Antioxidant Synergy of α-Tocopherol and Phospholipids

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ABSTRACT: The prevention of oxidation of a refined sardine oil by α-tocopherol at 0.04%, by several phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL)] at 0.5%, as well as by combinations of α-tocopherol with each phospholipid, was investigated. The evolution of the oxidation process during 1 mon at 40 ± 2°C was followed by a series of methods, measuring peroxide value (PV), diene, triene, and polyene index, and absorbance at 430 nm, while α-tocopherol and phospholipid content were being monitoried. Among these indices, PV was found to be the most adequate to follow the process. PC was the most effective individual antioxidant as shown by the PV values obtained at the end of the storage period, which were 54.0, 83.4, 87.9, and 97.7 meq O_2/kg for PC, CL, PE, and α-tocopherol, respectively. The highest synergistic effect was obtained with a mixture of α-tocopherol and PE, and the second and third best by mixtures made with PC and CL, respectively. The corresponding PV values recorded at the end of the period were 27.0, 35.0, and 58.0 meq O_2/kg. The high degree of synergy between PE and tocopherol is probably due to the occurrence of a simultaneous antioxidant mechanism involving Maillard compounds.

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KEY WORDS: Oxidation, phospholipids, sardine oil, synergistic effect, α-tocopherol.

Interest in fish oils is increasing due to perceived nutritional benefits. Eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are the major polyunsaturated n-3 fatty acid components responsible for this appeal (1,2). Nevertheless, the high unsaturation level of fish oils and the precarious characteristics of the captured biomass on the one hand and the multiple sequential operations involved in fish capture and processing on the other, contribute to the preservation of fish oil as an important technological objective. The addition of antioxidants is essential for the preservation of this product in order to extend its shelf life, which is accomplished by overcoming the easy oxidation of fish oil.

The first technological attempt to stabilize food products against oxidation was made 60 yr ago. Various natural substances were used, but they were soon replaced by synthetic chemicals, which are cheaper and more easily available (3). However, problems concerning the possible toxicity of synthetic antioxidants arose and were discussed and investigated for many years (4). Food safety legislation has gradually become more and more complex, requiring the use of toxicity tests of synthetic antioxidants. These requirements contribute to more expensive and time-consuming production. There is presently a tendency for consumers to use natural products as antioxidants, as these are perceived as safe and do not require prior testing (5).

Lipid oxidation follows a complicated set of mechanisms, and no single antioxidant is effective for all stages and circumstances under which it might occur, save the absence of available oxygen. It may be advisable to use antioxidant combinations in which the antioxidants produce a synergistic effect (6).Tocopherols are the most widely used natural antioxidants (7). They act by inhibiting the propagation step of the free-radical autoxidation mechanism by reacting with various free radicals. It has been reported that they are much more effective when used in combination with other antioxidants (8–10), and phospholipids have been widely used as synergists in combination with phenolic antioxidants (3). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) have received the most attention for this purpose (11–13). The mechanism responsible for the observed synergy of tocopherols and phospholipids against oxidation is not very well understood, but seems to be related to the involvement of the amino group of phospholipids in the regeneration of tocopherol, by hydrogen transfer.

The aim of this work was to obtain a deeper knowledge about the mechanism responsible for this synergistic effect and to select the most efficient combination of antioxidants for fish oil preservation. For this purpose, we decided to investigate the antioxidant and synergistic effect of different phospholipids with α-tocopherol, by comparing it with the effects shown by a good hydrogen donor, para-nitroaniline (pNA).

MATERIALS AND METHODS

Raw materials. Sardine oil was obtained from sardine mince, which was washed with cold water and used to prepare vari-

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TABLE 1
Concentrations of Added Tocopherol, Phospholipids, and pNA in the Various Experiments[a]

Treatment	Toc (mg/g)	PC (mg/g)	PE (mg/g)	CL (mg/g)	pNA (mg/g)
Oil					
Oil + Toc	0.4				
Oil + PC		5			
Oil + PC + Toc	0.4	5			
Oil + PE			5		
Oil + PE + Toc	0.4		5		
Oil + CL			5		
Oil + CL + Toc	0.4		5		
Oil + pNA				5	
Oil + pNA + Toc	0.4			5	

[a]Toc, tocopherol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; and pNA, para-nitroaniline.

ous samples by mixing with α-tocopherol, phospholipids, and pNA (Table 1).

