DETERMINATION OF THE INTERACTIONS BETWEEN BOUND AND FREE ANTIOXIDANTS NATURALLY OCCURRING IN FOODS

GIDALARDA BULUNAN SERBEST VE BAĞLI DOĞAL ANTİOKSİDANLAR ARASINDAKİ ETKİLEŞİMLERİN BELİRLENMESİ

ECEM EVRİM ÇELİK

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Supervisors

Submitted to Graduate School of Science and Engineering of Hacettepe University
as a Partial Fulfillment to the Requirements
for the Award of the Degree of Doctor of Philosophy
in Food Engineering

2017

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This work named **'Determination of the Interactions between Bound and Free Antioxidants Naturally Occurring in Foods'** by **ECEM EVRİM ÇELİK** has been approved as a thesis for the Degree of **DOCTOR OF PHILOSOPHY IN FOOD ENGINEERING** by the below mentioned Examining Committee Members.

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☐ **Serbest Seçenek/Yazarın Seçimi**

02 / 01 / 2018

Ecem Evrim Çelik

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- All cited studies have been fully referenced
- I did not do any distortion in the data set
- And any part of this thesis has not been presented as another thesis study at this or any other university

15/08/2017

ECEM EVRİM ÇELİK

PREFACE

This PhD study was carried out at the Department of Food Engineering, Faculty of Engineering, Hacettepe University and Department of Food Science, Faculty of Science, University of Copenhagen within the frame of a double degree PhD program. The project was partially funded by Hacettepe University Research Projects Coordination (*Project no: FBI-2015-5613*).

This thesis is dedicated to Prof. Dr. Yaşar Kemal Erdem,
the captain of pleasure, love and blues...

ABSTRACT

DETERMINATION OF THE INTERACTIONS BETWEEN BOUND AND FREE ANTIOXIDANTS NATURALLY OCCURRING IN FOODS

ECEM EVRİM ÇELİK

Doctor of Philosophy, Department of Food Engineering

Doctor of Philosophy, Department of Food Science

Supervisors: Prof. Dr. Vural GÖKMEN, Assoc. Prof. Jose Manuel Amigo RUBIO, Prof. Mogens Larsen ANDERSEN

December 2017, 99 pages

This thesis study aimed at investigating the interactions between free and macromolecule-bound antioxidants naturally occurring in foods, to estimate the possible status of antioxidant environment when they are found together. Chemometric tools were used both at the experimental design and multivariate data analysis steps.

Dietary fiber (DF), protein and lipid-bound antioxidants, obtained from whole wheat, soybean and olive oil products, respectively and Trolox were used for this purpose in the first and second parts of the experimental studies. In the first part, antioxidant capacity (AC) measurements were performed in aqueous ABTS radical medium by monitoring the absorbance in the presence of Trolox and macromolecule-bound antioxidants. Results revealed antagonistic interactions for Trolox with all macromolecule- bound antioxidants. The reason behind this antagonism was investigated through oxidation reactions of Trolox via mass spectrometry analysis. Consequently, a proof was obtained for inhibitory effect of bound-antioxidants on auto-regeneration reactions of Trolox. In the second part, experimental studies were carried out in an autoxidizing liposome medium by monitoring the inhibition of lipid oxidation via antioxidants. Results revealed synergistic interactions for DF and refined olive oil-bound antioxidants, and antagonistic interactions for protein and extra virgin olive oil-bound antioxidants with Trolox. A generalized version of logistic functions was successfully used for modelling the oxidation

curve of liposomes. Principal component analysis revealed two separate phases of liposome autoxidation.

Experiments in the third and fourth parts were performed with a structural approach to enlighten the mechanism behind the interactions of free and macromolecule- bound antioxidants. In the third part, the interactions between whole wheat (WW) DF- bound antioxidants and hydroxycinnamic acid/ hydroxybenzoic acid (HCA/ HBA) derivatives containing different amounts of –OH and -OCH_3 groups localized at different positions on their aromatic rings were investigated. Studies were performed in liposome and aqueous media by monitoring lipid oxidation and scavenging of DPPH radical respectively, in the presence of WW- bound antioxidants and HCA/ HBA derivatives. Predominantly synergistic interactions were observed in aqueous medium, while both synergistic and antagonistic interactions were seen in liposome medium. Behaviors of HCA/ HBA derivatives, linked with their substitutions were revealed. In the fourth part, the interactions of coffee and bread crust melanoidins with HCA/ HBA derivatives were investigated. Experimental studies were carried out in aqueous DPPH radical medium by monitoring the scavenging of radical via melanoidins and HCA/HBA derivatives. Synergistic interactions were revealed for both coffee and bread crust melanoidins with HCA/HBA derivatives. Phases of the radical scavenging reactions were revealed from the loadings plots.

In the fifth part, the interactions between insoluble fractions of different coffee infusions and major cocoa free antioxidants, catechin and epicatechin, as well as the interactions between different coffee infusions and dark chocolate were investigated. *Espresso*, filtered coffee, French press, and Turkish coffee were the coffee infusions used for this purpose. Antioxidant capacity measurements were performed in DPPH radical medium, by monitoring the absorbance in the presence of antioxidant components. Results revealed synergistic interactions for the insoluble fraction of *espresso*, and additive/antagonistic interactions for the insoluble fractions of rest of coffee infusions with catechin and epicatechin. Interactions between coffee infusions and chocolate were synergistic for French press and Turkish coffee and additive/antagonistic for the rest of coffee infusions.

Keywords: macromolecule- bound antioxidants, free antioxidants, interactions, chemometrics

ÖZET

GIDALARDA BULUNAN SERBEST VE BAĞLI DOĞAL ANTİOKSİDANLAR ARASINDAKİ ETKİLEŞİMLERİN BELİRLENMESİ

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Doktora, Gıda Mühendisliği Bölümü
Doktora, Gıda Bilimi Bölümü
Tez Danışmanları: Prof. Dr. Vural GÖKMEN, Assoc. Prof. Jose Manuel Amigo RUBIO, Prof. Mogens Larsen ANDERSEN
Aralık 2017, 99 sayfa

Bu tez calışması, gıdalarda doğal olarak görülen serbest ve makromoleküllere bağlı antioksidanlar arasındaki interaksiyonların, bir arada bulundukları zaman antioksidan çevrenin olası durumunu değerlendirmek amacıyla incelenmesini amaçlamıştır. Kemometrik araçlar, hem deneysel tasarım hem de çokyönlü veri analizi aşamalarında kullanılmıştır.

Bu amaçla deneysel çalışmaların ilk ve ikinci bölümlerinde sırasıyla tam buğday, soya fasulyesi ve zeytinyağı ürünlerinden elde edilen besinsel lif, protein ve lipide bağlı antioksidanlar ile Troloks kullanılmıştır. İlk bölümde, antioksidan kapasite ölçümleri sulu ABTS radikali ortamında, Troloks ve makromoleküllere bağlı antioksidanların varlığında absorbansın izlenmesi ile gercekleştirilmiştir. Sonuçlar, Troloks için tüm makromoleküllere bağlı antioksidanlarla birlikte antagonistik interaksiyonlar olduğunu ortaya çıkarmıştır. Bu antagonizmin arkasındaki sebep, Troloksun oksidasyon reaksiyonları yoluyla kütle spektrometresi analiziyle araştırılmıştır. Sonuç olarak, bağlı antioksidanların Troloksun oto-rejenerasyon reaksiyonu üzerine inhibitor etkisi için bir kanıt elde edilmiştir. İkinci bölümde, deneysel calışmalar otokside olan bir lipozom ortamında lipid oksidasyonunun antioksidanlarla

inhibisyonunun izlenmesiyle yürütülmüştür. Sonuçlar, Troloks ile besinsel liflere ve rafine zeytinyağına bağlı antioksidanlar arasında sinerjistik, protein ve sızma zeytinyağına bağlı antioksidanlar arasında antagonistik interaksiyonlar olduğunu ortaya çıkarmıştır. Lojistik fonksiyonunun genelleştirilmiş bir versiyonu lipozomlarin oksidasyon eğrisini modellemek amacıyla başarıyla kullanılmıştır. Temel bileşen analizi, lipozom otoksidasyonunun iki ayrı fazını ortaya çıkarmıştır.

Üçüncü ve dördüncü bölümlerdeki deneyler, serbest ve makromoleküllere bağlı antioksidanlar arasındaki interaksiyonların arkasındaki mekanizmanın aydınlatılması için, yapısal bir yaklaşımla yapılmıştır. Üçüncü bölümde, tam buğday besinsel liflerine bağlı antioksidanlar ve aromatik halkaları üzerinde farklı pozisyonlarda, farklı miktarlarda –OH ve -OCH_3 grupları içeren hidroksisinnamik/ hidroksibenzoik asitler arasındaki interaksiyonlar incelenmiştir. Çalışmalar, lipozom ortamı ve sulu ortamda tam buğdaya bağlı antioksidanlar ve hidroksisinnamik/ hidroksibenzoik asit türlerinin varlığında sırasıyla lipid oksidasyonu ve DPPH radikalinin sönümlenmesinin izlenmesiyle yürütülmüştür. Sulu ortamda ağırlıklı olarak sinerjistik interaksiyonlar gözlenirken, lipozom ortamında hem sinerjistik hem de antagonistik interaksiyonlar görülmüştür. Hidroksisinnamik/ hidroksibenzoik asit türlerinin, dallanma grupları ile ilişkili olarak davranışları ortaya çıkarılmıştır. Dördüncü bölümde, kahve ve ekmek kabuğu melanidinlerinin hidroksisinnamik/ hidroksibenzoik asit türleriyle interaksiyonları incelenmiştir. Deneysel çalışmalar sulu DPPH radikali ortamında radikalin melanoidinler ve hidroksisinnamik/ hidroksibenzoik asit türleri aracılığıyla sönümlenmesinin izlenmesi yoluyla yürütülmüştür. Hem kahve hem de ekmek kabuğu melanoidinleri icin hidroksisinnamik/ hidroksibenzoik asit türleri ile sinerjistik interaksiyonlar açığa çıkarılmıştır. Radikal sönümleme reaksiyonlarının aşamaları loading grafikleri ile ortaya çıkarılmıştır.

Beşinci bölümde, farklı kahve infüzyonlarının çözünmeyen kısımları ve temel kakao serbest antioksidanları, kateşin ve epikateşin ile farklı kahve infüzyonları ve bitter çikolata arasındaki interaksiyonlar incelenmiştir. Espresso, filtre kahve, French press ve Türk kahvesi bu amaçla kullanılan kahve infüzyonlarıdır. Antioksidan kapasite ölçümleri DPPH radikali ortamında antioksidan bileşenlerin varlığında absorbansın izlenmesiyle gerçekleştirilmiştir. Sonuçlar, kateşin ve epikateşin ile espressonun çözünmeyen kısmı için sinerjistik ve diğer kahve infüzyonlarının çözünmeyen kısımları için toplam/ antagonistik interaksiyonlar ortaya

koymuştur. Kahve infüzyonları ve çikolata arasındaki interaksiyonlar French press ve Türk kahvesi için sinerjistik, diğer kahve infüzyonları için toplam/ antagonistik olmuştur.
Anahtar Kelimeler: makromoleküllere bağlı antioksidanlar, serbest antioksidanlar, interaksiyonlar, kemometri

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SYMBOLS AND ABBREVIATIONS

AACC	American Association of Cereal Chemists
ABTS	2,2`-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
AC	Antioxidant Capacity
ADP	Adenosine 5′-diphosphate sodium salt
ANOVA	Analysis of Variance
ASCA	ANOVA Simultaneous Component Analysis
AUC	Area Under the Curve
CHD	Coronary Heart Disease
CI	Coffee Infusions
CVD	Cardiovascular Diseases
DF	Dietary Fiber
DoE	Design of Experiment
DPPH	2,2-Diphenyl-1-picrylhydrazil
GAB	Glycine Ascorbate Buffer
GI	Gastrointestinal
HBA	Hydroxybenzoic Acids
HCA	Hydroxycinnamic Acids
HDL	High Density Lipoprotein
MRPs	Maillard Reaction Products
OO	Olive Oil
PC	Principal Component
PCA	Principal Component Analysis
PB	Sodium Phosphate Buffer
PPB	Potassium Phosphate Buffer
PUFA	Polyunsaturated Fatty Acids
QUENCHER	Quick Easy New Cheap and Reproducible
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
TEAC	Trolox Equivalent Antioxidant Capacity

UV-Vis	Ultra Violet Visible
WW	Whole Wheat

LIST OF PUBLICATIONS

PEER REVIEWED PUBLICATIONS

PAPER I

Çelik, E. E., Rubio, J. M. A., Andersen, M. L., Gökmen, V. 2017. Interactions between macromolecule-bound antioxidants and Trolox during liposome autoxidation: A multivariate approach, Food Chemistry, 237, 989- 996.

PAPER II

Çelik, E. E., Rubio, J. M. A., Gökmen, V. 2017. Study of the Behavior of Trolox with Macromolecule-bound Antioxidants in Aqueous Medium: Inhibition of Auto-Regeneration Mechanism, Food Chemistry, Accepted Manuscript

PROCEEDING

MANUSCRIPT I

Çelik, E. E., Rubio, J. M. A., Andersen, M. L., Gökmen, V. Interactions between Dietary Fiber-Bound Antioxidants and Hydroxcinnamic / Hydroxybenzoic Acids in Aqueous and Liposome Media

MANUSCRIPT II

Çelik, E. E., Rubio, J. M. A., Andersen, M. L., Gökmen, V. Interactions of Coffee and Bread Crust Melanoidins with Hydroxycinnamic / Hydroxybenzoic Acids

MANUSCRIPT III

Çelik, E. E., Gökmen, V. Interactions between Antioxidants from Different Coffee Infusions and Major Free Antioxidants of Cocoa/Chocolate

ORAL PRESENTATIONS

Çelik, E. E., Rubio, J. M. A., Andersen, M. L., Gökmen, V. Interactions between Macromolecule- Bound Antioxidants and Free Antioxidant Trolox in Liposome Medium: A Multivariate Approach. EuroFoodChem XIX Conference, Budapest, Hungary, 2017.

POSTERS

Çelik, E. E., Rubio, J. M. A., Andersen, M. L., Gökmen, V. Interactions of Coffee Melanoidins with Hydroxycinnamic/ Hydroxybenzoic Acids. Fourth Internatıonal Conference on Cocoa Coffee and Tea, Turin, Italy, 2017

INTRODUCTION

Antioxidants are among the most important parts of human nutrition due to the correlation of their intake with the lower incidence for cardiovascular diseases (CVD), cancer, ageing and age-related degenerative processes [1]. Besides being free from physical or chemical interactions, a significant amount of dietary antioxidants are also bound to different macromolecules like dietary fibers (DFs), proteins or lipids [2]. Since both bound and free antioxidants constitutes a significant portion of our daily diet, it becomes essential to investigate their interactions in order to potentially take further advantage of their health effects after their consumption. If free and bound antioxidants, ingested with foods, exist in any part of the digestive system at the same time they may react, regenerate each other and create a synergistic effect; antagonistic or additive interactions may occur as well. In case of synergistic interaction, a greater total antioxidant activity will be created than the simple sum of the antioxidant activities of bound and free antioxidants separately. Thus, it will improve the health effects of these antioxidants in the gastrointestinal (GI) tract via improving the quality of the healthy antioxidant environment there. Contrarily, an antagonistic interaction will create a total antioxidant effect which is less than the sum of their individual antioxidant activities and impair the antioxidant environment. On the other hand, an additive interaction may not affect the total antioxidant activity of bound and free antioxidants as they act separately [3].

In this framework, the hypothesis and starting point of this thesis is the known regeneration reaction and synergistic interaction between free soluble and DF-bound antioxidants [4, 5]. Considering the diversity of macromolecule-bound antioxidants in our daily diet and the variety of interactions that can occur between these bound and free antioxidants that are consumed together, a necessity for a broader study comes out. Hence, the objective of this study is to investigate the interactions between free and macromolecule-bound antioxidants, obtained from different sources, to estimate the possible status of antioxidant environment when they found together.

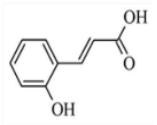
Three main groups of macromolecule-bound antioxidants, i.e.: DF-bound, protein-bound and lipid-bound antioxidants and a well-known free antioxidant, Trolox, were used in the first and second parts of the experimental studies. To represent DF, protein and lipid-bound antioxidants, processed (to various extents) and unprocessed versions of whole wheat, soybean and olive oil were used respectively, after some specific preparation steps. In the first part, antioxidant

capacities of Trolox and macromolecule- bound antioxidants separately and in mixtures, in different concentrations, were determined by measuring the absorbance of the aqueous ABTS radical in presence of these species. The pH of the radical media was also changed for DF and protein-bound antioxidant studies. In the second part, evaluation of antioxidant activities was performed by monitoring the oxidation of liposomes. In this way, the oxidation was investigated by the formation of lipid oxidation products stage. The sigmoid shaped oxidation curves of liposomes in the presence of the mixtures of macromolecule- bound antioxidants and Trolox were modelled according to the equation given, which is a generalized form of logistic equation:

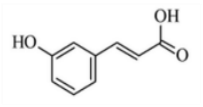
$$F = \frac{A}{B + \exp(-k * t)} \qquad (1)$$

The third and fourth parts of the experimental studies were designed in accordance with the need for a well- designed structural approach to make clearer explanations behind the interactions of free and macromolecule- bound antioxidants. In this framework, interactions between WW-bound antioxidants and 21 HCA/ HBA derivatives (Figure 1.1) containing different amounts of –OH and/or $–OCH_3$ groups localized at different positions of the aromatic ring were investigated in the third part. The experiments were performed both in aqueous and liposome media by monitoring the scavenging of DPPH radical and oxidation status of liposomes, respectively in the presence of WW- bound antioxidants and HCA/ HBA derivatives separately and in mixtures.

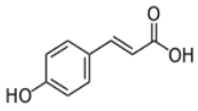
In the fourth part, the interactions between coffee and bread crust melanoidins with 20 different HCA/ HBA derivatives (except 2-hydroxy-3,4-dimethoxybenzoic acid in Figure 1.1) were investigated. Experimental studies were carried out in aqueous medium by monitoring the scavenging of DPPH radical in the presence of melanoidins and HCA/ HBA derivatives separately and in mixtures. In the fifth part, the interactions between bound and free antioxidants from food sources that are frequently consumed together were aimed to be examined. With this regard, the insoluble fractions of coffee infusions brewed with different methods and the major free antioxidants found in cocoa, namely catechin and epicatechin were investigated.



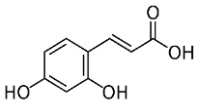
2- hydroxycinnamic acid
o-coumaric acid



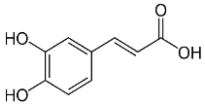
3- hydroxycinnamic acid
m-coumaric acid



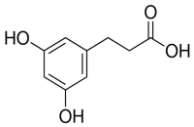
4- hydroxycinnamic acid
p-coumaric acid



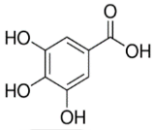
2,4-dihydroxycinnamic acid



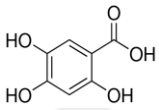
3,4-dihydroxycinnamic acid
caffeic acid



3,5-dihydroxyhydrocinnamic acid



3,4,5-trihydroxybenzoic acid
gallic acid



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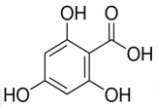

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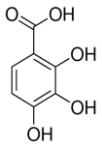

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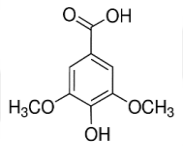
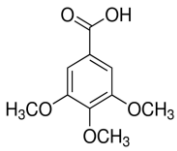


4-hydroxy-3,5-dimethoxybenzoic acid
syringic acid



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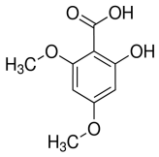

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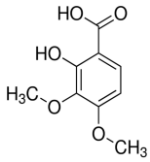

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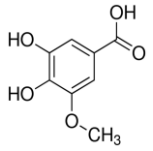

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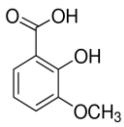

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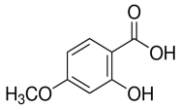

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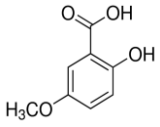

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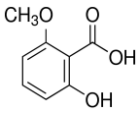
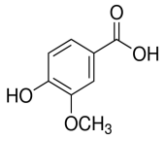
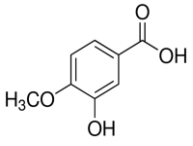


2-hydroxy-6-methoxybenzoic acid



4-hydroxy-3-methoxybenzoic acid
vanillic acid



3-hydroxy-4-methoxybenzoic acid
isovanillic acid

Figure 1.1. Hydroxycinnamic and hydroxybenzoic acid derivatives.

Also, the interactions between different coffee infusions and dark chocolate (60 % cocoa) directly as they are consumed were investigated. In this way, the background of the interactions, specifically whether they are dependent upon only the regeneration reaction between the insoluble fractions of coffee infusions and the free antioxidants of cocoa or the reactions between the total antioxidant content of these constituents were intended to be enlightened. In addition, the importance of brewing step on these interactions was aimed to be centered. Antioxidant capacity measurements were performed by measuring the absorbance of the aqueous ABTS radical in presence of these species.

Chemometic methods were used in this study both at the stage of Design of Experiment (DoE) and multivariate data analysis (Principal Component Analysis (PCA) and Anova Simultaneous Component Analysis (ASCA)) , in contrast to traditional chemical and physical relationships which usually consider just one or a very few variables at the same time [6].

1. GENERAL INFORMATION

1.1. Free Radicals and Oxidative Stress

Free radicals are the chemical species having one or more unpaired electron in their outer orbit [7]. They are inevitably unstable and reactive. The simplest free radical is a hydrogen atom, which contains one proton and a single unpaired electron. Once a free radical is formed, it has the ability to react with another radical or molecule through different interactions [8]. The selectivity and rate of these reactions may vary depending on: i) the concentration of radicals, ii) delocalization of the single electron of the radical, iii) existence of weak bonds in the molecules which present in the environment that the radical can react.

Examples of free radical species significant for living organisms include hydroxyl ($OH\cdot$), superoxide ($O_2\cdot-$), nitric oxide ($NO\cdot$), and peroxyl ($RO_2\cdot$). Besides, there are species, which can lead to free radical reactions in living organisms despite being non- radicals such as; singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO-$) and hypochorous acid ($HOCl$) [8]. Since the conversion of oxygen and nitrogen free radicals to the non-radical species is possible, the terms “reactive oxygen species” (ROS) and “reactive nitrogen species” (RNS) are not only used to express the radicals [9].

ROS and RNS are generated in animals and humans owing to biochemical reactions, overly exposure to environment and high levels of dietary xenobiotics [10]. They cause oxidative stress in different pathophysiological conditions [11, 12], which change the cellular constituents in the human body. Principally all biomolecules, especially the polyunsaturated fatty acids (PUFA) are affected by free radicals. Since the progress of the oxidative damage in PUFA, in other words lipid peroxidation is like a self-perpetuating chain reaction, it is destructive in particular [13, 14], contributing to the development of CVD like atherosclerosis and preeclampsia [8]. Moreover, the end products of lipid peroxidation can result in a damage on proteins and DNA, which is known to be a continuous process *in vivo* [8]. Also, deterioration of biological membrane functions such as decreasing fluidity and inactivating enzymes and receptors bound to membrane or altering the nonspecific calcium ion permeability are the effects of peroxidation [15, 16]. When proteins are damaged by oxidation, the function of enzymes, receptors and transport proteins may change. New antigens that can provoke immune responses may also be generated. Furthermore, products from the oxidative damage of proteins have the ability to cause secondary damage to other biomolecules

such as loss of faithfulness of DNA polymerases in DNA replication and inactivation of enzymes that repair DNA [17-19]. In addition to all these, oxidative stress induced by ROS can give rise to various diseases including coronary heart disease (CHD), cancer and osteoporosis [20].

Likewise, for foods, the main reason of chemical degradation is the lipid oxidation that is caused by free radical chain reactions, involving initiation, propagation and terminations steps. In short, these reactions start with an attack of a free radical to an unsaturated fatty acid to abstract a hydrogen atom from the site which needs the lowest energy [21] and propagates like a chain constructed by successive reactions, until a termination step is reached. These reactions are called as autoxidation, which results in development of rancidity in foods, diminishes shelf-life, deteriorates sensory properties and reduces consumer acceptability [22]. In addition, photooxidation, which is a lipoxygenase assisted process, and thermal oxidation, which occurs under high temperatures like in the frying process results in the impairment of lipid quality in foods [23].

As a matter of fact, oxidation is an inevitable process both for the human body and foods. All substrates of free radical oxidation, including PUFA, excessive amounts of oxygen and transition-metal catalysts, which are capable of generating chain- initiating radicals are present in human body. Besides, the physical conditions such as the body temperature (37 °C), moisture and exposition to light may favor free radical activity easily [24]. Also for foods, the conditions are always in favor of oxidation. Under these circumstances, the key to overcome the oxidative stress in human body is basically enhancing cellular defenses via antioxidants [25, 26]. Similarly, the protection of lipids from deterioration in food matrix can be provided by antioxidants. [22].

1.2. Antioxidants

Antioxidants were defined as `any substance, significantly delays or inhibits oxidation of an oxidizable substrate when present at low concentrations compared to that substrate` by Halliwell & Gutteridge (1989) [27]. This definition reveals two main classes of antioxidants as 1) chain breaking antioxidants, which intercept the primary chain reactions and 2) preventive antioxidants, which prevent or retard the formation of initiating radicals by using the mechanisms involving metal deactivation, UV absorption or peroxide decomposition [28].

In addition to this, antioxidants can be categorized as enzymatic and non-enzymatic according to their activities. [29]. The mechanism of action of enzymatic antioxidants is based on breaking

down and removing free radicals. They constitutively reduce the levels of lipid hydroperoxide and H_2O_2 within a multi-step process with the help of cofactors like copper, iron, manganese and zinc. In this way, inhibition of lipid peroxidation and maintenance of the function and structure of cell membranes are ensured. Protection of human body from ROS itself is provided by enzymatic antioxidants, such as catalase, glutathione peroxidase, superoxide dismutase and peroxiredoxin I-IV [12]. The amount of these enzymes may vary for different subcellular sites and different cell types [30].

On the other hand, the mechanism of action of non-enzymatic antioxidants is based on interrupting the free radical chain reactions, in which a compound carrying an unpaired electron reacts with another compound to leave an unpaired electron in that compound. After interrupting a free radical, they become able to interact with water- soluble compounds to regenerate themselves. In this way, the potential destructive effects of radicals are evaded from the target molecules. In such systems where an efficient regeneration is available, one to three molecules of antioxidants may be enough to preserve a thousand of target molecules[25]. Non-enzymatic antioxidants can be either natural or synthetic. Plant polyphenols, vitamin C, vitamin E, carotenoids and glutathione constitute the main representatives of the natural antioxidants [12].

1.2.1. Antioxidants Naturally Occurring in Foods

Despite the fact that the human body is able to produce a variety of antioxidants effective against ROS, we are still in need of at least two antioxidants, vitamins C and E, which the human body cannot produce itself, and hence must be ingested via foods regularly [31]. Besides those, a number of natural antioxidants are contained in foods, which may be; i) endogenous compounds, ii) substances formed during processing and iii) natural additives.

Generally, the natural antioxidants are of plant origin, which may be found in any part of the plant [32]. They are included in the group of phenolic compounds covering simple phenols, phenolic acids (rosmarinic acid), anthocyanins (deplhinidin), HCA (ferulic acid), HBA (vanillic acid), lignans (sesamol), tannins (tannic acid), stilbenes (resveratrol), coumarins (α-coumarin), essential oils (limonene, eugenol) and flavonoids (quercetin, catechin).

Plant polyphenols can act with various complementary and overlapping antioxidant mechanisms [33-38]. They function as reducing agents, hydrogen donors, metal chelators, singlet oxygen quenchers and ferryl hemoglobin reductants [39-44]. Numerous hydroxyl groups contained in their

structure gave them the ability to scavenge multiple free radicals. They can be divided into four major groups including phenolic acids (gallic, caffeic, rosmarinic acids), phenolic diterpenes (carnosol, carnosic acid), flavonoids (catechin, quercetin) and volatile essential oils (eugenol, menthol, thymol) [45]. Free radical scavenging and metal chelating constitutes the mechanism of actions for flavonoids broadly, while free radical trapping is the main action of phenolic acids [46].

Vitamin C, namely ascorbic acid is one of the principal water- soluble free radical scavengers existing in biological tissues. It is one of the most potent and least toxic antioxidant [47], which efficiently can scavenge free radicals and form new ones with low energies [48]. It functions as an oxygen scavenger, reducing agent and a metal chelator and is known to act synergistically with tocopherols. The synergistic action leads to the regeneration of tocopherols, which enables them to be used in lower amounts. Also when in use with synergistic antioxidants, ascorbic acid is an efficient stabilizing agent for oils and lipid containing foods [46].

Tocopherols, which belong to Vitamin E family together with tocotrienols [49] are antioxidants naturally found in plants [50]. They are subdivided into 4 groups as α- tocopherol (Vitamin E), which is biologically the most common and active form, β- tocopherol, δ- tocopherol and γ- tocopherol. The antioxidant activity of tocopherols is mainly supplied by the hydrogen atom bound at the C_6 position [46]. Besides being singlet oxygen quenchers [51], they show their fundamental effect by scavenging peroxyl and alkoxyl radicals [52]. They break free radical chain reactions by governing the free radical. This function is achieved by donating the hydrogen atom from the free hydroxyl group on their aromatic ring to the free radical and creating a rather stable form of Vitamin E [53, 54].

