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Antioxidative Activity of Amino Phospholipids and Phospholipid/Amino Acid Mixtures in Edible Oils As Determined by the Rancimat Method

FRANCISCO J. HIDALGO, M. MERCEDES LEÓN, AND ROSARIO ZAMORA*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Avenida Padre García Tejero 4, 41012 Seville, Spain

Phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysine (Lys), and mixtures of them were tested for antioxidative activity in refined olive oil by the Rancimat method to investigate the role of the chemical reactions produced in the Rancimat vessel on the induction periods (IPs) obtained. PE and Lys, but not PC, increased the IPs of the oil when tested alone. In addition, PE/Lys and PC/Lys mixtures, but not PC/PE mixtures, exhibited a synergistic effect. All these results can be understood considering the in situ formation of oxidized lipid/amino compound reaction products with antioxidative activities. Thus, the formation of pyrroles could be detected after derivatization with *p*-(dimethylamino)-benzaldehyde, and some of these compounds could be unambiguously identified by GC−MS after their conversion into volatile derivatives. In addition, the formed products contributed to the color developed, and a correlation was observed between the Rancimat IPs obtained and the yellowness index of the oxidized oils recovered from the Rancimat. Furthermore, the differences observed in the antioxidative activities of PE, PC, Lys, and their mixtures could be explained according to the lipophility and hydrophility of the oxidized lipid/amino compound reaction products formed. All these results suggest that chemical reactions are being produced in the Rancimat vessel and the Rancimat IPs obtained are a consequence of the antioxidative activities of the products formed in these reactions. Furthermore, Rancimat may be a valuable tool for testing antioxidative activities of antioxidants produced during food processing if favorable conditions for antioxidant formation are employed.

KEYWORDS: Amino/carbonyl reactions; food processing antioxidants; lipid oxidation; non-enzymatic browning; phospholipids; pyrroles; pyrrolized phospholipids; Rancimat

INTRODUCTION

Antioxidative properties of phospholipids have been demonstrated through their addition to processed vegetable oils and animal fats (*1*, *2*). These properties have been proposed to be a consequence of (i) synergism between phospholipids and tocopherol (*3*, *4*), (ii) chelation of pro-oxidant metals by phosphate groups (*5*, *6*), (iii) formation of Maillard-type products between amino phospholipids and oxidation products (*7*), and (iv) action as an oxygen barrier between oil and air interfaces (*8*). Among them, recent studies have shown that antioxidative activity of amino phospholipids increased when they were slightly oxidized, therefore confirming the contribution of carbonyl/amine reactions to the antioxidative properties of amino phospholipids (*9*). In fact, formation of carbonyl/amine reaction products, with antioxidative properties (*10*, *11*), was produced as a consequence of amino phospholipid oxidation (*9*). These studies also suggested that, if oxidation of phospholipids takes place in the presence of amino compounds, other phospholipids, such as phosphatidylcholine, might also exhibit antioxidative activities following oxidation. As a continuation of those studies, the present investigation describes the efficacy of phospholipids and phospholipid/amino acid mixtures for increasing the stability of a refined olive oil as determined by the Rancimat method.

Accelerated oxidation tests, such as Rancimat, are used frequently for predicting the oxidative stability (shelf life) of fats and oils and the efficacy of antioxidants for increasing their stabilities (*12*−*15*). However, the use of this methodology for the study of the antioxidative activity of oxidized lipid-amine reaction products is very scarce. Nevertheless, it may be very useful because this methodology might allow the formation of oxidized lipid-amine reaction products in situ. Thus, in the Rancimat method, the oil is exposed to a stream of atmospheric oxygen at elevated temperature. The oil is then oxidized, and the volatile decomposition products formed are trapped in a measuring vessel filled with distilled water and continuously detected with a conductivity cell. When amino compounds are present, such as amino phospholipids or amino acids, the oxidized lipids produced during oil oxidation can react with the amino compounds and produce in situ oxidized lipid-amine reaction products with antioxidative activities, which should protect the oil.

* Corresponding author. Phone: +(34) 954 611 550. Fax: +(34) 954 616 790. E-mail: rzamora@ig.csic.es.

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EXPERIMENTAL PROCEDURES

Materials. Phosphatidylethanolamine (PE) was isolated from soybean lecithin by column chromatography on silicic acid/silica gel (1:1) using chloroform/methanol (9:1). Phosphatidylcholine (PC) was isolated from soybean lecithin according to Singleton et al. (*16*). The identity and purity of the obtained phospholipids were confirmed by HPLC and ^{1}H and ^{13}C NMR spectroscopy. Each phospholipid exhibited a single peak in HPLC (*17*) and the characteristic ^{1}H and ^{13}C signals of their polar heads (*18*). The fatty acid composition of PE was as follows: palmitic acid (19.8%), stearic acid (3.2%), oleic acid (7.6%), linoleic acid (61.6%), and linolenic acid (7.8%). The fatty acid composition of PC was as follows: palmitic acid (3.6%), stearic acid (2.3%), oleic acid (8.3%), linoleic acid (75.9%), and linolenic acid (9.9%).