Phospholipids were dissolved in chloroform before they were added to the oil sample. Total evaporation of the solvent was confirmed by refractive index analysis. Sardine oil (5 g) with the appropriate additives was poured into a glass tube (10 mL) and allowed to oxidized for 1 mon in the dark at 40 ± 2ºC. Samples were taken at regular intervals for different analyses.

Analytical methods. Peroxide value (PV) determination was performed by a titrimetric method with potassium iodide and sodium thiosulfate (14). The amount of sample used was *ca.* 0.5 g oil.

For the determination of conjugated dienes, trienes, and Maillard compounds, sardine oil samples were dissolved in an isopropanol/heptane (1:1) mixture, to a concentration of 6 mg/mL. Absorbance was measured in a Hitachi U-2000 spectrophotometer (Tokyo, Japan) at 234, 268, and 430 nm, respectively.

Tocopherols were determined by normal-phase high-performance liquid chromatography (HPLC) following the method of Piironen *et al.* (15) with minor modifications. For sample preparation 9 mg of oil was dissolved in 250 µL of isohexane and 20 µL of the solution was injected in a system Jasco model PU 980 (Tokyo, Japan) equipped with an autosampler Jasco AS-950-10, and eluted withisohexane/isopropanol (99.8 : 0.2) using a flow rate of 0.5 mL/min in the HPLC system Beckman model 110A pump (München, Germany). The column used was a silica column Lichrosorb (250 × 3.0 mm i.d.) (Merck, Darmstadt, Germany). A fluorescence detector Jasco FP-920 was used with excitation wavelength at 292 nm and emission wavelength at 324 nm. Tocopherols were identified by comparison with standards (Merck, Darmstadt, Germany) and quantified by using a standard curve. Peaks were integrated using software from Borwin (version 1.2; Le Fontanil, France).

Samples of oil with PC and PE (100 mg) were dissolved in 500 µL of hexane/diethyl ether (1:1) and separated in a solid-phase Isolute silica column (Hengoed, United Kingdom). The nonpolar fraction was removed with 40 mL of hexane/diethyl ether (1:1), and the polar lipids were recovered with 20 mL of methanol and 20 mL of chloroform/methanol/water (3:5:2). Polar lipids were dissolved with hexane in a concentration of 2.5 mg/mL and injected (20 µL) into an HPLC Jasco PU 980 system (Japan) equipped with a YMC (Kyoto, Japan) PVA-Sil column (250 × 4.6 mm i.d.) using a method developed by Christie and Urwin (16). Detection was performed with an evaporative light-scattering SEDEX 65 equipment (Sedere, Alfortville, France). PC and PE were identified by comparison with standards (Merck), and peak areas were determined using software from Borwin (version 1.2).

Lipid samples were saponified and converted to methyl esters by heating in a nitrogen-flushed screw-cap tube with 5% CH_3COCl-MeOH for 1 h, according to Lepage and Roy (17). The fatty acid methyl esters were analyzed in a Varian 3400 gas chromatograph (Sugarland, TX) equipped with an autosampler and fitted with a flame-ionization detector. The separation was carried out with helium as the carrier gas in a fused-silica capillary column (Omegawax, 0.32 mm i.d. × 30 m, Supelco Inc., Bellefonte, PA) programmed from 180 to 200°C at 4°C/min, held for 10 min at 200°C, and heated to 210°C for 14.5 min, with the detector at 250°C. A split injector (100:1) at 250°C was used. Fatty acid methyl esters were identified by comparison of their retention time with those of Sigma (St. Louis, MO) chromatographic standards. Peak areas were determined using Varian software.

