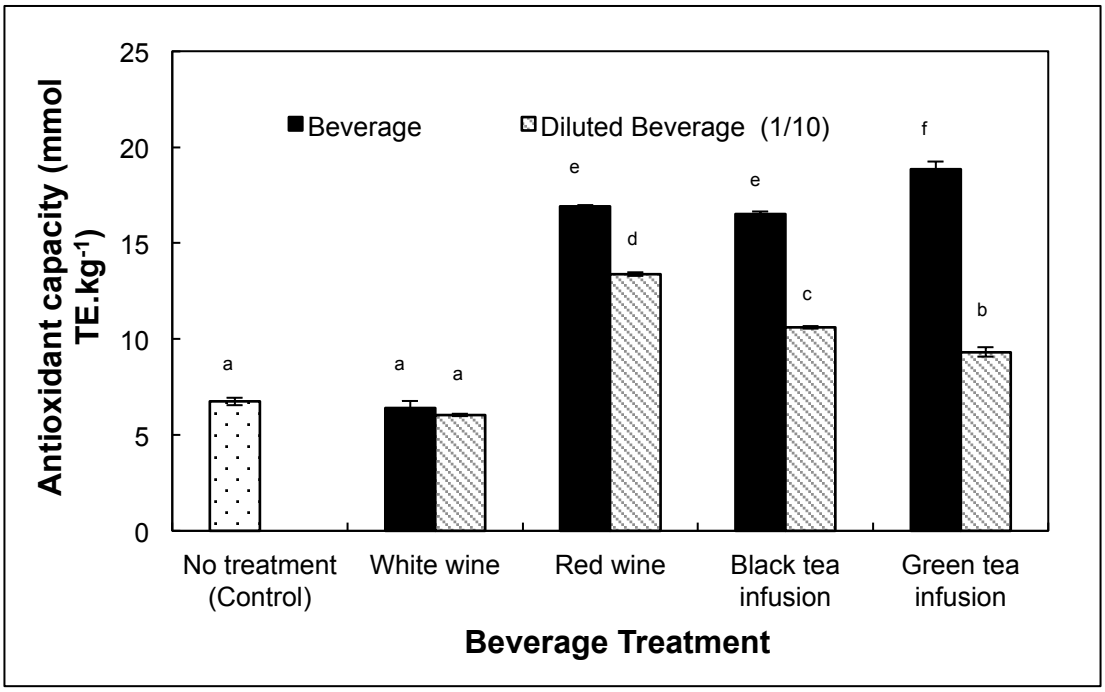


Figure 4.2. Increase of the antioxidant capacity of insoluble wheat bran during treatment with different concentrations of antioxidant-rich beverages at room temperature for 30 min

4.3. Effects of Radical Formation

According to the previous results, black tea and green tea infusions were found to be the most efficient beverages to increase antioxidant capacity of wheat bran. Therefore, these two beverages were used for further experiments to determine the effects of radical formation on the reaction between soluble phenolic compounds and insoluble dietary fiber.

As shown in Figure 4.3, radical formation caused less increase in antioxidant capacity of wheat bran due to the loss of antioxidant capacity of some functional groups on the surface of wheat bran. This result also showed that the increase in antioxidant capacity of wheat bran was dependent on the concentration of beverages. Green tea infusion at concentration of 1% was found to be as the most efficient beverage, and, therefore it was chosen as the source of soluble phenolic compounds for further reactions.

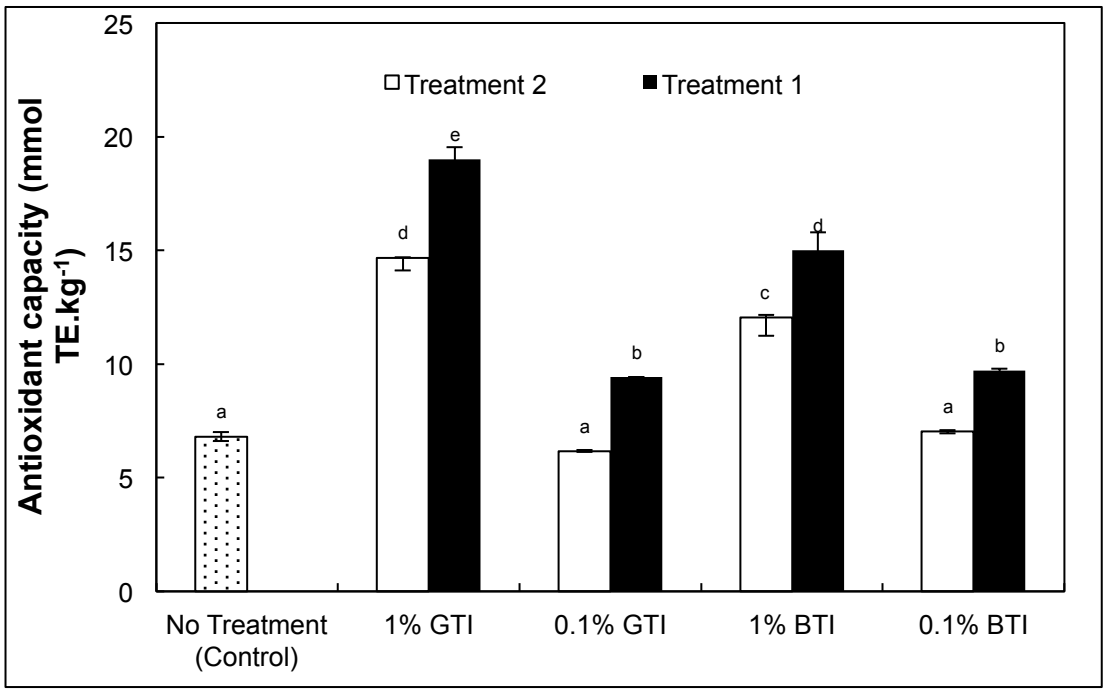


Figure 4.3. Increase of the antioxidant capacity of insoluble wheat bran and its radical pretreated form during treatment with different concentrations of black (BTI) and green tea infusion (GTI) at room temperature for 30 min (treatment 1: treatment with BTI or GTI without radical formation; treatment 2: treatment with BTI or GTI after radical formation on the surface)

