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Behaviour of Trolox with macromolecule-bound antioxidants in aqueous medium: Inhibition of auto-regeneration mechanism

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ABSTRACT

This work aimed at investigating the behaviour of Trolox, vitamin E analogue, in presence of macromolecule-bound antioxidants in aqueous radical medium. Three main groups of macromolecule-bound antioxidants were assayed: dietary fiber (DF), protein and lipid-bound antioxidants, represented by whole wheat, soybean and olive oil products, respectively. Experimental studies were carried out in aqueous ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) radical medium. Trolox and macromolecule-bound antioxidants were added to radical separately and together in different concentrations. Antioxidant capacities were determined using QUENCHER procedure. pH of radical media was altered for DF and protein-bound antioxidant studies to examine its effect. Chemometric tools were used for experimental design and multivariate data analysis. Results revealed antagonistic interactions for Trolox with all macromolecule-bound antioxidants. The reason behind this antagonism was investigated through oxidation reactions of Trolox via mass spectrometry analysis. Consequently, a proof was obtained for inhibitory effect of bound-antioxidants on auto-regeneration reactions of Trolox.

1. Introduction

Vitamin E is considered an important natural antioxidant based on its properties, such as the inhibition of lipid peroxidation, a contribution to antioxidant defense in biological membranes and being the first line of defense again polyunsaturated fatty acid peroxidation (Evstigneeva, Volkov, & Chudinova, 1998). Moreover, it has been introduced to have a cancer preventative potential for many cancer types, besides being related to a lower risk of ischemic heart disease (Pezeshk & Dalhouse, 2000; Shklar & Oh, 2000). Also, Vitamin E is used as a food protector for its preventative role against lipid oxidation (Eitenmiller, Lee, & Vitamin, 2004). In spite of this knowledge regarding Vitamin E, its mechanism of action is not yet completely understood (Lucio et al., 2009).

Investigating Vitamin E interactions with other components in possible reaction media has a big appeal. Its behaviour in the presence of other antioxidants constitutes a critical research topic. The potential synergetic, additive or antagonistic interactions between Vitamin E and other antioxidant species, namely the total effect greater, equal or lesser than the simple sum of the separate antioxidant effects, respectively (Wang, Meckling, Marcone, Kakuda, & Tsao, 2011) is an important question.

Vitamin E is a hydrophobic antioxidant, which is only soluble in organic solvents and membranes, and is difficult to handle in buffered reaction media (Lucio et al., 2009). This situation creates difficulties for the studies mentioned. This revives the use of Vitamin E analogues, which enables the ability to work in homogeneous, aqueous solutions, besides having a significant antioxidant activity (Thomas & Bielski, 1989).

Among them, Trolox, in which the polyisoprenoid tail of Vitamin E has been replaced by a carboxyl moiety, has the precedence of being moderately water soluble (Thomas & Bielski, 1989). The water solubility property is provided by the carboxyl group, while antioxidant activity is provided by the 6-chromanol moiety (Castle & Perkins, 1986; Cort et al., 1975). Trolox has been widely used as a model compound of α-tocopherol (Thomas & Bielski, 1989). Besides, Trolox is applied for the expression of antioxidant capacity of chemical compounds, food and biological matrices in terms of Trolox equivalent antioxidant capacity (TEAC), as a standard antioxidant compound (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). Hence, Trolox can be counted as a proper homologue of Vitamin E to investigate its behaviour in aqueous radical environments by itself alone and together with other

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antioxidant compounds.

The interactions of Vitamin E or Trolox with different free antioxidants, such as carotenoids, ascorbic acid, quercetin, (−)-epicatechin and (+)-catechin (Hamilton, Gilmore, Benzie, Mulholland, & Strain, 2000; Pedrielli & Skibsted, 2002; Schroeder, Becker, & Skibsted, 2006) and free antioxidant containing food matrices (fruit juices and green tea) (Graversen, Becker, Skibsted, & Andersen, 2008; Yin, Becker, Andersen, & Skibsted, 2012) have already been studied. In addition, the behaviour of Trolox itself in a radical environment has been assessed through its oxidation with Br_2^- which includes a step covering the auto-regeneration reaction of Trolox (Thomas & Bielski, 1989). However, the interactions of either Vitamin E or Trolox with macromolecule-bound antioxidants, which originally constitutes a significant portion of dietary antioxidants, has not been studied yet.

