Effect of Hydroxytyrosol Found in Extra Virgin Olive Oil on Oxidative DNA Damage and on Low-Density Lipoprotein Oxidation

Okezie I. Aruoma,*[,†,‡] Monica Deiana,[‡,§] Andrew Jenner,[‡] Barry Halliwell,[‡,||] Harparkash Kaur,[‡,⊥] Sebastiano Banni,[§] Francesco P. Corongiu,[§] M. Assunta Dessí,[§] and Robert Aeschbach[#]

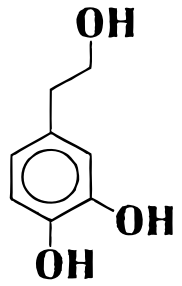
Department of Clinical Analysis, Toxicology and Bromatology, Faculty of Pharmaceutical Sciences, University of São Paulo-Ribeirão Preto, Avenida do Cafè s/n-Monte Alegre, CEP 14040-903, Ribeirão Preto-São Paulo, Brazil; Pharmacology Group, University of London King's College, Manresa Road, London SW3 6LX, United Kingdom; Dipartimento di Biologia Sperimentale, Sezione Patologia Sperimentale, Universitá degli Studi di Cagliari, Cittadella Universitaria, Km 4.500 SS 554, 09040 Monserrato, Cagliari, Italy; Department of Biochemistry, National University of Singapore, Kent Ridge Crescent, Singapore 119260; Vascular Biology Unit, Department of Surgical Research, Northwick Park Institute for Medical Research, Harrow, Middlesex HA1 3UJ, United Kingdom; and Nestlé Research Centre, Vers-Chez-les Blanc, CH-1000 Lausanne 26, Switzerland

Hydroxytyrosol found in extra virgin olive oil strongly inhibited low-density lipoprotein oxidation stimulated by 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), suggesting the ability to scavenge the AAPH-derived peroxyl radicals. Hydroxytyrosol inhibited iron-dependent phospholipid liposome peroxidation at low concentrations (IC_{50} = 50 ± 1.3 μM). In similar experiments, the calculated, IC_{50} values for other antioxidants compared are 1.5 ± 0.05 μM (carnosol), 2.25 ± 0.08 μM (carnosic acid), 65 ± 2.6 μM (Trolox C), and 250 ± 10 μM (vitamin E). Hydroxytyrosol and ascorbate reduced copper(II) ions to their copper(I) prooxidant form, but this was not reflected by their abilities to induce oxidative DNA damage in the complex copper−phenanthroline. Only high, nonphysiological, millimolar concentrations of pure hydroxytyrosol weakly stimulated copper-dependent chemical modification to DNA bases. The prooxidant (redox actions on metal ions) concentrations in vitro may never be achieved in vivo (following consumption of extra virgin olive oil). Thus, hydroxytyrosol may represent a useful diet-derived antioxidant depending on its bioavailability.

Keywords: *Extra virgin olive oil; hydroxytyrosol; low-density lipoprotein; rosemary; vitamin E; lipid peroxidation; plant antioxidants; DNA damage*

INTRODUCTION

The extent to which oxidation of fatty acids and their esters occurs in food depends on the composition of the fat, the type of food processing, the storage or cooking conditions, and the antioxidants present (Hudson, 1990; Loliger, 1991; Aruoma, 1996). Lipid peroxidation is also important in vivo. In particular, oxidation of low-density lipoprotein (LDL) may contribute to the pathology of atherosclerosis (Steinberg et al., 1989; Steinbrecher, 1987). This has led to increased interest in the role of natural antioxidant inhibitors of the oxidation of LDL and membrane lipids. Hydroxytyrosol is one of the many active compounds [see Boskou (1996) and Montedoro et al. (1992)] in extra virgin olive oil, and it has been suggested to possess antioxidant properties (Aeschbach et al., 1994; Salami et al., 1995; Visioli and Galli, 1995; Satue et al., 1995; Pearson et al., 1997; Visioli et al., 1998). The first objective in this paper was to assess the ability of hydroxytyrosol to inhibit LDL oxidation induced by the radical generator 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). The results suggest that the ability of hydroxytyrosol to inhibit the AAPH-induced LDL oxidation may be intrinsically linked to its ability to scavenge peroxyl radicals.



Hydroxytyrosol

Antioxidants that protect lipids against oxidative damage may actually accelerate damage to other biomolecules such as DNA and proteins (Aruoma et al., 1990) in vitro. Earlier work (Aeschbach et al., 1994) showed that hydroxytyrosol promoted oxidative deoxysugar damage and bleomycin−Fe(III)-dependent DNA

* Address correspondence to this author at the Department of Clinical Analysis, Toxicology and Bromatology, Faculty of Pharmaceutical Sciences, University of São Paulo-Ribeirão Preto, Avenida do Cafè s/n-Monte Alegre, CEP 14040-903, Ribeirão Preto-SãoPaulo, Brazil (e-mail oiaruoma@fcfrp.usp.br).
† University of São Paulo-Ribeirão Preto.
‡ University of London King's College.
§ Universitá degli Studi di Cagliari.
|| National University of Singapore.
⊥ Northwick Park Institute for Medical Research.
Nestlé Research Centre.

