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Soluble Antioxidant Compounds Regenerate the Antioxidants Bound to Insoluble Parts of Foods

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ABSTRACT: This study aimed to investigate the regeneration potential of antioxidant capacity of an insoluble food matrix. Investigations were performed in vitro with several food matrices rich in dietary fiber (DF) and bound antioxidants. After removal of the soluble fraction, the antioxidant capacity (AC) of the insoluble fraction was measured by the QUENCHER procedure using $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ radicals. After measurement, the insoluble residue was washed out to remove the excess of radicals and treated with pure antioxidant solution or antioxidant-rich beverage to regenerate depleted antioxidants on the fiber. Results revealed that the antioxidant capacity of compounds chemically bound to the insoluble moiety could be reconstituted in the presence of other hydrogen-donating substances in the liquid phase. Regeneration efficiency was found to range between 21.5 and 154.3% depending on the type of insoluble food matrix and regeneration agent. Among the food matrices studied, cereal products were found to have slightly higher regeneration efficiency, whereas antioxidant-rich beverages were more effective than pure antioxidants as regeneration agents. Taking wheat bran as reference insoluble material, the regeneration abilities of beverages were in the following order: green tea > espresso coffee > black tea > instant coffee > orange juice > red wine. These results highlighted the possible physiological relevance of antioxidants bound to the insoluble food material in the gastrointestinal tract. During the digestion process they could react with the free radicals and at the same time they can be regenerated by other soluble antioxidant compounds present in the meal.

KEYWORDS: *bound antioxidants, insoluble food matrix, dietary fibers, regeneration of antioxidant capacity*

INTRODUCTION

Antioxidant compounds contribute to the health potential of foods, and their intake has been correlated to lower incidence for cardiovascular diseases, cancer, aging, and age-related degenerative processes.[1] The measure of total antioxidant capacity (AC) is a useful tool for indicating the nutritional quality of antioxidant-rich foods.[2] However, 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.[3]

Antioxidants may be found in different forms in food microstructure: (1) free from chemical or physical interactions, (2) physically entrapped into food matrix, (3) chemically bound to other macromolecules, or (4) in insoluble form.[2] Antioxidants found as free form (vitamins C and E, carotenoids, low molecular weight polyphenols, and others) are solubilized and totally or partially absorbed either in the stomach or in the small intestine.[4] In complex food matrices they are mostly embedded in the matrix and somehow linked with different macromolecules such as carbohydrates, proteins, and lipids[5] and especially dietary fibers (DFs).

DFs are resistant to digestion and absorption in the small intestine,[6] and this affects the bioavailability of antioxidants by means of reducing the rate of their release.[7] DFs were also reported to retard phenolic antioxidant absorption besides carotenoids and probably α-tocopherol.[8] Hence, a significant amount of dietary antioxidants pass unchanged through the small intestine bound to DF and reach the colon, where they can be released from the fiber matrix by the action of the bacterial microbiota, producing bioactive metabolites that can be absorbed through the colon.[9,10] All nonabsorbable metabolites and nonfermented polyphenols remain in the colonic lumen and contribute to create a healthy antioxidant environment by scavenging free radicals and counteracting the effects of dietary pro-oxidants.[11] 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 gastrointestinal tract.[12] The slow and continuous release of polyphenols bound to DF after fermentation in the gut might contribute to the basal increase of some bioactive metabolites, eliciting anti-inflammatory responses by human bodies.[13] In this framework, bound antioxidants survived a considerable time in the gastrointestinal (GI) tract, and it was hypothesized that they played a central role in the prevention of some pathologies such as colon cancer by quenching free radicals that are continuously formed in the GI tract.[14]

The intestinal lumen should be considered as a dynamic system where, besides the absorption phenomena and the macronutrient degradation processes, also radicals and antioxidants, both soluble and not soluble, react with each other, and antioxidants bound to DF might have a pivotal role.

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This study aimed at investigating the regeneration behaviors of depleted antioxidants present in the insoluble materials, particularly those bound to the DFs from different food sources. Different pure antioxidant compounds and liquid foods rich in naturally occurring antioxidants were used to determine their efficiency to regenerate the in vitro antioxidant capacity of insoluble DFs.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents used were of analytical grade, unless otherwise stated. Potassium peroxydisulfate, cellulose powder, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox), (−)-epicatechin, chlorogenic acid, ascorbic acid, and dehydroascorbic acid were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethyl alcohol, methanol, and *n*-hexane were purchased from Merck (Darmstadt, Germany).

