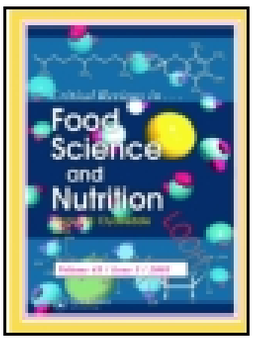

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REVIEW

Interactions between free and bound antioxidants under different conditions in food systems

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ABSTRACT

This review aimed to give comprehensive information about the interactions between free and bound antioxidants naturally found in different food matrices. In this context, firstly, the free and bound antioxidant terms are defined; their place in the daily diet, the path they follow in the body and their characteristics are explained. Factors affecting the interactions have been revealed as a result of the compilation of studies conducted until today, related to bound and free antioxidant interactions. Accordingly, it was observed that many factors such as reaction environment, concentration, pH, chemical structure, source and antioxidant/prooxidant nature of the compounds were effective on interactions. It has been emphasized that the interactions between free and bound antioxidants have a dynamic balance that can easily change under the influence of various factors, which in turn needs the interactions to be handled specifically for each case.

KEYWORDS

Free antioxidants; bound antioxidants; antioxidant activity; interaction; synergism; regeneration

Introduction

Antioxidants are defined as "any substance, significantly delays or inhibits oxidation of an oxidizable substrate, when present at low concentrations compared to that susbtrate" by Halliwell and Gutteridge (1989). This definition covers compounds of both enzymatic and non-enzymatic origin that can exist in the intracellular and extracellular environment. Enzymatic antioxidants, naturally found in the human body, includes glutathione, peroxidase and superoxide dismutase which are responsible for the self-defense of the body from reactive oxygen species. Their mechanism of action is based on breaking down or removing free radicals. On the other hand, non-enyzmatic antioxidants' action is based on interrupting the free radical chain reactions. In such reactions, a compound carrying an unpaired electron reacts with another compound, which in turn leaves the latter with an unpaired electron. This compound attacks to another one, creating a cycle, which continues until an antioxidant interrupts. After interrupting a radical, antioxidant compounds can regenerate themselves by reacting with some water-soluble compounds. With an efficient regeneration, one to three antioxidant molecules can preserve a thousand of target molecules (Nimse and Pal 2015). This kind of interaction is called synergism, in which the overall antioxidant effect is enhanced (Wang et al. 2011). Many antioxidant compounds can also act as prooxidants, depending on their concentrations, the oxidation mechanism, its mode of initiation and the O_2 tension in the medium (Zhang and Omaye 2001; Albertini and Abuja 1999; Tian et al. 2007). Moreover, antagonistic and additive effects, in which the overall antioxidant capacity (AC) is reduced and does not change, respectively may occur between antioxidant compounds.

Testing antioxidant/prooxidant effect of any substance before it is used in a particular environment for a specific purpose and its interactions with other substances (synergism, antagonism, additive effect) in the environment is very important in terms of optimizing the antioxidant effect to be obtained from that substance. In literature, many research exist, interested in the antioxidant/prooxidant properties of and interactions between some well known antioxidant compounds, including phenolic compounds. These studies largely focused on free soluble antioxidants, while bound antioxidants, the presence of which was noticed and manifested only about 20 years ago (Saura-Calixto 1998) were ignored in this period. With the increasing interest in bound antioxidant research in recent years, it is understood that they constitute an important part of human daily diet as well as free antioxidants. Considering that free and bound antioxidants coexist in different food matrices and consumed and act together in the gastrointestinal (GI) tract, their interactions with each other started to arouse curiosity. Thus, studies investigating their interactions have also started to appear in literature.

This review provides comprehensive information about the interactions between free and bound antioxidants naturally found in different food matrices. Within the scope of this article, the free and bound antioxidant terms, their occurrence in daily diet, their metabolic fates and physiological effects have been explained. The interactions between free and bound antioxidants have been discussed in detail

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through studies performed since free antioxidants have been proven to regenerate bound antioxidants. Research commenced on this area is believed to allow us to understand the effects of the interactions between free and bound antioxidants on human health.

Free antioxidants

Antioxidants may present in different forms in food microstructure as listed below.

1. Free from physical or chemical interactions with other macromolecules (low molecular weight compounds)
2. Physically entrapped into cellular structure or complex food matrix
3. Chemically bound to other macromolecules (low molecular weight compounds)
4. Insoluble (usually high molecular weight compounds)

The same antioxidant compound can be found in any forms listed above, depending on the food in which it is found. For example, ferulic acid is found in free form in many fruits, while in cereals it is attached to the cell wall polysachharide by ester bonds. In addition, processes applied to food can shift antioxidant compounds into their less soluble forms (Gökmen, Serpen, and Fogliano 2009).

Among these forms, the first group constitutes the focus of the majority of the antioxidant studies (Furr and Clark 1997; Lafay and Gil-Izquierdo 2008). This group includes all compounds that can be easily extracted with water or hydroalcoholic mixtures from foods and beverages. A major part of the antioxidants found in fruit and vegetable tissues are also free. Ascorbic acid and lipid-soluble tocopherols are usually found in free form. Moreover, phenolic acids, such as cinnamic and benzoic acids, flavonoinds family (with more than 500 compounds), stilbenes, lignans, hydrolizable tannins and polymerized flavonoids are included in this group. Nevertheless, polymerized flavonoids can easily shift to the macromolecule-bound antioxidants group, depending on their polymerization degree (Gökmen, Serpen, and Fogliano 2009).

Free antioxidants cause a significant increase in the plasma AC immediately after their consumption as indicated in various studies (Skrzydlewska et al. 2002; Rein et al. 2013). However, this does not last long for most of the free antioxidants and suddenly elevated plasma concentrations may decrease in a short time. For instance, the plasma concentration of the significant portion of flavonoids have been reported to reach a maximum within 1–3 hours after ingestion with foods or in pure form, but their half-life was reported to be completed within few hours (Manach et al. 2005). Moreover, there is no evidence that long term and regular consumption of flavonoid-rich foods ensure that flavonoids are stored in plasma in significant amounts (Moon et al. 2000). Especially gallic acid, catechins and flavanones do not have the opportunity to accumulate in plasma, considering their half-lifes. In a study on elderly women, it was reported that the consumption of strawberries, spinach, ascorbic acid and red wine increased the plasma AC to a maximum within 1–2 hours, but these levels decreased to the baseline level after 4 hours (Cao et al. 1998). Similarly, after chocolate consumption (either dark or milk), it was reported that the plasma antioxidant level reached a maximum within 1 hour and reached its initial level within 4 hours (Serafini et al. 2003). These situations severely limit the antioxidant activity of most of the free antioxidants *in vivo*.

Macromolecule-bound antioxidants

Antioxidants consumed in the daily diet can be also present as bound to macromolecules such as proteins, lipids and carbohydrates in food matrices (Palafox-Carlos, Ayala-Zavala, and González-Aguilar 2011). These antioxidants, which are mentioned in the third group, constitute an important part of the dietary antioxidants, although they have not received enough attention in the scientific studies up to date. Bound-phenolics in fruit, vegetable, legume and seed tissues constitute 20–60% of the total phenolics, which cannot be undervalued with respect to the soluble phenolics (Nayak, Liu, and Tang 2015). In plant tissues, the main macromolecules are carbohydrates (Manach et al. 2004), which constitute the cell wall material. Hence, most of the macromolecule-bound antioxidants in plant foods are covalently attached to cell wall materials including pectin, celuose, arabinoxylan and structural proteins. For instance, about 95% of the phenolic compounds in cereals are known to present as bound to cell wall polysaccharides. They are also known as dietary-fiber (DF) phenolic compounds or DF-antioxidants (Vitaglione, Napolitano, and Fogliano 2008). The most abundant bound-phenolic compound in cereals is ferulic acid, followed by diferulic, *p*-coumaric, sinapic, protocatechuic, vanilic, syringic, gallic, caffeic and 4-hydroxybenzoic acids (Irakli et al. 2012; Vitaglione, Napolitano, and Fogliano 2008). Substantial amounts of these phenolic acids are also found in bound form in legumes, including a variety of lentils (Alshikh, de Camargo, and Shahidi 2015), beans (Chen et al. 2015; Pająk et al. 2014; Ross, Beta, and Arntfield 2009), cowpeas, chickpeas (Gutiérrez-Uribe et al. 2010), some oilseeds (Pająk et al. 2014; Naczk and Shahidi 1989) and fruit seeds (Ayoub, de Camargo, and Shahidi 2016). Flavonoids such as quercetin, catechin, epicatechin and rutin are also known to be present in bound form in some plant foods.

In addition to their natural occurrence in foods, DF-bound antioxidants may also occur as a result of certain food preparation processes. Thermal treatment of foods, containing reducing sugars and free amino groups, yields melanoidins as a result of the Maillard reaction. Melanoidins are high molecular weight, brown colored complex molecules, containing nitrogen. They are compared with DFs due to their poor digestibility and are known to exert significant antioxidant activity, which makes them to be considered as DF-bound antioxidants. The chemical structures of melanoidins have not been elucidated yet, but it is known that various carbohydrate or protein based structures can be formed depending on the compounds included in the reaction (Borrelli and Fogliano 2005). Bread

crust and coffee are the major sources of melanoidins (Fogliano and Morales 2011) followed by cocoa (Hofmann, Bors, and Stettmaier 1999), malt (Obretenov et al. 1991), roasted barley (Milić et al. 1975), black beer (Kuntcheva and Obretenov 1996), roasted potatoes (Ponnampalam and Mondy, 1983), roasted pulses and seeds (Açar et al. 2009), meat (Obretenov et al. 1993), soy sauce (Sakurai, Hashiba, and Okuhara 1981), balsamic vinegar (Giudici et al. 2009), sweet vine (Ortega-Heras and González-Sanjosé 2009) and processed tomatoes (Adams et al. 2005).