10.1021/jf980649b CCC: $15.00
Published on Web 11/21/1998

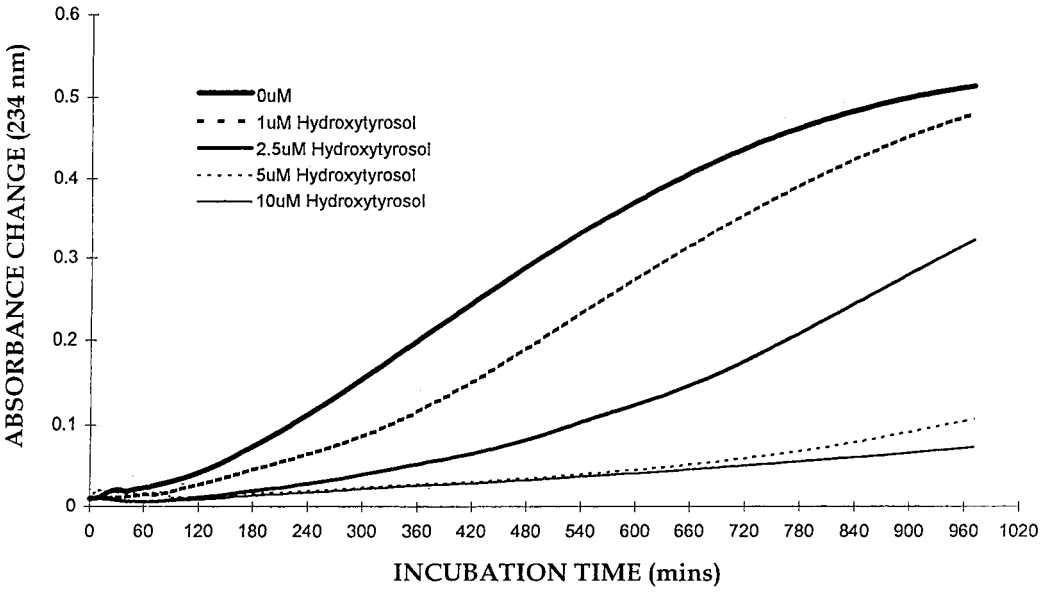


Figure 1. Changes in absorbance values, obtained by continuously subtracting the value of the blank (the absorbance of the incubation mixture without LDL but containing AAPH, soon after the addition of AAPH to the sample) from those recorded at 234 nm every 10 min, during the incubation period (16 h). Data are means of three independent experiments, and the calculated SD values were not more than 5−8%.

damage. Damage in the deoxyribose assay is mediated by $OH^{\bullet}$ generated in the Fenton type reaction (Aruoma, 1994), but in the bleomycin assay, $OH^{\bullet}$ involvement may be a minor side reaction. The copper ion−1,10-phenanthroline complex has nuclease activity and is a widely used tool to induce strand breakage in DNA (Sigman, 1986; Thederahm et al., 1989). The copper−phenanthroline complex in the presence of ascorbate induces extensive oxidative DNA base modification (Dizdaroglu et al., 1990). The pattern of base damage in this system is characteristic of $OH^{\bullet}$ attack. There is also minor deoxysugar damage in this system, making it an ideal model for assessing the prooxidant action of natural antioxidants (Aruoma, 1996). The second objective was to assess the ability of hydroxytyrosol to reduce copper(II) ions and to evaluate how this modulates copper ion-dependent oxidative base modification in DNA in vitro. The results from this study support the continuing debate that choice of model systems coupled with an understanding of the mechanisms of the reactions within the model is critical in evaluating antioxidant efficacy.

MATERIALS AND METHODS

Hydroxytyrosol was provided by Nestec SA, Lausanne, Switzerland. $CuCl_2$ and $FeCl_3$ were purchased from BDH Chemicals (Poole, Dorset, U.K.). 2,9-Dimethyl-1,10-phenanthroline (neocuproine) hydrochloride (NC), L-ascorbic acid, *dl*-α-tocopherol, bovine brain phospholipids, LDL in phosphate-buffered saline (pH 7.4) containing 0.01% EDTA, calf thymus DNA (Sigma type I), ascorbic acid, 1,10-phenanthroline, 6-azathymine, diaminopurine, 8-bromoadenine, 5-hydroxyuracil (isobarbituric acid), 4,6-diamino-5-formamidopyridine, 2,5,6-triamino-4-hydroxypyrimidine, and 5-(hydroxymethyl)uracil were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 8-Hydroxyguanine and Trolox C were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). AAPH was purchased from Polysciences, Inc. (Warrington, PA). 8-Hydroxyadenine and 2,6-diamino-5-formamidopyrimidine were synthesized and characterized using conditions previously described in Aruoma et al. (1989). The authentic standards 2-hydroxyadenine and 5-hydroxycytosine were kind gifts from Dr. Miral Dizdaroglu of the National Institute of Standards and Technology, Gaithersburg, MD. Silylation grade acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) (containing 1% trimethylchlorosilane, TMCS) were obtained from Pierce Chemical Co. (Rockford, IL).

LDL Oxidation with AAPH or $CuCl_2$. Before use, the LDL solution was dialyzed for 4 h at 4 °C in a 100-fold volume of 10 mM phosphate buffer (pH 7.4) and 0.16 M NaCl, changed every 30 min. The amount of LDL recovered after dialysis was determined using the Markwell protein assay (Markwell, 1981). The EDTA-free LDL solution was used for all oxidation studies and stored for not longer than 4 days at 4 °C.

For performing the oxidation experiments, the EDTA-free LDL solution was diluted with 10 mM phosphate buffer (pH 7.4) and 0.16 M NaCl, and the oxidation was initiated by the addition of a freshly prepared aqueous AAPH solution. The final conditions were in all experiments 37 °C, 83.3 μg of LDL/mL, and 0.5 mM AAPH. The progress of LDL oxidation was recorded using a Uvikon 944 plus spectrophotometer, by continuous monitoring of conjugated diene formation (Esterbauer et al., 1989). Spectra from 190 to 300 nm or absorbance values at 234 nm were recorded at 10 min intervals for several hours, using the incubation mixture soon after the addition of AAPH as blank. AAPH has an absorbance at 234 nm, which increases with time. The absorbance values were subtracted from those of the samples. Preliminary experiments indicated that the tendency of hydroxytyrosol to reduce copper(II) ions (discussed below) might interfere with the outcome of the assessment of antioxidant action.