Preparation of the Insoluble Fraction of Food Samples. A total of 16 DF-rich samples including cereal brans (wheat, oat), rye flakes, bread crust, nut skins (hazelnut, peanut, and pistachio), dried fruits (grape, prune, blueberry, black currant), roasted coffee beans, cocoa powder, black pepper, Mexican beans, and red beet skin were purchased from a local market.

On the other hand, the following beverages rich in free soluble antioxidants were used as regenerating agents: green tea, black tea, instant coffee, ground roasted coffee, orange juice, and red wine. All of them were purchased from a local market. Tea and coffee samples were appropriately brewed prior to regeneration treatment.

To remove water- and alcohol-soluble substances, ground DF-rich samples were consecutively washed with a large excess (10 mL mg^{-1} powder) of water, ethyl alcohol, and water. Fatty samples (coffee, cocoa, nut skins, cereal brans, rye flakes, black pepper) were first defatted by hexane to remove lipids and lipid-soluble substances from the samples. For colored samples (roasted coffee, cocoa powder, dried fruits, nut skins, black pepper, Mexican beans, and red beet skin), ethyl alcohol was replaced with the mixture of methanol/1 N HCl (85:15, v/v) in the washing procedure.

One gram of ground sample was mixed with 5 mL of distilled water. The mixture was homogenized for 3 min using an ultra turrax disperser (Heidolph Instruments GmbH, Schwabach, Germany). The mixture was further shaken at 350 rpm for 10 min using an orbital shaker (Edmund Bühler GmbH, Hechingen, Germany). The supernatant containing the soluble fraction was removed after centrifugation at 6080*g* for 5 min. The washing cycle was repeated three times with 5 mL of water, ethyl alcohol, and water, respectively. However, the homogenizer was replaced with a vortex mixer after the first washing cycle.

Washed samples were freeze-dried, ground with a ceramic mortar to a fine powder, and passed through a sieve (Endecotts Test Sieve, London, UK) with a mesh size of 40 (425 μm). The final insoluble powders were tested and found to be free of soluble antioxidant compounds. They were kept frozen (−18 °C) prior to analysis of AC.

Analysis of Phenolic Acids in the Insoluble Fraction. Phenolic acids bound to the insoluble fraction of food samples were analyzed according to the procedure described by Moore et al.[15] Bound forms were released by alkaline hydrolysis (4 N NaOH) for 4 h at room temperature prior to extraction. After the pH was adjusted to 2.0 (6 N HCl), the hydrolysates were extracted with ethyl acetate and diethyl ether (1:1, v/v) four times. The combined extract was evaporated under a N_2 stream at 30 °C to dryness. The final residue was dissolved in 1.5 mL of methanol, filtered through a 0.45 μm nylon filter prior to chromatographic analysis.

Chromatographic analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of photodiode array detector, a quaternary pump, an autosampler, and a column oven. Phenolic acids were separated on a Waters Atlantis C18 column (250 mm × 4.6 mm, 5 μm) (Waters Corp., Milford, MA, USA) using a linear gradient elution program with a mobile phase containing solvent A (formic acid/H_2O, 1:99, v/v) and solvent B (methanol) at a flow rate of 0.8 mL min^{-1}. The solvent gradient was programmed as described by Zilic et al.[16] The identification of phenolic acids was accomplished by comparing retention and spectral data of the peaks to those of standard compounds. The quantitation was based on calibration curves built for each compound identified in the samples.

Analysis of the AC of Insoluble Fraction. The AC of insoluble fractions was measured by using direct QUENCHER procedure with some modifications. ABTS and DPPH radical solutions were prepared as described by Serpen et al.[17] Ten milligrams of insoluble fraction was weighed into a test tube, and the reaction was initiated by adding 10 mL of $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ working solution. The tube was vigorously shaken in an orbital shaker in the dark, at 350 rpm for 27 min ,and centrifuged at 6080*g* for 2 min. The optically clear supernatant (2 mL) was transferred into a cuvette, and measurement was done exactly 30 min after the initiation of the reaction at 734 nm (for ABTS assay) or 525 nm (for DPPH assay) using a Shimadzu model 2100 variable-wavelength UV−visible spectrophotometer (Shimadzu Corp., Kyoto, Japan). When absorbance values fell outside the linear range of the calibration curve, the sample was diluted with cellulose, an inert material against the radical solution. The samples having high AC (dried fruits, nut skins, coffee, cocoa, Mexican beans, black pepper, and red beet skin) were mixed with powdered cellulose at a ratio of 1:10 (w/w). Dilution with cellulose was also useful for the samples having a sticky texture after freeze-drying[2] due to their high pectin content. This increased the success of the grinding step by preventing sample stickiness.

