Virgin Olive Oil: Free Radical Production Studied with Spin-Trapping Electron Paramagnetic Resonance Spectroscopy

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ABSTRACT: Spin trapping using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has been used to detect and distinguish free radicals in samples of Greek extra virgin olive oils. A number of the samples examined immediately after the addition of the spin trap showed a spontaneous complex electron paramagnetic resonance (EPR) signal. The majority of DMPO-radical adducts formed (80–90%) represented peroxyl and alkoxyl radical adducts. Similar spectra were recorded when DMPO was added in oxidized triolein and then treated with Fe^{2+}, Fe^{3+}, or Cu^{2+} or when EPR-silent olive oil samples were treated with these metallic ions. Metal ion-catalyzed decomposition of triolein hydroperoxides, as recorded by EPR signal intensity, increased with increasing metal ion concentration in the micromolar range. The relative concentration of alkoxyl-DMPO adducts increased with increasing Fe^{2+} or Fe^{3+} concentration, whereas that of peroxyl-DMPO species decreased. In contrast, the relative concentrations of alkoxyl and peroxyl species produced by Cu^{2+} were similar over the whole metal concentration range examined. Exposure of EPR-silent virgin olive oil or oxidized triolein to ultraviolet light in the presence of DMPO resulted in the detection of a three-line spectrum characterized by wide line widths.

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KEY WORDS: Copper, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), electron paramagnetic resonance spectroscopy (EPR), free radicals, iron, olive oil, spin trapping.

It has long been known that oxidative rancidity is the main deteriorative change of olive oil during storage and that it is due to the oxidation of unsaturated fatty acids and the subsequent formation of compounds possessing an unpleasant taste and odor (1). This general oxidation process affecting the stability of vegetable oils is often called autoxidation and involves a free radical mechanism. It is assumed that hydroperoxide groups attach to the carbon atom of unsaturated fatty compounds, and subsequently, the breakdown of hydroperoxides gives a chain reaction of autoxidation (2,3).

Olive oil oxidation is affected by a number of factors, such as oxygen, temperature, presence of metals and chromophores, light, and ionizing radiation, whereas the resistance of virgin olive oil to oxidation is related to the high levels of monounsaturated triacylglycerols and the presence of natural antioxidants (1). Transition metals, especially iron and copper, are known contaminants of virgin olive oil and may act as pro-oxidant factors because they catalyze both the generation of free radicals and the decomposition of hydroperoxides (1–3). The oxidation of unsaturated fats is also accelerated by exposure to light, and direct photo-oxidation is due to free radicals produced by ultraviolet (UV) irradiation, which catalyzes the decomposition of hydroperoxides and other oxygen complexes of unsaturated lipids (2,3).

Of the available methods for the detection of free radicals, only spin trapping offers the opportunity to simultaneously measure and distinguish among a variety of important chemically or biologically generated free radicals. Most of the spin-trapping agents used have a nitrone-type group that is able to form a nitroxide (spin adduct) during the trapping of the free radical, making it detectable in electron paramagnetic resonance (EPR) spectroscopy (4). Among several nitrones used as spin traps, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has received the most attention. Reaction of this spin trap with superoxide and hydroxyl radicals or radicals of a lipid nature derived from hydroxyl radicals produces spin-trapped adducts with characteristic EPR spectra (5,6). Moreover, spin trapping using DMPO has been used to detect and distinguish between carbon-centered, alkoxyl, and peroxyl radicals produced during metal ion- and lipoxygenase-catalyzed breakdown (7), as well as photolytic breakdown of peroxidized fatty acids (8).

In this study, DMPO has been used as a probe for monitoring metal ion-catalyzed production and photolytic generation of free radicals in virgin olive oil, and the EPR spectra recorded were compared with those obtained during decomposition of peroxidized triolein. The objective of this EPR approach was to understand metal ion-catalyzed production of free radicals in virgin olive oil, which will help to develop a methodology for the direct estimation of the oxidative stability of virgin olive oil samples against metal-induced decomposition.

EXPERIMENTAL PROCEDURES

Virgin olive oil samples were generously donated by ELAIS S.A.–Unilever, Greece, and by the National Agricultural Research Foundation, Subtropics and Olive Institute of Chania,

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Greece. DMPO, 99% triolein (T7140), and 65% triolein (T7752) were obtained from Sigma (St. Louis, MO).

