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Oxidative stability and radical scavenging activity of extra virgin olive oils: An electron paramagnetic resonance spectroscopy study

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Abstract

The oxidative stability of extra virgin olive oils (EVOO) from the Greek island of Crete was evaluated by electron paramagnetic resonance (EPR) spectroscopy and the spin trapping technique. The spin trap *N*-*t*-butyl-α-phenylnitrone (PBN) was added to the olive oil samples and the production of free radicals was monitored during heating at 70 °C. Induction time for the accelerated oxidation of virgin olive oils at 70 °C was determined. The EPR results were compared with the oxidative stability values provided by the Rancimat method at 110 °C and high linear correlations were found ($r = 0.922$). EPR spin trapping provides a sensitive and rapid method for evaluating the oxidative stability of EVOO. The same samples of Greek extra virgin olive oils were also examined for their radical scavenging activity (RSA) toward the stable galvinoxyl radical by EPR spectroscopy. The decrease of the intensity of the EPR signal upon incubation time was followed. Both oxidative stability and radical scavenging activity of EVOO samples were correlated to their content in polyphenols and tocopherols.

Keywords: Extra virgin olive oil; Antioxidants; Oxidative stability; Radical scavenging activity; Electron paramagnetic resonance spectroscopy

1. Introduction

One of the most severe quality problems of virgin olive oil is its oxidative rancidity due to oxidation of unsaturated fatty acids and subsequent formation of compounds that possess unpleasant taste and odor [1]. Virgin olive oil presents a remarkable resistance to oxidation, which has been related to both its fatty acid composition and the high levels of natural antioxidants, such as polar and non-polar phenols (polyphenols, tocophenols) and carotenoids [2,3]. The oxidative process affecting the stability of vegetable oils is often called autoxidation and involves a free radical formation mechanism [4]. Autoxidation process is characterized by a very slow initial stage (induction time) followed by a sudden increase in oxidation rate [5]. Free radicals produced during the oxidation process are very reactive and never reach a concentration high enough to be directly detected. The only adequate technique for such a determination is the electron paramagnetic resonance (EPR) spectroscopy and the use of spin traps. This class of compounds may react with transient radicals to yield stabilized species, which subsequently can be measured by EPR [6,7]. Most of the spin-trapping agents used have a nitrone-type group, which is able to form a nitroxide (spin adduct) during the trapping of the free radical. Among several nitrones used as spin traps, *N*-*t*-butyl-α-phenylnitrone (PBN) was preferred due to its lipophilic character and the stability of the resulting spin adducts [8]. PBN has been successfully used as a spin trap for the entrapment of lipid free radicals in food lipids [9], vegetable oils and their mixtures [10], mayonnaise [11] and fish oil [12].

Phenolic compounds present in virgin olive oils are strong free radical scavengers. Studies have shown that stable radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH). *N*,*N*-Dimethyl-*p*-phenylenediamine (DMPD) or the superoxide anion produced by the xanthine/xanthine oxidase system, were effectively scavenged by virgin olive oil polar and lipidic fractions [13–15]. In the present study, the stable galvinoxyl radical was preferred since no information was available concerning the scavenging ability of crude virgin olive oils toward this radical.

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The objective of this study was to evaluate whether EPR spin trapping technique can provide a sensitive and rapid method for evaluating the resistance of extra virgin olive oils (EVOO) to free radical formation under mild oxidation conditions. Quantification of free radicals was approached by external calibration using a stable lipophilic radical. A comparison of EPR spectroscopy with the Rancimat method was also made. Moreover, EPR spectroscopy of the stable galvinoxyl free radical was considered in order to estimate the radical scavenging activity (RSA) of extra virgin olive oils. Both oxidative stability and radical scavenging activity results were correlated to the content of olive oil samples in antioxidant compounds such as polyphenols and tocopherols.

2. Experimental

2.1. Chemicals

N-t-Butyl-α-phenylnitrone and 16-doxyl stearic acid (16-DSA) were obtained from Sigma. Galvinoxyl free radical was from Aldrich. The Folin Ciocalteau reagent, isooctane and *n*-hexane were from Merck. Triolein (65%) was from Sigma. Caffeic acid was from Fluka. Acetonitrile and methanol liquid chromatography (LC) grade were from Merck. Standards of α-, γ-, δ-tocopherols were purchased from Fluka.

