Electron Paramagnetic Resonance Investigations of Free Radicals in Extra Virgin Olive Oils

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Free radicals in olive oils were identified and quantified by EPR, by means of the spin-trapping technique making use of α-phenylbutylnitrone (PBN) as spin trap. The radical species were identified as PBN-trapped hydroxyl radicals (PBN-•OH) in the water microdroplets inside the fat medium. The largest radical concentration was 12.5 μM ≡ 100%. The following were the relative concentrations of the radicals under different conditions: (1) Two oils, produced by continuous centrifugation, aged for 1 year, showed a 25−30% increase in the radicals compared to nonaged oils; 1-year-old oil, produced by pressure, did not differ from the nonaged oil. (2) Radical production was markedly reduced by N_2 bubbling; it was increased by heating, whereas it showed a biphasic pattern by air bubbling over time. (3) Radical concentration as a function of the UV irradiation time increased up to a maximum, after which it decreased and finally remained constant. The phenolic and oxygen contents were related to the radical content. This study demonstrates that the EPR technique is suitably applied to the detection of free radicals in olive oil and that storage, handling, and stress conditions of the oils significantly influence the radical concentration.

Keywords: *EPR; olive oil; hydroxyl radical; oil handling; oil storage*

INTRODUCTION

The antioxidant compounds present in olive oil have been the focus of attention of food technologists and nutrition experts for several years. In contrast, scarce attention has been paid to the production of free radicals, which may accumulate in an olive oil during preparation, prolonged storage, or stress conditions.

Because of the wide use of extra virgin olive oils in the diet of the Mediterranean population, it is of paramount importance to identify and quantify free radicals in these oils and to prevent their formation or eliminate them by modifying storage conditions or procedures in oil production.

The formation of free radicals in olive oil has been studied by chemiluminometry upon UV and γ irradiation, heating, and different storage conditions (*1*−*3*). Other studies analyzed the formation of radical species in olive oils by high-performance liquid chromatography (HPLC) (*4*). These studies suggest that most of the radicals are produced by the oxidation of polyunsaturated acids and are mainly peroxyl radicals, which are scavenged by phenolic acids (*5*−*8*). Despite the effectiveness of the electron paramagnetic resonance (EPR) technique for radical identification and quantification and the well-known dangerous effect of free radicals to human health, the type and amount of radicals formed in extra virgin olive oil by different preparation procedures and storage conditions are still unknown. Therefore, we performed an EPR study on the radicals formed in some extra virgin olive oils, selected on the basis of different harvesting regions (Italian) and different preparation methods, subjected to different conditions such as aeration, heating, aging, and UV lighting.

The results provide evidence for the suitability of EPR in the study of the radicals of the olive oils, for the beneficial or detrimental effects of different handling or storage conditions on the radical concentration, and finally for the transient nature of the radicals, which are scavenged by the antioxidants of the oil when collisions of the two molecule types are provoked.

EXPERIMENTAL PROCEDURES

Materials. α-Phenylbutylnitrone (PBN) and 2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl (Tempo) were purchased from Sigma-Aldrich Srl (Milan, Italy) and used without any further purification; caffeic acid and Folin−Ciocolteu reagent were from Sigma. All other reagents were of analytical grade.

Sample Preparation and Method for EPR Analysis. The EPR spectra were recorded with the aid of EMX EPR instrumentation from Bruker, interfaced to a PC-IBM computer equipped with Bruker software for data recording and handling. Modulation of the magnetic field was 1 G. Each scan was repeated three times in 1.5 min, with a filter of 20 ms. A solid amount of PBN was dissolved into the olive oil to obtain a 10 mM solution. The PBN−oil mixture was left to equilibrate for 24 h, in the dark, and gently mixed in a sealed metal container under nitrogen atmosphere. A quartz flat cell fixed in the EPR cavity (to guarantee the reproducibility of the measurements and to allow intensity measurements) was filled by 0.5 mL of the oil mixture and immediately tested by EPR. However, the reproducibility of EPR measurements was verified by repeated measurements on different samples, and results were reported only in the case of reproducible measurements. Different concentrations of PBN provided the same results as the 10 mM concentration. Toluene and water solutions of PBN were tested as a control. In a few cases, a small EPR signal from these reference samples was subtracted from the EPR signal obtained by the oil sample.