Despite their influential presence in plant foods, the AC of DF-bound antioxidants has been largely underestimated due to their low water and organic solvent solubility. After introduction of the QUENCHER procedure (Gökmen, Serpen, and Fogliano 2009), which is based on direct solid-liquid surface reaction between radical and food matrix without any extraction, the notable antioxidant activity of DF-bound antioxidants were understood.

The most striking feature of macromolecule-bound antioxidants is that their functional properties, such as bioavailability and bioaccessibility are affected by the macromolecule that they are linked with. For instance, DFs, which are resistant to digestion in the upper GI tract, reduce the release rate and retard the absorption of antioxidants bound to them (Brownlee et al. 2006; Hoffmann et al. 1999). After ingestion, DF bound antioxidants pass the digestion in the upper GI tract and reach to colon in intact form. Antioxidants are slowly released from the fiber matrix in colon with the action of colonic microbiota (Arranz, Silván, and Saura-Calixto 2010). The long survival times of dietary antioxidants in colon during this period contributes to the formation of a steady antioxidant environment in colonic lumen, which will act against the radical species and prooxidants continuously formed there (Saura-Calixto 2011). This unique feature of DF-bound antioxidants was hypothesized to play a significant role in the prevention of some diseases including colon cancer and other digestive cancers (Babbs 1990; Adom and Liu 2002).

In addition to this, the nature of the DF-bound antioxidants helps to preserve the plasma antioxidant concentration at a stable level for longer periods, compared to free soluble antioxidants. After ingestion of wheat bran, it was reported that the plasma ferulic acid concentration of rats was created a small peak in 1 hour and remained at a constant level for 24 hours (Rondini et al. 2004). Similarly in a human study, after ingestion of wheat bran cereals, plasma ferulic acid concentration was reported to create a peak within 1–3 hours, decreased rapidly within 3–6 hours, which slowly continued until 24 hours (Kern et al. 2003). At this point, it should be noted that the health benefits of antioxidants could be only enhanced by their continuos presence at a constant level (even low) rather than high plasma concentrations followed by immediate elimination (Vitaglione, Napolitano, and Fogliano 2008).

Regeneration phenomenon

Regeneration of a radical scavenger may occur mostly when a radical scavenger with a lower reduction potential exist in the medium. In this phenomenon, the high-reduction potential radical scavenger acts as the primary antioxidant, while the low-reduction potential radical scavenger acts as the co-antioxidant or synergist. As a result, a higher antioxidant effect is obtained in total, compared to the simple sum of the individual effects, which is called as synergism (Decker 2002). A well-known example of this situation is ascorbic acid (330 mV)-tocopherol (500 mV) system (Liebler 1993). In this system, tocopherols act as the primary antioxidant and donate hydrogen to the radical species in the medium, generating tocopherol radicals. The radicals formed are then regenerated by taking hydrogen atoms from ascorbic acid molecules, yielding dehyrdoascorbic acid radicals (Buettner 1993). A similar, but more complex relationship exists between tocopherols and carotenoids, in which they are both able to regenerate each other (Mortensen and Skibsted 1997). However, carotenoids are more likely to be the principal antioxidant due to their higher reduction potential (700–1000 mV) (Liu, Gao, and Kispert 2000). There are a number of similar studies investigating the regeneration relations between a variety of pure phenolic compounds naturally found in foods including catechins, gallic acid, vanillic acid and quercetin (Pedrielli and Skibsted 2002; Jørgensen et al. 1999; Pazos et al. 2007).

However, it was only possible in 2013 that these researches expanded to include macromolecule-bound antioxidants. In this first study, the regeneration behavior of DF-bound antioxidants in various food matrices has been investigated by using a variety of pure antioxidant compounds and high-antioxidant beverages as co-antioxidants (Çelik, Gökmen, and Fogliano 2013). DF-bound antioxidants, contained in the insoluble fraction of different food matrices (wheat bran, oat bran, rye flakes, dried grape, hazelnut, peanut and pistachio skins) have been reported to be able to regenerate up to three times with ascorbic acid after radical treatment (ABTS or DPPH). Different regeneration efficiencies of different food matrices was attributed to their diverse DF structures (i.e. pectin, arabinoxylan or cellulose based). The resistance of DF structures was also believed to affect their regeneration behavior in the digestive system. Regeneration efficiency of wheat bran DF-bound antioxidants by different pure compounds (ascorbic acid, epicatechin, chlorogenic acid to their ternary mixture) and antioxidant-rich beverages (green tea, espresso, black tea, instant coffee, orange juice, red wine) was also investigated in the course of three step regeneration. No significant difference was observed between the regeneration efficiencies of different pure compounds. However, it was higher than pure compounds and different from each other for antioxidant-rich beverages.

With this study, it was shown for the first time that antioxidants bound to insoluble material are able to be regenerated by other hydrogen donating species present in the liquid phase. According to the proposed mechanism, DF-bound antioxidants, exposed to radical attack, lose hydrogen atoms during radical scavenging. Meanwhile, free antioxidants present in the medium come into contact with this depleted antioxidants and regenerate them by donating their hydrogen atoms, becoming radicals themselves. This has

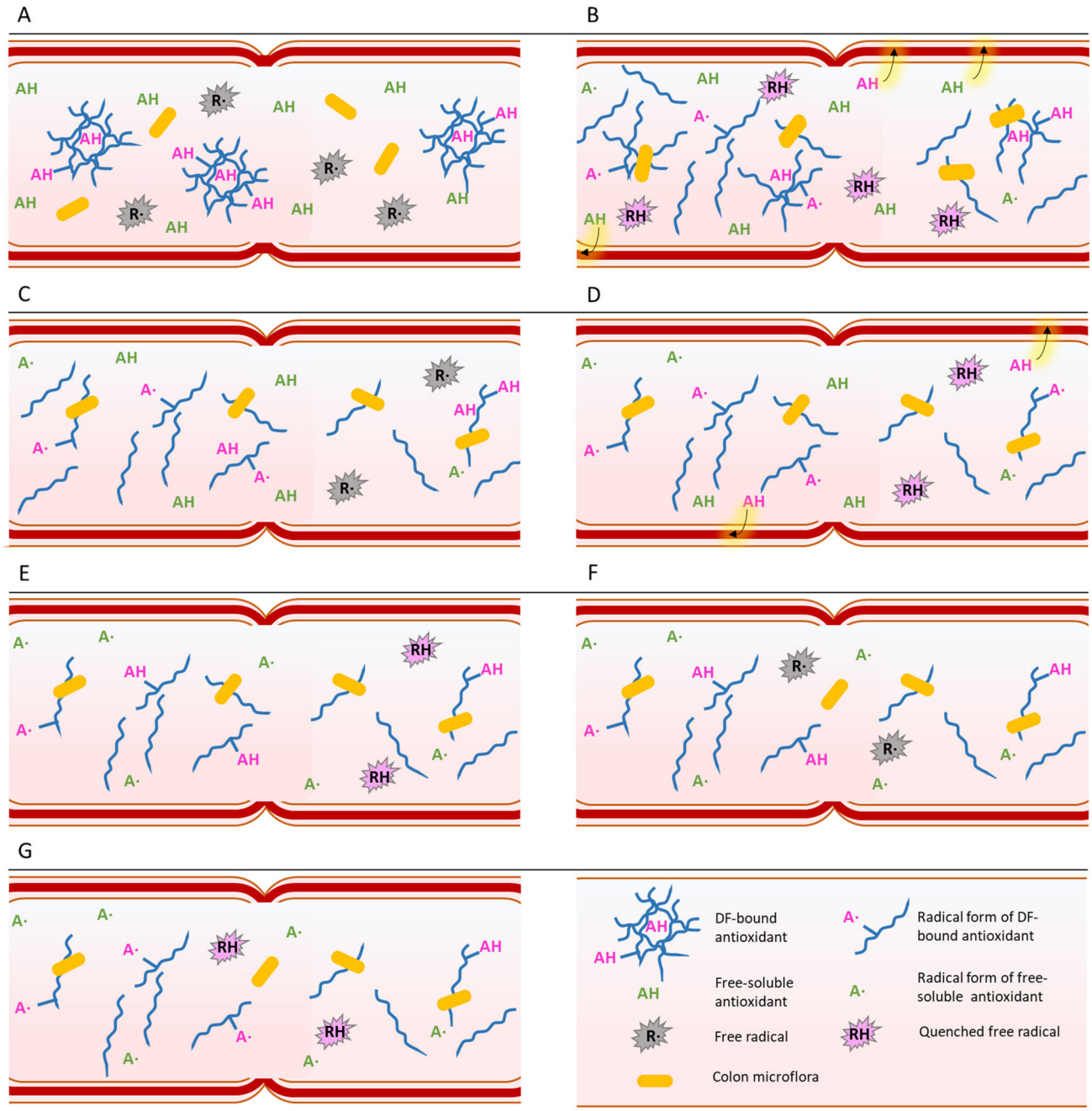

Figure 1. The fate of DF-bound and free soluble antioxidants in GI tract: the summary of the suggested regeneration mechanism. (A) DF-bound and free soluble antioxidants, free radicals and colonic microbiota are together in the colon after ingestion of DF-bound and free soluble antioxidant containing foodstuff. (B) Colonic microbiota starts to ferment DF matrix and convert the bound antioxidants into free form. While some of them pass into the blood stream together with free soluble antioxidants, some are used to quench the free radicals in the environment. (C) Colonic microbiota continues to ferment DFs and release bound antioxidants. Meanwhile, new free radicals are continuously formed as a result of the vital activities of the organism. (D) The new free radicals formed are quenched by the free and bound antioxidants. The bound antioxidants released from the fiber matrix continue to pass into the blood. (E) Bound antioxidants that became radicals themselves during radical quenching reactions are regenerated by free soluble antioxidants. (F) This phenomenon makes them ready to quench the newly formed free radicals. (G) A healthy colon environment is provided by quenching the continuously formed free radicals with bound antioxidants that are released slowly and can be regenerated and activated repeatedly.