Subsequently, different radical formation procedures were performed in order to investigate the effects of radical formation on the reaction between soluble phenolic compounds and insoluble fiber. In a previous study conducted by our group, it was indicated that tea catechins might react with radical forms of bound antioxidants and rapidly regenerate them by either giving one of their electrons/hydrogen atoms, becoming radical themselves or constituting a covalent bond with them and, therefore, the insoluble fiber material would have an increased total antioxidant capacity [121]. However, as shown in Figure 4.4, radical formation before reaction or at the interval of two green tea infusion treatments showed negative effects on increase of the antioxidant capacity of wheat bran as compared to non-radical forms of wheat bran treated with green tea

infusion. These results indicated that the radical formation had adverse effects on antioxidant capacity increase due to the loss of the activity of phenolic compounds via their radical scavenging mechanism. It was obvious that the binding of soluble phenolic compounds to the wheat bran surface was not the result of radical formation. Therefore, treatment 1, which was the simple reaction between soluble phenolic compounds and insoluble dietary fiber, was chosen as a target reaction.

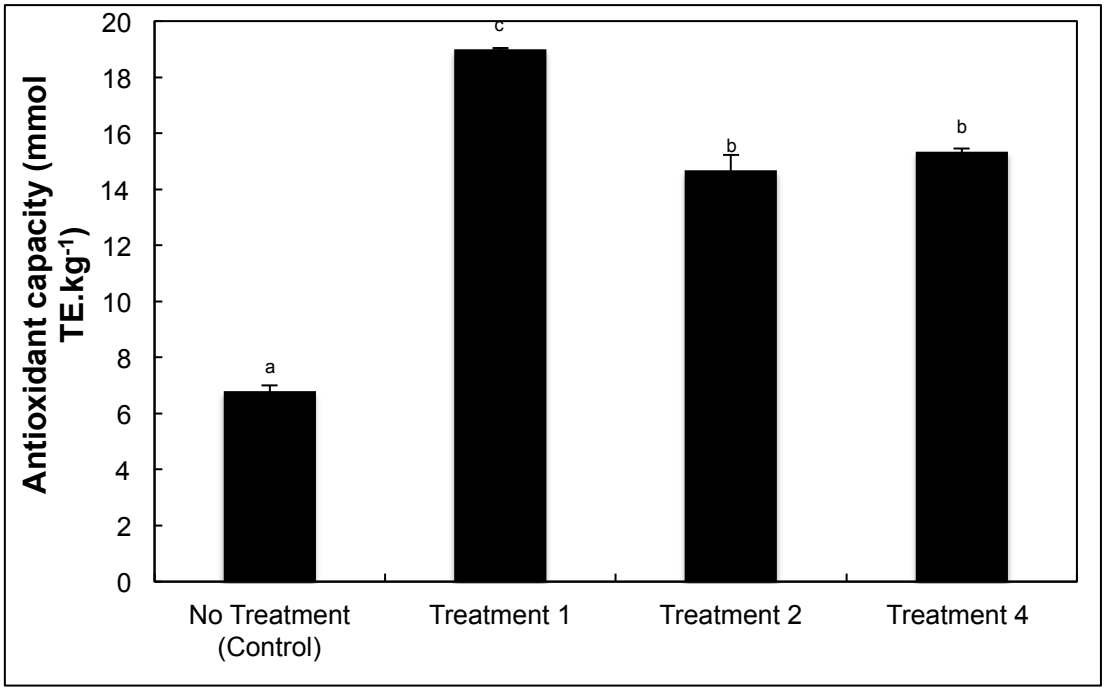


Figure 4.4. Effect of different radical formation treatments on increase of the antioxidant capacity of wheat bran after treatment with green tea infusion (GTI) at room temperature for 30 min (treatment 1: treatment wheat bran with GTI without radical formation; treatment 2: treatment radical pretreated wheat bran with GTI; treatment 4: after treatment wheat bran with GTI, radicals formed with $ABTS^{\bullet+}$ and followed by GTI adding to tube once again)

4.4. Determination of Optimum Reaction Conditions

Since green tea infusion was the most efficient beverage among others tested, it was used as the source of soluble phenolic compounds to determine the effects of different conditions (temperature, time, pH and air flow) on the antioxidant capacity of insoluble wheat bran. Increasing the treatment time from 30 min to 60 min

caused 1.49 times and 1.96 times increase at 25 °C and 50 °C, respectively, in the antioxidant capacity of insoluble wheat bran. The highest level of antioxidant capacity was attained after the treatment of insoluble wheat bran with green tea infusion at 50 °C for 60 min (Figure 4.5). At these conditions (50 °C, 60 min), changing the pH value of the reaction medium from 2.0 to 12.0 had a strong influence on the resulting antioxidant capacity (Figure 4.6). Increasing the pH of the reaction medium from 2.0 to 9.0 linearly increased the antioxidant capacity, reaching an apparent maximum level of 126.8 ± 0.6 mmol TE.kg^{-1}. However, further increase of pH to 10.0 and 12.0 caused a sharp decrease in the antioxidant capacity (Figure 4.6). It is a fact that polyphenols turn into phenolate ions at alkaline conditions followed by quinone formation [123]. This might be affecting their binding ability onto insoluble bran matrix. However, highly acidic and basic conditions (pH 2.0 or 12.0) were found as the least effective probably due to loss of bound phenolic acids as a result of hydrolysis.