Trolox was used as a standard reference to convert the inhibition percentage of each sample to the trolox equivalent antioxidant capacity (TEAC). The AC values were expressed as millimoles trolox per kilogram insoluble DF. The working solutions of trolox were prepared in methanol at a concentration range between 0 and 600 μg mL^{-1}. For each concentration, 0.1 mL standard solution was transferred into a test tube and mixed with 10 mL of $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ working solution. The mixtures were allowed to stand in the dark for 30 min, at room temperature. Subsequently, 2 mL of radical solution was transferred into a cuvette, and the absorbance was measured at 734 nm (for ABTS assay) or 525 nm (for DPPH assay) to build corresponding calibration curves as described by Serpen et al.[17]

Regeneration of the AC of Insoluble Fraction. The residue after AC measurement contains radical forms of the bound antioxidants and the excess of $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ radicals. Prior to regeneration, the remaining $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ radicals was washed out three times with 10 mL of water. For this purpose, the residue was mixed with water, the mixture was shaken for 10 min using an orbital shaker, and the supernatant was removed after centrifugation at 6080*g* for 5 min. To regenerate the radical forms of bound antioxidants, the residue after the washing process was mixed with 10 mL of aqueous pure antioxidant solution at a concentration of 100 mg L^{-1} or an antioxidant-rich beverage as the regeneration agent. The mixture was shaken for 1 h in the dark (room temperature) using an orbital shaker at a speed of 350 rpm to regenerate depleted antioxidants bound to the insoluble matrix. After that, the mixture was centrifuged at 6080*g* for 5 min, and the supernatant was removed. The residue after the regeneration process was washed out three times with 10 mL of water, ethyl alcohol, and water to remove remaining traces of the regeneration agent. The last washing water was tested and found to be free of soluble antioxidant compounds. Then, the insoluble residue was measured again for its AC using $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ assays to determine the regeneration efficiency of initial AC. The regeneration process was repeated up to three times using the procedure described above.

Aqueous solutions of ascorbic acid (AA), epicatechin (EC), and chlorogenic acid (CGA), and their mixture, at a final concentration of 100 mg L^{-1} were used as pure compounds. Orange juice, red wine, instant coffee, espresso coffee brew, and black and green tea infusions were used as regeneration agents. Forty milliliters of espresso coffee was prepared from 6 g of ground-roasted coffee bean. Instant coffee was prepared by adding 200 mL of hot water to 2 g of soluble coffee. Black and green teas were brewed for 15 min by adding 100 mL of hot

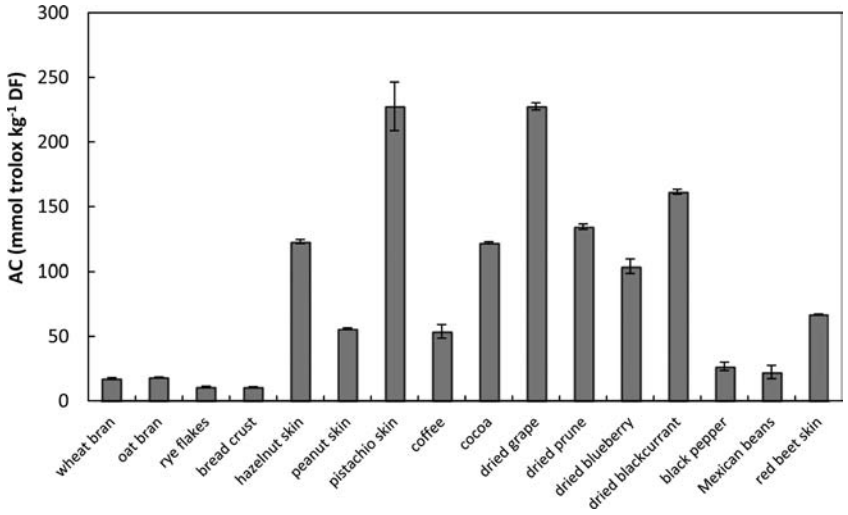

Figure 1. Initial AC values of the insoluble fractions of various food samples determined using the ABTS method.

water to 3 g of dry tea. Orange juice was filtered before use to remove solid particles.