Polyene index was calculated using the percentages of major polyunsaturated fatty acids EPA, 20:5n-3, and DHA, 22:6n-3, and the percentage of 16:0, according to the formula of Lin *et al.* (18):

$$\text{polyene index} = (\%\ \text{EPA} + \%\ \text{DHA}) \times 100/\%16{:}0 \qquad [1]$$

The diene index represents the absorbance at 234 nm per gram of oil, and the triene index is the absorbance at 268 nm per gram of oil.

Inhibition of lipid oxidation (%) was calculated using the PV results according to the following equations by Saito and Ishihara (19):

$$\%\ \text{inhibition} = (S_{\text{oil}} - S_{\text{oil + phospholipid}})/S_{\text{oil}} \qquad [2]$$

$$\%\ \text{synergistic effect} = \frac{(S_{\text{oil + tocopherol}} - S_{\text{oil + tocopherol + phospholipid}})}{S_{\text{oil + tocopherol}}} \qquad [3]$$

where S_{oil} is the slope of the plot of PV vs. time for the sample oil and $S_{\text{oil + phospholipid}}$ and $S_{\text{oil + tocopherol + phospholipid}}$ are the slopes of the plots of PV vs. time of treated oil with phospholipids and phospholipids and tocopherol, respectively.

Statistical analysis. All trials were done in duplicate and data are expressed as means ± standard deviation of three de-

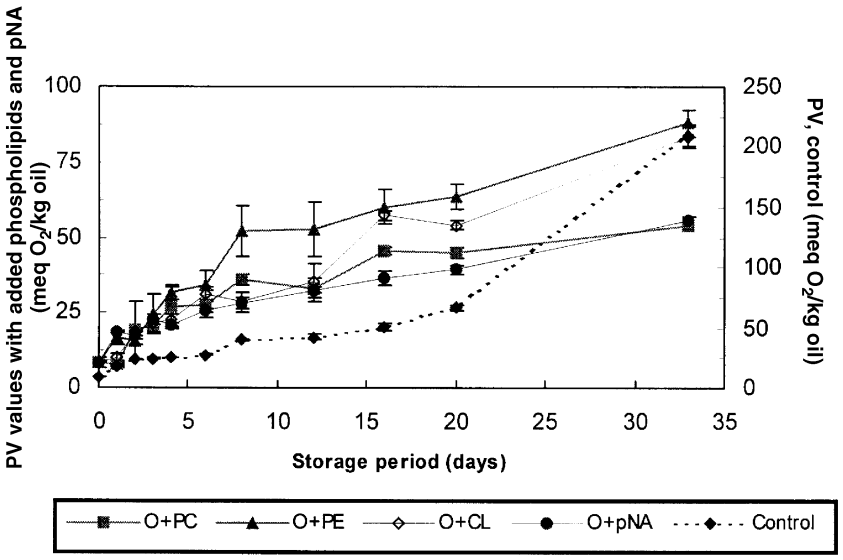

FIG. 1. Effect of added phospholipids and para-nitroaniline (pNA) on the peroxide value of sardine oil stored at 40°C. O, oil; PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; CL, cardiolipin. Data are expressed as means ± SD.

terminations. Data were assessed by one-way analysis of variance and values were considered statistically different for $P \leq 0.05$.

RESULTS AND DISCUSSION

Antioxidant effect of phospholipids. (i) PV. The evolution of PV of oil maintained at 40°C with and without the various phospholipids, tocopherol, and pNA is presented in Figure 1. PC was the most effective phospholipid antioxidant, presenting the lowest PV at the end of storage, similar to that obtained using pNA. CL, PE, and tocopherol were less effective, in that order. Segawa *et al.* (13) also reported on the greater effectiveness of PC compared with PE, phosphatidylglycerol, and phosphatidylinositol under similar circumstances. Pikul and Kummerow (20) reported a higher antioxidant effect for PC relative to PE, but considered this a consequence of the lesser unsaturation of fatty acids present in PC. However, the fatty acid profiles of PC and PE used in this work were similar, and linoleic acid was identified as the major fatty acid, consti-