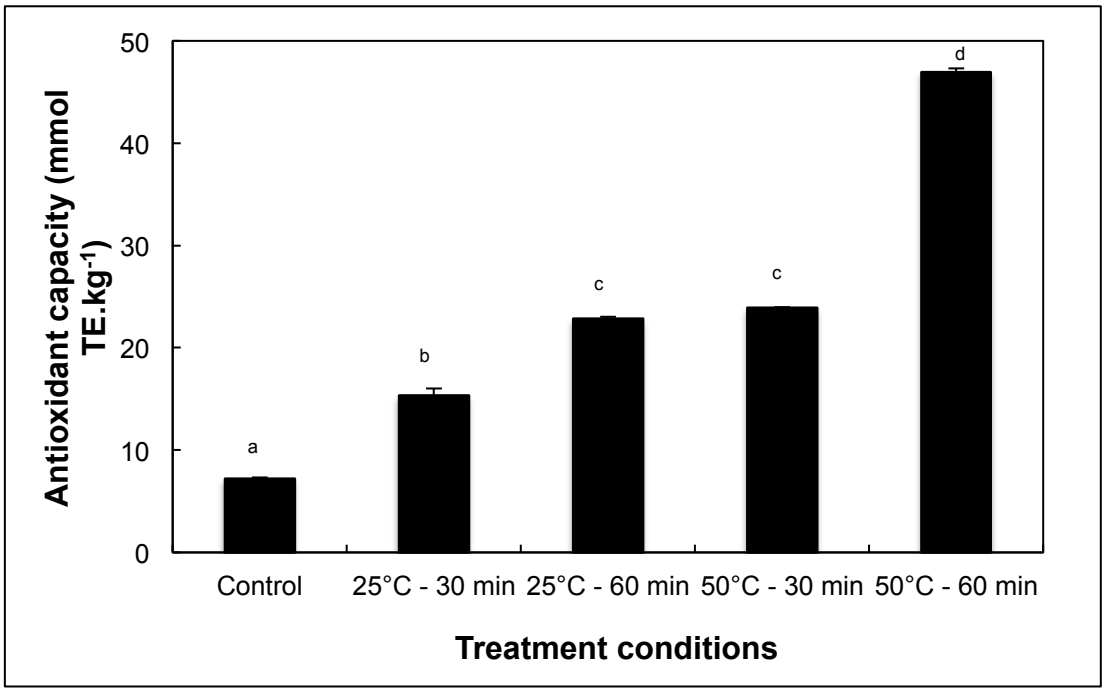


Figure 4.5. Effect of temperature (25 °C and 50 °C) and time (30 min and 60 min) on the increase of antioxidant capacity of insoluble wheat bran during treatment

with green tea infusion (3g/100 ml)

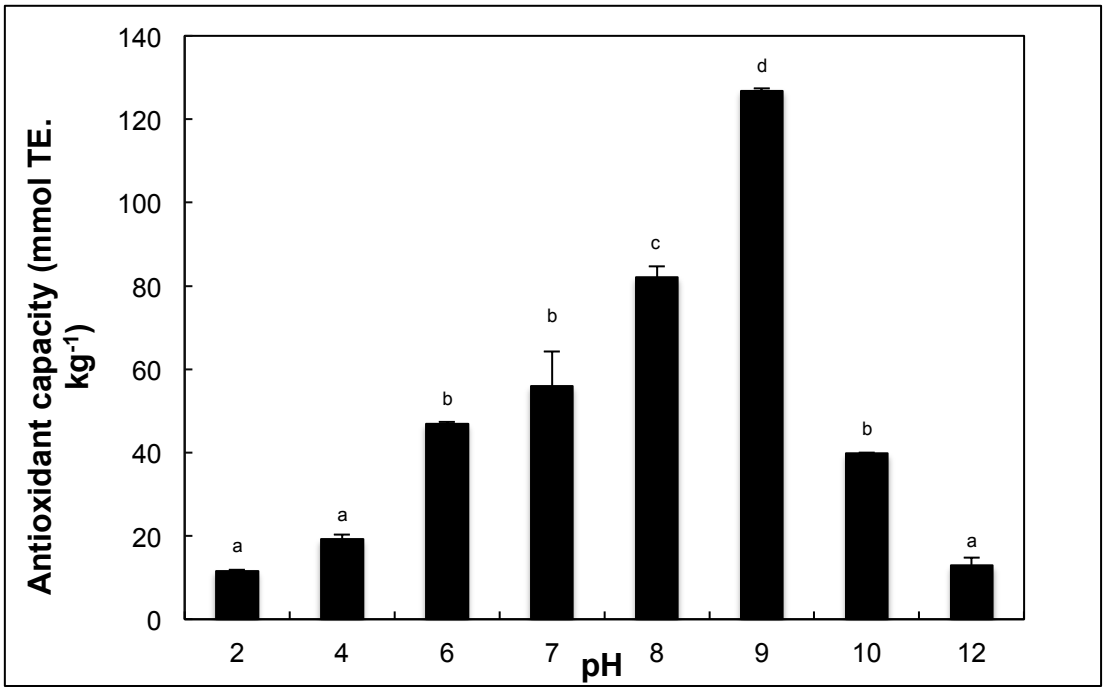


Figure 4.6. Effect of pH on the increase of antioxidant capacity of insoluble wheat bran during treatment with green tea infusion (3g/100 ml) at 50 °C for 60 min

The reaction medium was aerated to determine its effect on the resulting antioxidant capacity of insoluble wheat bran treated with green tea infusion. It was found that applying an air stream during the treatment adversely affected the binding ability of soluble phenolic compounds onto insoluble bran matrix (Figure 4.7). Compared to treatment with green tea infusion for 1 h at 50 °C and pH 9.0, the antioxidant capacity of insoluble wheat bran reached 81.3 ± 0.3 mmol TE.kg^{-1}, which was 35.9% lower when the reaction medium was aerated ($p<0.05$). It is thought that applying an air stream caused polymerization of soluble phenolic compounds in the liquid phase, but limited their binding to bran surface. The same procedure was repeated using black tea infusion instead of green tea infusion as a source of soluble antioxidants in polymeric forms. Applying aeration during treatment with black tea (at 50 °C and pH 9.0 for 1 hour) showed no effects on the binding ability of black tea phenolic compounds onto the insoluble wheat bran. In

this case, no statistically significant differences ($p > 0.05$) were observed due to the fact that black tea infusion had already polymeric compounds, so aeration did not cause polymerization in liquid phase.

