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Structure–antioxidant activity relationships of *o*-hydroxyl, *o*-methoxy, and alkyl ester derivatives of *p*-hydroxybenzoic acid

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ABSTRACT

Anti-DPPH radical effect as well as anti-peroxide activity of *o*-hydroxyl, *o*-methoxy, and alkyl ester derivatives of *p*-hydroxybenzoic acid in a bulk fish oil system and its O/W emulsion were investigated. Electronic phenomena, intra- and/or intermolecular hydrogen bonds, interfacial properties, and chemical reaction of the solvent molecules with phenolic compounds were considered to be mainly involved in the antiradical activities observed. Antioxidant activity of the phenolic acids derivatives as a function of these factors was variously affected by the environmental conditions which may occur in practice.

1. Introduction

Phenolic acids as a major class of bioactive phenolic compounds are widely distributed in plant kingdom and are known to have antioxidant activity (Frankel, 1998). Apart from other mechanisms that may be involved, the antioxidant activity of these compounds has been considered to be a very important cause of many biological capabilities, including anti-inflammatory, antiviral, antiatherogenic, antibacterial, and anticancer effects (Cos, Calomme, Pieters, Vlietinck, & Vanden Berghe, 2000; Middleton, Kandaswami, & Theoharides, 2000). *p*-Hydroxybenzoic acid (*p*-HBA) can be taken into account as the common monophenolic structure of a large number of derivatives obtained from phenolic acids. Monophenols have been shown to possess less efficient radical scavenging activity than polyphenols (Hsieh, Yen, & Chen, 2005). However, the structural variations due to the introduction of different electron-donating/withdrawing groups to the various positions of the phenolic ring can promote the antioxidant potency of the resulted compounds (Shahidi & Wanasundara, 1992). The functional groups substituted to the *ortho* or *para* positions of phenolic rings have been shown to be more effective than those attached to *meta* position in changing the performance of phenolic antioxidants (Chen & Ho, 1997). The majority of studies on the structure–antioxidant activity relationship have individually considered the influence of different functional groups or yielded the data which are less comparable because of the different methods used to evaluate the antioxidant capacity.

o-Hydroxyl, *o*-methoxy, and alkyl ester groups are among the functional groups that are frequently seen in a wide range of phenolic compounds. Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA), gallic acid (3,4,5-trihydroxybenzoic acid, GA), vanillic acid (3-methoxy,4-hydroxybenzoic acid, VA), syringic acid (3,5-dimethoxy,4-hydroxybenzoic acid, SA), methyl *p*-hydroxybenzoate (*p*-HBM), ethyl protocatechuate (EPC), and methyl gallate (MG) are recognized as the most well-known derivatives of *p*-HBA containing these functional groups (Fig. 1). In the present study, the antioxidant activity of these phenolic derivatives was evaluated by different methods, in order to get an insight into the structure–activity relationships responsible for the observed performances.

At the first step of the evaluation, the reactivity of the *p*-HBA derivatives was measured in terms of the strength to scavenge the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006), which is considered as the main mechanism of action of phenolic antioxidants. The second step was to investigate the inhibitory effect of the compounds on the formation of hydroperoxides, which is considered to be one of the most commonly used methods to evaluate the inhibitory effect of antioxidants, during the oxidation of fish oil as an edible oil with extremely beneficial effects on human health (Arkhipeko & Sazontova, 1995; Cleland, James, & Proudman, 2003; Hye-Kyeong, Della-Fera, Lin, & Baile, 2006) but high susceptibility to oxidative deteriorations. Finally, a dispersed system

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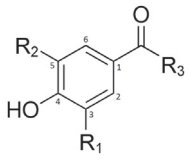

	R_1	R_2	R_3
p-Hydroxybenzoic acid	H	H	OH
Protocatechuic acid	OH	H	OH
Gallic acid	OH	OH	OH
Vanillic acid	OCH_3	H	OH
Syringic acid	OCH_3	OCH_3	OH
Methyl *p*-hydroxybenzoate	H	H	OCH_3
Methyl gallate	OH	OH	OCH_3
Ethyl protocatechuate	OH	H	OCH_2CH_3

Fig. 1. Molecular structure of *p*-hydroxybenzoic acid and its derivatives.

consisting of two immiscible phases (the fish oil-in-water, O/W, emulsion) was used to gain a better understand of the partitioning-based performance of the phenolic antioxidants in food matrices which are usually multicomponent systems.

2. Methods and materials

2.1. Materials

Crude Kilka fish oil (Table 1) was supplied by Khazar company (Babolsar, Iran). *p*-HBA and its derivatives (*p*-HBM, PCA, EPC, GA, MG, VA, and SA) as well as α-tocopherol (α-Toc) were purchased from Sigma–Aldrich (St. Louis, MO). All chemicals and solvents used in this study were of analytical reagent grade and purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (St. Louis, MO).