Brain Phospholipid Liposome Peroxidation. Hydroxytyrosol and ascorbic acid (dissolved in water) and Trolox C, α-tocopherol, carnosol, and carnosic acid (Nestec Research Centre) (dissolved in ethanol) were tested at different concentrations. In separate experiments, the inhibition of brain phospholipid liposomes peroxidation was assessed essentially as previously described (Aruoma et al., 1993), with a few modifications. Assay mixtures contained, in a final volume of 1 mL, PBS [3.4 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4), 0.15 M NaCl], 1 mg/mL brain phospholipid liposomes, 100 μM $FeCl_3$, various concentrations of the compounds (dissolved either in water or in ethanol), and 100 μM ascorbate (added last to start the reaction). Samples were incubated at 37 °C for 30 min; at the end of the incubation period, 0.1 mL of 2% (w/v) butylated hydroxytoluene was added to each sample. The extent of lipid peroxidation was measured as thiobarbituric acid (TBA) reactivity: 1 mL of 1% (w/v) TBA in 0.05 M NaOH and 1 mL of 2.8% (w/v) trichloroacetic acid were added to the

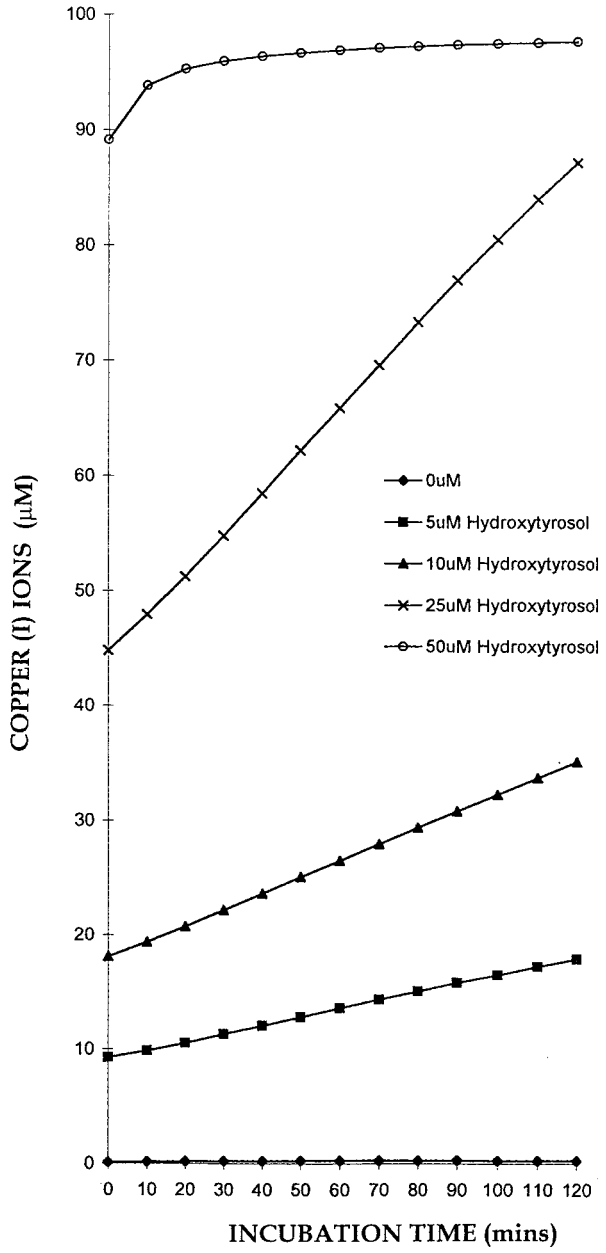


Figure 2. Ability of hydroxytyrosol to reduce Cu^{2+} ions. Experiments were conducted as described under Materials and Methods. The increase in absorbance was measured at 454 nm. Data are means of three independent experiments, and SD values were lower than 3−5%. At 50 μM hydroxytyrosol, the Cu^{2+} ions were fully consumed during the first phase of the reaction such that no further increases in the levels of Cu^{+} could be seen.

samples; tubes were then heated at 80 °C for 20 min, and the chromogen was extracted into 2 mL of butan-1-ol. The absorbance of the organic layer was read at 532 nm.

Reduction of Cu^{2+}. To measure the formation of Cu^{+} in the presence of hydroxytyrosol and ascorbic acid, 2 mL of samples of 100 μM $CuCl_2$, in the presence of 250 μM NC, were prepared in 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4). Hydroxytyrosol and ascorbic acid (in aqueous solutions), when used, were added, and the samples were incubated at room temperature. The time course of the formation of Cu(I)/NC complexes was assayed by measuring the absorbance value at 454 nm. Samples were blanked against NC alone in phosphate buffer. Absorbance readings for Cu(I)/NC complexes were converted into Cu(I) ion concentrations by using an extinction coefficient of 7.95×10^3 M^{-1} cm^{-1} essentially as described by Smith (1952).

Copper−Phenanthroline-Dependent DNA Damage. In a final volume of 1.2 mL the following reagents at the final concentration stated were added in the order indicated: 1,-10-phenanthroline (0.15 mM), copper ions (100 μM added as $CuCl_2$), DNA (0.42 mg mL^{-1}), KH_2PO_4/KOH buffer at pH 7.4 (10 mM), and various concentration of hydroxytyrosol or ascorbate to start the reaction. The mixtures were incubated at 37 °C for 1 h, at the end of which 0.1 mM EDTA was added to stop the reaction.

Assessment of DNA−Sugar Damage. At the end of the incubation, in one set of reaction mixtures, 0.5 mL of 1% (w/v) TBA in 0.05 M NaOH solution and 0.5 mL of 25% (v/v) HCl solution were added. The tubes were heated in a water bath maintained at 80 °C for 20 min. On cooling, the color formed in each tube was extracted into 2 mL of butan-1-ol and the absorbance measured at 532 nm.

Assessment of DNA Base Damage by GC/MS. The second set of reaction mixtures were placed in dialysis bags and dialyzed against water for 24 h. The amount of DNA recovered for each sample after dialysis was determined spectrophotometrically at 260 nm (A_{260} of 1.0 = 50 μg of DNA/mL). Preparation, hydrolysis, derivatization, and analysis of samples by gas chromatography/mass spectrometry (GC/MS) were performed as described previously (Aruoma et al., 1989; Jenner et al., 1998).

Statistical Analyses. Microcal Origin software (version 2.943D) was used to calculate the means and standard deviations (SD) of three independent experiments, involving triplicate analyses for each sample/condition. One-way ANOVA was used to test whether the group means differed significantly. This program uses the Bonferroni method: the threshold for statistical significance $p < 0.05$, divided by the number of comparisons. Thus, a more strict (lower) threshold of significance for each comparison was set in order to have an overall probability of 5% or less that random chance would make any one or more of the differences significant.