Refined olive oil was obtained from Koipe S. A. (Andujar, Jaén, Spain). This oil was free of phenols and phospholipids. Its tocopherol content was 124 ppm (120 ppm of α-tocopherol and 4 ppm of γ-tocopherol). 1-(4-Methoxyphenyl)-1*H*-pyrrole (**1**), 2-pentylfuran (**4**), *p*-anisidine, lysine (Lys), and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich. A solution of 0.1 N HCl in 2-propanol was obtained from Merck. Other reagents and solvents were of analytical grade and were purchased from reliable commercial sources.

Measurement of Antioxidative Activity. Oxidative stability of refined olive oil was compared with refined olive oil samples containing PE, PC, Lys, BHT, or mixtures of PE/Lys, PC/Lys, or PC/PE added at concentrations of 100−400 ppm. Antioxidants (5 mg) were dissolved in 2 mL of water containing 1% of tween 20 and added to the oil at the concentration indicated. The total amount of solvent added to the oil was always 400 μL, including the control oil.

Oil samples (2.5 g) were heated at 110 °C in a Metrohm Rancimat (Metrohm AG, Herisau, Switzerland). A continuous airstream (10 L/h) was passed through the heated sample, and the volatiles were absorbed in a conductivity cell. Conductivities were continuously monitored until a sudden rise signified the end of the induction period (IP). The color was determined in the oxidized oils obtained at the end of the IP. The presence of pyrroles was investigated in partially oxidized oil samples. Samples selected for pyrrole analyses were collected when the initial cell conductivity of 2 μS/cm increased to 10 μS/cm.

Spectrophotometric Determination of Pyrroles in Oxidized Oils. Pyrrole content in fully oxidized oils could not be determined because the presence of high amounts of oxidized lipids interfered in the method described by Zamora et al. (*19*). However, when only partially oxidized oils were employed, the presence of pyrroles in these oils could be studied by modifying this method. Briefly, oxidized oils (600 mg) were diluted with tetrahydrofuran (total volume 1.2 mL), and 600 μL of 0.1 N HCl in 2-propanol was added. The obtained solution was then treated with 300 μL of 0.1 N HCl in 2-propanol containing 23% of propyl gallate and 15% of *p*-(dimethylamino)benzaldehyde and rapidly incubated at 10 °C for 5 min. The absorbance spectrum between 450 and 600 nm was recorded.

Identification of Produced Pyrroles in Oxidized Oils by GC−MS. Some of the produced pyrrolic carbonyl/amine reaction products were identified after converting them into volatile derivatives. This reaction, which has been described very recently (*20*), is produced as a consequence of the reversion of the Paal−Knorr reaction suffered by 1*H*-pyrroles in acid media. Briefly, 500 mg of oxidized oil was treated successively with 2 mL of 0.3 M sodium citrate, pH 3, 1 mL of *p*-anisidine solution (1 mg/mL in 0.3 M sodium citrate, pH 3), and 50 μL of BHT solution (0.8% in acetic acid). The resulting mixture was stirred, bubbled with nitrogen, and heated at 110 °C for 20 h under an inert atmosphere. After that time, the resulting mixture was extracted three times with 2 mL of chloroform−methanol (3:2), and the organic layer was recovered and taken to dryness under nitrogen. The resulting residue was dissolved in 500 μL of chloroform and analyzed by GC−MS.

GC−MS analyses were conducted with a Hewlett-Packard 6890 GC Plus coupled with an Agilent 5973 MSD (Mass Selective Detector-Quadrupole type). A fused-silica HP5-MS capillary column (30 × 0.25 mm i.d.; coating thickness 0.25 μm) was used. Working conditions were: carrier gas helium (1 mL/min at constant flow); injector: 250 °C; oven temperature: from 70 (1 min) to 240 °C at 5 °C/min and then to 325 °C at 10 °C/min; transfer line to MSD: 280 °C; ionization EI 70 eV. Identification of 1-(4-methoxyphenyl)-1*H*-pyrrole (**1**), 1-(4-methoxyphenyl)-2-ethyl-1*H*-pyrrole (**2**), 1-(4-methoxyphenyl)-2-pentyl-1*H*-pyrrole (**3**), and 2-pentylfuran (**4**) was carried out by comparison of their retention indexes and mass spectra with those of authentic compounds. Compounds **1** and **4** were obtained from commercial sources (see above), and retention indexes and mass spectra of compounds **2** and **3** were described previously (*21*).