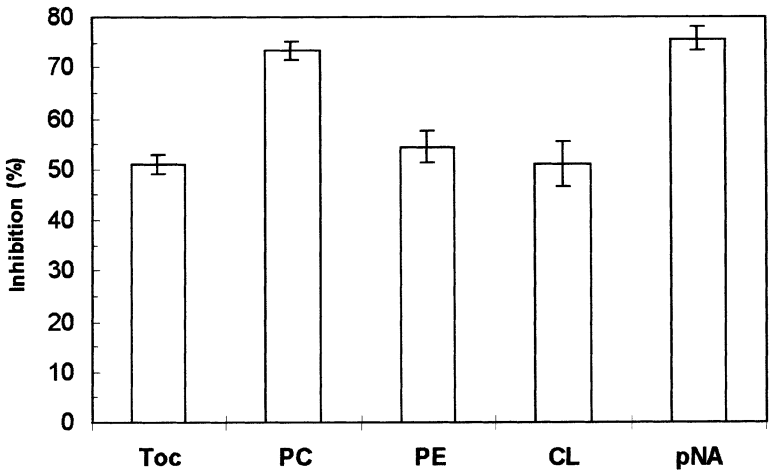

FIG. 2. Antioxidant effect of different antioxidants. Toc, tocopherol; see Figure 1 for other abbreviations. Data are expressed as means ± SD.

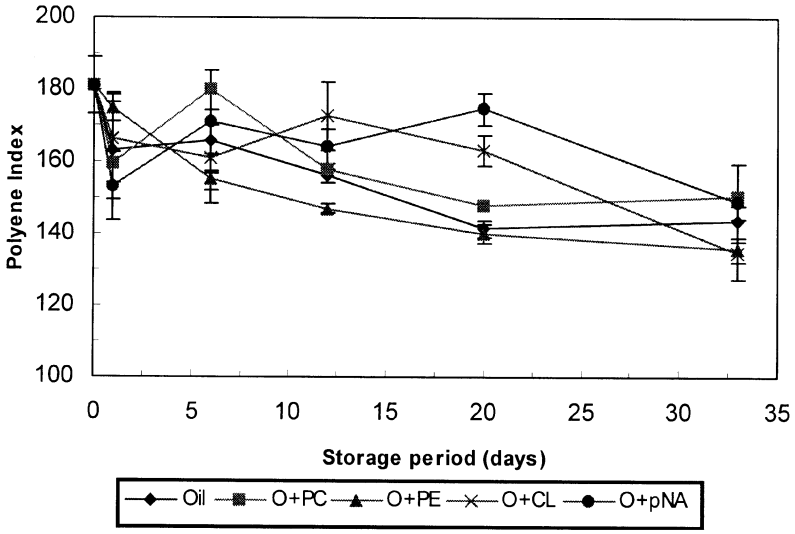

FIG. 3. Effect of added phospholipids and pNA on the polyene index of sardine oil stored at 40°C. See Figure 1 for abbreviations. Data are expressed as means ± SD.

tuting over 60% of both phospholipids. Similarly, Nwosu (21) found a high antioxidant effect in PC, concluding that the phospholipids with saturated fatty acids and a choline polar group were the most effective as antioxidants. Bhatia *et al.* (22), however, obtained diverse results, and they reported a higher antioxidant effect for PE than for PC.

The relative antioxidant inhibition for each phospholipid was calculated against that of tocopherol, and the results are presented in Figure 2. The highest inhibition at the end of storage was attained with pNA (75.8%) and PC (73.4%). The strong effect of pNA can be explained by the easy transference of hydrogen from the amino group to the lipid radicals, due to extensive delocalization of the resulting iminyl radical.

The lowest effect (*ca.* 50%) was obtained with CL and tocopherol. The inhibitory effect of CL could be due to the presence of two phosphate groups as referred to by Ishikawa *et al.* (23) in a study of the stability of lard with added phosphatidic acid. The poor antioxidant effect obtained with tocopherol alone (400 ppm) proved the need to use a combination of different antioxidants, especially for highly unsaturated fish oils. Koketsu and Satoh (24) also remarked on the low effectiveness of tocopherols (500 ppm) in the preservation of fish oils.

