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Effect of phospholipids on extraction of hydrophilic phenols from virgin olive oils

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ABSTRACT

Three methods, most frequently used in hydrophilic phenols extraction from virgin olive oils (liquid–liquid, solid-phase with C18 and solid phase with diol-bound sorbents), were applied on virgin olive oils enriched with 2.0–10.0 mg/kg of phospholipids (granular de-oiled soy lecithin). Phospholipids addition significantly decreased the total phenols concentration determined colorimetrically. Liquid–liquid extraction showed the best repeatability and recovery, and the lowest decrease of total phenols extracted in the presence of phospholipids. Extraction rate declined with the increase of phospholipids concentration, but showed a kind of saturation behaviour. Addition of phospholipids (5 g/kg) to various commercial extra virgin olive oils resulted in a different degree of total phenols decrease (from 1% to 45%), probably because of different affinity of phospholipids toward different classes of phenolic compounds. During prolonged contact time between phenols and phospholipids (60-days storage), oxidation monitored by K270 and a decrease of total phenols concentration proceeded more rapidly in the presence of phospholipids.

1. Introduction

Hydrophilic phenolic compounds from virgin olive oils (VOOs) are related to several beneficial health effects, such as prevention of cardiovascular diseases and some types of cancers, as well as favourable modifications of immune and inflammatory responses. Furthermore, they greatly contribute to the oxidative stability of virgin olive oil (VOO), due to their well studied antioxidant properties (Bendini et al., 2007). That is why numerous studies have focused on enhancing or preserving these compounds during olive growth and processing, as well as oil storage. However, hydrophilic phenols are responsible for the bitter taste of VOOs and, when present in higher concentrations, they could have negative impact on product acceptability (Mateos, Cert, Perez-Camino, & Garcia, 2004).

In a previous work we have demonstrated that addition of soy-derived phospholipids (de-oiled granular lecithin), at levels present in seed oils, decrease intensity of VOO bitterness, without causing significant changes of overall sensory quality grading (Koprivnjak, Škevin, Petričević, Brkić Bubola, & Mokrovčak, 2009). Addition of phospholipids (PL) could be furthermore interesting because some of them are considered antioxidant synergists, able to regenerate oxidised phenolic molecule by donation of hydrogen atom from amino groups. Synergism between soy lecithin and phenolic antioxidants has been demonstrated not only for tocopherols (Judde, Villeneuve, Rossignol-Castera, & Le Guillou, 2003; Koga & Terao, 1995), but even for the hydrophilic phenol quercetin in a triolein model system (Ramadan, 2008). However, we have reported recently that PL added to VOO inhibit its overall radical-scavenging activity and decrease the total content of hydrophilic phenols (Koprivnjak et al., 2008). This effect could be related to phenols degradation caused by oxidation products contained in de-oiled granular lecithin, as it is known that soy-derived PL are composed mostly of polyunsaturated fatty acids, which are easily oxidised (Weber, 1981). Nevertheless, the bipolar character of PL should be considered as well, especially regarding isolation of the phenolic fraction from an oil sample. Hydrophilic phenols in VOOs represent a complex group of different classes: phenolic acids, phenyl ethyl alcohols, hydroxyisochromans, flavonoids, lignans and secoiridoids (Bendini et al., 2007). Differences in molecular size, polarity and stability of those classes make phenols isolation a sensitive step in an analytical procedure, which could be easily influenced by the presence of bipolar molecules such as PL.

Isolation techniques used for this purpose are mostly based on liquid–liquid extraction (LLE) and solid-phase extraction (SPE) (Carrasco-Pancorbo et al., 2005). Bendini et al. (2003) compared the effectiveness of isolation methods applying three different SPE sorbents (alkylsilica C8, alkylsilica C18 and diol-bond) on VOO and lipid matrix spiked with standard phenolic compounds. They concluded that, among studied SPE methods, the best results were obtained using diol-bond sorbent, according to the method proposed by Mateos et al. (2001). The same diol-SPE and

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C18-SPE methods have been evaluated by Hrncirik and Fritsche (2004), analysing refined olive oil spiked with phenols extracted from VOO. They reported that the diol-SPE method had better repeatability and a significantly higher recovery of total phenolic compounds than C18-SPE.