been confirmed by the detection of the conversion of more than 70% of the ascorbic acid to dehydyoascorbic acid during regeneration. The in vitro regeneration concept proposed in this study, can be adapted to the GI tract as well. It is known that DF-bound antioxidants remain long times in the GI tract, especially in colon, during fermentation (24 hours). During this period, they may have the opportunity to exert their antioxidant effects by scavenging free radicals formed in the environment. At this stage, consumption of antioxidant rich beverages may lead to the regeneration of depleted antioxidants, which may help to maintain the healthy antioxidant environment created. The proposed mechanism for this concept is illustrated in Figure 1. The potential for this phenomenon to occur *in vivo* is thought to be important for building a healthy GI system awareness.

In a following study, the interactions between wheat bran DF-bound antioxidants and green tea infusions as well as pure soluble antioxidants in green tea infusions (epicatechin,

epigallocatechin gallate) have been investigated, without radical pretreatment (Çelik and Gökmen 2014). It has been observed that the AC of the insoluble fraction of wheat bran did not change after epicatechin treatment, but significantly increased after treatment with epigallocatechin gallate and green tea infusions. The difference observed for epicatechin was attributed to its lower effectiveness as a radical scavenger, comparing with epigallocatechin gallate (He et al. 2018). The lack of radical treatment before catechins suggested that some of the bound antioxidants may naturally exist in their radical form in wheat bran microstructure. This, in turn might give the opportunity to catechins to regenerate them. In addition to this, it was observed that the concentrations of pure epicatechin, epigallocatechin gallate and catechins in green tea infusions were decreased significantly after treatment with insoluble wheat bran. It is known that catechins were rather stable in experimental conditions and their self-degradation was limited (<10%). This had suggested that the consumption of catechins during treatment have arisen from their interactions with the depleted antioxidants available on the fiber matrix.

Nevertheless, the mechanism behind the interactions between insoluble wheat bran antioxidants and free antioxidants was reexamined in another study, with a different approach in 2015 (Doğan and Gökmen 2015). In this study, it has been observed that the AC of insoluble wheat bran and its radical pretreated form was increased at the same rate after treatment with tannic acid. This suggested that the reason for the increase of AC may not be the radicals formed on the DF matrix. Instead, it was considered that free antioxidants bind somewhat to the fiber structure. When insoluble wheat bran was treated with a variety of beverages, it has been observed that the AC increased most with green tea. Green tea infusions are known to be rich in flavan-3-ols, such as epicatechin, epigallocatechin and epigallocatechin gallate, which can be easily oxidized to quinones and polymerized during treatment (Wang and Ho 2009). It has been reported that the treatment of insoluble wheat bran with green tea under optimum conditions (60 minutes at 50° C and pH 9.0) increased the amount of bound phenolic compounds along with the AC of insoluble wheat bran. This has shown that green tea phenolics had been bound to the DF matrix during regeneration. It has been proposed that the phenolic compounds were oxidized to quinones under alkaline conditions and further bound to free amino groups available on the fiber matrix. This has been confirmed by the 59.5% reduction of free amino groups on the fiber matrix. In addition to this, it has been stated that polyphenols, like tannic acid, were more efficient than phenols, like gallic acid, in binding to fiber structures, probably due to the higher amount of quinones they produce.

Despite two different mechanisms proposed for the interaction of insoluble material and free antioxidants, it is a fact that free antioxidants are able to bind to DF-matrix and increase its effectiveness. Indeed, both mechanisms may play a role in increasing in the AC of the DF matrix. However, proving that the free amino groups in the fiber matrix are reduced increases the likelihood of the second mechanism to be more effective. In any case, this means reorganizing and enhancing, that is regenerating the antioxidant activity in the DF structure. The discovery of the regeneration relations between DF-bound antioxidants and free antioxidants has been the milestone for further researches, investigating the interactions between free and macromolecule-bound antioxidants.

Interactions between macromolecule-bound antioxidants and free antioxidants

The co-existence of antioxidants in complex food systems may result in antagonistic and additive interactions as well as the synergistic interactions observed during regeneration phenomenon. Antagonistic interaction is characterized by a smaller total effect, compared to the simple sum of the individual effects, while these effects are equal in additive interaction (Wang et al. 2011). Bound antioxidants are frequently consumed together with free antioxidants in different foods or even found in the same food matrix. Hence, understanding the interactions between free and macromolecule-bound antioxidants is important to estimate the possible status of antioxidant environment when they are found together. There are not many studies in literature on this subject, since importance and health effects of bound antioxidants have been understood in the recent past. However, a series of studies conducted in recent years provide an insight on the interactions between free and bound antioxidants found in various food matrices. Consequently, interactions between macromolecule-bound antioxidants and free soluble antioxidants were revealed to depend on many factors including source, chemical structure, concentration and antioxidant/prooxidant nature of antioxidants, reaction medium and pH.

In 2015, the interactions of DF-bound antioxidants in various grains with antioxidant-rich beverages or their pure antioxidants in aqueous radical (Fremy's salt) and liposome environments (Çelik, Gökmen, and Skibsted 2015) were examined. DF-bound antioxidants of wheat, oat and rye were found to exert synergistic effects in both media with high antioxidant beverages (green tea, red wine, pomegranate juice and espresso) and pure antioxidant compounds (chlorogenic acid, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate and resveratrol). This was revealed by comparing the simple sum of the individually measured AC values of free and bound antioxidants (estimated values) with the measured values for their real mixtures. Table 1 gives the intensity of the ESR signal values measured and estimated for the DF-bound antioxidant + beverage mixtures (in increasing amounts) in the Fremy's salt radical medium as an example. The fact that the measured values are higher than the estimated ones indicates the synergistic interaction. On the other hand, the interactions between DF-bound antioxidants in different whole wheat products (flour, paste and bread) and Trolox, a vitamin E analogue, was found to be antagonistic in a different aqueous radical environment (ABTS) (Çelik, Rubio, and Gökmen 2018). Lipid and protein-bound antioxidants, obtained from olive oil (extra virgin, refined) and soybean

Table 1. Relative decrease (%) in the intensity of estimated and measured ESR signal values for the mixtures of insoluble fibers from different grains and beverages rich in free antioxidants (Çelik, Gökmen, and Skibsted 2015).

	Decrease (%) in ESR signal with different mixture volume					
	100 μL		150 μL		200 μL	
Mixture	Estimated	Measured	Estimated	Measured	Estimated	Measured
Insoluble wheat + Green tea	23.0 ± 1.1^a	41.1 ± 1.0^b	31.7 ± 0.4^a	48.8 ± 1.4^b	42.1 ± 1.1^a	55.5 ± 0.3^b
Insoluble wheat + Red wine	20.8 ± 1.1^a	35.0 ± 0.2^b	28.7 ± 0.4^a	44.1 ± 0.7^b	37.4 ± 1.1^a	51.9 ± 0.2^b
Insoluble wheat + Pomegranate juice	19.4 ± 0.5^a	26.0 ± 2.9^a	27.1 ± 1.3^a	35.0 ± 1.7^b	37.8 ± 1.6^a	44.5 ± 4.5^a
Insoluble wheat + Espresso	18.8 ± 2.9^a	34.3 ± 6.1^a	26.0 ± 0.7^a	47.9 ± 2.2^b	37.0 ± 0.8^a	55.4 ± 5.1^b
Insoluble oat + Green tea	25.8 ± 1.3^a	47.8 ± 0.3^b	32.2 ± 1.2^a	64.5 ± 0.3^b	40.0 ± 0.8^a	77.7 ± 3.4^b
Insoluble oat + Red wine	23.6 ± 1.3^a	35.1 ± 3.7^a	29.3 ± 1.2^a	48.1 ± 0.7^b	35.3 ± 0.8^a	58.2 ± 2.6^b
Insoluble oat + Pomegranate juice	22.2 ± 0.6^a	36.1 ± 3.0^b	27.6 ± 2.0^a	49.0 ± 1.4^b	35.7 ± 1.3^a	58.3 ± 6.2^b
Insoluble oat + Espresso	21.6 ± 3.1^a	47.3 ± 6.2^b	26.5 ± 1.5^a	57.0 ± 3.8^b	34.9 ± 0.5^a	66.4 ± 4.5^b
Insoluble rye + Green tea	27.5 ± 1.5^a	41.6 ± 2.0^b	35.4 ± 0.3^a	63.9 ± 5.4^b	43.3 ± 1.0^a	69.5 ± 3.5^b
Insoluble rye + Red wine	25.3 ± 1.4^a	40.3 ± 0.6^b	32.5 ± 0.3^a	50.3 ± 3.7^b	38.5 ± 1.0^a	51.4 ± 3.8^b
Insoluble rye + Pomegranate juice	23.9 ± 0.8^a	45.0 ± 6.1^b	30.8 ± 1.1^a	52.0 ± 0.7^b	39.0 ± 1.6^a	67.5 ± 1.9^b
Insoluble rye + Espresso	23.3 ± 3.2^a	40.2 ± 1.7^b	29.7 ± 0.6^a	57.9 ± 0.5^b	38.2 ± 0.7^a	60.5 ± 0.1^b

[a,b]Different letters indicate the statistical significance of difference at $p < 0.05$ for each estimated-measured pair.