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Table 1. Characteristics of the Extra Virgin Olive Oils Used in This Study[a]

region of production	cultivar	period of harvest and milling	phenolic content (ppm)	free acidity (% w/w of oleic acid)	peroxide number (mequiv of peroxide/kg)	dissolved oxygen (ppm)
Apulia (new)	Coratina	December 1999	247	0.44	16.0	3.5
Apulia (old)	Coratina	December 1998	254	0.55	17.5	2.6
Umbria (new)	Frantoio	December 1999	135	0.50	18.0	4.2
Umbria (old)	Frantoio	December 1998	157	0.45	18.0	4.6
The Marches (new)	Leccino Frantoio Raggiola	December 1999	164	0.45	16.0	3.9
The Marches (old)	Leccino Frantoio Raggiola	December 1998	156	0.60	16.0	4.0

[a] Values are the means of four different assays. Each mean has a standard error of <10%. All parameters were measured before PBN addition, with minimal agitation of the oil.

The absolute concentrations were measured by comparing the intensities of the EPR spectra from oil solutions with standard toluene and water solutions of the nitroxide radical Tempo. Of course, the flat cell was used for these measurements, too. After identification of the type of radicals in the oil, the EPR analysis was carried out to evaluate the relative amounts of radicals formed in the experimental conditions. Double integration of the EPR signal allowed us to evaluate the intensity of the spectra. In case two components superimposed to produce the EPR line shape, a subtraction procedure of one component allowed us to separate the two components and calculate the intensity of each of them. The precision in the EPR parameters is ~3%.

Oils. The extra virgin olive oils used came from three Italian regions: The Marches, Umbria, and Apulia. Olive drupes of different cultivars were picked in the middle period of maturation in 1998 and 1999 and processed within 24−48 h of harvest. Olives from The Marches and Apulia were processed by the continuous centrifugation method (three-phase system), whereas Umbrian olives were processed by pressure. The oils analyzed in the present study are listed in Table 1; the oils made in 1999 were termed "new", whereas the 1-year-old oils, made in 1998, were termed "old". The old oils were aged in metal containers at temperatures ranging from 10 to 20 °C over 1 year. Having established that 24 h is needed to perfectly dissolve and equilibrate PBN in the oil matrix, all of the following experimental treatments were performed after 24 h from the PBN addition.

Experimental Treatment of the Oils. The oils were treated in the following ways:

1. Aliquots of old and new oils were stored in the dark under N_2 atmosphere for a further 48 h after the 24 h used for dissolution and equilibration of PBN. The N_2 atmosphere avoids further absorption of air (oxygen) in the oil mixture. Therefore, this experiment allows us to follow the fate over time of the spin-trapped radicals.

2. Air bubbling (oxygenation) with dry air at 25 °C was performed for increasing times varying from 5 s to 5 min, with pressure ranging from 0.2 to 0.6 bar. Longer air bubbling at lower or higher pressures did not produce any further variations in the radical concentration or EPR spectral features.

3. UV irradiation was performed at increasing times, from 5 s to 15 min, by means of a deuterium lamp, irradiating in the range of 200−400 nm. Longer irradiation times did not produce any further variations in the EPR results.

4. Dry N_2 bubbling at 0.4 bar was performed for increasing times, ranging from 5 s to 5 min, at 25 °C. Longer times did not produce any further variations in radical concentration or EPR spectral line shape.

5. Samples were heated in a dry oven at 50 and 90 °C for increasing times, ranging from 5 s to 5 min. Longer heating times did not produce any further variations in the EPR results.