As regards liquid–liquid extraction, the most common procedure is based on vigorous agitation of an oil solution in non-polar solvent with several portions of methanol or methanol/water mixture containing up to 40% of water (Carrasco-Pancorbo et al., 2005). In both above mentioned comparative studies, LLE method using hexane as non-polar solvent and methanol/water (60:40, v/v) has been evaluated as well. Although the two LLE procedures differed slightly in solvent volumes, oil concentrations and number of re-extractions, recoveries of total phenolic compounds were almost equal (around 93%) and in both cases significantly higher than for diol-SPE method (that was around 70%). Moreover, as regards individual compounds, Hrncirik and Fritsche (2004) have reported that secoiridoid aglycones were much better recovered by LLE than diol-SPE method.

Studying the influence of PL addition on antioxidant activity of VOO (Koprivnjak et al., 2008) we have hypothesised that emulsifying properties and bipolar character of these compounds could interfere with the extraction of hydrophilic phenols. Therefore, in this work we examined the effect of PL on total phenols concentration determined colorimetrically comparing three most frequently used isolation methods (LLE, diol-SPE and C18-SPE). After choosing the most appropriate method, the influence of increasing PL concentrations and behaviour of PL in different commercial VOO samples was checked. Considering high susceptibility of soy-derived lecithins to oxidation, the influence of prolonged contact time between phenols and PL was evaluated as well.

2. Materials and methods

2.1. Materials

A total of nine commercial extra virgin olive oil samples from various Croatian producers, deriving from the season 2007–2008, were used in this study. One of the samples was used for the preparation of the phenolic-free lipid matrix and isolation of hydrophilic phenols for recovery trial. De-oiled soy lecithin in the form of granules around 2 mm in diameter, containing <1% water and 97% phospholipids, was supplied from Life Time Nutritional Specialities Inc. (Anaheim, CA). Caffeic acid (purity 99%), ethyl acetate and methanol were purchased from Panreac (Barcelona, Spain). Folin-Ciocalteu reagent, *n*-hexane and sodium carbonate decahydrate were supplied from Merck (Darmstadt, Germany). Chromabond diol-modificated silica columns (500 mg/3 ml) and TLC plates silica gel 60 Alugram SIL G/UV254 (10×10 cm) were supplied by Macherey–Nagel (Düren, Germany), while Supelclean LC-18 columns (500 mg/1 ml) were from Supelco (Bellefonte, USA). Aluminium oxide type 507C neutral (100–125 mesh), acetic acid, acetone, sulphuric acid and perchloric acid were purchased from Sigma–Aldrich (Buchs, Switzerland), while ammonia, chloroform and iodine were from Kemika (Zagreb, Croatia).

2.2. Determination of PL composition

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) in the lecithin sample were separated by two-dimensional TLC and determined according to the method described by Parker and Peterson (1965). The first direction solvent system was chloroform/methanol/ammonia (65:35:5, v/v/v) and the second chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v/v/v/v). After visualisation of the phospholipids spots with iodine, individual classes were mineralised with sulphuric acid/perchloric acid (9:1, v/v) and analysed spectrophotometrically for phosphorus, using a Hach spectrophotometer DR/400 (Hach Company, Loveland, CO).

2.3. Preparation of oil samples enriched with PL

The appropriate amount of lecithin granules was added to VOO or lipid matrix in order to prepare the samples with a concentration range from 2.0 to 10.0 g/kg. Individual blends were tempered in a water bath at 40 °C and hand-mixed with a glass stick until the granules were dissolved. Samples with PL addition were prepared and analysed within 24 h, so as to minimise the possibility of phenols degradation by oxidation. The same procedure of mixing and heating of sample was applied on the pure VOO (without lecithin) or lipid matrix, in order to obtain fully comparable samples. For the trial of prolonged contact time between phenols and PL, the samples were stored after lecithin addition in fully filled and taped glass tubes, at ambient temperature in the dark, for 60 days.