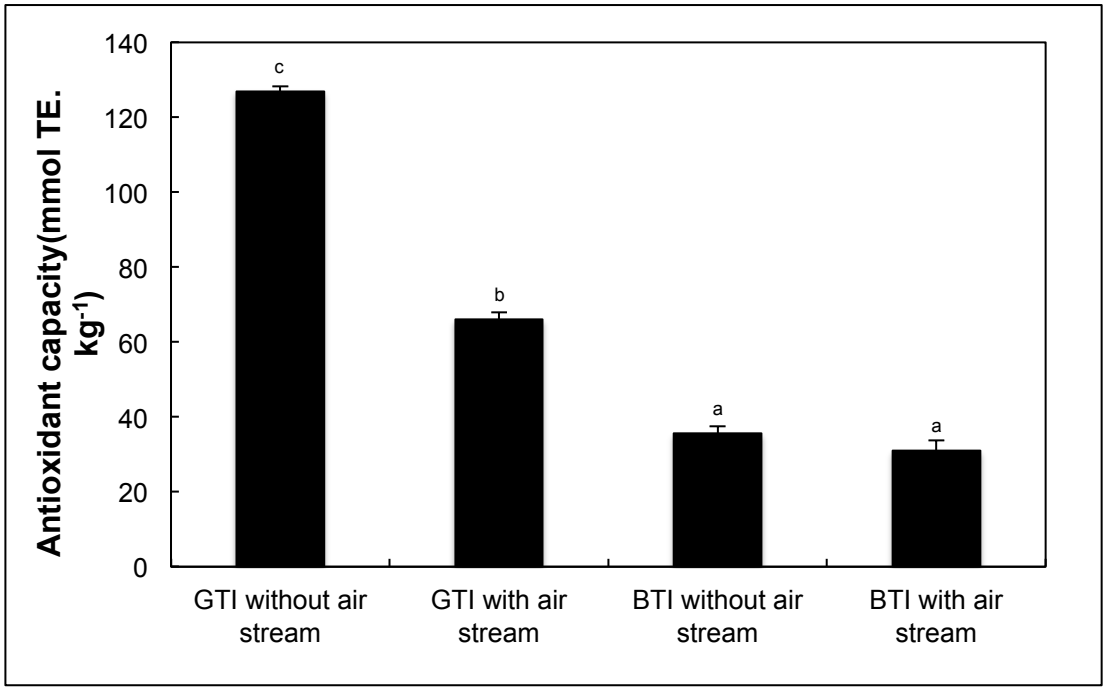


Figure 4.7. Effect of air stream on the increase of antioxidant capacity of insoluble wheat bran during treatment with black tea (BTI) or green tea (GTI) infusion (3g/100 ml) at pH 9.0 and 50 °C for 60 min.

After determination of optimum conditions for the reaction between insoluble fiber and soluble phenolic compounds, this reaction was performed with different dietary fiber sources (oat bran and rice bran) instead of wheat bran at two different conditions. Treatment rice bran and oat bran with green tea infusion at 25 °C for 30 min caused 4.78 and 3.23 times increase in their antioxidant capacities, while this treatment caused 2.09 times increase in antioxidant capacity of wheat bran. As shown in Figure 4.8, antioxidant capacities of wheat, oat and rice bran reached to 126.85 ± 1.37, 140.56 ± 2.62 and 177.16 ± 1.40 mmol TE.kg^{-1}, respectively, after reaction with green tea infusion at optimum conditions (at 50°C, pH 9.0 for 1 hour). These results indicated that this treatment caused 17.34 times increase in

antioxidant capacity of wheat bran, whereas it caused 9.29 and 7.65 times increase in antioxidant capacities of rice bran and oat bran. Therefore, wheat bran might be the most efficient matrix to bind the soluble phenolic compounds on to its surface.

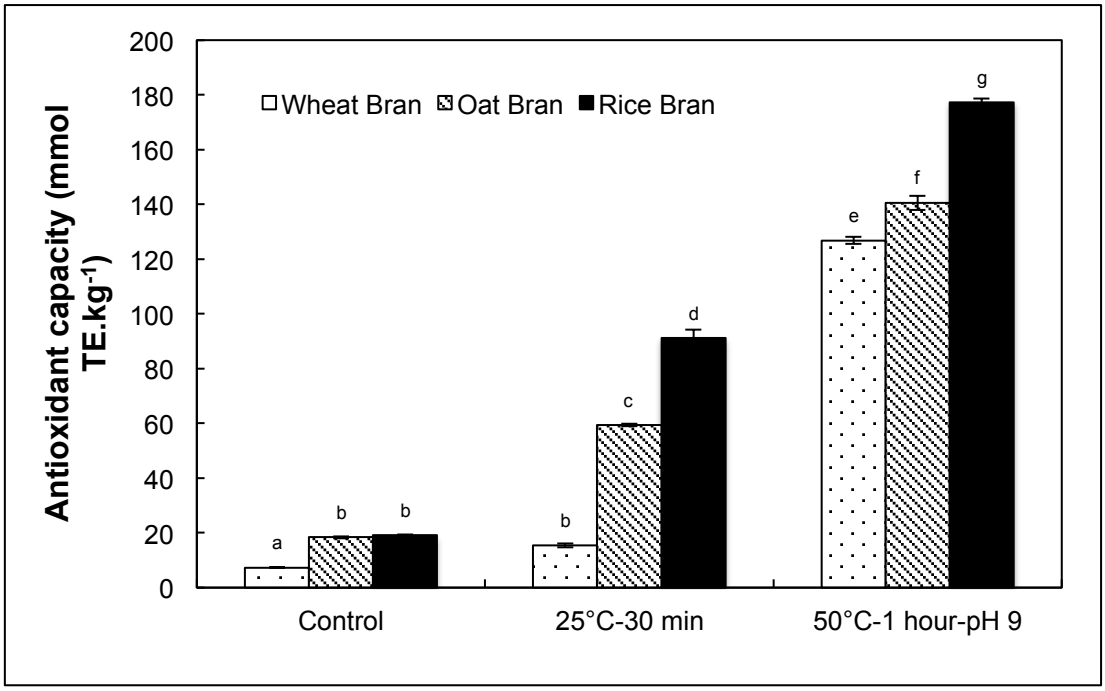


Figure 4.8. Increase of the antioxidant capacity of insoluble dietary fibers (wheat, oat, rice bran) during treatment with green tea infusion (3 g/100 mL) at two different conditions (at 25 °C for 30 min or at 50 °C and pH 9.0 for 1 hour)