(edamame, soybean, boiled soybean, soymilk and tofu) products respectively, also acted antagonistic with Trolox in the same medium. However, when the same materials were combined in liposome medium, Trolox exerted synergistic interactions with DF-bound antioxidants and antagonistic interactions with protein-bound antioxidants. Among lipid-bound antioxidants, those in extra virgin olive oil were antagonistic with Trolox, while those in refined olive oil were synergistic (Çelik et al. 2017). At this point, understanding the reason behind the interactions is of great importance.

Effect of antioxidant capacity

One of the basic rules that emerge as a result of these studies is that the matrices or compounds with high AC have high synergistic interaction abilities and high synergistic effects. For instance, according the measurements made with various free antioxidant sources (beverages, pure compounds) in Fremy's salt radical medium, the highest-ACsources were found to be green tea, EC and EGCG (individual results were not given in the related paper). In compliance, the highest synergistic effects were observed for these species with insoluble fractions of different cereals (Çelik, Gökmen, and Skibsted 2015). However, the combination of high antioxidant compounds or matrices may also result in concentration-dependent prooxidant and antagonistic effects in some cases. Detailed information on this issue is given under the title of "Effect of the Chemical Structure of Free Antioxidant."

Effect of antioxidant/prooxidant nature

Matrices or compounds acting as antioxidants have observed to yield higher synergistic interaction efficiencies than those acting as prooxidants in any medium. For instance, in the study mentioned above, the DF-bound antioxidants of wheat, oat and rye were found to act as antioxidant in aqueous radical environment (Fremy's salt) and prooxidant in liposome environment (individual results were not given in the related paper). Accordingly, an increasing synergistic effect has been observed with increasing concentrations of bound antioxidants in aqueous medium, while the opposite has been observed in liposome medium (Çelik, Gökmen, and Skibsted 2015). However, the situation may not always be observed as explained above. For instance DF bound antioxidants (of whole wheat-flour, -paste, -bread) exerting antioxidant properties in aqueous radical environment, were found to exhibit antagonistic relationship with Trolox; and exerting prooxidant properties in liposome environment were found to exhibit synergistic relationship with Trolox. At this point, it should be remembered that many factors have a combined effect on these synergistic/antagonist interactions (Çelik et al. 2017; Çelik, Rubio, and Gökmen 2018).

Effect of reaction medium

As mentioned above, the reaction medium have also revealed to affect the antioxidant/prooxidant behavior of free and bound antioxidants, and hence their interaction with other compounds or matrices. At this point, it is important to understand the reason behind the antioxidant/prooxidant behavior of a single matrix or compound in a certain media. This in turn may help to understand the reason behind the interactions with other matrices or compounds. In this context, it would be a great contribution to explain the prooxidant behaviors especially observed in the liposome medium.

Liposome medium

Liposomes are spherical vesicles consisting of one or more phospholipid bilayers. The similarity of their structures to human cell membrane locates them in an important place, in which they give an idea about the conditions *in vivo*. In liposome medium, oxidation is investigated at the formation of lipid oxidation products stage.

The primary reason for the prooxidant behavior of DF-bound antioxidants in liposome medium is considered to be the presence of redox-active metals. These may come from the fiber itself or already exist in the medium (an oxidizing agent including $FeCl_3$ is used to start the peroxidation of

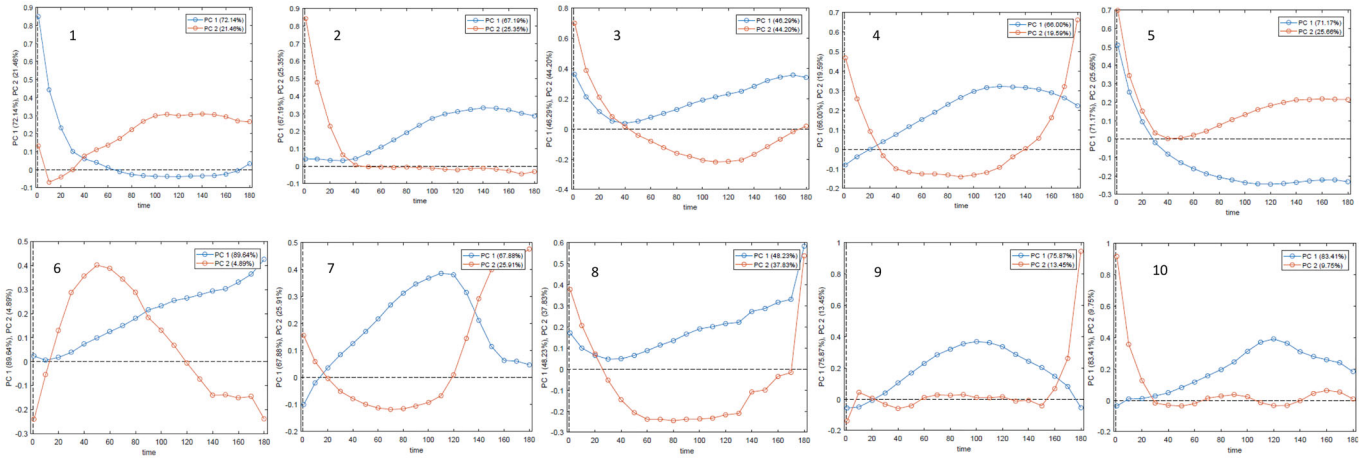

Figure 2. Loadings plots obtained for the mixtures of: (1) whole wheat flour, (2) whole wheat paste, (3) whole wheat bread, (4) soybean, (5) edamame, (6) boiled soybean, (7) soymilk, (8) tofu, (9) extra virgin olive oil, (10) refined olive oil with Trolox in liposome medium (Çelik et al. 2017).

liposomes in the studies mentioned). Redox active metals can cause ascorbate (present in the liposome system) and the phytophenolics to promote oxidation, which may result in prooxidant effect (Li and Trush 1994; Rahman et al. 1989; Yamanaka, Oda, and Nagao 1997). In addition to this, DF-phenolate groups may create a chain carrying effect by forming free radicals, which in turn will create a prooxidant effect (Çelik and Gökmen 2014). However, the interaction between DF-bound phenolics and Trolox in liposome media was found to be synergistic. This has been attributed to the ability of Trolox to regenerate DF-bound phenolics or higher reactivity of Trolox for reaction with Fe^{3+} or radical species present in the environment.

Similar to DF-bound antioxidants, protein-bound antioxidants of soybean products have also been observed to exert prooxidant effect in liposome medium (Çelik et al. 2017). The prooxidant behavior of protein-bound antioxidants may be caused by the tertiary structure of proteins. If amino acids, which are capable of interacting with free radicals, are trapped in such a way that they cannot reach the radicals, their antioxidant activities may be limited. In addition to this, the energy of protein radicals formed as a result of their antioxidant action is also important. If this energy is high enough to promote oxidation, the antioxidant activity displayed will be pointless (Elias, Kellerby, and Decker 2008). Also, metals linked to protein chelators can show a proxidative effect if they retain their ability to form a redox chain (Rahman et al. 1989). The prooxidant effect resulting from one or all of these reasons can easily produce an antagonistic effect with Trolox as well in the liposome medium.

On the other hand, the situation was rather complex for lipid-bound antioxidants. Extra virgin and refined olive oil bound antioxidants have found to be prooxidant themselves in liposome medium, while their interactions with Trolox was not the same of each other. Refined olive oil have found to act synergistic with Trolox while extra virgin olive oil was antagonistic. This was thought to be due to the fact that phenolic substances in olive oil exhibited prooxidant effect in the presence of Fe^{3+} in the liposome medium (Rahman et al. 1989). It has been introduced that refined olive oil, whose phenolic content is decreased with the refining process, has a less prooxidant effect, which in turn facilitating its regeneration by Trolox. Though, the prooxidant activity of phenolic substances, which are found in higher amount in extra virgin olive oil, was thought to be too high to be eliminated by Trolox.

As a result of the studies, it was observed that the oxidation curves for the interactions of macromolecule-bound antioxidants with Trolox in liposome environment were sigmoidal, including the lag phase before the onset of oxidation, initiation and propagation phases (Çelik et al. 2017). Therefore, the kinetic modeling of lipid oxidation has been successfully done using a generalized version of the logistic function given below (Özilgen and Özilgen 1990).

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

F is the intensity of the fluorescence signal, A is the initial level of oxidation, k is the reaction rate, t is time, B is the constant to adjust the model and the ratio of A/B represents the maximum level of oxidation.

In addition to this, it has been observed that liposome oxidation proceeds at two stages in the presence of Trolox and bound antioxidants. These phases were revealed through the loadings plots obtained as a result of the PCA (Principal Component Analysis) applied to the multivariate data, obtained by monitoring the secondary lipid oxidation products in liposomes for 180 min in 10 min intervals (Figure 2). The lag phase followed by the uninhibited oxidation phase were observed between 20–40 min and 60–120 min, respectively in the presence of different bound antioxidant + Trolox mixtures (Çelik et al. 2017).