(ii) Polyene index. Polyene index has been reported as a good index for the purpose of monitoring degradation of polyunsaturated fatty acids during storage (18,25,26). In order to characterize evolution of this index during storage, the fatty acid profile was determined and the calculated values are presented in Figure 3. Oil samples containing PC showed the highest polyene index at the end of storage period (150.7). This index was closely matched by the value obtained for pNA (148.8), indicating the presence of a higher proportion of n-3 fatty acids. The results obtained with this phospholipid are in agreement with the evolution of PV previously discussed. This index does not seem to be the most adequate to monitor the oxidation of fish oil because only a small decrease is observed during all the storage period.

(iii) Diene and triene indexes. Diene index was determined for every sample during the storage period and the results obtained were submitted to statistical analysis. The results obtained consist of nonsignificant alterations and suggest that this index is not adequate to monitor lipid oxidation in this case.

The triene index was recorded in all samples during storage and seen to increase in every case; the highest rate was obtained with the phospholipid samples. But various compounds absorb at this wavelength (27), and the numerical value of the index therefore reflects the sum of the absorbances due to species with three conjugated double bonds generated by oxidation, and also to those stemming from Maillard reactions of the amino group of phospholipids with carbonyl compounds (28). In fact, as may be seen in Figure 4, the darker samples were those from oil with PE and PC, where the latter reactions are favored, proving that the triene index cannot be used to measure oxidation in this case.

(iv) Phospholipids. The evolution of PC, PE, and of Maillard reaction products measured at 430 nm was monitored during the whole storage period (Fig. 5). PC was relatively more stable than PE, which was rapidly consumed, becoming nondetectable. This decrease could be due to the formation of Maillard addition compounds, in accordance with the higher absorbance measured at 430 nm in the oil with these phospholipids (Fig. 5), and these compounds could in turn be responsible for the enhanced antioxidant effect recorded for PE.

Synergy effects. (i) PV. The variation of PV during the storage of samples with different phospholipids combined with tocopherol can be observed in Figure 6. The evolution of PV during the initiation step was similar in all experiments,

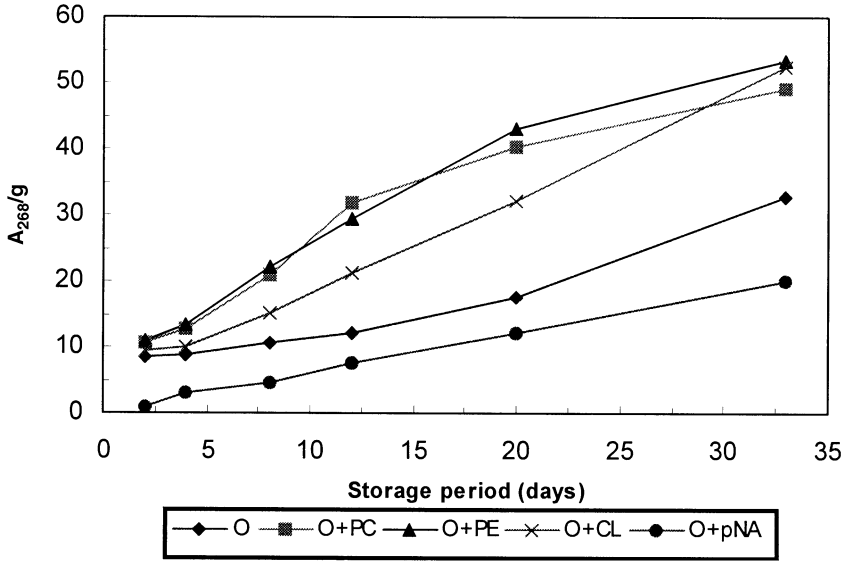

FIG. 4. Effect of added phospholipids and pNA on triene index (absorbance at 268 nm per gram of oil) of sardine oil stored at 40°C. See Figure 1 for abbreviations.

showing a very fast increase in the hydroperoxide formation.