To investigate the effects of optimum conditions on the structure of wheat bran, insoluble wheat bran reacted with water at optimum conditions (50 °C and pH 9.0 for 1 hour) instead of green tea infusion. As shown in Figure 4.9, antioxidant capacity of insoluble wheat bran reached only to 10.51 ± 0.37 mmol $TE.kg^{-1}$ from 7.31 ± 0.15 mmol $TE.kg^{-1}$ after its treatment with water. This slight increase in antioxidant capacity of insoluble wheat bran might be the result of high temperature and alkaline hydrolysis, which caused to reveal the antioxidant compounds that were previously buried in the structure of insoluble wheat bran. When the same reaction was repeated with green tea extract, the antioxidant

capacity of insoluble wheat bran reached to 137.03 ± 0.75 mmol TE.kg^{-1} due to the binding of green tea phenolic compounds to the insoluble wheat bran.

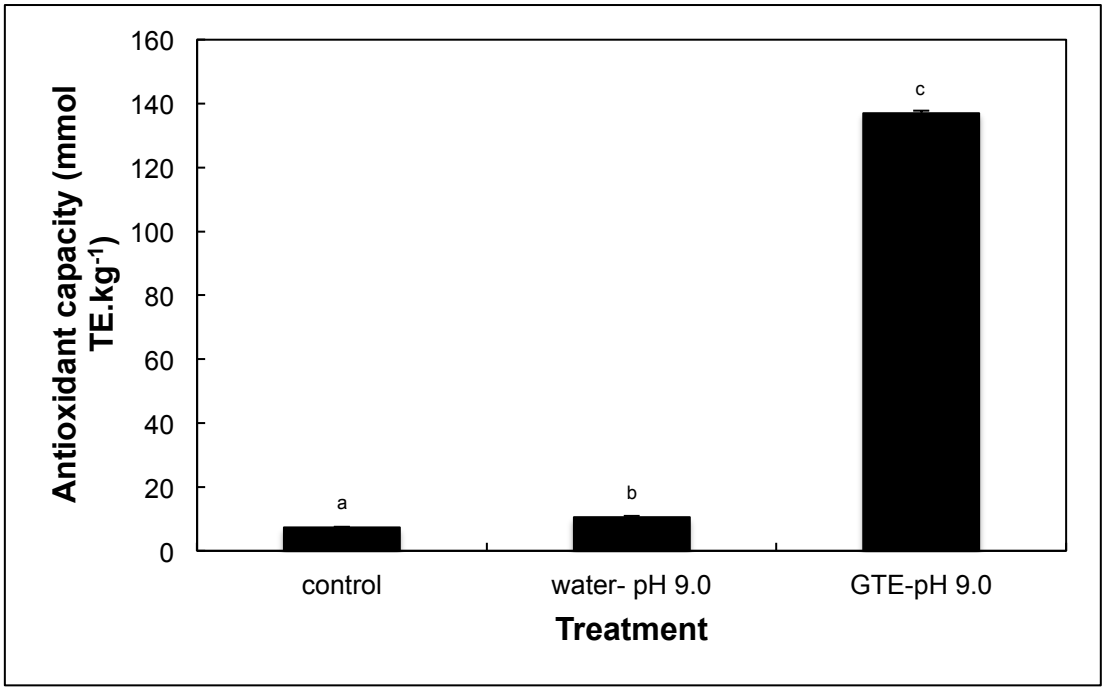


Figure 4.9. Increase of the antioxidant capacity of insoluble during treatment with green tea extract (10%) and water at optimum conditions (pH 9.0 at 50 °C for 1 hour).

4.5. Determination of Reaction Kinetics

The binding kinetics was investigated during the treatment of insoluble wheat bran with green tea extract (Figure 4.10). Aqueous solutions of green tea extract at two concentrations (0.5% and 1.0%) reacted with wheat bran at optimum conditions (at pH 9.0, 50 °C) for 3 hours to determine their effect on the binding rate. A biphasic kinetic behavior was observed for the increase of antioxidant capacity of insoluble wheat bran during treatment with green tea extract. In the first phase, the rate was independent of the concentration of soluble phenolic compounds in the liquid phase. There was a very sharp increase in the antioxidant capacity within the first 15 min. Afterwards, the antioxidant capacity continued to increase with a noticeably lower rate depending on the concentration of soluble phenolic

compounds. In the first phase, the sharp increase of the antioxidant capacity might be due to the binding of phenolic compounds via the formation of quinones that further reacted with free amine groups available in the bran matrix [122, 123, 128]. In the second phase, it was thought that soluble phenolic compounds continued to polymerize at the solid surface, which caused further increase in the bound antioxidant capacity of insoluble wheat bran.