EPR spectra were recorded on a Bruker ER 200D spectrometer system (Bruker, Rheinstetten Forchheim, Germany) operating at the X-band. Unless otherwise stated, the samples were contained in an aqueous WG-813-Q Wilmad (Buena, NJ) Suprasil cell at room temperature. In several experiments Wilmad 734-PQ-8 cylindrical Suprasil sample tubes were used. Typical instruments settings were as follows: center field, 3,471 G; scan range, 100 G; time constant, 500 ms; microwave frequency, 9.77 Ghz; and modulation amplitude, 1 G. In order to ensure comparable EPR quantitation, all spectra of DMPO-containing samples were measured under identical experimental conditions regarding both the samples (volume, concentration of the spin trap, the oxidizing agent, and the same central position in the cavity) and the EPR parameters (gain and modulation). Data acquisition and the measurement of hyperfine coupling constants were performed using DAT-200 software (University of Lubeck, Germany). Nitroxide production was monitored from the total area under the EPR spectrum curve. The simulations of the experimental spectra were conducted with a simulation program (Winsim) (9) developed at the National Institute of Environment and Health Sciences (Research Triangle Park, North Carolina), which is available through the Internet (http://epr.niehs.nih.gov).

Spin-trapping reaction mixtures (1 mL) containing olive oil sample or triolein, DMPO (18 mM), and various metal concentrations (when present, as indicated) were incubated for various time periods, at 25°C, under ambient light. When the breakdown of hydroperoxides by metal ions was examined, an aqueous metal solution (10 μL) was added to olive oil or triolein, and the mixture was incubated for 5 min or 15 h, at 25°C, in the absence of DMPO. EPR measurement started 5 min after the addition of the spin trap. Photolysis of olive oil or triolein was performed in the presence or absence of DMPO by exposing the sample contained in the EPR cell for 5 min to a 254- or 365-nm wave light of a Uniequip UVLS-26 lamp (Martinsried, Germany). No experimental differences were observed between using commercial DMPO and DMPO purified using the method of Buettner and Oberley (10). Both purified and unpurified DMPO samples by themselves, without any addition, showed no EPR signal. Triolein was peroxidized before use by exposure to air for 72 h at room temperature (11).

RESULTS AND DISCUSSION

Samples of Greek virgin olive oils were examined for the spontaneous production of free radicals by spin trapping using DMPO. Although most of the samples examined were EPR silent immediately after the addition of the spin trap, a number of samples showed a multiple EPR signal (Fig. 1). The composite spectrum revealed a mixture of four distinct radical adducts. After calculation of their respective hyperfine coupling constants (a_N, a_H in Gauss) by computer simulation, these adducts were assigned as peroxyl (DMPO-˙OOL; a_N = 13.5, $a_{H\beta}$ = 10.4, $a_{H\gamma}$ = 0.9), alkoxyl (DMPO-˙OL; a_N = 12.8, $a_{H\beta}$ = 7.4, $a_{H\gamma}$ = 2.0), alkyl (DMPO-ˆL; a_N = 14.4, $a_{H\beta}$ = 23.7), and an oxidized form of DMPO with a characteristic three-line EPR spectrum (a_N = 15.1) (12). As observed in Figure 1, some of the peaks, especially the inner peaks of the alkyl radicals, were not visible in the experimental spectrum owing to overlapping of the subspectra. The γ-hydrogen coupling constants of each DMPO adduct could not be revealed directly from the spectrum but were calculated in the simulations to obtain a better correlation. The above coupling constants appear to be consistent with those reported in the literature after trapping alkoxyl, peroxyl, and alkyl radicals generated from fatty acid suspensions in toluene (7,8). Semiquantitation of each radical adduct was achieved by double-integrating the simulated subspectra. Peroxyl, alkoxyl, alkyl, and oxidized DMPO radicals represented about 50 ± 3.5, 38 ± 2.7, 8 ± 0.6, and 4 ± 0.3%, respectively, of the total radical mixture [mean ± standard deviation (SD), 8 determinations].