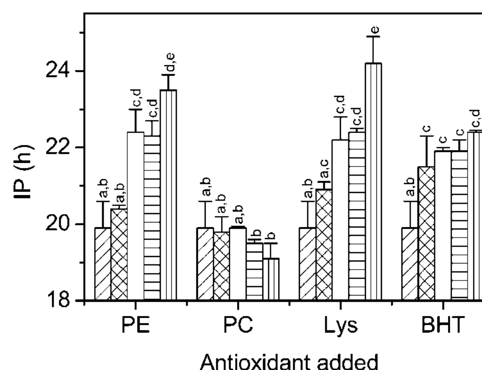


Figure 1. Rancimat IPs of refined olive oil treated with 0 (control oil, slashed bars), 100 (crosshatched bars), 200 (open bars), 300 (horizontally striped bars), or 400 ppm (vertically striped bars) of phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysine (Lys), or BHT. Values are means ± SD for, at least, two experiments. Means with different letters are significantly different ($p < 0.05$).

Determination of Oil Colors. Oil colors were determined spectrophotometrically employing a Shimadzu UV-2401 PC UV−vis spectrophotometer. Yellowness index (YI) was determined according to Francis and Clydesdale (*22*):

$$\mathrm{YI} = 142.86 \cdot b^*/L^*$$

Statistical Analysis. All determinations are expressed as mean values ± standard deviations (SD) of, at least, two independent experiments. Statistical comparisons among different groups were made using analysis of variance. When significant F values were obtained, group differences were evaluated by the Student−Newman−Keuls test (*23*). All statistical procedures were carried out using Primer of Biostatistics: The Program (McGraw-Hill, Inc., New York). The significance level is $p < 0.05$ unless otherwise indicated.

RESULTS

Antioxidative Activities of PE, PC, Lys, and BHT in Refined Olive Oil As Determined by the Rancimat Method. The addition of phospholipids, Lys, or BHT to a refined olive oil at a concentration of 100−400 ppm influenced the oil stability as determined by the Rancimat method (**Figure 1**). Thus, the addition of PE significantly increased oil stability when added at 200 ppm or more, and a correlation was observed between the induction period of the oil and the amount of PE added ($r = 0.98$, $p = 0.0033$). The IP of the oil increased 18% when 400 ppm of PE was added, and the protection offered by this phospholipid was similar to that of BHT when added at 200−400 ppm.

Different from PE behavior, the addition of PC did not contribute to oil stability. In fact, the IP seemed to be negatively correlated with the amount of PC added ($r = -0.92$, $p = 0.025$), and the IP of the oil decreased 4% when 400 ppm of PC was added.

The addition of Lys also increased oil stability when added at 200 ppm or more, and a correlation was observed between the IP of the oil and the amount of Lys added ($r = 0.98$, $p = 0.0044$). The addition of 400 ppm of Lys increased the IP of the oil 22%, and the protection offered by this amino acid was

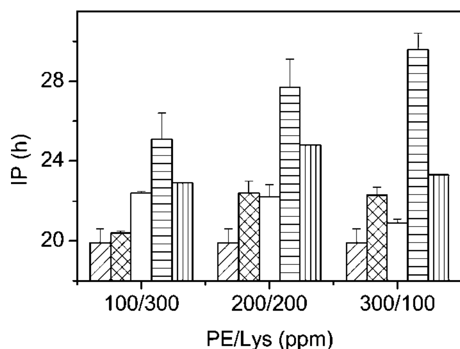


Figure 2. Rancimat IPs of refined olive oil treated with nothing (control oil, slashed bars), 100, 200, or 300 ppm of PE (crosshatched bars), 300, 200, or 100 ppm of Lys (open bars), and 100/300, 200/200, or 300/100 ppm of PE/Lys mixtures (horizontally striped bars). The theoretical additive IP of the mixture has also been included for comparison (vertically striped bars). Experimental IP values are means ± SD for, at least, two experiments.

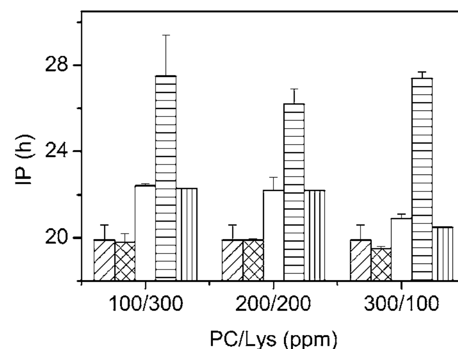


Figure 3. Rancimat IPs of refined olive oil treated with nothing (control oil, slashed bars), 100, 200, or 300 ppm of PC (crosshatched bars), 300, 200, or 100 ppm of Lys (open bars), and 100/300, 200/200, or 300/100 ppm of PC/Lys mixtures (horizontally striped bars). The theoretical additive IP of the mixture has also been included for comparison (vertically striped bars). Experimental IP values are means ± SD for, at least, two experiments.