RESULTS

Inhibition of Phospholipid Liposome Peroxidation. Ox brain phospholipid liposomes undergo rapid nonenzymic peroxidation when incubated in the presence of $FeCl_3$ and ascorbic acid. In line with previous observations, hydroxytyrosol inhibited this peroxidation at low concentrations. The calculated IC_{50} from the plot of concentration-dependent inhibition of oxidation was 50 ± 1.3 μM (Table 1). The calculated IC_{50} values for other antioxidants compared are 1.5 ± 0.05 μM (carnosol), 2.25 ± 0.08 μM (carnosic acid), 65 ± 2.6 μM (Trolox C, the water soluble analogue of vitamin E), and 250 ± 10 μM (vitamin E). Carnosol and carnosic acid from romemary (Aruoma et al., 1992) were some 130 times more potent than vitamin E in their abilities to protect the phospholipid liposomes against oxidation under the experimental conditions used.

Effect of Hydroxytyrosol on Peroxyl Radical-Dependent LDL Oxidation. Preliminary experiments on Cu^{2+}-dependent LDL oxidation suggested that hydroxytyrosol was reducing Cu^{2+} to Cu^{+}. It was then decided to use an alternative method that involved a peroxyl radical initiator. The azo compound AAPH is a water soluble generator of peroxyl radicals which can oxidize LDL (Noguchi et al., 1993). The oxidation of LDL was monitored by formation of conjugated dienes at 234 nm (Esterbauer et al., 1989, 1992). Hydroxytyrosol was found to be a powerful inhibitor of LDL oxidation (Figure 1). A concentration of 5 μM hydroxytyrosol increased the lag period, under the experimental condition described, to >2 h (Figure 1), indicating protection against oxidative damage to LDL.

Reduction of Cu^{2+} by Hydroxytyrosol. The ability of hydroxytyrosol to stimulate copper ion reduction

Table 1. Inhibition of Phospholipid Liposome Peroxidation by Antioxidants[a]

compound	mol wt	calcd IC_{50} (μM)	natural sources of the compounds
hydroxytyrosol	154	50 ± 1.3	olives
carnosol	340	1.5 ± 0.05	rosemary
carnosic acid	341	2.25 ± 0.08	rosemary
Trolox C	250	65 ± 2.6	synthetic
vitamin E	430	250 ± 10	various fruits and vegetables

[a] The experiments were conducted essentially as described under Materials and Methods. Carnosol, carnosic acid, and vitamin E were dissolved in 2% (v/v) ethanol—this level of ethanol had no effect on the outcome of the reactions. The IC_{50} values were calculated from the concentration-dependent percentage inhibition plot. Values are the means ± SD from three separate determinations.

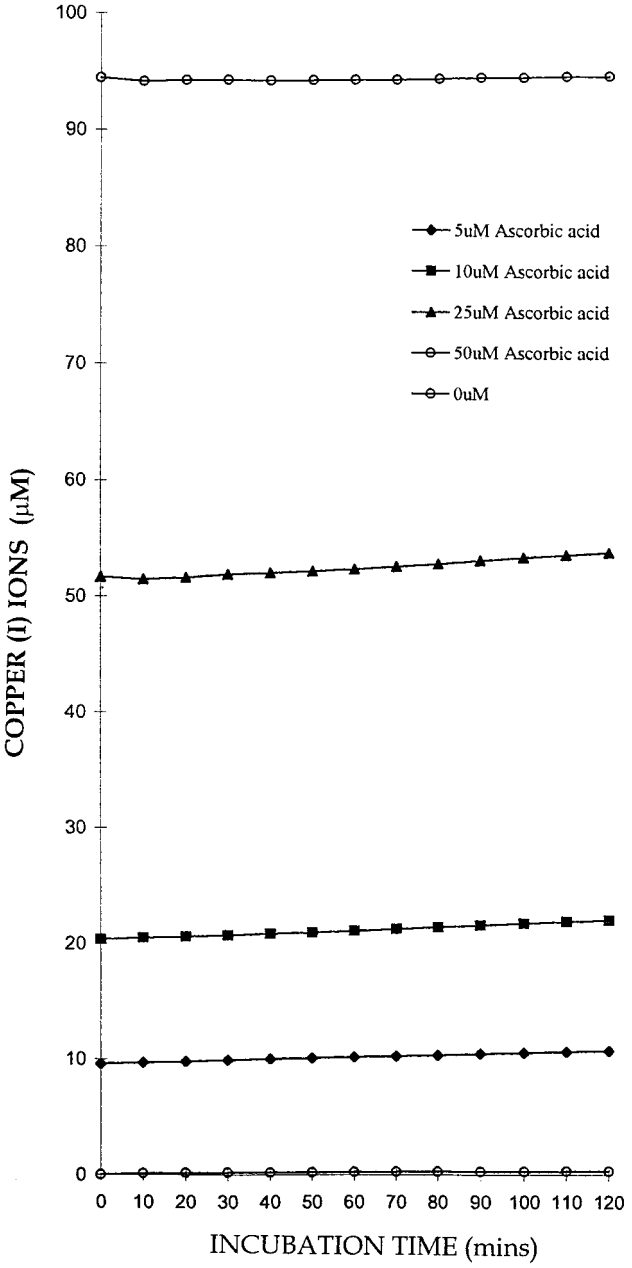


Figure 3. Ability of ascorbic acid to reduce Cu^{2+} ions. Details are as in the legend to Figure 2.

was investigated. Figure 2 shows time-dependent copper(II) reduction with increasing concentrations of hydroxytyrosol. Hydroxytyrosol (Figure 2) appears to have

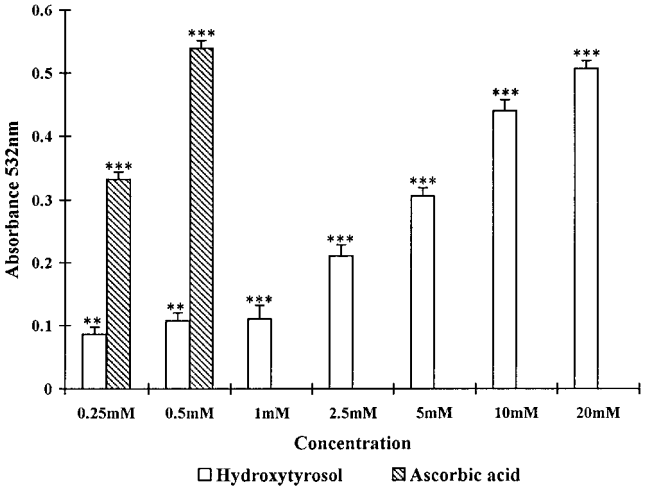


Figure 4. Deoxyribose damage to DNA in the copper–phenanthroline system. Data are means and SD of three independent experiments. The control absorbances in the absence and presence of copper ions were 0.02 ± 0.01 and 0.05 ± 0.01, respectively. ** = $p < 0.01$, *** = $p < 0.001$ versus samples that contained the copper ion–phenanthroline complex alone.