Further Characterization of the Oils. Phenolic compounds were assayed according to the Folin−Ciocolteu method (*9*). Free acidity and peroxide number were determined according to the Italian *Official Methods of Analysis of Oils and Fats* (*10*). Dissolved oxygen was measured with an O_2 transmitter 4100 (Mettler-Toledo, Milan, Italy). The water content of the various oils was measured by the weight drop till to stability during dehydration in the presence of sulfuric acid.

RESULTS AND DISCUSSION

Oil Characterization. Table 1 shows the acidity, total phenolic contents, peroxide value, and dissolved oxygen of the oils used in this study. The free acidity and the peroxide number are in the range indicated by international standards. The total content of phenols in Apulian oils is higher than in the other oils; however, all of the oils have phenolic values sufficiently high to guarantee significant antioxidant protection. Dissolved oxygen, which reached its maximum value (8.6 ppm) in oil samples transferred and handled without any special care, was quite low in our oils and reached the minimum in Apulian oils. This indicates a moderate stressing of all oils by agitation. Altogether, the data of Table 1 show that, following the standard parameters, the oils used for the EPR analysis were in optimal physical and chemical conditions. As we discuss hereafter, the discrepancies in the oxygen and phenolic contents among the different oils are related to the radical contents. The water amount is quite low for all oils, ranging from 1 to 3‰. The negligible discrepancies in the water content among the various oils used in this study indicated that the amount of radicals produced is not related to the water content, even if, as discussed below, the radicals mainly localized in the water microdroplets in the oil matrix.

Identification of the EPR Signals. All of the oil samples produced an EPR signal due to the spin-trapping of apparently transient radicals by PBN. The stable radicals produced by PBN spin-trapping are nitroxides (PBN + transient radicals ≡ nitroxides), which are stable and quite persistent (storage in liquid nitrogen prolongs the lifetime). Figure 1 shows some representative examples of the EPR spectra of the nitroxide radicals obtained from the oil samples in the presence of PBN. The spectra are characterized by three hyperfine lines for the coupling between the electron spin ($S = {}^1/_2$) and the nitrogen nuclear spin ($I = 1$). The nitrogen hyperfine coupling constant measured in the spectra is $A_N = 15.3$ G. No splitting from the β-hydrogen is found. A comparison with spectral profiles reported in the literature showed that the trapped radical is a hydroxyl radical (PBN-•OH) in water solution (*11*−*13*). Usually, EPR spectra of trapped radicals are considered to be diagnostic of the radical type. However, PBN is a very good spin trap for intensity measurements, but the magnetic parameters (A_N and A_H) are not so different for similar radical types. Fortunately, in the present case the correspondence between the parameters and the radical type is unique. Therefore, we are confident with the EPR evidence about the type of radical, even if it is well-known that the •OH radicals have a very short lifetime. To justify the presence and the persistence of hydroxyl radicals in water, despite the main lipid content of the oil and its antioxidant efficacy, we

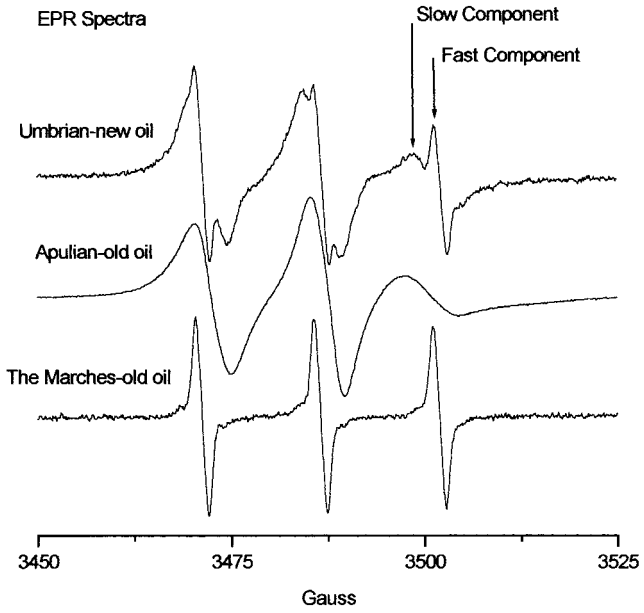

Figure 1. EPR spectra of Umbrian-new oil after UV irradiation for 180 s and of the untreated Apulian-old and The Marches-old oils. Spectra were carried out at room temperature and normalized at the same height. The spectrum of Umbrian-new oil is constituted by the superimposition of a slow EPR component, constituting only the spectrum of Apulian-old oil, and a fast EPR component, constituting only the spectrum of The Marches-old oil.