Analysis of Ascorbic Acid (AA) and Dehydroascorbic Acid (DHAA) by LC-MS/MS. An Agilent 1200 series HPLC system coupled to an Agilent 6460 triple-quadrupole mass spectrometer with electrospray ionization operated in negative mode was used to determine AA and DHAA in the samples before and after the regeneration treatment. The chromatographic separations were performed on an HIBAR Purespher-STAR RP-18e (150 mm × 4.6 mm i.d., 5 μm) column using 0.1% formic acid in water and 0.1% formic acid in methanol (95:5) as the mobile phase at a flow rate of 0.5 mL min^{-1} at 30 °C. The electrospray source had the following settings: capillary voltage of 4 kV; fragmentor voltage of 80 V; source temperature at 300 °C; source gas flow at 10 L min^{-1}; sheath gas temperature at 350 °C; sheath gas (N_2) flow of 11 L min^{-1}. AA and DHAA were identified by multiple reaction monitoring (MRM) of two channels. The precursor ion $[M + H]^+$ 175 was fragmented, and product ions 115 and 87 (collision energy of 8 and 16 V) were monitored for AA, and the precursor ion $[M + H]^+$ 173 was fragmented and product ions 113 and 71 (collision energy of 4 and 10 V) were monitored for DHAA. The product ions 115 and 113 were used for quantification of AA and DHAA, respectively. Concentrations of AA and DHAA were calculated by means of a calibration curve built in the range between 1 and 100 ng mL^{-1} (1, 2, 10, 50, and 100 ng mL^{-1}).

Statistical Analysis. The analytical data were reported as the mean ± standard deviation of duplicate independent measurements and were subjected to ANOVA. The significance of mean differences was determined by Duncan's post hoc test using SPSS version 17.0.

RESULTS AND DISCUSSION

The AC values of the insoluble fraction of 16 different food matrices including dried fruits, nut skins, cereal brans, rye flakes, bread crust, coffee, cocoa, black pepper, and Mexican beans are shown in Figure 1. The bound AC values were found to range between 10.8 and 227.5 mmol trolox kg^{-1} insoluble DF.

Wheat bran, oat bran, rye flakes, pistachio skin, hazelnut skin, peanut skin, and dried grape were selected among the samples to investigate their regeneration behavior due to their rich DF content and/or high AC. Ferulic and *o*-coumaric acids were identified as two dominant forms of bound phenolic acids in the insoluble fractions of food samples after alkaline hydrolysis (Table 1).

Ascorbic acid (AA) at a concentration of 100 mg L^{-1} was used to regenerate the initial AC of the above-mentioned samples. It should be noted here that AA at higher concentrations up to 1000 mg L^{-1} did not improve its regeneration efficiency. The AC values of the samples measured during the triple-step regeneration process are shown in Figure 2. There were remarkable differences in the regeneration efficiencies of the AC of different insoluble food matrices. By comparison to their initial AC values measured by the ABTS method (Figure 2a), the regeneration efficiency of the insoluble food matrices ranged from 21.5% (pistachio skin) to 50.5% (peanut skin) in the first step, from 6.3% (pistachio skin) to 32.2% (bread crust) in the second step, and from 6.7% (pistachio skin) to 27.5% (bread crust) in the third step when AA was used as the regeneration agent.

Table 1. Concentrations of Bound Phenolic Acids Identified in the Insoluble Fractions of Various Food Samples

sample	ferulic acid, mg kg^{-1}	*o*-coumaric acid, mg kg^{-1}
wheat bran	2179 ± 56	59 ± 4
oat bran	272 ± 21	23 ± 1
rye flake	134 ± 4	22 ± 1
hazelnut skin	22 ± 3	37 ± 3
peanut skin	231 ± 26	18 ± 3
pistachio skin	240 ± 12	38 ± 2
dried grape	108 ± 49	740 ± 6

As shown in Figure 2b, there were slight differences in the regeneration efficiencies when the DPPH method was used instead of the ABTS method. The regeneration efficiency ranged from 20.3% (pistachio skin) to 73.5% (wheat bran) in the first step, from 8.7% (pistachio skin) to 62.6% (peanut skin) in the second step, and from 9.9% (pistachio skin) to 73.5% (peanut skin) in the third step. This could be due to the fact that $DPPH^{\bullet}$ is solubilized better in less polar solvents, and it is more reactive toward hydrophobic antioxidants.[18]

In general, the regeneration efficiency was the highest in the first step for all food matrices. It tended to decrease sharply for the insoluble fractions of dried grape, peanut, and pistachio skins. Although their initial AC values were lower in comparison to other insoluble food matrices, cereal products were found to have slightly higher regeneration efficiency during triple-step regeneration process.