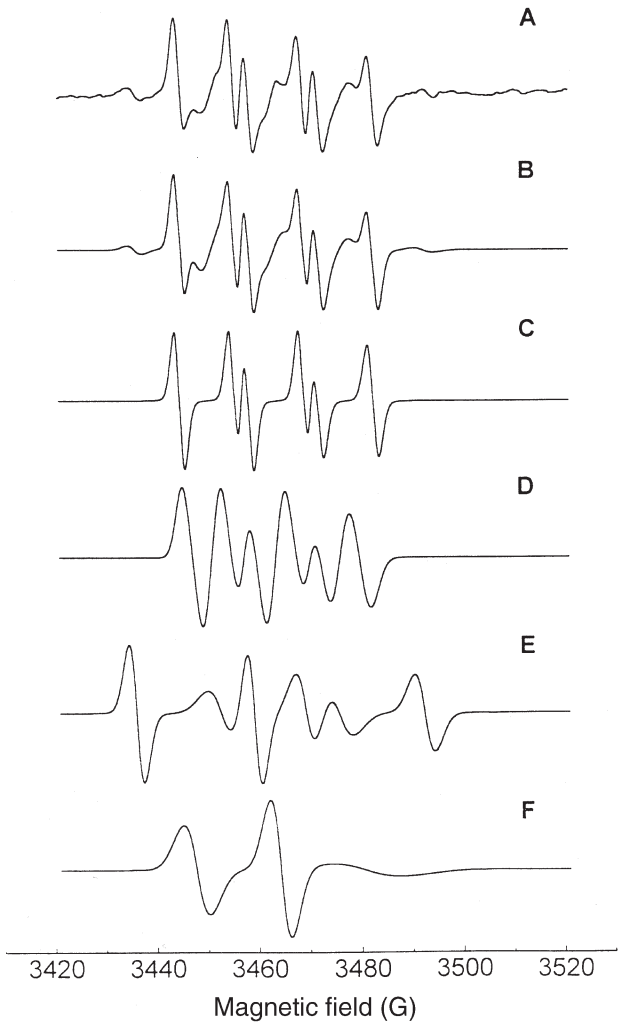

FIG. 1. Electron paramagnetic resonance (EPR) spectrum produced after addition of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (18 mM) to a spontaneously EPR-active virgin olive oil sample. (A) Experimental spectrum; (B) composite simulated spectrum; (C)–(F) simulated spectra of peroxyl adduct, alkoxyl adduct, alkyl adduct, and the oxidized form of DMPO, respectively.

It is already known that peroxidized fatty acids produce a number of DMPO-free radical adducts when reacted with metal ions and lipoxygenase (7,13,14). On the other hand, among a variety of olive oil pro-oxidant factors, iron and copper represent the major transition metal pro-oxidant factors of this oil (1), and we have recently reported the presence of lipoxygenase activity in virgin olive oil (15,16). In this respect, it is reasonable to assume that certain samples of virgin olive oil, such as the one in Figure 1, may contain a high ratio of pro-oxidant factors vs. antioxidant or chelating components, which will easily permit the production of free radicals detected by the DMPO spin trap.

Because oleic acid is the major constituent of olive oil triacylglycerols (55–83%) (1), we used triolein as a model system for detecting and distinguishing free radicals produced under various conditions, as compared to the system of virgin olive oil. When we treated oxidized triolein with low concentrations of Fe^{2+} (5 μM), a composite spectrum similar to that in Figure 1 was obtained (not shown). Analysis and resolution of this spectrum indicated the presence of the main radical species (peroxyl, alkoxyl, and alkyl) observed with olive oil, characterized by similar hyperfine coupling constants. In this case, peroxyl, alkoxyl, and alkyl radicals represented about 43 ± 3, 49 ± 3, and 8 ± 0.5% (mean ± SD, 8 determinations), respectively, of the total radical mixture. Because the 65% Sigma triolein normally used in our experiments may contain tocopherols, which could affect the nature of free radicals produced on oxidation, we also used highly purified Sigma triolein (99%) for comparison. In this case, no experimental differences were observed.

Metal ion-catalyzed decomposition of triolein hydroperoxides, as recorded by EPR signal intensity, increased with increasing metal ion concentration (Fig. 2). Fe^{2+} was more active than Fe^{3+} in producing radical adducts from oxidized triolein, which is in agreement with previous results with fatty acid hydroperoxides (7). In this respect, the signal intensity of the Fe^{2+}- induced EPR spectrum, as calculated by the total area of the spectra, reached a plateau value at a metal concentration of 5 μM, which is about threefold lower than the respective one for Fe^{3+}. On the other hand, Cu^{2+} was the least active metal ion in catalyzing breakdown of triolein hydroperoxides. Moreover, as shown in Figure 3, analysis of the simulated EPR spectra produced by Fe^{2+} or Fe^{3+} indicated that the relative concentration of alkoxyl-DMPO adducts increased with increasing metal concentration, whereas that of peroxyl-DMPO species decreased. In contrast, the relative concentrations of alkoxyl and peroxyl species produced by Cu^{2+} were similar over the whole metal concentration range examined.