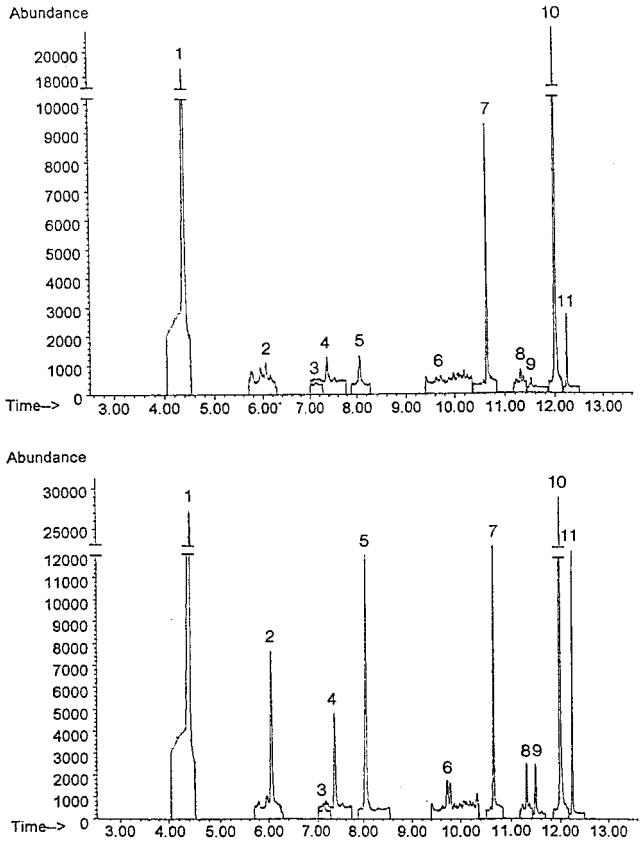


Figure 5. Selected ion current profiles obtained during GC/MS/SIM analysis: analysis of a trimethylsilylated hydrolysate of DNA treated with Cu/phen (top) and analysis of a trimethylsilylated hydrolysate of DNA treated with Cu/phen and 5 mM hydroxytyrosol (bottom). Peaks: 1, 6-azathymine (*m/z* 256); 2, 5-OH-uuracil (*m/z* 329); 3, 5-OHMe-uracil (*m/z* 357); 4, 5-OH-cytosine (*m/z* 328); 5, *cis*-thymine glycol (*m/z* 259); 6, FAPy-adenine (*m/z* 354); 7, 8-OH-adenine (*m/z* 352); 8, 2-OH-adenine (*m/z* 352); 9, FAPy-guanine (*m/z* 442); 10, 2,6-diaminopurine (*m/z* 351); 11, 8-OH-guanine (*m/z* 440).

a two-phase mechanism for reducing Cu^{2+} ions compared with ascorbate (Figure 3). Ascorbate reduction appears complete at the point of addition.

Table 2. DNA Base Modification in the Copper−Phenanthroline System: Effect of Ascorbate[a]

base product	control	SD	+ ph/Cu (mix)	SD	(mix) + 0.1 mM ascorbate	SD	(mix) + 0.25 mM ascorbate	SD
5-OH-uracil	0.04	0.00	0.03	0.00	0.58***	0.10	0.77***	0.09
5-(OHMe)-uracil	0.01	0.00	0.02	0.00	0.04	0.00	0.07	0.00
5-OH-cytosine	0.17	0.02	0.52	0.10	1.47***	0.09	1.71***	0.11
cis-thymine glycol	0.32	0.06	0.43	0.12	0.72***	0.09	1.60***	0.15
FAPy-adenine	0.08	0.01	0.07	0.02	0.14***	0.00	0.16***	0.01
8-OH-adenine	0.15	0.02	1.65	0.53	1.89	0.28	3.75***	1.17
2-OH-adenine	0.06	0.01	0.09	0.00	0.29***	0.07	0.30***	0.00
FAPy-guanine	0.16	0.06	0.44	0.16	0.53	0.07	1.07***	0.28
8-OH-guanine	0.33	0.03	1.72	0.48	3.62***	0.39	4.98***	1.70
total	1.32	0.21	4.97	1.41	9.28	1.09	14.41	3.51
modified bases per 10^6 DNA bases	420	67	1580	448	2951	347	4582	1116

[a] Data are the means ± standard deviations (SD) of three independent experiments ($n = 6$). Base product yields in nmol/mg of DNA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ versus samples that contained the copper ion−phenanthroline complex alone.

Table 3. Oxidative DNA Base Modification in the Copper−Phenanthroline System: Effect of Hydroxytyrosol[a]

base product	control	SD	+ ph/Cu (mix)	SD	(mix) + 0.1 mM H	SD	(mix) + 0.25 mM H	SD	(mix) + 0.5 mM H	SD	(mix) + 1 mM H	SD	(mix) + 5 mM H	SD
5-OH-uracil	0.04	0.00	0.03	0.00	0.18	0.02	0.62***	0.09	0.66***	0.08	1.00***	0.13	1.24***	0.21
5-(OHMe)-uracil	0.01	0.00	0.02	0.00	0.02	0.00	0.03	0.01	0.05***	0.01	0.07***	0.01	0.07***	0.01
5-OH-cytosine	0.17	0.02	0.52	0.10	0.47	0.01	0.85	0.28	1.17***	0.29	1.51***	0.08	1.65***	0.47
cis-thymine glycol	0.32	0.06	0.43	0.12	0.51	0.19	0.55	0.18	0.73	0.07	1.12***	0.32	2.08***	0.53
FAPy-adenine	0.08	0.01	0.07	0.02	0.09	0.02	0.13**	0.01	0.12*	0.04	0.20***	0.05	0.28***	0.02
8-OH-adenine	0.15	0.02	1.65	0.53	1.45	0.25	1.35	0.11	0.93	0.01	1.67	0.46	1.76	0.12
2-OH-adenine	0.06	0.01	0.09	0.00	0.10	0.02	0.15*	0.03	0.12	0.03	0.19***	0.09	0.27***	0.02
FAPy-guanine	0.16	0.06	0.44	0.16	0.20	0.00	0.58	0.19	0.65*	0.07	1.03***	0.04	2.48***	0.18
8-OH-guanine	0.33	0.03	1.72	0.48	1.43	0.54	1.57	0.11	1.84	0.02	2.49	0.80	3.28***	0.98
total	1.32	0.21	4.97	1.41	4.45	1.05	5.83	1.01	6.27	0.62	9.28	1.98	13.11	2.54
modified bases per 10^6 DNA bases	420	67	1580	448	1415	334	1854	321	1994	197	2951	630	4169	808