have to consider oil as a microemulsion of water in oil; that is, water microdroplets constitute protected pools for the trapped radicals. A large fraction of antioxidants is included in the lipid portion or at the lipid−water interface, but these antioxidants are not able to completely scavenge the •OH radicals and their adducts with PBN and avoid the formation of a stable concentration of PBN-•OH in the water droplets. The mechanism of the formation of the hydroxyl radicals, as reported in the literature (*14*), is shown in the following scheme: the lipid peroxidation of the oleic acid produces first peroxyl radicals (reaction 1), which, by a propagation reaction (2**)**, transform into hydroperoxide, and, finally, by reaction 3 produce the alkoxy radical, probably scavenged by the phenolic antioxidants in the oil matrix, and •OH, which is trapped by PBN in the water pools:

$$\mathrm{CH_3(CH_2)_7}\lceil\mathrm{R}\rceil\mathrm{CH{=}CHCH_2(CH_2)_6COOH} + \mathrm{O_2} \xrightarrow[\text{rearrangement}]{-\mathrm{H}^\bullet} \mathrm{CH_3(CH_2)_7}\lceil\mathrm{R}\rceil\mathrm{CH{=}CHCH(OO^\bullet)(CH_2)_6COOH} \quad (1)$$

$$\mathrm{R{-}CH{=}CHCH(OO^\bullet)(CH_2)_6COOH} + \mathrm{{-}CH_2{-}} \rightarrow \mathrm{R{-}CH{=}CHCH(OOH)(CH_2)_6COOH} + \mathrm{{-}C^\bullet H{-}} \quad (2)$$

$$\mathrm{R{-}CH{=}CHCH(OOH)(CH_2)_6COOH} \rightarrow \mathrm{R{-}CH{=}CHCH(O^\bullet)(CH_2)_6COOH} + {}^\bullet\mathrm{OH} \quad (3)$$

In Figure 1, the spectra of the Apulian-old and The Marches-old oils were obtained with untreated oils, whereas the spectrum of Umbria-new oil is recorded after UV irradiation for 180 s. Interestingly, this last spectrum is constituted by two spectral components, termed "slow" and "fast", which are superimposed over each other. Because the slow component transforms into the fast component with increasing temperature (results not shown), we believe that the two components arise from the same radical (PBN-•OH) localized in two different environments in the water microdroplets. The two EPR components are very interesting for their physical meaning. The fast component is due to nitroxide radicals rapidly moving in solution, corresponding to three narrow lines of almost the same width. A correlation time for the rotational mobility of the radicals, τ_c, was evaluated using the Kivelson theory (*15*). The procedure to evaluate τ_c is based on the relaxation mechanism and considers the dependence of the EPR line width (ΔH) on the magnetic spin quantum number for nitrogen (m_N):

$$\Delta H = A + Bm_N + Cm_N^2$$

where A, B, and C contain the components of the magnetic tensors and, in the fast motion regime of the radicals, are proportional to τ_c. The details of the method to evaluate the correlation time for the rotational motion of the nitroxides are described in the paper by Ottaviani (*16*). On the basis of this procedure we obtained $\tau_c = 5 \times 10^{-11}$ s for the fast component, which demonstrates the freedom of motion of the PBN-•OH radicals in the bulk of the water microdroplets. The slow component, the only one present for the EPR spectrum of Apulian-old oil (Figure 1), is due to nitroxide radicals slowly moving in the medium. The correlation time for the rotational mobility of the radicals was $\sim(2-3) \times 10^{-9}$ s, as determined by computation of the EPR line shape (*17*). The slowing of mobility may have two causes: (a) interaction of the radical with a surface, probably the liquid oil surface at high viscosity, offering interacting sites to the radicals; or (b) trapping of the radicals in a restricted space, such as the very small drops of water in the oil matrix. It is likely that both of these effects contribute to diminish radical mobility, as shown by the increase in the slow component with increased radical content and by the decrease in the slow component when the mixture is shaken.