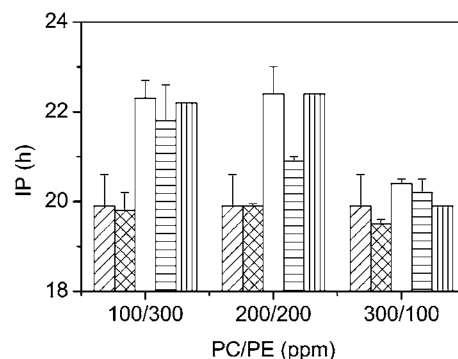


Figure 4. Rancimat IPs of refined olive oil treated with nothing (control oil, slashed bars), 100, 200, or 300 ppm of PC (crosshatched bars), 300, 200, or 100 ppm of PE (open bars), and 100/300, 200/200, or 300/100 ppm of PC/PE mixtures (horizontally striped bars). The theoretical additive IP of the mixture has also been included for comparison (vertically striped bars). Experimental IP values are means ± SD for, at least, two experiments.

similar, or superior, to that of BHT when added at 100−400 ppm.

Finally, BHT addition also increased the IP of the oil, and the protection offered by this synthetic antioxidant also seemed to be concentration-dependent, although a correlation poorer than those found for PE or Lys was observed ($r = 0.93$, $p = 0.023$). The addition of 400 ppm of BHT increased the IP of the control oil 12%. This increase was significantly lower than that of 400 ppm of Lys.

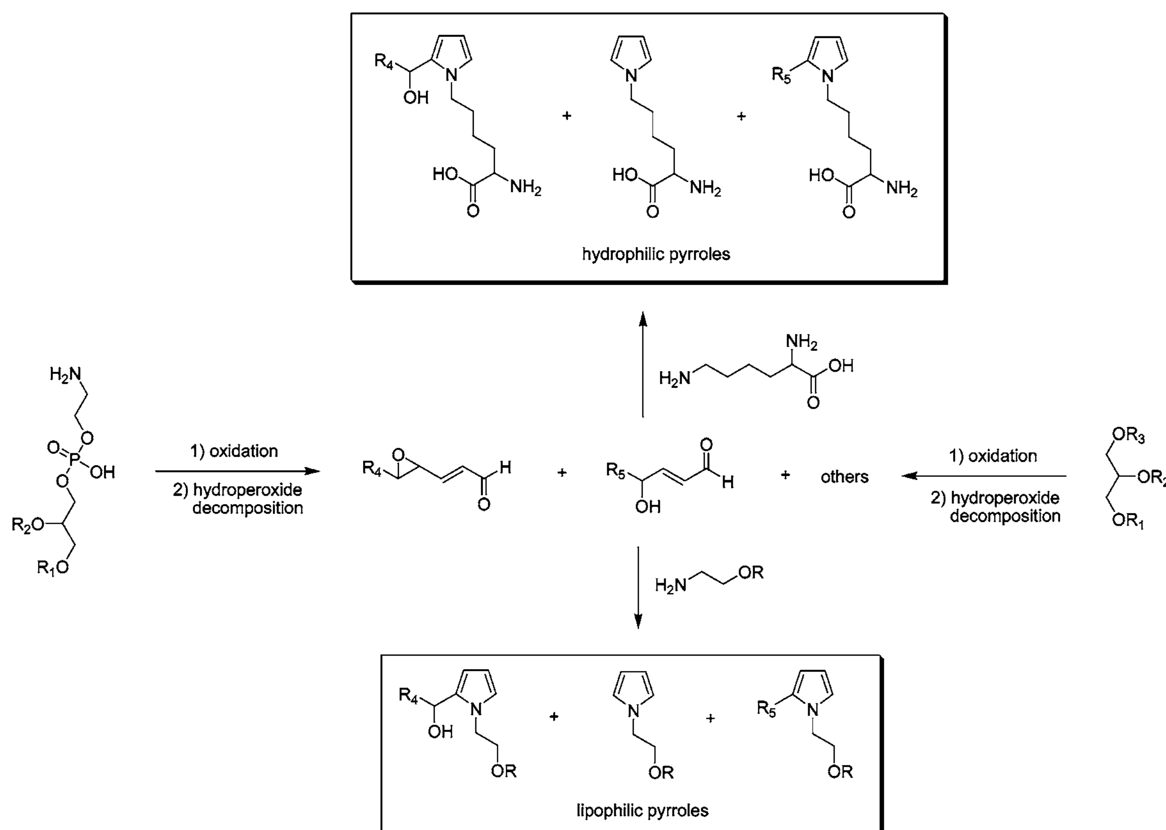
Antioxidative Activities of Phospholipid/Amino Acid Mixtures or Mixtures of Phospholipids As Determined by the Rancimat Method. When the antioxidants studied in the previous assay were tested simultaneously, the protection offered by their mixtures differed considerably from the additive effect that might be expected for the two added antioxidants. **Figure 2** shows the IPs of refined olive oil treated with three mixtures of PE/Lys: 100/300 ppm, 200/200 ppm, and 300/100 ppm. Although the three oils were treated with 400 ppm of antioxidants, the IPs of the oils treated with the three mixtures were different, and a higher amount of PE seemed to protect the oil more effectively. The figure also includes for comparison the IPs of PE and Lys at the added concentration and the hypothetical additive IP of the mixture, which was calculated by adding the protection exhibited by the components of the mixture at the concentrations added. As can be observed, when 100 ppm of PE and 300 ppm of Lys were added, the IP of the oil increased 5.2 h (26% higher than the IP of the control oil). The theoretical increase of the IP of this oil should have been 3.0 h (0.5 h for 100 ppm of PE and 2.5 h for 300 ppm of Lys). Therefore, the increase in the IP was 73% higher than expected. Something similar also occurred for the other two assayed mixtures. Thus, the IP of the oil treated with 200 ppm of PE and 200 ppm of Lys increased 7.8 h. This increase was 59% higher than the additive effect of the two assayed antioxidants (4.9 h). Finally, when the oil was treated with 300 ppm of PE and 100 ppm of Lys, the IP of the oil increased 9.7 h, which was 185% higher than the expected additive effect of 3.4 h. These results suggested a synergistic effect of both antioxidants. This effect was considerably higher when 300 ppm of PE and 100 ppm of Lys were used.