2.4. Preparation of lipid matrix

The phenolic-free lipid matrix was prepared according to the "solvent-free" procedure described by Yoshida, Kondo, and Kajimoto (1992). VOO sample (100 g) was passed through a glass chromatographic column (450×30 mm i.d.) filled with 100 g of aluminium oxide, previously activated 3 h at 200 °C. A glass column and collection vessel were connected to the vacuum and wrapped with an aluminium foil to the end of purification so as to reduce the risk of oxidation.

2.5. Preparation of lipid matrix spiked with phenolic extract

Phenolic compounds were isolated from the VOO sample using diol-bond phase cartridges, according to the SPE method reported by Mateos et al. (2001). After the determination of total phenols content, adequate aliquots of methanolic extract were transferred into a glass tube and evaporated under nitrogen steam to dryness. Dry residue was immediately dissolved in 2 g of the phenolic-free lipid matrix by vigorously shaking in a vortex apparatus, in order to prepare samples with 200 mg/kg and 400 mg/kg of total phenols.

2.6. Liquid–liquid extraction of hydrophilic phenols

Two grams of the VOO sample were weighed in a 20-ml screw-cap test tube and extracted two times with 5 ml of methanol/water (80:20, v/v). Each time the mixture was stirred for 5 min in a vortex apparatus and centrifuged at 3800 rpm for 4 min. Upper methanol layers were transferred to a 25-ml volumetric flask, which was then made up with methanol/water (80:20, v/v).

2.7. Solid-phase extraction of hydrophilic phenols

SPE with diol-bound columns was carried out according to the method described by Mateos et al. (2001). SPE with C18 columns was performed applying the method for bitter index determination of VOO, proposed by Gutiérrez Rosales, Perdiguero, Gutiérrez, and Olias (1992). Liquid–liquid or solid-phase extractions of each sample were run in triplicate, except for the repeatability trial where five repetitions were done.

2.8. Colorimetric determination of total phenols content

The determination of total phenols content in the extracts obtained from LLE and SPE was based on the procedure introduced

by Gutfinger (1981). A suitable aliquot of extract was diluted with 30 ml of distilled water, then Folin-Ciocalteau reagent (2.5 ml) and 7.5 ml sodium carbonate solution (25% w/v) were added. The reaction mixture was filled with deionised water to 50 ml and left in the darkness for 2 h. The absorbance at 765 nm (HACH spectrophotometer DR/400) was measured against a blank solution. The total phenols concentration was determined from a caffeic acid calibration curve and expressed in mg/kg, as caffeic acid equivalents.

2.9. Monitoring of oxidation products by UV spectrophotometry

Coefficients of specific extinction at 232 and 270 nm (K232 and K270) were determined according to EC Regulation 2568 (1991).

2.10. Statistical analysis

Differences among samples were tested by one-way analysis of variance at 5% or 1% significance level. The homogeneity of variance was tested by Levene's test and the mean values were compared by the Tukey's honest significant difference test. Statistical analyses were performed using the software package Statistica 7.1 (StatSoft Inc., Tulsa, OK).

3. Results and discussion

The isolation methods chosen were among the most frequently used and on the basis of their advantageous performances in comparative studies (Bendini et al., 2003; Hrncirik & Fritsche, 2004; Montedoro, Servili, Baldioli, & Miniati, 1992). However, some modifications were applied to the LLE method. In order to reduce emulsion formation, oil samples were not dissolved in hexane, but directly extracted with methanol/water (80:20, v/v). According to Montedoro et al. (1992), addition of non-polar solvents does not enhance the extraction yield of phenols. Besides, the ratio 80:20 was chosen because we achieved a better separation of layers after centrifugation, compared to the most frequently applied 60:40 (v/v) ratio. C18-SPE method for the bitter index determination was included in a comparative trial, since it has been already used in our previous work as an alternative to LLE in the determination of total phenols in lecithin-enriched VOOs (Koprivnjak et al., 2008). Suitable aliquots of methanolic extracts obtained by each of the three methods were directly used in the colorimetric reaction, equalizing the total reaction volume and methanol concentration, in order to obtain comparable data.