When EPR-silent olive oil samples reacted with Cu^{2+} (5 μM) for 5 min and were then treated with DMPO, a mixture of radical adducts was recorded containing mainly 55 ± 4.4%

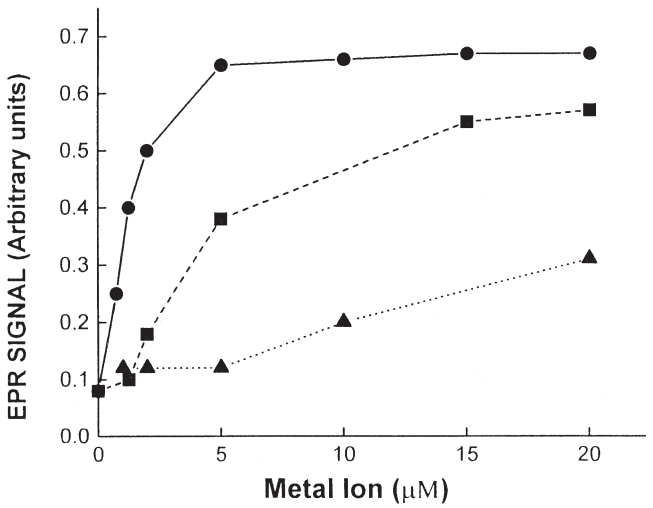

FIG. 2. Effect of metal ion concentration on metal ion-catalyzed decomposition of triolein hydroperoxides, as recorded by EPR signal intensity. Spin-trapping reaction mixtures (1 mL) containing triolein and various metal ion concentrations, as indicated, were incubated for 5 min, at 25°C, under ambient light. EPR measurement started 5 min after the addition of DMPO (18 mM). Signal intensity was calculated as the total area under the EPR curve. (●), Fe^{2+}; (■), Fe^{3+}; (▲), Cu^{2+}. See Figure 1 for abbreviations.

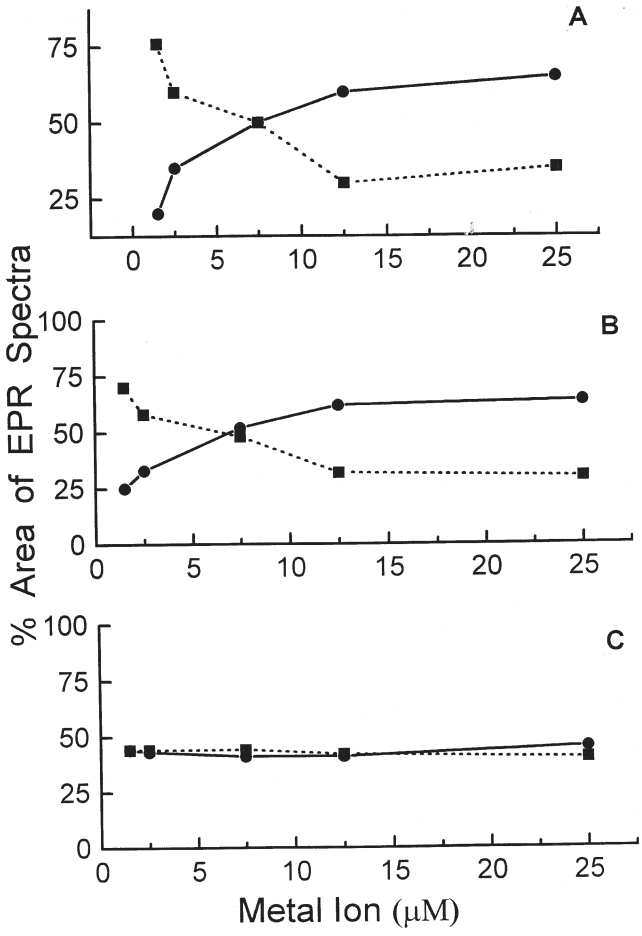

FIG. 3. Relative concentrations of alkoxyl (●) and peroxyl (■) radical adducts produced in triolein by Fe^{2+} (A), Fe^{3+} (B), or Cu^{2+} (C) as a function of metal ion concentration. Spin-trapping reaction mixtures (1 mL) containing triolein and various metal concentrations, as indicated, were incubated for 5 min, at 25°C, under ambient light. EPR measurement started 5 min after the addition of DMPO (18 mM). Relative concentrations of alkoxyl and peroxyl radical adducts were calculated from simulated spectra. See Figure 1 for abbreviations.