[a] Data are mean ± SD of three independent experiments ($n = 6$). Base product yields in nmol/mg of DNA. ** = $p < 0.01$, *** = $p < 0.001$ versus samples that contained the copper ion−phenanthroline complex. H, hydroxytyrosol; ph, phenanthroline.

Modulation of Metal Ion-Dependent Oxidative DNA Damage by Hydroxytyrosol. Incubation of calf thymus DNA with a system producing OH• radicals gives rise to extensive chemical modifications of the DNA bases, in a way that appears to be diagnostic for OH• radical (Aruoma et al., 1989; Dizdaroglu, 1991; Cadet et al., 1997). In the copper−phenanthroline-dependent oxidative DNA damage, both the sugar moiety and the bases are damaged. Figure 4 shows the effect of increasing concentration of hydroxytyrosol on DNA−sugar damage measured as absorbance at 532 nm. Ascorbate also exacerbated the sugar damage. A concentration of hydroxytyrosol (20 mM) 40 times that of ascorbate (0.5 mM) achieved the same extent of sugar damage (Figure 4).

The effects that hydroxytyrosol and ascorbate have on oxidative damage to DNA bases were examined using GC/MS. Figure 5 shows the selected ion chromatogram of the products measured, for example, 5-hydroxyuracil (5-OH-uracil), 5-(hydroxymethyl)-uracil (5-OHMe-uracil), 5-hydroxycytosine (5-OH-cytosine), *cis*-thymine glycol, 8-hydroxyadenine (8-OH-adenine), 2-hydroxyadenine (2-OH-adenine), 2,6-diamino-5-formamidopyrimidine (FAPy-guanine), 4,6-diamino-5-formamidopyridine (FAPy-adenine), and 8-hydroxyguanine (8-OH-guanine). Copper ions in the presence of a reducing agent such as ascorbate (Table 2) can damage DNA producing oxidized products in vitro (Aruoma et al., 1991; Dizdaroglu et al., 1990). Hydroxytyrosol (Table 3) increased the levels of the products. 8-OH-guanine, FAPy guanine, and *cis*-thymine glycol were the major products measured. A concentration of 5 mM hydroxytyrosol was required to produce the same level of base products as 0.25 mM ascorbate.

DISCUSSION

Plant extracts and plant-derived antioxidants are receiving wide attention in the food industry and in biomedical research primarily as a result of their ability to stabilize bulk oils, emulsions, and biological membranes against lipid peroxidation and their propensity to act as prophylactic agents (Johnson et al., 1994; Cook and Samman, 1996; Aruoma, 1997; Pezzuto, 1997; Shahidi, 1997). Extra virgin olive oil is widely consumed, and humans may thus consume hydroxytyrosol in the diet. In a study by Braga et al. (1998) on the role of olive oil and seasoning fats in the risk of colorectal carcinoma, it was concluded that "seasoning fats (sunflower, maize, peanut, and soya) did not appear to increase the risk of colorectal carcinoma, and that there was little evidence for a differential effect by fat type". It was further suggested that if a differential effect existed, this would be minor and could favor olive oil. Although the bioavailability of hydroxytyrosol in vivo is not known, the compound has been shown to inhibit platelet aggregation and to prevent the formation of the potent pro-inflammatory agent leukotriene B4 by stimulated leukocytes (Petroni et al., 1995; Galli et al., 1994; Visioli and Galli, 1995). This has led to the suggestion that pure compounds from extra virgin olive oil and olives may show protective effects impor-

tant to the pathologies of atherosclerosis and inflammation (Galli, 1997).

In line with previous observations, hydroxytyrosol was a powerful inhibitor of Fe-induced phospholipid liposome peroxidation (Aeschbach et al., 1994). When compared with other dietary components, hydroxytyrosol was less effective than carnosol and carnosic acid from rosemary (Aruoma et al., 1992) but better than vitamin E in inhibiting phospholipid liposome peroxidation. This could of course be different from their behavior in bulk phase lipids [see Frankel et al. (1994)], depending on the partition coefficient for each compound. Similarly, carnosol and carnosic acid were better than the water soluble analogue of vitamin E, Trolox C, in inhibiting the peroxidation of the phopholipid liposomes. Hydroxytyrosol shares the same solubility characteristics with Trolox C in that they are both liposoluble and also somewhat soluble in aqueous media. This makes both compounds useful for applications in stabilizing water/lipid emulsions against oxidation.

Although olive oil constituents have been reported to inhibit LDL oxidation in vitro (Visioli et al., 1995; Salami et al., 1995) and to be able to stabilize refined, bleached, and deodorized olive oil (Satue et al., 1995), the tendency of hydroxytyrosol to promote the Cu^{2+} reduction suggests that the use of the Cu^{2+}-dependent LDL oxidation may not be an accurate method for assessing the antioxidant action of compounds that can redox cycle metal ions. Nevertheless, hydroxytyrosol inhibited the AAPH-dependent oxidation. Indeed, Yamanaka et al. (1997) have shown that green tea catechins, (−)-epicatechin and (−)-epigallocatechin, depending on concentrations used, accelerate the Cu^{2+}-dependent LDL oxidation. In the AAPH system, the LDL oxidation was mediated by the AAPH-derived peroxyl radical. Hydroxytyrosol has been shown to scavenge the reactive organic peroxyl radical trichloromethylperoxyl (CCl_3O_2C) with a calculated rate constants for the reaction of 8.37×10^6 M^{-1} s^{-1} (Aruoma, 1994). Hydroxytyrosol would be expected to effectively scavenge the $ROO^{\bullet}$ from AAPH, thereby inhibiting LDL from oxidation.