2.2. Samples

Different extra virgin olive oil samples ($n = 15$) from the island of Crete were used in this study. All samples were provided by Cretan Unions of Agricultural Cooperatives (CUAC). The CUAC of Sitia provided seven samples (S1–S7), the CUAC of Merabelo provided three samples (MR1–MR3), three samples were by the CUAC of Mylopotamos (ML1–ML3) and two samples were from the CUAC of Peza (P1, P2). All olive oil samples were used as received.

2.3. Quantification of polyphenols in olive oils

The oil sample solutions were prepared by dissolving 50 g of oil in 50 ml *n*-hexane. The polyphenols were extracted from these solutions with three 30 ml portions of CH_3OH/H_2O (80/20, v/v). The mixture was shaken each time for 10 min, at 300 rpm. The separation of oil solution and methanol–water solution was achieved by centrifugation for 15 min, at 6000 rpm. The extracts were brought to dryness and then the residues were dissolved in 5 ml methanol. The resulting solutions were stored at −20 °C, until analysis.

The concentration of polyphenols in the methanolic extract was estimated with Folin Ciocalteau reagent [16]. The preparation of the samples consisted of dilution of 0.5 ml methanolic extract, 1 ml Folin Ciocalteau reagent and, after 3 min, 3.5 ml 10% Na_2CO_3 in a 50 ml volumetric flask, with nano-pure water. The absorbance of the samples was measured after 1 h and 15 min, at 725 nm against a blank sample with a UV–vis spectrophotometric detector, model SUV2120, Scinco. The calibration curve was constructed using standard solutions of caffeic acid. Results were expressed as microgram of caffeic acid equivalents (CAE) per gram of oil.

2.4. HP LC separation, identification and quantification of tocopherols of virgin olive oil

Tocopherols in olive oil were determined using HPLC. Oil sample solutions were prepared by dilution of 4 g oil into 50 ml *n*-hexane LiChrosolv 98%. Oil sample solutions were filtered through a 45 μm membrane filter (Gelman), before the analytical procedure [17]. The elution system was acetonitrile/methanol 75:25 v/v. Separation was achieved at 1.5 ml/min flow rate, on a Kromasil 100 C18, 5 μm, 250 mm × 4.6 mm column i.d. (Rigas Laboratories, Thessaloniki). The injection volume was 20 μl. The UV–vis detector set at 220 and 290 nm. The column remained at 23 °C, during the HPLC analysis. To determine measurements precision, each oil sample was injected three times. Calibration curves were prepared by using α-, γ-, δ-tocopherol standards.

2.5. EPR measurements

EPR measurements were carried out at constant room temperature 25 °C. using a Bruker ER 200D spectrometer operating at the X-band. The spectrometer was equipped with a Double Rectangular Cavity ER 4105 DR and samples were taken up in 734-PQ-8, thin wall suprasil, EPR sample tubes (Wilmad Glass Co., Buena, NJ, USA). Typical instrument settings were: centre field, 3470 G; scan range, 100 G; gain, 20,000; time constant, 500 ms; modulation amplitude, 1 G; phase 90°; microwave power, 3.1 mW (for the oxidative stability studies) and 6.3 mW (for the RSA studies). Data collection was performed using the computerized program DAT-200 (Data Acquisition Program, University Lubeck, Germany) and analysed with the GEP (Graphic Evaluation Program version 1.2) program for personal computer. Simulations of the experimental spectra were conducted with the simulation program WTNSIM (National Institute of Environment and Health Sciences).

2.6. Evaluation of oxidative stability

Free radical accumulation was measured by heating the EVOO samples in a water bath at 70 °C. The EVOO samples (1 g) were contained in EPR sample tubes. Prior to heating the lipophilic spin trap *N-t*-butyl-α-phenylnitrone was added (final concentration: 3 mg g^{-1} oil) to react with the free radicals as they formed during the incubation period. EVOO samples were withdrawn every 60 min periods, allowed to equilibrate in a water bath at 25 °C and then analysed in the EPR spectrometer. EPR signal intensities were approached by double integration of the spectra. Fig. 1 shows the EPR spectrum of the stable PBN spin adducts formed in olive oil during heating. Integral intensities were plotted against time to show the accumulation of free radicals. The induction time was determined as the period of time during which radicals are formed very slowly before a sudden sharp linear increase in signal intensity. Induction time was used for the evaluation of the oxidative stability of EVOO samples

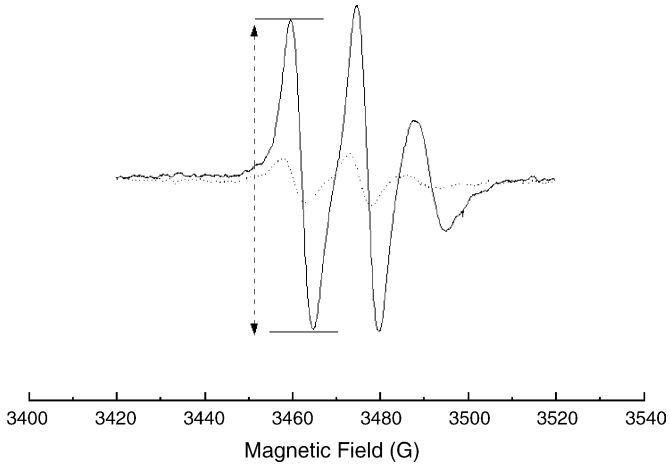

Fig. 1. EPR spectra of the PBN spin-adducts in EVOO samples after 6 h (dotted line) and 24 h (solid line) of incubation at 70 °C.

during heating. The oxidative stability of each EVOO sample was investigated by three independent experiments.

2.7. Quantification of radicals

The lipophilic spin probe 16-doxyl stearic acid dissolved in triolein was used as external standard for the quantitative analysis of PBN spin adducts formed during EVOO heating at 70 °C. Solutions of 16-DSA with concentrations ranging from 10 to 80 μM were prepared and EPR spectra were recorded and analysed as mentioned above. 16-DSA dissolved in triolein gave stable EPR spectra consisting of three peaks. All measurements were performed in triplicate. When the concentration of 16-DSA was increased, the signal intensity of the corresponding EPR spectrum, as determined by double integration, was increased A linear relationship of the integral intensity to 16-DSA concentration (μM) was observed. The regression equation is the following: integral intensity = 0.0403 + 0.043[16-DSA], $r = 0.998$, standard error = 0.102, $n = 6$.

2.8. Evaluation of radical scavenging activity

Samples of EVOO from Crete were examined for their radical scavenging activity toward the stable galvinoxyl radical (Galv-O•) by EPR spectroscopy. EVOO (20–80 mg) was added in a 0.12 mM solution of Galv-O• in isooctane and the mixture was transferred into an EPR sample tube for analysis. EPR spectra were recorded for 30–35 min at 25 °C. The EPR spectrum of galvinoxyl radical in isooctane consists of one broad peak. EPR signal intensity of galvinoxyl radical was decreased upon EVOO addition. The % remaining galvinoxyl radicals were calculated from the following equation:

$$\%\,\text{Remaining Galv} - \text{O}^{\bullet} = 100 - \frac{A_0 - A}{A_0} \times 100$$

where A_0 is the integral intensity of the EPR spectrum of a control sample (galvinoxyl solution which contains refined olive oil) and A is the integral intensity of the EPR spectrum in the presence of the same volume of EVOO. All experiments were performed in triplicate.

3. Results and discussion

3.1. Quantification of polyphenols and tocopherols

Total polyphenol and α-, γ-, δ-tocopherol content of EVOO samples used in the present study is given in Table 1. Total phenolic analysis showed that EVOO samples contained 73.8–147.5 μg (CAE)/g of oil. The relatively low content in total polyphenols can be possibly ascribed to the elapsed time between olive oil production and polyphenol determination. Also, the HPLC analysis showed that EVOO contained 142–278 μg total tocopherols/g of oil.

3.2. Oxidative stability of virgin olive oils

Virgin olive oil samples were examined for the production of free radicals after thermal treatment at 70 °C by spin trapping using PBN. The nitrone lipophilic compound, PBN, trapped these highly reactive species to form stable PBN spin adducts. The EPR spectra of PBN spin adducts exhibit restricted rotational motion. Simulation of the experimental EPR spectra indicated hyperfine splitting constants $\alpha_N = 14.73 \pm 0.02$ G and $\alpha_H = 2.50 \pm 0.1$ G. The width of the centre-line, Bpp, was found 5.09 ± 0.2 G (Fig. 1). This result could be possibly ascribed to the decreased mobility of the radicals due to the long chain of the oxidized lipids and/or the high viscosity of the reaction medium [6]. Because of line broadening, spectroscopic parameters of the trapped radicals cannot be determined with certainty. Broadened EPR lines are also obtained when several radical adducts are formed due to unresolved hyperfine splitting that causes line overlapping. As observed in Fig. 1, the intensity of the EPR signal was increased with incubation time, which is indicative of PBN spin adducts accumulation.

Furthermore, PBN was used as a spin trap for the evaluation of the oxidative stability of virgin olive oil samples. Oxidative stability was expressed as the period of time during which no EPR signal due to PBN spin adducts could be detected (induction

Table 1
Total polyphenol and α-, γ-, δ-tocopherol contents of the EVOO samples studied

Sample	Polyphenols (μg g^{-1} oil)	Tocopherols (μg g^{-1} oil)		
		α	γ	δ
P1	104.36	261	8.5	1.2
P2	125.56	209	8.2	0.4
ML1	73.824	132	9.3	0.9
ML2	100.02	255	20.7	2.7
ML3	111.18	236	21.5	n.d.
MR1	147.5	211	9.2	n.d.
MR2	140.4	231	11.0	n.d.
MR3	112.6	191	10.3	n.d.
S1	140.1	206	6.7	n.d.
S2	116.05	187	5.4	n.d.
S3	113.92	180	17.9	0.8
S4	104.94	168	7.0	0.9
S5	135.97	205	1.58	2.3
S6	132.84	195	7.1	n.d.
S7	120.08	178	7.4	n.d.

n.d., Not determined.

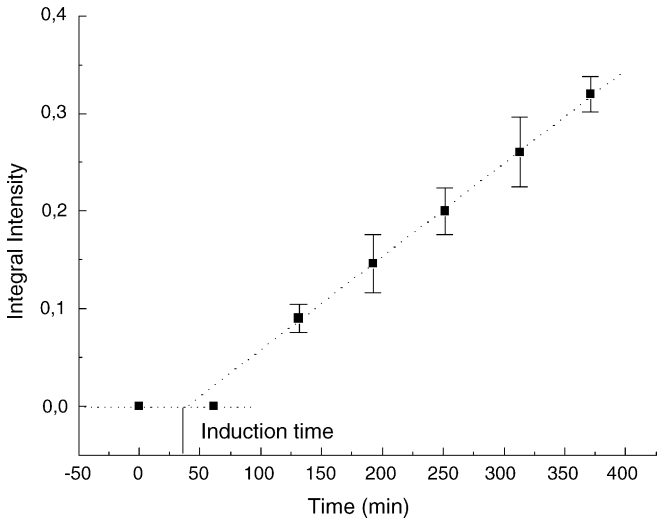

Fig. 2. EPR integral intensity against incubation time for radical formation from EVOO containing the spin trap PBN (3 μg g^{-1} oil) heated at 70 °C. The induction time is determined as the period of time before the sudden sharp increase of signal intensity. Error bars show the variations of three determinations in terms of standard deviation.

time). Fig. 2 shows the sharp linear increase of EPR integral intensity during storage of the olive oil sample at 70 °C.

Table 2 shows the induction times of all the VOO samples examined as determined by the EPR spin trapping technique. The induction times obtained were within the range 41–98.4 min. The sample, MR 1, with the highest induction time (98.4 ± 0.6 min) is the more resistant to the formation of free radicals under thermal treatment whereas the sample, ML 1, with the lowest induction time 41 ± 0 min) is the more susceptible to free radical formation. As it can be observed from Table 2, even small changes in oxidative stability were detected when the EPR spin trapping technique was considered.