Samples containing PC and PE, which presented the lowest level of hydroperoxides at the end of the experiment, evidenced approximately constant PV after 8 d. Stabilization of PV values for samples with CL and pNA occurred only after 15 d. However, this stabilization was never apparent for samples containing tocopherol alone.

The calculated synergy effect of these mixtures is presented in Figure 7. The highest synergistic effect was achieved with PE, in accordance with the results reported by Segawa *et al.* (29) on the use of PC and PE to stabilize fish oil. These authors explain the higher synergy of PE compared with that of PC by attributing it to the easier hydrogen transfer from the amino group to the tocopheroxyl radical, regenerating tocopherol. On the other hand, Weng and Gordon (30) attributed this synergy to a secondary antioxidant action of PE, reducing quinones formed during oxidation of phenolic compounds.

pNA, a good hydrogen donor, was used as a model to check the proposed mechanism of H transfer presumably in-

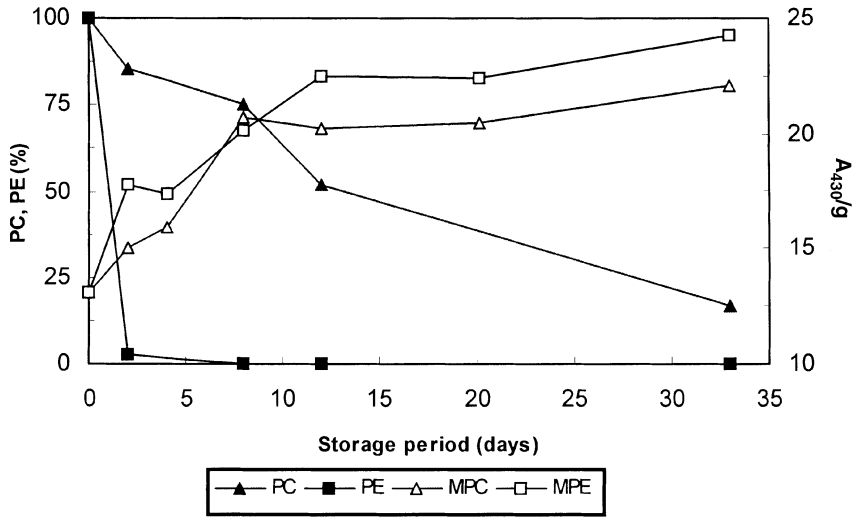

FIG. 5. Evolution of PC, PE, and Maillard (M) compound formation. MPC (MPE) are compounds resulting from the interaction between PC (PE) and secondary oxidation products. These polymeric brown products of ill-defined chemical structure absorb at 430 nm. For abbreviations see Figure 1.

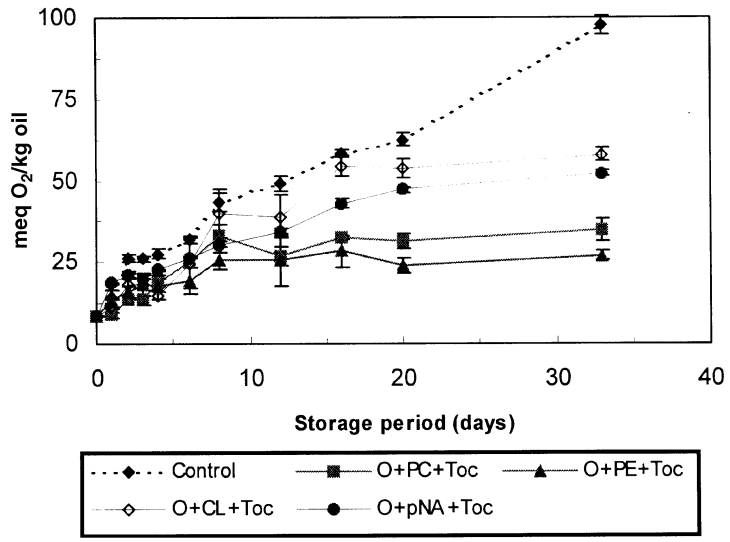

FIG. 6. Effect of added tocopherol, phospholipids, and pNA on peroxide value of sardine oil stored at 40°C. See Figures 1 and 2 for abbreviations.

volved in the synergy of tocopherol and phospholipids against oxidation. However, this compound in the presence of tocopherol shows a very low synergy (46.5%) compared with PE (72.4%). These results suggest that the secondary antioxidant action mentioned above, an additional effect of a fatty acid chain (12), or the formation of Maillard compounds (31) could be responsible for the synergy shown by PE and PC with tocopherol.