It is becoming clear that the so-called "antioxidants" could have both antioxidant and prooxidant activities depending on what is being tested. Metal chelation and its stabilization and redox activities are critical [see Aruoma (1994) and Halliwell (1990)]. The abilities of hydoxytyrosol and ascorbic acid to reduce copper ions were compared. Hydroxytyrosol first appears to rapidly reduce copper ions with a stoichiometry of 1:2. This is then followed by a second slower reduction at lower concentrations of hydroxytyrosol as there are still available copper(II) ions. This slower reduction was probably mediated by the para-hydroxyl function of hydroxytyrosol (Figure 2). At 50 μM hydroxytyrosol, the Cu^{2+} ions (100 μM) were fully consumed during the first phase of the reaction such that no further increases in the levels of Cu^{+} could be seen. The first rapid reduction probably retained Cu^{+} ions in a complex with partial bond between the 3′-OH function and the C-2 OH on the ethanolic moiety of hydroxytyrosol. By contrast, the first rapid reduction of copper(II) ions by hydroxytyrosol at higher concentrations, for example, 50 μM, was enough to consume all of the copper(II) ions; hence, no further increases were seen after $t = 0$. Ascorbic acid appeared to reduce copper ions with a stoichiometry of 2 molecules of copper ions being reduced by 1 molecule of ascorbic acid (Figure 3). For each point at $t = 0$, ascorbic acid is fully consumed and, therefore, no further increases in Cu(II) ion reduction could be seen.

It was decided to test if the abilities to reduce copper(II) ions could translate to the extent of oxidative damage (Table 3). Ascorbic acid as expected produced increased levels of DNA adducts. Hydroxyl radical attacking DNA can produce potentially mutagenic or lethal lesions; similarly, molecules that increase the formation of hydroxyl radicals would be mutagenic (Loeb, 1989; Newcomb and Loeb, 1998). The copper−phenanthroline complex has nuclease activity (Sigman et al., 1989), and the base modification produced in this system is characteristic of $OH^{\bullet}$ radical (Dizdaroglu et al., 1990). Although the in vitro data obtained may have important implications in vivo, DNA damage may not be the first line of cellular attack of free radicals. Nevertheless, hydroxytyrosol was prooxidant in this system. It is interesting, however, that a concentration of only 5 mM was required to produce the same level of products as 0.25 mM ascorbate. This concentration of hydroxytyrosol produced a 62% rise (based on the number of products measured) in the number of modified bases per 10^6 DNA bases. The finding here has implications for natural antioxidants and their bioavailability. It is not anticipated that the in vivo circulating concentration of hydroxytyrosol will be >500 μM. The high concentration required for the prooxidant activity is not physiological.

The complex relationship between the structure actions of plant-derived antioxidants and their radical scavenging inhibition of lipid peroxidation, pro-oxidant, and metal ion chelating abilities has implications for the consideration of the antioxidants as prophylactic agents in vivo *(*Aruoma, 1999*)*. It would now seem worthwhile to evaluate how human diets rich in spices, herbs, and extra virgin olive oil modulate indices of oxidative damage in vivo.

ACKNOWLEDGMENT

Dr. Elizabeth Offord is thanked for critical reading of the manuscript and for helpful suggestions. O.I.A. is Collaborador Professor of the University of São Paulo-Ribeirão Preto, Faculty of Pharmaceutical Sciences, Brazil.

LITERATURE CITED

Aeschbach, R.; Loliger, J.; Scott, B. C.; Murcia, A.; Butler, J.; Halliwell, B.; Aruoma, O. I. Antioxidant action of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food Chem. Toxicol.* **1994**, *32*, 31−36.

Aruoma, O. I. Nutrition and health aspects of free radicals and antioxidants. *Food Chem. Toxicol.* **1994**, *32*, 671−683.

Aruoma, O. I. Assessment of potential prooxidant and antioxidant actions. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1617−1625.

Aruoma, O. I. Extracts as antioxidant prophylactic agents. *Int. News Fats, Oils Relat. Mater.* **1997**, *8*, 1236−1242.

Aruoma, O. I. Free radicals, antioxidants and international nutrition. *Asia Pacific J. Clin. Nutr.* **1999**, *8* (Feb).

Aruoma, O. I.; Halliwell, B.; Dizdaroglu, M. Iron ion-dependent modification of bases in DNA by the superoxide radical generating system hypoxanthine/xanthine oxidase. *J. Biol. Chem.* **1989**, *264*, 13024−13028.

Aruoma, O. I.; Evans, P. J.; Kaur, H.; Sutcliffe, L.; Halliwell, B. An evaluation of the antioxidant and potential pro-

oxidant properties of food additives and trolox C, vitamin E and probucol. *Free Radical Res. Commun.* **1990**, *10*, 143−157.

Aruoma, O. I.; Halliwell, B.; Gajewski, E.; Dizdaroglu, M. Copper ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochem. J.* **1991**, *273*, 601−604.

Aruoma, O. I.; Halliwell, B.; Aeschbach, R.; Loliger, J. Antioxidant and prooxidant properties of active rosemary constituents: carnosol and carnosic acid. *Xenobiotica* **1992**, *22*, 257−268.

Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and pro-oxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem.* **1993**, *41*, 1880−1885.

Baraldi, P. G.; Simoni, D.; Manfredini, S.; Menziani, E. Preparation of 3,4-dihydroxy-1-benzeneethanol: A reinvestigation. *Liebigs Ann. Chem.* **1983**, *1983*, 684−686.

Boskou, D. *Olive Oil: Chemistry and Technology*; AOCS Press: Champaign, IL, 1996.

Braga, C.; Vecchia, C. L.; Franceschi, S.; Negri, E.; Parpinel, M.; Decarli, A.; Giacosa, A.; Trichopoulos, D. Olive oil, other seasoning fats and the riskof colorectal carcinoma. *Cancer* **1998**, *82*, 448−453.

Cadet, J.; Berger, M.; Douki, T.; Ravanat, J.-L. Oxidative damage to DNA: Formation, measurement and biological significance. *Rev. Biochem. Pharmacol.* **1997**, *131*, 1−87.