2.2. Fatty acid composition

The fatty acids methyl ester (FAME) preparation of the oil samples was carried out to determine the fatty acids composition in lipid fraction. The oil (0.1 ml) was pipetted into the clean 10 ml of screw-top glass bottles, dissolved in 1 ml of hexane and converted to the methyl esters by reaction with 0.5 ml of sodium methoxide. The mixture was homogenized using the vortex for 10–15 s. The clear upper phase layer was pipetted out and injected to the gas chromatograph (Sharina & Jumat, 2006). Menhaden oil was used as the standard of PUFA and the identity of individual FAME was compared after conversion to equivalent chain length.

Table 1
Fatty acid composition (%w/w) of the Kilka fish oil.

Parameter	
C14:0	6.22 ± 0.05
C16:0	17.31 ± 0.01
C16:1	13.23 ± 0.07
C17:0	1.89 ± 0.06
C18:0	3.23 ± 0.04
C18:1	27.51 ± 0.06
C18:2	8.16 ± 0.15
C18:3	1.17 ± 0.09
C20:0	1.16 ± 0.06
C20:4	0.21 ± 0.03
C20:5 (EPA)	6.35 ± 0.05
C22:6 (DHA)	5.89 ± 0.03

Mean ± SD (standard deviation) of triplicate determinations.

Routine GC analyses were performed on a Shimadzu GC-17A Gas Chromatography equipped with FID detector. The column used was BPX-70 (60 m length × 0.32 mm i.d × 0.25 μm thickness), split ratio 100:1. The analyses were performed using programmed temperature at the initial temperature of 120 °C, with the temperature increment rate at 3 °C min^{-1} and final temperature at 245 °C. The injection port temperature was set at 260 °C and detector temperature, was at 280 °C. Nitrogen gas was used as a carrier gas.

2.3. Purification of the fish oil sample

To remove indigenous antioxidants, 120 g of the oil sample were applied to a glass chromatographic column (50 × 5 cm i.d.) packed sequentially with three adsorbents. The bottom layer was aluminum oxide 60 (50 g, active, neutral) activated at 200 °C for 3 h immediately before use. The middle layer was 80 g of the activated silica gel (60–200 mesh) activated at 160 °C for 3 h immediately before use. The upper layer was 2 g of the activated carbon. The column and collection vessels were wrapped in aluminum foil, and the oil was drawn through the column by suction without solvent (Belhaj, Arab-Tehrany, & Linder, 2010).

2.4. Preparation of the fish oil-in-water emulsion

The O/W emulsion was prepared by gently adding 10% of the purified fish oil containing 200 ppm of each antioxidant into a solution containing 5% of soy protein isolate. To obtain a stable emulsion, the mixture was vortexed by ultra-Turrax (5 min, ~3000*g*), afterwards was sonicated for 4 min in an ice bath. To monitor lipid oxidation, the emulsion samples were kept in an oven at 55 °C. Oil extraction from the emulsions for analysis was carried out by mixing a chloroform/methanol solvent system (1:1, v/v) and the emulsion in a shaker (1 min) and then centrifuging for 1 min at ~700*g*. The lower lipid layer was collected and its solvent evaporated using a stream of nitrogen.

2.5. Partition coefficient (log P)

Solutions (0.3 mM) of each compound in 1-octanol were kept at 60 °C for 1 h. The maximum absorbance was read by UV spectrum (A_0). Equal volumes of this solution and acetate buffer (0.1 M, pHs 3.5 and 5.5) were vortexed (69.875 g) for 1 min. The UV spectrum of the 1-octanol layer was determined after 30 min (A_x). The partition coefficient (log *P*) of antioxidant was calculated according to the following equation (Gorden, Paivia-Martins, & Almeida, 2001):

$$P = A_x/(A_0 - A_x) \quad (1)$$

2.6. Radical scavenging activity

The antioxidants in different concentration ranges dependent on the antioxidative power were reacted with the stable DPPH free radical in a methanol solution (60 μM). After 30 min at room temperature in the dark, the absorbance of the samples was read against a blank at 517 nm. Inhibition of the DPPH free radical in percent (I%) was calculated as follows:

$$I\% = 100 \times (A_{Blank} - A_{Sample})/A_{Blank} \quad (2)$$

where A_{Blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the test compound. The concentration of antioxidant required for 50% inhibition of the DPPH free radical (IC_{50} value) was calculated by linear regression analysis of dose–response curve plotting between the % inhibition and concentrations (Lima et al., 2006). Radical scavenging activity (RSA) was calculated from the IC_{50} value as follows: