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The antioxidant capacity and oxidative stability of virgin olive oil enriched with phospholipids

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ABSTRACT

Virgin olive oil (VOO) enriched with phospholipids (soy lecithin) up to the levels present in seed oils (from 2.5 to 10.0 g/kg) was studied as a potential functional food. Lecithin addition slightly increased the concentration of tocopherols and considerably increased K270 values. In the fatty acid composition, an increase of linoleic and a slight decrease of oleic acid were observed, as the decrease of monounsaturated/polyunsaturated fatty acid ratio. The radical-scavenging activity was evaluated by two methods: electron spin resonance spectroscopy using galvinoxyl free radical and VIS spectroscopy measurement of the disappearance of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical. Results indicate that lecithin addition retards the scavenging activity of VOO that is ascribed to the bipolar character of lecithin and its ability to entrap hydrophilic antioxidants. The effect of lecithin addition on the oxidative stability of VOO was evaluated by the Rancimat method, and a positive linear correlation ($r = 0.9849$) with induction time was found.

1. Introduction

Phospholipids (PL) are minor constituents of seed oils, usually present in a concentration range of 10–20 g/kg (Bernardini, 1983). They are generally considered as synergists of phenolic antioxidants (Hidalgo, Nogales, & Zamora, 2005), but there is little information about those properties based on the determination of radical-scavenging activity. Ramadan, Kroh, and Mörsel (2003) have reported that PL could contribute to the radical-scavenging activity of different seed oils dissolved in toluene toward galvinoxyl free radical. The study of the lipid fraction of different vegetable oils by Espin, Soler-Rivas, and Wichers (2000) has concluded that PL have direct radical-scavenging activity toward the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical, although this is much lower than that of α-tocopherol.

In the context of functional food formulation, the addition of PL as synergists of phenolic antioxidants could be particularly interesting in the case of virgin olive oils (VOOs). They contain hydrophilic phenols, with known radical-scavenging activity (Carrasco-Pancorbo et al., 2005), in the range 40–800 mg/kg, as well as tocopherols in the range 100–400 mg/kg (Psomiadou, Tsimidou, & Boskou, 2000). On the other hand, the amounts of PL in VOOs are 300–400 times lower than those in seed oils (Koidis & Boskou, 2006).

The aim of this work was to investigate the possibility of PL enrichment of VOOs up to the concentration levels similar to those of seed oils. The influence of such high PL content on the antioxidant capacity of integral VOO was investigated using two direct determination methods. Electron spin resonance spectroscopy (ESR) applied on a sterically protected and resonance-stabilized galvinoxyl free radical was proposed by Quiles, Ramirez-Tortosa, Gomez, Huertas, and Mataix (2002) as a rapid and very sensitive method. The measurement of disappearance of DPPH free radical by VIS spectroscopy is the method widely used for the estimation of antioxidant activity of different foods, also applied to whole vegetable oils (Espin et al., 2000; Valavanidis et al., 2004). Besides, the influence of PL addition on the oxidative stability of VOO was evaluated by the accelerated oxidation test (Rancimat).

2. Materials and methods

2.1. Materials

A filtered extra VOO was purchased from the local Croatian olive oil producer. The commercial soy lecithin granules, containing

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<1% of water and 97% of phosphatides (approximately 70% of phosphatidylethanolamine, 20% of phosphatidylcholine and 7% of phosphatidylinositol) were supplied from Life Time Nutritional Specialities Inc. (Anaheim, USA). Ethyl acetate p.a., *n*-hexane p.a. and isooctane of UV-spectrophotometric quality were purchased from Panreac (Barcelona, Spain). Propan-2-ol for HPLC was obtained from Merck KGaA (Darmstadt, Germany). Methanol p.a. and toluene p.a. were supplied from Kemika (Zagreb, Croatia). DPPH and galvinoxyl free radicals, tocopherol standards and caffeic acid were from Sigma Chemical Co. (St. Louis, MO). Paraffin oil was bought from Ritosa d.o.o. (Porec, Croatia). Superclean TM LC-18 SPE Tubes (1 ml) were supplied from Supelco Inc. (Bellefonte, PA).

2.2. Sample preparation

Fifty grams of soy lecithin granules were mixed with 500 ml of oil heated to 40 °C, in order to prepare lecithin concentrate in extra VOO (primary solution). The blend was stirred for 30 min using a laboratory mixer with the propeller blade at 400–500 rpm, then, it was set aside for 10 min to allow precipitation of the undissolved part of lecithin. The same procedure of mixing and heating was applied on the pure extra VOO (without lecithin). The oil containing dissolved and dispersed lecithin was separated from the sediment and filtered over the quantitative filter paper with medium wide pores. The concentration of PL in the so obtained primary solution and in extra VOO was determined from the acetone insoluble matter, using the Lüde method described by Pardun (1964) and modified against the recommendation of List, Heakin, Evans, Black, and Mounts (1978). The real amount of the lecithin dissolved in primary solution was calculated as a difference between the PL concentration in the primary solution and extra VOO. The primary solution was used to prepare the samples in the concentration range of added lecithin, from 2.5 to 10.0 g/kg, by mixing the solution with appropriate portions of extra VOO.

2.3. Determination of standard quality and composition parameters

The free fatty acids, expressed as oleic acid, the peroxide value in mmol of oxygen per kg of oil, the coefficients of specific extinction at 232 and 270 nm (K232 and K270), and the fatty acid composition were determined according to EC Regulation 2568 (1991).

2.4. Determination of tocopherols

Tocopherols were analyzed by HPLC (Varian ProStar HPLC equipped with a Varian ProStar 363 fluorescence detector) according to the standard method (ISO, 1997). 0.1 g of oil sample was weighed into a 10 ml volumetric flask and diluted to the mark with *n*-hexane. The individual tocopherols were separated on a Restek Pinnacle II silica column, 15 cm × 4.6 mm i.d. (particle size 5 μm) which was held at 30 °C. Detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. A solution of 0.7% propan-2-ol in *n*-hexane was used as eluent at a rate of 0.60 ml/min. Tocopherol standards (α-, γ- and δ-tocopherol) were used for the setup of calibration curves that covered concentrations from 5 to 750 mg/kg of tocopherols in oil.

2.5. Determination of total phenol content

Hydrophilic phenols were extracted from the oil samples using the method for bitter index determination described by Gutiérrez Rosales, Perdiguero, Gutiérrez, and Olias (1992). A C18 SPE tube of 1 ml was activated with 6 ml of methanol and then washed with 6 ml of hexane; 1.00 ± 0.01 g of oil dissolved in 4 ml of hexane was passed through the column. After the elution, 10 ml of hexane were passed to eliminate the fat, and the retained compounds were eluted with 25 ml of methanol/water mixture (1:1, v/v). Folin–Ciocalteau reagent was added to a suitable aliquot of the eluate. The absorbance of the solution at 725 nm was measured after 2 h.

2.6. ESR measurements of radical-scavenging activity

ESR measurements were performed at room temperature (22 °C) using a Varian E-109 spectrometer equipped with a Bruker ER 041 XG microwave bridge. The spectroscopic parameters were: frequency 9.27 GHz, field sweep 10 mT, microwave power 4.9 mW and modulation amplitude 0.11 mT. Samples were prepared according to the method described by Papadimitriou et al. (2006). The freshly prepared 0.15 mM galvinoxyl free radical solution in isooctane was added to the VOO samples in order to obtain a 4% (w/v) oil solution. The oil solution was quickly mixed in the flask and immediately put into the standard ESR tube. ESR spectra were recorded during 30 min, starting from the first minute after the oil and free radical solution contact. Recording intervals were 1 min during the first 10 min of the reaction and 2 min during the rest of the measuring process. The signal intensities of galvinoxyl free radical were calculated by the double integration of ESR spectra, using the EW (EPRWare) Scientific Software Service program and expressed in arbitrary units. The signal intensity of the pure 0.15 mM galvinoxyl solution, measured immediately before starting the sample measurement, was taken as the reference signal intensity, i.e. signal intensity of the sample (A_0) for the reaction time zero ($t = 0$ min). The loss of the signal intensity (I) after the reaction time t was calculated as: $(I) = [(A_0 - A)/A_0] \times 100\%$, where A is the signal intensity of galvinoxyl radical in oil solution measured at time t. Each sample was analyzed in triplicate and the results are presented as mean values ± standard deviation.

2.7. Measurements of radical-scavenging activity using VIS spectroscopy

The reaction mixture of oil and DPPH· solution was prepared according to the procedure described by Kalantzakis, Blekas, Pegkildou, and Boskou (2006). One millilitre of oil solution (10% w/v) was added to 4 ml of a freshly prepared DPPH· solution (0.1 mM) in 20 ml test tube, which was immediately tapped and vigorously mixed during 10 s in a vortex apparatus. The ethyl acetate was used as a solvent. Absorbance of the mixture was measured during 30 min at 515 nm, using a HACH spectrophotometer DR/400, (Colorado, USA). Recording intervals were 1 min, starting from the first minute after the oil and free radical solution contact. The absorbance for $t = 0$ (A_0) was calculated as: $A_0 = A_B + A_S$, where A_B is the absorbance of blank solution (DPPH· in ethyl acetate, 0.1 mM) and A_S is absorbance of oil solution in ethyl acetate (10% w/v). The loss of DPPH· absorbance (I) was calculated as described for the loss of signal intensity in ESR measurements. Each sample was analyzed in triplicate and the results are presented as mean values ± standard deviation.

2.8. Determination of oxidative stability by Rancimat method

Oil samples having a mass of 3.0 g were heated in the Rancimat equipment at 120 °C (Metrohm Ltd., Herisau, Swiss) with a continuous air flow of 10 l/h passing through the samples. The conductivity cells were filled with 60 ml of deionised water (2 μS/cm). The time needed for the appearance of a sudden water conductivity rise, caused by the adsorption of volatiles deriving from oil oxidation, was registered as the induction time in hours.

2.9. Statistical analysis

One-way analysis of variance at 5% significance level was used to determine any significant difference in the radical-scavenging

activity and oxidative stability between VOO samples with different concentrations of PL. The mean values were compared by the Tukey honest significant difference test. The homogeneity of variance was previously tested by Levene's test. In the case of insufficient homogeneity, a logarithmic transformation of original data was carried out. All statistical analyses were performed using the software package Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. General

The VOO used in this experiment was of high quality, having low values of free fatty acids, peroxide and K-numbers (Table 1). The addition of lecithin did not cause any important changes of quality parameters, except for K270, that had a positive linear correlation with the lecithin concentration ($r = 0.9858$), suggesting the presence of secondary oxidation products and conjugated trienes in the commercial lecithin. In the sample with 10.0 g/kg of added lecithin, the value K270 exceeded the limit for extra VOO, that is 0.22. Oxidation products make oil more susceptible to oxidation and free radicals formation, which could react with antioxidants in VOO and, thus, reduce its antioxidant activity.

The used VOO had the usual value of total tocopherols. The addition of lecithin slightly increased the total content of tocopherols and decreased the α/γ tocopherol ratio. These changes are favourable for the antioxidant activity of lecithin-enriched VOOs, since γ-tocopherol is a more efficient antioxidant than is α-tocopherol.

Standard liquid/liquid extraction of phenolic compounds from the oils enriched with lecithin was not possible, due to the formation of a stable emulsion. Therefore, the total content of hydrophilic phenols was determined in the extract obtained by the solid phase extraction. Pure VOO had a high level of those compounds while, in the samples enriched with lecithin, their concentrations were 3–4 times lower (Table 1). This may not necessarily mean a degradation of phenols, since the bipolar character of lecithin could also have an effect on the extraction of those compounds during this analytical procedure. The polar moiety of lecithin can bind the hydrophilic phenols, hindering their retention on a solid phase during the fat elimination step by *n*-hexane. Thus, the total phenols content could not be reliably correlated with the results of oil antioxidant activity and oxidative stability.

According to the fatty acid composition (Table 2), the VOO sample had quite a high content of oleic acid. Taking into account soybean origin of the commercial lecithin used, a moderate reduction of the monounsaturated/polyunsaturated fatty acids ratio was expected. There was a small decrease of oleic acid and an increase of polyunsaturated acids (from 5.4% in pure VOO to 5.9% in the sample with 10 g/kg of lecithin). Tuberoso, Kowalczyk, Sarritzu, and Cabras (2007) found a positive linear correlation between antioxidant activity of vegetable oil lipophilic fractions and the polyunsaturated fatty acids ratio in the range 13–71%. In comparison with that range, a negligible contribution of such a small increase of polyunsaturated fatty acids to the antioxidant activity of VOO enriched with lecithin could be expected.

Table 2
Fatty acid composition (%) of VOOs enriched with lecithin[a]

Fatty acid	Lecithin concentration (g/kg)				
	0.0	2.5	5.0	7.5	10.0
C 14:0	0.0	0.0	0.0	0.0	0.0
C 16:0	11.4	11.3	11.2	11.7	11.3
C 16:1	1.0	1.0	1.0	1.1	1.0
C 17:0	0.1	0.1	0.1	0.1	0.1
C 17:1	0.1	0.1	0.1	0.1	0.1
C 18:0	2.6	2.6	2.6	2.7	2.6
C 18:1	78.2	78.2	78.2	77.4	77.9
C 18:2	4.8	4.9	4.9	5.1	5.2
C 18:3	0.6	0.6	0.6	0.6	0.7
C 20:0	0.5	0.5	0.5	0.5	0.5
C 20:1	0.3	0.3	0.4	0.4	0.4
C 22:0	0.1	0.1	0.1	0.1	0.1
C 22:1	0.1	0.1	0.0	0.1	0.1
C 24:0	0.1	0.1	0.2	0.2	0.1
MU/PU[b]	14.9	14.6	14.4	13.8	13.6

[a] Results are means of two replications.
[b] Monounsaturated/polyunsaturated fatty acids ratio.

3.2. Radical-scavenging activity

In ESR radical-scavenging measurements, galvinoxyl free radical was used as a scavenging object. The stability of its freshly prepared solution in a non-polar solvent (isooctane, polarity index $P' = 0.4$) was recorded during 30 min under the same conditions as for the sample measurement. Spontaneous loss of the galvinoxyl signal intensity was 1%; thus, it was necessary to prepare and use a fresh solution daily.

The direct radical-scavenging activity of commercial lecithin used in the experiment was evaluated in its 10.0 g/kg solution in paraffin oil, which is matrix free of any compound with such activity. The loss of the galvinoxyl signal intensity caused by lecithin was about 5% that is a much lower value than that of the VOO samples (Fig. 1). The inhibition of galvinoxyl free radical in the case of the VOOs was very rapid. During the first minute of scavenging reaction, 48% of the signal intensity was lost in the sample having 10.0 g/kg of lecithin and 68% in the pure VOO. It seems that during the first minute, the reaction followed linear time dependence, after which it slowed down abruptly and the signal intensity decreased exponentially. This strongly suggests that the scavenging process could be governed by two different reaction mechanisms. Therefore, the radical-scavenging activity of oil samples was considered at two key moments: the first one, for $t = 1$ min, which was the first technically possible point of measurement, and the second one, for $t = 30$ min, taken as the end-point (Fig. 2). The re-

Table 1
Standard quality parameters, tocopherols and total phenols of VOOs enriched with lecithin

Lecithin concentration (g/kg)	FFA[a,b] (%)	Peroxide no.[b] (mmol/kg)	K232[b]	K270[b]	Tocopherols[b] (mg/kg)				Total phenols[c] (mg/kg)
					α	γ	α/γ	Total	
0.0	0.3	2.0	1.70	0.13	146	8.5	17	155	525 ± 2.8
2.5	0.3	2.1	1.68	0.16	140	16.1	9	157	175 ± 0.8
5.0	0.4	2.5	1.65	0.20	146	14.3	10	162	125 ± 0.7
7.5	0.4	2.5	1.61	0.22	167	16.4	10	181	125 ± 0.8
10.0	0.4	2.4	1.73	0.25	162	16.1	10	178	176 ± 0.9

[a] Free fatty acids.
[b] Results are means of two replications.
[c] Results are means of three replications ± SD.