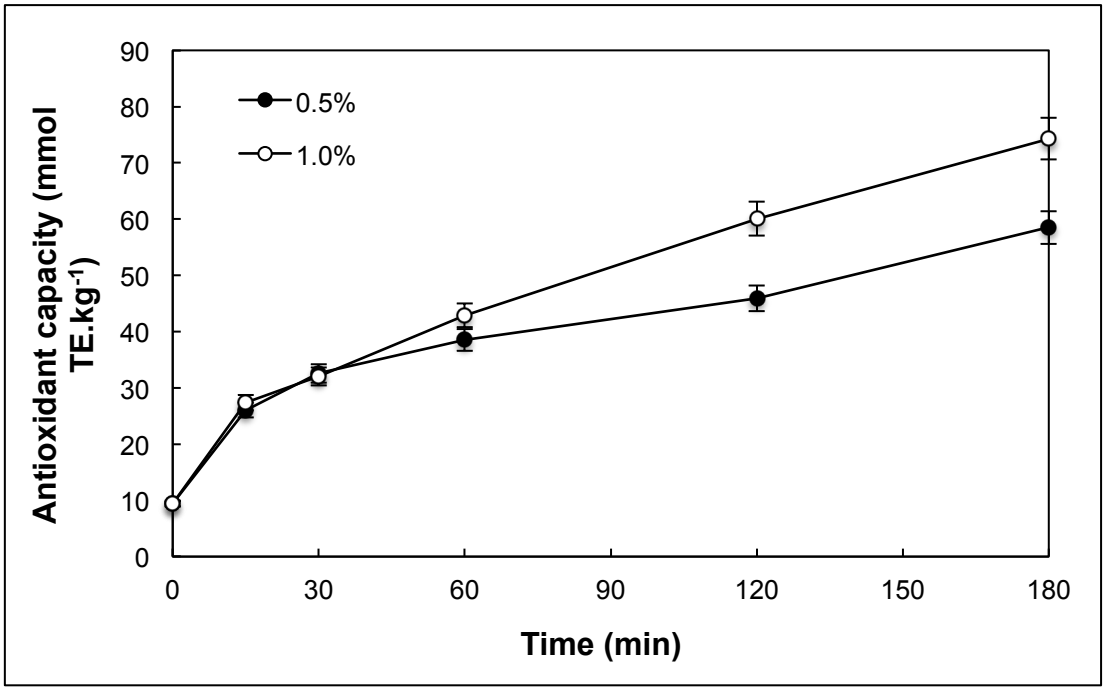


Figure 4.10. Change of the antioxidant capacity of insoluble wheat bran during treatment with two different concentration of green tea extract at 50 °C and pH 9.0

4.6. Determination of the Binding Mechanism

4.6.1. Ferulic Acid Analysis

In a previous study, it was reported that the reason of increase in antioxidant capacity of wheat bran might be the binding of soluble phenolic compounds to the ferulic acid or its radical form [121]. Therefore, the interaction between soluble phenolic compounds (green tea phenolics) and ferulic acid was investigated at optimum conditions. As given in Table 4.1, there were no statistically differences in

amount of ferulic acid of control and samples ($p > 0.05$). It was expected that the amount of ferulic acid might decrease after reaction with green tea phenolic compounds due to the binding. However, this result showed that green tea phenolic compounds could not bind to the ferulic acid even in soluble form. So, the binding mechanism of phenolic compounds onto the wheat bran surface might not depend on the bound ferulic acid in wheat bran.

Table 4.1. Ferulic acid content of GTE-ferulic acid system

	Amount of Ferulic Acid (mg L^{-1})
Control (ferulic acid-water mixture)	70.96 ± 0.09^{a}
GTE-ferulic acid mixture (before reaction)	71.29 ± 0.37^{a}
GTE-ferulic acid mixture (after reaction)	70.87 ± 0.23^{a}

* Values within rows having the same letter are not significantly different ($p > 0.05$)

4.6.2. Total Bound Phenolic Compounds

Total bound phenolic compounds were analyzed before and after the treatment of insoluble wheat bran with green tea infusion to confirm the binding of soluble phenolic compounds. As given in Table 4.2, treatment with green tea infusion at 50 °C and pH 9.0 for 1 h caused 10.5 times increase in the bound phenolic compounds concentration of insoluble wheat bran. Similarly, after treatment with green tea, antioxidant capacity of insoluble wheat bran also reached 126.8 ± 0.6 mmol TE.kg^{-1}, which is the 18.6 times of initial antioxidant capacity of insoluble wheat bran. The coherence between the increase of antioxidant capacity and bound phenolic compounds of insoluble wheat bran indicated the binding of phenolic compounds present in the green tea infusion (liquid phase) to the insoluble wheat bran (solid phase).

4.6.3. Free Amino Group Analysis

The initial concentration of free amino groups was found as 3.56 ± 0.06 mmol LE.kg^{-1} for the insoluble wheat bran fraction. It significantly decreased to 1.33 ± 0.17 mmol LE.kg^{-1} after the reaction of insoluble wheat bran with green tea infusion ($p < 0.05$) (Table 4.2). The reason for the 62.6% reduction in free amino groups of insoluble wheat bran should be due to binding of polyphenols present in green tea infusion via their oxidation to corresponding quinones. Generally, phenolic substances may be readily oxidized in alkaline solution or in presence of polyphenol oxidase [129, 130] and 1,2-dihydroxy or 1,2,3-trihydroxy phenolic groups are oxidized to quinone forms [131]. There are two reaction steps during quinone formation. The first step consists of the hydroxylation of monophenols into *o*-diphenols followed by oxidation of *o*-phenols into *o*-quinones. It has been previously reported that quinones, being a reactive electrophilic intermediate, could readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan moieties in a protein chain reversibly or irreversibly [122, 123]. As reported by others, insoluble wheat bran subjected to alkaline hydrolysis contained 1.4% of protein [128]. Therefore, the results suggest that green tea polyphenols are first oxidized to quinones under alkaline conditions, and then, they are further bound to free amino groups available on the surface of wheat bran matrix as shown in Figure 4.11.