Repeatability of the methods was tested on the VOO sample having a medium level of the total phenols concentration. Results ranged from 305 mg/kg to 396 mg/kg, depending on the extraction method (Fig. 1). Values obtained by LLE and diol-SPE were not significantly different ($p \leqslant 0.01$). Both methods had good repeatability in terms of relative standard deviation (RSD), although inferior for diol-SPE (4.7% *vs.* 2.5%). Repeatability achieved by C18-SPE was also good (RSD 2.5%) but the total phenols concentration was significantly lower ($p \leqslant 0.01$) compared to the other two methods. These results are in accordance with those reported by Bendini et al. (2003) and Hrncirik and Fritsche (2004), despite differences in applied LLE and C18-SPE methods.

Analysing the same VOO sample, but freshly enriched with 5 g/kg of granular de-oiled lecithin, a significant decrease ($p \leqslant 0.01$) of the total phenols concentration was observed for all the three methods, ranging from 21% (LLE) to 32% (diol-SPE) and 37% (C18-SPE) lower values, with respect to pure VOO. There are two possible explanations of such decreased values. The first should be attributed to oxidative degradation of phenols, and the second is the interference of their extraction caused by the bipolar nature of PL. The main components in used granular de-oiled lecithin were PC (37%) and PE (31%), followed by PI (18%) and PA

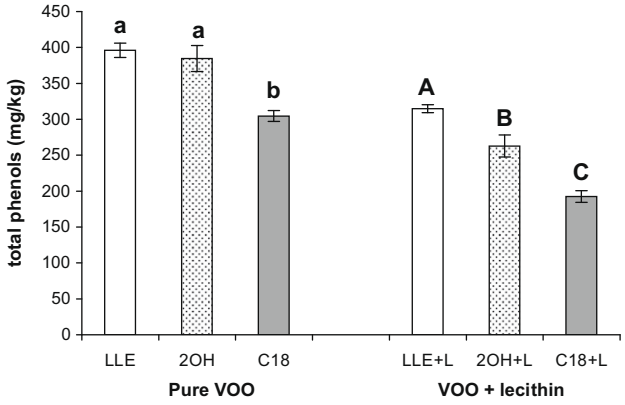

Fig. 1. Total phenols concentrations determined colorimetrically after LLE, diol-SPE (2OH) and C18-SPE from pure VOO and VOO sample freshly enriched with 5 g/kg of de-oiled granular lecithin; results are the means of five replications ± SD; the means within each sample labelled by different letters (lower cases for pure VOO, capitals for VOO + lecithin) are significantly different (Tukey's test, $p < 0.01$).

(11%). A high amount of phosphatidylamino alcohols PC and PE should be favourable in regeneration of oxidised phenols, given that hydroxy amino functions can act as hydrogen providers (Judde et al., 2003). Furthermore, considering the quite short time of contact between phenols and PL, only a minor contribution of oxidative degradation could be supposed. Hence, the bipolar nature of PL may have a more decisive influence, which was least expressed during LLE. In addition, the LLE method had the best repeatability (RSD 2.5%), comparable with that achieved analysing pure VOO. For both SPE methods, the critical point is most likely the fat elimination step, carried out by passing *n*-hexane through the column. The polar moiety of lecithin probably binds the hydrophilic phenols, hindering their retention on a solid phase. Taking into account the highest total phenols value, the lowest decrease in the presence of PL and the best repeatability, the LLE method was considered as the most appropriate for further evaluation.

Firstly, recovery was tested on the phenolic-free olive oil matrix (OOM) obtained by purification through alumina and spiked with hydrophilic phenols extracted from VOO, at concentration levels commonly found in VOOs (200 mg/kg and 400 mg/kg). For OOM without lecithin addition, recoveries were comparable to those reported in literature: 97% (200 mg/kg of phenols) and 93% (400 mg/kg of phenols). In OOM enriched with 5 g/kg of granular de-oiled lecithin, a significant decrease of recovery could be seen (Fig. 2), although at a different probability level (for 200 mg/kg of phenols at $p \leqslant 0.05$, while for 400 mg/kg at $p \leqslant 0.01$).