Differences observed in the regeneration behaviors may arise from differences in the fiber microstructure of insoluble food

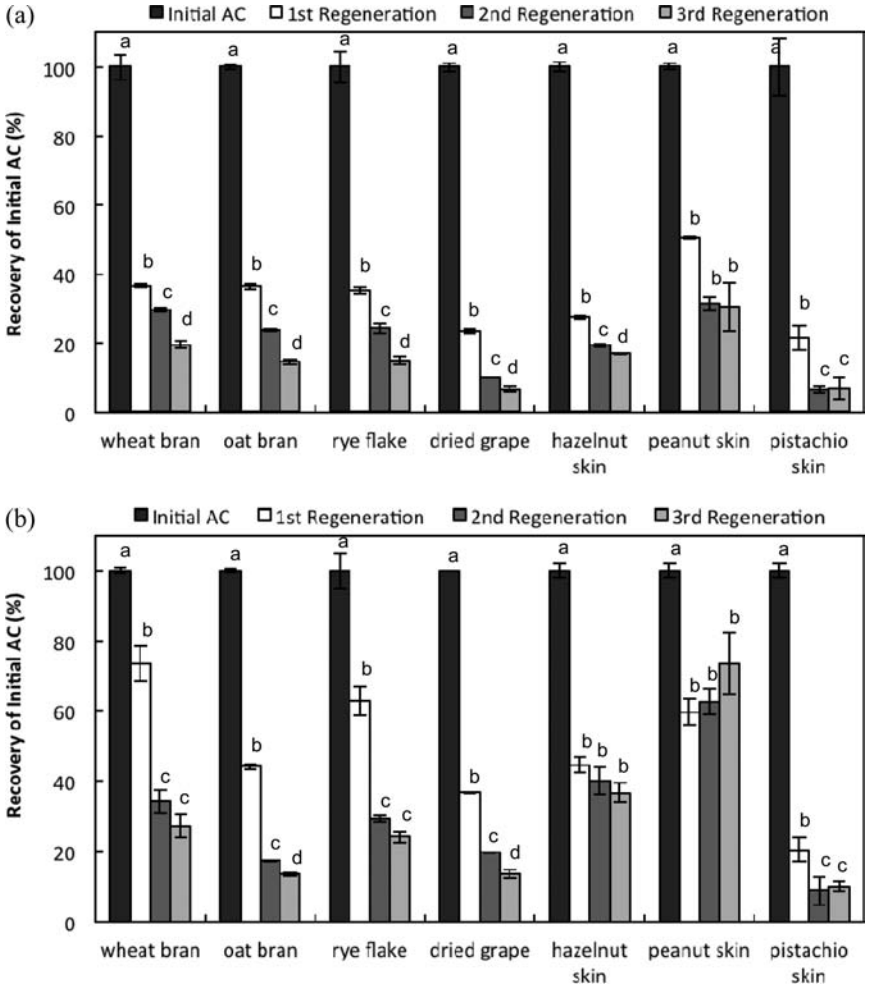

Figure 2. Recovery of initial AC values of the insoluble fractions of various food samples after the first, second, and third regeneration treatments with 100 mg L^{-1} of AA determined by (a) ABTS method and (b) DPPH method. Different letters on the bars indicate statistically significant differences.

matrices and the nature of bound antioxidants. Red-colored skins of pistachio and peanut kernels are known to contain high amounts of condensed tannins.[19] The insoluble tannins have been reported to comprise up to 95% of the total condensed tannins in the hulls of different seeds.[20] Insoluble DFs originating from dried fruits are mainly based on pectic substances, whereas cereal products are rich in arabinoxylans and cellulose-based substances. It is thought that the resistance of fiber structure will strongly influence its regeneration behavior in the digestion system.