Comparison of the Radical Concentration in the Oil Samples. The largest intensity value of the EPR signal was obtained in the present study for the air-treated (30 s at 0.4 bar) Apulian-old oil. This intensity was assumed, in order to choose a starting point, as a 100% radical content. It corresponds, by comparison with the standard Tempo solutions, to a concentration of radicals of $\sim 1.25 \times 10^{-5}$ M. Therefore, for the sake of clarity, the other quantities are referred to this "maximum" amount and indicated as relative percentages.

Figure 2 reports the relative percentages of the radicals obtained from the untreated old and new extra virgin olive oils. The histogram shows that Apulian and The Marches oils of the 1998 production contain a larger amount of radicals than the 1999 production. This is partly due to heating and shaking during the transfer, but aging also modifies the oils' chemical characteristics, making the double bonds of the polyunsaturated acids more fragile (*3*). On the contrary, Umbrian oil shows near equivalence in the radical content of the old and new products and the smallest amount of radicals found in our samples. Due to the small number of samples analyzed, no one interpretation of this equivalence and the small amounts of radicals between Umbrian oils can be suggested here; however, it is worth noting that both

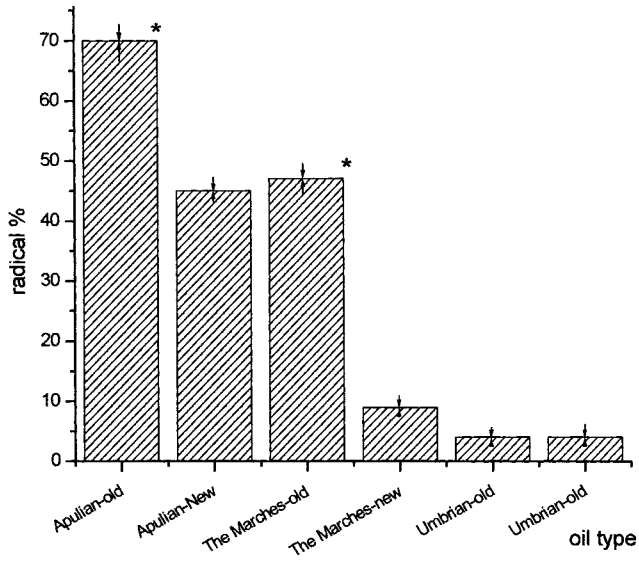

Figure 2. Histogram showing the relative percentages of radicals in old and new untreated oils. Values are means ± SD of four different experiments. Significance level was set at $p < 0.05$ by ANOVA.

Table 2. Relative Percentages of Radicals in Apulian-New and -Old Oils under Different Experimental Conditions[a]

	radicals (%)	
experimental conditions	new	old
24 h with PBN[b]	45 ± 5	70 ± 5
further 48 h with PBN	6 ± 2	5 ± 2
heating (90 °C) for 2 min	40 ± 5	73 ± 5
nitrogen bubbling (60 s)	2 ± 0.5	2 ± 0.5
air bubbling (30 s)	70 ± 4	100 ± 4[c]
UV irradiation (60 s)	40 ± 3	35 ± 4

[a] Each value is the mean ± SD of four different measurements. [b] PBN was equilibrated for 24 h in the oil before the EPR mesurement. [c] The largest intensity value of the EPR signals was found after 30 s of air bubbling in the old oil, and this intensity was considered the 100% value, corresponding to 12.5 μM radical concentration.

the old and the new oils were produced by pressure, a method which reduces both the amount of water (in this case we obtained <1% of water in the oil) and the time of contact between the oil and the water compared to the centrifugation method.