The low synergy obtained with CL (a phospholipid without an amino group), which could be construed as additional evidence for the importance of H transfer to tocopherol, remains unexplained.

(ii) Polyene index. The polyene index values of the various oil samples showed no significant difference (not shown), and this index was therefore of no practical use.

(iii) Diene and triene index. As mentioned before, the diene index did not show relevant differences during the storage period.

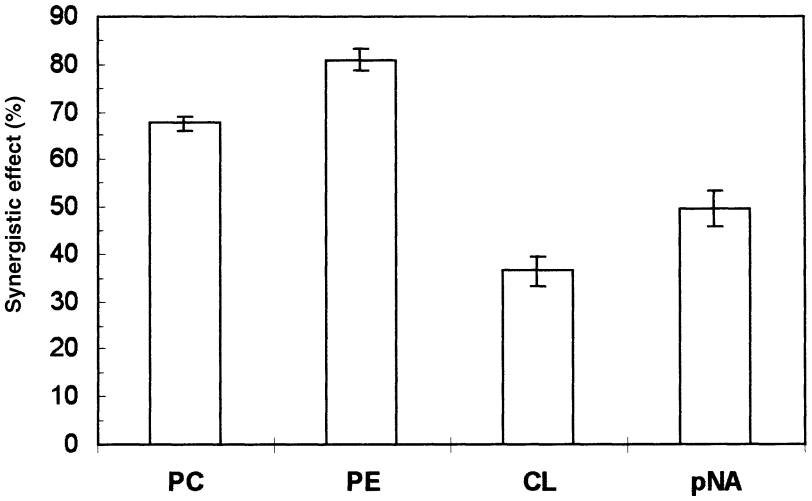

FIG. 7. Synergy of various phospholipids and pNA with tocopherol. For abbreviations see Figure 1.

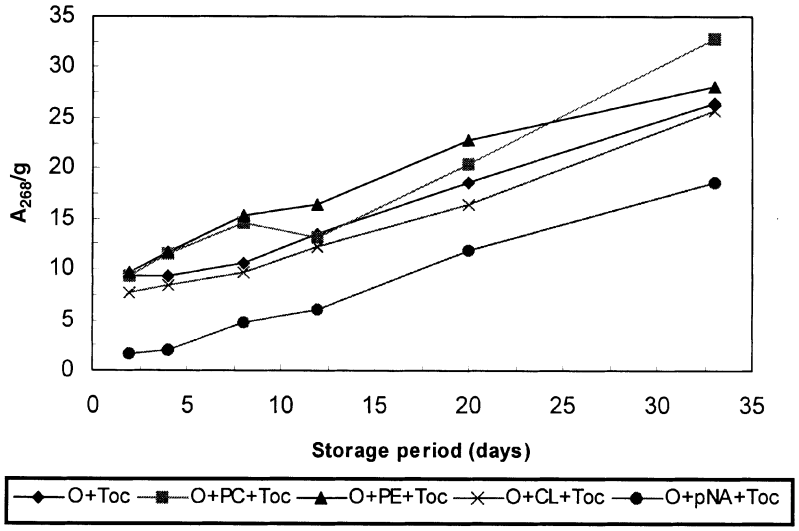

FIG. 8. Effect of added tocopherol, phospholipids, and pNA on triene index of sardine oil stored at 40°C. See Figures 1 and 2 for abbreviations.