Among the tested matrices, wheat bran insoluble material was selected for further investigation for the following reasons: (i) it is rich in DF, which is an essential component for regeneration concept; (ii) it is well-known for the ability to carry antioxidants along the GI tract; (iii) it is well characterized both for the polyphenol and for the carbohydrate moiety; (iv) it is widely consumed.

The effect of different antioxidant compounds on the regeneration of initial AC of wheat bran was determined using AA, EC, CGA, and their ternary mixture as regeneration agents. As shown in Figure 3, the regeneration behavior of wheat bran did not change significantly with the type of antioxidant compound used in this study ($p < 0.05$). At the first regeneration step, the AC of wheat bran was regenerated by

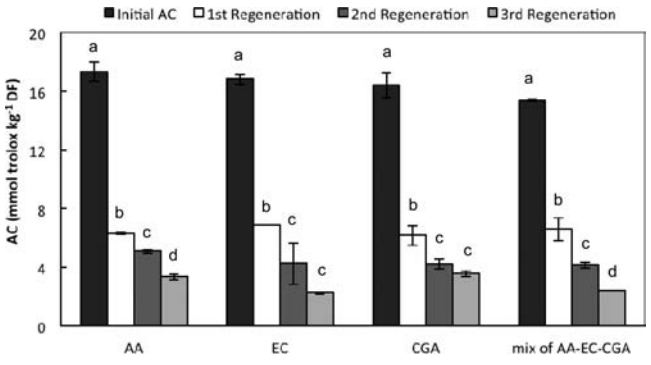

Figure 3. AC values of the insoluble fraction of wheat bran after the first, second, and third regeneration steps with 100 mg L^{-1} of AA, EC, CGA, and a ternary mixture of AA−EC−CGA measured by the ABTS method. Different letters on the bars indicate statistically significant differences.

36.5, 41.1, 37.9, and 43.1% with 100 mg L^{-1} of AA, EC, CGA, and the ternary mixture of AA−EC−CGA, respectively.

Replacing pure antioxidant compounds with antioxidant-rich beverages as the source of regenerating antioxidants caused remarkable changes in the regeneration behavior of wheat bran AC. In general, the regeneration efficiency for all beverages was much higher than that of pure antioxidants used in the present

study. The beverages also differed in their ability to regenerate the AC of wheat bran in the triple-step regeneration process. The AC value of wheat bran was found to be significantly higher ($p < 0.05$) than its initial AC value after the first regeneration step for red wine, instant coffee, black tea, and green tea as shown in Figure 4. After the first regeneration step,

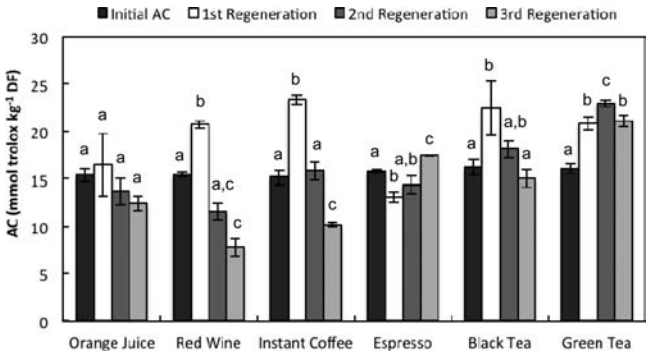

Figure 4. AC values of the insoluble fraction of wheat bran after the first, second, and third regeneration steps with various beverages (orange juice, red wine, instant coffee, espresso, black tea, and green tea) measured by the ABTS method. Different letters on the bars indicate statistically significant differences.

the regeneration efficiencies decreased sharply for red wine and instant coffee, but only slightly for orange juice and black tea at the second and third regeneration treatments. However, regeneration efficiency remained relatively stable for espresso coffee and green tea through the triple regeneration steps. On the basis of the AC of wheat bran measured through triple regeneration steps, regeneration abilities of the beverages were in the order green tea > espresso coffee > black tea > instant coffee > orange juice > red wine. One reason for this may be different reduction potentials of various antioxidants contained in these beverages. The reduction potential is regarded as an important parameter of low or high antioxidative activity of reducing agents as well as the ability of electron transfer from antioxidants to reactive oxygen species.[21]

Results revealed that the antioxidant capacity of compounds chemically bound to DF structure could be reconstituted in the presence of other hydrogen-donating substances in liquid phase. The proposed mechanism for the regeneration of antioxidant capacity bound to insoluble DF is shown in Figure 5a. When an antioxidant chemically bound to insoluble DF structure is exposed to a radical attack, for example, $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ radicals, it will give one of its electrons or a hydrogen atom to quench the radical and become an antioxidant radical itself. According to the proposed mechanism, if antioxidant synergist compounds are freely available in this environment, they will come into contact with the radicals formed on insoluble material and rapidly regenerate them by giving one of their electrons or a hydrogen atom, becoming radicals themselves. More than 70% of AA was converted to its oxidized form of DHAA during the regeneration treatment, providing an experimental confirmation of the mechanism illustrated in Figure 5a.