Table 2 shows the relative percentages of radicals obtained in different experimental conditions for the old and new Apulian oils. These oils are the most representative of the variations in radical concentration, because they proved to be more easily oxidized compared to the other oils. From the analysis of the data, the following conclusions can be drawn:

1. Storage of the oil after 1 day from PBN addition led to a progressive disappearance of the EPR signal. As reported under Experimental Procedures, 1 day was needed for radical equilibration in the PBN−oil mixture. Starting from 1 day after the mixture preparation, the marked decrease in radical concentration was seen upon 2 days (72 h from the initial addition of PBN). This experiment showed that the trapped radical species are still transient, probably due to migration in the fluid matrix and subsequent collisions with other paramagnetic species or antioxidants.

2. The heating of oil favored radical formation, as already found in previous studies (*1*−*3*). Heating at 90 °C for at least 2 min was needed to achieve a significant

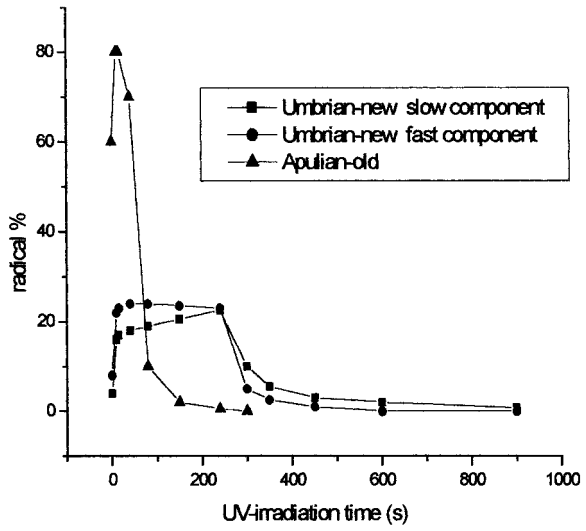

Figure 3. Variation of the radical percentage as a function of the UV irradiation time for Apulian-new and Umbrian-new oils. For the last oil, plots are reported for both the slow and fast EPR components. Values are the means of four different experiments. Each mean has a standard error of <10%. (*) Significantly different from the correspondent new oil with a significance level set at $p < 0.05$ by ANOVA.

increase in radicals in the old oil, whereas the increase for the new oil was not significant. Using a heating temperature of <90 °C did not succeed in modifying the EPR signal intensity. For instance, experiments performed with a prolonged heating (>20 min) at 40 and 50 °C did not increase the EPR signal intensity (data not shown). Heating at high temperature favors the oxidation and disruption of the unsaturated fatty acids with consequent increase in the radical formation.

3. N_2 bubbling provoked a decrease in EPR signal intensity. Two contemporaneous effects may contribute to this decrease: (a) nitrogen expels the oxygen species responsible for the radical formation from the oil; (b) the molecular motion created by the bubbling favors collisions among radicals or between radicals and antioxidants.

4. UV irradiation for 60 s did not significantly decrease the intensity of the radical signal in the Apulian-new oil, whereas in the Apulian-old oil the EPR signal was significantly reduced with respect to the untreated oil. These results are apparently contradictory with studies indicating UV irradiation as a possible source of radicals (*1*). However, EPR measurements in relation to irradiation time indicated that the signal intensity was not directly proportional to the time. Such results are reported in Figure 3, which shows the variation in the radical percentage as a function of the UV irradiation time for Apulian-old and Umbrian-new oils. For Apulian-old oil, only a few seconds (∼15 s) of UV irradiation causes an increase in the radical content, which suddenly decreases after ∼1 min. This decrease in intensity may be interpreted on the basis of structural modifications, which destroy the protection of radicals against antioxidants or favor the migration of the radicals in the oil matrix. For Umbrian-new oil, an increase in signal intensity occurs mainly in the first seconds of irradiation, then the signal intensities of the slow and fast components show a different pattern, which reaches in both cases a maximum at 4 min; the signal of both components then slowly decreases for longer irradiation time. The apparent different behavior of Apulia oil compared to Umbria oil may be related to