peroxyl, 40 ± 3.2% alkoxyl, and 5 ± 0.4% alkyl radicals (mean ± SD, 8 determinations) (Fig. 4A). In contrast, EPR-silent olive oil samples remained silent after similar treatment with Fe^{2+} (10 μM) or Fe^{3+} (10 μM). However, incubation of the same olive oil samples with Fe^{2+} or Fe^{3+} for 15 h produced very weak EPR spectra (Figs. 4B,4C); in both cases, the alkoxyl radicals were the predominant species calculated.

The complex EPR spectrum of Figure 1, originating from a spontaneous EPR-active olive oil sample or from triolein treated with Fe^{2+}, was transformed to a three-line EPR spectrum after storage of the sample—olive oil + DMPO (Fig. 5A) or triolein + Fe^{2+} + DMPO (not shown)—for 7 d. To explain this spectrum, one can speculate that a strong oxidizing agent, probably a peroxyl or alkoxyl radical, replaces the β-hydrogen on the DMPO adduct. In this case, the hyperfine splitting in the oxidized DMPO product is provided by the nitrogen atom only independently from the nature of the trapped radical(s) (11). This three-line spectrum was characterized by wide line widths and is reminiscent of that of an immobilized amphiphilic nitroxide free radical 5-doxyl-stearic acid (5-DSA) with anisotropic motion (Fig. 5B) (17). Apparently, the high viscosity of olive oil, as well as the hydrophobic interactions between the lipid chains of the trapped radicals (or the alkyl chains of 5-DSA) and the oil phase resulted in the anisotropic interaction between the free electron and the nitrogen nuclei and produced excessive line broadening in the spectrum.