When the results of Table 2 concerning the oxidative stability of the olive oil samples were compared to their content in antioxidant compounds, namely polyphenols and tocopherols (Table 1), the following conclusions can be drawn. The high oxidative stability of sample MR1 is mostly due to the high concentration of polyphenols (147.5 μg g^{-1} oil) and partly to the high concentration of tocopherols (220 μg g^{-1} oil). The olive oil sample with the lowest oxidative stability, ML1, had the lowest content in polyphenols (73.8 μg g^{-1} oil) and the lowest content in tocopherols (142 μg g^{-1} oil) among all samples tested. In general, VOO with high contents in total polyphenols and tocopherols are more resistant to free radical formation under accelerated oxidation conditions. Two olive oils with similar content in total polyphenols, P1 (10436 μg g^{-1} oil) and S4 (104.94 μg g^{-1} oil), exhibit different oxidative stabilities probably due to their different content in tocopherols (271 and 176 μg g^{-1}, respectively). Namely, sample P1 is more stable to thermal treatment (IT_{EPR} = 66.5 min) than sample S4 (IT_{EPR} = 44.2 min). This finding suggests a synergistic action between polyphenols and tocopherols in inducing oxidative stability. In addition, two samples with similar total tocopherol content MR3 (201 μg g^{-1}) and S6 (202 μg g^{-1}) have different oxidative stabilities (IT_{EPR} = 41.9 min and IT_{EPR} = 73.4 min, respectively), probably due the different content in total polyphenols (sample S6 is richer in polyphenols than sample MR3). When linear regression analysis was carried out, in order to evaluate the relationship between the EPR induction time and the concentration of total hydrophilic phenols (μg g^{-1} oil), a satisfactory correlation was obtained:

$$IT_{EPR} = -13.33 + 0.68\,[\text{Polyphenols}],$$

$$(r = 0.697,\ \text{standard error} = 14.08,\ n = 15)$$

When a similar regression analysis was carried out to evaluate the relationship between the EPR induction time and the concentration of total tocopherols (α-, γ- and δ-) (μg g^{-1} oil), the correlation was low (r = 0.276). Similar results have been reported by Baldioli et al. [2] concerning the antioxidant effect of hydrophilic phenols and tocopherols on the oxidative stability of VOO as determined by the Rancimat method. Recently, Mateos et al. [18] reported that α-tocopherols (the most abundant tocopherol in VOO) seem to have small contribution to VOO stability whereas *o*-diphenols are the most effective antioxidants.

Table 2 also shows the induction times of the same VOO samples as determined by the Rancimat method, which is widely used in industry for the determination of oxidative stability of fats and oils. As it can be observed from the values of Table 2, a very good agreement exists between the EPR and the Rancimat estimated induction times. The following equation shows the linear correlation between the induction times determined by EPR spectroscopy (IT_{EPR}) and by the Rancimat method ($IT_{Rancimat}$):

$$IT_{EPR}\,(\text{min}) = -64.5 + 6.19\,IT_{Rancimat}\,(\text{h}),$$

$$(r = 0.922,\ \text{standard error} = 7.3108,\ n = 13)$$

The high linear correlation found between the two methods, namely EPR spin trapping and Rancimat, is in agreement with the results reported by others [10] concerning the oxidative stability of several vegetable oils and their mixtures. This finding

Table 2
Evaluation of oxidative stability of virgin olive oils (VOO) based on induction times determined by Rancimat at 110 °C and by EPR spectroscopy at 70 °C (mean values ± S.D., n = 3)

Sample	EPR Induction time (min)	Rancimat Induction time (h)
P1	66.5 (±2.6)	21.23
P2	59.9 (±0.6)	20.86
ML1	41 (±0)	16.22
ML2	65.5 (±1.7)	18.43
ML3	52.6 (±4.7)	19.98
MR1	98.4 (±0.6)	24.94
MR2	85.5 (±5)	24.63
MR3	41.9 (±3)	17.55
S1	79.8 (±2.1)	23.11
S2	52 (±1)	19.88
S3	94.3 (±0.5)	19.86
S4	44.2 (±4.2)	19.12
S5	79.7 (±0)	23.59
S6	73.4 (±2.8)	21.59
S7	83.4 (±2.9)	18.98

indicates that EPR spin trapping spectroscopy can be applied as a mild, sensitive and rapid technique in order to evaluate the resistance of virgin olive oils to free radical formation.

3.3. Quantification of radicals

The concentrations of radicals formed in olive oil samples and trapped by PBN were determined, after incubation for 6 and 24 h at 70 °C (Table 3). In all samples the radical concentration after 24 h of treatment was find to be significantly higher than after 6 h. EVOO samples with high oxidative stability generally present a high radical concentration ratio (C_{24h}/C_{6h}), case of samples P1, ML2 and MR1 with ratios 16, 29 and 16, respectively, whereas the less stable ones showed a much lower ratio, case of samples ML1 and S4 with ratios 5 and 6, respectively. In the later case the low radical concentration ratio may be attributed to the high decomposition rate of the PBN radical adduct in the presence of low concentration of antioxidants.