Effect of PL addition was then tested on nine commercial extra VOOs having relatively similar total phenols concentrations, which ranged from 307 mg/kg to 396 mg/kg (Table 1). It is evident that, at the same level of lecithin concentration (5 g/kg), the decrease of total phenols expressed in percentage was not uniform, but varied from 1% to 45%. In this context, it is particularly interesting to compare OOM spiked with 400 mg/kg of phenols with VOO sample I, which possessed almost the same value (396 mg/kg). Addition of lecithin to this VOO sample caused a 21% decrease of the total phenol concentration, while for OOM, it was less emphasised and amounted to 12% regarding the matrix without lecithin addition. VOO is obviously a much more complex system than purified OOM. It contains some polar and bipolar molecules, such as free fatty acids, sterols or fatty acids oxidation products. It is possible that even those compounds have an interfering effect on hydrophilic phenols extraction, similar to that hypothesised for PL. However, it is noteworthy to mention the chemical and structural diversity of the phenolic compounds of VOOs and variability of

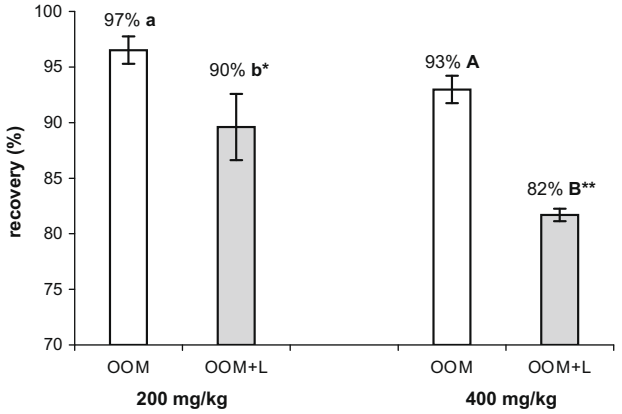

Fig. 2. Recoveries of total phenols determined colorimetrically after LLE from phenolic-free olive oil matrix (OOM) and OOM freshly enriched with 5 g/kg of de-oiled granular lecithin (OOM + L) spiked with 200 mg/kg and 400 mg/kg of VOO hydrophilic phenols; results are the means of three replications ± SD; the means within each hydrophilic phenols concentration labelled by different letters (lower cases for 200 mg/kg, capitals for 400 mg/kg) are significantly different (Tukey's test, $^{*}p < 0.05$; $^{**}p < 0.01$).

Table 1
Effect of lecithin addition (5 g/kg) on total phenols concentration determined colorimetrically after LLE of various commercial extra VOOs.

VOO sample[a]	Total phenols concentration[b] (mg/kg)		Decrease (%)
	Pure VOO	VOO with lecithin addition	
A	307 ± 0.5 a	235 ± 5.6 b	23
B	336 ± 2.4 a	185 ± 4.1 b	45
C	337 ± 8.2 a	306 ± 12.7 b	9
D	350 ± 10.0 a	250 ± 9.4 b	29
E	351 ± 3.2 a	347 ± 3.3 a	1
F	358 ± 16.8 a	226 ± 9.4 b	37
G	360 ± 2.5 a	315 ± 4.3 b	13
H	378 ± 5.1 a	291 ± 6.3 b	23
I	396 ± 9.9 a	315 ± 5.6 b	21

[a] Samples are listed in order of increasing total phenols concentration in pure VOO.
[b] Results are means of three replications ± SD; the means within each row labelled by different letters are significantly different (Tukey's test, $p < 0.01$).

phenols composition in various samples. As colour reaction of Folin-Ciocalteu reagent occurs with all phenolic hydroxyl groups, colorimetric assay cannot give information about possible different affinity of PL toward single phenolic classes or compounds. Nevertheless, it is most likely that the differences in the degree of decrease of the total phenols, shown in Table 1, are the consequence of the particular composition of polar fraction in each single VOO.