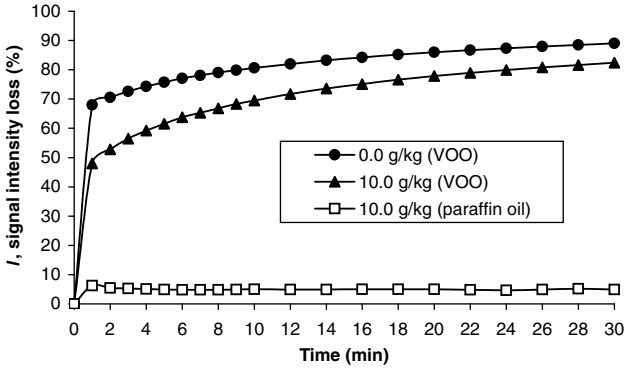

Fig. 1. Radical-scavenging activity (ESR method, galvinoxyl in isooctane) of VOO enriched with 0.0 and 10.0 g/kg of lecithin and paraffin oil enriched with 10.0 g/kg of lecithin.

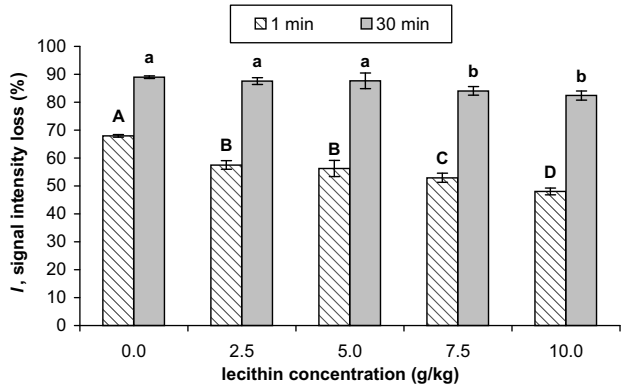

Fig. 2. Radical-scavenging activity (ESR method, galvinoxyl in isooctane) of VOOs enriched with lecithin at two reaction points (t = 1 min and t = 30 min); results are the means of three replications ± SD; the means within each reaction point (t = 1 min or t = 30 min) labelled by different letters are significantly different (Tukey's test, $p < 0.05$).

sults indicate that the addition of lecithin slowed down the scavenging activity of the virgin olive oil toward galvinoxyl free radical. The effect was more emphasized at the beginning of the reaction while, after 30 min, only the samples with higher lecithin concentrations (7.5 and 10.0 g/kg) showed significantly lower activities than those of pure VOO.

An additional investigation of the influence of lecithin on the radical-scavenging activity of virgin olive oil was done by VIS spectroscopy, using the DPPH free radical dissolved together with a VOO sample in a polar solvent (ethyl acetate, P' = 4.3). The radical-scavenging activity of integral vegetable oils toward the DPPH radical has been previously reported by Valavanidis et al. (2004) and Espin et al. (2000). Comparing different solvents used for this purpose, it has been concluded that ethyl acetate shows the best performance. In our experiment, the spontaneous loss of absorbance of a freshly prepared DPPH· solution, recorded during 30 min under the same conditions as for the sample measurement, was 0%, and this confirms the suitability of ethyl acetate.