Carotenoids are the fat-soluble pigments naturally found in yellow, orange or red color. They belong to the tetraterpene family characterized with a polyisoprenoid structure. Carotenes, which contain carbon and hydrogen atoms and xanthophylls (oxycarotenoids), which contain at least one oxygen atom in addition to carbon and hydrogen atoms constitute two major classes of carotenoinds. They are the protectives of plants against photooxidation [46]. Besides, they are efficient scavengers of free radicals and singlet oxygen molecules [55, 56]. The radical scavenging ability of carotenoids is originated from their extended electron delocalization. When the oxygen pressure is high in the medium, they act as singlet oxygen quenchers and hydrogen peroxide scavengers, while at low oxygen pressure when singlet oxygen is not present in the environment,

they act as chain breaking antioxidants and exert synergistic behavior with other antioxidant species [57, 58]. β- carotene, lycopene, lutein, zeaxanhtin, astaxanthin and β-cryptoxantin are the main carotenoids important for human nutrition [59].

Origin for plant polyphenols in foods can be counted as fruits, herbs, spices, seeds, nuts, grains, tea and coffee [46]. Among them, red fruits such as strawberry, blackberry, blueberry, sweet/ sour cherry, black/ red currant, chokeberry and red raspberry contain high amounts of HCA (caffeic, ferulic, p- coumaric acids), together with p-hydroybenzoic and ellagic acid [60]. Dietary spices including mint, sweet basil, oregano, rosemary, sage and thyme constitutes a good source for rosmarinic acid on the other hand [61]. Nuts and vegetable oils are the primary sources of tocopherols, while cereal grains and some specific vegetable oils like palm and rice bran oils are the main sources of tocotrienols. Carotenoids are contained in yellow or orange fruits and vegetables together with the green leafy vegetables. However, fruits like citrus fruits, cherries, kiwi and melon and vegetables like tomatoes, green leafy vegetables, cauliflower, cabbage, Brussel sprouts and broccoli constitute the main sources of ascorbic acid [46].

Flavonoids, with its 10 major subclasses, flavones, flavonols, flavanols, chalcones, flavanones, isoflavonoids, neoflavonoids, biflavonoids, flavanonols, and anthocyanins are contained in a wide range of foods as well. Flavonols, the most widespread subclass of flavonoids is present in onions, broccoli, tomato, apples, cherries, blueberries, kale, tea, red wine, caraway, cumin and buckwheat. Flavones are contained in parsley, broccoli, celery, legumes, thyme, tea and some other herbs [46], while some of their polymethoxylated flavones are stored in the skins of citrus fruits [62]. Flavanones (naringenin, hesperetin, eriodicytol) as well as their glycosides (naringin, hesperidin) are found in grapes, citrus fruits and medicinal herbs (from *Rutaceae, Rosaceae* and *Lagumunoseae* family) [63, 64]. Flavanols, the most complex flavonoids, such as catechin, epicatehin, epicatechin gallate, epigallocatechin and epigallocatechin gallate are contained in berries, apples, tea, cocoa [65], grapes and certain seeds of leguminous plants [66, 67]. Anthocyanins together with anthocyanidins are abundant in red, pink, purple or blue colored fruits such as grape skins, blueberries, red cabbages, beans, red/purple rice and corn and purple sweet potatoes. Chalcones are found in herbs, while isoflavones (genistein, daidzein) and their glycosides (genistin, daidzin) are mostly found in soya and its products. Fruits, vegetables, olive oil, wine, coffee and tea constitute the main sources of coumarins [68]. However, lignans are mainly

contained in flaxseed, seseme seeds and Brassica vegetables [69]. Olive oil is a good source for tyrosol, hydroxytyrosol and olueoropein [70-72], while resveratrol is mainly found in foods like grapes, peanuts, berries and turmeric.

In addition to this, there is considerable amounts of natural antioxidants in animal tissues such as carnosine, which is a dipeptide with the ability to act as a chelator and a free radical scavenger. Besides, there are antioxidant vitamins, minerals and enzymes that should not be ignored [23].

1.2.2. Antioxidants Formed During Processing

It is known that food processing like pasteurization, sterilization, dehydration and cooking together with storage results in a significant loss in the amount of antioxidants present in foods [73]. At the same time, especially during heat treatment, new molecules possessing antioxidant activity, namely melanoidins, may be formed via Maillard reactions, in which the sugars condense with free amino acids, peptides or proteins [74]. In this way, the loss of natural antioxidants can be balanced by the formation of new ones [75].

Melanoidins are brown- colored complex molecules which have the ability to scavenge free radicals such as hydroxyl, superoxide and peroxyl radicals and chelate metal ions, primarily iron [76, 77]. The molecular weights of melanoidins depend on the source of reactants together with the reaction conditions [78, 79] and their chemical structure is not clearly determined yet. However, there is a general opinion that they are anionic, high molecular weight nitrogen containing compounds [76, 80].

Melanoidins are contained in a wide variety of foods including roasted coffee beans [81], cocoa [82], bread [83], malt [84], roasted meat [85], roasted barley [86] and balsamic vinegar [87]. Among them, coffee and bread are considered as the main sources with a daily intake of approximately 10 g for the general population [88].

Melanoidins are responsible for the development of color [89] and texture in thermally processed foods, besides being able to bind flavors [90] and chelate metal ions [91]. Besides their antioxidant activity, [78, 92] they are known to exert antimicrobial [93] and prebiotic [83] activities. Furthermore, there is substantial evidence that they act as DF, being resistant to digestion in the upper GI tract and fermented in the gut [94].

Melanoidins do not fit into the definition of DF literally, since they are not "polysaccharides naturally present in raw foods", but formed during processing and include an amino acid/protein moiety in addition to the polysaccharides [94]. However, they are named as antioxidant- DF [95] since they are able to quench free radicals continuously formed in the GI tract via their reducing functional groups [96]. Carrying both antioxidant and DF characteristics sets them to an important place in the human nutrition like other DF- bound antioxidants naturally found in foods.

1.3. Structure- Activity Relationships of Antioxidants

Antioxidant activity of the major antioxidant components in human daily diet, namely phenolic compounds are dependent upon their molecular structure, broadly to the presence and number of -OH groups, double bond conjugation and resonance effects [97]. Especially the configuration and number of –OH groups can significantly affect the various mechanisms of antioxidant activity as conformed with most of the polyphenolic compounds [98-101]. As known, the stabilization of radicals such as hydroxyl, peroxyl and peroxynitrite occurs via donation of hydrogen atom or electron from the hydroxyl groups on the B-ring followed by a formation of relatively stable flavonoid radical. This radical scavenging activity increases with the number of –OH groups among species showing structural similarity [98].

A 3`4` catechol structure on the B-ring is evidenced to provide strong radical scavenging [98, 100, 102] and increased lipid peroxidation [103] activities. Flavonoids are oxidized, when catechol structure facilitates electron delocalization [104] by forming a fairly stable o-semiquinone radical [105]. However, flavones lacking catechol or o-trihydroxyl (pyrogallol) structures are weak scavengers [101] due to the formation of unstable radicals. Apart from increasing the total number of –OH groups, substitution of the A-ring has a little effect on antioxidant activity. A 5-OH is thought to contribute antioxidant activity [106], while a 5,7-m-dihydroxy structure is reported to increase TEAC [97]. Most importantly, the free radical scavenging activity of flavonoids strongly depends on the presence of a free 3-OH in their structure [101]. Together with 3`4` catechol design, flavonoids with 3-OH are shown to be 10 folds more potent than ebselen, which is a known RNS scavenger [100]. The main contribution of 3-OH to the antioxidant activity is the planarity that it creates in the three dimensional structure of flavonoids, which allows conjugation, electron dislocation and increase in the flavonoid phenoxyl radical scavenging stability [107]. However,

substitution of the 3-OH with a methyl of glycosyl group is reported to endamage the antioxidant activity as proven for quercetin and kaempferol against β-carotene oxidation in linoleic acid [101, 108].

When flavonoids are O-methylated instead of hydroxylation, the main factors affecting antioxidant activity are the hydrophobicity and molecular planarity changed. Generally, antioxidant activity is repressed through steric effects which subverts the planarity [101]. The ratio of methoxy to hydroxyl substituents has no effect on the radical scavenging capacity of flavonoids compounds. However, the position of the methoxy group on the B-ring is highly important. For instance, changing 6`-OH/4`-OMe structure with 6`-OMe/4`-OH, by encouraging coplanarity, can totally destroy the DPPH scavenging ability [109]. Creating a steric hindrance to the 3`4` catechol structure via 4`-O- methylation can easily destroy the antioxidant activity as well. Also, the presence of multiple methoxy groups on the A-ring is able to endamage the positive effect of a catechol structure on the B-ring. In any case, the effect of O- methylation is strongly dependent on the evaluation method and types of radical and substrate used [110].

Apart from these, 2-3 unsaturations together with a 4- carbonyl group have shown to have a significant effect on the antioxidant capacity. Nevertheless, this effect is of no standing unless other structural criteria such as 3`4`- catechol structure, 3-OH and other multi hydroxylation patterns are implemented. The TEAC values of quercetin and catechin constitutes a good example for this situation, quercetin being two folds of catechin, revealing the importance of 2-3 unsaturation and 4-carbonyl groups together [97]. Also, it was demonstrated that 2,3- unsaturation is less significant than 4-oxo itself.

On the other hand, it was proven that the antioxidant activity decreases when aglycones are converted to glycones [111]. Increasing number of glycosidic moieties further decreases the antioxidant activity as shown for flavonol gylcosides [112]. The structure of the sugar moiety together with its position also has an important role. Similar to O- methylation, steric hindrance created by 4`-glycosilation to the 3`4` catechol structure can destroy the antioxidant effect as well.

1.4. Health Effects of Antioxidants

It has been reported that diets rich in fruits and vegetables can be linked with a lower risk of CVD and cancer, which in turn is related with the high antioxidant contents in these foods [113]. In addition to this, antioxidants are known to protect human body from various disease processes including aging, allergies, algesia, arthritis, asthma, atherosclerosis, autoimmune diseases, bronchopulmonary dyspepsia, cataract, cerebral ischemia, diabetes mellitus, eczema, GI inflammatory diseases and genetic disorders [114].

Despite the fact that a wide range of antioxidants are included in the disease prevention, research studies have generally been focused on three antioxidants: Vitamin E, Vitamin C and carotenoids, which are known to be essential nutrients. Each of these antioxidants have its specific effects and they used to work synergistic with each other, enhancing the overall antioxidant capacity in the body.

Among them, Vitamin E is very well known by its singlet oxygen quenching and free radical scavenging abilities. Besides, it can protect β- carotene from oxidation, enhance the immune responses of human body at high concentrations and inhibit the conversion of nitrites to nitrosamines, a kind of cancer promoter, in stomach [10]. Indeed, there are contradictive research present in the literature regarding the effects of Vitamin E on cancer. For instance, the use of Vitamin E supplements has been associated with a 50% decrease in oral cancer risk [115], while an inverse relationship was revealed between Vitamin E supplements and dietary Vitamin E with breast cancer in another study [116]. On the other hand, there is strong evidence about the association between high intakes of Vitamin E and reduced risk of CHD. By inhibiting platelet aggregation and prostaglandin synthesis, Vitamin E has a significant effect on the development of atherosclerosis and other vascular diseases as well [10]. It was reported that Vitamin E supplementation is associated with a reduced rate in lipid peroxidation, which in turn is related with age related degenerative processes [117]. Together with Vitamin C and carotenoids, Vitamin E is linked with the reduced risk of cataract by retarding the lens opacity. Also, consumption of Vitamin C and Vitamin E are thought to be related with slowing the progression of Parkinson`s disease [10].

Vitamin C, namely ascorbic acid is another free radical scavenger and has the ability to regenerate Vitamin E from its radicals. It has a protective effect on aging, arthritis, cancer and coronary artery disease. Formation of carcinogenic compounds such as nitrosamines and quinones are suppressed by Vitamin C, which in turn reveals the protective effect of fruit and vegetable consumption on the risk of cancer [118]. Vitamin C reduces the risk of CVD by increasing HDL level and lowering the total cholesterol level in the blood. Besides, its consumption is associated with a lower risk of cataract and improved sperm quality in heavy smokers [10].

Among carotenoids, β-carotene is prominent by its singlet oxygen quenching and free radical scavenging properties [10]. Together with several other carotenoids, β-carotene is associated with a reduced risk of certain cancer types [119, 120]. For smokers, low concentrations of β-carotene is linked with a high risk of myocardial infarction [121]. Also, low levels of β-carotene in blood is associated with an increased risk of cataracts.

Other antioxidant species also have some beneficial health effects. For instance, selenium, an antioxidant mineral, is known to be a major prophylactic factor against cancer. Its consumption also has some linkages with myocardial ischemia and atherosclerosis. Likewise, flavonol and flavone intake was demonstrated to have an inverse relationship with CHD mortality [122]. Also in a cohort study performed with 500 middle- aged men, the risk of stroke was found to decrease by 60% in 15 years for men in the quartile with the highest intake of flavonol and flavones (> 30 mg/day) [123]. In addition to these, antioxidants found in garlic are related with its cardio-protective effect, anti- aging properties and blood cholesterol lowering effects, while antioxidants in red wine are linked with its protective effects against heart diseases [10].

1.5. Free and Macromolecule- Bound Antioxidants

The physical structure of antioxidant-rich food has a key role in determining the health beneficial effects of antioxidants. In fact, biological properties of antioxidants depend on their capability to react with free radicals, whereas their bioavailability and/or biotransformation can occur only after release from the food matrix during digestion or gut fermentation [124]. Antioxidants may be found in different forms in food microstructure: (i) free from chemical or physical interactions, (ii)

physically entrapped into food matrix, (iii) chemically bound to other macromolecules, or (iv) in insoluble form [5, 125].

Antioxidants found as free form such as vitamins C and E, carotenoids and low molecular weight polyphenols are solubilized and totally or partially absorbed either in the stomach or in the small intestine [5, 126]. They create a peak in plasma antioxidant level immediately after ingestion and disappear in a few hours [127].

However, in complex food matrices, antioxidants are mostly embedded in the matrix and somehow linked with different macromolecules such as carbohydrates, proteins, and lipids [2] and especially DFs [5], building up the "macromolecule-bound antioxidants" concept. These bound antioxidants, constituting a significant portion of dietary antioxidants, were shown to have the ability to quench free radicals as well as free antioxidants. In addition, they carry some noteworthy characteristics affecting their bioavailability and bioaccessibility derived from the macromolecules they bind to [128, 129].

For instance, DF- bound antioxidants have a relatively lower rate of release [130], owing to the resistance of DFs to digestion and absorption in the small intestine [131]. The absorption of phenolics, carotenoids and probably tocopherol are also reported to be retarded by DFs [132]. Indeed, a significant amount of dietary antioxidants pass unchanged through the small intestine bound to DF and reach to colon, where they can be released from the fiber matrix by the action of the bacterial microbiota. Consequently, bioactive metabolites that can be absorbed through the colon are produced [133, 134]. All non-absorbable metabolites and non-fermented polyphenols remain in the colonic lumen and contribute to create a healthy antioxidant environment there by scavenging free radicals and counteracting the effects of dietary pro-oxidants [135]. From this standpoint, DF can be considered the perfect tool to deliver antioxidant compounds to the intestinal microflora, avoiding the absorption in the initial part of the GI tract [128]. The slow and continuous release of polyphenols bound to DF after fermentation in the gut, gives them a considerable survival time in the GI tract on the contrary with free soluble antioxidants, maintaining the continuity of the healthy antioxidant environment there [5, 128]. Besides, it was hypothesized that DF- bound antioxidants play a central role in the prevention of various pathologies. They can contribute to the health effects attributed to DF as well as to dietary antioxidants [135]; such as

protection against CVD [136] and certain cancer types, diabetes, neurodegenerative diseases, and hypertension [137-140] as well as regulation of the insulin level in blood after food ingestion [141]. Moreover, they constitute a significant portion of dietary antioxidants and should not be counted as minor constituents of DF [135]. Grains, such as wheat, oat and rye, contributing about 50% of DF intake [142, 143] together with antioxidants, are principal carriers of DF- bound antioxidants. Besides, fruits and vegetables, which also contain various antioxidant compounds linked with DFs, the indigestible polysaccharides of cell walls, can be considered as a good source of DF- bound antioxidants [2].

Likewise, there are also naturally occurring lipid-bound phenolics present in foods, which in turn affect the bioaccessibility and bioavailability of the phenolics. Biologically active natural phenolic lipids may be generated through these interactions, which can be suitable for pharmaceutical and nutraceutical applications [129]. Phenolic lipids have a strong amphiphilic character due to the presence of separate hydrophilic and hydrophobic regions in their structure. They can interact with biological membranes and can incorporate into phospholipid bilayers, which results in considerable changes in the biophysical properties of these structures. They exert their antioxidant activity in various ways, such as retaining the transition metal ions from initiating oxidation, quenching intermediates of oxidation (ROS) and inhibiting some prooxidant enzymes, including lipoxygenases, cyclooxygenases and xanthine oxidase. Besides, they have antimutagenic, antigenotoxic, antibacterial, antitumor and fungustatic properties. They can also affect the structures and activities of proteins [144]. Such kind of interaction between phenolic compounds and lipid is known to exist in olive oil. Indeed, it is well known that phenolic compounds in virgin olive oil, including phenolic acids, phenolic alcohols, secoiridoids, hydroxy-isocromans, flavonoids and lignans, are highly bioavailable [145]. Also, it was demonstrated that they positively affect some physiological parameters, due to the fact that they reduce the development risk of chronic diseases [146-148]. However, there is no information regarding the nature or biological properties of those naturally occurring phenolic lipids [129]. Besides, it was reported that there are natural phenolic- lipid interactions in cashew [149] and propolis [150], whose functional health properties are increasingly gaining interest.

In a similar fashion, a significant amount of phenolic compounds are naturally found as bound to proteins. In contrast to DFs, which prevents the release, and thus the bioavailability of phenolics

during digestion [2], proteins allows the release of phenolic compounds during their digestion in the stomach and small intestine [151]. Binding of phenolic compounds to proteins are of non-covalent basis, in which the hydroxyl groups of phenolics form H-bonds with the carbonyl groups of peptides [152]. Oxidation to quinones also allows phenolics to bind with the reactive groups of proteins [153]. Besides, phenolic compounds can non- covalently bind on the surfaces of proteins via hydrophobic interactions [154]. Nevertheless, the binding may be also covalent between some oxidized phenolic compounds and proteins [155]. Such kind of interactions are reported to boost the antioxidant activity of proteins in plants [154, 156]. For instance, up to 82-85 % of total antioxidant activity in lentils is provided by phytochemicals, while it turns to be around 25-39 % for other leguminous foods including chickpeas, soybeans, green and yellow beans [157].

On the other hand, proteins, as vital macromolecules, contribute to the endogenous AC of foods themselves by containing various sites having antioxidant activity, without necessarily interfering with phenolics. They have the ability to inhibit lipid oxidation through inactivation of ROS, chelation of prooxidative transition metals, scavenging of free radicals, reduction of hydroperoxides and changing the physical properties of foods. In spite of all 20 amino acids are potentially oxidizable, those with nucleophilic sulfur- containing side chains such as cysteine and methionine or with aromatic side chains such as tryptophan, tyrosine, and phenylalanine from which the hydrogen atom is easily abstracted, are the most reactive ones. Soybean protein constitutes a good example in this case with its antioxidant capacity, which is mentioned to be originated from its tyrosine content [158]. Histidine with its imidazole- containing side chain is also labile to oxidation. In addition to this, amino acid residues like histidine, glutamic acid, aspartic acid, phosphorylated serine and threonine give some proteins the ability to bind metals, but do not have this function specifically. Nevertheless, the tertiary structure of proteins constraints the antioxidant activity of these amino acids mentioned, making them inaccessible to prooxidants when they are buried in the core [159]. Heating the proteins is reported to result in an increased antioxidant activity in some specific cases due to the exposure of antioxidative amino acid residues [160]. Furthermore, the antioxidant activity may increase through heating in the presence of sugars, due to the formation of antioxidative Maillard reaction products [159].

1.6. Multivariate Data Analysis

The nature of the food systems is multivariate itself. In other words, any particular phenomenon takes place with the simultaneous contribution from several different factors [161]. This complexity necessitates a multivariate approach when obtaining and analysing data. Design of experiment constitutes the first step in this context, since it allows the researcher to have the right type and sufficient amount of data to answer the questions of interest clearly [162]. When the right data is obtained, chemometric tools such as Principal Component Analysis (PCA), Partial Least Squares (PLS) or Anova Simultaneous Component Analysis (ASCA), among others, can be used to extract the most important information from the collected data.

1.6.1. Design of Experiment

Wagner et al. (2014) defined Design of experiment (DoE) as "a systematic approach to understand how process and product parameters affect response variables such as physical properties, product performance and processability" [163]. It should be noted that without a good experimental design, obtaining eligible conclusions from the data is difficult. Also, a well-designed experiment is the key to reach explicit data with less experimental runs when compared to ad hoc approaches [162].

The DoE process can be divided into 5 simple steps: i) definition of the problem, ii) planning the experiment, iii) running the experiment, iv) analysing the data with statistical methods and v) reporting results [163]. The definition of the particular questions to be answered before starting any experiment, in other words determining the aim of the experiment is the first and the most critical step. The aim may cover the estimation of some unknown parameters and/or revealing of connections between several factors and/or comparison of the effects of some parameters [162]. After clarifying the problem and aim, the dependent and independent variables are selected in the second step. Independent variables are the parameters of process or product, which can be controlled within the experimental design and can be either quantitative or qualitative. Dependent parameters on the other hand, are the responses that are measured to determine the effects of the independent variables. After that, the number of data points needed for each response is determined according to the number of tests required to provide statistical significance. The difference in the response variables is also adjusted by comparing the magnitudes of dependent responses at several experimental points.

The complexity of the real- world problems brings a natural difficulty to the experimental design problems. Consequently, the DoE can be in various forms. Among three experimental designs, namely mixture experiments, factorial design and response surface, factorial design is used to detect the factors that have a significant effect on the response. In general, factorial designs are comprised of two-levels. Fractional factorial designs reduce the number of experiments to be performed together with the potential information that can be gathered about the interactions of independent variables.

In the third step experiments are performed to collect data, always in a random order to minimise the systematic error. Replicate experiments are performed either at the center point and/or some selected points in the experimental domain. The fourth step includes data analysis, which can be easily done by some specific computer programs. Eventually, the prediction of independent factors and interactions which make significant effect on a specific response, generation of models predicting responses at any experimental point, visual comparison of the data via model equations, prediction of the conditions which allows to get the desired output can be done. After all, in the fifth step, the results are reported [163].

1.6.2. Principal Component Analysis

It is an ordination technique mainly used to display patterns in multivariate data. It displays the relative positions of data points in maps in less dimensions and investigates the relations between dependent variables [164]. By doing so, the similarity of the observations and the variables can be seen [165-167] and the description of the data set can be simplified [168]. While doing this, it also retains as much information as possible [164], leaving it in the so called principal components (independent between them); while the unwanted information is left outside (in the residuals).

Considering a data table comprised of I observations and J variables in the form of $I \times J$ matrix $\mathbf{X}$, is often preprocessed, prior to PCA. Mostly, the columns of $\mathbf{X}$ are centered providing the mean of each column to equal to 0. Besides centering, if the variables have different units, each variable is divided by its norm and standardized to unit norm. PCA computes a new set of orthogonal (independent) variables called Principal Components (PCs) from the original variables. The first PC contains the largest variance. The second component provide the condition of orthogonality to

the first component and has as large inertia as possible. The subsequent components are computed in the same way. The number of PCs generated increases with the number of variables. The values of the new variables created are named as factor scores, which can be shown geometrically as the projections of observations on the PCs [168]. Scores plot is considered as the main output of PCA and provides useful information about the data structure such as whether the samples are clustered or occupy a gradient. Also, contributory information is always available on the plot for interpretation. In addition to this, the values of the samples on the PC axes and the correlations between the original variables can form a reduced space plot in which the dependent variables are projected. These values are named as factor loadings. Loadings help explaining the importance of an axis to a variable [164].

1.6.3. Anova Simultaneous Component Analysis (ASCA)

Analysis of variance (ANOVA) is a useful tool when a single variable is measured with an underlying experimental design [169]. When several variables are measured at the same time like in metabolomics, proteomics or transcriptomics, generalizations of ANOVA are needed to consider the interrelation between variables. As there are several generalizations of ANOVA from univariate to multivariate case in literature such as MANOVA [170], PCA or PLS based ANOVA, there are also some hesitations or contradictive interpretations.

Among them, ANOVA Simultaneous Component Analysis (ASCA), combining ANOVA with PCA [171], was introduced as a novel method, generalizing some former methods. ASCA deals with a temporal and/or design structure of complex multivariate datasets obtained from a large spectrum of fields [172]. It basically uses the ANOVA model to decompose a data matrix into effect matrices, containing level averages of experimental factors and a matrix of residuals, which is not explained by the model. The significance of the experimental factors is quantified by calculating the p-values by a permutation test. The contribution of each variable on the PCs of the effect matrix is determined by loadings, which in turn allows easy interpretation of the factor levels by means of dependent variables [173]. The variation explained by the model is used as an indicator of quality of fit, as is common in multivariate data analysis [174].

2. EXPERIMENTAL

2.1. Interactions between Macromolecule- Bound Antioxidants and Free Antioxidant Trolox in Aqueous Medium

2.1.1. Chemicals

Potassium peroxydisulfate, 2,2`-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), monopotassium phosphate, disodium phosphate, sodium acetate trihydyrate, acetic acid, methanol, hexane, and ethanol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). All solvents were of analytical grade, unless otherwise stated. Water was purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA).

2.1.2. Food Samples

Whole-wheat (WW) flour, edamame, soybean, soymilk, tofu, extra virgin and refined olive oil were purchased from local markets in Ankara, Turkey. Paste was prepared from WW flour by heating the flour: water mixture, formed according to the ratio (3.5 g: 25 mL) given in the AACC method 72-21.01 "General Pasting Method for Wheat and Rye Flour Using the Rapid Visco Analyzer" [175], on a magnetic stirrer to 70 °C and leaving for set-back in the room temperature. Bread was prepared according to the AACC method 10-10B for "Straight-Dough Bread Making" [176]. Boiled soybeans were obtained by boiling 100 g raw soybeans in 600 ml water for 1 h.

2.1.3. Preparation of the DF-Bound Antioxidants

WW- flour, ground paste and bread samples were washed according to the procedure described by Çelik, Gökmen, & Fogliano (2013) [5] to remove water, alcohol and lipid-soluble fractions. The residues were freeze-dried, ground to a fine powder form and passed through a sieve (Endecotts Test Sieve, London, UK) of 40 mesh size. The powder obtained, containing DF-bound antioxidants, was tested to be free of soluble antioxidants and kept stable under -18 °C in a close-fitting vessel under nitrogen atmosphere prior to measurements.

2.1.4. Preparation of the Protein-Bound Antioxidants

Edamame, soybean, boiled soybean, soymilk and tofu proteins were subjected to isoelectric precipitation according to the method described by Dev, Quensel and Hansel (1986) [177] and Krause, Schultz and Dudek (2002) [178] with some modifications. Freeze dried and ground

samples (4 g) were first defatted with hexane (200 mL) in a soxhlet apparatus at 50 °C for 6 h, then dried at room temperature. Defatted samples (10 g) were mixed with NaOH (2.0 N, 100 mL) and the pH of the mixtures were adjusted to 11.0. Following 1 h stirring at room temperature, centrifugation was done at 8000 rpm for 30 min and supernatants were collected. The pH of the supernatants was adjusted to 4.6 using HCl (0.1 N) and the protein isolate precipitated was separated by centrifugation at 8000 rpm for 15 min. The isolates were freeze-dried and kept stable under -18 °C in a close-fitting vessel under nitrogen atmosphere prior to experiments.

2.1.5. Preparation of the Lipid-Bound Antioxidants

Extra virgin and refined olive oil samples (15 mL) were washed with methanol: water (70:30, v: v) mixture (25 mL) in three steps. Following vortexing of the olive oil: methanol: water mixtures, centrifugation was done at 8000 rpm for 3 min and the supernatants containing free phenolic compounds were removed in each step. The last supernatant was tested to be free of phenolic compounds via Folin-Ciocalteau [179] method and the final product containing lipid-bound antioxidants was kept stable under 4 °C in a close-fitting vessel under nitrogen atmosphere prior to experiments.

2.1.6. Antioxidant Capacity Measurement

Antioxidant capacity of DF, protein and lipid bound antioxidants and Trolox was measured separately and in mixtures by the QUENCHER [125] procedure using ABTS radical probe. ABTS•+ radical solution was prepared according to the method described by Serpen, Gokmen and Fogliano (2009) [180] with some minor modifications. Working solution of ABTS was prepared by diluting the stock solution in 0.1 M of sodium-potassium phosphate (pH 6.0) or sodium-acetate (pH 3.0 and 5.0) buffers for DF and protein-bound antioxidant studies. In this way, the pH of the ABTS radical media was ensured to stay in the mentioned values (3.0, 5.0 and 6.0) during the study for all samples. For lipid-bound antioxidant studies, the stock solution was diluted with ethanol: water (50:50, v: v) mixture to prepare working solution, disregarding the pH arrangement. The absorbance of the ABTS working solution was arranged a value between 0.7-0.8 at 734 nm. 10, 15 or 20 mg of DF/protein-bound antioxidant; 100, 150 or 200 µL of lipid-bound antioxidant or 100, 150 or 200 µL of Trolox (500 µM) was placed into a test tube and the reaction was initiated by adding 10 mL of ABTS radical solution. Following 27 min of orbital shaking at 350 rpm at room temperature in darkness, the tubes were centrifuged at 8000 rpm for 2 min. The optically

clear supernatants obtained were transferred into a cuvette and absorbance measurement was performed at 734 nm using a Shimadzu model 2100 variable-wavelength UV-visible spectrophotometer (Shimadzu Corp., Kyoto, Japan), exactly after 30 min from the initiation of the reaction. The absorbance measurements for bound antioxidant + Trolox mixtures were also performed according to the experimental matrix generated by DoE. The total antioxidant capacity of bound antioxidants and Trolox separately and in mixtures were expressed as inhibition % of the absorbance with respect to ABTS radical itself. Estimated inhibition % of the absorbance values were calculated by summing up the inhibition % values separately measured for the related concentrations of bound antioxidants and Trolox which were used to make the mixtures. Measured inhibition % of the absorbance values were the ones which obtained for the real mixtures.