The in vitro regeneration concept proposed in this study could be easily translated to the different parts of the GI tract and, in particular, to the lower gut lumen, where insoluble foods are present for many hours. Fairly high regeneration efficiencies were obtained especially for cereal products when highly consumed antioxidant-rich beverages were used as regeneration agents. Regeneration efficiencies varied for different insoluble food matrices and regeneration agents. Interestingly, beverages were found to have better regeneration

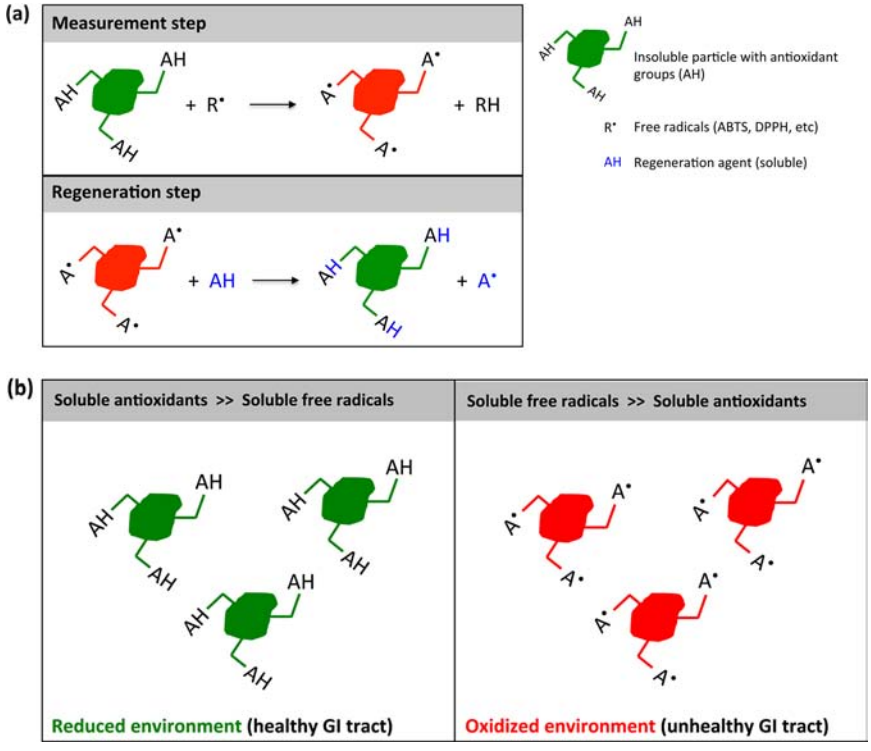

Figure 5. (a) Proposed regeneration mechanism of the AC of the insoluble fraction of foods; (b) potential role of soluble antioxidants and free radicals on DF-bound antioxidants and healthy status of the GI tract.

abilities in comparison to certain pure antioxidant compounds. This can be explained by a synergistic approach in which interactions between beverage antioxidants may occur. Although there was no clear synergism observed between AA, EC, and CGA, the situation might be different for beverages containing several types of antioxidants in different ratios that may lead to an increase in the regeneration efficiency. Because the insoluble fraction of foods remained for a long time (about 24 h) in the GI tract, it can contribute to maintaining a high antioxidant defense. As shown in Figure 5b, from a physiological point of view, regular consumption of high AC beverages may be very useful to regenerate the benefits provided by the consumption of food products rich in antioxidant DF. The beneficial effects of bound antioxidants will continue during their long survival period in the human GI tract.

In conclusion, the correct balance between the oxidative radical species produced during digestion process and by bacterial metabolism and the dietary antioxidants, both soluble and insoluble, could ensure a healthy antioxidant environment able to prevent many GI system diseases. Thus, the regeneration concept is a promising framework to develop functional foods and design functional dietary regimens to promote the health of the GI tract.

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Notes

The authors declare no competing financial interest.

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