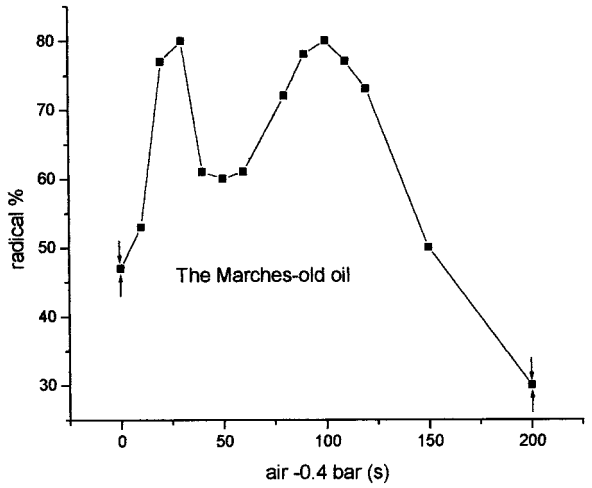

Figure 4. Variation in the radical percentage as a function of time during the aeration of The Marches-old oil at 0.4 bar. Values are the means of four different experiments. Each mean has a standard error of <10%.

their different compositions: the Apulia oils show a higher phenolic content and a lower oxygen content (Table 1) with respect to the other oils, mainly to the Umbria oils used in the present study. Of course, the amounts reported in Table 1 have to be considered as "final" amounts, after the preparation and period of storage of the oils and just before they were processed for EPR analysis. Therefore, we cannot know the starting amounts of phenols and oxygen in the oils, but we may assume that the low amount of phenols in the Umbria oils indicated that these antioxidants had already been consumed to scavenge the radicals formed during oil preparation and storage before EPR processing. As a result, the Umbria oil shows a lower content of radicals and a lower ability to form new radicals upon the treatments described here with respect to Apulia oils. In line with this hypothesis, the oxygen content in the Apulia oil is low because it has been already consumed to produce the radicals we identified in this oil. Finally, on this basis it is obvious that the old Apulia oil shows the minimum content of oxygen and the maximum content of radicals.

5. Air bubbling for 30 s at room temperature (0.4 bar) led to an increase in radical concentration. The same happened after 5−10 s of bubbling with pure oxygen (data not shown). Previous results on the oxidation processes of organic compounds, mainly of biological importance (*11*−*13*), largely promoted by oxygenation suggest that the oxygen contained in air is responsible for the formation of radicals. Figure 4 shows the variation of radical percentage as a function of the air bubbling time. The increase in signal intensity in the first 30 s is followed by a significant decrease, and then another maximum is reached after 2 min with a subsequent final decrease in intensity for longer air bubbling times. This biphasic pattern may be explained by two competing effects: the role of oxygen in promoting radical formation and the opposite role of the agitation of the oil mixture by air bubbling, which favors collisions among radicals and antioxidants.

In conclusion, this study demonstrates that EPR may be a suitable and sensible method to identify and quantify radical species in olive oil by means of the spin-trapping technique. The spin trap PBN must be perfectly dissolved and equilibrated in the oil matrix, and the EPR measurements must be performed within 12−24 h. The radical species identified are PBN-•OH in the water microdroplets inside the fat matrix. The concentration of free radicals varies according to treatment and storage conditions of the olive oil. N_2 bubbling provoked a decrease in the EPR signal intensity, whereas oxygenation and heating increased the concentration of radicals. UV irradiation shows immediately a maximum, which rapidly decreases to lower values.

Further studies are in progress to more thoroughly analyze the effects of the different technologies of production and storage methods, the addition of chemical additives often used in agriculture, and light irradiation at different wavelengths and at different doses.

ABBREVIATIONS USED

PBN, α-phenylbutylnitrone; TEMPO, 2,2,6,6-tetramethyl-1-piperidine-*N*-oxyl; EPR, electron paramagnetic resonance; •OH, hydroxyl radical; HPLC, high-performance liquid chromatography.

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