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Antioxidative activity of phenolic compounds on the metal-ion breakdown of lipid peroxidation system

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The generation of a lipid alkoxyl (LO$^{\bullet}$) radical as a result of metal-ion breakdown of hydroperoxide-enriched methyl linoleate of sunflower oil has been established by a combination of spin trapping and electron spin resonance (ESR) spectroscopy. Antioxidative activities of gallic, caffeic, chlorogenic, vanillic and salicylic acids at concentrations of 0.5, 1.0 and 2.0 mmol were studied in a lipid peroxidation system. The results show that the investigated phenolic compounds scavenge alkoxyl radical. This activity depends on the structure of the molecules, and number and position of the hydroxyl group in the molecules; it is increased in the order gallic > caffeic > > chlorogenic > vanillic > salicylic acids. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

During recent years, considerable attention has been given to the involvement of oxyl radicals, oxidizing lipids and metal-ions in oxidative damage in living tissue and food systems. Alkoxyl (RO$^{\bullet}$), peroxyl (ROO$^{\bullet}$) and hydroxyl ($^{\bullet}$OH) radicals, have been suggested as intermediates in the breakdown of lipid hydroperoxide, on the basis of the product studies, oxygen uptake measurements and other techniques, though very little direct evidence for the occurrence of these species has been obtained (Cheeseman and Slater, 1993; Halliwell and Chirico, 1993).

Electron spin resonance (ESR) spectroscopy is one of a few ways of directly detecting oxyl types of radicals. Using the spin trap, *N*-t-butyl-α-phenyl-nitrone (PBN), it is possible to convert oxygen-centered radicals, which are short-lived and at low concentrations, to stable nitroxide radicals (adducts) with spectral hyperfine splittings that reflect the nature and structure of these radicals (Davies, 1987; Davies and Slater, 1986). Lipid oxidation, as a free radical reaction, is a major cause of deterioration in the quality of food products and can directly affect many characteristics such as flavour, colour, texture and nutritive value (Hamilton, 1983).

Many antioxidants both artificial and natural, have been examined in attempts to control lipid oxidation in food products (Namiki, 1990).

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are well known for their ability to inhibit the catalytic chain reactions which initiate and propagate lipid peroxidation (Kahl, 1984). On the other hand, BHT has been shown to enhance carcinogenesis, cause pulmonary damage in mice and liver necrosis and hemorrhagic death in rats, while BHA induces neoplasia in rat forestomach (Branen, 1975; Ito *et al.*, 1985).

Numerous investigations have been carried out to discover naturally occurring phenolic compounds which not only prolong the preservation of food products but also participate as radical scavengers in living organisms. If phenolic compounds are used as antioxidants for food this will present several essential requirements: effectiveness at low concentration, physical and chemical compatibility with the substrate, absence of sensory influence on the food product and absence of toxicology.

The task of this study is to evaluate the scavenger ability of some naturally occurring phenolic compounds for alkoxyl radicals formed during the ferrous ion breakdown of hydroperoxide-enriched methyl linoleate.

MATERIALS AND METHODS

Methyl esters of sunflower oil were purchased from Fluka Chemie A.G. (Buchs, Switzerland). They were exposed to air for 72 h at room temperature in order to be peroxidized before use. The oil prepared in this way contains about 60% hydroperoxide-enriched methyl linoleate.

N-t-butyl-α-phenylnitrone (PBN), 2-amino-2-hydroxymethyl-1,3-propane-diol (TRIS), ferrous chloride and all phenolic compounds were supplied by Sigma Chemical Co (St Louis, MO). The solutions of these compounds were prepared daily, fresh in argon-sparged water.

Reactions were conducted by adding reactants in the following order: 0.6 g hydroperoxide enriched methyl linoleate of sunflower oil, phenolic compounds (gallic, chlorogenic, caffeic, vanillic and salicylic acids) at concentrations 0.5 mmol, 1.0 mmol or 2.0 mmol, 48 mmol PBN and 0.1 mmol ferrous chloride. After 3 h, the mixtures were shaken and transferred to a quartz ESR flat cell ER-160FC for analyses.

ESR spectra were obtained with a Bruker ESR-300E spectrometer. Magnetic field scanning and *g*-value determinations were calibrated using Fremy's salt (peroxylamine disulphonate). Splitting constants were calculated from computer-generated second derivatives of the spectra, after optimizing signal-to-noise ratios and were verified by computer simulations.

The trapping of lipid alkoxyl ($LO^{\bullet}$) radical by phenolic compounds, led to a declination in the formation of PBN–alkoxyl radical adduct (PBN–LO). The scavenging activity of the phenolic compounds was estimated by the percentage decrease of the relative intensity (RI) of the signal of PBN–alkoxyl radical adduct with reference to the control without phenolic compounds (control, RI = 100%). All the experiments were repeated three times.