2.1.7. High Resolution Mass Spectrometry Analysis of Auto-Regeneration Reaction End Product of Trolox

10 mg WW bound antioxidants + 100 µL Trolox (500 µM) mixture and 100 µL of Trolox (500 µM) was separately mixed with 10 mL of ABTS working solution, prepared by diluting the stock solution in ethanol: water (50:50, v: v) mixture. After 27 min of reaction by orbitally shaking the tubes at 350 rpm, at room temperature, in darkness the tubes were centrifuged at 8000 rpm for 2 minutes. The optically clear supernatants were placed into vials and the measurements were done immediately. ABTS working solution in the same conditions without any antioxidant added was analyzed as well.

The measurements were carried out by using a Thermo Scientific Accela Liquid Chromatography System (San Jose, CA, USA) coupled to a Thermo Scientific Q-Exactive Orbitrap High Resolution Mass Spectrometry (San Jose, CA, USA) in heated electrospray ionization (HESI) mode. Chromatographic separations were performed on Thermo Hypersil Gold aQ column (20 x 2.1 mm i.d., 1.9 µm) (San Jose, CA, USA). An isocratic mixture (70:30) of 0.1 % formic acid having 2 mM ammonium formate in water (A) and 0.1 % formic acid having 2 mM ammonium formate in (methanol: ACN (50:50, v: v)): water (99.5:0.5, v: v) mixture (B) was used as the mobile phase at a flow rate of 0.3 mL/min. The injection volume was 10 µL. The scan analyses were performed in an m/z range between 70 and 300 at ultra-high resolving power (R = 70000). The automatic gain control target and maximum injection time were set to 1×10^6 and 100 ms respectively. The HESI source parameters were as follows: sheath gas flow rate 45 (arbitrary units), auxiliary gas flow rate

10 (arbitrary units), spray voltage 3.70 kV, capillary temperature 320 °C, auxiliary gas heater temperature 250 °C.

2.1.8. Design of Experiment (DoE)

Experiments for the absorbance measurements of DF and protein-bound antioxidants + Trolox mixtures were performed according to a fractional factorial design with 3 factors (bound antioxidant concentration, Trolox concentration, pH) at 3 levels, while a full factorial design with 2 factors (bound antioxidant concentration, Trolox concentration) at 3 levels was used for lipid-bound antioxidants + Trolox mixtures (Table 2.1). The experiments were performed randomly by using two replicate samples for each bound antioxidant matrix, and each measurement was done in triplicate.

Table 2.1. Experimental matrices for DF/protein-bound antioxidants + Trolox and lipid-bound antioxidants + Trolox mixtures studies in aqueous medium

Experiment	[DF/Protein-Bound Antioxidant] mg	[Trolox] µL	pH	[Lipid-Bound Antioxidant] µL	[Trolox] µL
1	10	100	3	100	100
2	10	150	6	100	150
3	10	200	5	100	200
4	15	100	6	150	100
5	15	150	5	150	150
6	15	200	3	150	200
7	20	100	5	200	100
8	20	150	3	200	150
9	20	200	6	200	200

2.1.9. Statistical Analysis

Multi-way ANOVA was performed by the statistical toolbox working under Matlab v. 2016a (The Mathworks, Inc. Massachusetts, USA). Duncan´s post-hoc test was perfomed at IBM SPSS Statistics version 24. Student`s t-test and Anova-Single Factor tests were perfomed in EXCEL v. 2016 (Microsoft Corporation, Washington, USA).

2.2. Interactions between Macromolecule- Bound Antioxidants and Free Antioxidant Trolox in Liposome Medium

2.2.1. Chemicals

L-α-phosphatidylcholine from soybean (99%), sodium phosphate dibasic, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), iron(III) chloride, adenosine 5′-diphosphate sodium salt (ADP), L-(+)-Ascorbic acid, methanol, chloroform, hexane, and ethanol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and glycine were purchased from Merck (Darmstadt, Germany). All solvents used were of analytical grade, unless otherwise stated. Water was purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA).

2.2.2. Food Samples

WW flour, edamame, soybean, soymilk, tofu, extra virgin and refined olive oil were purchased from local markets in Ankara, Turkey. Paste, bread and boiled soybean were prepared as described above in Section 2.1.2.

2.2.3. Preparation of the DF-Bound Antioxidants

DF- bound antioxidants from WW flour, paste and bread were prepared as described above in Section 2.1.3. Samples obtained in powder form, which are free of soluble antioxidants were kept stable under -18 °C in a close-fitting vessel under nitrogen atmosphere prior to measurements.

2.2.4. Preparation of the Protein- Bound Antioxidants

Isoelectric precipitation of edamame, soybean, boiled soybean, soymilk and tofu proteins were carried out according to the method described above in Section 2.1.4. Freeze dried protein isolates obtained were kept stable under -18 °C in a close-fitting vessel under nitrogen atmosphere prior to experiments.

2.2.5. Preparation of the Lipid-Bound Antioxidants

Lipid- bound antioxidants from extra virgin and refined olive oil samples were obtained according to the procedure described above in Section 2.1.5. Samples were kept stable under 4 °C in a close-fitting vessel under nitrogen atmosphere prior to experiments.

2.2.6. Preparation and Peroxidation of Liposomes

Liposomes were prepared with minor modifications [181] according to the method described by Tirmenstein, Pierce, Leraas, & Fariss (1998) [182]. Forty milligrams of soybean

phosphatidylcholine were dissolved in chloroform and evaporated to dryness under nitrogen flow. The residual dry phosphatidylcholine was subsequently rehydrated with 5 mL of 50 mM sodium phosphate buffer (PB) (pH 7.4), vortexed and the suspension was sonicated at 100 W with 30 s intervals 10 times to obtain a homogeneous suspension of multilamellar liposomes. Between the intervals, the suspension was left to cool in an ice bath for at least 2 min. The sonicated suspension was centrifuged at 16000g for 30 min, and the supernatant containing the liposomes was collected. The liposomes were stored under 4 °C for 1 week prior to experiments to increase the level of lipid hydroperoxides (about 5−10% of the phosphatidylcholine). These will react with Fe^{3+} to produce free radicals.

Liposomes prepared weekly prior to measurement were pipetted into a 96-well transparent plate as 10 µL for each well and mixed with 50 µL of sample dilutions, whereas Milli-Q water was used as zero sample. Sample dilutions were prepared as 5, 10 and 50 µM for Trolox 1, 5 and 10 mg/mL aqueous solutions for DF and protein-bound antioxidant samples (wheat and soybean products) and as 300, 600, 900 µL/mL hexane for lipid-bound antioxidant samples (olive oil products). Mixtures of DF, protein and lipid bound antioxidant sample derivatives with Trolox were prepared by combining different concentrations of bound antioxidants and Trolox at a ratio of 1:1 to give 50 µL sample. Glycine was added to the reaction medium as glycine ascorbate buffer (GAB) as 120 µL per well to react with aldehydes generated by lipid peroxidation to produce fluorescence. GAB was was prepared as 50 mM potassium phosphate buffer (PPB) with 100 mM glycine and 450 µM ascorbate at pH 7.4. Lipid peroxidation was initiated by adding 20 µL of an oxidizing agent composed of 25 µM $FeCl_3$ in 50 mM PPB (pH 7.4) with 1 mM ADP to the wells.

The plate was inserted into the plate reader of a Tecan GENios plus fluorometer (Tecan Trading AG, Switzerland) immediately after addition of oxidizing agent. Oxidation of liposome system was monitored for 180 min at 37 °C (excitation at 360 nm, emission at 460 nm) in 10 min intervals, and samples were analyzed in triplicate.

From the oxidation curves obtained after measurements for each well, AUC (area under the curve) for samples as well as control was calculated between 0-180 min. To avoid differences originating from different starting levels of oxidation, all fluorescent values were subtracted from the lowest value, considering the lowest point as zero. The AUC was calculated by the trapezoid rule. Then by using AUC values, inhibition % of lipid oxidation value for each sample was calculated with

reference to the control sample. Estimated inhibition % of lipid oxidation values were calculated by summing up the halves of the inhibition % of lipid oxidation values separately measured for the related concentrations of bound antioxidants and Trolox, which were used to make a 1:1 combination.

2.2.7. Design of Experiment (DoE)

In order to perform the experimental studies regarding the percentage of inhibition of lipid oxidation measurements for the mixtures of free and bound antioxidants, a full factorial design with 2 factors (bound-antioxidant concentration and free antioxidant concentration) at 3 levels, was performed (Table 2.2). The experiments were performed randomly by using two replicate samples for each bound antioxidant matrix, and each measurement was done in triplicate.

Table 2.2. Experimental matrices for DF/protein-bound antioxidants + Trolox and lipid-bound antioxidants + Trolox mixtures studies in liposome medium

Experiment	[DF/Protein-Bound Antioxidants] mg/mL	[Trolox] µM	[Lipid-Bound Antioxidants] µL/mL
1	1	5	300
2	1	10	300
3	1	50	300
4	5	5	600
5	5	10	600
6	5	50	600
7	10	5	900
8	10	10	900
9	10	50	900

2.2.8. Statistical Analysis

The curve fitting was done with Matlab, based on the theoretical model given by truncating the data from the point when it reaches zero oxidation level after noisy part at the beginning of the peroxidation. The fitting was performed by using the well-known Levenberg-Marquard algorithm. PCA and ASCA were performed by using PLS_Toolbox v. 8.0 (Eigenvector Research, Inc. Manson, WA, USA) working under Matlab v. 2016a (The Mathworks, Inc. Massachusetts, USA). Duncan´s post-hoc test was performed at IBM SPSS Statistics version 24. Student`s T-Test and Anova-Single Factor tests were performed at EXCEL v. 2016 (Microsoft Corporation, Washington, USA).

2.3. Interactions between Whole Wheat Dietary Fiber- Bound Antioxidants and Hydroxycinnamic/ Hydroxybenzoic Acids in Aqueous and Liposome Media

2.3.1. Chemicals

L-α-phosphatidylcholine from soybean (99%), sodium phosphate dibasic, iron(III) chloride, adenosine 5′-diphosphate sodium salt (ADP), 2,2-Diphenyl-1-picrylhydrazil (DPPH), 2-hydroxycinnamic (o-coumaric) acid, 3-hydroxycinnamic (m-coumaric) acid, 4-hydroxycinnamic (p-coumaric) acid, 3,4-dihydroxycinnamic (caffeic) acid, 3,5-dihydroxyhydrocinnamic acid, 2,4-dihydroxycinnamic acid, 3,4,5-trihydroxybenzoic (gallic) acid, 2,4,5-trihydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 2,3,4-trihydroxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic (syringic) acid, 3,4,5-trimethoxybenzoic acid, 2-hydroxy-4,6-dimethoxybenzoic acid, 2-hydroxy-3,4-dimethoxybenzoic acid, 3,4-dihydroxy-5-methoxybenzoic acid, 2-hydroxy-3-methoxybenzoic acid, 2-hydroxy-4-methoxybenzoic acid, 2-hydroxy-5-methoxybenzoic acid, 2-hydroxy-6-methoxybenzoic acid, 4-hydroxy-3-methoxybenzoic (vanillic) acid, 3-hydroxy-4-methoxybenzoic (isovanillic) acid, L- (+)-ascorbic acid, methanol, chloroform, hexane, and ethanol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and glycine were purchased from Merck (Darmstadt, Germany). All solvents used were of analytical grade, unless otherwise stated. Water was purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA).

2.3.2. Preparation of the DF- Bound Antioxidants from Whole Wheat

WW- flour was purchased from a local factory in Ankara, Turkey. DF-bound antioxidants from WW- flour were obtained according to the procedure described above in Section 2.1.3. The final product in powder form was tested to be free of soluble antioxidant compounds and kept under -18 °C in a close- fitting vessel under nitrogen atmosphere prior to experiments.

2.3.3. Antioxidant Capacity Measurement in Liposome Medium

Antioxidant capacity measurement in liposome medium was carried out as described above in Section 2.2.6 with minor modifications. Sample dilutions were prepared as 1, 5 and 10 mg/mL aqueous solutions for WW- bound antioxidants and 10, 50 and 100 µM for o-coumaric, m-coumaric, p-coumaric, 2,4 dihydroxycinnamic, 3,5 dihydroxyhydrocinnamic, caffeic and gallic acids. Milli-Q water was used as control sample and combinations of HCA with WW- bound antioxidants were prepared by mixing their different concentrations at a ratio of 1:1 to give 50 µL of sample. Sample were analyzed in triplicate. AUC and measured/ estimated inhibition % values

were calculated as mentioned above (Section 2.2.6) for each sample.

2.3.4. Antioxidant Capacity Measurement in Aqueous Medium

Antioxidant capacity measurements of WW- bound antioxidants and HCA/ HBA derivatives separately and in mixtures were performed by using direct QUENCHER procedure with minor modifications. DPPH stable radical was used in the method since it works at around physiological pH unlike the other well-known antioxidant activity assays.

The DPPH solution was prepared according to the method described by Serpen et al. (2009) [180]. Dilutions of WW- bound antioxidant samples and HCA / HBA derivatives were pipetted into 96-well transparent plates as 40 µL and mixed with 260 µL of DPPH radical, whereas Milli-Q water was used as zero sample. Sample dilutions were prepared as 10, 30, 50 mg/mL for WW- bound antioxidants; 25, 50, 100 µM for Caffeic, Gallic, 2,4,5- trihydroxybenzoic, 2,4,6-trihydroxybenzoic, 2,3,4- trihydroxybenzoic and 3,4-dihydroxy-5-methoxybenzoic acids and 100, 300, 500 µM for the rest of the HCA/ HBA derivatives. Mixtures of WW- bound antioxidants with HCA/ HBA derivatives were prepared by combining different dilutions at a ratio of 1:1 to give 40 µL sample. After mixing the radical with samples, the plate was immediately inserted into the plate reader of the SpectraMax i3x Multi-Mode Detection Platform. The absorbance was measured at 525 and 850 nm, the wavelengths giving maximum absorbance for DPPH and WW- bound antioxidants respectively, for 120 min with 5 min intervals. The absorbance values measured at 850 nm were subtracted from that at 525 nm to eliminate the interference of the insoluble WW-bound antioxidant fraction in the wells. Scavenging % of DPPH radical was calculated with respect to the zero sample at each time point and oxidation rate curves were constructed by plotting scavenging % data vs time. The area under the curve (AUC) values were then calculated with trapezoid rule for each sample. Estimated AUC values for WW- bound antioxidants + HCA/ HBA mixtures were calculated by summing up the halves of the AUC values separately obtained for the related concentrations of WW- bound antioxidants and HCA/ HBA.

2.3.5. Design of Experiment

A full factorial design with 2 factors (WW- bound antioxidant concentration, HCA/ HBA concentration) at 3 levels was used for the experimental studies performed in both liposome and aqueous media (Table 2.3). The experiments were performed randomly by using two replicate samples of WW- bound antioxidants with each HCA/ HBA variety, and each measurement was

done in triplicate.

Table 2.3. Experimental matrices for WW-bound antioxidants + HCA/ HBA derivatives mixtures studies in liposome (I) and aqueous (II) media.

Experiment	I		II		
	[WW-Bound] mg/mL	[HCA /HBA] µM	[WW-Bound] mg/ mL	[HCA / HBA] µM	
1	1	10	10	25	100
2	1	50	10	50	300
3	1	100	10	100	500
4	5	10	30	25	100
5	5	50	30	50	300
6	5	100	30	100	500
7	10	10	50	25	100
8	10	50	50	50	300
9	10	100	50	100	500

2.3.6. Statistical Analysis

PCA and ASCA were performed by using the PLS_Toolbox v. 8.0 (Eigenvector Research, Inc. Manson, WA, USA) working under Matlab v. 2016a (The Mathwotks, Inc. Massachusetts, USA). Duncan`s post hoc test was performed at IBM SPSS Statistics version 24. Student`s t-test and Anova-Single Factor tests were performed in EXCEL v. 2016 (Microsoft Corporation, Washington, USA).

2.4. Interactions between Coffee/ Bread Crust Melanoidins and Hydroxycinnamic/ Hydroxybenzoic Acids in Aqueous Medium

2.4.1. Chemicals

2,2-Diphenyl-1-picrylhydrazil (DPPH), p-Coumaric acid, o-Coumaric acid, m-Coumaric acid, 3,4-Dihydroxycinnamic acid (Caffeic acid), 3,5-Dihydroxyhydrocinnamic acid, 2,4-Dihydroxycinnamic acid, Gallic acid, 2,4,5-Trihydroxybenzoic acid, 2,4,6-Trihydroxybenzoic acid, 2,3,4-Trihydroxybenzoic acid, Syringic Acid, 3,4,5-Trimethoxybenzoic Acid, 2-Hydroxy-4,6-dimethoxybenzoic acid, 2-Hydroxy-3,4-dimethoxybenzoic acid, 3,4-Dihydroxy-5-methoxybenzoic acid, 2-Hydroxy-3-methoxybenzoic acid, 2-Hydroxy-4-methoxybenzoic acid, 2-Hydroxy-5-methoxybenzoic acid, 2-Hydroxy-6-methoxybenzoic acid, Vanillic acid, Isovanillic acid and ethanol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Water was

purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA). Instant coffee (Nescafe Gold) and bread was purchased from a local market in Ankara.

2.4.2. Isolation of Coffee Melanoidins

Coffee melanoidins was isolated according to the procedure described by Delgado-Andrade et al. (2005) [78] with minor modifications. Two grams of instant coffee was resuspended in 100 mL of hot water (50-60 °C) and the solution obtained was filtered through a coarse filter paper. An aliquot of the filtered sample was subjected to ultrafiltration using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with 10 kDa nominal molecular mass cut off membrane. The retantate was filled up to 200 mL deionized water and the ultrafiltration process was continued to wash it. The washing procedure was repeated at least three times. The residual high molecular weight fraction corresponding to melanoidins was collected, freeze-dried and stored under -18 °C prior to analysis.

2.4.3. Isolation of Bread Crust Melanoidins

Bread crust melanoidins was isolated according to the procedure described by Borrelli & Fogliano (2005) [183]. Bread crust was separated from bread manually using kitchen type knives, ground and freeze- dried. 500 mg freeze- dried bread crust was mixed with 6 mL of 0.2 M Tris- HCl buffer (pH 8.0) containing Pronase E (0.1 mg/mL). The mixture was kept under 37 C for 70 h using a water bath equipped with a shaker. The brown supernatants containing hydrolisates were collected after centrifugation at 4000 rpm for 15 min. The high molecular weight fraction containing melanoidins was separated by ultrafiltration using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with 10 kDa nominal molecular mass cut off membrane. The melanoidins obtained was freeze- dried and stored under -18 °C prior to analysis.

2.4.4. Antioxidant Capacity Measurement

Antioxidant capacity measurements of bread crust and coffee melanoidins samples together with HCA/ HBA separately and in mixtures were performed according to the method described above in Section 2.3.4 with minor modifications. Sample dilutions were prepared as 0.1, 0.5, 1.0 mg/mL for coffee melanoidins; 10, 50, 100 mg/ mL for bread crust melanoidins; 25, 50, 100 µM for Caffeic, Gallic, 2,4,5- Trihydroxybenzoic, 2,4,6- Trihydroxybenzoic, 2,3,4- Trihydroxybenzoic and 3,4-Dihydroxy-5-methoxybenzoic Acids and 100, 300, 500 µM for the rest of the HCA/ HBA derivatives. Mixtures of melanoidins with HCA/ HBA were prepared by combining different

concentrations of melanoidins and HCA/ HBA at a ratio of 1:1 to give 40 µL sample. The wavelength giving maximum absorbance for the melanoidins was also 850 nm, hence the absorbance was measured at 525 and 850 nm for 120 min with 5 min intervals, as done before. Samples were analyzed in triplicate. Inhibition % of the DPPH radical and measured/ estimated AUC values were calculated as mentioned in Section 2.3.4.

2.4.5. Design of Experiment (DoE)

A full factorial design with 2 factors (melanoidins concentration, HCA/ HBA concentration) at 3 levels was performed (Table 2.4). Experimental studies were performed according to the matrix constructed randomly and each measurement was done in triplicate.

Table 2.4. Experimental matrices for CM + HCA/ HBA derivatives and BCM + HCA/ HBA derivatives mixtures studies.

Experiment	[CM] mg/mL	[HCA / HBA] µM		[BCM] mg/ mL	[HCA / HBA] µM	
1	0.1	25	100	100	25	100
2	0.1	50	300	300	50	300
3	0.1	100	500	500	100	500
4	0.5	25	100	100	25	100
5	0.5	50	300	300	50	300
6	0.5	100	500	500	100	500
7	1	25	100	100	25	100
8	1	50	300	300	50	300
9	1	100	500	500	100	500

2.4.6. Statistical Analysis

PCA and ASCA were performed by using PLS_Toolbox v. 8.0 (Eigenvector Research, Inc. Manson, WA, USA) working under Matlab v. 2016a (The Mathworks, Inc. Massachusetts, USA). Duncan´s post-hoc test was performed at IBM SPSS Statistics version 24. Student`s t-test and Anova-Single Factor tests were performed at EXCEL v. 2016 (Microsoft Corporation, Washington, USA).

2.5. Interactions between the Insoluble Fractions of Different Coffee Infusion & Major Cocoa Free Antioxidants and Different Coffee Infusions & Dark Chocolate

2.5.1. Chemicals

2,2-Diphenyl-1-picrylhydrazil (DPPH), (+)-Catechin, (-)-Epicatechin and ethanol were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). All solvents used were of analytical grade, unless otherwise stated. Water was purified through a Millipore Q-plus purification train (Millipore Corp., Bedford, MA, USA).

2.5.2. Coffee and Chocolate Samples

Ground roasted coffee (Arabica) and dark Turkish chocolate containing 60 % cocoa were purchased from a local market in Ankara, Turkey. All different types of coffee infusions were prepared from the same ground roasted coffee. *Espresso* was prepared by brewing 6 g of coffee in 30 mL of water using a kitchen type coffee machine (DeLonghi icona vintage) as well as by brewing 15 g of coffee in 150 ml of water in a moka pot (this coffee will be mentioned as moka in the text hereafter) on a hot plate at constant temperature (350 °C). French press was prepared by brewing 10 g of coffee in 180 mL water for 4 min in a French press mechanism. Filtered coffee was prepared by brewing 10 g of coffee in 180 mL of water in a filtered coffee machine. Turkish coffee was prepared by cooking 5 g of coffee in 75 mL of water in a Turkish coffee machine. After 4 min of sedimentation, the brew was separated from the coffee ground.

2.5.3. Isolation of the Insoluble Fractions of Coffee Infusions and Coffee

Coffee infusions prepared with different methods were freeze dried, ground and subjected to a washing procedure to remove water, alcohol and lipid-soluble fractions as described by Çelik et al. (2013) [5]. Ground roasted coffee beans were also washed following the same procedure. The ratio of samples to washing solvent was 1 g: 5 mL. Samples were first defatted by hexane twice and then washed thrice with water. Methanol: 1 N HCl (v:v, 85:15) was used to remove the colored components (3 washes) and a final wash with water was carried out 3 times. Washed samples were freeze- dried, ground to a fine powder form and passed through a sieve (Endecotts Test Sieve, London, UK) of 40 mesh size. The final products containing the insoluble fractions of coffee infusions and coffee were kept under -18 °C prior to experiments.

2.5.4. Antioxidant Capacity Measurement

AC measurement was performed with direct QUENCHER procedure, which does not need any extraction or hydrolysis steps for samples prior to measurement (Gokmen, Serpen & Fogliano,

2009) [184]. DPPH was used as the radical probe, since it allows measurements at physiological pH unlike other well-known radical probes. DPPH radical solution was prepared according to the method described by Serpen, Gökmen & Fogliano (2009). Sample dilutions were prepared as follows. The insoluble fractions of coffee infusions/coffee were diluted with cellulose powder, which does not possess AC in the ratio of 1:10. Freeze dried coffee infusions/coffee samples were re-suspended in water to a final concentration of 1 mg/ ml. Chocolate was melted with bain-marie technique and diluted in water to a final concentration of 10 mg/ ml, while catechin/epicatechin was prepared as a 300 µM solution. The insoluble fractions of coffee infusions/coffee dilutions at 10, 15, 20 mg corresponding to 1.0, 1.5 and 2.0 mg sample were added to test tubes. Coffee infusions/coffee, chocolate and catechin/ epicatechin dilutions at 100, 150, 200 µL, corresponding to 0.10, 0.15, 0.20 mg; 1.0, 1.5, 2.0 mg and 30, 45, 60 nmol of sample, respectively, were added to the test tubes. The reaction was initiated by adding 10 mL of DPPH radical solution. After 27 min of reaction in dark in an orbital shaker (350 rpm) at room temperature, tubes were centrifuged at 6080 g for 2 min. The optically clear supernatants were transferred into a cuvette and absorbance measurement was performed at 525 nm using a Shimadzu model 2100 variable-wavelength UV-visible spectrophotometer (Shimadzu Corp., Kyoto, Japan), exactly after 30 min from the initiation of the reaction. Measurements for the mixtures of the insoluble fractions of coffee infusions and catechin/epicatechin together with coffee infusions and chocolate samples were also performed by mixing them at a ratio of 1:1 in the reaction medium, according to the experimental matrices generated by DoE. The percentage of inhibition of DPPH radical for the individual samples and their mixtures were calculated with respect to the absorbance of the DPPH radical itself. Estimated percentage of inhibition values for coffee infusions + chocolate mixtures together with the insoluble fractions of coffee infusions + catechin/epicatechin mixtures were calculated by summing up the percentage of inhibition values separately obtained for the related concentrations of the constituent samples. The estimated inhibition values calculated were then compared with the measured values for the real combinations to reveal the type of interactions. The total AC of the individual samples of coffee infusions/coffee and the insoluble fractions of coffee infusions/coffee were also expressed in terms of Trolox equivalents to compare with each other.

2.5.5. Design of Experiment

Design of Experiment (DoE) was used to construct the experimental design for the mixture experiments. A full factorial design with 2 factors (the insoluble fraction of coffee infusions

concentration and catechin/epicatechin concentration or coffee infusions concentration and chocolate concentration) at 3 levels was used for this purpose (Table 2.5). Experiments were performed randomly by using two replicate samples for coffee infusions and the insoluble fractions of coffee infusions and each measurement was done in triplicate.

Table 2.5. Experimental matrices for the investigation of the interactions between: the insoluble fractions of coffee infusions/coffee and catechin/epicatechin; coffee infusions/coffee and chocolate.

	Factors		
Insoluble Fractions of Coffee Infusions/Coffee	**Experiment**	**Insoluble Fractions of Coffee Infusions (mg)**	**Catechin/Epicatechin (nmol)**
	1	1.0	30
	2	1.0	45
	3	1.0	60
	4	1.5	30
	5	1.5	45
	6	1.5	60
	7	2.0	30
	8	2.0	45
	9	2.0	60
Coffee Infusions/ Coffee	**Experiment**	**Coffee Infusions (mg)**	**Chocolate (mg)**
	1	0.10	1.0
	2	0.10	1.5
	3	0.10	2.0
	4	0.15	1.0
	5	0.15	1.5
	6	0.15	2.0
	7	0.20	1.0
	8	0.20	1.5
	9	0.20	2.0

2.5.6. Statistical Analysis

Multi-way ANOVA was performed to determine the significance of the effects of the concentrations of coffee infusions, insoluble fractions of coffee infusions, catechin, epicatechin and chocolate on the AC by using the statistical toolbox working under Matlab v. 2016a (The

Mathworks, Inc. Massachusetts, USA). Duncan`s post hoc test was performed at IBM SPSS Statistics version 24. Student`s t-test and Anova-Single Factor tests were performed in EXCEL v. 2016 (Microsoft Corporation, Washington, USA).

3. RESULTS & DISCUSSION

3.1. Interactions between Macromolecule- Bound Antioxidants and Free Antioxidant Trolox in Aqueous Medium

Relative inhibition % values of ABTS radical measured and estimated in the presence of the mixtures of DF, protein or lipid-bound antioxidants with Trolox is given in Table 3.1 Generally, the difference between estimated and measured values was significant, clearly revealing the antagonistic interactions between Trolox and macromolecule-bound antioxidants. Combinations of some bound antioxidants with 200 µL Trolox at pH 3.0 and 5.0 showed up exceptions giving either synergistic or additive interactions. Among DF-bound antioxidants, mixtures of whole wheat flour with Trolox exerted the lowest antioxidant capacity, while mixtures of paste with Trolox being higher than or equal to the bread`s. Among protein or lipid- bound antioxidant samples on the other hand, there were no gradation in between themselves by means of their antioxidant capacities in their mixtures with Trolox.

To demonstrate the significance of free and bound antioxidant concentrations, pH and their 2 and 3-way interactions on the inhibition % values of ABTS radical measured, a multiple comparison test was performed by using one-way ANOVA. The *p* values obtained within 0.95 confidence interval from the test were shown in Table 3.2. Accordingly, bound antioxidant concentration was found to make a significant effect on the inhibition % of ABTS radical measured, except the mixtures of boiled soybean, soymilk and tofu with Trolox. Trolox concentration was only found to have a significant effect when mixed with whole wheat flour, edamame, extra virgin and refined olive oil samples. Meanwhile change of pH was found to make a significant effect for all mixtures of Trolox with DF and protein-bound antioxidants except whole wheat flour. On the other hand, all 2 and 3-way interactions between these variables were found to make a significant effect on the inhibition % of ABTS radical measured. In general, the resultant antioxidant activity showed a tendency to increase with the increasing concentrations of Trolox and bound antioxidants. However, the situation was not as simple for the effect of pH. For the mixtures of Trolox with paste, bread and boiled soybean`s bound antioxidants, there was a significant difference between pH 3.0 and 5.0.

Table 3.1. Measured and estimated values of relative inhibition % of ABTS radical for the mixtures of DF, protein or lipid- bound antioxidants with Trolox.