The next question to ascertain was how the increasing concentrations of phospholipids influence the extraction rate of total phenols from virgin olive oils. This was examined on the VOO sample containing 361 mg/kg of total phenols, freshly enriched with granular de-oiled lecithin in a range from 0 to 10 g/kg. As it is evident from Fig. 3, a significant decrease of the total phenols concentration ($p \leqslant 0.05$) was obtained upon addition of up to 4 g/kg of lecithin, while no significant changes were found at higher concentrations. This kind of saturation behaviour suggests selective action of PL, i.e., their possible different affinity toward different classes of phenolic compounds.

Considering the low stability of lecithin and possible degradation of VOO phenols caused by oxidation products deriving from de-oiled granular lecithin, it was also important to check the influence of prolonged contact time between phenols and PL. Results in Fig. 4 show a decreasing trend of the total phenols concentration

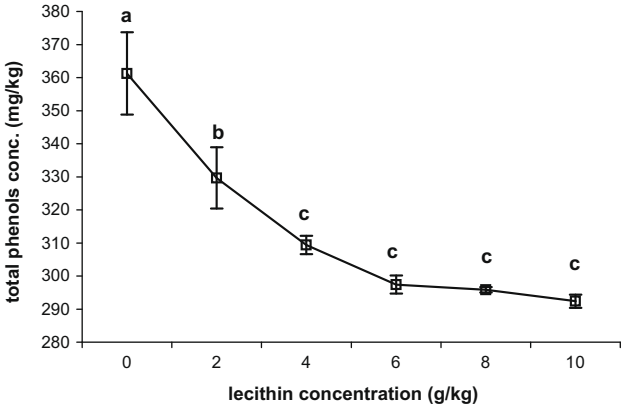

Fig. 3. Extraction rate of total phenols from virgin olive oil in relation to increasing concentration of granular de-oiled lecithin in range from 0 to 10 g/kg; results are the means of three replications ± SD; the means labelled by different letters are significantly different (Tukey's test, $p < 0.05$).

during 60 days of oil storage. This was somewhat more emphasised for VOO with lecithin addition (gradient of −0.955) than for pure VOO (gradient of −0.744), although the granular lecithin used contained a high ratio of PL with antioxidant properties (PC and PE).

To monitor the oxidation status of stored oil samples, UV spectrophotometry was used as a tool that is simple and suitable for that purpose. Oxidation of polyunsaturated fatty acids is typically accompanied by an increase in the UV absorption at 232 nm (due to formation of conjugated hydroperoxide dienes) and 270 nm (formation of trienes and secondary oxidation products). At time 0, *K*-values of both pure and lecithin-enriched VOO sample were lower than maximum limits for extra VOO (K232 ⩽ 2.50 and K270 ⩽ 0.22), established by Codex Alimentarius (1981) (Figs. 5 and 6). During 60 days of storage, K270 of pure VOO increased very slowly, while the increase of K232 was much faster, although without exceeding the extra VOO maximum limit. This indicates accumulation of primary oxidation products and negligible formation of secondary products, characteristic of the initial phase of oxidative degradation. Addition of granular de-oiled lecithin caused an immediate increase of K270, suggesting it contained secondary oxidation products and conjugated trienes. As a consequence, the oxidation proceeded more rapidly than in pure VOO, so that after 60 days of storage, the K270 value of lecithin-enriched sample attained the extra VOO maximum limit. In these circumstances oxidative degradation of phenolic antioxidants is more pronounced