The results of VIS spectroscopy are in agreement with those of ESR measurements. The direct radical-scavenging activity of the commercial lecithin dissolved in paraffin oil was even lower in this case: the loss of DPPH· absorbance was about 2% (Fig. 3). Within the first minute of the scavenging reaction, the results of VIS spectroscopy related to the VOO samples showed a much slower inhibition of the DPPH· radical than was observed for galvinoxyl in the ESR method. Only 14% of DPPH· absorbance was lost for the sample

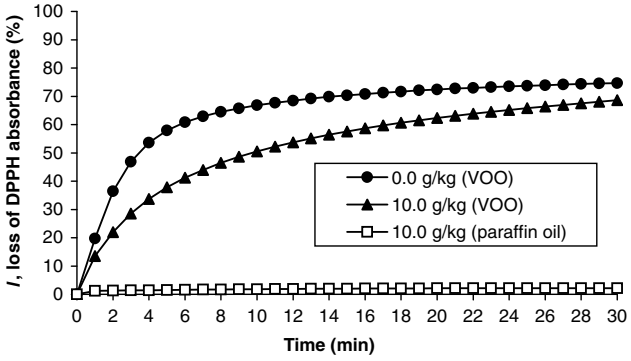

Fig. 3. Radical-scavenging activity (VIS method, DPPH· in ethyl acetate) of VOO enriched with 0.0 and 10.0 g/kg of lecithin and paraffin oil enriched with 10.0 g/kg of lecithin.

having 10.0 g/kg of lecithin and 20% for the pure virgin olive oil sample (Fig. 4). Espin et al. (2000) have obtained a similar curve of DPPH· disappearance in the presence of olive oil that was fitted by nonlinear regression to the double-exponential equation. They assume that this could be due to the presence of two groups of antioxidants, dissimilar in their scavenging velocity. These facts support our hypothesis of two reaction mechanisms during the scavenging of galvinoxyl radical. Differences in the reaction dynamics obtained by the ESR and VIS methods may be caused by different affinities of the antioxidants present in VOO toward the used free radicals and different polarities of the used solvents. According to the "polar paradox" cited by Ramadan et al. (2003), non-polar media (isooctane) used in ESR measurements could enhance the activity of hydrophilic antioxidants from VOO (phenols). This can partly explain a sharp loss of the ESR signal intensity at the beginning of the reaction. On the other hand, in the case of VIS spectroscopy, the polar solvent (ethyl acetate) favours activity of lipophilic antioxidants (tocopherols) that could be associated with a slower process of DPPH free radical-scavenging. In addition, the free electron of DPPH· seems to be sterically more protected than that of galvinoxyl.

Nevertheless, the main observation deriving from the obtained results is that, in both cases, the addition of lecithin at levels similar to those of seed oils slows down the scavenging activity of virgin olive oil. Possible reasons for the radical-scavenging inhibition behaviour of lecithin could be related to the oxidation products contained in it. However, the impact of the bipolar character of lec-

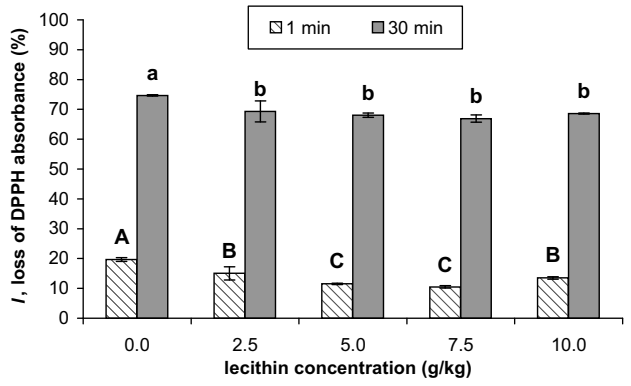

Fig. 4. Radical-scavenging activity (VIS method, DPPH· in ethyl acetate) of VOOs enriched with lecithin at two reaction points (t = 1 min and t = 30 min); results are the means of three replications ± SD; the means within each reaction point (t = 1 min or t = 30 min) labelled by different letters are significantly different (Tukey's test, $p < 0.05$).

ithin could be even more important. Koga and Terao (1995) claim that bipolar molecules, when dissolved in an organic solvent, form reverse micelles with polar groups directed toward the interior. The hydrophilic antioxidants may be entrapped in such polar cores that make some type of physical barrier between them and free radicals. Applying the trans-electron microscopy to the bulk oil with an addition of different commercial emulsifiers, Schwarz et al. (2000) observed, in some cases, the formation of lamellar structures. They reported that those emulsifiers could decrease the activity of polar phenolic antioxidants (gallic acid and propyl gallate), and that supports the hypothesis of the physical barrier, mentioned above.