3.4. Radical scavenging activity of virgin olive oils

The antiradical properties of the EVOO samples examined were estimated by EPR spectroscopy of the stable galvinoxyl free radical, isooctane was used throughout the experiment to dissolve both free radicals and olive oil samples. Galvinoxyl free radical has a well-defined EPR spectrum (Fig. 3). EPR signal intensity was decreased upon EVOO addition due to the scavenging effect induced by olive oil antioxidant compounds. The scavenging reaction taking place between the stable Galv-O$^{\bullet}$ radical and the antioxidants is the following [19]:

$$\text{Galv-O}^{\bullet} + \text{A-OH} \leftrightarrow \text{Galv-OH} + \text{A-O}^{\bullet}$$

where A-OH is hydrogen donating compound such as polyphenols and tocopherols and A-O$^{\bullet}$ the resulting unstable radicals. A rapid decrease in EPR signal intensity was observed within the first 10 min of the scavenging reaction.

Table 3
Concentration of radicals formed and trapped by PBN after 6 and 24 h heating at 70 °C as determined by using a standard curve based on 16-DSA dissolved in triolein (mean values ± S.D., $n = 3$)

Sample	Concentration of PBN spin-adducts (μM)	
	6 (h)	24 (h)
P1	3 (±1)	47 (±3)
P2	2 (±0)	60 (±2)
ML1	7 (±1)	36 (±8)
ML2	2 (±0)	58 (±1)
ML3	4 (±1)	76 (±4)
MR1	3 (±0)	49 (±2)
MR2	4 (±1)	54 (±2)
MR3	5 (±1)	75 (±4)
S1	3 (±0)	65 (±1)
S2	5 (±1)	75 (±5)
S3	3 (±0)	66 (±8)
S4	6 (±1)	24 (±4)
S5	4 (±0.5)	59 (±4)
S6	3 (±0.5)	52 (±3)
S7	2 (±1)	42 (±3)

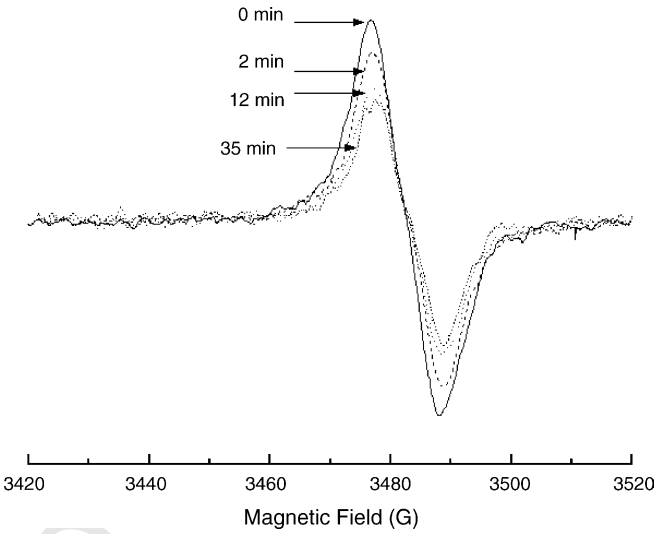

Fig. 3. EPR spectra of galvinoxyl radicals in the presence of 2% (v/v) EVOO at different incubation times: (solid line) 0 min, (dashed line) 2 min, (dotted line) 12 min, (short dashed line) 35 min.