Similar results were obtained when mixtures of PC and Lys were added to the refined olive oil (**Figure 3**). Thus, the mixture of 100 ppm of PC and 300 ppm of Lys increased the IP of the oil 7.6 h, which was 217% higher than the additive effect of both antioxidants (2.4 h). Analogously, the addition of 200 ppm of PC and 200 ppm of Lys increased the IP of the oil 6.3 h. This was 174% higher than the additive effect of both antioxidants. Finally, the addition of 300 ppm of PC and 100 ppm of Lys increased the IP of the oil 7.5 h, and this was 1150% higher than the additive effect of both antioxidants (0.6 h). Therefore, a synergistic effect was also observed between PC and Lys, and this was higher than the above-described effect for the mixtures of PE and Lys. In addition, and analogously to that found for the mixtures of PE and Lys, the highest effect was observed for the mixture of 300 ppm of phospholipid and 100 ppm of the amino acid.

Different from PE/Lys and PC/Lys mixtures, the mixtures of PC and PE did not exhibit any synergism (**Figure 4**). Thus, the oil treated with 100 ppm of PC and 300 ppm of PE increased the IP of the oil 1.9 h, and the additive effect of both antioxidants is 2.3 h. The oil treated with 200 ppm of PC and 200 ppm of PE increased the IP 1.0 h, and the additive effect of both antioxidants at 200 ppm is 2.5 h. Finally, the addition of 300 ppm of PC and 100 ppm of PE increased the IP 0.3 h, and the theoretical increase is 0.0 h. Therefore, when the amino acid was not present, no synergism was observed although an amino phospholipid was present.

Formation of Carbonyl/Amino Reaction Products in the Oil Samples Oxidized in the Rancimat. Lipid oxidation is a complex process in which hydroperoxides are the initial

Scheme 1. Production of Reactive Carbonyls during PE and Triacylglycerol Oxidation and the Later Formation of Pyrrolized Phospholipids or Amino Acids by Carbonyl/Amine Reactions[a]



[a] The scheme only includes the phospholipid PE because PC cannot produce pyrrolized phospholipids although it also contributes to reactive carbonyls production. In addition, only the production of pyrroles in the ϵ-amino group of lysine is shown. Nevertheless, analogous pyrroles are also produced in the α-amino group of the amino acid. R is the phospholipid without the polar head; R_1, R_2, and R_3 are fatty acid chains; R_4 and R_5 are alkyl chains.