3.3. Oxidative stability

To investigate the influence of lecithin addition on the oxidative stability of the VOO samples, the Rancimat method was applied. This accelerated oxidation test includes air flux of 3–20 l/h across oil heated at 100–130 °C. Results from Fig. 5 show that, in such conditions, the addition of lecithin significantly increases oil stability, despite a somewhat less favourable mono and polyunsaturated fatty acid ratio in the VOOs enriched with lecithin. A positive linear correlation ($r = 0.9849$) between the induction time and lecithin concentration is in accordance with the results of Pokorny, Davidek, Vierecklova, Ranny, and Sedlacek (1990). They concluded that lecithin present in higher concentrations (0.5–2.0%) has a pronounced autooxidation inhibition activity toward seed oils, but only a moderate one at low levels (0.02–0.10%).

Likewise, Hidalgo, Leon, and Zamora (2006) have reported that the addition of pure phosphatidylethanolamine at levels of 200–400 mg/kg significantly increased the oil stability determined by the Rancimat method. They demonstrated that reactive carbonyls, produced during the oil heating, could react with the primary amino group in phosphatidylethanolamine, giving pyrolized PL, heterocyclic residues with antioxidant properties. Furthermore, the amino group of phosphatidylethanolamine and phosphatidylcholine can facilitate hydrogen or electron donation to tocopherols (Ramadan et al., 2003). Since, in our experiment, the addition of lecithin increased the total content of tocopherols and decreased the α/γ tocopherol ratio, this could be an additional contribution to higher oxidative stability of enriched VOOs. Besides, other PL with no primary amino group could act by chelating metals via phosphate groups and creating a barrier between the oil and air interface.

These mechanisms explain a negative linear correlation between the oxidative stability determined by the Rancimat method and the radical-scavenging activities of samples enriched with lecithin. The correlation coefficients in the case of the ESR method were $r = -0.9297$ ($t = 1$ min) and $r = -0.7745$ ($t = 30$ min), while for VIS spectroscopy they were $r = -0.7772$ ($t = 1$ min) and $r = -0.7123$ ($t = 30$ min).

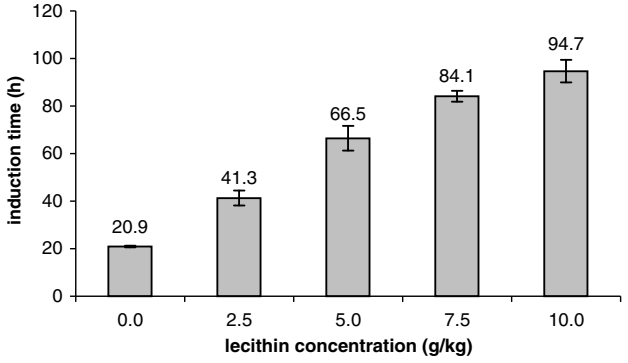

Fig. 5. Oxidative stability of VOOs enriched with lecithin determined by the Rancimat method; results are the means of three replications ± SD.

4. Conclusion

Contrary to the expectations based on the previously reported synergism between PL and liposoluble phenolic antioxidants, the addition of commercial soy lecithin (up to concentrations similar to those of seed oils) decreased the antioxidant activity of VOO. Since the main antioxidants in VOOs are hydrophilic phenols, bipolar molecules of PL could obstruct their activity, surrounding and isolating them from the matrix in which they are present. Therefore, from the nutritional point of view, VOO enriched with PL could be interesting as a functional food, but its possible lower stability during storage at ambient temperature must be taken into account. This will be the objective of our further research.

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