The "Macromolecule-bound antioxidants" concept includes dietary antioxidants bound to different macromolecules, like dietary fibers (DFs), proteins or lipids in complex food matrices (Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011). These bound antioxidants were shown to have the ability to quench free radicals as well as free antioxidants. In addition, they carry some noteworthy characteristics affecting their bioavailability and bioaccessibility derived from the macromolecules they are bound to Alu'datt et al. (2014) and Vitaglione, Napolitano, and Fogliano (2008). Hence, investigating their interactions with Vitamin E, nominately Trolox as its water-soluble analogue, constitutes a challenging topic for understanding the possible effect when they are found together.

Indeed, the auto-regeneration reaction of Trolox is thought to have a key role in antioxidant capacity measurements, such as ABTS and DPPH based assays, which express the results in terms of Trolox equivalent. Trolox in a radical environment may exaggerate its antioxidant activity through auto-regeneration leading to an underestimation of the antioxidant capacity of food samples.

In this context, this study investigates the behaviour of the free antioxidant, Trolox, in an aqueous radical medium in the presence of macromolecule-bound antioxidants. The interactions between Trolox and different macromolecule-bound antioxidants were aimed to be present as well. For this purpose, whole wheat, soybean and olive oil products were used in the experimental studies after specific preparation steps, to represent three main groups of macromolecule-bound antioxidants: DF, protein and lipid bound antioxidants, respectively. Experimental studies were carried out in the aqueous ABTS radical medium, by using different concentrations of Trolox and macromolecule-bound antioxidants. The pH of the radical media was also changed for DF and protein-bound antioxidant studies. Antioxidant capacities of Trolox and macromolecule bound antioxidants separately and in mixtures were determined by measuring the absorbance of the radical in presence of these species at 734 nm according to the QUENCHER procedure (Gokmen, Serpen, & Fogliano, 2009). Results are given in terms of percentage of inhibition values, which are calculated by using absorbance values measured with respect to the absorbance of ABTS radical. The experimental matrices for Trolox + macromolecule-bound antioxidant mixture experiments were constructed by using Design of Experiment (DoE). Multi-way ANOVA was performed to determine the significance of the effects of macromolecule-bound antioxidants, free antioxidants and pH.

2. Materials & methods

2.1. Materials

2.1.1. Chemicals

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

Table 1
Experimental matrix for DF/protein-bound antioxidants + Trolox and lipid-bound antioxidants + Trolox mixtures studies.

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

2.1.2. Food samples

Whole-wheat flour, edamame, soybean, soymilk, tofu, extra virgin and refined olive oil were purchased from local markets in Ankara, Turkey. Paste was prepared from whole wheat flour by heating the flour:water mixture, formed according to the ratio (3.5 g:25 ml) given in the AACC method 72-21.01 "General Pasting Method for Wheat and Rye Flour Using the Rapid Visco Analyzer" (1999), on a magnetic stirrer to 70 °C and leaving for set-back at room temperature. Bread was prepared according to the AACC method 10-10B for "Straight-Dough Bread Making" (1985). Boiled soybeans were obtained by boiling 100 g raw soybeans in 600 ml water for 1 h.

2.2. Methods

2.2.1. Preparation of the DF-bound antioxidants

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

2.2.2. Preparation of the protein-bound antioxidants

Edamame, soybean, boiled soybean, soymilk and tofu proteins were subjected to isoelectric precipitation according to the method described by Dev, Quensel, and Hansen (1986) and Krase, Schultz, and Dudek (2002) with some modifications. Freeze dried and ground samples (4 g) were first defatted with hexane (200 ml) in a soxhlet apparatus at 50 °C for 6 h, then dried at room temperature. Defatted samples (10 g) were mixed with NaOH (2.0 N, 100 ml) and the pH of the mixtures were adjusted to 11.0. Following 1 h stirring at room temperature, centrifugation was done at 8000 rpm for 30 min and supernatants were collected. The pH of the supernatants was adjusted to 4.6 using HCl (0.1 N) and the protein isolate precipitated was separated by centrifugation at 8000 rpm for 15 min. The isolates were freeze-dried and kept stable under −18 °C in a close-fitting vessel under nitrogen atmosphere prior to experiments.

2.2.3. Preparation of the lipid-bound antioxidants

Extra virgin and refined olive oil samples (15 ml) were washed with a methanol:water (70:30, v:v) mixture (25 ml) in three steps. Following vortexing of the olive oil:methanol:water mixtures, centrifugation was done at 8000 rpm for 3 min and the supernatants containing free phenolic compounds were removed in each step. The last supernatant was