products, and then they enter into numerous interlaced reactions involving substrate degradation and interaction, which results in myriad compounds of various molecular weights, flavor thresholds, and biological significance (*24*). When this oxidation takes place in the presence of amino compounds, including amino phospholipids, amino acids, and proteins, the formation of oxidized lipid/amino compound reaction products is an unavoidable process, which should be considered as a last step in the lipid oxidation process (*17*, *25*, *26*) and that contributes to the changes of colors, flavors, and antioxidative activities produced in foods during processing and storage (*9*, *19*, *27*−*30*). Therefore, during oil oxidation, the carbonyl derivatives produced in the Rancimat vessel should react with the amino compounds present there and form carbonyl/amino reaction products. Among these products, pyrrole derivatives with antioxidant properties have been shown to be produced in different oxidized lipid/amino compound mixtures (*25*, *26*), and they can be determined spectrophotometrically with the Ehrlich reagent (*19*, *31*, *32*). Pyrrole formation is shown in **Scheme 1**.

Fully oxidized samples, recovered from the Rancimat, exhibited some interferences in the Ehrlich procedure, and their pyrrole content could not be determined. However, when partially oxidized oils were treated with the Ehrlich reagent, the formation of Ehrlich adducts was observed. Thus, **Figure 5** shows the absorbance spectra of Ehrlich adducts produced in (**a**) the control oil, (**b**) the oil treated with 400 ppm of PE, and (**c**) the oil treated with 300 ppm of PE and 100 ppm of Lys. The spectrum of the control oil exhibited a maximum at ~480 nm that was produced by the propyl gallate added to avoid destruction of Ehrlich adducts by lipid radicals. In addition, a small shoulder was observed at ~515 nm. An absorbance at this wavelength is characteristic of Ehrlich adducts of pyrroles (*19*). This shoulder may correspond to trace amounts of pyrroles formed in the oil between the lipid oxidation products produced and the minute amounts of amino compounds that are present among the minor components of the oil (*33*, *34*).

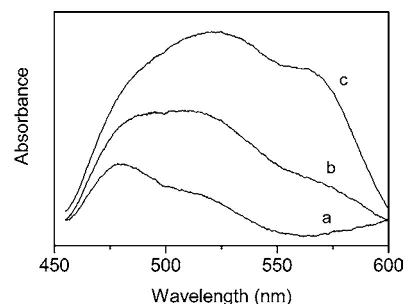
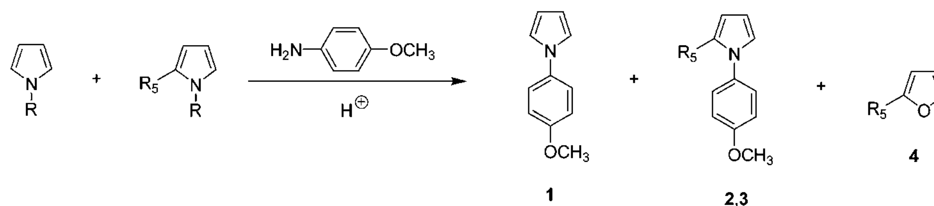


Figure 5. Absorbance spectra of Ehrlich adducts obtained by treating partially oxidized (**a**) control refined olive oil, (**b**) oil treated with 400 ppm of PE, and (**c**) oil treated with 300 ppm of PE and 100 ppm of Lys, with *p*-(dimethylamino)benzaldehyde.

When the oil was oxidized in the presence of 400 ppm of phosphatidylethanolamine, the band at ~515−520 nm increased considerably and a shoulder also appeared at ~570 nm. Both absorption bands are characteristic of Ehrlich adducts of pyrrole derivatives (*19*, *31*, *32*). When studying these samples, the absorption band produced by propyl gallate was only a shoulder at ~480 nm in the absorption spectra obtained.

Finally, when the oil was oxidized in the presence of 300 ppm of phosphatidylethanolamine and 100 ppm of lysine, the

Scheme 2. Conversion of Amino Acid- and Amino Phospholipid-Bound Pyrroles into Volatile Pyrroles[a]



[a] R is the amino acid or amino phospholipid chain. For **2**: R_5 = ethyl. For **3** and **4**: R_5 = pentyl.