	Bound (mg)	10			15			20		
	Trolox (µL)	100	150	200	100	150	200	100	150	200
	pH	3	6	5	6	5	3	5	3	6
Whole Wheat Flour	measured	30.7±1.2^{*,a}	42.5±4.2^{*,a}	47.2±1.5^{*,a,c}	50.2±2.3^{*,a}	46.3±2.3^{a}	50.5±0.9^{*,a}	44.9±2.7^{*,a}	49.1±0.4^{*,a}	62.8±3.8^{*,a}
	estimated	36.0±0.6	65.5±1.6	35.7±1.2	76.7±0.2	50.2±1.0	40.8±1.2	55.8±1.6	53.7±0.9	80.5±1.5
Whole Wheat Paste	measured	40.3±1.0^{*,b,d}	67.9±2.1^{*,b}	58.3±1.2^{b}	76.3±0.3^{*,b}	62.7±1.9^{*,b}	61.0±0.6^{*,b}	66.2±1.4^{*,b}	64.5±0.8^{*,b}	86.1±0.1^{*,b}
	estimated	52.9±0.9	95.5±1.2	60.4±0.9	107.1±0.6	77.4±1.5	63.1±0.7	86.6±1.5	77.9±0.8	112.3±1.6
Whole Wheat Bread	measured	41.2±0.9^{*,b}	59.8±3.1^{*,c,e}	57.6±1.5^{b}	66.5±0.7^{*,c}	59.1±1.7^{*,c}	62.3±1.0^{b}	63.3±1.4^{*,c}	65.3±1.5^{*,b}	78.8±1.5^{*,c,e}
	estimated	50.4±0.5	91.2±1.3	56.7±0.5	102.8±1.4	73.8±1.1	60.8±1.1	83.3±1.4	74.3±0.9	108.7±1.7
Soybean	measured	46.5±2.0^{*,c}	56.2±2.4^{*,c,d}	41.8±1.8^{c}	59.1±2.1^{*,d}	39.2±0.4^{*,d}	68.8±1.5^{c}	38.4±0.9^{*,d}	75.3±1.8^{*,c}	71.7±1.7^{*,d}
	estimated	57.4±1.0	81.6±2.0	41.6±1.4	98.7±1.2	51.3±2.0	68.7±1.4	56.0±1.4	84.0±2.0	104.3±0.7
Edamame	measured	45.8±1.0^{*,c}	54.0±1.8^{*,d}	44.3±1.9^{a,c}	57.6±2.0^{*,d}	42.8±1.1^{*,e}	66.8±1.1^{*,c,d}	39.9±0.8^{*,d,e}	70.2±1.2^{*,d}	70.7±1.7^{*,d}
	estimated	55.3±1.2	80.5±0.6	44.5±1.1	95.5±0.6	114.4±2.0	69.7±0.5	59.8±0.5	81.7±1.7	103.1±0.5
Boiled Soybean	measured	24.9±4.3^{*,e}	68.7±1.1^{*,b}	50.1±0.7^{*,d}	75.3±1.0^{*,b}	46.7±0.5^{*,a}	49.7±3.8^{a}	48.8±1.0^{*,f}	51.1±5.8^{*,a}	81.4±0.2^{*,e}
	estimated	42.6±0.7	111.5±1.1	52.1±0.9	119.0±0.9	57.8±2.6	49.5±0.8	67.9±1.0	63.1±1.8	122.6±0.6
Soymilk	measured	43.1±1.8^{*,b,c}	62.0±0.7^{*,e}	45.1±2.5^{*,a,c}	66.9±1.8^{*,c}	43.1±2.1^{*,e}	65.5±0.8^{*,d}	41.7±0.8^{*,e}	69.5±0.8^{*,d}	76.5±1.2^{*,c}
	estimated	58.2±0.7	99.5±2.4	49.4±1.1	109.6±1.3	60.1±1.6	76.3±1.2	66.0±1.4	90.4±1.2	114.4±0.9
Tofu	measured	36.9±2.2^{*,d}	69.0±1.2^{*,b}	45.0±2.9^{*,a,c}	73.4±0.7^{*,b}	41.7±1.4^{*,d,e}	57.0±2.8^{*,e}	35.4±2.1^{*,g}	61.2±2.3^{*,b}	79.0±1.9^{*,c,e}
	estimated	49.5±0.9	103.2±0.8	50.6±1.2	116.0±0.9	57.4±1.9	63.9±0.8	70.8±1.5	75.7±1.2	117.9±0.4
	Bound (µL)	100			150			200		
	Trolox (µL)	100	150	200	100	150	200	100	150	200
Extra Virgin Olive Oil	measured	49.3±1.4^{*,a}	60.8±1.7^{*,a}	75.7±0.3^{*,a}	48.6±0.5^{*,a}	48.4±1.0^{*,a}	56.8±0.1^{*,a}	38.7±1.4^{*,a}	49.4±0.4^{*,a}	64.5±1.0^{*,a}
	estimated	54.9±1.4	68.9±0.9	82.6±0.5	64.2±1.9	78.2±1.4	91.9±1.0	76.5±2.1	90.5±1.5	104.2±1.1
Refined Olive Oil	measured	50.3±0.9^{*,a}	59.5±0.3^{*,a}	71.2±0.9^{*,b}	35.1±0.6^{*,b}	51.8±1.3^{*,b}	55.1±0.8^{*,b}	40.0±1.0^{*,a}	49.4±0.4^{*,a}	66.2±0.0^{*,a}
	estimated	57.4±1.5	71.4±1.0	85.1±0.6	69.5±1.1	83.5±0.5	97.2±0.1	81.6±1.8	152.9±1.3	109.3±0.9

Different letters indicate the statistical significance of difference at $p<0.05$ between the measured inhibition values of bound antioxidant + Trolox mixtures, for each combination lined up in columns. The * symbols indicate the statistical significance of difference at $p<0.05$ for each measured-estimated pair.

Table 3.2. The p values within 0.95 confidence interval calculated by using anova1 function in Matlab for [B]: Bound antioxidant concentration, [T]: Trolox concentration and pH; [B]*[T], [B]*pH, [T]* pH: the 2- way interactions between [B], [T] and pH; [B]*[T]*pH: the 3- way interaction between [B], [T] and pH.

	[B]	[T]	pH	[B]*[T]	[B]*pH	[T]*pH	[B]*[T]*pH
Whole Wheat Flour	0.0014	0.0018	0.0731	4.77E-11	4.77E-11	4.77E-11	4.77E-11
Whole Wheat Paste	0.0136	0.4612	0.0002	3.71E-18	3.71E-18	3.71E-18	3.71E-18
Whole Wheat Bread	3.13E-05	0.1094	0.0068	3.61E-17	3.61E-17	3.61E-17	3.61E-17
Soybean	0.0252	0.1223	1.50E-07	2.97E-19	2.97E-19	2.97E-19	2.97E-19
Edamame	0.0461	0.0322	2.95E-07	2.24E-21	2.24E-21	2.24E-21	2.24E-21
Boiled Soybean	0.2856	0.4491	9.30E-11	1.27E-17	1.27E-17	1.27E-17	1.27E-17
Soymilk	0.0829	0.1189	1.59E-08	3.22E-22	3.22E-22	3.22E-22	3.22E-22
Tofu	0.4024	0.3928	1.21E-11	8.52E-20	8.52E-20	8.52E-20	8.52E-20
Extra Virgin Olive Oil	0.0054	8.09E-08		2.50E-24			
Refined Olive Oil	0.003	4.45E-08		7.86E-24			

Antioxidant activity was higher at pH 3.0 than at pH 5.0 for the mixtures of paste and bread and the vice versa was valid for boiled soybean. Meanwhile the results for pH 3.0 and 6.0 was significantly different for the mixtures of Trolox with soybean, edamame and soymilk. pH 3.0 gave higher antioxidant activity results compared to pH 6.0 for the mixtures of soybean and edamame and the opposite was valid for soymilk. The change of pH did not make any significant difference on the antioxidant activity of flour-Trolox mixture while it made sense for all different pH values for tofu- Trolox mixtures.

At this point it is important to understand the reason behind the antagonistic behavior of Trolox with different kinds of macromolecule-bound antioxidants (Table 3.1). Oxidation reactions of Trolox induced by Br_2^-, which was revealed by Thomas et al. (1989) [185], was considered as a reference for this purpose. In this study, Trolox c (3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid) (I) had been converted into its phenoxyl radical (II) via oxidation with Br_2^- following a specific procedure [186, 187]. Then, the phenoxyl radical (II) had disproportionated to Trolox c (I) and a cross conjugated ketone (4,5-dihydro-3,6,8,9-tetramethyl-2H-3,9a-epoxy-l-benzoxepin-2,7 (3H)-dione) (III). Finally, the intermediate ketone (III) had been hydrolyzed to a quinone (2-hydroxy-2-methyl-4-(2,5,6-trimethyl-2,4-dioxo-2,5-cyclohexadienyl) butanoic acid) (IV). Disproportionation of the phenoxyl radical (II), formed by the oxidation of

Trolox c (I) with Br_2^-, to Trolox c (I) and a cross conjugated ketone (III), namely the auto-regeneration reaction of Trolox, constituted the basis for the hypothesis of the experimental studies. The antagonism observed was thought to be originated from the interference of bound antioxidants to the auto-regeneration step. Evidence for this thesis was tried to be collected by monitoring the level of the stable end product, quinone, in the ABTS radical media containing Trolox alone or together with DF-bound antioxidants, via mass spectrometry analysis. The results obtained were as shown in Figure 3.1.

The initial level of the ions of end product, which measured immediately after mixing Trolox and ABTS radical was around 2.79E6 (Figure 3.1.a). After 30 min of reaction in darkness at room temperature, the ion level increased by around 36 %, to 3.81E6 (Figure 3.1.b). However, the mixture of Trolox with whole wheat bound antioxidants in the same reaction conditions yielded an ion level of 3.44E6 (Figure 3.1.c), giving around a 10 % decrease for the amount of stable end product.

After 1 h, all measurements were repeated and the initial ion level of the end product was found to be increased to 3.24×10^6 (Figure 3.1.d). However, the level after 90 min of reaction with Trolox and Trolox + whole wheat bound antioxidants mixture was found to decrease down to 3.70×10^6 (Figure 3.1.e) and 3.33×10^6 (Figure 3.1.f), respectively, when compared to the levels after 30 min of reaction.

The results centered an inhibitory role for whole wheat bound antioxidants on the oxidation reaction of Trolox depending upon the 10 % decrease on the level of ions of the stable end product in the reaction medium. This might be originated from the regenerative activities of Trolox molecules on depleted bound antioxidants during reaction in the ABTS radical environment, retaining themselves from the auto-regeneration reactions. This phenomenon for the regeneration of bound antioxidants via soluble antioxidants indeed was revealed in one of our previous study [5]. This situation consequently might lead to antagonistic interactions between Trolox and whole wheat bound antioxidants. On the other hand, the increase on the ion level of the stable end product after 1 h (Figure 3.1.d) and the decrease after 1.5 h (Figure 3.1.e) indicates that there is a breaking point for the auto-regeneration reaction even if Trolox is the only antioxidant species in the radical environment.

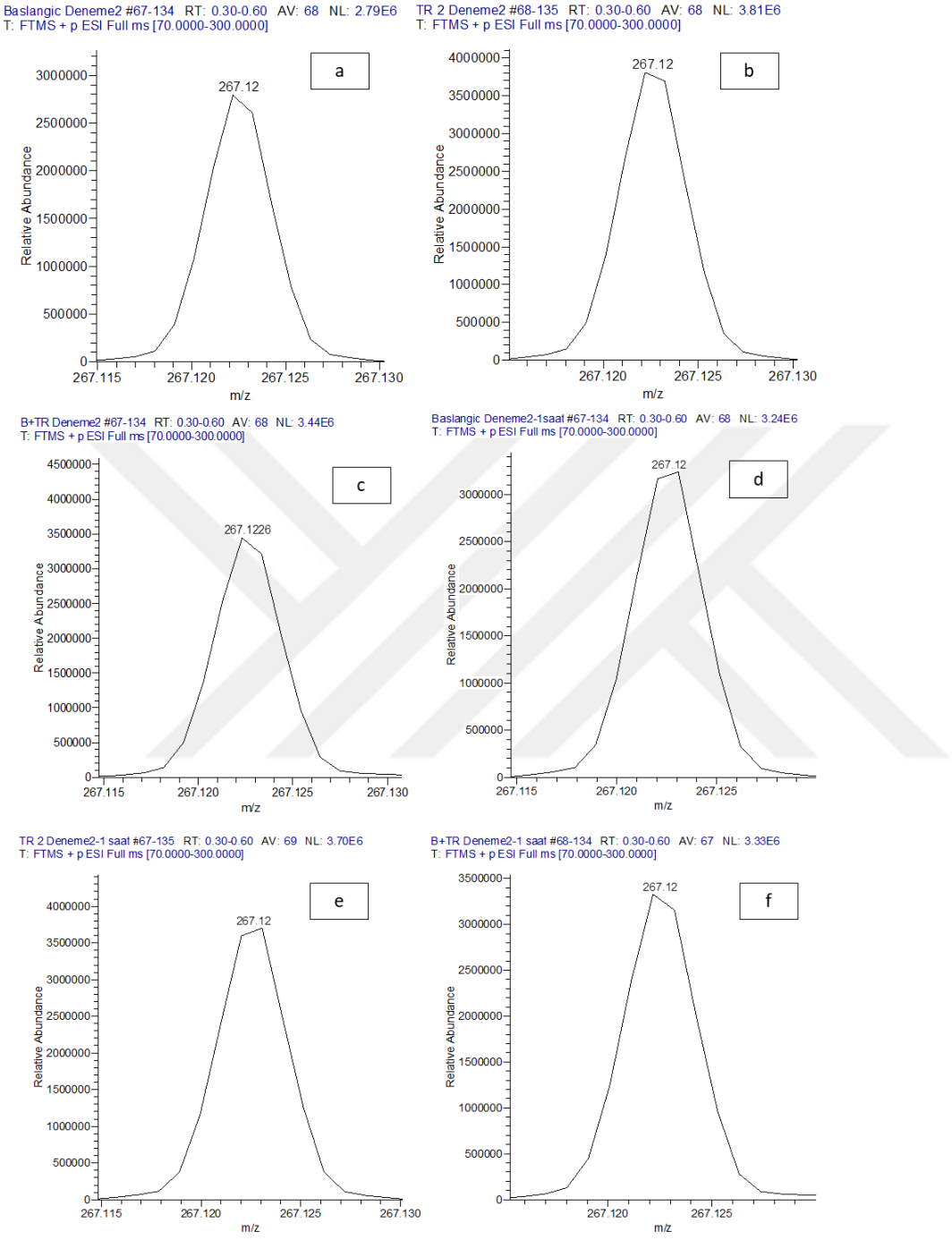


Figure 3.1. Mass spectra obtained for 2-hydroxy-2-methyl-4-(2,5,6-trimethyl-2,4-dioxo-2,5-cyclohexadienyl) butanoic acid, the stable end product of the oxidation reaction of Trolox, for the following reaction conditions: a) Trolox + ABTS, 0 min b) Trolox + ABTS, 30 min c) Trolox + whole wheat bound antioxidants + ABTS, 30 min, d) Trolox + ABTS, 60 min e) Trolox + ABTS, 90 min, f) Trolox + whole wheat bound antioxidants + ABTS, 90 min.

On the other hand, in order to understand whether antagonistic interactions of Trolox are only attributable to bound antioxidants, interactions of Trolox with free forms of the DF, protein and lipid-bound antioxidants used in this study was investigated. For this purpose, ferulic, caffeic and p- coumaric acids were used as the free forms of whole wheat DF- bound antioxidants. Cysteine, methionine, tryptophan, tyrosine, phenylalanine and histidine were tested as the free amino acids found in soybean products, while rutin and quercetin were tested as the free forms of olive oil bound antioxidants. Consequently, antagonistic interactions were also observed with the free forms of macromolecule- bound antioxidants (results are not given). This situation suggests a general inhibitory role for the other antioxidant types on the auto-regeneration reaction of Trolox regardless of being free or bound. However, further investigations are needed to prove this inference.

3.2. Interactions between Macromolecule- Bound Antioxidants and Free Antioxidant Trolox in Liposome Medium

The time course for the oxidation of liposomes in the presence of 5 µM Trolox and either aqueous solutions of DF/protein-bound or hexane solutions of lipid-bound antioxidants were monitored during 180 min (Figure 3.2.A). The levels of oxidation were evaluated by the formation of fluorescent compounds derived from the secondary lipid oxidation products generated [181]. Although the lag phase of the oxidation curves remained similar in general, the levels of oxidation depended on the added compounds. Mixtures of extra virgin and refined olive oil samples with Trolox gave higher levels of oxidation than the control sample indicating a pro-oxidative behavior. On the other hand, the mixtures of DF and protein bound antioxidants with Trolox, gave lower levels of oxidation than the control suggesting antioxidant effects. Adding Trolox alone to the liposomes gave an antioxidant effect, while the addition of whole wheat flour-bound antioxidants only gave weak extra antioxidant effects (Figure 3.2.B).

The antioxidative and prooxidative effects were evaluated quantitatively based on area under the curves (AUC) of the fluorescence changes. The measured values of relative inhibition % of lipid oxidation, based on the AUC values were calculated for the different combinations of DF, protein and lipid-bound antioxidants with Trolox, and have been compared to estimated values calculated based on the sum of the values of the individual components (Table 3.3). Accordingly, the measured inhibition % values for the mixtures of whole wheat flour, paste and bread`s DF-bound

antioxidants with Trolox were significantly higher than the estimated ones, indicating synergistic interactions. Only the combination of 10 mg/mL whole wheat flour with 5 µM Trolox and the combinations of 5 mg/mL whole wheat flour with 5 and 10 µM Trolox acted antagonistically.

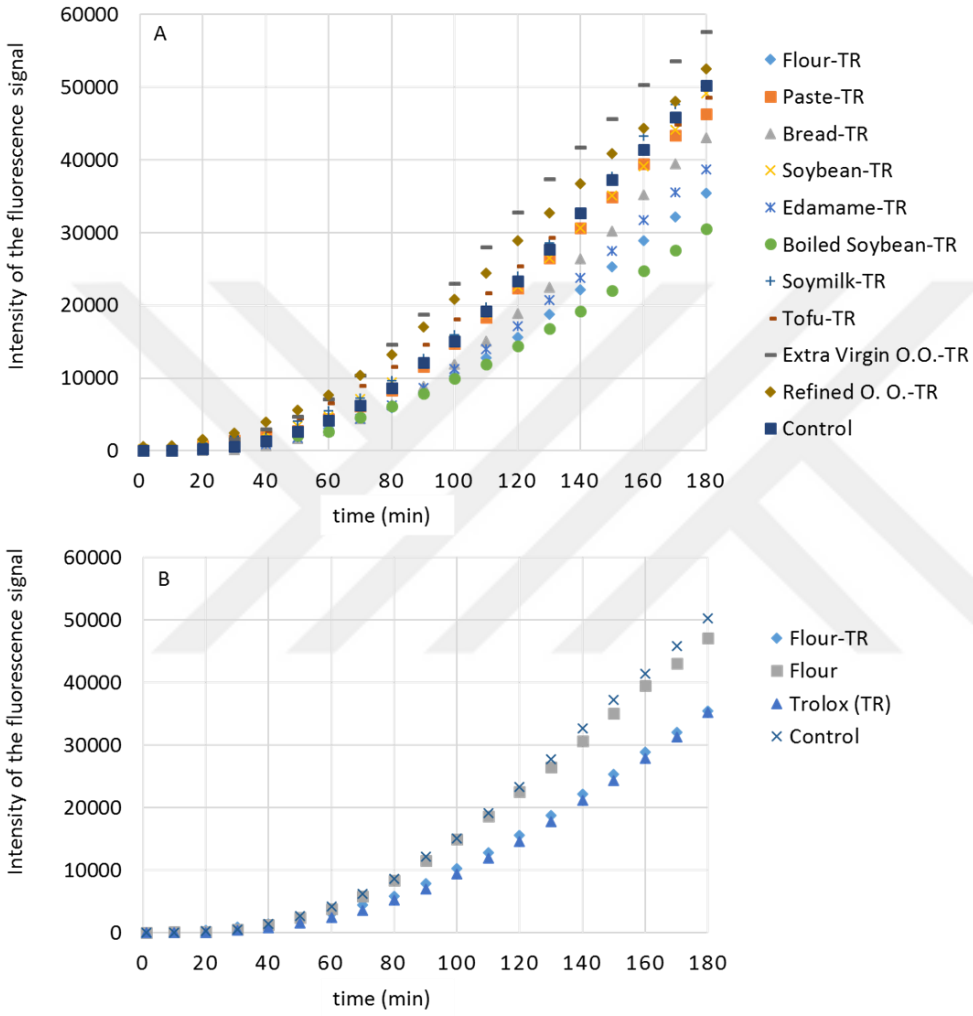


Figure 3.2. Time course of the fluorescence change in the liposome systems containing: A) the mixtures of 5 µM Trolox with 1 mg/mL aqueous solutions of DF/protein-bound and 300 µL/mL hexane solutions of lipid-bound antioxidants. B) 5 µM Trolox and 1 mg/mL aqueous solution of whole wheat flour separately and mixed.

For combinations of protein-bound antioxidants with Trolox, more complex results were observed. Especially for 5 and 10 mg/mL protein-bound antioxidant together with 10 and 50 µM Trolox blends gave a systematically antagonistic behavior. Moreover, for almost all combinations of tofu

with Trolox, the interactions were significantly antagonistic. On the contrary with DF and protein-bound antioxidants, lipid bound antioxidants did not show a specific group behavior. The interaction of Trolox with extra virgin olive oil was significantly antagonistic, while it was synergistic with refined olive oil.

Liposomes are spherical vesicles consisting of one or more lipid bilayers that is generally created from phospholipids. Trolox, a water soluble analogue of Vitamin E, locates mainly in the aqueous phase and partly in the lipid surface of this phospholipid membranes [188]. It is known to exert a significant antioxidant activity when the peroxidation reaction is initiated in the aqueous phase, which is also observed in our measurements (results regarding the separate measurements for bound antioxidants and Trolox are not shown).

DF-bound antioxidants of whole wheat products exerted a pro-oxidant behavior themselves. This can be due to the known pro-oxidant effect of phytophenolics in the presence of redox-active metals such as Cu and Fe. With the help of oxygen, these metals can catalyze the redox cycling of phenolics, leading to the formation of reactive oxygen species and other organic radicals that can damage DNA, lipids and other biological molecules [189-192]. Besides, DF phenolate groups may create a chain-carrying effect, by forming free radicals, that will lead to a pro-oxidant behavior [193]. Ferulic acid, as the predominant form of bound antioxidants in whole wheat DF matrix may probably be responsible for such kind of effect. In most combinations with Trolox synergistic antioxidant behavior of whole wheat products was observed, suggesting that Trolox has the capability of regenerating DF-bound phenolics, or is more reactive to react with either Fe^{3+} or radical species created, improving antioxidant capacity.

Table 3.3. Measured and estimated values of relative inhibition % of liposome oxidation values for the mixtures of DF, protein or lipid-bound antioxidants with Trolox.

	Bound (mg/mL)	1			5			10		
	Trolox (µM)	**5**	**10**	**50**	**5**	**10**	**50**	**5**	**10**	**50**
Whole Wheat Flour	measured	11.7±2.3*,a	15.6±4.3*,a	57.6±4.0*,a,c	-15.6±0.2*,a	-13.3±2.6*,a	56.0±3.2*,a,b	-17.6±2.9*,a	24.1±0.7*,a	49.3±2.4*,a
	estimated	-11.3±1.1	-4.8±1.1	17.1±1.1	-8.1±1.7	-0.5±0.9	21.8±1.8	-2.6±0.7	5.3±1.8	27.4±1.5
Whole Wheat Paste	measured	21.1±4.2 *,b	37.1±3.4*,b	61.8±1.0*,a,b	11.7±1.8b	30.9±1.9*,b	61.2±4.4*,b	21.0±1.5*,b	32.3±1.9*,a	66.0±4.3*,b
	estimated	6.8±1.4	13.3±1.9	28.5±2.5	11.9±1.5	21.23±1.7	35.0±2.3	14.2±1.2	24.2±2.4	36.3±3.0
Whole Wheat Bread	measured	24.1±2.5 *,b	30.9±2.8*,c	56.7±4.0*,a,c,d	27.9±2.4*,c	33.4±1.1*,b	56.6±0.3*,a,b	20.9±2.8b	33.6±3.7b	65.4±4.0*,b
	estimated	10.5±1.0	16.8±2.2	30.2±2.6	15.4±3.2	22.6±3.1	33.8±3.4	23.6±3.7	30.3±2.3	49.0±2.9
Soybean	measured	2.3±0.8 *,c	21.6±2.2*,d	46.5±3.6 *,e	-16.8±3.4*,a	3.4±0.9c	48.8±2.0*,c	-4.5±0.3*,c	5.6±1.2*,c	35.4±4.4*,c
	estimated	-16.2±2.7	4.2±0.6	18.1±2.1	3.7±0.1	7.7±2.6	24.1±3.1	5.7±0.5	11.5±2.5	28.3±0.9
Edamame	measured	7.6±1.6*,a	22.3±2.8*,d	54.4±4.2*,c,d	-28.6±3.0*,d	-8.1±0.8*,d	48.0±2.7*,c	-23.7±2.4*,d	-12.6±1.7*,d	52.0±1.9*,a,d
	estimated	-16.1±2.3	-1.6±0.9	19.4±2.1	-20.3±3.4	-3.3±0.9	16.3±2.7	-10.5±2.0	14.2±1.7	38.1±0.8
Boiled Soybean	measured	26.1±3.0 *,b	35.6±3.9*,b,c	62.9±2.5*,b	9.9±2.4*,b	23.0±1.5*,e	53.9±4.1*,a,c	19.8±3.5*,b	26.7±3.4*,a	56.0±2.0*,d
	estimated	21.1±3.3	29.3±3.3	42.6±3.3	24.2±2.1	29.9±2.0	46.7±3.0	24.0±3.6	36.7±2.4	50.0±2.4
Soymilk	measured	-18.3±2.1d	1.0±0.9*,e	51.9±2.3 *,d	-75.3±5.2*,e	-67.9±4.1*,f	23.1±1.9*,d	-46.9±3.7*,e	-64.6±4.2*,e	-8.5±1.5*,e
	estimated	-28.8±5.4	-18.4±5.4	21.1±3.5	-47.7±2.9	-37.3±2.9	-15.3±2.2	-22.3±0.7	-11.8±0.7	8.2±0.7
Tofu	measured	-36.3±2.3*,e	-17.3±1.3*,f	41.7±3.1 *,f	-27.7±5.9*,d	-19.3±2.7*,g	8.2±4.3d	-39.7±2.7*,f	-33.4±0.9*,f	-7.6±5.2*,e
	estimated	-27.7±3.6	-12.2±3.6	-22.1±3.6	-15.8±2.6	-2.1±0.1	15.9±2.6	2.6±0.3	18.5±0.6	36.3±2.9
	Bound (µL/mL)	**300**			**600**			**900**		
	Trolox (µM)	**5**	**10**	**50**	**5**	**10**	**50**	**5**	**10**	**50**
Extra Virgin Olive Oil	measured	-64.7±6.9*,a	-53.6±2.8*,a	-6.8±1.4*,a	-48.9±4.6*,a	-59.0±4.1*,a	6.1±1.5a	52.1±3.0*,a	-42.8±3.2*,a	9.8±3.7*,a
	estimated	-18.6±1.3	-8.3±1.3	9.2±1.3	-23.4±0.6	-13.1±0.6	4.0±0.6	-30.0±2.5	-19.7±2.5	-4.2±1.2
Refined Olive Oil	measured	-7.4±1.7b	7.8±2.5b	50.2±4.1*,b	-5.7±2.0b	-26.0±1.5*,b	53.7±1.4*,b	-10.8±3.0b	6.5±0.3*,b	51.3±2.1*,b
	estimated	-6.0±1.9	8.1±0.8	23.8±2.7	-7.1±0.8	5.3±0.9	22.9±0.8	-14.4±2.2	-3.4±0.9	15.6±2.2

Different letters indicate the statistical significance of difference at $p<0.05$ between the measured inhibition values of bound antioxidant in mixtures with Trolox for each combination lined up in columns. * indicate the statistical significance of difference at $p<0.05$ for each measured-estimated pair.

The prooxidant behavior of soybean protein-bound antioxidants themselves and the antagonistic behavior of them with Trolox is not surprising if we consider the nature of proteins and protein-phenolic interactions. Indeed, all 20 amino acids have the potential to interact with free radicals, some of them (cysteine, methionine, tryptophan, tyrosine, phenylalanine…etc.) being more reactive. However, the antioxidant activity of these amino acid residues can be limited by the tertiary structure of the polypeptide: they can be buried within the protein core, being inaccessible to pro-oxidants. Besides, the ability of a protein to act as an antioxidant strongly depends on the energy of the resulting protein radical. If it serves and transports high energy free radicals promoting oxidation, it will have no value as an antioxidant as well [159]. Moreover, metals bound to non-specialized protein chelators can remain pro-oxidative if they retain their ability to redox cycle, which will be valid for our system with Fe^{3+} as the oxidizing agent. In the presence of transition metals, phenolic compounds have the potential to act as pro-oxidants as mentioned earlier [189].

In case of lipid-bound antioxidants, both extra virgin and refined olive oil samples acted pro-oxidant themselves. However, the fact that their interactions with Trolox being totally opposite of each other was remarkable. With the washing procedure applied, free phenolics were removed from the oil samples, remaining lipid-bound phenolics [194]. Refined olive oil have less phenolics than extra virgin olive oil, hence should have less antioxidative potential. The synergistic behavior of refined olive oil and antagonistic behavior of extra virgin olive oil here suggests that the lipid-bound phenolics exerted a pro-oxidant behavior in the presence of Fe^{3+} [189]; Trolox regenerated this phenolics, being more effective in the case of refined olive oil, since it has less phenolics, and remaining incapable for extra virgin olive oil, hence not being able to prevent the antagonistic behavior.