Aqueous radical medium

Interactions between macromolecule-bound and free antioxidants have been investigated in various aqueous radical media, consisting of ABTS, DPPH and Fremy's salt radicals until today. The AC measurements in these media have been made according to the QUENCHER procedure.

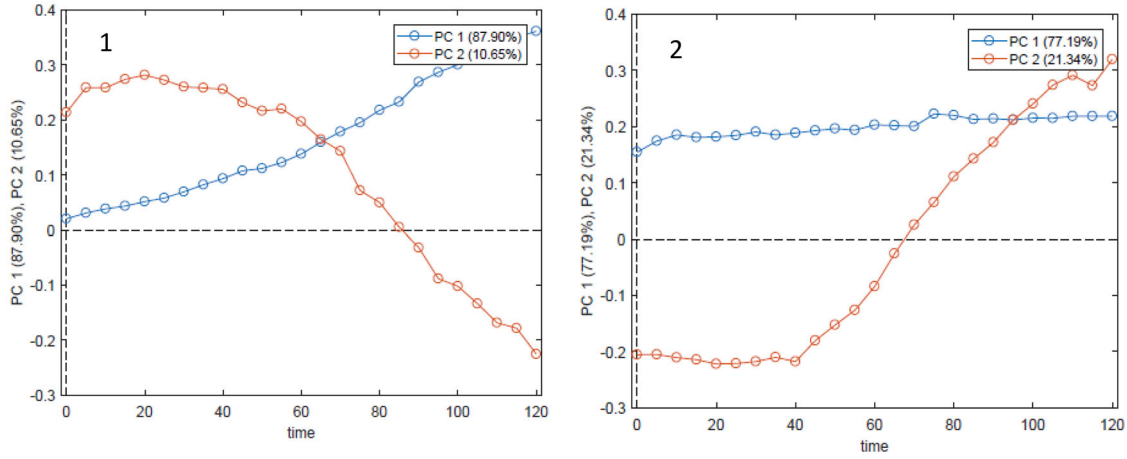

Figure 3. Loadings plots obtained for the mixtures of (1) 2-hydroxy-4,6-dimethoxybenzoic acid, (2) 2,4,5-trihydroxybenzoic acid with whole wheat-bound antioxidants in DPPH radical medium (Çelik et al. 2019).

Oxidation in the aqueous radical medium is investigated at the radical scavenging stage, unlike liposome medium.

In general, it has been observed that the antioxidant/prooxidant behaviors or interaction types of the compounds or matrices differ in the aqueous radical environment and liposome environment. For instance, when DF, protein and lipid-bound antioxidants used in the liposome studies were examined in the aqueous ABTS radical environment, an antioxidant behavior was observed for each, in contrast to their prooxidant effects in liposome environment. However, it has been observed that the interactions of the same bound antioxidants with Trolox were antagonistic, except few cases.

When the cause of this antagonism was investigated, strong evidence was obtained that the autoregeneration mechanism of Trolox was inhibited by bound antioxidants. However, before discussing this, it will be useful to briefly explain the autoregeneration mechanism of Trolox. In 1989, Thomas and Bielski showed that Trolox c, which was induced to oxidize with Br_2^-, regenerated Trolox c as a result of a series of reactions: oxidation of Trolox c first forms its phenoxyl radical, which converts into Trolox c and a cross-conjugated ketone. The conjugated ketone formed is then oxidized to form a quinone (Thomas and Bielski 1989). Inhibition of this reaction in the presence of bound antioxidants has been demonstrated by mass spectrometry analysis of the final product quinone, in the ABTS radical medium containing Trolox alone or together with DF-bound antioxidants. Consequently, it was confirmed that the amount of quinone formed is decreased by 10%, in the presence of DF-bound antioxidants in aqueous radical media. This is thought to be due to the inability of Trolox to undergo autoregeneration reaction as a result of its effort to regenerate the depleted bound antioxidants. At this point, it should be noted that the regeneration phenomenon may result in an antagonistic effect as well as a synergistic effect.

On the other hand, interactions between Trolox and free forms of the DF, protein and lipid-bound antioxidants used in this study were also tested to determine whether inhibition of autoregeneration reaction of Trolox only occur due to DF-bound antioxidants. As a result, antagonistic interactions were also observed for the free forms of some whole wheat DF-bound (ferulic, caffeic, p-coumaric acid) and olive oil-bound antioxidants (rutin, quercetin) as well as soybean free amino acids (cysteine, methionine, tryptophan, tyrosine, phenylalanine, histidine).

Each antioxidant has its individual dynamic system. The autoregeneration reaction of Trolox is an example of this situation. The dynamic systems of antioxidants in itself are one of the important factors that determine their interaction with other compounds or matrices.

Apart from this, aqueous radical media was found to be less efficient than a liposome medium in revealing differences between antioxidant/prooxidant behaviors and interactions between different compounds or matrices. Because, in aqueous radical media, the same behavior tends to be seen for all tested samples. For instance, when interactions between whole wheat-bound antioxidants and some hydroxycinnamic acid (HCA)/hydroxybenzoic acid (HBA) derivatives were examined in aqueous radical (DPPH) and liposome media, synergistic interactions were found to be dominant in aqueous medium while both synergistic and antagonistic interactions were observed in liposome medium (Çelik et al. 2019). The main reason of this situation is considered to be the difference between the nature of the antioxidant mechanisms in these two systems.

As a result of free and bound antioxidant interaction studies, carried out in various aqueous radical environment, oxidation has been shown to proceed at two stages. This was done through the loadings plots obtained from the PCA. In this context, the loading plots obtained as a result of the interaction studies carried out in the DPPH radical environment of whole wheat-bound antioxidants and two different HBA derivatives are given as an example in Figure 3 (Çelik et al. 2019). The intersection of principal component (PC)-1 and PC-2 around 60–100 min have been thought to correspond to the depletion of the HBA derivatives in the medium, either by scavenging DPPH radicals or regenerating whole wheat-bound antioxidants. After this point, the majority of the antioxidant action was thought to be due to the whole wheat-bound antioxidants. This regeneration phenomenon of DF-bound antioxidants by free

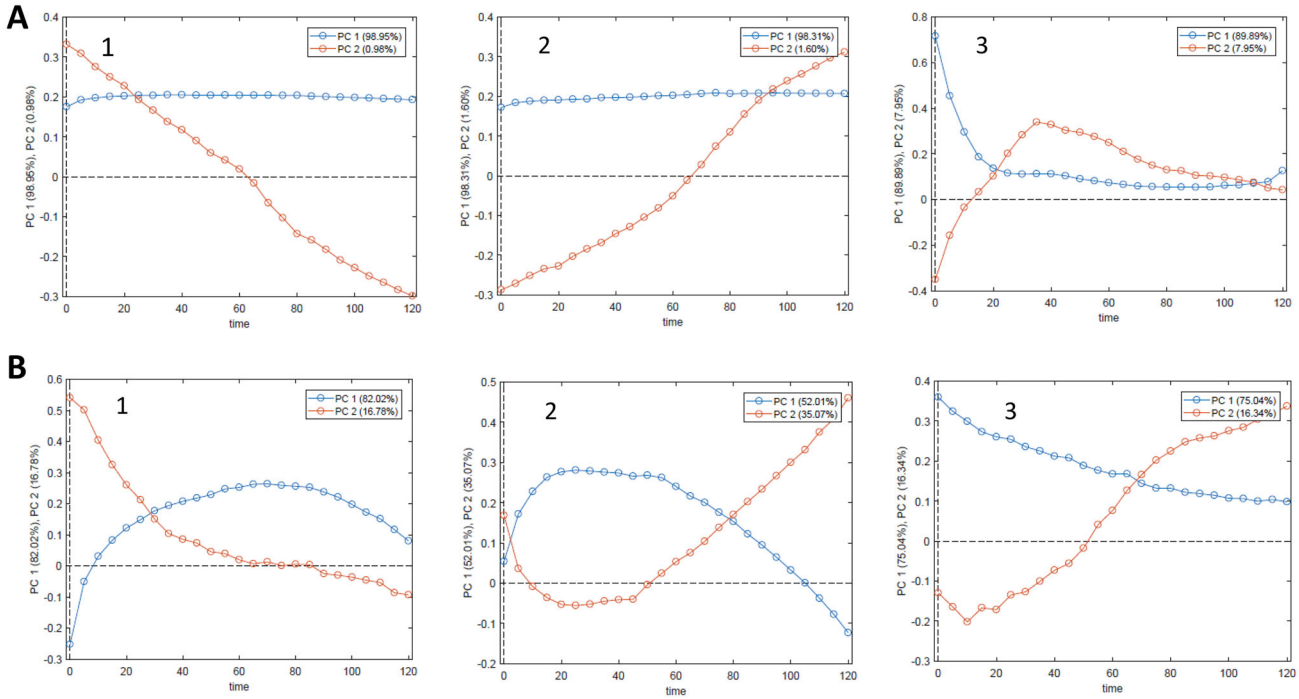

Figure 4. Loadings plots obtained for the mixtures of (A) coffee melanoidins with (A.1) o-coumaric acid, (A.2) p-coumaric acid, (A.3) syringic acid; (B) bread crust melanoidins with (B.1) m-coumaric acid, (B.2) caffeic acid, (B.3) 2,4,5-trihydroxybenzoic acid in DPPH radical medium (Çelik, Rubio, Andersen, et al. 2018).

soluble antioxidants was revealed in a previous study (Çelik, Gökmen, and Fogliano 2013).