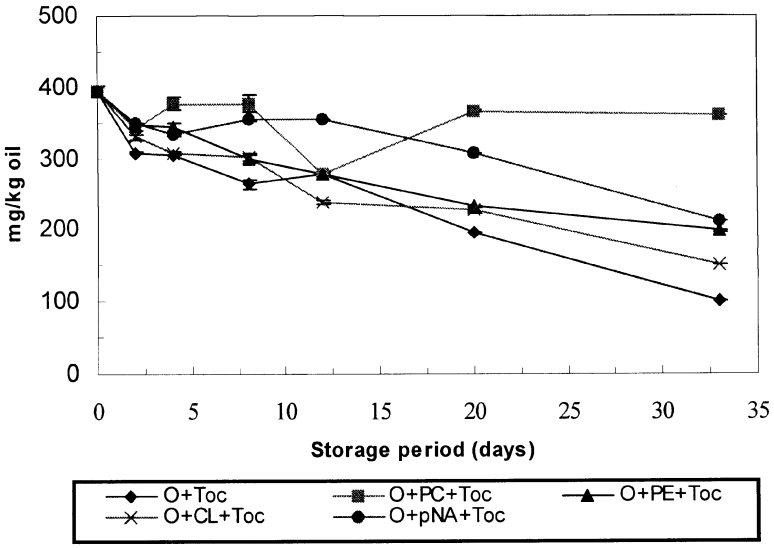

FIG. 9. Evolution of tocopherol content in various experiments. See Figures 1 and 2 for abbreviations.

The results obtained for the triene index are presented in Figure 8. During storage, a significant increase of this index occurred in all experiments. The highest values were recorded in the experiments with PC and PE and were attributed to the formation of Maillard compounds as previously mentioned. These compounds render this index inadequate in monitoring the evolution of oxidation.

(iv) Tocopherols. The evolution of tocopherol content during the different experiments is presented in Figure 9. No significant differences among trials were detected during the first

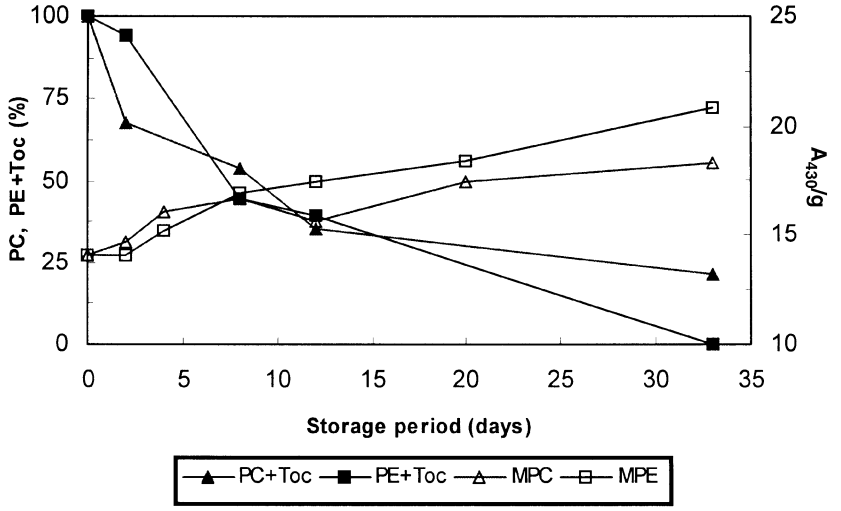

FIG. 10. Evolution of PC, PE and Maillard compound formation in samples with tocopherol. M, Maillard; see Figures 1 and 2 for abbreviations.

week. However, at the end of the experiment, the content of tocopherol in the oil with PC was significantly higher than in the other experiments.

The content of tocopherol was fairly constant, particularly in the oil with PC, during all the storage period which suggests a better interaction between tocopherol and this phospholipid.

(v) Phospholipids. Figure 10 shows the evolution of phospholipid contents in samples with tocopherol, as well as the formation of Maillard compounds. As can be seen, the presence of tocopherol contributed to increased PE stability, and a smaller formation of Maillard compounds with PE than was observed in the system containing this phospholipid alone. The low content of Maillard compounds also showed the lower availability of reactive carbonyl compounds than in the system where PE alone was used. This is a consequence of the lower rate of formation of hydroperoxides due to the presence of tocopherol.

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