Considering the same concentration combinations of DF and protein-bound antioxidants with Trolox, the relative inhibition % values did not follow a certain trend (Table 3.3). This situation proved that the interactions differs not only depending on the type of the bound antioxidant, but also on the concentration combinations with Trolox.

Modelling of the Oxidation Reaction in Liposomes

The fluorescence developed during the oxidation of the liposome were further analyzed by fitting to the sigmoidal function given by equation 1 (Figure 3.3). All the experimental data gave good fits with high R^2-values (Table 3.4).

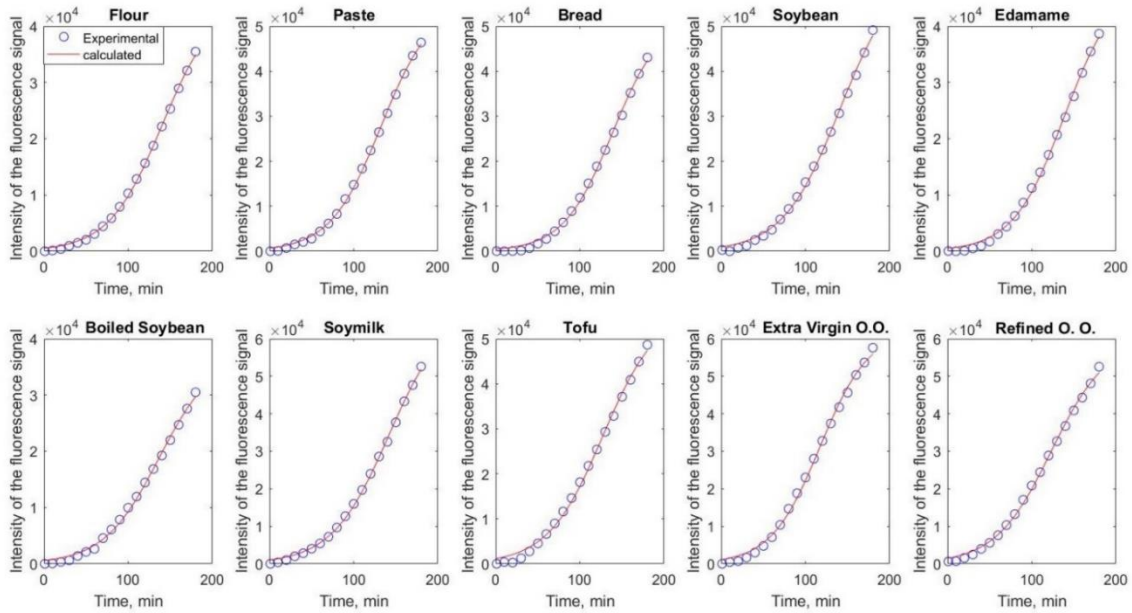


Figure 3.3. Curve fitting performed by using the sigmoidal model given for experimental data regarding the liposome systems containing the mixtures of 5µM Trolox with 1 mg/mL aqueous solutions of DF/protein-bound and 300 µL/mL hexane solutions of lipid-bound antioxidants.

The oxidation rates of liposomes, which are represented by the fitted *k* values, were not significantly different from each other for the mixtures of Trolox with DF, protein-bound antioxidants and for lipid bound antioxidants (Table 3.4). However, for most of these systems the reaction rates were faster than the control indicating prooxidant effects. The initial levels of oxidation, *A*, were found to have the highest values for liposomes containing the mixtures of Trolox with tofu and extra virgin olive oil, being significantly different from the others. Liposomes containing the mixtures prepared with whole wheat flour, paste, bread, edamame, boiled soybean and refined olive oil had similar initial oxidation levels. The *A*/*B* values, which are equivalent to the maximum levels of oxidation when time reaches infinity, were for most samples equal to the

control or slightly lower. Liposomes containing the mixtures of Trolox with whole wheat flour, edamame and boiled soybean was found to reach a lover maximum than the remaining samples.

Table 3.4. The fitted constants of equation 1 modelling the oxidation reaction of liposomes in mixtures of Trolox with DF, protein or lipid-bound antioxidants.

	A	B	A/B	k (min^{-1})	R^2
Whole Wheat Flour	431 ±110[a]	0.0097±0.0020[a]	44244 ± 14418	0.0334±0.0022 [a,b]	0.9994
Whole Wheat Paste	652 ± 50[a,b,c]	0.0117±0.0008[a]	55548 ± 5752	0.0325±0.0002 [a,b,c]	0.9993
Whole Wheat Bread	498 ± 43[a,b]	0.0095±0.0005[a]	52620 ± 5172	0.0336±0.0002[a,b]	0.9990
Soybean	816 ± 168[c]	0.0139±0.0018[a]	58834 ± 14236	0.0307±0.0011[a,c]	0.9991
Edamame	491 ± 19[a,b]	0.0104±0.0002[a]	47163 ± 2062	0.0328±0.0001[a,b,c]	0.9992
Boiled Soybean	463 ± 133[a]	0.0120±0.0035[a]	38468 ± 13338	0.0321±0.0023[a]	0.9987
Soymilk	766 ± 211[b,c]	0.0115±0.0026[a]	66295 ± 23748	0.0326±0.0025[a,b,c]	0.9994
Tofu	1123 ± 77[d]	0.0201±0.0009[c]	55790 ± 4590	0.0306±0.0004[a,c]	0.9983
Extra Virgin Olive Oil	1161 ± 231[d]	0.0180±0.0038[c]	64381 ± 18618	0.0343±0.0001[a,b]	0.9990
Refined Olive Oil	515 ± 59[a,b]	0.0095±0.0012[a]	54128 ± 8994	0.0353±0.0005[b]	0.9993
Control	858 ± 0.1[c]	0.0135±0.0002[a]	63760 ± 1173	0.0291±0.0000[c]	0.9979

Different letters indicate the statistical significance of difference at $p < 0.05$ between mixtures composed of different bound antioxidants + Trolox for each constant.

Principal Component Analysis (PCA)

The lipid oxidation fluorescence curves were also analyzed using PCA. For this purpose, the intensity of the fluorescence signal data obtained from the measurements performed at each 10 min for 180 min for the mixtures of bound antioxidants with Trolox was preprocessed by using the first derivative (window size of 5 points and polynomial degree of 2) and then mean centered. The first 2 PCs contained the highest amount of variance (supplementary information).

According to scores plots, which were marked according to Trolox concentration (a plots in Figure 3.4), samples mixed with 50 µM Trolox were clearly discriminated, whereas samples with 5 and 10 µM Trolox were found to be grouped together for all bound antioxidants. Except whole wheat flour, the majority of this variance was explained by PC-1. Considering scores plots, which were marked according to concentrations of bound antioxidants (b plots in Figure 3.4), there were no clear discrimination among concentrations, although for 10 mg/mL bound antioxidant

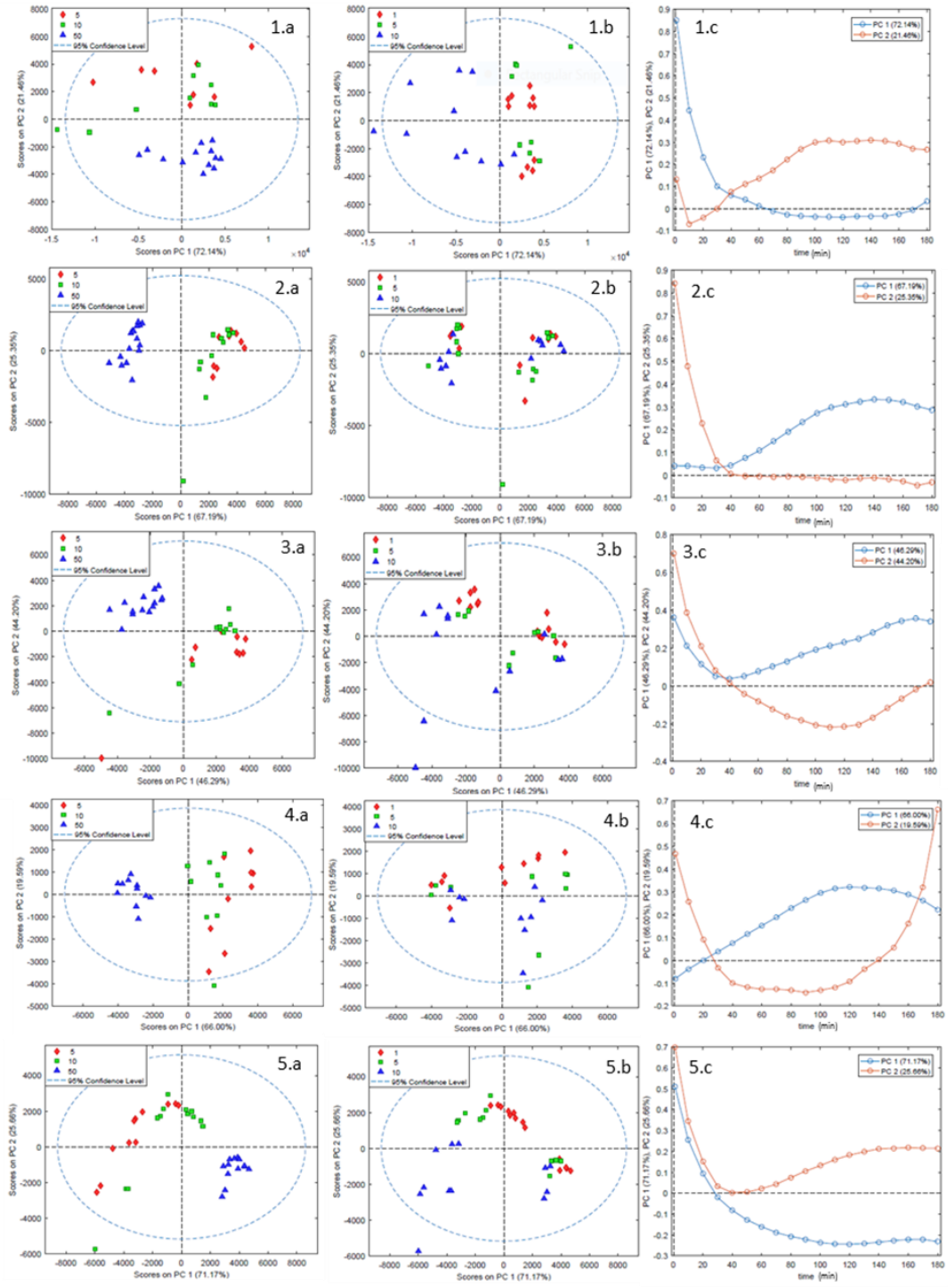
concentration a grouping was slightly obvious for some samples. Also, a perceptible discrimination among different concentrations was obvious for boiled soybean samples.

The reason for the data being nested always for samples mixed with 5 and 10 µM Trolox will probably because these concentrations were too close to each other, restraining the discrimination. This may also be the case for the effect of bound antioxidant concentrations.

The most striking characteristics of the loadings plots (c plots in Figure 3.4), were the intersections of the PC-1 and PC-2 around 20-40 min and the peaks observed for both PC-1 and PC-2 between 60-120 min. Accordingly, it is suggested that the curve intersections in the 20-40 min interval correspond to the end of the lag phase, and after that point the initiation of uninhibited lipid oxidation occur in the liposome system. For most of the bound antioxidants one of the two PCs have a positive maximum after the intersections while the other PC is close to zero or have negative values.

The increase in PC-1 or PC-2 between 60-120 min interval may thus provide information about the uninhibited oxidation phase. The edamame system did not give intersecting PCs, which probably represents a reverse situation with a totally reversed order of its data in the PC-1 scale with respect to the others. Similarly, for whole wheat the behavior of the PC-1 and PC-2 appears to be interchanged compared.

For samples showing discrimination due to a specific PC, according to free antioxidant concentration for instance, the data grouped in the negative part of that specific PC, correlates the negative part of the loading of that PC and the data on the positive part with the positive part of that PC in the loading: i.e. the mixtures of edamame samples with 50 µM Trolox correlates with the positive part of PC-1, which is suggested to provide information related to the lag phase before the onset of the oxidation, indicating the mixtures with 50 µM Trolox were more effective during the lag phase. Likewise, for whole wheat flour, data grouped on the negative part of the PC-2, mainly the mixtures with 50 µM Trolox, correlates with the negative part of the loading of PC-2, again corresponding to the lag phase before the onset of the oxidation. The opposite situation was also valid for other bound antioxidants` data.


1.a
1.b
1.c
2.a
2.b
2.c
3.a
3.b
3.c
4.a
4.b
4.c
5.a
5.b
5.c
95% Confidence Level
Scores on PC 1 (72.14%)
Scores on PC 2 (21.46%)
PC 1 (72.14%)
PC 2 (21.46%)
Scores on PC 1 (67.19%)
Scores on PC 2 (25.35%)
PC 1 (67.19%)
PC 2 (25.35%)
Scores on PC 1 (46.29%)
Scores on PC 2 (44.20%)
PC 1 (46.29%)
PC 2 (44.20%)
Scores on PC 1 (66.00%)
Scores on PC 2 (19.59%)
PC 1 (66.00%)
PC 2 (19.59%)
Scores on PC 1 (71.17%)
Scores on PC 2 (25.66%)
PC 1 (71.17%)
PC 2 (25.66%)
time (min)

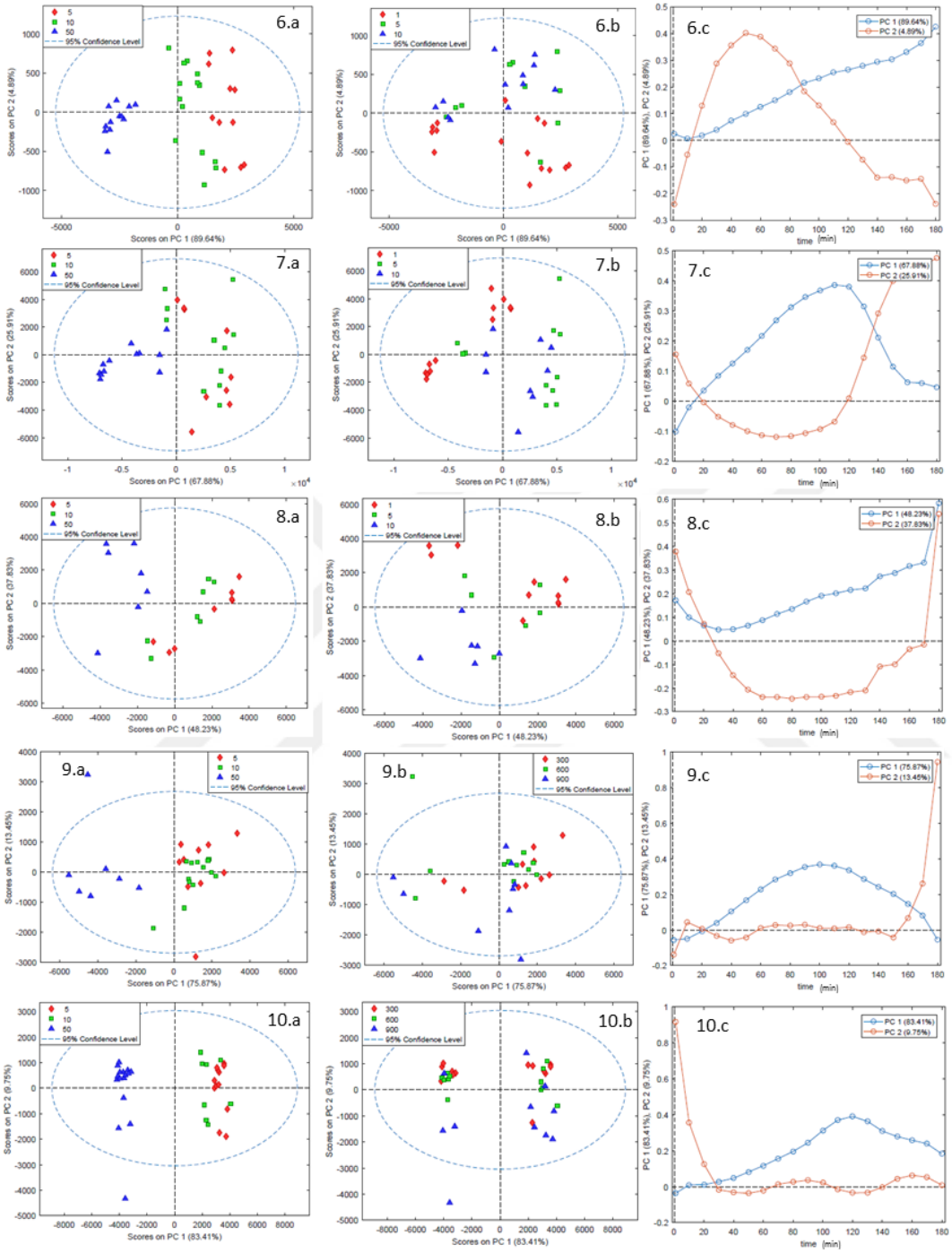


Figure 3.4. Scores and loadings plots for the mixtures of: 1) Whole-wheat flour, 2) Whole-wheat paste, 3) Whole-wheat bread, 4) Soybean, 5) Edamame, 6) Boiled soybean, 7) Soymilk, 8) Tofu, 9) Extra virgin olive oil, 10) Refined olive oil with Trolox. The letters indicate are as follows: (a) scores plot colored by Trolox concentration, (b) scores plot colored by bound antioxidant concentration, (c) loadings plot.

Anova Simultaneous Component Analysis (ASCA)

The significance of the effects of bound antioxidant and Trolox concentrations on the measured inhibition % values were tested with ANOVA-Simultaneous Component Analysis (ASCA) applied on the matrix with the same preprocessing as before. The number of permutations was 500 and two-way interactions were included in the model (Table 3.5). The Trolox concentration had a significant effect on the inhibition % of lipid oxidation values measured for all bound antioxidant samples. Also, the effect of bound antioxidant concentration was significant, for the mixtures of all bound antioxidants with Trolox, except whole wheat paste. The interaction between bound and free antioxidant concentrations however, was only significant for soybean, boiled soybean, soymilk, tofu and refined olive oil samples.

Table 3.5. The p values within 0.95 confidence interval calculated by using Anova Simultaneous Component Analysis (ASCA) in Matlab for [T]: Trolox concentration, [B]: Bound antioxidant concentration, [T] * [B]: the 2-way interaction between [B] and [T].

	[T]	**[B]**	**[T] * [B]**
Whole Wheat Flour	0.0020	0.0100	0.1780
Whole Wheat Paste	0.0020	0.5120	0.2580
Whole Wheat Bread	0.0020	0.0020	0.3820
Soybean	0.0020	0.0020	0.0260
Edamame	0.0020	0.0020	0.1980
Boiled Soybean	0.0020	0.0020	0.0020
Soymilk	0.0020	0.0020	0.0020
Tofu	0.0020	0.0020	0.0120
Extra Virgin Olive Oil	0.0020	0.0020	0.3620
Refined Olive Oil	0.0020	0.0020	0.0020

Indeed, for most phytochemicals, the antioxidant and pro-oxidant behaviors are concentration-dependent. Several polyphenols previously counted as antioxidants such as quercetin, catechins and gallic acid have been reported to have pro-oxidant activity at high doses [195-198]. Besides, most amino acids are known to exert antioxidant activity at very low concentrations, but became

pro-oxidative at increased concentrations [199]. This is in agreement with the synergistic behavior of soybean proteins at low concentrations and antagonistic behavior of high concentrations.

3.3. Interactions between Whole Wheat Dietary Fiber- Bound Antioxidants and Hydroxycinnamic/ Hydroxybenzoic Acids in Aqueous and Liposome Media

The percentage of inhibition of lipid oxidation values measured for the real combinations of WW-bound antioxidants and HCA/ HBA and estimated by summing up their separate inhibition % values are given in Table 3.6.1. Both pro- oxidant and antioxidant behaviors were observed in the liposome medium depending on the type of HCA/ HBA mixed with the WW-bound antioxidants. The comparison of the measured values with the estimated ones revealed a barely synergistic behavior for o-, m-, and p-coumaric acids with WW- bound antioxidants at low concentrations. The interaction was rather antagonistic with the increasing concentrations of both types in the mixture, except m-coumaric acid being synergistic at 10 mg/mL-100µM combination. Meanwhile, 3,5-dihydroxyhydrocinnamic acid exerted an opposite behavior, i.e. acting antagonistic at low concentrations and synergistic at high concentrations of both free and bound antioxidants in the mixture. On the other hand, 2,4-dihydroxycinnamic acid was significantly antagonistic, while Gallic and Caffeic acids were significantly synergistic with WW- bound antioxidants at almost all combinations.

The AUC values calculated from the oxidation rate curves of DPPH radical for the real combinations of WW- bound antioxidants and HCA/ HBA and the related estimated values are given in Table 3.6.2. The results were demonstrated only for the combinations of 100 µM HCA/ HBA, the common concentration for all HCA/ HBA derivatives, with WW-bound antioxidants. On the contrary to the liposome medium, a rather antioxidant behavior was observed in the aqueous medium. Almost all HCA/ HBA derivatives were significantly synergistic with WW-bound antioxidants except 2,4,5-trihydroxybenzoic acid. Especially Isovanillic, 2-hydroxy-3,4-dimethoxybenzoic and 3,4,5-trimethoxybenzoic acids were considerably synergistic.

There was a different gradation for the mixtures of different HCA/ HBA derivatives with WW-bound antioxidants at a certain concentration combination by means of percentage of inhibition or AUC values measured for both for liposome and aqueous media studies. Thus, the multivariate nature of the antioxidant activity, basically depending on the concentrations and types of antioxidants contained in the mixture was proven once more.

Table 3.6. Measured and estimated values of 1) relative inhibition % of lipid oxidation and 2) AUC calculated based on the inhibition % rate of DPPH radical for the mixtures of WW- bound antioxidants with HCA / HBA

1	**[WW- Bound], mg/mL**	**1**			**5**			**10**		
	[HCA/ HBA], µM	**10**	**50**	**100**	**10**	**50**	**100**	**10**	**50**	**100**
o-coumaric acid	**measured**	-3.44±2.69*^{,b}	-26.39±1.23*^{,b}	-30.66±3.10*^{,b}	-7.84±1.84^{b}	-22.82±2.56*^{,b}	-29.84±1.41*^{,b}	-13.48±2.26^{a}	-13.14±2.45^{a}	-17.76±0.39*^{,b}
	estimated	-9.88±1.75	-8.59±1.75	-6.34±1.75	-8.47±0.52	-7.18±0.52	-4.93±0.52	-10.22±1.01	-8.93±1.01	-6.68±1.01
m-coumaric acid	**measured**	-6.22±1.71*^{,b,c}	-4.77±1.40*^{,c}	-14.50±2.67*^{,a}	-19.62±1.32*^{,c}	-10.42±1.53^{a,c}	-14.78±0.24*^{,c}	-13.50±3.55^{a}	-12.20±1.60*^{,a}	-1.02±1.46*^{,c}
	estimated	-11.40±1.97	-7.95±1.97	-9.02±1.97	-15.68±0.78	-12.24±0.78	-13.31±0.78	-12.52±1.38	-9.08±1.38	-10.15±1.38
p-coumaric acid	**measured**	5.03±1.73*^{,a}	4.86±0.61*^{,ai}	-10.30±2.57*^{,a}	-12.25±2.06*^{,a}	-13.46±3.50*^{,a}	-0.47±2.15^{a}	-12.26±3.14*^{,a}	-10.88+2.49*^{,a}	-7.22±2.13*^{,a}
	estimated	-2.47+0.47	-2.94+0.47	1.39±0.47	-1.35±0.63	-1.82±0.63	2.51±0.63	-4.94±1.57	-5.41±1.57	-1.08±1.57
2,4- dihydroxycinnamic acid	**measured**	-22.11±1.05*^{,e}	-62.30±3.24*^{,e}	-119.44±0.84*^{,d}	-19.46±1.48*^{,c}	-62.02±2.28*^{,e}	-118.62±2.31*^{,e}	-13.60±2.37^{a}	-81.73±2.28*^{,c}	-95.35±2.50*^{,e}
	estimated	-13.31±0.68	-47.31±0.68	-71.31±0.68	-11.75±0.25	-45.75±0.25	-69.75±0.25	-14.45±1.26	-48.45±1.26	-72.45±1.26
Caffeic acid	**measured**	24.01±0.60*^{,d}	24.57±2.73*^{,d}	48.13±5.90*^{,c}	0.46±2.00*^{,d}	46.51±2.78*^{,d}	75.03±5.32*^{,d}	-13.03±2.02*^{,a}	54.45±6.77*^{,b}	48.46±1.82*^{,d}
	estimated	11.00±1.12	19.46±1.12	29.86±1.12	9.30±2.04	17.75±2.04	28.15±2.04	17.49±1.09	25.95±1.09	36.34±1.09
3,5- dihydroxyhydrocinnamic acid	**measured**	-9.05±1.88^{c}	-12.63±0.41*^{,f}	-15.06±1.41*^{,a}	-26.12±1.94*^{,e}	-7.56±0.52*^{,c}	-4.81±1.60*^{,a}	-5.15±1.99*^{,b}	-3.21±1.09^{d}	3.28±0.26*^{,c}
	estimated	-10.38±1.64	-3.83±1.64	-9.16±1.64	-11.07±1.36	-4.52±1.36	-9.85±1.36	-8.94±1.01	-2.39±1.01	-7.72±1.01
Gallic acid	**measured**	1.51±1.39*^{,a}	23.52±3.37*^{,d}	40.39±4.15*^{,e}	-9.69±1.17^{a,b}	15.41±0.48*^{,f}	32.90±3.73*^{,f}	-9.84±2.82*^{,a,b}	13.71±1.86^{e}	40.22±6.09*^{,f}
	estimated	-2.29±1.33	17.43±1.33	27.42±1.33	-8.84±1.83	10.88±1.83	20.87±1.83	-5.63±2.15	14.10±2.15	24.08±2.15
2	**[HCA/ HBA], µM**	**100**								
	[WW- Bound], mg/mL	**10**			**30**			**50**		
o-coumaric acid	**measured**	1486.46 ± 26.74*^{,a}			1848.44 ± 458.48^{a}			2491.48 ± 187.40*^{,a}		
	estimated	616.35 ± 23.91			783.41 ± 126.87			685.96 ± 161.52		
m-coumaric acid	**measured**	1903.93 ± 280.29^{a,b}			2170.55 ± 30.78*^{,a,b}			3272.87 ± 455.09*^{,a,b}		
	estimated	867.63 ± 76.23			990.39 ± 37.84			867.55 ± 125.79		
p-coumaric acid	**measured**	3874.16 ± 293.29*^{,c,i}			4522.22 ± 122.01*^{,c}			5392.31 ± 41.43*^{,c,j}		
	estimated	1674.54 ± 23.91			1841.60 ± 126.87			1744.15 ± 161.52		
2,4-dihydroxycinnamic acid	**measured**	10172.14 + 107.95*^{,d}			10640.82 ± 31.64*^{,d}			11202.99 ± 4.11*^{,d}		
	estimated	7833.51 ± 112.23			7949.97 ± 4.84			7641.88 ± 73.66		
Caffeic acid	**measured**	8648.84 ± 243.21^{e,j}			7799.89 ± 186.32^{e}			9474.40 ± 372.25*^{,e}		
	estimated	7873.06 ± 148.73			7466.20 ± 137.88			6988.39 ± 334.12		
3,5-dihydroxyhydrocinnamic acid	**measured**	2990.29 ± 114.79^{c,f,k,l}			3701.46 ± 49.60*^{,f,k}			4635.61 ± 38.50*^{,b,c,f}		
	estimated	2770.74 ± 43.86			2849.42 ± 59.13			2039.17 ± 62.85		
2,3,4-trihydroxybenzoic acid	**measured**	12328.04 ± 52.36*^{,g}			13824.33 ± 212.88*^{,g}			14102.19 ± 434.00*^{,g}		

	estimated	9423.68 ± 148.73	9016.82 ± 137.88	8539.01 ± 334.11
2,4,5-trihydroxybenzoic acid	**measured**	6708.12 ± 157.12*,h	6627.61 ± 65.44*,h	7667.22 ± 156.30*,h,k
	estimated	9063.02 ± 131.11	9258.38 ± 215.57	9275.71 ± 59.62
2,4,6-trihydroxybenzoic acid	**measured**	4451.86 ± 142.54i	6191.63 ± 11.37*,h	4844.36 ± 72.07*,c,f
	estimated	4052.15 ± 44.69	3949.78 ± 4.94	2633.31 ± 203.17
Gallic acid	**measured**	9437.15 ± 1115.01d,j	16104.00 ± 86.71*,i	16135.70 ± 49.20*,i
	estimated	12163.69 ± 76.23	12286.45 ± 37.84	12163.61 ± 125.79
3,4-dihydroxy-5-methoxybenzoic acid	**measured**	6679.09 ± 170.07*,h	6149.01 ± 56.05h	7439.46 ± 168.14*,h,k
	estimated	5763.71 ± 21.96	5995.26 ± 50.60	5437.95 ± 1.06
2-hydroxy-3-methoxybenzoic acid	**measured**	2072.00 ± 16.40*,a,b,f	2552.88 ± 153.63*,b	2933.58 ± 2.97*,a
	estimated	1506.05 ± 26.68	1510.80 ± 28.09	1018.98 ± 54.45
2-hydroxy-4-methoxybenzoic acid	**measured**	3143.58 ± 24.46*,c,k,l	5441.34 ± 194.19*,j	5216.21 ± 343.71*,c
	estimated	1990.09 ± 26.68	1994.84 ± 28.09	1503.01 ± 56.45
2-hydroxy-5-methoxybenzoic acid	**measured**	2032.23 ± 53.39a,b	4203.53 ± 53.10*,c,k	4615.56 ± 11.78*,b,c,f
	estimated	1085.15 ± 249.49	1331.58 ± 52.05	364.87 ± 96.83
2-hydroxy-6-methoxybenzoic Acid	**measured**	3664.53 ± 102.64*,c,i	6246.10 ± 269.90*,h	5781.10 ± 212.26*,c,j
	estimated	1596.79 ± 249.49	1843.22 ± 52.05	876.50 ± 96.83
Isovanillic acid	**measured**	3302.85 ± 261.27*,c,l	3498.01 ± 354.61*,f	2477.09 ± 208.51*,a
	estimated	732.74 26.95	739.84 ± 43.64	761.54 ± 133.31
Vanillic acid	**measured**	3504.24 ± 200.36*,c,l	4472.11 ± 304.86*,c	6689.91 ± 191.55*,h,j
	estimated	2156.82 ± 99.83	2514.14 ± 212.54	2473.65 ± 264.79
2-hydroxy-3,4-dimethoxybenzoic acid	**measured**	1560.80 ± 20.79*,a	2355.24 ± 307.76*,a,b	3564.97 ± 31.61*,a,b,f
	estimated	262.19 ± 15.39	498.07 ± 14.14	30.32 ± 85.02
2-hydroxy-4,6-dimethoxybenzoic acid	**measured**	2307.92 ± 34.85*,a,b,f,k	2226.13 ± 40.33*,a,b	2753.85 ± 282.43*,a
	estimated	1209.56 ± 106.64	942.65 ± 23.80	953.30 ± 240.40
Syringic acid	**measured**	8304.56 ± 7.98*,e	9981.99 ± 61.23*,l	8594.00 ± 536.35e,k
	estimated	7676.75 ± 99.83	8034.07 ± 212.54	7993.58 ± 264.79
3,4,5-trimethoxybenzoic acid	**measured**	2621.77 ± 262.96*,b,f,k,l	4261.87 ± 92.63*,c,k	5145.00 ± 360.15*,c
	estimated	500.11 ± 109.01	393.35 ± 19.34	220.24 ± 125.05

Different letters indicate the significance of the difference at $p<0.05$ between the measured inhibition % values of WW- bound antioxidant + hydroxycinnamic acid mixtures for each combination, specified in columns. * indicate the significance of the difference at $p<0.05$ for each measured-estimated pair.