The same pattern has also been observed for the interactions between melanoidins, the thermal process sourced DFs, and HCA/HBA derivatives. An example of the loadings plots obtained from a study investigating the interactions between coffee and bread crust melanoidins with some HCA/HBA derivatives in DPPH radical medium is given in Figure 4 (Çelik, Rubio, Andersen, et al. 2018). The intersection of PC-1 and PC-2 at around; 20–40 min in Figure 9(A.1) and 9(B.1), 80–100 min in Figure 9(A.2) or 60–80 min in Figure 9(B.3) were thought to correspond to the depletion of HCA/HBA derivatives in the medium as explained before. This was probably due to the scavenging of DPPH stable radicals or the regenerative activities of HCA/HBA derivatives on melanoidins. After this point, the majority of the antioxidant action was thought to be exerted by the melanoidins. The two different timings observed for the depletion of HCA/HBA derivatives on the other hand, were thought to indicate the early and later stages of the radical scavenging reactions. In addition to this, a different pattern was observed in the loadings plot including two different intersection points of PC-1 and PC-2 as shown in Figure 9(A.3) and 9(B.2). In this pattern, the first intersection point around 20–30 or 10–20 min is thought to correspond to the depletion of HCA/HBA derivatives, after which the majority of the antioxidant effect is started to be exerted by melanoidins. On the other hand, the second intersection point is thought to indicate the moment where the antioxidant action is started to be exerted by regenerated melanoidins.

Effect of concentration

The concentration of a compound or matrix is an important factor both in the emergence of its antioxidant/prooxidant effect and for its interaction with other compounds or matrices. In this context, several phytochemicals including quercetin, catechins and gallic acid have been reported to exert prooxidant effect at high doses, although they have previously counted as antioxidants (De Marchi et al. 2009; Galati et al. 2006). Likewise, most amino acids have been reported to show antioxidant activity at very low concentrations, but act prooxidative at increased concentrations (White and Xing 1997).

This was also demonstrated for the interactions between macromolecule-bound and free antioxidants, both in aqueous and liposome media (Çelik et al. 2017; Çelik, Rubio, Andersen, et al. 2018, Çelik et al. 2019; Çelik and Gökmen 2014). However, the effect of concentration may not be the same for every compound or matrix in all cases. It can be customized for the reaction environment and conditions and other species in the medium. For example, it has been observed that the AC of insoluble wheat bran does not change significantly as a result of the treatment with increasing concentrations of EC, but increases linearly from 9.56 to 25.76 mmol Trolox/kg insoluble wheat bran when EC was replaced with EGCG. On the other hand, it has been observed that the AC of insoluble wheat bran increases as a result of treatment with green tea, but this increase decreases as the green tea concentration increases from 1 g/100 ml to 5 g/100 ml (Çelik and Gökmen 2014). In addition to this, the interaction between 3,5-dihydroxycinnamic acid

Table 2. *p* values within 0.95 confidence interval obtained with ASCA for [WW-bound aox]: Whole wheat-bound antioxidant concentration, [HCA/HBA]: HCA/HBA concentration, [HCA/HBA] * [WW-bound aox]: The interaction between HCA/HBA and whole wheat-bound antioxidant concentrations (Çelik et al. 2019).

	[WW-bound aox]	[HCA/HBA]	[HCA]*[WW-bound aox]
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

and whole wheat bound antioxidants were found to be synergistic at high concentrations and antagonistic at low concentrations of both types in liposome media. However, in aqueous radical environment, it was observed that this interaction was synergistic for all concentration combinations (Çelik et al. 2019).

The effect of concentration on AC has been demonstrated by ASCA (Anova Simultaneous Component Analysis), which enables the application of Anova (analysis of variance) to multivariate data. The fundamental role of ASCA is to reveal the effect of variables on the response (Smilde et al. 2005). In addition to this, it has been demonstrated by PCA, which is used to extract the most important information in multivariate data and reveals the similarities between observations (Jackson 1991).

In this context, ASCA results obtained from a study performed in the liposome environment with different concentrations of whole wheat-bound antioxidants and some HCA/HBA derivatives are given in Table 2 as an example. Here, it is possible to see the individual effects of HCA/HBA and whole wheat-bound antioxidant concentrations as well as their two-way interaction effect. The results revealed that both HCA/HBA and whole wheat-bound antioxidant concentrations have an important effect on the resultant AC (with some exceptional combinations), in 0.95 confidence interval. The interaction of HCA/HBA and whole wheat-bound antioxidant concentrations also had a significant effect on AC, except the combination of o-coumaric acid with whole wheat-bound antioxidants (Çelik et al. 2019).

Scores plot examples obtained as a result of PCA performed within the scope of the same study are also given in Figure 5. Accordingly, no clear distinction have been observed in the plots colored by whole wheat-bound antioxidant concentration (Figure 10.A.1 and 10.A.2), while a clearer distinction could be observed in the plots colored by HCA/HBA concentration (Figure 10.B.1 and 10.B.2). This was probably due to the narrow concentration range choosed for whole wheat-bound antioxidants, which prevent observation of a more pronounced difference (Çelik et al. 2019). In any case, PCA results should be considered as supportive information for ASCA results.

Effect of pH

pH is known to be a determinant factor on the AC of many compounds. Phenolic acids including gallic, caffeic, syringic, sinapic, ferulic and vanillic acids and quercetin have been reported to have a higher AC at pH 7.4 comparing with pH 3.5 (Di Majo et al. 2011). Similarly, the AC of cysteine and histidine have been reported to decrease at acidic conditions (Patterson and Leake 1998). In another study, phenolic compounds including caffeic, protocatechuic and chlorogenic acids and α-tocopherol were found to exert weak antioxidant properties at pH 4.0, but exert increased antioxidant properties with increasing pH to 8.0 (Amorati et al. 2006). Accordingly, the radical scavenging efficiencies of the phenolics tested are considered to be low in acidic environments such as gastric lumen, but high at pH 7.0–8.0 in the intestinal tract, blood, extracellular fluid and within the cell.

The pH of the reaction medium is also important in terms of the interactions between free and bound antioxidants. This situation was clearly observed when interactions of Trolox with various macromolecule-bound antioxidants were examined at different pH values of the ABTS radical environment.

In these studies, the pH of the radical medium was altered as 3.0, 5.0 and 6.0, together with the changes in the concentrations of Trolox and DF and protein-bound antioxidants. The multivariate data obtained was then subjected to a multiple comparison test by using one-way Anova to determine the significance of the effects of concentration, pH and their two and three-way interactions. The *p* values calculated in 0.95 confidence interval are given in Table 3. Accordingly, bound antioxidant and Trolox concentrations were found to make a significant effect on the percentage inhibition of ABTS radical for certain combinations (except boiled soybean, soymilk, tofu + Trolox mixtures for bound antioxidant concentration; for whole wheat flour, edamame, extra virgin olive oil, refined olive oil + Trolox mixture for Trolox concentration). On the other hand, pH was shown to make a significant effect on the inhibition of ABTS radical, for all combinations of Trolox with DF and protein-bound antioxidants, except whole wheat flour. All 2 and 3-way interactions of pH with Trolox and bound antioxidant concentrations were also significant. Especially, pH 3.0 yielded a higher overall AC in general, being significantly different from either pH 5.0 or 6.0 depending on the matrix tested.

The most striking outcome of this study, comparing with the literature, is that the effect of pH on the AC is different for compounds or matrices, individually and in case of interactions. Although the AC of compounds have been reported to decrease individually at acidic pH, they have been shown to exert a higher total antioxidant effect at low pHs when they interact with each other. At this point, it is believed that the interactions between bound and free