Table 4.2. Phenolic compounds, free amino groups analysis and antioxidant capacity measurement

	Before Reaction	**After Reaction**
Phenolic Compounds (mg gallic acid.kg^{-1})	1099.1 ± 61.4[a]	12593± 170.5[b]
Free Amino Groups (mmol LE.kg^{-1})	3.56 ±0.06[c]	1.33 ± 0.17[d]
Antioxidant Capacity (mmol TE.kg^{-1})	6.8 ± 0.2[e]	126.8 ± 0.6[f]

* Values within rows having the same letter are not significantly different ($p > 0.05$)

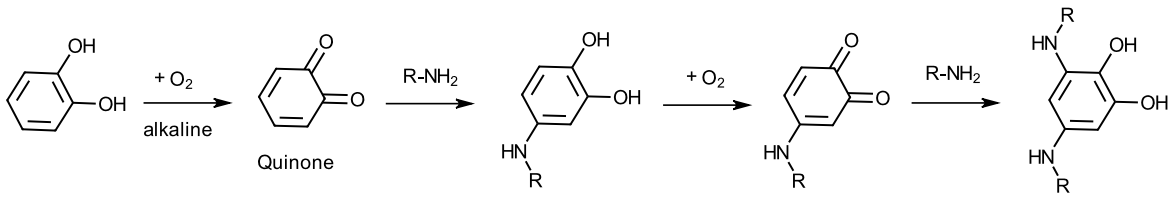


Figure 4.11. Proposed mechanism for reaction between soluble antioxidants and insoluble wheat bran

The results of free amino group analysis of oat bran and rice bran were also similar with wheat bran. As given in Table 4.3, 41.9% and 58.4% reduction in free amino groups of oat bran and rice bran was coherence with 7.7 and 9.3 fold increase in their antioxidant capacity. It was clearly indicated that the more increase in antioxidant capacity of cereal bran the more decrease in free amino groups. These results were evidence for binding of soluble phenolic compounds to the free amino groups of dietary fiber.

Table 4.3. Decrease in free amino groups and increase in antioxidant capacity of dietary fibers after reaction with green tea infusion (3 g/100 mL) at pH 9.0, 50 °C for 1 hour

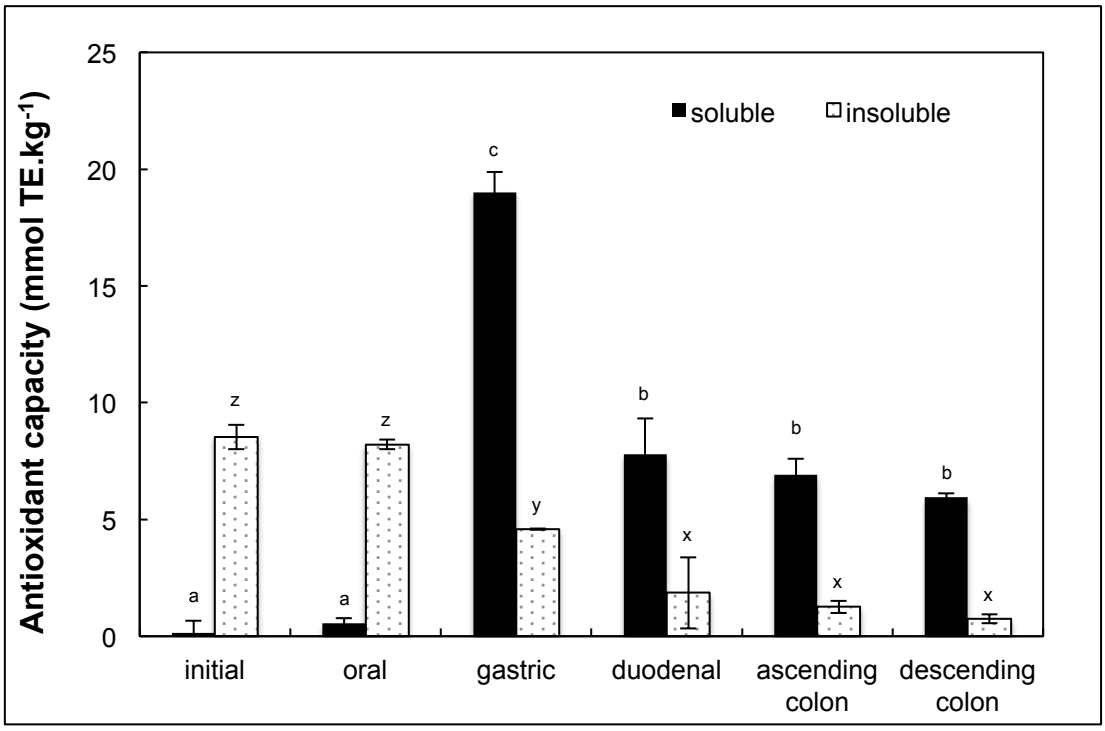
Sample	Before Reaction (mmol LE.kg^{-1})	After Reaction (mmol LE.kg^{-1})	Amino Group Decrease (%)	Antioxidant Capacity Fold Increase
Wheat Bran	3.56 ± 0.06^{a}	1.33 ± 0.17^{b}	62.6	17.3
Oat Bran	2.15 ± 0.09^{c}	1.25 ± 0.10^{d}	41.9	7.7
Rice Bran	3.44 ± 0.05^{e}	1.43 ± 0.09^{f}	58.4	9.3

* Values within rows having the same letter are not significantly different ($p > 0.05$)