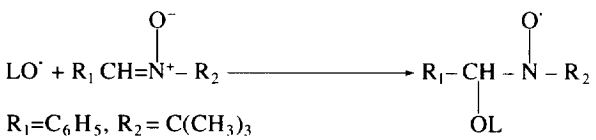
The oxygen-centered antioxidant radicals were determined in the lipid peroxidation system with 5.0 mmol of each phenolic compound and 5.0 mmol Tris buffer (100 mmol litre^{-1}, pH 7.5) without PBN.

RESULTS AND DISCUSSION

It has been suggested that metal ions such as iron play an important role in the peroxidation of lipid. Ferrous ions decompose lipid hydroperoxide (LOOH) by the hemolytic cleavage of the O—O bond and give lipid alkoxyl radicals, which initiate free radical chain oxidation (Yamamoto *et al.*, 1987).

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + OH^- + LO^{\bullet}$$

Using the spin trap PBN, it is possible to trap the obtained alkoxyl radical and verify this by ESR.



The ESR spectrum of the PBN–alkoxyl radical adduct, obtained in the reaction of sunflower oil, PBN and ferrous ions is shown in Fig. 1.

These six peaks are characteristic of an interaction between the unpaired electron and one ^{14}N atom (spin I = 1) and one ^{1}H atom (spin I = 1/2) and they are typical of radical adducts of PBN in which the unpaired electron is primarily located on the nitroxide and proton splitting originates from the CH- group adjacent to the nitroxide.

The parameters, $a(^{14}N)$ = 1.485 mT and $a(^{1}H)$ = 0.225 mT, derived from the spectra on the Fig. 1 correspond to a lipid alkoxyl radical adduct. Because of the solvent effects (Schaich and Borg, 1990), the lipid-derived alkoxyl radical from the sunflower oil produces a PBN–alkoxyl radical adduct with hyperfine splittings that differs significantly from the values in the literature for such an adduct.

In the absence of phenolic compounds, the relative intensity of the signal in the ESR spectra of the spin adduct of the lipid alkoxyl radical is increased dramatically, especially during the first 3 h, because it was rapidly oxidized (Fig. 2).

The influence of different amounts of the gallic, chlorogenic, caffeic, vanillic and salicylic acids, respectively, on the relative intensity of the signal in the ESR spectra of the spin adduct of the lipid alkoxyl radical is presented in the diagrams of Fig. 3.

No change in the shapes of ESR spectra, in all examined cases, was detected, but the relative intensity (RI) of ESR signals, corresponding to the concentration of formed free radical, was decreased with higher concentrations of the added phenolic compounds. The $LO^{\bullet}$ scavenging activities of phenolic compounds tested in this study depended on their concentrations and structures. At a lower concentration (0.5 mmol) of phenolic compounds, only gallic, caffeic and chlorogenic acids scavenged $LO^{\bullet}$ radical. Higher concentrations (1.0 mmol or 2.0 mmol) more or less significantly increased scavenging activity of phenolic compounds. The highest scavenging activity was observed in the reaction mixture containing gallic acid at the concentration of 2.0 mmol

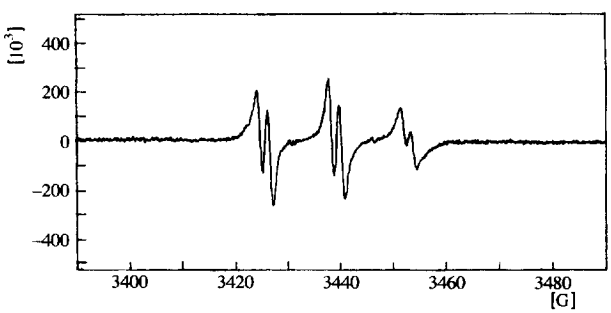


Fig. 1. ESR spectrum of PBN–alkoxyl radical adduct formed during reaction of 0.6 g hydroperoxide-enriched methyl esters of sunflower oil, 48 mmol PBN with 0.1 mmol ferrous chloride. Spectrometer conditions were as follows: receiver gain, 2.5×10^5 time constant 327.68 ms, modulation amplitude 0.204 G, microwave power 19.9 mW, centre-field 3440G, conversion time 1310.72 ms.

(%RI of the PBN–alkoxyl adduct was 8.42%). A minor scavenging activity was shown in the reaction mixtures containing vanillic acid (from RI = 59.7% to RI = 37.2%) and salicylic acid (from RI = 79.7% to RI = 45.3%).

Some generalizations can be drawn from these results. The $LO^{\bullet}$ scavenging ability of the phenolic compound tested seems directly correlated to the number of hydroxyl groups substituted in the aromatic ring and to the nature of the substituent at the *o*-position. The major inhibition effect on the free radical formations was shown by gallic acid, a three hydroxy compound, then caffeic acid, a two hydroxy compound and vanillic acid, a monohydroxy compound. A minor effect was manifested by salicylic acid, a monohydroxy compound.