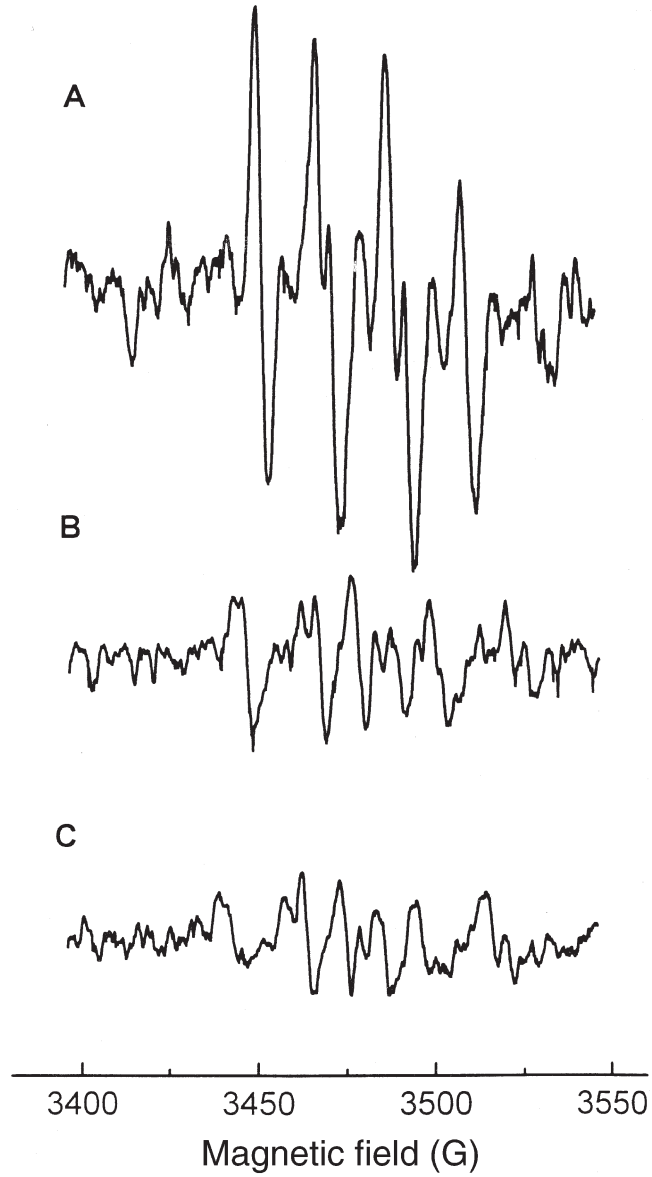

FIG. 4. EPR spectra of virgin olive oil treated with metal ions in the presence of DMPO. Spin-trapping reaction mixtures (1 mL) containing olive oil were treated with Cu^{2+} (5 μM) (A), or Fe^{2+} (10 μM) (B), or Fe^{3+} (10 μM) (C) for 15 h (A,B) or 5 min (C), at 25°C, under ambient light. EPR measurement started 5 min after the addition of DMPO (18 mM). See Figure 1 for abbreviations.

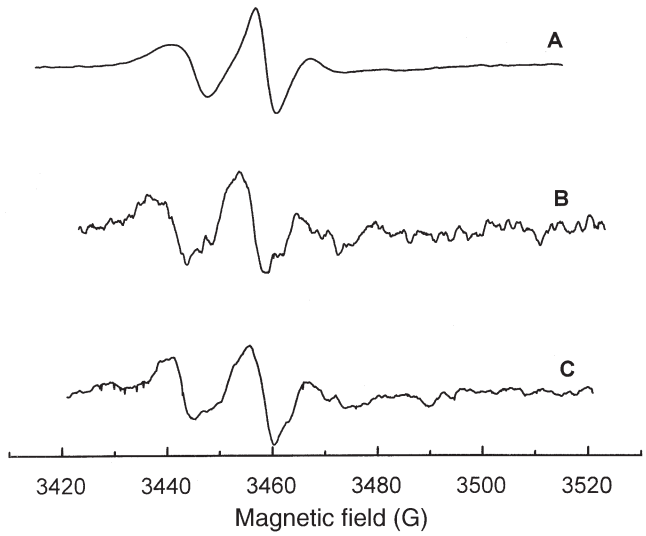

FIG. 5. EPR spectra of (A) 5-doxyl-stearic acid (1 mM) in EPR-silent olive oil, under ambient light, in transparent glass bottles; (B) spontaneously EPR-active virgin olive oil stored for 7 d in presence of DMPO (18 mM); (C) ultraviolet-irradiated (5 min) EPR-silent virgin olive oil. See Figure 1 for other abbreviations.

A similar three-line EPR spectrum characterized by wide line widths (Fig. 5C) was also obtained when EPR-silent virgin olive oil was exposed to UV light (254 nm) in the presence of DMPO. Unlike the results in Figure 5C, it has been reported that photolysis of peroxidized fatty acids in toluene in the presence of DMPO produced mostly alkoxyl and peroxyl radical adducts as well as weak signals from unidentified carbon-centered radicals (8). The above discrepancy may be due either to the intensity and the wavelength profile of the UV lamp used or to the irradiated sample itself, which in our case is consisted primarily of triacylglycerols and not free fatty acids (1). In this respect, a well-known difference between hydroperoxides of unsaturated fatty acids and hydroperoxides of unsaturated triacylglycerols is the tendency for dimerization of oxidized free fatty acids or their methyl esters only (3). Because the UV spectrum of sunlight does not contain the component of 254 nm, the possibility of 254-nm UV exposure during production and storage of olive oil is not realistic. However, photolysis was carried out using this high energy radiation in order to

drastically accelerate the photolytic breakdown of peroxidized fatty acids. Accordingly, by using a 365-nm source for the irradiation of our samples (20 min), a multicomponent EPR spectrum was recorded, consisting mainly of alkoxyl and peroxyl species (not shown). We also have to emphasize that UV-irradiated DMPO does not show any EPR activity.

We conclude that, because concentrations of iron and copper present in virgin olive oil range between 0.5 and 3 ppm and 0.001 and 0.2 ppm, respectively (1), it is reasonable to assume that the contamination of virgin olive oil with intermediate concentrations of iron or relatively high concentrations of copper (according to the above range), together with the presence in the oil of low amounts of free radical scavengers and metal chelators, is responsible for the production of alkoxyl, peroxyl, and carbon-centered free-radical DMPO adducts observed in several samples of this vegetable oil. In addition, direct photo-oxidation of virgin olive oil may gradually induce the formation of strong oxidizing agents such as peroxyl and alkoxyl radicals. All the above free-radical species produced will negatively affect the quality of olive oil.

ACKNOWLEDGMENTS

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