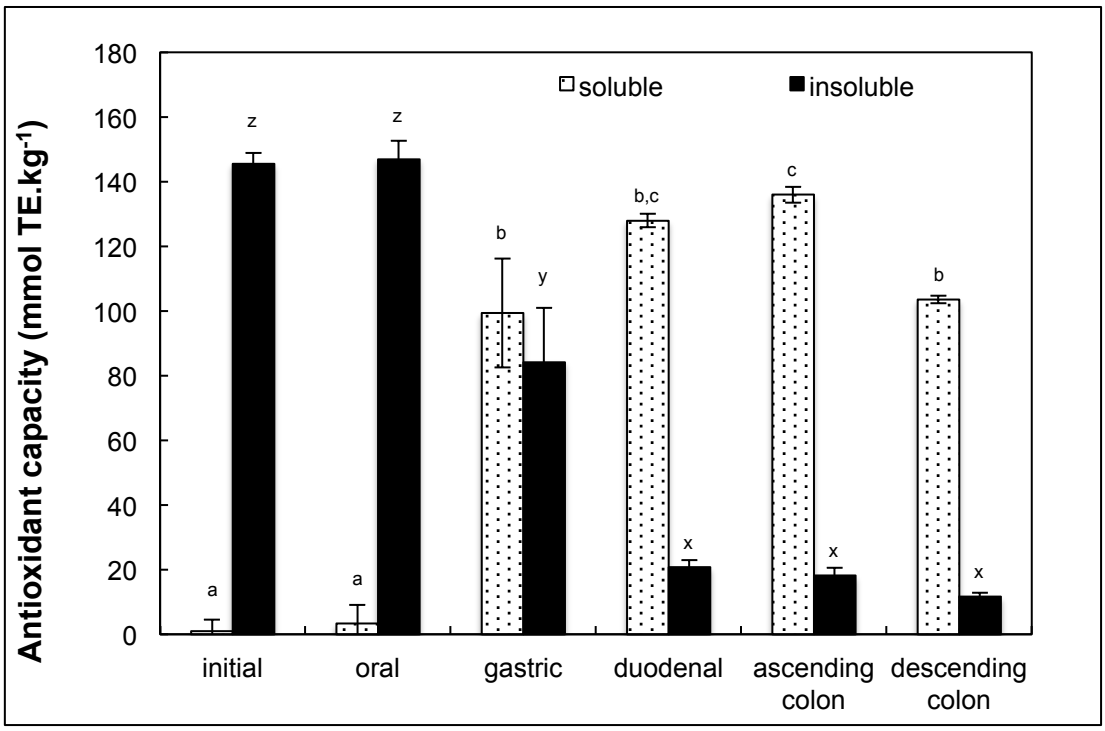
4.7. *In vitro* Digestion Behaviors of Bound Antioxidant Compounds

To investigate the digestion behavior of bound antioxidant compounds, insoluble wheat bran and green tea infusion (3 g/100 ml) treated (at 50 °C and pH 9.0 for 1 h) wheat bran were subjected to *in vitro* digestion protocol. According to the results, oral phase did not cause any statistically change in antioxidant capacity of insoluble wheat bran (Figure 4.12.a). The antioxidant capacity of soluble fraction increased dramatically at the end of the gastric phase, while there was a significant decrease in antioxidant capacity of insoluble fraction. This result indicated that most of the antioxidant compounds in insoluble wheat bran transferred to soluble fraction after gastric phase. As a result of the acid hydrolysis (pH 2.0 in gastric phase), which made some antioxidant compounds previously buried in the structure of the insoluble material bioaccesible, total antioxidant capacity of insoluble wheat bran also increased. Following the gastric phase, no change was observed in antioxidant capacities of both soluble and insoluble fractions of wheat bran during duodenal and colon phases. Green tea treated wheat bran had the similar digestion trend with insoluble wheat bran (Figure

4.12.b). The antioxidant capacities of insoluble fraction of green tea treated wheat bran and untreated insoluble wheat bran decreased to about 20 mmol TE kg^{-1} and 1 mmol TE kg^{-1}, respectively, after duodenal and colon phases. These results indicated that green tea treated wheat bran having more bound antioxidant compounds in its structure, showed 20 times more antioxidant activity than untreated insoluble wheat bran. This is in agreement with previous studies highlighting the role of bound antioxidants in transporting their activity through the colon [126, 132].



(a)



(b)

Figure 4.12. Changes in antioxidant capacities of (a) insoluble wheat bran and (b) green tea infusion treated wheat bran during in vitro digestion

5. CONCLUSION AND RECOMMENDATIONS

The required daily intake of dietary antioxidants is estimated as 9–11 mmol TE.kg^{-1} to prevent postprandial oxidative stress relative to a daily energy intake of 2000–2500 kcal [133]. Researchers previously reported that green tea polyphenols inhibit cyclooxygenase and lipoxygenase activities in human colon mucosa cells and human colon cancer cells [134]. On the other hand, the importance of green tea polyphenols as radical and oxidant scavengers *in vivo* might be minor, based on their limited concentrations achieved in plasma and tissues for a few hours [135]. However, fiber-bound antioxidants may reach the colon without being digested, and contribute to the formation of a reduced environment. They are also extensively degraded by the colon microflora to produce simpler phenolic compounds. The health benefits of bound whole grain phytochemicals are more effective in the colon. This may partly explain the mechanism of grain consumption in the prevention of colon cancer and other digestive cancers, which is supported by epidemiological studies. Moreover, fiber-bound antioxidants have a potential importance in food processing due to the inhibition of lipid oxidation via releasing of phenolic compounds slowly and continuously. Nevertheless, the levels of antioxidant capacity bound to naturally occurring dietary fibers are very limited. Therefore, increasing the bound antioxidant capacity of fiber is vital for both human health and food processing. The present study revealed that the antioxidant capacity of insoluble wheat bran could be increased by means of its treatment with different beverages. Among the beverages tested (white and red wines, black and green tea infusions), treatment with green tea infusion caused the highest increase in the total antioxidant capacity of insoluble wheat bran. Temperature, time, air and pH were found to be significantly affecting the reaction between insoluble wheat bran and polyphenols.

Antioxidant capacity of insoluble wheat bran increased to a remarkably high level exceeding 100 mmol TE.kg^{-1} from a level less than 10 mmol TE.kg^{-1} after treatment with green tea infusion under optimum conditions (50 °C, pH 9.0, no airflow). Concentration of free amino groups available in wheat bran significantly decreased while the total bound phenolic compounds in wheat bran increased after the treatment. These results suggested that the reaction between oxidized polyphenols and free amino groups available on the surface of bran might be responsible for the increased antioxidant capacity of insoluble wheat bran. According to *in vitro* digestion results, green tea treated wheat bran preserve some of its antioxidant capacity in its insoluble fraction through the colon. It shows 20 times more antioxidant activity than untreated insoluble wheat bran at the end of the duodenal and colon phases, since it has more bound antioxidant compounds than insoluble wheat bran.

This master thesis contributed greatly to understanding the reaction between dietary fiber and soluble antioxidant compounds and digestion behaviors of bound antioxidant compounds in *in vitro* digestion model. These findings can be used to develop functional dietary fibers rich in bound antioxidant capacity and to design foods with a controlled release of phytochemicals in digestion system.

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