Fig. 4 shows the effect of EVOO concentration on the decay curves of the scavenging reaction mentioned above. By increasing the amount of EVOO the reaction rate was increased. Radical scavenging activity of EVOO samples based on the % remaining galvinoxyl activity after 30 min of incubation at 25 °C is shown in Table 4. EVOO samples MR1, MR2, ML3, S1 and S2 exhibited high radical scavenging activities. After 30 min of incubation, 60.1%, 61.9%, 58%, 58.4% and 59.6% of the galvinoxyl radicals were quenched by the above-mentioned samples, respectively. All these samples were found rich in total polyphenols and tocopherols (Table 1). EVOO samples with very low radical scavenging activities were the samples S3, S7 and ML1, which after 30 min of incubation quenched only 33.9%. 39.8% and 39.9% of the galvinoxyl radicals, respectively. All these samples were among the poorest in polyphenols and tocopherols (Table 1). When linear regression analysis was carried out, in

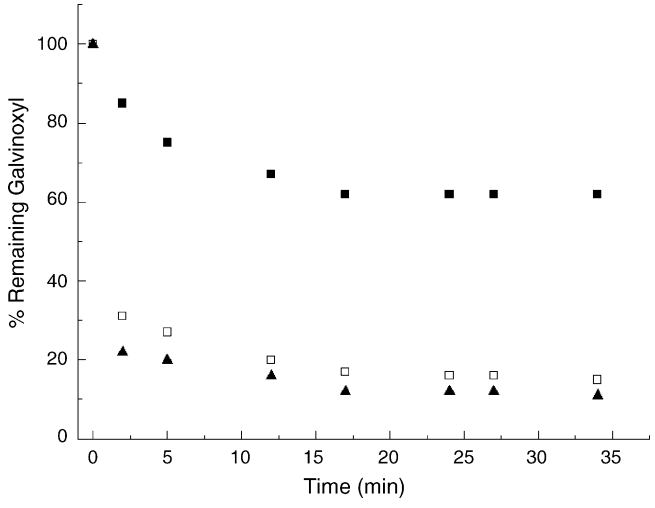

Fig. 4. Scavenging effect of EVOO on the galvinoxyl radical as a function of incubation time at different VOO concentrations: (■) 2% (v/v), (□) 5% (v/v) and (▲) 9% (v/v).

Table 4
Radical scavenging activity of EVOO from Crete based on the % remaining activity of Galv-O• radicals after 30 min incubation, (mean values ± S.D., $n = 3$)

Sample	% Remaining Galv-O• activity
P1	57.9 (±1.4)
P2	58.5 (±2.6)
ML1	60.3 (±1)
ML2	47.1 (±1)
ML3	42 (±1)
MR1	39.9 (±0.1)
MR2	38.1 (±1)
MR3	49.7 (±1.1)
S1	41.6 (±1)
S2	40.4 (±1.1)
S3	66.1 (±2.5)
S4	47.4 (±2.2)
S5	44.7 (±2.2)
S6	45.5 (±0.5)
S7	60.2 (±0.1)

Galv-O• = 0.120 mM, EVOO = 2% (v/v).

order to evaluate the relationship between the RSA for galvinoxyl radical and the EPR or Rancimat estimated induction times and the concentration of total hydrophilic phenols ($\mu g\, g^{-1}$ oil), the linear correlation obtained was not satisfactory (data not shown). This may be due to the fact that radical scavenging was determined with two different types of radicals. Either a stable exogenous free radical (galvinoxyl) or endogenous free radicals generated in olive oil during oxidative decomposition. Moreover, within a biological system where a number of polyphenols, tocopherols and other hydrogen-donating compounds exist, radical scavenging efficacy may be governed by reaction kinetics of a specific radical with various antioxidants, rather than antioxidant concentrations. In this respect, among the phenolic compounds of the polar fraction different scavenging activities toward the galvinoxyl radical may exist. As it has been shown by McPhail et al. [19] marked differences existed between 15 different flavonoid compounds in the kinetics of the reduction of the galvinoxyl radical. On the other hand, Ramadan et al. [20] showed that the level of polyunsaturated fatty acids, the initial peroxide value and the levels of polar lipids also affect radical scavenging activity of crude oils.

4. Conclusion

EPR spin trapping provides a sensitive and simple method for evaluating the resistance of extra virgin olive oils to free radical formation under mild oxidation conditions. Relative small changes in oxidative stability of extra virgin olive oils were detected when the EPR spin trapping technique was considered, hi spite of the different experimental approaches the two methods considered, EPR spectroscopy and Rancimat, predict the same oxidative stabilities of extra virgin olive oils. Oxidative stability of virgin olive oils correlates with their concentration in polyphenols and tocopherols. On the other hand, galvinoxyl free radical quenching followed by EPR spectroscopy can provide a useful method for estimating radical scavenging activity of extra virgin olive oils.

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