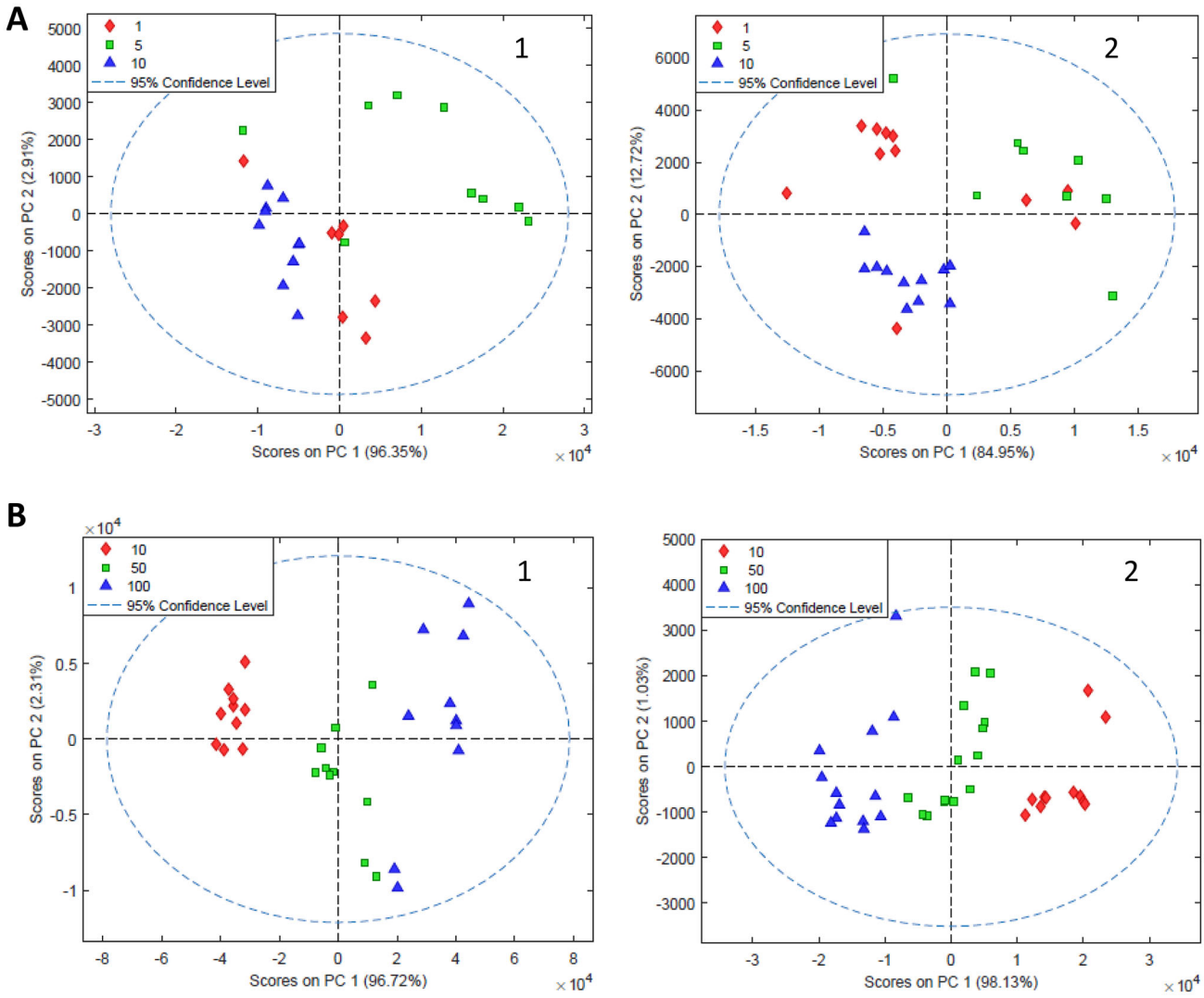

Figure 5. Scores plots colored by (A) whole wheat-bound antioxidant concentration and (B) HCA/HBA concentration, obtained for the mixtures of (A.1) o-coumaric acid, (A.2) p-coumaric acid, (B.1) 2,4-dihydroxycinnamic acid, (B.2) gallic acid, with whole wheat-bound antioxidants in liposome medium (Çelik et al. 2019).

Table 3. 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 (Çelik, Rubio, and Gökmen 2018).

	[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

antioxidants will also differentiate at different parts of the GI tract, with the changing pH.

Effect of the chemical structure of free antioxidant

The effect of the chemical structure of free antioxidant, which interacts with the bound-antioxidant, on the resultant AC was also investigated. Evidence for these statement have been obtained from the studies investigating the interactions between HCA/HBA derivatives having different substitution patterns of -OH and -OCH_3 groups on their aromatic rings and whole wheat bound antioxidants in liposome and DPPH radical media. The interactions of the same HCA/HBA derivatives have also been investigated with coffee and bread crust melanoidins, which are counted as DF, in DPPH radical medium. All these studies have contributed to the understanding of the structural nature of the interactions. Within the scope of these studies, 21 different HCA/HBA derivatives have been examined as illustrated in Figure 6.

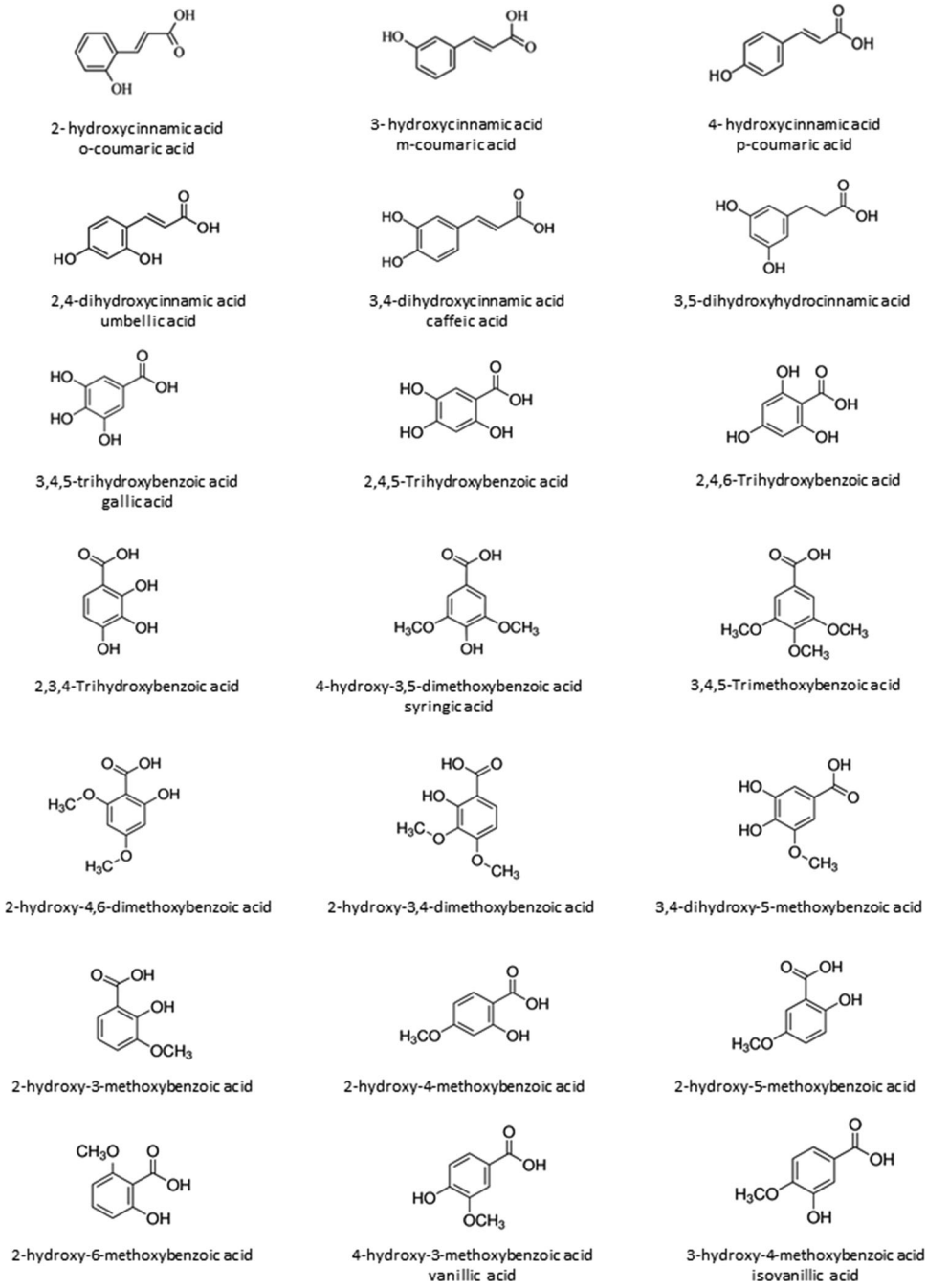

Figure 6. HCA/HBA derivatives (Çelik et al. 2019).

Consequently, the same free antioxidant compound was shown to have different effects in different radical media (liposome or aqueous), the behavioral difference being more pronounced in liposome medium. In aqueous medium, almost all HCA/HBA derivatives (except 2,4,5 trihydroxybenzoic acid) exerted a synergistic effect with whole wheat-bound antioxidants, despite their chemical structure. Similar synergistic table was also observed for the interactions between the great majority of HCA/HBA derivatives and coffee and bread crust melanoids. Only some combinations of 2,4,5 trihydroxycinnamic and gallic acids with coffee melanoidins; and some combinations of 2,4-dihydroxycinamic, 2,3,4-trihydroxycinnamic, 2,4,5-trihydroxycinnamic, gallic and syringic acids with bread crust melanoidins have exerted antagonistic interactions.

In DPPH radical medium, both coffee and bread-crust melanoidins and the whole wheat-bound antioxidants have been found to act as antioxidants themselves. Meanwhile, the antioxidant/prooxidant properties of the HCA/HBA derivatives have changed according to their –OH and -OCH_3 substitutions. As a rule of thumb, HCA/HBA derivatives with one –OH group exerted weak antioxidant or prooxidant properties. When the number of -OH group increased to two, the strength of antioxidant activity increased, and when it reached to three, the antioxidant behavior was stimulated more (except 2,4,6-

trihydroxybenzoic acid acting as a weak antioxidant), in good agreement with the literature (Arora, Nair, and Strasburg 1998). Substitution with $-OCH_3$ groups either have decreased the antioxidant strength or lead to a prooxidative behavior, in accordance with the existing knowledge (Rice-Evans, Miller, and Paganga 1996). In this context, 3,4,5-trimethoxybenzoic acid was found to be the only strong prooxidant among all HCA/HBA derivatives tested. Nevertheless, syringic acid have exerted a strong antioxidative behavior despite its two $-OCH_3$ groups. This was thought to be due to the hydrogen enhancing effect of the$-OCH_3$ groups located next to the (3^{rd} and 5^{th} positions) –OH (4^{th} position) group on the aromatic ring (Rice-Evans, Miller, and Paganga 1996). Similarly, 3,4-dihydroxy-5-methoxybenzoic acid have exerted a strong antioxidant behavior, probably due to the location of its –OH groups (3^{rd} and 4^{th} positions), which were together mentioned to induce high AC.