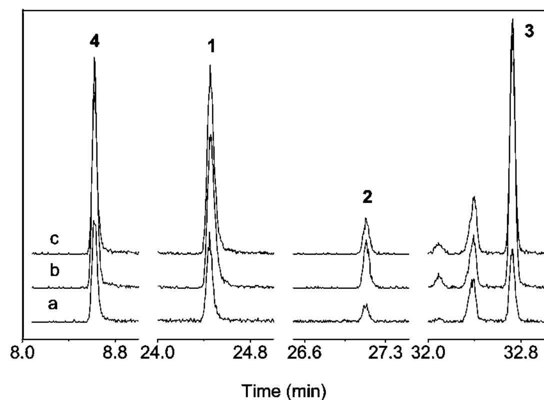


Figure 6. Ion chromatograms of GC–MS analysis of pyrrole derivatives produced in (**a**) control oil, (**b**) oil treated with 400 ppm of PE, and (**c**) oil treated with 300 ppm of PE and 100 ppm of Lys, after oxidation in the Rancimat vessel. Pyrroles detected were 1-(4-methoxyphenyl)-1*H*-pyrrole (**1**), 1-(4-methoxyphenyl)-2-ethyl-1*H*-pyrrole (**2**), and 1-(4-methoxyphenyl)-2-pentyl-1*H*-pyrrole (**3**). 2-Pentylfuran (**4**) was always produced. The *m*/*z* ions employed for the different compounds were 138 (**4**), 158 (**1**), and 186 (**2** and **3**). 2-Pentylpyridine was employed as internal standard to standardize the obtained chromatograms.

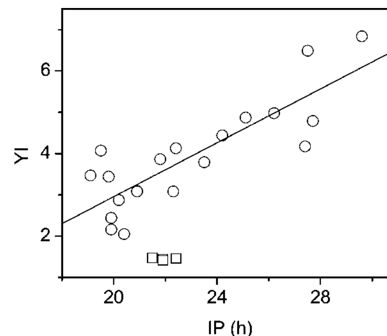


Figure 7. Plot of Rancimat IPs obtained for the refined olive oils treated with 100–400 ppm of PE, PC, Lys, and their mixtures, against the YI of the fully oxidized oils recovered from the Rancimat. The squares are the data obtained for the oil samples treated with 100–400 ppm of BHT.

intensity of the bands corresponding to the Ehrlich adducts achieved the highest values, and the spectra exhibited two maxima at ~520−525 and ~570 nm, respectively.

Although the concentration of pyrrole derivatives could not be calculated, the relative intensity of Ehrlich adducts maxima was related to the antioxidative activity exhibited by the different samples. Thus, 400 ppm of phosphatidylamine protected the oil (**Figure 1**), but the protection offered by the mixture of 300 ppm of phosphatidylethanolamine and 100 ppm of lysine was much higher (**Figure 2**). In fact, there was a correlation (r = 0.9999, p = 0.01) between the absorbance at 515 nm of the adducts obtained by treating these partially oxidized oils with *p*-(dimethylamino)benzaldehyde and the IPs obtained for the same oils.

An unambiguous confirmation of the formation of these pyrrole derivatives was obtained by GC−MS. As indicated in **Scheme 1**, the produced pyrroles should be bound to either the amino phospholipid or the amino acid. These either low volatile or large pyrrole derivatives could not be determined directly. However, a recently described reaction allowed their conversion into volatile pyrrole and furan derivatives (*20*). This reaction is based on the opening and latter closure of pyrrole rings in acid media and in the presence of an aromatic amine (**Scheme 2**). Thus, when oxidized oils obtained from the Rancimat vessel were heated overnight at pH 3 in the presence of *p*-anisidine, the formation of compounds **1**−**4** was observed (**Figure 6**), therefore confirming the presence of N-substituted 1*H*-pyrroles and N-substituted 2-(ethyl or pentyl)-1*H*-pyrroles in the oxidized oils. Although no quantitative procedure has been yet developed for the determination of these compounds, they were always present in a certain extent in oil samples treated with PE, Lys, and their mixtures. In addition, they were also present in a lower extent in the control oils, therefore confirming the formation of small amounts of pyrroles in these oils, which were previously described by using the Ehrlich reagent (see above).

A further step in the pyrrolation process suffered by amino compounds as a consequence of lipid oxidation is the polymerization of some of these pyrrole derivatives (*35*). The mechanism for this polymerization reaction is mostly known, and it has been related to browning development (*35*−*37*). Therefore, the study of the browning developed in the oxidized samples should be an additional proof of the formation of these carbonyl/amine reaction products. In fact, when the YI of the oxidized oil samples obtained from the Rancimat was plotted against the IPs obtained for the different oils, a linear correlation (r = 0.84, $p < 0.0001$) was observed (**Figure 7**). Exceptions were the BHT-treated samples (squares in **Figure 7**). The antioxidative activity of these samples is not a consequence of carbonyl/amine reaction products development, and, therefore, they were far from the straight line obtained for the other samples.

DISCUSSION

Lipid oxidation in foods is a serious problem, often difficult to overcome, that leads to loss of shelf life, palatability, functionality, and nutritional quality (*30*, *38*, *39*). This process may be delayed by antioxidants, which influence either the onset of oxidation or the rate at which it proceeds (*30*, *40*). Antioxidants can occur as natural constituents of foods, but they can also be intentionally added to products or formed during processing. The effectiveness of these compounds depends on different factors, including their innate potency and the occurrence of complex interfacial phenomena in oils and food emulsions (*8*).