Even though the results for individual measurements are not given here, it is important to mention that WW- bound antioxidants acted as pro-oxidants in the liposome medium, which was also revealed in our previous studies [4]. Among HCA/ HBA derivatives studied in liposome medium, p-coumaric acid was found to be a weak antioxidant whereas o-coumaric, m-coumaric and 3,5-dihydroxyhydrocinnamic acids were weak pro-oxidants. Conversely, 2,4-dihydroxycinnamic acid was a strong pro-oxidant, while caffeic and gallic acids were strong antioxidants. In literature, it was reported that the o- and p- substitution with another hydroxyl group increases the stability of the phenoxyl radical, which in turn determines the antioxidant activity of a compound [200]. The importance of the –OH group at third position for a high antioxidant activity was also mentioned [104]. This knowledge together explains the high antioxidant activity of caffeic and gallic acids among others. The evidence reported for the significant contribution of 3`,4` dihydroxyphenyl (catechol) substitution on the B- ring to antioxidant activity, provided by catechin, epicatechin and their various epimers also supports this situation [201].

On the other hand, in aqueous medium WW- bound antioxidants undertook an antioxidant role. o-coumaric, m- coumaric, 2-hydroxy-3-methoxybenzoic, 2-hydroxy-4-methoxybenzoic, 2-hydroxy-5-methoxybenzoic, 2-hydroxy-6-methoxybenzoic, 2-hydroxy-3,4-dimethoxybenzoic and 2-hydroxy-4,6-dimethoxybenzoic acids acted as weak antioxidants at the early stages of the reaction with DPPH radical and turned to be weak pro-oxidants at the later stages. p-coumaric, vanillic, isovanillic and 2,4,6-trihydroxybenzoic acids were weak antioxidants while caffeic, 3,5-dihydroxyhydrocinnamic, 3,4-dihydroxy-5-methoxybenzoic, 2,3,4-trihydroxybenzoic, 2,4,5-trihydroxybenzoic acids were moderate and syringic acid, 2,4-dihydroxycinnamic and gallic acids were strong antioxidants. 3,4,5-trimethoxybenzoic acid itself was pro-oxidant in the aqueous medium. Indeed, our results were in good in agreement with the existing knowledge in literature indicating that the DPPH radical scavenging activity increase depending on the number of active groups substituted in compounds [104]. Also, the common knowledge about the mitigation effect of methylation on both antioxidant and pro-oxidant activities of flavonoids [97] were observed for the methoxylated species in our study, except 3,4-dihydroxy-5-methoxybenzoic and syringic acids. The reason of the relatively higher antioxidant activity of 3,4-dihydroxy-5-methoxybenzoic acid can be attributed to its–OH groups located at the third and fourth positions, which together are known to induce high antioxidant activity [201]. For syringic acid, even though the substitution of third and fifth –OH groups with methoxy groups leads to a decrease in antioxidant activity

compared to its trihydroxy derivative, the presence of two methoxy groups next to the –OH group at fourth position significantly enhances the hydrogen availability [97].

According to the individual antioxidant/ pro- oxidant effects, in liposome medium it can be seen that HCA/ HBA derivatives which exerted strong antioxidant characteristics like gallic and caffeic acids, acted determinedly synergistic with WW- bound antioxidants. 2,4-dihydroxycinnamic acid showing strong pro-oxidant characteristics, acted distinctly antagonistic on the other hand. HCA/ HBA derivatives having weak pro-oxidant or antioxidant characteristics revealed both synergistic and antagonistic interactions with WW- bound antioxidants depending on their concentrations in the mixtures. This situation showed that the antioxidant/pro-oxidant power of a HCA/ HBA had a significant role on the determination and dominance of its synergistic or antagonistic interactions with other types in the liposome medium. On the other hand, in the aqueous medium the pro-oxidant/ antioxidant nature of the HCA/ HBA derivatives had no concern with the synergistic/ antagonistic behavior with WW- bound antioxidants. In fact, 3,4,5-trimethoxybenzoic acid, acting pro-oxidant itself, exerted strongly synergistic behavior and 2,4,5-trihydroxybenzoic acid, as an antioxidant, exerted antagonistic behavior with WW- bound antioxidants enigmatically.

The chemical structure of HCA/ HBA derivatives also gave some insights regarding their synergistic or antagonistic behaviors. For example, in liposome medium, –OH groups linked at the third and fourth positions of the phenol ring together, like as in caffeic and gallic acids, thought to make a significant contribution to the synergistic behavior, probably with a similar mechanism. However, a single –OH group at the mentioned positions, like as in p- and m-coumaric acids, were not enough to create a synergistic effect, even prone to act antagonistic. The synergistic behavior of 3,5-dihydroxyhydrocinnamic acid at high concentration combinations also strengthened the possibility that –OH group linked at third position, may have an important role on the synergy behavior when it is not single. –OH groups at fifth position, which are not single, like as in gallic acid might have a synergistic effect as well. On the other hand, the strong antagonistic effect of 2,4-dihydroxycinnamic acid and the general antagonistic profile of o-coumaric acid suggests that, either single or not, -OH linkage from second position may contribute to the antagonistic behavior. At this point, two different scenarios appear for the behavior of –OH groups bound at the fourth position of the phenol ring. If there is a neighboring –OH group bound at the: (i) second position, or in m- position, it acts antagonistic, (ii) third position, or in o-position it acts synergistic. However, the predominant synergistic attitude in the aqueous medium did not allow any structure-

behavior relationship to be manifested like as in the liposome medium.

PCA was applied to the data collected from liposome and aqueous media studies for the mixtures of WW-bound antioxidants and HCA/ HBA derivatives to better understand the nature of the lipid oxidation and radical scavenging reactions, respectively. For this purpose, the intensity of the fluorescence data obtained from the measurements performed at each 10 min during 180 min and the scavenging % of DPPH data obtained during 120 min via measurements in each 5 min was used for liposome and aqueous medium studies respectively. The first derivative (window size:5 points, polynomial degree:2) and mean centering was applied as pre-processing for the intensity of the fluorescence data, while only mean centering was used for scavenging % of DPPH data. The number of components explaining the highest amount of variance was found to be two for both datasets.

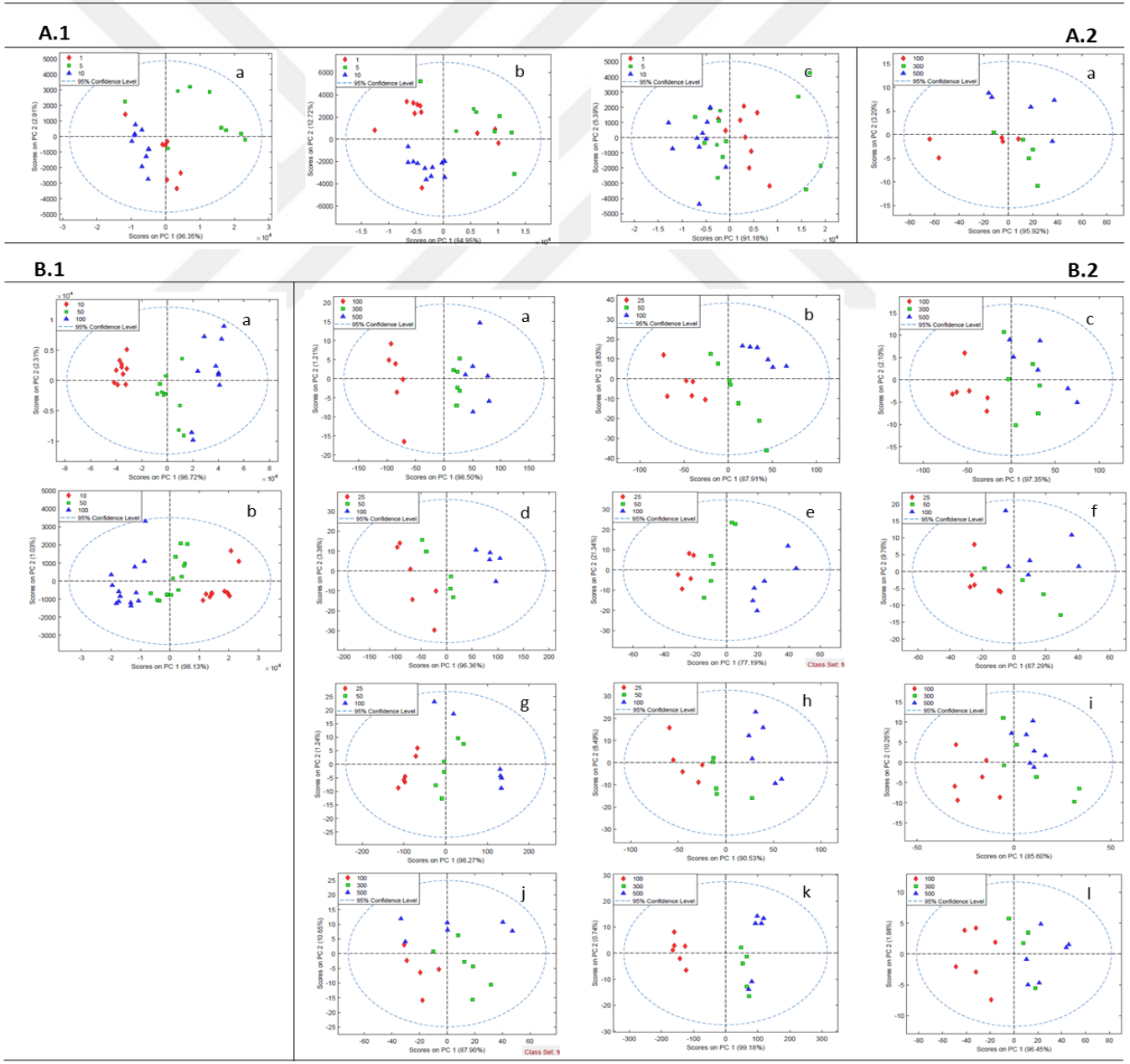
The results are shown as scores (A, B) and loadings (C) plots for liposome (1) and aqueous medium (2) studies for the mixtures of some HCA/HBA derivatives with WW-bound antioxidants, giving the clearest discriminations, in Figure 3.5. According to the scores plots colored by WW-bound antioxidant concentration (A plots), the grouping was yet unclear for both liposome (1) and aqueous medium (2) studies. The discrimination according to HCA/ HBA concentration (B plots) on the other hand was more pronounced in both media with clear boundaries between different concentrations for given examples. In any case, the reason for the ambiguous discriminations was thought to be the concentrations studied being very close to each other.

The scores plot for the mixtures of different HCA/ HBA derivatives with WW- bound antioxidants in aqueous medium gave the same pattern in general (Figure 3.5.B.2). However, in liposome medium, the scores plots for the mixtures of 2,4-dihydroxycinnamic acid (Figure 3.5.B.1.a) and Gallic (Figure 3.5.B.1.b) acid with WW-bound antioxidants was the mirror image of each other. In the loadings plots (C plots), there were two types of systematical behavior for PCs in each media for the mixtures of different HCA/ HBA derivatives with WW- bound antioxidants. In liposome medium, PC-1 always followed an increasing trend within the positive range. However, either by moving from positive to negative scale or vice versa, PC-2 interrupted PC-1 line at between 10-30 min interval, gave a peak around 60-80 min and either moved to the positive or negative scale. o-coumaric, m- coumaric and 3,5-dihydroxycinnamic acids exerted the same type of behavior with p-coumaric acid (Figure 3.5.C.1.a), while Caffeic and Gallic acids exerted the opposite behavior as illustrated for 2,4-dihydroxycinamic acid (Figure 3.5.C.1.b).

In the aqueous medium, PC-1 followed an increasing trend within the positive range similar to that of liposome medium. Meanwhile PC-2 either by moving from positive to negative scale or vice versa interrupted PC-1 line at around 60-100 min interval and either moved to the positive or negative scale. Among HCA/ HBA derivatives Gallic and p-coumaric acids exerted the same behavior with 2,4,5-trihydroxybenzoic acid (Fig. 3.5.C.2.b), while the rest of the HCA/ HBA derivatives exerted the opposite behavior as illustrated for 2-hydroxy-4,6-dimethoxybenzoic acid (Fig. 3.5.C.2.a)

The loadings plots were holding a key role by explaining the kinetic nature of the lipid oxidation and DPPH radical scavenging reactions in this research. For example, in liposome medium, the intersection of PC-1 and PC-2 around 10-30 min and the peak of PC-2 around 60-80 min, thought to coincide with the end of lag phase and the initiation of the uninhibited oxidation phases respectively. The different trends observed for PC-2 in general may add up to a denominational distinction between different HCA/ HBA derivatives, by means of their antioxidant behaviors. On the other hand, the intersection of PC-1 and PC-2 around 60-100 min in aqueous medium was thought to correspond to the depletion of the HCA/ HBA derivatives either by scavenging DPPH radicals or regenerating WW-bound antioxidants. After this point the majority of the radical scavenging activity is thought to be undertaken by WW- bound antioxidants. This phenomenon for the regeneration of DF- bound antioxidants via free soluble antioxidants and the synergistic interaction between them was indeed revealed in our former studies [4, 97, 104, 200-202].

In general, divergent behaviors were observed for the mixtures of different HCA/ HBA derivatives with WW- bound antioxidants, with different groupings between each other. To better understand the effect of amount and positions of the –OH and -OCH_3 groups on the aromatic ring on lipid oxidation and DPPH radical scavenging %, PCA was applied to the pooled datasets. The intensity of the fluorescence data and the scavenging % of DPPH data obtained from the measurements for all HCA/ HBA + WW- bound antioxidants mixtures were collected in itself and used for this purpose. As shown in Figure 3.6.1.a for liposome studies, data for the mixtures of o- and p-coumaric acids were overlapped, located at the center of the plot and diverged from the m-coumaric acid data on the right side. 2,4 and 3,5-dihydroxycinnamic acid data was also located on the right side of the plot, while caffeic and gallic acid data was appeared on the left being apart from each other.


A.1
A.2
B.1
B.2
a
b
c
d
e
f
g
h
i
j
k
l
95% Confidence Level
Scores on PC 1 (96.35%)
Scores on PC 1 (84.95%)
Scores on PC 1 (91.18%)
Scores on PC 1 (95.92%)
Scores on PC 1 (96.72%)
Scores on PC 1 (98.13%)
Scores on PC 1 (98.50%)
Scores on PC 1 (87.91%)
Scores on PC 1 (97.35%)
Scores on PC 1 (96.36%)
Scores on PC 1 (77.19%)
Scores on PC 1 (87.29%)
Scores on PC 1 (98.27%)
Scores on PC 1 (90.53%)
Scores on PC 1 (85.60%)
Scores on PC 1 (87.90%)
Scores on PC 1 (99.18%)
Scores on PC 1 (96.45%)

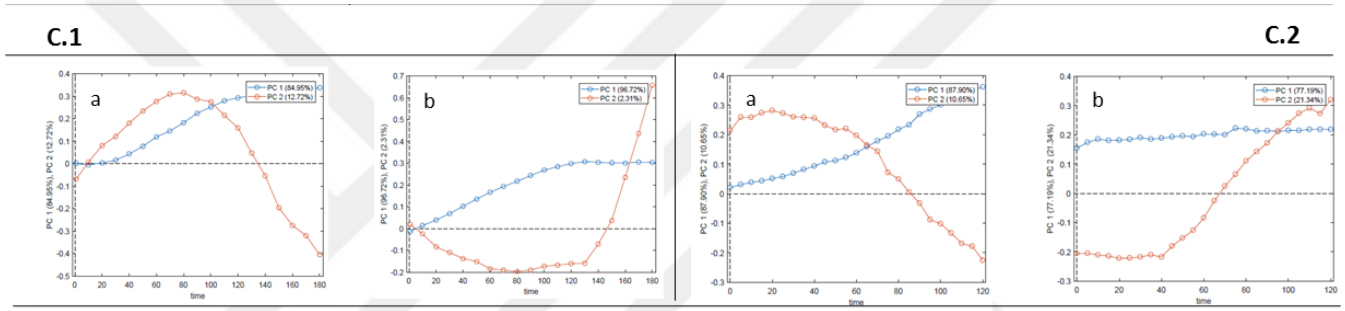


Figure 3.5. Scores plots colored according to A) WW-bound antioxidant and B) HCA/ HBA concentrations and C) loadings plots for the mixtures of some HCA/ HBA derivatives with WW- bound antioxidants obtained from 1) liposome and 2) aqueous medium studies. A.1. a) o-coumaric acid, b) p-coumaric acid, c) 3,5-dihydroxyhydrocinnamic acid; A.2. a) 2-hydroxy-6-methoxybenzoic acid; B.1.a) 2,4-dihydroxycinnamic acid, b) Gallic acid; B.2.a) 2,4-dihydroxycinnamic acid, b) Caffeic acid, c) 3,5-dihydroyhydrocinnamic acid, d) 2,3,4-trihydroxybenzoic acid, e) 2,4,5-trihydroxybenzoic acid, f) 2,4,6-trihydroxybenzoic acid, g) Gallic acid, h) 3,4-dihydroxy-5-methoxybenzoic acid, i) 2-hydroxy-3-methoxybenzoic acid, j) 2-hydroxy-4,6-dimethoxybenzoic acid, k) Syringic acid, l) Isovanillic acid; C.1.a) p-coumaric acid, b) 2,4-dihydroxycinnamic acid; C.2.a) 2-hydroxy-4.6-dimethoxybenzoic acid, b) 2,4,5-trihydroxybenzoic acid.

On the other hand, data obtained from aqueous medium studies (Figure 3.6.2.a) was mostly overlapped on the left part of the plot giving divergent patterns for only 2,4-dihydroxycinnamic, 3,4- dihydroxycinnamic (caffeic), 2,3,4- trihydroxybenzoic, 2,4,5-trihydroxybenzoic, 3,4,5-trihydroxybenzoic (gallic), 4-hydroxy-3,5-dimethoxybenzoic (syringic) and 3,4-dihydroxy-5-methoxybenzoic acids on the right side.

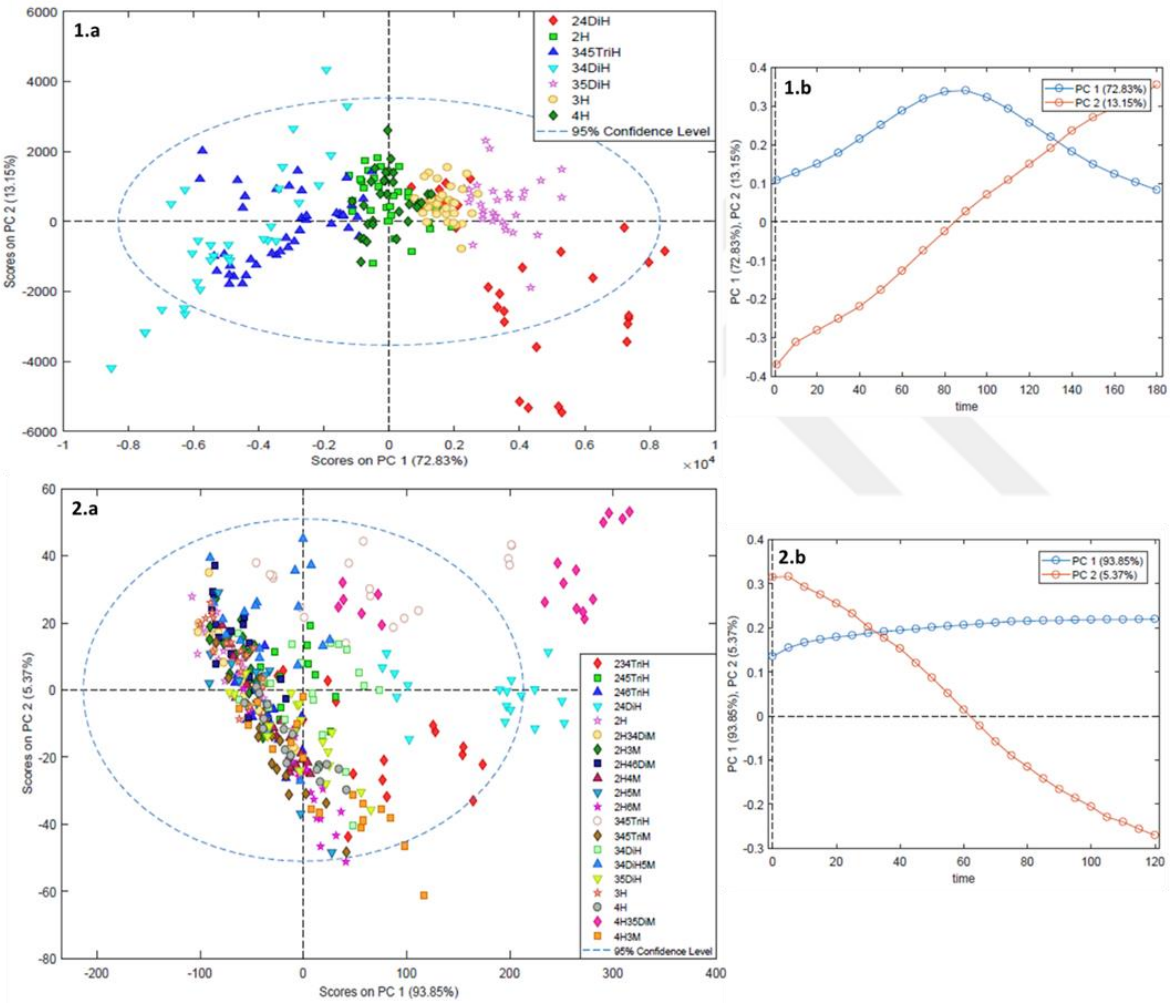


Figure 3.6. Scores plot obtained from the PCA of pooled data of the mixtures of HCA/ HBA derivatives with WW- bound antioxidants, colored by HCA/ HBA type for 1) liposome and 2) aqueous medium studies.

Considering the related loadings plots, PC-1 was found to stay in the positive range for both liposome and aqueous media. Meanwhile, PC-2 cut the PC-1 line at around 130 and 30 min by moving from negative to positive scale and vice versa in liposome and aqueous media, respectively.

In liposome medium, HCA/ HBA derivatives having m- positioned –OH groups like m-coumaric, 2-4 and 3,5-dihydroxycinnamic acids were deemed to have a close relationship with each other by aligning close in the scores plot. HCA/ HBA derivatives with o-positioned –OH groups like gallic and caffeic acids established a similar relationship for themselves as well.

Interestingly, HCA/ HBA derivatives, which appeared as divergent groups in aqueous medium were the ones which also gave the highest AUC values in Table 3.6.2. This situation indicates a correlation between the antioxidant capacity and behavioral characteristics of the mixtures of HCA/ HBA derivatives with WW- bound antioxidants. In other words, it could be said that determined and discrete behaviors necessitates relatively higher antioxidant capacities in aqueous medium for HCA/ HBA + WW- bound antioxidant mixtures. The related loadings plots for the pooled datasets centered general lipid oxidation and DPPH radical scavenging characteristics Accordingly, the end of lag phase and the depletion of free HCA/ HBA derivatives in the radical medium were determined as 130 and 30 min, respectively, as discussed before for the individual loadings plots. Among PCs, PC-1, which explains a significant part of the variation in both media is thought to be related with the use of HCA/ HBA derivatives either by scavenging DPPH radicals or by preventing the oxidation of liposomes. This attitude constitutes a good explanation for the junction points of PCs in both media, which indeed are both related with the depletion of HCA/ HBA derivatives available in the environment. Thus, PC-2, explaining the minor part of the variation, is thought to be related with the use of WW- bound antioxidants, which are also thought to have relatively minor effect on the radical scavenging and inhibition of lipid oxidation reactions compared to HCA/ HBA derivatives.

Apart from that, the significance of the effects of HCA/ HBA and WW- bound antioxidant concentrations on lipid oxidation and scavenging % of DPPH was investigated by performing ASCA. Datasets were treated with the same preprocessing as before and models, including two-way interactions were obtained after 500 permutations.

Table 3.7. The p values within 0.95 confidence interval obtained by using Anova Simultaneous Component Analysis (ASCA) in Matlab for [HCA/ HBA]: HCA/ HBA concentration, [WW]: WW-bound antioxidant concentration, [HCA/ HBA] * [WW]: the 2-way interaction between [HCA/ HBA] and [WW] in 1) liposome and 2) aqueous media.

1	[WW]	[HCA/ HBA]	[HCA]*[WW]
o-coumaric acid	0.0020	0.0020	0.1540
m-coumaric acid	0.0040	0.2240	0.0020
p-coumaric acid	0.0020	0.8060	0.0020
2,4-dihydroxycinnamic acid	0.3060	0.0020	0.0020
Caffeic acid	0.0680	0.0020	0.0020
3,5-dihydroxyhydrocinnamic acid	0.0020	0.0020	0.0020
Gallic acid	0.0020	0.0020	0.0020
2	[WW]	[HCA/ HBA]	[HCA]*[WW]
o-coumaric acid	0.0020	0.0020	0.0140
m-coumaric acid	0.0020	0.0020	0.0320
p-coumaric acid	0.0020	0.0020	0.0140
2,4-dihydroxycinnamic acid	0.0580	0.0020	0.0100
Caffeic acid	0.1920	0.0020	0.0020
3,5-dihydroxyhydrocinnamic acid	0.0020	0.0020	0.0020
2,3,4-trihydroxybenzoic acid	0.0020	0.0020	0.0020
2,4,5-trihydroxybenzoic acid	0.0180	0.0020	0.0020
2,4,6-trihydroxybenzoic acid	0.1580	0.0060	0.0020
Gallic acid	0.5720	0.0020	0.0020
3,4-dihydroxy-5-methoxybenzoic acid	0.0020	0.0020	0.0020
2-hydroxy-3-methoxybenzoic acid	0.3880	0.0020	0.0020
2-hydroxy-4-methoxybenzoic acid	0.0020	0.5060	0.0320
2-hydroxy-5-methoxybenzoic acid	0.2220	0.2540	0.0020
2-hydroxy-6-methoxybenzoic acid	0.0020	0.0020	0.0600
Isovanillic acid	0.0640	0.0020	0.0080
Vanillic acid	0.0020	0.0020	0.0020
2-hydroxy-3,4-dimethoxybenzoic acid	0.0020	0.1120	0.0020
2-hydroxy-4,6-dimethoxybenzoic acid	0.0280	0.0240	0.0020
Syringic acid	0.0060	0.0020	0.0020
3,4,5-trimethoxybenzoic acid	0.0020	0.0120	0.0020

The results revealed that HCA/ HBA concentration made a significant effect on the inhibition % of lipid oxidation values measured, except for the mixtures prepared with m- and p-coumaric acids, having single –OH group at the third and fourth positions of the phenol ring. The concentration of WW- bound antioxidants was also significant for the inhibition % of lipid oxidation, except for the mixtures of caffeic and 2,4-dihydroxycinnamic acids. (Table 3.7.1). In aqueous medium, the

concentration of HCA/ HBA made a significant effect on DPPH radical scavenging % except the mixtures prepared with 2-hydroxy-4-methoxybenzoic, 2-hydroxy-5-methoxybenzoic, 2-hdroxy-3,4-dimethoxybenzoic acids. The concentration of WW- bound antioxidants was also significant for DPPH radical scavenging % except in the mixtures of 2,4-dihydroxycinnamic, caffeic, 2,4,6-trihydroxybenzoic, gallic, 2-hydroxy-3-methoxybenzoic, 2-hydroxy-5-methoxybenzoic, and isovanillic acids (Table 3.7.2). The interaction between WW- bound antioxidants and HCA/ HBA concentrations was significant in both liposome and aqueous media except mixtures prepared with o-coumaric and 2-hydroxy-6-methoxybenzoic acids, respectively.