From the structure characteristics it may be concluded that *o*-dihydroxyl groups in the investigated phenolic compounds increased antioxidative activity. Similar results were reported by Namiki (1990). The author showed that methoxylation of a hydroxyl group at the position resulted in a drastic decrease in scavenging activity.

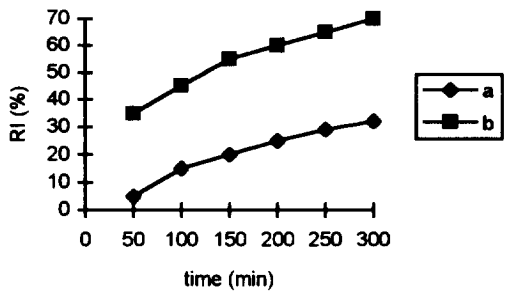


Fig. 2. The change of relative intensity of the signal in the ESR spectrum of the spin adduct of lipid alkoxyl radical during the oxidation of sunflower oil, (a) no Fe^{2+}, no phenolic compounds; (b) in the presence of Fe^{2+}, without phenolic compounds.

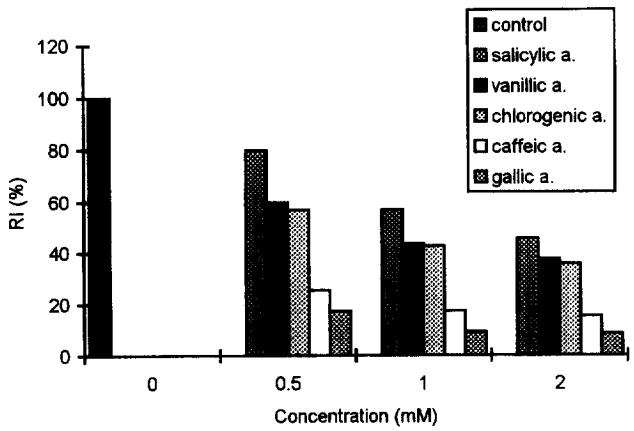


Fig. 3. The influence of different amounts of gallic, chlorogenic, caffeic, vanillic and salicylic acids, respectively, on the relative intensity of the signal in the ESR spectrum of the spin adduct of the lipid alkoxyl radical.

The phenolic compounds were almost stable in aqueous solutions, while in the presence of a lipid alkoxyl radical they degraded rapidly (Chimi *et al.*, 1991; Milić *et al.*, 1995). Phenolic antioxidants (R-OH) inhibited lipid peroxidation by trapping the lipid alkoxyl radical ($LO^{\bullet}$):

$$LO^{\bullet} + R - OH \rightarrow LOH + RO^{\bullet}$$

However, the mechanisms of their radical scavenging as well as the physico–chemical properties (acid-base, redox properties) which determine the activities of these phenolic compounds are scarcely known (Jovanović and Simić, 1994). The results obtained from scavenging effects of phenolic compounds indicated that the compounds react directly with $LO^{\bullet}$, and donate a hydrogen atom, causing termination of the radical chain reaction.

In the presence of a high concentration (5.0 mmol) of the phenolic compounds and without addition of the spin trap, PBN, new ESR signals developed in all the investigated lipid systems. Computer simulation was then carried out and it was revealed that this signal is attributable to the oxygen-centered antioxidant radicals. These radicals are insufficiently reactive and they can disappear by several mechanisms (Halliwell and Gutteridge, 1989).

Relatively stable semiquinone radicals were observed at pH 7.5. Different types of oxygen-centered antioxidant radicals are shown in Fig. 4. The gallic, caffeic and chlorogenic acids produced different types of semiquinone anion radicals. Gallic acid gave a spectrum with a pyrogallol-type anion-radical. The ESR spectrum of caffeic acid consisted of eight signals, which were attributable mainly to an interaction of an unpaired electron with three unequivalent protons at position 6, 5 and 2, respectively. Eight peaks, which could be observed in ESR spectrum of chlorogenic acid, were characteristic of an interaction between an unpaired electron with three unequivalent protons at positions 5, 6 and 2, respectively. The line pattern and splitting constants of all these radicals are comparable to those previously reported (Neta and Fessenden, 1974; Kalyanaraman *et al.*, 1987; Djilas *et al.*, 1996). Salicylic and vanillic acids also formed semiquinone radicals, as secondary type radicals. These radicals are produced by oxidation of the dihydroxy compounds formed by hydroxylation of the initial compound at positions *o*- and *p*- to the hydroxyl group.