The method employed to determine antioxidative effectiveness also influences the obtained results (*41*). This is particularly important in accelerated stability tests because these methods use elevated temperatures and some of the tested antioxidants

may either be volatile or be decomposed at tested temperatures. On the other hand, these tests might be useful for testing natural antioxidants formed during processing. If conditions employed during the test favored antioxidant formation, these substances might be evaluated at the same time that they were being produced.

The results obtained in this study suggest that the Rancimat method may be appropriate to evaluate the antioxidants produced by reaction of amine compounds with lipid oxidation products. Thus, when PE was tested by employing the Rancimat method, carbonyl/amine compounds were produced in situ, and these compounds, which previous studies have shown to have antioxidative properties (*10*, *42*−*45*), protected the oil. These results were analogous to those obtained when antioxidative activity of slightly oxidized PE samples was tested by the Schaal oven test (*9*). On the other hand, PC, which cannot produce carbonyl/amine reactions, did not protect the oil, which is also in accordance with that previously determined by the test at 60 °C (*9*). Therefore, for the study of antioxidative activity of PC and PE, the Rancimat method and the Schaal oven test produced analogous results when the oxidation of the sample was needed to produce carbonyl/amine reaction products with antioxidative properties. Additional data of antioxidative properties of phospholipids in olive oils have been described by Velasco and Dobarganes (*46*).

Although PE exhibited a significant antioxidative activity, its effectiveness increased considerably when small amounts of lysine were added. This may be a consequence of the relative effectiveness of antioxidants in oils and emulsions. When PE is oxidized in the presence of Lys, two different groups of reactions are competing: those produced between the oxidized lipids and the amino group of the phospholipid and those produced between the oxidized lipids and the amino group of Lys (**Scheme 1**). Both groups of pyrroles are identical, but those produced by reaction of the oxidized lipids with the amino group of the amino phospholipid are lipophilic, and those produced with the amino group of the amino acid are hydrophilic. Therefore, their antioxidative activities in oils may be different. In fact, different studies have shown that lipophilic antioxidants are more effective in an oil-in-water emulsion system than in bulk oil, while the opposite trend is found for hydrophilic antioxidants (*47*, *48*). Thus, the oxidation of PE alone should only produce lipophilic antioxidants that should be less effective than the hydrophilic antioxidants produced in the presence of Lys.

This may also be the reason for the antioxidant activity found for Lys. The lipid oxidation products formed during triacylglycerol oxidation should react with the amino groups of the amino acid to produce hydrophilic pyrroles with strong antioxidative activity. However, because the triacylglycerols of the employed olive oil are more stable than the fatty acid chains of PE (olive oil is a high oleic oil and the employed PE was high linoleic), the oxidized products of fatty acid chains of PE should be produced earlier than the lipid oxidation products derived from triacylglycerols. This may be contributing to the higher antioxidative effect of the mixture of 300 ppm of PE and 100 ppm of Lys in comparison with the antioxidative effect of 400 ppm of Lys. In fact, the mixture of 300 ppm of phospholipid and 100 ppm of amino acid was always the most effective, therefore suggesting that a higher concentration of easily oxidizable lipids was more important than a higher concentration of primary amino groups.

A further confirmation of all these conclusions was obtained when different mixtures of PC and Lys were tested. Thus, the highly unsaturated fatty acid chains of PC can be easily oxidized and the oxidized products would react with the amino groups of Lys producing hydrophilic antioxidants. Because in the PE/Lys mixtures there is a competence for forming both lipophilic and hydrophilic antioxidants, and PC/Lys mixtures can only produce hydrophilic antioxidants, the synergism of PC/Lys mixtures was always higher than the synergism of PE/Lys mixtures at the three phospholipid/amino acid ratios assayed. The competence for forming pyrroles bound to either the phospholipid or the amino acid in PE/Lys mixtures was studied previously (*27*, *37*).

Different from PE/Lys or PC/Lys mixtures, PC/PE mixtures cannot produce hydrophilic pyrroles. Therefore, these mixtures should only produce the same pyrroles than PE alone, and the antioxidative effect of PC/PE mixtures was mostly the antioxidative activity of the employed PE.

All these results confirm that reactions are occurring during antioxidative activity evaluation by Rancimat and the IPs obtained are a consequence of the antioxidative activities of the products formed in the different processes involved. These results also point to the Rancimat as a valuable tool for testing antioxidative activities of antioxidants produced during food processing.

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