3.4. Interactions between Coffee/ Bread Crust Melanoidins and Hydroxycinnamic/ Hydroxybenzoic Acids in Aqueous Medium

The AUC values based on the oxidation rate curves obtained for the real mixtures of CM and BCM with HCA/ HBA were calculated and have been compared to the estimated values calculated based on the sum of the AUC values of individual components (Table 3.8). Accordingly, a general synergistic interaction was observed between CM and HCA/ HBA derivatives, except some combinations of 2,4,5- trihydroxybenzoic and Gallic acids, being antagonistic. Likewise, the interactions were generally synergistic with BCM. However, some combinations of BCM with 2,4- dihydroxycinnamic, 2,3,4- trihydroxycinnamic, 2,4,5- trihydroxycinnamic, Gallic and Syringic acids were antagonistic. The measured AUC values, hence the AC exerted increased with the increasing concentrations of CM and BCM as a rule. The AC exerted was changed by changing HCA/ HBA derivatives mixed with CM and BCM. However, there were not certain gradations among different mixtures of either CM or BCM with HCA/ HBA derivatives by means of AC considering their different concentration combinations.

Indeed, both CM and BCM acted as antioxidants themselves, as already known. However, the antioxidant/ pro-oxidant properties of HCA/ HBA derivatives were changed according to their –OH and -OCH_3 contents. For instance, HCA/ HBA derivatives having only one –OH group acted as either weak antioxidants or pro-oxidants. When the number of –OH groups increased to two, the behavior turned to be strongly antioxidant. Three –OH groups boosted the antioxidant behavior even more, just except 2,4,6-trihydroxybenzoic acid acting as a weak antioxidant. Including –OCH_3 groups to the aromatic ring, either decreased the antioxidant strength of the compounds or shifted the behavior to pro- oxidant in general. 3,4,5- trimethoxybenzoic acid was the only strong pro-oxidant through all HCA/ HBA derivatives. Among the compounds with - OCH_3 groups only

Syringic acid exerted strong antioxidant characteristics. The aromatic structure including one –OH group at fourth position and two $–OCH_3$ groups at third and fifth positions, might suggest an encouraging effect on the AC.

HCA/ HBA derivatives, acting against the general synergistic picture with their antagonistic behaviors were 2,4,5- trihydroxybenzoic and Gallic acids for CM studies and 2,4-dihydroxycinnamic, 2,3,4- trihydroxycinnamic, 2,4,5- trihydroxycinnamic, Gallic and Syringic acids for BCM studies. The common property of these antagonistic acting compounds is the amount of –OH group on their aromatic ring either being equal to or more than two. This situation creates a contradiction for the effects of –OH- rich HCA/ HBA derivatives. Although these compounds are strong antioxidants, their antagonist efficacy suggests that they repress the antioxidants of CM and BCM, leading $-OCH_3$ groups to be pronounced as the promoting groups.

PCA was applied to interpret the nature of radical scavenging reactions of the mixtures of CM and BCM with HCA/ HBA derivatives. For this purpose, the percentage inhibition of DPPH radical data, obtained by monitoring the absorbance of DPPH radical in the presence of the mixtures of HCA/ HBA derivatives with CM and BCM for 120 min in each 5 min was used. The pre-processing was applied as mean centering for both CM and BCM data. The number of principal components were 2 and 3 for CM and BCM datasets, respectively. Results are given in Figure 3.7 as scores (A, B) and loadings (C) plots for CM (1) and BCM (2) studies. Only explicit discriminations, which are also representative for other datasets are shown.

According to the scores plots colored by CM concentration, there were two different types of discrimination, both being clear. Among them Figure 3.7.A.1.a, obtained for o- coumaric acid data, showed the same pattern with Caffeic, 3,4- dihydroxy-5-methoxybenzoic, 2,4,6-trihydroxybenzoic, Gallic, 2- hydroxy-4-methoxybenzoic, 2-hydroxy-6-methoxybenzoic, Vanillic and 3,4,5- trimethoxybenzoic acids. Figure 3.7.A.1.b, obtained for m- coumaric acid data, showed the same pattern with the rest of the HCA/ HBA derivatives. However, for the scores plots colored by BCM concentration, there were 6 different types of discrimination patterns. Figure 3.7.A.2.d and e were mirror images of each other, Figure 3.7.A.2.e representing both Caffeic and 2,3,4-trihydroxybenzoic acids. Also Figure 3.7.A.2.h and j were likely to be the mirror images, Figure 3.7.A.2.j representing Syringic acid in addition to Gallic acid. Figure 3.7.A.2.n, obtained for 2-

hydroxy-5-methoxybenzoic acid showed the same pattern with the rest of the HCA/ HBA derivatives except 3,4-dihydroxy-5-methoxybenzoic acid (Figure 3.7.A.2.k).

On the other hand, there were no clear discrimination on the scores plots colored by HCA/ HBA concentration for CM studies as illustrated for p- coumaric acid in Figure 3.7.B.1.c. Also the discrimination was not clear for most of the HCA/ HBA derivatives in BCM studies as illustrated for Isovanillic acid in Figure 3.7.B.2.p. The mixtures of 2,4-dihydroyxcinnamic and Caffeic acid with BCM showed relatively clear discriminations (Figure 3.7.B.2.d and e respectively), Caffeic acid sharing the same pattern with 2,3,4- trihydroxybenzoic, 2,4,5- trihydroxybenzoic, 3,4-dihydroxy-5-methoyxbenzoic, Gallic and Syringic acids.

Table 3.8. Measured and estimated values of the AUC of the oxidation rate curves for the mixtures of 1) CM and 2) BCM with HCA/ HBA

1	HCA/ HBA (μM)	100					
	CM (mg/mL)	0.1		0.5		1.0	
		measured	estimated	measured	estimated	measured	estimated
o-Coumaric Acid		5349.99 ± 97.45*,a	3225.25 ± 41.31	13661.96 ± 248.01*,a	9075.56 ± 158.91	19772.11 ± 150.07*,a,d	9308.02 ± 53.83
m-Coumaric Acid		2512.48 ± 259.57b	2684.93 ± 122.68	14492.06 ± 61.88*,b	9480.34 ± 43.94	18970.01 ± 23.81*,b	9333.08 ± 9.78
p-Coumaric Acid		4601.63 ± 161.61c,d	4424.017 ± 41.31	14873.09 ± 473.25*,b	10274.33 ± 158.91	18543.96 ± 94.80*,c,f	10506.79 ± 53.83
2,4-Dihydroxycinnamic Acid		11676.14 ± 115.09*,e	11002.43 ± 51.59	18462.24 ± 202.97*,c	16604.41 ± 12.01	20034.40 ± 198.44*,d,g,i	16121.13 ± 80.83
3,4-Dihydroxycinnamic (Caffeic) Acid		11489.61 ± 311.74e	10951.76 ± 51.58	19999.37 ± 38.67*,d	16553.75 ± 12.01	20531.49 ± 75.65*,e,j	16070.46 ± 80.83
3,5-Dihydroxycinnamic Acid		3118.52 ± 315.18f	4229.04 ± 53.07	12760.27 ± 232.29*,e	10341.54 ± 87.18	18593.67 ± 82.84*,f	10856.65 ± 9.60
2,3,4-Trihydroxybenzoic Acid		12138.16 ± 63.11*,g	11319.49 ± 28.20	20022.16 ± 83.05*,d	17398.86 ± 41.49	19948.59 ± 96.36*,a,d,g,i	16983.60 ± 28.68
2,4,5-Trihydroxybenzoic Acid		9962.84 ± 113.13*,h	10911.31 ± 19.28	18110.48 ± 93.86*,c	16906.14 ± 71.19	20305.00 ± 238.33*,e,h	17537.82 ± 24.85
2,4,6-Trihydroxybenzoic Acid		6036.51 ± 5.98i	6148.74 ± 41.99	17529.64 ± 10.24*,f	11932.80 ± 83.21	20198.48 ± 153.19*,g,h,i	11386.29 ± 29.19
3,4,5-Trihydroxybenzoic (Gallic) Acid		17657.33 ± 58.44*j	15572.90 ± 41.99	21029.92 ± 396.63g	21356.95 ± 83.21	20710.76 ± 37.28*,j	20810.44 ± 29.19
3,4-Dihydroxy-5-Methoxybenzoic Acid		4521.80 ± 82.48*c,d	3487.94 ± 12.23	16198.06 ± 172.95*,h,k	8312.03 ± 7.21	20345.03 ± 39.09*,e,h	9065.36 ± 98.59
2-Hydroxy-3-Methoxybenzoic Acid		4672.27 ± 230.63*c,d	3538.24 ± 120.97	12547.64 ± 161.14*,e	7238.07 ± 120.97	19453.96 ± 1.22*,k	9275.65 ± 55.17
2-Hydroxy-4-Methoxybenzoic Acid		4876.87 ± 61.36*c,d	4094.78 ± 35.53	17224.52 ± 145.71*,f,i	10747.07 ± 12.13	19781.24 ± 83.28*,a,d	10379.50 ± 93.55
2-Hydroxy-5-Methoxybenzoic Acid		4491.36 ± 128.53c,k	4069.31 ± 120.97	15650.04 ± 225.61*,h,j	7769.14 ± 120.97	14205.29 ± 25.43*,l	9806.72 ± 55.17
2-Hydroxy-6-Methoxybenzoic Acid		3931.17 ± 62.99*l	3486.57 ± 37.02	17686.66 ± 144.51*,f	7264.43 ± 129.14	19356.65 ± 44.23*,k	9268.46 ± 11.01
3-Hydroxy-4-Methoxybenzoic (Isovanillic) Acid		4809.46 ± 64.49c,d	4688.88 ± 64.06	15556.67 ± 4.09*,j	10973.65 ± 29.01	19857.97 ± 97.87*,a,d	10600.95 ± 21.41
4-Hydroxy-3-Methoxybenzoic (Vanillic) Acid		4956.21 ± 350.64*,a,d	4038.42 ± 11.02	15892.23 ± 372.39*,h,j	10270.15 ± 29.01	20253.75 ± 25.40*,h	9897.44 ± 21.41
2-Hydroxy-4,6-Dimethoxybenzoic Acid		2290.96 ± 84.38b	2932.77 ± 162.66	10874.57 ± 206.40*l	9162.06 ± 16.97	18335.67 ± 28.50*,c	9653.37 ± 28.06
4-Hydroxy-3,5-Dimethoxybenzoic (Syringic) Acid		11458.56 ± 47.91e	11445.70 ± 28.20	19207.18 ± 141.03*m	17525.08 ± 41.49	19744.54 ± 39.58*,a	17109.82 ± 28.68
3,4,5-Trimethoxybenzoic Acid		3876.12 ± 225.42l	3488.70 ± 12.23	13953.60 ± 305.75*,a	8312.78 ± 7.21	19900.54 ± 62.33*,a,d	9066.11 ± 98.59
2	HCA/ HBA (μM)	100					
	BCM (mg/mL)	10		50		100	
		measured	estimated	measured	estimated	measured	estimated
o-Coumaric Acid		3142.36 ± 39.95*,a,m,n	867.14 ± 147.78	2945.74 ± 203.21a	2023.51 ± 73.17	5364.04 ± 242.02*,a	3102.02 ± 183.81
m-Coumaric Acid		2908.57 ± 48.97*,a,b,m	1817.06 ± 34.56	5711.32 ± 58.52*,b	3027.33 ± 73.47	6131.19 ±24.32*,b	3407.54 ± 90.72
p-Coumaric Acid		1843.68 ± 145.33c	2169.10 ± 147.78	3653.36 ± 1.46*,c	3325.47 ± 73.17	4908.29 ± 130.21a	4403.99 ± 183.81

2,4-Dihydroxycinnamic Acid	7648.73 ± 310.45[d]	7246.20 ± 5.08	7166.69 ± 121.91*[,d]	8991.73 ± 9.99	6669.86 ± 19.34*[,b,h]	8712.32 ± 109.97
3,4-Dihydroxycinnamic (Caffeic) Acid	9012.89 ± 26.67*[,e,g]	6803.55 ± 5.08	10561.03 ± 109.82*[,e]	8549.08 ± 9.99	9297.31 ± 38.58*[,c]	8269.67 ± 109.97
3,5-Dihydroxycinnamic Acid	3966.69 ± 123.19[f]	4313.69 ± 0.57	6623.61 ± 16.37[f]	6676.59 ± 103.71	7425.37 ± 130.29*[,d,f,g]	6280.17 ± 192.64
2,3,4-Trihydroxybenzoic Acid	9295.85 ± 149.44*[,g]	7725.32 ± 122.48	8263.75 ± 81.69*[,g]	10343.63 ± 256.41	10113.88 ± 19.07*[,e]	11391.80 ± 21.39
2,4,5-Trihydroxybenzoic Acid	8826.94 ± 126.43*[,e,h]	11521.52 ± 9.27	8642.05 ± 168.19*[,g]	13598.54 ± 35.28	8839.88 ± 47.97*[,c]	14273.78 ± 23.60
2,4,6-Trihydroxybenzoic Acid	4126.02 ± 85.81*[,f]	3344.57 ± 62.40	4511.12 ± 161.65[h,l]	4155.54 ± 15.68	7396.64 ± 62.41*[,d,f,g]	3516.58 ± 100.22
3,4,5-Trihydroxybenzoic (Gallic) Acid	17956.30 ± 46.83*[,i]	13420.54 ± 38.08	12787.64 ± 92.61*[,i]	15775.60 ± 39.64	7573.14 ± 690.15*[,f,g]	15041.00 ± 107.36
3,4-Dihydroxy-5-Methoxybenzoic Acid	7197.30 ± 60.26*[,j]	6036.01 ± 62.40	8290.71 ± 60.20*[,g]	6846.98 ± 15.68	8100.84 ± 310.70*[,g]	6208.02 ± 100.22
2-Hydroxy-3-Methoxybenzoic Acid	2625.04 ± 242.44[b,k,o]	2037.70 ± 69.25	5062.13 ± 641.42*[,j]	3560.15 ± 17.63	7708.36 ± 118.95*[,f,g]	2270.09 ± 24.80
2-Hydroxy-4-Methoxybenzoic Acid	2504.86 ± 81.10*[,k,l,o]	2045.04 ± 69.25	6058.23 ± 43.42*[,b,k]	3567.49 ± 17.63	6821.99 ± 146.96*[,d,h]	2277.43 ± 24.80
2-Hydroxy-5-Methoxybenzoic Acid	2343.55 ± 136.60*[,k,l]	1441.63 ± 4.48	4199.33 ± 123.35[h]	4119.01 ± 45.80	7642.44 ± 135.76*[,f,g]	4630.07 ± 100.57
2-Hydroxy-6-Methoxybenzoic Acid	2997.47 ± 89.51*[,a,b,m,n]	2134.46 ± 21.20	6114.47 ± 31.19*[,b,k]	3701.39 ± 37.03	7716.05 ± 20.05*[,f,g]	1795.08 ± 83.16
3-Hydroxy-4-Methoxybenzoic (Isovanillic) Acid	3197.54 ± 186.40[m,n]	2474.90 ± 21.20	6047.19 ± 38.67*[,b,k]	4041.83 ± 37.03	7179.22 ± 65.80*[,d,f,h]	2135.52 ± 83.16
4-Hydroxy-3-Methoxybenzoic (Vanillic) Acid	3312.82 ± 9.23*[,n]	3047.12 ± 15.21	6213.23 ± 100.19*[,f,k]	4872.16 ± 21.75	7415.30 ± 98.65*[,d,f,g]	3087.37 ± 373.65
2-Hydroxy-4,6-Dimethoxybenzoic Acid	2223.21 ± 28.62[l]	2344.06 ± 19.55	5033.85 ± 19.22*[,j]	4083.02 ± 120.58	7734.77 ± 62.19*[,f,g]	3358.53 ± 183.99
4-Hydroxy-3,5-Dimethoxybenzoic (Syringic) Acid	8467.80 ± 54.71*[,h]	7722.07 ± 122.48	8609.95 ± 17.64*[,g]	10340.38 ± 256.41	9269.24 ± 456.34*[,c]	11388.55 ± 21.39
3,4,5-Trimethoxybenzoix Acid	2780.09 ± 228.52*[,a,b,o]	1352.81 ± 19.55	4828.92 ± 35.98*[,j,l]	3091.76 ± 120.58	7819.75 ± 82.11*[,f,g]	2367.27 ± 183.99

Different letters indicate the statistical significance of difference between samples for each concentration combination illustrated in columns.
*shows the statistical significance of difference between each estimated-measured pair

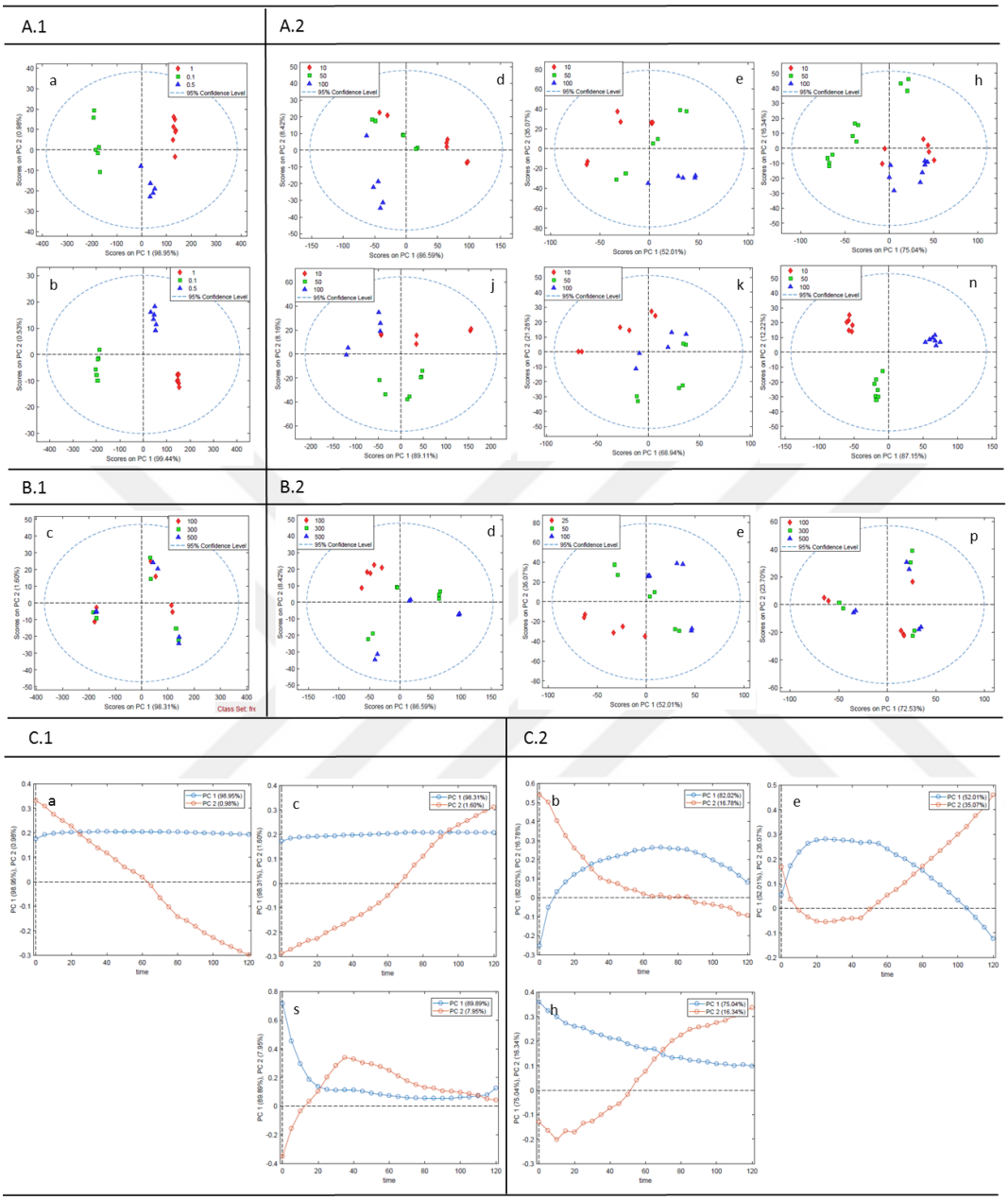


Figure 3.7. Scores plots colored according to A) melanoidins and B) HCA/ HBA concentrations and C) loadings plots for the mixtures of 1) CM and 2) BCM with some HCA/ HBA derivatives. A.1. a) o- coumaric acid, b) p- coumaric acid; A.2. d) 2,4- dihydroxycinnamic acid, e) Caffeic acid, h) 2,4,5- trihydroxybenzoic acid, j) Gallic acid, k) 3,4- dihydroxy-5-methoxybenzoic acid, n) 2-hydroxy-5-methoxybenzoic acid; B.1. c) p- coumaric acid; B.2. d) 2,4- dihydroxycinnamic acid, e) Caffeic acid, p) Isovanillic acid; C.1. a) o- coumaric acid, c) p- coumaric acid, s) Syringic acid; C.2. b) m- coumaric acid, e) Caffeic acid, h) 2,4,5- trihydroxybenzoic acid.

Considering loadings plots obtained for CM studies, there were three different types of behavior as illustrated in Figure 3.7.C.1.a, c and s, for o- coumaric, p- coumaric and Syringic acids, respectively. In Figure 3.7.C.1.a and c, the trend of PC1 was the same staying on the positive range of PCs. PC-2, moved either from positive to negative scale or vice versa by cutting PC-1 line at around 20-50 or 50-100 mins, for o- and p- coumaric acids, respectively. However, in Figure 3.7.C.1.s, while PC-1 was staying on positive scale, PC-2 cut it two times from around 20-30 and 110-120 minutes. For the loadings plots obtained in BCM studies, there were 3 different patterns as well, as illustrated in Figure 3.7.C.2.b, e and h, for m-coumaric, Caffeic and 2,4,5-trihydroxybenzoic acids. In Figure 3.7.C.2.b and e PC-1 created a slight curve by moving between positive and negative scales, while it stayed only in the positive scale in Figure 3.7.C.2.h. PC-2 cut the PC-1 line at around 10-30 min or 70-110 min for m-coumaric and 2,4,5- trihydroxybenzoic acids respectively, either by decreasing or increasing. However, PC-2 line cut the PC-1 line twice, first by decreasing at around 10-20 min and then by increasing at around 80-110 min for Caffeic acid.

In general, evaluation of the scores plots revealed a number of groupings between different HCA/ HBA derivatives, indicating some behavioral associations among them. Discriminations according to CM and BCM concentrations were notably clear when compared with the discriminations according to HCA/ HBA concentrations, which were rather ambiguous. Nevertheless, there were some HCA/ HBA derivatives which gave relatively clear discriminations according to HCA/ HBA concentration. These compounds were all known to exert strong antioxidant characteristics themselves, except 3,4- dihydroxy-5-methoxybenzoic acid. This situation uncovered the linkage between the strength of antioxidant power and the significance of the effect of concentration on AC once more.

On the other hand, the different patterns seen in the loadings plots are thought to give some clues about the progression of the radical scavenging reactions for different HCA/ HBA and CM or BCM combinations. Considering Figures 3.7.C.1.a and 3.7.C.1.c, the loadings plots obtained from CM studies, the intersection of PC-1 and PC-2 at around 20- 50 or 50- 100 min is thought to correspond to the depletion of HCA/ HBA derivatives in the medium. This could be happened either by scavenging the DPPH stable radical or by regenerating the depleted antioxidants of melanoidins. After intersections, the majority of the antioxidant effect is thought to be exerted by the melanoidins. The same explanation will also be valid for Figures 3.7.C.2.b and 3.7.C.2.h,

obtained from BCM studies, which have the intersections at around 10-30 and 10-110 min respectively. At this point, two different timing by means of depletion of HCA/ HBA derivatives draws attention, pointing the early and late stages of the radical scavenging reactions.
Besides, there was a third pattern seen on the loadings plots, which includes two different intersection points for PC-1 and PC-2 as illustrated in Figure 3.7.C.1.s and 3.7.C.2.e, obtained from CM and BCM studies. In these plots, the first intersection point which is around 20-30 or 10-20 mins is thought to correspond to the depletion of HCA/ HBA derivatives which are easily accessible considering their relatively small structure. After that point, the majority of the antioxidant activity is thought to be carried out by melanoidins. When the second intersection point is reached, the antioxidant activity is thought to be exerted by the regenerated melanoidins.

The significance of the effects of CM, BCM and HCA/ HBA concentrations and their 2- way interactions on the percentage of scavenging of DPPH radical was investigated with ANOVA-Simulatenos Component Analysis (ASCA). The preprocessing was used as mean centering as used before for the PCA and the number of permutations were 500. The p values obtained within 0.95 confidence interval were illustrated in Table 3.9. The CM and BCM concentrations had a significant effect on the percentage of scavenging of DPPH radical measured for all HCA/ HBA derivatives. The concentration of HCA/ HBA derivatives were significant except o-, m-, p-coumaric, 2- hydroxy- 4- methoxybenzoic and 2- hydroxy- 5- methoxybenzoic acids when combined with CM. For the combinations with BCM, the effectsf of HCA/ HBA derivatives were also significant except 2,4,6- trihydroxybenzoic, 2- hydroxy- 3- methoxybenzoic, 2- hydroxy- 4- methoxybezoic and 2- hydroxy- 5- methoxybenzoic acids. The 2- way interactions between either CM or BCM concentrations and HCA/ HBA concentrations were significant in general, except the combinations of 2- hydroxy- 4- methoybenzoic acid with BCM.

Table 3.9. The p values within 0.95 confidence interval obtained by using Anova Simultaneous Component Analysis (ASCA) in Matlab for [HCA/ HBA]: HCA/ HBA concentration, [CM]: CM concentration, [BCM]: BCM concentration; [CM]*[HCA/ HBA], [BCM]*[HCA/ HBA]: the 2-way interaction between [HCA/ HBA] and [CM] or [BCM], respectively.

	[CM]	[HCA/ HBA]	[CM]* [HCA/ HBA]	[BCM]	[HCA/ HBA]	[BCM]* [HCA/ HBA]
o-Coumaric Acid	0.0020	0.5020	0.0020	0.0020	0.0020	0.0020
m-Coumaric Acid	0.0020	0.9660	0.0340	0.0020	0.0020	0.0020
p-Coumaric Acid	0.0020	0.0820	0.0440	0.0020	0.0020	0.0020
2,4-Dihydroxycinnamic Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
3,4-Dihydroxycinnamic (Caffeic) Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
3,5-Dihydroxycinnamic Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
2,3,4-Trihydroxybenzoic Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
2,4,5-Trihydroxybenzoic Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
2,4,6-Trihydroxybenzoic Acid	0.0020	0.0160	0.0020	0.0020	0.0680	0.0020
3,4,5-Trihydroxybenzoic (Gallic) Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
3,4-Dihydroxy-5-Methoxybenzoic Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
2-Hydroxy-3-Methoxybenzoic Acid	0.0020	0.0040	0.0020	0.0020	0.3600	0.0020
2-Hydroxy-4-Methoxybenzoic Acid	0.0020	0.0020	0.0020	0.0020	0.1160	0.0320
2-Hydroxy-5-Methoxybenzoic Acid	0.0020	0.0960	0.0020	0.0020	0.0600	0.0200
2-Hydroxy-6-Methoxybenzoic Acid	0.0020	0.2400	0.0020	0.0020	0.0380	0.0020
3-Hydroxy-4-Methoxybenzoic (Isovanillic) Acid	0.0020	0.0020	0.0020	0.0020	0.0060	0.0020
4-Hydroxy-3-Methoxybenzoic (Vanillic) Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
2-Hydroxy-4,6-Dimethoxybenzoic Acid	0.0020	0.0020	0.0020	0.0020	0.0400	0.0680
4-Hydroxy-3,5-Dimethoxybenzoic (Syringic) Acid	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020
3,4,5-Trimethoxybenzoic Acid	0.0020	0.0020	0.0020	0.0020	0.0280	0.0020

The common general feature of HCA/ HBA derivatives which play a role in the insignificance of the effect of concentration is containing only one –OH group and sometimes an additional -OCH_3 group on their aromatic ring, except 2,4,6- trihydroxybenzoic acid. However, it should be remembered that all the HCA/ HBA derivatives included in this situation exerted weak antioxidant/ pro- oxidant behavior themselves. Hence, it can be deduced that the strength of the antioxidant/ pro- oxidant effect of a chemical compound may also influence the significance of its effect of concentration on the AC. In any case, the concentration dependency of the AC was proven once more with the rest of the HCA/ HBA derivatives, CM and BCM.

PCA was also applied to the pooled datasets obtained from CM and BCM studies, by using same PCA models as applied before for each dataset. The scores plots obtained were colored by the type of HCA/ HBA derivatives and shown in Figure 3.8.1 and 4.8.2 for CM and BCM studies, respectively. Accordingly, no clear distinction was seen between the data points of different HCA/ HBA derivatives, all being overlapped, for the combinations with both CM and BCM.