It has been demonstrated that the common property of the antagonistic acting HCA/HBA derivatives is their strong antioxidant characteristics. This have suggested that there could be a concentration-dependent prooxidative effect in the co-existence of these compounds and especially high AC melanoidins. This phenomenon has been demonstrated for the phenolic compounds in biological systems (Dintcheva et al. 2017). The metal chelating or metal reducing properties of these compounds at high concentrations can be responsible from this situation, which retains or increases their catalytic activity or increases their ability to form free radicals, respectively (Croft 1998). Besides, the quinones formed as a result of the oxidation reactions of high-AC HCA/HBA derivatives can attack to melanoidins and prevent their radical scavenging activities. The redox-active properties of quinones, which leads to the formation of reactive oxygen species is well-known in literature as well (O'Brien 1991).

In liposome environment the interaction of whole wheat-bound antioxidants with o-, m- and p-coumaric acids have been shown to be barely synergistic at low concentrations of both types, which shifted to the antagonistic site with their increasing concentrations. The opposite behavior has been demonstrated for 3,5-dihydroxycinnamic acid. Meanwhile, the interaction of whole wheat-bound antioxidants have been demonstrated to be strongly antagonistic with 2,4-dihydroxycinnamic acid and significantly synergistic with gallic and caffeic acids.

This divergent pattern observed in liposome medium has been explained by the nature of the HCA/HBA derivatives. Among HCA/HBA derivatives tested, o- and p-coumaric acids have been found to act as weak antioxidants, while m-coumaric and 3,5-dihydroxycinnamic acids have been found to act as weak prooxidants. On the other hand, 2,4-dihydroxycinnamic acid have been found to act as a strong prooxidant, while caffeic and gallic acids have been found to act as strong antioxidants. It is known that orto- and para-substitution with another –OH group increases the stability of phenoxyl radical and thus the AC (Ma et al. 2011). This explains the weak antioxidant or prooxidant nature of o-, m- and p-coumaric acids comparing with their 2 –OH containing derivatives like caffeic and gallic acids. Prooxidant behaviors of the meta-substituted 3,5-dihydroxycinnamic and 2,4-dihydroxycinnamic acids also becomes meaningful in this context. Besides, the fact that an –OH group at 3^{rd} position and 3′,4′-dihydroxyphenyl (catechol) structure on B ring contribute to high antioxidant activity also explains the high AC values of caffeic and gallic acids (Arora, Nair, and Strasburg 1998; Bors et al. 1990). At this point, attention should be paid to the fact that strong antioxidants such as gallic and caffeic acids and strong prooxidants such as 2,4-dihydroxynnamic acid exerted a determinedly synergistic or antagonistic behavior, respectively. On the contrary, HCA/HBA derivatives having weak antioxidant or prooxidant properties were able to exert both synergistic and antagonistic behaviors according to their concentrations.

In liposome medium, it has been observed that an additional –OH group in the 3rd position contributes to the synergistic behavior. The synergistic behavior of 3,5-dihydroxycinnamic acid at high concentrations also supported this observation. Besides, it has been thought that an additional –OH group in the 5^{th} position, as in gallic acid, is also able to contribute to the synergistic effect. On the contrary, it has been observed that presence of an –OH group in 2^{nd} position can support antagonistic behavior, as in the case of 2,4-dihydroxycinnamic and o-coumaric acids. In this regard, two scenarios have been appeared for the –OH groups in 4^{th} position. If there is an accompanying –OH group in; (i) meta-position, it acts antagonistic, (ii) ortho-position, it acts synergistic.

However, the dominant synergistic interactions observed in the aqueous radical medium prevented such interpretations. The main reason for this may be the difference in antioxidant mechanisms in these reaction media. In liposome system, oxidation is induced by metal ions and the antioxidant effect consist of the combination of metal chelating and radical scavenging activities (Arora, Nair, and Strasburg 1998). However, in DPPH radical system, the antioxidant effect only includes radical scavenging either by H-atom or electron transfer. Accordingly, it has been thought that metal-induced oxidation reactions may be more effective in determining the AC than radical-induced ones. For metal chelating, flavonoids have been reported to need a 3′,4′-dihydroxy configuration, a C-4 carbonyl group and a C-3 or C-5 -OH group (Charles 2013). These specifications may help to explain the behavioral differences of HCA/HBA derivatives in the liposome system compared to the aqueous radical system.

Interactions between the antioxidants in foodstuffs

Investigating the interactions between free and bound antioxidants found in frequently consumed foodstuffs is an important attempt for estimating the antioxidant environment that may form after their consumption. In this context, the interactions between coffee, as one of the most widely consumed beverages in the world, and dark chocolate, as a highly preferred, phenolics-rich accompaniment

have been investigated. In addition to this, interactions between the insoluble fractions of coffee infusions and the major cocoa free antioxidants, catechin and epicatechin, have been investigated. The latter helped to understand whether interactions occurred especially between the insoluble fractions of coffee infusions and cocoa free antioxidants, or between the total antioxidant contents of these components. Coffee infusions, obtained with various brewing methods; expresso, French press, filter coffee and Turkish coffee have been used in these studies to reveal the effect of brewing step as well. All AC measurements have been performed in the aqueous DPPH radical medium (Çelik and Gökmen 2018).

The results have demonstrated that the amount of dry matter passed through the infusions and the insoluble fraction contained in this dry matter has changed depending on the brewing method. The contact time between water and coffee, and the coffee: water ratio was thought to be the determinant factors for this. Increase in the contact time and decrease in the coffee: water ratio, probably by increasing the contact surface between coffee and water, might have increased the extraction efficiency. In this context, the highest amount of insoluble fraction per 1 gram of ground roasted coffee was detected in Turkish coffee, whose preparation took longest. Meanwhile the lowest amount was detected in espresso, probably due to the difficulty of water reaching the coffee grounds through the pressed coffee cake.

It is known that the amount of phenolic compounds and the AC values of the infusions change according to the brewing method (Sánchez-González, Jiménez-Escrig, and Saura-Calixto 2005). However, conflicting studies on the literature have not yet produced a definitive judgment on the effects of brewing methods (Sánchez-González, Jiménez-Escrig, and Saura-Calixto 2005; Niseteo et al. 2012). When the AC values measured in the total dry matter and insoluble fraction of the coffee infusions were compared, it was observed that the AC value in the dry matter was approximately 10–30 times the insoluble fraction. This showed that AC is mostly provided by free antioxidants. It has been observed that the interactions between the insoluble fractions of coffee infusions and the catechin/epicatechin are synergistic only for espresso and antagonistic/additive for the insoluble fractions of other infusions. It is thought that this may be due to the fact that the catechin/epicatechin regenerates the insoluble fraction of espresso. However, it will be surprising that only the regeneration reaction is effective in the synergistic interaction of espresso, which contains the lowest insoluble fraction per 1 gram of coffee. On the other hand, it has been observed that the interactions between dark chocolate and coffee infusions are synergistic for Turkish coffee and French press and antagonistic/additive for the rest of the infusions. Free antioxidants in Turkish coffee and French press and/or other antioxidants or components other than catechin/epicatechin in chocolate may be the reason for this situation. Similarly, free antioxidants found in espresso and other antioxidants or components found in chocolate may also be responsible for the additive effect (Çelik and Gökmen 2018). This is the only study that examines the interaction between free and bound antioxidants found in prepared foodstuffs. This is why only coffee and chocolate interactions are discussed under this heading. However, research on this subject needs to be expanded to include other frequently consumed food items and *in vivo* conditions.

Conclusion

In this review, interactions between macromolecule-bound antioxidants, whose importance in human nutrition is recently interiorized, and free antioxidants commonly consumed with them in the daily diet are discussed. After understanding that DF-bound antioxidants can be regenerated by free antioxidants, literature on the interactions between free and bound antioxidants has begun to form. Through the limited number of studies, that have been performed to clarify the potential reaction mechanisms and cause-effect relationships, the point reached in today's understanding of the interactions between bound and free antioxidants has been centered. It has been revealed that interactions between free and bound antioxidants have a dynamic balance that can easily change under the influence of various factors. Food is a dynamic system per se, with all the components it contains, their proportions and its rapidly changing structure. Considering that other factors such as antioxidant concentrations, reaction environment and conditions contributed to this dynamism, it is better understood how special the interactions should be handled for each situation. Thus, information obtained as a result of each study is considered to be unique for that specific conditions. However, in the light of the information obtained, an overall view for the interactions between free and bound antioxidants, without speculations, has been tried to be established.

Despite this complex system, it is still essential to investigate the state of the mechanisms, illuminated under *in vitro* conditions, *in vivo* with further studies. Revealing the health effects in colon that may be caused by the interactions of macromolecule-bound and free antioxidants will carry the acquired theoretical knowledge one step further. After this point, nutritional designs should be on the agenda through suggested interaction mechanisms. In this context, meal arrangements can be made, including the consumption of compounds or matrices that are known to act synergistically. An example of this is the combination of black tea and whole grain cookies containing gallic and acid whole wheat-bound antioxidants, respectively, which are known to act synergistically in the liposome environment. In addition to this, new functional food designs including ingredients having bound and free antioxidants that are proven to act synergistically can be worked on.

Disclosure statement

No potential conflict of interest was reported by the authors.

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