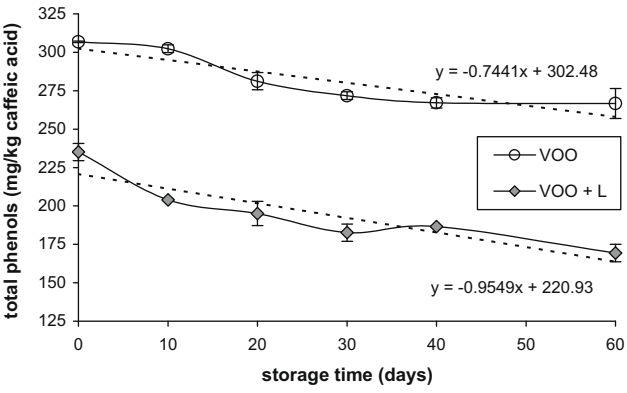

Fig. 4. Changes of total phenols during 60 days storage (darkness, ambient temperature) of pure VOO (VOO) and VOO sample enriched with 5 g/kg of de-oiled granular lecithin (VOO + L); results are the means of three replications ± SD.

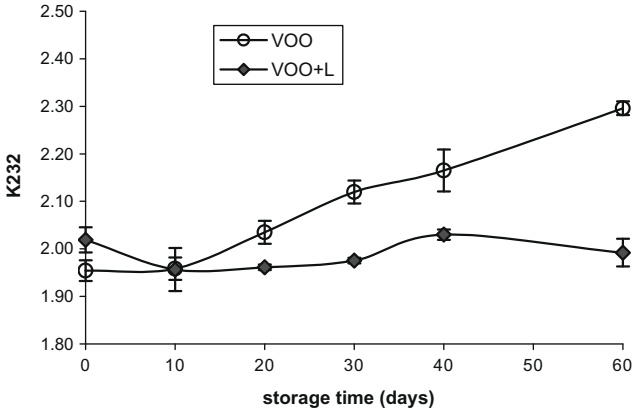

Fig. 5. Changes of K232 during 60 days storage (darkness, ambient temperature) of pure VOO (VOO) and VOO sample enriched with 5 g/kg of de-oiled granular lecithin (VOO + L); results are the means of three replications ± SD.

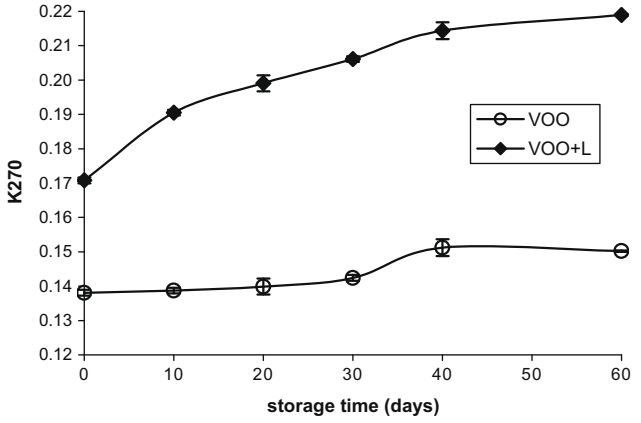

Fig. 6. Changes of K270 values during 60 days storage (darkness, ambient temperature) of pure VOO (VOO) and VOO sample enriched with 5 g/kg of de-oiled granular lecithin (VOO + L); results are the means of three replications ± SD.

and this could explain a higher decreasing rate of total phenols concentration compared to the sample without lecithin addition.

4. Conclusions

This study confirmed our previous observation that PL interfere with the extraction of hydrophilic phenols, although great differences exist in the efficiency of the tested methods. LLE is the best option in terms of good repeatability and recovery, as well as the lowest decrease of total phenols extracted in the presence of PL. The extraction rate of total phenols declines with an increase of PL concentration, but it shows a kind of saturation behaviour that suggests selective action of PL toward different classes of phenolic compounds. Addition of PL to various commercial extra VOOs resulted in a different degree of total phenols decrease. Since this could be correlated with the particular composition of phenolic compounds in each single VOO, further study could be directed to this issue. During oil storage, the decrease of total phenols concentration in the presence of PL is higher than in pure VOO, due to the presence of oxidation products in the granular de-oiled lecithin used as a source of PL. As a whole, it can be concluded that an immediate decrease of the total phenols concentration in lecithin-enriched VOO is mainly the consequence of PL interference in their extraction, while during oil storage, oxidative degradation of phenols should be taken into account.

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