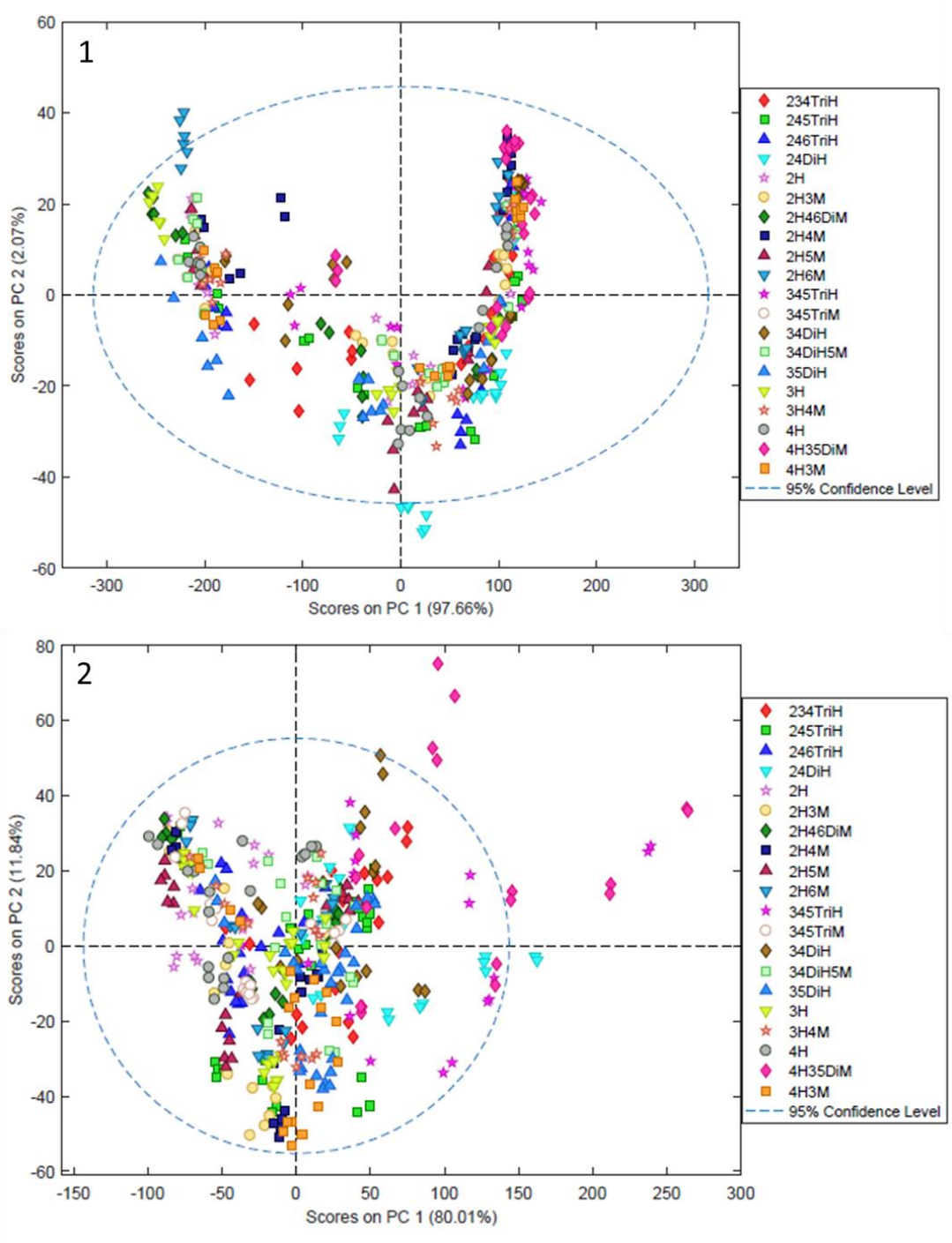


Figure 3.8. Scores plot obtained from the PCA of pooled data of the mixtures of HCA/ HBA derivatives with 1) CM and 2) BCM colored by HCA/ HBA type.

3.5. Interactions between the Insoluble Fractions of Different Coffee infusions & Major Cocoa Free Antioxidants and Different Coffee infusions & Dark Chocolate

Interactions between the insoluble fractions of different types of coffee infusions and major free antioxidants of cocoa as well as different types of coffee infusions and chocolate were investigated in this study. Ground roasted coffee was brewed using different methods to obtain a variety of coffee infusions. Amounts of coffee infusions (ml), total dry weight of coffee infusions (g) and the insoluble part of coffee infusions (mg) per gram of ground roasted coffee and per serving are given in Table 3.10.

Table 3.10. Amounts of coffee infusion (mL), total dry weight of coffee infusion (g) and the insoluble fraction of coffee infusion/coffee (mg) per gram of ground roasted coffee and per serving size.

	Per 1 g of Coffee			Per serving size		
Infusion type	**Coffee Infusion (mL)**	**Total Dry Weight (g)**	**Insoluble Fraction (mg)**	**Coffee Infusion (mL)**	**Total Dry Weight (g)**	**Insoluble Fraction (mg)**
Espresso	4.02 ± 0.003^{a}	0.20 ± 0.001^{a}	1.07 ± 0.002^{a}	24.13 ± 0.003^{a}	1.20 ± 0.001^{a}	1.07 ± 0.002^{a}
Moka coffee	15.83 ± 0.003^{b}	0.18 ± 0.001^{b}	9.90 ± 0.002^{b}	122.36 ± 0.003^{b}	4.55 ± 0.001^{b}	9.37 ± 0.002^{b}
French press	14.89 ± 0.003^{c}	0.25 ± 0.001^{c}	8.76 ± 0.002^{c}	148.97 ± 0.003^{c}	2.54 ± 0.001^{c}	14.15 ± 0.002^{c}
Filtered coffee	8.15 ± 0.003^{d}	0.30 ± 0.001^{d}	5.86 ± 0.002^{d}	158.35 ± 0.003^{d}	1.82 ± 0.001^{d}	33.52 ± 0.002^{d}
Turkish coffee	8.97 ± 0.003^{e}	0.26 ± 0.001^{e}	9.98 ± 0.002^{e}	44.88 ± 0.003^{e}	1.28 ± 0.001^{e}	15.64 ± 0.002^{e}
Coffee	-	-	448.60 ± 0.002^{f}	-	-	-

Different letters indicate the statistical significance of difference between samples for each measurement illustrated in columns.

The volume of infusions obtained per gram of ground roasted coffee was highest in moka and French press coffee, followed by Turkish coffee, filtered coffee and *espresso*. However, the total amount of dry matter transferred from one gram of coffee to the infusion was higher for filtered coffee, Turkish coffee and French press. After removal of water, alcohol and lipid soluble fractions, the insoluble fraction that remained was found to be highest in Turkish coffee followed by moka and French press, while *espresso* was found to contain the lowest amount of insoluble fraction. The amount of insoluble fraction was considerably higher (45–420 times) in one gram of ground roasted coffee than in coffee infusions obtained from one gram of coffee. Considering one serving size, the coffee infusion volumes obtained followed the following decreasing order: filtered coffee, French press, moka, Turkish coffee, and *espresso*. The total amount of dry matter in freeze-dried infusions was relatively higher for moka, French press and filtered coffee compared

to *espresso* and Turkish coffee. The insoluble fraction that remained after specific washing procedure was higher in filtered coffee, Turkish coffee and French press followed by moka and *espresso*.

Consequently, it was revealed that the amount of dry matter which passed through the final infusions brewed with different methods using the same coffee and the insoluble fraction remained in those dry matter after the washing step were different. Considering one gram of ground roasted coffee, the amount of insoluble fraction present was relatively higher in the coffee infusions whose preparation took longer times. In this context, Turkish coffee was the first one followed by moka, French press and filtered coffee. *Espresso*, which took only few seconds to prepare had the least amount of insoluble fraction. The contact time of water with coffee is thought to be the main reason for this.

However, when serving size is taken into account, following is the decreasing order of the different types of coffee infusions in terms of the amount of their insoluble fractions: filtered coffee, Turkish coffee, French press, moka and *espresso*. In addition to contact time, the coffee: water ratio in the coffee infusions is also considered to have an effect. The amounts of insoluble fractions present in the infusions were relatively higher in those whose coffee: water ratio was lower in general. A probable reason for this could be the enhancement of the extraction process due to increase in the contact surface between coffee and water. Similarly, a short contact time and the compressed coffee cake which inhibits water from reaching coffee grounds deeply might be the reason for *espresso* having less insoluble fraction.

Nevertheless, Turkish coffee contained higher amount of insoluble fraction despite its high coffee: water ratio. This could be an effect of the preparation technique. As is known, the content of bioactive compounds and AC of coffee infusions is primarily affected by the brewing procedure [203]. However, there are conflicting reports about the effect of different preparation techniques. For example, Niseteo, Komes, Belščak-Cvitanović, Horžić & Budeč (2012) [204] determined the total phenolic content of different coffee infusions and found it to be in the following decreasing order: instant coffee, Turkish coffee, *espresso*, filtered coffee. In contrast, Sánchez-González et al. (2005) [203] revealed that the total phenolic content of filtered coffee was the highest followed by *espresso*. Sacchetti, Di Mattia, Pittia & Mastrocola (2009) [205] have reported that the MRPs formed during roasting, which can be counted as bound- antioxidants found in the insoluble

fraction, do not contribute significantly to the AC of coffee infusions. Hence, reaching a definite judgement based on existing knowledge on this subject, beyond speculations is still difficult.

The AC of coffee and different types of coffee infusions in terms of Trolox equivalents, for their total dry weight and insoluble fractions are given in Table 3.11. In general, a deep difference was obvious between the AC values measured for the total dry weight and insoluble fractions, the total contents exerting at around 10- 30 folds of insoluble fractions. Among different types of coffee infusions, the insoluble fractions of Turkish coffee and French press were found to exhibit the highest AC, while those of moka exhibited the weakest antioxidant properties. *Espresso* and moka showed the highest AC on the total dry weight basis. In addition, the AC measured for the total dry weight of ground roasted coffee was much lower than the AC of different types of coffee infusions.

The fact that AC of the total contents being much higher than the insoluble fractions indicates that the AC of the different types of coffee infusions was mainly provided by their free soluble antioxidants rather than the bound ones found in the insoluble fraction. Especially moka, whose insoluble fraction exerted the lowest AC while its total dry content was exerting one of the highest ones constituted a proof for this phenomenon. On the other hand, the fact that the AC of coffee being much lower than that of the different types of coffee infusions showed that the brewing process constituted an enriching role on the AC. The main reason for this is thought to be the heat assisted extraction process applied during different brewing procedures.

Table 3.11. Trolox equivalent AC (mmol Trolox/ kg sample) measured for the total dry weight and insoluble fractions of different types of coffee infusions and coffee.

	Trolox Equivalent AC (mmol Trolox/kg sample)	
	Total dry weight	**Insoluble fraction**
Espresso	$1095 \pm 42.1^{*,a}$	78 ± 7.9^{a}
Filtered coffee	$1067 \pm 5.2^{*,a}$	$86 \pm 8.7^{a,b}$
French press	1076 ± 21.0^{a}	103 ± 7.6^{b}
Moka coffee	$1184 \pm 11.3^{*,b}$	45 ± 0.0^{c}
Turkish coffee	$953 \pm 2.6^{*,c}$	105 ± 1.3^{b}
Coffee	$581 \pm 5.2^{*,d}$	68 ± 1.6^{a}

Different letters indicate the statistical significance of difference at $p < 0.0.5$ between Trolox Equivalent AC values measured for the total dry weight and the insoluble fractions of different types of coffee infusions/ coffee.

A comparison of the percentage inhibition of DPPH radical (measured and estimated values) of the mixtures of (a) insoluble fraction of coffee infusions and catechin/epicatechin (Table 3.12) and (b) coffee infusions and chocolate (Table 3.13) was done so as to understand the type of interactions occurring between these components. Accordingly, a synergistic interaction was evident between catechin and the insoluble fraction of espresso, whereas both antagonistic and additive interactions were observed for the mixtures of catechin with the rest of the insoluble fractions obtained from different types of coffee infusions (Table 3.12.a). A similar synergistic action was observed for epicatechin and the insoluble fraction of espresso while the reaction was predominantly antagonistic with the insoluble fractions obtained from other coffee infusions (Table 3.12.b). On the other hand, when the experiments were performed with coffee infusions and chocolate instead of the insoluble fraction of coffee infusions and free antioxidants of cocoa, the status of the interactions changed dramatically. French press and Turkish coffee acted synergistic with chocolate for almost all concentration combinations, whereas espresso and filtered coffee were additive in general. However, moka exerted both additive and antagonistic behaviors with chocolate, depending on the concentration combinations (Table 3.13).

In this regard, the synergistic interactions between *espresso* and catechin/epicatechin indicates the possibility that the regeneration reaction has taken place between bound antioxidants of *espresso* and catechin/epicatechin. However, the amount of insoluble fraction and in turn the amount of bound antioxidants contained in the infusions prepared with 1 g of coffee was the least in *espresso*. Under these conditions, it would be surprising that the synergistic interactions only occur in conjunction with regeneration reactions. On the other hand, either the free soluble antioxidants contained in Turkish coffee and French press or the other antioxidant species and components found in chocolate are thought to be the responsible for this synergistic behavior. Contrarily, the free soluble antioxidants found in *espresso* or the other constituents in chocolate rather than catechin/epicatechin are thought to be the reason of the additive behavior.

Table 3.12. Measured and estimated values of inhibition % of DPPH radical in the presence of insoluble fractions of coffee infusions and (a) catechin, (b) epicatechin mixtures.

(a)

Insoluble fractions of coffee infusions (mg)	Catechin (nmol)		Espresso	Filtered Coffee	French Press	Moka Coffee	Turkish Coffee
1.0	30	measured	25.29±1.46^{a}	41.03±0.32^{b}	43.77±0.64^{c}	38.27±0.00*^{,d}	46.65±0.57*^{,e}
		estimated	22.09±1.13	46.13±1.80	50.26±2.19	35.92±0.33	50.77±0.65
	45	measured	30.83±0.76^{a}	49.05±1.46^{b}	50.25±0.25^{b,c}	45.39±0.28^{d}	51.84±0.32*^{,c}
		estimated	26.50±1.13	54.94±1.80	59.07±2.19	44.73±0.33	59.58±0.65
	60	measured	34.59±0.06^{a}	53.82±0.25*^{,b}	56.11±0.89*^{,b}	54.05±0.14^{b}	60.32±1.33*^{,c}
		estimated	30.77±1.13	63.49±1.80	67.61±2.19	53.27±0.33	68.13±0.65
1.5	30	measured	33.21±0.38*^{,a}	47.77±2.10^{b}	51.46±0.70^{c}	40.37±0.48^{d}	61.17±0.00^{e}
		estimated	24.55±0.32	55.67±1.03	56.64±1.35	39.86±0.00	61.66±1.48
	45	measured	33.59±0.00*^{,a}	54.33±0.51*^{,b}	56.04±0.32*^{,c}	46.38±0.48*^{,d}	59.84±1.19*^{,e}
		estimated	28.95±0.32	64.48±1.03	65.44±1.35	48.67±0.00	70.47±1.48
	60	measured	39.21±0.26*^{,a}	58.72±1.59*^{,b}	61.77±1.21*^{,b,d}	51.64±0.68*^{,c}	63.32±0.00*^{,d}
		estimated	33.23±0.32	73.03±1.03	73.99±1.35	57.22±0.00	79.02±1.48
2.0	30	measured	31.56±0.70^{a}	55.47±0.38*^{,b}	57.06±0.06*^{,b}	43.22±0.75^{c}	59.78±1.46*^{,d}
		estimated	29.94±0.39	64.43±0.39	68.75±0.45	43.28±0.97	70.75±0.26
	45	measured	37.00±0.26*^{,a}	59.29±0.89*^{,b}	62.21±0.00*^{,c}	50.00±0.27^{d}	67.15±1.19*^{,e}
		estimated	34.35±0.39	73.24±0.39	77.56±0.45	52.09±0.97	79.56±0.26
	60	measured	41.55±0.19*^{,a}	65.46±1.46*^{,b}	65.33±0.83*^{,b}	54.79±0.82*^{,c}	72.43±0.00*^{,d}
		estimated	38.62±0.39	81.79±0.39	86.11±0.45	60.64±0.97	88.10±0.26

(b)

Insoluble fractions of coffee infusions (mg)	Epicatechin (nmol)		Espresso	Filtered Coffee	French Press	Moka Coffee	Turkish Coffee
1.0	30	measured	28.43±0.00*^{,a}	48.51±0.81^{b}	46.09±0.40*^{,c}	36.23±0.32*^{,d}	50.20±0.27*^{,e}
		estimated	24.37±0.94	50.68±2.19	54.80±1.81	40.46±0.06	55.32±0.26
	45	measured	31.85±0.63^{a}	56.33±0.31^{b}	54.37±0.60*^{,c}	45.88±0.32*^{,d}	57.57±0.86*^{,c}
		estimated	29.80±1.19	61.56±1.69	65.68±2.30	51.34±0.43	66.20±0.75
	60	measured	36.99±0.70^{a}	62.42±0.31^{b}	60.86±1.13*^{,b}	53.62±0.06*^{,c}	62.15±0.40*^{,b}
		estimated	34.04±0.66	70.02±2.74	74.15±1.25	59.81±0.62	74.66±0.30
1.5	30	measured	36.42±1.02*^{,a}	56.89±0.37*^{,b}	55.78±1.73^{b}	44.97±0.40^{c}	58.27±1.20^{b}
		estimated	26.82±0.22	60.22±0.65	61.18±0.97	44.40±0.38	66.21±1.87
	45	measured	39.15±0.70*^{,a}	62.55±0.68*^{,b}	62.08±0.46*^{,b}	51.34±0.34*^{,c}	63.67±0.60*^{,b}
		estimated	32.26±0.02	71.09±1.14	72.06±1.46	55.28±0.11	77.09±1.37
	60	measured	44.04±0.13*^{,a}	67.45±0.12*^{,b}	69.19±1.20*^{,b}	58.72±0.07*^{,c}	69.73±0.67*^{,b}

		estimated	36.49±0.50	79.56±0.09	80.53±0.41	63.75±0.94	85.55±2.42
	30	**measured**	38.12±0.00*,a	57.83±1.80*,b	63.50±1.00*,c	37.69±0.51*,a	64.62±0.40*,c
		estimated	32.21±0.20	68.98±0.00	73.30±0.07	47.83±0.58	75.29±0.13
	45	**measured**	43.14±0.83*,a	64.78±0.06*,b	69.28±0.66*,c	48.73±1.40*,d	71.23±0.87*,c
2.0		**estimated**	37.65±0.44	79.86±0.50	84.17±0.56	58.70±1.08	86.17±0.37
	60	**measured**	46.95±0.06*,a	70.31±0.00*,b	73.60±1.66*,b	54.06±0.38*,c	78.24±1.87*,d
		estimated	41.89±0.08	88.32±0.55	92.64±0.49	67.17±0.03	94.64±0.68

Different letters indicate the statistical significance of difference at p< 0.0.5 between measured inhibition values of the mixtures of the insoluble fractions of coffee infusions and catechin or epictechin for each combination lined in rows. The * symbols indicates the statistical significance of difference at p< 0.05 between each estimated- measured pair.

Table 3.13. Measured and estimated values of inhibition % of DPPH radical in the presence of coffee infusions and chocolate mixtures.

Coffee infusions (mg)	Chocolate (mg)		Espresso	Filtered Coffee	French Press	Moka Coffee	Turkish Coffee
0.10	**1.0**	**measured**	27.18±0.26[a]	25.71±0.26*,[a]	32.29±0.06*,[b]	26.07±0.46*,[a]	33.12±0.90*,[b]
		estimated	27.48±0.68	27.14±0.10	27.26±0.42	28.58±0.02	25.74±0.13
	1.5	**measured**	33.29±0.13[a]	32.79±0.19[a]	38.55±0.19*,[b]	31.47±0.26*,[c]	42.55±0.19*,[d]
		estimated	32.28±0.78	31.93±0.20	32.05±0.52	33.37±0.10	30.53±0.23
	2.0	**measured**	38.69±0.07[a]	38.51±1.10[a]	45.78±0.51*,[b]	36.28±0.26*,[a]	49.23±0.58*,[c]
		estimated	37.20±0.55	36.86±0.03	36.97±0.29	38.30±0.11	35.46±0.00
0.15	**1.0**	**measured**	33.49±0.59[a]	32.27±0.32[a]	36.45±0.13*,[b]	31.99±0.39[a]	36.33±0.00*,[b]
		estimated	33.36±0.62	32.50±0.06	33.09±0.20	33.94±0.51	30.00±0.13
	1.5	**measured**	38.49±0.91[a]	36.62±0.39[b]	45.20±0.19*,[c]	37.52±0.20[a,b]	44.93±0.26*,[c]
		estimated	38.15±0.71	37.29±0.04	37.88±0.29	38.73±0.61	34.79±0.23
	2.0	**measured**	43.76±0.59[a]	40.78±0.13*,[a]	49.62±0.13[b]	44.54±1.63[a]	53.15±1.93*,[b]
		estimated	43.08±0.48	42.21±0.19	42.80±0.06	43.65±0.38	39.71±0.00
0.20	**1.0**	**measured**	39.47±0.46[a]	38.61±0.07*,[a]	44.10±0.00[b]	37.94±0.65[a]	42.11±0.00*,[c]
		estimated	38.50±0.65	37.48±0.22	37.99±0.45	39.74±0.48	34.28±0.48
	1.5	**measured**	43.17±0.26[a]	41.93±1.17[a]	53.08±1.15[b]	42.63±0.26[a]	50.19±0.51*,[c]
		estimated	43.29±0.75	42.27±0.12	42.78±0.55	44.54±0.57	39.07±0.38
	2.0	**measured**	48.18±0.85[a]	46.42±0.85[a]	59.81±0.45[b]	46.81±0.00*,[a]	58.99±0.96*,[b]
		estimated	48.21±0.52	47.19±0.35	47.70±0.32	49.46±0.35	43.99±0.61

Different letters indicate the statistical significance of difference at p< 0.0.5 between measured inhibition values of the mixtures of coffee infusions and chocolate for each combination lined in rows. The * symbols indicates the statistical significance of difference at p< 0.05 between each estimated- measured pair.

Significance of the effects of the concentrations of the insoluble fractions of coffee infusions and catechin/epicatechin together with the concentrations of coffee infusions and chocolate in their mixtures on the AC were also investigated and the p values calculated were illustrated in Table 3.14. The results were statistically significant for all coffee infusions except moka when considering the interactions between the insoluble fractions and catechin. With epicatechin, the results were not significant for only filtered coffee and moka.

The concentrations of catechin and epicatehin and the two- way interactions between the concentrations of the insoluble fractions of coffee infusions and catechin/epicatechin was significant for all different types of coffee infusions. On the other hand, the concentration of coffee infusions was significant except Turkish coffee, while the concentration of chocolate and the two- way interaction between the concentrations of coffee infusions and chocolate was significant for all different types of coffee infusions.

Table 3.14. The p values within 0.95 confidence interval calculated by using anova1 function in Matlab for [CI]: coffee infusions concentration, [Insoluble CI]: Concentration of the insoluble fraction of coffee infusions, [Epictechin]: Epicatechin concentration, [Catechin]: Catechin concentration, [Chocolate]: Chocolate concentration; [CI] * [Chocolate], [Insoluble CI] *[Catechin], [Insoluble CI] *[Epicatechin]: the 2 way interactions between [CI] and chocolate; [Insoluble CI] and [Catechin]; [Insoluble CI] and [Epicatechin].

	Espresso	**Filtered Coffee**	**French Press**	**Moka Coffee**	**Turkish Coffee**
[Insoluble CI]	0.0475	0.0097	0.0030	0.7913	0.0008
[Catechin]	1.97E-5	0.0010	0.0047	2.53E-10	0.0178
[Insoluble CI]* [Catechin]	1.36E-11	1.93E-13	9.59E-16	1.47E-19	8.31E-17
[Insoluble CI]	0.0156	0.0921	0.0169	0.9020	0.0076
[Epicatechin]	7.03E-6	3.31E-5	0.0004	4.90E-14	0.0013
[Insoluble CI]* [Epicatechin]	3.90E-13	4.28E-16	8.98E-15	3.80E-19	6.44E-16
[CI]	0.0007	0.0011	0.0010	0.0007	0.0975
[Chocolate]	0.0031	0.0026	0.0009	0.0025	8.33E-7
[CI]* [Chocolate]	2.80E-13	1.35E-13	5.19E-17	5.34E-14	6.09E-14

4. CONCLUSIONS AND PERSPECTIVES

The interactions between macromolecule- bound antioxidants from different sources and various free antioxidants naturally occurring in foods were investigated in different reaction media with a multivariate approach throughout the thesis work.

Examination of the interactions between macromolecule- bound antioxidants and Trolox in aqueous medium revealed antagonistic relations in general. An inhibitory effect was evidenced for bound antioxidants on the auto- regeneration reaction of Trolox, with the mass spectra obtained for the stable end product of the oxidation reaction of Trolox. This finding was crucial to fully understand the mechanism behind the antagonistic interactions of Trolox. Besides, a significant effect for pH was revealed for the combinations of Trolox with almost all macromolecule- bound antioxidants. Especially, pH 3.0 inspired a higher overall antioxidant capacity for Trolox + bound antioxidant mixtures in general, being significantly different from either pH 5.0 or 6.0 due to samples. In this context, the behavior of Trolox is believed to change at different parts of the GI tract with the changing pH values. Also, the behavior of Trolox was evidenced to be particular due to the food matrix and the concentrations of both Trolox and macromolecule- bound antioxidants in the medium. Apart from these, an argument was centered for the constraints created in the standard antioxidant capacity measurement methods by the auto- regeneration reaction of Trolox, namely the potential underestimation of the antioxidant capacity of food samples, which should be considered carefully for further studies.

The interactions between macromolecule- bound antioxidants and Trolox were also investigated in liposome medium to understand the effect of reaction media on the type of interactions and were found to be varied depending on the macromolecule- bound antioxidant source. Synergistic interactions were observed for DF and refined olive oil-bound antixodants, while antagonistic interactions were observed for protein and extra virgin olive oil-bound antioxidants with Trolox. A generalized version of the logistic function was successfully used for modelling the oxidation curve of liposomes, while the two components describing the time course of the oxidation reactions of liposomes were revealed with PCA. Also, the significance of the effects of free and bound antioxidant concentrations on the resultant antioxidant activity was revealed with PCA and ASCA.

In addition to these, the interactions between WW-bound antioxidants and HCA/ HBA derivatives having different amounts of –OH and –OCH_3 groups at different positions on their aromatic ring

were investigated in both liposome and aqueous media to explain the background of the interactions with a mechanistical approach. The types of interactions were different from each other in liposome and aqueous media. Synergistic interactions were predominated in the aqueous medium, while both synergistic and aqueous interactions were observed in liposome medium. Inhibition of lipid oxidation and DPPH radical scavenging characteristics in the presence of the mixtures of WW- bound antioxidants and HCA/ HBA derivatives, and the behavioral similarities between different HCA/ HBA derivatives were revealed. Broadly, liposome medium was more informative by means of revealing and explaining the mechanism behind different types of interactions with a structural approach.

Coffee and bread crust melanoidins were also used as DF- bound antioxidant sources during this work. Synergistic interactions were revealed between coffee/ bread crust melanoidins and HCA/ HBA derivatives in aqueous medium in general, pointing a stimulating effect for HCA/ HBA- rich foods for the AC exerted by coffee and bread crust. The characteristics of radical scavenging reactions in the presence of coffee/ bread crust melanoidins and HCA/ HBA derivatives in DPPH radical scavenging reactions was presented, by means of the usage sequence of this species. Since HCA/ HBA derivatives are known to be present in a variety of foods, the last two set of studies are also believed to be meaningful by considering the human diet.

Apart from these the interactions between the insoluble fractions of different types of coffee infusions and major cocoa free antioxidants together with different types of coffee infusions and chocolate was investigated in this study. It was revealed that the brewing procedure can change the AC of coffee infusions, therefore the interactions between both coffee infusions and their insoluble fractions with either catechin/epicatechin or chocolate antioxidants. The importance of brewing step in coffee preparation by means of the interactions of its antioxidants with other antioxidant species was also proven. When jointly consumption of coffee and chocolate is considered, this situation becomes crucial.

In conclusion, this thesis study is an inception for the further studies investigating the interactions of macromolecule- bound antioxidants with free soluble antioxidants with a multivariate and structural approach. Further in vivo studies are encouraged to explain the behavior of macromolecule- bound antioxidants in human body after consumption with foods containing free soluble antioxidants.

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- Maillard reaction products (Melanoidins)

Structure-Activity Relationships of Antioxidants

Reaction kinetics

- Kinetic modelling

Chemometrics

- Principal Component Analysis (PCA)
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Publications

**indicates publications from this PhD thesis*

[1]* Çelik, E. E., Rubio, J. M. A., Gökmen, V., Behaviour of Trolox with macromolecule-bound antioxidants in aqueous medium: Inhibition of auto-regeneration mechanism, Food Chemistry, 243, 428–434, **2018.**

[2]* Çelik, E. E., Rubio, J. M. A., Andersen. M. L., Gökmen, V., Interactions between macromolecule-bound antioxidants and Trolox during liposome autoxidation: A multivariate approach, Food Chemistry, 237, 989–996, **2017.**

[3] Çelik, E. E., Gökmen, V., Skibsted, L. H., Synergism between soluble and dietary fiber bound antioxidants, *Journal of Agricultural and Food Chemistry*, 63, 2338–2343, **2015.**

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[5] Çelik, E. E., Gökmen, V., Fogliano, V., Soluble antioxidant compounds regenerate the antioxidants bound to insoluble parts of foods, *Journal of Agricultural and Food Chemistry,* 61, 10329–10334, **2013.**

Oral Presentations

**indicates publications from this PhD thesis*

[1]* Çelik, E. E., Rubio, J. M. A., Andersen, M. L., Gökmen, V., Interactions between macromolecule- bound antioxidants and free antioxidant trolox in liposome medium: a multivariate approach, *EuroFoodChem XIX Conference,* 3-7 October, Budapest, Hungary, **2017.**

[2] Çelik, E. E., Gökmen, V., Skibsted, L. H., Study of the Synergy Between Soluble and Dietary Fiber Bound Antioxidants**,** *249th American Chemical Society National Meeting & Exposition,* 22-26 March, Denver, CO, USA, **2015.**

[3] Çelik, E. E., Gökmen, V., Skibsted, L. H., Study of the Synergy Between Free and Bound Antioxidants, 29th EFFoST International Conference, 9-12 December, Athens, Greece, **2015.**

[4] Çelik, E. E., Gökmen, V., Regeneration behavior of bioactive compounds bound to insoluble food matrices, *EuroFoodChem XVII Conference,* 7-10 May, Istanbul, Turkey, **2013.**

Poster Presentations

**indicates publications from this PhD thesis*

[1]* Çelik, E. E., Rubio, J. M. A., Andersen. M. L., Gökmen, V., Interactions of coffee melanoidins with hydroxycinnamic/ hydroxybenzoic acids, *4th International Congress on Cocoa Coffee and Tea,* 25-28 June, Turin, Italy, **2017.**

[2] Çelik, E. E., Gökmen, V., Determination of antioxidant capacity and regeneration behavior of bioactive compounds bound to insoluble dietary fibers, *International Conference on Chemical Reactions in Foods VII,* 14-16 December, Prague, Czech Republic, **2012.**

HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
THESIS/DISSERTATION ORIGINALITY REPORT

HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
TO THE DEPARTMENT OF FOOD ENGINEERING

Date: 02/01/2018

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