Cook, N. C.; Samman, S. Flavonoids—Chemistry, metabolism, cardioprotective effects and dietary sources. *Nutr. Biochem.* **1996**, *7*, 66−76.

Dizdaroglu, M. Chemical determination of free radical-induced damage to DNA. *Free Radical Biol. Med.* **1991**, *10*, 225−242.

Dizdaroglu, M.; Aruoma, O. I.; Halliwell, B. Modification of bases in DNA by copper-ion 1,10-phenanthroline complexes. *Biochemistry* **1990**, *29*, 8447−8451.

Esterbauer, H.; Striegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **1989**, *6*, 67−75.

Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **1992**, *13*, 341−390.

Frankel, E. N.; Huang, S. W.; Kanner, J.; German, J. B. Interfacial phenomena in the evaluation of antioxidants: bulk oils vs. emulsions. *J. Agric. Food Chem.* **1994**, *42*, 1054−1059.

Galli, C. Letter to the editor on olive oil. *Int. News Fats, Oils Relat. Mater.* **1997**, *8*, 232.

Galli, C.; Petron, A.; Visioli, F. Natural antioxidants with special reference to those in olive oil and cell protection. *Eur. J. Pharm. Sci.* **1994**, *21*, 67−68.

Halliwell, B. How to characterize a biological antioxidant. *Free Radical Res. Commun.* **1990**, 1−32.

Hudson, B. J. F. *Food Antioxidants*; Elsevier Applied Science: London, U.K., 1990.

Jenner, A.; England, T. G.; Aruoma, O. I.; Halliwell, B. Measurement of oxidative DNA damage by gas chromatography-mass spectrometry. The use of ethanethiol to prevent artefactual generation of oxidized DNA bases. *Biochem. J.* **1998**, *331*, 365−369.

Johnson, I. T.; Williamson, G.; Musk, S. R. R. Anticarcinogenic factors in plant foods. A new class of nutrients. *Nutr. Res. Rev.* **1994**, *7*, 1−30.

Loeb, L. A. Endogenous carcinogenesis: molecular oncology into the twenty-first century presidential address. *Cancer Res.* **1989**, *49*, 5489−5496.

Loliger, J. The use of antioxidants in food. In *Free Radicals and Food Additives*; Aruoma, O. I., Halliwell, B., Eds.; Taylor and Francis: London, U.K., 1991; pp 121−150.

Markwell, M. A. K.; Has, S. M.; Tolbert, N. E.; Bieger, L. L. Protein determination in membrane and lipoprotein samples: manual and automased procedures. *Methods Enzymol.* **1981**, *72*, 296−303.

Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable phenolic compounds in virgin olive oil: their extraction separation and quantitative and semi-quantitative evaluation by HPLC. *J. Agric. Food Chem.* **1992**, *40*, 1571−1576.

Newcomb, T. G.; Loeb, L. A. Mechanism of mutagenicity of oxidatively-modified bases. In *Molecular Biology of Free Radicals in Human Diseases*; Aruoma, O. I., Halliwell, B., Eds.; Saint Lucia: OICA International: Saint Lucia, 1998; pp 137−166.

Noguchi, N.; Gotoh, N.; Niki, E. Dynamics of the oxidation of low density lipoprotein induced by free radicals. *Biochim. Biophys. Acta* **1993**, *1168*, 348−357.

Pearson, D. A.; Frankel, E. N.; Aeschbach, R.; German, J. B. Inhibition of endothelial cell-mediated oxidation of low density lipoprotein of rosemary and plant phenolics. *J. Agric. Food Chem.* **1997**, *45*, 578−582.

Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G. F.; Galli, C. Inhibition of platelet aggregation and eicosanoid production by a phenolic component of olive oil. *Thromb. Res.* **1995**, *78*, 151−160.

Pezzuto, J. M. Plant-derived anticancer agents. *Biochem. Pharmacol.* **1997**, *53*, 121−133.

Salami, M.; Galli, C.; De Angelis, L.; Visioli, F. Formation of F_2-isoprostanes in oxidized low density lipoproteins: inhibitory effect of hydroxytyrosol. *Pharmacol. Res.* **1995**, *31*, 1−5.

Satue, M. T.; Huang, S. W.; Frankel, E. N. Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached and deodorized olive oil. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1131−1137.

Shahidi, F. *Natural Antioxidants. Chemistry, Health Effects and Applications*; AOCS Press: Champaign, IL, 1997.

Sigman, D. S. Nuclease activity of 1,10-phenanthroline copper ion. *Acc. Chem. Res.* **1986**, *19*, 180−186.

Smith, G. F.; McCurdy, W. H. 2,9-dimethyl-1,10-phenanthroline. New specific in spectrophotometric determination of copper. *Anal. Chem.* **1952**, *24*, 371−373.

Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztem, J. L. Beyond cholesterol modification of low density lipoprotein that increase its atherogenicity. *New Engl. J. Med.* **1989**, *320*, 915−924.

Steinbrecher, U. P. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein b by lipid peroxide decomposition products. *J. Biol. Chem.* **1987**, *262*, 3603−3608.

Thederahn, T. B.; Kuwabra, D. M.; Larson, T. A.; Sigman, D. S. Nuclease activity of 1,10-phenanthroline copper: kinetic mechanism. *J. Am. Chem. Soc.* **1989**, *111*, 4941−4946.

Visioli, F.; Galli, C. Natural antioxidants in prevention of coronary heart disease: the potential role of olive oil and its minor constituents. *Nutr. Metab. Cardiovasc. Dis.* **1995**, *5*, 306−314.

Visioli, F.; Bellomo, G.; Montedoro, G. F.; Galli, C. Low density lipoprotein oxidation is inhibited *in vitro* by olive oil constituents. *Atherosclerosis* **1995**, *117*, 25−32.

Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60−64.

Yamanaka, N.; Oda, O.; Nagao, S. Green tea catechins such as (−)-epicathechin and (−)-epigallocatechin accelerate Cu^{2+}-induced low density lipoprotein oxidation in propagation phase. *FEBS Lett.* **1997**, *401*, 230−234.

Received for review June 15, 1998. Revised manuscript received October 6, 1998. Accepted October 6, 1998. We are grateful to Nestec Research Center, Switzerland, the World Cancer Research Fund, and the U.K. Ministry of Agricultural Fisheries and Food for research support.

JF980649B