In summary, we have shown that the ESR spin-trapping technique can be successfully employed to detect the changes in lipid peroxidation systems which contain phenolic compounds. These investigated phenolic compounds scavenge an alkoxyl radical, resulting in the occurrence of antioxidative activity. This activity depends on the number and position of the hydroxyl group in the molecules.

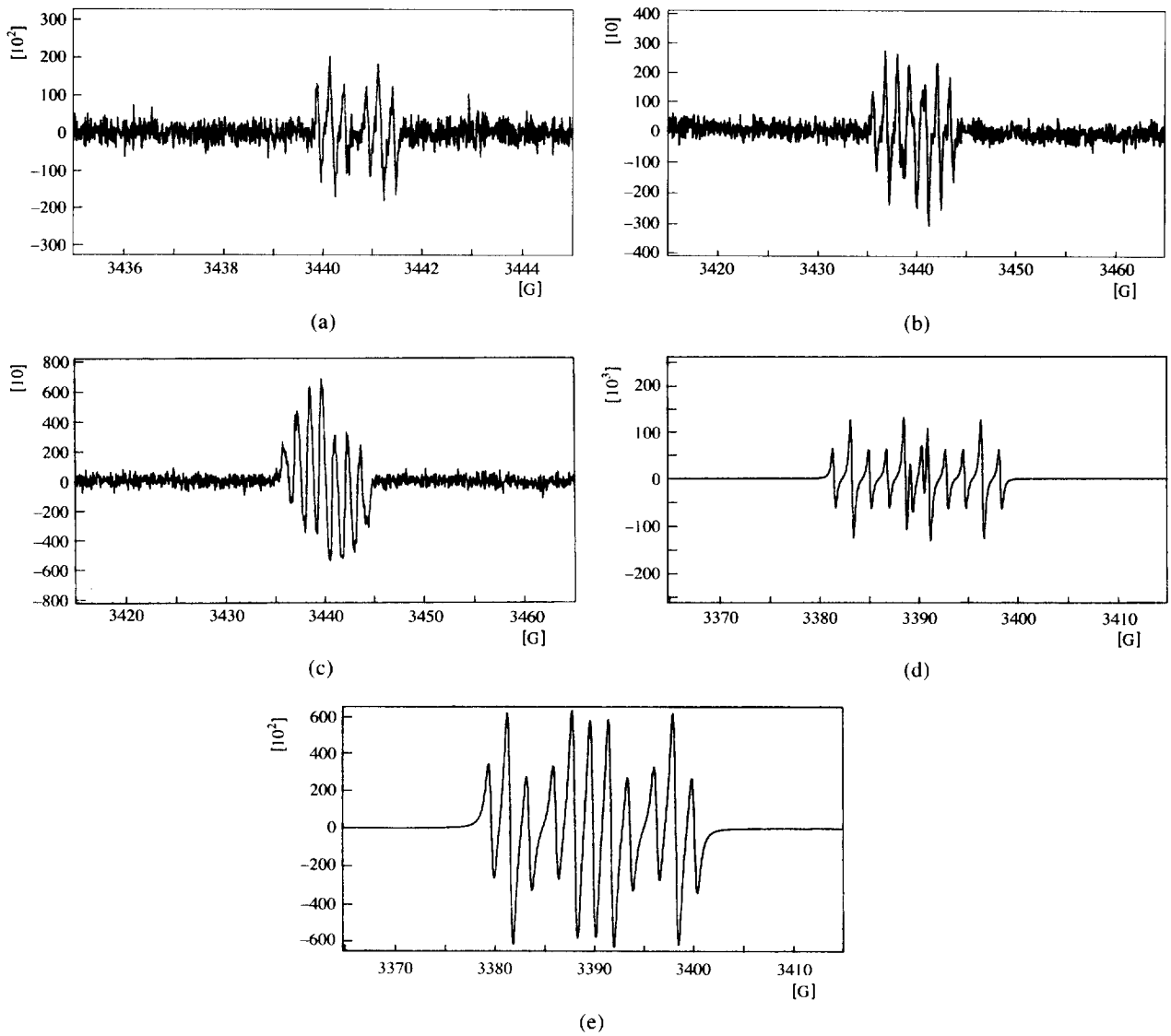


Fig. 4. ESR spectra of oxygen-centered antioxidant radicals obtained during the metal ion breakdown of the lipid peroxidation system which contains: (a) 5.0 mmol gallic acid in Tris pH 7.5, (b) 5.0 mmol caffeic acid in Tris, pH 7.5, (c) 5.0 mmol chlorogenic acid in Tris, pH 7.5, (d) 5.0 mmol vanillic acid in Tris, pH 7.5, (e) 5.0 mmol salicylic acid in Tris, pH 7.5 Spectrometer conditions were as follows: receiver gain, 2.5×10^5 time constant 81.92 ms, modulation amplitude 0.144 G, microwave power 0.632 mW, conversion time 163.84 ms.

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